

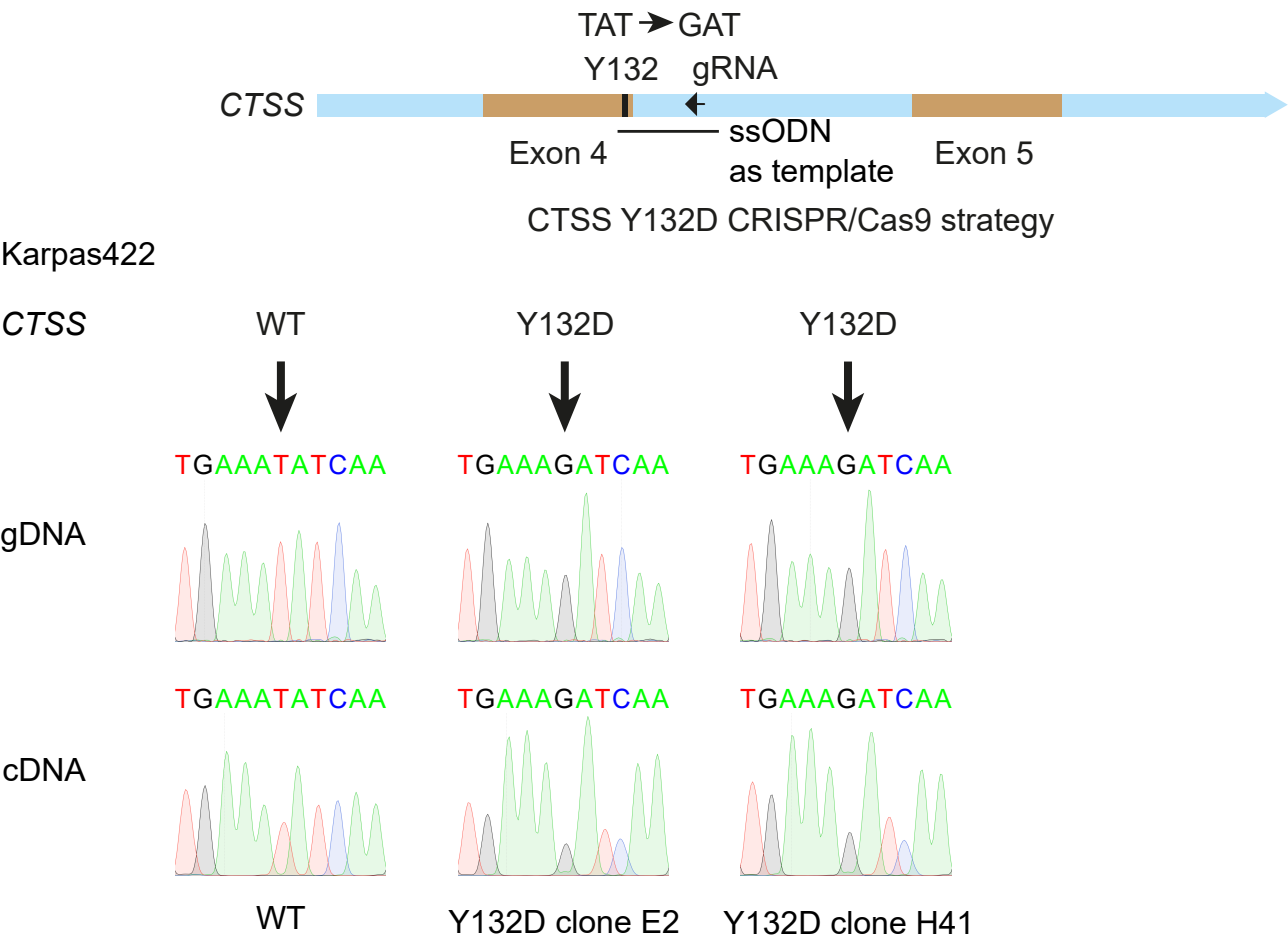
## Supplemental Information

### ***Cathepsin S* Alterations Induce a Tumor-Promoting Immune Microenvironment in Follicular Lymphoma**

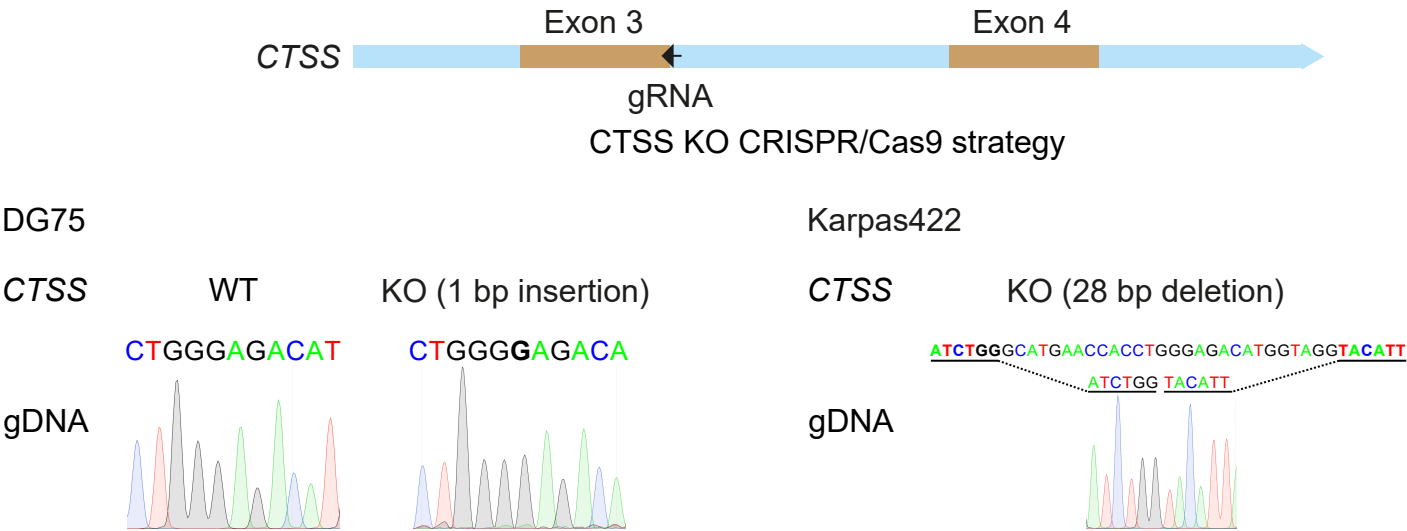
Deepak Bararia, Johannes A. Hildebrand, Sebastian Stolz, Sarah Haebe, Stefan Alig, Christopher P. Trevisani, Francisco Osorio-Barrios, Michael D. Bartoschek, Michael Mentz, Alessandro Pastore, Erik Gaitzsch, Michael Heide, Vindi Jurinovic, Katharina Rautter, Jay Gunawardana, Muhammed B. Sabdia, Monika Szczepanowski, Julia Richter, Wolfram Klapper, Abner Louissaint Jr., Christina Ludwig, Sebastian Bultmann, Heinrich Leonhardt, Sebastian Eustermann, Karl-Peter Hopfner, Wolfgang Hiddemann, Michael von Bergwelt-Baildon, Christian Steidl, Robert Kridel, Joshua W.D. Tobin, Maher K. Gandhi, David M. Weinstock, Marc Schmidt-Supprian, Menyhárt B. Sárosi, Martina Rudelius, Verena Passerini, Josef Mautner, and Oliver Weigert

