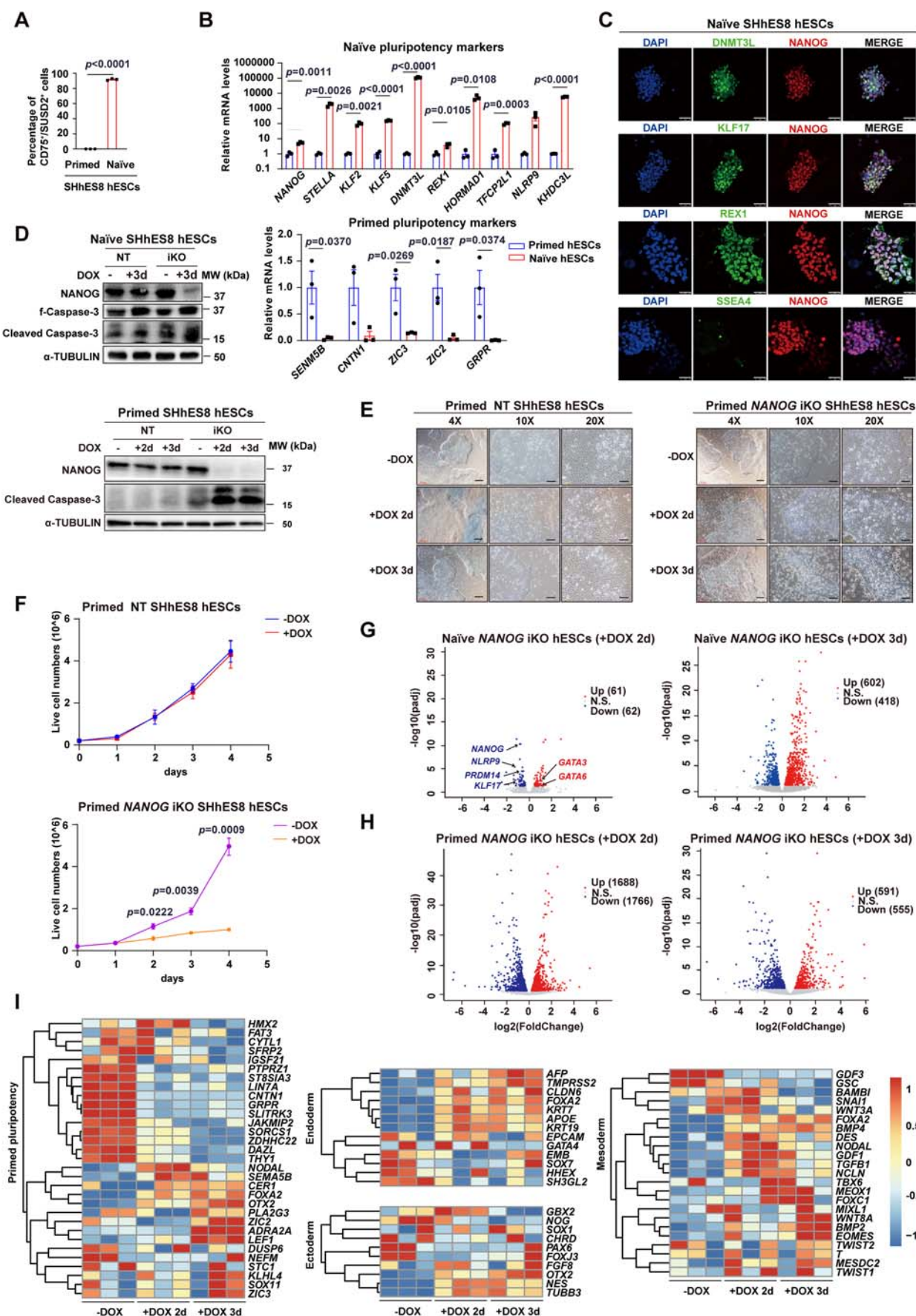
