



Published in final edited form as:

Nat Cell Biol. ; 13(7): 762–770. doi:10.1038/ncb2283.

Opposing Effects of Tcf3 and Tcf1 Control Wnt-Stimulation of Embryonic Stem Cell Self Renewal

Fei Yi^{1,2}, Laura Pereira^{1,2}, Jackson A. Hoffman¹, Brian R. Shy¹, Courtney M. Yuen³, David R. Liu³, and Bradley J. Merrill^{1,4}

¹Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, IL 60607

³Howard Hughes Medical Institute, Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138

Abstract

The observation that Tcf3 (MGI name: Tcf711) bound the same genes as core stem cell transcription factors, Oct4 (MGI name: Pou5f1), Sox2 and Nanog, revealed a potentially important aspect of the poorly understood mechanism whereby Wnts stimulate self renewal of pluripotent mouse embryonic stem (ES) cells. Although the conventional view of Tcf proteins as the β -catenin-binding effectors of Wnt signaling suggested Tcf3- β -catenin mediated activation of target genes would stimulate ES cell self renewal, here we show that an antagonistic relationship between Wnt3a and Tcf3 on gene expression is important for regulating ES cell self renewal. Genetic ablation of Tcf3 replaced the requirement for exogenous Wnt3a or GSK3-inhibition for self renewal of ES cells, demonstrating that inhibition of Tcf3-repressor is the necessary downstream effect of Wnt signaling. Interestingly, the molecular mechanism underlying Wnt's effects required both Tcf3- β -catenin and Tcf1- β -catenin interactions, as they each contributed to Wnt stimulation of self renewal and gene expression. Finally, the combination of Tcf3 and Tcf1 was necessary to recruit Wnt-stabilized β -catenin to Oct4 binding sites in ES cell chromatin. These results elucidate the molecular link between the effects of Wnt and the regulation of the Oct4/Sox2/Nanog network.

The Oct4/Sox2/Nanog core self renewal circuit was defined primarily by experiments showing physical interactions between proteins^{1, 2}, substantially overlapping sites of chromatin occupancy^{3, 4}, and similar effects in loss of function experiments^{5–9}. Several data indicated that Tcf3 was an integral component of the Oct4/Sox2/Nanog self renewal circuit. ChIP-seq experiments showed that of the 1369 known genes identified as Tcf3-bound, 1173

Users may view, print, copy, download and text and data- mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: http://www.nature.com/authors/editorial_policies/license.html#terms

⁴correspondence should be addressed to: Bradley J. Merrill – merrillb@uic.edu, Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, 900 S Ashland Ave, MBRB 2270, M/C 669, Chicago, IL 60607, ph: 312-996-0346, fax: 312-413-0353.

²These authors contributed equally to this manuscript.

AUTHOR CONTRIBUTIONS

All authors designed and analysed experiments. F.Y., L.P., J.A.H., B.S. and C.M.Y. performed experiments. F.Y., L.P. and B.J.M. wrote the manuscript. B.J.M and D.R.L. supervised the project.

were also bound by Oct4, and 942 were bound by all four factors (Tcf3, Oct4, Sox2, and Nanog)¹⁰. The co-occupancy of Tcf3 with Oct4, Sox2 and Nanog on a genome-wide scale was also independently determined with ChIP-chip experiments^{11, 12}. Whereas knockdown of Nanog or Oct4 caused largely similar effects on gene expression⁴, the ablation of Tcf3 caused strongly opposite effects on gene expression compared to Nanog knockdown and Oct4 knockdown¹³.

One intriguing aspect of Tcf3's function in stem cells has been its potential role as a downstream effector of the canonical Wnt signaling pathway. An important consequence of Wnt ligand binding its receptor is the inhibition of GSK3 phosphorylation of β -catenin¹⁴. This stabilizes β -catenin from ubiquitin-mediated degradation and promotes signaling by allowing β -catenin to form transcription-activating complexes with DNA-binding proteins, most notably Tcf factors^{15–17}. Treating cells with Wnts¹⁸ or blocking GSK3 activity by genetic ablation¹⁹ or small molecule inhibitors^{18, 20, 21} all effectively stimulated ES cell self renewal. Indeed, inhibition of GSK3 combined with inhibition of ERK activity in so-called two-inhibitor (2i) conditions was sufficient to promote ES cell self renewal in the absence of serum and extrinsic cellular signals^{20, 21}.

The role of Tcf- β -catenin complexes as downstream mediators of Wnt and GSK3 effects has been complicated by the facts that GSK3 affects many cellular activities in addition to destabilizing β -catenin, and it is difficult to experimentally control for the broad effects of GSK3 inhibition. As such, it is not clear what biochemical activities downstream of Wnt and GSK3-inhibition were most important for ES cell self renewal; however, previously published examination suggested three intriguing hypotheses: either, 1. the effects of Wnt-stimulated β -catenin needed to function through an Oct4- β -catenin interaction and Tcf- β -catenin interactions only secondarily affected self renewal²², 2. Tcf3- β -catenin activator complexes directly stimulated self renewal and the expression of self renewal genes¹¹, or 3. Tcf3 transcriptional-repressor activity inhibited self renewal and was suppressed by Wnt^{13, 23}.

To test these hypotheses, a series of cell-based experiments were completed. First, the ability of Wnt signaling to replace LIF and to support self renewal in *Tcf3*^{+/+} cells was confirmed by showing that Oct4 promoter activity and protein levels were maintained in ES cells by adding recombinant Wnt3a to LIF deficient cultures (Supplementary Fig. S1a,b). This effect required an intact Oct4/Sox2/Nanog circuit, as Wnt3a was unable to rescue LIF deficiency in cells genetically lacking Nanog (Supplementary Fig. S1c,d). Since a similar experiment using *Tcf3*^{-/-} would not be informative because these cells self renew in the absence of exogenous LIF even without Wnt stimulation¹³, we tested the possibility that forced overexpression of Tcf3 could inhibit self renewal by using a doxycycline-regulated system to increase Tcf3 levels in *Tcf3*^{+/+} cells (Supplementary Fig. S2a)²⁴. If the hypothesis of a Tcf3- β -catenin transactivator complex was correct, one would expect that ectopic expression of Tcf3 would enhance ES cell self renewal upon Wnt3a stimulation. Instead, a twofold increase in Tcf3 protein efficiently blocked self renewal, as no alkaline phosphatase (AP)⁺ ES colonies were present four days after induction of Tcf3 in +LIF cultures (Fig. 1a). Flow cytometry of propidium iodide-stained cells showed a conversion from the high S-phase, ES cell cycle profile to a G1-enriched, somatic cell cycle profile

within 72 hours of Tcf3 overexpression (Fig. 1a). Wnt3a sharply inhibited the differentiation-promoting effects of Tcf3 by restoring AP+ colonies and an ES cell cycle profile to Tcf3-overexpressing cells in both +LIF and LIF-deficient conditions (Fig. 1a,b); however, colony formation of Wnt3a-treated, Tcf3-overexpressing cells was diminished relative to the non-overexpressing controls. These results best support the hypothesis that Tcf3 represses self renewal and Wnt3a antagonizes Tcf3-repression.

Recently, Tcf- β -catenin-independent effects were suggested to provide a mechanism whereby inhibition of GSK3 stimulated the self renewal of ES cells^{21, 22}. These conclusions were based primarily on the inability of heterologously expressed Lef1 N²¹ or Tcf4 N²² proteins to rescue differentiation defects caused by stable β -catenin. These observations suggested that the self-renewal stimulating effects of Wnt or GSK3-inhibition were independent of Tcf protein function^{21, 22}, which appeared to be inconsistent with the ability of full length Tcf3 to inhibit self-renewal when overexpressed (Fig. 1a,b). To provide a better test of the role of endogenous Tcf proteins in ES cells, we performed experiments using loss of function techniques. First, we tested whether ablation of Tcf3 could replace the requirement for GSK3 inhibition for self renewal in serum free culture conditions²¹. After a single passage in serum free conditions, *Tcf3*^{-/-} cells generated a high percentage of AP+ colonies when only PD0325901 (ERK Inh) or both PD0325901 and CHIR99021 (2i) were added to media (Fig. 1c). In contrast, *Tcf3*^{+/+} cells generated a high percentage of AP+ colonies only in 2i conditions and differentiated in ERK Inh only (Fig. 1c). While both *Tcf3*^{+/+} and *Tcf3*^{-/-} cells generated AP+ colonies in the presence of CHIR99021 alone (GSK3 Inh) for a single passage (Fig. 1c), both cell lines lost viability and differentiated in GSK3 Inh conditions after being passaged (Fig. 1d). Importantly, *Tcf3*^{-/-} cells were serially passaged in ERK Inh conditions and maintained a high level of proliferation and AP + morphology (Fig. 1d). Thus, ablation of Tcf3 effectively replaced the need for GSK3-inhibition in serum free culture conditions, suggesting that antagonism of Tcf3 is a critical downstream effect of GSK3-inhibitors in 2i culture conditions.

To define better how Tcf3 and Wnt signaling functioned together in ES cells, we compared effects of recombinant Wnt3a-treatment (W) or control media (C) on gene expression in *Tcf3*^{+/+} and *Tcf3*^{-/-} ES cells. Principal component analysis (PCA) was used to explore correlations among gene expression microarray datasets for three biological replicates for each of the four different conditions (*Tcf3*^{+/+}(C), *Tcf3*^{-/-}(C), *Tcf3*^{+/+}(W), *Tcf3*^{-/-}(W)) (see Methods)²⁵. Plotting each replicate according to its principal component (PC)1 and PC2 values effectively separated each of the four conditions while grouping replicates according to their condition (Fig. 2a). Interestingly, *Tcf3*^{-/-}(C) and *Tcf3*^{+/+}(W) conditions shared similar PC2 scores, suggesting these two conditions shared a common set of variations that differed in the other conditions. *Tcf3*^{-/-}(C) and *Tcf3*^{+/+}(W) had different PC1 scores, suggesting that these two conditions differed from each other based on the variations comprising PC1. Thus, Tcf3-ablation and Wnt3a-treatment cause one set of common changes and one set of different changes.

