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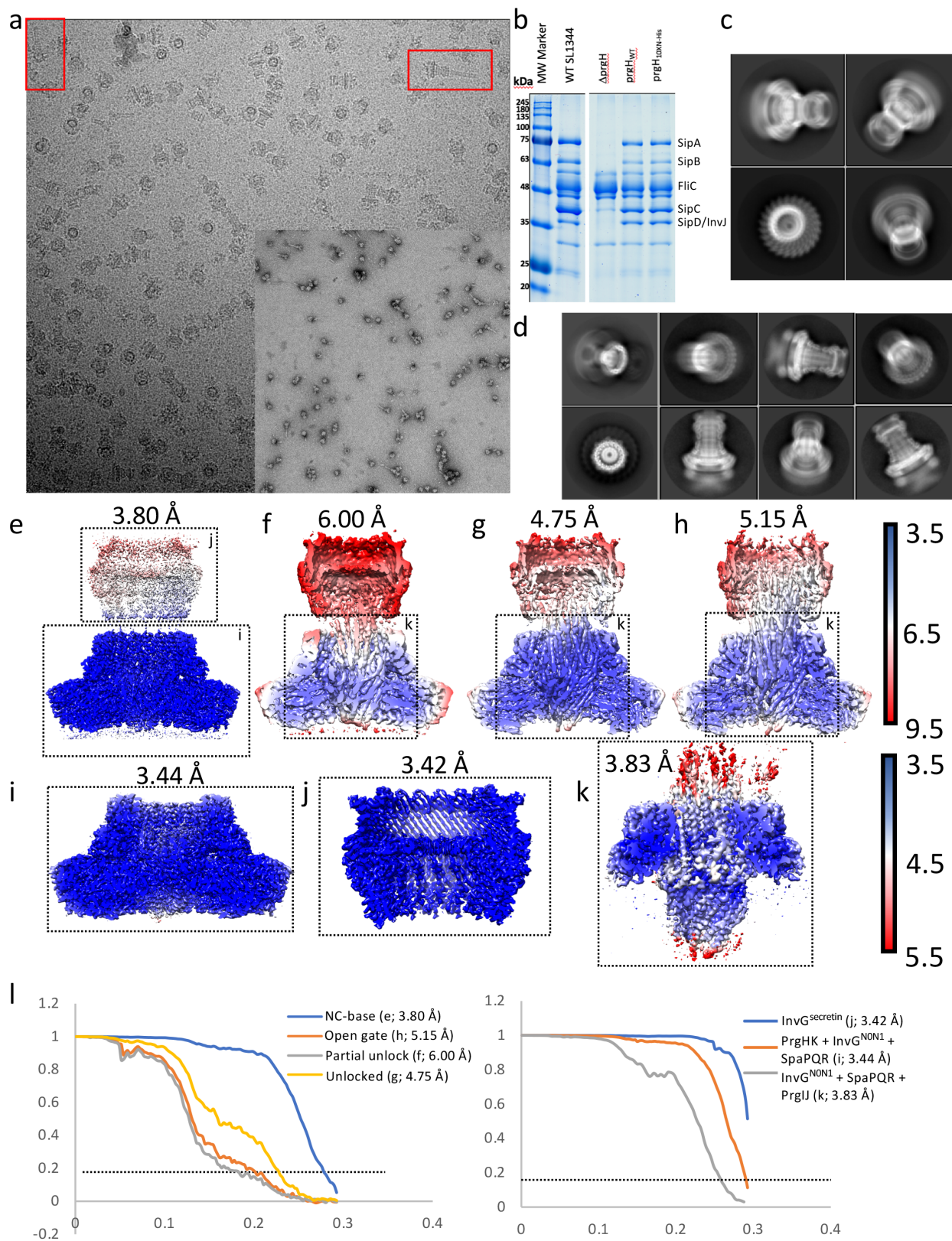
T3S injectisome needle complex structures in four distinct states reveal the basis of membrane coupling and assembly

