

Expanded View Figures

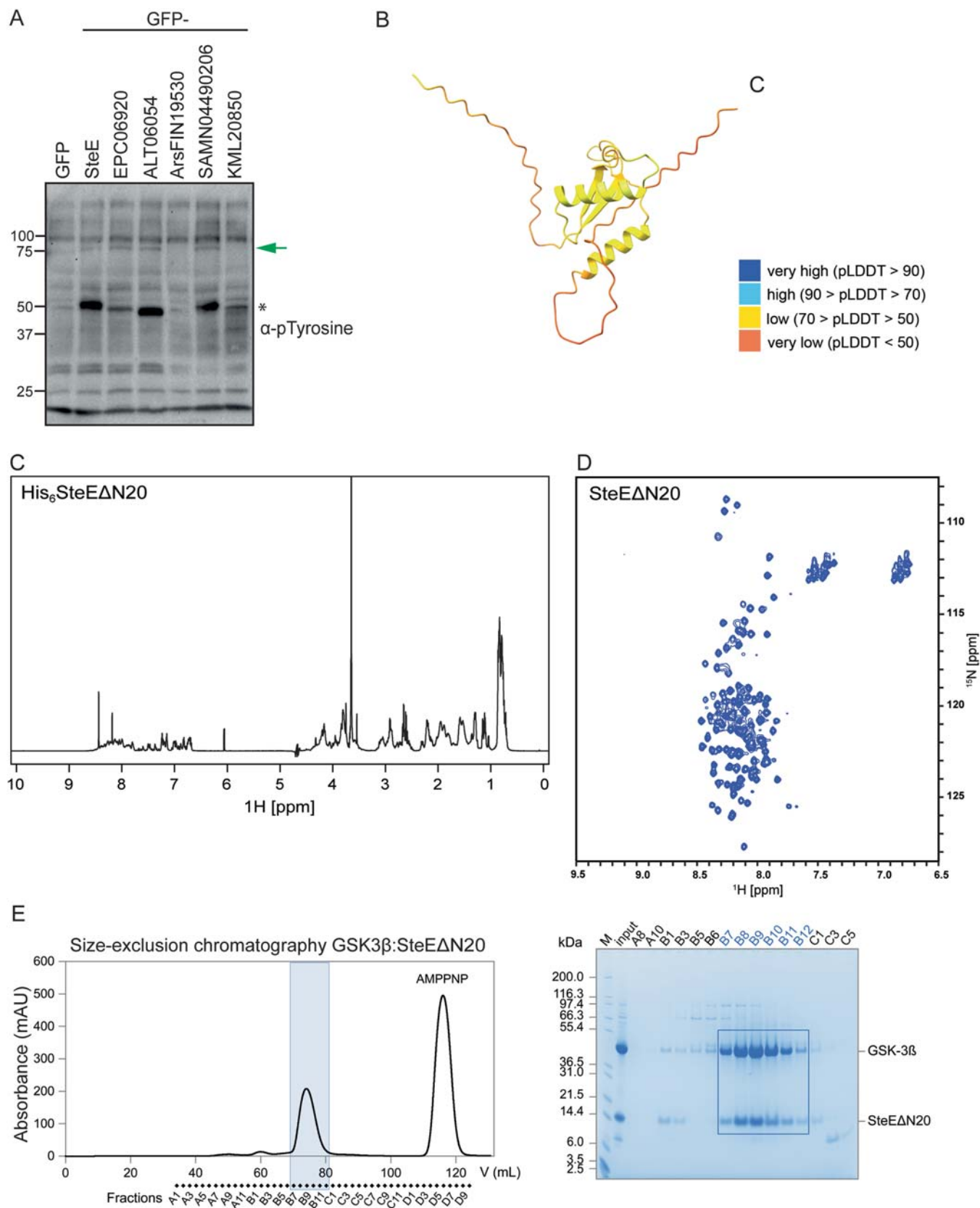
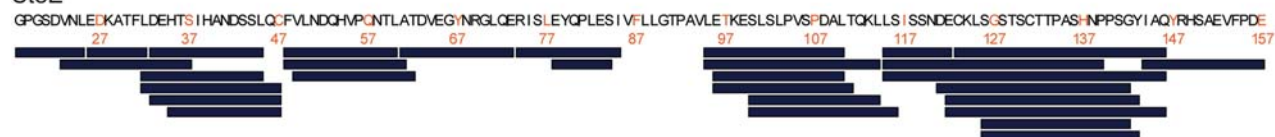


Figure EV1. SteE is a mainly disordered protein in solution.

