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Transcriptional Targets of Foxd3 in murine ES Cells

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Abstract

Understanding gene regulatory networks controlling properties of pluripotent stem cells will facilitate development of stem cell-based therapies. The transcription factor Foxd3 is critical for maintenance of self-renewal, survival, and pluripotency in murine embryonic stem cells (ESCs). Using a conditional deletion of Foxd3 followed by gene expression analyses, we demonstrate that genes required for several developmental processes including embryonic organ development, epithelium development, and epithelial differentiation were misregulated in the absence of Foxd3. Additionally, we identified 6 novel targets of Foxd3 (*Sox4*, *Safb*, *Sox15*, *Fosb*, *Pmaip1* and *Smarcd3)*. Finally, we present data suggesting that Foxd3 functions upstream of genes required for skeletal muscle development. Together, this work provides further evidence that Foxd3 is a critical regulator of murine development through the regulation of lineage specific differentiation.

Keywords

ES cells; transcription; Foxd3; Sox15; skeletal muscle

1. Introduction

Embryonic stem cells (ESCs) are a unique cell type with the ability to self-renew and differentiate into all embryonic lineages. Due to multiple issues surrounding the feasibility and ethical considerations of using human ESCs (hESCs), one goal of stem cell biologists is to determine the transcriptional networks controlling stem cell properties in other models of pluripotent stem cells including murine ESCs and/or induced pluripotent stem cells (iPSCs). Our lab determined that the Forkhead transcription factor Foxd3 is required for self-renewal and potency of ESCs[1]. Without Foxd3, several signature stem cell proteins and their corresponding mRNAs (including Oct4, Sox2, and Nanog) are maintained at relatively normal levels suggesting that Foxd3 is not required for their expression. Despite the maintained expression of these genes, ESCs lacking Foxd3 lose key stem cell properties.

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All experiments were carried out at Vanderbilt University Medical Center.

They are no longer pluripotent; they differentiate into mesendoderm and trophectoderm lineages under conditions that normally maintain pluripotency. Additionally, inducible-mutant ESCs lose self-renewal capacity and undergo aberrant apoptosis[1]. While Foxd3 is not one of the "core" transcription factors sufficient for reprogramming somatic cells into iPSCs[2], it is indispensable for generating iPSCs; mouse embryonic fibroblasts lacking Foxd3 cannot be reprogrammed into pluripotent stem cells (Suflita, Labosky, and Ess, 2013 unpublished data). Together, these data demonstrate that Foxd3 functions downstream of, or in a pathway parallel to, other stem cell factors and is required for self-renewal and pluripotency of ESCs.

Because Foxd3 regulates stem cell properties in multiple lineages[1,3–7], Foxd3 target genes must regulate self-renewal, pluripotency, and/or survival of stem cells. Currently, only two direct targets of Foxd3 have been identified (*Alb1* and the *λ5-preB* locus)[8,9]. Therefore, we sought to identify additional targets of Foxd3. Using microarrays, qRT-PCR, and ChIP assays, we identified 6 novel targets of Foxd3: *Sox4*, *Safb*, *Sox15*, *Fosb*, *Pmaip1* and *Smarcd3.* Additionally, we present data that Foxd3 functions upstream of genes required for skeletal muscle differentiation.

2. Materials and Methods

2.1 Cell Culture

Foxd3 inducible-mutant ESCs lines were previously characterized[1]. The cells were maintained using standard procedures[10]. To generate EBs, ESCs were dissociated into a single cell suspension, preplated to deplete feeder cells, and diluted to a final concentration of 20,000 cells/mL in ESC medium lacking LIF. Tamoxifen (TM, 2μM) was added to mutant cultures, and 400 cells (20 uL) were placed on the underside of a culture dish lid to form hanging drops[11]. After 3 days in culture, EBs were harvested for RNA analysis.

2.2 Immunocytochemistry

Immunocytochemistry to detect Foxd3 protein was performed following standard techniques[1] with the Foxd3 primary antiserum[7] diluted in blocking (5% normal donkey serum in PBS) solution (1:1000).

2.3 RNA Isolation and qRT-PCR

ESCs were harvested, RNA extracted as described[1], and cDNA generated using the GoScript Reverse Transcription System (Promega). cDNA samples were amplified in an Applied Biosystems 7900HT Real-Time PCR system using GoTaq qPCR Master Mix (Promega). Relative gene expression was calculated as described[12]. Primer sequences are listed in Table S1. Statistical significance was determined using a two-tailed Student's t-test.

