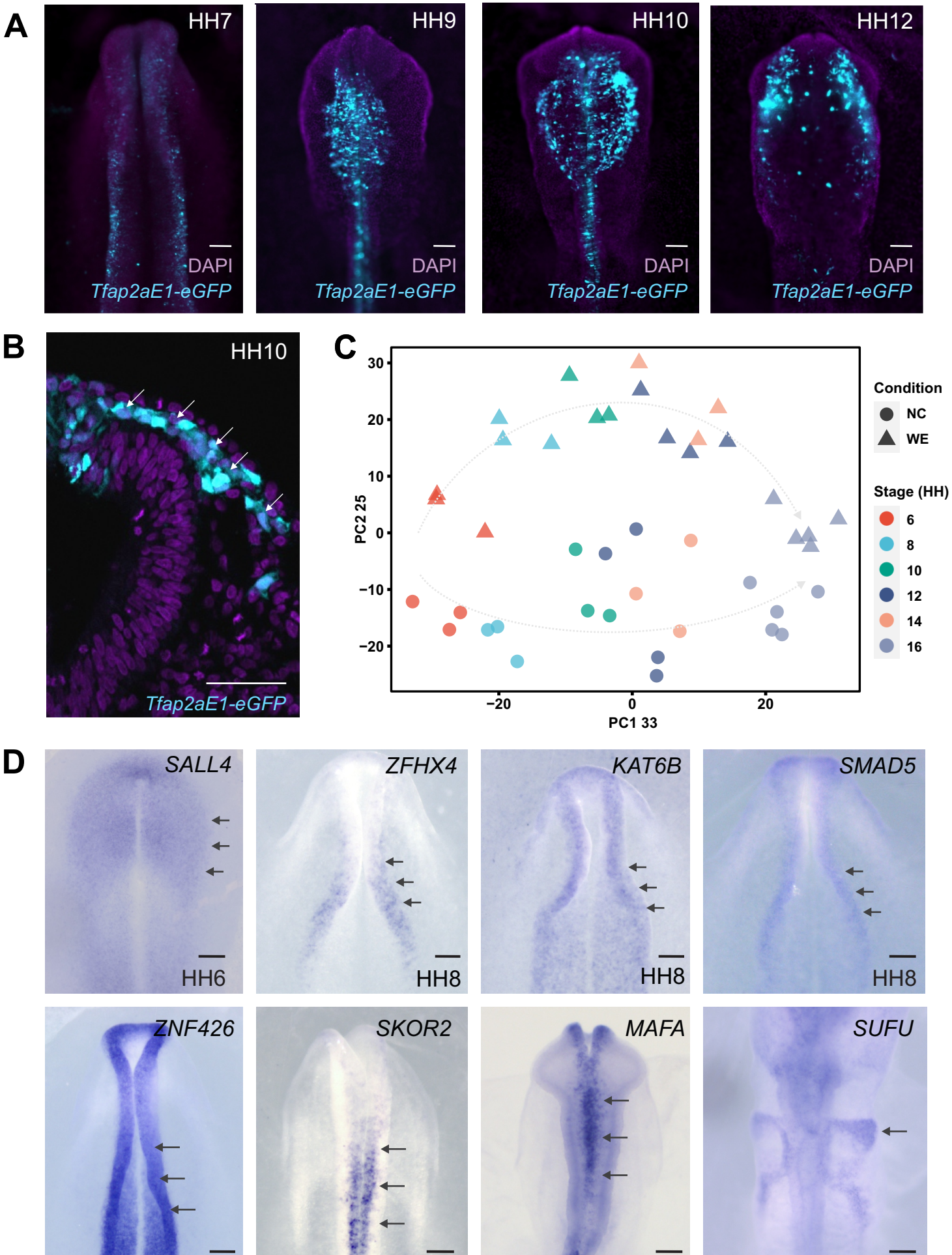

A
HH7
HH9
HH10
HH12
DAPI
Tfap2aE1-eGFP
B
HH10
Tfap2aE1-eGFP
C
PC2 25
PC1 33
30
20
10
0
−10
−20
−20
0
20
Condition
NC
WE
Stage (HH)
6
8
10
12
14
16
D
SALL4
ZFHX4
KAT6B
SMAD5
HH6
HH8
HH8
HH8
ZNF426
SKOR2
MAFA
SUFU
HH8+
HH9-
HH9
HH12

Fig. S1. (Related to figure 1) Characterization of *TFAP2AE1* expression, RNA-Seq quality control and enriched factor in-situ hybridization

(A) *Ex ovo* electroporation of *Tfap2aE1-GFP* shows enhancer activity in the dorsal neural folds (HH7), and in the early migrating (HH9-10) and late migrating (HH12) cranial neural crest. Scale bar 100 μm.

(B) Cross-section of a HH10 chicken embryo, showing that activity of the *Tfap2aE1-GFP* enhancer is specific to the neural crest. Scale bar 50 μm.

(C) Principal component analysis of both neural crest (GFP+) and whole embryo (GFP-) RNA-Seq samples. Principal component 1 (33% of variance) separates both conditions by developmental time, while principal component 2 (25% of variance) separates neural crest and whole embryo samples.

(D) Whole mount *in situ* hybridizations for significantly enriched factors expressed in the neural crest lineage. Scale bar 100 μm.

