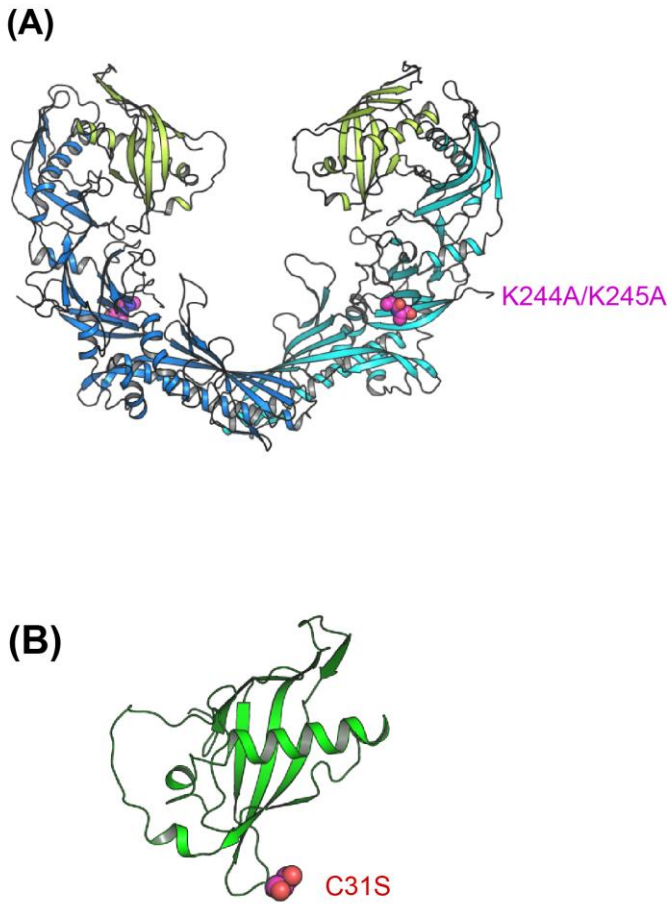

Figure S1. The locations of the K244A/K245A mutations on the OrfX1–OrfX3 complex and the C31S mutation on OrfX1. (A) Crystal structure of the OrfX1–OrfX3 tetrameric complex with K244A/K245A mutations in OrfX3 shown as sphere representations. These two residues are located on the OrfX3 surface, which are not involved in OrfX3 dimerization nor its interaction with OrfX1. **(B)** The structure of OrfX1 with C31S mutation shown as a sphere representation.

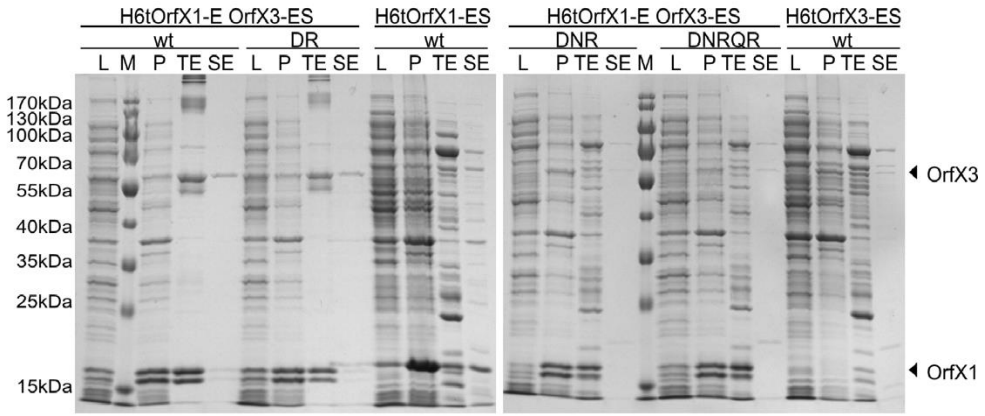

Figure S2. Validation of interactions between OrfX1 and OrfX3 in the OrfX1–OrfX3 complex. Co-expression analysis of the WT H6tOrfX1-ES, WT H6tOrfX3-ES, H6tOrfX1-OrfX3-ES WT complex and the respective OrfX3 mutants D152R/R162A (DR), D57A/N60A/R62A (DNR), D57A/N60A/R62A/Q93A/R162A (DNRQR). Protein load of WT and DR eluates (TE, SE) were 10-fold reduced to account for the high protein expression. L, clear lysate; P, cell pellet after lysis; TE, eluate from Co^{2+}-Talon matrix; SE, eluate from StrepTactin matrix. Samples were analyzed by SDS-PAGE analysis and Coomassie stain.

