Electronic supplementary material (ESM)

RFX6 haploinsufficiency predisposes to diabetes through impaired beta cell function

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This PDF file includes:

ESM Methods

Tools and software used in bulk RNA-seq analysis

Fastq filtering: cutadapt (version 2.6)

Mapping / alignment software: STAR (version 2.7.6a)

RNA-seq analysis software: R (version 4.1) using Bioconductor (version 3.14)

Read counting: Rsubread (version 2.8.2)

Venn diagram: ggvenn (version 0.1.8) https://github.com/yanlinlin82/ggvenn

Heatmap figures: ComplexHeatmap (version 2.10.0)

Volcano plot, Boxplot, and PCA figures: ggplot2 (version 3.3.5)

Genome annotations: GENCODE GRCh38.p13

RNA-seq adapter sequences:

5′-AGATCGGAAGAGCACACGTCTGAACTCCAGTCA-3′ (R1),

5′-AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-3′ (R2)

ESM Table 1 Primers for PCR, CRISPR off-targets and NGS sequencing

Primer	Sequence
RFX6 Fw	GGCAGTGTTAAAAGGATGCTTTGG
RFX6 Rv	GGGTGCCTAATTCTGCTTTCTG
RFX6 dsDNA Mutation TseI 200bp Fw	TATTATATATACTTTCATGTTCTGTTCTTAATGAGTTCATGT TAAAGAAAAAAATCTTAACATACTTTTTACTCTGCAACTA GATCCAGCATTTTTTGCTGCTTTTGG
RFX6 dsDNA Mutation TseI 200bp Rv	TACCTTATAAAGAATTGAGTCACAAACACAGAAAATATCA ATGATAACAGGATTTTCCAATAAGGGAAGCAAGTGATCAG GCATTCCTTGCCAAAAGCAGCAAAAAAT
RFX6 Cpf1 Off-target1 Fw	TCCCAGGATTAAGTGTGGGG
RFX6 Cpf1 Off-target1 Rv	TCAATATTTGGTACCTGCCAACT
RFX6 Cpf1 Off-target2 Fw	TCCTGCACAGAGGTCCTACA
RFX6 Cpf1 Off-target2 Rv	AGCATCATGGGTGTTCGACT
RFX6 Cpf1 Off-target3 Fw	CAGGGAACGAGGGAGAACTG
RFX6 Cpf1 Off-target3 Rv	GAGTTTCCTAGGTGGGTGCC
RFX6 cDNA NGSseq Fw	*ACACTCTTTCCCTACACGACGCTCTTCCGATCT*ACTCTGCAAC TAGATCCAGCATTTT
RFX6 cDNA NGSseq Rv	*AGACGTGTGCTCTTCCGATCT*AAGAATTGAGTCACAAACAC AGAAA

ESM Table 2 Differentiation protocol and media formulations for hESCs

Stage	Reagent	Medium
Stage 1 3 days	d0: 100 ng/mL human Activin A, 3 μmol/l CHIR d1: 100 ng/ml human Activin A, 0.3 μmol/l CHIR d2: 100 ng/ml human Activin A	Basal 1: MCDB131 (10372-019, Life Technologies), 2 mmol/l Glutamax, 1.5 g/l NaHCO3 (Sigma-Aldrich), 0.5% BSA fraction V Fatty acid free (Sigma-Aldrich), 10 mmol/l glucose
Stage 2 3 days	0.25 mmol/l Ascorbic acid, 50 ng/ml FGF7	
Stage 3 2 days	0.25 mmol/l Ascorbic acid, 50 ng/ml FGF7, 0.25 μmol/l SANT1, 1 μmol/l Retinoic Acid, 100 nmol/l LDN and 200 nmol/l TPB	Basal 2: MCDB131, 2 mmol/l Glutamax, 2.5 g/l NaHCO3, 2% BSA fV, 10 mmol/l glucose, 1:200 ITS-X (51500-056, Life Technologies)
Stage 4 4 days	0.25 mmol/l Ascorbic acid, 2 ng/ml FGF7, 0.25 μmol/l SANT1, 0.1 μmol/l Retinoic Acid, 200 nmol/l LDN, 100 ng/ml EGF, 10 mmol/l Nicotinamide, 100 nmol/l TPB and 10 μmol/l ROCKi	
Stage 5 4 days	0.25 μmol/l SANT1, 0.05 μmol/l Retinoic acid, 100 nmol/l LDN, 10 μmol/l ALK5inhII, 1 μmol/l GC1, 20 ng/ml Betacellulin, 100 nmol/l GSiXX and 5 μmol/l ROCKi	Basal 3: MCDB131, 2 mmol/l Glutamax, 1.5 g/l NaHCO3, 2% BSA fV, 20 mmol/l glucose, 1:200 ITS-X, 10 μg/ml Heparin (H3149, Sigma-Aldrich), 10 μmol/l Zinc Sulfate
Stage 6 8 days	100 nmol/l LDN, 10 μmol/l ALK5inhII, 1 μmol/l GC1 and 100 nmol/l GSiXX	
Stage 7 21 days	1 mmol/l N-Acetylcysteine, 10 nmol/l Tri-iodothyronine (T3) and 0.5 μmol/l Aurora kinase inhibitor ZM447439	CMRL-modified: CMRL 1066 (15-110-CVR, Corning), 2% BSA fV, 2 mmol/l Glutamax, 1:200 ITS-X, Heparin 10 μg/ml, 10 μmol/l Zinc Sulfate, 5 mmol/l Sodium Pyruvate (Lonza), 1:2000 chemically defined lipid concentrate, 1:2000 medium trace elements A, 1:2000 medium trace elements B

ESM Table 3 Differentiation protocol and media formulations for iPSCs

Stage	Reagent	Medium
Stage 1 2 days	d0: 100 ng/ml human Activin A, 3 μmol/l CHIR d1: 100 ng/ml human Activin A, 0.3 μmol/l CHIR	Basal 1: MCDB131 (10372-019, Life Technologies), 2 mmol/l Glutamax, 1.5 g/l NaHCO3 (Sigma-Aldrich), 0.5% BSA fraction V Fatty acid free (Sigma-Aldrich), 10 mmol/l glucose
Stage 2 3 days	0.25 mmol/l Ascorbic acid, 50 ng/ml FGF7	
Stage 3 2 days	0.25 mmol/l Ascorbic acid, 50 ng/ml FGF7, 0.25 μmol/l SANT1, 1 μmol/l Retinoic Acid, 100 nmol/l LDN and 200 nmol/l TPB	Basal 2: MCDB131, 2 mmol/l Glutamax, 2.5 g/l NaHCO3, 2% BSA fV, 10 mmol/l glucose, 1:200 ITS-X (51500-056, Life Technologies)
Stage 4 4 days	0.25 mmol/l Ascorbic acid, 2 ng/ml FGF7, 0.25 μmol/l SANT1, 0.1 μmol/l Retinoic Acid, 200 nmol/l LDN, 100 ng/ml EGF, 100 nmol/l TPB and 10 mmol/l Nicotinamide	
Stage 5 4 days	0.25 μmol/l SANT1, 0.05 μmol/l Retinoic acid, 100 nmol/l LDN, 10 μmol/l ALK5inhII, 1 μmol/l GC1, 20 ng/ml Betacellulin and 100 nmol/l GSiXX	Basal 3: MCDB131, 2 mmol/l Glutamax, 1.5 g/l NaHCO3, 2% BSA fV, 20 mmol/l glucose, 1:200 ITS-X, 10 μg/ml Heparin (H3149, Sigma-Aldrich), 10 μmol/l Zinc Sulfate
Stage 6 12 days	100 nmol/l LDN, 10 μmol/l ALK5inhII, 1 μmol/l GC1, 100 nmol/l GSiXX and 10 μmol/l ROCKi	

ESM Table 4 Differentiation growth factors and reagents

Chemical/Reagent	Company name	Catalog#
ROCKi Y-27632	Selleckchem; Houston, USA	Cat# S1049
Activin A	QKine Ltd; Cambridge, UK	Cat# Qk001
CHIR	Tocris; Bristol, UK	Cat# 4423
Ascorbic acid	Sigma-Aldrich; USA	Cat# A4544
FGF7	Genscript; USA	Cat# Z03047
SANT1	Sigma-Aldrich	Cat# 4572
LDN	Selleckchem	Cat# S2618
Retinoic acid	Sigma-Aldrich	Cat# R2625
TPB	Santa Cruz; USA	Cat# sc-204424
EGF	Peprotech; USA	Cat# AF-100-15
Nicotinamide	Sigma-Aldrich	Cat# N0636
ALK5inhII	Selleckchem	Cat# S7223
GC1	Tocris	Cat# 4554
GSiXX	Millipore; Burlington, USA	Cat# 56578
N-Acetylcysteine	Sigma-Aldrich	Cat# A9165
Tri-iodothyronine (T3)	Sigma-Aldrich	Cat# T6397
Aurora kinase inhibitor ZM447439	Selleckchem	Cat# S1103
Glutamax	Life Technologies; USA	Cat# 35050038
Heparin	Sigma-Aldrich	Cat# H3149
Zinc Sulfate	Sigma-Aldrich	Cat# Z0251
Chemically defined lipid concentrate	Invitrogen; USA	Cat# 11905-031
Medium trace elements A	Corning; USA	Cat# 25-021-CI
Medium trace elements B	Corning	Cat# 99-175-CI
Cycloheximide	Sigma-Aldrich	Cat# 01810-1G
Exendin-4	Tocris	Cat# 1933

ESM Table 5 Antibodies used for immunofluorescence, flow cytometry and immunoblotting

