A common assembly module in injectisome and flagellar type III secretion sorting platforms

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Translocating proteins across the double membrane of Gram-negative bacteria, type III secretion systems (T3SS) occur in two evolutionarily related forms: injectisomes, delivering virulence factors into host cells, and the flagellar system, secreting the polymeric filament used for motility. While both systems share related elements of a cytoplasmic sorting platform that facilitates the hierarchical secretion of protein substrates, its assembly and regulation remain unclear. Here we describe a module mediating the assembly of the sorting platform in both secretion systems, and elucidate the structural basis for segregation of homologous components among these divergent T3SS subtypes sharing a common cytoplasmic milieu. These results provide a foundation for the subtype-specific assembly of T3SS sorting platforms and will support further mechanistic analysis and anti-virulence drug design.
Type III secretion systems (T3SS) allow the transport of protein substrates directly across the double membrane of Gram-negative bacteria. There are two evolutionarily related, yet functionally distinct subtypes of T3SS: ‘injectisomes’, which deliver effector proteins into the cytoplasm of eukaryotic host cells\(^1\), and the flagellar apparatus, which secretes the polymeric filament used for motility\(^2\). Despite their functional divergence, injectisomes and the flagella share a common core of homologous gene products and possess ultrastructural similarities\(^3\). For example, both systems share related elements of a ‘sorting platform’ that facilitates the hierarchical secretion of protein substrates\(^4\).

Proteomic analyses have identified the major components of the sorting platform for the Salmonella typhimurium SPI-1 injectisome: the AAA\(^{+}\) ATPase InvC, its regulator OrgB and the proteins SpaO and OrgA\(^5\). While SpaO has been shown to be necessary for formation of the sorting platform\(^4\), little is known about its molecular structure. In Yersinia, the SpaO homologue is expressed as a full-length protein as well as a carboxy-terminal fragment translated from an internal translation start site\(^5\); this carboxy-terminal fragment dimerizes and can interact with the full-length protein. The crystal structure of the Yersinia carboxy-terminal dimer is similar to that of its Pseudomonas\(^6\) and flagellar\(^7\) homologues, together characterizing a structural class known as the surface presentation of antigens (SPOA) domain.

In the flagellar apparatus, the SpaO homologues FliM and FliN form a robust, stable ring (the ‘C-ring’) at the cytoplasmic face of the basal body\(^8\). Electron microscopic analyses have similarly localized the SpaO homologue to the cytoplasmic face of the Shigella injectisome\(^9\), and recent cryo-electron tomographic studies in the same organism identified SpaO homologue-dependent ‘pods’ of density beneath the injectisome\(^10\).

In contrast to the flagellar C-ring, this sub-injectisome structure is less robust\(^10\), and fluorescence microscopic analysis of the Yersinia SpaO homologue show that there is dynamic exchange between cytoplasmic- and injectisome-associated forms\(^11\). How SpaO and its homologues interact with other elements of the T3SS has yet to be shown at high resolution, and how homologous flagellar and injectisome components are properly segregated to their cognate secretion systems remains an open question.

Here we show that a novel, heterotypic interaction between SPOA domains serves as a scaffold for sorting platform assembly in both injectisome and flagellar T3SS. Solution nuclear magnetic resonance (NMR) data support the crystallographic model, and structure-guided mutagenesis shows that this interaction is necessary for formation of the SpaO–OrgB–InvC complex, the proper localization of SpaO to the bacterial inner membrane and T3SS function. Structures of the flagellar SpaO–OrgB homologues FliM, FliN and FliH reveal a mechanism for the proper segregation of homologous sorting platform components among T3SS subtypes sharing a common cytoplasmic milieu. Together, these structures define a common module utilized in sorting platform assembly and provide insight into the subtype-specific assembly of T3SS.

**Results**

SpaO contains two bona fide SPOA domains. To dissect the structural basis for the sorting platform assembly, we determined the structures of individual domains of S. typhimurium SpaO and then characterized their interactions with other sorting platform components. Preliminary bioinformatic analyses suggested the presence of two putative SPOA domains in the carboxy-terminal half of SpaO, which we denote SPOA1 and SPOA2 (Fig. 1a). We first determined the structure of the SPOA2 homodimer to 1.35 Å resolution (Fig. 1b,c; Table 1; Supplementary Fig. 1). The SPOA2 homodimer structure is architecturally similar to its

