RESEARCH ARTICLE

Figures and figure supplements

Recognition of discrete export signals in early flagellar subunits during bacterial type III secretion

Owain J Bryant *et al*

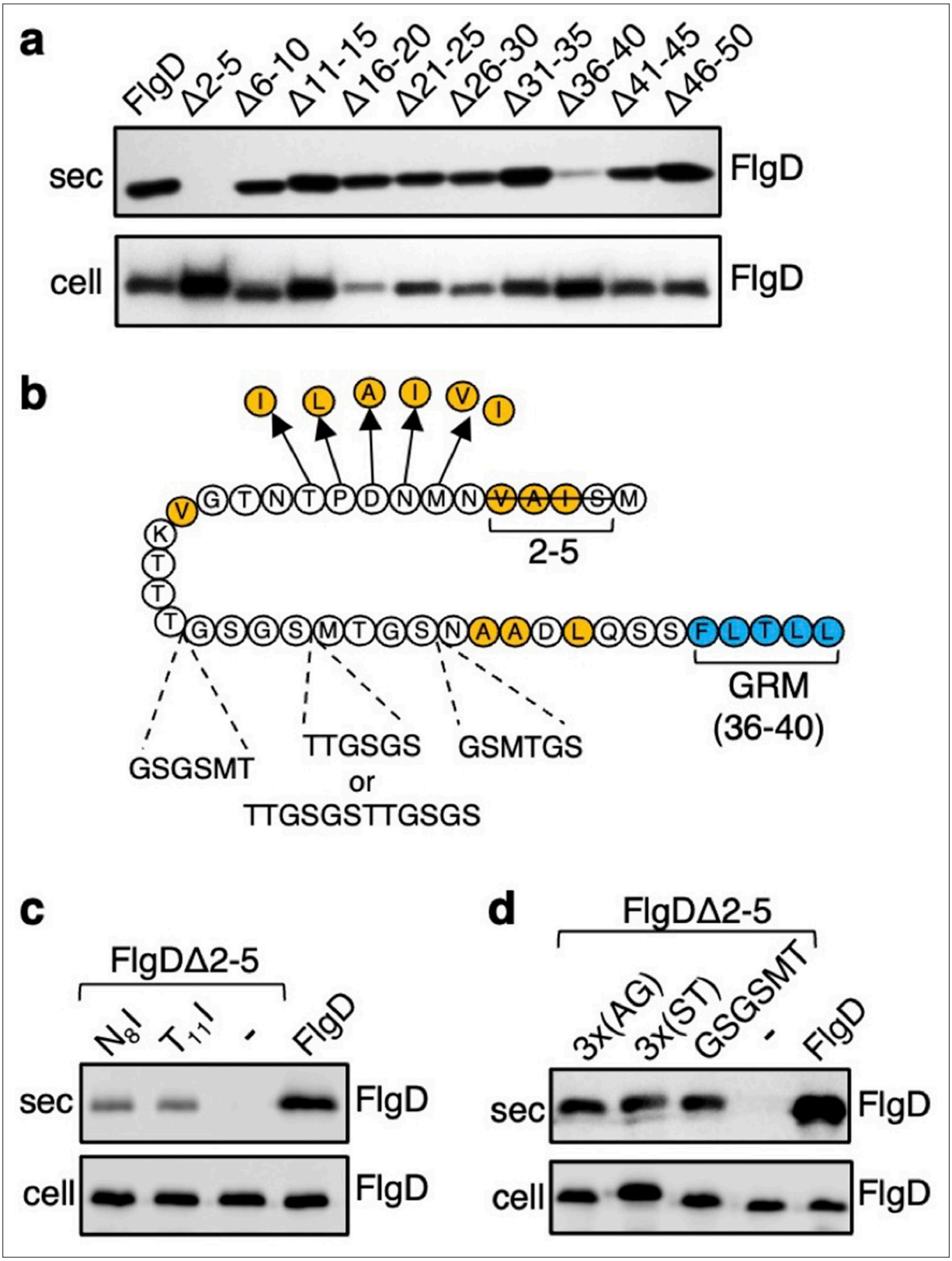

Figure 1. Screening for export-defective FlgD variants.

(**a**) Whole cell (cell) and supernatant (sec) proteins from late exponential phase cultures of a *Salmonella flgD* null strain expressing plasmid-encoded wild type FlgD (FlgD) or its variants (Δ2–5, Δ6–10, Δ11–15, Δ16–20, Δ21–25, Δ26–30, Δ31–35, Δ36–40, Δ41–45 or Δ46–50) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera. (**b**) A schematic displaying all intragenic suppressor mutations within amino

Figure 1 continued on next page

Figure 1 continued

acids 1–40 of FlgD isolated from the FlgDΔ2–5 variant. Small non-polar residues are highlighted in orange. All suppressor mutations were located between the gate-recognition motif (GRM, blue) and the extreme N-terminus, and can be separated into two classes: insertions or duplications that introduced additional sequence between valine-15 and the gate-recognition motif, or missense mutations that re-introduce small non-polar residues at the N-terminus. All intragenic suppressors isolated from FlgDΔ2–5 are displayed in this figure. (**c**) Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of *Salmonella flgD* null strains expressing plasmid-encoded: suppressor mutants isolated from the FlgDΔ2–5 variant (FlgDΔ2–5-N_8I or FlgDΔ2–5-T_{11}I), FlgDΔ2–5 variant (-) or wild type FlgD (FlgD) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera. (**d**) Whole cell (cell) and supernatant (sec) proteins from late exponential phase cultures of *Salmonella flgD* null strains expressing plasmid-encoded wild type FlgD (labelled FlgD), FlgDΔ2–5 (labelled as DΔ2–5) or variants of FlgDΔ2–5 containing between residues 19 and 20 a six-residue insertion of either small non-polar (AGAGAG) residues (labelled as 3 x(AG)), polar (STSTST) residues (labelled as 3 x(ST)), or the sequence from an isolated insertion suppressor mutant (GSGSMT) (labelled as GSGSMT), were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera.

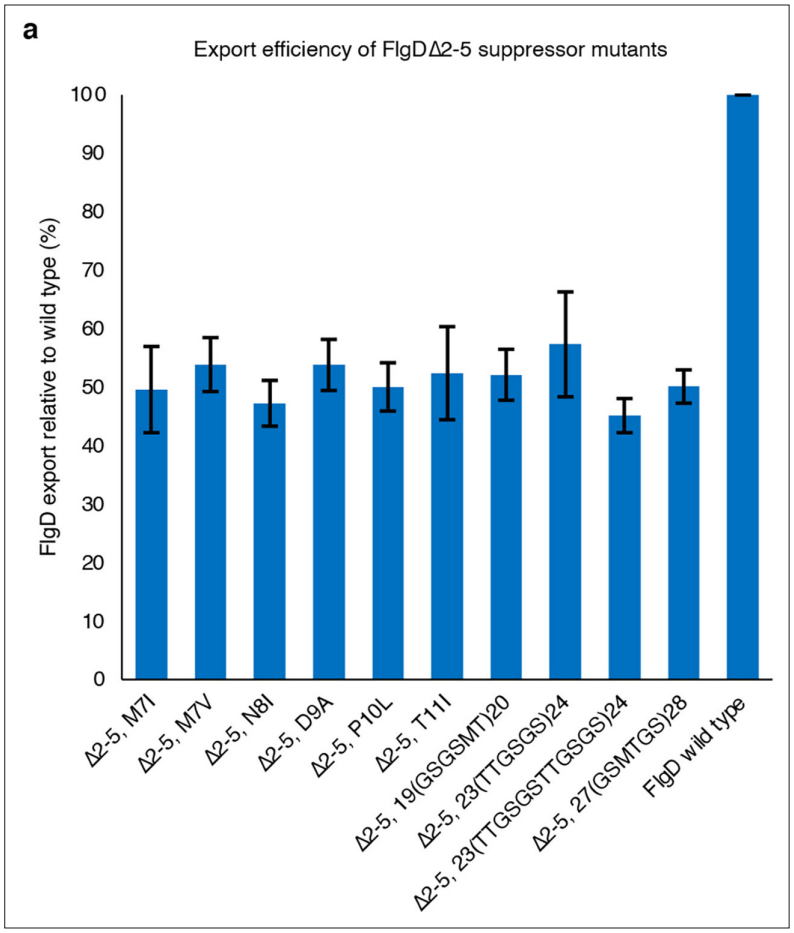

Figure 1—figure supplement 1. Relative amounts of FlgD secreted into culture supernatants from a recombinant *Salmonella flgD* null strain expressing plasmid-based FlgDΔ2–5 suppressor mutants and wild type FlgD. Relative amounts of FlgD secreted into culture supernatants were quantified using image Studio Lite software and normalised to the level of wild type FlgD in culture supernatants. Error bars represent the standard error of the mean calculated from at least three biological replicates.

