Stem Cell Reports, Volume *5*

Supplemental Information

A Quantitative Proteomic Analysis of Hemogenic Endothelium Reveals Differential Regulation of Hematopoiesis by SOX17

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SUPPLEMENTAL FIGURE TITLES AND LEGENDS

Figure S1. Proteomic analysis of VE-CADHERIN regulated proteins. Related to Figure 1. A. Protein expression of VEC^+ vs VEC^- populations was analyzed by unsupervised hierarchal cluster analysis [HC]. Green denotes high, relative protein expression and blue low, relative protein expression. B. Protein expression of E10.5 VEC^+ vs E11.5 VEC^+ was analyzed by HC. C. GO-terms linked to proteins enriched in E10.5 VEC^+ populations were analyzed in G-profiler using the cocoa algorithm.

Figure S2. Regulation of SOX17-dependent protein expression during a time course of directed differentiation. Related to Figure 2. A. Schematic outlining inducible SOX17 time course comparison. B. Volcano plot of differentially expressed proteins in D7_dox+ vs D7_dox-. Significant hits are shown in red (FDR<0.05). C. SOX17 protein expression was quantified by nano-LC-MS/MS on a Q Exactive. D. SOX17-regulated changes in protein expression were measured by nano-LC-MS/MS during a time course of directed differentiation to hemogenic endothelium. E. Expression of proteins linked to erythrocyte function is displayed. F. GO-terms linked to proteins regulated by SOX17 overexpression were analyzed in G-profiler using the cocoa algorithm.

Figure S3. *Stat1* is not a direct target of SOX17 in HE. Related to Figure 3. A. STAT3, STAT5A, STAT5B protein expression was quantified by nano-LC-MS/MS on a Q Exactive. B. Luciferase assays using HEK293T cells co-transfected with pGL3 control vector or the *Stat1* promoter contructs and increasing concentrations of the pCAG

expression plasmids. Bars represent mean luciferase intensity relative to pGL3-empty +/- SEM, N=3 independent experiments. C. qRT-PCR analysis of D5.25 Flk-1^{+} cells cultured for 2 days with (+DOX) or without (-DOX) 1ug/mL doxycycline to induce *Sox17* cDNA expression P > 0.05 D. Representative flow cytometric analysis of VE-CADHERIN (VEC), CD45 and CD41 on D7 cells derived from wildtype and *Sox17*$^{-/-}$ mESCs.

SUPPLEMENTAL TABLE TITLES AND LEGENDS

Table S1. All quantified proteins. Related to Figure 1. Peptides corresponding to 4044 proteins were measured by nano-LC-MS/MS on a Q Exactive mass spectrometer and quantified using label free methods across both the *in vivo* and in vitro samples. Common contaminants and reverse hits were removed.

Table S2. Proteins enriched in VE-CADHERIN^{+} or VE-CADHERIN^{-} cell populations in vivo. Related to Figure 1. Proteins that were differentially expressed between conditions were identified using a permutation-based t-test (S1, FDR 5%) in Perseus 1.4.1.3. Comparison of E10.5/E11.5 VEC^{+} to E10.5/E11.5 VEC^{-} populations.

Table S3. Proteins enriched in VE-CADHERIN^{+} or VE-CADHERIN^{-} cell populations in vitro. Related to Figure 1. Proteins that were differentially expressed between conditions were identified as in Table S2. Analysis of proteins expressed in mESC VEC^{+} versus mESC VEC^{-} cell populations.

Table S4. Proteins enriched in VE-CADHERIN$^+$ cell populations in vivo. Related to Figure 1. Proteins that were differentially expressed between conditions were identified as in Table S2. Comparison of proteins measured from E10.5 VEC$^+$CD45$^-$ and E11.5 VEC$^+$CD45$^-$ populations.

Table S5. Proteins regulated by inducible expression of SOX17 in mESCs. Related to Figure S2. D5.25 Flk-1$^+$ endothelial progenitors were treated with doxycycline for 1-3 days to induce the expression of SOX17. Samples were isolated from D6-D9 and analyzed for total protein expression using label free proteomics as in Table S2.