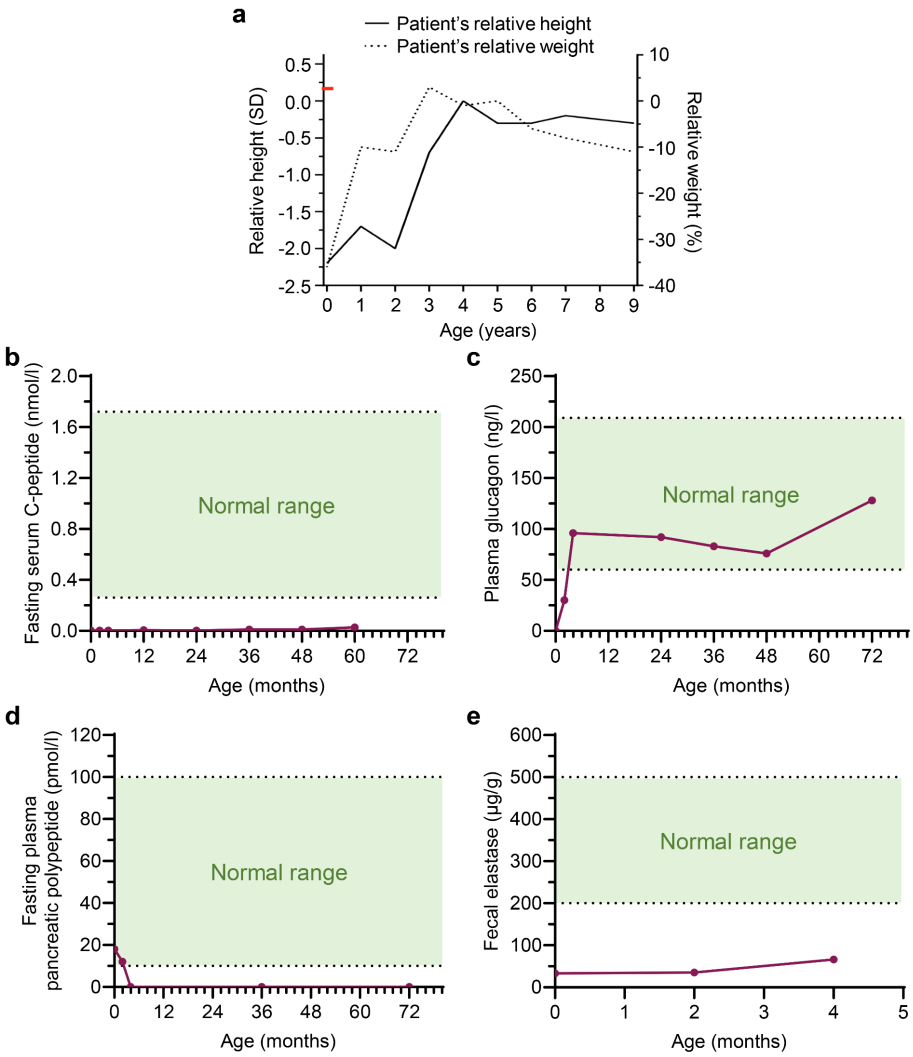
Antibody	Company	Catalog#	Dilution
Rabbit anti-OCT4 Rabbit	Santa Cruz	Cat# sc-9081; RRID: AB_2167703	1:250 ICC
Rabbit anti-SSEA4 mAb DyLight™ 488	Thermo Fisher Scientific; USA	Cat# MA1-021-D488; RRID: AB_2536688	1:100 ICC
Mouse anti-TRA 1-60 mAb	Thermo Fisher Scientific	Cat# MA1-023; RRID: AB_2536699	1:100 ICC
Goat anti-PDX1	R&D systems; USA	Cat# AF2419; RRID: AB_355257	1:500 ICC, IHC
Mouse anti-NKX6.1	DSHB Hybridoma; USA	Cat# F55A10; RRID: AB_532378	1:250 ICC, IHC
Sheep anti Human Neurogenin-3	R&D systems	Cat# AF3444; RRID: AB_2149527	1:250 ICC, IHC
Rabbit anti-SOX9	Sigma-Aldrich	Cat# AB5535; RRID: AB_2239761	1:500 ICC, IHC
Mouse anti-GCG antibody	Sigma-Aldrich	Cat# G2654; RRID: AB_259852	FC-1:160 IHC- 1:500
Guinea pig anti-Insulin	Dako; USA	Cat# A0564; RRID: AB_10013624	1:500 ICC, IHC
Rabbit anti-Somatostatin	Dako	Cat# A0566; RRID: AB_2688022	1:500 ICC, IHC
Goat anti-Pancreatic polypeptide	Sigma-Aldrich	Cat# SAB2500747; RRID: AB_10611538	1:500 ICC, IHC
Goat anti-Ghrelin	Santa Cruz	Cat# sc-10368; RRID: AB_2232479	1:500 ICC, IHC
Mouse anti-CHGA	Dako	Cat# M0869; RRID: AB_2081135	FC-1:160 IHC- 1:500
Rabbit anti-SLC18A1	Sigma-Aldrich	Cat# HPA063797; RRID: AB_2685125	1:250 IHC
Mouse anti-human Mitochondria clone 113-1	Sigma-Aldrich	Cat# MAB1273; RRID: AB_94052	1:150 IHC
Mouse Anti-CD184 (CXCR4) Monoclonal Antibody	BD Biosciences; USA	Cat# 555974; RRID: AB_396267	1:10 FC
Mouse IgG2a, kappa Isotype Control, Phycoerythrin Conjugated	BD Biosciences	Cat# 5555749; RRID: AB_396091	1:10 FC
PE-Mouse anti PDX1	BD Biosciences	Cat# 562161; RRID: AB_10893589	1:80 FC
Alexa Fluor 647 Mouse anti NKX6-1	BD Biosciences	Cat# 563338; RRID: AB_2738144	1:80 FC
Alexa Fluor 647 Mouse IgG1 k isotype control	BD Biosciences	Cat# 557714; RRID: AB_396823	1:80 FC
Alexa Fluor 647 Rabbit anti Insulin (C27C9)	Cell Signaling Technology; USA	Cat# 9008; RRID: AB_2687822	1:80 FC
Alexa Fluor 647 Rabbit IgG Isotype Control	Cell Signaling Technology	Cat# 3452S; RRID: AB_10695811	1:80 FC

Alexa FluoR 488 Donkey anti-Mouse IgG secondary antibody	Thermo Fisher Scientific	Cat# A-21202; RRID: AB_141607	1:500
Alexa FluoR 594 Goat anti-Guinea Pig IgG secondary antibody	Thermo Fisher Scientific	Cat# A-11076; RRID: AB_2534120	1:500
Alexa FluoR 594 Donkey anti-Sheep IgG secondary antibody	Thermo Fisher Scientific	Cat# A- 11016; RRID: AB_2534083	1:500
Alexa FluoR 488 Donkey anti-Rabbit IgG secondary antibody	Thermo Fisher Scientific	Cat# A-21206; RRID: AB_2535792	1:500
Alexa FluoR 488 Donkey anti-Mouse IgG secondary antibody	Thermo Fisher Scientific	Cat# A-21203; RRID: AB_141633	1:500
Alexa FluoR 488 Donkey anti-Goat IgG secondary antibody	Thermo Fisher Scientific	Cat# A-11055; RRID: AB_2534102	1:500
Mouse anti-alpha-Tubulin Mouse mAb	Sigma-Aldrich	Cat# T5168; (B-5-1-2); RRID: AB_477579	1:2000 WB
Mouse anti-β-actin-HRP mAb	Santa Cruz	Cat# sc-47778; (C4); RRID: AB_626632	1:1000 WB
Rabbit anti-RFX6	Sigma-Aldrich	Cat# HPA037696; RRID: AB_2675616	1:100 WB
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling Technology	Cat# 7074; RRID: AB_2099233	1:2000 WB
Anti-mouse IgG, HRP-linked Antibody	Cell Signaling Technology	Cat# 7076; RRID: AB_330924	1:5000 WB

ESM Table 6 Primers for quantitative RT-PCR

Primer	NCBI Reference	Sequence and amplicon size
PPIG primer pair Housekeeping gene	NM_004792	Fw: TCTTGTCAATGGCCAACAGAG Rv: GCCCATCTAAATGAGGAGTTG; 84 bp
OCT4 primer pair	NM_002701	Fw: TTGGGCTCGAGAAGGATGTG Rv: TCCTCTCGTTGTGCATAGTCG; 91 bp
SOX2 primer pair	NM_003106	Fw: GCCCTGCAGTACAACTCCAT Rv: TGCCCTGCTGCGAGTAGGA; 85 bp
NANOG primer pair	NM_024865.2	Fw: CTCAGCCTCCAGCAGATGC Rv: TAGATTTCATTCTCTGGTTCTGG; 94 bp
RFX6 primer pair	NM_173560.1	Fw: GCATGTCGAACTCCAGTCCT Rv: GGAGTCCAGGGTTTCTTGAG; 114 bp
PDX1 primer pair	NM_000209.3	Fw: AAGTCTACCAAAGCTCACGCG Rv: CGTAGGCGCCGCCTGC; 52 bp
SOX9 primer pair	NM_000346	Fw: ATCAAGACGGAGCAGCTGAG Rv: GGCTGTAGTGTGGGAGGTTG; 100 bp
NEUROG3 primer pair	NM_020999	Fw: GACGACGCGAAGCTCACCAA Rv: TACAAGCTGTGGTCCGCTAT; 98 bp
INS primer pair	NM_000207	Fw: CAGAAGCGTGGCATTGTGGA Rv: GCTGCGTCTAGTTGCAGTAG; 82 bp
GCG primer pair	NM_002054	Fw: GAAGGCGAGATTTCCCAGAAG Rv: CCTGGCGGCAAGATTATCAAG; 113 bp
GHRL primer pair	NM_016362	Fw: TGAACACCAGAGAGTCCAGCA Rv: GCTTGGCTGGTGGCTTCTT; 53 bp
PPY primer pair	NM_002722	Fw: ATGCCACACCAGAGCAGATG Rv: CAGCGTGTCCTCTTTGTGTC; 104 bp
SST primer pair	NM_001048	Fw: CCCAGACTCCGTCAGTTTCT Rv: ACAGCAGCTCTGCCAAGAAG; 88 bp

