Functional analysis of the ubiquitin-like modifier FAT10 in autophagy

Dissertation zur Erlangung des akademischen Grades eines Doktors
der Naturwissenschaften (Dr. rer. nat.)

vorgelegt von
Valentina Spinnenhirn
an der

Universität Konstanz

Mathematisch-Naturwissenschaftliche Sektion
Fachbereich Biologie

Tag der mündlichen Prüfung: 9.3.2015

1. Referent: Prof. Dr. Marcus Groettrup, Universität Konstanz

2. Referent: Dr. Felix Randow, University of Cambridge

3. Referent: Prof. Dr. Christof Hauck, Universität Konstanz

Konstanzer Online-Publikations-System (KOPS)
URL: http://nbn-resolving.de/urn:nbn:de:bsz:352-0-287368
Danksagung

Bei Marcus Groettrup möchte ich mich für die Bereitstellung des Themas und besonders für die fachliche und menschliche Unterstützung bedanken. Seine ansteckende, wissenschaftliche Begeisterung hat mich immer wieder motiviert.

Ebenfalls danke ich...

Dr. Felix Randow sowie Prof. Dr. Christof Hauck für die Erstellung der Gutachten und die Bereitschaft als mündliche Prüfer einzutreten.

der „chemical biology graduate school“ der Universität Konstanz für die finanzielle Unterstützung.

Michael Basler für seine Unterstützung und Zusammenarbeit, insbesondere bei in vivo Experimenten, bei denen er mir tatkräftig zur Seite stand.


den Mitarbeitern des „bioimaging center“ der Universität Konstanz für die wertvolle Unterstützung beim Mikroskopieren.

all meinen Kollegen auf P1, die ich aus Platzgründen nicht alle persönlich benennen kann. Die tolle Atmosphäre und die durchweg schöne Zeit waren Grund genug immer gerne zur Arbeit zu kommen. Insbesondere danke ich Kathrin, Andrea, Stella, Gretl, Richi, Valerie und Annette S. die nicht nur tolle Kollegen sind, sondern die ich auch als gute Freunde schätze. Vielen Dank für die schöne Zeit!

meiner Familie und meinen Freunden, die mich während meiner gesamten Doktorandenzeit unterstützt haben. Besonderer Dank gilt meinen Eltern, für das Vertrauen und die seelische Unterstützung sowie Patrick, für seine Liebe und unendliche Geduld.
Table of content

Danksagung .................................................................................................................. I
Table of content ............................................................................................................. II
Abstract .......................................................................................................................... VI
Zusammenfassung ........................................................................................................... VII
Preface ............................................................................................................................ IX

1 Introduction ................................................................................................................ 1
   1.1 Protein degradation by the ubiquitin-proteasome system (UPS) ....................... 1
       1.1.1 Ubiquitin and ubiquitin like modifiers (ULMs) ..................................... 2
       1.1.2 The proteasome ...................................................................................... 16
       1.1.3 The immunoproteasome ....................................................................... 19
   1.2 Protein degradation by macroautophagy ......................................................... 21
       1.2.1 Initiation .................................................................................................. 23
       1.2.2 Elongation ............................................................................................... 25
       1.2.3 Maturation ............................................................................................... 30
       1.2.4 Autophagy and immunity ................................................................... 30
   1.3 Protein aggregation ............................................................................................ 36

Aim of this study ............................................................................................................. 39

2 Material and methods ............................................................................................... 40
   2.1 Cell lines and cell culture ................................................................................ 40
   2.2 Generation of stable cell lines ......................................................................... 40
   2.3 Generation of primary cells ............................................................................. 41
   2.4 Transfection and electroporation .................................................................... 41
   2.5 siRNA ............................................................................................................. 42
   2.6 Quantitative real-time RT-PCR ..................................................................... 42
   2.7 Plasmids ........................................................................................................ 44
2.8 Generation of competent *E. coli* ................................................................. 45
2.9 Transformation .............................................................................................. 45
2.10 Cloning and site directed mutagenesis ......................................................... 46
2.11 *In vitro* FAT10ylation ................................................................................ 47
2.12 *In vitro* infection ......................................................................................... 47
2.13 Gentamicin protection assay ......................................................................... 48
2.14 Mouse strains ............................................................................................... 48
2.15 Mouse genotyping ......................................................................................... 49
2.16 *In vivo* infection ........................................................................................ 50
2.17 Determination of bacterial load ..................................................................... 50
2.18 Radiolabelling and pulse chase .................................................................... 50
2.19 Sample preparation and immunoprecipitation .............................................. 52
2.20 SDS-PAGE and immunoblot ....................................................................... 54
2.21 Immunohistochemistry ................................................................................ 55
2.22 Spinning disc microscopy ............................................................................. 57
2.23 Flow cytometry ............................................................................................ 57
2.24 Statistical analysis ....................................................................................... 58
3 Results ............................................................................................................. 59
3.1 The role of FAT10 in autophagy ................................................................. 59
  3.1.1 The FAT10-p62 interaction ...................................................................... 59
  3.1.2 Autophagosomal targeting of mCherry-EGFP-FAT10 ............................ 60
  3.1.3 Autophagosomal targeting of endogenous FAT10 ................................. 66
  3.1.4 FAT10 as a regulator of mCherry-EGFP-p62 autophagosomal targeting 67
3.2 FAT10 in xenophagy ..................................................................................... 69
  3.2.1 Characterization of *Salmonella* strain SHF2 ........................................ 70
  3.2.2 FAT10 decorates autophagy targeted *S. Typhimurium* ....................... 72
  3.2.3 FAT10 localization in microdomains ...................................................... 75
3.2.4 Characterization of the FAT10 decoration on SHF2........................................77
3.2.5 Autophagosomal targeting and FAT10 decoration of SHF2 follow the same kinetic.................................................................80
3.2.6 FAT10 deficiency or overexpression does not change bacterial replication in vitro.................................................................83
3.2.7 FAT10 deficiency in NRAMP1<sup>−/−</sup> mice reveals a higher susceptibility to S. Typhimurium.................................................................87
3.3 Characterization of the pool of ULM substrates.................................................90
  3.3.1 The pool of FAT10 substrates........................................................................91
  3.3.2 The pool of ubiquitin substrates ...................................................................94
3.4 Analysis of immunoproteasome-dependent protein turnover .........................96
  3.4.1 Ubiquitin conjugate degradation during immunoproteasome neosynthesis........................................................................96
  3.4.2 ALIS induction and degradation during immunoproteasome neosynthesis 98
4 Discussion ........................................................................................................100
  4.1 Putative FAT10 associated pathways..............................................................100
  4.2 FAT10 and cytosolic protein aggregates .........................................................101
  4.3 FAT10 as a potential autophagosomal targeting mechanism ..........................103
  4.4 FAT10 as a potential regulator of autophagy..................................................104
  4.5 FAT10 and xenophagy ....................................................................................106
    4.5.1 Characterization of FAT10 decoration on cytosolic S. Typhimurium .......106
    4.5.2 The functional role of FAT10 in xenophagy..............................................114
    4.5.3 Critical aspects of xenophagy during S. Typhimurium pathology ..........119
    4.5.4 FAT10 and p62-signaling in response to infection.................................119
    4.5.5 Other putative and confirmed FAT10 interacting proteins involved in pathogen or aggregate degradation..........................................................121
  4.6 Immuno- and constitutive proteasome degradation .......................................123
    4.6.1 Immuno- and constitutive proteasomes and the proposed differences in...
the proteolytic activity ...............................................................124

4.6.2 Mechanistic aspects of the immunoproteasome function ..........127

Tables and figures ......................................................................129

Abbreviations ............................................................................131

References ..................................................................................135

Eidesstattliche Erklärung ..............................................................161

Record of contribution ................................................................161
Abstract

The ubiquitin like modifier HLA-F adjacent transcript 10 (FAT10) resembles ubiquitin in many ways. FAT10 is, analogous to ubiquitin, covalently conjugated to substrate proteins and was shown to target substrates for proteasomal degradation in a ubiquitin independent manner. Importantly, FAT10 is constitutively expressed only in lymphoid organs but can be induced by pro-inflammatory cytokines like interferon-gamma and tumor necrosis factor-alpha in various other cell types. Additionally, FAT10 was found to interact covalently and non-covalently with p62. This protein can function as an adapter for autophagosomal degradation and as a scaffolding protein in diverse signaling pathways.

The first part of this study revealed the co-localization of FAT10 with p62 in cytosolic aggregates. Substrates for antigen presentation like defective ribosomal products (DRiPs) are known to accumulate in these cytosolic structures. But the analysis of FAT10 conjugated substrates revealed no preferential conjugation of FAT10 to newly synthesized proteins. Furthermore, the role of FAT10 in autophagosomal substrate targeting as well as its potential regulatory function in autophagosomal turnover was analyzed, but no functional relevance could be revealed. However, when the elimination of cytosolic pathogens via autophagy was investigated, FAT10 staining was detectable on the bacterial surface in immune fluorescence experiments. FAT10 positive S. Typhimurium were simultaneously decorated with ubiquitin and other autophagy markers. A kinetic analysis revealed an early but transient recruitment of FAT10 to bacteria which resembled that of p62. Bacterial replication was not detectably altered in FAT10-depleted or overexpressing cells in vitro. But survival experiments revealed a higher, though not significantly increased, susceptibility of FAT10-deficient mice compared to wild type NRAMP1-transgenic mice to orally inoculated S. Typhimurium. This study thereby describes FAT10 as a new player potentially associated with autophagosomal capturing and elimination of cytosolic S. Typhimurium.

In the second part of the study, the role of interferon-gamma inducible immunoproteasomes in protein homeostasis was reinvestigated. Recently, a novel function of immunoproteasomes was proposed. It was reported to have a higher proteolytic capacity compared to constitutively expressed proteasomes. The performed experiments in this part, however, revealed no differences in the degradation rate of polyubiquitin conjugates between immunoproteasome-deficient and wild type cells. Similarly, no differences were detectable with respect to elimination of cytosolic, polyubiquitin containing aggregates in these cells.
Zusammenfassung


Im zweiten Teil der Arbeit wurde die Rolle des Immunproteasoms im Zusammenhang mit der Proteinhomöostase untersucht. Kürzlich wurde eine neue Funktion für das
Preface


Most parts of section 3.2 and additional data from Hesso Farhan (Focal Area Infection Biology, Biozentrum, University of Basel), Michael Basler and Annette Aichem (both at the Biotechnology Institute Thurgau, Switzerland) have been published in Spinnenhirm, V. *et al.* The ubiquitin-like modifier FAT10 decorates autophagy-targeted Salmonella and contributes to Salmonella resistance in mice. *J. Cell Sci.* 127, 4883–93 (2014). I planned and conducted the experiments and the manuscript was written by me with some editing by Marcus Groettrup (University of Konstanz).

Data in chapter 3.4 has been published in Nathan, J. *et al.* Immuno- and constitutive proteasomes do not differ in their abilities to degrade ubiquitinated proteins. *Cell* 152, 1184–94 (2013). I planned and conducted the experiments with the exception of Figure 37 B, where Michael Basler (Biotechnology Institute Thurgau, Switzerland) performed three of the four quantified immunoblot kinetics.
1 Introduction

From protozoans to multicellular, complex organisms there is a need for each cell to maintain protein homeostasis. Protein synthesis is therefore constantly accompanied by protein degradation. This preserves not only the equilibrium state of protein homeostasis but simultaneously bears a level of regulation that influences cellular processes with equal potency than transcriptional and translational control. Therefore recycling of proteins is a complex process and is executed by several diverse catabolic pathways. These include two major pathways: proteasomal and lysosomal degradation. Both pathways are associated with preparative and completing processes like substrate modification, substrate delivery and further processing of degradation products.

1.1 Protein degradation by the ubiquitin-proteasome system (UPS)

Proteasomal degradation allows the breakdown of endogenous translation products in contrast to exogenous material that is primarily degraded via lysosomes. Proteins designated for proteasomal degradation are in most cases post translationally modified with ubiquitin. However, also ubiquitin independent proteasomal degradation is known for several substrates. The close proximity to the degradative machinery as well as an unstructured region is thereby sufficient for degradation. Additionally, conjugation of the ubiquitin-like modifier (ULM) HLA-F adjacent transcript 10 (FAT10) was shown to mediate proteasomal degradation in a ubiquitin independent manner. Proteasomal degradation products are further processed by cytosolic proteases that allow recycling of amino acids or trimming of the peptides for loading onto major histocompatibility complex (MHC) class I molecules. The entire process from substrate identification to peptide generation by the proteasome will be introduced in greater detail in this chapter.
1.1.1 Ubiquitin and ubiquitin like modifiers (ULMs)

1.1.1.1 Ubiquitin

Ubiquitin is a globular protein of ~8.5 kDa in size and is highly conserved as only three amino acids differ between yeast, plants and animals. It was initially discovered as a ubiquitously expressed and ATP dependent proteolysis factor (Ciechanover et al., 1980). The protein is transcribed from several genes throughout the genome either as a N-terminal fusion protein with ribosomal proteins or as a polyubiquitin precursor. Processing of ubiquitin fusion proteins and the polyubiquitin precursor into a functional, monomeric, 76 residue peptide with a C-terminal diglycine motive is mediated by specific hydrolases (Shabek and Ciechanover, 2010). The liberated diglycine motive is a prerequisite for ubiquitin and other ULMs to be conjugated covalently but reversibly to specific substrates. The conjugation, based on an isopeptide linkage, requires the action of a multistep enzyme cascade, catalyzing the activation and ligation of the C-terminal glycine carboxylate to an ε-amino group of a specific lysine residue within the substrate sequence (Hershko and Ciechanover, 1998). In a first step, the ubiquitin-activating enzyme termed E1 binds ATP and ubiquitin to form a ubiquitin adenylate and free pyrophosphate. This intermediate step results in a thioester bond between the active site cysteine and the C-terminal glycine carboxylate of ubiquitin. In a second ATP consuming step the E1-ubiquitin thioester recruits another ubiquitin to the adenylation site. This ternary complex is now competent to transfer the thioester-linked ubiquitin moiety to a cysteine residue on the ubiquitin-conjugating enzyme, termed E2, in a so called transthiolation reaction. Finally, a ubiquitin protein ligase, termed E3, catalyzes the transfer of ubiquitin from the E2 enzyme to the substrate lysine (Fang and Weissman, 2004) (Figure 1).

1.1.1.1.1 Ubiquitin conjugate formation

E2s can be charged with activated ubiquitin by two E1 enzymes called ubiquitin-activating enzyme 1 (UBA1) or UBA6 (Chiu et al., 2007; Handley et al., 1991; Jin et al., 2007; Pelzer et al., 2007). As UBA6 deficiency in mice was shown to be lethal, one can assume that there is no redundancy in ubiquitin activation. The general question, why there is a need for two E1 enzymes to activate ubiquitin remains unanswered (Chiu et al., 2007). There are several E2s encoded in the genome of all eukaryotes, ranging from about eight to over 50 in some multicellular plants and animals (Burroughs et al., 2008). In human, more than 40 are assumed to be ubiquitin conjugating enzymes (Merbl et al., 2013; Michelle et al., 2009). Importantly, one E2 enzyme called UBA6 specific E2 1 (USES1) accepts activated ubiquitin only from UBA6 (Chiu et al., 2007; Jin et al., 2007). This specificity of E2 binding to the
respective E1 is regulated by the ubiquitin-fold domain of the cognate E1. After the nucleophilic attack by the conserved E2 cysteine on the carbonyl group of the ubiquitin/ULM-E1 thioester linkage, the activated modifier is transferred either to the active site cysteine of an E3 enzyme or directly to a lysine of the substrate mediated by the E3 enzyme as an adapter.

There are hundreds of E3 ligases encoded in the human genome (Li et al., 2008). This number reflects their main function within the ubiquitination process, which is the assurance of substrate specificity. Through direct interaction or with the help of adaptor proteins, specific protein substrates are identified and the transfer of ubiquitin from a thioester intermediate of the cognate E2 enzyme to a target protein is mediated. Furthermore, it was reported that also preassembled polyubiquitin chains can be generated on E2s prior to their transfer to the target substrate (Li et al., 2007). For the transfer reaction, E3 ligases facilitate close proximity of the E2-ubiquitin thioester conjugate and the specific substrate. Distinct E3 families containing conserved protein domains have been identified (Figure 1). Homologous to E6-AP carboxyl terminus (HECT) domain E3s form thioester intermediates with ubiquitin, leading to ubiquitination of substrates. A cysteine close to the C-terminus accepts the ubiquitin and the amino terminal part of the ligase determines cellular localization and substrate specificity (Kamadurai et al., 2013). Other E3 ligases are categorized as really interesting new gene (RING) E3s. All RING E3 ligases coordinate two zinc ions via eight cysteine and histidine residues in a cross-brace formation. They are lacking a catalytic cysteine residue and therefore merely mediate the direct transfer of activated ubiquitin from the E2 to the substrate (Joazeiro and Weissman, 2000). A similar scaffolding function is described for U-box E3 ligases, which are modified RING-E3s without coordinated zinc ions (Aravind and Koonin, 2000). Another structurally related group of E3 enzymes comprises ligases known as the RING-between-RING (RBR) E3s (Marin and Ferrus, 2002). They differ from HECT and RING like E3s since they are complex multi-domain proteins with two RING like domains and an in between RING (IBR) domain. Functionally, these E3s combine the catalytic mechanisms of both RING and HECT E3 ligases, since one of the RING like domains contains an active site cysteine (Spratt et al., 2014).

1.1.1.1.2 Ubiquitin chains

Ubiquitin conjugation to a specific lysine residue within the substrate sequence can remain as a simple monoubiquitination. Additionally, this posttranslational modification can be accompanied by multiple cycles of ubiquitin activation and conjugation to the same substrate
by the respective E1, E2, E3 enzyme cascade. These ongoing rounds of conjugation can target additional lysines as well as the N-terminus within the substrate, resulting in multi-monoubiquitination modifications or target lysine residues within the previously conjugated ubiquitin (Figure 1). Mono- and multi monoubiquitination is of importance in signaling events and involved in endocytosis regulation (Mukhopadhyay and Riezman, 2007). Modifications of the first substrate-attached ubiquitin at specific lysines (K7, K11, K27, K29, K33, K48, K63 and N-terminus) lead to the formation of polymeric ubiquitin chains of diverse appearance (Komander, 2009). They can be built of only a single linkage type as well as a mixture of different linkages. Furthermore, a single ubiquitin moiety within the chain can be modified at two different lysines simultaneously, resulting in a branched chain (Kulathu and Komander, 2012). Of note, the exclusiveness of lysine modification has recently been challenged by “non-canonical” ubiquitination. The observation that even cysteine, serine or threonine residues can be targeting sites within the substrate further increases the complexity of this modification system (Kravtsova-Ivantsiv and Ciechanover, 2012; McDowell and Philpott, 2013).

Investigations to elucidate the function of specific chain types initially focused on K48-likages, which were the first to be characterized and are known to target substrate proteins for proteasomal degradation. Additionally, K29, K11, K27, and K6 linkages have been demonstrated, besides non-degradative functions, to reduce the half-life of substrates through proteasomal targeting in yeast and mammalian cells (Dammer et al., 2011; Johnson et al., 1995; Xu et al., 2009). K63 polyubiquitin chains and monoubiquitination serve diverse cellular functions, manly through proteasome-independent mechanisms, although also these modifications have been proposed to be sufficient for proteasomal degradation in vitro (Saeki et al., 2009). Conversely, with the help of proteomic analysis of cells, the K63 linkage was excluded from all other chain types to be a proteasome dependent, degradative signal (Nathan et al., 2013a; Xu et al., 2009). Undebated are non-degradative functions of K63 polyubiquitin chains similar to monoubiquitination that modify multiple signaling pathways, like protein kinase activation, DNA repair, membrane trafficking and chromatin remodeling. Simultaneously, K63 linked substrates have been implicated in the targeting of misfolded and aggregated proteins for autophagosomal degradation (Tan et al., 2008a; Tan et al., 2008b). Induced K63 ubiquitination during periods of proteasome impairment can even help to maintain cellular protein homeostasis (Lim et al., 2013). The function of linear ubiquitin chains (Met1) as a critical step in the nuclear factor of kappaB (NF-κB) pathway has recently been elucidated by several groups (Bianchi and Meier, 2009; Haas et al., 2009; Kirisako et
al., 2006). Surprisingly, also this atypical chain conformation is sufficient to be recognized by the proteasome and therefore all known ubiquitin chains have been shown to target substrates to the proteasome. To mention, some data relies on artificial model substrates and cell-free systems only. Therefore, these findings necessarily have to be confirmed in cells (Ciechanover and Stanhill, 2014).

The E2 binding specificity of the E1 enzyme at the beginning of the conjugation cascade ensures that ubiquitin or the appropriate ULM enters the respective conjugation pathway (Schulman and Harper, 2009). Additionally, the huge number of E3 ligases and their binding partners preserves the specificity with respect to the correct substrate. The complexity of the pathway is even extended by the diversity of polyubiquitin chains, which is mainly regulated by the E2 enzymes, with the exception of linear chain formation. E2s, which are dependent on RING E3 ligases, usually have a fixed linkage specificity and form only a single type of chains (Eddins et al., 2006; Petroski and Deshaies, 2005). Other E2s can generate multiple linkage types dependent on the E3 ligase they interact with (Kim et al., 2007b; Kirkpatrick et al., 2006). A fourth enzyme category, termed E4, can bind to ubiquitin moieties of preformed conjugates and catalyze ubiquitin chain assembly in conjunction with E1, E2, and E3 thereby editing preexisting ubiquitin chains (Koegl et al., 1999).

1.1.1.1.3 Ubiquitin deconjugation

The half-life of about ten hours measured for monomeric ubiquitin, which mediates the majority of cellular protein turnover, defines this protein as relatively stable (Carlson and Rechsteiner, 1987). This is in line with the observation that polyubiquitin chains are not degraded along with their substrates but get mostly recycled prior to proteolysis. But a minor portion of ubiquitin gets proteasomally degraded via at least two modes: as a free monomer or in a conjugated form as part of the targeted substrate (Shabek and Ciechanover, 2010). Recycling of the major part of polyubiquitin chains at the proteasome level is carried out by deubiquitinating enzymes (DUBs) (Figure 1), classified into either the ubiquitin C-terminal hydrolases (UCH) or the ubiquitin-specific processing proteases (UBP). They can likewise modify existing ubiquitin chains by trimming of single ubiquitin moieties or even deconjugate whole chains from a substrate at the “trunk”. Especially when proteasomal targeting is completed, ubiquitin chains are cleaved off by the action of a proteasome intrinsic regulator subunit and ubiquitin is released to maintain the cellular ubiquitin pool (Amerik and Hochstrasser, 2004). This group of enzymes renders the complex system of ubiquitin conjugation into a reversible modification which bears a further level of regulation. Especially
in the context of the half-life of substrates and signaling cascades regulated by ubiquitin modifications, DUBs play a major regulatory role.

**Figure 1: Ubiquitin conjugate formation and ubiquitin linkage types.** Ubiquitin is activated in an ATP dependent manner by an E1 enzyme followed by a transthiolation reaction mediated by an E2 enzyme. Conjugation and ligation to a specific substrate are accomplished via a thioester bond between the activated ubiquitin and a HECT E3 ligase or by direct transfer of activated ubiquitin from an E2 enzyme facilitated by a RING E3 ligase. Substrate deconjugation or disassembly of unanchored ubiquitin chains is mediated by deubiquitinating enzymes (DUBs). Catalysis of an isopeptide bond between the C-terminal glycine carboxylate of ubiquitin with the ε-amino group of a Lys-residue within the substrate can result in mono- or multiple monoubiquitination. Further rounds of modification targeting the previously conjugated ubiquitin at one of its seven lysines or the N-terminus lead to homotypic or mixed linkage polyubiquitination. Heterologous modifications can occur in the case of ubiquitin and SUMO. The image is adapted from (Husnjak and Dikic, 2012).
1.1.1.2 Ubiquitin like modifiers

Ubiquitin folds into a compact, globular β-grasp fold, termed “ubiquitin fold” (Vijay-Kumar et al., 1987). Dozens of proteins with varying levels of sequence similarity resemble this characteristic structure. These proteins are collectively called ubiquitin like proteins (UBLs) which can be further subdivided into two groups. The first group comprises proteins that are conjugated to substrates in a manner analogous to ubiquitin itself, termed ubiquitin like modifiers (ULMs). Members of the second group contain an integral element that forms a so called ubiquitin-like domain, and are therefore called ubiquitin-domain proteins (UDPs) (Jentsch and Pyrowolakis, 2000; Pelzer and Groettrup, 2010).

Several ULMs characterized today are listed in Table 1 (van der Veen and Ploegh, 2012). They are translated as precursors, processed, activated via an enzyme cascade, and attached to target proteins analogous to ubiquitin.

<table>
<thead>
<tr>
<th>ULM</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMOs</td>
<td>(Small ubiquitin like modifiers) -1, -2 and -3</td>
</tr>
<tr>
<td>NEDD8</td>
<td>(Neural precursor cell-expressed, developmentally downregulated 8)</td>
</tr>
<tr>
<td>Atg8 and Atg12</td>
<td>(Autophagy related gene 8 and 12)</td>
</tr>
<tr>
<td>UFM-1</td>
<td>(Ubiquitin-fold modifier-1)</td>
</tr>
<tr>
<td>HUB-1</td>
<td>(Homology to ub-1)</td>
</tr>
<tr>
<td>ISG15</td>
<td>(Interferon stimulated gene 15)</td>
</tr>
<tr>
<td>FAT10</td>
<td>(HLA-F locus associated transcript 10)</td>
</tr>
<tr>
<td>MNSFβ</td>
<td>(Monoclonal nonspecific suppressor factor β)</td>
</tr>
<tr>
<td>URM-1</td>
<td>(Ubiquitin-related modifier-1)</td>
</tr>
<tr>
<td>MUB</td>
<td>(Membrane anchored UBL-fold)</td>
</tr>
</tbody>
</table>

In contrast to other ULMs, URM1, Atg12 and FAT10 are translated with a preserved, free C-terminal glycine and have no requirement for processing. Further exceptions are Atg8 and MUB concerning the substrate molecule of conjugation, which is in their case a lipid (Downes et al., 2006; Geng and Klionsky, 2008). Whether HUB-1, which completely lacks a C-terminal glycine, is at all covalently conjugated is still a matter of debate (van der Veen and Ploegh, 2012). Many of the enzymes involved in conjugation of ULMs have been discovered and the enzymatic cascades resemble ubiquitination, although generally limited numbers of
E2 conjugating enzymes and E3 ligases are required. Notably, most ULMs preserve their own E1 enzyme, again with several exceptions. As mentioned above, UBA6 serves as E1 for ubiquitin and FAT10 simultaneously (Chiu et al., 2007). Similarly, Atg8 and Atg12 share the E1 enzyme Atg7 (Geng and Klionsky, 2008) and the heterodimer of the SUMO-activating enzyme subunits 1 and 2 (SAE1/2) activate the SUMO1 and SUMO2/3 paralogs, despite severe sequence differences between the three ULMs (Tatham et al., 2001). Moreover, the in vitro study by Chiu et al. revealed a weak activation of ISG15 by the ubiquitin activating enzyme UBA1 (Chiu et al., 2007).

Functional consequences of ULM modifications are diverse. Besides ubiquitin itself, proteasomal degradation of conjugates has also been reported to be mediated via FAT10 (Hipp and Kalveram, 2005). Similarly, targeting of NEDD8 conjugated substrates for proteasomal degradation via NEDD8 ultimate buster 1 (NUB1) has been described (Kamitani et al., 2001; Kito et al., 2001). However, whether this pathway is independent of ubiquitin was not shown. Additionally, SUMOylation and NEDDylation can alter the ubiquitination status of substrates or E3 ligase activities respectively, thereby rendering the proteasome dependent half-life of certain proteins indirectly (Tanaka et al., 2012; Uzunova et al., 2007). Also mixed chains of ubiquitin with NEDD8 and SUMO have been described (Grabbe and Dikic, 2009) (Figure 1). It was additionally suggested that SUMO chains can target substrates for proteasomal degradation, though in a ubiquitin dependent manner, since proteasomal inhibition led to the accumulation of SUMO containing chains. Some ubiquitin E3 ligases were shown to be dependent on preceding SUMOylation of the substrate. Other E3s can mediate the modification of a substrate with either SUMO or ubiquitin or both ULMs that compete for targeting sites (Matafora et al., 2009; Schimmel et al., 2008). Furthermore, ULM modifications can result in changes of cellular localization as well as substrate enzymatic turnover, signaling translation or transcription activity. Taken together, a high level of cooperation and reciprocal regulation of the different ULM pathways can be observed. This increasing complexity demands further investigation.

1.1.1.3 FAT10

HLA-F adjacent transcript 10 (FAT10) represents the youngest member within the family of ULMs and was initially termed di-ubiquitin or ubiquitinD (Fan et al., 1996). Although not crystallized yet, the protein is assumed to comprise two ubiquitin-like domains in a head to tail formation connected with a short linker built of five amino acids (Figure 2). Both ubiquitin like domains resemble the characteristic ubiquitin fold, with 29% and 36% sequence identity.
The identity between the two FAT10 domains remains at a lower level (Bates et al., 1997). This led to the suggestion by Bates and colleagues that the evolution of FAT10 aims towards domains with separate functions. The lysines corresponding to K27, K33, K48 and K63 within the ubiquitin protein sequence are conserved in both human FAT10 UBL-domains as well as the C-terminal glycine carboxylate as a site of substrate conjugation (Bates et al., 1997). In mice, however, only the lysines corresponding to K48 are conserved in both FAT10 domains (Raasi et al., 1999). Therefore Raasi et al. suggested that K27, K33 and K63 might be functionally irrelevant. Furthermore, both FAT10 domains show similar sequence identity between human and mouse, indicating that both domains are essential for the exertion of the protein function. Several groups have reported that FAT10 is targeted by post translational modifications (PTM). First of all, it was shown that conserved lysines in FAT10 are ubiquitinated, and thereby might serve as targeting sites for chain formation (Buchsbaum et al., 2012a; Hipp and Kalveram, 2005). Furthermore, Kalveram et al. found FAT10 to be acetylated at lysines (Kalveram et al., 2008), a modification that was suggested earlier by Raasi et al. due to the observation of differently charged FAT10 specific spots in 2D gel electrophoresis (Raasi et al., 2001).

Figure 2: Ribbon diagram of the resolved ubiquitin and predicted FAT10 structure. The model structure of FAT10 shows two domains of which each resembles the typical β-grasp fold of ubiquitin. This structure typically consists of a α-helix (turquoise) surrounded by β-sheets (purple). Both FAT10 domains are connected by a linker in a head to tail orientation. The image is adapted from (Groettrup et al., 2008)

1.1.1.3.1 FAT10 expression

In mice and human constitutive FAT10 expression is mainly restricted to primary and secondary lymphatic organs. Relevant amounts of FAT10 expression were reported for
thymus, spleen, lymph nodes, fetal liver and the gastrointestinal tract (Canaan et al., 2006; Lee et al., 2003; Liu et al., 1999; Lukasiak et al., 2008). Additionally, FAT10 transcript is induced during activation of monocyte derived DCs and constitutively expressed to a lower extend also in primary human B-cells (Bates et al., 1997; Lukasiak et al., 2008). Uregulation of FAT10 was furthermore observed in HIV infected renal tubular epithelial cells (RTECs) (Ross et al., 2006). Similarly Epstein-Barr virus (EBV) infection has been associated with FAT10 expression in B-cells (Bates et al., 1997; Fan et al., 1996). In most other tissues FAT10 is not expressed unless pro-inflammatory cytokines like IFN-γ and TNF-α are present to act in a synergistic manner (Liu et al., 1999; Raasi et al., 1999). The presence or prediction of binding sites for multiple transcription factors, such as STAT1/3, NF-κB, p53, IRF, AP-1 and MZF-1 on the promoter region and the 5`untranslated region of the FAT10 gene implicates that additional mechanisms of regulation are possible (Canaan et al., 2006; Choi et al., 2014; Zhang et al., 2006). Indeed, FAT10 induction was observed in response to other diverse stimuli like CD40L, LPS and polyI:C during DC activation (Bates et al., 1997; Lukasiak et al., 2008), IL-6 and TNF-α in a synergistic manner (Choi et al., 2014), RANKL in follicle associated epithelial cells (Kobayashi et al., 2012) and retinoids in MCF-7 breast carcinoma cells (Dokmanovic et al., 2002). For p53 and FAT10 a covalent conjugate was described as well as contrary models of negative and positive reciprocal regulation (Choi et al., 2014; Li et al., 2011; Peng et al., 2013; Zhang et al., 2006). A negative transcriptional regulation of FAT10 by p53, as seen by Choi et al., confirms the earlier observed correlation between FAT10 and mutant p53 expression in gastric cancer biopsies (Ji et al., 2009).

The classical characterization that defines cellular localization of a certain protein is not satisfactorily resolved in the case of FAT10. Several studies have performed microscopic analysis of various FAT10 constructs and endogenous FAT10 expressed in different cell lines with divergent outcomes. Tagged and untagged FAT10 was either observed primarily in the nucleus (Lee et al., 2003; Ren et al., 2006) or in the cytosol (Hipp et al., 2004; Raasi et al., 1999) or with localization to both compartments (Kalveram et al., 2008). In the latter study, Kalveram et al. additionally have shown that FAT10 localizes to aggresomes under conditions of proteasomal inhibition. Similarly, endogenous FAT10 was reported to localize to cytosolic protein aggregates (Aichem et al., 2012; Buchsbaum et al., 2012b). In one study, FAT10 was detected in the nucleus in HeLa S3 cells, surprisingly, however, without cytokine induction or overexpression (Merbl et al., 2013).
1.1.1.3.2 FAT10 conjugate formation

FAT10, as a member of the ULM family, is conjugated to substrates in a ubiquitin like manner. As mentioned above, ubiquitin and FAT10 share the activating enzyme UBA6 (Chiu et al., 2007). In in vitro conjugation assays, UBA6 preferentially formed a thioester with ubiquitin, although in binding affinity measurements a higher affinity of UBA6 for FAT10 over ubiquitin was measured (Chiu et al., 2007; Gavin et al., 2012). Still, both authors suggest that the regulation of the two pathways is probably controlled by the massive induction of FAT10 through pro-inflammatory cytokines, changing the ratio of ubiquitin and FAT10 severely. Some known corresponding E2 enzymes specific for UBA6, namely ubiquitin-conjugating enzyme E2 5 and E2 13 (UBC5 and UBC13), can be charged with activated ubiquitin, but not with FAT10 in vitro (Chiu et al., 2007). Activated FAT10 is exclusively transthiolation to the E2 enzyme Uba6-Specific E2 conjugating Enzyme 1 (USE1). This E2 enzyme is, similarly to UBA6, bi-specific for ubiquitin and FAT10. In Addition, USE1 accepts both modifiers only from UBA6. The pathway specificity therefore relies on the recognition of the ULM and the respective E2 by the E1. Therefore no discrimination between the ULMs occurs during transthiolation or any subsequent step (Gavin et al., 2012). This E1 specificity assures that FAT10 is directed to the correct conjugation and ligation cascade.

