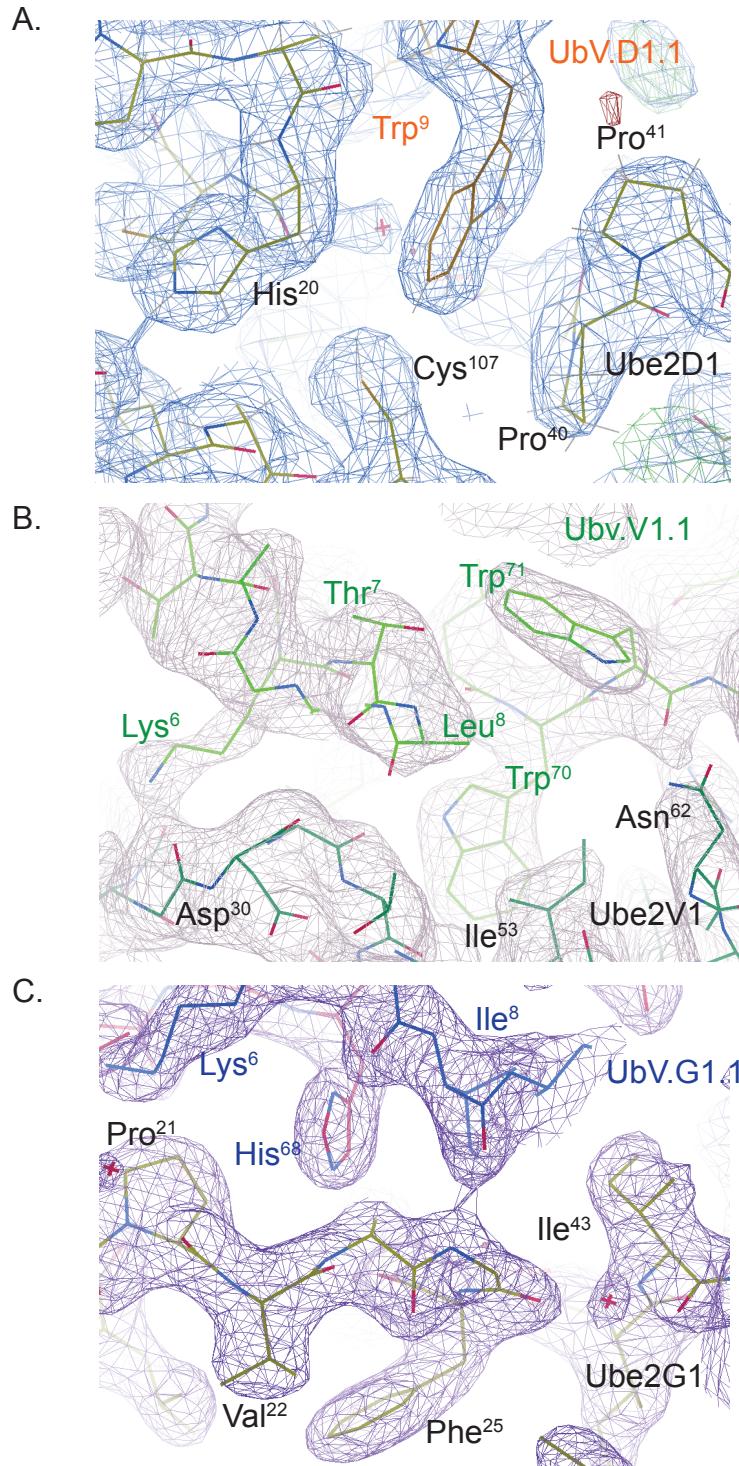
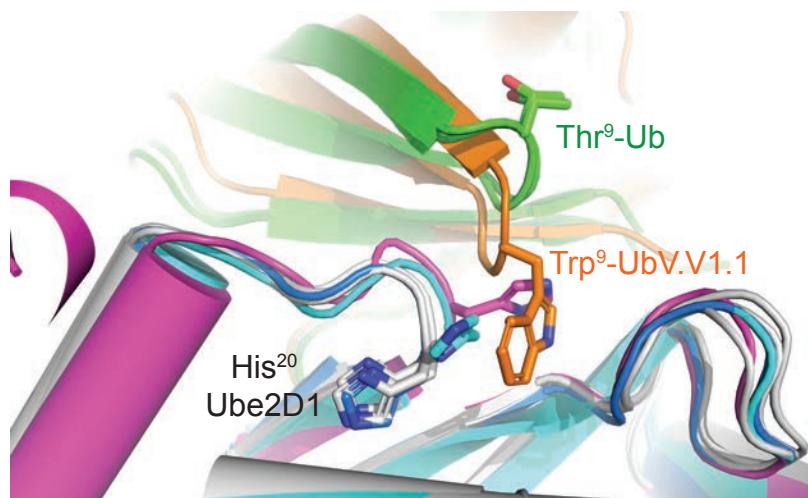
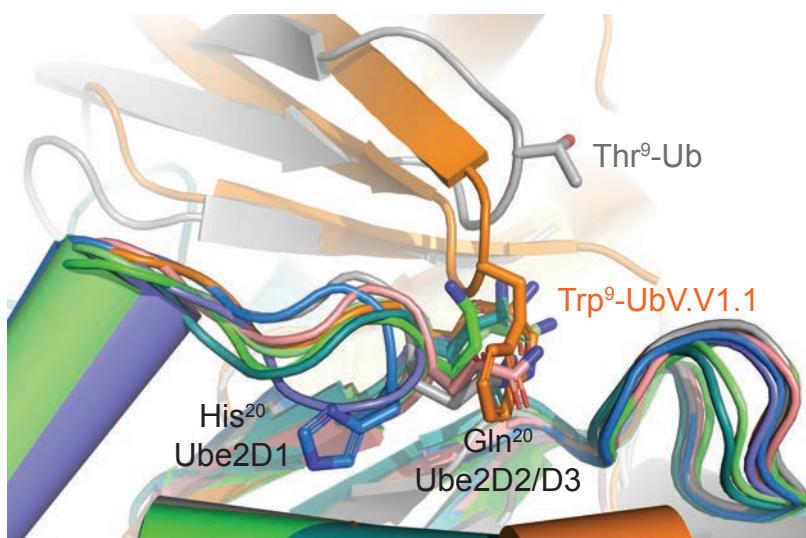


**Figure S1**

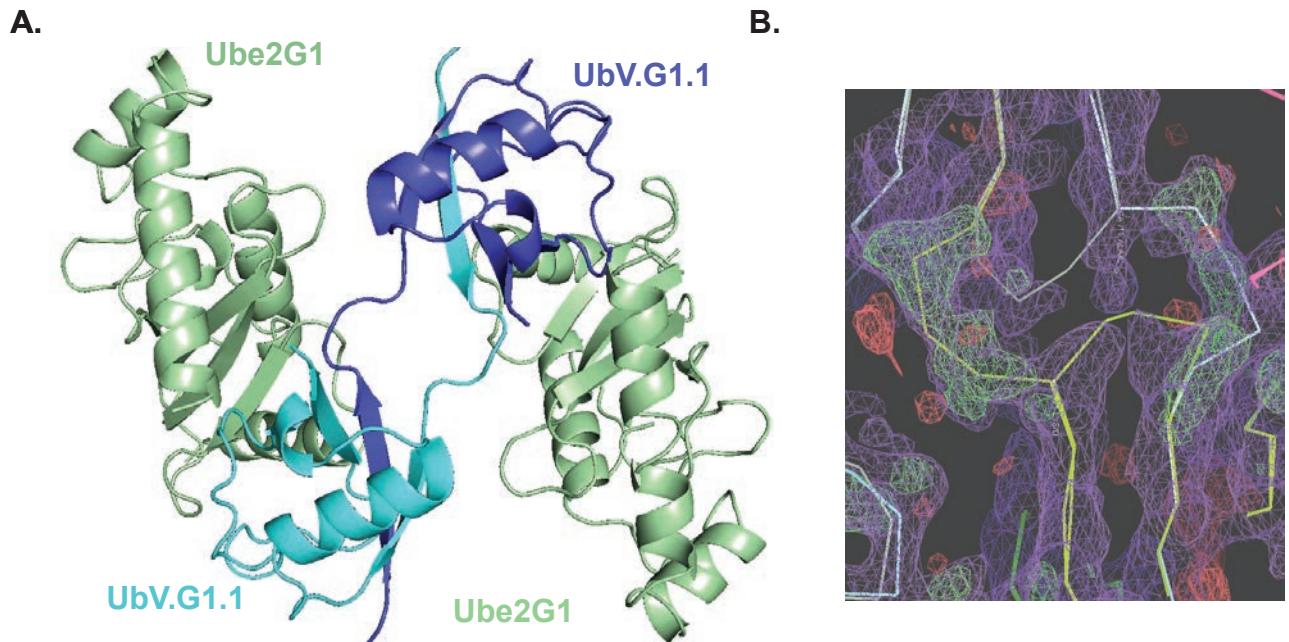
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Ube2D1	L	Q	P	P	A	H	C	S	A	G	P	Q	A	T	M	P	Q	G	V	F	M
Ube2D2	L	A	P	P	A	Q	C	S	A	G	P	Q	A	T	M	N	Q	G	V	F	M
Ube2D3	L	A	P	P	A	Q	C	S	A	G	P	Q	A	T	M	N	Q	G	V	F	M
Ube2D4	L	Q	P	P	A	Q	C	S	A	G	P	Q	A	T	M	N	Q	G	V	F	M
Ube2V1	L	Q	G	D	G	T	V	S	W	G	G	T	G	M	I	P	E	N	I	S	-
Ube2V2	L	Q	G	D	G	T	V	S	W	G	L	T	G	M	I	P	E	N	I	S	-
Ube2G1	L	N	P	V	E	G	F	S	A	G	L	E	V	L	I	P	E	G	V	K	K
Ube2G2	Y	T	P	P	E	G	I	V	A	G	P	E	A	L	M	E	E	F	V	P	K