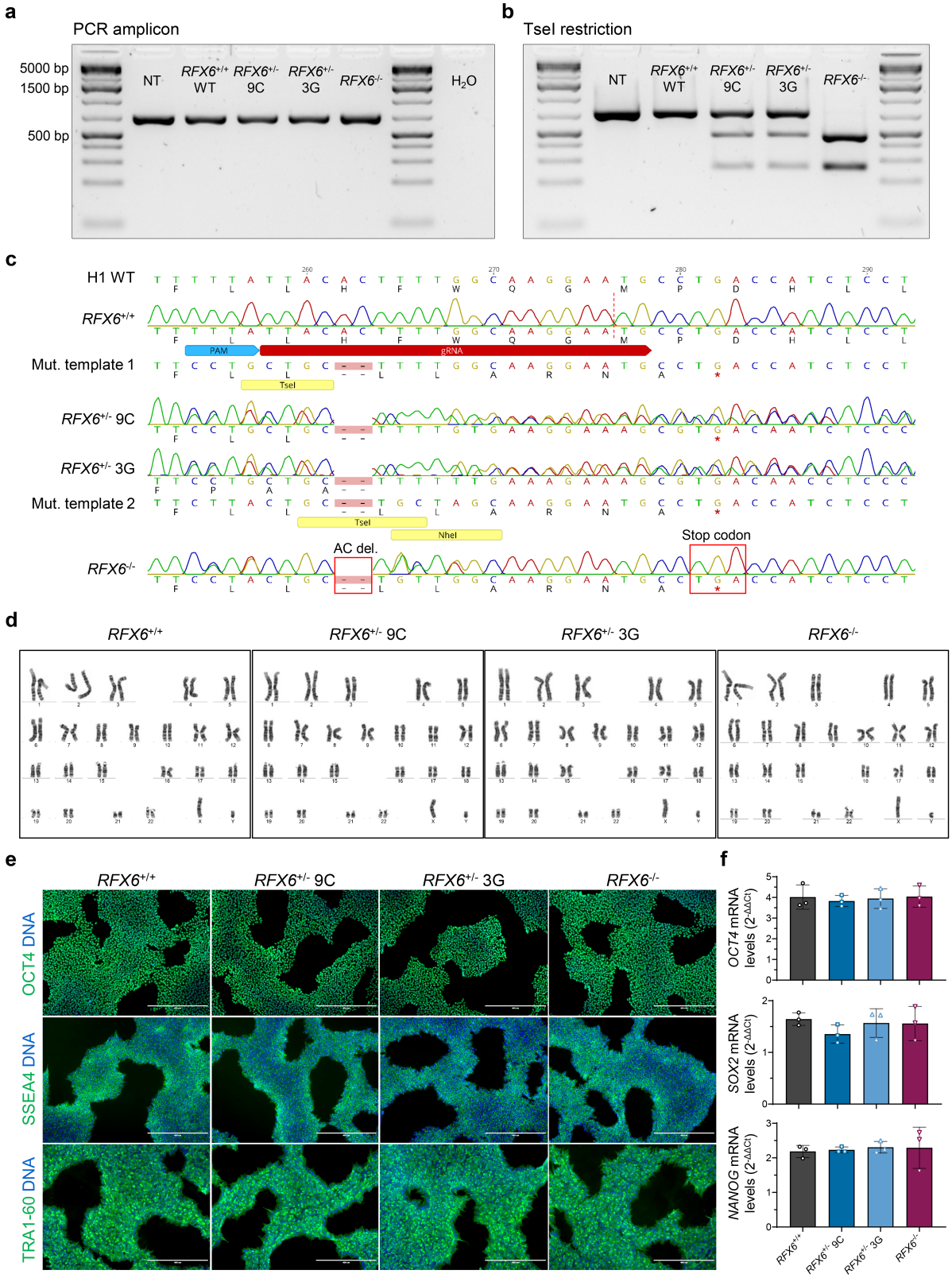
ESM Fig. 1



ESM Fig. 1 Clinical manifestations of the homozygous *RFX6* patient.

(**a**) Growth chart showing the patient's relative height and weight. Patient was born at gestation week 38+1, height; 47 cm and weight; 1800 g. Parental target relative height (+0.3 SD; red mark on left y axis). Relative height is depicted with solid line (left y axis) and relative weight (i.e. weight-for-height) with dotted line (right y axis).
(**b**) Fasting serum c-peptide levels in nmol/l during the first 5 years of age (normal range 0.26–1.72 nmol/l). Normal range in green represents clinical laboratory reference values.
(**c**) Plasma glucagon levels in ng/l during the first 6 years of age (normal range 60–209 ng/l).
(**d**) Fasting plasma pancreatic polypeptide levels in pmol/l during the first 6 years of age (normal range 10–100 pmol/l). Normal range in green represents clinical laboratory reference values.
(**e**) Fecal elastase levels in µg/g for the first 4 months after birth (normal level >200 µg/g). Normal range in green represents clinical laboratory reference values.

ESM Fig. 2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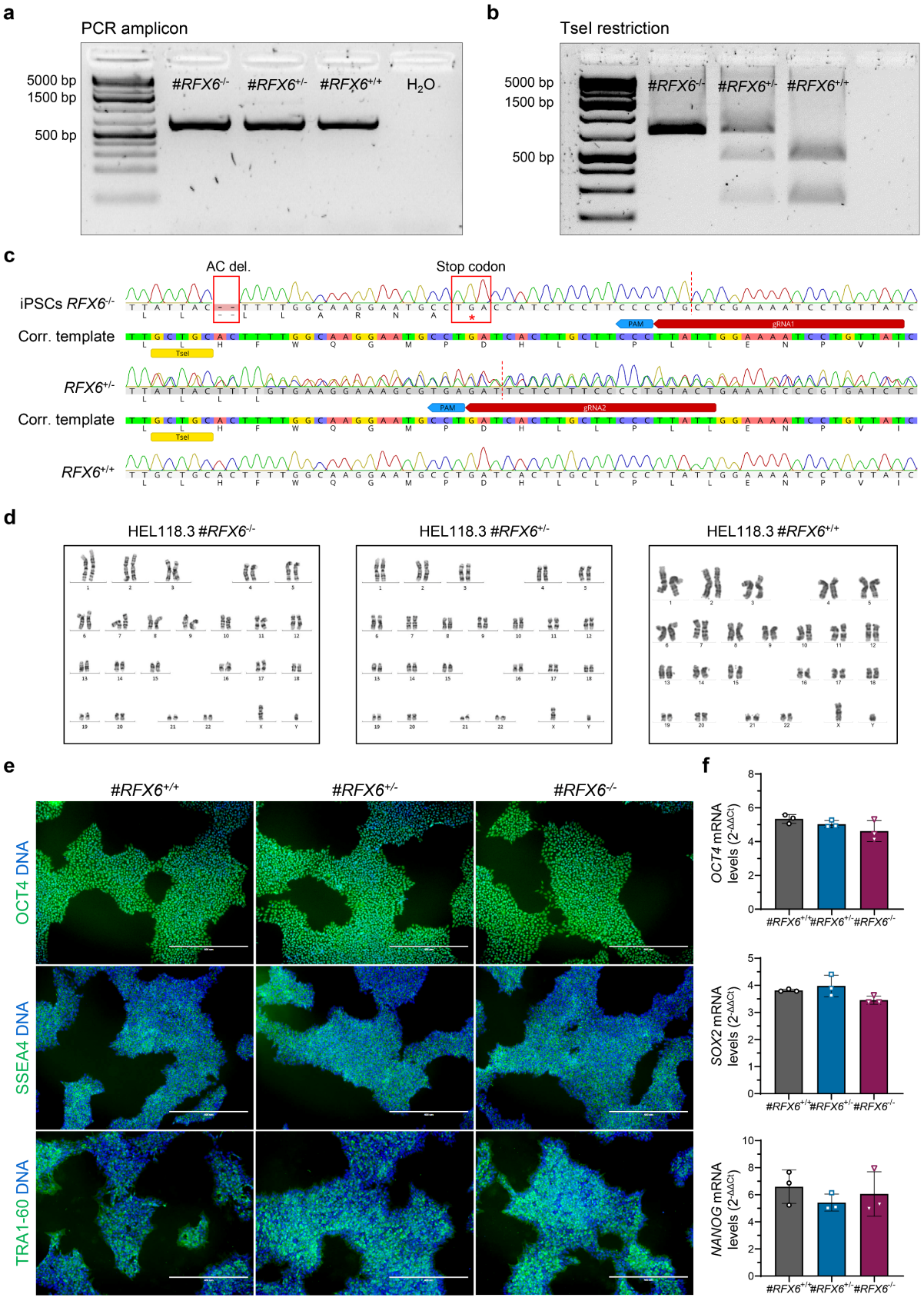
PCR amplicon
5000 bp
1500 bp
500 bp
NT
RFX6+/+ WT
RFX6+/- 9C
RFX6+/- 3G
RFX6-/-
H2O
b
TseI restriction
NT
RFX6+/+ WT
RFX6+/- 9C
RFX6+/- 3G
RFX6-/-
c
H1 WT
RFX6+/+
PAM
gRNA
Mut. template 1
TseI
RFX6+/- 9C
RFX6+/- 3G
Mut. template 2
TseI
NheI
AC del.
Stop codon
RFX6-/-
d
RFX6+/+
RFX6+/- 9C
RFX6+/- 3G
RFX6-/-
e
RFX6+/+
RFX6+/- 9C
RFX6+/- 3G
RFX6-/-
OCT4 DNA
SSEA4 DNA
TRA1-60 DNA
f
OCT4 mRNA levels (2-ΔΔCt)
SOX2 mRNA levels (2-ΔΔCt)
NANOG mRNA levels (2-ΔΔCt)
RFX6+/+
RFX6+/- 9C
RFX6+/- 3G
RFX6-/-

ESM Fig. 2 *RFX6* patient frameshift variant introduction in H1 hESCs using CRISPR-Cpf1.

