PRC2 Inhibitors Overcome Glucocorticoid Resistance Driven by NSD2 Mutation in Pediatric Acute Lymphoblastic Leukemia

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Supplementary Methods

DNA Methylation

DNA methylation analysis of the *BCL2L11* GR-binding enhancer region was performed as previously described {Jing, 2018 #23}. Genomic DNA from isogenic ALL cell lines (RCH-ACV, SEM, RPMI-8402 and CEM) was extracted using the Quick-DNA Miniprep Plus Kit (Zymo Research, #D4069) and bisulfite converted using the EZ DNA Methylation-Gold™ Kit (Zymo Research, #D5005). The CT converted DNA was amplified using Hot Start ZymoTaq™ DNA Polymerase (Zymo Research, #E2001) with the following PCR conditions: 95°C for 10 min; 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min for 40 cycles; 72°C for 1 min. PCR products were separated by agarose gel electrophoresis and purified with Zymoclean Gel DNA Recovery Kit (Zymo Research, #D4007) and cloned using the TOPO TA Cloning Kit for sequencing (Invitrogen, #K457501). Ten cloned vectors of each cell line were sent for Sanger sequencing in Genewiz (NJ, USA). The primers were shown in **Supplementary Table S7.**

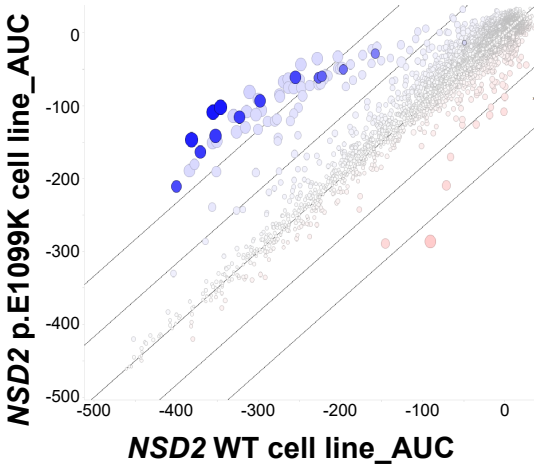
Knockdown of EZH2 in ALL Cell Line with *NSD2* p.E1099K Mutation

HEK 293T cells were transfected with a control scrambled shRNA and directed against EZH2 shRNAs (TRCN0000040074 and TRCN0000010474, Millipore-Sigma) (gift of Wendy Beguelin, Weill Cornell Medical College) and lentiviral packaging plasmids (psPAX2 RRID: Addgene #12260 and VSV-G RRID: Addgene #12259; both gifts of Didier Trono) using the FuGENE 6 Kit

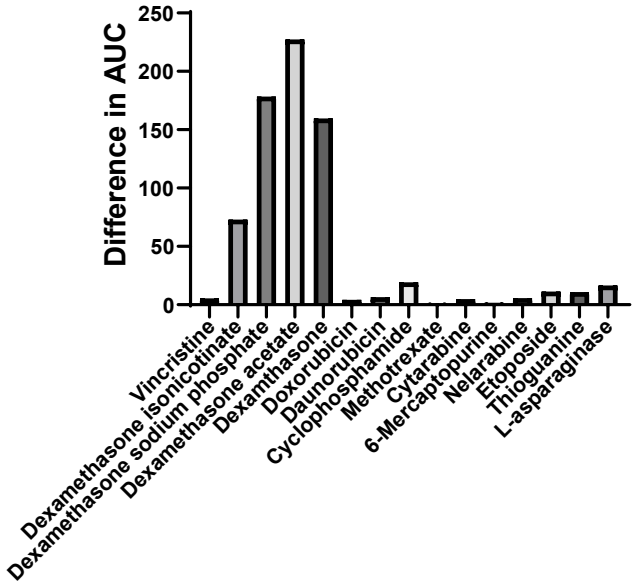
(Promega, #E2312). ALL cell line RCH-ACV with *NSD2* p.E1099K mutation were infected by packaged shRNA lentivirus. The cells were selected using Puromycin (Gibco, #A11138-03) and used for further experiments including immunoblotting, CellTiter-Glo and apoptosis analysis.

Supplementary Figures

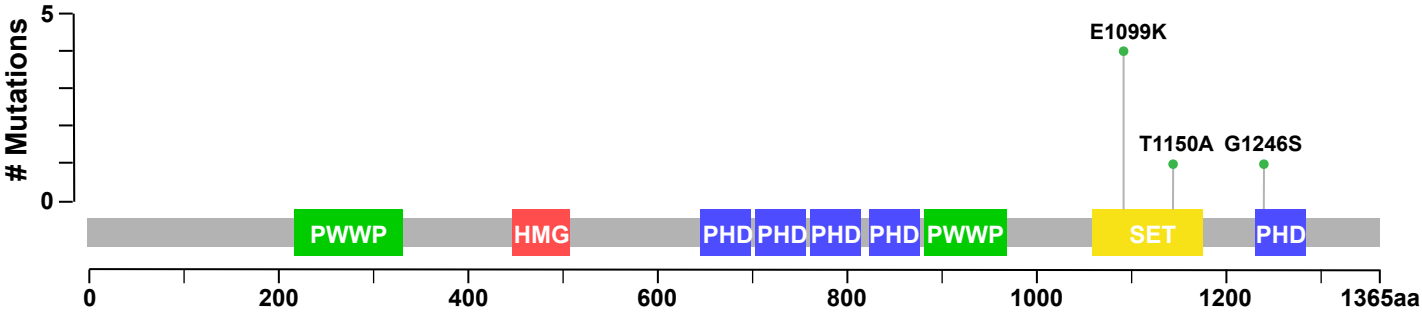
A


NSD2 p.E1099K cell line_AUC
NSD2 WT cell line_AUC
0
-100
-200
-300
-400
-500

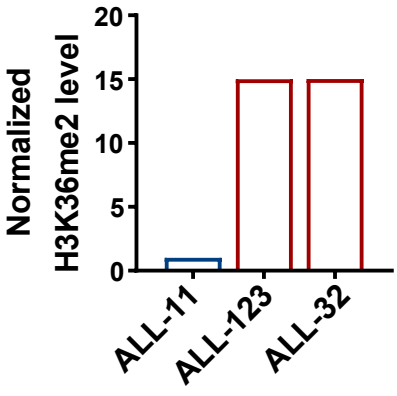
B

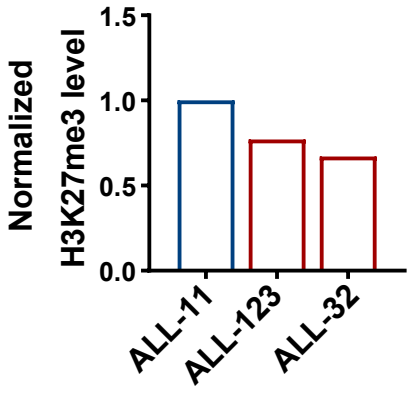

Difference in AUC
250
200
150
100
50
0
Vincristine
Dexamethasone isonicotinate
Dexamethasone sodium phosphate
Dexamethasone acetate
Dexanthasone
Doxorubicin
Daunorubicin
Cyclophosphamide
Methotrexate
Cytarabine
6-Mercaptopurine
Nelarabine
Etoposide
Thioguanine
L-asparaginase

C

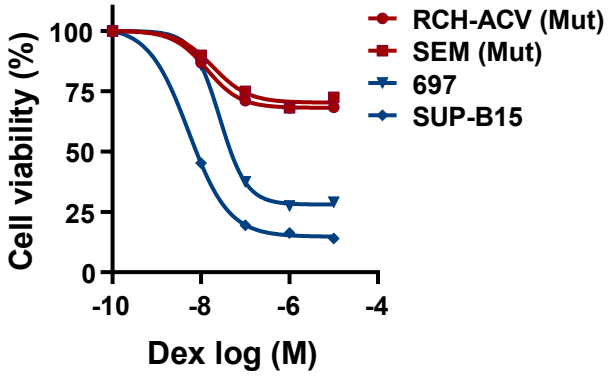

Mutations
5
0
E1099K
T1150A
G1246S
PWWP
HMG
PHD
PHD
PHD
PHD
PWWP
SET
PHD
0
200
400
600
800
1000
1200
1365aa