Ube2A	F	Q	P	P	A	G	V	S	G	A	P	N	A	V	F	E	E	D	T	K	A
Ube2B	F	Q	P	P	V	G	V	S	G	A	P	N	A	V	F	E	E	D	T	K	A
Ube2E1	L	T	P	P	P	N	C	S	A	G	P	R	S	T	L	P	E	G	V	F	T
Ube2E2	L	T	P	P	P	N	C	S	A	G	P	R	S	T	L	P	E	G	V	F	T
Ube2E3	L	T	P	P	P	N	C	S	A	G	P	R	S	T	L	P	E	G	V	F	T
Ube2C	L	M	G	D	K	G	I	S	A	F	P	V	G	T	H	A	E	D	R	K	S
Ube2F	V	E	L	P	C	T	C	K	V	F	P	Q	L	T	-	D	Q	G	K	Q	-
Ube2H	V	I	S	K	H	E	V	T	I	L	G	V	V	K	Y	Q	E	G	V	K	T
Ube2I	R	R	H	P	F	G	F	V	A	V	P	E	C	A	P	K	E	G	L	K	A
Ube2J1	A	K	P	T	D	H	Y	H	A	Q	P	H	F	T	R	P	D	G	V	H	G
Ube2J2	Y	K	P	V	P	Y	I	C	A	E	P	H	Y	V	R	E	E	G	Y	H	A
Ube2K	F	L	S	K	N	Q	I	K	V	D	L	R	G	E	A	P	E	G	R	Q	G
Ube2L3	L	R	G	M	K	N	F	R	N	Q	V	Q	G	L	-	D	D	K	A	R	E
