Supplementary Material

Gene editing to prevent ventricular arrhythmias associated with cardiomyocyte cell therapy

Marchiano S., Nakamura K., Reinecke H., Neidig L., Lai M., Kadota S., Perbellini F., Yang X., Klaiman J. M., Blakley L. P., Karbassi E., Fields P. A., Fenix A. M., Beussman K. M., Jayabalu A., Kalucki F. A., Futakuchi-Tsuchida A., Weber G. J., Dupras S., Tsuchida H., Pabon L., Wang L., Knollmann B. C., Kattman S., Thies R. S., Sniadecki N., MacLellan W. R., Bertero A., and Murry C. E.

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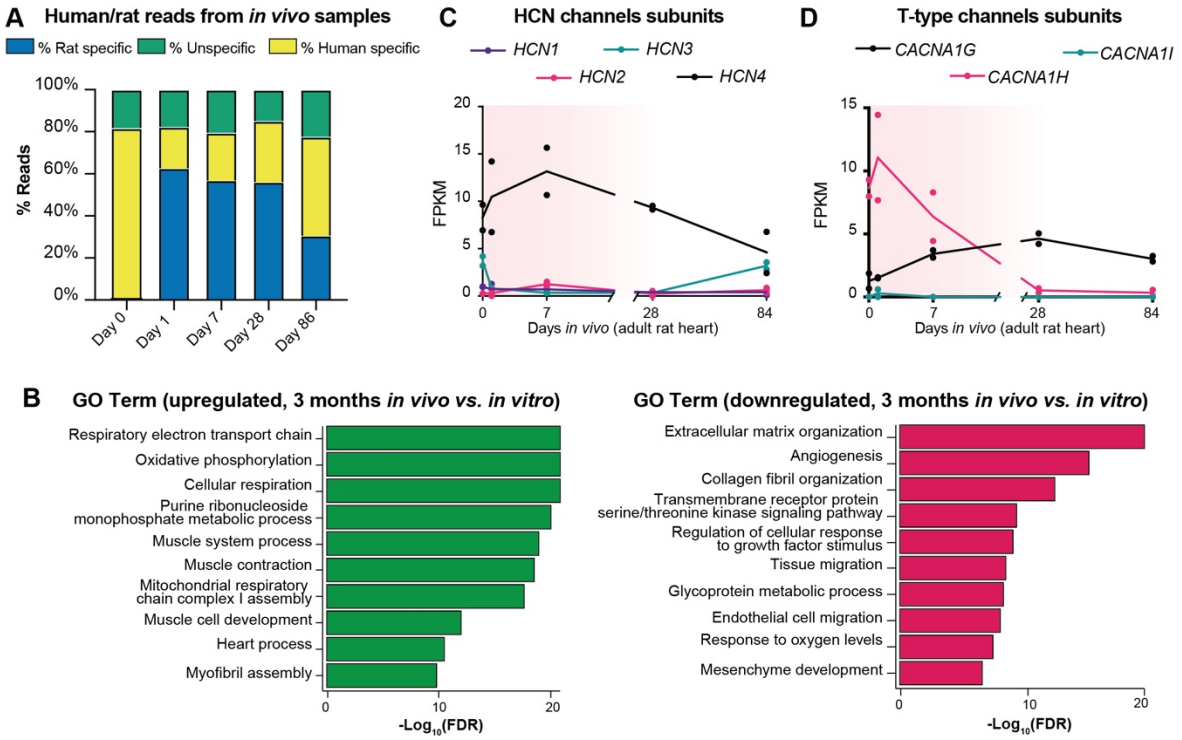
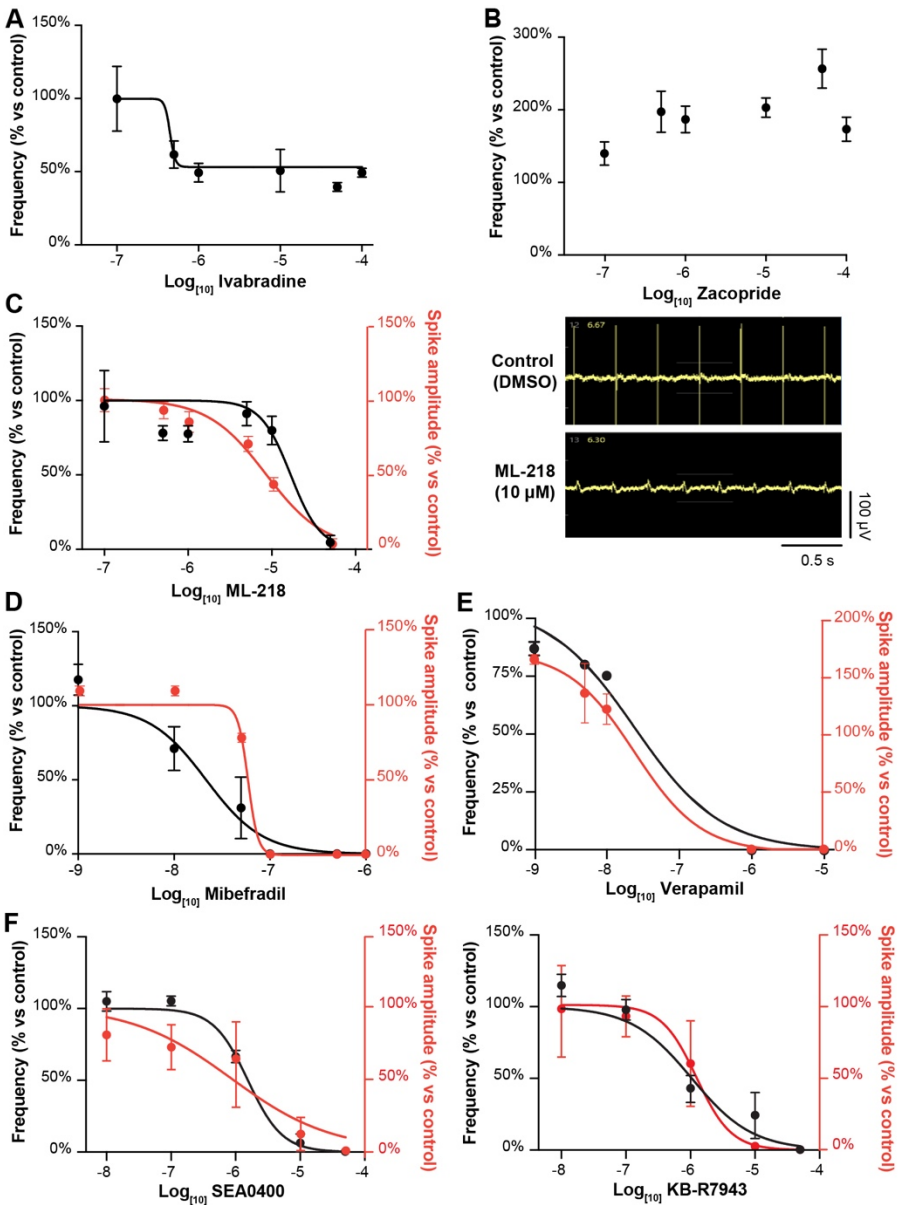
Supplementary Video 2. Point stimulation of host myocardium and calcium transient elicited in graft (related to Figure 7D).

