



Interactions of bacterial proteins with host eukaryotic ubiquitin pathways

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Ubiquitination is a post-translational modification in which one or more 76 amino acid polypeptide ubiquitin molecules are covalently linked to the lysine residues of target proteins. Ubiquitination is the main pathway for protein degradation that governs a variety of eukaryotic cellular processes, including the cell-cycle, vesicle trafficking, antigen presentation, and signal transduction. Not surprisingly, aberrations in the system have been implicated in the pathogenesis of many diseases including inflammatory and neurodegenerative disorders. Recent studies have revealed that viruses and bacterial pathogens exploit the host ubiquitination pathways to gain entry and to aid their survival/replication inside host cells. This review will summarize recent developments in understanding the biochemical and structural mechanisms utilized by bacterial pathogens to interact with the host ubiquitination pathways.

Keywords: ubiquitin, E3 ligase, F-box, DUB, bacterial pathogen

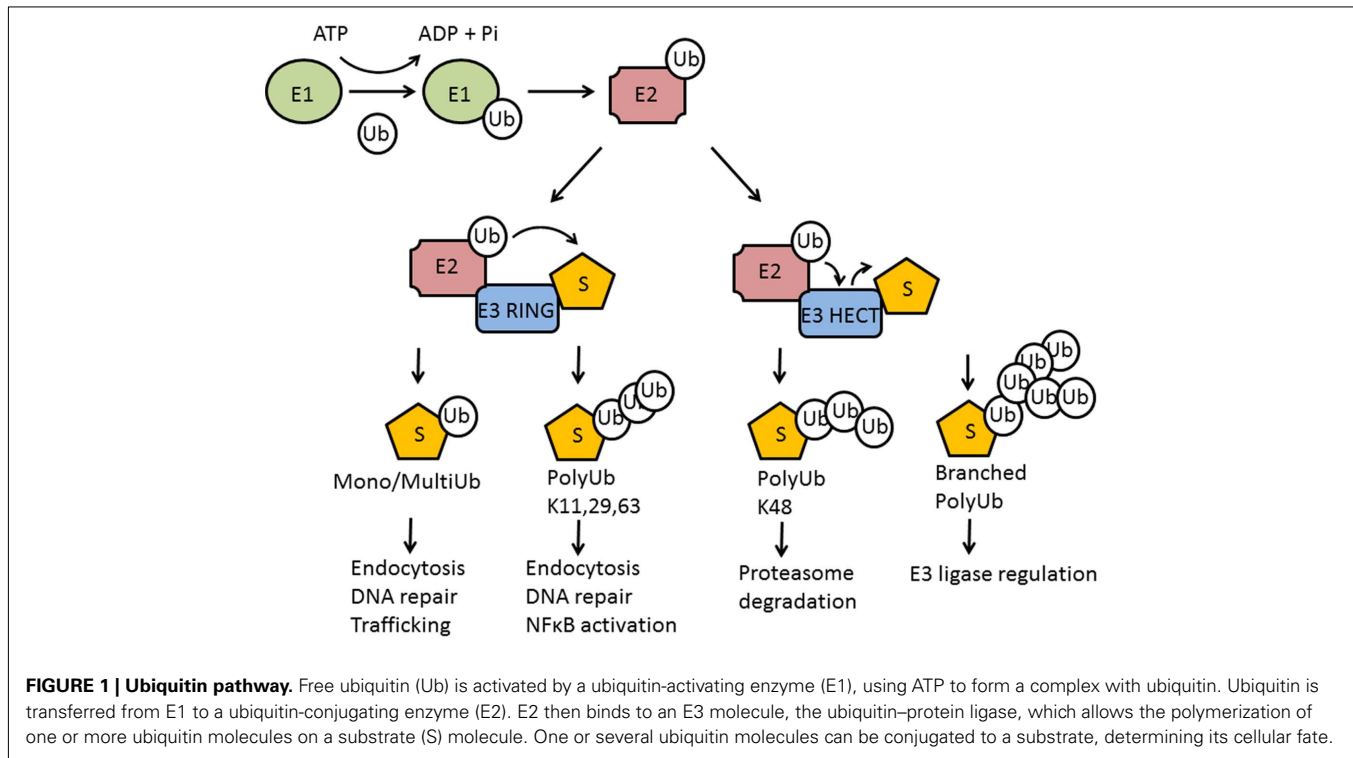
INTRODUCTION

Discovery of the ubiquitination system follows an elegant classical biochemistry story, culminating with the award of the 2004 Nobel Prize in Chemistry to three contributors: Dr. Avram Hershko, Dr. Aaron Ciechanover, and Dr. Irwin Rose. The story started with a search for a non-lysosomal pathway for protein degradation. The lysosome was initially given credence for the responsibility of protein turnover in a cell. However, several aspects of the lysosomal mode of proteolysis did not fit data that was emerging, and thus a search for an alternative mechanism began. The breakthrough came with the use of crude extracts from reticulocytes: immature red blood cells which lack lysosomes. Protein degradation was shown to require two complementing fractions resolved from the crude extract: one containing protease activity and the other containing a small heat-stable protein, ATP-dependent proteolytic factor 1 (APF-1), which stimulated proteolysis (Ciechanover et al., 1978). Studying the mechanism of action of APF-1 led to the discovery that this protein is in fact the polypeptide ubiquitin (Goldstein et al., 1975; Ciechanover et al., 1978; Wilkinson et al., 1980). Additional study led to the elucidation of the multi-enzyme cascade which allows ubiquitin modification of proteins, and which firmly established the key steps involved in the ubiquitin–proteasome system (UPS; Ciechanover et al., 1981; Hershko et al., 1983; Reiss et al., 1989).

The UPS consists of three classes of enzymes known as ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin–protein ligases (E3; **Figure 1**). The ubiquitination reaction is initiated when ubiquitin is activated by the ubiquitin-activating enzyme, E1. A thioester bond forms between the active cysteine residue of E1 and the C-terminus of ubiquitin in an ATP-dependent reaction. Following ubiquitin activation, activated ubiquitin is transferred to a ubiquitin-conjugating enzyme, E2 (also known as Ubc), in another ATP-dependent reaction. Finally, with the help of a

ubiquitin–protein ligase, E3, a covalent bond is formed between the C-terminus of ubiquitin and the ϵ -amino group of a lysine residue on the protein substrate. One round of ubiquitination produces a monoubiquitinated protein. Typically, successive rounds occur, producing a protein with a polyubiquitinated chain. Each additional ubiquitin moiety is attached via the internal lysine of the previously conjugated ubiquitin molecule. There are seven lysine residues within the ubiquitin molecule: K6, K11, K27, K29, K33, K48, and K63, and all seven lysines can be used to link ubiquitin subunits into polyubiquitin chains *in vitro* (Kim et al., 2007). *In vivo*, the specific lysine modification used in the polyubiquitin chain translates into the fate of the protein (**Figure 1**). The formation of a K48-linked polyubiquitin chain usually leads to the substrate being targeted for proteasome degradation (Hough et al., 1986; Ganoth et al., 1988). The formation of a K63-linked polyubiquitin chain provides the substrate with the ability to act as a scaffold, allowing it to assemble signaling complexes and regulate protein localization, protein kinase activation, DNA repair, or transcription through proteasome-independent mechanisms (Pickart, 2001; Weissman, 2001).

The E1 ubiquitin-activating enzymes are well conserved in eukaryotes (Hershko et al., 1983; Chiu et al., 2007). For many years it was believed that only one E1 existed in eukaryotic cells (Zacksenhaus and Sheinin, 1990; Mcgrath et al., 1991). Recently, however, an E1-like protein, termed E1–L2, was also found to activate ubiquitin (Chiu et al., 2007; Jin et al., 2007; Pelzer et al., 2007). Unlike the E1 family, there are many more members of the E2 and E3 family of proteins, with the E3 family being the largest (Hershko and Ciechanover, 1998). E3s play pivotal roles in defining the specificity of target proteins to be ubiquitinated. There are two major families of E3 ubiquitin ligases; the homologous to E6–AP carboxyl terminus (HECT) domain family and the really interesting new gene (RING) family. The U-box family possess a finger domain that is a close relative of the RING motif and are



therefore often categorized as members of the RING family. Each family is differentiated by their distinct sequences, structures, and catalytic properties (Huibregtse et al., 1995; Lorick et al., 1999; Pickart, 2001). The HECT family E3 ubiquitin ligases contain a 350-residue region that maintains a strictly conserved cysteine residue located approximately 35 residues from the C-terminus (Huibregtse et al., 1995; Schwarz et al., 1998). This cysteine forms an essential thioester intermediate during catalysis (Huibregtse et al., 1995). The RING-finger family E3 ubiquitin ligases feature a set of cysteine and histidine residues that have a distinctive spacing to stabilize their globular conformation by interacting with two zinc ions (Lorick et al., 1999). Whereas RING ligases function as molecular scaffolds to bring E2 and the substrates into close proximity, HECT E3s participate directly in the chemistry of ubiquitination by accepting ubiquitin from an E2 enzyme, in the form of a ubiquitin–thioester intermediate, and thus directly catalyze protein ubiquitination (Huibregtse et al., 1995; **Figure 1**). Both HECT and RING proteins can be modular, single proteins, or multi-protein complexes, such as the Skp, Cullin, F-box (SCF)-containing complexes (Ardley and Robinson, 2005).

Ubiquitin modification of proteins is a process restricted to eukaryotes. Consequently, all of the enzymes that act on ubiquitin were originally believed to be confined to eukaryotes. However, given the importance of ubiquitination to such a large number of cellular processes required for normal cell function, it is perhaps no surprise that pathogens have developed mechanisms to manipulate the host ubiquitin pathway to their advantage. Viruses are known to modulate the host antigen presentation and immune responses by modulating the ubiquitination system (Shackelford and Pagano, 2005; Barry and Fruh, 2006; Gao and Luo, 2006; Lindner, 2007). It is now recognized that bacterial pathogens also manipulate the host ubiquitination system.