Ube2L6	L	Q	P	P	P	Y	L	R	N	S	S	H	A	L	-	D	H	L	A	N	V
Ube2M	-	E	L	P	K	T	C	D	I	F	S	K	L	V	-	D	K	S	K	V	-
Ube2N	T	L	P	V	P	G	I	K	A	E	P	H	V	V	A	Q	E	G	T	K	M
Ube2Q1	L	Y	K	G	G	N	Y	A	V	E	L	N	V	K	L	D	H	A	F	L	I
Ube2Q2	L	Y	K	T	G	I	Y	S	V	E	L	H	V	K	Q	D	H	I	Y	L	I
Ube2R1	L	Q	P	V	E	G	F	R	V	T	L	E	V	A	F	P	E	G	Y	K	K
Ube2R2	L	Q	P	V	E	G	F	R	I	T	L	E	V	A	F	P	E	G	Y	K	K
Ube2S	Y	T	P	P	D	G	I	K	V	F	P	Q	V	T	E	E	A	G	L	R	G
Ube2T	L	A	P	P	P	G	I	T	C	W	Q	R	A	Q	L	A	E	K	V	K	R
Ube2U	F	K	N	Y	K	G	I	T	A	K	P	E	V	E	E	Q	Q	G	V	Q	M
Ube2W	L	Q	P	P	P	G	M	T	L	K	S	I	V	D	E	P	E	G	K	Q	T
Ube2Z	I	Y	P	P	P	G	M	F	V	V	P	H	A	L	T	F	E	G	F	L	V



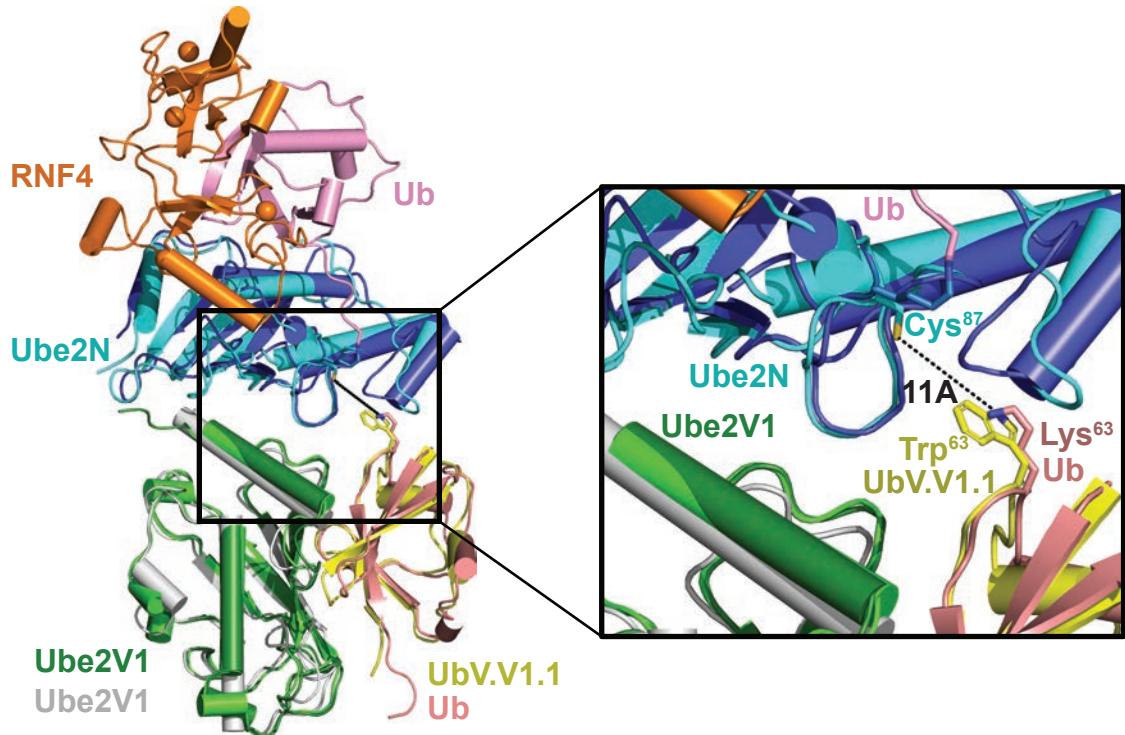
**Supplementary Figure 2.** Representative electron density of each UbV in complex with the backside of its cognate E2 protein. A) Residues of Ube2D1 and UbV.D1.1 are labelled in black or orange respectively. The electron density 2FoFc map at 2.1 Å resolution was contoured at 1.0 $\sigma$  and colored blue. B) Residues of Ube2V1 and UbV.V1.1 are labelled in black or green respectively. The electron density 2FoFc map at 2.55 Å resolution was contoured at 1.0 $\sigma$  and colored gray. C) Residues of Ube2G1 and UbV.G1.1 are labeled in black or blue respectively. The electron density 2FoFc map at 2.35 Å resolution was contoured at 1.0 $\sigma$  and colored purple.

**A.****B.**

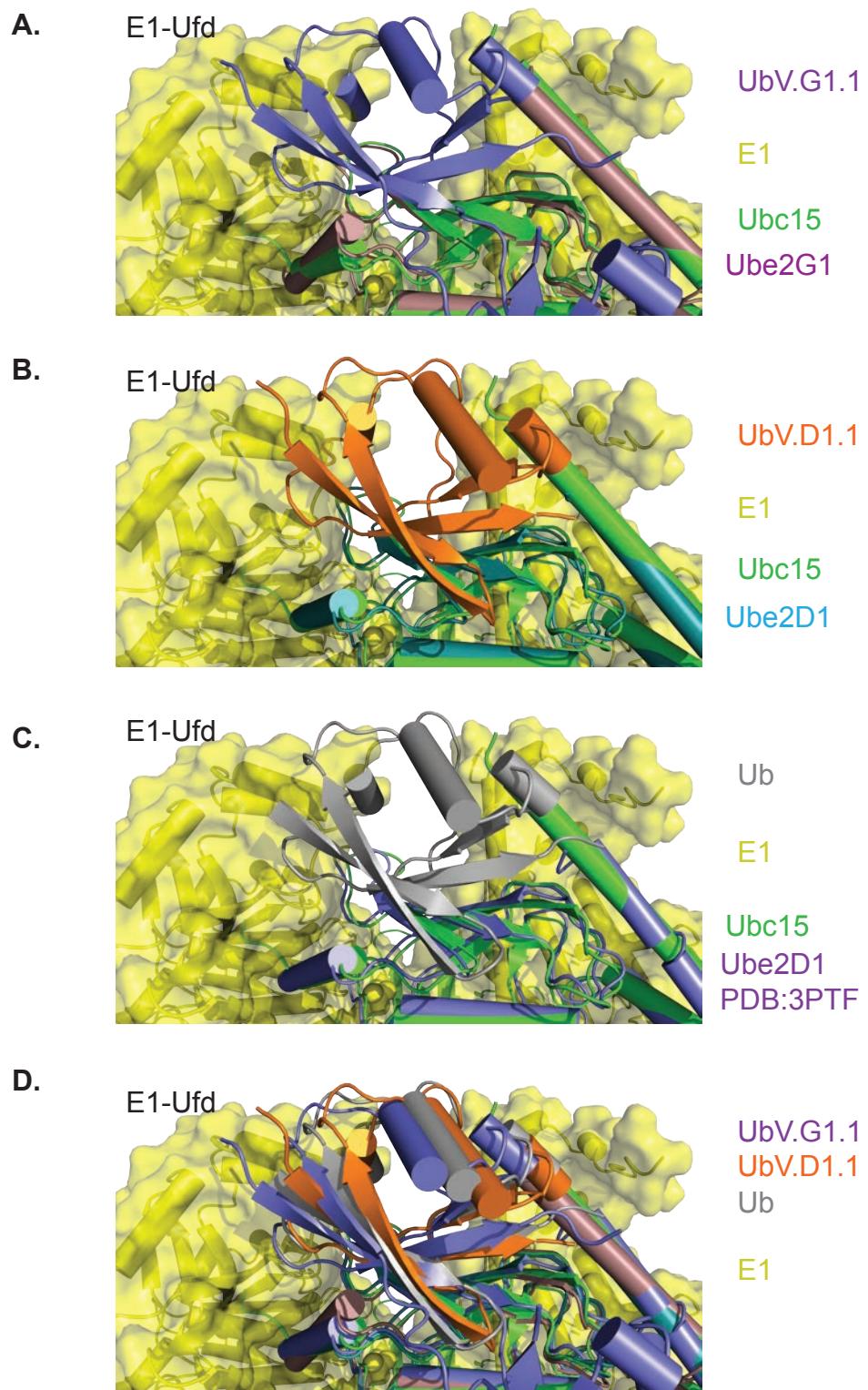
**Supplementary Figure 3. Structural basis for the specificity of UbV.D1.1 for Ube2D1 over other Ube2D family members.** (A) Trp<sup>9</sup> of UbV.D1.1 projects into a cleft on the Ube2D1 surface with His<sup>20\*</sup> in the open conformation. The same orientation of His<sup>20\*</sup> is observed in the Ube2D1~Ub donor complexes (PDB: 3OJ4, cyan; 4AP4, gray; 5TUT, gray) and Ube2D1-Ub backside complex (PDB: 3PTF, gray). An alternative closed conformation of His<sup>20\*</sup> that is observed in the Ube2D1 apo structure (PDB: 2C4P, pink) would clash with Trp<sup>9</sup> of UbV.D1.1 but not with Thr<sup>9</sup> of Ub.wt bound to the backside (PDB: 3OJ4 and 3PTF in green). (B) Trp<sup>9</sup> of UbV.D1.1 clashes with the closed conformation of Gln<sup>20\*</sup> observed in Ube2D2/3 structures (PDB: 3A33, pink; 3TDG, orange, 3UGB, light green, 3JVZ, green, 4V3L, gray and 5DIL, cyan). Thr<sup>9</sup> of Ub bound to the backside of Ube2D2 (PDB: 4V3L, gray) would not clash with the closed conformation of Gln<sup>20\*</sup>.



**Supplementary Figure 4.