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Supplemental Information

SIX2 Regulates Human β Cell Differentiation from Stem Cells and Functional Maturation *In Vitro*

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Supplementary Figures

A

HUES8 hESC

Relative SIX2 Expression

1.5 1.0 0.5 0.0

sh-ctrl sh-SIX2-1 sh-SIX2-2

1013-4FA hiPSC

SIX2 Relative Expression

1.5 1.0 0.5 0.0

sh-ctrl sh-SIX2-1

B

CP SIX2 DAPI

20 μm

sh-ctrl sh-SIX2-1

C

20mM Glucose

Insulin Secretion (μIU / μg DNA)

600 400 200 0

sh-ctrl sh-SIX2-2

0 10 20 30 40 50

Time (Minutes)

D

Glucose

2mM 20mM

Insulin Secretion (μIU/ 10^6 Cells)

8,000 6,000 4,000 2,000 0

sh-ctrl sh-SIX2-2

E

1013-4FA

20mM Glucose

Insulin Secretion (μIU/μg DNA)

800 600 400 200 0

sh-ctrl sh-SIX2-1

0 10 20 30 40 50

Time (Minutes)

F

1013-4FA

Insulin Secretion (μIU/10^6 cells)

6,000 4,000 2,000 0

2mM 20mM

sh-ctrl sh-SIX2-1

G

Created with SnapGene

Deletion.Primer.F 5' gRNA

SIX2 Coding Sequence

Deletion.Primer.R

Inside.Primer.F Inside.Primer.R

3' gRNA

H

Deletion Primers

Inside Primers

100bp wt KO1 KO2 wt KO1 KO2

I

ggggttcggagcttcgtgggacctgtcagccaacctcgtggacctgggctcctagaacccatt

ccccaagcctcgaagcaccctggacagtcggttggagcacctggacccgaggatcttgggtaa

285 290

S A N L V D L G S

5' gRNA

SIX2

3' gRNA

ggggttcggagcttcgtgggacctgtcagccaacctcgtggacctgggctcctagaacccatt

Indels

GGGGTTCGGAGCTTCGTGG--------------------ACCTGGGCTCCTAGAACCCATT KO-SIX2-1 allele #1

GGGGTTCGGAGCTTCGTGGGACCTG-------------------GGCTCCTAGAACCCATT KO-SIX2-1 allele #2

GGGGTTCGGAGCTTCGTGGGACC----AGCCAACCTCGTGGACCTGGGCTCCTAGAACCCATT KO-SIX2-2 allele #1

GGGGTTCGGAGCTTCGTGGGAC--GTCAGCCAACCTCGTGGACCTGGGCTCCTAGAACCCATT KO-SIX2-2 allele #2

J

SIX2 Relative Expression

1.5 1.0 0.5 0.0

SIX2 WT SIX2 KO

K

CP SIX2 DAPI

20 μm

SIX2-WT SIX2-KO

Figure S1. Validation of SIX2 KD and KO assessments. Related to Figure 1. (**A**) Real-time PCR measurements of SIX2 gene expression for Stage 6 cells transduced with sh-ctrl, sh-SIX2-1, or sh-SIX2-2 for differentiated HUES8 (left) or 1013-4FA, made with protocol 2, (right) cells. n=3. (**B**) C-peptide and SIX2 immunostaining of stage 6 cells made with protocol 2 transduced with sh-ctrl or sh-SIX2-1 in the 1013-4FA background. (**C**) Dynamic glucose-stimulated insulin secretion of Stage 6 HUES8 cells transfected with control shRNA (sh-ctrl; n=4) or shRNA targeting SIX2 (sh-SIX2-2; n=4). Cells are perfused with 2 mM glucose except when indicated in a perifusion chamber. (**D**) Static glucose-stimulated insulin secretion of sh-ctrl or sh-SIX2-2 transduced Stage 6 HUES8 cells. n=5. (**E**) Dynamic glucose-stimulated insulin secretion of Stage 6 1013-4FA cells made with protocol 2 transfected with control shRNA (sh-ctrl; n=4) or shRNA targeting SIX2 (sh-SIX2-1; n=4). Cells are perfused with 2 mM glucose except when indicated in a perifusion chamber. (**F**) Static glucose-stimulated insulin secretion of sh-ctrl or sh-SIX2-1 transduced Stage 6 1013-4FA cells made with protocol 2. n=5. (**G**) CRISPR knock out strategy for the generation of the SIX2 KO HUES8 cell lines KO-SIX2-1 and KO-SIX2-2. HUES8 homozygous SIX2 KO clones were generated by deleting the SIX2 coding sequence using two gRNAs target flanking regions of the SIX2 coding sequence. Also shown are the primers used to validate deletion, "deletion primers" and "inside primers". **(H)** PCR of SIX2 KO clones confirming SIX2 coding sequence deletion. "Deletion primers" will produce 3368 bp amplicon for wt but only ~300 bp amplicon with successful deletion. "Inside primers" will produce ~300 bp amplicon for wt but fail to amplify with successful deletion. (**I**) Next generation sequencing confirming deletion of SIX2 coding sequence in KO cell lines. The entire sequence between the 5' and 3' gRNA was absent (marked with red dash in reference). 1,085 total reads for KO-SIX2-1 clone and 840 total reads for KO-SIX2-2 clone were sequenced and 0% wt gRNA target sequence were detected and frame shifting indels totaled 100% for both clones. A few bp of SIX2 coding are leftover. (**J**) Real-time PCR measurements of SIX2 gene expression for Stage 6 cells made with protocol 2 from wt or KO SIX2 HUES8 backgrounds. n=5. (**K**) C-peptide and SIX2 immunostaining of Stage 6 cells made with protocol 2 from wt or KO SIX2 HUES8 backgrounds. Error bars represent s.e.m.

