Expanded View Figures

Figure EV1. Transcriptomics of *Hdac3*$^{-/-}$ cells.

A Anti-Hdac3 Western blot of naïve *Hdac3*$^{-/-}$ TNG-A clones. Specific Hdac3 band is indicated.

B, C Pairwise Pearson correlations similar to Fig 1B, but using different embryo RNAseq samples (Mohammed *et al*, 2017) (B) or *in vitro* cell types (Anderson *et al*, 2017) (D). Primitive streak (PS) Extraembryonic endoderm cell states (nEnd, XEN), anterior definitive endoderm (ADE).

D Similar to Fig 1C with a magnified view of cluster 5. Canonical PrE marker genes are highlighted in red.

E GO terms enriched in cluster 5. Modified Fisher exact *P*-values as determined by DAVID (Huang *et al*, 2008) are shown.

F–H Indicated mRNA levels relative to *WT* cells of indicated genotypes and treatments after 3 days in SL^{RA} (F) and 2 days in SL (H). Average and standard deviation (SD) of at least two independent clones. Indicated PrE marker mRNA expression relative to negative control siRNA transfection of cells transfected with indicated siRNAs after 2 days in SL (G).

I Similar to Fig 1C with a magnified view of a panel of naïve and general pluripotency, and post-implantation markers.

Source data are available online for this figure.