Supplementary Video 3. Point stimulation of graft and calcium transient elicited in host myocardium (related to Supplementary Fig. 6E).

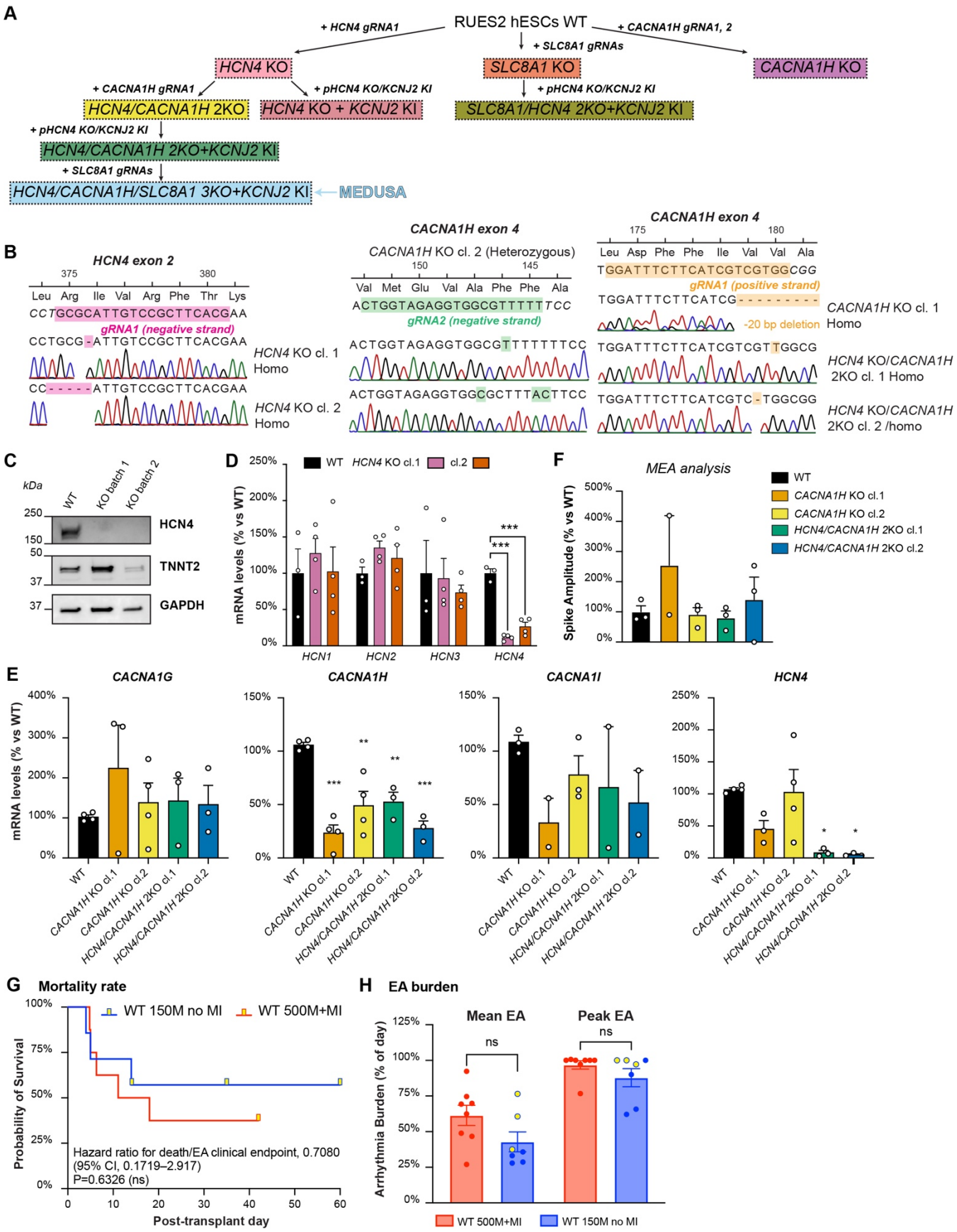
Supplementary Figures



Supplementary Fig. 1 – RNA-seq analysis of *in vivo* transplanted hiPSC-CMs. (**A**) Percentage of human/rat reads from *in vivo* samples after laser capture microdissection in the experiment described in Figs. 1B-D. "Unspecific" indicates reads that do not uniquely map to either genome. (**B**) GO term analysis of selected upregulated and downregulated pathways at 3 months after hiPSC-CMs transplantation. See also Supplementary Table 1. **(C)** RNA-seq expression dynamics of HCN channel isoforms and **(D)** T-type calcium channel isoforms during *in vivo* maturation of hiPSC-CMs. See also Fig. 1F.

