Foxn1 regulates key target genes essential for T cell development in postnatal thymic epithelial cells

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Thymic epithelial cell differentiation, growth and function depend on the expression of the transcription factor Foxn1; however, its target genes have never been physically identified. Using static and inducible genetic model systems and chromatin studies, we developed a genome-wide map of direct Foxn1 target genes for postnatal thymic epithelia and defined the Foxn1 binding motif. We determined the function of Foxn1 in these cells and found that, in addition to the transcriptional control of genes involved in the attraction and lineage commitment of T cell precursors, Foxn1 regulates the expression of genes involved in antigen processing and thymocyte selection. Thus, critical events in thymic lympho-stromal cross-talk and T cell selection are indispensably choreographed by Foxn1.

The thymic microenvironment is unique in its ability to promote the development and selection of naive T cells with a repertoire purged of vital ‘self’ specificities, but that are prepared to react to injurious non-self. Thymic epithelial cells (TECs), which can be categorized into separate cortical (cTEC) and medullary (mTEC) lineages1,2, are essential for this competence. cTECs attract blood-borne precursor cells, commit them to a T cell fate and foster their differentiation to express an αβ T cell antigen receptor (TCR). Reactivity to major histocompatibility complex (MHC)-peptide complexes presented by TECs authorizes the generation of a bespoke TCR repertoire. Because TCRs are initially generated pseudo-randomly, their specificity is scrutinized during thymocyte development to establish a selected repertoire that is tailored for an individual, whereby cTEC positively select thymocytes that express a TCR with a sufficient affinity for self-antigens3. Subsequently, both cTECs and mTECs deplete thymocytes with substantial reactivity to self-antigens, a process that is known as negative selection4.

TEC differentiation and growth are dependent on the transcription factor Foxn1, which is present in the thymus exclusively in TECs, but is not required there for the cells’ initial fate specification5-7. Foxn1 is continuously expressed in the thymus and may therefore be required for the maintenance of cTECs and mTECs, both in the embryo and in postnatal mice8-10. This finding suggests that Foxn1 constantly controls diverse aspects of TEC biology, ranging from steps that are essential in early epithelial cell differentiation to the transcriptional control of genes that are important for thymus function and maintenance. Although several genes have been implicated as being transcriptionally controlled by Foxn1, none have been physically identified as direct targets. Using different static and inducible genetic mouse model systems, we determined, in a genome wide-manner, the direct target genes of Foxn1. Among these are genes that contribute to antigen processing and presentation, including threonine peptidases, components of the proteasome complex, protein transporters and CD83.

RESULTS
A Foxn1 transgenic rescue of the nude thymus phenotype
The direct identification of Foxn1 target genes in TEC using chromatin immunoprecipitation sequencing (ChIP-seq) has been impeded by a lack of suitable anti-Foxn1 antibodies. We therefore generated nude (Foxn1nu/nu) mice transgenic for a bacterial artificial chromosome (BAC)11 that encodes a Foxn1 protein tagged with a Flag-octapeptide, which is expressed under the normal control of Foxn1 regulatory elements and permits immunoprecipitation (Supplementary Fig. 1a,b). Homozygous for the BAC, these mice (designated Foxn1wt/−/wt) had a regular coat and a gross anatomically normal thymus with an ordered stromal architecture and normal Foxn1 protein expression. Total thymus and TEC cellularity were mildly reduced in mice 4 weeks or older when compared with age-matched wild-type controls (Fig. 1a and Supplementary Fig. 1c,f). Thus, the Foxn1-Flag protein in TECs of Foxn1wt/−/wt mice almost completely rescued the nude phenotype. In contrast with Foxn1wt/−/wt mice, nude mice heterozygous for the BAC allele (designated Foxn1wt/−/−) had a significantly smaller thymus that was marked by reduced Foxn1 protein expression, fewer mTECs,
multiple large cysts and a severely disorganized cortex-medulla segregation with medullary islands located adjacent to the organ's capsule (Fig. 1a–c and Supplementary Fig. 1c,f). Furthermore, Foxn1<sup>wt/wt</sup> mice had fewer mature (MHC<sup>II</sup>CD80<sup>+</sup>CD86<sup>+</sup>) mTECs and their MHCII cell surface expression was reduced in comparison with wild-type and Foxn1<sup>wt/wt</sup> mice (Fig. 1f–h). Thus, our results establish the recombinant BAC as a hypomorphic Foxn1 allele with expression of Foxn1-Flag from a single allele that only partially rescues the nude thymus phenotype.

