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Bacterial virulence mediated by orthogonal post-translational modification

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Many bacterial pathogens secrete virulence factors, also known as effector proteins, directly into host cells. These effectors suppress pro-inflammatory host signaling while promoting bacterial infection. A particularly interesting subset of effectors post-translationally modify host proteins using novel chemistry that is not otherwise found in the mammalian proteome, which we refer to as 'orthogonal post-translational modification' (oPTM). In this Review, we profile oPTM chemistry for effectors that catalyze serine/threonine acetylation, phosphate β-elimination, phosphoribosyl-linked ubiquitination, glutamine deamidation, phosphocholination, cysteine methylation, arginine *N*-acetylglucosaminylation, and glutamine ADP-ribosylation on host proteins. AMPylation, a PTM that could be considered orthogonal until only recently, is also discussed. We further highlight known cellular targets of oPTMs and their resulting biological consequences. Developing a complete understanding of oPTMs and the host cell processes they hijack will illuminate critical steps in the infection process, which can be harnessed for a variety of therapeutic, diagnostic, and synthetic applications.

During bacterial infection, host cells recognize extracellular stimuli from invading bacteria and in response activate pro-inflammatory signals that protect the host[1,2]. To subvert these defenses, many bacteria have evolved elaborate secretion systems that directly inject host cells with virulence factors, also known as effectors[3]. Some bacteria, like *Shigella flexneri*, secrete at least 25 discrete effectors, whereas others, like *Legionella pneumophila*, secrete more than 300 (refs. [4,5]). These virulence factors wage a biochemical war that interrupts or rewires host signaling, thereby silencing the host innate immune response while promoting bacterial entry, survival, and replication (Fig. 1)[1,2,6].

Just over 50 years ago, it was reported that an effector secreted by *Corynebacterium diphtheriae* mediated toxicity via post-translational modification (PTM) of host proteins[7]. Specifically, diptheria toxin was found to ADP-ribosylate host elongation factor 2, inhibiting protein synthesis and ultimately leading to host cell death. This discovery provided the first evidence that bacterial effectors could enable infection through the chemical modification of host proteins. Since then, many effectors have been found to modify host proteins, often using familiar chemistry to install or remove functional groups, including phosphate, ubiquitin, and ADP-ribose[6]. During infection, effectors use these well-recognized transformations to mimic, and thereby hijack, the host's own intracellular processes[1,2,6].

A particularly interesting subset of bacterial effectors modify host proteins using chemistry that is not otherwise found in the eukaryotic proteome (Fig. 2a–h). We have termed the resulting set of modifications 'orthogonal post-translational modifications' (oPTMs). A PTM can be considered orthogonal if its chemical transformation does not occur as part of the healthy, eukaryotic post-translational repertoire and only transpires once the effector enters the host cell. Herein we summarize the current knowledge of the eight known oPTMs relevant to human infection, including serine/threonine acetylation (Fig. 2a), phosphate β-elimination (Fig. 2b), phosphoribosyl-linked ubiquitination (Fig. 2c), glutamine deamidation (Fig. 2d), phosphocholination (Fig. 2e), cysteine methylation (Fig. 2f), arginine *N*-acetylglucosaminylation (Fig. 2g), and glutamine ADP-ribosylation (Fig. 2h). In some cases, oPTMs result in novel modifications that have not been described in humans (Fig. 2b-e). In other instances, oPTM effectors repurpose known chemistries using altered substrate specificities to produce unusual modifications that have not been observed in uninfected human cells (Fig. 2a,f–h).

The enzymes responsible for catalyzing oPTMs target relatively few cellular pathways. The mitogen-activated protein kinase (MAPK) and nuclear factor κB (NFκB) pathway are both frequent targets that control expression of immune response genes and coordinate defenses against pathogens (Fig. 3). Host ubiquitination and the ubiquitin-proteasome system (UPS) are also common targets, allowing pathogens to broadly disrupt the immune response and cell cycle (Fig. 4). Finally, modulating GTPase activity allows pathogens to interfere with actin dynamics and has important implications for wound repair and vesicle trafficking (Fig. 5). Thus, although oPTM effectors catalyze distinct chemistry, they use a shared approach to interfere with host biochemical processes.

oPTMs represent a distinct class of pathogen-mediated host protein modifications that occur within the confines of the host cell. Host cells are incapable of catalyzing oPTMs on their own and often lack the enzymatic capability to reverse or remove oPTMs. Additionally, many of the bacteria known to mediate oPTMs cause serious, life-threatening infections, and some are known bioterror threats. Thus, oPTMs represent an intriguing group of modifications not only from a chemical perspective, but also with respect to their biology and relevance to infectious disease. Developing a complete understanding of oPTM effectors and the host cell processes they coopt is the first step toward harnessing their activities to illuminate the infection process, identify new opportunities for therapeutic intervention, and discover new biomarkers for human infection.

Examples of oPTMs

Serine/threonine acetylation. Mammalian acetyltransferases modify lysine residues, the protein N terminus, or other cellular amines. Acetylation of these amines removes the positive charge, thereby altering the electrostatic protein surface that is important

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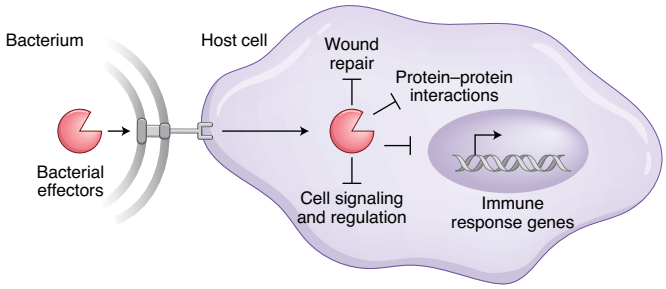

Fig. 1 | Secreted bacterial effectors wage a biochemical war in host cells. During the course of infection, some bacterial pathogens inject effector proteins directly into the host mammalian cell via a secretion system. These effector proteins interfere with vital cellular pathways, working in concert to promote bacterial growth and survival while suppressing the host immune response.

for recognition by 'reader' proteins. In contrast, the serine/threonine acetyltransferase family of effectors catalyzes the transfer of acetyl from acetyl-CoA to hydroxyls on serine and threonine residues (Fig. 2a)[8,9]. The serine/threonine acetyltransferase family is small, consisting mostly of effectors from plant pathogens[10]. Three members belong to human pathogens, including YopJ, which is secreted by *Yersinia pestis*, the causative agent of the plague[8,9]. The other two are AvrA and VopA, from *Salmonella enterica* Typhimurium and *Vibrio parahaemolyticus*, respectively, which are both human pathogens that cause gastrointestinal distress[11,12]. YopJ, AvrA, and VopA acetylate serine (Ser) and threonine (Thr) residues of proteins within the MAPK pathway, a central hub for immune signaling (Fig. 3).