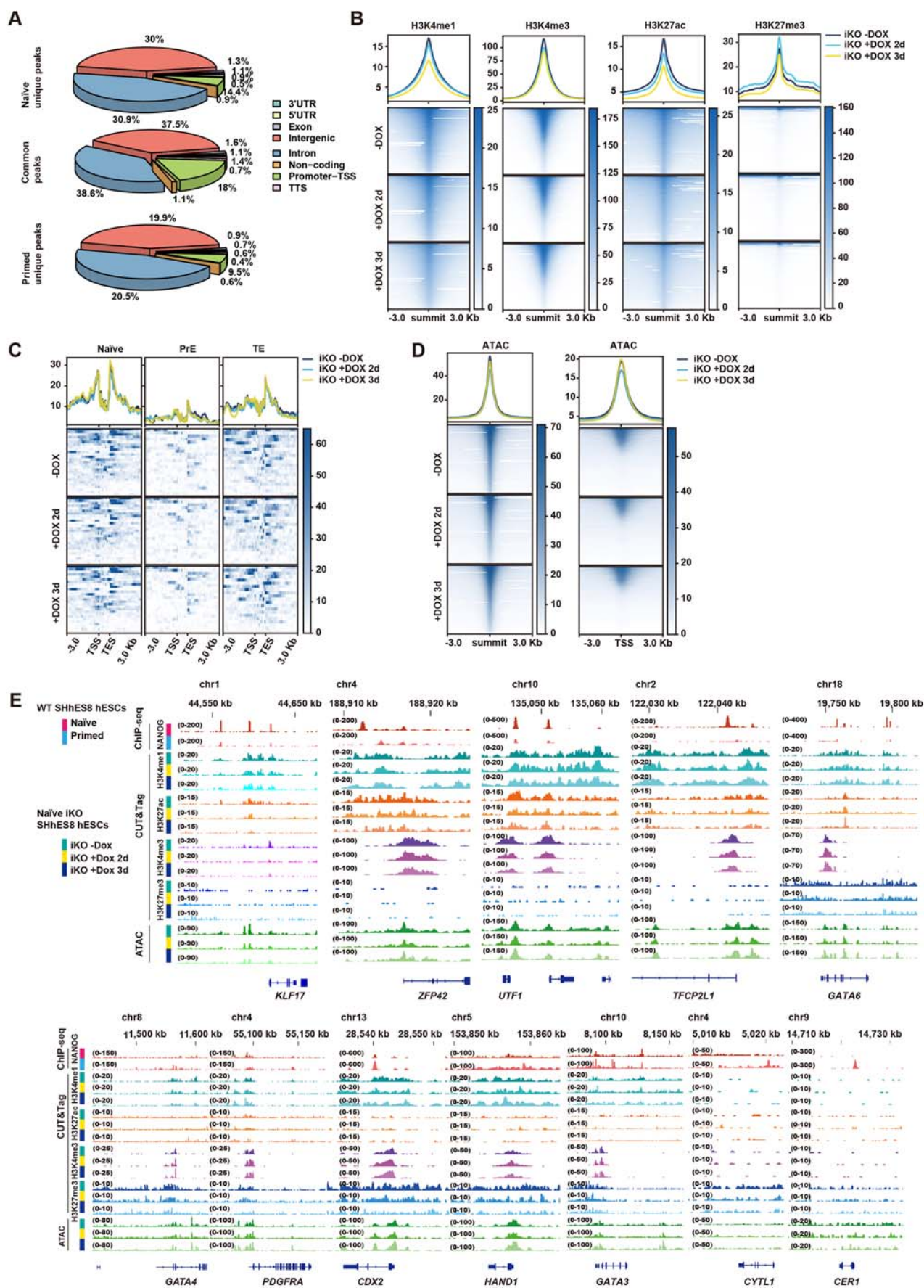