**Foxn1 acts at multiple stages of thymocyte development**

We next evaluated the thymopoiesis of 4–6-week-old Foxn1<sup>wt/wt</sup>, Foxn1<sup>wt/−</sup> and wild-type mice. The progression of early thymic precursors (ETPs, which are Lin<sup>−</sup>CD4<sup>−</sup>CD8<sup>−</sup>CD44<sup>+</sup>c-kit<sup>+</sup> thymocytes, whereby lineage negativity, Lin<sup>−</sup>, refers to the absence of TCRβ, CD4, CD8, CD19, CD11c, CD11b, F4/80, TCRγδ, NK1.1 and TER119 expression) to CD4<sup>+</sup>CD8<sup>+</sup> (double positive, DP) thymocytes was comparable between Foxn1<sup>wt/wt</sup> and wild-type mice (Fig. 2a–c), indicating that a homozygous BAC transgene rescued the early thymopoietic competence of Foxn1<sup>mu/mu</sup> TECs. In contrast, the frequency of ETPs, a population that seeds the thymus in response to chemokines CCL19, CCL21 and CCL25 expressed by TECs<sup>12</sup>, was reduced 32-fold in Foxn1<sup>wt/−</sup> mice (Fig. 2a), whereas B cells were 30-fold more frequent in Foxn1<sup>wt/−</sup> mice than in wild-type animals (Supplementary Fig. 2a). The frequency of the subsequent stages of Lin<sup>−</sup> thymocytes increased in Foxn1<sup>wt/−</sup> mice relative to ETPs, but did not reach the values observed in wild-type animals (Fig. 2b).

The frequency of mature CD4<sup>+</sup> single-positive (CD4SP) and CD8<sup>+</sup> single-positive (CD8SP) thymocytes was mildly reduced in Foxn1<sup>wt/wt</sup> and was strongly diminished in Foxn1<sup>wt/−</sup> mice when compared with wild-type animals (Fig. 2c). These changes in Foxn1<sup>wt/wt</sup> and Foxn1<sup>wt/−</sup> mice correlated with a reduced positive selection, as measured by the upregulation of CD69 expression on thymocytes with an intermediate expression of the TCR, despite an increased frequency of
Figure 2 Foxn1 availability in TECs determines T cell developmental defects. Flow cytometric analysis of 4-week-old mice with indicated genotype for (a) CD44+c-kit+ thymocytes. Right, frequencies of CD44+c-kit+CD25+ thymocytes. (b-h) CD44 and CD25 expression on Lin+CD4+CD8+ thymocytes (b), CD4 and CD8 expression on total thymocytes (c), CD69 and TCR β-chain expression on total thymocytes (d), Helios and PD-1 expression on Foxp3+CCR7+ thymocytes (e), CD24 and CCR7 expression on CD4+CD8+ TCR+CD5+Foxp3+ thymocytes (f), CD24 and CCR7 expression on CD4+CD8+ TCR+CD5+ thymocytes (g), and Foxp3 and CD25 expression by CD4 cells (h). Representative contour plots for h are displayed in Supplementary Figure 2c. *P < 0.05. (Student’s t test, a-h). Data in bar graphs are from one experiment representative of two independent experiments with four replicates each. Contour plots (a-g) are representative of data in bar graphs. Numbers shown in individual gates and quadrants of flow cytometry plots represent the frequencies observed in a representative experiment.

cTECs relative to that observed in wild-type mice (Figs. 1e and 2d and Supplementary Fig. 2b). Because a decrease in CD4SP and CD8SP thymocytes could also be a consequence of increased clonal deletion, we next determined among cortical TCRβhi DP thymocytes the frequency of negatively selected thymocytes marked by coexpression of PD-1 and Helios in the absence of the transcription factor Foxp3 and the chemokine receptor CCR7 (refs. 13,14). Their frequency was diminished in both Foxn1wt/−mice and Foxn1wt/+mice compared with wild-type animals (Fig. 2e).

We next investigated the post-selection maturation of medullary thymocytes. The upregulation of CCR7 on CD4SP thymocytes was reduced in Foxn1wt/−mice (Fig. 2f), whereas the expression of Helios, a marker identifying cells undergoing Bim-mediated negative selection14, was diminished at initial stages, but was increased at later stages during maturation in Foxn1wt/−mice relative to wild-type mice (Fig. 2f), revealing increased negative selection of these cells.

Medullary CD8SP thymocytes demonstrated a decreased downregulation of CD24 in Foxn1wt/− mice, thereby also revealing a partial block in maturation (Fig. 2g). Moreover, the differentiation of immature and mature regulatory T cells (Treg cells) was also diminished in Foxn1wt/− and Foxn1wt/+ mice compared with wild-type animals (Fig. 2h and Supplementary Fig. 2c). Thus, an incomplete complementation of Foxn1 altered T cell development marked by a lower frequency of ETPs, reduced positive selection, partial maturation blocks and a variance in negative thymocyte selection that resulted in fewer mature, post-selection thymocytes.

Identification of Foxn1 binding sites and motifs

ChIP-seq analyses employing a Flag-tag specific antibody (Supplementary Fig. 3a) and TEC nuclear extracts from Foxn1wt/wt mice revealed 9,012 peaks at an irreproducible discovery rate (IDR) of <0.05. A third of these peaks were located immediately 5′ and within 5 kb of Foxn1 binding sites and motifs.
upstream of transcriptional start sites (TSSs; Fig. 3a,b). Overlap of these peaks with H3K4me3 marks was enriched approximately 23-fold (Supplementary Fig. 3b) and 75-fold enhanced in open chromatin regions, as measured by ATAC-seq in cTECs (Fig. 3b). Approximately two-thirds (67.3%) of Foxn1-binding sites intersected ATAC-seq peaks, and this value increased to 92.8% when considering only binding sites in regions 5 kb upstream and 100 bases downstream of the TSSs of genes. In contrast, Foxn1 showed a 0.53-fold reduction ($P < 0.0001$) in binding near the TSSs of genes encoding tissue-restricted antigens relative to all others.

