
Supplementary information

Phosphoantigens glue butyrophilin 3A1 and 2A1 to activate V γ 9V δ 2 T cells

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Supplementary Information for

Phosphoantigens glue butyrophilin 3A1 and 2A1 to activate V γ 9V δ 2 T cells

Linjie Yuan^{1*}, Xianqiang Ma^{1*}, Yunyun Yang^{1,2*}, Yingying Qu¹, Xin Li¹, Xiaoyu Zhu³, Weiwei Ma¹, Jianxin Duan⁴, Jing Xue¹, Haoyu Yang¹, Jian-Wen Huang², Simin Yi², Mengting Zhang², Ningning Cai¹, Lin Zhang¹, Qingyang Ding¹, Kecheng Lai², Chang Liu², Lilan Zhang², Xinyi Liu⁵, Yirong Yao⁶, Shuqi Zhou⁶, Xian Li², Panpan Shen², Qing Chang^{6,7}, Satish R. Malwal⁸, Yuan He⁹, Wenqi Li^{6,7}, Chunlai Chen⁶, Chun-Chi Chen², Eric Oldfield⁸, Rey-Ting Guo^{2†}, Yonghui Zhang^{1†}

¹ Tsinghua-Peking Center for Life Sciences, State Key Laboratory of Membrane Biology, School of Pharmaceutical Sciences, Tsinghua University; Beijing 100084, China.

² State Key Laboratory of Biocatalysis and Enzyme Engineering, Hubei Hongshan Laboratory, Hubei Collaborative Innovation Center for Green Transformation of Bio-Resources, Hubei Key Laboratory of Industrial Biotechnology, School of Life Sciences, Hubei University, Wuhan 430062, China.

³ Department of Hematology, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, 230001, P.R. China.

⁴ Schrödinger, GmbH, Glücksteinallee 25, Mannheim, Germany.

⁵ School of Medicine, Tsinghua University, Beijing, 100084, China.

⁶ School of Life Sciences, Tsinghua University, Beijing, 100084, China.

⁷ Beijing Advanced Innovation Center for Structural Biology, Technology Center for Protein Sciences, Tsinghua University, Beijing 100084, China.

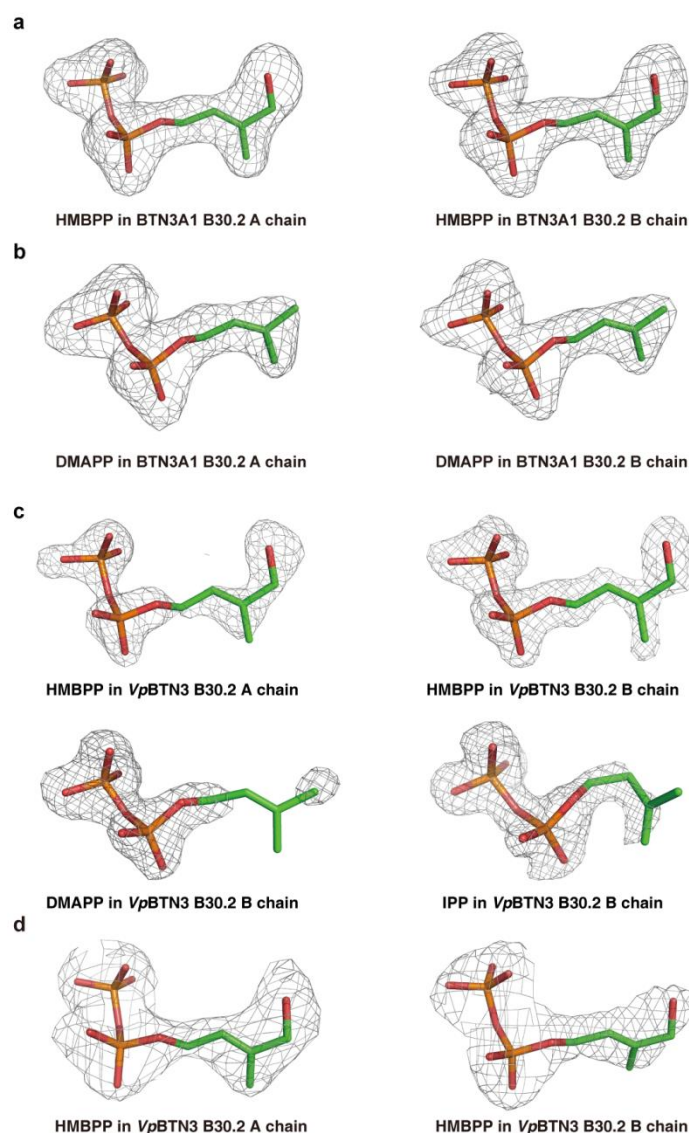
⁸ Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA.

⁹ Research Beyond Borders, Boehringer Ingelheim (China), Shanghai 200040, China.

*These authors contributed equally to this work.

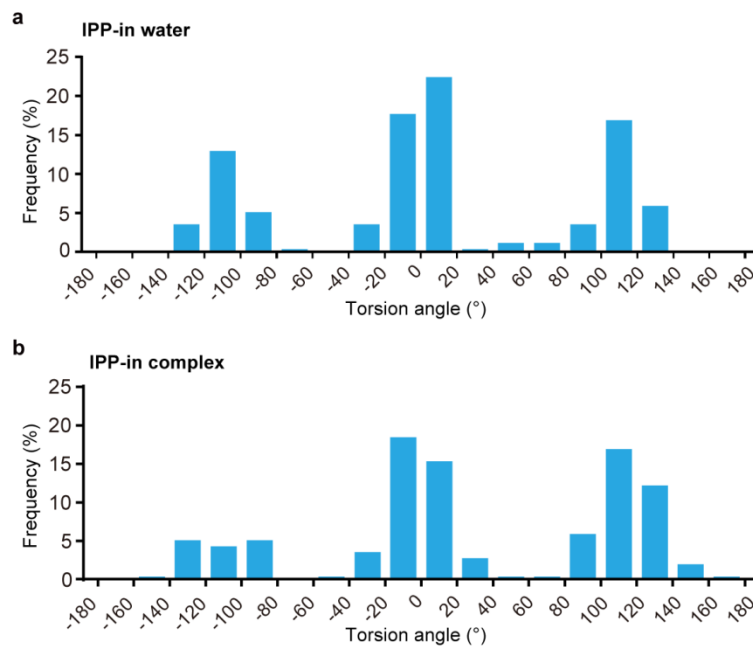
†Corresponding author. Email: zhangyonghui@tsinghua.edu.cn (Y.Z.) (lead contact); guoreyting@hubu.edu.cn (R.-T.G.)

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Supplementary Fig. 1| Electron density maps. a, Electron density maps of HMBPP in the 3A1 B30.2-HMBPP-2A1 B30.2 complex structure (PDB : 8JYE). The Fo-Fc omit maps of HMBPP in the A and B chains were each contoured at 2.5 σ (gray). **b**, Electron density maps of DMAPP in the 3A1 B30.2-DMAPP-2A1 B30.2 complex structure (PDB: 8JYC). The Fo-Fc omit maps of DMAPP in the A and B chains were each contoured at 2.5 σ (gray). **c**, Electron density maps of HMBPP in the VpBTN3 B30.2-HMBPP complex structure (PDB: 8JY9); DMAPP in the VpBTN3 B30.2-DMAPP complex structure (PDB: 8JYF); and IPP in the VpBTN3 B30.2-IPP complex structure (PDB: 8JYA). The Fo-Fc omit maps of HMBPP in the A and B chains, DMAPP in the

