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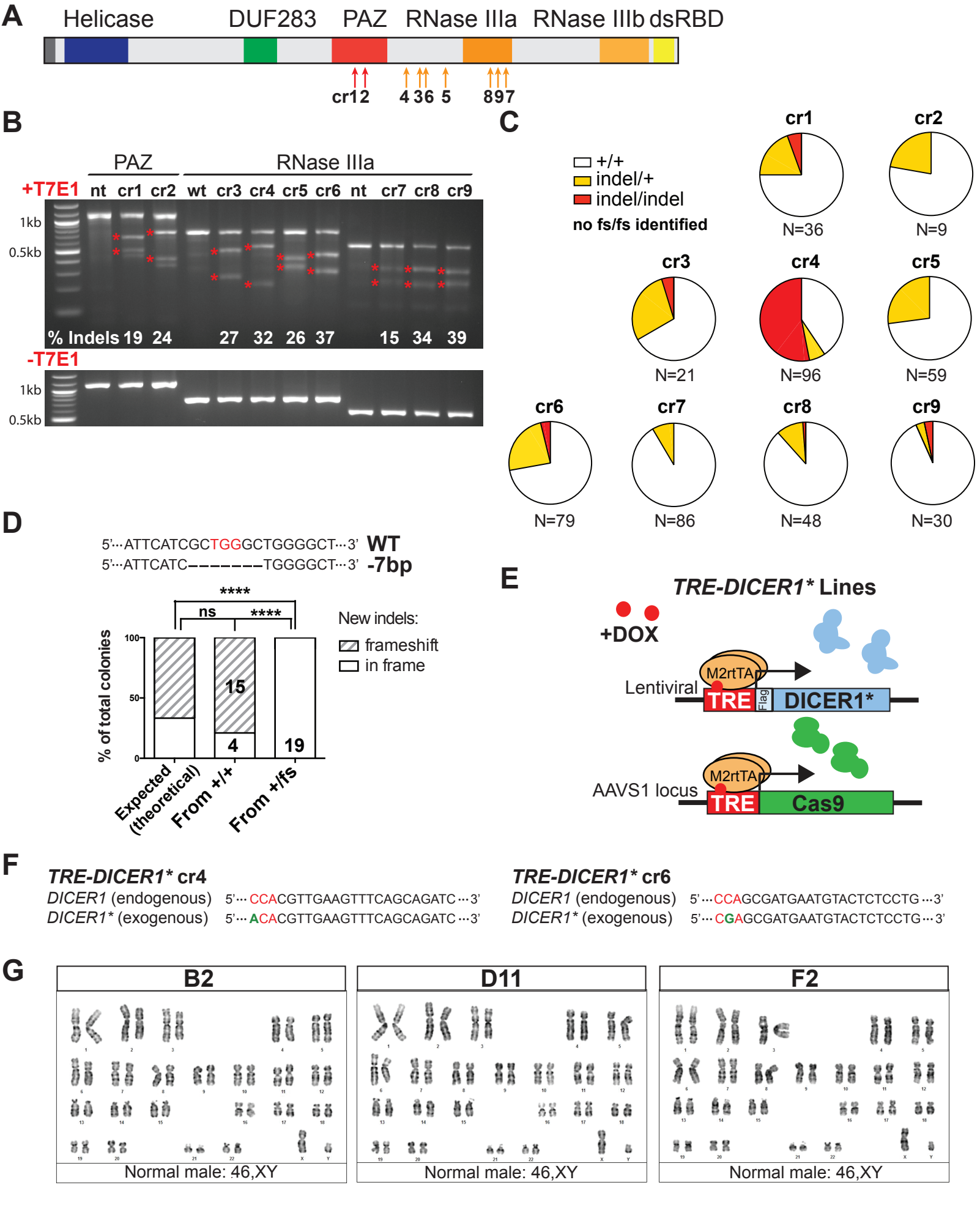
Supplemental Information

DICER1 Is Essential for Self-Renewal of Human Embryonic Stem Cells

Virginia Teijeiro, Dapeng Yang, Sonali Majumdar, Federico González, Robert W. Rickert, Chunlong Xu, Richard Koche, Nipun Verma, Eric C. Lai, and Danwei Huangfu

Supplemental Figure 1. Related to Figure 1.



Supplemental Figure 1. Generation of *DICER1* Knockout hESC lines, Related to Figure 1.

(A) Schematic representation of DICER1 protein with domains. Red and orange arrows represent gRNA-targeting loci. Red arrows represent gRNAs targeting the PAZ domain, and orange arrows represent gRNAs targeting the RNase IIIa domain. cr, CRISPR; DUF, Domain of Unknown Function; dsRBD, double-stranded RNA-Binding Domain.

(B) T7E1 assay in HUES8 iCas9 cells transfected with nine different gRNAs targeting the functional domains of PAZ and RNase IIIa in feeder-based condition. nt, non-transfected control; cr, CRISPR; T7E1, T7 endonuclease 1.

(C) CRISPR/Cas9 targeting results of HUES8 iCas9 lines in feeder-based condition. White, wildtype lines; Yellow, monoallelic mutant lines; Red, biallelic mutant lines. fs, frameshift allele. N indicates the number of clones analyzed.