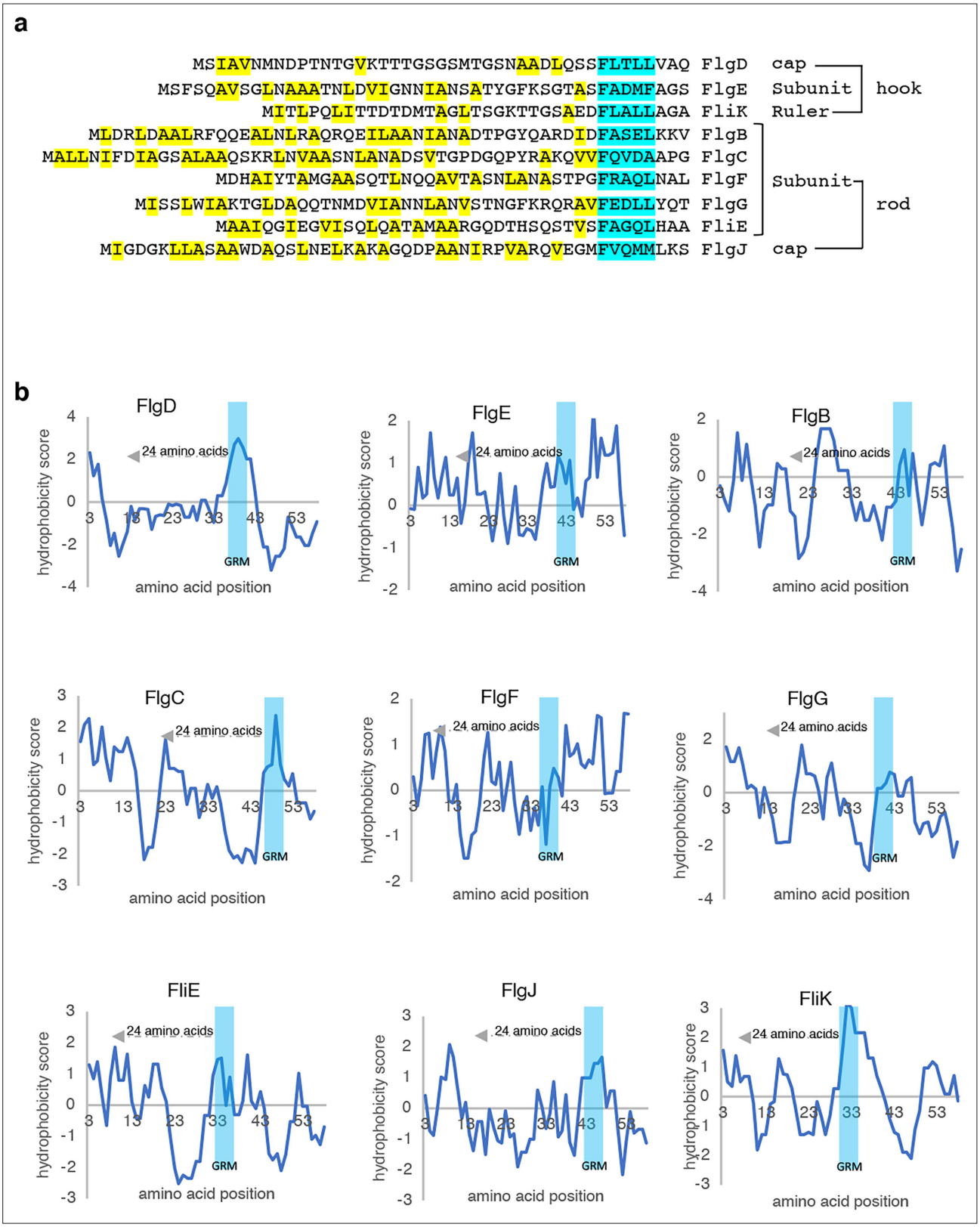

Figure 1—figure supplement 2. Hydrophobicity of N-terminal sequences of flagellar rod and hook subunits. (**a**) N-terminal sequences of all *Salmonella* flagellar rod and hook subunits aligned to their gate-recognition motif (GRM, blue). Small non-polar residues upstream from the gate-recognition motif are highlighted (yellow). (**b**). Hydrophobicity plots for the N-terminal 60 residues of each *Salmonella* flagellar rod and hook subunit were generated by ExPASy tools using the Kyte and Doolittle method (***Kyte and Doolittle, 1982***). The *x* axis of the plot indicates the amino acid position, starting from the

Figure 1—figure supplement 2 continued on next page

Figure 1—figure supplement 2 continued

N terminus. The *y* axis of the plot indicates the hydrophobicity of the amino acid sequence, where higher values represent higher hydrophobicity. Amino acid sequence corresponding to the gate-recognition motif of each subunit is highlighted in blue.

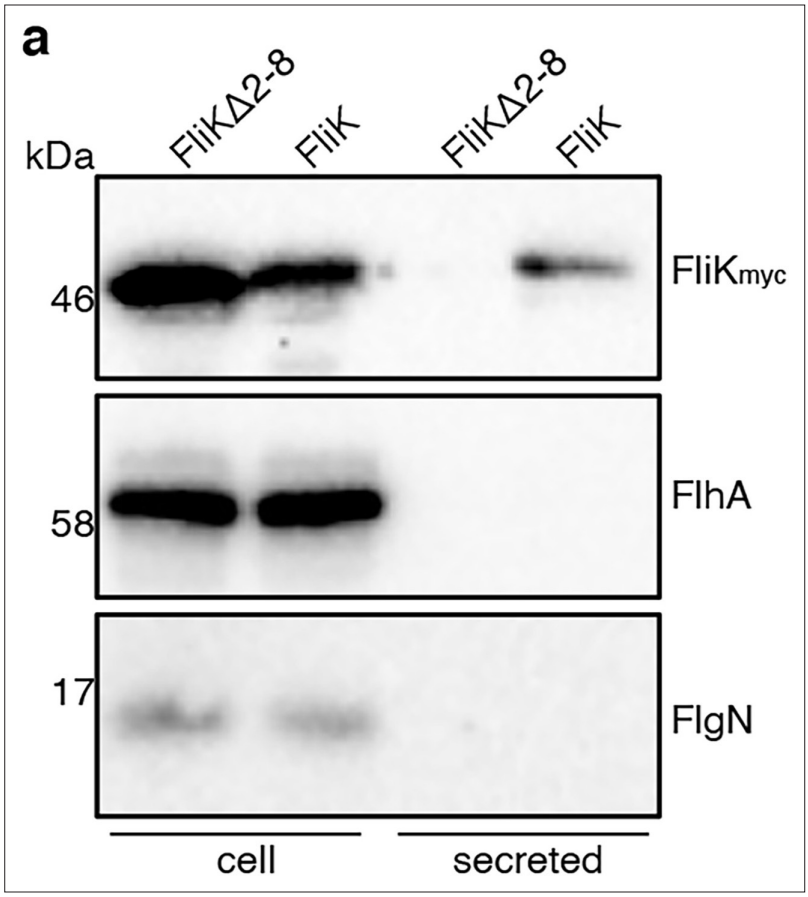

Figure 1—figure supplement 3. Deletion of the FliK N-terminal signal attenuates export.
Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of *Salmonella flgD* null strains expressing wild type FliK containing a C-terminal myc tag for immunodetection (FliK) or its variant in which residues two to eight are deleted (FliKΔ2–8) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-myc monoclonal antisera (top), anti-FlhA (middle), or anti-FlgN (bottom) polyclonal anti-sera. Apparent molecular weights are in kilodaltons (kDa).

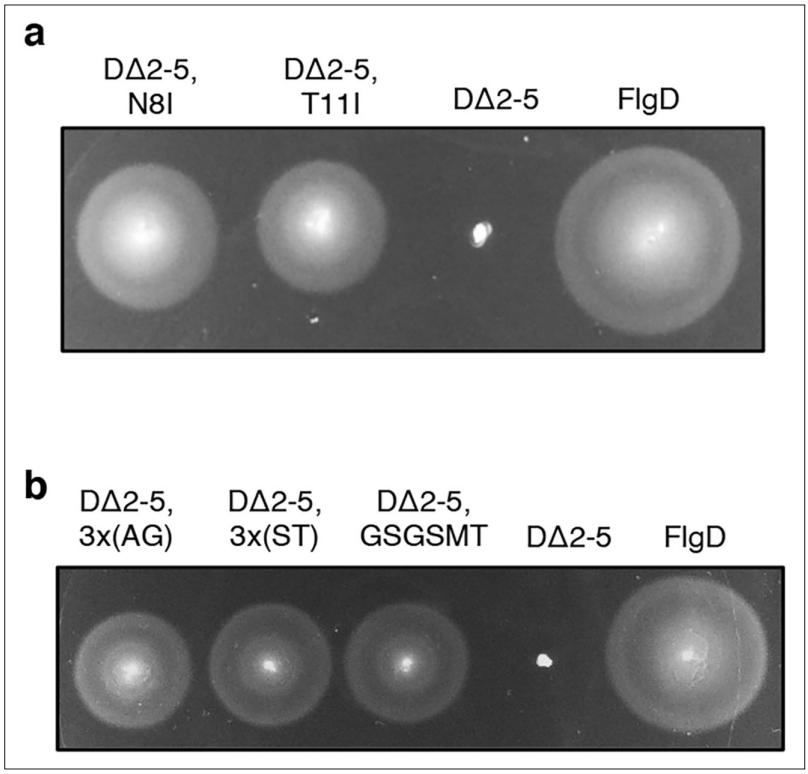

Figure 1—figure supplement 4. Swimming motility of FlgDdelta2-5 suppressor mutant strains. (**a**) Swimming motility of a *Salmonella* Δ*recA* strain expressing: suppressor mutants isolated from the FlgDΔ2–5 variant (labelled as FlgDΔ2–5 N_8I or FlgDΔ2–5 T_{11}I), the parent FlgDΔ2–5 variant (labelled as DΔ2–5) or wild type FlgD (labelled as FlgD). Motility was assessed in 0.25% soft-tryptone agar containing 100 µg/ml ampicillin and 50 µM IPTG and incubated 4–6 hours at 37 °C. (**b**). Swimming motility of a *Salmonella* Δ*recA* strain expressing wild type FlgD (labelled as FlgD), FlgDΔ2–5 (labelled as DΔ2–5) or its variants containing a six-residue insertion between residues 19 and 20 of either small non-polar (AGAGAG) residues (labelled as DΔ2–5 3 x(AG)), polar (STSTST) residues (labelled as DΔ2–5 3 x(ST)), or the sequence from an isolated insertion suppressor mutant (GSGSMT) (labelled as DΔ2–5 GSGSMT). Motility was assessed in 0.25% soft-tryptone agar containing 100 µg/ml ampicillin and 50 µM IPTG and incubated 4–6 hr at 37 °C.