Since Tcf3-ablation and Wnt-activation both stimulated self renewal of ES cells^{11, 18, 23}, common effects were likely to be mediators of self renewal. To identify these genes, we focused on the 831 genes that were significantly increased (Benjamini-Hochberg adjusted

FDR < 0.05; Supplementary Fig. S3a) in any of three comparisons: 1. Increased in *Tcf3*^{+/+} (W) vs *Tcf3*^{+/+} (C), 2. Increased in *Tcf3*^{-/-} (C) vs *Tcf3*^{+/+} (C), and 3. Increased in *Tcf3*^{-/-} (W) vs *Tcf3*^{-/-} (C) (Supplementary Table S1). Each gene was given three scores corresponding to their ranked fold-increase for each of the three comparisons (Supplementary Table S2). To populate a group of common changes, a gene was assigned to group 1 (Red; Fig. 2b,c) when the effect (i.e. ranked fold-increase) caused by Wnt3a in *Tcf3*^{+/+} cells (score #1) was closer to the effect caused by Tcf3 ablation (score #2) than to the effect of Wnt3a in *Tcf3*^{-/-} cells (score #3). For example, *Tbx3* was placed in group 1 because its ranked fold-increases for comparisons 1–3 were 29|20|336, respectively; *Cdx1* was placed in group 2 with ranked fold-increases of 10|257|17 (Supplementary Table S2). Performing this relatively simple calculation separated all genes into two strikingly distinct groups, which was evident when ranked-fold increase values were used to plot individual genes (Fig. 2b). For group 1 genes, Wnt3a effects on *Tcf3*^{+/+} cells displayed a strong correlation (Pearson correlation coefficient $\rho = 0.90$) when compared to effects of ablating Tcf3 (Fig. 2b, bottom left). Wnt3a had different effects on group 1 genes in *Tcf3*^{+/+} cells compared to *Tcf3*^{-/-} cells ($\rho = -0.23$) (Fig. 2b, top left). In contrast, for group 2 genes (Blue; Fig. 2b,c), Wnt3a effects on *Tcf3*^{+/+} cells displayed a strong correlation ($\rho = 0.91$) when compared to Wnt3a effects on *Tcf3*^{-/-} cells (Fig. 2b; top right). Thus, Wnt3a stimulated one set of genes similarly to the ablation of Tcf3 (group 1), and stimulated a second set of genes regardless of Tcf3's presence (group 2).

When the effects of Wnt3a and Tcf3 were compared to the effects of Oct4 shRNA for the two groups of genes (Fig. 2c), a moderate to strong anti-correlation was observed only for the Tcf3-dependent group 1 genes ($\rho = -0.65$ for Wnt3a on *Tcf3*^{+/+} cells, and $\rho = -0.72$ for Tcf3 ablation). No relationship with Oct4 was apparent for the Tcf3-independent group 2 genes (ρ values from -0.40 to 0.32), or with the effects of Wnt3a on Tcf3-dependent group 1 genes in the absence of Tcf3 (i.e. in *Tcf3*^{-/-} cells; $\rho = 0.31$). Thus, group 1 genes correlated well with regulation by Oct4, consistent with these genes being most relevant for self renewal. In addition, a separate, independent set of 311 genes was selected because they were decreased by Nanog and Oct4 shRNA⁴; most of these genes (81%) were increased in *Tcf3*^{-/-} cells compared to *Tcf3*^{+/+} cells. Wnt3a stimulation of these genes frequently achieved a higher level (Fig. 2d, green bar, 44% of genes) or nearly the same level (Fig 2d, blue bar; 12% of genes) in *Tcf3*^{-/-} cells compared to *Tcf3*^{+/+} cells. Wnt3a-stimulation of genes rarely achieved a higher level in *Tcf3*^{+/+} cells compared to *Tcf3*^{-/-} cells (Fig. 2d, orange bar, 2% of genes; Supplementary Fig. S3b). The overlapping chromatin occupancy of Nanog and Tcf3 at common genes^{10, 11} and Wnt3a/Tcf3-regulation of Nanog levels (Fig. 3a,c; Supplementary Fig S2a,b)²³ suggested the possibility that Wnt3a/Tcf3 effects could occur secondarily through deregulated Nanog. We tested this by reducing Nanog transiently in *Tcf3*^{+/+} cells by siRNA (Fig3a) or stably in *Tcf3*^{-/-} cells by shRNA (Fig 3b) and by increasing Nanog in *Tcf3*^{+/+} cells by stable transfection of a Nanog cDNA (Fig. 3c). Measuring levels of group 1 gene products after either loss or gain of function experiments indicated that Wnt3a/Tcf3 effects did not correlate with increased Nanog levels (Fig. 3d), indicating that increased Nanog levels did not mediate Wnt3a/Tcf3 effects on gene expression. Taken together, these data show that Tcf3-inhibits whereas Wnt3a stimulates a

common set of genes, and these common genes are most closely related to the effects of the core Oct4/Sox2/Nanog self renewal circuit.

We were intrigued by the observation that most Wnt3a-stimulated genes did not require Tcf3 for activation, and instead were stimulated to a higher level in *Tcf3*^{-/-} cells than *Tcf3*^{+/+} cells. Consistent with results from Fig. 1, this observation suggested that Tcf3-mediated repression was only inhibited by Wnt3a, and that Tcf3 did not function as an activator of target genes. These observations suggested Tcf3-β-catenin may not be important for actively stimulating Nanog/Oct4/Sox2/Tcf3 co-occupied genes, as suggested by Cole et al.¹¹. To test this possibility and to determine the role of Tcf3-β-catenin interaction in ES cells, we sought to block the Tcf3-β-catenin interaction in ES cells. Given the differentiation-inducing effects of twofold overexpression of Tcf3 (Fig. 1), we reasoned that experiments utilizing heterologous expression of Tcf3 mutants would be prone to neomorphic effects; therefore, we engineered *Tcf3*^{N/N} ES cells in which nine residues necessary for β-catenin binding were deleted from the endogenous Tcf3 gene product by homologous recombination (Supplementary Fig. S4a,b). When grown in self renewal culture conditions (+LIF, +serum) without exogenous Wnt3a, *Tcf3*^{N/N} ES cells were indistinguishable from *Tcf3*^{+/+} cells in terms of cell proliferation, cell cycle kinetics, and expression of Tcf3, Oct4, and Nanog (Supplementary Fig.S4c, S5c). Interestingly, Wnt3a stimulation of AP+ colony formation in the absence of LIF was reduced, but not eliminated, in *Tcf3*^{N/N} cells (56% for *Tcf3*^{N/N} compared to 77% for *Tcf3*^{+/+}) (Fig. 4a) and, treating *Tcf3*^{-/-} cells with Wnt3a also stimulated AP+ colony formation (from 70% to 86%) (Fig. 4a). Similar results were obtained with experiments performed using serum-free, 2i conditions (Supplementary Fig. S4d). Moreover, ablation of the Tcf3-β-catenin interaction diminished but did not eliminate the ability of cells to activate Tcf-β-catenin responsive promoter reporters, SuperTOPFlash, Nanog, and Nr5a2 (also called LRH-1) (Fig. 4b). These data are not consistent with a previously described mechanism¹¹ suggesting a key role for Tcf3-β-catenin as an activator. Moreover, the only partial inhibition of Wnt3a stimulation of self renewal and target gene activation in *Tcf3*^{N/N} cells revealed that Wnt3a stimulation of self renewal involves a combination of Tcf3-β-catenin dependent and previously uncharacterized Tcf3-independent mechanisms.

The possibility that another Tcf protein could contribute Tcf3-independent effects of Wnt3a was supported by several observations. First, the highly conserved HMG domain shared by Tcf proteins allows all Tcf proteins to bind to the same sequences as Tcf3^{26, 27}. The more severe phenotypes of double mutant mouse embryos (*Tcf1*^{-/-}; *Tcf4*^{-/-}, *Tcf1*^{-/-}; *Lef1*^{-/-} and *Tcf3cKO*, *Tcf4*^{-/-}) compared to single mutants demonstrated that one Tcf protein can compensate for the absence of a family member, thus indicating the conserved HMG domains allow Tcf family members to affect an overlapping set of target genes²⁸⁻³⁰. Finally, since the SuperTOPFlash reporter is specific for Tcf proteins³¹, reporter activation in *Tcf3*^{-/-} and *Tcf3*^{N/N} cells showed that another endogenous Tcf protein was present in ES cells, and that this Tcf was capable of activating target genes.

To determine which Tcf protein was activating Tcf3-target genes, we first measured levels of Tcf gene products in ES cell lines. Tcf3 mRNA was the most abundant family member in *Tcf3*^{+/+} and *Tcf3*^{N/N} cells (Fig. 5a), Tcf1 and Tcf4 were also detected at levels that we

reasoned could have a biological effect (Fig. 5a). The ability of each Tcf protein to activate gene expression in response to stable β -catenin was tested by measuring the activation of SuperTOPFlash (Fig. 5b). Tcf1 and Lef1 displayed a similar activation of Tcf- β -catenin signaling, whereas Tcf3 and Tcf4 both repressed SuperTOPFlash and Nanog reporter activity in ES cells (Fig. 5b, Supplementary Fig. S6b). Since, Tcf1 was the best candidate to function endogenously in ES cells to activate Tcf3-repressed genes in response to Wnt-stabilized β -catenin, we used stable lentiviral shRNA knockdown to inhibit the Tcf1 gene product in each *Tcf3*^{+/+}, *Tcf3*^{-/-}, and *Tcf3*^{N/N} cells. This approach was effective at reducing Tcf1 mRNA and protein levels (Fig. 5c), and it affected >90% of cells for each cell line as indicated by GFP reporter gene expressed from the lentiviral shRNA vector (Supplementary Fig. S5a). Control cell lines harboring a scrambled shRNA sequence (SCRM) were also generated for each *Tcf3* line. In the absence of exogenous Wnt3a, each of the Tcf1 shRNA cell lines were similar to corresponding SCRM cell lines in terms of cell proliferation, cell cycle kinetics, and expression of Tcf3 and Oct4 (Fig. 5c, Supplementary Fig. S5b,c). Thus, Tcf1 knockdown did not cause an apparent cellular defect under normal self renewal conditions, without the addition of exogenous Wnt to the media. In contrast, Tcf1 shRNA substantially reduced transcriptional activation by stable β -catenin in each *Tcf3*^{+/+}, *Tcf3*^{-/-}, and *Tcf3*^{N/N} ES cells (Fig. 5d). Note that promoter activity was higher in the absence of Tcf3 (in *Tcf3*^{-/-} cells) (Fig. 5d) without an increase in Tcf1 or Lef1 mRNA or protein or β -catenin protein (Fig 5a, Supplementary Fig. S6a), which was consistent with Tcf3 functioning as a repressor and directly interfering with Tcf1- β -catenin mediated transcriptional activation. Interestingly, whereas *Tcf3*^{N/N}; Tcf1 shRNA and *Tcf3*^{+/+}; Tcf1 shRNA cells displayed very low levels of activity, the *Tcf3*^{-/-}; Tcf1 shRNA cells displayed promoter activity comparable to *Tcf3*^{+/+}; SCRM cells. These data suggest that even residual levels of Tcf1 remaining after shRNA knockdown (Fig. 5c), and possibly the low endogenous levels of Lef1 (Fig. 5a,b), were sufficient to activate SuperTOPFlash when Tcf3-repression is eliminated by genetic ablation.