Using a non-supervised method on all Foxn1 ChIP-seq peaks with an IDR < 0.05, we identified, based on our results, a consensus Foxn1-binding motif of 5 nucleotides (GA-a/c-GC) (Supplementary Fig. 3c,d). Focusing our analysis on peaks between 5 kb upstream and 100 bases downstream of the TSS refined the prediction of the Foxn1 binding motif to GACGC (Fig. 3c). This motif was associated with a higher binding affinity across all peaks, even after adjustment for GC content ($\beta = 6.1, P < 0.0001$; GAAGC: $\beta = 0.8, P = 0.44$) and was strongly enriched (3.2-fold) around the summit of Foxn1 ChIP-seq peaks when compared with surrounding sequences (Fig. 3d). This Foxn1 core binding sequence is identical to a previously suggested motif to GACGC ($\beta = 6.1, P < 0.0001$; GAAGC: $\beta = 0.8, P = 0.44$) and was strongly enriched (3.2-fold) around the summit of Foxn1 ChIP-seq peaks when compared with surrounding sequences (Fig. 3d). This motif was associated with a higher binding affinity across all peaks, even after adjustment for GC content ($\beta = 6.1, P < 0.0001$; GAAGC: $\beta = 0.8, P = 0.44$) and was strongly enriched (3.2-fold) around the summit of Foxn1 ChIP-seq peaks when compared with surrounding sequences (Fig. 3d). This Foxn1 core binding sequence is identical to a previously suggested motif to GACGC ($\beta = 6.1, P < 0.0001$; GAAGC: $\beta = 0.8, P = 0.44$) and was strongly enriched (3.2-fold) around the summit of Foxn1 ChIP-seq peaks when compared with surrounding sequences (Fig. 3d).

**Identification of Foxn1 target genes**

Because transcription factor binding does not necessarily result in the transcriptional regulation of nearby genes, we analyzed the gene expression profiles of cTECs and mTECs isolated by FACS from 1-week-old Foxn1$^{wt/wt}$ and Foxn1$^{wt/-}$ mice, respectively (Supplementary Fig. 4a,b). Large numbers (8,378 and 11,690, respectively) of genes were differentially expressed in cTECs and mTECs of Foxn1$^{wt/-}$ relative to Foxn1$^{wt/wt}$ mice (Supplementary Table 1), with the TEC transcriptional profiles of Foxn1$^{wt/wt}$ mice highly correlating with previously published data of wild-type TECs (mean ± s.d. Spearman’s $r$: cTEC, 0.94 ± 0.00; mTEC, 0.92 ± 0.00).

To address the issue of which of the TEC transcriptomic differences observed in Foxn1$^{wt/-}$ mice result from a change in direct Foxn1-mediated gene regulation, we generated triple mutant mice (Psmb11-rtTA::tetO-Cre::Foxn1$^{7,loxP/loxP}$, designated iFoxn1$^{A7,8}$, in which exons 7 and 8 of the Foxn1 locus can be deleted in a doxycyclin (Dox)-induced, cTEC-restricted fashion (Supplementary Fig. 5a–c). The thymi of these mice displayed a regular architecture, epithelial composition and lymphopoietic function in the absence of Dox treatment (Supplementary Fig. 6 and data not shown). The thymus of these mice had largely lost Foxn1 expression by 3 d after Dox exposure (Supplementary Fig. 6a) and displayed a decreased cellularity despite normal intrathymic T cell development, as assessed by CD4 and CD8 expression (Supplementary Fig. 6b,c). However, there was a marked decrease in Lin$^-$CD44$^+$CD25$^+$CD4$^-$$^-$CD8$^-$ and Lin$^-$CD44$^+$CD25$^+$CD4$^-$$^-$CD8$^-$ thymocytes when comparing 3-d Dox-treated with untreated iFoxn1$^{A7,8}$ mice (Fig. 4a). These results indicate that continuous and steady-state Foxn1 expression in postnatal cTECs is required for normal T cell development.

Transcriptome analysis of cTECs from 1-week-old iFoxn1$^{A7,8}$ mice isolated 3 d after Dox treatment identified 2,506 differentially regulated genes compared with cTECs isolated from Dox-treated iFoxn1$^{A7,8}$ mice deficient for the TetO-Cre transgene, and thus unresponsive to Dox (false discovery rate (FDR) < 0.05; Supplementary Table 1 and Supplementary Fig. 4c). Many of the downregulated genes (566) most likely represented direct targets because their Foxn1 binding sites were placed between 5 kb upstream and 100 bases downstream of their TSS, and their transcripts decreased coincident with a loss of full-length Foxn1 transcripts, as was found for several previously suspected Foxn1 target genes that are essential for early thymocyte development, including Cd25 (ref. 12), Cxcl12 (ref. 17) and Dll4 (ref. 18) (Fig. 4b). Samples from Foxn1$^{wt/wt}$ and Foxn1$^{wt/-}$

![Figure 3](image-url) Foxn1 ChIP-seq analysis. (a) Proportion of Foxn1 ChIP-seq peaks falling in different RefSeq meta-gene regions. (b) Left, enrichment of Foxn1 ChIP-seq signal across a meta-gene profile comprising all RefSeq mouse genes relative to control. TES, transcriptional end site. Right, relative coverage of cTEC ATAC-seq transposon insertions relative to the summit of Foxn1 ChIP-seq peaks. (c) MEME-ChIP-derived Foxn1-binding site motif for TSS-associated peaks (~5 kb before and 100 bp after TSS, $E < 10^{-80}$). (d) Motif coverage relative to the summit of Foxn1 ChIP-seq peaks for TSS-associated peaks.
mice segregated in principal component analysis first by cell type and then by genotype (Supplementary Fig. 7a), and samples from Foxn11Δ7.8 mice separated in this analysis according to the presence or absence of the TetO-Cre transgene (Supplementary Fig. 7b). These observations therefore support the validity of the mouse model to probe Foxn1 biology.