2.4 Microarray Analysis

Microarray images were scanned with an Affymetrix high resolution GenePix 4000B scanner. Raw .CEL files were uploaded into Partek Genomics Suite version 6.6 (Partek Incorporated), processed using Robust Multi-chip Average (RMA) normalization[13], and all three possible individual pairwise comparisons of average group values were analyzed with one-way ANOVA. Probes that showed at least 1.5-fold change with a *p*-value less than 0.05 were considered significantly altered.

Gene functions were determined using NCBI Entrez Gene, Stanford SOURCE, Aceview, and Pubmed databases. Sequences for differential probes not associated with transcripts, based on Affymetrix database annotations, were retrieved from the Affymetrix NetAffx Analysis Center web site. Statistical analyses (including corrections for multiple hypothesis

testing) for identification of overrepresented functional categories and pathways were performed using Partek Genomics Suite and DAVID[14].

2.5 Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) assays were performed using established methods[8]. DNA was immunoprecipitated, purified, and amplified using qPCR (as above). The ΔΔCt method was used to calculate enrichment of Foxd3 at putative binding sites and Ser5-PolII enrichment at the proximal promoter of target genes. First, the Ct of the immunoprecipitated sample was normalized to input DNA for each amplicon (ΔCt). Next, the ΔCt values obtained from the Foxd3 (Millipore) and Ser5 PolII (Abcam) immunoprecipitated samples were normalized to the ΔCt of non-specific IgG (Santa Cruz) immunoprecipitated sample (ΔΔCt). In the case of PolII ChIP, samples were normalized by dividing the ΔΔCt value of the TM-treated samples by the ΔΔCt value of the untreated samples. Primer sequences are listed in Table S1. Statistical significance was determined using a Student's t-test comparing the enrichment of not-treated and TM-treated ESCs.

3. Results

3.1 Foxd3 regulates developmental processes

To characterize the function of Foxd3, we used ESCs carrying two conditional alleles of *Foxd3* with the entire coding sequence flanked by LoxP sites[6]. To delete the locus, the ESCs also carried a ubiquitously expressed Cre recombinase transgene[1]. Upon addition of Tamoxifen (TM), the Foxd3 coding sequence was deleted. Using qRT-PCR, we determined that *Foxd3* mRNA levels were not significantly reduced until 12 hours after the addition of TM, and this reduction in *Foxd3* mRNA was more pronounced following 24 hours of TM treatment (Fig. 1A). To determine when Foxd3 protein was diminished, we performed fluorescent immunocytochemistry. After 12 hours of culture with TM, Foxd3 protein was reduced but could still be detected (Figs. 1B–C). However, 24 hours after TM addition, Foxd3 protein was only rarely detected (Figs. 1D–E), suggesting that Foxd3 protein persists after loss of *Foxd3* mRNA, presumably due to the half-life of the protein. Therefore, to enable us to detect gene regulatory differences due to the loss of Foxd3, we completed our experiments after ESCs were cultured with TM for at least 24 hours.

To characterize genes misregulated in the absence of Foxd3, we used Affymetrix Gene/Exon microarrays to determine which genes were misregulated in the absence of Foxd3 after 24 hours of TM treatment (n=3 hybridizations of each group). Statistical analysis of the TM treated versus not treated cells yielded 423 significantly differentially expressed probes (Table S2). Hierarchical clustering of normalized hybridization signals for these 423 probes successfully separated the TM-treated from untreated cells based on gene expression patterns (Fig. 2A), suggesting that the findings from each experiment were highly reproducible and gene expression patterns between control cells and TM-treated cells were distinct.

