SUPPLEMENTAL DATA

Specific covalent inhibition of MALT1 paracaspase suppresses B-cell lymphoma growth

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Supplemental methods

In vitro MALT1 inhibition assay

The preparation of LZ-MALT1 (340-789) was described previously (26). *In vitro* assay was performed in 20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1mM DTT, 0.01% TritonX-100, and 10% Glycerol. After mixing 50-100 nM LZ-MALT1 with compound and 0.2 mM substrate Ac-LRSR-AMC (Peptides International), fluorescence signal was immediately measured using a SpectraMax M5e plate reader (Molecular Devices). The Ki of covalent compounds was calculated using equation $[\mathrm{P}] = \mathrm{Vi}\,(1 - \mathrm{e}^{-\mathrm{k_{obs}t}})/\mathrm{k_{obs}}$ and $\mathrm{k_{obs}} = \mathrm{k_{inact}}\,[\mathrm{I}]/(\mathrm{K_i}(1 + [\mathrm{S}]/\mathrm{K_m}) + [\mathrm{I}])$. $\mathrm{K_i^*} = \mathrm{K_i}(1 + [\mathrm{S}]/\mathrm{K_m})$ was used as the apparent K_i to compare compound potency. Data processing was performed using the Origin software (OriginLab Corporation).

Protease panel

All reaction components were diluted to work concentrations with assay buffer. Reactions were assembled in plates by adding test compound, then enzyme; and incubated for 20 minutes at RT. Reaction was initiated by addition of the appropriate substrate for each protease (panel description in Supplemental Table 2). Plate reading was carried out with PHERAstar PLUS (BMG) or FlexStation 3 (MD) using kinetics setting. Data acquisition and analyses were performed using Excel 2007 and GraphPad Prism 6. The percentage inhibition was calculated from the following equation: % inhibition= [1-(sample activity - Min)/ (Max-Min)]*100; where Max is enzyme, substrate and buffer in reaction buffer and Min is substrate and buffer in reaction buffer with no enzyme added.

Growth inhibition assay

Cell proliferation was determined by ATP quantification using CellTiter-Glo (Promega). Briefly, 2,000-5,000 cells/well were plated in 384 well plates and treated every 48 hours with either vehicle or inhibitor. Luminescence was measured at 96 hours. Results were calculated as fold to vehicle treated cells and used to compute GI_{50} values.

Molecular Mass Measurement by Multi-Angle Light Scattering (MALS)

After MALT1-inhibitor complexes were reconstituted and purified, the peak fraction containing 0.2 mg protein were loaded onto a Superdex 200 gel filtration column coupled to a three-angle light scattering detector (mini-DAWN TRISTAR) and a refractive index detector (Optilab DSP) (Wyatt Technology). Data analysis was carried out using ASTRA V.

Flow Cytometry

To test the effects of compound 3 on cell proliferation, we used the CellTrace™ CFSE Cell Proliferation Kit (Invitrogen). Briefly, cells were labeled according to the manufacturer's protocol with CFSE dye that covalently labels intracellular molecules. CFSE dilutes with each cell division. Reference samples were analyzed 24h after staining (day 1 control). Cells were split and treated every two days and analyzed after 6 days of treatment. $DAPI^-$ cells were used for flow cytometric analysis. To analyze cell cycle distribution, we used the FITC BrdU Flow Kit (BD Pharmingen). Briefly, cells were treated with vehicle or compound 3 for 24 hours and then pulsed with BrdU for 15 minutes, fixed and stained according to manufacturer's protocol. Apoptosis was assessed by Annexin V-APC/DAPI (BD Pharmingen) staining.

Western Blot and ELISA

Equal amounts of protein extracts (20-80 μg) from tissue lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies: MALT1 (B-12, Santa Cruz Biotechnology), RelB (C1E4, Cell Signaling Technology), uncleaved Bcl10 (EP606Y, Abcam), Roquin (3F12, EMD Millipore Corporation), β-Actin (AC-15, Sigma-Aldrich) and β-Tubulin (D66, Sigma-Aldrich).

Human IL10 in mouse serum was quantified using the human IL-10 ELISA kit from eBioscience following manufacturer's instructions.

MALT1 knockdown

TMD8 and OCI-Ly3 cells were infected with pLKO.1-U6-shRNA-YFP. Hairpin sequences were shMALT1_1 (CCTCACTACCAGTGGTTCAAA), shMALT1_2 (CCTCACTACCAGTGGTTCAAA) and a non targeting shRNA (CAACAAGATGAAGAGCACCAA). YFP+DAPI- cells were sorted and RNA extracted as indicated below.