USE1 has been revealed as one of the first FAT10 substrates, as it FAT10ylates itself in cis (Aichem et al., 2010). This conjugation does not change the capability of USE1 to form a reducible “on-top” thioester with activated ubiquitin or FAT10, but rather targets USE1 for proteasomal degradation (Aichem et al., 2014). Another enzyme of the ubiquitin activation cascade is targeted by FAT10ylation, UBA1 (Rani et al., 2012). Also this enzyme has been shown to be targeted for proteasomal degradation via FAT10 conjugation (Rani et al., 2012) (Johanna Bialas, Biotechnology Institute Thurgau, Switzerland, submitted manuscript). Both enzymes represent attractive targets for a potential regulation of the engaged ubiquitin and FAT10 conjugation pathways by FAT10 itself. The necessity of substrate recognition seems to be negligible in the case of FAT10 conjugation, since in vitro FAT10ylation is successful in the presence of UBA6 and USE1 only (Aichem et al., 2010) (Johanna Bialas, Biotechnology Institute Thurgau, Switzerland, submitted manuscript). Nevertheless, the identification of potential E3 ligases and also deconjugating enzymes is of certain interest and under current investigation.

Proteomic analysis of FAT10 conjugation revealed hundreds of putative substrates as well as non-covalent interaction partners (Aichem et al., 2012; Leng et al., 2014). The functional distribution of these hits gave no rise to which cellular pathway FAT10 might be associated.
In the study of Aichem et al., the most eminent group of FAT10 interacting proteins represented DNA and RNA binding proteins followed by cancer related proteins and E3 ligases. Protein array data from Merbl et al., however, elucidated a functional classification of FAT10 interaction partners within the pathways of cytokinesis, lipid metabolic process and antigen presentation for MHC class II (Merbl et al., 2013). By means of FAT10 conjugates, no general conserved FAT10 consensus sequence could be revealed. So far, only rudimentary information exists showing that the modified sites were enriched with hydrophilic amino acids (Leng et al., 2014). Furthermore, the function of FAT10 conjugation seems not necessarily dependent on the lysine position within the substrate sequence since mutation of the FAT10ylated lysine does not completely abrogate FAT10 conjugation (Aichem et al., 2014; Buchsbaum et al., 2012b).

1.1.1.3.3 FAT10 function

One of the first functional observations regarding FAT10 and its conjugates was the stabilization during proteasomal inhibition (Liu et al., 1999; Raasi et al., 1999). Within the ULM family, FAT10 has been shown to be the only one capable of directly targeting substrates for proteasomal degradation in a ubiquitin independent manner (Hipp and Kalveram, 2005; Schmidtke et al., 2009). Although ubiquitination of FAT10-fusion proteins has been observed in cells, no high molecular weight conjugates were detectable. Furthermore, the FAT10 mutant lacking all lysines still showed the same degradation capability, excluding the necessity of ubiquitin for proteasomal degradation (Hipp and Kalveram, 2005). Another hint provides the study by Schmidtke et al. that shows ubiquitin independent degradation of FAT10 fusion proteins in an in vitro degradation assay (Schmidtke et al., 2009). However, the model of ubiquitin independent proteasomal degradation of FAT10 conjugates was challenged by a study from Buchsbaum et al.. They observed efficient FAT10 degradation only with the participation of ubiquitin, although they didn’t entirely exclude a ubiquitin independent mechanism (Buchsbaum et al., 2012a). The identification of the precise mechanism, how FAT10 conjugates are targeted for proteasomal degradation, was initiated by the observation that NEDD8 ultimate buster 1-long (NUB1L) accelerates the degradation rate of FAT10 and its conjugates (Hipp et al., 2004). FAT10 is thereby able to bind to a subunit of the proteasome regulatory particle (RP) by its own. However, an accelerated degradation can be observed when NUB1L simultaneously binds to another subunit of the RP. As a trimeric complex between NUB1L, FAT10, and the RP exists, two models are suggested: either NUB1L acts like a soluble FAT10 receptor to transfer FAT10ylated substrates to the proteasome or NUB1L accelerates the degradation by
conformational changes within the RP (Rani et al., 2012). In addition, no deconjugating enzymes are known for FAT10. Together with the observation that FAT10 and its conjugates have a relatively short half-life of only about two hours it is assumed, that FAT10 is not recycled but degraded along with its substrates by the proteasome (Schmidtke et al., 2014).

Besides the observation that FAT10 is involved in proteasomal degradation of various substrates other, partially divergent, functional implications of FAT10 were reported. Initially, FAT10 expression was supposed to have pro-apoptotic functions (Li et al., 2011; Liu et al., 1999; Raasi et al., 1999; Ross et al., 2006; Snyder et al., 2009) but at the same time it was known that FAT10 is expressed in various carcinoma, including hepatocellular, gastrointestinal, gynecological cancers (Ji et al., 2009; Lee et al., 2003; Liu et al., 2014; Lukasiak et al., 2008; Qing et al., 2011; Yan et al., 2010; Zhang et al., 2006), glioma (Yuan et al., 2012) and lymphoma (Hartmann et al., 2013). Carcinoma cells have been shown to co-express other pro-inflammatory cytokine inducible proteins like the proteasomal subunit low molecular mass protein 2 (LMP2) and therefore still respond to cytokine stimulation (Lukasiak et al., 2008). However, Lee et al. found cytokine inducible genes not induced in FAT10 overexpressing human colon carcinoma samples (Lee et al., 2003). Therefore, the question, whether FAT10 is induced in these tissues either due to the pro-inflammatory environment of tumors, or due to the lack of p53 negative regulation, or because it has indeed oncogenic properties during chronic inflammation is highly debated. Recently, however, direct action of FAT10 in the process of carcinogenesis was reported (Chen et al., 2014; Gao et al., 2014; Liu et al., 2014) and FAT10 has been proposed as an independent prognostic factor for tumor progression in hepatocellular carcinoma and pancreatic ductal adenocarcinoma patients (Liu et al., 2014; Sun et al., 2014). Moreover, in aging colonies of FAT10 wild type and knockout mice, Canaan et al. found beneficial global effects of FAT10 abrogation on tumorogenesis (Canaan et al., 2014). The carcinogenic function of FAT10 was suggested to be a consequence of increased chromosomal instability mediated by the non-covalent mitotic arrest deficient 2-like protein 1 (MAD2)-FAT10 interaction (Ren et al., 2011). In this study, FAT10 was suggested to protect cells from TNF-α induced cell death. Similarly, knocking down FAT10 in carcinoma cells reduced cell cycle progression through inhibiting cell cycle S-phase entry and induced apoptosis (Chen et al., 2014; Liu et al., 2014). Additionally, FAT10 expression and conjugation was reported to be regulated in a cell cycle dependent manner and thereby FAT10ylation might play a role in mitotic regulation (Lim et al., 2006; Merbl et al., 2013). Merbl et al., however, point out that growing evidence for a key role of FAT10 in mitosis nevertheless seems to be surprisingly. They argue that first of all
FAT10 knockout mice are viable and secondly FAT10 appears only late during evolution. But they also mention that compensation in mitosis is common, and this might also be the case for FAT10 and ubiquitin.

FAT10 was also shown to interact non-covalently with histone deacetylase 6 (HDAC6) (Kalveram et al., 2008), whose function is essential for formation and autophagosomal elimination of cytosolic protein inclusions. Excess protein aggregates are trafficked and disposed via HDAC6 to the aggresome, a microtubule organizing center (MTOC) localized inclusion body (Iwata et al., 2005; Lee et al., 2010). In accordance with this, FAT10 localized to aggresomes under proteasome inhibitory conditions. This observation was dependent on HDAC6 expression as well as on a functional tubulin network (Kalveram et al., 2008).

Several in vitro studies revealed a positive feedback mechanism for FAT10 in the NF-κB pathway (Gao et al., 2014; Gong et al., 2010). This is in line, for example, with the observed elevated IL-10 production in skeletal muscle in aged FAT10 knockout mice, since IL-10 is the major immune suppressive mediator that down regulates the expression of pro-inflammatory cytokines and antagonizes NF-κB signaling (Canaan et al., 2014). In contrast, however, Canaan et al. observed previously a hypersensitivity phenotype of FAT10 knockout mice against LPS, which leads to sepsis and death. This fact rather implicates a negative feedback mechanism of FAT10 within the NF-κB pathway in response to Toll-like receptor 4 (TLR4) (Canaan et al., 2006). Such a negative regulation of NF-κB signaling by FAT10 was indeed observed by Buchsbaum et al. They found FAT10 to be covalently conjugated to the endoplasmic reticulum (ER) membrane protein lumenal domain-like LAP1 (ULL1) and the inflammatory mediator leucine-rich repeat fli-I-interacting protein 2 (LRRFIP2) (Buchsbaum et al., 2012b). The latter one is implicated in NF-κB activation following stimulation of TLR4. FAT10 modification led to the retention of LRRFIP2 to cellular aggregates, which resulted in an inhibitory effect of FAT10 on NF-κB activation (Buchsbaum et al., 2012b). These divergent observations implicate a highly complex and apparently tissue and stimuli specific FAT10 function.

Recently, FAT10 and its conjugates were also found to interact with aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1) (Bett et al., 2012). This protein, which is involved in inherited Leber’s congenital amaurosis (LCA) blindness, has previously been shown to interact with NUB1 (Akey et al., 2002). The three proteins together can form a ternary complex whereby AIPL1 inhibits the acceleration of FAT10 degradation by NUB1. Interestingly, AIPL1 also co-immunoprecipitated together with UBA6. Therefore, Bett et al.
suggested that AIPL1 may also play a role in regulating the FAT10 conjugation machinery in addition to inhibit its degradation pathway.

Interestingly, several studies analyzing single nucleotide polymorphisms (SNPs) within the FAT10 locus found associations with human diseases. One genome-wide association study (GWAS) identified genes, that influence natural fertility in human. Among the nine associated loci, candidate genes included FAT10 and ubiquitin-specific-processing protease 8 (USP8), a DUB (Kosova et al., 2012). The authors suggest a relationship between immune regulation and reproductive function in the testicular tissue. Additionally, a regulatory polymorphism, located downstream of the FAT10 gene and resulting in upregulation of FAT10 in the intestinal mucosa, was associated with celiac disease, an immune-mediated disorder of the small intestine (Castellanos-Rubio et al., 2010). In a follow-up study Frank et al. tested the hypothesis whether polymorphisms within the FAT10 gene might affect colorectal cancer (CRC) risk. Therefore they analyzed FAT10 I68T (rs2076485) and FAT10 S160C (rs8337) in a population-based case-control study and found the minor allele of FAT10 I68T to be significantly associated with advanced stages of CRC and with CRC below 65 years of age (Frank et al., 2010). Furthermore, regulatory polymorphisms in the region of the FAT10 gene were found to be associated with type 1A diabetes (Aly et al., 2008). Forkhead box protein P3 (FOXP3) mutations similarly lead to the development of diabetes, most likely due to the lack of regulatory T-cells (Wildin et al., 2002). Since FAT10 was additionally reported to act downstream of FOXP3 (Ocklenburg et al., 2006), it might potentially be involved in mutant FOXP3 associated diabetes as a downstream effector molecule.
1.1.2 The proteasome

Regulatory, short lived or damaged, soluble proteins with the recognition site of at least four K48 linked ubiquitin moieties represent the typical proteasomal substrate. They are degraded by the proteasome, a multicatalytic, ATP- and ubiquitin-dependent, proteolytic complex located in the cytoplasm and nucleus. The eukaryotic 26S proteasomal holoenzyme consists of a proteolytic 20S core particle (CP) and a 19S regulatory particle (RP, PA700), that can associate to both sides of the CP (Peters et al., 1993) (Figure 3). Comparable complexes in prokaryotes are known, though multimeric, ATP-dependent proteases are less complex in bacteria and archaea (Baker and Sauer, 2006).

Figure 3: The eukaryotic 26S proteasome holoenzyme. The cryoelectron microscopy density of the 26S proteasome from yeast depicts the 19S regulatory particle (RP) lid subcomplex (yellow), the RP base subcomplex (blue) and the 20S core particle (CP, gray). The image is adapted from (Lander et al., 2012).

1.1.2.1 The constitutive Proteasome

The CP is a highly conserved, stacked structure that contains 28 subunits arranged in four heptameric rings. Each outer ring contains the α-subunits 1-7. Similarly, each of the two inner rings is built of seven different β-subunits. Three of these β-subunits (β1, β2, β5) harbor the actual catalytic activity, which is constrained within the “barrel” shaped cylinder (Groll et al., 1997). The proteolytic specificity between the three subunits differs. The β1 subunit preferentially cleaves peptide bonds C-terminally after acidic amino acids, β2 after basic
residues, and β5 after hydrophobic residues. The cleavage specificities are also referred to as caspase-like (β1), trypsin-like (β2) and chymotrypsin-like (β5) activity (Coux et al., 1996). As the catalytic sites face the interior of the CP, substrates designated for proteasomal degradation have to get access to the inner chamber. On the other hand, uncontrolled entry and subsequent random proteolysis has to be prevented. Therefore the outer α-rings serve as a barrier. They form a closed gate on both sides by N-terminally protrusions of each of the seven subunits that direct into the lumen of the cylinder (Groll and Huber, 2003). For this reason, the 20S CP on its own shows limited proteolytic activity, though exceptions are described (Baugh et al., 2009; Shringarpure et al., 2001).

To activate the CP, the proteasomal activators (PA) and the RP bind to the outer α-rings. Besides the opening of the CP gate, the 19S RP additionally regulates recruitment, deubiquitination and unfolding of substrates. Different PAs, besides the 19S RP, can associate with the proteasome and will be introduced in more detail in the next paragraph. Although they all share the ability to induce a conformational change within the CP, different mechanisms have evolved to open the gate. The binding site at the CP is formed by the α-ring subunits, which form binding pockets between the individual subunits. Protrusions of different shape, as part of the PA or RP binding site, insert into these inter-α-α-binding pockets and mediate thereby a most effective asymmetric, structural change in the N-termini of all α-subunits that leads to the opening of the gate. As soon as the PA or RP binds to the CP, this opened conformation is induced and the regulation of substrate entry or exit is then covered by the activator itself (Schmidt and Finley, 2014). Within the CP cylinder translocation and subsequent proteolysis takes place in a processive manner. Combined with the different cleavage specificities of β1, β2 and β5, each substrate is entirely degraded into short peptides. The processive character of the proteolysis guarantees that proteins are not degraded partially. Otherwise, this could result in altered protein function and be potentially harmful to the cell. However, in some cases, partial degradation of proteins, known as endoproteolysis, is permitted and even necessary to regulate the activity of certain transcription factors (Hoppe et al., 2000; Hoppe et al., 2001; Tian et al., 2005).

1.1.2.1.1 The 19S regulatory particle

The 19S RP, which exceeds the CP (Figure 3, grey) in size, consists of a proximal base (Figure 3, blue) and a distal lid (Figure 3, yellow). The base directly interacts with the outer α-ring of the CP. Similarly, also this complex has the shape of a ring, formed by six regulatory particle tripleA-ATPases (Rpt1-6) as well as the regulatory particle non-ATPase 1
(Rpn1) and Rpn2, serving as scaffolding proteins, and the substrate receptor Rpn13 (Förster et al., 2013). The actual arrangement of the ATPase ring reveals a narrow translocation channel to unfold substrate proteins (Lander et al., 2012; Lasker et al., 2012). This translocation of the unfolded peptide chain into the CP is dependent on ATP-hydrolysis. The lid of the 19S RP is formed by nine subunits, and recently Lander et al. suggested that the substrate receptor Rpn10 also belongs to this complex. Initially it was thought to be part of the base. The best characterized and most important function of the lid is performed by the subunit Rpn11, a DUB. As soon as the substrate is committed for ATP-dependent unfolding and translocation to the base, Rpt11 deconjugates the whole polyubiquitin chain en bloc (Förster et al., 2013).

1.1.2.1.2 Proteasomal activators

Besides the 19S RP, two PAs have been described to modify proteasomal activity, but without ATPase activity. These are PA200/bleomycin resistance protein (BLM10) and PA28/REG. Similarly to the RP, they also bind to the outer α-ring of the CP with the same outcome of an opened CP gate. One PA can bind to the CP opposite of the RP, thereby forming a hybrid proteasome (Cascio et al., 2002; Schmidt et al., 2005; Shibatani et al., 2006). Yeast BLM10 (former BLM3) and its homolog PA200 in mammalia is the most conserved PA. In contrast to the multimeric complexes PA28/REG and RP, this activator binds to the CP as a ~250 kDa monomer. It is functionally necessary for efficient proteasome activity during DNA or oxidative damage repair and spermatogenesis (Schmidt and Finley, 2014) as well as for the maturation of the CP (Fehlker et al., 2003; Marques et al., 2007). The second and best characterized PA is PA28/REG. It adopts the CP structure of a heptameric ring composed of α and β subunits. The closely related PA REGγ, from which PA28 has probably evolved, similarly consists of seven γ subunits forming a heptameric ring. PA28αβ differs from REGγ as it is restricted to vertebrates only and its basal expression is inducible by IFN-γ. Furthermore, both PAs are restricted to different subcellular compartments since REGγ localizes to the nucleus and PA28αβ is retained within the cytosol. A role of PA28αβ in MHC class I restricted antigen presentation was supposed and has been confirmed for pathogenic and self-antigens (Groettrup et al., 1996b; Sun et al., 2002; Textoris-Taube et al., 2007; van Hall T et al., 2000). The dependence of certain antigens on PA28/CP/RP hybrid proteasomes is probably mediated by an altered retention time of peptides within the CP due to the gate opening function of PA28. Thereby, MHC-class I peptides are secured from further processing by the proteasome and are released with the appropriate size of 8-10 amino acids (Förster et al., 2005; Whitby et al., 2000). Furthermore, REGγ has been described to mediate ubiquitin independent proteasomal degradation of several substrates, which contain loosely
folded parts within their protein sequence (Chen et al., 2007; Li et al., 2006).

Proteasomal degradation is a constant process within the cytosol and also this essential task is likewise adjusted to changing situations by transcriptional regulation. When proteotoxic stress increases, the transcription factors nuclear factor erythroid 2-related factor 1 and 2 (NRF1 and NRF2) are essential to induce the transcription of proteasome subunits (Sykiotis and Bohmann, 2010).

1.1.3 The immunoproteasome

Two decades after the discovery of the multicatalytic proteasome complex, several groups reported about the existence of IFN-γ inducible catalytic subunits encoded in the MHC class II region, that can substitute two of the catalytic subunits, β1 and β5, within the CP (Brown et al., 1991; Glynne et al., 1991; Kelly et al., 1991; Ortiz-Navarrete et al., 1991). Several years later, a third inducible subunit was discovered and described as a β2 replacement (Groettrup et al., 1996a; Nandi et al., 1996) encoded outside the MHC class II region (Cruz et al., 1997). The latest member of these “non-canonical” proteasome catalytic subunits was identified by Murata et al. in 2007 and termed β5t, due to the fact that it is expressed exclusively in thymus epithelial cells (Murata et al., 2007). Proteasomes that contain the inducible subunits β1i (LMP2), β2i (MECL-1) and β5i (LMP7) were termed immunoproteasomes (IP) in contrast to standard proteasomes (SP).

The induction of the IP is predominantly mediated by the major transcription factors involved in IFN-γ signal transduction. Other stress but non-cytokine related inducers are also described, which is in line with multiple transcription factor binding sites identified within the promoter regions of the three inducible subunits (Ferrington and Gregerson, 2012). Considering conservative substitutions, the similarity between amino acid sequences of IP and SP catalytic subunits shows high correlation with about 76% to 83%. Together with high sequence similarities between different species, a strong evolutionary pressure to preserve the structure of the catalytic subunits is assumed (Ferrington and Gregerson, 2012). The structure of the murine IP 20S complex has been resolved recently and gives insight into properties of the binding pockets that might help to explain differences between IP and SP cleavage specificity (Huber et al., 2012). The β1i subunit shows chymotrypsin-like activity in contrast to caspase-like activity of constitutive β1, a functional variation that can be explained by substitution of Thr and Arg by Val and Leu that changes the overall charge from positive to neutral along the binding channel of β1i. This structural difference could enhance the production of peptides with a small, hydrophobic C-terminal residue serving as an anchor. For
β5i an enhanced kinetic of peptide bond cleavage is suggested due to changes within the active site. However, the resolved structure of the IP reveals no explanation for the catalytic differences between β2 and β2i, thereby leaving the observed phenotype of β2i deficient mice without clarification (Basler et al., 2013).

The assembly of proteasomes follows a rigid organization, accompanied and regulated by several assembly factors. The incorporation of inducible subunits occurs only during de novo proteasomal complex formation in a cooperative manner. Thereby the formation of homogeneous IP particles containing all three inducible subunit is favored as well as the formation of IP over SP (De et al., 2003; Griffin et al., 1998; Groettrup et al., 1996a). Still in accordance with these assembly rules, the existence of CPs containing a mixture of standard- and immunoproteasome subunits has been described (Dahlmann et al., 2000; Drews et al., 2007; Klare et al., 2007). These so called intermediate proteasomes were further characterized as symmetrical β1-β2-β5i and β1i-β2-β5i intermediate proteasomes, although asymmetric intermediate proteasomes could not be excluded (Guillaume et al., 2010). This finding further complicates the picture of existing proteasome subtypes, their different properties and specialized functions. Importantly, the turnover of IP and SP also differs. Additionally to the preferred and therefore faster assembly of IP, this complex shows also a 4-5 times reduced half-life, emphasizing the transient and flexible exertion of IP complexes (Heink et al., 2005).

1.1.3.1 The immunoproteasome function

Researchers immediately suggested a role for the inducible IP complex in antigen processing. Indeed, the best described function of IPs is to generate peptides with a hydrophobic C-terminus that can be processed to fit in the groove of MHC class I molecules. In recent years, however, knowledge of other functions has emerged, mainly based on studies of IP-deficient mice and the characterization of polymorphisms within IP subunits. MHC class I peptide generation by the proteasome serves each individual cell to present intracellular antigens to CD8+ cytotoxic T-cells and secondly determines the specificity of the CD8+ T-cell repertoire in the first place. This fundamental concept of the immune system was shown to have substantial overlap, but also significant differences based on the IP or SP proteolytic action. This was shown by use of IP subunit knockout mice by several groups (Basler et al., 2013; Ferrington and Gregerson, 2012). Interestingly, findings achieved by the usage of specific IP subunit inhibitors led to the suggestion that rather the subunit structure than the actual proteolytic activity is responsible for differences in peptide generation. Thereby the IP subunits might rescue peptides from cleavage by SP subunits (Basler et al., 2012; Sijts et al.,
Other functions for the IP have been described. Although still controversial, a role of β1i in inhibitor of kappaB α (IκBα) degradation and also in signaling events upstream of NF-κB activation was proposed by several groups (Ferrington and Gregerson, 2012). Also the Akt signaling inhibitor phosphatase and tensin homolog (PTEN) was reported to be regulated by IP proteolytic activity (Cai et al., 2008; Zu et al., 2010). Furthermore, in two studies the inability of IP subunit deficient T-cells to expand after transfer into virus infected wild type mice was observed (Chen et al., 2001; Moebius et al., 2010). If a graft rejection by the wild type mice could be generally excluded, this would give rise to a new function of IP in T-cell proliferation and would therefore be an interesting target to counteract excessive T-cell responses like autoimmune reactions. Indeed, in several models for autoimmune diseases like experimental colitis, experimental arthritis, murine lupus like disease and Hashimoto’s thyroiditis, a positive influence of the β5i specific inhibitor could be observed (Basler et al., 2010; Ichikawa et al., 2012; Muchamuel et al., 2009; Nagayama et al., 2012). Also for IP subunit deficiency in mice a beneficial effect on disease progression could be observed in experimental inflammatory bowel disease (Basler et al., 2010; Fitzpatrick et al., 2006; Schmidt et al., 2010). In contrast, evidence for an IP protecting function was elucidated with respect to other autoimmune diseases. For example diabetes mellitus and Nakajo-Nishimura syndrome as well as lipodystrophy and CANDLE syndrome in human were shown to be associated with polymorphisms in the β5i gene (Agarwal et al., 2010; Arima et al., 2011; Liu et al., 2012; Zaiss et al., 2011). Additionally, controversial data exists concerning the role of IP in establishment and progression of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis (EAE). Seifert et al. suggested a function of IP subunits in EAE by increased proteasomal activity and maintaining of protein homeostasis during inflammation and increased oxidative stress (Seifert et al., 2010). Similar observations have been made by Opitz et al. during acute enterovirus myocarditis (Opitz et al., 2011). However, no differences were observable with the same mouse model performed with LMP2, LMP7 or MECL-1 deficient mice (Basler et al., 2014; Frausto et al., 2007; Nathan et al., 2013b). Additionally, the crystal structure of murine IP reveals no mechanistic evidence that would implicate a role of IP in the rate limiting step of proteolysis, which is the entry of substrate into the CP regulated by PAs (Basler et al., 2013; Huber et al., 2012).

1.2 Protein degradation by macroautophagy

As already mentioned, protein degradation can occur via the proteasome or the lysosome. In
contrast to cytosolic proteasomal degradation, lysosomal degradation of endocytosed material occurs within isolated, acidified compartments. However, the delivery of cytosolic components for lysosomal degradation is likewise possible, described as a process called autophagy. Different autophagy pathways are known today, including chaperone-mediated autophagy (CMA), microautophagy, macroautophagy (here referred to as autophagy) and the yeast cytoplasm-to-vacuole targeting pathway (Cvt). More than thirty genes have been identified as essential for Cvt, which are called autophagy-related genes (Atg) (Klionsky et al., 2011). Autophagy, in contrast to CMA and Cvt has originally been considered to degrade bulk cytosolic material in a non-specific manner, especially to restore energy homeostasis and amino acid levels during nutritional deprivation. However, increasing evidence exists that autophagy represents a highly selective degradation process (Johansen and Lamark, 2011). Autophagy is unrestricted by its size or complexity, since it regulates for example the elimination of depolarized mitochondria (mitophagy) (Elmore et al., 2001), peroxisomes (pexophagy) (Dunn et al., 2005), ribosomes (ribophagy) (Kraft et al., 2008), aggregated proteins (aggrephagy) (Øverbye et al., 2007) or cytosol colonizing microbes (xenophagy) (Knodler and Celli, 2011). In principle, three stages are involved: (i) initiation (a crescent membrane called phagophore is formed), (ii) elongation and closure (a double membrane autophagosome encloses sequestered cargo) and (iii) maturation (fusion with late endosomal and lysosomal organelles to form a degradative autolysosome) (Figure 4).
1.2.1 Initiation

Autophagosomal delivery of cytosolic material to lysosomes occurs at a low, but constant level in most cells, thereby fulfilling the important “housekeeping” function of eliminating superfluous or damaged cytosolic components and organelles. Upon divergent stimuli autophagy can additionally be induced to meet special requirements counteracting extra- and intracellular stress situations. These include for example starvation, hypoxia, absence of growth factors, increase in cytosolic Ca$^{2+}$ concentrations and unfolded protein response...
Introduction

(UPR) resulting from ER stress. In most situations autophagy fulfills a pro-survival function, but it was also reported that ER stressors may cause autophagic cell death. Despite these autophagy inducing stimuli, the innate immune system has evolved a cell autonomous strategy to combine pathogen detection with autophagy inducing signaling to combat pathogenic cytosol colonization (reviewed in He and Klionsky, 2009).

Besides other functions, mammalian target of rapamycin complex 1 (mTORC1) has been well-characterized as a regulator of autophagy, with mTOR as the central molecule linking nutrient sensing and basic cellular metabolic functions (Figure 5). Autophagy is induced by the initial signaling complex containing uncoordinated-1-like kinase 1 (ULK1) or ULK2 amongst others and forms at the phagophore assembly site (PAS). In the presence of nutrients, mTORC1 phosphorylates ULKs, thus inhibiting the recruitment of the ULK complex to the PAS. When mTORC1 is inactivated, the ULK complex can assemble and recruit other Atg proteins to the PAS. The function of mTORC1 is known to be inhibited by the immunosuppressant rapamycin, and rapamycin is therefore generally used to activate autophagy. The subsequent nucleation step of autophagosome formation in either yeast or mammals requires the modification of the membrane with phosphatidylinositol-3-phosphate (PtdIns(3)P; PI3P) by a complex consisting of the type III PI3 kinase PIK3C3/VPS34, PIK3R4/p150 (Vps15 in yeast), Atg14L and Beclin 1 (Vps30/Atg6 in yeast) (Figure 5). This complex is recruited to the PAS by the ULK complex (reviewed in Huang and Brumell, 2014). These PI3P enriched membranes at the PAS can form as subdomains at the ER which are called omegasomes (Hayashi-Nishino et al., 2009; Ylä-Anttila et al., 2009). Regulation of the PI3 kinase complex is mediated by binding partners of the Beclin 1 subunit, the master regulator of autophagy initiation. Negative regulation is mediated by run domain Beclin 1 interacting and cysteine-rich containing protein (RUBICON) and B-cell lymphoma 2 (BCL2), since they prevent the association of the PI3 kinase complex (Matsunaga et al., 2009; Pattingre et al., 2005). Binding partners that induce kinase activity of the complex are activating molecule in Beclin 1 regulated autophagy protein 1 (AMBRA1) and Bax-interacting factor 1 (Bif-1) (Fimia et al., 2007; Takahashi et al., 2011).
1.2.2 Elongation

The source of membranes for subsequent elongation is still a matter of debate, but has been described to arise from ER (Hayashi-Nishino et al., 2009), endosomes (Puri et al., 2013), plasma membrane (Ravikumar et al., 2010), Golgi complex (Takahashi et al., 2011) and mitochondria (Hailey et al., 2010). The elongation machinery consists of two ubiquitin-like conjugation systems (reviewed by Ohsumi, 2001). The first conjugation system, Atg7 (E1-like) and Atg10 (E2-like), accomplish the activation and covalent conjugation of the ubiquitin like modifier Atg12 to Atg5. This conjugate is directed to precursor vesicles by Atg16L1. The second conjugation cascade, consisting of Atg7 (E1-like) and Atg3 (E2-like), activates the ubiquitin like modifier Atg8 in yeast. The human genome encodes for six functional Atg8 orthologs and several pseudogenes belonging to three different subfamilies: the light chain 3 (LC3), Golgi-associated ATPase enhancer of 16 kDa (GAT16) and γ-aminobutyrate receptor-associated protein (GABARAP) family (Shpilka et al., 2011). Together, the Atg5-Atg12 Atg16L1 complex mediates in a proposed E3-like manner the target specific conjugation of processed and activated LC3 to phosphatidylethanolamine (PE) within the autophagosomal membrane (Fujita et al., 2008; Hanada et al., 2007). Liberation of the conserved C-terminal glycine of the LC3 and GABARAP family precursors as well as their deconjugation and recycling from the target membrane is mediated by four homologous enzymes, Atg4A-D (Kuang et al., 2012). The Atg4-processed form of LC3 is referred to as LC3-I and the PE-
conjugated form is called LC3-II. The precise function of LC3 conjugation is not well understood, but marking the nascent autophagosomal membrane with PE-LC3 has been shown to be essential for hemifusion of vesicles and might thereby be involved in the expansion of autophagosomal membranes (Nakatogawa et al., 2007). Depending on the cargo, the diameter of autophagosomes usually ranges from 0.4 to 0.9 µm in yeast, and 0.5 to 1.5 µm in mammals (Parzych and Klionsky, 2014). Furthermore, it was suggested that the conjugation of the different LC3 paralog is timely orchestrated during elongation and closure of autophagosomes (Weidberg et al., 2010). Additionally, deconjugation of LC3 from the outer autophagosomal membrane is essential for fusion with late endosomes and lysosomes and has therefore been elucidated as an important maturation step (Nair et al., 2012; Yu et al., 2012).

1.2.2.1 Autophagosomal adapters

Ubiquitin constitutes a universal degradation signal for both major proteolytic systems in the cytosol: the proteasome and autophagy. Typical for selective autophagosomal capture, however, is the usage of adapter proteins. All known autophagy adapters share the LC3 interacting region (LIR) as well as different UBDs (Kirkin et al., 2009c; Pankiv et al., 2007; von Muhlinen et al., 2012; Wild et al., 2011). With this characteristic combination, each adapter is equipped to target nascent autophagosomes to their specific cargo. Today four such classical adapters with similar, but not redundant, functions are characterized: p62 which was formerly known as sequestosome-1 (p62/SQSTM1), nuclear dot protein 52 (NDP52), neighbor of BRCA1 gene 1 protein (NBR1) and Optineurin (OPTN) (Figure 6). Consistent with the described difference in the ubiquitin binding capability of each autophagy adapter is the observation, that after long term inhibition of autophagy all kinds of polyubiquitin chains accumulate in cells (Riley et al., 2010).

Recent evidence, however, reviewed by Rogov and colleagues, challenge the initial, straightforward concept of autophagy adapters as crucial bridging factors between cargo and the nascent phagophore during selective autophagy (Rogov et al., 2014). For example, direct recruitment of the Atg5-Atg12-Atg16 complex to the autophagosomal cargo, namely ubiquitinated cytosolic Salmonella enterica Typhimurium (S. Typhimurium), independent of adapters was reported (Fujita et al., 2013). Other studies have also described variants of the recruitment hierarchy of autophagy components around the cargo, which are incompatible with the original concept (Itakura et al., 2012a; Kageyama et al., 2011). Therefore, Rogov et al. proposed a model, where the nucleation complex is established directly at the ubiquitinated
cargo either via autophagosomal adapters, via the Atg5-Atg12-Atg16 complex or directly via LC3 conjugated phagophores, which are already present on the cargo (Rogov et al., 2014). Nevertheless, autophagosomal adapters are still considered to be important factors for selective autophagy. The two adapter p62 and NDP52 will be introduced in more detail in the next paragraph.

**Figure 6: Domain structure of autophagy receptors.** The characteristic binding sites for cargo signals (red) and Atg8 family members (green) are depicted for each receptor. NDP52 binds Galectin-8 (Gal8) (violet) in addition to the shared ubiquitin binding capability. p62, NBR1 and OPTN bind non-selectively to LC3 and all its GABARAB paralogs (LC3/GBR) via their LC3-interactin regions (LIRs), while NDP52 preferentially interacts with LC3C via a LC3C-specific binding site (CLIR). Abbreviations: CC, coiled-coil; Gal8IR, Galectin-8 interacting region; PB1, Phox and Bem1P; SKICH, skeletal muscle and kidney enriched inositol phosphatase carboxyl homology; UBA, ubiquitin-associated domain; UBAN, ubiquitin binding in ABIN and NEMO domain; ZnF, zink finger domain. Image adapted from (Boyle and Randow, 2013).