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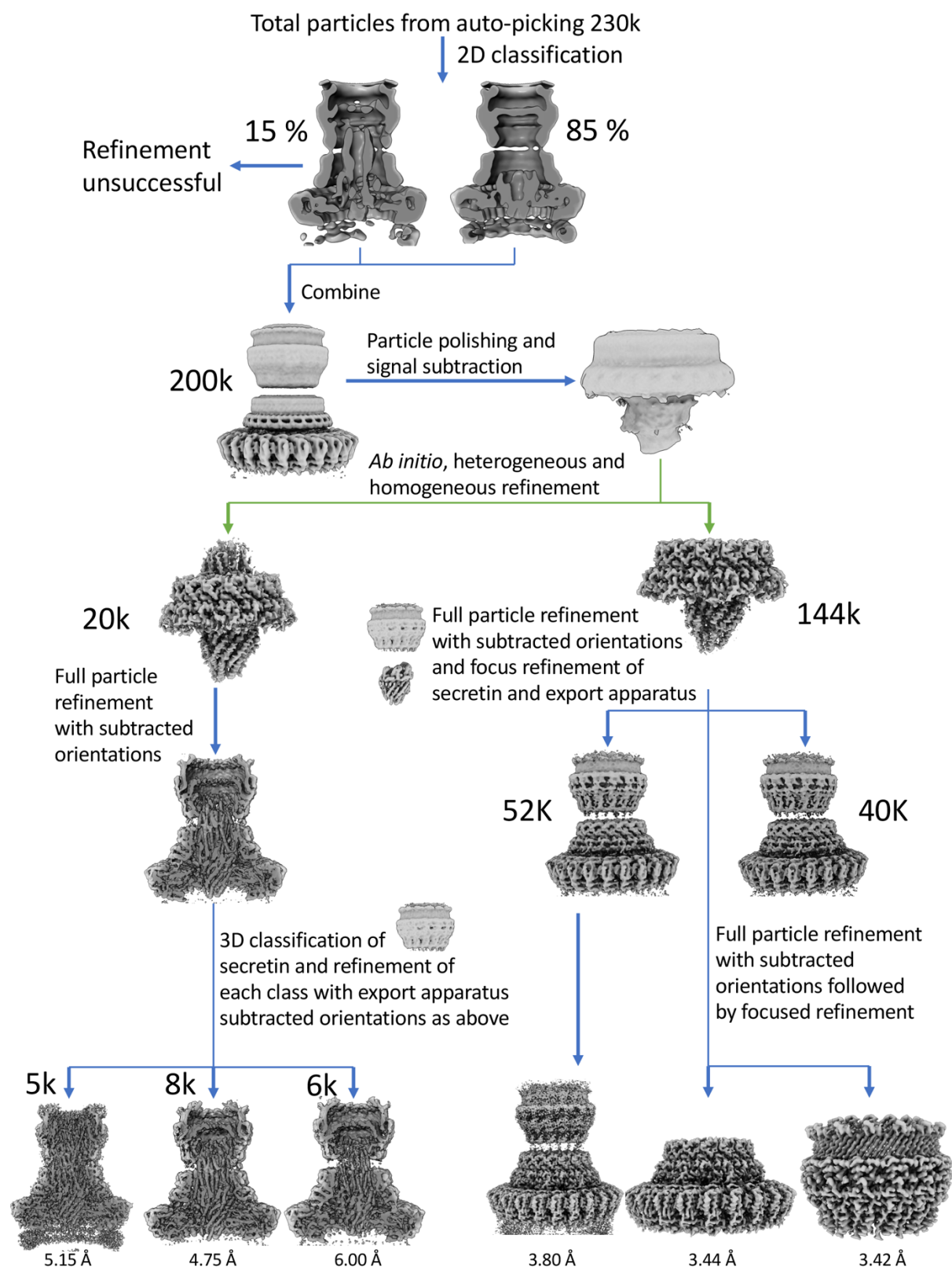
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	#1 NC-base (EMDB- 20310)	#2 NC partially unlocked (EMDB- 20313)	#3 NC unlocked (EMDB- 20314)	#4 NC open (EMDB- 20312)	#5 InvG ^{secretin} (EMDB- 20315) (PDB 6PEE)	#6 PrgHK, InvG ^{NON1} , SpaPQR (EMDB- 20316) (PDB 6PEM)	#7 InvG ^{NON1} , SpaPQR, PrgIJ (EMDB- 20317) (PDB 6PEP)
Data collection and processing							
Magnification	29240	29240	29240	29240	29240	29240	29240
Voltage (kV)	300	300	300	300	300	300	300
Electron exposure (e-/Å ²)	15	15	15	15	15	15	15
Defocus range (μm)	1.5-3	1.5-3	1.5-3	1.5-3	1.5-3	1.5-3	1.5-3
Pixel size (Å)	1.71	1.71	1.71	1.71	1.71	1.71	1.71
Symmetry imposed	C1	C1	C1	C1	C15	C1	C1
Initial particle images (no.)	235618	235618	235618	235618	235618	235618	235618
Final particle images (no.)	51816	5319	7249	5018	185855	144097	25666
Map resolution (Å)	0.143	0.143	0.143	0.143	0.143	0.143	0.143
FSC threshold							
Map resolution (Å)	3.80	6.00	4.75	5.15	3.42	3.44	3.83
Refinement							
Initial model used (PDB code)					5TCR	5TCR	5TCR, 6DWB
Model resolution (Å)					0.143	0.143	0.143
FSC threshold							
Model resolution (Å)					3.42	3.44	3.83
Map sharpening <i>B</i> factor (Å ²)					-68	-39	-47
Model composition							
Non-hydrogen atoms					40440	108060	39841
Protein residues					5175	13503	5051
Ligands					60	n/a	n/a
<i>B</i> factors (Å ²)							
Protein					58.91	65.19	120.07
Ligand					97.71	n/a	n/a
R.m.s. deviations							
Bond lengths (Å)					0.0058	0.0045	0.0066
Bond angles (°)					1.16	1.09	1.17
Validation							
MolProbity score					1.50	1.28	1.72
Clashscore					3.17	2.60	5.42
Poor rotamers (%)					0.34	0.19	1.47
Ramachandran plot							
Favored (%)					94.21	96.40	95.72
Allowed (%)					5.79	3.58	4.20
Disallowed (%)					0.00	0.02	0.08

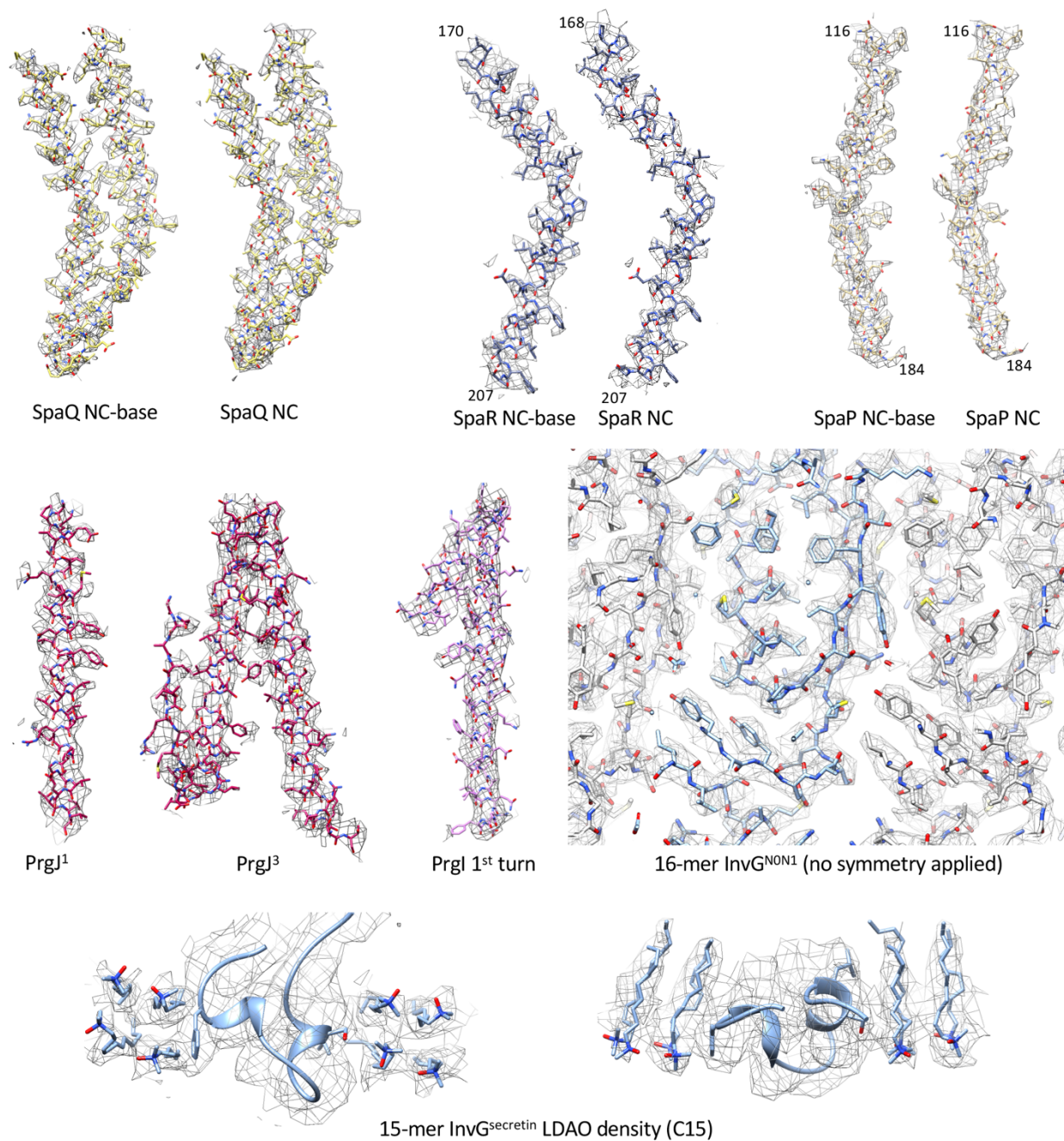
Supplementary Table 1. Cryo-EM data collection, refinement and validation statistics.



