LETTER

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Pathogen blocks host death receptor signalling by arginine GlcNAcylation of death domains

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The tumour necrosis factor (TNF) family is crucial for immune homeostasis, cell death and inflammation. These cytokines are recognized by members of the TNF receptor (TNFR) family of death receptors, including TNFR1 and TNFR2, and FAS and TNF-related apoptosis-inducing ligand (TRAIL) receptors[1]. Death receptor signalling requires death-domain-mediated homotypic/heterotypic interactions between the receptor and its downstream adaptors, including TNFR1-associated death domain protein (TRADD) and FAS-associated death domain protein (FADD)[2]. Here we discover that death domains in several proteins, including TRADD, FADD, RIPK1 and TNFR1, were directly inactivated by NleB, an enteropathogenic *Escherichia coli* (EPEC) type III secretion system effector known to inhibit host nuclear factor-κB (NF-κB) signalling[3,4]. NleB contained an unprecedented *N*-acetylglucosamine (GlcNAc) transferase activity that specifically modified a conserved arginine in these death domains (Arg 235 in the TRADD death domain). NleB GlcNAcylation (the addition of GlcNAc onto a protein side chain) of death domains blocked homotypic/heterotypic death domain interactions and assembly of the oligomeric TNFR1 complex, thereby disrupting TNF signalling in EPEC-infected cells, including NF-κB signalling, apoptosis and necroptosis. Type-III-delivered NleB also blocked FAS ligand and TRAIL-induced cell death by preventing formation of a FADD-mediated death-inducing signalling complex (DISC). The arginine GlcNAc transferase activity of NleB was required for bacterial colonization in the mouse model of EPEC infection. The mechanism of action of NleB represents a new model by which bacteria counteract host defences, and also a previously unappreciated post-translational modification.

EPEC contains several type-III-secreted effectors, including NleC/D[5–8], Tir[9,10], NleE and NleB[3,4,11,12], all of which can inhibit host NF-κB signalling and pro-inflammatory cytokine production. Among these effectors, NleB is required for virulence *in vivo*[13–15]. NleB homologues are present in *Salmonella* and pathogenic *E. coli* strains[16]. Consistent with previous studies[4,9], the expression of NleB (E2348C_3231) in HeLa cells selectively blocked TNF-α but not interleukin (IL)-1β activation of NF-κB signalling (Supplementary Fig. 1a, b). TNF-α-induced IκB-α phosphorylation and degradation were both severely inhibited (Supplementary Fig. 1c). NleB blocked TRAF2- but not TRAF6-induced NF-κB activation (Supplementary Fig. 2a). TNF-α- but not IL-1β-stimulated TAK1 phosphorylation, downstream of the receptor and TRAF complex, was diminished by NleB (Supplementary Fig. 2b). TNFR can also induce apoptosis or necroptosis (in cells deficient in caspase activity), playing an important role in microbial infection and inflammation[17]. NleB efficiently blocked TNF-α plus cycloheximide-induced 293T cell apoptosis (Fig. 1a), and also blocked necroptosis of HeLa cells stably expressing the RIP3 protein kinase (HeLa-RIP3 cells) (Fig. 1b). This agrees with NleB targeting upstream of TAK1, as TAK1 is not required for TNF-α-induced cell death[18]. Thus, NleB differs from other NF-κB-targeting effectors and can block both NF-κB signalling and TNF-α-induced cell death.

A yeast two-hybrid screen of a HeLa complementary DNA library identified a cDNA clone encoding the death domain of TRADD, a universal component of the TNFR1 but not IL-1R complex[19–21]. NleB also interacted with full-length TRADD, but not with components of the TAK1 and IκB kinase (IKK) complexes; no interaction occurred between NleE and TRADD (Fig. 1c). Endogenous TRADD was readily precipitated by NleB (Supplementary Fig. 3a). The TRADD death domain (residues 195–312) was required and sufficient for precipitation by enhanced green fluorescent protein (eGFP)–NleB (Fig. 1d and Supplementary Fig. 3b). Similar to that observed with TNF-α stimulation, NleB completely abolished TRADD overexpression-induced NF-κB activation (Fig. 1e) and apoptosis in 293T cells (Fig. 1f and Supplementary Fig. 4a). Thus, NleB can target TRADD and disrupt multiple signalling pathways downstream of TNFR1.

NleB did not affect TRADD stability or turnover (Supplementary Fig. 5a). TRADD contains an amino-terminal TRAF2-binding domain and a carboxy-terminal death domain. The TRADD death domain oligomerizes with itself and also with death domains of TNFR1 and FADD[22]. Death-domain-mediated TRADD recruitment to TNFR1 initiates TNF-α signalling, and TRADD–FADD complex formation triggers apoptosis[23]. TRADD readily precipitated TNFR1c (the intracellular death-domain-containing region) from 293T cells, and this was abolished by NleB (Supplementary Fig. 5b). A similar blocking effect was observed in TRADD–FADD but not in TRADD–TRAF2 co-immunoprecipitation (Supplementary Fig. 5c, 6). TRADD oligomerization is crucial for TNFR1 complex assembly[22]. TRADD or TRADD death domain expressed in 293T cells appeared as a large oligomer on a blue native polyacrylamide gel electrophoresis (PAGE) gel, and NleB co-expression shifted TRADD and also the TRADD death domain to a lower molecular mass position that roughly corresponded to the monomer form (Fig. 2a). Consistently, NleB inhibited the recruitment of TRADD, TRAF2, HOIP, SHAPPIN, HOIL-1L and ubiquitinated RIPK1 to TNFR1 (Fig. 2b). Thus, NleB inactivates the TRADD death domain and disrupts homotypic/heterotypic death domain interactions and the TNFR1 complex assembly.

