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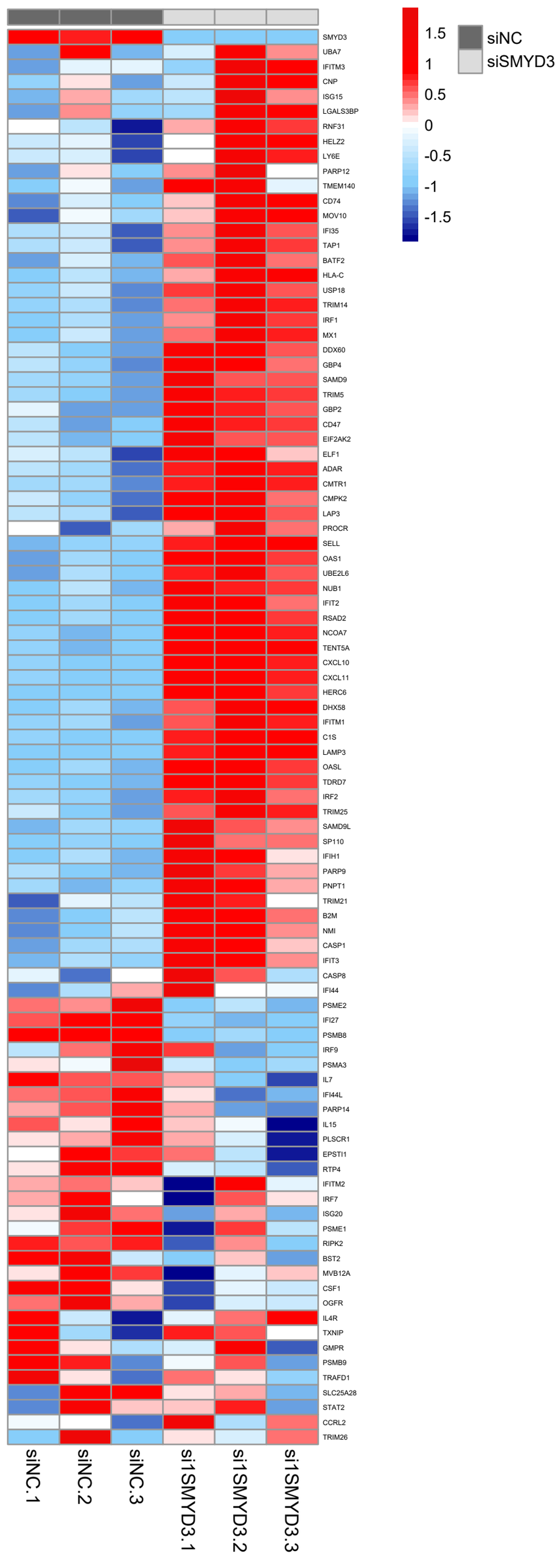
Supplemental information

SMYD3 represses tumor-intrinsic interferon response in HPV-negative squamous cell carcinoma of the head and neck

Nupur Nigam, Benjamin Bernard, Samantha Sevilla, Sohyoung Kim, Mohd Saleem Dar, Daniel Tsai, Yvette Robbins, Kyunghee Burkitt, Cem Sievers, Clint T. Allen, Richard L. Bennett, Theophilus T. Tettey, Benjamin Carter, Lorenzo Rinaldi, Mark W. Lingen, Houssein Sater, Elijah F. Edmondson, Arfa Moshiri, Abbas Saeed, Hui Cheng, Xiaolin Luo, Kevin Brennan, Vishal Koparde, Chen Chen, Sudipto Das, Thorkell Andresson, Abdalla Abdelmaksoud, Madhavi Murali, Seiji Sakata, Kengo Takeuchi, Raj Chari, Yusuke Nakamura, Ravindra Uppaluri, John B. Sunwoo, Carter Van Waes, Jonathan D. Licht, Gordon L. Hager, and Vassiliki Saloura

Supplementary Figure 1. Comprehensive RNA-seq heatmaps of HN-6 cells treated with siSMYD3 for 72h and IFN-β exposure for 24h. **(A)** IFNa GSEA gene set, **(B)** APM GSEA gene set.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LY6E
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CD74
MOV10
IFI35
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BATF2
HLA-C
USP18
TRIM14
IRF1
MX1
DDX60
GBP4
SAMD9
TRIM5
GBP2
CD47
EIF2AK2
ELF1
ADAR
CMTR1
CMPK2
LAP3
PROCR
SELL
OAS1
UBE2L6
NUB1
IFIT2
RSAD2
NCOA7
TENT5A
CXCL10
CXCL11
HERC6
DHX58
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C1S
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OASL
TDRD7
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SP110
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TRIM21
B2M
NMI
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IFIT3
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PSMA3
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PLSCR1
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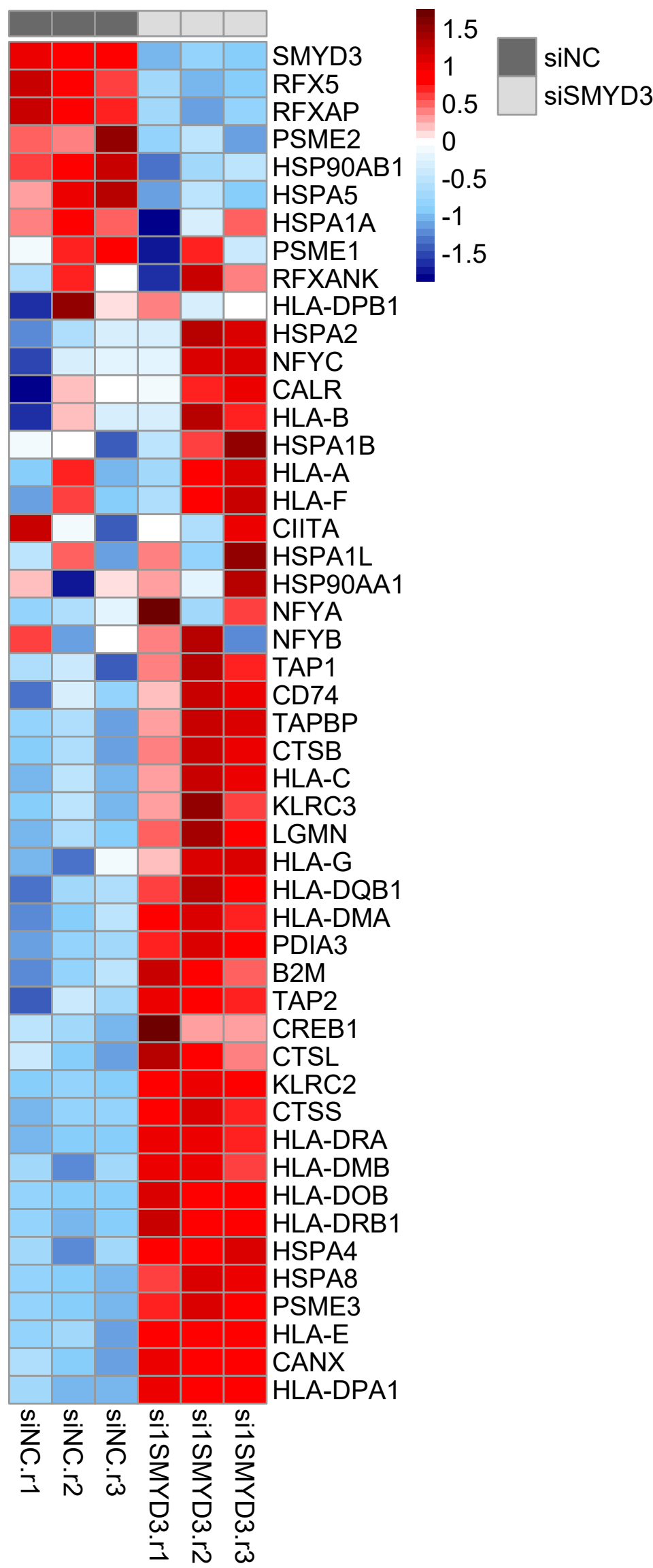
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PSME1
RFXANK
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NFYC
CALR
HLA-B
HSPA1B
HLA-A
HLA-F
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HSPA1L
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NFYA
NFYB
TAP1
CD74
TAPBP
CTSB
HLA-C
KLRC3
LGMN
HLA-G
HLA-DQB1
HLA-DMA
PDIA3
B2M
TAP2
CREB1
CTSL
KLRC2
CTSS
HLA-DRA
HLA-DMB
HLA-DOB
HLA-DRB1
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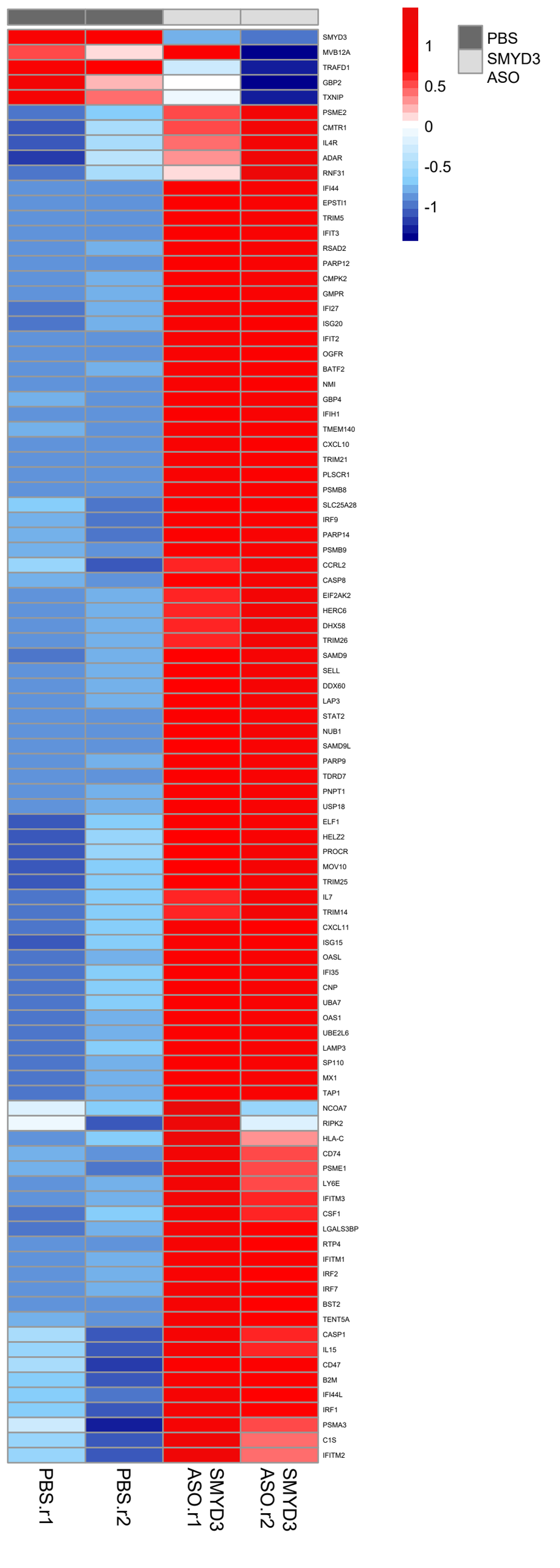
si1SMYD3.r3

