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Slam is an outer membrane protein that is required for the surface display of lipidated virulence factors in *Neisseria*

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Lipoproteins decorate the surface of many Gram-negative bacterial pathogens, playing essential roles in immune evasion and nutrient acquisition. In *Neisseria* spp., the causative agents of gonorrhoea and meningococcal meningitis, surface lipoproteins (SLPs) are required for virulence and have been extensively studied as prime candidates for vaccine development. However, the machinery and mechanism that allow for the surface display of SLPs are not known. Here, we describe a transposon (Tn5)-based search for the proteins required to deliver SLPs to the surface of *Neisseria meningitidis*, revealing a family of proteins that we have named the surface lipoprotein assembly modulator (Slam). *N. meningitidis* contains two Slam proteins, each exhibiting distinct substrate preferences. The Slam proteins are sufficient to reconstitute SLP transport in laboratory strains of *Escherichia coli*, which are otherwise unable to efficiently display these lipoproteins on their cell surface. Immunoprecipitation and domain probing experiments suggest that the SLP, TbpB, interacts with Slam during the transit process; furthermore, the membrane domain of Slam is sufficient for selectivity and proper surface display of SLPs. Rather than being a *Neisseria*-specific factor, our bioinformatic analysis shows that Slam can be found throughout proteobacterial genomes, indicating a conserved but until now unrecognized virulence mechanism.

Gram-negative bacteria are encapsulated by a double membrane that is separated by a thin peptidoglycan layer and periplasmic space[1]. The outer membrane confers protection against toxic compounds and host defence molecules. However, this benefit creates unique challenges for the bacteria with respect to the delivery of effector proteins to the cell surface. The molecular processes required to insert proteins into the outer membrane are well described[2], but many Gram-negative organisms also display peripherally associated nutrient scavenging and immune defence proteins on their surface via a covalently associated lipid anchor inserted into the outer membrane, collectively termed bacterial surface lipoproteins (SLPs)[3–5].

The human pathogen *Neisseria meningitidis* uses SLPs to acquire nutrients and evade the host immune defences to achieve a sustained infection[6]. For example, the neisserial factor H-binding protein (fHbp) is an SLP that binds the human serum protein factor H to avoid the complement-mediated bactericidal activity of the blood[7]. SLPs involved in iron acquisition have also been studied extensively, with the uptake process being initiated by a bipartite receptor composed of a surface-exposed lipoprotein (transferrin or lactoferrin binding protein B, TbpB or LbpB, or haemoglobin–haptoglobin using protein A, HpuA) and an integral outer membrane protein[8,9]. In accordance with their roles in these processes and the fact that their functions depend on direct, specific interactions with host proteins on the bacterial cell surface[10], these SLPs elicit bactericidal antibodies[11–13] and have been successfully used as vaccine antigens to prevent meningococcal disease[14–16].

To properly traffic to the cell surface, the neisserial SLPs require signalling motifs to translocate across the bacterial cell envelope (Supplementary Fig. 1a–d). SLPs contain the canonical signal peptide and consensus lipobox motif that are necessary for translocation through the Sec secretion machinery and lipidation of their mature amino-terminal cysteine[17]. This was confirmed for neisserial SLPs by the detection of unprocessed full-length TbpB build-up in *N. meningitidis* and *Escherichia coli* with globomycin treatment[18] (Supplementary Fig. 1b). Lipoproteins can traverse the periplasm through the Lol system. The *E. coli* LolA R43L mutation inhibits the transfer of lipoproteins to LolB and effectively traps LolA–lipoprotein complexes[19]. Expression of TbpB together with LolA R43L stalls lipoprotein translocation and the complex of TbpB bound to LolA R43L can be detected in pulldowns (Supplementary Fig. 1c), confirming the use of the Lol system by neisserial SLPs. Although TbpB expressed in laboratory strains of *E. coli* is able to reach the outer membrane (Supplementary Fig. 1d), it is not displayed on the surface (Supplementary Fig. 1e). This suggests that neisserial SLPs undergo an additional translocation step that 'flips' them across the outer membrane to the surface of the cell. Until now, it remained unknown how this latter step occurrred.

Results

Identification of surface lipoprotein assembly modulator (Slam) in *N. meningitidis*. To identify the component(s) required for SLP translocation to the *N. meningitidis* cell surface, we constructed a genome-wide random Tn5-based transposon mutant library within the *N. meningitidis* strain B16B6. Using a whole cell solid-phase binding assay, 4,000 mutants from this library were analysed for the ability to display TbpB on the cell surface (Supplementary Fig. 2). One of the defective mutants had incorporated a Tn5 into a genetic region that aligns with the ORF *nmb0313* of the published *N. meningitidis* MC58 genome sequence[20]. This *nmb0313*::Tn5 mutant (*nmb0313/tn5*) has effectively lost TbpB from its cell surface when compared with the wild-type

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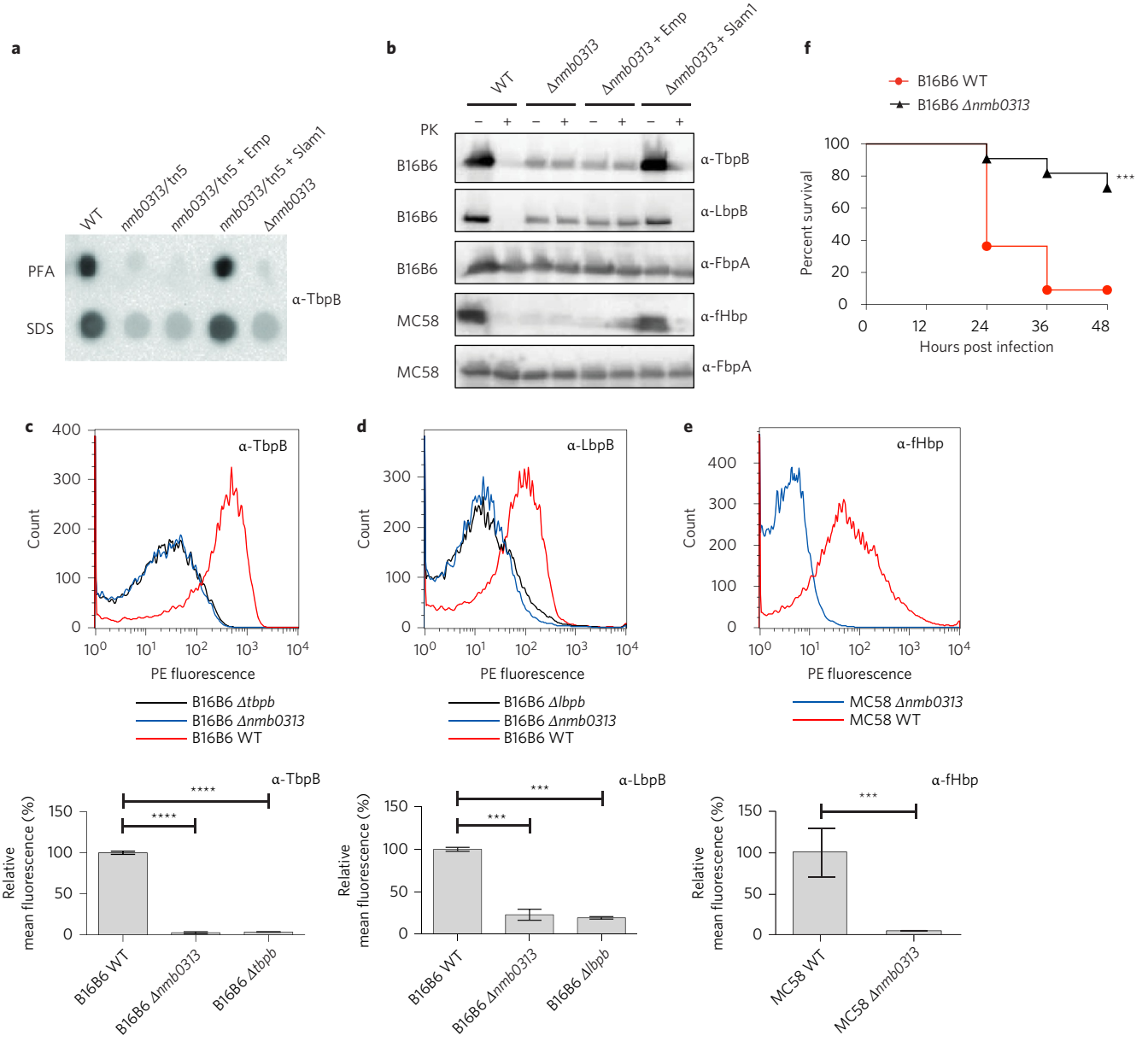

