Exploitation of eukaryotic subcellular targeting mechanisms by bacterial effectors

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Abstract | Several bacterial species have evolved specialized secretion systems to deliver bacterial effector proteins into eukaryotic cells. These effectors have the capacity to modulate host cell pathways in order to promote bacterial survival and replication. The spatial and temporal context in which the effectors exert their biochemical activities is crucial for their function. To fully understand effector function in the context of infection, we need to understand the mechanisms that lead to the precise subcellular localization of effectors following their delivery into host cells. Recent studies have shown that bacterial effectors exploit host cell machinery to accurately target their biochemical activities within the host cell.

Several bacterial species that infect eukaryotic hosts have evolved specialized protein secretion systems that mediate the transport of bacterial effector proteins into the cytosol of host cells. These effectors can modulate a variety of host cell functions to promote the survival and replication of the bacteria. The best studied of the bacterial protein secretion systems are the so called type III secretion system (T3SS) and type IV secretion system (T4SS), which are present in a wide range of bacteria that are symbionts or pathogens of mammals, plants or even insects[1,2] (BOX 1). These systems often deliver multiple proteins with a variety of biochemical activities that, in a coordinated manner, modulate complex host cell processes, including cytoskeletal dynamics, membrane trafficking, transcription, cell cycle progression, signal transduction and protein ubiquitylation[3–5]. There has been significant progress in understanding the biochemical activities of many bacterial effectors, but how these diverse activities are ultimately coordinated after the effectors have been delivered into the eukaryotic host cell is less well understood. A growing number of studies have revealed that to exert their functions in a spatially coordinated manner, effector proteins must be accurately targeted to their place of action. The precise targeting of bacterial effectors to specific subcellular compartments presumably has many benefits. As bacterial effectors are delivered in low concentrations, targeting to precise subcellular locations can increase the effective concentration of these proteins. Perhaps more importantly, the subcellular targeting of effectors ensures the engagement of the correct targets for their various biochemical activities.

Eukaryotic cells are organized into discrete compartments that are most often membrane bound. Therefore, eukaryotic proteins destined for these locations have evolved specific sorting signals or targeting motifs that determine their final destination. These signals are often recognized by multiprotein machines that directly mediate protein transport or further modify the target proteins so that they can become transport competent. The compartmentalized organization of eukaryotic cells also presents a major challenge to the precise subcellular targeting of bacterial effectors, which are usually delivered directly into the cytosol of the target cell. However, bacterial effectors have evolved various strategies to reach their final destinations. These strategies often involve exploitation of the eukaryotic host cell machinery; some strategies require signal sequences embedded within the amino acid sequence of the effectors themselves, and other strategies entail post-translational modification of the effector before it is targeted to its final destination. Here, we discuss different examples of effector-targeting mechanisms (TABLE 1). Instead of a comprehensive review of the literature, we focus on a few of the most well-characterized examples that illustrate the main themes which have been revealed as a result of studying the subcellular targeting of bacterial effectors.

Membrane targeting by host-mediated lipidation

An increasing number of bacterial effectors have been shown to be covalently modified by the attachment of a variety of lipid groups following translocation into the cytoplasm of eukaryotic cells (FIG. 1). The addition

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Box 1 | **Type III and type IV secretion systems**

Many bacteria that are pathogens or symbionts of animals, plants or insects have evolved complex multiprotein machines known as type III and type IV secretion systems to deliver bacterial effector proteins into target eukaryotic cells[1,2]. Proteins delivered by these machines possess specific targeting signals that selectively deliver the proteins to the transport machines. Inside the target cell, the various biochemical activities of these effector proteins can modulate a variety of eukaryotic host cell functions. Although these secretion machines have a common function, they clearly evolved independently, presumably from flagella in the case of the type III secretion system or from the bacterial conjugation systems in the case of the type IV secretion system. Both machines are composed of supramolecular structures that cross the entire bacterial envelope to provide a passageway for proteins travelling through this secretory pathway. It is believed that these machines must have evolved out of the need to deliver multiple proteins to the same target cell, presumably to modulate complex cellular processes in a manner in which a single biochemical activity might not be able to do.

of lipid groups increases the hydrophobicity of proteins, thus facilitating their tethering to intracellular membranes and, as a result, affecting protein localization and function. Some proteins are exclusively modified by a single lipid group, whereas others are sequentially modified with different lipids, allowing for multiple layers of targeting information. Lipidation of proteins not only promotes their membrane association, but also directs their association with liquid-order domains of the membrane, such as lipid rafts and caveolae. The major forms of protein lipidation in eukaryotes are *S*-palmitoylation, *N*-myristoylation and prenylation.

S-*palmitoylation.* *S*-palmitoylation has an important role in regulating protein stability and activity, as well as protein–protein interactions[6–8]. This modification is unique among lipid modifications in that it is generally reversible and can therefore affect protein function by dynamically controlling protein–protein interactions and/or the association of the protein with the membrane. *S*-palmitoylation is characterized by the addition of a saturated 16-carbon palmitic acid to a specific Cys residue in a protein through a thioester bond[8] (FIG. 1a). Unlike other lipid modifications, *S*-palmitoylation has no clear amino acid sequence requirement other than the presence of a Cys residue. In eukaryotic cells, *S*-palmitoylation is catalysed by a family of 23 Asp-His-His-Cys (DHHC) motif-containing proteins that have *S*-palmitoyltransferase activity[9]. DHHC proteins (also known as ZDHHC proteins) are polytopic membrane proteins that are found on a range of cellular membranes and have a conserved cytoplasmic-facing Cys-rich domain that includes the DHHC catalytic motif.

Salmonella enterica causes gastroenteritis and typhoid fever, a life-threatening systemic disease. A recent study has shown that host-mediated *S*-palmitoylation of an amino-terminal Cys residue of the *Salmonella enterica* T3SS effector SspH2 results in targeting of this effector to the host cell plasma membrane[10] (FIG. 1a). On the basis of amino acid sequence similarity, many other potentially *S*-palmitoylated *S. enterica* T3SS effectors were identified in this study, including SseI. Both SspH2 and SseI are targeted to the plasma membrane in an *S*-palmitoylation-dependent manner. Screening the DHHC family identified a subset of enzymes able to catalyse the *S*-palmitoylation of SspH2 and SseI[10]. Two of these enzymes, DHHC3 and DHHC7, can *S*-palmitoylate SspH2 and SseI directly *in vitro*. DHHC3 and DHHC7 are phylogenetically related and have been shown to be active towards other N-terminally *S*-palmitoylated substrates. Although both SspH2 and SseI are stably *S*-palmitoylated, they are targeted to different domains of the plasma membrane of polarized cells[10] (FIG. 1a). This suggests that although *S*-palmitoylation is essential for plasma membrane localization, it is not sufficient to target these effectors to their final destination. SspH2 belongs to a family of bacterial effectors that possess a C-terminal NEL (new E3 ligase) domain and have been implicated in immune response modulation during infection[11,12]. The C-terminal domain of SseI has amino acid sequence similarity with a family of bacterial deamidases[13] and has been reported to regulate cell migration[13,14], a function that requires SseI *S*-palmitoylation and plasma membrane targeting[10]. Thus, *S. enterica* has evolved to exploit host-mediated *S*-palmitoylation to target at least two distinct biochemical activities to their sites of action at the host cell plasma membrane.

N-*myristoylation.* *N*-myristoylation results in the covalent attachment of 14-carbon myristic acid, via an amide bond, primarily to the α-amino group of an N-terminal Gly residue[15] (FIG. 1b). This modification is catalysed by an *N*-myristoyltransferase following exposure of a GXXX(S/T/C) N-terminal consensus sequence. Although *N*-myristoylation has been historically described as a co-translational modification that occurs following the removal of the initiator Met residue of a protein, there are known examples of post-translational *N*-myristoylation[15,16]. In many instances, protein *N*-myristoylation is necessary but not sufficient to promote stable and permanent membrane association. Consequently, this modification often occurs together with *S*-palmitoylation of proximal Cys residues or with a polybasic amino acid domain next to the N terminus of the protein.

Pseudomonas syringae, which causes a range of diseases in plants, encodes many T3SS effectors that promote virulence in susceptible hosts or are recognized by host resistance proteins and thus trigger defence responses in resistant plants[17,18]. A subset of these effectors, including avirulence protein B (AvrB), AvrRpm1, HopF2 and multiple members of the HopZ family, encode N-terminal eukaryotic consensus sites for *N*-myristoylation[19–21]. When expressed in susceptible plant cells, AvrB and AvrRpm1 are targeted to the plasma membrane[19]. Substitution of the crucial N-terminal Gly2 residue with an Ala in both proteins results in their mislocalization to the cytosol. In addition, both effectors are also *S*-palmitoylated on a Cys immediately following the Gly, and this modification increases effector association with the membrane[19]. Therefore, *N*-myristoylation and *S*-palmitoylation of these Avr effectors are required for correct plasma membrane localization. Both AvrB and AvrRpm1 have been shown to interact with and induce phosphorylation of RPM1-interacting protein 4

Lipid rafts
Small (~70 nm width), dynamic microdomains of the plasma membrane that are enriched in cholesterol, and in sphingolipids and phospholipids with saturated acyl chains.

Caveolae
Specialized lipid raft regions of the plasma membrane that contain the protein caveolin and form flask-shaped, cholesterol-rich invaginations of the membrane.

