Genome-wide CRISPR screens identify ferroptosis as a novel therapeutic vulnerability in acute lymphoblastic leukemia

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Supplementary Information

Supplemental Methods

Gene network analysis

Key pathways associated with ALL specific genes were determined by mapping ALL enriched essential genes onto the STRING protein database[1]. High confidence protein-protein interactions were retained (score > 0.7). We used Cytoscape and maximum clique centrality (MCC) to identify the top 100 hub genes[2,3]. For each hub (cluster), a KEGG analysis was performed to highlight key pathways in the network[4].

Plasmid /library preparation

pLenti-Cas9-2A-Blast was a gift from Jason Moffat (Addgene plasmid # 73310). pLKV2-U6gRNA5-PGKpuro2ABPF-W human whole-genome library (Addgene # 67989), pLKV2-U6gRNA5(gGFP)-PBKBFP2AGFP-W (Addgene plasmid #67980), pKLV2-U6gRNA5(BbsI)-PGKpuro2ABFP-W (Addgene plasmid #67974) and pLKV2-U6gRNA5(empty)-PBKBFP2AGFP-W (Addgene plasmid #67978) were a gift from Kosuke Yusa. pLenti-FSP1-myc-DDK-P2A-puro was obtained from Origene (#RC204934L3). All plasmids were amplified for maxiprep at 30ºC in NEB® Stable bacteria (NEB, ON) and CIRCLEGROW™ media (MP Biomedicals™, OH) except pLKV2 library plasmids that were amplified in electrocompetent Endura™ bacteria (Lucigen, WI), at a 250-fold colony representation on LB-agar (BD) plates with carbanecillin (Bioshop Canada, ON). DNA was extracted using a proprietary in-house low-endotoxin ion exchange method. Library representation was validated using next generation sequencing of 8 pmoles library on NextSeq 500 System using a NextSeq 500 mid output Reagent kit v2, 75 cycles (Illumina, CA).

Cell lines and Cell Culture

B-ALL cell lines used for this study were NALM6, HAL-01, REH, 697, RS4;11, RCH-ACV, Tanoue, SEM (DSMZ, Germany). A549 (lung carcinoma), MCF7 (breast adenocarcinoma) and NCI-H226 (lung squamous cell carcinoma) were obtained from ATCC. Cell lines were cultured in RPMI 1640 media (HyClone™) + 10% heat-inactivated FBS (Gibco™) + 2 mM glutamine (Gibco™) at 37°C in 5% CO_2 incubators. HEK293SF-3F6

(ATCC) were grown in HyCell TransFx-H media (Hyclone™, Fisher Scientific) + 4 mM L-glutamine (HyClone™) + 0,1% Kolliphor (Sigma) in shaker flasks, at 120 rpm, 37°C in 5% CO_2 incubators.

Lentiviral production

HEK293SF-3F6 cells were transfected at a concentration of $1x10^6$ cells/mL with pLKV2 library, pLKV2-U6gRNA5(gGFP)-PBKBFP2AGFP-W, pLKV2-U6gRNA5(empty)-PBKBFP2AGFP-W, pLenti-FSP1-myc-DDK-P2A-puro (Origene # RC204934L3) or lenti-Cas9-2A-blast plasmid using PEIpro® (Polyplus, NY) together with packaging plasmids for lentiviral production (pMDLg-Gag/Pol, pCMV-CuO-VSVG, pRSV-Rev) at a ratio of 50 (gene of interest):25:15:10. Sodium butyrate was added at a final concentration 5mM at 16h post-transfection and supernatants were concentrated 72 hours post-transfection 200-fold on 20% sucrose cushion at 37,000g for 3hrs at 4°C.

Sequencing of screen samples

Genomic DNA (gDNA) extraction was performed using Blood & Cell Culture DNA Maxi Kit (Qiagen) and RNase A (Qiagen). gDNA was amplified by a two-step nested PCR reaction, first using primers CAGCGGTGCTGTCCATCTG and CCATTTGGTTAGTACCGGGC for PCR1 with the NEBNext® Ultra™ II Q5® Master Mix (NEB, ON, Canada) and by performing 20 reactions per sample each containing 2.5 µg of gDNA. A single PCR2 reaction was done similarly using 20 µL of PCR1 and with an equimolar mix of D501_F1-F8 primers and a specific D800R primer for indexing (sequences shown in Supplemental Table 1). PCR2 was purified on agarose gel using Gel extraction kit (Qiagen) followed by SPRIselect (Beckman Coulter, IN) bead purification. Purified fragments were quantified using NEBNext® Library Quant Kit for Illumina® (NEB, ON, Canada) and 14.3 fmoles of each PCR2 was multiplexed for sequencing on a NextSeq 500 system using NSQ 500 v2 75 cycle Hi Output kit (Illumina, CA).

Sequencing data processing and statistical analyses

Reads from Illumina sequencing were trimmed at both ends with the cutadapt package to extract the 20 bases sgRNA sequences and MAGeCK was used to count and normalize the reads[6]. Counts were corrected for copy number with CRISPRcleanR[2]. Guides targeting genes expressed below 0.5 TPM based on our RNAseq results or with average guide count below 30 at the initial time

point were discarded. Finally, MAGeCK was used to evaluate gene essentiality for each cell lines separately with the Robust Rank Analysis (RRA) algorithm, choosing the best p-value from the positive and negative test. ROC curves were generated using CRISPRcleanR package using BAGEL essential and BAGEL non-essential gene sets as true positive and true negative reference, respectively[7]. Principal component analysis (PCA) was done with the prcomp function of the R stats package (version 3.6.2)[8]. We identified ALL-enriched genes by comparing our significant gene list (present in >4/7 cell lines, 5% FDR) to the Broad and Sanger essential gene lists[9]. Wikipathway analysis of gene and pathway enrichment was performed using Molecular Signatures Database (MSigDB) investigation tool[10,11].