Table S6. Proteins enriched in *Sox17*$^{-/-}$ mESCs. Related to Figure 2. Total protein expression of D7 VEC$^+$CD45$^-$ cell populations from WT or *Sox17*$^{-/-}$ mESCs were analyzed as in Table S2.

Table S7. Primer Sequences. A list of the sequences used in primers for QPCR.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

mESC maintenance and differentiation

mESC lines were maintained in serum-free/feeder-free culture conditions (SF-ES) supplemented with 2i (Gadue et al., 2006; Ying et al., 2008). mESC-1 is a derivative of the E14 mESC line from the 129/Ola mouse strain, mESC-2 is derived from the C57BL/6J x 129S6 mouse strain. mESCs were differentiated in a serum-free media

protocol. mESCs were trypsinized using Trypsin-LE (Invitrogen) and cultured in serum-free differentiation (SF-D) media in the absence of growth factors at a concentration of $2x10^5$/ml for 48hrs to form EBs. At day 2 (D2) EBs were dissociated with Typsin-LE and reaggregated at a concentration of $2x10^5$/ml in SF-D media containing recombinant human (rh) VEGF (5ng/ml, R&D, cat # 293-VE), rhBMP4 (1ng/ml, R&D, cat # 314-BP), recombinant mouse (rm) Wnt3a (3ng/ml, R&D, cat # 1324-WN) and ActivinA (1ng/ml, R&D, cat# 338-AC) unless otherwise indicated for 30hrs. At D3+6hr EBs were dissociated and reaggregated at a concentration of $5x10^5$/ml in 6 well tissue culture pluronic treated plates in SF-D containing ActA (1ng/ml) for 24hrs. At day 4+6 a final volume rhVEGF (5ng/ml), rhBMP4 (10ng/ml) and SB431452 (6uM, Sigma, cat# S-4317) were added to the cultures. At day 5+6 EBs were dissociated using TrypLE and Flk-1^+ cells were isolated by FACS. Flk-1^+ cells were aggregated overnight in 96 well locluster plates at a concentration of $3x10^4$/30uL in SF-D containing KL (2%vol/vol)-conditioned medium, rhVEGF (5ng/ml) and rhbFGF (10ng/ml, R&D, cat# 233-FB). The next morning aggregates were spoted on Matrigel (VWR, cat# 354230)-coated 24 well plates for 12hrs to allow adherance and then the wells were filled with SF-D containing KL (2%), rhVEGF (5ng/ml) and rhbFGF (10ng/ml) to form HE. At day 7+6 monolayers were trypsinized using Trypsin-LE and VEC^+CD45^- HE cells were isolated by FACS or maintained as monolayers and induced to undergo the EHT by culturing in SF-D containing KL (2%), rhVEGF (10ng/ml), rhbFGF (10ng/ml), rmTPO (50ng/ml, R&D, cat3 488-TO), rmIL6 (10ng/ml, R&D, cat# 406-ML), hIL11 (10ng/ml, R&D, cat# 218-IL), rmFlt3L (10ng/ml, R&D, cat3 427-FL) and rhBMP4 (30ng/ml) to induce hematopoiesis.

Embryo generation

Animals were maintained and bred the Animal Resource Center in the MaRS complex according to institutional guidelines. Embryos were generated through timed matings with the day of vaginal plug designated embryonic day 0.5. Pregnant dams were sacrificed and the developmental stage of the embryos was determined by counting the number of somite pairs.

Embryo cell preparation

Embryonic cells for methylcellulose assays were prepared as follows. The yolk sac and fetal livers from embryos were dissected and dissociated in 0.2 Collagenase B for 20' at 37°C. Cells were washed twice in IMDM and plated in methylcellulose assays at the following concentrations; E8.5 yolk sacs 1 yolk sac was plated per 1ml of methylcellulose, E14 fetal livers $5x10^4$ total cells was plated per 1 ml of methylcellulose.

