The ubiquitin ligation machinery in the defense against bacterial pathogens

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Abstract

The ubiquitin system is an important part of the host cellular defense program during bacterial infection. This is in particular evident for a number of bacteria including Salmonella Typhimurium and Mycobacterium tuberculosis which—invintively as part of their invasion strategy or accidentally upon rupture of seized host endomembranes—become exposed to the host cytosol. Ubiquitylation is involved in the detection and clearance of these bacteria as well as in the activation of innate immune and inflammatory signaling. Remarkably, all these defense responses seem to emanate from a dense layer of ubiquitin which coats the invading pathogens. In this review, we focus on the diverse group of host cell E3 ubiquitin ligases that help to tailor this ubiquitin coat. In particular, we address how the divergent ubiquitin conjugation mechanisms of these ligases contribute to the complexity of the anti-bacterial coating and the recruitment of different ubiquitin-binding effectors. We also discuss the activation and coordination of the different E3 ligases and which strategies bacteria evolved to evade the activities of the host ubiquitin system.

Keywords E3 ligase; innate immunity; Salmonella; ubiquitin; xenophagy

Introduction

Most mammalian cell types have the capacity to defend and protect themselves against pathogens, thereby contributing to cell-autonomous innate immunity. Over the last two decades, macroautophagy has emerged as a critical innate immunity pathway that detects a variety of cytosolic pathogens and initiates their disposal.

Originally, macroautophagy has been described as a catabolic process that sequesters unwanted intracellular components, including misfolded proteins and dysfunctional organelles, and eliminates them through the lysosomal degradation machinery (Fig. 1A). A hallmark of macroautophagy is the de novo formation of a double-membrane vesicle called autophagosome (Nakatogawa, 2020). Autophagosome biogenesis proceeds from an isolated crescent-shaped membrane structure (called phagophore or isolation membrane) that is derived from preexisting endomembranes including the ER and that grows around, and ultimately encloses cytosolic components. To complete macroautophagy, autophagosomes fuse with the lysosome, forming an autolysosome to allow disintegration of the inner membrane and the degradation of the sequestered material by diverse lysosomal hydrolases, such as lipases, proteases, glycosylases, and nucleases (Glick et al., 2010; Rubinsztain et al., 2012). To date, an increasing number of autophagy-related (ATG) proteins have been identified that are essential for the formation of the autophagosome and its fusion with the lysosome. Their molecular details and mechanistic interactions have been worked out over the last decades, their description, however, would exceed the scope of this review and the interested reader is referred to some excellent recent reviews (Noda & Inagaki, 2015; Nakatogawa, 2020).

Macroautophagy comes in two flavors, depending on how degradation targets are sequestered. During bulk macroautophagy, the forming autophagosome engulfs randomly cytoplasmic components that are close by. This form of macroautophagy is active at a basal level in nutrient-rich conditions, but can be upregulated in response to several stress conditions, such as starvation, thereby playing an integral part in recycling nutrients to maintaining energy homeostasis. By contrast, during selective macroautophagy, certain molecules, structures, and organelles are recognized via eat-me signals by target-specific proteins named autophagy receptors, which in turn mediate a stepwise recruitment of different macroautophagy machinery components to initiate and scaffold autophagosome formation exclusively around a distinct cargo (Reggiori et al., 2012; Stolz et al., 2014; Zaffagnini & Martens, 2016). Selective macroautophagy can be classified according to its cargo, including mitophagy (mitochondria), ribophagy (ribosomes), ER-phagy (ER), lipophagy (lipid droplets), lysophagy (lysosomes), and pexophagy (peroxisomes) (Gatica et al., 2018).

However, cells utilize selective macroautophagy not only to clear and recycle their own cellular material, but can also direct the macroautophagy machinery to eliminate invaded cellular pathogens, a process commonly known as antimicrobial macroautophagy.
or xenophagy—from Greek for ‘foreign/strange’ and ‘eating’ (Hu et al., 2020). Non-canonical forms of autophagy that do not involve the formation of double-membrane autophagosomes such as LC3-associated phagocytosis (LAP) also contribute to eliminate pathogens (Sanjuan et al., 2007; Mehta et al., 2014; Heckmann et al., 2017; Martinez, 2018). However, in this review, we will focus on our current mechanistic understanding of xenophagy and discuss recent advances on the role of the ubiquitin (Ub) machinery in targeting pathogenic bacteria for their clearance and how bacteria developed ways to fend off this process for survival or to even hijack it for their own benefit.

**Mechanism of ubiquitin-mediated xenophagy**

Intracellular pathogens, such as the bacteria *Shigella flexneri* (*S. flexneri*), *Mycobacterium tuberculosis* (*M. tuberculosis*), *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), and *Listeria monocytogenes* (*L. monocytogenes*), are able to reside and reproduce inside the host cell and are responsible for many severe human diseases. Besides passive engulfment by macrophages and neutrophils via phagocytosis, bacteria evolved different strategies to actively invade endothelial and epithelial host cells (Cossart & Sansonetti, 2004). Following their entry, pathogens can either reside inside membranous compartments (vacuoles) or escape to the cytosol. Nevertheless, xenophagy can target these bacteria to restrict their growth and replication. Since *S. Typhimurium* is one of the best-studied examples of bacteria that are targeted and eliminated by xenophagy (Herhaus & Dikic, 2018), we will use this model substrate to describe the different steps and key features of antimicrobial macroautophagy (Fig. 1B). After internalization, *S. Typhimurium* resides in an acidic compartment called the *Salmonella*-containing vacuole (SCV). SCVs are used by *S. Typhimurium* as a permissive niche for survival and growth (LaRock et al., 2015). Numerous bacterial genes, including the effector SifA, are required for the extension of the membranous surface of the SCV to accommodate the replicating bacteria. However, in the early phase of infection (usually within the first hour), a subset of SCV membranes is damaged and specific signature molecules become exposed on the bacterial cell wall as well as on the luminal surface of the SCV. Specifically, β-galactosides—which normally modify the extracellular side of the plasma membrane and the luminal surface of endosomes—are recognized on the damaged SCV membranes by the fast recruitment of the β-galactoside-binding lectin, galectin-8 which acts as an essential damage and/or pattern-recognition receptor and directs the phagophore receptor NDP52 (also known as CALCCOCO2) via its the galectin-interacting region (GIR) motif (Thurston et al., 2012).

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**Glossary**

- **ARIH1**: ARiadne homolog
- **ATG**: Autophagy-related
- **BMDM**: Bone marrow-derived macrophages
- **BMP**: Bone morphogenic protein
- **CALCOCO**: Calcium-binding and coiled-coil domain-containing protein
- **CC1**: Coiled-coil 1
- **CRL**: Cul5 RING ligase
- **DUB**: Deubiquitinating enzyme
- **GAS**: Galectin interacting region
- **GIR**: Galectin-interacting region
- **GWAS**: Genomewide association study
- **HECT**: Homologous to E. coli HECT family
- **HHAR1**: Homologues to human ARiadne 1
- **HOIL-IL**: Heme-oxidized IRP2 Ub ligase 1L
- **HOIP**: HOIL-1-interacting protein
- **IBR**: In between RING
- **IKK**: IkB kinase
- **LAP**: LC3-associated phagocytosis
- **LDD**: Linear Ub-determining domain
- **LIR**: LC3-interacting region
- **LPS**: Lipopolysaccharide
- **LRR**: Leucine-rich repeat
- **LRSAM1**: Leucine-rich repeat and sterile alpha motif-containing protein 1
- **LUBAC**: Linear Ub assembly complex
- **MAP1LC3B**: Microtubule-associated proteins 1A/1B light-chain 3B
- **NBR1**: Neighbor of BRCA1
- **NDP52**: Nuclear dot protein 52
- **NEL**: Novel E3 ligase
- **NEMO**: Neighbour of BRCA1 gene 1
- **NF-kB**: Nuclear factor kappa-B
- **NOD1**: Nucleotide-binding oligomerization domain-containing protein 1
- **OMP**: Outer membrane protein
- **OPTN**: Optineurin
- **OTULIN**: OTU domain DUB with L1NAR linkage specificity
- **PAMP**: Pathogen-associated molecular pattern
- **PE**: Phosphatidylethanolamine
- **PINK1**: PTEN-induced kinase 1
- **RBR**: RING-between-RING
- **RING**: Really interesting new gene
- **RNF213**: Ring finger protein 213
- **SAM**: Sterile alpha motif-containing domain
- **SCV**: *Salmonella*-containing vacuole
- **SHARPIN**: Shank-associated RH domain interacting protein
- **SIM**: Structured illumination microscopy
- **SLO**: Streptolysin O
- **Smurf2**: SMAD Ub regulator factor 1
- **SQSTM1**: Sequestosome-1
- **TAK1**: Transforming growth factor-β-activated kinase 1
- **TBK1**: TANK-binding kinase 1
- **TGF-β**: Transforming growth factor beta
- **TLR**: Toll-like receptor
- **TRA2**: TNF receptor-associated factor 2
- **TRIM21**: Tripartite motif-containing protein 21
- **TXN**: Thioredoxin
- **Ub**: Ubiquitin
- **UBA**: Ubiquitin-associated
- **UBL**: Ubiquitin-like domain
- **UVRAG**: UV radiation resistance associated
- **ZF**: Zinc finger
Figure 1. Selective macroautophagy.