CCLE RNAseq and proteomic data analysis

Data from the Broad Institute Cancer Cell Line Encyclopedia (CCLE) version 19Q4 was downloaded from the depmap portal (https://depmap.org/portal/download/). Essentiality scores were used to classify cell lines into either GPX4 sensitive (< -0.7) or non-sensitive (> -0.3). Comparisons of RNA expression or protein levels between the two groups were done using two-sided unpaired Student's t-test, whose results were corrected for multiple testing using Benjamini-Hochberg. Only proteins detected in at least 20 cell lines in each group were considered for protein level.

Antibodies and western blotting

Anti-GPX4 (ab125066, Abcam, ON, Canada), anti-SLC7A11 (ab175186, Abcam, ON, Canada), anti-β-actin (sc-47778, Santa Cruz Biotechnologies, Dallas, TX), anti-GAPDH (G8795, Millipore Sigma, Oakville, ON, Canada) and anti-FSP1 (sc377120, Santa Cruz Biotechnologies, Dallas, TX) antibodies were used for western blotting (WB), followed by either goat anti-rabbit IgG HRP (Santa Cruz Biotechnologies, Dallas, TX) or mIgGκ BP-HRP (sc-516102, Santa Cruz Biotechnologies, Dallas, TX) respectively. Whole cell extracts were prepared by direct lysis in 4X Laemmli buffer + DTT. SDS-PAGE 4-15% gradient gels (BioRad, Mississauga, ON) were transferred on nitrocellulose membranes (BioRad, Mississauga, ON), blocked with 5% milk (BioRad, Mississauga, ON) and the signal detected with Clarity ECL (BioRad, Mississauga, ON).

Flow Cytometry analysis

Cells treated with 1 µM RSL3 for 4h were stained with 200 µM C11 581/591 BODIPY™ or Annexin V Pacific Blue™ conjugate (Molecular Probes®) as per manufacturer protocol. A 20h 8 µM etoposide treatment was used as a control for apoptosis induction. For each condition, approximately 30,000 cells were analyzed by flow cytometry with a BD LSRFortessa™ system (BD Biosciences, CA). Data were analyzed using the BD FACSDiva™ Software (BD Biosciences).

Fsp1 rescue experiments

Lentiviruses expressing FSP1-myc-DDK-P2A-puro were used to infect NALM6 and REH cell lines. FSP1 expression levels were validated by WB. Following puromycin selection, pools were used in parallel with parental cells to perform RSL3 IC50 curves as described above.

Patient-derived xenografts and drug treatments

PDX were generated as described in details in Nicolas Montpas *et al*. (manuscript in preparation). Briefly, bone marrow samples of B-ALL patients were collected at diagnosis and cells were isolated by Ficoll-Paque. 0.6-5 x 10^6 cells were injected in the tail vein of NOD-*scid*IL2Rgamma^{null} (NSG) mice and blast percentage (blast%) in the blood was monitored by monthly bleeding using human CD45, CD10 and CD19 vs CD45 murine antibodies (Biolegend, CA). Cells were expanded until blast% >1. Mice were then sacrificed, and PDX were isolated by extracting pre-B cells from mice spleen. Drug treatments were done for 36h before cell viability assessment by CellTiter-Glo® (Promega, WI). Detailed information on each PDX can be found in Suppl. Table 7.

Drug treatments

Ferroptosis-modulating drugs were procured from Millipore Sigma (Oakville, ON, Canada). DMSO was from Santa Cruz Biotechnologies (Dallas, TX). IC50 curves and drug rescue were measured using CellTiter-Glo® (Promega, WI) and luminescence was measured as described above. For drug rescue experiments, both drugs were added simultaneously.

GSH level measurements

GSH levels from 10,000 cells/well plated the day of the experiment were measured using GSH-Glo® (Promega, WI) according to the manufacturer's recommendation. Luminescence was measured as described above.

GPX4 KO clone generation

GPX4 sgRNAs (#1 CACGCCCGATACGCTGAGTG and #2 CTTGGCGGAAAACTCGTGCA) were subcloned in pKLV2-U6gRNA5(BbsI)-PGKpuro2ABFP-W, using BbSI (NEB, ON, Canada) digestion and ligation. Lentiviral productions of these plasmids were done as described above. SEM and REH Cas9 stable pools were infected with GPX4#2 and GPX4#1 sgRNA lentivirus respectively and cells were immediately treated with 4 µM ferrostatin and selected with 1-7.5 µg/mL puromycin (Gibco™). Cells resistant to puromycin were plated by limiting dilution into 384-well plates and clones were grown for two weeks (with addition of ferrostatin after about a week), before being transferred in two replicates 96-well plates, one without and one with 4 µM ferrostatin, respectively. After four days, a fraction of each well (+/- ferrostatin) was measured for cell viability using CellTiter-Glo® (Promega, WI). Luminescence of cells treated with or without 4 µM ferrostatin were compared to identify clones that had completely died without ferrostatin treatment. These clones were further evaluated by Sanger Sequencing for GPX4 KO, using TCCCTGCTCAGCTTCCTTTG and GCCCTTGGGTTGGATCTTCA primers for PCR on extracted gDNA.