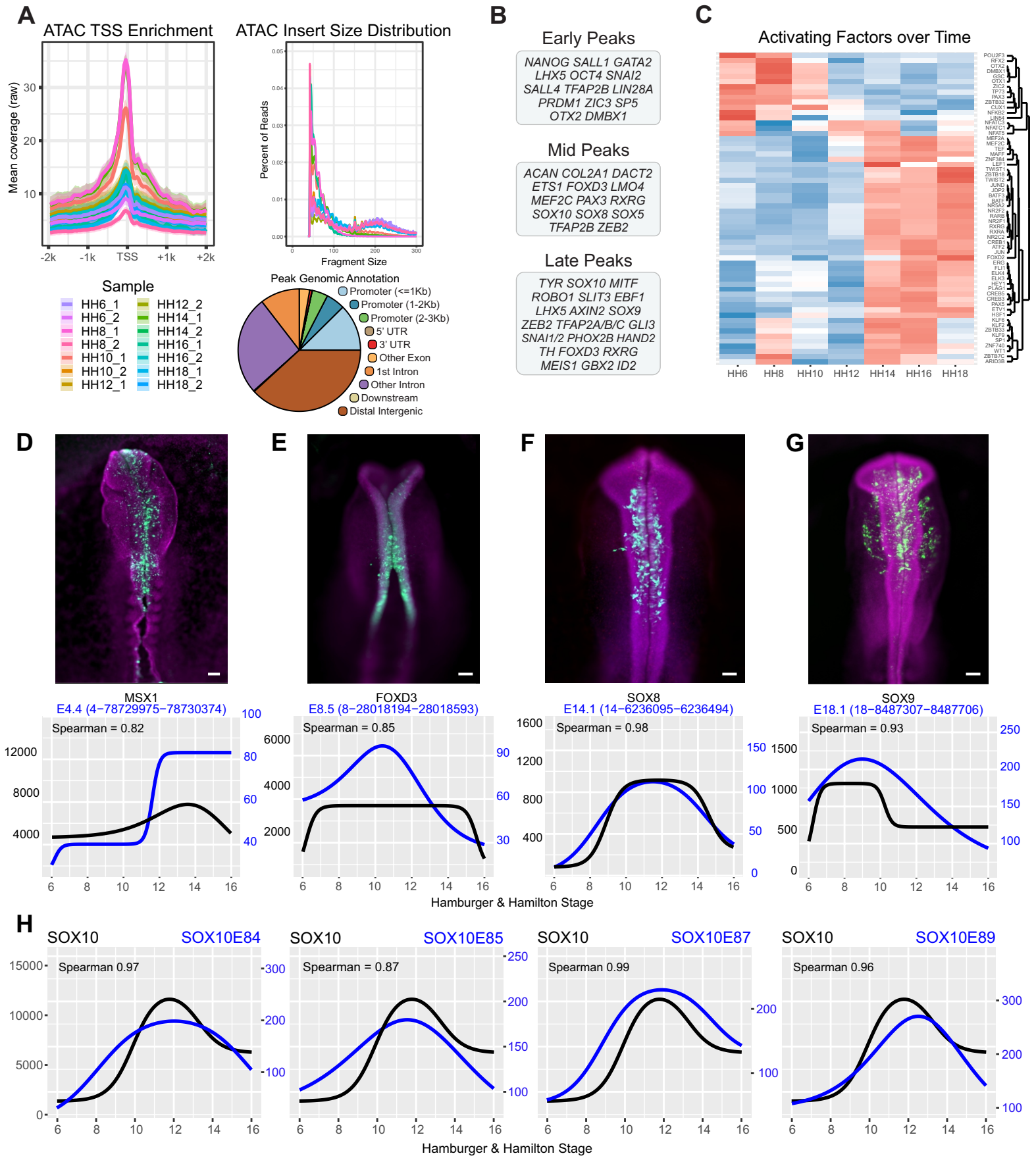

A
ATAC TSS Enrichment
Mean coverage (raw)
-2k
-1k
TSS
+1k
+2k
ATAC Insert Size Distribution
Percent of Reads
Fragment Size
Sample
HH6_1
HH6_2
HH8_1
HH8_2
HH10_1
HH10_2
HH12_1
HH12_2
HH14_1
HH14_2
HH16_1
HH16_2
HH18_1
HH18_2
Peak Genomic Annotation
Promoter (<=1Kb)
Promoter (1-2Kb)
Promoter (2-3Kb)
5' UTR
3' UTR
Other Exon
1st Intron
Other Intron
Downstream
Distal Intergenic
B
Early Peaks
NANOG SALL1 GATA2 LHX5 OCT4 SNAI2 SALL4 TFAP2B LIN28A PRDM1 ZIC3 SP5 OTX2 DMBX1
Mid Peaks
ACAN COL2A1 DACT2 ETS1 FOXD3 LMO4 MEF2C PAX3 RXRG SOX10 SOX8 SOX5 TFAP2B ZEB2
Late Peaks
TYR SOX10 MITF ROBO1 SLIT3 EBF1 LHX5 AXIN2 SOX9 ZEB2 TFAP2A/B/C GLI3 SNAI1/2 PHOX2B HAND2 TH FOXD3 RXRG MEIS1 GBX2 ID2
C
Activating Factors over Time
HH6
HH8
HH10
HH12
HH14
HH16
HH18
D
E
F
G
MSX1
E4.4 (4−78729975−78730374)
Spearman = 0.82
FOXD3
E8.5 (8−28018194−28018593)
Spearman = 0.85
SOX8
E14.1 (14−6236095−6236494)
Spearman = 0.98
SOX9
E18.1 (18−8487307−8487706)
Spearman = 0.93
Hamburger & Hamilton Stage
H
SOX10
SOX10E84
Spearman 0.97
SOX10
SOX10E85
Spearman = 0.87
SOX10
SOX10E87
Spearman 0.99
SOX10
SOX10E89
Spearman 0.96
Hamburger & Hamilton Stage

Fig. S2 (Related to figure 2) ATAC-Seq quality control, DiffTF and chromVAR integration, and in-vivo verification of peak to gene correlation analysis.