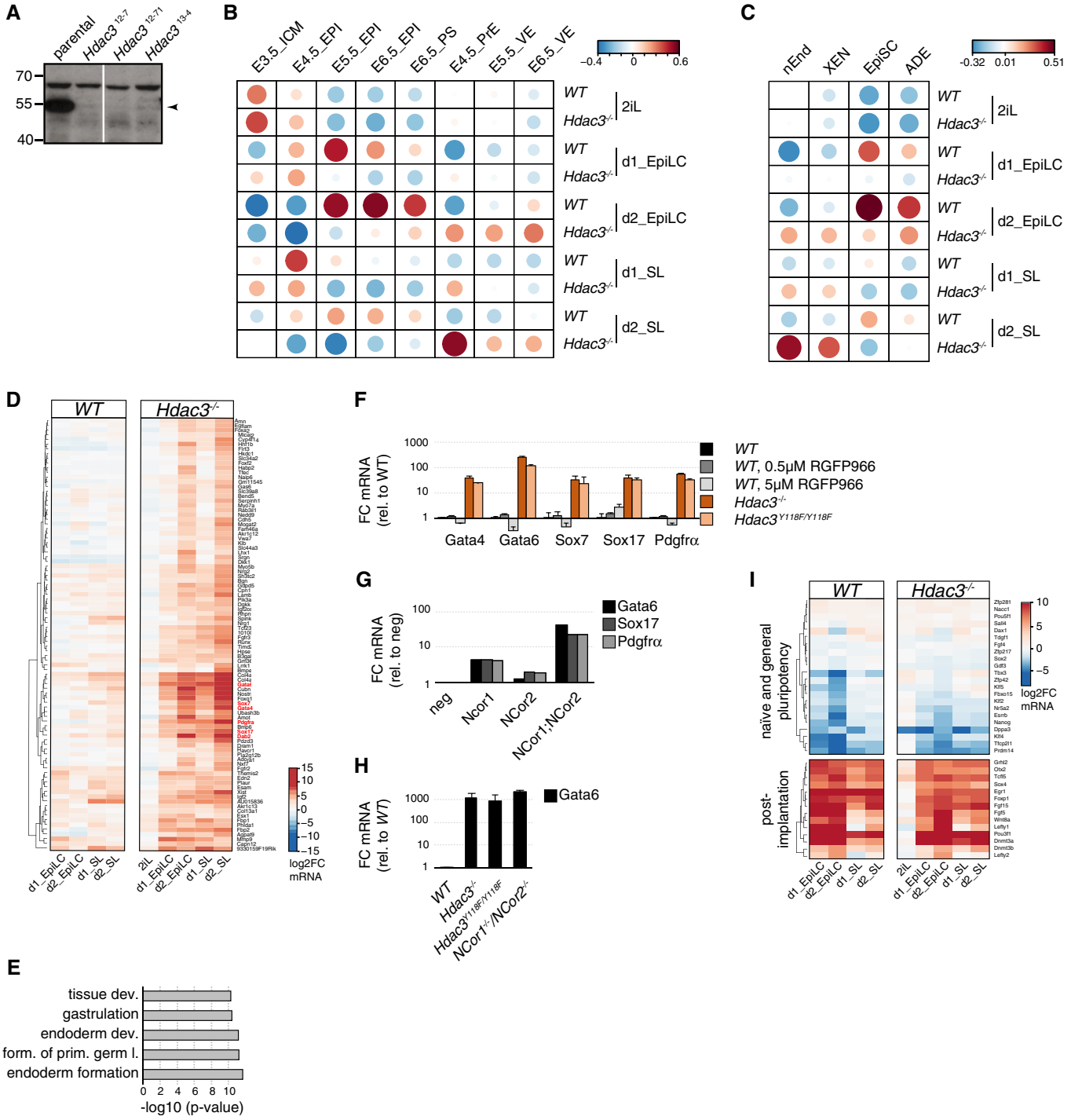

Figure EV1.

Figure EV2. Characterization of *Hdac3*$^{-/-}$ and *Dax1*$^{-/-}$ cells.

A Anti-Hdac3 Western blot of naïve *Hdac3*$^{-/-}$ G6C18 clones. Specific Hdac3 band is indicated.
B Representative *Nanog*>GFP and *Gata6*::mCherry fluorescence intensity plots in indicated genotypes after 3 days that were used for quantifications shown in Fig 2B.
C Quantification of *Gata6*::mCherry reporter signal, background staining, and Sox17, Dab2, and Lama1 immunofluorescence intensities in single, 3 days SL^{RA}-differentiated *Hdac3*$^{-/-}$ cells binned by *Gata6* reporter activity.
D Immunofluorescence and reporter expression in spheroids derived from single WT and *Hdac3*$^{-/-}$ naïve mESCs. DNA counterstain is Hoechst. Dashed white lines indicate the embryonic *Gata6*::mCherry negative part. Scale bar: 10 μm.
E Similar to Fig 1D. Arrowheads indicate Dab2- and *Gata6*::mCherry-positive PrE cells. Scale bar: 10 μm.
F Anti-Dax1, anti-Esrrb, and anti-Hdac3 Western blots for the genotyping of G6C18 mutant clones. Migration behavior of targeted proteins is indicated.
G Representative *Nanog*>GFP and *Gata6*::mCherry fluorescence intensity plots in indicated genotypes and conditions that were used for quantifications shown in Fig 2C.
H Quantification of *Nanog*>GFP geometric mean intensities in 2iL. Average and SD of at least two independent clones.
I Representative *Nanog*>GFP and *Gata6*::mCherry fluorescence intensity plots in *Dax1*$^{-/-}$ cells after 3 days that were used for quantifications shown in Fig 2E.
J Ratios of *Nanog*>GFP and *Gata6*::mCherry intensity (periphery/center) in spheroids of indicated genotypes. Dashed lines indicate the mean in *WT* cells.