A role for endogenous Tcf1 in Wnt-stimulation of self renewal was tested with colony formation in serum-containing media lacking LIF. Without exogenous Wnt3a, Tcf1 shRNA and SCRM cell lines differentiated similarly to form primarily AP-negative colonies in *Tcf3*^{+/+} and *Tcf3*^{N/N} cells and primarily AP+ colonies in *Tcf3*^{-/-} cells (Fig. 6a). When exogenous Wnt3a was added, Tcf1 shRNA decreased the generation of AP+ colonies by half for both *Tcf3*^{+/+} and *Tcf3*^{N/N} cells (Fig. 6a). In contrast, Tcf1 shRNA had a less substantial effect on Wnt3a treated *Tcf3*^{-/-} cells, which still generated AP+ colonies from 68% of Tcf1 shRNA-containing cells. To confirm that self renewal effects were caused by Tcf1 knockdown and not off target effects, transfection experiments using siRNA molecules targeting a different Tcf1 sequence were performed; Tcf1 siRNA caused a similar 50% reduction in AP+ colonies for *Tcf3*^{+/+} and *Tcf3*^{N/N} cells compared to control (Supplementary Fig. S5d,e). Considering both the clonal nature of the assay using shRNA cell lines and that 9% of *Tcf3*^{N/N}; Tcf1 shRNA cells plated were GFP-negative (Supplementary Fig. 5a), the 14% of *Tcf3*^{N/N}; Tcf1 shRNA colonies displaying an AP+ ES colony morphology suggested that Wnt3a stimulation of self renewal primarily relies on the combination of Tcf1 and Tcf3- β -catenin interaction. These results show that endogenous Tcf1 is a key mediator of Wnt stimulation of self renewal in ES cells.

To determine if endogenous Tcf1 was important for Wnt-effects on Tcf3-regulated genes, we measured expression of several Wnt3a-stimulated and Tcf3-repressed genes by qPCR. Expression levels of endogenous genes were measured for 12 conditions (+/- Tcf1 shRNA and +/- Wnt3a treatment in each *Tcf3*^{+/+}, *Tcf3*^{-/-}, and *Tcf3*^{N/N} ES cells). The relative level of each gene is shown by heat map (Fig. 6b); numerical values and standard deviations are shown in Supplementary Table S5. For previously characterized Wnt/Tcf target genes (T, Cdx1, Axin2), Wnt3a stimulated high expression levels in *Tcf3*^{+/+} and *Tcf3*^{-/-} cells, and much lower levels in *Tcf3*^{N/N} cells (Fig. 6b, top). In each *Tcf3*^{+/+}, *Tcf3*^{-/-} and *Tcf3*^{N/N} cells, Tcf1 shRNA reduced the level of Wnt3a stimulation. These results were consistent with Axin2, T, and Cdx1 being predominantly regulated by Tcfs and Wnt signaling in ES cells. Moreover, they showed that Tcf1 was necessary for full Wnt3a-stimulation of gene expression. Genetically blocking Tcf3-β-catenin interaction (in *Tcf3*^{N/N} ES cells) inhibited both Wnt3a-mediated suppression of Tcf3-repressor activity and Tcf1-activator activity.

To determine if Wnt3a-effects on self renewal (Fig. 1, 4, 6a) were caused by the combined activities of Tcf1 and Tcf3 described above, expression of several genes previously implicated in ES cell self renewal was examined. These genes behaved in one of three manners with respect to the 12 conditions (Fig. 6b, bottom). First, Gpa33, Tcf1, Nanog, Prdm14, Jam2, Nr6a1, Nr5a2, Eomes, Kit, and Tnfrsf19 were increased both by Wnt3a and by Tcf3 ablation. Tcf1 shRNA decreased the Wnt3a-stimulation of these genes in each *Tcf3*^{+/+}, *Tcf3*^{-/-}, and *Tcf3*^{N/N} cells, demonstrating a role for Tcf1 in Wnt3a activation of gene expression. Second, Esrrb, Tbx3, Klf4, Nodal, and Nfib were increased by Tcf3 ablation, but not increased by Wnt3a. Interestingly, Tcf1 shRNA reduced the levels of these genes, suggesting a Wnt3a-independent role for Tcf1. However, the effect of Tcf1 on expression was small and did not correspond to a cellular effect on self renewal (Fig. 6a). Third, Oct4, Sox2, Rif1 and Mycn gene products were only modestly (<1.5 fold) increased by any combination of treatments. As expected, Tcf1 shRNA did not substantially affect expression of these genes. Together, these data were consistent with Tcf3 repression of endogenous genes being inhibited by Wnt through a combination of Tcf3-β-catenin-interaction-dependent and Tcf1-dependent mechanisms, and full Wnt-stimulation of genes requires both mechanisms.

Finally, to determine if the mechanism for Wnt stimulation of self renewal involves β-catenin recruitment to sites of Tcf/Oct4/Sox2/Nanog co-occupancy in ES chromatin, a series of ChIP-qPCR experiments was performed. The genomic binding sites for analysis were selected from previously published datasets^{1, 4, 10-12}. Criteria for choosing common sites (Tcfcp2l1, Mycn, Nodal, Oct4, Sox2, Tcf3, Zic5) included: multiple studies reporting a high ChIP signal for Oct4 and Nanog occupancy, a known relevance of the gene to ES cell self-renewal, and reproducible enrichment of the site in our ChIP-qPCR experiments. In addition, previously characterized Tcf-β-catenin responsive sites (Cdx1 and Axin2) were included as positive controls for Tcf3-binding^{1, 32}. Tcf3 and Oct4 antibodies both enriched all common sites as well as the Tcf3-binding controls (Fig. 6c,d), confirming the high level of overlapping binding discovered with ChIP-chip and ChIP-seq techniques¹⁰⁻¹². Interestingly, the relative amount of Oct4 occupancy on chromatin was increased for 8 of 10

sites in *Tcf3*^{-/-} cells compared to *Tcf3*^{+/+} cells (Fig 6d), suggesting the possibility that Oct4 and Tcf3 compete for occupancy. This observation was important as it provided an excellent context to test the relative contribution of Oct4 and Tcf proteins to the recruitment of Wnt-stabilized β -catenin to target genes. If Oct4- β -catenin complexes mediate Wnt-stimulation of self renewal²², then the increased Oct4 occupancy should enhance β -catenin recruitment in *Tcf3*^{-/-} cells. Instead, β -catenin occupancy at common sites was reduced in *Tcf3*^{-/-} cells, and was further reduced by Tcf1 shRNA for all 10 sites (Fig 6e). Taken together, these ChIP data do not support a substantial role for Oct4 directly binding to and recruiting β -catenin to chromatin in Wnt-stimulated ES cells. Instead they show Tcf3- β -catenin and Tcf1- β -catenin interactions recruit Wnt-stabilized β -catenin to chromatin.

The data shown here are most consistent with Tcf proteins being the critical regulators of Wnt's stimulation of pluripotent cell self renewal. That said, shRNA knockdown of Tcf1 did not completely eliminate Wnt3a-stimulation of AP⁺ colony formation in *Tcf3*^{N/N} cells, as a small percentage of these Wnt3a treated cells formed AP⁺ colonies (Fig. 6a). This may have been caused by incomplete Tcf1 knockdown or activity of endogenous Lef1; however, the low levels of reporter activity (Fig. 5d) and endogenous gene expression (Fig. 6b) in *Tcf3*^{N/N}; Tcf1 shRNA cells makes this possibility appear unlikely. Alternatively, a Tcf-independent effect of Wnt-stabilized β -catenin²² may contribute to stimulating self renewal. A primary role for Tcf-independent effects of β -catenin was suggested by Kelly et al., as overexpression a Δ Tcf4 protein failed to rescue differentiation defects in GSK3 double knockout ES cells²². While our results do not rule out the possibility of a secondary effect of Tcf-independent mechanisms, they strongly suggest that Tcf proteins play the primary role because: Overexpressing full length, wild-type Tcf3 blocked ES cell self renewal (Fig 1a,b). Self renewal was restored in Tcf3-overexpressing cells with exogenous Wnt3a (Fig. 1 a,b). Conversely, ablating Tcf3 replaced the need to inhibit GSK3 for ES cell self renewal in serum free conditions (Fig. 1c,d). The common effects of Wnt3a and Tcf3 ablation of gene expression correlated with Nanog/Oct4 regulation, whereas Tcf3-independent effects of Wnt3a did not (Fig. 2). The effect of Wnt3a on *Tcf3*^{N/N}; Tcf1 shRNA AP colony formation was small compared to Tcf3 ablation (Fig 6a). Finally, the Tcf-independent mechanism of β -catenin was suggested to work through an Oct4- β -catenin interaction²², but Oct4 did not recruit β -catenin to chromatin in the absence of Tcf3 and Tcf1 (Fig. 6c-e). We conclude that Wnt stabilized β -catenin stimulates ES cell self renewal primarily by inhibiting Tcf3-mediated repression of target genes.