Supplementary Figure 2. Comprehensive RNA-seq heatmaps of HN-6 cells treated with PBS or SMYD3 ASOs for 72h and IFN-β exposure for 24h. (A) IFNa GSEA gene set, **(B)** APM GSEA gene set.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CMTR1
IL4R
ADAR
RNF31
IFI44
EPSTI1
TRIM5
IFIT3
RSAD2
PARP12
CMPK2
GMPR
IFI27
ISG20
IFIT2
OGFR
BATF2
NMI
GBP4
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TMEM140
CXCL10
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PARP14
PSMB9
CCRL2
CASP8
EIF2AK2
HERC6
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SELL
DDX60
LAP3
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MX1
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RIPK2
HLA-C
CD74
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LY6E
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CSF1
LGALS3BP
RTP4
IFITM1
IRF2
IRF7
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TENT5A
CASP1
IL15
CD47
B2M
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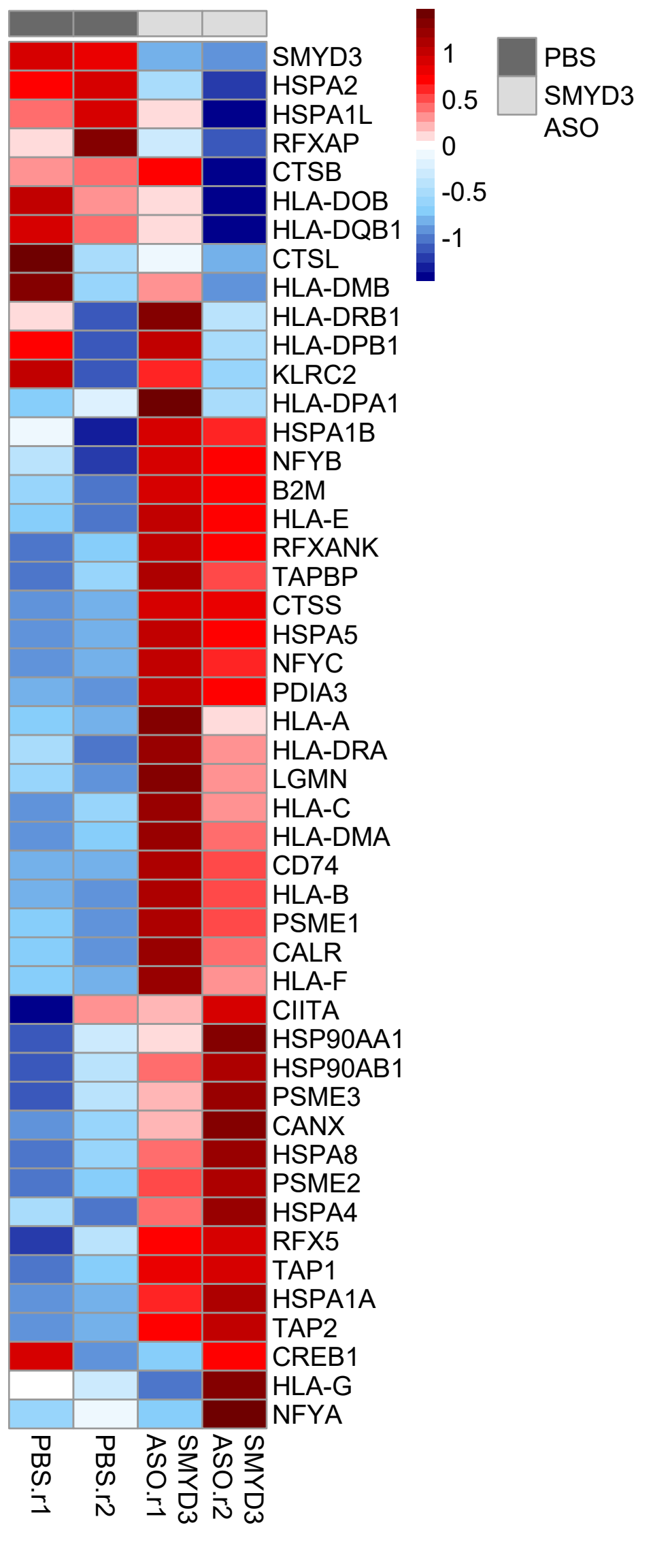
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HLA-DQB1
CTSL
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KLRC2
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HSPA1B
NFYB
B2M
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RFXANK
TAPBP
CTSS
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NFYC
PDIA3
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LGMN
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TAP2
CREB1
HLA-G
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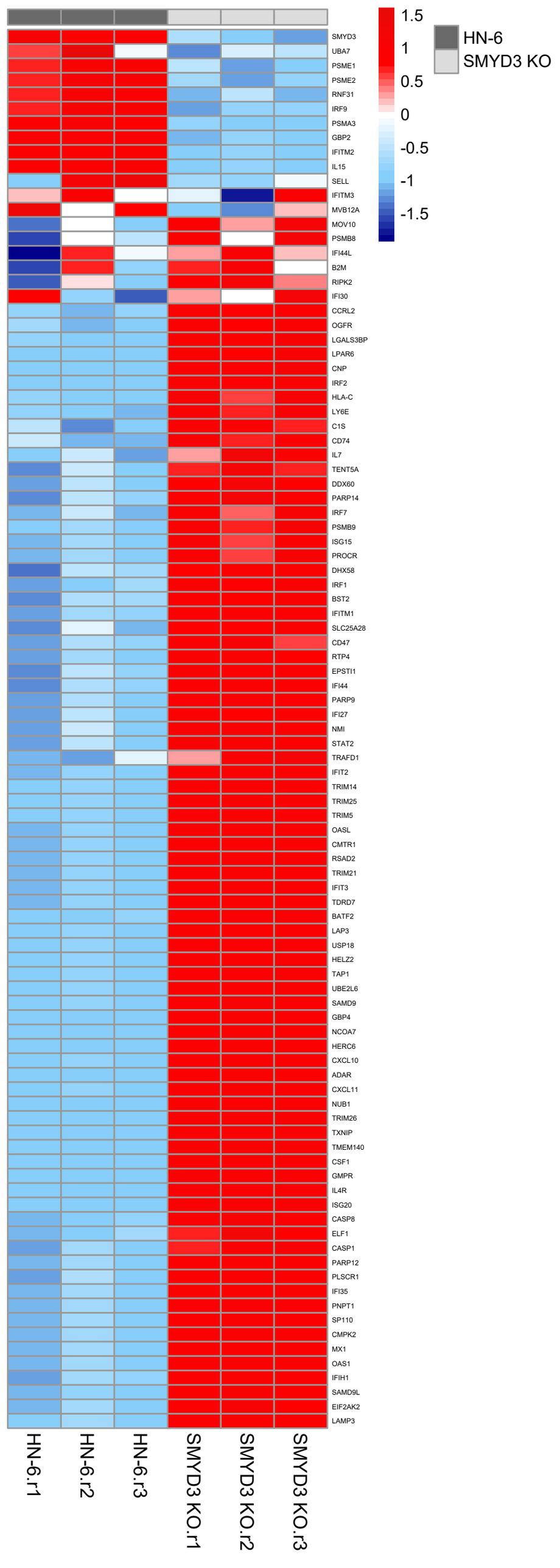
PBS.r1
PBS.r2
ASO.r1
SMYD3
ASO.r2
SMYD3

Supplementary Figure 3. Comprehensive RNA-seq heatmaps of a CRISPR SMYD3 KO cell line (clone 5-3) exposed to IFN-β for 24h. (A) IFNa GSEA gene set, **(B)** APM GSEA gene set.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SELL
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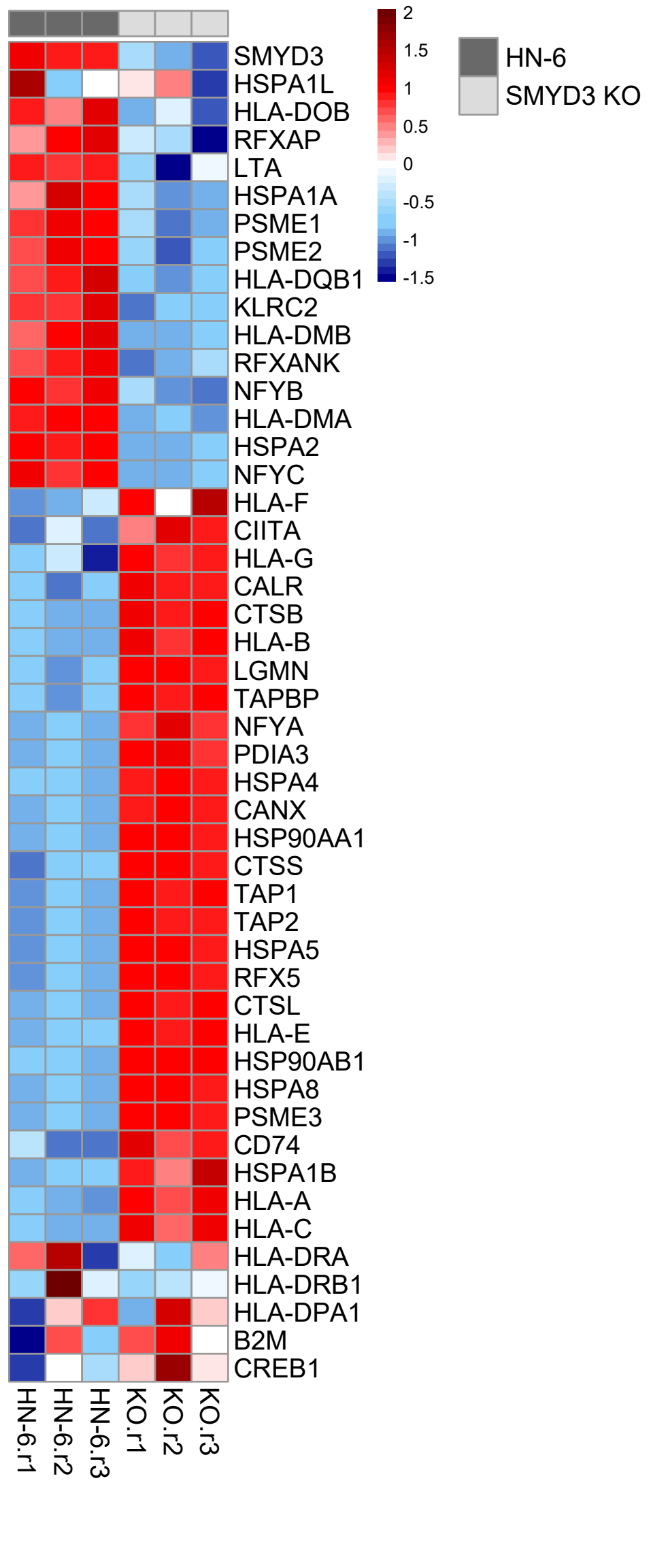
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PSME2
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KLRC2
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CTSL
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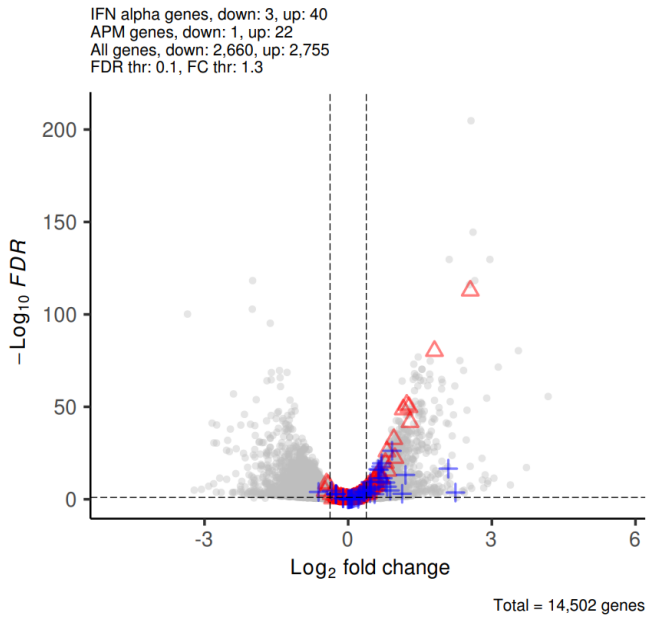
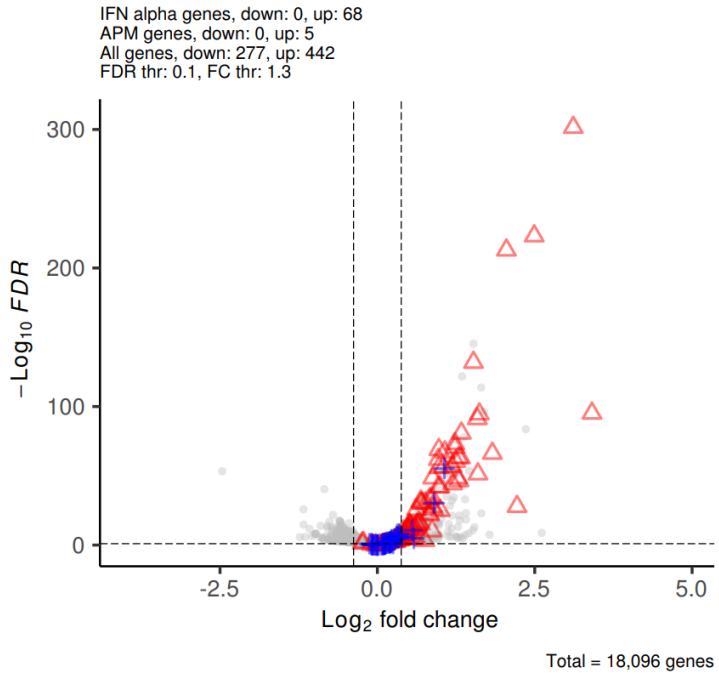
HLA-DRB1
HLA-DPA1
B2M
CREB1
HN-6.r1
HN-6.r2
HN-6.r3
KO.r1
KO.r2
KO.r3

Supplementary Figure 4. Volcano plots showing DESeq2 results of RNA-seq in HN-6 cells treated with siSMYD3 or SMYD3 ASOs. Volcano plot in siSMYD3 **(A)** and SMYD3 ASO **(B)** treated **(B)** HN-6 cells for 72h, exposed to IFN-β for 24h. FDR: 0.1, log2FC threshold: log2 (1.3). Red triangles: IFNa genes (from GSEA gene set), blue crosses: APM genes (from GSEA gene set), gray circles: other genes.

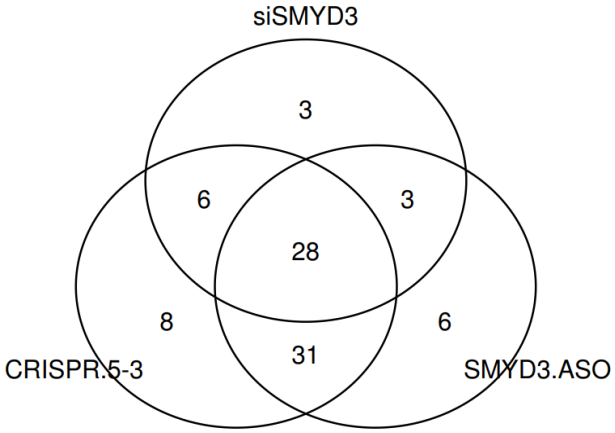
(A) siSMYD3 volcano plot:



(B) SMYD3 ASO volcano plot:

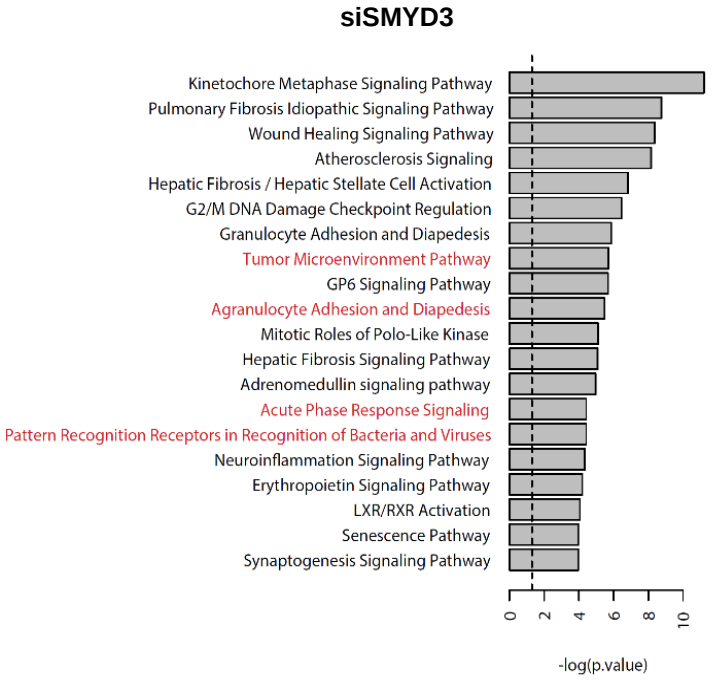


Supplementary Figure 5. Commonly upregulated IFNa response genes in HN-6 cells treated with siSMYD3 or SMYD3 ASOs for 72h, and in a SMYD3 KO cell line (CIRPSR.5-3), exposed to IFN-β for 24h. Number of common, significantly (FDR<0.1, log2 fold change>abs(log2(1.3)) upregulated IFNa response genes among 97 Hallmark IFNa response genes (GSEA).