YopJ family effectors are atypical, as they exhibit no sequence similarity with known acetyltransferases. Initially, they were thought to be cysteine proteases due to a conserved catalytic triad and homology with the adenovirus protease and yeast ubiquitin-like protein protease-1 (ref. [13]), though evidence for such activity could not be recapitulated in vitro. In 2006, Mukherjee et al. reported the discovery of hydroxyacetylation as an explanation for the elusive mechanism by which YopJ inhibits MAPK and NFκB pathways[8,13,14]. Tandem mass spectrometry (MS) analysis of tryptic peptides obtained after co-expression of MAPKK6 with YopJ revealed multiple acetylated sites, each exhibiting a +42 Da shift, within the conserved MAPKK6 activation loop[8]. Furthermore, an in vitro experiment with ^{14}C-labeled acetyl-CoA revealed that MAPKK6 gains a labeled acetyl group when co-incubated with YopJ[8].

Later that year, Mittal et al. corroborated this acetyltransferase activity and confirmed another YopJ target using multiple antibodies specific for MAPKK2 (ref. [9]). The first, which recognized an internal protein sequence, showed a YopJ-dependent loss of signal by western blot. In contrast, the second anti-MAPKK2 antibody, raised against a terminal sequence, showed unchanging protein levels. This finding confirmed that YopJ chemically modifies MAPKK2, masking the epitope recognized by the first antibody. Subsequent MS analysis revealed a +42 Da shift on Ser residues within the MAPKK2 activation loop[9]. Building on reports that YopJ also affects NFκB and interacts with inhibitor of nuclear factor-κB (IκB) kinase (IKK)[14], researchers analyzed IKK proteins exposed to YopJ and found that IKKβ was acetylated on Thr180 in its activation loop[8,9]. Further experiments have expanded the YopJ substrate scope to include the kinases TAK1 and RIP2, both upstream of MAPK and NFκB[15,16].

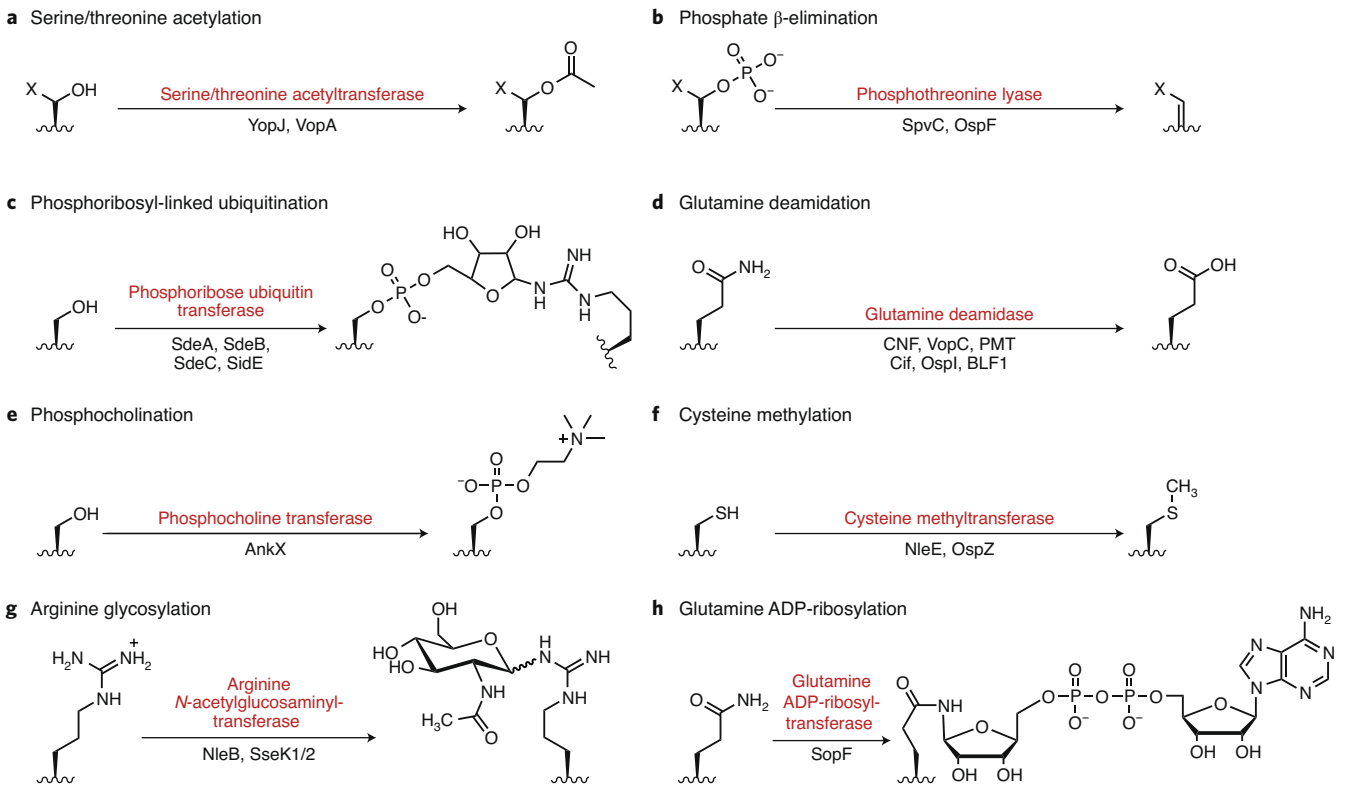

Fig. 2 | A subset of bacterial effectors catalyze orthogonal post-translational modifications (oPTMs) in host cells. A relatively small subset of bacterial effectors catalyzes orthogonal post-translational modifications (oPTMs) that are not found in uninfected host cells. Such modifications include Ser/Thr acetylation (X = H, Ser; X = CH_3, Thr) (**a**), phosphate β-elimination (X = H, Ser; X = CH_3, Thr) (**b**), phosphoribosyl-linked ubiquitination (**c**), glutamine deamidation (**d**), phosphocholination (**e**), cysteine methylation (**f**), arginine *N*-acetylglucosaminylation (**g**), and glutamine ADP-ribosylation (**h**).