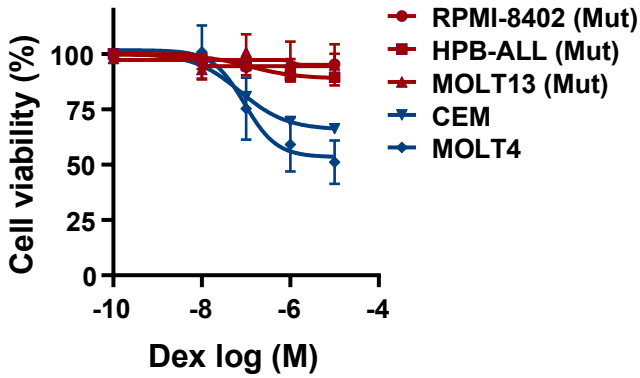
D


Normalized H3K36me2 level
20
15
10
5
0
ALL-11
ALL-123
ALL-32


Normalized H3K27me3 level
1.5
1.0
0.5
0.0
ALL-11
ALL-123
ALL-32

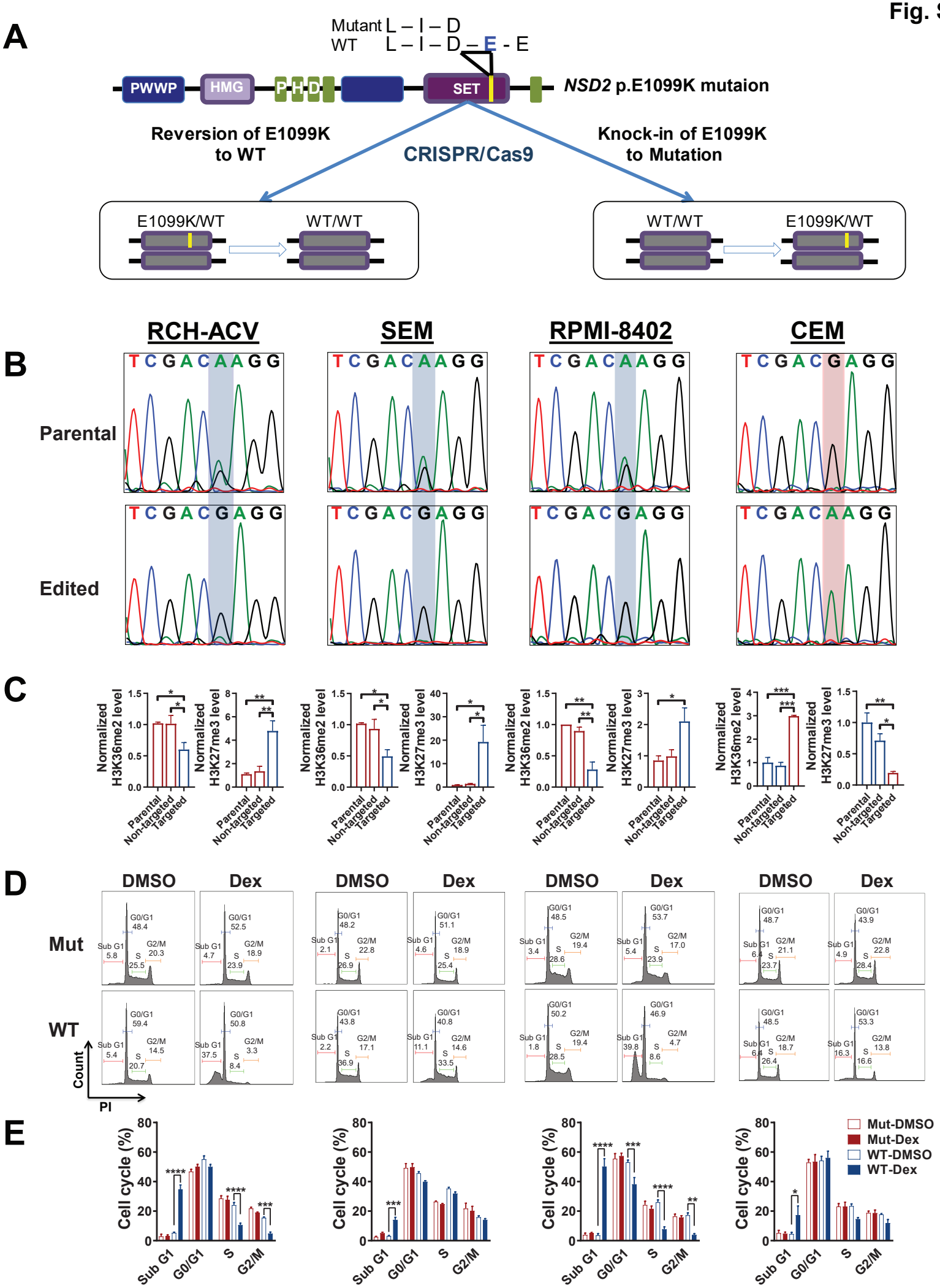
E


Cell viability (%)
100
75
50
25
0
-10
-8
-6
-4
Dex log (M)
RCH-ACV (Mut)
SEM (Mut)
697
SUP-B15


Cell viability (%)
100
75
50
25
0
-10
-8
-6
-4
Dex log (M)
RPMI-8402 (Mut)
HPB-ALL (Mut)
MOLT13 (Mut)
CEM
MOLT4

Supplementary Figure S1. ALL cell lines and PDX-ALL cells with *NSD2* p.E1099K mutation are resistant to glucocorticoids. **A,** Blue dots indicate glucocorticoids among drugs that preferentially inhibit growth of *NSD2* WT cells. **B,** Inhibition difference in AUC for conventional chemotherapeutic agents used for ALL in isogenic RCH-ACV cell lines. **C,** Distribution of mutations in the *NSD2* gene in a cohort of pediatric ALL. **D**, Quantification of immunoblotting analysis of H3K36me2 and H3K27me3 in *NSD2* p.E1099K (Red) and WT PDX cells (Blue). **E,** Viability of *NSD2* p.E1099K (Red) or *NSD2* WT (Blue) ALL cell lines after treatment with increasing doses of dexamethasone for 48 hours compared to DMSO control as determined by CellTiter-Glo assay (biological triplicate). Blue, *NSD2* WT; Red, *NSD2* p.E1099K; Dex, Dexamethasone.

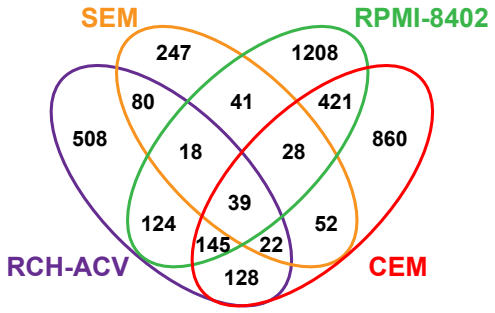
Fig. S2


A
Mutant L – I – D
WT L – I – D – E - E
PWWP
HMG
P-H-D
SET
NSD2 p.E1099K mutaion
Reversion of E1099K to WT
CRISPR/Cas9
Knock-in of E1099K to Mutation
E1099K/WT
WT/WT
WT/WT
E1099K/WT
B
RCH-ACV
SEM
RPMI-8402
CEM
Parental
Edited
T C G A C A A G G
T C G A C G A G G
C
Normalized H3K36me2 level
Normalized H3K27me3 level
Parental
Non-targeted
Targeted
D
DMSO
Dex
Mut
WT
Count
PI
Sub G1
G0/G1
S
G2/M
E
Cell cycle (%)
Sub G1
G0/G1
S
G2/M
Mut-DMSO
Mut-Dex
WT-DMSO
WT-Dex

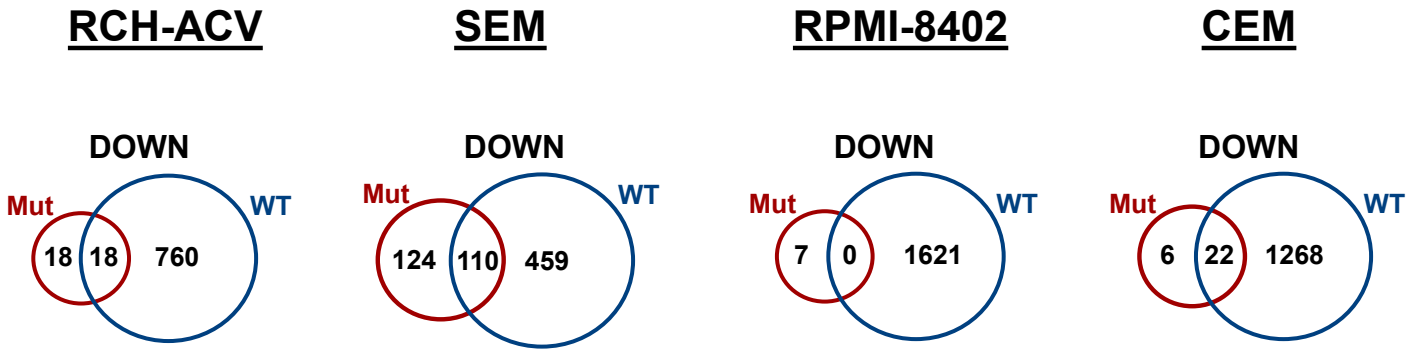
Supplementary Figure S2. CRISPR/Cas9 gene editing and cell cycle of isogenic ALL cell lines with *NSD2* p.E1099K mutation in response to glucocorticoids. A, Schematic strategy of CRISPR/Cas9 editing in ALL cell lines with or without *NSD2* p.E1099K mutation. **B,** Sequencing of clones showed three ALL cell lines with *NSD2* p.E1099K mutation (RCH-ACV, SEM and RPMI-8402) were reverted to *NSD2* WT cells (blue box) while the *NSD2* p.E1099K mutation was edited into CEM (red box) using the CRISPR/Cas9 gene editing technology. **C**, Immunoblotting quantification of H3K36me2 and H3K27me3 in isogenic *NSD2* mutant and WT ALL cell lines. **D and E,** Representative plots and quantification of the cell cycle profiles of isogenic ALL cell lines as determined by propidium iodide (PI) staining and flow cytometry after 24 hours of dexamethasone (1μM) treatment, compared to DMSO controls (biological triplicates). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, *NSD2* WT; Mut, *NSD2* p.E1099K; Dex, Dexamethasone.