(**a**) PCR gel electrophoresis showing 729 bp amplicons in all the cell-lines.
(**b**) PCR gel electrophoresis of the TseI restricted amplicons showing the restricted bands of the edited alleles 264+465 bp in heterozygous and homozygous cell-lines.
(**c**) Sanger sequencing showing the variant introduction heterozygously and homozygously using CRISPR-Cpf1 gRNAs and mutation templates.
(**d**) Normal karyotype visualised with G-banding for the four generated clones.
(**e**) Immunocytochemistry for pluripotency factors OCT4, TRA 1-60 and SSEA4 for the four clones. Scale bar, 400 μm.
(**f**) Relative gene expression levels of pluripotency factors *OCT4*, *SOX2* and *NANOG* for the four clones (n=3).
Statistical significance was measured using one-way ANOVA with Tukey's test for multiple comparisons correction in (f). Data are presented as means ± SD.

ESM Fig. 3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PCR amplicon
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1500 bp
500 bp
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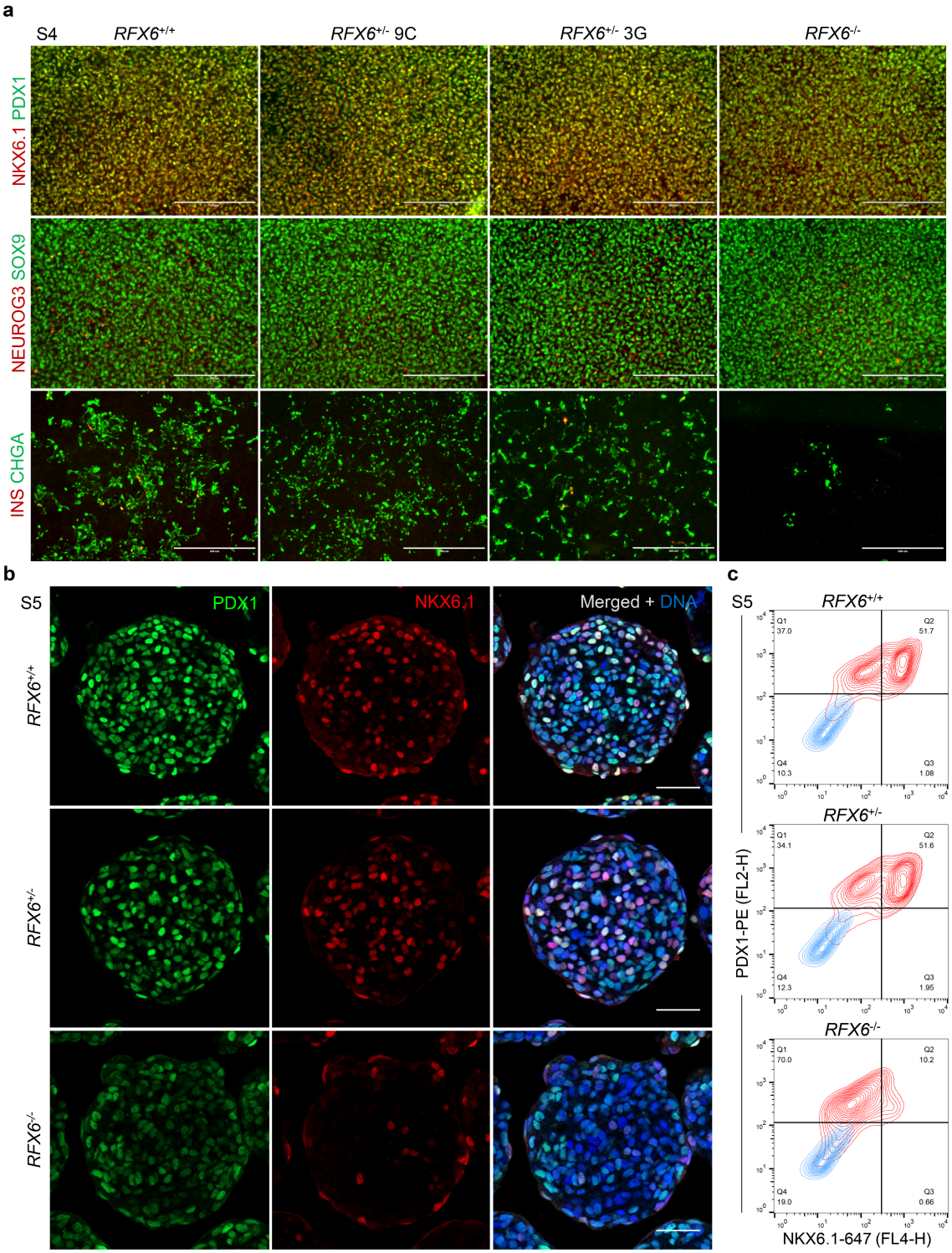
#RFX6+/-
#RFX6+/+
H2O
b
TseI restriction
5000 bp
1500 bp
500 bp
#RFX6-/-
#RFX6+/-
#RFX6+/+
c
AC del.
Stop codon
iPSCs RFX6-/-
Corr. template
RFX6+/-
Corr. template
RFX6+/+
PAM
gRNA1
gRNA2
TseI
d
HEL118.3 #RFX6-/-
HEL118.3 #RFX6+/-
HEL118.3 #RFX6+/+
e
#RFX6+/+
#RFX6+/-
#RFX6-/-
OCT4 DNA
SSEA4 DNA
TRA1-60 DNA
f
OCT4 mRNA levels (2-ΔΔCt)
SOX2 mRNA levels (2-ΔΔCt)
NANOG mRNA levels (2-ΔΔCt)
#RFX6+/+
#RFX6+/-
#RFX6-/-

ESM Fig. 3 *RFX6* frameshift variant correction in patient derived iPSCs using CRISPR-Cas9.

(**a**) PCR gel electrophoresis showing 729 bp amplicons of *RFX6* in all the cell-lines.
(**b**) PCR gel electrophoresis of the TseI restricted amplicons showing the restricted bands of the corrected alleles 264+465 bp in heterozygous and homozygous corrected cell-lines.
(**c**) Sanger sequencing showing the variant correction of the patient derived iPSCs using CRISPR-Cas9 gRNAs and a correction template.
(**d**) Normal karyotype visualised with G-banding for the three clones.
(**e**) Immunocytochemistry for pluripotency factors OCT4, TRA 1-60 and SSEA4 for the three clones. Scale bar, 400 μm.
(**f**) Relative gene expression levels of pluripotency factors *OCT4*, *SOX2* and *NANOG* for the three clones (n=3).
Statistical significance was measured using one-way ANOVA with Tukey's test for multiple comparisons correction in (f). Data are presented as means ± SD.

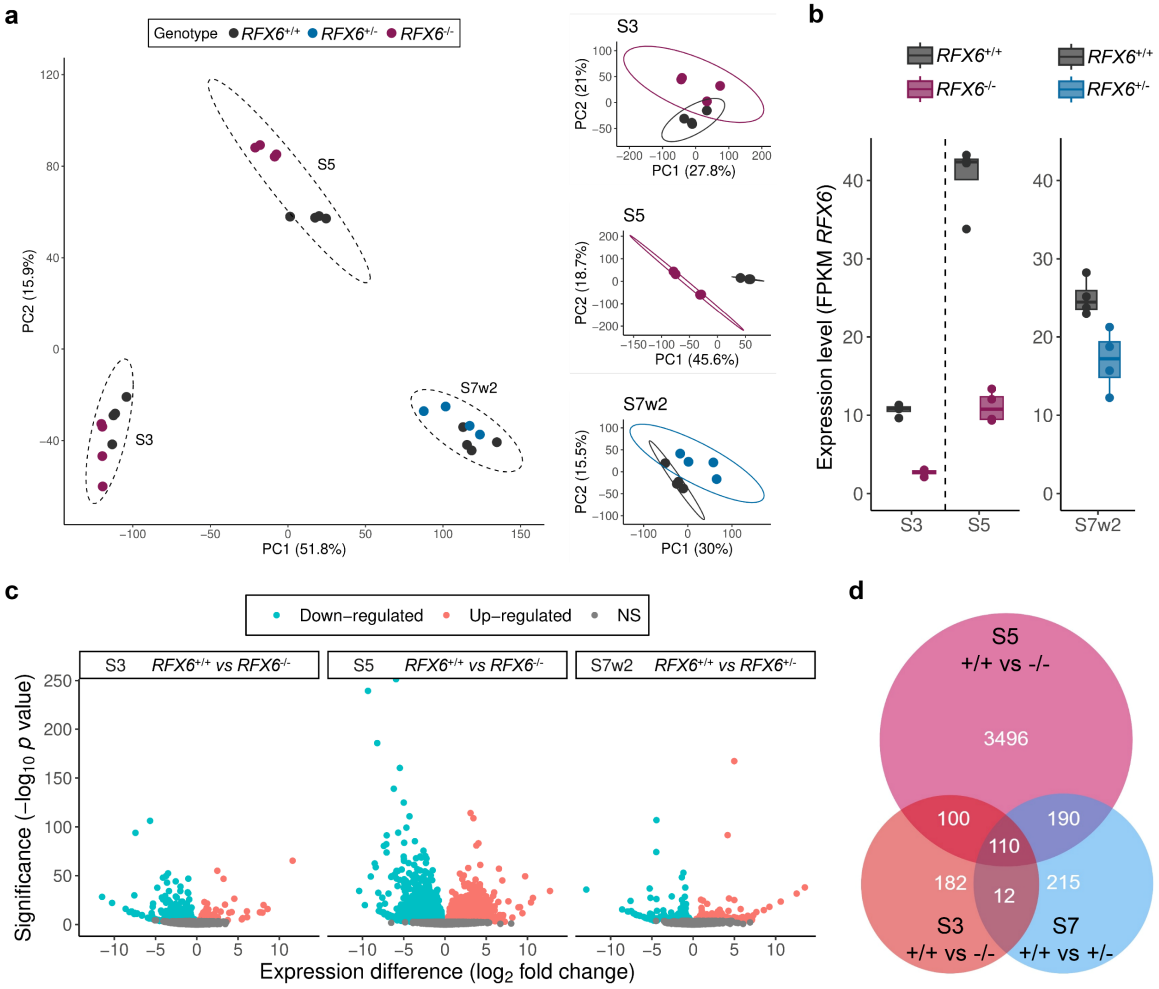
ESM Fig. 4



ESM Fig. 4 Reduced NKX6.1^{+} cells at the endocrine precursor stage in *RFX6*$^{-/-}$.