### 1.2.2.1.1 p62/SQSTM1

p62 expression seems to be restricted to metazoans and, as a multifunctional adapter protein, it is implicated in diverse cellular pathways (Johansen and Lamark, 2011). The 440 amino acid containing protein bears several characterized protein interaction domains like Phox and Bem1 (PB1), the ZZ type zinc finger domain (ZnF) and a UBA and LIR domain (Figure 6). The UBA domain of full length p62 seems to have highest affinity for K63 linked polyubiquitin chains in cells, although *in vitro* studies with isolated p62-UBA domains only showed a weak, non-selective interaction (Kirkin et al., 2009b; Raasi et al., 2005; Seibenhener et al., 2004; Wooten et al., 2008). Additionally the p62-UBA domain was reported to be phosphorylated (Matsumoto et al., 2011; Pilli et al., 2012). This modification, which was observed during proteasomal inhibition and xenophagy, further increased the
affinity of the p62-UBA domain to K48 and K63 ubiquitin chains and therefore its adapter function during xenophagy. p62 transcription has been shown to be regulated by Nrf2. This transcription factor is usually associated with kelch-like ECH-associated protein 1 (Keap1), a negative regulator that rapidly leads to proteasomal degradation of Nrf2. In response to oxidative, electrophilic and proteotoxic stimuli, Keap1 and Nrf2 dissociate and genes regulated by the antioxidant response element (ARE), like p62, are transcriptionally activated (Kaspar et al., 2009). Furthermore, p62 contains a Keap1 interacting region (KIR) to compete with Nrf2 for Keap1 binding, thereby representing a positive feedback mechanism during Nrf2 activation (Ichimura et al., 2013; Jain et al., 2010; Komatsu et al., 2010). This feedback mechanism was shown to be functionally involved in coupling autophagy with the Keap1-Nrf2 system. Ichimura et al. reported that during xenophagy p62 is phosphorylated at the KIR domain. This induced its affinity for Keap1 and thereby the expression of cytoprotective Nrf2 target genes (Ichimura et al., 2013).

Proteolytic degradation of misfolded and damaged proteins by p62 has been extensively analyzed. This occurs primarily via autophagosomal degradation (Bjørkøy et al., 2005; Pankiv et al., 2007), although proteasomal targeting of substrates by p62 via direct interaction with subunits of the RP has been reported (Seibenhener et al., 2004). p62 itself is continuously degraded by autophagy via its LIR domain (Bjørkøy et al., 2005; Pankiv et al., 2007), but it has additionally been reported to be targeted for proteasomal degradation in a ubiquitin and FAT10 dependent manner (Aichem et al., 2012; Lee et al., 2012). Furthermore, the ability of p62 to homo- and heterooligomerize in cytosolic aggregates via the PB1 domain is required for efficient selective autophagy (Ichimura et al., 2008). This was shown to be as important for autophagosomal targeting as its ability to specifically recognize substrates via the UBA domain or the interaction with Atg8 orthologs (Bjørkøy et al., 2005; Pankiv et al., 2007). Additionally, p62 has been proposed as a histochemical marker for several protein aggregation diseases like neurodegenerative and liver diseases, since it aggregates in ubiquitin positive cytosolic and nuclear inclusions (Kuusisto et al., 2008). Interestingly, Kirkin et al. have shown that other autophagy adapters can compensate for the p62 function with regard to protein aggregation and degradation, which is in line with the relatively mild phenotype of p62 knockout mice (Kirkin et al., 2009b). Besides protein aggregates also various intracellular pathogens and their remnants have been shown to be degraded via autophagy in a p62 dependent manner (Dupont et al., 2009; Ponpuak et al., 2010; Zheng et al., 2009). This process will be introduced in more detail in 1.2.4.1.

Oligomerization and multiple binding domains that preserve the interaction with several
kinases, signaling molecules and ubiquitin mediated pathways, predestine p62 as a signaling scaffold besides its autophagosomal adapter function (Moscat et al., 2007). Through the interaction with the ubiquitin E3 ligase tumor necrosis factor receptor-associate factor 6 (TRAF6), receptor interacting protein 1 (RIP1) and the atypical protein kinase C (aPKC), p62 is known to modulate NF-κB signaling in response to IL-1β, TNF-α, RANKL and downstream of T-cell receptor (TCR) activation during Th2 differentiation (Durán et al., 2004; Martin et al., 2006; Sanz et al., 1999; Sanz et al., 2000). The role of p62 as a signaling scaffold is underscored by functional analysis of p62 mutations within the UBA domain that are associated with Paget’s disease of the bone. This disease is accompanied by upregulated osteoclastogenesis. The p62P392L mutation within the UBA domain seems to be a prerequisite therefore, since this mutation was shown to be responsible for increased osteoclast differentiation due to hyperactivation of the NF-κB pathway in response to RANKL (Cavey et al., 2005; Kurihara et al., 2007).

1.2.2.1.2 NDP52

NDP52, also named calcium binding and coiled-coil domain 2 (CALCOCO2), shares the two essential binding domains with all other autophagy adapters, a zink finger (ZnF) as UBD and a LIR domain (Figure 6). Though, there are characteristic features uniquely observed for NDP52. First of all, NDP52 additionally contains a Galectin-8 interacting region (Gal8IR) (Thurston et al., 2012). The cytosolic lectin Galectin-8 binds sugar molecules located at the extracellular sheath of the plasma membrane and serves therefore as a danger receptor for damaged vesicles by surveying the integrity of the entire endolysosomal compartment. Damaged, vesicular structures are recognized and subsequently eliminated by the autophagy machinery through the NDP52-Galectin-8 interaction (Li et al., 2013a; Thurston et al., 2012). Secondly, the LC3 interacting region of NDP52 was shown to specifically bind to the paralog LC3C and was therefore named LC3C-specific LIR domain (CLIR) (von Muhlinen et al., 2012). This unique feature allows NDP52 to orchestrate the recruitment of other Atg8 orthologs since LC3C is required for the recruitment of the other Atg8 orthologs to bacteria, thereby establishing a hierarchy amongst Atg8 family members. This selective NDP52-LC3C interaction furthermore seems to be essential during xenophagy of S. Typhimurium (von Muhlinen et al., 2012). Besides the Gal8IR region NDP52 can additionally recognize its cargo via the ZnF domain which was suggested to be specific to a wide variety of polyubiquitin linkages (Husnjak and Dikic, 2012). NDP52 is expressed in a wide variety of tested human tissues and was shown to be regulated by the Nrf2 transcription factor, similar to p62 (Jo et al., 2014; Thurston and Ryzhakov, 2009). NDP52 was shown to localize to
nuclear dots (Korioth et al., 1995), to the Golgi network (Morriswood et al., 2007) and also to cytosolic pathogens like S. Typhimurium and Group A streptococcus. In the latter case, NDP52 functions, similar to p62, as an autophagosomal adapter in addition to indirectly recruiting the TANK-binding kinase 1 (TBK1) (Thurston and Ryzhakov, 2009), a non-canonical IκB kinase (IKK) family member that activates non-canonical NFκB signaling and directly enhances autophagosomal elimination of pathogens (Pilli et al., 2012; Wild et al., 2011). In contrast, Ellinghaus et al. identified an association between Crohn’s disease (CD) and a common missense mutation in the NDP52 gene (Ellinghaus et al., 2013). This mutation is suggested to impair a proposed inhibitory effect of NDP52 on NF-κB induced activation of genes. Thereby, the study rather implicates a role for NDP52 in controlling pro-inflammatory signaling during chronic inflammation. This was further confirmed by the observed downregulating effect of NDP52 on TLR-signaling (Inomata et al., 2012). Furthermore, NDP52, similar to p62, has been shown to facilitate degradation of the phosphorylated tau protein, which induces Alzheimer disease (Babu et al., 2005; Jo et al., 2014).

1.2.3 Maturation

The last step of autophagy involves fusion with late endosomes and lysosomes to mature into a degradative compartment capable of destructing the engulfed cargo by lysosomal hydrolases. Several facilitators for membrane fusion in mammalian cells have been elucidated to be required for this specific process which are the small GTPase RAB7 (Ypt7 in yeast), the autophagosomal soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) protein syntaxin 17 and the lysosomal SNARE vesicle-associated membrane protein 8 (VAMP8) as well as lysosomal membrane proteins such as lysosomal-associated membrane glycoprotein 2 (LAMP2) (Itakura et al., 2012b; Jäger et al., 2004; Tanaka et al., 2000). After completion of lysosomal degradation the recycled small molecules, amino acids in particular, are transported back to the cytosol for protein synthesis and maintenance of cellular functions during starvation.

1.2.4 Autophagy and immunity

Autophagy is a highly conserved process and its role in immunity has reached a high level of complexity in higher eukaryotes. From the simple pathogen degradation and elimination machinery it has evolved into a regulator of diverse immunological processes like cytokine secretion, inflammation and shaping the T-cell repertoire. Consequently, pathogens that are highly adapted to their host have co-evolved various evasion strategies to subvert autophagy. This further strengthens the importance of autophagy in general, and xenophagy in particular,
as a defense mechanism during infectious diseases.

1.2.4.1 Microbial elimination

Pathogens can invade eukaryotic cells. Some pathogens reside within endocytic or phagocytic vesicles, but others are capable to replicate within the cytosol. As a consequence eukaryotes developed various defense mechanisms to counteract cytosolic replication. Effector mechanisms, that are often cytokine inducible, defend the host cell against infection and rely on oxidative, nitrosative and protonative chemistries. Similarly, restricting cytosolic nutrients by compartmentalization of pathogens by autophagosomal membranes turned out to be an effective mechanism. Therefore, xenophagy carried out by the autophagy machinery, developed into a cell autonomous defense mechanism (Birmingham et al., 2006; Gutierrez et al., 2004; Nakagawa et al., 2004; Ogawa et al., 2005).

A well-studied in vitro and in vivo infection model represents the Gram-negative, facultative intracellular bacterium *S.* Typhimurium. Infection by this pathogen usually leads to severe food poisoning with symptoms of gastroenteritis in human. In contrast, *S.* Typhi, which is restricted to human, can distribute to extraintestinal tissues and establish a systemic infection (Bhan et al., 2005). *S.* Typhimurium can also infect other animals and in most inbred mouse strains it leads to systemic infections, similar to *S.* Typhi in human. Therefore, infection of susceptible mice with *S.* Typhimurium represents the gold standard for studying thyphoidal infections (Tam et al., 2008). *S.* Typhimurium actively invades non-phagocytic cells and penetrates the gut epithelium predominantly via Peyer’s patches and other organized lymphoid structures in the small intestine (Halle et al., 2007; Jones et al., 1994). Bacterial distribution in mice has been described to occur intracellularly via neutrophils, DCs and macrophages (reviewed in Tam et al., 2008). Systemic infection usually affects extraintestinal organs like liver, spleen and bone marrow. Moreover, persistent *S.* Typhimurium colonies in mesenteric lymph nodes and the gall bladder of mice were documented (Monack et al., 2004). The importance of autophagy in host resistance against *S.* Typhimurium revealed by in vitro experiments was recently confirmed by in vivo studies with diverse model organisms (Benjamin et al., 2013; Conway et al., 2013; Jia et al., 2009).

Active invasion of non-phagocytic epithelial cells by *S.* Typhimurium (Figure 7) is triggered by translocation of virulence factors via the Type-III secretion system (T3SS) encoded by the *Salmonella* pathogenicity island-1 (SPI-1) leading to host cell mediated bacterial uptake (Brumell et al., 1999). Subsequent translocation of virulence factors into the host cell transforms the phagosome into an acidic compartment that supports bacterial replication, the
Salmonella-containing vacuole (SCV) (Arpaia et al., 2011; Hensel et al., 1998). The maturation and maintenance of this replicative niche largely relies on the expression of effector molecules encoded within the SPI-2 (Beuzón et al., 2000). Some intracellular bacteria like Listeria and Shigella escape into the cytosol where they obtain nutrients to replicate and subvert host immune responses like xenophagy (reviewed by Ray et al., 2009). S. Typhimurium is considered as a facultative, intracellular pathogen, which can be released from the SCV and enters the cytosol of macrophages and epithelial cells. Whether bacteria actively damage the vacuole, whether this happens accidently or whether host factors induce vacuole damage is still a matter of debate (Birmingham et al., 2006; Meunier et al., 2014). S. Typhimurium can also subvert cytosolic defense mechanisms like xenophagy, but not as efficiently as Listeria and Shigella (Le Negrate et al., 2008; Tattoli et al., 2012a; Ye et al., 2007). Therefore, it remains an open question why S. Typhimurium enters the host cytosol. Nevertheless, bacterial entry into the cytosol does not remain unnoticed by the host cell as ubiquitin accumulates in close vicinity to this fraction of bacteria (Perrin et al., 2004). It is unclear whether pathogens are either directly ubiquitinated or ubiquitinated host proteins accumulate on their surface (Huett et al., 2012; Manzanillo et al., 2013; Perrin et al., 2004). Recently, the two E3 ligases leucine-rich repeat and sterile alpha motif-containing protein 1 (LRSAM1) and parkin have been reported to contribute to polyubiquitination of different Gram-positive and Gram-negative bacteria, however, the actual bacterial targets still remain unknown (Huett et al., 2012; Manzanillo et al., 2013). Genetic polymorphisms in the gene encoding for parkin, an E3-ligase with a well-established role in mitophagy, is associated with increased susceptibility to intracellular bacterial pathogens in human, including Mycobacterium leprae and S. Typhi (Manzanillo et al., 2013). In addition to the ubiquitin detection system, other targeting mechanisms are known for xenophagy. As mentioned above, also Galectin-8 can bind to bacteria associated membranes to identify phagosomes containing bacteria with diacylglycerol (DAG) was reported to facilitate autophagosomal elimination of S. Typhimurium (Figure 7) (Shahnazari et al., 2010). Polyubiquitin chains as well as Galectin-8 lead to the subsequent identification by autophagy adapter p62, NDP52 and OPTN that recruit the nascent autophagosomes to cytosolic bacteria. The observation that linear as well as K63 linked ubiquitin chains were detected around S. Typhimurium (van Wijk et al., 2012) is in line with autophagy adapters having different ubiquitin chain specificities and the fact, that they localize to distinct microdomains within the bacterial coat (Cemma et al., 2011; Thurston et al., 2012; Wild et al., 2011).
Another non-canonical autophagy pathway exists that similarly engages the function of LC3/GABARAP proteins but does not include classical cargo engulfment. This process is termed LC3-associated phagocytosis (LAP) (Figure 7) (reviewed by Mehta et al., 2014). LC3 is recruited and inserted into the membrane of phagosomes and facilitates their maturation by fusion with lysosomes. LAP has been observed to play a role in antibacterial phagocytosis and

Figure 7: Model of S. Typhimurium infection. After bacterial uptake into the host cell, bacteria translocate effector proteins via the type III secretion system (T3SS). This allows the maturation of phagosomes into salmonella containing vacuoles (SCV) which provide a replicative niche for S. Typhimurium. A fraction of intracellular bacteria enter the cytosol where they can be identified by host defense mechanisms to be sequestered in autophagosomes and degraded by the lysosome. Alternatively, the association of LC3 with phagocytosed S. Typhimurium can likewise lead to lysosomal degradation in an autophagy independent manner via LC3 associated phagocytosis (LAP). Image adapted from (Huang and Brumell, 2014)
clearance of apoptotic cells. To insert LC3 into phagosomal membranes only a minor part of the autophagy machinery like Atg7 (E1 like) and Atg5 as well as the type III PI3 kinase complex containing Beclin 1 are necessary. Both pathways have been shown to exist in parallel. The striking difference between LAP and autophagosomal structures is detectable by the lack of a double membrane in LAP, though they are marked by LC3-II. These structures can therefore be distinguished from autophagy only by electron microscopy but not by LC3 co-localization (Levine et al., 2011). Mechanistically, LAP is induced by pathogen associated molecular patterns (PAMPs) and biochemical signs of cell death like phosphatidylycerine (PtdSer) present on the phagocytosed material (Martinez et al., 2011; Sanjuan et al., 2007). Furthermore, the accumulation of DAG at the membrane of SCVs has recently been implicated in inducing LAP of SCVs (Huang and Brumell, 2014; Shahnazari et al., 2010). In addition to LAP, where no double membrane structure is formed, other non-canonical autophagy pathways have been observed. It was for example reported that Atg5 and Atg7 knockout cells were able to form double membrane, autophagosome like structures around cytosolic bacteria, although they were clearly not able to activate and conjugate LC3 or its paralogs to these membranes. As a consequence, the lack of LC3-II conjugation to the double membrane led to the inability to restrict bacterial growth, most probably because LC3 is needed for autophagosomal closure (Kageyama et al., 2011; Nakatogawa et al., 2007). Not only Gram-positive and -negative bacteria are targeted for xenophagy but also a variety of DNA and RNA viruses (reviewed by Dong and Levine, 2013) and even protozoan parasites like Toxoplasma and Leishmania (reviewed by Skendros and Mitroulis, 2012). Furthermore, many of these pathogens have evolved strategies to modulate autophagy to their benefit. This includes the inhibition of autophagy initiation signaling, interference with the activity of autophagy components, evasion of autophagy recognition, blockage of autophagosome fusion with the lysosome and hijacking autophagy as a replicative niche (reviewed by Huang and Brumell, 2014).

1.2.4.2 Autophagosomal regulation of signaling and cytokine response

Repression and induction of autophagy initiation via immune signaling has been studied extensively. Negative regulation of autophagy was observed for example in response to T-helper 2 (Th2) cytokines like IL-4 (Harris et al., 2007). In contrast, pro-inflammatory cytokines like IFN-γ and TNF-α can have inducing effects (Djavaheri-Mergny et al., 2006; Gutierrez et al., 2004). Similarly, pathogen-associated molecular patterns (PAMPs) can trigger autophagy via pattern-recognition receptors (PRRs) like TLRs (Delgado et al., 2008).
and NOD-like receptors (NLRs) (Travassos et al., 2010) at the plasma membrane, within endosomes and in the cytosol upon pathogen-derived ligand binding. TLR-activation induces the NF-κB pathway (reviewed by Into et al., 2012). Following ligand binding to the respective TLR, signaling pathways are initiated via two key adaptor proteins, myeloid differentiation factor 88 (MyD88) and Toll/interleukin (IL)-1 receptor homology domain (TIR)-containing adaptor inducing interferon (IFN)-β (TRIF). The adaptor MyD88 is employed by all TLRs except TLR3 and upon receptor-ligand binding it eventually initiates signaling pathways resulting in the canonical activation of the transcriptional factor NF-κB and the cascades of mitogen-activated protein kinases (MAPKs). Thereby MyD88 forms a signaling complex with IL-1 receptor-associated kinase-4 (IRAK4) and IRAK1 or IRAK2. IRAKs are regulated and activated by polyubiquitination in order to recruit the K63-linkage specific E3 ubiquitin ligase TNF receptor-associated factor 6 (TRAF6) to the complex. TRAF6-generated K63 ubiquitin chains lead to the activation of TGFβ-activated kinase 1 (TAK1) and also bind to the scaffold protein NF-κB essential modulator (NEMO/IKKγ), a regulatory component of the canonical IκB kinase (IKK) complex, thereby activating IKKα and IKKβ. The transcription factor NF-κB, which is retained in the cytoplasm by IκB proteins is subsequently released following ubiquitination and proteasomal degradation of IκB through phosphorylation of the activated IKK complex. The transcriptional activation via TLR-signaling results in the activation of several transcription factors and consequently induces a broad array of genes, which include cytokines, adhesion molecules, antimicrobial molecules, co-stimulatory factors, anti-apoptotic molecules, signaling modulators and transcriptional regulators. In macrophages, autophagy has been shown to be regulated via TLR-signaling by the interaction of MyD88 or TRIF with Beclin 1. Shi and Kehrl suggested that these interactions reduced the autophagy inhibiting binding of Beclin 1 to Bcl-2 (Shi and Kehrl, 2008). The induction of autophagy by TLR adaptor proteins as well as pro-inflammatory cytokines was additionally supposed to be mediated by TRAF6 ubiquitination of Beclin 1 (Shi and Kehrl, 2010). Similarly, during viral infection the IFN-inducible, double-stranded RNA-dependent protein kinase R (PKR) is activated and upregulates autophagy (Lussignol et al., 2013).

On the other hand, autophagy can modulate inflammation, resulting in a reciprocal regulation between the two systems. Thereby, autophagy represents an important pathway to control the host response to infection and other inflammatory stimuli (Saitoh and Akira, 2010). An emerging role for autophagy in limiting uncontrolled immune activation and inflammation has been described in several studies. First of all, autophagy has been implicated in negatively
regulating inflammasome activation, a PRR complex that responds to danger-associated molecular patterns (DAMPs) and PAMPs. Activation of caspase-1 by this complex, as indicated by increased IL-1β and IL-18 secretion in response to TLR4 stimulation, was observed in autophagy impaired mice (Saitoh et al., 2008). Likewise, vacuolar membrane remnants from *Shigella* were shown to be recognized by p62 and targeted for autophagic degradation thereby dampening pro-inflammatory and promoting pro-survival pathways (Dupont et al., 2009). Similarly, activation of the pro-inflammatory pathway of NF-κB has also been described as a result of increasing levels of p62 due to autophagy deficiency (Moscat and Diaz-Meco, 2009). p62 bodies have been suggested to act as signaling hubs where p62 interacts with TRAF6 and may affect pro-survival signaling pathways. This was observed by Lee et al., who found autophagy to suppresses IL-1β signaling by increased p62 degradation via autophagosomal and proteasomal pathways (Lee et al., 2012).

Besides, the activation of the key extrinsic apoptosis initiation factor caspase-8 is regulated by p62 polymerization and induces pro-apoptotic pathways (Jin et al., 2009). Autophagosomal membranes thereby serve as a platform for intracellular death-inducing signaling complex (iDISC)-mediated caspase-8 activation (Young et al., 2012). Furthermore, autophagy is necessary for clearance of apoptotic cells which otherwise trigger inflammation by secondary, post-apoptotic necrosis (Qu et al., 2007).

### 1.3 Protein aggregation

Several cytosolic and nuclear protein aggregates are characterized and differ for example in composition, mobility and localization. Aggresomes and aggresome like inducible structures (ALIS) for example contain ubiquitinated, aggregated proteins, but they differ in several ways. The existence of ALIS was first described during maturation of dendritic cells (DCs) and they were therefore termed dendritic ALIS (DALIS) (Lelouard et al., 2002). However, ALIS can be observed in several cell types and are inducible in response to diverse stress stimuli (Pankiv et al., 2007; Szeto et al., 2006). The formation of these structures is typically dependent on translation and induced by puromycin treatment, indicating that misfolded proteins, also known as defective ribosomal products (DRiPs) account for the majority of proteins stored in ALIS. Furthermore, ALIS are not localized to the MTOC and they are not static like aggresomes, although the actual way of movement is unclear, as they circulate within cells independently of microtubules and actin cytoskeleton (Lelouard et al., 2002; Lelouard et al., 2004; Szeto et al., 2006). Interestingly, the regulation of (D)ALIS elimination is still a matter of debate and was likewise suggested to be proteasomal (Lelouard et al., 2002;
Seibenhener et al., 2004; Szeto et al., 2006) as well as autophagy dependent (Bjørkøy et al., 2005; Fujita et al., 2011; Pankiv et al., 2007; Szeto et al., 2006). The two major constituent of ALIS, NBR1 and p62, mediate the selective degradation of misfolded proteins by autophagy (Kirkin et al., 2009a; Pankiv et al., 2007). Their role in aggregate formation and protein clearance has been studied intensively leading to the following model proposed by Kirkin et al. (Kirkin et al., 2009b). Misfolded proteins are ubiquitinated and preferentially destroyed by the 26S proteasome. However, under stress conditions, which eventually increase the production of misfolded proteins, these soluble but potentially toxic oligomeric proteins accumulate and are polyubiquitinated. In a next step, these substrates, designated for degradation, are recognized by NBR1 and p62 and delivered to the forming autophagosome. If the production of misfolded proteins further increases and degradation of these soluble complexes is incomplete, NBR1 and p62 oligomerize and form ALIS (Pankiv et al., 2007; Szeto et al., 2006).

Histone deacetylase 6 (HDAC6) can bind dynein molecular motors, and is thereby involved in the microtubuli dependent transport of ubiquitin and FAT10 conjugated substrates to aggresomes (Kalveram et al., 2008; Kawaguchi et al., 2003). Additionally, it was likewise suggested to be involved in other stages of aggrephagy, like recruitment of autophagy components and the facilitation of autophagosome lysosome fusion (Figure 8) (Iwata et al., 2005; Lee et al., 2010). Besides direct substrate and dynein binding, the main function of HDAC6 in these processes is to ensure microtubule dynamics and F-actin assembly by its deacetylation activity. Interestingly, p62 and HDAC6 are likewise phosphorylated by casein kinase 2 (CK2). Both modifications have been shown to facilitate efficient clearance of ubiquitinated cargo in autophagosomes (Matsumoto et al., 2011; Watabe and Nakaki, 2011).

Of interest is the proposed function of DALIS as a source of MHC class I peptides via direct presentation. Philippe Pierre suggested that antigens in form of DRiPs are stored in DALIS and are subsequently degraded via the proteasome in matured DCs (Pierre, 2005). The main substrates for DALIS formation and subsequent proteasomal MHC class I peptide generation are DRiPs. This pool of proteins represents the major part of rapidly degraded proteins (RDPs) in contrast to fully translated and properly folded, long-lived proteins (Yewdell and Nicchitta, 2006). However, it has to be stressed that this model was previously challenged by Kenneth Rock and colleagues (Farfán-Arribas et al., 2012; Rock et al., 2014). They argue in favor of stable, slowly degraded proteins to represent a source for MHC class I peptides with equal efficiency than DRiPs.
Figure 8: The multiple roles of HDAC6 in proteostasis. HDAC6 is involved in several stages of aggrephagy. Aggregate formation is regulated via the direct interaction with dyneine motor protein complexes and ubiquitinated cargo. A further function of HDAC6 in this process is to ensure microtubule dynamics and F-actin assembly by its deacetylation activity. Prior to aggresome formation, HDAC6 also influences aggregation of proteasome substrates via its interaction with valosin-containing protein (VCP). In a later step of aggregation prone protein degradation HDAC6 facilitates the autophagosome lysosome fusion by recruitment of autophagy components. The image is adapted from (d’Ydewalle et al., 2012)
**Aim of this study**

FAT10 was shown to localize to aggresomes and to interact with p62. The aim of this study was to further investigate functional aspects of the covalent and non-covalent interaction between FAT10 and p62. Both interaction partners, HDAC6 and p62, are involved in aggregation of ubiquitinated proteins designated for proteasomal as well as autophagosomal degradation. The role of FAT10ylation in targeting substrates for proteasomal degradation was already described. However, the question arose, whether FAT10ylation might additionally mediate the targeting of substrates for autophagosomal elimination via binding to p62. Thereby the interesting question, whether FAT10 might be relevant for the immunological function of autophagy to eliminate cytosolic pathogens should be investigated. Several aspects within the process of xenophagy are thereby of special interest. First of all, the question whether FAT10 is covalently or non-covalently associated with xenophagy substrates should be addressed. Furthermore, the functional relevance *in vitro* and *in vivo* should be investigated. Additionally, since p62 represents not only an autophagy adapter but is also involved in autophagosomal regulation, the potential impact of FAT10 on the p62 regulator function should be addressed.

In the second part, the recently proposed immunoproteasome function, that describes an increased proteolytic capacity compared to the standard proteasome, should be reinvestigated. Therefore, two different aspects should be addressed. On the one hand, the polyubiquitin conjugate degradation and secondly, the elimination of cytosolic aggregates, both in dependence of the immunoproteasome subunit LMP7.
2 Material and methods

2.1 Cell lines and cell culture

HeLa cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) containing 10% (v/v) FBS (Gibco) and 1% (v/v) penicillin and streptomycin (Gibco). Stable transfected human embryonic kidney (HEK293) cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) containing 10% (v/v) FBS (Gibco), 1% (v/v) penicillin and streptomycin (Gibco) and 1 mg/ml geneticin (Gibco) for selection. Human umbilical vein endothelial cells (HUVECs) from single donors (PromoCell) were cultured in endothelial cell growth medium (PromoCell) containing endothelial cell growth supplement (PromoCell) without antibiotics. Cell culture dishes for HUVECs cultivation were coated prior to seeding with 0.2% (w/v) porcine skin gelatin (Sigma-Aldrich) in water. Aliquots of primary cells and cell lines were frozen in liquid nitrogen for long term storage in 90% FBS supplemented with 10% DMSO. All cell lines were cultured at 37°C and 5% CO₂. Endogenous FAT10-expression was induced by treatment of cells with 400 U/ml TNF-α and 200 U/ml IFN-γ (both Peprotech, Hamburg, Germany).

2.2 Generation of stable cell lines

HEK293 cells were transiently transfected with pSV2-neo (ATCC® 37149™) using TransIT®-LT1 Transfection Reagent (Mirus, Madison, WI) according to the manufacturer’s instructions. 20 hours after transfections, transfected cells were seeded, together with wild type HEK293 cells in the concentrations 1:1, 1:10 and 1:100 in 96-well plates. After 24 hours, medium containing 1 mg/ml geneticin (Gibco) was added to select single clones.
2.3 Generation of primary cells

For mouse embryonic fibroblast (MEFs) preparation pregnant, female C57BL/6 mice were sacrificed 13.5 days post coitum (dpc). Embryos were dissected, rinsed with PBS and kept in a dish containing PBS. Head and liver of each embryo were cut off before mincing the remaining tissue in a minimal volume of PBS. Subsequently, the remaining tissue was resuspended in 1-2 ml trypsin/EDTA (Gibco) and incubated for 15 min at 37°C with gentle shaking. Trypsination was stopped with DMEM containing 10% (v/v) FBS (Gibco) and 1% (v/v) penicillin and streptomycin (Gibco). The cell suspension without remaining larger tissue pieces was centrifuged and resuspended in fresh DMEM before plating at one embryo/10cm dish. Prepared cells were cultured at 37°C and 5% CO₂. Peritoneal macrophages (pMΦs) were prepared by intra peritoneal (i.p.) injection of 3% thioglycollate solution into C57BL/6 mice. After three days, peritoneal cells were washed out of the abdominal cavity with 10 ml PBS. Cells were cultured in RPMI containing 10% (v/v) FBS (Gibco) and 1% (v/v) penicillin and streptomycin (Gibco) for two days and adherent cells were used for gentamicin protection assays.

2.4 Transfection and electroporation

HEK293T cells were transiently transfected with TransIT®-LT1-transfection reagent (Mirus, Madison, WI) according to the manufacturer’s instruction using a 3:1 ratio of transfection reagent volume (µl) and DNA (µg). HeLa cells were transiently transfected with FuGENE® HD Transfection Reagent (Promega, Madison, WI) according to the manufacturer’s instruction using a 3:1 ratio of transfection reagent volume (µl) and DNA (µg).

HUVECs were transiently transfected with GeneTransII (MoBiTec, Goettingen; Germany) using the DNA Diluent B protocol. Cells were washed three times prior to transfection and cultured in Optimem (Gibco) for transfection. A ratio of 2.6:1 of transfection reagent volume (µl) and siRNA (20 µM) volume (µl) instead of DNA was used, resulting in a final concentration of 30 nM siRNA during transfection. After four hours of incubation at 37°C and 5% CO₂ cells were washed two times and endothelial cell growth medium (PromoCell) containing endothelial cell growth supplement (PromoCell) without antibiotics was added afterwards.

For electroporation with the Nucleofector™ II device (LONZA, Basel, Switzerland) the Amaza® Nucleofector™ Kit for HUVECs (LONZA, Basel, Switzerland) was used according to the manufacturer’s instruction. 100 µl HUVEC Nucleofector® Solution was mixed with
4 µg of DNA and transferred together with 1x10^6 HUVECs into a certified cuvette and processed for the Nucleofector® Program A-034. Cells were subsequently resuspended in 500 µl endothelial cell growth medium (PromoCell) containing endothelial cell growth supplement (PromoCell) without antibiotics and 5x10^5 transfected HUVECs were plated in 24-wells. After two hours non-adherent cells were aspirated and new medium was added.

## 2.5 siRNA

### Table 2: siRNA for knock down experiments

<table>
<thead>
<tr>
<th>name</th>
<th>gene</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs_UBE2Z_2</td>
<td>USE1</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>Hs_UBE2Z_3</td>
<td>USE1</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>Hs_UBE2Z_5</td>
<td>USE1</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>Hs_FLJ13855_4</td>
<td>USE1</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>Hs_UBA6_1</td>
<td>UBA6</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>Hs_UBA6_2</td>
<td>UBA6</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>Hs_FLJ10808_2</td>
<td>UBA6</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>Hs_FLJ10808_4</td>
<td>UBA6</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>Hs_UBD_1</td>
<td>FAT10</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>Hs_UBD_2</td>
<td>FAT10</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>Hs_UBD_3</td>
<td>FAT10</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>Hs_UBD_5</td>
<td>FAT10</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>AllStars Negative Control siRNA</td>
<td>scrambled</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
</tbody>
</table>

## 2.6 Quantitative real-time RT-PCR

Total RNA was extracted from cells with RNeasy® Plus Micro or Mini Kit (Qiagen) according to the manufacturer’s instructions. The following reverse transcription of mRNA into single stranded cDNA with Oligo(dT)$_{15}$ primers was performed with the Reverse Transcription System (Promega). The cDNA was used for PCR amplification using the LightCycler Fast Start DNA Master SYBR Green I Kit (Roche) according to the manufacturer’s instructions. Samples were measured with the LightCycler instrument (Roche) with the corresponding LightCycler Software Version 3.5. Target specific sense and antisense primers are listed in Table 3. Cycling programs for human FAT10, GAPDH, mouse FAT10 and HPRT are listed in Table 4, Table 5 and Table 6, respectively. Relative gene expression was normalized to mHPRT for mouse samples and huGAPDH for human samples and
evaluated according to the Pfaffl method using the Excel-based software tool REST-384-beta (Pfaffl et al., 2002).