Figure S1

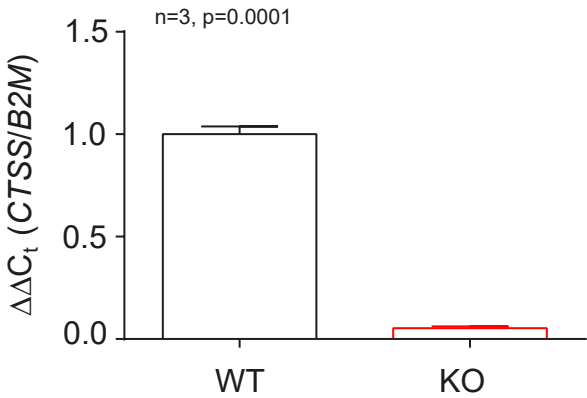
A



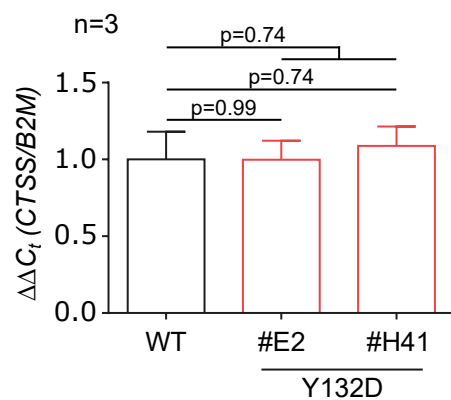
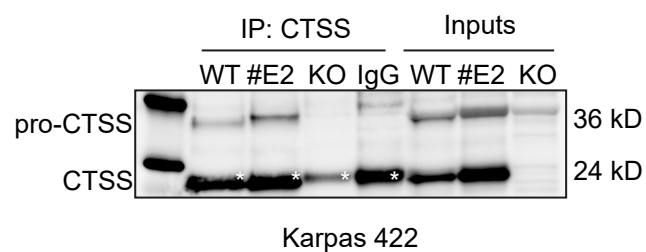
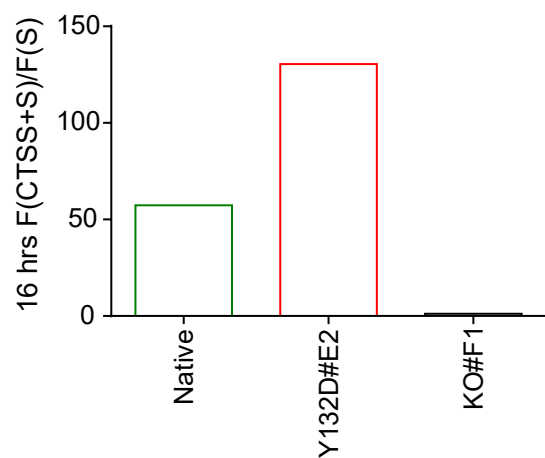
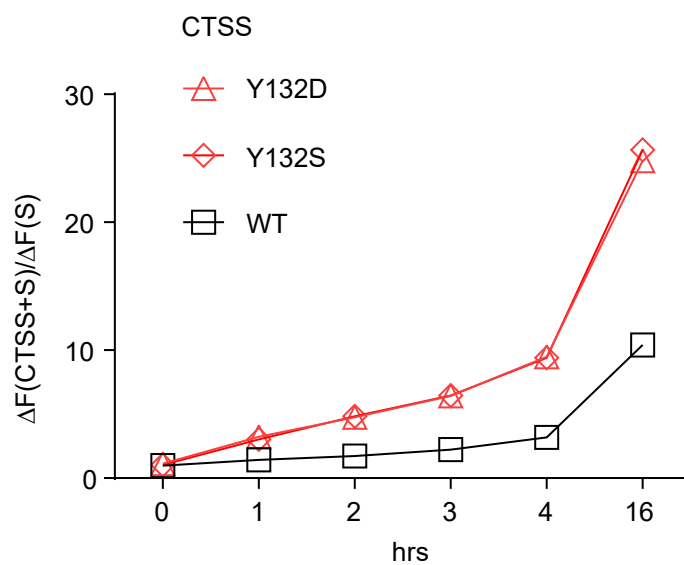
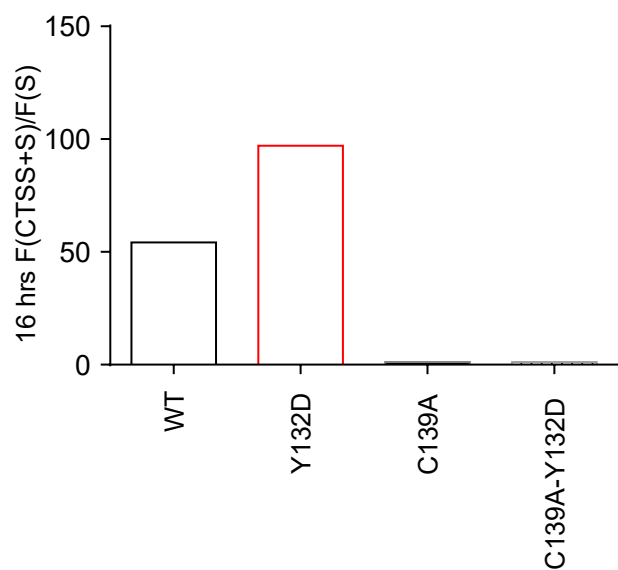
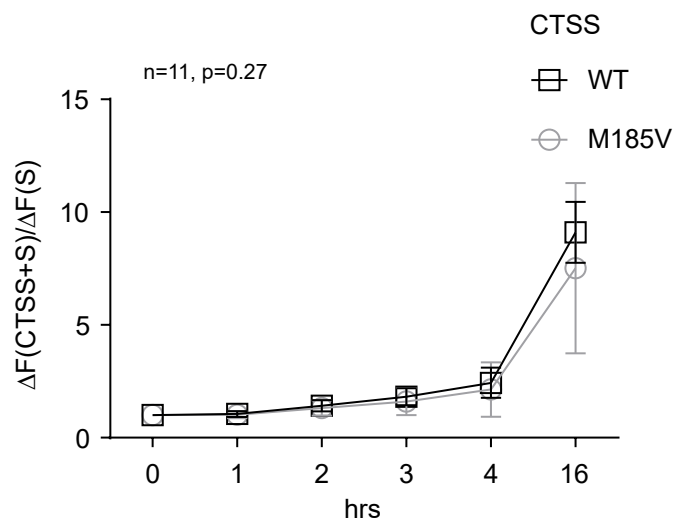
B



C



**Supplementary Figure 1. CRISPR/Cas9 mediated *CTSS* Y132D knock-in and *CTSS* knock-out (KO) mutant cells. Related to Figure 2.** **A)** Schematic overview of the target site in the *CTSS* locus showing Y132 in exon 4, the guided RNA binding orientation, and single stranded oligonucleotide donor (ssODN) as a template. Single base substitution (T to G) at the genomic (gDNA) and mRNA (c-DNA) levels were verified by Sanger sequencing. **B)** Schematic overview of the target site in the *CTSS* locus showing the position of the guided RNA for exon-intron boundary disruption. DG75 *CTSS*<sup>KO</sup> (clone #2A1) has a single base insertion in exon 3 resulting in a frame-shift as detected by Sanger sequencing at the gDNA level. Karpas422 *CTSS*<sup>KO</sup> clone (#F1) has a 28 bp deletion resulting in a non-functional protein. **C)** qRT-PCR for *CTSS* expression in DG75 native cells (*CTSS* WT) and in the DG75 *CTSS*<sup>KO</sup> clone (#2A1).  $\beta$ -2 microglobulin (*B2M*) was used as a reference gene. Pooled data from biological replicates (N) are represented as mean  $\pm$  SD; p-values from unpaired Student's t test.

