Defining super-enhancers by highly ranked histone H4 multi-acetylation levels identifies transcription factors associated with glioblastoma stem-like properties

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Supplementary Figures

Figure S1

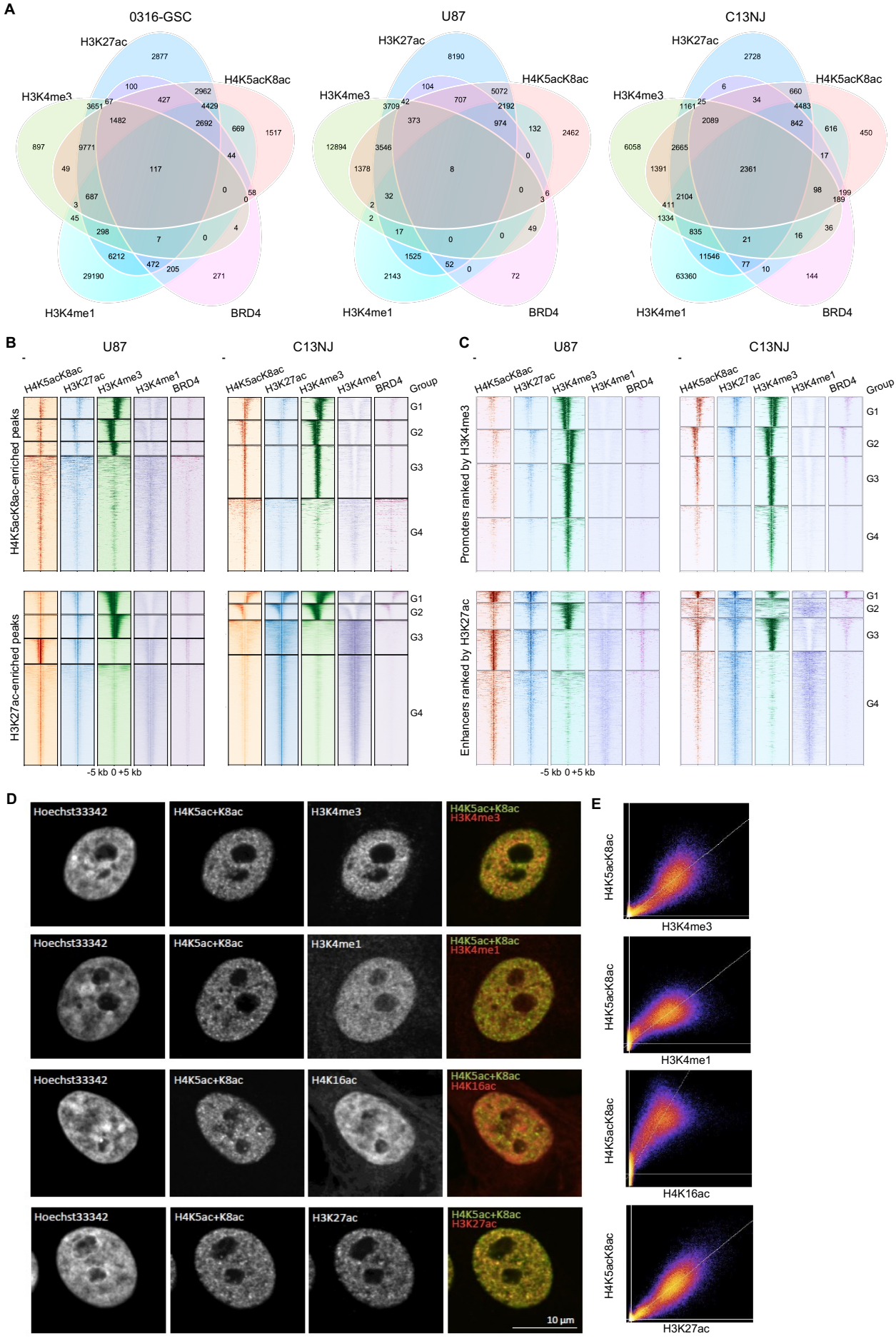

A
0316-GSC
U87
C13NJ
H3K27ac
H4K5acK8ac
H3K4me3
H3K4me1
BRD4
B
U87
C13NJ
H4K5acK8ac
H3K27ac
H3K4me3
H3K4me1
BRD4
Group
G1
G2
G3
G4
H4K5acK8ac-enriched peaks
H3K27ac-enriched peaks
-5 kb 0 +5 kb
C
Promoters ranked by H3K4me3
Enhancers ranked by H3K27ac
D
Hoechst33342
H4K5ac+K8ac
H3K4me3
H4K5ac+K8ac H3K4me3
H3K4me1
H4K5ac+K8ac H3K4me1
H4K16ac
H4K5ac+K8ac H4K16ac
H3K27ac
H4K5ac+K8ac H3K27ac
10 µm
E
H4K5acK8ac
H3K4me3
H3K4me1
H4K16ac
H3K27ac

Figure S1 (continued)

