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Supplemental Information

The Long Non-coding RNA *Cyrano* Is Dispensable for Pluripotency of Murine and Human Pluripotent Stem Cells

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Supplemental Information

SUPPLEMENTAL FIGURES

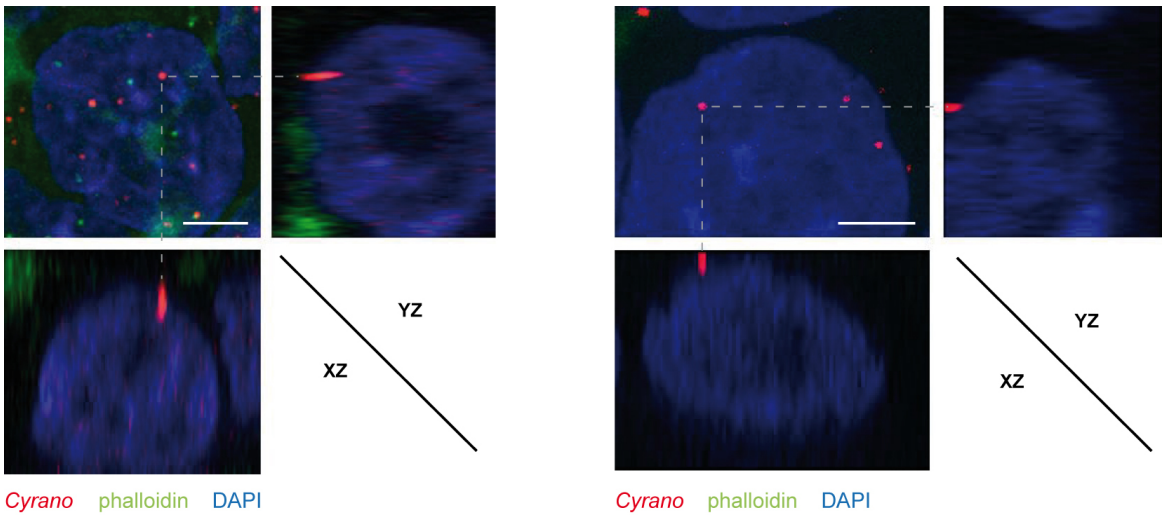


Figure S1 *Cyrano* is localised in the cytoplasm within a cell. Related to Figure 1.
Representative RNA-FISH images stained with a *Cyrano*-specific probe and phalloidin in unmodified human iPSCs as 3D reconstruction. Scale 5 µm.