(A) Key steps in selective macroautophagy. (1) The process of macroautophagy is initiated by isolation membranes and vesicles that gradually expand and mature to phagophores decorated with membrane-anchored LC3 and GABARAPs (orange hexagons). (2) These act as a binding site for autophagy cargo receptors (brown) allowing direct delivery and accumulation of cellular cargo. Finally, the phagophore closes forming a double-membraned vesicle called autophagosome (3), which subsequently fuses with the lysosome forming an autolysosome (4), where the content is degraded by lysosomal enzymes.

(B) Xenophagy, selective macroautophagy of *Salmonella*. (1) *Salmonella* enters host cells by forming a *Salmonella*-containing vacuole (SCV) that protects it from the host surveillance system and serves as a replicative niche. (2) SCVs can rupture allowing access of host cytosolic proteins. (3) The exposed glycans, that are normally present on the outer side of the cell membrane, serve as a danger signals that are recognized by galectins (purple triangles). Besides, *Salmonella* is detected by E3 ligases that generate a dense Ub coat, consistent of different linkage-type Ub chains (red and orange circles), around *Salmonella*. (4) Both, galectins and Ub recruit a variety of autophagy receptors (brown) that mediate *Salmonella* capture by LC3-conjugated phagophores and *Salmonella*-containing autophagosomes (5) are targeted for lysosomal degradation (6).
Besides β-galactosides/galectin-8-initiated autophagy, damaged SCVs and exposed Salmonella activate the cellular machinery and trigger the formation of a dense Ub coat dressing the whole Salmonella (Perrin et al., 2004). Ubiquitination, the covalent attachment of Ub to substrate proteins, requires the orchestrated action of at least three enzymes working in a relay, an Ub-activating enzyme (E1), an Ub-conjugating enzyme (E2), and an Ub ligase (E3) (Hershko et al., 2000; Butow & Huang, 2016). The E3s play a central part in substrate specificity and can be classified into three main families depending on the presence of characteristic domains and on the mechanism of Ub transfer to usually lysine residues of substrate proteins: really interesting new gene (RING) E3 ligases catalyze via allostERIC activation of the E2·Ub conjugate (i.e., indicating a thioester bond) the direct Ub transfer from the E2 to the substrate, whereas homologous to the E6AP carboxyl terminus (HECT) E3 ligases and Ring-Between-Ring (RBR) E3 ligase utilize a catalytic cysteine forming a transient E3·Ub thioester before mediating Ub transfer onto substrates (Zheng & Shabek, 2017). Substrates can be modified by single Ub molecules on one or multiple lysins (mono- or multi-monoubiquitylation) and/or polyubiquitin chains (polyubiquitylation) can be formed by linking Ub molecules via one of their seven lysine residues (K6, K11, K27, K29, K33, K48, K63) or N-terminal methionine (M1). These Ub chains are structurally distinct and can come in different variations, homotypic (single linkage type) or heterotypic (multiple, mixed linkage types) including branched polyubiquitin chains, resulting in a complex Ub code (Komander & Rape, 2012). Identity and origin of ubiquitination targets forming the Ub coat were assessed by quantitative proteomics of S. Typhimurium-infected epithelial cells and revealed ubiquitination sites on several outer membrane-associated and integral outer membrane proteins of Salmonella (Fiskin et al., 2016). Moreover, several chain types are associated with the Ub coat of S. Typhimurium including M1- (also known as linear Ub chains), K48- , and K63-polyubiquitin chain linkages, suggesting a complex structure and signaling platform of the Ub coat (van Wijk et al., 2012; Manzanillo et al., 2013). Over the last two decades, several E3 ligases like Smurf1, Parkin, ARIH1, LRSAM1, RNF213, and LUBAC, were identified to ubiquitinate more than one type of bacteria, and in some cases, the bacteria are targeted simultaneously by multiple E3 ligases. For instance, ARIH1, LRSAM1, RNF213, and LUBAC are known to colocalize with damaged SCVs and promote cytosolic S. Typhimurium Ub coat formation. There is also a group of E3 ligases like RNF166 (Heath et al., 2016), MARCh8 (Jin et al., 2017), TRIM21 (Hos et al., 2020), TRIM22 (Lou et al., 2018), and TRIM16 (Chauhan et al., 2016) that do not recognize the pathogen itself but recognize other aspects of infection like exposed glycan (Chauhan et al., 2016).

Similar to Galectin-8, the bacterial Ub coat functions as a xenophagic eat-me signal (Birmingham et al., 2006) and triggers the binding of autophagy receptors such as p62 (alias SQSTM1), NDP52 (alias CALCOCO2), and optineurin (OPTN) which are essential for the engulfment of bacteria by autophagosomes (Zheng et al., 2009; Wild et al., 2011; Thurston et al., 2012). These receptors are multifunctional proteins harboring distinct ubiquitin-binding domains (UBD) with inherent specificities toward different ubiquitin chain types, as well as protein–protein interaction motifs that mediate the recruitment of autophagy machinery components to induce the formation and elongation of phagophore membranes proximal to bacteria (Kirkin & Rogov, 2019). The modular nature of these receptors is best understood in the case of NDP52 which employs a GIR and an Ub-binding zinc finger (ZF) to associate with bacteria, a SKICH domain to recruit the autophagy promoting ULK1 and TBK1 kinases via their respective adaptors FIP200 and SINBAD/NAP1 as well as an LC3-interacting region (LIR) to guide the engulfment of bacteria by the nascent autophagosome via LC3 (von Muhlen et al., 2012; Thurston et al., 2012; Ravenhill et al., 2019). While NDP52’s ZF seems to promiscuously bind mono- and polyubiquitin (Xie et al., 2015), the UBAN domain of OPTN specifically recognizes M1- and K63-linked ubiquitin chains of the Salmonella Ub coat (Wild et al., 2011). TBK1-mediated phosphorylation of OPTN fosters its interaction with LC3, thereby supporting phagosome/autophagosome formation and restriction of Salmonella growth (Wild et al., 2011). Notably, the actions of multifunctional Ub-binding autophagy receptors are not limited to xenophagy, but shared among other selective autophagy processes including aggrephagy and mitophagy (Turco et al., 2019; Vargas et al., 2019; Shi et al., 2020; Yamano et al., 2020). There are also some special cases, like xenophagy of Mycobacterium tuberculosis, where cytosolic Ub is directly binding to the UBA domain of the M. tuberculosis surface protein Rv1468c leading to the recruitment of p62 and LC3-mediated delivery of the bacteria to lysosomes (Chai et al., 2019). Hence, Ub plays a central role as danger signal and sensor of cytosolic bacteria, highlighting the importance of the Ub machinery in xenophagy.

