Foxd3 suppresses NFAT-mediated differentiation to maintain self-renewal of embryonic stem cells

Lili Zhu^{1,†}, Shiyue Zhang^{1,2,†} & Ying Jin^{1,3,*}

Abstract

Pluripotency-associated transcription factor Foxd3 is required for maintaining pluripotent cells. However, molecular mechanisms underlying its function are largely unknown. Here, we report that Foxd3 suppresses differentiation induced by calcineurin–NFAT signaling to maintain the ESC identity. Mechanistically, Foxd3 interacts with NFAT proteins and recruits co-repressor Tle4, a member of the Tle repressor family highly expressed in undifferentiated ESCs, to suppress NFATc3's transcriptional activities. Furthermore, global transcriptome analysis shows that Foxd3 and NFATc3 co-regulate a set of differentiation-associated genes in ESCs. Collectively, our study establishes a molecular and functional link between a pluripotency-associated factor and an important ESC differentiation-inducing pathway.

Keywords embryonic stem cells; Foxd3; NFAT

Subject Categories Development & Differentiation; Stem Cells

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Introduction

Mouse embryonic stem cells (ESCs) derived from the preimplantation embryo are self-renewing [1]. Pluripotency and unlimited self-renewal of ESCs are under the control of an intrinsic transcriptional regulatory network and several extrinsic signaling pathways [2,3]. Transcription factors such as Oct4, Sox2, Nanog, Tbx3, Esrrb, Klf4 and Foxd3 constitute the important component of the intrinsic circuitry, being essential for establishing and maintaining the unique properties of ESCs [2–9]. Fgf/Ras/Mek/MAPK and calcineurin–NFAT signaling pathways have been demonstrated to be required and sufficient to trigger the transition of mouse ESCs from an undifferentiated self-renewal state to differentiation. Interestingly, inhibiting one of the two pathways inactivates the other one [10–12]. Moreover, both pathways are shown to be critical for early lineage specification during mouse development [10]. One of

the important questions in the developmental and stem cell biology fields is how pluripotency-associated transcriptional factors counteract differentiation-inducing signaling to orchestrate the balance between self-renewal and differentiation in ESCs. Answers to this question will help to develop more efficient strategies to expand undifferentiated stem cells and to generate therapeutically useful differentiated cells.

Foxd3 is a transcriptional factor belonging to the Fox family, whose members play critical roles in development processes [13]. Foxd3 is highly expressed in the undifferentiated ESCs and is dramatically downregulated when ESCs undergo differentiation [14]. It was demonstrated to be required for the maintenance of pluripotent progenitor cells in early mouse embryos and *Foxd3*^{−/−} embryos die shortly after the implantation [15]. *Foxd3*-deficient ESCs show increased apoptosis and extensive differentiation toward multiple lineages [16]. These studies indicate that Foxd3 is required for the maintenance of pluripotency and self-renewal properties in ESCs. However, the molecular mechanisms underlying its function are largely unknown.

Calcineurin–NFAT signaling has been studied in T-lymphocyte activation and other diverse biological processes [17–21]. Among five members of the NFAT family, four members (NFATc1 to c4) are controlled at a posttranslational level by calcineurin phosphatase [22,23]. NFAT proteins in the cytoplasm are dephosphorylated by calcineurin and translocated into the nucleus, where they regulate the expression of target genes together with their cofactors, such as AP1 [22,24,25]. We previously reported that calcineurin–NFAT signaling is a pivotal pathway for mouse ESC pluripotency and early lineage specification during development [10]. The activation of calcineurin–NFAT signaling triggers mouse ESC to exit from a self-renewal state into differentiation, while inhibition of this pathway can maintain ESC self-renewal independent of leukemia inhibitory factor (LIF), which is regarded as a necessary supplement for conventional ESC culture [26]. Furthermore, we showed that calcineurin–NFAT signaling activates the expression of *Src*, which in turn induces the epithelial–mesenchymal transition (EMT) and ESC differentiation. However, it remains unclear how the transcriptional activity of NFAT proteins is regulated in the ESCs.