### Table 3: primer for quantitative RT-PCR

<table>
<thead>
<tr>
<th>gene</th>
<th>sense (5’→3’)</th>
<th>antisense (3’→5’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>huFAT10</td>
<td>AAT GAC CTT TGA TGC CAA CC</td>
<td>GCC GTA ATC TGC CAA CAT CAT</td>
</tr>
<tr>
<td>huGAPDH</td>
<td>GAA GGT GAA GGT CGG AGT C</td>
<td>GAA GAT GGT GAT GGG ATT TC</td>
</tr>
<tr>
<td>mFAT10</td>
<td>GCT TCT GTC CGC ACC TGT GTT GT</td>
<td>TGG GGC TTG AGG ATT TTG GAG TCT</td>
</tr>
<tr>
<td>mHPRT</td>
<td>CCA GCA GGT CAG CAA AGA ACT TA</td>
<td>TGG ACA GGA CTG AAA GAC TTG</td>
</tr>
</tbody>
</table>

### Table 4: LightCycler program for huFAT10 and GAPDH

<table>
<thead>
<tr>
<th>step</th>
<th>duration</th>
<th>temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>10 min</td>
<td>95°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>10 sec</td>
<td>95°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>5 sec</td>
<td>60°C</td>
</tr>
<tr>
<td>Amplification</td>
<td>11 sec</td>
<td>72°C</td>
</tr>
<tr>
<td>reading of the fluorescence</td>
<td>62°C to 95°C transition with 0.1°C increment/s</td>
<td></td>
</tr>
<tr>
<td>melting curve analysis</td>
<td>95°C for 0 s; 65°C for 15 s, 95° for 0 s with a temperature transition rate of 0.1°C/s (continuous mode)</td>
<td></td>
</tr>
</tbody>
</table>

### Table 5: LightCycler program for mHPRT

<table>
<thead>
<tr>
<th>step</th>
<th>duration</th>
<th>temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>10 min</td>
<td>95°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>10 sec</td>
<td>95°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>5 sec</td>
<td>59°C</td>
</tr>
<tr>
<td>Amplification</td>
<td>5 sec</td>
<td>72°C</td>
</tr>
<tr>
<td>reading of the fluorescence</td>
<td>62°C to 95°C transition with 0.1°C increment/s</td>
<td></td>
</tr>
<tr>
<td>melting curve analysis</td>
<td>95°C for 0 s; 65°C for 15 s, 95° for 0 s with a temperature transition rate of 0.1°C/s (continuous mode)</td>
<td></td>
</tr>
</tbody>
</table>
### Material and methods

#### Table 6: LightCycler program for mFAT10

<table>
<thead>
<tr>
<th>step</th>
<th>duration</th>
<th>temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>10 min</td>
<td>95°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>10 sec</td>
<td>95°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>10 sec</td>
<td>68°C</td>
</tr>
<tr>
<td>Amplification</td>
<td>7 sec</td>
<td>72°C</td>
</tr>
<tr>
<td>reading of the fluorescence</td>
<td></td>
<td>62°C to 95°C transition with 0.1°C increment/s</td>
</tr>
<tr>
<td>melting curve analysis</td>
<td></td>
<td>95°C for 0 s; 65°C for 15 s, 95°C for 0 s with a temperature transition rate of 0.1°C/s (continuous mode)</td>
</tr>
</tbody>
</table>

#### 2.7 Plasmids

#### Table 7: plasmids

<table>
<thead>
<tr>
<th>name</th>
<th>published</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.1His-3xFlag-FAT10</td>
<td>(Chiu et al., 2007)</td>
<td>Ph.D. Zhijian J. Chen, Dallas</td>
</tr>
<tr>
<td>pcDNA3.1His-3xFlag-FAT10ΔGG</td>
<td>(Aichem et al., 2010)</td>
<td>University of Konstanz</td>
</tr>
<tr>
<td>pIRESmCherry-EGFP-p62</td>
<td>unpublished</td>
<td>This study</td>
</tr>
<tr>
<td>pIRESmCherry-EGFP-p62-FAT10</td>
<td>unpublished</td>
<td>This study</td>
</tr>
<tr>
<td>pDest-mCherry-EGFP-p62</td>
<td>(Pankiv et al., 2007)</td>
<td>T. Johansen, Tromsø University</td>
</tr>
<tr>
<td>pcDNA3.1mCherry-EGFP-HIS-Flag-FAT10</td>
<td>(Aichem et al., 2012)</td>
<td>This study</td>
</tr>
<tr>
<td>pDest-mCherry-EGFP-4xUbiquitin</td>
<td>unpublished</td>
<td>T. Johansen, Tromsø University</td>
</tr>
<tr>
<td>pcDNA3.1mCherry-EGFP</td>
<td>(Aichem et al., 2012)</td>
<td>This study</td>
</tr>
<tr>
<td>pcDNA3.1His-3xFlag-FAT10ΔGG-C0</td>
<td>unpublished</td>
<td>This study</td>
</tr>
<tr>
<td>pcDNA3.1His-3xFlag-FAT10-C0</td>
<td>unpublished</td>
<td>This study</td>
</tr>
<tr>
<td>HA-p62 (HA-tag repaired by K. Kluge)</td>
<td>(Lamark et al., 2003)</td>
<td>T. Johansen, Tromsø University</td>
</tr>
<tr>
<td>pcDNA3.1-HA-ubiquitin GG</td>
<td>unpublished</td>
<td>Michael Basler, University of Konstanz</td>
</tr>
<tr>
<td>pcDNA3.1-HA-ubiquitin GA</td>
<td>unpublished</td>
<td>Michael Basler, University of Konstanz</td>
</tr>
</tbody>
</table>
2.8 Generation of competent E. coli

**LB**
- 1% (w/v) tryptone (BD)
- 0.5% (w/v) yeast extract (BD)
- 1% (w/v) NaCl (Roth)

**LB-agar**
- LB medium
- 1.5% (w/v) agar (BD)

A 2.5 ml LB pre-culture was inoculated with E. coli (strain XL10 gold) and grown overnight at 37°C and 200 rpm. This culture was used to inoculate 250 ml LB for growth until OD<sub>600nm</sub>=0.5-0.6 measured with the SmartSpec™ Plus (BioRad). Subsequently, the bacterial culture was incubated on ice for 15 min and kept on ice for the following procedure. After centrifugation at 3500 x g at 4°C the pellet was resuspended in 100 ml 0.1 M ice cold MgCl<sub>2</sub> (Acros Organics). After a second centrifugation step, bacteria were resuspended in 100 ml ice cold 0.1 M CaCl<sub>2</sub> (Roth). This solution was again centrifuged and subsequently resuspended in CaCl<sub>2</sub>/Glycerol (86 mM CaCl<sub>2</sub> (Roth), 10% (v/v) glycerol (VWR)), aliquoted in pre-cooled tubes and stored at -80°C.

2.9 Transformation

**Super Optimal Broth with Catabolite repression (SOC)**
- 2% (w/v) tryptone (BD)
- 0.5% (w/v) yeast extract (BD)
- 0.05% (w/v) NaCl (Roth)
- 0.0186% (w/v) KCl (Merck)
- 0.095% (v/v) MgCl<sub>2</sub> (Acros Organics)

50 µl of chemically competent E. coli were thawed on ice and about 100 ng of plasmid-DNA or ligation reaction were added. The mixture was further incubated on ice for 30 min followed by a heat shock for 45 sec at 42°C. Bacteria were placed back on ice and 500 ml of SOC medium was added prior to further incubation for 1 h at 37°C. 50 µl and 450 µl culture was plated on separate agar-plates containing the corresponding selection antibiotics and incubated overnight at 37°C. To analyze individual clones, several colonies were inoculated with 5 ml of LB medium overnight and plasmid DNA was purified with the NucleoSpin® Plasmid MiniPrep kit (Macherey & Nagel) according to the manufacturer’s instruction. Prior to centrifugation, 800 µl of culture was mixed with 200 µl sterile glycerol (VWR) and stored at -80°C.
2.10 Cloning and site directed mutagenesis

The mutagenesis primers for generation of cysteine-less Flag-FAT10 (G20C_G26C, G401C, G485C) listed in Table 8 and mCherry-EGFP-HIS-Flag-FAT10 (Ins_2nt_sense, Ins_2nt_anti tf) listed in Table 9 were designed with the web-based QuikChange primer design program which is provided by Agilent on their homepage (www.agilent.com/genomics/qcpd). Both mutagenesis kits were used according to the manufacturer’s protocol.

For generation of cysteine-less Flag-FAT10 and cysteine-less Flag-FAT10ΔGG the primers listed in Table 8 were used in combination with the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer’s instructions. For generation of pcDNA3.1mCherry-EGFP, mCherry-EGFP was excised from pDest-mCherry-EGFP-p62 by the restriction enzymes Nhel and HindIII (Fermentas / Fisher Scientific, Schwerte, Germany), purified by the NucleoSpin® Gel and PCR Clean-up Kit (Macherey & Nagel, Dueren, Germany) and inserted into pcDNA3.1-3xFlag-FAT10 by the use of the T4 DNA ligase (Promega, Mannheim, Germany). For the generation of pcDNA3.1mCherry-EGFP-Flag-FAT10 the stop codon of pcDNA3.1mCherry-EGFP was eliminated by the QuikChangeII Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) with the primers listed in Table 9 according to the manufacturer’s instructions. All constructs were verified by sequencing (GATC Biotech, Konstanz, Germany). For Plasmid purification the NucleoSpin® Plasmid MiniPrep Kit (Macherey & Nagel, Dueren, Germany) was used according to the manufacturer’s instruction.

<table>
<thead>
<tr>
<th>name</th>
<th>sense (5´→3´)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G20C_G26C</td>
<td>GGCTCCCAATGCTTCTCCCTCTCTCTGATGTC</td>
</tr>
<tr>
<td>G401C</td>
<td>GACCCAGATTGACTTCCATGGAAAGAGACTGG</td>
</tr>
<tr>
<td>G485C</td>
<td>CTTACTTCTCTGGGAGGAGGATG</td>
</tr>
</tbody>
</table>

Table 8: QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands)

<table>
<thead>
<tr>
<th>name</th>
<th>sense (5´→3´)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ins_2nt_sense</td>
<td>CTCAGATCTCAGCTCAAGCTTACCTGGGCATGCATC</td>
</tr>
<tr>
<td>Ins_2nt_anti tf</td>
<td>GATGATGGCCCAGGTAAGCTTGAGCTGAGATCTGAG</td>
</tr>
</tbody>
</table>

Table 9: QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands)
2.11 In vitro FAT10ylation

**In vitro reaction buffer**

- 20 mM Tris-HCl (Roth), pH 7.6
- 50 mM NaCl (Roth)
- 10 mM MgCl₂ (Acros Organics)
- 4 mM adenosine triphosphate (ATP) (Sigma-Aldrich)
- 0.1 mM dithiothreitol (DTT) (Sigma-Aldrich)
- 5 U/ml inorganic pyrophosphatase (Sigma-Aldrich)
- 20 mM creatine phosphate (Sigma-Aldrich)
- 4 µg/ml creatine phosphokinase (Sigma-Aldrich)
- 1 x protease inhibitor mix (complete Mini EDTA-free, Roche, Germany)

**Recombinant proteins**

- FAT10 (0.4 mg/ml)
- HIS-USE1 (0.59 mg/ml)
- HIS-UBA6 (0.29 mg/ml)

For *in vitro* FAT10ylation *S. Typhimurium* (strain SHF2) were used. SHF2 bacteria were grown overnight in LB-LS containing 90 µg/ml streptomycin (Sigma-Aldrich) and 100 µg/ml ampicillin (Roche) and sub-cultured (1:33) in fresh LB-LS for 3 h (OD₆₀₀ₙ₉ > 1) (SmartSpec™ Plus (BioRad)). SHF2 cultures were pelleted for 10 min at 3500 x g and washed twice with PBS. 2 x 10⁸ cfu SHF2 were resuspended in *in vitro* reaction buffer containing the respective recombinant proteins as indicated. In a final volume of 40 µl of 1 x reaction buffer the following amounts of protein was added: 2.9 µg HIS-UBA6, 3 µg; His-USE1 and 4 µg FAT10. The *in vitro* reaction was incubated for 50 min at 30°C with constant shaking (1150rpm). Subsequently reactions were centrifuged at 20000 x g for 5 min at room temperature. 4 x SDS-sample buffer with or without 10% β-mercaptoethanol (Merck) was added as indicated to the supernatant and incubated at 95°C for 5 min. The bacterial pellet was washed with PBS and likewise incubated with 10% β-mercaptoethanol (Merck) containing 2 x SDS-sample buffer at 95°C for 5 min.

2.12 In vitro infection

**LB-LS**

- 1% (w/v) tryptone (BD)
- 0.5% (w/v) yeast extract (BD)
- 0.5% (w/v) NaCl (Roth)

**BHI**

Brain Heart Infusion, Porcine (Difco)

For *in vitro* infections with *S. Typhimurium* (strain SHF2), bacteria were grown overnight in
Material and methods

LB-LS containing 90 µg/ml streptomycin (Sigma-Aldrich) and 100 µg/ml ampicillin (Roche) and sub-cultured (1:33) in fresh LB-LS for 3 h (OD\(_{600nm}\)>1) (SmartSpec\textsuperscript{TM} Plus (BioRad)). SHF2 cultures were pelleted for 10 min at 3500 x g, washed twice with PBS and cells in 24-well plates were infected with the respective multiplicity of infection (MOI). The infected cells were centrifuged for 5 minutes at 1000 x g and incubated for 30 min (HEK293, MEFs) or 60 minutes (HUVECs, peritoneal macrophages) at 37°C to allow cellular uptake.

L. monocytogenes (Lm10403S) was cultured in BHI broth over night at 37°C to stationary phase. This culture was subsequently diluted 1:10 with fresh BHI broth and cultured until OD\(_{600nm}\)~0.5. Listeria was incubated with 10 µg/ml Fluorescein 5(6)-isothiocyanate (Sigma) or 0.2 µg/ml 5-(6)-carboxytetramethylrhodamine-succinylester (Molecular Probes, Eugene, OR) for 15 min at room temperature. Labeled bacteria were extensively washed with PBS prior to use. Listeria was added to host cells at a multiplicity of infection of 100. Bacteria and cells were centrifuged at 1000 x g for 5 min at room temperature and then incubated at 37°C and 5% CO\(_2\) for 1 h. For following incubation times 50 µg/ml gentamicin (Gibco) was added which was further diluted after 30 min to 25 µg/ml gentamicin.

2.13 Gentamicin protection assay

**LB-agar**

LB-LS medium

1.5% (w/v) agar (BD)

To enumerate intracellular SHF2, cells were plated in 24-well plates and infected with SHF2 with the MOI=100 if not stated otherwise. Following two washes with PBS and incubation with 100 µg/ml gentamicin (HEK293, MEFs, peritoneal macrophages) or 25 µg/ml gentamicin (HUVECs) for 30min, cells were cultured in 25 µg/ml gentamicin for the indicated time period. For harvesting, cells were washed twice with PBS and lysed in 0.5 ml cold PBS containing 1% (v/v) Triton X100 (Fluka). The lysates were diluted up to 1:10\(^6\) and 50 µl of three different dilutions were plated on 90 µg/ml streptomycin (Sigma-Aldrich) and 100 µg/ml ampicillin (Roche) containing LB-LS agar plates. Cfu were counted for each time point and normalized to 0 h of infection. This sample was harvested right after invasion, before gentamicin was added.

2.14 Mouse strains

All mice were purchased from the animal facility of the University of Konstanz. C57BL/6
mice were originally obtained by Charles River Laboratories, Kisslegg, Germany. FAT10-deficient C57BL/6 mice (FAT10-/-) were a kind gift from Sherman M. Weissman and have been described elsewhere (Canaan et al., 2006). C57BL/6 wild type and FAT10-/- mice (University of Konstanz) were intercrossed with C57BL/6 NRAMP1 transgenic mice (Forschungszentrum Borstel) to generate NRAMP1 transgenic C57BL/6 wild type and FAT10-/- mice.

### 2.15 Mouse genotyping

**Tail digestion buffer**

10 mM Tris-HCl (Roth) pH 8.4,  
50 mM KCl (Merck)  
2.5 mM MgCl₂ (Acros Organics)  
0.45% (v/v) NP-40/Igepal® (Sigma-Aldrich),  
0.45% (v/v) Tween20 (Sigma-Aldrich)  
0.2 mg/ml Proteinase K (Sigma-Aldrich)

Tail biopsies were digested with tail digestion solution and incubated overnight at 55°C under constant shaking followed by incubation at 95°C for 5 min. Samples were centrifuged at 20000 x g for 10 min and 1 µl of the supernatant was used as PCR template. PCR was performed with GoTaq polymerase (Promega, Mannheim, Germany) according to manufacturer’s instructions using the Thermocycler comfort (Eppendorf, Hamburg, Germany). The primer pairs displayed in Table 10 were used, which were all synthesized by Microsynth (Balgach, Switzerland). The PCR programs for FAT10 and for NRAMP1 are listed in Table 11 and Table 12, respectively. PCR-products were visualized by agarose gel electrophoresis.

**Table 10: primer for genotyping**

<table>
<thead>
<tr>
<th>name</th>
<th>sense (5’→3’)</th>
<th>antisense (3’→5’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAT10</td>
<td>GGGATTGACAAGGAAACCACTA</td>
<td>TTCACAACCTGCTTCTTAGGG</td>
</tr>
<tr>
<td>NRAMP lytR</td>
<td>ACGCATCCCCTGCTGGGG</td>
<td>ACAGCCGGACAGGTGGG</td>
</tr>
<tr>
<td>NRAMP lytS</td>
<td>ACGCATCCCCTGCTGGGA</td>
<td>ACAGCCGGACAGGTGGG</td>
</tr>
</tbody>
</table>
Material and methods

2.16 In vivo infection

For survival and cfu enumeration experiments, sex matched mice with the age of 8-12 weeks were fasted for 4 h followed by oral gavage with $1.6 \times 10^8$ cfu S. Typhimurium (SL1344) in 100 µl PBS. The body weight, fur appearance, activity and food and water uptake of mice was monitored four times a week and mice were sacrificed when their body weight loss exceeded 20%.

2.17 Determination of bacterial load

For cfu enumeration, organs were harvested and kept in PBS on ice. Whole organs were homogenized in PBS using a Polytron PT2100 homogenizer (Kinematica). Cecum specimens were homogenized with content. The lysates were diluted up to 1:10^6 and 50 µl of each dilution was plated on 90 µg/ml streptomycin (Gibco) containing LB-LS agar plates.

2.18 Radiolabelling and pulse chase

HEK293T cells were radio labelled with $^{35}$S-L-cysteine and $^{35}$S-L-methionine after 24 h of incubation with 200 U/ml IFN-γ and 400 U/ml TNF-α (both from Peprotech, Hamburg,

<table>
<thead>
<tr>
<th>Table 11: FAT10 genotyping</th>
</tr>
</thead>
<tbody>
<tr>
<td>step</td>
</tr>
<tr>
<td>Denaturation</td>
</tr>
<tr>
<td>Denaturation</td>
</tr>
<tr>
<td>Annealing</td>
</tr>
<tr>
<td>Amplification</td>
</tr>
<tr>
<td>Amplification</td>
</tr>
<tr>
<td>pause</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 12: NRAMP1 genotyping</th>
</tr>
</thead>
<tbody>
<tr>
<td>step</td>
</tr>
<tr>
<td>Denaturation</td>
</tr>
<tr>
<td>Denaturation</td>
</tr>
<tr>
<td>Annealing</td>
</tr>
<tr>
<td>Amplification</td>
</tr>
<tr>
<td>Amplification</td>
</tr>
<tr>
<td>pause</td>
</tr>
</tbody>
</table>
Germany) and 8-18 h with 100 nM bafilomycin A1 (ENZO life science, Loerrach, Germany). Cells were washed with PBS and incubated prior to labelling for 1 h with RPMI lacking L-glutamine, cysteine and methionine (Sigma-Aldrich) supplemented with 10% (v/v) dialyzed FBS (Gibco), 1% (v/v) penicillin and streptomycin (Gibco) and 300 µg/ml L-glutamine (Sigma-Aldrich). This starvation medium was afterwards replaced by fresh RPMI lacking L-glutamine, cysteine and methionine (Sigma-Aldrich) supplemented with 10% (v/v) dialyzed FBS (Gibco), 1% (v/v) penicillin and streptomycin (Gibco), 300 µg/ml µM L-glutamine and 0.25 µCi/ml Met-[S35]-label (Hartmann Analytics, Braunschweig, Germany) and incubated for 1 h. Subsequently, cells were detached with PBS-EDTA (0.8 mM EDTA), washed with PBS and seeded into 6-wells with equal cell numbers for each time point. After the indicated time points cells were harvested and lysed with Ripa buffer.

HEK293T cells were radiolabeled with [35S]-L-cysteine after 8 h (early labelling) and 22 h (late labelling) of transient transfection. The procedure was performed as described for [35S]-L-cysteine and [35S]-L-methionine labelling except for the cells being incubated with RPMI lacking L-glutamine, cysteine and methionine (Sigma-Aldrich) supplemented with 10% (v/v) dialyzed FBS (Gibco), 1% (v/v) penicillin and streptomycin (Gibco), 300 µg/ml L-glutamine, 15 µg/ml L-methionine (Sigma-Aldrich) and 0.25 µCi Cys-[S35] (Hartmann Analytics, Braunschweig, Germany) for 2 h instead of 1 h. The labelling medium was either replaced by DMEM containing 10% (v/v) FBS (Gibco) and 1% (v/v) penicillin and streptomycin (Gibco) (early labelling) or cells were lysed with NP-40-lysis buffer subsequently after labelling (late labelling). Samples were processed for SDS-PAGE and gels were subsequently dried with the Gel dryer 583 (BioRad, Muenchen). After exposure of 1-3 days the imaging plates (Fuji Film, Duesseldorf) were read by the PMI personal molecular image reader (BIO RAD).
## 2.19 Sample preparation and immunoprecipitation

<table>
<thead>
<tr>
<th>Ripa lysis buffer</th>
<th>Tris/Triton lysis buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris (Roth), pH 7.5</td>
<td>20 mM Tris (Roth) pH 7.6</td>
</tr>
<tr>
<td>1 mM EDTA (Roth)</td>
<td>50 mM NaCl (Roth) (added after lysis)</td>
</tr>
<tr>
<td>150 mM NaCl (Roth)</td>
<td>0.1% (v/v) Triton-X-100 (Fluka)</td>
</tr>
<tr>
<td>0.1% SDS (Serva)</td>
<td>1 x protease inhibitor cocktail (complete Mini EDTA-free, Roche, Germany)</td>
</tr>
<tr>
<td>1% NP-40/Igepal® (Sigma-Aldrich)</td>
<td></td>
</tr>
<tr>
<td>5 µM MG-132 (Sigma-Aldrich)</td>
<td></td>
</tr>
<tr>
<td>10 mM NEM (Sigma-Aldrich)</td>
<td></td>
</tr>
<tr>
<td>1 x protease inhibitor cocktail (complete Mini EDTA-free, Roche, Germany)</td>
<td></td>
</tr>
</tbody>
</table>

### 4 x SDS sample buffer

<table>
<thead>
<tr>
<th>40% Glycerol (Roth)</th>
<th>240 mM Tris (Roth) pH 6.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>8% SDS (Serva)</td>
<td>8% SDS (Serva)</td>
</tr>
<tr>
<td>0.04% Bromophenol blue (Applichem)</td>
<td>0.04% Bromophenol blue (Applichem)</td>
</tr>
<tr>
<td>10% β-mercaptoethanol (Merck)</td>
<td>10% β-mercaptoethanol (Merck)</td>
</tr>
</tbody>
</table>

For sample preparation of HeLa cells Tris/Triton lysis was used (Figure 12 A). For lysis of HUVECs and HEK293T cells (Figure 14 A) Ripa buffer was used. MEFs were lysed in Ripa lysis buffer without SDS. Cells were harvested 24 h after transient transfection or cytokine treatment and lysed in 70-100 µl lysis buffer per 6-well. Cells were kept on ice for 30 min and in the case of Tris/Triton-lysis the lysate was afterwards sonicated with a Sonoplus sonicator (Bandelin, Berlin, Germany) three times (10 s, 5 cycles, 70% intensity). Lysates were cleared by centrifugation at 20000 x g for 15 min at 4°C. Protein concentration was measured by DCT™ Protein Assay (BioRad, Hercules, CA) and protein levels of each sample were adjusted to equal concentrations. Radioactively labeled lysates were measured on Luma-Plates-96 (Canberra Packard, Schwadorf, Austria) with the scintillation counter TOPcount NXT (Canberra Packard, Schwadorf, Austria). Lysates were then adjusted to the same relative radioactivity. For analysis of total cell lysate, samples were subsequently boiled with 4 x SDS sample buffer with a final concentration of 10% (v/v) β-mercaptoethanol (Merck) for 5 min at 95°C. For immunoprecipitation analysis, lysates were incubated with 30 µl pre-equilibrated affinity matrix that was additionally blocked with 1 mg/ml BSA (Fisher Scientific, Pittsburgh, PA). The affinity matrixes used in this study were EzView™ Red anti-HA affinity gel.
(Sigma-Aldrich), EzViewTM Red anti-Flag affinity gel (Sigma-Aldrich) and EzViewTM Red protein G affinity gel (Sigma-Aldrich). The latter one was used in combination with 8 µg of the respective primary antibody. After incubation overnight at 4°C, the beads were washed three times with the respective lysis buffer and once with 20 mM Tris pH 7.6. After aspiration of the remaining washing buffer, beads were boiled with 2 x SDS sample buffer with a final concentration of 10% (v/v) β-mercaptoethanol (Merck) for 5 min at 95°C.

NP-40 lysis buffer
20 mM Tris (Roth) pH7.8
50 mM NaCl (Roth)
10 mM MgCl2 (Acros Organics)
1% (v/v) NP-40/Igepal® (Sigma-Aldrich)
1x protease inhibitor cocktail
(complete Mini EDTA-free, Roche, Germany)

4 x SDS sample buffer
40% Glycerol (Roth)
240 mM Tris (Roth) pH 6.8
8% SDS (Serva)
0.04% Bromophenol blue (Applichem)
10% β-mercaptoethanol (Merck)

NET-TN
50 mM Tris (Roth) pH 8
650 mM NaCl (Roth)
5 mM EDTA (Roth)
0.5% (v/v) Triton X-100 (Fluka)

NET-T
50 mM Tris (Roth) pH 8
150 mM NaCl (Roth)
5 mM EDTA (Roth)
0.5% (v/v) Triton X-100 (Fluka)

For sample preparation of HEK293T cells in Figure 35 and Figure 36 NP-40 lysis buffer was used. Cells were harvested 24 h after transient transfection and radioactive labelling. After two cycles of PBS washing the cells of one 10 cm dish were lysed in 500 µl of NP-40 lysis buffer for 30 min on ice after rigorous vortexing. The lysate was cleared at 20000 x g for 15 min at 4°C and 2 µl of each sample were measured on Luma-Plates-96 (Canberra Packard, Schwadorf, Austria) with the scintillation counter TOPcount NXT (Canberra Packard, Schwadorf, Austria) to measure the radioactive labelling efficacy. Lysates were adjusted to the same relative radioactivity and incubated with 30 µl pre-equilibrated EzViewTM Red anti-Flag affinity gel (Sigma-Aldrich) at 4°C for 3 h. In the next step, the Flag-beads were washed twice with NP-40 lysis buffer and subsequently incubated with 500 µl of NP-40 lysis buffer containing 100 µg/ml 3 x Flag-peptide(Sigma-Aldrich) for 45 min at 30°C. This step was repeated twice and the eluate was pooled. A total of 1.5 ml eluate was subsequently incubated with 30 µl pre-equilibrated EzViewTM Red Protein G affinity gel (Sigma-Aldrich)
in the presence of 8 μg monoclonal mouse anti-human FAT10 (4F1) overnight at 4°C. The beads were washed twice with 1 ml NET-TN and twice with 1 ml NET-T. After aspiration of the entire washing buffer, the beads were boiled for 5 min at 95°C in 2xSDS sample buffer with a final concentration of 10% (v/v) β-mercaptoethanol (Merck).

2.20 SDS-PAGE and immunoblot

<table>
<thead>
<tr>
<th>10 X Running buffer</th>
<th>Running buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 mM Tris (Roth) pH 8.3</td>
<td>1 x running buffer</td>
</tr>
<tr>
<td>1.92 M Glycine (Roth)</td>
<td>0.1% (w/v) SDS (Serva)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blotting buffer</th>
<th>4 x stacking gel buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x running buffer</td>
<td>0.5 M Tris pH 6.8</td>
</tr>
<tr>
<td>10% (v/v) methanol</td>
<td>0.4 % (w/v) SDS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>4 x separating gel buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M Tris pH 8.8</td>
</tr>
<tr>
<td>0.4% (w/v) SDS</td>
</tr>
</tbody>
</table>

Equal amounts of protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Mini Protean® cells (Bio-Rad) under denaturing conditions. The precise procedure has been described elsewhere (Laemmli, 1970). 10 to 15% polyacrylamide (4K solution, 30% (w/v); Applichem) gels were prepared with polymerization induction by ammoniumpersulfate (APS) and TEMED (both Sigma-Aldrich). PageRuler Prestained Protein Ladder (10-170 kDa, Thermo Scientific, Rockford, IL) was used as molecular weight marker. SDS-PAGEs were run with running buffer at 60 V for about 30 min until the samples had entered the separating gel. For sample separation, gels were run at 120 V. Subsequently, samples were transferred to Protran BA85 nitrocellulose membranes (pore size 0.45 μm, GE healthcare) with Criterion™ Blotter or Mini Trans-Blot® cells (both Bio-Rad) according to the manufacturer’s instruction for 1 h at 110V. Membranes were blocked in 1 x Roti-Block (Roth) for 1 h at room temperature and subsequently incubated at 4°C overnight with the respective antibodies listed in Table 13 which were diluted in 1 x Roti-Block (Roth). HRP-coupled secondary antibodies listed in Table 13 were likewise diluted in 1 x Roti-Block (Roth) and incubated with membranes for 1 h at room temperature. To detect specific antibody signals, SuperSignal West Pico or SuperSignal West Femto (both Thermo Scientific) developing reagent was used according to the manufacturer’s instruction.
Chemiluminescent signals were detected by the ChemiDoc system in conjunction with Quantity One Software (both Bio-Rad).

**Table 13: antibodies used for immunoblot**

<table>
<thead>
<tr>
<th>antibody</th>
<th>host</th>
<th>origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HA-HRP</td>
<td>HA-7</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Anti-Flag HRP</td>
<td>M2</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Anti-human FAT10</td>
<td>4F1</td>
<td>University of Konstanz</td>
</tr>
<tr>
<td>Anti-human USE1</td>
<td>519-1</td>
<td>(Aichem et al., 2010), University of Konstanz</td>
</tr>
<tr>
<td>Anti-human UBA6</td>
<td>PW0525</td>
<td>BioMol, Hamburg, Germany</td>
</tr>
<tr>
<td>Anti-ubiquitin</td>
<td>FK2</td>
<td>ENZO life science, Loerrach, Germany</td>
</tr>
<tr>
<td>Anti-GAPDH</td>
<td>71.1</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Anti-α tubulin</td>
<td>B-5-1-2</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Anti-human p62</td>
<td>GP62-C</td>
<td>Progen, Heidelberg, Germany</td>
</tr>
<tr>
<td>Anti-HIS HRP</td>
<td>mouse</td>
<td>Roche, Penzberg, Germany</td>
</tr>
<tr>
<td>Anti-mouse LMP7</td>
<td>mouse</td>
<td>University of Konstanz</td>
</tr>
<tr>
<td>Anti-guinea pig-HRP</td>
<td>rabbit</td>
<td>Dako, Hamburg, Germany</td>
</tr>
<tr>
<td>Anti-rabbit-HRP</td>
<td>swine</td>
<td>Dako, Hamburg, Germany</td>
</tr>
<tr>
<td>Anti-mouse-HRP</td>
<td>goat</td>
<td>Dako, Hamburg, Germany</td>
</tr>
</tbody>
</table>

**2.21 Immunohistochemistry**

<table>
<thead>
<tr>
<th>1 x PBS pH 7.4</th>
<th>washing buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>137 mM NaCl (Roth)</td>
<td>0.1 mM CaCl₂ (Roth),</td>
</tr>
<tr>
<td>8.1 mM Na₃H₂PO₄ x 2H₂O (Sigma-Aldrich)</td>
<td>1 mM MgCl₂ (Acros Organics),</td>
</tr>
<tr>
<td>1.5 mM KH₂PO₄ (Sigma-Aldrich)</td>
<td>2 mM NaN₃ (Riedel-de Haen),</td>
</tr>
<tr>
<td>2.7 mM KCl (Roth)</td>
<td>1 x PBS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>blocking buffer</th>
<th>permeabilization buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2% (v/v) fish gelatin (Sigma-Aldrich)</td>
<td>0.2% (v/v) Triton X-100 (Fluka),</td>
</tr>
<tr>
<td>in washing buffer</td>
<td>in blocking buffer</td>
</tr>
</tbody>
</table>

Cells were grown on glass cover slips (Ø13 mm; VWR, Thermo Fisher). After treatment, cells were fixed for 15 min with 4% paraformaldehyde, washed three times with washing buffer and incubated with permeabilization buffer for 10 min at room temperature. After three times
of washing cells were blocked with blocking buffer for 30 min. Immunostaining was performed with primary antibodies listed in Table 14. Cells were first labelled with primary antibodies, followed by three washing steps for 5 min, 10 min and 15 min and incubation with the respective Alexa Fluor™ labelled secondary antibodies (F(ab)_2) (dilution 1:400, Invitrogen). All antibodies were diluted in 0.2% gelatin (Sigma-Aldrich). 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, AL). Images were acquired and analyzed with a LSM 510 confocal laser-scanning microscope (Carl Zeiss, Jena, Germany) using a 63x plan-apochromat, oil-immersion objective (NA=1.4). To determine the Pearson’s coefficient, a 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. To quantify ALIS, confocal pictures were taken of random fields from cells stained for ubiquitin with the FK2 antibody, and the number of ALIS per cell was assessed by counting extranuclear, ubiquitin positive structures larger than 0.5 µm^2. The number of ALIS per cell was calculated by dividing the total area of fluorescence of all ALIS per cell by the minimum ALIS-size of 0.5 µm^2.