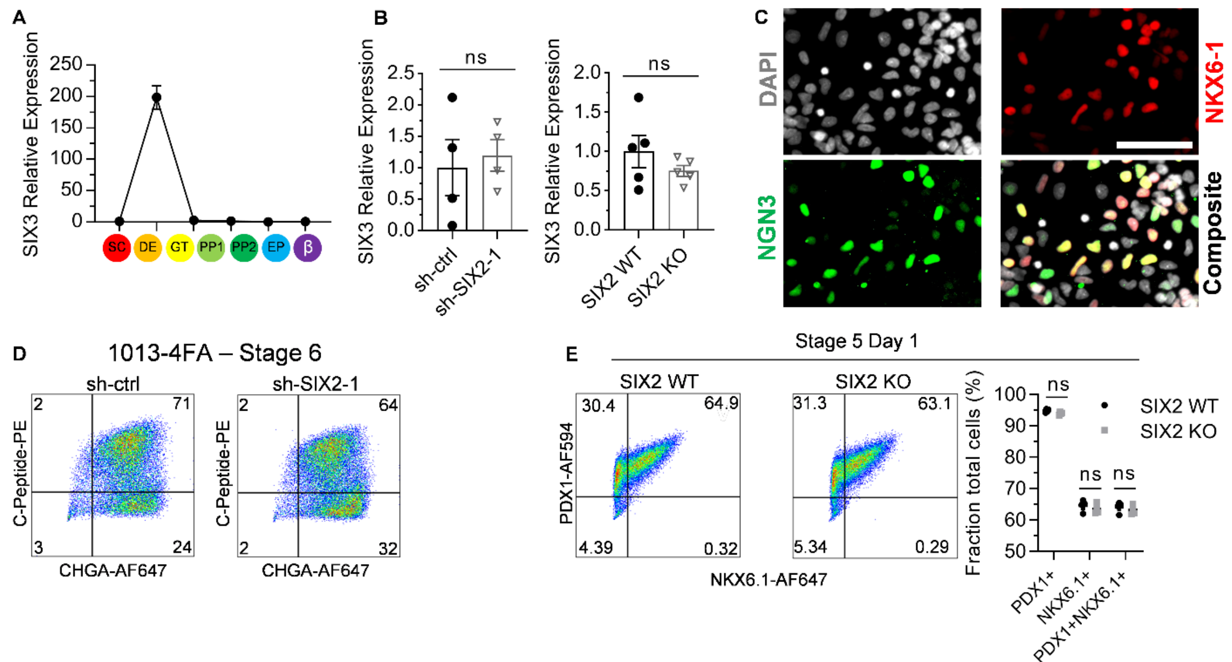

Figure S2. Additional evaluation of SIX3 and SIX2. Related to Figure 2. (**A**) Real-time PCR measurements of SIX3 in undifferentiated hESCs and at the end of each stage of the differentiation. Data is presented as the fold change relative to Stage 6 cells. All n=6, except PP2 n=3. (**B**) Real-time PCR measurements of SIX3 gene expression for Stage 6 cells transduced with sh-ctrl or sh-SIX2-1 (n=4; left) or SIX2 wt and KO cells (n=5; right). ns=p>0.05. (**C**) Immunostaining of NGN3 and NKX6-1 3 days into Stage 5. Scale bar=25 µm. (**D**) Flow cytometry plots of Stage 6 cells made with protocol 2 from the 1013-4FA background transduced with shRNA against GFP (control) and SIX2. (**E**) Flow cytometry plots of Stage 5 day 1 cells made with protocol 2 from the HUES8 background wt or KO for SIX2. n=4. ns by unpaired two-way t test. Error bars represent s.e.m.

