A20 and RBX1 regulate brentuximab vedotin sensitivity in Hodgkin Lymphoma identified by a sensitization CRISPR screen

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Supplementary Information

Supplemental Experimental Procedures

Cell line Authentication.

All the cell lines used in this study, including all the parental and the engineered cell lines (Cas9 single cell clones and BV-resistant single cell clones) have been authenticated and verified by the STR analysis (DNA fingerprinting) method described in (1). In brief, 16 pairs of primers are designed to recognize 16 different single- and multiple-locus variable numbers of tandem repeats (VNTRs) in human genomics. Then the Polymerase chain reaction (PCR) was used to amplify the fragment length polymorphism (AmpFLP) of these 16 prominent and highly polymorphic minisatellite VNTR loci. The generated banding pattern (by electrophoretic system) was compared to the original banding pattern of this cell line in our databases of all original lymphoma cell lines.

Cell Culture.

The Hodgkin lymphoma cell lines L428, KMH2, L1236 and L540 were grown in RPMI-1640 medium (Invitrogen) +10%FBS (Hyclone, Defined) +1%pen/strep (Invitrogen). All cell lines were grown to log phase at 37$_o$C, 5% CO2 when experiments were started.

Generation of the inducible Cas9 expression cell lines.

L428, KMH2, L1236 and L540 cell lines were transduced with an inducible Cas9 vector, single-cell cloned, and tested for Cas9 cutting efficiency and inducibility.

Ubiquitin regulator-focused sgRNA library.

A 10-sgRNA-per-gene CRISPR–Cas9 deletion library was designed to target ~1300 genes in the human genome focused on ubiquitin regulators, including all E3 ligases, deubiquitinating enzymes (DUB), and immune cell signaling components as controls. The library contained 100 negative control sgRNAs: non-targeting control sgRNA with no binding sites in the genome.

Brentuximab Vedotin (BV) sensitization CRISPR library screen.

The Cas9 inducible L428 cell line was used for the BV sensitization CRSIPR library screen. In brief, 40 million cells were infected with the pooled lentiviral ubiquitin regulators-focused sgRNA library at a multiplicity of infection of 0.3 and selected with 2 μg/ml puromycin for 72-96 hours, commencing 48 hours after transduction. GFP percentage was examined to ensure the completion of selection. Doxycycline was then added to induce sgRNAs expression. Six days after Dox induction, library transduced cells were treated with an IC50 dosage of BV for 6 days or left untreated. After 6 days of a treatment period, the BV treated cells and untreated cells were collected for genomic DNA extraction (Qiagen). sgRNA sequences were amplified by two rounds of PCR, with the second-round primers containing adaptors for Illumina sequencing. The resulting libraries were sequenced with single end read with dual-index 75 bp on a HiSeq2500. The sequence reads were trimmed to remove the constant portion of the sgRNA sequences with a Fastx clipper, then mapped to the reference sgRNA library with bowtie2. After filtering to remove multi-aligning reads, the read counts were computed for each sgRNA.

Lentiviral production and transduction.

Lentivirus was produced by triple transfection of HEK-293FT cells with a lentiviral transfer vector, and the packaging plasmids psPAX2 and pMD2.G. Transfection was performed using TransIT-293 Transfection Reagent as recommended by the manufacturer. The viral supernatant was

collected 48 hours following transfection, filtered through a 0.45 μm filter, concentrated by Lenti-X concentrator (TaKaRa), and added to target cells.

Lentiviral construct used for ectopic expression.

A20, ABCB1, IKKβ WT and IKKβ S177/181E cDNA were cloned into the inducible lentiviral expression vector pCW57.1. A20 cDNA was obtained by RT PCR. ABCB1 cDNA was obtained from Addgene (#10957). IKKβ WT and IKKβ S177/181E cDNAs were described as in (2).

