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Repression of Nanog Gene Transcription by Tcf3 Limits Embryonic Stem Cell Self-Renewal[▽]

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The dual function of stem cells requires them not only to form new stem cells through self-renewal but also to form lineage-committed cells through differentiation. Embryonic stem cells (ESC), which are derived from the blastocyst inner cell mass, retain properties of self-renewal and the potential for lineage commitment. To balance self-renewal and differentiation, ESC must carefully control the levels of several transcription factors, including Nanog, Sox2, and Oct4. While molecular mechanisms promoting transcription of these genes have been described, mechanisms preventing excessive levels in self-renewing ESC remain unknown. By examining the function of the TCF family of transcription factors in ESC, we have found that Tcf3 is necessary to limit the steady-state levels of Nanog mRNA, protein, and promoter activity in self-renewing ESC. Chromatin immunoprecipitation and promoter reporter assays showed that Tcf3 bound to a promoter regulatory region of the Nanog gene and repressed its transcriptional activity in ESC through a Groucho interaction domain-dependent process. The absence of Tcf3 caused delayed differentiation of ESC in vitro as elevated Nanog levels persisted through 5 days of embryoid body formation. These new data support a model wherein Tcf3-mediated control of Nanog levels allows stem cells to balance the creation of lineage-committed and undifferentiated cells.

The unlimited potential of the fertilized egg to contribute all differentiated cell types becomes progressively restricted in cell progeny as embryogenesis proceeds. Transplantation experiments and genetic lineage tracing have shown that the cells of the blastocyst inner cell mass (ICM) and their progeny in postimplantation epiblast maintain lineage potential to differentiate into each of the cell types in the adult organism, a property termed pluripotency (38, 53). Remarkably, the pluripotency and unlimited proliferative capacity of mouse ICM cells were discovered to be maintained during the in vitro culture of so-called embryonic stem cells (ESC) (15, 35). While in vitro self-renewal of ESC initially required coculture with feeder cells or conditioned media (15, 35), identification of the leukemia inhibitory factor (LIF) cytokine as the factor secreted by feeders allowed feeder-independent ESC self-renewal in LIF-conditioned media (60).

A combination of cell culture and gene ablation studies in mouse embryos has been used to identify intracellular determinants of ICM and ESC pluripotency. Ablation of the gene encoding the homeobox-containing Oct4 (MGI name, POU5F1) transcription factor in mouse embryos prevented proliferation of ICM cells and promoted differentiation into trophectoderm (43, 45). Reducing Oct4 expression in ESC to 50% promoted trophectoderm differentiation, and increasing Oct4 expression to 150% promoted endoderm differentiation (45). Ablation of the Sox2 HMG domain-containing protein produced embryos that failed to form an epiblast and ESC that failed to self-renew in vitro (2). A third transcription factor, the homeodomain-containing Nanog protein, has proven to be both necessary and sufficient for promoting ESC self-renewal (9, 39). Unlike Oct4, overexpression of Nanog did not induce differentiation but instead was sufficient to maintain pluripotency in the absence of LIF (LIF^-) (9, 39). Gene ablation studies showed that Nanog was required for the proliferation and pluripotency of mouse ICM and ESC (9, 39). Moreover, differentiation displayed by $Nanog^{+/-}$ ESC and RNA interference (RNAi)-mediated knockdown of Nanog to 30% of its normal levels showed that reduction of Nanog promoted differentiation in vitro (22, 23). Thus, although the levels of Oct4 must be maintained between a high and low threshold, Nanog protein must be maintained only above a threshold to promote ESC self-renewal.

Elucidation of extensive molecular genetic interactions between these three intrinsic determinants of ESC characteristics (Oct4, Nanog, and Sox2) has led to the hypothesis that these factors regulate a core transcriptional circuit for ESC self-renewal (4). This model was supported by the heterodimerization of Oct4 and Sox2 proteins on conserved DNA elements in promoter regulatory regions of several target genes expressed in ESC, including Fgf4, Oct4, Sox2, Nanog, Utf1, Opn, and Fbx15 (3, 30, 44, 46, 49, 55, 63). Analysis of the promoter regions for each Oct4, Sox2, and Nanog gene showed that the presence of an Oct4-Sox2 DNA-binding site was required for the activation of each promoter in ESC or embryonic carcinoma cells (11, 30, 49). In addition, small interfering RNA-mediated knockdown of Oct4 or Sox2 reduced the activity of Nanog, Oct4, and Sox2 promoters in ESC (11). A genome-wide assessment of chromatin binding sites for Oct4, Sox2, and Nanog in human ESC revealed that they not only bound one another's promoter region but also bound a highly overlapping set of target gene promoters (4). Taken together, these data suggested the existence of a feed-forward circuit whereby Oct4,

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Sox2, and Nanog all positively regulate one another's promoter activity, thus promoting self-renewal. Given that the embryonic function of ICM and epiblast cells is to differentiate into lineage-committed cells according to spatiotemporal constraints and molecular stimuli, the existence of such a feed-forward circuit would predict inherent stabilizing mechanisms limiting levels of these core transcription factors. In particular, Nanog's ability to promote self-renewal when overexpressed in ESC could require negative feedback to prevent differentiation defects; such a mechanism has not yet been described as functioning within self-renewing ESC.

Tcf proteins (Tcf1, Tcf3, Tcf4, and Lef1 in mammals) are the DNA-binding transcriptional regulators of the canonical Wnt signaling pathway. Through a highly conserved HMG domain and an amino-terminal β-catenin interaction domain, each Tcf protein can promote transcription of downstream targets when Wnt-stabilized β-catenin accumulates intracellularly (6, 40, 57). In the absence of stabilized β-catenin, Tcf proteins have been shown to function as transcriptional repressors by interacting with corepressor proteins, such as Groucho, CtBP, and HIC-5 (5, 7, 8, 17, 51). Direct relationships between the biochemical properties of Tcf proteins and their physiological effects have been demonstrated by several studies expressing mutated forms of the proteins in model organisms (28, 36).

Although a role for Tcfs in ESC has not yet been identified, the effects of alterations to Wnt signaling on ESC characteristics have suggested that these downstream components of the pathway could play an important role in regulating stem cell characteristics. Treatment of mouse ESC (mESC) with Wnts or Wnt pathway stimulators, such as the glycogen synthase kinase 3-β inhibitor, BIO, promoted self-renewal under LIF^- conditions (1, 20, 52). Similarly, activation of the Wnt pathway by mutations affecting the adenomatous polyposis coli protein inhibited in vitro mESC differentiation and neural differentiation in teratoma assays (27). In contrast, Wnt3a stimulation or β-catenin overexpression promoted neural differentiation in mESC grown at high densities (47). In addition, treatment with Wnts provided a transient stimulation of human ESC proliferation, but it did not prevent differentiation during long-term culture (14). The contradictory nature of these observations has suggested that Wnt signaling could have multiple and/or complex effects on ESC characteristics and that elucidation of the molecular mechanisms affecting Wnt signaling in ESC could provide a better understanding of how these effects occur. It remains unknown whether the Wnt signaling pathway functionally affects transcription factors Nanog, Oct4, and Sox2 in regulating stem cell self-renewal.

The purpose of this study was to determine whether Tcf factors affect the process of self-renewal and differentiation of ESC. Although expression of all four Tcf mRNAs was detected in mESC, Tcf3 accounted for nearly two thirds of total Tcf expression. Removal of Tcf3 by genetic ablation caused ESC to delay differentiation in favor of self-renewal. These defects were associated with elevated levels of Nanog protein, mRNA, and promoter activity in self-renewing ESC. The mechanism causing increased Nanog required Tcf3 repressor activity and Tcf3 binding to the Nanog promoter DNA. The effects of Tcf factors on Nanog promoter activity link Wnt signaling to the proposed feed-forward circuit regulating ESC self-renewal. By limiting the level of Nanog in self-renewing ESC, Tcf3 functions in ESC to allow appropriate and efficient responses to differentiation-promoting cues.

MATERIALS AND METHODS

Embryonic stem cell culture and differentiation. ESC were maintained under standard culture conditions (37). For maintenance of ESC lines, cells were propagated on a feeder layer of gamma-irradiated mouse embryonic fibroblasts in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum (Atlanta Biologicals), 2 mM L-glutamine (Gibco), 10 mM HEPES (Gibco), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 0.1 mM nonessential amino acids (Gibco), 0.1 mM β-mercaptoethanol (Gibco), and 1,000 U/ml LIF (Chemicon). Media were replaced daily, and every 2 to 3 days single-cell suspensions of ESC were created by trypsin treatment (0.25% trypsin-EDTA; Gibco) and passaged onto new feeders. A blastocyst-derived (BD) $TCF3^{-/-}$ ESC line was created by recovering 4.5-day embryos from $TCF3^{+/-}$ double heterozygous mating. Blastocysts were recovered in phosphate-buffered saline (PBS) and placed on mitotically inactivated feeders with LIF-containing embryonic stem (ES) media. Outgrowths from the ICM were isolated and cultured as described previously (42). The genetic background for the mouse lines consisted of a mixture of C57/Bl6 and 129/Sv strains.

