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Supplemental Information

Structure and Functional Binding Epitope of V-domain Ig Suppressor of T Cell Activation

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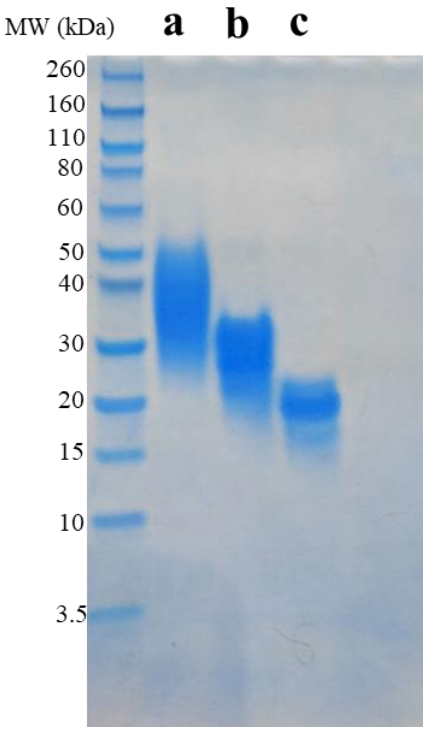

Figure S1. Deglycosylation of VISTA, related to Figure 1. SDS-PAGE gel of VISTA ECD at different stages of deglycosylation. **(a)** Wild-type VISTA (Met1-Ala194), **(b)** VISTA with three asparagine to glutamine mutations (N59Q, N76Q, N158Q), **(c)** VISTA with 3 N→Q mutations, Kifunensine added to the culture media, and Endo Hf enzymatic cleavage. The predicted molecular mass of VISTA ECD is 19 kDa. Only the combination of genetic mutations and enzymatic cleavage produced a distinct band at the estimated molecular mass.

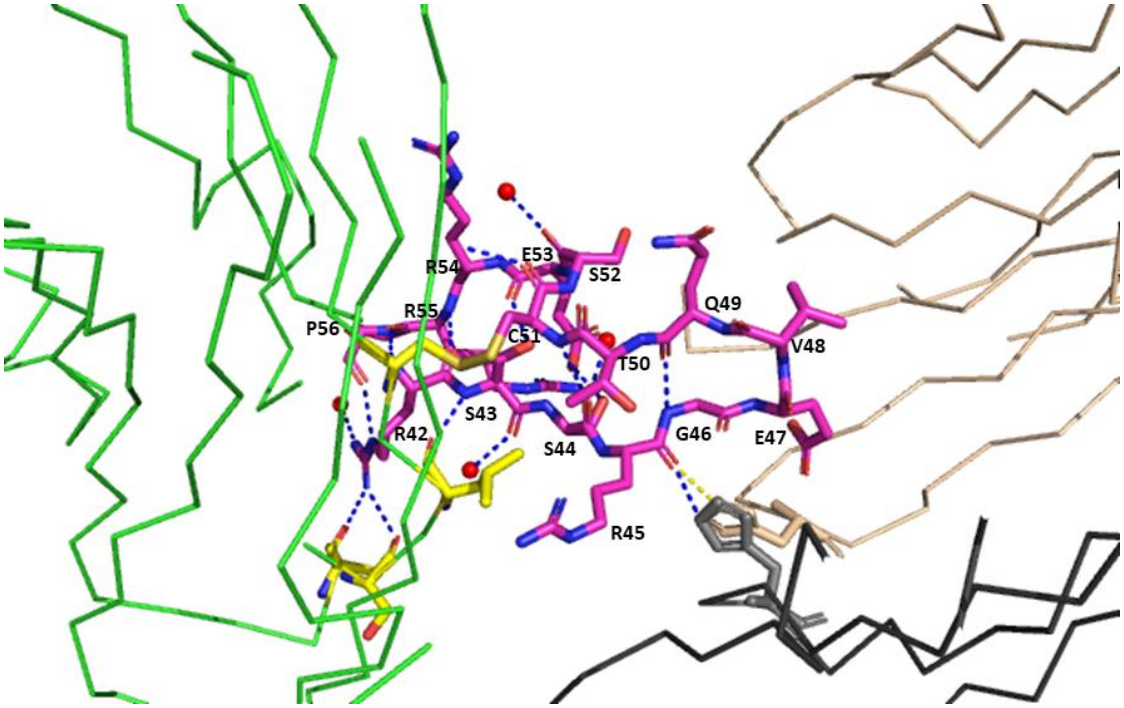

Figure S2: Hydrogen bonds originating from C-C' loop, related to Figure 1.
A single VISTA ECD molecule is shown in green with the extended C-C' loop in pink and two surrounding symmetry molecules in beige and black. Hydrogen bonds from the extended portion of the C-C' loop (residues 42-56) are depicted as dashed lines. Minor hydrogen bonding contacts between loop side chains and symmetry molecules are shown compared to the extensive hydrogen bonding within a single VISTA monomer.

