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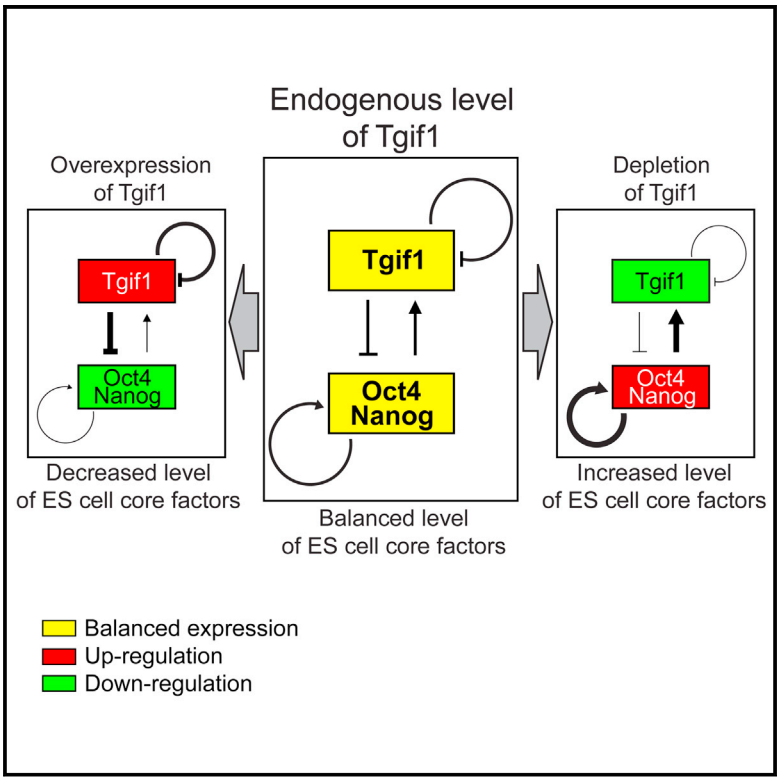
Report

Tgif1 Counterbalances the Activity of Core Pluripotency Factors in Mouse Embryonic Stem Cells

Graphical Abstract



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In Brief

Although many positive regulators have been identified, little is known about negative regulations that are also critically important in sustaining self-renewing status of embryonic stem cells (ESCs). Lee et al. reveal Tgif1 as an integral negative regulator of the core regulatory circuitry, which precisely controls the levels of the ESC core factors.

Highlights

- The level of Tgif1 is regulated by embryonic stem cell (ESC) core pluripotency factors
- Tgif1 is an integral component of the ESC core regulatory network
- Tgif1 counterbalances the levels of the core pluripotency factors
- Tgif1 functions independently of the TGFβ/activin/nodal signaling in mouse ESCs

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Tgif1 Counterbalances the Activity of Core Pluripotency Factors in Mouse Embryonic Stem Cells

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SUMMARY

Core pluripotency factors, such as Oct4, Sox2, and Nanog, play important roles in maintaining embryonic stem cell (ESC) identity by autoregulatory feedforward loops. Nevertheless, the mechanism that provides precise control of the levels of the ESC core factors without indefinite amplification has remained elusive. Here, we report the direct repression of core pluripotency factors by Tgif1, a previously known terminal repressor of TGFβ/activin/nodal signaling. Overexpression of Tgif1 reduces the levels of ESC core factors, whereas its depletion leads to the induction of the pluripotency factors. We confirm the existence of physical associations between Tgif1 and Oct4, Nanog, and HDAC1/2 and further show the level of Tgif1 is not significantly altered by treatment with an activator/inhibitor of the TGFβ/activin/nodal signaling. Collectively, our findings establish Tgif1 as an integral member of the core regulatory circuitry of mouse ESCs that counterbalances the levels of the core pluripotency factors in a TGFβ/activin/nodal-independent manner.

INTRODUCTION

Embryonic stem cells (ESCs) infinitely self-renew in vitro while maintaining their pluripotency. Core pluripotency transcription factors (TFs), including Oct4, Sox2, and Nanog, play critical roles in maintaining ESC identity by forming an intricate regulatory circuitry where they cooperatively preserve the pluripotency, in part, by self-activating and by silencing lineage-specific regulators (Kim et al., 2008; Niwa et al., 2000). The maintenance of optimal levels of these core factors has been suggested to be a critical prerequisite for sustaining stem cell identity (Kopp et al., 2008; Niwa et al., 2000), but progress has been made mostly in elucidating the positive regulatory mechanisms (e.g., autoregulatory and feedforward regulatory loops) among TFs within the core pluripotency network (Jaenisch and Young, 2008; Young, 2011). Little is known about the negative regulatory mechanisms stabilizing the entire pluripotency network without overwhelming the activation of the ESC core factors.

Signaling pathways converge external signals onto transcriptional regulatory networks, thereby affecting pluripotency and differentiation of ESCs (Pera and Tam, 2010). Among these, the TGFβ/activin/nodal-signaling pathway has been implicated in the maintenance of human ESCs and mouse epiblast stem cells (James et al., 2005). Notably, previous studies have suggested that this pathway is not crucial for mouse ESC maintenance (Fei et al., 2010; James et al., 2005). The TGFβ signaling is transduced through phosphorylation of cytoplasmic effectors, such as Smad2 and Smad3 (Smad2/3), by forming a Smads complex. The Smads complex then interacts with tissue-specific TFs and subsequently regulates downstream targets (Liu, 2008).