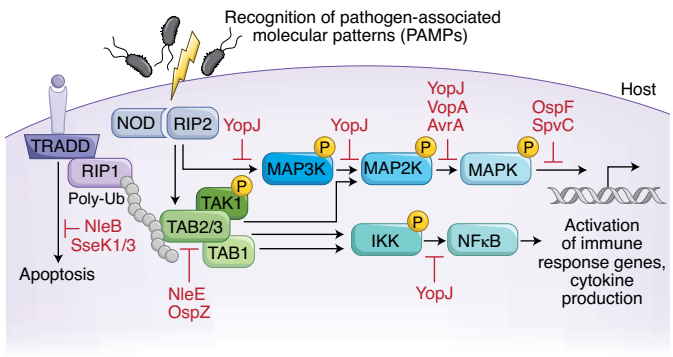

Fig. 3 | Bacterial effectors use oPTMs to silence pro-inflammatory signaling through the MAPK and NFκB pathways. Invading bacteria display pathogen-associated molecular patterns (PAMPs) that are sensed by pattern recognition receptors on the host cell surface and can activate pro-inflammatory signaling through the MAPK and NFκB pathways, as shown for signaling through nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and the receptor-interacting serine/threonine-protein kinase 2 (RIP2, also known as RIPK2 or RICK). Exposure to bacterial pathogens also stimulates cytokine production that further activates pro-inflammatory signaling. For instance, activation of the tumor necrosis factor receptor 1 (TNFR1) and the tumor necrosis factor receptor type 1-associated death domain protein (TRADD) promotes death receptor signaling that can lead to apoptosis or activation of the MAPK and NFκB pathways. Ser/Thr acetyltransferases, such as YopJ (from *Y. pestis*), VopA (from *V. parahaemolyticus*), and AvrA (from *S. enterica* Typhimurium) block phosphorylation to suppress inflammation, modulate apoptosis, impair wound healing, and disrupt the intestinal barrier. Phosphothreonine lyases, such as SpvC (*S. enterica* Typhimurium) and OspF (*S. flexneri*) irreversibly remove phosphorylation to inhibit the pro-inflammatory response and decrease cytokine release. Effectors such as Cys methyltransferases, like NleE (*E. coli*) and OspZ (*S. flexneri*), inhibit NFκB signaling by interfering with a critical polyubiquitin binding interaction, thereby preventing cytokine release. Arg *N*-acetylglucosaminyl transferases, including NleB (*E. coli*) and SseK1/3 (*S. enterica* Typhimurium) interfere with both apoptosis and NFκB signaling via modification of a critical Arg residue within death domains. These actions ultimately suppress inflammation, thereby encouraging microbial survival and pathogenesis. RIP1, receptor interacting protein kinase 1; TAK1, TGF-β-activated kinase; TAB1/2/3, TGF-β-activated kinase 1 binding protein 1/2/3; MAP3K, mitogen-activated protein kinase kinase kinase; MAP2K, mitogen-activated protein kinase kinase; IKK, inhibitor of nuclear factor-κB (IκB) kinase.

YopJ and its homologs do not resemble known acetyltransferases, and instead share homology with cysteine proteases[17]. Two recent crystallographic studies have yielded substantial structural and mechanistic insights into YopJ function (PDB IDs: 5KLQ, 5KLP, 5W40, 5W3Y, 5W3T)[18,19]. HopZ1a, a YopJ effector from *Pseudomonas syringae*, was found to be allosterically activated by inositol hexakisphosphate (IP_6), a cofactor found in eukaryotic hosts[18]. Only after bacteria secrete YopJ-family effectors into their hosts, where IP_6 is present, can they bind acetyl-CoA with high affinity. A subsequent study with PopP2, from *Ralstonia solanacearum*, provided long-sought evidence in support of a 'ping-pong' mechanism in which acetylation occurs through sequential transfer of acetyl, first from acetyl-CoA to a covalent acetyl-enzyme intermediate and next onto the substrate[19]. The active site excludes water to avoid undesired acetyl-CoA hydrolysis that would waste a valuable cellular metabolite[17–19]. This mechanism is reminiscent of proteolysis facilitated by cysteine proteases, suggesting that the conserved catalytic triad acquired acetyltransferase activity in part through the addition of IP_6-sensing domains that modulate acetyl-CoA binding. Thus, YopJ and its family members highlight the challenge of identifying enzymes that catalyze novel chemistries, as their activities can be misclassified on the basis of homology alone.

The majority of serine/threonine acetylation sites modified by YopJ are known, or immediately adjacent to, phosphorylation sites[8,9,12,15,16]. During bacterial infection, a cascade of phosphorylation events activates MAPK and NFκB pathways[1,2]. Disengaging these pathways inhibits the pro-inflammatory response (Fig. 3). Furthermore, YopJ modification of host RIP2 and TAK1 kinases leads to altered nucleotide-binding oligomerization domain-containing protein 2 (Nod2) signaling upon exposure to *Yersinia pseudotuberculosis*, which increases intestinal permeability, allowing bacterial entry[15]. As a result, YopJ is crucial for *Y. pestis* and *Y. pseudotuberculosis* entry and residence in host cells and is required for full virulence[20]. In contrast, AvrA does not acetylate TAK1 and instead inhibits c-Jun N-terminal kinase (JNK), but not NFκB, signaling by acetylation of MKK4 and MKK7 (refs. [11,21]). Likewise, investigations into *V. parahaemolyticus* infections have demonstrated that VopA inhibits the JNK arm of the MAPK pathway as well as apoptosis, which is also crucial for *V. parahaemolyticus* pathogenesis[12]. Thus, bacteria use Ser/Thr acetylation to block phosphorylation-dependent host cell signaling.

Phosphate β-elimination. Typical phosphorylation involves kinase-catalyzed addition of phosphate to protein hydroxyls on Ser, Thr, and tyrosine (Tyr). Phosphorylation is readily reversed by phosphatase-mediated hydrolysis to regenerate unmodified hydroxyls. In contrast, bacterial phosphothreonine lyases, or phospholyases catalyze β-elimination of phosphate from phosphothreonine (pThr), resulting in the formation of dehydrobutyrine (Dhb) (Fig. 2b)[22]. Members of this family involved in human pathogenesis include SpvC from *S. enterica* Typhimurium, OspF from *S. flexneri*, and VirA from *Chromobacterium violaceum*[22,23].