CRISPR mediated gene disruption.

sgRNA oligonucleotides (IDT) were phosphorylated and annealed and cloned into lentiviral expression vectors: pLKO.1-puro/GFP U6 sgRNA BfuAI stuffer for inducible sgRNA expression. For CRISPR mediated gene disruption, the inducible Cas9 expression cell lines were transduced with the lentiviral sgRNA expression vector. Knockout clones were identified by flow cytometry analysis or immunoblot. *The sgRNA sequences used in this study are listed as following:*

NAME	**Sequence**
CTRL	GTAGCGAACGTGTCCGGCGT
A20#1	TGGATGATCTCCCGAAACTG
A20#2	GCCCCACATGTACTGAGAAG
CD30#1	AAGTCACGCAGGCTGTACAG
CD30#2	TTGCAGATTCCAGACCCAGG
RBX1#1	TCCATTGGACAACAGAGAGT
RBX1#2	AGTGGAATGCAGTAGCCCTC
ABCB1#1	CTGGAGAGATCCTCACCAAG
ABCB1#2	TTTATAGTAGGATTTACACG

Cell viability MTS assay.

Cells were seeded in triplicate 96-well tissue culture plates at 8000 cells/well in 100 μL of media under various treatment conditions. 50 μL of fresh medium (drug containing) were added to each well at day 2. After 4 days of treatment, 20 μL of CellTiter Aqueous one solution cell proliferation reagent (Promega) were added to each well and mixed for 5 minutes. The absorbance was read on

the Spark multimode microplate reader (Tecan) after 2-4 h incubation. The background was subtracted using a media only control. All experiments were performed in triplicate.

Establishment of BV-resistant single cell clones of HL.

To generate BV-resistant single cell clones in HL, the KMH2 and L428 cells were first treated at the IC90 concentration of BV for one week then recovered in BV-free medium for another week. This BV IC90 – BV free cycle was repeated for another 3 times (in a total of a 7-week period) to generate BV-resistant pools. These BV-resistant pools were confirmed by MTS assay and cell counting assay before the next single-cell cloning step. To make the BV-resistant single cell clones, the BV BV-resistant pools were single-cell isolated using a 96-well plate (0.5 cells/well) with a BV (IC90) containing medium. Expanded clones were collected once they reached a population of 10_6 cells, and were retested for drug resistance before any further studies.

Antibody and reagents.

The antibodies used in this study were purchased as follows: anti-ABCB1(D3H1Q) and anti-IKKβ (D30C6) from Cell Signaling Technologies; anti-RBX1 (E-11), anti-TNFAIP3 (A-12), anti-β-actin (C4,) and anti-GDPDH (0411) from Santa Cruz Biotechnology; anti-CD30 (Ber-H2) from Sigma-Aldrich. Secondary horseradish peroxidase (HRP)-conjugated antibodies were obtained from Invitrogen. FASC antibodies anti-CD83 (HB15e), anti-CD30 (BY88), anti-ABCB1 (UIC2) from BioLegend. Isotype-matched control antibodies were obtained from the same company as each experimental antibody.

Brentuximab Vedotin (BV) was obtained from Fox Chase Cancer Center Pharmacy. HeFi-1 was described as in (3). The ABCB1 inhibitor cyclosporine (CsA) and NAE inhibitor Pevonedistat were obtained from Selleckchem. The IKKβ inhibitor MLN120B and Monomethyl auristatin E (MMAE) was purchased from MedChemExpress. Tissue culture grade DMSO vehicle control was

obtained from Sigma-Aldrich.

Western blotting.

Cell pellets were lysed in the modified RIPA buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1% NP40, 0.25% deoxycholic acid, 1mM EDTA) supplemented with a protease inhibitor tablet and a phosphatase inhibitor tablet (Roche), 1mM DTT, 1mM Na_3VaO_4, 1mM PMSF. Protein concentrations were measured by BCA Protein Assay Kit (Thermo Scientific). Total proteins were separated on 4% to 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes.

Real-Time Quantitative PCR.

Total RNA was extracted from Hodgkin cell lines with RNeasy mini kit (Qiagen). Total RNA was reverse transcribed with random primers and multiscribe reverse transcriptase (Invitrogen). ABI7500 instrument (Applied Biosystems) was performed using the resultant cDNA. Gene expression was normalized to the expression of GAPDH for all samples. The following quantitative PCR primers were used:

	FW	RV
ABCB1	TTGCTGCTTACATTCAGGTTTCA	AGCCTATCTCCTGTCGCATTA
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG

IL-6 primer was obtained from ABI (Hs00985639-m1).

Flow cytometry.

To examine the surface markers CD30, ABCB1, and CD83 expression, cells were pelleted and washed one time with ice cold FASC buffer (1% FBS in PBS) and incubated with APC anti-CD30 and APC anti-CD83 antibodies (Biolegend) at 4°C for at least 30 min. Stained cells were washed twice, resuspended in 200 μL FASC buffer and then analyzed using a flow cytometer.