Expanded View Figures

Figure EV1. *NANOG* deficiency impairs self-renewal and transcriptional programs of hESCs.

(A–C) Characterization of naïve hESCs by the flow cytometric analysis of the percentage of CD75 and SUSD2 double-positive cells (A), RT-qPCR analyses of relative mRNA levels of naïve (top) and primed (bottom) pluripotency markers (B), and representative immunofluorescence staining images for NANOG as well as naïve (DNMT3L, KLF17, and REX1) and primed (SSEA4) pluripotency markers in SHhES8 hESCs (C). Scale bar, 25 μ m. Data are presented as mean $\pm$ SEM ($n = 3$) (A, B). $p = 1.09 \times 10^{-8}$ (A). $KLF5$, $p = 1.56 \times 10^{-5}$; $DNMT3L$, $p = 5.76 \times 10^{-5}$; $KHDC3L$, $p = 1.33 \times 10^{-5}$ (B). (D) The representative western blot analysis result showing protein levels of NANOG and cleaved Caspase-3 in naïve (top) and primed (bottom) NT or *NANOG* iKO SHhES8 hESCs, either untreated or treated with DOX for the indicated time lengths. Alpha-TUBULIN served as a loading control. (E) Representative phase contrast images displaying cell colonies of primed NT (left) and *NANOG* iKO (right) SHhES8 hESCs, either untreated or treated with DOX for two or three days. (F) The line chart depicting live cell counts of primed NT (top) or *NANOG* iKO (bottom) SHhES8 hESCs, either untreated or treated with DOX over the indicated days. Data are presented as mean $\pm$ SEM ($n = 3$). (G, H) Volcano plots depicting upregulated or downregulated DEGs in naïve (G) and primed (H) *NANOG* iKO SHhES8 hESCs induced by *NANOG* depletion. Upregulated genes (red dots) were defined by an adjusted p value (padj) < 0.05 and $\log_2$ (FoldChange) > 0 ; Downregulated genes (blue dots) are shown with $\text{padj} < 0.05$ and $\log_2$ (FoldChange) < 0 . Comparisons were made between DOX-treated cells and their untreated counterparts. NS, not significantly changed. DESeq2 was used for the statistical analysis of DEGs. Three biological replicates were used for each condition ($n = 3$). (I) Heatmaps displaying normalized RNA-seq levels of selected markers for three germ layers and primed pluripotency state in primed *NANOG* iKO SHhES8 hESCs, either untreated or treated with DOX for two or three days. The color represents Z-scores. Three biological replicates were used for each condition ($n = 3$). The unpaired two-tailed Student's t -test was used for the statistical analysis in (A, B, F).





◀ **Figure EV2. NANOG depletion results in distinct epigenetic changes for marker genes of different lineages.**

(A) Pie charts displaying the genomic distribution of the three groups of NANOG binding peaks. (B) Heatmaps depicting changes in the abundance (normalized by total reads) of histone modifications (H3K27ac, H3K27me3, H3K4me1, and H3K4me3) across the whole genome in naïve *NANOG* iKO SHhES8 hESCs, either untreated or treated with DOX for two or three days, as analyzed by the deepTools. (C, D) Heatmaps illustrating the abundance (normalized by total reads) of ATAC-seq signals within a 3 kb range upstream and downstream of marker genes associated with naïve pluripotency, TE, or PrE (C), and of ATAC-seq signals across the whole genome (left) or TSS regions (right) (D) in naïve *NANOG* iKO SHhES8 hESCs, either untreated or treated with DOX for three days, as analyzed by the deepTools. (E) Genome browser snapshots of NANOG occupancy at the vicinity of naïve pluripotency markers (*KLF17*, *ZFP42*, *UTF1*, and *TFCP2L1*), PrE markers (*GATA6*, *GATA4*, and *PDGFRA*), TE markers (*GATA3*, *CDX2*, and *HAND1*), and primed pluripotency markers (*CYT11* and *CERT1*) in primed and naïve SHhES8 hESCs, as well as histone modifications and chromatin openness in naïve *NANOG* iKO SHhES8 hESCs, either untreated or treated with DOX for two or three days.