SpvC, an effector protein produced by *S. enterica* Typhimurium, was the first phospholyase to be reported. At the time, nothing was known about its function, but the gene encoding SpvC was located on a plasmid required for virulence[24]. In 2007, Arbibe et al. demonstrated that OspF, a SpvC homolog, could remove phosphate from the MAPK ERK1/2, presumably through phosphatase activity[23]. Shortly thereafter, Li et al. reported the modification of ERK1/2 by OspF through an entirely novel mechanism: phosphate β-elimination. This conclusion was supported by MS data that revealed a −98 Da shift from pThr, ruling out phosphatase-mediated hydrolysis, which would cause only a −80 Da shift[22].

Most enzyme-catalyzed β-eliminations require the assistance of cofactors, but phospholyases do not. Instead, two structural and mechanistic studies revealed that a neutral Lys residue in the active site is the catalytic base that deprotonates the α-proton and that a nearby histidine protonates the β-phosphate group as it leaves (PDB IDs: 2P1W, 2Q8Y, 2Z8M, 2Z8N, 2Z8O, 2Z8P)[25,26]. Subsequent computational studies suggested that the mechanism occurs through a step-wise $E1_{CB}$ mechanism in which the resulting carbanion intermediate is stabilized by an oxyanion hole comprised of Y158 and K104, both identified as important residues for catalysis in an earlier study[27].

It is thought that phospholyases rely on multiple discrete interactions that target them to proteins within the MAPK cascade[22]. The N-terminal domains of SpvC and OspF are largely disordered and possess a canonical D motif that recognizes MAPK-family substrates[25]. Moreover, phospholyase active sites possess abundant positive charge, which is critical for binding the conserved pT-X-pY MAPK activation loop. Indeed, unphosphorylated substrates have substantially poorer binding affinity[22,25,28]. Investigations into *S. flexneri* pathogenesis have shown that when OspF is secreted into host cells, there are dramatic, widespread changes in host gene expression and phosphorylation[29,30]. In fact, when compared to the

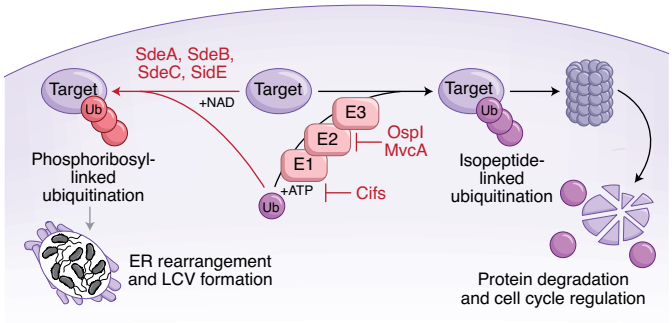

Fig. 4 | Bacterial effectors use oPTMs to interfere with the host ubiquitin-proteasome system. The ubiquitin-proteasome system (UPS) is central to virtually all aspects of cellular signaling and regulation. For this reason, bacterial effectors that catalyze oPTMs can impede or hijack host ubiquitination to promote infection. Glutamine deamidases, such as Cifs (from *E. coli* and *B. pseudomallei*), OspI (from *S. flexneri*) and MvcA (from *L. pneumophila*), inhibit host mono- and polyubiquitination (black arrows), thereby interrupting the cell cycle and inhibiting NFκB-mediated pro-inflammatory signaling. Phosphoribose ubiquitin transferases from *L. pneumophila* (SdeA, SdeB, SdeC, SidE) have been shown to interfere with host ubiquitination through the direct attachment of ubiquitin by novel linkages (red arrows) that rewire and/or silence the host UPS while promoting ER rearrangement and formation of the *Legionalla*-containing vacuole (LCV).

other *S. flexneri* effectors, OspF causes the largest change in host gene expression and phosphorylation. It is thought that many of these changes are mediated by OspF-dependent silencing of the MAPK signaling hub. However, both host gene expression analysis and phosphorylation profiling show OspF-dependent changes that are not fully explained by targeting MAPK alone, suggesting that OspF, and other phospholyases, may have additional targets within the host[29–31].

Phosphoribosyl-linked ubiquitination. Canonical ubiquitination occurs through isopeptide formation that links the ubiquitin C terminus to lysine residues on target proteins. This ATP-dependent process requires the sequential activity of E1, E2, and E3 enzymes. Atypical ubiquitination has been occasionally reported in mammalian cells, occurring through ester formation linking the ubiquitin C terminus to Ser or Thr residues[32,33]. In contrast, a very unusual linkage of ubiquitin to host target proteins was recently reported for the SidE family of effectors secreted by *L. pneumophila*, the causative agent of Legionnaire's disease and pneumonia[34,35]. SidE, or Sde, family proteins (SdeA, SdeB, SdeC, and SidE) are secreted by a type IVB secretion system and catalyze the direct linkage of a ubiquitin arginine (Arg) to substrate hydroxyls using a mechanism that does not engage or mimic host ubiquitination machinery (Figs. 2c and 4). The reaction is sequential, starting first with the attachment of ADP-ribose to ubiquitin, followed by hydrolysis of AMP and attachment of a phosphoribosylated ubiquitin to substrate hydroxyls[34,35].

A flurry of studies from multiple groups led to the discovery of phosphoribosyl-linked ubiquitination[34–36]. In early 2016, Qiu et al. reported that a mono-ADP-ribosyltransferase (mART) domain in SdeA was essential for *L. pneumophila* virulence[36]. However, ADP-ribosylated host proteins could not be found upon SdeA treatment. Instead, SdeA induced ubiquitination of Rab GTPases through an unknown mechanism that was independent of host E1 or E2 enzymes[36]. This ubiquitination was attributed to SdeA's mART activity, although the exact chemistry remained unknown[36]. Within a year of this report, two groups independently reported that SdeA and SdeC use their mART domains to catalyze ADP-ribosylation at R42 of ubiquitin and subsequently use their dual phosphodiesterase (PDE) activity to remove AMP and crosslink ubiquitin to hydroxyl groups on target proteins[34,35].