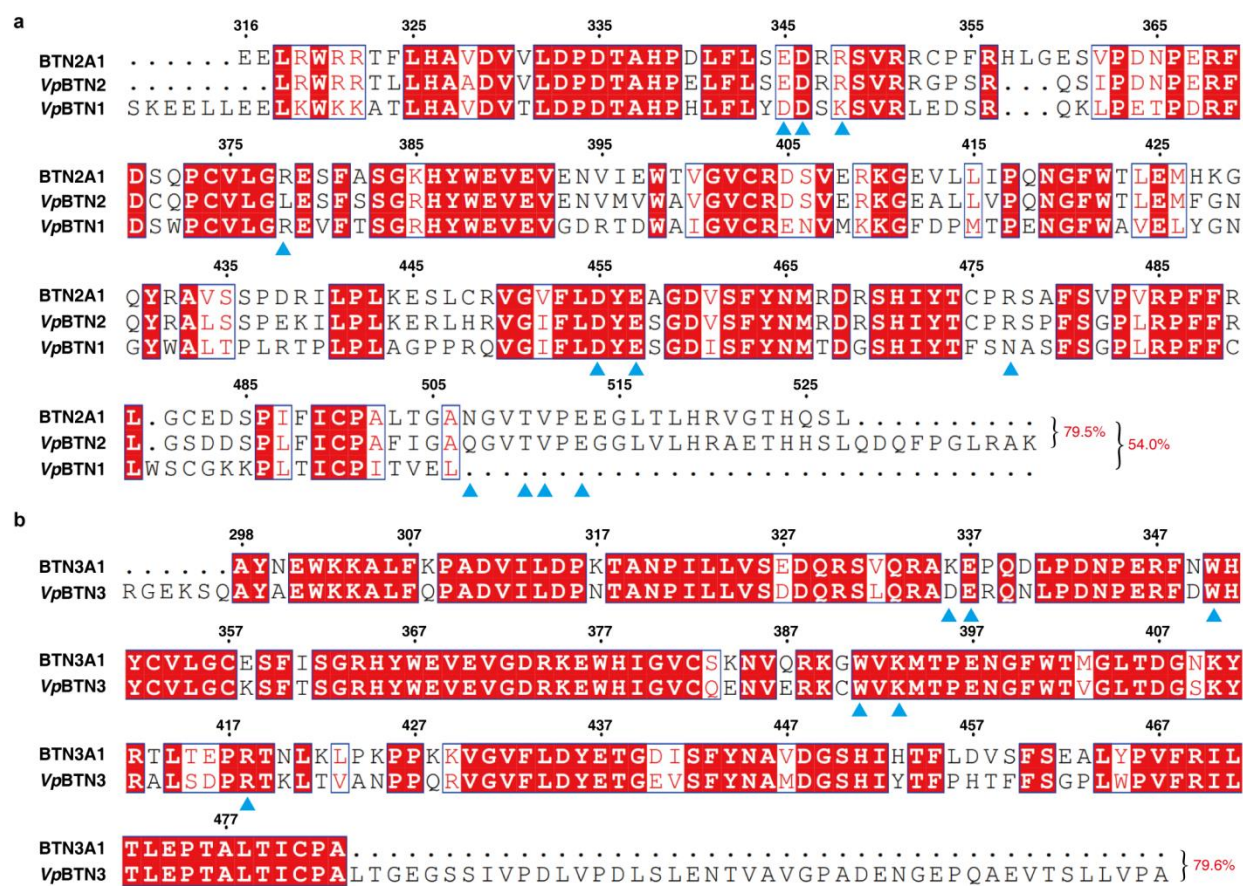
38 B chain and IPP in the B chain were each contoured at 2.5σ (gray). There is partial density loss of
39 the alkyl side chain for both DMAPP and IPP, possibly owing to the lack of the stabilizing 1-OH
40 group in HMBPP. **d**, Electron density maps of HMBPP in the *Vp*BTN3 B30.2-HMBPP-*Vp*BTN2
41 B30.2 complex structure (PDB: 8HJT). The Fo-Fc omit maps of HMBPP in the A and B chains
42 were each contoured at 2.5σ (gray).



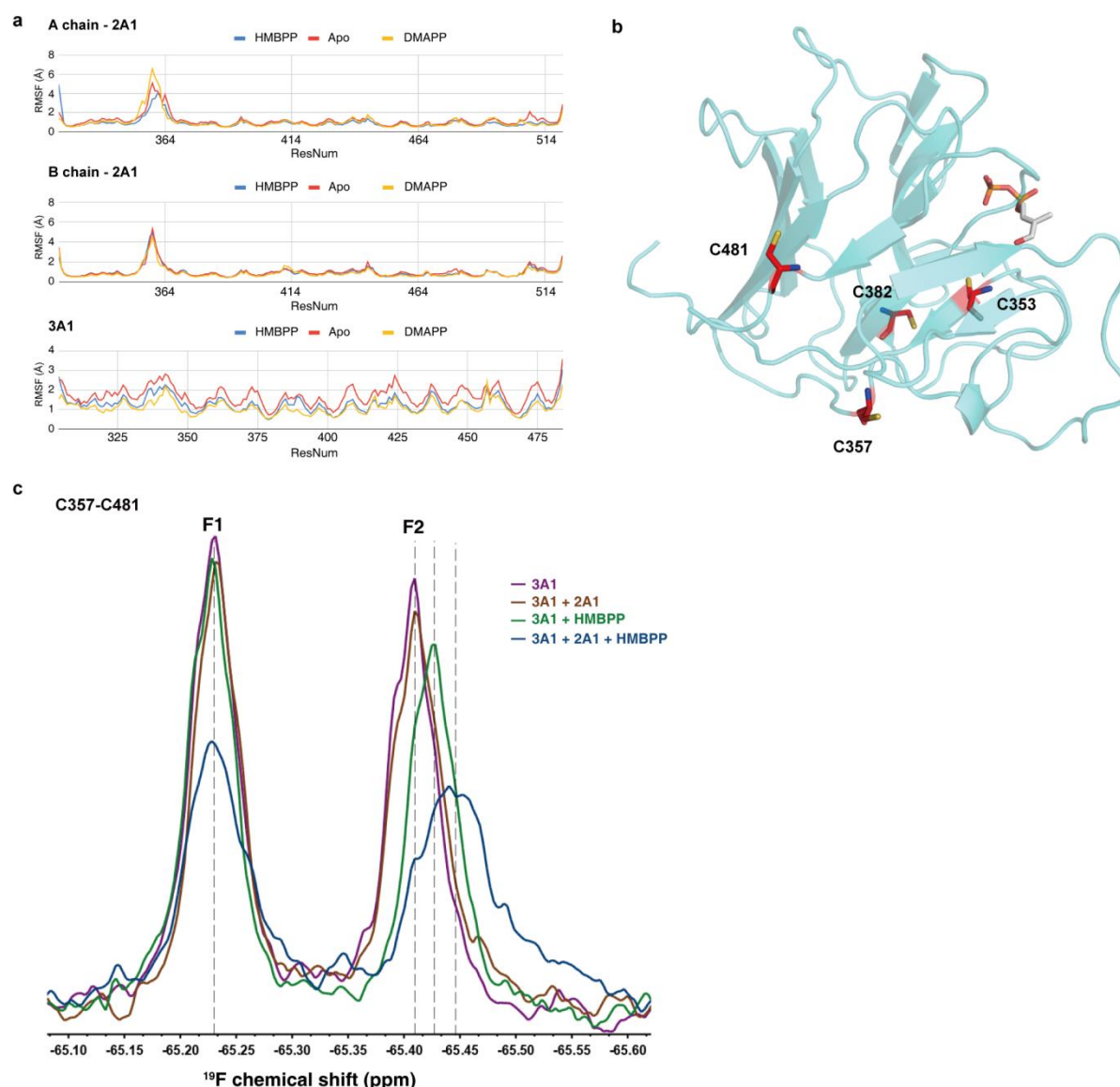
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44 **Supplementary Fig. 2| The torsion angle frequency of the 2-methyl-butenyl group in IPP. a,**