Supplementary References

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Supplemental Figure 1. A) Example of Cas9 reporter assay in SEM Cas9 stable pool using pLKV2-U6gRNA5(empty)-PBKBFP2AGFP-W and pLKV2-U6gRNA5(sgGFP)-PBKBFP2AGFP-W plasmids. GFP/BFP ratio in cells infected with sgGFP plasmid reflects Cas9 activity. B) List of screened B-ALL cell lines with the respective % Cas9 activity of derived pools. C) Precision / Recall curves for screened cell lines. ROC (receiver operating characteristics) curves at sgRNA level with BAGEL essential and non-essential genes as reference. D) Principal Component analysis of screen results (T0 and Tf) for B-ALL cell lines. E) GPX4 sgRNA sequencing counts in screened B-ALL cell lines. Data are shown for each sgRNA targeting GPX4 in the pLKV2 library, with light colors for T0 counts and dark colors for Tf counts (14 doublings).

Supplemental Figure 2. A) C11 BODIPY™ vs Annexin V staining of RSL3 treated RS4;11 cells. 8 uM Etoposide +/- ZVADK caspase inhibitor treatments are shown as positive controls. B) Lipid Ros level (C11 BODIPY™) measurement at steady-state in ALL cell lines compared to non-GPX4 sensitive (non-ALL) cell lines. ** Unpaired t-test pvalue <0,01. C) Sensitivity of ALL vs non- ALL cell lines to deferoxamine treatment. Cell viability was evaluated after 24h treatment.

Supplemental Figure 3. A) C11 BODIPY™ vs Annexin V staining of RS4;11 treated for 24h with ferroptosis inducing drugs. B) Scheme of +/- Ferrostatin-1 screens in REH^{Cas9} and SEM^{Cas9} pools. C) Selenocompound metabolism gene knock-out induces lipid peroxidation. RS4;11 cells were infected with lentiviruses encoding sgRNAs targeting the indicated selenocompound gene and lipid peroxidation was measured using C11 BODIPY™ 120h post-infection. D) Distribution of GPX4 essentiality across CCLE cell lines. CRISPR screen data from CCLE cell lines were analyzed for GPX4 KO essentiality. Cell lines with a GPX4 essentiality score <-0.7 were classified as GPX4 essential and cell lines with a score >-0.3 were deemed GPX4 non-essential. E) Volcano plot of gene co-essential with GPX4 in screened CCLE cell lines. Selenocompound genes show high co-essentiality with GPX4.

Supplemental Figure 4. A) GPX4 mRNA and B) protein levels distribution across CCLE cell lines in GPX4 sensitive and non-sensitive cells. C) Anti-GPX4 WB on cell lines tested for RSL3 sensitivity. B-actin is showed for normalization. Quantification of bands was performed for comparison purposes. D) FSP1 mRNA level comparison between GPX4 sensitive and non-sensitive CCLE cell lines by cancer type.

Supplemental Table 1. List of primers used for NGS.

D501-F	AATGATACGGCGACCACCGAGATCTACAC TATAGCCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT TTGTGGAAAGGACGAAACACCG
D501-F_1	AATGATACGGCGACCACCGAGATCTACAC TATAGCCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT C TTGTGGAAAGGACGAAACACCG
D501-F_2	AATGATACGGCGACCACCGAGATCTACAC TATAGCCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT GC TTGTGGAAAGGACGAAACACCG
D501-F_3	AATGATACGGCGACCACCGAGATCTACAC TATAGCCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT AGC TTGTGGAAAGGACGAAACACCG
D501-F_4	AATGATACGGCGACCACCGAGATCTACAC TATAGCCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT CAAC TTGTGGAAAGGACGAAACACCG
D501-F_6	AATGATACGGCGACCACCGAGATCTACAC TATAGCCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT TGCACC TTGTGGAAAGGACGAAACACCG
D501-F_7	AATGATACGGCGACCACCGAGATCTACAC TATAGCCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT ACGCAAC TTGTGGAAAGGACGAAACACCG
D501-F_8	AATGATACGGCGACCACCGAGATCTACAC TATAGCCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT GAAGACCC TTGTGGAAAGGACGAAACACCG
D801-R	CAAGCAGAAGACGGCATACGAGAT CGAGTAAT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTAAAGCGCATGCTCCAGAC
D802-R	CAAGCAGAAGACGGCATACGAGAT TCTCCGGA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTAAAGCGCATGCTCCAGAC
D803-R	CAAGCAGAAGACGGCATACGAGAT AATGAGCG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTAAAGCGCATGCTCCAGAC
D804-R	CAAGCAGAAGACGGCATACGAGAT GGAATCTC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTAAAGCGCATGCTCCAGAC
D805-R	CAAGCAGAAGACGGCATACGAGAT TTCTGAAT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTAAAGCGCATGCTCCAGAC
D806-R	CAAGCAGAAGACGGCATACGAGAT ACGAATTC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTAAAGCGCATGCTCCAGAC
D807-R	CAAGCAGAAGACGGCATACGAGAT AGCTTCAG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTAAAGCGCATGCTCCAGAC
D808-R	CAAGCAGAAGACGGCATACGAGAT GCGCATTA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTAAAGCGCATGCTCCAGAC
D809-R	CAAGCAGAAGACGGCATACGAGAT CATAGCCG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTAAAGCGCATGCTCCAGAC
D810-R	CAAGCAGAAGACGGCATACGAGAT TTCGCGGA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTAAAGCGCATGCTCCAGAC
D811-R	CAAGCAGAAGACGGCATACGAGAT GCGCGAGA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTAAAGCGCATGCTCCAGAC
D812-R	CAAGCAGAAGACGGCATACGAGAT CTATCGCT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTAAAGCGCATGCTCCAGAC

Supplemental Table 2. Cas9 activity in each Cas9 stable pool based on reporter assay developed by [5]

Cas9 pools	cas9 reporter assay (cas9 activity)
HAL-01	48,4%
tanoue	96.6%
RCH-ACV	93,6%
NALM6	80,5%
Jurkat	34,4%
Molt4	4,7 %
TOM1	40,5%
REH	85,5%
RPMI8402	75,3%
KOPN-8	46,8%
RS4;11	80,7%
697	82,6%
molt3	88,0 %
SEM	84,0%

Supplemental Table 3. B-ALL KO CRISPR screen data analysis with MAGeCK.

Supplemental Table 4. Venn diagram data used for Figure 1C.

Supplemental Table 5. Wikipathways enriched in B-ALL CRISPR screens.

Wikipathway	Nb. genes	qValue
Electron Transport Chain (OXPHOS system in mitochondria)	11/106	$1,8X10^{-7}$
Oxidative phosphorylation	9/62	$2,3X10^{-7}$
TCA Cycle	6/18	$4,8X10^{-7}$
Amino Acid metabolism	9/91	$3,2X10^{-6}$
One carbon metabolism and related pathways	7/54	$1,2X10^{-5}$
Pentose Phosphate Metabolism	3/7	$4,4X10^{-4}$
Ferroptosis	4/40	$4,7X10^{-3}$
Cell Cycle	6/122	$6,6X10^{-3}$
PI3K-Akt Signaling Pathway	10/345	$6,8X10^{-3}$
Glutathione Metabolism	3/22	$8,5X10^{-3}$
B Cell Receptor Signaling Pathway	5/98	$1,3X10^{-2}$

Supplemental Table 6. B-ALL KO CRIPSR screen data +/- ferrostatin analysis with MAGeCK

Supplemental Table 7. B-ALL patient-derived xenografts characteristics.

PDX #	Gender, age at diagnosis (year)	Genetic characteristics	Amplification days in mice
#1	Female, 2	n/a	116
#2	Female, 4	ETV6 rearrangement, 3 RUNX1 copies, t(12;21)	168
#3	Female, 7	AML1 amplification (4-10 copies)	68
#4	Female, 4	Hyperdiploidy for chromosomes 4, 6, 8, 10 and 17.	103
#5	Female, 3	n/a	62
#6	Female, 3	n/a	141
#7	Male, 16	TCF3 (19p13) loss of a copy	43
#8	Male, 3	n/a	69
#9	Female, 4	n/a	116

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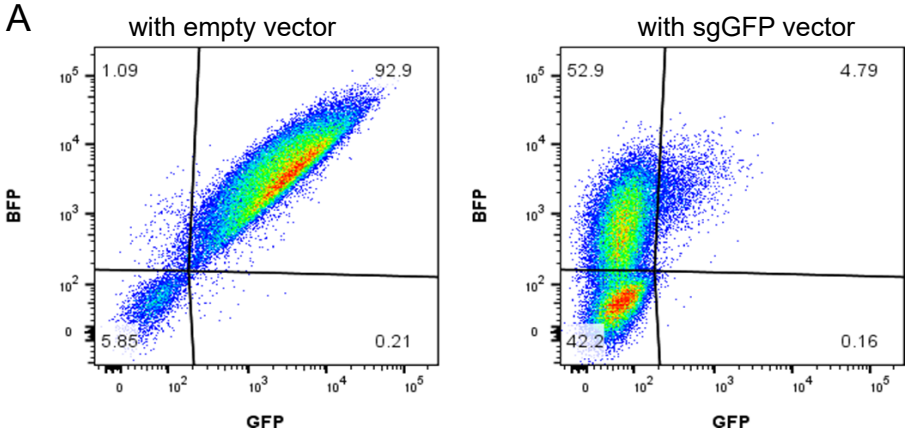

B

Screened cell lines	% Cas9 activity of pool
Nalm6	80,5%
RS4;11	80,7%
REH	85,5%
Tanoue	96,6%
RCH-ACV	93,6%
SEM	84,0%
697	82,6%

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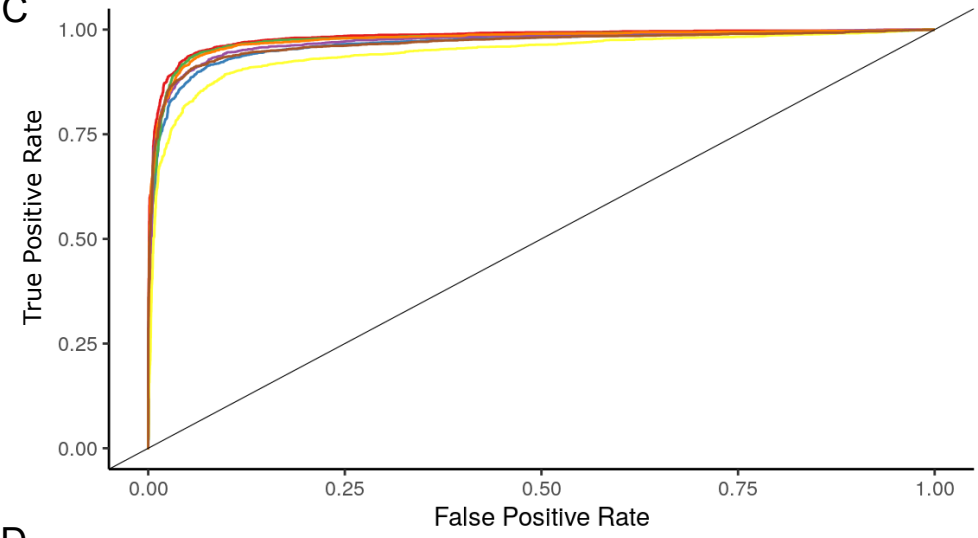