To generate $TCF3^{-/-}$ ESC lines with a pure 129/Sv background, GS1 ($TCF3^{+/+}$) ESC (Research Genetics) were subjected to reiterative rounds of homologous recombination as described for the creation of the $TCF3^{+/-}$ ESC line used for generating the TCF3 knockout mouse strain (37). Southern blot analysis and PCR-based screening to identify gene targeting events were identical to those previously described (37).

Tcf3 expression was restored in the BD $TCF3^{-/-}$ and HR-G4 $TCF3^{-/-}$ ESC lines by electroporation (280 mV, 500 μF) of 20 μg of linearized pCDNA3-Tcf3 expression plasmid DNA with a Gene Pulser apparatus (Bio-Rad). Selection was started 24 h after electroporation of 10^7 ESC by adding G418 (300 μg/ml) to media. Media were replaced daily, and individual colonies were isolated after 5 days of growth. Each clone was screened for expression of Tcf3 protein by Western blot analysis of protein extracts (36). Tubulin protein levels were used as an internal control for gel loading and transfer efficiency.

For the embryoid body (EB) differentiation assay, ESC maintained on feeders were trypsinized and plated on gelatin for 30 min to deplete contaminating mouse embryonic fibroblasts. At the end of this preplating step, 1×10^6 nonadherent cells were induced to differentiate into EBs by plating them in bacteriological-grade petri dishes without LIF. Media were replaced every second day. Samples (1/10 volume of total culture) were taken daily and processed for protein or RNA extraction.

Stealth RNAi duplexes (Invitrogen) designed against mouse Nanog (CCAG UGGAGUAUCCCAGCAUCCAUU) or a control RNAi (GGAAGACUAGA GGCGGUCAUGAGUU) were used to specifically downregulate Nanog expression levels. For transfection, ESC were trypsinized, pelleted by centrifugation, resuspended in ES media without antibiotics, and combined with Lipofectamine 2000 transfection complexes of control or increasing amounts of Nanog RNAi (1 to 15 nM) following the manufacturer's protocol (Invitrogen). Transfected cells were plated on gelatinized plates and either processed for protein samples or used in a colony differentiation assay 12 h after transfection. For the alkaline phosphatase colony assay, transfected ESC were plated on gelatinized wells of a six-well dish (1,000 cells/well) in media without LIF and fed daily. Colonies were stained for alkaline phosphatase activity after 4 days. Briefly, cells were washed once with PBS, fixed in citrate-acetate-formaldehyde (18 mM sodium citrate, 9 mM NaCl, pH 3.6, 65% acetone, 37% formaldehyde) for 45 seconds, washed three times with water, and stained with nitro blue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) solution (Promega). For each well, 100 colonies were scored for levels of alkaline phosphatase activity and morphology into three categories of undifferentiated (high alkaline phosphatase activity and tight colony borders), partially differentiated (intermediate alkaline phosphatase activity), and differentiated (loss of activity and absence of ES colony morphology) colonies.

Quantitative and semiquantitative PCR analysis. For PCR analysis of ESC or lineage markers, cells were collected and washed twice with 1× PBS. Total RNA was isolated with TRIzol reagent (Invitrogen), and 4 μg was used as a template for cDNA synthesis. Reverse transcription was carried out with oligo(dT) primers (0.5 μg/μl), using a SuperScript III first-strand cDNA synthesis kit (Invitrogen). PCRs for the lineage markers were optimized to allow semiquantitative comparisons within the log phase of amplification and typically used 1 μl of cDNA sample. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers were used to control for loading. PCR products were analyzed by standard agarose gel electrophoresis, visualized by ethidium bromide staining, and pho-

todocumented with the Gel Doc XR system (Bio-Rad). Control reactions using mock cDNA preparations lacking reverse transcriptase were run in parallel for each analysis to ensure the absence of genomic DNA contamination.

Quantitative real-time PCRs (qPCRs) were performed with the iTaq SYBR green supermix (Bio-Rad) and an iCycler apparatus (Bio-Rad). Amplification was achieved by 40 cycles of 95°C for 30 s followed by 60°C for 30 s. To identify potential amplification of contaminating genomic DNA, control reactions using mock cDNA preparations lacking reverse transcriptase were run in parallel for each analysis. To ensure specificity of PCR, melt curve analyses were performed at the end of all PCRs. The relative amount of target cDNA was determined from the appropriate standard curve and divided by the amount of GAPDH cDNA present in each sample for normalization. For Tcf/Lef qPCR, genomic DNA was used to establish a standard curve where 1 μg of DNA was calculated to contain 3×10^5 copies of each gene and relative copy number was calculated as described before. All PCRs had an efficiency of 80% or higher. Each sample was analyzed in duplicate, and results were expressed as means ± standard deviations. Primer sequences and reaction conditions are available for each primer set upon request.

Luciferase assays. Cells were plated 24 h before transfection on 24-well plates coated with 0.1% gelatin. They were transfected with Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. After 24 h, cells were harvested and lysed in 1× passive lysis buffer (Promega). Luciferase activity was measured using a Clarity luminometer (Bio-Tek) and a dual-luciferase reporter assay system (Promega). Each transfection was carried out in duplicate and repeated at least twice. Relative activity was calculated as the ratio of the reporter plasmid to *Renilla* luciferase activity (pRL-CMV), which was cotransfected as a control.

Western blot analysis. ESC or EB samples were collected and washed twice with cold PBS. Lysates were made using RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% sodium dodecyl sulfate [SDS], 1.0% Triton X-100, 1.0% deoxycholate, 5 mM EDTA, and protease inhibitors). Protein concentration was determined using the Coomassie Plus protein assay reagent (Pierce). Protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% Carnation brand nonfat dry milk in PBS-0.05% Tween and incubated with primary antibody in PBS-0.05% Tween-5% milk overnight at 4°C.

The primary antibodies and dilutions used were mouse antitubulin at 1/3,000 (E7; DSHB), mouse anti-Oct4 at 1/1,500 (AB/POU31-M; BD Biosciences), rabbit anti-Nanog at 1/500 (ab21603; Abcam), rabbit anti-STAT3 (catalog no. 9132; Cell Signaling Technologies), rabbit anti-P-STAT3 (catalog no. 9131; Cell Signaling Technologies), and guinea pig anti-Tcf3 (36). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit, -mouse, and -guinea pig antibodies, all at a 1/3,000 dilution (The Jackson Laboratory). Signals were detected with ECL Western blotting detection reagents (Amersham Biosciences).

ChIP assays. BD TCF3$^{-/-}$ and BD TCF3$^{-/-}$-plus-pCDNA3-Tcf3 ESC strains were cultured on inactivated feeders in LIF-containing ES media and processed for chromatin immunoprecipitation (ChIP) analysis. A total of 2×10^7 cells were cross-linked in situ by the addition of 37% formaldehyde (Fisher Scientific) to a final concentration of 1% and incubated at 25°C for 10 min. The reaction was quenched by the addition of glycine (0.125 M final concentration) for 5 min, and cells were washed twice with ice-cold PBS. Cells were scraped in cold PBS containing protease inhibitors (Sigma) and resuspended in 2.0 ml lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, protease inhibitors) for 10 min on ice. Chromatin was sheared to a 300-bp average size (more than 90% of DNA fragments were smaller than 700 bp) by sonication on ice with a Misonix 600W sonicator fitted with a 3-mm stepped microtip for 25 pulses of 15 seconds at 10% power. Cellular debris was removed by centrifugation (13,000 rpm, 4°C, 10 min), and the supernatant was diluted 1:10 in 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris-HCl, pH 8.

All samples were processed for immunoprecipitation essentially as described for the Upstate cell ChIP assay protocol (catalog no. 17-295; Lake Placid, NY). For immunoprecipitation of Tcf3, 10 μl of guinea pig anti-Tcf3 antisera was used (36). Control precipitations using goat anti-Sox2 (Y-17; Santa Cruz) were used to ensure that chromatin samples were intact and that the Nanog proximal promoter region could be detected in all samples (not shown). Following elution of immunoprecipitated chromatin, cross-links were reversed by the addition of proteinase K (10 μg) and NaCl (200 mM) and incubation at 65°C for 4 h. Following phenol-chloroform extraction and ethanol precipitation, DNA was dissolved in 50 μl Tris-EDTA. For each PCR, 2.5 μl of DNA sample was used.

RESULTS

Tcf3 is the most abundant Tcf factor in ESC. Although Wnt-stabilized β-catenin has been shown to support self-renewal of several types of stem cells (16, 34, 48, 59), it has also been found to promote differentiation in several cell lineages (12, 29, 31). One important molecular mechanism that is frequently overlooked in analyses is the function of the Tcf proteins, which are biochemically affected by stabilized β-catenin. Tcf proteins can have multiple effects on target genes in terms of transcriptional repression and activation. The functions of individual Tcf factors depend upon protein interactions that are either common to all members (e.g., β-catenin and DNA binding) or variable among family members (e.g., Groucho and CtBP binding) (5–8, 36, 51). In the skin, for example, Tcf3 functions primarily as a transcriptional repressor whereas Lef1 functions as an activator (36). Importantly, cells expressing either Lef1 or Tcf3 behave differently when they encounter stabilized β-catenin; Lef1-expressing cells of the precortex terminally differentiate, whereas Tcf3-expressing stem cells self-renew (36). Therefore, to determine the function of Tcf factors within a cellular context, it is important to identify which Tcf family members are expressed and the relative level of expression for each Tcf gene.

To determine the level of each Tcf mRNA in ESC, a qPCR assay was developed. Primers were designed to amplify regions corresponding to sequences within a single exon of each TCF gene. Since the mouse genome contains an equal number of copies of each TCF gene (approximately 3×10^5 copies of each gene per microgram of DNA), genomic DNA was used to generate a standard curve for each Tcf qPCR assay. This approach allowed the absolute copy number (relative to GAPDH loading control) for each Tcf mRNA to be calculated; thus, the relative abundance of each Tcf mRNA was directly compared. Under self-renewal conditions, expression of each TCF gene was detected in ESC with this assay (Fig. 1A). Tcf3 mRNA was the predominant TCF family member, comprising 60% of all Tcf mRNA detected. Tcf1 (23%) and Lef1 (12%) were expressed at lower levels than Tcf3. Under self-renewal conditions, Tcf4 (5%) was nearly undetectable in ESC.

To identify individual Tcf factors as candidate regulators of either self-renewal or differentiation of ESC, the level of each Tcf transcript was detected in ESC subjected to in vitro differentiation. EB formation used cell aggregation and the absence of LIF to promote differentiation into cell types corresponding to the primary germ layers of ectoderm, mesoderm, and endoderm. Total RNA was purified from samples of ESC subjected to a standard EB differentiation assay, and cDNA was made for qPCR analysis. As EB differentiation progressed, Tcf1 and Lef1 decreased with similar kinetics (Fig. 1B). Tcf3 remained at pre-EB levels during the first day and then diminished similarly to Tcf1 and Lef1. In contrast, Tcf4 expression rose sharply during EB differentiation; the first significant increase was detected at the third day of differentiation. Comparison of the relative level of each Tcf mRNA during EB differentiation showed that the Tcf3/Tcf1/Lef1 ratio remained relatively constant throughout the 7 days of EB differentiation (Fig. 1C). In contrast, Tcf4 increased substantially (5% on day 0 to 27% on day 5). Based on these expression patterns, Tcf1, Lef1, and Tcf3 each had a potential role in the self-renewal and

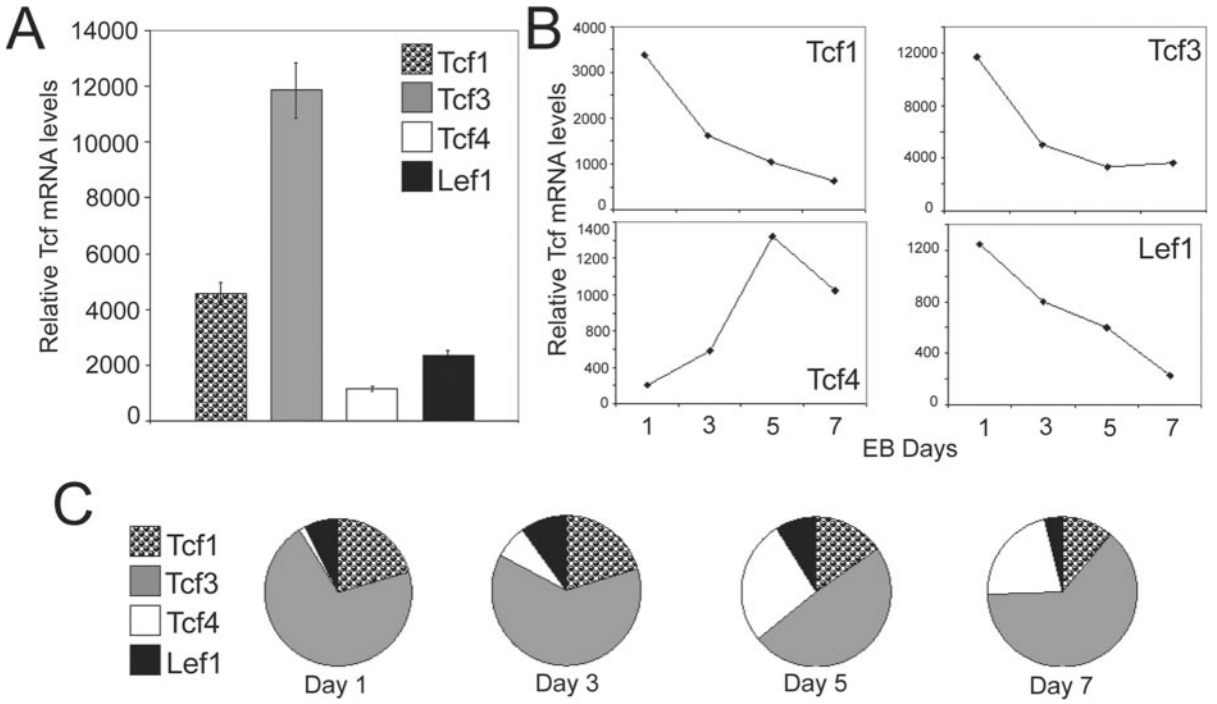

FIG. 1. TCF expression in embryonic stem cells. qPCR was used to measure the levels of each TCF gene in wild-type cells. The relative efficiency of each qPCR was accounted for by calculating copy numbers of TCF mRNA from a standard curve of known target concentrations. Values are expressed as the means ± standard deviations of the TCF mRNA divided by GAPDH levels, which were also determined by qPCR. (A) Amount of each TCF mRNA expressed in self-renewing ESC. (B) Amount of each TCF mRNA expressed during embryoid body differentiation. (C) Pie charts displaying the proportion of each TCF mRNA to the total amount of all TCF genes detected from differentiating embryoid body samples isolated at the indicated days.

maintenance of ESC characteristics, while Tcf4 was more likely to function during EB differentiation.

Creation of multiple, independently derived TCF3$^{-/-}$ ESC lines. In order to determine whether Tcf factors affect ESC characteristics, it was logical to focus on the function of Tcf3 for several reasons. Tcf3 was the most abundantly expressed Tcf family member in both self-renewing ESC and differentiating EB samples (Fig. 1). Analysis of knockout mouse mutants showed that Tcf3 was the only Tcf factor uniquely required for early mouse embryogenesis; in TCF3$^{-/-}$ embryos, the progenitor of the ICM, the epiblast, displayed defective differentiation during gastrulation (37). Tcf3 has been shown to be expressed in several types of stem cells, including those from neural, hematopoietic, and hair follicle origins (26, 56). These data suggested that examining the function of Tcf3 could elucidate a role in ESC maintenance that could also relate to other types of stem cells.

To determine whether Tcf3 played a role in ESC self-renewal or differentiation, mutant ESC lines lacking a functional TCF3 gene were created. Since individual ESC lines can display distinctly different characteristics caused by artifacts of culture conditions and/or complicated genetic contributions from different mouse strain backgrounds, several independently derived TCF3$^{-/-}$ ESC lines were created and used for the experiments in this study. One set of TCF3$^{-/-}$ ESC lines was generated using homologous recombination (HR) with the previously described TCF3 knockout vector and a TCF3$^{+/-}$ ESC line that had been used to engineer mouse mutants (36). Integration of the knockout vector DNA into the TCF3 locus introduced a LoxP site between exons 1 and 2 and a PGK-NEO cassette flanked by LoxP sites between exon 2 and exon 3 (36). Of 288 G418-resistant clones picked, 7 had integrated the knockout vector sequence into the TCF3 locus. Three of those seven positive clones were determined by PCR analysis to have integrated into the wild-type allele, resulting in a TCF3$^{-/Neo}$ genotype (36). One clone was transiently transfected with a Cre recombinase expression DNA, and PCR analysis of 288 new clones picked under nonselective conditions identified five clones that had successfully undergone Cre-mediated excision of exon 2 and the PGK-NEO cassette, resulting in ESC lines with an HR TCF3$^{-/-}$ genotype (not shown). Protein samples from the four mutants shown in Fig. 2A demonstrated that they no longer produced the Tcf3 protein.

An independent TCF3$^{-/-}$ ESC line was recovered from an outgrowth of blastocyst-stage embryos from matings between two TCF3$^{+/-}$ mice with a mixed C57Bl6 and 129Sv genetic background (36). The single BD TCF3$^{-/-}$ ESC line derived from a blastocyst was confirmed to lack exon 2 of the TCF3 gene by PCR (not shown) and Tcf3 protein by Western blot analysis (Fig. 2A). Tcf3 expression was returned to BD TCF3$^{-/-}$ by stable transfection with the pCDNA3-mycTcf3 expression construct (Fig. 2A). The level of expression of Tcf3 was similar to that of the TCF3$^{+/+}$ ESC line, as determined by Western blot and qPCR analyses of mRNA (Fig. 2A and B).

Previous studies have shown that Tcf proteins can regulate the expression of other Tcf family members. In particular,

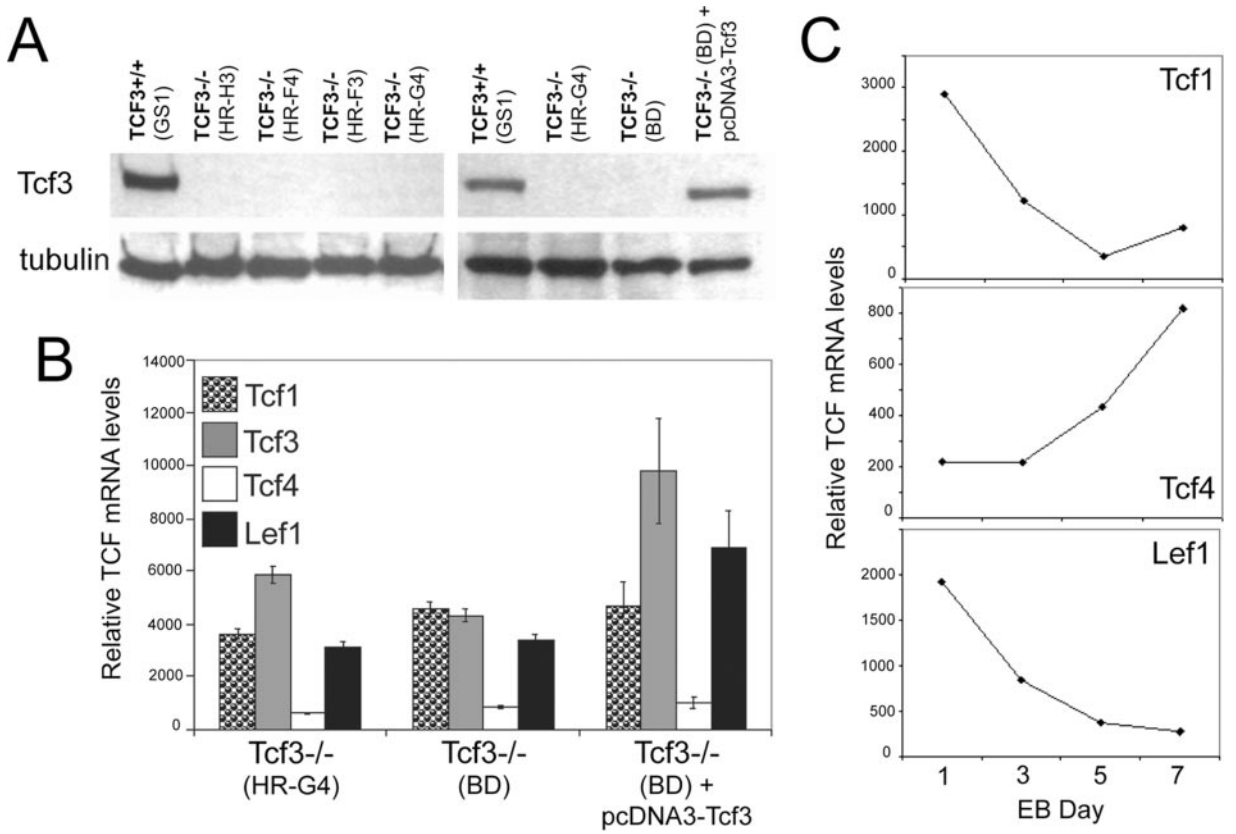

FIG. 2. TCF expression in TCF3$^{-/-}$ embryonic stem cell lines. Several TCF3$^{-/-}$ ESC lines were generated by homologous recombination (HR) or blastocyst derivation (BD). (A) Western blot analysis using guinea pig anti-Tcf3 (36) or mouse antitubulin (DSHB) antibodies to determine levels of Tcf3 protein in protein extracts from the indicated cell lines. HR-H3, -H4, -F3, and -G4 refer to individual HR-derived TCF3$^{-/-}$ ESC lines. "TCF3$^{-/-}$ (BD) + pCDNA3-Tcf3" refers to an ESC line in which Tcf3 expression was returned to the BD TCF3$^{-/-}$ cells by selection of stable transfection of the pCDNA3-Tcf3 expression construct. (B) Quantitative PCR measurement of each TCF mRNA relative to GAPDH in TCF3$^{-/-}$ strains under self-renewal conditions (compare to TCF3$^{+/+}$ ESC in Fig. 1A). (C) Quantitative PCR measurement of indicated TCF mRNA in HR-G4 TCF3$^{-/-}$ embryoid bodies (compare to TCF3$^{+/+}$ ESC in Fig. 1B).

ectopic Wnt pathway stimulation caused the increased expression of Tcf1 and Lef1 in intestine (25, 50). Since all Tcf proteins can bind the same DNA sequence and interact with β-catenin, interpretation of the results regarding the role of Tcf3 by mutational analysis necessitated the quantitation of Tcf gene expression in TCF3$^{-/-}$ ESC lines. Potential effects on Tcf mRNA levels were examined by qPCR in ESC (Fig. 2B) and differentiating EB (Fig. 2C). Tcf3 mRNA was detected in TCF3$^{-/-}$ ESC because the qPCR analysis did not involve exon 2, which was deleted by mutation. Note that the levels of Tcf3 mRNA diminish by approximately 50% in TCF3$^{-/-}$ ESC; it remains unknown whether the decrease is caused by mRNA instability or decreased transcription. Comparison of the relative levels of Tcf1, Tcf4, and Lef1 mRNAs in TCF3$^{-/-}$ ESC (Fig. 2B) to those in TCF3$^{+/+}$ ESC (Fig. 1A) revealed that they were similar between the different lines. Lef1 was increased in both TCF3$^{-/-}$ lines by approximately 50%; however, this was most likely not directly caused by the absence of Tcf3, since Lef1 is increased threefold in the BD TCF3$^{-/-}$-plus-pCDNA3-Tcf3 cell line. Upon differentiation of ESC, the levels of Tcf mRNAs changed similarly in TCF3$^{+/+}$ and TCF3$^{-/-}$ EBs; however, there were potentially important differences. Tcf4 expression does not reach the same peak levels by day 7, and its first increase is delayed until day 5 in TCF3$^{-/-}$ ESC (Fig. 2C). Taken together, these data show that independently derived TCF3$^{-/-}$ ESC lines are similar to TCF3$^{+/+}$ ESC in the levels of Tcf mRNAs and the changes in these mRNAs during EB differentiation. Therefore, any differences between TCF3$^{-/-}$ ESC compared to TCF3$^{+/+}$ controls likely reflect the absence of Tcf3 directly as opposed to compensatory effects of other Tcf gene products.

Tcf3 is not required for ESC self-renewal. Activation of the Wnt pathway by either chemical treatments (BIO, purified Wnt) or genetic effect (adenomatous polyposis coli mutation) inhibited mESC differentiation in vitro (27, 52). These data suggested that Wnt is sufficient to promote self-renewal in some contexts. Although treatment with a Wnt inhibitor, sFRP, caused ESC to adopt characteristics of neural differentiation, there has been little direct evidence suggesting that Wnt signaling is required for self-renewal (1). Since Tcf3 was found to be the most abundant Tcf in ESC and since Tcfs directly regulate Wnt signaling activity, we tested the hypothesis that Tcf3 was required for ESC self-renewal. In order to rule out effects that could be caused by strain-specific and Tcf3-independent contributions, multiple ESC lines were examined. The proliferation of each TCF3$^{-/-}$ line, BD and HR-

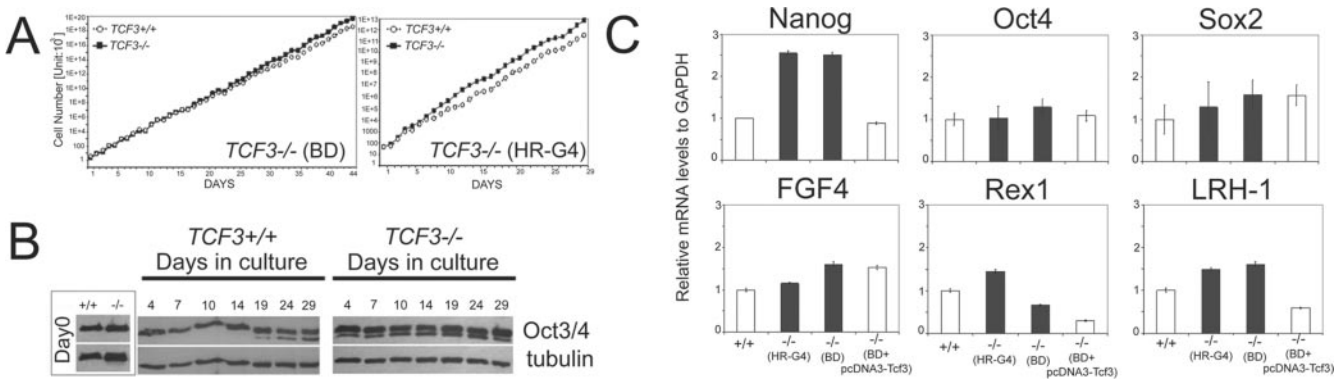

FIG. 3. Ability of TCF3$^{-/-}$ embryonic stem cells to self-renew in vitro. (A) Population doubling of cultures of indicated TCF3$^{+/+}$ and TCF3$^{-/-}$ ESC lines grown on gelatinized plates in ES media containing 1,000 U/ml LIF. (B) Protein samples were isolated from HR-G4 TCF3$^{+/+}$ and TCF3$^{-/-}$ ESC cultures used to measure cell proliferation in panel A, and the amount of Oct4 was measured by Western blot analysis using a mouse anti-Oct3/4 antibody (Chemicon). Tubulin was used as a loading control. (C) Quantitative PCR analysis of previously identified stem cell marker genes expressed in TCF3$^{+/+}$, HR-G4 TCF3$^{-/-}$, BD TCF3$^{-/-}$, and BD TCF3$^{-/-}$-plus-pCDNA3-TCf3 ESC lines is shown. Each value is expressed as a mean ± standard deviation for the stem cell gene divided by the GAPDH level.

