Review

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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—inventively 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
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See the Glossary for abbreviations used in this article.

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, autophagosome fuses 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; Rubinsztein *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

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Glossary

ARIH1	ARIadne 1 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	Cullin RING ligase
DUB	Deubiquitinating enzyme
GAS	Group A streptococcus
GIR	Galectin interacting region
GWAS	Genomewide association study
HECT	Homologous to E6AP carboxyl terminus
HHAR1	Homologues to human ARIadne 1
HOIL-IL	Heme-oxidized IRP2 Ub ligase 1L
HOIP	HOIL-1-interacting protein
IBR	In between RING
IKK	IκB 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	NF-kB essential modifier
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 LINear 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
Smurf1	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
TRAF2	TNF receptor-associated factor 2
TRIM21	Tripartite motif-containing protein 21
TXN	Thioredoxin
Ub	Ubiquitin
UBA	Ubiquitin-associated
UBL	Ubiquitin-like domain
UPD	Unique Parkin domain
UVRAG	UV radiation resistance associated
ZF	Zinc finger

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 directly associates with the autophagy receptor NDP52 (also known as CALCOCO2) via its the galectin-interacting region (GIR) motif (Thurston *et al*, 2012).

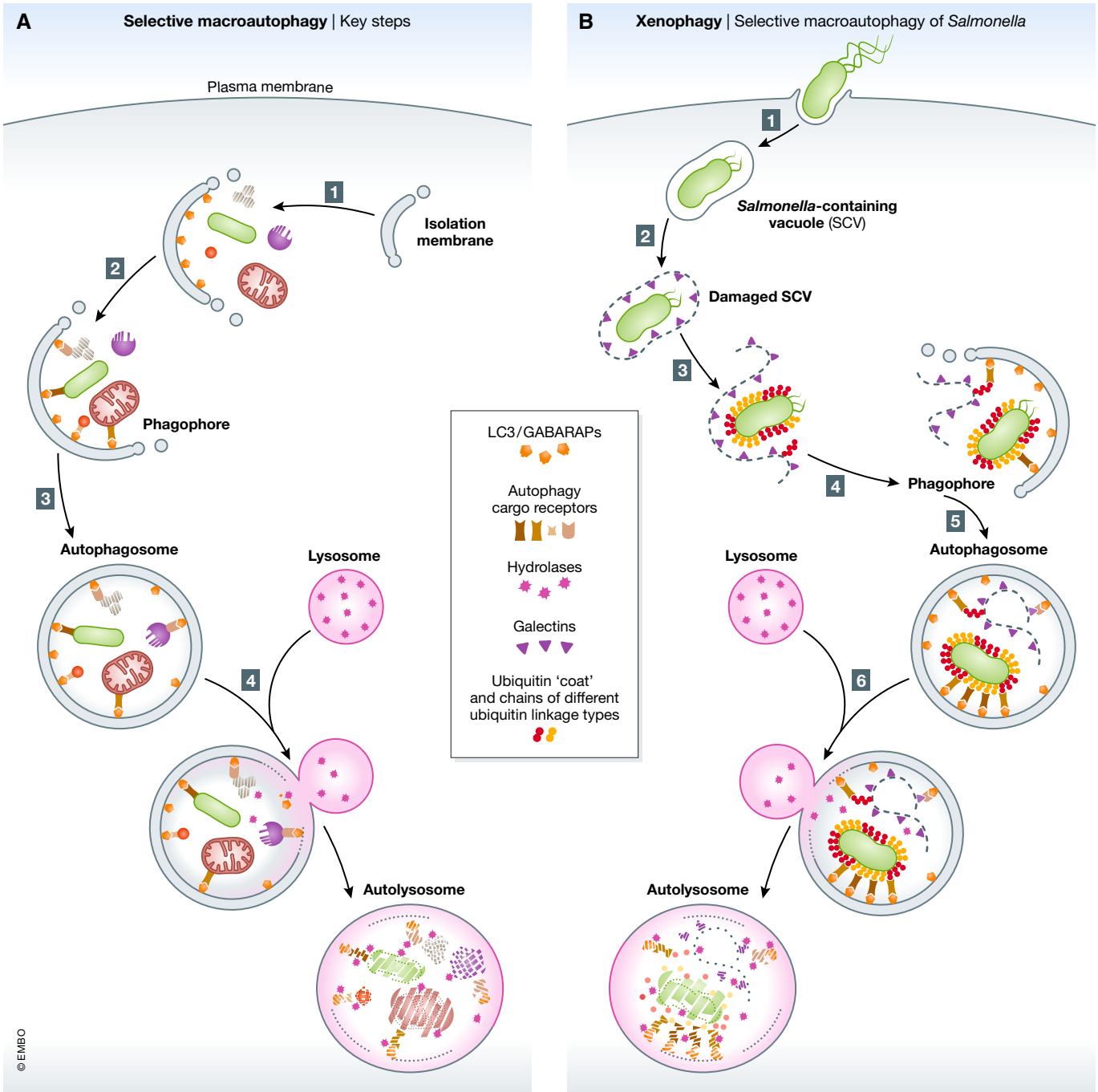

Figure 1. Selective macroautophagy.

(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 Ub machinery and trigger the formation of a dense Ub coat dressing the whole *Salmonella* (Perrin *et al*, 2004). Ubiquitylation, 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; Buetow & 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 (˜ 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 ubiquitylation targets forming the Ub coat were assessed by quantitative proteomics of *S*. Typhimurium-infected epithelial cells and revealed ubiquitylation 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 ubiquitylate 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 glycans (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 LC3C (von Muhlinen *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 (Deshaies & 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*,

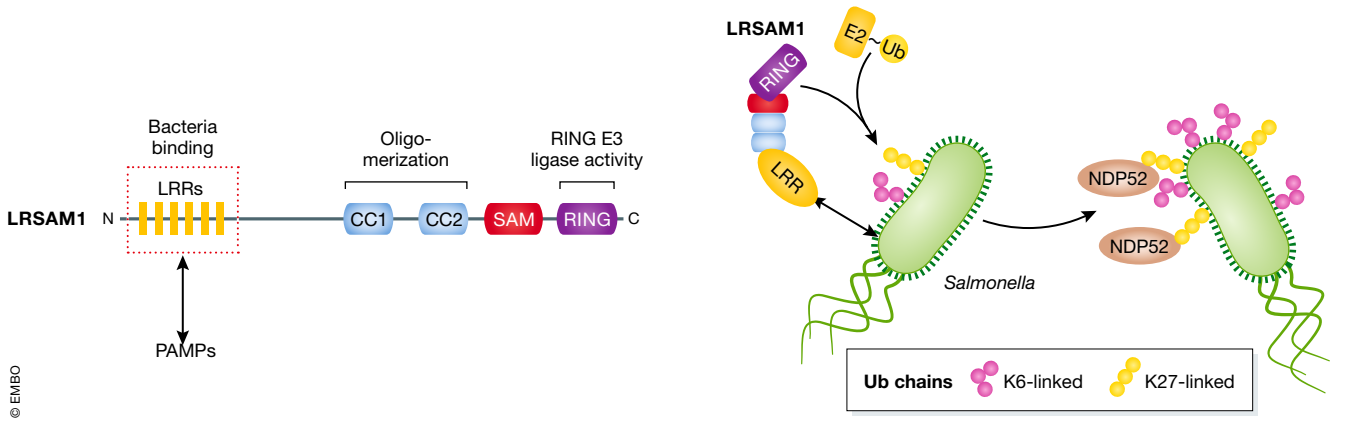

Figure 2. The RING-type E3 ligase LRSAM1 in Ub coat formation of cytosolic *Salmonella*.

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.

2020). Apart from the C-terminal RING domain, LRSAM1 contains a leucine-rich repeat (LRR) domain at the N terminus, a sterile alpha motif-containing domain (SAM domain), and two coiled-coil domains (CC1 and CC2), which are known to regulate the activity of E3 ligases by forming self-associated oligomers (Bian *et al*, 2017).