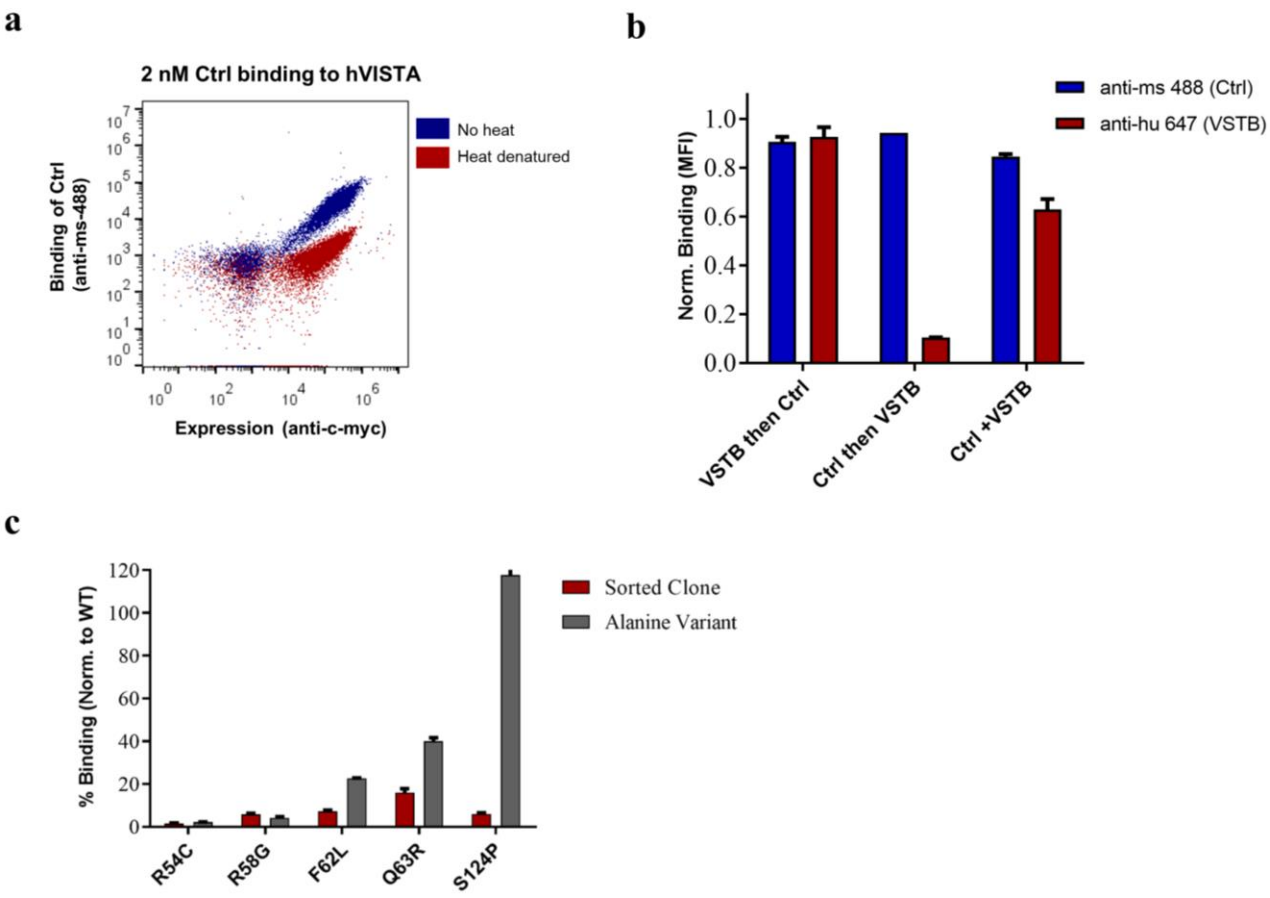

Figure S3. Antibody verification and individual clone analysis, related to Figure 3. (a) Flow cytometry plot of Ctrl antibody binding to yeast-displayed hVISTA with and without heat denaturation of yeast. Decreased binding after heat denaturation confirms conformational (rather than linear) epitope binding. **(b)** Relative binding plot of 30 nM Ctrl and 300 nM VSTB antibodies to yeast-displayed hVISTA. Antibodies were added sequentially, where the first antibody was allowed to reach equilibrium, then the second antibody was added for 15 min, or together where both antibodies were allowed to reach equilibrium. Binding of Ctrl antibody = blue; binding of VSTB antibody = red, normalized to binding signal when antibodies are bound individually. Means ± standard deviation for duplicate measurements are shown in b and c. **(c)** The five hVISTA mutations identified from screening (red) and alanine variants at the same position (gray) were displayed as individual clones on yeast and measured for binding to VSTB (200 μM). Binding was normalized to wild-type (WT) hVISTA binding to 200 μM VSTB.