The role for Tcf3 as a limiter of ES cell self renewal appears to be unique among Tcf factors. Moreover, endogenous Tcf1 opposed Tcf3-repression and was necessary for the complete Wnt3a-stimulation of target genes and self renewal (Fig 6a,b). In light of the need to suppress Tcf3-repression for LIF-independent self renewal, it is interesting to consider why overexpression of Δ Lef1²¹, Δ Tcf1²² and Δ Tcf4²² each failed to inhibit self renewal or failed to rescue differentiation defects caused by β -catenin stabilization. We believe the simplest explanation is that Tcf3 possesses a biochemical activity not shared by other Tcf/Lefs, and this transcriptional repressor activity inhibits ES cell self renewal. Expression of another Tcf/Lef would effectively inhibit Tcf3-repression by competing for DNA binding via virtually identical HMG domains shared by all Tcf/Lefs. As such,

overexpression of Tcf1, Tcf4 or Lef1 could have a similar effect as downregulating or inhibiting Tcf3. This mechanism is consistent with the observation that endogenous Tcf1 was necessary for maximal Wnt3a-suppression of Tcf3-repressed target genes (Fig 6b). Comparing biochemical properties of Tcf/Lef proteins in ES cells has potential to reveal a unique co-repressor that limits activity of the self renewal circuitry in pluripotent cells.

METHODS

Construction of Nanog promoter reporter plasmid

Fragments of the murine Nanog promoter and 5' UTR encompassing genomic region chr6:122652598–122657800 were amplified by PCR from a mouse genomic BAC (clone RP23-406B15, BACPAC Resources, Oakland, CA) and cloned into a pGL3b luciferase reporter vector (Promega, Madison, WI).

Alkaline phosphatase staining assay

One thousand ES cells were plated on each well of gelatinized 6-well plate and media were replaced daily. Colonies were stained for alkaline phosphatase (AP) activity after 4 days. Briefly, cells were washed once with PBS, fixed in citrate-acetate-formaldehyde (18mM NaCitrate, 9mM NaCl, pH 3.6, 65% acetone, 3.7% formaldehyde) for 45 seconds, washed three times with water, and stained with NBT/BCIP solution (Promega). For each well, 100 random colonies were scored as AP activity positive (AP+) or negative (AP-). Experiments were performed in duplicate and results were expressed as means +/- standard deviations.

Flow cytometry

Single cell suspensions were made by trypsin and resuspended in 100 μ l PBS. Cells were fixed by drop-wise addition of 1ml cold (-20°C) 70% ethanol while vortexing. Cell cycle profile was checked by analyzing DNA content. Briefly, fixed cells were rehydrated and washed twice with 10 ml PBS before finally being suspended in 200 μ l PBS. Cells were stained by incubating with of 5 $\mu\text{g}/\text{ml}$ propidium iodide (Sigma) and 1 mg/ml RNase A for 20 min at room temperature and analyzed on Beckman-Counter Cell Lab Quanta SC MPL using Cell Lab Quanta Collection program (Beckman Coulter).

Microarray and Data Analysis

For each condition, three independent *Tcf3*^{+/+} and *Tcf3*^{-/-} ESC cultures were treated with or without 50ng rmWnt3A (Recombinant mouse Wnt3A, #1324-WN, R&D System) for 24 hours. Total RNA were extracted by Trizol reagent (Invitrogen, Carlsbad, CA) and purified using RNeasy Mini Kit (Qiagen, Hilden, Germany). Quality of RNA sample was assessed by spectrophotometer (A_{260}/A_{280} between 1.9 and 2.1). 10 μg (1 $\mu\text{g}/\mu\text{l}$) total RNA of each sample were used for microarray analysis. Labeling of the RNA, hybridization onto Affymetrix mouse GeneChip 430 2.0 microarrays (Affymetrix, Santa Clara, CA), and scanning of samples were performed by the Functional Genomics Facility, University of Chicago. Raw data were normalized by quantiles and summarized by robust multiarray average. Pairwise comparisons between groups of three replicates were performed, and the *t* test was used to rank each probe set on the basis of its likely statistical significance using the Comparative Marker Selection module of GenePattern software (Broad Institute,

Cambridge, MA). Probe sets displaying a significant difference as defined by a Benjamini-Hochberg adjusted *t* test *p* value < 0.05 were used for subsequent analyses. Principal component analysis (PCA) was performed using the PCA module of GenePattern software. All microarray data points were subjected to PCA to create a set of principal experimental components to identify relevant patterns or variations across the four conditions: *Tcf3*^{+/+} (C), *Tcf3*^{-/-} (C), *Tcf3*^{+/+} (W), *Tcf3*^{-/-} (W). The analysis identifies patterns by finding principal components (PC) that account for variance in data points among all samples. Each PC thus represents a combination of gene expression values, and each PC is mutually uncorrelated and orthogonal. The first two PC scores (PC1 and PC2) resolved 99% of variance amongst all samples. A two dimension plot of PC1 and PC2 was generated using the PCViewer module of GenePattern.

Quantitative real-time PCR (qPCR)

Total RNA was isolated with TRIZOL reagent (Invitrogen, Carlsbad, CA), and 3.0 µg total RNA of each sample was used as template for cDNA synthesis. Reverse transcription was carried out with oligo-dT primers (0.5 mg/ml) using SuperScript™ III First-Strand cDNA Synthesis kit (Invitrogen). Quantitative real-time PCR reactions were performed with the iTaq SYBR Green Supermix (BioRad) and an iCycler apparatus (BioRad). Amplification was achieved by the following protocol: 1× 95°C for 2 min; 40× 95°C for 30 sec; 60°C for 30 sec. 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 PCR reactions. 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. All PCRs had an efficiency of 85% or higher. Each sample was analyzed in duplicate, and results were expressed as means ± standard deviations. Primer sequences used for qPCR are specified in Supplementary Table S5.

Manipulation of Nanog expression in ESC

Transient knockdown of Nanog expression in wild-type ESC was achieved as previously described by transfection of siRNA targeting Nanog or SCRM siRNA²³. Twelve hours after siRNA transfection, cells were treated with or without 50 ng/ml Wnt3a for 24 hours. Overexpression of Nanog in *Tcf3*^{+/+} ESC was achieved by transfection of the ppyCag-Nanog-IP expression vector⁸ or empty ppyCagIP vector control and G418 selection for transfectants. Nanog knockdown in *Tcf3*^{-/-} ESC was performed by stable integration of a pSuper shRNA vector (OligoEngine) designed with a target sequence directed to the Nanog transcript (GAGACAGTGAGGTGCATAT) as previously described³³ or empty pSuper vector control. The pSuper vectors were linearized and electroporated for stable insertion into the genome. After puromycin selection, colonies were picked and clonally expanded to generate individual cell lines. RNA isolation and quantitative real-time PCR were performed as described above. Verification of Nanog knockdown or overexpression was performed by SDS-Page gel electrophoresis and western blotting with Nanog specific antibodies (Bethyl Labs A300-397A, CosmoBio RCAB0002P-F, and Abcam ab21603) and by quantitative real-time PCR.

Luciferase reporter assay

Cells were plated 24 hours before transfection on gelatinized 24-well plates at a density of 1×10^5 cells/ml. Transient transfection of DNA vectors were conducted by Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. 24 hours after transfection, cells were lysed by 1X passive lysis buffer (Promega). Luciferase activities were measured by Clarity luminometer (Bio-Tek) with Dual-luciferase reporter assay system (Promega). Each transfection was carried out in duplicate. Relative activity was calculated as the ratio of the reporter plasmid Firefly luciferase activity to Renilla luciferase activity (pRL-CMV), which was co-transfected as control.

Lentivirus particle generation and lentivirus mediated shRNA gene knockdown

Tcf1 shRNA sequence was subcloned into lentiviral vector pLVTHM. Lentivirus were produced following previously established procedures. Briefly, 293T cells were plated 3 days before transfection. Tcf1 shRNA pLVTHM plasmid were mixed with envelop plasmid (pMD2G) and packaging plasmid (psPAX2) in desired ratio and transfected 293T cells by calcium phosphate. Fresh media were changed 14–16 hours post-transfection and supernatant containing lentivirus were collected 12 hours later. Supernatants can be harvested 3 times every 12 hours. Titers of virus were determined using 293T and ES cells by FACS analysis for percentage of GFP expressing cells. Three targeting sequences for Tcf1 were used to generate shRNA lentivirus (#1 TCTCCACTCTACGAACATT; #2 GGAGCTGCAGCCATATGAT; #3 GCCAAGGTCATTGCTGAGT) and tested for Tcf1 knockdown. The #3 sequence (GCCAAGGTCATTGCTGAGT) had the highest Tcf1 knockdown efficiency and was used to generate stable Tcf1 ES cell lines in this study. A scramble shRNA sequence (GTCACGATAAGACAATGAT) was used to generate control lentivirus (SCRM). Clonal Tcf1 knockdown ES cell lines and SCRM control lines were established by single colony picking and following expansion. Efficiency of Tcf1 knockdown was confirmed by qPCR and Western blot.

Chromatin Immunoprecipitation (ChIP)

Approximately 2×10^7 ES cells were cultured and processed for each ChIP experiment. Cells were chemically crosslinked by the addition of one-tenth volume of fresh formaldehyde solution (11% formaldehyde, 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Hepes pH 8) for either 10 minutes at room temperature (Tcf3) or overnight at 4°C (Oct4, β -catenin). The reaction was inactivated by the addition of 2.5 M Glycine (0.125 M final concentration) and incubated at room temperature for 10 minutes. Cells were rinsed twice with cold 1xPBS, harvested using a silicon scraper, and flash frozen in liquid nitrogen. Cells were stored at -80°C until the day of use.