UBIQUITIN MODULATING EFFECTORS PROTEINS

Many Gram-negative bacteria encode sophisticated protein secretion and translocation systems to deliver bacterial virulence determinants across both the bacterial and host cell membranes into the infected cells. These bacterial virulence factors are often referred to as effectors due to their ability to exploit host cellular functions. Two of the most studied transport apparatuses are the type III and type IV secretion and translocation systems (Cornelis, 2006; Hayes et al., 2010). The biochemical and cellular functions of these effector proteins are subjects of intense study since they are likely to shed light on the molecular mechanisms utilized by bacterial pathogens to mount successful infections. In the last few years, it is becoming increasingly evident that many of these effectors interact with eukaryotic ubiquitination pathways to exploit host functions regulated by ubiquitination. We designate this group of effectors as ubiquitin modulating effectors (UME) Proteins.

E2-TARGETING UME

So far, only one example of a bacterial protein targeting a ubiquitin-conjugating/E2 enzyme exists: OspG in *Shigella flexneri* (Kim et al., 2005). *Shigella* is the etiological agent of human bacillary dysentery. Virulence is dependent on a type three secretion system (T3SS) encoded on a 200-kb plasmid (Buchrieser et al., 2000). Effectors encoded on the plasmid or on the chromosome are secreted and translocated by this T3SS to enable invasion of the colonic epithelium. *Shigella* is then able to induce the intense inflammatory response that is characteristic of *Shigella* infection.

OspG is a 196 residue protein secreted by the *Shigella* T3SS. A yeast two hybrid screen identified several E2 proteins as binding partners of OspG. This included UbcH5b, a component of the SCF^{β-TrCP} E3 complex, which promotes ubiquitination of phospho-IκBα and its subsequent degradation by the proteasome

(Karin and Ben-Neriah, 2000). I κ B α binds the transcription factor NF κ B, sequestering it in an inactive state in the cytoplasm. Phosphorylation of I κ B α , by I κ B kinase (IKK), allows I κ B α to be ubiquitinated and targeted for proteasome degradation. This releases NF κ B, allowing its translocation to the cell nucleus and the regulation of genes involved in inflammation, immunity, cell survival, and other pathways. OspG was shown to delay the degradation of phospho-I κ B α , and consequently reduce the transcription of NF κ B-regulated genes (Kim et al., 2005). *In vivo*, an *ospG* mutant enhances inflammatory responses. Thus *Shigella*, by using OspG to bind host E2 proteins, appears to reduce host inflammatory responses at early time points during infection. This may allow *Shigella* time to establish itself inside the host (Figure 2). An avirulent *Salmonella* strain has also been shown to block SCF $^{\beta}$ -TrCP function and prevent ubiquitination of I κ B α (Neish et al., 2000). The authors hypothesized that this may be one of the mechanisms by which gastrointestinal microflora are tolerated by the host. Such a mechanism is therefore likely to be used by other bacteria to promote their colonization of a host. As OspG was also shown to bind other E2 proteins, in addition to UbcH5b, there may be further unknown consequences to the ubiquitination processes during *Shigella* infection.

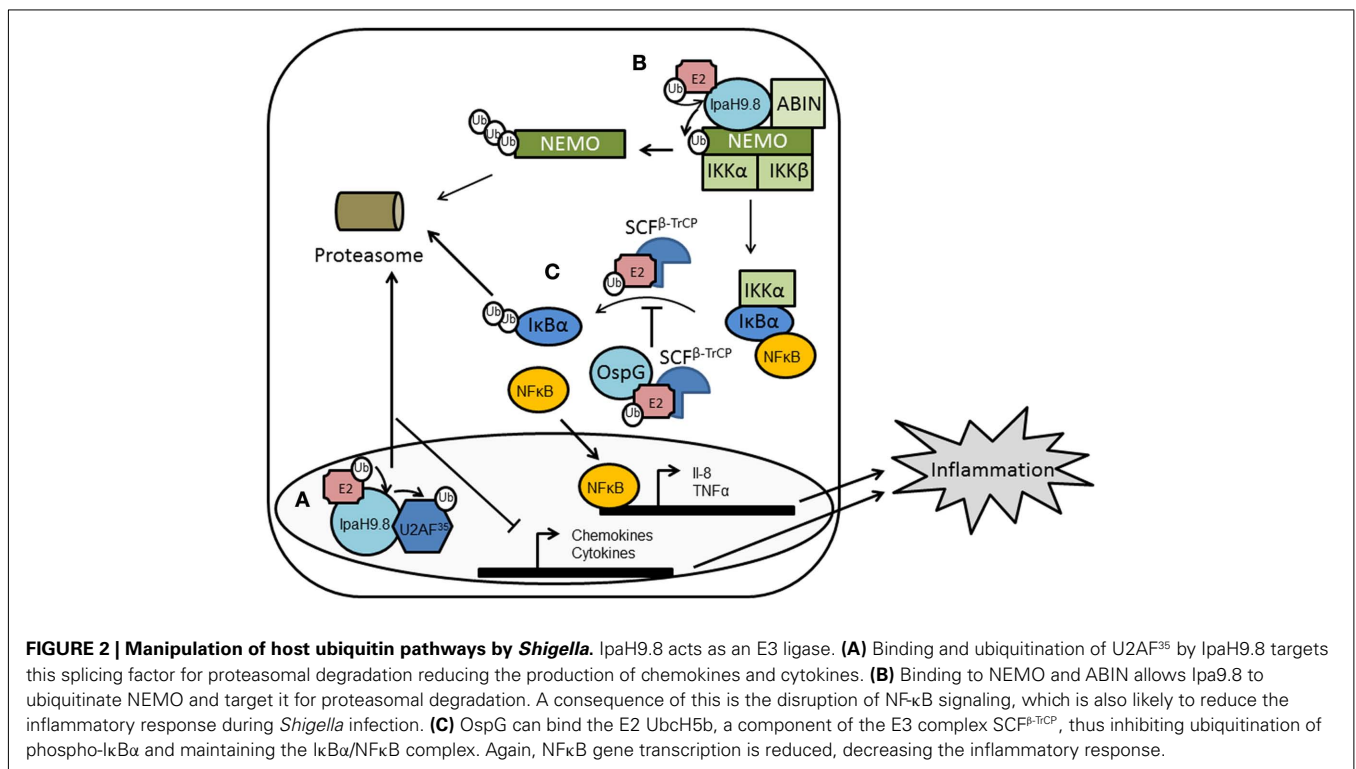
E3-TARGETING UME SALMONELLA

Salmonella utilizes two T3SS to establish itself inside host cells; the first, encoded by *Salmonella* Pathogenicity Island-1 (SPI-1), facilitates invasion of non-phagocytic cells, while the second, encoded by SPI-2, enables intracellular survival and replication (Lostro and Lee, 2001; Knodler and Steele-Mortimer,

2003). Multiple effectors are translocated through each T3SS to exploit host cell functions, and a major focus of *Salmonella* research is identifying and characterizing the function of each effector.

Salmonella SopA was initially identified as a SPI-1 effector in *Salmonella dublin* with a key role in the induction of enteritis (Wood et al., 2000; Zhang et al., 2002). However, the biochemical mechanism by which SopA induced this effect was unknown. The first clue suggesting SopA exploits host ubiquitination pathways came from a yeast two hybrid screen searching for host cellular proteins that interact with SopA. The yeast two hybrid assay indicated SopA interacts with human RMA1 (HsRMA1), a membrane-bound RING ubiquitin E3 ligase (Zhang et al., 2005). Bacterial SopA was shown to be ubiquitinated and degraded by the HsRMA1-mediated ubiquitination pathway *in vitro* and in a cell culture model (Zhang et al., 2005). One consequence of SopA ubiquitination and degradation by the proteasome appeared to be the escape of *Salmonella* into the cytoplasm of HeLa cells, where it could rapidly replicate. However, it is unclear whether this occurs *in vivo* during infection since *Salmonella* remains mostly within a *Salmonella*-containing vacuole (SCV; Knodler and Steele-Mortimer, 2003).

