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Supplemental Information

The Polycomb Group Protein L3MBTL1 Represses a SMAD5-Mediated Hematopoietic Transcriptional Program in Human Pluripotent Stem Cells

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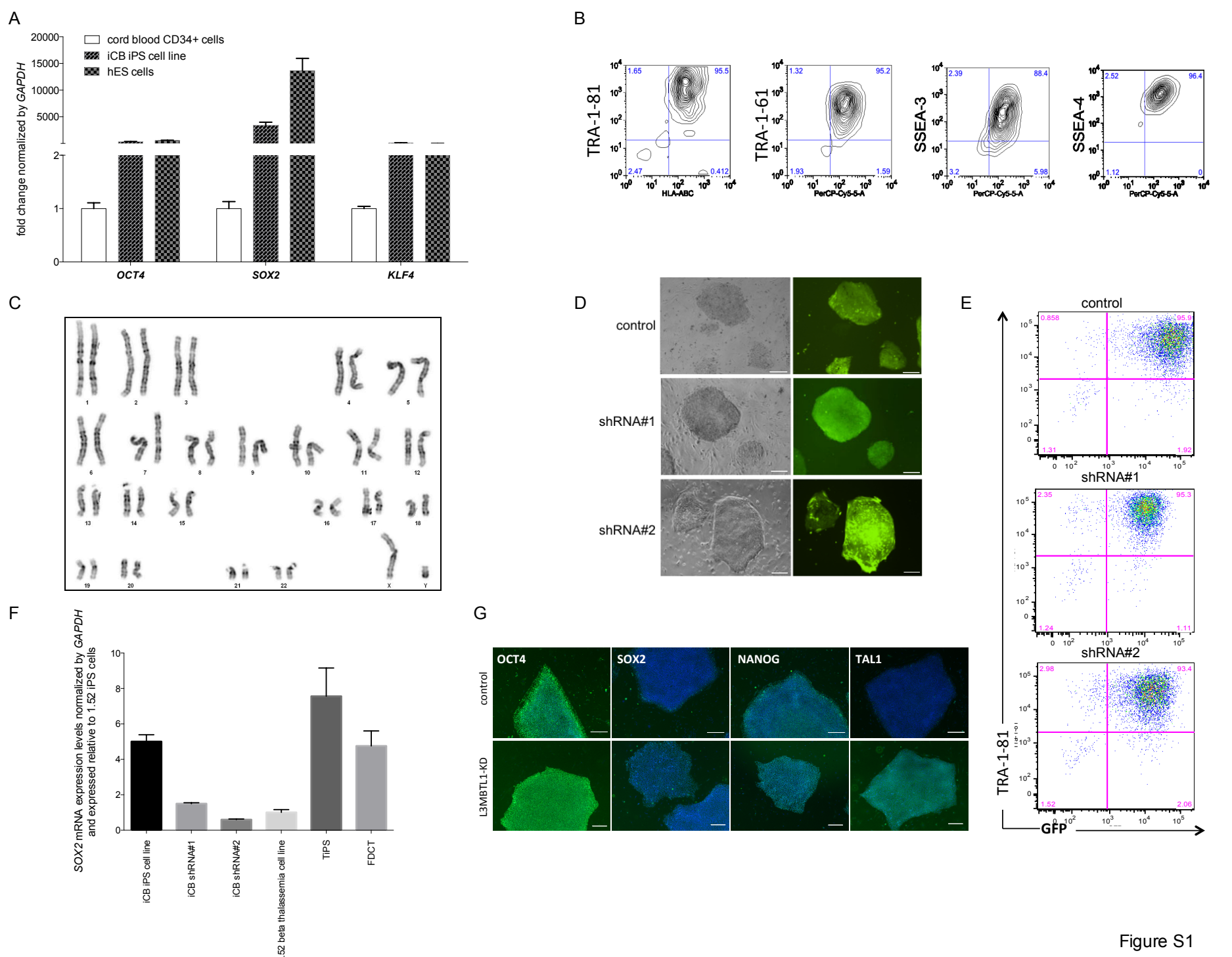
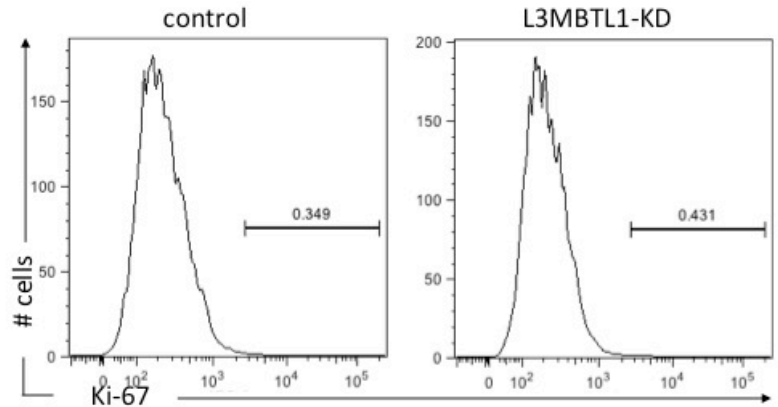
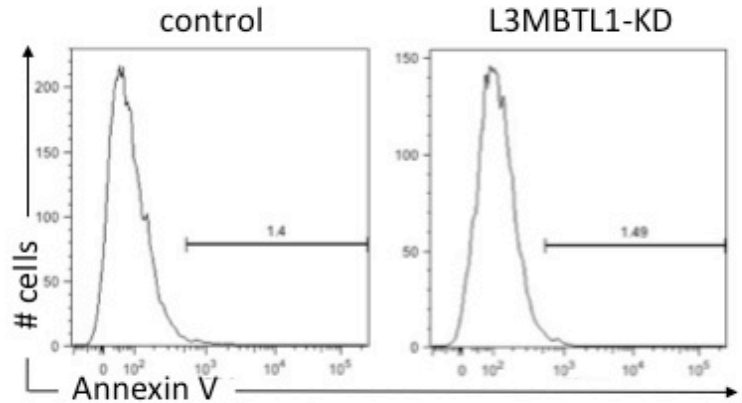

