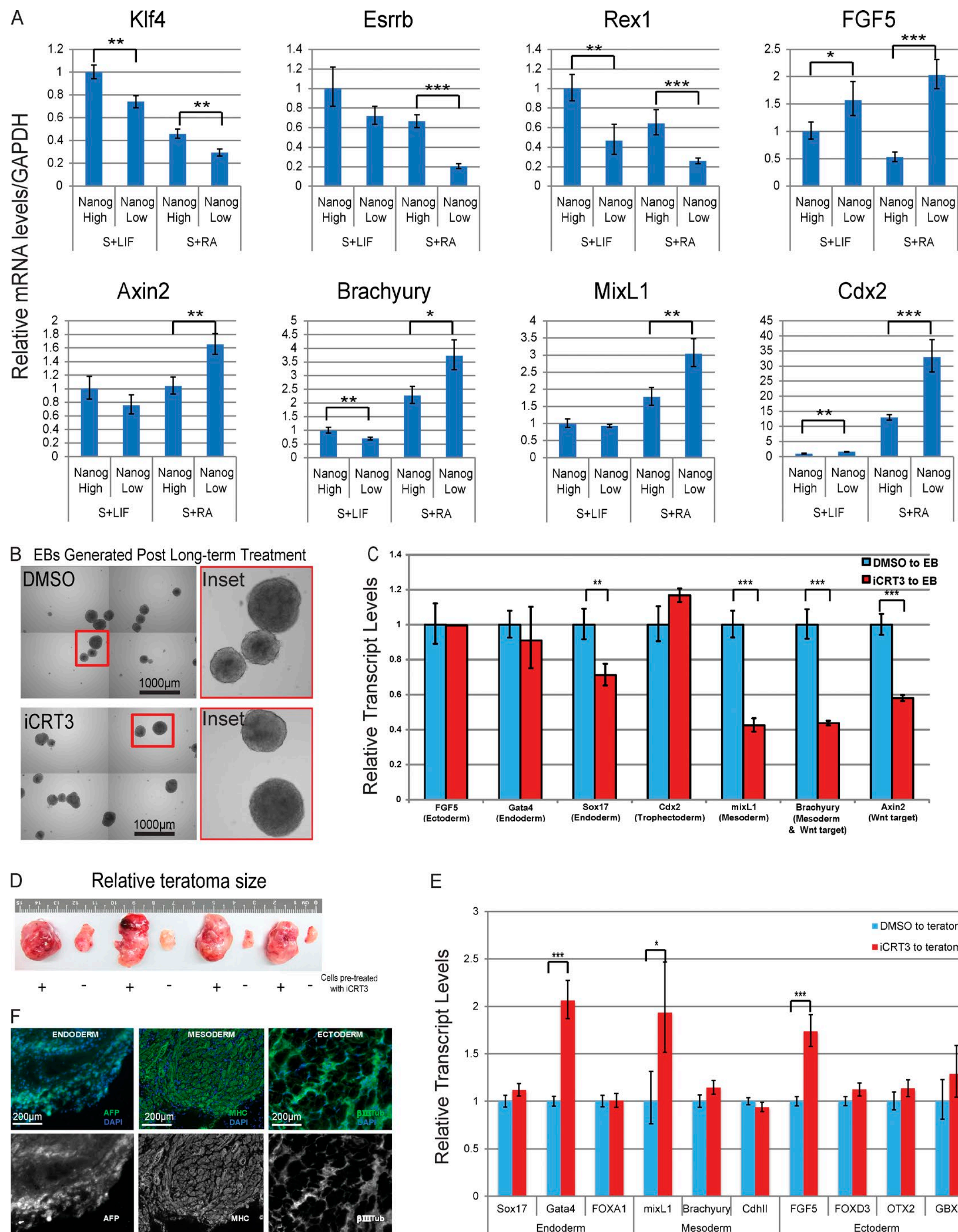

Figure S1. **Inhibition of TCF transcriptional activity enhances pluripotency of cultured mESCs (related to Fig. 2).** (A) qPCR analysis of pluripotency and differentiation markers along with TCF target genes in sorted Nanog^{High} and Nanog^{Low} mESCs from serum plus LIF (S+LIF) and serum + RA (S+RA). Mean ± SD of three replicates. (B) Long-term culture of mESCs with iCRT3 did not influence their potency for multilineage differentiation, as evident by their ability to form EBs in vitro (bottom panel), similar to DMSO controls (top panel). Resized original images and additional 4× digitally magnified insets shown are from a single representative experiment (for NG4) out of three replicates for each of NG4 and Rex1-GFP cell lines. (C) qPCR analysis of differentiation marker genes in EBs generated from cells cultured for 14 d with iCRT3 or DMSO showed comparable differentiation potential, with the exception of mesoderm specific genes. Mean ± SD of three replicates. (D) Representative teratomas of eight pairs dissected from mice showed that iCRT3-treated mESCs readily differentiated in vivo. (E) qPCR analysis of germ layer markers revealed similar expression levels in teratomas derived from cells that had been maintained with DMSO and iCRT3 for 15 d. Mean ± SD of three replicates. (F) Representative immunostaining of teratomas derived from ESCs treated with iCRT3 showing expression of lineage markers for endoderm (AFP), mesoderm (MHC), and ectoderm (βIIITubulin). Resized original images shown are from a single representative experiment out of eight pairs of teratomas generated from cells treated with DMSO/iCRT3 for 14 d. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