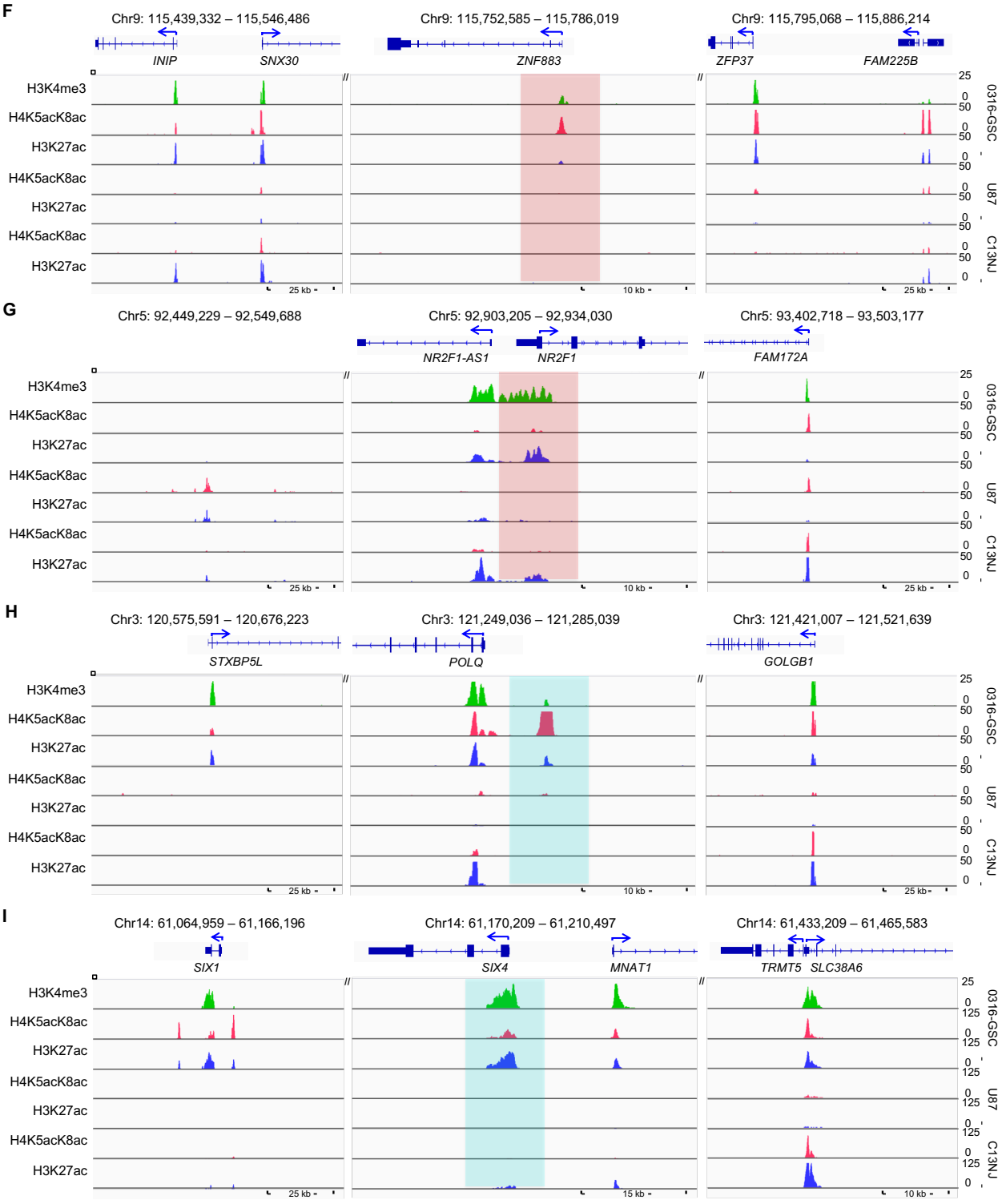


Figure S1. Identification of regions that bind H4K5acK8ac preferentially over H3K27ac. A Venn diagrams representing the overlap of ChIP-seq peaks of H4K5acK8ac with H3K27ac, H3K4me1, H3K4me3, and BRD4 across 0316-GSC, U87, and C13NJ cell lines. **B** and **C** Heatmaps representing different ChIP-seq datasets (H4K5acK8ac, H3K27ac, H3K4me3, H3K4me1, and BRD4) in U87 and C13NJ cell lines. Data are from within ± 5-kb from the summit of H4K5acK8ac-enriched peaks (upper, **B**) and H3K27ac-enriched peaks (lower, B), and promoters defined by H3K4me3 at the promoters (upper, **C**) and enhancers defined by H3K27ac located outside a promoter (lower, **C**). Each row represents a single peak. Color density indicates the average enrichment of each mark at the selected regions. H4K5acK8ac- or H3K27ac-enriched peaks (**B**) and promoters or enhancers (**C**) are each clustered into four groups (G1 to G4) according to the ChIP-seq profiles. **D** Immunostaining analysis demonstrating the colocalization of H4K5acK8ac with H3K4me3, H3K4me1, H4K16ac, and H3K27ac marks in U87 cells. Hoechst 33342 was used for nuclear staining. Scale bar, 10 μm. **E** Correlation of immunofluorescence signal between H4K5acK8ac and other specific marks in U87 cells. **F**–**I** Representative 0316-GSC-specific ChIP-seq tracks of H4K5acK8ac-preferred (log_2 fold-change (FC) [H4K5acK8ac/H3K27ac] > 1) and H3K27ac-preferred (log_2FC [H4K5acK8ac/H3K27ac] < −1) promoters (**F** and **G**) and enhancers (**H** and **I**).

Figure S2

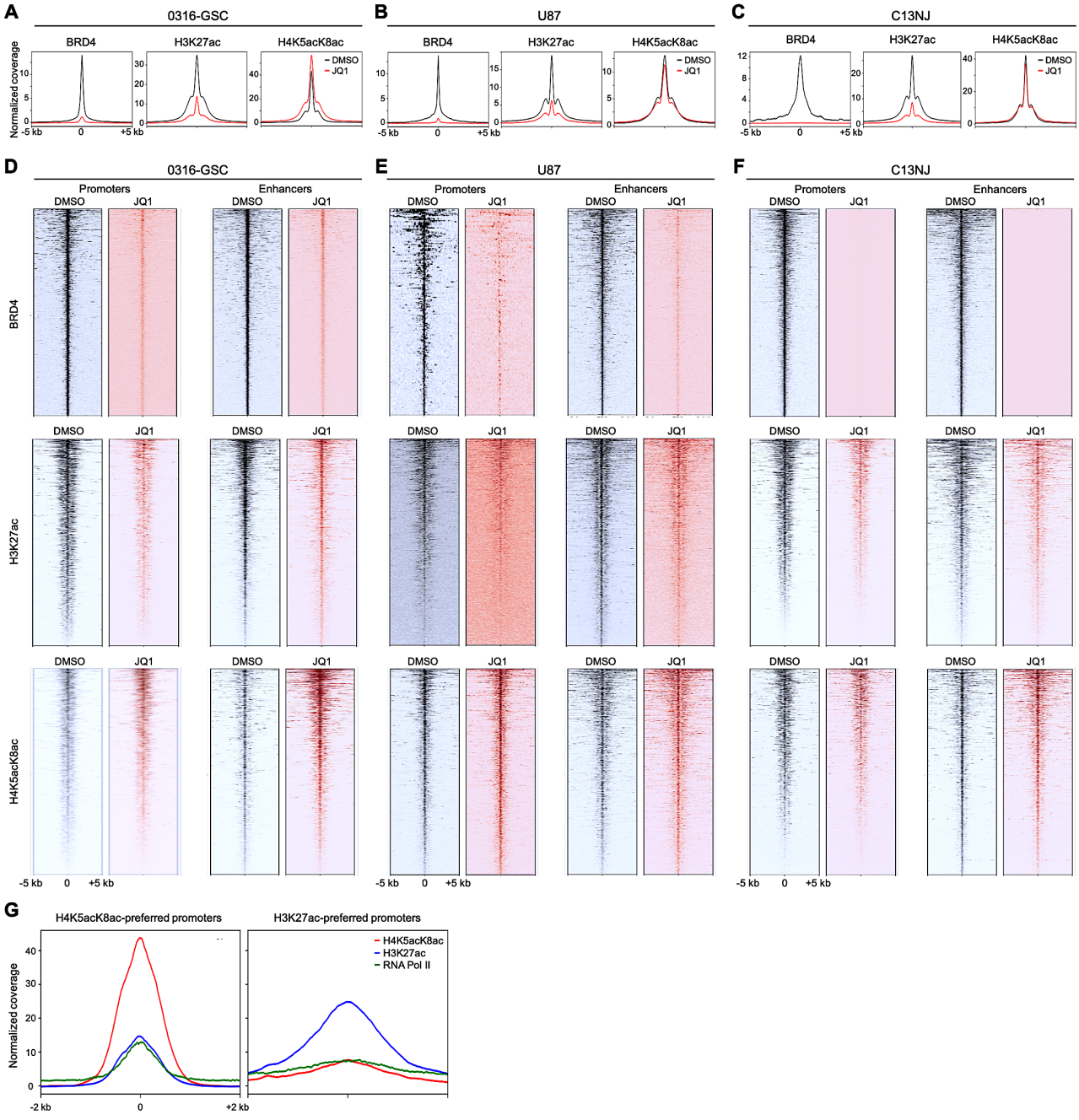


Figure S2. Effect of JQ1 on genome-wide enrichment of BRD4, H3K27ac, and H4K5acK8ac across glial cell lines. **A–C** ChIP-seq meta-profiles for dimethyl sulfoxide (DMSO; vehicle control) and JQ1-treated cells showing the average read counts (reads per million; RPM) of ± 5 kb regions from the summit of BRD4-, H3K27ac-, and H4K5acK8ac-enrichment. Compared with treatment with DMSO, treatment with 5 µM JQ1 for 24 h led to a global loss of BRD4 and H3K27ac across the three cell lines, and the enrichment of H4K5acK8ac in 0316-GSC but not U87 or C13NJ cells. **D–F** Heatmaps of ChIP-seq datasets of BRD4, H3K27ac, and H4K5acK8ac upon JQ1 treatment in 0316-GSC cells (**D**), U87 (**E**), and C13NJ (**F**) cells within ± 5 kb from the summit of active promoters (defined by cell type–specific H3K4me3) (left) or active enhancers (defined by cell type–specific H3K27ac located outside promoter) (right). Each row represents a single peak. Color density indicates the average enrichment of each mark at promoters and enhancers. **G** Colocalization of RNAP II with promoters. ChIP-seq meta-profiles show the association of RNAP II (green) with H4K5acK8ac-preferred (red) and H3K27ac-preferred (blue) promoters.