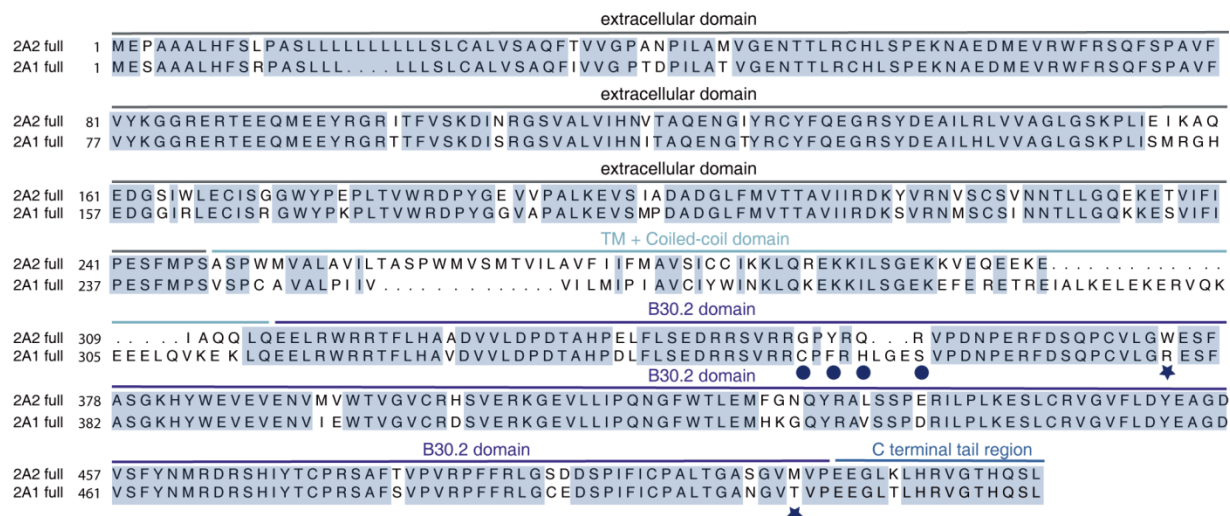
45 **IPP alone in water solvent. b,** When IPP is in the complex of 3A1 and 2A1 B30.2 domains.



Supplementary Fig. 3| Sequence alignment of human and alpaca BTN proteins. a, Sequence alignment indicating 79.5% similarity between VpBTN2 B30.2 and 2A1 B30.2, and 54.0% similarity between VpBTN1 B30.2 and BTN2A1 B30.2. Blue triangles highlight the residues in 2A1 B30.2 that involved in the interaction with 3A1 B30.2. **b,** Sequence alignment indicating 79.6% similarity between VpBTN3 and 3A1 B30.2. Blue triangles highlight the residues in 3A1 B30.2 that involved in the interaction with 2A1 B30.2.

