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Lab Resource: Multiple Genetically-Modified Cell Lines

Title: Generation of three human induced pluripotent stem cell lines with IRX5 knockout and knockin genetic editions using CRISPR-Cas9 system.

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Abstract:

Studies on animal models have shown that *Irx5* is an important regulator of cardiac development and that it regulates ventricular electrical repolarization gradient in the adult heart. Mutations in *IRX5* have also been linked in humans to cardiac conduction defects. In order to fully characterize the role of *IRX5* during cardiac development and in cardiomyocyte function, we generated three genetically-modified human induced pluripotent stem cell lines: two knockout lines (heterozygous and homozygous) and a knockin HA-tagged line (homozygous).

Resource Table:

Unique stem cell lines identifier	<ol style="list-style-type: none">1. ITXi002-A2. ITXi002-A-13. ITXi002-A-24. ITXi002-A-3
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Alternative name(s) of stem cell lines	<ol style="list-style-type: none"> 1. IRX5-Wt (ITXi002-A) 2. IRX5-Het (ITXi002-A-1) 3. IRX5-KO (ITXi002-A-2) 4. IRX5-KI (ITXi002-A-3)
Institution	l'institut du thorax (INSERM UMR1087, CNRS UMR6291)
Contact information of the reported cell line distributor	Nathalie Gaborit, nathalie.gaborit@univ-nantes.fr
Type of cell lines	iPSC
Origin	Human
Additional origin info (applicable for human ESC or iPSC)	IRX5-Wt (ITXi002-A) <ul style="list-style-type: none"> • Age: 15 • Male
Cell Source	Skin Fibroblasts
Method of reprogramming	Sendai virus (<i>OCT4</i> , <i>KLF4</i> , <i>SOX2</i> and <i>c-MYC</i>)
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	RT/q-PCR
Cell culture system used	StemMACS™ iPS Brew XF Medium on Matrigel® hESC-Qualified Matrix
Type of Genetic Modification	CRISPR/Cas9
Associated disease	N/A
Gene/locus	IRX5/16q12.2
Method of modification/site-specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	Electroporation of RNP
All genetic material introduced into the cells	IRX5-KI: single-stranded oligo DNA nucleotide (ssODN)
Analysis of the nuclease-targeted allele status	Sequencing of the targeted alleles
Method of the off-target nuclease activity surveillance	N/A
Name of transgene	N/A
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	N/A
Inducible/constitutive system details	N/A
Date archived/stock date	IRX5-Wt: 04-05-2013
Cell line repository/bank	IRX5-Wt: https://hpscereg.eu/cell-line/ITXi002-A IRX5-Het: https://hpscereg.eu/cell-line/ITXi002-A-1 IRX5-KO: https://hpscereg.eu/cell-line/ITXi002-A-2 IRX5-KI: https://hpscereg.eu/cell-line/ITXi002-A-3
Ethical/GMO work approvals	A signed informed consent has been received. Authorization MESRI Authorization IE 2009-505(1) has been obtained from competent ethics review board and approval authority (French Health Ministry and French ethical committee) allowing the generation of these lines.
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

Resource utility

The three generated hiPSC lines provide a (1) heterozygous or (2) homozygous deletion of *IRX5* gene and (3) an HA-tagged IRX5 protein which are a useful resource to investigate IRX5 transcription factor function in any human cell type, including mature and differentiating cardiomyocytes.

Resource Details

IRX5 transcription factor (TF) of the Iroquois homeobox family has been described to participate to murine cardiac development and cardiomyocyte functions, especially the regulation of cardiac ventricular electrical repolarization gradient (Costantini et al., 2005; Gaborit et al., 2012; Kim et al., 2012). Several mutations in *IRX5* have been found in patients with Hamamy syndrome presenting a severe clinical phenotype, with cardiac structural and electrical defects (Bonnard et al., 2012; Al Sayed et al., 2020).