Cells were resuspended in lysis buffer LB1 containing protease inhibitors (50 mM Hepes pH 7.7, 140 mM NaCl, 1 mM EDTA, 10% Glycerol, 0.5% NP-40, 0.25% Triton-X-100), incubated for 10 minutes on ice and centrifuged at 4°C for 10 minutes at 2500 rpm. The cell pellet was resuspended in LB2 (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris pH 7.5), incubated for 10 minutes on ice and centrifuged at 4°C for 10 minutes at 2500 rpm. Finally the cell pellet was resuspend in sonication buffer LB3 (1 mM EDTA, 0.5 mM EGTA, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Na-deoxycholate) and sonicated to

solubilize and shear crosslinked DNA. Sonication was performed using a Branson sonicator for 20 μ 30 second pulses (60 second pause between pulses) at a 60% amplitude. Triton-X-100 was added to the lysates after sonication to a final concentration of 1%. Cellular debris was removed by centrifugation. Samples were kept on ice at all times.

The resulting chromatin extract containing DNA fragments with an average size of 500 bp were immunoprecipitated overnight at 4°C using magnetic beads (Protein G Dynabeads, Invitrogen) that had been pre-incubated with 5 μ g of the appropriate antibody. The antibodies used for immunoprecipitation were Tcf3 (Merrill Lab), Oct4 (Santa Cruz sc8628) and β -catenin (Invitrogen 71–2700). Beads were washed 3 times with RIPA ChIP buffer (50 mM HEPES, 1mM EDTA, 0.7% Na-deoxycholate, 1% NP-40, 0.5 M LiCl) and 1 time with TBS (50 mM Tris, 150 mM NaCl, pH 7.6). Bound complexes were eluted from the beads by heating at 65°C with occasional vortexing, and crosslinks were reversed by overnight incubation at 65°C. Whole cell extract DNA (reserved from the sonication step) was also treated for crosslink reversal. Eluted protein-DNA complexes were treated with RNase A (0.2 μ g/ μ l) and proteinase K (0.2 μ g/ μ l). Following phenol-chloroform extraction and ethanol precipitation, DNA was dissolved in Tris-EDTA.

siRNA downregulation of Tcf1 for AP colony assays

Stealth siRNA duplexes (Invitrogen) designed against mouse Tcf1 (AGAGACUGUCUACUCUGCCUCAAU) or a control siRNA (GGAAGACUAGAGGCGUCAUGAGUU) were used to specifically downregulate Tcf1 mRNA expression levels. For transfection, ES cells were trypsinized, pelleted by centrifugation and resuspended in media without antibiotics. For each phenotype, 100,000 cells per well were combined with Lipofectamine 2000 transfection complexes of control or Tcf1 siRNA (50 nM) following the manufacturer's protocol (Invitrogen) and plated on gelatin on a 6-well dish. Twelve hours after transfection cells were split onto new plates and used in a colony differentiation assay.

Accession Numbers

The complete microarray dataset was submitted to NCBI GEO repository and is available as GSE27455.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Kathrin Plath for her helpful discussions and thoughtful comments on the manuscript, and Dr. Minoru Ko and Dr. Akira Nishiyama for the inducible Tcf3 overexpression cell line. The work was funded by grants from the American Cancer Society (RSG GGC 112994, B.J.M.), the National Institutes of Health (R01-CA128571, B.J.M. and R01-GM065400, D.R.L.) and the Howard Hughes Medical Institute (D.R.L.).

REFERENCES

1. Kim J, Chu J, Shen X, Wang J, Orkin SH. An extended transcriptional network for pluripotency of embryonic stem cells. *Cell*. 2008; 132:1049–1061. [PubMed: 18358816]

2. Liang J, et al. Nanog and Oct4 associate with unique transcriptional repression complexes in embryonic stem cells. *Nat Cell Biol.* 2008; 10:731–739. [PubMed: 18454139]
3. Boyer LA, et al. Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell.* 2005; 122:947–956. [PubMed: 16153702]
4. Loh YH, et al. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet.* 2006
5. Nichols J, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell.* 1998; 95:379–391. [PubMed: 9814708]
6. Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet.* 2000; 24:372–376. [PubMed: 10742100]
7. Avilion AA, et al. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev.* 2003; 17:126–140. [PubMed: 12514105]
8. Chambers I, et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell.* 2003; 113:643–655. [PubMed: 12787505]
9. Mitsui K, et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell.* 2003; 113:631–642. [PubMed: 12787504]
10. Marson A, et al. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell.* 2008; 134:521–533. [PubMed: 18692474]
11. Cole MF, Johnstone SE, Newman JJ, Kagey MH, Young RA. Tcf3 is an integral component of the core regulatory circuitry of embryonic stem cells. *Genes Dev.* 2008; 22:746–755. [PubMed: 18347094]
12. Tam WL, et al. T-cell factor 3 regulates embryonic stem cell pluripotency and self-renewal by the transcriptional control of multiple lineage pathways. *Stem Cells.* 2008; 26:2019–2031. [PubMed: 18467660]
13. Yi F, Pereira L, Merrill BJ. Tcf3 Functions as a Steady State Limiter of Transcriptional Programs of Mouse Embryonic Stem Cell Self Renewal. *Stem Cells.* 2008
14. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell.* 2009; 17:9–26. [PubMed: 19619488]
15. Brannon M, Gomperts M, Sumoy L, Moon RT, Kimelman D. A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in *Xenopus*. *Genes Dev.* 1997; 11:2359–2370. [PubMed: 9308964]
16. Molenaar M, et al. XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. *Cell.* 1996; 86:391–399. [PubMed: 8756721]
17. van de Wetering M, et al. Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. *Cell.* 1997; 88:789–799. [PubMed: 9118222]
18. Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med.* 2004; 10:55–63. [PubMed: 14702635]
19. Doble BW, Patel S, Wood GA, Kockeritz LK, Woodgett JR. Functional redundancy of GSK-3alpha and GSK-3beta in Wnt/beta-catenin signaling shown by using an allelic series of embryonic stem cell lines. *Dev Cell.* 2007; 12:957–971. [PubMed: 17543867]
20. Nichols J, Silva J, Roode M, Smith A. Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. *Development.* 2009; 136:3215–3222. [PubMed: 19710168]
21. Ying QL, et al. The ground state of embryonic stem cell self-renewal. *Nature.* 2008; 453:519–523. [PubMed: 18497825]
22. Kelly KF, et al. beta-catenin enhances Oct-4 activity and reinforces pluripotency through a TCF-independent mechanism. *Cell Stem Cell.* 2011; 8:214–227. [PubMed: 21295277]
23. Pereira L, Yi F, Merrill BJ. Repression of nanog gene transcription by tcf3 limits embryonic stem cell self-renewal. *Mol Cell Biol.* 2006; 26:7479–7491. [PubMed: 16894029]
24. Nishiyama A, et al. Uncovering early response of gene regulatory networks in ESCs by systematic induction of transcription factors. *Cell Stem Cell.* 2009; 5:420–433. [PubMed: 19796622]

25. Raychaudhuri S, Stuart JM, Altman RB. Principal components analysis to summarize microarray experiments: application to sporulation time series. *Pac Symp Biocomput.* 2000;455–466. [PubMed: 10902193]
26. Arce L, Yokoyama NN, Waterman ML. Diversity of LEF/TCF action in development and disease. *Oncogene.* 2006; 25:7492–7504. [PubMed: 17143293]
27. van de Wetering M, Oosterwegel M, Dooijes D, Clevers H. Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence-specific HMG box. *Embo J.* 1991; 10:123–132. [PubMed: 1989880]
28. Galceran J, Farinas I, Depew MJ, Clevers H, Grosschedl R. Wnt3a^{-/-}-like phenotype and limb deficiency in Lef1^(-/-)Tcf1^(-/-) mice. *Genes Dev.* 1999; 13:709–717. [PubMed: 10090727]
29. Gregorieff A, Grosschedl R, Clevers H. Hindgut defects and transformation of the gastro-intestinal tract in Tcf4^(-/-)/Tcf1^(-/-) embryos. *Embo J.* 2004; 23:1825–1833. [PubMed: 15057272]
30. Nguyen H, et al. Tcf3 and Tcf4 are essential for long-term homeostasis of skin epithelia. *Nat Genet.* 2009; 41:1068–1075. [PubMed: 19718027]
31. Biechele TL, Adams AM, Moon RT. Transcription-based reporters of Wnt/beta-catenin signaling. *Cold Spring Harb Protoc.* 2009; 2009 pdb prot5223.
32. Jho EH, et al. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol Cell Biol.* 2002; 22:1172–1183. [PubMed: 11809808]
33. Wang J, et al. A protein interaction network for pluripotency of embryonic stem cells. *Nature.* 2006; 444:364–368. [PubMed: 17093407]