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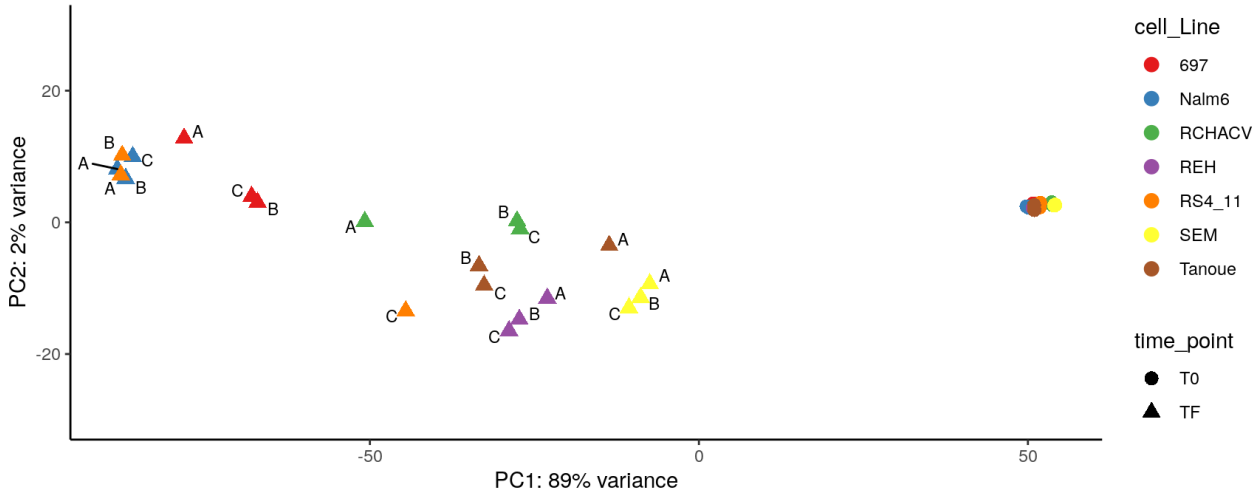