Hematopoietic progenitor colony assay

Cells were plated in 1% methylcellulose (wt/vol, Sigma) containing 10% (vol/vol) plasma-derived serum (PDS; Antech), 5% (vol/vol) protein-free hybridoma medium (PFHM-II; Invitrogen) and the following cytokines: KL (2% (vol/vol) conditioned medium), TPO (50ng/ml), erythropoietin (2 U/ml), IL11 (25ng/ml) IL3 (1% (vol/vol) conditioned medium), GM-CSF (1% (vol/vol) conditioned medium), Transferrin (0.5%vol/vol) and IL6 (5 ng/ml). Cultures were maintained at 37°C, 5% CO_2 for 5-12 days. Mean and standard errors of 3 independent experiments were calculated.

Flow cytometry and cell sorting

EBs generated during differentiations were dissociated using Trypsin-LE and then stained in FACS buffer (PBS+3%FCS+0.02%NaN3) with the following antibodies: anti-Flk-1 (hybridoma), anti-CD41 APC (cat# 17-0411, eBioscience), anti-CD144 PECy7 (cat# 25-1441, eBioscience), anti-CD45 eF450 (cat# 48-0451, eBioscience). Isotype control antibodies were used for the negative control (cat# 16-4301, eBioscience,). Cells were analyzed on an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo (Tree Star, Ashland OR). Cells were sorted on a FACSAria (BD Biosciences).

Luciferase assays

Approximately 2.5KB of the *Stat1* sequence upstream of the transcriptional start site was clone into the pGL3-basic vector (Kindly provided by Dr. John Crispino, Northwestern University, Chicago IL). HEK293 ($5x10^4$) were plated in triplicate and transfected with 100ng of the indicated promoter/reporter constructs, 10ng pRL-TK, 3-300ng pCag-Sox17 or vector control using X-tremeGENE 9 (Roche). 24hrs after stimulation firefly and *Renilla* substrate activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Firefly values were normalized to *Renilla* and then all normalized values set relative to the pGL3 control vector.

SUPPLEMENTAL REFERENCES

Gadue, P., Huber, T.L., Paddison, P.J., and Keller, G.M. (2006). Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation

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Ying, Q.L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P., and Smith, A. (2008). The ground state of embryonic stem cell self-renewal. Nature *453*, 519-523.