In these TRADD oligomerization and binding assays, the amount of NleB was considerably lower than those of TRADD, indicating that NleB may act in a catalytic manner on the TRADD death domain. Mass spectrometry analysis identified a 203-dalton (Da) mass increase on TRADD death domain co-expressed with NleB in either *E. coli* (Supplementary Fig. 7) or 293T cells (Fig. 2c), whereas control TRADD death domain showed the theoretic molecular mass. The 203-Da mass increase indicated a GlcNAc modification. Consistently, recombinant NleB efficiently transferred ^{3}H-GlcNAc, but not ^{3}H-glucose, from radiolabelled sugar donors onto purified TRADD (Fig. 2d). Cold UDP-GlcNAc, but not UDP-GalNAc, UDP-glucose, UDP-galactose and UDP-GlcA,

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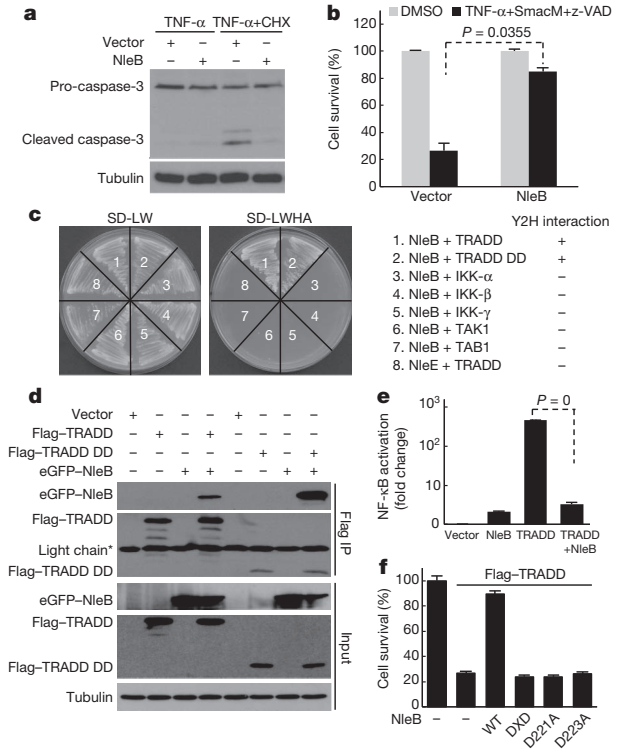

Figure 1 | NleB blocks TNF-α signalling by directly targeting the TRADD death domain. **a**, Effects of NleB transfection on TNF-α-induced caspase-3 activation in 293T cells. CHX, cycloheximide. **b**, Vector- or NleB-transfected HeLa-RIP3 cells were stimulated with TNF-α plus Smac mimetic (SmacM) and z-VAD, and cell viability was determined by measuring ATP levels. DMSO, dimethylsulphoxide. **c**, Yeast strain was transformed with indicated plasmid combinations (bait plus prey) to assay the interaction of NleB with TRADD or other components downstream of TNFR. Yeast strains were grown on SD-LW (non-selective) and SD-LWHA (selective) media. DD, death domain; Y2H, yeast two-hybrid. **d**, Co-immunoprecipitation of NleB with TRADD and TRADD death domain in 293T cells. **e**, NF-κB luciferase activity of NleB- and/or TRADD-transfected 293T cells is shown as fold change normalized to that in vector-transfected cells. **f**, Viability of NleB- and/or TRADD-transfected 293T cells was determined by measuring ATP levels. $P < 0.0001$ for all (TRADD plus NleB versus TRADD plus DXD, versus TRADD plus Asp221Ala, and versus TRADD plus Asp223Ala). For **b**, **e** and **f**, $n = 3$; data are mean ± s.d.; P value determined by Student's t-test. Data in **a**, **c** and **d** are representative from at least three repetitions.

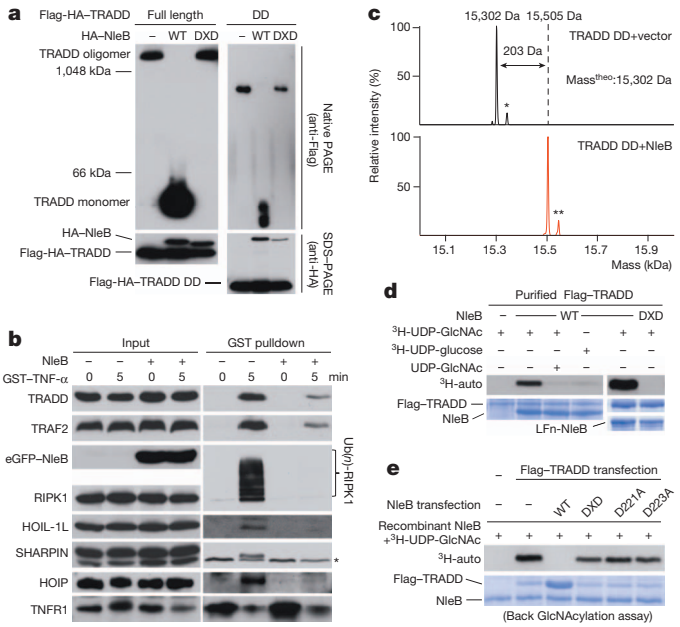

Figure 2 | NleB GlcNAcylates the TRADD death domain and disrupts its oligomerization. **a**, Effects of NleB transfection on TRADD oligomerization in 293T cells. Shown are immunoblots of cell lysates loaded onto native (top) and SDS–PAGE (bottom) gels. HA, haemagglutinin; WT, wild type. **b**, Effects of NleB transfection on TNFR1 complex formation in HeLa cells. Ub(n)-RIPK1 denotes polyubiquitinated RIPK1; asterisk marks a nonspecific band. GST, glutathione *S*-transferase. **c**, Electrospray ionization (ESI)-mass spectrometry determination of the total mass of TRADD death domain immunopurified from NleB- or vector-transfected 293T cells. Single and double asterisks denote the acetylated TRADD death domain. **d**, *In vitro* ^{3}H-UDP-GlcNAc labelling of TRADD by recombinant NleB. LFn-NleB refers to NleB protein fused C-terminally to the N-terminal domain of anthrax lethal factor. **e**, TRADD immunopurified from NleB-transfected 293T cells was subjected to further *in vitro* ^{3}H-UDP-GlcNAc labelling by recombinant NleB. Data in **a**, **b**, **d** and **e** are representative from at least three repetitions.

abolished ^{3}H-GlcNAc labelling of TRADD by NleB (Fig. 2d and Supplementary Fig. 8). TRADD purified from NleB-expressing cells was resistant to further *in vitro* ^{3}H-GlcNAc labelling by recombinant NleB in the 'back glycosylation' assay[24] (Fig. 2e), confirming the complete modification of TRADD in NleB-expressing 293T cells. Thus, NleB is a glycosyltransferase that GlcNAcylates the TRADD death domain.

Extensive mutagenesis of potentially catalytic residues identified NleB Asp221Ala and Asp223Ala mutants that lost the activity of inhibiting TNF-α–NF-κB signalling (Supplementary Fig. 9). Interestingly, the GT-A family of glycosyltransferases[25], including the large clostridial toxins that modify Rho/Ras small GTPases[26], feature a catalytic Asp-X-Asp (DXD) motif that coordinates manganese (Mn^{2+}) and/or the sugar donor. The NleB(Asp221Ala/Asp223Ala) (DXD) mutant could not block the death-domain-mediated TRADD interaction with TNFR1c and FADD (Supplementary Fig. 5b, c) or TRADD self-oligomerization (Fig. 2a). Recombinant NleB DXD mutant exhibited no catalytic activity of GlcNAcylating TRADD (Fig. 2d); these two aspartate residues were required for NleB interaction (Supplementary Fig. 3a) and modification of TRADD in transfected 293T cells (Fig. 2e), as well as for inhibiting TRADD-induced apoptosis (Fig. 1f and Supplementary Fig. 4a). Concurrent to our analysis, another study[27] also proposes that NleB is a GT-A-type glycosyltransferase according to fold recognition prediction. This study identifies glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a host binding partner of NleB. In *O*-GlcNAc antibody blotting, ^{3}H-GlcNAc labelling and mass spectrometric assays, GAPDH was not glycosylated by NleB *in vitro* and *in vivo* (Supplementary Fig. 10). GAPDH binding may represent a regulatory mechanism of NleB function, or be a target of NleB homologue. EPEC contains an NleB paralogue known as NleB2 (E2348C_1041) and an NleB homologue, SseK1, is present in *Salmonella enterica* serovar Typhimurium. Although SseK1 functioned comparably to NleB in inhibiting TNF-α–NF-κB signalling and GlcNAcylating the TRADD death domain, NleB2 showed a lower enzymatic activity and only a fraction of TRADD was modified by NleB2 in 293T cells (Supplementary Fig. 11).

Protein *O*-GlcNAcylation is an *O*-linked GlcNAc modification generally occurring on serine/threonine residues of intracellular proteins. *O*-GlcNAcylation is abundant from bacteria to mammals, and has been proposed to be a phosphorylation-like modification in regulating transcription or signalling in response to nutrients and cellular stresses[28,29]. The 203-Da mass increase indicates a single GlcNAc modification to the TRADD death domain by NleB (Fig. 2c and Supplementary Fig. 7a). To pinpoint the exact modification site, mutagenesis analyses were first used given that mapping the *O*-GlcNAcylation site by the conventional collision-assisted dissociation (CAD) mass spectrometry is technically difficult owing to the labile nature of GlcNAc modification[29]. Surprisingly, no single mutation in all the ten serine/threonine residues in the TRADD death domain disrupted its modification by NleB (Supplementary Fig. 12). The ST10A mutant (all ten serine/threonine residues

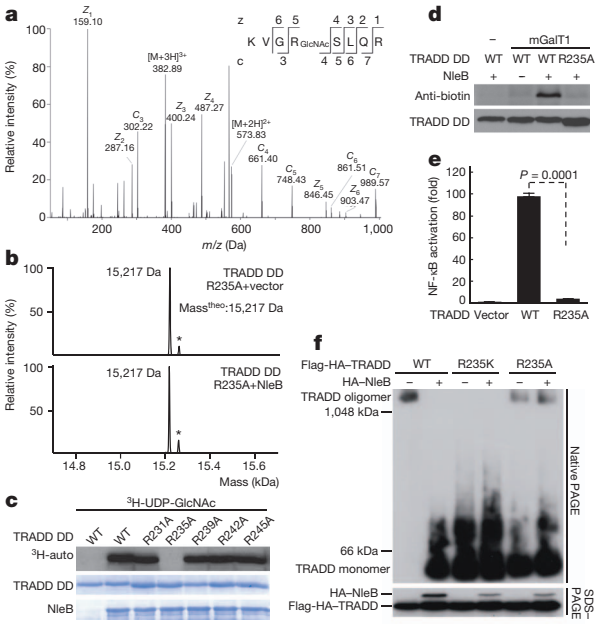

Figure 3 | NleB GlcNAcylates Arg 235 that is required for TRADD function. **a**, ETD-tandem mass spectrum of a triply charged Arg-235-containing tryptic peptide from NleB-modified TRADD death domain in bacteria. The fragmentation patterns that generate the observed *c* and *z* ions are illustrated along the peptide sequence shown on top of the spectrum. **b**, ESI-mass spectrometry determination of the total mass of TRADD arginine mutants immunopurified from NleB- or vector-transfected 293T cells. All arginine residues in the TRADD death domain were individually mutated into alanine (also see Supplementary Fig. 17). Asterisk denotes the acetylated TRADD death domain Arg235Ala mutant. **c**, *In vitro* ^{3}H-UDP-GlcNAc labelling of TRADD death domain arginine mutants by recombinant NleB. **d**, TRADD death domain or its Arg235Ala mutant immunopurified from NleB-transfected 293T cells was subjected to chemoenzymatic UDP-GalNAz labelling followed by reaction with an alkyne-biotin derivative. **e**, **f**, TRADD or its Arg 235 mutants were expressed in TRADD-knockdown 293T cells. **e**, NF-κB luciferase activity (fold change) is normalized to that in vector-transfected cells ($n = 3$; mean ± s.d.; Student's *t*-test). Data in **c**, **d** and **f** are representative from at least three repetitions.

were mutated simultaneously) was still GlcNAcylated to a considerable extent (Supplementary Fig. 12). Thus, NleB-induced modification may not be a canonical *O*-GlcNAcylation. The NleB-modified TRADD death domain (Supplementary Fig. 13) was then subjected to in-depth mass spectrometry analyses. Among the 13 tryptic peptides that cover the large majority of the TRADD death domain sequence, two overlapping ones (232-KVGRSLQR-239 and 233-VGRSLQR-239) showed a 203-Da mass increase (Supplementary Fig. 14). Electron-transfer dissociation (ETD) tandem mass spectrometry, which works better in preventing loss of the labile GlcNAc during peptide fragmentation[29], revealed that the 203-Da mass increase occurred on Arg 235 (Fig. 3a).

When the Arg-235-containing tryptic peptide was purified and sequentially digested by proteinase K and carboxypeptidase, the GlcNAc-modified dipeptide (234-Gly-Arg-235) and even Arg 235 alone were identified by higher-energy collision dissociation tandem mass spectrometry (Supplementary Fig. 15). When the modification was performed in cells metabolically labelled with 2-(acetyl-d_3-amino)-2-deoxy-1,3,4,6-tetra-*O*-acetyl-D-glucopyranose ($Ac_4GlcNAc$-d_3), a mass increase of 206 Da, corresponding to modification by the deuterium-labelled GlcNAc, occurred on Arg 235 in the TRADD death domain (Supplementary Fig. 16).