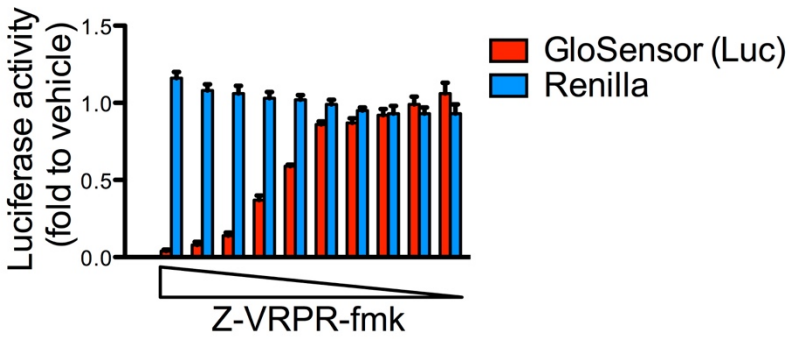
RNA extraction and qPCR

RNA was isolated from cell lines or tumor samples using TRIzol (Invitrogen) and RNeasy kit (Qiagen). All extracts were treated with DNase in column during RNA purification.

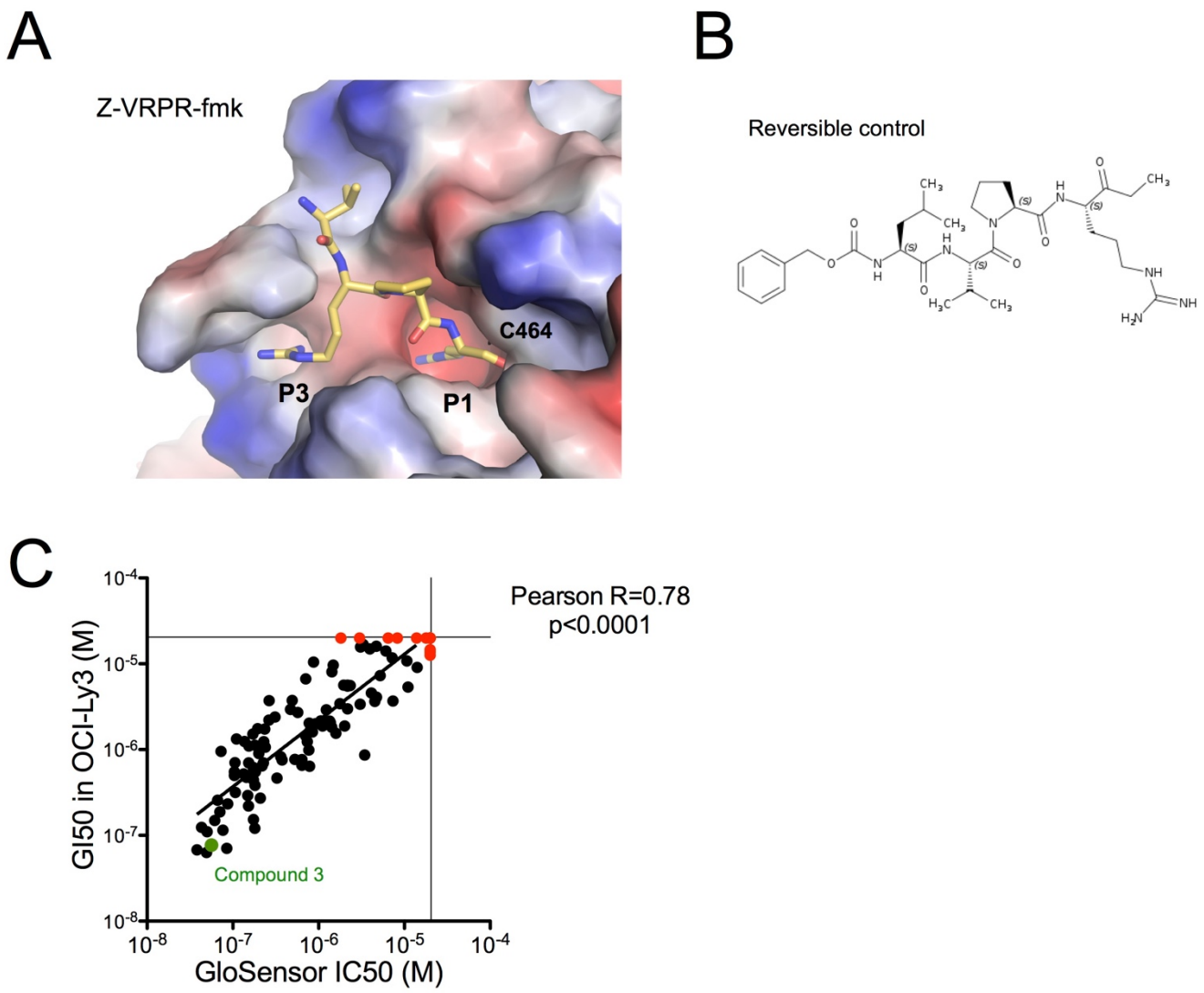
cDNA was synthesized from 1 μg total RNA with Verso DNA Synthesis Kit (Thermo Scientific). qPCR primers sequence are listed in Supplemental Table 5. All samples were run in triplicate using SYBR Green (Thermo Fisher Scientific) on a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). Gene expression

was normalized to HPRT1 and then relative to the average ΔCt of control group (ΔΔCt method). Results are represented as mean relative expression ± SEM.