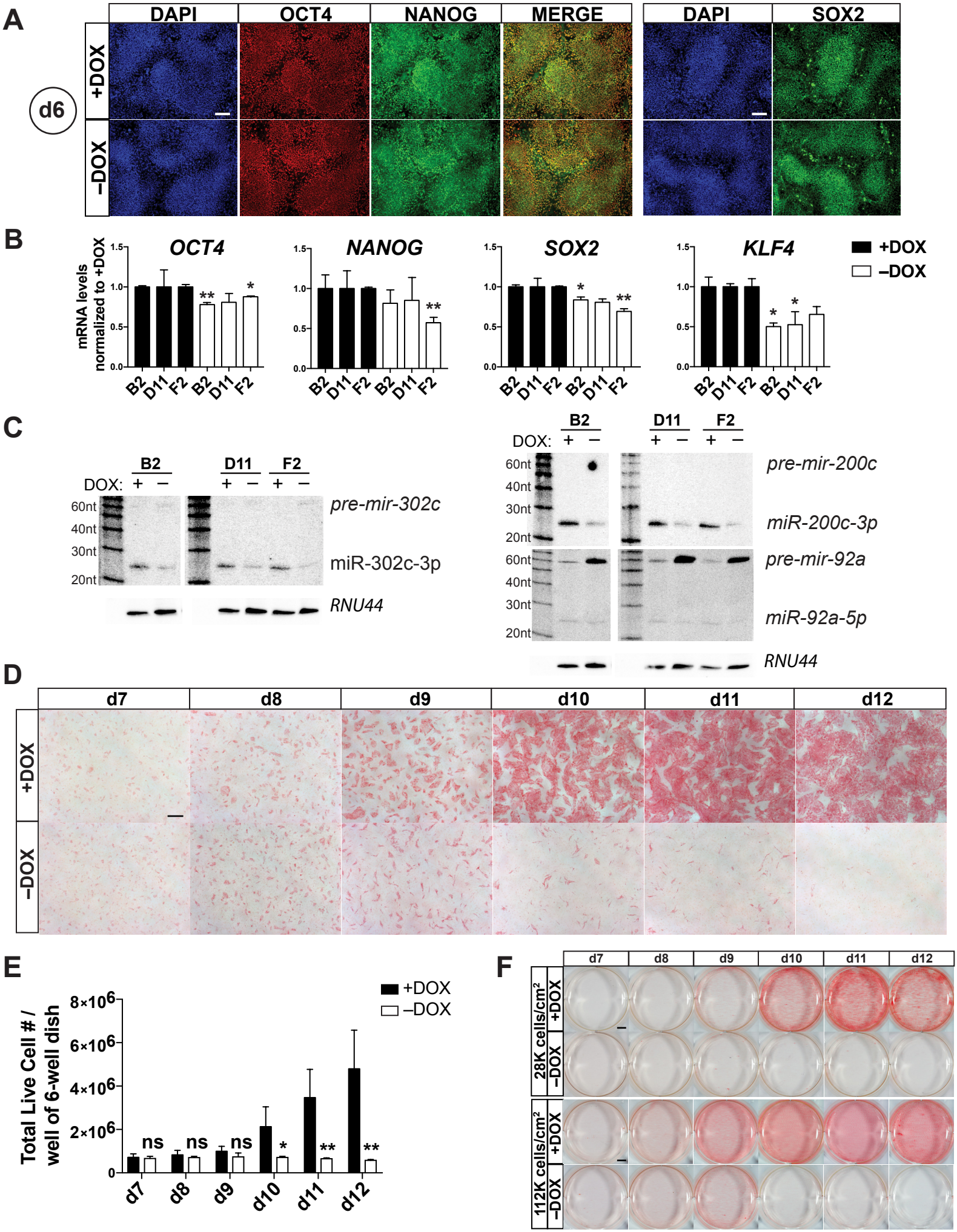
(D) Re-targeting of a *DICER1* heterozygous line generated using cr6 gRNA in feeder-based condition. This line has a 7 bp deletion spanning the NGG PAM sequence (in red) and a wildtype allele. cr6 gRNA was used to target the wildtype allele of this *DICER1* heterozygous line. Results are shown comparing frameshift versus in-frame mutations generated experimentally when targeting a wildtype line versus a heterozygous line, and compared to the theoretical expectation. Chi-test was used for statistical analysis.

(E) Diagram showing the conditional *DICER1* hESC lines expressing DICER1* and Cas9 upon doxycycline treatment.

(F) Sequences of the endogenous *DICER1* and the cr4 and cr6 gRNA-immune *DICER1** transgenes. NGG sequence is in red. Silent mutation base pair change is in green.

(G) Karyotype results of conditional *DICER1* knockout lines (B2 from cr4; D11 and F2 from cr6).

Supplemental Figure 2. Related to Figure 2.



Supplemental Figure 2. *DICER1*-depleted hESCs Display Self-Renewing Defects, Related to Figure 2.

(A) Immunofluorescence staining of pluripotency markers (OCT4, NANOG, SOX2) in conditional B2 *DICER1* knockout hESCs in feeder-based condition with and without doxycycline treatment for 6 days.

(B) qRT-PCR analysis of pluripotency marker expression (*OCT4*, *NANOG*, *SOX2*, and *KLF4*) in three conditional *DICER1* knockout lines in feeder-based condition with and without doxycycline treatment for 6 days. Levels are relative to *GAPDH*. Statistical analysis was performed comparing to +DOX samples. n=3 independent experiments.

(C) Northern blots of *miR-302c-3p*, *miR-200c-3p*, *miR-92a-3p* in conditional *DICER1* knockout hESC lines in feeder-based condition and maintained with or without doxycycline for 6 days. *RNU44* was used as a loading control. Pre-mir, precursor microRNA; miR, mature microRNA.