A

Genes only UP in WT with Dex

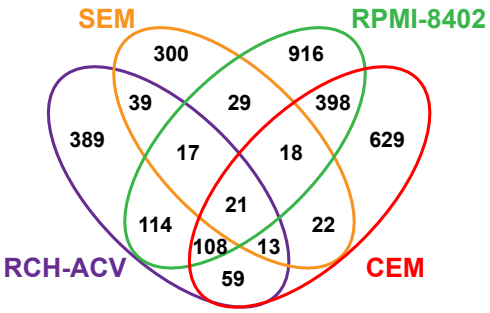


B

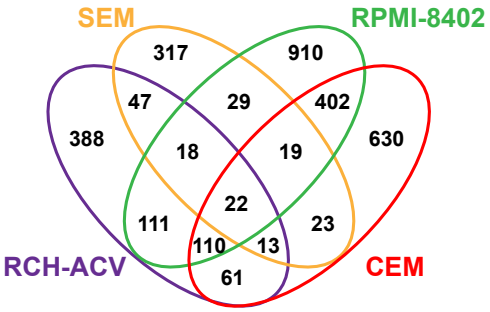


C

Genes only DOWN in WT with Dex

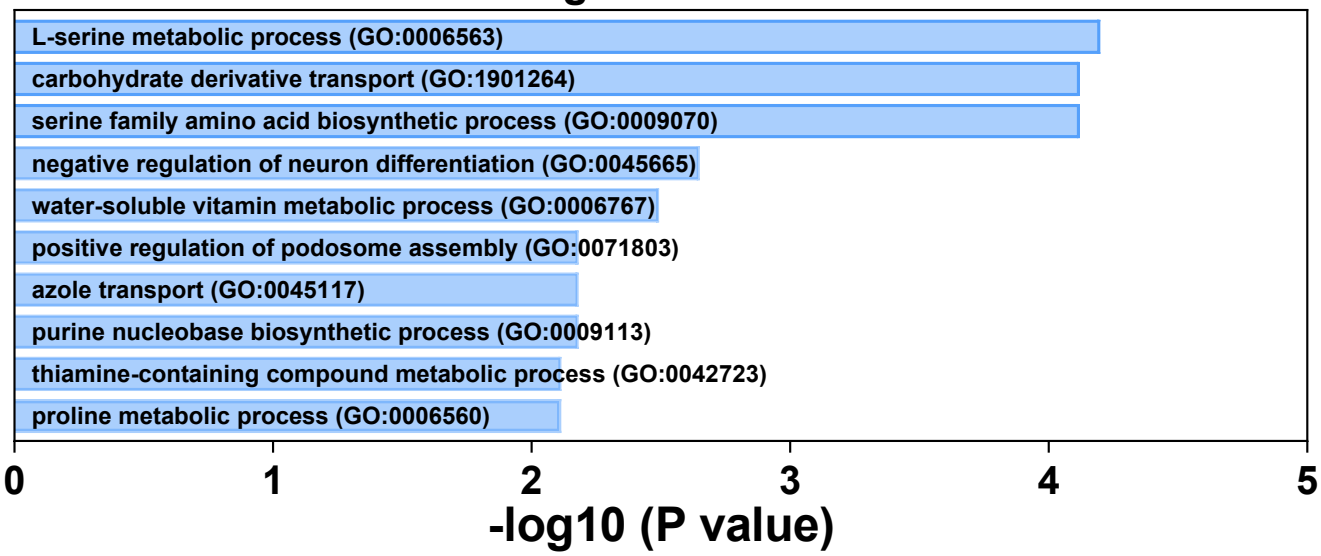


Genes DOWN in WT and Genes DOWN in both WT and Mut (WT/Mut: 1.5 fold) with Dex

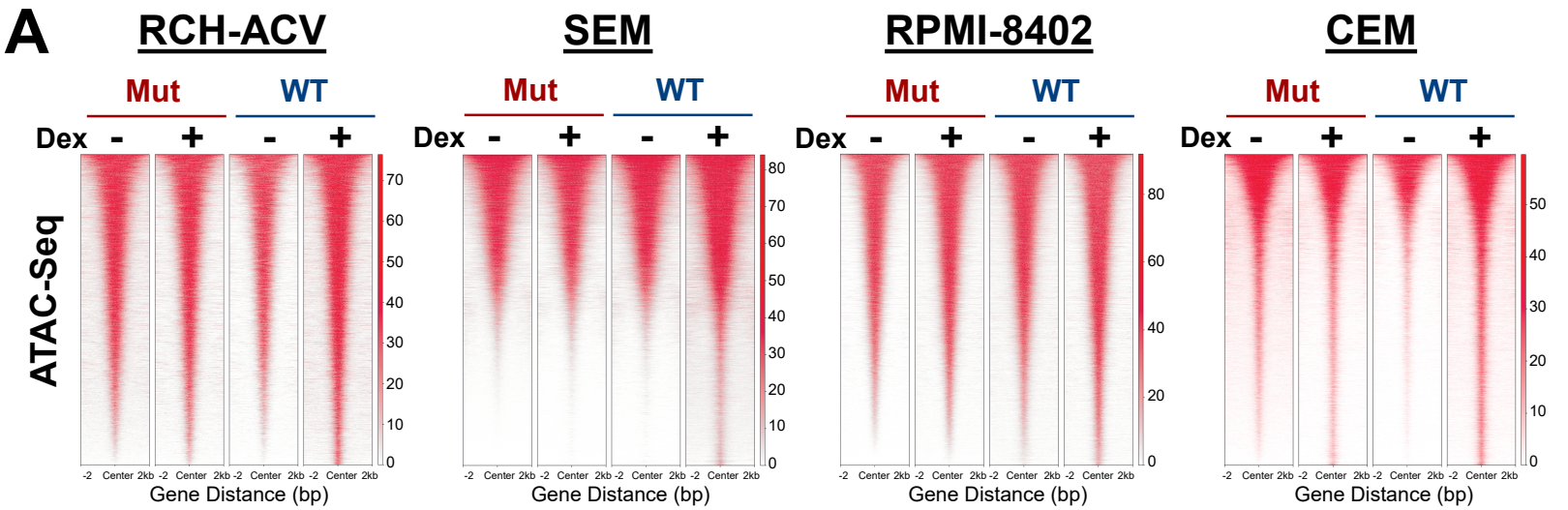


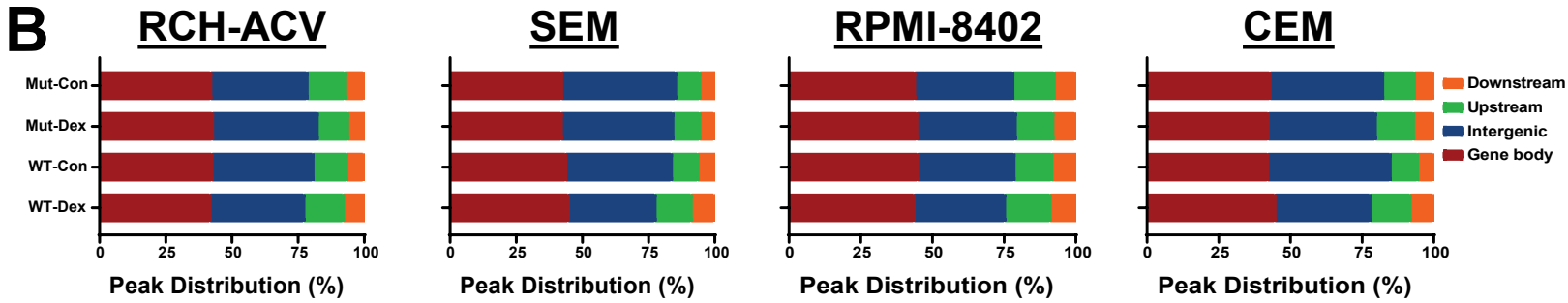
D

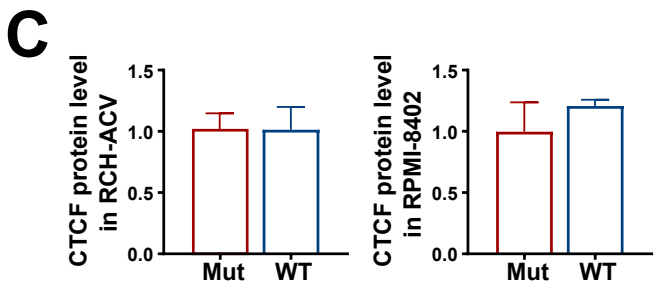
GO Biological Process 2018

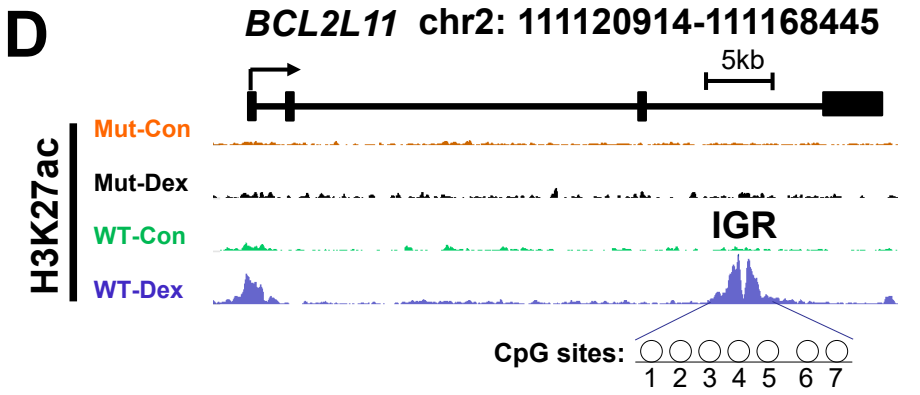


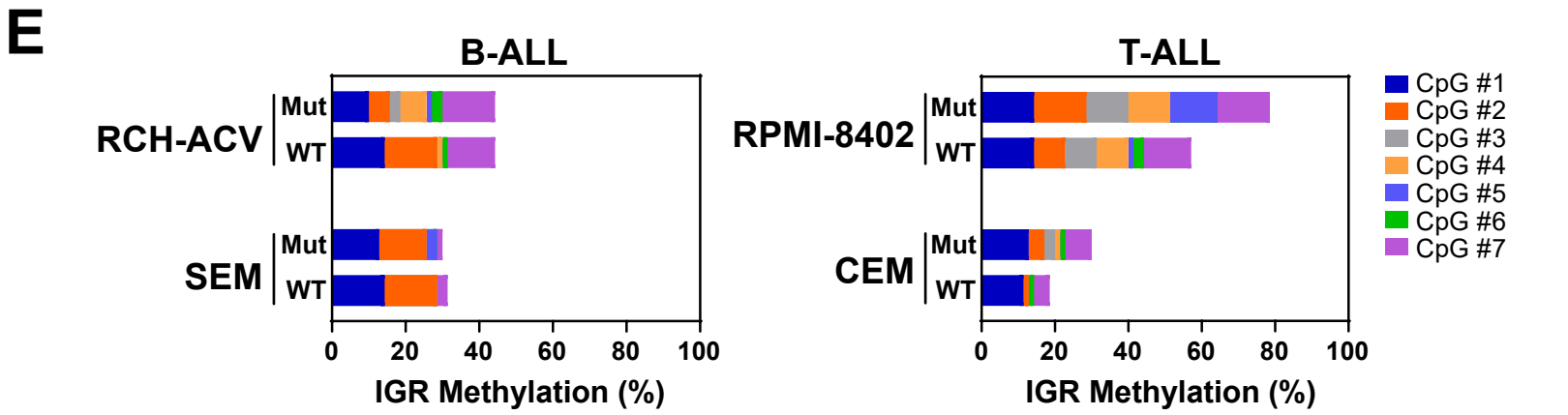
Supplementary Figure S3. *NSD2* p.E1099K mutation blocks the gene transcriptome in response to glucocorticoid in ALL cell lines. **A,** Venn diagram of the overlap of genes upregulated only in *NSD2* WT cells after 24 hours of treatment with dexamethasone (1μM) as determined by RNA-Seq analysis (biological triplicate) of isogenic ALL cell lines (RCH-ACV, SEM, RPMI-8402, CEM). **B,** Overlap analysis of genes