NleB GlcNAcylation of TRADD Arg 235 was supported by four additional evidences. Arg235Ala and Arg235Lys mutations in the TRADD death domain abolished the GlcNAc modification by NleB in 293T cells, whereas mutation in any of the other ten arginine residues had no such effect (Fig. 3b and Supplementary Fig. 17). The purified TRADD(Arg235Ala) mutant resisted NleB-catalysed *in vitro* GlcNAcylation (Fig. 3c). When chemoenzymatic labelling of the terminal GlcNAc was used to detect GlcNAcylation, the wild-type TRADD

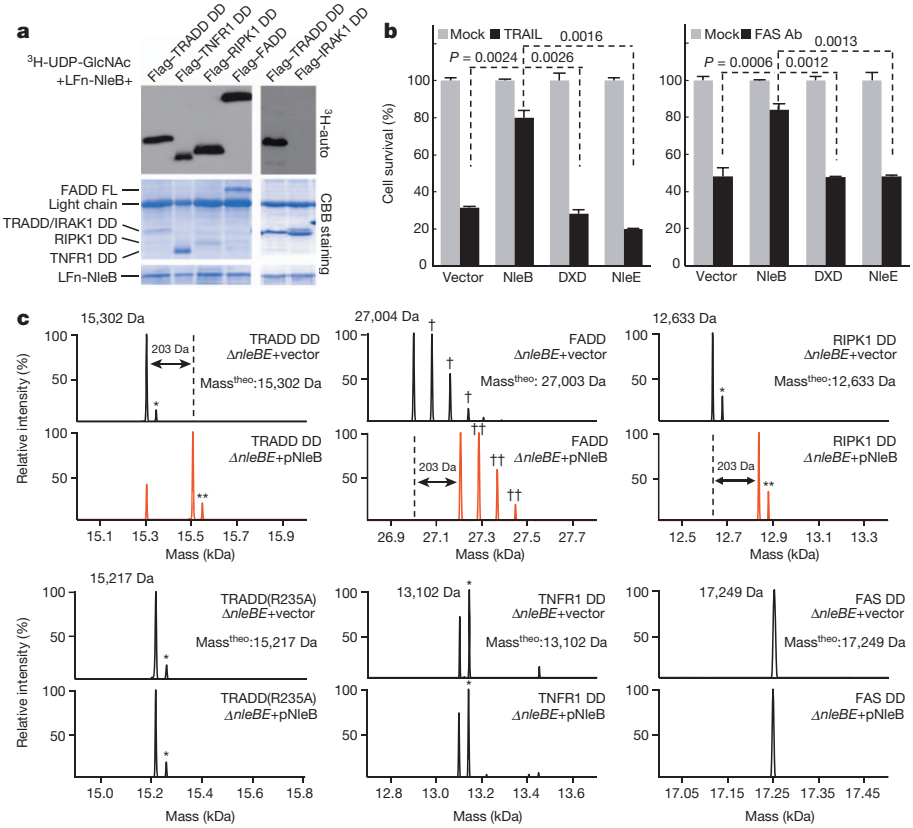

Figure 4 | NleB GlcNAcylates FADD and other death domain proteins, and blocks FAS- and TRAIL-induced apoptosis. **a**, *In vitro* ^{3}H-UDP-GlcNAc labelling of indicated death domains or death-domain-containing proteins by recombinant NleB. Data represent at least three repetitions. **b**, NleB-, NleE- or vector-transfected HeLa cells were stimulated with FAS antibody (Ab) or TRAIL, and cell viability was determined by measuring ATP levels ($n = 3$; mean ± s.d.; Student's *t*-test). **c**, ESI-mass spectrometry determination of the total mass of indicated death domain proteins purified from EPEC-infected 293T cells. Δ*nleBE*, NleB and NleE double-deletion EPEC E2348/69 strain; pNleB, NleB rescue plasmid. Dagger symbols denote the phosphorylated FADD; asterisks mark the corresponding acetylated proteins.

death domain from NleB-expressing 293T cells was readily labelled with *N*-azidoacetylgalactosamine (GalNAz) by an engineered galactosyltransferase (Tyr289Leu mutant of GalT1), whereas no evident GalNAz labelling occurred on TRADD(Arg235Ala) (Fig. 3d). Lastly, NleB-catalysed GlcNAc modification was not reversed by *O*-GlcNAcase, β-*N*-acetylglucosaminidase and β-*N*-acetylhexosaminidase$_f$ (a recombinant protein fusion of β-*N*-acetylhexosaminidase and maltose binding protein) (Supplementary Fig. 18), enzymes that are capable of removing *O*-linked or terminal GlcNAc.

TRADD-knockdown 293T cells were generated (Supplementary Fig. 19a). Knockdown of endogenous TRADD minimized its hetero-oligomerization and interference with transfected TRADD mutants. In contrast to wild-type TRADD, expression of TRADD(Arg235Ala) in TRADD-knockdown cells was largely deficient in activating the NF-κB signalling (Fig. 3e and Supplementary Fig. 19b). Consistently, lysine or alanine substitution of Arg 235 in TRADD completely or nearly completely disrupted TRADD self-oligomerization, and the residual Arg235Ala oligomer was insensitive to further NleB expression (Fig. 3f). Thus, Arg 235 is important for TRADD function and activity.

Arg 235 is conserved in one-third of the total of more than 30 death-domain-containing proteins in humans[22] (Supplementary Fig. 20), including FADD, TNFR1, RIPK1, FAS and death receptor-3/4/5 (DR3/4/5) that function in death receptor signalling. NleB completely modified the death domains of TNFR1 and RIPK1 as well as full-length FADD in bacteria (Supplementary Fig. 21) or 293T cells (Supplementary Fig. 22); a considerable portion of FAS death domain was also GlcNAc-modified. Death domains of MYD88 and IRAK1 devoid of the conserved arginine were not modified by NleB (Supplementary Fig. 22). In the ^{3}H-GlcNAc labelling assay, FADD and death domains of TNFR1 and RIPK1 were GlcNAcylated by recombinant NleB, with efficiency comparable to that of the TRADD death domain (Fig. 4a). Induction of apoptosis by FAS ligand (FasL) and TRAIL require death-domain-mediated FADD interaction with the receptors and formation of a caspase-8-containing death-inducing signalling complex (DISC). Consistently, anti-FAS antibody and TRAIL-stimulated HeLa cell apoptosis was inhibited by NleB, but not by the GlcNAc-transferase-deficient DXD mutant or the NleE effector (Fig. 4b).