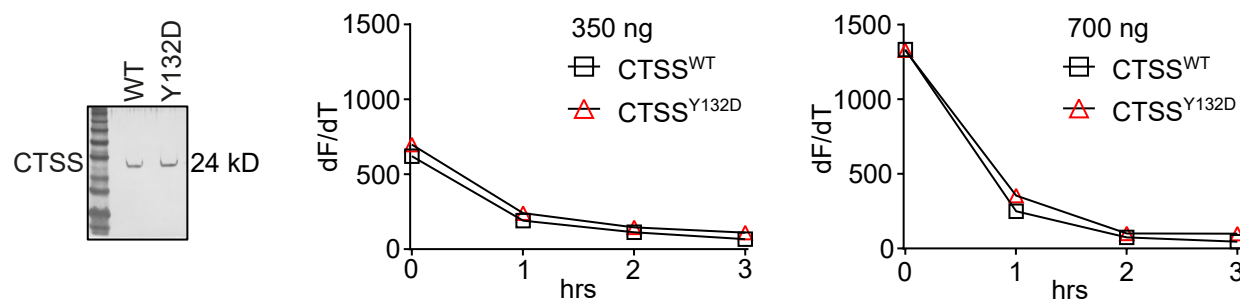
**Figure S2****A****B****C****D****E****F**

**Supplementary Figure 2. *In vitro* substrate cleavage activity from further CTSS mutants. Related to Figure 2.**

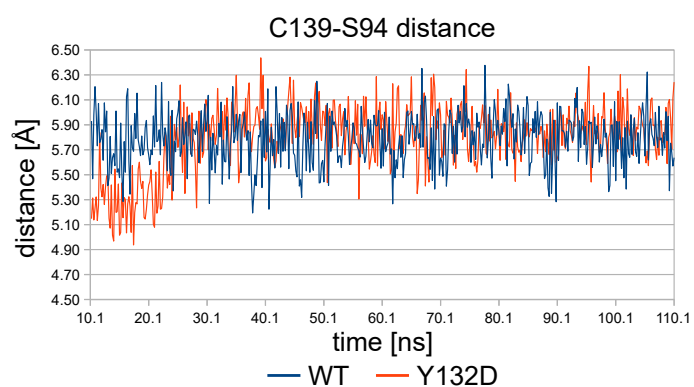
**A)** qRT-PCR of respective *CTSS* mRNA;  $\Delta\Delta C_t$ , difference (*CTSS* vs *B2M* reference gene). **B)** Western blot for CTSS immunoprecipitates (IP) from Karpas422 cells. Single cell derived Karpas422 *CTSS*<sup>KO</sup> cells (clone #F1) were used as negative control. Asterisks indicate IgG light chains slightly above CTSS. **C)** CTSS substrate cleavage activity at 16 hours (hrs) with immunoprecipitates from B). F(CTSS)/F(S) is the fold-change after 16 hrs compared to AFC-signal without enzyme added (IP control). **D)** Substrate cleavage activity of immunoprecipitated CTSS Y132D and CTSS Y132S proteins from pCIG-*CTSS* (Y132D vs Y132S) transfected HEK293T cells.  $\Delta F(CTSS)/\Delta F(S)$  is the fold-change over time compared to AFC-signal without enzyme added. **E)** CTSS substrate cleavage activity at 16 hours (hrs) with immunoprecipitated CTSS C139A and C139A-Y132D. F(CTSS)/F(S) is the fold-change after 16 hrs compared to AFC-signal without enzyme added (IP control). **F)** Substrate cleavage activity over time from N=11 biological replicates using immunoprecipitated CTSS M185V and CTSS WT from Karpas422 with or without CTSS M185V introduced by CRISPR/Cas9; data are represented as mean  $\pm$  SD; p-values from unpaired Student's t test.

**Figure S3**

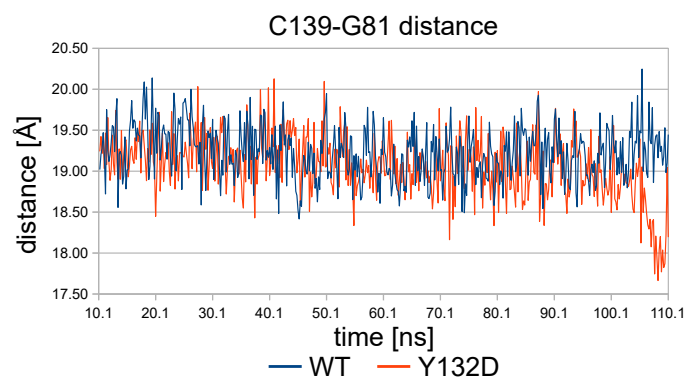
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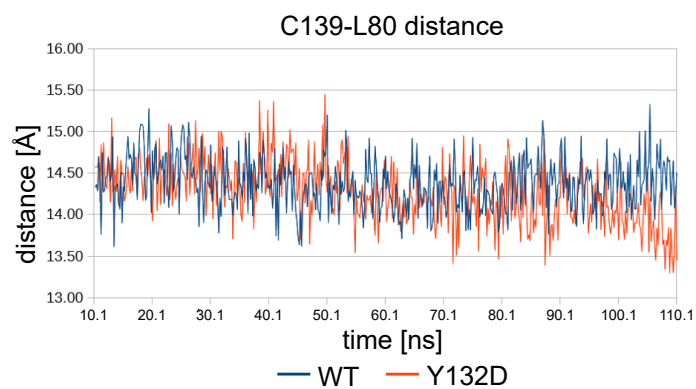
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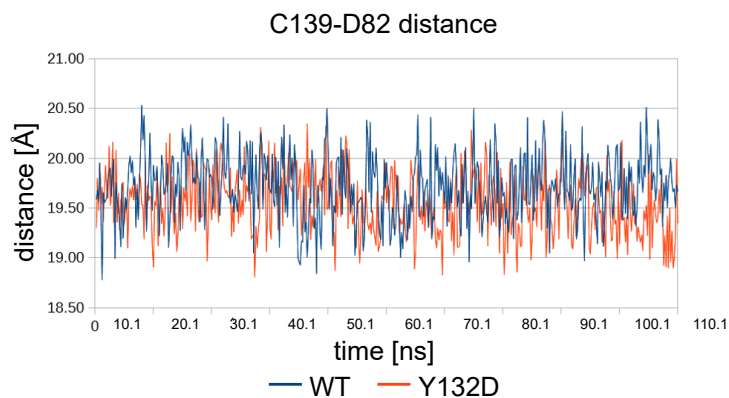
**C**



