Stem Cell Reports, Volume *9*

Supplemental Information

Long Noncoding RNA Moderates MicroRNA Activity to Maintain Self-Renewal in Embryonic Stem Cells

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FIGURE S1

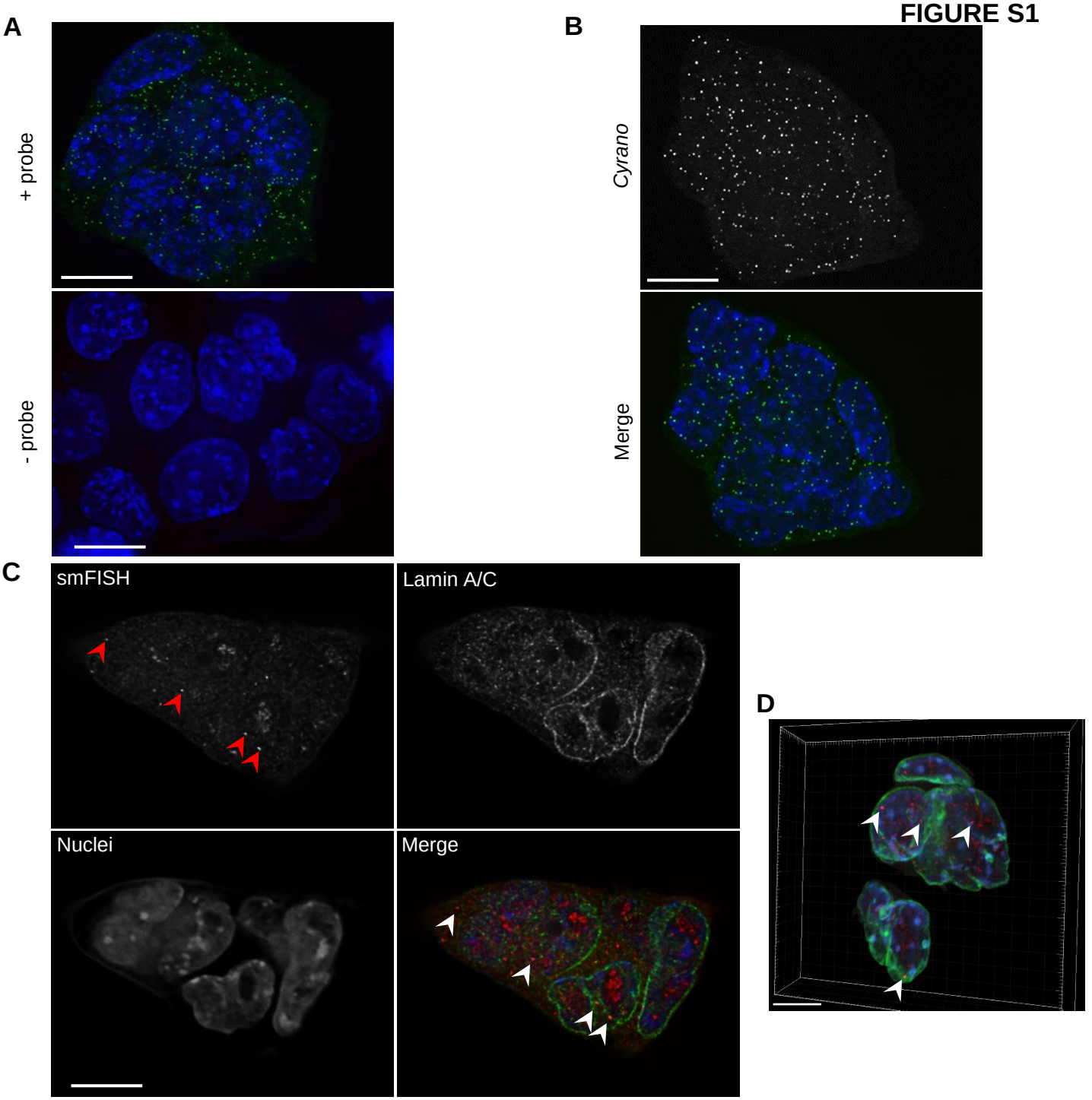
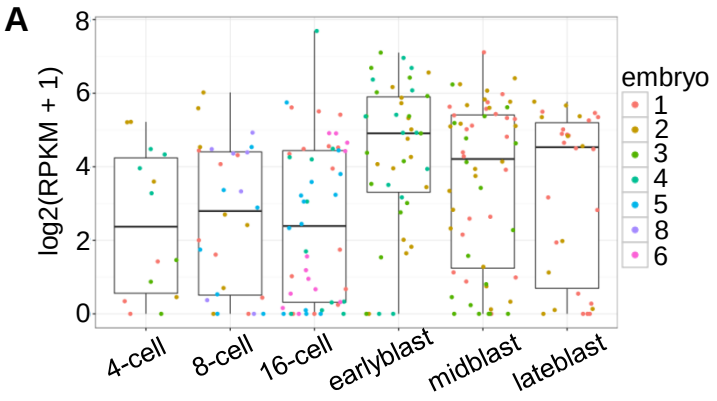
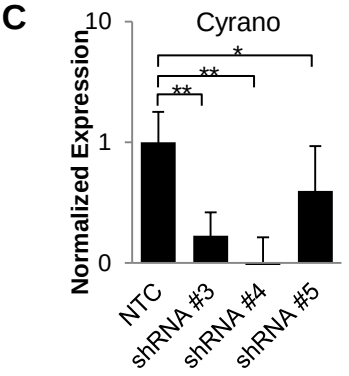
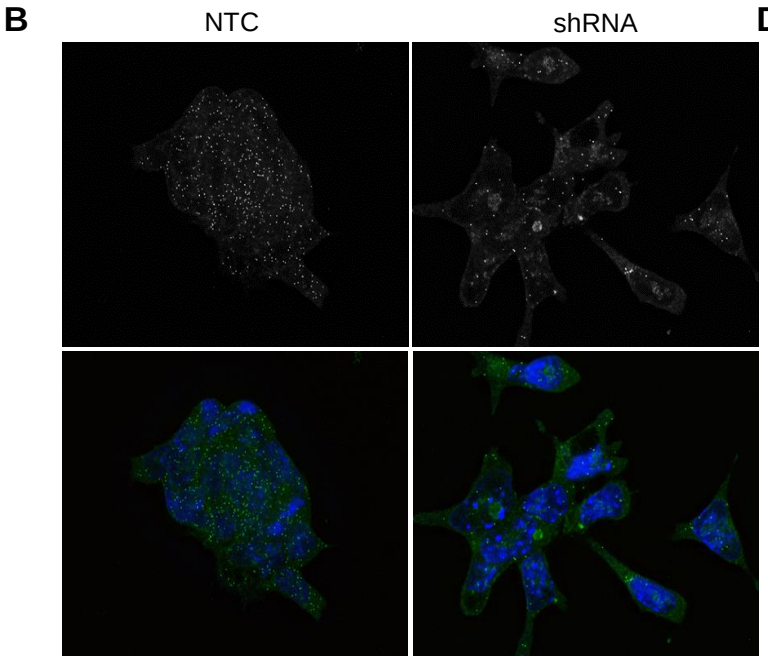
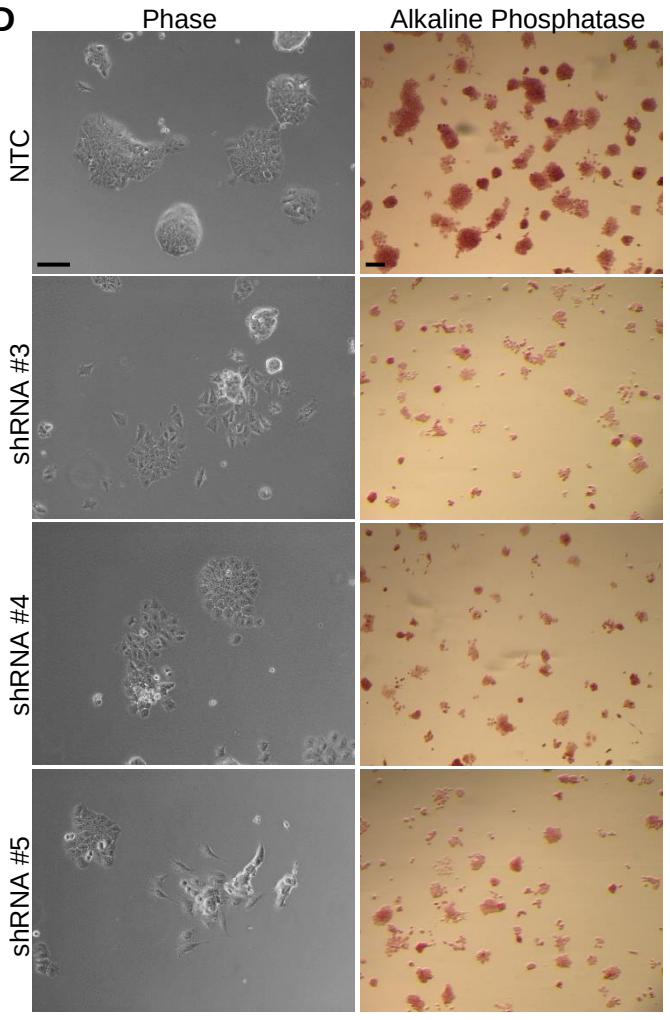