293T cells expressing various death domains or death domain proteins were infected with an NleB-proficient or -deficient EPEC strain. FADD, RIPK1 death domain and a portion of TRADD death domain from NleB-positive infection showed a 203-Da mass increase (Fig. 4c). Modification of these proteins was not observed with the NleB-deficient strain. Importantly, no GlcNAc modification occurred on TRADD(Arg235Ala) (Fig. 4c). Consistent with these observations, expression of NleB, but not the DXD mutant, in a NleB/E double-deletion EPEC strain blocked p65 nuclear translocation in infected mouse embryonic fibroblast (MEF) cells (Fig. 5a and Supplementary Fig. 23a). GlcNAc-transferase-active NleB inhibited apoptosis in EPEC-infected HeLa cells after stimulation by TNF-α, FasL or TRAIL (Fig. 5b and Supplementary Fig. 4b). Type-III-delivered NleB, but not the DXD mutant, also effectively blocked TNF-α-induced necroptosis in HeLa-RIP3 or HT-29 cells (Supplementary Fig. 24). Furthermore, TNF-α-induced recruitment of TRADD, TRAF2, SHARPIN and ubiquitinated RIPK1 to TNFR1 as well as TRAIL-induced DISC formation were both disrupted by EPEC expressing the catalytically active NleB, and disruption of DISC formation was more severe (Fig. 5c and Supplementary Fig. 23b). When expressed in the type III secretion-deficient Δ*escN* strain, NleB did not affect cell death and death receptor complex assembly (Fig. 5a–c and Supplementary Figs 23b and 24).

Deletion of *nleB* from *Citrobacter rodentium* results in severely reduced bacterial colonization in infected mice[13]. *C. rodentium* NleB (NleBc) inhibited TNF-α signalling to an extent comparable to that of EPEC NleB (Supplementary Fig. 25a); NleBc also GlcNAcylated several death domain proteins, including FADD, TNFR1 and RIPK1, despite a slightly different substrate preference (Supplementary Fig. 25b). In *C.-rodentium*-inoculated C57BL/6 mice, the Δ*nleB* mutant showed a significantly reduced colonization compared to the wild-type strain,

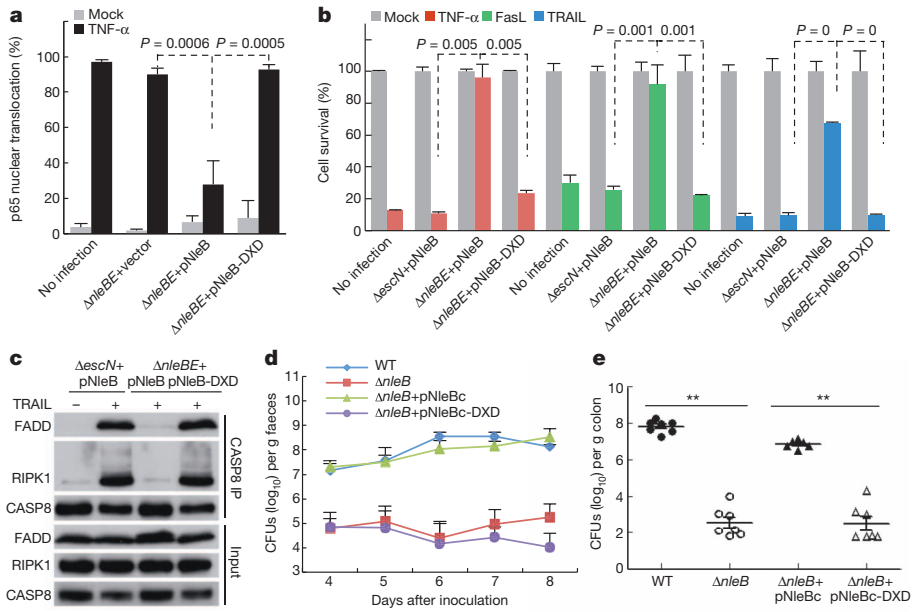

Figure 5 | Disrupting several death receptor pathways by NleB GlcNAc transferase activity that is required for bacterial colonization *in vivo*. **a**, MEF cells infected with indicated EPEC strains were stimulated with TNF-α, and statistics of cells with nuclear-localized p65 are shown. Representative fluorescence images are in Supplementary Fig. 23a. **b**, HeLa cells infected with indicated EPEC strains were stimulated with TNF-α, FasL or TRAIL, and cell viability was determined by measuring ATP levels. For **a** and **b**, $n = 3$; mean ± s.d. **c**, Lysates of EPEC-infected HeLa cells stimulated with TRAIL were subjected to anti-caspase-8 (CASP8) immunoprecipitation. Data represent at least three repetitions. **d**, **e**, Five–six-week-old C57BL/6 mice were oral gavaged with indicated *C. rodentium* DBS100 strains. Viable stool bacterial counts (**d**), measured at indicated points after inoculation, are shown as mean ± s.e.m. of $\log_{10}$ colony-forming units (CFU) per gram faeces, and bacterial colonization of the intestine 8 days after infection (**e**) is shown as the mean ± s.e.m. of $\log_{10}$ CFU per gram colon ($n > 6$). $^{**}P < 0.01$ (Student's *t*-test). pNleB(c) and pNleB(c)-DXD are rescue plasmids expressing wild-type and the DXD mutant of NleB(c), respectively.

demonstrated by colony-forming units of bacteria recovered from stool samples and colons from infected mice (Fig. 5d, e). Complementation of Δ*nleB* with NleBc or EPEC NleB, but not the DXD mutant, recovered stool counts to the level of the wild-type strain and restored bacterial colonization in the intestinal tract of mice (Fig. 5d, e and Supplementary Fig. 26). Thus, the arginine GlcNAc transferase activity of NleB is crucial for bacterial colonization and virulence in mice.

We discover that the EPEC type III effector NleB targets several death-domain-containing proteins in TNFR, FAS and TRAIL death receptor complexes to block host cell death. NleB probably functions as part of a network of effectors during EPEC interaction with the host. NleB is the first bacterial virulence factor hijacking the death receptor complex. Such distinguished feature may account for the unique virulence activity of NleB[13–15]. TRADD and FADD are central adaptors in death receptor signalling. TRADD also mediates Toll-like receptor signalling in macrophages[19–21], suggesting an even broader function of NleB in EPEC modulation of host defences. NleB modifies its death domain targets by GlcNAcylation on a conserved arginine. Discovering arginine GlcNAcylation is conceptually unexpected despite one early preliminary study proposing such modification on a corn protein[30]. The labile GlcNAc modification is refractory to conventional mass-spectrometry-based proteomic identifications that are performed in the absence of an arginine GlcNAcylation hypothesis. Thus, arginine GlcNAcylation might be widely used and represent a previously unappreciated mechanism in signalling.