Figure S3

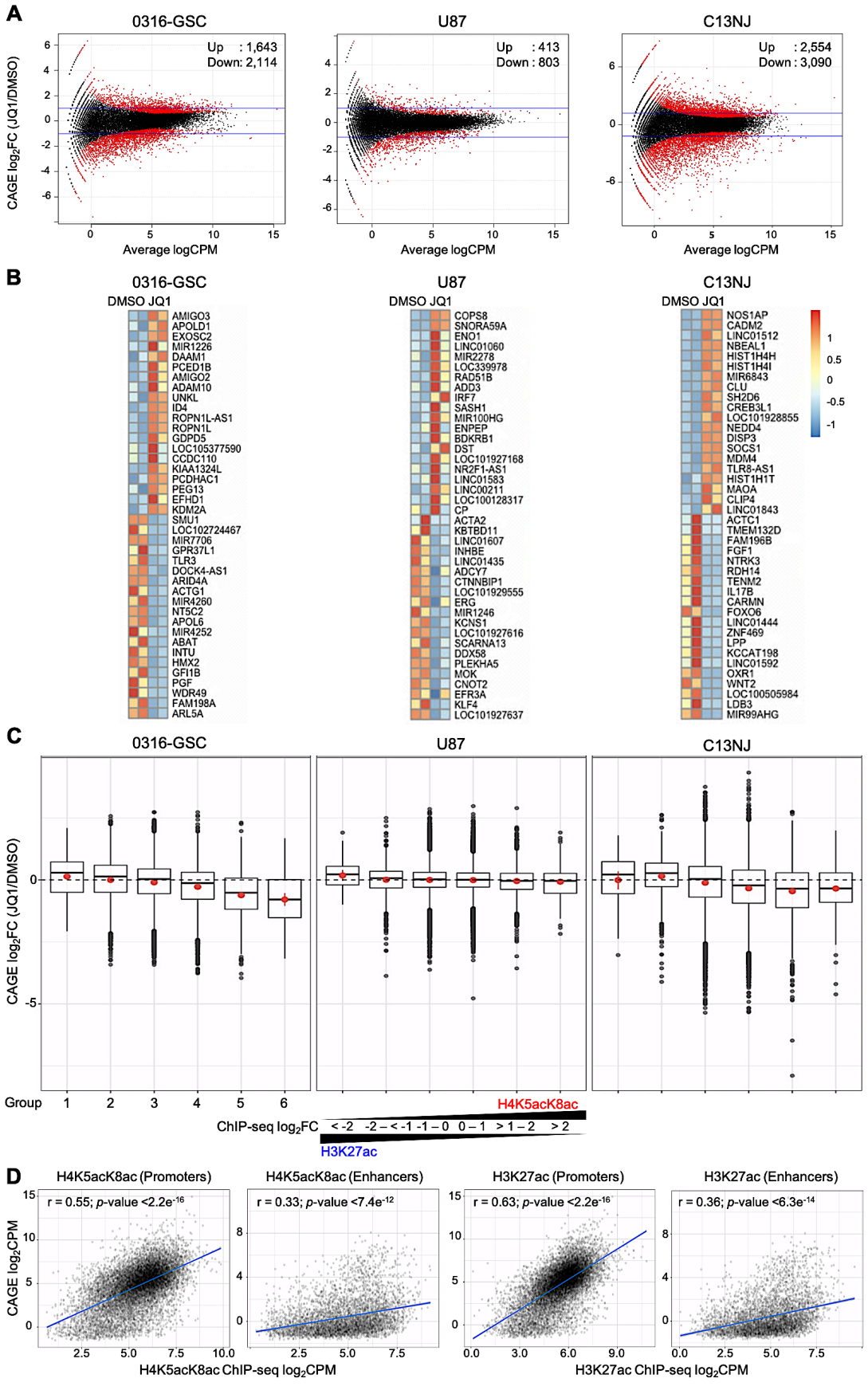
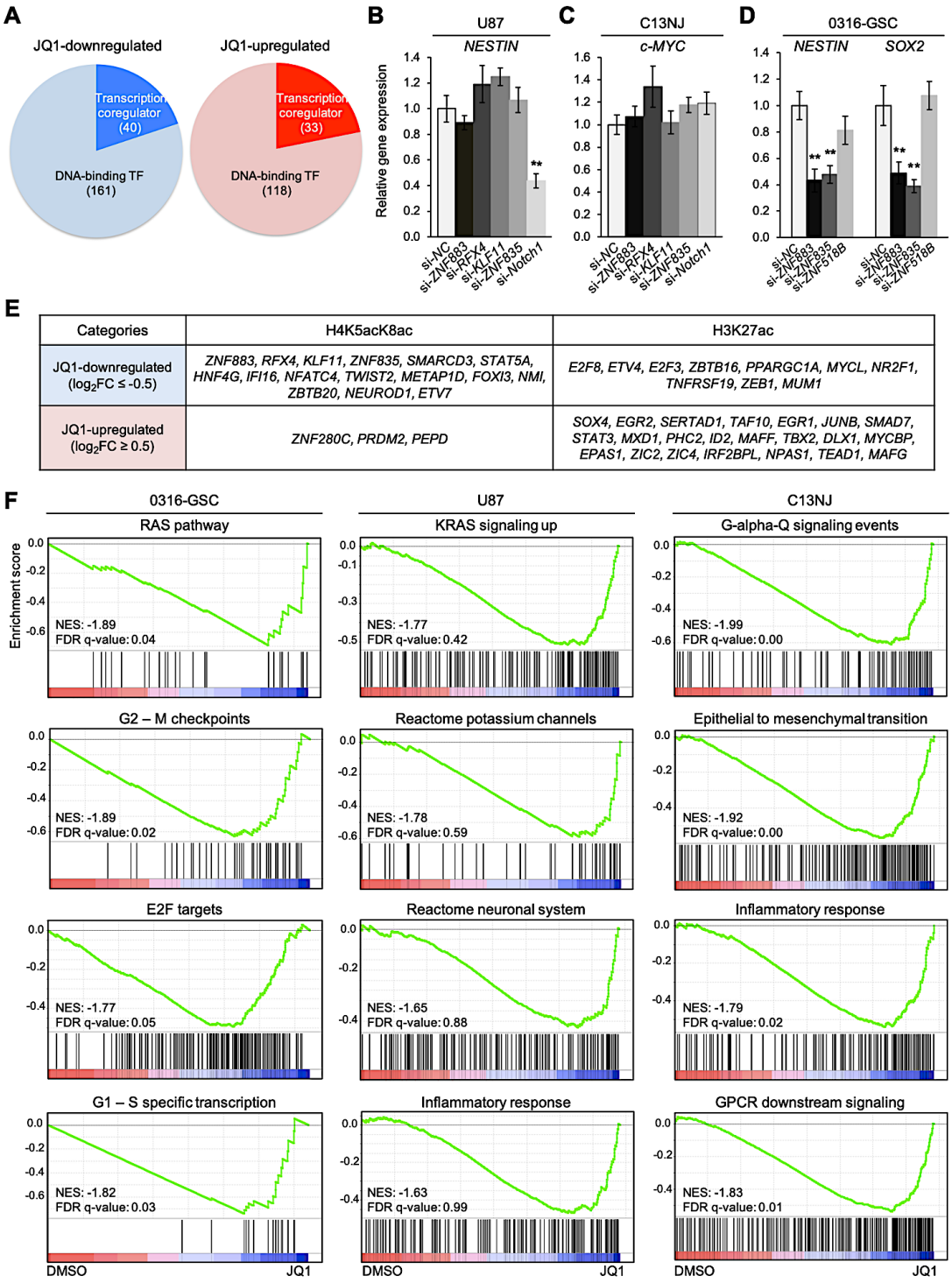