(A) Quality control plots for chicken ATAC-Seq samples. Left, an ATAC-Seq TSS enrichment plot with mean coverage around the transcription start site and +/- 2 kilobases of all genes. Right top, the insert size distribution plot. Fragments < 120bp indicate nucleosome free binding. Right bottom, a genomic annotation plot of all ATAC-Seq peaks, demonstrating promoter, intronic, and distal intergenic enrichment.

(B) Genes identified from peak to gene correlation analysis in the early, mid, and late peak groups.

(C) A heatmap of DiffTF classified activating transcription factors variability over time. Transcription factors are clustered hierarchically, resulting in two large clusters.

(D) *Ex ovo* electroporation of the *MSX1 E4.4* enhancer and the correlation plot between the gene (black) and the enhancer (blue). Scale bar 100 μm.

(E) *Ex ovo* electroporation of the *FOXD3 E8.5* enhancer and the correlation plot between the gene (black) and the enhancer (blue). Scale bar 100 μm.

(F) *Ex ovo* electroporation of the *SOX8 E14.1* enhancer and the correlation plot between the gene (black) and the enhancer (blue). Scale bar 100 μm.

(G) *Ex ovo* electroporation of the *SOX9 E18.1* enhancer and the correlation plot between the gene (black) and the enhancer (blue). Scale bar 100 μm.

(H) Correlation plots for *SOX10* and four associated enhancer regions, demonstrating that this method can robustly capture known enhancers.