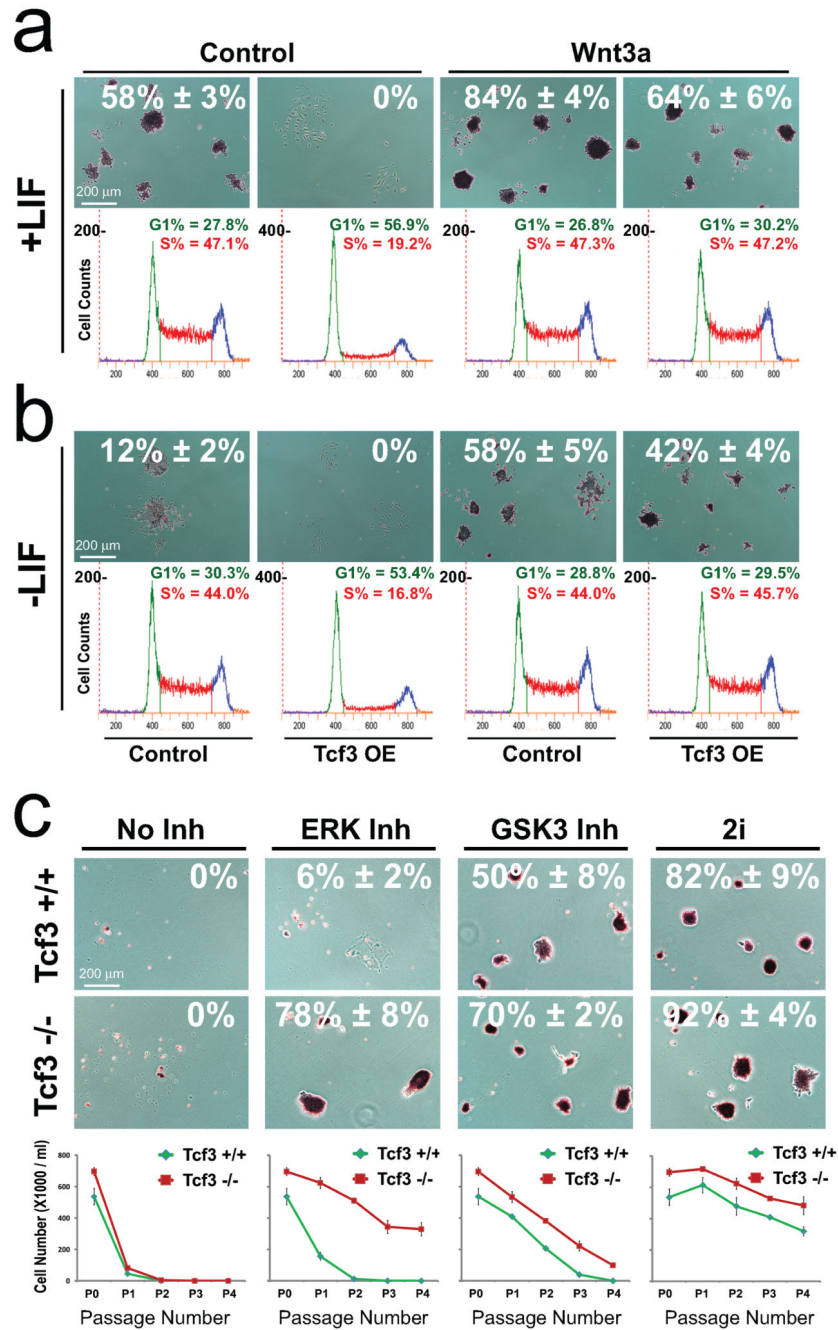


Figure 1. Tcf3 regulates the Wnt stimulation and GSK3-inhibition of ES cell self renewal (a–b) Alkaline phosphatase (AP) staining of ES cell colonies after four days in control or Wnt3a conditioned media (indicated at top). Overexpression of full-length Tcf3 protein (indicated at bottom) was induced at the onset of the experiment. Supplementary Fig. S2 shows Western blot analysis of protein lysates taken from Tcf3-overexpressing cells. The percentage of AP+ ES colonies (yellow number atop each image) represents mean ± standard deviation of biological duplicated experiments. Below each image, flow cytometry analysis of cell cycle progression by propidium iodide staining was performed on separate

cell cultures subjected to the same conditions for three days. Results shown in (a) were performed in media containing 1000 U/ml of exogenous LIF, whereas those shown in (b) did not contain exogenous LIF.

(c) ES colony formation from *Tcf3*^{+/+} or *Tcf3*^{+/+} cells (indicated on left) subjected to serum-free culture conditions for four days and stained for AP activity. Colonies were treated with N2B27 media without inhibitors (No Inh), N2B27 media + PD0325901 (ERK Inh), N2B27 media + CHIR99021 (GSK3 Inh), or N2B27 + PD0325901 and CHIR99021 (2i). The percentage of AP⁺ ES colonies (yellow number atop each image) represents mean \pm standard deviation of biological duplicated experiments.

(d) Number of dye-excluding cells following each serial passage of *Tcf3*^{+/+} (green) and *Tcf3*^{-/-} (red) ES cells in serum free conditions as noted in (c).

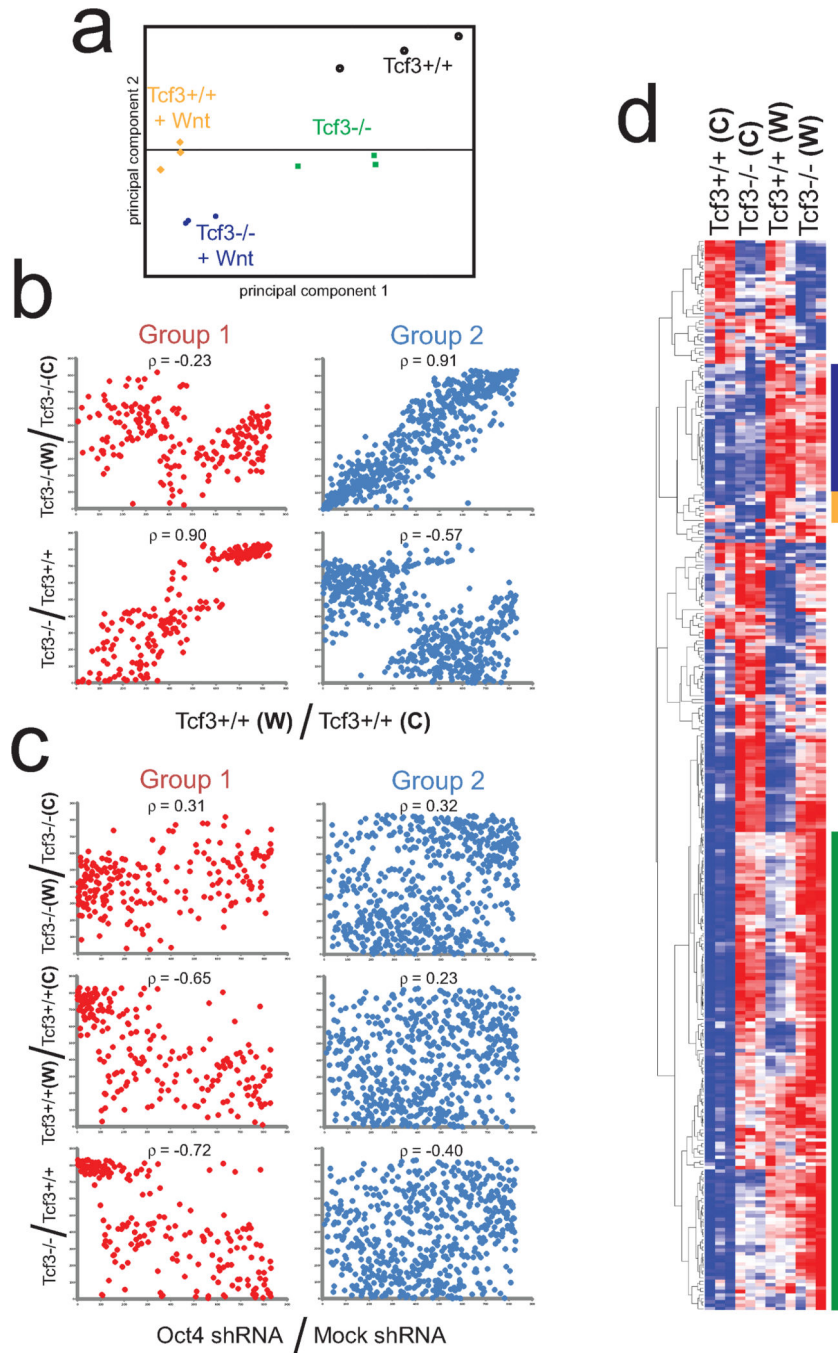


Figure 2. Wnt3a stimulates and Tcf3 inhibits expression of Oct4 and Nanog regulated genes
 (a) Principle component analysis of gene expression in four conditions in ES cells: Tcf3^{+/+} (black); Tcf3^{-/-} (green); Tcf3^{+/+} + Wnt3a (orange); Tcf3^{-/-} + Wnt3a (blue). Each point represents one of three biological replicates for each condition.
 (b) Each point on a graph represents one of the 831 genes significantly increased in any of the three comparisons (Tcf3^{-/-} vs. Tcf3^{+/+}; Tcf3^{+/+} + Wnt3a vs. Tcf3^{+/+}; or Tcf3^{-/-} + Wnt3a vs. Tcf3^{-/-}). Individual genes are listed in Supplementary Table S1. Genes were separated into group 1 (red) and group 2 (blue) genes and plotted according to their rank fold effect in

the comparisons listed on each axis. Pearson correlation coefficients (top of each graph) are shown for each comparison. Calculations for formation of groups and determination of correlation coefficients are presented in Supplementary Table S2.

(c) Individual genes were plotted based on rank fold effect of Oct4 shRNA treatment⁴ (x-axis) and the effect of Tcf3 and/or Wnt3a (y-axis). Group 1 or group 2 genes are the same as in (b). Pearson correlation coefficients (top of each graph) are shown for each comparison. Calculations for formation of groups and determination of correlation coefficients are presented in Supplementary Table S2.