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**Figure 1 | Homotypic and heterotypic SPOA interactions.** (a) Bioinformatic analysis of SpaO. PSIPRED secondary-structure predictions and sequence homology suggest the presence of two putative SPOA domains in SpaO. Probability of helical character is plotted in red, strand in blue and disorder in yellow. The arrow at codon 203 represents a predicted Val\(_{203}\) internal translation start site, as has been shown for YscQ Met218 in Yersinia pseudotuberculosis\(^8\). (b,c) Comparison of homotypic SPOA2–SPOA2 and heterotypic SPOA1–SPOA2 structures from SpaO. (b) Ribbon diagrams show the similar organization of secondary-structural elements in both SPOAs. Asterisks denote the antiparallel beta-sheet ‘floor’, (c) Amino (blue) to carboxy terminus (red) Cx traces of SPOA2 (top) and SPOA1 (bottom) reveal a similar topology in interaction with SPOA2 (grey surface representation, top and bottom).
homologues\textsuperscript{5,6}: like two left hands grasping one another, an
interacting protomer each bury about 1,800 Å\textsuperscript{2} against their
(Fig. 1b,c). In both SPOA\textsubscript{1,2} and the SPOA\textsubscript{2} homodimer,
antiparallel beta-sheet and fingers-to-palm architecture
backbone follows that of the prototypical SPOA fold, retaining
topology similar to that of the SPOA\textsubscript{2} homodimer. The SPOA\textsubscript{1}
distinct, heterotypic SPOA–SPOA interaction with an overall
Table 1; Supplementary Fig. 3). SPOA\textsubscript{1} and SPOA\textsubscript{2} form a
its structure was determined to 2.9 Å resolution (Fig. 1b,c;
could be co-refolded with SPOA\textsubscript{2}. This complex crystalized, and
consistent with this hypothesis, a SPOA\textsubscript{1} construct (145–213)
connected by a flexible linker (Supplementary Fig. 2b). We
amide resonances suggested a secondary-structure pattern similar
SPOA\textsubscript{1} and SPOA\textsubscript{2} (residues 140–297) were stable and soluble.

While SPOA\textsubscript{1} alone was insoluble, constructs containing both
SPOA\textsubscript{1} and SPOA\textsubscript{2} (residues 140–297) were stable and soluble.
SpaO(1–219) was analysed by solution NMR (Supplementary Fig. 2a), and chemical shift deviation (CSD) analysis of backbone
amide resonances suggested a secondary-structure pattern similar
to that predicted by bioinformatic analyses: two SPOA domains
connected by a flexible linker (Supplementary Fig. 2b). We
hypothesized that SPOA\textsubscript{2} interacts with and stabilizes SPOA\textsubscript{1};
consistent with this hypothesis, a SPOA\textsubscript{1} construct (145–213)
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antiparallel beta-sheet and fingers-to-palm architecture
(Fig. 1b,c). In both SPOA\textsubscript{1,2} and the SPOA\textsubscript{2} homodimer,
interacting protomers each bury about 1,800 Å\textsuperscript{2} against their
binding partner. SPOA\textsubscript{1,2} and the SPOA\textsubscript{2} homodimer
superspose with 2.47 Å r.m.s.d. (Supplementary Fig. 3b), and the conformation
of SPOA\textsubscript{2} in association with SPOA\textsubscript{1} is grossly similar to
that seen in the homodimer, supersposing with an r.m.s.d. of
1.67 Å (Supplementary Fig. 3c).

Further supporting the hypothesis that SPOA\textsubscript{1} and SPOA\textsubscript{2}
interact in solution, a post hoc analysis of the three dimensional
\textsuperscript{15}N-edited nuclear Overhauser enhanced spectroscopy-(3D
NOESY-HSQC) for SpaO\textsubscript{140–297} revealed long-range amide
proton correlations between SPOA\textsubscript{1} and SPOA\textsubscript{2} (Supplementary
Fig. 4). Given the <20 residue linker connecting SPOA\textsubscript{1} and
SPOA\textsubscript{2}, they would experience a low millimolar-range relative
concentration and would likely interact in an intramolecular
fashion (Fig. 2a). However, at high local SpaO concentrations (for
example, in association with the T3SS), intermolecular hetero-
typic SPOA interactions might explain the apparent oligomeric
nature of the sorting platform (Fig. 2a). Indeed, a similar model of
intermolecular domain swapping was recently suggested for the
ring-forming injectisome protein PrgK\textsuperscript{12}.

Hypothetical SpaO oligomerization driven by intermolecular
heterotypic SPOA interactions would be dependent on the
covalent linkage of SPOA\textsubscript{1} and SPOA\textsubscript{2}. Thus, we tested whether
genomic deletion of the SpaO amino-terminal domain and
SPOA\textsubscript{1} can be complemented in trans, as assayed by
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SPOA\textsubscript{1} can be complemented in trans, as assayed by
Salmonella

| Data collection and refinement statistics for injectisome structures. |
|--------------------------------------|--------------------|
| SpaO(232–297, SeMet) | SpaO(145–213, SeMet) + SpaO(232–297, SeMet) | SpaO(145–213) + SpaO(232–297) + OrgB(1–30): lysozyme | SpaO(145–213) + SpaO(232–297) + OrgB(1–30): lysozyme |
| Data collection |
| Space group | P 2\textsubscript{1} | P 4\textsubscript{1} 2\textsubscript{1} 2 | P 4\textsubscript{1} 2\textsubscript{1} 2 | P 2\textsubscript{1} |
| Cell dimensions |
| \(a, b, c \text{ (Å)}\) | 35, 41.27, 48 | 66.38, 66.38, 95.21 | 65.76, 65.76, 95.65 | 62.092, 89.07, 95.21 |
| Resolution (Å) | 31.26–1.35 (1.37–1.35) | 46.94–3.00 (3.18–3.00) | 38.68–2.9 (3.08–2.9) | 47.59–2.0 (2.05–2.0) |
| \(R_{ave}\) | 0.146 (1.281) | 0.221 (1.463) | 0.166 (1.447) | 0.102 (0.530) |
| \(I/\sigma I\) | 8.6 (2.1) | 11.8 (3.1) | 14.4 (2.7) | 10.5 (3.2) |
| Completeness (%) | 99.7 (100) | 100 (100) | 99.3 (99.3) | 99.5 (99.8) |
| Redundancy | 7.0 (7.1) | 25.6 (27.2) | 24.6 (26.2) | 5.1 (5.2) |
| Refinement |
| No. of reflections | 29,246 | 4,964 | 41,183 | 25,740 |
| \(R_{free}/R_{ave}\) | 0.1724/0.2053 | 0.2085/0.2795 | 0.1571/0.2096 | 0.1984/0.2618 |
| No. of atoms | 1,286 | 1,024 | 5,769 | 4,940 |
| Protein | 1,062 | 1,023 | 5,112 | 4,818 |
| Ligand/ion | 2 | 1 | 0 | 0 |
| Water | 222 | 0 | 657 | 122 |
| B factors |
| Protein | 14.70 | 74.20 | 33.10 | 46.90 |
| Ligand/ion | 14.20 | 105.00 | 39.60 | 45.60 |
| Water | 32.10 | | | |
| r.m.s.d. |
| Bond lengths (Å) | 0.007 | 0.010 | 0.008 | 0.011 |
| Bond angles (°) | 1.09 | 1.33 | 1.16 | 1.46 |