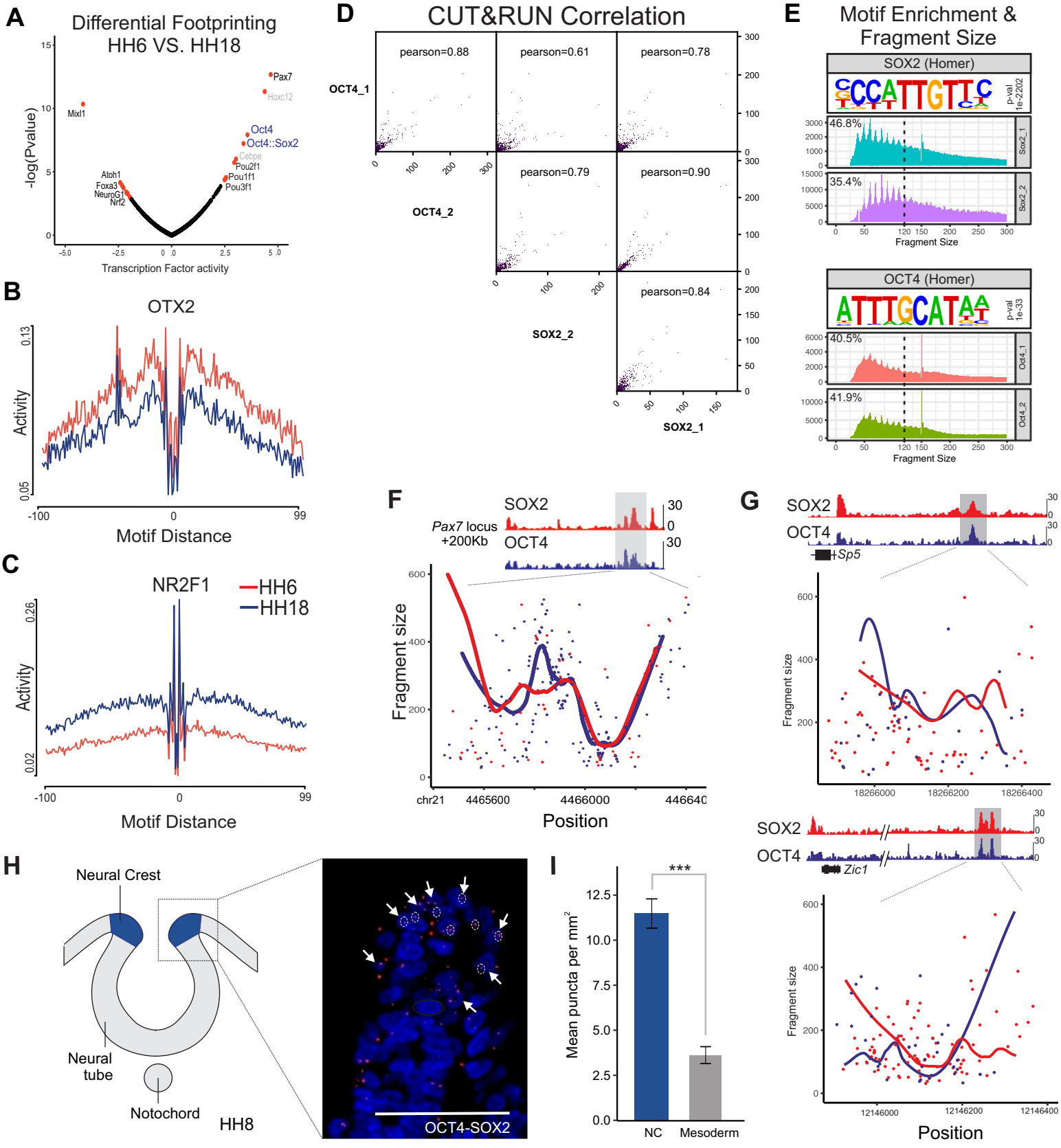

A
Differential Footprinting
HH6 VS. HH18
-log(Pvalue)
Transcription Factor activity
Pax7
Hoxc12
Mixl1
Oct4
Oct4::Sox2
Cebpe
Pou2f1
Pou1f1
Pou3f1
Atoh1
Foxa3
NeuroG1
Nrf2
B
OTX2
Activity
Motif Distance
C
NR2F1
HH6
HH18
Activity
Motif Distance
D
CUT&RUN Correlation
pearson=0.88
pearson=0.61
pearson=0.78
pearson=0.79
pearson=0.90
pearson=0.84
OCT4_1
OCT4_2
SOX2_2
SOX2_1
E
Motif Enrichment &
Fragment Size
SOX2 (Homer)
P-val
1e-2202
46.8%
35.4%
Sox2_1
Sox2_2
Fragment Size
OCT4 (Homer)
P-val
1e-33
40.5%
41.9%
Oct4_1
Oct4_2
Fragment Size
F
SOX2
Pax7 locus
+200Kb
OCT4
Fragment size
chr21
4465600
4466000
446640
Position
G
SOX2
OCT4
Sp5
Fragment size
18266000
18266200
18266400
SOX2
OCT4
Zic1
Fragment size
12146000
12146200
12146400
Position
H
Neural Crest
Neural
tube
Notochord
HH8
OCT4-SOX2
I
Mean puncta per mm2
***
NC
Mesoderm

Fig. S3 (Related to figure 3) CUT&RUN quality control, transcription factor footprinting and EChO analysis.

(A) Differential footprinting analysis between HH6 and HH18 ATAC-Seq samples shows OCT4 and OCT4-SOX2 are two of the top motifs whose footprint are enriched in HH6.

(B) A motif footprinting plot for OTX2, showing enrichment in HH6 ATAC-Seq samples.

(C) A motif footprinting plot for NR2F1, showing enrichment in HH18 ATAC-Seq samples.

(D) Bin-wise correlation quality control plots for the OCT4 and SOX2 CUT&RUN libraries.

(E) Motif enrichment (from Homer) and fragment size distribution quality control plots for the SOX2 and OCT4 CUT&RUN libraries.

(F) A genome browser plot of a putative regulatory region for *SP5.* EChO analysis of OCT4 and SOX2 CUT&RUN data shows co-binding events (local minima).

(G) A genome browser plot of a putative regulatory region for *ZIC1*. EChO analysis of OCT4 and SOX2 CUT&RUN data shows co-binding of the two factors in the *ZIC1* locus.