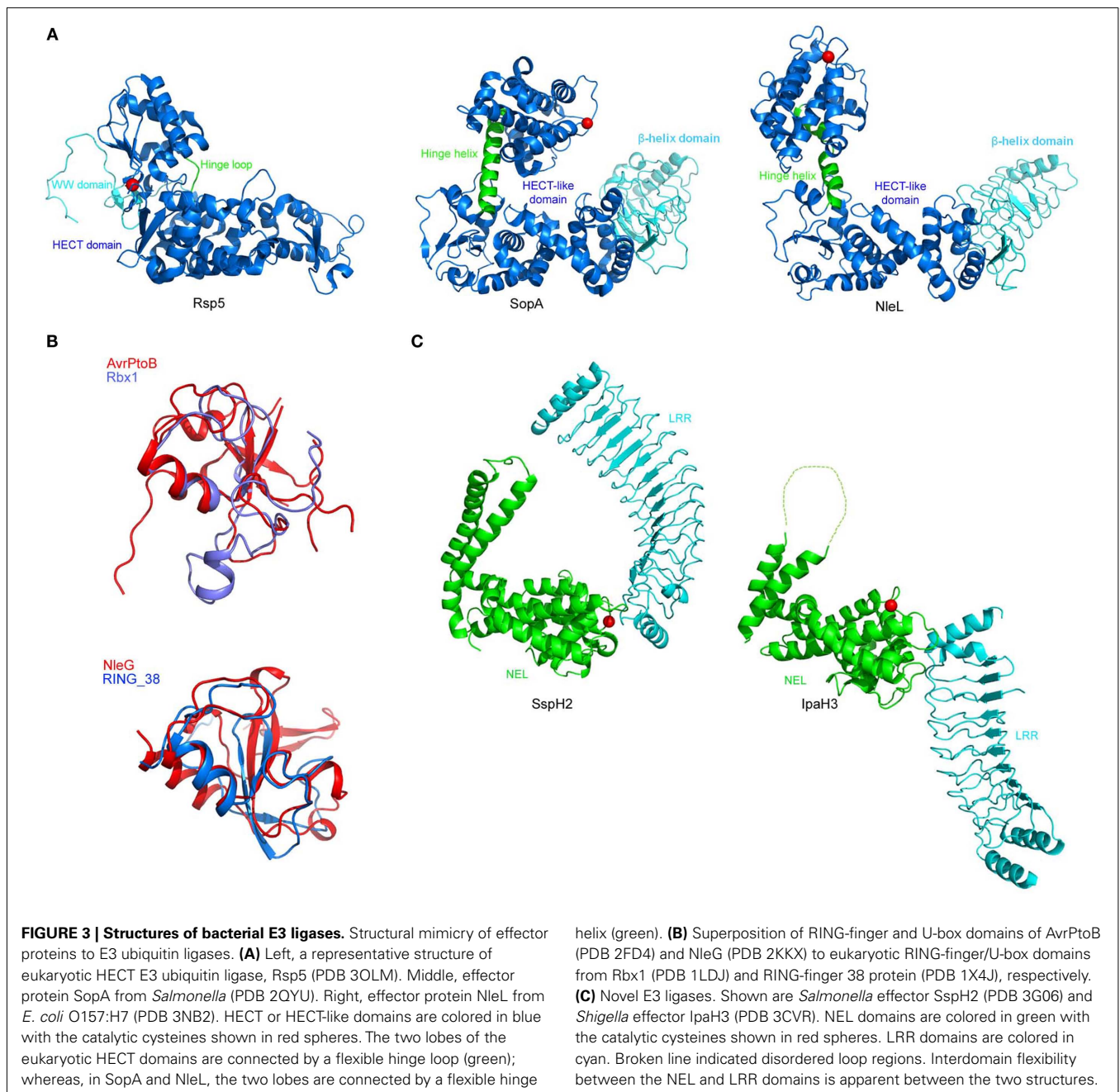
The E3 ubiquitin ligase activity of SopA itself was discovered accidentally when a mysterious banding pattern was observed in a routine ubiquitination assay in the absence of HsRMA1 (Zhang et al., 2006). This showed SopA possessed auto-ubiquitination activity: a key feature of most E3 ligases. *In vitro*, the ubiquitination kinetics of SopA is similar to other known E3 ubiquitin ligases (Coscoy et al., 2001; Lostroh and Lee, 2001; Matsuda et al., 2001; You and Pickart, 2001; Yamanaka et al., 2003).



Amino acid comparisons of SopA with HECT domain and RING-finger E3s did not reveal significant primary sequence similarities. However, it was determined that SopA has a cysteine residue (C753) 30 residues away from the C-terminus. Members of the HECT family E3 ubiquitin ligases feature a strictly conserved catalytic cysteine residue positioned approximately 35 residues from the C-terminus (Huibregtse et al., 1995; Scheffner et al., 1995; Dietrich et al., 1997; Hatakeyama et al., 1997; Yamamoto et al., 1997; Bates and Vierstra, 1999; Yanase and Ishi, 1999). This indicated SopA may also belong to this family. Biochemical assays confirmed that Cys753 is the active site of SopA for E3 ubiquitin ligase activity (Zhang et al., 2006). The crystal structure of

SopA further confirmed it as a mimic of eukaryotic HECT E3s (Diao et al., 2008). SopA shares structural similarity to HECT E3s, including the sequence of the active site loop, the bilobal architecture, and the conformational flexibility of the C lobe. However, it also possesses many unique characteristics (**Figure 3**). For example, the folding of the N and C lobes is different and the putative substrate binding domains are in closer proximity than most eukaryotic E3 HECT ligases, which may indicate SopA in fact belongs to a novel class of E3 ligases; only time will tell.

SopA was implicated in the induction of enteritis by *Salmonella*, in part by stimulating efficient polymorphonuclear leukocyte (PMN) transepithelial migration (Wood et al., 2000). The



catalytically inactive SopA^{C753S} mutant strain was found to induce significantly less PMN transepithelial migration as compared to wild-type *Salmonella* (Zhang et al., 2006). Thus, SopA E3 ligase activity appears to play a role in SopA-mediated enteritis. The SopA substrate(s) remain unknown. It has been postulated that both host proteins and bacterial T3SS translocated effectors are potential targets of SopA. Host proteins may be directly involved in *Salmonella*-induced inflammatory responses. Alternatively, they may regulate the activity/level of *Salmonella* effectors that are involved in inducing such inflammation (Zhang et al., 2006).

SopA is not the only ubiquitin E3 ligase *Salmonella* possesses (Figure 4). It has three others: *Salmonella* leucine-rich repeat protein (SlrP), *Salmonella* secreted protein H1 (SspH1) and SspH2 (Rohde et al., 2007). SlrP and SspH2 are found in most *Salmonella enterica* strains, while SspH1 is limited to serovar Typhimurium strain 14028s (Miao et al., 1999). SlrP, SspH1, and SspH2 were first identified as belonging to a group of T3SS effectors from various human, animal, and plant pathogens that contain LPX repeats; a subtype of the leucine-rich repeat (LRR) superfamily of protein binding domains (Miao et al., 1999; Tsolis et al., 1999). IpaH from *Shigella* (Venkatesan et al., 1991) and YopM from *Yersinia* (Boland et al., 1996) also belong to this group. It was assumed LRRs were required for protein–protein interactions, but the precise functions were unclear. SspH1 and SspH2 had together been shown to reduce mortality in calves due to reduced intestinal lesions, although single mutants were not attenuated in this or cell culture models of invasion or replication (Miao et al., 1999). SlrP was required for virulence in mice but not bovine models of infection (Tsolis et al., 1999).

The discovery that *Shigella* IpaH9.8 was a ubiquitin E3 ligase (Rohde et al., 2007) provided the key to the activity of SlrP, SspH1, and SspH2. Rohde et al. (2007) confirmed SspH1 as an E3 ligase; endowed with the classic activities of E3 ubiquitin ligases, i.e., the ability to remove ubiquitin from ubiquitinated UbcH5B, to autoubiquitinate, and to polyubiquitinate HA-tagged ubiquitin. SspH1 had previously been shown to localize to the nucleus of host cells (Haraga and Miller, 2006). Here, SspH1 binds the human serine/threonine kinase protein kinase PKN1 and leads to NFκB inhibition and reduction in IL-8 secretion (Haraga and Miller, 2006). PKN1 was confirmed as a substrate of SspH1 (Rohde et al., 2007), and thus SspH1 appears to use its E3 activity to down-regulate inflammatory responses.

Salmonella secreted protein H2 and SlrP were also confirmed as ubiquitin E3 ligases. SspH2 is known to localize to the cell periphery, particularly to microvilli (Miao et al., 2003; Quezada et al., 2009). This localization to areas of dynamic actin polymerization may be through the ability of SspH2 to interact with filamin and profilin (Miao et al., 2003). So far, no role has been identified for SspH2 during infection and the substrate(s) for SspH2 are unknown (Quezada et al., 2009). SlrP appears to be involved with inducing cell death. This is achieved through modulating two independent pathways, only one of which is dependent on SlrP E3 activity (Bernal-Bayard and Ramos-Morales, 2009; Bernal-Bayard et al., 2010). SlrP uses cysteine residue 546 to ubiquitinate thioredoxin (Trx), a protein antioxidant. Ubiquitination does not appear to target Trx for proteasome degradation. Instead, it reduces Trx activity, leading to increase in cell death (Bernal-Bayard and Ramos-Morales, 2009). This may be important for the escape of

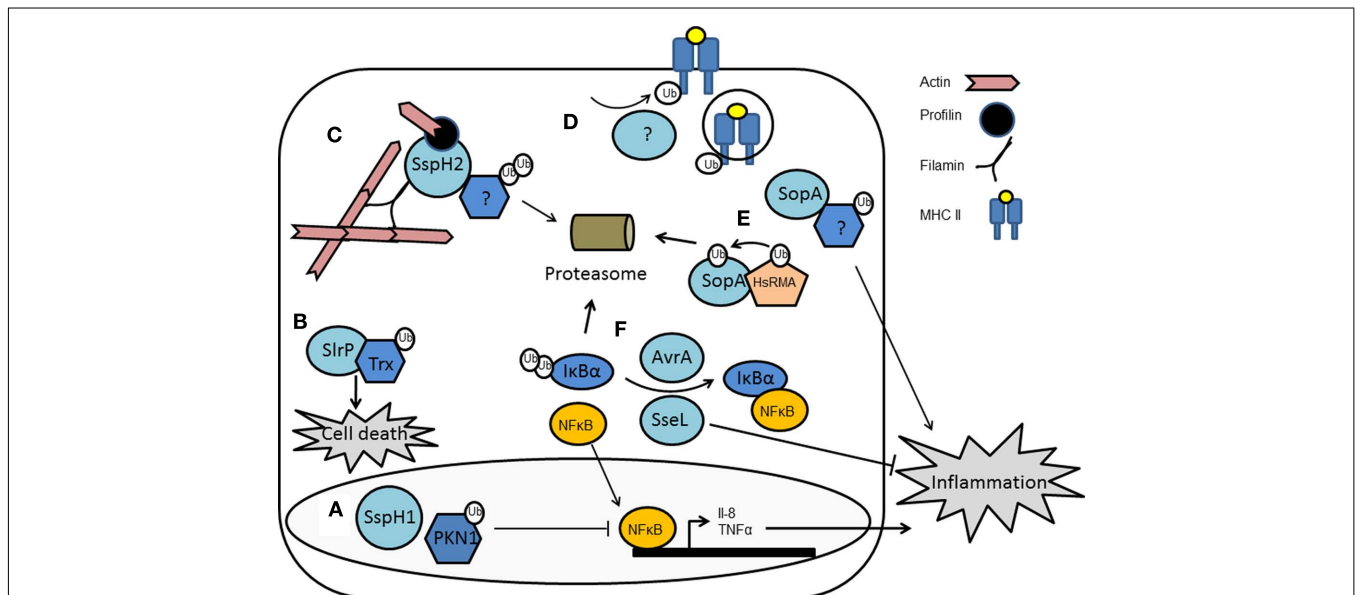


FIGURE 4 | Manipulation of host ubiquitin pathways by *Salmonella enterica*. Four effector proteins have been identified to be translocated into host cells where they can act as ubiquitin E3 ligases. **(A)** SspH1 ubiquitinates PKN1 in the cell nucleus which inhibits expression of NFκB-regulated genes. **(B)** SlrP targets thioredoxin (TRX) to trigger cell death. **(C)** SspH2 binds profilin and filamin but the substrate of its E3 activity has not been identified. **(D)** An unknown effector may be responsible for the ubiquitination of MHCII

receptors and their internalization, reducing CD4⁺ T-cell response. **(E)** SopA ubiquitinates an unknown substrate, the downstream effect of which is induction of inflammation. SopA is also a substrate of the host E3, HsRMA, being targeted for proteasomal degradation. **(F)** AvrA and SseL both act as DUBs. Both deubiquitinate IκBα and allow it to form an inhibitory complex with NFκB, thus reducing transcription of NFκB genes and therefore decreasing inflammatory responses.