A
cord blood CD34+ cells
iCB iPS cell line
hES cells
fold change normalized by GAPDH
OCT4
SOX2
KLF4
B
TRA-1-81
TRA-1-61
SSEA-3
SSEA-4
HLA-ABC
PerCP-Cy5-5-A
C
D
control
shRNA#1
shRNA#2
E
control
shRNA#1
shRNA#2
TRA-1-81
GFP
F
SOX2 mRNA expression levels normalized by GAPDH and expressed relative to 1.52 iPS cells
iCB iPS cell line
iCB shRNA#1
iCB shRNA#2
1.52 beta thalassemia cell line
TiPS
FDCT
G
OCT4
SOX2
NANOG
TAL1
control
L3MBTL1-KD

Figure S1

A



B



C

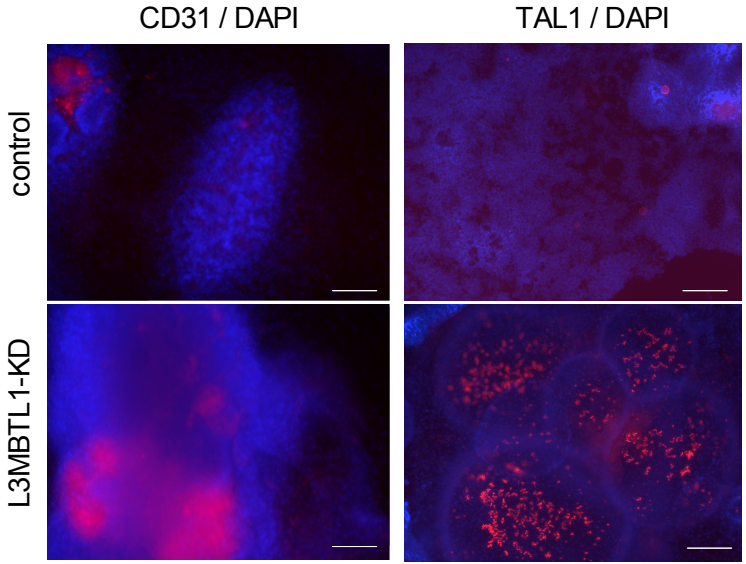


Figure S2

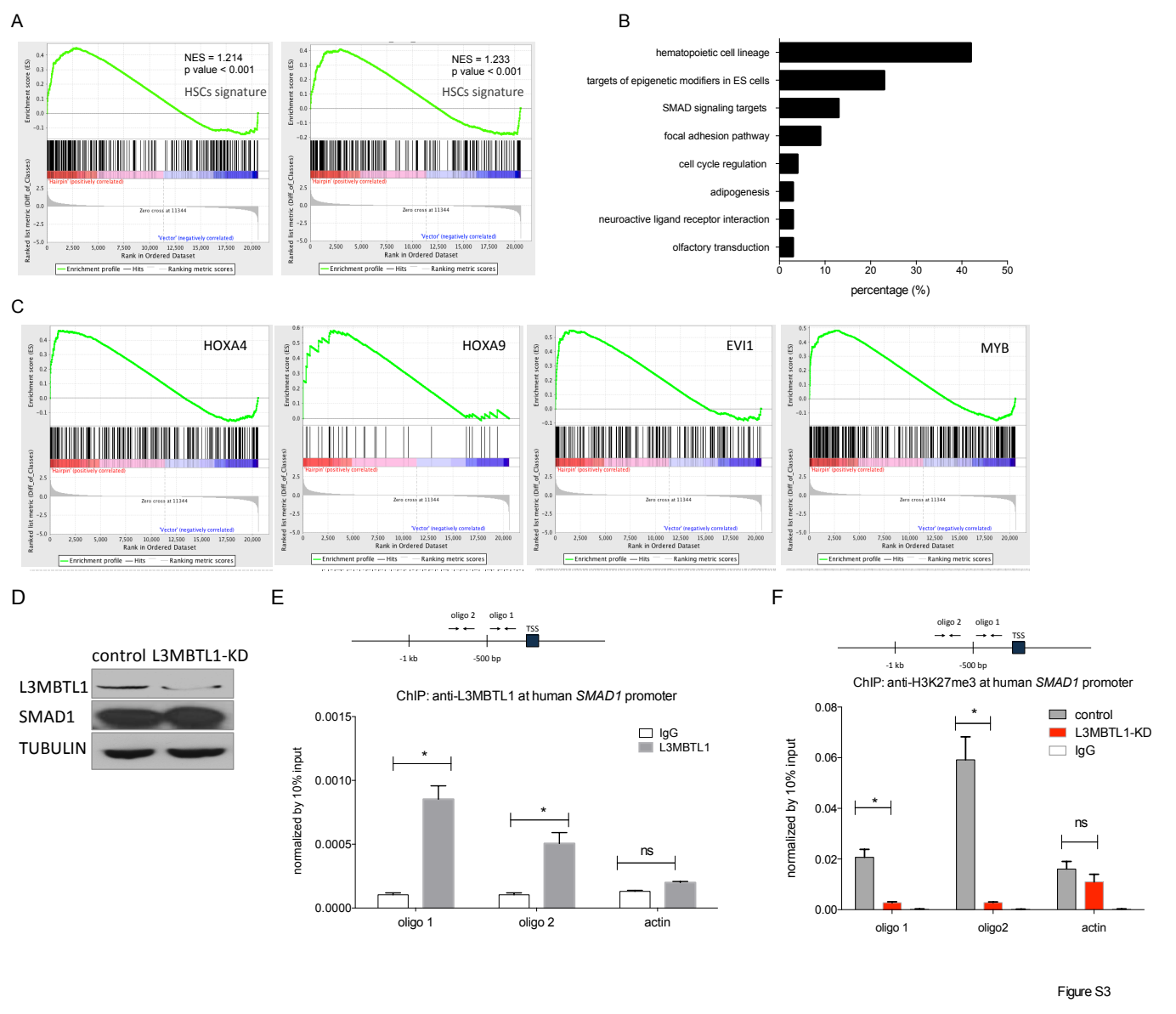

A
NES = 1.214
p value < 0.001
HSCs signature
NES = 1.233
p value < 0.001
HSCs signature
B
hematopoietic cell lineage
targets of epigenetic modifiers in ES cells
SMAD signaling targets
focal adhesion pathway
cell cycle regulation
adipogenesis
neuroactive ligand receptor interaction
olfactory transduction
percentage (%)
C
HOXA4
HOXA9
EVI1
MYB
D
control L3MBTL1-KD
L3MBTL1
SMAD1
TUBULIN
E
oligo 2
oligo 1
TSS
-1 kb
-500 bp
ChIP: anti-L3MBTL1 at human SMAD1 promoter
normalized by 10% input
IgG
L3MBTL1
oligo 1
oligo 2
actin
ns
F
oligo 2
oligo 1
TSS
-1 kb
-500 bp
ChIP: anti-H3K27me3 at human SMAD1 promoter
normalized by 10% input
control
L3MBTL1-KD
IgG
oligo 1
oligo2
actin
ns