We next integrated the Foxn1 ChIP-seq data with TEC RNA-seq data from Foxn11wt/wt, Foxn11wt/−, and Foxn11Δ7.8 mice. This enabled us to identify a set of high-confidence genes both directly bound and transcriptionally regulated by Foxn1. We identified 5,415 genes by ChIP-seq that had Foxn1 peaks between 5 kb upstream or 100 bases downstream of the TSS (Fig. 4c). Of these, 1,749 genes contained at least one Foxn1-binding peak and were upregulated in TECs of Foxn11wt/wt relative to Foxn11wt/− mice (Fig. 4c). Finally, 450 of the 1,148 genes bound by Foxn1 and upregulated in cTECs of Foxn11wt/wt relative to Foxn11wt/− mice were also upregulated in iFoxn11Δ7.8 mice that lacked the TetO-Cre transgene compared with iFoxn11Δ7.8 mice in which Foxn1 was deleted in response to Dox treatment (Fig. 4c, Table 1 and Supplementary Table 2). Using the binding and expression target analysis (BETA) software package on our ChIP-seq and cTEC transcriptomic data revealed that Foxn1 functions predominantly as a transcriptional activator \((P < 10^{-7})\), as Foxn1 ChIP-seq peaks were 1.92-fold more likely \((P < 0.01)\) to mark genes whose expression was decreased in cTEC of iFoxn11Δ7.8 mice in which Foxn1 had been deleted (Fig. 4d and Supplementary Fig. 8).

We next identified Foxn1 ChIP-seq peaks of cTEC genes that were placed near TSSs and intersected an ATAC-seq peak, and compared these to ChIP-seq peaks that did not have such an association. There were no significant difference in the log2 fold change of gene expression between cTECs from iFoxn11Δ7.8 mice or iFoxn11Δ7.8 mice that lacked the TetO-Cre transgene \((P = 0.14)\). However, the presence of an ATAC-seq peak in cTECs was predictive in iFoxn11Δ7.8 mice of the magnitude of expression for genes with a nearby Foxn1 ChIP-seq peak (ATAC-seq peak versus no ATAC-seq peak: 2.4-fold, \(P < 0.0001\)).

The high-confidence Foxn1 gene targets that we identified demonstrated a higher median chromatin accessibility in cTECs when compared with all other genes \((P < 0.0001; \text{Fig. 4e})\). mRNA expression from high-confidence genes revealed a significant linear relationship with the relative proportion of full-length, wild-type Foxn1 transcripts in iFoxn11Δ7.8 mice (Supplementary Fig. 9). Most of these high-confidence Foxn1 targets were highly expressed in cTECs of Foxn11wt/wt mice (fragments per kilobase of exon per million fragments mapped, FPKM) > 10, 360 genes, although many of them were also expressed in mTECs of Foxn11wt/wt mice (FPKM > 1, 428 genes; Fig. 4f).
suggesting that our approach identified genes of importance to general TEC biology. To check that these high-confidence Foxn1 targets were not driven by contamination with other thymic stromal cells, we screened the Foxn1 target genes that we identified in TECs against a list of potential contaminants derived from a previous microarray study of cell types resident in the thymus.\(^{20,21}\) None of the high-confidence Foxn1 targets were identified as being derived from contaminating cell types.

We next assessed whether the comparison between cTEC from Foxn1\(^{wt/wt}\) and Foxn1\(^{wt/-}\) mice captured similar underlying transcriptional changes, as was observed for cTECs from Dox-treated ifoxn1\(^{17,8}\) mice compared with cTECs from Dox-treated ifoxn1\(^{17,8}\) mice lacking the TetO-Cre transgene. Positive fold change indicates higher expression in Foxn1\(^{wt/wt}\) mice compared with Foxn1\(^{wt/-}\) mice (1.10-fold, \(P < 0.05\) for all pair-wise comparisons). Given that Aire expression was comparable in mTECs of Foxn1\(^{wt/wt}\) and Foxn1\(^{wt/-}\) mice (1.10-fold, \(P = 0.23\)), these findings suggest that Foxn1 expression facilitates promiscuous gene expression in a fashion that is independent, but complementary to, Aire.

We then used existing TEC transcriptomic data sets to place our high-confidence Foxn1 targets in the context of a cTEC-specific coexpression network constructed using microarray data generated at multiple developmental time points.\(^{20,21}\) We used weighted gene coexpression network analysis to identify gene modules containing more Foxn1 targets than would be expected by chance (Supplementary Fig. 11 and Supplementary Table 4). Dll4, Ccl25 and several other putative high-confidence Foxn1 targets were significantly enriched in a gene module associated with the regulation of T cell activity (\(P < 0.002\); Supplementary Table 4). The high-confidence Foxn1 targets formed a biological coexpression network in cTECs (\(P < 0.002\)) in which Foxn1 was identified as being present in a highly connected central hub (Supplementary Fig. 12a). Thus, Foxn1 target genes formed an interconnected coexpression hub that is associated with antigen processing and presentation.

