

RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Ectopic TBX1 suppresses thymic epithelial cell differentiation and proliferation during thymus organogenesis

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ABSTRACT

The thymus and parathyroid glands arise from a shared endodermal primordium in the third pharyngeal pouch (3rd pp). Thymus fate is specified in the ventral 3rd pp between E9.5 and E11, whereas parathyroid fate is specified in the dorsal domain. The molecular mechanisms that specify fate and regulate thymus and parathyroid development are not fully delineated. Previous reports suggested that *Tbx1* is required for thymus organogenesis because loss of *Tbx1* in individuals with DiGeorge syndrome and in experimental *Tbx1* deletion mutants is associated with thymus aplasia or hypoplasia. However, the thymus phenotype is likely to be secondary to defects in pharyngeal pouch formation. Furthermore, the absence of *Tbx1* expression in the thymus-fated domain of the wild-type 3rd pp suggested that *Tbx1* is instead a negative regulator of thymus organogenesis. To test this hypothesis, we generated a novel mouse strain in which expression of a conditional *Tbx1* allele was ectopically activated in the thymus-fated domain of the 3rd pp. Ectopic *Tbx1* expression severely repressed expression of *Foxn1*, a transcription factor that marks the thymus-fated domain and is required for differentiation and proliferation of thymic epithelial cell (TEC) progenitors. By contrast, ectopic *Tbx1* did not alter the expression pattern of *Gcm2*, a transcription factor restricted to the parathyroid-fated domain and required for parathyroid development. Ectopic *Tbx1* expression impaired TEC proliferation and arrested TEC differentiation at an early progenitor stage. The results support the hypothesis that *Tbx1* negatively regulates TEC growth and differentiation, and that extinction of *Tbx1* expression in 3rd pp endoderm is a prerequisite for thymus organogenesis.

KEY WORDS: TBX1, FOXN1, Thymus, Mouse

INTRODUCTION

T-cell development takes place in the thymus, which provides a unique and indispensable stromal microenvironment composed of thymic epithelial cells (TECs), fibroblasts, endothelial cells, macrophages and dendritic cells. TECs are the predominant cell type in the thymic microenvironment and perform a number of nonredundant functions that are essential for proper thymocyte

growth and maturation (reviewed by Boehm, 2008; Rodewald, 2008; Manley et al., 2011; Thompson and Zuniga-Pflucker, 2011; Anderson and Takahama, 2012). Cortical TECs (cTECs) and medullary TECs (mTECs) present self-peptides that mediate the positive and negative selection processes required to produce T cells that express a diverse array of antigen-specific receptors, yet are self-tolerant. TECs also produce cytokines that promote growth and survival of thymocytes, chemokines that direct thymocyte migration and Notch ligand that activates signaling pathways required for thymocyte differentiation and T-cell lineage commitment. Given the various crucial roles played by TECs in T-cell development and the loss of TECs that occurs during age-related thymus involution, there is considerable interest in elucidating the molecular mechanisms that commit primitive endodermal progenitors to a TEC fate and regulate thymus organogenesis.

The thymus and parathyroid glands arise from shared primordia that develop bilaterally from third pharyngeal pouch (3rd pp) endoderm. Correct patterning and cell specification within the shared pouch are essential for development of both organs. The ventral posterior region of the 3rd pp is specified to a thymus fate, whereas the dorsal anterior region is specified to a parathyroid fate. Although the thymus and parathyroid domains are specified by E9.5–10.5, they cannot be morphologically distinguished (Gordon et al., 2001, 2004; Patel et al., 2006). By E11.5, the thymus- and parathyroid-fated domains can be recognized by *Foxn1* and *Gcm2* expression respectively. However, although these transcription factors regulate differentiation, they do not specify thymus versus parathyroid fate (Bleul et al., 2006; Liu et al., 2007; Nowell et al., 2011). A *Hox-Pax-Eya-Six* cascade is implicated upstream of thymus specification, but has not been directly linked to the establishment of thymus fate (Manley and Condle, 2010). Additional transcription factors with restricted expression patterns in the 3rd pp have been identified, but their role in thymus fate is not yet established (Wei and Condle, 2011). Thus, the transcription factors and molecular pathways that specify thymus fate and regulate thymus organogenesis are not yet defined.