(**a**) Immunocytochemistry showing PDX1^{+} NKX6.1^{+}, SOX9^{+} NEUROG3^{+} and CHGA^{+} INS^{+} cells at S4. Scale bar, 200 µm.
(**b**) Immunohistochemistry showing PDX1^{+} and NKX6.1^{+} cells at S5. Scale bar, 50 µm.
(**c**) Flow cytometry analysis for PDX1^{+} and NKX6.1^{+} cells at S5. PDX1 and NKX6.1 sample is shown in red and IgG isotype negative control is shown in blue.

ESM Fig. 5



ESM Fig. 5 RNAseq analysis of H1 *RFX6* differentiated cells.

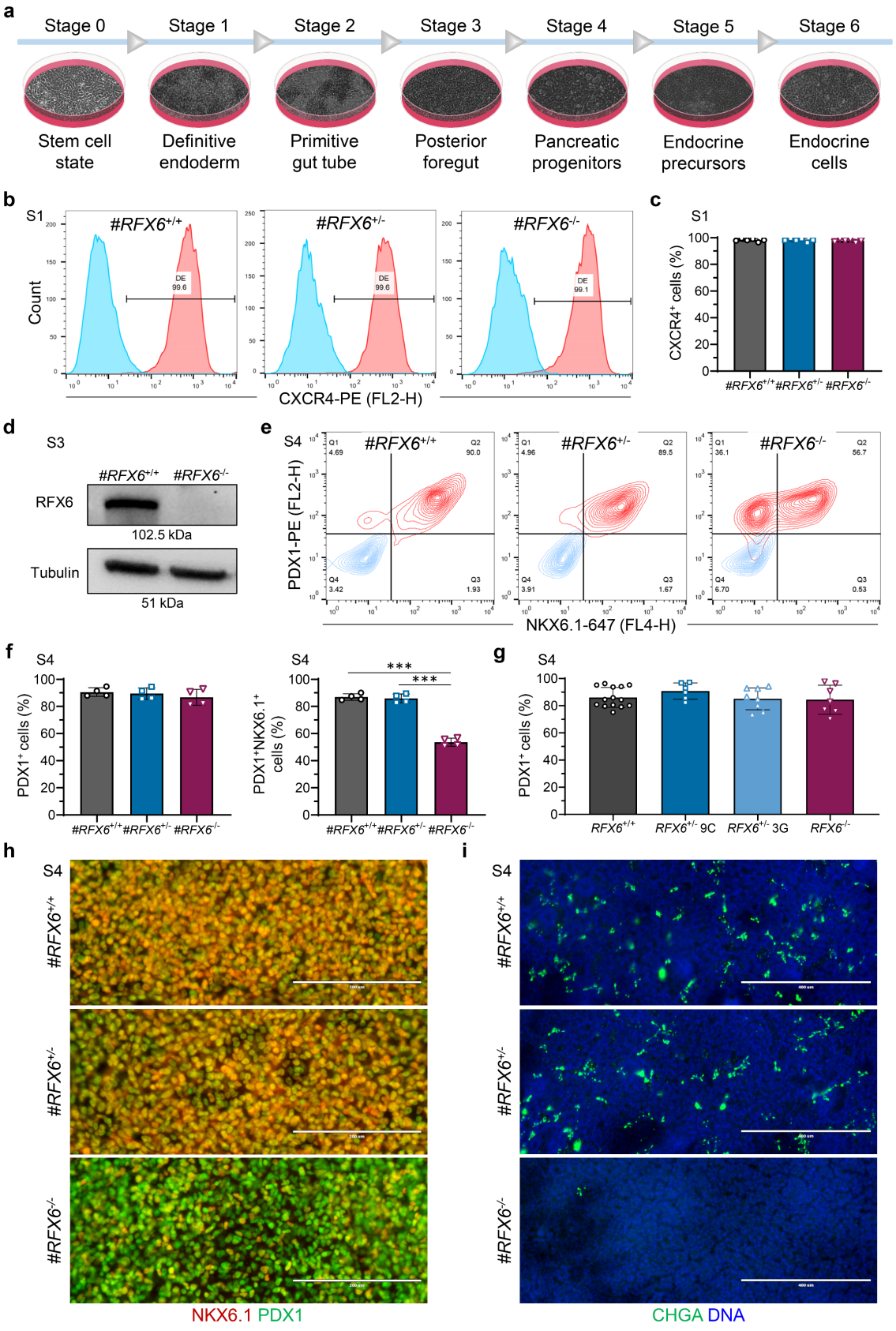
(**a**) Principal component analysis (PCA) of the RNA-seq data (using normalised read counts). The principal components that account for the majority of variation, separate the samples by developmental stage. The sub-plots show a PCA separately for each stage highlighting the differences between the genotypes; *RFX6*$^{+/+}$ vs *RFX6*$^{-/-}$ at S3 and S5, and *RFX6*$^{+/+}$ vs *RFX6*$^{+/-}$ at stage 7 week 2 (S7w2) (n=4).
(**b**) Boxplot of *RFX6* expression levels (FPKM) at different developmental stages for the tested genotypes (n=4).
(**c**) Volcano plots for differentially expressed genes for *RFX6*$^{+/+}$ vs *RFX6*$^{-/-}$ at S3 and S5, and for *RFX6*$^{+/+}$ vs *RFX6*$^{+/-}$ at S7w2. Significantly downregulated genes, iris blue; upregulated genes, soft red.
(**d**) Venn diagram showing the number and overlap of differentially expressed genes (FDR<0.01) between the genotypes within each developmental stage.

ESM Fig. 6

ESM Fig. 6 Reduced gene expression levels of pancreatic markers in *RFX6*$^{-/-}$.

(**a**) Barplots of average gene expression (FPKM) of the heatmap in (Fig. 2g). Statistical significance is indicated as False Discovery Rate corrected *p* value, *FDR<0.05, **FDR<0.01, ***FDR<0.001. Data are presented as means ± SD.

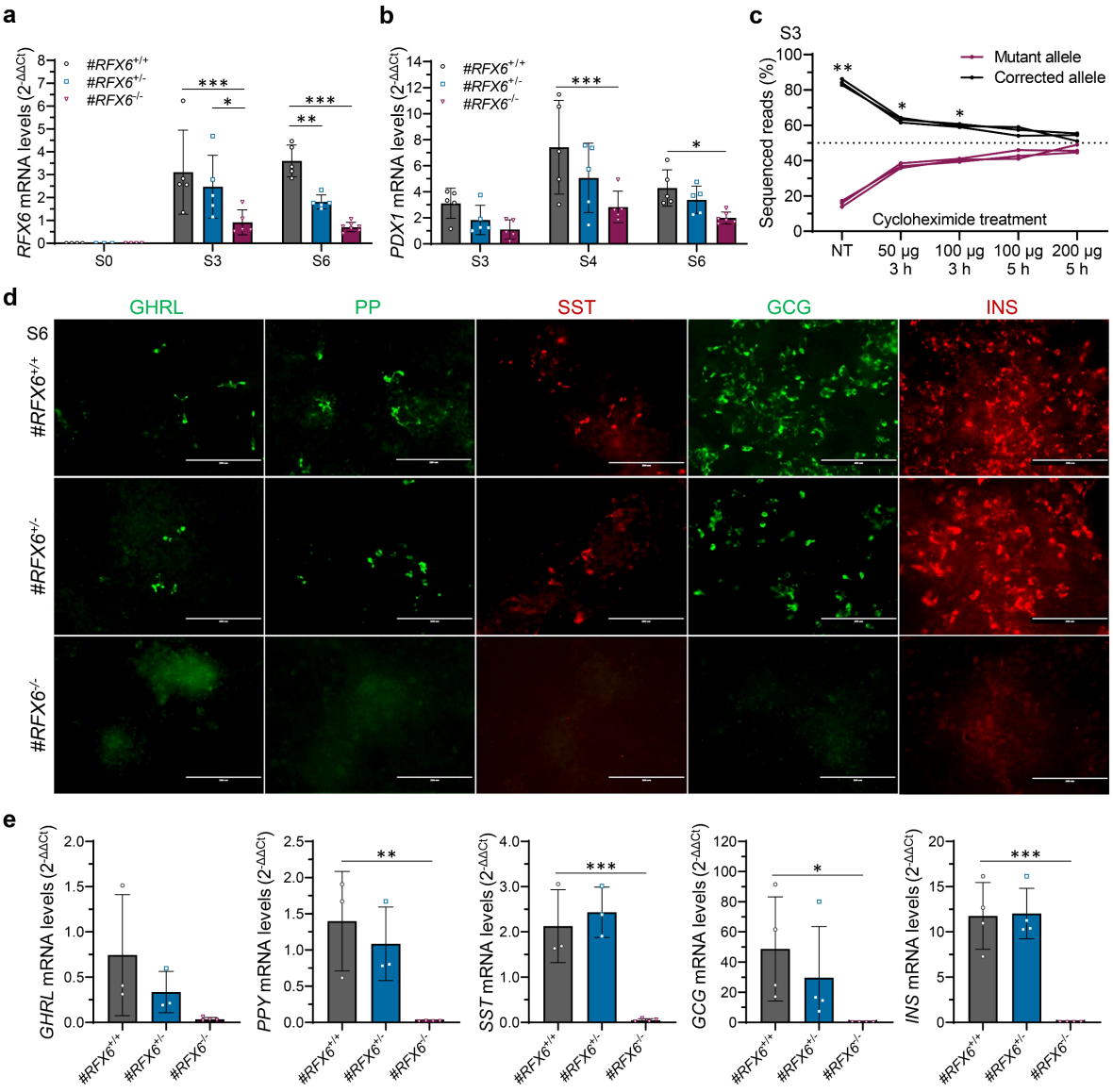
ESM Fig. 7


a
Stage 0
Stage 1
Stage 2
Stage 3
Stage 4
Stage 5
Stage 6
Stem cell state
Definitive endoderm
Primitive gut tube
Posterior foregut
Pancreatic progenitors
Endocrine precursors
Endocrine cells
b
S1
#RFX6+/+
#RFX6+/-
#RFX6-/-
Count
DE 99.6
DE 99.6
DE 99.1
CXCR4-PE (FL2-H)
c
S1
CXCR4+ cells (%)
#RFX6+/+ #RFX6+/- #RFX6-/-
d
S3
#RFX6+/+ #RFX6-/-
RFX6
102.5 kDa
Tubulin
51 kDa
e
S4
#RFX6+/+
#RFX6+/-
#RFX6-/-
PDX1-PE (FL2-H)
NKX6.1-647 (FL4-H)
f
S4
PDX1+ cells (%)
PDX1+NKX6.1+ cells (%)
***
***
#RFX6+/+ #RFX6+/- #RFX6-/-
g
S4
PDX1+ cells (%)
RFX6+/+ RFX6+/- 9C RFX6+/- 3G RFX6-/-
h
S4
#RFX6+/+
#RFX6+/-
#RFX6-/-
NKX6.1 PDX1
i
S4
#RFX6+/+
#RFX6+/-
#RFX6-/-
CHGA DNA

ESM Fig. 7 Reduced numbers of pancreatic progenitor cells in patient-derived iPSCs.

(**a**) Schematic of pancreatic endocrine differentiation protocol for 6 stages in monolayer.
(**b**) Flow cytometry analysis for the expression of C-X-C motif chemokine receptor 4-positive ($CXCR4^+$) definitive endoderm cells at the end of S1. CXCR4 sample is shown in red and IgG isotype negative control is shown in blue.
(**c**) Quantification of (b) (n=6).
(**d**) Immunoblot for RFX6 and α-Tubulin at S3 of control HEL46.11 ($\#RFX6^{+/+}$) compared to the patient cell-line ($\#RFX6^{-/-}$).
(**e**) Flow cytometry analysis for $PDX1^+$ and $NKX6.1^+$ cells in the iPSC-lines at S4. PDX1 and NKX6.1 sample is shown in red and IgG isotype negative control is shown in blue.
(**f**) Quantification of (e) (n=4).
(**g**) Flow cytometry quantification for $PDX1^+$ cells in the hESC-lines at S4 (n=7–14)
(**h**) Immunocytochemistry for $PDX1^+$ and $NKX6.1^+$ at the end of S4. Scale bar, 200 μm.
(**i**) Immunocytochemistry for $CHGA^+$ at the end of S4. Scale bar, 400 μm.
Statistical significance was measured using one-way ANOVA with Tukey's test for multiple comparisons correction in (c, f and g). Data are presented as means ± SD, ***p<0.001.

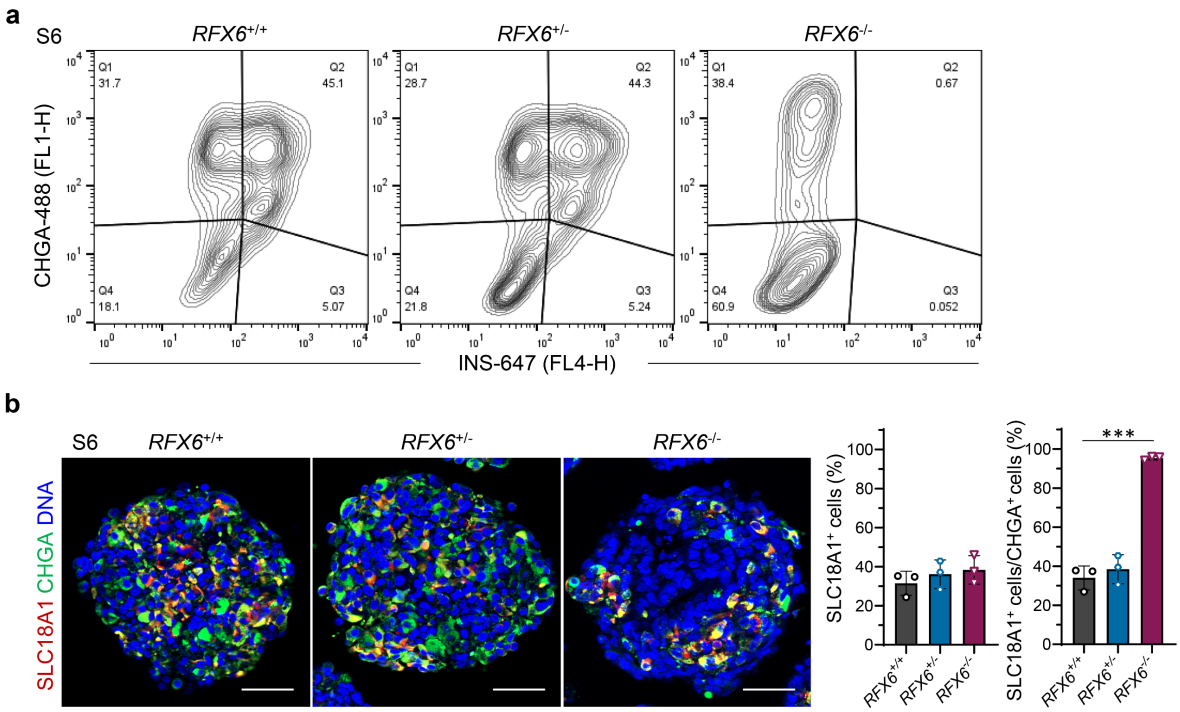
ESM Fig. 8



ESM Fig. 8 *RFX6* frameshift variant nonsense-mediated mRNA decay and diminished production of pancreatic endocrine cells from patient-derived iPSCs.

(**a**) Relative gene expression levels of *RFX6* at S0, S3 and S6 (n=3–7).
(**b**) Relative gene expression levels of *PDX1* at S3, S4 and S6 (n=5–6).
(**c**) Percentage of *RFX6* cDNA reads of the variant and corrected alleles at S3 of *#RFX6*$^{+/-}$ cell-line treated with different doses of the NMD inhibitor cycloheximide (n=3).
(**d**) Immunocytochemistry at S6 for the five pancreatic endocrine cell types. Ghrelin (GHRL), pancreatic polypeptide (PP), somatostatin (SST), glucagon (GCG) and insulin (INS). Scale bar, 200 µm.
(**e**) Relative gene expression levels of the five pancreatic hormones *GHRL*, *PPY*, *SST*, *GCG* and *INS* for the three isogenic cell-lines at S6 (n=3–4).
Statistical significance was measured using two-way ANOVA with Tukey's test for multiple comparisons correction in (a and b), using paired parametric multiple t-tests in (c), and using one-way ANOVA with Tukey's test for multiple comparisons correction in (e). Data are presented as means ± SD, *p<0.05, **p<0.01, ***p<0.001.

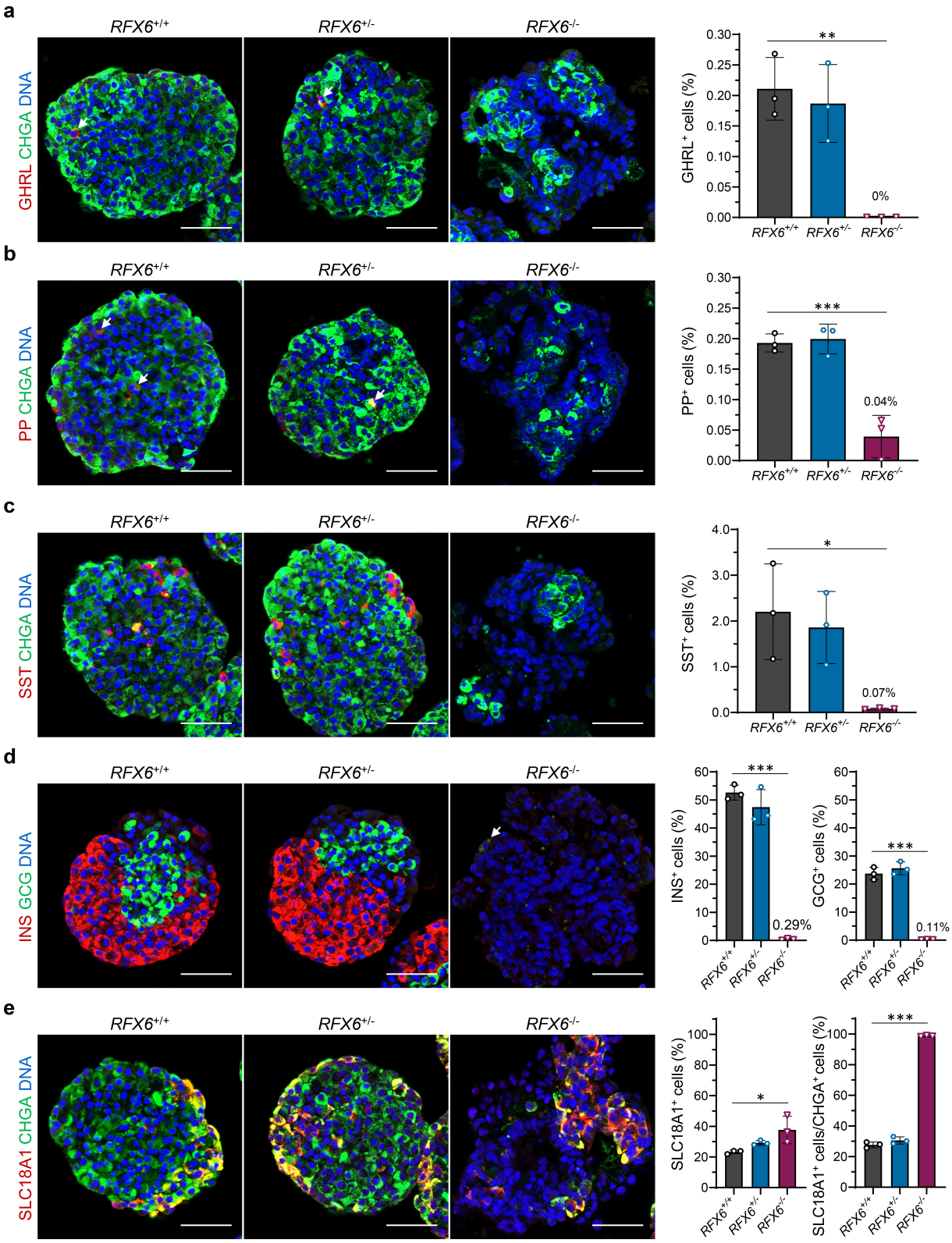
ESM Fig. 9



ESM Fig. 9 Reduced CHGA$^+$ endocrine cells in *RFX6*$^{-/-}$ at S6.

(**a**) Flow cytometry analysis for CHGA$^+$ cells (y-axis) and INS$^+$ cells (x-axis) at S6.
(**b**) Immunohistochemistry showing CHGA$^+$ (green) and SLC18A1$^+$ (red) cells in SC-islets at S6 (scale bar, 50 µm) and quantification of SLC18A1$^+$ cells per total cells and per CHGA$^+$ cells (n=3). Statistical significance was measured using one-way ANOVA with Tukey's test for multiple comparisons correction. Data are presented as means ± SD, ***p<0.001.

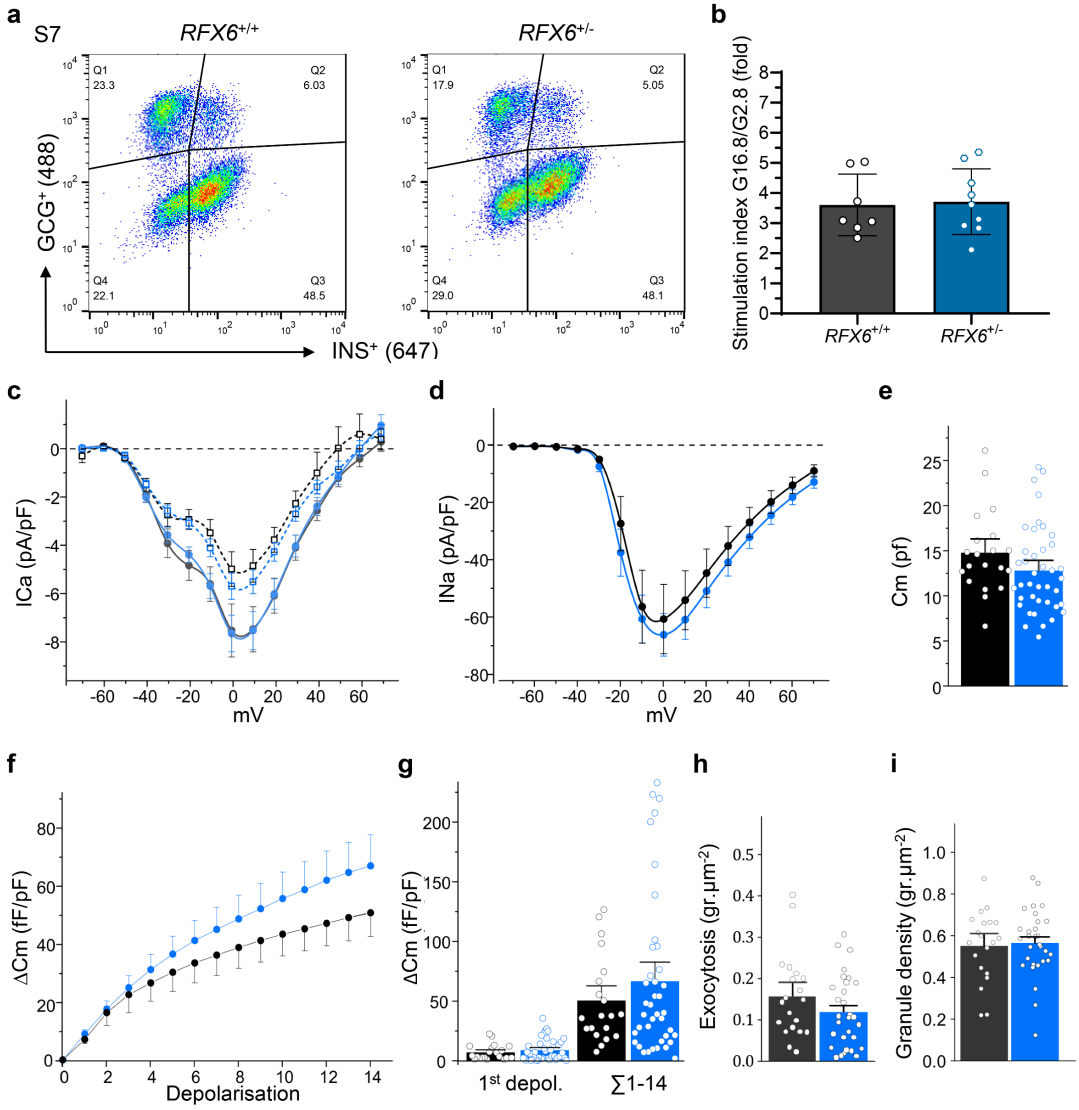
ESM Fig. 10


a
RFX6+/+
RFX6+/-
RFX6-/-
GHRL CHGA DNA
GHRL+ cells (%)
**
0%
b
PP CHGA DNA
PP+ cells (%)
***
0.04%
c
SST CHGA DNA
SST+ cells (%)
*
0.07%
d
INS GCG DNA
INS+ cells (%)
***
0.29%
GCG+ cells (%)
***
0.11%
e
SLC18A1 CHGA DNA
SLC18A1+ cells (%)
*
SLC18A1+ cells/CHGA+ cells (%)
***

ESM Fig. 10 Scarce pancreatic endocrine cells in *RFX6*$^{-/-}$.

(**a**) Immunohistochemistry showing CHGA^{+} (green) and GHRL^{+} (red with white arrows) cells in SC-islets at stage 7 week 2 (S7w2) for *RFX6*$^{+/+}$ and *RFX6*$^{+/-}$, and at stage 7 day 2 (S7d2) for *RFX6*$^{-/-}$ (scale bar, 50 μm), and quantification of GHRL^{+} cells (n=3).
(**b**) Immunohistochemistry showing CHGA^{+} (green) and PP^{+} (red with white arrows) cells in SC-islets at S7w2 for *RFX6*$^{+/+}$ and *RFX6*$^{+/-}$, and at S7d2 for *RFX6*$^{-/-}$ (scale bar, 50 μm), and quantification of PP^{+} cells (n=3).
(**c**) Immunohistochemistry showing CHGA^{+} (green) and SST^{+} (red) cells in SC-islets at S7w2 for *RFX6*$^{+/+}$ and *RFX6*$^{+/-}$, and at S7d2 for *RFX6*$^{-/-}$ (scale bar, 50 μm), and quantification of SST^{+} cells (n=3).
(**d**) Immunohistochemistry showing GCG^{+} (green) and INS^{+} (red) cells in SC-islets at S7w2 for *RFX6*$^{+/+}$ and *RFX6*$^{+/-}$, and at S7d2 for *RFX6*$^{-/-}$ (white arrow points at a GCG^{+} cell) (scale bar, 50 μm), and their quantification (n=3).
(**e**) Immunohistochemistry showing CHGA^{+} (green) and SLC18A1^{+} (red) cells in SC-islets at S7w2 for *RFX6*$^{+/+}$ and *RFX6*$^{+/-}$, and at S7d2 for *RFX6*$^{-/-}$ (scale bar, 50 μm), and quantification of SLC18A1^{+} cells per total cells and per CHGA^{+} cells (n=3).
Statistical significance in (a–e) was measured using one-way ANOVA with Tukey's test for multiple comparisons correction. Data are presented as means ± SD, *p<0.05, **p<0.01, ***p<0.001.