E3 ligase
An enzyme that is required to attach the molecular tag ubiquitin to proteins, This tag modifies protein function or targets the protein for proteosomal degradation.

Table 1 | **The subcellular localization of bacterial effectors**

Species	Effector	Subcellular localization	Targeting mechanism	Refs
Agrobacterium tumefaciens	VirE2	Nucleus	Bipartite NLS	112,115
	VirD2	Nucleus	Bipartite NLS	96,97
Escherichia coli	Map	Plasma membrane and mitochondria	Protein–protein interaction and mitochondrial targeting pre-sequence	80,82,86
	EspF	Mitochondria, nucleus and plasma membrane	Mitochondrial targeting pre-sequence?	81,88,116
	Tir	Plasma membrane	Unknown	117–119
Legionella spp.	AnkB	LCV	Prenylation	32
	Lpl2806*	LCV	Prenylation	31
	SidC	LCV	PtdIns4P-binding domain	60,61
	SidM	LCV	PtdIns4P-binding domain	62,63,120
	LidA	LCV	PtdIns3P- and PtdIns4P-binding domain	62,63
	SetA	LCV	PtdIns3P-binding domain	64
Pseudomonas spp.	AvrB	Plasma membrane	*N*-myristoylation and *S*-palmitoylation	19
	AvrRpm1	Plasma membrane	*N*-myristoylation and *S*-palmitoylation	19
	HopF2	Plasma membrane	*N*-myristoylation and *S*-palmitoylation	21
	HopZ	Plasma membrane	*N*-myristoylation and *S*-palmitoylation	20
	AvrPphB	Plasma membrane	*N*-myristoylation and *S*-palmitoylation	24
	ORF4	Plasma membrane	*N*-myristoylation and *S*-palmitoylation	24
	AvrPto	Plasma membrane	*N*-myristoylation and *S*-palmitoylation	121
	ExoU	Plasma membrane	PtdIns(4,5)P_2-binding domain	66,67
	Multiple effector families‡	Mitochondria or chloroplasts	Mitochondrial or chloroplast targeting pre-sequence	77
	HopG1	Mitochondria	Unknown	79
Rhizobium spp.	NopT	Plasma membrane	*N*-myristoylation and *S*-palmitoylation	24
Salmonella spp.	SspH2	Plasma membrane	*S*-palmitoylation	10
	SseI	Plasma membrane	*S*-palmitoylation	10
	SifA	SCV and *Salmonella*-induced filaments	Prenylation	34–36
	SipB	Mitochondria	Unknown	122
	SopA	Mitochondria	Unknown	123
	SopB	SCV	Ubiquitylation	51,52
Xanthomonas spp.	AvrBs3	Nucleus	Monopartite NLS	107,109,110
	AvrXa5	Nucleus	Monopartite NLS	109,124
	AvrXa7	Nucleus	Monopartite NLS	125
	AvrXa10	Nucleus	Monopartite NLS	109
	PthA	Nucleus	Monopartite NLS	109
Shigella spp.	IpaH1	Plasma membrane	*S*-palmitoylation	10
	IpaH4.5	Plasma membrane	*S*-palmitoylation	10

AnkB, ankyrin B; Avr, avirulence; LCV, *Legionella*-containing vacuole; NLS, nuclear localization signal. PtdIns3P, phosphatidylinositol-3-phosphate; PtdIns4P, PtdIns-4-phosphate; PtdIns(4,5)P2, PtdIns-4,5-bisphosphate; SCV, *Salmonella*-containing vacuole. *Ivanov *et al.*[31] showed that eight different *Legionella* effectors require the CaaX motif for membrane localization; one example is given here. ‡Subcellular localization of multiple *Pseudomonas syringae* effectors, including Hop, Avr, Hol, and Hrp family members, was predicted by the program TargetP.

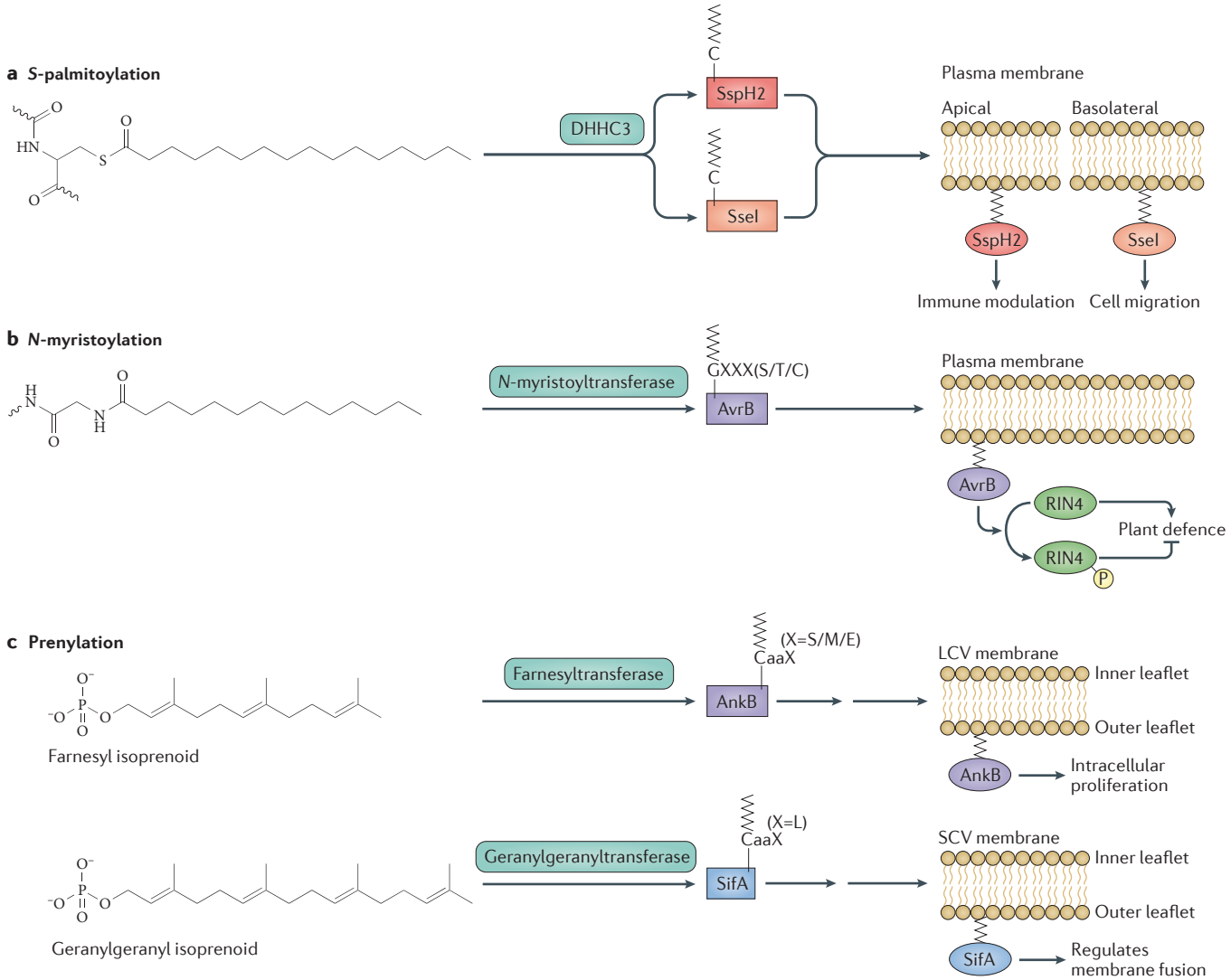

Figure 1 | **Membrane targeting of effectors by host-mediated lipidation.** **a** | *S*-palmitoylation involves the addition of a saturated 16-carbon palmitic acid to a specific Cys residue through a thioester bond. Host-mediated *S*-palmitoylation of the *Salmonella enterica* type III secretion system (T3SS) effectors SspH2 and SseI by Asp-His-His-Cys (DHHC) motif-containing host *S*-palmitoyltransferases results in effector targeting to the host cell plasma membrane. As these effectors are targeted to different plasma membrane domains, *S*-palmitoylation might not be sufficient to target these effectors to their final destination. SspH2 might be involved in modulating the host immune response, and SseI has been reported to regulate cell migration. **b** | *N*-myristoylation involves the covalent attachment of a 14-carbon myristic acid, via an amide bond, to the α-amino group of the Gly in a GXXX(S/T/C) N-terminal consensus sequence. The attachment is catalysed by an *N*-myristoyltransferase. The *Pseudomonas aeruginosa* T3SS effector avirulence protein B (AvrB) is attached to the host plant cell membrane by *N*-myristoylation and *S*-palmitoylation. AvrB has been shown to inhibit plant immunity in susceptible plants through phosphorylation of the immune regulator RPM1-interacting protein 4 (RIN4). **c** | Prenylation involves the covalent addition of a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid group to a Cys residue within a conserved carboxy-terminal CaaX motif (in which a represents an aliphatic residue and X is one of four amino acids). When X is Ser, Met or Gln, the protein is recognized by a farnesyltransferase, and when X is Leu, the protein is modified by a geranylgeranyl transferase. The *Legionella pneumophila* effector ankyrin B (AnkB), which is secreted by the type IV secretion system, is essential for *L. pneumophila* proliferation within the *Legionella*-containing vacuole (LCV). Following AnkB translocation into the host cell, the C-terminal CaaX motif is farnesylated, allowing the effector to associate with the cytosolic face of the LCV. The *S. enterica* T3SS effector SifA is geranylgeranylated on a C-terminal CaaX motif. SifA is required for *S. enterica* pathogenesis and is thought to regulate membrane fusion as the *Salmonella*-containing vacuole (SCV) expands.