(A) 293ET cells expressing GFP or GFP-tagged SteE homologues were lysed and analysed by immunoblotting for tyrosine phosphorylation status. Data represent at least three independent biological repeats. * Indicates bands at the expected molecular weight of the GFP-tagged SteE homologues whilst the green arrow represents a band at the expected molecular weight of STAT3. (B) AlphaFold3 prediction of SteE (STM2585), coloured according to pLDDT values. (C) 1D ^1H NMR spectrum of ^{15}N -isotopically labelled His₆SteE $\Delta\text{N}20$ at 266 μM in 18 mM Tris-HCl, 90 mM NaCl, 0.9 mM DTT, 0.9 mM MgCl_2^{2+} , 10% (v/v) D₂O, pH 7.0 was recorded at 700 MHz at 298 K. (D) 2D ^1H , ^{15}N -sofast HMQC spectrum of ^{15}N -His₆SteE $\Delta\text{N}20$ at 266 μM (same buffer as 1B), recorded at 700 MHz at 298 K. (E) Size-exclusion chromatography of SteE $\Delta\text{N}20$ incubated with GSK3 β . Data represent two independent biological repeats.

SteE



Total: 30 peptides, 93.6 % Coverage, 3.66 Redundancy

GSK3 β 

Total: 107 peptides, 93.6 % Coverage, 4.96 Redundancy

Figure EV2. Peptide coverage for HDX-MS.

Amino acid sequence and peptide coverage obtained for SteE and GSK3 β . Related to Fig. 1E.

STM2585	-----MFTIN-STNRVAST-----IAP	16
KML20850	MVE-----FVYNELNLTNRKGTALGSGVWESNMFTIITNADRM-A-----AAA	41
EPC06920	MLR-----SQRFL--SIPKLDYICDHVMEENMLAI-HNLNRI--E-----TAV	38
ALT06054	-----	0
ArsFIN19530	-----	0
SAMN04490206	MLSSRFDSVISSNQVLSSEHTVEGAGSQRASVLSSENTLSVQQTSRSMCKANDAGVDRQCIT	60
STM2585	YACVSDVNLEDKATFLDEHTSI-----HANDSSLQCFVLNDQHPQNTLATDVEGYN-	68
KML20850	DTHLNHVNVVEGKSGVNLVSVKNDPINMLRPDNFSQCVILNNLHVPGALVTDIDNYN-	100
EPC06920	VKHVNCANVEGKPTFVASVSSVENVSTDMKPKIFAQCVILNNLHVQNIPDTDIEGYN-	97
ALT06054	-----MISNNMIKCNVFDLHI PNDAAESDVETIK-	30
ArsFIN19530	--MSNKINITKDRPLIKNSQ--QQNQLKTNALISKIKNCFVLNNIHISTIGSSKAKFYHQ	57
SAMN04490206	NACEVNAENNGVRVLM SALVSVGNVSTGFVRQEINKVIVRDDIHVPQGSMPHDKMCD-	119
	. : : . : *	
STM2585	-----RGLQERISLEYQPLESIVFLLGT TPAVLETKESLSLPVSPDAL	110
KML20850	-----KGLQLRINLEYNPKGSIVFLLGSPEALDANESLSLP IFSHVL	142
EPC06920	-----KGMQVRINQYQPGKTVFLLGSPEVLEPDESLSLPASPHIL	139
ALT06054	-----NDLSERISCEYHTDSNEVFILASPEELED TESLSLAVSPKAL	72
ArsFIN19530	LMLRLNLDSSIQIKNYHHYHLTSRLNLEYNSTTENVALQGT PKNIHSAESLSLPVCPFL	117
SAMN04490206	-----EGQRIRLNDEYHPLHHTVFLQGT PERLGIHHQLSLPVSPSML	161
	* : . ** : * : . : * : . . *** *	
STM2585	TQKL LSISSNDECKLSGSTCTPASHNPP SGY IAQYRHSAEVFPDE-----	157
KML20850	TQKL LNISNSKLCESLVKSNGYV-----	165
EPC06920	AQKLSSIANIKACAFSFSNGYVQRSE---DNFI--YRNGTS-LPL-----	179
ALT06054	HQAISC-----ELAKMTDHDLRERD-----MVETGKEIKPEEDVTKLHEYIIRAN	117
ArsFIN19530	SQKLIQVKNKFT---KKKKCKMKELD-----	140
SAMN04490206	TEKL IEVIREKNE-----KEQRASQAENGYVCQVGSVMTT-----	197
	: :	
STM2585	---	157
KML20850	---	165
EPC06920	---	179
ALT06054	GYV	120
ArsFIN19530	---	140
SAMN04490206	---	197

Figure EV3. Sequence alignment between SteE and its putative homologues.

Amino acid sequence alignment between SteE (STM2585) and its putative homologues as generated by Clustal Omega. Conserved motifs within residues 95–116 of SteE are shown with shading. Known phosphosites in SteE are shown in bold. Asterisk (*) = fully conserved residue, Colon (:) = amino acid residues with similar properties, Period (.) = amino acid with weakly similar properties. Amino acid sequences are listed in Appendix Table S3.