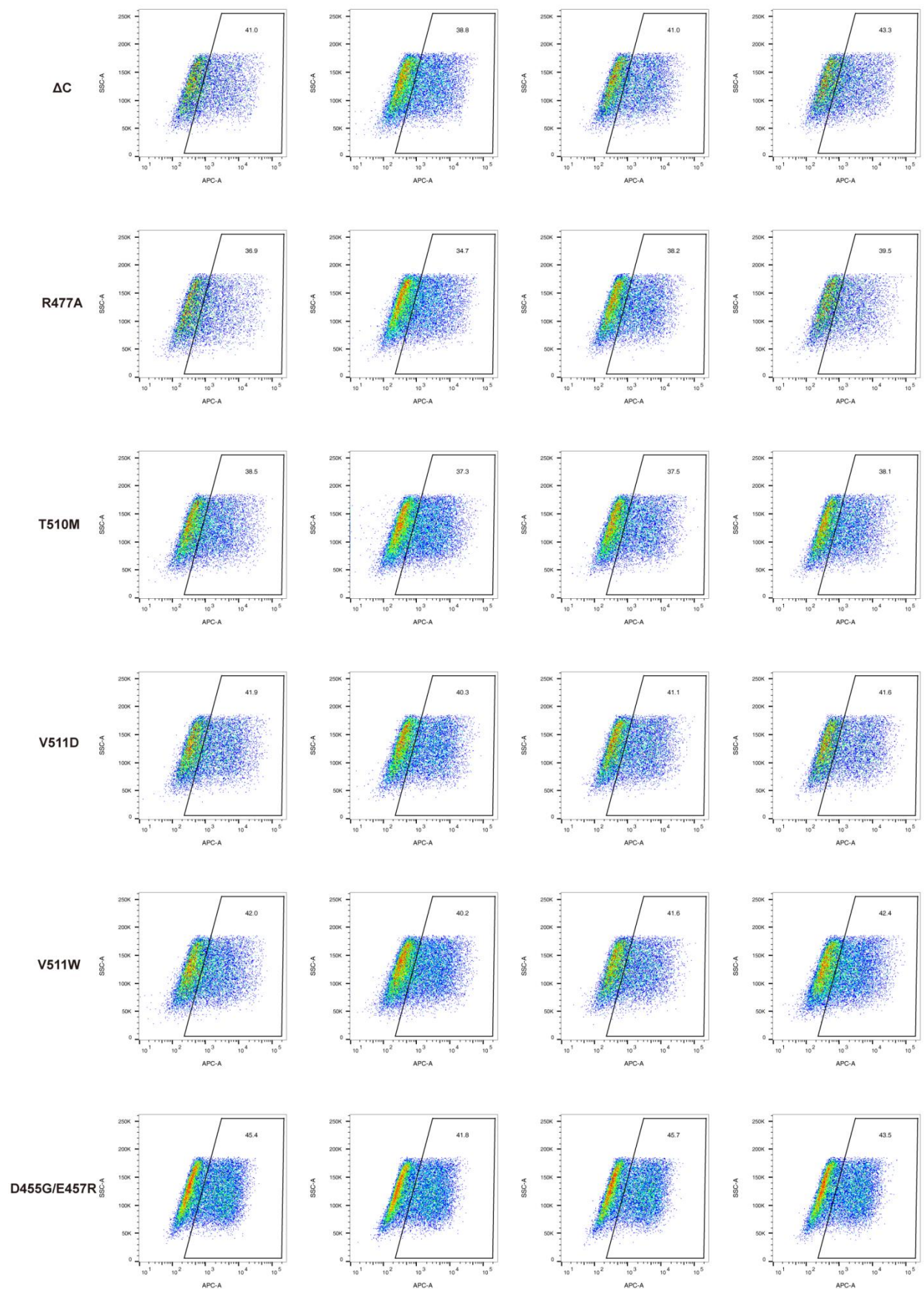


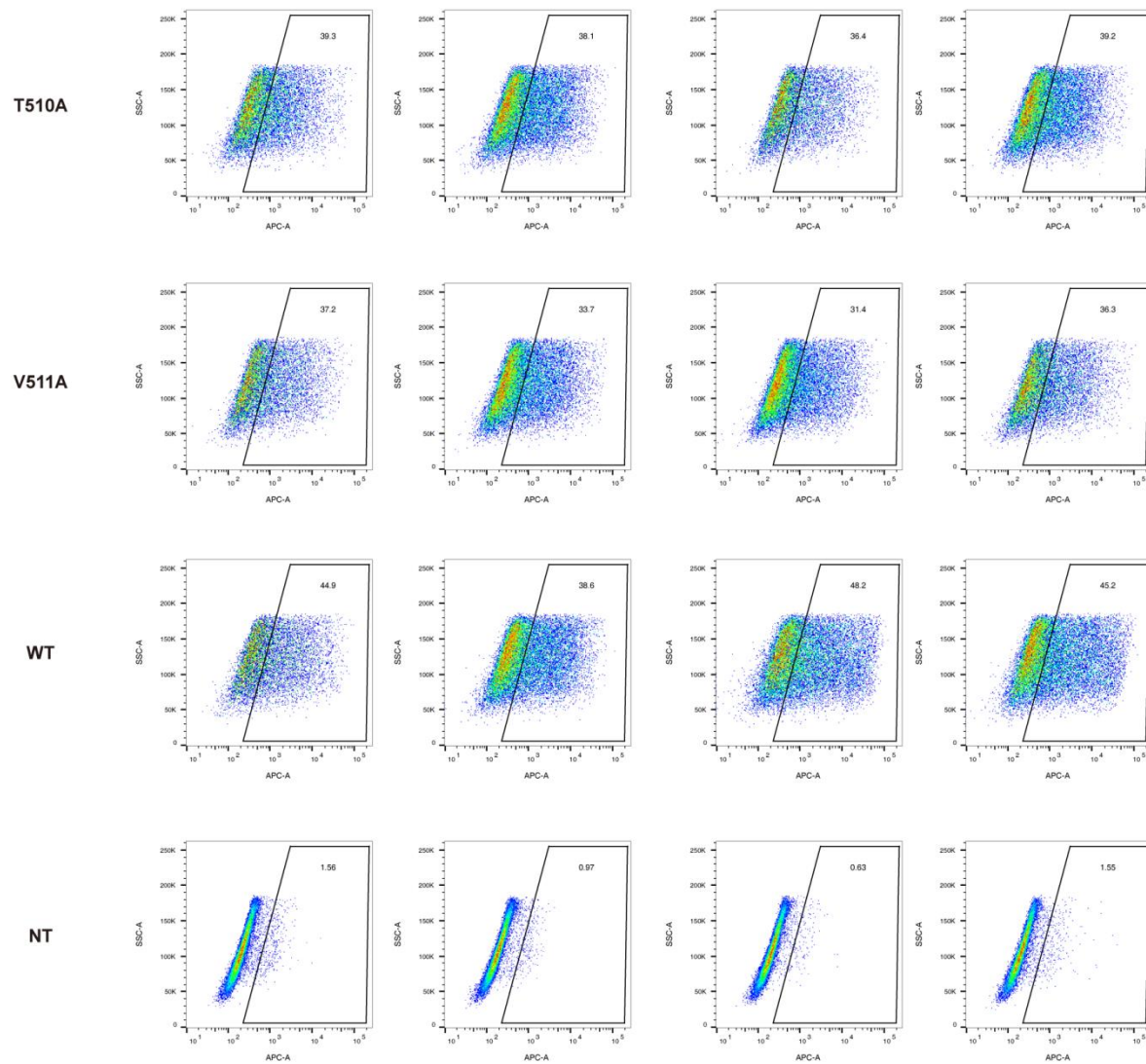
Supplementary Fig. 4| MD simulations and ^{19}F -NMR studies of 3A1 B30.2 fluctuations induced by pAg binding and upon association with 2A1 B30.2. **a**, $\text{C}\alpha$ -RMSF profiles for simulations with the apo-complex as well as for HMBPP-bound and DMAPP-bound complexes. **b**, There are two deeply buried and non-reactive residues (C353 and C382) and two exposed cysteine residues (C357 and C481) in HMBPP-bound 3A1 B30.2 (in cyan, PDB: 5ZXK). **c**, ^{19}F -NMR spectra showing that upon addition of HMBPP and 2A1 30.2. to 3A1 B30.2, one TET signal F1 (-65.23 ppm) remained unchanged, while F2 (-65.41 ppm) changed.



Supplementary Fig. 5| Sequence alignment of the full-length 2A1 and 2A2 proteins. BTN2A1

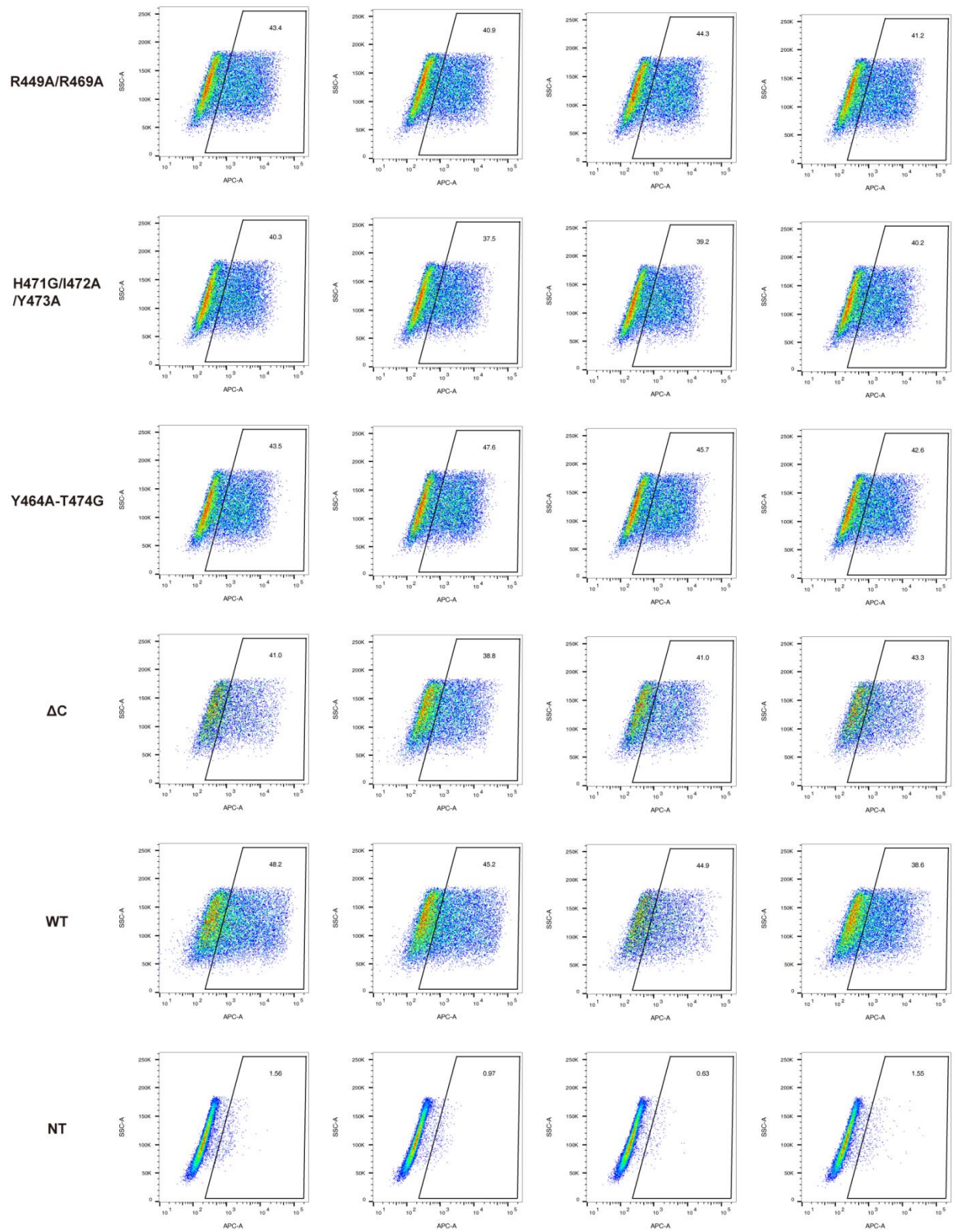
B30.2 and BTN2A2 B30.2 share 88.7% similarity. Blue stars indicate the residues in 2A2 (W374 and M506) analogous to 2A1 B30.2 residues (R378 and T510) identified as functionally relevant for intracellular association with 3A1. Note that 2A1 has an additional loop region (C353F355HLGES, shown by the blue circles) that is not present in the 2A2 structure (G352Y354QR). Based on these structural analyses, we generated the following 2A2 mutations: G352Y354QR, W374R, M506T, W374R/M506T, and G352Y354QR/W374R/M506T. But only two 2A2 B30.2 mutants (M506T and W374R/M506T) bind to the 3A1 B30.2 domain in the presence of HMBPP.