(RIN4), a regulator of basal plant defence that localizes to the plasma membrane of plant cells[22,23] (FIG. 1b). Consistent with the importance of these modifications for effector function, *N*-myristoylation and, to a lesser extent, *S*-palmitoylation were shown to be required for AvrB- and AvrRpm1-mediated triggering of defence responses in resistant plants and for the virulence phenotype of AvrRpm1 in susceptible hosts[19].

Both AvrB and AvrRpm1 possess conventional *N*-myristoylation sites like those found at the very N terminus of eukaryotic proteins, but another subset of Avr type III effectors, including AvrPphB (also known as HopAR1) and ORF4 from *P. syringae*, and nodulation protein NopT from *Rhizobium* spp., possesses internal *N*-myristoylation sites within their full-length protein sequences[24]. Interestingly, following translocation into host cells, these effectors undergo an autoproteolytic processing event that exposes a new N terminus which contains the *N*-myristoylation consensus sequence. This process is analogous to the *N*-myristoylation of certain eukaryotic proteins following cleavage by caspases to reveal cryptic *N*-myristoylation consensus sites[25]. The processed bacterial effectors are then *N*-myristoylated, as well as *S*-palmitoylated, by the host and targeted to the plasma membrane[24]. AvrPphB specifically cleaves the *Arabidopsis thaliana* plasma membrane-localized protein kinase PBS1 to inhibit the RPS5 (resistance to *Pseudomonas syringae* protein 5)-dependent immune response in susceptible hosts; in resistant plants, this PBS1 cleavage triggers defence responses[26,27]. Dual lipidation is required not only for the plasma membrane localization of AvrPphB, but also for the activity of this effector[24]. Therefore, *P. syringae* encodes proteins bearing both conventional and cryptic eukaryotic *N*-myristoylation motifs that can functionally target bacterial effectors to the plasma membrane of the host cell during infection.

Prenylation. Prenylation is a permanent post-translational modification at the C terminus of a protein and is required for protein stability. This modification results in the localization of a protein to an intracellular membrane through attachment of the modified C terminus to the membrane. Prenylation involves the covalent addition of a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid group to a Cys residue within the conserved C-terminal tetrapeptide CaaX motif (in which 'a' represents an aliphatic residue and X is one of four amino acids)[28]. The residue located at position X determines whether the protein will be farnesylated or geranylgeranylated: when X is Ser, Met or Gln, the protein is recognized by farnesyltransferases, and when X is Leu, the protein is modified by geranylgeranyltransferases[28] (FIG. 1c). These prenyltransferases are found only in eukaryotic cells. Following prenylation, the terminal aaX sequence is cleaved off by RAS-converting enzyme 1 (RCE1), which is located in the ER membrane[29]. The C-terminal prenylated Cys residue is then methylated by isoprenyl-Cys carboxyl methyltransferases[30].

Legionella pneumophila, the cause of Legionnaires' disease, encodes several T4SS effectors with CaaX motifs within their sequences, and these CaaX motifs are required for subcellular localization of the T4SS effectors[31–33]. For example, following translocation into the host cytosol, the *L. pneumophila* effector ankyrin B (AnkB) is farnesylated on the Cys residue of a conserved CaaX motif by the host protein farnesyltransferase and, as a result, becomes associated with the cytosolic face of the *Legionella*-containing vacuole (LCV)[32] (FIG. 1c). Mutation of the crucial Cys residue to Ala or inhibition of host protein farnesyltransferase results in loss of farnesylation and failure to target to the LCV. The LCV membrane is derived from the ER, which contains the eukaryotic enzymes required for processing farnesylated proteins. It has therefore been proposed that immediately following translocation into the LCV, AnkB is locally prenylated, anchoring it into the LCV membrane[32]. AnkB also contains a eukaryotic F-box domain that is thought to recruit polyubiquitylated proteins to the LCV. AnkB is required for rapid acquisition of polyubiquitylated proteins and is essential for intracellular *L. pneumophila* proliferation. *L. pneumophila* strains expressing prenylation-deficient AnkB are unable to recruit polyubiquitylated proteins to the LCV and are attenuated in a mouse model of Legionnaires' disease[32,33].

The *S. enterica* T3SS effector SifA contains a C-terminal non-traditional CaaX motif (^{331}CLCCFL) and is geranylgeranylated on Cys333 by a host geranylgeranyltransferase[34,35]. SifA localizes to the *Salmonella*-containing vacuole (SCV) (FIG. 1c) as well as to tubular membrane structures known as *Salmonella*-induced filaments (SIFs), which extend from the SCV during infection[36]. Inhibition of SifA prenylation leads to the mislocalization of SifA to the cytosol[34]. Although prenylation alone can facilitate interaction of the protein with cellular membranes, some prenylated proteins require either a stretch of basic amino acids or *S*-palmitoylation upstream of the prenylation site for their stable association with membranes. It was shown that Cys331 of SifA is *S*-palmitoylated, which presumably strengthens the association with membranes[34]. SifA is required for *S. enterica* pathogenesis and is thought to regulate membrane fusion to help provide sufficient membrane as the SCV expands[36]. Deletion of SifA results in a bacterial replication defect in macrophages and reduced bacterial colonization in mouse studies; however, prenylation- and *S*-palmitoylation-deficient SifA strains do not show this phenotype[34]. Therefore, although targeting of SifA to the SCV and SIFs requires prenylation, the contribution of this localization to SifA function remains unclear.

Membrane targeting by ubiquitylation

Ubiquitylation results in the covalent attachment of ubiquitin to a Lys residue on a target protein and constitutes a common and important post-translational modification in eukaryotic cells[37]. Following the initial conjugation, a substrate can remain monoubiquitylated at a single Lys residue, or additional ubiquitin molecules can be ligated either to one of the seven Lys residues in the first ubiquitin molecule, resulting in polyubiquitylation, or to multiple Lys residues in the original protein, resulting in multiubiquitylation. The type of ubiquitylation and the topology of the ubiquitin chains that are formed direct the fate of the substrate[38].

Protein ubiquitylation can be a signal for proteasome-dependent degradation of the protein, but it can also modulate protein function by, for example, changing protein localization or allowing the formation of multiprotein complexes[39–43]. Many bacterial effector proteins have evolved to directly modulate the ubiquitylation

Caspases
A family of Cys-Asp proteases involved in apoptosis, necrosis and inflammasome activation.

Legionella-containing vacuole
A host cell membrane-dervived compartment in which intracellular growth of *Legionella* spp. occurs.

Salmonella-induced filaments
A tubular network of membranes that extend from the *Salmonella*-containing vacuole.

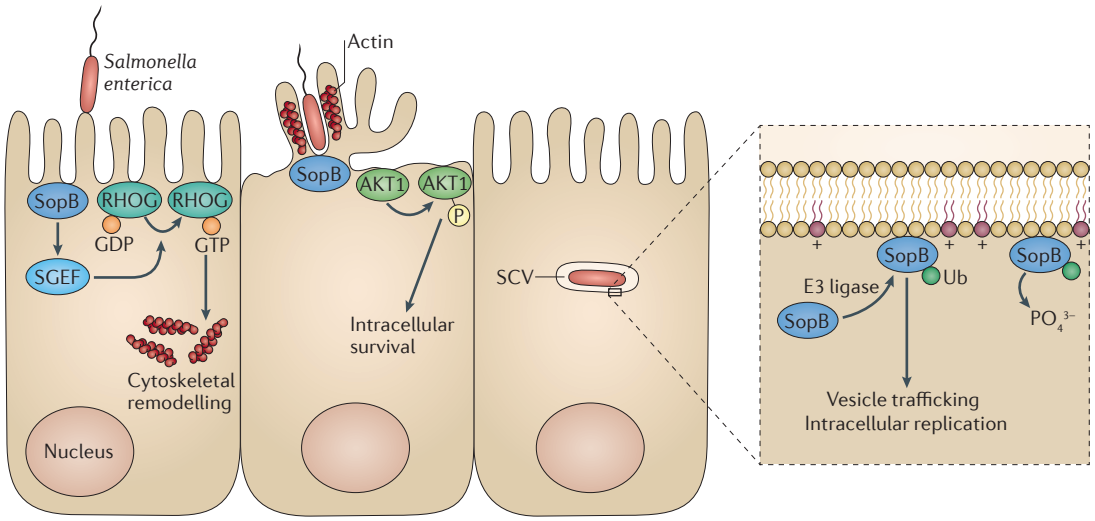

Figure 2 | **Membrane targeting of effectors by host-mediated ubiquitylation.** The *Salmonella enterica* phosphoinositide phosphatase SopB is secreted into the host cell by the type III secretion system. Temporal regulation of the SopB cellular localization leads to three distinct activities. Early during infection, SopB localizes to the plasma membrane, where it stimulates the RHOG exchange factor SGEF (SRC homology 3 domain-containing guanine nucleotide exchange factor), thus mediating actin-dependent *S. enterica* internalization into non-phagocytic cells. At the plasma membrane. SopB also promotes intracellular survival by activating the protein kinase AKT1. Later in infection, SopB is monoubiquitylated, and this modification facilitates SopB localization to the *Salmonella*-containing vacuole (SCV), where the effector modulates the phosphoinositide composition and charge distribution of the membrane, thus affecting the membrane trafficking of this compartment. Ub, ubiquitin.