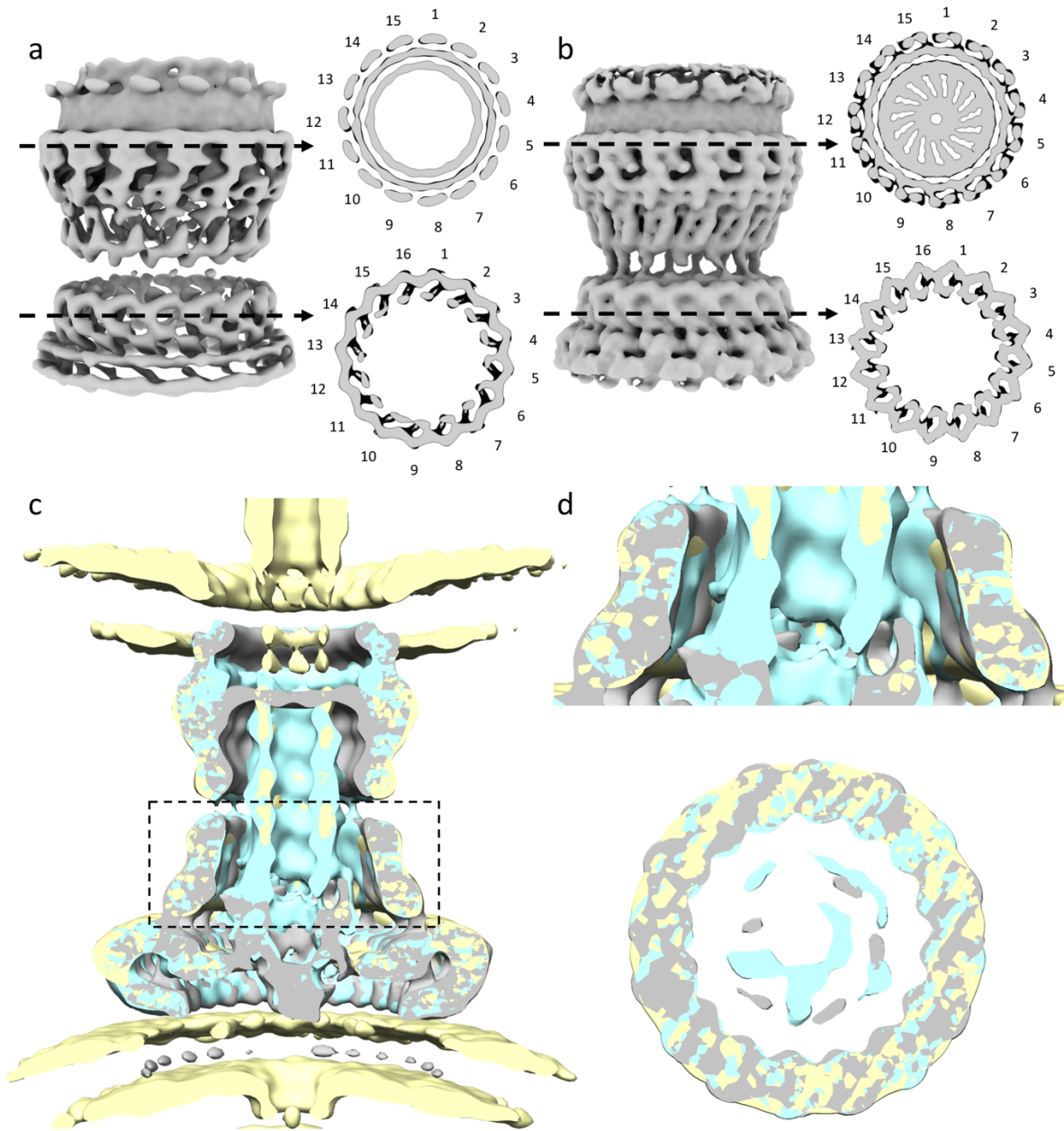
Supplementary Figure 1: Single particle cryo-EM data processing. **a**, Representative micrograph of the sample from overnight culture. Fully assembled NCs boxed. Inset is a negative stain EM micrograph of the purified sample after 4-hour expression showing increased proportion of NCs with assembled needles. We propose the variation in needle length is due to shearing/depolymerization and increased levels of immature NC-bases due to insufficient membrane and cytosolic components. Similar assembly distributions were noted in two separate sample preparations. **b**, Complementation of a *prgH* knockout with an N-terminally His-tagged PrgH plasmid (*prgH* 10xNhis) restores secretion as effectively as the full-length *prgH* plasmid (*prgH*_{WT}) compared to the wild-type strain (WT SL1344). SDS-PAGE of secreted proteins, with the most abundant proteins labelled on right. Assays were repeated three times with the same results. **c**, Selected 2D class averages of the data. **d**, Selected 2D class averages of (from left to right) the subtracted SpaPQR and InvG^{N0N1} region, open state, unlocked state, and partially unlocked state. **e-h**, Local resolution of the four NC assembly snapshots as in Fig. 1b-e. The boxed regions correspond to the focused refinements in **i-k** as labelled. Resolution color indicated on right. **i-k**, Focused refinement reconstructions of the different regions boxed in **e-h**. Resolution color indicated on right. **l**, FSC curves for reconstructions in **e-k**.



Supplementary Figure 2: Single particle cryo-EM data processing strategy. Blue arrows correspond to processing in Relion, green cryoSPARC.