SeMet, selenomethionine.
necessary for T3SS function. Thus, if intermolecular heterotypic SPOA interactions do occur in vivo, they are not explicitly necessary for secretion. It should be noted that SpaO(1–219) does not complement a full genomic deletion of spaO, demonstrating that SpaO SPOA2 is also necessary for T3SS function (Fig. 2c).

Similarly, insertion of a double stop codon after spaO codon 219, SpaOc indicates the cryptically expressed SpaOc (‘FL’, full length) and SpaOc(140–297, Val203GTT) are each sufficient to co-affinity purify the SpaO–OrgB–InvC complex formation. Asterisk denotes nonspecific proteins, likely chaperones. SpaOc indicates the cryptically expressed SPOA2-containing carboxy-terminal fragment.

Data shown are representative of three experiments.

Figure 2 | Intermolecular SPOA1–SPOA2 interactions are not necessary for T3SS function. (a) Schematic models for putative intra- and intermolecular SPOA1–SPOA2 interactions and their implications for the SpaO oligomerization state. The numbers 1 and 2 indicate the SpaO SPOA1 and SPOA2, respectively, and N indicates the SpaO amino-terminal domain(s). (b) Coomassie-stained polyacrylamide gel electrophoresis (PAGE) of S. typhimurium culture supernatants grown under T3SS-stimulating conditions (0.3 M NaCl, strain SB1741). Bands previously identified by Mizusaki et al.15 are noted and colour coded by the T3SS subtype—injectisome in red and flagellar in blue. (c) Coomassie-stained PAGE of S. typhimurium culture supernatants grown under T3SS-stimulating conditions. Red asterisks indicate injectisome-specific secretion substrates. WT, wild type; ΔO, deletion of spaO; Δ1–203, deletion of spaO codons 1–203, 1–219, complementation with SpaO(1–219); 1–219stop, insertion of two stop codons following spaO codon 219. SpaO was 3 x FLAG tagged at its amino terminus in each S. typhimurium strain (except ΔO) and complementation construct. Data shown are representative of three experiments.

SpaO SPOA1,2 is a scaffold for interaction with OrgB–InvC. Double-hexahistidine-tagged SpaO is able to co-affinity purify the sorting platform components OrgB and InvC when co-expressed in Escherichia coli (Fig. 3a). Formation of the SpaO–OrgB–InvC ternary complex is OrgB dependent, as SpaO alone is insufficient to co-affinity purify InvC (Fig. 3a). We hypothesized that SPOA1,2 might serve as a scaffold for the interaction of SpaO with OrgB–InvC. Indeed, SPOA1,2 is sufficient to co-affinity purify OrgB–InvC (Fig. 3a). This construct contains a Val203GTT to Val203GTT mutation to prevent the duplicitous translation of SPOA2 from its cryptic internal translation start site, demonstrating that the SPOA2 homodimer is dispensable for SpaO–OrgB–InvC complex formation.

OrgB and its homologues are predicted to share a common amino-terminal organization: a disordered region followed by a coiled coil. In the flagellar system, the unstructured region at the amino terminus of OrgB and its homologues as the adaptor peptide of the ATPase regulator (APAR).

The SPOA1,2–OrgB(APAR): lysozyme complex was crystalized and its structure solved to 2.0 Å resolution (Fig. 3b; Table 1;
The two data sets are left white. The same view of SpaO is shown with and without OrgB (grey surface). The crystal structure. Surface residues are colour coded by the size of their weighted CSD in units of s.d. Residues not assigned an amide resonance in one of the structures are left white. The same view of SpaO is shown with and without OrgB (grey surface). The crystal structure. Surface residues are colour coded by the size of their weighted CSD in units of s.d. Residues not assigned an amide resonance in one of the structures are left white. The same view of SpaO is shown with and without OrgB (grey surface). The crystal structure. Surface residues are colour coded by the size of their weighted CSD in units of s.d. Residues not assigned an amide resonance in one of the structures are left white. The same view of SpaO is shown with and without OrgB (grey surface). The crystal structure. Surface residues are colour coded by the size of their weighted CSD in units of s.d. 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Independent NMR analyses of SpaO(140–297) bound to OrgB(APAR) in solution are consistent with the interface defined in the crystal (Fig. 4). Compared with apo-SpaO, the largest CSDs of backbone amide resonances in SpaO–OrgB map on the crystal structure to residues involved in the interface, which are highly conserved across both the Salmonella/Shigella and Yersinia/Pseudomonas clades (Fig. 4; Supplementary Fig. 7). In the crystal, these residues form the docking site for OrgB residues Ile17, Leu18 and Ile19 (Fig. 5a). The OrgB surface area buried by these three residues (360 Å²) accounts for approximately one-third of the APAR’s total buried area. Here, the APAR shows noteworthy sequence homology: immediately following a conserved glycine (Gly16, pseudo-lariat apex) is a string of aliphatic and basic amino acids in each homologue (Fig. 5a).