pathway by acting as deubiquitinases or E3 ligases, thus altering the function or stability of host target proteins[12,44,45]. Other effector proteins, however, use ubiquitylation to modulate their own function. For example, it has been shown that the half-life of some effectors is controlled by ubiquitin-mediated degradation[46,47]. In other cases, ubiquitylation can be crucial for targeting the effector protein to its final destination. SopB is an *S. enterica* T3SS effector that is involved in at least three distinct phenotypes, all of which are strictly dependent on SopB phosphoinositide phosphatase activity. By stimulating the host RHOG exchange factor SGEF (SRC homology 3 domain-containing guanine nucleotide exchange factor), SopB mediates actin-dependent *S. enterica* internalization into non-phagocytic cells[48] (FIG. 2). In addition, SopB is required for activation of the protein kinase AKT1 by still poorly understood mechanisms, and this promotes intracellular survival of the bacterium[49] (FIG. 2). Last, SopB can modulate the phosphoinositide composition of the SCV, thus modulating the membrane trafficking of this compartment[50] (FIG. 2). This functional diversification correlates with the temporal regulation of SopB localization. Early during infection, SopB localizes to the plasma membrane, where it activates RHOG and AKT. Later, it localizes to the SCV, where it modulates the phosphoinositide composition of the SCV membrane. The differential localization of SopB is strictly dependent on monoubiquitylation of the protein, as a SopB mutant that cannot be ubiquitylated remains at the plasma membrane[51,52]. Although this mutant can mediate RHOG-dependent entry and AKT activation, it is unable to localize to the SCV, resulting in a defect in intracellular growth. The ubiquitin-mediated relocalization of SopB is reminiscent of the ubiquitin-mediated relocalization of epidermal growth factor receptor (EGFR) from the plasma membrane to an endocytic compartment in eukaryotes[53,54]. However, although both proteins are relocalized in a ubiquitin-dependent manner, the cellular machinery necessary for this relocalization seems to be different for each protein, as depletion of proteins required for EGFR delivery to an endosomal compartment (that is, components of the ESCRT (endosomal sorting complex required for transport) complex) does not affect SopB delivery to the SCV[52].

ESCRT
(Endosomal sorting complex required for transport). A conserved cellular machinery for the sorting of ubiquitylated cargo proteins into vesicles and the subsequent scission of the membrane neck.

Membrane targeting by phospholipid binding

Phosphoinositides play an essential part in regulating a variety of cellular processes ranging from membrane trafficking to actin dynamics[55–57]. The reversible phosphorylation of the inositol group of phosphoinositides at different positions and in various combinations can generate seven phosphoinositide species, which concentrate in different intracellular membranes. This differential distribution is highly regulated and allows for effective subcellular targeting of host membrane-associated signalling events. Phosphoinositides serve as anchor moieties for a variety of proteins with binding domains that are selective for different phosphoinositide species. Many bacterial effectors have been shown to use phosphoinositides to accurately target their biochemical activities[58,59]. For example, several *L. pneumophila* T4SS effectors are targeted to the LCV by binding different phosphoinositides: SidC and SidM (also known

as DrrA) are targeted by binding phosphatidylinositol-4-phosphate (PtdIns4P)[60–63], SetA by interacting with PtdIns3P[64], and LidA by binding both of these phosphoinositides[62,63]. Together, these effectors influence the remodelling and maturation of the LCV by promoting the interaction of the LCV with host vesicles and organelles. Interestingly, the discrete domains of these effectors that bind PtdIns4P or PtdIns3P do not exhibit any detectable sequence or structural similarity to equivalent domains in eukaryotic proteins, suggesting that these domains are the result of convergent evolution, a common theme in the evolution of effector proteins[65].

The *Pseudomonas aeruginosa* T3SS effector protein ExoU is another example of an effector that is targeted to its final destination by phosphoinositide binding. ExoU is a phospholipase that is targeted to the plasma membrane by a discrete α-helical bundle domain at its C terminus; this domain has a high affinity for PtdIns-4,5-bisphosphate (PtdIns(4,5)P_2), which is abundant at the cytoplasmic side of the plasma membrane[66,67]. Targeting of the ExoU phospholipase activity to the plasma membrane via the ExoU C-terminal phosphoinositide-binding domain leads to rapid necrotic host cell death by disrupting host cell membranes, and this promotes *P. aeruginosa* pathogenesis[67–69].

Helicobacter pylori infections are associated with the development of stomach cancer[70]. Central to the pathogenesis of this bacterium is the function of a T4SS that, through the delivery of the effector CagA, modulates a variety of host cell functions[71]. Following delivery by the T4SS, CagA localizes to the inner surface of the plasma membrane of polarized cells[72]. Targeting of CagA to this location involves binding to phosphatidylserine through a non-canonical PH (pleckstrin homology) domain. However, in nonpolarized cells, CagA is targeted to the plasma membrane by a C-terminally located EPIYA (Glu-Pro-Ile-Tyr-Ala) motif. It has been proposed that this targeting mechanism must involve an EPIYA motif-binding protein that interacts with CagA[73], although such a protein has not yet been identified.

Targeting to mitochondria and chloroplasts

Eukaryotic cells use similar mechanisms to target proteins to mitochondria and chloroplasts. Most eukaryotic mitochondrial and chloroplast proteins are synthesized in the cytosol as pre-proteins that encode either an N-terminal pre-sequence or an internal targeting signal[74,75]. The pre-sequence forms an amphiphilic α-helix with positively charged residues on one side of the helix and hydrophobic residues aligned on the other side. During synthesis, many mitochondrial and chloroplast pre-proteins associate with molecular chaperones that maintain them in a partially unfolded conformation before targeting to the organelle.

The targeting mechanisms for mitochondrial proteins have been examined in detail. The targeting signal is recognized by receptor subunits of the TOM (transport outer membrane) and TIM (transport inner membrane) complexes present in mitochondrial membranes. Following binding of the pre-sequence to these complexes, the pre-protein is imported across the mitochondrial membranes in an unfolded state. After translocation, the pre-sequence is usually cleaved off, and chaperones in the mitochondrial matrix mediate protein folding[76]. Mitochondrial import is generally described as a post-translational process, implying that proteins are completely synthesized before import starts[74].

Amino acid sequence analysis revealed that the N-terminal T3SS signals of most *P. syringae* effectors have a similar amino acid composition to chloroplast and mitochondrial targeting pre-sequences[77]. As a result, most *P. syringae* effectors are predicted to localize to chloroplasts or mitochondria[77]. However, this similarity could merely imply an evolutionarily conserved mechanism used by these organelles and the T3SS to recognize and target substrates. Therefore, caution should be exercised before assigning a mitochondrial or chloroplast localization to bacterial T3SS effectors on the basis of bioinformatic analysis alone. Nevertheless, studies have shown that, when transiently expressed in plants, the *P. syringae* effectors HopL1 and HopG1 are targeted to chloroplasts and mitochondria, respectively, altering thylakoid structures (in the case of HopL1) and suppressing host defences (in the case of both proteins)[78,79]. The targeting of HopL1 and HopG1 seems to involve non-canonical mechanisms, as these particular effectors do not have canonical targeting signals and do not undergo proteolytic processing following targeting. Whether these effectors are targeted to chloroplasts or mitochondria during bacterial infection remains unknown.

In addition to *P. syringae* effectors, the enteropathogenic *Escherichia coli* (EPEC) T3SS effectors Map, EspF and EspZ encode putative N-terminal mitochondrial pre-sequences with cleavage signals[80,81]. After Map delivery into the host cell by the T3SS, the N terminus of the protein can be cleaved, and Map then localizes to host mitochondria, indicating that the host cell mitochondrial targeting machinery can recognize Map as a substrate[80]. *In vitro* assays showed that mitochondrial import of Map involves components of the mitochondrial import pathway (TOM22, TOM40 and mtHSP70 (also known as GRP75)), which is consistent with Map import into the mitochondria matrix via the classical import mechanism[82] (FIG. 3). In addition, it has been reported that Map disrupts the mitochondrial membrane potential, leading to mitochondrial fragmentation[80,82]. More recent structural and functional studies, however, have shown that EPEC Map is a guanine nucleotide exchange factor (GEF)[83,84]. In addition to a GEF domain, Map possesses a canonical C-terminal PDZ-binding motif that interacts with the PDZ domains of ezrin–radixin–moesin–binding phosphoprotein 50 (EBP50; also known as NHERF1)[85,86]. Following translocation, Map localizes to the plasma membrane at the bacterium–host interface[86]. This localization results in cell division cycle 42 (CDC42) activation, leading to the localized formation of actin-rich membrane protrusions[86] (FIG. 3). Although this particular activity seems to be at odds with the reported mitochondrial localization of Map, and the mitochondrial membrane disruption

Guanine nucleotide exchange factor
(GEF). A protein that induces a GTPase to exchange GTP for GDP, resulting in activation of the GTPase.