### E3 ubiquitin ligases associated with xenophagy

The Ub coat on the surface of cytosolic bacteria including S. Typhimurium and the nonmotile ΔactA mutant of L. monocytogenes was first described by Perrin and colleagues in 2004 (Perrin et al., 2004). This seminal discovery initiated extensive research efforts in the past two decades to identify the Ub-targeting machinery that recognizes and targets cytosol bacteria for Ub-mediated xenophagy and innate immune responses. In this chapter, we will describe in a historic order the identification of xenophagy-associated E3 ligases. We will discuss our current knowledge of their E3-typical features and mechanisms in context of (i) sensing/recognition of cytosolic bacteria (ii) E3 ligase activation and (iii) type of Ub substrate modification.

#### LRSAM1

In 2011, the first E3 ligase, leucine-rich repeat and sterile alpha motif-containing protein 1 (LRSAM1), was functionally linked to Ub coat synthesis and xenophagy (Ng et al., 2011). LRSAM1, first shown to play a role in the endosomal sorting machinery (Amit et al., 2004; McDonald & Martin-Serrano, 2008), belongs to the RING-type E3 ligases that share the presence of a RING domain (Fig. 2). Canonical RING domains comprise conserved cysteines and histidines that allow coordinate binding of two zinc ions thereby stabilizing an overall globular three-dimensional domain structure (Deshaias & Joazeiro, 2009). RING domains themselves lack Ub transfer activity, but accommodate the charged E2·Ub in a distinct architecture known as the closed conformation that allosterically activates E2·Ub for the nucleophilic attack by a suitable placed substrate lysine to form an isopeptide bond (Dou et al., 2012, 2013; Plechanovova et al., 2012; Pruneda et al., 2012; Branigan et al,
S. flexneri pathogens including S. (Perrin already proposed at the time when the Ub coat was first observed designed E3 for recognizing and ubiquitylating cytosolic bacteria. associated molecular patterns (PAMPs) making LRSAM1 a suitably controlling bacterial replication of the less virulent protein including the entire RING domain, are less efficient in lack expression of LRSAM1 due to a frameshift that truncates the strain (i.e. NTCC12023; (Huett et al, 2004). LRSAM1 colocalizes with xenophagy-susceptible pathogens including S. Typhimurium, L. monocytogenes EGD-e, S. flexneri, and invasive Escherichia coli, and colocalization is mediated by the LRR domain (Huett et al, 2012). The nature of PAMPs that are recognized by LRR is not characterized yet (Fig. 2). The RING domain of LRSAM1 is dispensable for bacterial association; however, it is essential to promote bacteria-associated ubiquitylation with preference for K6- and K27-linked chain formation in vitro. Whether the Ub coat is formed by bacteria-bound autoubiquitylated LRSAM1 and/or by LRSAM1-mediated ubiquitylation of bacterial surface proteins is not entirely clear. LRSAM1 accumulation with cytosolic Salmonella is peaking 40 min post-infection coinciding with transient galectin-8/NDFP52 colocalization. However, LRSAM1 and NDFP52 mark spatially different subdomains around Salmonella and are recruited independently (Huett et al, 2012; Thurston et al, 2012), Ub detection follows LRSAM1 occurring an hour post-infection and is thought to foster NDFP52 accumulation necessary for the recruitment of the autophagy machinery (Thurston et al, 2012).

The functional association of LRSAM1 with xenophagy is essential to control bacterial infection. Depletion of LRSAM1 in epithelial cells promotes cytoplasmic growth of S. Typhimurium. Lymphoblasts derived from Charcot–Marie–Tooth disease patients, which lack expression of LRSAM1 due to a frameshift that truncates the protein including the entire RING domain, are less efficient in controlling bacterial replication of the less virulent S. Typhimurium strain (i.e. NTCC12023; (Huett et al, 2012)). Conversely, the bactericide Biochanin A, a plant isoflavone derivate, was shown to enhance killing of Salmonella in infected HeLa cells and macrophages by reinforced LRSAM1/NDFP52 accumulation and autophagosome formation (Zhao et al, 2018). The underlying mechanism of Biochanin A’s bactericidal activity is not known, though. LRSAM1, as a pattern-recognition E3, may play a central role in the early step of anti-bacterial defense by the establishment of the initial Ub coat on S. Typhimurium; however, it became clear that the Ub coat is further modified and re-modeled by other xenophagy-associated E3s to create an even more complex signaling platform on cytosolic bacteria. PARKIN Genomewide association studies (GWAS) identified some genetic polymorphism in the parkin gene (PRKN) that are also associated with increased susceptibility to intracellular pathogens, such as S. Typhimurium and Mycobacterium leprae, suggesting a potential connection between mitochondrial homeostasis and xenophagy via Parkin (Mira et al, 2004; Ali et al, 2006). However, Parkin is primarily known for its well-established role in mitophagy and got much attention as it is frequently mutated in autosomal recessive juvenile Parkinsonism, a neurological disorder that leads to the progressive loss of dopaminergic neurons.

Parkin belongs to the RBR family of E3 ligases that are defined by common domain organization and catalytic mechanism (reviewed in (Cotton & Lechtenberg, 2020)). The catalytic RBR module consists of three zinc-binding motifs: a RING1 domain with a canonical RING fold mediating E2 binding, followed by an In-Between-RING (IBR) domain, and finally a Rcat (also known as RING2) domain containing the catalytic cysteine (Fig. 3A). Specific for Parkin is the presence of an N-terminal Ub-like (UBL) domain that shares a structural fold similar to Ub and an additional zinc-coordinating unique parkin domain (UPD) (also known as RING0) (Trempe et al, 2013). Both, UBL and UPD play a central role in regulating Parkin’s E3 ligase activity. The apo-form of Parkin adopts a multilayered autoinhibited conformation, whereby the UBL and a repressor element (REP) occlude the E2 binding site in RING1, and

Figure 2. The RING-type E3 ligase LRSAM1 in Ub coat formation of cytosolic Salmonella.

The domain architecture of LRSAM1 showing the leucine-rich repeats (LRR) domains (red dotted box) that detects PAMPs of Salmonella and mediates bacterial binding, coiled-coil (CC) domains, a sterile alpha motif (SAM) containing domain, and the C terminus RING domain with E3 ligase activity. LRSAM1-mediated ubiquitylation of cytosolic Salmonella, preferentially K6- and K27-linked chains, leads to the accumulation of autophagy cargo receptor NDP52 mediating the recruitment of the autophagy machinery.
the UPD domain masks the catalytic cysteine 431 (C431) in Rcat preventing the formation of the E3’Ub thioester intermediate (Chau-gule et al., 2011; Trempe et al., 2013; Wauer & Komander, 2013). Several elegant studies revealed a striking activation mechanism of Parkin in mitophagy. Parkin activation depends on PINK1 kinase, which itself accumulates and is activated on the outer membrane of damaged mitochondria (Matsuda et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010; Kondapalli et al., 2012). PINK1 phosphorylates Ub on serine 65 (pS65-Ub) which increases Ub’s affinity for an allosteric site—a pS65-Ub-binding pocket—in Parkin. Once bound, pS65-Ub induces a conformational change in the IBR domain resulting in the release of the inhibitory UBL from the RBR module, overall destabilizing the autoinhibitory conformation and making the Rcat accessible for translocation. Subsequently, freed UBL becomes a substrate of PINK1 and pS65-UBL further fosters an open, active conformation (Ordureau et al., 2014; Kazlauskaite et al., 2015; Kumar et al., 2015; Sauve et al., 2015; Wauer et al., 2015; Yamano et al., 2015; Aguirre et al., 2017; Gladkova et al., 2018).