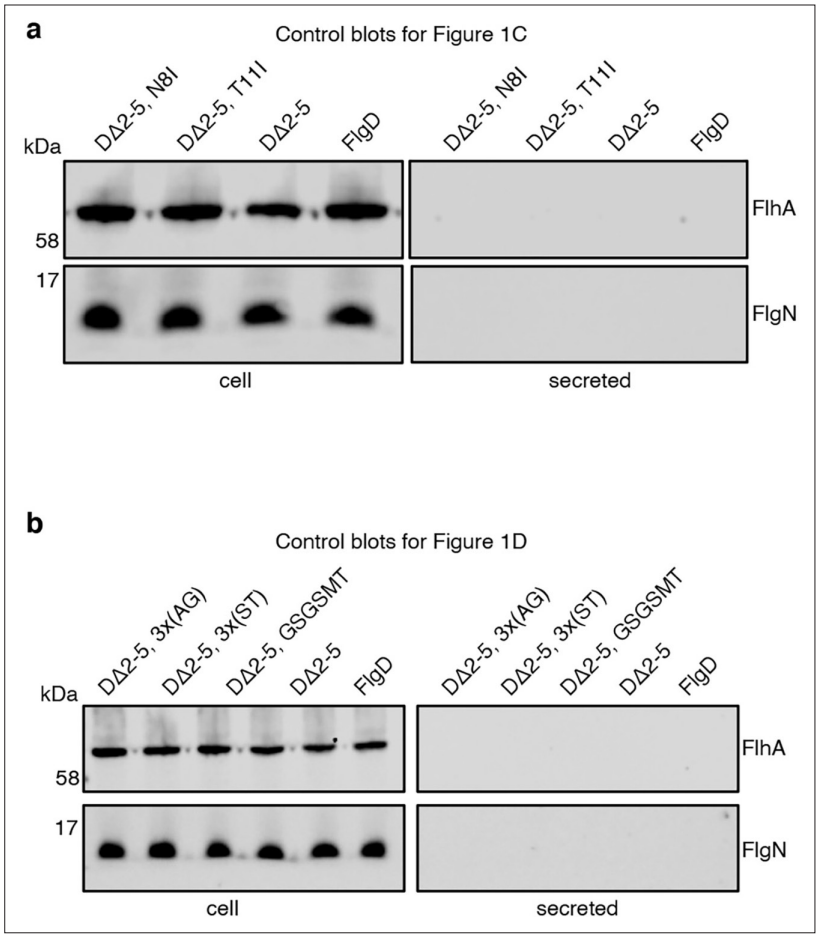

Figure 1—figure supplement 5. Control blots for ***Figure 1C-D*** showing that membrane embedded FlhA and cytoplasmic FlgN proteins are expressed (cell) but absent from supernatant fractions (sec). (**a**) Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of *Salmonella flgD* null strains expressing: suppressor mutants isolated from the FlgDΔ2–5 variant (FlgDΔ2–5 N_8I or FlgDΔ2–5 $T_{11}I$), FlgDΔ2–5 variant (-) or wild type FlgD (FlgD) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlhA or FlgN polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa). (**b**). Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of *Salmonella flgD* null strains expressing wild type FlgD (FlgD), FlgDΔ2–5 (labelled as DΔ2–5) or its variants containing a six-residue insertion between residues 19 and 20 of either small non-polar (AGAGAG) residues (labelled as DΔ2–5 3 x(AG)), polar (STSTST) residues (labelled as DΔ2–5 3 x(ST)), or the sequence from an isolated insertion suppressor mutant (GSGSMT) (labelled as DΔ2–5 GSGSMT) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa).

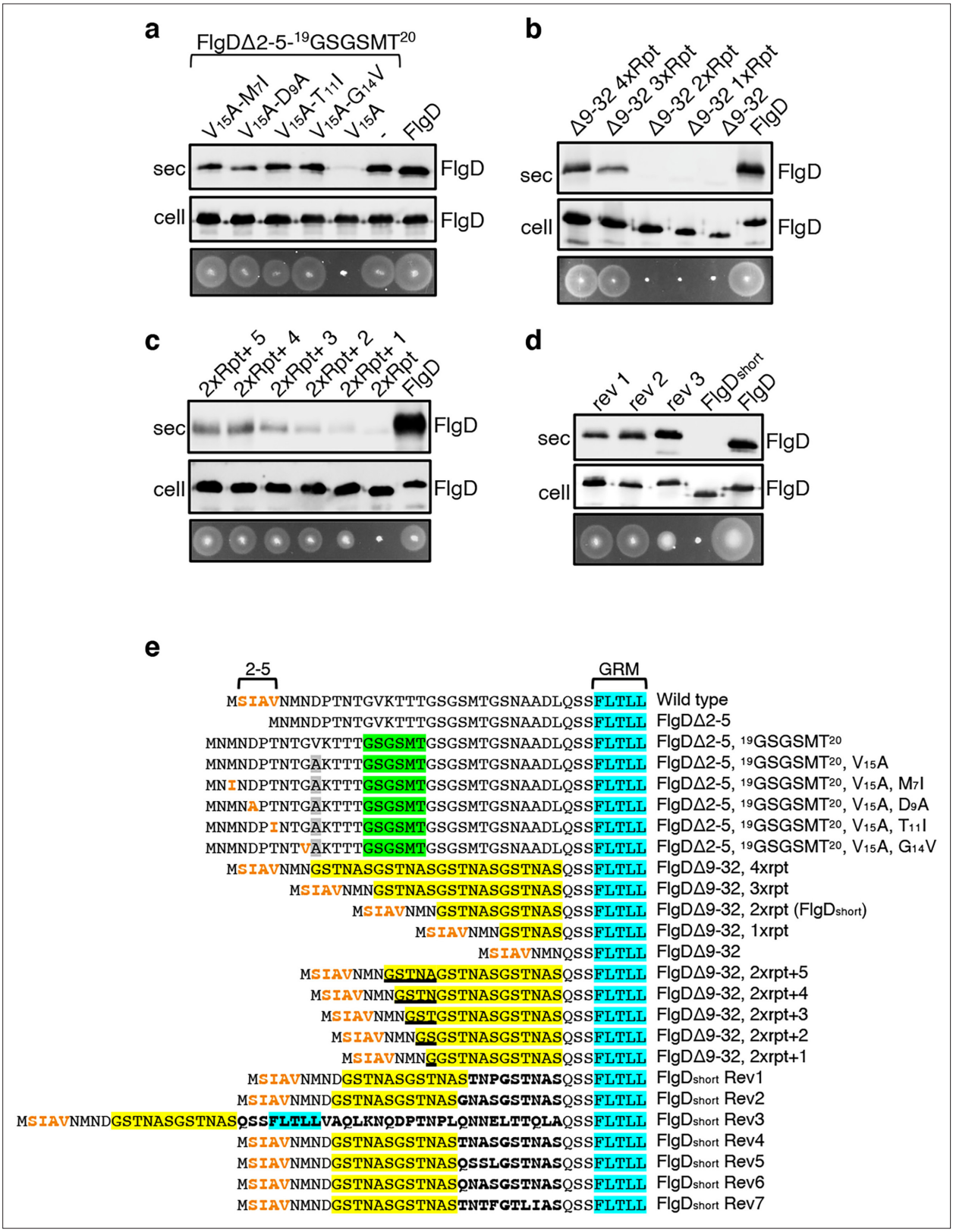

Figure 2. Export of FlgD variants in which the position of the hydrophobic export signal is varied relative to the gate recognition motif (GRM). Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing plasmid encoded suppressor mutants isolated from the FlgDΔ2-5-19(GSGSMT)20 $V_{15}A$ variant ($V_{15}A$-M_7I, $V_{15}A$-D_9A, $V_{15}A$-$T_{11}I$, $V_{15}A$-$G_{14}V$), their parent FlgD variant FlgDΔ2-5-19(GSGSMT)20 $V_{15}A$ (labelled as $V_{15}A$), FlgDΔ2-5-19(GSGSMT)20 (labelled as -) or wild type FlgD (FlgD) were separated by SDS (15%)-PAGE and

Figure 2 continued on next page

Figure 2 continued

analysed by immunoblotting with anti-FlgD polyclonal antisera. Swimming motility (bottom panel; 0.25% soft tryptone agar) of the same strains were carried out at 37 °C for 4–6 hours. (**b**). Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing plasmid-encoded wild type FlgD (labelled as FlgD), FlgDΔ9–32 or its variants in which residues 9–32 were replaced by between one and four six-residue repeats of Gly-Ser-Thr-Asn-Ala-Ser (GSTNAS): (Δ9–32 4xRpt, Δ9–32 3xRpt, Δ9–32 2xRpt or Δ9–32 1xRpt) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera. Swimming motility (bottom panel; 0.25% soft tryptone agar) of the same strains were carried out at 37 °C for 4–6 hours. (**c**). Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing plasmid-encoded wild type FlgD (labelled as FlgD), a FlgD variant in which residues 9–32 were replaced by two repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (labelled as 2xRpt) or its variants containing between one and five additional residues inserted directly after the two repeats (labelled as 2xRpt + 1, 2xRpt + 2, 2xRpt + 3, 2xRpt + 4 or 2xRpt + 5) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera. Swimming motility (bottom panel; 0.25% soft tryptone agar) of the same strains were carried out at 37 °C for 4–6 hr. (**d**). Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing plasmid-encoded wild type FlgD (labelled as FlgD), a FlgD variant in which residues 9–32 were replaced by two repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (labelled as $FlgD_{short}$) or suppressor mutants isolated from this strain (labelled as rev1, rev2 or rev3) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera. Swimming motility (bottom panel; 0.25% soft tryptone agar) of the same strains were carried out at 37 °C for 4–6 hr. (**e**). N-terminal sequences of wild type FlgD and its variants aligned to their gate-recognition motif (GRM; blue). The following sequence features or residues are displayed: The N-terminal hydrophobic signal (residue 2–5; orange), the Gly-Ser-Gly-Ser-Met-Thr (GSGSMT) insertion (green) isolated from the FlgDΔ2–5 suppressor screen, the valine-15 to alanine mutation (grey), small non-polar mutations (M7I, D9A, T11I, G14V; orange) isolated from the FlgDΔ2–5, ^{19}GSGSMT^{20} suppressor screen, FlgDΔ9–32 and its variants in which residues 9–32 are replaced with one, two, three or four repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (GSTNAS; yellow), FlgDΔ9–32, 2xrpt (hereafter termed $FlgD_{short}$) containing five, four, three, two, or one additional residues (underlined) inserted between the GRM and N-terminal hydrophobic signal, and suppressor mutants (Rev 1–7) isolated from $FlgD_{short}$ that introduced additional residues (bold) between the N-terminal hydrophobic signal (orange) and the gate-recognition motif (blue).