Figure 1 | NMB0313 is required for surface localization of TbpB, fHbp and LbpB, and is essential for virulence in *N. meningitidis*. **a**, Solid-phase binding analysis of TbpB in cells treated with paraformaldehyde (PFA) or lysed with SDS. B16B6 WT, NMB0313 transposon mutants (*nmb0313/tn5*), complemented strains with empty vector (Emp) or Slam1 and NMB0313 complete knockout cells (*Δnmb0313*) were examined. **b**, Whole-cell proteinase K (PK) surface shaving assays of B16B6 or MC58 WT, *Δnmb0313* and complemented strains. Samples were analysed by immunoblotting for TbpB, LbpB and fHbp or periplasmic FbpA. Representative blots of at least three independent experiments are shown. **c–e**, Flow cytometry profiles and quantification of WT (red) or *Δnmb0313* (blue) cells for TbpB, LbpB or fHbp surface display. For TbpB and LbpB surface profiles, *Δtbpb* (black) and *Δlbpb* (black) strains are shown as controls. Relative mean fluorescence was calculated by scaling the averaged mean fluorescence intensity of the WT to 100 and applying the same scaling factor to the other samples. Error bars represent the standard error of the mean (s.e.m.) from three biological replicates. Statistical significance was determined by ANOVA. $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$. **f**, Survival curve of mice following intraperitoneal injection with 1×10^6 c.f.u. of B16B6 WT or *Δnmb0313*. Mice ($n = 11$, combined from two independent experiments) were monitored for survival every 12 h starting 48 h pre-infection to 48 h post-infection and additionally monitored at 3 h post-infection. Statistical significance was based on the Mantel–Cox log rank test: $^{***}P \leq 0.001$.

(WT) strain (Fig. 1a). Complementation with a genomic copy of *nmb0313* under the control of a *lac* promoter (*nmb0313/tn5* + Slam1), together with a complete deletion of the entire gene (*Δnmb0313*) (Fig. 1a), confirmed the importance of the gene in TbpB surface display.

We further assessed the effects of NMB0313 on other SLPs. Exposure of WT *N. meningitidis* or the *nmb0313* complemented strains to proteinase K led to degradation of the surface-exposed TbpB, LbpB and fHbp, whereas these proteins remained intact within the *Δnmb0313* strain due to their retention within the cell (Fig. 1b). To quantify this effect at the single-cell level, antibodies to TbpB, LbpB and fHbp were used to probe WT and *Δnmb0313* strains of *N. meningitidis* by flow cytometry (Fig. 1c–e). The *Δnmb0313* strain effectively presented as knockouts of the SLPs, similar to *Δtbpb* or *Δlbpb* (Fig. 1c,d), because they could not be detected on the cell surface. Together, these data show that NMB0313 is required for *N. meningitidis* to efficiently display TbpB, fHbp and LbpB on the cell surface. We named the protein