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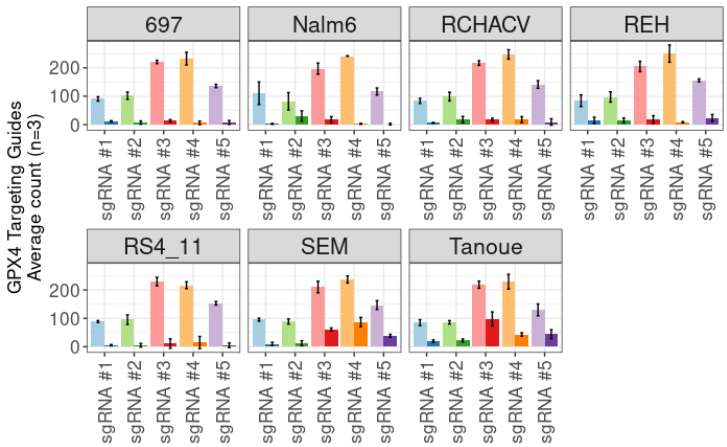
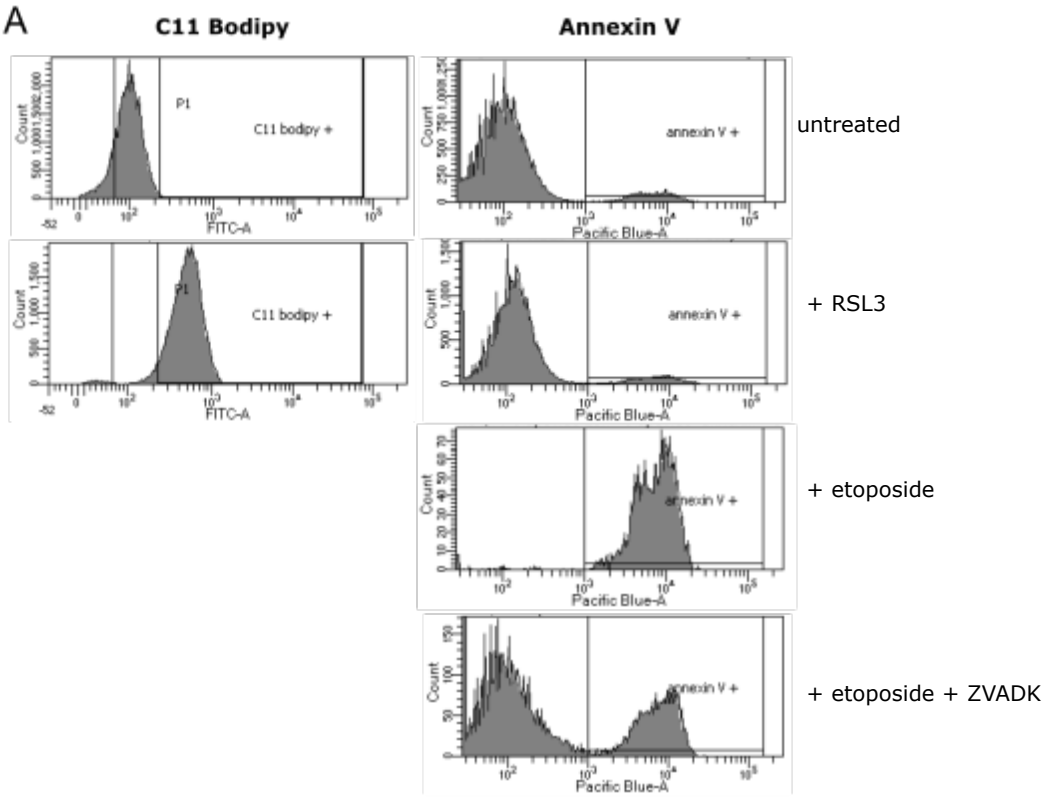
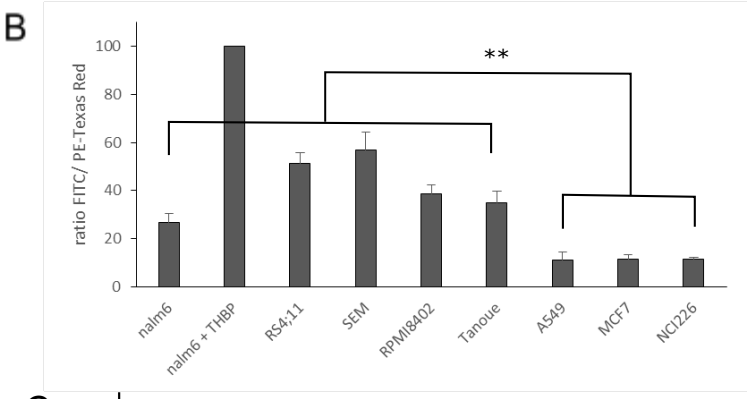
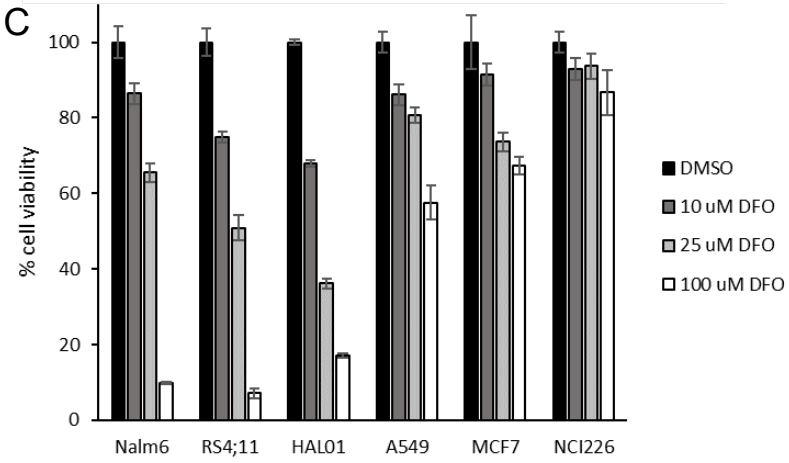