**D**



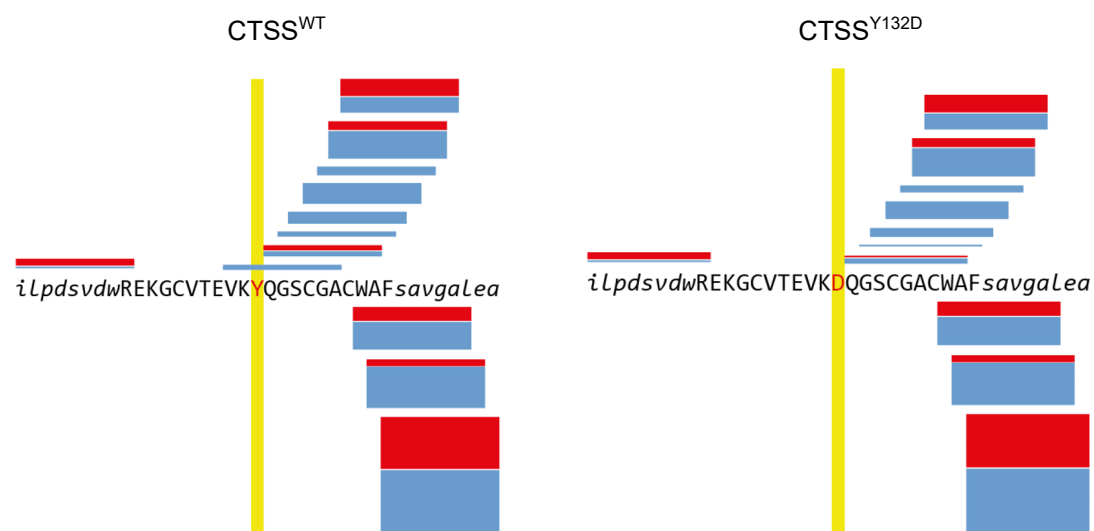
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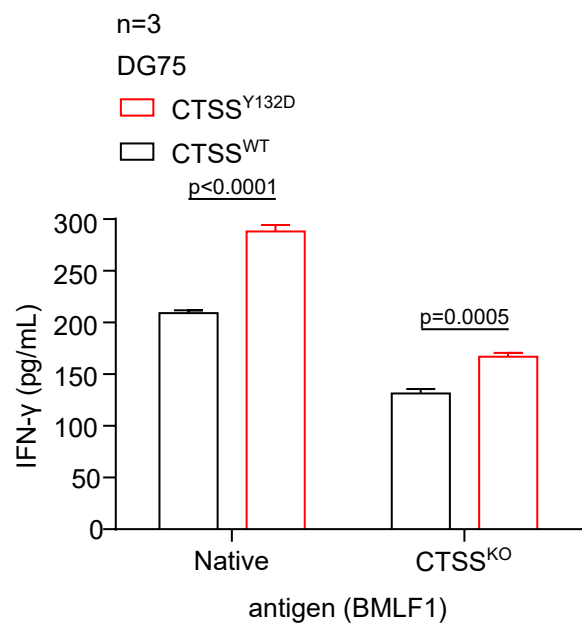
**Supplementary Figure 3. Mature CTSS activity and structural modelling of CTSS WT vs CTSS Y132D. Related to Figure 3. A)** CTSS substrate cleavage assay over 3 hrs using purified and completely converted mature CTSS (WT and Y132D), visualized in a silver stained gel. Two different starting concentrations are shown;  $dF/dt$  = rate of reaction. **B-E)** MD simulations of molecular distances between the center of mass of atoms: C139 and S94 (**B**), C139 and G81 (**C**), C139 and L80 (**D**), and C139 and D82 (**E**) (Y132D vs WT); Å = Ångström, ns = nanoseconds.

**Figure S4**

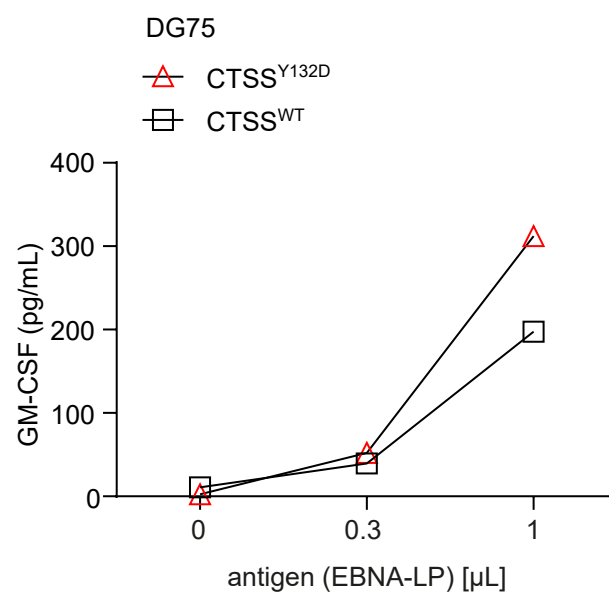
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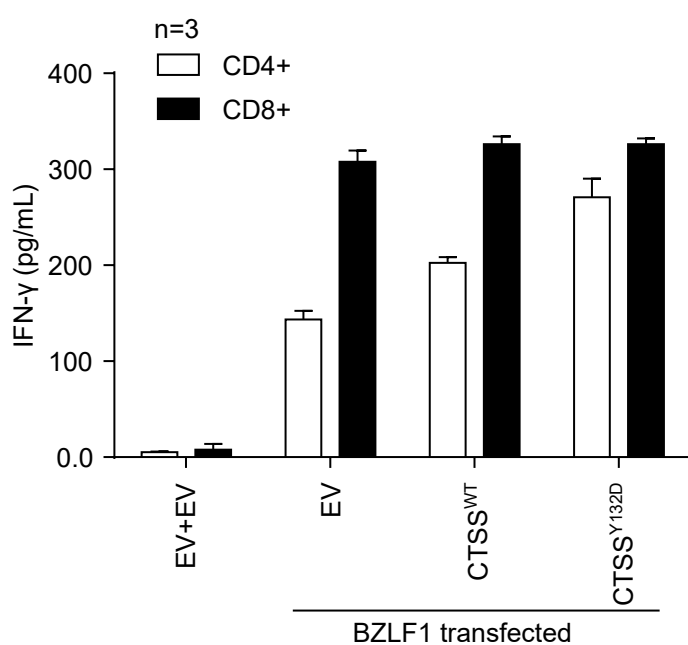
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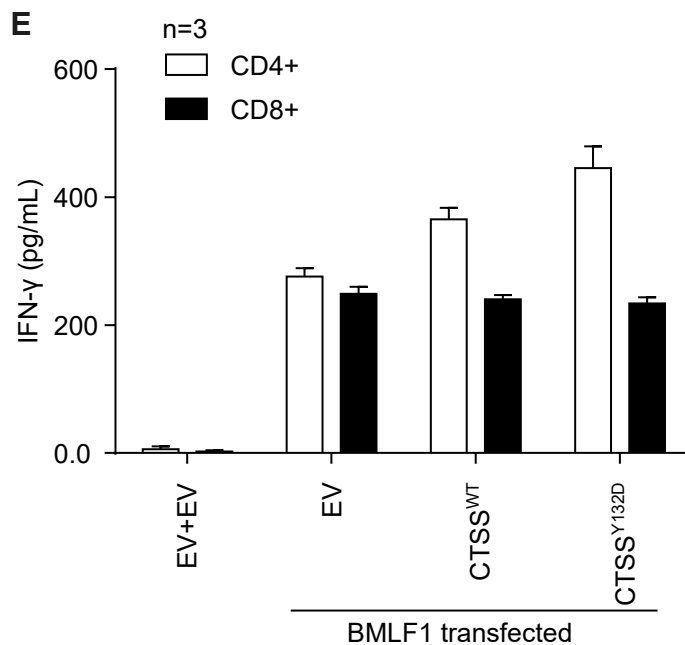
**C**