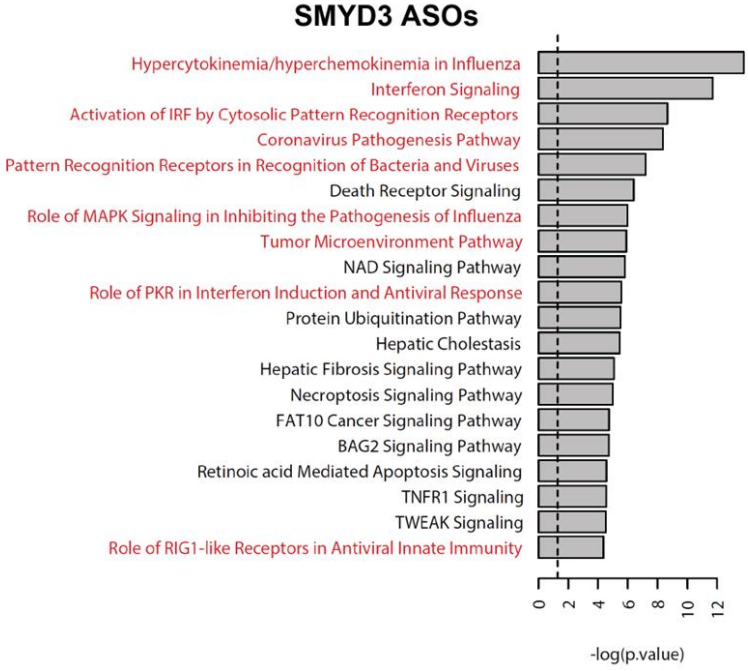


Supplementary Figure 6. Ingenuity Pathway Analysis (IPA) of RNA-seq of HN-6 cells after SMYD3 depletion. IPA reveals enrichment of pathways related to inflammation in HN-6 cells treated with siSMYD3 **(A)** or SMYD3 ASOs **(B)** for 72h and IFN-β exposure for 24h.

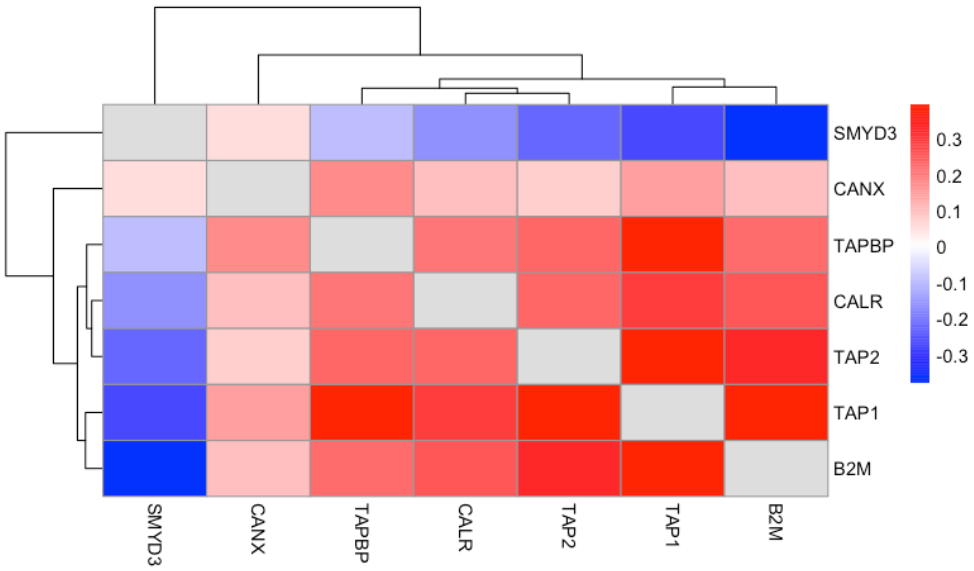
(A)



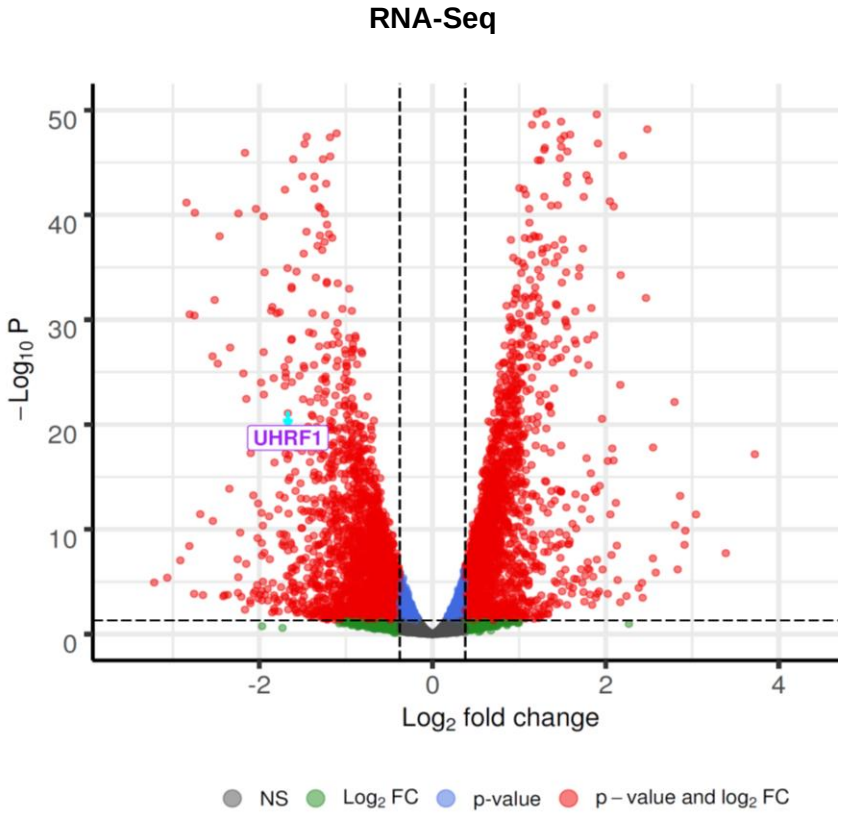
(B)



Supplementary Figure 7. Pairwise correlations between *SMYD3* and *APM* mRNA levels in HPV-negative cancer cells. A publicly available single cell RNA-seq database of HPV-negative HNSCC tumors was utilized to associate mRNA levels of *SMYD3* with *APM* genes in HPV-negative HNSCC cancer cells.

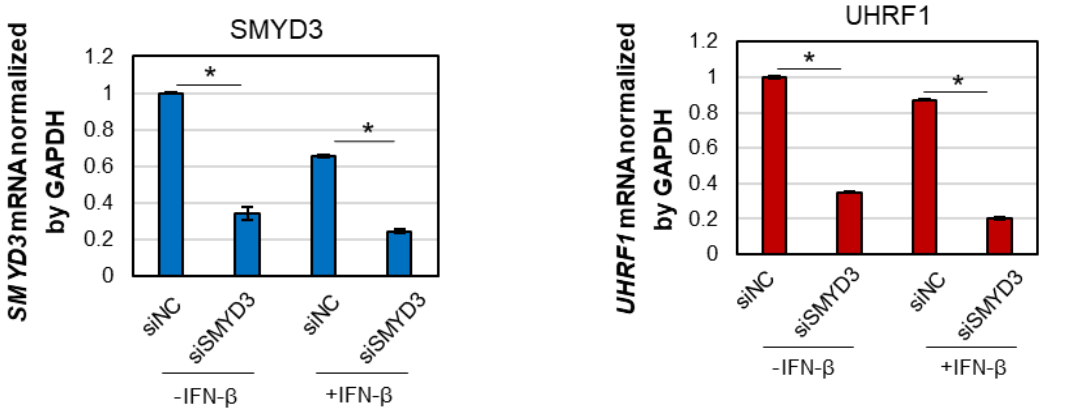


Supplementary Figure 8. Volcano plot of RNA-seq in HN-6 cells treated with negative control or SMYD3-targeting siRNA for 3 days in the presence of IFN-β. *UHRF1* mRNA highlighted in purple font. Log2 FC: log2 fold change>1.3.

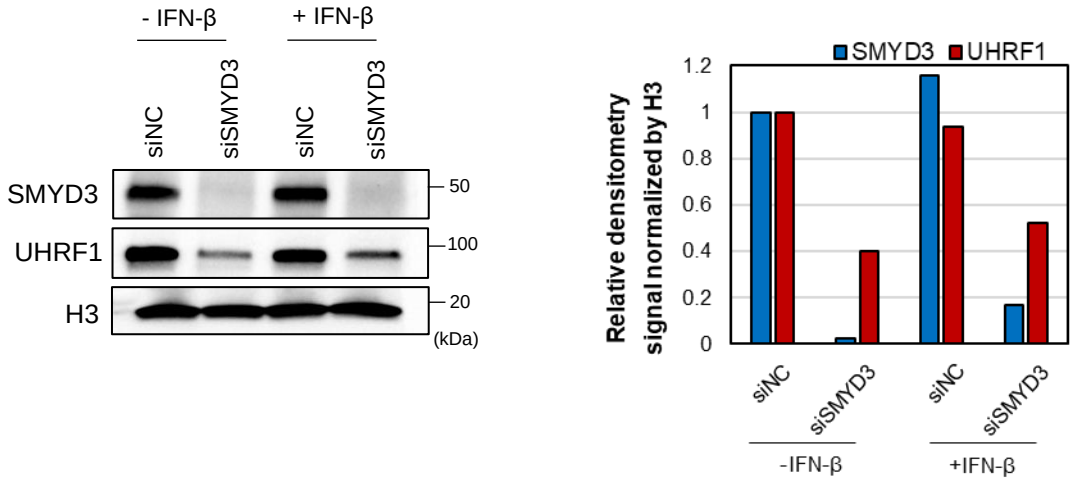


Supplementary Figure 9. SMYD3 knockdown is associated with downregulation of UHRF1 in HN-SCC-151 cells at the mRNA and protein levels. HN-SCC-151 cells were treated with siNC or siSMYD3 for 72h, in the presence or absence of IFN-β for 24h prior to cell collection. RNA and nuclear protein extraction (Active Motif) were conducted in two separate biological replicates. **(A)** qPCR for *SMYD3* and *UHRF1* mRNA in cells treated with siNC or siSMYD3 for 72h in the presence or absence of IFN-β. mRNA levels were normalized by GAPDH. Data represent the mean +/- SEM of three technical replicates. **(B)** Western blotting of nuclear extracts for SMYD3 (10ug) and UHRF1 (15ug). H3 was used as a loading control. Densitometry results are shown on the right side of the blot. Similar results were obtained in a separate biological replicate.

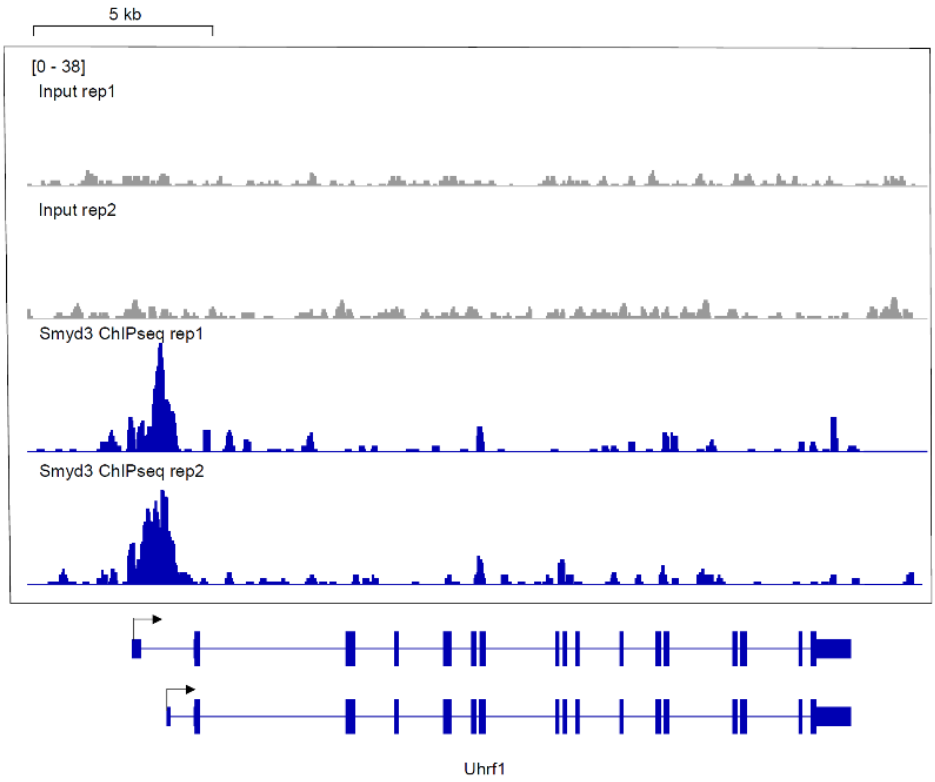
(A)



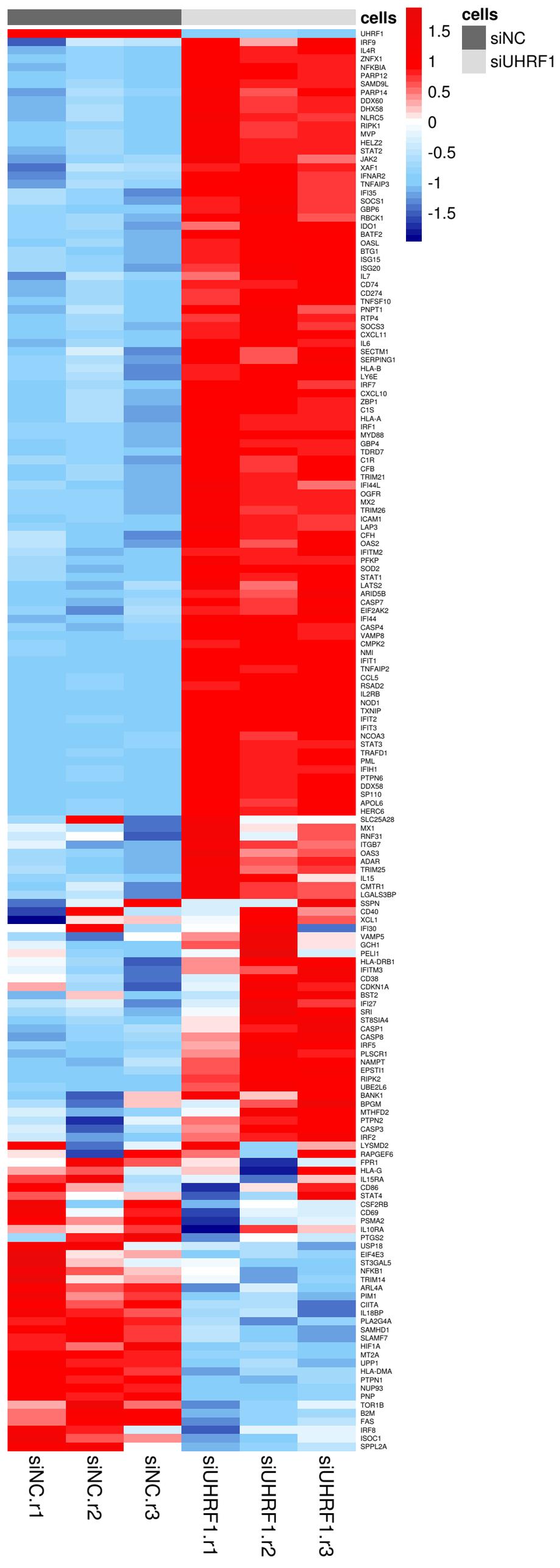
(B)



Supplementary Figure 10. Mouse Smyd3 occupies the TSS of *Uhrf1* in mouse hepatocellular carcinoma cells. USCS tracks of Smyd3 ChIP in mouse hepatocellular carcinoma cells focusing on the mouse *Uhrf1* gene locus. Tracks show enrichment of Smyd3 at the *Uhrf1* TSS.



