Supporting Information
for

Structural basis for the glycosyltransferase activity of the Salmonella effector SseK3

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Figure S1. Binding affinity of UDP-GlcNAc for $SseK3_{14-335}$. S-2

Figure S2. Structural features of SseK3 and its ligand. S-3

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Figure S4. Arg-GlcNacylation gel assay of SseK3 mutants. S-5

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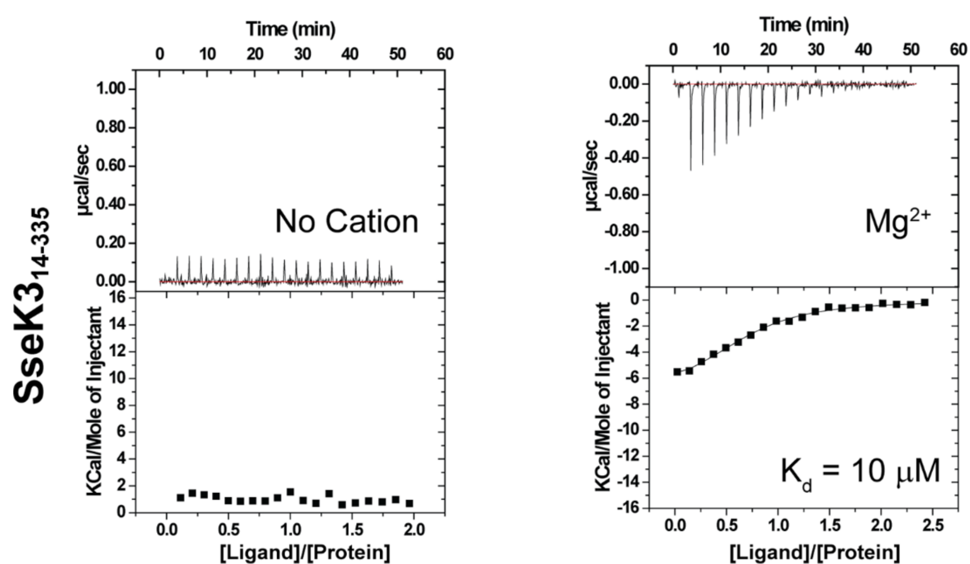

Figure S1. The presence of a divalent cation is necessary for binding of UDP-GlcNac to SseK3. Isothermal Titration Calorimetry curves for the interaction of SseK3$_{14-335}$ with UDP-Glucosamine (UDP-GlcNAc) in the absence of coordinating divalent cation (left panel) and in the presence of 5 mM $MgCl_2$ (right panel). The integrated heat for the binding interaction was integrated as a function of the molar ratio of titrant to protein in the cell. The data was fitted to a 1:1 binding model and the dissociation constant is reported in each panel.