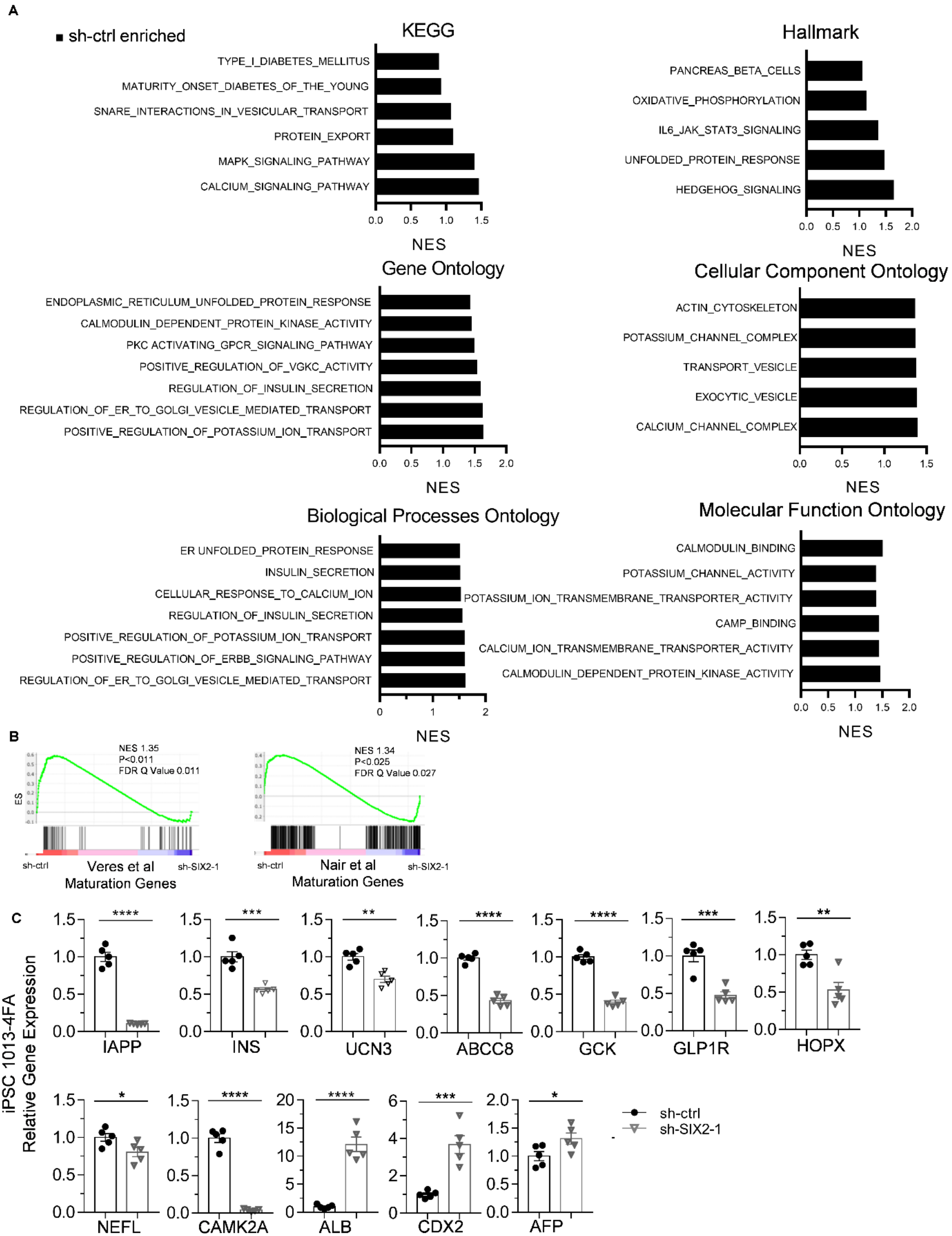

Figure S3. Additional RNA sequencing analysis. **Related to Figure 3.** (**A**) Enriched gene sets for important β cell processes from the Molecular Signatures Database. (**B**) Additional enrichment plots. These are made with genes from

the individual custom gene sets comprising 76 genes identified in Veres et al (Veres et al., 2019) and the top 424 genes identified in Nair et al (Nair et al., 2019) positively correlating with time and maturation in vitro (Tables S2-S3). (**C**) Real-time PCR measurements of Stage 6 cells made with protocol 2 transduced with sh-ctrl or sh-SIX2-1 in the 1013-4FA background. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ by two-way unpaired t test. Error bars represent s.e.m.

