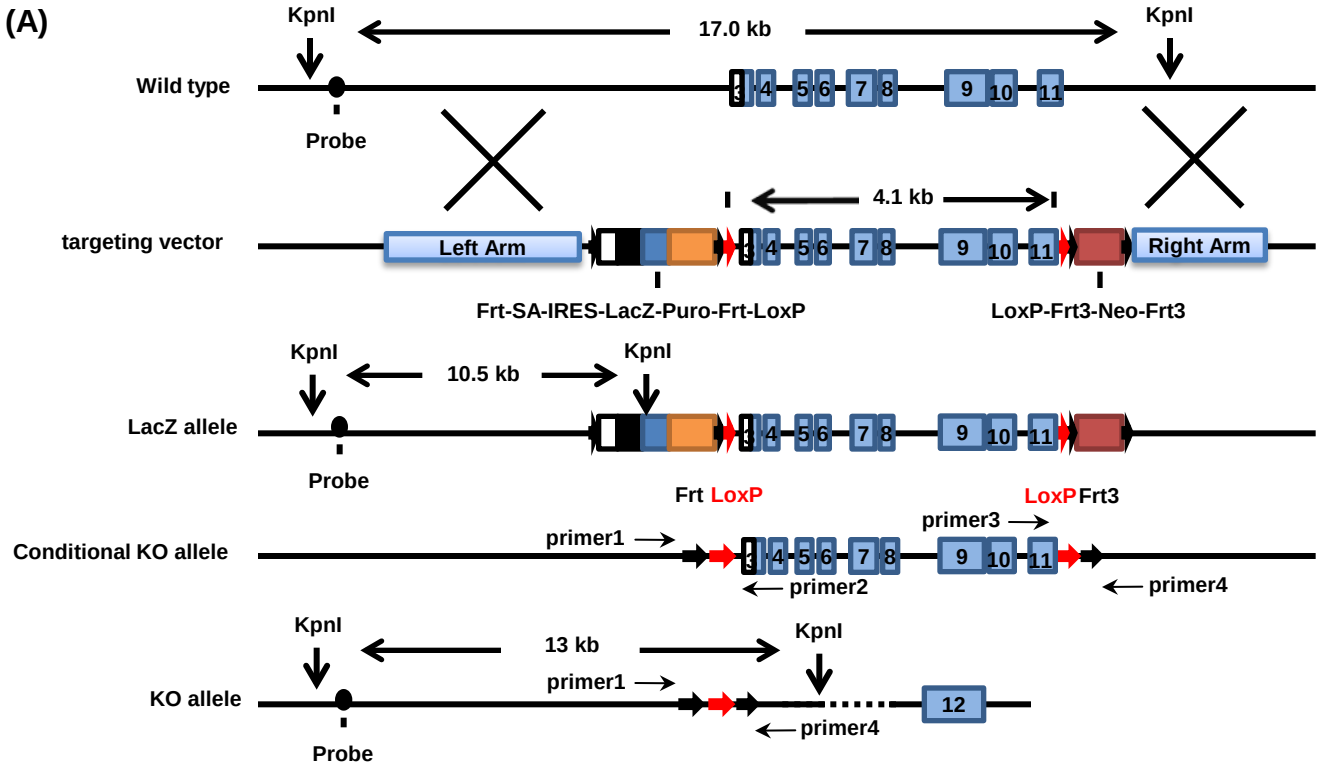
Stem Cell Reports, Volume *7*

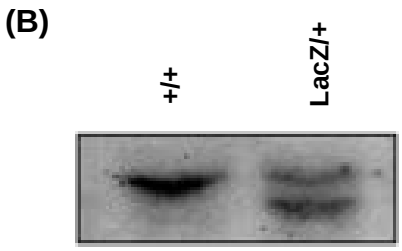
Supplemental Information

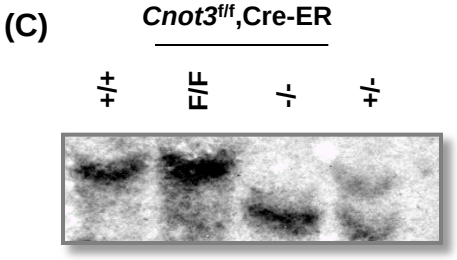
CNOT3-Dependent mRNA Deadenylation Safeguards the Pluripotent State