downregulated in response to 1μM dexamethasone in *NSD2* WT and mutant isogenic cells as determined by RNA-Seq. **C,** Left- Venn diagram of the overlap of genes downregulated only in *NSD2* WT RCH-ACV, SEM, RPMI-8402, CEM cells in response to dexamethasone. Right- Venn diagram of the overlap of genes downregulated only in *NSD2* WT cells plus genes downregulated in both WT and mutant cells but decreased $\geq$ 1.5-fold more in WT cells in response to dexamethasone. **D,** Enriched pathways of downregulated genes in RCH-ACV, SEM, RPMI-8402 and CEM cell lines in response to dexamethasone determined by Enrichr. WT, *NSD2* WT; Mut, *NSD2* p.E1099K; UP, upregulated genes; DOWN, downregulated genes.


A
RCH-ACV
SEM
RPMI-8402
CEM
Mut
WT
Dex - + - +
ATAC-Seq
Gene Distance (bp)

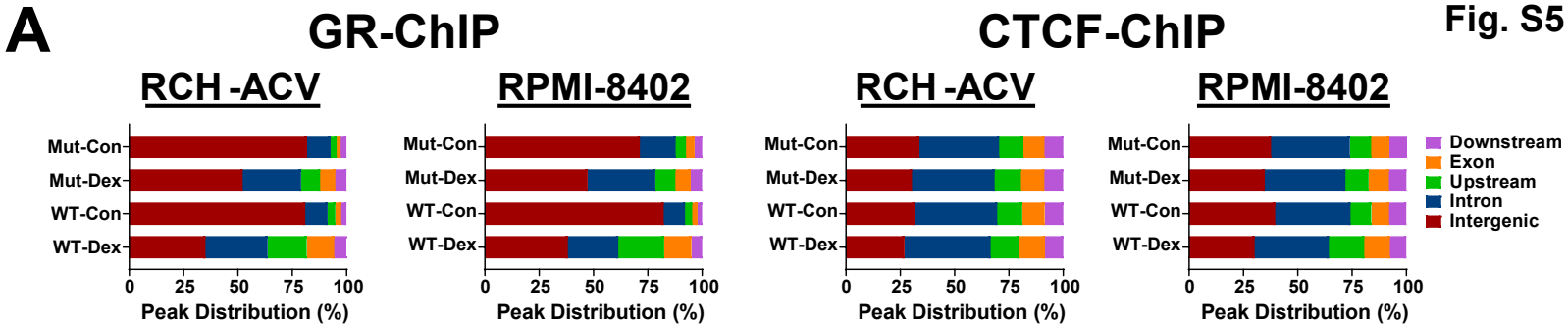

B
RCH-ACV
SEM
RPMI-8402
CEM
Mut-Con
Mut-Dex
WT-Con
WT-Dex
Downstream
Upstream
Intergenic
Gene body
Peak Distribution (%)