Supplementary Figure 11. **Comprehensive RNA-seq heatmaps of HN-6 cells treated with siUHRF1 for 72h and IFN-β exposure for 24h. (A)** IFNa GSEA gene set, **(B)** APM GSEA gene set.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PARP12
SAMD9L
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DDX60
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NLRC5
RIPK1
MVP
HELZ2
STAT2
JAK2
XAF1
IFNAR2
TNFAIP3
IFI35
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ZBP1
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IL15
CMTR1
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SSPN
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XCL1
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VAMP5
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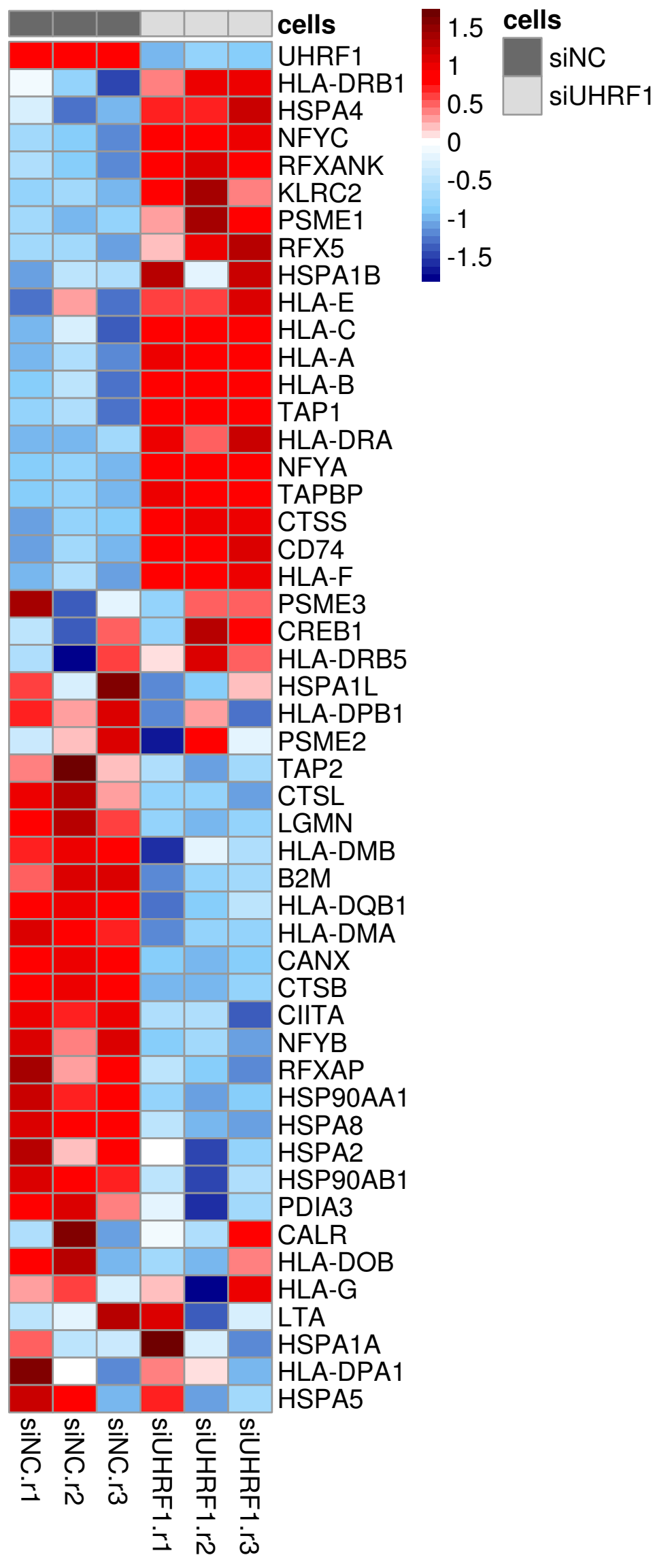
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NFYC
RFXANK
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PSME1
RFX5
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HLA-C
HLA-A
HLA-B
TAP1
HLA-DRA
NFYA
TAPBP
CTSS
CD74
HLA-F
PSME3
CREB1
HLA-DRB5
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CTSL
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CTSB
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HLA-DPA1
HSPA5
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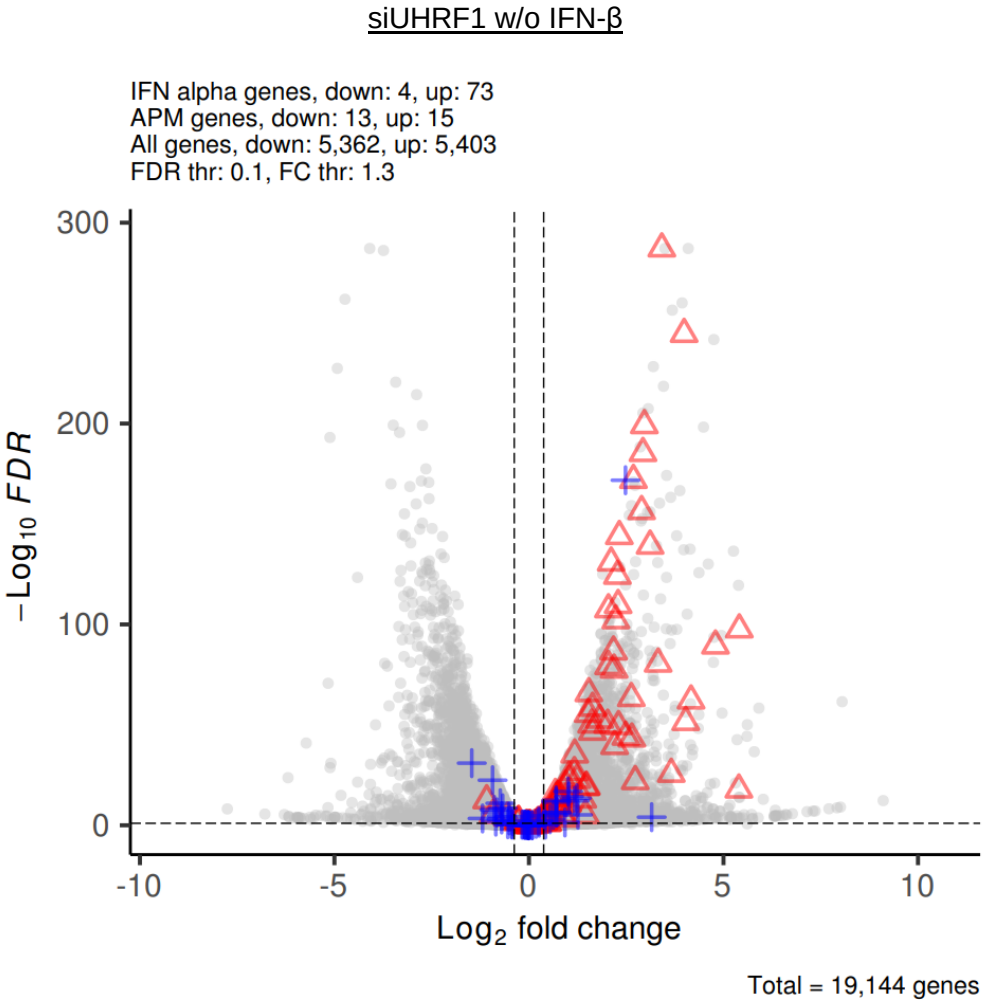
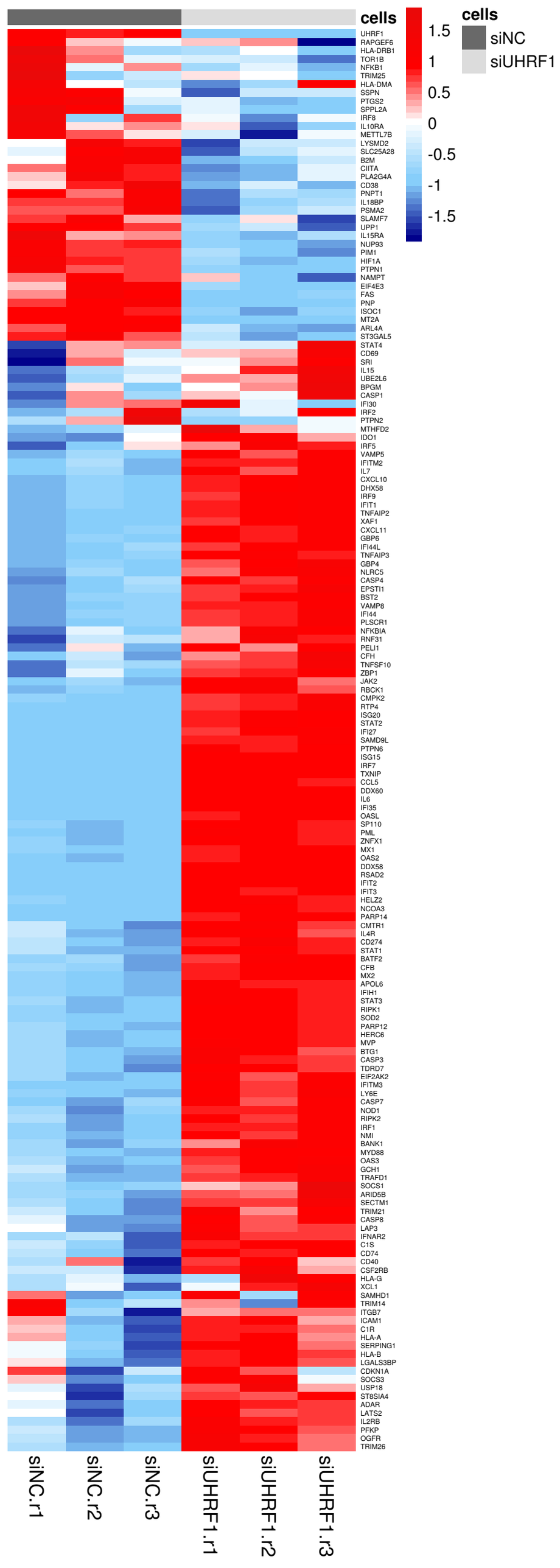
siNC.r2
siNC.r3
siUHRF1.r1
siUHRF1.r2
siUHRF1.r3

Supplementary Figure 12. Upregulation of immune-related genes after UHRF1 depletion in HN-6 cells in the absence of IFN-β. (A) Volcano plot showing DESeq2 results of RNA-seq of HN-6 cells transfected with a UHRF1-targeting siRNA compared to control for 72h without exposure to IFN-β. FDR < 0.1, log2FC threshold: log2 (1.3). Red triangles: IFNa genes (from GSEA gene set, upregulated: 73, downregulated: 4), blue crosses: APM genes (from GSEA gene set, upregulated: 15, downregulated: 13), gray circles: other genes. Total number of genes=19,144 (upregulated: 5,403, downregulated: 5,362). **(B)** Comprehensive RNA-seq heatmaps of type I IFN response **(B)** and APM genes **(C)** in HN-6 cells after UHRF1 depletion for 3 days without IFN-β. HN-6 cells were treated with control siRNAs or a UHRF1-targeting siRNA for 72h (three biological replicates per condition, siNC.r : control replicate, siUHRF1.r: UHRF1 siRNA replicate). Cells were collected at 72h and RNA-seq was conducted. Heatmaps showing z-score of variance stabilizing transformed expression values.

(A)