METHODS SUMMARY

The EPEC E2348/69 Δ*nleB/E* SC3909 strain (ΔIE2::*kan* and *nleBE IE6*::*tet*)[3] was used for cell culture infection. A single bacterial colony was inoculated into 0.5 ml of LB medium and statically cultured overnight at 37 °C. Bacterial cultures were then diluted by 1:40 in DMEM supplemented with 1 mM isopropyl-β-D-thiogalactoside (IPTG) and cultured for an additional 4 h at 37 °C in the presence of 5% CO_2. Infection was performed at a multiplicity of infection of 200:1 in the presence of 1 mM IPTG for 2 h. Cells were washed four times with PBS and bacteria were killed by 200 μg ml^{-1} gentamicin. To assay NleB-induced modification, 293T cells were transfected with pCS2-Flag-TRADD death domain plasmids 24 h before infection. z-VAD (Sigma) (20 μM) was added to inhibit apoptosis. Lysates of infected cells were subjected to anti-Flag immunoprecipitation to purify the TRADD death domain for mass spectrometry analysis. All independent experiments carried out in this study and indicated in the figure legends were biological replicates.

Full Methods and any associated references are available in the online version of the paper.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.C. (chenshe@nibs.ac.cn) or F.S. (shaofeng@nibs.ac.cn).

METHODS

Plasmid, antibodies and reagents. DNA for NleB and NleB homologue genes was amplified from genomic DNA of EPEC E2348/69, *C. rodentium* DBS100 and *S. enterica* Typhimurium LT2 strains, and inserted into pCS2-EGFP, pCS2-HA and pcDNA4-Flag-HA vectors for mammalian expression, and pGEX-6P-2, pET28a-LFn and pSUMO for expression in *E. coli*. NleB DNA was also ligated into the pTRC99A vector for complementation in EPEC (under the *trc* promoter) and pET28a for complementation in *C. rodentium* (under the *C. rodentium nleB* promoter). cDNAs for TRADD, RIPK1, TRAF2/5/6, IKK-α/β/γ, TAK1, TAB/23 and IκB-α were amplified from a HeLa cDNA library as previously described[12,31]. TRADD death domain refers to residue 195–312 of human TRADD. cDNAs for cIAP1/2 and TNFRI were gifts from X. Wang and H. Wu, respectively. cDNAs for FAS, FADD, IRAK1, GAPDH, MYD88 and OGT were amplified from human ultimate ORF clones (Invitrogen). For mammalian expression, cDNAs were cloned into pCS2-EGFP and pCS2-3Flag vectors. DNA encoding residues 31–624 of *O*-GlcNAcase was amplified from the *Clostridium perfringens* genome, and cloned into the pGEX6p-2 vector for recombinant expression. For yeast two-hybrid analysis, DNAs encoding NleB, TAK1 and NleE were cloned into the bait vector pGBKT7; cDNAs for TRADD, RIPK1, TRAF2/5, IKKα/β/γ, TAK1, TAB1, cIAP1/2 and FADD were cloned into the prey vector pGAD-GH. The yeast two-hybrid interaction assay was performed using the Matchmaker 2-hybrid system (Clontech) following the manufacturer's instruction. All single point mutations were generated by QuickChange site-directed mutagenesis kit (Stratagene), and multiple point mutations and truncation mutants were generated by standard molecular biology procedures. NF-κB reporter plasmids were previously described[32]. Plasmids were prepared by GoldHi endofree plasmid maxi kit (CW2104, Beijing CoWin Bioscience) for transfection. All plasmids were verified by DNA sequencing.

Antibodies for IκB-α (44D4, catalogue number 4812), p-IκB-α (S32) (14D4, 2589), PARP (9542), caspase-8 (1C12, 9746, for immunoblotting) and caspase-3 (9662) were purchased from Cell Signaling Technology. Antibodies for GFP (sc-8334), p65 (sc-372), TNFRI (H271, sc-7895), TRAF2 (C-20, sc-876), TRAF6 (H274, sc-7221), haemagglutinin probe (Y-11, sc-805), caspase-8 (C-20, sc-6136, for immunoprecipitation), FADD (H-181, sc-5559) and TAK1 (sc-7162) were all from Santa Cruz Biotechnology. Antibodies for TRADD (610573) and RIPK1 (610459) were from BD Transduction Laboratories. Flag (M2), tubulin and actin antibodies were from Sigma. Horseradish peroxidase (HRP)-conjugated anti-biotin antibody was from Abcam (ab19221); p-TAK1 (Thr187) antibody was a gift from H. Sakurai; antibodies for HOIL-1L (2E2), HOIP (N1) and SHARPIN were provided by K. Iwai. Homemade SmacM compound was described previously[33]. Caspase-8 peptide (FFIQACQGDNYQKGIPVETD) was commercially synthesized (SciLight-Peptide). Cell culture products were from Invitrogen, all other reagents were Sigma unless noted.

Cell culture and luciferase reporter assay. 293T and HeLa cells obtained from the American Type Culture Collection (ATCC) and MEF cells from S. Ghosh were grown in DMEM (HyClone) supplemented with 10% FBS, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin. HT-29 cells (ATCC) were maintained in McCoy's 5A media supplemented with 10% FBS and 2 mM L-glutamine. Cells were cultivated in a humidified atmosphere containing 5% CO2 at 37 °C. Vigofect (Vigorus) was used for transfection following the manufacturer's instructions. Luciferase activity was determined 24 h after transfection by using the dual luciferase assay kit (Promega) according to the manufacturer's instructions. Detailed procedures were previously described[31,32]. One-hour CHX pretreatment (1 μg ml^{-1}) was used to sensitize TRAIL (200 ng ml^{-1}) and FAS antibody (1 μg ml^{-1} FAS) stimulation of apoptosis, and the concentration of CHX used for TNF-α (10 ng ml^{-1}) was 4 μg ml^{-1}. SmacM (100 μM) and z-VAD (20 μM; Sigma) were used to induce necroptosis.

Stable cell-line construction. To generate NleB stable expression cells, empty pCDNA4 vector or pCDNA4-NleB plasmid was transfected into 293T cells. Forty-eight hours after transfection, cells were subcultured in the complete DMEM medium supplemented with 100 μg ml^{-1} zeocin (Invitrogen). Two-to-three weeks later, clones were lifted and tested for expression of the transgene. To generate TRADD stable knockdown cells, pLKO.1 or pLKO.1-shTRADD (TRC number TRCN0000008020, Sigma) was transfected into 293T cells. Forty-eight hours after transfection, cells were subcultured in the complete DMEM medium containing 1 μg ml^{-1} puromycin. Two-to-three weeks later, clones were lifted, the culture was expanded, and expression of endogenous TRADD was tested by immunoblotting analysis. The HeLa-RIP3 stable cell line used for TNF-α induction of necroptosis was generated as previously described[33,34] and maintained in DMEM medium supplemented with 10% tetracycline-free FBS, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, 10 μg ml^{-1} blasticidin and 1 mg ml^{-1} G418.