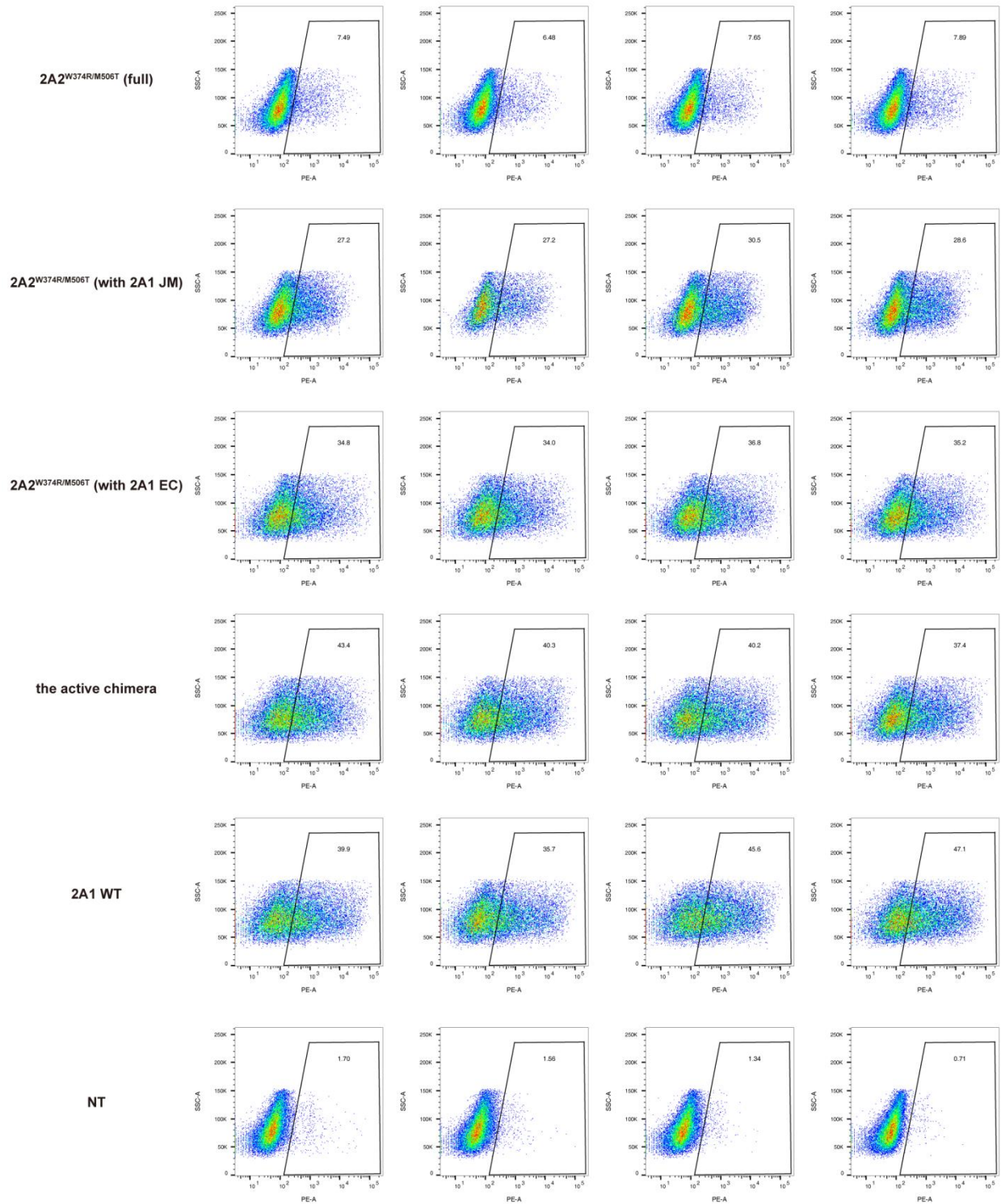


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73 **Supplementary Fig. 6| Gating strategy for flow cytometry analysis shown in Extended Data**
 74 **Fig. 2h. The percentage of His-tagged 2A1 mutant variants expression in $BTN2A^{-/-}$ 293T cells.**



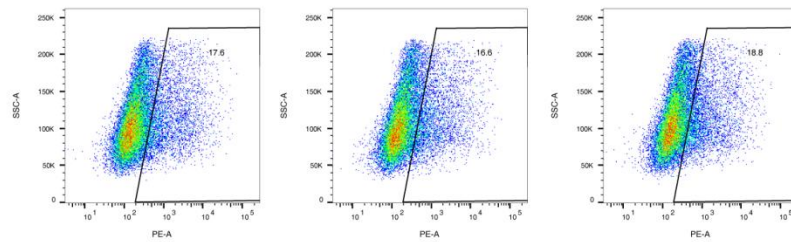
Supplementary Fig. 7| Gating strategy for flow cytometry analysis shown in Extended Data Fig. 3e. The percentage of His-tagged 2A1 mutant variants expression in $BTN2A^{-/-}$ 293T cells.



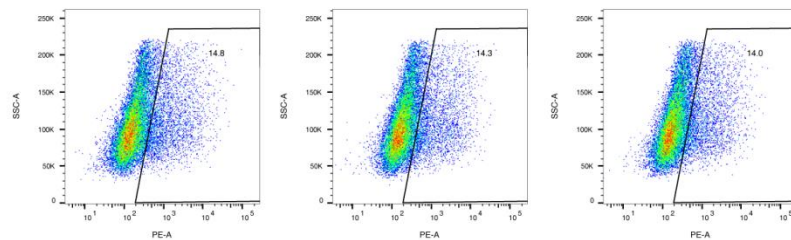
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79 **Supplementary Fig. 8| Gating strategy for flow cytometry analysis shown in Extended Data**
 80 **Fig. 6f.** The percentage of His tagged chimeric variants of 2A2 (with the 2A1 JM, EC, or both JM
 81 and EC, as indicated).

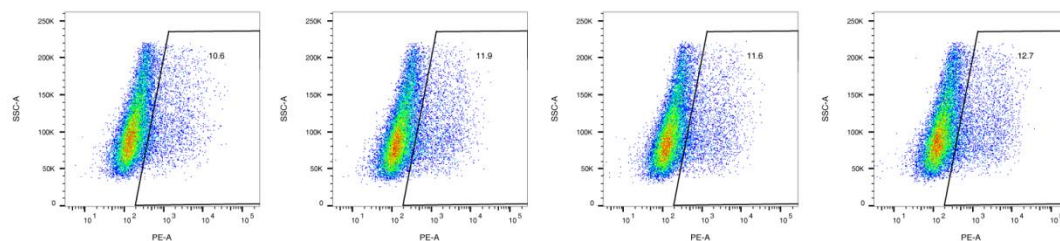
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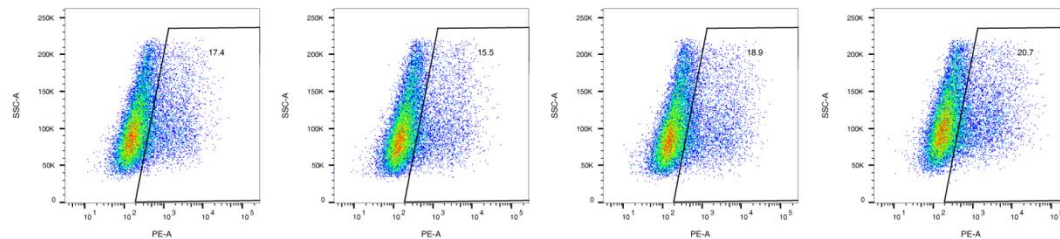
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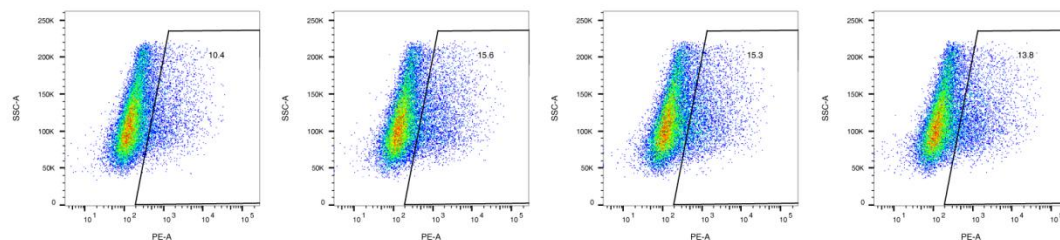
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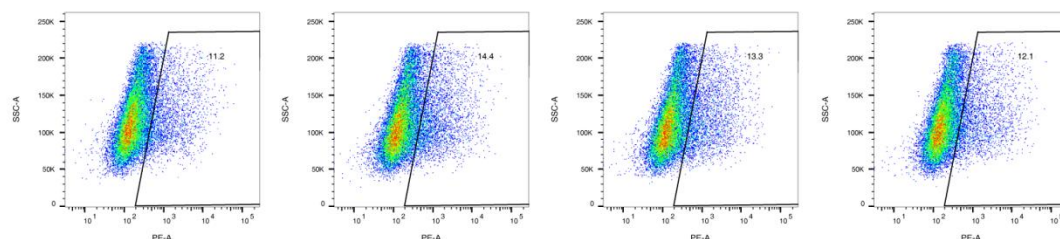
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E286G