G4, was assessed by counting cells after growth on gelatinized plates for a minimum of nine passages under normal self-renewal conditions. No significant difference in proliferation was observed between any of these lines when LIF was included in the culture media (Fig. 3A). Western blot analysis revealed that all ESC lines tested maintained expression of the stem cell marker Oct4 throughout the course of the experiment (Fig. 3B).

To determine whether Tcf3 was required to maintain stem cell characteristics during proliferation, the expressions of several stem cell markers were examined by qPCR (Fig. 3C). Nanog, Oct4, and Sox2 have been proposed to compose a feed-forward circuit promoting ESC characteristics and allowing differentiation (4). Nanog expression was maintained in TCF3$^{-/-}$ ESC; moreover, it displayed a 2.5-fold increase in the TCF3$^{-/-}$ lines compared to that in each of the Tcf3^{+} lines. Oct4 and Sox2 were similarly expressed in each Tcf3^{+} and Tcf3^{-} ESC line examined. Fgf4 and Rex1 are two markers of ESC, although they have not been shown to be genetically required for self-renewal. Fgf4 expression was higher in BD TCF3$^{-/-}$ and its Tcf3^{+} derivative than in TCF3$^{+/+}$ and HR-G4 TCF3$^{-/-}$ lines, suggesting that this effect was likely caused by strain differences and not the absence of Tcf3. In contrast, Rex1 was 1.4-fold higher in HR-G4 TCF3$^{-/-}$ than in TCF3$^{+/+}$ and 2.2-fold higher in BD TCF3$^{-/-}$ than in its pCDNA3-Tcf3-containing derivative, suggesting that the increased Rex1 levels depended upon the absence of Tcf3. The effects of Tcf–β-catenin complexes on Rex1 promoter activity have previously been described, and these results are consistent with those previously reported data (52). In addition, we have observed Tcf3-mediated repression of Rex1 promoter activity in ESC (data not shown).

LRH-1 has been shown to positively regulate expression of Oct4 in ESC and ICM. The absence of LRH-1 in ESC resulted in the increased rapidity of differentiation and loss of Oct4 (18). In addition, it binds to the same DNA sequences as germ cell nuclear factor (GCNF), which is required for repression of Nanog during retinoic acid-induced differentiation of ESC (18, 19). The expression of LRH-1 was elevated 1.4- and 2.7-fold in TCF3$^{-/-}$ ESC compared to that in controls, suggesting that Tcf3 limited LRH-1 levels (Fig. 3C). However, this increase in LRH-1 was not sufficient to affect Oct4 levels in self-renewing ESC.

Since TCF3$^{-/-}$ ESC lines continued to self-renew and express stem cell marker genes, we concluded that Tcf3 was not required for ESC self-renewal. Oct4 and Sox2 mRNA levels remained unaffected by the loss of Tcf3. Moreover, TCF3$^{-/-}$ ESC contained abnormally high levels of the Nanog transcription factor, Rex1, and LRH-1. These data suggest the possibility that Tcf3 is required not for self-renewal but instead for proper differentiation of ESC. The effects of these alterations in expression and their molecular mechanism are elucidated below.

Stem cell differentiation is delayed by the absence of Tcf3. To determine the effects of Tcf3 absence on lineage commitment, TCF3$^{+/+}$ and TCF3$^{-/-}$ ESC were subjected to in vitro differentiation. EB assays were performed to differentiate cells by aggregation in the absence of LIF. No apparent differences in the efficiency of forming EBs were observed between TCF3$^{-/-}$ and TCF3$^{+/+}$ cell lines. Total RNA was isolated from EBs daily and used to make cDNA for semiquantitative PCR analysis of markers defining developmental lineages (Fig. 4A). Endoderm markers (GATA4, GATA6, α-fetoprotein, and FoxA2) were first detected in TCF3$^{+/+}$ EBs after 3 days of differentiation. The mesodermal marker, Brachyury, was first detected after 7 days of EB differentiation. Embryonic ectoderm (FGF5) and neural (Sox1) markers were detected in TCF3$^{+/+}$ EBs starting at days 3 and 6, respectively. In TCF3$^{-/-}$ EBs, GATA4 and GATA6 remained at very low levels throughout 8 days of differentiation and α-fetoprotein displayed a delay in its appearance such that levels of α-fetoprotein expressed by day 5 in TCF3$^{+/+}$ EBs were not detected until day 8 in TCF3$^{-/-}$ EBs. Brachyury expression was not detected throughout the course of the EB experiment (Fig. 4A); however, it was detected in longer-term EB differentiation experiments (not shown). The expression levels of Sox1, FGF5, and FoxA2 were also delayed in TCF3$^{-/-}$ EBs compared to those in the TCF3$^{+/+}$ control. Thus, the absence of

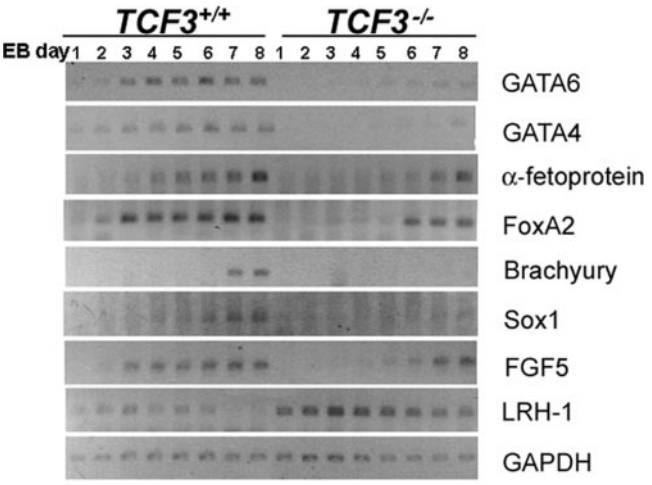

FIG. 4. Delayed differentiation of TCF3$^{-/-}$ embryonic stem cells. TCF3$^{+/+}$ and TCF3$^{-/-}$ ESC were subjected to differentiation through aggregation in EBs. RNA isolated from EB samples at the indicated days (1 to 8) was used to make cDNA and subjected to semiquantitative PCR analysis with the primers detecting genes listed on the right. Endoderm (GATA6, GATA4, α-fetoprotein, and FoxA2), mesoderm (Brachyury), ectoderm (FGF5), neural (Sox1), and stem cell (LRH-1) markers and loading control (GAPDH) are shown. Mock reactions containing mRNA processed without reverse transcriptase (not shown) demonstrated that the signal shown was dependent on cDNA.

Tcf3 caused a significant delay in differentiation, but it did not completely prevent differentiated cell types from occurring in these in vitro assays. These results are consistent with observations from the TCF3$^{-/-}$ mutant mouse embryos that are capable of forming differentiated cell types (mesoderm and endoderm) but display defects in the appearance of these cell types within a three-dimension basic body plan (37).

Nanog is required for delayed differentiation. The analysis of ESC markers indicated that Nanog mRNA was increased in each of the TCF3$^{-/-}$ ESC lines examined (Fig. 3C). LRH-1 and Rex1 levels were also elevated, but neither of those factors has been shown to be sufficient to prevent ESC differentiation when overexpressed. Nanog is the only transcription factor shown to be necessary for self-renewal and sufficient to prevent differentiation of ESC in the absence of LIF/Jak/Stat signaling (9, 39). To examine the possibility that increased Nanog mRNA expression could cause the differentiation delay in TCF3$^{-/-}$ EBs, the amount of Nanog protein in the collection of TCF3$^{-/-}$ ESC lines was examined by Western blot analysis (Fig. 5A). There was a direct correlation between the presence of Tcf3 and the level of Nanog protein; each TCF3$^{-/-}$ ESC line contained between 1.8- and 2.4-fold-higher levels of Nanog protein than its corresponding Tcf3-expressing control (Fig. 5A).

To examine how the absence of Tcf3 could affect Nanog expression during differentiation, the amount of Nanog mRNA was determined by qPCR during EB formation. Nanog levels diminish sharply in the first 2 days of differentiation in TCF3$^{+/+}$ samples (Fig. 5B). By comparison, Nanog mRNA diminished slowly in TCF3$^{-/-}$ samples. Combined with the higher level of Nanog prior to differentiation (Fig. 3C), the slower decline in Nanog levels caused TCF3$^{-/-}$ EBs to maintain higher levels of Nanog mRNA until day 5 of differentiation. The appearance of differentiation markers in TCF3$^{-/-}$ EBs (Fig. 4) coincided with the reduction of Nanog in TCF3$^{-/-}$ EBs (Fig. 5) to levels below those observed for self-renewing TCF3$^{+/+}$ ESC (Fig. 3 and 5).