The PINK1/Parkin pathway is tightly linked with damaged mitochon-dria, however, and rather surprising, it was shown that Parkin is required for M. tuberculosis xenophagy in vitro. M. tuberculosis is a vascular pathogen that resides inside a phagosomal compartment after entering the cell. Due to the activity of mycobacterial ESX-1 secretion system, the phagosomal membrane becomes permeable allowing components of the Ub-xenophagy pathway to access M. tuberculosis (Watson et al., 2012). In infected murine bone marrow-derived macrophages (BMDMs), Parkin colocalizes with M. tuberculosis and mediates a predominantly K63-linked Ub coat surrounding M. tuberculosis phagosomes. This contrasts findings by Ordureau et al. (2014) indicating that Parkin does not exhibit specificity for single chain types (Manzanillo et al., 2013; Ordureau et al., 2014). Parkin-mediated ubiquitylation subsequently leads to the recruitment of autophagy receptors p62 and NDP52 via UBA and UBZ Ub-binding domains, respectively, that facilitate the delivery of mycobacteria to autophagosomes (Ichimura et al., 2008; Watson et al., 2012; Manzanillo et al., 2013). In vivo data further support an important role of Parkin against a range of intracellular bacterial infections. Park2−/− mice have an extreme susceptibility to M. tuberculosis and L. monocytogenes infections. Moreover, D. melanogaster and C. elegans strains deficient for Park2 homologues are highly susceptible to infection by pathogens including L. monocytogenes, S. Typhimurium, and Mycobacterium marinum, suggesting a conserved role of Parkin in innate immunity across metazoans (Manzanillo et al., 2013). Importantly, while studies in cultured cells show involvement of many autophagy factors including ATG5, ATG12, ATG16L, and LC3 in restricting M. tuberculosis replication (Gutierrez et al., 2004; Dutta et al., 2012; Watson et al., 2012; Seto et al., 2013; Sakowski et al., 2015), comprehensive genetic investigations in mice revealed a unique and autophagy-independent role of ATG5 in controlling resistance to M. tuberculosis infections (Kimmey et al., 2015). Given the lack of a clear correlation between in vitro and in vivo findings, Parkin’s precise in vivo role in killing of M. tuberculosis, with a possible functional link to ATG5 in early events of innate immunity, needs further investiga-

The gram-positive Group A streptococcus (GAS) was the first bacterium with definitive evidence to be degraded by autophagy (Nakagawa et al., 2004). Generally, GAS is extracellular but has the ability to trigger its internalization into non-immune cells (endothelial and epithelial cells) to evade immune surveillance (Molinari et al., 2000; Nakagawa et al., 2001). Intracellular GAS colocalizes to a large extent with LC3-positive compartments which is dependent on the pore-forming streptolysin O (SLO) (Nakagawa et al., 2004). SLO promotes the escape of GAS from the phagolysosome, which gives access to galectin-8-dependent Parkin-mediated ubiquitylation of the GAS/phagosome compartment (Cheng et al., 2017). The autophagy receptors p62, NDP52, and NBR1—a p62 homolog, containing a PB1 domain to interact with p62, a UBA domain to interact with Ub, and a LIR motif (Kirkin et al., 2009; Lamark et al., 2009)—are essential for the recognition of Ub decorated GAS and initiation of LC3-dependent xenophagic degradation (Thurston et al., 2009; Barnett et al., 2013). Consistent with the key role of galectin-8 in this process, the efficiency of Parkin recruitment and subsequent GAS ubiquitylation is highly dependent on the abundance of galectin-8. While exposed glycans can be sensed by other galectins in cells with low amounts of galectin-8, the compensatory recognition of GAS by for example galectin-3 does not lead to GAS elimination (Cheng et al., 2017). However, the differential roles of these two galectins in response to GAS remain poorly understood.

Although there are striking parallels between xenophagy and mitophagy pathways downstream of Parkin/ubiquitylation, tempting questions regarding how Parkin is recruited to cytosolic pathogens and what relieves Parkin’s autoinhibition, processes that might be mechanistically interconnected, remain unanswered with room for speculations. Recently, similar to findings for PRKN (Mira et al., 2004; Ali et al., 2006), common variants in PINK1 gene were associated with increased risk of leprosy, a chronic infectious, and neurological disease caused by Mycobacterium leprae (Wang et al., 2016). Hence, PINK1, in conjunction with Parkin, might have a role in M. leprae innate immune response via xenophagy. However, no
study addressed yet, whether PINK1 accumulates on M. leprae/ phagophores and initiates pS65-Ub and/or pS65-UBL-mediated Parkin activation. At least, no pS65-Ub was found to colocalize with S. Typhimurium (Thurston et al., 2012). Alternatively, Parkin activation could be PINK1-independent in the context of xenophagy, and other sensor and activating molecules exist leading to recruitment as well as activation of Parkin (Singh et al., 2018). Addressing Parkin activation and sensing mechanism of cytosolic bacteria will be instrumental to further our understanding of Parkin’s role in xenophagy.

SMURF1

SMAD Ub regulator factor (Smurf1) was originally identified and characterized as negative regulator of bone morphogenetic protein (BMP) and transforming growth factor beta (TGF-β) (sequence L/P-P-x-Y) and phosphorylated Ser/Thr-Pro sites (Chen & Sudol, 1995; Lu et al., 1999; Leon & Haguenauer-Tsapis, 2009), and an N-terminal C2 domain mediating membrane association via interactions with phospholipids and inositol phosphates (Plant et al., 1997; Angers et al., 2004; Dunn et al., 2004; Scott et al., 2020). In agreement with a role of C2 in regulating subcellular localization, the C2 domain is essential for the accumulation of Smurf1 at sites of M. tuberculosis, whereas the E3 ligase activity of Smurf1 is dispensable. Importantly, the C2 domain also participates in Smurf1 homodimerization and intramolecular autoinhibition, whereby the C2 domain of one Smurf1 interacts in trans with the HECT domain of another Smurf1 (Fig. 4). It is tempting to speculate that C2 binding to M. tuberculosis and/or phagophore relieves the autoinhibitory conformation by liberating the HECT domain. Hence, coupling of recruitment and activation mechanisms might ensure spatiotemporal control of Smurf1-mediated ubiquitylation. Similar to reports for Parkin (Watson et al., 2012), Smurf1 colocalization with a M. tuberculosis strain, which lacks the virulence factor and type VII secretion system ESX-1, is reduced, suggesting that only damaged and permeabilized phagosomes promote the recruitment and activation of xenophagic Ub machineries.

Smurf1 mediates preferentially K48-linked Ub chains on M. tuberculosis as well as L. monocytogenes that lead to binding of the autophagy receptor NBR1 (Franco et al., 2017). In addition, Smurf1 was reported to promote autophagosome maturation by K29/K33 ubiquitylation of UV radiation resistance associated (UVRAG), which is a key component of the phosphatidylinositol 3-kinase (PI3K) pathway.

Figure 4. The HECT-type E3 ligase Smurf1 in Ub coat formation of cytosolic Mycobacteria.

Domain schematic of Smurf1 highlighting the C2 domain required for bacterial binding (red dotted box) and C-terminal HECT domain with E3 ligase activity. In its apo state, Smurf2 forms inactive homodimers. Interaction between the C2 domain and cytosolic Mycobacterium might relieve Smurf1’s autoinhibition and trigger K48-linked Ub chain synthesis. Autophagy adaptor protein NBR1 recruits phagophores and initiates xenophagy.
phosphate kinase complex localized in the endosomal compartment. Whether this Smurf1 activity contributes to xenophagy remains to be tested (Feng et al., 2019). Using a murine model of tuberculosis, it was shown that Smurf1 deficiency during the chronic, but not in the acute phase of M. tuberculosis infection, increased bacterial growth in lung and spleen causing increased pulmonary inflammation (Franco et al., 2017). Moreover, Smurf1 is essential to limit mycobacterial replication in human macrophages and associates with bacteria in lungs of patients with pulmonary tuberculosis. Together with the aforementioned E3s LRSAM1 and Parkin, Smurf1-mediated K48-linked ubiquitin chains and the recruitment of NBR1 contributes further to the pattern of the signaling platform by the Ub coat mediating xenophagic elimination of cytosolic bacteria.