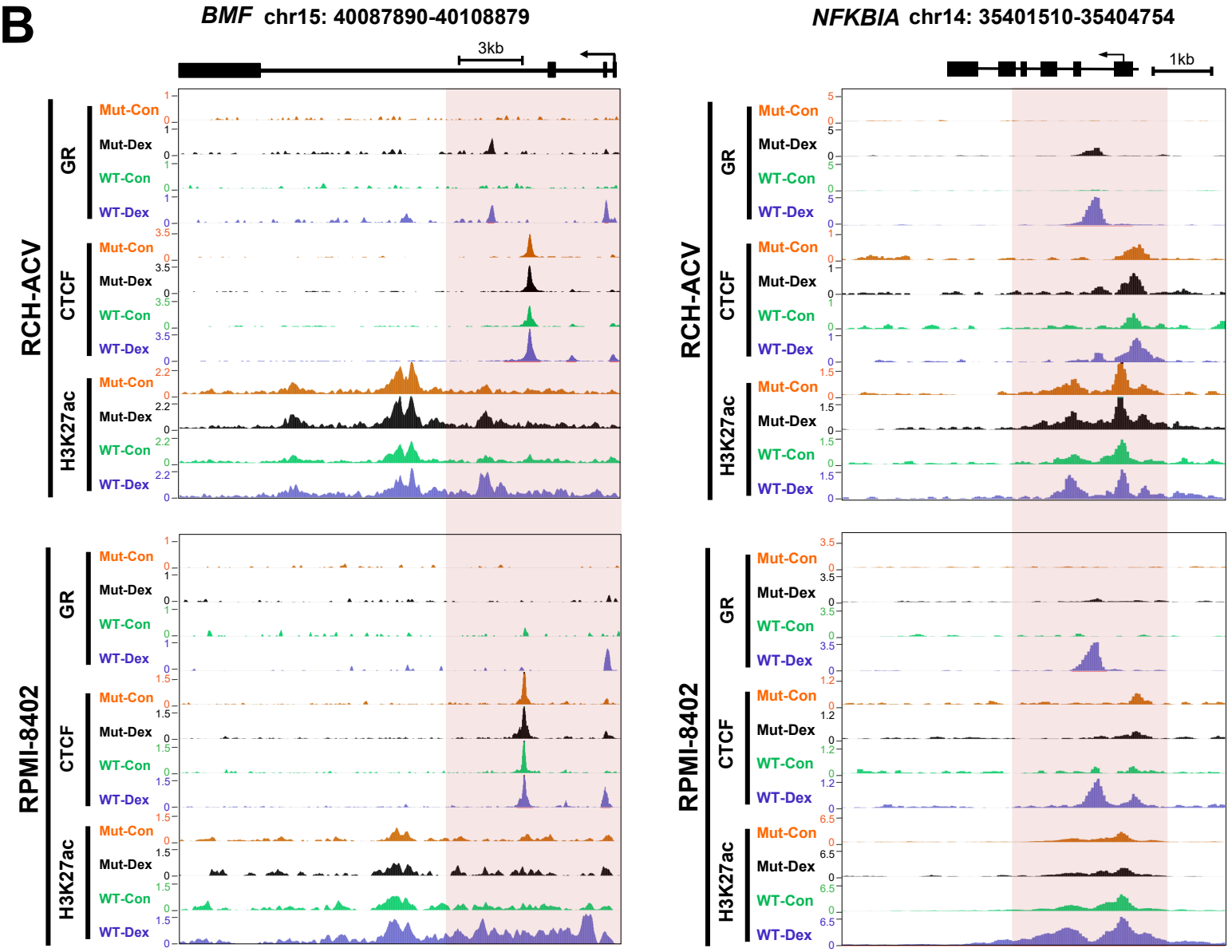

C
CTCF protein level in RCH-ACV
CTCF protein level in RPMI-8402
Mut
WT

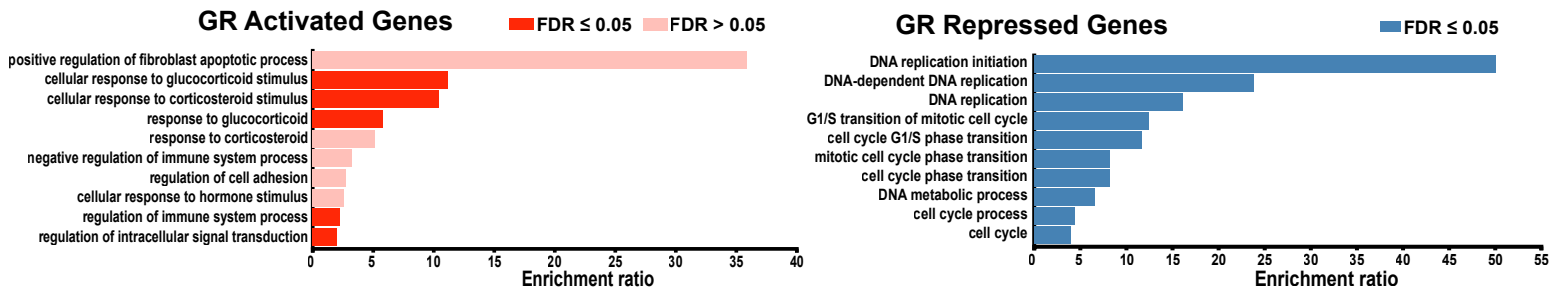

D
BCL2L11 chr2: 111120914-111168445
5kb
H3K27ac
Mut-Con
Mut-Dex
WT-Con
WT-Dex
IGR
CpG sites:
1 2 3 4 5 6 7


E
B-ALL
T-ALL
RCH-ACV
SEM
RPMI-8402
CEM
Mut
WT
CpG #1
CpG #2
CpG #3
CpG #4
CpG #5
CpG #6
CpG #7
IGR Methylation (%)

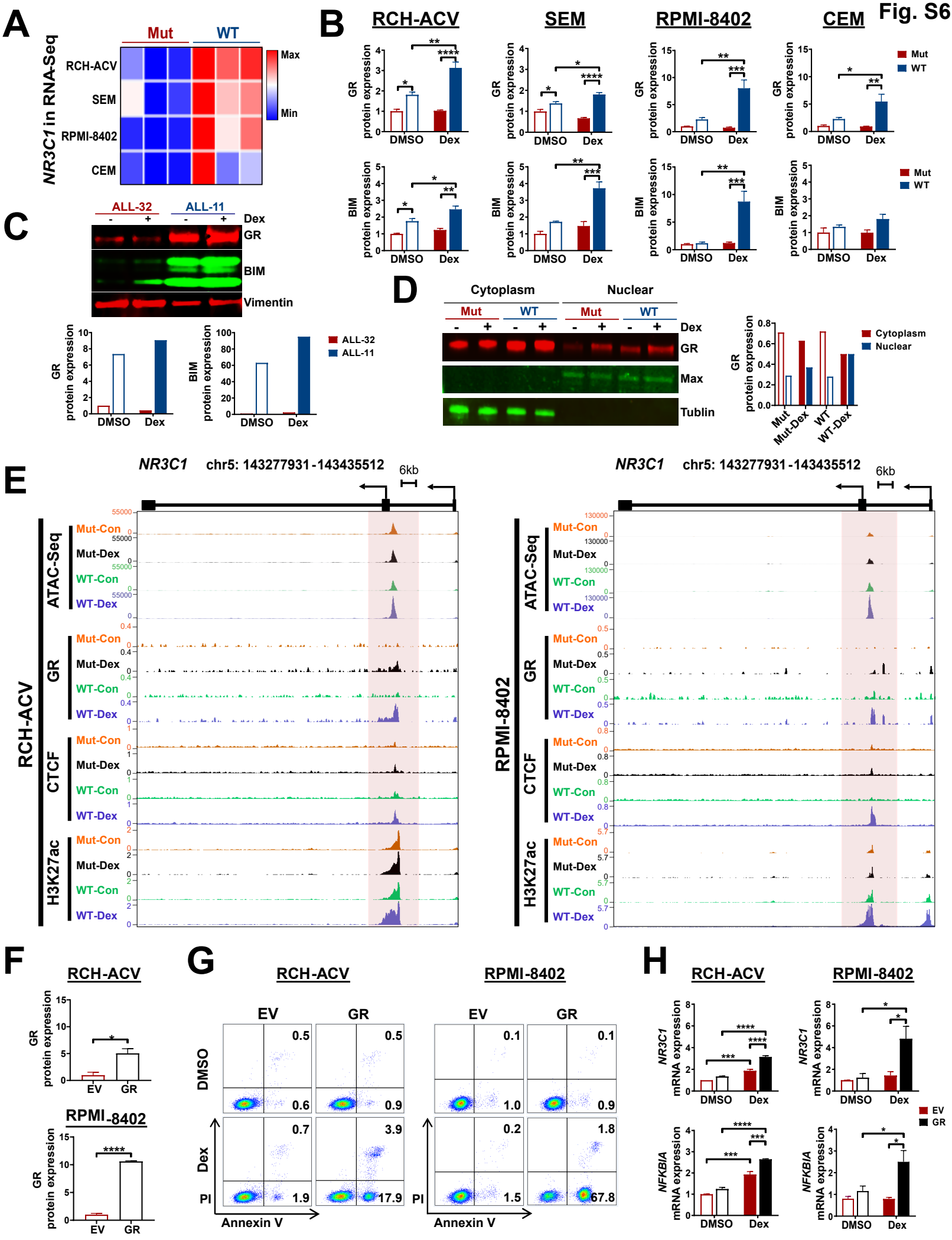
Supplementary Figure S4. *NSD2* p.E1099K mutation alters chromatin accessibility in response to glucocorticoid in ALL cell lines. A, ATAC-Seq heatmaps of differential peaks (up) in isogenic ALL cell lines (RCH-ACV, SEM, RPMI-8402 and CEM) in response to dexamethasone. **B,** Distribution of differential peaks (up) in the ATAC-Seq of isogenic ALL cell lines (RCH-ACV, SEM, RPMI-8402 and CEM) in response to dexamethasone. **C**, Immunoblotting quantification of CTCF protein levels in isogenic RCH-ACV and RPMI-8402 cell lines. **D,** H3K27ac ChIP-Seq track of *BCL2L11/BIM* in RCH-ACV cells in the presence and absence of dexamethasone with schematic indicating location of methylated CpG sites present in the BIM IGR (intronic glucocorticoid receptor-binding region). **E**, Histograms displaying the percentage of methylation of 7 CpG sites in the BIM IGR in isogenic RCH-ACV, SEM, RPMI-8402 and CEM cell lines as detected by sequencing ten clones of PCR-amplified DNA derived from bisulfite converted DNA from each cell line. WT, *NSD2* WT; Mut, *NSD2* p.E1099K; Con, Control (DMSO); Dex, Dexamethasone.