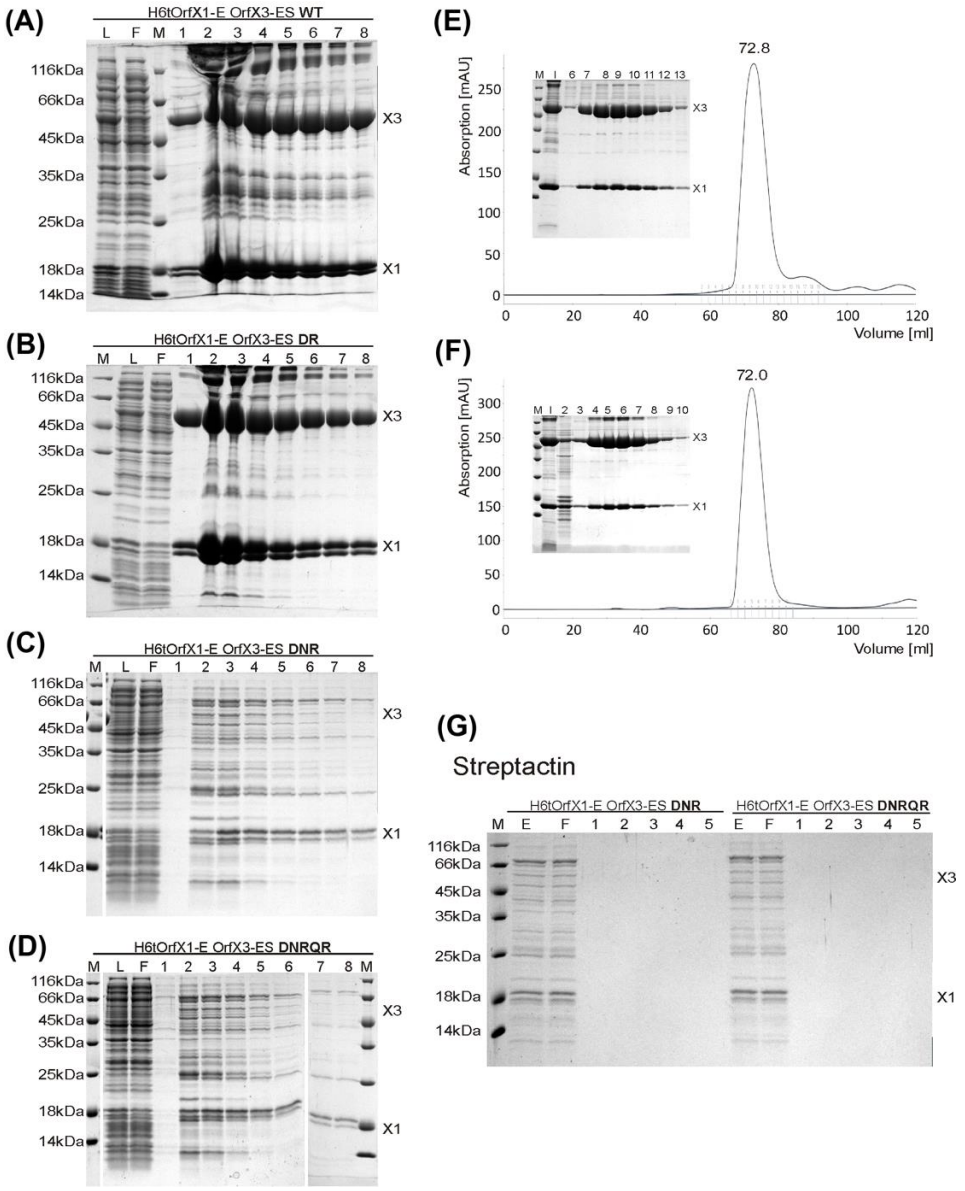

Figure S3. Validation of interactions between OrfX1 and OrfX3 in the OrfX1–OrfX3 complex. Immobilized metal affinity chromatography (IMAC) purification of the H6tOrfX1-OrfX3-ES WT complex (**A**) and the respective OrfX3 mutants D152R/R162A (DR) (**B**), D57A/N60A/R62A (DNR) (**C**), D57A/N60A/R62A/Q93A/R162A (DNRQR) (**D**). L, clear lysate; F, flow through; the numbers indicate fractions eluted from Co^{2+}-Talon matrix. (**E–F**) The pooled IMAC fractions of the WT and the DR mutants were treated with thrombin to remove the His tag and further subjected to size-exclusion chromatography (Superdex-200 16/60PG). Please note the His-tagged OrfX1 shows as double bands on SDS-PAGE, but a single band after His tag removal. (**G**) The pooled IMAC fractions of the DNR and DNRQR mutants were subjected to StrepTactin binding targeting the Strep-tagged OrfX3, which did not yield detectable OrfX3 or the OrfX1–OrfX3 complex. E, eluate from Co^{2+}-Talon matrix; F, flow through; the numbers indicate eluate from StrepTactin matrix. All samples were analyzed by SDS-PAGE and Coomassie stain.