**D**



**E**



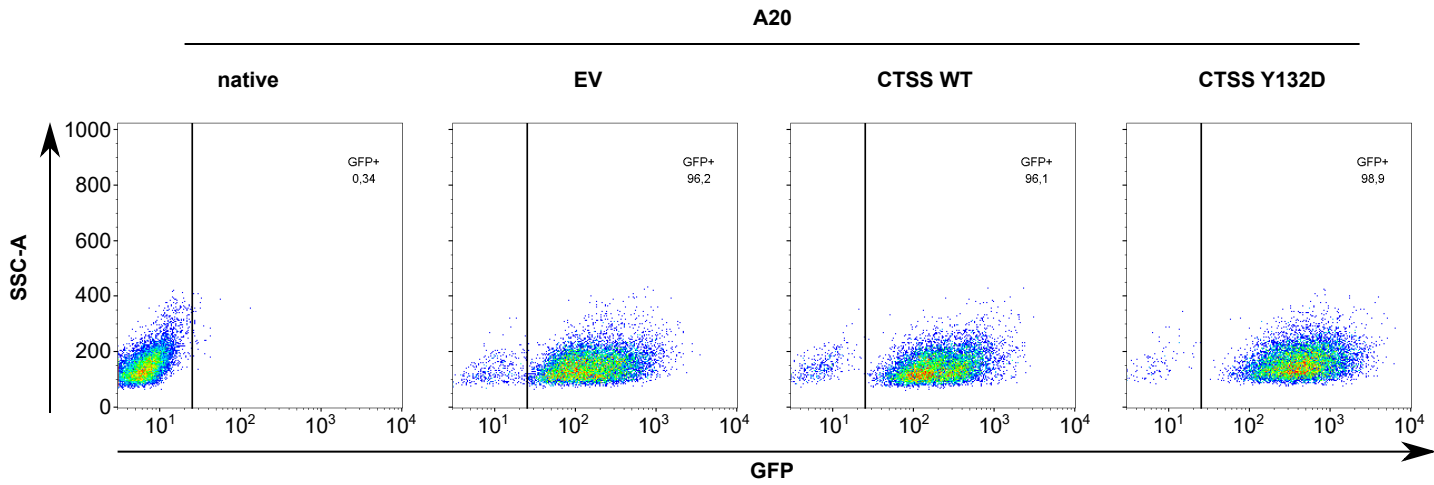


**Supplementary Figure 4. CTSS Y132D and CTSS WT overexpression increase antigen-specific CD4<sup>+</sup> but not CD8<sup>+</sup> T-cell responses *in vitro*. Related to Figure 4. A)** CD4<sup>+</sup> T-cell epitope prediction (NetMHCII 2.3) within CTSS (WT vs Y132D) potentially binding to representative class II (25 HLA-DR, 20 HLA-DQ and 9 HLA-DP) alleles. Predicted epitopes are depicted as blue (predicted weak binders) or red (predicted strong binders) lines above and below the amino acid sequence of human CTSS. The thickness of the line represents the number of HLA alleles (1-37 alleles) to which the predicted epitope may bind. The position of amino acid 132 (Y) is marked in color, the adjacent 10 amino acids are in capital letters and the 8 N- and C-terminal flanking amino acids are shown in italics. **B)** DG75 native (*CTSS* WT) cells were transfected with *CTSS* WT (*CTSS*<sup>WT</sup>) or Y132D (*CTSS*<sup>Y132D</sup>) expression constructs along with MHC-II. Cells were incubated with increasing antigen concentrations (EBNA-LP) and co-cultured with antigen-specific CD4<sup>+</sup> T-cells. GM-CSF in the supernatant was measured by ELISA. **C)** Similar to Figure 4G using BMLF-1 antigen. In this experiment, *CTSS* native and *CTSS*<sup>KO</sup> cells were transfected in parallel. Data is representative of 3 independent experiments. **D)** DG75 native (*CTSS* WT) cells were transfected with *CTSS* WT (*CTSS*<sup>WT</sup>) or Y132D (*CTSS*<sup>Y132D</sup>) expression constructs along with BZLF1 and co-cultured with antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in separate wells. Differences between CD8<sup>+</sup> T-cell activation (black bars) are not significant. **E)** Similar to D) with BMLF1. Again, differences between CD8<sup>+</sup> T-cell activation are not significant,  $p > 0.6$  by 2-tailed Student's t-test.

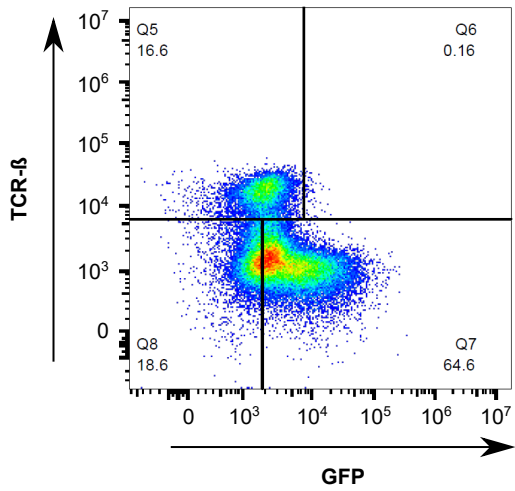
Pooled data from biological replicates (N) are represented as mean  $\pm$  SD; p-values from unpaired Student's t test.

Figure S5

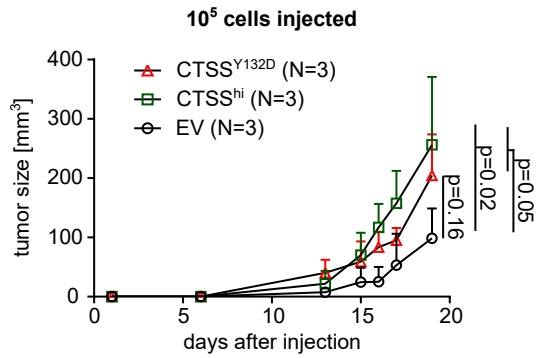
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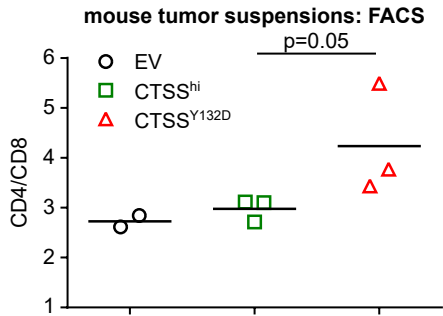
B