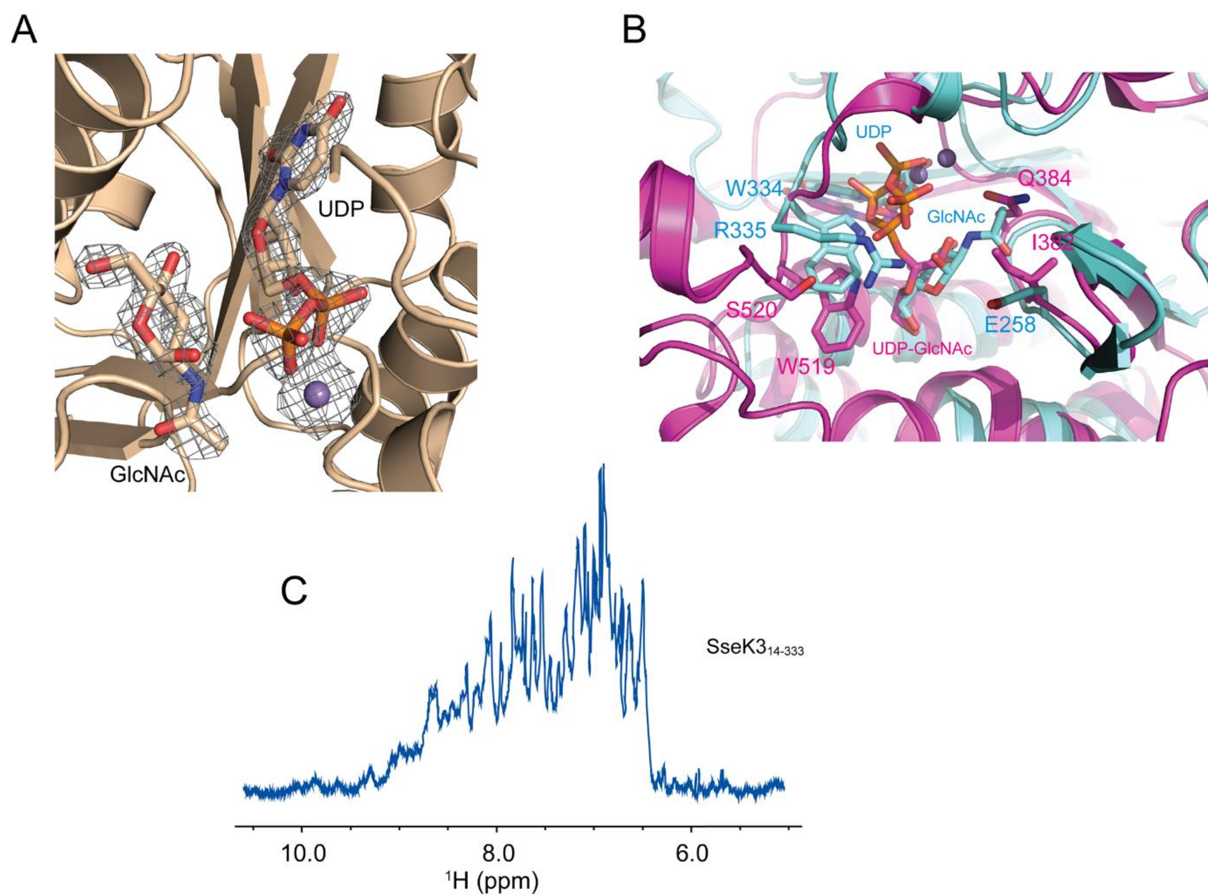

Figure S2. Ligand binding to SseK3. (A) 2Fo-Fc electronic density map around UDP and GlcNAc in the active site pocket of $SseK3_{14\text{-}335}$. The map is plotted at 2σ around the UDP and 1σ around the N-acetyl α-D-glucosamine. (B) Overlap of the structure of the active site of *C. difficile* toxin A (PDB code 3SRZ) with its ligand UDP-Glucose (magenta) and SseK3 with UDP/GlcNAc (cyan). Residues in the different chains are coloured accordingly. (C) Downfield region of the proton NMR spectra of $SseK3_{14\text{-}333}$.

