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Supplemental Information

Cell-Cycle Control of Developmentally Regulated Transcription Factors Accounts for Heterogeneity in Human Pluripotent Cells

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Inventory of Supplemental Information

Figure S1, related to Figure 2. This figure evaluates cell cycle genes using the Fucci system, and also using a cell cycle dye to independently verify findings.

Figure S2, related to Figure 3. This figure evaluates newly synthesized transcript levels to demonstrate that cell cycle changes are due to transcriptional regulation. This Figure also shows that pluripotency and differentiation markers are co-expressed, and that neuroectoderm genes are cell cycle regulated.

Figure S3, related to Figure 4. This figure provides evidence that Fucci cells differentiate from G1.

Figure S4, related to Figure 4. This figure demonstrates that late-G1 sorted cells can re-establish cell cycle regulated developmental genes.

Figure S5, related to Figure 5. This figure provides additional amplicons of 5hmC-capture qPCR for GATA6 and SOX17.

Table S1, related to Figure 2. This table provides RNA-seq findings for all cell cycle regulated genes.

Table S2, related to Figure 2. This table provides detailed GO analysis for cell cycle regulated genes by RNA-seq and further broken down by cluster analysis.

Table S3, related to Figure 5. This table provides the complete list of 5hmC cell cycle regulated genomic loci, and detailed base resolution analysis from TABseq.

Table S4, related to Figure 5. Primers used in 5hmC-capture qPCR and TABseq.