A
+ probe
- probe
B
Cyrano
Merge
C
smFISH
Lamin A/C
Nuclei
Merge
D
E
Human ES cells
Scale
10 kb
hg19
chr15:
41,590,000
OIP5-AS1
Ensembl Genes
H1hSC cel pA- + 2
H1hSC cel pA+ + 2
H1hSC nuc pA- + 2
H1hSC nuc pA+ + 2
H1hSC cyt pA- + 2
H1hSC cyt pA+ + 2

FIGURE S2


A
log2(RPKM + 1)
embryo
1
2
3
4
5
8
6
4-cell
8-cell
16-cell
earlyblast
midblast
lateblast


C
Cyrano
Normalized Expression
NTC
shRNA #3
shRNA #4
shRNA #5


B
NTC
shRNA


D
Phase
Alkaline Phosphatase
NTC
shRNA #3
shRNA #4
shRNA #5

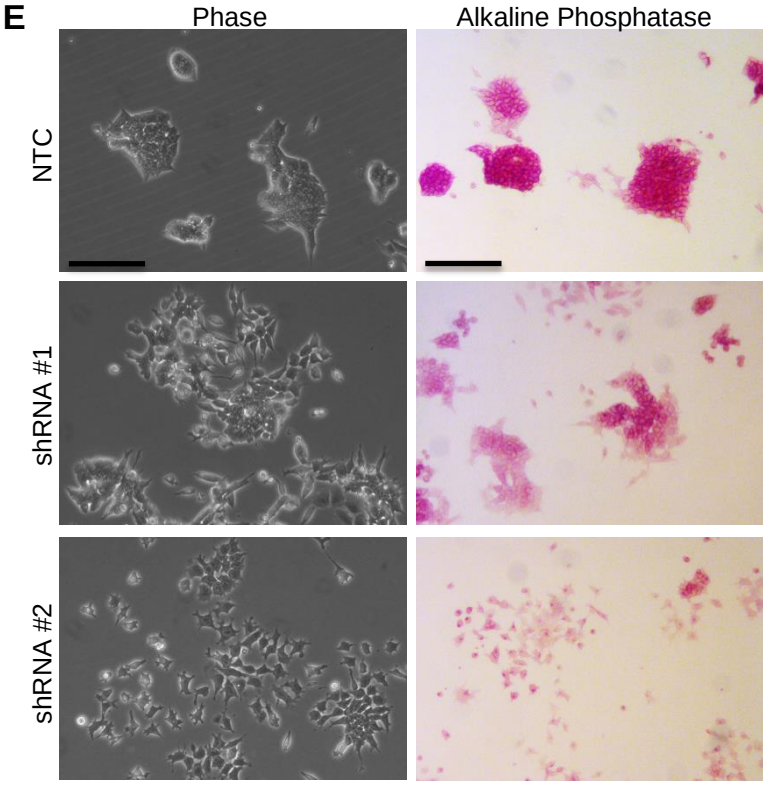
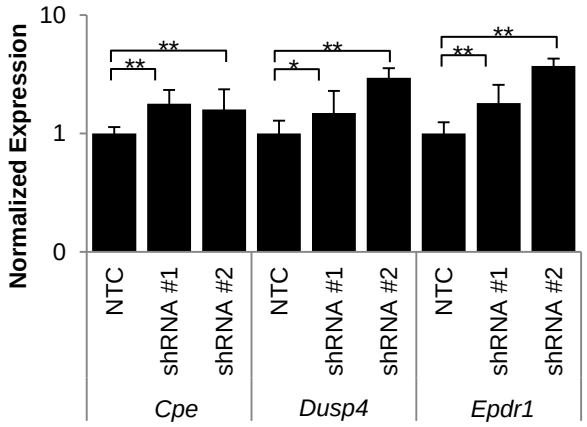

E
Phase
Alkaline Phosphatase
NTC
shRNA #1
shRNA #2

FIGURE S3

A


Normalized Expression
10
1
0
**
**
**
*
**
**
NTC
shRNA #1
shRNA #2
NTC
shRNA #1
shRNA #2
NTC
shRNA #1
shRNA #2
Cpe
Dusp4
Epdr1