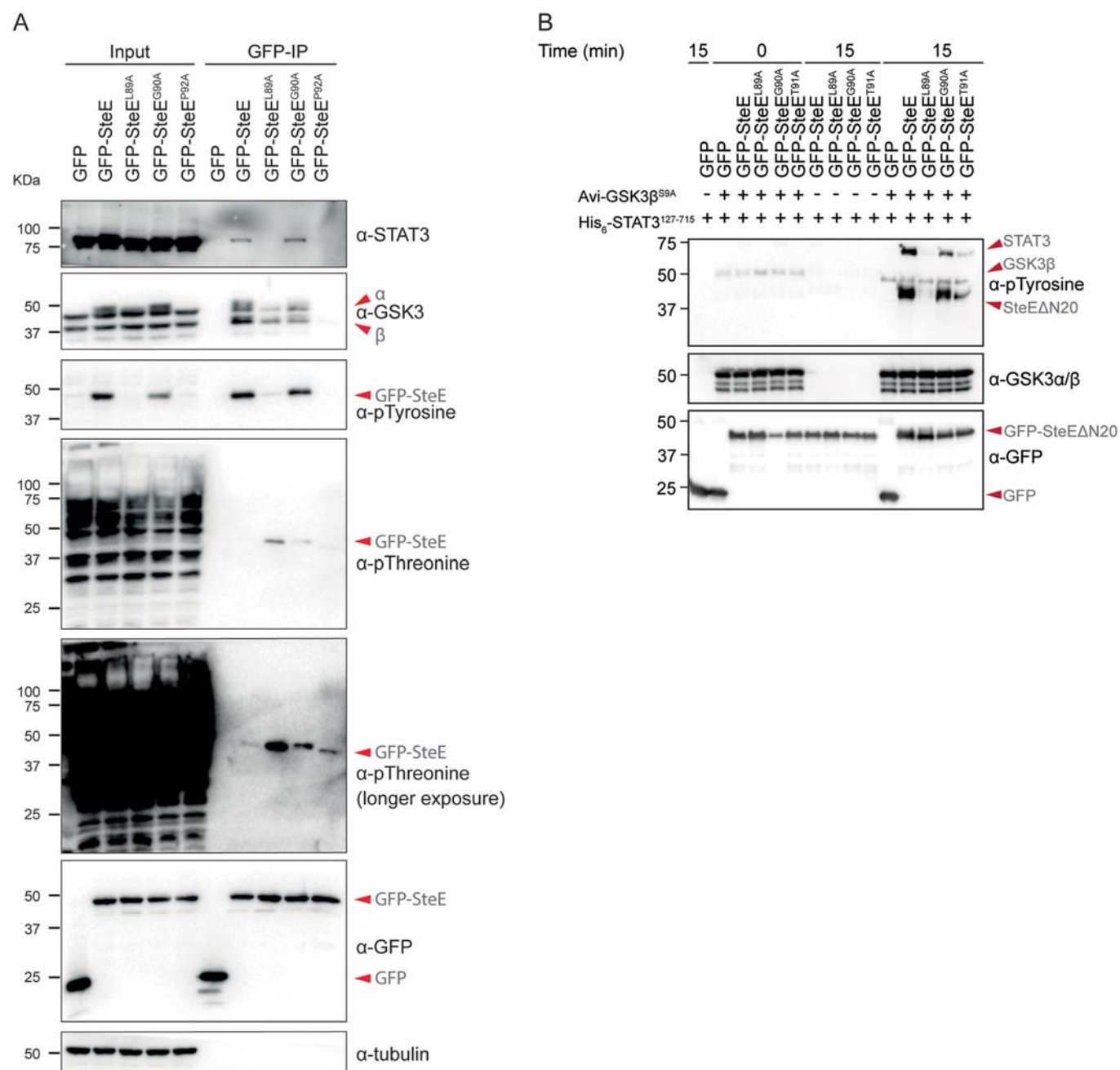


Figure EV4. Residue L89 of SteE is required for tyrosine phosphorylation mediated by GSK3.

(A) 293ET cells expressing GFP or the indicated GFP-tagged SteE variant were lysed, and post-nuclear supernatants were subjected to GFP-TRAP immunoprecipitation (GFP:IP). Samples were analysed by immunoblotting. Data represent three independent biological repeats. (B) The indicated GFP-tagged proteins were immobilised on beads after expression from *GSK3α/β*^{-/-} 293ET cells. Tyrosine phosphorylation by GSK3β was analysed in an in vitro kinase reaction containing 1 mM ATP, 0.2 μM His₆-STAT3¹²⁷⁻⁷¹⁵, with or without recombinant Avi-GSK3β^{S9A} (0.2 μM). Data represent two biological repeats.