The increased levels of Nanog and the timing of reduction during EB differentiation suggested that increased Nanog could be necessary for TCF3$^{-/-}$ ESC to display delayed differentiation. To test this possibility directly, the levels of Nanog were reduced in TCF3$^{-/-}$ ESC cultures by transfection with small interfering RNA molecules (RNAi). Transfection of TCF3$^{-/-}$ ESC with increasing concentrations of Nanog-specific RNAi complexes reduced the level of Nanog protein in a dose-dependent manner (Fig. 5C). The Nanog and mock RNAi-transfected ESC were trypsinized to single-cell suspensions and plated onto gelatinized dishes at a clonal density in LIF^{-} media. The retention of the ESC marker, alkaline phosphatase activity, was used to assess the level of colony differentiation. After 4 days of colony growth, a substantial difference (32%) in the number of undifferentiated colonies was observed in TCF3$^{+/+}$ ESC compared to that in TCF3$^{-/-}$ ESC (Fig. 5D). Comparison of the ratio of alkaline phosphatase-positive colonies derived from each of the treatments revealed that RNAi-mediated knockdown of Nanog in TCF3$^{-/-}$ ESC inhibited LIF-independent self-renewal of ESC in this colony assay. Interestingly, although the RNAi treatments caused a clear dose-dependent reduction of Nanog protein, the effects on self-renewal were not clearly dose dependent. For example, the TCF3$^{-/-}$ ESC transfected with 15 nM Nanog RNAi had approximately fivefold-lower Nanog levels than TCF3$^{+/+}$ ESC, yet they each produced similar levels of differentiated colonies. Taken together, we interpret these data to suggest that increased Nanog levels are necessary for TCF3$^{-/-}$ ESC to exhibit delayed differentiation and that the absence of Tcf3 likely causes additional effects that are Nanog independent.

Molecular mechanism limiting Nanog levels in Tcf3^{+} ESC. Whether a Tcf protein functions as an activator or as a repressor of target gene transcription depends upon its molecular context in terms of the protein interactions and upstream signaling pathways within a cell. In previously described in vivo contexts, Tcf3 has consistently functioned as a transcriptional repressor (13, 24, 28, 36, 37); however, it is capable of activating model target genes in some contexts, such as in primary human keratinocytes (36). To determine whether Tcf3 functions as a repressor in ESC, transient transfection experiments were performed with the model Tcf–β-catenin reporter, SuperTOPFlash (58). Experiments were performed with TCF3$^{-/-}$ ESC to prevent interference from endogenous Tcf3. The addition of a stable form of β-catenin (ΔNβcat) stimulated reporter activity 100-fold, suggesting that the Tcf1 and Lef1 proteins in TCF3$^{-/-}$ ESC can act as potent activators (Fig. 6A). The lack of activation of the control reporter (SuperFOPFlash) containing mutated Tcf-binding sites demonstrated that the activity was specific for Tcf–β-catenin complexes. This activation was inhibited in a dose-dependent manner by expression of full-length Tcf3 protein (Fig. 6A). Note that repression was never complete. This is consistent with β-catenin having an activator function in TCF3$^{+/+}$ ESC and Tcf3 being capable of inhibiting but not completely inactivating this activity (not shown). In contrast to Tcf3-mediated repres-

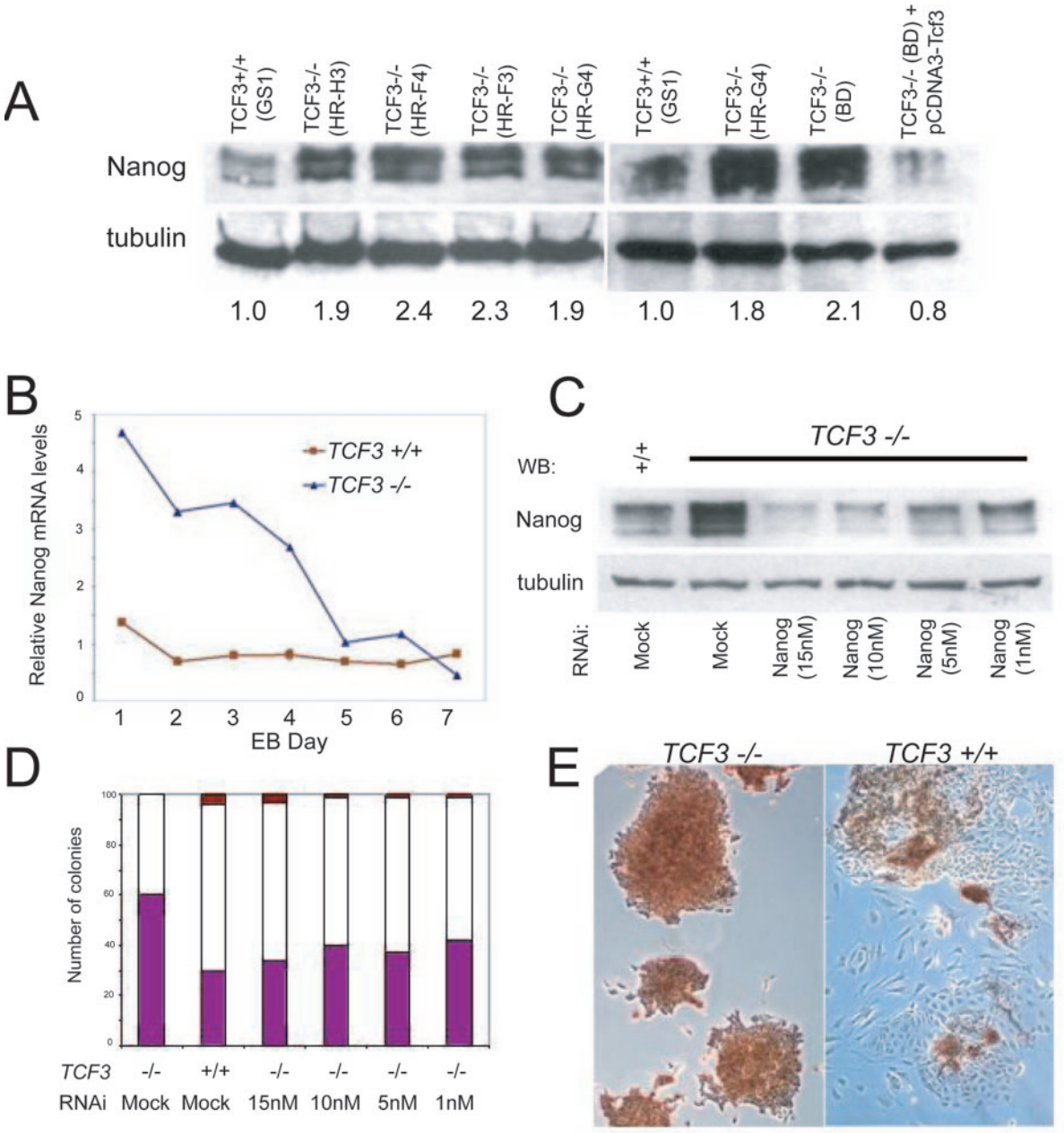

FIG. 5. Increased Nanog in TCF3$^{-/-}$ embryonic stem cell lines. (A) Western blot analysis of protein extracted from Tcf3^{+} and Tcf3^{-} ESC lines by using a rabbit anti-Nanog antibody (Abcam). Numbers are the difference (*n*-fold) of Nanog/tubulin levels compared to those of TCF3$^{+/+}$ samples. Note that Nanog is phosphorylated in ESC, resulting in the detection of several bands after SDS-polyacrylamide gel electrophoresis separation (62) (B) Quantitative PCR measurement of Nanog mRNA levels in TCF3$^{+/+}$ and TCF3$^{-/-}$ cells undergoing embryoid body differentiation. Nanog mRNA was higher in TCF3$^{-/-}$ ESC prior to differentiation (Fig. 3C) and remained higher than TCF3$^{+/+}$ ESC levels until day 5 of differentiation. (C) Western blot (WB) analysis of Nanog RNAi-transfected TCF3$^{-/-}$ ESC showed decreased Nanog levels compared to analysis of mock RNAi-transfected ESC. Tubulin was used as a loading control. (D) Single-cell suspensions of ESC transfected with the indicated concentrations of mock (15 nM) or Nanog RNAi molecules were placed onto gelatinized plates in media without LIF. After 4 days, colonies were fixed and stained for alkaline phosphatase activity. The ratio of intensely stained undifferentiated colonies (purple), lightly stained colonies (white), and completely differentiated colonies (red) is displayed after 4 days of colony growth. The graph shown is representative of three independent experiments. (E) The morphologies of the majority of TCF3$^{-/-}$ colonies and TCF3$^{+/+}$ colonies after 4 days are shown.

sion, expression of human Lef1 protein increased β-catenin-dependent activation.