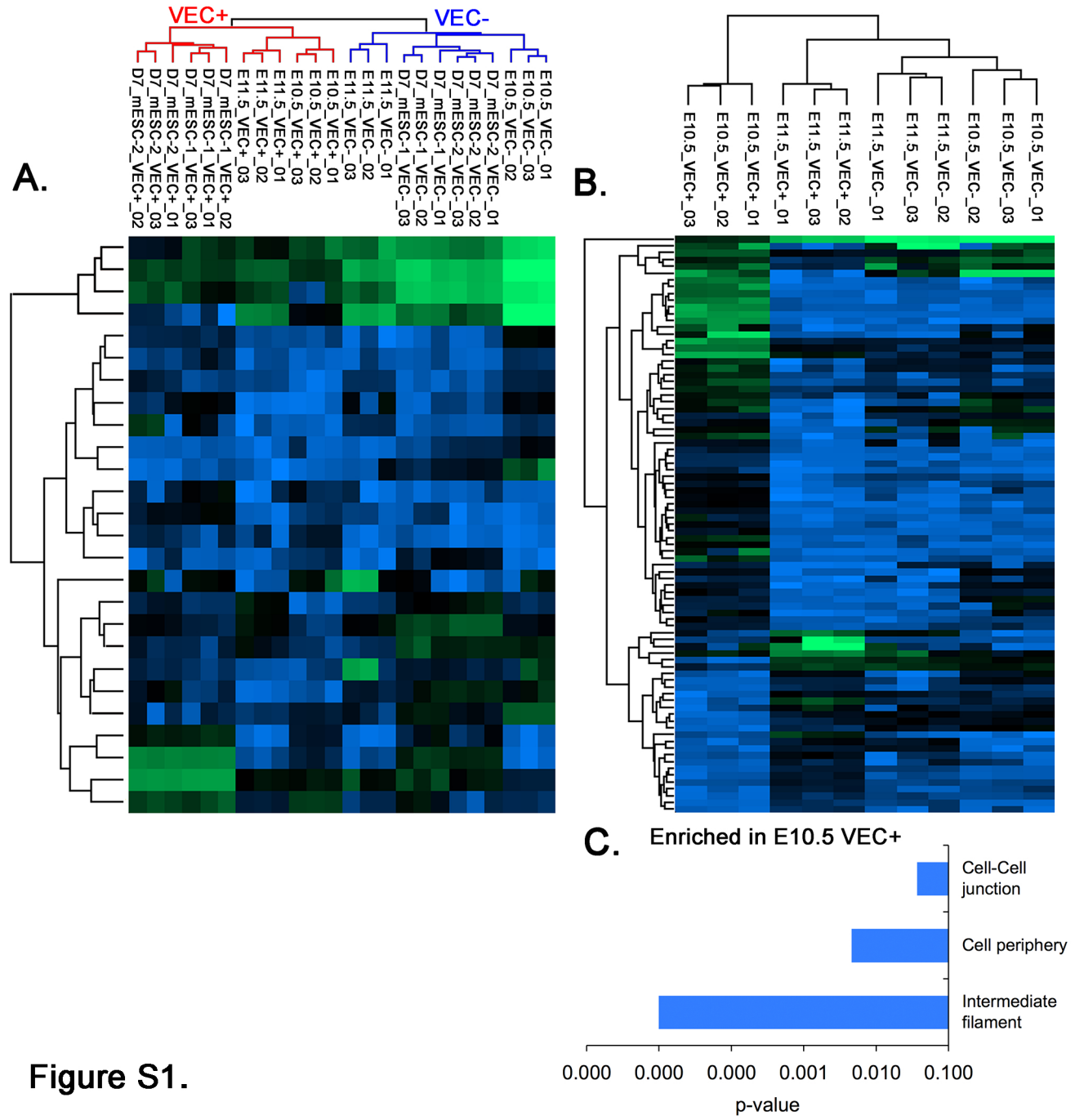
A.
VEC+
VEC-
D7_mESC-2_VEC+_02
D7_mESC-2_VEC+_03
D7_mESC-2_VEC+_01
D7_mESC-1_VEC+_03
D7_mESC-1_VEC+_01
D7_mESC-1_VEC+_02
E11.5_VEC+_03
E11.5_VEC+_02
E11.5_VEC+_01
E10.5_VEC+_03
E10.5_VEC+_02
E10.5_VEC+_01
E11.5_VEC-_03
E11.5_VEC-_02
E11.5_VEC-_01
D7_mESC-1_VEC-_03
D7_mESC-1_VEC-_02
D7_mESC-1_VEC-_01
D7_mESC-2_VEC-_03
D7_mESC-2_VEC-_02
D7_mESC-2_VEC-_01
E10.5_VEC-_03
E10.5_VEC-_02
E10.5_VEC-_01
B.
E10.5_VEC+_03
E10.5_VEC+_02
E10.5_VEC+_01
E11.5_VEC+_01
E11.5_VEC+_03
E11.5_VEC+_02
E11.5_VEC-_01
E11.5_VEC-_03
E11.5_VEC-_02
E10.5_VEC-_02
E10.5_VEC-_03
E10.5_VEC-_01
C.
Enriched in E10.5 VEC+
Cell-Cell junction
Cell periphery
Intermediate filament
0.000
0.000
0.000
0.001
0.010
0.100
p-value

Figure S1.