The SPOA1,2–APAR interaction is necessary for T3SS function. To test whether the SPOA1,2–APAR interaction per se is necessary for T3SS function, we constructed an OrgB triple mutant (I17D, L18D, I19D) to disrupt its interaction with SpaO. As predicted, spaO failed to co-affinity purify OrgB(I17D, L18D, I19D)–InvC when co-expressed in E. coli (Fig. 5b), and the aspartate triple mutation completely abolished T3SS in vivo (Fig. 5c). Fluorescence microscopic analyses of the Yersinia SpaO homologue have shown it to localize in discrete perimembranous punctae11. Might the SPOA1,2–APAR interaction function to localize SpaO to the bacterial inner membrane? In an otherwise wild-type genomic background, an EGFP::3 FLAG::SpaO fusion exhibits punctate, perimembranous localization, consistent with its recruitment to injectisome basal body channels (Fig. 5d). Deletion of orgB disrupts proper SpaO localization, producing a more diffuse, cytoplasmic pattern, and the aspartate triple mutation was sufficient to phenocopy the orgB deletion mutant (Fig. 5d). Together, these data suggest that the SpaO(SPOA1,2)–APAR assembly is necessary for the proper localization of SpaO to discrete perimembranous puncta, and that this arrangement is required for T3SS function.

A divergent SPOA1,2–APAR assembly in the flagellar T3SS. The flagellar C-ring is primarily composed of three proteins: FliM, FliN and FliG8. The SpaO homologues FliM and FliN are predicted to contain one SPOA domain each, which we designate as SPOA1 and SPOA2, respectively. Paralleling the injectisome, FliN is known to interact with the OrgB homologue FliH14. The evolutionary relationship between injectisomes and flagella creates a practical conundrum: how are homologous T3SS components segregated to their corresponding secretion systems within a common cytoplasmic milieu? To qualitatively assess the subtype specificity of SPOA–APAR interactions, we co-affinity purified a panel of Salmonella SPAO domains with hexahistidine-tagged APAR::lysozyme fusions (Fig. 6). Indeed, the OrgB and FliH APARs robustly co-affinity purify their cognate SPOA1,2 but not that of the other T3SS subtype (Fig. 6, red asterisks). Neither SpaO nor FliM–FliN are pulled down by the APAR from a second pathogenic T3SS found in S. typhimurium (SPI-2 SsaK). Consistent with the observation that the OrgB APAR interacts with surfaces on both SPOA1 and SPOA2, the OrgB and FliH APARs more robustly pull down their cognate SPOA1,2 than homodimeric SPOA2 (Fig. 6).

We hypothesized that divergence of the SPOA1,2–APAR assembly architecture contributes to proper component segregation among T3SS subtypes, and sought to structurally

Figure 4 | The APAR-binding site of SpaO. (a) The SpaO residues at the APAR interaction site are highly conserved across homologues in other species. Excerpts of the M-COFFEE alignment of SpaO, Shigella flexneri Spa33, Yersinia enterocolica YscQ and Pseudomonas aeruginosa PscQ are shown with conserved APAR-interacting residues highlighted in red. Symbols beneath the alignment indicate the degree of conservation: asterisks denote full conservation, colons denote strong similarity, and dots denote weak similarity. (b) A surface representation of SpaO with the conserved interface residues identified in a are coloured red and the OrgB APAR backbone is yellow. (c) Overlaid 15N-heteronuclear single quantum coherence spectra of apo- (green) and APAR-bound (violet) SpaO(140–297). The five largest peak shifts are noted. (d) The solution interaction data from c are mapped onto the SpaO–OrgB crystal structure. Surface residues are colour coded by the size of their weighted CSD in units of s.d. Residues not assigned an amide resonance in one of the two data sets are left white. The same view of SpaO is shown with and without OrgB (grey surface).
characterize the flagellar SPOA–APAR interactions. While complexes of FliM and FliN were stable, they were resistant to crystallization. Interestingly, FliM and FliN can be fused and still support flagellin secretion (Supplementary Fig. 8a) and some swarming motility15. We crystallized the SPOA of FliM (residues 245–334) fused to FliN(5–137), and its structure was solved to 2.56 Å (Table 2; Supplementary Fig. 8). Architecturally, the FliM(SPOA1)–FliN(SPOA2) interaction is similar to that of SpaO (Supplementary Fig. 8e, 2.28 Å r.m.s.d.), with the exception of additional helices present at the carboxy terminus of each SPOA, as observed in FliN homodimers from Thermotoga maritima7. The similarity of these structures is consistent with the SPOA heterotypic interaction being generalizable across T3SS subtypes.