Supplemental Information

Figure S1

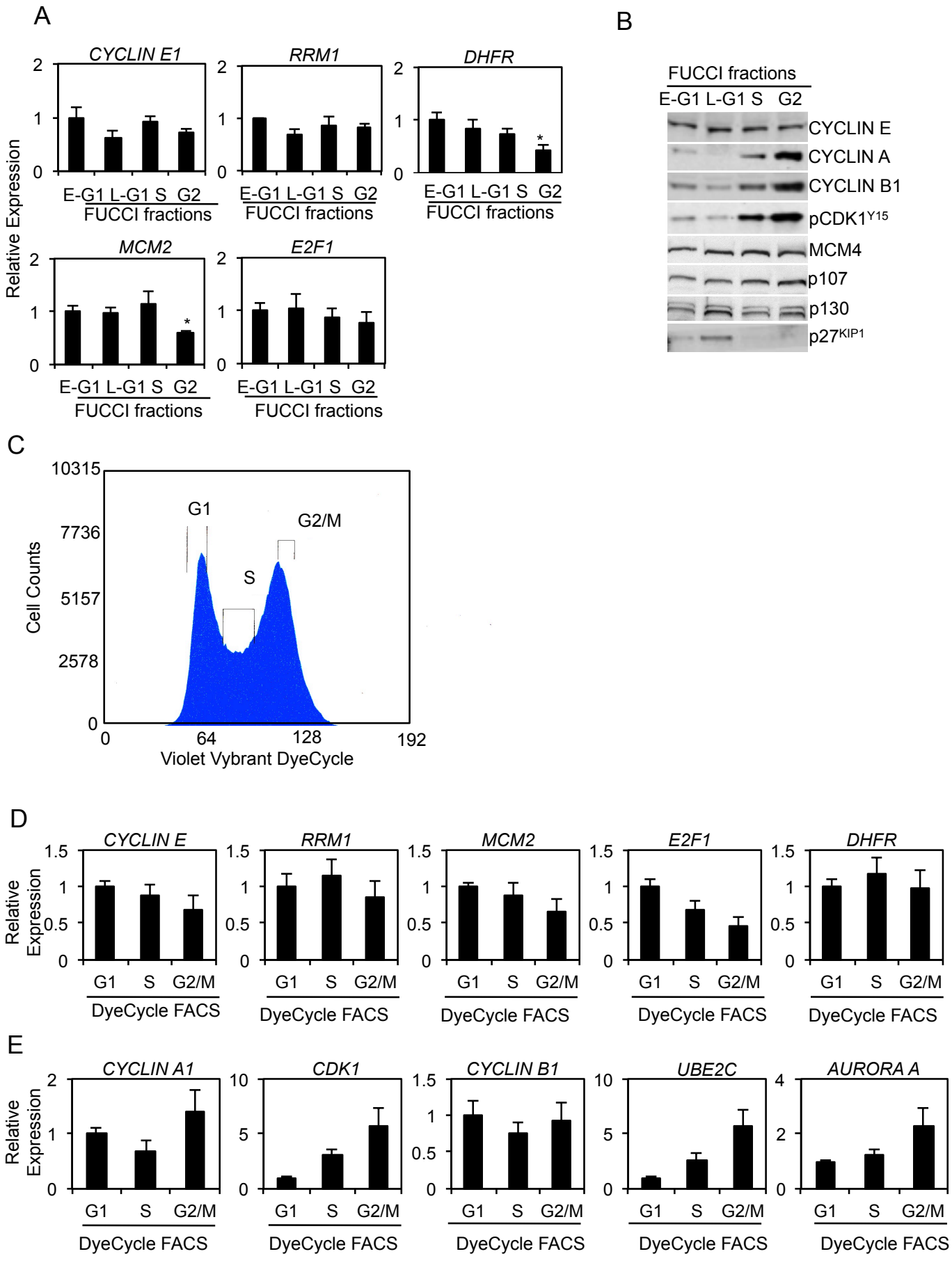

Figure S1, related to Figure 2. hESCs lack E2F-transcriptional regulation. (A) WA09 Fucci hESCs were separated into cell cycle phases by FACS and RNA expression analysis by qRT-PCR was performed for E2F target genes. All experiments were performed in technical triplicate and are representative of multiple experiments. (B) WA09 Fucci hESCs were separated into cell cycle phases by FACS and immunoblotting was performed for cell cycle proteins. (C) WA09 hESCs were incubated with Violet Vybrant DyeCycle for 2 hours and cell cycle fractions were isolated by FACS. (D) RNA expression analysis for