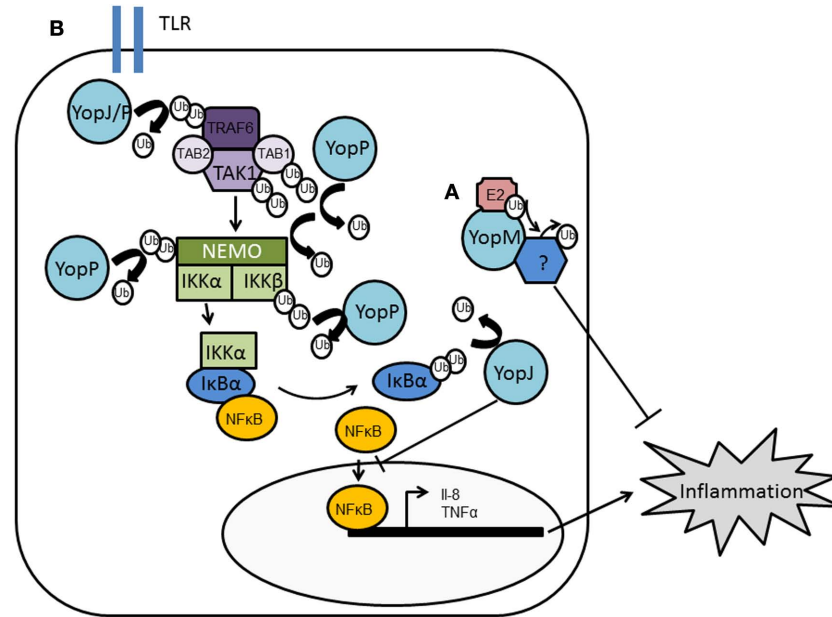


FIGURE 5 | Manipulation of host ubiquitin pathways by *Yersinia*. *Yersinia* translocates YopM and YopJ into host cells. (A) YopM is an E3 ligase although its substrate has not been identified but is likely to moderate host cell pathways to reduce immune system function. (B) YopJ is a DUB, with the

ability to deubiquitinate TRAF2, TRAF6, and IκBα. In *Y. enterocolitica*, the homologous protein YopP has been shown to deubiquitinate TAK1, TAB1, IκBβ, and NEMO. Both YopJ and YopP therefore disrupt NFκB signaling and reduce the inflammatory response.

Salmonella should the cell become unviable either through damage or due to maximum numbers of *Salmonella* being achieved. It is also likely to contribute to the inflammation associated with *Salmonella* pathogenesis. The non-proteolytic use of ubiquitin by SlrP mimics the interaction between SspH1 and PKN1, indicating these two proteins may use a similar mechanism to manipulate host cell proteins. SspH2 meanwhile, has been shown to synthesize K48-linked poly-Ub chains, implying that its targets are destined for proteasomal destruction (Levin et al., 2010).

The crystal structure of SspH2 has been determined and reveals a two-domain arrangement (Figure 3). The N-terminal domain is composed of 12 LRRs; the C-terminus is globular, with a unique fold unrelated to known HECT or RING-finger E3 ligases, which the authors termed novel E3 ligase (NEL; Quezada et al., 2009). The LRR domain sequesters the catalytic cysteine residue of the NEL domain thereby auto-inhibiting the ligase activity. This may prevent cellular toxicity until SspH2 comes into contact with the appropriate activated-E2 and its substrate, whereupon there is a large conformational shift, reorienting the domains (Quezada et al., 2009). A similar fold and a similar auto-inhibitory mechanism appears to be used by the related *Shigella* IpaH proteins (Singer et al., 2008; Zhu et al., 2008), and thus likely represents a common feature in this novel class of ubiquitin E3 ligases.

In addition to belonging to a novel group of E3s, SspH2 is further unique in its binding of its cognate E2. Distinct from classical E2–E3 interactions, binding between SspH2 and UbcH5 is via both the ubiquitin and a region in the UbcH5 not previously considered important (Levin et al., 2010). A see-saw mechanism of ubiquitin chain synthesis has been proposed to explain the mechanism of ubiquitin chain formation, whereby the growing ubiquitin chain is reciprocally transferred between the E2 and E3 active sites. To grow

the chain, a new E2–Ub is recruited to SspH2–Ub_n. The chain is subsequently added to the E2–Ub, creating E2–Ub_{n+1}, before the chain is passed back to SspH2.

In addition to the four E3 ligases already identified in *Salmonella*, there is the potential that further E3s are encoded on the *Salmonella* chromosome. In particular, it has been shown that *Salmonella* enhances the ubiquitination of human leukocyte antigen–DR (HLA–DR). This leads to increased internalization of MHC class II antigens, and subsequent lower expression on the surface of antigen presenting cells, such as DCs (Lapaque et al., 2009). Reducing the ability of cells to present antigen to CD4-restricted T-cells, the most important T-cell subset required for resolution of infection (Hess et al., 1996), may provide a mechanism by which *Salmonella* can influence the initiation of adaptive immune responses, and enhance its survival. Ubiquitination of HLA–DR and its subsequent internalization were dependent on the T3SS encoded by SPI-2, although no specific effector was identified to be involved (Lapaque et al., 2009). However, this does not rule out the possibility that these or other putative effectors may be involved, especially since, as highlighted above, the currently known *Salmonella* E3 ubiquitin ligases are mimics of host E3s and share little primary sequence similarity with their host counterparts, and in the case of SlrP, SspH1, and SspH2 share little structural homology. It will be interesting to see as more extensive research is conducted on the *Salmonella* proteome whether additional E3 ligases exist.

SHIGELLA

The IpaH family of proteins were originally identified as five homologous genes carried on the virulence plasmid: IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, and IpaH9.8 (Hartman et al., 1990;

Venkatesan et al., 1991). Subsequently, it was shown that there were copies of IpaH on the *Shigella* chromosome which were also secreted and translocated into host cells and important to pathogenesis (Ashida et al., 2007), bringing the total to nine IpaH proteins. Each IpaH protein has a variable N-terminal domain containing six to eight LRR segments and an approximately 300-residue C-terminal domain (CTD) that is virtually identical in all IpaH proteins (Ashida et al., 2007). It is this CTD which carries the E3 activity.

IpaH E3 ubiquitin ligase activity was identified in a yeast surrogate model. *S. flexneri* IpaH9.8 was shown to inhibit yeast pheromone response signaling through ubiquitination and targeting of the mitogen-activated protein kinase kinase (MAPKK) Ste7 for degradation by the proteasome (Rohde et al., 2007). Identification of a conserved cysteine residue, C337 in IpaH9.8, within all the IpaH proteins and other homologous LRR-containing bacterial effectors, e.g., *Salmonella* SspH1 and *Yersinia* YopM characterized this family of proteins as a novel family of bacterial E3 ubiquitin ligases.

The two-domain architecture of IpaH and the presence of a cysteine residue in the CTD (IpaH-CTD) suggested IpaH may belong to the HECT E3 family. However, IpaH-CTD has no sequence similarity with HECT domain E3s. It also lacks structural similarity with either HECT domain E3s, including *Salmonella* SopA, or RING-finger proteins (Singer et al., 2008; Zhu et al., 2008). Instead, the IpaH E3 ubiquitin ligase consisted of a new all-helical fold in which the conserved and essential cysteine residue is located in a surface-exposed flexible loop surrounded by conserved acidic residues (CXD motif; Singer et al., 2008; Zhu et al., 2008). This fold shares similarity to the NEL domain identified in *Salmonella* SspH2 (Quezada et al., 2009). Unsurprisingly, while the cysteine residue acts as a thiol nucleophile to catalyze ubiquitin transfer through a transthioleation reaction, analogous to the catalytic cysteine in HECT E3s, other features of the reaction are different to the HECT family. For example IpaH requires the neighboring Asp365 for thiol-catalyzed polyubiquitin chain formation (Zhu et al., 2008). The N-terminal domain of the protein is also inhibitory for the auto-ubiquitination activity of the full-length IpaH9.8 with substrate binding releasing this inhibition (Singer et al., 2008; Seyedarabi et al., 2010).

How does the IpaH E3 ligase activity contribute to *Shigella* pathogenesis? Substrates of IpaH9.8 have been identified as U2AF³⁵, a mammalian splicing factor, and NF- κ B essential modulator (NEMO)/IKK γ (Ashida et al., 2007, 2010; Seyedarabi et al., 2010; Figure 2). It was previously shown that IpaH9.8 binds U2AF³⁵ and led to down-regulation of pro-inflammatory chemokine and cytokine production during infection (Okuda et al., 2005). Given that Lys48 is the predominant ubiquitin linkage used by IpaH (Zhu et al., 2008), it is likely that U2AF³⁵ is targeted for degradation and falling levels of this splicing factor prevent production of mRNA which can be translated. Interestingly, it has been discovered that IpaH9.8-CTD can exist *in vitro* as a monomer able to catalyze ubiquitination of U2AF³⁵, and as a dimer that does not possess this activity (Seyedarabi et al., 2010). The dimerization of IpaH9.8-CTD occurs in response to oxidizing conditions and leads to domain swapping which switches off

IpaH9.8 E3 ligase activity. Thus it appears the activity of IpaH9.8 can be regulated in response to the level of damage of the host cell, and may allow injured cells to remain in a benign state suitable for bacterial survival and unable to mount an attack (Seyedarabi et al., 2010).