### Foxn1 targets of antigen processing and presentation

We used gene ontology analysis to identify the biological categories to which high-confidence Foxn1 target genes contribute. A significant enrichment was observed for categories involving threonine peptidases (including Tasp1), the components of the proteasome complex (such as Psmb9, Psmb10, Psmb4 and Psmad) and protein transporters (including Tap2) for antigen presentation by MHCII. In addition, we found that expression of Prrs16, which encodes a thymus-specific serine protease that is expressed in cTECs and required for CD4 lineage selection and high MHCII expression,\(^{23}\) was regulated by Foxn1 (Table 1). Thus, Foxn1 controls multiple mechanisms that are critical for self-antigen processing and presentation.

Given this association of Foxn1 expression with pathways involved in antigen presentation, we next tested whether there was a differential expression of genes regulated by the autoimmune regulator Aire between mTECs from Foxn1\(^{wt/wt}\) and Foxn1\(^{wt/-}\) mice. As a group, Aire-regulated genes tended to be more highly expressed in mTECs of Foxn1\(^{wt/wt}\) than Foxn1\(^{wt/-}\) mice when compared with either Aire-independent tissue-restricted antigens or other protein-coding genes (median fold change: Aire-induced tissue-restricted antigens, 2.93-fold; other Aire-induced genes, 2.61-fold; Aire-independent tissue-restricted antigens, 1.14-fold; other protein-coding genes, 1.00-fold; \(P < 0.05\) for all pair-wise comparisons). Given that Aire expression was comparable in mTECs of Foxn1\(^{wt/wt}\) and Foxn1\(^{wt/-}\) mice (1.10-fold, \(P = 0.23\)), these findings suggest that Foxn1 expression facilitates promiscuous gene expression in a fashion that is independent, but complementary to, Aire.

### Identification of Foxn1 co-factors

To identify DNA-binding factors that could cooperate with Foxn1 in controlling gene expression, we next searched for known transcription factor recognition motifs in and adjacent to Foxn1 ChIP-seq peaks. This analysis identified several motifs that were significantly and centrally overrepresented when compared with a background of mixed promoter and enhancer regions (Supplementary Fig. 12b,c). Among these were binding sites for the transcription factors CREB1 and TP63, which are known to regulate proliferation and TEC aging, respectively.\(^{25,26}\) Foxn1 binding in TEC was 11.7-fold enriched near TP63-binding sites that were previously recognized in Foxn1-expressing keratinocytes (\(P < 0.0001\)), supporting the notion that a Foxn1-TP63 axis regulates TEC homeostasis.\(^{26,27}\) Thus, we identified a number of potential Foxn1 co-factors, of which TP63 is a particularly biologically plausible candidate.
Psmb11 and Cd83 are direct targets of Foxn1 targets

Psmb11, encoding the proteasome component β5t, and Cd83 were identified as two high-confidence Foxn1 target genes (Table 1). As early as 24 h post-Dox treatment, transcripts for both genes were significantly reduced in cTEC from iFoxn11Δ7,8 mice when compared with untreated iFoxn11Δ7,8 mice (Fig. 5a), suggesting that the genes direct transcriptional control by Foxn1. Psmb11 is expressed almost exclusively in cTECs and is pivotal for positive selection of CD8+ T cells.18,29, CD83 is a surface marker expressed in cTECs that is required for the development of CD4SP cells.38, A single Foxn1-binding peak was identified upstream of the TSS of Psmb11 that contained two copies of the Foxn1-binding motif GACGC and two copies of the predicted alternative motif GAAGC. The Cd83 promoter contained a proximal GACGC and three distal GAAGC motifs (Fig. 5a). The functional importance of these binding sites was investigated in HEK293 cells co-transfected with a Foxn1 expression plasmid and a luciferase construct containing the Psmb11 and the Cd83 promoters, respectively, each with or without disrupted consensus motifs. Mutations of the proximal and, to a lesser degree, distal GACGC motifs of the Psmb11 promoter significantly decreased luciferase activity, whereas changes to both ablated transcription altogether (Fig. 5b). Deletion of the single GACGC motif in the promoter of Cd83 decreased the transcriptional activity by half, and removal of the promoter sequence containing the distal two GAAGC motifs reduced transcription by two-thirds (Fig. 5c). Mutating the proximal GAAGC in the truncated promoter, in contrast with removal of GAAGC, had no effect on the transcriptional activity (Fig. 5c). Thus, the provision of Foxn1 in heterologous HEK293 cells is sufficient to activate the minimal Psmb11 and Cd83 promoters, each containing intact GACGC Foxn1-binding motifs.