In 2018, four separate structural studies published in the same month revealed the mechanism of atypical ubiquitination mediated by Sde proteins (PDB IDs: 5YIM, 5YIJ, 5YIK, 5ZQ5, 5ZQ4, 5ZQ7, 5ZQ6, 5ZQ3, 5ZQ2, 6B7M, 6B7P, 6B7O, 6B7Q, 6G0C)[37–40]. The catalytic cycle initiates with binding of ubiquitin (Ub) and NAD^+ to the mART domain. Sde family proteins are the first mARTs known to target Ub. They do so by engaging in an unprecedented binding interaction with the C terminus of Ub, mediated by R72 and R74 of Ub[37]. This binding is specific for Ub, as the ubiquitin-like protein SUMO, which shares the canonical Ub fold and R42, but lacks R72 and R74, is not a substrate[37,38]. Intriguingly, in the crystal structure of SdeA bound to Ub and NADH, the residue that becomes ADP-ribosylated (R42) is positioned outside of the active site. Using molecular dynamics, Dong et al. resolved this incongruous finding by revealing that nicotinamide release triggers a conformational change, allowing R42 to enter the active site as R72 is excluded[37]. This swap enables R42 to attack the ADP-ribosyl oxocarbenium ion in a putative S_N1-type mechanism.

After ADP-ribosylation, a negatively charged glutamate (E340) facilitates the positioning of ADP-ribosylated Ub in the phosphodiesterase active site. E340 is also thought to activate a histidine (H277), which cleaves the phosphodiester linking phosphoribosylated Ub to AMP[39,40]. Using low-energy higher-energy collisional dissociation (HCD) MS and a catalytically impaired mutant of SdeA (H407N), Kalayil et al. further showed that H277 forms a covalent phosphoramidate intermediate[40]. Subsequently, H407 facilitates nucleophilic attack on this intermediate by substrate hydroxyl groups. This can occur with a water molecule to result in release of phosphoribosylated Ub or with Ser (or potentially Tyr) residues on substrate proteins to result in phosphoribosyl-linked ubiquitinated products.

The mART and PDE domains found within Sde proteins are biochemically independent. The two active sites face away from one another and are roughly 55 Å apart[39]. Interdomain interactions have been captured crystallographically and were shown to influence catalysis[38], but it is unclear whether the mechanism involves a considerable conformational change that has not yet been captured. As an alternative, there is evidence that Sde effectors function as dimers, which, with further study, could provide a plausible model for how these two activities are connected[38]. Thus, despite recent progress, it remains an open question as to how these two activities are coupled to result in phosphoribosyl-linked ubiquitination.

The primary targets of Sde family proteins appear to be host reticulon proteins and Rab GTPases[34,35]. SdeC was found to induce ubiquitination of host reticulon 4. This modification resulted in a substantial morphological rearrangement of tubular endoplasmic reticulum that promotes *L. pneumophila* replication (Fig. 4)[35]. Further, Ub phosphoribosylation is thought to broadly inhibit conventional ubiquitin transfer through the ubiquitin-proteasome system (UPS), albeit through an unknown mechanism[34]. However, Kalayil et al. determined that only phosphoribosyl-mediated crosslinking of Ub to target proteins, not phosphoribosylation of Ub itself, contributes to acute *L. pneumophila* pathogenicity[40]. Recent work has begun to identify additional *L. pneumophila* effectors that regulate phosphoribosyl-linked ubiquitination via novel deubiquitinase activity[41–43] or glutamylation[44,45], suggesting that there is more to be learned about this atypical ubiquitin linkage. Thus, through this novel ubiquitin linkage, bacteria have found a way to accomplish in one multistep reaction a feat that human cells achieve through an elaborate enzymatic cascade.

Glutamine deamidation. Protein deamidation is a non-enzymatic PTM that is typically limited to asparagine (Asn), as glutamine

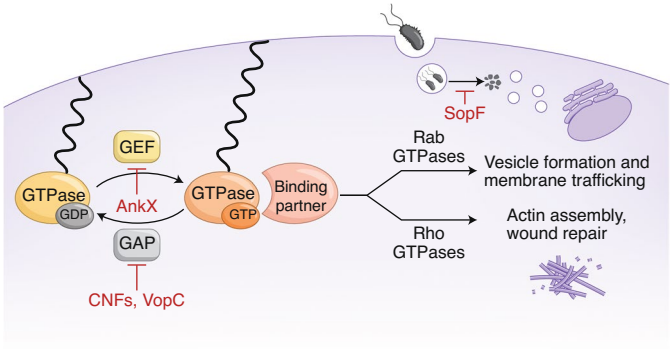

Fig. 5 | Bacterial effectors use oPTMs to alter host GTPase activity. GTPases act as molecular switches that control many aspects of cell signaling, including actin dynamics and vesicle trafficking. Numerous oPTMs disrupt GTPase activity to promote infection. Glutamine deamidases, such as CNFs (from *E. coli* and *Y. pseudotuberculosis*) and VopC (from *V. parahaemolyticus*) modulate Rho GTPase activity to interfere with actin dynamics and wound repair, helping to mediate pathogen invasion. The phosphocholine transferase from *L. pneumophila*, AnkX, disrupts Rab GTPase activity to interfere with host vesicle formation and trafficking. SopF, a Gln ADP-ribosyltransferase from *S. enterica* Typhimurium, impairs vacuolar ATPase activity, thus inhibiting autophagy induced by invading bacterial pathogens. GAP, GTPase-activating protein; GEF, Guanine nucleotide exchange factor.

(Gln) residues are recalcitrant to non-enzymatic deamidation under biological conditions[46]. Asn deamidation is typically observed only in aging or diseased cells and is highly dependent on the surrounding microenvironment[46]. In contrast, Gln deamidation is an oPTM that results from the enzymatic transformation of Gln to glutamate (Glu) through amide hydrolysis, generating a carboxylic acid and liberating ammonia (Fig. 2d)[47–49].

The first deamidases discovered were the cytotoxic necrotizing factor (CNF) family of effectors, found in *Escherichia coli*[47,48]. A few years later, the cycle inhibiting factor (Cif) family of deamidases was discovered in enteropathogenic and enterohaemorrhagic strains of *E. coli*, and is so named for interfering with the host cell cycle[50]. Subsequent studies demonstrated that Cif effectors lead to cell cycle arrest at both G_2/M[50,51] and G_1 (ref. [52]) phases without engaging the DNA damage checkpoint used by other bacterial toxins[51]. Two structural studies subsequently revealed that Cif homologs from *Burkholderia pseudomallei* and enteropathogenic *E. coli* had a papain-like fold and a catalytic triad that is homologous among cysteine proteases (PDB IDs: 3EIT, 3EIR, 3EFY)[53,54]. Moreover, both studies demonstrated that mutation of these residues resulted in Cif variants that did not induce cell cycle arrest. Accordingly, it was proposed that Cif effectors possessed proteolytic activity, though such activity was not observed using a general protease substrate like casein in vitro[54]. Thus, the search continued for an alternative hydrolytic activity that could explain Cif function.