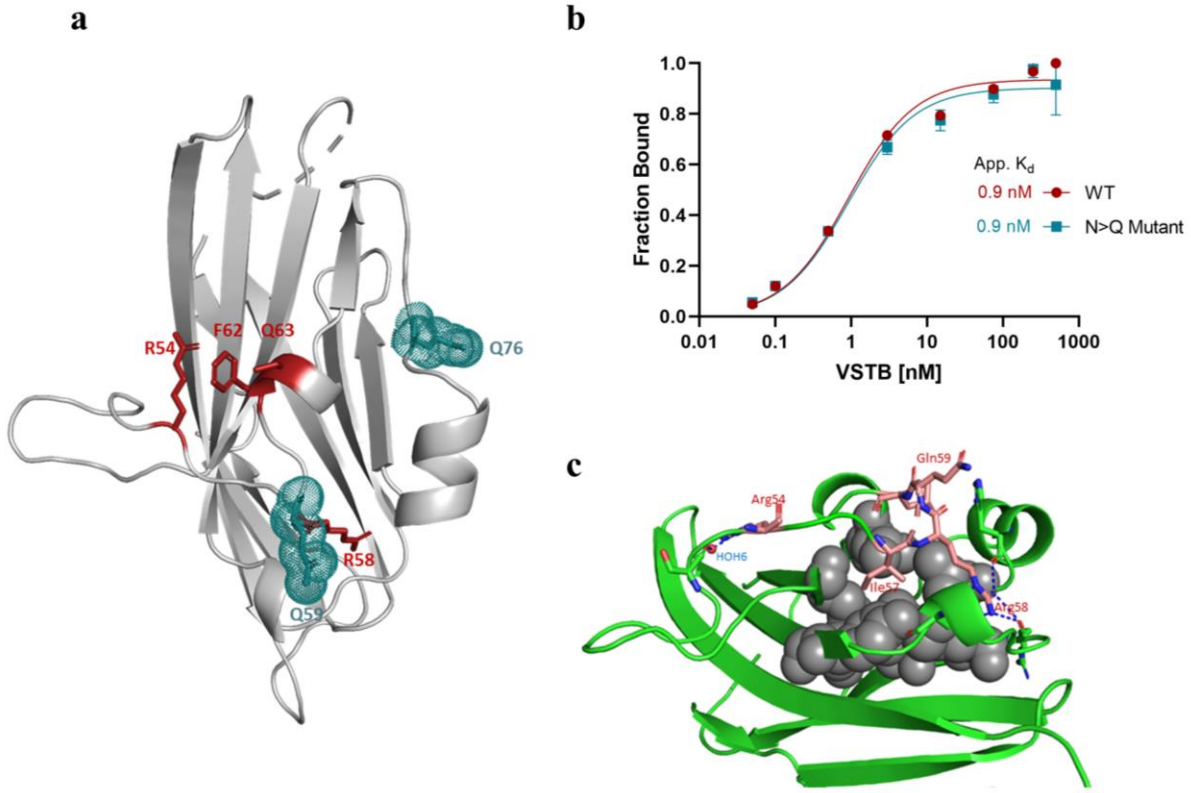

Figure S4: The proximity of N→Q mutations and effects on VSTB Binding, related to Figure 3. (a) Isolated epitope residues (red) and the sites of N→Q mutations (cyan). Three N→Q mutations, N59Q/N76Q/N158Q, were introduced to facilitate crystallization of the hVISTA ECD domain. N158 is part of the C-terminal section that could not be resolved and is therefore not shown in the structure. **(b)** Yeast-displayed WT VISTA or N59Q/N76Q/N158Q triple mutant VISTA was measured for affinity to soluble VSTB. Means ± standard deviation for duplicate measurements are shown. **(c)** Hydrophobic packing of the C-C' loop turn region. Gray spheres indicate hydrophobic packing and hydrogen bonds are shown as dashed lined.