In comparison with wild-type animals, Foxn11Δ7Δ8 mice had fourfold fewer CD8SP thymocytes (Fig. 5d), which correlated with a partial block in thymocyte selection, as measured by CD69 and TCR surface expression (Fig. 5e). These changes closely, but not completely, mirrored those observed in Psmb11-deficient mice (Fig. 5d,e). We therefore investigated the negative selection of cortical (that is, CCR7+) DP
DISCUSSION

Drawing on newly created mouse models, we identified a core set of 450 high-confidence Foxn1 target genes in postnatal TECs that, among other functions, control multiple mechanisms that are indispensable for self-antigen processing and presentation. Our findings demonstrate that Foxn1 controls essential functions in TEC biology in addition to those previously noted to be important for initial stages in thymus organogenesis and early thymopoiesis.

Our in vivo ChIP-seq data using nuclear lysates from primary TEC of Foxn1$^{wt/−}$ mice established the Foxn1 consensus binding motif, 5′- a/g/t-G-A-C-G-C, with a core identical to that previously identified by in vitro studies,16,32. Although our studies predicted GAAGC as an alternative motif, mutational analyses of the Psmbl1 and Cd83 promoters revealed that there were no substantial functional contributions of Foxn1 binding to these sequences. However, productive Foxn1 binding to this alternative motif may occur in other cellular or developmental contexts and could depend on co-factors to specify tissue-contextual gene expression profiles, as has been suggested for other Fox family members.32,33 Although specific co-factors that physically or functionally associate with Foxn1 have not thus far been identified, our ChIP-seq data recognized several transcription factor binding motifs enriched in the vicinity of canonical Foxn1-binding sites, for example, those for the transcriptional regulator TP63. An association of Foxn1 with TP63 is interesting because TP63 maintains TEC stemness,24, is positioned upstream of and physically interacts with the PRC1 (polycomb repressive complex) component CBX4, which we identified as a high-confidence Foxn1 target. Notably, the lack of Cbx4 expression impaired TEC generation, proliferation and thymopoietic function.35 In this context, it is also noteworthy that Tbata, which also controls TEC proliferation,36, is another Foxn1 target gene that we identified.

Differences in gene expression profiles between TECs isolated from Foxn1$^{+/+}$ and wild-type mice have previously and by inference been understood to be transcriptionally controlled by Foxn1, including MHCII (ref. 9), Pax1 (ref. 9) and FGFR2 (ref. 37). However, our data exclude these loci as direct Foxn1 targets (at least in postnatal TECs) and suggest that their transcriptional changes are the indirect consequences of alterations in Foxn1 complementation. In contrast, our experimental approach combining ChIP-seq and gene expression...
profiling confirmed that the previously suspected targets Dll4, Cxcl12 and Ccl25 are indeed controlled by Foxn1. These findings provide a validated molecular explanation for the failure of Foxn1-deficient TECs to attract ETPs and to assure their subsequent commitment to a T cell fate.

Our approach also identified thymic Foxn1 target genes that function at stages beyond the early arrest in thymus organogenesis characteristic for Foxn1mut/−/− mice and marked by a lack of regular homing of ETP and their commitment to the T cell lineage. Many of these hitherto unrecognized Foxn1 target genes are involved in thymocyte development and selection, with functions extending from self-antigen processing to T cell activation and post-selection maturation. Investigating the function of Pomb11 and Cdx83-deficient TEC, respectively, revealed the most likely molecular cause of some of the major phenotypic features observed in Foxn1wt/wt and Foxn1wt/wt−/− mice. However, determining the extent of the contributions of Pomb11 and Cdx83 to the observed phenotypes will necessitate transgenic reconstitution experiments in Foxn1−/− mice similar to those that established a critical role of the direct Foxn1 targets Dll4 and Cxcl12 in early T cell development.

Notably, transgenic expression of Dll4 and Cxcl12 in Foxn1−/− TEC is only sufficient to support thymocyte development up to the DP stage.

The iFoxn1−/− mouse model also demonstrated the need for continuous Foxn1 expression to maintain regular thymopoietic activity by cTECs. Dox treatment of these mice deleted exons encoding the forkhead and the transcriptional activation domains and resulted in a twofold reduction of full-length Foxn1 transcripts within 24 h and a decreased expression of hundreds of cTEC genes within 72 h (threefold, 406 genes; ≥ twofold, 1,192 genes), affecting total thymocyte cellularity and early thymocyte differentiation at that point in time. Although at variance with a previous observation for reasons yet to be elucidated, our results clearly demonstrate the importance of continuous Foxn1 expression for the functional competence of postnatal cTECs.

Taken together, our results demonstrate for the first time, to the best of our knowledge, that Foxn1 directly binds to and controls the transcription of several hundred genes that, in aggregate, control essential checkpoints during intrathymic T cell development. These genes are critical for the steady-state function of adult TECs, and their expression needs to be preserved for the continued thymopoietic competence of the thymus.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GEO: microarray data, GSE75219.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS


COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. C57BL/6 and Foxn1tm1Bnd mice were obtained from Janvier and Taconic, respectively. The generation of β5t-rTα, and TetO-Cre animals has previously been reported.[4,40] Mice transgenic for a BAC encoding a Foxn1-Flag fusion protein under the regulatory control of the Foxn1 locus were generated similarly to the previously reported Foxn1-Cre mice.[41] Foxn1-Flag transgenic mice were subsequently backcrossed onto a Foxn1tm1Bnd background to obtain mice homozygous or heterozygous for the BAC transgene, designated Foxn1tm1Bnd/wt* and Foxn1wt*/wt*, respectively. Mice with a conditional Foxn1 locus where exons 7 and 8 are flanked by loxP sites were generated by homologous recombination, and subsequently crossed to β5t-rTα and TetO-Cre transgenic animals to obtain triple transgenic mice, designated ifoxn1147.8. Sex-matched male and female mice aged 4–7 weeks were used. No specific exclusion criteria were used in mouse experiments. All animals were kept under specific pathogen-free conditions and experiments were carried out in accordance with local and national regulations and permissions approved by Kantonales Veterinäramt BS.