To elucidate the mechanism of FliH-specific assembly with FliM–FliN, we co-crystalized the FliM(SPOA)::FliN fusion with a FliH(1–18)::lysozyme fusion (Fig. 7; Table 2; Supplementary Fig. 9). As with its injectisome counterparts, the FliM–FliN SPOA1,2 did not undergo large conformational changes upon APAR binding (Supplementary Fig. 9c, 1.11 Å r.m.s.d.); however, the binding mode for the FliH APAR is radically different. In contrast to the OrgB pseudo-lariat, the FliH APAR adopts a near-linear conformation along the ‘top’ of FliM–FliN (Fig. 7a). As observed in the SpaO–OrgB assembly, the FliH APAR makes

**Figure 5** | Structure-guided disruption of the SPOA1,2-APAR interaction. (a) Clustal Omega alignment of the APAR regions of OrgB, S. flexneri MxiN, Y. enterocolica YscL and P. aeruginosa PscL. The conserved pseudo-lariat apex glycine is indicated by a green asterisk and the subsequent patch of aliphatic (red) and basic (blue) amino acids is highlighted with a purple bar. Beneath, the binding site for OrgB(17–19) (grey) is shown as an electrostatic surface. OrgB(1–15) have been removed for clarity. (b) Coomassie-stained polyacrylamide gel electrophoresis of the protein elution from NiNTA resin shows that double-hexahistidine-tagged SpaO can co-affinity purify wild-type InvC–OrgB but not InvC–OrgB(I17D,L18D,I19D) when co-expressed in E. coli. 3 × D indicates the OrgB(I17D,L18D,I19D) triple mutant. Asterisk denotes nonspecific co-purifying E. coli proteins, likely chaperones. SpaOc indicates the cryptically expressed SPOA2-containing carboxy-terminal fragment. (c) Coomassie-stained culture supernatant from wild-type (WT, strain SB1741), orgB deletion(Δ) and orgB(I17D,L18D,I19D) (3 × D) S. typhimurium shows loss of injectisome substrate (red asterisks) secretion in the mutants, while flagellar secretion remains intact. (d) Widefield microscopic imaging of fixed S. typhimurium shows exclusive perimembranous localization of SpaO in the WT background, but cytoplasmic localization in the orgB mutants (scale bar, 2 μm, single z-slices shown). Data shown in b–d are representative of three experiments.

**Figure 6** | APARs preferentially interact with their cognate SPOA1,2. Coomassie-stained gels showing the input and imidazole elution for APAR–SPOA co-affinity purification experiments. Red asterisks indicate the cognate SPOA1,2 band(s). T4lyso, T4 lysozyme; O1,2, SpaO(140–297); O2, SpaO(232–297); M, FliM(245–320); N, FliN (1–137); n, co-purifying amino-terminal FliN degradation product. Data shown are representative of three experiments.
extensive contact with both SPOA1 and SPOA2 (Fig. 7a), supporting the observation that the FliH APAR interacts more strongly with the FliM–FliN heterodimer than the FliN homodimer (Fig. 6).

The FliH–FliM–FliN assembly is characterized by the burial of several highly conserved hydrophobic FliH side chains. Two tryptophan side chains form an aromatic clamp, which binds hydrophobic pockets on opposite faces of the FliN thumb (Fig. 7b). These residues are critical for flagellar function14 and are highly conserved (Fig. 7c). Similarly, the bulky side chain of FliH Leu15 fills a hydrophobic pocket on the thumb of FliM (Supplementary Fig. 10). The binding interfaces for these three residues are formed by both FliM and FliN and are highly conserved across species (Supplementary Fig. 10). This structure presents a conserved model for FliH–FliM–FliN interaction, which is distinct from that of SpaO–OrgB.

Discussion

We present here a series of structures that yield critical mechanistic insights into T3SS sorting platform assembly across multiple species and secretion subtypes. The existence of heterotypic SPOA interactions provides a structural explanation for the observed ∼1:3 stoichiometry of SPOA1 to SPOA2 in SpaO homologues5. While two of these SPOA2 domains could be accounted for by a homodimer interacting with full-length SpaO, the conformation of the third SPOA2 (located in the full-length protein) was unclear. Previous reports had proposed the existence of an alternate autostabilizing conformation for the third SPOA2 (ref. 5). We show here that the third SPOA2 can be stabilized by a SPOA1–SPOA2 interaction.

Similar to SpaO and its injectisome homologues, the ratio of FliM to FliN in situ is estimated to be 1:3 (ref. 8). In the context of our FliM–FliN structure, this suggests a model for FliM–FliN interaction similar to that of SpaO. FliM(SPOA1) would engage FliN(SPOA2) in a heterotypic SPOA–SPOA interaction, and additional homodimeric FliN would interact with FliM–FliN in an as of yet undetermined fashion (analogous to the SpaO SPOA2 homodimer interaction with full-length SpaO). However, reports of FliN tetramerization and FliM:FliN ratios between 1:3 and 1:4 suggest that more complicated higher-order structures may be used by the flagellar apparatus7. It should also be noted that while previous investigations of the flagellar T3SS have focused on the interaction between FliH and FliN specifically14, our structures and biochemical data show that the FliH APAR more strongly interacts with the FliM–FliN complex than with FliN alone.