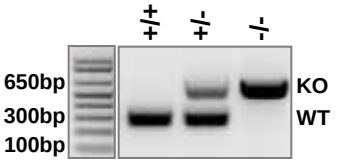
Xiaofeng Zheng, Pengyi Yang, Brad Lackford, Brian D. Bennett, Li Wang, Hui Li, Yu Wang, Yiliang Miao, Julie F. Foley, David C. Fargo, Ying Jin, Carmen J. Williams, Raja Jothi, and Guang Hu

Zheng_Figure S1

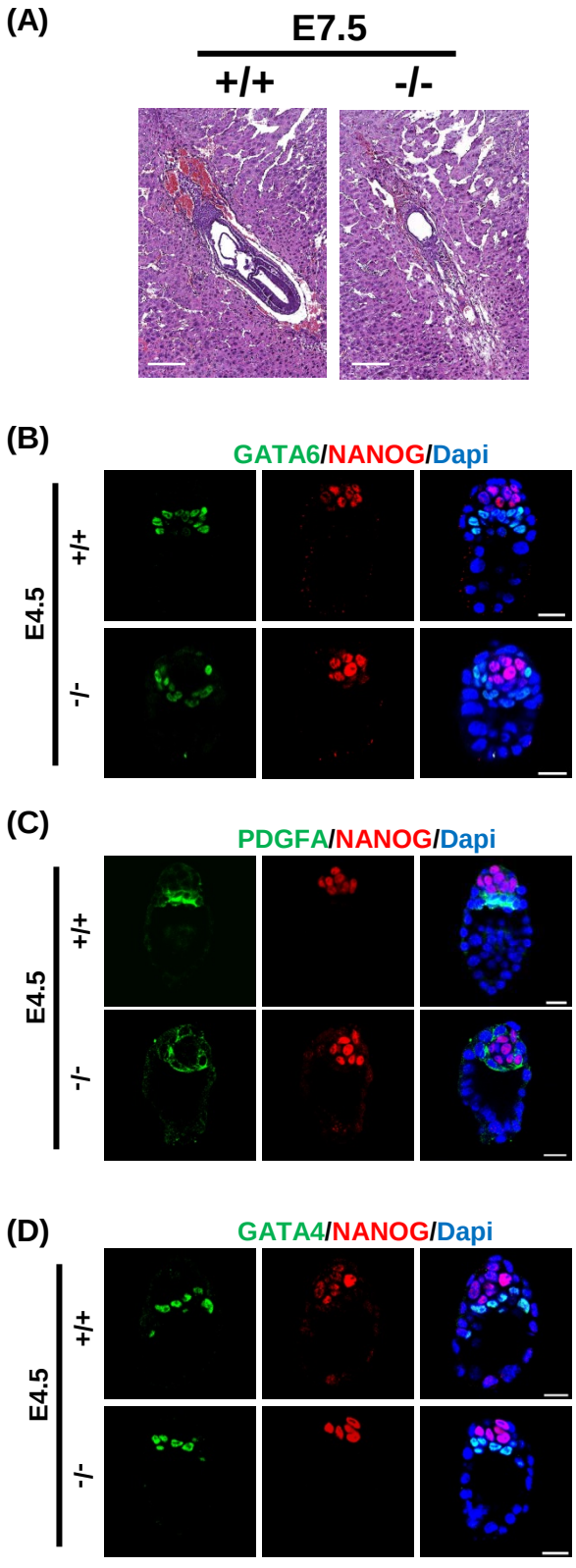




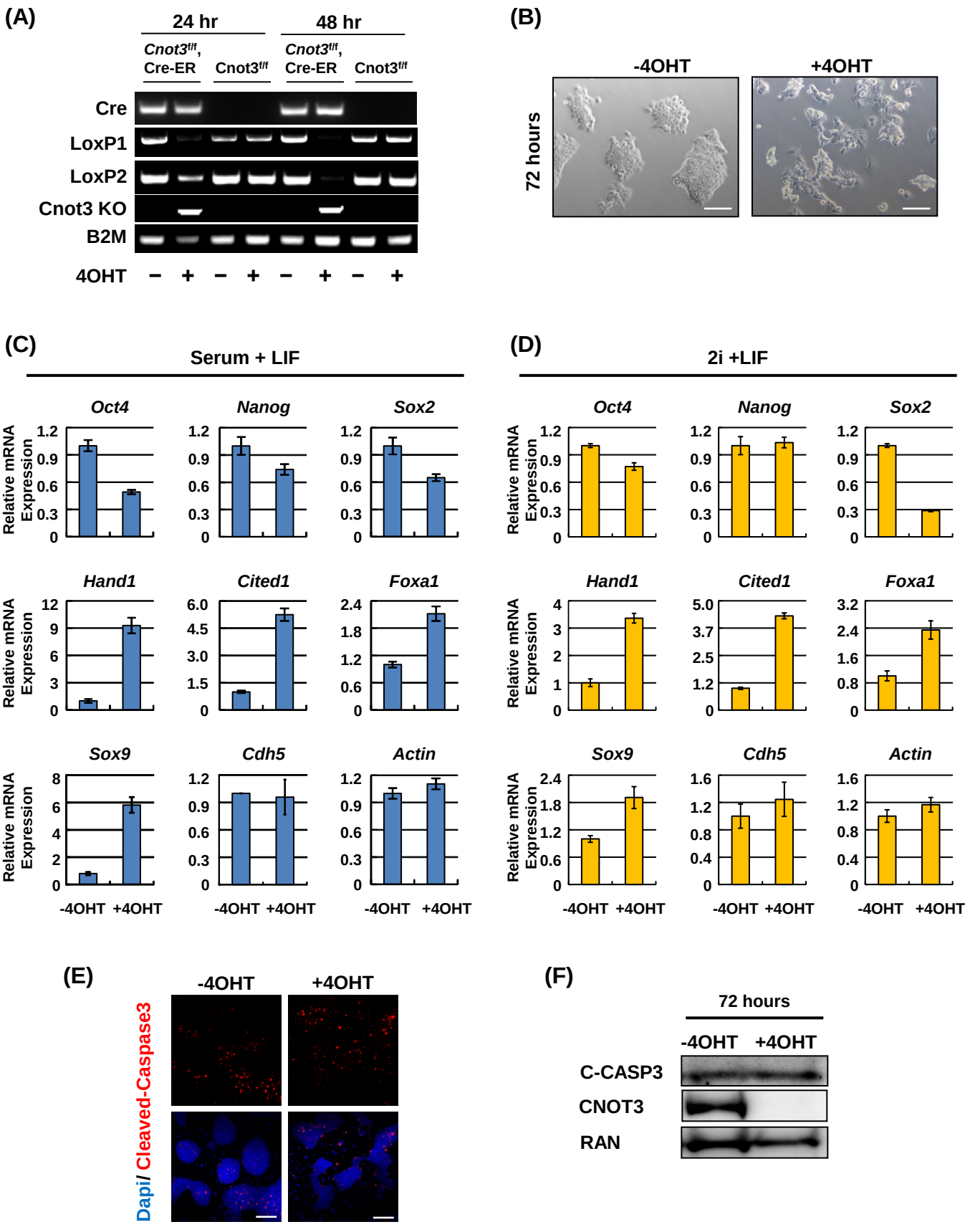




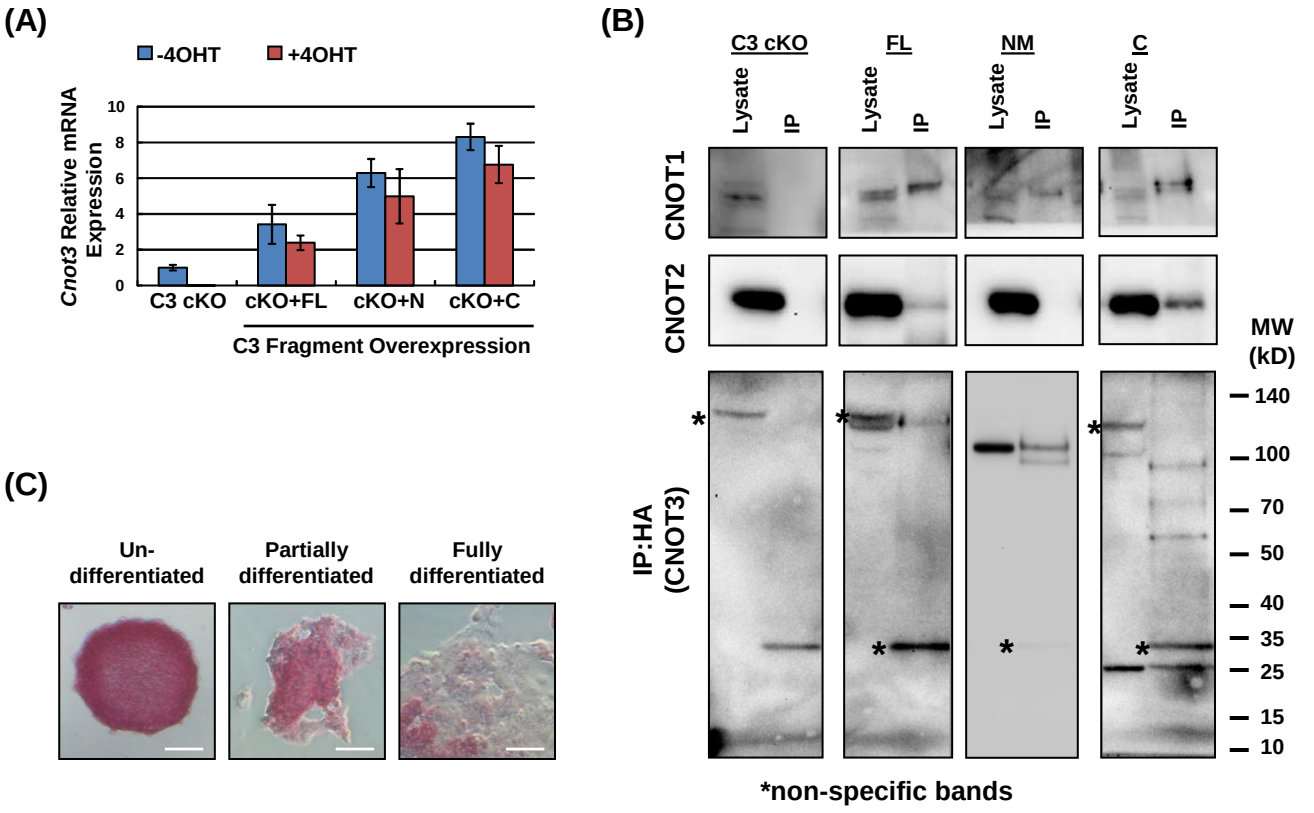
Zheng_Figure S2



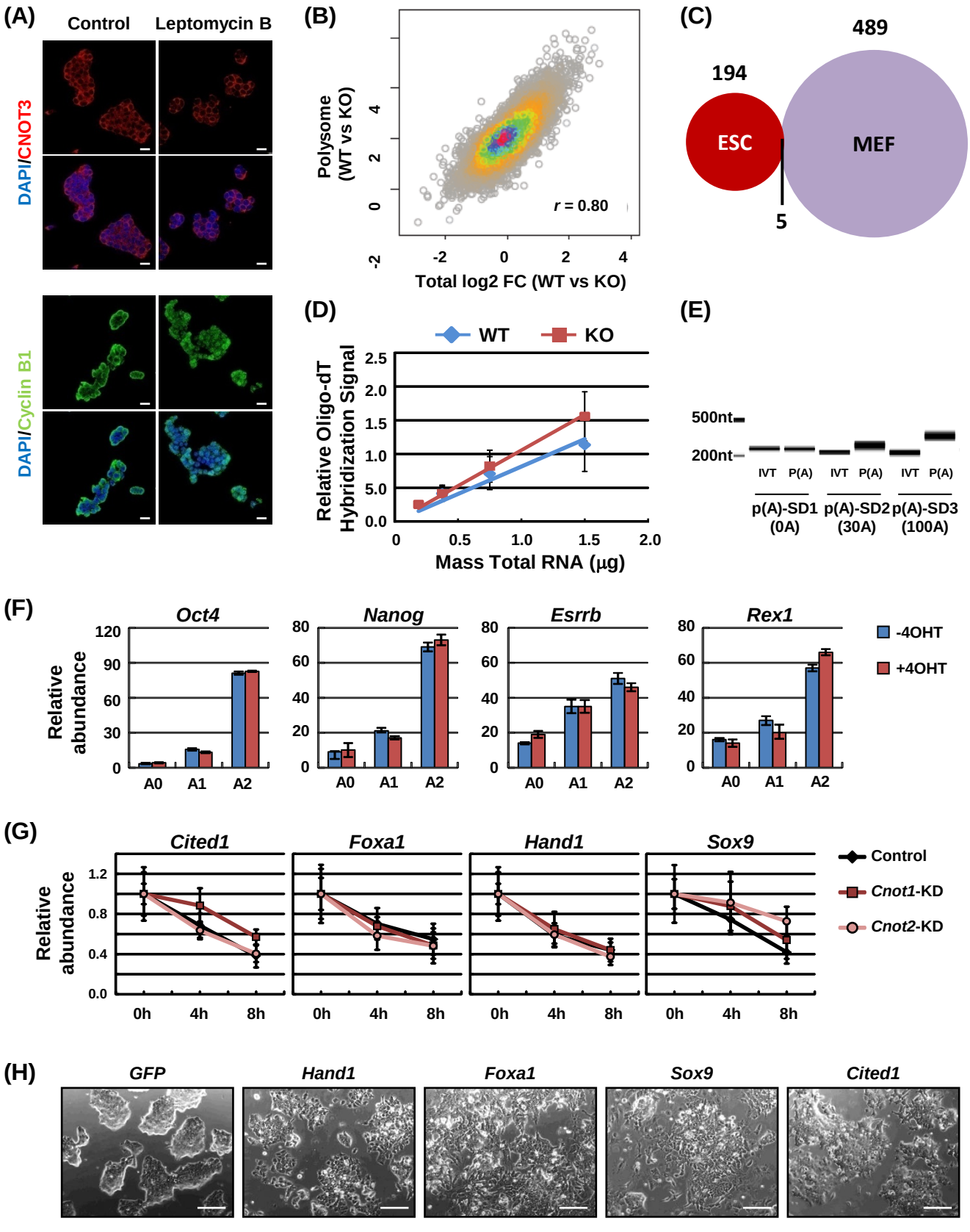
Zheng_Figure S3



Zheng_Figure S4



Zheng_Figure S5



Supplemental Figure Legends

Figure S1. *Cnot3* gene deletion strategy. (A) Gene targeting strategy for *Cnot3*. **(B-D)** Genotyping of *Cnot3* cKO mice **(B)** and ESCs **(C)** by Southern blotting, and *Cnot3* deletion embryos by genomic PCR **(D)**.