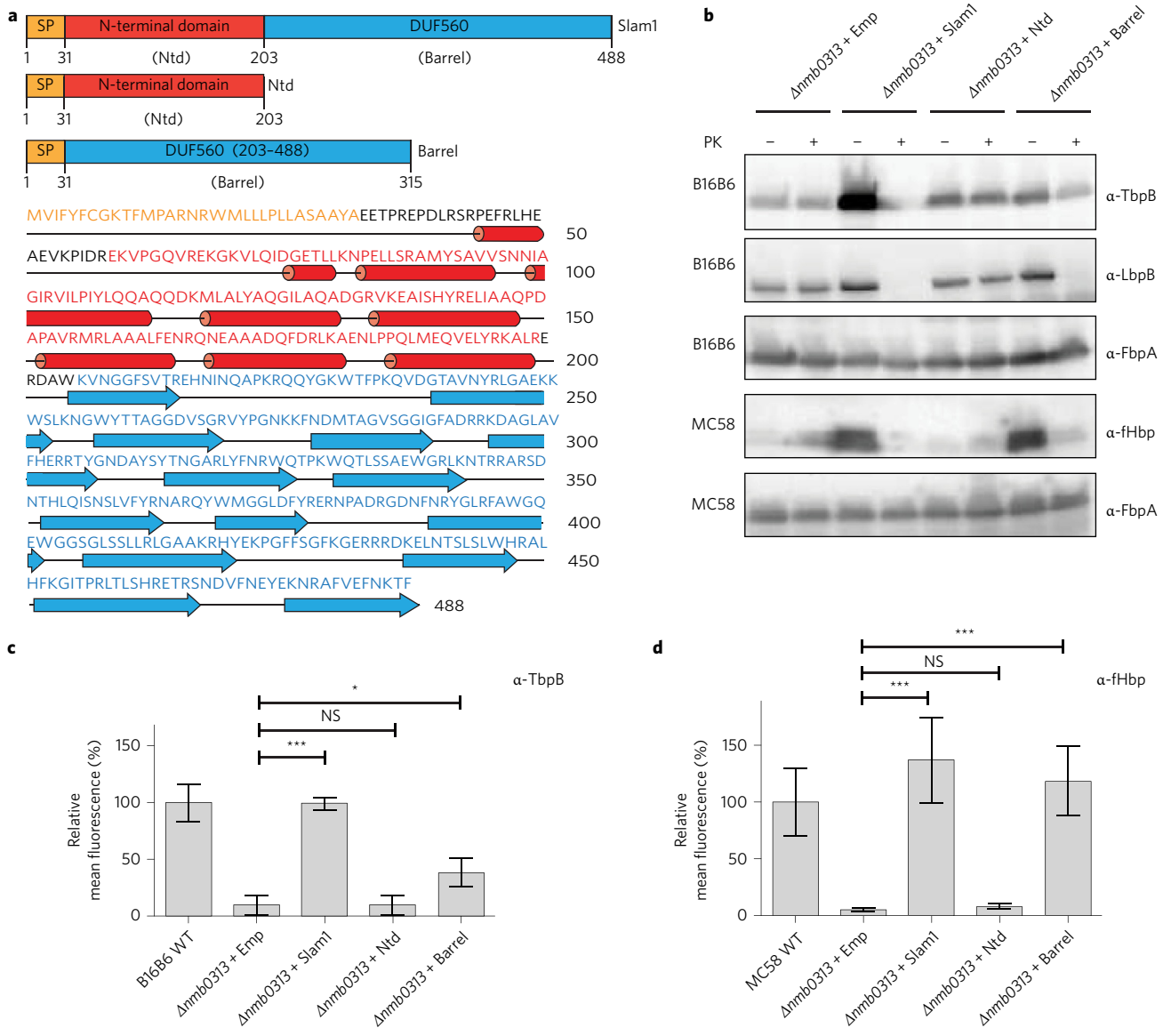

Figure 2 | The barrel domain of Slam1 is sufficient to translocate SLPs in *N. meningitidis*. **a**, Structural features of Slam1. Slam1 contains a signal peptide (SP), N-terminal domain (Ntd) and C-terminal DUF560 domain (β-barrel). Predicted helices are represented by cylinders in the Ntd and strands are represented by arrows in the β-barrel domain. **b**, Whole-cell PK surface shaving assays of B16B6 or MC58 strains complemented with empty vector (Emp), full length (Slam1) or domains (Ntd or Barrel) of Slam1. Samples were analysed for TbpB, LbpB and fHbp or periplasmic localized FbpA. One representative blot of at least three independent experiments is shown. **c**,**d**, Quantification of TbpB or fHbp surface display in WT and complemented strains, as determined by flow cytometry. Relative mean fluorescence was calculated by scaling the averaged mean fluorescence intensity of the WT to 100 and applying the same scaling factor to the other samples. Error bars represent the s.e.m. from three biological replicates. Statistical significance was determined by ANOVA. $^{*}P < 0.05$, $^{***}P < 0.001$; NS, not significant.

encoded by *nmb0313* 'Slam', for 'surface lipoprotein assembly modulator'.

Slam is essential for neisserial virulence. As Slam had a profound effect on the surface display of these key neisserial virulence factors, we examined the *Δnmb0313* strain for its pathogenic properties and infectivity. Previously, a genome-wide approach used by Exley *et al.* revealed that Slam was required for meningococcal colonization of nasopharyngeal organ cultures[21], and a distant homologue of Slam (NilB) in *Xenorhabdus nematophila* was shown to be required for the colonization of nematodes[22]. To examine the effects of Slam within a mammalian infection, we introduced isogenic WT or mutant *N. meningitidis* strains into a mouse sepsis model. WT meningococci caused severe sepsis, but mice infected with B16B6 *Δnmb0313* progressed well through to the endpoint of the experiment (Fig. 1f). To confirm that bacterial fitness did not cause this reduction in infectivity for B16B6 *Δnmb0313*, *in vitro* growth rates were measured and showed no differences with the WT (Supplementary Fig. 3). Taken together, these results confirm the essential nature of Slam in the establishment of bacterial colonization and promotion of meningococcal disease.

The β-barrel domain of Slam is necessary for SLP surface display. To examine the regions within Slam that were required for SLP surface display, we initially used PSIPRED[23] to define secondary structure elements and domain architecture. Further analysis using SignalP[24], XtalPred[25], InterProScan[26] and Boctopus[27] predicted that Slam encodes an outer membrane protein that consists of a

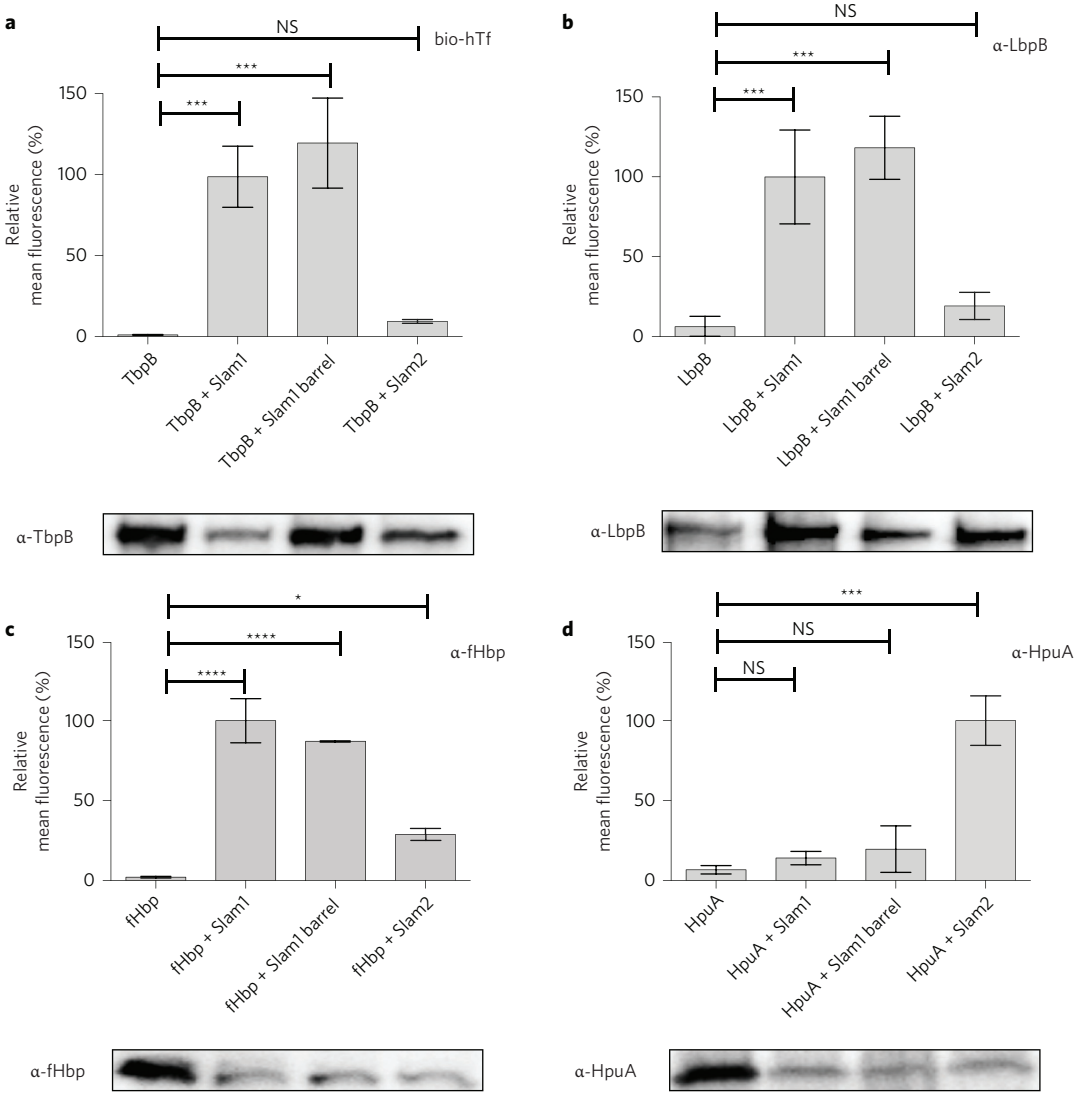

Figure 3 | Slam1 and Slam2 demonstrate substrate-specific translocation of SLPs in an *E. coli* reconstituted translocation assay. a–d, Quantification of surface TbpB, LbpB, fHbp or HpuA in *E. coli*, as analysed by flow cytometry. *E. coli* was transformed as labelled and grown in auto-induction media. Cells were labelled with biotinylated human transferrin (bio-hTf) to detect TbpB surface expression, or antibodies to detect LbpB, fHbp or HpuA. Relative mean fluorescence was calculated by scaling the averaged mean fluorescence intensity of cells reconstituted with full length Slam1 (**a–c**) or Slam2 (**d**) to 100 and applying the same scaling factor to the other samples. Error bars represent the s.e.m. from three biological replicates. Statistical significance was determined by ANOVA. $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$; NS, not significant. Western blot analyses of TbpB, LbpB, fHbp or HpuA on lysates prepared from cells of equal density that were used for labelling are shown under the main panels. Blots are representative of three independent experiments.