To further analyze the function of genes misregulated in the absence of Foxd3, we used functional analysis program, DAVID, to identify significantly enriched gene ontologies (Table 1). These data suggested that Foxd3 regulates genes controlling several developmental processes including embryonic organ development, epithelium development, and epithelial differentiation. On a pathway level, Foxd3 regulates components of the Wnt and FGF signaling pathways (Table 1), specifically *Fgf4* and its receptor *Fgf2r*[15]. Strikingly, mice lacking β-catenin, a downstream mediator of canonical Wnt signaling, die at approximately 6.5 days post coitum (dpc) with disrupted embryonic tissues and morphologically normal extraembryonic tissue[16], and *Fgf4* null embryos die around

implantation due to impaired expansion of the epiblast[17]. The timing of lethality and phenotype of both the Wnt and FGF4 signaling mutants is similar to the lethality of *Foxd3* null embryos, consistent with the possibility that Foxd3 regulates these pathways *in vivo*[3]. Finally, loss of Foxd3 in ESCs also impacts expression of transcription factors as indicated by enrichment of three functional categories: transcription regulator activity, transcription factor complex, and positive regulation of transcription. Interestingly, as summarized in Table 1, expression of some misregulated genes is increased in the absence of Foxd3 (red) while expression of others is decreased (blue) suggesting that Foxd3 both activates and represses these biological processes. Our findings also suggest that Foxd3 functions in stem cells by primarily regulating development and differentiation.

Analysis of the results obtained from the microarray allowed us to prioritize the verification of changes in expression of genes encoding proteins known to control stem cell properties in addition to those with unknown function. Using qRT-PCR to assay gene expression, we confirmed 10 genes of interest that were significantly misregulated (p-value < 0.05) after 24 hours of TM treatment (Fig. 2B, purple). We also assayed gene expression after 12 hours of TM treatment, and unsurprisingly, found that no genes of interest were misregulated (Fig. 2B, green). Eight of the misregulated genes (Eras, Fosb, Clip2, Smarcd3, Ngfr, Sox4, Tub, and Safb) were downregulated while 2 (Pmaip1 and Sox15) were upregulated, suggesting that Foxd3 both positively and negatively regulates expression of putative target genes.

3.2 Identification of direct targets of Foxd3

To determine whether the misregulated genes are direct targets of Foxd3, we used rVista to identify putative Foxd3 binding sites less than 20 kb away from the misregulated genes, and verified Foxd3 occupancy using chromatin immunoprecipitation (ChIP) assays followed by quantitative PCR (qPCR). Using this assay, we determined that 6 of the 10 misregulated genes (*Sox4*, *Safb*, *Sox15*, *Fosb*, *Pmaip1* and *Smarcd3*) were direct targets of Foxd3 (Fig. 2C, grey bars). Foxd3 occupancy at the *λpreB* locus served as a positive control[8], while the *Hprt* coding sequence served as a negative control. To validate specificity of the antibody, we analyzed Foxd3 occupancy in TM treated ESCs and, as expected, did not detect Foxd3 at any loci (Fig. 2C, green bars). Together, these results indicate that the identified binding sites are novel targets of Foxd3. The Foxd3 binding sites near Fosb, Safb, Smarcd3, and Sox4 are conserved among mice, rats, and humans; however, the antibody used for ChIP assays did not provide reproducible results in hESCs (data not shown).

To further characterize the role of Foxd3 in regulating ES cell properties, we chose to analyze developmental processes misregulated in the absence of Foxd3. Furthermore, published data demonstrates that Foxd3 and Sox2 interact in ESCs and are replaced by Foxp1 and Sox4 in differentiating B cells[8]. Therefore, we focused our analyses on the misregulated Sox family members, Sox15 and Sox4. We detected Foxd3 bound at regions 4.8 kb upstream of the Sox15 transcriptional start site and 9.5 kb downstream of the Sox4 gene (Fig. 2C). To determine if *Sox15* and *Sox4* transcription is altered in the absence of Foxd3, we used ChIP assays to analyze the occupancy of RNA Polymerase II phosphorylated at Serine5 (Ser5-PolII). There was a significant increase in Ser5-PolII occupancy at the *Sox15* proximal promoter in TM-treated cells compared to controls. Alternatively, Ser5-PolII occupancy was drastically decreased at the *Sox4* promoter in ESCs lacking Foxd3 (Fig. 2D). These data suggest that Foxd3 directly regulates the transcription of these two target genes.