We previously generated a control human induced pluripotent stem cell (hiPSC) line, IRX5-Wt, from skin fibroblasts collected from a healthy control individual (Al Sayed et al., 2020), that we used to perform various genetic modifications of *IRX5* gene. Using CRISPR-Cas9 technology we first generated two knockout hiPSC lines: heterozygous (IRX5-Het) and homozygous (IRX5-KO). For that, we electroporated the IRX5-Wt hiPSC line with ribonucleoproteins (RNP) that combined the Cas9 protein, a fluorescent transactivator-ATTO488, the gRNA1 and gRNA2 (targeting respectively *IRX5* first and third exons), in order to delete the entire gene. Dissociated fluorescent hiPS cells were FACS-sorted, replated by wells to allow clonal expansion and genotyped to validate the *IRX5* deletions (Figure 1A). IRX5 being more expressed in cardiomyocytes than in hiPSCs, using western-blot on cardiomyocytes derived from IRX5-Wt, IRX5-Het and IRX5-KO hiPSC lines, we confirmed the reduction or complete absence of IRX5 protein expression (Figure 1B). Second, to generate the hiPSC line expressing HA-tagged IRX5 protein (IRX5-KI) IRX5-Wt cells were transfected with an RNP combining Cas9 protein, a fluorescent transactivator-ATTO488 and the gRNA2, together with a fluorescent ssODN-Alexa660 that carried the HA tag sequence. The double fluorescent hiPSC clones sorted by FACS and genotyped by PCR, were sequenced to confirm HA tag insertion (Figure 1C). The four isogenic hiPSC lines were cultured on Matrigel®-coated plates with StemMACS™ iPS-Brew XF medium. They were tested negative for mycoplasma contamination by luminescence analysis (Supplementary Figure 1E). They all showed typical colony morphology assessed by brightfield microscopy (Figure 1D-Left panels). They also expressed pluripotency markers OCT3/4 and TRA-1-60 as evaluated by immunofluorescence experiments (Figure 1D-right panels). Pluripotency status of all four cell lines was also quantitatively assessed by RT-qPCR on *NANOG* and *POU5F1* markers (Figure 1E). Regarding specifically the control line, IRX5-Wt, the absence of Sendai virus persistence following reprogramming process was proved (Figure 1F). We then validated the ability of the three genetically modified cell lines and their isogenic control to differentiate into derivatives of the three germ layers by RT-qPCR analysis after 20 days of spontaneously differentiating embryoid bodies (Figure 1G). Furthermore, no major chromosomal aberrations were found between the IRX5-Wt isogenic control and (1) its corresponding parental fibroblasts (Supplemental Figure 2A) and (2) its CRISPR-Cas9 resulting cell lines, IRX5-Het, IRX5-KO and IRX5-KI (Figure 1H-J). Finally, evidence of genetic identity between the control parental fibroblasts and the four hiPSC lines was shown by single nucleotide polymorphism (SNP) analysis (Supplemental Figure 2B). Detailed informations for all lines are provided in Table 1 and Table2.

Materials and Methods

hiPSC generation and culture

Following Sendai virus reprogramming and CRISPR-based editing, hiPSC isogenic lines were maintained at 37°C, 5% CO₂, 21% O₂ in StemMACS™ iPS Brew XF Medium (Miltenyi Biotec) on Matrigel® hESC-Qualified Matrix (0.05mg/mL, Corning). At 75% confluency, cells were passaged as clumps using Gentle Cell Dissociation Reagent (STEMCELL Technologies).

CRISPR-Cas9 genome editing

Two gRNAs were used to delete *IRX5* gene in IRX5-Wt hiPSC line to generate IRX5-Het and IRX5-KO lines. Regarding IRX5-KI, one gRNA and a fluorescent HA-tag ssODN-Alexa660 were used to introduce HA-tag before *IRX5* STOP codon. Cas9/gRNA/transactivator/±ssODN were delivered by electroporation with P3 Primary Cell 4D-Nucleofector™ X kit, CA137 pulse program (Lonza). Cells were FACS-sorted 48h post-electroporation (BD FACSMelody™ cell sorter). Single or double positive cells were replated as single-cells and maintained in iPSC Brew medium until they reached 80% confluency, prior duplication for genotyping and banking.

Immunofluorescence analysis

Cells were fixed with 4% paraformaldehyde for 15min at room temperature (RT) in Matrigel®-coated μ-Slide 8 Well (IBIDI) prior permeabilisation with 0.5% PBS-Triton (15min) and blocking with 3% PBS-BSA (RT, 30min). Cells were then incubated with primary antibodies (4°C, overnight). Finally, cells were washed and incubated with secondary antibodies and DAPI (RT, 1h) and conserved in 0.5% paraformaldehyde (4°C). Images were acquired by confocal microscopy (MicroPICell core facility).