LRRs are typically involved in the detection of pathogen-associated molecular patterns (PAMPs) making LRSAM1 a suitably designed E3 for recognizing and ubiquitylating cytosolic bacteria. Notably, an LRR-mediated recognition mechanism by E3 ligases was already proposed at the time when the Ub coat was first observed (Perrin *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/NDP52 colocalization. However, LRSAM1 and NDP52 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 NDP52 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/NDP52 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

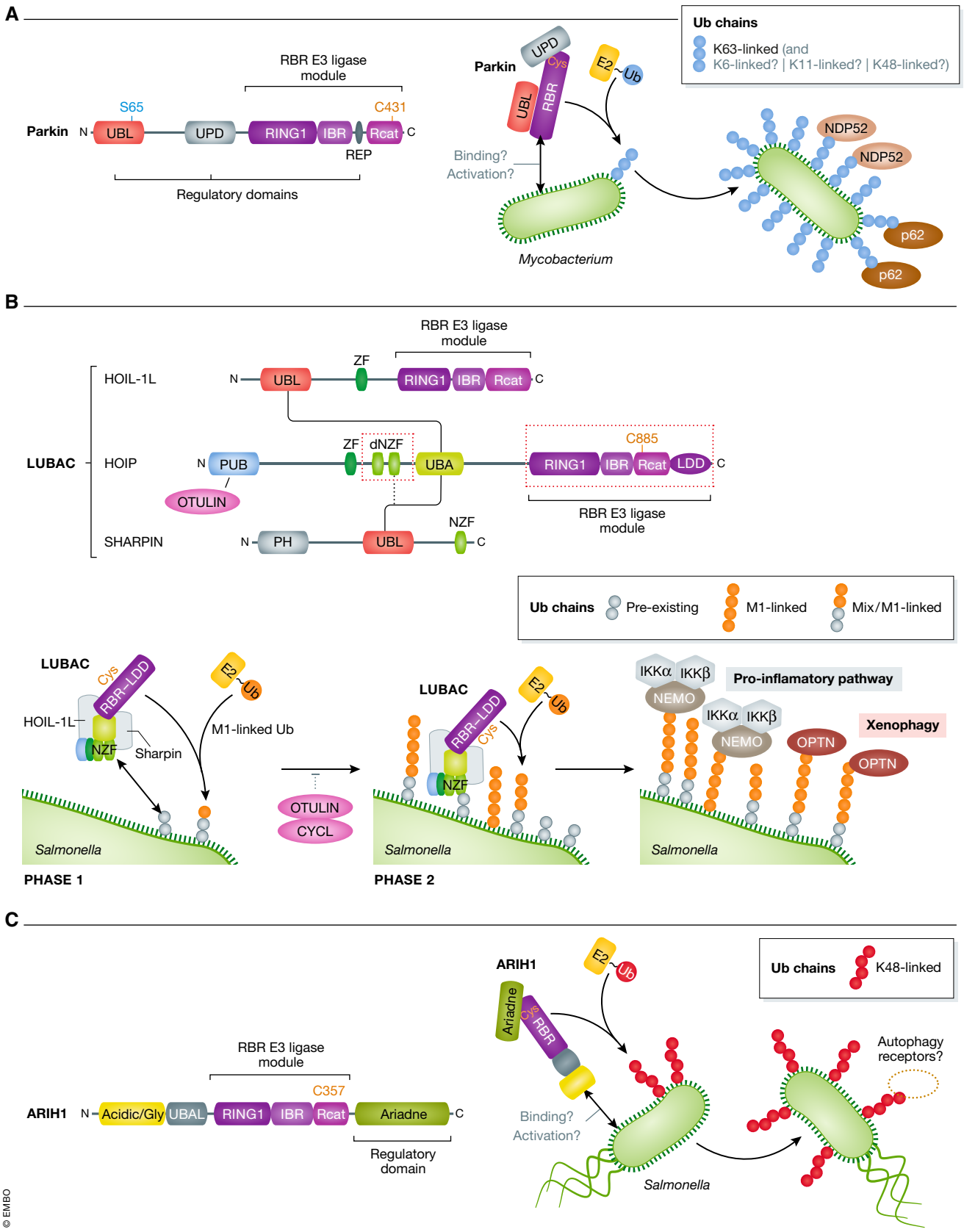

Figure 3.

Figure 3. RBR-type E3 ligases involved in coating cytosolic bacteria with ubiquitin.