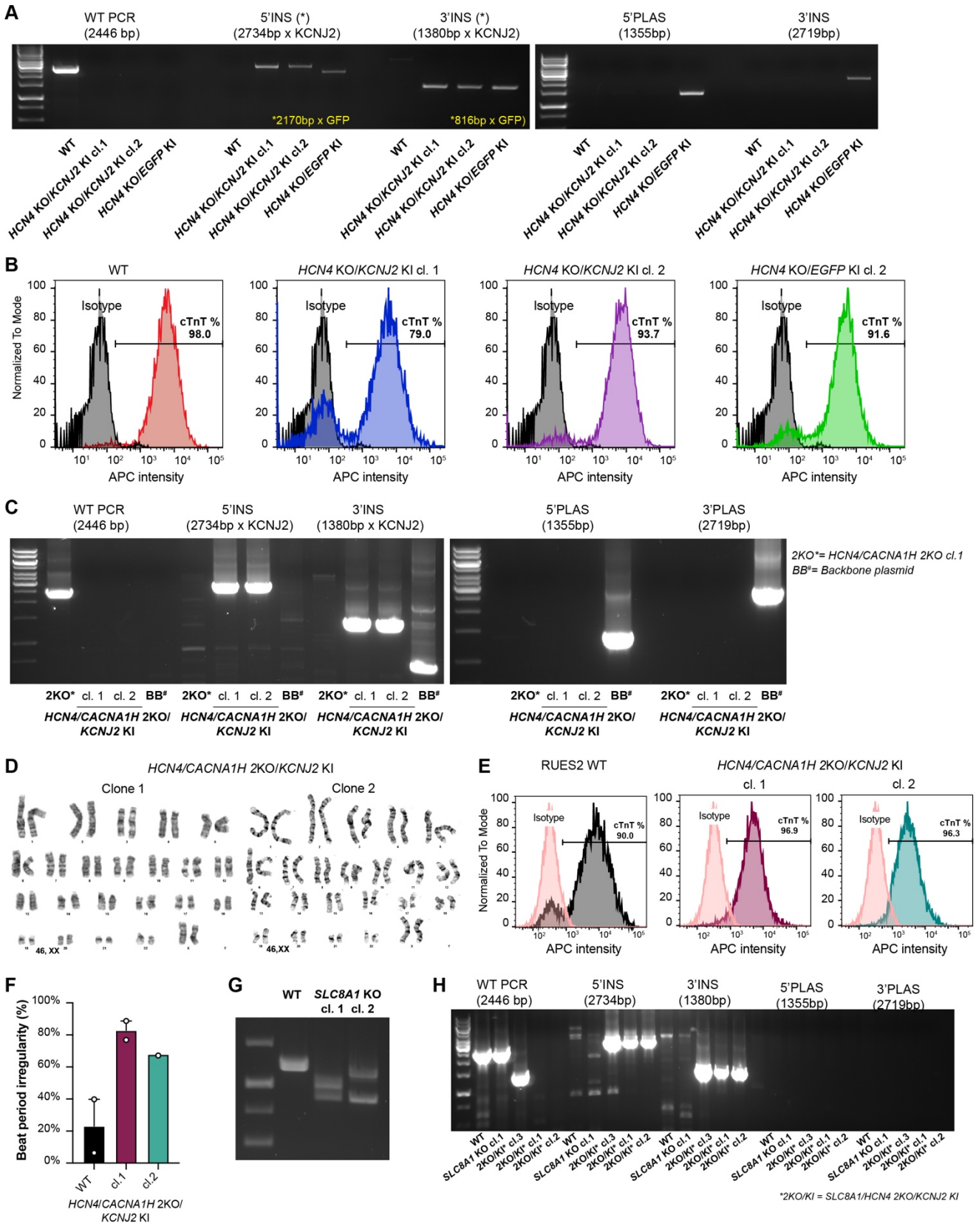


Supplementary Fig. 2 – Pharmacological inhibition of hESC-CMs automaticity *in vitro*. (**A, B**) Dose-response curves of Ivabradine (I_f inhibitor, A) and Zacopride (I_{K1} agonist, B) on MEA system. (**C-F**) Frequencies (left y-axis) and spike amplitude (right y-axis) dose-response curves and representative traces of ML218, Mibefradil (I_{CaT} inhibitors, C, D); Verapamil (I_{CaL} inhibitor, E) and SEA0400 and KB-R7943 (I_{NCX} inhibitors, F). Data shown as mean ± SEM of 2 independent experiments each with 6 technical replicates, normalized on baseline and expressed as % *vs.* control (DMSO). Lines represents nonlinear regression of normalized response.