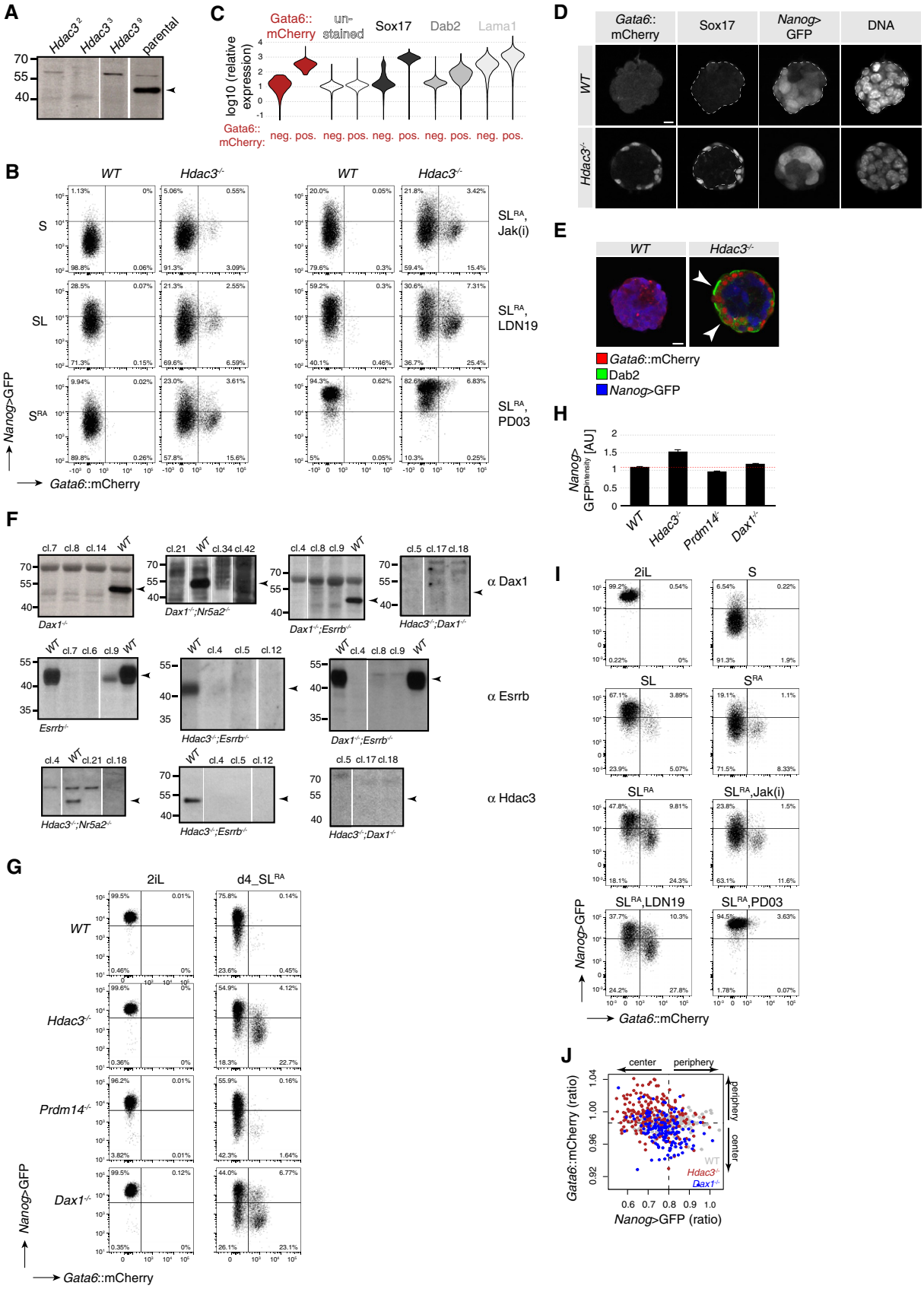

Figure EV2.