**Table 14: antibodies used for immunohistochemistry**

<table>
<thead>
<tr>
<th>antibody</th>
<th>host</th>
<th>origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human FAT10</td>
<td>4F1</td>
<td>mouse</td>
</tr>
<tr>
<td></td>
<td></td>
<td>University of Konstanz</td>
</tr>
<tr>
<td>Anti-human USE1</td>
<td>519-1</td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Aichem et al., 2010)</td>
</tr>
<tr>
<td>Anti-human UBA6</td>
<td>PW0525</td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BioMol, Hamburg, Germany</td>
</tr>
<tr>
<td>Anti-human p62</td>
<td>D10E10</td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cell signaling technology, Danvers, MA</td>
</tr>
<tr>
<td>Anti-human p62</td>
<td>H290</td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Santa Cruz, Heidelberg, Germany</td>
</tr>
<tr>
<td>Anti-LC3B</td>
<td>PM036</td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MBL, Woburn, MA</td>
</tr>
<tr>
<td>Anti-NDP52</td>
<td>Calcoco2</td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Anti-LPS-Salmonella</td>
<td>TS 1624</td>
<td>rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sifin, Berlin, Germany</td>
</tr>
<tr>
<td>Anti-ubiquitin</td>
<td>rabbit</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Invitrogen, Heidelberg, Germany</td>
</tr>
<tr>
<td>Anti-ubiquitin</td>
<td>FK2</td>
<td>mouse</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ENZO life science, Loerrach, Germany</td>
</tr>
<tr>
<td>Anti-rabbit-IgG (H+L) - alexa 546 (F(ab)_2)</td>
<td>goat</td>
<td>Invitrogen, Life Technologies, Darmstadt, Germany</td>
</tr>
<tr>
<td>Anti-mouse-IgG (H+L) - alexa 546 (F(ab)_2)</td>
<td>goat</td>
<td>Invitrogen, Life Technologies, Darmstadt, Germany</td>
</tr>
<tr>
<td>Anti-rabbit-IgG (H+L) - alexa 647 (F(ab)_2)</td>
<td>goat</td>
<td>Invitrogen, Life Technologies, Darmstadt, Germany</td>
</tr>
<tr>
<td>Anti-mouse-IgG (H+L) - alexa 647 (F(ab)_2)</td>
<td>goat</td>
<td>Invitrogen, Life Technologies, Darmstadt, Germany</td>
</tr>
</tbody>
</table>
2.22 Spinning disc microscopy

HeLa cells were grown in CELLview™ glass bottom dishes (Greiner bio one, Greiner, Frickenhausen, Germany) with IMDM glutamax containing 25 mM HEPES. After 24-42 h of transient transfection (FuGene HD, Roche) cells were incubated with proteasome inhibitor (MG132, 5 µM) for additional 6 h, starved in Earle's Balanced Salt Solution (EBSS, Sigma-Aldrich) for 2-3 h or left untreated. Live cell microscopy was performed with a Zeiss Cellobserver HS microscope equipped with a Zeiss Spinning Disc Scanning Unit and a Zeiss Axiocam MRm detector using a 63x plan-apochromat, oil-immersion objective (NA=1.4). An incubation chamber with temperature control was used for stable live cell conditions. Single confocal pictures or merged Z-stacks (12 slices á 0.8 µm) were analyzed for quantification of red dots per cell by ImageJ. Therefore, mCherry and EGFP positive dots (> 0.4 µm²) were quantified and the number of EGFP positive structures was subtracted from the number of mCherry positive structures to obtain the amount of mCherry single positive structures per cell.

2.23 Flow cytometry

FACS buffer
2% FCS (Gibco)
2 mM EDTA (Roth)
2 mM NaN₃ (Riedel-de Haen)
1 x PBS

2x10⁵ cells of peritoneal lavage were incubated with Fc-block solution (CD16/CD33) for 10 min at 4°C. These cells were subsequently stained in FACS buffer with the indicated antibodies diluted 1:150 for 30 min at 4°C. After two times of washing with FACS buffer, cells were analyzed by flow cytometry (Accuri C6, BD Bioscience, San Jose, CA).

Table 15: antibodies used for flow cytometry

<table>
<thead>
<tr>
<th>antibody</th>
<th>label</th>
<th>host</th>
<th>origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-F4/80</td>
<td>APC</td>
<td>rat</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Anti-CD11b (M1/70)</td>
<td>FITC</td>
<td>rat</td>
<td>eBioscience</td>
</tr>
</tbody>
</table>
2.24 Statistical analysis

Statistical analysis was performed with GraphPad Prism 4 (GraphPad Software Inc., La Jolla, USA) using unpaired student’s t-test with unequal variances, one-way ANOVA with Tukey’s multiple comparison test or log-rank curve comparison test.
3 Results

3.1 The role of FAT10 in autophagy

FAT10 co-localized with HDAC6 to aggresomes upon proteasomal inhibition (Kalveram et al., 2008). Additionally, HDAC6 was identified as a FAT10 interacting protein by mass spectrometry (Aichem et al., 2012). Most importantly, HDAC6 augmented the attention to another aggregation prone interaction partner identified by the same mass spec analysis: the autophagy adapter p62. In the following section functional aspects of this interaction were analyzed.

3.1.1 The FAT10-p62 interaction

Aichem et al. confirmed the initial mass spectrometry results by immunoprecipitation since four stable, non-reducible endogenous p62-FAT10 conjugates were detectable in cytokine treated cells (Aichem et al., 2012). A covalent p62-FAT10 conjugate was also visible in overexpression studies as well as a non-covalent interaction by GST pull down assays (Kluge, 2014, University of Konstaz). In a further step the p62 and FAT10 localization in cytokine induced HeLa cells was analyzed. Therefore HeLa cells were treated with IFN-γ and TNF-α for 24 hours and stained for endogenous FAT10 and p62 (Figure 9). Endogenous FAT10 staining co-localized with endogenous p62 staining in cytosolic dots. p62 positive inclusion bodies were detected in most cells, but co-localization with FAT10 was restricted to a minor fraction of cells only. p62 and FAT10 co-localized also in untreated control cells. The staining for p62 showed no changes upon cytokine treatment. This immunofluorescence staining confirmed the previously observed p62-FAT10 interaction in immunoprecipitation experiments. Based on these observations we hypothesized that FAT10 might be, analogous to ubiquitin, involved in p62 mediated, autophagosomal targeting of substrates.
3.1.2 Autophagosomal targeting of mCherry-EGFP-FAT10

Autophagosomal targeting of a specific protein can be monitored by tandem fluorescence (tf) tagging (Figure 10) (Pankiv et al., 2007). This tag consists of two fluorescent proteins, mCherry and EGFP. Besides differences in the fluorescence spectra and emission intensities between these two proteins, they also differ in their pH-sensitivity (Bjørkøy et al., 2009). This effect is advantageous when analyzing protein localization to cytosolic compartments, which differ in pH levels. A tf-tagged autophagosomal substrate is expressed and sequestered for autophagy within the cytosol at a neutral pH. At this stage, both fluorescence proteins emit at their characteristic wavelength and the two emitted signals co-localize. During maturation of the autophagosome into an autolysosome the compartment acidifies by fusion with lysosomal vesicles. At this stage, captured, tf-tagged proteins lose their EGFP emission and only mCherry emission remains within these structures. As recommended by the “Guidelines for the use and interpretation of assays for monitoring autophagy” (Klionsky et al., 2012) these structures can be quantified best by live-cell microscopy, since the protonation of EGFP is a reversible effect and can only be efficiently quantified in intact, unpermeabilized cells. Additionally, autolysosomes are very motile. Therefore transiently transfected, living cells were analyzed by spinning disc microscopy, a technique that allows simultaneously fast and
confocal microscopic data acquisition. As a negative control for monitoring autophagosomal flux, the tf-tag on its own was expressed. To monitor efficient autophagosomal targeting tf-p62 was used as a positive control. As an appropriate control for monitoring autophagosomal targeting of a potential substrate, tf-tagged tetra-ubiquitin (tf-4xUb) was additionally used. This fusion protein was reported to be targeted specifically to autophagosomes in earlier experiments (personal communication with T. Johansen). All three constructs as well as tf-tagged FAT10 were transiently transfected in HeLa cells plated on glass bottom dishes.

![Image of autophagic flux](#)

**Figure 10: Monitoring autophagosomal flux.** A tandem fusion construct of mCherry and GFP fused to the gene of interest (GOI) generates a pH-sensitive sensor that can be used to monitor autophagosomal targeting in living cells. The GFP tag is acid-sensitive while the mCherry tag is acid-resistant. In the neutral surrounding of the cytosol and in autophagosomes both tags emit fluorescent light resulting in yellow emitted fluorescence. Fusion of autophagosomes with late endosomes or lysosomes results in acidic amphisomes or autolysosomes where the green fluorescence from GFP is lost due to protonation in a reversible manner. Image was adapted from (Hansen and Johansen, 2011)

Transient expression of the four tf-constructs in HeLa cells showed different localization in live-cell spinning disc microscopy (Figure 11). mCherry and EGFP fluorescent signals of the tf-tag, tf-Flag-HIS-FAT10 as well as tf-4xUb were evenly distributed throughout the cytosol and the nucleus, whereas the tf-p62 expression was exclusively cytosolic, which is in line with endogenous p62 expression (Figure 9). However, endogenous FAT10 expression was less in the nucleus compared to the cytosol which was different for overexpressed tf-Flag-HIS-FAT10 in HeLa cells being evenly distributed in all compartments. Furthermore, tf-
p62 expression was barely visible as soluble protein but was mostly associated with small, cytosolic aggregates or dots. This corresponds to endogenous p62 expression (Figure 9).

The tf-Flag-HIS-FAT10 construct was furthermore tested for its non-covalent interaction with p62 to rule out steric hindrance of the large fluorescent tag. As seen in Figure 12 A the tf-Flag-HIS-FAT10 co-immunoprecipitation with HA-p62 was similarly efficient than with Flag-FAT10 confirming the persistence of interaction between tf-Flag-HIS-FAT10 and p62. To confirm the functionality of the tf-tag as a pH sensor, all three constructs were expressed in the presence and absence of the potent inhibitor of vacuolar H+ ATPases (V-ATPase) bafilomycin A1 (baf A1). Quantification of the percentage of cells that contained structures which were only positive for mCherry revealed a reduction for bafilomycin A1 treated cells,
thereby confirming the blocking of EGFP protonation by this reagent (Figure 12 B).

Figure 12: The mCherry-EGFP tag allows tracking of FAT10 into acidified compartments. (A) HeLa cells were transfected with expression plasmids for HA-p62, mCherry-EGFP-Flag-HIS-FAT10 or HIS-FLAG-FAT10 and subsequent immunoprecipitation was performed against human FAT10 (4F1). Proteins were detected using a direct horseradish peroxidase-labelled anti-HA (HA7) or anti-Flag antibody. (B) HeLa cells were transiently transfected with the tandem fluorescence tag mCherry-EGFP (tf), mCherry-EGFP-HIS-FlagFAT10 (tf-HIS-Flag-FAT10), mCherry-EGFP-4xUbiquitin (tf-4xUbiquitin) or mCherry-EGFP-p62 (tf-p62), grown in CELLview™ glass bottom dishes (Greiner bio one), and left untreated or incubated with 100 nM bafilomycin A1 for six hours. The percentage of cells that contained mCherry (red) fluorescent dots was enumerated by live-cell confocal microscopy.

When the specificity of the tool and the proper expression of all constructs were successfully tested, mCherry and EGFP positive structures per cell were quantified (Figure 13 A, C, E). The number of EGFP positive structures was subtracted from the number of mCherry positive structures to obtain the amount of mCherry single positive structures per cell (Figure 13 B, D, F). The amount of mCherry single positive structures can be used to compare the rate of autopahgosomal targeting of either different constructs or different conditions. As seen in Figure 13 B quantification of mCherry single positive structures revealed a significantly higher autophagosomal targeting rate of tf-p62 and tf-4xUbiquitin compared to the tf-tag itself. In contrast, tf-Flag-HIS-FAT10 showed no significant increase of mCherry single positive dots per cell compared to the tf-tag. To additionally investigate autophagosomal targeting of these constructs under autophagy induced conditions, the same experiments were performed with starved cells cultured in Earle's balanced salt solution (EBSS) (Figure 13 C) as well as in cells impaired in proteasomal degradation due to MG132 treatment (Figure 13 E). Proteasome inhibition was shown to increase autophagy flux by inducing autophagosomal degradation of substrate aggregates in contrast to diffuse, soluble substrates (Myeku and
Figueiredo-Pereira, 2011). For tf-Flag-HIS-FAT10 and tf-4xubiquitin as well as for the tf-tag itself an increase in mCherry single positive structures in starved cells was observed but statistics revealed the same differences for all of the three constructs compared to untreated control cells (Figure 13 D). Of note, during proteasomal inhibition the quantity of EGFP positive structures was strongly increased for all constructs (Figure 13 E). This resulted partially in negative values within the mCherry single positive dots quantification (Figure 13 F). As a result we were not able to confirm our initial hypothesis that FAT10 might function as an autophagy targeting mechanism, since we could not detect a significantly higher autophagosomal targeting of tf-Flag-HIS-FAT10 compared to the tf-tag itself. This was the case under normal as well as under autophagy induced conditions.
Figure 13: mCherry-EGFP-FAT10 is not specifically targeted to autolysosomes. HeLa cells were grown in CELLview™ glass bottom dishes (Greiner bio one) in full medium (A and B), in 5 μM MG132 for six hours (C and D) or starved in EBSS for three hours (E and F). (A, C and E) Cells were analyzed 24-48 hours after transient transfection of the tandem fluorescence tag mCherry-EGFP (tf), mCherry-EGFP-HIS-Flag-FAT10 (tf-HIS-Flag-FAT10), mCherry-EGFP-4xUbiquitin (tf-4xUbiquitin) or mCherry-EGFP-p62 (tf-p62). EGFP and mCherry positive dots per cell were quantified by spinning disc microscopy. (B, D and F) Red dots per cell were calculated by subtraction of EGFP positive dots from mCherry positive dots measured in (A, C and E). The total number of mCherry (red) and EGFP (green) dots per cell (> 0.4 μm²) was measured by ImageJ. The box plot shows median, box (25th to 75th percentiles) and whiskers (5-95%). One-way ANOVA with Tukey’s multiple comparison test, * p<0.05.
3.1.3 Autophagosomal targeting of endogenous FAT10

The overexpressed tf-Flag-HIS-FAT10 fusion protein was not specifically targeted to autophagosomes (Figure 13). With the following experiments, the initial hypothesis of FAT10 being an autophagosomal degradation signal, was further investigated by means of endogenous FAT10. FAT10 expression was induced by IFN-γ and TNF-α treatment for 24 hours in HEK293T cells which were subsequently radioactively pulse labelled. Endogenous FAT10 was immunoprecipitated after 30 minutes, 1, 2 and 4 hours to determine the degradation rate by autoradiography (Figure 14 A). When comparing cells that were treated with bafilomycin A1 and which are therefore impaired in lysosomal degradation with untreated control cells, no difference in the kinetic of FAT10 degradation was detectable by means of densitometric analysis (Figure 14 B). Additionally, impaired lysosomal acidification during 24 hours of bafilomycin A1 treatment was confirmed by increasing levels of the autophagosomal substrate p62 (Figure 14 C). The observation, that monomeric FAT10 degradation was not impaired during bafilomycin A1 treatment further strengthened the previous statement that the non-covalent FAT10-p62 interaction does not represent a general autophagy targeting mechanism.
Results

3.1.4 FAT10 as a regulator of mCherry-EGFP-p62 autophagosomal targeting

FAT10 overexpression and conjugation to p62 leads to its proteasomal degradation and was suggested to reduce thereby the excess of p62 protein subsequently to its upregulation with pro-inflammatory cytokines (Aichem et al., 2012). The kinetic of FAT10 and p62 induction after IFN-γ and TNF-α treatment analyzed in the same study supported this hypothesis. To further explore the functional relevance of this observation the tf-fluorescent tag was used. In the following experiment tf-p62 was expressed together with untagged FAT10 in HeLa cells for live-cell spinning disc microscopy (Figure 15 A). Both proteins were translated from a
single mRNA transcript, since both genes of interest were cloned in a bicistronic pIRES vector. In this system FAT10 translation is driven by conventional cap dependent translation and tf-p62 is driven by the cap independent internal ribosomal entry site (IRES). The expression of both proteins was confirmed by immunoblot (Figure 15 B) and revealed an expression level for tf-p62 that was less than endogenous p62 (data not shown).

**Figure 15: pIRES-mCherry-EGFP-62-FAT10 expression in HeLa cells.** (A) Live-cell micrographs of HeLa cells grown in CELLview™ glass bottom dishes expressing IRES driven mCherry-EGFP-p62 with (IRES tf-p62 FAT10) or without (IRES tf-p62) untagged FAT10. Scale bar 16 µm. (B) Immunoblot of total lysates of HeLa cells transfected with constructs as in (A). GAPDH served as loading control.

mCherry-EGFP double positive and mCherry single positive structures were detectable in HeLa cells comparable to the expression of tf-p62 in Figure 15 A. mCherry and EGFP positive structures were quantified by live-cell microscopy (Figure 16 A) and mCherry single positive structures (red dots per cell) were calculated to quantify tf-p62 targeting to autolysosomes in the presence or absence of FAT10 (Figure 16 B). As a result, no difference in tf-p62 containing, acidified autolysosomes could be observed. This indicates that FAT10 does not mediate a substantial reduction in targeting tf-p62 to lysosomes by proteasomal
targeting and degradation instead. As a conclusion FAT10 and tf-p62 co-expression had no measurable effect on tf-p62 autophagosomal targeting. Additionally, we can exclude the involvement of FAT10 in tf-p62 localization.

![Figure 16: FAT10 does not influence lysosomal targeting of tf-p62.](image)

Figure 16: FAT10 does not influence lysosomal targeting of tf-p62. (A) HeLa cells were grown in CELLview™ glass bottom dishes expressing IRES driven mCherry-EGFP-p62 with (IRES tf-p62 FAT10) or without (IRES tf-p62) untagged FAT10. EGFP and mCherry positive dots per cell were quantified by spinning disc microscopy. Therefore Z-stack (12 slices à 0.8 µm) were merged and dots (>0.4 µm²) were quantified with imageJ software. (B) Red dots per cell were calculated by subtraction of EGFP positive dots from mCherry positive dots measured in (A). The box plot shows median, box (25th to 75th percentiles) and whiskers (5-95%) for at least 180 cells for each condition in two independent experiments.

### 3.2 FAT10 in xenophagy

Functioning as an autophagosomal adapter, p62 has been described in several studies to be implicated in the capture of cytosolic bacteria or its remnants (Johansen and Lamark, 2011). We speculated that the induction of FAT10 expression during inflammation as well as the interaction with p62, which is involved in the elimination of pathogens, might be of functional interest. To follow this question, in vitro infection experiments with *Salmonella* were performed to investigate the potential recruitment of FAT10 to cytosolic bacteria by confocal microscopy.
3.2.1 Characterization of \textit{Salmonella} strain SHF2

A prerequisite to analyze the fate of cytosolic bacteria \textit{in vitro} is the ability to differentiate between cytosolic and vacuolar bacteria. Different markers like ubiquitin and LAMP1 have been used to distinguish these populations in immune fluorescence. A tool to precisely identify cytosol-exposed \textit{S}. Typhimurium was designed by Hesso Farhan and named Hesso Farhan \textit{Salmonella} 2 (SHF2) (Hesso Farhan, Focal Area Infection Biology, Biozentrum, University of Basel; unpublished data). HeLa cells were infected with this modified \textit{Salmonella} SL1344 strain (SHF2) that expresses GFP tagged hexose phosphate transport protein (GFP-UhpT). UhpT is a transmembrane protein within the inner cell membrane of

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure17.png}
\caption{Model of the conditional GFP expressing S. Typhimurium strain SHF2. The S. Typhimurium strain \textit{Salmonella} Hesso Farhan (SHF2) expresses GFP-UhpT under the control of glucose-6-phosphate. This glucose metabolite is exclusively present in the cytoplasm and excluded from the lumen of phagosomes, including the \textit{Salmonella} containing vacuole (SCV). Expression of GFP by SHF2 is indicative of the exposure to glucose-6-phosphate in the cytosol or within damaged SCVs. These can be sequestered in nascent autophagosomes and degraded in autolysosomes.}
\end{figure}
various Gram-negative bacteria as part of the sugar phosphate transport system and is regulated by external glucose-6-phosphate through the action of three linked regulatory genes (Island et al., 1992). Glucose-6-phosphate is exclusively present in the host cytoplasm and excluded from the lumen of endomembranes, including the SCV as depicted schematically in Figure 17. Expression of GFP in SHF2 *Salmonella* is therefore indicative of an exposure to glucose-6-phosphate. To test whether green fluorescence in SHF2 *Salmonella* indeed faithfully reports cytotoxic bacteria, HUVECs were infected with SHF2 and GFP positive bacteria were enumerated during the first three hours of infection (Figure 18 A). Up to 20% of total SHF2 induced GFP expression within that time period. In addition, ubiquitin decoration of *Salmonella* which is indicative for cytosolic exposure strongly correlated with induced GFP expression (~35%). In contrast, GFP negative SHF2 showed only a minor decoration with ubiquitin (Figure 18 B). These data are in line with another experimental approach by Knodler et al. to precisely assess cytotoxic *Salmonella* (Knodler et al., 2014). They revealed similar kinetics and absolute numbers than the quantification of GFP positive SHF2 in Figure 18 A shows. In summary, this tool can therefore be used to readily distinguish *Salmonella* in intact SCVs from those which are located in the cytosol or are found in damaged SCVs.

**Figure 18: Characterization of SHF2 during early infection.** (A) HUVECs were infected with SHF2 (MOI=100) for up to three hours, fixed and stained for total SHF2 (anti LPS). The fraction of GFP+ SHF2 was quantified by fluorescence microscopy. The average +/- SD is shown for at least 1000 bacteria analyzed for each condition in two independent experiments. (B) HUVECs were infected as in (A) and cells were fixed and stained for total SHF2 (anti LPS) and ubiquitin (FK2). The percentage of ubiquitin+/GFP+ and ubiquitin+/GFP- SHF2 was enumerated by fluorescence microscopy. The average +/- SD is shown for a total of at least 1000 bacteria analyzed for each condition in two independent experiments.
3.2.2 FAT10 decorates autophagy targeted *S. Typhimurium*

Different cell types were infected with SHF2 *Salmonella*. After one hour of infection, cells were fixed, permeabilized and stained for endogenous NDP52, p62, LC3B, FAT10 and ubiquitin (Figure 19 A, red). At this time point, a minor fraction of SHF2 already showed induced GFP-UhpT expression (Figure 19 A, blue). In HUVECs (Figure 19 A) and HeLa cells (Figure 19 B) FAT10 decoration of cytosolic, GFP expressing SHF2 was indeed visible, similar to NDP52, p62, LC3B and ubiquitin decoration, as indicated by arrow heads and magnifications in insets.
This decoration was observable one hour after infection and 24 hours after cytokine treatment. Importantly, differences in the quantity of bacterial FAT10 decoration between these two cell types were observed (data not shown). 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; Lukasiak et al., 2008;...
Raasi et al., 1999). Therefore subsequent infection experiments were performed in HUVECs. To further characterize the FAT10 decoration of cytosolic SHF2, in vitro infections were performed again for one hour and FAT10 was co-stained with either LC3B, NDP52, p62 or ubiquitin. GFP and FAT10 double positive SHF2 were quantified and further analyzed for simultaneous decoration with any of the other mentioned markers (Figure 19 C). Thereby FAT10 decoration was almost exclusively observed with bacteria that were simultaneously decorated with LC3B, NDP52, p62 or ubiquitin. Remarkably, FAT10 decorated bacteria with no autophagy adaptor or LC3B present could not be detected. In summary, a certain pool of cytosolic SHF2 was indeed decorated with FAT10. In particular, these bacteria were indeed targeted for autophagy since they were at the same time decorated with LC3B, NDP52, p62 and in most cases also with ubiquitin.

Some xenophagy targeted pathogens have evolved strategies to escape this cytosolic defense mechanism, especially the detection via ubiquitination. Therefore FAT10 decoration might represent a backup mechanism for ubiquitin to target these pathogens in particular. The Gram-positive pathogen L. monocytogenes is well known to evade autophagy and was therefore used for HUVEC infection. IFN-γ and TNF-α treated HUVECs were infected with FITC labelled L. monocytogenes for up to three hours, fixed and immunostained for FAT10 (Figure 20). However, no FAT10 decoration on these bacteria could be detected.

![Confocal micrographs of IFN-γ and TNF-α stimulated HUVECs infected with FITC labelled L. monocytogenes (Lm10403S) for the indicated time period, fixed and immunostained as indicated for FAT10 (4F1) followed by secondary antibodies goat anti-mouse-Alexa546. DAPI, 4,6-diamidino-2-phenylindole. Scale bar 10 µm.](image)

**Figure 20:** FAT10 does not decorate L. monocytogenes.
3.2.3 FAT10 localization in microdomains

Cemma et al. suggested that the decoration of cytosolic bacteria with p62 and NDP52 is not homogenous, but has the characteristics of patches (Cemma et al., 2011). Moreover, they report that in immunostainings p62 and NDP52 specific fluorescent signals are rather segregated. In addition, the recruitment of the two adaptors to cytosolic bacteria was found to be independent of each other. Similar observations have been made by Thurston et al. who found NDP52 and p62 present on segregated patches where they correlated with Galectin-8 and ubiquitin signals respectively (Thurston et al., 2012). Therefore it was investigated whether the FAT10 specific fluorescent signal also correlates with certain microdomains. To address this question co-immunostainings of SHF2 infected, IFN-γ and TNF-α treated HUVECs were performed (Figure 21 A). After one hour post infection cells were fixed, permeabilized and stained for FAT10 together with either ubiquitin, LC3B, p62 or NDP52. With confocal microscopy a heterogeneous staining for FAT10 (Figure 21 A, red) and the other markers (Figure 21 A, blue) around the GFP positive bacteria (Figure 21 A, green) was observable. In co-staining, these microdomains showed a varying degree of correlation (Figure 21 A, merge). This was additionally illustrated with the help of intensity histograms in Figure 21 B. To be able to evaluate these observations, co-localizing fluorescent signals were quantified and the Pearson’s correlation coefficient of each co-staining was calculated as shown in Figure 21 C. As a result we found the FAT10 specific fluorescent signal to co-localize best with p62 and LC3 but significantly less with NDP52. Co-localization with FAT10 and ubiquitin revealed a correlation factor higher than NDP52 but still significantly lower than p62. We therefore concluded that FAT10 is preferentially recruited to microdomains where p62 and LC3B are present.
Figure 21: Fluorescent FAT10 signals correlate with p62-positive but less with NDP52-positive microdomains. (A) Confocal micrographs of IFN-γ and TNF-α stimulated HUVECs infected with SHF2 (green) for one hour, fixed and co-immunostained as indicated for NDP52 (Calcoco2), p62 (D10E10), LC3B (PM036) and ubiquitin (rabbit mAb) (red) together with human FAT10 (4F1) (blue) followed by secondary antibodies goat anti-mouse-Alexa546 or goat anti-rabbit-Alexa647. (B) Fluorescence line scan along the white line in the merge picture of corresponding micrographs in (A) recorded with imageJ software. Value= relative intensity of fluorescent signal; Distance= white line (scale bar) in merged pictures in (A). (C) Co-localization of immunostained NDP52, p62, LC3B and ubiquitin together with FAT10 as in (A). The cooc2 plugin of imageJ software was used to calculate 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 three independent experiments. One-way ANOVA with Tukey’s multiple comparison test, * p<0.05.
3.2.4 Characterization of the FAT10 decoration on SHF2

To address the question, whether the presence of FAT10 on the bacterial surface is due to covalent conjugation or as a consequence of non-covalent interaction, we tried to *in vitro* FAT10ylate SHF2. Therefore, the recombinant proteins FAT10, HIS-UBA6 and HIS-USE1 were incubated with living SHF2 bacteria under *in vitro* FAT10ylation conditions. *In vitro* FAT10 conjugation has been successfully shown for USE1 without the addition of an E3 ligase (Aichem et al., 2010). After the completion of *in vitro* conjugation bacteria were centrifuged down and the pellet as well as the supernatant samples were subsequently boiled with (Figure 22 A and C) or without (Figure 22 B) 10% β-mercaptoethanol. All samples were separated by SDS-PAGE and analyzed by immunoblot with anti HIS-antibody (Figure 22 A and B) or anti human FAT10 antibody (Figure 22 C). Control reactions lacking bacteria or any of the other components were simultaneously performed. A HIS-USE1~FAT10 conjugate on the anti HIS immunoblot of the supernatant under non-reducing conditions was detectable in samples were all three components were present (Figure 22 B, lane 5 and 8). These HIS-USE1~FAT10 conjugates were not visible under reducing conditions, thereby confirming the successful activation of FAT10 by HIS-UBA6 and HIS-USE1. However, pellet samples analyzed under reducing conditions revealed no prominent, covalent FAT10 conjugate appearing in the *in vitro* reaction where viable SHF2 were present (Figure 22 C, lane 5) compared to the sample without SHF2 (Figure 22 C, lane 8).
To further address the same question whether the presence of FAT10 on the bacterial surface is due to covalent conjugation or as a consequence of non-covalent interaction, HUVECs were transiently transfected with either control siRNA or siRNA against the FAT10 activating and conjugating enzymes UBA6 and USE1 respectively. These cells were afterwards infected for one hour with SHF2 and the percentage of FAT10 decorated SHF2 was quantified in order to elucidate whether FAT10 decoration of SHF2 is dependent on covalent conjugation (Figure 23 A). As a control, USE1, UBA6 and FAT10 protein levels were detected by immunoblot in total lysate of siRNA treated and control cells (Figure 23 B). Several quantification experiments of FAT10 decoration on SHF2 revealed strong variances and no significant differences.

**Figure 22: FAT10 is not covalently conjugated to S. Typhimurium in vitro.** Recombinant expressed FAT10, HIS-UBA6 and HIS-USE1 were incubated with viable SHF2 for 50 minutes under in vitro FAT10ylation conditions. Bacteria were pelleted by centrifugation and the supernatant was separated on SDS-PAGE under reducing (A) and non-reducing (B) conditions, and subsequently analyzed by immunoblot for HIS-tagged proteins (HIS-HRP). (C) The bacterial pellet was lysed and separated on SDS-PAGE under reducing conditions and subsequently analyzed by immunoblot with human FAT10 antibody (4F1).
Results

To address the same question whether FAT10 is covalently or no-covalently associated with SHF2, constructs for Flag-HIS-FAT10 (Figure 24 A) and a Flag-HIS-FAT10ΔGG were used. These constructs were transfected in HUVECs in order to detect differences in FAT10 decoration on SHF2 in immunofluorescence, potentially dependent on the C-terminal diglycine motive of FAT10. HUVECs were transfected by electroporation and the transfection efficiency was enumerated by counting positive cells with fluorescence microscopy. The control pmaxGFP-plasmid (Lonza) reached transfection efficiency of about 70% (Figure 24 A). In the case of Flag-HIS-FAT10 (Figure 24 A) and Flag-HIS-FAT10ΔGG, however, only few transfected cells were detectable (~5%). Flag-FAT10 transfected HUVECs were nevertheless infected with SHF2 for one hour (Figure 24 B). No Flag-FAT10 decorated SHF2 were detectable. However, only few cells expressed Flag-FAT10, and hardly any of those simultaneously harbored GFP positive SHF2. In summary, no conclusion could be drawn from these experiments regarding a covalent or a non-covalent interaction of FAT10 with cytosolic bacteria.

Figure 23: FAT10 decoration of SHF2 in dependence of UBA6 and USE1 expression. (A) HUVECs were transfected with control siRNA, UBA6 specific siRNA or USE1 specific siRNA as indicated. 24 hours after the first transfection, cells were transfected again and stimulated with IFN-γ and TNF-α for further 24 hours. Cells were infected with SHF2 for one hour, fixed and immunostained for human FAT10 (4F1) followed by secondary antibody goat anti-mouse-Alexa546. The fraction of FAT10 positive SHF2 of at least 600 GFP+ SHF2 was enumerated by fluorescence microscopy. (B) Samples treated as in (A) but not infected were also analyzed by immunoblot. GAPDH served as loading control.

To address the same question whether FAT10 is covalently or no-covalently associated with SHF2, constructs for Flag-HIS-FAT10 (Figure 24 A) and a Flag-HIS-FAT10ΔGG were used. These constructs were transfected in HUVECs in order to detect differences in FAT10 decoration on SHF2 in immunofluorescence, potentially dependent on the C-terminal diglycine motive of FAT10. HUVECs were transfected by electroporation and the transfection efficiency was enumerated by counting positive cells with fluorescence microscopy. The control pmaxGFP-plasmid (Lonza) reached transfection efficiency of about 70% (Figure 24 A). In the case of Flag-HIS-FAT10 (Figure 24 A) and Flag-HIS-FAT10ΔGG, however, only few transfected cells were detectable (~5%). Flag-FAT10 transfected HUVECs were nevertheless infected with SHF2 for one hour (Figure 24 B). No Flag-FAT10 decorated SHF2 were detectable. However, only few cells expressed Flag-FAT10, and hardly any of those simultaneously harbored GFP positive SHF2. In summary, no conclusion could be drawn from these experiments regarding a covalent or a non-covalent interaction of FAT10 with cytosolic bacteria.
Results

3.2.5 Autophagosomal targeting and FAT10 decoration of SHF2 follow the same kinetic

Ubiquitination has been shown to be implicated in the recognition of *S. Typhimurium* in the cytosol of mammalian cells (Perrin *et al.*, 2004). However, the actual bacterial targets still remain unknown. Additionally, ubiquitin decoration has been shown to be an early event in autophagosomal targeting of *Salmonella* (Fujita *et al.*, 2013; Huett *et al.*, 2012; Perrin *et al.*, 2004; Zheng *et al.*, 2009). Similarly, the subsequent process of xenophagy has been described to be an early and transient cellular reaction (Birmingham *et al.*, 2006; Tattoli *et al.*, 2012a; Travassos *et al.*, 2010; Zheng *et al.*, 2009). Infection experiments revealed that FAT10 and ubiquitin decorate the same fraction of cytosolic bacteria (Figure 19 C). Next, it was investigated whether the two modifiers are additionally recruited to cytosolic bacteria with the same kinetic. To test this, cytokine stimulated and untreated HUVECs were infected for three hours with SHF2 and the percentage of FAT10 or ubiquitin positive cytosolic bacteria was quantified (Figure 25). Ubiquitin (50.6% +/-3.6) but no FAT10 decoration of SHF2 was
detected in control cells (Figure 25 A). Whereas the highest percentage for FAT10 (11.7% +/- 8.5) and Ubiquitin (51.05% +/- 0.6) decoration in IFN-γ and TNF-α treated cells occurred as early as one hour after infection (Figure 25 B). During the time course of three hours post infection the values decreased for both modifiers under each condition (Figure 25 A and B). Besides, similar kinetics for the autophagy adapter p62 and NDP52 as well as for LC3B were measured, as each of these markers showed the highest percentage of decoration within the first hour of infection (Figure 25).

One can therefore conclude that FAT10 and ubiquitin associate not only to the same fraction of cytosolic S. Typhimurium but also with similar kinetics. Additionally, FAT10 induction did not reduce p62 decoration of S. Typhimurium. Therefore we can exclude that the reported proteasomal degradation of the p62-FAT10 conjugate (Aichem et al., 2012) influences the level of p62 decoration on cytosolic SHF2. Instead, p62 decoration and xenophagy of cytosolic bacteria was prolonged in cytokine treated HUVECs (Figure 25 B).