A
0316-GSC
U87
C13NJ
Up : 1,643
Down: 2,114
Up : 413
Down : 803
Up : 2,554
Down : 3,090
CAGE log2FC (JQ1/DMSO)
Average logCPM
B
0316-GSC
U87
C13NJ
DMSO JQ1
AMIGO3
APOLD1
EXOSC2
MIR1226
DAAM1
PCED1B
AMIGO2
ADAM10
UNKL
ID4
ROPN1L-AS1
ROPN1L
GDPD5
LOC105377590
CCDC110
KIAA1324L
PCDHAC1
PEG13
EFHD1
KDM2A
SMU1
LOC102724467
MIR7706
GPR37L1
TLR3
DOCK4-AS1
ARID4A
ACTG1
MIR4260
NT5C2
APOL6
MIR4252
ABAT
INTU
HMX2
GFI1B
PGF
WDR49
FAM198A
ARL5A
COPS8
SNORA59A
ENO1
LINC01060
MIR2278
LOC339978
RAD51B
ADD3
IRF7
SASH1
MIR100HG
ENPEP
BDKRB1
DST
LOC101927168
NR2F1-AS1
LINC01583
LINC00211
LOC100128317
CP
ACTA2
KBTBD11
LINC01607
INHBE
LINC01435
ADCY7
CTNNBIP1
LOC101929555
ERG
MIR1246
KCNS1
LOC101927616
SCARNA13
DDX58
PLEKHA5
MOK
CNOT2
EFR3A
KLF4
LOC101927637
NOS1AP
CADM2
LINC01512
NBEAL1
HIST1H4H
HIST1H4I
MIR6843
CLU
SH2D6
CREB3L1
LOC101928855
NEDD4
DISP3
SOCS1
MDM4
TLR8-AS1
HIST1H1T
MAOA
CLIP4
LINC01843
ACTC1
TMEM132D
FAM196B
FGF1
NTRK3
RDH14
TENM2
IL17B
CARMN
FOXO6
LINC01444
ZNF469
LPP
KCCAT198
LINC01592
OXR1
WNT2
LOC100505984
LDB3
MIR99AHG
1
0.5
0
-0.5
-1
C
0316-GSC
U87
C13NJ
CAGE log2FC (JQ1/DMSO)
Group 1 2 3 4 5 6
H4K5acK8ac
ChIP-seq log2FC < -2 -2 – < -1 -1 – 0 0 – 1 > 1 – 2 > 2
H3K27ac
D
H4K5acK8ac (Promoters)
H4K5acK8ac (Enhancers)
H3K27ac (Promoters)
H3K27ac (Enhancers)
r = 0.55; p-value <2.2e-16
r = 0.33; p-value <7.4e-12
r = 0.63; p-value <2.2e-16
r = 0.36; p-value <6.3e-14
CAGE log2CPM
H4K5acK8ac ChIP-seq log2CPM
H3K27ac ChIP-seq log2CPM

Figure S3. Effect of H4K5acK8ac level in regulatory elements on changes in gene expression upon JQ1 treatment in three glial cell lines. A Relative expression of genes upon JQ1 treatment. Numbers of genes significantly upregulated or downregulated by 24-h treatment with 5 μM JQ1 relative to DMSO (false discovery rate < 0.05; CAGE $\log_2$FC [JQ1/DMSO] > 0.5 or < −0.5, respectively) are shown for each of the three cell lines. **B** Heatmaps of the top 20 genes differentially upregulated and top 20 genes differentially downregulated by JQ1 are shown based on the ranking of CAGE $\log_2$FC in each cell line. **C** Box plots of relative expression of genes upon JQ1 treatment for promoters or enhancers grouped by their level of enrichment of H4K5acK8ac over H3K27ac. Groups 1–3 are defined by ChIP-seq $\log_2$FC (H4K5acK8ac/H3K27ac) of < −2, −2 to < −1, and −1 to 0, respectively (H3K27ac-preferred; $\log_2$FC < −1). Groups 4–6 are defined by ChIP-seq $\log_2$FC 0 to 1, >1 to 2, and >2, respectively (H4K5acK8ac-preferred; $\log_2$FC > 1). **D** Scatter plots of the change in gene expression upon JQ1 treatment (CAGE $\log_2$FC) versus levels of histone modifications (ChIP-seq $\log_2$CPM) for genes associated with H4K5acK8ac-preferred and H3K27ac-preferred promoters or enhancers in 0316-GSC cells. The blue line indicates the linear fit; *r* indicates Spearman's correlation (n = 2 biological replicates for each histone modification and FC value of each gene is the average of the FCs of the two biological replicates).

Figure S4



Categories	H4K5acK8ac	H3K27ac
JQ1-downregulated ($log_2FC \leq -0.5$)	*ZNF883, RFX4, KLF11, ZNF835, SMARCD3, STAT5A, HNF4G, IFI16, NFATC4, TWIST2, METAP1D, FOXI3, NMI, ZBTB20, NEUROD1, ETV7*	*E2F8, ETV4, E2F3, ZBTB16, PPARGC1A, MYCL, NR2F1, TNFRSF19, ZEB1, MUM1*
JQ1-upregulated ($log_2FC \geq 0.5$)	*ZNF280C, PRDM2, PEPD*	*SOX4, EGR2, SERTAD1, TAF10, EGR1, JUNB, SMAD7, STAT3, MXD1, PHC2, ID2, MAFF, TBX2, DLX1, MYCBP, EPAS1, ZIC2, ZIC4, IRF2BPL, NPAS1, TEAD1, MAFG*

Figure S4. Effect of JQ1 on specific genes and various biological pathways and molecular functions across the three glial cell lines. A JQ1-downregulated and upregulated genes involved in transcriptional activity were categorized as transcription coregulators or DNA-binding transcription factors (TFs). **B** and **C** Unlike siRNA knockdown of *NOTCH1*, siRNA knockdown of TF candidate genes with H4K5acK8ac-preferred promoters did not result in significant changes in expression of the stem cell marker gene *NESTIN* in U87 (**B**) cells. Neither siRNA knockdown of *NOTCH1* nor siRNA knockdown of the set of TF candidate genes with H4K5acK8ac-preferred promoters resulted in significant changes in c-*Myc* expression in C13NJ (**C**) cells (n = 3). **D** Comparative siRNA knockdown of selected JQ1-unaffected and JQ1-downregulated genes encoding zinc-finger TFs with H4K5acK8ac-preferred promoters in 0316-GSC. siRNA knockdown of the JQ1-unaffected gene, *ZNF518B*, did not suppress the expression of stem cell marker genes *NESTIN* and *SOX2* (n = 3), but siRNA knockdown of JQ1-downregulated genes, *ZNF833* and *ZNF835*, did significantly suppress the expression of these genes. Data are means ± SEM. **, $P < 0.01$ (two-tailed Student's *t*-test). **E** JQ1-downregulated and upregulated TF candidate genes are shown categorized according to whether they have H4K5acK8ac- or H3K27ac-preferred promoters in 0316-GSC cells. **F** GSEA results of gene ontology (GO) biological pathways significantly over-represented in the sets of JQ1-downregulated genes in 0316-GSC, U87, and C13NJ are shown (NES; Normalized Enrichment Score). **G** and **H** GO molecular functions (**G**) and biological pathways (**H**) that are significantly over-represented in JQ1-downregulated genes associated with H4K5acK8ac- (upper) or H3K27ac-preferred (lower) promoters in 0316-GSC cells are shown.