The T-box transcription factor TBX1 is widely considered to play an indispensable role in thymus development. During ontogeny, *Tbx1* is expressed throughout the pharyngeal endoderm and arch mesenchyme, but is not expressed by neural crest-derived mesenchyme (Vitelli et al., 2002). Deletion or mutation of *Tbx1* is a common denominator in the human chromosome 22q11.2 deletion syndrome (22q11DS) that results in DiGeorge and velocardiofacial syndromes (Jerome and Papaioannou, 2001; Merscher et al., 2001; Yagi et al., 2003; Baldini, 2005). In addition to cardiovascular defects and craniofacial abnormalities, these patients typically have thymus aplasia or hypoplasia (Hollander et al., 2006). Furthermore, thymus aplasia is a characteristic feature of *Tbx1* homozygous deletion mutants, a finding consistent with the prevalent view that TBX1 potentiates thymus development (Jerome and Papaioannou,

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2001; Baldini, 2005; Hollander et al., 2006). However, this interpretation is complicated by the fact that *Tbx1* is required for segmentation of the pharyngeal apparatus; in its absence, the pharyngeal pouches do not develop (Chapman et al., 1996; Jerome and Papaioannou, 2001; Lindsay et al., 2001; Vitelli et al., 2002; Arnold et al., 2006). Therefore, the athymia observed in the absence of *Tbx1* is a secondary consequence of defective pouch formation; as a result, the potential role of *Tbx1* in thymus organogenesis cannot be determined from analysis of *Tbx1*-null embryos. Moreover, by E10.5 *Tbx1* expression in the 3rd pp is restricted to the parathyroid domain and excluded from the thymus domain (Vitelli et al., 2002; Manley et al., 2004; Dooley et al., 2007), suggesting that *Tbx1* expression may be necessary for parathyroid formation, but may antagonize rather than promote thymus development. A previous study reported the occurrence of thymus hypoplasia resulting from *Tbx1*^{Cre}-mediated activation of a conditional *Tbx1* transgene during embryogenesis; however, the mechanism responsible for this phenotype was not determined (Vitelli et al., 2009).

To test the hypothesis that *Tbx1* expression in 3rd pp endoderm suppresses thymus fate and/or TEC differentiation, we generated a novel mouse strain in which a stop-floxed *Tbx1* allele was knocked into the ubiquitously expressed *Rosa26* locus. We used *Foxn1*^{Cre} to activate *Tbx1* expression in the thymus-fated domain of the 3rd pp. Here, we show that ectopic *Tbx1* expression in the ventral 3rd pp suppresses *Foxn1* expression, inhibits TEC proliferation and blocks TEC differentiation at an early progenitor stage, but does not reverse thymus fate. The results support the hypothesis that *Tbx1* negatively regulates TEC growth and differentiation, and that extinction of *Tbx1* expression in 3rd pp endoderm is a prerequisite for thymus organogenesis.

RESULTS

Foxn1^{Cre} activates ectopic expression of the *R26*^{*Tbx1*} allele in the ventral domain of the 3rd pp

Tbx1 is expressed throughout 3rd pp endoderm at E9.5, but by E10.5 is restricted to the dorsal, parathyroid-fated domain (Vitelli et al., 2002; Manley et al., 2004; Zhang et al., 2005; Liu et al., 2007). *Foxn1*-expressing cells are present in the dorsal 3rd pp and extend into pharyngeal endoderm in the absence of SHH, a positive regulator of *Tbx1* expression (Garg et al., 2001; Yamagishi et al., 2003; Moore-Scott and Manley, 2005). Taken together, these data suggest that TBX1 antagonizes thymus development. Therefore, we used a gain-of-function approach to test this hypothesis. *Tbx1* cDNA and an IRES-GFP tag were inserted into a modified *Rosa26* targeting vector containing a floxed stop cassette to generate a knock-in strain hereafter referred to as *R26*^{*Tbx1*} (supplementary material Fig. S1). This allele activates expression of both *Tbx1* and *EGFP* after Cre-mediated deletion of the stop cassette.