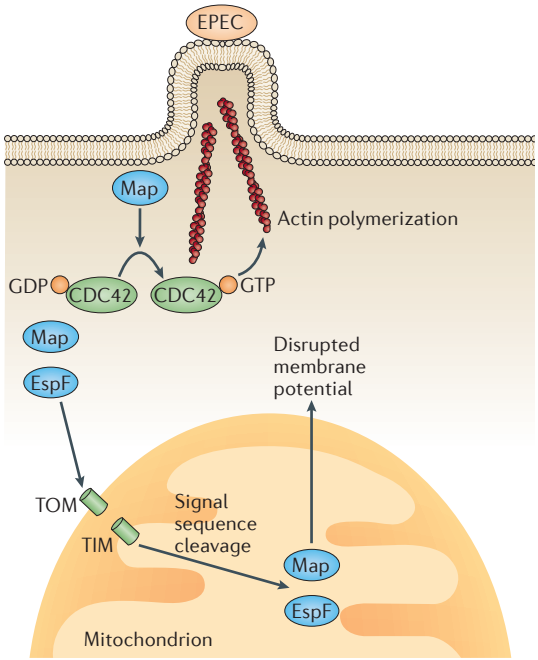

Figure 3 | **Effector targeting to mitochondria.** The enteropathogenic *Escherichia coli* (EPEC) effectors Map and EspF are secreted into host cells by the type III secretion system. These effectors contain putative amino-terminal mitochondrial targeting signals, and when the effectors are transiently expressed in eukaryotic cells, they localize to mitochondria by interacting with components of the classical mitochondrial transport out membrane (TOM) import machinery. Map has been shown to disrupt the mitochondrial membrane potential. Map also localizes to the plasma membrane at the bacterium–host interface and, as a guanine nucleotide exchange factor, activates cell division cycle 42 (CDC42), leading to the localized formation of actin-rich membrane protrusions. TIM, transport inner membrane.

caused by the protein, it has been observed that at later time points, Map colocalizes with the mitochondria. It is therefore possible that Map has a dual function, working as a GEF for CDC42 to mediate actin rearrangements at the plasma membrane early during infection, thus promoting bacterial attachment, and disrupting mitochondrial function later in infection.

Some studies have suggested that the EPEC T3SS effector EspF is targeted to host mitochondria[81,85,87–89] (FIG. 3). However, other studies have also observed that EspF can be targeted to the nucleus and/or plasma membrane[87,88]. Some of these disparate subcellular localizations could be a result of the use of epitope tags or, in some instances, overexpression to visualize the protein, as these factors can alter the physiological localization of proteins. Alternatively, the disparate locations for EspF could be a reflection of functional diversification, as several functions have been attributed to this effector, including disruption of tight junctions, inhibition of phagocytosis, remodelling of the plasma membrane, rearrangement of the cytoskeleton, disruption of the nucleolus, disruption of mitochondrial function, and apoptosis[90,91]. Finally, it should be observed that when ectopically expressed within mammalian cells, some bacterial proteins can localize to the host mitochondria despite clear evidence that during infection this is not their final destination[92], suggesting that cryptic mitochondrial targeting signals are common in bacterial proteins. Therefore, the observation that an effector protein targets to mitochondria should be interpreted with caution in the absence of functional evidence to support the physiological relevance of such an observation.

Nuclear localization

Given the central importance of nuclear functions in cellular physiology, it is not surprising that many bacterial effectors have evolved to carry out their function within the nucleus of an infected cell[93–100]. Most proteins that function in the nucleus are selectively imported from the cytosol through nuclear pore complexes, which are large structures that form channels allowing proteins smaller than the diameter of the channel to passively diffuse into the nucleus. Globular proteins larger than 50 kDa are unable to passively diffuse through the nuclear pore complex and therefore require active import, which is dependent on the presence of a nuclear localization signal (NLS) in the protein.

Several pathways for nuclear import have been described. The classical NLS consists of either one (monopartite) or two (bipartite) short amino acid sequences rich in the positively charged amino acids Lys and Arg. NLSs can be present in multiple copies, usually forming a loop on the surface of the fully folded protein. NLSs are recognized by soluble carriers of the importin-α and importin-β superfamilies[101]. In classical nuclear import, importin-α recognizes and binds both the nuclear cargo and importin-β in the cytoplasm, and importin-β then mediates the interaction of the complex with the nuclear pore as it translocates into the nucleus[102–104]. When the complex reaches the nucleus, RAN·GTP binds to importin-β, causing a conformational change that results in the release of the importin-α–cargo complex into the nucleus[105].

In most instances, the transport of bacterial effector proteins into the nucleus is carried out by the cellular nuclear import machinery. For example, members of the plant pathogen genus *Xanthomonas* encode a large family of transcription activator-like (TAL) T3SS effectors, including AvrBs3, AvrXa5, AvrXa7, AvrXa10 and PthA, which localize to the plant nucleus and regulate plant gene expression during infection[94]. TAL effectors are characterized by a central DNA-binding region consisting of a nearly identical tandem of 34 amino acid repeats, followed by a classic monopartite NLS and an acidic transcriptional activation domain (AAD)[94,95]. In susceptible host species, the TAL effector AvrBs3 of *Xanthomonas campestris* pv. *vesicatoria* modulates gene expression to induce hypertrophy of plant host cells[106]. By contrast, in resistant species, AvrBs3 triggers defence responses, leading to localized host cell death. Both responses require the NLS and AAD of AvrBs3, supporting a functional role for AvrBs3 in the host nucleus[106–108]. Sequence analysis revealed three putative NLSs within

the C-terminal domain of AvrBs3 and found that these sequences are conserved in other members of the TAL family[109]. NLS2 and NLS3 of AvrBs3 were shown to be required for AvrBs3 to be fully active, whereas NLS1 is insufficient for AvrBs3 activity[107,110]. In *Capsicum annuum* plants expressing the Bs3 protein, AvrBs3 was shown, by both yeast two-hybrid and *in vitro* binding assays, to interact with the importin-α proteins CALMPα1 and CALMPα2, two members of the classical nuclear import pathway that mediate nuclear import by binding the NLS of a substrate protein in the cytosol[110]. The interaction of AvrBs3 with CALMPα1 and CALMPα2 was shown to be dependent on NLS2 (REF. 110). Therefore, following translocation into the host cell cytosol, AvrBs3 is targeted to the nucleus by the classical eukaryotic nuclear import pathway, and there it alters host gene expression.

Agrobacterium tumefaciens pathogenesis requires the transfer of bacterial tumour-inducing DNA (transfer DNA (T-DNA)) into the host plant cell[111]. The T-DNA is then imported into the cell nucleus and integrates into the plant cell genome. Expression of T-DNA-encoded genes leads to tumour formation in the host plant. The nuclear import of T-DNA requires the function of two *A. tumefaciens* T4SS effectors, VirD2 and VirE2 (FIG. 4). VirD2 associates with the 5′ end of the single-stranded T-DNA, and VirE2 coats the rest of the T-DNA molecule[112,113]. Both VirD2 and VirE2 possess functional NLSs. Of the two NLSs in VirD2, the C terminus-proximal NLS has been shown to target reporter proteins to the nucleus in plant, animal and yeast cells[96,97]. VirD2 binds the *A. thaliana* importin-α subunit KAPα1 in a manner dependent on the presence of the C terminus-proximal NLS[114], consistent with KAPα1 acting as an import receptor for the classical nuclear import pathway.

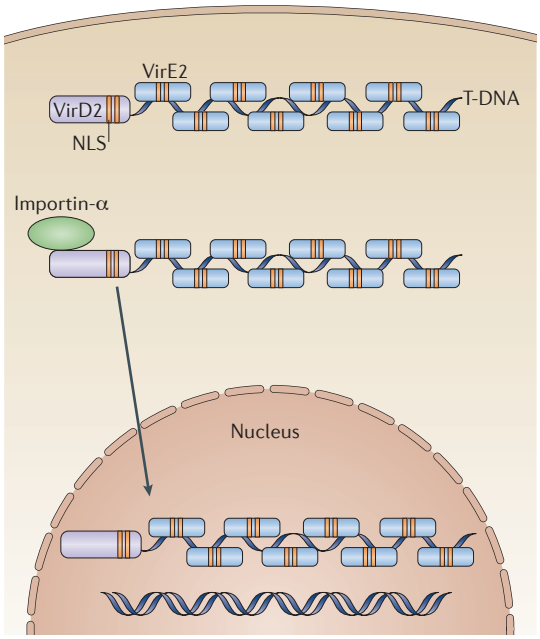

Figure 4 | **Effector targeting to the nucleus.** *Agrobacterium tumefaciens* must transfer the bacterial tumour-inducing DNA (known as transfer DNA (T-DNA)) into the host plant cell, where it is imported into the nucleus and integrates into the plant cell genome. The nuclear import of T-DNA requires two *A. tumefaciens* effectors, VirD2 and VirE2, which are secreted into the host cell via the type IV secretion system. VirD2 associates with the 5′ end of the single-stranded T-DNA, and VirE2 coats the rest of the T-DNA. Both effectors contain nuclear localization signals (NLSs). VirD2 has been shown to bind the *A. thaliana* importin-α subunit KAPα1 in an NLS-dependent manner, suggesting that KAPα1 is an import receptor for the classical nuclear import pathway.