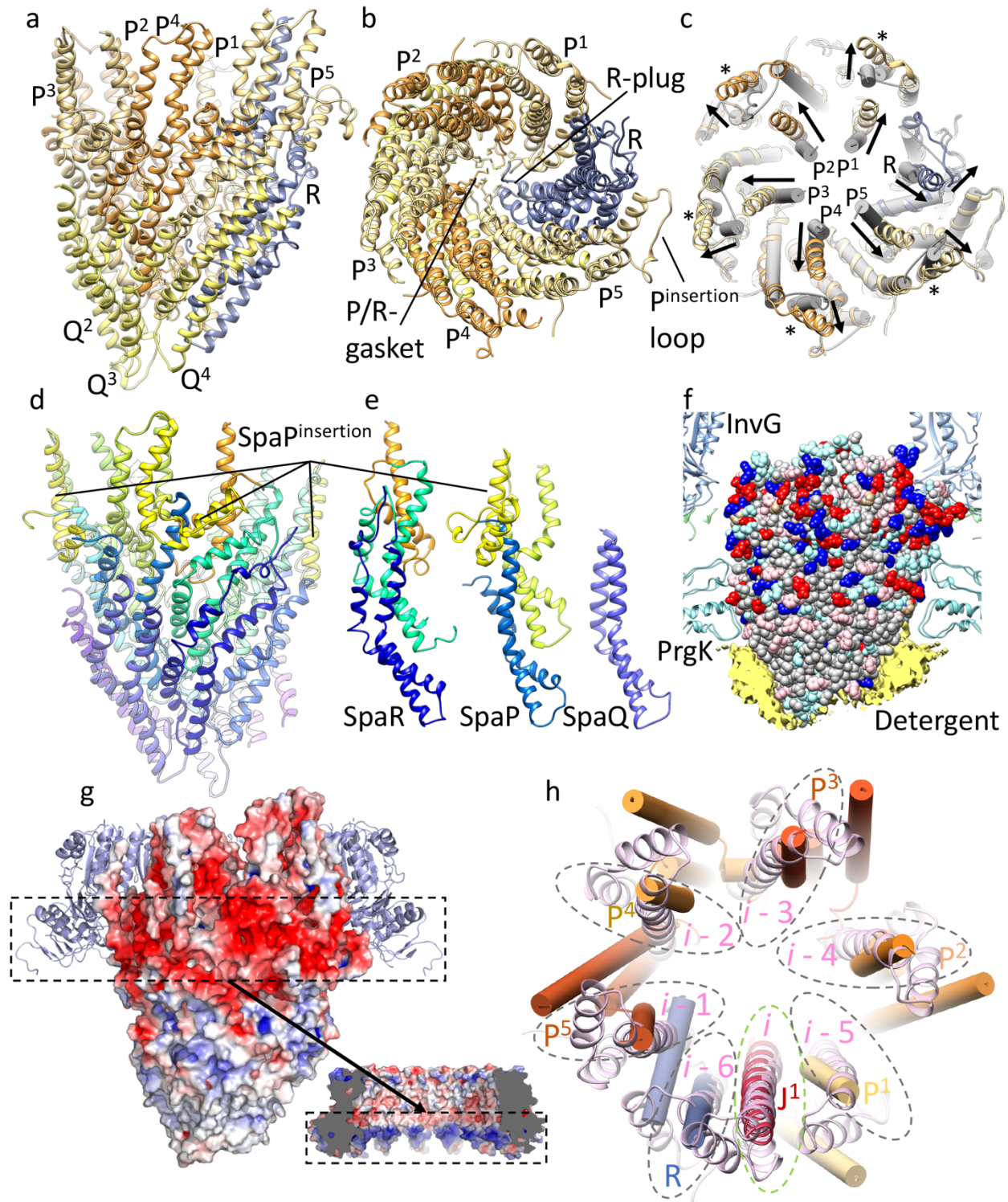
Supplementary Figure 3: Sample cryo-EM density. The SpaPQR and PrgIJ components from the focused refinement reconstructions in the absence (3.44 Å resolution) and presence (3.83 Å resolution) of PrgIJ. 16-mer InvG^{NON1} region with no symmetry applied at 3.44 Å resolution. C15 InvG^{secretin} reconstruction at 3.42 Å resolution showing AHL with bound LDAO.



Supplementary Figure 4: Mixed secretin stoichiometry in other purified NC samples and

comparison to the *in situ* injectisome. a, C1 InvG focused refinement from intact NC sample data (left), and top slab views (marked with dashed arrows) at the $\text{InvG}^{\text{secretin}}$ showing 15 copies (top right) and the $\text{InvG}^{\text{NON1}}$ region showing 16 copies (bottom right). **b**, C1 InvG focused refinement from the $\text{PrgH}_{130-392}$ mutant NC-base data¹ (left), and top slab views (marked with dashed arrows) at the $\text{InvG}^{\text{secretin}}$ showing 15 copies (top right) and the $\text{InvG}^{\text{NON1}}$ region showing 16 copies (bottom right). **c**, Superposition of the *in situ* cryo-ET reconstruction² (yellow), and the low-pass filtered NC-base (grey) and NC reconstructions (cyan). **d**, Side and top zoomed views of the 16-mer $\text{InvG}^{\text{NON1}}$ region from c showing near perfect agreement between high resolution cryo-EM and lower resolution cryo-ET reconstructions.