RB/E2F target genes, and (E) other cell cycle genes on Vybrant DyeCycle FACS-isolated cell cycle fractions. All experiments were performed in technical triplicate and are representative of multiple experiments. *$p<0.05$, **$p<0.01$.

Figure S2

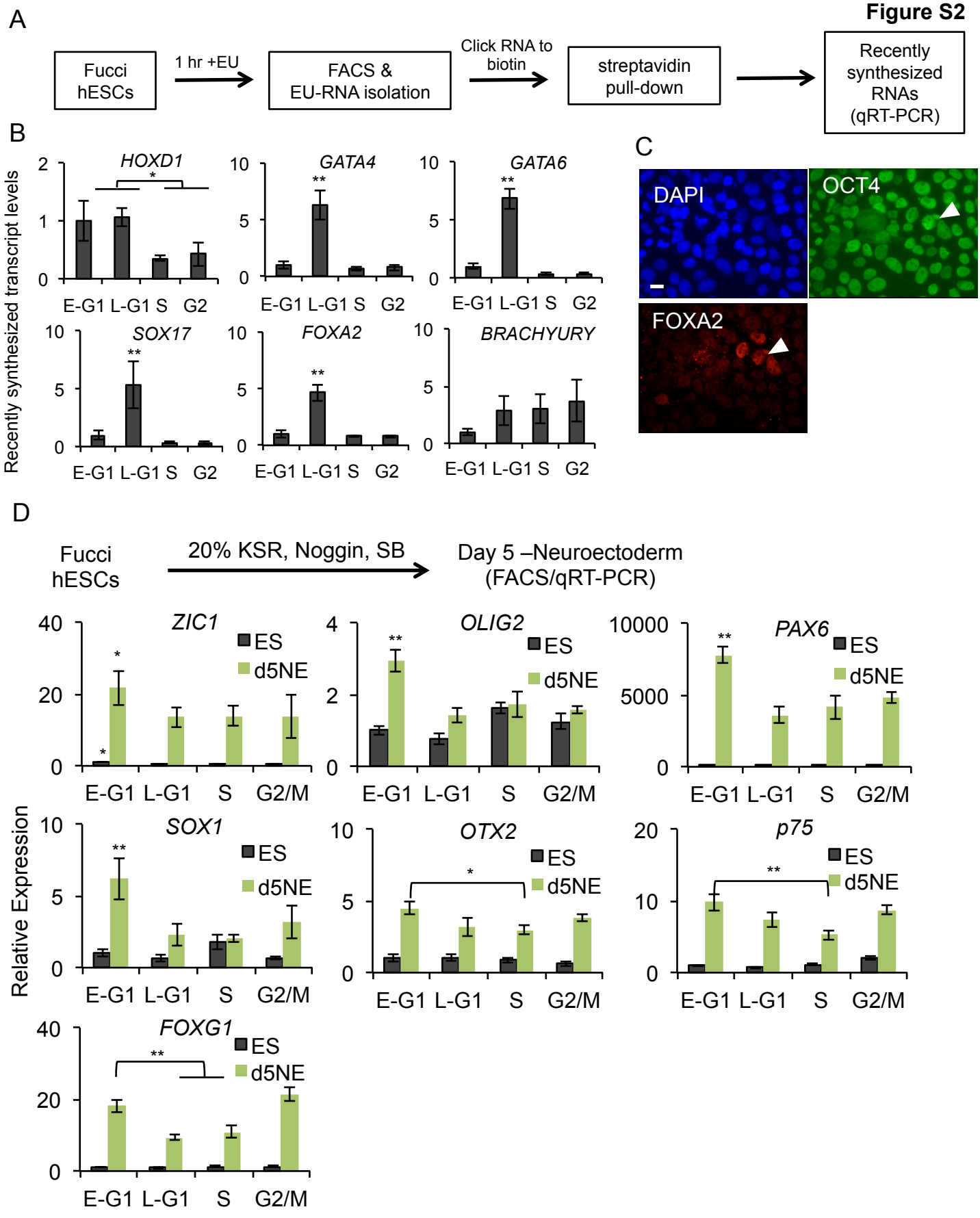
A
Fucci hESCs
1 hr +EU
FACS & EU-RNA isolation
Click RNA to biotin
streptavidin pull-down
Recently synthesized RNAs (qRT-PCR)
B
Recently synthesized transcript levels
HOXD1
GATA4
GATA6
SOX17
FOXA2
BRACHYURY
E-G1 L-G1 S G2
C
DAPI
OCT4
FOXA2
D
Fucci hESCs
20% KSR, Noggin, SB
Day 5 –Neuroectoderm (FACS/qRT-PCR)
ZIC1
OLIG2
PAX6
SOX1
OTX2
p75
FOXG1
ES
d5NE
Relative Expression
E-G1 L-G1 S G2/M