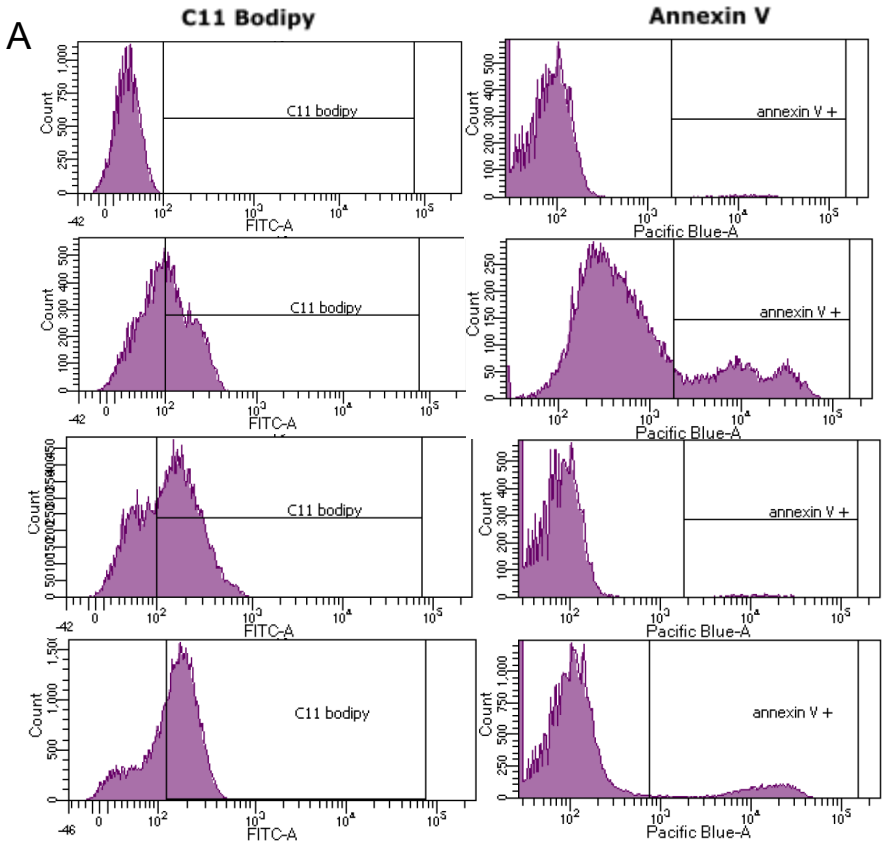
suppl. Figure 1

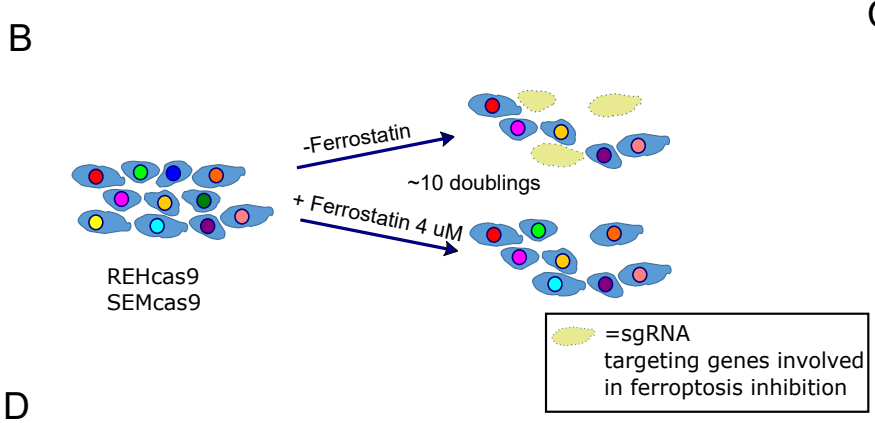


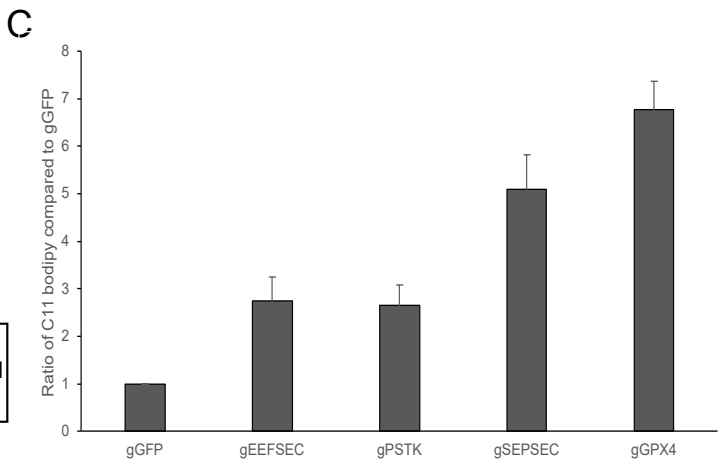


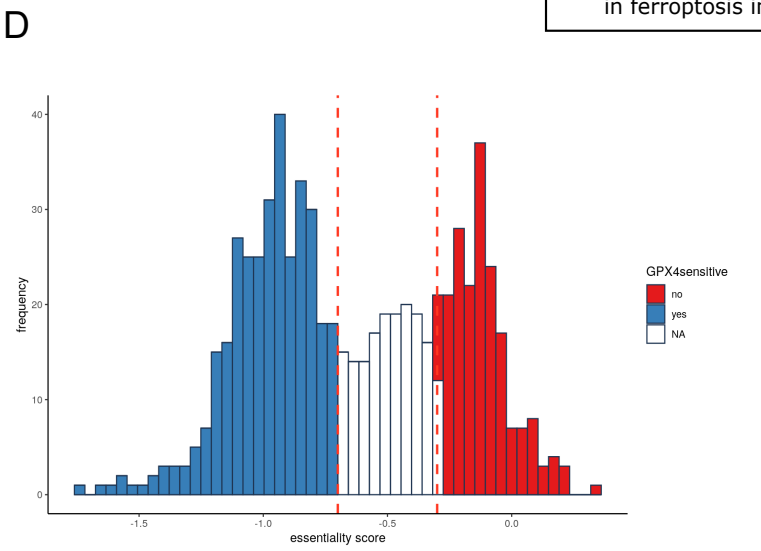


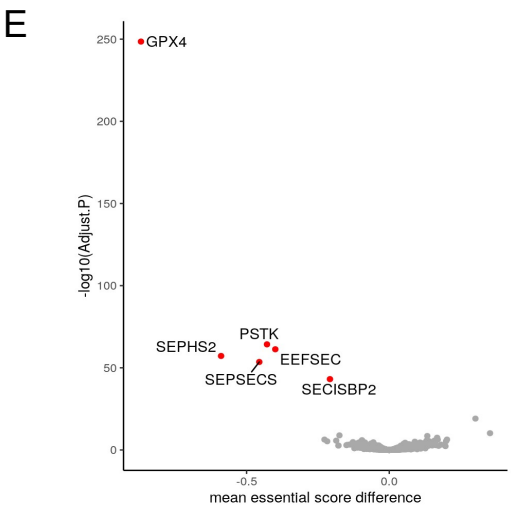
suppl. Figure 2











suppl. Figure 3

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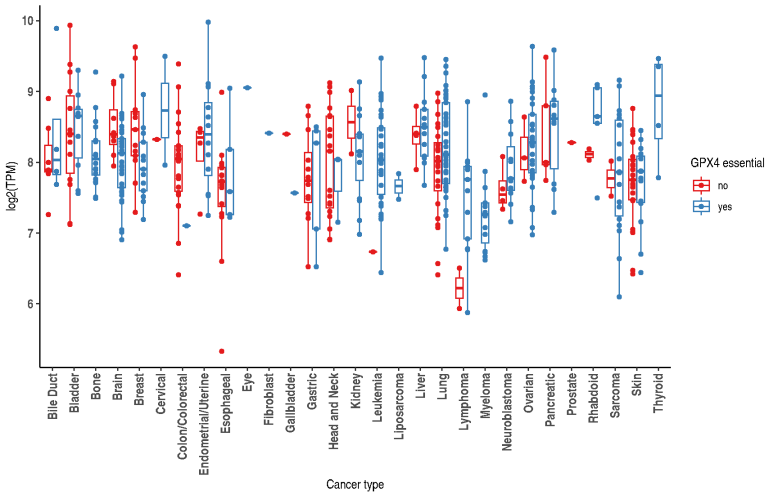