C



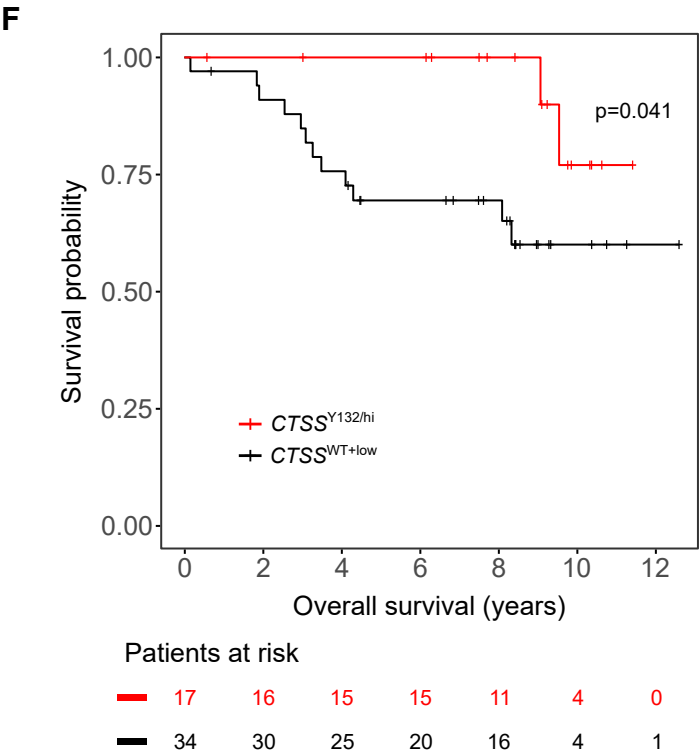
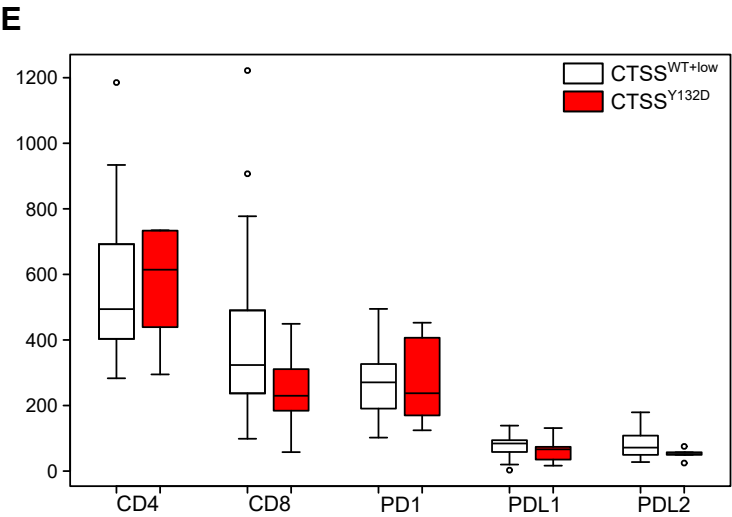
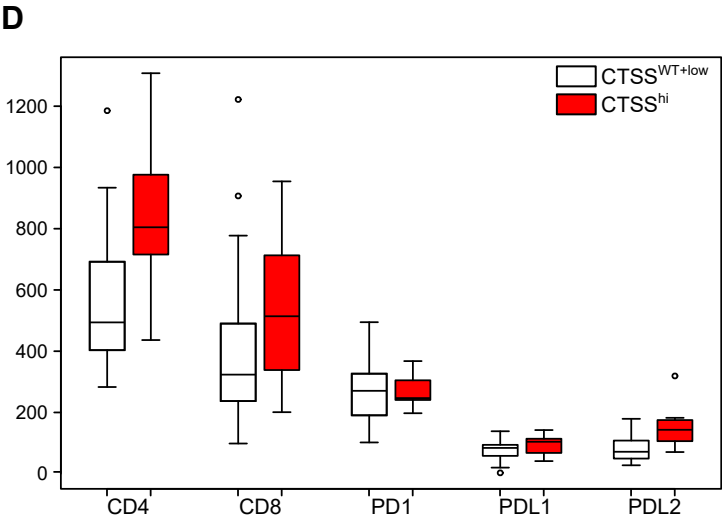
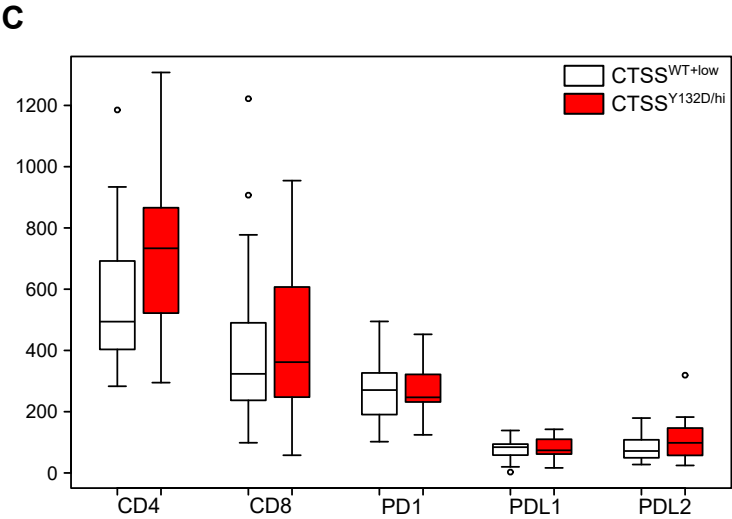
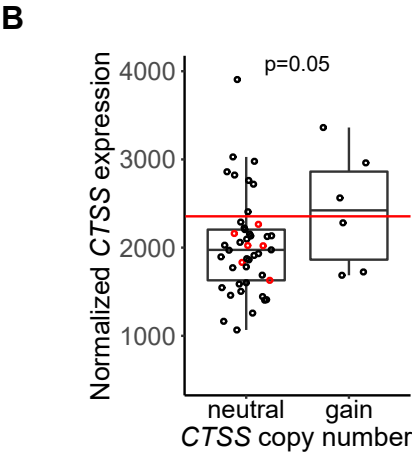
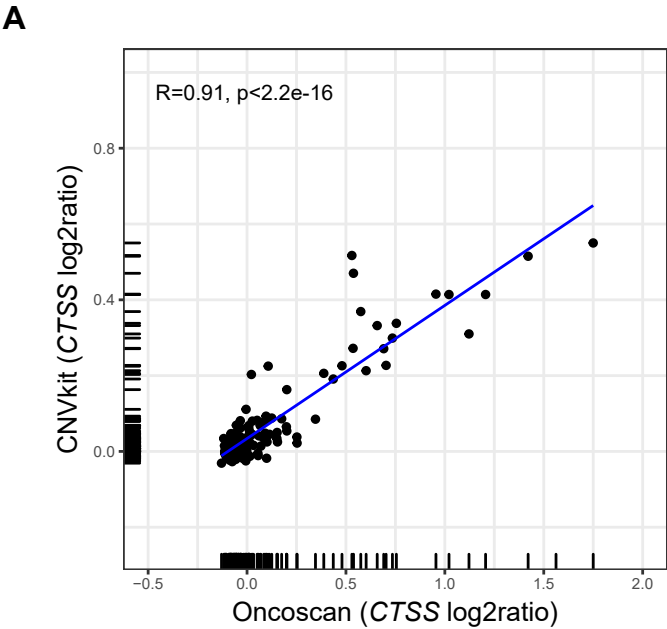
D



**Supplementary Figure 5. Supplementary data for *in vivo* experiment. Related to Figure 5.** **A)** FACS of native, empty vector (EV), *CTSS* WT and *CTSS* Y132D transduced A20 cells. **B)** FACS of a pre-injection mix consisting of splenocytes and transduced A20 cells in a 1 to 5 ratio. **C)** Tumor growth in BALB/c mice with N=3 mice per construct. Splenocytes ( $0.2 \times 10^5$ ) from sheep red blood cell (SRBC)-immunized BALB/c mice were mixed 1-to-5 with stably transduced A20 cells ( $1 \times 10^5$ ).  $1.2 \times 10^5$  cells were then injected per BALB/c mouse (N=3 mice per group). **D)** FACS analysis of isolated tumor suspensions from C).