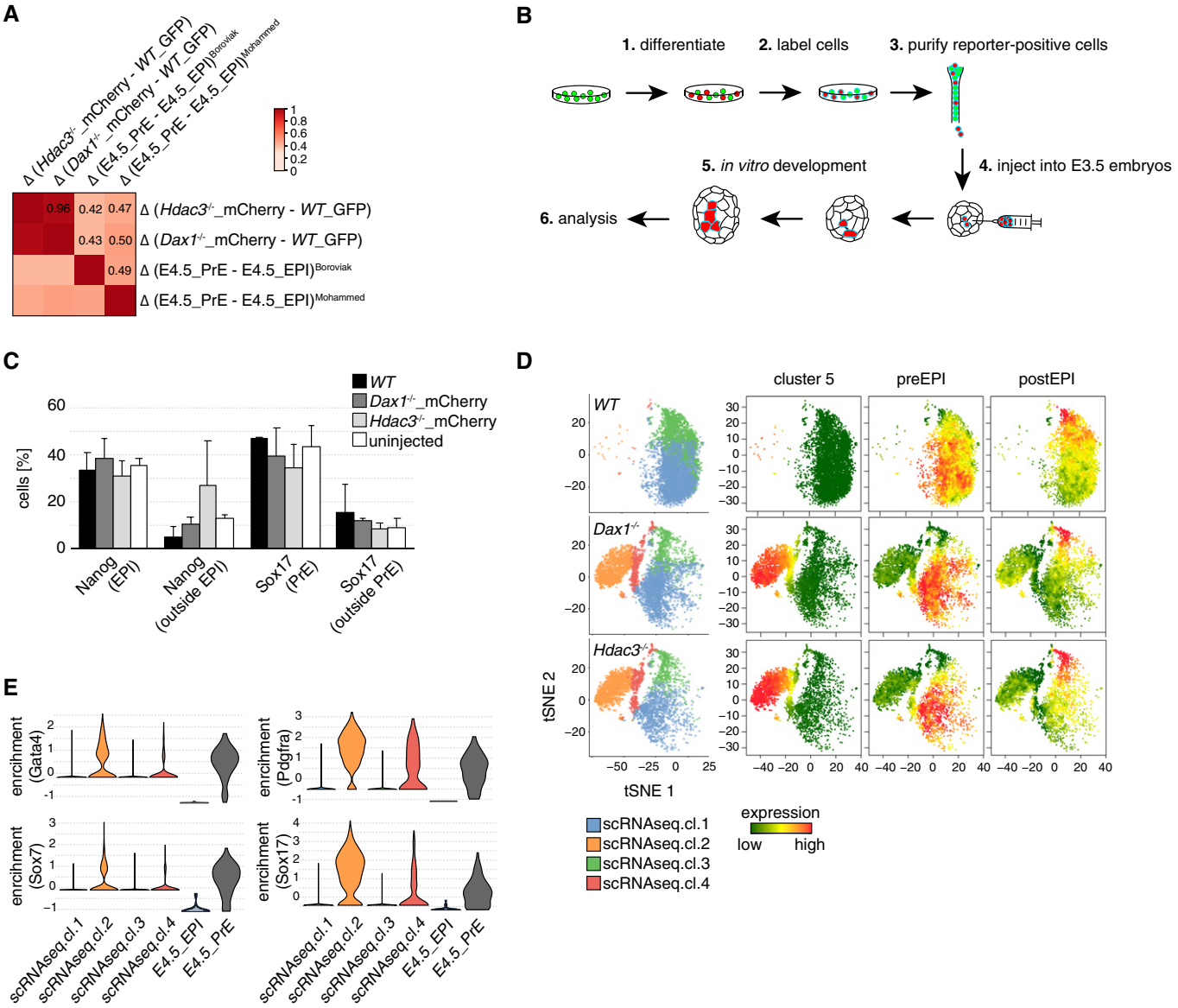

Figure EV3. Characterization of mESC-generated PrE cells.

A Pairwise Pearson correlation of transcriptional differences between indicated samples. Individual correlation coefficients are indicated.

B Experimental scheme used to determine developmental competence of subpopulations.

C Quantification of the cell fate (based on expression of Nanog and Sox17) and the localization of unlabeled endogenous cells. Average and SD of two independent experiments with at least 5 embryos per condition and experiment.

D Similar to Fig 3E. Only cells of indicated genotypes are shown.

E Similar to Fig 3F, showing expression distribution of indicated transcripts.

Source data are available online for this figure.

Figure EV4. Characterization of compound mutant cells.

A Representative *Nanog*>GFP and *Gata6*::mCherry intensity plots of genotypes and conditions quantified in Fig 4C.
B Geometric mean intensity of *Nanog*>GFP reporter in 2iL. Average and SD of three independent clones.
C Similar to Fig 2F. Scale bar: 25 μm.
D Similar to Fig 2G.
E Similar to Fig 4D, but focusing on cluster 5 genes.
F Similar to Fig EV1I with a magnified view of a panel of naïve and general pluripotency, and post-implantation markers in indicated genotypes and conditions relative to naïve *WT* cells in 2iL.

Source data are available online for this figure.

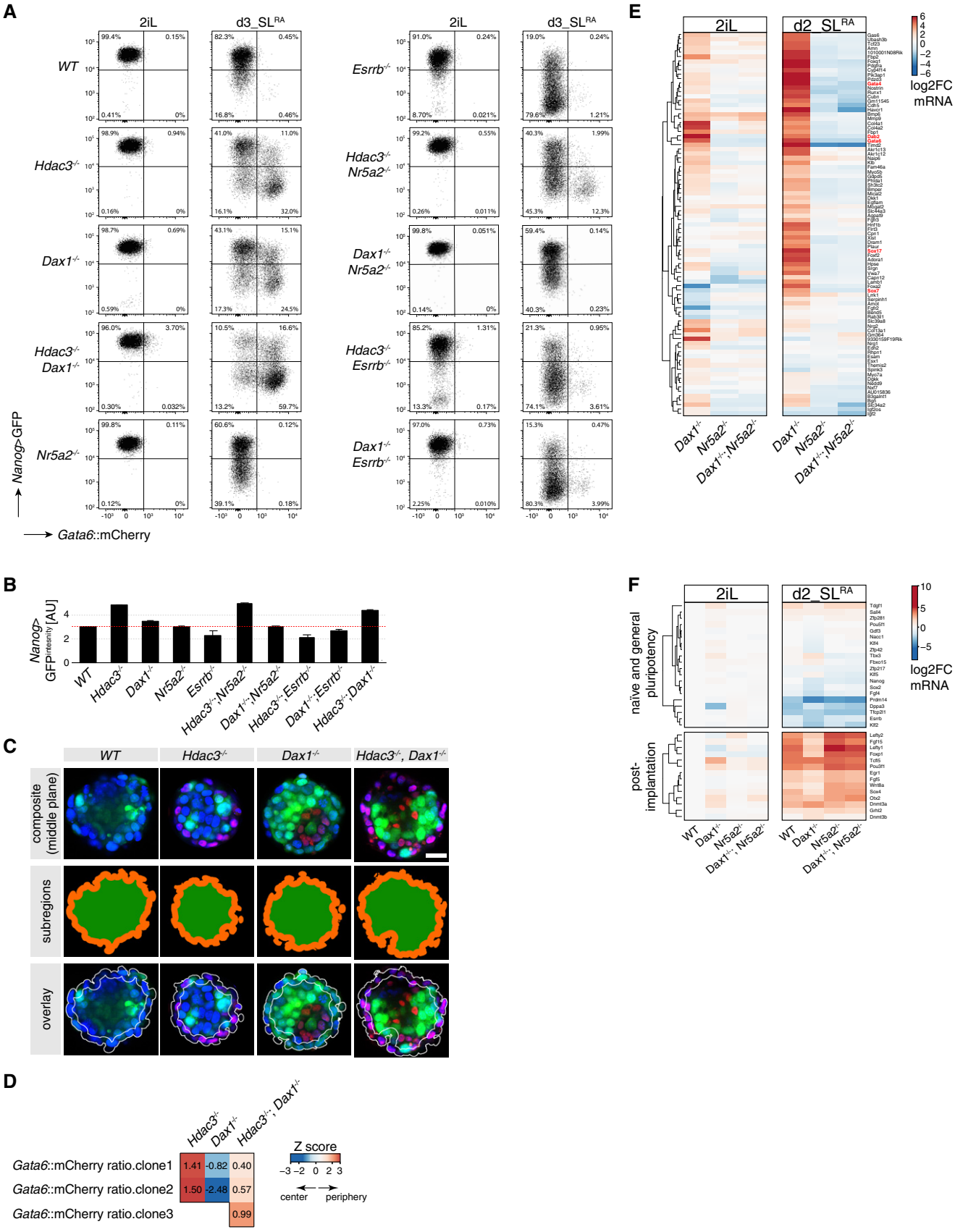
A
2iL
d3_SL^RA
WT
Hdac3-/-
Dax1-/-
Hdac3-/- Dax1-/-
Nr5a2-/-
Esrrb-/-
Hdac3-/- Nr5a2-/-
Dax1-/- Nr5a2-/-
Hdac3-/- Esrrb-/-
Dax1-/- Esrrb-/-
Nanog>GFP
Gata6::mCherry
B
Nanog> GFPintensity [AU]
WT
Hdac3-/-
Dax1-/-
Nr5a2-/-
Esrrb-/-
Hdac3-/-;Nr5a2-/-
Dax1-/-;Nr5a2-/-
Hdac3-/-;Esrrb-/-
Dax1-/-;Esrrb-/-
Hdac3-/-;Dax1-/-
C
WT
Hdac3-/-
Dax1-/-
Hdac3-/-, Dax1-/-
composite (middle plane)
subregions
overlay
D
Hdac3-/-
Dax1-/-
Hdac3-/-; Dax1-/-
Gata6::mCherry ratio.clone1
Gata6::mCherry ratio.clone2
Gata6::mCherry ratio.clone3
1.41
-0.82
0.40
1.50
-2.48
0.57
0.99
Z score
center
periphery
E
2iL
d2_SL^RA
log2FC mRNA
Dax1-/-
Nr5a2-/-
Dax1-/-;Nr5a2-/-
F
naïve and general pluripotency
post-implantation
log2FC mRNA
WT
Dax1-/-
Nr5a2-/-
Dax1-/-; Nr5a2-/-

Figure EV4.

Figure EV5. Epigenomic analysis and enh^{-45} characterization.

A Pairwise Pearson correlation of Hdac3, Dax1, Nr5a2, Esrrb occupancy at regions bound by Hdac3 and/or Dax1. Individual correlation coefficients are indicated.