TG-interacting factor 1 (Tgif1), a homeodomain-containing TF, has been identified as a terminal repressor of TGFβ signaling in somatic cells (Wotton et al., 1999a). Loss-of-function studies revealed that Tgif1 is critical in diverse developmental processes, such as gastrulation and craniofacial, neuronal, and placental development (Bartholin et al., 2008; Powers et al., 2010). Tgif1 also plays diverse roles in cell-cycle regulation and repression of retinoic acid signaling (Bartholin et al., 2006; Mar and Hoodless, 2006). TGFβ signaling was suggested to be insignificant in mouse ESCs, but Tgif1 was identified as a common target of multiple mouse ESC core factors (Kim et al., 2008). Preferential interactions between mouse-ESC-specific enhancers and the regulatory elements of Tgif1 have also been observed (Kieffer-Kwon et al., 2013). These findings suggest Tgif1 may play important roles in mouse ESCs, but its functions have not been thoroughly investigated.

Here, we report that Tgif1 is a member of the mouse ESC core regulatory network and that it negatively controls the levels of ESC core factors. Perturbations of the Tgif1 level lead to abnormal growth of ESCs. Integrative analyses of chromosomal targets and global gene expression profiles following knockdown (KD) and overexpression (OE) of Tgif1 reveal that Tgif1 directly represses the ESC core factors. We confirm physical associations between Tgif1 and ESC core factors and the negligible effect of TGFβ signaling in mouse ESCs. All together, we propose that Tgif1 acts as a molecular rheostat responsible for precise control of the levels of ESC core factors with a role independent from TGFβ signaling.

RESULTS

Abnormal Level of Tgif1 Promotes Loss of Normal Mouse ESC Phenotype

We have previously predicted Tgif1 as a common target of multiple core TFs in mouse ESCs using chromatin immunoprecipitation (ChIP) followed by microarrays (Kim et al., 2008). The result was confirmed by ChIP, followed by massive parallel sequencing (ChIP-seq) of Oct4 and Nanog (Figure 1A), suggesting a direct regulatory role of Oct4 and Nanog on the expression of Tgif1. Consistently, OE of Oct4 or Nanog increased the level of Tgif1 (both mRNA and protein), whereas KD of Oct4 or Nanog reduced the level of Tgif1 (Figures 1B, S1A, and S1B), indicating that Oct4 and Nanog directly activate Tgif1. In mouse ESCs, Tgif1 is robustly expressed and exclusively resides in the nucleus (Figures 1C and S1C). Its expression gradually diminishes following differentiation, similar to Oct4, implying Tgif1 may have important roles in ESCs (Figure 1D).

We delineated the roles of Tgif1 by first performing KD of Tgif1 using short hairpin RNAs (shRNAs). Tgif1 KD in ESCs resulted in a flattened cell morphology, monolayer growth, and a decreased alkaline phosphatase (AP) activity within 4 days (Figures 1E and S1D–S1F). This aberrant morphology was consistently observed in Tgif1-knockout (KO) ESCs generated by the CRISPR-Cas9 system (Figures 1F, S1G, and S1H), as well as in two other ESC lines (E14 and CJ7) following Tgif1 KD (Figure S1I). We rescued the Tgif1 KD phenotype by reconstituting the Tgif1 protein (Figure 1G), indicating that Tgif1 is responsible for loss of the normal ESC phenotype. We then investigated the effects of Tgif1 OE. An approximately 2-fold induction of Tgif1 was sufficient to abrogate the convex morphology of ESC colonies and to trigger a substantial loss of AP activity (Figures 1H and S1J). A positive correlation was also observed between the extent of Tgif1 OE and the severity of phenotypic change (Figures S1J and S1K). These results, together with the morphological differences observed following KD and OE of Tgif1 (Figures 1E and 1H), suggest that Tgif1 induces distinct and dosage-dependent phenotypic changes.

We investigated the self-renewal capacity following perturbations of Tgif1 (KD, KO, and OE) by conducting colony-forming assays as well as cell growth assays. The Tgif1 KD and KO cells both formed flattened colonies with reduced AP activity, and more than 60% of the Tgif1 OE cells were differentiated at least partially (Figure 1I). Consistently, cell proliferation rate was significantly reduced by OE of Tgif1, whereas a slight reduction was observed in KD and KO cells (Figure S1L). We further tested the requirement of Tgif1 during somatic cell reprogramming using Oct4, Sox2, and Klf4 (Nakagawa et al., 2008) and found that KD of Tgif1 significantly impeded the generation of induced pluripotent stem cells (iPSCs) (Figures 1J and S1M), suggesting that Tgif1 is required for the acquisition of induced pluripotency. Taken together, these results indicate a proper level of Tgif1 is critical for maintenance of the normal ESC phenotype and induced pluripotency.

Abnormal Levels of Tgif1 Induce Lineage Marker Genes

We examined the effect of Tgif1 perturbations on the nature of ESCs by performing global gene expression profiling. We found many differentially expressed genes upon perturbation of Tgif1 (Tables S1 and S2). Gene Ontology (GO) analyses revealed KD or OE of Tgif1 induced several genes that are implicated in developmental processes (Figure S2A), but only a small number of genes upregulated were in common (Figure S2B). This result may account for the morphological differences between Tgif1 KD and Tgif1 OE cells (Figure 1). Accordingly, gene set enrichment analysis (GSEA) (Subramanian et al., 2005) revealed that KD of Tgif1 preferentially induces endoderm markers, whereas OE of Tgif1 induces markers of other lineages (mesoderm, ectoderm, and trophectoderm; Figure 2A), which were validated by qPCR (Figure S2C; Table S3).