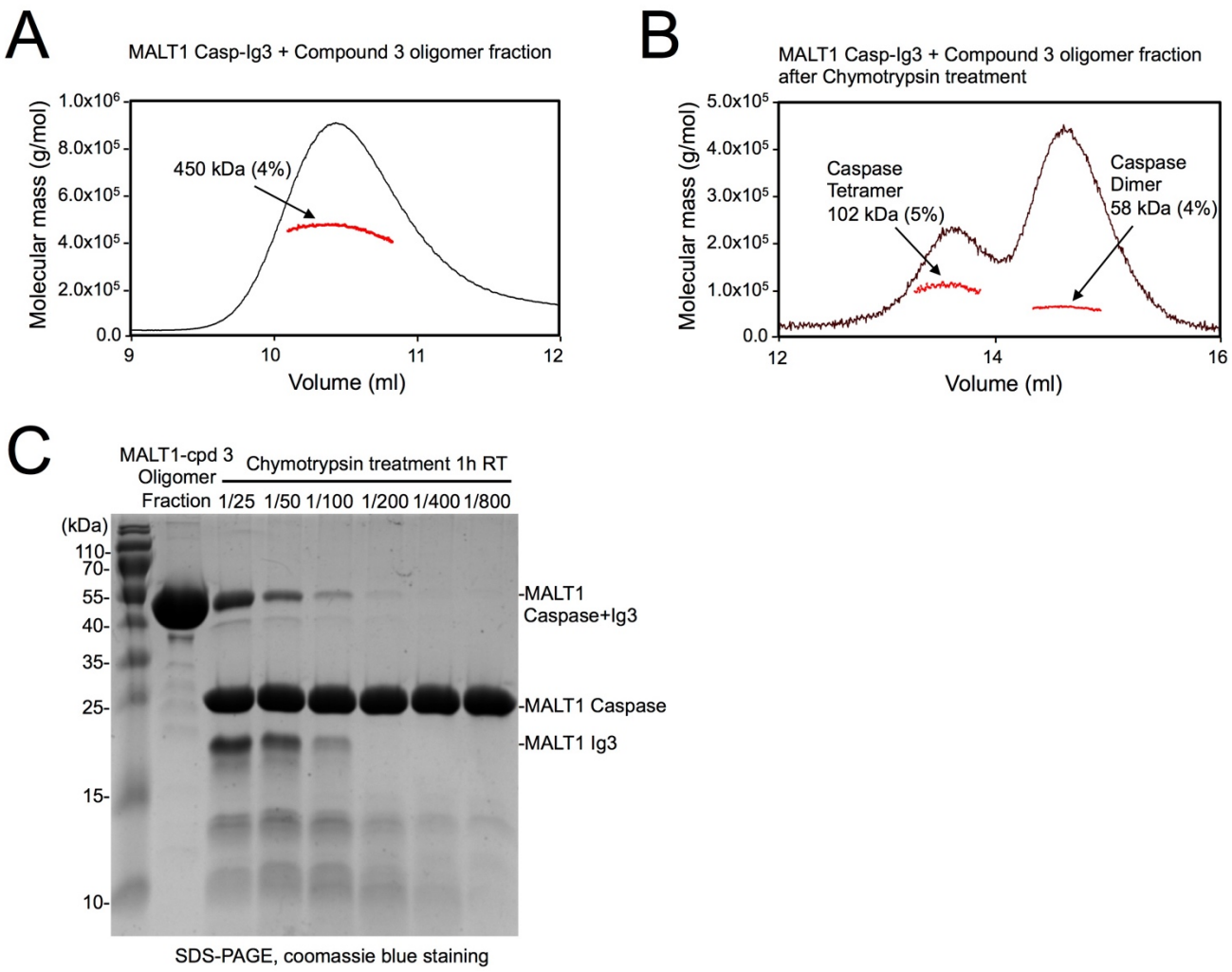
Supplemental Figures



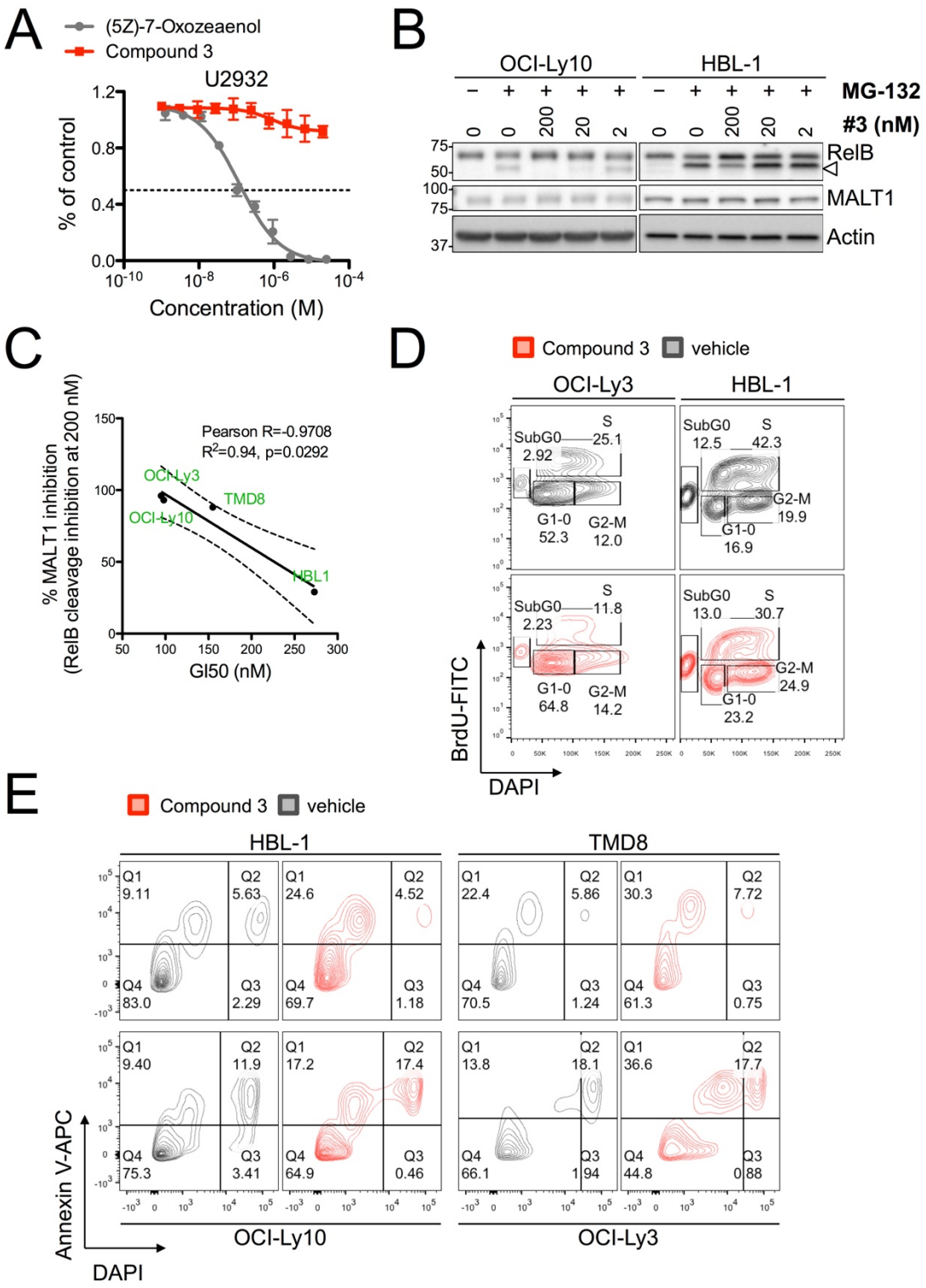
Supplemental Figure 1. MALT1-GloSensor reporter and Renilla control luciferase activities in response to escalating doses of Z-VRPR-fmk in Raji cells.