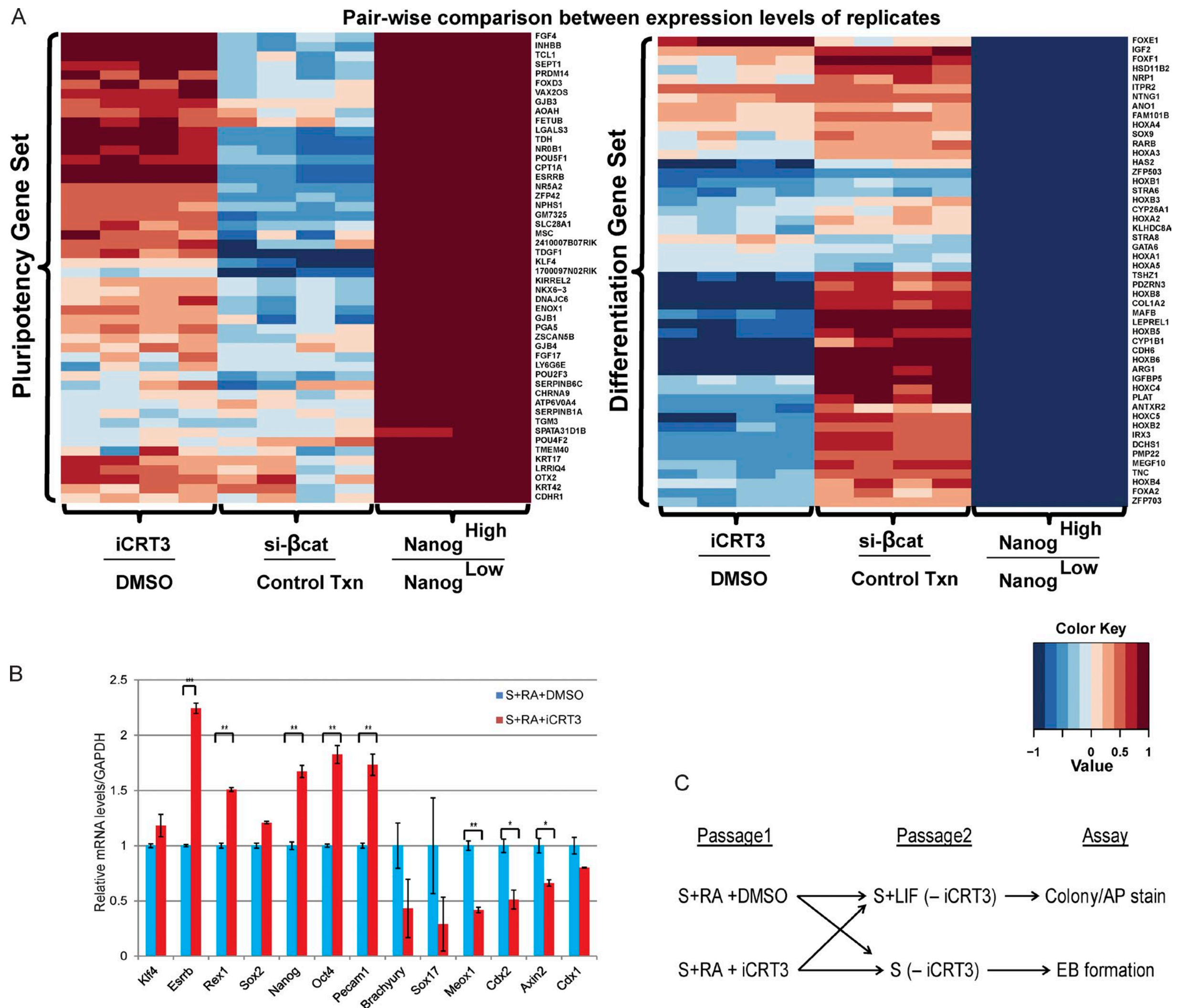

Figure S2. **Transcriptome profiles of iCRT3 and si-βcat–treated mESCs (related to Fig. 3).** (A) Heat map representation for trends of log2 fold change expression values of genes for every possible pairwise comparison of a treatment with its respective control/opposite treatments. All four possible comparisons between two independent replicates each are shown in clustered columns as labeled along bottom of heat map. Rows (genes) have been reordered by hierarchical clustering with complete linkage with Euclidean distance (as implemented by the heatmap.2 function in the R package gplots). Relative changes in the gene expression between conditions are displayed in colors scaled by row (gene) as highlighted in key. The heat map has been split into two major clusters to highlight relative expression levels of top 50 genes (in terms of absolute log2FC values) from the pluripotency (up-regulated genes in $Nanog^{High}$ cells in serum plus LIF [S+LIF]) and differentiation (up-regulated genes in $Nanog^{Low}$ cells in serum plus RA [S+RA]) gene sets. The cluster analysis suggests that the gene signature profile of cells treated with iCRT3 during RA-mediated differentiation (relative to DMSO control) is similar to self-renewing mESCs ($Nanog^{High}$ in S+LIF). By contrast, cells treated with si-β-cat (relative to Scramble control transfection) reveal an mRNA profile more similar to the differentiating cells. For complete list of gene identification numbers used for the related GSEA analysis, please refer to