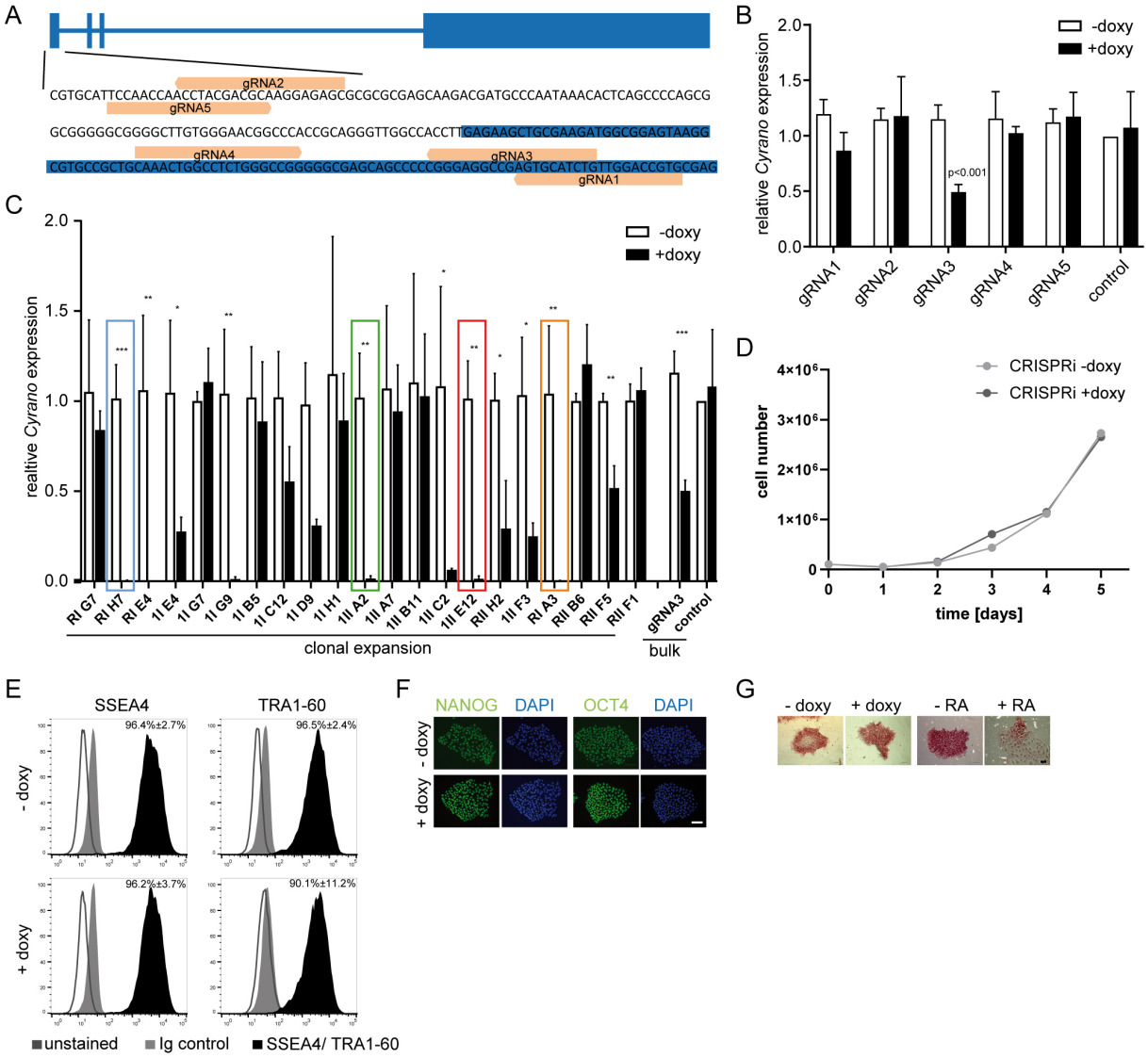


Figure S2 Generation of *Cyrano* KD human iPSC cell lines using CRISPRi approach where doxycycline itself has no effect on pluripotency. Related to Figure 3.

A Five gRNAs were tested, located between 150 bp up- and downstream of the transcription start site of *Cyrano*. gRNAs in red, exon depicted in blue. **B** Expression of *Cyrano* after doxycycline treatment in polyclonal populations. Expression levels were measured by RT-qPCR, mean ± SD of 3-5 independent experiments are shown, unpaired *t*-test for statistical analysis was performed. **C** Expression of *Cyrano* after doxycycline treatment in monoclonal populations. Expression levels were measured by RT-qPCR, mean ± SD of 3-5 independent experiments are shown unpaired *t*-test was performed for statistical analysis, * p<0.05, ** p<0.01, *** p<0.001. **D** Growth curve of CRISPRi iPSCs (without gRNA) with and without doxycycline (doxy) treatment. Mean of 3 independent experiments is shown. **E** Flow cytometry analysis of SSEA4 and TRA1-60 of CRISPRi iPSCs. Isotype controls and unstained cells were used as controls. Representative plots with mean ± SD of 3 independent experiments are depicted. **F** Fluorescence immunocytochemistry of *NANOG* and *OCT4* was performed in CRISPRi iPSCs after doxycycline treatment. Scale 100 µm. **G** Representative images of alkaline phosphatase staining with and without doxycycline treatment. Cells were also treated with 100nM retinoic acid (RA). Scale 500 µm.