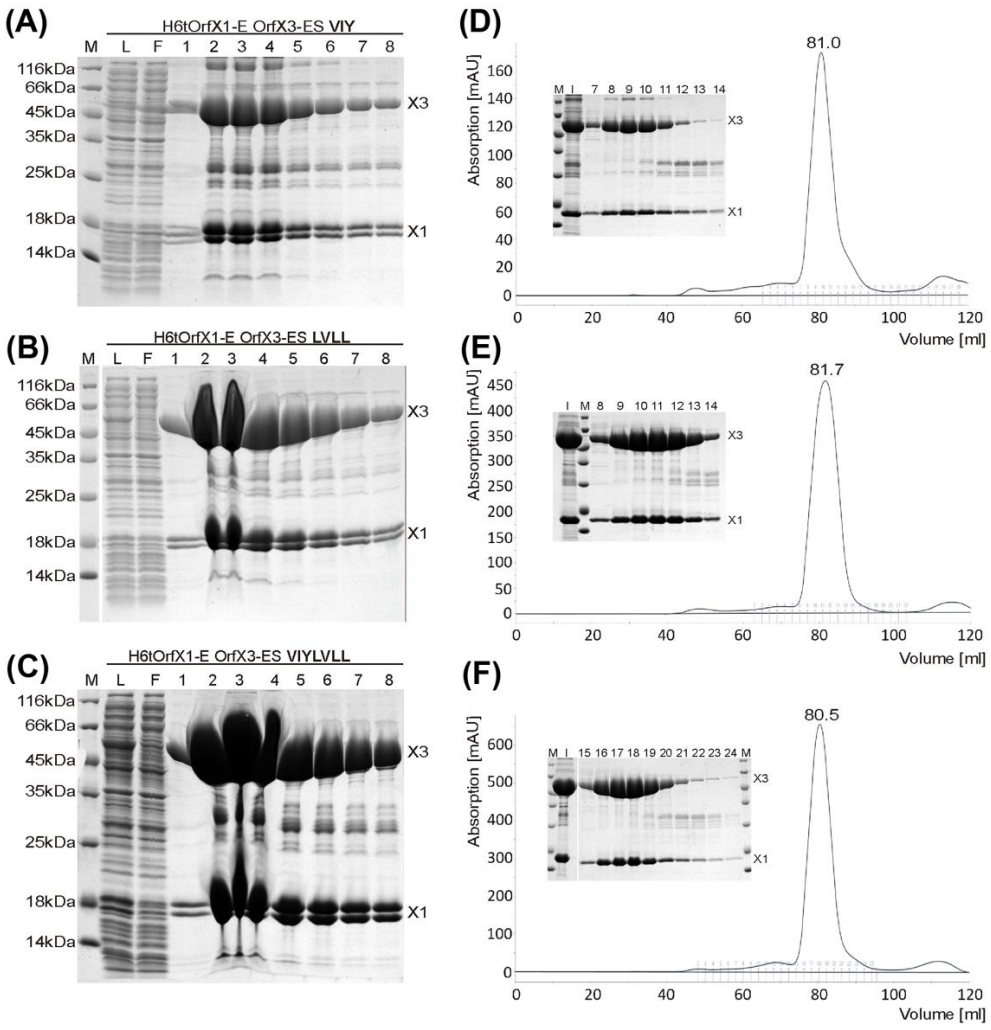

Figure S4. Validation of interactions between the homodimeric OrfX3 in the OrfX1–OrfX3 complex. (A–C) IMAC purification of the respective H6tOrfX1-OrfX3-ES complex mutants V377E/I379E/Y381S (VIY), L390E/V394D/L401E/L405E (LVLL), and (VIY/LVLL); L, clear lysate; F, flow through, the numbers indicate fractions eluted from Co^{2+}-Talon matrix. All samples were analyzed by SDS-PAGE and Coomassie stain. (**D–F**) The pooled IMAC fractions of the three mutants were treated with thrombin to remove the His tag and further subjected to size-exclusion chromatography (Superdex-200 16/60PG).