We then examined the effects of Tgif1 perturbation on global gene expression by monitoring the activity of three previously defined ESC modules (Core, Myc, and PRC; Kim et al., 2010) and bivalent genes (Bernstein et al., 2006). As predicted from their morphology, Tgif1 OE cells showed decreased activity of Core and Myc modules, with increased activity of the PRC module and bivalent genes (Figures 2B and S2D). Surprisingly, an unexpected elevation of Core module activity was observed in the Tgif1 KD cells (Figures 2B and S2D), suggesting that Tgif1 may negatively affect the levels of core module factors. We validated the results by qPCR of self-renewal-related genes following acute KD (for 48 hr) and KO of Tgif1, as well as acute OE of Tgif1 using a Tet-inducible system (for 24 hr; Figure 2C). E14 and CJ7 cells also showed similar induction of the core factors following KD of Tgif1 (for 48 hr; Figure S2E). These findings collectively suggested that Tgif1 negatively affects ESC core factor levels. Notably, Tgif1-mediated repression of ESC core factors was modest in 2i culture condition where ESCs show more morphologically uniform and homogenous expression of ESC core factors (Marks et al., 2012) (Figures S2F and S2G). This may be due to context-dependent regulatory roles of Tgif1 under serum/LIF versus 2i culture conditions.

Tgif1 Shares Common Genomic Targets with ESC Core Factors

We identified genomic targets of Tgif1 by performing ChIP-seq (16,400 target sites and 9,857 target genes; Table S4). Tgif1 occupies the proximal regions of the transcription start sites (TSSs) of genes, but it also occupies many distal regions (>50% of targets; Figure 3A). This result was similar to the target occupancy patterns of many ESC core factors. Further GO analysis revealed that Tgif1 significantly occupies many stem cell maintenance/development-related genes (Figure 3B). A global target correlation analysis also showed that Tgif1 shares many targets with Oct4, Sox2, and Nanog (Figures 3C, S3A, and S3B). Consistently, the Core module genes were strongest Tgif1 targets (Figures S3C and S3D), and motif analysis further confirmed a significant enrichment of motifs for Oct4, Sox2, and Klf4 near the Tgif1 sites (Figure S3E). All these findings indicate that Tgif1 is tightly interconnected with the ESC core regulatory circuitry.

Tgif1 Directly Attenuates the Levels of ESC Core Factors

To examine the direct transcriptional influence of Tgif1 on its targets, we ranked the expression values following OE of Tgif1