Figure S5

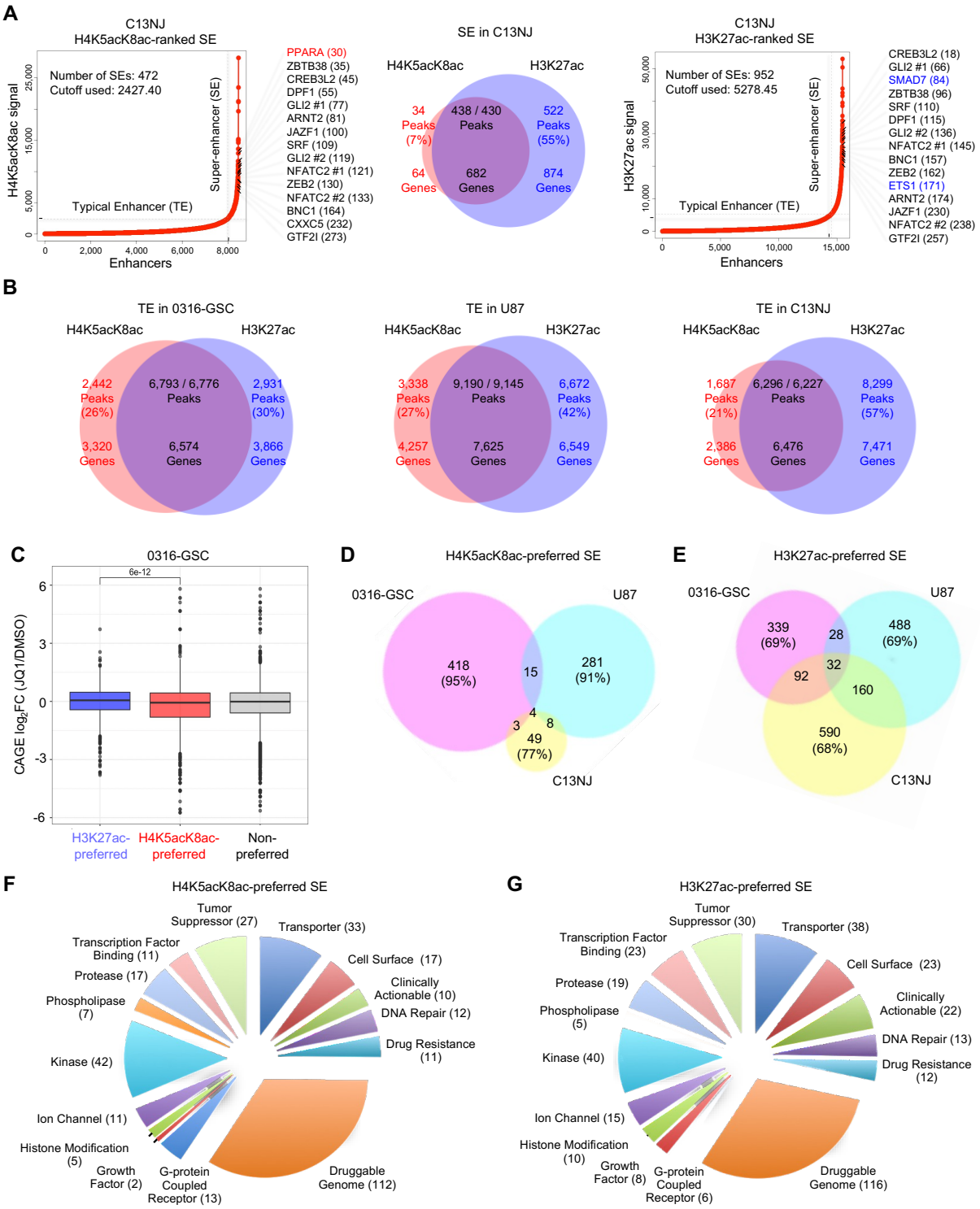
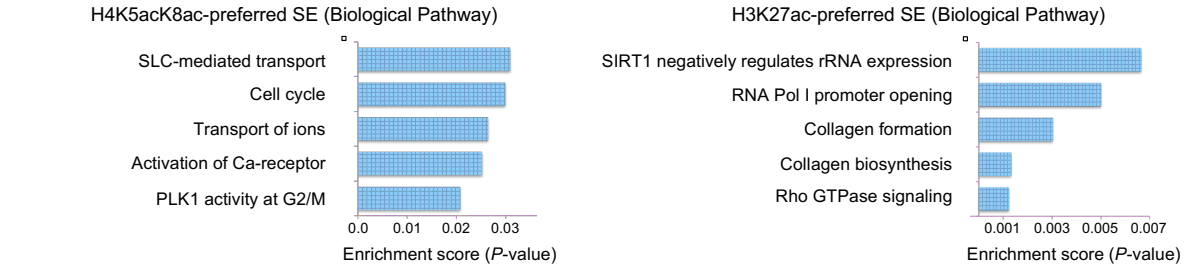

A
C13NJ
H4K5acK8ac-ranked SE
Number of SEs: 472
Cutoff used: 2427.40
H4K5acK8ac signal
Super-enhancer (SE)
Typical Enhancer (TE)
Enhancers
PPARA (30)
ZBTB38 (35)
CREB3L2 (45)
DPF1 (55)
GLI2 #1 (77)
ARNT2 (81)
JAZF1 (100)
SRF (109)
GLI2 #2 (119)
NFATC2 #1 (121)
ZEB2 (130)
NFATC2 #2 (133)
BNC1 (164)
CXXC5 (232)
GTF2I (273)
SE in C13NJ
H4K5acK8ac
H3K27ac
34 Peaks (7%)
438 / 430 Peaks
522 Peaks (55%)
64 Genes
682 Genes
874 Genes
C13NJ
H3K27ac-ranked SE
Number of SEs: 952
Cutoff used: 5278.45
H3K27ac signal
Super-enhancer (SE)
Typical Enhancer (TE)
Enhancers
CREB3L2 (18)
GLI2 #1 (66)
SMAD7 (84)
ZBTB38 (96)
SRF (110)
DPF1 (115)
GLI2 #2 (136)
NFATC2 #1 (145)
BNC1 (157)
ZEB2 (162)
ETS1 (171)
ARNT2 (174)
JAZF1 (230)
NFATC2 #2 (238)
GTF2I (257)
B
TE in 0316-GSC
H4K5acK8ac
H3K27ac
2,442 Peaks (26%)
6,793 / 6,776 Peaks
2,931 Peaks (30%)
3,320 Genes
6,574 Genes
3,866 Genes
TE in U87
H4K5acK8ac
H3K27ac
3,338 Peaks (27%)
9,190 / 9,145 Peaks
6,672 Peaks (42%)
4,257 Genes
7,625 Genes
6,549 Genes
TE in C13NJ
H4K5acK8ac
H3K27ac
1,687 Peaks (21%)
6,296 / 6,227 Peaks
8,299 Peaks (57%)
2,386 Genes
6,476 Genes
7,471 Genes
C
0316-GSC
6e-12
CAGE log2FC (JQ1/DMSO)
H3K27ac-preferred
H4K5acK8ac-preferred
Non-preferred
D
H4K5acK8ac-preferred SE
0316-GSC
U87
418 (95%)
15
281 (91%)
3
4
8
49 (77%)
C13NJ
E
H3K27ac-preferred SE
0316-GSC
U87
339 (69%)
28
488 (69%)
92
32
160
590 (68%)
C13NJ
F
H4K5acK8ac-preferred SE
Tumor Suppressor (27)
Transporter (33)
Transcription Factor Binding (11)
Cell Surface (17)
Protease (17)
Clinically Actionable (10)
Phospholipase (7)
DNA Repair (12)
Drug Resistance (11)
Kinase (42)
Ion Channel (11)
Histone Modification (5)
Growth Factor (2)
G-protein Coupled Receptor (13)
Druggable Genome (112)
G
H3K27ac-preferred SE
Tumor Suppressor (30)
Transporter (38)
Transcription Factor Binding (23)
Cell Surface (23)
Protease (19)
Clinically Actionable (22)
Phospholipase (5)
DNA Repair (13)
Kinase (40)
Drug Resistance (12)
Ion Channel (15)
Histone Modification (10)
Growth Factor (8)
G-protein Coupled Receptor (6)
Druggable Genome (116)

Figure S5 (continued)

H



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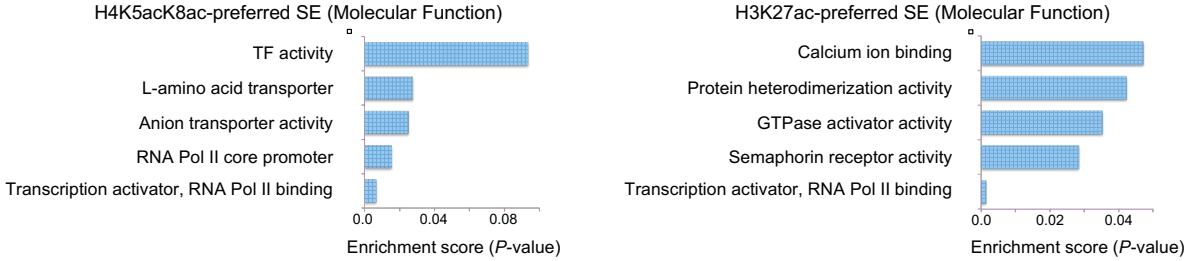

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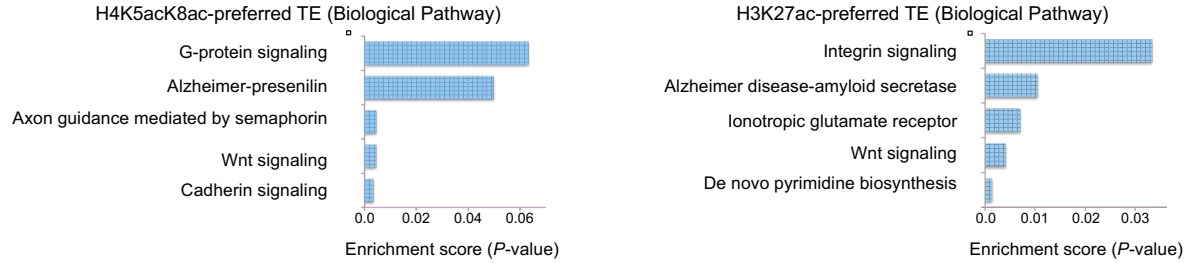