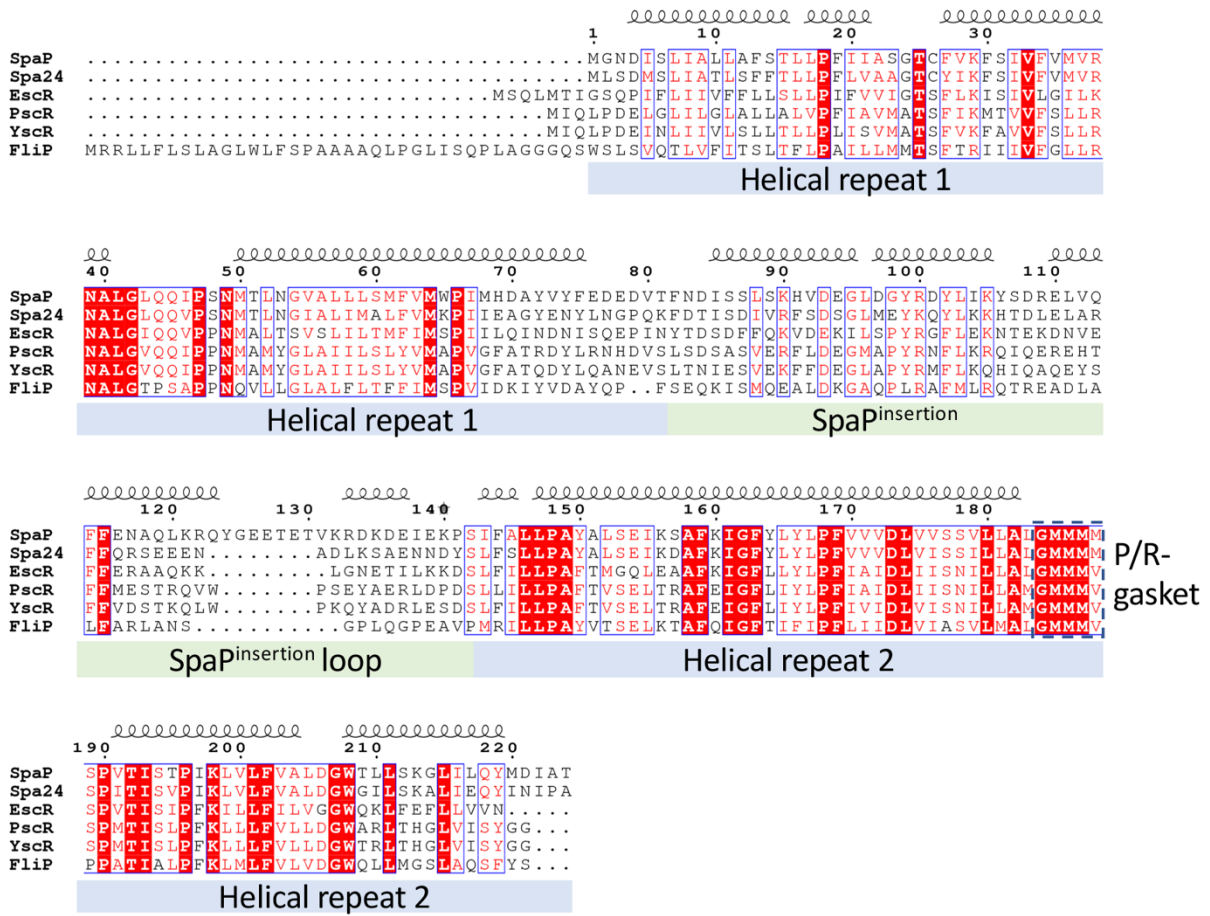
interface between InvG and PrgH also explains the secretion and assembly deficient phenotype associated with deletion of the C-terminal 6 residues from PrgH⁴. We further probed this interface, demonstrating Trp³⁸⁶ and Phe³⁸⁸ are critical determinants of the interaction (see **b**). **b-c**, Secretion assays for PrgH (**b**) and PrgJ mutants (**c**), carried out in a *prgH* or *prgJ* deletion SL1344 strain complemented with plasmids containing the wild-type *prgH* or *prgJ* sequences (*prgH*_{WT}, *prgJ*_{WT}) or the labelled mutants. SDS-PAGE showing secreted effectors SipA (late substrate), translocators SipBCD (middle substrates), and InvJ (early substrate). Assays were repeated three (**b**) or two (**c**) times with the same results. **d**, The structure of PrgJ, initiating needle polymerization on SpaPR, locates mutants^{5,6} shown to abrogate secretion and PrgJ binding (Asp³¹ and Tyr⁷⁷ on the N-terminal and C-terminal helices respectively); result in weakly attached needles (Asp⁶³ at the interface with the N-terminus of a PrgI monomer one full helical turn downstream); specifically abrogate translocator secretion (Thr²⁴, Ile²⁶, Leu²⁹ and Ile⁴², at the interface of the N-terminal loop/helix with the SpaP^{insertion}); and, identified here, selectively abolish late effector secretion (Arg⁸⁹ and Lys⁹⁰ on interior surface, see **c**, **e** and **f**). PrgJ mutants shown as ball-and-stick, key interacting residues on SpaR or SpaP shown as stick. We note these *prgJ* mutants were identified using plasmid-complementation^{5,6}, which has been suggested to result in artifacts when assaying for needle length (due to secretion competition between early substrates)⁷, with further experiments required to fully probe the role of PrgJ assembly in regulation of needle assembly and secretion. **e-f**, Top view of the SpaPQR-PrgJ complex colored according to calculated electrostatic surface for the native structure (**e**) and the Arg89Ala, Lys90Ala double mutant (**f**), which abolishes secretion of late substrates (effector SipA) but not early (InvJ) or middle substrates (SipBCD), see **c**.



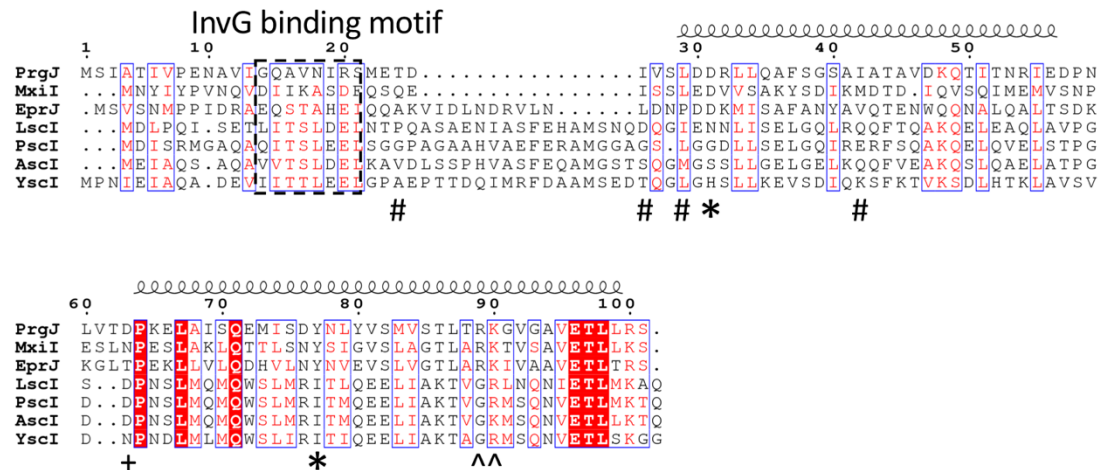
Supplementary Figure 6: SpaPQR complex and bound PrgIJ. **a**, Side view of atomic model of the SpaPQR complex, colored as in Fig. 3a. **b**, top view of **a**. **c**, Comparison of SpaPQR in the presence (cartoon) and absence (tubes) of bound PrgJ and PrgI. PrgJ binding is coupled with significant change to the N-terminal helices of SpaP and SpaR, and the SpaP^{insertion} (*) indicated with arrows. **d**, The individual

repeating helix-turn-helix motifs that make up SpaPQR and generate the pseudo-helical assembly are individually colored from blue-green-orange (SpaP^{insertion} colored yellow). The kinked nature of the repeats narrows the assembly towards the IM (bottom). **e**, Individual repeat motifs in SpaP, SpaQ, and SpaR monomers. **f**, Space filling representation of SpaPQR complex (positive charge: blue, negative charge: red, hydrophobic: grey, aromatic: pink, polar: cyan) and its surrounding detergent (map in yellow), PrgK (cyan), and InvG^{N0N1} (light blue). **g**, Electrostatic surface of SpaPQR-PrgJ complex. Surrounding InvG^{N0N1} (blue) shown and corresponding, complimentary electrostatic surface of the interacting N0 domain boxed. **h**, The pseudo-hexameric SpaR and SpaP N-terminal helices, which bind PrgJ, superpose with the 6 subunit turn of the PrgI needle (labelled $i - 1$ to $i - 6$ and circled, the green circle corresponds to the location of PrgJ^I or superposed PrgI^I). The SpaPR scaffold thus mimics the homotypic polymerization interfaces of the PrgI needle and explicitly defines its helical parameters.