In 2010, Cui et al. reported that CHBP, a Cif homolog from *B. pseudomallei*, was a glutamine deamidase that directly modifies Ub[49]. A faster migration of CHBP-treated Ub was observed by native polyacrylamide gel electrophoresis, indicating that CHBP-treated Ub possessed additional negative charge compared to wild type. Subsequent tandem MS confirmed that CHBP converts the conserved Gln40 on both Ub and the Ub-like protein NEDD8 to Glu, thereby establishing glutamine deamidation as a pathogenic strategy for interfering with host cell processes[49].

The UPS is one of the primary targets for pathogenic deamidation (Fig. 4). Ub deamidation diminishes its ability to form chains, whereas deamidation of NEDD8 inhibits its transfer to target proteins by cullin-RING E3 ligases[49,55]. These oPTMs interfere with UPS-dependent degradation of cell cycle regulators, thus contributing to cell cycle arrest[49–52]. Alternatively, deamidation by the *S. flexneri* effector OspI targets the E2 ubiquitin-conjugating enzyme UBC13 (ref. [56]). Deamidation of Gln100 on UBC13 impairs its ability to polyubiquitinate TRAF6, a protein upstream of NFκB signaling, ultimately dampening the immune response (Figs. 3 and 4)[56]. Two recently identified Cif homologs from *L. pneumophila*, MavC and MvcA, also target Ub and UBC13 (ref. [57]). In this case, MavC is a transglutaminase that monoubiquitinates Ubc13, which can be reversed through MvcA deamidase activity[58,59]. These studies provide yet another mechanism through which *L. pneumophila* circumvents host ubiquitin machinery. In addition, deamidases also interfere with protein translation. BLF1, an effector secreted by *B. pseudomallei*, deamidates translation initiation factor eIF4A at Gln339 (ref. [60]). This modification dramatically decreases protein translation, proving toxic to the host cell[60].

Host GTPases are also common targets for pathogenic deamidation (Fig. 5). In particular, CNF deamidases from *E. coli* (CNF1) and *Y. pseudotuberculosis* (CNFY) target host GTPases. Multiple studies have found that CNF1 and CNFY catalyze deamidation at Gln63 of RhoA, a GTPase[47,48,61]. This modification disrupts the interaction between GTPase-activating proteins (GAPs) and RhoA, thereby leading to constitutive activation of downstream pathways tied to host cytoskeleton modification and cytotoxicity[47,48,61]. Similarly, VopC is a bacterial deamidase, secreted by *V. parahaemolyticus*, that deamidates Rac and CDC42 (ref. [62]). Deamidation of Rho, Rac, and CDC42 on their conserved Gln blocks their interactions with GAPs, stimulating actin rearrangement believed to aid in bacterial entry (Fig. 5)[47,48,61,62].

Glutamine deamidation is catalyzed by the largest number of human pathogens compared to the other oPTMs. Unlike the other oPTMs discussed, this modification leads to a small mass change of just 1 Da. If deamidation happens non-stoichiometrically, detection by MS could be difficult, particularly when looking for new targets. As the number of known deamidases grows, it will be interesting to discern their other targets and learn how such a modest chemical change leads to profound changes in host signaling.

Phosphocholination. The enzymatic addition of phosphocholine to hydroxyl groups is catalyzed by phosphocholine transferases (Fig. 2e)[63]. To date, there is only a single effector known to catalyze this transformation: AnkX, which is secreted by *L. pneumophila*. AnkX contains a conserved Fic (filamentation induced by cAMP) domain and is homologous to proteins that promote AMPylation, particularly for small GTPases[64,65]. This observation led researchers to suspect that AnkX would catalyze AMPylation of host GTPases. Co-expression of AnkX with members of the Rab GTPase family did confirm that Rab proteins are AnkX targets. However, tandem MS revealed only +183 Da shifts to Ser in Rab1A and Rab1B, considerably smaller than expected for AMPylation[63]. A search through metabolite databases revealed that the mass shift could correspond to the addition of phosphocholine. Co-incubation of AnkX and Rab with CDP-choline recapitulated the modification, providing definitive evidence that AnkX catalyzes phosphocholination[63].

A subsequent structural study of AnkX revealed that its Fic domain binds to the phosphocholine substrate in an orientation that facilitates phosphocholine rather than nucleotide transfer (PDB IDs: 4BES, 4BEP, 4BER, 4BET)[66]. Fic and pseudokinase domains that catalyze AMPylation bind their substrates in the opposite orientation, enabling nucleotide transfer[67,68]. An active site histidine in AnkX is essential and, in the crystal structure, placed proximally to the scissile bond[66]. A series of biochemical and spectroscopic studies[69,70] suggest that AnkX uses a ping-pong mechanism, although a covalent phosphoramidate intermediate has not been observed experimentally. AnkX-mediated phosphocholination blocks association of Rab GTPases with connecdenn, a Rab activator, inhibiting

the activation of GTPase signaling and ultimately disrupting normal vesicular trafficking (Fig. 5)[63,70]. Thus, addition of the phosphocholine group, a never-before-seen protein modification, is an innovative bacterial strategy for masking critical binding interactions that can interfere broadly with host cell signaling.