Additionally, protein levels of total lysate were monitored by immunoblot. Unexpectedly, during the first hours of infection, monomeric FAT10 levels started to decline (Figure 26 A and B). In parallel, FAT10 transcript was monitored during the first seven hours post infection, revealing no significant reduction (Figure 26 C). A rescue of monomeric FAT10 by treatment with inhibitors for either proteasomal (MG132) or lysosomal (baf A1) degradation was only partially successful (Figure 26 B).
Figure 26: Monomeric FAT10 levels decrease during infection with S. Typhimurium. (A) IFN-γ and TNF-α induced HUVECs infected with S. Typhimurium for the indicated time points were left untreated, incubated with 10 µM MG132 or with 100 nM baf A1. Cells were lysed and analyzed by immunoblot for FAT10 (4F1) and p62 (GP62) protein levels. GAPDH served as a loading control. (B) Densitometric quantification of FAT10 specific immunoblot signal in (A) from three independent experiments. (C) Quantitative real-time RT-PCR of mRNA extracted from IFN-γ and TNF-α induced HUVECs which were left untreated or infected with S. Typhimurium for the indicated time points. Bar graph depicts three independent experiments. One way ANOVA with Tukey’s multiple comparison test, * p<0.05.
3.2.6 FAT10 deficiency or overexpression does not change bacterial replication in vitro

Although the percentage of FAT10 decorated SHF2 is less than for ubiquitin, it was investigated whether this might have an impact on intracellular replication of SHF2. In HUVECs, FAT10 decoration was observable with highest percentages. Hence, in a first approach, FAT10 was knocked down in cytokine treated HUVECs and bacterial replication was quantified (Figure 27 A). Intracellular replication can be determined by means of gentamicin protection assays. Therefore gentamicin is added to the cells after invasion is completed. This kills extracellular bacteria but does not harm intracellular bacterial replication. In HUVECs rapid replication of SHF2 was observable in uninduced control cells within six hours of infection. In contrast, cells that were primed with IFN-γ and TNF-α for FAT10 induction could control bacterial replication, whether they were depleted of FAT10 expression or not (Figure 27 A). The efficiency of the FAT10 siRNA targeting during infection was controlled by monitoring FAT10 protein levels at the time point of infection (Figure 27 B). Additionally FAT10 mRNA levels were analyzed by quantitative real-time RT-PCR (Figure 27 C).
When the same gentamicin protection assays were performed with wild type and FAT10 knockout murine embryonic fibroblasts (MEFs) the same outcome was observable (Figure 28 A). Also for MEFs the induction of FAT10 expression was controlled by real-time RT-PCR at the time point of infection (Figure 28 B).
Primary fibroblasts and epithelial cell lines are commonly used for in vitro infection assays, although they represent tissues with less physiological relevance. Macrophages are considered to be the principal cell type involved in the activation of the immune system by S. Typhimurium, which can invade these cells and survive within them (Schwan et al., 2000; Vazquez-Torres et al., 2000). Schwan et al. observed that bacterial survival inside tissue culture cells can offer some useful information on pathogenesis but may not always reflect what occurs in host primary macrophage cells. Therefore, the expression of FAT10 and its potential impact on bacterial replication in peritoneal macrophages was likewise investigated.

In comparison to MEFs and HUVECs, bacterial replication was rapidly blocked and macrophages reduced the bacterial load over time (Figure 29 A and B). Minor differences between cytokine primed and untreated peritoneal macrophages were detectable but less severe (Figure 29 A and B). But more important, no significant difference in bacterial cfu could be observed in FAT10 knockout compared to wild type macrophages in Figure 29 A and B. The induced expression of FAT10 in cytokine treated macrophages in comparison to untreated cells was controlled by real time RT-PCR (Figure 29 C). Additionally, specific markers for the macrophage lineage were used in FACS analysis to confirm the enrichment of macrophages from peritoneal lavage (Figure 29 D). Both preparations from C57BL/6 wild type and FAT10-deficient mice revealed about 70% F4/80 and CD11b double positive cells, therefor representing cells of the macrophage linage.

Figure 28: FAT10 deficiency in MEFs does not change bacterial replication in vitro. (A) SHF2 infected MEFs were incubated with gentamicin and subsequently lysed at indicated time points. For cfu enumeration dilutions of lysates were plated on agar plates. The average +/- SEM is shown for at least three independent experiments and triplicate colony counts. (B) Quantitative real-time RT-PCR of IFN-γ and TNF-α stimulated and untreated wild type and FAT10 KO MEFs. FAT10 expression was normalized to HPRT.
Figure 29: FAT10 deficiency in macrophages does not change bacterial replication in vitro.
SHF2 infected murine peritoneal macrophages treated with IFN-γ and TNF-α (A) or left untreated (B) were incubated with gentamicin and subsequently lysed at indicated time points. For cfu enumeration, dilutions of lysates were plated on agar plates. The average +/- SEM is shown for at least three independent experiments and triplicate colony counts. (C) Quantitative real-time RT-PCR of IFN-γ and TNF-α stimulated and untreated wild type and FAT10-deficient peritoneal macrophages. FAT10 expression was normalized to HPRT. (D) Cells obtained by peritoneal lavage were stained for CD11b and F4/80 and analyzed by flow cytometry.

To address the same question whether FAT10 influences intracellular bacterial replication without the need of cytokines, stable Flag-FAT10 overexpressing HEK293 cells were infected (Figure 30 A). Since these cells are under geneticin selection pressure and prokaryotes like SHF2 are much more sensitive to this agent than eukaryotic cells, geneticin was withdrawn three day prior to infection. Additionally, to have suitable control cells for the gentamicin protection assay we generated stable geneticin resistant HEK293 clones. Gentamicin protection assays with two Flag-FAT10 expressing (A2 and D2) and two control (B12 and F12) clones revealed no significant result but a tendency for reduced bacterial replication in the FAT10 overexpressing clones A2 and D2 compared to control clones (Figure 30 A). The
Flag-FAT10 expression levels were monitored by immunoblot to confirm persistent FAT10 expression after three days without selection pressure (Figure 30 B). In summary, primary cells of different origin with FAT10 knock down or knockout phenotypes as well as FAT10 overexpressing cells were analyzed. But no significant effect of FAT10 expression by enumeration of bacterial replication could be shown.

3.2.7 FAT10 deficiency in NRAMP1<sup>−/−</sup> mice reveals a higher susceptibility to S. Typhimurium

Experiments performed with the in vitro experimental setup could so far not reveal a significant, functional impact of FAT10 expression on intracellular Salmonella replication. To test our hypothesis in vivo, we generated FAT10 wild type and knockout NRAMP1-transgenic C57BL/6 mice and orally infected them with Salmonella SL1344. The extreme susceptibility of inbred C57BL/6 mice to different intracellular pathogens due to the expression of mutant, non-functional NRAMP1 could mask any effect of FAT10 deficiency because all infected mice die within a week. To monitor bacterial load, body weight and survival for an extended period of time, the NRAMP1 transgenic C57BL/6 Salmonella resistance mouse model (NRAMP<sup>−/−</sup>) was employed. At day 4 and 14 after infection the bacterial load in liver, mesenteric lymph nodes, spleen and cecum was enumerated by harvesting, homogenization.
and plating of organ lysates in serial dilutions on agar plates (Figure 31 A-D). The bacterial burden increased from 4 to 14 days in all tested organs. After 14 days significant differences in mesenteric lymph nodes between wild type and FAT10 knockout mice were detectable (Figure 31 B).

![Figure 31](image)

**Figure 31**: NRAMP1 transgenic FAT10 knockout mice show a higher bacterial load in mesenteric lymph nodes. Groups of 8–12 week-old male and female NRAMP1 transgenic mice were orally inoculated with $1.6 \times 10^8$ cfu *S. Typhimurium* (SL1344) in 100 µl PBS. 4 and 14 days post-infection liver (A), mesenteric lymph nodes (B), spleen (C) and cecum (D) were harvested and homogenized for cfu enumeration. Data represent two independent experiments, N=9. * p<0.05 by student’s t-test.

Additionally, three independent survival experiments with groups of 5 to 13 animals were performed. Mice were orally infected with $1.6 \times 10^8$ cfu *S. Typhimurium* (SL1344). During 30 days post infection death events and the body weight was monitored. Individual mice that lost more than 20% of body weight were sacrificed. In each of the three independent survival experiments more death events for FAT10 knockout mice than for wild type mice were
counted (Figure 32).

Figure 32: Survival curve of NRAMP1 transgenic wild type and FAT10 knockout mice after orally inoculated S. Typhimurium. Groups of 8–12 week-old male and female NRAMP1 transgenic mice were orally inoculated with $1.6 \times 10^8$ CFU S. Typhimurium (SL1344) in 100 µl PBS. (A), (B) and (C) show survival plots of three independent survival experiments with indicated group size. Death events also represent mice with body weight loss of more than 20%.

When combining all three experiments comprising 23 NRAMP1-transgenic C57BL/6 mice and 23 NRAMP1-transgenic FAT10 knockout mice a reduced, though not significant, survival rate of the FAT10 knockout mice was calculated with a p-value of 0.06 (Figure 33 A). Moreover, FAT10 knockout mice in total showed a tendency to lose more body weight than the FAT10 proficient wild type control mice (Figure 33 B). To conclude, FAT10 contributes to the resistance of mice against S. Typhimurium infection.
Results

Characterization of the pool of ULM substrates

Conjugation of ubiquitin-like modifiers to target proteins generates, similar to ubiquitin itself, characteristic conjugate “smears” when detected by immunoblot. This was shown for example for SUMO1, 2, and 3, ISG15 and also for FAT10 (Aichem et al., 2012; Becker et al., 2013; Durfee et al., 2010). A major effort during the last years concentrated on the characterization of individual target proteins of ULMs and the functional aspects of this modification. A recent study by Huibregtse and coworkers followed an alternative approach by analyzing the whole pool of proteins conjugated to ISG15. Thereby they revealed a co-translational and, most importantly, substrate independent function of ISG15. The authors could show that the

Figure 33: 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 x 10^8 cfu *S. Typhimurium* (SL1344) in 100 µl PBS. (A) Survival plots of three combined survival experiments depicted in Figure 32. Death events also represent mice with body weight loss of more than 20%. * p<0.05 by log-rank curve comparison test. (B) Bodyweight curve of N=23 mice monitored during the 30 day survival experiment in (A).

3.3 Characterization of the pool of ULM substrates

Conjugation of ubiquitin-like modifiers to target proteins generates, similar to ubiquitin itself, characteristic conjugate “smears” when detected by immunoblot. This was shown for example for SUMO1, 2, and 3, ISG15 and also for FAT10 (Aichem et al., 2012; Becker et al., 2013; Durfee et al., 2010). A major effort during the last years concentrated on the characterization of individual target proteins of ULMs and the functional aspects of this modification. A recent study by Huibregtse and coworkers followed an alternative approach by analyzing the whole pool of proteins conjugated to ISG15. Thereby they revealed a co-translational and, most importantly, substrate independent function of ISG15. The authors could show that the
ISGylation machinery shows a preference for newly translated proteins in general as substrates for conjugation (Durfee et al., 2010). In the case of FAT10, no such investigation has been performed so far. FAT10 can in principle be conjugated to hundreds of substrates in different cell lines. Interestingly, proteomic approaches identified substrates of diverse biological categories, without functional or structural similarity. As FAT10 is expressed in most tissues only after cytokine treatment and serves as a proteasomal targeting molecule it is tempting to speculate whether FAT10 might be involved in the degradation of newly translated proteins, which are suggested to be a major source of antigen processing and presentation (Yewdell and Nicchitta, 2006). Other antigen specific approaches to address this question revealed so far no evidence for this hypothesis. Nevertheless, characterizing the pool of proteins conjugated to FAT10 with regard to their half-life might give an epitope independent answer to this question.

3.3.1 The pool of FAT10 substrates

To address the question whether FAT10 is preferentially conjugated to newly translated proteins, radioactive pulse labelling experiments with $^{35}\text{S}$-cysteine in HEK293T cells were performed. To assure that the radioactive signal is incorporated in substrates only but not in FAT10 itself, the cysteine-less FAT10 mutant HIS-Flag-FAT10-C0-GG was generated. This construct was transiently transfected in HEK293T cells. The labelling was performed eight hours after transfection, when no FAT10 conjugation took place yet. At the time point of harvest, the labelled proteins in this early labelled sample (E) represent mostly stable proteins. A second sample (L) was labelled 22 hours after transfection, right before harvest and therefore it contained labelled proteins that were newly translated, including DRiPs (Figure 34). As controls, untransfected samples were processed along to monitor general unspecific binding as well as the cysteine- and diglycine-less FAT10 mutant HIS-Flag-FAT10-C0-AV to be able to discriminate between covalent substrates and non-covalent binding to FAT10.
Results

In a first approach FAT10-conjugates were immunoprecipitated by an anti-Flag immunoprecipitation (data not shown). In this experiment no signal over background was detectable in HIS-Flag-FAT10-C0-GG transfected samples. FAT10 overexpression results in a much higher ratio of ULM monomer to conjugate compared to ubiquitin or ISG15. Therefore conjugate purification is less efficient as the excess of FAT10 monomer covers the binding capacity of the matrix with the same ratio. To optimize the “signal to noise” ratio we extended the procedure by a second immunoprecipitation to gain a higher yield and purity of FAT10 conjugates. Therefore the conjugates bound to the anti-Flag-beads were eluted three times with 3xFlag peptide and a second immunoprecipitation against human FAT10 (4F1) was performed. With this setup labelled FAT10 conjugates separated on SDS-PAGE could be successfully visualized by autoradiography (Figure 35 A, lane 2 and 4). Importantly, the autoradiographic signal represented covalent conjugates, as the HIS-Flag-FAT10-C0-AV sample showed no signal over background (Figure 35 A, lane 5). This FAT10 conjugate “smear” was quantified by densitometric analysis and normalized to load signals (Figure 35 A, lane 6 to 10). Two individual experiments showed no difference between the early (E) and late (L) labelled samples (Figure 35 C) indicating that the FAT10 conjugation machinery does not preferentially target newly translated proteins. To control, whether FAT10 conjugation took place in both samples with similar efficiency, immunoblot analysis of the immunoprecipitated samples were additionally performed (Figure 35 B). E- and L-samples contained comparable amounts of Flag-FAT10-C0-GG conjugates (Figure 35 B, lane 2 and 4) and no conjugates were detectable, as expected, in the Flag-FAT10-C0-AV transfected sample (Figure 35 B, lane 5).

Figure 34: Schematic illustration of the \(^{35}\)S-cystein labelling. HEK293T cells were transiently transfected with HIS-Flag-FAT10-C0-GG/AV or with HA-ubiquitin-GG/GA. Cells were either labelled early (8 hours) or late (22 hours) after transfection and lysed for subsequent immunoprecipitation followed by autoradiography or immunoblot analysis.
Figure 35: FAT10 is not preferentially conjugated to newly translated proteins. HEK293T cells were transiently transfected with HIS-Flag-FAT10-C0-GG or HIS-Flag-FAT10-C0-AV, labelled with $^{35}$S-cysteine at the indicated time points and lysed 24 hours after transfection. Load samples were taken and a first immunoprecipitation was performed with anti-Flag beads. After elution with 3xFlag peptide a second immunoprecipitation against human FAT10 (4F1) was performed. Immunoprecipitation and load samples were subsequently analyzed by (A) autoradiography and (B) immunoblot. (C) Autoradiographs of two independent experiments were quantified by densitometric analysis and depicted as bar graph.
3.3.2 The pool of ubiquitin substrates

Ubiquitin, a modifier which mediates degradation of the majority of short lived ribosomal products (Qian et al., 2006), was likewise tested for preferential conjugation to newly translated proteins with the same experimental setup. For this purpose pulse labelling experiments with $^{35}$S-cysteine were performed as for FAT10. But in the case of ubiquitin, no cysteine to serine mutation was necessary as wild type ubiquitin lacks cysteine in the first place. Additionally, since the ratio of transiently overexpressed HA-ubiquitin monomer to conjugate is lower than for FAT10, there was no need for two immunoprecipitations in succession. As a control untransfected samples as well as the non-conjugatable ubiquitin mutant HA-Ubiquitin-GA were used to monitor unspecific binding to the matrix and non-covalent ubiquitin binding respectively. The analysis of the ubiquitination machinery revealed, similarly to FAT10, no preferential targeting of newly translated proteins. This was visualized by the same intensity of autoradiographic signal in E- as well as L- labelled HA-ubiquitin-GG transfected and immunoprecipitated samples (Figure 36 A, lane 2 and 4). Control immunoblots showed comparable levels of ubiquitin conjugation in both E- and L- labelled HA-ubiquitin-GG samples (Figure 36 B, lane 2 and 4). Densitometric analysis of autoradiographic signals of two independent experiments revealed no differences between E- and L- samples (Figure 36 C). To conclude, there is no overall preference in ubiquitin conjugation towards newly translated proteins.
Figure 36: Ubiquitin is not preferentially conjugated to newly translated proteins. HEK293T cells were transiently transfected with HA-ubiquitin-GG or HA-ubiquitin-GA, labelled with $^{35}$S-cysteine at the indicated time points and lysed 24 hours after transfection. Load samples were taken and an anti-Flag immunoprecipitation was performed and samples were subsequently analyzed by autoradiography (A) and immunoblot (B). (C) Autoradiographs of two independent experiments were quantified by densitometric analysis and depicted as bar graph.
3.4 Analysis of immunoproteasome-dependent protein turnover

It was reported that in response to IFN-γ treatment, polyubiquitination of newly translated proteins is enhanced, accompanied by remodeling of the UPS (Seifert et al., 2010). Thereby, the authors observed an accumulation of polyubiquitin conjugates which they claim are only efficiently cleared in IP proficient cells. As a consequence they suggested that this enhanced proteolytic capacity of the IP preserves cell viability during IFN-γ induced oxidative stress. This proposed new function of the immunoproteasome raised a number of fundamental questions that are difficult to resolve with the present understanding of proteasome function. Therefore some key findings of Seifert et al. were reinvestigated.

3.4.1 Ubiquitin conjugate degradation during immunoproteasome neosynthesis

Seifert et al. reported that IFN-γ induced polyubiquitin conjugates accumulate due to a transient decrease in proteasome activity during the shift from 26S SP to IP (Seifert et al., 2010). Within this time period of up to 48 hours of IFN-γ treatment the amount of ubiquitin conjugates of total lysate from wild type and LMP7 deficient MEFs was monitored. Cell lysates were separated by SDS-PAGE and analyzed by immunoblot (Figure 37 A). Densitometric analysis of total polyubiquitin specific signal normalized to the loading control is shown in Figure 37 B for four independent experiments (three experiments were performed by Michael Basler, Biotechnology Institute Thurgau, Switzerland). With this experiment no significant differences between wild type and LMP7 deficient MEFs, with respect to ubiquitin conjugates during 48 hours of cytokine treatment, visualized by ubiquitin “smear” in immunoblot were detectable in contrast to Seifert et al.. The efficiency of IFN-γ induction as well as LMP7 deficiency by the induction or lack of expression of LMP7 in wild type and LMP7-deficient MEFs respectively was controlled by immunoblot. As seen in Figure 37 C, immunoblots of total cell lysates of both wild type and LMP7 deficient MEFs treated with IFN-γ for up to 48 hours showed LMP7 induction, as expected.
Figure 37: The amount of high molecular weight polyubiquitin conjugates does not change in response to IFN-γ. (A) Immunoblot analysis of polyubiquitin conjugates in MEFs from LMP7 knockout and C57BL/6 wild type mice at different times after exposure to IFN-γ; α-tubulin served as loading control. One representative experiment out of four independent experiments with similar outcomes is shown. (B) Bar graph showing the ubiquitin levels determined by densitometric analyses of four different immunoblot reproductions comparable to the one shown in (A); shown are the mean values ± SD obtained after normalization to α-tubulin and relative to the value for C57BL/6 wild type mice before IFN-γ stimulation (BL6 WT 0 hour). Unpaired student's t-test. * p<0.05. (C) The induction of LMP7 with 200 U/ml IFN-γ was confirmed by immunoblot analysis; α-tubulin served as loading control.
3.4.2 ALIS induction and degradation during immunoproteasome neosynthesis

Seifert et al. reported that, similar to the induction of polyubiquitin conjugates, the formation of ALIS is induced by IFN-γ. Therefore it was analyzed by immunofluorescence whether inclusions are formed differently in MEF cells generated from wild type and LMP7-deficient mice. MEF cells from C57BL/6 wild type and LMP7-deficient mice were grown on coverslips and treated with IFN-γ (200 U/ml) for up to 48 hours. Cells were fixed, permeabilized and stained for ubiquitin with an anti-ubiquitin antibody (FK2) which detects mono- and polyubiquitinated conjugates (Figure 38 A). With confocal microscopy ubiquitin positive structures were quantified by measuring the total area of extranuclear, FK2 positive structures larger than 0.5 µm². The number of ALIS per cell was calculated by dividing the total area of FK2 fluorescence of all ALIS per cell by the minimum ALIS-size of 0.5 µm². Statistical analysis was performed for three independent experiments resulting in a total of 210 analyzed cells (Figure 38 B). As reported by Seifert et al., we could also detect inclusion formation after IFN-γ treatment in both wild type and LMP7 deficient MEFs. However, in contrast to their data, we found that the number of aggregates per cell increased to the same extent in LMP7-deficient and wild type MEFs.
Figure 38: The number of ALIS per cell increased to the same extent in LMP7-deficient and wild type MEFs. (A) LMP7 knockout and wild type C57BL/6 MEFs were treated with IFN-γ for the indicated time period. Formation of ALIS was visualized after fixation by immune staining for ubiquitin (FK2) (green). Accumulation of ubiquitin positive aggregates (arrow heads) was detectable after IFN-γ exposure. Scale bar 10 µm. Pictures were analyzed with ImageJ software. (B) Statistical evaluation of ALIS per cell was performed for three independent experiments; N>210 cells. Unpaired student’s t-test. * p<0.05.
4 Discussion

No well-defined immunological function for FAT10 has been reported to date. However, FAT10 was shown to exert important pathophysiological roles as it was reported to promote obesity, aging and tumorogenesis (Canaan et al., 2014; Gao et al., 2014). Because induced FAT10 expression was found to mediate these adverse effects, it is tempting to speculate that this might be the reason for its strict regulation. The transient nature of FAT10 expression, its assumed irreversible conjugation and subsequent degradation undermines the apparent necessity of a timely and locally restricted expression. On the other hand, however, it remains the question for what reason FAT10 expression is induced in the first place that would warrant the potential risk? The expression profile of FAT10 indicates that this benefit might be of immunological relevance and might be considered as the primary function of FAT10.

4.1 Putative FAT10 associated pathways

As a member of the ULM family, FAT10 was shown to be covalently attached to a large number of cellular proteins. A reasonable approach to investigate the FAT10 function seemed to be the identification and characterization of its substrate conjugates. Several studies followed this question but failed to define a function for FAT10 on the basis of the identified substrates (Aichem et al., 2012; Leng et al., 2014; Merbl et al., 2013). Merbl et al. analyzed putative FAT10 substrates by protein microarray analysis. They suggested several molecular functions and biological processes based on some proteins that were specifically enriched in the pool of FAT10 substrates. However, one has to consider that in their experimental setup recombinant FAT10, together with its cognate E1 and E2 enzymes instead of FAT10 inducing cytokines were added. Therefore, the identified putative substrates in uninduced cells might not fully reflect the regular targets of FAT10ylation. Especially, since E3 ligases for
FAT10ylation are still not identified and one cannot exclude that, if they exist, they might be cytokine inducible as well. The approach by Aichem et al. does not bear this drawback as in this study the analyzed cells were treated with IFN-γ and TNF-α. The induced, endogenous FAT10 conjugates were enriched and analyzed by mass spectrometry (Aichem et al., 2012). During this study, hundreds of FAT10 interacting proteins were identified, including putative FAT10 substrates. Categorization of these proteins revealed the highest incidence for RNA/DNA binding proteins over cancer related proteins and E3-ligases. Though, it would be interesting to perform further in silico analysis of these data in order to potentially confirm the pathways identified by Merbl et al.. Several newly identified FAT10 interacting proteins within this mass spectrometry analysis, validated or invalidated, supported the different working hypotheses described in the results section and will be discussed.

4.2 FAT10 and cytosolic protein aggregates

FAT10 was previously found to localize to aggresomes together with ubiquitin in dependence of HDAC6 (Kalveram et al., 2008), it non-covalently and covalently interacts with p62 (Aichem et al., 2012) and co-localized with p62 in cytosolic aggregates of variable size in HeLa cells (Figure 9). Furthermore, Buchsbaum et al. similarly found that FAT10, together with one of its substrates, localized to cytosolic aggregates (Buchsbaum et al., 2012b). In contrast to these findings was the observation by Nagashima et al. who found FAT10 conjugation to be an important solubility and proteasomal targeting factor for aggregate prone polyQ huntingtin (Nagashima et al., 2011).

The question arose, what role the ubiquitin-like modifier FAT10 might play in protein aggregation and clearance. Because both TNF-α and IFN-γ strongly and synergistically up-regulate MHC class I cell surface expression and because FAT10 is encoded in the class I locus, it was previously speculated that FAT10 expression might affect the generation of MHC class I peptide ligands, thus leading to a change in MHC class I cell surface expression or antigen presentation (Raasi et al., 2001). However, no quantitative or qualitative differences for two viral epitopes and three examined MHC class I molecules dependent on FAT10 expression were detectable. A further interesting aspect is the fact that FAT10 is induced in activated DCs, which simultaneously induce the formation of DALIS (Lukasiak et al., 2008). Indeed, FAT10 and ubiquitin co-localization in DALIS was detected in human monocyte derived DCs (Richard Schregle, University of Konstanz, unpublished data). Furthermore, in a previous study, the proteasome dependent MHC class I peptide generation of a FAT10 fusion protein was described to be similar efficient than the analogous ubiquitin
fusion \textit{in vitro}. However, \textit{in vivo}, during DNA vaccination and recombinant vaccinia virus infection, differences in the immunogenicity could be revealed, since the FAT10 fusion protein gave even better CD8$^+$ T-cell activation results than the ubiquitin fusion (Schliehe \textit{et al.}, 2012). Additionally, FAT10 might have an epitope dependent effect on the T-cell repertoire since FAT10 has been shown to have an impact on thymic selection (Buerger, 2013, University of Konstanz). Michael Basler (Biotechnology Institute Thurgau, Switzerland) currently further investigates this question by comparing isolated MHC class I peptides from FAT10 overexpressing and wild type HEK293 cells by mass spectrometry. First experiments, however, revealed a reduction in the peptide repertoire comparing FAT10 overexpressing with wild type cells (unpublished data).

These observations raised the interesting question whether FAT10 might be involved in proteasomal degradation of DRiPs. These are newly synthesized, misfolded proteins that aggregate during situations of stress, impaired degradation or can be used for antigen storage in DALIS (Pierre, 2005). This hypothesis was further supported by the observed FAT10-BAG6 interaction (Aichem \textit{et al.}, 2012; Buerger, 2013, University of Konstanz). BAG6 was reported to be essential for targeting defective proteins specifically for proteasomal degradation (Minami \textit{et al.}, 2010). Interestingly, BAG6, like FAT10, has been reported to interact with the Rpn10 subunit of the 26S proteasome (Kikukawa \textit{et al.}, 2005; Rani \textit{et al.}, 2012). Thus, it was tested whether FAT10 is conjugated preferentially to newly synthesized proteins. As a result neither for FAT10 (Figure 35) nor for ubiquitin (Figure 36) a preferential conjugation to these substrates could be revealed. But both results have to be interpreted with caution. A strong autoradiographic signal in the late compared to the early labelled sample, as shown for ISG15 in the study by Durfee \textit{et al.} (Durfee \textit{et al.}, 2010), would clearly argue in favor of a preferential conjugation to newly synthesized proteins. Instead, the same intensity was detected in the early and late labelled samples as seen in Figure 35 A and Figure 36 A. This does, however, not allow the conclusion that conjugation to newly synthesized proteins is generally excluded. Therefore, no signal over background in late labelled compared to early labelled samples should be detectable. The results for HA-ubiquitin in Figure 36 A are therefore in line with existing data, showing that ubiquitination is indeed necessary for degradation of the majority of DRiPs (Qian \textit{et al.}, 2006). The results for FAT10 in Figure 35 A have to be interpreted similarly. They likewise suggest that it is not the superior function of FAT10 to be conjugated to newly translated proteins as it was shown for ISG15 (Durfee \textit{et al.}, 2010). But on the other hand, the results don’t allow the reverse interpretation that FAT10 is entirely excluded from being conjugated to DRiPs.
Furthermore, one has to consider the reduced physiological relevance of the experimental system. An important issue might be the fact that certain stimuli to induce DRiP synthesis as well as ALIS formation are missing in the first place. A possibility would be to induce the formation of DRiPs by puromycin during the two hours of late labelling, right before harvest. This would simultaneously concentrate the $^{35}$S-Cys incorporation specifically in DRiPs and not in newly translated proteins in general. On the other hand, puromycin treatment might reduce comparability of early and late labelled samples since puromycin might generally influence the rate of protein synthesis and thereby the incorporation of radioactive cysteine.

Another reason that complicates the interpretation of negative results in Figure 35 is the problem, that whenever FAT10 is expressed exogenously, the potential role of IFN-γ and TNF-α inducible factors involved in FAT10 conjugation could be of importance. Repeating the experiment for FAT10 conjugation under cytokine inducing conditions might, however, result in the conjugation of endogenous, cysteine containing and thus labelled FAT10. Therefore, this approach would not be compatible with the general experimental setup. As FAT10 and ubiquitin were shown to be not preferentially conjugated to newly translated proteins, a positive control should additionally be included. To confirm, that the experimental setup is appropriate to visualize differences in conjugation preferences in general, one could repeat the experiments with the positively reported cysteine-less ISG15.

4.3 FAT10 as a potential autophagosomal targeting mechanism

Since ubiquitin has been shown to be a targeting signal for autophagosomal degradation and FAT10 similarly interacts with the autophagosomal adapter p62, several questions concerning a potential role for FAT10 in autophagosomal targeting of p62 substrates were investigated (3.1). Encouraged by the endogenous p62-FAT10 co-localization to cytosolic aggregates (Figure 9), experiments to elucidate the potential autophagosomal targeting function of FAT10 were performed. Surprisingly, FAT10 exhibited no specific recruitment of FAT10 to autolysosomes (Figure 13). Instead, the tf-Flag-HIS-FAT10 autophagosomal targeting was significantly decreased during proteasomal inhibition. The negative values calculated for some cells in Figure 13 F might be due to the fact, that the absolute EGFP fluorescence emission is about two times higher compared to mCherry fluorescence (Bjørkøy et al., 2009). As a result, a fraction of EGFP positive structures might not be detectable over background in the mCherry fluorescence channel. Since the number of EGFP positive structures strongly increased during proteasomal inhibition this might have led to the calculated negative values. The observation that FAT10 is significantly less targeted to autolysosomes during
Discussion

Proteasomal inhibition is therefore doubtful and most probably biologically irrelevant.

Furthermore, the question, whether FAT10 is degraded in autolysosomes was addressed by radioactive pulse labelling. These experiments revealed also no impact of lysosomal degradation on FAT10 levels (Figure 14 B). In contrast, additional experiments even revealed a decrease of bulk endogenous FAT10 conjugates after bafilomycin A1 treatment (Annette Aichem, Biotechnology Institute Thurgau, Switzerland; unpublished data). Furthermore, this decrease was partially rescued by proteasome inhibition. In line with these observations was the outcome of experiments conducted with overexpressed, tagged FAT10 and p62 (Kluge, 2014, University of Konstanz). Flag-FAT10 and its conjugates co-expressed with HA-p62 were similarly reduced within five hours during cycloheximide chase experiments, independent of bafilomycin A1 treatment. As expected, proteasomal inhibition rescued FAT10 disappearance in all described experiments. In her thesis work, Kathrin Kluge could furthermore show that the PB1 domain of p62, which is essential for polymerization and effective autophagosomal targeting, is no prerequisite for the non-covalent interaction with FAT10. This indicates, that the interaction of FAT10 with p62 is independent on the aggregation capability of p62 (Kluge, 2014, University of Konstanz). Furthermore, it could be successfully shown with the same experimental set up that tf-4xUb was specifically targeted to autolysosomes (Figure 13 B). However, one has to consider the artificiality of the tf-tag detection system itself. In the case of FAT10, this might also account for the discrepancy between endogenous (Figure 9) and tf-tagged FAT10 (Figure 11, second line) localization. But differences in FAT10 localization dependent on expression tags as well as on cell types were reported earlier (1.1.1.3). Steric hindrance by the large tf-tag was excluded by confirming the persistent interaction of tf-Flag-HIS-FAT10 and HA-p62 (Figure 12 A). Still, it remains the question whether the activation and conjugation of tf-Flag-HIS-FAT10 might be impaired, resulting in less conjugation. To address this issue, an alternative approach could be the generation and analysis of FAT10-AV-mCherry-EGFP, an uncleavable, C-terminally tf-tagged FAT10 construct. Such a construct wouldn’t need activation and conjugation but would represent by itself a model substrate, though an artificial one. In summary, these data argue against a preferential role of FAT10 in autophagosomal targeting and aggrephagy, but in contrast strengthen its role in proteasomal degradation. Finally, the possibility, that FAT10 localization to p62 positive inclusions might merely be a consequence of their passive interaction cannot be excluded.

4.4 FAT10 as a potential regulator of autophagy

The observation that FAT10 can target the autophagy adaptor and regulator p62 for
proteasomal degradation (Aichem et al., 2012) raised the question whether FAT10 might be a regulator of autophagosomal degradation. Since the p62 protein level was initially shown to be regulated by autophagy (Matsumoto et al., 2011), the FAT10 modification might bear a new, UPS dependent, regulatory mechanism in autophagy in addition to the recently elucidated ubiquitination of p62 (Lee et al., 2012). Besides p62, other proteins, which are similarly involved in autophagy regulation were identified as putative FAT10 interacting proteins by mass spectrometry (Aichem et al., 2012). This is for example the regulatory-associated protein of mTOR (Raptor), an integral part of the mTORC1 complex that is activated in response to high nutrient abundance and therefore represents a negative regulator of autophagy. A crucial step in nutrient induced mTORC1 activation is its assembly at the lysosomal membrane, a process that was shown to be mediated by p62 binding to TRAF6 and to Raptor (Linares et al., 2013). The FAT10-Raptor interaction identified by mass spectrometry has so far not been confirmed by other methods. Therefore it cannot be excluded that the FAT10-Raptor is indirect and mediated by p62. The covalent as well as non-covalent interaction of p62 with FAT10 might nevertheless influence the p62 binding capability with respect to mTORC1 activation. Additionally, a further autophagy regulator, AMBRA1 that activates autophagy induction was identified as a FAT10 interacting protein. This non-covalent interaction was confirmed by Stella Ryu (Ryu, 2012, University of Konstanz), who found an accelerated degradation of Myc-Flag-tagged AMBRA1 in cells co-expressing HA-FAT10. Surprisingly, the degradation did not seem to be proteasome dependent. Altogether, the observation that FAT10 interacts with several autophagy regulators raised the question whether FAT10 might be involved in autophagy regulation. The analysis revealed, however, that FAT10 and tf-p62 co-expression resulted in no difference with regard to tf-p62 autolysosomal targeting (Figure 16 B). This indicates that FAT10 expression has first of all no influence on the autolysosomal targeting of tf-p62 and secondly also not on general autolysosomal turnover. To alternatively address the same question, whether FAT10 influences autophagy regulation, one could additionally check for differences in the autophagosomal flux. This could be monitored by visualizing the LC3-I to LC3-II conversion in HEK293 wild type and stable Flag-FAT10 expressing cells with immunoblot. Similarly, one could quantify EGFP-positive structures in stable EGFP-LC3 expressing cells in the presence or absence of FAT10 expression. As a conclusion, no role of FAT10, neither in general autophagosomal targeting nor in autophagy regulation could be determined in HeLa and HEK293T cells, thereby not supporting our original hypothesis.
4.5 FAT10 and xenophagy

Autophagy is involved in several processes directly influencing the immune system. A functional role of autophagy has been described, for example during pathogen degradation, during cytokine secretion and in inflammasome and NF-κB signaling (1.2.4.). The role of the autophagy machinery during pro-inflammatory conditions is of certain importance, since these stimuli are likewise important for FAT10 expression and consequently also for its functional relevance. To further analyze a potential role of FAT10 in autophagy, it appeared reasonable to investigate these specialized, immunological effector functions of autophagy. Thereby, the correlation between IFN-γ and TNF-α secretion during bacterial infection, the inducible expression of FAT10 and p62 in response to these cytokines, and the reported role of p62 in pathogen elimination, namely xenophagy seemed to be of interest. Furthermore, S. Typhimurium and its components like LPS have been shown to induce (D)ALIS in macrophages, DCs and non-immune cells (Canadien et al., 2005; Lelouard et al., 2002; Mesquita et al., 2012).