31-residue signal peptide and a 457-amino-acid (52.6 kDa) mature protein with two domains: a soluble N-terminal domain (Ntd) containing two tetratricopeptide repeats (TPR, residues 118–151, 152–185) and a C-terminal β-barrel domain (residues 204–488) consisting of 14 outer membrane-spanning strands that is annotated as a DUF560 (Fig. 2a). To examine the role of each Slam domain, the β-barrel and N-terminal domains were individually expressed in *Δnmb0313* strains of *N. meningitidis* B16B6 and MC58, and the SLP surface display was examined by proteinase K digestion and flow cytometry (Fig. 2b–d). The β-barrel domain of Slam is sufficient to rescue TbpB, LbpB and fHbp surface translocation when expressed in *N. meningitidis*, whereas the N-terminal TPR-containing domain was unable to complement the SLP translocation deficiency of the knockout strain. By flow cytometry, we confirmed that the β-barrel domain is able to confer approximately half of TbpB translocation activity (Fig. 2c) and equivalent levels of fHbp surface display (Fig. 2d) compared to WT strains. These results suggest that the β-barrel domain is critical for SLP translocation while the TPR-containing N-terminal domain may have an ancillary role in SLP translocation by stabilizing the SLP substrate and/or acting as a plug for the barrel domain.

Discovery of Slam2 in *N. meningitidis*. Given the importance of Slam in the translocation of SLPs, we performed an extensive bioinformatics search for β-barrel membrane proteins containing a DUF560 domain with an N-terminal TPR-containing domain. Homologues of Slam (*nmb0313*) are found in all *N. meningitidis* strains sequenced to date, but, more broadly, Slam proteins were found throughout the proteobacteria phylum and notably within many Gram-negative pathogens: *Moraxella catarrhalis*, *Acinetobacter baumannii*, *Haemophilus influenzae* and *Vibrio cholerae* (Supplementary Fig. 4). Unexpectedly, we discovered an additional Slam homologue in *Neisseria*. The second Slam paralogue (*nmb1971*) also contains a DUF560 and a TPR domain, and is located directly upstream of another known SLP, HpuA

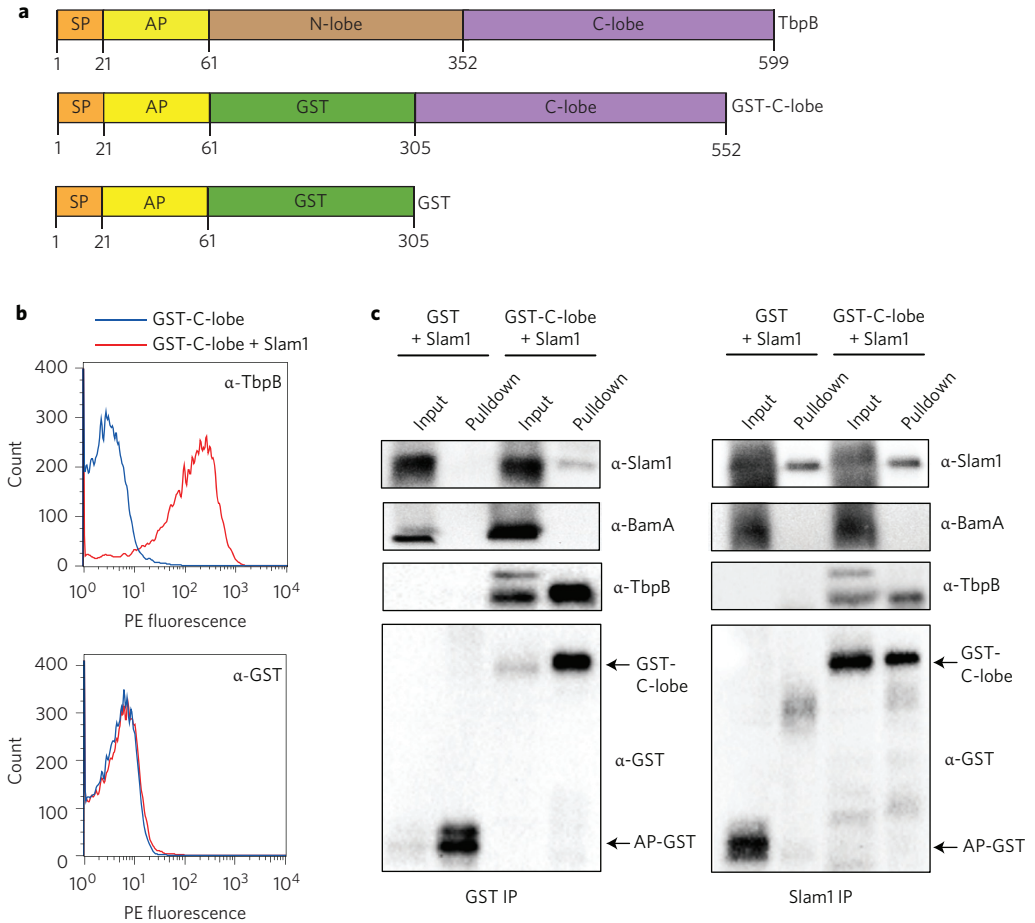

Figure 4 | Slam1 interacts with TbpB in *E. coli*. **a**, Schematic representation of TbpB constructs used in the translocation assays. TbpB has a signal peptide (SP), anchoring peptide (AP), N- and C-lobes. GST-TbpB chimeric fusion constructs were designed as illustrated. **b**, Flow cytometry profiles of *E. coli* cells expressing GST-C-lobe with or without Slam1 using α-TbpB or α-GST antibodies. Cell counts are shown against PE fluorescence for each antibody. One representative histogram of three independent experiments is shown. **c**, Interaction assay of GST-C-lobe with Slam1. *E. coli* cells transformed with GST-C-lobe or GST and Slam1 were immunoprecipitated with glutathione resin (left) or α-Slam1 antibodies conjugated to sepharose beads (right). Whole-cell lysate (input) and eluted fractions (pulldown) were subjected to western blot analysis probed with α-Slam1, α-BamA, α-TbpB and α-GST antibodies. Blots are representative of at least three independent experiments.

(Supplementary Fig. 5). Hence, we refer to NMB1971 as Slam2 and NMB0313 as Slam1.