(d) Hierarchical clustering comparison of effects caused by ablation of Tcf3 and Wnt3a treatment. The heat map shows only genes that were decreased by Oct4 and Nanog RNAi experiments⁴. Genes that were increased in *Tcf3*^{-/-} and further increased in *Tcf3*^{-/-} + Wnt3a are highlighted with the green bar. Genes that were increased by Wnt3a in a partially Tcf3-dependent manner are highlighted with blue and orange bar. Expression values for the 311 genes are presented in Supplementary Table S3

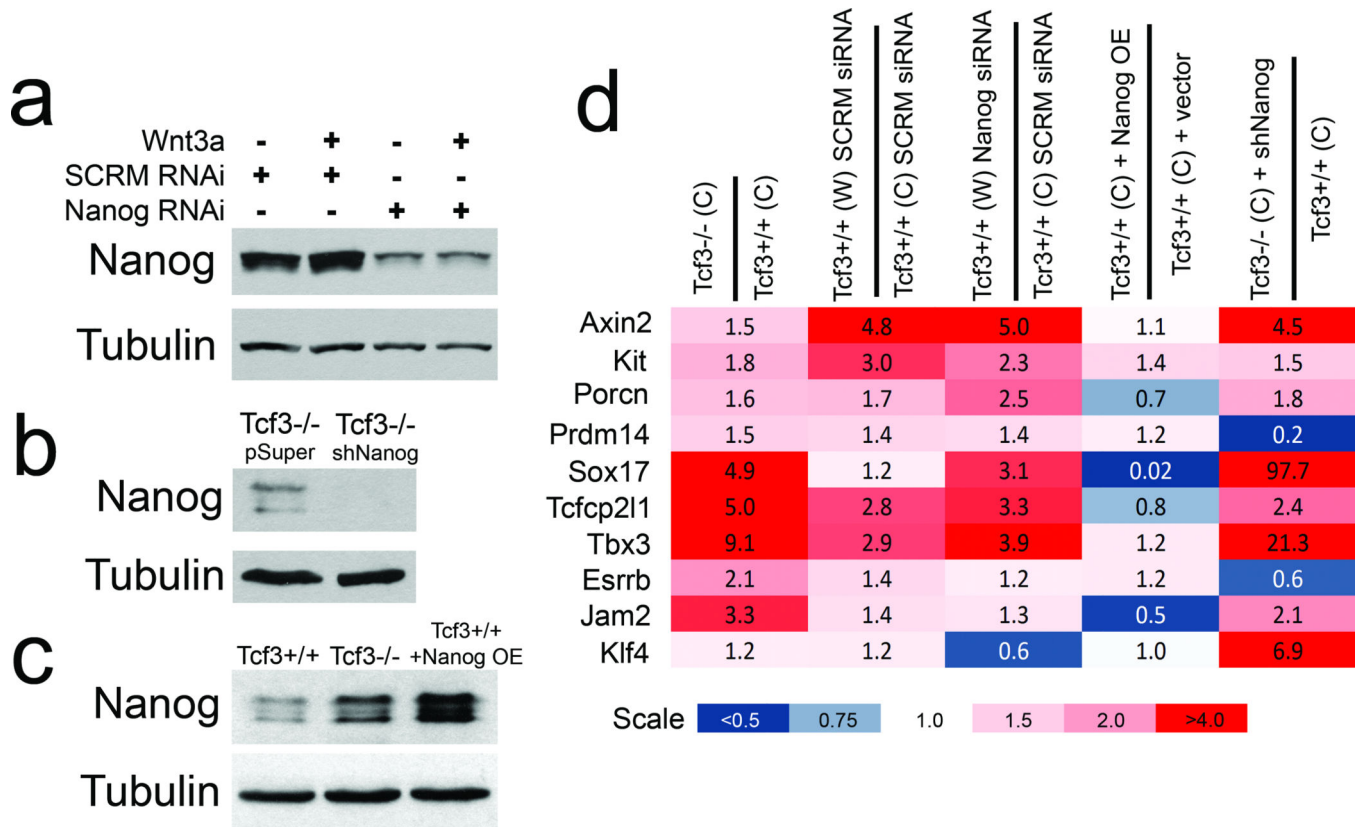


Figure 3. Nanog-independence of Wnt3a/Tcf3-mediated effects on gene expression

(a) *Tcf3*^{+/+} ES cells were transfected with Nanog siRNA or control (SCRM) siRNA and treated for 24hr with 50 ng/ml Wnt3a. Protein lysates were used for Western blot analysis to detect Nanog and Tubulin proteins. Identically treated cells were used for RNA isolation for qPCR data shown in Fig 3d.

(b) *Tcf3*^{-/-} ES cells were stably transfected with either a Nanog shRNA or control pSuper vector. Protein lysates were used for Western blot analysis to detect Nanog and Tubulin proteins. Identically treated cells were used for RNA isolation for qPCR data shown in Fig 3d.

(c) *Tcf3*^{+/+} ES cells were stably transfected with a Nanog-overexpression plasmid. Protein lysates from cells grown in LIF⁺, self renewal conditions were used for Western blot analysis to detect Nanog and Tubulin proteins. *Tcf3*^{+/+} and *Tcf3*^{-/-} without the Nanog expression plasmid were included as controls. Identically treated cells were used for RNA isolation for qPCR data shown in Fig 3d.

(d) Quantitative PCR analysis measuring mRNA levels of 10 group 1 genes (Fig 2). The level of each gene (rows) relative to GAPDH was used to determine fold change effects shown in the heatmap. Raw data are shown in Supplementary Table S4. Fold changes are shown for each the comparisons depicted atop each column, and numbers within each box equal fold-changes for each gene. Note that for most genes, knockdown of Nanog did not reduce the stimulation of gene expression caused by Wnt3a treatment or by Tcf3 ablation. In addition, overexpression of Nanog in *Tcf3*^{+/+} cells was not sufficient to activate expression of group 1 genes as well as either Wnt3a treatment or Tcf3 ablation.

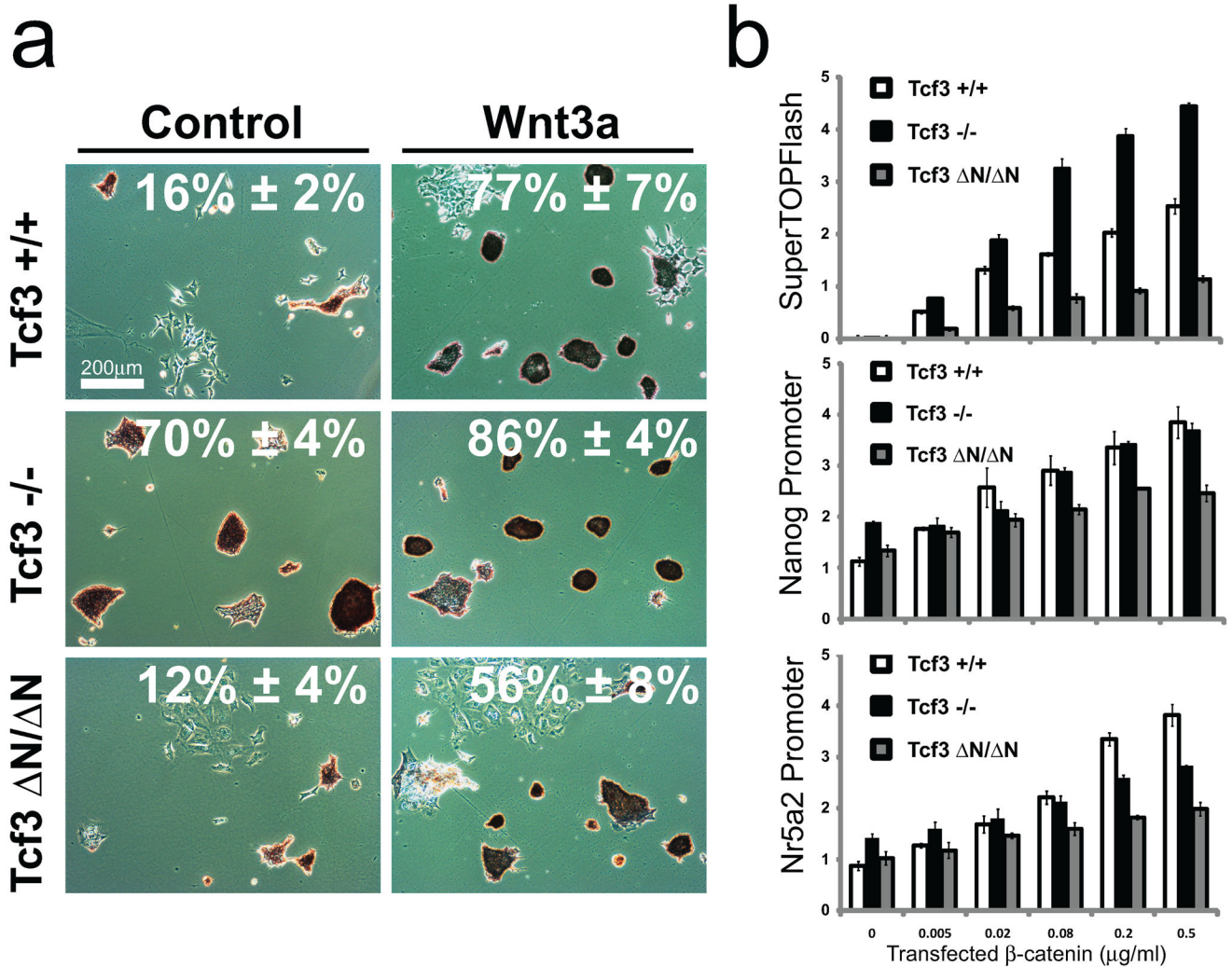


Figure 4. A combination of Tcf3- β -catenin-dependent and Tcf3- β -catenin-independent mechanisms mediate Wnt3a stimulation of self renewal and target gene expression

(a) Alkaline phosphatase staining of *Tcf3*^{+/+}, *Tcf3*^{-/-} and *Tcf3* ^{$\Delta N/\Delta N$} ES colonies after four days in serum containing media without LIF, and without (Control; left) or with 50 ng/ml recombinant mouse Wnt3a (Wnt3a; right). The percentage of AP⁺ ES colonies (white number atop each image) represents mean \pm standard deviation of biological duplicate experiments.

(b) Luciferase reporter assays for β -catenin stimulation of SuperTOPFlash (top graph), Nanog promoter (middle graph) and Nr5a2 promoter (bottom graph). Increasing amount of stable β -catenin expression plasmid differentially activated reporter activities in *Tcf3*^{+/+} (white), *Tcf3*^{-/-} (black) and *Tcf3* ^{$\Delta N/\Delta N$} (gray) ES cells. Data supporting the generation and primary characterization of *Tcf3* ^{$\Delta N/\Delta N$} ES cells are shown in Supplementary Fig. S4. Values represent mean \pm standard deviation of biological duplicates.