Embryoid bodies (EB) generation

hiPSC colonies were detached with Dispase (STEMCELL Technologies) and replated in Costar® 6 Well Plate Low Attachment (Corning). Medium was changed the next day and unsupervised EB differentiation was started a day after, by incubating cells in EB medium: 78% (v/v) KnockOut™ DMEM/F-12 (Gibco) + 1% (v/v) GlutaMAX™ Supplement (Gibco) + 20% (v/v) KnockOut™ Serum Replacement (Gibco) + 1X MEM Non-Essential Amino Acids Solution + 0.1mM 2-Mercaptoethanol (Gibco). The EB medium was changed daily during 21 days before RT-qPCR analysis.

SNP and CNV analysis

To confirm isogenic hiPSC line genome integrity we compared their single nucleotide polymorphisms (SNP). Genomic DNA was extracted from somatic and hiPSC samples using the QIAGEN QiaAmp kit. 200ng of gDNA were outsourced to Integragen Company (Evry, France) for karyotype analysis using HumanCore-24-v1 SNP arrays. Analysis was performed with GenomeStudio software. Chromosome abnormalities were visually determined on logR ratios values and comparing parental cells with hiPSC-derived samples. SNP data were used to compute CNV and validate cell line parentality.

RT-qPCR

Total RNAs were extracted using NucleoSpin RNA kit (MACHEREY-NAGEL) and reverse transcribed using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems™). PCR amplification was performed using (1) FAM-labeled TaqMan probes (Applied Biosystems) for pluripotency and differentiation potential, and (2) SYBR-green technology (Applied Biosystems) for SeV loss (p23-MS368; p25-MS381). Data were normalized to ACTB or GAPDH.

Western blot

60μg proteins were denatured using NuPAGE Sample Reducing Agent and NuPAGE LDS Sample Buffer and ran in 4–15% precast polyacrylamide gel (Bio-Rad). Then, proteins were transferred on nitrocellulose membrane prior blocking (5% milk) and antibodies incubations. Revelation was performed using chemiluminescence camera (Bio-Rad), and the Stainfree technology was used as loading control.

Mycoplasmas test

Mycoplasma contamination was assessed with MycoAlert™ Mycoplasma Detection Kit (Lonza).

Acknowledgments

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Additional files:

Table 1: Characterization and validation

Classification	Test	Result	Data
Morphology	Photography	Typical morphological aspect of hiPSCs and colonies	Figure 1D, left panels
Pluripotency status evidence for the described cell line	Qualitative analysis (immunofluorescence)	Assess staining of pluripotency markers: OCT3/4 and TRA-1-60	Figure 1D, right panels
	Quantitative analysis (RT-qPCR)	Assess transcript expression of pluripotency markers: POU5F1 and NANOG	Figure 1E
Karyotype	Molecular karyotyping (SNP array; >300000 markers)	IRX5-Wt: 46,XY IRX5-Het: 46,XY IRX5-KO: 46,XY	Figure 1H-J and Supplementary Figure 2A