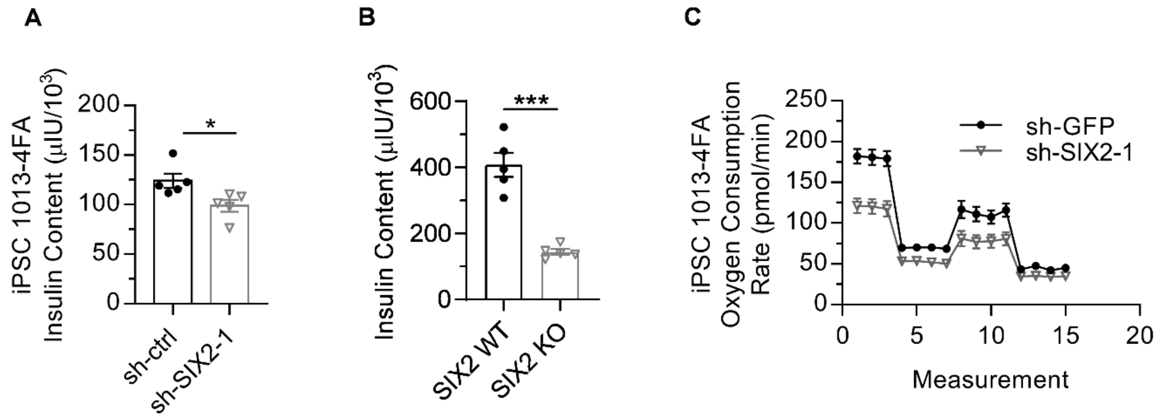

Figure S4. Additional data on SIX2 KD and KO effects on SC-β cells. Related to Figure 4. (**A**) Insulin content for Stage 6 cells transduced with sh-ctrl or sh-SIX2-1 in the 1013-4FA background. n=5. (**B**) Insulin content for Stage 6 cells wt or KO for SIX2. n=5. (**C**) OCR measurements for Stage 6 cells transduced with sh-ctrl or sh-SIX2-1 in the 1013-4FA background under basal conditions and after sequential injections of Oligomycin (OM), Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP), and Antimycin A with rotenone (AA/R). n=9 for sh-ctrl and n=10 for sh-SIX2-1. *p<0.05,***p<0.001 by two-way unpaired t test. Error bars represent s.e.m.