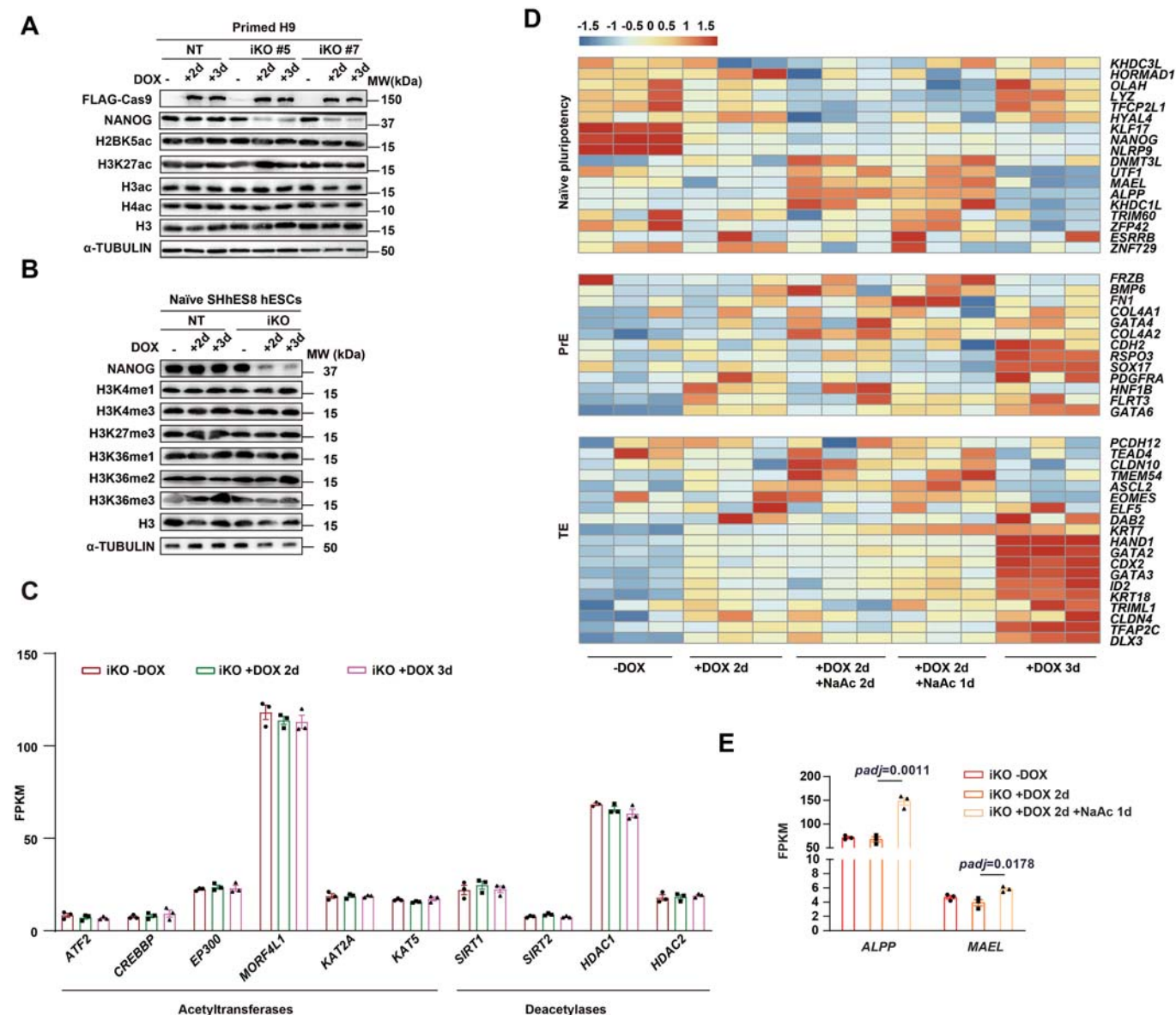


Figure EV3. NANOG maintains histone acetylation modifications via controlling acetyl-CoA production in naïve hESCs.

(A) The representative western blot analysis result for levels of NANOG proteins and histone acetylation modifications in primed NT and NANOG iKO H9 hESCs, either untreated or treated with DOX for two or three days. Alpha-TUBULIN and histone 3 served as loading controls. Single clonal culture of NANOG iKO #5 and #7 was used. (B) The representative western blot analysis result for levels of NANOG and histone methylation modifications in naïve NT and NANOG iKO SHhES8 hESCs, either untreated or treated with DOX for two or three days. Alpha-TUBULIN and histone 3 served as loading controls. (C) The FPKM values from our RNA sequencing data for a set of genes related to histone acetylation modifications in naïve NANOG iKO hESCs, either untreated or treated with DOX for two or three days. Data are presented as mean $\pm$ SEM ($n = 3$). The unpaired two-tailed student's t -test was used for statistical analysis. (D) Heatmaps showing normalized mRNA levels, measured by our RNA-seq assay, of selected naïve pluripotency, PrE and TE marker genes in naïve NANOG iKO SHhES8 hESCs cultured under indicated conditions. The color represents Z-scores. Three biological replicates were used for each culture condition. (E) The FPKM values from our RNA sequencing data for two naïve pluripotency marker genes, ALPP and MAEL, either untreated or treated with DOX or DOX and NaAc in naïve NANOG iKO hESCs. Data are presented as mean $\pm$ SEM ($n = 3$). DESeq2 was used for the statistical analysis.