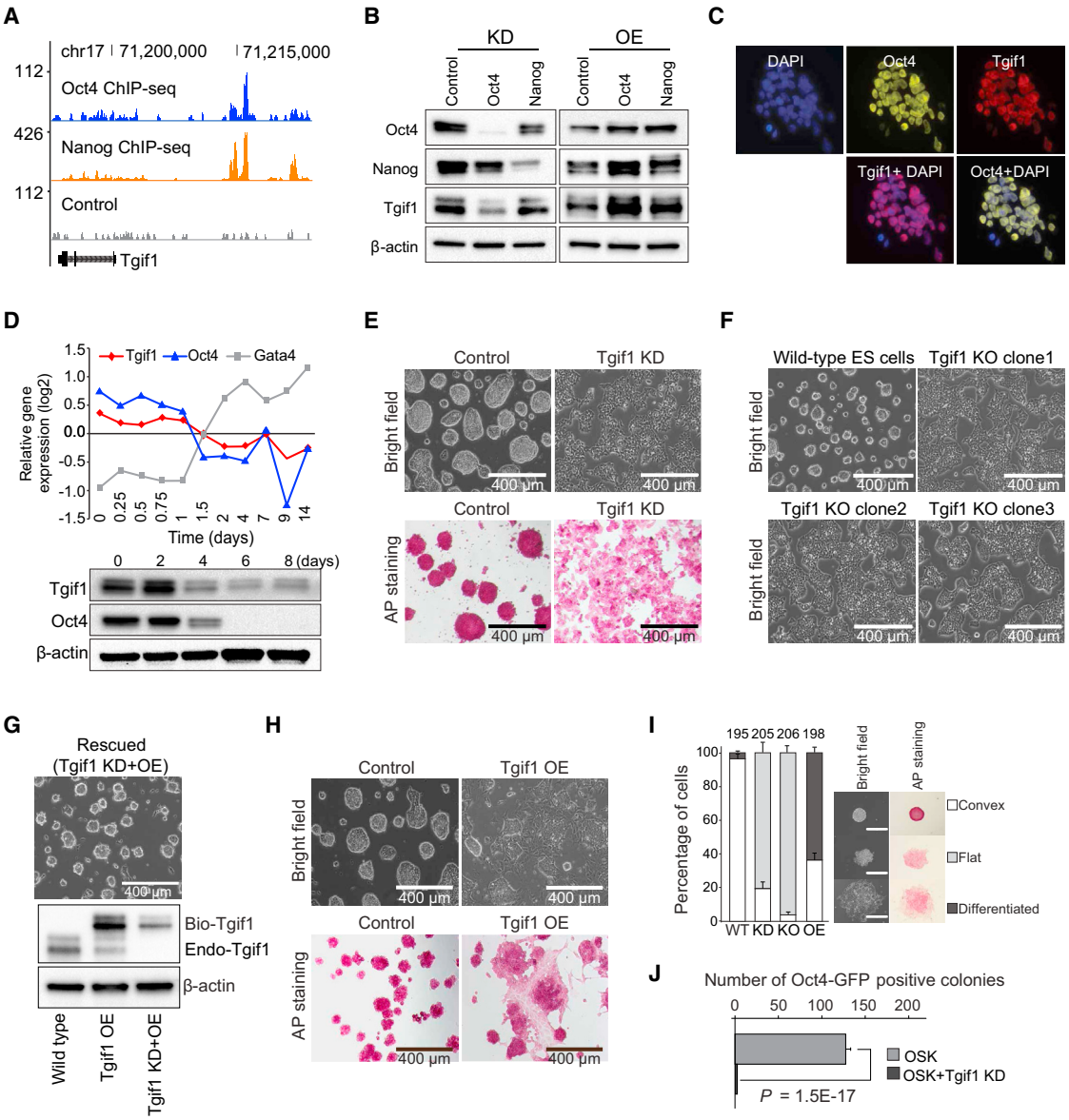


Figure 1. Aberrant Expression of Tgif1 Disrupts Mouse ESC Morphology

(A) ChIP-seq signal tracks showing Oct4 and Nanog occupancy at the regulatory regions of *Tgif1*.

(B) Protein levels of Tgif1, Oct4, and Nanog upon KD (left panel) and OE of Oct4 and Nanog (right panel).

(C) Immunofluorescence (IF) images depicting nuclear localization of Tgif1 and Oct4.

(D) Relative mRNA and protein levels of Tgif1 and Oct4 upon differentiation of ESCs. y axis, relative gene expression to an averaged expression value across the time points.

(E) Colony morphology and AP staining upon Tgif1 KD.

(F) ESC morphologies in Tgif1 KO cells in normal culture conditions.

(G) Rescued morphology and Tgif1 protein levels upon OE and KD+OE in ESCs.

(H) Colony morphology and AP staining upon Tgif1 OE.

(I) Colony-forming assays of wild-type (WT) and Tgif1-perturbed cells (OE, KD, and KO). Bar graphs show percentage of self-renewing ESCs forming round-shape colony and cells with flattened or more-differentiated morphology. Error bars indicate SEM (n = 3). Averaged total number of colonies in each condition is shown on the top of each bar.

(J) A bar graph showing the number of Oct4-GFP-positive colonies from three-factor reprogramming (OSK) and reprogramming with KD of Tgif1. p value was calculated using Fisher's exact test.

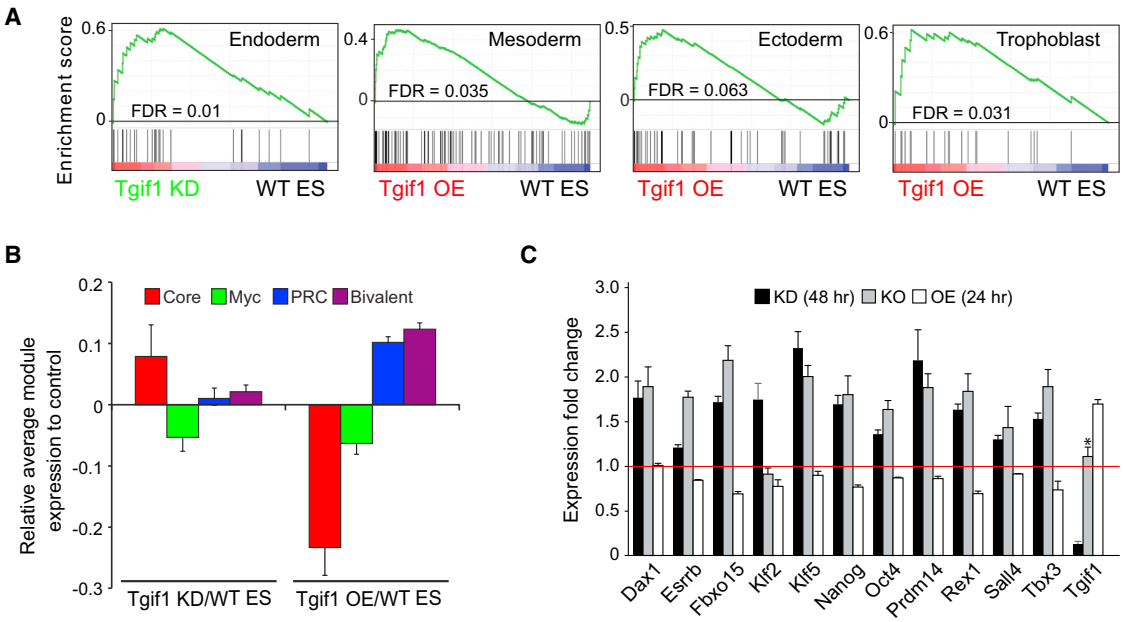


Figure 2. Abnormal Level of Tgif1 Promotes Induction of Lineage Marker Genes

(A) GSEA with gene sets representing specific lineage markers upon KD or OE of Tgif1. WT ES indicates control ESCs.