NF- κ B essential modulator, also known as inhibitor of NF κ B kinase (IKK γ), is the regulatory subunit of the inhibitor of IKK complex, which activates NF- κ B. IpaH9.8 interacts with NEMO/IKK γ and A20 binding inhibitor of NF- κ B (ABIN-1), a ubiquitin-binding adaptor protein (Ashida et al., 2010). ABIN-1 promotes IpaH9.8-mediated NEMO ubiquitination leading to proteasome degradation of NEMO and disruption of NF- κ B signaling. Thus, together with its ubiquitination of U2AF³⁵ it appears the main role of the IpaH9.8 E3 ligase domain is to inhibit the inflammatory response, which presumably allows *Shigella* to initiate colonization at early stages of infection (Figure 2).

YERSINIA

Yersinia pestis is the causative agent of plague, while *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* cause gastrointestinal disease. Each utilizes a T3SS to inject virulence proteins, known as *Yersinia* outer proteins (Yops), into host cells to subvert their function (Cornelis, 1998). Host cells targeted by Yops are typically cells of the immune system such as macrophages, PMNs, and dendritic cells. Impeded in phagocytosis and signaling by the Yop proteins, these cells fail to mount a sufficient immune response to remove the pathogen, subsequently leading to disease.

YopM is one of the six Yop effectors of *Yersinia* and has an important role for virulence of *Yersinia* spp. (Trulzsch et al., 2004). The size of YopM differs between different strains and serotypes ranging from 42 to 54 kDa due to a variable number and composition of LRRs (Evdokimov et al., 2001). These LRRs give the protein homology to *Salmonella* SlrP, SspH1, and SspH2 and the *Shigella* IpaH proteins. YopM appears to be most similar to SspH1 since it too seems to localize to the nucleus and co-immunoprecipitates with kinases, in this case ribosomal s6 kinase 1 (RSK1) and PKN2 (Mcdonald et al., 2003). However, the role of this interaction is unknown during *Yersinia* infection, as are other aspects of how YopM aids in eliciting disease.

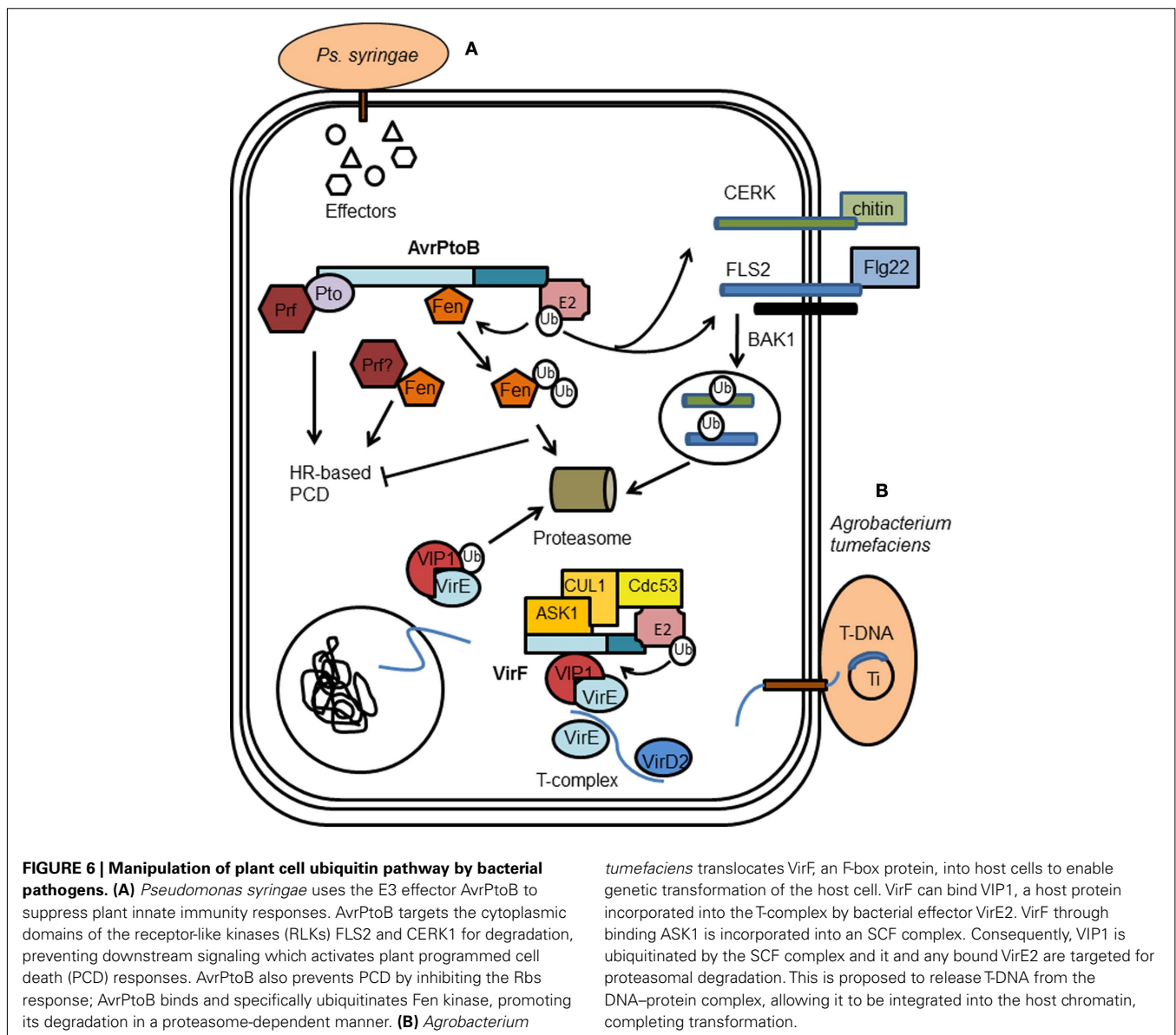
Soundararajan et al. (2010) using related *Salmonella* and *Shigella* effectors as templates, were able to use fold identification and homology-based modeling [protein core atomic interaction network (PCAIN) methodology] to distinguish the structure of full-length YopM. This confirmed the presence of a NEL domain in YopM. The YopM NEL shares the conserved catalytic site, conserved molecular surface electrostatics, and LRR-based auto-inhibition of E3 ligase activity found in *Salmonella* SspH2 and *Shigella* IpaH NELs (Soundararajan et al., 2010, 2011). The ability of YopM to act as an E3 ligase has not been empirically tested by biochemical means. Furthermore, it has not been translated into a defined role for YopM during infection. However, given that the YopM homologs from *Salmonella* and *Shigella* are involved in the proteolysis of host HLA-DR and NEMO (see above, Figures 2 and 4) it appears plausible that YopM may also modulate human immunity in similar ways (Figure 5).

PSEUDOMONAS SYRINGAE

Pseudomonas syringae pathovar tomato causes bacterial speck disease of tomato by using its T3SS to deliver about 30 effectors into the plant cell (Buell et al., 2003). One of these effectors is AvrPtoB, a 59-kDa protein with a modular architecture. The N-terminal region (amino acids 1–387) is recognized by the Pto kinase in tomato varieties that are immune to speck disease. Pto interacts with the resistance (R) protein Prf and upon binding of AvrPtoB activates the hypersensitive response (HR): an event characterized by rapid, localized programmed cell death (PCD), and which ultimately limits pathogen growth. The CTD of AvrPtoB (comprising amino acids 308–553) has anti-PCD activity, and this is correlated with the ability of this domain to act as an E3 ligase (Abramovitch et al., 2006). AvrPtoB–CTD exhibits homology to the eukaryotic RING-finger and U-box families of proteins, and includes conservation of a core fold and a spatially clustered three-amino acid

surface patch required for E2-binding (Figure 3). Such a structure suggested AvrPtoB may function as a mimic of host E3 ubiquitin ligases, which was subsequently demonstrated biochemically (Abramovitch et al., 2006).

There appear to be two distinct mechanisms that lead to AvrPtoB-mediated PCD (Figure 6). The first, mentioned above, is dependent on both the Pto and Prf proteins in resistant tomato. AvrPtoB has been shown unable to suppress PCD via this pathway (Pedley and Martin, 2003). The second mechanism, termed “resistance suppressed by AvrPtoB C-terminus” (Rsb), is Pto-independent and is speculated to involve another putative resistance protein and Prf. It was shown by Janjusevic et al. (2006) that in plants lacking Pto and Prf, infection with a mutant of AvrPtoB that lacks E3 activity triggers the HR response, i.e., it can no longer suppress the Rsb response. This indicated that a likely substrate for AvrPtoB was the putative resistance protein



that leads to Rsb-mediated PCD. The Pto family member, Fen kinase, was subsequently found to fulfill this role and to physically interact with AvrPtoB¹⁻³⁸⁷ (Rosebrock et al., 2007). The AvrPtoB E3 ligase specifically ubiquitinates Fen, promoting its degradation in a proteasome-dependent manner and leading to disease susceptibility in Fen-expressing tomato lines (Rosebrock et al., 2007).