Figure S3

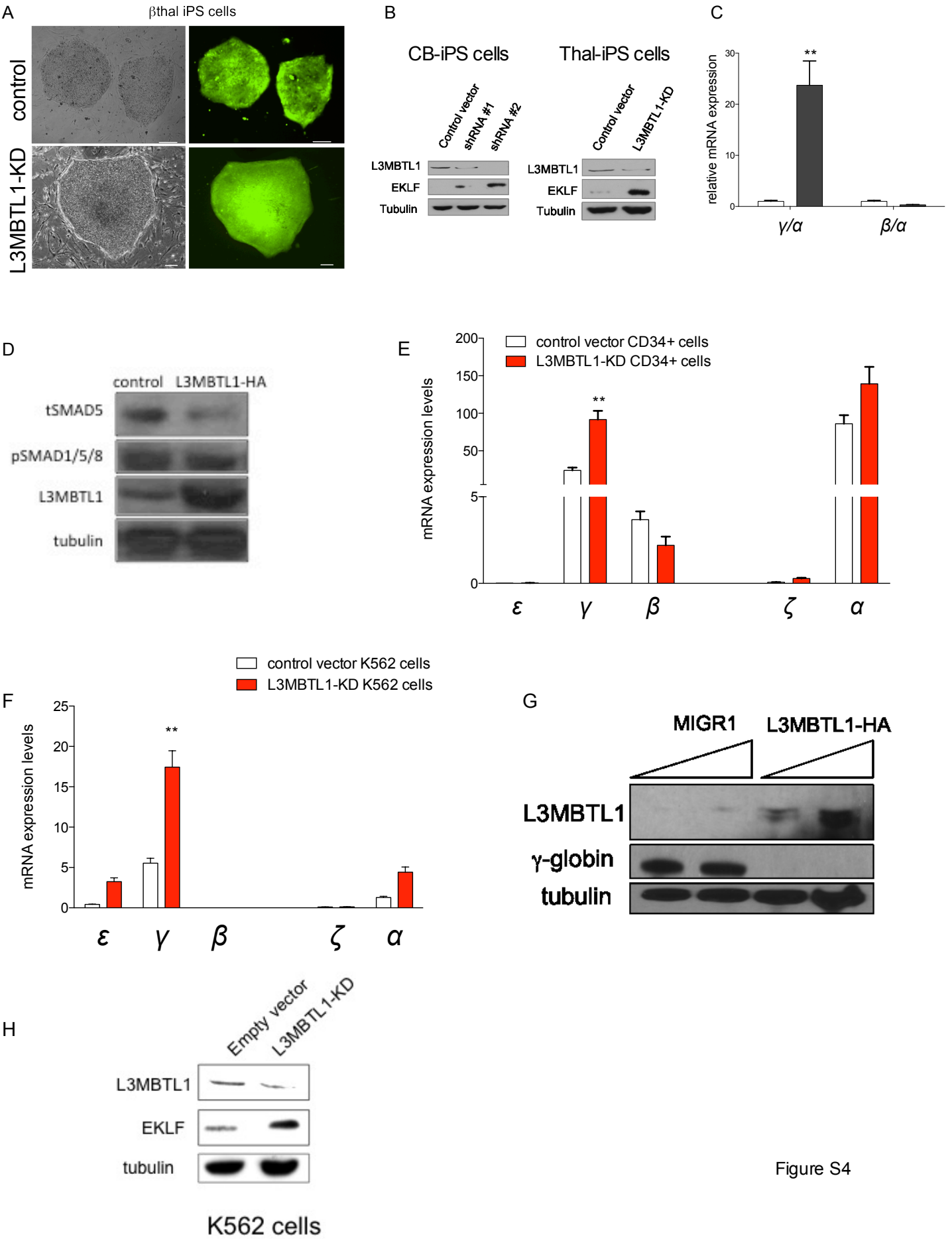

A
βthal iPS cells
control
L3MBTL1-KD
B
CB-iPS cells
Thal-iPS cells
Control vector
shRNA #1
shRNA #2
L3MBTL1-KD
L3MBTL1
EKLF
Tubulin
C
relative mRNA expression
γ/α
β/α
D
control
L3MBTL1-HA
tSMAD5
pSMAD1/5/8
L3MBTL1
tubulin
E
control vector CD34+ cells
L3MBTL1-KD CD34+ cells
mRNA expression levels
ε
γ
β
ζ
α
F
control vector K562 cells
L3MBTL1-KD K562 cells
mRNA expression levels
G
MIGR1
L3MBTL1-HA
L3MBTL1
γ-globin
tubulin
H
Empty vector
L3MBTL1-KD
L3MBTL1
EKLF
tubulin
K562 cells