(B) Relative average expression levels of three ESC modules (Core, Myc, and PRC) and bivalent genes upon KD or OE of Tgif1 to the levels in control cells. Expression of genes in each module upon either KD or OE of Tgif1 was averaged and divided with the averaged module expression of control.

(C) Bar graphs showing relative expression of multiple ESC pluripotency-related genes upon acute KD (for 48 hr), KO, and acute OE (for 24 hr; inducible system) of Tgif1 to wild-type ESCs. Error bars indicate SEM (n = 3). Unchanged Tgif1 transcript level upon Tgif1 KO; CRISPR-Cas9 system introduces premature stop codons (*).

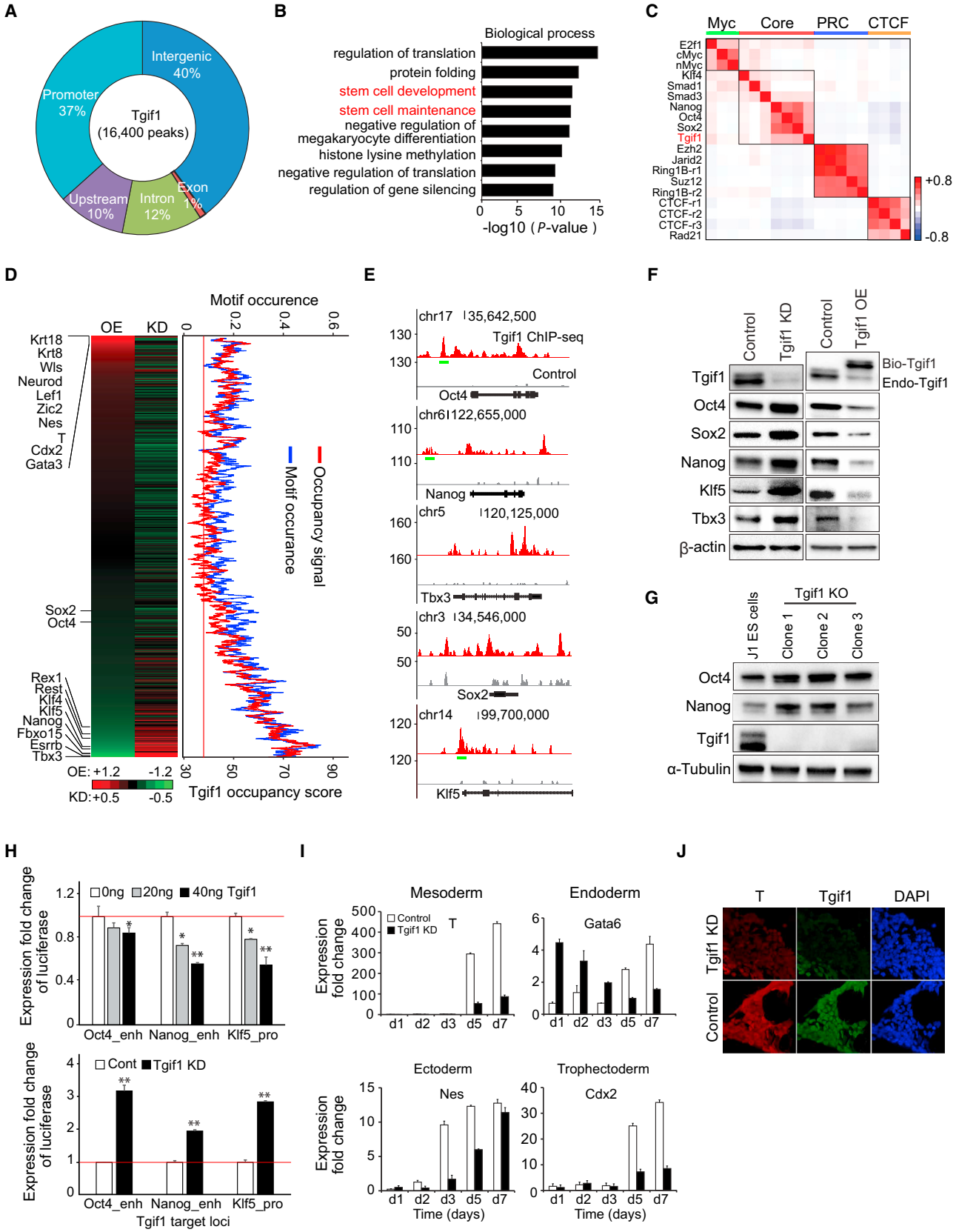
relative to control cells and plotted the corresponding Tgif1 target occupancy signals, motif occurrence scores, and gene expression profile following Tgif1 KD (Figure 3D). Some genes activated by OE of Tgif1 showed slightly higher occupancy signals over the background, but the genes strongly repressed by OE of Tgif1 were the strongest Tgif1 targets and showed the highest motif occurrence (Figures 3D and S3F). Consistently, the top 5% of strong Tgif1 target genes were dramatically downregulated following OE of Tgif1 (Figure S3G), indicating a direct repressive role of Tgif1 on its targets. The genes downregulated by Tgif1 OE were also significantly induced by Tgif1 KD, and many ESC-specific TFs (e.g., Nanog, Klf5, Rex1, Esrrb, and Tbx3) belonged to this group (Figures 3D and S3H). Figure 3E shows that Tgif1 occupies the regulatory regions of the ESC core factors, and western blotting confirmed the direct Tgif1-mediated repression of the core factors (Figures 3F, 3G, and S3I). We further validated the repressive roles of Tgif1 by luciferase assays, revealing that the activities of the promoter and enhancer target loci of Tgif1 were indeed decreased following OE of Tgif1, whereas KD of Tgif1 increased the luciferase activity (Figures 3H and S3J). We also observed a direct auto-repressive role of Tgif1 (Figures 3F and S3K).