Concluding remarks

Some bacteria have evolved simple mechanisms to deliver a limited number of biochemical activities into eukaryotic cells in the form of exotoxins. The delivery mechanisms are so simple that all the necessary information is contained within a single polypeptide in some instances and in just a handful of proteins in the more complex cases. However, the need to deliver multiple (in some cases, more than 100) biochemical activities into the same cell in the form of effector proteins and in a temporally and spatially restricted manner has demanded the evolution of the T3SS and T4SS, which are much more complex delivery machines. The diversity of the biochemical activities of these effectors and the coordination that is necessary for them to exert their appropriate functions demand that the effectors are targeted precisely to the appropriate subcellular location. It is becoming increasingly clear that this requires host cell machinery which is usurped by these effectors to reach their final destination. The challenge for the future will be to visualize the fate of the translocated effectors in live cells during bacterial infection. This is difficult because, in addition to the intrinsic limitations of the protein delivery systems themselves, the effector proteins are delivered at very low concentrations. Therefore, more sensitive live-imaging techniques will be required to address this important issue. It is also evident that in order to understand the contribution of the different effectors to bacterial pathogenesis, we need a better understanding of their subcellular localizations following translocation. It is also possible that information gained by studying the targeting mechanisms used by different effectors will lead to the development of novel therapeutic strategies to combat infections by these pathogens.

1. Christie, P., Atmakuri, K., Krishnamoorthy, V., Jakubowski, S. & Cascales, E. Biogenesis, architecture, and function of bacterial type IV secretion systems. *Annu. Rev. Microbiol.* **59**, 451–485 (2005).
2. Galán, J. E. & Wolf-Watz, H. Protein delivery into eukaryotic cells by type III secretion machines. *Nature* **444**, 567–573 (2006).
3. Galán, J. Common themes in the design and function of bacterial effectors. *Cell Host Microbe* **5**, 571–579 (2009).
4. Ashida, H. *et al.* A bacterial E3 ubiquitin ligase IpaH9.8 targets NEMO/IKKγ to dampen the host NF-κB-mediated inflammatory response. *Nature Cell Biol.* **12**, 66–73 (2010).
5. Xin, D. W. *et al.* Functional analysis of NopM, a novel E3 ubiquitin ligase (NEL) domain effector of *Rhizobium* sp. strain NGR234. *PLoS Pathog.* **8**, e1002707 (2012).
6. Linder, M. E. & Deschenes, R. J. Palmitoylation: policing protein stability and traffic. *Nature Rev. Mol. Cell Biol.* **8**, 74–84 (2007).