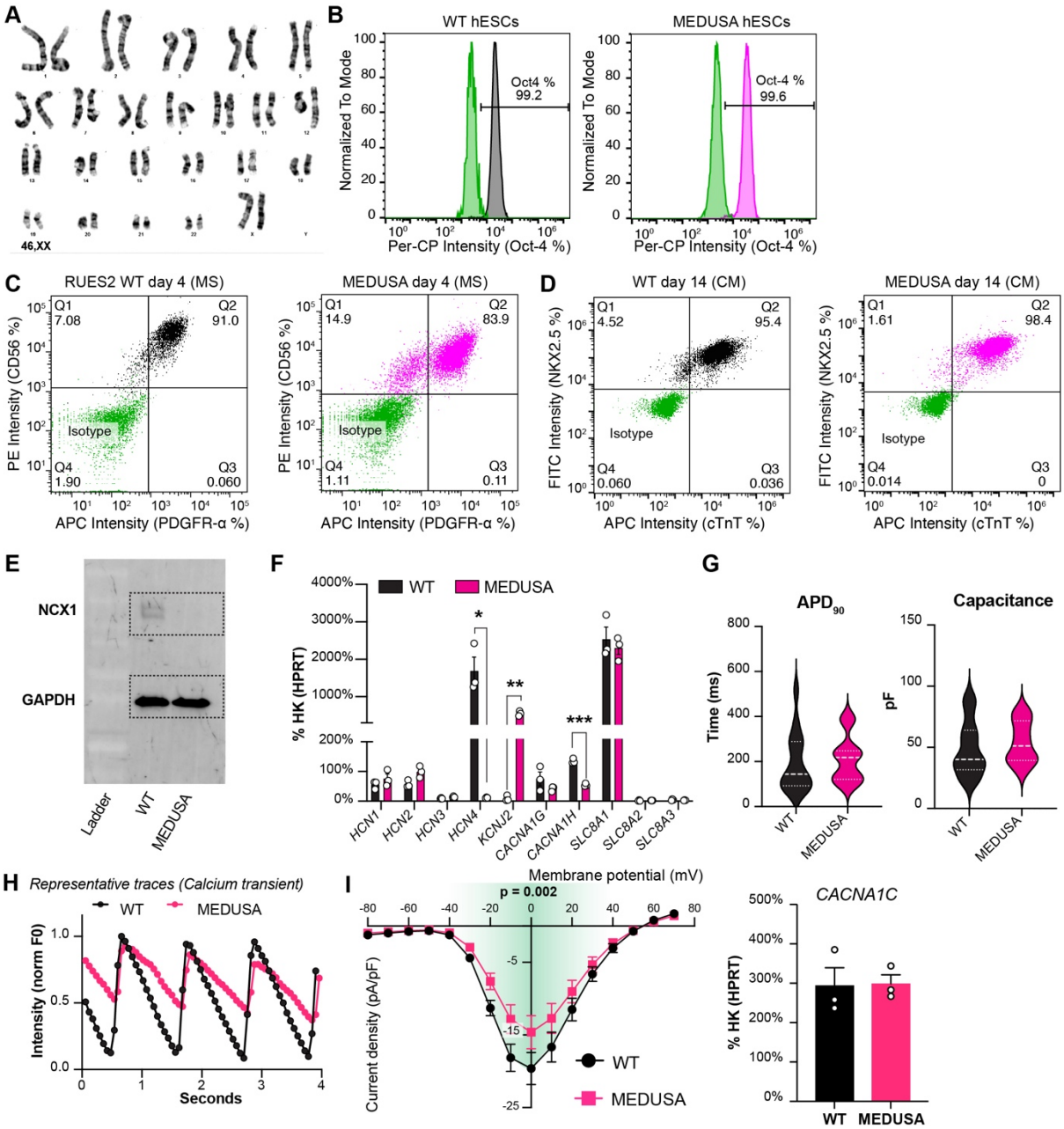
Supplementary Fig. 3 – Characterization of gene-edited cell lines targeting phase 4 of action potential. (**A**) Gene editing approach for the generation of the different cell lines. *pHCN4/KCNJ2 KI*

indicates the targeting vector described in Fig. 3A. (**B**) Sanger sequencing validation of *HCN4* KO clones (*HCN4* KO cl.1 = 1 bp homozygous deletion; *HCN4* KO cl. 2 = 5 bp homozygous deletion), *CACNA1H* KO clones (*CACNA1H* KO cl.1= 20 bp homozygous deletion, *CACNA1H* KO cl.2= compound heterozygous indels leading to frameshift on the different alleles) and double edited cells (*HCN4*/*CACNA1H* KO cl.1= 1 bp homozygous insertion, *HCN4*/*CACNA1H* KO cl.2= 1 bp homozygous deletion). (**C**) Western blot for HCN4, TNNT2 (cardiac troponin T) and GAPDH (loading control) in different batches of MEDUSA hESC-CMs, reported here with the parental line for simplicity. (**D, E**) Gene expression analyses by qRT-PCR of HCN channel subunits in *HCN4* KO, *CACNA1H* KO and *HCN4*/*CACNA1H* 2KO clones compared to WT in 4 independent batches of CMs. In some batches, *CACNA1I* was undetectable (cycle threshold > 40). Differences quantified by one-way ANOVA with Sidak correction for multiple comparisons (** $p < 0.01$, *** $p < 0.001$). (**F**) Spike amplitude of *CACNA1H* KO and *HCN4/CACNA1H* 2KO clones from MEA analysis. Data shown as mean ± SEM of 2-3 independent experiments normalized on WT Spike amplitude. (**G**) Kaplan-Meier curve for freedom from EA mortality (described as death, unstable VT or heart failure necessitating either euthanasia or pharmacological intervention) in uninjured animals receiving 150M WT cells (n=7) or infarcted animals receiving 500M WT cells (n=8,). Yellow-colored marks on the treatment line indicate non-cardiac deaths due to opportunistic infection or planned euthanasia. (**H**) EA burden in uninjured and injured animals shown as mean and peak EA of animals receiving WT cells (Mann-Whitney test). Yellow-colored symbols represent animals that reached endpoint as described in the Method section. Data for 500M + MI in G and H from *Nakamura K. et al., Stem Cell Reports 2021.*