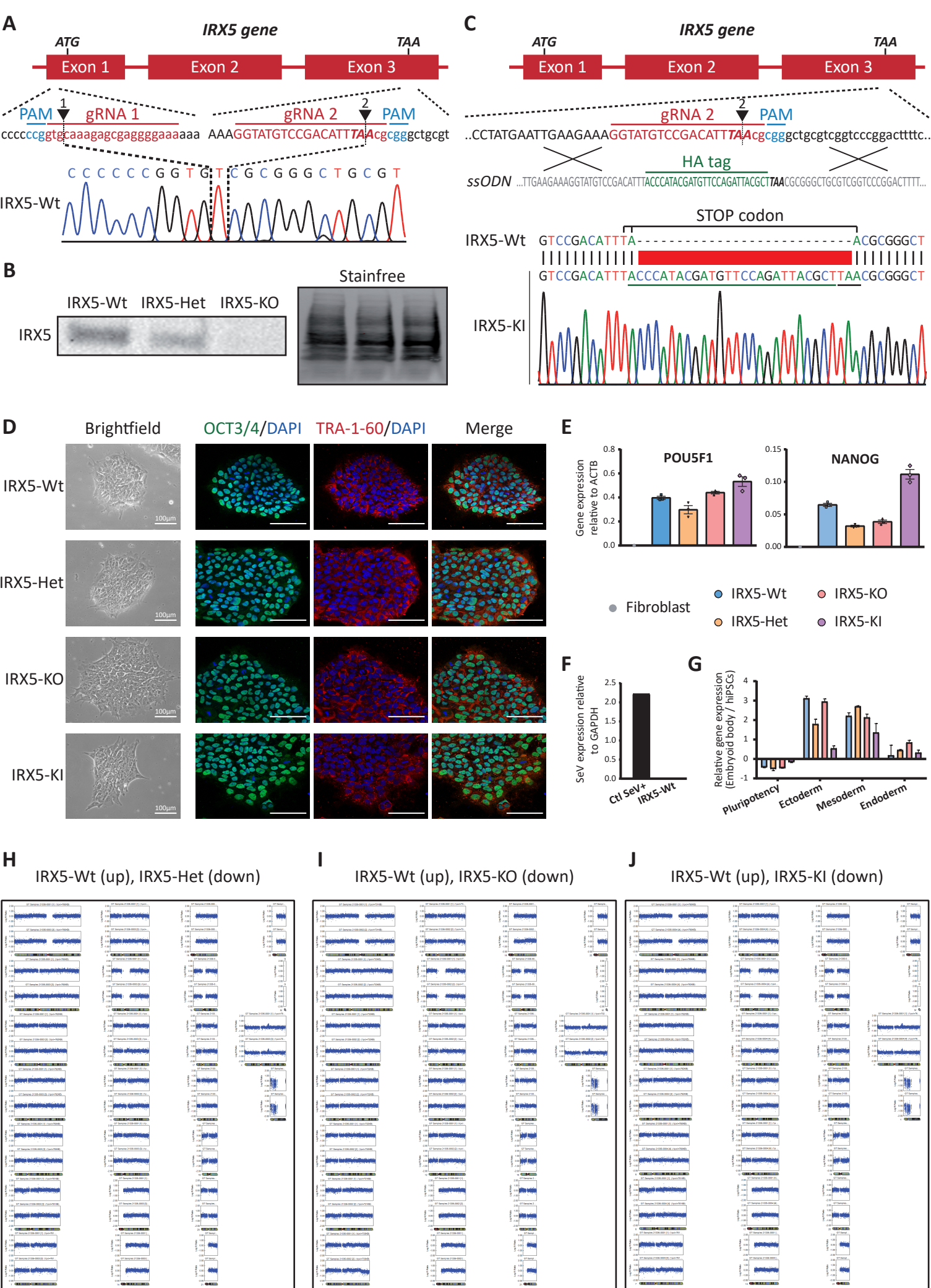
		IRX5-KI: 46,XY	
Verification of the absence of random plasmid integration events	PCR/Southern	N/A	N/A
Parental and modified cell line genetic identity evidence	CNV analysis	SNP array to compare IRX5-Wt to parental fibroblast and CRISPR-edited lines to IRX5-Wt line. Matching genotypes confirmed the origins of iPSC lines	Supplementary Figure 2B and archived with the journal
		190 tested sites	Archived with the journal
Mutation analysis	PCR across the edited site and sequencing	Complete IRX5 deletion validated for IRX5-Het and IRX5-KO cell lines. Validated insertion of the HA-tag at the end of <i>IRX5</i> 's ORF for the IRX5-KI cell line.	Figure 1A, C and Supplementary Figure 1A-D
	Western blot	No IRX5 protein expression in IRX5-KO cell line	Figure 1B
Off-target nuclease analysis-	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing	N/A	N/A
Specific pathogen-free status	Mycoplasma	Mycoplasma testing by luminescence: no contamination for all cell lines	Supplementary figure 1E
Multilineage differentiation potential	Embryoid body formation and differentiation followed by RT-qPCR analysis	Expression of genes in embryoid bodies: Ectoderm (<i>PAX6</i>); Mesoderm (<i>HAND1</i>); Endoderm (<i>SOX17</i>)	Figure 1G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

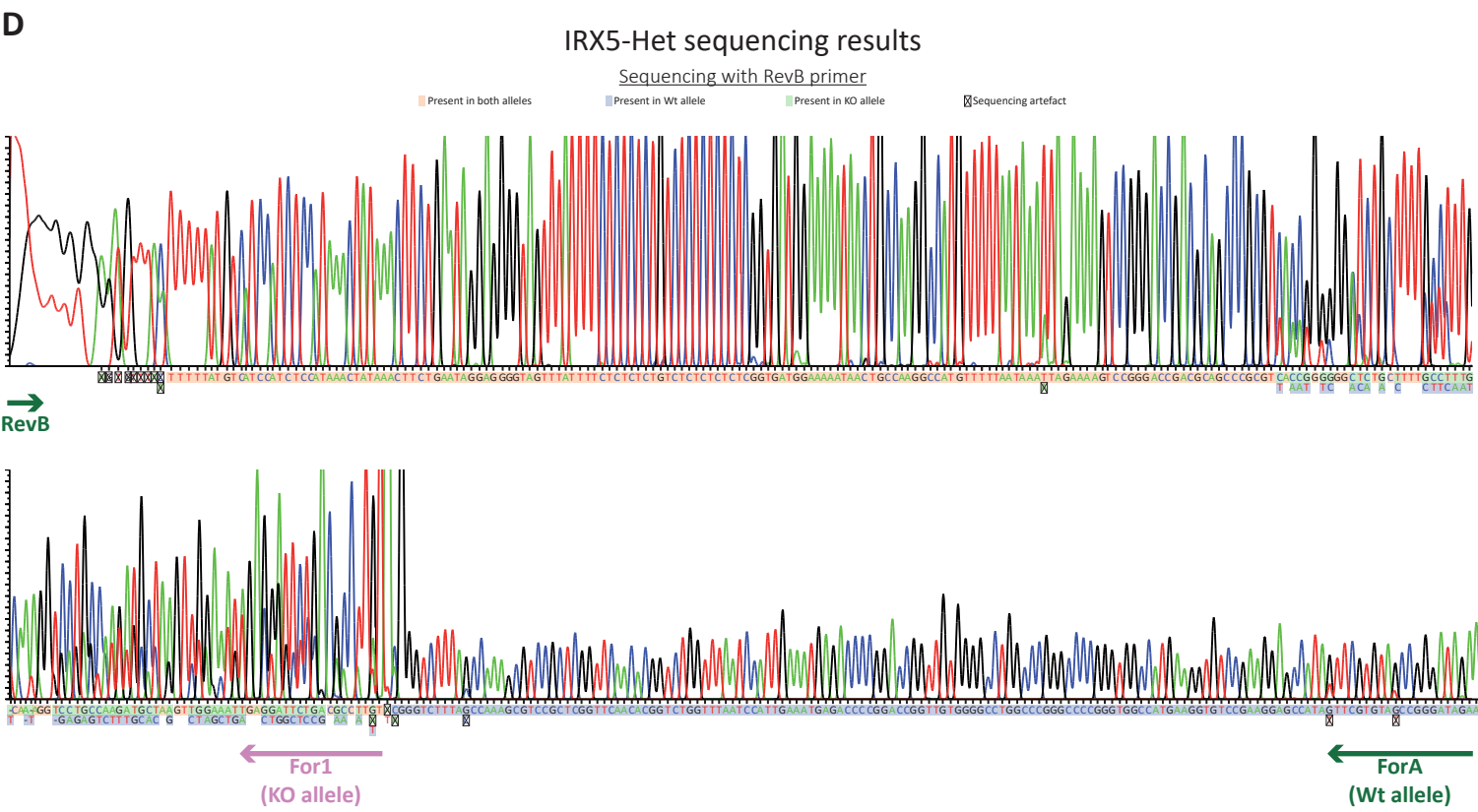
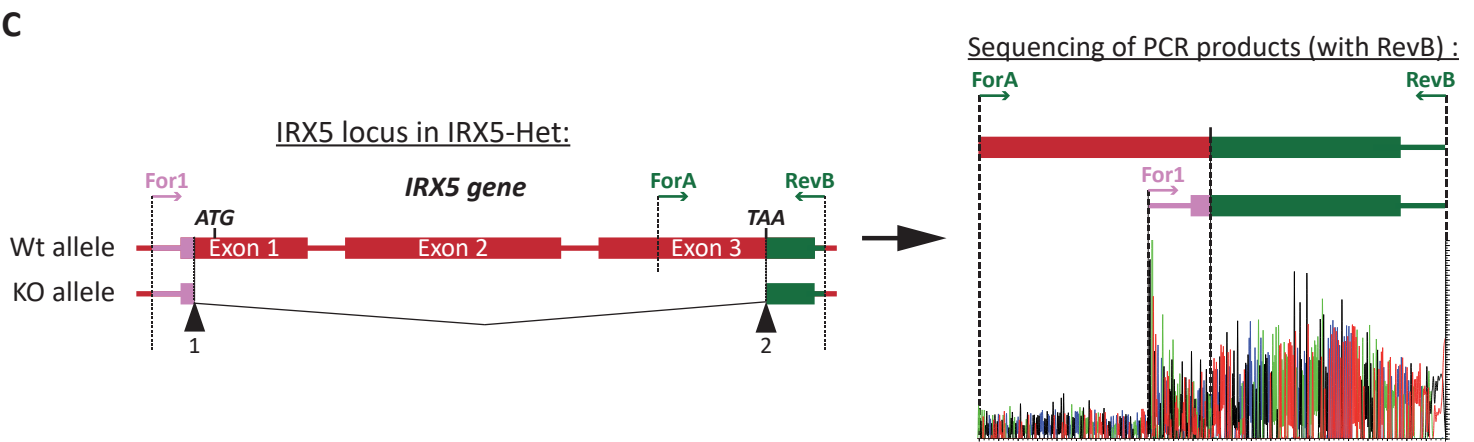