7. Resh, M. D. Palmitoylation of ligands, receptors, and intracellular signaling molecules. *Sci. STKE* **2006**, re14 (2006).
8. Smotrys, J. E. & Linder, M. E. Palmitoylation of intracellular signaling proteins: regulation and function. *Annu. Rev. Biochem.* **73**, 559–587 (2004).
9. Greaves, J. & Chamberlain, L. H. Palmitoylation-dependent protein sorting. *J. Cell Biol.* **176**, 249–254 (2007).
10. Hicks, S. W., Charron, G., Hang, H. C. & Galán, J. E. Subcellular targeting of *Salmonella* virulence proteins by host-mediated *S*-palmitoylation. *Cell Host Microbe* **10**, 9–20 (2011).
The first study to demonstrate *S*-palmitoylation of a bacterially translocated effector during infection. This study also shows the direct *S*-palmitoylation of these effector *in vitro* by host cell enzymes.
11. Quezada, C. M., Hicks, S. W., Galán, J. E. & Stebbins, C. E. A family of *Salmonella* virulence factors functions as a distinct class of autoregulated E3 ubiquitin ligases. *Proc. Natl Acad. Sci. USA* **106**, 4864–4869 (2009).
12. Hicks, S. W. & Galán, J. E. Hijacking the host ubiquitin pathway: structural strategies of bacterial E3 ubiquitin ligases. *Curr. Opin. Microbiol.* **13**, 41–46 (2010).
13. McLaughlin, L. M. *et al.* The *Salmonella* SPI2 effector SseI mediates long-term systemic infection by modulating host cell migration. *PLoS Pathog.* **5**, e1000671 (2009).
14. Worley, M. J., Nieman, G. S., Geddes, K. & Heffron, F. *Salmonella typhimurium* disseminates within its host by manipulating the motility of infected cells. *Proc. Natl Acad. Sci. USA* **103**, 17915–17920 (2006).
15. Farazi, T. A., Waksman, G. & Gordon, J. I. The biology and enzymology of protein *N*-myristoylation. *J. Biol. Chem.* **276**, 39501–39504 (2001).
16. Towler, D. A., Eubanks, S. R., Towery, D. S., Adams, S. P. & Glaser, L. Amino-terminal processing of proteins by *N*-myristoylation. Substrate specificity of *N*-myristoyl transferase. *J. Biol. Chem.* **262**, 1030–1036 (1987).
17. Block, A. & Alfano, J. Plant targets for *Pseudomonas syringae* type III effectors: virulence targets or guarded decoys? *Curr. Opin. Microbiol.* **14**, 39–46 (2011).
18. Feng, F. & Zhou, J. Plant–bacterial pathogen interactions mediated by type III effectors. *Curr. Opin. Plant Biol.* **15**, 469–476 (2012).
19. Nimchuk, Z. *et al.* Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae*. *Cell* **101**, 353–363 (2000).
An investigation which shows that AvrB and AvrRpm1 are both *S*-palmitoylated and *N*-myristoylated in host cells and that these modifications are required for virulence in susceptible hosts.
20. Lewis, J. D., Abada, W., Ma, W., Guttman, D. S. & Desveaux, D. The HopZ family of *Pseudomonas syringae* type III effectors require myristoylation for virulence and avirulence functions in *Arabidopsis thaliana*. *J. Bacteriol.* **190**, 2880–2891 (2008).
21. Robert-Seilaniantz, A., Shan, L., Zhou, J. M. & Tang, X. The *Pseudomonas syringae* pv. tomato DC3000 type III effector HopF2 has a putative myristoylation site required for its avirulence and virulence functions. *Mol. Plant Microbe Interact.* **19**, 130–138 (2006).
22. Thornbrough, J. M. & Worley, M. J. A naturally occurring single nucleotide polymorphism in the *Salmonella* SPI-2 type III effector *srfH*/*sseI* controls early extraintestinal dissemination. *PLoS ONE* **7**, e45245 (2012).
23. Marathe, R. & Dinesh-Kumar, S. P. Plant defense: one post, multiple guards?! *Mol. Cell* **11**, 284–286 (2003).
24. Dowen, R. H., Engel, J. L., Shao, F., Ecker, J. R. & Dixon, J. E. A family of bacterial cysteine protease type III effectors utilizes acylation-dependent and -independent strategies to localize to plasma membranes. *J. Biol. Chem.* **284**, 15867–15879 (2009).
25. Martin, D. D., Beauchamp, E. & Berthiaume, L. G. Post-translational myristoylation: fat matters in cellular life and death. *Biochimie* **93**, 18–31 (2011).
26. Shao, F. *et al.* Cleavage of *Arabidopsis* PBS1 by a bacterial type III effector. *Science* **301**, 1230–1233 (2003).
27. Shao, F., Merritt, P. M., Bao, Z., Innes, R. W. & Dixon, J. E. A. *Yersinia* effector and a *Pseudomonas* avirulence protein define a family of cysteine proteases functioning in bacterial pathogenesis. *Cell* **109**, 575–588 (2002).
28. Zhang, F. L. & Casey, P. J. Protein prenylation: molecular mechanisms and functional consequences. *Annu. Rev. Biochem.* **65**, 241–269 (1996).
29. Boyartchuk, V. L., Ashby, M. N. & Rine, J. Modulation of Ras and a-factor function by carboxyl-terminal proteolysis. *Science* **275**, 1796–1800 (1997).
30. Dai, Q. *et al.* Mammalian prenylcysteine carboxyl methyltransferase is in the endoplasmic reticulum. *J. Biol. Chem.* **273**, 15030–15034 (1998).
31. Ivanov, S. S., Charron, G., Hang, H. C. & Roy, C. R. Lipidation by the host prenyltransferase machinery facilitates membrane localization of *Legionella pneumophila* effector proteins. *J. Biol. Chem.* **285**, 34686–34698 (2010).
32. Price, C. T., Al-Quadan, T., Santic, M., Jones, S. C. & Abu Kwaik, Y. Exploitation of conserved eukaryotic host cell farnesylation machinery by an F-box effector of *Legionella pneumophila*. *J. Exp. Med.* **207**, 1713–1726 (2010).
Work finding that AnkB is targeted to the LCV via prenylation, and that AnkB prenylation influences disease severity.
33. Price, C. T., Jones, S. C., Amundson, K. E. & Kwaik, Y. A. Host-mediated post-translational prenylation of novel Dot/Icm-translocated effectors of *Legionella pneumophila*. *Front. Microbiol.* **1**, 131 (2010).
34. Reinicke, A. T. *et al.* A *Salmonella typhimurium* effector protein SifA is modified by host cell prenylation and *S*-acylation machinery. *J. Biol. Chem.* **280**, 14620–14627 (2005).
35. Boucrot, E., Beuzon, C. R., Holden, D. W., Gorvel, J. P. & Meresse, S. *Salmonella typhimurium* SifA effector protein requires its membrane-anchoring C-terminal hexapeptide for its biological function. *J. Biol. Chem.* **278**, 14196–14202 (2003).
36. Stein, M. A., Leung, K. Y., Zwick, M., Garcia-del Portillo, F. & Finlay, B. B. Identification of a *Salmonella* virulence gene required for formation of filamentous structures containing lysosomal membrane glycoproteins within epithelial cells. *Mol. Microbiol.* **20**, 151–164 (1996).
37. Kerscher, O., Felberbaum, R. & Hochstrasser, M. Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu. Rev. Cell Dev. Biol.* **22**, 159–180 (2006).
38. Ikeda, F. & Dikic, I. Atypical ubiquitin chains: new molecular signals. *EMBO Rep.* **9**, 536–542 (2008).
39. Haglund, K. & Dikic, I. Ubiquitylation and cell signaling. *EMBO J.* **24**, 3353–3359 (2005).
40. Hicke, L. & Dunn, R. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu. Rev. Cell Dev. Biol.* **19**, 141–172 (2003).
41. Huang, J., Xu, L. G., Liu, T., Zhai, Z. & Shu, H. B. The p53-inducible E3 ubiquitin ligase p53RFP induces p53-dependent apoptosis. *FEBS Lett.* **580**, 940–947 (2006).
42. Thrower, J. S., Hoffman, L., Rechsteiner, M. & Pickart, C. M. Recognition of the polyubiquitin proteolytic signal. *EMBO J.* **19**, 94–102 (2000).
43. Xu, P. *et al.* Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell* **137**, 133–145 (2009).
44. Kubori, T. & Nagai, H. Bacterial effector-involved temporal and spatial regulation by hijack of the host ubiquitin pathway. *Front. Microbiol.* **2**, 145 (2011).
45. Collins, C. A. & Brown, E. J. Cytosol as battleground: ubiquitin as a weapon for both host and pathogen. *Trends Cell Biol.* **20**, 205–213 (2010).
46. Kubori, T. & Galan, J. E. Temporal regulation of *Salmonella* virulence effector function by proteasome-dependent protein degradation. *Cell* **115**, 333–342 (2003).
47. Kubori, T., Shinzawa, N., Kanuka, H. & Nagai, H. *Legionella* metaeffector exploits host proteasome to temporally regulate cognate effector. *PLoS Pathog.* **6**, e1001216 (2010).
48. Patel, J. C. & Galán, J. E. Differential activation and function of Rho GTPases during *Salmonella*–host cell interactions. *J. Cell Biol.* **175**, 453–463 (2006).
49. Steele-Mortimer, O. *et al.* Activation of Akt/protein kinase B in epithelial cells by the *Salmonella typhimurium* effector SigD. *J. Biol. Chem.* **275**, 37718–37724 (2000).
50. Hernandez, L. D., Hueffer, K., Wenk, M. R. & Galán, J. E. *Salmonella* modulates vesicular traffic by altering phosphoinositide metabolism. *Science* **304**, 1805–1807 (2004).
51. Knodler, L. A., Winfree, S., Drecktrah, D., Ireland, R. & Steele-Mortimer, O. Ubiquitination of the bacterial inositol phosphatase, SopB, regulates its biological activity at the plasma membrane. *Cell. Microbiol.* **11**, 1652–1670 (2009).
52. Patel, J. C., Hueffer, K., Lam, T. T. & Galán, J. E. Diversification of a *Salmonella* virulence protein function by ubiquitin-dependent differential localization. *Cell* **137**, 283–294 (2009).
An investigation which demonstrates that SopB is monoubiquitylated and that this results in SopB removal from the plasma membrane and delivery to the SCV.
53. Sorkin, A. & Goh, L. K. Endocytosis and intracellular trafficking of ErbBs. *Exp. Cell Res.* **315**, 683–696 (2009).
54. von Zastrow, M. & Sorkin, A. Signaling on the endocytic pathway. *Curr. Opin. Cell Biol.* **19**, 436–445 (2007).
55. De Matteis, M. A. & Godi, A. PI-loting membrane traffic. *Nature Cell Biol.* **6**, 487–492 (2004).
56. Di Paolo, G. & De Camilli, P. Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443**, 651–657 (2006).
57. Michell, R. H. Inositol derivatives: evolution and functions. *Nature Rev. Mol. Cell Biol.* **9**, 151–161 (2008).
58. Pizarro-Cerda, J. & Cossart, P. Subversion of phosphoinositide metabolism by intracellular bacterial pathogens. *Nature Cell Biol.* **6**, 1026–1033 (2004).
59. Weber, S. S., Ragaz, C. & Hilbi, H. Pathogen trafficking pathways and host phosphoinositide metabolism. *Mol. Microbiol.* **71**, 1341–1352 (2009).
60. Weber, S. S., Ragaz, C., Reus, K., Nyfeler, Y. & Hilbi, H. *Legionella pneumophila* exploits PI(4)P to anchor secreted effector proteins to the replicative vacuole. *PLoS Pathog.* **2**, e46 (2006).
61. Ragaz, C. *et al.* The *Legionella pneumophila* phosphatidylinositol-4 phosphate-binding type IV substrate SidC recruits endoplasmic reticulum vesicles to a replication-permissive vacuole. *Cell. Microbiol.* **10**, 2416–2433 (2008).
62. Brombacher, E. *et al.* Rab1 guanine nucleotide exchange factor SidM is a major phosphatidylinositol 4-phosphate-binding effector protein of *Legionella pneumophila*. *J. Biol. Chem.* **284**, 4846–4856 (2009).
63. Machner, M. P. & Isberg, R. R. Targeting of host Rab GTPase function by the intravacuolar pathogen *Legionella pneumophila*. *Dev. Cell* **11**, 47–56 (2006).
64. Jank, T. *et al.* Domain organization of Legionella effector SetA. *Cell. Microbiol.* **14**, 852–868 (2012).
65. Stebbins, C. E. & Galán, J. E. Structural mimicry in bacterial virulence. *Nature* **412**, 701–705 (2001).
66. Rabin, S. D., Veesenmeyer, J. L., Bieging, K. T. & Hauser, A. R. A. C-terminal domain targets the *Pseudomonas aeruginosa* cytotoxin ExoU to the plasma membrane of host cells. *Infect. Immun.* **74**, 2552–2561 (2006).
67. Gendrin, C. *et al.* Structural basis of cytotoxicity mediated by the type III secretion toxin ExoU from *Pseudomonas aeruginosa*. *PLoS Pathog.* **8**, e1002637 (2012).
68. Finck-Barbancon, V. *et al.* ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. *Mol. Microbiol.* **25**, 547–557 (1997).
69. Allewelt, M., Coleman, F. T., Grout, M., Priebe, G. P. & Pier, G. B. Acquisition of expression of the *Pseudomonas aeruginosa* ExoU cytotoxin leads to increased bacterial virulence in a murine model of acute pneumonia and systemic spread. *Infect. Immun.* **68**, 3998–4004 (2000).
70. Marshall, B. & Windsor, H. The relation of *Helicobacter pylori* to gastric adenocarcinoma and lymphoma: pathophysiology, epidemiology, screening, clinical presentation, treatment, and prevention. *Med. Clin. North Am.* **89**, 313–344 (2005).
71. Murata-Kamiya, N. Pathophysiological functions of the CagA oncoprotein during infection by *Helicobacter pylori*. *Microbes Infect.* **13**, 799–807 (2011).
72. Murata-Kamiya, N., Kikuchi, K., Hayashi, T., Higashi, H. & Hatakeyama, M. *Helicobacter pylori* exploits host membrane phosphatidylserine for delivery, localization, and pathophysiological action of the CagA oncoprotein. *Cell Host Microbe.* **7**, 399–411 (2010).
73. Higashi, H. *et al.* EPIYA motif is a membrane-targeting signal of *Helicobacter pylori* virulence factor CagA in mammalian cells. *J. Biol. Chem.* **280**, 23130–23137 (2005).
74. Neupert, W. Protein import into mitochondria. *Annu. Rev. Biochem.* **66**, 863–917 (1997).
75. Schatz, G. The protein import system of mitochondria. *J. Biol. Chem.* **271**, 31763–31766 (1996).