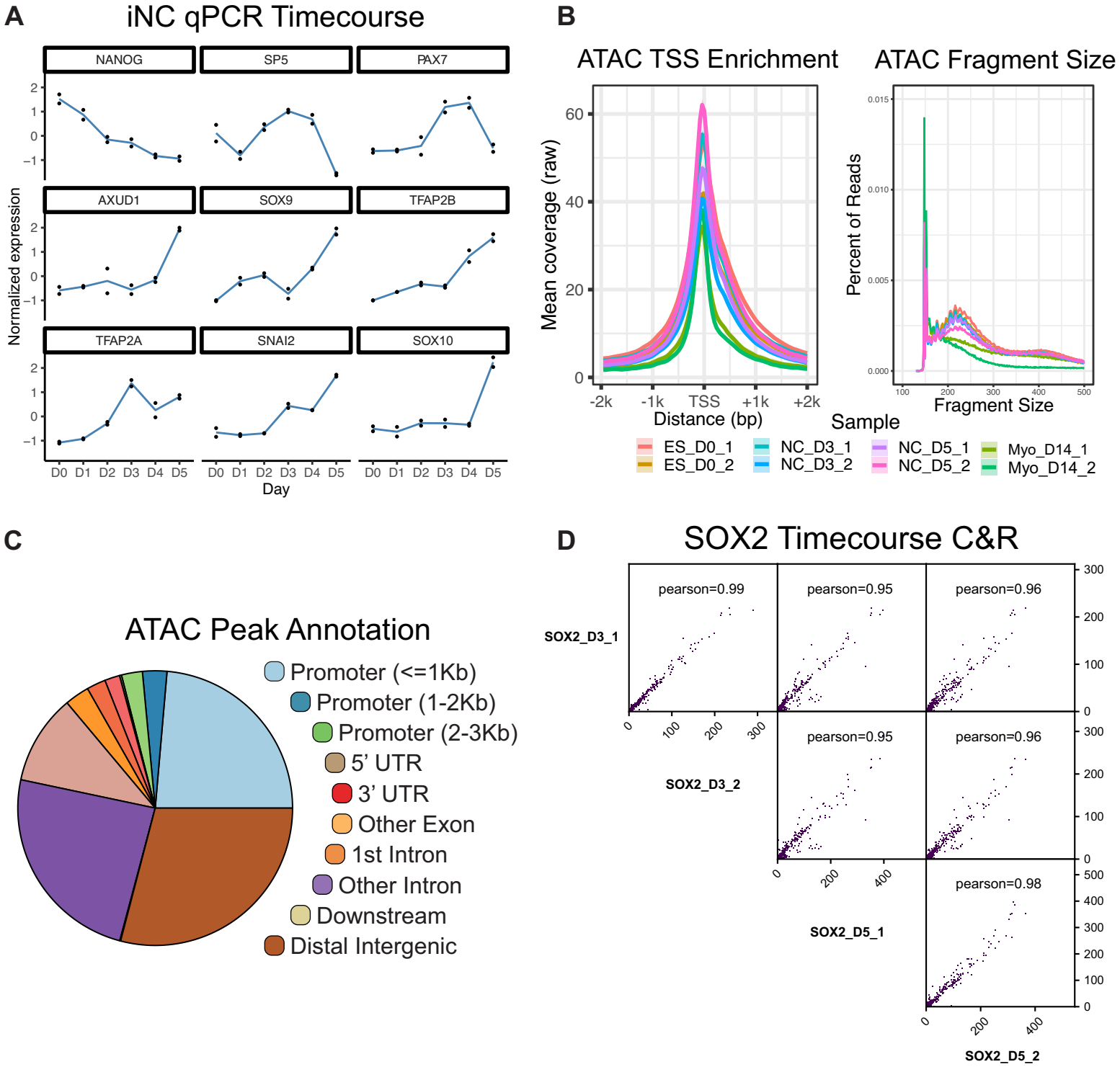

A
iNC qPCR Timecourse
NANOG
SP5
PAX7
AXUD1
SOX9
TFAP2B
TFAP2A
SNAI2
SOX10
Normalized expression
D0 D1 D2 D3 D4 D5
Day
B
ATAC TSS Enrichment
ATAC Fragment Size
Mean coverage (raw)
-2k -1k TSS +1k +2k
Distance (bp)
Percent of Reads
Fragment Size
Sample
ES_D0_1
ES_D0_2
NC_D3_1
NC_D3_2
NC_D5_1
NC_D5_2
Myo_D14_1
Myo_D14_2
C
ATAC Peak Annotation
Promoter (<=1Kb)
Promoter (1-2Kb)
Promoter (2-3Kb)
5' UTR
3' UTR
Other Exon
1st Intron
Other Intron
Downstream
Distal Intergenic
D
SOX2 Timecourse C&R
pearson=0.99
pearson=0.95
pearson=0.96
pearson=0.95
pearson=0.96
pearson=0.98
SOX2_D3_1
SOX2_D3_2
SOX2_D5_1
SOX2_D5_2
E
D3
SOX2 (Homer)
p-val 1e-1631
51.6%
50.0%
SOX2_D3_1
SOX2_D3_2
Occurrences
Size
D5
SOX2 (Homer)
p-val 1e-723
51.4%
52.8%
SOX2_D5_1
SOX2_D5_2
Size

Fig. S4. (Related to figure 4) hiNCC expression characterization and genomic library quality control.

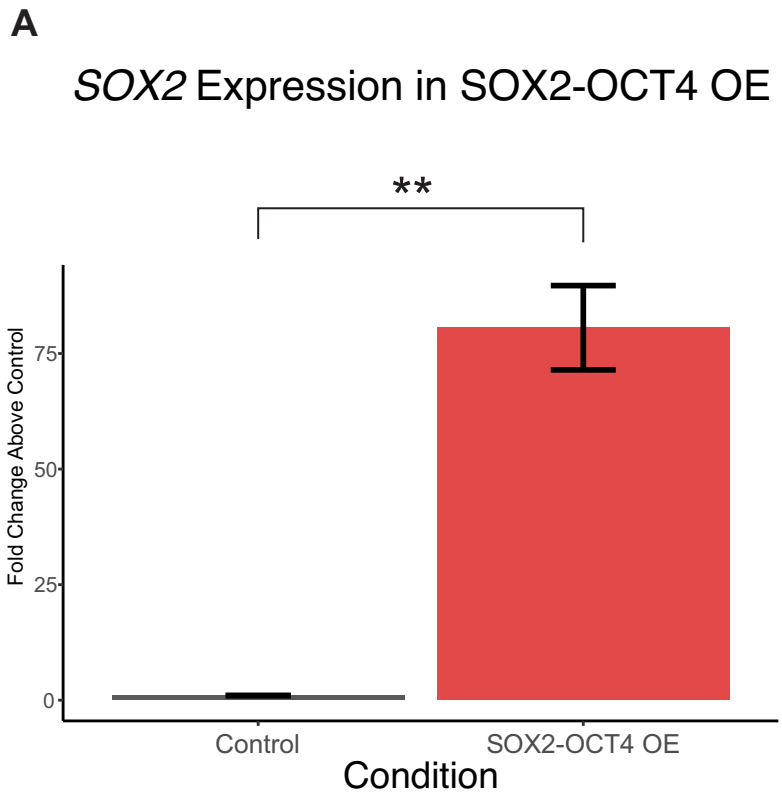
(A) qPCR expression plots for various genes during the human embryonic stem cell (D0) to induced neural crest cell (D5) differentiation process.

(B) Quality control plots for ATAC-Seq performed across the iNC time course. Left, an ATAC-Seq TSS enrichment plot with mean coverage around the transcription start site and +/- 2 kilobases of all genes. Right, the insert size distribution plot. Fragments < 120bp indicate nucleosome free binding.

(C) Genomic annotation of all ATAC-Seq peaks, demonstrating enrichment at promoters, introns, and distal intergenic regions.

(D) Bin-wise correlation quality control plot for SOX2 CUT&RUN for D3 and D5 iNC CUT&RUN libraries.

(E) Motif enrichment for SOX2 (from Homer) and fragment size distribution for the D3 and D5 iNC CUT&RUN libraries.


A
SOX2 Expression in SOX2-OCT4 OE
**
Fold Change Above Control
75
50
25
0
Control
SOX2-OCT4 OE
Condition

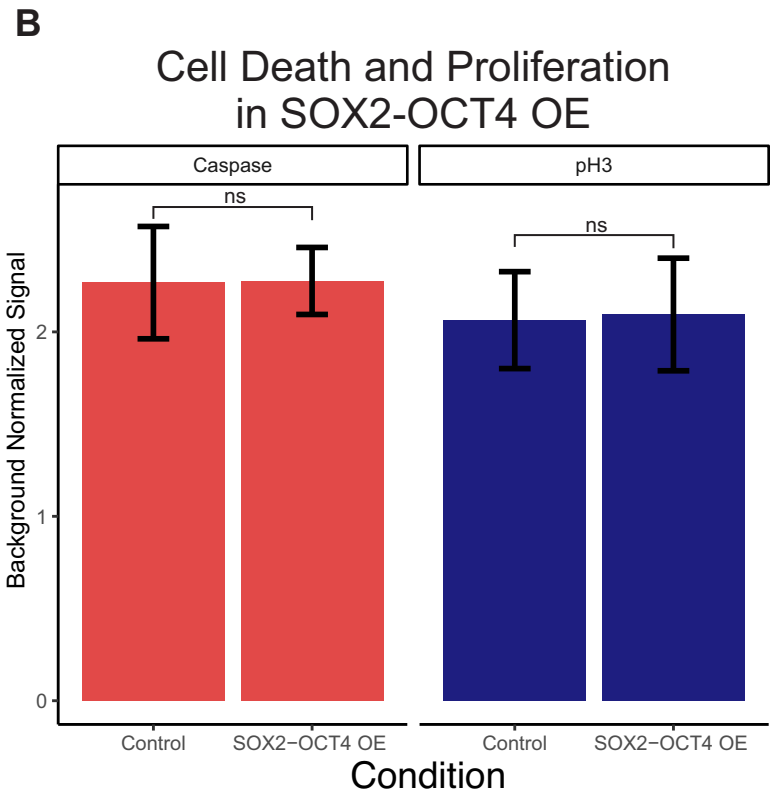

B
Cell Death and Proliferation
in SOX2-OCT4 OE
Caspase
pH3
ns
ns
Background Normalized Signal
2
1
0
Control
SOX2−OCT4 OE
Control
SOX2−OCT4 OE
Condition

Fig. S5. (Related to figure 5) Quality control of SOX2/OCT4 in-vivo overexpression.

(A) Verification of SOX2 overexpression via qPCR for the SOX2-OCT4 overexpression experiment. qPCR was performed with five independent biological replicates with three technical replicates. SOX2-OCT4 OE condition had significantly higher (**, p-val 0.018) from a student's two-sided t-test.

(B) Quantification of immunohistochemistry for cell death (Caspase) and proliferation (pH3) in control versus SOX2-OCT4 overexpression conditions (ns, p-val > 0.05).