To determine whether distinct domains of Tcf3 were responsible for its repressor activity in ESC, a mutational analysis was performed. Mutant Tcf3 expression constructs, containing mutations designed to affect specific Tcf3 interactions, were used to replace wild-type Tcf3 in SuperTOPFlash reporter assays (Fig. 6B). These transfections showed that the carboxy-terminal domain, including CtBP interaction (not shown), was not required for repression. β-Catenin interaction was also not

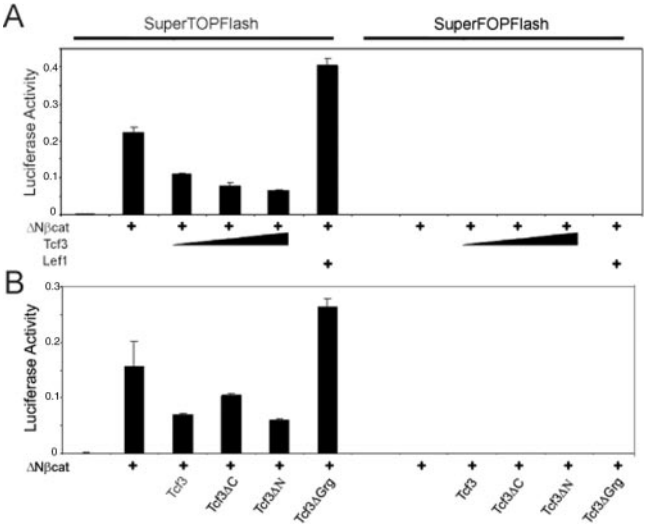

FIG. 6. Tcf3 functions as a repressor in embryonic stem cells. TCF3$^{-/-}$ ESC were transiently transfected with SuperTOPFlash or SuperFOPFlash reporter constructs and the indicated expression constructs. A pRL-CMV reporter construct was included in each to measure transfection efficiency. Values represent means ± standard deviations of duplicate transfection. Each graph is representative of three separate experiments. (A) Expression of a stable form of β-catenin (ΔNβcat)-activated SuperTOPFlash but not the SuperFOPFlash control containing mutated Tcf-binding sites. Increasing amounts of Tcf3 expression construct (0.2 to 0.6 μg) repressed activity. Expression of a human Lef1 increased activation. (B) Expression of the mutated Tcf3 proteins indicated that amino (ΔN)- and carboxy (ΔC)-terminal regions of Tcf3 were not required for repression, but the Groucho interaction domain (ΔGrg) was required for repression.

required for repression. Interestingly, the level of repression was the same between full-length Tcf3 and ΔNTcf3, suggesting that β-catenin's effect on Tcf3 repressor activity on this artificial promoter reporter was independent of a physical interaction with the Tcf3 amino-terminal domain. In contrast, the Groucho interaction domain was required for repression; mutation of this domain (amino acids 143 to 318) converted Tcf3 from a repressor to an activator. These transfection experiments indicate that β-catenin can activate target genes in ESC through interaction with Tcf proteins, and Tcf3 functions as a transcriptional repressor in ESC that counteracts the Tcf–β-catenin activation of target genes.

The mechanisms required to stimulate Nanog expression have been described previously. Oct4 and Sox2 proteins coordinately bind to a DNA site within 200 bp of the transcription start site (30, 49, 61). Oct4-Sox2 binding has been shown to be both necessary and sufficient for activation of Nanog promoter fragments and induction of endogenous Nanog gene transcription. The mechanisms involved in the retinoic acid-induced and DNA damage-induced repression of Nanog promoter activity have been identified for p53 and GCNF proteins (19, 32). However, there have been no reports of mechanisms limiting Nanog within self-renewing ESC. In considering a molecular mechanism by which Tcf3 limits Nanog expression, we tested expression of both p53 and GCNF by qPCR and found them to be normal in TCF3$^{-/-}$ ESC (not shown). Since the Nanog promoter activity was increased by the absence of Tcf3 and Tcf3 was found to function as a transcriptional repressor in ESC, a parsimonious mechanism was that Tcf3 could directly regulate Nanog promoter activity in ESC.

To determine whether Tcf3 repressed Nanog promoter activity, we began by examining the effects of Tcf3 on a previously characterized reporter construct containing bp −4828 to +190 of the upstream Nanog gene DNA (61). A cluster of five optimal Tcf recognition sequences, (A/T)(A/T)CAAAG, was found between kb −3 to −5 of the Nanog promoter fragment shown in Fig. 7A. This region was tested for activity and Tcf3 binding in ESC. Increasing levels of Tcf3 expression construct repressed Nanog reporter activity in a dose-dependent manner (Fig. 7B). A twofold repression of Nanog promoter activity was effected by the highest Tcf3 concentrations; similar differences were observed when promoter activities were compared between TCF3$^{+/+}$ and TCF3$^{-/-}$ ESC cells (not shown). Tcf3 expression did not affect a proximal promoter fragment (bp −270 to +190) containing the Sox2-Oct4 binding element (not shown). In contrast, transfection of a stable form of β-catenin increased Nanog promoter activity in TCF3$^{+/+}$ (Fig. 7C) and TCF3$^{-/-}$ (not shown) ESC. Note that the effects of β-catenin occurred in both TCF3$^{+/+}$ and TCF3$^{-/-}$ ESC, suggesting that they did not require Tcf3.

To determine whether repression was due to direct binding of Tcf3, two experiments were performed. First, ChIP assays using a Tcf3-specific antibody (36, 37) were used to determine whether Tcf3 occupied DNA fragments required for Tcf3-mediated repression of Nanog promoter activity (Fig. 7D). Control PCRs consisting of mock-precipitated chromatin, precipitations performed with TCF3$^{-/-}$ ESC, and PCRs directed to other DNA regions (TCF4 locus is shown) together demonstrated specific binding of Tcf3 protein to the upstream regulatory region of the Nanog promoter. Binding was most effectively detected by PCR 2, and flanking regions detected by PCRs 1 and 3 indicated lower levels of Tcf3 binding (Fig. 7D). In the second experiment, we tested whether expression of a Tcf3 protein (Tcf3*) harboring two point mutations in its DNA-binding domain could cause repression. This mutant form was previously shown to be capable of interacting with β-catenin and Grg proteins but incapable of binding Tcf recognition sequences in DNA (36). In comparison to wild-type Tcf3, the Tcf3* mutant failed to repress Nanog reporter activity in transfected ESC (Fig. 7E). These experiments showed that Tcf3 is associated with chromatin in the Nanog promoter regions and that the DNA-binding activity of Tcf3 was required for repression.

To identify the domains of the Tcf3 protein required to limit Nanog expression in ESC, mutated Tcf3 proteins were tested for their ability to repress Nanog promoter activity (Fig. 7E). The Groucho interaction domain was required for repression of the Nanog reporter; however, neither the carboxy terminus nor the β-catenin interaction domain was required for repression of reporter activity (Fig. 7E). To determine whether these effects could be recapitulated with the endogenous Nanog gene in ESC, Tcf3 mutant expression plasmids were used to generate stably expressing clones as described earlier for wild-type Tcf3 (Fig. 2A). Using qPCR to measure Nanog expression in the ESC showed that both the Groucho interaction domain and an intact DNA-binding domain were required to limit the levels of endogenous Nanog gene expression (Fig. 7F). Western blot analysis of protein extracts showed that the effects on

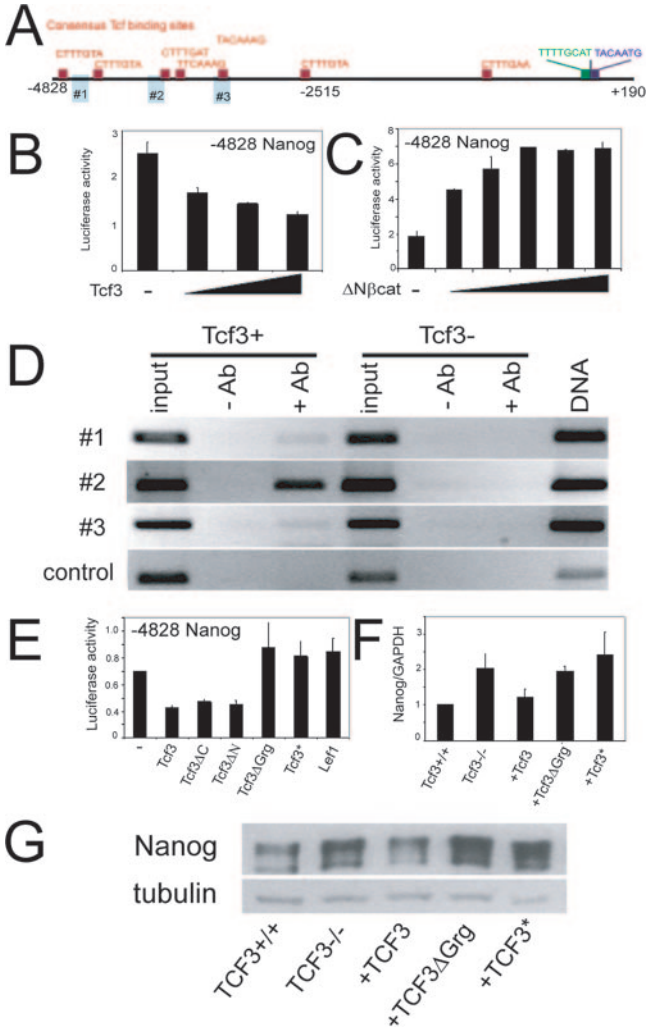

FIG. 7. Nanog is a direct target of Tcf3 repression. (A) Diagram of Nanog promoter region showing Oct-Sox2 site required for activation, potential Tcf-binding sites (red), and region corresponding to PCR products 1 to 3 in panel D. (B and C) Nanog promoter (bp −4828 to +190) luciferase construct was transfected into ESC. (B) Increasing amounts (0.2 to 1.0 μg) of Tcf3 expression construct repress Nanog promoter activity in $TCF3^{-/-}$ ESC. (C) Increasing amounts (0.2 to 1.0 μg) of stable β-catenin activate Nanog promoter activity in $TCF3^{+/+}$ ESC. (D) Results of chromatin immunoprecipitation using anti-Tcf3 antibody (+Ab) or preimmune serum (−Ab) with cross-linked, sheared chromatin from BD $TCF3^{-/-}$ plus pCDNA3-Tcf3 ($Tcf3^{+}$) and BD $TCF3^{-/-}$ ($Tcf3^{-}$) self-renewing ESC. Input lanes equal 1% of DNA samples prior to immunoprecipitation. (E) Relative Nanog promoter activity in $TCF3^{-/-}$ ESC cotransfected with the indicated Tcf expression construct. (F) Quantitative PCR measurement of Nanog mRNA isolated from HR-G4 $TCF3^{-/-}$ ESC and derivatives created by stable transfection of pCDNA3 expression constructs containing the indicated Tcf3 wild-type or mutant protein. Nanog levels are reported relative to those of GAPDH. Each value is the mean ± standard deviation of three measurements performed in independent assays. (G) Western blot analysis of Nanog and tubulin protein levels in ESC lines shown in panel F.