B

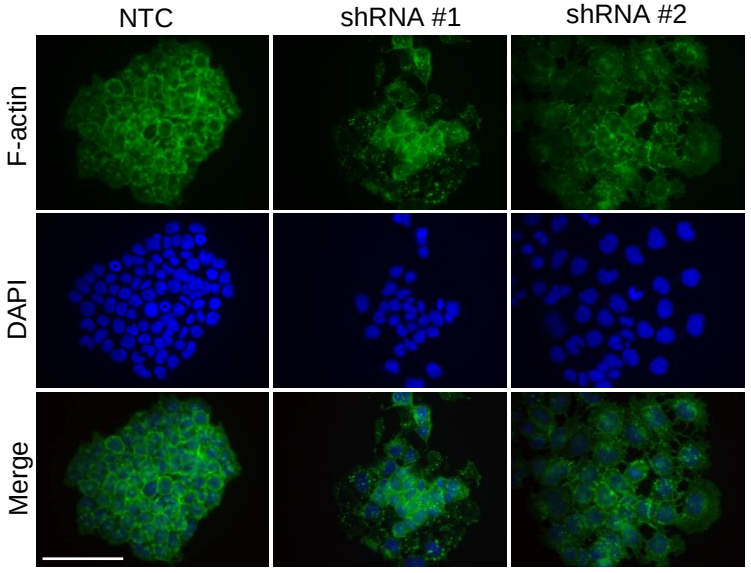

NTC
shRNA #1
shRNA #2
F-actin
DAPI
Merge

FIGURE S4

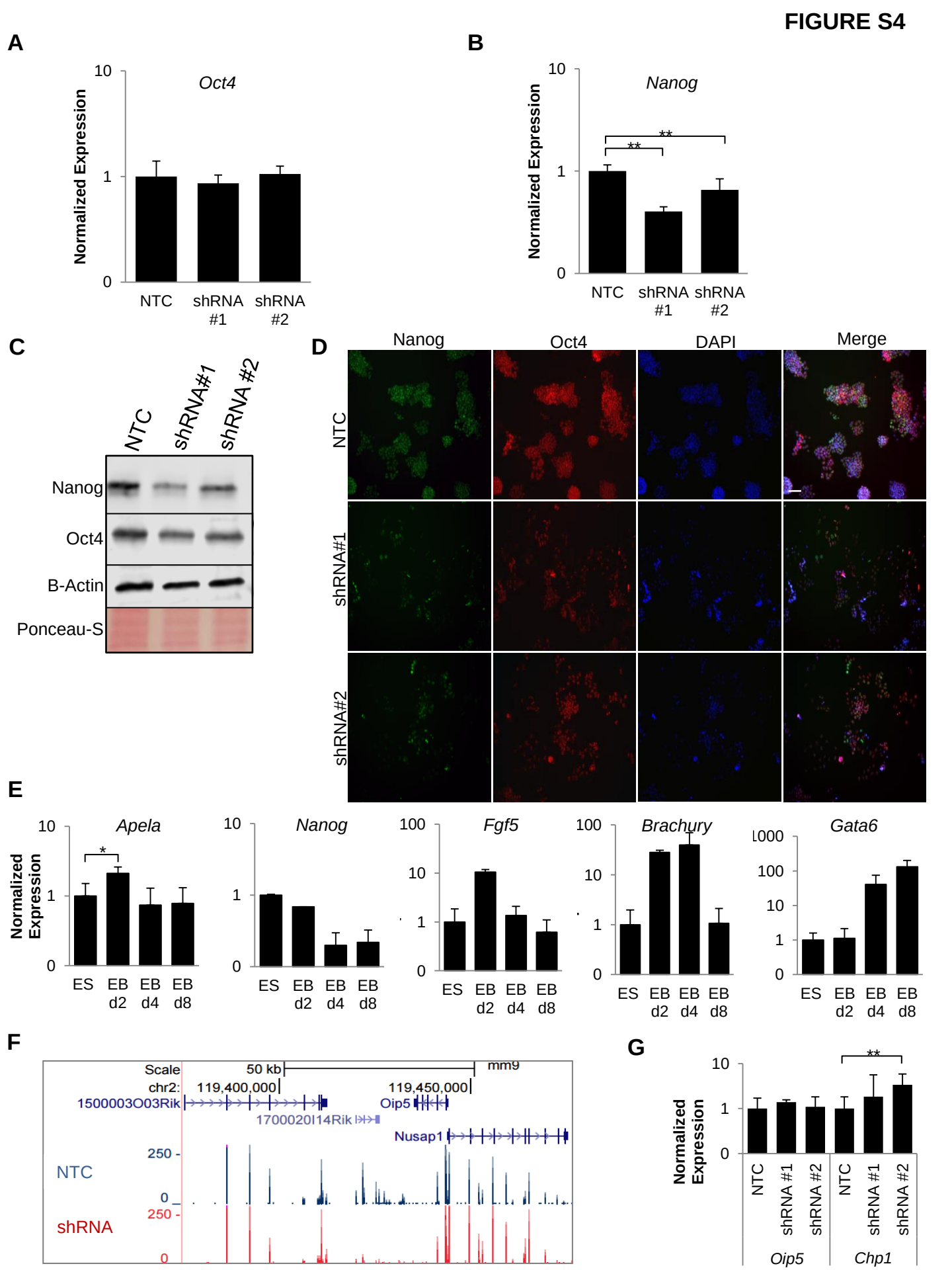
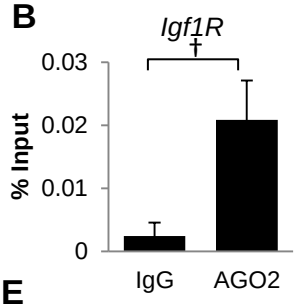

A
Oct4
Normalized Expression
10
1
0
NTC
shRNA #1
shRNA #2
B
Nanog
Normalized Expression
**
**
C
NTC
shRNA#1
shRNA #2
Nanog
Oct4
B-Actin
Ponceau-S
D
Nanog
Oct4
DAPI
Merge
NTC
shRNA#1
shRNA#2
E
Apela
Nanog
Fgf5
Brachury
Gata6
Normalized Expression
ES
EB d2
EB d4
EB d8
F
Scale
50 kb
mm9
chr2:
119,400,000
119,450,000
1500003O03Rik
Oip5
1700020I14Rik
Nusap1
NTC
shRNA
250
0
G
Normalized Expression
**
NTC
shRNA #1
shRNA #2
Oip5
Chp1

FIGURE S5

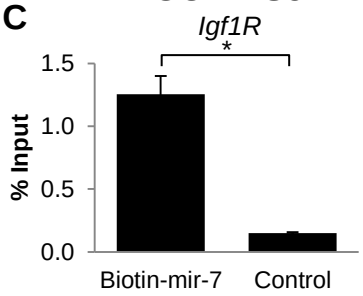
A

Ensembl Transcript ID	Transcript name	Seed length	Start	End
ENSMUST00000153581	***1700020I14Rik-001***	***8***	***2959***	***2952***
ENSMUST00000147425	*1700020I14Rik-002*	8	606	599
ENSMUST00000153581	***1700020I14Rik-001***	***7***	***2958***	***2952***
ENSMUST00000147425	*1700020I14Rik-002*	7	605	599