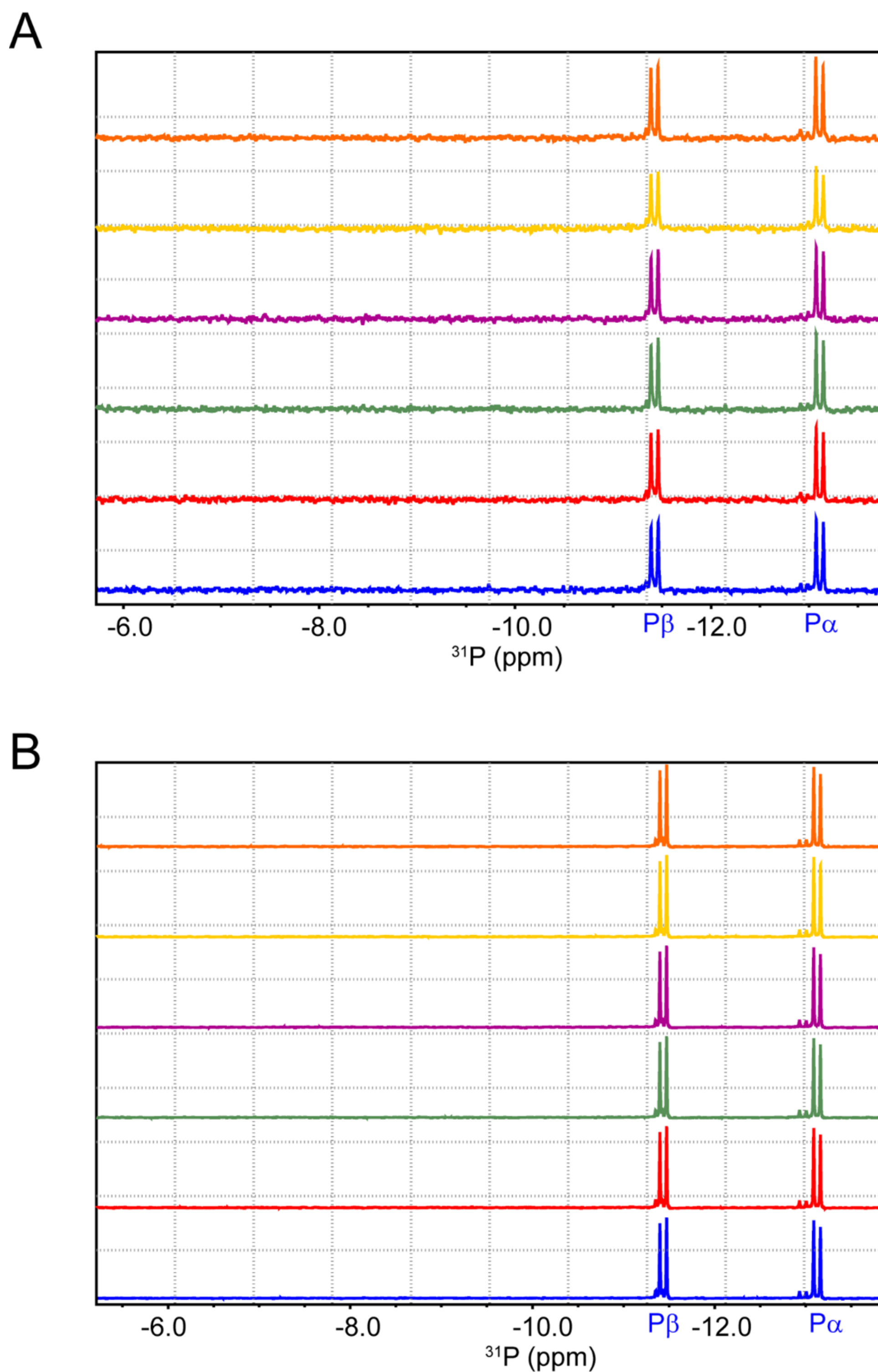

Figure S3. Ligand hydrolysis by $SseK3_{14-333}$. ^{31}P NMR spectra of a 500 μM solution of UDP-GlcNAC in the presence (A) and absence (B) of 10 μM $SseK3_{14-333}$. The spectra were recorded at different time points: 0 min (blue), 5 min (red), 15 min (green), 30 min (purple), 90 min (yellow) and 12 hours (orange). The UDP-GlcNAc Pα and Pβ doublets are assigned in the relative spectra.