◀ **Figure EV4. NANOG activates a set of genes involved in acetyl-CoA synthesis in naïve hESCs.**

(A) The GO analysis of 473 NANOG-occupied and NANOG depletion- upregulated (top) as well as 362 NANOG-occupied and NANOG depletion- downregulated (bottom) DEGs in naïve hESCs. The top 20 enriched terms are presented. (B) The heatmap depicting normalized mRNA levels of metabolism- related genes in enriched pathways shown in Fig. 4B, measured by our RNA-seq assays. The color represents Z-scores. Three biological replicates were used for each condition ($n = 3$). (C) A schematic diagram of metabolic pathways of acetyl-CoA synthesis and putative NANOG targets associated with the pathway. (D) The representative western blot analysis result for protein levels of NANOG, histone acetylation modifications, and proteins related to acetyl-CoA synthesis in naïve H9 NANOG iKO hESCs cultured under the 5iLA condition, either untreated or treated with DOX for two or three days. Alpha-TUBULIN and histone 3 served as loading controls (left). The quantitative analysis results of relative protein levels are shown on the right side. Data are presented as mean $\pm$ SEM ($n = 3$). DLAT, Row 1, $p = 2.93 \times 10^{-7}$; NANOG, Row 1, $p = 2.60 \times 10^{-5}$. (E) The representative western blot analysis result for levels of proteins indicated in naïve NT and NANOG iKO SHhES8 hESCs cultured under the PXGL condition, either untreated or treated with DOX for two or three days. Alpha-TUBULIN and histone 3 served as loading controls (left). The quantitative analysis results of relative protein levels are shown on the right side. Data are presented as mean $\pm$ SEM ($n = 3$). CPT1A, $p = 1.08 \times 10^{-5}$; NANOG, Row 1, $p = 1.06 \times 10^{-6}$. (F) RT-qPCR analysis results for relative mRNA levels of the indicated genes in naïve NANOG iKD SHhES8 hESCs, either untreated or treated with DOX for 3 days. Data are presented as mean $\pm$ SEM ($n = 3$). NANOG, $p = 9.39 \times 10^{-5}$; ALDH2, $p = 9.35 \times 10^{-6}$; MPC2, $p = 3.62 \times 10^{-5}$. (G) The representative western blot analysis result for levels of indicated proteins in naïve NANOG iKD SHhES8 hESCs, either untreated or treated with DOX for 3 days. dCas9 was tagged by HA. Alpha-TUBULIN and histone 3 served as loading controls. The unpaired two-tailed student's *t*-test was used for the statistical analysis in (D-F).