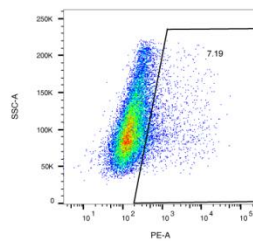
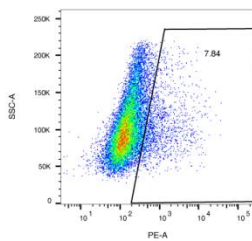
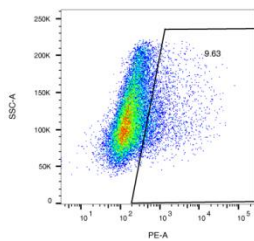
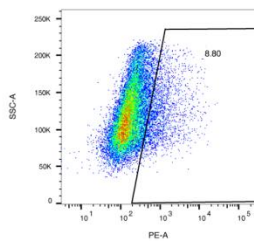
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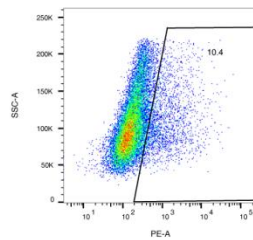
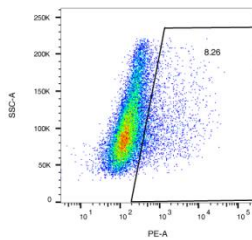
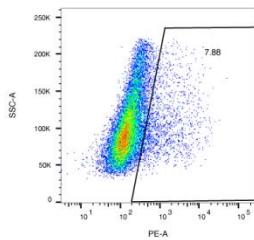
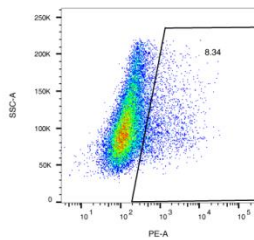
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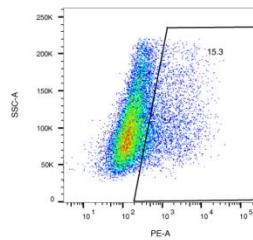
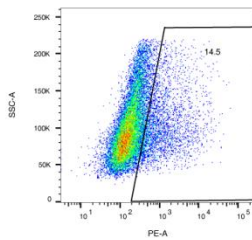
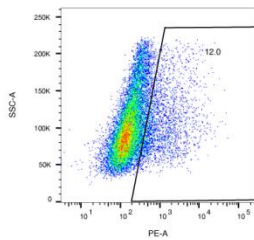
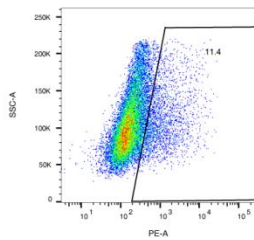
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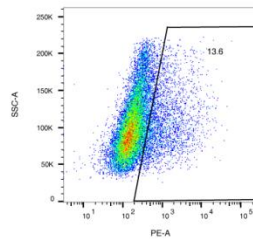
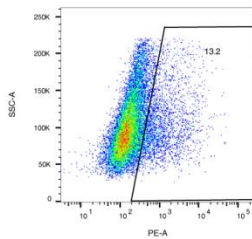
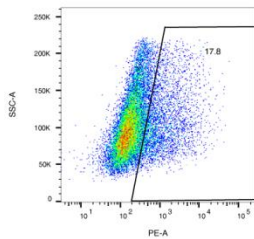
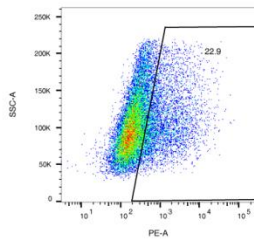
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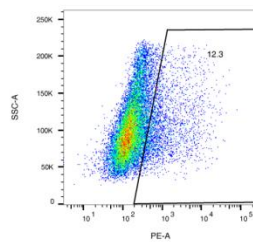
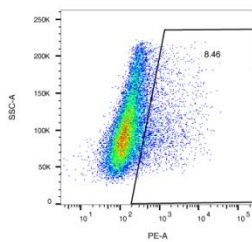
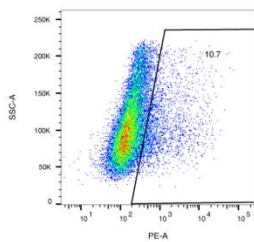
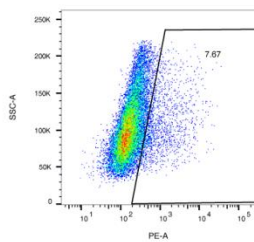
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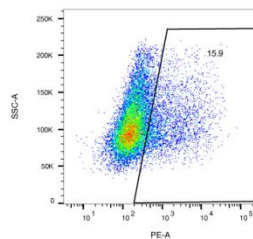
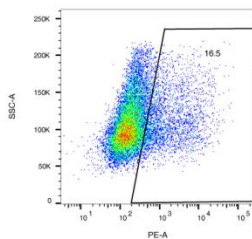
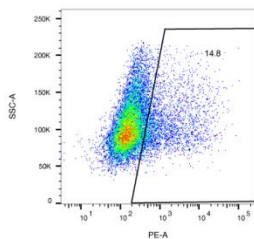
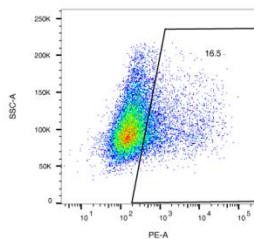
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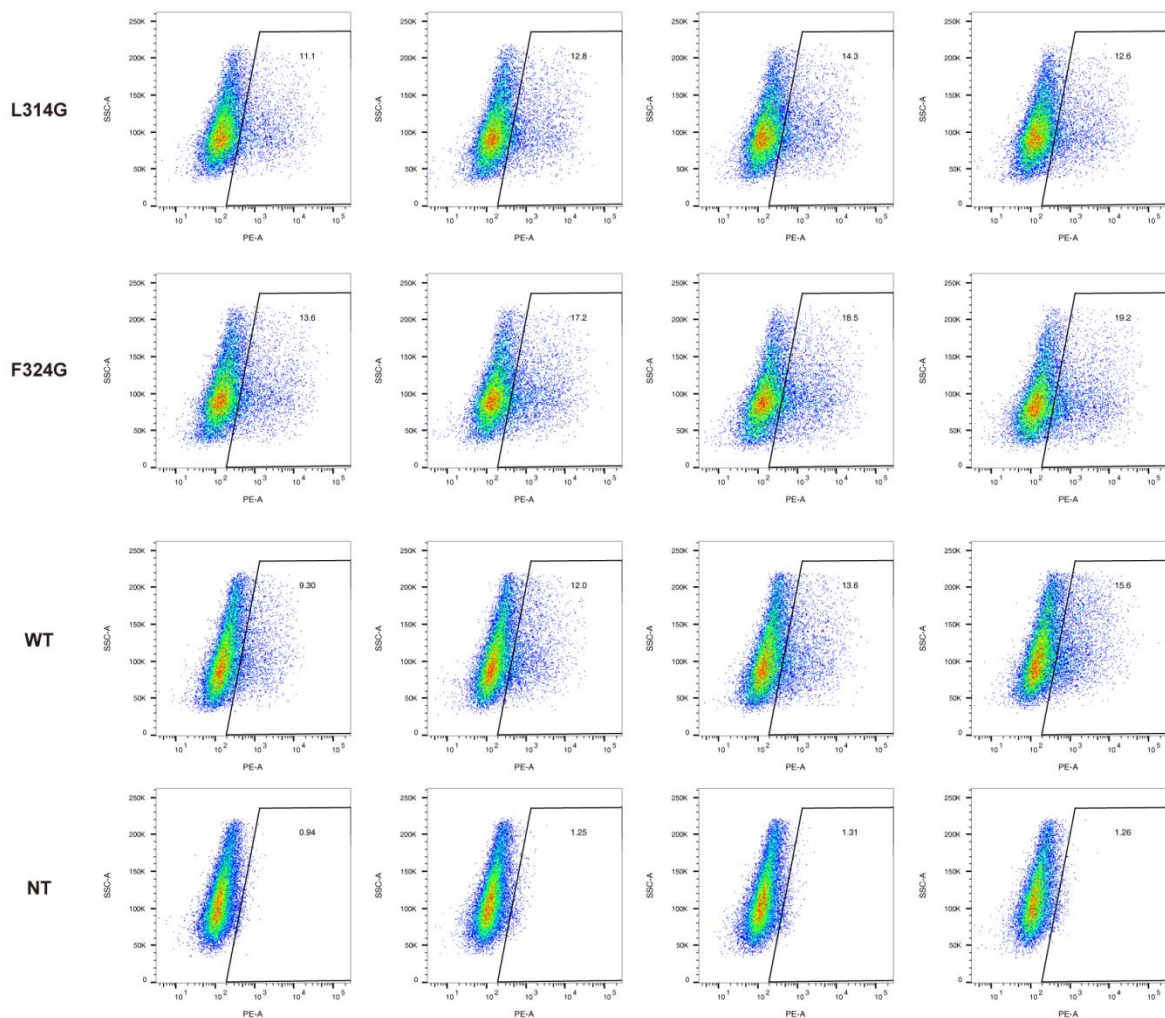


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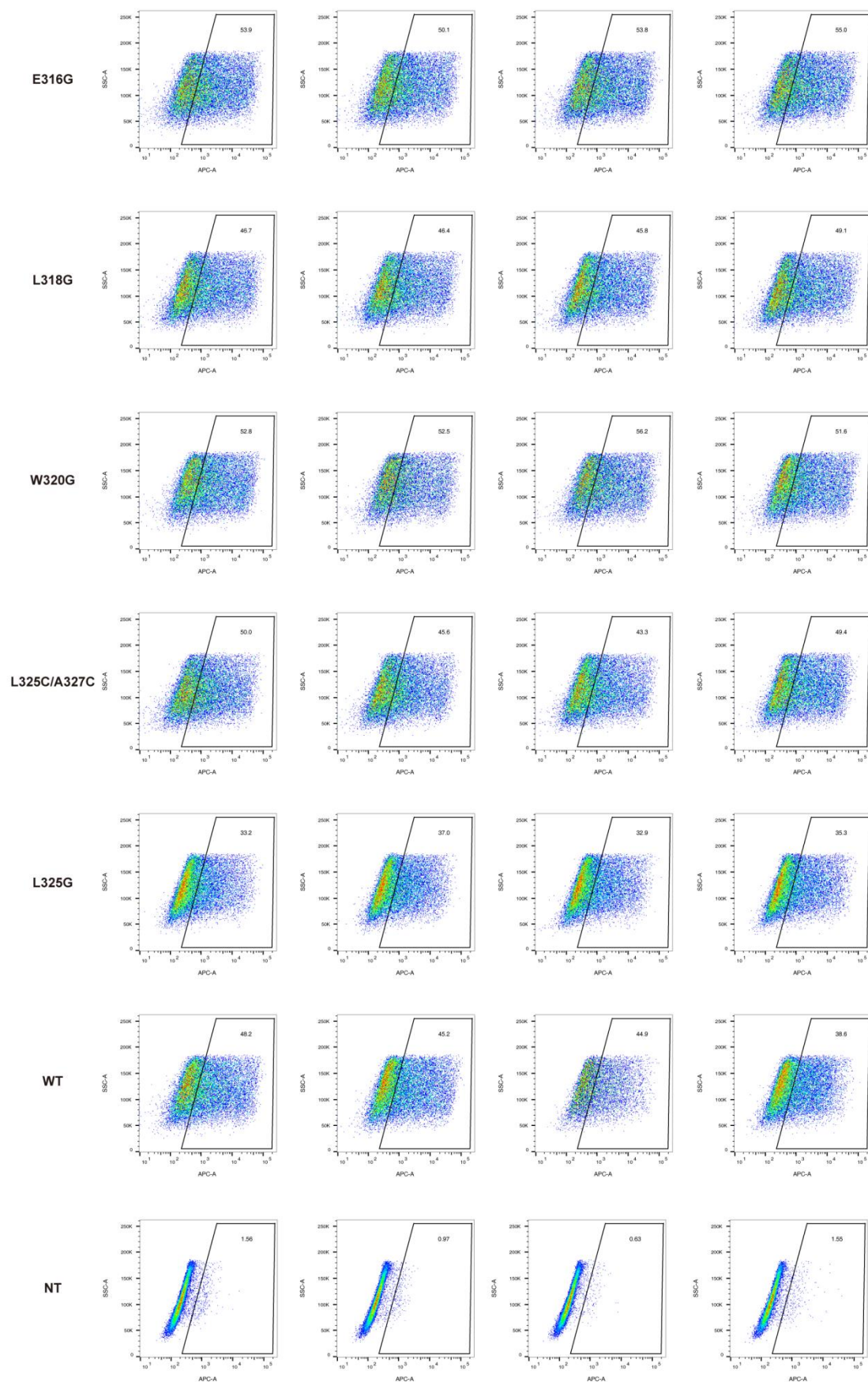


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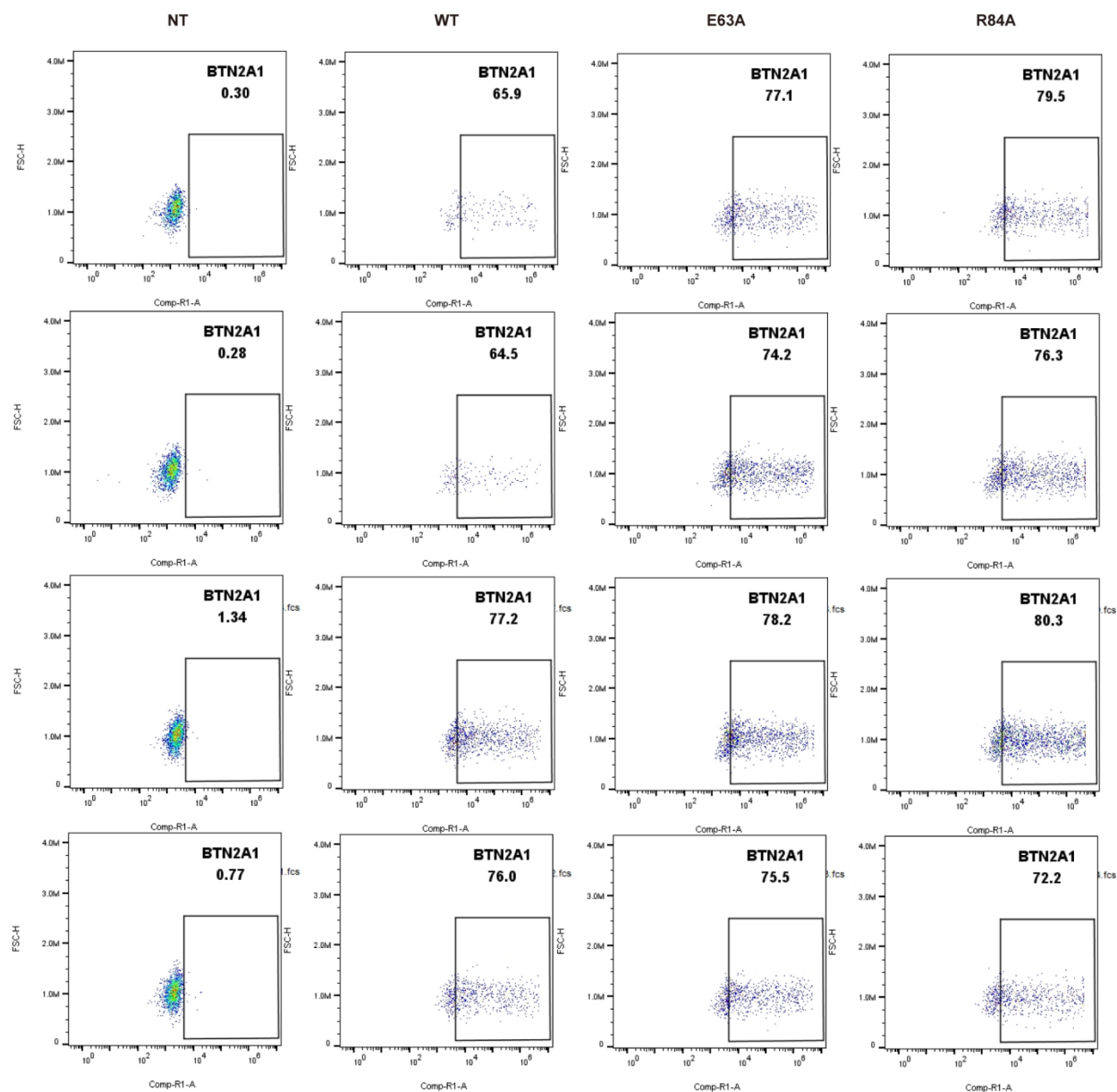




Supplementary Fig. 9| Gating strategy for flow cytometry analysis shown in Extended Data Fig. 7c. The percentage of His-tagged 2A1 JM mutant variants in *BTN2A*^{-/-} 293T cells.



89 **Supplementary Fig. 10| Gating strategy for flow cytometry analysis shown in Extended Data**
90 **Fig. 7d.** The percentage of His-tagged 2A1 JM mutant variants in $BTN2A^{-/-}$ 293T cells.



Supplementary Fig. 11| Gating strategy for flow cytometry analysis shown in Extended Data Fig. 7g. The percentage of His-tagged 2A1 EC mutant variants (NT, WT, E63A and R84A) expression in $BTN2A1^{-/-}$ MIA PaCa-2 cells.

Supplementary Table 1| The BTN2A1-BTN3A1 interaction in the presence of HMBPP or DMAPP

PAG	BTN	Residue	Bond type
HMBPP	3A1 B30.2	H351, Y352, W391, M394, R412, R418, R469	H-bonding, salt bridge
	2A1 B30.2 A Chain	T510, V511	H-bonding
	2A1 B30.2 B Chain	R477	H-bonding, salt bridge
DMAPP	3A1 B30.2	H351, Y352, W391, M394, R412, R418, R469	H-bonding, salt bridge
	2A1 B30.2 A Chain	T510, V511	H-bonding
	2A1 B30.2 B Chain	R477	H-bonding, salt bridge

Supplementary Table 2| Interactions between 3A1 B30.2 and 2A1 B30.2

3A1 B30.2	2A1 B30.2 A Chain	2A1 B30.2 B Chain	Bond type
K336	E345, D346	-	H-bonding (water-mediated)
E337	R378	-	Salt bridge
K393	E513	-	Salt bridge
R418	E513	-	H-bonding
W350	-	D455	H-bonding (water-mediated)
W391	-	E457	H-bonding

Supplementary Table 3| Interactions between BTN2A1 A chain and B chain

2A1 A chain	2A1 B chain	Distance (Å)	Bond type
F324	H326	3.58	Pi-pi stacking
L325	A327	2.8	H-bonding
H326	F324	4.07	Pi-pi stacking
A327	L325	2.9	H-bonding
Y387	Y387	2.5	H-bonding
E389	R469	3.2	H-bonding
R439	E514	3.2	H-bonding
R449	M466, R467	2.6, 2.8	H-bonding
D460	A506	2.7	H-bonding
Y464	G505	2.7	H-bonding
M466	R449	2.7	H-bonding
R467	R449	3.0	H-bonding
R469	E389, P501	2.9, 3.0	Salt bridge, H-bonding
H471	H519, R520	3.0, 2.9	H-bonding
I472	L518	2.9	H-bonding
Y473	E514	2.6	H-bonding
T474	L516	3.0, 3.0	H-bonding
R477	A506, V509	2.9, 2.9	H-bonding
P501	R469	3.0	H-bonding
G505	Y464	2.7	H-bonding
A506	D460, R477	2.8, 2.8	H-bonding
V509	R477	2.7	H-bonding
E514	Y473, R439	2.7, 3.2	H-bonding
L516	T474	2.9, 2.9	H-bonding
L518	I472	2.8	H-bonding
H519	H471	3.0	H-bonding
R520	H471	2.9	H-bonding

**Supplementary Table 4| Summary of cell activities and binding affinities of HMBPP
analogs**

Compounds	EC ₅₀ (M) ^a	K _D (M) ^b	CLogP ^c
1	3.875E-09	4.68E-08	-2.241
2	3.378E-05	3.45E-05	-0.454
3	4.934E-03	6.25E-04	-0.654
4	4.498E-07	3.26E-07	-1.712
5	1.302E-08	2.06E-07	-1.667
6	1.798E-06	1.69E-05	-1.932
7	1.461E-06	4.62E-05	-2.640
8	9.280E-07	1.25E-05	-0.741
9	3.499E-05	4.97E-05	-1.241
10	5.325E-06	1.15E-05	-1.041
11	8.710E-07	2.81E-05	-1.441

^a The EC₅₀ for MIA PaCa-2 cell lysis mediated by HMBPP analogs (Extended Data Fig. 5a) and Vγ9Vδ2 T cells.

^b K_D for phosphoantigen binding to the pre-conditioned 2A1 B30.2 and 3A1 B30.2 as determined by isothermal titration calorimetry.

^c The log₁₀ of the oil/water partition coefficient P, computed by using ChemDraw.

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	Reverse	TCTCCATCGCCCTTCTTCTTGAAGTTTCTCTTTT	
W320A	Forward	AAGAATTGCGAGCGAGAAGAACATTCTTACATGC	
	Reverse	ATGTTCTTCTCGCTCGCAATTCTTCTTGAAGTTTCTC	
F324G	Forward	GAAGAACAGGCTTACATGCTGTTGATGTGGTC	
	Reverse	GCATGTAAGCCTGTTCTTCTCCATCGCAATTC	
L325G	Forward	AAGAACATTCCGACATGCTGTTGATGTGGTCCTG	
	Reverse	ATCAACAGCATGTCCGAATGTTCTTCTCCATCGCA	
D455G/E457R	Forward	CGGGTGGGCGTCTTCTGCGGATATCGGGCTGGAG	
	Reverse	GAAGGAGACATCTCCAGCCCGATATCCAGGAAG	
E63A	Forward	TGAGGACATGGCGGTGCGGTGGTTCCGGTCT	
	Reverse	ACCACCGCACCCGCCATGTCTCTCAGCATTTTCT	
R84A	Forward	AGGTGGCAGAGAGGCAACAGAGGAGCAGATGGAGGA	
	Reverse	CTCCATCTGCGCCTCTGTTCTCTCTGCCACCT	
BTN2A2	Forward	TGCCGCGCGGCAGCCATATGGAGAATCTTTATTTTCAGGGCGCTGGTGCTG	pET28a
	Reverse	GTGCTGAAGAAATGCGATGG	
	Forward	CGGAGCTCGAATTCGGATCCCTATAGGCTCTGGTG	
	Reverse	TGCCCCCTTCAGGCACCTAGGGGAGAGCGTGCCTGACAACCCAGAGAGATT	
	Forward	TGCCCCCTTCAGGCACCTAGGGGAGAGCGTGCCTGACAACCCAGAGAGATT	
	Reverse	TCCTGGGACGGGAGAGCTTCGCCTCAGGGAACATTAC	pET28a, pcDNA3.1
	Reverse	AAGCTCTCCCGTCCCAGGACACAAGGCTGACTGTGCGAA	
	Forward	GTGGGGTCACGGTGCCTGAAGAGGGCCTGAAACTTCAC	
	Reverse	TCAGGCACCGTGACCCCACTGGCTCCTGTGAGTGCAGG	
	Forward	TCCTGGGACGGGAGAGCTTCGCCTCAGGGAACATTACGTGGGGTCACGG	
CRISPR Cas9	Reverse	TGCCTGAAGAGGGCCTGAAACTTCAC	
	Reverse	AAGCTCTCCCGTCCCAGGACACAAGGCTGACTGTGCAATCAGGCACCGTG	
	Reverse	ACCCCACTGGCTCCTGTGAGTGCAGG	
	Forward	CACCGGCCATCCTGCACCTCGTAG	
	Reverse	AAACCTACGAGGTGCAGGATGGCC	
	Forward	CACCGTGCCACCACGAGGCGTAGGA	PX458- pSpCas9(B B)-2A- GFP-MCS
	Reverse	AAACTCCTACGCCTCGTGGTGGCA	
	Forward	CACCGCCATCCAGGGAGATGCGGTC	
	Reverse	AAACGACCGCATCTCCCTGGATGG	