Figure S2, related to Figure 3. Recently synthesized transcript levels of differentiation genes are cell cycle regulated and developmental factors are co-expressed with pluripotency factors. (A) Newly transcribed RNA of Fucci hESCs were labeled for 1 hour, isolated and used for qRT-PCR. (B) Developmental genes have increased expression levels of new transcripts in late G1 (KO2+) cells. All experiments were performed in technical triplicate and are representative of multiple experiments. (C) Immunostaining reveals that FOXA2 and OCT4 are co-expressed in WA09 hESCs. Arrowhead shows FOXA2 and OCT4 double positive cell. Scale: 50μM. (D) RNA expression of neuroectoderm developmental regulators following 5 days of differentiation and FACS-isolation of Fucci fractions. All experiments were performed in technical triplicate and are representative of multiple experiments. *$p<0.05$, **$p<0.01$.

Figure S3

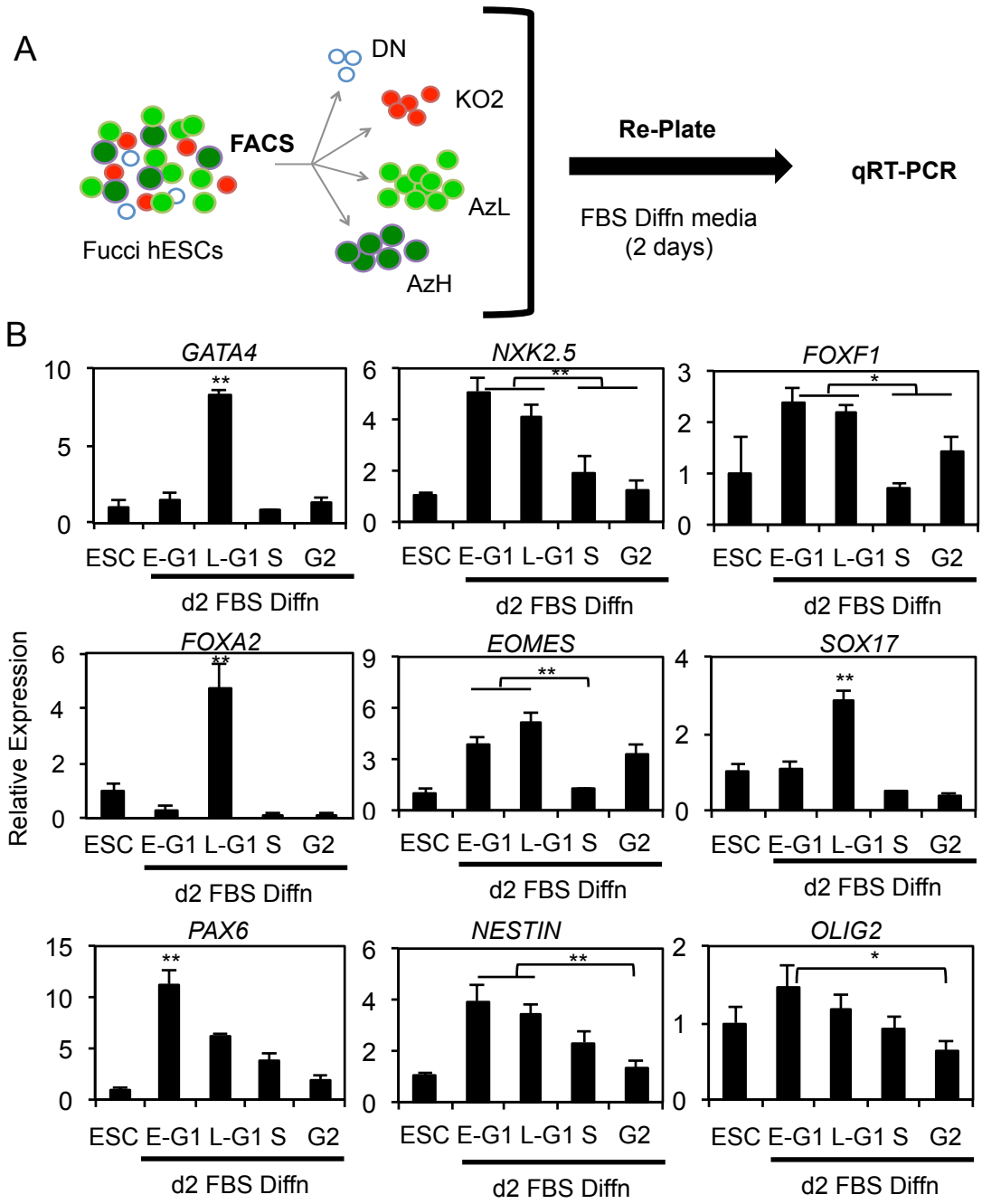
A
DN
KO2
AzL
AzH
FACS
Fucci hESCs
Re-Plate
FBS Diffn media
(2 days)
qRT-PCR
B
GATA4
NXK2.5
FOXF1
FOXA2
EOMES
SOX17
PAX6
NESTIN
OLIG2
Relative Expression
ESC E-G1 L-G1 S G2
d2 FBS Diffn
C
Fucci hESCs
DE - Diffn
3 hours
FACS/qRT-PCR
GATA4
GATA6
SOX17
ESC
DE-3hr
Relative Expression
E-G1 L-G1 S G2

Figure S3, related to Figure 4. Pluripotent stem cells have an increased propensity to differentiate from G1. (A) Fucci hESCs cell cycle fractions were isolated by FACS and re-plated in differentiation media consisting of 20% fetal calf serum (FCS) for 48 hours and cells were collected for RNA analysis. (B) RNA expression analysis by qRT-PCR for mesoderm, endoderm or neurectoderm markers comparing undifferentiated Fucci cells (ESC) to Fucci isolated cell fractions differentiated for 2 days in FCS. Cells in early G1 or late G1 (DN or KO2) have increased levels of differentiation markers compared to S-phase or G2 cells (AzL or AzH), demonstrating that cells in G1 are more prone to differentiation. All experiments were performed in technical triplicate and are representative of multiple experiments. (C) RNA expression for endoderm markers following a 3-hour treatment with DE differentiation conditions and FACS-isolation of Fucci fractions. All experiments were performed in technical triplicate and are representative of multiple experiments. *$p<0.05$, **$p<0.01$.