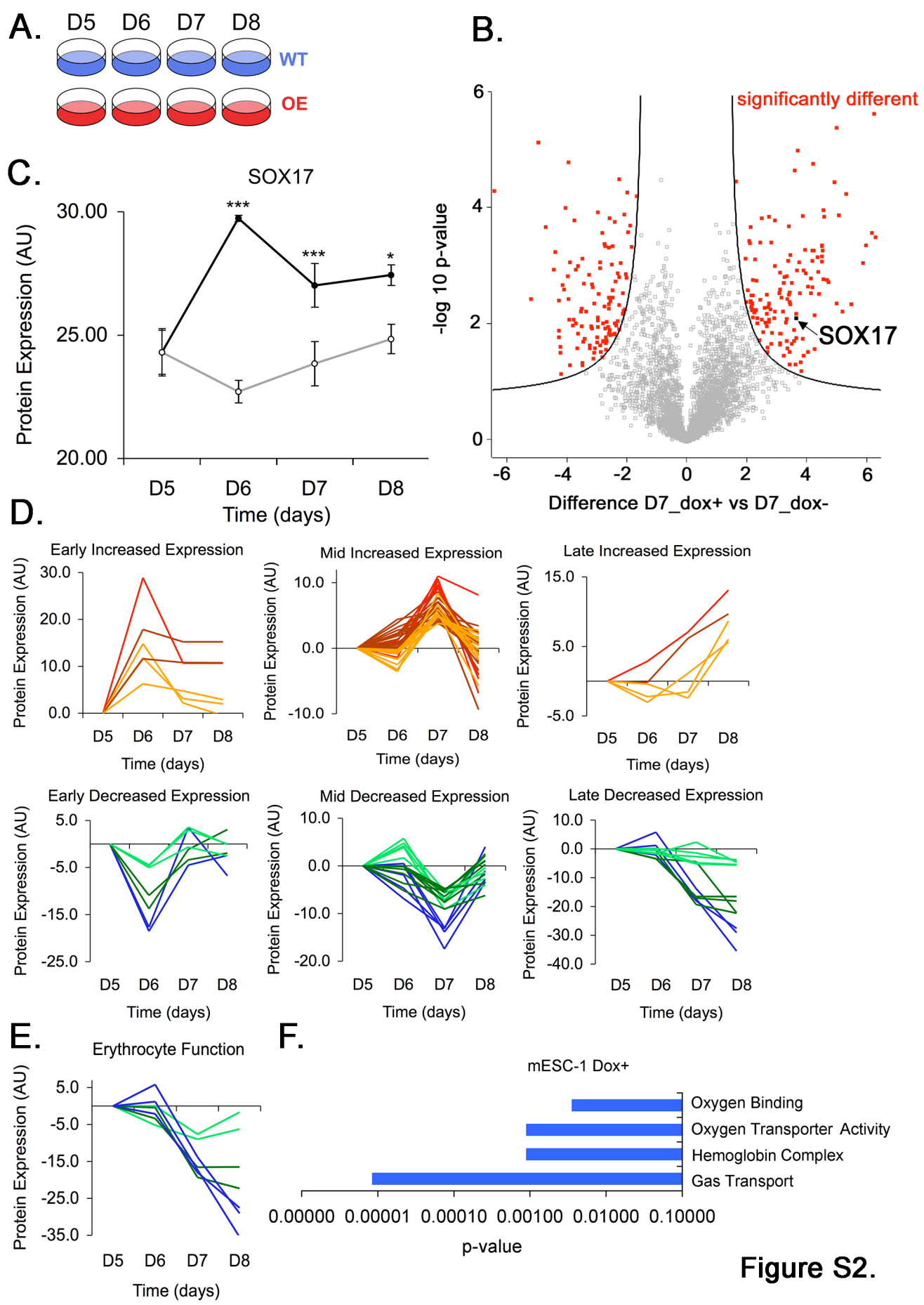
A.
D5
D6
D7
D8
WT
OE
B.
significantly different
SOX17
-log 10 p-value
Difference D7_dox+ vs D7_dox-
C.
SOX17
Protein Expression (AU)
Time (days)
D.
Early Increased Expression
Mid Increased Expression
Late Increased Expression
Early Decreased Expression
Mid Decreased Expression
Late Decreased Expression
E.
Erythrocyte Function
F.
mESC-1 Dox+
Oxygen Binding
Oxygen Transporter Activity
Hemoglobin Complex
Gas Transport
p-value

Figure S2.