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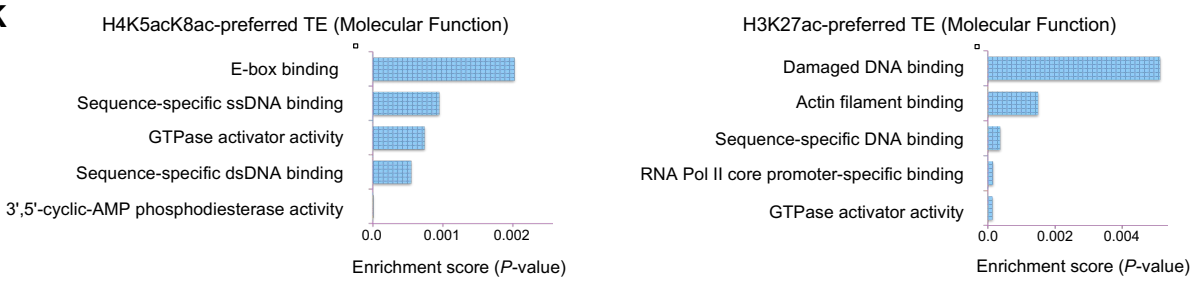

L

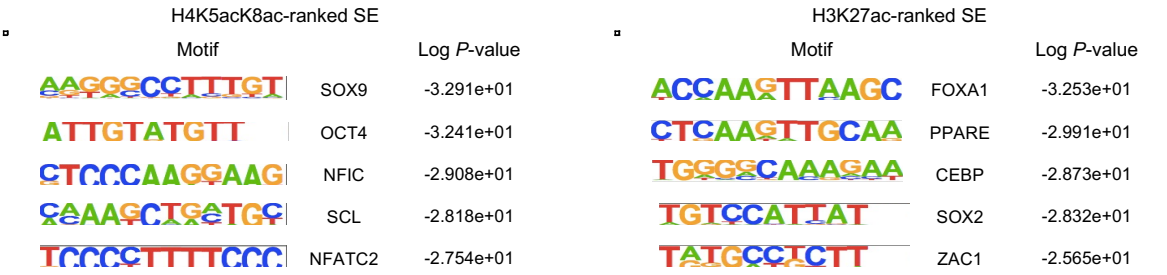

Figure S5. Defining super-enhancers (SEs) by H4K5acK8ac enrichment ranking in 0316-GSC, U87, and C13NJ cells. A Enhancers in C13NJ cells were ranked by H4K5acK8ac (left) or H3K27ac (right) signal level by using the ROSE algorithm; those with extremely high signals were defined as SEs (see Methods in the main text for threshold calculations). JQ1-downregulated (*i.e.*, CAGE $\log_2$FC < −0.5) transcription factor (TF) candidate genes with H4K5acK8ac- (red) or H3K27ac-preferred (blue) SEs are shown. Venn diagrams (middle) show the number of peaks and associated genes with H4K5acK8ac- or H3K27ac-preferred SEs or both (the intersection, purple). **B** Venn diagrams of genes with H4K5acK8ac- and/or H3K27ac-preferred typical enhancers (TEs) across three cell lines. **C** Box plots of change in gene expression levels upon JQ1 treatment in 0316-GSC. Genes associated with enhancers (peaks > 1-kb from the TSS) are grouped by the enrichment level of H4K5acK8ac over H3K27ac. The H3K27ac-, H4K5acK8ac-, and non-preferred peaks associated with enhancers are defined by ChIP-seq $\log_2$FC (H4K5acK8ac/H3K27ac): $\log_2$FC < −1, $\log_2$FC > 1, and −1 ≤ $\log_2$FC ≤ 1 (n = 2 biological replicates for each histone modification and FC value of each gene is the average of the FCs of the two biological replicates). **D** and **E** Venn diagrams showing cell-type specificities of genes with H4K5acK8ac-preferred (**D**) and H3K27ac-preferred (**E**) SEs. **F** and **G** Pie charts of genes with H4K5acK8ac-preferred (**F**) and H3K27ac-preferred (**G**) SEs in 0316-GSC are shown; the genes were identified by integrating the SE-associated genes with information in the Drug-Gene Interaction Database. **H**–**K** The indicated gene ontology (GO) molecular function and biological pathway categories were over-represented in JQ1-downregulated genes associated with H4K5acK8ac-preferred (left) or H3K27ac-preferred (right) SEs and TEs (**H** and **I**: SEs; **J** and **K**: TEs) in 0316-GSC cells. **L** TF-binding motifs within the H4K5acK8ac (left)- and H3K27ac (right)-ranked SEs. The motifs were found *de novo* by HOMER and then linked to the best matching known motifs. The occurrence panels represent the enrichment of the motif within 500 bp of H4K5acK8ac- and H3K27ac-ranked SEs.

Figure S6

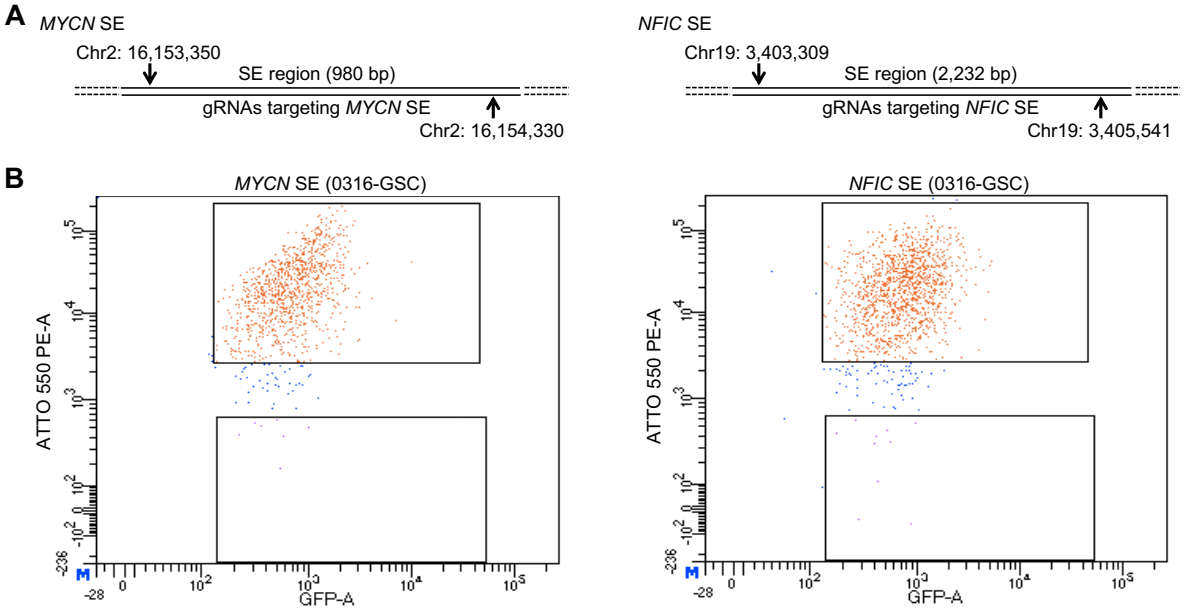

Figure S6. CRISPR-Cas9–mediated genome editing strategy of H4K5acK8ac-preferred super-enhancers (SEs) in 0316-GSC cells. A CRISPR-Cas9 knockout strategy for the SEs associated with *MYCN* and *NFIC* in 0316-GSC cells. Guide RNAs (gRNAs; vertical arrows) show the position for deletion of each SE. **B** Fluorescence-activated cell sorting of the SE-edited cells. CRISPR-Cas9–mediated knockout was performed by transfection of a ribonucleoprotein complex consisting of Cas9, crRNA (gRNA), and tracrRNA conjugated with ATTO 550. The sorted ATTO 550 positive cells (upper box) were subjected for further analysis.