We used *Foxn1*^{Cre} to activate ectopic *Tbx1* expression in the ventral thymus-fated domain of the 3rd pp beginning at ~E11.25 (Gordon et al., 2007). Thymus fate is specified in 3rd pp endoderm as early as E9.5–10.5 prior to expression of *Foxn1*, which is essential for TEC differentiation and proliferation (reviewed by Manley and Condie, 2010; Gordon and Manley, 2011). Therefore, *Foxn1*^{Cre} activates expression of the *R26*^{*Tbx1*} allele in ventral 3rd pp endoderm after the cells commit to a thymus fate and are beginning to differentiate. Immunohistochemical (IHC) analysis of GFP expression in the *Foxn1*^{Cre/+}; *R26*^{*Tbx1*} 3rd pp verified the predicted ectopic expression of *R26*^{*Tbx1*} in the ventral, but not in the dorsal, domain and confirmed the absence of a GFP signal in *Foxn1*^{Cre/+}; *R26*^{+/+} controls (supplementary material Fig. S2). Consistent with previous reports, 3rd pp and thymus development

were normal in *Foxn1*^{Cre/+}; *R26*^{+/+} controls (supplementary material Fig. S2; see Fig. 3) (Gordon et al., 2007; Chen et al., 2009). Continued expression of the *R26*^{*Tbx1*} allele in fetal thymus throughout ontogeny was confirmed by FACS analysis of GFP expression, which also verified restriction of the GFP signal to EpCAM⁺ CD45[–] TECs (supplementary material Fig. S2).

Ectopic TBX1 expression suppresses FOXN1 but does not alter GCM2 expression

We asked whether ectopic expression of *Tbx1* in the ventral domain altered patterning of the 3rd pp at E11.5. IHC staining of serial sections from *Foxn1*^{Cre}; *R26*^{+/+} controls confirmed that endogenous TBX1 is restricted to the dorsal parathyroid-fated domain (Fig. 1A), whereas FOXN1 is restricted to the ventral thymus-fated domain (Fig. 1A,C). FOXN1 and TBX1 co-expressing cells were not found in the control 3rd pp. In contrast to the restricted expression of TBX1 in the control 3rd pp at this stage, we found an increased number of TBX1-positive cells located in both the ventral and dorsal regions of the *Foxn1*^{Cre}; *R26*^{*Tbx1*} 3rd pp (Fig. 1B,E). The presence of ectopic TBX1 protein in the ventral 3rd pp is consistent with the GFP staining pattern observed in the *Foxn1*^{Cre}; *R26*^{*Tbx1*} 3rd pp (supplementary material Fig. S1). Ectopic TBX1 in the ventral domain resulted in a profound and rapid reduction in the frequency of 3rd pp cells containing detectable levels of FOXN1 (Fig. 1B,D,E). The few cells in the ventral 3rd pp that did express detectable levels of FOXN1 generally co-expressed TBX1 (Fig. 1B).