Table S2 (first and second tabs). (B) Relative mRNA levels revealed that cells maintained with iCRT3 in differentiating conditions (S+RA) exhibited enhanced expression of pluripotency markers such as Rex1, Esrrb, Pecam1, and the core PTN members. Meanwhile, differentiation markers (Sox17, Meox1, and Cdx2) and TCF targets (Brachyury, Cdx1, and Axin2) were significantly reduced. Mean ± SD of two replicates. (C) Schematic representation of experimental outline (refer to Fig. 3, G and H) to test the effect of iCRT3 treatment during RA-induced differentiation in modulating functional pluripotency of mESCs in colony forming efficiency and teratoma assays. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

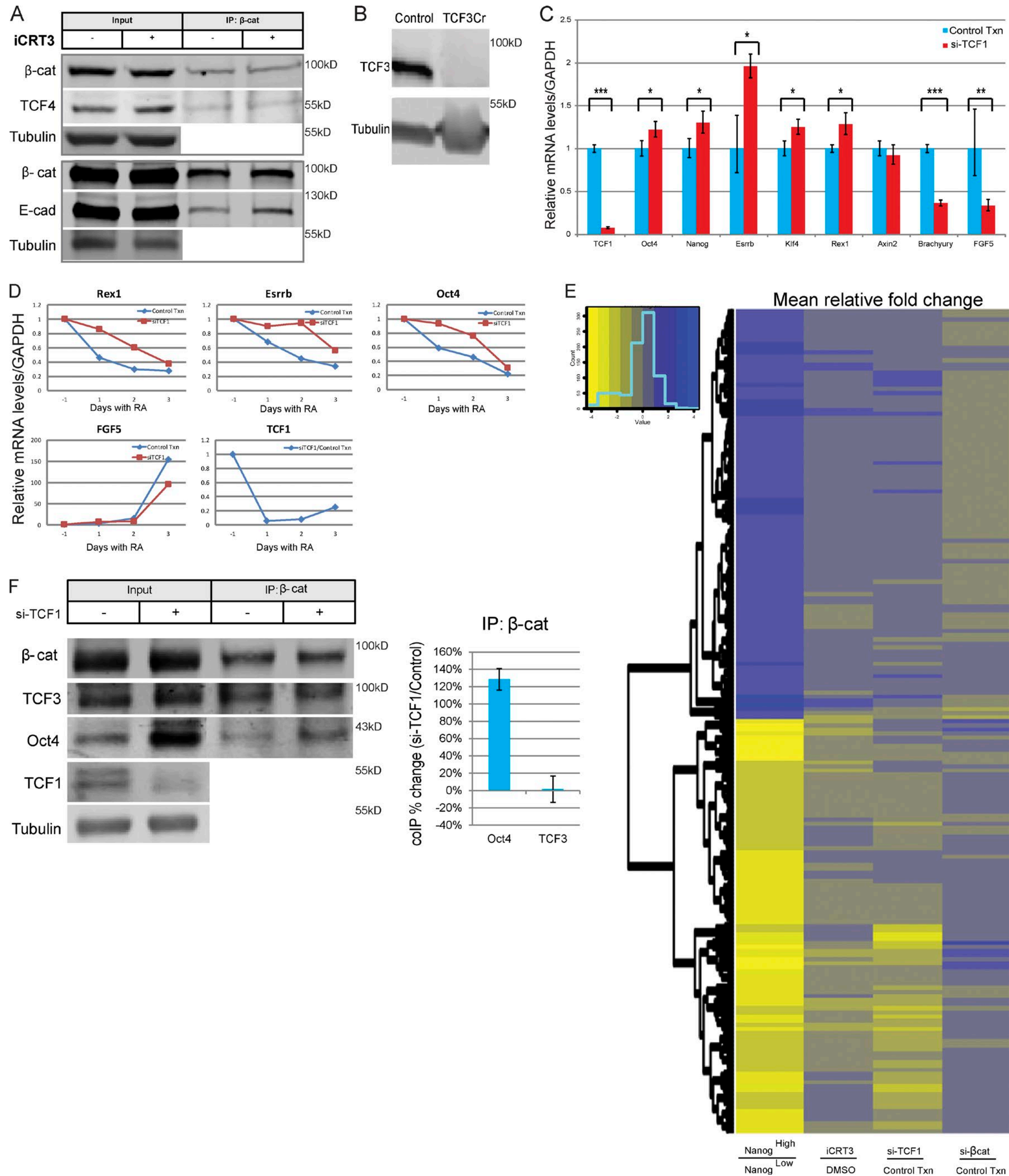

Figure S3. **Characterization of altered gene expression and β-catenin/TCF interactions mediated by iCRT3 and loss of TCF1 (related to Fig. 4 and Fig. 5).** (A) Representative Western blots of three CoIP experiments for TCF4 and E-cadherin proteins upon pull down with mouse anti–β-catenin from total cell lysates of differentiating cells (serum plus RA [S+RA]) maintained with iCRT3 or DMSO for 48 h. Tubulin is used as loading control. For a representative bar plot of quantification, refer to Fig. 3 B. (B) Western blot showing CRIPR/Cas9-mediated loss of TCF3 protein in clonal NG4 cells (TCF3Cr). Tubulin is used as loading control. (C) qPCR analysis for expression of pluripotency and differentiation markers upon RNAi-mediated loss of TCF1 in RA-treated mESCs. Mean ± SD of three replicates. (D) qPCR analysis shows that si-TCF1 treatment progressively delays loss of pluripotency markers