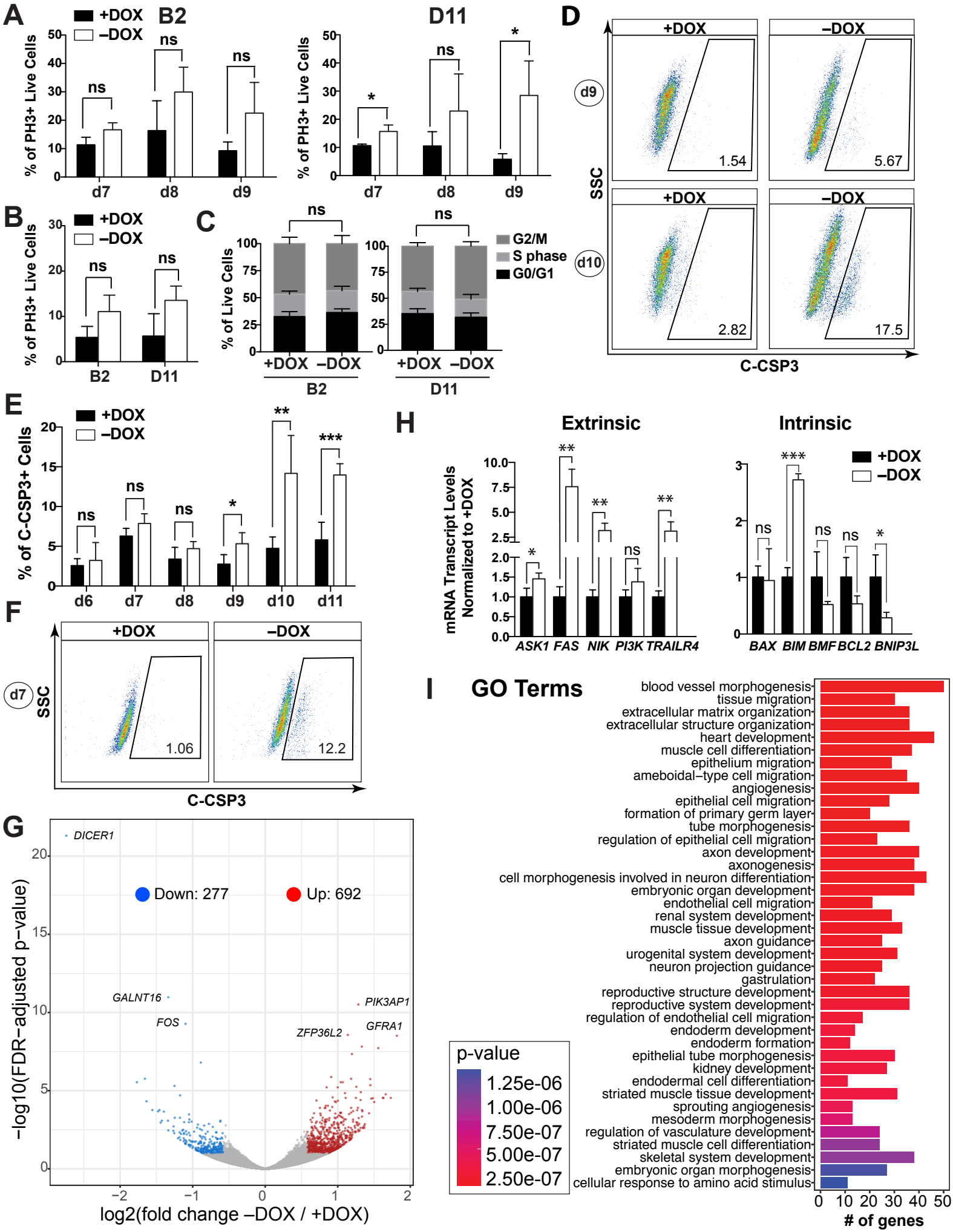
(D) Representative alkaline phosphatase (AP) staining of the B2 *DICER1* knockout line from days 7 to 12 treated with and without doxycycline in feeder-based condition. Scale bar represents 1 mm. n=3 independent experiments.

(E) Cell number quantification of the B2 conditional *DICER1* knockout line after replating between days 7 and 12 of doxycycline withdrawal in feeder-based condition. For each time point, statistical analysis was performed comparing to +DOX samples. n=5 for d7-9, n=4 for d10, and n=3 for d11-12. All from independent experiments.

(F) Representative whole-well view of alkaline phosphatase stained B2 conditional *DICER1* knockout after being replated at different densities (28,000 and 112,000 cells/cm^2) from days 7 to 12 with or without doxycycline treatment in feeder-based condition. 28,000 cells/cm^2 is the density at which we passage our lines regularly. Scale bar represents 5 mm.

Error bars indicate SD, and significance is indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; ****$p < 0.0001$; ns, not significant ($p \geq 0.05$).

Supplemental Figure 3. Related to Figure 3.



Supplemental Figure 3. *DICER1*-depletion in hESCs Causes Apoptosis instead of Proliferation Defects, Related to Figure 3.

(A) Flow cytometry of phospho histone-3 (PH3) from days 7 through 9 in the B2 and D11 conditional *DICER1* knockout lines with and without doxycycline in feeder-based condition. n=3 independent experiments.

(B) Flow cytometry of PH3 from day 7 in the B2 and D11 conditional *DICER1* knockout lines maintained with and without doxycycline in feeder-free condition. n=3 independent experiments.

(C) Cell cycle analysis by propidium iodide staining on day 7 in B2 and D11 conditional *DICER1* knockout lines maintained with or without doxycycline in feeder-free condition. n=4 independent experiments.

(D) Representative flow plots of the D11 conditional *DICER1* knockout line maintained with and without doxycycline at days 9 and 10 in feeder-based condition. n=3 independent experiments.

(E) Flow cytometry of C-CSP3 from days 6 through 11 in the B2 conditional *DICER1* knockout line maintained with and without doxycycline in feeder-based condition. n=3 independent experiments.

(F) Representative flow plots of the D11 conditional *DICER1* knockout line maintained with and without doxycycline at day 7 in feeder-free condition. n=4 independent experiments.