Figure S4

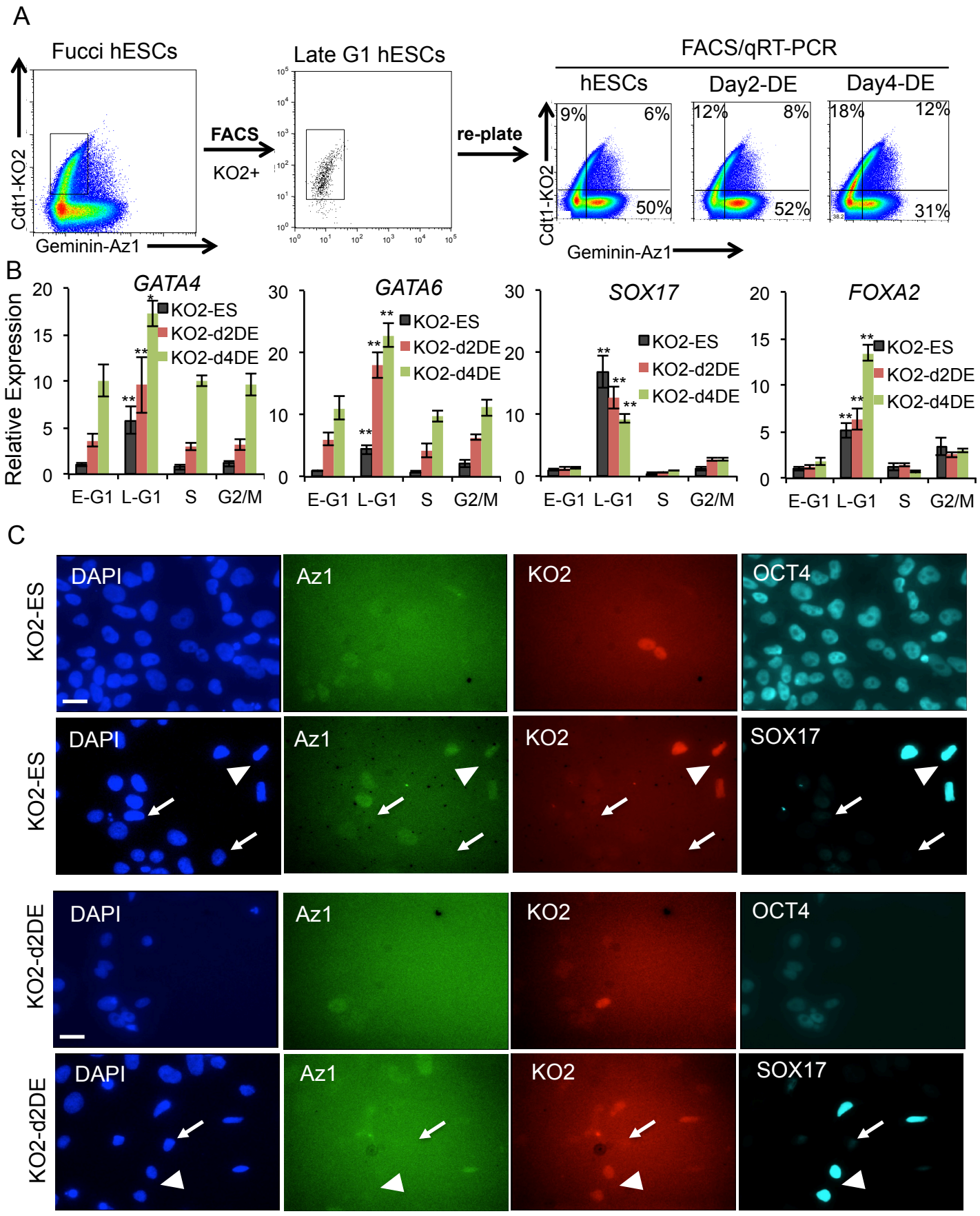
A
Fucci hESCs
Cdt1-KO2
Geminin-Az1
FACS
KO2+
Late G1 hESCs
re-plate
FACS/qRT-PCR
hESCs
Day2-DE
Day4-DE
9%
6%
12%
8%
18%
12%
50%
52%
31%
Cdt1-KO2
Geminin-Az1
B
Relative Expression
GATA4
GATA6
SOX17
FOXA2
KO2-ES
KO2-d2DE
KO2-d4DE
E-G1
L-G1
S
G2/M
C
KO2-ES
KO2-d2DE
DAPI
Az1
KO2
OCT4
SOX17

Figure S4, related to Figure 4. KO2-sorted Fucci cells re-establish heterogeneity in self-renewal and differentiation media. (A) Late-G1 (KO2+) cells were isolated from Fucci hESCs and re-plated in either ESC media or DE-differentiation media for 2 or 4 days, and then re-sorted by FACS for (B) qRT-PCR of endoderm markers. All experiments were performed in technical triplicate and are representative of multiple experiments. (C) Late-G1 (KO2+) cells were isolated by FACS and re-plated in ESC or differentiation media, and immunostained after 2 days for OCT4 or SOX17. Arrows denote DN cells (early-G1) that are negative for SOX17. Arrowheads denote KO2+ cells (late G1) or double positive cells that express Sox17. Scale: 50μM. *$p<0.05$, **$p<0.01$.