1 Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences/Shanghai JiaoTong University School of Medicine, Shanghai, China

2 University of Chinese Academy of Sciences, Beijing, China

3 Shanghai Stem Cell Institute, Shanghai JiaoTong University School of Medicine, Shanghai, China

*Corresponding author. Tel: +86 21 5492 3342; E-mail: yjin@sibs.ac.cn

†These authors contributed equally to this study

In the present study, we report that Foxd3 interacts with NFATc3 directly and inhibits calcineurin–NFAT-induced ESC differentiation. Mechanistically, Foxd3 recruits Tle4, a co-repressor capable of recruiting histone deacetylase [27], to repress transcriptional activities of NFAT. Our global microarray analysis further supports the notion that Foxd3 may safeguard mouse ESC self-renewal through suppressing expression of differentiation-associated genes induced by NFAT signaling.

Results and Discussion

Calcineurin–NFAT signaling is implicated in *Foxd3* depletion-induced ESC differentiation

Studies by Liu and Labosky [16] clearly documented that the deficiency of *Foxd3* resulted in aberrant ESC differentiation along multiple lineages, indicating that Foxd3 may maintain the pluripotency through suppressing important differentiation induction signaling pathways. Our previous work showed that the calcineurin–NFAT signaling pathway was essential and sufficient for triggering mouse ESC differentiation [10]. Therefore, we asked whether *Foxd3* deficiency-induced ESC differentiation was associated with the activation of the calcineurin–NFAT pathway. To answer this question, we first examined whether *Foxd3* deletion could activate the calcineurin–NFAT pathway by utilizing a *Foxd3* conditional knockout ESC line generated previously by Liu *et al* [16], in which tamoxifen treatment resulted in an almost complete loss of *Foxd3* expression (Fig 1A and Supplementary Fig S1A). We found that *Foxd3* deletion increased the mRNA level of *Src*, a previously reported NFAT direct downstream target gene (Fig 1B), implying the activation of NFAT transcriptional activities in *Foxd3*-depleted cells. Moreover, a specific calcineurin–NFAT pathway inhibitor (FK 506 [28]) treatment markedly reduced *Src* expression induced by *Foxd3* deletion, implicating a role of calcineurin–NFAT signaling for *Foxd3* deletion-induced *Src* expression. Nevertheless, we do not rule out the possibility that *Foxd3* depletion-induced ESC differentiation also accounted for the increased *Src* mRNA level. Secondly, we test the role of calcineurin–NFAT in *Foxd3* deletion-caused ESC differentiation. Consistent with previous report, the loss of *Foxd3* led to extensive ESC differentiation, exhibited by the differentiated cell morphology (Fig 1C) and increased levels of lineage-specific markers, such as trophoblast markers (*Cdx2*, *Hand1*), primitive markers (*LaminB*, *Dab2*), epiblast marker (*Fgf5*) as well as EMT-related genes (*Fgfr2c*, *Igf2*). However, FK506 treatment efficiently diminished ESC differentiation in *Foxd3*-depleted cells (Fig 1D). It appears that FK506 treatment made ESC colonies more compact even in the absence of tamoxifen. It could be due to the effect of FK506 on the basal NFAT activity in ESCs. Although FK506 reduced tamoxifen-induced differentiation gene expression, the effect was neither statistically significant nor complete. This could be attributed to the following two reasons. One could be relatively large variation in gene expression levels among three independent experiments. Second, *Foxd3* depletion might also lead to changes independent of NFAT signaling. Collectively, our results suggest that the calcineurin–NFAT pathway may be implicated in *Foxd3* deletion-induced ESC differentiation.

Foxd3 counteracts ESC differentiation induced by NFATc3 overexpression

Fore-mentioned results hinted at an important role of an appropriate balance between Foxd3 expression and the activity of calcineurin–NFAT signaling for maintaining the ESC identity. To test the assumption, we overexpressed the constitutively active form of NFATc3 (CA-NFATc3), with or without concomitant overexpression of *Foxd3* into ESCs. As expected, active NFATc3 resulted in extensive ESC differentiation, evidenced by the loss of typical round and compact ESC colonies and appearance of flat and loosely distributed differentiated cells (Fig 1E). Consistent results were obtained when the culture was stained for the expression of alkaline phosphatase, an often used marker for undifferentiated ESCs (Supplementary Fig S1B). Simultaneously, transcript levels of various germ layer markers such as *Cdx2*, *Hand1*, *Fgf5* were significantly increased (Fig 1F). Notably, differentiation phenotypes were evidently inhibited when NFATc3 and *Foxd3* were co-expressed (Fig 1E and F, and Supplementary Fig S1B). Therefore, Foxd3 appears capable of counteracting NFATc3-induced ESC differentiation.

Foxd3 and NFATc3 form protein complexes both in ESCs and *in vitro*

We next set out to explore the relationship between Foxd3 and NFATc3 at a molecular level. Western blot analysis for co-immunoprecipitation (Co-IP) experiments showed that exogenously expressed NFATc3 and Flag-Foxd3 interacted in COS7 cells, where there was not endogenous expression of Foxd3 and NFATc3 (Fig 2A). The specificity of the interaction was indicated by the presence of NFATc3 proteins in Flag antibody-precipitated proteins only when Flag-Foxd3 and NFATc3 were co-expressed. To test whether Foxd3 could interact with endogenous NFATc3, we then carried out Co-IP experiments using cell extract prepared from ESCs overexpressing Flag-Foxd3 under a LIF withdrawal condition, which was previously shown to induce the expression of NFATc3, for the easiness of detecting the interaction [10]. Endogenously expressed NFATc3 proteins were found to form protein complexes with Foxd3 (Fig 2B). To explore whether NFATc3 could directly interact with Foxd3, we conducted an *in vitro* GST pull-down assay using recombinant GST-Foxd3 and His-NFATc3 proteins. GST-Foxd3, not GST alone, was able to pull down His-NFATc3 (Fig 2C), providing an evidence for the specific and direct interaction between Foxd3 and NFATc3 proteins.

In order to identify the regions of Foxd3 proteins involved in the physical association with NFATc3, we performed Co-IP experiments using truncated forms of Foxd3 (Fig 2D). Western blot analysis showed that both Forkhead domain (110 amino acids) and C-terminus (251 amino acids) of Foxd3 were capable of binding NFATc3, while N-terminus of Foxd3 failed to do so (Fig 2E). In addition, we mapped the region of NFATc3 proteins mediating its interaction with Foxd3. Accordingly, a series of plasmids containing truncated NFATc3 were made (Fig 2F). We found that the region encompassing residues 1–595 in NFATc3 (designated as NFATc3-N) was required for its specific interaction with Foxd3 (Fig 2G). Taken together, Foxd3 and NFATc3 formed protein complexes in both ESCs and *in vitro*. The Forkhead domain and C-terminus of Foxd3

Figure 1. Foxd3 inhibits NFATc3-induced ESC differentiation.

A Western blot (WB) analysis of Foxd3 protein levels in *Foxd3^{fl/fl};Cre-ER* cells grown for 3 days with or without 2 μ M of tamoxifen (TM). Tubulin was used as a loading control.

B *Src* mRNA levels were determined by qRT-PCR analysis in *Foxd3^{fl/fl};Cre-ER* cells grown for 3 days with DMSO or 2 μ M of TM and in the absence or the presence of calcineurin inhibitor FK506. Data are shown as the mean $\pm$ SD ($n = 3$).

C Phase contrast images of *Foxd3^{fl/fl};Cre-ER* cells after treated with 2 μ M TM or DMSO for 3 days, with or without FK506. Scale bars, 100 μ m.

D qRT-PCR analysis of marker gene expression levels in cells described in (C). Data are shown as the mean $\pm$ SD ($n = 3$). * $P < 0.05$.

E Phase contrast images of ESCs transfected with the indicated plasmids for 2 days. Scale bar, 100 μ m. CA-NFATc3 is a constitutively active form of NFATc3.

F qRT-PCR analysis of marker expression levels in cells described in (E). Data are shown as the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$.

and the N-terminus of NFATc3 were most likely responsible for their interaction, respectively.

Foxd3 suppresses the transcriptional activity of NFATc3

We next determined whether the interaction between Foxd3 and NFAT could affect NFAT transcriptional activities. A luciferase reporter construct containing conserved binding motifs for NFAT

and AP1 (NFAT:AP1-luc) [29] was used to address this question. Substantial activation of the NFAT:AP1-Luc reporter by CA-NFATc3 verified the robustness of the reporter in ESCs. Importantly, the activation was dramatically suppressed by co-transfection of *Foxd3* (Fig 3A), revealing the negative regulation of Foxd3 on the transcriptional activities of NFATc3. To test the role of Foxd3 for the control of the endogenous NFAT activity, we overexpressed *Foxd3* in ESCs, which were then cultured with or without LIF for 4 days.

Figure 2. Foxd3 and NFATc3 interact through distinct domains.

- A** Foxd3 and NFATc3 interact in COS7 cells. Flag-Foxd3 and CA-NFATc3 (a constitutively active form of NFATc3) were transfected separately or co-transfected into COS7 cells. After 2 days, whole-cell lysates (WCL) were collected, immunoprecipitated (IP) with anti-Flag antibody and analyzed by Western blotting (WB) with the indicated antibodies.
- B** Foxd3 and NFAT interact in ESCs. Flag-Foxd3 or control vector were transfected into ESCs. After cells were cultured without LIF for 3 days, they were lysed, IP with anti-Flag antibody and analyzed by WB with the indicated antibodies.
- C** Foxd3 directly binds NFATc3 *in vitro*. Bacterially expressed GST-Foxd3 fusion proteins were incubated with His-NFATc3 proteins. His-NFATc3 proteins in glutathione-sepharose pulled-down proteins were detected by WB (upper panel) with His antibody. WB was conducted to determine amounts of GST and GST-Foxd3 used in the reaction (lower panel).
- D** Schematic representation of subdomains in Foxd3 proteins. Foxd3-N, N-terminal domain. Foxd3-FH, Forkhead domain. Foxd3-C, C-terminal domain. Foxd3-FL, full-length Foxd3.
- E** FH and C-terminal domains of Foxd3 bind NFATc3. Various subdomains of Flag-Foxd3 proteins were co-transfected with NFATc3, respectively, into COS7 cells as indicated. Flag antibody was used to IP Flag-Foxd3 and its associated proteins, which was further analyzed by WB using NFATc3 antibody.
- F** Schematic representation of subdomains of NFATc3 proteins. NFATc3-N, N-terminal domain. NFATc3-M, middle domain. NFATc3-C, C-terminal domain. NFATc3-FL, full-length NFATc3.
- G** N-terminal domain of NFATc3 interacts with Foxd3. Various subdomains of HA-NFATc3 were co-transfected with Flag-Foxd3, respectively, into COS7 cells as indicated. Flag antibody-precipitated proteins were analyzed by WB using HA antibody.

Our previous study demonstrated that NFATc3 proteins are highly expressed in undifferentiated ESCs but predominantly stay in the cytoplasm at an inactive form. During LIF withdrawal-induced differentiation, total protein levels do not change, but dephosphorylated NFAT proteins translocate into the nucleus [10]. Indeed, our reporter assay results showed that LIF withdrawal activated NFAT activities substantially and that Foxd3 significantly suppressed LIF withdrawal-activated reporter activities (Fig 3B). We then studied whether the suppressive effect of Foxd3 on the NFAT transcriptional activity was dependent on its interaction with NFAT proteins. The truncated forms of Foxd3 were used in reporter assays. In line with results of Co-IP assays, Forkhead and C-terminal domains of Foxd3 significantly repressed NFATc3-activated reporter activities, while N-terminus of Foxd3 did not (Fig 3C), suggesting that the physical association with NFAT is probably important for Foxd3 to inhibit NFAT's transcriptional activities.

Last, we examined whether Foxd3 and NFATc3 could form protein complexes at the promoter sequence of known NFAT target gene, *Src* [10]. To this end, electrophoretic mobility shift assays (EMSAs) were conducted with a biotinylated *Src* oligo probe (containing the NFAT consensus sequence from the *Src* promoter), and nuclear extracts from COS7 cells overexpressing Foxd3 or NFATc3, or both. A slow migration band was observed in lanes 2 and 3 on the short exposure film, suggesting of the association of NFATc3 and Foxd3 with the labeled probe, respectively (Fig 3D). Notably, a lower migration band appeared in lane 4, where the nuclear extract contained overexpressed NFATc3 and Foxd3, indicating the association of NFAT–Foxd3 protein complexes with the probe. The specificity of the association was verified by the vanishing of the slow migration bands when excessive amounts of unlabeled probe were present to compete with the labeled probe (lane 5). An even slowly migrating super-shifted band was seen in lanes 6 and 7, when specific NFAT or Foxd3 antibody was included, respectively, on the long exposure film, validating the presence of NFATc3 and Foxd3 in the slower migrating protein–DNA complexes. Moreover, chromatin immunoprecipitation (ChIP) assays showed significant enrichment of Foxd3 and NFATc3 at the *Src* gene (Fig 3E) at a similar amplitude as to their binding at the promoter of *Sox15*, a reported Foxd3 target gene (Supplementary Fig S2), verifying an association of endogenously expressed NFATc3 and Foxd3 with *Src* gene in the undifferentiated ESCs. Together with finding that Foxd3 deletion activated *Src* expression in ESCs (Fig 1B), we propose that Foxd3 might repress *Src* expression through its interaction with and suppression of NFAT transcriptional activities.

Foxd3 recruits Tle4 to form Tle4–Foxd3–NFAT complexes suppressing NFAT transcriptional activities

Our observation that Foxd3 represses the NFAT's transcriptional activity is in agreement with previous studies in *Xenopus*, which demonstrated that Foxd3 acted as a transcriptional repressor [30,31]. Further investigation by Yaklichkin *et al* revealed that the suppressive function of Foxd3 was dependent on the recruitment of transcriptional co-repressors of the transducin-like enhancer of split (Tle)/Groucho family [31,32]. Therefore, we attempted to explore whether Tle members would play a role in Foxd3-mediated inhibition of NFAT activities in ESCs. We first profiled the mRNA

levels of *Tle1*, *Tle2*, *Tle3* and *Tle4* in ESCs by qRT-PCR and found that *Tle4* was the only member of its family highly expressed in ESCs (Supplementary Fig S3A). Moreover, among COS7, HEK293T cells and E14T mouse ESCs, abundant Tle4 proteins were only detected in ESCs (Supplementary Fig S3B). Furthermore, Western blot analysis showed that, similar to Foxd3, the Tle4 expression level had a mild rise after 1 day of LIF withdrawal and decreased drastically afterward until day 5 (Supplementary Fig S3C). The distinct expression pattern of Tle4 placed it as a potential co-repressor of Foxd3 in undifferentiated and early differentiated ESCs. Therefore, we examined whether Foxd3 could form protein complexes with Tle4. The results of Co-IP experiments showed that exogenously expressed Flag-Foxd3 interacted with Tle4 and the C-terminal domain of Foxd3 was likely responsible for the interaction (Fig 4A and B). Importantly, we detected the interaction between endogenous Tle4, NFATc3 and Foxd3 in undifferentiated ESCs (Fig 4C). The presence of protein complexes containing the three proteins was further supported by Co-IP experiments in Flag-Foxd3 or Flag-NFATc3 overexpressed ESCs, respectively. We found that Flag-Foxd3 protein complexes contained endogenous NFATc3 and Tle4, whereas Flag-NFATc3 complexes contained Tle4 and Foxd3 (Fig 4D and E). Additionally, our ChIP assays indicated that Tle4 could be recruited to the *Src* promoter, in a way similar to NFATc3 and Foxd3 (Fig 3E), further validating the association of NFATc3, Foxd3 and Tle4 at the NFAT target gene.

To examine the role of Tle4 for Foxd3-mediated suppression on the NFAT activity, we performed the NFAT:AP1 luciferase reporter assays in COS7 cells. Overexpression Foxd3, *Tle4* or *Aes* (a dominant inhibitory Tle protein) had no significant effect on the reporter activity, whereas NFATc3 activated the reporter activity profoundly, about 800-fold activation (Fig 4F). Although co-expression of Foxd3 and NFATc3 reduced reporter activities modestly (about 30%), inclusion of *Tle4* suppressed the reporter activity dramatically (about 70%). In contrast, *Aes* overexpression abolished the effect of Tle4 on transcriptional repression of Foxd3. These observations support the notion that Tle4 is important for Foxd3 to suppress NFAT transcriptional activities. To further explore the role of Tle4, the same reporter assay was carried out in ESCs (Fig 4G). NFATc3 overexpression led to about 100-fold increase in the reporter activity. Notably, Foxd3 repressed the NFATc3's transcriptional activity by about 80%, a much stronger suppression than in COS7 cells. However, exogenously expressed Tle4 did not enhance the effect of Foxd3 transcriptional repression further in ESCs, as it did in COS7 cells. This phenomenon could be due to a much higher level of Tle4 in ESCs than in COS7 cells (Supplementary Fig S3B). The differential expression levels of Tle4 between ESCs and COS7 cells could also account for the different extent for Foxd3 to repress NFAT transcriptional activities between the two types of cells. Furthermore, *Aes* acted in a dominant-negative manner to interfere the transcriptional suppression of Foxd3 on NFATc3 activities in ESCs. As *Aes* might also function via other members of the Tle family, we knocked down *Tle4* expression by two specific *Tle4* siRNA oligos in ESCs. The efficiency of knock-down at 50–70% was obtained (Supplementary Fig S4A). Indeed, silencing of *Tle4* expression significantly reduced the inhibitory effect of Foxd3 on the NFAT:AP1 reporter activity (Supplementary Fig S4B), further supporting the specific role of Tle4 in Foxd3-mediated suppression of NFAT activities.

Figure 3. Foxd3 suppresses the transcriptional activities of NFATc3.

- A** Luciferase reporter assays were performed after various constructs, including NFAT:AP1 reporter, vector, constitutively active form of NFATc3 (CA-NFATc3) with or without Foxd3, were transiently transfected into ESCs. The luciferase activity in cells transfected with the vector and without Foxd3 was set as 1.0. Data are shown as the mean $\pm$ SD ($n = 3$). ** $P < 0.01$.
- B** Foxd3 inhibits LIF withdrawal-induced activation of NFAT:AP1 reporter activity. After ESCs were cultured with or without LIF for 2 days, vector or Foxd3 was transfected. 48 h after transfection, luciferase activities were measured. The activity in the cells cultured with LIF and transfected with the vector was set as 1.0. Data are shown as the mean $\pm$ SD ($n = 3$). ** $P < 0.01$.
- C** FH and C-terminal domains of Foxd3 repress NFATc3-activated reporter activity. ESCs were transiently transfected with NFAT:AP1 reporter and the indicated constructs (vector, various subdomains of Foxd3) with or without CA-NFATc3. The activity in the cells transfected with the vector was set as 1.0. Data are shown as the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$.
- D** Foxd3 and NFATc3 form complexes with the *Src* promoter probe. Electrophoretic mobility shift assay (EMSA) was carried out using the biotinylated *Src* probe and nuclear extracts of COS7 cells transiently overexpressing CA-NFATc3 and Foxd3 alone or in combination of both Foxd3 and CA-NFATc3 for 2 days. Cold probes are the unlabeled *Src* probes. Supershift bands were observed when antibodies against Foxd3 or NFATc3 were included in the reaction mixture. The left panel is for the short exposure film, and the right panel is for the long exposure film.
- E** ChIP assays demonstrate the capacity of Foxd3 (left), NFATc3 (middle) and Tle4 (right) to bind the *Src* promoter sequence in ESCs. The *HPRT* coding sequence served as a negative control (Supplementary Table S1). Data are presented as the fold of enrichment compared to the input genomic DNA and shown as mean $\pm$ SD ($n = 3$). ** $P < 0.01$.

Figure 4. Foxd3 recruits Tle4 to suppress NFAT transcriptional activities.

- A** Foxd3 interacts with Tle4 in COS7 cells. Flag-Foxd3 and Tle4 were transfected into COS7 cells. After 2 days, whole-cell lysates (WCL) were collected, immunoprecipitated (IP) with Flag antibody and analyzed by Western blotting (WB) using Tle4 and Flag antibodies, respectively (top two panels). Proteins in WCL were also analyzed by WB using Tle4 and Flag antibodies, respectively (bottom two panels).
- B** The C-terminal domain of Foxd3 interacts with Tle4. Various subdomains of Flag-Foxd3 were co-transfected with Tle4 into COS7 cells. After 2 days, WCL was collected and Flag antibody was used to immunoprecipitate Flag-Foxd3 and associated proteins, which were analyzed by WB using Tle4 and Flag antibodies, respectively (left). Proteins in WCL were also analyzed by WB (right). Foxd3-N, N-terminal domain. Foxd3-FH, Forkhead domain. Foxd3-C, C-terminal domain.
- C** Endogenous Tle4, Foxd3 and NFATc3 form protein complexes in undifferentiated ESCs. Proteins in WCL of ESCs were immunoprecipitated with specific Tle4 antibody. IgG was used as a negative control for IP. The precipitated proteins were analyzed by WB using Tle4, Foxd3 or NFATc3 antibody, respectively.
- D** Flag-Foxd3 forms protein complexes with endogenous Tle4 and NFATc3 in ESCs. Proteins in WCL of cells stably expressing Flag-Foxd3 were immunoprecipitated using Flag antibody and further analyzed by WB using antibodies against NFATc3, Tle4 and Foxd3, respectively (left). Protein expression analysis in WCL is shown on the right.
- E** Flag-NFATc3 forms protein complexes with endogenous Foxd3 and Tle4 in ESCs. Proteins in WCL of cells expressing Flag-NFATc3 induced by withdrawal of tetracycline for 3 days were immunoprecipitated by Flag antibody and were further analyzed by antibodies against NFATc3, Tle4 and Foxd3, respectively (left). The protein expression analysis in WCL is shown on the right.
- F** Tle4 increases Foxd3-mediated inhibition of the NFATc3 transcriptional activity. COS7 cells were transiently transfected with NFAT:AP1 reporter and the indicated constructs (vector, Foxd3, Tle4 and Aes) with or without NFATc3. Aes is a dominant-negative form of Tle. The activity in the cells transfected with vector was set as 1.0. Data are shown as the mean $\pm$ SD ($n = 3$). * $P < 0.05$.
- G** Aes compromises the repressive effect of Foxd3 on the NFATc3 transcriptional activity in ESCs. ESCs were transiently transfected with NFAT:AP1 reporter and the indicated constructs as in (F). Data are shown as the mean $\pm$ SD ($n = 3$). * $P < 0.05$.

Figure 5. Foxd3 and NFATc3 co-regulate a set of genes to maintain the balance between self-renewal and differentiation in ESCs.

- A The left heat map shows differentially expressed genes in ESCs overexpressing vector (vector), CA-NFATc3 (NFATc3 OE), Foxd3 (Foxd3 OE) and NFATc3 + Foxd3 (NFATc3/Foxd3 OE). The right heat map shows differentially expressed genes in *Foxd3*^{fl/fl} and *Foxd3*^{-/-} ESCs.
- B A Venn diagram shows the numbers of differentially expressed genes in NFATc3 OE versus vector, Foxd3 KO versus control KO, and NFATc3 OE versus NFATc3/Foxd3 OE. Co-regulated genes are indicated by the overlapped area in the diagram.
- C Gene ontology analysis of 55 genes, which were shared by differentially expressed genes in all three groups including NFATc3 OE UP, Foxd3 KO UP and NFATc3/Foxd3 OE DOWN.
- D qRT-PCR analysis of mRNA levels of some shared genes in ESCs at different days of LIF withdrawal differentiation. *Gapdh* was used as an internal control. The mRNA level in ESCs (D0) was set as 1. Data are shown as the mean $\pm$ SD ($n = 3$).
- E The same gene expression analysis as in (D) was conducted in ESCs at different days of embryoid body (EB) formation. Data are shown as the mean $\pm$ SD ($n = 3$).
- F qRT-PCR analysis of genes expression levels in ESCs transfected with the indicated constructs. The mRNA level of ESCs transfected with vector was set as 1. Data are shown as the mean $\pm$ SD ($n = 3$).

Foxd3 and NFATc3 co-regulate a set of genes to maintain the balance between self-renewal and differentiation in ESCs

To obtain a global view for the impact of the interaction between Foxd3 and NFATc3 on the ESC state and identify their common target genes, we conducted microarray assays for cells from 4 experimental groups with biological triplicated samples: a control group transfected with an empty vector; a Foxd3 overexpression group; a NFATc3 overexpression group; and a combination group with Foxd3 and NFATc3 co-overexpression. In addition, we also compared transcriptomes between wild-type and *Foxd3*-deleted ESCs (Fig 5A). From analysis of these data, three sets of differentially expressed genes were selected: NFATc3-induced genes (NFATc3 OE UP), Foxd3 repressed NFATc3 OE-induced genes (NFATc3 Foxd3 OE DOWN) and Foxd3 deletion-induced genes (Foxd3 KO UP) (Fig 5B). When these 3 sets of genes were overlapped, 55 common genes were identified (Supplementary Table S2). We considered these genes as the candidates of Foxd3 repressed/NFAT-activated genes. A gene ontology (GO) analysis of these genes revealed that they were enriched for genes involved in the regulation of cell apoptosis, JAK-STAT cascade, cellular response to fibroblast growth factor stimulus, BMP signaling pathway as well as cell adhesion and cell proliferation (Fig 5C). These functions are known closely associated the maintenance of balance between self-renewal and differentiation in ESCs. As the most of these genes had not been studied for their role in ESC self-renewal and differentiation, we assessed the expression pattern for some of the candidate genes and verified their expression regulation by Foxd3 and NFAT. The expression of candidate genes, such as *Cd44*, *Fhl2* and *Col3a*, increased gradually along with LIF withdrawal and embryoid body formation (Fig 5D and E, Supplementary Fig S5A–C), hinting at possible roles in ESC differentiation. Moreover, their expression was activated by NFATc3 overexpression, whereas *Foxd3* compromised the activation of NFATc3, revealing that they could be common target genes of NFATc3 and Foxd3. Furthermore, *Aes* overexpression could diminish Foxd3 suppression on NFATc3 to various extents (Fig 5F). These observations implicate that Foxd3 and NFATc3 might co-regulate a set of genes to maintain the balance between self-renewal and differentiation in ESCs. Nevertheless, it is difficult to ascertain the target genes of either NFATc3 or Foxd3 or both with only microarray analysis without simultaneous ChIP-sequencing data. Further, systemically investigating the transcriptional regulation for genes potentially regulated by Foxd3 and NFATc3 will facilitate our understanding of how these two factors interact and function.

Counteracting differentiation-associated signaling or master factors could be a common strategy for pluripotency-associated factors to maintain ESC at an undifferentiated state. However, few being counteracted factors or signals have been identified. *Cdx2* has been demonstrated to be sufficient to induce ESCs to differentiate into the trophectoderm lineage and *Oct4* interacts with *Cdx2* to antagonize *Cdx2*'s action, albeit their reciprocal expression pattern [33]. Similarly, Foxd3 is highly expressed in undifferentiated ESCs, and its expression level declines quickly upon differentiation, whereas NFATc3 is at an inactive state distributed predominantly in the cytoplasm in undifferentiated ESCs and it becomes activated through translocation into the nucleus to induce ESC differentiation.

Although predominant NFATc3 proteins located in the cytoplasm in the undifferentiated ESCs, we did detect approximately 20% of NFATc3 proteins in the nucleus (Supplementary Fig S6). In addition to identification of interaction between Foxd3 and NFAT, we provide experimental evidence that Foxd3 recruits co-repressor Tle4 to suppress the transcriptional activity of NFAT proteins in ESCs. In a previous study, Foxd3 was shown to physically interact with *Grg4*, and the interaction was required for Foxd3 to induce mesoderm in *Xenopus* [31]. However, the roles of Tle4, the murine ortholog of *Grg4*, for the regulation of ESC pluripotency and the function of Foxd3 had not been examined. We found that knock-down of *Tle4* in ESCs caused ESC differentiation with the decrease in the expression of pluripotency-associated markers, such *Rex1* and *Oct4*, and the increase of differentiation-associated markers including *B(T)*, *Fgf5* and *Gata6* (Supplementary Fig S4C and D). Thus, the role of the Tle/Groucho family member in Foxd3-mediated functions seems evolutionally conserved. Uncovering the balance between NFAT signaling and FoxD3 and the role of Tle4 is important for understanding how ESC self-renewal is controlled.

Materials and Methods

Cell culture

E14T and CGR8 mouse ESCs (gifts of Dr. Austin Smith) were grown as previously described [34]. *Foxd3*^{fl/fl}:*Cre-ER* cells were provided by Dr. Labosky, and the *Foxd3* coding region was deleted when cells were treated with 4-hydroxytamoxifen (TM) [16]. Neo and *Foxd3* stable ESC lines were generated in our laboratory. Tetracycline (Tc)-inducible iNFATc3 ESCs were established with the Rosa-Tet system [35] maintained in the medium containing Tc (0.5 mM). Flag-NFATc3 gene was induced after removal of Tc. Transient transfection in ESCs were conducted with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts isolated from COS7 cells overexpressing CA-NFATc3 or Flag-Foxd3 alone or both for 2 days were prepared, and EMSA was performed as described previously [36]. In brief, oligonucleotide probes were synthesized and labeled with biotin at the 5' end of the forward oligonucleotide. For competitive assay, an additional 200-fold molar excess of the unlabeled probe was added. For super-shift analysis, NFATc3 and Foxd3 antibodies (2 μ l per reaction) were added. The list of probe sequences, primers and RNAi oligo sequences are provided in Supplementary Table S1.

Statistical analysis

Data are shown as means $\pm$ SD. To determine the significance of differences between two groups, an unpaired Student's *t*-test was used. The 0.05 confidence level was considered statistically significant. In all figures, *P < 0.05, **P < 0.01 and ***P < 0.001.

Supplementary information for this article is available online:

<http://embo.embopress.org>

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Author contributions

LZ and SZ designed and performed experiments shown in the manuscript, analyzed data and wrote the manuscript. YJ analyzed data and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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