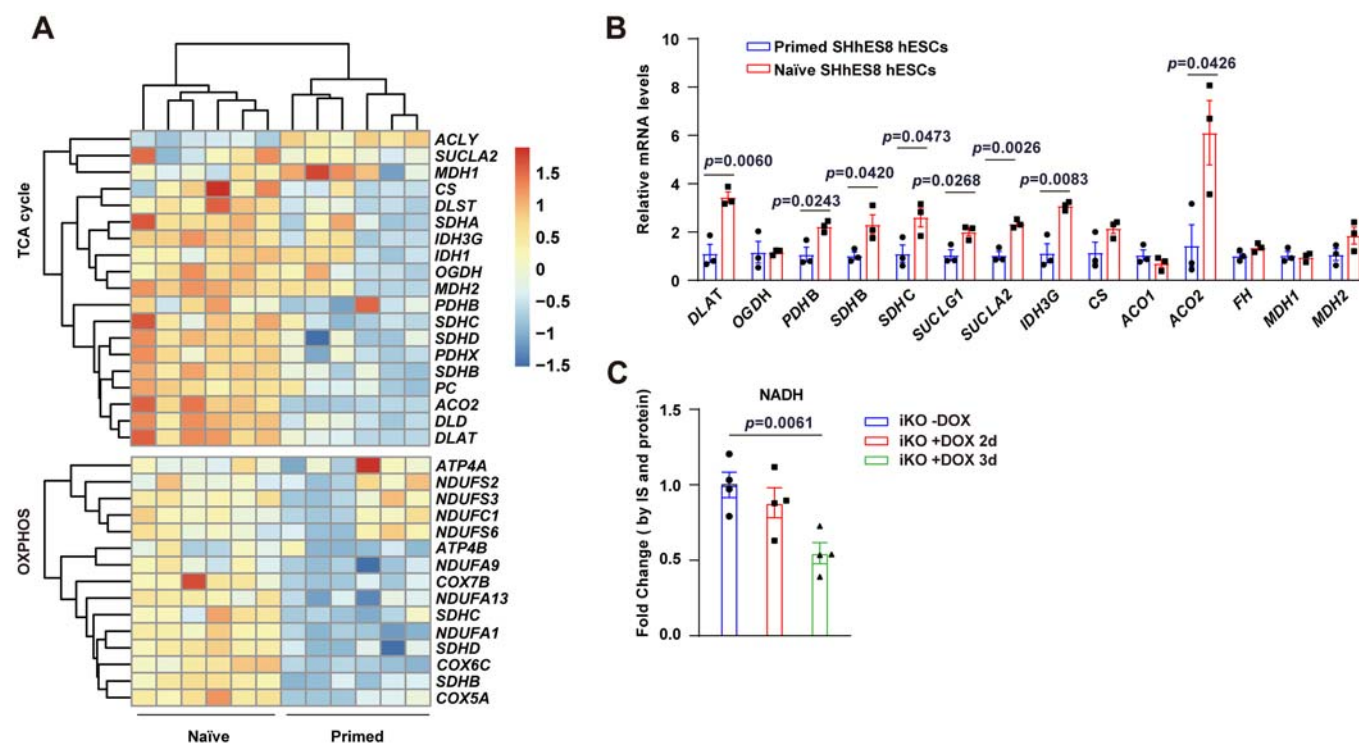


Figure EV5. Expression profiles of metabolism-associated genes and abundance of NADH.

(A) Heatmaps showing mRNA levels of genes related to the TCA cycle and OXPHOS in naïve and primed hESCs. Public RNA-seq datasets (GEO: [GSE169678](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE169678)) and our RNA-seq datasets (SRA: PRJNA1258868) were used. The color represents Z-scores. Six biological replicates were used for each state of hESCs. (B) Results of RT-qPCR assays for the comparison in relative mRNA levels of TCA cycle-related genes between primed and naïve hESCs. Data are presented as mean $\pm$ SEM ($n = 3$). (C) Bar charts showing the relative abundance of NADH in naïve NANOG iKO SHhES8 hESCs, either untreated or treated with DOX for two or three days, as measured by the LC-MS analysis. Data are shown as mean $\pm$ SEM ($n = 4$). The unpaired two-tailed student's *t*-test was used for the statistical analysis in (B, C).