B Pairwise Pearson correlation of changes in H3K27ac and H4K5ac or accessibility in mutant relative to *WT* cells and in indicated conditions at regions bound by Hdac3 and/or Dax1. Individual correlation coefficients are indicated.

C Similar to Fig 5B. CRE bins according to activation and repression in *Dax1* and *Hdac3* mutants during differentiation are colored.

D Heat map of high-confidence TF motifs, enrichments, and false discovery rates (FDRs) at these bins.

E Similar to Fig 5B with individual regions colored by ChIPseq signal for indicated TFs (upper panels). The same scatterplots with log2 fold H3K27ac changes in naïve *Hdac3* mutants instead of accessibility changes in differentiating *Dax1*$^{-/-}$ cells plotted on the y-axis (lower panels).

F Expression changes of PrE markers in naïve mESCs of indicated genotypes relative to *WT* cells. Average and SD of three independent clones.

G Scatterplots of log2 fold changes in H4K5ac and H3K27ac in *Hdac3* mutants at regions bound by Dax1 and/or Hdac3 in naïve and differentiation conditions. Regions colored in red are associated with Gata6.

H, I Representative *Nanog*>GFP and *Gata6*::mCherry intensity plots (H) and quantification of *Gata6*::mCherry-positive cells (I) in indicated genotypes after 3 days in SL^{RA}. Average and SD of at least three independent clones.

J Model. In *WT* cells, Hdac3 and Dax1 form a regulatory network that inhibits lineage conversion. Hdac3/NCor1/NCor2 bind and silence enh^{-45}, while Dax1 interacts with and antagonizes Nr5a2, and indirectly influences enh^{-45}. Derepression of *Gata6* in *Dax1* and *Hdac3* mutants causes Gata6 to associate with Hdac3- and Dax1/Nr5a2-bound CREs, together with Esrrb forming a positive feedforward loop for PrE conversion.

Source data are available online for this figure.