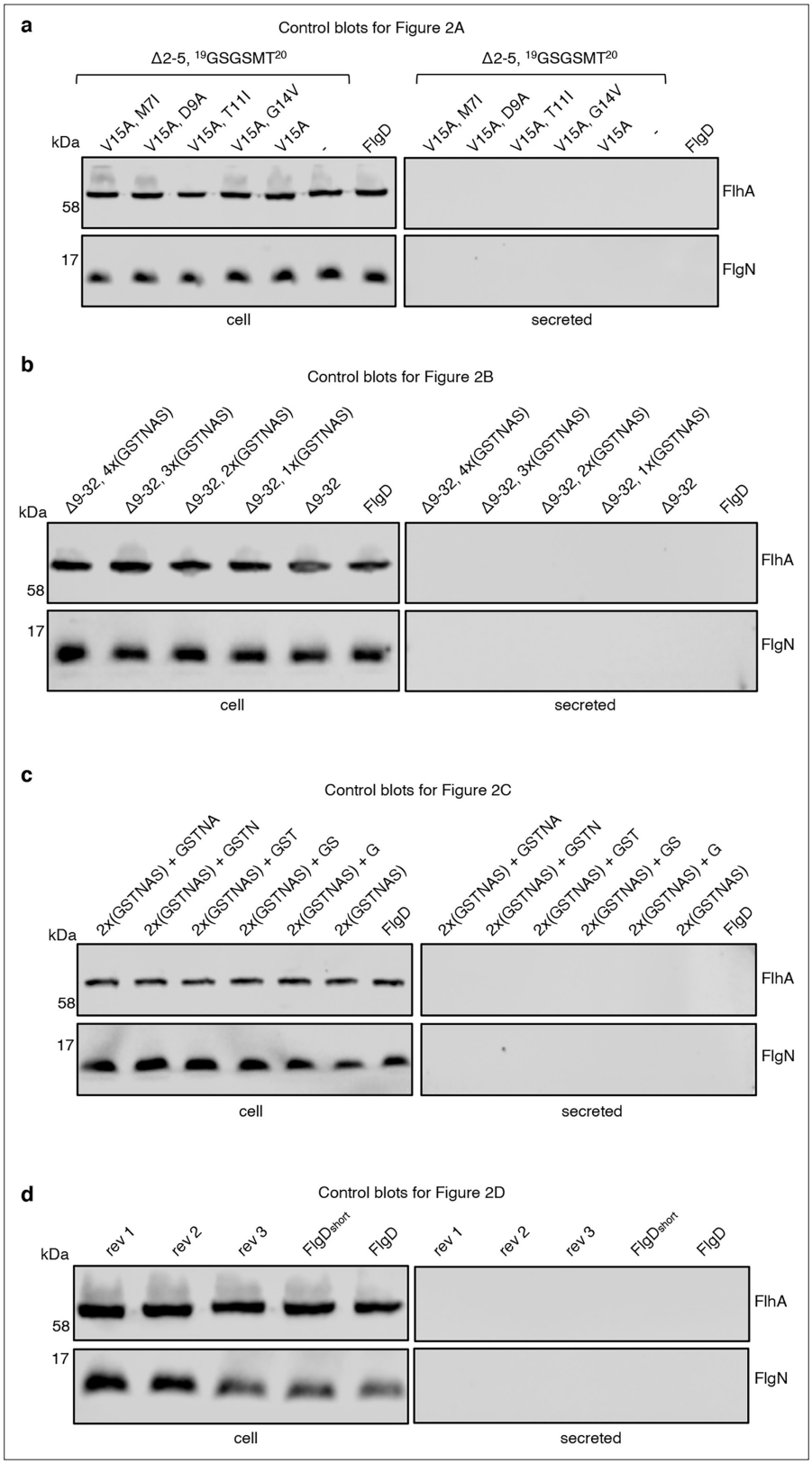

Figure 2—figure supplement 1 Control blots for Figure 2A-C showing that membrane-embedded FlhA and cytoplasmic FlgN are expressed (cell) but absent from supernatant fractions (sec). (**a**) Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing suppressor mutants isolated from a FlgDΔ2-5-19(GSGSMT)20 V_{15}A variant (V_{15}A-M_7I, V_{15}A-D_9A, V_{15}A-T_{11}I, V_{15}A-G_{14}V),

Figure 2—figure supplement 1 continued on next page

Figure 2—figure supplement 1 continued

their parent FlgD variant FlgDΔ2-5-19(GSGSMT)20 $V_{15}A$ (labelled as $V_{15}A$), FlgDΔ2-5-19(GSGSMT)20 (-) or wild type FlgD (FlgD) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlhA and FlgN polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa). (**b**). Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing wild type FlgD (FlgD), FlgDΔ9–32 or its variants in which residues 9–32 were replaced by between one and four six-residue repeats of Gly-Ser-Thr-Asn-Ala-Ser (GSTNAS): (Δ9–32 4xRpt, Δ9–32 3xRpt, Δ9–32 2xRpt, Δ9–32 1xRpt) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlhA and anti-FlgN polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa). (**c**). Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing wild type FlgD (labelled as FlgD), a FlgD variant in which residues 9–32 were replaced by two repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (labelled as 2xRpt) or its variants containing between one and five additional residues inserted directly after the two repeats (labelled as 2xRpt + 1, 2xRpt + 2, 2xRpt + 3, 2xRpt + 4 or 2xRpt + 5) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa). (**d**). Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing wild type FlgD (labelled as FlgD), a FlgD variant in which residues 9–32 were replaced by two repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (labelled as $FlgD_{short}$) or suppressor mutants isolated from this strain (labelled as rev1, rev2 or rev3) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlhA and anti-FlgN polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa).

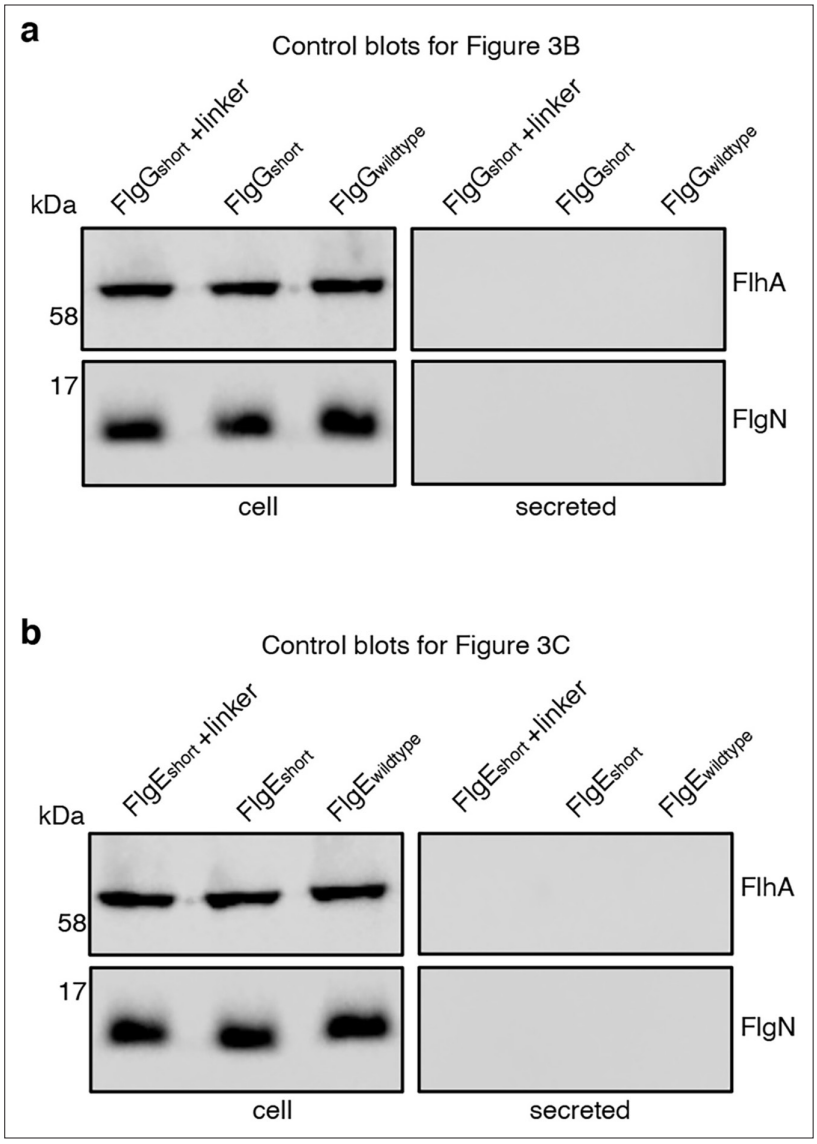

Figure 2—figure supplement 2. Control blots for ***Figure 3B-C*** showing that membrane embedded FlhA and cytoplasmic FlgN proteins (cell) are expressed but absent from supernatant fractions (sec). (**a**) Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of a *Salmonella flgE* null strain expressing wild type FlgG (labelled as $FlgG_{wild\ type}$), a FlgG variant in which residues 11–35 were deleted (labelled as $FlgG_{short}$) or a FlgG variant in which residues 11–35 were replaced by four repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (labelled as $FlgG_{short}$ + 4 Rpt). All FlgG variants were engineered to contain an internal 3xFLAG tag for immunodetection. Proteins were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlhA or anti-FlgN polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa). (**b**). Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing wild type FlgE (labelled as $FlgE_{wild\ type}$), a FlgE variant in which residues 9–32 were deleted (labelled as $FlgE_{short}$) or a FlgE variant in which residues 9–32 were replaced by four repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (labelled as $FlgE_{short}$ + 4 Rpt). All FlgE variants were engineered to contain an internal 3xFLAG tag for immunodetection. Proteins were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FLAG monoclonal antisera.