4.5.1 Characterization of FAT10 decoration on cytosolic S. Typhimurium

4.5.1.1 Specific FAT10 decoration of cytosolic S. Typhimurium

Xenophagy has been described for several pathogens which are used quite frequently for in vitro studies. However, many of these pathogens have evolved escape mechanism and are targeted and degraded by autophagy with lower efficiency. The autophagosomal targeting and elimination of the facultative, intracellular pathogen S. Typhimurium, however, has been studied extensively and represents a well characterized model for xenophagy. A fraction of cytosol-exposed bacteria was indeed found to be decorated by endogenous FAT10 in primary HUVECs as well as HeLa cells infected with the conditional, GFP-expressing SHF2 strain (Figure 19 A and B). An important issue denoted at this point was the characterization data of the monoclonal, human FAT10 antibody (4F1), which was used in this study (Kalveram, 2009; University of Konstanz). Birte Kalveram tested other ULMs for cross-reactivity with the 4F1 antibody and observed a weak binding to LC3. This raised the general question about the specificity of the endogenous FAT10 staining. However, several aspects argue against unspecific antibody binding of the 4F1. First of all, FAT10 decoration of SHF2 was observable almost exclusively in IFN-γ and TNF-α induced cells (Figure 25 A and B). Furthermore, the 16 kDa protein LC3 was not identified within the ~17 kDa SDS-PAGE gel slice of 4F1 enriched proteins analyzed by mass spectrometry (Aichem et al., 2012). In addition, about five times more LC3B decorated SHF2 were detectable compared to FAT10.
In co-staining many of these bacteria were heavily decorated with LC3B but did not give any signal for the 4F1 antibody. A further issue that might indicate unspecific binding of 4F1 is the lack of SHF2 that are decorated with FAT10 only but no other markers, as quantified in Figure 19 C. This can be explained by the fact that xenophagy induction takes place almost simultaneously with 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, has the length of only a few minutes. Importantly, the temporal kinetics of decoration with ubiquitin and p62 are very similar (Fujita et al., 2013). Therefore, it is possible, that as soon as a defined FAT10 decoration is detectable by confocal microscopy, adaptors are already present on the bacterial surface. As a conclusion, the FAT10 decoration of SHF2 can be considered as a specific signal. To verify the specificity of the 4F1 staining one could, however, confirm the FAT10 decoration of SHF2 with another human FAT10 specific antibody.

As already mentioned, other pathogens have evolved strategies to escape or prevent autophagic capturing. For example Listeria has been shown to be able to colonize the cytosol as it expresses effector molecules that counteract ubiquitination (Dortet et al., 2011; Yoshikawa et al., 2009). The interesting question, whether FAT10 might be an alternative tagging system for autophagy in the case of Listeria was followed, especially because pro-inflammatory cytokines have been shown to repress Listeria replication (Mostowy et al., 2011). Infection of HUVECs with this Gram-positive pathogen, however, resulted in no detectable FAT10 decoration (Figure 20). This result does not support the idea, that FAT10 might have a non-redundant function in the way, that it targets pathogens that can escape the ubiquitination machinery. It rather implicates that FAT10 decoration depends on the same cytosolic detection mechanism than ubiquitin.

4.5.1.2 Characterization of the FAT10 decoration

A remaining question in the field of autophagic clearance of Salmonella is whether ubiquitin is directly conjugated to these bacteria. The same question was addressed for FAT10. Initial in vitro FAT10ylation experiments performed with living SHF2 did not reveal any evidence for covalent conjugation of recombinant FAT10 to SHF2 (Figure 22 C, lane 5). As a positive control, USE1 activation could be shown without the addition of an E3 ligase (Figure 22 B, lane 5 and 8). However, one cannot rule out, that a specific E3 ligase is needed for substrate identification of a potential SHF2 surface protein in Figure 22 C, lane 5. An analogous experiment to analyze in vitro ubiquitin conjugation was published in a study by Huett et al.
who identified the E3 ligase LRSAM1 to mediate the ubiquitination of S. Typhimurium and other Gram-negative and -positive bacteria (Huett et al., 2012). A second E3 ligase, parkin, influences ubiquitination of mycobacteria but not of S. Typhimurium, although bacterial burden of both pathogens was increased in parkin deficient worms and flies (Manzanillo et al., 2013). The role of ubiquitin E3 ligases in directly modifying bacterial targets is, however, insufficiently explored. In the case of FAT10, no direct conclusion could be drawn from the negative result in the in vitro FAT10ylation assay, as the potential involvement of E3 ligases in this process needs to be further investigated. An alternative approach to clarify the existence of covalent FAT10 conjugates with bacterial substrates might be mass spectrometry analysis. Therefore, one could isolate and enrich SHF2 from cytokine treated and infected HUVECs. If FAT10 conjugates from lysates of these bacteria could be immune-precipitated in sufficient amounts, they could be identified by mass spectrometry.

Another approach to elucidate the nature of the FAT10-SHF2 interaction was performed by infecting HUVECs overexpressing wild type Flag-FAT10 or a Flag-FAT10ΔGG mutant to allow the detection of differences in FAT10 decoration, potentially dependent on the C-terminal diglycine motive (Figure 24). In HUVECs, FAT10 overexpression reached lower transfection efficiencies than other control plasmids. The efficiency could be slightly increased by fixing cells at earlier time points (data not shown). This indicates that FAT10 might lead to a reduction in cell viability and therefore negatively influences the transfection efficiency. Difficulties with cell viability after transient FAT10 expression or when generating stable FAT10 expressing cell lines have been reported earlier (Liu et al., 1999; Merbl et al., 2013; Raasi et al., 1999). However, inhibition of FAT10 transfected cells with caspase inhibitors did not increase the electroporation efficiency (data not shown). Furthermore, individual cells that managed to overexpress Flag-HIS-FAT10 showed an unexpected localization of wild type FAT10 in nuclear speckles, which resembled the nucleolus (Figure 24 A and B). A similar localization for FAT10 was observed by members of the group of Terje Johansen, when endogenous FAT10 was stained in HeLa cells treated with leptomycin B, an inhibitor of the nuclear export machinery (Terje Johansen, University of Tromsø; personal communication). The problem of transfection efficiencies and unexpected expression patterns was further exacerbated by the fact that the rare Flag-HIS-FAT10 expressing cells were infected less efficiently compared to untransfected cells. In summary, no Flag-HIS-FAT10 decorated SHF2 were detectable. Therefore, no evidence for diglycine dependent FAT10 decoration of SHF2 could be revealed. Furthermore, one can again not exclude the necessity of an E3 ligase or other factors involved in FAT10 decoration of SHF2
which might be cytokine inducible.

To further address the same question, whether FAT10 is covalently or non-covalently attached to cytosolic bacteria, a second, but indirect approach was chosen. By knocking down the FAT10 conjugation machinery, namely UBA6 and USE1, we expected to gain evidence, whether FAT10 needs to be conjugated to a substrate to localize around bacteria or whether it is recruited non-covalently. The knockdown of both enzymes was successful, though in each of three experiments variations in the level of FAT10 expression in immunoblot analysis were detected (Figure 23 B). Due to these controls, it seemed problematic to interpret the quantification of FAT10 decoration, since varying amounts of FAT10 expression in individual samples could affect the quantitative analysis of decoration (Figure 23 A). Reduced levels of monomeric FAT10 after siRNA treatment have been observed previously (Aichem et al., 2012; Rani et al., 2012). A possibility to draw a conclusion from this data would be to normalize the quantification of SHF2 decoration to FAT10 mRNA levels, like Rani et al. did (Rani et al., 2012). Alternatively, one could normalize the quantified decoration directly to the FAT10 protein levels detected in the corresponding immunoblot shown in Figure 23 B. However, normalization might thereby lead to a higher rate of FAT10 decoration on SHF2 in cells treated with UBA6 and USE1 siRNA compared to untreated cells. This interpretation of the results would contradict the current knowledge about FAT10 and its conjugation machinery. In general, one has to be careful when interpreting effects on the basis of FAT10 levels, as translational regulation might influence the protein level of especially short lived proteins such as FAT10. An explanation for varying FAT10 protein levels might be sequence independent off-target effects of siRNA treatment, like the induction of signaling events affecting translation. siRNA is an established and specific tool for gene silencing (Caplen et al., 2001; Elbashir et al., 2001). Though, sequence independent off-target effects like TLR and dsRNA dependent protein kinase (PKR) signaling in response to siRNA treatment have been reported (Karikó et al., 2004; Persengiev et al., 2004; Sledz and Williams, 2004). An alternative explanation might be the fact that cells expressing high amounts of FAT10 have been shown to be prone to apoptosis (Raasi et al., 2001). Low FAT10 expression might therefore be a prerequisite to survive the cellular stress during siRNA knock down experiments.

Reduced levels of FAT10 decoration on SHF2 as a consequence of knocking down the conjugation machinery would have revealed indirect evidence that FAT10 is directly conjugated to SHF2. Additionally, such a result could likewise have indicated that FAT10 decoration on SHF2 represents FAT10-p62 conjugates. However, this approach would not
Discussion

give any answer to the question, whether FAT10-conjugates are recruited to cytosolic SHF2 non-covalently via p62. In the case of bulk ubiquitin conjugates such an observation was made by Ponpuak et al., who described their recruitment to autolysosomes in macrophages where they were proteolytically converted into products, capable of killing M. tuberculosis (Ponpuak et al., 2010). These substrates are targeted to autophagosomes in a p62 dependent manner, where antimicrobial peptides are liberated by lysosomal enzymes and subsequently delivered to pathogen containing phagosomes. A similar mechanism is thinkable for FAT10 and SHF2 infected cells, though the described active peptides in ubiquitin are not conserved in FAT10 (Foss et al., 2012). Nevertheless, as an initial experiment, one could digest recombinant FAT10 or bulk endogenous FAT10-conjugates by lysosomal enzymes and test the antimicrobialicidal activity of the produced peptides in an in vitro killing assay similar to KEPPA (in vitro killing assay with extracts from phagosomes purified from cells induced for autophagy) performed by Ponpuak et al. (Ponpuak et al., 2010). Additionally, one could knock down p62 in FAT10 expressing HUVECs and quantify the decoration of SHF2 in order to elucidate a non-covalent targeting mechanism of FAT10 conjugates to autophagocytosed SHF2. Also the quantification of ubiquitin decoration on SHF2 in dependence of p62 could be of interest, since the same approach revealed significantly less ubiquitin co-localization with M. tuberculosis in p62 siRNA treated RAW264.7 macrophages (Ponpuak et al., 2010). In summary, several approaches could not clarify the important issue whether FAT10 localizes to SHF2 in a non-covalent or covalent manner. However, both possibilities are of essential interest and need further clarification.

4.5.1.3 Qualitative aspects of the FAT10 decoration

To gain further insight into the characteristics of FAT10 decoration on SHF2, the precise staining pattern and co-localization of FAT10 with other markers was analyzed. Since FAT10 was reported to interact with p62 (Aichem et al., 2012), it was expected to associate with p62-positive microdomains on SHF2. Indeed, FAT10 signals were found to correlate best with LC3B and p62 positive domains and the correlation was significantly reduced for ubiquitin and NDP52 signals (Figure 21 B and C). This might indicate that FAT10 can partially replace ubiquitin in certain microdomains, which might therefore be functionally distinct. More important, the significantly lower correlation of FAT10 signals with NDP52 compared to p62 positive domains implicated that FAT10 is probably not involved in sensing damaged host membranes by the NDP52-Galectin-8 axis. It rather implicated a role in cargo recognition via the p62-ubiquitin axis. In general one has to be careful when analyzing microdomains, as these structures have been suggested to be dynamic in their composition during the time
course of infection (Thurston et al., 2012). NDP52 is reported to be recruited by Galectin-8 in an early phase to damaged SCVs. But also Galectin-8 binding-deficient NDP52 is recruited to bacteria, thus only at later time points and thereby in a ubiquitin dependent manner. In contrast, no early NDP52 recruitment followed by a delayed ubiquitin and probably also delayed p62 recruitment at later stages of infection was observable (Figure 25). In HUVECs, both signaling events appeared at a maximum already one hour after infection and decoration with NDP52 as well as p62 declined with similar kinetics. A similar kinetic of an early peak within the first two hours of infection has also been reported for p62 (Cemma et al., 2011; Zheng et al., 2009), NDP52 (Huett et al., 2012; Tattoli et al., 2012a; Tattoli et al., 2012b), ubiquitin (Huett et al., 2012; Perrin et al., 2004) and LC3 (Birmingham et al., 2006; Tattoli et al., 2012a). In contrast, a prolonged ubiquitin decoration (Birmingham et al., 2006; Tattoli et al., 2012b), p62 decoration (Tattoli et al., 2012b) and NDP52 decoration (Thurston et al., 2012) have been reported in some of these studies. Taken together, xenophagy quantification of intracellular Salmonella seems to be highly divergent throughout literature. It should be noted, however, that we counted only S. Typhimurium that had contact with cytosol and therefore expressed GFP, in contrast to other studies, where markers like ubiquitin and LAMP1 were used instead. To conclude, the co-localization analysis of FAT10 and other markers within microdomains of the bacterial coat are nevertheless conclusive, since the analysis was performed at one hour post infection. At this time point, NDP52 recruitment is clearly dependent on Galectin-8 and is not supposed to interfere with ubiquitin positive domains, regardless of the ongoing kinetic.

The functional relevance of microdomains on S. Typhimurium has not been revealed so far. It was suggested, that different ubiquitin linkages might mediate the recruitment to distinct domains, since the known adapters have shown to have different binding affinities for different ubiquitin linkages (Cemma et al., 2011). Thereby p62, NDP52 and Optineurin might recruit the different signaling complexes they coordinate into separate microdomains.

4.5.1.4 Quantitative aspects of the FAT10 decoration

It was further analyzed whether FAT10 decoration on SHF2 follows the same kinetic than the association of p62, NDP52 and LC3B. Therefore, the fraction of marker and GFP positive SHF2 was determined over the time period of three hours post infection (Figure 25). The results implicated that about 50% of cytosolic bacteria are targeted by ubiquitin, p62, NDP52 and LC3 simultaneously. From these data, however, one cannot conclude that the decorated bacterial population of a certain marker correlates with the same population of another
marker, since the correlation of p62, NDP52 and LC3B with each other was not quantified in this study. The fact that all analyzed markers followed the same kinetic supported, however, the idea that the fraction of bacteria is indeed the same for all markers. This is also in line with the observed recruitment of p62 and NDP52 at the same time to the same population of bacteria (Cemma et al., 2011). Additionally, about 75% of LC3 decorated bacteria have been shown to co-localize with p62 and NDP52 (Thurston and Ryzhakov, 2009; Zheng et al., 2009). Therefore, one can assume that also LC3B decoration in Figure 25 applies to the same population of bacteria than the decoration with autophagy adapters. Furthermore, in both studies, about 80-90% of ubiquitin decorated bacteria are already decorated with the adapter p62 and NDP52, respectively. On the other hand, Birmingham et al. reported that only about 50% of LC3 positive bacteria were positive for ubiquitin (Birmingham et al., 2006). Again, varying numbers of S. Typhimurium decoration with certain markers are published, most probably dependent on cell type, bacterial strains and variations in infection protocols. Most importantly, the correlation between FAT10 and other markers was quantified (Figure 19 C). This analysis revealed that almost 100% of FAT10 decorated bacteria were also positive for all four markers, ubiquitin, p62, NDP52 and LC3B. Furthermore, FAT10 followed the same kinetic than the other markers. In summary, this might indicate that FAT10 decoration is indeed functionally associated with xenophagy.

When kinetics of FAT10 decoration were analyzed in comparison to ubiquitin, the two autophagy adapters and LC3B, it was remarkable to find the great majority of cytosolic bacteria undetected by the host cell after several hours of infection, independent of cytokine treatment (Figure 25). It remains the question, why xenophagy decreases that fast within three hours to leave the majority of cytosolic bacteria completely unharmed. An interesting finding by Tattoli et al. has recently uncovered a mechanism of S. Typhimurium to evade autophagy. This mechanism relies not on subversion of cellular detection strategies but on favoring the normalization of metabolic stress (Tattoli et al., 2012a). They describe this metabolic stress as a host induced amino acid (AA) starvation response due to pathogen induced membrane damage. This resulted in a general induction of autophagy via a reduction in mTOR activity. Simultaneously, the binding of NDP52 and Galectin-8 ensures the coordinated recruitment of the autophagy machinery to damaged membranes in order to concentrate the induced autophagic degradation capacity to the actual scene where it is needed. In this study S. Typhimurium has been shown to subvert AA starvation induced xenophagy by reactivating the mTOR activity. However, it remains unclear whether this host defense mechanism is transient either because SCVs are damaged only at early times of infection or because the
bacterium has developed strategies to interfere with the host detection system of membrane
damage at later time points. Increasing numbers of cytosolic S. Typhimurium at later time
points were recently reported, arguing in favor of autopahgosomal evasion by
S. Typhimurium (Knodler et al., 2014; Yu et al., 2014). The GFP positive bacteria in the
present study represent either bacteria still within damaged vacuoles or bacteria that have
escaped xenophagy early after invasion and are replicating within the cytosol. To explain the
reduction of xenophagy targeting at three hours after infection, S. Typhimurium could either
reduce membrane damaging and cytosolic escape or inhibit the prolonged host induction of
xenophagy. But it remains an open question why the membrane damage independent
pathogen detection mechanism, namely ubiquitination and subsequent p62 binding, is
likewise impaired at later time points. This might be due to the specific function of NDP52
during xenophagy. It has been shown to specifically bind the LC3C paralog, which was
shown to be essential for recruitment of other LC3 proteins to the nascent phagophore
(von Muhlinen et al., 2012). Additionally, NDP52 can bind the E3 ligase LRSAM1, although
this enzyme can bind pathogens also directly via its LRR-domain (Huett et al., 2012). These
special features implicate a prerequisite function of NDP52 during xenophagy of
S. Typhimurium. On the other hand, it has been shown that the p62 and NDP52 pathways act
independently (Cemma et al., 2011). This observation contradicts the possibility that NDP52
localization with pathogens is a prerequisite for p62 recruitment. During later stages of
infection S. Typhimurium must therefore have evolved a strategy to simultaneously prevent
its identification via membrane damage mediated by Galectin-8 as well as PAMP recognition
followed by ubiquitination.

Another unexpected observation was the reduction of monomeric FAT10 levels during
infection (Figure 26 A and B). This could not be explained by a simultaneous reduction of
FAT10 messenger RNA since no significant difference was measured (Figure 26 C).
Unexpectedly, a full rescue of monomeric FAT10 by either inhibition of proteasomal
(MG132) or lysosomal (baf A1) degradation could not be observed (Figure 26 A and B).
Whether the reduction of monomeric FAT10 is specifically mediated by Salmonella or
whether a host cell specific mechanism in response to cellular stress during infection is
responsible could not be revealed. Several causes could account for a reduction in FAT10
protein levels. First, monomeric FAT10 might be reduced due to increased conjugation. This
possibility is interesting and needs further clarification. Secondly, FAT10 represents a protein
with a very short half-life of only two hours (Hipp and Kalveram, 2005; Schmidtke et al.,
2006). Therefore, interference with translation combined with an unchanged proteasomal
degradation rate might result in a rapid, though unspecific reduction of FAT10 levels. Indeed, Tattoli et al. have recently described a pathogen induced, translational arrest mediated by *S. Flexneri* and *S. Typhimurium* during infection (Tattoli et al., 2012a; Tattoli et al., 2012b). Interference with the eIF4 and eIF2 control mechanisms was reported in this study, though some of these effects were only transient in *Salmonella* infected cells compared to *Shigella* infection. Therefore, this can only partially explain the sustained reduction of monomeric FAT10. To test whether FAT10 is specifically targeted by SHF2 one could repeat the infection kinetic in HUVECs with and without chloramphenicol, a plasma membrane permeable antibiotic which prevents intracellular, bacterial replication. If FAT10 levels would still decrease to the same extent during infection one could exclude bacterial effector molecules to specifically interfere with FAT10 expression.

4.5.2 The functional role of FAT10 in xenophagy

4.5.2.1 *In vitro* infection experiments

Since FAT10 was found to associate with xenophagy targeted, intracellular bacteria, though low in quantity, the reasonable question, whether this has an impact on intracellular replication, was addressed. However, a significant impact of FAT10 on bacterial replication, tested with either overexpression or FAT10 deficient cells, could not be determined (Figure 27) (Figure 28) (Figure 29) (Figure 30). Strikingly, in these experiments, a strong impact of IFN-γ and TNF-α treatment on cfu enumeration was observed. Cytokine treatment, which is essential to induce FAT10 expression, simultaneously activated HUVECs and MEFs for efficient bacterial elimination (Figure 27 A) (Figure 28 A).

The critical role for IFN-γ and TNF-α in infectious diseases is well established (Henry et al., 2009; Jouanguy et al., 1999; Mastroeni, 2002; Monack et al., 2004; van de Vosse et al., 2009). IFN-γ is implicated in the induction of the NADPH oxidase system known as “respiratory burst”, priming for NO production and up-regulation of lysosomal enzymes that promote phagosome maturation and microbe destruction during macrophage activation (Decker et al., 2002; Vazquez-Torres et al., 2000). TNF-α enhances some of these IFN-γ induced functions (Sedgwick et al., 2000). Importantly, the induction of cell autonomous immunity allows microbicidal defense not only in professional phagocytic cells but also in most other nucleated cells (Flannagan et al., 2009; MacMicking, 2012; Nathan and Shiloh, 2000). Additionally, IFN-γ and TNF-α have been demonstrated to directly induce autophagy in phagocytic and non-phagocytic cells (reviewed in Harris, 2011). However it remains the question, to which extent autophagy contributes to the enhanced bacterial clearance in
Discussion

phagocytic and, even more strikingly, in non-phagocytic cells observed in Figure 27, Figure 28 and Figure 29. Actually, only a minor increase in ubiquitin, p62, NDP52 and LC3 decorated cytosolic S. Typhimurium could be measured in IFN-γ and TNF-α treated HUVECs during the first hours of infection (Figure 25 A and B). These observations implicate only a minor contribution of xenophagy to the overall antimicrobial capacity in cytokine induced cells. Thus, measuring only GFP positive SHF2 in Figure 25, these quantifications contain no information concerning LAP, which targets bacteria in intact SCVs. Randow and co-authors suggested this LC3 dependent mechanism as a reasonable explanation for the observed LC3 localization with intact SCVs, as seen by other groups (Boyle and Randow, 2013; Huang et al., 2009; Kageyama et al., 2011; Randow and Münz, 2012; Shahnazari et al., 2010). In conclusion, the inducing effect of pro-inflammatory cytokines on xenophagy in HUVECs seems to be rather low. It should additionally be mentioned that the infection efficiency was reduced in cytokine treated cells. Cell autonomous immunity also includes the prevention of invasion, an effect known at least for epithelial cells (Botteaux et al., 2009; Gattas et al., 2009). Therefore it is likely that the reduced invasion efficiency additionally contributed to a reduced intracellular replication of S. Typhimurium (Figure 27 A and Figure 28 A).

Within this cytokine primed cellular situation, one might face a sensitivity problem when using gentamicin protection assays. Especially, since FAT10 was shown to target only a minor proportion of cytosolic bacteria and no robust phenotype can be expected. In order to confirm the proposed sensitivity problem, one could knock down other characterized mediators of xenophagy, for example p62 or NDP52, or chemically inhibit autophagy by 3-Methyladenine (3-MA), and perform gentamicin protection assays with and without IFN-γ and TNF-α treatment. If the effect on bacterial replication would likewise be detectable only without cytokines, one could assume that this is due to other cytokine inducible, autophagy independent mechanisms. Moreover, redundancy of FAT10 and ubiquitin in xenophagy function cannot be ruled out. Ideally, one would perform these experiments in the absence of ubiquitin, but as ubiquitin is absolutely essential for cell survival this is not possible.

As an alternative for the cytokine biased experimental setup, FAT10 overexpressing HEK293 cells were chosen for gentamicin protection assays. A tendency of reduced, bacterial replication in two tested, FAT10 overexpressing clones was detectable. Though, the effect was not significant (Figure 30 A). Again, any negative results gained by FAT10 overexpression experiments might be influenced by the absence of potential cytokine inducible factors relevant for the endogenous FAT10 function. The conclusion that FAT10 has no or limited functional relevance for intracellular replication, based on these gentamicin
protection assays, is therefore debatable, due to the described experimental drawbacks.

4.5.2.2 *In vivo* infection experiments

A large body of literature exists that describes autophagy related genes in the elimination of cytosolic *S. Typhimurium* *in vitro*. But only few studies started to examine the relevance of this pathway during *S. Typhimurium* infection *in vivo* (Benjamin *et al.*, 2013; Conway *et al.*, 2013; Jia *et al.*, 2009). Choosing the right mouse model has been shown to be of particular importance, since there are considerable differences between different mouse as well as pathogen strains. Many susceptibility host genes have been identified that are important in controlling infectious disease progression (Mastroeni, 2002). Especially many inbred mouse strains like C57BL/6 and BALB/c mice have gained loss of function mutations in the chromosome 1 locus Bcg/Ity/Lsh. This locus encodes for the divalent cation transporter solute carrier family 11 member 1 (Slc11a1), also known as natural resistance-associated macrophage protein 1 (NRAMP1). Initial studies identified Slc11a1 as a risk locus for different intracellular pathogens (Bradley and Kirkley, 1977; Plant and Glynn, 1976; Skamene *et al.*, 1982; Skamene *et al.*, 1984). It is thought that Slc11a1 functions as a pH dependent divalent cation efflux pump within the phagosomal membrane, thereby restricting the access of pathogens to iron and manganese (Jabado *et al.*, 2000; Schaible and Kaufmann, 2004; Weinberg, 1992). Severe differences in the pathophysiology in mice dependent on Slc11a1 have been revealed. The immunologic function of Slc11a1 and its role in activation of macrophages and DCs has been analyzed extensively, including the up-regulation of MHC class II expression, antigen presentation (Lang *et al.*, 1997; Stober *et al.*, 2007) as well as the release of reactive oxygen and nitrogen intermediates (Barton *et al.*, 1995). Importantly, several studies emphasized a stronger pro-inflammatory cytokine response during *Salmonella* infection or DSS induced colitis as well as in *in vitro* infection experiments (Fritsche *et al.*, 2008; Jiang *et al.*, 2009; Roach *et al.*, 1991; Valdez *et al.*, 2008; Valdez *et al.*, 2009). Of special interest is the observation by Brown *et al.* who observed differences between naturally Slc11a1 expressing Sv129S6 mice and Slc11a1 transgenic C57BL/6 mice, with respect to pro-inflammatory cytokine responses (Brown *et al.*, 2013). They found the pro-inflammatory response in C57BL/6 transgenic mice was more pronounced and Th1-polarized compared to Sv129S6 mice. Therefore, a robust IFN-γ and TNF-α mediated FAT10 induction during *S. Typhimurium* infection in these Slc11a1 transgenic C57BL/6 mice can be expected.

Of interest, M-cells and adjacent follicle associated epithelial cells (FAE) express FAT10 in response to RANKL induced differentiation (Hase *et al.*, 2005; Kobayashi *et al.*, 2012). This
specialized tissue, that overlays the Peyer’s patches throughout the intestine, is a crucial entry point of invasive *Salmonella* (Jepson and Clark, 2001; Jones *et al.*, 1994). Therefore Kobayashi *et al.* have previously suggested a role for FAT10 in pathogen degradation (Kobayashi *et al.*, 2012).

Increasing systemic colonization by *S. Typhimurium* within 14 days after infection was observable with significant differences in mesenteric lymph nodes (Figure 31 A-D). This implicates, that FAT10 expression seems to have no relevant impact on bacterial replication in other tested organs *in vivo* like liver, spleen and cecum. Nevertheless, in three independent experiments, a lower survival rate for FAT10 deficient mice was observed (Figure 32). The overall onset of disease was observable around ten days after infection. This time point implicates a role for FAT10 in adaptive responses rather than in innate immunity. Since FAT10 expression is not detectable in most tissues under physiological conditions, one can expect a role for FAT10 only during a Th1 polarized, adaptive immune response, when cytokines like IFN-γ and TNF-α are secreted. During *Salmonella* infection, it was reported that Th1 cells contribute to bacterial growth restriction only three weeks after infection (Hess *et al.*, 1996; Ravindran *et al.*, 2005). This can be explained by active inhibition of the early Th1 effector response by replicating *Salmonella* dependent on SPI-2 genes *in vivo* (Srinivasan *et al.*, 2009). This inhibitory effect is on expanded effector Th1 cells rather than on the initial activation of naive T cells (Griffin and McSorley, 2011). These observations could explain why differences in the pathology of infected wild type and FAT10-deficient mice occur only within two to three weeks after infection, although the proposed function of FAT10 in xenophagy contributes to cell autonomous, innate immunity.

Of special interest is a study from Monack *et al.*. They found *S. Typhimurium* to persist in macrophages within mesenteric lymph nodes of chronically infected Slc11a1 proficient Sv129 mice. Most importantly this was dependent on IFN-γ, since IFN-γ neutralization reactivated the persistent pathogen (Monack *et al.*, 2004). The authors suggested that IFN-γ might stimulate infected macrophages to suppress bacterial replication. However, although we found increased levels of bacterial replication in MLN, no direct evidence could be revealed by means of gentamicin protection assays with wild type and FAT10 knockout primary peritoneal macrophages, indicating that FAT10 might be such an IFN-γ induced factor (Figure 29 A and B). Another study revealed a critical role of MLNs in systemic dissemination of *S. Typhimurium* (Voedisch *et al.*, 2009). They found that increased DC trafficking promotes the dissemination of *S. Typhimurium* to MLN, but not into spleen and liver. Furthermore, dissection of MLNs increased the bacterial load in liver and spleen of resistant 129Sv mice.
Thus, they concluded that confinement of S. Typhimurium in MLN delays massive extraintestinal dissemination and at the same time allows for the establishment of protective adaptive immune responses. Griffin and MacSorley conclude that, although the MLN is often considered a potential site of bacterial persistence, it actually provides an important protective function as a firewall, preventing bacterial dissemination in primary and relapsing Salmonella infection (Griffin and McSorley, 2011). FAT10 might be involved in fine tuning innate or adaptive immunity within the MLN.

The characterization of the C57BL/6 FAT10 knockout mouse by Canaan et al. revealed a higher sensitivity of these mice to low doses of endotoxin. They administered LPS from E. coli intraperitoneal and determined a lethal dose three times lower for knockout mice compared to wild type animals (Canaan et al., 2006). The authors suggest that in vivo FAT10 plays an important role in resisting sepsis from Gram-negative bacteria, as it is mediated by endotoxin. This raised the question, whether the tendency of reduced resistance observed in the present study (Figure 33 A) might be mediated by sepsis. However, oral infection with S. Typhimurium seems not likely to result in endotoxic shock from high levels of soluble LPS since it is known that S. Typhimurium resides intracellularly during infection, preferably in macrophages and DCs (Richter-Dahlfors et al., 1997; Vazquez-Torres et al., 1999; Worley et al., 2006). On the other hand, it cannot be excluded, since dissemination of two other S. enterica serovars has been reported to occur via blood and lymph extracellularly, though not in mice but in cattle (Pullinger et al., 2007). Furthermore, Canaan et al. established aging colonies of FAT10 deficient mice and wild type littermates to further investigate effects of aging on the observed LPS hypersensitivity (Canaan et al., 2006). Surprisingly, in older FAT10 knockout mice, they could document an unexpected phenotype of delayed aging and extended lifespan (Canaan et al., 2014). At the age of 12 weeks they measured significantly less body fat. They suggested a role of FAT10 in immune metabolic regulation that might have an impact on aging and chronic diseases. Canaan et al. concluded that FAT10 expression has a promoting effect on adiposity, insulin resistance, and inflammation. Furthermore, they suggested that this proposed FAT10 function might enhance survival in response to starvation or pathogen challenge. However, in an environment of over nutrition and reduced energy expenditure, FAT10 expression might represent a risk factor that promotes obesity and chronic inflammation (Canaan et al., 2014). These observations have to be taken into account when interpreting the mouse experiments shown in Figure 33. Body weight was documented to determine the progression of infection in 8-12 week old mice. At this age, differences in body fat and total body weight of FAT10 deficient and wild type mice were first documented.
by Canaan et al. (Canaan et al., 2014). Reduced body weight and the proposed systemic, anti-inflammatory milieu in FAT10 knockout mice could therefore indeed account for the observed tendencies in Figure 33. To determine whether the FAT10 deficiency itself or the infection with Salmonella is responsible for reduced body weight one could include an uninfected control group during the survival experiments.

Finally, some aspects that interfere with the generalization of the respective mouse model in the present study have to be mentioned. In rodents, expression of LC3C is lost and NDP52, which is present only during early development, has lost its Galectin-8 and ubiquitin binding sites, though it still binds to M. Tuberculosis and restricts its proliferation (Boyle and Randow, 2013; Watson et al., 2012). Furthermore, Slc11a1 determines susceptibility and resistance of mouse strains to S. Typhimurium and other intracellular pathogens, while polymorphisms at this locus do not appear to influence human susceptibility to the comparable systemic S. Typhi infection (Blackwell, 2001; Dunstan et al., 2001). In conclusion, when interpreting in vivo data concerning FAT10 deficiency phenotypes, one has to bear in mind that, although FAT10 expression is regulated the same way in mice and human, S. Typhimurium infection is obviously divergent in both species.