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-1.5
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TOR1B
NFKB1
TRIM25
HLA-DMA
SSPN
PTGS2
SPPL2A
IRF8
IL10RA
METTL7B
LYSMD2
SLC25A28
B2M
CIITA
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CD38
PNPT1
IL18BP
PSMA2
SLAMF7
UPP1
IL15RA
NUP93
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EIF4E3
FAS
PNP
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ARL4A
ST3GAL5
STAT4
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VAMP5
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IL7
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IRF9
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GBP4
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CASP4
EPSTI1
BST2
VAMP8
IFI44
PLSCR1
NFKBIA
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PELI1
CFH
TNFSF10
ZBP1
JAK2
RBCK1
CMPK2
RTP4
ISG20
STAT2
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PTPN6
ISG15
IRF7
TXNIP
CCL5
DDX60
IL6
IFI35
OASL
SP110
PML
ZNFX1
MX1
OAS2
DDX58
RSAD2
IFIT2
IFIT3
HELZ2
NCOA3
PARP14
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IL4R
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STAT1
BATF2
CFB
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APOL6
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STAT3
RIPK1
SOD2
PARP12
HERC6
MVP
BTG1
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TDRD7
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IFITM3
LY6E
CASP7
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IRF1
NMI
BANK1
MYD88
OAS3
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TRAFD1
SOCS1
ARID5B
SECTM1
TRIM21
CASP8
LAP3
IFNAR2
C1S
CD74
CD40
CSF2RB
HLA-G
XCL1
SAMHD1
TRIM14
ITGB7
ICAM1
C1R
HLA-A
SERPING1
HLA-B
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CDKN1A
SOCS3
USP18
ST8SIA4
ADAR
LATS2
IL2RB
PFKP
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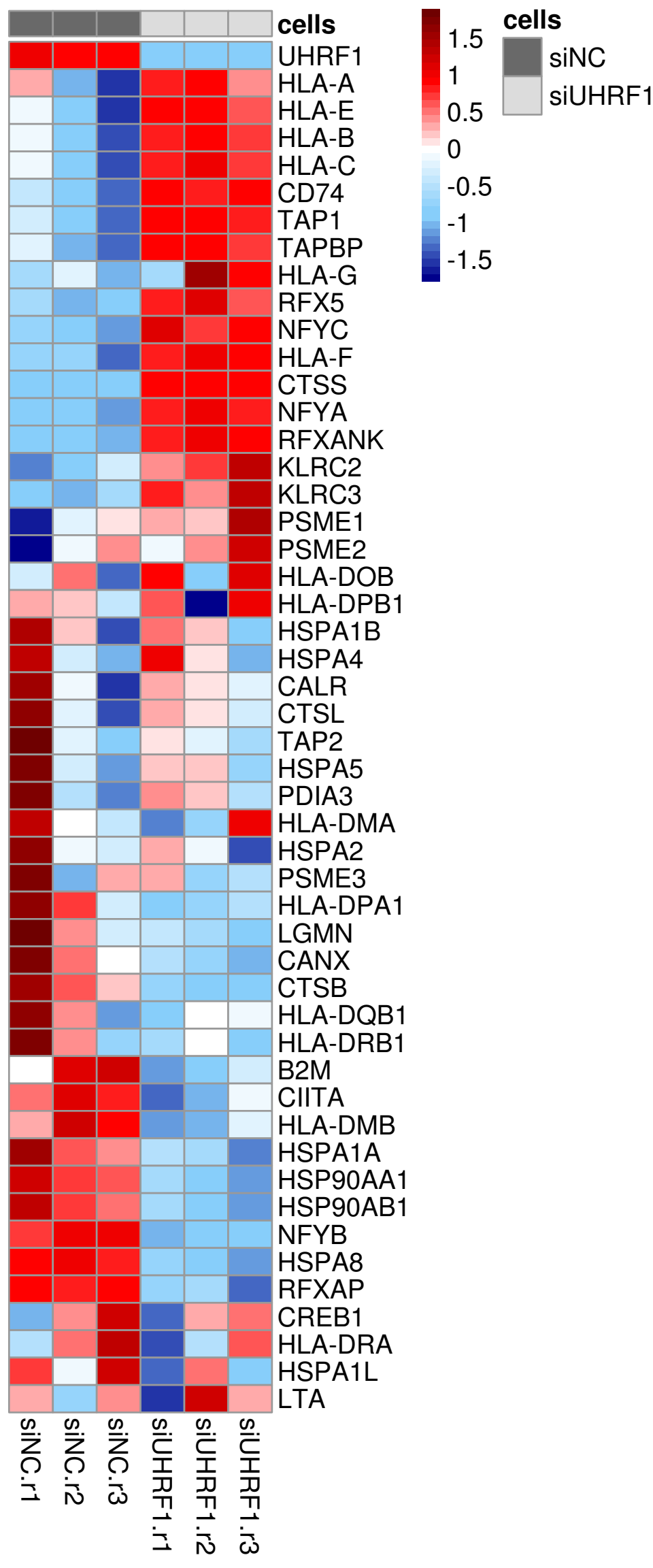
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0.5
0
-0.5
-1
-1.5
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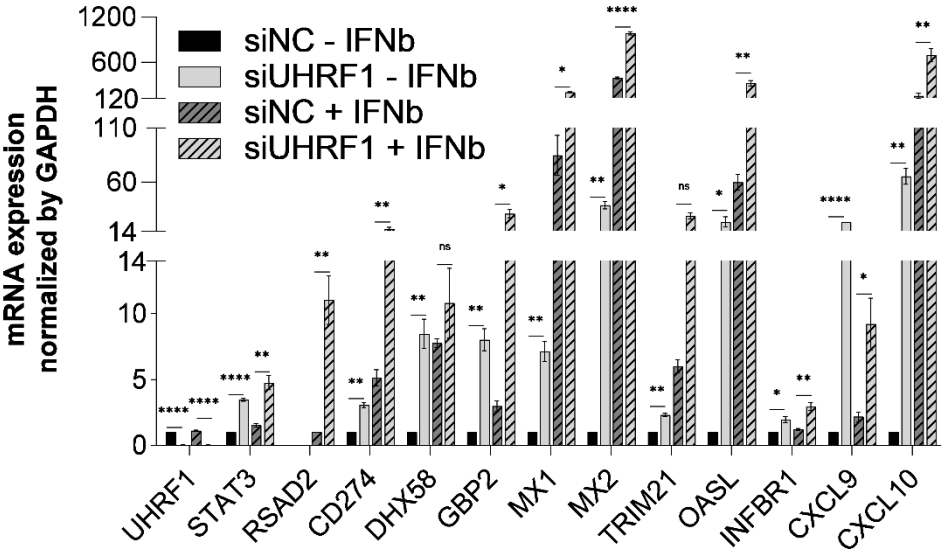
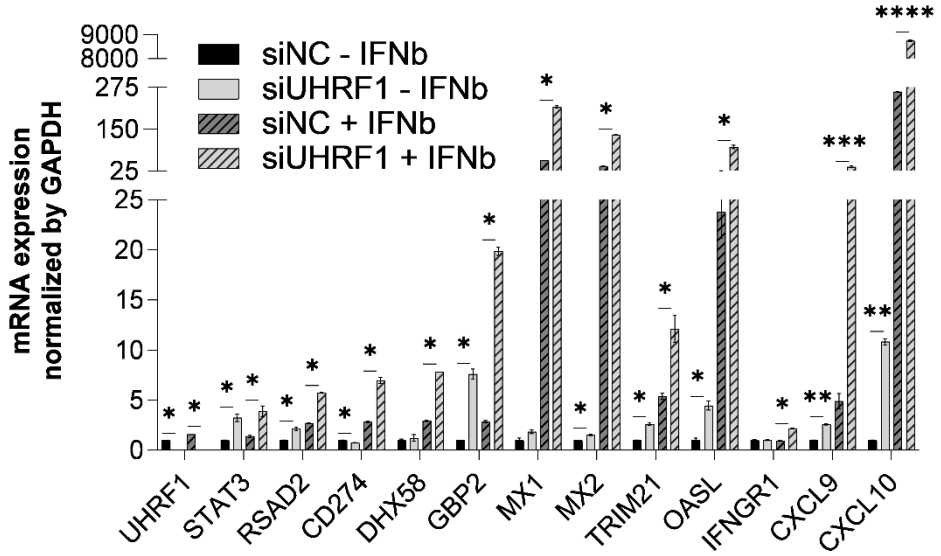
HLA-E
HLA-B
HLA-C
CD74
TAP1
TAPBP
HLA-G
RFX5
NFYC
HLA-F
CTSS
NFYA
RFXANK
KLRC2
KLRC3
PSME1
PSME2
HLA-DOB
HLA-DPB1
HSPA1B
HSPA4
CALR
CTSL
TAP2
HSPA5
PDIA3
HLA-DMA
HSPA2
PSME3
HLA-DPA1
LGMN
CANX
CTSB
HLA-DQB1
HLA-DRB1
B2M
CIITA
HLA-DMB
HSPA1A
HSP90AA1
HSP90AB1
NFYB
HSPA8
RFXAP
CREB1
HLA-DRA
HSPA1L
LTA
siNC.r1
siNC.r2
siNC.r3
siUHRF1.r1
siUHRF1.r2
siUHRF1.r3

Supplementary Figure 13. qPCRs confirming upregulation of immune-related genes after UHRF1 depletion in HN-6 and HN-SCC-151 cells in the presence or absence of IFN-β. HN-6 **(A)** or HN-SCC-151 **(B)** cells were transfected with siNC or siUHRF1 siRNAs for 72h, and exposed to 1000U/ml of IFN-β or not at 48h after transfection. Cells were collected for RNA extraction and cDNA synthesis. SYBR green qPCR was conducted. Data represent mean +/- SEM. Similar results were obtained with two biological replicates.

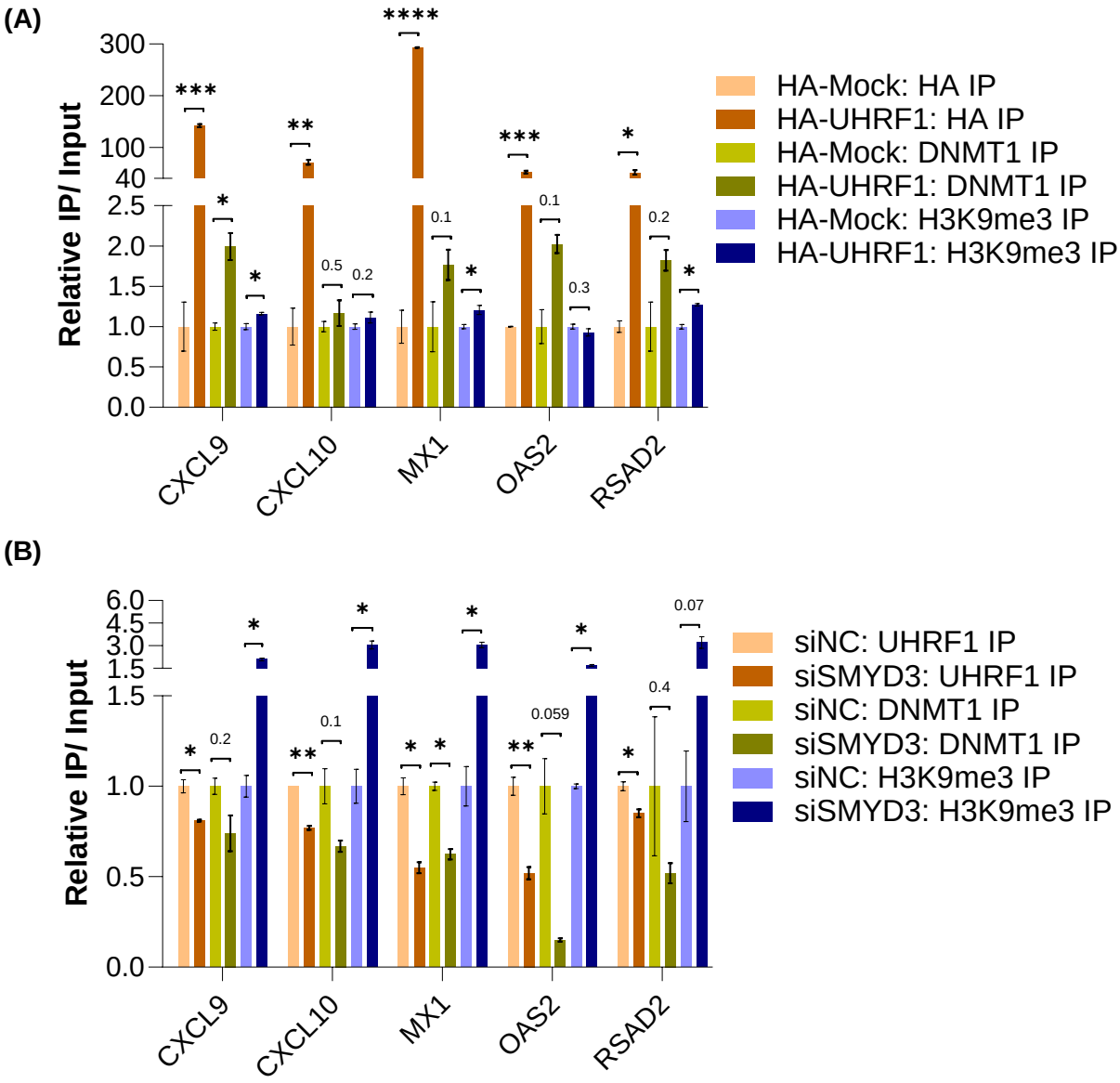
(A) HN-6



(B) HN-SCC-151

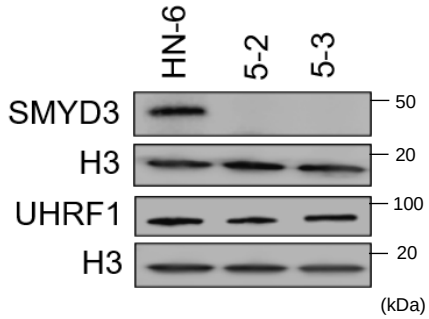


Supplementary Figure 14. Second biological replicate of ChIP assays for HA-UHRF1, DNMT1 and H3K9me3 in an HA-UHRF1 overexpression system, and for UHRF1, DNMT1 and H3K9me3 in a UHRF1 knockdown system. (A) Separate biological replicate of ChIP assay for HA (shades of brown), endogenous DNMT1 (shades of green) and endogenous H3K9me3 (shades of purple) followed by qPCR for *CXCL9*, *CXCL10*, *MX1*, *OAS2* and *RSAD2*. HN-6 cells were transfected with HA-Mock or HA-UHRF1 for 48h and exposed to IFN-β for 24h prior to cell collection. Standard error (SE) bars represent the SE of two technical replicates per reaction. Student t-test, *p<0.05, ** p<0.01, ***p<0.001. **(B)** Separate biological replicate of ChIP assay for endogenous UHRF1 (shades of brown), DNMT1 (shades of green) and H3K9me3 (shades of purple) followed by qPCR for *CXCL9*, *CXCL10*, *MX1*, *OAS2* and *RSAD2*. HN-6 cells were transfected with negative control (siNC) or a SMYD3-targeting siRNA (siSMYD3) for 72h and exposed to IFN-β for 24h prior to cell collection. Standard error (SE) bars represent the SE of two technical replicates per reaction. Data represent mean +/- SEM. Similar results were obtained in a second biological replicate. Student t-test, *p<0.05, ** p<0.01, ***p<0.001.

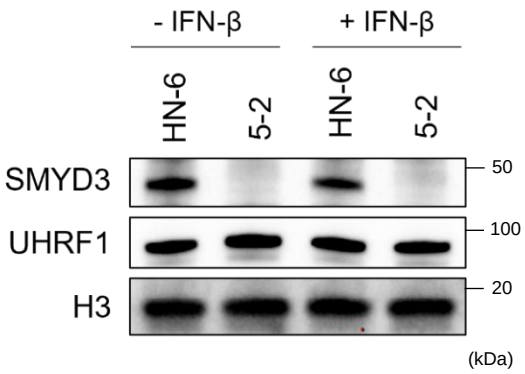


Supplementary Figure 15. UHRF1 protein levels are stable SMYD3 KO cell lines compared to the parental cell line regardless of the presence or absence of IFN-β. Western blotting for SMYD3 and UHRF1 in HN-6 and SMYD3 KO cell lines 5-2 and 5-3 in the absence of IFN-β **(A)** and in the presence and absence of IFN-β in 5-2 cells **(B)**. Cells were collected and nuclear extraction was conducted followed by Western blotting for SMYD3 and UHRF1. 10ug for SMYD3 and 15ug for UHRF1 of nuclear extract were loaded for all conditions. H3 was used as a loading control.