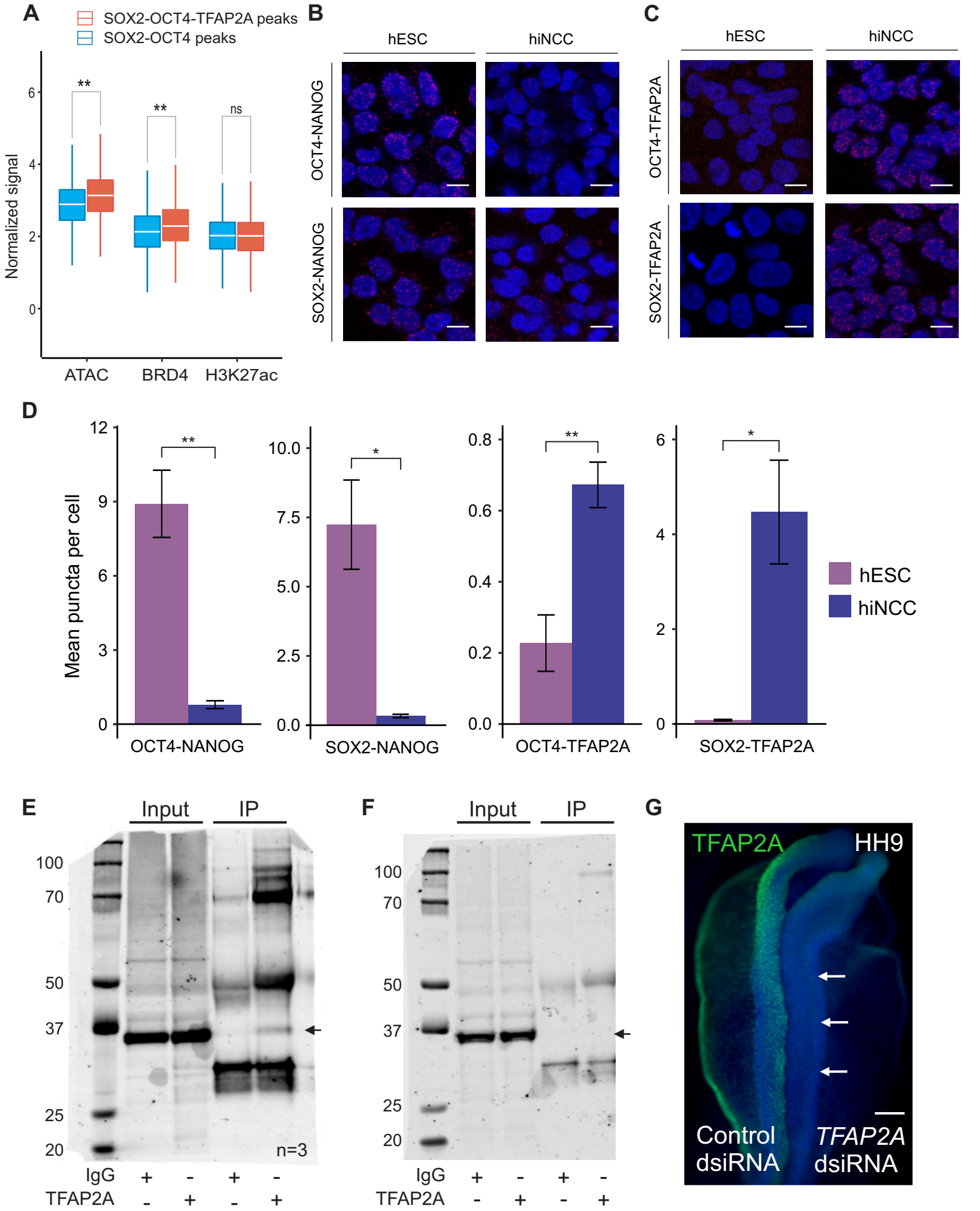

A
SOX2-OCT4-TFAP2A peaks
SOX2-OCT4 peaks
Normalized signal
**
**
ns
ATAC
BRD4
H3K27ac
B
hESC
hiNCC
OCT4-NANOG
SOX2-NANOG
C
hESC
hiNCC
OCT4-TFAP2A
SOX2-TFAP2A
D
Mean puncta per cell
OCT4-NANOG
SOX2-NANOG
OCT4-TFAP2A
SOX2-TFAP2A
hESC
hiNCC
E
Input
IP
100
70
50
37
25
20
n=3
IgG
TFAP2A
F
Input
IP
100
70
50
37
25
20
IgG
TFAP2A
G
TFAP2A
HH9
Control dsiRNA
TFAP2A dsiRNA

Fig. S6. (Related to figure 6) Endogenous Co-IP, TFAP2A dsiRNA validation, and proximity ligation assays.

(A) Boxplot showing that ATAC signal and BRD4 binding is higher in peaks bound by all 3 factors than those only bound by SOX2 and OCT4. H3K27ac signal at these regions was not significantly different.

(B) Proximity Ligation Assays (PLAs) for SOX2/OCT4-NANOG interactions. Positive interactions appear as nuclear, red puncta. Scale bar 10 μm.

(C) Proximity Ligation Assays (PLAs) for SOX2/OCT4-TFAP2A interactions. Positive interactions appear as nuclear, red puncta.

(D) Quantification of parts D&E. Samples were compared using a single tailed Student's t-test (* p-val < 0.05; ** p-val < 0.01)

(E) A western blot for SOX2 in an endogenous (chicken) co-immunoprecipitation assay. Under IP conditions, pulling down with TFAP2A results in detection of SOX2.

(F) A western blot for SOX2 in an endogenous (human embryonic stem cell) co-immunoprecipitation assay. Pulldown of TFAP2A in unable to recover SOX2 in D0 human ESCs.

(G) A HH9 embryo that has been bilaterally transfected with the *TFAP2A* dsiRNA on the right side of the embryo and immunostained for the TFAP2A protein. Scale bar 100 μm.