Immunoprecipitation and receptor complex pulldown. For co-immunoprecipitation, 293T cells at a confluency of 60–70% in 6-well plates were transfected with a total of 5 μg plasmids. Twenty-four hours after transfection, cells were washed once in PBS and lysed in buffer A containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10% glycerol and 1% Triton X-100, supplemented with a protease inhibitor mixture (Roche Molecular Biochemicals). Pre-cleared lysates were subjected to anti-Flag M2 immunoprecipitation following the manufacturer's instructions. The beads were washed four times with lysis buffer and the immunoprecipitates were eluted by 2× SDS sample buffer followed by standard immunoblotting analysis. All the immunoprecipitation assays were performed more than three times and representative results are shown.

To purify death domains or death-domain-containing proteins for mass spectrometry, 293T cells overexpressing death domain or death-domain-containing proteins were collected in buffer B containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM *n*-octyl-β-D-glucopyranoside (INALCO) and 5% glycerol, supplemented with an EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals). Cells were lysed by ultrasonication. The supernatant was pre-cleared by protein G–sepharose at 4 °C for 1 h and subjected to anti-Flag (M2) immunoprecipitation. After 4 h incubation, the beads were washed once with buffer B and then four times with TBS buffer (50 mM Tris-HCl, pH 7.5, and 150 mM NaCl). Bound proteins were eluted with 600 μg ml^{-1} Flag peptide (Sigma) in TBS buffer. The eluted protein was verified by Commassie brilliant blue staining on an SDS–PAGE gel before mass spectrometry analysis.

For TNFR complex pulldown, HeLa cells were treated with 1 μg ml^{-1} recombinant GST–TNF-α for indicated lengths of time (for $t = 0$, GST–TNF-α was added after the cells were lysed). Cells were lysed in the GST pulldown buffer containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10% glycerol, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1% Triton X-100, supplemented with the protease inhibitor mixture. Total cell lysates were incubated with glutathione sepharose 4B beads and mixed at 4 °C for 4 h. The beads were washed once with PBS plus 1% Triton X-100, and twice with PBS plus 0.5% Triton X-100. Bead-bound proteins were analysed by immunoblotting using indicated antibodies. For DISC complex pulldown, HeLa cells infected with indicated EPEC strains were stimulated with 200 ng ml^{-1} TRAIL for 2 h. Anti-caspase-8 immunoprecipitation was then performed according to published literatures with minor modifications[35,36]. Cells were lysed in DISC immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol and 1% Triton X-100) supplemented with 1% protease inhibitor, 10 mM NaF, 2 mM Na_3VO_4, 0.1 mM PMSF and 1 mM EDTA. Caspase-8 antibody (1.5 μg) was coupled to 20 μl protein G–sepharose in TBS (supplemented with 7 mg ml^{-1} BSA). The pre-coupled sepharose was then washed and incubated with 2 mg of cell lysates overnight at 4 °C. Following extensive washes with DISC immunoprecipitation buffer (four times) and TBS (once), the immunocomplex were eluted using 40 μl of TBS containing 1 mg ml^{-1} caspase-8 peptide and 1% protease inhibitor at room temperature for 1 h. The eluted proteins were analysed by SDS–PAGE and immunoblotting.

Immunofluorescence. For immunofluorescence, rhodamine-phalloidin (Invitrogen) staining of actin in transfected HeLa cells or pedestal in EPEC-infected HeLa cells and p65 staining of NF-κB activation in TNF-α-treated HeLa cells were performed as previously described[31,37].

Expression and purification of recombinant proteins. Protein expression was induced in *E. coli* BL21 (DE3) strain (Novagen) at 23 °C for 15 h with 0.4 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) after absorbance at 600 nm ($A_{600\,nm}$) reached 0.8–1.0. Affinity purification of GST–TRADD death domain or 6×His–SUMO-NleB and LFn-NleB proteins was performed using glutathione sepharose (GE Healthcare) and Ni-NTA agarose (Qiagen), respectively, following the manufactures' instructions. GST–TRADD death domain was further purified by ion exchange chromatography and concentrated in a buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl and 5% glycerol.

Native PAGE. Blue native gel electrophoresis was performed to examine TRADD oligomerization using the NativePAGE Bis-Tris gel system from Invitrogen. In brief, Flag–TRADD was transfected into intact 293T or TRADD knockdown 293T cells for 24 h. Transfected cells were washed twice with cold PBS and lysed in 1% digitonin-containing native lysis buffer (50 mM Bis-Tris, pH 7.2, 50 mM NaCl, 10% (w/v) glycerol, 0.001% Ponceau S, 1% digitonin, 2 mM Na_3VO_4 and 25 mM NaF) supplemented with the EDTA-free protease inhibitor cocktail. Cell lysis was performed on ice for 30 min, and cell debris was removed by centrifugation (16,000*g*, 20 min) at 4 °C. Lysates were separated by NativePAGE using the Novex Bis-Tris gel system (Invitrogen). Native gels were soaked in 10% SDS for 5 min before transfer to PVDF membrane (Millipore) for immunoblotting analysis.

Chemoenzymatic labelling-based GlcNAcylation detection. To detect NleB GlcNAcylation of TRADD death domain in 293T cells, immunopurified TRADD death domain was chemoenzymatically labelled using the Click-iT *O*-GlcNAc enzymatic labelling system (Invitrogen) and GlcNAc modification was detected by Click-iT protein analysis detection kits (Invitrogen) following the manufacturer's

protocol. In brief, 1×10^8 293T cells were transfected with indicated plasmids and anti-Flag immunoprecipitation was carried out as described earlier. The immunoprecipitates (~200 μg) were eluted with 1% SDS in 20 mM HEPES, pH 7.9, at 95 °C for 5 min, and then subjected to UDP-GalNAz labelling using the mGalT1 enzyme (Tyr289Leu mutant of galactosyltransferase GalT1) and a biotinylated-alkyne/azide click chemistry conjugation. The GlcNAc modification was detected by immunoblotting analysis using an anti-biotin antibody.

***In vitro* ^{3}H-UDP-GlcNAc labelling.** Flag–TRADD or other death domain proteins expressed in 293T cells were immunopurified and immobilized on the Flag M2 beads. The beads were incubated with 5 μg NleB for 2 h at 37 °C in 40 μl buffer containing 20 mM HEPES, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.4 μCi (0.2 μM) of ^{3}H-UDP-GlcNAc (Perkin Elmer). The reaction mixtures were separated on a 12% SDS–PAGE gel followed by Coommasie blue staining. Incorporation of ^{3}H-UDP-GlcNAc was visualized by ^{3}H autoradiography. For the ligand competition, 10 μM cold UDP-activated sugars were included in the GlcNAcylation reaction.