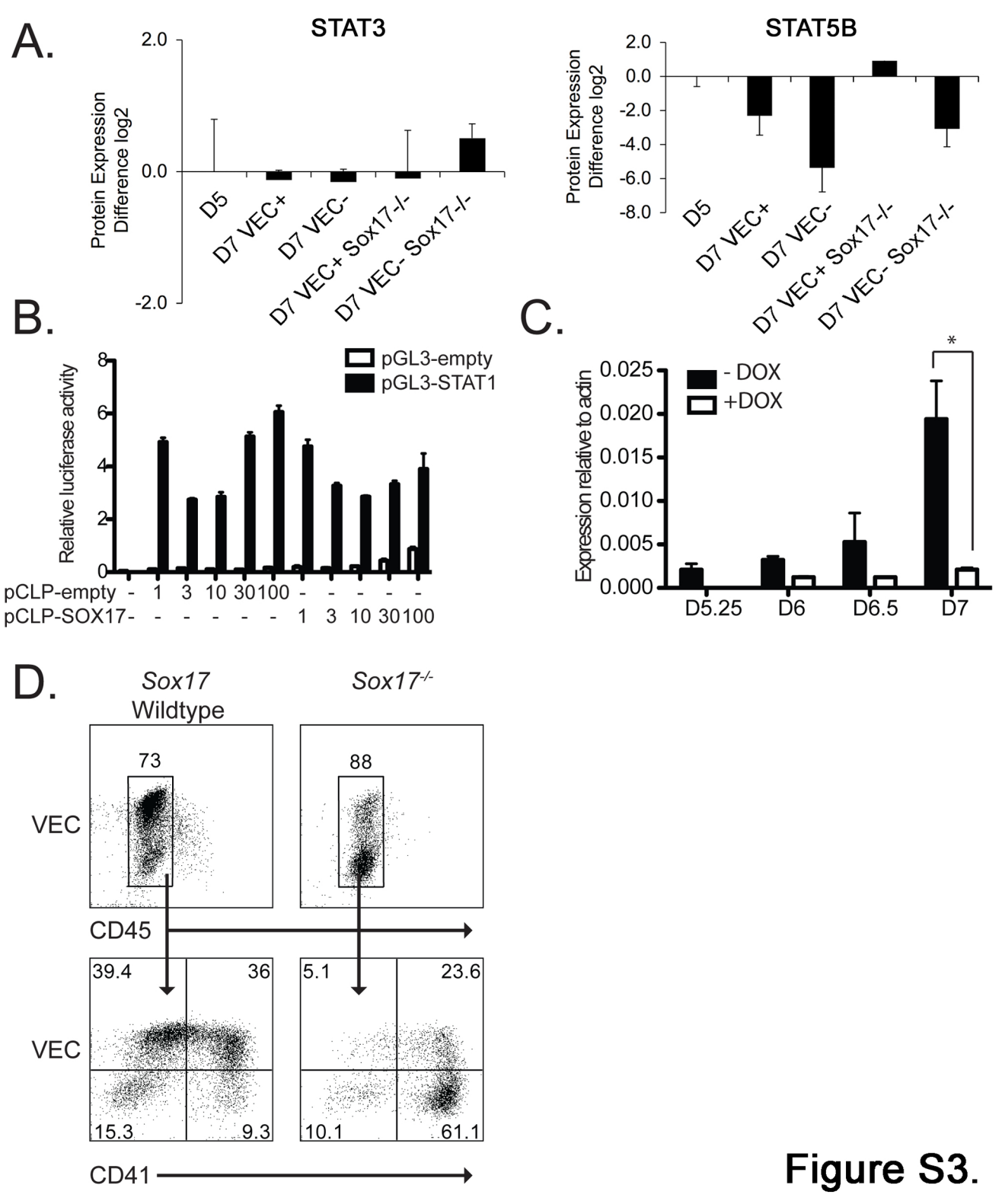
A.
STAT3
Protein Expression Difference log2
2.0
0.0
-2.0
D5
D7 VEC+
D7 VEC-
D7 VEC+ Sox17-/-
D7 VEC- Sox17-/-
STAT5B
Protein Expression Difference log2
2.0
0.0
-2.0
-4.0
-6.0
-8.0
D5
D7 VEC+
D7 VEC-
D7 VEC+ Sox17-/-
D7 VEC- Sox17-/-
B.
pGL3-empty
pGL3-STAT1
Relative luciferase activity
8
6
4
2
0
pCLP-empty - 1 3 10 30 100 - - - - -
pCLP-SOX17- - - - - - 1 3 10 30 100
C.
*
- DOX
+DOX
Expression relative to actin
0.025
0.020
0.015
0.010
0.005
0.000
D5.25
D6
D6.5
D7
D.
Sox17
Wildtype
Sox17-/-
73
88
VEC
CD45
39.4
36
15.3
9.3
5.1
23.6
10.1
61.1
VEC
CD41

Figure S3.