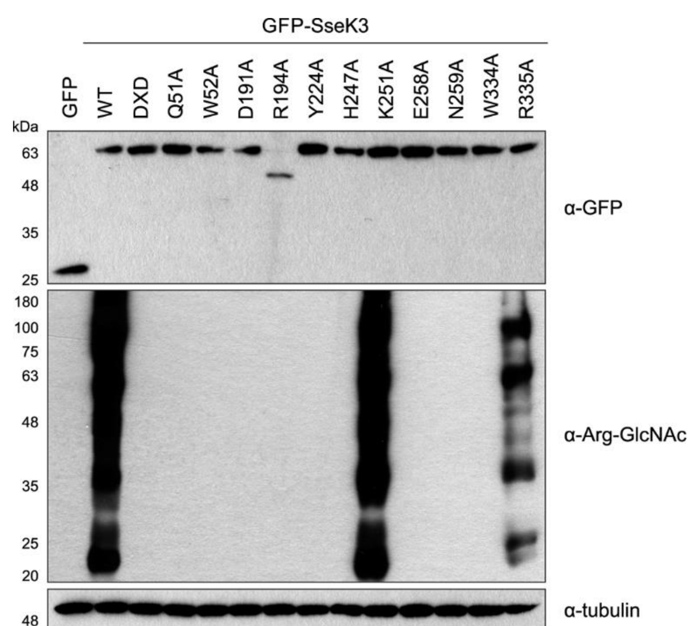

Figure S4. Expression of and Arg-GlcNAcylation induced by putative SseK3 catalytic mutants. 293ET whole cell lysates from Figure 5B were immunoblotted with anti-GFP antibody to test for expression of the GFP-tagged SseK3 variants during NF-κB reporter experiments. Lysates were also blotted with anti-Arg-GlcNAc antibody and anti-tubulin antibody as a loading control. Data shown is representative of 5 independent experiments. DXD corresponds to the SseK3 D226A/D228A mutant.

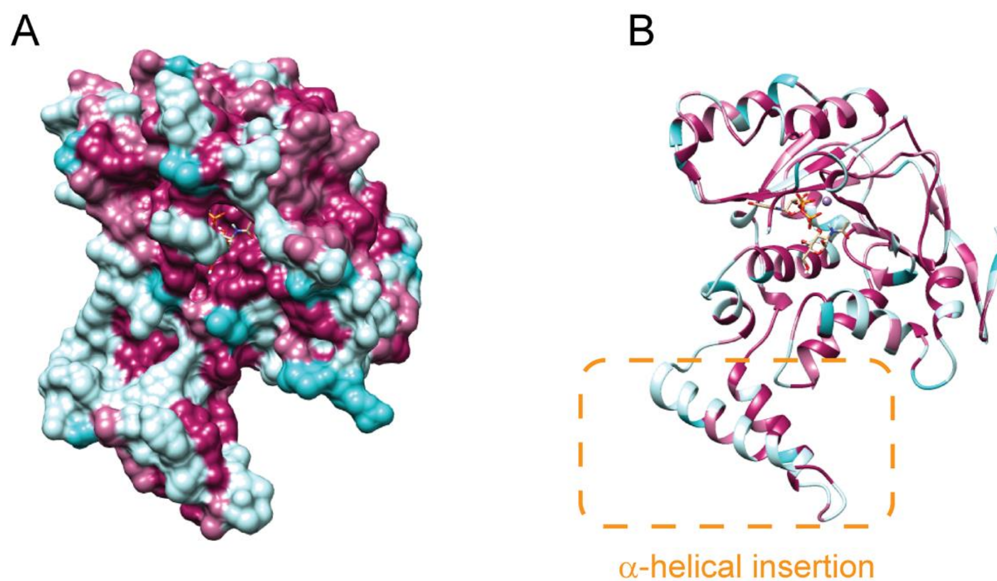

Figure S5. Sequence conservation among the SseK effector family. SseK3 solvent-accessible surface coloured according to sequence conservation among the SseK proteins and their ortholog in *E. coli* NleB. The poorly conserved α-helical insertion is highlighted on the structure of $SseK3_{14-335}$.