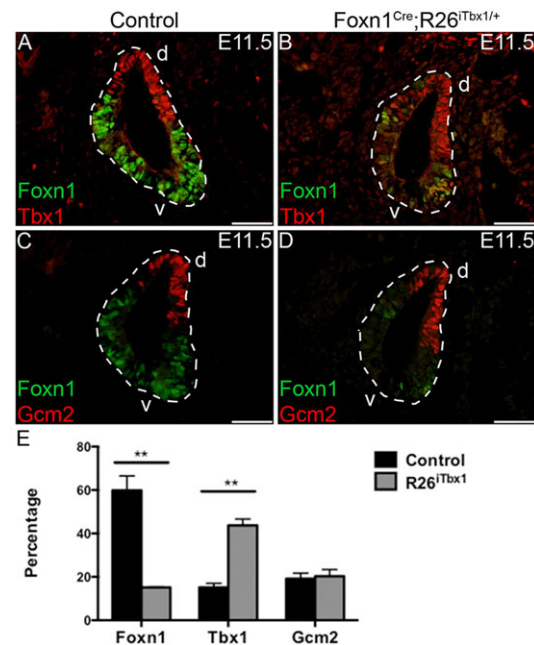


Fig. 1. Ectopic TBX1 in the ventral 3rd pp reduces the number of FOXN1-positive cells. (A–D) Representative immunohistochemical stains of sagittal sections from the 3rd pp of *Foxn1*^{Cre}; *R26*^{+/+} control and *Foxn1*^{Cre}; *R26*^{*Tbx1*} E11.5 embryos. (A,B) TBX1 (red) is restricted to the dorsal (d) parathyroid-fated domain of the control, but is present throughout the mutant 3rd pp. There is a marked reduction in FOXN1-positive cells (green) in the mutant 3rd pp. A few cells in the mutant 3rd pp are FOXN1 positive and co-stain for TBX1 (yellow). (C) GCM2 (red) is present in parathyroid-fated cells in the dorsal domain of the control 3rd pp. (D) Ectopic TBX1 does not affect localization or frequency of GCM2-positive cells in the mutant 3rd pp. Scale bars: 50 μ m. (E) The percentage \pm s.d. of 3rd pp cells that are positive for FOXN1, TBX1 or GCM2 ($n=4$ control and $n=4$ *Foxn1*^{Cre}; *R26*^{*Tbx1*} 3rd pps). ** $P<0.02$. v, ventral.

Gcm2 was previously proposed to be a downstream target of *Tbx1* (Ivins et al., 2005; Liu et al., 2007). However, ectopic *Tbx1* expression had no discernible effect on the number or localization of GCM2-positive cells (Fig. 1D,E). GCM2 was localized to the dorsal anterior region of the control 3rd pp, and this restricted expression pattern was maintained in the *Foxn1^{Cre};R26^{iTbx1/+}* 3rd pp. Therefore, these data demonstrate that ectopic expression of *Tbx1* is not sufficient to induce *Gcm2* expression in the ventral domain of the 3rd pp, suggesting that additional regulators are involved in controlling *Gcm2* expression.

Ectopic TBX1 expression in the E11.5 3rd pp does not reverse thymus fate

Owing to the severe reduction in FOXN1-positive cells and failure to expand GCM2, the majority of cells in the ventral domain of the *Foxn1^{Cre};R26^{iTbx1/+}* 3rd pp express neither FOXN1 nor GCM2 (Fig. 1B,D,E). To determine whether these cells are no longer committed to a thymus fate, we examined expression of additional factors that are expressed by thymus-specified cells. We previously reported that at E11.5, thymus-, but not parathyroid-fated, cells in the 3rd pp express IL7, a cytokine that is essential for thymocyte differentiation and survival (Zamisch et al., 2005; Repass et al., 2009). Moreover, IL7 is expressed by immature TECs in the thymic

rudiment of FOXN1-deficient nude (*Foxn1^{nu/nu}*) mice (Zamisch et al., 2005). Therefore, IL7 is a *Foxn1* independent marker of thymus-fated cells. Quantitative RT-PCR analysis demonstrated comparable levels of *Il7* mRNA in 3rd pp cells of *Foxn1^{Cre};R26^{iTbx1/+}* and control embryos (Fig. 2A).

FOXG1 is a transcription factor that is initially expressed in the ventral domain of the 3rd pp by E10.5, preceding *Foxn1* (Wei and Condie, 2011). Similar to *Il7*, *Foxg1* is expressed in the *Foxn1^{nu/nu}* thymic rudiment (Q. Wei and B. Condie, unpublished). IHC analysis of serial sections in the control 3rd pp showed that FOXG1 overlaps with FOXN1 in the ventral domain and is excluded from the dorsal GCM2 domain (Fig. 2B,D). Interestingly, FOXG1 is abundantly expressed by the FOXN1-negative cells in the *Foxn1^{Cre};R26^{iTbx1/+}* ventral 3rd pp domain (Fig. 2C). The robust expression of IL7 and FOXG1 in the *Foxn1^{Cre};R26^{iTbx1/+}* ventral 3rd pp demonstrates that ectopic expression of *Tbx1* does not repress or reverse thymus fate, but instead is selectively acting through repression of *Foxn1*.

Ectopic TBX1 expression reduces proliferation of GCM2-negative cells in the ventral 3rd pp

Despite the severely reduced levels of *Foxn1* expression in the 3rd pp of *Foxn1^{Cre};R26^{iTbx1/+}* embryos, thymic primordia formed, detached from the pharynx and migrated to a normal position above the heart in a timely manner (Fig. 3) (Gordon and Manley, 2011). These data are consistent with the conclusion that cells in the ventral 3rd pp remain specified to a thymus fate after ectopic *Tbx1* expression. However, *Foxn1^{Cre};R26^{iTbx1/+}* fetal thymi are severely hypoplastic throughout ontogeny and remain so in the postnatal period (Fig. 3; data not shown). The hypoplastic thymus phenotype is not surprising given that FOXN1 is essential for TEC proliferation (Itoi et al., 2001; Su et al., 2003; Chen et al., 2009; Nowell et al., 2011).

To determine the cellular mechanism(s) by which ectopic TBX1 expression results in thymus hypoplasia, we analyzed the frequency

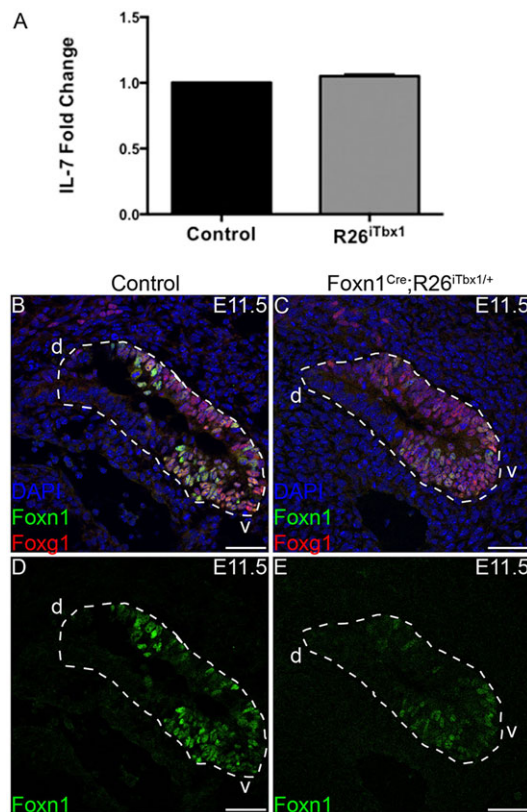


Fig. 2. Ectopic TBX1 does not affect expression of IL7 or FOXG1 in the ventral 3rd pp. (A) Real-time quantitative PCR analysis shows equivalent *Il7* mRNA levels in the 3rd pp dissected from E11.5 control or *Foxn1^{Cre};R26^{iTbx1/+}* embryos ($n=6$ control and $n=6$ *Foxn1^{Cre};R26^{iTbx1/+}*). (B) Representative immunohistochemical stain showing a sagittal section of E11.5 3rd pp. FOXG1 (red) and FOXN1 (green) are co-expressed in the ventral (v) domain; DAPI (blue). (C) Representative immunohistochemical stain of a sagittal section of the mutant 3rd pp showing abundant FOXG1 in the ventral domain despite the severe reduction in FOXN1. (D,E) Single-color images of FOXN1 staining. $n=4$ control and $n=4$ *Foxn1^{Cre};R26^{iTbx1/+}* 3rd pps). Scale bars: 50 μm. d, dorsal.

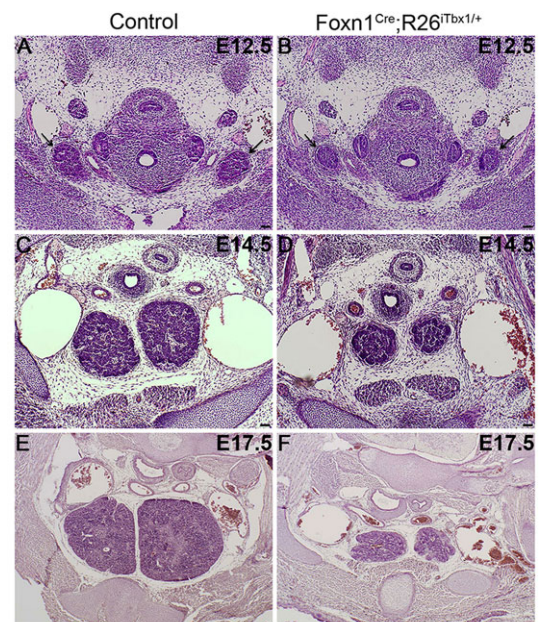


Fig. 3. Thymic lobes are hypoplastic throughout ontogeny in *Foxn1^{Cre};R26^{iTbx1/+}* embryos. (A–F) Hematoxylin and Eosin-stained transverse sections show that *Foxn1^{Cre};R26^{iTbx1/+}* thymic lobes (arrows) are smaller than controls at each developmental stage. (A,B) E12.5; (C,D) E14.5; (E,F) E17.5. $n=5$ control and $n=5$ *Foxn1^{Cre};R26^{iTbx1/+}* 3rd pps. Scale bars: 50 μm.

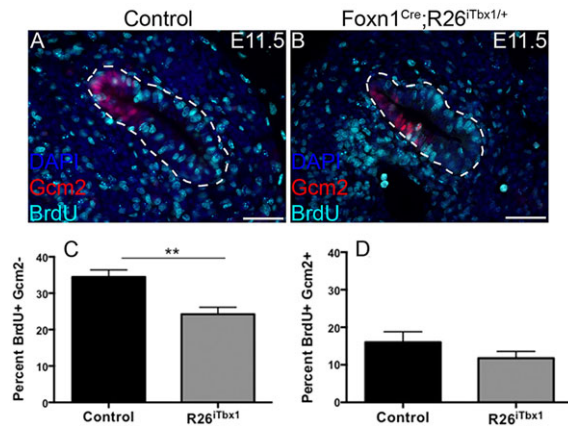


Fig. 4. Proliferation is reduced in the *Foxn1*^{Cre};*R26*^{iThx1/+} thymus-fated 3rd pp domain. (A,B) BrdU (10 mg/ml) was injected into pregnant females 1.5 h prior to obtaining E11.5 embryos. Immunohistochemical staining for BrdU (teal) identifies proliferating cells in the GCM2-positive (red), parathyroid-fated domain and the GCM2-negative thymus-fated domain of the 3rd pp from control (A) and *Foxn1*^{Cre};*R26*^{iThx1/+} (B) embryos. Scale bars: 50 μ m. (C,D) Bar graphs showing the frequency of BrdU-labeled cells that are GCM2 negative or GCM2 positive. Data show percentage \pm s.d. for eight control and eight *Foxn1*^{Cre};*R26*^{iThx1/+} 3rd pps. ***P* < 0.005.

of proliferating and apoptotic cells in the ventral domain of the 3rd pp. Owing to the paucity of FOXN1-positive cells caused by ectopic TBX1, we determined the percentage of BrdU-incorporating cells in GCM2-positive compared with GCM2-negative 3rd pp domains. Ectopic *Tbx1* expression decreased the frequency of proliferating cells in the GCM2-negative domain (Fig. 4A–C). By contrast, we found no difference in the frequency of proliferating GCM2-positive cells. To determine whether ectopic *Tbx1* expression enhanced apoptosis in the 3rd pp, we stained sections for cleaved caspase 3. No difference was observed in the frequency of apoptotic 3rd pp cells in *Foxn1*^{Cre};*R26*^{iThx1/+} compared with control embryos (data not shown). These data demonstrate that TEC proliferation, but not survival, is compromised by ectopic expression of *Tbx1* in the thymus-fated domain.