Additionally, using an embryoid body (EB) assay to analyze gene expression in differentiating cells, we determined that *Sox15* mRNA levels quickly decreased while *Sox4* mRNA levels gradually increased upon differentiation of untreated ESCs (Fig. 2E–F, grey). Consistent with increased *Sox15* mRNA levels in ESCs lacking Foxd3, *Sox15* mRNA is

maintained in EBs lacking Foxd3 (Figure 2E, green), while *Sox4* mRNA was decreased in TM-treated cells (Figure 2F, green). Together, these data indicate that *Sox4* and *Sox15* expression is altered in EBs lacking Foxd3.

3.3 Foxd3 functions upstream of genes required for skeletal muscle development

While some data suggest that Foxd3 functions as a transcriptional activator[18–20], compelling evidence indicates that Foxd3 functions as a transcriptional repressor in mesoderm induction in *Xenopus*[21,22]. Therefore, we focused our analyses on Sox15, which is repressed by Foxd3. Because *Sox15* is regulated by Foxd3, and Sox15 is a critical regulator of skeletal muscle differentiation *in vitro*[23–25], we sought to first characterize the effects of loss of Foxd3 on genes functioning downstream of Sox15 required to regulate skeletal muscle development. Skeletal muscle is derived from paraxial mesoderm and requires the myogenic bHLH transcription factors MyoD and Myf5 for differentiation[26]. Following determination to the skeletal muscle lineage, myoblast progenitor cells divide, align, and fuse to generate multinucleated myotubes, resulting in mature muscle fibers that also contain muscle stem cells. A putative stem cell population, the satellite cells, is capable of proliferating to generate new myoblasts that fuse with mature muscle fibers[27].

The transcription factors Pax3 and Sox15 function upstream of Myf5 and MyoD[23–25]. *Sox15* null animals cannot regenerate skeletal muscle following injury, while overexpression of Sox15 results in increased Pax3 expression, decreased Myf5 expression, and an expansion of immature myoblasts[25,28]. Because Sox15 is upregulated in Foxd3 induced mutant ESCs and Sox15 inhibits myogenesis, we hypothesized that ESCs lacking Foxd3 cannot be directed to produce mature skeletal muscle. Additionally, Foxd3 is expressed in the paraxial mesoderm of mouse embryos, further suggesting that Foxd3 may be an important regulator of skeletal muscle development[29]. To determine if genes functioning downstream of Sox15 are misregulated in the absence of Foxd3, we assayed mRNA levels of these myogenic genes in differentiating ESCs using qRT-PCR. Consistent with our hypothesis, *Myf5* expression decreased while *Pax3* expression increased (Fig. 3A), suggesting that Foxd3 functions upstream of Sox15 to regulate myogenesis.

4. Discussion

We identified several genes, pathways, and biological functions that are misregulated in ESCs lacking Foxd3. Additionally, we identified 6 novel targets of Foxd3: *Sox4*, *Safb*, *Sox15*, *Fosb*, *Pmaip1*, and *Smarcd3*. We further characterized the expression of genes that function downstream of Sox15, and we showed that Foxd3 directly or indirectly regulates genes required for skeletal muscle development and regeneration, uncovering a novel role for Foxd3.

The data presented in Figure 3, together with previous work in the lab[1], suggest that Foxd3 induced mutant ESCs precociously express genes required for mesoderm induction, but they are likely unable to differentiate into skeletal muscle. These data are consistent with the model shown in Fig. 3B in which Foxd3 represses *Sox15* transcription resulting in increased *Pax3* and decreased *Myf5* expression in ESCs undergoing differentiation. An increase in *Pax3* in skeletal muscle progenitors may result in increased self-renewal and decreased differentiation, limiting the number of mature skeletal muscle fibers[30,31]. Additionally, decreased *Myf5* may result in decreased generation of skeletal muscle. The data presented here are consistent with a recent publication demonstrating the function of FOXD3 in hESCs; overexpression of FOXD3 in hESCs induces differentiation to paraxial mesoderm, including differentiation into skeletal myoblasts[29]. Together, these data suggest a conserved function for Foxd3 in regulating skeletal muscle development in mammals.

We hypothesize that the other targets of Foxd3 (*Sox4*, *Safb*, *Fosb*, *Pmaip1* and *Smarcd3*) also regulate ES cell properties, and based on published accounts, several of these targets are of future interest. The transcription factor Sox4 is required for cardiac outflow tract development[32–34], a process regulated by the cardiac neural crest, another multipotent progenitor population in which Foxd3 function is critical[5,6,35]. The transcription factor FBJ osteosarcoma oncogene B (Fosb) promotes osteoblast differentiation while inhibiting adipogenesis[36] suggesting that inhibition of Fosb by Foxd3 regulates differentiation of these lineages. Smarcd3 (also called Baf60c), a member of the Swi/Snf chromatin remodeling complex, associates with MyoD to promote transcription of genes required for myogenesis[37,38]. While Sox15, Sox4, Fosb, and Smarcd3 have been implicated in regulating differentiation of disparate lineages, no one has carefully investigated the role of these proteins in maintaining ESC properties, and it is possible that Sox4, Fosb, and Sox15 are involved in maintaining pluripotency in ESCs.

In addition to genes regulating pluripotency, two novel Foxd3 targets have the potential to regulate self-renewal of ESCs. Smarcd3 is a component of a Swi/Snf complex and is required to regulate self-renewal of neural stem cells[39]. While *Smarcd3* mRNA can be detected in ESCs (Figure 2B), to date, no one has analyzed the requirement for this protein in regulating self-renewal of ESCs. In addition, the function of the ubiquitously expressed nuclear scaffolding protein, Safb, has yet to be determined. It has been suggested that Safb may regulate the cell cycle, consistent with the possibility that Safb is required for ES cell proliferation and/or self-renewal[40–42]. Together, this evidence from the literature is consistent with the hypothesis that these new targets of Foxd3 may regulate self-renewal in ESCs.

Lastly, some targets of Foxd3 are also required to prevent aberrant apoptosis. Safb indirectly represses apoptotic genes in breast cancer cells[43,44]. Therefore, decreased *Safb* expression in Foxd3 mutant ESCs may lead to an increase in apoptosis. Finally, Pmaip1 (also called Noxa) is a direct target of Foxd3 and is a critical regulator of cell death. Pmaip1 is required for the activation of caspases and contributes to p53-dependent apoptosis[45–47].

Altogether, these data from our laboratory and others suggest that Foxd3 functions upstream of critical regulators of stem cell properties. Prior to this manuscript, only two direct targets of Foxd3 were identified, and the work reported here has uncovered several factors that function downstream of Foxd3. Additional characterization of the function of these factors in ESCs will further elucidate gene regulatory networks controlling stem cell properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Foxd3 regulates expression of genes controlling developmental processes
- Foxd3 both positively and negatively regulates expression of downstream genes
- Newly identified direct targets of Foxd3 include: Sox4, Safb, Sox15, Fosb, Pmaip1, and Smarcd3
- Foxd3 functions upstream of genes required for skeletal muscle development

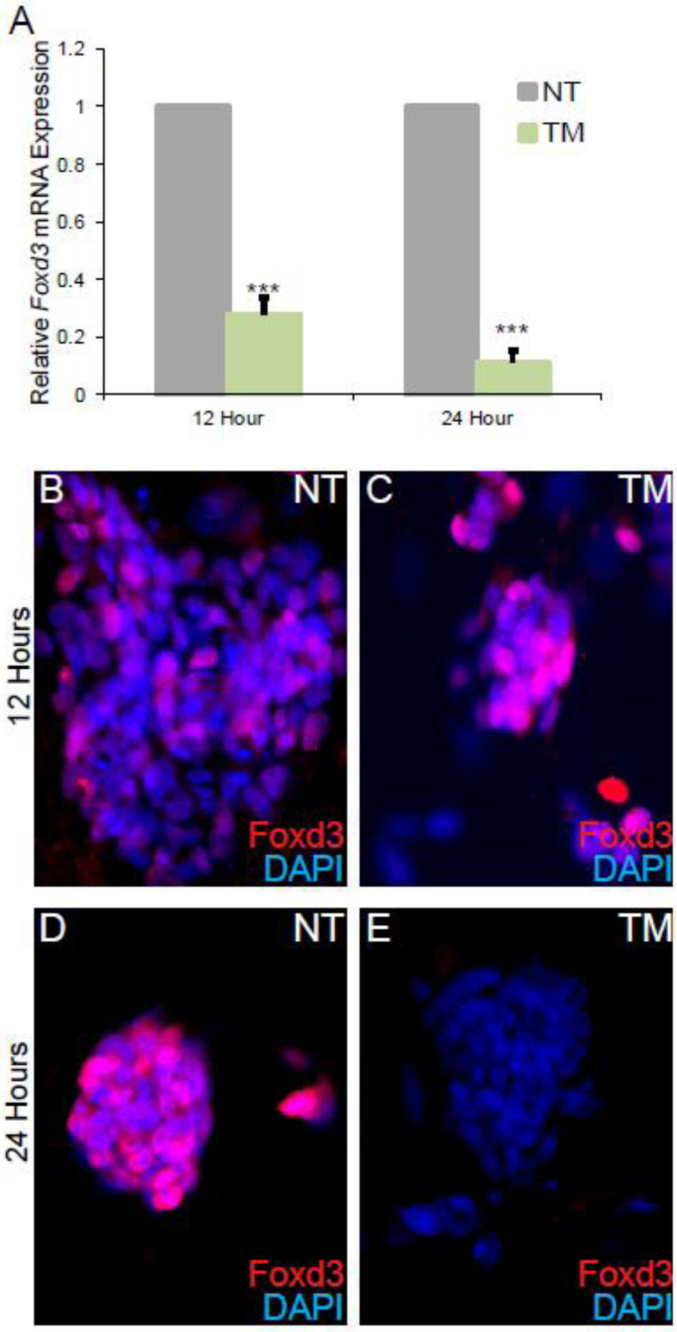

Figure 1. Foxd3 protein cannot be detected after 24 hours in culture with Tamoxifen (TM)

A. qRT-PCR analysis of *Foxd3* mRNA levels after 12 and 24 hours of culture with TM. Relative *Foxd3* expression is decreased in TM-treated ESCs (green) at both time points compared to untreated controls (grey). Error bars indicate SEM. *** $p<0.001$. N=3 experiments. The expression of *Foxd3* in NT cells is set to 1. B–E. Immunocytochemistry analysis of Foxd3 protein expression (red) after 12 (B–C) and 24 (D–E) hours in culture in NT (B,D) and TM-treated (C,E) ESCs. Nuclei are indicated by DAPI (blue).

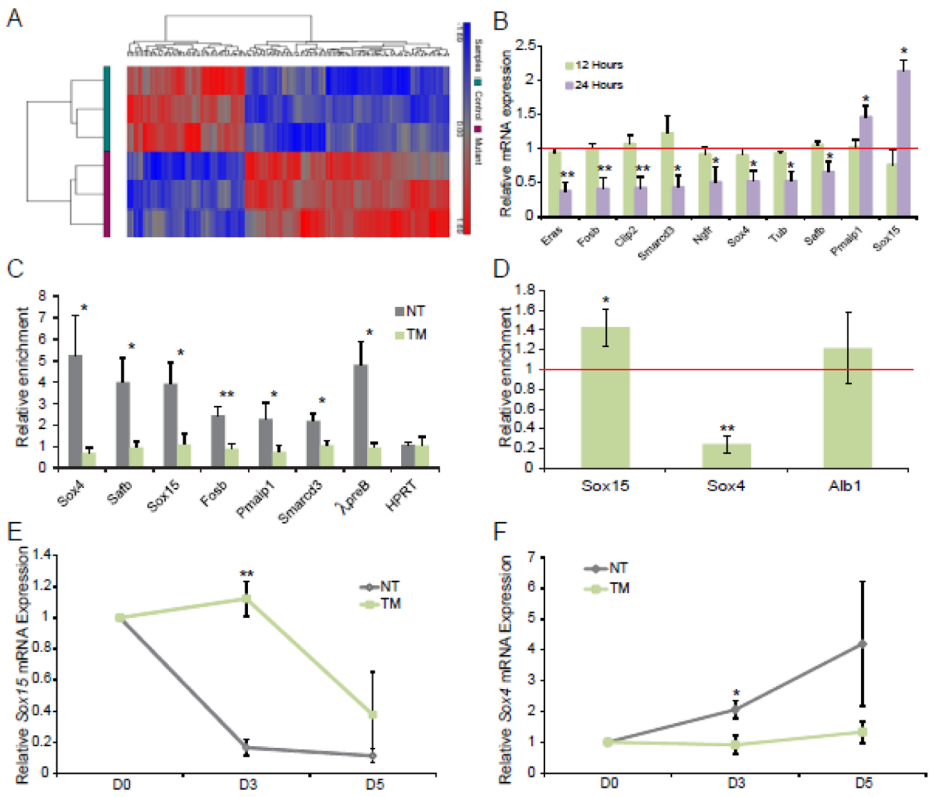

Figure 2. Identification of direct targets of Foxd3

A. Hierarchical clustering of 423 probes detected as significantly differential (at least 1.5-fold, *p*-value < 0.05) between NT and TM treated ESCs. Values shown are $\log_2$, and bright red, bright blue, and gray indicate the highest, lowest, and median normalized signal values, respectively. Vertical dendrograms represent the individual samples, of which there are three replicates for each sample type. B. qRT-PCR validation of misregulated genes identified using microarrays at 12 (green) and 24 (purple) hours post TM treatment. The red line indicates the relative gene expression levels in NT control ESCs. N=4 experiments. C. qPCR following ChIP experiments was used to identify novel targets of Foxd3. The data are portrayed as enrichment over a non-specific rabbit IgG antibody in untreated (NT, grey) and TM treated (green) ESCs after 24 hours in culture. The *λ5preB* locus serves as a positive control while the *HPRT* coding sequence serves as a negative control. N=5 experiments. D. qPCR following ChIP experiments to determine if Ser5-PolII occupancy is altered at the proximal promoters of *Sox15* and *Sox4* in the absence of Foxd3. The data depict the enrichment of Ser5-PolII in induced mutant (TM) ESCs after 48 hours in culture normalized to untreated cells (red line). The enrichment at a sequence −7kb from the *Alb1* gene serves as a negative control; enrichment in both TM and untreated cells are at least 3-fold less at this sequence than at *Sox4* and *Sox15* proximal promoters. E–F. qRT-PCR analysis of *Sox15* (E) and *Sox4* (F) expression in untreated (control, grey) and TM treated (induced mutant, green) EBs at Day 0 (D0) and Day 3 (D3) normalized to expression in untreated ESCs at D0. N=3 experiments. In all panels, error bars indicate SEM. *p<0.05 **p<0.01, ***p<0.001.

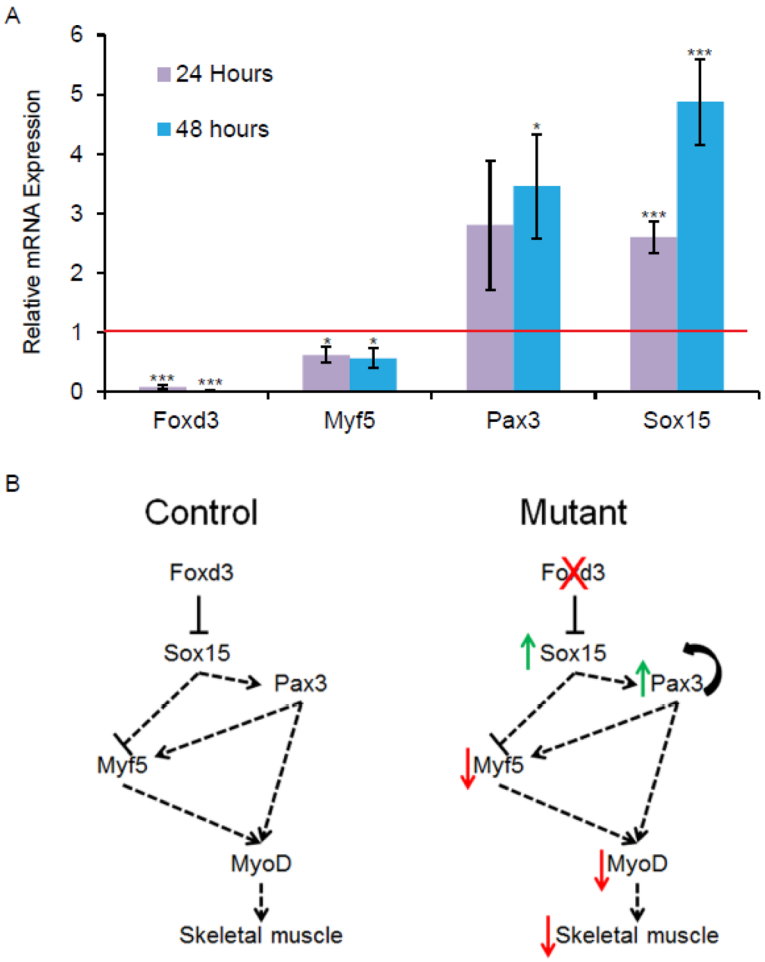

Figure 3. Foxd3 functions upstream of genes required for skeletal muscle development