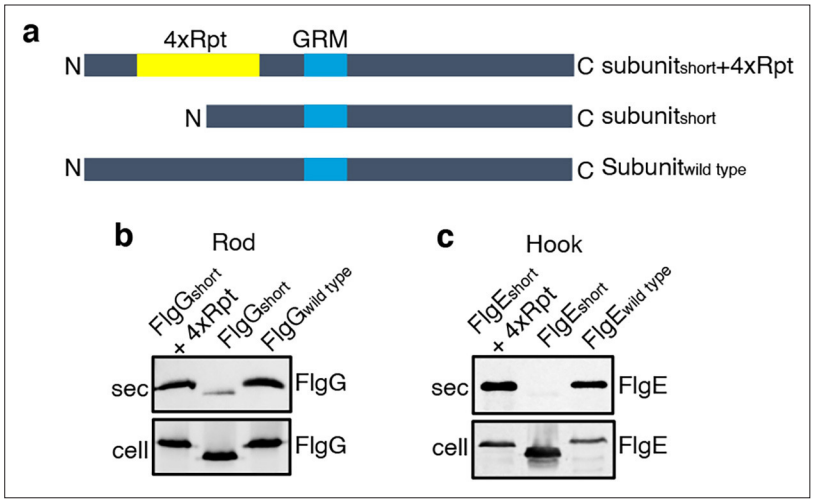

Figure 3. Effect of the relative position of the N-terminus and GRM on the export of other rod and hook subunits. (**a**) Schematic representation of a wild-type subunit (labelled as subunit$_{wild\ type}$), a subunit containing a deletion of sequence from between the N-terminus and GRM (labelled as subunit$_{short}$) and a subunit in which the deleted sequence was replaced by four repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (yellow, labelled as subunit$_{short}$ +4 Rpt). (**b**). Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of a *Salmonella flgE* null strain expressing plasmid-encoded wild type FlgG (labelled as FlgG$_{wild\ type}$), a FlgG variant in which residues 11–35 were deleted (labelled as FlgG$_{short}$) or a FlgG variant in which residues 11–35 were replaced by four repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (labelled as FlgG$_{short}$ + 4 Rpt). All FlgG variants were engineered to contain an internal 3xFLAG tag for immunodetection. Proteins were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FLAG monoclonal antisera. (**c**). Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing plasmid-encoded wild type FlgE (labelled as FlgE$_{wild\ type}$), a FlgE variant in which residues 9–32 were deleted (labelled as FlgE$_{short}$) or a FlgE variant in which residues 9–32 were replaced by four repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (labelled as FlgE$_{short}$ + 4 Rpt). All FlgE variants were engineered to contain an internal 3xFLAG tag for immunodetection. Proteins were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FLAG monoclonal antisera.

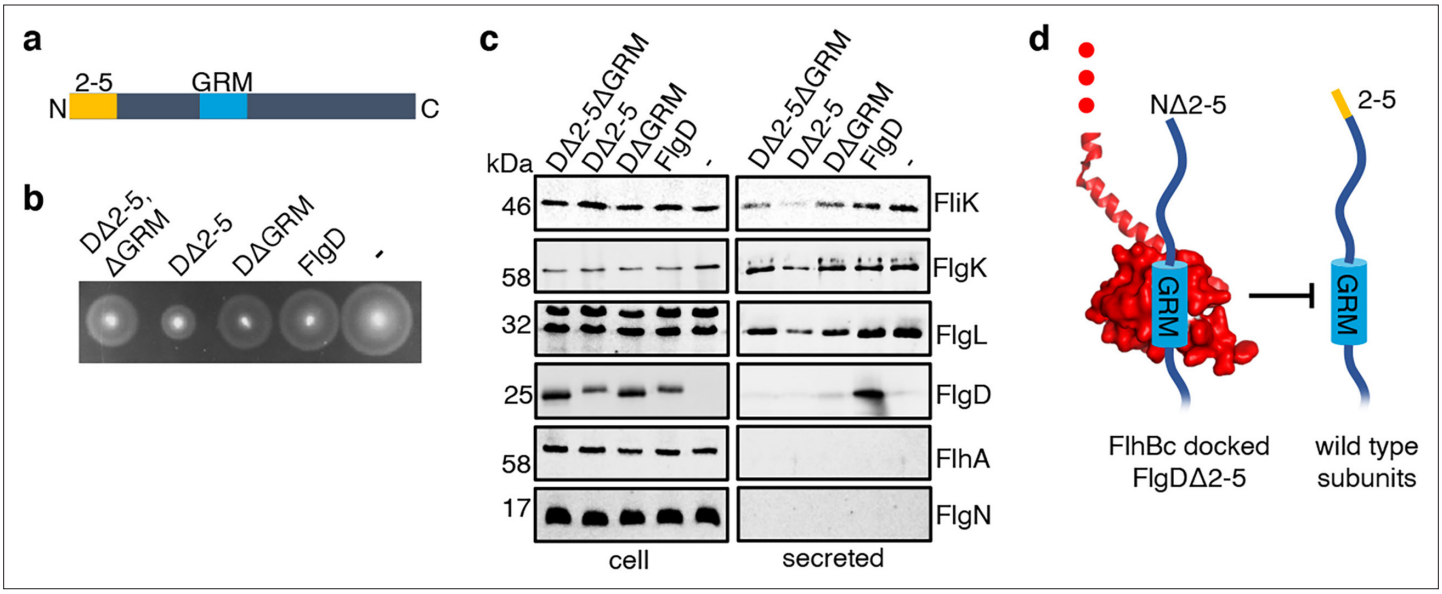

Figure 4. Effect on subunit export of overexpressed FlgDΔ2–5 and variants.
(**a**) Schematic representation of a FlgD subunit containing a N-terminal hydrophobic signal (orange, labelled as 2–5) and a gate-recognition motif (blue, labelled as GRM). (**b**) Swimming motility of a *Salmonella* Δ*recA* strain expressing plasmid-encoded wild type FlgD (FlgD), its variants (DΔ2-5ΔGRM, DΔ2–5 or DΔGRM) or empty pTrc99a vector (-). Motility was assessed in 0.25% soft-tryptone agar containing 100 µg/ml ampicillin and 100 µM IPTG and incubated for 4–6 hr at 37 °C. (**c**) Whole cell (cell) and secreted proteins (secreted) from late-exponential-phase cultures were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FliK (hook ruler subunit), anti-FlgK and anti-FlgL (hook-filament junction subunits), anti-FlgD hook cap subunit, anti-FlhA (component of the export machinery) and anti-FlgN (export chaperone for FlgK and FlgL) polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa).(**d**). A model depicting a FlgDΔ2–5 subunit (left) docked *via* its gate-recognition motif (GRM, blue) at the subunit binding pocket on $FlhB_C$ (PDB: 3B0Z ***Kuhlen et al., 2020***, red), preventing wild type subunits (right) from docking at $FlhB_C$.