Figure S4

Supplementary figure titles and legends

Figure S1. Characterization of the iPS cell line generated from CB $CD34^+$ cells.
A) Expression of pluripotency markers in an iPS cell line derived from cord blood (iCB) by qPCR. *GAPDH* was used as housekeeping gene and the data are compared to hES cells and definitive cord blood CD34+ cells. The data represent the mean ± SD of the three independent experiments. B) Expression of the TRA-1-81, TRA-1-60, SSEA-3, SSEA-4 pluripotency markers was evaluated in the CB1.9 cells by FACS analysis. C) Chromosome analysis was performed on a minimum of 15 DAPI-banded metaphases. All metaphases were fully karyotyped. This culture has a normal male karyotype (46XY) in the majority of cells analyzed. D) Undifferentiated iPS cells, transduced with lentiviral vectors expressing shRNA targeting L3MBTL1 or luciferase (and GFP). GFP^+ cells maintain the undifferentiated morphology. Scale bars indicate 100μm. E) GFP^+ cells maintain an undifferentiated cell immunophenotype, based on TRA-181 positivity. F) Relative *SOX2* mRNA expression levels in control and L3MBTL1-KD iPS cells, which we derived from CB $CD34^+$ cells, in comparison to the 1.52 β thalassemic iPS cell line that we previously characterized (Papapetrou et al., 2011). T-iPS and FDCT cell lines were previously characterized (Papapetrou et al., 2011; Themeli et al., 2013). Data were normalized to *GAPDH* and analyzed by ΔΔCt and expressed as fold change. The data represent the mean ± SD of the three independent experiments. G) Expression of pluripotency (OCT4, SOX2 and NANOG) and hematopoietic-specific markers (TAL1) was assessed in undifferentiated control and L3MBTL1-KD iPS cells by flow cytometry. Scale bars indicate 100μm.

Figure S2. Neural development in L3MBTL1-KD pluripotent stem cells.
A) Representative FACS scatter plot measuring KI-67 expression as a marker of cell proliferation B) Representative FACS scatter plot measuring Annexin V expression as marker of apoptosis. C) L3MBTL1-KD hEBs show enhanced expression of hematopoietic markers. CD31 and TAL1 expression were assessed by IF in EBs and showed over DAPI staining. Scale bars indicate 100μm.

Figure S3. Transcriptional analyses in undifferentiated L3MBTL1-KD iPS cells

A) Two hematopoietic stem cells gene sets are enriched in KD cells, in addition to the gene set showed in Figure 3C. B) Top 30 most significant curated pathway gene sets from GSEA (FDR<0.001; p-value <0.0002), were ranked based on NES (normalized enrichment score), and grouped in eight main categories based on lineage and biological pathways, with the "hematopoietic cell lineage" category being the most represented. 12 out of 30 (40%) gene sets were found statistically (FDR<0.001; p value <0.0002) enriched. For example, the hematopoietic category included gene sets of hematopoietic stem cells, hematopoietic cell lineage, hematopoietic transcription factor targets, endothelial cells and angiogenesis. The epigenetic signature category included targets of (7/30) SUZ12, EZH2, SWI/SNF and MLL in ES cells, genes involved in transcription elongation and genes with low CpG density promoters bearing H3K4me3. This is in agreement with the role of L3MBTL1 in affecting the chromatin state and transcriptionally repressing gene expression. C) Gene Set Enrichment Analysis for several combined hematopoietic transcription factor signatures. D) L3MBTL1 and SMAD1 protein expression levels were assessed in iPS cells by WB assay. Tubulin served as loading control. E) iPS cells were crosslinked with 1% formaldehyde and immunoprecipitated with an anti-L3MBTL1 antibody or an IgG antibody as a negative control. Data were normalized by 10% input. The data represent the mean ± SD of the three independent experiments. * $p < 0.05$ by Student's t test. F) iPS cells were crosslinked with 1% formaldehyde and immunoprecipitated with an anti-H3K27me3 antibody or an IgG antibody as a negative control. Data were normalized by 10% input. The data represent the mean ± SD of the three independent experiments. * $p < 0.05$ by Student's t test. ns indicates not significant.