** The crystal structure of the Ube2G1-UbV.G1.1 complex. Two Ube2G1 chains (light green) bound to two UbV.G1.1 chains (cyan and blue) [RMSD of Ube2G1-UbV.G1.1 superimposed on the second complex is 0.624 Å]. The UbV.G1.1 protomers exchange  $\beta$ 1-strands, which results in a higher order complex observed in the crystal structure lattice. (B) Electron density 2fofc map (contoured at 1.0  $\sigma$ ) is shown in purple and the fofc difference density map (contoured at 2.5  $\sigma$ ) is shown in green. UbV.G1.1  $\beta$ 1- $\beta$ 2 loop regions are superimposed as separate Ub chains or as the  $\beta$ 1-strand exchanged dimer.



**Supplementary Figure 5. The crystal structure of the Ube2N-Ube2V1-UbV.V1.1 complex.** The complex of Ube2V1 (green), Ubv.V1.1 (yellow) and Ube2N (cyan) was superimposed on the complex of Ubc13 (blue), Ub (pink), RNF4 (orange) and Ube2V2 (green) (PDB: 5AIT) and the complex of Ube2V1 (gray) and Ub (pink) (PDB: 1ZGU). The trimeric arrangement positions Trp<sup>63</sup> of UbV.V1.1 (equivalent to Lys<sup>63</sup> of Ub positioned on Ube2V1) approximately 11 Å from the catalytic Cys<sup>87</sup> of Ube2N. A zoomed view of the interface is shown on the right.