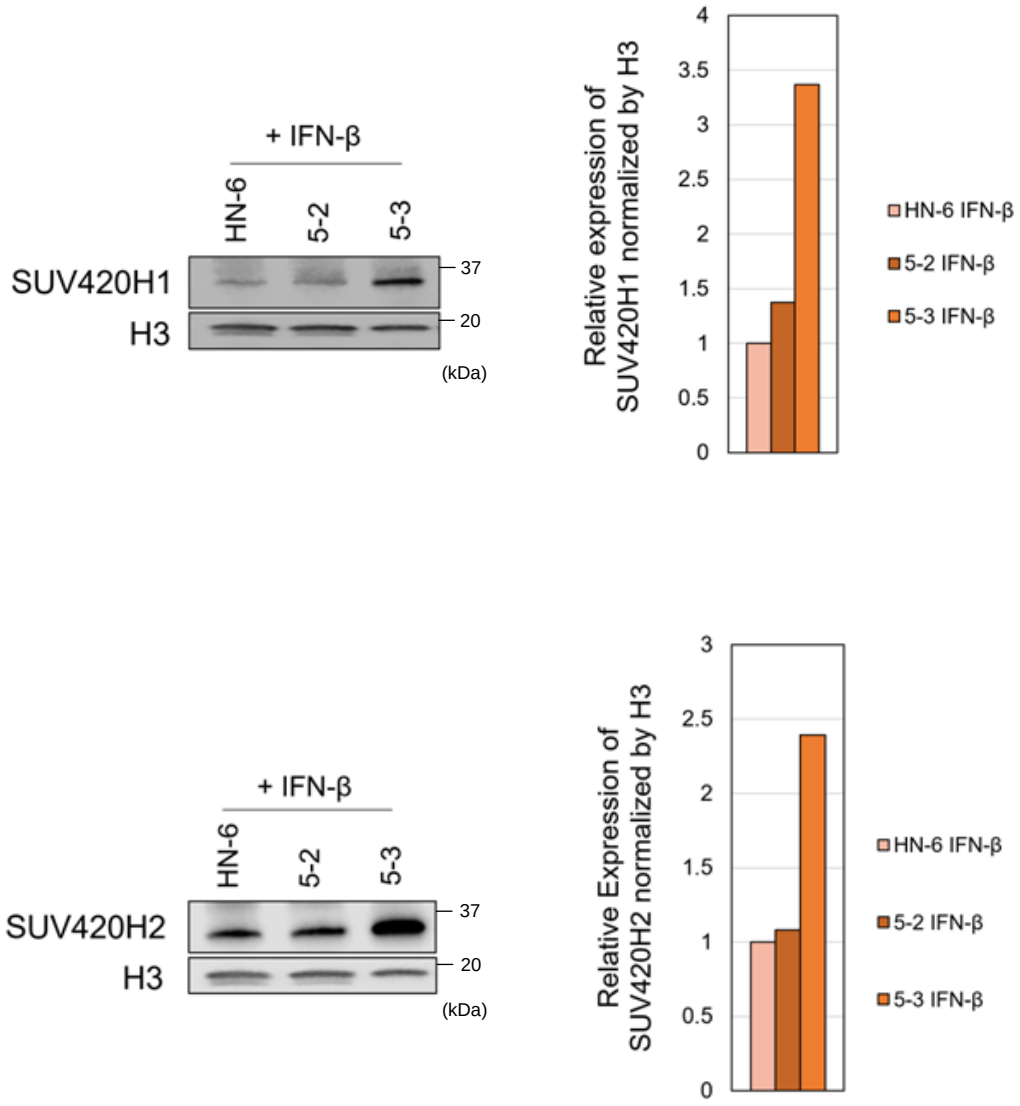
(A)



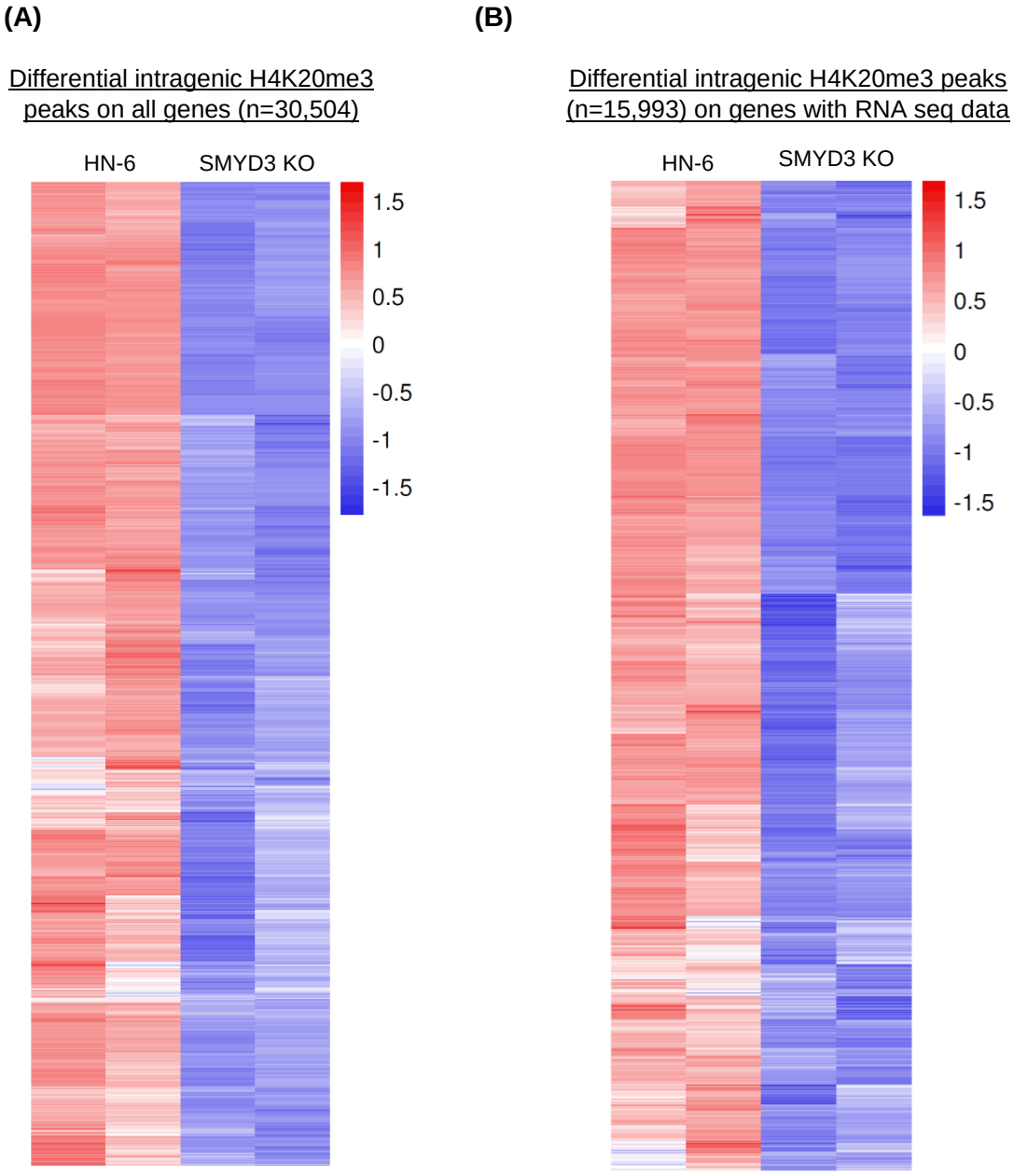
(B)



Supplementary Figure 16. Protein expression levels of SUV420H1/2 in HN-6, 5-2 and 5-3 cells. Western blotting for SUV420H1 (KMT5B) and SUV420H2 (KMT5C) in HN-6 and two SMYD3 KO cell lines, 5-2 and 5-3. 15ug of nuclear extract were loaded, and H3 was used as a loading control. Densitometry results are shown on the right side of each blot.

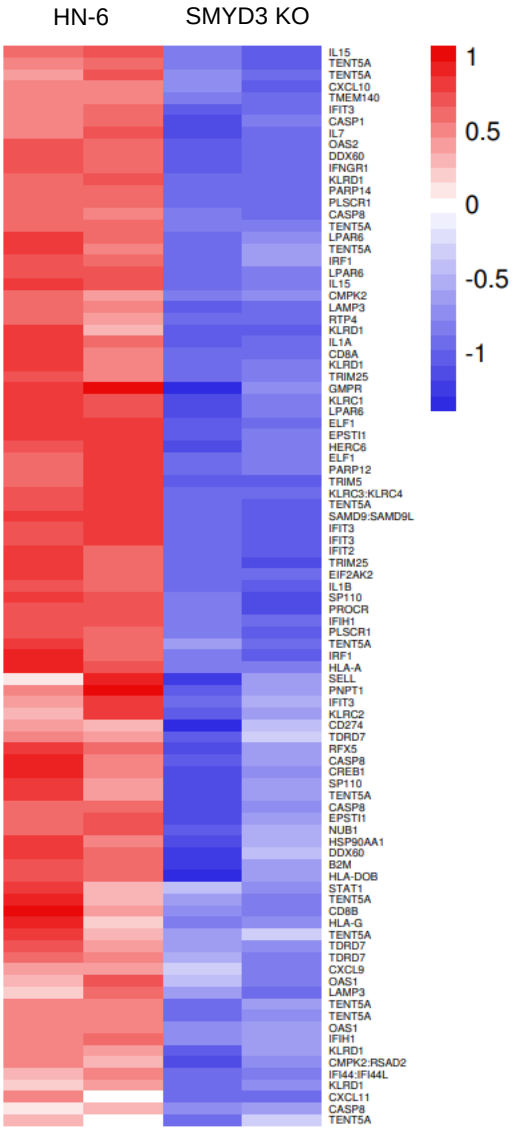


Supplementary Figure 17. Heatmaps of differential intragenic H4K20me3 peaks corresponding to genes with evaluable RNA-seq data. (A) Heatmap of all differential intragenic peaks (n=30,504) annotated to 9,273 genes. **(B)** Heatmap of differential intragenic H4K20me3 peaks (n=15,993) annotated to the promoters, TSS, introns, exons and 5' and 3'UTRs of 4,874 genes with evaluable RNA-seq expression data, comparing HN-6 and 5-3. Two biological replicates are shown. **(C)** Heatmap of differential intragenic peaks (n=93) annotated to 60 immune-related genes. **(D)** Heatmap of differential H4K20me3 peaks (n=84) annotated to the promoters, TSS, introns, exons and 5' and 3'UTRs of 54 immune-related genes with evaluable RNA-seq expression data comparing HN-6 and 5-3. One representative peak per gene is shown. Two biological replicates are shown. FDR<0.05, abs.log2FC (1.3).



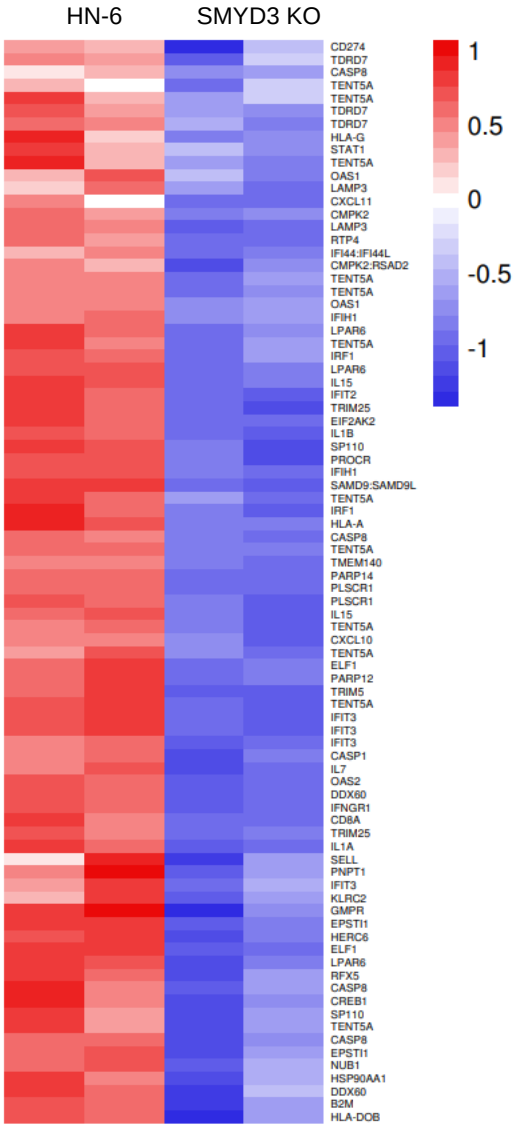
(C)

Differential intragenic H4K20me3 peaks on immune-related genes (n=93)

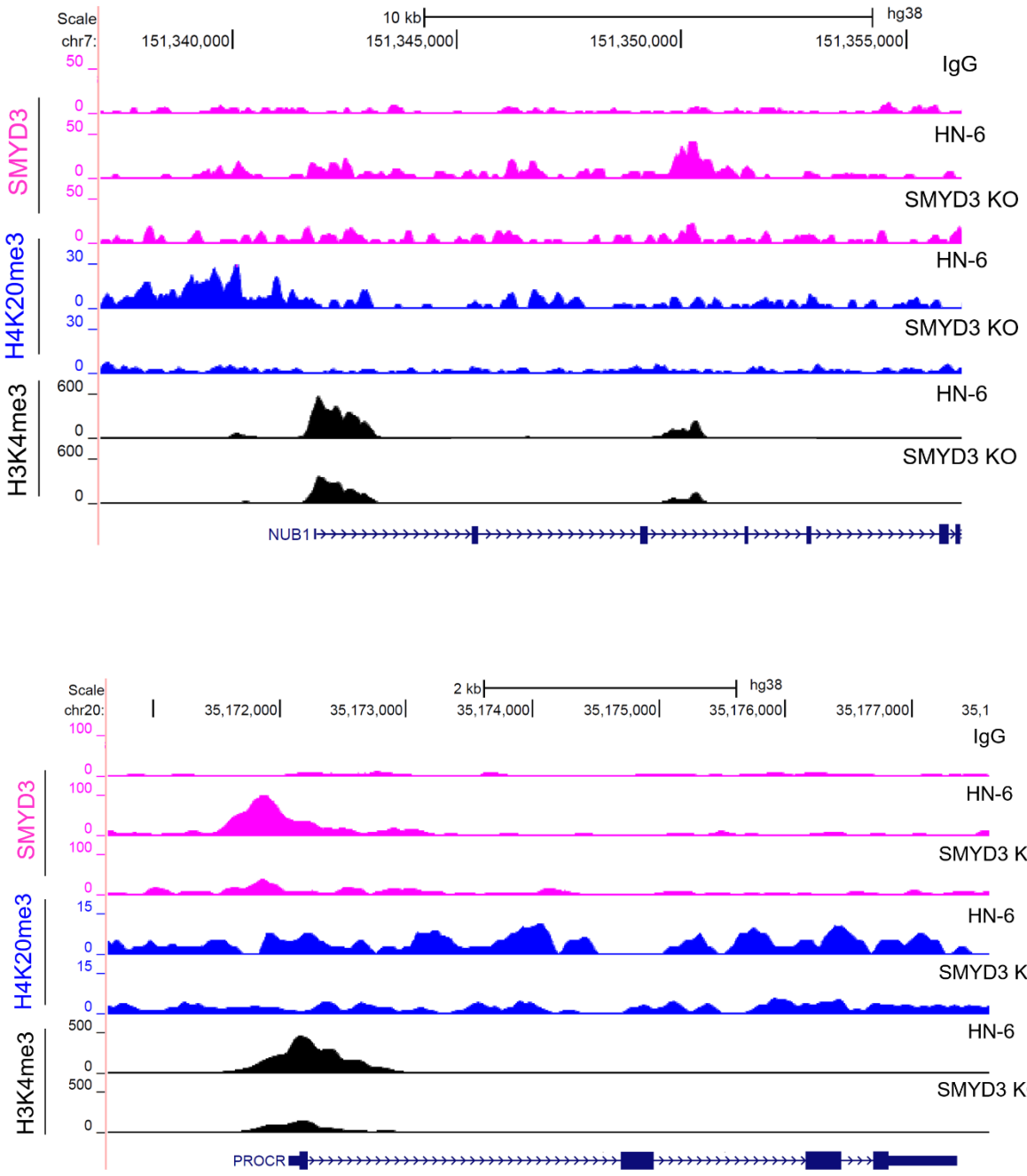


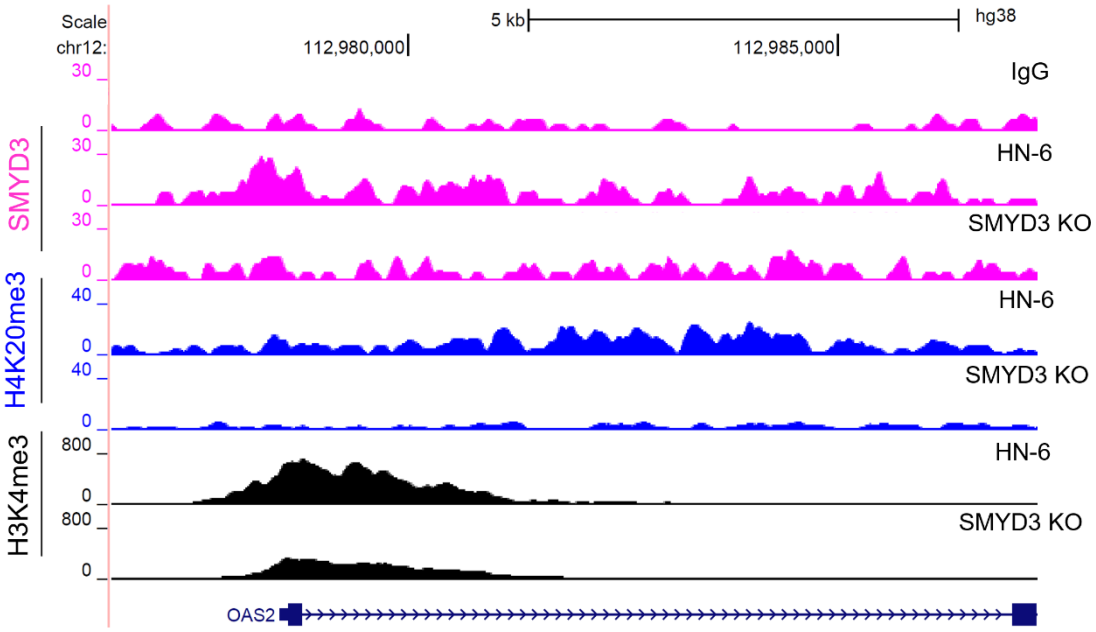
(D)

Differential intragenic H4K20me3 peaks (n=84) on immune-related genes with RNA-seq data



Supplementary Figure 18. Examples of UCSC tracks of representative immune-related genes. The tracks of *NUB1*, *PROCR* and *OAS2* are shown below.

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112,985,000
IgG
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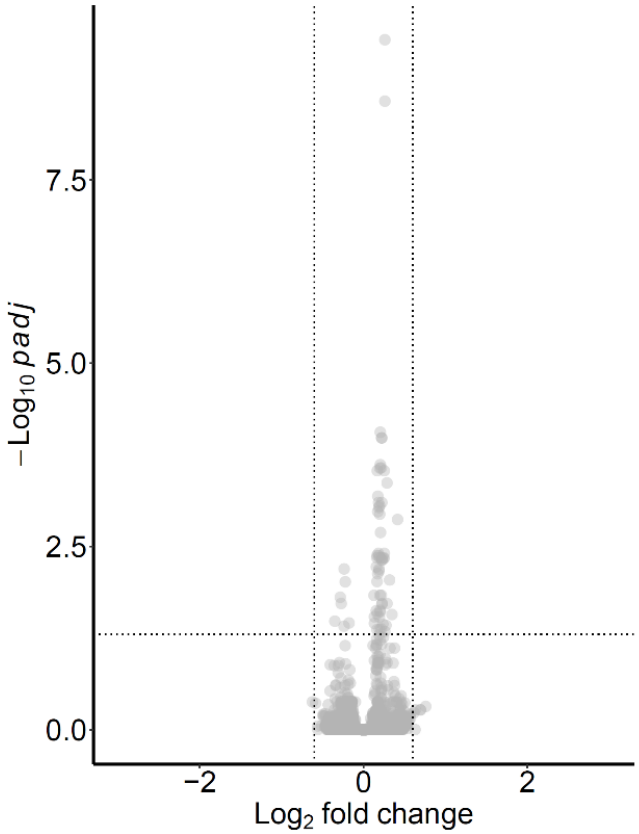
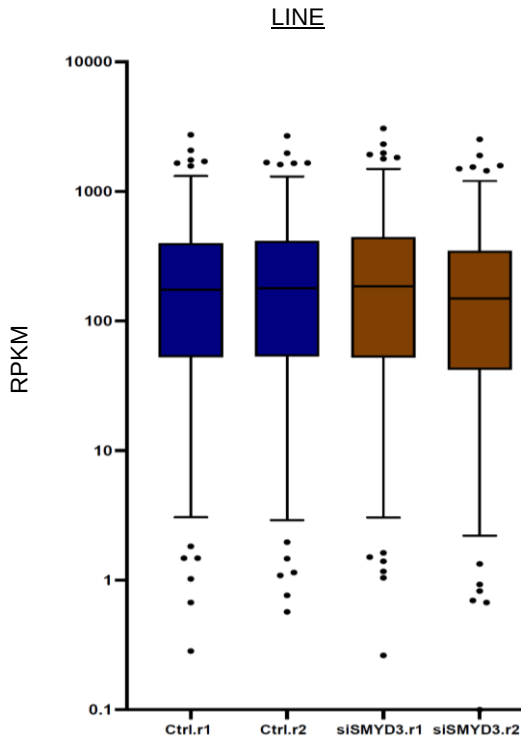
SMYD3 KO
HN-6
SMYD3 KO
HN-6
SMYD3 KO
SMYD3
H4K20me3
H3K4me3
30
0
30
0
30
0
40
0
40
0
800
0
800
0
OAS2

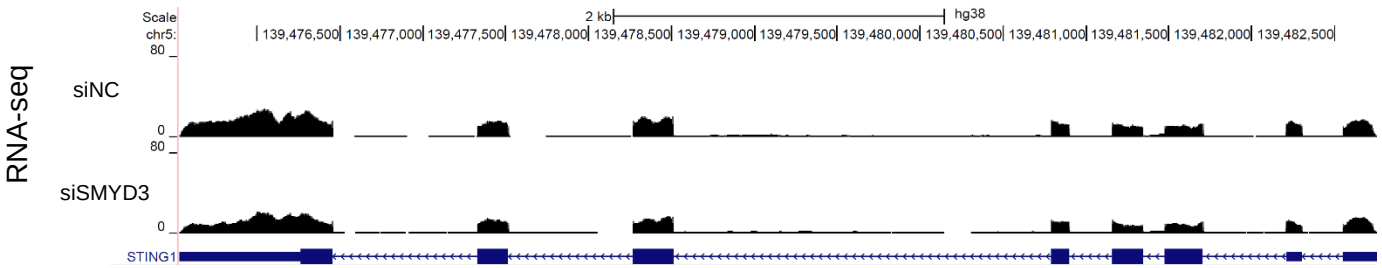
Supplementary Figure 19. Volcano plot of DESeq2 results of H4K20me3 intragenic peaks. CUT&RUN assay for H4K20me3 was conducted in HN-6 cells treated with siNC or siSMYD3 siRNAs for 3 days and exposed to IFN-β for 24h. FDR threshold: 0.05.



Supplementary Figure 20. Boxplot of LINE transposable elements in HN-6 cells treated with siNC or siSMYD3 in the presence of IFN-β. RNA-seq retrotransposon quantification and analysis was performed using homer and bedtools. HN-6 cells were treated with siNC or siSMYD3 siRNAs for 3 days and for 24h with IFN-β prior to collection. RNA was extracted and mRNA-seq was performed. RPKM; per million mapped reads.

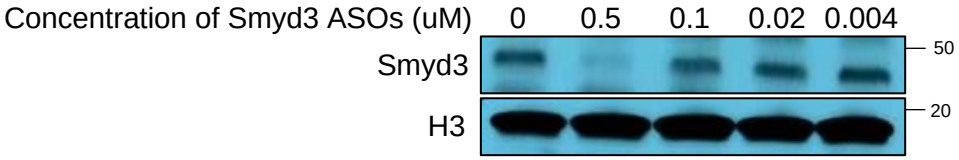


Supplementary Figure 21. Tracks representing mRNA expression levels of *STING1*. HN-6 cells were treated with siNC or siSMYD3 siRNAs for 3 days and after 24h of IFN-β exposure. Cells were then collected and RNA extraction and RNA-seq were conducted.

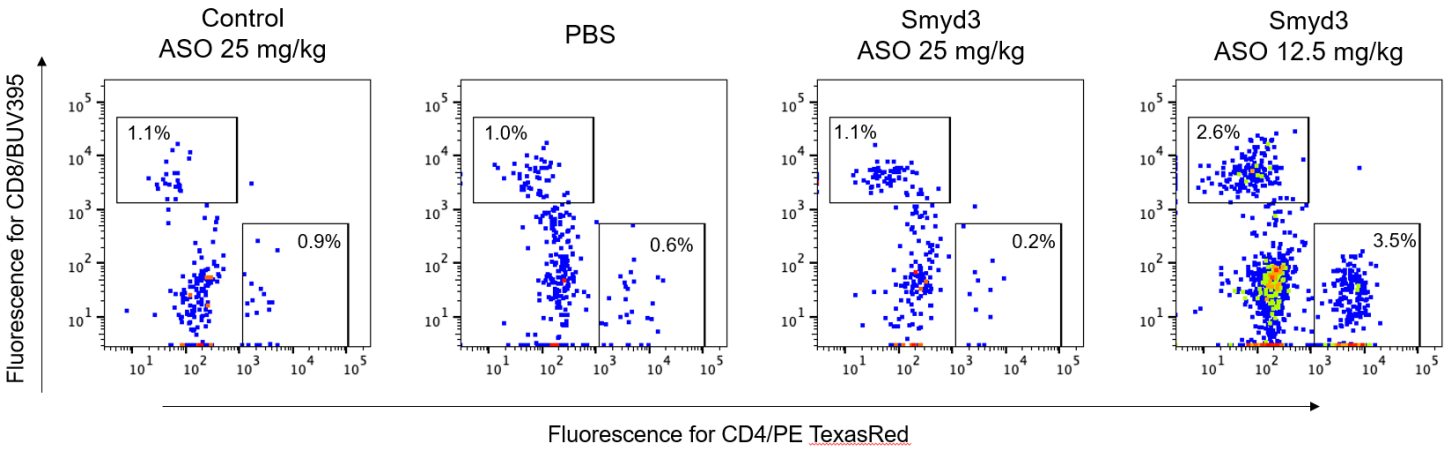


Supplementary Figure 22. Effect of Smyd3 ASO treatment on Smyd3 protein expression levels in MOC1 cells and on CD4+ and CD8+ T-cell infiltration in MOC1 tumors. (A) Western blotting for Smyd3 in MOC1 cells treated with increasing molar concentrations of Smyd3 ASOs. MOC1 cells were plated in 10cm dishes and treated with 0, 0.5, 0.1, 0.02 or 0.004uM of Smyd3 ASOs for 72h. Cells were collected and nuclear extraction was conducted. 10ug of nuclear extract were loaded for Smyd3 blotting, and H3 was used as a loading control. 0.5uM of Smyd3 ASOs induced near complete knockdown of Smyd3. **(B)** Examples of multicolor flow cytometry graphs of MOC1 tumors treated with control ASOs (25mg/kg), PBS, Smyd3 ASOs at 25mg/kg or Smyd3 ASOs at 12.5mg/kg. CD3+/CD4+ and CD3+/CD8+ T-cells expressed as % of CD45+ cells. Anti-CD4: PE TexasRed, anti-CD8: BUV395.