such as Rex1, Esrrb, and Oct4 and induction of differentiation marker FGF5 during RA-mediated differentiation. Day (−)1 of RA treatment on the *x* axis marks day 0 of transfection with Scramble (control) and si-TCF1. Plots represent mean of two replicates. (E) Clustered heat map plot showing relative mRNA expression levels of 200 significantly differentially expressed genes between $Nanog^{High}$ and $Nanog^{Low}$ mESCs, during RA-induced differentiation upon treatment with iCRT3, si-TCF1, and si-β-catenin (relative to the respective controls). The heat map represents the mean fold changes of two replicates for $Nanog^{High}/Nanog^{Low}$, iCRT3/DMSO, and si-β-cat/control transfection and three replicates for si-TCF1/control transfection. For a list of genes, calculated significance, and corresponding mean log2 fold change values, refer to Table S3. (F) Representative Western blots for CoIP from total cell lysates of differentiating cells (S+RA) treated with si-TCF1. Tubulin is used as the loading control. Bar graph representing quantification of CoIP showing changes in protein–protein interactions of β-catenin with TCF3 and Oct4 respectively. Mean ± SD of two replicates. Refer to the Materials and methods for details. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table S1. **Primers used for expression analysis (qPCR primers)**

qPCR primer name	Primer pair sequences
GAPDH	5′-AACTTTGGCATTGTGGAAGG-3′
	5′-GGATGCAGGGATGATGTTCT-3′
NANOG	5′-TACCTCAGCCTCCAGCAGAT-3′
	5′-GCAATGGATGCTGGGATACT-3′
Oct4	5′-CCAATCAGCTTGGGCTAGAG-3′
	5′-CTGGGAAAGGTGTCCCTGTA-3′
REX1	5′-GGTGCAAGAAGAAGCTGAGG-3′
	5′-GTTTCGAGCTCTCCGTGAAG-3′
KLF4	5′-TGGTGCTTGGTGAGTTGTGG-3′