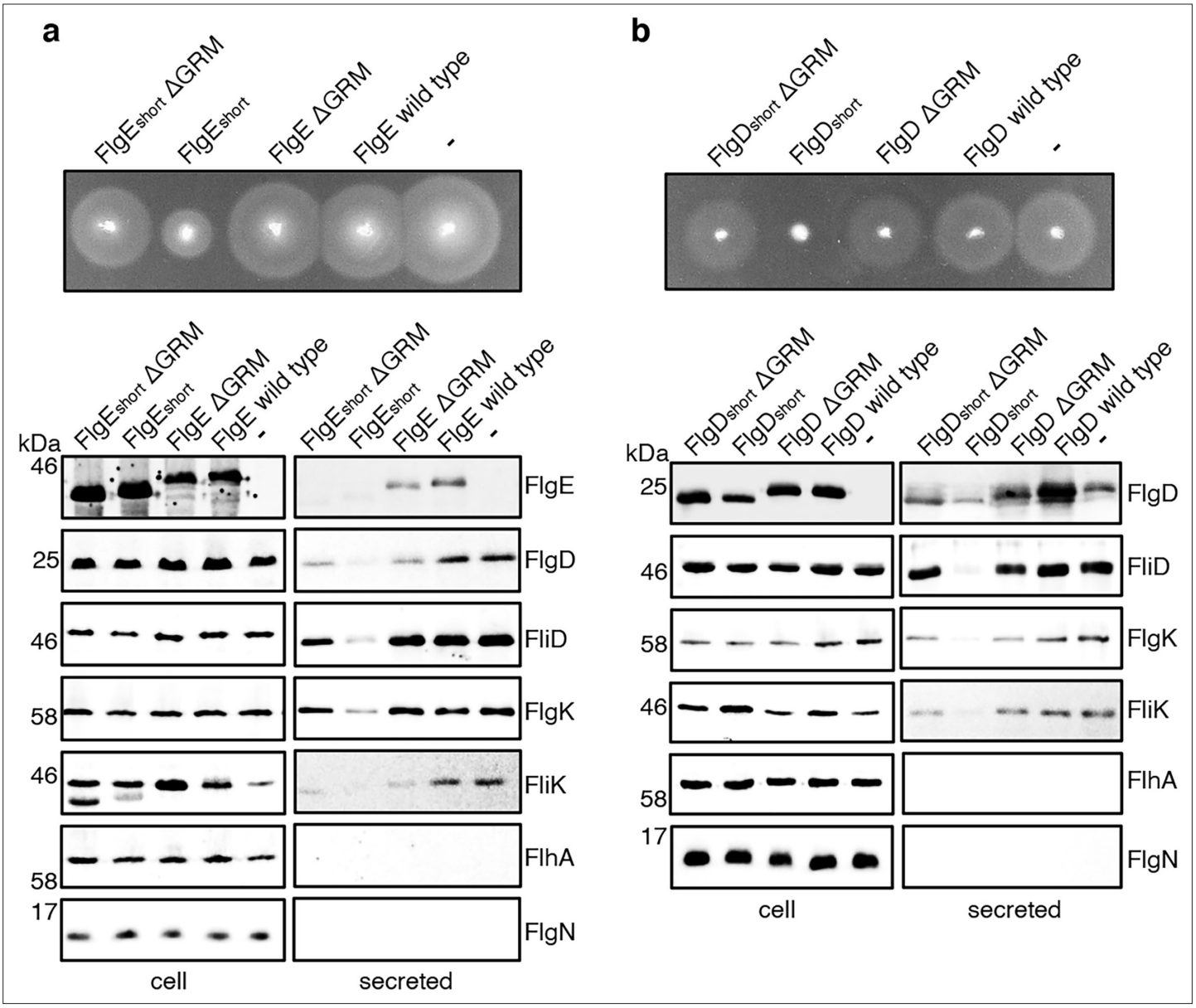

Figure 5. Effect on subunit export of overexpressed $FlgE_{short}$, $FlgD_{short}$ and variants.
(**a**) Swimming motility of a *Salmonella* Δ*recA* strain expressing plasmid-encoded wild type FlgE (labelled as FlgE wild type), a FlgE variant in which residues 9–32 were deleted (labelled as $FlgE_{short}$), a FlgE variant in which residues 9–32 and residues 39–43 (corresponding to the gate-recognition motif) were deleted (labelled as $FlgE_{short}$ΔGRM), a FlgE variant in which residues 39–43 were deleted (labelled as FlgEΔGRM) or empty pTrc99a vector (labelled as -). All FlgE variants were engineered to contain an internal 3xFLAG tag for immunodetection. Motility was assessed in 0.25% soft-tryptone agar containing 100 µg/ml ampicillin and 100 µM IPTG and incubated for 4–6 hr at 37 °C (top panel). Whole cell (cell) and secreted proteins (secreted) from late-exponential-phase cultures were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FLAG monoclonal antisera (to detect the flag tagged hook subunit FlgE) or anti-FlgD (hook cap subunit), anti-FliD (filament cap subunit), anti-FlgK (hook-filament junction subunit), anti-FliK (hook-ruler subunit), anti-FlhA (component of the export machinery) and anti-FlgN (chaperone for FlgK and FlgL) polyclonal antisera (bottom). Apparent molecular weights are in kilodaltons (kDa).(**b**) Swimming motility of a *Salmonella* Δ*recA* strain expressing plasmid-encoded wild type FlgD (labelled as FlgD wild type), a FlgD variant in which residues 9–32 were replaced with two repeats of the six amino acid sequence Gly-Ser-Thr-Asn-Ala-Ser (labelled as $FlgD_{short}$), a FlgD variant in which residues 9–32 were replaced with two repeats of the six amino acid sequence Gly-Ser-Thr-Asn-Ala-Ser and residues 36–40 were deleted (labelled $FlgD_{short}$ΔGRM), a FlgD variant in which residues 36–40 were deleted (labelled as FlgDΔGRM) or empty pTrc99a vector (labelled as -). Motility was assessed in 0.25% soft-tryptone agar containing 100 µg/ml ampicillin and 100 µM IPTG and incubated for 4–6 hours at 37 °C (top panel). Whole cell (cell) and secreted proteins (secreted) from late-exponential-phase cultures were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD (hook cap subunit), anti-FliD (filament cap subunit), anti-FlgK (hook-filament junction subunit), anti-FliK (hook ruler subunit), anti-FlhA (component of export machinery), and anti-FlgN (chaperone for FlgK and FlgL) polyclonal antisera (bottom). Apparent molecular weights are in kilodaltons (kDa).

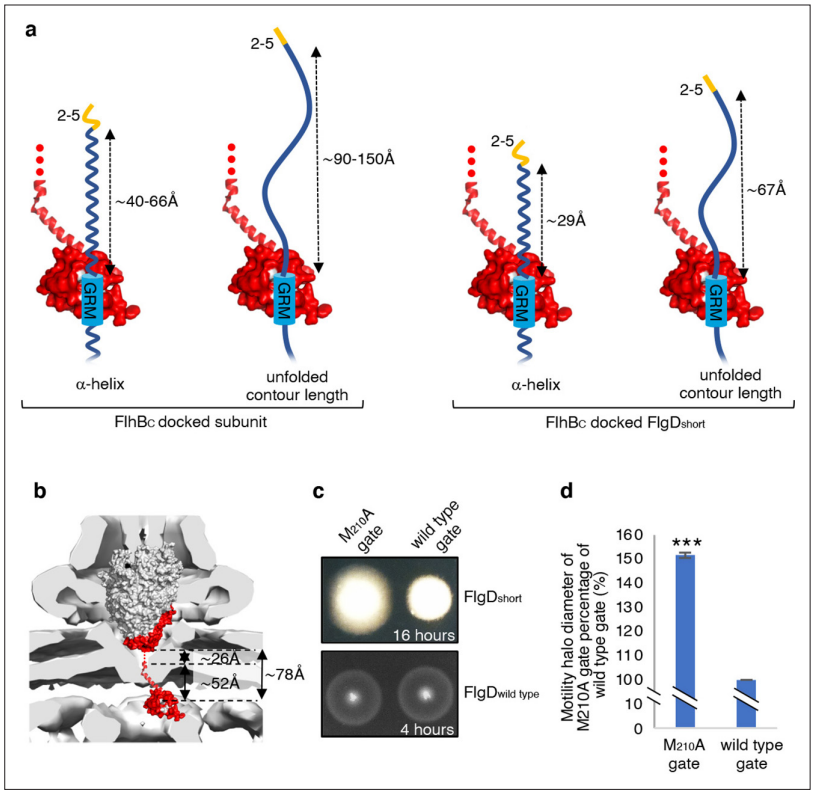

Figure 6. Suppression of the $FlgD_{short}$ motility defect by mutations in FliP. (**a**) A model depicting subunits docked via their gate-recognition motif (GRM, blue) at the subunit binding pocket on $FlhB_C$ (PDB: 3B0Z ***Meshcheryakov et al., 2013***, red) with N-termini of early flagellar subunits adopting either an α-helical conformation separating the N-terminal hydrophobic signal (2–5, orange) and gate-recognition motif (GRM, blue) by ~40–60 ångstrom (where each amino acid is on average separated by ~1.5 Å, left) or an unfolded conformation where the unfolded contour length separating the N-terminal hydrophobic signal (2-5) and gate-recognition motif (GRM) is ~90–150 ångstrom (where each amino acid is on average separated by ~3.5 Å, middle left). Values corresponding to the distance separating the N-terminal hydrophobic signal (2–5, orange) and gate-recognition motif (GRM, blue) of a FlgD subunit variant in which residues 9–32 are replaced with two repeats of the six amino acid sequence Gly-Ser-Thr-Asn-Ala-Ser ($FlgD_{short}$) indicate that the N-terminal hydrophobic signal (2–5, orange) and gate-recognition motif (GRM, blue) are separated by ~29 ångstrom (α-helical conformation, middle right) or ~67 ångstrom (unfolded contour length, right). (**b**) Placement of the crystal structure of FlhBc (PDB:3BOZ ***Kuhlen et al., 2020***; red) and the cryo-EM structure of FliPQR-FlhB (PDB:6S3L ***Ward et al., 2018***) in a tomographic reconstruction of the *Salmonella* SPI-1 injectisome (EMD-8544 [60]; grey). The minimum distance between the subunit gate-recognition motif binding site on $FlhB_C$ (grey) to $FlhB_N$ (defined as *Salmonella* FlhB residue 211 [61]; ~ 78 Å) was estimated by combining: the value corresponding to the distance between the subunit binding pocket on $FlhB_C$ (***Evans et al., 2013***) (grey) and the N-terminal visible residue (D_{229}) in the $FlhB_C$ structure (PDB:3BOZ ***Kuhlen et al., 2020***; ~ 52 Å) with the value corresponding to the minimum distance between FlhB residues 211 and 228 (based on a linear α-helical conformation; ~ 26 Å). (**c**) Swimming motility of recombinant *Salmonella flgD* null strains producing a chromosomally-encoded FliP-M_{210}A variant (M_{210}A gate, left) or wild type FliP (wild-type gate, right). Wildtype FliP and FliP-M_{210}A were engineered to contain an internal HA tag positioned between residue 21 and 22 to allow immunodetection of FliP. Both strains produced either a pTrc99a plasmid-encoded FlgD subunit variant in which residues 9–32 were replaced with two repeats of the six amino acid sequence Gly-Ser-Thr-Asn-Ala-Ser ($FlgD_{short}$; top panel) or a pTrc99a plasmid-encoded wild-type FlgD subunit ($FlgD_{wild\ type}$; bottom panel). Motility was assessed in 0.25% soft-tryptone agar containing 100 µg/ml ampicillin and 50 µM IPTG and incubated for 16 hr (top panel) or 4–6 hr at 37 °C (bottom panel). (**d**). The mean motility halo diameter of recombinant *Salmonella flgD* null strains producing a chromosomally-encoded FliP-M_{210}A variant (M_{210}A gate, left) or wild-type FliP (wild-type gate, right). Wild-type FliP and FliP-M_{210}A were engineered to contain an internal HA tag positioned between residue 21 and 22 to allow immunodetection of FliP. Both strains produced a pTrc99a plasmid-encoded FlgD subunit variant in which residues 9–32 were replaced with two repeats of the six amino acid sequence Gly-Ser-Thr-Asn-Ala-Ser ($FlgD_{short}$). Error bars represent the standard error of the mean calculated from at least three biological replicates. *** indicates a p-value < 0.001.