a

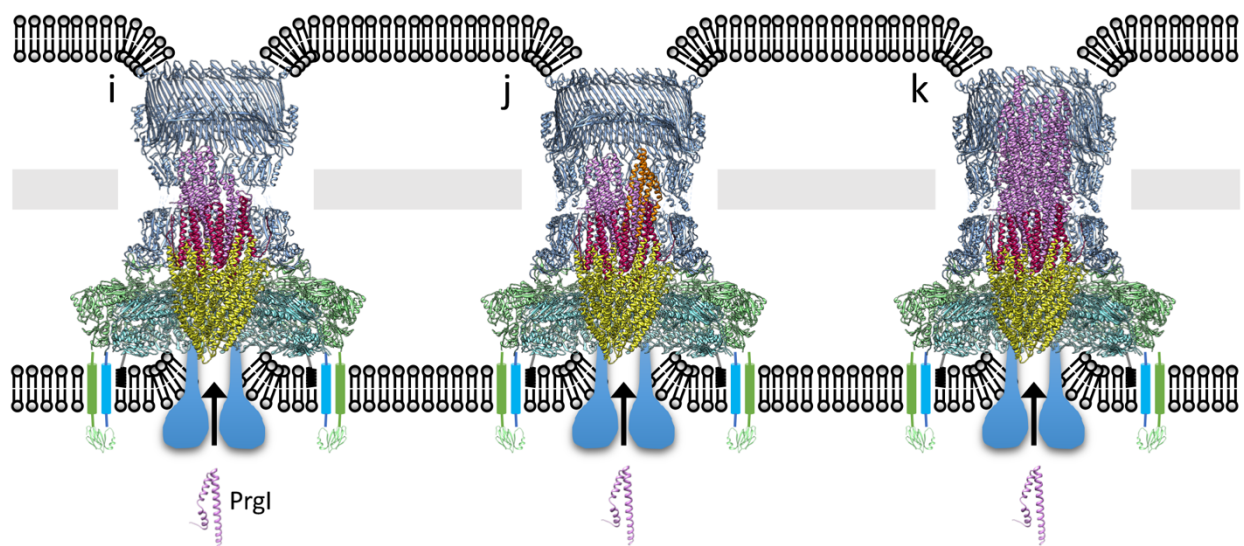
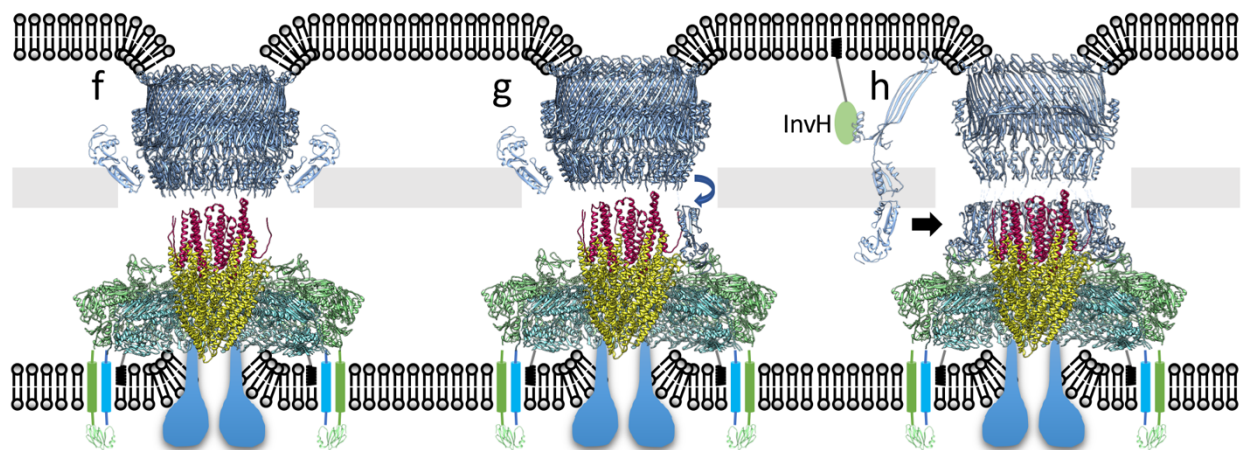
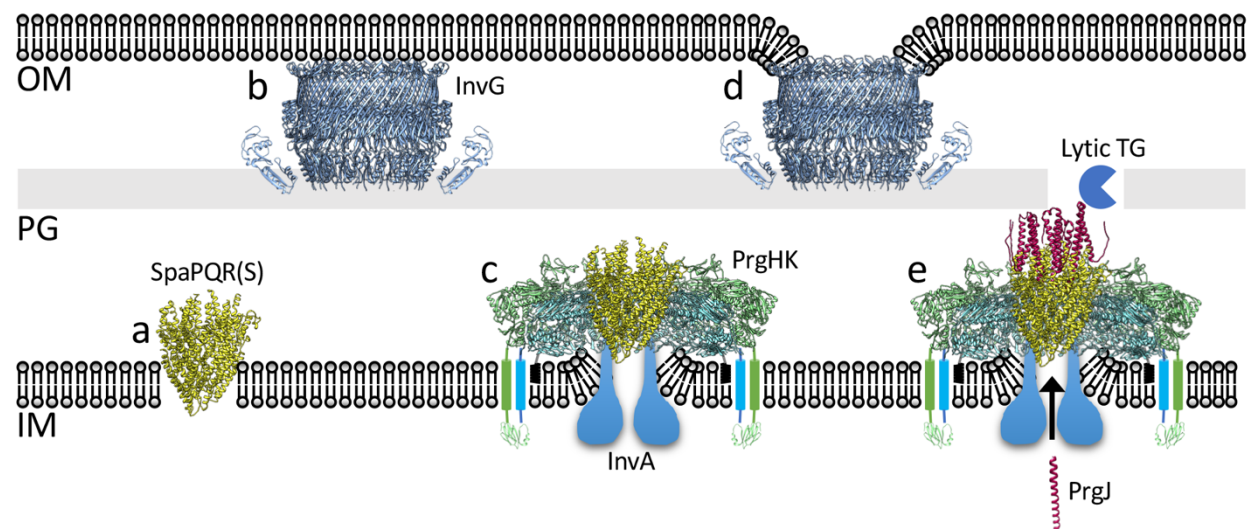