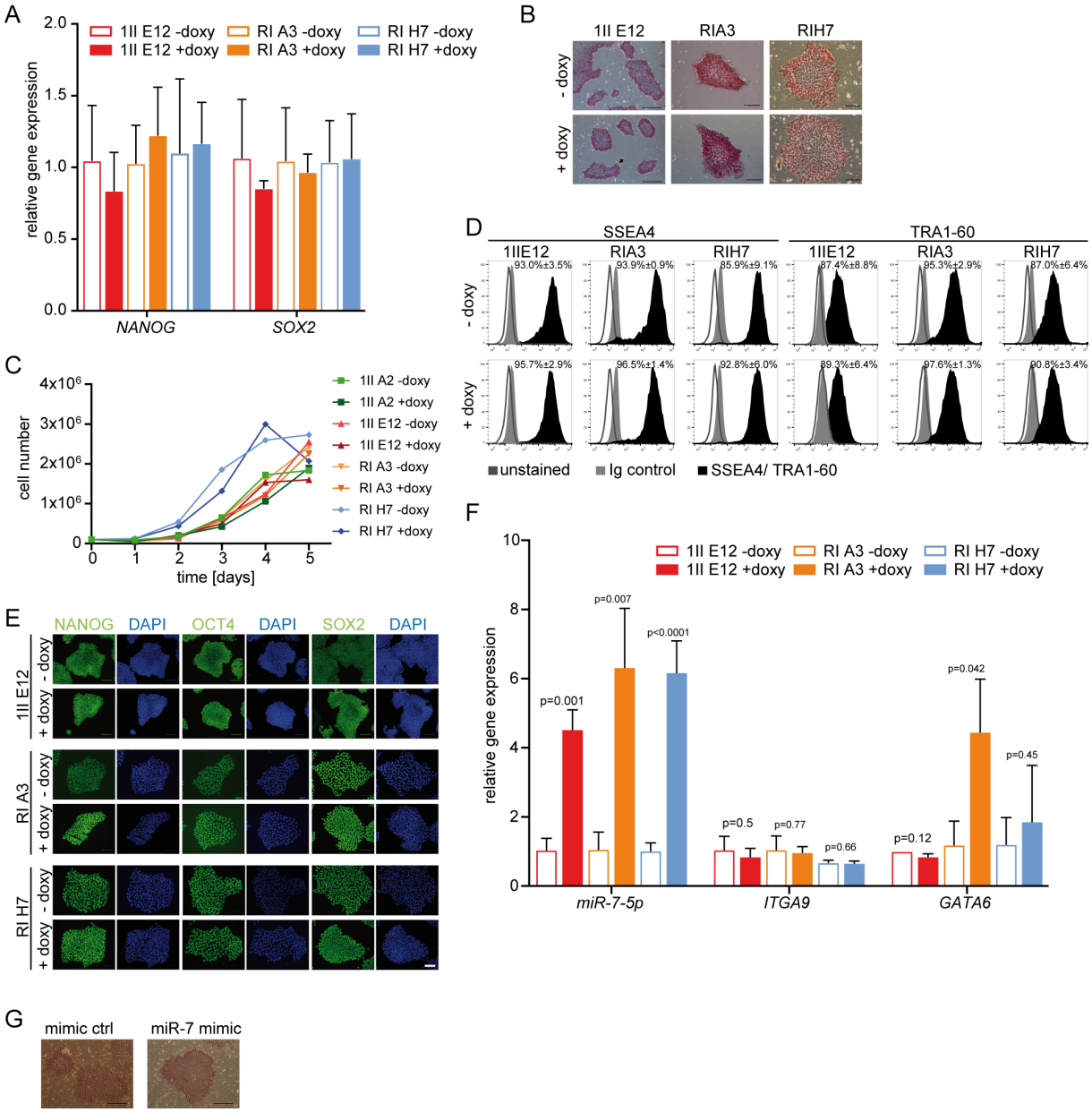