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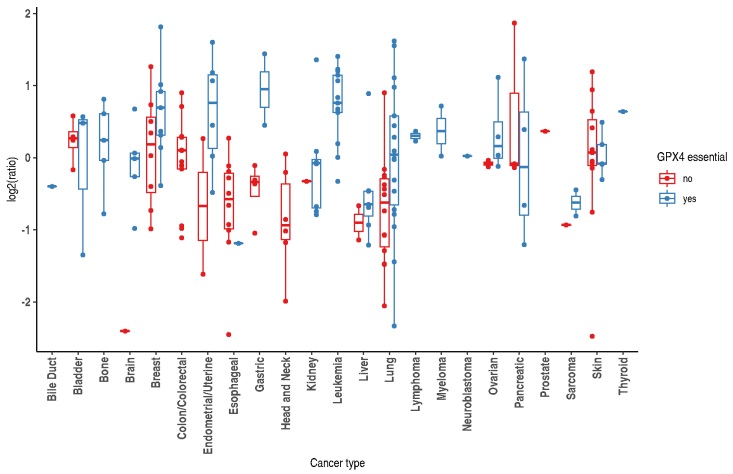

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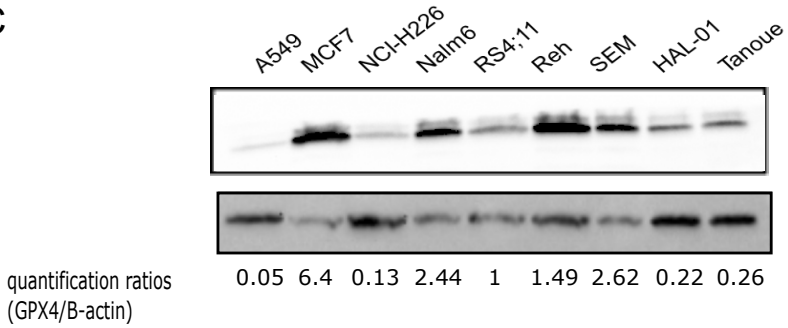

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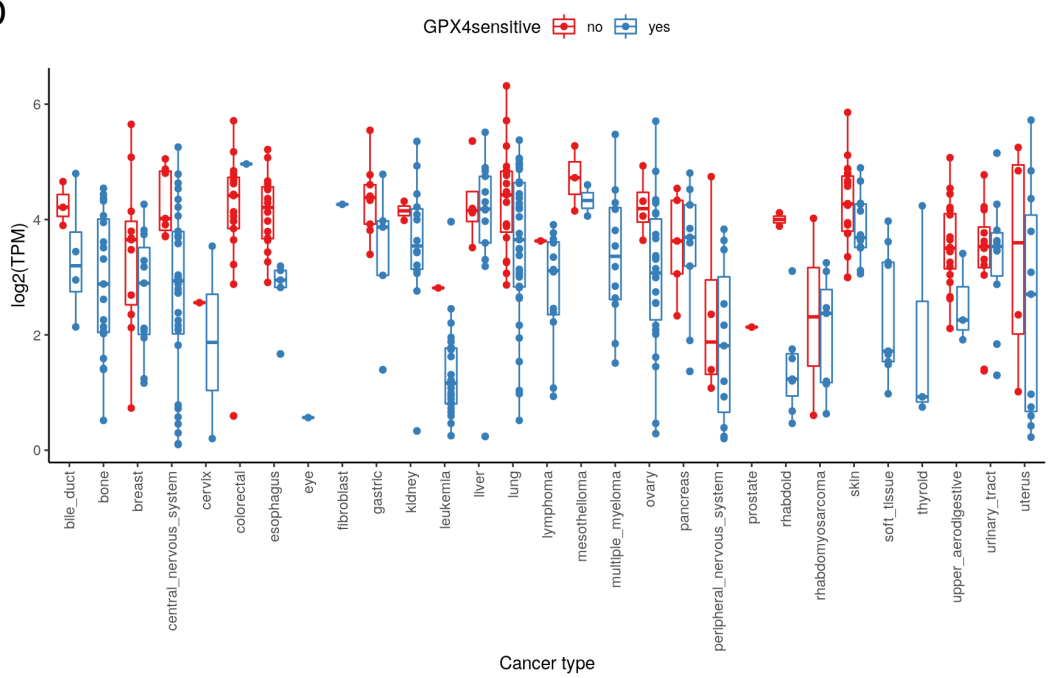

suppl. Figure 4