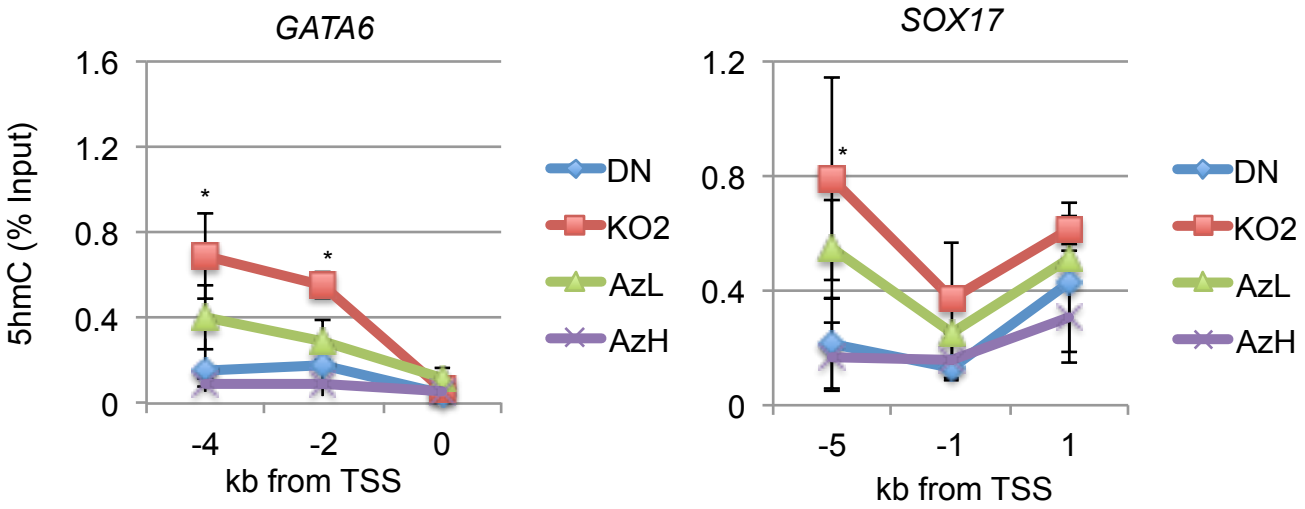

Figure S5, related to Figure 5. 5hmC-capture qPCR for *GATA6* and *SOX17* across their upstream promoter regions. All experiments were performed in technical triplicate and are representative of multiple experiments. *p<0.05 (KO2 vs DN).

Table S1, related to Figure 2. Cell cycle regulated transcripts (>2-fold cutoff) from experiment performed in biological triplicate identified by RNA-Seq of WA09 Fucci hESCs.

Table S2, related to Figure 2. Gene Ontology (GO) analysis of cell cycle regulated transcripts and GO analysis according to heat map clusters.

Table S3, related to Figure 5. Differentially 5-hydroxymethylated regions (DhMR) identified by 5hmC-capture-sequencing and TAB-seq analysis.

Table S4, related to Figure 5. Primers used for 5hmC-capture qPCR and TAB-Seq.

Gene	Approx. Position	Forward primer	Reverse primer
GATA6	-4kb	GACCTGCAAGTTTCCGCC	GTAGGCCGTCGACCAGAG
GATA6	-2kb	TTCTGCAAAACCCAGTCCAC	AGAAGAGATAGCCAGGCGTC
GATA6	+46bp	CGCGGACCAACTTCTAGTCT	TCTCTGCCTGCCTAACTACC
GATA6 (TABSeq)	+150bp	TAGTYGGAGGAGATGTATTAGA	CRAACAACATAAAACCCA
SOX17	-5kb	AAAGCCCTGCCTTCTCCTAG	GTACTGTCTTGCTCACCCCT
SOX17	-1kb	ACACAGGCAAGTTGAGTCCT	GTGTTTCAGTGGTGGGTGAC
SOX17	+1kb	CACATGGGCGGCCACTAC	GCGAAGAAAGCCGGGTCG
SOX17 (TABSeq)	+1kb	GTAAGTAGGTGAAGYGGTTGAAG	CCAAACTCTAACAATCRCRATA
CDK1	-15kb	CTTTTGGGTCTCCAGCTTTG	TGGGTGAGCAGCAGGTACTA
SOX2	-7kb	TCCAACCCTGACACAGACAA	GGGCTCAATGGTGTCAAGTT