(A) Diagram of Parkin showing the Ub-like (UBL) domain, the unique parkin domain (UPD), and the repressor element (REP), which form the regulatory part of Parkin, and the catalytic part comprising the RBR module at the C terminus. The mechanism for activation of Parkin in response to bacterial infection remains to be addressed but upon *Mycobacterium* infection, activated Parkin decorates the cytosolic bacteria with an Ub coat (predominantly K63 chains) that recruits p62 and NDP52 autophagy adaptors triggering xenophagy. (B) Domain organization of the three subunits of LUBAC: HOIP, HOIL-IL, and SHARPIN. HOIL-IL and SHARPIN interact via their respective UBL domain with the UBA domain of HOIP (solid lines), with potential contribution of the double NZF (dNZF) domain (dotted line). The dNZF and RBR domains of HOIP (red dotted boxes) are required for binding *Salmonella* in a two-phase mechanism: in the first phase, LUBAC binds to preexisting Ub chains on *Salmonella*, in the second phase, enhanced HOIP activity mediates M1- and mixed M1-linked Ub chains synthesis on these preexisting chains and triggers further LUBAC binding. The M1-Ub chains recruit adapter proteins OPTN and NEMO which initiate xenophagy and pro-inflammatory response, respectively. DUB activity of OTULIN and CYLD may counterbalance LUBAC activity. (C) Schematic view of ARIH1 domains showing the acidic/glycine region, UBA-like domain (UBAL), the RBR module, and the regulatory Ariadne domain at the C terminus. ARIH1 decorates the *Salmonella* with K48-Ub chains. The mechanism of its activation, binding to the cytosolic *Salmonella,* and the downstream action of ARIH1-mediated K48-Ub chains are not understood.

the UPD domain masks the catalytic cysteine 431 (C431) in Rcat preventing the formation of the E3˜Ub thioester intermediate (Chaugule *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 transthiolation. 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 mitochondria, however, and rather surprising, it was shown that Parkin is required for *M. tuberculosis* xenophagy *in vitro*. *M. tuberculosis* is a vacuolar 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 *Parc2* 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 investigation.

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 phagophore, which gives access to galectin-8-dependent Parkin-mediated ubiquitylation of the GAS/phagophore 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

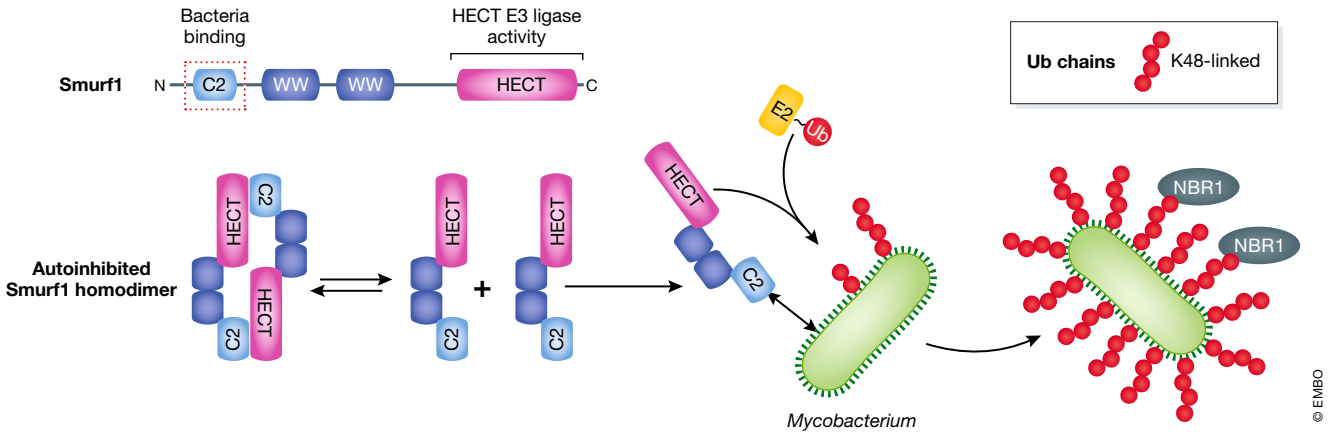

Figure 4. The HECT-type E3 ligase Smurf1 in Ub coat formation of cytosolic *Mycobacterium*.