Pooled data from biological replicates (N) are represented as mean  $\pm$  SEM in C); p-values from unpaired Student's t test.

Figure S6

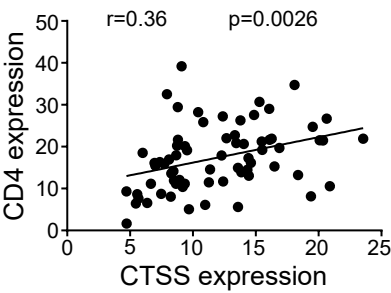


**Supplementary Figure 6. CTSS hyperactivity in primary patient samples from the GLSG2000 cohort. Related**

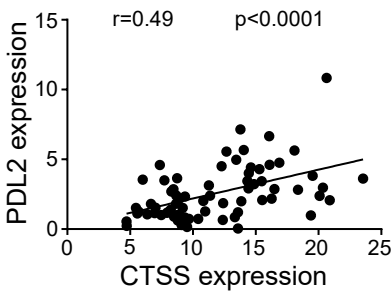
**to Figure 6. A)** Inferred copy number (CN) analysis from targeted DNA sequencing data. *CTSS* log2 values obtained from targeted DNA sequencing data and analyzed via the CNVkit pipeline plotted against the *CTSS* log2 values obtained from chromosome genomic array testing (CGAT, OncoScan) for a subset of our study cohort (N=146); Pearson's  $r$  and p-value are shown.. **B)** Normalized *CTSS* gene expression by *CTSS* CN status (neutral vs gain). Samples with high *CTSS* expression (above red line) were identified by *k*-mean clustering. FL with *CTSS* Y132 mutations are indicated by red circles; p-value from unpaired Student's *t* test. **C)** Boxplots comparing indicated immune genes in  $CTSS^{WT+low}$  to  $CTSS^{Y132D/hi}$  patients. **D)** Boxplots comparing indicated immune genes in  $CTSS^{WT+low}$  to  $CTSS^{hi}$  patients only. **E)** Boxplots comparing indicated immune genes in  $CTSS^{WT+low}$  to  $CTSS^{Y132D}$  patients only. The 50% quantile and the mean  $\pm$  SD are shown in C)-E). **F)** Kaplan-Meier plot for overall survival (OS) of patients with  $CTSS^{Y132D/hi}$  FL (N=17) vs  $CTSS^{WT+low}$  (N=34); p-value from log-rank test.

Figure S7

A

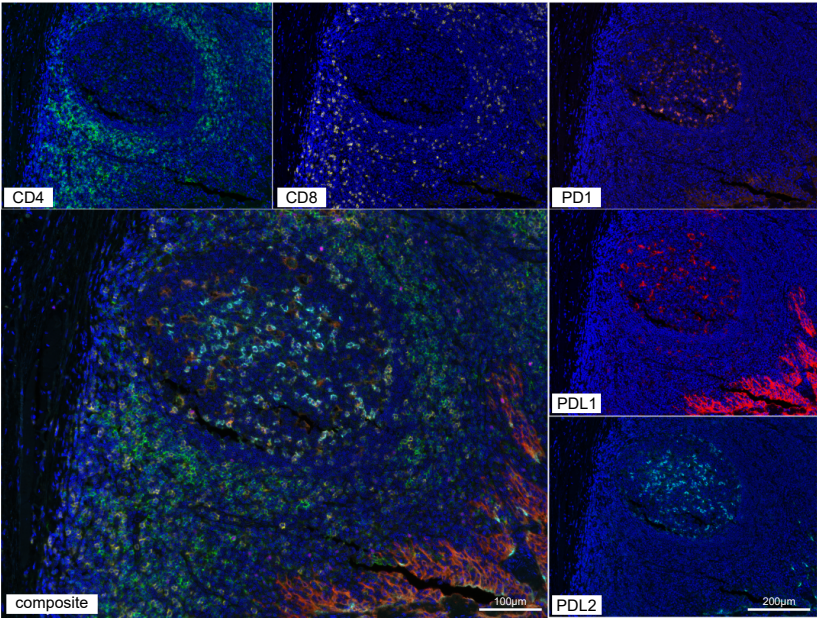


B



C

Tonsil



**Supplementary Figure 7. CTSS hyperactivity in primary patient samples from independent validation cohort (Tobin et al., 2019). Related to Figure 7. A)** Correlation of *CTSS* expression with *CD4* and **B)** *PDL2* expression. Pearson's  $r$  and  $p$ -value are shown. Data was generated with the Elements XT kit from NanoString. **C)** Multispectral imaging of a healthy human tonsil used as a control for staining patterns. Of note,  $CD4^+$  cells surround the germinal center.

**Table S1.** Oligonucleotides used for PCR, Sanger sequencing, CRISPR/Cas9 of CTSS. Related to STAR Methods.

Oligonucleotides		
CTSS_KO_sgRNA_Forward: CACCGtacctaccatgtctcccagg	This paper	N/A
CTSS_KO_sgRNA_Reverse: AAACcctgggagacatggttagtac	This paper	N/A
CTSS_Y132D_repair template: agccagtggcagagaaatcacatataagtcaaaccctaatcgatattgcctgattct gtggactggagagagaaagggtgtgttactgaagtgaatGatcaagtgaagtattacca tccaagcGtctgaaccctaggtagagctctttaaagcttagtctagctacagaatttga cagtgaatcttacagtcccac	This paper	N/A
CTSS_Y132D_sgRNA_Forward: CACCGgagctctacctagggttcag	This paper	N/A
CTSS_Y132D_sgRNA_Reverse: AAACctgaaccctaggtagagctcC	This paper	N/A
Primer for PCR screening CTSS KO forward: AACACTTATTTGTCCAGGATGCAG	This paper	N/A
Primer for PCR screening CTSS KO reverse: TTCTGTACACGTTCTTACGTCCTT	This paper	N/A
Primer for PCR screening CTSS Y132D forward: GATGAGTTCCTGAGAGTTCCC	This paper	N/A
Primer for PCR screening CTSS Y132D reverse: ACACGTGGGACTGTAAGATTC	This paper	N/A
Primer for Sanger sequencing of plasmids CTSS forward: TGTGCTCTTGGTGTGCTCCT	This paper	N/A
Primer for Sanger sequencing of plasmids CTSS reverse: CATTGCAGCCTTTGTTTCCA	This paper	N/A
Primers to validate cDNA after CRISPR forward: CTGAGAGTTCCCAGCCAGTG	This paper	N/A
Primers to validate cDNA after CRISPR reverse: CAGGTTCTGGGCACTGAGAG	This paper	N/A



**Table S2.** Antibodies used for immunohistochemistry (IHC), flow cytometry (FACS) and immunoprecipitation (IP). Related to STAR Methods.