	5′-GCTCCCCCGTTTGGTACCTT-3′
Esrrb	5′-CAGGCAAGGATGACAGACG-3′
	5′-GAGACAGCACGAAGGACTGC-3′
E-cad	5′-CAGTTCCGAGGTCTACACCTT-3′
	5′-TGAATCGGGAGTCTTCCGAAAA-3′
FGF5	5′-TCTGGATCTCCTTTGCGTTT-3′
	5′-GGGCTTCGAAAGCACATTTA-3′
Gata4	5′-TTCCTCTCCCAGGAACATCAA-3′
	5′-GCTGCACAACTGGGCTCTACTT-3′
Sox17	5′-TTCTGTACACTTTAATGAGGCTGTTC-3′
	5′-TTGTGGGAAGTGGGATCAAG-3′
Cdx2	5′-TGGTGTACACAGACCATCAGC-3′
	5′-CCTTGGCTCTGCGGTTCT-3′
MixL	5′-AGTTGCTGGAGCTCGTCTTC-3′
	5′-TTCTGGAACCACACCTGGAT-3′
Brachyury	5′-CAGCTGTCGGGGAGCCTGG-3′
	5′-TGCTGCCTGTGAGTCATAAC-3′
Axin2	5′-GAAGAAATTCCATACAGGAGGAT-3′
	5′-GTCACTCGCCTTCTTGAAATAA-3′
Meox1	5′-GGAAGGAGAGGACAGCCTTC-3′
	5′-CCCTTCACACGTTTCCACTT-3′
Pdgfra	5′-AATCCTGCAGACGAGAGCAC-3′
	5′-GCCACCAAGGGAAAAGATTT-3′
Foxa1	5′-ACAGGGTTGGATGGTTGTGT-3′
	5′-TGTTGCTGACAGGGACAGAG-3′
Foxd3	5′-TCTTACATCGCGCTCATCAC-3′
	5′-TCTTGACGAAGCAGTCGTTG-3′
Otx2	5′-GGAGAGGACGACATTTACTAGG-3′
	5′-TTCTGACCTCCATTCTGCTG-3′
Gbx2	5′-ATGGCGCTCACCTCCACGC-3′
	5′-CATCTGAGCTGTAATCCA-3′
TCF1 (TCF7)	5′-AGCTTTCTCCACTCTACGAACA-3′
	5′-AATCCAGAGAGATCGGGGGTC-3′
TCF3(TCF7L1)	5′-ACGAGCTGATCCCCTTCCA-3′
	5′-CAGGGACGACTTGACCTCAT-3′

Available online as Excel files are Table S2, showing alphabetically organized identification numbers used for GSEA for RNA-seq data and functional enrichment analysis for differentially expressed genes in Nanog^{High} and Nanog^{Low} mESCs, and Table S3, showing the top 200 significantly differentially expressed (refer to padj values) genes organized by their log2 fold change in a comparison of Nanog^{High} versus Nanog^{Low} and the corresponding mean log2 fold change values for iCRT3, si-TCF1, and si-βcat relative to respective controls.