Figure S2. *Cnot3* deletion does not impact trophectoderm and primitive endoderm formation. (A) Hematoxylin and eosin stain of E7.5 WT and *Cnot3* KO embryo sagittal sections (white bar = 200 μm). Genotypes of the embryos were determined by laser capture micro-dissection. **(B-C)** Immunofluorescence staining of primitive endoderm markers GATA6, PDGFRα, GATA4 and epiblast marker NANOG in E4.5 WT and *Cnot3* deletion embryos (white bar = 20 μm).

Figure S3. *Cnot3* is required for ESC maintenance. (A) Genotyping by genomic PCR in *Cnot3* cKO ESCs treated with (KO) or without (WT) 0.1 μM 4-OHT for 24 and 48 hours. B2M primers amplify a genomic regions present in both WT and KO cells and serves as a control for PCR. **(B)** Morphological changes of *Cnot3* cKO ESCs 72 hours after 4-OHT treatment (white bar = 200 μm). **(C-D)** Changes in marker gene expression 72 hrs after 4-OHT treatment in the serum+LIF medium **(C)** or the 2i+LIF medium **(D)**, as determined by RT-qPCR. Expression was normalized by *Actin*, and values were plotted as Mean ± SEM from 3 independent experiments. **(E)** Immunofluorescence staining (white bar = 10 μm) and western blot **(F)** of cleaved-Caspase3 in WT and KO ESCs.

Figure S4. C-terminal domain in CNOT3 is required for ESC maintenance. (A) Overexpression of CNOT3 fragments in *Cnot3* cKO ESCs. Cells were treated with or without 4-OHT for 48 hrs, and the expression of total *Cnot3* (endogenous *Cnot3* and exogenous *Cnot3* fragment) was determined by RT-qPCRs. Expression was normalized by *Actin*, and values were plotted as Mean ± SEM from 3 independent experiments. **(B)** Interaction between CNOT3 fragments and CNOT1 or CNOT2. HA-tagged CNOT3 fragments were expressed in *Cnot3* cKO ESCs and affinity purified by HA-beads. Co-purified endogenous CNOT1 and CNOT2 were detected by western blot. Cropped images of the same blots were shown in Figure 3D. **(C)** Representative images of alkaline phosphatase stained colonies from Figure 3G (white bar = 100 μm).