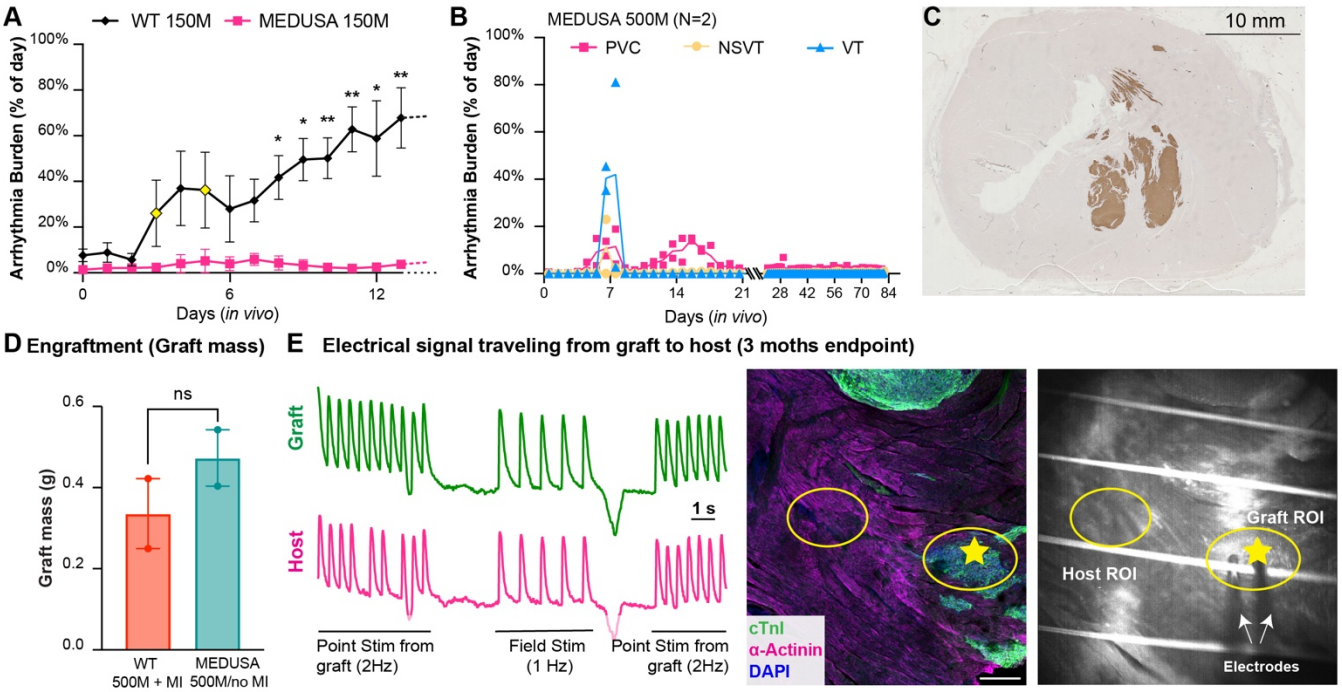
Supplementary Fig. 4 – Genotyping and characterization of triple-edited cell lines. (**A**) Genotyping of *HCN4* KO/*KCNJ2* KI clones generated with CRISPR/Cas9-mediated homology directed repair using

the targeting plasmid described in Fig. 3A. (**B**) Representative flow cytometry data of *HCN4* KO/*KCNJ2* KI clones at day 14 of differentiation stained for cardiac troponin T (cTnT, pan-CM marker). (**C**) Genotyping of *HCN4*/*CACNA1H* 2KO/*KCNJ2* KI clones generated with the targeting plasmid described in Fig. 3A. Refer also to Supplementary Fig. 3A for parental lines. (**D**) Karyotype analyses of *HCN4*/*CACNA1H* 2KO/*KCNJ2* KI clones. (**E**) Representative flow cytometry analysis of *HCN4*/*CACNA1H* 2KO/*KCNJ2* KI CMs at day 14 of differentiation stained for cTnT. (**F**) Beat period irregularity quantified as standard deviation of the beat period record in 100 sec, divided by the mean of the beat period in that same period. Data shown as mean ± SEM of 2 independent experiment (for *HCN4*/*CACNA1H* 2KO/*KCNJ2* KI cl.2, n=1 due to lack of spontaneous activity in the second experiment that prevented calculation of beat irregularity). (**G**) Genotype of *SLC8A1* KO clones generated via combination of 3 gRNAs. (**H**) Genotype of *SLC8A1*/*HCN4* 2KO/*KCNJ2* KI clones generated with same approach described in Fig. 3A. Due to heterozygosity of clone 3, this clone was not used for further experiment.