**LUBAC**

Super-resolution microscopy (by structured illumination microscopy, SIM) of the Ub coat on cytosolic S. Typhimurium revealed microdomains/clusters of M1-linked Ub chains that occur later than other linkage types (Noad et al., 2017; van Wijk et al., 2017). The concept of microdomains on bacterial surfaces was originally proposed for autophagy receptor proteins. The Brumell laboratory described that p62 and NDP52 are recruited independently, marking non-overlapping microdomains on bacteria (Cemma et al., 2011). The discovery of Ub linkage-specific microdomains suggests thus that this Ub code defines the discrete receptor pattern by recruiting Ub linkage-specific receptors.

The only enzyme known so far to be specific for the synthesis of M1 linkages is the linear Ub assembly complex (LUBAC), a multi-meric E3 Ub ligase composed of heme-oxidized IRP2 Ub ligase 1L (HOIL-1L), HOIL-1 interacting protein (HOIP), and Shank-associated RH domain interacting protein (SHARPIN) (reviewed in (Rittinger & Ikeda, 2017)) (Fig. 3B). Assembly of LUBAC is mediated by domain-defined protein–protein interactions. HOIP’s UBA domain is required to associate with the respective UBL domains of HOIL-1L and SHARPIN. The NZF1 domain of HOIP might additionally mediate HOIP-SHARPIN binding. Moreover, HOIP was shown to associate via the PUB domain with the deubiquitinating (DUB) enzyme OTU domain DUB with LINear linkage specificity (OTULIN), and via the adapter protein SPARTA to the M1- and K63-chain cleaving DUB CYLD (Keusekotten et al., 2013; Rivkin et al., 2013; Elliott et al., 2016; Hrdinka et al., 2016; Kupka et al., 2016), Both, HOIP and HOIL-1L subunits contain the RBR domain cluster; however, HOIL-1L’s activity is non-essential for M1-Ub chains synthesis by LUBAC (Kirisako et al., 2006). In fact, the RBR domain of HOIP plus a C-terminal extension, known as the linear Ub-determining domain (LDD), contains the minimal catalytic machinery required for the highly specific synthesis of M1 linkages (Smit et al., 2012; Stiegitz et al., 2012, 2013). However, apo full-length HOIP is inactive. Detailed structural and mechanistic studies of inactive HOIP are still missing, but it is thought that the catalytic C885 is occluded involving the UBA domain. The autoinhibitory conformation is relieved in LUBAC assembly whereby HOIL-1L via its UBL associates with the UBA domain of HOIP (Kirisako et al., 2006). Structural insight into HOIP’s active form was recently provided by the crystal structure of the RBR-LDD fragment in complex with thecharged E2, UBE2D7Ub (Lechtenberg et al., 2016). This structure uncovered how UBE2D7Ub is tightly embraced by the RBR, orientating the E2’Ub thioester bond in close proximity to C885 primed to promote trans-thiolation.

Furthermore, an allosteric Ub-binding site was revealed. Ub or M1-linked di-Ub binding to this site apparently promotes binding efficiency of UBE2D7Ub donor Ub, HOIP’Ub thioester, and polyubiquitin chain formation (Lechtenberg et al., 2016).

Recruitment of LUBAC to cytosolic S. Typhimurium was dissected in detail by the Randow laboratory proposing a two-phase mechanism (Noad et al., 2017) (Fig. 3B): First, LUBAC binds on preexisting Ub chains on S. Typhimurium mediated by the Ub-binding dNIFZ domain of HOIP. Second, efficient LUBAC accumulation depends on a feed forward loop requiring HOIP’s synthesis of M1-linked Ub chains (likely on the preexisting chains), that in turn take part in the aforementioned allosteric activation of HOIP as well as the accelerated recruitment of yet more HOIP/LUBAC. This raises the tempting question whether the feed forward loop is counterbalanced by the activity of LUBAC-associated DUBs, OTULIN and/or CYLD. Recent findings suggest that HOIP and OTULIN cooperate in regulating autophagy, whereby HOIP is required for autophagy initiation, whereas OTULIN is necessary for autophagy maturation steps (Chu et al., 2021; Weinelt & van Wijk, 2021). Curiously, OTULIN-depleted cells had fewer LC3B-positive autophagosome-associated Salmonella, suggesting that OTULIN is not simply competing with HOIP and M1-linked Ub chain synthesis to control xenophagy.

Originally, it was suggested that LUBAC recognizes the substrate target and attaches the first Ub on N-terminal M1. Subsequently, this priming ubiquitin becomes substrate for M1-linkage synthesis (Tokunaga et al., 2009; Smit et al., 2013; Fujita et al., 2014). However, an alternative model has been proposed by Emmerich et al. (2013) whereby HOIP recognizes preexisting K63-linked chains that serve as substrates for HOIP-mediated M1-linkage extensions that result in mixed (heterotypic) K63/M1 chains (Emmerich et al., 2013). Hence, the Ub coat surrounding cytosolic bacterial might not be limited to homotypic Ub chains, but also heterotypic chains of K63/M1 type. The M1-linked Ub chain deposits on S. Typhimurium recruit the Ub-binding effector proteins OPTN and NF-kB Essential Modifier (NEMO) and thereby transform the bacterial surface into a signaling platform coordinating two cellular defense pathways: xenophagy and the NF-kB pro-inflammatory pathway, respectively (Noad et al., 2017) (Fig. 3B). OPTN serves as an autophagy receptor protein that requires phosphorylation-mediated activation by kinase TBK1 and recruits LC3-coated phagophores to eliminate cytosolic S. Typhimurium (Wild et al., 2011; Rogov et al., 2013). Independent of OPTN, NEMO restricts together with the other subunits of the IKK complex (IKKα and IKKβ) proliferation of cytosolic S. Typhimurium via activation of the pro-inflammatory NF-kB pathway. This signaling axis has striking similarities to Toll-like receptor (TLR)-mediated signaling and might converge with other innate immune response pathways (Fiil & Gyrd-Hansen, 2021).

**ARIH1**

The fifth E3 ligase entering the stage of Ub-mediated xenophagy was ARIH1 (also known as Homologues to Human ARIde1 (HHAR)) (Fig. 3C). We isolated ARIH1 in a focused image-based RNAi screen comprised of 14 known RBR E3 ligases that aimed to identify E3 ligases controlling replication of cytosolic S. Typhimurium and Ub coat formation (Polajnar et al., 2017). The recruitment of ARIH1 to cytosolic Salmonella is a fast response post-infection, with kinetics comparable to galectin-8 binding to damaged SCVs (Thurston et al., 2012; Polajnar et al., 2017). The molecular mechanism explaining of
how ARIH1 recognizes *Salmonella* has not been determined, but ARIH1 is detected by super-resolution microscopy in a patch-like pattern around *Salmonella* cells even before the occurrence of the pattern-recognition E3 LRSAM1 (see above), and mediates K48-linked chain formation. ARIH1 together with LRSAM1 likely constitutes the first phase of E3 action and ubiquitylation on damaged SCVs (Polajnar et al., 2017). The observation that ARIH1 is also recruited to depolarized mitochondria might point to common molecular cues exposed on damaged membranes of these two compartments (Villa et al., 2017).