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, Smurf1 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.

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 morphogenic protein (BMP) and transforming growth factor beta (TGF-β) signaling pathways, which play an essential role in embryogenesis and adult tissue homeostasis (Cao & Zhang, 2013). However, it is now evident that Smurf1 regulates a plethora of different cellular and developmentally important signaling pathways and networks impacting on a wide spectrum of biological roles including cell proliferation, DNA damage response, chromatin organization, and selective autophagy (recently reviewed in Koganti *et al* (2018)). The first hint linking Smurf1 to xenophagy came from Orvedahl *et al* (2011) who identified Smurf1 in a genomewide small interfering RNA screen for genes essential for autophagy of viruses and damaged mitochondria (Orvedahl *et al*, 2011). Drawing parallels between mitophagy and xenophagy, Franco *et al* (2017) subsequently showed that Smurf1 colocalizes with *M. tuberculosis*-containing compartments mediating xenophagic degradation and suppression of *M. tuberculosis* replication in macrophages (Franco *et al*, 2017).

Smurf1 is a member of the HECT E3 Ub ligase family (Huibregtse *et al*, 1995; Scheffner & Staub, 2007; Scheffner & Kumar, 2014; Lorenz, 2018). HECT E3s have a modular architecture characterized by the C-terminal HECT domain that consists of 2 lobes: the larger N-lobe associates with the E2 enzyme (Huang *et al*, 1999) and the C-lobe contains the catalytic cysteine and interacts with the Ub during the transfer reaction from the E2 (Scheffner *et al*, 1995; Kamadurai *et al*, 2009; Lorenz *et al*, 2013). While the HECT domain represents the catalytic module, substrate recruitment and regulation of the catalytic activity of HECT E3s are determined by their respective N-terminal extensions (Sluimer & Distel, 2018). Specifically, Smurf1 belongs to the C2-WW-HECT (also known as NEDD4-like) subfamily (Fig. 4) with two central WW domains which usually control protein–protein interaction by recruiting substrates and adaptor proteins containing proline-rich PY motifs (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 spaciotemporal 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-

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 Ub 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 know so far to be specific for the synthesis of M1 linkages is the linear Ub assembly complex (LUBAC), a multimeric 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; Stieglitz *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 the charged E2, UBE2D˜Ub (Lechtenberg *et al*, 2016). This structure uncovered how UBE2D˜Ub is tightly embraced by the RBR, orientating the E2˜Ub thioester bond in close proximity to C885 primed to promote trans-thiolyation. Furthermore, an allosteric Ub-binding site was revealed. Ub or M1-linked di-Ub binding to this site apparently promotes binding efficiency of UBE2D˜Ub 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 dNFZ 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 chain extension (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 bacteria 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-κB 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-κB 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 ARIadne 1 (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 ARHI1 binds directly to neddylated cullins (cullin-1, -2, -3, 4a, and -4b) and to numerous neddylated cullin 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 E2˜Ub (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 dynein-like AAA+ motor domain and a RING-type domain-containing E3 ligase module (Ahel *et al*, 2020) (Fig. 5). Interestingly, the dynein-like AAA+ motor domain is required for LPS ubiquitylation, whereas—unexpectedly—no such requirement was found for the RING domain (Ahel *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: Ahel *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