Supplementary Fig. 5 – Characterization of MEDUSA cell line. (**A**) Karyotype analysis of MEDUSA hESCs. (**B**) Representative flow cytometry analysis of MEDUSA hESCs and WT hESCs stained with Oct3/4 as pluripotency marker, as compared to respective isotype control. (**C, D**) Representative flow cytometry analysis of mesoderm markers (CD56/PDGFRα) and ventricular markers (NKX2.5/cTnT) during cardiac differentiation in WT and MEDUSA, compared to isotype controls. MS= Mesoderm state. (**E**) Western blotting for NCX1 and GAPDH (loading control) in WT and MEDUSA hESC-CMs. Dotted squares represent inserts shown in Fig. 5A. (**F**) qRT-PCR analysis of genes encoding for I_f, I_{CaT}, I_{K1} and I_{NCX} in MEDUSA hESC-CMs, compared to WT-matched controls. Note the absence of nonsense-mediated decay for *SLC8A1.* Data shown as mean ± SEM of 3 independent biological replicates. Differences *vs.* WT by multiple unpaired t test (* p <0.05, ** p <0.01 and *** p <0.001). See also Fig. 5A. (**G**) Action potential duration at 90% of repolarization (APD_{90}) and capacitance current of WT and MEDUSA CMs. Data shown as described in Figs. 5E. (**H**) Representative calcium transient trace from WT and MEDUSA hESC-CMs. See also Fig. 5H. (**I**) Current density plot for I_{CaL} currents and qRT-PCR for *CACNA1C* in MEDUSA hESC-CMs, compared to WT controls. For I/V plot, data shown as mean ± SEM of n = 22 for WT and n = 15 for MEDUSA hESC-CMs. Green-shaded area indicates difference vs.

WT hESC-CMs by two-way ANOVA with Sidak correction for multiple comparison ($p = 0.002$). For qRT-PCR. Data shown as mean ± SEM of 3 independent biological replicates.



Supplementary Figure 6. Characterization of transplanted MEDUSA hESC-CMs. (**A**) Daily arrhythmia burden focused on the first 2 weeks after transplantation for animals receiving 150 x 10^6 (150M) WT or MEDUSA hESC-CMs (See also Fig. 6A for arrhythmia burden over 7-weeks observation period). Differences vs. WT by multiple unpaired t-test (* $p < 0.05$ and ** $p< 0.01$). Yellow-colored symbols indicate animals that reached EA endpoint as detailed in Fig. 6A and Methods section. (**B**) Quantification of EA burden divided in PVCs, VT and NSVT for both 500M MEDUSA animals in the first 3 weeks after transplantation. See also Methods section for details on EA characterization. (**C**) Whole-slide scanning showing MEDUSA grafts at 500M dose at 3 months after transplantation. (**D**) Graft mass of 500M MEDUSA hESC-CMs transplanted into the uninjured pig heart compared to 500M WT hESC-CMs transplanted into infarcted animals. Data shown as average ± SEM, differences by Mann-Whitney test. For 500M WT hESC-CMs + MI, data points from *Nakamura K., et al., Stem Cell Reports 2021*.
(**E**) Cardiac slice model of 12 weeks old MEDUSA-CMs grafts. Left panel showing Fluo-4 traces of cardiac slice paced with field and point stimulation from graft region with relative immunofluorescence and monochromatic images on the right side. Yellow circle indicates ROI for Fluo-4 recording, yellow star indicates point stimulation location. Scale bar = 500 μm. See also Supplementary Video 3.

Supplementary Table 1. Extended top 40 GO terms from Supplementary Fig. 1B.

Top 40 upregulated GO terms 3 months in vivo vs in vitro

GO term	***-LogFDR***
mitochondrial ATP synthesis coupled electron transport	26.7593509
ATP synthesis coupled electron transport	26.7593509
respiratory electron transport chain	26.7593509
oxidative phosphorylation	26.7593509
cellular respiration	26.7593509
muscle system process	24.905479
electron transport chain	24.905479
muscle contraction	24.3724063
energy derivation by oxidation of organic compounds	23.8385322
ATP metabolic process	23.7816273
mitochondrial electron transport, NADH to ubiquinone	23.4889897
purine ribonucleotide metabolic process	22.4583209
purine ribonucleoside monophosphate metabolic process	22.4583209
purine nucleoside monophosphate metabolic process	22.400329
purine ribonucleoside triphosphate metabolic process	22.1572909
mitochondrial respiratory chain complex assembly	21.8021029
ribonucleoside triphosphate metabolic process	21.6997652
purine nucleoside triphosphate metabolic process	21.6435556
nucleoside triphosphate metabolic process	21.6148587
ribonucleoside monophosphate metabolic process	21.4475971
NADH dehydrogenase complex assembly	21.0603809
mitochondrial respiratory chain complex I assembly	21.0603809
nucleoside monophosphate metabolic process	20.6662339
generation of precursor metabolites and energy	19.0256188
muscle cell development	16.1158982
striated muscle contraction	15.5888635
mitochondrion organization	15.1695254
striated muscle cell development	14.9634709
heart process	13.3913741
heart contraction	13.3913741
myofibril assembly	12.7808906
striated muscle tissue development	11.6790402
muscle tissue development	10.8758565
cardiac muscle contraction	10.8758565
cellular component assembly involved in morphogenesis	10.5252677
mitochondrial translational elongation	10.5027401
mitochondrial translational termination	10.3981337
striated muscle cell differentiation	10.3179452
muscle cell differentiation	9.92716966
sarcomere organization	9.89901292