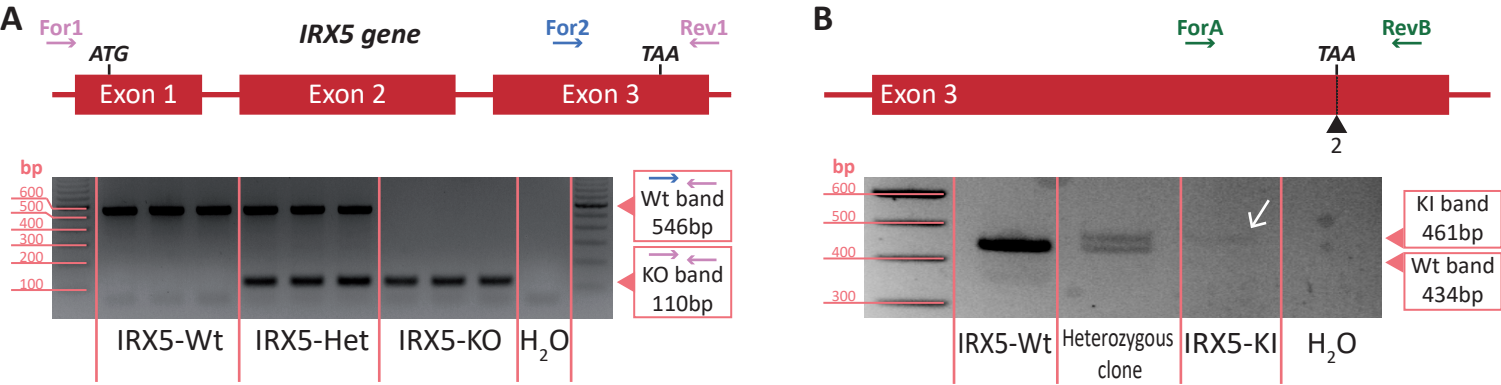
Table 2: Reagents details