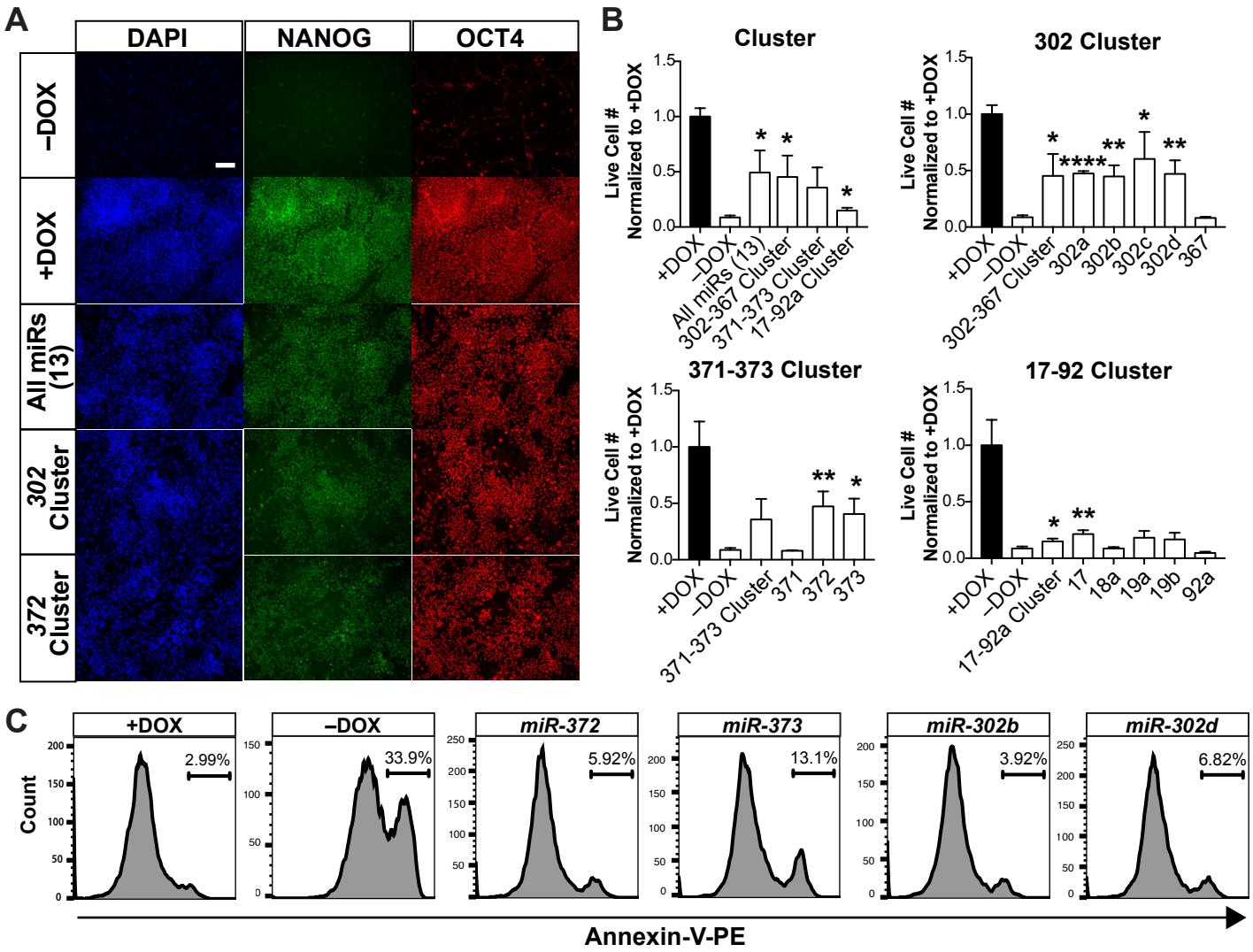
(G) Volcano plot of RNAseq data from B2 *DICER1* knockout line maintained with and without doxycycline at day 9 in feeder conditions. In blue, downregulated genes in -DOX condition (n=277). In red, upregulated genes in -DOX condition (n=692). Up or downregulated genes were decided by logFC > 1.5 and adjusted p-value < 0.1. The top three up or downregulated genes are labeled. n=3 independent experiments.

(H) qRT-PCR analysis of representative pro-apoptotic genes belonging to either the extrinsic (*ASK1*, *FAS*, *NIK*, *PI3K*, *TRAILR4*) or intrinsic pathways (*BAX, BIM, BMF, BCL2, BNIP3L*) in the B2 line maintained in feeder-free condition with and without doxycycline treatment for 8 days. n=3 independent experiments.

(I) Gene ontology analysis was performed on 692 upregulated genes and 277 downregulated genes in B2 -DOX vs. +DOX samples using the clusterProfiler (v3.6.0) package. Benjamini-Hochberg adjusted q value of less than 0.05 was set as the cutoff, and the top 40 terms were plotted. n=3 independent experiments.

Error bars indicate SD, and significance is indicated as *p < 0.05, **p < 0.01, and ***p < 0.001; ****p< 0.0001; ns, not significant (p ≥ 0.05).

Supplemental Figure 4. Related to Figure 4.



Supplemental Figure 4. Members of the *mir-371-373* and *mir-302-367* Clusters Partially Reverse the Apoptosis Defects in *DICER1*-deficient hESCs, Related to Figure 4.

(A) Representative immunofluorescence staining pictures of pluripotency markers NANOG and OCT4 in the D11 conditional *DICER1* knockout line transfected with miRNA clusters *302-367* and *371-373* in feeder-based condition. Positive control is +DOX, negative control is –DOX.

(B) Quantification of microRNA rescue by cell counting of 24-well plates and normalized to +DOX control in feeder-based condition. Statistics compare individual miRNA transfections to -DOX condition. n=3 independent experiments.

(C) Representative histograms of Annexin-V flow cytometry of the D11 conditional *DICER1* knockout line transfected with miRNA mimics or control miRNA mimics in feeder-free condition. n=3 independent experiments.

Error bars indicate SD, and significance is indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; ****$p < 0.0001$; ns, not significant ($p \geq 0.05$).

Supplementary Table 1. miRNA Target Predictions, Related to Figure 4.

Targets with a target score ≥ 50 are shown in this table. The TargetScanHuman7.2 version (http://www.targetscan.org/vert_72/, Agarwal et al., 2015) and MirTarget (http://www.mirdb.org/, Wong and Wang, 2015) were used for the analysis.

miRNA	Position in 3'UTR	Target Score	Bioinformatic Tool	Conservation
hsa-miR-372-3p	2901-2907 of *FAS*	95	TargetScan	Human
	298-304 of *TRAILR4*	76	TargetScan	Human
	829-835 of *TRAILR4*	77	TargetScan	Human
	1033-1039 of *NIK*	64	MirTarget	Human
	1998-2004 of *BIM*	82	TargetScan	Human, Mouse
hsa-miR-373-3p	2901-2907 of *FAS*	94	TargetScan	Human
	298-304 of *TRAILR4*	73	TargetScan	Human
	829-835 of *TRAILR4*	81	TargetScan	Human
	1033-1039 of *NIK*	64	MirTarget	Human
	1998-2004 of *BIM*	77	TargetScan	Human, Mouse
hsa-miR-302a-3p	2901-2907 of *FAS*	95	TargetScan	Human
	298-304 of *TRAILR4*	75	TargetScan	Human
	829-835 of *TRAILR4*	80	TargetScan	Human
	1033-1039 of *NIK*	64	MirTarget	Human, Mouse
	1998-2004 of *BIM*	84	TargetScan	Human, Mouse
hsa-miR-302b-3p	2901-2907 of *FAS*	95	TargetScan	Human
	298-304 of *TRAILR4*	75	TargetScan	Human
	829-835 of *TRAILR4*	80	TargetScan	Human
	1033-1039 of *NIK*	64	MirTarget	Human, Mouse
	1998-2004 of *BIM*	84	TargetScan	Human, Mouse
hsa-miR-302c-3p	2901-2907 of *FAS*	95	TargetScan	Human
	298-304 of *TRAILR4*	75	TargetScan	Human
	829-835 of *TRAILR4*	78	TargetScan	Human
	1033-1039 of *NIK*	64	MirTarget	Human, Mouse
	1998-2004 of *BIM*	84	TargetScan	Human, Mouse
hsa-miR-302d-3p	2901-2907 of *FAS*	95	TargetScan	Human
	298-304 of *TRAILR4*	75	TargetScan	Human
	829-835 of *TRAILR4*	78	TargetScan	Human
	1033-1039 of *NIK*	64	MirTarget	Human, Mouse
	1998-2004 of *BIM*	84	TargetScan	Human, Mouse

Supplementary Table 2. CRISPR Sequence Information, Related to Figure S1.

CRISPR gRNA sequences:

CRISPR gRNA	Targeted Region	CRISPR gRNA Target Sequence (5' → 3')	Oligo Sequence for Cloning into piCRg Entry (5' → 3')
cr1	PAZ	GAGAAGTCTGAAGCTCGCAT	**F:** CACCGAGAAGTCTGAAGCTCGCAT **R:** AAACATGCGAGCTTCAGACTTCTC
cr2	PAZ	TTCCATTTAAATACCTACCT	**F:** CACCGTCCATTTAAATACCTACCT **R:** AAACAGGTAGGTATTTAAATGGAC
cr3	RNase IIIa	GCTAACAGAGACTTTTGCCA	**F:** CACCGCTAACAGAGACTTTTGCCA **R:** AAACTGGCAAAAGTCTCTGTTAGC
cr4	RNase IIIa	GATCTGCTGAAACTTCAACG	**F:** CACCGATCTGCTGAAACTTCAACG **R:** AAACCGTTGAAGTTTCAGCAGATC
cr5	RNase IIIa	CCTGTGATGGCCGTAATGCC	**F:** CACCGCTGTGATGGCCGTAATGCC **R:** AAACGGCATTACGGCCATCACAGC
cr6	RNase IIIa	CAGGAGAGTACATTCATCGC	**F:** CACCGAGGAGAGTACATTCATCGC **R:** AAACGCGATGAATGTACTCTCCTC
cr7	RNase IIIa	ACTTACCCTGATGCGCATGA	N/A, generated through *in vitro* transcription from ssDNA templates (see below)
cr8	RNase IIIa	ACTCTGTCAAACGCTAGTGA	
cr9	RNase IIIa	CGCTAGTGATGGATTTAACC	

PCR primers for generating *in vitro* transcription template from piCRg gRNA vectors:

CRISPR gRNA	Oligo (5' → 3')
cr1	TAATACGACTCACTATAGGGAGAAGTCTGAAGCTCGCAT
cr2	TAATACGACTCACTATAGGTTCCATTTAAATACCTACCT
cr3	TAATACGACTCACTATAGGGCTAACAGAGACTTTTGCCA
cr4	TAATACGACTCACTATAGGGATCTGCTGAAACTTCAACG
cr5	TAATACGACTCACTATAGGCCTGTGATGGCCGTAATGCC
cr6	TAATACGACTCACTATAGGGAGGAGAGTACATTCATCGC
Universal reverse primer	AAAAGCACCGACTCGGTGCC

Oligonucleotides for *in vitro* transcription from ssDNA templates:

CRISPR gRNA	**110nt Oligo Template for *in vitro* transcription (5' → 3')**
Cr7	TAATACGACTCACTATAGGGACTTACCCTGATGCGCATGAGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGT
Cr8	TAATACGACTCACTATAGGGACTCTGTCAAACGCTAGTGAGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGT
Cr9	TAATACGACTCACTATAGGGCGCTAGTGATGGATTTAACCGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGT
Forward and reverse PCR primers for generating PCR product for *in vitro* transcription: TAATACGACTCACTATAGGG, AAAAGCACCGACTCGGTGCC	

PCR Primers for T7E1 and Sanger Sequencing:

Targeted locus	**Forward Primer (5' → 3')**	**Reverse Primer (5' → 3')**	**Sequencing Primer (5' → 3')**
PAZ domain (Cr1-2)	TCAACTTTAGAAGGCGGAAGCTC	TACAATGCTAAAATCACAGCCCAC	GATCGAGGTGCCTCTTCTATT
RNase IIIa (Cr3-6)	CGGGTGGAAAAAATCTATTGACAG	CTCATATATGAAAGGCGGCCC	CGGGTGGAAAAAATCTATTGACAG
RNase IIIa (Cr 4&6 for TRE-DICER1*)	CGGGTGGAAAAAATCTATTGACAG	GCATGATACGTTCTCATCCTC	CGGGTGGAAAAAATCTATTGACAG
RNase IIIa (Cr7-9)	GCGATGAATGTACTCTCCTGA	CAATACTCATCAACTGCCAGG	GCGATGAATGTACTCTCCTGA

Supplementary Table 3. PCR Primer Information, Related to Figures 4 and S2.

Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
*DICER1** (transgene)	TGGACTACAAAGACGATGACG	TTGTTGCCATGGCAGTCCAAA
OCT4	TGGTCCGAGTGTGGTTCTGTAA	TGTGCATAGTCGCTGCTTGAT
NANOG	GCTGGTTGCCTCATGTTATTATGC	CCATGGAGGAAGGAAGAGGAGAGA
SOX2	GGCAGCTACAGCATGATGCAGGAGC	CTGGTCATGGAGTTGTACTGCAGG
KLF4	TATGACCCACACTGCCAGAA	TGGGAACTTGACCATGATTG
BAX	CAAACTGGTGCTCAAGGCCC	GAGACAGGGACATCAGTCGC
BIM	AGACAGAGCCACAAGCTTCC	ACCATTCGTGGGTGGTCTTC
BMF	CCCTCCTTCCCAATCGAGTC	GATGGCTCCATCTCTCCTGG
BCL2	CTGCACCTGACGCCCTTCACC	CACATGACCCCACCGAACTCAAAGA
BNIP3L	TCGTGTTTGCCTGTAGCTGA	CACCCAGGAACCTTGTGAACT
ASK1	AGAGGCTTGCTGGCATAAACCC	GCTGCTTTTCCGTAGCCTCTTG
TRAILR4	CTGCTGGTTCCAGTGAATGACG	TTTTCGGAGCCCACCAGTTGGT
PI3K	TGCCAAACCACCTCCCATTCCT	CATCTCGTTGCCGTGGAAAAGC
FAS	GGACCCAGAATACCAAGTGCAG	GTTGCTGGTGAGTGTGCATTCC
NIK	GGAATACCTCCACTCACGAAGG	CTGTGAGCAAGGACTTTCCCAG
GAPDH	GGAGCCAAACGGGTCATCATCTC	GAGGGGCCATCCACAGTCTTCT

Supplemental Experimental Procedures

Generation of gRNAs for CRISPR/Cas9 Targeting

To generate CRISPR/Cas9 gRNA expression vectors targeting specific genomic loci, 20 base pairs (bp) of sequence located 5' of the PAM sequence was cloned into piCRg Entry (Gonzalez et al., 2014) following an established protocol (Cong et al., 2013). In brief, piCRg Entry was digested with BbsI, dephosphorylated and gel purified. A pair of oligos including the 20bp homology (**Table S2**) were annealed and phosphorylated, generating BbsI overhangs that can be cloned into the BbsI-digested and dephosphorylated vector. For production of gRNAs, we first generated the *in vitro* transcription template by adding the T7 promoter to the gRNA sequence in the piCRg Entry vector through PCR amplification using CRISPR-specific forward primers and a universal reverse primer (**Table S2**). Alternatively, for *Cr7-9* gRNAs we designed a 120-nt oligo that included the T7 promoter, and 20bp target gRNA sequence, and the tracr RNA sequence. This oligo was used as a template for PCR amplification using universal forward and reverse primers (**Table S2**). T7-gRNA PCR products were used as templates for IVT using the MEGAshortscript T7 kit (Thermo Fisher Scientific, AM1354). The resulting gRNAs were purified using the MEGAclear kit (Thermo Fisher Scientific, AM1908), eluted in RNase-free water and stored at −80 °C until use.

Construction of *TRE-DICER1** plasmids

Construction of low copy pBR_ENTRY vector: pCR-Blunt II-TOPO vector (Thermo Fisher, K280002) was partially digested with PvuII and BspHI to replace the high copy pUC origin of replication and the Zeocin resistance gene by a PvuII-BspHI fragment obtained from pMC1403 vector (http://seq.yeastgenome.org/vectordb) containing the low copy pBR322 origin of replication. The resulting vector was then opened with ApaI and SacI in order to insert an ApaI-SacI digested (in bold) GenScript-synthetized DNA fragment (Piscataway, NJ, USA):

TTTA**GGGCCC**CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTG

ATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTCCGCGGCCGCCAG
TGTGATGGATATCTGCAGAATTCAAGGGTGGGCGCGCCGACCCAGCTTTCTTGTACAAAGT
TGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAA
ATAAAATCATTATTTGCCATCCAGATCC**GAGCTC**CCTT. This fragment contains attL1 and attL2 Gateway cloning sequences flanking a polylinker including SacII, NotI, EcoRV, PstI and EcoRI unique restriction sites.

Construction of pBR_ENTRY_DICER1 vector: pBR_ENTRY was digested with EcoRV and dephoshorylated to clone the Xho-Acc65I digested, Klenow blunt ended Flag-tagged DICER1 cDNA from pCAGGS-Flag-DICER1 (Addgene plasmid 41584, a gift from Phil Sharp) (Gurtan et al., 2012).

Construction of the pIND_DICER1* vectors: We used the QuickChange II Site-Directed Mutagenesis Kit to introduce silent mutations in *DICER1* that disrupt the PAM sequences corresponding to cr4 and cr6, respectively (Agilent, 20052), using pBR_ENTRY_DICER1 as the template and the following primers: 5'-GCGAGTCCCCTGGTAAGCTACACGTTGAAGTTTCAGC-3' and 5'-GCTGAAACTTCAACGTGTAGCTTACCAGGGGACTCGC-3' for cr4 targeting, and 5'-CGAGAACCAGCCCCAGCCGAGCGATGAATGTACTCTCCTG-3' and 5'-CAGGAGAGTACATTCATCGCTCGGCTGGGGCTGGTTCTCG-3' for cr6 targeting. We then used Gateway LR Clonase (Thermo Fisher Scientific, 11791-020) to transfer the DICER1* inserts into pINDUCER21 (Addgene plasmid 46948, a gift from Stephen Elledge & Thomas Westbrook) (Meerbrey et al., 2011).

Generation of *TRE-DICER1 Lines**

We first prepared pIND_DICER1* viruses for inducible expression of *DICER1** transgenes with a silent mutation in the PAM sequence corresponding to cr4 or cr6 gRNAs (Figure S1E, S1F). 14 million 293T cells were plated in 15cm dishes pre-coated with collagen.

Cells were allowed to grow in the absence of antibiotics for two days. Thirty minutes before transfection, fresh media was added to the cells. Transfection was performed using JetPRIME Polyplus transfection reagent (VWR, 114-01) as per manufacturer's recommendations. Briefly, 20.4 µg of pIND_DICER1* (with specific PAM-sequence mutations for cr4 or cr6) lentiviral vector was mixed with 8.16 µg of psPAX2 and 2.04 µg of pCMV-VSVG. This mix was then diluted in 1,500 µL of JetPRIME buffer, followed by the addition of 60 µL of JetPRIME transfection reagent. The mix was incubated at room temperature for 10 minutes, and then added dropwise to one 15cm dish. Media was changed the following day, and the supernatant was harvested on days 2, 3, and 4 after the transfection. The three harvests were pooled together and passed through a 0.45 µm filter. Virus was concentrated by ultracentrifugation for 1.5 hours at 4 °C and 250,000 rpm, and resuspended in 400 µL of hESC medium overnight at 4 °C. The virus was then aliquoted and stored at -80 °C.

To generate *TRE-DICER1** Lines, HUES8 iCas9 cells were grown to 60-70% confluency, and then disaggregated using TrypLE, counted, and resuspended at 1 million cells/mL in hESC medium with 5 µm Y-27632 and 10 µg/mL of Protamine Sulfate (MP Biomedicals). 1 mL cell suspension was plated per well of a 6-well dish that was pre-seeded with iMEFs. Infection was carried out overnight using 150 µL of virus. Media was changed every day until the cells were confluent and ready for FACS sorting of GFP+ colonies. GFP+ cells were plated at low density (2,500 cells) in 10cm dishes previously seeded with iMEFs, and grown for ~2 weeks until colonies were visible and ready to pick. Five colonies were picked for inducible *DICER1** expression*: TRE-DICER1** cr4 and *TRE-DICER1** cr6, respectively (Figure S1F); and one each was chosen for making DICER1 conditional knockout lines. 2 µg/mL doxycycline was used when maintaining the inducible DICER1* lines unless otherwise specified.

Establishment of *DICER1* Knockout Lines

A detailed description on how to establish clonal mutant lines from HUES8 iCas9 hESCs has been described previously (Gonzalez et al., 2014; Zhu et al., 2014). To generate mutant lines, iCas9 hESCs were treated with doxycycline (2 μg/mL) for 1 day before gRNA transfection, to induce Cas9 expression. The *TRE-DICER1** lines, were kept on doxycycline continuously. Cells were dissociated using TrypLE Select, replated onto iMEF-coated plates and transfected in suspension with gRNA using Lipofectamine RNAiMAX (Thermo Fisher Scientific) per manufacturer's instructions. gRNAs were added at a final concentration of 10nM. A second transfection was performed a day later. Two days after the last gRNA transfection, hESCs were dissociated into single cells, passed through a 40 μm cell strainer, and replated at a low density (2,000 cells/10cm dish). Genomic DNA was also extracted at this stage to perform T7E1 assay to verify that the gRNAs had generated indels. Cells were grown until clonal colonies became visible (~12-14 days). 48-96 colonies were picked when colonies were in excess. Colonies were mechanically disaggregated and replated into individual wells of 96-well plates. Clonal lines were expanded and analyzed by Sanger sequencing to identify mutant clones. PCR and sequencing primers are listed in **Table S2**. Selected clonal lines carrying desired mutations were further expanded and frozen down.

RNA Processing and Quantitative qRT-PCR

miRNA and total RNA were isolated using miRNeasy Mini Kit (Qiagen, 217004). For mRNA qRT-PCR, this was followed by cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368814), and qRT-PCR was performed with QPCR SYBR Green low ROX Kit (Thermo Fisher Scientific, AB4322B). For miRNA qRT-PCR, we used Taqman MicroRNA Assays (Thermo Fisher Scientific, 4427975) per manufacturer's instructions. RNU-44 was used for normalization. We used the Applied Biosystems 7500 Real-Time PCR System for all qRT-PCRs. Primer sequences are specified in **Table S3**.

RNA Sequencing, Gene Ontology, and Pathway Enrichment Analysis

Conditional *DICER1* knockout cells (line B2) cultured in the feeder-based condition were collected at day 9 after doxycycline withdrawal along with the control doxycycline-treated cells with three biological replicates each. hESCs were sorted from potential contaminating iMEFs using TRA1-60 staining. RNA was isolated using the above-mentioned RNA isolation method. RNA-seq was performed by the MSKCC Integrated Genomics Operation Core. RNA-seq reads were filtered for quality and 3' trimmed for adapter sequences using TrimGalore (0.4.5). Reads were aligned to human genome assembly HG19 using STAR (v2.5.3a) with default parameters. Read count tables were created using HTSeq v0.9.1. Normalization and differential expression analysis was performed with DESeq2. Analyzed RNA-seq data (normalized counts) can be found in **Table S4**. Differentially expressed genes were identified based on a fold change of greater than 1.5 and a q-value of less than 0.1, and analyzed using the clusterProfiler (v3.6.0) package in R (v3.4.2) to identify enriched gene ontology (GO) terms and KEGG pathways. The Benjamini-Hochberg adjusted q value of less than 0.05 was set as the cutoff, and the top 40 terms were plotted.

Western Blotting

Protein samples were collected from cell lysate homogenized in RIPA buffer in the presence of proteinase inhibitor cocktail (Cell Signaling Technology) and stored in -80**º**C until use. Proteins were separated on a NuPAGE Novex Bis-Tris 3-8% Tris-Acetate protein gel (Life Technologies) and transferred to a PVDF membrane (Life Technologies) overnight at 4 **º**C. Blocking was performed with 5% milk in TBST. Membranes were incubated with primary antibodies overnight at 4 **º**C and HRP-conjugated secondary antibodies for 1 hour at room temperature. ECL reagents (Pierce) were obtained from GE Healthcare. Primary antibodies

used were DICER1 (1:1,000, Cell Signaling, 5325), Flag (1:5,000, Sigma Aldrich, F1804), and GAPDH (1:5,000, Cell signaling, 5174).

Northern Blotting

Total RNA was isolated using the miRNeasy Mini Kit as per manufacturer's recommendation (Qiagen, 217004). 20 µg of total RNA per lane were separated on 15% polyacrylamide 7 M urea gels and transferred onto GeneScreen Plus membrane (Perkin Elmer) using A Trans-Blot SD Semi-Dry Cell (Biorad). The blots were UV crosslinked (Stratagene), baked at 80 °C for 1 hour and probed with γ-[^{32}P]-ATP labeled DNA oligonucleotide probes. The probe sequences are 5'-CCACTGAAACATGGAAGCACTTA-3', 5'-TCCATCATTACCCGGCAGTATTA-3', 5'-AGCATTGCAACCGATCCCAACCT-3', 5'-AGTTAGAGCTAATTAAGACCT-3' for *miR-302c-3p*, *miR-200c-3p*, *miR-92a-5p*, and *RNU44*, respectively. The blots were stripped and re-probed for multiple miRNAs and a loading control (*RNU44*). Decade Marker RNA (Thermo Fisher Scientific) was labeled with γ-[^{32}P]-ATP and used as a size standard (10-100 bases).

Alkaline Phosphatase and Immunofluorescence Staining

Cells were fixed with 4% paraformaldehyde in PBS for ≤10 minutes. For nuclear immunostaining, we washed once with PBS, and then permeabilized in PBS with 0.1% Triton (PBS-T) for 15 minutes. Blocking was done using donkey blocking solution at room temperature for 5 minutes (5% donkey serum in PBS-T). Primary and secondary antibodies were diluted in blocking solution and incubated at room temperature for 1 hour. The following primary antibodies were used: NANOG (1:100, Cosmobio Japan, REC-RCAB0004P-F), OCT3/4 (1:100, Santa Cruz, sc-8628), Sox2 Y-17 (1:100, Santa Cruz, sc-17320), Ki67 (1:1,000, Vector Laboratories, VP-K451), PH3 (1:100, Cell Signaling, Ser10 9701), and C-CSP3 (1:400, Cell

Signaling, Asp175 9661). AP staining was performed using Vector Red Alkaline Phosphatase Substrate Kit following manufacturer's guidelines (Vector Laboratories, SK-5100).

Flow Cytometry

Cells were dissociated using 1X TrypLE, then collected and washed with cold FACS buffer (5% FBS in PBS). For intracellular staining, cells were stained with live/dead staining (LIVE/DEAD Fixable Violet Dead Cell Stain Kit from Molecular Probes, L34955, 1:1,000) for 30 minutes on ice in FACS buffer. Intracellular staining was then performed using the Foxp3 Staining Buffer Set (eBioscience, 300-5523-00) as per manufacturer's instructions. Fixation and permeabilization were carried out on ice for 1 hour and followed by antibody staining: Cleaved Caspase-3 (1:200, Cell Signaling Technology, 9661S), and Phospho-Histone H3 (1:200, Cell Signaling Technology, 9701). FlowJo software was used for analysis.

Annexin V staining was performed using the PE Annexin V Apoptosis Detection Kit I as per manufacturer's instructions (BD Biosciences, 559763). For cell cycle analysis, cells were resuspended at $1x10^6$ cells/mL in wash buffer (PBS with 0.1% FBS). 1 million cells were chilled in an ice bucket, and then 3 mL cold (-20 °C) absolute ethanol were added dropwise while vortexing. Cells were fixed overnight at 4 °C. Cells were then washed twice with PBS. 1 mL of propidium iodide (Sigma Aldrich, P4170 Sigma) was added to the cell pellet and mixed well. 50 µL RNase A (Thermo Fisher Scientific, EN0531) stock solution (10 mg/mL) was added and incubated at 4 °C for 3 hours. Cells were protected from light and were not washed prior to flow analysis.

Supplemental References

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