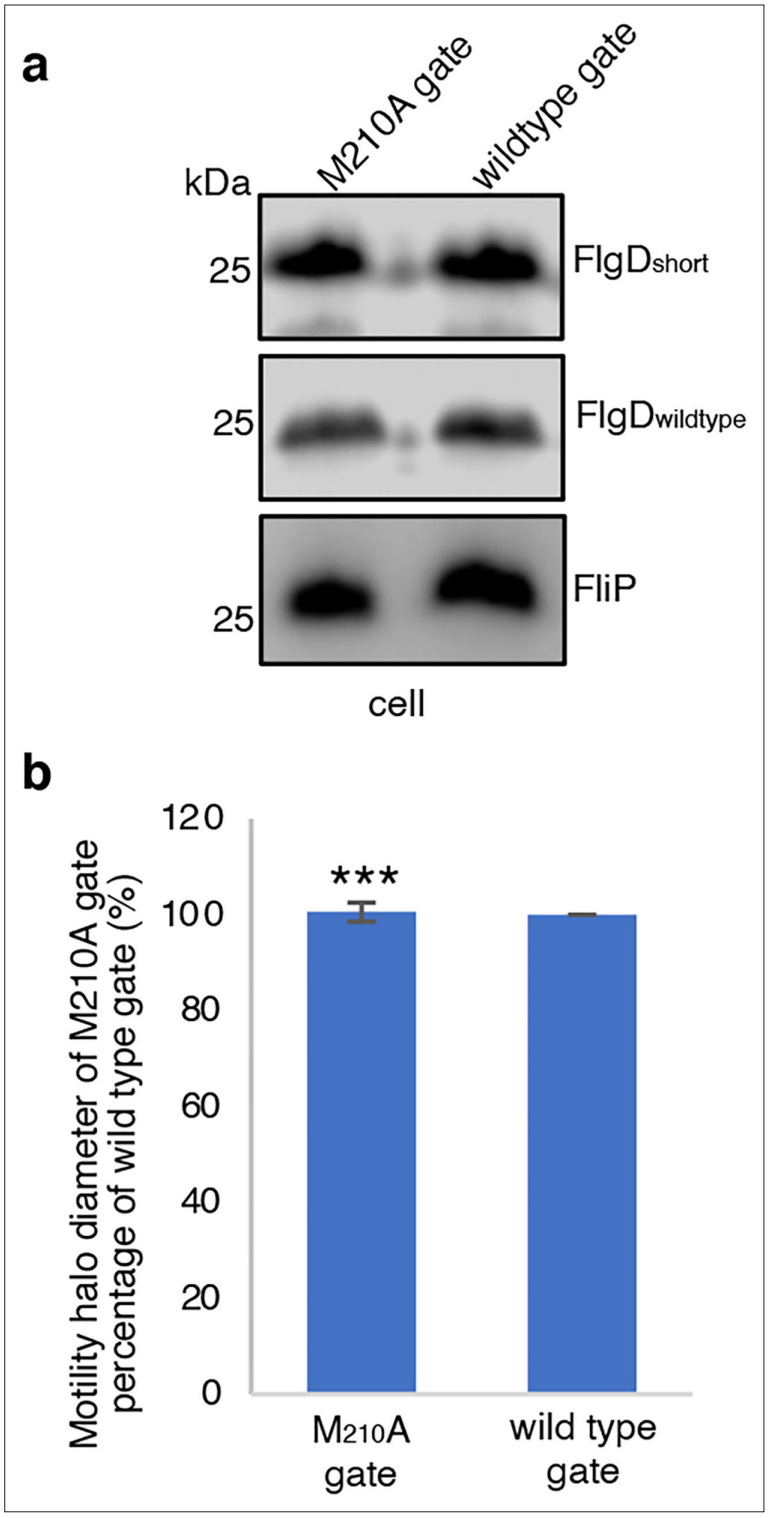

Figure 6—figure supplement 1. Mutations in FliP suppress the motility defect associated with FlgDshort but do not change the motility of strains producing wild type FlgD. (**a**) Whole cell (cell) proteins from late exponential-phase cultures of recombinant *Salmonella flgD* null strains producing a chromosomally-encoded FliP-M_{210}A variant (M_{210}A gate, left) or wild-type FliP (wild-type gate, right). Wild-type FliP and FliP-M_{210}A were engineered to contain an internal HA tag positioned between residue 21 and 22 to allow immunodetection of FliP (***Ward et al., 2018***) (bottom panel). Both strains produced either a pTrc99a plasmid-encoded FlgD subunit variant in which residues 9–32 were replaced with two repeats of the six amino acid sequence Gly-Ser-Thr-Asn-Ala-Ser ($FlgD_{short}$; top panel)

Figure 6—figure supplement 1 continued on next page

Figure 6—figure supplement 1 continued

or a pTrc99a plasmid-encoded wild type FlgD subunit ($FlgD_{wild\ type}$; middle panel). Proteins were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera or anti-HA monoclonal antisera. (**b**). The mean motility halo diameter of recombinant *Salmonella flgD* null strains producing a chromosomally-encoded FliP-M_{210}A variant (M_{210}A gate, left) or wild-type FliP (wild-type gate, right). Wild-type FliP and FliP-M_{210}A were engineered to contain an internal HA tag positioned between residue 21 and 22 to allow immunodetection of FliP. Both strains produced a pTrc99a plasmid-encoded wild type FlgD. Error bars represent the standard error of the mean calculated from at least three biological replicates. *** indicates a p-value < 0.001.

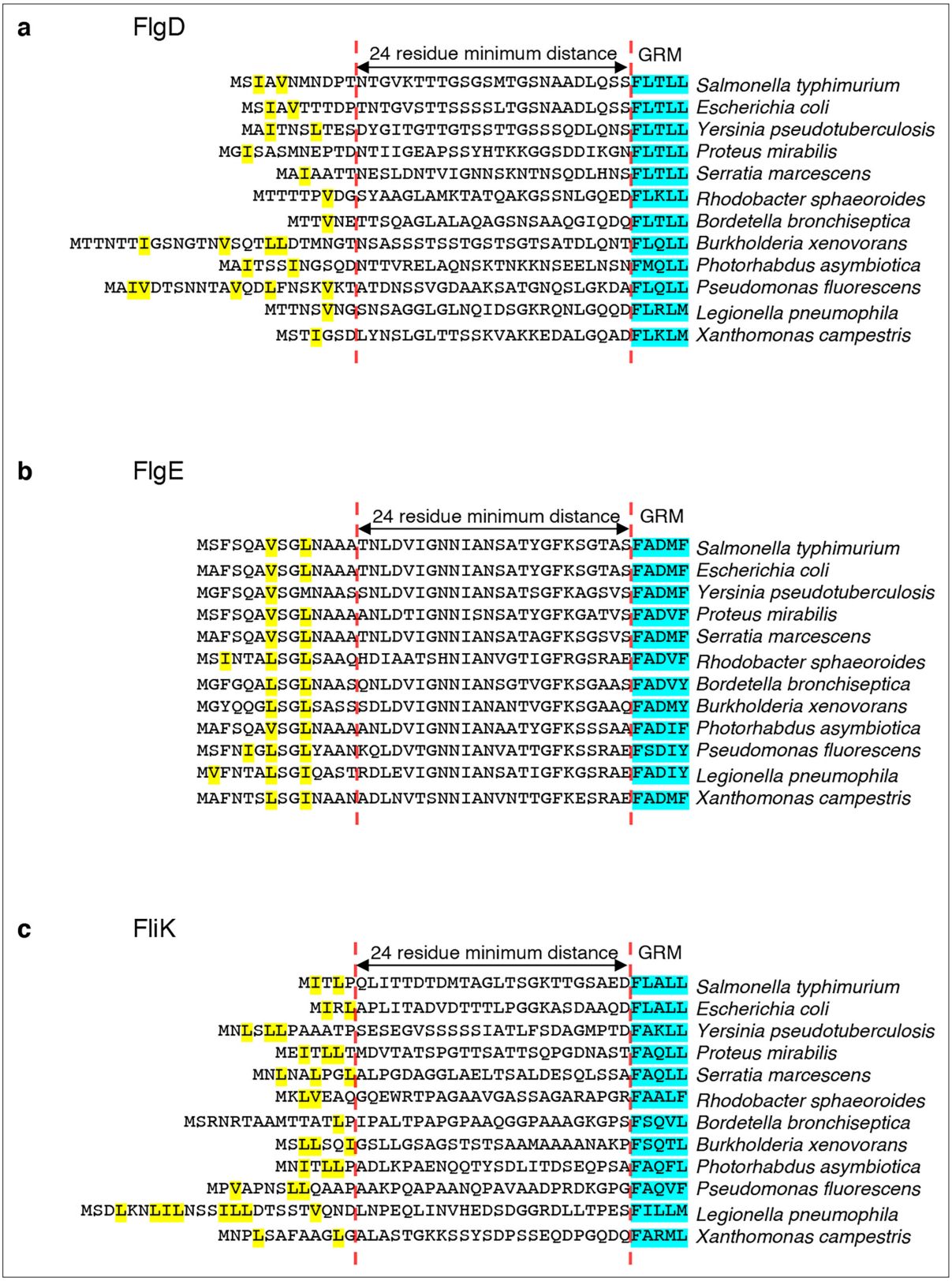

Figure 6—figure supplement 2 Amino acid sequence alignments of N-terminal regions of *Salmonella* FlgD cap, FlgE hook and FliK ruler with flagellar subunits from other bacterial species. (**a**) The N-terminal sequence of the *Salmonella* FlgD cap subunit aligned by the conserved gate-recognition motif (GRM, blue) with N-terminal sequences of FlgD from other bacterial species. Small non-polar residues located N-terminal from the minimum distance threshold of 24 residues from the gate-recognition motif are highlighted (yellow). (**b**). The N-terminal sequence of the *Salmonella* FlgE hook subunit

Figure 6—figure supplement 2 continued on next page

Figure 6—figure supplement 2 continued

aligned by the conserved gate-recognition motif (GRM, blue) with N-terminal sequences of FlgE from other bacterial species. Small non-polar residues located N-terminal from the minimum distance threshold of 24 residues from the gate-recognition motif are highlighted (yellow). (**c**). The N-terminal sequence of the *Salmonella* FliK ruler subunit aligned by the conserved gate-recognition motif (GRM, blue) with N-terminal sequences of FliK from other bacterial species. Small non-polar residues located N-terminal from the minimum distance threshold of 24 residues from the gate-recognition motif are highlighted (yellow).

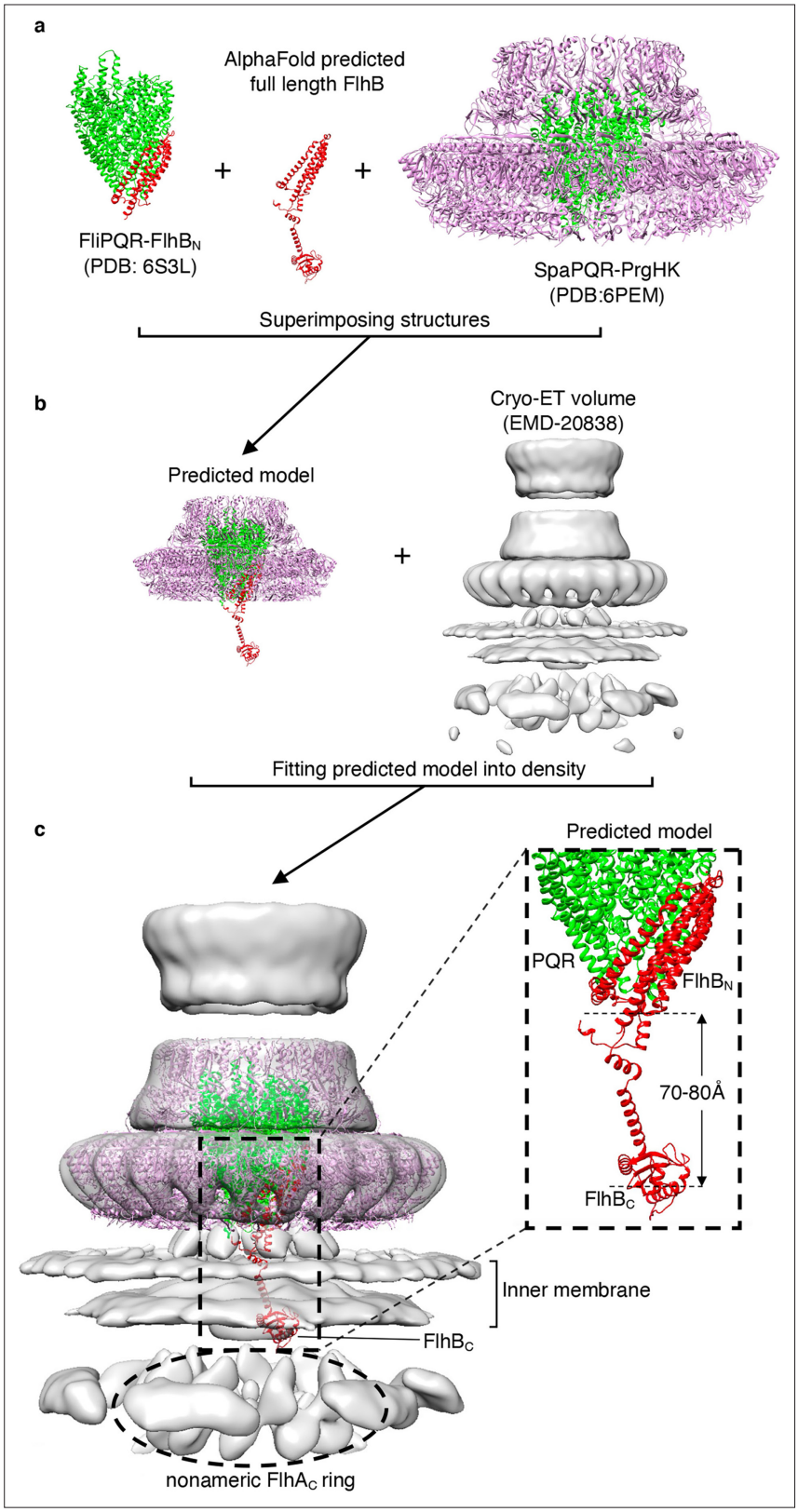

Figure 6—figure supplement 3. The substrate binding cytoplasmic domain of FlhB is predicted to be positioned between the plane of the inner membrane and cytoplasmic domain of FlhA. (**a**) Cryo-EM structure of the FliPQR-FlhB complex (PDB:6S3L ***Kuhlen et al., 2020***, left) displaying FliPQR (green) and FlhB residues 1–221 (red), the alphafold predicted structure of full-length FlhB (red, middle) and the Cryo-EM structure of the PrgHK-SpaPQR

Figure 6—figure supplement 3 continued on next page

Figure 6—figure supplement 3 continued

complex (PDB:6PEM) (***Hu et al., 2019***) displaying PrgHK (magneta) in complex with the FliPQR homologue, SpaPQR (green). (**b**). Structures from (**a**) were superimposed in chimera using MatchMaker to generate a predicted model of the PrgHK - FliPQR - full-length FlhB complex (left). A tomographic reconstruction of the *Salmonella* SPI-1 vT3SS (EMD-20838) (***Butan et al., 2019***) is shown in grey (right). (**c**). Placement of the PrgHK – FliPQR – full-length FlhB predicted model in the tomographic reconstruction of the Salmonella SPI-1 vT3SS, which suggests that the substrate binding cytoplasmic domain of FlhB ($FlhB_C$) is positioned below the plane of inner membrane and above the visible nonameric ring formed by the cytoplasmic domain of FlhA ($FlhA_C$). Using the predicted model, the distance between the GRM binding site on $FlhB_C$ and the base of the FliPQR-$FlhB_N$ complex is estimated to be between 70 and 80 angstrom.

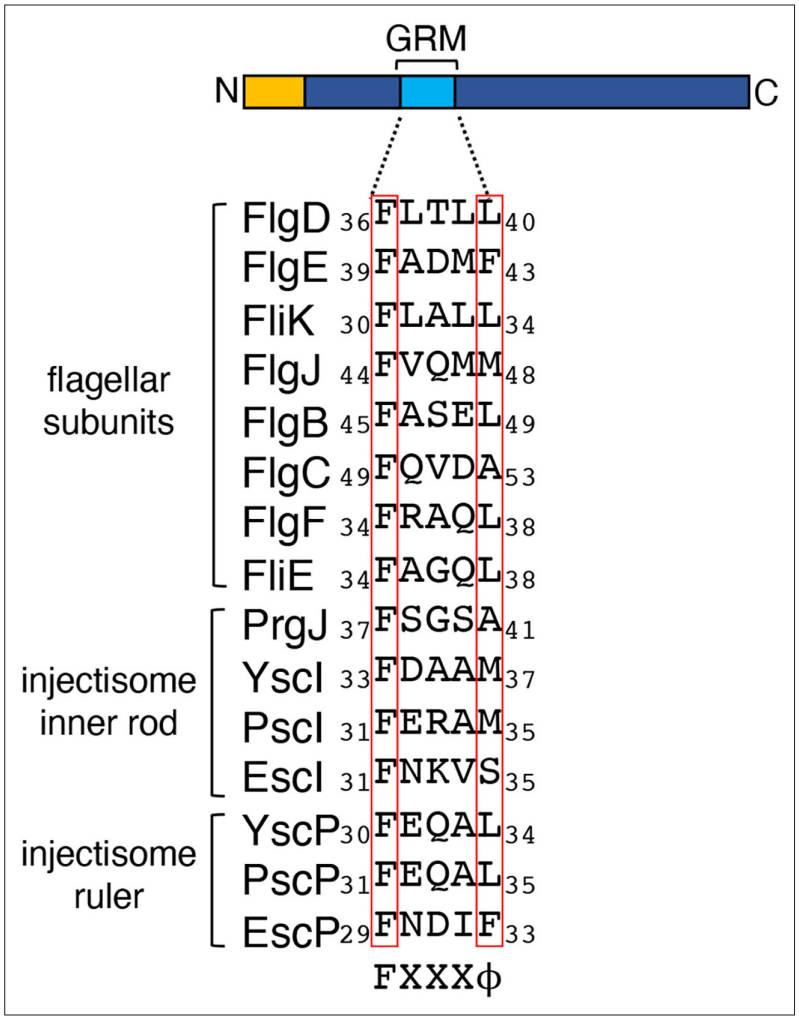

Figure 6—figure supplement 4. A schematic of an early flagellar subunit containing an N-terminal export signal (yellow) and gate-recognition motif (GRM, blue). The essential gate recognition motif (GRM) of *Salmonella* FlgD (residues 36–40) was aligned with homologous regions in other early flagellar subunits and with the inner rod and ruler subunits of the injectisome from *Salmonella* and other bacterial species.