4.5.3 Critical aspects of xenophagy during S. Typhimurium pathology

A previous report by Huang et al. showed that knockdown of RAB1, a GTPase required for autophagy of Salmonella, slightly decreased intracellular Salmonella replication in HeLa cells (Huang et al., 2011). In line with this observation is a recent study by the group of Brett Finlay, where they challenge the current model of S. Typhimurium being eliminated by autophagy (Yu et al., 2014). They reported a beneficial effect of autophagy on intracellular bacterial replication in HeLa cells. These observations are in contrast to the current model of xenophagy representing a defense mechanism during S. Typhimurium infection. Of note, Yu et al. infected HeLa cells with a MOI=10, which is ten times less than frequently used. However, it is unclear why different results were obtained with similar experimental approaches. Similarly, Mostowy and Cossart emphasized previously the multiple evasion strategies of pathogens that allow them to evade or even to use autophagy to their own benefit. Therefore, they suggested that completion of the current understanding of bacterial autophagy in vivo is critical to exploit autophagy and its therapeutic potential (Mostowy and Cossart, 2012).

4.5.4 FAT10 and p62-signaling in response to infection

p62 is involved in several pathways activated during infection. Any p62 function, besides
autophagosomal targeting, could likewise be influenced by FAT10 conjugation or non-covalent interaction, resulting for example in steric hindrance or in accelerated proteasomal degradation.

4.5.4.1 Autophagy and the UPS in PRR signaling regulation

Bacterial infection can be detected by PAMPs like LPS via TLR4. Importantly, TLR4 is not expressed ectopically, but detectable on monocytes, macrophages, dendritic cells and several types of T-cells. However, also non-immune cells can express TLR4, as it is the case for endothelial cells like HUVECs. PRR signaling activation on endothelial cells, including TLR4 and TLR2 specific activation, are considered as an early event in innate immunity (Reed et al., 2014).

Given the crucial roles of autophagy and the innate immune system in front line defense against infection, it is no surprise that a complex cross-talk exists between the two pathways (1.2.4.2). PAMPs can trigger autophagy via TLRs and NLRs upon pathogen-derived ligand binding. On the other hand, PAMP signaling is fine-tuned by autophagy in order to prevent harmful immune responses or severe inflammation. Other regulatory mechanisms commonly involved in downstream PRR-signaling are polyubiquitinated proteins which are effectively deactivated by negative regulators, such as DUBs, or which are alternatively degraded by the proteasome. This UPS regulated, host immune signaling has been shown to be subverted by virulence factors of various pathogens. Also S. Typhimurium encodes two effector proteins, avirulence factor for Salmonella (AvrA) and Salmonella effector L (SseL) that resemble host DUBs and manipulate immune signaling after translocation into the host cytosol. Both effector proteins are able to deubiquitinate one of the key host factors in TLR-signaling, phosphorylated IκBα (Le Negrate et al., 2008; Ye et al., 2007).

The role of p62 as a signaling integrator via its interaction with several kinases, signaling molecules and ubiquitin mediated pathways was already described in 1.2.2. It was reported that p62 has an activating effect on NF-κB signaling in response to several stimuli (Durán et al., 2004; Martin et al., 2006; Sanz et al., 1999; Sanz et al., 2000). However, p62 plays also a crucial role in autophagy mediated negative regulation of immune activation and inflammation in response to PAMPS and DAMPs as described in 1.2.4.2. This includes the autophagy mediated TLR-signaling regulation where p62 and NDP52 accumulate signaling proteins like MyD88, TRAF6 and TRIF, to form cytosolic aggregates, which are ultimately degraded through selective autophagy (Inomata et al., 2012; Into et al., 2010).

p62 localization to intracellular pathogens might therefore not exclusively be the consequence
of acting as an autophagosomal adapter but it simultaneously could fulfill a signal integrating or regulatory function on the bacterial entry site. Interestingly, Dupont et al. found the NLR nucleotide-binding oligomerization domain-containing protein 1 (NOD1) bound to damaged *Shigella* containing vacuolar membranes and similarly observed TRAF6 and NEMO being recruited to these membranes. Both proteins were recruited in association with polyubiquitinated proteins (Dupont et al., 2009). It is tempting to speculate whether FAT10 might be involved in the p62 and autophagy regulation of PAMP-signaling. With respect to bacterial subversion of host signaling events, FAT10 represents a most probably irreversible and therefore attractive alternative to ubiquitin, since no FAT10 deconjugating enzymes are known so far. Additionally, FAT10 decoration was observed only for a minor fraction of GFP positive SHF2, an observation that argues against a substantial role in autophagosomal degradation. It rather implicates either a transient nature of FAT10 recruitment to *S. Typhimurium* or, alternatively, FAT10 localizes only to a fraction of cytosolic bacteria with certain, unknown characteristics. A role for FAT10 in NF-κB signaling was already described in 1.1.1.3. Most interestingly, Buchsbaum et al. confirmed a covalent interaction between FAT10 and LRRFIP2, which resulted in a negative feedback of FAT10 on NF-κB activation downstream of TLR4 (Buchsbaum et al., 2012b). Furthermore, FAT10 putatively interacts with IRAK1 (Aichem et al., 2012) and IRAK4 (Merbl et al., 2013), two essential kinases to activate the NF-κB pathway downstream of TLR and IL-1 receptor. Additionally, the non-covalent interaction of FAT10 in mass spectrometry with Toll-interacting protein (Tollip) (Aichem et al., 2012) was confirmed by Stefanie Buerger (Buerger, 2013, University of Konstanz). Tollip was identified as the human homolog of coupling of ubiquitin conjugation to ER degradation protein 5 (CUE5), a protein recently described as the first autophagosomal adapter in yeast (Lu et al., 2014). Furthermore, Tollip has been shown to inhibit IRAK1 phosphorylation and kinase activity (Zhang and Ghosh, 2002). It might be of interest to determine whether the IRAK1- and IRAK4-FAT10 non-covalent or putative covalent interaction can be confirmed by immune precipitation experiments. Furthermore it might be worthwhile to test whether LRRFIP2, IRAK1, IRAK4 or Tollip likewise decorate cytosolic SHF2.

### 4.5.5 Other putative and confirmed FAT10 interacting proteins involved in pathogen or aggregate degradation

The mechanism of FAT10 recruitment to cytosolic *Salmonella* and its functional significance remain unclear. The possibility of covalent FAT10 conjugation to SHF2 is challenged by non-covalent interactions of FAT10 with p62 and other validated or putative interaction partners.
So far, only a minor fraction of putative covalent and non-covalent FAT10 interacting proteins identified by mass spectrometry (Aichem et al., 2012) has been validated, and some of them were already discussed. Other putative interaction partners represent additional, promising and interesting, though hypothetic, discussion points.

4.5.5.1 The 26S proteasome decorates S. Typhimurium

The 26S proteasomal interaction with FAT10 via Rpn10 is robust and well characterized (Rani et al., 2012). Interestingly, proteasome recruitment to intracellular Salmonella has been demonstrated for macrophages, though not for epithelial cells (Perrin et al., 2004). A function for this observation has so far not been described. It might be of interest to see whether FAT10 co-localizes together with the 26S proteasome on cytosolic SHF2 in HUVECs. Especially, since it was reported that p62 delivers substrates to the proteasome as well (Seibenhener et al., 2004).

4.5.5.2 Autophagy and the exocyst

An interesting hit within the mass spectrometry data by Aichem et al. was the identification of several components of the exocyst complex. The subunits Sec3, Sec5, Sec8 and Sec10 were identified as FAT10 interacting proteins (Aichem et al., 2012). Additionally, proteins like 14-3-3zeta/delta, Tsg101 and moesin, which are associated with the exocyst complex in drosophila, were likewise identified, though only moesin has been validated as a FAT10 interacting protein yet (Buerger, 2013, University of Konstanz). A recent study revealed a scaffolding function of the exocyst complex for autophagy induction in response to starvation and intracellular pathogens (Bodemann et al., 2011). Different exocyst complexes have been shown to be either inducing or inhibiting, with respect to LC3 recruitment to S. Typhimurium (Ishikawa et al., 2009). This led to the suggestion by Bodemann et al. that the exocyst might represent a regulatory hub during autophagy initiation, as it can split upstream signaling events via two sub-complexes in dependence of Sec5 (Bodemann et al., 2011). They claim that this might help to support pathogen recognition and clearance. In general, immunoprecipitation of the core exocyst subunits recovers all characterized components of the exocyst complex (Grindstaff et al., 1998). FAT10 might therefore putatively interact with one subunit within the complex leading to the identification of several subunits by mass spectrometry. The potential interaction of FAT10 with Sec5 in particular, which was described to have a regulatory function during intracellular pathogen defense, provides therefore another hypothetical link for FAT10 being recruited to cytosolic SHF2. Since FAT10 was reported to target its substrates for proteasomal degradation, it might be involved
4.5.5.3 Aggregate degradation via the ESCRT complex

Aichem et al. identified two other, though not validated FAT10 interaction partners: Tumor susceptibility gene 101 protein (Tsg101) and vacuolar protein sorting-associated protein 24 (Vps24). Both proteins are known subunits of endosomal sorting complexes required for transport (ESCRT). ESCRT complexes are important for trafficking of ubiquitinated proteins from endosomes to lysosomes via multivesicular bodies (MVBs) (Williams and Urbé, 2007). Importantly, these MVBs can fuse to form a pre-autolysosomal compartment, the so-called “amphisome”, that contains both, autophagic and endocytic material (Gordon and Seglen, 1988). Filimonenko et al. found that number and size of p62-positive structures were dramatically increased in Tsg101- and Vps24-depleted cells due to a decrease in autophagosomal degradation (Filimonenko et al., 2007; Watabe et al., 2014). Of special interest is therefore the identified, but putative interaction between FAT10 and Mahogunin RING finger protein 1 (MGRN1) (Aichem et al., 2010), the E3 ligase that monoubiquitinates Tsg101 (Kim et al., 2007a). The potential influence of FAT10 induction on activity or expression levels of Tsg101 and Vps24 could represent a further hypothetical mechanism of autophagosomal regulation.

Interestingly, two mutated gene products have been identified in cytosolic aggregates of Vps24 deficient cells, which are a pathological hallmark of amyotrophic lateral sclerosis (ALS) (Watabe et al., 2014). These two proteins, transactivation response (TAR) DNA-binding protein 43 (TDP-43) and fused in sarcoma (FUS) have also been identified as putative FAT10 interacting proteins (Aichem et al., 2012). TDP-43 and FUS are RNA-binding proteins that self-associate through their prion-like domains during cellular stress (Li et al., 2013b). These induced stress granules (SGs) have been shown to be cleared by autophagy (Buchan et al., 2013) and HDAC6 was suggested to be involved in their formation (Kwon et al., 2007) (Figure 8). Although no FAT10 mediated autophagosomal targeting could be observed by means of mCherry-EGFP-tagged FAT10 expression (Figure 13), it might still be worthwhile to validate these putative interaction partners, and to further investigate a role of FAT10 in autophagosomal regulation or degradation.

4.6 Immuno- and constitutive proteasome degradation

MHC-I peptides are generated by the UPS and transported via the transporter associated with antigen processing (TAP) into the ER for loading onto nascent MHC-I molecules. Differences
in cleavage specificity, due to IFN-\(\gamma\) inducible catalytic subunits, increase the efficient generation of peptides that are preferentially transported and loaded onto MHC-I molecules. This concept was in addition corroborated by several *in vivo* studies analyzing the T-cell response in IP deficient mice (Basler *et al.*, 2006; Kincaid *et al.*, 2012; Van Kaer *et al.*, 1994).

4.6.1 Immuno- and constitutive proteasomes and the proposed differences in the proteolytic activity

A new attempt to explain the higher efficiency of IPs with regard to peptide generation was recently published (Seifert *et al.*, 2010). Seifert *et al.* suggested an enhanced proteolytic activity of IPs compared to SPs. They suggested that this leads to an increase in peptide supply for immune surveillance and simultaneously preserves protein homeostasis during cytokine induced oxidative damage. In their proposed model, they stated that IFN-\(\gamma\) induces oxidative stress, which affects most frequently newly translated proteins. As the translation was likewise induced by IFN-\(\gamma\) via mTOR activation, this led to an increase in DRiPs. As a consequence, Seifert *et al.* proposed a broad upregulation of the UPS in order to meet the enhanced degradation requirement. This includes ubiquitination as well as a higher proteolytic activity of nascent formed IPs (Seifert *et al.*, 2010). The accumulation of polyubiquitinilated proteins in response to IFN-\(\gamma\) treatment was observable for example by an increase of ubiquitin “smear” in total lysates on immunoblots and also in increasing numbers of ALIS quantified by immunofluorescence microscopy. Both effects were transient in wild type cells, but persisted in IP deficient cells. Since the accumulation of polyubiquitinilated proteins was diminished after IPs formed, the authors concluded that IPs have a higher proteolytic capacity than SPs. This new, proposed role for IPs, however, raised some concerns. Most strikingly, the question about mechanistic explanations, leading to this drastic effect, remains unanswered. Therefore several experiments performed by Seifert and coauthors were repeated with the same conditions, in order to confirm these results (Nathan *et al.*, 2013b). Furthermore, experimental setups were modified and additional experiments were performed in order to verify the proposed model.

4.6.1.1 Polyubiquitination does not change after IFN-\(\gamma\) treatment

Several human and mouse cell types showed accumulation of polyubiquitinilated proteins within eight hours of IFN-\(\gamma\) treatment analyzed by immunoblotting performed by Seifert *et al.* (Seifert *et al.*, 2010). This approach was reexamined with human HeLa cells, BALB/c-derived mouse fibroblast cells (B8) and mouse embryonic fibroblasts (MEFs) (Figure 37 A) in two different laboratories (Nathan *et al.*, 2013b). But the effect of IFN-\(\gamma\) stimulation on these
cells seen by Seifert et al. could not be reproduced. Densitometric analysis of ubiquitin signals revealed no significant increase in any cell type, including MEFs (Figure 37 B). More importantly, the lack of increased polyubiquitin conjugates resulted consequently in no detectable differences regarding conjugate degradation in wild type and IP deficient cells (Figure 37 B and C). Several technical concerns were raised by Seifert and colleagues (Ebstein et al., 2013) with regard to these experimental repetitions (Nathan et al., 2013b). First of all, the importance of cell viability was pointed out, which was claimed to be critical for detecting an increase in polyubiquitin conjugates in response to IFN-γ treatment. IFN-γ signaling induced apoptosis in wild type and LMP7 deficient murine embryonic cardiomyocytes, though this seemed to happen earlier and with higher incidence in LMP7 deficient cells (Ebstein et al., 2013). The level of apoptosis induction was not measured in parallel to the experiments performed in Figure 37. However, no noticeable morphological changes or detachment of cells was observable during IFN-γ stimulation of MEFs in Figure 37. Furthermore, induction of apoptosis was observed by Ebstein et al. at time points 72 and 96 hours of IFN-γ treatment. Though, within the first 48 hours, when polyubiquitin conjugates were analyzed, the apoptosis rate was nonetheless low (Ebstein et al., 2013). Besides, differences in lysis protocols were pointed out by Seifert and colleagues to be a reason for the undetectable increase of ubiquitin conjugates with immunoblot analysis. It was mentioned that deubiquitination and degradation during cell lysis must be counteracted (Ebstein et al., 2013). Therefore, proteasome inhibitor (MG132) and DUB inhibitor (N-ethylmaleimide, NEM) were added to the lysis buffer in addition to the combined protease inhibition cocktail (cOmplete Protease Inhibitor Cocktail Tablets, Roche) (Seifert et al., 2010). The same reagents were added to the lysis buffer used in Figure 37 A. However, three of the four experiments included in the quantification in Figure 37 B were performed by Michael Basler (Biotechnology Institute Thurgau, Switzerland) without MG132 and NEM. However, since polyubiquitin conjugates were in principle detectable with any of the lysis conditions, it remains the question why the lack of MG132 and NEM would preferentially reduce the IFN-γ induced fraction of polyubiquitin conjugates. One could rather expect that ingredients of the lysis buffer would only lead to systematic differences, apparent in reduced conjugates in any sample.

4.6.1.2 The kinetic of ALIS formation is not dependent on immunoproteasomes

After having observed a transient increase of polyubiquitin conjugates, Seifert et al. hypothesized that the conjugate degradation might be accelerated by the IP, since the incorporation of LMP7, LMP2 and MECL-1 timely coincided with the reduction in
polyubiquitin conjugates. Similar to the increase of polyubiquitin “smear” they also observed an increase of ALIS in MEFs, which were degraded in wild type cells, but persisted in LMP7 knockout cells (Seifert et al., 2010). As shown in Figure 38 A, ALIS were indeed visible by fluorescence microscopy but the number of ALIS increased for up to 48 hours in wild type and LMP7 knockout MEFs (Figure 38 B). This increase in ALIS is presumably due to accumulation of aberrant proteins through the de novo production or free radical damage. In any case, because the loss of LMP7 did not enhance inclusion formation, the SPs and IPs seem to have similar capacities to eliminate ubiquitinated proteins in cells (Nathan et al., 2013b). It was criticized by Seifert and colleagues that differences in ALIS quantification might have been missed in Figure 38 A since the quantification of ALIS in MEFs was not extended to later time points. But since the induction of LMP7, visualized by immunoblot, was almost at maximum 24 hours after IFN-γ induction (Figure 37 C), it seemed not reasonable to expect the proposed increase in proteasomal activity through IP neosynthesis with more than 24 hours delay. Additionally, the actual number of ALIS per cell in Figure 38 B was assessed by measuring the total signal of extranuclear, ubiquitin positive structures (FK2 positive) larger than 0.5 µm². The number of ALIS per cell was than calculated by dividing the total area of FK2 fluorescence of all ALIS per cell by the minimum ALIS-detection-size of 0.5 µm². These calculated values reflected the load of aggregated ubiquitin positive proteins per cell more adequately, since the size difference of ALIS in this experiment varied from the exclusion size of 0.5 µm² to full-blown aggresome like structures. The enumeration of ALIS in this case, without taking their size into account, might be misleading. In case that Seifert et al. detected ALIS with similar variances in size, it remains the possibility that Seifert et al. observed less but larger ALIS 48 hours after IFN-γ treatment in wild type cells compared to LMP7 knockout cells (Seifert et al., 2010). But this would not necessarily argue in favor of an increased IP protease activity, since the overall amount of aggregated proteins remains similar. However, since the precise method of ALIS quantification was not further explained, ALIS size variations seemed not to be an issue in the experiments performed by Seifert et al..

A further issue that may seem contradictory is the number of ALIS that increases in MEFs after IFN-γ treatment (Figure 38 B), even though the amount of polyubiquitinated proteins in total lysates remains unchanged (Figure 37 B). In Figure 37 A, the same buffer conditions as noted by Seifert et al. were used, which contained the detergent NP-40. In the additional three experiments, which were performed by Michael Basler (Biotechnology Institute Thurgau, Switzerland) for densitometric quantification in Figure 37 B, Triton X-100 was used instead
to lye the cells. However, both nonionic-detergents are not supposed to solubilize cytosolic aggregates. Therefore, it might be possible that cytosolic inclusions are detectable in response to IFN-γ by confocal microscopy but cell lysates, generated with non-ionic detergents are cleared from ALIS, and only the soluble fraction is further processed for SDS-PAGE and immunoblot. This issue was commented by Seifert et al. in the way that they corrected the announced buffer conditions used for polyubiquitin conjugates. In the method section of the initial publication the use of only nonionic detergents was documented (Seifert et al., 2010). In the subsequent correspondence, however, the need of 0.1% SDS was emphasized for detecting polyubiquitin conjugates (Ebstein et al., 2013). It remains therefore possible that the addition of 0.1% SDS during lysis allows the detection of polyubiquitinated conjugates by immunoblot. In this case, differences in the degradation rate between wild type and LMP7 deficient cells should be re-investigated, especially in combination with the supplementation of NEM and MG132 to the lysis buffer. Nevertheless, other experimental approaches like ALIS formation, in vitro peptidase activity measurements of total lysate or purified proteasome species as well as in vivo studies revealed no evidence for an accelerated degradation rate of IPs compared to SPs (Nathan et al., 2013b).

4.6.2 Mechanistic aspects of the immunoproteasome function

Some of the findings published by Seifert et al. were not fully reproducible (Figure 37) (Figure 38) (Nathan et al., 2013b). The proposed altered function of IPs in proteostasis during an ongoing immune response compared to SPs remains therefore questionable. Most strikingly, it remains an open question how, mechanistically, changes inside the CP due to the incorporation of LMP7, LMP2 and MECL-1 could influence the degradation process, including substrate recognition and processing. The binding of substrates is mainly mediated by the RP subunits Rpn10 and Rpn13, which are the same in 26S SPs and IPs (Husnjak et al., 2008; Saeki et al., 2002). In addition, the gating mechanism for processing and translocation of the unfolded substrate into the CP, which is regulated by the PA, was suggested to be similar for IPs and SPs, based on recent structural analysis (Huber et al., 2012). Huber et al., however, also suggested that peptide bond hydrolysis might be favored by an increased hydrophilicity of the active site of LMP7 in the IP. Altered peptide hydrolysis for the different subunits are well known, but it remains doubtful whether this leads to a decrease or increase in total proteasome capacity. Especially, since other peptidase activities are reduced, as it is known for the caspase-like activity of β1 due to the replacement by MECL-1 (Rock and Goldberg, 1999). It is well accepted, that IPs generate immunodominant peptides with higher efficiency and faster kinetics during infection (Deol et al., 2007). But these findings can be
Likewise explained by the well-known alterations in the quality of peptidase activity between SPs and IPs. An overall enhancement of the degradation capacity does not contribute to a better understanding of the known role of IPs in MHC-I peptide generation.

Currently, IP inhibitors are tested in preclinical studies in the treatment of rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis and cancer undermining the beneficial effect of IP inhibition (Basler et al., 2010; Basler et al., 2014; Muchamuel et al., 2009; Singh et al., 2011). On the other hand the identification of point mutations in IP subunits that are associated with specific diseases provides direct evidence that a loss in IP function can lead to severe sickness. Knowledge about the actual impact of these mutations, not only on the catalytic activity itself but also on structural changes in the CP, is of special need. Being aware of the heterogeneity of proteasome subtypes, their immunologic and non-immunologic functional differences and diverse expression profiles, further insight into functional relevance of this heterogeneous proteolytic complex will be necessary.
Tables and figures

Table 1: characterized ULMs (van der Veen and Ploegh, 2012) ....................................................... 7
Table 2: siRNA for knock down experiments ......................................................................................... 42
Table 3: primer for quantitative RT-PCR ............................................................................................... 43
Table 4: LightCycler program for huFAT10 and GAPDH ................................................................. 43
Table 5: LightCycler program for mHPRT ......................................................................................... 43
Table 6: LightCycler program for mFAT10 ......................................................................................... 44
Table 7: plasmids ............................................................................................................................... 44
Table 8: QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) ........................................................................................................ 46
Table 9: QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) .................................................................................................................... 46
Table 10: primer for genotyping .......................................................................................................... 49
Table 11: FAT10 genotyping .............................................................................................................. 50
Table 12: NRAMP1 genotyping ........................................................................................................... 50
Table 13: antibodies used for immunoblot ............................................................................................ 55
Table 14: antibodies used for immunohistochemistry .......................................................................... 56
Table 15: antibodies used for flow cytometry ..................................................................................... 57

Figure 1: Ubiquitin conjugate formation and ubiquitin linkage types. 6
Figure 2: Ribbon diagram of the resolved ubiquitin and predicted FAT10 structure. 9
Figure 3: The eukaryotic 26S proteasome holoenzyme. 16
Figure 4: Orchestration of autophagy. 23
Figure 5: Regulation of autophagy induction. 25
Figure 6: Domain structure of autophagy receptors. 27
Figure 7: Model of S. Typhimurium infection. 33
Figure 8: The multiple roles of HDAC6 in proteostasis. 38
Figure 9: Endogenous p62 co-localizes with endogenous FAT10. 60
Figure 10: Monitoring autophagosomal flux. 61
Figure 11: Expression of mCherry-EGFP tagged FAT10, ubiquitin and p62. 62
Figure 12: The mCherry-EGFP tag allows tracking of FAT10 into acidified compartments. 63
Figure 13: mCherry-EGFP-FAT10 is not specifically targeted to autolysosomes. 65
Figure 14: FAT10 is not degraded via the lysosomal degradation pathway 67
Figure 15: pIRES-mCherry-EGFP-62-FAT10 expression in HeLa cells. 68
Figure 16: FAT10 does not influence lysosomal targeting of tf-p62. 69
Figure 17: Model of the conditional GFP expressing S. Typhimurium strain SHF2. 70
Figure 18: Characterization of SHF2 during early infection. 71
Figure 19: FAT10 decorates autophagy targeted S. Typhimurium. 71
Figure 20: FAT10 does not decorate L. monocytogenes. 74
Figure 21: Fluorescent FAT10 signals correlate with p62-positive but less with NDP52-positive microdomains. 76
Figure 22: FAT10 is not covalently conjugated to S. Typhimurium in vitro. 78
Figure 23: FAT10 decoration of SHF2 in dependence of UBA6 and USE1 expression. 79
Figure 24: Exogenous FAT10 expression in HUVECs. 80
Figure 25: FAT10 decoration of S. Typhimurium and xenophagy follow the same kinetic. 81
Figure 26: Monomeric FAT10 levels decrease during infection with S. Typhimurium. 82
Figure 27: FAT10 deficiency in HUVECs does not change bacterial replication in vitro. 84
Figure 28: FAT10 deficiency in MEFs does not change bacterial replication in vitro. 85
Figure 29: FAT10 deficiency in macrophages does not change bacterial replication in vitro. 86
Figure 30: FAT10 overexpression does not significantly change bacterial replication in vitro. 87
Figure 31: NRAMP1 transgenic FAT10 knockout mice show a higher bacterial load in mesenteric lymph nodes. 88
Figure 32: Survival curve of NRAMP1 transgenic wild type and FAT10 knockout mice after orally inoculated S. Typhimurium. 89
Figure 33: NRAMP1 transgenic FAT10 knockout mice are more sensitive to orally inoculated S. Typhimurium. 90
Figure 34: Schematic illustration of the $^{35}$S-cystein labelling. 92
Figure 35: FAT10 is not preferentially conjugated to newly translated proteins. 93
Figure 36: Ubiquitin is not preferentially conjugated to newly translated proteins. 95
Figure 37: The amount of high molecular weight polyubiquitin conjugates does not change in response to IFN-γ. 97
Figure 38: The number of ALIS per cell increased to the same extent in LMP7-deficient and wild type MEFs. 99
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-MA</td>
<td>3-Methyladenine</td>
</tr>
<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>AIPL1</td>
<td>aryl hydrocarbon receptor-Interacting Protein-Like 1</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>ARE</td>
<td>antioxidant response element</td>
</tr>
<tr>
<td>Atg8</td>
<td>autophagy related gene</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CANDLE</td>
<td>chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature</td>
</tr>
<tr>
<td>CMA</td>
<td>chaperone-mediated autophagy</td>
</tr>
<tr>
<td>CP</td>
<td>core particle</td>
</tr>
<tr>
<td>Cvt</td>
<td>cytoplasm-to-vacuole targeting</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sodium sulfate</td>
</tr>
<tr>
<td>DUB</td>
<td>de-ubiquitinating enzyme</td>
</tr>
<tr>
<td>EAE</td>
<td>autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ESCRT</td>
<td>endosomal sorting complexes required for transport</td>
</tr>
<tr>
<td>FAE</td>
<td>follicle associated epithelia</td>
</tr>
<tr>
<td>FAT10</td>
<td>HLA-F locus associated transcript 10</td>
</tr>
<tr>
<td>FOXP3</td>
<td>forkhead box protein P3</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome-wide association study</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone de-acetylase</td>
</tr>
<tr>
<td>HECT</td>
<td>homologous to E6-AP carboxyl terminus</td>
</tr>
<tr>
<td>HUB-1</td>
<td>homology to UB-1</td>
</tr>
<tr>
<td>IBR</td>
<td>in between RING</td>
</tr>
<tr>
<td>IP</td>
<td>immune precipitation</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
</tr>
<tr>
<td>ISG15</td>
<td>interferon stimulated gene 15</td>
</tr>
<tr>
<td>IκBα</td>
<td>inhibitor of kappaB α</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LAP</td>
<td>LC3 associated phagocytosis</td>
</tr>
<tr>
<td>LC3</td>
<td>light chain 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LCA</td>
<td>Leber’s congenital amaurosis</td>
</tr>
<tr>
<td>LIR</td>
<td>LC3 interacting region</td>
</tr>
<tr>
<td>LMP</td>
<td>low molecular mass protein</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LRRFIP2</td>
<td>leucine-rich flI-interacting protein 2</td>
</tr>
<tr>
<td>LULL1</td>
<td>luminal domain-like LAP1</td>
</tr>
<tr>
<td>MAD2</td>
<td>mitotic arrest deficient 2-like protein 1</td>
</tr>
<tr>
<td>MECL-1</td>
<td>multicatalytic endopeptidase complex subunit-1</td>
</tr>
<tr>
<td>MNSFβ</td>
<td>monoclonal nonspecific suppressor factor β</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule organizing center</td>
</tr>
<tr>
<td>mTORC</td>
<td>mammalian target of rapamycin complex</td>
</tr>
<tr>
<td>MUB</td>
<td>membrane anchored UBL-fold</td>
</tr>
<tr>
<td>MVB</td>
<td>multivesicular body</td>
</tr>
<tr>
<td>MZF-1</td>
<td>myeloid zinc finger 1</td>
</tr>
<tr>
<td>NDP52</td>
<td>nuclear dot protein 52</td>
</tr>
<tr>
<td>NEDD8</td>
<td>neural precursor cell-expressed, developmentally downregulated 8</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
</tr>
<tr>
<td>NeoR</td>
<td>neomycin resistance</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa b</td>
</tr>
<tr>
<td>NRAMP1</td>
<td>natural resistance-associated macrophage protein 1</td>
</tr>
<tr>
<td>PA</td>
<td>proteasome activator</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PAS</td>
<td>phagosome assembly site</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PI3P</td>
<td>phosphatidylinositol-3-phosphate</td>
</tr>
<tr>
<td>PIK</td>
<td>PI3 kinase</td>
</tr>
<tr>
<td>PKR</td>
<td>double-stranded RNA-dependent protein kinase R</td>
</tr>
<tr>
<td>pMΦ</td>
<td>peritoneal macrophage</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>PtdSer</td>
<td>phosphatidyl serine</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue</td>
</tr>
<tr>
<td>PTM</td>
<td>post translational modifications</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator of nuclear factor kappa-B ligand</td>
</tr>
<tr>
<td>RBR</td>
<td>RING-between-RING</td>
</tr>
<tr>
<td>RING</td>
<td>really interesting gene</td>
</tr>
<tr>
<td>RP</td>
<td>regulatory particle</td>
</tr>
<tr>
<td>RTECs</td>
<td>renal tubular epithelial cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SAE</td>
<td>SUMO-activating enzyme</td>
</tr>
<tr>
<td>SG</td>
<td>stress granules</td>
</tr>
<tr>
<td>SIM</td>
<td>SUMO interacting motive</td>
</tr>
<tr>
<td>Slc11a1</td>
<td>solute carrier family 11 member 1</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SP</td>
<td>standard proteasomes</td>
</tr>
<tr>
<td>SPI</td>
<td><em>Salmonella</em> pathogenicity island</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
</tr>
<tr>
<td>SUMO</td>
<td>small ubiquitin like modifiers</td>
</tr>
<tr>
<td>T3SS</td>
<td>type III secretion system</td>
</tr>
<tr>
<td>TLR</td>
<td>toll like receptor</td>
</tr>
<tr>
<td>TRAF</td>
<td>tumor necrosis factor receptor-associate factor</td>
</tr>
<tr>
<td>UBA</td>
<td>ubiquitin activating enzyme</td>
</tr>
<tr>
<td>UBC</td>
<td>ubiquitin conjugating enzyme</td>
</tr>
<tr>
<td>UBL</td>
<td>ubiquitin like proteins</td>
</tr>
<tr>
<td>UDP</td>
<td>ubiquitin domain proteins</td>
</tr>
<tr>
<td>UFM-1</td>
<td>ubiquitin-fold modifier-1</td>
</tr>
<tr>
<td>UIM</td>
<td>ubiquitin interacting motive</td>
</tr>
<tr>
<td>ULK</td>
<td>uncoordinated-1-like kinase</td>
</tr>
<tr>
<td>ULM</td>
<td>ubiquitin like modifier</td>
</tr>
<tr>
<td>URM-1</td>
<td>ubiquitin-related modifier 1</td>
</tr>
<tr>
<td>USE1</td>
<td>UBA6-specific E2-enzyme</td>
</tr>
<tr>
<td>USP</td>
<td>ubiquitin-specific-processing protease</td>
</tr>
<tr>
<td>VCP</td>
<td>valosin-containing protein</td>
</tr>
<tr>
<td>VPS</td>
<td>vacuolar protein sorting-associated protein</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>Amino acid</td>
<td>Three letter code</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Alanine</td>
<td>Ala</td>
</tr>
<tr>
<td>Arginine</td>
<td>Arg</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Glu</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln</td>
</tr>
<tr>
<td>Glycine</td>
<td>Gly</td>
</tr>
<tr>
<td>Histidine</td>
<td>His</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Ile</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
</tr>
<tr>
<td>Proline</td>
<td>Pro</td>
</tr>
<tr>
<td>Serine</td>
<td>Ser</td>
</tr>
<tr>
<td>Threonine</td>
<td>Thr</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Trp</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
</tr>
<tr>
<td>Valine</td>
<td>Val</td>
</tr>
</tbody>
</table>
References


References


References


References


References


References

346–51.


References

602–10.


References


References
References


Eidesstattliche Erklärung

Ich versichere hiermit, dass ich die vorliegende Arbeit mit dem Thema

**Functional analysis of the ubiquitin-like modifier FAT10 in autophagy**

selbständig verfasst und keine anderen Hilfsmittel als die angegebenen benutzt habe. Die Stellen, die anderen Werken dem Wortlaut oder dem Sinne nach entnommen sind, habe ich in jedem einzelnen Falle durch Angabe der Quelle kenntlich gemacht. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

___________________________   ________________________
Ort, Datum                        Unterschrift

Record of contribution

I designed and performed all experiments except for the results depicted in Figure 37 B, were Michael Basler (Biotechnology Institute Thurgau, Switzerland) performed three of the four quantified immunoblots. The plasmids pIRESmCherry-EGFP-p62 and pIRESmCherry-EGFP-p62-FAT10 were obtained in the context of the bachelor thesis of Theresa Birke at the University of Konstanz, which was performed under my supervision.