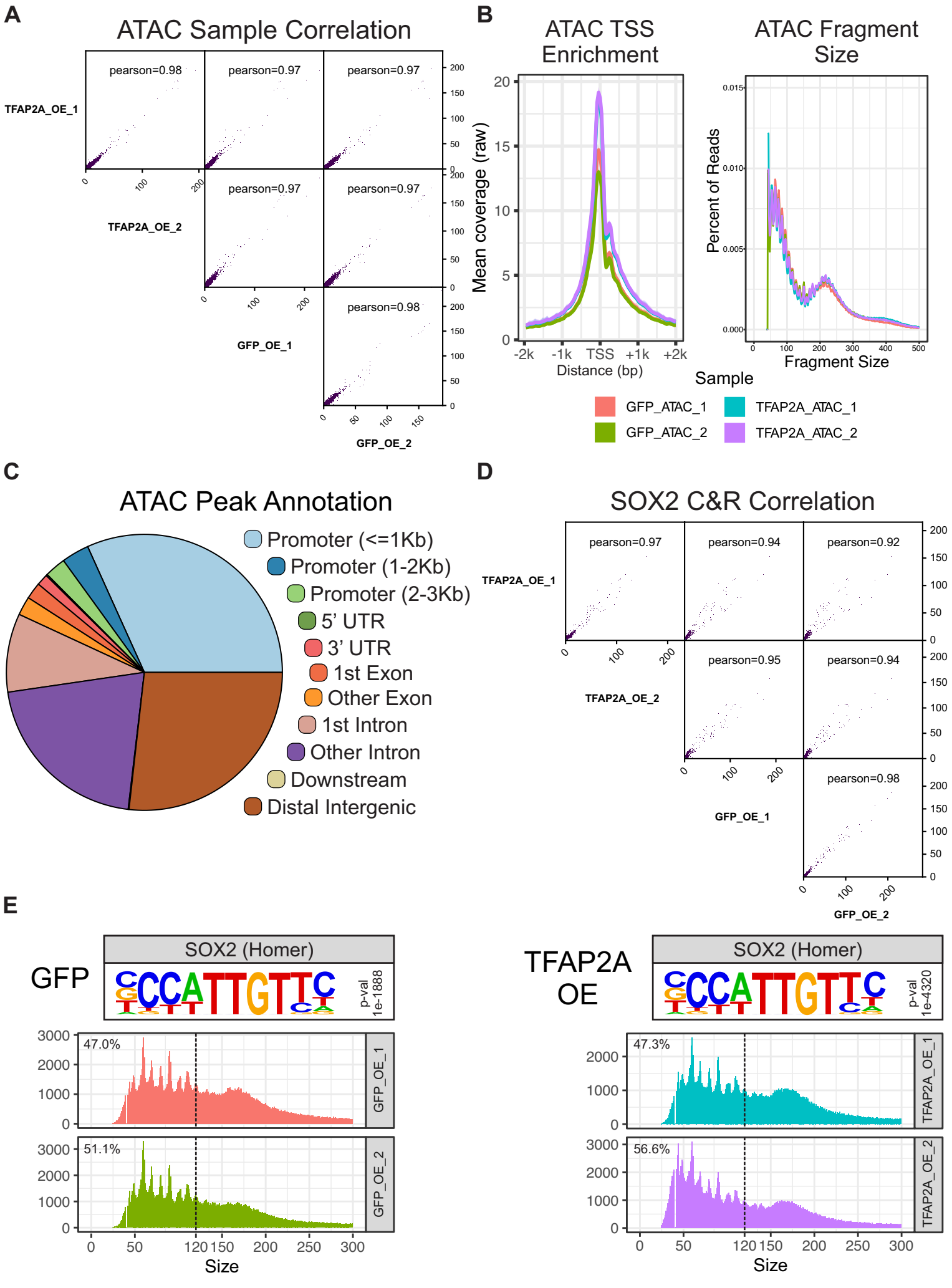

A
ATAC Sample Correlation
pearson=0.98
pearson=0.97
pearson=0.97
pearson=0.97
pearson=0.97
pearson=0.98
TFAP2A_OE_1
TFAP2A_OE_2
GFP_OE_1
GFP_OE_2
B
ATAC TSS Enrichment
Mean coverage (raw)
-2k
-1k
TSS
+1k
+2k
Distance (bp)
ATAC Fragment Size
Percent of Reads
Fragment Size
Sample
GFP_ATAC_1
GFP_ATAC_2
TFAP2A_ATAC_1
TFAP2A_ATAC_2
C
ATAC Peak Annotation
Promoter (<=1Kb)
Promoter (1-2Kb)
Promoter (2-3Kb)
5' UTR
3' UTR
1st Exon
Other Exon
1st Intron
Other Intron
Downstream
Distal Intergenic
D
SOX2 C&R Correlation
pearson=0.97
pearson=0.94
pearson=0.92
pearson=0.95
pearson=0.94
pearson=0.98
TFAP2A_OE_1
TFAP2A_OE_2
GFP_OE_1
GFP_OE_2
E
GFP
SOX2 (Homer)
p-val 1e-1888
47.0%
51.1%
GFP_OE_1
GFP_OE_2
Size
TFAP2A OE
SOX2 (Homer)
p-val 1e-4320
47.3%
56.6%
TFAP2A_OE_1
TFAP2A_OE_2
Size

Fig. S7. (Related to figure 7) Quality control of genomic datasets in hESCs with TFAP2A and GFP overexpression.

(A) Bin-wise correlation quality control plots for TFAP2A and GFP overexpression ATAC libraries.

(B) Quality control plots for TFAP2A and GFP overexpression ATAC-Seq libraries. Left, an ATAC-Seq TSS enrichment plot with mean coverage around the transcription start site and +/- 2 kilobases of all genes. Right, an insert size distribution plot demonstrating nucleosome patterning.

(C) A genomic annotation plot for ATAC-Seq peaks, with strong enrichment in promoters, introns, and distal intergenic regions.

(D) A bin-wise correlation quality control plot for the TFAP2A and GFP overexpression SOX2 CUT&RUN libraries.

(E) SOX2 motif enrichment (from Homer) and fragment size distribution for TFAP2A and GFP overexpression CUT&RUN libraries.