**Supplementary Figure 6. Superposition of E2-UbV backside complexes with the E1-Ubc15 complex (PDB: 5KNL).** (A) The Ub fold domain (Ufd) of E1 in yellow clashes with UbV.G1.1 in complex with the backside of Ube2G1 when superimposed on the Ubc15 E2 domain. (B) UbV.D1.1 bound to the backside of Ube2D1 exhibits minor clashes with the E1 Ufd. (C) Ub bound to the backside of Ube2D1 (PDB: 3PTF) exhibits a minor clash with the E1 Ufd. (D) Overlay of the above panels reveals that the most significant modeled clash is between UbV.G1.1 and the E1 Ufd due to a rotation of the UbV relative to Ub in the backside position.



Supplementary Table 1 continued

Target	Library	Ubv	Ub variant sequence																												
			wt	2	4	6	7	8	9	10	11	12	14	42	44	46	47	48	49	62	63	64	65	66	68	70	71	72	73	74	75
Ube2V1	2	V1.41	- - - - M	-	-	-	-	-	-	-	-	-	-	- -	D	F	G	L	W	-	-	-	-	-	-	-	-	-	-		
	2	V1.42	- - - - -	-	-	-	-	-	-	-	-	-	-	- -	D	F	V	L	W	M	-	-	-	-	-	-	-	-	-	-	
	2	V1.43	- - - - -	-	-	-	-	-	-	-	-	-	-	- -	G	F	-	L	W	-	-	I	P	R	D	-	-	-	-	-	
	2	V1.44	- - - - -	-	-	-	-	-	-	-	-	-	-	- -	G	F	-	L	W	-	-	P	W	S	-	-	-	-	-	-	
	2	V1.45	- - - - -	-	-	-	-	-	-	-	-	-	-	- -	Q	F	-	L	W	V	K	F	P	S	S	-	-	-	-	-	
	2	V1.46	- - - - -	-	-	-	-	-	-	-	-	-	-	- -	S	F	A	L	W	D	-	I	-	-	-	-	-	-	-	-	
	2	V1.47	- - - I	-	-	-	-	-	-	-	-	-	-	- -	Y	A	L	W	W	-	P	-	S	V	-	-	-	-	-	-	
	2	V1.48	- - - N	-	-	A	-	-	I	-	-	-	L	- -	H	-	L	W	W	-	T	-	S	-	-	-	-	-	-		
	2	V1.49	- - - P	-	S	S	-	-	-	-	-	-	-	- -	D	Y	V	L	W	-	-	P	-	-	-	-	-	-	-	-	
	2	V1.50	- - - S	-	-	-	-	-	-	-	-	-	-	- -	F	-	L	W	T	-	-	E	D	-	-	-	-	-	-	-	
	2	V1.51	- - - S	-	-	-	-	-	-	-	-	-	-	- -	V	A	-	L	W	F	Q	-	-	-	-	-	-	-	-	-	
	2	V1.52	- - - N	K	G	K	-	-	-	-	-	-	-	- -	Y	A	N	G	V	S	I	-	-	-	-	-	-	-	-	-	
	2	V1.53	- - - Q	I	-	Y	-	-	-	A	-	-	-	- -	R	F	G	L	W	F	T	-	-	-	-	-	-	-	-	-	
	2	V1.54	- - - Q	S	-	S	-	-	-	-	-	-	-	- -	D	F	N	-	W	F	-	I	F	-	-	-	-	-	-	-	
	2	V1.55	- - - T	-	P	-	-	-	-	-	-	-	-	- -	F	R	I	-	G	-	F	H	S	-	-	-	-	-	-	-	