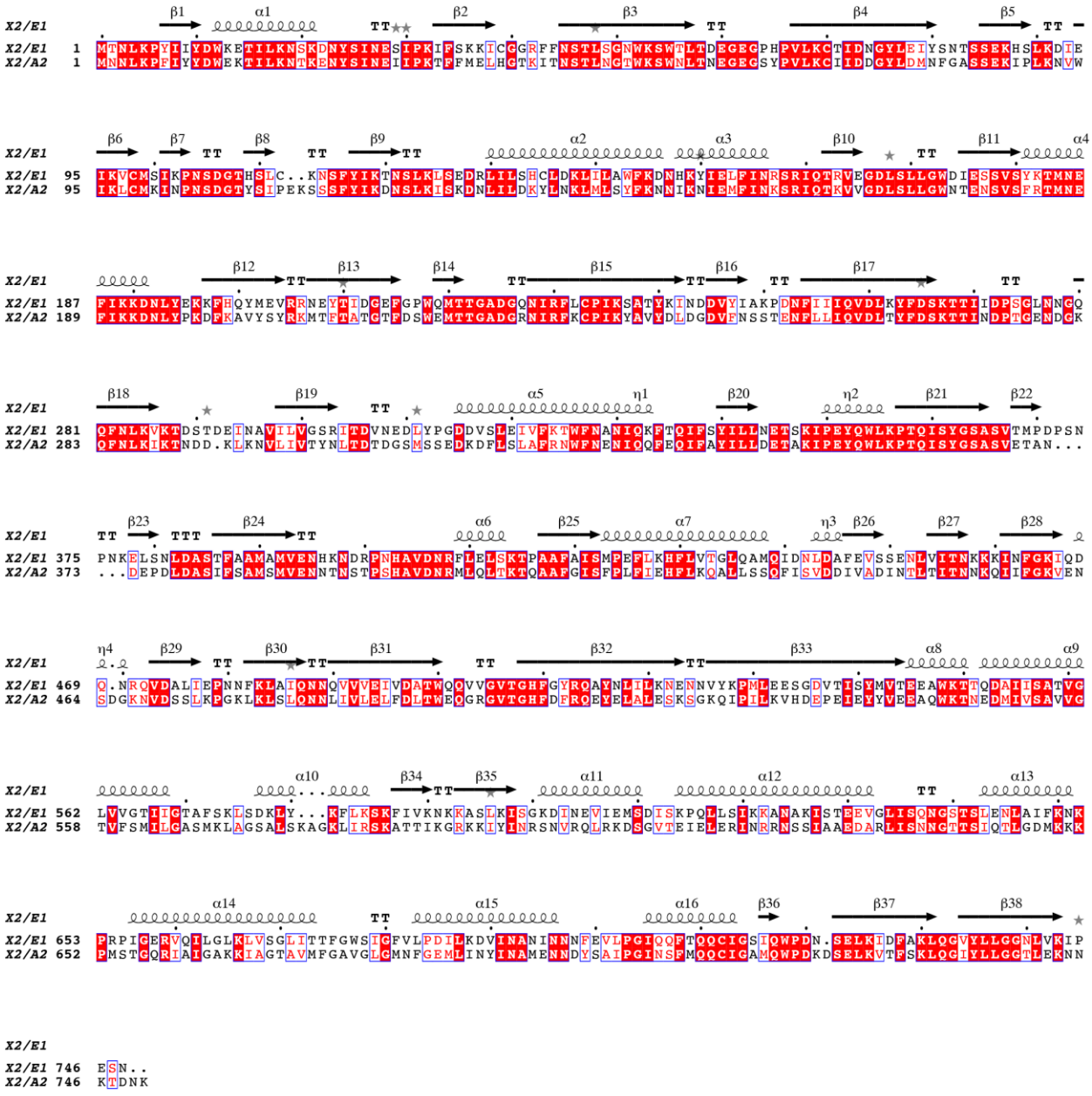

Figure S5. Sequence alignment between BoNT/E1 OrfX2 and BoNT/A2 OrfX2.

Table S1. Data collection and refinement statistics.