As discussed for the RBR E3s Parkin and HOIP, isolated ARIH1 is also autoinhibited, raising the question of how ARIH1 is activated during *Salmonella* infection. Structural studies of ARIH1 revealed that the C-terminal Ariadne domain occludes the catalytic site cysteine C357 on the Rcat domain (Duda et al., 2013). To relieve the autoinhibition and to gain full Ub transfer activity, large-scale conformational changes of ARIH1 are needed. Recently, an intriguing strategy/mechanism of ARIH1 activation was proposed. An analysis of the ARIH1 interactome revealed that ARIH1 binds directly to neddylated cullins (cullin-1, -2, -3, 4a, and -4b) and to numerous neddylated Cull ring ligase (NEDD8-CRLs) complexes (Kelsall et al., 2013; Scott et al., 2016). Recent cryo-EM structural work and elegant biochemical studies suggest that binding of NEDD8-CRL complexes repositions the inhibitory Ariadne domain and opens the structure making the catalytic C357 accessible to react with E2Ub (Kelsall et al., 2013; Scott et al., 2016; Horn-Ghetko et al., 2021). Further, ARIH1 in complex with NEDD8-CRLs complexes promotes fast and efficient monoubiquitylation of CRL clients, thus priming them for subsequent CRL-mediated Ub chain elongation (Scott et al., 2016; Kelsall et al., 2019). Polajnar et al. (2017) addressed whether NEDD8-CRL-dependent activation of ARIH1 plays a role in *Salmonella* xenophagy. Neither cullin depletion via RNAi nor treatment of cells with the NEDD8-activating E1 enzyme inhibitor MLN4924, which abolishes all neddylation conjugations in the cell, did impact on Ub coat formation or did increase *Salmonella* replication, suggesting another activator and/or alternative activation mechanism of ARIH1 in the context of xenophagy. Despite the elusive activation mechanism, ARIH1-mediated K48-type Ub chains synthesis adds to the aforementioned, K63 and M1 Ub chain types to an unprecedented complexity of the Ub code on cytosolic bacteria. Notably, while K63 and M1 chains have apparently overlapping functions in the recruitment of autophagy receptors, ARIH1 is dispensable for p62-, OPTN-, and NDP52-mediated xenophagy (Polajnar et al., 2017). The identification of downstream effectors of the K48-type Ub chains on *Salmonella* is exciting goals for future studies.

**RNF213**

Besides the growing number of E3 enzymes and their contribution to the formation of the Ub coat that dress cytosolic bacteria, the identity of bacterial molecules that are conjugated with Ub was previously restricted to *Salmonella* outer membrane proteins (Fiskin et al., 2016). Otten and colleagues extended the landscape of ubiquitylation targets to lipopolysaccharide (LPS) of the outer surface of *S. Typhimurium* (Otten et al., 2021). LPS consists of Lipid A, which is anchored in the bacterial outer membrane, and oligosaccharides termed as inner-, outer-core, and O-antigen. Using a set of *Salmonella* mutants that synthesize truncated versions of LPS in combination with chemical approaches that allowed discrimination between protein and non-protein ubiquitin ligation, it was shown that lipid A is the minimal subunit to form an ester-linked Ub conjugate with its hydroxy and/or phosphate groups (Fig. 5). To identify the enzyme that ubiquitylates LPS, Otten and colleagues fractionated HeLa cell lysates by applying sequential biochemical purification steps and isolated RNF213 as the cognate LPS-active E3 ligase (Otten et al., 2021). With a mass of nearly 600 kDa, RNF213 is the largest identified E3 ligase in mammalian proteomes. A recent cryo-EM structure of mouse RNF213 revealed distinct domain architectures of a dynacine-like AAA+ motor domain and a RING-type domain-containing E3 ligase module (Abel et al., 2020) (Fig. 5). Interestingly, the dynacine-like AAA+ motor domain is required for LPS ubiquitylation, whereas—unexpectedly—no such requirement was found for the RING domain (Abel et al., 2020; Otten et al., 2021). However, Otten and colleagues mapped a molecular motif within the E3 module, named RZ-finger, that is typical for zinc-binding domains, and showed that it is required for LPS ubiquitylation. Recent biochemical studies pinpointed an active site cysteine within the RZ finger that accepts Ub from the E2 enzyme UBCH7, suggesting that RNF213 represents an undescribed type of transthiolation E3 enzyme (preprint: Abel et al., 2021). RNF213 knockout mouse embryonic fibroblasts (MEFs) or cells expressing RNF213 mutations corresponding to the RZ-finger are severely defective in Ub coat formation around cytosolic *Salmonella* and also failed to recruit LUBAC and lacked LUBAC-mediated M1 Ub chain synthesis. In agreement with the aforementioned dependency of LUBAC activity on pre-formed Ub chains on *Salmonella* (Fig. 3B), RNF213 is a likely candidate for the first-line E3 ligase activity, priming *Salmonella* with ubiquitylated LPS for xenophagy and innate immune response pathways. However, RNF213-mediated immune response might not be limited to the gram-negative bacterium *Salmonella*. A recent study suggested that RNF213 exerts a much broader antimicrobial activity, counteracting infections with the gram-positive bacterium *L. monocytogenes* as well as several viral pathogens *in vitro* (preprint: Thery et al., 2021). Importantly, this finding would imply that LPS is unlikely the only target of RNF213 and other substrates might exist.

Despite compelling evidence of a central role of RNF213’s E3 ligase in cell-autonomous immunity studying cell systems, there are only limited data from *in vivo* studies. RNF213-deficient mice have an increased susceptibility to *L. monocytogenes* infections (reprint: Thery et al., 2021); however, the function of RNF213 in innate immunity remains poorly understood *in vivo*. Several studies describe RNF213 as a major susceptibility gene for Moyamoya disease, a cerebrovascular disorder characterized by arterial stenosis and abnormal development of collateral blood vessels that frequently leads to brain strokes (Kamada et al., 2011; Liu et al., 2011). The physiological and pathophysiological role of RNF213 is little understood, but RNF213 was shown to be involved in lipid metabolism, lipid droplet formation, and signaling associated with cell death and immune-system function (Banh et al., 2016; Piccolis et al., 2019; Sugihara et al., 2019). The majority of RNF213 mutations found in Moyamoya patients cluster in the composite E3 module; however, none of the tested mutations affected LPS ubiquitylation (Otten et al., 2021), suggesting that defective bacterial ubiquitylation might not play a role in Moyamoya disease. Still, to address whether bacterial infections are implemented in the development of the disease is worthy of future investigations. Moreover, it is tempting to speculate that RNF213 ligase activity for lipid
substrates might regulate other types of selective macroautophagy, such as lipophagy. RNF213 as a potential immune sensor adds a new exciting player to the xenophagy machinery with interesting questions regarding precise enzymatic mechanism and substrate recognition/specificity for the future.