Doxycycline treatment. 1-week-old ifoxn1147.8 mice were treated with a single intraperitoneal injection of Dox (0.3 mg).

Flow cytometry. Thymocytes were incubated with antibodies against TCR beta (1:400, H57-597; BioLegend), CD4 (1:100, GK1.5; BioLegend), CD5 (1:400, 53-7.3; eBioscience), CD8 (1:500, 53-67; BioLegend), CD25 (1:1,000, PC61.5; BioLegend), CD44 (1:500, IM7; BioLegend), c-kit (1-200, 20B8; BioLegend), CD24 (1:1,000, M1/69; BioLegend), CD69 (1:200, H1.2F3; BioLegend), PD-1 (1-200, 29E1A12, BioLegend), CCRT (1:200, 4B12, BioLegend). For intracellular staining, cells were fixed, permeabilized (Fixation/Permeabilization kit, eBioscience) and stained for Foxp3 (1:70, FJK-16S, eBioscience) and Helios (1:70, 22F6, eBioscience). TECs were stained using antibodies against CD45 (1:400, 30F11; BioLegend), EpCAM (1:1,000, G8.8; BioLegend), MHCII (1:1000, M5/114.15.2; BioLegend), Ly51 (1:1,000, 6C3; BioLegend), UEA-1 (1:1,000, Reactolab), CD80 (1:500, 16-10A1, BioLegend), CD86 (1:500, GL-1, BioLegend), CD83 (1:500, Michel 19, BioLegend). For intracellular staining, cells were fixed, permeabilized (Cytofix/Cytoperm Kit, BD Biosciences) and labeled for the expression of CD83. Stained samples were acquired on a FACSaria II or BD LSRFortessa flow cytometers and the data was analyzed using the FlowJo (Treestar software).

Quantitative PCR analysis. Total RNA was isolated from sorted cells with the RNeasy Kit (Qiagen), cDNA was synthesized using SuperScriptIII (Live Technologies) and assessed by qPCR (SensiMix; Bioline).

ChiP-seq. Thymic lobes from 1-week-old Foxn1tm1Bnd/wt* mice were digested with 0.2 mg/ml Liberase TM (Roche Diagnostics) and 30 μg/ml DNaseI (Roche Diagnostics) in PBS at 37 °C for 30–60 min. TECs were enriched to 15–20% using magnetic beads (autoMACS Pro Separator, Miltenyi Biotech) and then subjected to DNA crosslinking. Nuclear isolation and chromatin fragmentation were performed with 20–30 × 10^6 cells enriched for TEC using the trueChiP High Cell Chromatin Shearing Kit with nonionic Shearing Buffer according to the manufacturer's recommendations (Covaris). Chromatin was immunoprecipitated using the M2 anti-FLAG antibody (F1804; Sigma) with parallel input samples prepared from non-immunoprecipitated chromatin. To increase the specificity of ChiP-seq analysis, it would have been ideal to include immunoprecipitated chromatin from wild-type TEC as negative control but the large amount of material required made this approach infeasible. Precipitated DNA from TEC-enzriched (15–20%) and control thymocyte only samples was subjected to qPCR analysis using primers specific to promoter regions of Psmb11 and Dll4 (both TEC-specific genes) and Foxp3 (thymocyte specific gene) (Supplementary Fig. 3a). DNA samples from multiple ChiP showing successful enrichment of TEC candidate genes promoters were pooled to generate two replicates (5 ng each) for library generation and sequencing. Contaminating adaptor sequences were removed from fastq sequences using Trimmomatic (version 0.32). Reads were aligned against the mouse genome (UCSC build mm10) using pre-alignment with BWA (version 0.7.5 with the options -q10 -t4) and final alignment was achieved with Stampy (version 1.0.23 with the options -t4 -bamkeepgoodreads -M4243). Peaks were called on deduplicated aligned sequences (paired ChiP and input samples) using MACS2 (version 2.0.10) with a relaxed P value setting of 0.1 (ref. 44). Peaks from replicates were then pooled and analyzed using irreproducible discovery rate analysis (IDR < 0.05) (https://sites.google.com/site/anshulkundaje/projects/idi). Peaks were filtered against the ENCODE blacklist regions (https://sites.google.com/site/anshulkundaje/projects/blacklists).

RNA-seq. TECs were enriched as detailed above and then sorted using a FACSaria II (BD Bioscience). RNA was isolated using RNeasy kit (Qiagen) and subjected to sequencing (TrueSeq, BGI). Reads were aligned against the Ensembl transcriptome and genome (GRCh38) using TopHat2 (version 2.0.10). Reads were allocated to protein-coding gene meta-features using Rsубread (requiring both pairs of reads to be aligned and excluding multi-mapping reads). Exon-level expression data was estimated using BEDTools coverage. Differential expression analysis on genes with at least 1 aligned fragment was conducted using general linear modelling in edgeR, correcting for common, trended and tagwise dispersion, with additional batch correction for the conditional Foxn1 knockout model.[45] Genes were identified as differentially expressed using the default edgeR threshold (FDR < 0.05).

ATAC-seq. A cell pellet of ~10,000 cTEC was lysed, treated with transposase and DNA tagmented in accordance with a previously published protocol.[46] Tagmented fragments were amplified by PCR and sequenced using an Illumina MiSeq on 75bp paired end reads. Trimmomatic was used to trim Kmer biases from reads (version 0.32). Fragments were aligned to the mm10 genome using Bowtie2 (version 2.2.3 with the options--no-discordant--no-mixed -X 2000). Read positions were corrected for transposon insertion offset. The significance of differences in coverage between sets of genes was estimated using Wilcoxon rank sum tests. ATAC-seq peaks were called using MACS2 (with the options--nomodel--nolambda--keep-dup all--call-summits; FDR < 0.05). Peaks were screened against blacklisted regions.

Statistical analysis of ChiP-seq and RNA-seq. De novo motif discovery within Foxn1 ChiP-seq peaks was carried out using MEMEChIP on the 200 base-pairs surrounding the peak summit. Background nucleotide content was controlled for using a first-order Markov model. Motif location was identified using FIMO with a threshold of P < 0.05 on repeat-masked fasta files using a first-order Markov background. The sequence within 100 bases of each ChiP-seq summit was classified as either containing or not containing a version of each candidate Foxn1 recognition motif. General linear regression, adjusting for GC content within 100 bases of each ChiP-seq summit, was performed to test the predictive power of each motif’s presence for the significance score assigned to each summit by MACS2. Central enrichment was tested by permuting the position of motifs within 1kb of peak summit 10,000 times and assessing the number of motifs falling into a 200-base central window. Foxn1 ChiP-seq peaks were scanned for co-factor motifs using PScanChiP and a mixed background.[44] Genomic enrichment was calculated using GAT with 10,000 randomizations, controlling for GC content and using an appropriate workspace as background. Genomic features were annotated using HOMER. ChiP-seq peaks were allocated to genes using a window of 5 kb upstream and 100 bases downstream of the TSS. This gene definition was selected to be comparable to ChiP-seq annotation methods such as GREAT but using greater stringency. To establish whether Foxn1 functioned predominantly as a repressor or activator of transcription, we used BETAto integrate the Foxn1 ChiP-seq peaks and cTEC RNA-seq data sets from ifoxn1147.8 mice (using default settings).[48] We then restricted Foxn1 direct gene targets to those significantly differentially expression in the appropriate direction as a result of Foxn1 deficiency and with a Foxn1 binding site within 5 kb upstream or 100 bases downstream of the TSS.

Coexpression analysis. Microarray expression data was downloaded from GSE6928 (ref. 21). This was quantile normalized and a thymic epithelial cell-specific (cTEC, mTEC[40] and mTEC[40]) signed coexpression network constructed using bidirectional weighted correlation in WGCNA[40]. The optimal soft threshold power was chosen to achieve r^2 > 0.9 for the network. Individual sets of genes were analyzed by permutation analysis, controlling for expression decimals. This data set was also used to generate a list of genes

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likely to be primarily driven by non-TEC contaminating thymic stromal cells. To this end, the data set was screened for genes displaying significant differences on ANOVA testing between TECs and non-TECs, fourfold higher expression in non-TECs than TECs and an expression level in TECs in the bottom quintile of genes.

Gene ontology analysis. Gene ontology analysis was conducted using DAVID\textsuperscript{22}. All enriched GO terms with Benjamini-Hochberg corrected $P < 0.05$ and twofold enrichment are shown in the relevant figures.

Histological analyses. Frozen thymus tissue sections (8 μm) were fixed in acetone and stained using antibodies specific for Psmb11 (1:500, PD021, MBL), CD4 (1:200, GK1.5; BioLegend), CK5 (1:500, PRB-160P; Covance), CK8 (1:200, TROMA-1, NICHD supported Hybridoma Bank), ERTR7 (1:50, provided by W. van Ewijk, Erasmus Medical Centre, Rotterdam, the Netherlands), Foxn1 (1:1000, provided by T. Amagai, Meiji University of Integrative Medicine, Hiyoshi-cho, Nantan, Japan) and Aire (1:200, 5H12, eBioscience). Images were acquired using a Zeiss LSM510 (Carl Zeiss).

Luciferase assay. Promoter fragments of Psmb11 and Cld83 were subcloned into the pGL4.10(luc2, Promega) reporter plasmid and co-transfected with an expression vector encoding Foxn1 into HEK293 cells using FuGENE HD (Promega). Luciferase activity was measured 24 h later using the Dual-Luciferase Assay kit (Promega). Motifs were mutated using Q5 Site-Directed Mutagenesis Kit (NEB).

Statistical analyses. Statistical analyses for data presented were performed using Students $t$ test (unpaired, two-tailed). $P < 0.05$ was considered to be significant. The statistical evaluation of the ChIP-seq and RNA-seq data is described in separate statistical analysis section above. The sample size used and estimates of variation within groups were based on published results using similar approaches. No randomization was done for animal studies and investigators were not blinded to experimental group allocations.