Additional oPTMs. There are a few additional examples of oPTMs that repurpose known PTMs to modify residues that are not otherwise substrates in human cells. The effectors NleE, from enteropathogenic and enterohaemorrhagic *E. coli* (EPEC/EHEC), and OspZ, from *S. flexneri*, catalyze *S*-adenosyl-L-methionine-dependent methylation at cysteine, rather than arginine or lysine (Fig. 2f)[71–73]. Cysteine methylation within the zinc-finger domain of TAB2 and TAB3 impairs their ability to bind linear ubiquitin chains, suppressing NFκB-mediated proinflammatory signaling (Fig. 3a,b). Similarly, NleB, from *E. coli*, and SseK1/3, from *S. enterica* Typhimurium, catalyze *N*-acetylglucosaminylation (GlcNAcylation) at arginine (Fig. 2g)[74–77], in contrast to the O-linked GlcNAcylation that is common for mammalian proteins. GlcNAcylation of Arg residues within the death domains of TRADD, FADD, RIPK1, and TNFR1 inhibits death receptor signaling, allowing bacteria to impede apoptosis and broadly block host inflammation (Fig. 3). Finally, SopF, from *S. enterica* Typhimurium, catalyzes ADP-ribosylation on glutamine rather than arginine (Fig. 2h)[78]. ADP-ribosylation of Gln in host vacuolar ATPase inhibits vesicle acidification, impairing pathogen-induced autophagy and silencing yet another innate immune defense mechanism (Fig. 5).

Exploiting oPTMs as biomarker candidates

Because oPTMs are not present in healthy tissue, they are promising biomarkers for bacterial infection. Many bacteria that secrete oPTM effectors cause serious infections and are easily spread through air, water, and food sources. Infected individuals can be asymptomatic and difficult to diagnose during early stages of infection, making early detection critical for rapid treatment and containment[79]. oPTM effectors are largely employed during early phases of infection, and their products are constantly amplifying. Thus, developing tools for oPTM detection could be a fruitful diagnostic strategy[80]. A number of bacteria that secrete oPTMs are considered bioterrorism threats by the Centers for Disease Control and Prevention (CDC), including *B. pseudomallei*, *Shigella* species, *Salmonella* species, and *Yersinia* species. Intriguingly, nearly all of these pathogens catalyze multiple oPTMs in unique combinations, suggesting that discrete groups of oPTMs could be diagnostic for specific infections (Table 1).

For instance, *B. pseudomallei*, the causative agent of melioidosis, secretes two effectors that catalyze oPTMs, including CHBP and BLF1 (refs. [49,60]). These effectors catalyze Gln deamidation of NEDD8 and Ub, and eIF4A, respectively. *S. flexneri*, the causative agent of dysentery, secretes three oPTM effectors: OspF catalyzes phosphate β-elimination from ERK1/2 and p38, OspI catalyzes deamidation of UBC13 (refs. [22,56]), and OspZ catalyzes methylation of TAB2/3 (ref. [73]). Additionally, *S. enterica* Typhimurium is responsible for foodborne illness that can result in severe, life-threatening infection. The *Salmonella* effector AvrA catalyzes Ser/Thr acetylation of MKK4 and MKK7 (ref. [11]), whereas SpvC catalyzes phosphate β-elimination from ERK1/2 and p38 (refs. [22,25]). *Salmonella* also secretes SseK1/3, which catalyzes GlcNAcylation of TRADD and FADD[77], and SopF, which catalyzes ADP-ribosylation on ATP6V0C[78]. Similarly, enteropathogenic or enterohaemorrhagic *E. coli* strains secrete effectors that catalyze deamidation of RhoA (CNF1), as well as NEDD8 and ubiquitin (Cif)[47–52,61]. They also secrete NleE, which catalyzes methylation of TAB2/3, and NleB, which catalyzes GlcNAcylation of TRADD, FADD, RIPK1, and TNFR1 (refs. [72–76]).

Although it is not considered a biological warfare agent by the CDC, *L. pneumophila* is a serious infective threat that causes Legionnaire's disease and pneumonia[81]. *L. pneumophila* is particularly well-poised to be detected by oPTMs, as it not only catalyzes multiple oPTMs, but also catalyzes two that have not been identified in other species. These include phosphocholination of Rab GTPases by AnkX and phosphoribosyl-linked ubiquitination by Sde family members[34,35,63]. Based on our current understanding, detection of these oPTMs in a human sample would unambiguously indicate the presence of *L. pneumophila*. Taken together, these specific complements of orthogonally modified proteins may offer a unique collection of biomarkers that could be useful for detecting a variety of serious bacterial infections.

Table 1 | Many pathogenic bacteria secrete multiple effectors that catalyze oPTMs acting on diverse host targets

Pathogen	oPTM effector	Known target(s)
Burkholderia pseudomallei	**BLF1** Gln deamidase	eIF4A
	CHBP Gln deamidase	NEDD8, ubiquitin
***Escherichia coli* (EPEC/EHEC)**	**CNF1** Gln deamidase	RhoA
	Cif Gln deamidase	NEDD8, ubiquitin
	NleE Cys methyltransferase	TAB2/3
	NleB Arg GlcNAc-transferase	TRADD, FADD, RIPK1, TNFR1
Legionella pneumophila	**AnkX** Phosphocholine transferase	Rab1A, Rab1B, Rab35
	MavC, MvcA Gln deamidase	UBC13, ubiquitin
	Sde family Phosphoribose Ub transferase	Reticulon 4, Rab1, Rab33b
Shigella flexneri	**OspF** Phosphothreonine lyase	Erk, p38
	OspI Gln deamidase	UBC13
	OspZ Cys methyltransferase	TAB2/3
***Salmonella enterica* Typhimurium**	**AvrA** Ser/Thr acetyltransferase	MKK4, MKK7
	SpvC Phosphothreonine lyase	Erk, p38
	SseK1/3 Arg GlcNAc-transferase	TRADD, FADD
	SopF Gln ADP-ribosyltransferase	ATP6V0C
Vibrio parahaemolyticus	**VopC** Gln deamidase	eIF4A
	VopA Ser/Thr acetyltransferase	MKK6
Yersinia pestis	**YopJ** Ser/Thr acetyltransferase	MKK6, MKK2, IKKβ, RICK, TAK1
Yersinia pseudotuberculosis	**Cif** Gln deamidase	NEDD8, ubiquitin
	CNFY Gln deamidase	RhoA

Harnessing the unique chemistry of oPTMs

Another useful aspect of oPTMs is that their reactions are unique, enzyme catalyzed, and typically absent in mammalian biochemical processes. Thus, effectors that catalyze oPTMs can orthogonally modify cellular systems. Phospholyases have been the focus of a number of reports that use them to specifically inactivate MAPK proteins[82,83]. Members of the Lim laboratory used OspF to rewire kinase signaling[84], demonstrating that a switchable OspF could inactivate MAPK signaling and prevent cytokine release and proliferation in T cells[84]. Thus, a synthetic OspF gene circuit can introduce a pause switch to improve the safety of therapeutic T cells. To our knowledge, no other oPTMs have been used for synthetic signaling, but many could be useful for such applications, as they are tailor-made to rewire existing pathways.