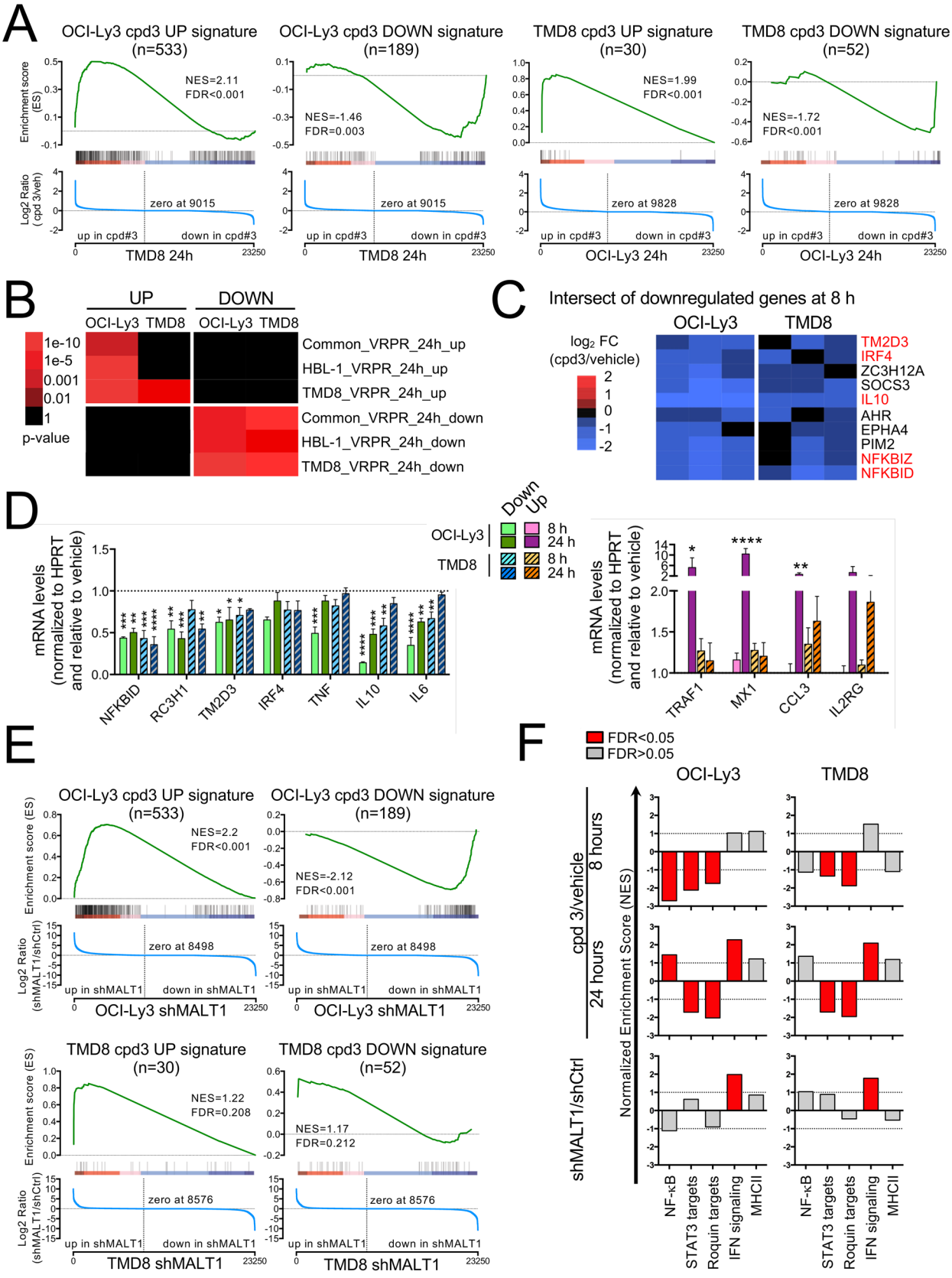
Supplemental Figure 2. **(A)** Crystal structure of Z-VRPR-fmk and MALT1 showing P1 and P3 interactions. **(B)** Structure of reversible analog of compound 1. **(C)** Correlation plot between growth inhibition in OCI-Ly3 and Raji MALT1-GloSensor assays for 111 Z-VRPR-fmk derivatives.



Supplemental Figure 3. (A) MALS measurement using MALT1 oligomer peak sample. **(B)** MALS measurement of MALT1 oligomer peak treated by Chymotrypsin. Compared with Figure 3B, the decreased tetramer/dimer ratio may be due to sample dilution in gel filtration. **(C)** SDS-PAGE showing MALT1 sample before and after Chymotrypsin treatment.

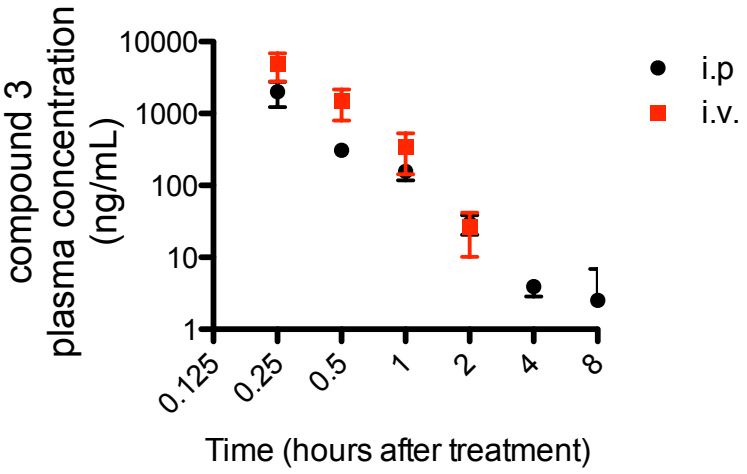


Supplemental Figure 4. (A) Growth response of U2932 to TAK1 inhibition by (5Z)-7-Oxozeaenol compared to MALT1 inhibition by compound 3. **(B)** Western blot analyses of RelB and MALT1 after 30 min pretreatment with 200 nM compound 3 or vehicle, followed by proteasome inhibitor MG-132 at 5 μM for 1.5 h in the indicated cell lines. Arrowhead indicates cleaved product. **(C)** Correlation between % inhibition of MALT1 activity (measured as % RelB cleavage inhibition at 200 nM compound 3) and GI_{50} value for MALT1 sensitive cell lines. Plotted are linear correlation and 90% confidence band (dotted lines). **(D)** Representative flow cytometry charts of BrdU pulse-labeled OCI-Ly3 and HBL1. **(E)** Annexin V-DAPI labeling representative flow cytometry charts for the indicated cell lines. Cells were treated every 48 hours for 6 days with 1 μM compound 3.