76. Wiedemann, N., Frazier, A. E. & Pfanner, N. The protein import machinery of mitochondria. *J. Biol. Chem.* **279**, 14473–14476 (2004).
77. Guttman, D. S. *et al.* A functional screen for the type III (Hrp) secretome of the plant pathogen *Pseudomonas syringae*. *Science* **295**, 1722–1726 (2002).
78. Jelenska, J. *et al.* A J domain virulence effector of *Pseudomonas syringae* remodels host chloroplasts and suppresses defenses. *Curr. Biol.* **17**, 499–508 (2007).
79. Block, A. *et al.* The *Pseudomonas syringae* type III effector HopG1 targets mitochondria, alters plant development and suppresses plant innate immunity. *Cell. Microbiol.* **12**, 318–330 (2010).
80. Kenny, B. & Jepson, M. Targeting of an enteropathogenic *Escherichia coli* (EPEC) effector protein to host mitochondria. *Cell. Microbiol.* **2**, 579–590 (2000).
A study showing that the *E. coli* effector Map is found in the host cell mitochondrial matrix and that this can disrupt mitochondrial membrane potential.
81. Nougayrede, J. P. & Donnenberg, M. S. Enteropathogenic *Escherichia coli* EspF is targeted to mitochondria and is required to initiate the mitochondrial death pathway. *Cell. Microbiol.* **6**, 1097–1111 (2004).
82. Papatheodorou, P. *et al.* The enteropathogenic *Escherichia coli* (EPEC) Map effector is imported into the mitochondrial matrix by the TOM/Hsp70 system and alters organelle morphology. *Cell. Microbiol.* **8**, 677–689 (2006).
83. Alto, N. M. *et al.* Identification of a bacterial type III effector family with G protein mimicry functions. *Cell* **124**, 133–145 (2006).
84. Bulgin, R. *et al.* Bacterial guanine nucleotide exchange factors SopE-like and WxxxE effectors. *Infect. Immun.* **78**, 1417–1425 (2010).
85. Hodges, K., Alto, N. M., Ramaswamy, K., Dudeja, P. K. & Hecht, G. The enteropathogenic *Escherichia coli* effector protein EspF decreases sodium hydrogen exchanger 3 activity. *Cell. Microbiol.* **10**, 1735–1745 (2008).
86. Simpson, N. *et al.* The enteropathogenic *Escherichia coli* type III secretion system effector Map binds EBP50/NHERF1: implication for cell signalling and diarrhoea. *Mol. Microbiol.* **60**, 349–363 (2006).
87. Alto, N. M. *et al.* The type III effector EspF coordinates membrane trafficking by the spatiotemporal activation of two eukaryotic signaling pathways. *J. Cell Biol.* **178**, 1265–1278 (2007).
88. Dean, P. *et al.* The enteropathogenic *E. coli* effector EspF targets and disrupts the nucleolus by a process regulated by mitochondrial dysfunction. *PLoS Pathog.* **6**, e1000961 (2010).
89. Marches, O. *et al.* EspF of enteropathogenic *Escherichia coli* binds sorting nexin 9. *J. Bacteriol.* **188**, 3110–3115 (2006).
90. Wong, A. R. *et al.* Enteropathogenic and enterohaemorrhagic *Escherichia coli*: even more subversive elements. *Mol. Microbiol.* **80**, 1420–1438 (2011).
91. Holmes, A., Muhlen, S., Roe, A. J. & Dean, P. The EspF effector, a bacterial pathogen's Swiss army knife. *Infect. Immun.* **78**, 4445–4453 (2010).
92. Pistor, S., Chakraborty, T., Niebuhr, K., Domann, E. & Wehland, J. The ActA protein of *Listeria monocytogenes* acts as a nucleator inducing reorganization of the actin cytoskeleton. *EMBO J.* **13**, 758–763 (1994).
93. Zurawski, D., Mumy, K., Faherty, C., McCormick, B. & Maurelli, A. *Shigella flexneri* T3SS effectors OspB and OspF target the nucleus to down-regulate the host inflammatory response via interactions with retinoblastoma protein. *Mol. Microbiol.* **71**, 350–368 (2009).
94. Bogdanove, A. J., Schornack, S. & Lahaye, T. TAL effectors: finding plant genes for disease and defense. *Curr. Opin. Plant Biol.* **13**, 394–401 (2010).
95. Boch, J. & Bonas, U. *Xanthomonas* AvrBs3 family-type III effectors: discovery and function. *Annu. Rev. Phytopathol.* **48**, 419–436 (2010).
96. Howard, E. A., Zupan, J. R., Citovsky, V. & Zambryski, P. C. The VirD2 protein of *A. tumefaciens* contains a C-terminal bipartite nuclear localization signal: implications for nuclear uptake of DNA in plant cells. *Cell* **68**, 109–118 (1992).
97. Rossi, L., Hohn, B. & Tinland, B. The VirD2 protein of *Agrobacterium tumefaciens* carries nuclear localization signals important for transfer of T-DNA to plant. *Mol. Gen. Genet.* **239**, 345–353 (1993).
98. Gelvin, S. Finding a way to the nucleus. *Curr. Opin. Microbiol.* **13**, 53–58 (2010).
99. Daniel, V. *et al. Shigella flexneri* T3SS effectors OspB and OspF target the nucleus to down-regulate the host inflammatory response via interactions with retinoblastoma protein. *Mol. Microbiol.* **71**, 350–368 (2009).
100. Haraga, A. & Miller, S. I. A. *Salmonella* type III secretion effector interacts with the mammalian serine/threonine protein kinase PKN1. *Cell. Microbiol.* **8**, 837–846 (2006).
101. Mattaj, I. W. & Englmeier, L. Nucleocytoplasmic transport: the soluble phase. *Annu. Rev. Biochem.* **67**, 265–306 (1998).
102. Gorlich, D. *et al.* Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. *Curr. Biol.* **5**, 383–392 (1995).
103. Gorlich, D., Prehn, S., Laskey, R. A. & Hartmann, E. Isolation of a protein that is essential for the first step of nuclear protein import. *Cell* **79**, 767–778 (1994).
104. Gorlich, D., Vogel, F., Mills, A. D., Hartmann, E. & Laskey, R. A. Distinct functions for the two importin subunits in nuclear protein import. *Nature* **377**, 246–248 (1995).
105. Lee, S. J., Matsuura, Y., Liu, S. M. & Stewart, M. Structural basis for nuclear import complex dissociation by RanGTP. *Nature* **435**, 693–696 (2005).
106. Marois, E., Van den Ackerveken, G. & Bonas, U. The *Xanthomonas* type III effector protein AvrBs3 modulates plant gene expression and induces cell hypertrophy in the susceptible host. *Mol. Plant Microbe Interact.* **15**, 637–646 (2002).
107. Van den Ackerveken, G., Marois, E. & Bonas, U. Recognition of the bacterial avirulence protein AvrBs3 occurs inside the host plant cell. *Cell* **87**, 1307–1316 (1996).
108. Lahaye, T. & Bonas, U. Molecular secrets of bacterial type III effector proteins. *Trends Plant Sci.* **6**, 479–485 (2001).
109. Yang, Y. & Gabriel, D. W. *Xanthomonas* avirulence/pathogenicity gene family encodes functional plant nuclear targeting signals. *Mol. Plant Microbe Interact.* **8**, 627–631 (1995).
110. Szurek, B., Marois, E., Bonas, U. & Van den Ackerveken, G. Eukaryotic features of the *Xanthomonas* type III effector AvrBs3: protein domains involved in transcriptional activation and the interaction with nuclear import receptors from pepper. *Plant J.* **26**, 523–534 (2001).
The discovery that AvrBs3 interacts with two importin-α proteins via the AvrBs3 NLS.
111. Pitzschke, A. & Hirt, H. New insights into an old story: *Agrobacterium*-induced tumour formation in plants by plant transformation. *EMBO J.* **29**, 1021–1032 (2010).
112. Citovsky, V., Zupan, J., Warnick, D. & Zambryski, P. Nuclear localization of *Agrobacterium* VirE2 protein in plant cells. *Science* **256**, 1802–1805 (1992).
113. Zupan, J. R., Citovsky, V. & Zambryski, P. *Agrobacterium* VirE2 protein mediates nuclear uptake of single-stranded DNA in plant cells. *Proc. Natl Acad. Sci. USA* **93**, 2392–2397 (1996).
114. Ballas, N. & Citovsky, V. Nuclear localization signal binding protein from *Arabidopsis* mediates nuclear import of Agrobacterium VirD2 protein. *Proc. Natl Acad. Sci. USA* **94**, 10723–10728 (1997).
A paper reporting that VirD2 binds to the *A. thaliana* nuclear import receptor, KAPα1, via the VirD2 NLS.
115. Citovsky, V., Warnick, D. & Zambryski, P. Nuclear import of *Agrobacterium* VirD2 and VirE2 proteins in maize and tobacco. *Proc. Natl Acad. Sci. USA* **91**, 3210–3214 (1994).
116. Nagai, T., Abe, A. & Sasakawa, C. Targeting of enteropathogenic *Escherichia coli* EspF to host mitochondria is essential for bacterial pathogenesis: critical role of the 16th leucine residue in EspF. *J. Biol. Chem.* **280**, 2998–3011 (2005).
117. Hartland, E. L. *et al.* Binding of intimin from enteropathogenic *Escherichia coli* to Tir and to host cells. *Mol. Microbiol.* **32**, 151–158 (1999).
118. Liu, H. *et al.* Point mutants of EHEC intimin that diminish Tir recognition and actin pedestal formation highlight a putative Tir binding pocket. *Mol. Microbiol.* **45**, 1557–1573 (2002).
119. Luo, Y. *et al.* Crystal structure of enteropathogenic *Escherichia coli* intimin-receptor complex. *Nature* **405**, 1073–1077 (2000).
120. Murata, T. *et al.* The *Legionella pneumophila* effector protein DrrA is a Rab1 guanine nucleotide-exchange factor. *Nature Cell Biol.* **8**, 971–977 (2006).
121. de Vries, J. S., Andriotis, V. M. E., Wu, A.-J. & Rathjen, J. P. Tomato Pto encodes a functional *N*-myristoylation motif that is required for signal transduction in *Nicotiana benthamiana*. *Plant J.* **45**, 31–45 (2006).
122. Hernandez, L. D., Pypaert, M., Flavell, R. A. & Galán, J. E. A. *Salmonella* protein causes macrophage cell death by inducing autophagy. *J. Cell Biol.* **163**, 1123–1131 (2003).
123. Layton, A. N., Brown, P. J. & Galyov, E. E. The *Salmonella* translocated effector SopA is targeted to the mitochondria of infected cells. *J. Bacteriol.* **187**, 3565–3571 (2005).
124. Zou, H. *et al.* Identification of an avirulence gene, *avrxa5*, from the rice pathogen *Xanthomonas oryzae* pv. oryzae. *Sci. China Life Sci.* **53**, 1440–1449 (2010).
125. Yang, B., Zhu, W., Johnson, L. B. & White, F. F. The virulence factor AvrXa7 of *Xanthomonas oryzae* pv. *oryzae* is a type III secretion pathway-dependent nuclear-localized double-stranded DNA-binding protein. *Proc. Natl Acad. Sci. USA* **97**, 9807–9812 (2000).

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