transcription and mRNA levels also affected protein level (Fig. 7G). Taken together, these results showed that Tcf3 directly repressed Nanog promoter activity to limit the steady-state levels of Nanog protein in self-renewing ESC.

DISCUSSION

A common feature defining stem cells is their ability to undergo both self-renewal and lineage commitment. It is this dual role that sets stem cells apart in terms of their importance for the morphogenesis and maintenance of organs and tissues. For embryonic stem cells, a molecular network of transcription factors, including Nanog, Oct4, and Sox2, has been shown to provide a positive feed-forward system that maintains ESC self-renewal (4, 33). Forced expression of Nanog in ESC showed that its levels must be diminished for ESC to undergo lineage commitment in vitro (9, 39). Here, we find that Tcf3's repressor activity on the Nanog promoter represents the first described mechanism to limit Nanog levels in self-renewing ESC. We find that failure to limit Nanog in ESC was associated with delayed lineage commitment in $TCF3^{-/-}$ embryoid bodies. Given the requirement for Tcf3 in the gastrulating mouse embryo to restrict formation of the primitive streak, it is interesting to suggest a role for Tcf3-mediated Nanog regulation in the control of cell fate decisions during postimplantation embryonic development.

Combined with the requirement for Tcf3 to limit Nanog expression in ESC, recently published data suggest that Tcfs may function within an intrinsic stem cell network that regulates the decision between self-renewal and differentiation. Chromatin from the upstream region of the human gene encoding Tcf3 (TCF7L1) was found to be bound by each Sox2, Oct4, and Nanog in human stem cells through ChIP-chip analysis (4). Identification of Oct4 and Nanog chromatin binding sites in mESC also identified TCF3 as 1 of only 18 targets found in both mouse and human ESC (33). Although it remains unknown whether the binding of these factors to the Tcf3 promoter region affects transcription in ESC, it suggests an interesting possibility that Tcf3 functions as a negative feedback regulator of the Oct4-Sox2-Nanog feed-forward regulatory circuit; as Nanog levels rise, stimulation of the Tcf3 promoter produces a repressor to limit Nanog. Consistent with this possibility, Tcf3 mRNA was elevated by Nanog overexpression in retinoic acid-treated ESC, and Tcf3 mRNA was decreased by Nanog RNAi treatment (33). The fact that increased Oct4 and Sox2 levels were not detected in $TCF3^{-/-}$ ESC suggested that Tcf3 does not provide negative feedback for those factors (Fig. 3C). It also suggests that the increased Nanog levels were caused by loss of Tcf3-mediated repression and not simply increased Oct4-Sox2 activation of promoter activity. This conclusion was also consistent with the Nanog proximal promoter region containing the Oct4-Sox2 sites displaying similar activities in $TCF3^{+/+}$ and $TCF3^{-/-}$ ESC (not shown).

Despite the importance of Tcf3 for limiting Nanog expression below a differentiation-inhibiting threshold, the twofold repression of Nanog reporter activity by Tcf3 is relatively modest in comparison to other transcriptional regulators. Considering that Nanog expression is diminished far greater than twofold in differentiated progeny compared to that in ESC and that Tcf3 expression diminishes as ESC differentiate, there must be Tcf3-independent molecular mechanisms that shut off Nanog expression and allow differentiation to proceed. This conclusion is consistent with the previously identified repressors of Nanog, p53 and GCNF (19, 32). It is important to point

out that these previous examples differ from Tcf3 in that neither p53 nor GCNF was reported to repress Nanog in self-renewing ESC (19, 32). GCNF and p53 were found to be either absent or expressed at relatively low levels in self-renewing ESCs; it was only when ESC were subjected to in vitro differentiation or DNA damage that levels of p53 and GCNF repressed Nanog promoter activity (19, 32, 54). In addition, Nanog expression was expanded into extraembryonic trophectoderm cells of the $CDX2^{-/-}$ blastocyst whereas it was restricted to ICM cells in $CDX2^{+/+}$ blastocysts (9, 39, 54). Although the downstream molecular mechanism by which Cdx2 represses Nanog has remained unknown, it is unlikely to directly involve Tcf proteins because (i) Oct4 is also upregulated in $CDX2^{-/-}$ embryos but not in $TCF3^{-/-}$ ESC (54) and (ii) $TCF3^{-/-}$ mouse embryos progress past blastocyst formation without apparent differentiation defects (37). We conclude that a function for Tcf3 in regulating Nanog expression is in an important way distinct from the repression involving p53, GCNF, or Cdx2 during differentiation. Whereas those previously described mechanisms were shown to be necessary for shutting off Nanog expression in response to differentiation-inducing signals, Tcf3 is required to function in Nanog-expressing cells to allow efficient initiation of lineage commitment by lowering steady-state levels of Nanog.

Although we found that Tcf3-mediated repression attenuates Nanog promoter activity in ESC, the precise molecular mechanism whereby it does so remains unknown. Analysis of the effects of several Tcf3 mutants showed that repression of Nanog required both the DNA-binding and the Groucho interaction domain (Fig. 7E and F). The association of histone deacetylase activity with Groucho-containing complexes provides a potential mechanism (10); however, an examination of the changes to chromatin and histone modifications within the Nanog regulatory regions will be required for precise biochemical understanding of Tcf3-mediated effects. Interestingly, the expression of the mutant Tcf3 defective in DNA binding resulted in increased levels of Nanog promoter activity (Fig. 7E) and endogenous mRNA levels (Fig. 7F). In addition, expression of a form of Lef1 that does not interact with Groucho proteins (36, 64) also caused an increase in Nanog promoter activity (Fig. 7E). These results suggest that Tcf proteins binding DNA but not Groucho or binding Groucho but not DNA could function as a dominant negative in terms of repressing Nanog activity. Given that both Tcf1 and Lef1 were expressed at detectable levels in ESC and that they can all recognize the same DNA-binding sites, either Tcf1 or Lef1 is a good candidate to provide a partial repression of Nanog in the absence of Tcf3 and the absence of stabilized β-catenin.

It is interesting to note that Tcf3 is expressed at higher levels in ESC than in differentiated progeny, yet the function identified through examination of $TCF3^{-/-}$ ESC suggests that Tcf3 is required to limit the levels of a protein, Nanog, which is absolutely required for ESC self-renewal (9, 39). Ostensibly, this conclusion may appear to present a paradox as one might expect that one "stem cell gene," such as Tcf3, should not repress the activity of a second "stem cell gene," such as Nanog. However, the importance and relevance of this relationship become clear when one considers that the in vivo function of the epiblast cells requires them to become lineage committed in a timely and efficient manner. Classic teratoma experiments showed that epiblast cells self-renew and can maintain pluripotency until lineage restriction occurs during gastrulation (38, 53). For the formation of the primitive streak, epiblast cells must process signals (i.e., Wnt, BMP, and Nodal) directing them to committed lineages for the formation of mesoderm, endoderm, and neuro-/ectoderm germ layers. Epiblast cells exhibiting ectopic resistance to lineage-inducing signals in favor of self-renewal would likely disrupt the dynamics of normal cell movements and patterning that govern gastrulation and the formation of the basic body plan. Based upon the ability of even relatively modest increases in Nanog expression to promote resistance of ESC differentiation, ectopic expression of Nanog in epiblast cells prior to primitive streak formation could cause significant morphogenetic defects (62). In support of this model, Nanog expression has been detected in epiblast cells lateral to the primitive streak and absent in cells in the primitive streak as they commit to a mesoderm lineage (21, 22, 41). Examination of Nanog expression in $\beta\text{-catenin}^{-/-}$ and $WNT3^{-/-}$ embryos showed that it required both of these Wnt signaling activators (41). Determining the role of Tcf3 in regulating Nanog expression in embryos and the ability of increased Nanog levels to cause morphogenetic defects will be important for determining whether the molecular framework described here is also important for providing a molecular link between lineage commitment and the formation of the three-dimensional architecture in mammals.

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