A. qRT-PCR data demonstrating the relative expression of *Foxd3, Myf5*, and *Pax3* mRNA in Foxd3 induced mutant ESCs cultured for 24 (purple) and 48 (blue) hours. Red line indicates expression of these mRNAs in untreated ESCs. Error bars indicate SEM. *p< 0.05, ***p< 0.001 B. Model of Foxd3 and Sox15 in mESCs in the process of skeletal muscle differentiation. In a control progenitor cell (left), Foxd3 represses *Sox15* allowing precise regulation of *Pax3* and *Myf5* and proper skeletal muscle development. In the absence of Foxd3 (right), *Sox15* is upregulated, resulting in an increase in *Pax3* and a decrease in *Myf5* expression.

Table 1
Enriched Functional Categories in Mutant Cells

Functional classification of genes misregulated after 24 hours of TM treatment. The *p*-value was calculated by the online database and functional analysis program, DAVID using Fisher's Exact Test. Upregulated genes are indicated by red text while downregulated genes are indicated by blue text.

Gene Ontology Category	P value	Genes
Biological Process		
Epithelium development	6.3×10^{-5}	Fgf42, Cobl, Lama1, Vegfc, Sfrp1, Pou2f3, Tgm1, Car9, Sfn, Sprr2i
Tissue morphogenesis	8.8×10^{-4}	Fgfr2, Cobl, Lama1, Vegfc, Sfrp1, Wnt3a, Car9, Ngfr*
Positive regulation of transcription	9.7×10^{-4}	Zbtb7b, Hoxa1, Smarcd3*, Pou2f3, Ebf1, Pax8, Tead1, Sox4*, Sox15, Smarca1, Foxd3
Epithelial cell differentiation	1.2×10^{-3}	Fgfr2, Lama1, Pou2f3, Tgm1, Sfn, Sprr2i
Embryonic organ development	4.7×10^{-3}	Fgfr2, Hoxa1, Gcm1, Otx1*,Wnt3a, Ttpa, Foxd3
Morphogenesis of an epithelium	5.3×10^{-3}	Fgfr2, Cobl, Lama1, Vegfc, Sfrp1, Car9
Embryonic morphogenesis	8.6×10^{-3}	Fgfr2, Cobl, Hoxa1, Vegfc, Gcm1, Otx1*, Wnt3a, Fgf4
Wnt receptor signaling pathway	1.0×10^{-2}	Dact1, Sfrp1, Wnt3a, Sfrp4, Sox4*
Regulation of RNA metabolism	1.4×10^{-2}	Calcr, Zbtb7b, Zfp345, Tcfap2c, Otx1*, Tead1, Sox4*, Fosb*, Obox6, Hoxa1, Gcm1, Pou2f3, Ebf1, Pax8, Sox15, Zfp820, Smarca1, Foxd3
Morphogenesis of a branching structure	4.7×10^{-2}	Fgfr2, Lama1, Gcm1, Sfrp1
Cellular Component		
Cell-cell junction	1.6×10^{-2}	Tgm1, Scn2a1, Esam, Jam2, Calb2
Transcription factor complex	4.2×10^{-2}	Gcm1, Pou2f3, Pax8, Tead1, Foxd3
Molecular Function		
Transcription regulator activity	3.5×10^{-3}	Zbtb7b, Tcfap2c, Otx1*, Tead1, Sox4*, Fosb*, Obox6, Msc, Hoxa1, Gcm1, Olig3, Smarcd3*, Pou2f3, Pax8, Ebf1, Sox15, Smarca1, Foxd3
Signaling Pathways		
WNT signaling pathway	2.1×10^{-2}	Dact1, Sfrp1, Smarcd3*, Wnt3a, Sfrp4, Prkch, Smarca1
FGF signaling pathway	5.6×10^{-2}	Fgfr2, Prkch, Sfn, Fgf4

*These genes fell below the cut-off criteria chosen for analysis of microarray data but were validated by qRT-PCR.