Notably, several lineage markers (i.e., Wls, Brachyury [T], Nes, Gata3, and Cdx2) induced by OE of Tgif1 were not the strongest targets of Tgif1 (Figure 3D). The levels of these genes were not significantly changed by Tgif1 KD, suggesting that upregulation of lineage markers following OE of Tgif1 was not due to a direct activation by Tgif1. Instead, upregulation of lineage markers might be triggered by the differentiation of ESCs upon downregulation of ESC core factors.

Because Tgif1 KD increased the level of ESC core factors (Figure 2A), we sought to test to what extent the KD of Tgif1 prevents ESC from normal differentiation. A significant impairment was noted in the induction of markers from the mesoderm (T and Wls), ectoderm (Nes), and trophectoderm (Cdx2, Gata3, and Krt8) during differentiation of Tgif1 KD ESCs (Figures 3I, 3J, and S3L). Concomitantly, Tgif1 KD cells showed relatively slower downregulation of ESC-specific genes with less-differentiated morphology during differentiation when compared to wild-type ESCs (Figures S3M–S3O). Collectively, these results indicate that Tgif1 in self-renewing ESCs directly attenuates the level of ESC core factors. By contrast, its depletion causes defects in the proper induction of lineage markers during differentiation due to a failure in the timely reduction of ESC core factors.

Tgif1 Is an Integral Component of the Core Pluripotency Network

Previous studies showed that the factors within the core regulatory circuit of ESCs co-occupy many common targets and physically interact with each other (Kim et al., 2008; Wang et al., 2006). Tgif1 shares many targets with the core TFs (Figure 3C), so we examined their mutual associations using co-immunoprecipitation (coIP) of biotin-tagged Tgif1, followed by western blots (WBs). Figure 4A shows that Tgif1 interacts with Oct4 and Nanog, and the Oct4-Tgif1 interaction was cross-validated by



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the coIP of biotin-tagged Oct4 with Tgif1 antibody (Figure S4A). As Tgif1 exerts repressive functions in somatic cells (Liu, 2008), together with Smad2 and HDAC1/2 (Wotton et al., 1999b), we found associations of Tgif1 with pSmad2, HDAC1/2, and Mta1 as well as their common target sharing (Figures 3C and 4B). These results all strongly suggest that Tgif1 is an integral member of the ESC core regulatory circuitry and balances the level of ESC-specific factors, possibly through HDAC1/2.

Tgif2 Has Redundant Functions with Tgif1 but Does Not Compensate for Tgif1 KD in ESCs

It was shown that double KO of Tgif1 and Tgif2 causes lethality during early embryogenesis, suggesting a functional redundancy between Tgif1 and Tgif2 (Powers et al., 2010). We investigated this potential redundancy in ESCs by performing KD and OE of Tgif2. Unlike the effects of KD of Tgif1, KD of Tgif2 did not cause any alterations in phenotype or AP activity; this was possibly due to the very low expression level of Tgif2 in ESCs (Figure S4B). On the other hand, OE of Tgif2 showed a similar phenotype and gene expression pattern to those seen in Tgif1 OE cells (Figures S4C and S4D). BioChIP-seq (Beck et al., 2014) of overexpressed Tgif2 revealed that Tgif2 and Tgif1 share many common targets (Figures S4E and S4F), and suppressed levels of the core factors were also observed upon OE of Tgif2 (Figure S4G), collectively suggesting that Tgif2 has redundant functions with Tgif1. However, Tgif2 was not upregulated upon KD/KO of Tgif1, failing to compensate for the loss of Tgif1 in mouse ESCs.

The Roles of Tgif1 Are Independent of the TGFβ Signaling in Mouse ESCs

The restriction of pSmad2/3 levels by Tgif1 has been reported in non-ESC contexts (Liu, 2008). Because we detected a Tgif1-pSmad2 association in ESCs (Figure 4A), we examined the level of pSmad2 following perturbations of Tgif1. Consistent with previous reports, OE of Tgif1 decreased the level of pSmad2 in mouse ESCs, whereas KD of Tgif1 increased the pSmad2 levels (Figure 4C), indicating conserved negative regulatory roles of Tgif1 on pSmad2. Tgif1 forms a complex with pSmad2/3, but our analysis of the target occupancy between Tgif1 and pSmad2 (and Smad3) indicated that the targets of Smad2/3 were only a small subset of Tgif1 targets (Figures 4B, S4H, and S4I). This implies that Tgif1 may have a pSmad2/3-independent function in mouse ESCs.