Enzymes catalyzing oPTMs can also be adapted for bioconjugation. For example, a genetically encodable AnkX recognition motif enabled site-specific protein labeling with phosphocholine analogs in vitro[85,86]. Crucial to this work was the discovery that AnkX can accept many variants of choline, including those bearing fluorophores, affinity tags, or chemoselective handles. Additionally, our group has recently reported a phosphine probe that selectively labels Dhb-modified proteins in cell lysates[31]. Notably, the reduced reactivity of Dhb relative to dehydroalanine may be beneficial for protein labeling in cellular environments, as it avoids competition with abundant cellular metabolites. Thus, further exploration of the chemical space surrounding oPTMs and their effectors could lead to new and useful methods for selective protein functionalization that are readily accomplished within cells.

Targeting oPTMs to fight bacterial infection

The emergence of bacterial resistance to antibiotic therapies remains an ongoing menace to global health[87]. To combat antibiotic resistance, some new approaches to fight infection focus on antivirulence strategies[88–90]. Antivirulence agents selectively disarm pathogens by targeting only bacterial proteins and pathways necessary for virulence. For instance, the type III secretion system (T3SS) is highly conserved among many strains of Gram-negative bacteria, and has thus received attention as a potential antivirulence target[90,91]. The most well-studied class of T3SS inhibitors are the salicylidenes[92], which impair infection but do not otherwise affect bacterial growth[92,93]. Targeting of secreted effectors, rather than the T3SS itself, has also been explored as an antivirulence strategy[94], though oPTM effectors remain underappreciated targets. Still, blocking oPTM effector activities could be a particularly efficacious antivirulence strategy. First, genetic knockdown or catalytic impairment of oPTM effectors often leads to diminished pathogenicity[13,23,36]. Additionally, oPTM effectors catalyze chemistry that is absent from the healthy human proteome, suggesting that successful inhibitors could be specific and free of off-target effects. Lastly, oPTM effectors act within human cells, thus circumventing resistance mechanisms involving bacterial uptake and efflux of small-molecule drugs. Thus, we anticipate that oPTM effectors may become an exciting class of new antivirulence targets.

Presaging new, widespread PTMs in humans?

Recent advances in genomics and proteomics have unlocked the ability to search widely for previously unknown PTMs and map their locations in the proteome. As a result, nearly all oPTMs have been identified within just the last 10–15 years, suggesting that additional effectors and entire classes of oPTMs will continue to be revealed. Yet, one lingering question remains: are the oPTMs described herein truly orthogonal, or have they simply not yet been discovered in humans?

Historically, the discovery of novel enzyme activities from viral or bacterial pathogens has predated or predicted their discovery in eukaryotes. The discovery of v-Src, a viral oncogene, revealed the whole family of non-receptor tyrosine kinases, and the discovery of E6-AP, a viral E3 ubiquitin ligase, originated the entire class of HECT E3 ligases[95]. The unfolding story of hydroxyl AMPylation—a PTM that could be considered orthogonal until only recently—offers some possible clues. AMPylation was first discovered in 1967 as a regulatory mechanism for *E. coli* glutamine synthetase-adenylyltransferase[96]. More than four decades later, effectors from *V. parahaemolyticus* (VopS) and, later that same year, *Histophilus somni* (IbpA) were found to mediate cytotoxicity through AMPylation of host Rho GTPases, causing cell rounding and interference with host actin assembly (Fig. 5a)[64,65]. One year later, DrrA, an effector from *L. pneumophila*, was also found to be an AMPylator that modifies host Rab1b, a small GTPase crucial for vesicle trafficking[97]. Thus, at first, AMPylation appeared to be used exclusively by bacteria, either as a self-regulatory PTM or oPTM to aid in infection.

However, the Fic domains that catalyze AMPylation are conserved among bacteria, archaea, and eukaryotes, including humans. In 2009, the only human protein known to contain a Fic domain, FICD, was found to be an AMPylator[65]. FICD activity initially appeared to be limited to ER-localized proteins[98,99], though chemoproteomic advances later enabled the identification of many FICD substrates[100,101]. In 2020, FICD was found to modulate neuronal differentiation, providing critical evidence in support of widespread native AMPylation in human cells[102].

Intriguingly, the human pseudokinase, selenoprotein-O (SelO), was also discovered to be a human AMPylator despite lacking the Fic domain shared by most other AMPylators[68]. Instead, crystallographic studies revealed that SelO has a protein kinase-like fold, but ATP binding is reversed, burying the γ-phosphate and positioning AMP for transfer to protein substrates. These recent studies provide long-sought evidence that AMPylation is widely utilized in human cells[68,102]. In particular, pseudokinases, which constitute roughly 10% of all human kinases, are so named because they lack conserved residues essential for ATP binding and γ-phosphate transfer[68]. By identifying the pseudokinase SelO as an AMPylator, Sreelatha et al. reveal that human AMPylation has the potential to be far more prevalent than previously thought.

Conclusions and outlook

To date, it remains unclear whether a similar narrative will unfold for the eight oPTMs discussed. For instance, phosphocholination is catalyzed by the same, highly conserved, Fic domain used for AMPylation. Perhaps further study will reveal phosphocholination to be widespread, just like AMPylation. On the other hand, the enzymes that catalyze phosphate β-elimination remain a small family that does not extend to eukaryotes, suggesting that this activity could remain orthogonal. Deamidases and Ser/Thr acetyltransferases share homology with protease domains but accomplish distinct chemistry. Thus, further study of currently unannotated protease-like domains may reveal Gln deamidase or Ser/Thr acetyltransferase activity. Similarly, phosphoribosyl-linked ubiquitination relies on enzymes that use known mono-ADP-ribosyltransferase and phosphodiesterase domains but connects them to catalyze an unprecedented ubiquitin linkage. Thus, it is possible that other proteins with unknown functions possessing two (or more) domains not typically found together could uncover new enzymes capable of novel chemistry. So far, such activities have not been reported in healthy human cells, but only time and continued research efforts will reveal whether this remains true. In the meantime, identifying new effectors and their oPTMs will continue to reveal essential aspects of the infection process that can be harnessed for a variety of therapeutic, diagnostic, and synthetic applications.

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Competing interests

The authors declare no competing interests.

Additional information

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