Plant innate immunity relies on a subset of receptor-like kinases (RLKs) called pattern recognition receptors (PRRs), which respond to ligands known as pathogen-associated molecular patterns (PAMPs). For example, perception of bacterial flagellin or flg22, a peptide containing the flagellin epitope, is through an induced receptor complex composed of the LRR receptor kinases flagellin-sensing 2 (FLS2) and BRI1-associated kinase 1 (BAK1). Fungal chitin is detected by the *Arabidopsis* receptor kinase chitin elicitor receptor kinase 1 (CERK1; **Figure 6**). Upon PAMP-induced activation, PRRs stimulate a plethora of defense responses, one of which is the MAP kinase activation discussed above. AvrPtoB has now also been found to target the cytoplasmic domains of RLKs for degradation (Gohre and Robatzek, 2008; Gimenez-Ibanez et al., 2009). AvrPtoB associates with FLS2 via its N-terminal domain while it uses the E3 ligase activity of its C-terminal to preferentially target activated FLS2 for degradation (Gohre and Robatzek, 2008). AvrPtoB also ubiquitinates the CERK1 kinase domain *in vitro* and targets CERK1 for degradation *in vivo* (Gimenez-Ibanez et al., 2009). Since the RLK/Pelle gene family, which includes the known targets of AvrPtoB, makes up 60% of kinases in plants, there are likely to be further potential targets of AvrPtoB within plants. It may also suggest that AvrPtoB targets the plant proteome somewhat non-specifically (Gimenez-Ibanez et al., 2009).

ENTEROHEMORRHAGIC *ESCHERICHIA COLI*

Enterohemorrhagic *Escherichia coli* (EHEC) causes bloody diarrhea and hemolytic uremic syndrome (HUS). EHEC possesses a T3SS encoded by a region of its chromosome termed the locus of enterocyte effacement (LEE). Effectors encoded in LEE, and also a large number of additional non-LEE-encoded (Nle) effector proteins, are transmitted by the T3SS into host cells, where they interfere with host cellular processes to induce disease. In part this includes subverting actin polymerization to form a “pedestal” on the host cell surface which facilitates adherence and colonization of the pathogen, and leads to attaching and effacing (A/E) lesions.

NleG is one recently identified Nle effector, with 24 homologs identified (mainly in the genomes of pathogenic *E. coli* and *Salmonella*). This group of proteins form a distinct family which does not share any significant sequence similarity with other proteins. However, all NleG proteins contain a conserved region of ~100 residues localized to the C-terminus. Analysis of this region showed the presence of a motif similar to RING-finger/U-box domains, and was shown to act as a functional E3 ligase in the presence of ubiquitin, E1 and E2 (UBE2D2; Wu et al., 2010). E2 interacts with NleG effectors following the general architecture established for E2/eukaryotic RING-finger complexes. Indeed, there is significant structural similarity between NleG and the CTD of AvrPtoB from *P. syringae* (**Figure 3**), further indicating NleG may function in part as an E3 ligase (Wu et al., 2010). However, no substrate has been reported for NleG; a

major hindrance being that the N-terminal is not conserved between NleG family members and none has an identifiable substrate binding motif. The authors therefore suggest NleG effectors may primarily associate with specific host E2 enzymes rather than transfer ubiquitin onto a substrate protein, and in this way manipulate the host ubiquitination pathway (Wu et al., 2010). This is an intriguing possibility but requires much further work to confirm. Alternatively, the NleG effectors may contain novel binding motifs or use scaffolding proteins to interact with their substrates.

Another Nle effector has been identified in EHEC as an E3 ligase: EspX7, renamed as non-Lee-encoded effector ligase (NleL) to reflect its novel biochemical activity (Piscatelli et al., 2011). This effector was identified as an E3 ligase given its similarity to SopA from *Salmonella* (Zhang et al., 2006; Lin et al., 2011). Similar to SopA, NleL contains a protease-sensitive N-terminus, a β -helix domain, and a C-terminal catalytic domain that resembles the bilobal architecture of eukaryotic HECT E3s (**Figure 3**). The ubiquitin ligase activity of NleL is dependent upon a cysteine residue (Cys⁷⁵³) that forms an intermediate with ubiquitin through a thioester bond, thus NleL, like SopA, acts as a bacterial mimic of eukaryotic HECT E3s (Lin et al., 2011; Piscatelli et al., 2011).

In vitro ubiquitination assays show NleL preferentially forms unanchored polyUb chains using Lys⁶ and Lys⁴⁸ linkages. This suggests NleL plays a role in moderating signaling pathways rather than targeting proteins for degradation, and is supported by the observation that NleL E3 ubiquitin ligase activity is involved in modulating Tir-mediated pedestal formation (Piscatelli et al., 2011). Interestingly, an EHEC mutant strain deficient in E3 ligase activity induced more pedestals than the wild-type strain. It therefore appears that EHEC may use specific effector proteins to ensure there is control in the manipulation of host pathways so a balance is achieved between bacterial and cell survival. Consistent with the hypothesis that unregulated pedestal formation may alter disease, it was demonstrated that the *Citrobacter rodentium* NleL homolog, which also acts as an E3 ubiquitin ligase, was required for efficient infection of murine colonic epithelial cells *in vivo* (Piscatelli et al., 2011). So far the target of NleL is unknown. It appears Tir is not ubiquitinated by NleL (Piscatelli et al., 2011), and thus it is reasonable to suppose that NleL ubiquitinates an unknown bacterial or host protein(s) involved in pedestal formation, with a decrease in protein level or an alteration in localization resulting in a check in pedestal formation.

LEGIONELLA

The *Legionella* chromosome encodes a Dot/Icm type IV secretion system (T4SS) which is essential for a number of virulence traits, including replication within host cells (Segal et al., 1998; Vogel et al., 1998). In *Legionella* infections, it has been shown that anti-polyubiquitin antibodies decorate *Legionella*-containing vacuoles (LCVs), suggesting a significant amount of polyubiquitinated proteins exist on LCVs (Dorer et al., 2006). As the proteasome inhibitor MG132 was reported to adversely affect the intracellular growth of *Legionella* in mouse macrophages, it appears that *Legionella* may be an additional pathogen that exploits the host UPS (Dorer et al., 2006).

In a search for novel translocated effectors of *Legionella*, Lpg2830 was identified (Kubori et al., 2008). Lpg2830 contains two-domains with striking similarity to U-boxes (U-box 1 and U-box 2), and therefore was named LubX (*Legionella* U-box protein). LubX was shown to exhibit E3 ligase activity *in vitro*, and required U-box 1 for this activity (Kubori et al., 2008). U-box 2 was shown to play a non-canonical role, being required for binding of the LubX substrate: Cdc2-like kinase 1 (Clk1). Clk kinases phosphorylate serine/arginine-rich proteins (SR proteins), which in turn modulate alternative splicing-site selection (Prasad et al., 1999; Schwertz et al., 2006). Clk1 is shown to be required for the growth of *Legionella* in macrophages, indicating *Legionella* targets this host protein during infection. While it was demonstrated that LubX does indeed polyubiquitinate Clk1, it was not clear what type of linkage was used and whether Clk1 was targeted for degradation (Kubori et al., 2008).

More recently, it has been shown that another substrate of LubX is the *Legionella* effector protein SidH (Kubori et al., 2010). SidH levels within host cells were shown to decline over time and were concurrent with increasing levels of LubX. It was then shown that LubX can bind SidH and direct its ubiquitination, targeting it for degradation by the proteasome (Kubori et al., 2010). This is the first example of a bacteria effector targeting and regulating another bacterial effector within host cells, and the authors have designated such an effector as a “metaeffector.” When it is considered that *Legionella* possesses 275 confirmed Dot/Icm substrates (Zhu et al., 2011), it is perhaps not surprising that some of these may be utilized to coordinate the function of other effector proteins and ensure the infection proceeds as desired by the bacterium. It will not only be interesting to uncover further metaeffectors in the *Legionella* effector repertoire but to determine what other metaeffectors are used by other bacteria, if at all.

F-BOX PROTEINS

In addition to U-boxes, it is known that proteins containing F-boxes have an important role in protein ubiquitination. The F-box motif is ~50 amino acids long, although with only a few conserved residues, and mediates protein–protein interactions. F-box proteins form one of the constituents of the SCF complex, a multi-protein E3 ubiquitin ligase (Zimmerman et al., 2010). The SCF complex works in the following way to target proteins for proteasomal degradation. Firstly, F-box proteins bind independently to a target protein via the variable protein–protein interaction domain. S-phase kinase-associated protein 1 (Skp1) binds the F-box protein via the F-box motif and in this way targets the substrate recognition component of the complex to the scaffold protein Cullin-1. Cullin-1 links the Skp domain with the RING-finger domain of Rbx1/Roc1/Roc2. This allows ubiquitin to be passed from the E2 molecule, bound to Rbx1, to the target protein bound by the F-box containing protein.

AGROBACTERIUM

One of the first bacterial F-box proteins identified was VirF, from the plant pathogen *Agrobacterium tumefaciens*. *A. tumefaciens* genetically transforms plants during infection leading to the formation of crown galls, which are essentially plant tumors. Genetic transformation is achieved by transporting a single-stranded copy

of the bacterial transferred DNA (T-DNA) from the *A. tumefaciens* tumor-inducing (Ti) plasmid into the plant cell nucleus followed by integration into the host genome (Gelvin, 2000). During genetic transformation, virulence (Vir) proteins are also transferred into the host cell, many of which play a role in mediating T-DNA integration. VirE2, associates with T-DNA to create a nucleoprotein complex (T-complex; Citovsky et al., 2007), and through its association with the host VIP1 protein facilitates nuclear import of the T-complex. Before integration into the host genome, the T-DNA must be uncoated from its cognate proteins. VirF, an F-box protein, had been shown to bind the plant homolog of Skp1, ASK 1 (Schrammeijer et al., 2001). As part of the Skp1–Cdc53/Cullin–F-box (SCF) complex, VirF has been shown to bind VIP1, thereby targeting both VIP1 and its associated VirE2 for proteasomal degradation (Figure 6). This leads to the release of T-DNA from the DNA–protein complex, allowing it to be integrated into the host chromatin (Tzfira et al., 2004).

Interestingly, VirF is not required for genetic transformation of some plant species (Hirooka et al., 1987). Instead, the VirF function is performed by a host plant protein(s). The F-box protein, VIP1-binding F-box (VBF), has recently been identified as fulfilling this role (Zaltsman et al., 2010). VBF recognizes and binds VIP1 and its associated VirE2, forming ternary VBF–VIP1–VirE2 complexes. VBF can then act to destabilize both VIP1 and VirE2 via the SCF–VBF pathway (Zaltsman et al., 2010). Thus, *Agrobacterium* subverts a host defense pathway induced in response to infection, to facilitate infection.