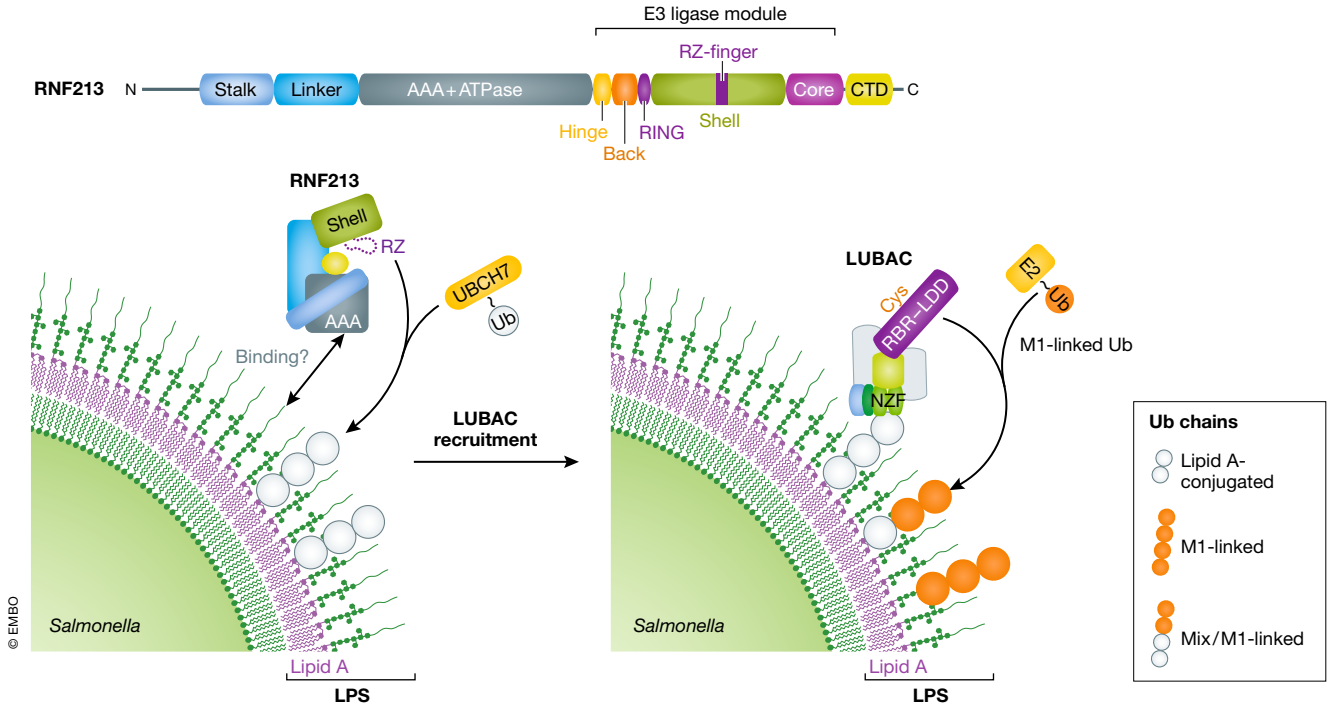

Figure 5. The atypical E3 ligase RNF213 ubiquitylates LPS on *Salmonella*.

Schematic representation of the domain organization of RNF213 consistent of N-terminal stalk, the central AAA+ ATPase, and the E3 ligase module. The AAA+ and RZ-finger domains are required for ubiquitylation of lipid A/LPS on *Salmonella's* outer surface. These Ub chains recruit LUBAC-mediated xenophagy and pro-inflammatory response pathways (see also Fig 3B).

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

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, *Coxiella* 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). *Rickettsia* 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,

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-kB 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-kB	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	Baxt 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 methlytransferase	Modifies the lysine of outer membrane proteins with methyl groups and prevents their recognition by the host ubiquitin machinery	Engstrom *et al*, (2021)

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 ligases 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 & Ramos-Morales, 2009; Bhavsar *et al*, 2013; Keszei

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-κB pathway activation (de Jong *et al*, 2016). In parallel, ubiquitylation and degradation of the NF-κB 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-κB 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-κB 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 Randow 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.

Box 1. In need of answers

- What determines the recognition and association of the E3 ligase machineries with cytosolic bacteria? What constitutes the specificity of xenophagic E3 ligases?
- What is the full substrate spectrum on cytosolic bacteria that is conjugated with ubiquitin? Which bacterial outer membrane proteins are ubiquitylated by which E3 ligase? Does RNF213 ubiquitylate other non-protein targets than LPS which is only present in gram-negative bacteria?
- What is the precise Ub chain type composition and architecture of the Ub coat on cytosolic bacteria? Is the Ub coat bacteria-specific?
- How are Ub and autophagy receptor microdomains on bacterial surfaces established and what are their roles in orchestrating xenophagic and innate immune response pathways?

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Conflict of interest

The authors declare that they have no conflict of interest.

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