Figure S4. Erytrhoid differentiation is promoted in L3MBTL1-KD cells

A) Thalassemic iPS colonies were efficiently infected with lentiviral vectors expressing shRNA against *L3MBTL1* and GFP. Scale bars indicate 100μm. B) Knock-down of L3MBTL1 increases EKLF protein expression levels, as shown by WB assay in normal iPS cells. Tubulin served as loading control. EKLF

expression was consistently increased in β-thalassemic iPS cells, as shown by WB assay. Tubulin served as loading control. C) *Fetal globin* gene expression was upregulated in erythroid progeny derived from L3MBTL1-KD β-thalassemic iPS cells. The data represent the mean ± SD of the three independent experiments. ** $p < 0.01$ by Student's t test. D) Total SMAD5, phospho SMAD1/5/8, L3MBTL1 expression levels were assessed in K562 overexpressing L3MBTL1-HA by WB. Tubulin served as loading control. E) Globin gene expression in L3MBTL1-KD CB $CD34^+$ cells compared to controls by qRT-PCR. Data were normalized by *GAPDH*. The data represent the mean ± SD of the three independent experiments. ** $p < 0.01$ by Student's t test. F) Globin gene expression in L3MBTL1-KD K562 cells compared to controls by qRT-PCR. Data were normalized by *GAPDH*. The data represent the mean ± SD of the three independent experiments. ** $p < 0.01$ by Student's t test. G) L3MBTL1 and gamma globin protein expression was measured in L3MBTL1-overexpressing K562 cells by WB assay. Tubulin served as loading control. H) EKLF expression in L3MBTL1-KD K562 cells by WB assay. Tubulin served as loading control.

Table S1. Gene Ontology analysis, related to Figure S3.

Term		P value
Developmental process	(GO:0032502)	1.71E-04
Endothelial cell proliferation	(GO:0001935)	4.99E-04
Positive regulation of pathway-restricted SMAD protein phosphorylation	(GO: 0010862)	5.48E-04
Embryonic hematopoiesis	(GO:0035162)	5.76E-04
Positive regulation of hematopoietic progenitor cell differentiation	(GO:1901534)	8.55E-04
Negative regulation of astrocyte differentiation	(GO:0048712)	1.12E-03
Regulation of myeloid leukocyte differentiation	(GO:0002761)	1.36E-03
Positive regulation of blood vessel-endothelial cell migration	(GO:0043536)	3.14E-03
Regulation of vasculogenesis	(GO:2001212)	3.84E-03
Histone H4-K20 methylation	(GO: 0034770)	4.58E-03
Regulation of vasculature development	(GO:1901342)	6.18E-03
Regulation of hippo signaling	(GO:0035330)	6.24E-03
Positive regulation of erythrocyte differentiation	(GO:0045648)	6.38E-03
Negative regulation of T cell differentiation	(GO:0045581)	6.66E-03
Erythrocyte differentiation	(GO:0043353)	7.35E-03
Skeletal muscle satellite cell differentiation	(GO:0014816)	7.70E-03
Regulation of mesodermal cell fate specification	(GO:0042661)	7.73E-03

Gene Ontology analysis was performed on 419 differentially expressed genes by Panther (Mi et al., 2013). Ontology: biological process. Correction: Bonferroni.

References:

Mi, H., Muruganujan, A., Casagrande, J.T., and Thomas, P.D. (2013). Large-scale gene function analysis with the PANTHER classification system. Nature protocols *8*, 1551-1566.

Papapetrou, E.P., Lee, G., Malani, N., Setty, M., Riviere, I., Tirunagari, L.M., Kadota, K., Roth, S.L., Giardina, P., Viale, A.*, et al.* (2011). Genomic safe harbors permit high beta-globin transgene expression in thalassemia induced pluripotent stem cells. Nature biotechnology *29*, 73-78.

Themeli, M., Kloss, C.C., Ciriello, G., Fedorov, V.D., Perna, F., Gonen, M., and Sadelain, M. (2013). Generation of tumor-targeted human T lymphocytes from induced pluripotent stem cells for cancer therapy. Nature biotechnology *31*, 928-933.