Figure S5. CNOT3 regulates differentiation gene mRNA expression and deadenylation. (A) CNOT3 localization in ESCs. ESCs were treated with or without leptomycin B, and protein localization was determined by immunofluorescence staining. Localization of Cyclin B1 served as a positive control (white bar = 20 μm). **(B)** Comparison of changes in polysome-associated vs. total RNAs between WT and *Cnot3* KO ESCs. *Cnot3* cKO ESCs were treated with (KO) or without 4-OHT (WT) for 72 hrs, and both total and polysome-associated RNAs were extracted and sequenced. Log2 fold-changes were calculated for all detected total or polysome-associated RNAs in WT and KO cells and plotted. **(C)** Venn diagram comparing CNOT3 target genes in ESCs (194 genes) and MEFs (486 genes). p-value represents the test for depletion in overlapping genes, and was calculated by the Fisher Exact test. **(D)** Quantitation of global polyadenylation in WT and *Cnot3* KO ESCs. *Cnot3* cKO ESCs were treated with (KO) or without 4-OHT (WT) for 48 hrs, and global polyadenylation in total RNAs were determined by biotinylated Oligo-dT

hybridization. Relative hybridization signal intensity values were plotted as Mean ± SEM from 3 independent experiments. **(E) S**tandards used for poly(A)-tail length determination. Each poly(A) standard was synthesized by in vitro transcription followed by poly(A) tailing, and the length of the poly(A) tail was estimated by the size difference between the in vitro transcribed and the polyadenylated standard using bioanalyzer. The estimated poly(A)-tail length for the standards are: p(A)-SD1 = 0 A; p(A)-SD2 = 30 A; p(A)-SD3 = 100 A. **(F)** Measurements of poly(A)-tail length of pluripotency gene mRNAs based on the Oligo-dT fractionation method. Values were plotted as Mean ± SEM from 3 independent experiments. **(G)** Examination of mRNA stability for the indicated genes. E14 ESCs were transfected with *Cnot1* or *Cnot2* siRNAs. Actinomycin-D was added to the cells 48 hrs after transfection, and mRNA level was measured by RT-qPCR at the indicated time points after Actinomycin-D treatment. Relative expression values were plotted as Mean ± SEM from 3 independent experiments. **(H)** Overexpression of CNOT3 target genes in ESCs. E14 cells were transfected with plasmids expressing the indicated genes using Lipofectamine 2000. Cell morphology was imaged 72 hrs after transfection (white bar = 200 μm).

Supplemental Tables

Table S1: Changes in mRNA half-life and steady-state level after *Cnot3* deletion (excel attached)

Table S2: Gene ontology analysis for up-regulated genes in *Cnot3* KO ESCs

GO term	p-value	-LOG(p-value)
developmental process	8.58E-12	11.06662882
multicellular organismal development	2.07E-11	10.68417222
system development	2.71E-10	9.566792523
organ development	4.15E-10	9.381632278
anatomical structure development	1.04E-09	8.981101734
cell differentiation	7.84E-09	8.105426255
cellular developmental process	2.06E-08	7.685927377
Wnt receptor signaling pathway, calcium modulating pathway	2.80E-08	7.553361502
placenta development	8.41E-06	5.075135325
anatomical structure morphogenesis	9.44E-06	5.024928977
embryonic placenta development	2.59E-05	4.586413727
heart development	3.12E-05	4.505344342
embryonic organ development	1.04E-04	3.981376088
tissue development	1.55E-04	3.809826638
anatomical structure formation involved in morphogenesis	1.56E-04	3.805510393
vasculature development	1.81E-04	3.743337562
angiogenesis	2.79E-04	3.55503242
cell adhesion	2.93E-04	3.533834593
biological adhesion	3.00E-04	3.522609248
blood vessel development	3.31E-04	3.479932407
cell development	4.46E-04	3.351019422
organ morphogenesis	4.77E-04	3.321027603
cellular amino acid derivative biosynthetic process	5.74E-04	3.241170939
cellular process	6.69E-04	3.17483647
negative regulation of cellular process	7.12E-04	3.147793718