RALSTONIA

Ralstonia solanacearum is another plant pathogen that appears to utilize F-box proteins during infection. A group of seven genes with homology to plant-specific LRRs have been identified and named GALA proteins after a conserved GAXALA sequence in their LRR (Cunnac et al., 2004). These seven GALA family effectors have been shown to contain F-box motifs in their N-terminus and mediate binding to host Skp1 proteins, ASK1 and ASK2 in *Arabidopsis* (Angot et al., 2006). Thus, GALA family proteins function as SCF E3s, and this was shown to be important for virulence of *Ralstonia*. A mutant in which all of the seven GALA genes were deleted or mutated did not cause wilt in *Arabidopsis*, and slowed the progression of wilting in tomato (Angot et al., 2006).

LEGIONELLA

Of human pathogens, all strains of *Legionella pneumophila* sequenced to date encode multiple genes with predicted F-box motifs, including three F-box genes in *L. pneumophila* strain Paris (Lomma et al., 2010) and five F-box genes in *L. pneumophila* strain Philadelphia-1 (Ensminger and Isberg, 2010). Since single, double, and triple F-box mutations in the Paris strain F-box genes have been shown to cause impaired intracellular replication and defective colonization of the lungs of A/J mice, it indicates F-box proteins and their potential interactions with host ubiquitin pathways are important to *Legionella* infection (Lomma et al., 2010).

The *L. pneumophila* F-box containing proteins have a structure that mimics that of their eukaryotic homologs, with an

N-terminal F-box domain and a second C-terminal protein interaction motif. However, they are unique in that the second interaction domain is neither a WD40 repeat, nor a LRR region as reported for eukaryotic F-box proteins (Skaar et al., 2009a,b). One F-box protein in particular has been studied due to its conservation between *Legionella* strains: AnkB (Lpg2144) in strain AA100/130b, LegAU13 in the Philadelphia-1 strain, and Lpp2082 in the Paris strain. AnkB has been shown to interact with Skp1. The target of the Skp1–Cullin–AnkB complex was found to be ParvB (Lomma et al., 2010). ParvB belongs to the Parvin family of proteins that are involved in linking integrins and associated proteins with intracellular pathways that regulate actin cytoskeletal dynamics and cell survival. Overexpression of AnkB in A549 cells and infection with *L. pneumophila* wild-type, but not the *ankB* mutant strain, decreased ubiquitination of ParvB (Lomma et al., 2010). It appears that AnkB inhibits the endogenous ubiquitin ligase of ParvB perhaps by competing for the interaction site (Lomma et al., 2010). The authors also hypothesized that by decreasing ubiquitination of ParvB, AnkB might stabilize the integrin-linked kinase (ILK)–PINCH–ParvB complex leading to caspase-3 activation and apoptosis. Alternatively, ParvB may act as an accessory protein to direct AnkB to the plasma membrane, where the E3 ligase activity of SCF directs the ubiquitination of one or more additional proteins. Since inhibition of ParvB by RNA interference resulted in reduced intracellular growth of *L. pneumophila*, this reveals a new mechanism by which *L. pneumophila* may employ translocated effector proteins to promote bacterial survival.

Another *Legionella* F-box protein, LegU1 (Lpg0171), has also been shown to form a complex with both Skp1 and Cullin, and to therefore possess E3 ligase activity (Ensminger and Isberg, 2010). Using a two-step enrichment strategy in which immunoprecipitates of 3XFLAG-tagged LegU1 were subjected to a second round of immunoprecipitation to isolate ubiquitinated species, HLA–B-associated transcript 3 (BAT3) was identified as a target of the SCF–LegU1 complex (Ensminger and Isberg, 2010). The translocated *Legionella* effector, Lpg2160, was also found to associate with the SCF–LegU1–BAT3 complex although it does not get ubiquitinated. As two *Legionella* effectors target BAT3, it appears that modulation of BAT3 activity is crucial during *Legionella* infection. BAT3 plays a role in the ER stress response and host apoptotic pathways (Desmots et al., 2008; Tsukahara et al., 2009), thus, LegU1 and Lpg2160 may interfere with BAT3 function to mitigate the effects of disrupting normal vesicular trafficking in the host cell.

LicA, is a third translocated *L. pneumophila* Philadelphia effector with an F-box motif. While it associates with Skp1 it does so in the absence of Cullin1, and lacks auto-ubiquitination activity. This suggests LicA does not form an E3 ligase, but may also suggest the ability of LicA to form a non-canonical SCF complex using alternate eukaryotic components (Ensminger and Isberg, 2010).

CRYPTOCOCCUS

Another human pathogen utilizing an F-box protein is *Cryptococcus neoformans*, the leading cause of fungal meningitis in the immunocompromised. Fbp1 in *C. neoformans* contains a putative

F-box domain and 12 LRRs and physically interacts with Skp1, suggesting it may function as part of a SCF E3 ligase (Liu and Shen, 2011). As Fbp1 is essential for *C. neoformans* virulence, this indicates the SCF E3 ubiquitin ligase-mediated proteolysis pathway is likely to be important during infection, although its substrates remain to be identified.

NON-PATHOGENIC BACTERIA

F-box proteins are not only associated with pathogens. The amebal symbiont *Amoebophilus asiaticus* encodes 15 proteins with predicted F-box motifs and 9 proteins with E3 ligase-associated U-box domains in its chromosome, indicating it too may interfere with the host ubiquitination system (Schmitz-Esser et al., 2010).

DE-UBIQUITINATING UME

In addition to being able to moderate the activities of a protein by adding ubiquitin, the removal of ubiquitin can also be a powerful tool to control cellular functions. De-ubiquitinating enzymes (DUBs) cleave the isopeptide bond between ubiquitin and its target protein. This produces free ubiquitin which can then be recycled by the cell. Thus, ubiquitination is a reversible post-translational modification, and as such, is often compared to the post-translational modification of phosphorylation. Both modifications require ATP, are irreversible and alter the recognition of proteins by the cell (Orth, 2002). Since bacteria have evolved to possess effectors with the ability to modify proteins with ubiquitin, it is not surprising that they have also evolved effectors that act as DUBs.

YERSINIA

Yersinia effector YopJ (YopP in *Y. enterocolitica*), is essential for the ability of Yersiniae to kill macrophages and to establish a systemic infection in mice (Monack et al., 1998). YopJ appears to have several activities in infected cells that contribute to its importance to establish infection. YopJ inhibits the pro-inflammatory mitogen-activated protein kinase (MAPK) and NFκB pathways (Palmer et al., 1998; Ruckdeschel et al., 1998; Schesser et al., 1998), both of which play roles in innate and adaptive immunity. *Yersinia* appears to target both pathways to reduce the inflammatory response during infection; the production of protective cytokines, such as tumor necrosis factor-α (TNF-α) and IL-8, are inhibited, and apoptosis in macrophages is induced. Sequence analysis of YopJ showed no homology to known proteins, but secondary structure analysis indicated similarity to adenovirus protease (AVP; Orth et al., 2000). A conserved catalytic cysteine was identified between these two proteins, mutation of which in YopJ abolishes its ability to inhibit the MAPK and NFκB pathways, and inhibit TNF-α production (Orth et al., 2000). Thus, the protease activity of YopJ is key to its function.

Adenovirus protease had been indicated to resemble the cysteine protease, ubiquitin-like protein protease 1 (Ulp1) in yeast (Li and Hochstrasser, 1999), which is able to remove small ubiquitin-related modifier (SUMO) from modified proteins. YopJ was shown by Orth et al. (2000) to share this activity. Overexpression of YopJ in 293 cells results in a decrease in proteins bound to SUMO. This activity of YopJ has been questioned by Zhou et al. (2005) who found that YopJ did not deSUMOlyate proteins. However,

both authors agree that YopJ possesses deubiquitination activity, dependent on cysteine 172, and as such was the first bacterial effector shown with this property (Orth, 2002; Zhou et al., 2005).

YopJ appears to be a potent de-ubiquitinating protease, being able to cleave ubiquitin moieties from many substrates including TNF receptor-associated factor 2 (TRAF2), TRAF6, NF- κ B-inducing kinase (NIK), and I κ B α (Figure 5). TRAF2 and TRAF6 undergo auto-ubiquitination and acquire K63 polyubiquitin chains which are proposed to promote the assembly of a multi-protein complex that activates the IKK complex and hence, NF- κ B signaling. YopJ can remove both K48 and K63 linkages. By reversing the ubiquitination of TRAF2, TRAF6 and/or phosphorylated I κ B α during *Yersinia* infection, YopJ maintains the I κ B-NF κ B complex. This keeps NF κ B sequestered in the cytoplasm thereby attenuating the NF κ B pathway which is observed during wild-type *Yersinia* infections (Figure 5). *Y. enterocolitica* YopP has also been shown to bind and deubiquitinate IKKb, TRAF6, and NEMO *in vitro* (Carter et al., 2003; Haase et al., 2005). However, Thiefes et al. (2006) reported TRAF6 was not deubiquitinated by YopP but rather TAK1 and TAB1 are deubiquitinated, inhibiting TAK1 autophosphorylation and activity (Thiefes et al., 2006). There is no evidence that MAPKKs require ubiquitin or SUMO conjugates for activation, and thus it was not clear how YopJ/P could suppress the MAPK pathway simultaneously with the NF κ B pathway. By placing the inhibitory mechanism of YopP at TAK1, i.e., downstream from TRAF6 and upstream from MAPKK6/p38 MAPK, it does provide a mechanism whereby YopJ/P can activate both the MAPK and NF κ B pathways to down-regulate innate immune signaling. However, it is unclear whether these observations are specific to the IL-1 activation pathway or universal. Recently an alternative mechanism by which YopP/J suppresses the two pathways has been identified: YopJ acts not only as a DUB but as an acetyltransferase (Mittal et al., 2006; Mukherjee et al., 2006). YopJ binds and acetylates critical serine or threonine residues, using acetyl-coenzyme A (CoA), in the activation loop of MAPKKs and IKKb, respectively. This prevents these residues from being phosphorylated and from being activated, blocking signaling through the MAPKK and NF κ B pathways (Mukherjee et al., 2006). Inositol hexakisphosphate (IP₆) acts as an activation cofactor for YopJ acetyltransferase activity (Mittal et al., 2006). Thus, it appears YopJ has two different enzymatic capabilities and may explain why *Yersinia* is so efficient at inhibiting the MAPK and NF κ B pathways; acting on multiple components in a multitude of ways.