Table S1: Data collection and refinement statistics, related to Figure 1

	VISTA
Data collection	
Space group	$P2_1$
Cell dimensions	
***a*, *b*, *c* (Å)**	29.17, 64.09, 37.69
α, β, γ (°)	90.0, 91.7, 90.0
Resolution (Å)	40-1.85 (1.90-1.85) *
R_{sym} **or** R_{merge}	10.1 (156.2)
$I / \sigma I$	13.0 (1.5)
Completeness (%)	98.4 (96.2)
Redundancy	9.2 (8.4)
Refinement	
Resolution (Å)	40-1.85
No. reflections	11694
R_{work} / R_{free}	0.18/0.22
No. atoms	
Protein	1219
Ligand/ion	0
Water	41
***B*-factors (Å^2)**	
Protein	41.2
Ligand/ion	
Water	43.3
R.m.s. deviations	
Bond lengths (Å)	0.006
Bond angles (°)	1.002

*Values in parentheses are for highest-resolution shell.

Table S2: Hydrogen bonds within C-C' loop, related to Figure 1

42 Arg N --- O (55 Arg) = 2.96 O --- N (55 Arg) = 2.76 NE --- O (56 Pro) = 2.99 NH1 --- O (107 Leu) = 2.87 NH1 --- O (109 Ser) = 2.97 NH2 --- O (1 HOH) = 3.12 O (1 HOH) --- N (59 Glu) = 2.96	54 Arg N --- O (148 Val) = 2.97 O --- N (148 Val) = 3.18 O --- O (7' HOH) = 2.84 O (7' HOH) --- O (5 HOH) = 2.69 O (5 HOH) --- N (62 Phe) = 2.92 O (5 HOH) --- O (55 Arg) = 3.01 NE --- O (6 HOH) = 2.50 O (6 HOH --- O (52 Ser) = 2.67
43 Ser N --- O (111 Leu) = 2.76 O --- O (4 HOH) = 2.78 O (4 HOH) --- N (111 Leu) = 2.93 Og --- O (53 Glu) = 2.67	55 Arg N --- O (42 Arg) = 2.76 O --- N (42 Arg) = 2.96 NH2 --- OE2 (53 Glu) = 2.70 **NH2 --- OD1 (33' Asp) = 3.06** **NH2 --- OD2 (33' Asp) = 3.09**
44 Ser O --- O (2 HOH) = 2.80 O (2 HOH) --- OE1 (53 Glu) = 2.88 O (2 HOH) --- N (52 Ser) = 3.05 O --- N (51 Cys) = 3.36 Og --- OE2 (53 Glu) = 2.47 OE2 (53 Glu) ---NH2(55 Arg) = 2.70	56 Pro O --- NE (42 Arg) = 2.99
45 Arg **O --- NE2 (122' His) = 2.95** **O --- NE2 (94' His) = 2.92**	57 Ile N --- O (60 Leu) = 2.81 O --- O (60 Leu) = 2.84
46 Gly N --- O (49 Gln) = 2.69	58 Arg NE --- OD1 (108 Asp) = 3.08 NH2 --- OD2 (108 Asp) = 2.80 NH2 --- O (102 Arg) = 2.75 NH1 --- O (102 Arg) = 2.84 NH1 --- O (85 His) = 2.92
51 Cys S --- S (113 Cys) Disulfide bond O --- OH (41 Tyr) = 2.81	
52 Ser O --- O (6 HOH) = 2.67 O (6 HOH) --- NE (54 Arg) = 2.50	
53 Glu OE2 --- NH2 (55 Arg) = 2.70 NH2 (55 Arg) --- OD1 (33 Asp) = 3.06	

Supplementary Table 2. Hydrogen bonds within C-C' loop. Intermolecular interactions from side chains of C-C' loop residues are listed (Residues 42-59). Hydrogen bonds acting through secondary interactions are shown in blue. There are 30 unique hydrogen bonds originating from the C-C' loop side chains compared to four between symmetry molecules (bold).

Table S3: Epitope mapping sequencing results, related to Figure 3

Mutation	No. Samples
F62L	10
R54C	9
S124P	9
Q63R	5
R58G	4
L67P	3
F1L	2
S8P	2
C51R	2
F62S	2
D64G	2
E118G	2
C113R	2

Supplementary Table 3. Epitope mapping sequencing results. Table of repeat mutations found from sequencing 50 clones isolated from the yeast population after Sort 5 of epitope mapping. The top five mutations by frequency are highlighted in yellow. Mutations that appeared four times or more were selected for single clone analysis and site-directed mutagenesis.