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rat anti-OCT3/4	1:200	Thermo Fisher Scientific Cat# 14-5841-80, RRID:AB_914299

	Mouse anti-TRA-1-60	1:200	Thermo Fisher Scientific Cat# 14-8863-80, RRID:AB_891612
Nuclear stain	DAPI	1µg/mL	Merck Cat# D9564
Secondary antibodies	Goat Anti-Mouse IgG (H+L) Antibody, Alexa Fluor 568 Conjugated	1:1000	Molecular Probes Cat# A-11004, RRID:AB_2534072
	Goat Anti-Rat IgG (H+L) Antibody, Alexa Fluor 488 Conjugated	1:1000	Molecular Probes Cat# A-11006, RRID:AB_141373
	Goat anti-mouse IgG-HRP antibody	1:10 000	Santa Cruz Biotechnology Cat# sc-2055, RRID:AB_631738
Mutation analysis - WB	Mouse anti-IRX5	1:250	Santa Cruz Biotechnology Cat# sc-81102, RRID:AB_1124818
Site-specific nuclease			
Nuclease information	Alt-R® S.p. HiFi Cas9 Nuclease V3	Integrated DNA Technologies (IDT) Cat# 1081061	
Delivery method	Nucleofection	P3 Primary Cell 4D-Nucleofector™ X, Lonza Cat# V4XP-3024	
Selection/enrichment strategy	FACS sorting using transactivator-associated fluorescence with (if present) ssODN-associated fluorescence	<u>Transactivator</u> : Alt-R® CRISPR-Cas9 tracrRNA ATTO™ 488, IDT <u>ssODN</u> : Single-stranded DNA oligo Alexa Fluor® 660, IDT	
Primers and Oligonucleotides used in this study			
	Target	Forward/Reverse primer (5'-3')	
Sendai Virus Detection (primer)	SeV	Forward: 5'-GGA TCA CTA GGT GAT ATC GAG C-3' Reverse: 5'-ACC AGA CAA GAG TTT AAG AGA TAT GTA TC-3'	
House-Keeping Genes (primer)	<i>GAPDH</i>	Forward: 5'-AAT CCC ATC ACC ATC TTC CA-3' Reverse: 5'-TGG ACT CCA CGA CGT ACT CA-3'	
Pluripotency Markers (TaqMan probe)	<i>NANOG</i>	Thermo Fisher Scientific Cat# Hs02387400	
Pluripotency Markers (TaqMan probe)	<i>POU5F1</i>	Thermo Fisher Scientific Cat# Hs04260367	
Ectoderm Marker (TaqMan probe)	<i>PAX6</i>	Thermo Fisher Scientific Cat# Hs01088114	
Endoderm Marker (TaqMan probe)	<i>SOX17</i>	Thermo Fisher Scientific Cat# Hs00751752	
Mesoderm Marker (TaqMan probe)	<i>HAND1</i>	Thermo Fisher Scientific Cat# Hs02330376	
House-Keeping Genes (TaqMan probe)	<i>ACTB</i>	Thermo Fisher Scientific Cat# Hs01060665	
IRX5 gDNA PCR	<i>IRX5</i>	For1: 5'-ACAAGGCGTCAGAATCCTCA-3' For2: 5'-GGGCCCTCGGTTATCCATTC-3' ForA: 5'-TCTATCCCGGCTACACGAAC-3' Rev1: 5'-GTCGGTCCCGGACTTTTCTA-3' RevB: 5'-TGTCTTAACCAACCGAAAAGAAA-3'	
Sequencing	<i>IRX5</i> deletion	RevB: 5'-TGTCTTAACCAACCGAAAAGAAA-3'	

	HA tag sequence insertion	ForA: 5'-TCTATCCCGGCTACACGAAC-3'
crRNA IRX5-Exon1 (gRNA 1)	<i>IRX5</i>	5'-GTGCAAAGAGCGAGGGGAAA-3'
crRNA IRX5-Exon3 (gRNA 2)	<i>IRX5</i>	5'-GGTATGTCCGACATTTAACG-3'
Genomic target sequence	<i>IRX5</i> gene	Human, GRCh38.p13, Chr16: 54,930,865-54,934,485
ssODN HA tag	<i>IRX5</i>	5'- TATGAATTGAAGAAAGGTATGTCCGACATTTACCCATACGA TGTTCCAGATTACGCTTAACGCGGGCTGCGTCGGTCCCGGA CTTTTCTAATTTATTTAAA-3'



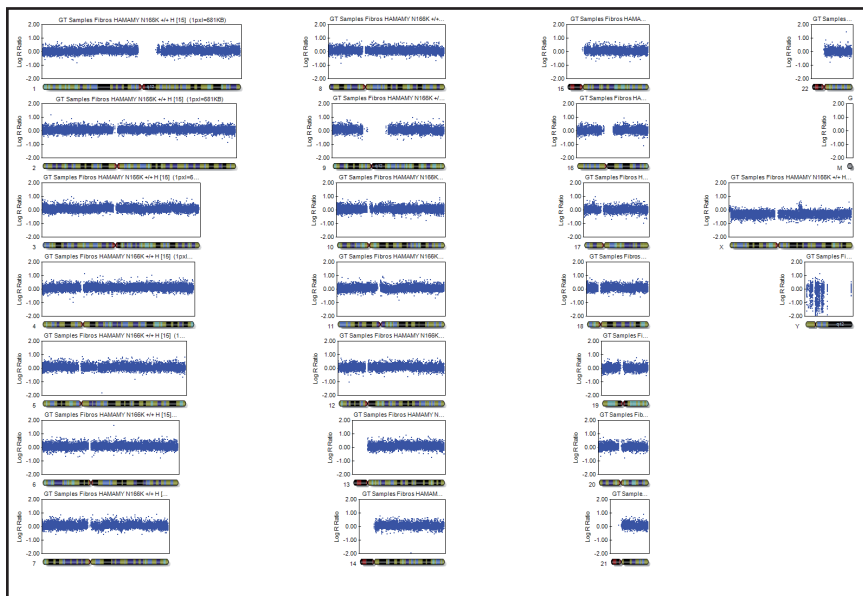


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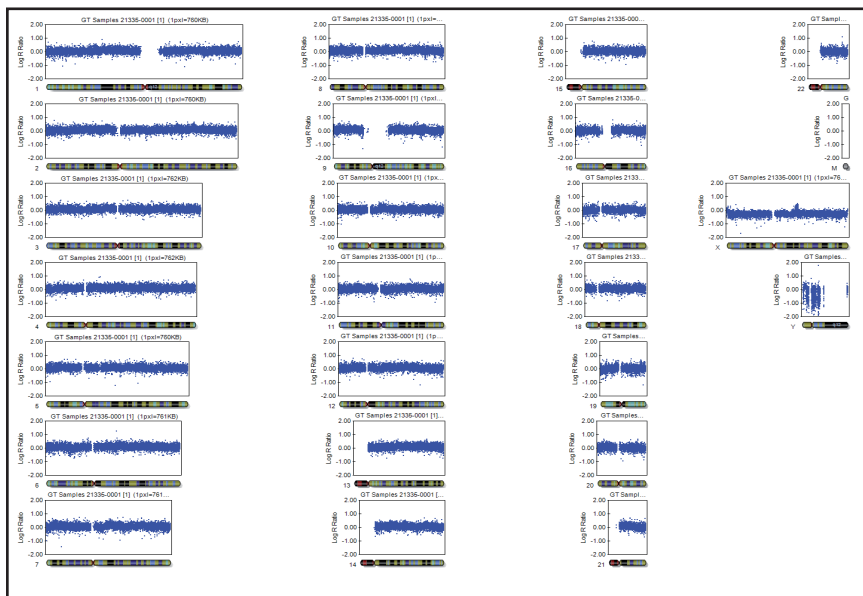
	IRX5-Wt	IRX5-Het	IRX5-KO	IRX5-KI	Positive control	H2O
Read A	188.9	230.3	172.4	223.5	113.9	282.3
Read B	121.8	164.2	104.8	121.3	1156	96.41
Ratio B/A	0.6448	0.7130	0.6079	0.5427	10.15	0.3415
Mycoplasma	Negative	Negative	Negative	Negative	Positive	Negative

A

IRX5-Wt - parental skin fibroblasts



IRX5-Wt - hiPSC line



B

Percentage CNV identity

iPS-ITXi002-A_vs_Fibroblast-ITXi002-A	iPS-ITXi002-A_vs_Unrelated_PBMC
84.02	45.56

Percentage CNV identity

iPS-ITXi002-A_vs_iPS-ITXi002-A-1	iPS-ITXi002-A_vs_iPS-ITXi002-A-2	iPS-ITXi002-A_vs_iPS-ITXi002-A-3
89.35	87.57	91.12

Percentage CNV identity

iPS-ITXi002-A-1_vs_Unrelated_iPS	iPS-ITXi002-A-2_vs_Unrelated_iPS	iPS-ITXi002-A-3_vs_Unrelated_iPS
47.93	49.70	47.34