**Table 2 | Data collection and refinement statistics for flagellar structures.**

|                      | FliM(245–334)::FliN(5–137), SeMet | FliM(245–334)::FliN(5–137) + FliH(1–18)::lysozyme |
|----------------------|----------------------------------|-----------------------------------------------------|
| **Data collection**   |                                  |                                                     |
| Space group          | P 2; 2; 2; 2;                     | P 2; 2; 2; 2;                                       |
| a, b, c (Å)          | 75.15, 81.50, 89.96               | 43.21, 76.37, 119.4                                 |
| Resolution (Å)       | 57.67–2.56 (2.67–2.56)            | 64.33–2.30 (2.38–2.30)                              |
| Rmerge               | 0.079 (1.215)                     | 0.070 (0.923)                                       |
| completeness (%)      | 100 (100)                         | 99.8 (99.8)                                        |
| Redundancy           | 13.8 (14.3)                       | 12.9 (12.9)                                        |
| **Refinement**       |                                  |                                                     |
| No. of reflections   | 18,372                           | 18,174                                              |
| Rwork/Rfree          | 0.2175/0.2593                     | 0.1967/0.2620                                       |
| No. of atoms         | 2,633                            | 2,739                                               |
| Protein              | 2,605                            | 2,668                                               |
| Ligand/ion           | 5                                | 0                                                   |
| Water                | 23                               | 71                                                  |
| B factors            |                                   |                                                     |
| Protein              | 68.30                            | 69.70                                               |
| Ligand/ion           | 73.40                            | 65.00                                               |
| Water                | 64.50                            | 65.00                                               |
| r.m.s.d.             |                                   |                                                     |
| Bond lengths (Å)     | 0.010                            | 0.009                                               |
| Bond angles (°)      | 1.31                             | 1.15                                                |

SeMet, selenomethionine.

Figure 7 | Structure of the SPOA1–2–APAR interaction in the flagella. (a) Ribbon diagram (left) and surface representation (right) of the FliM–FliN–FliH structure. T4 lysozyme has been omitted. N and C indicate the amino and carboxy termini of the FliH–FliN–FliH structure. (b) A zoomed view of the FliH aromatic clamp, with the side-chain atoms of FliH W7 and W10 represented as spheres. (c) Excerpted M-COFFEE alignment of FliH with its homologues from S. flexneri, Y. enterocolitica and P. aeruginosa. Highly conserved residues of interest are noted (S. typhimurium numbering). Symbols beneath the alignment indicate the degree of conservation: asterisks denote full conservation, colons denote strong similarity, and dots denote weak similarity.
sugest that the FliM–FliN complex is the physiologically relevant binding partner for FliH.

Our structures suggest a partial model for the subtype-specific assembly of the T3SS sorting platforms: the heterotypic interaction between SPOA domains within a given T3SS subtype functions as an adaptor for ATPase and its regulator through interaction with the APAR peptide (Fig. 8). However, a number of questions remain regarding the higher-order architecture of the sorting platform in situ. We hypothesize that the puncta formed by SpaO in vivo represent the high-molecular weight sorting platforms described by Lara-Tejero et al.4. Diepold et al. have quantified the stoichiometry and dynamics of these puncta in Yersinia, showing them to possess ~22 copies of the SpaO homologue per punctum and to be in dynamic exchange with the cytoplasm11. In contrast, the recent tomographic reconstruction of Shigella injectisomes by Hu et al. revealed the presence of only six SpaO homologue-dependent pods of density beneath the injectisome, and their localization was OrgB homologue independent10. Taken together with our findings, these results suggest that there may be two subpopulations of SpaO in vivo: one stably associated with the injectisome, and a second dynamic population in exchange with the cytoplasm, requiring the SPOA1,2–APAR interaction to form high-molecular weight, perimembranous sorting platforms. Recent analyses of Fli ATPase dynamics by Bai et al. suggest a similar two-population model, which they hypothesize functions to deliver secretion substrates to the assembling flagella16.

What might be the mechanism for sorting platform targeting, and how might this factor into T3SS machine function? Perhaps APAR binding to the SPOA1,2 scaffold induces conformational changes in OrgB, InvC and/or the amino terminus of SpaO, which facilitates interaction with the membrane integral components of the T3SS. Alternatively, the SPOA1,2–APAR assembly might function simply by inducing proximity between sorting platform components. Intriguingly, the FliH APAR region has been shown to possess two subpopulations of density beneath the injectisome, and their localization was OrgB homologue independent10. Taken together with our findings, these results suggest that there may be two subpopulations of SpaO in vivo: one stably associated with the injectisome, and a second dynamic population in exchange with the cytoplasm, requiring the SPOA1,2–APAR interaction to form high-molecular weight, perimembranous sorting platforms. Recent analyses of Fli ATPase dynamics by Bai et al. suggest a similar two-population model, which they hypothesize functions to deliver secretion substrates to the assembling flagella16.

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For purification under denaturing conditions, guanidinium chloride was added to the lysate to a final concentration of 6 M. The post-extraction lysate was clarified by centrifugation at 30,000g for 15 min at 4°C and loaded onto NiNTA resin in a batch at 25°C. Still at 25°C, the resin was washed with denaturing wash buffer (8 M urea, 500 mM NaCl, 20 mM Tris-Cl pH 8.0, and 30 mM imidazole-Cl pH 8.0) and eluted in denaturing elution buffer (8 M urea, 200 mM NaCl, 20 mM Tris-Cl pH 8.0 and 300 mM imidazole-Cl pH 8.0). The elution was supplemented with 5 mM EDTA and 5 mM dithiothreitol (DTT), and the protein refolded by dialysis against 200 mM NaCl, 20 mM Tris-Cl pH 8.0 and 1 mM DTT (3–4 changes, dialysis time of 24 h total, 4°C). For T4 lysozyme fusions, Hepes-Na pH 7.0 was substituted for Tris-Cl pH 8.0. Insoluble material was removed by centrifugation or filtration and affinity tags were removed by cleavage with HRV 3C protease.

Affinity-purified proteins were further purified by ion-exchange chromatography using an AKTA FPLC and the following columns (GE Healthcare): T4 lysozyme fusions were purified by cation exchange on a SourceQ column; all other constructs were purified by an exchange on a Source60Q column. For cation-exchange chromatography, proteins were loaded in a batch in 10 mM Hepes-Na pH 7.0, 50–100 mM NaCl and eluted by a NaCl gradient (from 0 to 1,000 mM) in the same buffer. For anion exchange, proteins were loaded in a batch in 20 mM Tris-Cl pH 8.0, 50–100 mM NaCl and eluted by a NaCl gradient (from 0 to 1,000 mM) in the same buffer. Prior to crystallization, ion-exchange-purified proteins were further purified by gel filtration chromatography on a Superdex 75 column (GE Healthcare) in final buffer (200 mM NaCl, 20 mM Tris-Cl pH 8.0 and 2 mM DTT) and concentrated using centrifugal concentrators (Amicon). To form the SpaO–OrgB:lysozyme complex, the crystallization, cation-exchange-purified OrgB(1–30):T4 lysozyme was mixed with an excess of anion-exchange-purified Spa(145–213) and Spa(232–297) and allowed to incubate overnight at 4°C. The SpaO–OrgB:lysozyme complex was then purified by gel filtration chromatography. To form the FliM(245–334):FliN(5–137) complex, anion-exchange-purified FliM(245–334):FliN(5–137) was washed with cation-exchange-purified FliH(1–18):T4 lysozyme and allowed to incubate overnight at 4°C. The FliM:FliN–FliH::T4 lysozyme complex was then purified by gel filtration chromatography.

Crystallization. All proteins were crystallized by hanging-drop vapour diffusion with 1:1 and 2:1 ratios of protein (in final buffer) to precipitant at 25°C (except where noted). For crystallization, SpaO(232–297) and Flm(245–334):FlN(5–137) were concentrated to 18 mg ml\(^{-1}\) and pressed overnight with HRV 3C protease. One mg of hexahistidine-tagged SpaO(232–297, SeMet) was co-refolded with 50 mM maltose and 5 mM EDTA and they were further purified by one mg of hexahistidine–T4 lysozyme (PDB 2LZM). Structures were built in Phenix Autosol. The SPOA1,2–APAR::lysozyme structures were solved by molecular replacement in Phaser\(_{MR}\) using the experimentally phased cognate SPOA1,2 structure and T4 lysozyme (PDB 2LZM). Structures were built in Phenix (Autobuild) with additional manual model building performed in Coot\(_{26}\).

Structures were refined and validated in Phenix (Tables 1 and 2), SpaO(145–213) + SpaO(232–297) + OrgB(1–30):T4 lysozyme crystals exhibited twinning and were refined in Phenix using the twin law \( l-kh \). Ramachandran statistics for all models are as follows: SpaO(232–297, SeMet): 98% favoured, 0% outliers; SpaO(145–213) + SpaO(232–297): 98% favoured, 3% outliers; SpaO(145–213) + SpaO(232–297) + OrgB(1–30): T4 lysozyme: 94% favoured, 0.8% outliers; SpaO(145–213, SeMet) + SpaO(232–297, SeMet) + OrgB(1–30):T4 lysozyme: 89% favoured, 1.8% outliers; Flm(245–334):FlN(5–137): 92% favoured, 0.9% outliers; Flm(245–334):FlN(5–137) + FlH(1–18):T4 lysozyme: 94% favoured, 0.9% outliers.

ANOPE\(_{29}\) was used to perform post hoc analysis of anomalous scatterers in SpaO(145–213, SeMet) + SpaO(232–297, SeMet) + OrgB(1–30):T4 lysozyme crystals, providing additional empirical support for the SpaO–OrgB model coordinates (Supplementary Fig. 6b). Except where indicated, all representations of models and maps for figures were produced in QM\(_{26,28}\).

NMR spectroscopy. The NMR sample of refolded SpaO(140–297) consisted of 0.5 mg ml\(^{-1}\) in 100 mM NaCl, 400 mM NaCl, 10% (v/v) deuterium oxide, 10 mM Tris-Cl pH 7.0 and 35% glycerol. SpaO(145–213, SeMet) was concentrated to 8 mg ml\(^{-1}\) in 100 mM NaCl and 1 mM dithiothreitol. For comparison of the apo and APAR-bound forms, \(^{1}N^{15}C\)-labelled SpaO(140–297) was co-refolded with an excess of unlabelled thioredoxin-OrgB(2–30). The thioredoxin solubilization tag was cleaved off by overnight incubation with HRV 3C protease. Protease and affinity tags were removed on NiNTA resin and the SpaO–OrgB complex was separated from the majority of free thioredoxin by Superdex 75 gel filtration chromatography. The final concentration of the protein complex was 0.2 mM in 10 mM citrate buffer at pH 5.6 supplemented with 10% v/v deuterium oxide, 100 mM NaCl and 2 mM dithiothreitol.