Figure S7

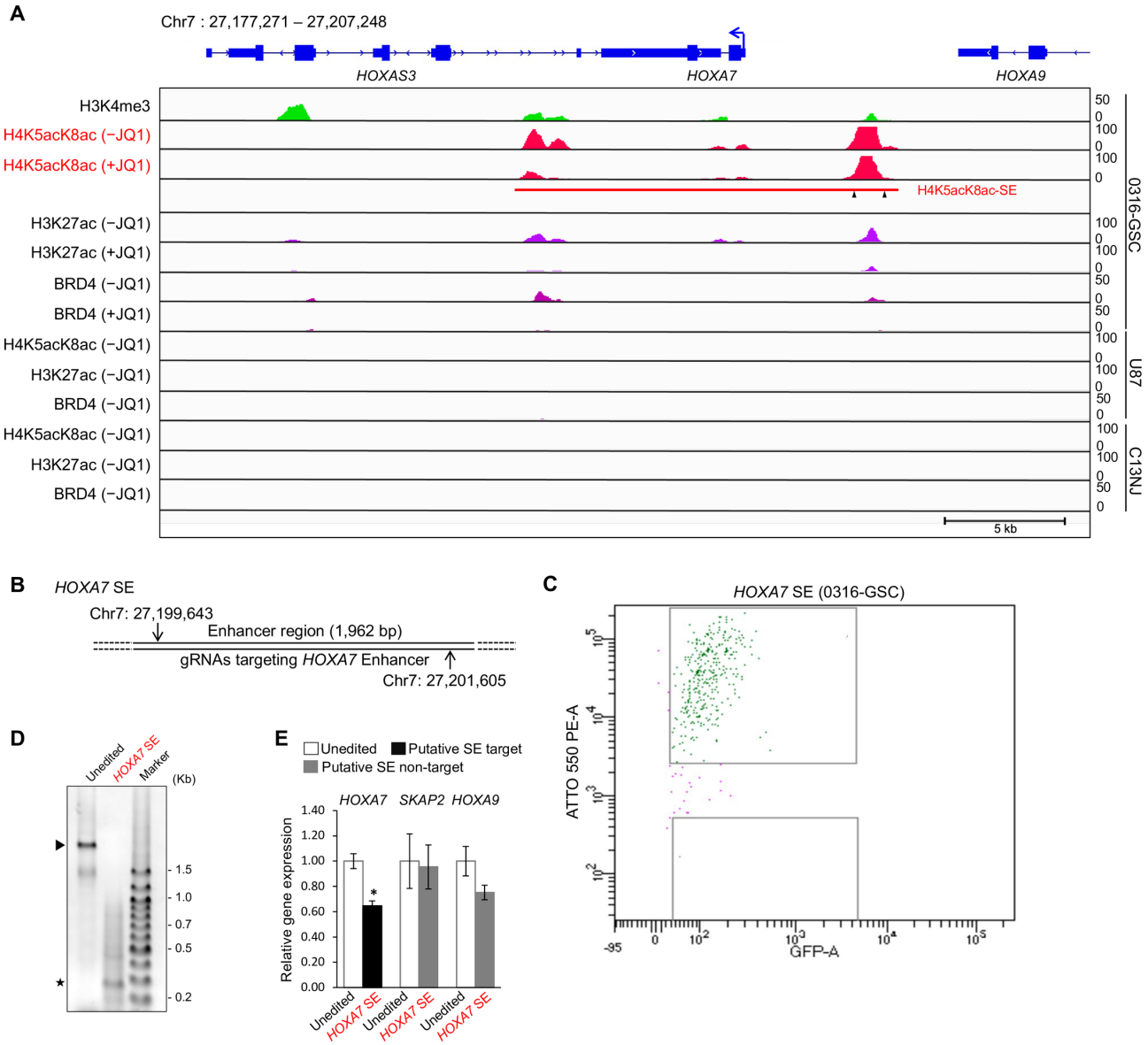

Figure S7. CRISPR-Cas9–mediated genome editing of *HOXA7* SE in 0316-GSC cells. A Representative ChIP-seq occupancy tracks of genes with H4K5acK8ac-preferred SE associated with *HOXA7*. The arrowheads under the SE bar show the position for CRISPR-Cas9–mediated deletion of the SE region. ChIP-seq reads were averaged from two biological replicates. **B** CRISPR-Cas9 knockout strategy. Guide RNAs (gRNAs; vertical arrows) show the position for deletion of each enhancer in SE. **C** Fluorescence-activated cell sorting of the SE-edited cells. CRISPR-Cas9–mediated knockout was performed by transfection of a ribonucleoprotein complex consisting of Cas9, crRNA (gRNA), and tracrRNA conjugated with ATTO 550. The sorted ATTO 550 positive cells (upper box) were subjected for further analysis. **D** Expected band sizes of genomic DNA for unedited (arrowhead) and enhancer-edited (asterisk) samples: the results show the deletion of the *HOXA7* SE. Image of the uncropped gel is shown in Figure S9B. **E** RT-qPCR analysis of the expression of putative target TF genes associated with SE and non-target genes (n= 3). Data are means ± SEM. *, $P < 0.05$ (two-tailed Student's *t*-test).

Figure S8

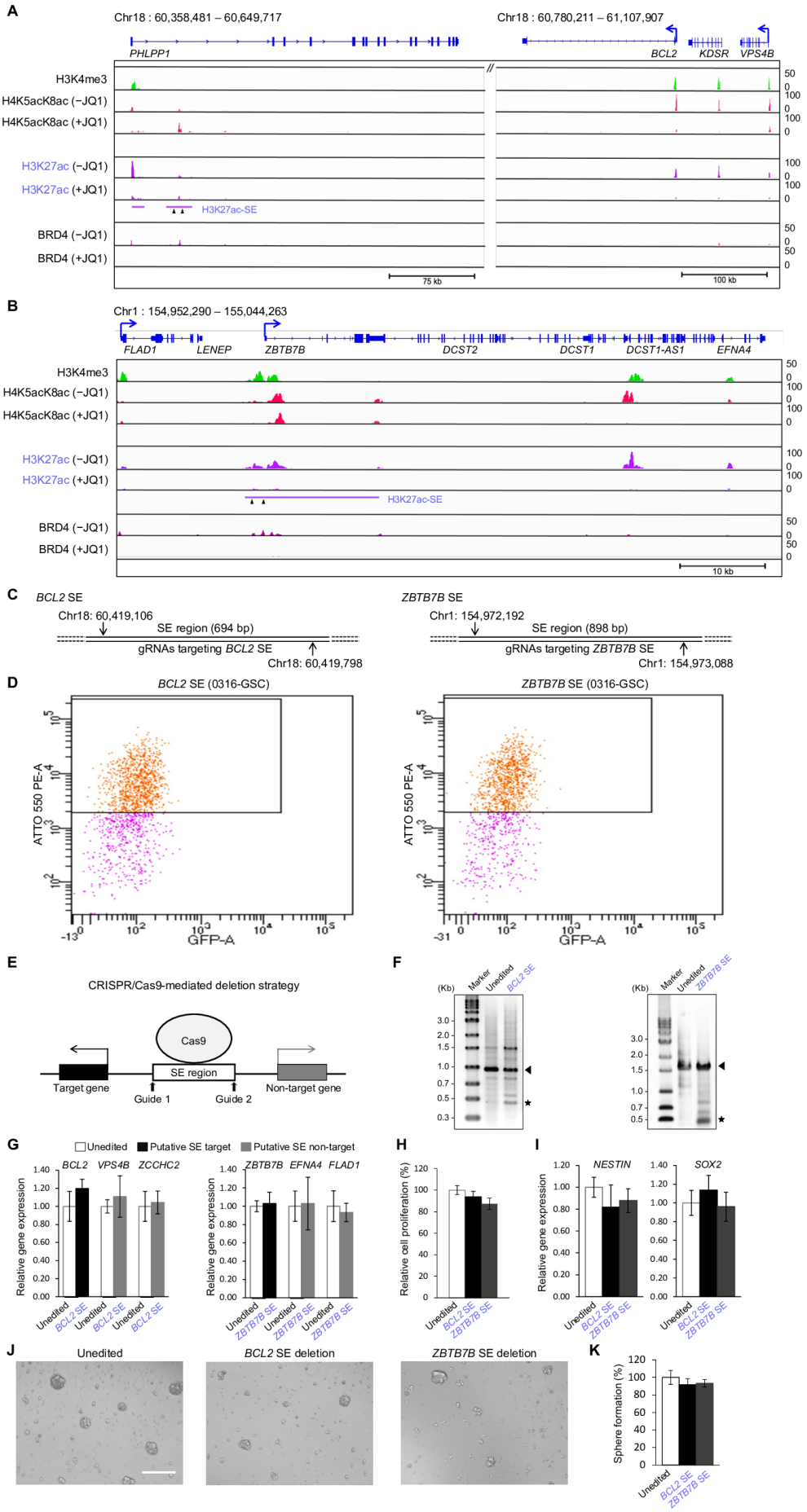
A
Chr18 : 60,358,481 – 60,649,717
Chr18 : 60,780,211 – 61,107,907
PHLPP1
BCL2
KDSR
VPS4B
H3K4me3
H4K5acK8ac (−JQ1)
H4K5acK8ac (+JQ1)
H3K27ac (−JQ1)
H3K27ac (+JQ1)
H3K27ac-SE
BRD4 (−JQ1)
BRD4 (+JQ1)
75 kb
100 kb
B
Chr1 : 154,952,290 – 155,044,263
FLAD1
LENEP
ZBTB7B
DCST2
DCST1
DCST1-AS1
EFNA4
H3K4me3
H4K5acK8ac (−JQ1)
H4K5acK8ac (+JQ1)
H3K27ac (−JQ1)
H3K27ac (+JQ1)
H3K27ac-SE
BRD4 (−JQ1)
BRD4 (+JQ1)
10 kb
C
BCL2 SE
Chr18: 60,419,106
SE region (694 bp)
gRNAs targeting BCL2 SE
Chr18: 60,419,798
ZBTB7B SE
Chr1: 154,972,192
SE region (898 bp)
gRNAs targeting ZBTB7B SE
Chr1: 154,973,088
D
BCL2 SE (0316-GSC)
ZBTB7B SE (0316-GSC)
ATTO 550 PE-A
GFP-A
E
CRISPR/Cas9-mediated deletion strategy
Cas9
SE region
Target gene
Guide 1
Guide 2
Non-target gene
F
(Kb)
Marker
Unedited
BCL2 SE
ZBTB7B SE
G
Unedited
Putative SE target
Putative SE non-target
BCL2
VPS4B
ZCCHC2
ZBTB7B
EFNA4
FLAD1
Relative gene expression
H
Relative cell proliferation (%)
I
NESTIN
SOX2
Relative gene expression
J
Unedited
BCL2 SE deletion
ZBTB7B SE deletion
K
Sphere formation (%)