Intracellular flow of p-p65.

To examine the intracellular p-p65 expression, cells were pelleted and washed one time with ice cold FASC buffer (0.5% BSA in PBS) and then fixed with 2% paraformaldehyde (PFA) at room temperature for 10 min. Cells were pelleted, washed twice with FASC buffer containing 0.03% Saponin and PhosStop (Sigma-Aldrich). Then, cells were incubated with anti-phospho-NF-κB p65 antibody (93H1, Cell Signaling Technologies) at 4°C for at least 30 min. Stained cells were

Data access.

The high-throughput RNA sequencing data from this study have been submitted to the NCBI Sequence Read Archive (SRA) under accession number: SUB6466040

Gene set enrichment analysis.

The expression dataset was analyzed with Gene Set Enrichment Analysis (GSEA) (http://software.broadinstitute.org/gsea/index.jsp) using the Molecular Signatures Database, MSigDB (http://www.broad.mit.edu/gsea/msigdb/index.jsp). GSEA identified significant sets of genes that were over-represented at the top or bottom of the ranked set of genes that were differentially expressed between comparison groups. For our analyses, we used the MSigDB hallmark gene sets and C2 KEGG curated gene sets. Gene sets with FDR p-value < 0.05 were considered significant.

Tumor Model and Therapy Study.

For L428 xenograft tumor model, the human Hodgkin lymphoma L428 cells were subcutaneously (s.c.) injected ($1.0x10^7$ cells in 200μL PBS) into the flanks of female nonobese diabetic/severe combined IL2Rgamma$_{null}$ immunodeficient (NSG) mice (8 weeks old, NCI-Frederick, Frederick, MD). Tumor growth was monitored by measuring tumor size in two orthogonal dimensions. The tumor volume was calculated by using the formula 1⁄2 (long dimension) x (short dimension)^2. When the average tumor volume reached 200 mm^3 the therapies were started. Mice were divided

into 6 groups, with comparable tumor volume among groups. MLN120B was dissolved in vehicle (0.5% methylcellulose) at a final concentration of 6 mg/mL. Group 1 received vehicle alone by oral administration as a control. Group 2 received MLN20B (50 mg/kg, daily) orally. Group 3 received HeFi-1 (100 μg/mouse, weekly) by intravenous injection (i.v.). Group 4 received BV (0.4 mg/kg, every 4 days) by i.v. Group 5 received a combination of MLN120B with HeFi-1 at the same doses and dosing schedules as those in the MLN120B and HeFi-1 groups. Group 6 received a combination of MLN120B with BV at the same doses and dosing schedules as those in the MLN120B and BV groups. Tumor volume was assessed by caliper measurement and calculated. Mice were euthanized (killed) once the tumor reached 2 cm (in any dimension). The tumor volumes and number of dead mice were recorded to calculate the survival rate during the growth of xenograft tumor. All animal experiments were approved by the National Cancer Institute Animal Care and Use Committee (NCI ACUC) and were performed in accordance with NCI ACUC guidelines.

For KMH2 xenograft tumor model, the human Hodgkin lymphoma KMH2 cells were subcutaneously (s.c.) injected ($2.0x10^7$ cells in 200μL PBS) into the flanks of female NSG mice (8 weeks old, Fox Chase Cancer Center). Tumor growth was monitored by measuring tumor size in two orthogonal dimensions. The tumor volume was calculated by using the formula $1/2$(long dimension) x $(\text{short dimension})^2$. When the average tumor volume reached 200-400 mm^3, the therapies were started. Mice were divided into 5 groups, with comparable tumor volumes among groups. Group 1 received vehicle alone by oral administration as a control. Group 2 received BV (2.5 mg/kg, every 4 days, i.v.). Group 3 received BV (5 mg/kg, every 4 days, i.v.). Group 4 received a combination of MLN120B (50 mg/kg, daily, oral) with BV (2.5 mg/kg, every 4 days, i.v.). Group 5 received a combination of MLN120B (50 mg/kg, daily, oral) with BV (5 mg/kg,

every 4 days, i.v.). Treatment continued until the tumor disappeared or tumor (original or relapsed) reached 2cm (in any dimension) and the animal was euthanized. The tumors were resected for further qPCR analysis after the relapsed tumor appeared again. All animals were maintained in the LAF at Fox Chase Cancer Center and all experiments were performed in accordance with procedures approved by the Fox Chase Cancer Center Animal Care and Use Committee.

IL-6 ELISA.

Human IL-6 was measured using ELISA kit from BioLegend, according to manufacturer instructions.

Statistical analysis.

All experiments have been repeated and results reproduced. Where possible, error bars or P values are shown to indicate statistical significance. In some figures, error bars are not visible due to their short heights relative to the size of the symbols. $P < 0.05$ was considered statistically significant.

Supplementary Figure Legends

Supplementary Figure 1: A20 promotes BV and MMAE sensitivity in HL cell lines.

A. Overview of the BV sensitization CRISPR library screen results in the L428 cell line. Shown are results of all sgRNA log2 counts in untreated (BV-) and treated (BV+) population. The red dashed lines indicate log2(BV- counts / BV+ counts) = ±1. **B.** HL cell line L428 was transduced with CD30 or Ctrl sgRNAs, selected and expression induced, then treated with BV at the indicated concentrations for 4 days. Viability was measured by the MTS assay and normalized to PBS-treated cells. *Error bars denote SEM of triplicates. P were calculated comparing Ctrl and CD30 sgRNA transduced groups; ** indicates P < 0.01.* Lysates of each selected and induced line was also analyzed by immunoblotting for the indicated proteins (right). **C.** Lysates of each selected and induced line in **Fig. 1D** were analyzed by immunoblotting for the indicated proteins to ensure the sgRNAs depletion effects. **D.** Lysates of each selected and induced line in **Fig. 1H** and **Fig. 1I** were analyzed by immunoblotting for the indicated proteins.

Supplementary Figure 2: NF-κB activity and ABC transporters expression regulate BV sensitivity.

A. IKKβ cDNA expression in **Fig. 2D**. The HL cell line L428 was transduced with cDNA of IKKβ WT, IKKβ S177/181E or empty control, selected and expression induced. Lysates of each selected and induced lines were analyzed by immunoblotting for the indicated proteins. **B.** KEGG pathway emrichment analysis of all the significant enriched ($p<0.05$) in four BV-resistant clones (KMH2 and L428). ABC transporters pathway (red) was the only common pathway in all the clones. The dashed line indicates $p=0.05$.

Supplementary Figure 3: ABCB1 expression is essential for BV sensitivity.

A. ABCB1 sgRNAs depletion effect. L428 was transduced with ABCB1 or Ctrl sgRNAs, selected and expression induced. Lysates of each selected and induced lines were analyzed by immunoblotting for the indicated proteins. **B.** ABCB1 cDNA expression. L428 was transduced with ABCB1 cDNA or empty control, selected and expression induced. Lysates of each selected and induced lines were analyzed by immunoblotting for the indicated proteins. **C.** Viability of HL cell lines L1236 and L540 after treatment (4 days) with the indicated concentrations of BV,

ABCB1 inhibitor CsA, or both. Data are normalized to PBS-treated cells. Error bars denote SEM of triplicates.

Supplementary Figure 4: NF-κB and A20 regulate ABCB1 expression in HL cells.

A. HL cell line L428 was transduced with cDNA of IKKβ WT, IKKβ S177/181E or empty control, selected and expression induced. ABCB1 expressions were measured by immunoblot. **B.** The L428 line was transduced with A20 or Ctrl sgRNAs, selected and expression induced. ABCB1 expressions were measured by immunoblot. **C.** The L428 line was transduced with cDNA of IKKβ WT, IKKβ S177/181E or empty control, selected and expression induced, then treated with the indicated concentrations of MMAE, ABCB1 inhibitor CsA, or both for 4 days. Viability was measured by MTS assay and normalized to DMSO-treated cells. *Error bars denote SEM of triplicates. ** indicates $P < 0.01$; n.s indicates no statistical difference.*

Supplementary Figure 5: RBX1 regulates BV sensitivity.

A. HL cell lines KMH2, L1236 and L540 were transduced with RBX1 or Ctrl sgRNAs, selected and expression induced, then treated with BV at the indicated concentrations for 4 days. Viability was measured by MTS assay and normalized to PBS-treated cells. **B.** Lysates of each selected and induced line in **Fig. 5G** and **panel A** were analyzed by immunoblotting for the indicated proteins to ensure the RBX1 sgRNAs depletion effects. **C.** The L428 line was transduced with RBX1 or Ctrl sgRNAs along with GFP. Surface CD30 expression in uninfected (GFP-) cells and sgRNA infected (GFP+) cells was measured by flow cytometry. One of the representative experiments is shown. **D.** The L428 line was transduced with RBX1 or Ctrl sgRNAs, selected and expression induced. Surface CD83 and intracellular p-p65 expression were measured by flow cytometry. The relative CD83 and p-p65 MFI were normalized to the Ctrl sgRNA transduced cells. **E.** HL cell lines KMH2, L1236 and L540 were transduced with RBX1 or Ctrl sgRNAs, selected and expression induced, then treated with MMAE at the indicated concentrations for 4 days. Viability was measured by MTS assay and normalized to DMSO-treated cells. **F.** The L428 line was transduced with RBX1 or Ctrl sgRNAs, selected and expression induced. ABCB1 expressions were measured by immunoblot. *For all the panels, Error bars denote SEM of triplicates. * indicates $P < 0.05$; ** indicates $P < 0.01$.*

Supplementary Figure 6: CRLs/SCF E3 ligase activity is required for ABCB1 expression in HL cells.

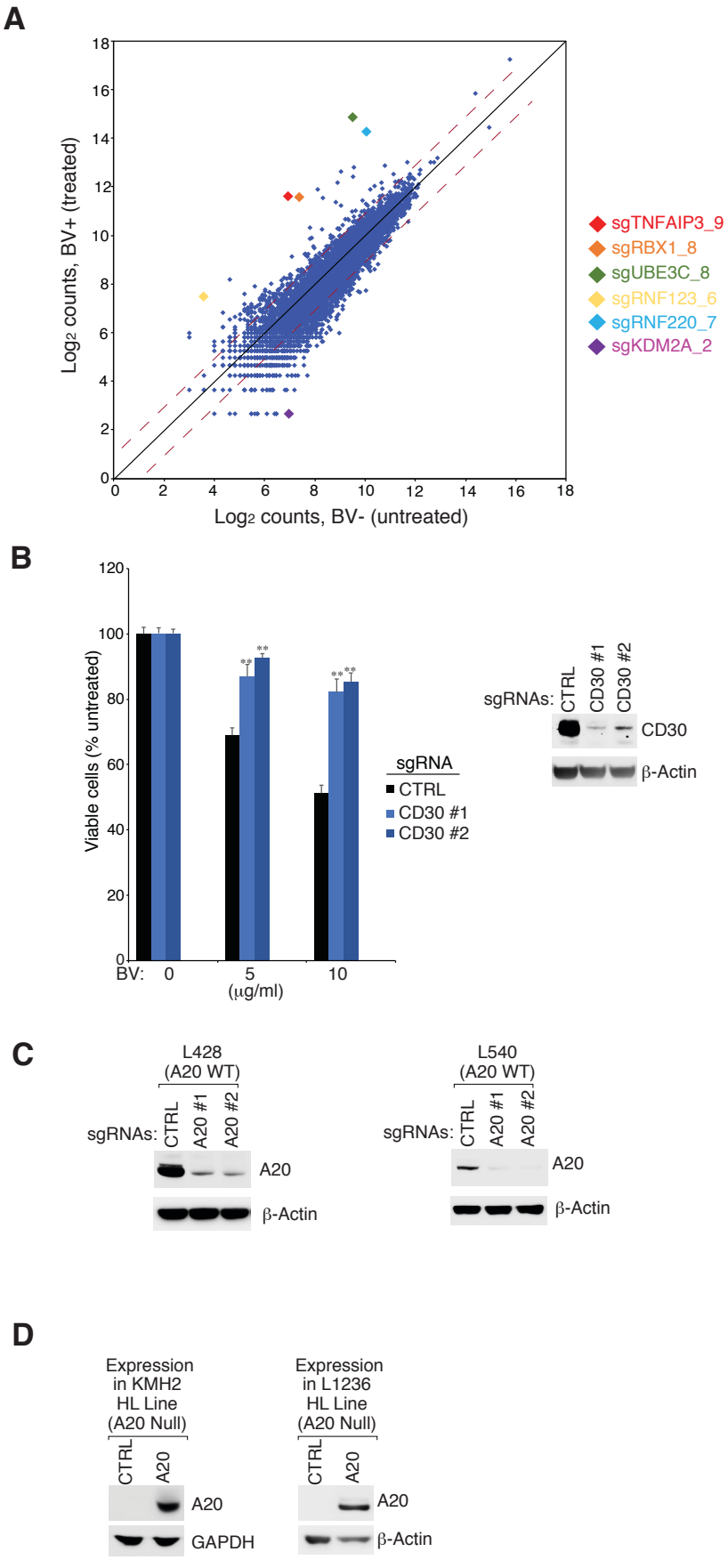
A. HL cell lines KMH2 and L428 were treated with NAE inhibitor Pevonedistat at the indicated concentrations for 24 hours. Lysates were analyzed by immunoblotting for the indicated proteins.

B. Viability of HL cell lines KMH2 and L428 after treatment (4 days) with the indicated concentrations of BV, NAE inhibitor Pevonedistat, or both. Data are normalized to PBS-treated cells. Error bars denote SEM of triplicates.

Reference

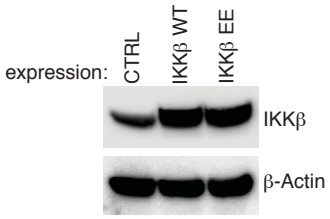
1. Dirks WG, Drexler HG. Authentication of scientific human cell lines: easy-to-use DNA fingerprinting. Methods Mol Biol **2005**;290:35-50.
2. Ceribelli M, Kelly PN, Shaffer AL, Wright GW, Xiao W, Yang Y*, et al.* Blockade of oncogenic IkappaB kinase activity in diffuse large B-cell lymphoma by bromodomain and extraterminal domain protein inhibitors. Proc Natl Acad Sci U S A **2014**;111(31):11365-70 doi 10.1073/pnas.1411701111.
3. Zhang M, Yao Z, Zhang Z, Garmestani K, Goldman CK, Ravetch JV*, et al.* Effective therapy for a murine model of human anaplastic large-cell lymphoma with the anti-CD30 monoclonal antibody, HeFi-1, does not require activating Fc receptors. Blood **2006**;108(2):705-10 doi 10.1182/blood-2005-11-4607.

Supplemental Fig. 1



Supplemental Fig. 2

A



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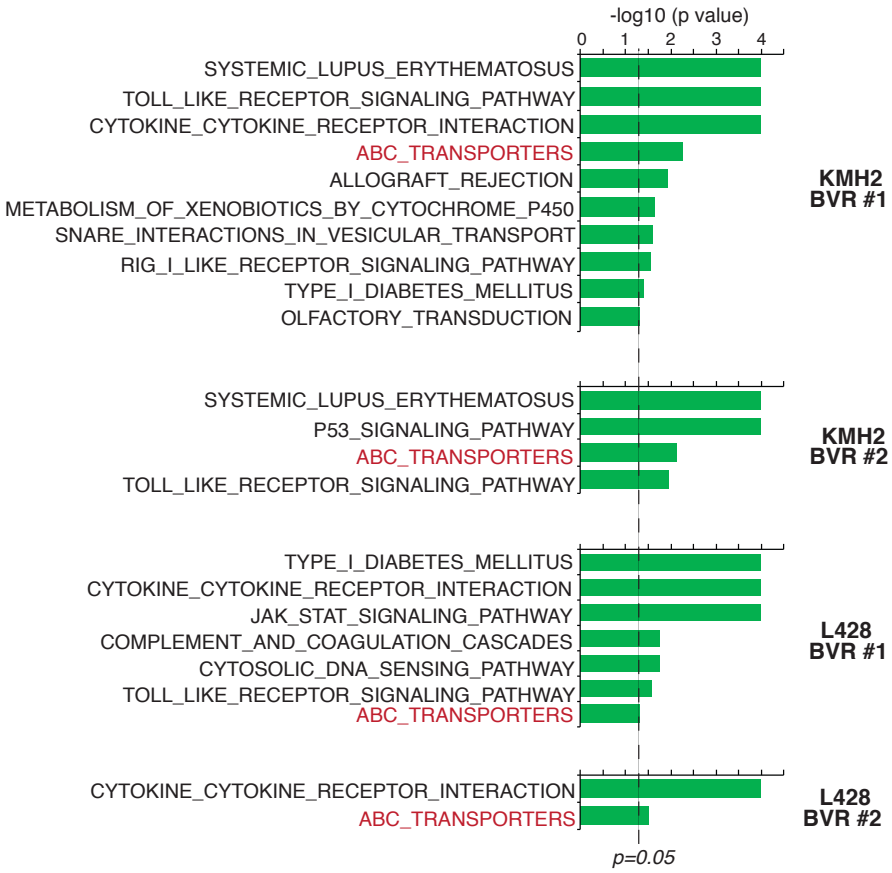

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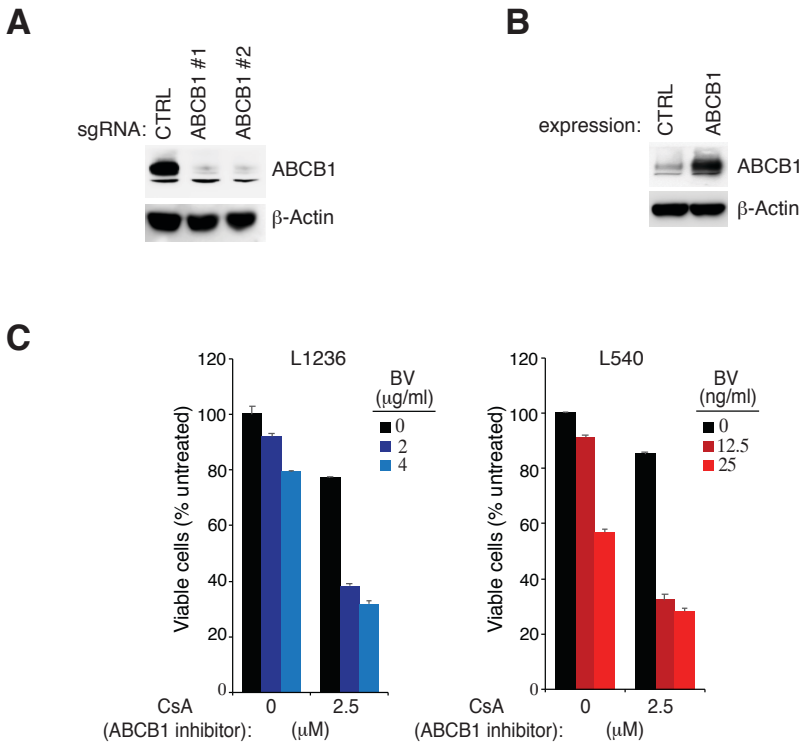

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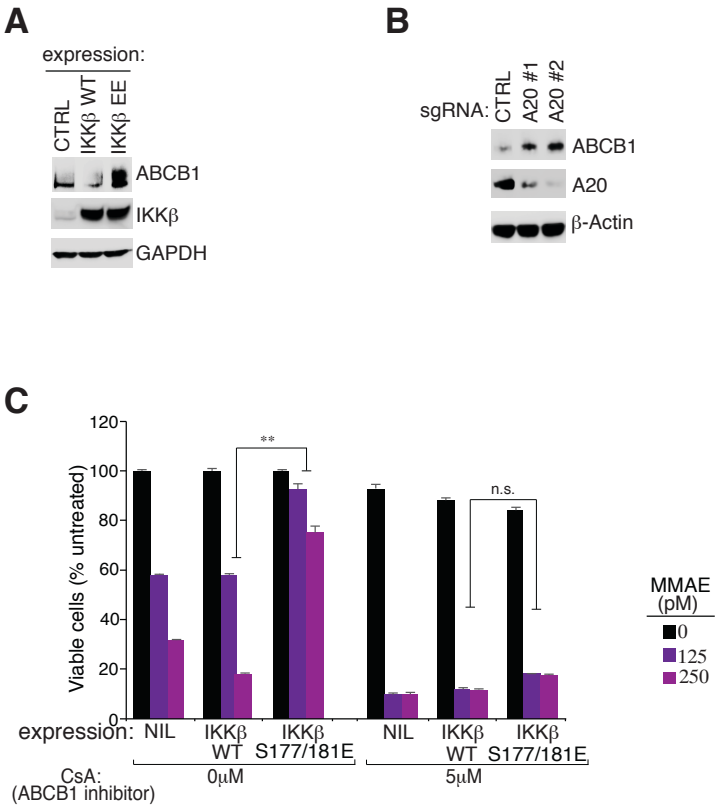

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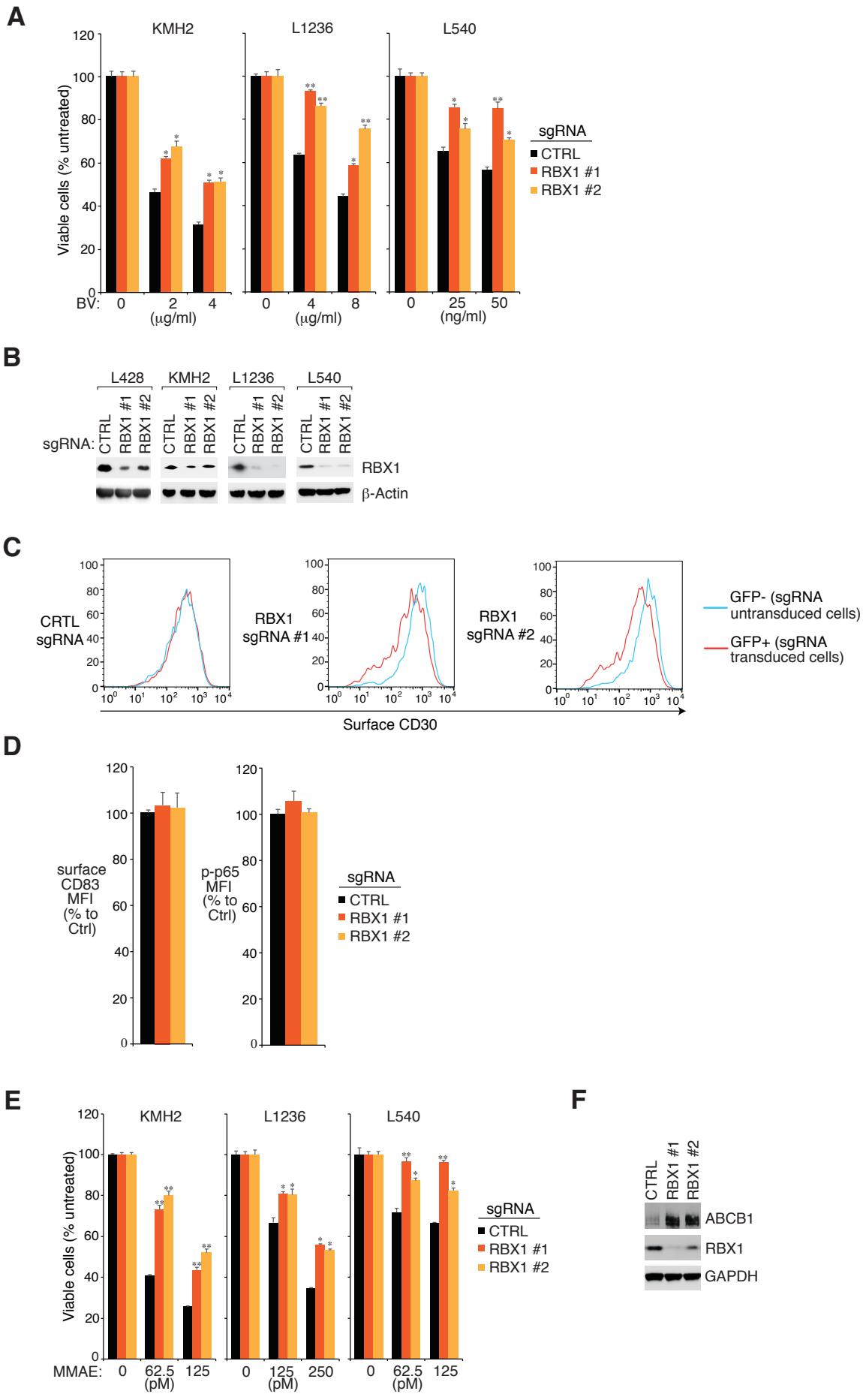
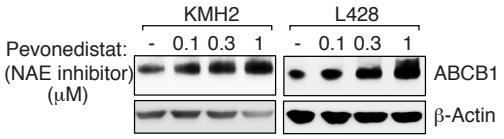


Supplemental Fig. 6

A



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