The NMR data were acquired on Bruker 600, 800 and 900 MHz AVANCE spectrometers equipped with TCI/TXI Cryoprobes at 20°C for the apo-SpaO and 30°C for the APAR-bound forms. For resonance assignments of apo-SpaO, transverse relaxation-optimized triple-resonance\(_{31}\) experiments including trHNCQ, trHNC(A)CQ, trHNCQ(C), trHNCACB and trHNCQ(C)ACB were acquired at 600 and 900 MHz. A three dimensional \(^{15}N\)-edited nuclear Overhauser enhanced spectroscopy-heteronuclear single quantum coherence spectrum with 100 ms mixing time was also acquired at 900 MHz. To assign APAR-bound SpaO, a suite of conventional backbone experiments\(_{32}\) were acquired at 600 and 800 MHz.

The data were processed in Topspin 2.1 spectra and analysed using the Autolink module in CARA 1.5 (ref. 33). In both apo-SpaO and its complex with APAR, we were able to successfully assign > 95% of the backbone resonances. The heteronuclear chemical shifts were analysed using the TALOS \(_{34}\) database to predict the secondary structure of the protein. The weighted CS\(_{D}\) were calculated from amide proton (H) and nitrogen chemical shifts (\(^{15}N\)) using the following equation: \(CS_D = \sqrt{{(\Delta H^N) + (\Delta H^N/2)^2}}\).

Co-affinity purification assay. For co-affinity purification of the SpaO–OrgB-Invc complex (Figs 3 and 5), the proteins indicated were co-expressed and purified under native conditions as described above. For the SPOA-APAR-lysozyme pull-down experiment (Fig. 6), the indicated SPOA-containing proteins were Ni-affinity purified under native conditions, their affinity tags were removed by overnight incubation with HRV protease 3C and they were further purified by anion-exchange chromatography (as above). APAR:lysozyme fusions were then separately purified under denaturing conditions and were subjected to cation-exchange chromatography after refolding (as above). One mg of hexahistidine-tagged APAR:lysozyme fusion protein was mixed with 2 mg of the indicated SPOA-containing protein in 0.2 M NaCl and 20 mM Tris-Cl pH 8.0 (final volume of 0.35 ml) and incubated on ice overnight. The mixture was passed twice over 2 ml of NiNTA resin, washed with 8 ml wash buffer (200 mM NaCl, 20 mM Tris-Cl pH 8.0, 5% v/v glycerol and 30 mM imidazole-Cl pH 8.0) and then eluted in 3.5 ml elution buffer (200 mM NaCl, 20 mM Tris-Cl pH 8.0, 5% v/v glycerol and 360 mM imidazole-Cl pH 8.0).

In vitro secretion assay. S. typhimurium of the indicated genotype were grown for 6 h at 37°C in LB medium with NaCl supplemented to a final concentration of 0.5 M. Cells were pelleted at 3,400 g for 2 min. The supernatants were 0.22-µm filtered. Secreted proteins were precipitated from the filtered supernatants with 15% trichloroacetic acid overnight at 4°C. The precipitate was
pellet by centrifugation at 3,400 g for 1 h at 4 °C, resuspended in ice-cold acetone and transferred to a microfuge tube. After 0.25 h on ice, the precipitate was harvested by centrifugation at 16,000g for 0.75 h at 4 °C and resuspended in 0.2 M Tris-Cl pH = 8.0 and 0.2 M NaCl to neutralize any residual acid before the addition of SDS-polyacrylamide gel electrophoresis loading buffer. For plasmid complementation analysis, S. typhimurium were electroporated with SpA O sequences cloned into the pBAD vector and expression was induced with 0.01% arabinose for the entire duration of the experiment.

Fluorescence microscopy. S. typhimurium were grown as for the in vitro secretion assay. Cells were harvested by centrifugation, washed three times in PBS and fixed overnight with 4% formaldehyde in PBS at 4 °C. Cells were again washed three times in PBS, counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich) and immobilized on poly-L-lysine (Sigma-Aldrich)-coated coverslips. Covers were mounted in Prolong Diamond (Life Technologies) and sealed with nail polish. Slides were imaged on a DeltaVision Image Restoration Microscope with a ×100 objective (Applied Precision). Images were deconvoluted in Softworx (Applied Precision) and processed identically in ImageJ (NIH) and Photoshop (Adobe).

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Author contributions
R.Q.N. conceived of the project, designed and performed experiments, analysed data and wrote and revised the paper. S.B. designed and performed experiments, analysed data and revised the paper. M.L. performed experiments and revised the paper. C.E.S. conceived the project, analysed data and wrote and revised the paper.

Additional information
Accession codes: Atomic coordinates have been deposited in the Protein Data Bank under the following deposition codes: 4XVX, SpaO(322–297, SeMet); 4YX, SpaO(145–213) + SpaO(232–297); 4YXT, SpaO(145–213) + SpaO(232–297) + OrgB(1–30):T4 lysozyme; 4YXA, SpaO(145–213, SeMet) + SpaO(223–297, SeMet) + OrgB(1–30):T4 lysozyme (native); 4YXB, Flim(245–344):FlI(5–137).
SeMet: 4YXC, FliM(245–334):FliN(5–137) + FliH(1–18):T4 lysozyme. NMR chemical shifts are deposited in the BMRB under ID 26543 (apo-SpaO) and 26546 (APAR-bound SpaO).

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