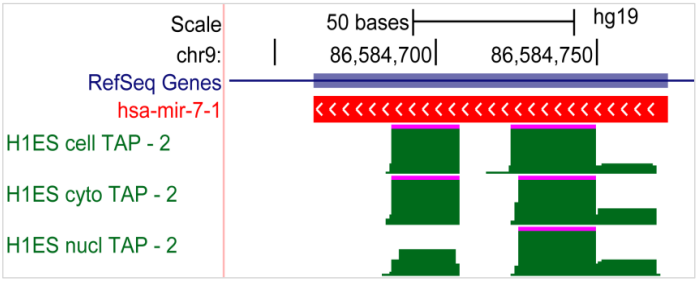
B



C



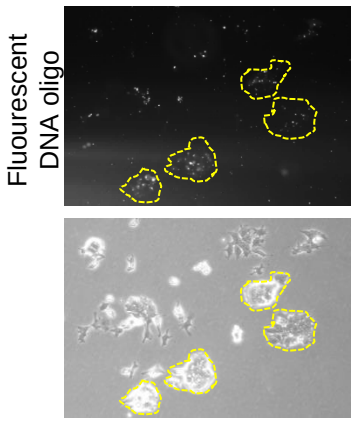
D



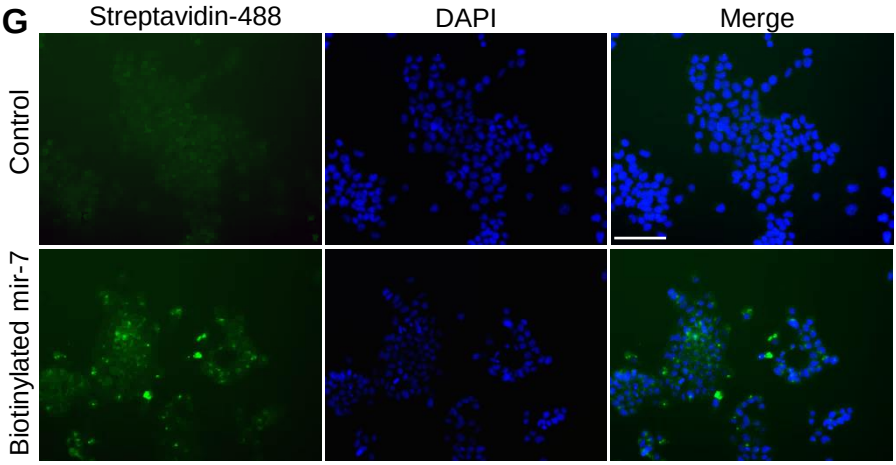
E

Gene	RefSeq ID	Algorithms	Region
Itga9	NM_133721	6/7	3′ UTR
Itga9	NM_001113514	4/7	3′ UTR
Itga9	NM_133721	4/7	CDS
Itga9	NM_001113514	2/7	CDS
Nanog	NM_028016	6/7	3′ UTR
Nanog	NM_028016	3/7	CDS