Slams reconstitute the translocation of neisserial SLPs to the surface in *E. coli*. In contrast to *N. meningitidis*, our bioinformatic investigation did not reveal any Slam homologue in genome sequences of prototypical *E. coli*. Thus, to decipher the specificity and mechanism of Slam translocation, we examined the cell surface display of neisserial SLPs in laboratory strains of *E. coli* (BL21–C43(DE3)). Surprisingly, the simple addition of Slam1 to *E. coli* C43 cells co-expressing TbpB, LbpB or fHbp facilitated their display on the bacterial surface, demonstrating a 'gain of function' phenotype in *E. coli* cells (Fig. 3a–c). Importantly, the ability of TbpB to recognize human transferrin (used for labelling in flow cytometry) suggests that these translocated SLPs are fully functional (Fig. 3a). To ensure that this translocation activity was not due to the aberrant expression of SLPs in the presence of Slams, we performed western blots and found no correlation between surface display and expression (Fig. 3a–c), nor do we see differences in the localization of TbpB (Supplementary Figs 1d and 6a). Finally, to confirm that Slams are specific to neisserial SLPs, we tested for the surface display of *E. coli* PgaB[28], an inner-leaflet outer-membrane lipoprotein, when co-expressed with Slams (Supplementary Fig. 6b). No surface PgaB was observed, illustrating that Slams have specificity for SLPs and do not translocate periplasmic-facing *E. coli* lipoproteins. Collectively, these findings suggest a key role played by Slam1 in transporting SLPs across the outer membrane.

In contrast to Slam1, Slam2 was ineffective at translocating TbpB, LbpB and fHbp to the *E.coli* surface (Fig. 3a–c). In considering that Slams may have cargo specificity, we tested the surface translocation potential of another neisserial SLP, HpuA, which is encoded directly adjacent to Slam2 within the neisseral genome (Supplementary Fig. 5). Consistent with this model, HpuA was surface-exposed in *E. coli* co-expressing Slam2 but not in cells with Slam1 (Fig. 3d). Furthermore, this cargo specificity resides primarily within the β-barrel domains as the Slam1 β-barrel alone can translocate TbpB, LbpB and fHbp but not HpuA to the surface of *E. coli* (Fig. 3). Taken together, this establishes that Slams are necessary and sufficient for these *E. coli* strains to translocate specific neisserial SLPs across the outer membrane to the cell surface.

TbpB interacts with Slam1 during its transit to the cell surface. To establish a direct role of Slam in SLP translocation, we investigated potential interactions between Slam and an SLP. Evident from the lack of remaining SLPs after proteinase K shaving in *Neisseria* (Fig. 1b), we suspected that the SLPs spend minimal time transiting through the periplasm and interacting

with their translocon. In fact, co-immunoprecipitations using α-Slam antibodies in *N. meningitidis* or *E. coli* were not fruitful in revealing a direct interaction with the SLPs. We hypothesized that a rapidly folding soluble domain like gluthione S-transferase (GST) fused to an SLP may be unable to pass easily through the translocon and thereby trap the SLP in complex with its transporter in a similar manner to the stalling of the outer membrane translocon FhaC[29]. As such, we constructed a GST fusion of TbpB where the GST domain replaced the N-lobe of TbpB (Fig. 4a) and transformed the plasmid into *E. coli* strain C43 with or without Slam1. Although flow cytometry experiments performed with α-TbpB antibodies detected GST-C-lobe on the cell surface (Fig. 4b), experiments performed with α-GST antibodies revealed that the GST-tag could not be detected on the surface of the cell (Fig. 4b), although the GST could be detected in cells with permeabilized outer membranes (Supplementary Fig. 7). Furthermore, to confirm the stalled transport of GST-C-lobe in *Neisseria*, we complemented the B16B6 *Δtbpb* strain with full-length TbpB, GST-C-lobe or GST fused to the TbpB signal and anchor peptide (Fig. 4a). Confirming the *E. coli* experiments, the C-lobe was surface-exposed while the GST was not (Supplementary Fig. 8a–c). Hence, we were able to obtain a GST-TbpB chimera that is partially stalled at the outer membrane translocation step.

To determine whether Slam1 interacts with this chimera, immunoprecipitations were performed with α-Slam1 antibodies in *E. coli* (Fig. 4c) and *N. meningitidis* (Supplementary Fig. 8d). The interaction between the C-lobe of TbpB and Slam1 was observed and is specific, as lysates from cells expressing lipidated-GST and Slam1 do not immunoprecipitate together. Collectively, this illustrates that TbpB interacts with Slam1 at some point during its transit across the outer membrane.

Previous work has shown that Omp85-family members such as BamA or TamA may be involved in transport of cell-surface proteins across the outer membrane[30–33]. To investigate if BamA plays a direct role in neisserial SLP transport as part of the translocation complex, we tested whether BamA co-immunoprecipitated along with the GST-chimera and Slam1 (Fig. 4c). The absence of detectable signals in pulldown fractions suggest that BamA does not interact strongly with Slam1 and GST-C-lobe, although we have not yet ruled out more transient interactions. To examine the Tam complex, *E. coli* strains lacking either TamA or TamB (ytfM and ytfN) were tested for surface display of TbpB using flow cytometry, which demonstrated that neither Tam components are required to translocate TbpB to the surface in a Slam-dependent manner (Supplementary Fig. 9). Taken together, our investigations have so far only validated Slam1 as an outer membrane protein participant in the translocation of SLPs.

Discussion

In recent years, surface lipoproteins with different topologies and biological functions have been identified in Gram-negative bacteria and these SLPs (for example, pullulanase and Rcsf) use distinct translocation machineries for moving across the outer membrane[32,34,35]. Here, we reveal a previously uncharacterized integral outer membrane protein, Slam, that is necessary to translocate neisserial-derived virulence factors to the bacterial cell surface. Indeed, Slams can reconstitute SLP translocation in laboratory strains of *E. coli* that are otherwise unable to translocate neisserial SLPs to their surface. Our analysis reveals Slam homologues in a number of Gram-negative bacteria presumably functioning to aid in the surface display of distinct SLPs. Using GST-TbpB fusions, we were able to show that Slam1 interacts with TbpB as it crosses the outer membrane; however, whether Slams act as conduits for transport, or SLPs require other outer membrane complexes to move across the outer membrane, is still unknown. Nonetheless, Slams are a newly identified family of outer membrane proteins dedicated to SLP translocation and hence represent a novel unexplored target for antimicrobial therapeutics directed at the SLP family of virulence factors. Moreover, the ability of Slam-expressing *E. coli* to translocate surface lipoproteins en masse to the bacterial cell surface represents a powerful new strategy for protein engineering, including protein purification strategies and, importantly, the presentation of antigens for vaccine production.

Methods

Bacterial strains and growth conditions. Strains used in this study are summarized in Supplementary Table 1. *N. meningitidis* strain B16B6 was grown on brain heart infusion (BHI) plates and MC58 was grown on Difco GC base plates, supplemented with 1% Isovitalex Enrichment (BD), at 37 °C with 5% CO_2. Where appropriate, antibiotics were used (80 μg ml^{-1} kanamycin for both *N. meningitidis* strains, 30 μg ml^{-1} erythromycin for B16B6 and 5 μg ml^{-1} erythromycin for MC58). B16B6 and MC58 complemented strains were grown on 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) plates to induce expression of the complemented gene. *E. coli* were grown in Luria-Bertani (LB) medium containing antibiotics when necessary (50 μg ml^{-1} kanamycin, 100 μg ml^{-1} ampicillin and 10 μg ml^{-1} gentamycin). Cloning procedures were carried out using *E. coli* MM294 competent cells. Protein expression was performed using C43(DE3) cells or Keio collection[36] strains BW25113 WT, JW4179-1 (*ΔytfM*) and JW4180-1 (*ΔytfN*). *E. coli* strains E11 WT and *ΔpgaB*[37] were used for the validation of α-PgaB antibodies.