SALMONELLA

Salmonella possesses two DUBs with homology to YopJ. AvrA is encoded by the SPI-1 region of the *Salmonella* genome (Hardt and Galán, 1997), and cleaves ubiquitin moieties from I κ B α and β -catenin, thus regulating both the NF κ B and β -catenin signaling pathways (Collier-Hyams et al., 2002; Sun and Chen, 2004; Ye et al., 2007; Figure 4). Stabilized I κ B α leads to inhibition of NF- κ B signaling and consequently inhibition of the inflammatory response. Stabilization of β -catenin leads to increased β -catenin

transcriptional activity, activating cell proliferation and inhibiting cell apoptosis (Ye et al., 2007). Like YopJ, AvrA also exhibits acetyltransferase activity which is targeted on MAPKKs (Jones et al., 2008). However, interestingly, AvrA is anti-apoptotic not pro-apoptotic like YopJ and its homolog AopP of *Aeromonas salmonicida* (Jones et al., 2008). These differences may be accounted for by the selectivity of substrates. For example AvrA acts as a c-Jun N-terminal kinase (JNK) inhibitor at the level of MKK4/7, preventing JNK stimulating a pro-apoptotic response (Jones et al., 2008; Du and Galan, 2009), while YopJ has a more promiscuous range of MAPKK inhibitory activity (Mukherjee et al., 2006). Recently, the number of pathways on which AvrA potentially has an impact has been expanded to include: the p53 pathway, mTOR signaling, oxidative phosphorylation, VEGF, and JAK-STAT (Liu et al., 2010; Wu et al., 2010). Their roles in the anti-apoptotic function of AvrA have yet to be characterized.

Examining *Salmonella* genes expressed under SPI-2 inducing conditions led to the identification and characterization of another YopJ family member in *Salmonella*: SseL (Coombes et al., 2007; Rytönen et al., 2007). Biochemical assays show SseL functions as a DUB and, like AvrA, impairs I κ B α ubiquitination and degradation, suppressing NF κ B activation (Le Negrate et al., 2008). This appears important for the systemic spread of *Salmonella* in mice, since by competitive index a *sseL* null mutant is attenuated in virulence, although no defect was observed *in vitro* (Coombes et al., 2007; Rytönen et al., 2007). However, while AvrA, through its activity on the β -catenin pathway, inhibits apoptosis, it appears SseL may induce cytotoxicity in macrophages at later stages of infection (Rytönen et al., 2007); although the importance of SseL in this process has been challenged by Le Negrate et al. (2008) who observed only a slight cytotoxic effect due to SseL. Since both AvrA and SseL act on I κ B α , this appears to be an important stage at which the NF κ B pathway proteins and *Salmonella* effectors intersect. The activity of AvrA and SseL is consistent with the *in vivo* ability of *Salmonella* to dampen innate immune signaling while preventing the apoptotic elimination of infected, compromised cells. This may allow *Salmonella* more extensive tissue invasion, extending its long-term survival as it replicates in viable host cells.

In addition to the *Salmonella* DUBs, several other bacterial proteins have been discovered with similar homology to YopJ (Table 1). This “YopJ family” includes effectors from animal and plant pathogens and plant symbionts. Each shares a conserved catalytic core, consisting of three key amino acid residues (histidine, glutamic acid, and cysteine), and designates them as such to the C55 family of cysteine proteases (Orth et al., 2000; Barrett and Rawlings, 2001). Each protein appears to share at least one catalytic activity prescribed to YopJ, i.e., the ability to deubiquitinate, deSUMOylate, or acetylate. Interestingly, *Xanthomonas* possesses not only YopJ-like effectors which exhibit similarity to a ubiquitin-like protein protease (Ulp1), but also the effector XopD which is a *bona fide* member of the Ulp1 protein family with deSUMOylating activity (Hotson et al., 2003). This may suggest that after acquiring an Ulp1/Ulp-1 like hydrolase, pathogenic bacteria have adapted this protein to increase/modify its range of activity, thus making it a more potent tool during pathogenesis.

Table 1 | YopJ family members.

Bacterium	Effector	Activity	Target(s)	Role during infection	Reference
<i>Yersinia pseudotuberculosis</i>	YopJ	DUB	TRAF2, TRAF6 NIK, I κ B α	Inhibits NF κ B and MAPKK pathways	Orth et al. (2000), Zhou et al. (2005), Mukherjee et al. (2006)
<i>Yersinia enterocolitica</i>	YopP	DUB	Acetyltransferase MAPKKs and IKKb	Inhibits NF κ B and MAPKK pathways	Carter et al. (2003), Haase et al. (2005), Thiefes et al. (2006)
<i>Salmonella enterica</i>	AvrA	DUB Acetyltransferase	IKKb, TRAF6, TAK1, TAB1, NEMO I κ B α , β -catenin MAPKKS; MKK4/7	Inhibits NF κ B and MAPKK pathways	Collier-Hyams et al. (2002), Sun et al. (2004), Ye et al. (2007), Jones et al. (2008), Du and Galan (2009)
<i>Vibrio parahaemolyticus</i>	SseL	DUB	I κ B α	Inhibits NF κ B pathways	Le Negrate et al. (2008)
<i>Aeromonas salmonicida</i>	VopA	Acetyltransferase	MAPKKs	Inhibits MAPK signaling pathways (but not NF κ B pathway)	Trosky et al. (2004, 2007)
<i>Xanthomonas campestris</i>	AopP		I κ B α	Inhibits NF κ B pathway	Fehr et al. (2006)
	AvrRxv	deSUMOylation	AvrRxv interactor 1 (ARI1)	Inhibit HR response	Whalen et al. (1993), Ciesiolka et al. (1999), Whalen et al. (2008)
	AvrXv4	deSUMOylation		Inhibit HR response	Roden et al. (2004)
	AvrBst	deSUMOylation	SNF1-related kinase 1 (SnRK1)	Inhibit HR response	Ciesiolka et al. (1999), Szczesny et al. (2010)
	XopJ	deSUMOylation		Inhibition of cell wall-based defense responses	Noel et al. (2003), Bartetzko et al. (2009)
<i>Ralstonia solanacearum</i>	PopP1				Lavie et al. (2002)
	PopP2	Acetyltransferase	RRS1-R R protein	Stabilizes RRS1-R; elicits RRS1-R-mediated disease resistance in <i>Arabidopsis</i>	Deslandes et al. (2003), Tasset et al. (2010)
<i>Pseudomonas syringae</i>	HopZ1 (HopPmaD)		Isolflavone biosynthesis enzyme, 2- hydroxyisoflavanone dehydratase (GmHID1)		Alfano et al. (2000), Deng et al. (2003), Zhou et al. (2011)
	HopZ2 (AvrPpiG1)				Alfano et al. (2000), Deng et al. (2003)
	HopZ3 (HopPsyV)				Alfano et al. (2000), Deng et al. (2003)
<i>Rhizobium</i> sp. strain NGR234	Y4IO			Symbiotic determinant required for the formation of functional nitrogen-fixing nodules	Yang et al. (2009)
<i>Erwinia amylovora</i>	ORFB				Oh et al. (2005)

CHLAMYDIA

Chlamydia trachomatis is a bacterium which possesses DUBs distinct from those of the YopJ family. Instead, the predicted catalytic domains of *ChlaDub1* and *ChlaDub2* share similarity with

the Ulp domain of SUMO-specific proteases (SENPs), a family of proteases that possess either deneddylating or desumoylating activities (Misaghi et al., 2006). *ChlaDub1* and *ChlaDub2* have been shown *in vitro* to hydrolyse the thioester bonds that

attach ubiquitin and NEDD8 (a ubiquitin-like protein) to target proteins (Misaghi et al., 2006). It appears for *ChlaDub1* this translates into impairment of I κ B α ubiquitination and its subsequent proteasome-mediated degradation, thereby preventing NF κ B activation and potentially providing one mechanism by which *Chlamydia* is able to evade immune responses and establish long-term carrier states in infected individuals (Le Negrate et al., 2008). Since several viruses encode DUBs (Balakirev et al., 2002; Kattenhorn et al., 2005; Schlieker et al., 2005), it is not surprising that *Chlamydia* with its similar intracellular replication cycle, has developed tools to modulate the host ubiquitin–proteasome pathway. Further studies into the cellular function of *ChlaDub1* and particularly *ChlaDub2* will no doubt provide more information as to how *C. trachomatis* uses DUBs to manipulate its host.

CONCLUSION

Many bacterial pathogens have developed sophisticated secretion and translocation apparatus to transfer a set of proteins (effectors) directly into the cytoplasm of the host cell. These effectors manipulate host cellular responses, promoting the progression of bacterial infection and pathogenesis. It appears that interfering with ubiquitin signaling in the host is an important and common strategy used by pathogens to promote their survival in the host. In many of the examples reviewed here, exploiting the ubiquitin pathways to evade the host immune system during the course of infection is of particular importance to bacteria; *Salmonella*, *Shigella*, and *Yersinia* all target the NF κ B pathway to control the inflammatory response elicited during their infection. However, while each bacterium may target similar pathways, in many instances they appear to have developed different tools, and in doing so may target different stages in a specific host pathway. For example while *Shigella* uses OspG, an E2 targeting UME, to prevent signaling through the NF κ B pathway, *Salmonella* and *Yersinia* use DUBs, albeit DUBs which act at different stages of the pathway. Thus, while there are many common themes in how bacteria manipulate host ubiquitin pathways, each bacterium has often developed one or more unique effectors allowing them to exquisitely manipulate the host to suite their particular lifestyle.

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