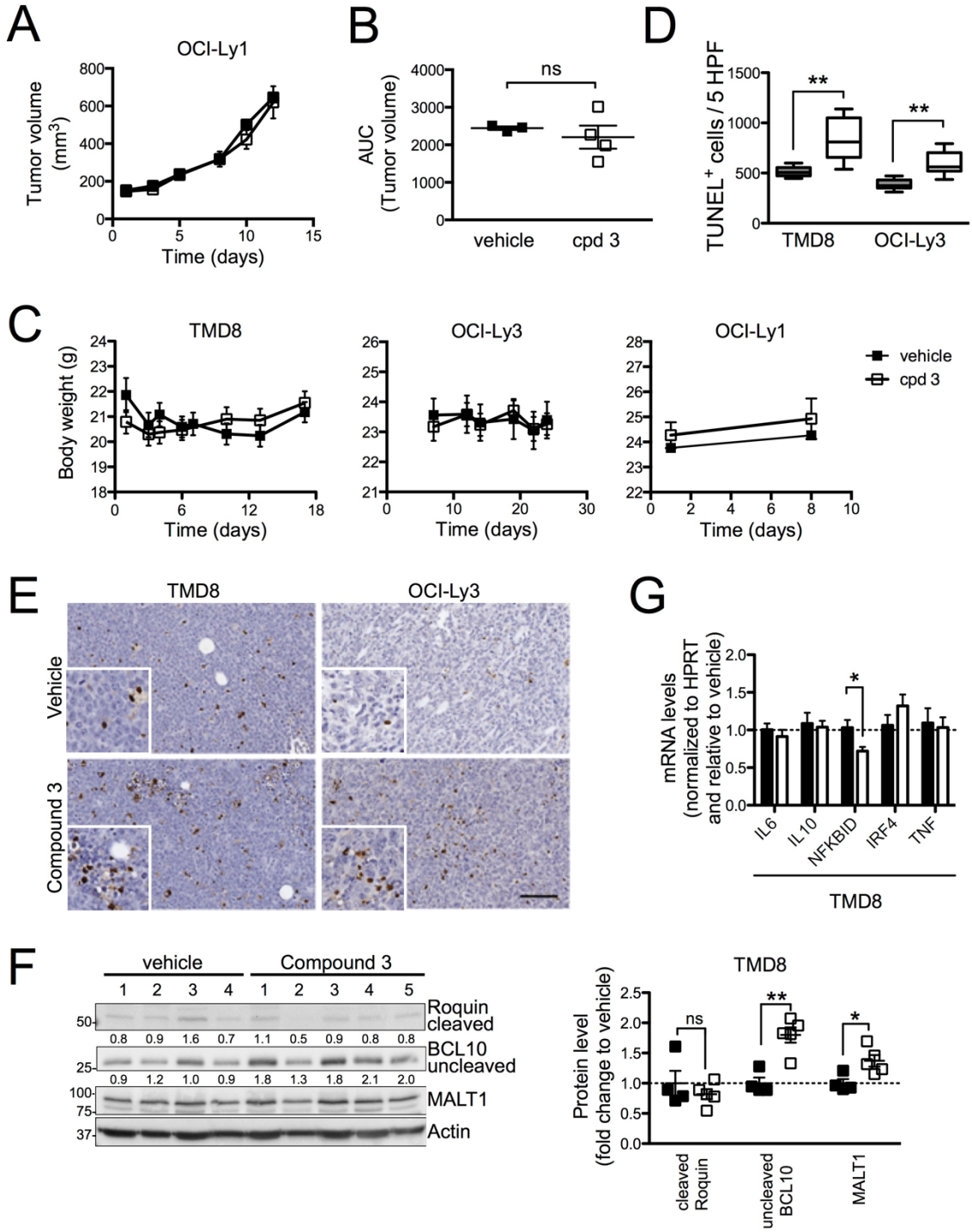


Supplemental Figure 5. (A) Gene Set Enrichment Analysis for the OCI-Ly3 24 hours compound 3 UP (n=533 genes) and DOWN-regulated (n=189 genes) gene signatures in TMD8 cells treated with compound 3 at 24 hours; and *vice versa*, Gene Set Enrichment Analysis for the TMD8 24 hours compound 3 UP (n=30 genes) and DOWN-regulated (n=52 genes) gene signatures in OCI-Ly3 cells treated with compound 3 at 24 hours. Normalized enrichment scores and FDR values are shown. **(B)** Hypergeometric test results for pathway enrichment analysis of Z-VRPR-fmk signatures in OCI-Ly3 and TMD8 compound 3-treated cells at 24 hours. **(C)**