<b>Table S2: Antibodies</b>							
<b>Purpose</b>	<b>ID</b>	<b>Company</b>	<b>Species</b>	<b>Clone</b>	<b>Dilution</b>	<b>Cat#</b>	<b>RRID</b>
IHC on human tissue	CD34	Beckman-Coulter	mouse	QBEnd10	1:700	IM1185	AB_131602
	CD4	Leica Biosystems	mouse	4B12	1:25	PA0371	AB_1055443
	CTSS	R&D	goat	polyclonal	1:50	AF1183	AB_2087994
	CD8	DAKO	mouse	C8/144B	1:200	M7103,	AB_2075537
	PD1	CellMarque	mouse	MRQ-22	1:200	315M-95	AB_1160824
	PDL1	Cell Signaling	rabbit	E1L3N	1:200	13684	AB_2687655
IHC on mouse tumor tissue	CD4	abcam	rabbit	EPR19514	1:500	ab183685	AB_2686917
	CD45R/B220	BD	rat	RA3-6B2	1:200	553084	AB_394614
	ki67	Cell Marque	rabbit	SP6	1:200	275R-15	AB_1158033
Vectra Polaris on human tissue	PDL2	Cell Signaling	rabbit	D7U8C	1:50	82723	AB_2799999
	PD1	Medac	mouse	MRQ22	1:80	315M-95	AB_1160824
	PDL1	DCS	rabbit	QR1	1:150	PI516C002	N.A.
	CD4	Sigma-Aldrich	rabbit	EP204	1:200	104R-26	N.A.
	CD8	Cell Marque	mouse	C8/144B	1:300	108M-94	AB_1158205
FACS on mouse tumor suspensions	CD19 eFluor660	eBioscience	rat	eBio1D3	1:200	50-0193-82	AB_11218286
	NK1.1 PE	eBioscience	mouse	PK136	1:100	A26000	AB_2536060
	TCR $\beta$ AF 700	eBioscience	hamster	H57-597	1:1500	56-5961-82	AB_2802349
	CD4 PerCP-Cy 5.5	eBioscience	rat	RM4-5	1:1000	45-0042-82	AB_1107001
	CD8 $\alpha$ Bv 763	eBioscience	rat	53-6.7	1:100	14-0081-82	AB_467087
Western blot	CD74	Abcam	mouse	2D1B3	1:1000	AB181465	N.A.
	CTSS (Peptide: Q17-I331)	R and D Systems	goat	polyclonal	1:2000	AF1183	AB_2087994
	FLAG-M2	Sigma-Aldrich	mouse	M2	1:5000	F3165	AB_259529
	Goat-IgG-HRP	Santa Cruz	donkey	polyclonal	1:2000	sc-2020	AB_631728
	HSP 90	Cell Signaling	rabbit	C45G5	1:5000	4877	AB_2233307
	Goat anti-Mouse IgG-HRP	Thermo Fisher	goat	Polyclonal	1:5000	31430	AB_228307
	Rabbit-IgG-HRP	Thermo Fisher	goat	polyclonal	1:2000	31460	AB_228341
	$\beta$ -tubulin	Cell Signaling	rabbit	9F3	1:5000	2128	AB_823664
IP	Normal Goat IgG Control	R and D Systems	goat	polyclonal	1:200	AB-108-C	AB_35426
	CTSS (Peptide: Q17-I331)	R and D Systems	goat	polyclonal	1:100	AF1183	AB_2087994

**Table S3.** DNA sequencing details of CTSS mutations.

Gene	Patient ID	Type	Effect	Amino acid change	Reference base	Alternate base	Coverage	VAF	Cohort	Reported in
CTSS	1607	SNP	Missense	Y132D	A	C	293	0.38	GLSG	Pastore et al. 2015
CTSS	1653	SNP	Missense	Y132D	A	C	349	0.44	GLSG	Pastore et al. 2015
CTSS	1812	SNP	Missense	Y132D	A	C	278	0.22	GLSG	Pastore et al. 2015
CTSS	1890	SNP	Missense	M185V	T	C	180	0.31	GLSG	Pastore et al. 2015
CTSS	2025	SNP	Missense	Y132S	T	G	366	0.16	GLSG	Pastore et al. 2015
CTSS	2085	SNP	Missense	Y132N	A	T	128	0.08	GLSG	Pastore et al. 2015
CTSS	2209	SNP	Missense	G251A	C	G	311	0.36	GLSG	Pastore et al. 2015
CTSS	2223	SNP	Missense	Y132D	A	C	81	0.38	GLSG	Pastore et al. 2015
CTSS	2567	SNP	Missense	Y132D	A	C	199	0.07	GLSG	Pastore et al. 2015
CTSS	2659	SNP	Missense	Y132D	A	C	247	0.12	GLSG	Pastore et al. 2015
CTSS	2676	SNP	Missense	Y132N	A	T	172	0.07	GLSG	Pastore et al. 2015
CTSS	2772	SNP	Missense	Y132D	A	C	181	0.12	GLSG	Pastore et al. 2015
CTSS	2803	SNP	Missense	Y132D	A	C	212	0.12	GLSG	Pastore et al. 2015
CTSS	3018	SNP	Missense	Y132D	A	C	403	0.40	GLSG	Pastore et al. 2015
CTSS	3145	SNP	Missense	Y132D	A	C	93	0.27	GLSG	Pastore et al. 2015
CTSS	3175	SNP	Missense	Y132D	A	C	228	0.22	GLSG	Pastore et al. 2015
CTSS	3421	SNP	Missense	Y132D	A	C	92	0.23	GLSG	Pastore et al. 2015
CTSS	RG100	SNP	Missense	L80V	G	C	74	0.76	BCCA	Pastore et al. 2015
CTSS	RG136	SNP	Missense	Y267D	A	C	137	0.20	BCCA	Pastore et al. 2015
CTSS	RG62	SNP	Missense	Y132D	A	C	95	0.09	BCCA	Pastore et al. 2015
CTSS	RG64	SNP	Missense	Y132D	A	C	81	0.19	BCCA	Pastore et al. 2015
CTSS	RG76	SNP	Missense	Y132D	A	C	203	0.05	BCCA	Pastore et al. 2015
CTSS	24	SNP	Missense	Y132D	A	C	364	0.11	PAH	Tobin et al. 2019
CTSS	65	SNP	Missense	Y132D	A	C	884	0.22	PAH	Tobin et al. 2019
CTSS	124	SNP	Missense	Y132D	A	C	340	0.19	PAH	Tobin et al. 2019
CTSS	130	SNP	Missense	Y132D	A	C	297	0.15	PAH	Tobin et al. 2019
CTSS	188	SNP	Missense	Y132D	A	C	514	0.18	PAH	Tobin et al. 2019
CTSS	207	SNP	Missense	M185V	T	C	515	0.32	PAH	Tobin et al. 2019

SNP = Single Nucleotide Polymorphism, VAF = Variant Allele Frequency, GLSG = German Low-Grade Lymphoma Study Group, BCCA = British Columbia Cancer Agency, PAH = Princess Alexandra Hospital