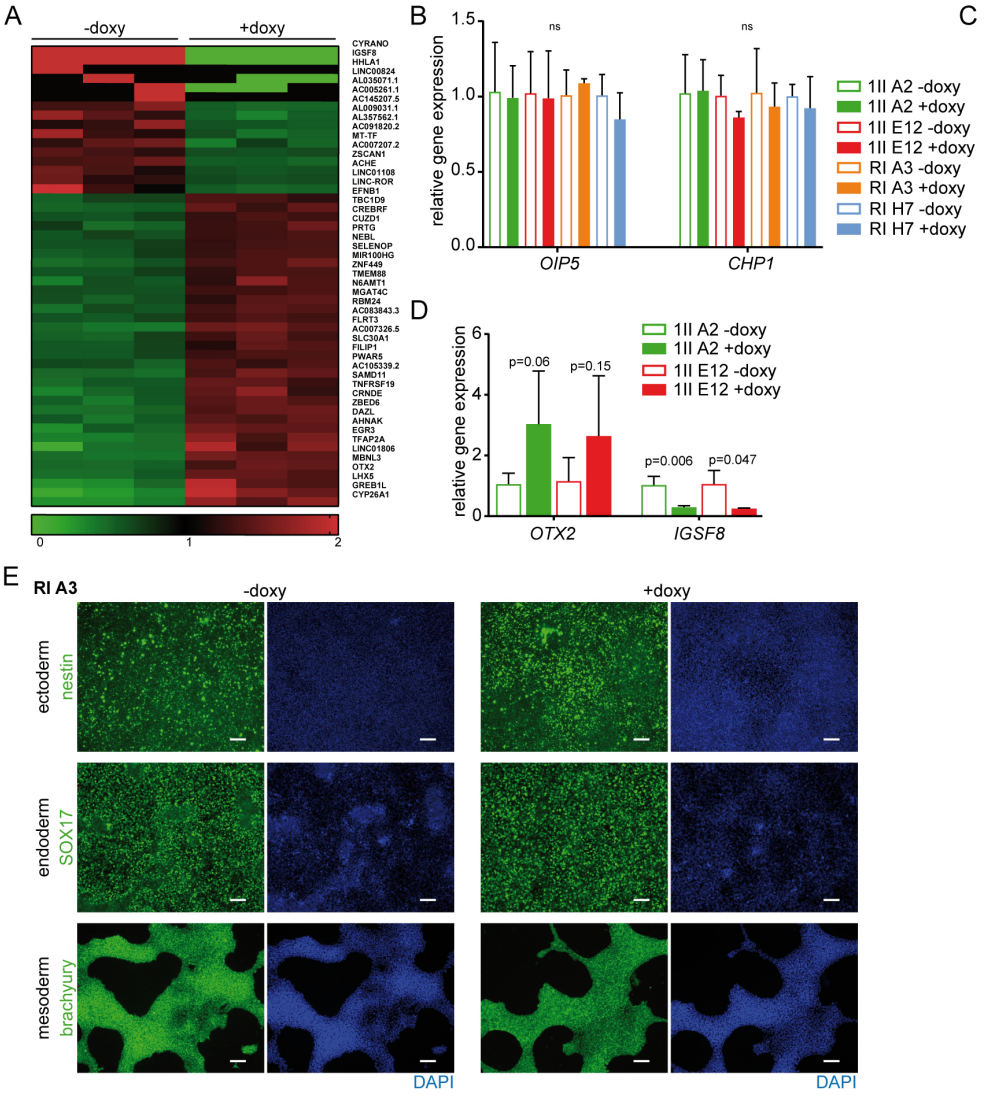


Figure S3 Pluripotency analysis of three further human CRISPRi *Cyrano* iPSCs. Related to Figure 3.

A Expression levels were measured by RT-qPCR, mean ± SD of 3 independent experiments, unpaired *t*-test was performed for statistical analysis. **B** Representative images of alkaline phosphatase staining of KD clones with and without doxycycline treatment. Scale 200 µm. **C** Growth curve of CRISPRi iPSCs knockout clones with and without doxycycline (doxy) treatment. Mean of 3 independent experiments is shown. **D** Flow cytometry analysis of SSEA4 and TRA1-60 of three *Cyrano* KD iPSCs. Isotype controls and unstained cells were used as controls. Representative plots with mean ± SD of 3 independent experiments are depicted. **E** Fluorescence immunocytochemistry of NANOG, OCT4 and SOX2 was performed in *Cyrano* KD cells after doxycycline treatment. Scale 100 µm. **F** Expression levels were measured by RT-qPCR, mean ± SD of 3 independent experiments, unpaired *t*-test was performed for statistical analysis. **G** Representative images of alkaline phosphatase staining of CRISPRi iPSCs after miR-7 overexpression are shown. Scale 500 µm.



C

Geneset (REACTOME database): transcriptional regulation of PSCs			
gene	normalised read counts		log2(FC)
	-doxy	+doxy	
PRDM14	2098	1323	-0.65
SALL4	9145	6275	-0.54
HIF3A	1055	778	-0.43
ZSCAN10	8237	6446	-0.35
STAT3	5163	4079	-0.33
EPAS1	146	114	-0.31
TDGF1	25192	20292	-0.31
FOXD3	512	422	-0.27
TSC22D1	5956	5251	-0.18
NR5A1	18	15	-0.16
EPHA1	5036	4519	-0.15
NR6A1	8395	8253	-0.02
LIN28A	61688	61685	0.00
PBX1	9715	10120	0.06
FGF2	9013	9468	0.07
SMAD2	3387	3640	0.10
ZIC3	10556	11546	0.13
DKK1	13	17	0.19
FOXP1	422	506	0.24
SALL1	2625	3509	0.41
HHEX	92	138	0.47

Figure S4 Silencing of *Cyrano* did not influence the transcriptomics nor differentiation capacity. Related to Figure 3 and 4.

A Top 50 deregulated genes are shown in a heatmap. **B** Expression levels were measured by RT-qPCR, mean ± SD of 3 independent experiments, unpaired *t*-test was performed for statistical analysis. **C** Genes from RNAseq analysis showing up in transcriptional regulation of pluripotent stem cells (PSCs) pathway in REACTOME pathway database after doxycycline (doxy) treatment. **D** Expression levels were measured by RT-qPCR, mean ± SD of 3 independent experiments, unpaired *t*-test was performed for statistical analysis. **E** Fluorescence immunocytochemistry of germ layer marker nestin, SOX17 and brachyury was performed a *Cyrano* KD clone after doxycycline (doxy) treatment and trilineage differentiation. Nuclei were stained with DAPI. Scale 100 µm.

SUPPLEMENTAL TABLES

Table 1 Murine and human primer sequences used in RT-qPCR. Related to Experimental Procedures.

gene	species	forward sequence (5' - 3')	reverse sequence (5' - 3')
Cyrano + quantification	mouse	CTGAGCTGTCCTGCACCTTG	ACACACACCAAGCAATTAGGGC
Cyrano check for KO	mouse	GGGTGTAGTGTTAGTCCGGGT	GTGACGCCTTCGGTAAAGGG
Klf4	mouse	GTGCAGCTTGCAGCAGTAAC	AGCGAGTTGGAAAGGATAAAGTC
Nanog	mouse	CTGGGAACGCCTCATCAATG	AGAAGAATCAGGGCTGCCTT
Sox2	mouse	CAGGAGAACCCCAAGATGCACAA	AATCCGGGTGCTCCTTCATGTG
Tbp	mouse	TGGAATTGTACCGCAGCTTCA	CTGCAGCAAATCGCTTGGGA
CHP1	human	GGGACCGGATCATCAATGCC	TCGCATGAATCCACGGAAGT
Cyrano	human	GCGAAGAGACCACCAAACAG	ACTGGGTAAAAGAAGCAGGAC
Cyrano quantification	human	GGGGAAAAGAGATAAGGCCCA	GACACAAAGACAGCTGGTTTCC
GATA6	human	CCAGGAAACGAAAACCTAAG	CCATCTTGACCCGAATACTT
GUSB	human	GACACCCACCACCTACATCG	CTTAAGTTGGCCCTGGGTCC
IGSF8	human	TACATGCATGCCCTGGACAC	ACCTGGGGAGTAAGGGATCA
ITGA9	human	TGCCTATGATGCCAACGTGT	AAGTCCGATTCCAGCAGCTC
NANOG	human	GGATCCAGCTTGTCCCCAAA	TCTGCTGGAGGCTGAGGTAT
OIP5	human	CTGAGAGGTGCGCTGTGTTC	GAGTGAACCTTCAATGCCAACT
OTX2	human	GCAAATCTCCCTGAGAGCGG	TGGGTTTGGAGCAGTGGAAC
SOX2	human	ATGCACCGCTACGACGTGA	CTTTTGCACCCCTCCCATTT

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Murine iPSC culture and generation

Murine induced pluripotent stem cells have been reprogrammed from OG2 mice expressing EGFP under the control of an Oct3/4 promoter (Kensah et al., 2013). The murine iPSCs were grown in Dulbecco's modified Eagle's Medium knock out (DMEM knock out) (Gibco) supplemented with 15% knockout serum replacement (KSR) (Gibco), 2 mM L-glutamine (Gibco), 1% MEM non-essential amino acids 100x (Gibco), 0.1 mM 2-mercaptoethanol (Gibco) and 1:1000 Leukaemia inhibitory factor (LIF) 1000x (Institute of Technical Chemistry, Leibniz University, Hanover). The iPSCs were grown on irradiated mitotic inactive mouse embryonic fibroblasts (MEF) seeded one day before in 4.5 g/L glucose DMEM (Gibco) supplemented with 10% heat-inactivated FBS (56°C for 30 min) (Gibco) on 0.1% gelatine (Sigma-Aldrich) coated plates.

To knock out the *Cyrano* gene in iPSCs the dual guide RNA (sgRNA) CRISPR/Cas9 approach was used (Heckl et al., 2014). The first exon of *Cyrano* was excised with two sgRNAs knocking out both murine transcripts. The sgRNAs were designed using CCTop - CRISPR/Cas9 target online predictor(Stemmer, Thumberger, Del Sol Keyer, Wittbrodt, & Mateo, 2015). Primers were purchased with 5´ overhang specific for the plasmid. The two sgRNAs, which were upstream, were cloned into pL40C (Addgene plasmid # 89392), sgRNA located downstream of the exon was ligated into pSGL40C (Addgene plasmid # 69147). Oligos were annealed, phosphorylated and ligated into the digested vectors. Competent Stbl3 E. coli were transformed and the construct was checked via sequencing. The two verified vectors were combined to one dual vector pL40C coding for two sgRNAs and Cas9. 500 ng/mL CRISPR/Cas9 dual vector was transfected with Lipofectamine 2000 (Thermo Fisher). Two days after transfection single cells were sorted for EGFP and dTomato double positive cells and seeded as single cells on 96-well with feeder cells at the Research Facility Cell Sorting of the Hannover Medical School. The genomic DNA was isolated with the DNeasy Blood & Tissue Kit (Qiagen) and subsequently used for PCR and sequencing analysis.

Human iPSC culture and generation

The gRNA design, cloning and nucleofection was performed as described previously (Mandegar et al., 2016). Cells were selected with 5 µg/ml blasticidin. After three passages gRNA efficiency was analysed by RT-qPCR after doxycycline (doxycycline hyclate, D9891, Sigma-Aldrich) treatment. The polyclonal population with the most efficient gRNA, analysed by RT-qPCR, was subcloned by serial dilution. Cells were diluted to 0.7 cells/well in mTeSR supplemented with 1x CloneR (STEMCELL Technologies), 100 U/ml Penicillin-Streptomycin (Gibco) or 1 cell/well in mTesR, 100 U/ml Penicillin-Streptomycin. Clones were treated with doxycycline and KD efficiency was analysed by RT-qPCR.

Gene expression

For RNA isolation of murine iPSCs, iPSCs were from the feeder cells by cultivating them in feeder free conditions for two passages prior to RNA isolation.

For DNase treatment RNase-free DNase set (Qiagen) was used. In 20 µl isolated RNA 1x RDD buffer, 10 U RNaseOut (Invitrogen) and 0.3 U DNase I were incubated at 37°C for 30 min and stopped by

adding 1.25 mM EDTA and incubated at 65°C for 5 min. 100 to 1000 ng were reverse transcribed using Biozym cDNA synthesis kit.

Real-time quantitative PCR was performed in CFX384 Touch Real-Time PCR Detection System (Bio-Rad) or QuantStudio 7 Flex System (Applied Biosystems). cDNA was mixed for mRNA and lncRNA expression analysis with iQ SYBR Green Supermix (Bio-Rad), ROX Reference dye and primer of interest. For miRNA expression analysis cDNA was mixed with ABsolute Blue qPCR Mix (ThermoFisher Scientitic), ROX Reference Dye and Taqman probe. The expression levels of human samples were normalized to the endogenous control gene beta-glucuronidase (GUSB), the miRNA expression to small nucleolar RNA C/D box 48 (RNU48) the expression of murine genes to TATA-binding protein (TBP). Primer sequences are listed in Table 1.

Absolute quantification of *Cyrano*

The copy number of *Cyrano* was quantified absolutely by qPCR and a plasmid standard curve as described before (Feretzaki 2019). The plasmid contained approximately 700 bp of the murine or human sequence of the long terminal exon of *Cyrano*, respectively. Briefly, the plasmids were quantified by Qubit dsDNA High Sensitivity Assay Kit (Thermo Fischer Scientific) and 2 to $2x10^8$ molecules were used for the standard curve. The number of *Cyrano* copies in different cell lines was calculated using the standard curve. RNA of different cell lines (murine: iPSC, 3T3, HL-1, SVEC, RAW264.7; human: iPSC, human cardiac fibroblasts (HCF), iPSC-derived cardiomyocytes (iPSC-CM), HUVEC (human umbilical vein endothelial cells), macrophages from peripheral blood mononuclear cells (PBMC)) was digested twice with DNase as described above and subsequently used for reverse transcription. RNA isolation and cDNA synthesis were assumed to be 100% efficient.

Flow cytometry

For flow cytometry cells were separated to single cells with Accutase and 100 000 cells were suspended in FACS buffer (PBS, 1% heat-inactivated FBS, 2.5 mM EDTA). The cells were stained for 30 min at 4°C for SSEA4 (SSEA4 Monoclonal Antibody MC-813-70, Alexa Fluor 488, eBioscience #53-8843-42), TRA 1-60 (TRA-1-60 Monoclonal Antibody, PE, eBioscience #12-8863-82) or isotype controls (mouse IgG3 FITC eBioscience #11-4742-41; mouse IgM PE R&D #IC015P). Subsequently cells were washed with PBS twice and suspended in FACS buffer. Flow cytometry was performed at Guava easyCite 5 flow cytometer (Millipore) and data analysed with FlowJo (BD).

Human ESCs were fixed and permeabilised with FIX&PERM kit 1000 (Nordic MUbio). Staining was performed as described above.

Immunocytochemistry

iPSCs were washed three times with PBS and fixed with 4% formaldehyde for 20 min at room temperature. For staining key pluripotency markers cells were washed with PBS three times and permeabilised with 0.5% Tween-20, 0.1% Triton X-100, 0.1% IGEPAL in TBS for 20 min. Before blocking cells were washed with TBS three times and blocked with 5% donkey serum in TBS-T (0.1% Tween-20 in TBS) for 1 hour. Afterwards washed once with TBS-T and incubated with the primary antibody OCT4 (rabbit-anti-OCT4 C30A3, Cell signalling #2840, 1:400), SOX2 (rabbit-anti-SOX2 D6D9, Cell signalling #3579, 1:400) or NANOG (rabbit-anti-NANOG D73G4, Cell signalling #4903,

1:200) respectively in 1% donkey serum in TBS-T at 4 °C overnight. Cells were washed three times with TBS-T for 5-10 min, and then incubated with secondary antibody (donkey-anti-rabbit Alexa488, Invitrogen #A21206, 1:400) in 1% donkey serum in TBS-T for 1 hour at room temperature. Before staining with DAPI cells were washed three times with TBS-T for 5-10 min, then stained with 5 ng/ml DAPI (Sigma) in TBS for 5 min, afterwards washed twice with TBS.

For staining of germ layer markers cells were washed with PBS three times and permeabilised with 0.2% Tween-20 and 0.1% IGEPAL in TBS for 20 min. Before blocking cells were washed with TBS three times and blocked with 1%BSA and 2% donkey serum in TBS for 1 hour. After washed once with TBS the primary antibody for nestin (R&D MAB1259, 1:62.5), SOX17 (R&D AF1924, 1:400) or brachyury (R&D AF2085, 1:20) was added in 0.25% BSA and 0.5% donkey serum in TBS and incubated overnight at 4 °C. Cells were washed three times with TBS and then incubated with secondary antibody (donkey-anti-mouse Alexa488, Invitrogen #A2120 or donkey-anti-goat Alexa488, Invitrogen #A11055, 1:400) in 0.25% BSA and 0.5% donkey serum in TBS for 1 hour at room temperature. Before staining with DAPI cells were washed three times with TBS, then stained with 5 ng/ml DAPI (Sigma) in TBS for 5 min, afterwards washed twice with TBS.