	SeMet-OrfX1–OrfX3 (K244A/K245A)	OrfX1–OrfX3 (K244A/K245A) PDB: 8FBD	SeMet-OrfX1-C31S PDB: 8FBE	SeMet-OrfX2 PDB: 8FBF
Data collection				
Wavelength (Å)	0.97918	0.97918	0.97918	0.9795
Resolution (Å)	173.01-2.35 (2.40-2.35)	141.28-2.05 (2.09-2.05)	49.02-1.73 (1.76-1.73)	65.05-1.85 (1.92-1.85)
Space group	*P* $2_12_12_1$	*P* $2_12_12_1$	*P* 3_2	*P* 2_1
Cell dimensions				
a, *b*, *c* (Å)	39.2, 173.0, 242.8	39.3, 174.0, 242.0	56.6, 56.6, 84.3	106.3, 83.2, 108.4
α, *β*, *γ* (°)	90, 90, 90	90, 90, 90	90, 90, 120	90, 105.8, 90
Completeness (%)	99.9 (99.8)	99.2 (95.4)	99.8 (100.0)	99.8 (99.5)
Redundancy	3.7 (3.7)	4.7 (4.5)	3.5 (3.5)	6.7 (6.2)
R_{merge} (%)	14.0 (136.2)	8.9 (77.8)	5.3 (77.8)	14.7 (75.8)
R_{pim} (%)	8.2 (80.1)	6.5 (61.7)	3.4 (49.7)	6.2 (31.8)
Mean *I*/σ(*I*)	12.0 (1.5)	11.1 (1.6)	17.3 (1.8)	22.2 (2.3)
$CC_{1/2}$	0.996 (0.543)	0.994 (0.545)	0.999 (0.555)	0.966 (0.892)
Refinement				
Resolution (Å)		49.67-2.05	19.94-1.73	19.89-1.85
No. reflections		104, 662	31, 471	154, 831
R_{work}/R_{free} (%)		17.99/21.90	17.21/21.65	19.12/23.95
No. atoms				
Protein		9, 958	2, 379	11, 855
Ligand/ion		8	0	20
Water		572	238	848
B-factors (Å^2)				
Protein		48.4	35.2	35.2
Ligand/ion		45.5	-	48.6
Water		46.7	42.1	39.1
r.m.s. deviations				
Bond lengths (Å)		0.008	0.007	0.006
Bond angles (°)		1.073	1.080	1.008

Values in parentheses are for the highest resolution shell.

Table S2. Interacting residues at the OrfX1–OrfX3 heterodimer interface.

Interacting residues at the OrfX1–OrfX3 heterodimer interface				
Type of interaction	**Chain ID**	**Residues in OrfX1**	**Chain ID**	**Residues in OrfX3**
Salt Bridge	A, C	D58	B, D	R78
Salt Bridge	A, C	E108	B, D	R162
Salt Bridge	A, C	E122	B, D	R162
H-bond	A	N14	B	E151
H-bond	C	N14	D	D152
H-bond	A, C	N30	B, D	Q233
H-bond	A, C	C31	B, D	S59
H-bond	A, C	S32	B, D	D57
H-bond	A, C	N34	B, D	D57
H-bond	A, C	Y35	B, D	G55
H-bond	A, C	Y37	B, D	N60
H-bond	A	K52	B	Y147
H-bond	A, C	N102	B, D	G55
H-bond *	A, C	N102	B, D	R62
H-bond	A, C	Y104	B, D	N60
H-bond	A, C	Y104	B, D	Q93
H-bond	A, C	N107	B, D	P124
H-bond	A, C	N126	B, D	S133
Hydrophobic	A, C	I119	B, D	Y40, Y159, L163
Hydrophobic	A, C	L120, V123, I124	B, D	F155
Cation-pi	A, C	Y104	B, D	R62
Van der Waals	A, C	N30	B, D	Q235
Van der Waals	A, C	P33	B, D	Q234, Q235, K236
Van der Waals	A, C	V105	B, D	I130
Van der Waals	A, C	I106	B, D	P124, E125
Van der Waals	A, C	K116, N117, G118	B, D	Y40
Van der Waals	A, C	L120	B, D	F155
Van der Waals	A, C	L130	B, D	S133

* indicates a water-mediated H-bond.

Table S3. Interacting residues at the OrfX3 homodimer interface in the OrfX1–OrfX3 complex.

Interacting residues at the OrfX3 homodimer interface in the OrfX1–OrfX3 complex				
Type of interaction	**Chain ID**	**Residues OrfX3**	**Chain ID**	**Residues in OrfX3'**
H-bond	B	S375	D	N387
H-bond	B, D	N378	D, B	S382
H-bond	B, D	Q380	D, B	Q380
H-bond	B, D	S382	D, B	N378
Van der Waals	B, D	V377, I379	D, B	E383, Y381
Hydrophobic	B, D	S398, L401, S402 L405	D, B	L390, V393, V394, L397, S398