A
GR-ChIP
CTCF-ChIP
RCH -ACV
RPMI-8402
RCH -ACV
RPMI-8402
Mut-Con
Mut-Dex
WT-Con
WT-Dex
0 25 50 75 100
Peak Distribution (%)
Downstream
Exon
Upstream
Intron
Intergenic


B
BMF chr15: 40087890-40108879
NFKBIA chr14: 35401510-35404754
3kb
1kb
RCH-ACV
RPMI-8402
GR
CTCF
H3K27ac
Mut-Con
Mut-Dex
WT-Con
WT-Dex

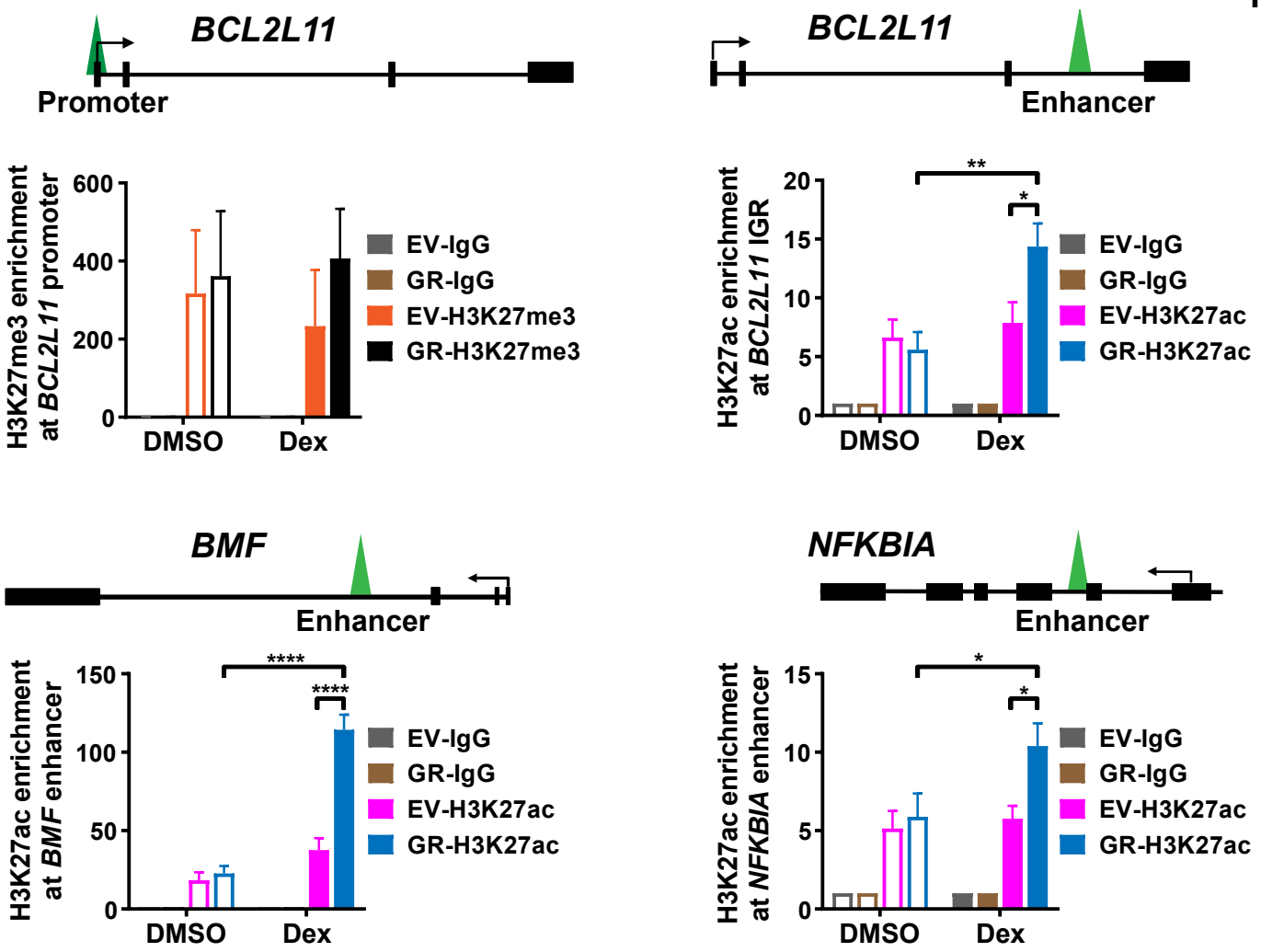

C
Ontogeny of Genes Bound by GR in NSD2 WT RCH-ACV and RPMI-8402 Cells
GR Activated Genes
FDR ≤ 0.05
FDR > 0.05
positive regulation of fibroblast apoptotic process
cellular response to glucocorticoid stimulus
cellular response to corticosteroid stimulus
response to glucocorticoid
response to corticosteroid
negative regulation of immune system process
regulation of cell adhesion
cellular response to hormone stimulus
regulation of immune system process
regulation of intracellular signal transduction
0 5 10 15 20 25 30 35 40
Enrichment ratio
GR Repressed Genes
FDR ≤ 0.05
DNA replication initiation
DNA-dependent DNA replication
DNA replication
G1/S transition of mitotic cell cycle
cell cycle G1/S phase transition
mitotic cell cycle phase transition
cell cycle phase transition
DNA metabolic process
cell cycle process
cell cycle
0 5 10 15 20 25 30 35 40 45 50 55
Enrichment ratio

Supplementary Figure S5. *NSD2* p.E1099K mutation alters GR and CTCF binding, and H3K27ac enrichment to glucocorticoid response elements (GREs). A, Peak distribution of GR and CTCF ChIP-Seq in different regions of isogenic ALL cell lines (RCH-ACV and RPMI-8402) in response to dexamethasone (1 μM, 24 hours). **B,** Representative GR and CTCF binding and H3K27ac enrichment at the gene *BMF* and *NFKBIA* loci of isogenic ALL cell lines (RCH-ACV and RPMI-8402) in response to dexamethasone (red box). **C,** Ontogeny of genes (WebGestalt) directly bound by GR in *NSD2* WT cells identified using RNA-Seq, ATAC-Seq and ChIP-Seq datasets of isogenic RCH-ACV and RPMI-8402 cell lines in response to GC. WT, *NSD2* WT; Mut, *NSD2* p.E1099K; Con, Control (DMSO); Dex, Dexamethasone.