	2	V1.56	- - - T	L	V	M	-	-	G	-	-	K	- -	R	-	S	Y	-	-	S	T	L	-	-	-	-	-	-	-	-	
Ube2G1	2	G1.1	- - - P	I	R	V	-	-	-	-	S	-	L	- -	Y	-	-	L	-	-	R	H	-	-	-	-	-	-	-	-	
	2	G1.2	- - - V	F	W	-	-	-	-	-	-	-	L	- -	K	F	A	-	L	-	-	-	-	-	-	-	-	-	-	-	
	2	G1.3	- - - N	I	R	-	-	-	G	-	G	-	W	- -	Y	S	-	-	-	-	Y	-	-	-	-	-	-	-	-	-	
	2	G1.4	- - - V	F	W	-	-	-	-	-	-	-	L	- -	A	L	A	-	K	-	H	-	L	-	-	-	-	-	-	-	
	2	G1.5	- - - A	G	R	-	-	-	-	-	-	-	L	- -	F	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	2	G1.6	- - - H	-	R	A	-	-	T	-	-	-	L	- -	F	A	Q	-	-	-	-	R	-	-	-	-	-	-	-	-	
	2	G1.7	- - - A	F	Y	-	-	-	-	-	-	-	-	- -	F	A	-	L	-	-	P	-	-	-	-	-	-	-	-	-	
	2	G1.8	- - - A	F	Y	-	-	-	-	-	-	-	-	- -	L	Y	-	L	M	-	V	-	V	-	-	-	-	-	-	-	
	2	G1.9	- - - K	G	Y	-	-	-	G	-	-	N	- -	Y	R	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Ube2G2	1	G2.1	- - R	-	-	K	W	-	I	S	-	Y	S	-	-	R	M	G	-	N	-	-	F	-	I	L	-	K	-	-	
	1	G2.2	- - R	-	V	N	R	S	-	-	S	-	H	-	K	-	Q	-	A	L	I	-	M	-	N	-	-	-	-	-	
	1	G2.3	- - - -	K	W	Q	I	-	-	-	-	-	-	K	-	K	-	-	L	-	Q	-	-	G	V	-	-	-	-	-	
	1	G2.4	T	-	-	P	K	W	-	I	N	I	F	-	-	K	-	Q	-	-	L	G	-	-	-	-	-	-	-	-	-
	1	G2.5	- - - -	W	-	L	-	-	-	-	-	-	Y	-	R	-	N	-	-	Y	-	-	I	-	Q	-	-	-	-	-	
	1	G2.6	H	-	-	I	-	W	Y	-	-	F	-	T	P	R	-	G	-	N	-	-	V	-	-	S	Y	-	-	-	-
	1	G2.7	- - R	-	N	R	N	-	-	-	-	-	H	-	K	-	Q	-	Y	L	-	-	M	-	D	-	-	-	-	-	
	1	G2.8	- - - -	W	-	I	I	-	F	-	-	D	-	R	-	-	L	-	-	L	-	-	-	-	-	-	-	-	-	-	
	1	G2.9	- - R	-	I	W	-	-	-	-	-	-	-	R	N	R	-	F	-	-	T	-	-	I	-	-	-	-	-	-	
	1	G2.10	- - - -	W	-	V	-	-	-	-	-	Y	-	H	K	-	L	-	-	L	-	-	F	-	I	-	-	-	-	-	
	1	G2.11	- - - I	W	-	I	-	-	F	S	-	-	-	-	-	-	-	-	-	L	-	-	F	-	R	-	-	-	-	-	
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	1	G2.13	D	-	-	-	W	-	I	P	-	F	-	-	P	H	-	G	-	L	-	-	-	-	-	-	-	-	-	-	
	1	G2.14	- - T	-	-	W	D	V	-	M	F	-	-	K	-	K	-	N	-	-	F	T	P	-	T	-	-	-	-	-	
	1	G2.15	- - R	-	N	R	S	-	-	S	-	H	-	-	H	-	H	-	A	L	I	-	I	-	N	-	-	-	-	-	
	1	G2.16	- - R	-	R	L	L	-	-	-	-	-	K	-	-	Y	L	-	F	-	-	V	-	-	-	-	-	-	-	-	
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	1	G2.18	- - R	-	-	W	-	I	-	N	-	Y	-	-	K	-	N	L	-	-	I	L	-	E	-	-	-	-	-	-	