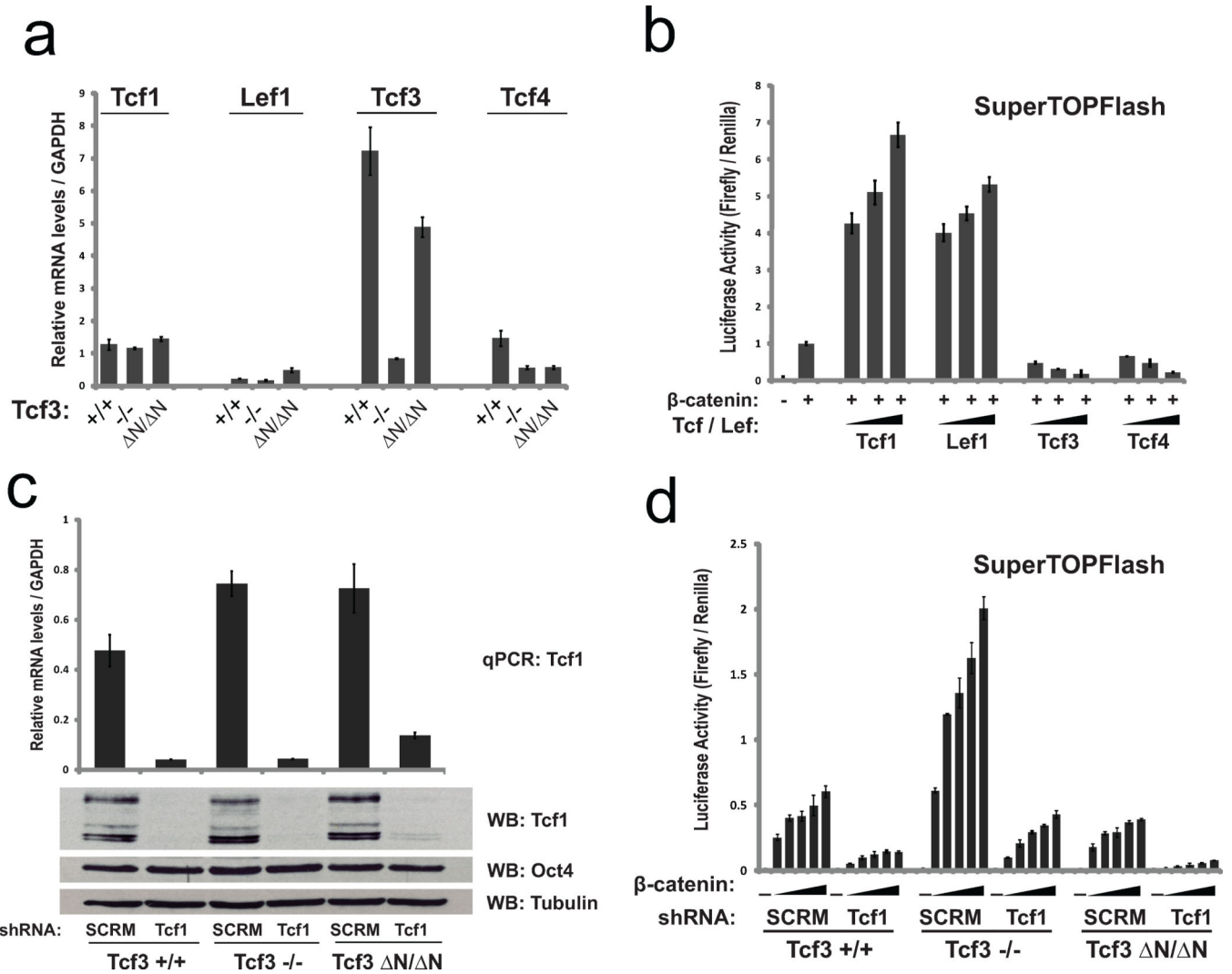


Figure 5. Endogenous Tcf1 stimulates Tcf3-β-catenin-independent activation of Wnt target genes
 (a) Tcf cDNAs were measured by qPCR from RNA isolated from *Tcf3*^{+/+}, *Tcf3*^{-/-} and *Tcf3*^{ΔN/ΔN} ES cells.
 (b) Stable β-catenin expression plasmid was cotransfected into ES cells with increasing concentrations of the indicated Tcf-expression plasmids. Relative SuperTOPFlash luciferase levels were measured and values represent mean of biological duplicates +/- standard deviation.
 (c) qPCR measurement of Tcf1 mRNA (graph) from Tcf1 shRNA and SCRIN shRNA cell lines identified at the bottom of the panel. Western blot analysis of protein lysates isolated from indicated ES cell lines was performed using antibodies identified at the right. Data supporting the effectiveness of shRNA knockdown of Tcf1 are shown in Supplementary Fig. S5.
 (d) SuperTOPFlash luciferase reporter assay using cell lines also used in (c) for transfection of increasing levels of stable β-catenin expression plasmid.

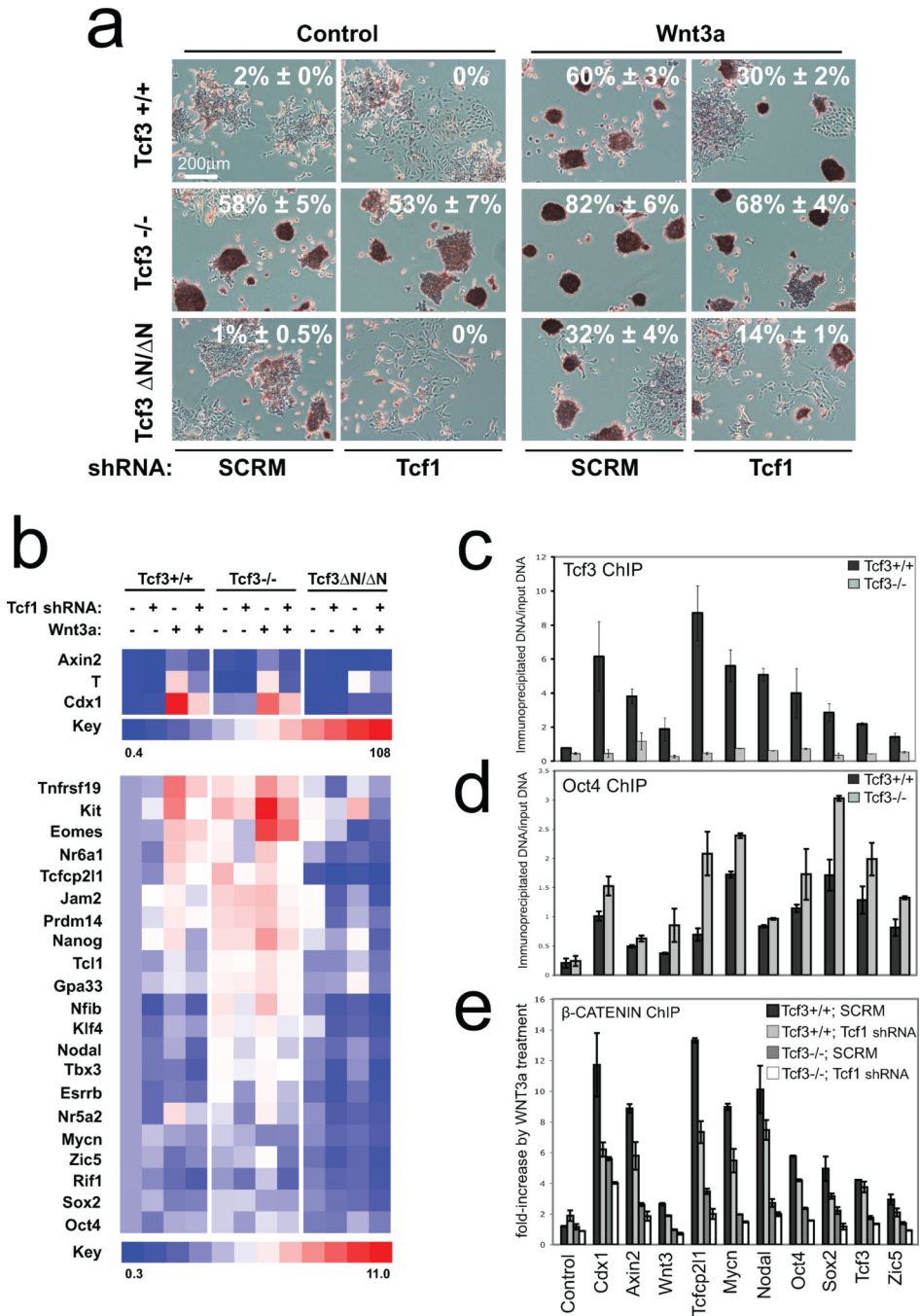


Figure 6. Combined effect of Tcf1 and Tcf3-β-catenin for the Wnt3a response of ES cells
 (a) Alkaline phosphatase staining of colonies from ES cell lines with different Tcf3 genotypes (indicated on left) and expressing an shRNA (indicated on bottom). Colonies were formed for four days in serum containing media without LIF, and with control or Wnt3a (indicated on top). Percentage of AP+ colonies (white number) represents mean ± standard deviation of biological duplicates. Wnt3a stimulated self-renewal at different levels in *Tcf3*^{+/+}, *Tcf3*^{-/-} and *Tcf3*^{N/N} ES cells, while knockdown of Tcf1 significantly reduced stimulations in all three ES cell lines.

(b) Gene expression analysis in different Tcf3 ES cell lines \pm Tcf1 shRNA and \pm recombinant Wnt3a. The relative level of mRNA for each gene is compared to untreated *Tcf3*^{+/+} cells set at 1.0. Numerical values \pm standard deviations are shown in Supplementary Table S5.

(c–e) Chromatin immunoprecipitated with antibodies against Tcf3 (c), Oct4 (d), or β -catenin (e) was measured by qPCR using primers (Supplementary Table S5) specific for upstream regions of the ten genes labeled at the bottom of graph shown in (e). A representative graph from three biological replicates is shown for each experiment.

(c,d) Assays were performed using chromatin from *Tcf3*^{+/+} (black) and *Tcf3*^{-/-} (gray) ES cells. Values represent the mean percentage of DNA immunoprecipitated relative to input \pm standard deviations of replicate measurements.

(e) Assays were performed using chromatin from *Tcf3* ES cell lines \pm Tcf1 shRNA and \pm Wnt3a conditioned media. The percentage of β -catenin immunoprecipitated was determined for control and +Wnt3a treatments, and values represent the mean fold increase in β -catenin occupancy stimulated by Wnt3a \pm standard deviations of replicate measurements.