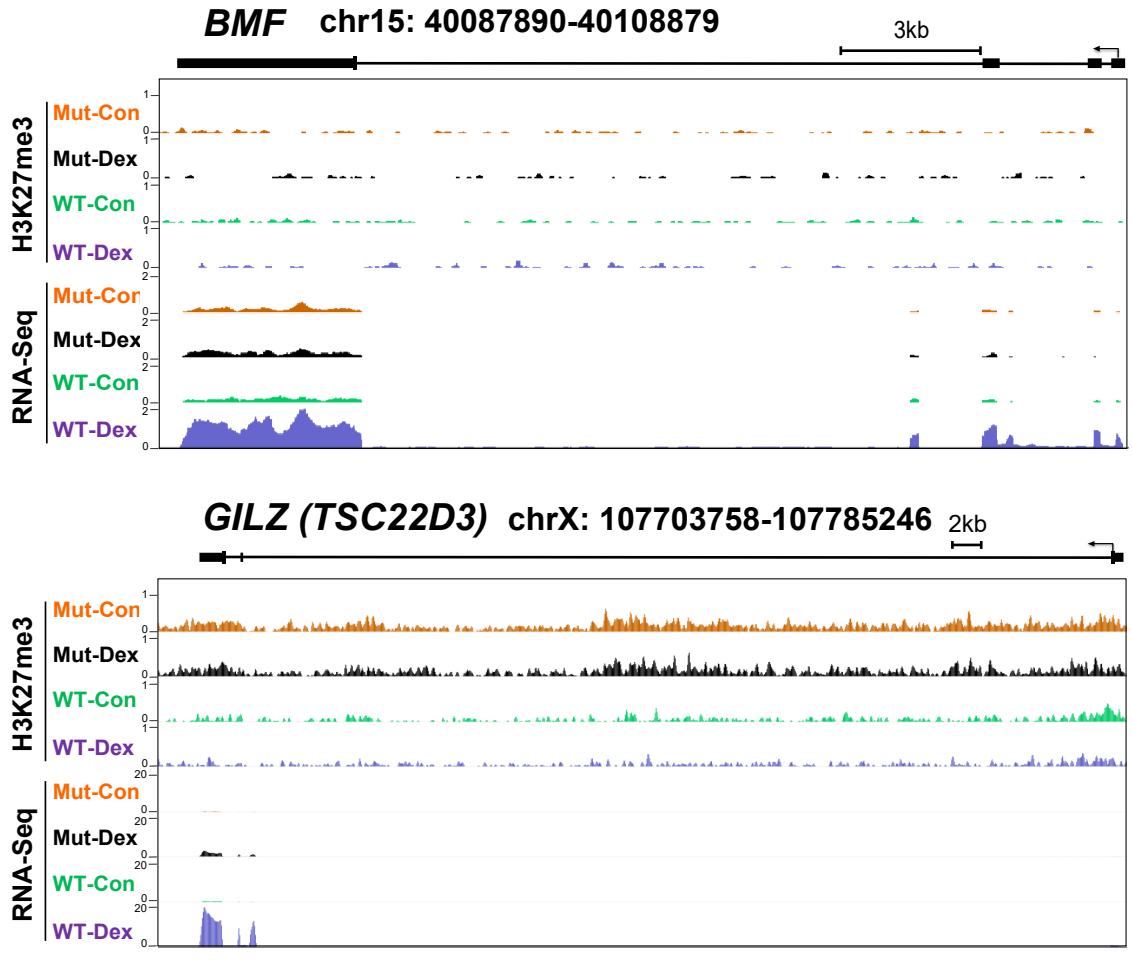

A
NR3C1 in RNA-Seq
Mut
WT
RCH-ACV
SEM
RPMI-8402
CEM
Max
Min
B
RCH-ACV
SEM
RPMI-8402
CEM
GR protein expression
BIM protein expression
DMSO
Dex
Mut
WT
C
ALL-32
ALL-11
Dex
GR
BIM
Vimentin
ALL-32
ALL-11
D
Cytoplasm
Nuclear
Mut
WT
Dex
GR
Max
Tublin
Cytoplasm
Nuclear
Mut
Mut-Dex
WT
WT-Dex
E
NR3C1
chr5: 143277931-143435512
6kb
RCH-ACV
RPMI-8402
ATAC-Seq
GR
CTCF
H3K27ac
Mut-Con
Mut-Dex
WT-Con
WT-Dex
F
RCH-ACV
RPMI-8402
GR protein expression
EV
GR
G
RCH-ACV
RPMI-8402
EV
GR
DMSO
Dex
PI
Annexin V
0.5
0.5
0.6
0.9
0.7
3.9
1.9
17.9
0.1
0.1
1.0
0.9
0.2
1.8
1.5
67.8
H
RCH-ACV
RPMI-8402
NR3C1 mRNA expression
NFKBIA mRNA expression
DMSO
Dex
EV
GR

Supplementary Figure S6. *NSD2* p.E1099K mutation inhibits GR expression and auto-activation but not translocation. A, *NR3C1* (encoding GR) mRNA expression plotted as a heatmap from RNA-Seq data of isogenic ALL cell lines (RCH-ACV, SEM, RPMI-8402 and CEM) before dexamethasone treatment. **B**, Immunoblotting-based quantification of GR and BIM protein expression in isogenic *NSD2* mutant and WT ALL cell lines in response to dexamethasone (1μM, 24 hours). **C,** Immunoblotting of GR and BIM protein expression (top), quantified (bottom) in PDX ALL cells in response to dexamethasone (5nM, 24 hours). **D,** GR translocation from cytoplasm to nucleus and normalization in isogenic RCH-ACV cell lines detected by immunoblotting of subcellular fractions. Max- nuclear marker; Tubulin- cytoplasmic marker. **E,** Genome browser tracks of chromatin accessibility (ATAC-Seq), GR and CTCF binding and H3K27ac enrichment at the *NR3C1* gene in isogenic RCH-ACV and RPMI-8402 ALL cell lines in response to dexamethasone (red box). **F**, Immunoblotting quantification of GR overexpression in *NSD2* p.E1099K RCH-ACV and RPMI-8402 cell lines. **G,** Representative flow analysis of apoptosis as determined by Annexin V/PI staining in *NSD2* p.E1099K RCH-ACV and RPMI-8402 cell lines overexpressing GR or empty vector (EV) after treatment with 1 μM dexamethasone for 24 hours compared to DMSO control. **H,** Expression of *NR3C1* and *NFKBIA* in *NSD2* p.E1099K RCH-ACV and RPMI-8402 cell lines overexpressing GR after treatment with 1μM dexamethasone or DMSO for 24 hours was measured using real time PCR in biological triplicate, normalizing to housekeeping gene *PPP1R15B*. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, *NSD2* WT; Mut, *NSD2* p.E1099K; EV, empty vector; GR, GR vector; Con, Control (DMSO); Dex, Dexamethasone.

A


BCL2L11
Promoter
BCL2L11
Enhancer
H3K27me3 enrichment at BCL2L11 promoter
600
400
200
0
DMSO
Dex
EV-IgG
GR-IgG
EV-H3K27me3
GR-H3K27me3
H3K27ac enrichment at BCL2L11 IGR
20
15
10
5
0
**
*
DMSO
Dex
EV-IgG
GR-IgG
EV-H3K27ac
GR-H3K27ac
BMF
Enhancer
H3K27ac enrichment at BMF enhancer
150
100
50
0
****
****
DMSO
Dex
EV-IgG
GR-IgG
EV-H3K27ac
GR-H3K27ac
NFKBIA
Enhancer
H3K27ac enrichment at NFKBIA enhancer
15
10
5
0
*
*
DMSO
Dex
EV-IgG
GR-IgG
EV-H3K27ac
GR-H3K27ac

B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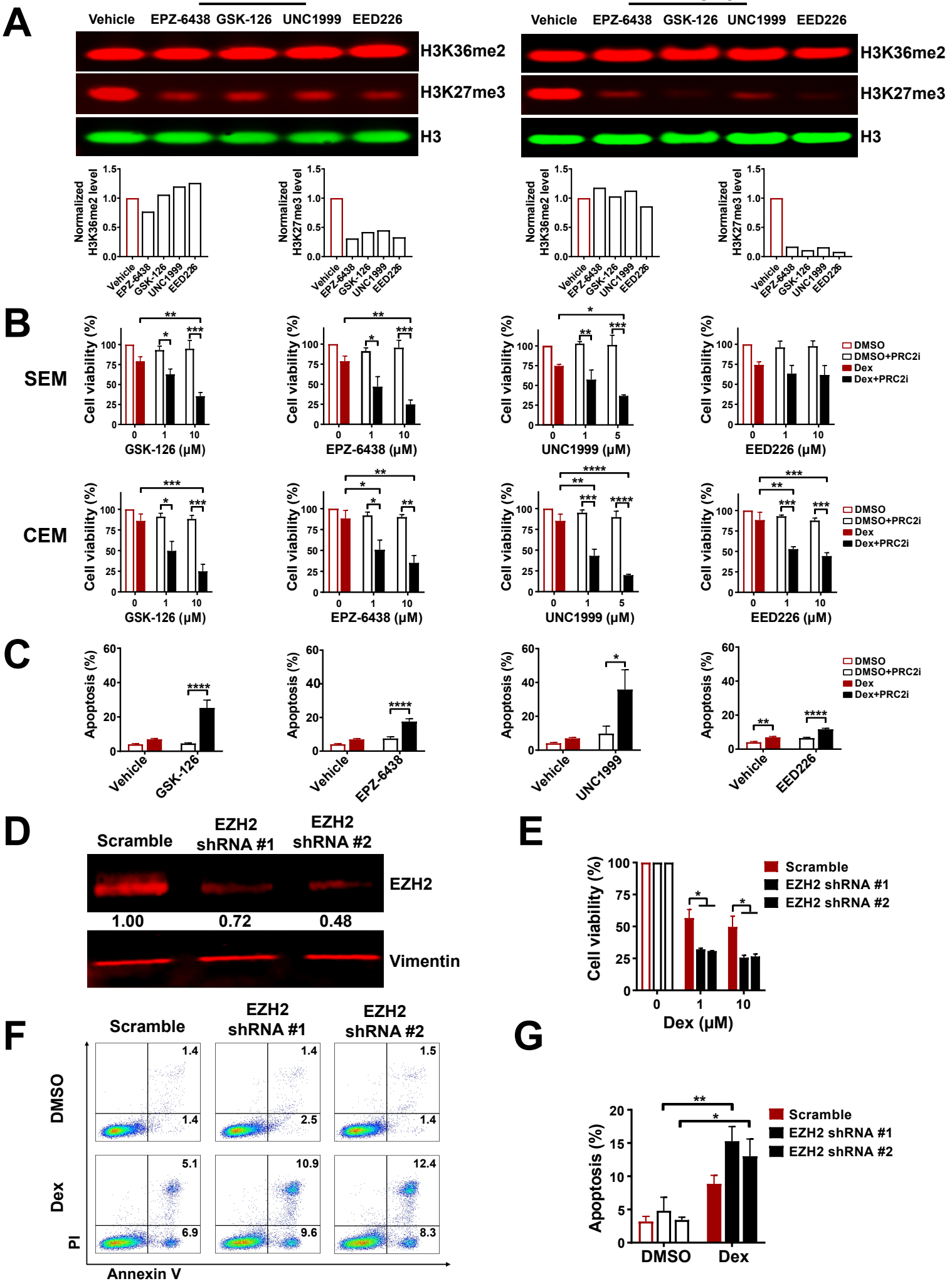
3kb
H3K27me3
Mut-Con
Mut-Dex
WT-Con
WT-Dex
RNA-Seq
Mut-Con
Mut-Dex
WT-Con
WT-Dex
GILZ (TSC22D3) chrX: 107703758-107785246
2kb
H3K27me3
Mut-Con
Mut-Dex
WT-Con
WT-Dex
RNA-Seq
Mut-Con
Mut-Dex
WT-Con
WT-Dex

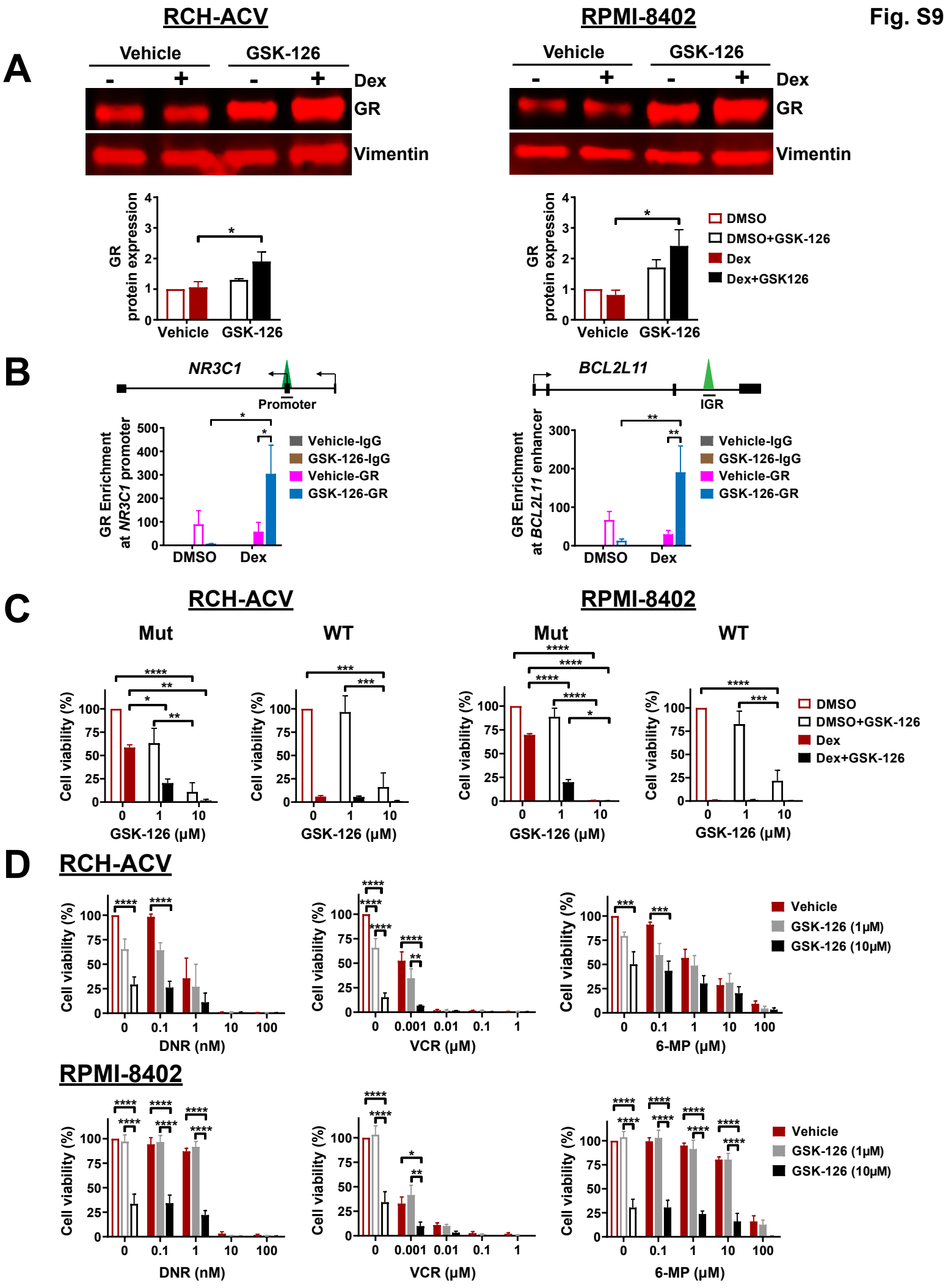
Supplementary Figure S7. Chromatin changes in response to GR overexpression in *NSD2* p.E1099K ALL cell lines. **A,** H3K27me3 enrichment at the promoter of *BCL2L11* and H3K27ac enrichment at the IGR/enhancer of *BCL2L11*, and *BMF* and *NFKBIA* enhancers in *NSD2* p.E1099K cells (RCH-ACV) with GR overexpression in response to dexamethasone (1μm, 24 hours) compared to DMSO control. **B,** H3K27me3 ChIP-Seq and RNA-Seq track at *BMF* and *GILZ (TSC22D3)* genes of isogenic *NSD2* p.E1099K and WT cell lines. WT, *NSD2* WT; Mut, *NSD2* p.E1099K; EV, empty vector; GR, GR vector; Con, Control (DMSO); Dex, Dexamethasone.

Fig. S8


A
RCH-ACV
RPMI-8402
Vehicle
EPZ-6438
GSK-126
UNC1999
EED226
H3K36me2
H3K27me3
H3
Normalized H3K36me2 level
Normalized H3K27me3 level
B
SEM
CEM
Cell viability (%)
GSK-126 (μM)
EPZ-6438 (μM)
UNC1999 (μM)
EED226 (μM)
DMSO
DMSO+PRC2i
Dex
Dex+PRC2i
C
Apoptosis (%)
D
Scramble
EZH2 shRNA #1
EZH2 shRNA #2
EZH2
Vimentin
1.00
0.72
0.48
E
Cell viability (%)
Dex (μM)
F
DMSO
Dex
PI
Annexin V
G
Apoptosis (%)

Supplementary Figure S8. PRC2 inhibitors or EZH2 knockdown restore glucocorticoid sensitivity of *NSD2* p.E1099K ALL cells. A, Immunoblotting quantification of H3K36me2 and H3K27me3 levels in RCH-ACV and RPMI-8402 *NSD2* p.E1099K cells treated with PRC2 inhibitors (GSK-126, EPZ-6438, UNC1999 and EED226) for 7 days. **B,** Cell viability of *NSD2* p.E1099K cells (SEM and CEM) after combinational treatment with PRC2 inhibitors (7 days) and dexamethasone (1μM, 48 hours) as determined by CellTiter-Glo assay. **C,** Quantification of apoptosis of *NSD2* p.E1099K RCH-ACV cells after sequential treatment with PRC2 inhibitors (7 days) followed by dexamethasone (1μM, 24 hours). **D,** Immunoblotting quantification of EZH2 protein levels in *NSD2* p.E1099K RCH-ACV cell line after shRNA-mediated knockdown. **E,** Viability of *NSD2* p.E1099K RCH-ACV cells with EZH2 knockdown was determined by CellTiter-Glo after treatment with dexamethasone (1μM, 48 hours) or DMSO. **F and G,** Representative flow cytometric plots and statistical analysis of apoptosis detected using Annexin V/PI staining in *NSD2* p.E1099K RCH-ACV cell line with EZH2 knockdown after dexamethasone treatment (1μM, 48 hours) or DMSO. CellTiter-Glo and flow cytometric assays were performed in biological triplicates. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. PRC2i, PRC2 inhibitors (GSK-126, EPZ-6438, UNC1999 and EED226); Vehicle, DMSO; Dex, Dexamethasone.

Fig. S9


RCH-ACV
RPMI-8402
A
Vehicle
GSK-126
Dex
GR
Vimentin
GR protein expression
DMSO
DMSO+GSK-126
Dex
Dex+GSK126
B
NR3C1
Promoter
BCL2L11
IGR
GR Enrichment at NR3C1 promoter
GR Enrichment at BCL2L11 enhancer
Vehicle-IgG
GSK-126-IgG
Vehicle-GR
GSK-126-GR
DMSO
Dex
C
Mut
WT
Cell viability (%)
GSK-126 (μM)
D
DNR (nM)
VCR (μM)
6-MP (μM)
Vehicle
GSK-126 (1μM)
GSK-126 (10μM)

Supplementary Figure S9. Effect of PRC2 inhibitors on glucocorticoid and chemotherapy response in *NSD2* p.E1099K ALL cells. A, Immunoblotting (top) and quantification (bottom) of GR protein expression in *NSD2* p.E1099K RCH-ACV and RPMI-8402 cells after treatment with GSK-126 (7 days) followed by dexamethasone (1μM, 24 hours). **B,** ChIP-qPCR analysis of GR binding at the gene *NR3C1* promoter and *BCL2L11* IGR/enhancer in *NSD2* p.E1099K RPMI-8402 cells after pretreatment with GSK-126 (7 days) followed by dexamethasone (1μM, 24 hours). **C,** Viability of isogenic *NSD2* mutant and WT RCH-ACV and RPMI-8402 cells, as determined by CellTiter-Glo, after combination treatment with GSK-126 (14 days) followed by dexamethasone (1μM, 48 hours). **D,** Viability of *NSD2* p.E1099K RCH-ACV and RPMI-8402 cells, as determined by CellTiter-Glo, after combination treatment with GSK-126 (7 days) followed by 48 hours with the indicated chemotherapeutic agents. All the experiments were performed in biological triplicates. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, *NSD2* WT; Mut, *NSD2* p.E1099K; Vehicle, DMSO; Dex, Dexamethasone; DNR, Daunorubicin; VCR, Vincristine; 6-MP, 6-mercaptopurine.