Top 40 downregulated GO terms 3 months in vivo vs in vitro

GO term	***-LogFDR***
extracellular matrix organization	17.7593509
extracellular structure organization	17.1853197
angiogenesis	12.9354422
collagen fibril organization	11.8842897
response to transforming growth factor beta	8.36141092
cellular response to transforming growth factor beta stimulus	8.31441435
regulation of cellular response to growth factor stimulus	8.31441435
transmembrane receptor protein serine/threonine kinase signaling pathway	8.20004291
glycoprotein metabolic process	7.97323075
negative regulation of cellular component movement	7.77955431
tissue migration	7.77955431
ameboidal-type cell migration	7.54849756
epithelial cell migration	7.25524618
negative regulation of locomotion	7.19507949
endothelial cell migration	7.19507949
epithelium migration	7.18531966
epithelial cell proliferation	7.14470181
regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	7.11153344
ossification	7.1086856
urogenital system development	6.99968308
transforming growth factor beta receptor signaling pathway	6.93544218
glycoprotein biosynthetic process	6.8465011
kidney development	6.82004877
response to oxygen levels	6.79713949
skeletal system development	6.28222967
aminoglycan biosynthetic process	6.24490535
mesenchyme development	6.21299108
renal system development	6.12732771
response to endoplasmic reticulum stress	6.09141515
negative regulation of cell motility	6.0323522
glycosaminoglycan biosynthetic process	5.99544011
mesenchymal cell differentiation	5.99166511
cholesterol biosynthetic process	5.99166511
secondary alcohol biosynthetic process	5.99166511
negative regulation of cellular response to growth factor stimulus	5.99166511
regulation of vasculature development	5.92648734
response to decreased oxygen levels	5.80131543
response to hypoxia	5.80131543
cell-substrate adhesion	5.7220266
chondrocyte differentiation	5.66680572

Supplementary Table 2. Graft size quantification.

Animal	*Procedure*	*Cell Prep*[a]	*%Area graft*[b]	*%Graft size*[c]	*Endpoint*	*EA observed*[d]
WT #1	Catheter	SC	0.245%	0.00310%	Endpoint 2 weeks	Yes
WT #2	Catheter	SC	0.742%	0.00932%	Euthanized day 6	Yes, rapid
WT #3	Catheter	SC	1.676%	0.01275%	Endpoint 2 weeks	Yes
WT #4	Direct thoracotomy	SC	NA	NA	Amiodarone at day 4	Yes, rapid
WT #5	Direct thoracotomy	SC	NA	NA	Endpoint 5 weeks	Yes
WT #6	Direct thoracotomy	SC	NA	NA	Amiodarone at 2 weeks	Yes, rapid
WT #7	Direct thoracotomy	SC	NA	NA	Endpoint 3 months	Yes
HCN4 *KO #1*	Catheter	SC	0.913%	0.00953%	Endpoint 4 weeks	Yes
HCN4 *KO #2*	Catheter	SC	2.351%	0.02200%	Endpoint 4 weeks	Yes
HCN4 *KO*/KCNJ2 *KI #1*	Catheter	SC	0.986%	0.01574%	Euthanized day 7	Yes, rapid
HCN4 *KO*/KCNJ2 *KI #2*	Catheter	SC	0.120%	0.00128%	Euthanized day 9	Yes, rapid
HCN4 *KO*/CACNA1H *2KO*/KCNJ2 *KI #1*	Catheter	SC	4.116%	0.04128%	Euthanized day 8	Yes, rapid
HCN4 *KO*/SLC8A1 *2KO*/KCNJ2 *KI #1*	Direct thoracotomy	Mono	2.444%	0.03165%	Euthanized day 7	Yes, rapid
HCN4 *KO*/SLC8A1 *2KO*/KCNJ2 *KI #2*	Direct thoracotomy	Mono	0.294%	0.00367%	Endpoint 4 weeks	No
HCN4 *KO*/SLC8A1 *2KO*/KCNJ2 *KI #3*	Direct thoracotomy	Mono	6.281%	0.05714%	Endpoint 4 weeks	No
MEDUSA #1	Direct thoracotomy	Mono	0.747%	0.00837%	Endpoint 4 weeks	No
MEDUSA #2	Direct thoracotomy	Mono	2.701%	0.02459%	Endpoint 4 weeks	No
MEDUSA #3	Direct thoracotomy	SC	9.284%	0.07453%	Endpoint 7 weeks	No
MEDUSA 500M #1	Direct thoracotomy	SC	36.94%	0.29081%	Endpoint 3 months	Yes, self-terminating
MEDUSA 500M #2	Direct thoracotomy	SC	28.70%	0.24330%	Endpoint 3 months	Yes, self-terminating

[a] SC: suspension culture differentiation; Mono: monolayer differentiation
[b] %Area graft expressed as percentage of the relative myocardial section.
[c] %Graft size: (% area graft x block weight) / left ventricle mass.
[d] Rapid indicates extreme tachycardia over 300 beats per minute necessitating euthanasia

Supplementary Table 3. Oligonucleotides sequences for cloning gRNAs and genotyping.