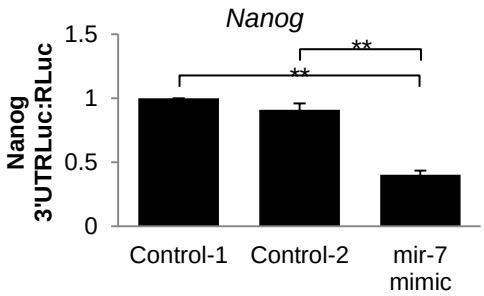
F



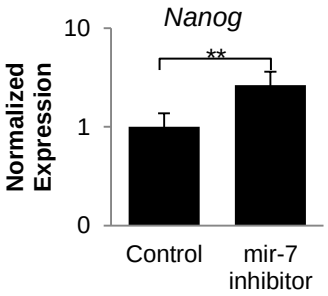
G



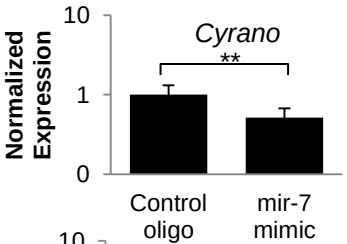
H



I



J



K

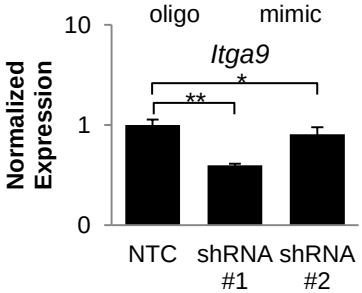


FIGURE S6

A

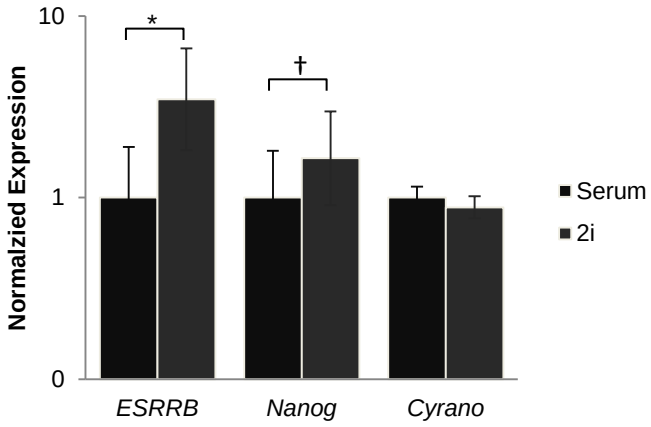


Figure S1, Related to Figure 1. Subcellular localization of *Cyrano*. (A) Specificity of the smFISH probe used to detect *Cyrano* in R1 mouse ES cells. Nuclei are shown in blue (DAPI); Scale bar, 10 μm. (B) Similar subcellular localization of *Cyrano* is observed by smFISH in ES 2-1 mouse ES cells. Nuclei are shown in blue (DAPI); Scale bar, 10 μm. (C) Immunofluorescence at a position of 5.2μm within an ES cell colony, using a lamin A/C antibody to localize the nuclear periphery, coupled with smFISH and DAPI staining confirmed the presence of smFISH signals (red arrowheads for contrast, left upper panel; white arrowheads, right lower panel) in the nucleus as well as the cytoplasm. (D) 3D reconstruction of nuclear volume only, indicates a fraction of *Cyrano* molecules localize to the nucleus (white arrowheads); Scale bar, 3 μm. (E) UCSC genome browser plots illustrating RNA-Seq results which indicate *Oip5-AS1's (Cyrano*'s ortholog) expression and subcellular localization in human ES cells.

Figure S2, Related to Figure 2. Embryonic Expression and Independent Validation of *Cyrano* knockdown and phenotype. (A) Single cell RNA-Seq analysis (GSE45719) of *Cyrano*, in pre-implantation development. (B) Significant reduction in *Cyrano* expression is confirmed with smFISH. Independent knockdown using three additional shRNAs (C). Experiments were performed in triplicate, normalized to *GAPDH*, with error bars representing 95% CI. This confirms reduction in the capacity to maintain self-

renewing ES cell colonies upon *Cyrano* loss (D). (E) Independent validation of the *Cyrano*-deficient phenotype in the ES 2-1 ES cell line.

Figure S3, Related to Figure 3. Gene expression analysis and differential colony structure upon *Cyrano* knockdown. (A) qRT-PCR validation of the differentially expressed non-pluripotency related genes *Cpe*, *Dusp4* and *Epdr1* (approximate positions circled in Fig. 3A) after *Cyrano* knockdown. Experiments were performed in triplicate, normalized to *GAPDH*, with error bars representing 95% CI. *p<0.05, **p<0.01. (B) Immunofluorescence examination of phalloidin staining to examine F-actin localization in control and knockdown cells. Nuclei, blue (DAPI); Scale bar, 100 μm. Data are from 3 independent experiments.

Figure S4, Related to Figure 4. Aberrant gene expression suggests *Cyrano* functions in trans to impact ES cell self-renewal. qRT-PCR monitoring of *Oct3/4* (A) and *Nanog* (B) levels upon *Cyrano* knockdown. Experiments were performed in triplicate, normalized to *GAPDH*, with error bars representing 95% CI. (C-D) Significant decreases in NANOG protein levels occur with *Cyrano* KD. (E) Assessment of *Apela* expression in embryoid body differentiation relative to marker gene expression. Experiments were performed in triplicate, normalized to *GAPDH*, with error bars

representing 95% CI. (F-G) No significant and consistent changes in expression are seen for neighboring genes (*Oip5* and *Chp1/1500003O03Rik)* of *Cyrano*. Experiments were performed in triplicate, normalized to *GAPDH*, with error bars representing 95% CI. *p<0.05, **p<0.01. Data are from 3 independent experiments.

Figure S5, Related to Figure 5. Regulatory loop involving *Cyrano* and *mir-7.* (A) Positions of seed sequences in *Cyrano* as identified by miRWalk (the position corresponding to the conserved atypical mir-7 binding sequence is bold). See Table S1 for further details. *Note, despite not being called by miRWalk, the additional *mir-7* seed can also be found in the *1700020I14Rik*-001 isoform (approximate nucleotide position, 768; blue box in Figure 5A). (B) RNA-immunoprecipitation showing the *mir-7* target *Igf1R* being bound by Ago2 in ESCs. Experiments were performed in triplicate with error bars representing S.E.M. (C) miRNA pulldown and qPCR indicates a physical interaction between *Igf1R* and *mir*-7 in ESCs, with experiments performed in triplicate and error bars representing S.E.M. (D) UCSC genome browser plot showing subcellular localization of *mir-7,* similar to *Cyrano* (see Figure S1E). (E) Number of algorithms as assessed by miRWalk which identify mir-7 seed sequences in *Itga9* and *Nanog.* Transfections with a fluorescent DNA oligo (F) and biotinylated *mir-7* (G) were used to elucidate the success of *mir-7* transfection in ES cells. (H) Despite having only a

7mer-A1 seed sequence, *mir-7* inhibited luciferase activity based on the *Nanog* 3'UTR. Experiments were performed in triplicate, error bars represent S.E.M. (I) *mir-7* inhibition increases *Nanog* levels. (J) *mir-7* overexpression reduces *Cyrano* levels. (K) *Itga9* levels decrease with *Cyrano* knockdown. Experiments were performed in triplicate, normalized to *GAPDH*, with error bars representing 95% CI. Data are from 3 independent experiments. *$p<0.05$, **$p<0.01$, $^{\dagger}p<0.1$.

Figure S6, Related to Figure 6. Examination of *Cyrano* in 2i+LIF medium. (A) No significant changes are seen in *Cyrano* levels in 2i medium relative to serum containing medium. Experiments were performed in triplicate, normalized to *GAPDH*, with error bars representing 95% CI. Data are from 3 independent experiments. *$p<0.05$, $^{\dagger}p<0.1$.

Table S1, Related to Figure 5. Table S1 provides a summary of lncRNA targets of *mir-7.* SPMS: Starting Position of a miRNA seed; SL: Seed length; SeedS: Seed start; SeedE: Seed end.

Table S2, Related to Figure 5. Table S2 provides a summary of miRNA site predictions in the 3'UTR and CDS of Itga9 and Nanog as provided by miRWalk which includes

assessments using the following algorithms: miRWalk, Microt4, miRanda, PITA, RNA22, RNAhybrid and Targetscan. The 'SUM' column indicates how many algorithms were able to identify the miRNA target site.

Table S3, Oligos used in this study

Supplemental Experimental Procedures

Cell Culture and RNAi

Mouse R1 (XY, Nagy et al., 1993) ESCs were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 10% Knockout Serum Replacement, 2mM L-glutamate, 1mM sodium pyruvate, 0.1mM β-mercaptoethanol, and 100U/ml penicillin-streptomycin and LIF on gelatin-coated dishes. Mouse ES2-1 cells (XX, Royce-Tolland et al., 2010) were maintained in Dulbecco's Modified Eagle Medium supplemented with 15% FBS, 2mM L-glutamate, 1mM sodium pyruvate, 0.1mM β-mercaptoethanol, 1x MEM non-essential amino acids,100U/ml penicillin-streptomycin and LIF on feeders or gelatin-coated dishes. Embryoid body differentiation was carried out on low adherent dishes under LIF withdrawal conditions.

Apoptosis was monitored using the ReadyProbes Cell Viability Imaging Kit two days post-transfection (ThermoFisher).

Cyrano cDNA was obtained from the RIKEN cRNA collection (Source BioScience, clone ID M5C1004K09), transferred to pCAGEN or pCAGIG (Gift from Connie Cepko, Addgene plasmid #11159) and transfected into ESCs using Lipofectamine LTX reagent (ThermoFisher). Similarly, shRNA constructs in pSicoR-Ef1a-mCh-Puro (Gift from Bruce Conklin, Addgene plasmid # 31845) (Salomonis et al., 2010), pSicoR PGK puro (Gift from Tyler Jacks, Addgene plasmid # 12084) (Ventura et al., 2004) or SmartVector (ThermoFisher) targeting *Cyrano* and non-targeting control were transfected into ESCs using Lipofectamine LTX reagent (ThermoFisher), followed by selection/enrichment upon passaging at d1 post-transfection using puromycin selection or flow cytometry at the UNC Flow Cytometry Core Facility to enrich for transfectants. Alternatively, lentiviral particles at a MOI of 50-100 were used. Cell death assays were carried out without selection at d2 post-transfection. Gene expression assays were carried out upon selection on d2 and d3 post-transfection and phenotypic assays including morphological assessments and alkaline phosphatase activity determination were carried out on d3 post-transfection.

miR-7 overexpression was carried out using the Lipofectamine RNAiMax reagent and the mmu-miR-7a-5p miRIDIAN microRNA mimic (Dharmacon) compared to control

oligos, (Dharmacon, ThermoFisher), while inhibition was carried out with a mmu-miR-7a-5p inhibitor (Dharmacon).

RNA Extraction and Quantitative RT-PCR

To investigate RNA stability, actinomycin D (10µg/ml) was added to cell culture medium and cells incubated for the stated periods.

To prepare nuclear and cytoplasmic fractions, ESCs were incubated in hypotonic buffer (10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA), 1mM DTT and protease inhibitors. NP-40 (1.6%) was added and the sample vortexed briefly and centrifuged to pellet nuclei, which permitted removal of the cytoplasmic supernatant. Nuclear proteins were extracted from the pellet with 20mM HEPES, 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT and protease inhibitors.

smFISH

A pool of FISH probes, comprising 36 20-mer probes that show complementarity to *Cyrano* were designed with the Stellaris Probe designer. Hybridization was carried out according to the manufacturer's recommendations (Biosearch Technologies). Briefly, cells were cultured on coverslips for approximately 24-30h before colonies became

densely structured to enable visualization of individual cells. Cells were fixed with 4% PFA in PBS and permeabilized with 70% ethanol for 1 hr at 4°C. After incubation in wash buffer (10% formamide, 2xSSC), cells were hybridized in 100mg/ml dextran sulphate, 1% formamide and 2xSSC overnight at 37°C. Slides were subsequently submerged in wash buffer twice for 30 min at 37°C, followed by a brief incubation in 2xSSC.

To enable visualization of the nuclear periphery, immunofluorescence to detect lamin A/C localization was carried out prior to FISH described above. After fixing with 4% PFA in PBS and permeabilizing with 0.5% Triton-X100/PBS for 10 min at room temperature, primary antibody incubations were carried out for 1hr at room temperature followed by washes and incubation with fluorescent-conjugated Alexa Fluor secondary antibodies for 1h at room temperature (ThermoFisher). After washes, a post-fix step was carried out for 10 min at room temperature using 4% PFA in PBS prior to washing with wash buffer in preparation for FISH. Coverslips were mounted with Prolong Gold Antifade Mountant containing DAPI (ThermoFisher) for nuclear visualization. Z-stack images were acquired by AxioVision software, using a 63x or 100x objective on a Zeiss Axio Imager 2, and deconvolution carried out using an iterative-constrained algorithm. Imaris (Bitplane) was used for 3D reconstruction and surface reconstruction of the nucleus to

enable specific identification of the nuclear volume (Hooker Imaging Core, University of North Carolina at Chapel Hill).

Quantitation of smFISH signals was carried out using the StarSearch software (Raj Laboratory, University of Pennsylvania). The average count per cell was derived by the total number of FISH signals/colony relative to the total number of cells as determined by non-overlapping DAPI stained nuclei.

Transcriptomics

Libraries for RNA-Seq were prepared using 30 µg total RNA with a modified dUTP Strand Specific method (Zhong et al., 2011) from cells three days post-knockdown with shRNA#1. Poly A+ selection was carried out using Dynabeads (ThermoFisher), and RNA fragmentation carried out with the Ambion Fragmentation reagent (ThermoFisher) for 4 min at 70 ºC. Fragmented RNA was purified using the RNA Clean and Concentrator Kit (Zymo), and used for first strand synthesis with Superscript III (ThermoFisher) and random primers (NEB). After purification with the RNA Clean and Concentrator Kit (Zymo), second strand synthesis with dUTP was carried out for 2h at 16 ºC. End repair (NEBNext Kit), purification with 1.8 volumes of Ampure beads (Beckman-Coulter), followed by A-tailing with Klenow exo- and purification with 1.8 volumes of Ampure beads, followed by standard Illumina Library preparation. Here,

adapters were ligated using the Quick Ligation Module (NEBNext Kit). Libraries were purified twice with Ampure beads (1 volume, followed by 0.8 volumes), followed by amplification, uracil-DNA glycosylase treatment and quantitation for submission at the University of North Carolina at Chapel Hill High Throughput Sequencing Facility. qPCR validation was carried out on independent samples with both shRNA #1 and shRNA #2.

Western blot Analysis & Immunofluorescence

Antibodies were used for lamin A/C (E-1, Santa Cruz), Oct4 (H10, Santa Cruz), Nanog (A300-397A, Bethyl Laboratories; 8822, Cell Signaling Technologies) and e-Cadherin (13-1900, Zymed). Cell extracts for immunoblot analysis were prepared using a modified RIPA buffer (50mM Tris pH 8.0, 150mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, dithothreitol) containing protease inhibitors (Roche), electrophoresed and blotted onto a nitrocellulose/PVDF membrane (BioRad) before incubation in the appropriate primary antibody. After incubation with HRP-conjugated secondary antibody (Santa Cruz), membranes were developed with SuperSignal West Dura Chemiluminescent substrate (Pierce).

In immunofluorescence experiments, cultured cells were fixed with 4% PFA in PBS and permeabilized with 0.3% Triton-X100/PBS for 5 min at room temperature. After blocking

for 1 hr, primary antibody incubations were carried out for 1hr at room temperature or overnight at 4°C, followed by washes and incubation in fluorescent-conjugated Alexa Fluor secondary antibodies (ThermoFisher). DAPI (ThermoFisher) was used for nuclear visualization.

Supplemental References

Murakami, K., Araki, K., Ohtsuka, S., Wakayama, T., and Niwa, H. (2011). Choice of random rather than imprinted X inactivation in female embryonic stem cell-derived extra-embryonic cells. Development *138,* 197-202.

Rosengren, L., Vasilcanu, D., Vasilcanu, R., Fickenscher, S., Sehat, B., Natalishvili, N., Naughton, S., Yin, S., Girnita, A., Girnita, L., *et al.* (2006). IGF-1R tyrosine kinase expression and dependency in clones of IGF-1R knockout cells (R-). Biochem. Biophys. Res. Commun. *347*, 1059-1066.

Salomonis, N., Schlieve, C.R., Pereira, L., Wahlquist, C., Colas, A., Zambon, A.C., Vranizan, K., Spindler, M.J., Pico, A.R., Cline, M.S.*, et al.* (2010). Alternative splicing regulates mouse embryonic stem cell pluripotency and differentiation. Proc. Natl. Acad. Sci. U. S. A. *107,* 10514-10519.

Ventura, A., Meissner, A., Dillon, C.P., McManus, M., Sharp, P.A., Van Parijs, L., Jaenisch, R., and Jacks, T. (2004). Cre-lox-regulated conditional RNA interference from transgenes. Proc. Natl. Acad. Sci. U. S. A. *101,* 10380-10385.