Because Tgif1 is a previously known terminal repressor of TGFβ signaling, we also examined the roles of Tgif1 in the context of the TGFβ signaling in mouse ESCs. We treated the cells with an activator (activin A) or inhibitor (SB431542) of the TGFβ pathway and then monitored the levels of pSmad2, Tgif1, Oct4, and Nanog. Figure 4D shows that the activator or inhibitor treatment significantly increased or decreased the level of pSmad2, respectively. These patterns were similar to the results obtained from the KD or OE of Tgif1 (Figure 4C). However, we did not detect significant changes in ESC morphology or alterations in mRNA and protein levels of Oct4, Nanog, and Tgif1 following activation or inhibition of the TGFβ signaling. Only subtle reductions in Nanog and Tgif1 were observed following SB431542 treatment (Figures 4D and S4J). These results, along with the dramatic effects of KD or OE of Tgif1 on the levels of ESC core factors, suggest that the fine tuning of the levels of core TFs by Tgif1 is mostly independent of the TGFβ signaling in mouse ESCs.

DISCUSSION

Both positive and negative regulations are critical for balancing the proper levels of the ESC core factors in order to sustain the self-renewing status of ESCs (Niwa et al., 2000; Young, 2011). Nevertheless, only a few negative regulators of the core factors have been reported. Here, we show Tgif1 is a member of the core pluripotency regulatory circuit and directly suppresses ESC core factors, eventually counterbalancing activity of the core regulatory network in mouse ESCs (Figure 4E).

Similar to Tgif1, Tcf3 is a terminal effector of the canonical Wnt-signaling pathway and has been suggested as a repressor of the ESC factors (Cole et al., 2008). Despite their similar repressive roles, the KD of Tcf3 did not cause any obvious morphological changes in ESCs, whereas the KD of Tgif1 generated a flattened morphology. Notably, no direct associations between Tcf3 and ESC core factors have been reported either. Dax1 and Zfp281 have been also suggested as negative regulators of the core factors (Fidalgo et al., 2011; Sun et al., 2009). Both, however, have been implicated in the activation of ESC core factors

Figure 3. Tgif1 Shares Many Common Targets with ESC Core Factors and Directly Attenuates Their Levels

(A) A pie chart presenting the distribution of Tgif1-binding sites. Promoters, regions within ±2 Kb from the TSSs; upstream, regions between 2 Kb and 20 Kb upstream of the TSSs; intergenic, regions except promoters, upstream, exons, and introns.

(B) A bar graph showing enriched GO terms of top 5,000 strong Tgif1 target genes.

(C) A binding site correlation heatmap of TFs indicated. "r" indicates a biological replicate.

(D) Heatmaps showing expression values for 13,687 genes. Genes were sorted from high to low expression upon OE of Tgif1. Corresponding expression values upon KD of Tgif1 (for 4 days), motif occurrences, and Tgif1 occupancy signals are also shown. For motif occurrences and occupancy signals, moving average (window: 100; bin: 1) was applied.

(E) Tgif1 ChIP-seq track images for ESC pluripotency genes. Green boxes indicate cloned loci for luciferase assay.

(F) WB showing increased and decreased protein levels of the core factors upon KD and OE of Tgif1, respectively.

(G) WB of Oct4, Nanog, and Tgif1 in wild-type and Tgif1-KO ESCs.

(H) Luciferase assays using Tgif1 target loci upon OE (upper panel) and KD (bottom panel) of Tgif1. Klf5_pro, Klf5 promoter; Nanog_enh, Nanog enhancer; Oct4_enh, Oct4 enhancer. p values were calculated using Student's t test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ ($n = 4$).

(I) Bar graphs showing relative mRNA levels of lineage marker genes in control and Tgif1-deficent cells during time course differentiation. Each time point was normalized to day 0.

(J) IF of lineage marker T upon differentiation of wild-type and Tgif1 KD ESCs.