Figure S8. CRISPR-Cas9–mediated genome editing of H3K27ac-preferred super-enhancers (SEs) in 0316-GSC cells. A ChIP-seq occupancy tracks of H3K27ac-preferred SEs of *BCL2*. **B** ChIP-seq occupancy tracks of H3K27ac-preferred SEs of *ZBTB7B*. Arrowheads under the SE bar show the position for CRISPR-Cas9–mediated deletion of the SE region. ChIP-seq reads were averaged from two biological replicates. **C** CRISPR-Cas9 knockout strategy. Guide RNAs (gRNAs; vertical arrows) show the position for deletion of each SE. **D** Fluorescence-activated cell sorting of the SE-edited cells. CRISPR-Cas9–mediated knockout was performed by transfection of a ribonucleoprotein complex consisting of Cas9, crRNA (gRNA), and tracrRNA conjugated with ATTO 550. The sorted ATTO 550 positive cells (upper box) were subjected for further analysis. **E** Schematic representation showing the CRISPR-Cas9–mediated genome editing approach for SEs. Guide indicates gRNA. **F** Expected band sizes of genomic DNA for unedited (arrowhead) and SE-edited (asterisk) samples: the results show the deletion of the SE associated with *BCL2* and *ZBTB7B*. Images of the uncropped gel are shown in Figure S9C. **G** RT-qPCR analysis of the expression of putative target TF genes associated with enhancers (En) in SEs and non-target genes (n= 3). **H** Cell proliferation rates at 4 days after SE deletion (n = 4). **I** Expression of stem cell marker genes, *NESTIN* (left) and *SOX2* (right), at 4 days after SE deletion (n = 3). **J** Phase-contrast images of 0316-GSC cells at 14 days after SE deletion. Images are representative of three independent experiments. Scale bar, 50 μm. **K** *In vitro* sphere formation efficiency of 0316-GSC cells at 2 weeks after SE deletion (n = 3).

Figure S9

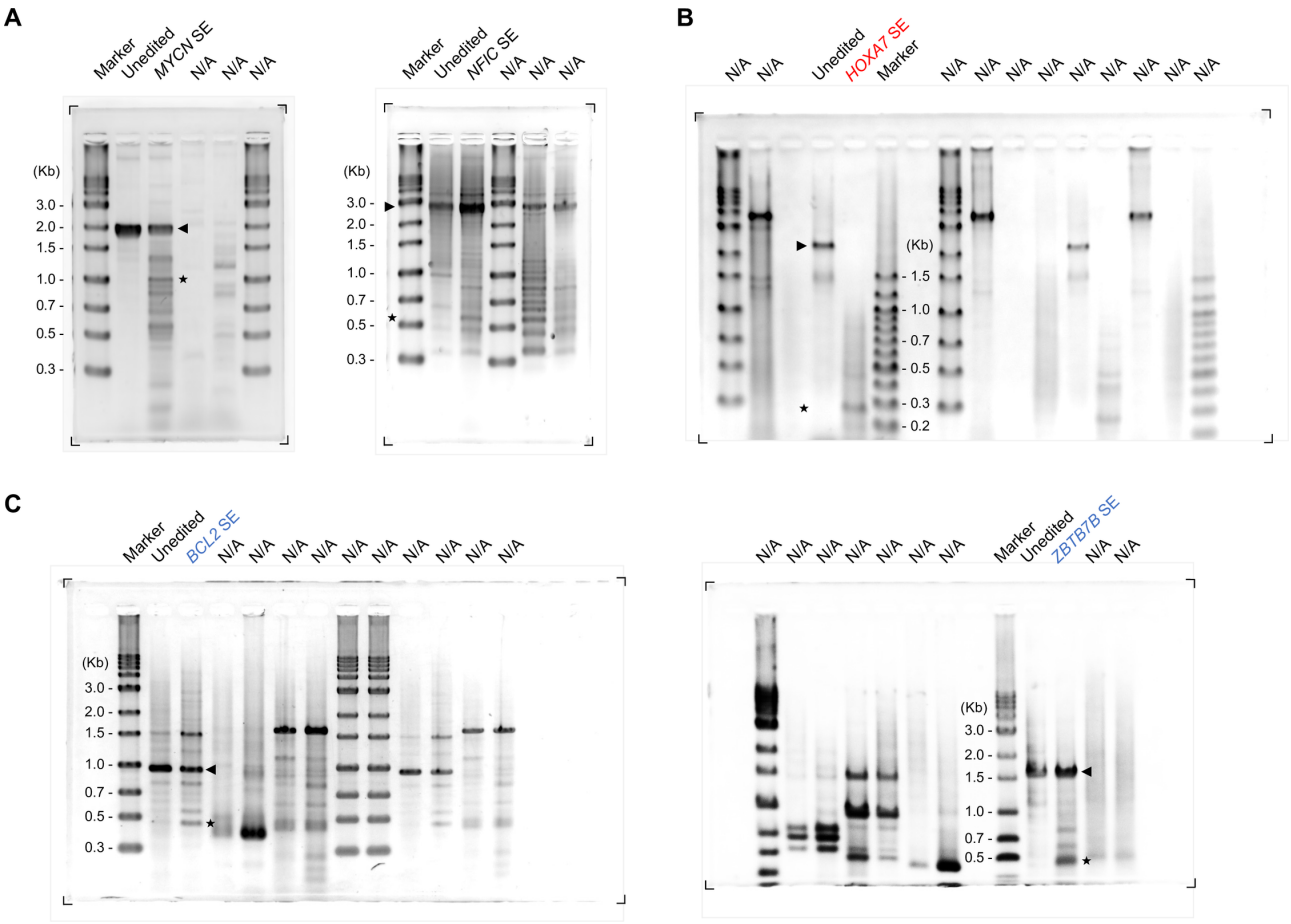

Figure S9. Source data for cropped gel images. A Figure 7B. **B** Figure S7D. **C** Figure S8F. N/A denotes not applicable. Edges of the blots are indicated by black corner brackets.