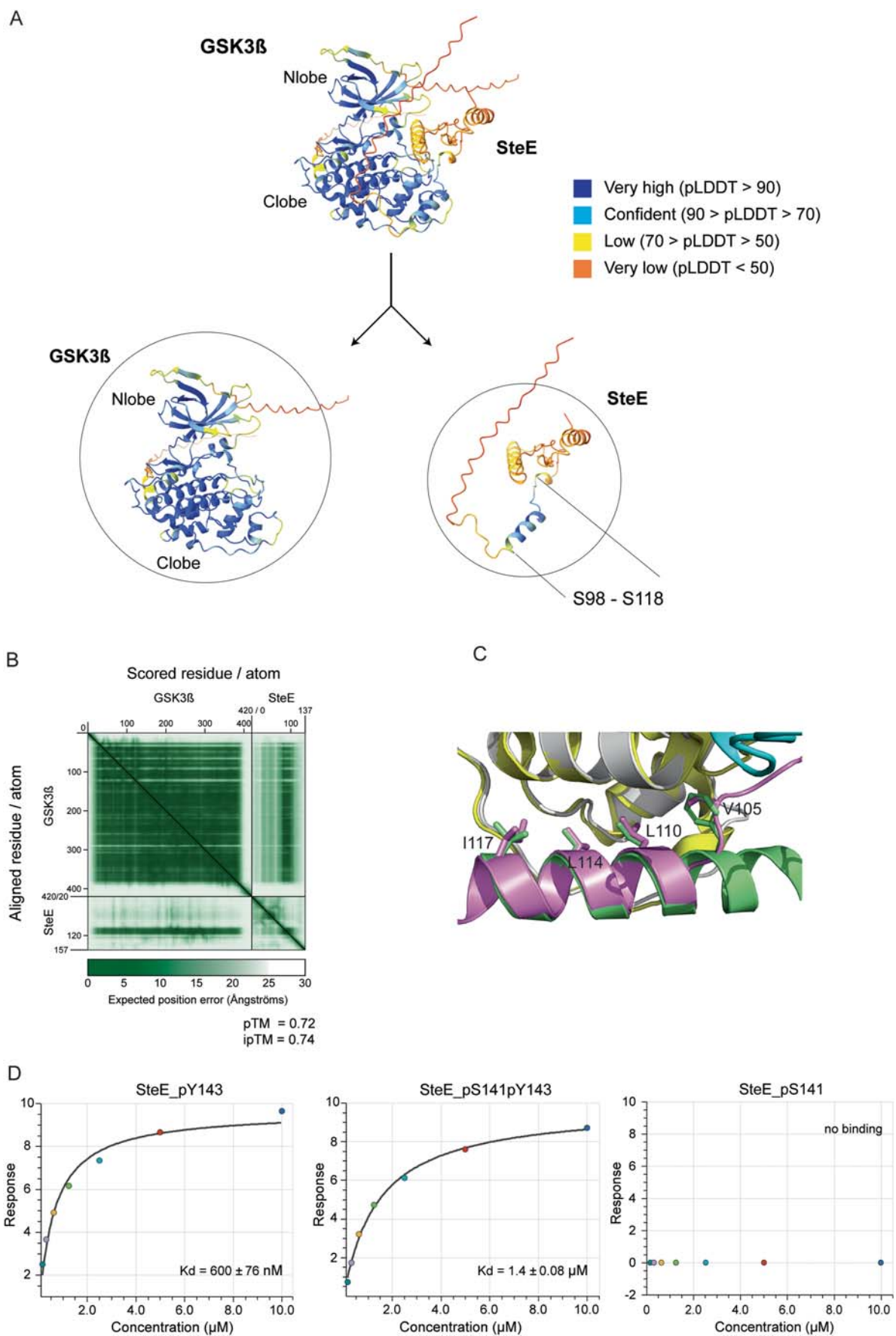


Figure EV5. Model of the GSK3 β -SteE complex and role of SteE phosphorylation on Y143.

(A) The polypeptide chains are coloured according to pLDDT values, shown in the same orientation as in Fig. 4B. For clarity, GSK3 β and SteE are also shown separately to highlight regions on both proteins at various pLDDT confidence levels. (B) Expected position error matrix of the GSK3 β -SteE complex AlphaFold3 prediction. Black lines indicate chain boundaries, with darker green regions showing higher confidence interactions. (C) Structure of GSK3 β -axin peptide (yellow/green) superimposed with the predicted GSK3 β -SteE interacting helix (magenta), with key hydrophobic interface residues of SteE highlighted. (D) Affinity of interaction between the indicated SteE¹³⁸⁻¹⁴⁸ phosphorylated peptides and His₆STAT3¹²⁷⁻⁷¹⁵ was determined by biolayer interferometry.