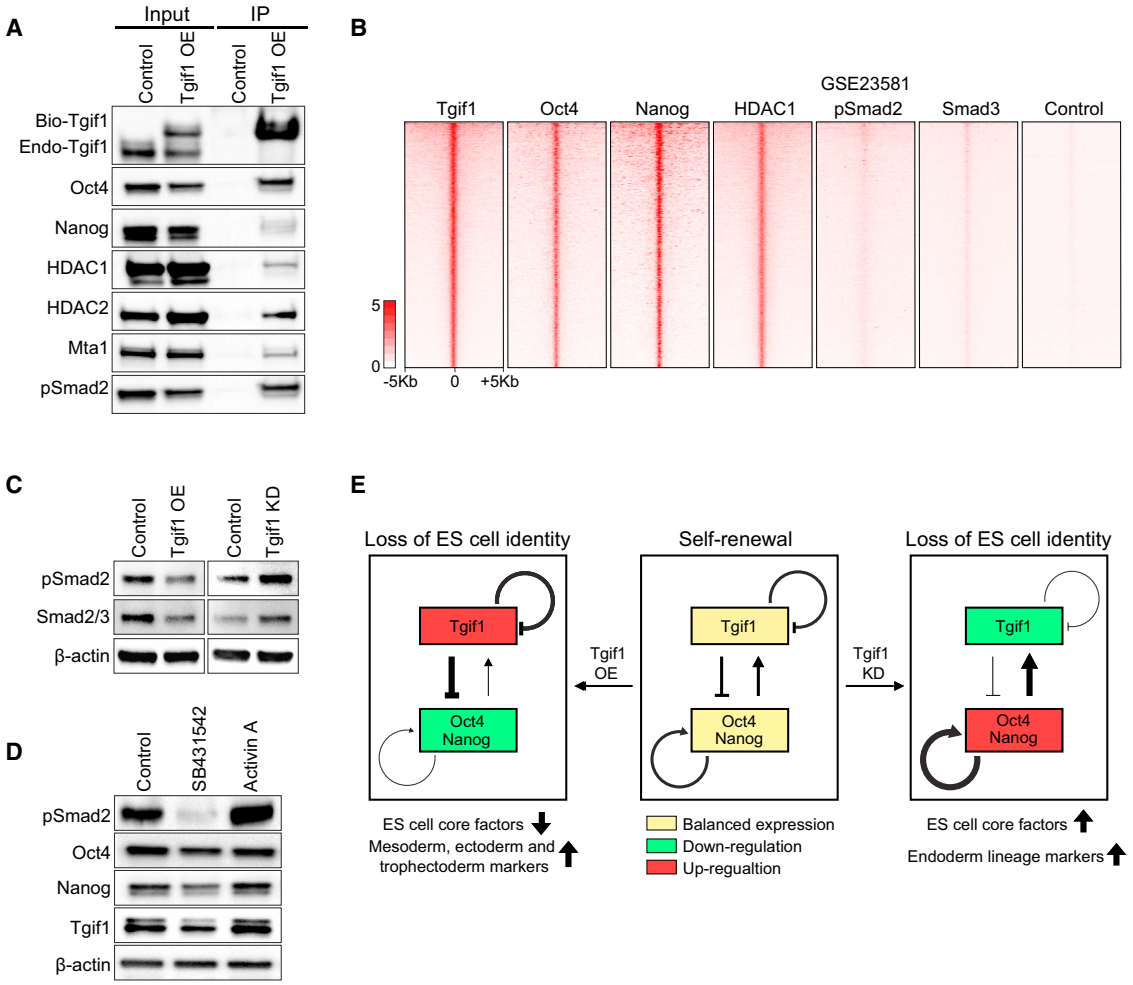

Figure 4. Tgif1 Is an Integral Member of the ESC Core Regulatory Circuitry

(A) CoIP followed by WB showing physical associations between Tgif1 and other factors.

(B) Heatmaps showing co-localization of indicated factors at the center of Tgif1-binding sites. Occupancy signals within ±5 Kb of the center of Tgif1-binding sites are shown.

(C) Protein levels of Smad2/3 and pSmad2 upon OE and KD of Tgif1.

(D) Protein levels of indicated factors upon treatment of SB431542 or activin A.

(E) Protein levels of pSmad2 upon OE and KD of Tgif1. A model illustrating the roles of Tgif1 in mouse ESCs is shown. Red, green, and yellow colors indicate activation, repression, and no change of gene activity, respectively. Line thickness indicates relative degree of transcriptional influence. Under the self-renewing condition (center), Tgif1 activated by the core factors (Oct4 and Nanog) attenuates the core factors via a negative feedback, in turn sustaining the proper level of the core pluripotency network. Upon OE of Tgif1 (left), the core network is repressed by Tgif1, leading to differentiation of ESCs with the activation of multiple lineage markers. Upon depletion of Tgif1 (right), the core network is released from the suppression, resulting in upregulation of ESC core factors.

as well (Kelly et al., 2010; Wang et al., 2008), indicating that they may function differently from Tgif1.

Whereas we observed dramatic effects on morphology, gene expression, and differentiation potential of mouse ESCs upon perturbation of Tgif1, previous studies in human and mouse model system showed inconsistency regarding Tgif1 functions. In humans, mutations in TGIF1 are associated with a genetic disease holoprosencephaly (HPE) (Gripp et al., 2000), whereas a targeted disruption of Tgif1 in mice does not result in HPE (Shen and Walsh, 2005). With our observations of functional redundancy between Tgif1 and Tgif2 as well as moderate effects of Tgif1 KD in 2i conditions, these results suggest that Tgifs may have species- and/or context-dependent regulatory roles that need to be further characterized.

In accordance with previous studies showing that modulation of TGFβ signaling does not alter the morphology or level of Oct4 in mouse ESCs (Fei et al., 2010; James et al., 2005), we observed only a minor reduction in Nanog or Tgif1 following treatment of mouse ESCs with SB431542. Conversely, KD or OE of Tgif1 induced significant changes in the level of core TFs, global gene expression profile, and ESC morphology. Notable alterations in the level of pSmad2/3 were detected

following perturbations of the Tgif1 level and activation or inhibition of the TGFβ signaling. These results clearly show that alterations in the level of pSmad2/3 do not recapitulate the dramatic changes in the levels of ESC core factors following KD or OE of Tgif1 and that Tgif1 mostly acts independently of the TGFβ pathway in mouse ESCs.

EXPERIMENTAL PROCEDURES

ESC Lines and Culture

Mouse J1 ESC lines were maintained in ES medium as described in the Supplemental Information. ESC lines expressing biotin-tagged TFs were generated as previously described (Kim et al., 2008).

Microarrays

Gene expression profiles were conducted using Affymetrix GeneChip Mouse Genome 430A 2.0 arrays. Data analysis procedures are described in the Supplemental Information. The raw and processed data sets are available at GEO: GSE55401.

ChIP

ChIP assays were performed as described previously (Kim et al., 2008), and further details are available in the Supplemental Information. ChIP-seq data sets are available at GEO: GSE55404.

ACCESSION NUMBERS

The accession number for the raw and processed data sets reported in this paper is GEO: GSE55401. The accession number for the ChIP-seq data reported in this paper is GEO: GSE55404.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article at http://dx.doi.org/10.1016/j.celrep.2015.08.067.

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