**Interplay of xenophagic E3 ligases**

Whether the different pathogen-associated E3 ligases act in a hierarchically coordinated or independent random manner is not fully understood since systematic, time-resolved analyses with comparable infection conditions across different bacteria are lacking. So far, studies have only examined pairs (Smurf1/Parkin, LRSAM1/Parkin, and RNF213/HOIP) and trios (ARIH1/LRSAM1/HOIP and Parkin/LRSAM1/HOIP) of these ligases in response to infection with *M. tuberculosis* and *S. Typhimurium*, respectively. Therefore, the emerging picture is sketchy at best. ARIH1 seems to translocate to *Salmonella* as soon as they evade into the cytosol followed by LRSAM1. Conversely, LRSAM1 tends to persist longer on these bacteria compared to ARIH1 (Polajnar *et al.*, 2017). Given that ARIH1 is not required for the bacterial recruitment of LRSAM1 and that both ligases do not interact (Polajnar *et al.*, 2017), it seems plausible that both ligases translocate to cytosolic *Salmonella* independent from each other as part of a first line of defense. While the kinetics of Smurf1, Parkin, HOIP, and RNF213 colocalization to cytosolic bacteria in relation to ARIH1 and LRSAM1 have not been monitored in detail, HOIP is only recruited to bacteria that already carry an initial Ub coat (Noad *et al.*, 2017). Notably, binding to ubiquitylated bacteria is mediated by HOIP’s dNZF domain which has an *in vitro* preference for K63 chains (Noad *et al.*, 2017). However, Parkin and LRSAM1 turned out to be dispensable for HOIP recruitment (Noad *et al.*, 2017), whereas ARIH1 and Smurf1 have not been tested due to their preference to generate K48 chains. In the search of a ligase whose activity is responsible for the downstream recruitment of HOIP, the Randow laboratory recently provided compelling evidence that ubiquitylation of bacterial LPS by RNF213 is the priming event of this process (Otten *et al.*, 2021). In the absence of RNF213, the recruitment of HOIP, M1-Ub, NEMO, OPTN, and LC3 to bacteria was dramatically reduced and bacterial replication increased at 4 h after infection with *S. Typhimurium*. The observation that loss of RNF213-mediated ubiquitylation also ablated the translocation of p62 and NDP52 to *Salmonella* suggests that RNF213 is a major driver of xenophagy at this time point of infection. Consistent with a rather late arrival of RNF213 at cytosolic bacteria 2-3 h post-infection, cells lacking RNF213 were still able to initially coat *Salmonella* with ubiquitin at 1 h post-infection (Otten *et al.*, 2021). This indicates that RNF213 constitutes a second but massive response wave to fend off cytosolic bacteria. Nevertheless, it remains to be clarified whether RNF213—like HOIP—requires a priming ubiquitylation event for its recruitment to *Salmonella* or depends on other cellular or bacterial factors for its translocation to cytosolic bacteria.
bacteria. Conversely, it is fully possible that Ub moieties generated by RNF213 on bacterial LPS are extended by the other xenophagic ligases.

While the chain type preference of RNF213 is not known (Otten et al., 2021) and ablation of ARIH1, Smurf1, Parkin or HOIP reduces the bacterial ubiquitin coat by the chain type that is specifically generated by these E3 ligases (Manzanillo et al., 2013; Franco et al., 2017; Noad et al., 2017; Polajnar et al., 2017) their impact on other chain topologies has not been assessed comprehensively. However, the analysis of M1 chain decorated cytosolic bacteria in cells depleted of ARIH1 unveiled a first glance on an unexpected interplay between the different anti-bacterial ligases. Surprisingly, HOIP’s activity compensated for a reduction of ARIH1 (Polajnar et al., 2017). While it remains to be seen whether loss of LRSAM1, Smurf1, or Parkin likewise triggers a robust increase in M1 chains on Salmonella and whether RNF213 plays a role in this compensatory ubiquitylation axis, the finding from ARIH1 depleted cells suggests that different E3 ligases may be competent for available Ub acceptor sites within the bacteria coat. Even more surprising, ARIH1 recruitment to Salmonella was found to be increased in the absence of LRSAM1 or HOIP which both tend to follow ARIH1 on cytosolic bacteria (Polajnar et al., 2017). This observation raises the question about the existence of feedback loops or hand-over mechanisms between ARIH1, LRSAM1, and HOIP. A systematic mutual analysis of recruitment and activity dependencies would be highly informative to understand the coordinated action of the different anti-bacterial ligases. However, such a study is currently lacking.

With the advantages in super-resolution microscopy in the last years, it became increasingly clear that the Ub coat is a heterogenic assembly composed of at least K48, K63, and M1 chain types (van Wijk et al., 2012, 2017; Noad et al., 2017), which serve as specific direct binding sites on cytosolic bacteria for distinct cellular factors. These include selective autophagy receptors such as NDP52, p62, OPTN, and NBR1, innate immunity components such as NEMO and LUBAC as well as proteasomal subunits (Thurston et al., 2009; Cemma et al., 2011; Wild et al., 2011; Manzanillo et al., 2013; Franco et al., 2017; Noad et al., 2017; van Wijk et al., 2017). From this array of Ub-binding partners, it is evident that the bacterial Ub coat integrates a number of different anti-bacterial functions ranging from xenophagy to NF-κB signaling which cooperatively ensures an efficient defense against cytosolic pathogens. Given that the pan-Ub antibody FK2 recognizes almost all chains except K6 (Noad et al., 2017) and that the bacterial Ub coat has not been comprehensively sampled with chain type-specific antibodies, we are still far away from grasping the full repertoire of host processes triggered by this assembly.

Hijacking the ubiquitin machinery by pathogenic bacteria

Bacterial pathogens, that invade and reside in host cells, have evolved various strategies to divert the host pathways for their advantage. They achieve these feats by secreting effector proteins or toxins that modify or interact with the host protein. Secretion of these effector proteins differs between species of bacteria, the diversity of the strain within species, and the exposure to different cytosolic proteins in different host cell types. Mediated by these proteins, Salmonella directs macroautophagy to facilitate its replication (Yu et al., 2014), Listeria modulates host actin protein to escape LC3-positive membranes in macrophages (Cheng et al., 2018), Shigella modifies histone to inhibit activation of NF-κB signaling (Arbibe et al., 2007), and many more. In this section of the review, we will only focus on the strategies employed by the cytosolic bacteria to evade or hijack the Ub machinery.

To prevent modification by E3 ligases and subsequent host responses, cytosol-dwelling bacteria have evolved a number of different strategies: First, some bacteria actively avoid leaving their protective vacuoles. For example, at an early stage of infection when Salmonella resides in endosomes the bacteria divert several autophagy pathway components to repair damage on their surrounding membranes. This prevents the exposure of bacteria to the cytosol and allows a stepwise maturation of SCVs via recruitment of early and late endosomal markers as well as acidification (Kreibich et al., 2015). Likewise, Coxella decorates its vacuole with LC3 to delay fusion with lysosomes (Beron et al., 2002; Gutierrez et al., 2005).

Second, other bacteria prevent ubiquitylation of their surfaces by molecular disguises (Table 1). Francisella tularensis modifies its surface with polysaccharide O-antigen (Case et al., 2014), whereas Shigella secretes the effector protein IscB to camouflage its own outer membrane protein VirG (Ogawa et al., 2005) and Listeria evades detection by recruiting actin via its secreted effector ActA (Yoshikawa et al., 2009). Ricettettia uses its two protein–lysine methyltransferases (PKMTs) to methylate lysine residues of outer membrane proteins (Engstrom et al., 2021) whereas its outer membrane protein B (OmpB) physically shields other surface proteins in macrophages (Engstrom et al., 2019), thereby preventing their recognition and subsequent ubiquitylation.

Third, still other bacteria interfere with the various components of the xenophagy machinery. M. tuberculosis employs the effector SapM to hydrolyze phagosomal PI3P and bind Rab7 to inhibit maturation of phagosomes and autophagosomes to phagolysosomes and autolysosomes, respectively (Vergne et al., 2005; Hu et al., 2015). In contrast, GAS serotype M1T1 secretes the cysteine protease SpeB that degrades p62, NBR1, and NDP52 (Barnett et al., 2013) and Legionella’s RavZ effector protein irreversibly deconjugates LC3 from phosphatidylethanolamine (PE), thereby preventing engulfment by autophagosomes (Choy et al., 2012).

Fourth, inflammatory signaling is commonly targeted by various pathogens since multiple innate immunity sensing pathways including detection of bacterial or damaged host membranes converge on this response. Bacterial survival strategies often involve the concerted action of several different effectors. Intriguingly, a number of bacteria including Salmonella, Shigella, and Legionella have developed E3-like enzymes which specifically utilize the host ubiquitin system in their favor. This is remarkable since these pathogens do not possess genes encoding for ubiquitin themselves. An emerging theme of these mimicry effectors is to interact with host E3 ligases and modulate their functions in different ways to inhibit innate immune signaling. The Salmonella HECT-like E3 ligase SopA engages two tripartite motif-containing (TRIM) E3 ligases TRIM56 and TRIM65 which are involved in type I interferon expression (Kamanova et al., 2016). SopA inhibits both ligases via binding to their respective RING domains and triggering the proteasomal degradation of TRIM56 and TRIM65 by mediating their K48 and K11 polyubiquitylation (Fiskin et al., 2017). Moreover,
Salmonella SspH1, SspH2, and SlrP represent so-called novel E3 ligases (NELs) which do not share structural homology with any member of the three eukaryotic ligase families. NELs bind to host E2 enzymes, transfer the charged ubiquitin to a catalytic cysteine residue within their conserved ligase domain and use LRRs of varying lengths to interact with host substrates (Hicks & Galan, 2010; Maculins et al, 2016). By SspH1-, SspH2- and SlrP-mediated ubiquitylation of serine/threonine-protein kinase N1 (PKN1), nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and thioredoxin (TXN), respectively, Salmonella counteracts NF-κB signaling and promotes host cell death (Haraga & Miller, 2006; Bernal-Bayard and Ramos-Morales, 2009; Bhavsar et al, 2013; Keszei

Table 1. Effector proteins employed by bacterial pathogens to escape or hijack components of the host ubiquitin machinery

| Pathogen                     | Effector | Protein type     | Mechanism of action                                                                 | Reference                        |
|------------------------------|----------|------------------|-------------------------------------------------------------------------------------|----------------------------------|
| *Salmonella Typhimurium*     | SopA     | HECT-like        | Ubiquitylates host E3 ligases TRIM65 and TRIM56 thereby blocking the expression of interferon-γ and nucleic acid sensing receptor STING | Fiskin et al, (2017)            |
|                              | SspH1    | Novel E3 ligase family | Ubiquitylates serine/threonine kinase PKN1 resulting in its proteasomal degradation and suppression of NF-κB pathway | Keszei et al, (2014)             |
|                              | SspH2    | Novel E3 ligase family | Ubiquitylates and activates NOD1 signaling                                             | Bhavsar et al, (2013)           |
|                              | SlrP     | Novel E3 ligase family | Ubiquitylates host thioredoxin (TXN) inducing host cell death                         | Bernal-Bayard and Ramos-Morales (2009) |
|                              | SseL     | Deubiquitinase    | Removes Ub aggregates on *Salmonella* and SCVs                                        | Mesquita et al, (2012)          |
| *Legionella pneumophila*     | RavZ     | Cysteine protease | Inhibits recruitment of LC3 to LCV by irreversibly deconjugating LC3 from PE; Inhibits recruitment of Ub to LCV via unknown mechanism | Choy et al, (2012); Kubori et al, (2017) |
|                              | RavD     | Deubiquitinase    | RavD specifically hydrolyzes Met1-linked Ub chains directly antagonizing LUBAC activity and thus activation of NF-κB | Wan et al, (2019) |
| *Shigella flexneri*          | IpaH 1.4 | Novel E3 ligase family | Interacts with HOIL and conjugate K48 chains to the catalytic domain of HOIP, thereby triggering its proteasomal degradation | de Jong et al, (2016)           |
|                              | IpaH 2.5 | Novel E3 ligase family | Interacts with HOIL and conjugate K48 chains to the catalytic domain of HOIP, thereby triggering its proteasomal degradation | de Jong et al, (2016)           |
|                              | IpaH 4.5 | Novel E3 ligase family | Binds to p65 subunit of NF-κB and modulates inflammatory response; Promotes K48 polyubiquitylation mediated degradation of TBK1, thereby shutting down the host anti-bacterial response | Wang et al, (2013) and Zheng et al, (2016) |
|                              | IpaH 9.8 | Novel E3 ligase family | Ubiquitylates NEMO leading to its proteasomal degradation, thereby inhibiting the NF-κB signaling pathway | Ashida et al, (2010)            |
|                              | IpaH 0722 | Novel E3 ligase family | Inhibits PKC-mediated activation of NF-κB by ubiquitylating TRAF2 for proteasomal degradation | Ashida et al, (2013)            |
|                              | IcsB     | Fatty acyltransferase | Represses the recruitment of LC3 and NDP52 to *Shigella*; Escapes host autophagy indirectly by mediating fatty acylation and inactivation of CHMP5 protein | Bax and Goldberg (2014); Liu et al, (2018) |
| *Listeria monocytogenes*     | ActA     | Actin assembly-inducing protein | Recruits Arp2/3 complex and Ena/VASP to disguise the bacterial surface from autophagic recognition | Yoshikawa et al, (2009)          |
| *Mycobacterium tuberculosis* | SapM     | Acid phosphatase | Prevents fusion of lysosomes with mycobacterium containing phagosomes | Zulauf et al, (2018)            |
|                              | PknG     | Ser-Thr protein kinase | Ubiquitylation of TRAF2 and TAB1 resulting in their proteasomal degradation | Wang et al, (2021)              |
| *Group A Streptococcus (GAS)*| SpeB     | Cysteine protease | Degrades p62, NDP52, and NBR1 in the host cytosol                                      | Barnett et al, (2013)           |
| *Rickettsia parkeri*         | OmpB     | Outer membrane protein | Acts as a protective shield obstructing recognition by the autophagy machinery       | Engstrom et al, (2019)          |
|                              | PKMT1/2  | Lysine methyltransferase | Modifies the lysine of outer membrane proteins with methyl groups and prevents their recognition by the host ubiquitin machinery | Engstrom et al, (2021)          |
et al., 2014). In contrast, Shigella secretes the NEL effector IpaH1.4 and IpaH2.5 that directly interact with HOIL-1L and attach K48 chains to HOIP causing its proteasomal degradation and inhibition of NF-kB pathway activation (de Jong et al., 2016). In parallel, ubiquitylation and degradation of the NF-kB signaling components TRAF2, NEMO, and p65 are mediated by the Shigella NELs IpaH0722, IpaH9.8, and IpaH4.5, respectively (Ashida et al., 2013; Ashida & Sasakawa, 2015). In another notable example, M. tuberculosis protein kinase G (PknG) effector protein, which is a eukaryotic-type serine/threonine kinase, inhibits NF-kB signaling by acting as both E1 and E3 enzyme to trigger ubiquitylation and degradation of TRAF2 and TAK1 (Wang et al., 2021).

Lastly, Legionella RavD has deubiquitinase activity, which exclusively cleaves M1-linked Ub chains in a similar manner than the host DUB OTULIN, and hence directly antagonizes the activity of LUBAC. Secreted RavD removes M1 chains from Legionella-containing vacuoles and thereby suppresses NF-kB signaling (Wan et al., 2019). Given the importance of Ub modifications, in particular M1 chains, for the defense against pathogens it is somewhat surprising that RavD is currently the only bacterial DUB known to hydrolyze M1 chains. However, effectors with deubiquitinase activity from other intracellular pathogens might have gone unrecognized since bacterial DUBs are very divergent from eukaryotic DUBs (Hermanns & Hofmann, 2019).

Concluding remarks and outlook

Since the discovery of the first bacteria-targeting Ub E3 ligase, our knowledge about the role of Ub in host cell responses against cytosol intruding pathogens increased substantially—both at the level of the involved conjugation machinery and at the level of generated Ub chains. Since the in vitro and in vivo activities of the identified ubiquitin E3 ligases (ARIH1, LRSAM1, LUBAC, Smurf1, Parkin, and RNF213) do not exactly match the spectrum of chain types decorating cytosolic bacteria, the emerging picture is likely to become even more complex. A challenging task will be to systematically decipher the interdependence of these different E3 ligases with regard to their recruitment to bacteria and activation of ubiquitylation (Box 1). With the recent work from the Rainbow and Dikic laboratories (Noad et al., 2017; van Wijk et al., 2017), it becomes increasingly clear that Ub is not only attached to mark bacteria for clearance by xenophagy. Therefore, another important task will be to identify the full array of Ub chain type-specific binding effector proteins and their anti-bacterial activities. Lastly, it will be crucial to test these Ub-mediated host cell detection and response cascades in (patho)physiological relevant settings across a number of different cytosol-dwelling pathogens.

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Conflict of interest

The authors declare that they have no conflict of interest.

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