b

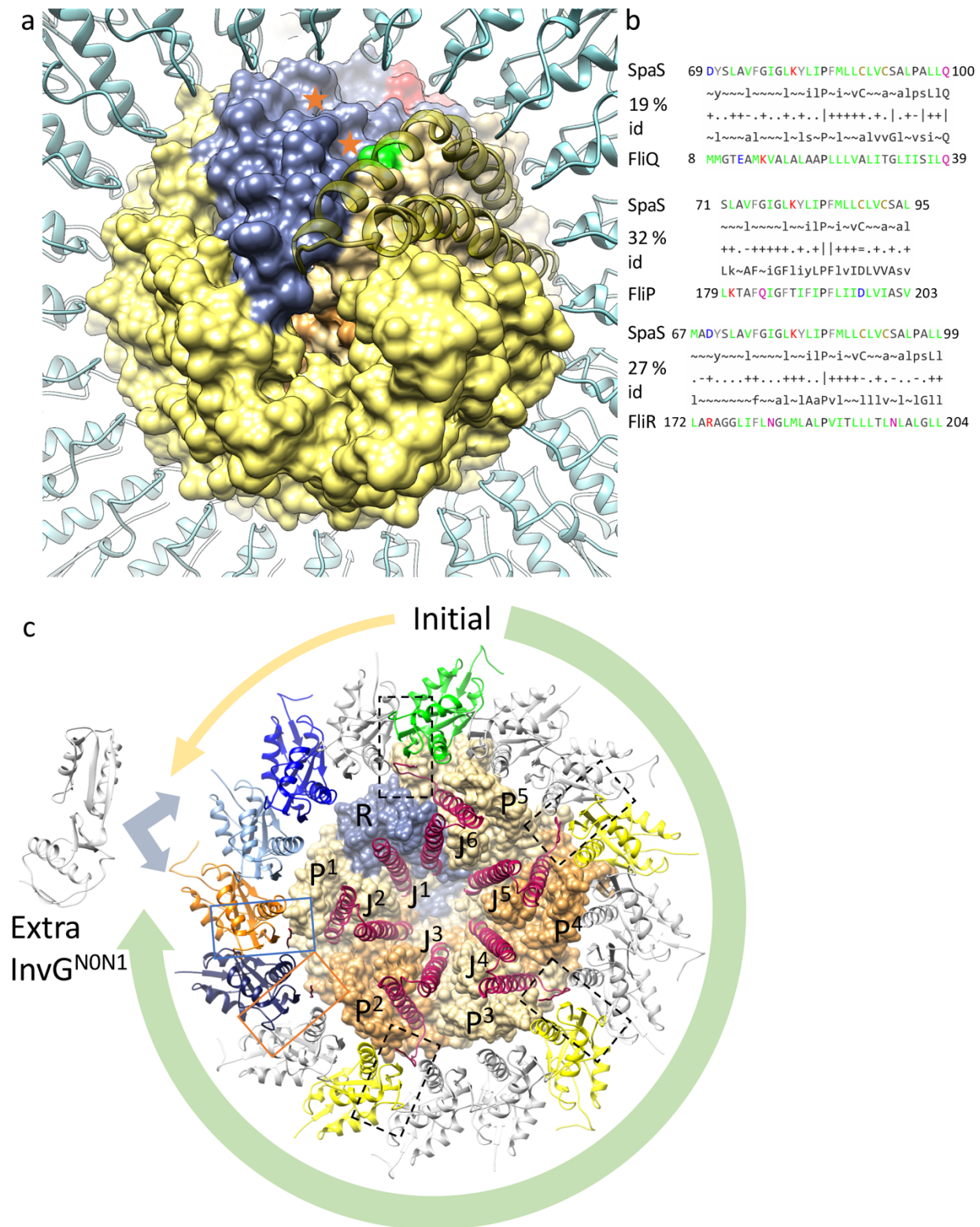


Supplementary Figure 7: Sequence alignments of SpaP and PrgJ. a, Sequence alignment of SpaP homologues. Secondary structure from SpaP annotated above. Spa24, *S. flexneri*; EscR, EPEC; PscR, *P. aeruginosa*; YscR, *Y. pestis*; FlpI, *S. Typhimurium*. The position of the helical repeats and SpaP^{insertion}

indicated. **b**, Sequence alignment of PrgJ homologues. PrgJ secondary structure annotated above. MxiI, *S. flexneri*; EprJ, EHEC; LscI, *P. luminescens*; PscI, *P. aeruginosa*; AscI, *A. salmonicida*; YscI, *Y. pestis*. Key mutants labelled: Asp³¹ and Tyr⁷⁷ which abolish PrgJ function (*)^{5,6}; Thr²⁴, Ile²⁶, Leu²⁹, Ile⁴², which selectively abolish middle substrate (translocases) secretion (#)⁶; and Arg⁸⁹ and Lys⁹⁰, which selectively abolish late substrate (effectors) secretion (^), see Supplementary Fig 6c-e). Blue boxes indicate global similarity determined according to physio-chemical property with a score of 0.7.