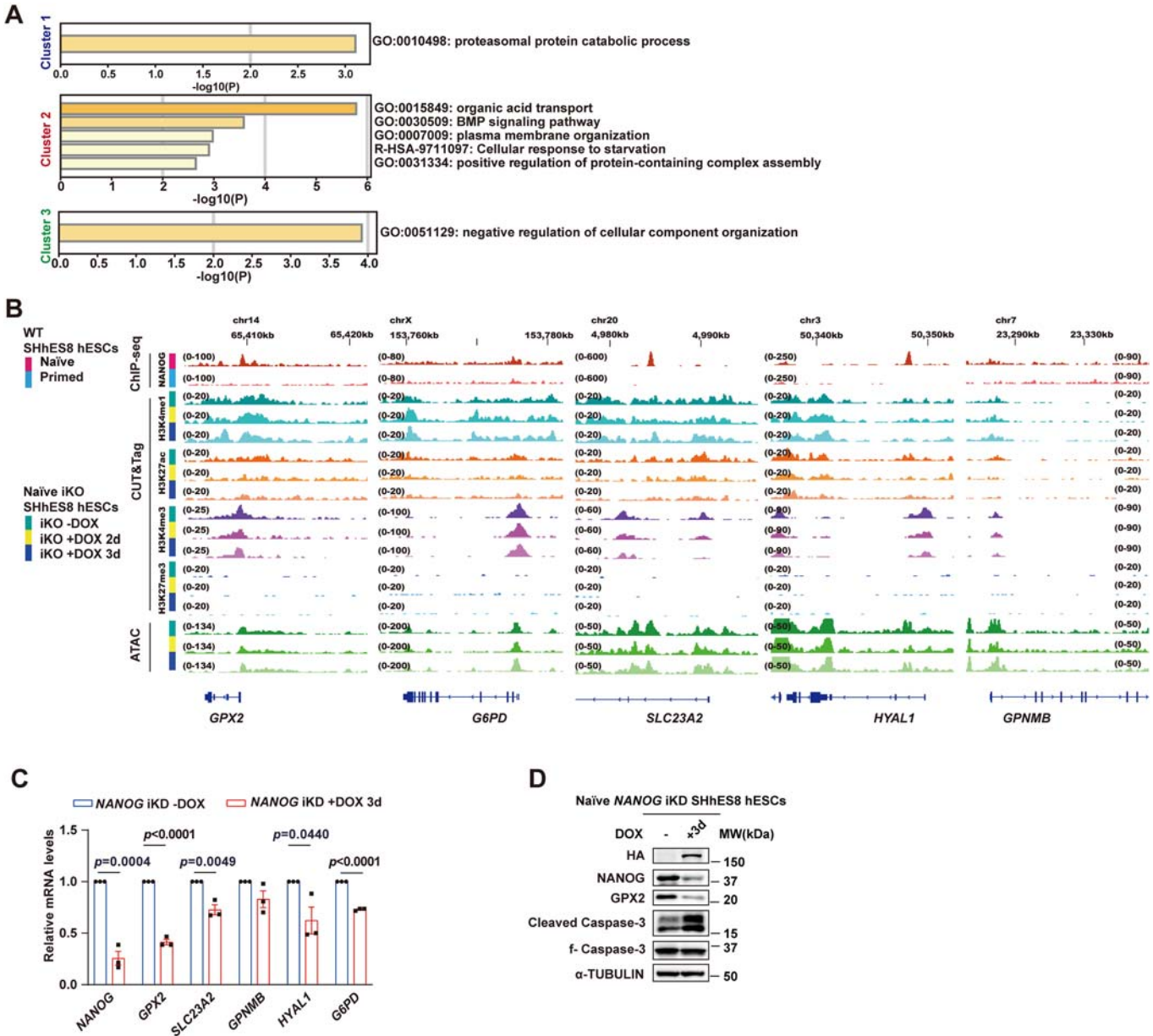


Figure EV6. NANOG directly regulates certain oxidative stress- related genes in naïve hESCs.

(A) Bar charts showing functional annotation of genes from the three clusters described in Fig. 6C. The hypergeometric test was used for statistical analysis. (B) Genome browser snapshots of NANOG occupancy at the vicinity of *GPX2*, *G6PD*, *SLC23A2*, *HYAL1*, and *GPNMB* in primed and naïve SHhES8 hESCs, as well as histone modifications and chromatin openness in naïve *NANOG* iKO SHhES8 hESCs, either untreated or treated with DOX for two or three days. (C) RT-qPCR analysis results for relative mRNA levels of indicated genes in naïve *NANOG* iKO SHhES8 hESCs, either untreated or treated with DOX for three days. Data are presented as mean $\pm$ SEM ($n = 3$). *GPX2*, $p = 2.48 \times 10^{-5}$; *G6PD*, $p = 2.31 \times 10^{-6}$. The unpaired two-tailed student's *t*-test was used for statistical analysis. (D) The representative western blot analysis result for levels of indicated proteins in naïve *NANOG* iKO SHhES8 hESCs, either untreated or treated with DOX for three days. dCas9 was tagged by HA. Alpha-TUBULIN served as a loading control.