Table S3: Gene expression datasets used for data analysis

Citation	PMID	Accession	Gene (s)
Yamaji et al. (2013)	23333148	GSE42580	Prdm14 KO
Oldfield et al. (2014)	25132174	GSE56840	NF-YA,B,C triple KD
Cinghu et al. (2014)	24711389	GSE47872	Ncl KD
Loh et al. (2006)	16518401	GSE4189	Oct4 KD
Leeb et al. (2010)	20123906	GSE19076	Eed, Ring1B KO
Shen et al. (2008)	19026780	GSE12982	Ezh2 KO
Jiang et al. (2008)	18264089	GSE9775	Klf2,4,5 triple KD
Ho et al. (2009)	19279218	GSE14344	Brg KO
Ho et al. (2011)	21785422	GSE27708	LIF withdrawal (Stat3)
Freudenberg et al. (2012)	22210859	GSE34887	Tet1 KD
Ivanova et al. (2006)	16767105	GSE4679	Esrrb, Nanog, Sox2, Tbx3, Tcl1 KD
Merrill et al. (2011)	21685894	GSE27455	Tcf3 KO
Ding et al. (2009)	19345177	GSE12078	Paf1 KD
Lim et al. (2008)	18804426	GSE12482	Sall4 KD
Yamamizu et al. (2011)	25371362	GSE31381	Gata2, Gata3, Dtx3, Eomes, T, Ascl2, Cdx2, Sox9, Ascl1, Rhox6, Foxa1, Sox7, Tbx5 OE

Table S4: Primers used in this study

qPCR primers		
mCnot3	Cnot3-F	AGAGGCCGATCTACAGATAGTGA
	Cnot3-R	GACAGGCTTGGAGCCATTT
mHand1	Hand1-F	TCTGGCTCGCTCTCTCGTCC
	Hand1-R	CTCGAGAAGGCATCAGGGTA
mCited1a	Cited1a-F	TCGAGGCCTGCACTTGATGTCAAG
	Cited1a-R	ATCCTTCACTCCAAGGTTGGAGTAG
mSox9	Sox9-F	ATCTGCACAACGCGGAGCTCA
	Sox9-R	CTCTTCTCGCTCTCGTTCAGCAG
mFoxa1	Foxa1-F	GAACTCCATCCGCCACTCGCTG
	Foxa1-R	GCGCAAGTAGCAGCCGTTCTCG
mOct4	Oct4-F	CCTCCTCTGAGCCCTGTGC
	Oct4-R	CTCCTTCTGCAGGGCTTTCAT
mSox2	Sox2-F	TCGGGCTCCAAACTTCTCT
	Sox2-R	TGCTGCCTCTTTAAGACTAGGG
mActin	Actin-F	AAGGCCAACCGTGAAAAGAT
	Actin-R	GTGGTACGACCAGAGGCATAC
mCdx2	Cdx2-F	CCTGTGCGAGTGGATGCGGAAG
	Cdx2-R	CTCCAGCTCCAGCCGCTGA
mActin2	Actin2-F	AGTACTCTGTCTGGATCGGTGGCTC
	Actin2-R	TCGTCGTATTCCTGTTTGCTGATC
mCnot3-N-term	Cnot3-N-term-F	GAGGCTGACCTAAAGAAGGAGA
	Cnot3-N-term-R	TCATTTGATGCTACCCATGTCT
mCnot3-C-term	Cnot3-C-term-F	GAGTTCTACCAGCGCCTGTC
	Cnot3-C-term-R	AATCGCCAGGACTGCTTCT
Poly(A) standards (*Primers for cloning poly(A) standards also work as qPCR primers)		
Hygro*	P(A)-SD1-F	GTATTGACTGGAGCGAGGCGAT
	P(A)-SD1-R	CTGCTGCTCCATACAAGCCAACCA
GFP*	P(A)-SD2-F	GAACGGCATCAAGGTGAACTTCAAGAT
	P(A)-SD2-R	GTGTTCGCTGGTAGTGGTCGGCGA
Luciferase*	P(A)-SD3-F	GTGCCAGAGTCCTTCGATAGGGACAA
	P(A)-SD3-R	CGACACCTTTAGGCAGACCAGTAGATCCA
Cloning primers for Cnot3		
mCnot3 aa249 pDNR5	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCACCATGGAGGACG AGATCTTCAACCAGTC	
mCnot3 aa605 pDNR5	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCACCATGCTCACCAA GGAGCAGCTATACCAACAGG	
mCnot3 aa636 pDNR3	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGGGAGGTACTGCCGAAT GCG	
mCnot3 pDNR5	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCACCATGGCGGACAA GCGCAAACTCC	
mCnot3 pDNR3	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGGAGGTCCCGGTCCTC CAGG	