## **Supplemental Figures and Tables**



**Figure S1**. (**A**) Impact of SARS-CoV-2 infection on the subcellular localization of host SUMO-related proteins. A549-hACE2 cells were infected with CoV-2 (SH01,

MOI=1) for 24 h, followed by immunofluorescence analysis using the antibodies as indicated in figure. The scale bar represents 5 µm. Enlarged views of both infected and non-infected cells are shown, with nuclei stained with DAPI. (**B**) Expression level of SUMO protein in A549-hACE2 SUMO-KO cell lines. Whole cell lysates from wild-type (WT) or SUMO-knockout (SU1-KO, SU2-KO, and SU3-KO) A549-hACE2 cells were subjected to immunoblotting using the antibodies indicated in the figure. (**C**) Validation of SUMO1/2/3 gene knockout in the A549hACE2SUMO-KO cell lines. Total DNA was extracted from A549-hACE2 SU1-KO, SU2-KO and SU3-KO cell lines, reverse-transcribed, and second-generation sequencing. The image shows the alignment of small guide RNA (sgRNA) targeting sequences for CRISPR-Cas9 with the original genomic sequences, confirming the successful knockout of the SUMO1/2/3 genes. A

						2
ID	Position	Peptide	Score	Cut-off	P-Value	Туре
S	510 - 514	VGYQPYR <mark>VVVLS</mark> FELLHAP	44.466	29.92	0.03	SUMO interaction
Ν	130 - 134	YGANKDG <mark>IIWVA</mark> TEGALNT	44.416	29.92	0.036	SUMO interaction
ORF1ab	7086 - 7090	IIRENNR <mark>VVISS</mark> DVLVNN*	42.485	29.92	0.039	SUMO interaction



**Figure S2**. (**A**) The top three ranked viral proteins encoded by SARS-CoV-2 related to SUMO modification predicted by GPS-SUMO software. The entire genome sequence of SARS-CoV-2 WHU1 strain was subjected to GPS-SUMO (version 1.0) prediction tool (http://sumo.biocuckoo.cn/advanced.php) for SUMOylated sites and SIM motifs. (**B**) Overexpression of CoV-2-encoding N and ORF6 protein or Poly I: C treatment does not enhance SUMO2/3 perinuclear localization as puncta. A549-hACE2 WT or A549-hACE2 SU1-KO cells were individually transfected with the CoV-2-encoding N and ORF6 protein, or treated with Poly I: C for 18 h at 24h post-transfection, followed by immunofluorescence using antibodies against Flag tag and SUMO2/3. Mock is untreated group.



**Figure S3**. (**A**) Expression level of SUMO protein in HEK293T SUMO-KO cell lines. Whole cell lysates from wild-type (WT) or SUMO-knockout (SU1-KO, SU2-KO, and SU3-KO) HEK293T cells were subjected to immunoblotting using the antibodies indicated in the figure. (B) Validation of SUMO1/2/3 gene knockout in the HEK293T SUMO-KO cell lines. Total DNA was extracted from HEK293T SU1-KO, SU2-KO and SU3-KO cell lines, reverse-transcribed, and second-generation sequencing. The image shows the alignment of small guide RNA (sgRNA) targeting sequences

for CRISPR-Cas9 with the original genomic sequences, confirming the successful knockout of the SUMO1/2/3 genes.



**Figure S4**. SUMO2 KO in target cells reduces viral cell-to-cell transmission. (**A**) Schematic representation of SUMO modification's effect of Spike protein on cell-to-cell transmission. Donor HEK293T cells were co-transfected with plasmids encoding the Spike protein, an NL4.3-inNluc reporter, and eGFP. At 24 h post-transfection, donor cells were co-cultured with target cells A549-hACE2 either wild-

type (WT) or SUMO KO (SU1-KO, SU2-KO, SU3-KO) for 72 h, after which luciferase activity was measured. For cell-free infection, virus was harvested from an equivalent number of transfected donor cells and used to infect equal numbers of A549-hACE2 cells and untransfected donor cells. (B) SUMO2 KO in target cells reduces Spike-mediated syncytia formation. A representative image of immunofluorescence analysis of donor cells at 48 h post-co-culture with target cells (as described in panel A). (C) SUMO2 KO in target cells dominantly reduces Spikemediated cell-to-cell transmission. Data is from three independent experiments in panel A. (D) SUMO3 KO in target cells exclusively induces Spike-mediated cellfree infection. Three independent experiments in panel A show that SUMO3 KO specifically enhances cell-free infection mediated by the Spike protein. (E) SUMO2 KO in target cells dominantly reduces the ratio of cell-to-cell to cell-free infection mediated by the Spike protein. The relative ratio of cell-to-cell to cell-free infection was calculated from data in panels C and D. Each bar represents the mean  $\pm$ standard deviation. \*\**p*< 0.01; \*\*\* *p*< 0.001; and \*\*\*\* *p* < 0.0001.

Α

В

Position	Peptide	Score	Cut-off	Туре
510-514	VGYQPYR <mark>VVVLS</mark> FELLHAP	0.9973	0.85	SUMO interaction
1176-1180	ISGINAS <mark>VVNIQ</mark> KEIDRLN	0.9653	0.85	SUMO interaction
117-121	LDSKTQS <b>LLIVN</b> NATNVVI	0.8973	0.85	SUMO interaction
126-130	IVNNATN <b>VVIKV</b> CEFQFC	0.8602	0.85	SUMO interaction
1264-1268	DEDDSEP <mark>VLKGV</mark> KLHYT**	0.8586	0.85	SUMO interaction
1269	EPVLKGVKLHYT***	0.7903	0.72	SUMOylation
1149	QPELDSFKEELDKYF	0.7737	0.72	SUMOylation
986	DILSRLD <mark>K</mark> VEAEVQI	0.7502	0.72	SUMOylation
129	NATNVVIKVCEFQFC	0.746	0.72	SUMOylation
462	LFRKSNL <mark>K</mark> PFERDIS	0.7293	0.72	SUMOylation
933	QFNSAIGKIQDSLSS	0.7208	0.72	SUMOylation





with myc-tagged WT Spike or its mutants, either in the presence or absence of HAtagged SUMO. At 36 h post-transfection, cells were treated with MG132 for 12 h before being harvested for directly immunoblotting assays using the antibodies against Spike (600-700).  $\alpha$ -tubulin was used as an internal control.



**Figure S6**. The cp129K/R peptides derived from the SUMOylated sites K129 of Spike protein dramatically promotes SARS-CoV-2 replication. (**A**) The relative viral replication within host cells were analyzed by qPCR targeting the CoV-2 N or E gene. All analyses were conducted using the same A549-hACE2 cells as in Figure 6A. (**B**) Immunoblotting was performed on cell lysates from panel B using antibodies against the Spike protein (600-700). The relative density (RD) of the cleaved Spike form (S1) band was quantified and is shown in the middle panel. The  $\alpha$ -tubulin served as an internal control.





(A) Schematic of cpSIM2 peptide injection in mice. C57BL/6 mice (n=5) were intraperitoneally injected with PBS or cpSIM2 at dosage of 12.5, 25, or 50 mg/kg daily for 4 days. On day 5 post-treatment, mice were euthanized, and blood and

organ tissues were individually collected for analysis. (**B**) Comparison of blood parameters. Blood samples were analyzed for biochemical and hematological parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), alkaline phosphatase (ALP), white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), and platelet (PLT) counts. Data are presented as mean ± SD (n=5). (**C**) Histopathological analysis. Representative H&E-stained sections of major organs (liver, kidney, heart, spleen, and lung) were prepared to evaluate tissue morphology and potential pathology. Staining was performed twice independently with consistent results.

Primer	Oligo Sequence
S-pA3M-F	5'-CAAGCTTGGTACCGAGCTCGGCCACCATGTTCGTGTTCCTGGT-
	3'
S-pA3M-R	5'-GCCAGTGTGATGGATATCTGCAGGGTGTAGTGCAGCTTCACGC-
	3'
K129R-F	5'-CAACGCTACAAATGTGGTGATCAGGGTGTGCGAGTT-3'
K129R-R	5'-AACTCGCACACCCTGATCACCACATTTGTAGCGTTG-3'
K462R-F	5'-ACTGTTCAGAAAGAGCAATCTGAGGCCTTTCGAGAG-3'
K462R-R	5'-CTCTCGAAAGGCCTCAGATTGCTCTTTCTGAACAGT-3'
K933R-F	5'-AGCGCCATCGGCAGGATCCAGGACAGC-3'
K933R-R	5'-GCTGTCCTGGATCCTGCCGATGGCGCT-3'
K986R-F	5'-CTGAGCAGACTGGACAGGGTGGAGGCCG-3'
K986R-R	5'-CGGCCTCCACCCTGTCCAGTCTGCTCAG-3'
K1149R-F	5'-CAGCTCCTCCCTGAAGCTGTCCAGCTCAGG-3'
K1149R-R	5'-CCTGAGCTGGACAGCTTCAGGGAGGAGCTG-3'
K1269R-F	5'-CTGTGCTGAAGGGCGTGAGGCTGCACTAC-3'
K1264R-R	5'-TGTGATGGATATCTGCAGGGTGTAGTGCAGCCTC
	ACGCCCTT-3'
mSIM1-F	5'-CACCACCTTGGATTCCAAGACTCAGAGCGCGGCGGC
	TGCAAACAACGCTACAAATGTGGTGATCAAG-3'
mSIM1-R	5'-CTTGATCACCACATTTGTAGCGTTGTTTGCAGCCGCCGCG
	CTCTGAGTCTTGGAATCCAAGGTGGTG-3'
mSIM1'-F	5'-GCTGATTGTAAACAACGCTACAAATGCGGCGGCCGCGG
	CGTGCGAGTTCCAGTTCTGCAATGACC-3'
mSIM1'-R	5'-GGTCATTGCAGAACTGGAACTCGCACGCCGGGCC
	GCCGCATTTGTAGCGTTGTTTACAATCAGC-3'
mSIM2-F	5'-AGCGTGCAGCAGCTCGAAGGCCGCCGCCGCCGCTCTG
	TAAGGCTGGTAGCC-3'
mSIM2-R	5'-GGCTACCAGCCTTACAGAGCGGCGGCGGCGGCCTTCG
	AGCTGCTGCACGCT-3'
mSIM3-F	5'-TTCGGGAATCAATGCCAGCGCGGCGAATGCCCAGAAGGA

 Table S1. Oligo sequence for plasmid construction

	AATTGATCGGC-3'
mSIM3-R	5'-GCCGATCAATTTCCTTCTGGGCATTCGCCGCGCTGGCATTG
	ATTCCCGAA-3'
mSIM4-F	5'-GACAGCGAGCCTGCGGCGAAGGGCGCGAAGCTGCACTAC-3'
mSIM4-R	5'-GTAGTGCAGCTTCGCGCCCTTCGCCGCAGGCTCGCTGTC-3'
ORF9-	5'-CTGGCTAGCGTTTAAACTTAATGGACCCCAAAATCAGCGA-3'
pA3F-F	
ORF9-	5'-AGTGTGATGGATATCTGCAGTTTTACCGTCACCACCACGA-3'
pA3F-R	

 Table S2. Oligo sequence for qPCR

Primers	Oligo sequence
N qPCR Forward	5'-GACCCCAAAATCAGCGAAAT-3'
N qPCR Reverse	5'-CTGGTTACTGCCAGTTGAATCTG-3'
E qPCR Forward	5'-CGATCTCTTGTAGATCTGTTCTC-3'
E qPCR Reverse	5'-ATATTGCAGCAGTACGCACACA-3'
GAPDH qPCR Forward	5'-TGCCTTCTTGCCTCTTGTCT-3'
GAPDH qPCR Forward	5'-GGCTCACCATGTAGCACTCA-3'

Group	Number	TBIL (μM)	ΤΒΑ (μΜ)	Urea (mM)	Crea (μM)	Ca2+ (mM)	P (mM)	TC (mM)	TG (mM)	CK (U/L)	GLOB (g/L)
	1	4.44	146.1	6.50	31.6	2.61	2.55	1.26	.95	1436.3	21.5
	2	2.28	6.2	8.99	33.5	2.70	2.67	2.12	1.18	1609.8	26.4
PBS	3	1.10	3.9	9.92	27.0	2.71	2.74	2.25	1.38	2462.2	24.3
	4	1.58	3.4	7.34	28.7	2.56	3.00	2.15	1.15	1587.4	24.5
	5	1.77	6.2	8.39	40.7	2.62	2.40	2.26	1.42	2248.6	24.6
	1	0.6	8.4	6.9	28.6	2.6	3.0	2.8	1.9	2750.8	21.4
CpSIM2 12.5 mg/kg	2	-0.2	9.0	6.3	29.0	2.6	2.9	2.6	1.6	1774.6	21.4
	3	1.3	10.8	7.0	29.2	2.6	2.8	2.4	1.2	1673.2	20.8
	4	1.3	6.2	7.0	30.0	2.5	2.4	2.2	1.1	1418.8	20.8
	5	0.2	9.5	7.5	31.0	2.6	2.5	2.6	1.6	2720.8	21.3

**Table S3**. Biochemical and hematological parameters of cpSIM2 peptide in mice

CpSIM2 25 mg/kg	1	-0.9	11.5	5.2	44.5	2.7	3.9	2.4	1.3	4814.0	19.0
	2	0.4	5.0	8.4	37.4	2.6	3.6	2.2	1.2	5547.0	20.2
	3	1.6	8.2	5.6	29.6	2.6	2.5	2.1	1.0	2358.0	22.2
	4	0.9	5.6	6.4	40.2	2.6	2.8	2.2	1.0	2246.0	23.2
	5	1.0	5.6	5.8	27.6	2.6	2.6	2.3	0.9	1644.0	21.6
CpSIM2 50 mg/kg	1	3.6	191.6	4.2	26.0	2.6	4.1	1.6	1.2	2375.2	18.2
	2	2.21	8.1	8.01	36.7	2.75	2.17	2.27	1.02	1664.9	27.0
	3	2.5	14.4	7.0	27.6	2.7	2.7	2.5	1.2	1821.0	18.8
	4	2.6	6.6	5.6	31.8	2.7	2.5	2.2	1.0	2090.6	20.8
	5	1.92	8.0	8.29	32.9	2.77	2.49	2.20	1.00	1238.8	25.7