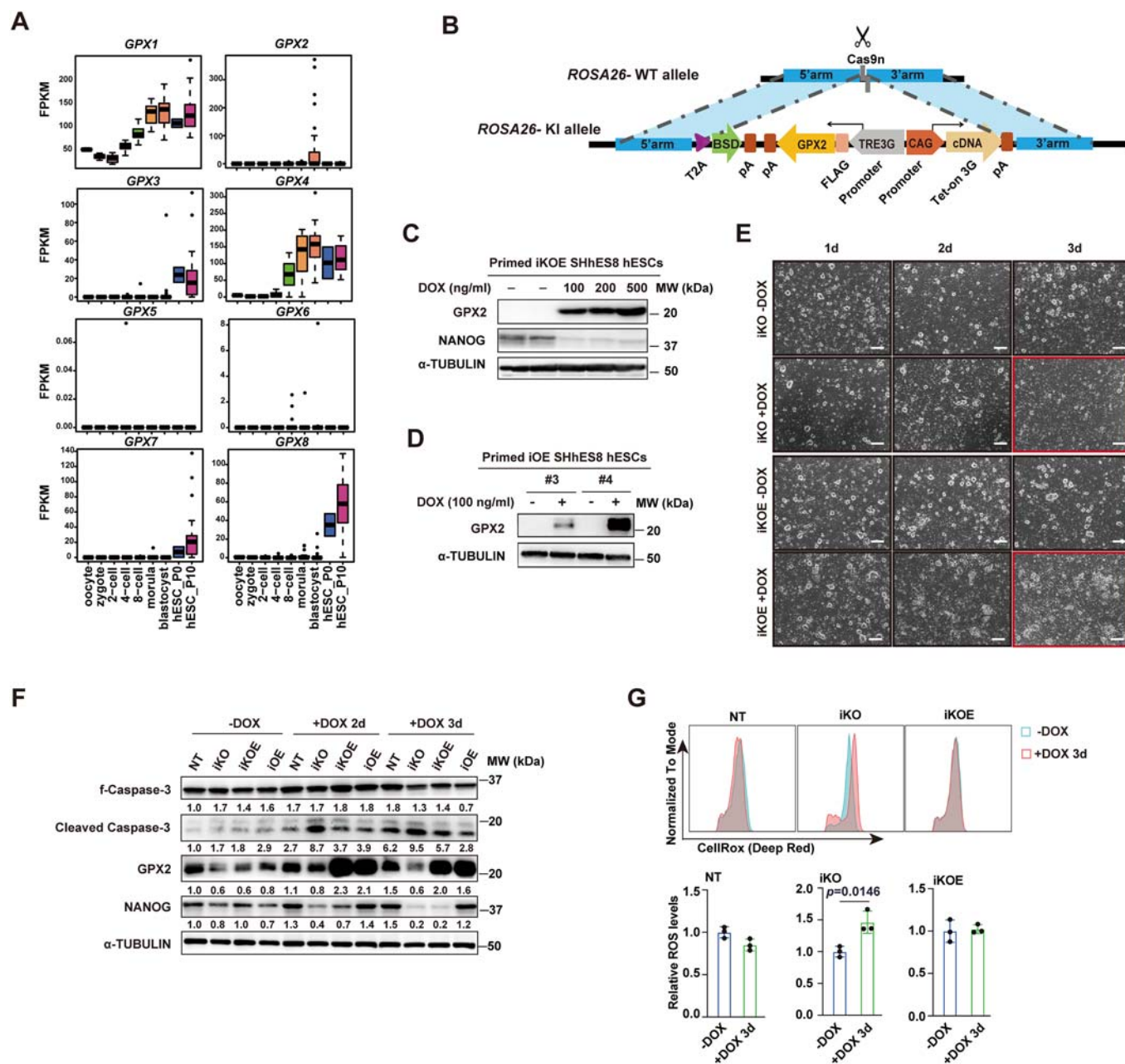


Figure EV7. GPX2 expression profiles and its rescue effect on NANOG depletion- induced oxidative stress and cell death in naïve hESCs.

(A) The expression profile of glutathione peroxidase family members during human early embryonic development and in cultured hESCs. Data were obtained from the published dataset (GEO: [GSE36552](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36552)). (B) The schematic diagram showing the insertion of the cassette for DOX inducible overexpression of GPX2 into the ROSA26 locus of hESCs. (C, D) The representative western blot analysis results of GPX2 and NANOG protein levels in primed iKOE (C) and GPX2 in iOE (D) SHhES8 hESCs, either untreated or treated with DOX for three days. Alpha-TUBULIN served as a loading control. (E) Phase contrast images of naïve NANOG iKO and iKOE SHhES8 hESCs, either untreated or treated with DOX. Scale bar, 200 μ m. (F) The representative western blot analysis result for levels of indicated proteins in naïve NT, iKO, iKOE, and iOE SHhES8 hESCs, either untreated or treated with DOX for two or three days. Alpha-TUBULIN served as a loading control. The number under each row indicates the relative protein abundance measured by ImageJ. (G) The representative ROS peak graph calculated by the CellROX staining combined with flow cytometric analysis in naïve NT, iKO, and iKOE hESCs cultured in the PXGL medium, treated without or with DOX (top). The quantitative analysis of relative ROS levels from three independent experiments are shown at the bottom. Data are shown as mean $\pm$ SD ($n = 3$). The unpaired two-tailed Student's t -test was used for statistical analysis.