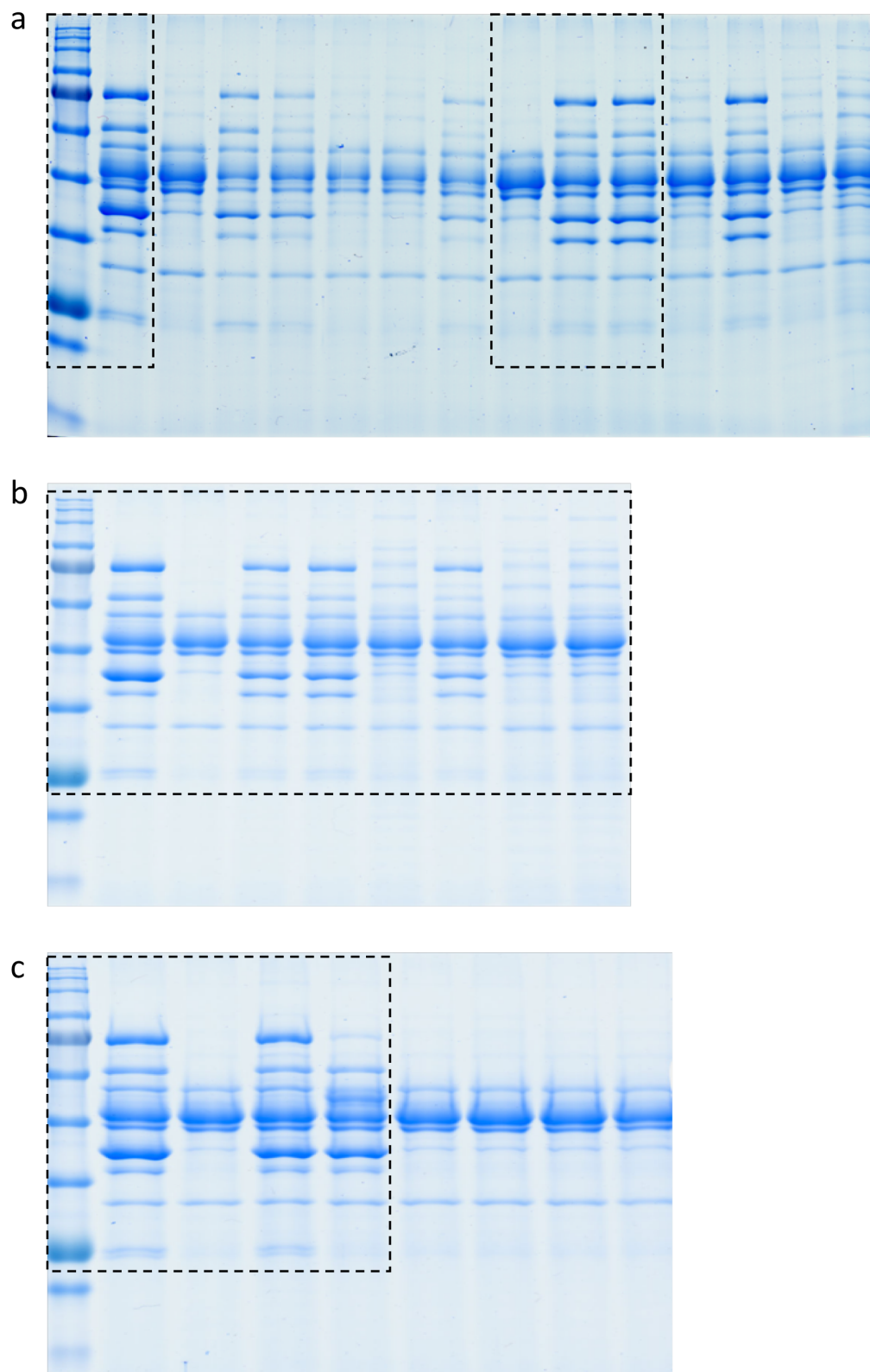
Supplementary Figure 8: Assembly of the needle complex. Different assembly pathways have been proposed for the NC with an inside-out model in the *Salmonella* SPI-1 system⁸, where assembly starts with the IM export apparatus, and an outside-in model alternatively suggested in *Yersinia*⁹, where assembly is dependent instead on the OM secretin YscC (InvG) and interaction with PrgH orthologue YscD. This possibly reflects differences in the architecture of the two systems: the *in situ* structure of the *Yersinia* injectisome¹⁰ appears elongated compared to *Salmonella*¹¹ and *Shigella*¹², with YscD correspondingly showing an extended structure with a predicted less intimate/stable interface with YscJ compared to PrgHK¹⁰. Together with published data, our novel structures are more consistent with the inside-out pathway and we propose the following model for NC assembly: Assembly is initiated at the IM with formation of the SpaPQR(S) complex (a) and at the OM by the 15-mer InvG^{secretin} pore (b). Export gate InvA (blue) is recruited and assembly of the PrgHK rings is nucleated around the SpaPQR(S) complex, facilitating its extraction from the IM (c) and resulting in the membrane deformation observed in *in situ* cryo-ET¹¹. The OM secretin spontaneously inserts into the OM mediated by the conserved amphipathic helical loop and “lip” beta-barrel¹ (d). Assembly of the cytoplasmic components results in a T3S competent apparatus, secretion and assembly of PrgJ serves to recruit a T3S lytic transglycosylase to locally clear peptidoglycan (e). The OM InvG^{secretin} localizes to the IM complex (f) and coupling of the InvG^{NON1} domains to PrgH is initiated via interaction with SpaP⁵ (g). InvG^{NON1} coupling proceeds and is terminated by recruitment of a 16th InvG^{NON1} domain to complete the symmetric interface with PrgH (h). PrgI secretion and assembly atop PrgJ causes the allosteric gating changes in the InvG^{NON1} ring to unlock the periplasmic gate (i and j) with subsequent steric gate opening via continued PrgI polymerization (k).



Supplementary Figure 9: Composition of the SpaPQR complex and coupling of the IM/OM rings.

a, Space sufficient for additional components binding to the SpaPQR complex is observed around the

exterior surface of SpaR (blue) and SpaP¹ (light orange). This is consistent with a FliP₅Q₅R₁ species observed using native mass spectrometry¹³, with the additional SpaQ (transparent yellow ribbon) binding the exterior of SpaP¹ as the other SpaP:SpaQ interactions. The peripheral location and reduced interaction with PrgK would explain its ready dissociation. We also propose that the exterior surface of SpaR (orange stars) represents the binding site for SpaS, with cross-linking¹⁴ locating it near the C-terminal helix of SpaP (green) and gene-fusion¹⁵ experiments showing it can be linked to the C-terminus of SpaR (red). This would locate the SpaS C-terminal domain, implicated in regulation of secretion hierarchy, near the cytoplasmic facing entrance to the SpaPQR entry portal to the PrgIJ needle channel. **b**, Similarity of the SpaS predicted N-terminal transmembrane region to the helical repeat motifs of FliP, FliQ, and FliR, supporting a similar helical repeat structure. **c**, We propose coupling of the 15-mer OM InvG^{secretin} pore and 24-mer IM PrgH is initiated by interaction of an InvG^{N0N1} domain (green) with SpaP⁵, the most extensive interface observed here. Subsequent assembly proceeds primarily in a clockwise direction (viewed from OM perspective; green arrow) driven by interaction with SpaP, PrgH, and PrgJ, with secondary counter-clockwise assembly (orange arrow). Accordingly, incorporation of the 15 InvG^{N0N1} domains belonging to the InvG^{secretin} pore with the observed PrgH₃:InvG₂ symmetrical interface results in a vacant position around SpaP¹. To complete IM/OM ring coupling, an extra InvG^{N0N1} domain is recruited (blue arrow), and we observe this here in neighboring, alternate locations (light blue or orange InvG^{N0N1}, colored as Fig. 2). The interaction of the PrgJ N-terminal loop with InvG^{N0N1} is boxed; PrgJ³⁻⁶ interact with evenly distributed (every third) InvG^{N0N1} domains (green/yellow). PrgJ², by contrast, is observed to bind in alternate locations: the expected, evenly spaced InvG^{N0N1} domain (orange) and its neighbor (dark blue). We interpret this to suggest that PrgJ is secreted and assembled prior to IM/OM coupling, with the PrgJ N-terminal loops binding to the InvG^{N0N1} domains of the InvG^{secretin} pore on coupling, prior to recruitment of the extra 16th InvG^{N0N1} domain to complete the coupling interface: when the light blue InvG^{N0N1} is the extra 16th recruited domain, PrgJ² binds its preferred site (blue box), however, when this preferred InvG^{N0N1} is instead the extra 16th (see Fig. 2), PrgJ² instead binds the immediate neighbor (orange box).



Supplementary Figure 10: Full length blots for gels shown in Supplementary Figure 1b (a), Supplementary Figure 5b (b) and c (c).

Supplementary Video 1: Allosteric step of InvG periplasmic gate opening. Morph between the InvG closed gate state of the NC-base (grey) and the unlocked state on initial needle assembly (yellow). Refer to Fig. 5 for annotation of key structural changes.

Supplementary Video 2: Steric step of InvG periplasmic gate opening. Morph between the InvG unlocked gate state (yellow) and the open state on continued needle polymerization (cyan). Refer to Fig. 5 for annotation of key structural changes.

References

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