Liquid chromatography–mass spectrometry analysis. *E. coli* or 293T-cell purified death domain proteins were loaded onto a homemade capillary column (150 μm ID, 3-cm long) packed with Poros R2 media (AB-Sciex), and eluted by an Agilent 1100 binary pump system with the following solvent gradient: 0–100% B in 60 min (A = 0.1 M acetic acid in water; B = 0.1 M acetic acid, 40% acetonitrile and 40% isopropanol). The eluted proteins were sprayed into a QSTAR XL mass spectrometer (AB-Sciex) equipped with a Turbo Electrospray ion source. The instrument was acquired in mass spectrometry mode under 5 K volts spray voltage. The protein charge envelop was averaged across the corresponding protein elution peaks, and de-convoluted into non-charged forms by the BioAnalyst software provided by the manufacturer.

MALDI–TOF mass spectrometry analysis of tryptic peptides. Purified GST–TRADD death domain proteins were in-solution cleaved by PreScission, and GST was depleted by glutathione sepharose 4B. TRADD death domain co-expressed with SUMO-NleB was completely soluble, whereas TRADD death domain expressed alone aggregated to some extent after PreScission cleavage. After centrifugation at 3,200*g* for 1 min to remove the beads, the supernatant containing TRADD death domain was digested with sequencing-grade modified trypsin (Promega) at 37 °C for at least 3 h in 50 mM ammonium bicarbonate. The digested peptide samples were analysed on an Autoflex II MALDI–TOF/TOF (matrix-assisted laser desorption/ionization–time of flight) mass spectrometer (Bruker) equipped with a nitrogen pulsed laser. In brief, equal volumes of the peptide samples and 2,5-dihydroxybenzoic acid solution (Agilent) were mixed together and spotted on a Bruker MTP 384 massive stainless steel MALDI target. The matrix spots were allowed to dry at room temperature and then washed on-target with 0.1% trifluoroacetic acid to remove salts and other water soluble contaminations. Peptide mass fingerprinting spectra were acquired in the positive reflector mode with pulsed ion extraction.

To identify GlcNAcylated arginine and arginine-containing peptides, the trypsin-digested TRADD death domain (modified by NleB in bacteria) was separated on an Agilent Ellipse C18 reversed phase column (4.6 × 150 mm) using the Agilent 1260 Infinity HPLC system. The HPLC gradient was as follows: 0–20% B in 10 min, 20–100% B in 20 min (solvent A = 10 mM ammonium acetate, solvent B = 10 mM ammonium acetate in 80% acetonitrile). The ultraviolet detector wavelength was 215 nm and the fractionation size was 300 μl. Peptides from each fraction were detected by MALDI–TOF on Autoflex II mass spectrometer. The HPLC fraction containing peptide with *m*/*z* matching 233-VGR-GlcNAc_SLQR-239 with GlcNAc modification was concentrated to ~50 μl and then digested with proteinase K (New England Biolabs) overnight at 37 °C. To obtain the single arginine with GlcNAc modification, the proteinase-K-treated peptides were digested further with carboxypeptidase A (Sigma) overnight at 37 °C. The digests were loaded into a nanoES emmiter and sprayed directly into LTQ Orbitrap Velos mass spectrometer equipped with a nanoESI ion source. The dipeptide GR_{GlcNAc} and the single arginine with a GlcNAc modification were manually selected for high-energy collision dissociation tandem mass analysis.

ETD–MS analysis of GlcNAc-modified peptides. To determine the exact GlcNAcylation site on TRADD proteins after NleB modification, purified TRADD death domain protein co-expressed with SUMO-NleB in *E. coli* was trypsin digested and the resulting tryptic peptides were analysed on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher) equipped with an ETD ion source and a nanoESI ion source. The peptide solution was loaded into a nanoES emitter and sprayed directly into the instrument. The peptides with *m*/*z* matched to calculated GlcNAc-modified TRADD peptides were manually selected for ETD fragmentation with a 2-Da mass selection window.

Stable isotope labelling. Isotope-labelled $Ac_4GlcNAc$ (2-(acetyl-d_3-amino)-2-deoxy-1,3,4,6-tetra-*O*-acetyl-D-glucopyranose) ($Ac_4GlcNAc$-d_3) was synthesized as previously described[38] and the spectroscopic data were consistent with that in the literature[38]. 293T cells (5×10^8) were pretreated with 100 μM isotope-labelled $Ac_4GlcNAc$-d_3 for 12 h and transfected with Flag–TRADD death domain and NleB for another 12 h. Flag–TRADD death domain was immunopurified as that for mass spectrometry measurement of the total mass and prepared into the 50 mM NH_4HCO_3, pH 8.0, buffer. Flag peptide was depleted with Flag M2 beads and the supernatant was subjected to trypsin digestion at 37 °C for 1 h. The resulting tryptic peptides were analysed by ETD as described earlier.

Mice infection and *C. rodentium* colonization assays. All animal experiments were conducted following the Ministry of Health national guidelines for housing and care of laboratory animals and performed in accordance with institutional regulations after review and approval by the Institutional Animal Care and Use Committee at National Institute of Biological Sciences. Male C57BL/6 mice, 5–6 weeks old, maintained in the specific pathogen-free environment were used. All animals were housed in individually high efficiency particulate air (HEPA)-filtered cages with sterile bedding. Mice were randomized into each experimental group with no blinding. Independent experiments were performed using 7–8 mice per group.

Deletion of the gene encoding NleBc in *C. rodentium* strain DBS100 (ATCC51459; ATCC) was generated by standard homologous recombination using the suicide vector pCVD442, as described previously[32]. The DNA of pNleBc was amplified by PCR from genomic DNA of DBS100 strain to include a putative upstream promoter sequence (~300 bp), using the forward primer 5′-TCAGGGCCGGCCGACTGGAACATATGCGGG-3′ and reverse primer 5′-TGACGGCGCGCCTTACCATGAACTGTTGGTATACATACTG-3′. For oral inoculation and harvesting, *C. rodentium* wild-type strain and represented derivatives were prepared by overnight shaking of bacterial culture at 37 °C in LB broth. Mice were orally inoculated using a gavage needle with 200 μl suspension of bacteria in PBS (~2×10^9 CFU). The number of viable bacteria used as the inoculum was determined by retrospective plating onto LB agar containing the appropriate antibiotics. Stool samples were recovered aseptically at various time points after inoculation, and the number of viable bacteria per gram of stool was determined after homogenization in PBS and plating onto LB agar containing the appropriate antibiotics. Eight days after inoculation, colons were removed aseptically, weighed and homogenized in PBS. Homogenates were serially diluted and plated to determine CFU counts. Colonization data were analysed using a Student's *t*-test in the commercial software GraphPad Prism. $P < 0.05$ was considered significant.

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