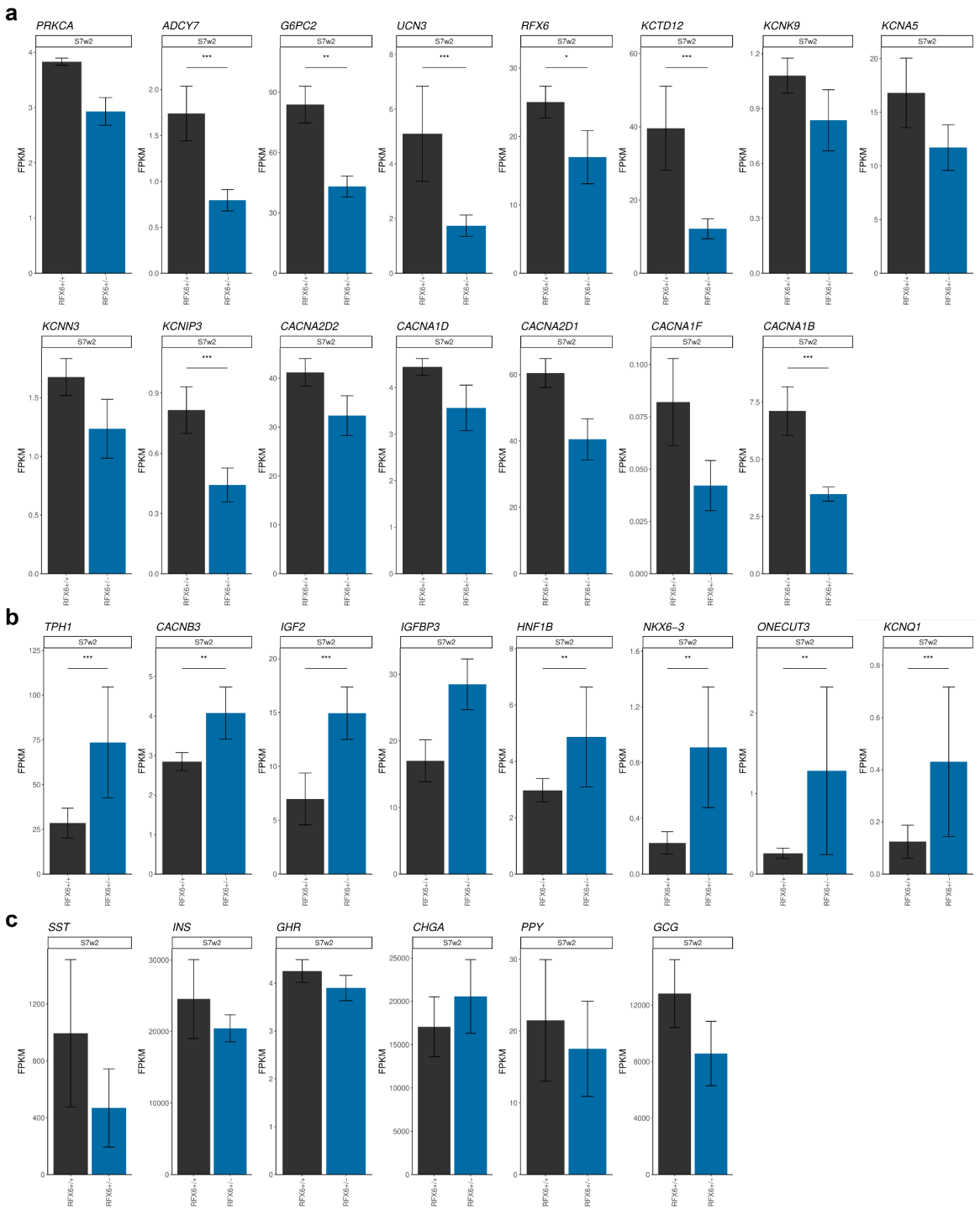
ESM Fig. 11



ESM Fig. 11 Voltage-dependence and exocytosis of *RFX6* SC-islets.

(**a**) Flow cytometry analysis for GCG^+ cells (y-axis) and INS^+ cells (x-axis) at stage 7 week 2 (S7w2).
(**b**) Insulin secretion stimulation index from static GSIS assays (G16.8 mmol/l / G2.8 mmol/l) (n=7–9).
(**c**) Voltage-dependence of Ca^{2+}-currents in *RFX6*$^{+/+}$ (black, n=20 cells) and *RFX6*$^{+/-}$ (blue, n=37 cells) beta cells, in absence (solid lines) or presence of 20 µM nifedipine.
(**d**) Voltage-dependence of Na^+-currents in the same cells as in (c).
(**e**) Initial whole cell capacitance, as measure of cell size.
(**f**) Exocytosis stimulated by a train of voltage-clamp depolarisations, measured as capacitance increase in *RFX6*$^{+/+}$ (black) and *RFX6*$^{+/-}$ (blue, n=40 cells) beta cells.
(**g**) Quantfication of capacitance changes in F during the first (left) and the sum of all depolarisations.
(**h-i**) Exocytosis (**h**) and initial docked granules (**i**) measured by TIRF microscopy of NPY-EGFP labelled insulin granules in *RFX6*$^{+/+}$ (black, n=20 cells) and *RFX6*$^{+/-}$ (blue, n=30 cells) beta cells. Cells were stimulated by elevating K^+ for 40s; data are normalised to cell footprint area.

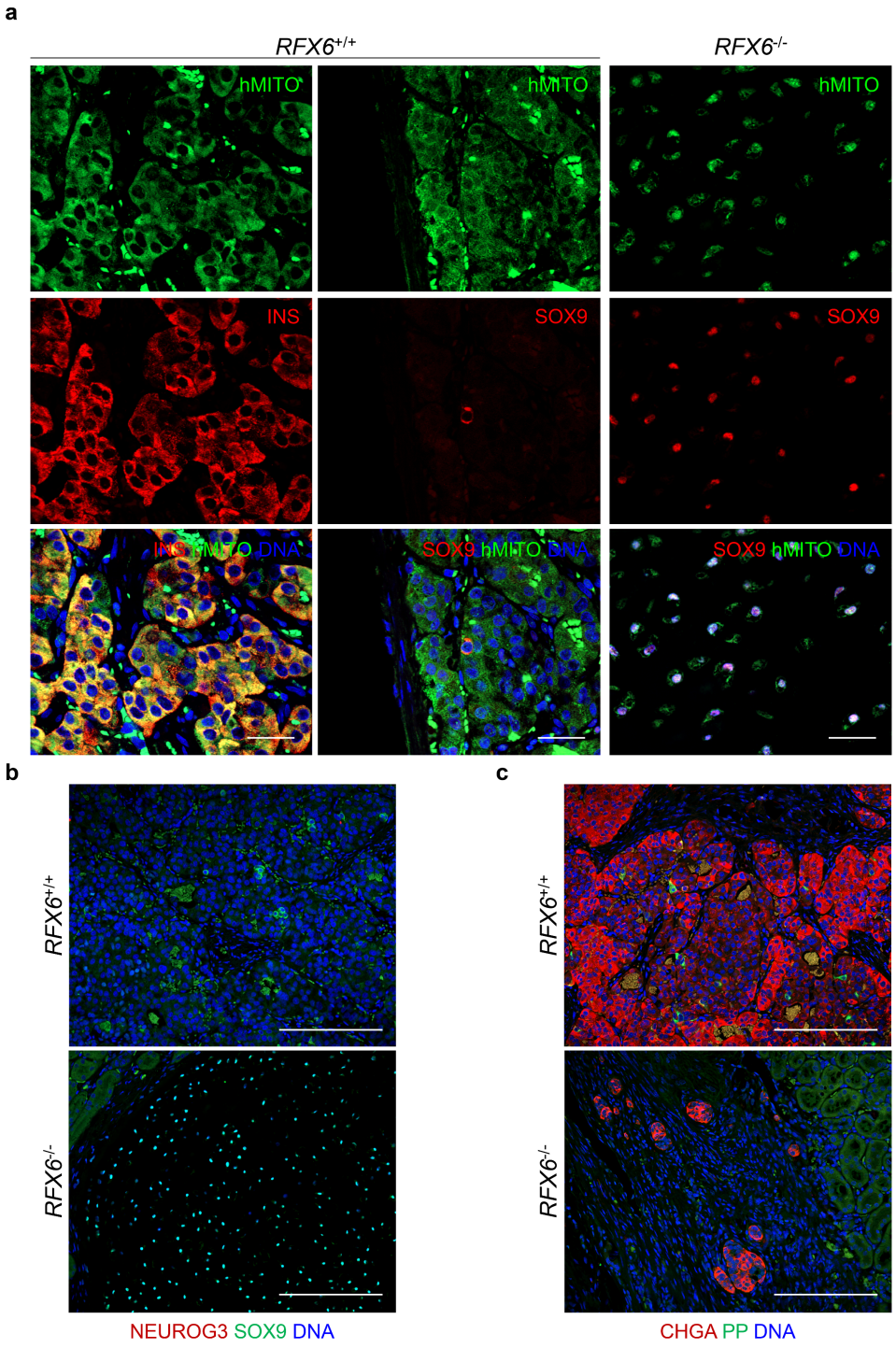
ESM Fig. 12



ESM Fig. 12 Altered gene expression levels in *RFX6*$^{+/-}$ at S7.

(**a**) Barplots of average gene expression (FPKM) of the upregulated genes heatmap in (Fig. 5c).
(**b**) Barplots of average gene expression (FPKM) of the downregulated genes heatmap in (Fig. 5d).
(**c**) Barplots of average gene expression (FPKM) of the hormone genes heatmap in (Fig. 5e).
Statistical significance is indicated as False Discovery Rate corrected *p* value, *FDR<0.05, **FDR<0.01, ***FDR<0.001. Data are presented as means ± SD.

ESM Fig. 13



ESM Fig. 13 Persistent expression of SOX9 but not NEUROG3 in homozygous *RFX6*$^{-/-}$ cells post implantation.

(**a**) Immunohistochemistry showing human specific mitochondria (hMITO^{+}), INS^{+} and SOX9^{+} cells at month 3 post implantation. Scale bar, 50 µm.
(**b**) Immunohistochemistry showing SOX9^{+} and NEUROG3^{+} cells at month 3 post implantation. Scale bar, 200 µm.
(**c**) Immunohistochemistry showing PP^{+} and CHGA^{+} cells at month 3 post implantation. Scale bar, 200 µm.