Images were acquired with microscope (Nikon) and NIS-Elements software (Nikon).

RNA fluorescence in situ hybridisation (FISH)

RNA FISH was performed with ViewRNA Cell plus Assay (ThermoFisher Scientific) according to manufacturer's guidelines in Nunc Lab-Tek 8 well chamber slides (Merck) with a specific *Cyrano* probe (ID VA1-3020478-VCP, ThermoFisher Scientific). To visualise the subcellular localisation of *Cyrano* actin was stained with phalloidin-FITC (Sigma P5282, 1:200). Images were acquired with confocal microscope Leica SP8 and LAS X (Leica) software.

Transfection of siRNA

The downregulation was achieved by transfecting 200 nM siRNAs (eurofins) with Lipofectamine 2000 (Invitrogen) was used according to manufacturer's guidelines. After 6 h medium was changed. Two siRNAs targeting *Cyrano* were used as a mix (CAUGCAGUGCCAUCUGACUUUAU(dTdT) and AGAAGCUGCGAAGAUGGCGGAGUAA(dTdT)) and a scrambled sequence as control (AGGUAGUGUAAUCGCCUUG(dTdT)).

RNA sequencing

Library generation, quality control, and quantification:

500 ng of total RNA per sample were utilized as input for mRNA enrichment procedure with 'NEBNext® Poly(A) mRNA Magnetic Isolation Module' (E7490L; New England Biolabs) followed by stranded cDNA library generation using 'NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina' (E7760L; New England Biolabs). All steps were performed as recommended in user manualE7760 (Version 1.0_02-2017; NEB) except that all reactions were downscaled to 2/3 of initial volumes. Furthermore, one additional purification step was introduced at the end of the standard procedure, using 1x 'Agencourt® AMPure® XP Beads' (#A63881; Beckman Coulter, Inc.).

cDNA libraries were barcoded by dual indexing approach, using 'NEBNext Multiplex Oligos for Illumina – 96 Unique Dual Index Primer Pairs' (6440S; New England Biolabs). All generated cDNA libraries were amplified with 6 cycles of final PCR.

Fragment length distribution of individual libraries was monitored using 'Bioanalyzer High Sensitivity DNA Assay' (5067-4626; Agilent Technologies). Quantification of libraries was performed by use of the 'Qubit® dsDNA HS Assay Kit' (Q32854; ThermoFisher Scientific).

Library denaturation and Sequencing run:

Equal molar amounts of nine individually barcoded libraries were pooled. Accordingly, each analyzed library constitutes 8.3% of overall flowcell capacity. The library pool was denatured with NaOH and was finally diluted to 1.8 pM according to the Denature and Dilute Libraries Guide (Document # 15048776 v02; Illumina). 1.3 ml of denatured pool was loaded on an Illumina NextSeq 550 sequencer using a High Output Flowcell for paired-end reads (20024907; Illumina). Sequencing was performed with the following settings: Sequence reads 1 and 2 with 76 bases each; Index reads 1 and 2 with 8 bases each.

BCL to FASTQ conversion:

BCL files were converted to FASTQ files using bcl2fastq Conversion Software version v2.20.0.422 (Illumina).

Raw data processing and quality control:

Raw data processing was conducted by use of nfcore/rnaseq (version 1.3) which is a bioinformatics best-practice analysis pipeline used for RNA sequencing data at the National Genomics Infrastructure at SciLifeLab Stockholm, Sweden. The pipeline uses Nextflow, a bioinformatics workflow tool. It pre-processes raw data from FastQ inputs, aligns the reads and performs extensive quality-control on the results. The genome reference and annotation data were taken from GENCODE.org (Homo sapiens; GRCh38; release 29).

Normalization and differential expression analysis:

Normalization and differential expression analysis was performed with DESeq2 (Galaxy Tool Version 2.11.40.2) with default settings except for "Output normalized counts table" which was set to "Yes".

SUPPLEMENTAL REFERNCES

Stemmer, M., Thumberger, T., Del Sol Keyer, M., Wittbrodt, J., & Mateo, J. L. (2015). CCTop: An intuitive, flexible and reliable CRISPR/Cas9 target prediction tool. *PLoS ONE*, *10*(4), 1–11. https://doi.org/10.1371/journal.pone.0124633