Heatmap of log2 fold change compound 3 to vehicle treated cells showing the overlap between significantly enriched genes in TMD8 and OCI-Ly3 cell lines at 8 hour treatment with compound 3. In red, Roquin targets. **(D)** qPCR validation of genes found downregulated (*IL10*, *IL6*, *NFKBID*, *RC3H1*, *TM2D3*, *IRF4* and *TNF*) or upregulated (*TRAF1*, *MX1*, *CCL3* and *IL2RG*) by RNAseq in OCI-Ly3 cells. Y-axis: mRNA levels were normalized to HPRT and are presented as fold change to vehicle treated cells at the indicated time points. Statistics, ANOVA with Dunnett's correction for multiple comparison. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. **(E)** Gene Set Enrichment Analysis for the OCI-Ly3 24 hours compound 3 UP (n=533 genes) and DOWN-regulated (n=189 genes) gene signatures in shMALT1 OCI-Ly3 cells vs control; and Gene Set Enrichment Analysis for the TMD8 24 hours compound 3 UP (n=30 genes) and DOWN-regulated (n=52 genes) gene signatures in shMALT1 TMD8 cells vs control. Normalized enrichment scores and FDR values are shown. **(F)** GSEA Normalization Enrichment Scores for the indicated signatures in OCI-Ly3 or TMD8 treated with compound 3 for 8 or 24 hours or expressing shMALT1 *vs* a non targeting control.



Supplemental Figure 6. Compound 3 was administered i.v. (red) or i.p. (black) to C57BL/6 mice (n=3 mice per time point) at a dose of 2 mg/Kg. Compound 3 was formulated as 0.5mg/mL solution in 10/10/80 DMSO/Tween 80/Water. Y-axis is compound 3 concentration in plasma in ng/mL. X-axis is time after injection. Results are given as mean ± SD.



Supplemental Figure 7. (A) Tumor growth curve for xenografts of OCI-Ly (n=4/group) following compound 3 treatment. Mice were treated with 30 mg/kg BID compound 3 or same volume of vehicle for 14 consecutive days. **(B)** Tumor volumes in control and compound 3 treated animals bearing OCI-Ly xenografts. Growth of each tumor was measured as area under the curve (AUC). Mean ± SEM, 2-tailed unpaired t test. **(C)** Body weight of OCI-Ly1-xenografted NOD-SCID mice over the course of the treatment with compound 3. **(D)** Bar plot of differences in TUNEL$^+$ staining in histologic sections of TMD8 and OCI-Ly3 xenografts. The y-axis represents number of TUNEL$^+$ cells per 5 high power fields (n=6-9 evaluable tumors/treatment). Statistical significance was calculated using t-test. **(E)** Representative image of TUNEL staining in vehicle and compound 3 treated TMD8 and OCI-Ly3 xenografts. Bar: 100 µm. Insets are 2X. **(F)** Western blot analyses of MALT1, uncleaved BCL10 and cleaved Roquin in xenografted TMD8 tumors at the end point of the experiment. Bar plot shows protein level normalized to Actin and relative to average of vehicle controls. **(G)** mRNA levels for Roquin targets in TMD8 xenografted compound 3 treated *vs.* vehicle treated tumors. Y-axis: mRNA levels normalized to HPRT and relative to the average of vehicle treated mice. Statistics, t-test. *, $p<0.05$; **, $p<0.01$; ns, $p>0.05$.

Supplemental Tables

Supplemental Table 1. Summary of biochemical and cellular data for series of compounds.

	LZ-MALT1	GloSensor	OCI-Ly3	OCI-Ly1
	IC50 (nM)	IC50 (nM)	GI50 (nM)	GI50 (nM)
z-VRPR-fmk	140	1,056	2,500	>20,000
Compound 1	90	528	768	>20,000
Reversible control	na	>20,000	>20,000	>20,000
Compound 2	30	1,960	5,620	>20,000
Compound 3	10	56	87	>20,000

Supplemental Table 2. %Inhibition of activity in a panel of Cysteine proteases. Data for Compound 3 at 10 μM are shown for 26 proteases. All enzymes that showed over 50% inhibition at 10 μM were assayed in a dose-response assay.

		Compound 3			
Protease	**Compound 3**	**10**μM	**1**μM	**0.1**μM	**Assay substrate**
Calpain	101.3	105.3	25.6	23.6	5-FAM/QXL 520
Caspase-1	93				Ac-YVAD-AMC
Caspase-2	24.9				Caspase 2 (ICH-1) substrate
Caspase-3	11.5				Ac-DEVD-AMC
Caspase-4	12.5				Ac-LEHD-AMC
Caspase-5	-22.9				Ac-LEHD-AMC
Caspase-6	-81.7				Ac-VEID-AMC
Caspase-7	1.3				Ac-DEVD-AMC
Caspase-8	-10.7				Ac-IETD-AMC
Caspase-9	-18.0				Ac-LEHD-AMC
Caspase-10	-17.6				Ac-IETD-AMC
Cathepsin B	97.5	99.6	92.5	34.2	Z-FR-AMC
Cathepsin D	-42.2				5-FAM/QXL 520
Cathepsin G	-9.4				Suc-AAPF-pNA
Cathepsin K	98.4	98.7	97.1	63.2	Z-FR-AMC
Cathepsin L	57.3	44.9	-33.4	-23.6	Z-FR-AMC
Cathepsin S	10.7	99.1	83.9	78.2	Z-VVR-AMC
Cathepsin V	86.3	89.4	56.9	-28.7	Z-LR-AMC
Factor Xa	7.6				MeOCO-D-CHA-GR-pNA-AcOH
KLK-1	-1.1				D-VLR-AFC
KLK-2	-2.3				PFR-AMC
Legumain	31.5				Z-AAN-AMC
Thrombin	76.9	83	25.2	14.8	Z-GPR-AMC
Trypsin	97.9	95.3	98	92.9	Z-GPR-AMC
Tryptase BII	85.8	79	14	6.1	Z-GPR-AMC
Urokinase	50.6				Z-GGR-AMC

Supplemental Table 3. Crystallographic statistics.

	MALT1 Caspase-Ig3 Plus Compound 2	MALT1 Caspase Plus Compound 3
Data collection		
Space group	$P4_1$	$P6_122$
Cell dimensions		
a, b, c (Å)	76.2, 76.2, 146.9	74.0, 74.0, 197.3
α, β, γ ($^\circ$)	90.0, 90.0, 90.0	90, 90, 120
Resolution (Å)	147.0 – 2.2	64.1 – 1.9
R_{pim} (%)	2.9 (43.2)[†]	3.2 (62.6)[†]
Mean I/σ (I)	12.3 (2.5)[†]	20.1 (2.0)[†]
Completeness (%)	99.1 (99.1)[†]	99.2 (98.6)[†]
Redundancy	5.8 (5.9)[†]	37.7 (40.0)[†]
Refinement		
Resolution (Å)	43.4 - 2.2	64.1 - 1.9
Molecules/au[*]	2	1
No. reflections	41,912	27,491
R_{work} / R_{free} (%)	20.4 / 23.5	15.4 / 19.6
No. atoms		
Protein	5,942	1,850
Compound	72	35
Average B-factors (Å^2)		
Protein	67.5	32.8
Compound	59.8	34.9
R.M.S. deviations		
Bond lengths (Å)	0.007	0.015
Bond angles (°)	1.082	1.525
Ramachandran plot (%)		
Most favored regions	96.1	96.1
Additional allowed regions	3.9	3.9

[†]Values in parentheses are for the highest-resolution shell.
[*]Asymmetric unit.

Supplemental Table 4. Summary of growth inhibition data for compound 3 and MALT1 activity in a panel of 11 cell lines.

	DLBCL subtype	GI_{50} (nM)	% GI at 20 μM	MALT1 active
HBL1	ABC	273±64	77%	Yes
TMD8	ABC	155±17	78%	Yes
OCI-Ly10	ABC	98±32	64%	Yes
OCI-Ly3	ABC	87±6	91%	Yes
U2932	ABC	>3,000	19%	Yes
SU-DHL-2	ABC	>3,000	26%	No
HLY1	ABC	>3,000	-2%	No
RC-K8	ABC	>3,000	15%	No
OCI-Ly7	GCB	>3,000	14%	No
OCI-Ly1	GCB	>3,000	11%	No
SU-DHL-4	GCB	>3,000	-7%	No

Supplemental Table 5. qPCR primers.

Name	Sequence (5'-3')
IL6-F	GAAAGCAGCAAAGAGGCACT
IL6-R	TTTCACCAGGCAAGTCTCCT
IL10-F	CCAAGACCCAGACATCAAGG
IL10-R	GGCCTTGCTCTTGTTTTCAC
NFKBID-F	AGACAGGCTGGATTGTGTCC
NFKBID-R	AGCAGCAGCTGAACCAGAGT
IRF4-F	AGAAGAGCATCTTCCGCATC
IRF4-R	CCTTTAAACAGTGCCCAAGC
TNF-F	CTGCTGCACTTTGGAGTGAT
TNF-R	CGGGGTTCGAGAAGATGAT
TM2D3-F	GGGAAGCCTGTCACTTTTGA
TM2D3-R	CCAGCAAAATCTGCAAGTCA
RC3H1-F	TGGACAACCAGAACCACAAA
RC3H1-R	GATCCATTTGGTACATCACTGCT
CCL3-F	TGCAACCAGTTCTCTGCATC
CCL3-R	TGGCTGCTCGTCTCAAAGTA
MX1-F	ACCACAGAGGCTCTCAGCAT
MX1-R	CTTCAGGTGGAACACGAGGT
IL2RG-F	AATTCCCACCCTGAAGAACC
IL2RG-R	ACGAGGCAGAGTCGTTCACT
TRAF1-F	AGGACCGTCAGCCTCTTCTC
TRAF1-R	GAGCGACAGATGGGTTCTCT
HPRT1-F	AAAAGGACCCCACGAAGTGTT
HPRT1-R	TCAAGGGCATATCCTACAACAA