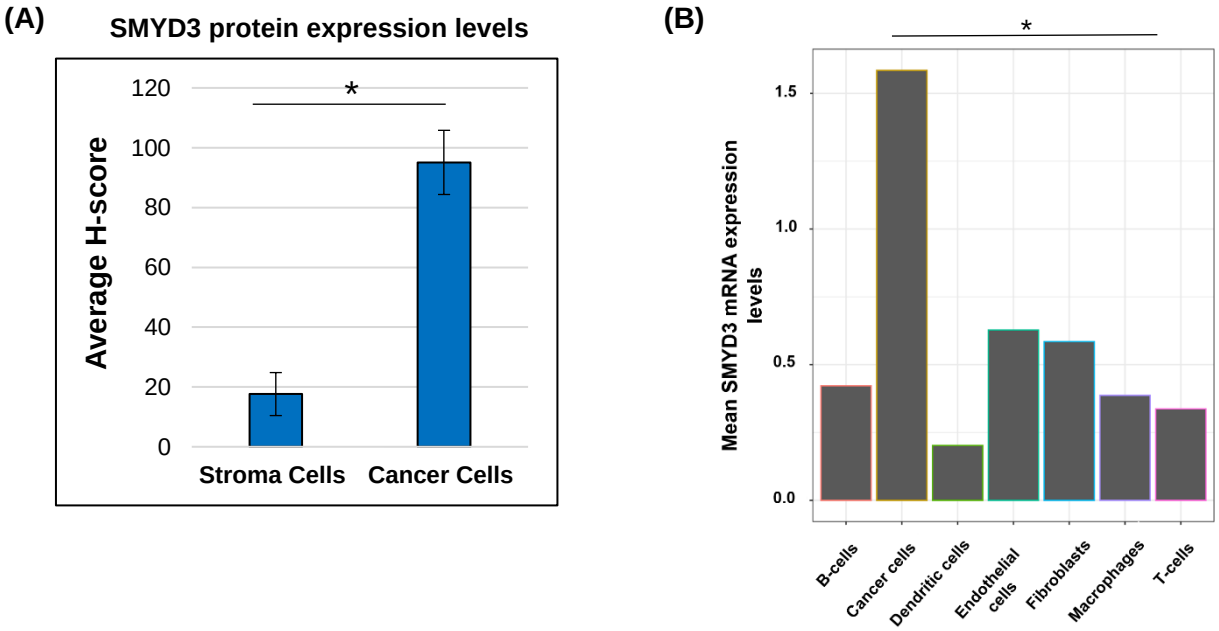
(A)



(B)

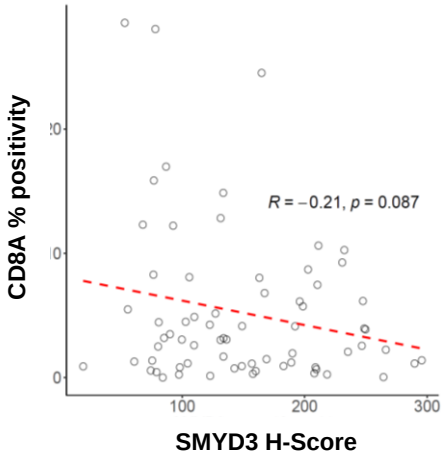


Supplementary Figure 23. Relative protein and mRNA expression levels in cell populations within HPV-negative HNSCC tumors. (A) Comparison of SMYD3 protein expression levels in the cancer cell versus stroma cell compartment. IHC for SMYD3 was conducted in HPV-negative HNSCC tumors. H-score was determined by QuPath in 13 regions of interest (ROIs) captured in the stroma and 89 ROIs captured in the cancer cell compartment of 32 HPV-negative HNSCC tumors. Data represent mean +/- SEM. Wilcoxon rank sum test, $p=3.6x10^{-6}$. **(B)** Mean *SMYD3* mRNA expression levels in different cell types assessed from a publicly available single-cell RNA-seq database of HPV-negative HNSCC tumors. Wilcoxon rank sum test with continuity correction, $p<0.0001$.

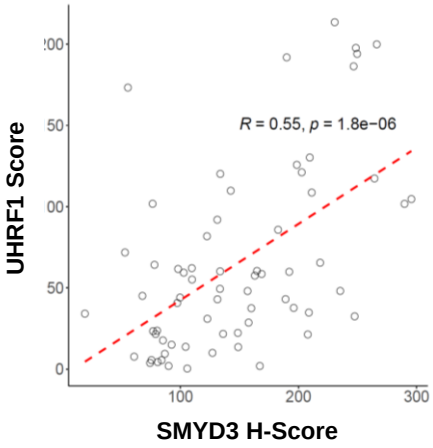


Supplementary Figure 24. Correlation between SMYD3, UHRF1 and CD8A protein expression levels in HPV-negative HNSCC tumors. IHC for SMYD3, UHRF1 and CD8A was conducted in 64 HPV-negative HNSCC tumors (University of Chicago cohort). H-score and % CD8A cell positivity were determined by QuPath. Pearson's correlations between SMYD3 and CD8A (*R*=-0.21, p=0.087) **(A)**, SMYD3 and UHRF1 (*R*=0.55, p<0.001) **(B)**, and UHRF1 and CD8A (*R*=-0.011, p=0.93) **(C)** protein levels in 64 HPV-negative HNSCC tumors.

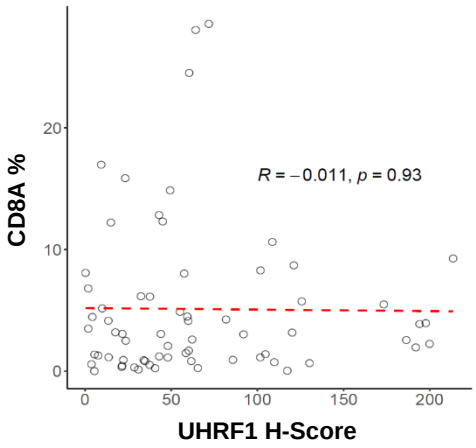
(A)



(B)

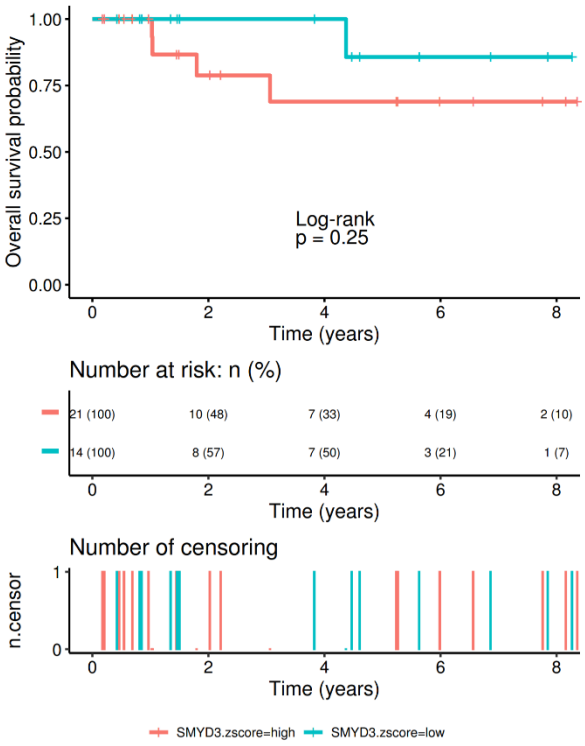


(C)

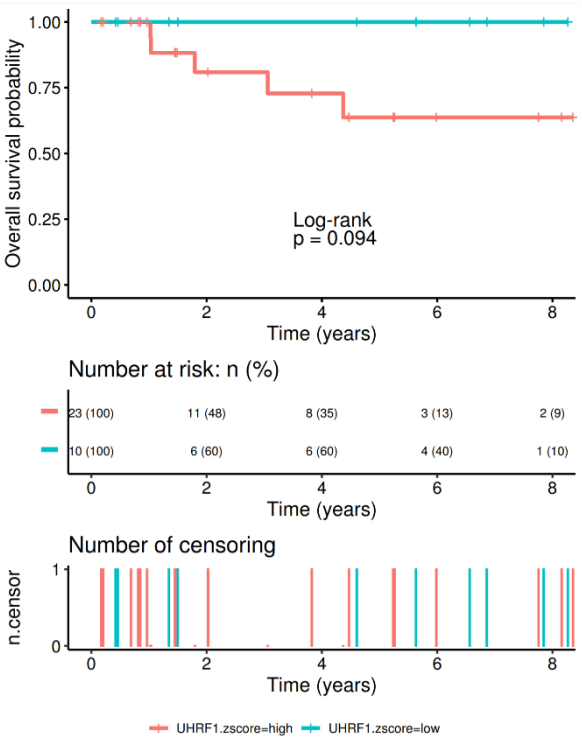


Supplementary Figure 25. Survival analysis based on SMYD3, UHRF1 and CD8A protein expression levels in 35 patients with HPV-negative HNSCC (University of Chicago patient database). Kaplan Meier curves for overall survival based on QuPath scores for SMYD3 **(A)**, UHRF1 **(B)**, CD8A % positivity **(C)**, combined SMYD3/UHRF1 **(D)** and combined SMYD3/UHRF1/CD8A **(E)**. Kaplan Meier curves for progression free survival based on QuPath scores for combined SMYD3/UHRF1/CD8A **(F)**. Log-rank p-values.

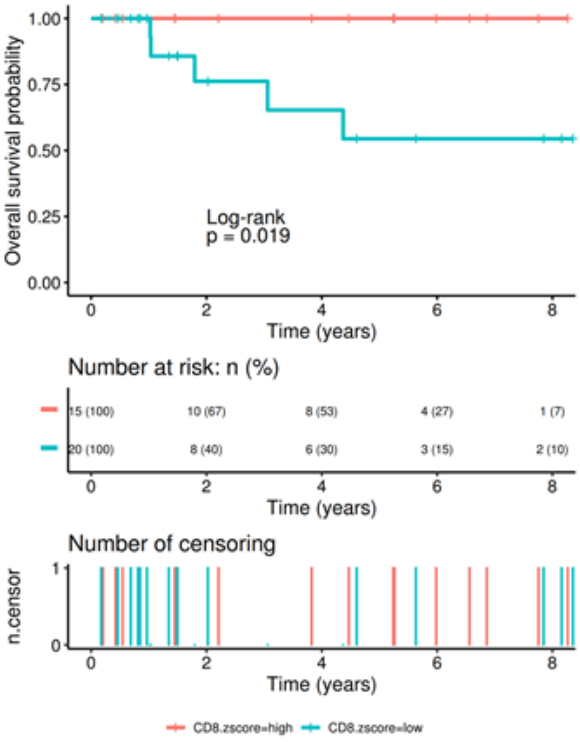
(A) SMYD3



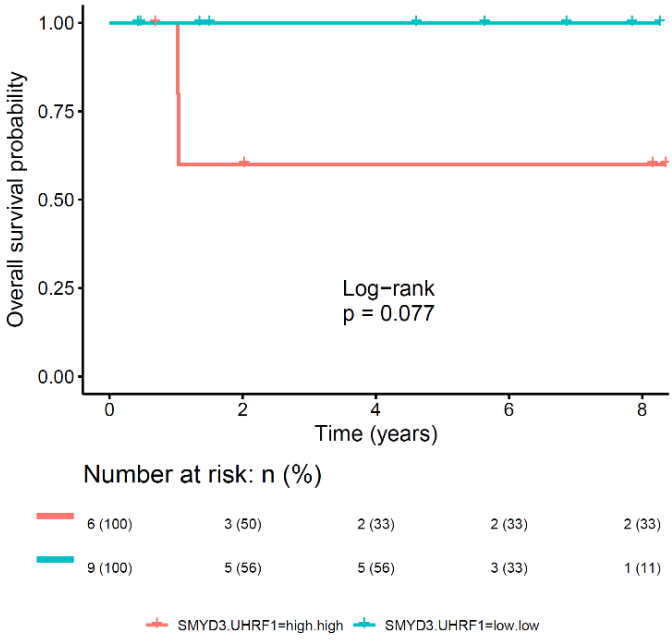
(B) UHRF1



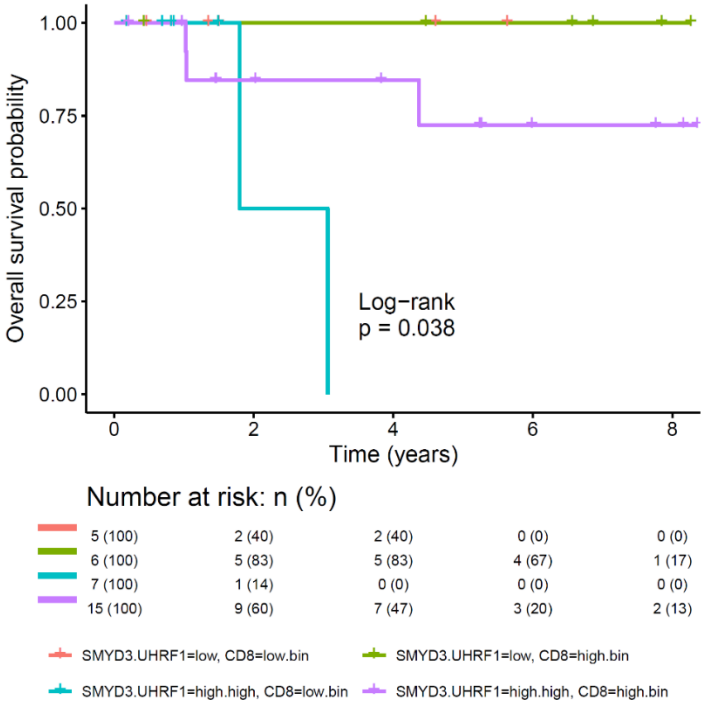
(C) CD8A



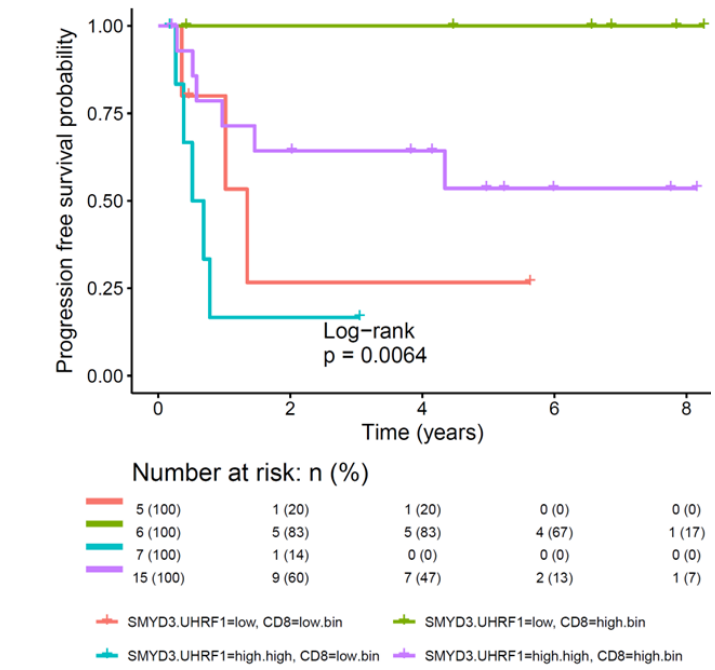
(D) Combined SMYD3/UHRF1



(E) Combined SMYD3/UHRF1/CD8A



(F) Combined SMYD3/UHRF1/CD8A



Supplementary Figure 26. Genomic alterations of *SMYD3* in HPV-negative tumors. Oncoprint for *SMYD3*, TCGA dataset (Firehose Legacy, n=432).