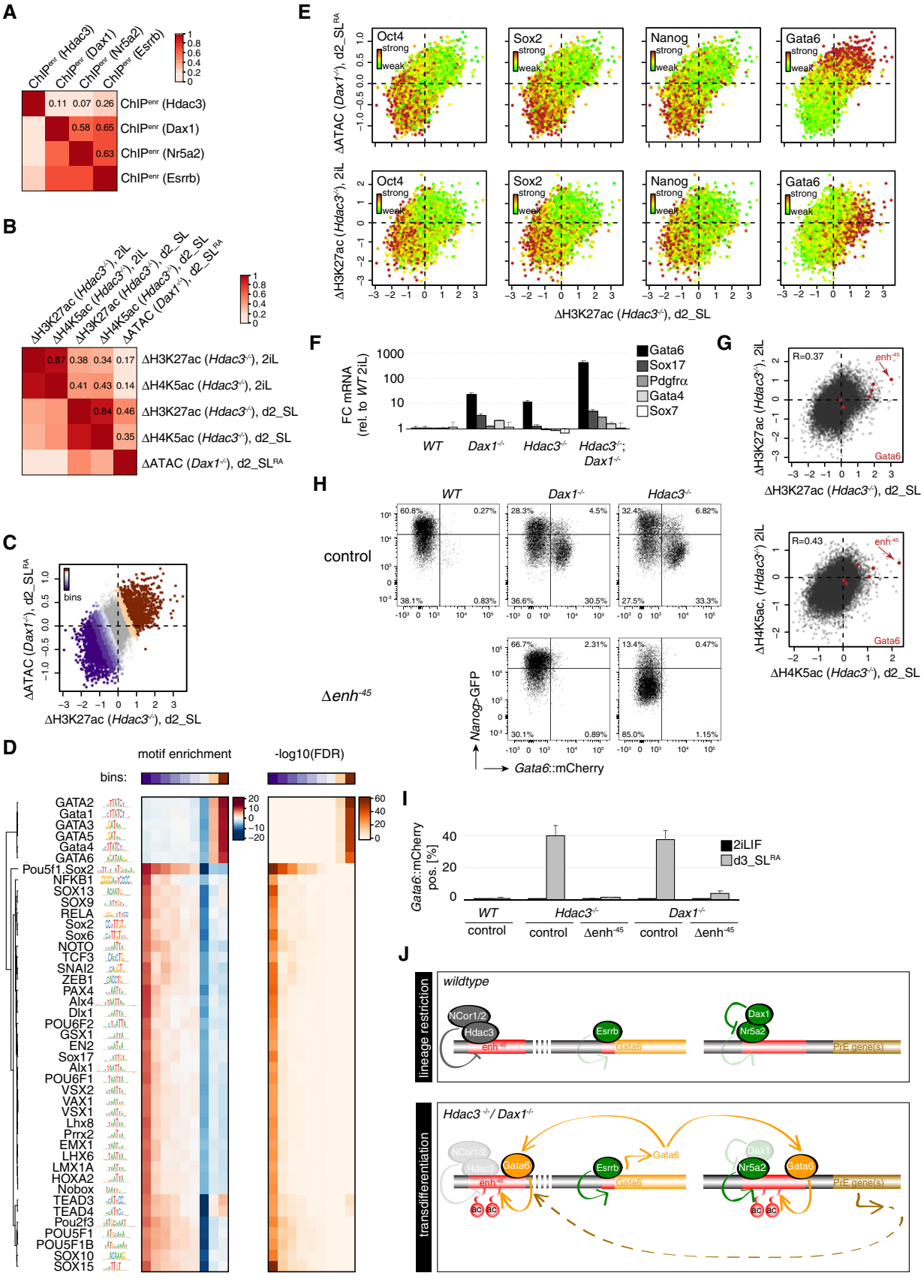
A
ChIPenr (Hdac3)
ChIPenr (Dax1)
ChIPenr (Nr5a2)
ChIPenr (Esrrb)
0.11 0.07 0.26
0.58 0.65
0.63
B
ΔH3K27ac (Hdac3-/-), 2iL
ΔH4K5ac (Hdac3-/-), 2iL
ΔH3K27ac (Hdac3-/-), d2_SL
ΔH4K5ac (Hdac3-/-), d2_SL
ΔATAC (Dax1-/-), d2_SLRA
0.87 0.38 0.34 0.17
0.41 0.43 0.14
0.84 0.46
0.35
C
bins
ΔATAC (Dax1-/-), d2_SLRA
ΔH3K27ac (Hdac3-/-), d2_SL
D
motif enrichment
-log10(FDR)
bins:
GATA2
Gata1
GATA3
GATA5
Gata4
GATA6
Pou5f1,Sox2
NFKB1
SOX13
SOX9
RELA
Sox2
Sox6
NOTO
TCF3
SNAI2
ZEB1
PAX4
Alx4
Dlx1
POU6F2
GSX1
EN2
Sox17
Alx1
POU6F1
VSX2
VAX1
VSX1
Lhx8
Prrx2
EMX1
LHX6
LMX1A
HOXA2
Nobox
TEAD3
TEAD4
Pou2f3
POU5F1
POU5F1B
SOX10
SOX15
E
Oct4
Sox2
Nanog
Gata6
strong
weak
ΔATAC (Dax1-/-), d2_SLRA
ΔH3K27ac (Hdac3-/-), 2iL
ΔH3K27ac (Hdac3-/-), d2_SL
F
FC mRNA (rel. to WT 2iL)
Gata6
Sox17
Pdgfrα
Gata4
Sox7
WT
Dax1-/-
Hdac3-/-
Hdac3-/-; Dax1-/-
G
R=0.37
enh-45
Gata6
ΔH3K27ac (Hdac3-/-), 2iL
ΔH3K27ac (Hdac3-/-), d2_SL
R=0.43
ΔH4K5ac, (Hdac3-/-) 2iL
ΔH4K5ac (Hdac3-/-), d2_SL
H
WT
Dax1-/-
Hdac3-/-
control
Δenh-45
60.8% 0.27% 38.1% 0.83%
28.3% 4.5% 36.6% 30.5%
32.4% 6.82% 27.5% 33.3%
66.7% 2.31% 30.1% 0.89%
13.4% 0.47% 85.0% 1.15%
Nanog>GFP
Gata6::mCherry
I
Gata6::mCherry pos. [%]
2iLIF
d3_SLRA
WT
Hdac3-/-
Dax1-/-
control
Δenh-45
J
lineage restriction
wildtype
NCor1/2
Hdac3
enh-45
Esrrb
Gata6
Dax1
Nr5a2
PrE gene(s)
transdifferentiation
Hdac3-/-/Dax1-/-
ac

Figure EV5.