Transposon library generation and screening by solid-phase binding (dot blot). For construction of the *N. meningitidis* B16B6 transposon library, the EZ::TN Transposon kit (Epicentre) was used following the manufacturer's protocol. Briefly, 400 ng of sonicated B16B6 genomic DNA (gDNA) was mixed with EZTn5 transposon and transposase in a 100 μl reaction and incubated at 37 °C for 2 h. Following inactivation with EZTn5 Stop solution and incubation at 70 °C, the reaction was concentrated by ethanol precipitation and resuspended in 14 μl water. Gaps created by the transposon reaction were filled by T4 DNA polymerase (NEB) and T4 ligase (NEB) in a similar method to that of Pelicic and co-authors[38]. The concentrated gDNA was incubated with 1 μl of T4 DNA polymerase (NEB) and 1 μl of 10 mM dNTP in a 20 μl reaction at 12 °C for 30 min. The DNA polymerase was inactivated at 75 °C for 20 min and the reaction was further incubated with 1 μl of T4 ligase (NEB) at 16 °C for 16 h.

The entire transposon reaction was used to transform *N. meningitidis* strain B16B6 by spot transformation[39]. *N. meningitidis* B16B6 was streaked on BHI plates spotted with the transposon reaction and the plate was incubated at 37 °C with 5% CO_2 until colonies appeared. The meningococci, grown on the spots, were collected and plated on kanamycin plates to select for mutants that had incorporated the transposons into their chromosomes. Transposon mutants were collected into microtiter plates and grown in liquid BHI for 6 h before storage in BHI containing 20% glycerol.

The library was screened for mutants lacking surface TbpB by solid-phase binding analysis. Transposon mutants were grown in BHI containing 0.1 mM deferoxamine for 3 h, collected by centrifugation, fixed with 2% formaldehyde in PBS for 20 min, and further washed and resuspended in PBS. Whole fixed cells were spotted on nitrocellulose membrane for α-TbpB western blot analysis.

To map the transposon insertion sites, gDNA was extracted from mutants by boiling a swab of colonies in 5% chelex-100 (Biorad) for 15 min and collecting the supernatant. The gDNA was used for random amplification of transposon ends (RATE) PCR[40] or splinkerette PCR[41] to isolate the genomic fragments flanking the transposon insertion site. For RATE PCR, gDNA was mixed with a single primer (inv1 or inv2[40]) for a three-step PCR reaction consisting of stringent annealing temperatures in the first round, low annealing temperatures in the second and stringent annealing temperatures in the third. The resulting product was sequenced with Kan F or Kan R primers (Epicentre). For splinkerette PCR, gDNA was digested by restriction enzymes (BstY1, BglII, or HindIII) separately, producing sticky ends that could be ligated to the spinkerette oligonucleotide. The resulting product is used for two nested PCRs to amplify the genomic sequence between the TN5 insertion and the splinkerette. The product was used for sequencing with another nested primer.

Gene deletion and complementation of Slam. Restriction free (RF) cloning[42] was used for the following plasmid constructs (outlined in Supplementary Table 1). To completely replace the *nmb0313* gene with a kanamycin cassette, pUC19 Slam1-Kan was cloned to contain the upstream and downstream 500 bp flanking regions of *nmb0313* with *kan2* from the EZ::TN transposon kit (Epicentre) introduced between the fragments. The plasmid was used to transform *N. meningitidis* strains B16B6 and MC58 by spot transformation. Knockouts were selected on BHI or gonococcal (GC) media plates containing kanamycin and verified by polymerase chain reaction (PCR) using primers that flank *nmb0313*. To generate growth curves, B16B6 WT and *Δnmb0313* were grown on BHI plates overnight, collected into liquid BHI, diluted to an optical density (OD_{550}) of 0.1 and grown at 37 °C with OD_{550} measurements performed every 2 h.

Complementation vector pGCC4 Slam1 was constructed by cloning the B16B6 *nmb0313* gene into the PacI/FseI site of pGCC4[43] by RF cloning. Using inverse PCR

cloning[44], an 8xHis-tag was inserted after the signal peptide by amplifying the whole vector with phosphorylated primers containing the His-tag sequence and re-circularizing with T4 ligase. Complementation vector pGCC4 Slam1Ntd was cloned by amplifying the signal peptide, 8xHis and Ntd domain (amino acids 31–203) from pGCC4 Slam1 and inserting the PCR product into an empty pGCC4 vector. The complementation vector pGCC4 Slam1barrel was constructed by amplifying the barrel domain (amino acids 204–488) of Slam1 and inserting the amplicon after the His-tag in a manner that replaced the Ntd domain in pGCC4 Slam1Ntd.

These complementation plasmids and the empty pGCC4 vector were used for transforming the Slam1 transposon and knockout mutants. Transformants were selected on erythromycin plates and insertion was verified by PCR. Expression of Slam1 was induced by growth on 1 mM IPTG plates and confirmed by α-Slam1 (1:5,000) western blots.

Solid-phase binding analysis (dot blot). *N. meningitidis* B16B6 WT, mutant and complemented strains were grown overnight on BHI plates containing appropriate antibiotics or IPTG. Cells were collected by centrifugation and either fixed with 2% formaldehyde in PBS for 20 min or lysed in SDS loading buffer. Both fixed and lysed samples were spotted on nitrocellulose membrane for detection with α-TbpB antibodies.

Solid-phase binding analyses of *E. coli* C43(DE3) cells transformed with pETDuet TbpB (as described in the '*E. coli* expression plasmids' section) were performed as described above, except that cells were grown overnight in 1 ml of auto-induction medium[45] before being collected.

Proteinase K assay. *N. meningitidis* WT, mutant and complemented cells were grown overnight on BHI plates containing the appropriate antibiotics or IPTG. Whole cells were collected into PK buffer (50 mM Tris pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$) and washed once before incubation with or without proteinase K (500 μg ml^{-1}, Bioshop) for 30 min at room temperature. Cells were washed twice with PK buffer containing 2 mM phenylmethylsulfonyl fluoride (PMSF) before lysis for SDS–polyacrylamide gel electrophoresis (PAGE) and western blot analysis with α-TbpB (1:10,000), α-LbpB (1:5,000), α-fHbp (1:5,000) and α-FbpA (1:5,000).