Primer Name	Sequence	Target
HCN4_gRNA1_FWD	CACCGTCGTGAAGCGGACAATGCGC	HCN4 gRNA1
HCN4_gRNA1_RVS	AAACGCGCATTGTCCGCTTCACGAC	
CACNA1H_gRNA1_FWD	CACCGGATTTCTTCATCGTCGTGG	CACNA1H gRNA1
CACNA1H_gRNA1_RVS	AAACCCACGACGATGAAGAAATCC	
HCN4_gRNA1_KI_FWD	CACCGCAGCTTGTCCATGGCGCCAG	HCN4 KO_KCNJ2 gRNA1
HCN4_gRNA1_KI_RVS	AAACCTGGCGCCATGGACAAGCTGC	
HCN4_gRNA2_KI_FWD	CACCGGCAGCTTGTCCATGGCGCC	HCN4 KO_KCNJ2 gRNA2
HCN4_gRNA2_KI_RVS	AAACGGCGCCATGGACAAGCTGCC	
SLC8A1_gRNA1	GGAUCAUAUUACUGUAAGAA	SLC8A1 gRNA1
SLC8A1_gRNA2	CAGCAAUUACAUGGUCCACA	SLC8A1 gRNA2
SLC8A1_gRNA3	UGAAAUCCCAUUGAAAAGGU	SLC8A1 gRNA3
HCN4-KI_5HA_FW	gaatgcgtcgagatATTGGGTCgcggccgcACGAACC CGGTCGCCTCCCA	Cloning HCN4 5' HA
HCN4-KI_5HA_REV	TGGCTGATCATTAATTAAGCGGGTTTAAACG GGCCCATGGCGCCAGGGGCCGG	
HCN4-KI_3HA_FW	AAAGAGAGAGCAATATTTCAAGAATGCAtgcgt caattttacgcagactatctttctagggTTAAGACAAGCTG CCGCCGTCC	Cloning HCN4 3' HA
HCN4-KI_3HA_REV	ggtcccggcatccgatACCCAATggcgcgccGGCTGAA TGACCCGGAGCTG	
pA_FW	AAACCCGCTTAATTAATGATCAGCCATCGATT CGActgtgccttctagttgccag	Cloning polyA
pA_REV	AATTTTACGCATGATTATCTTTAACgtacgtcaca atatgattatctttctagggTTAAacccatagagcccaccgc	
EGFP_Amp_FW	GACCTTAACCatggtgagcaaggg	Cloning EGFP
EGFP_Amp_REV	GGTTCCATCGATttacttgtacagctcgtccatg	
eSpCas9_seq	GGCCTATTTCCCATGATTCCT	Sequencing pX459 and pX330 cloned plasmids
HCN4_gen_FWD	GAGAACACCACACCCTGGATT	Genotype HCN4 KO clones
HCN4_gen_RVS	TGCCACAATCTGACAGCCTAT	
CACNA1H_gen_FWD	TTTCCTGATGAGCCAACGCC	Genotype CACNA1H KO clones
CACNA1H_gen_RVS	CCGGTCACTTACTAGGCACG	
SLC8A1_gen_FWD	TCATGTACAACATGCGGCGA	Genotype SLC8A1 KO clones
SLC8A1_gen_RVS	CTCCGTCAGGCACCACATAA	
WT_PCR_FWD	CACCCTGCCCATGTCACAGG	Genotype HCN4 KO_KCNJ2 KI clones
WT_PCR_RVS	GTGACTTCGGTCCTCCAGGG	
5'INS_FWD	CACCCTGCCCATGTCACAGG	
5'INS_RVS	CGTCAATTTTACGCATGATTATCTTTAAC	
3'INS_FWD	GCGACGGATTCGCGCTATTTAGAAAG	
3'INS_RVS	GTGACTTCGGTCCTCCAGGG	
5'PLAS_FWD	GAGCGGATAACAATTTCACACAGG	
5'PLAS_RVS	GCGACGGATTCGCGCTATTTAGAAAG	

3'PLAS_FWD	CGTCAATTTTACGCATGATTATCTTTAAC	
3'PLAS_RVS	AGGGTTTTCCCAGTCACGACGTT	

Supplementary Table 4. Oligonucleotides sequences for RTqPCR

Gene target	Sequence
HCN4_FWD	GATCCTCAGCCTCTTACGCC
HCN4_RVS	CCCCAGGAGTTGTTCACCAT
HCN1_FWD	ATGGTAATCCAGAGGTCAGACA
HCN1_RVS	TTCTTCGAGGCGGCAGTATC
HCN2_FWD	GGCATGGTGAACCACTCGT
HCN2_RVS	TGTACTGCTCCACCTGCTTG
HCN3_FWD	GGTTCCTGGTTGACCTCATCT
HCN3_RVS	GTGAAAGATCTCCTCCCACTGGT
CACNA1H_FWD	ATGCTGGTAATCATGCTCAACTG
CACNA1H_RVS	AAAAGGCGAAAATGAAGGCGT
CACNA1G_FWD	CGCCATCTTCCAGGTCATCA
CACNA1G_RVS	TCTGAGAACTGCGTGGCAAT
CACNA1I_FWD	GGATGGAGCTGATCCTCATGT
CACNA1I_RVS	AAGATGAAGTCATCAAAGACCTGC
KCNJ2_FWD	GTGCGAACCAACCGCTACA
KCNJ2_RVS	CCAGCGAATGTCCACACAC
SLC8A1_FWD	AGACCTGGCTTCCCACTTTG
SLC8A1_RVS	TGGCAAATGTGTCTGGCACT
SLC81A2_FWD	GTCACTGCCTCTTGGAGCAT
SLC81A2_RVS	CACACCGGGAAGAAGACCAG
SLC81A3_FWD	CCGAAATGGATGGAACGTGG
SLC81A3_RVS	GAATGGGTCCCCACAACCAA
TNNT2_FWD	TTCACCAAAGATCTGCTCCTCGCT
TNNT2_RVS	TTATTACTGGTGTGGAGTGGGTGTGG
HPRT_FWD	TGACACTGGCAAAACAATGCA
HPRT_RVS	GGTCCTTTTCACCAGCAAGCT