Flow cytometry assay. *N. meningitidis* B16B6 and MC58 WT, deletions or complemented strains were grown overnight on BHI or GC plates, harvested and washed in PBS, then fixed in 2% formaldehyde for 20 min. Labelling was performed with α-TbpB (1:200), α-LbpB (1:200), α-fHbp (1:500) or α-HpuA (1:200), followed by staining with R-phycoerythrin (PE) conjugated α-rabbit IgG (Rockland) or R-PE conjugated α-mouse IgG (Thermo Scientific) at 25 μg ml^{-1} for 1 h at 4 °C. Flow cytometry was performed with a Becton Dickinson FACSCalibur and the results were analysed using FLOWJO software. Mean fluorescence intensity (MFI) was used to compare the surface exposure of a given SLP between WT and mutant strains. For each SLP, WT MFI averages from at least three biological replicates were obtained and a scaling factor was calculated by normalizing these averages to 100. The scaling factor was then applied to each sample to obtain a relative MFI. These relative MFIs were plotted and analysed using GraphPad Prism 5 software. Statistical significance was calculated using the one-way analysis of variance (ANOVA) test.

For flow cytometry experiments performed in *E. coli*, pairs of SLP and Slam plasmids (as described in the '*E. coli* expression plasmids' section) were transformed into C43(DE3) cells and grown in 1 ml of auto-induction medium[45] for 18 h. Cells were collected, washed twice in PBS containing 1 mM $MgCl_2$ and labelled with the antibodies described above as well as with α-PgaB (1:200), α-GST (1:100, Cell Signalling Technologies) or biotinylated human transferrin (0.05 mg ml^{-1}, Sigma) and R-PE conjugated streptavidin (0.5 mg ml^{-1}, Cedarlane). Following staining, cells were fixed in 2% formaldehyde for 20 min and further washed with PBS. Flow cytometry analysis was performed as described above. The scaling factor was calculated by scaling MFI averages obtained for cells co-expressing SLP and Slam genes to 100.

Mouse infection study. Sepsis modelling was performed as described by Gorringe *et al.*[46] and Johswich and co-authors[47].

Eight-week-old male C57BL/6 mice (Charles River Laboratories) were randomly assigned to two infection groups and the experimenter was not blinded. Mice were inoculated via intraperitoneal injection with *N. meningitidis* strains B16B6 WT or B16B6 *Δnmb0313*. A total of 11 mice per group were chosen based on previous studies[46,47] in two independent experiments (five in the first experiment and six in the second). To prepare inoculums for infection, bacterial strains were grown overnight on GC agar, resuspended and then grown for 4 h in 10 ml of BHI medium at 37 °C with moderate shaking. Cultures were adjusted such that each final 500 μl inoculum contained 1×10^6 colony forming units (c.f.u.) and 10 mg human holo-transferrin. Mice were monitored at least every 12 h starting 48 h before infection to 48 h after infection for changes in weight, clinical symptoms and bacteraemia. Mice were scored on a scale of 0–2 based on the severity of the following clinical symptoms: grooming, posture, appearance of eyes and nose, breathing, dehydration, diarrhoea, unprovoked behaviour and provoked behaviour. Animals reaching endpoint criteria were humanely euthanized. Animal experiments were conducted in accordance with the protocols approved by the Animal Ethics Review Committee of the University of Toronto.

Immunoprecipitation assay with Slam1 and GST-C-lobe. *E. coli* C43(DE3) cells transformed with pETDuet Slam1 and pGCC4 GST or GST-C-lobe were grown for 3 h at 37 °C, induced with 1 mM IPTG and grown overnight in 200 ml LB with the required antibiotics. The cells were resuspended in assay buffer (40 mM Tris pH 8.0, 200 mM NaCl) supplemented with fresh lysozyme (1 mg ml^{-1}), 2 mM PMSF and DNase I (0.05 mg ml^{-1}) and lysed by sonication. The samples were centrifuged twice: first, at 20,000 r.c.f., 4 °C for 40 min to remove the cell debris and then at 125,000 r.c.f., 4 °C for 1 h to extract the cellular membranes. The membranes were collected, homogenized and extracted overnight at 4 °C in assay buffer with 2% Elugent (Millipore). The detergent-extracted membranes were used for immunoprecipitation experiments.

NHS agarose beads (GE Healthcare) conjugated to rabbit α-Slam1 serum or glutathione-sepharose 4B beads (GE Healthcare) were equilibrated in assay buffer with 1% Elugent. The samples were incubated with the beads for 2 h at 4 °C with gentle mixing. The beads were washed at least thrice in assay buffer with 1% Elugent and eluted in SDS loading buffer for SDS–PAGE and western blot analysis using α-Slam1, α-BamA (1:5,000), α-GST (1:5,000, GenScript) and α-TbpB antibodies.

***E. coli* expression plasmids used in flow cytometry experiments.** Genes were cloned into expression vectors by RF cloning[42] and signal peptides and tags were inserted using round the horn cloning.

For flow cytometry experiments performed in *E. coli* C43(DE3), the four SLPs (TbpB, LbpB, fHbp and HpuA) were cloned into pETDuet (to make pETDuet TbpB, LbpB, fHbp or HpuA) and Slams were inserted into pET26b (pET26 Slam1, pET26 Slam1barrel and pET26 Slam2). Genes *tbpb* and *hpua* were amplified from the genome of *N. meningitidis* strain B16B6, and *lbpb* and *fhbp* were amplified from *N. meningitidis* strain MC58 (due to the availability of genetic information or antibodies for these proteins) and inserted into pETDuet by RF cloning. The *E. coli pelB* signal peptide was inserted to replace the endogenous neisserial signal peptides. The mature *slam1* gene was cloned from B16B6 and *slam2* from MC58 to be in frame with the *pelB* signal peptide of pET26b. A 7xHis-tag was inserted between the *pelB* and the mature Slam sequences. The *pelB* Slam1 was also subcloned into the MCS site 1 of pETDuet for immunoprecipitation assays. The pET26 Slam1 barrel construct was cloned by amplifying the β-barrel domain (coding region from amino acid 204 to the end) and inserting the amplicon in frame with the 7xHis-tag of pET26 Slam1 while replacing the mature full-length sequence.

For flow cytometry experiments performed in *E. coli* Keio strains, Slam1 and TbpB were expressed from an arabinose and a pT5-lac promoter, respectively, because Keio strains do not carry the lambda DE3 construct. *slam1* from pET26 was subcloned into pHERD30T to make pHERD30T pelB Slam1 and the pT5-lac promoter from pQE30 (Qiagen) was inserted by assembly PCR[48] upstream of TbpB in pETDuet TbpB to make pETDuet pT5 TbpB.

To clone the pETDuet GST-C-lobe construct for the spheroplast assays, the GST tag was amplified from pGEX-6p-1 with primer ends complementary to the anchor peptide (forward) or C-lobe (reverse) of TbpB so that the amplicon could replace the N-lobe by RF cloning.

Globomycin assay. *N. meningitidis* strain B16B6 WT and *Δnmb0313* were grown on BHI plates overnight and then resuspended in BHI media containing 0.1 mM of the iron chelator deferoxamine mesylate (Sigma) for 1 h. Cells were dosed with 10 μg ml^{-1} of globomycin (a gift from L. Howell) for 2 h, collected, lysed in SDS loading buffer and analysed for TbpB build-up by western blot using α-TbpB antibodies.

E. coli C43(DE3) cells transformed with pETDuet TbpB alone or pETDuet TbpB with pET26 Slam1 were used to inoculate 5 ml of LB supplemented with appropriate antibiotics. The starter cultures were diluted 1:50 with LB to start fresh 1 ml cultures. After reaching an OD_{600} of 0.8, cells were dosed with 1 mM IPTG and 10 μg ml^{-1} of globomycin and further incubated for 3 h at 37 °C. Cells were then collected by centrifugation, lysed in SDS loading buffer and analysed using western blots probed with α-TbpB antibodies.

Pulldown of TbpB with LolA R43L mutant. *E. coli lolA* was cloned into pETDuet MCS site 1 of pETDuet TbpB (TbpB in site 2). Further, a 6xHis-tag was inserted at the C-terminal end of LolA. Site-directed mutagenesis was performed to generate the R43L mutation, producing pETDuet LolA R43L_TbpB. The pETDuet TbpB and pETDuet LolA R43L_TbpB plasmids were separately transformed into *E. coli* C43 (DE3) and a swab of at least ten colonies was used to inoculate 5 ml of auto-induction media[45] with appropriate antibiotics. Cells were grown for 18 h, collected and lysed in 1 ml of lysis buffer (50 mM Tris pH 8.0, 200 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mg ml^{-1} lysozyme, 0.05 mg ml^{-1} DNase I and 2 mM PMSF) for 15 min at room temperature. After cell debris was removed by centrifugation, 400 μl of lysate was diluted ten times in resuspension buffer (50 mM Tris pH 8.0, 200 mM NaCl, 10 mM imidazole, 0.1% Triton X-100) and then incubated with 25 μl of Ni-NTA resin (pre-equilibrated with resuspension buffer) for 1 h of batch binding at 4 °C. Ni-NTA resin was washed three times with resuspension buffer before mixing with SDS loading buffer for analysis by SDS–PAGE and western blots using α-His (1:5,000) or α-TbpB antibodies.

Localization of TbpB by sucrose density gradient centrifugation. *E. coli* C43(DE3) cells were transformed with pETDuet TbpB with or without pET26 Slam1. A 5 ml

overnight starter culture was used to inoculate 750 ml LB with the appropriate antibiotic and was grown to an OD_{600} of 0.8. The culture was dosed with 1 mM IPTG and grown for an additional 18 h. The cells were pelleted, resuspended in 20 mM Tris pH 8.0, 200 mM NaCl supplemented with fresh lysozyme (1 mg ml^{-1}), 2 mM PMSF and DNase I (0.05 mg ml^{-1}), lysed by sonication and then centrifuged at 10,000 r.c.f. to remove cell debris.

The inner and outer membrane were separated following the protocol described by Fan and co-authors[49]. All subsequent steps were carried out at 4 °C. Briefly, the supernatant was centrifuged at 125,000 r.c.f. for 1 h to collect the cellular membranes. The membrane pellet was resuspended in 5 ml of 20 mM Tris pH 8.0, 200 mM NaCl using a mini-glass homogenizer and then applied on top of a 38.5 ml thin-wall polyproplyene tube containing step gradients of 10 ml of 2.02 M, 14 ml of 1.44 M and 10 ml of 0.77 M sucrose. The tubes were centrifuged at 83,000 r.c.f. for 16 h. The outer membrane and inner membranes were seen as discrete bands at the interface of the 2.02 M and 1.44 M sucrose cushions and 1.44 M and 0.77 M sucrose layers, respectively. Twelve 3 ml fractions were collected and subjected to SDS–PAGE followed by western blotting with α-TbpB, α-LepB (1:10,000), α-OmpA (1:40,000) and α-Slam1 antibodies.

Identification of slam homologues. Homologues of Slam1 were identified using psi-blast searches performed against NCBI's non-redundant (NR) protein database (performed on 18 May 2014) using the B16B6 Slam1 sequence as query. Representative strains from each bacterial species were identified and their Slam1 homologues were totalled. Slam2 (*nmb1971*) was identified in B16B6 and in other *N. meningitidis* strains. HpuA and HpuB genes were identified as neighbours by visual inspection of the *N. meningitidis* genomes.

Flow cytometry analysis of TamA- and TamB-deficient *E. coli*. *E. coli* Keio collection[36] strains BW25113 WT, JW4179-1 (*ΔytfM*) and JW4180-1 (*ΔytfN*) were transformed with plasmids expressing pETDuet pT5 TbpB and pHERD30 T pelB Slam1. Transformants were grown in 0.01% arabinose overnight to induce expression of Slam1 and then in 1 mM IPTG for 3 h for TbpB expression before cells were collected and prepared for flow cytometry, as described in the 'Flow cytometry assay' section.

Flow cytometry analysis of *E. coli* spheroplasts. To obtain spheroplasts, non-transformed *E. coli* C43(DE3) and cells transformed with pETDuet GST-C-lobe were grown overnight in auto-induction media[45] with appropriate antibiotics. Cells were collected and resuspended in 500 µl of 100 mM Tris pH 7.5 and 0.5 M sucrose to an OD_{600} of 2.0. Spheroplast formation was initiated by adding 500 µl of 8 mM EDTA pH 8.0 and 0.2 mg ml^{-1} lysozyme to the samples and incubating for 30 min on ice. The spheroplasts were collected by centrifugation at 10,000 r.c.f. for 5 min at 25 °C and used for labelling.

Sample preparation and flow cytometry analysis were performed as described previously, with two differences: first, all centrifugation steps were performed at 10,000 r.c.f. for 5 min at 25 °C; second, 0.25 M sucrose was added to all downstream buffers to prevent spheroplast lysis.

Immunoprecipitation assays in *Neisseria*. Full-length TbpB and TbpB-GST chimaeras were cloned into pGCC4 for neisserial complementation assays and *E. coli* and *N. meningitidis* immunoprecipitation assays. The B16B6 *tbpb* gene was cloned into the PacI/FseI sites of pGCC4 to produce pGCC4 TbpB. GST chimaeras pGCC4 GST-C-lobe and pGCC4 GST were cloned by amplifying *GST* from pGEX-6p-1 and replacing the N-lobe or both N- and C-lobes by RF cloning.

N. meningitidis strain B16B6 *Δtbpb* was complemented with pGCC4 TbpB, GST-C-lobe or GST. Expression of complemented genes was induced by growth on BHI plates containing 1 mM IPTG and verified by α-GST or α-TbpB western blots.

NHS sepharose beads were conjugated to either rabbit α-Slam1 serum or rabbit pre-immune serum. Neisserial cells were swabbed from BHI plates into PBS, washed once and lysed in lysis buffer (50 mM Tris pH 8.0, 200 mM NaCl, 2 mM EDTA, 1% Elugent) supplemented with fresh lysozyme (1 mg ml^{-1}), 2 mM PMSF and DNase I (0.05 mg ml^{-1}) for 20 min on ice. Cell debris was removed by centrifugation at 18,000 r.c.f. for 20 min and the supernatant was incubated with α-Slam1 or pre-immune beads for 1 h at 4 °C. After three washes in lysis buffer, SDS loading buffer was added to the beads to release bound materials. Samples were resolved in an 8% SDS–PAGE for western blot analysis with α-GST antibodies.

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Author contributions

T.F.M. and S.D.G. conceived and supervised the study. Y.H., C.C.L., A.J. and H.E.S. designed and performed the experiments. C.M.B. completed the mice infection studies. Y.H., C.C.L., A.J. and T.F.M. prepared the manuscript.

Additional information

Supplementary information is available online. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to T.F.M.

Competing interests

The authors declare no competing financial interests.