TEC differentiation is impaired by ectopic TBX1 expression

Given that FOXN1 is required for TEC differentiation (Blackburn et al., 1996; Bleul et al., 2006; Chen et al., 2009; Corbeaux et al., 2010; Nowell et al., 2011), we predicted that this process would be impaired in *Foxn1*^{Cre};*R26*^{iThx1/+} thymi. To test this hypothesis, we first assessed expression of MHC Class II (MHCII), an early

differentiation marker that is expressed on wild-type TECs beginning at E12.5 in a *Foxn1*-dependent manner (Chen et al., 2009; Shakib et al., 2009; Xiao and Manley, 2010; Nowell et al., 2011). Whereas most TECs in E15.5 control thymi expressed high levels of MHCII, there were five to ten times fewer MHCII^{hi} TECs in *Foxn1*^{Cre};*R26*^{iThx1/+} mutants, indicating an early block in TEC differentiation (Fig. 5A). We isolated TECs by cell sorting into MHCII^{hi} and MHCII^{neg-lo} populations for qRT-PCR analysis using the sort gates shown in Fig. 5A. As previously reported (Chen et al., 2009), MHCII^{hi} TECs from controls expressed higher levels of *Foxn1* transcripts than MHCII^{neg-lo} TECs (Fig. 5B). Neither TEC subset isolated from control thymi expressed significant levels of *Tbx1* (Fig. 5C). By contrast, both MHCII^{hi} and MHCII^{neg-lo} TECs from *Foxn1*^{Cre};*R26*^{iThx1/+} thymi expressed very low levels of *Foxn1* and high levels of *Tbx1* mRNA (Fig. 5B,C). These data are consistent with the notion that TBX1 suppression of *Foxn1* causes decreased TEC differentiation.

FOXN1-positive TECs express cTEC and mTEC markers, and segregate from TBX1-positive TECs

By E15.5, a small number of FOXN1-positive TECs were present in the *Foxn1*^{Cre};*R26*^{iThx1/+} thymi; these TECs had low TBX1 levels, presumably owing to downregulation of or failure to activate *R26*^{iThx1}. The small number of FOXN1-positive TECs in the E15.5 mutant thymus localize predominantly to the central region of each lobe (Fig. 6B), whereas FOXN1-expressing TECs are distributed throughout control thymi (Fig. 6A). The majority of TECs in E15.5 *Foxn1*^{Cre};*R26*^{iThx1/+} thymic lobes continued to express high levels of TBX1 and low to undetectable levels of FOXN1. These cells were concentrated towards the outer region of each lobe (Fig. 6B). A similar pattern of TBX1 and FOXN1 expression was observed at E17.5 (supplementary material Fig. S3).

To determine whether TECs in *Foxn1*^{Cre};*R26*^{iThx1/+} thymi were committed to either cTEC or mTEC lineages, we analyzed additional lineage-specific TEC markers. The thymoproteosome subunit β 5t, as well as the surface receptor CD205, are expressed in a FOXN1-dependent manner at early stages of cTEC differentiation and are therefore expressed poorly or not at all in the *Foxn1* null (nude) thymus (Nowell et al., 2008; Shakib et al., 2009; Ripen et al., 2011). As expected, the majority of cTECs in the control thymus were positive for both CD205 and β 5t (Fig. 6C,E). By contrast, only a subset of TECs in *Foxn1*^{Cre};*R26*^{iThx1/+} thymi expressed β 5t or CD205. TECs expressing these markers were primarily found in the center of each lobe, where FOXN1-positive cells were also located (Fig. 6D,F).

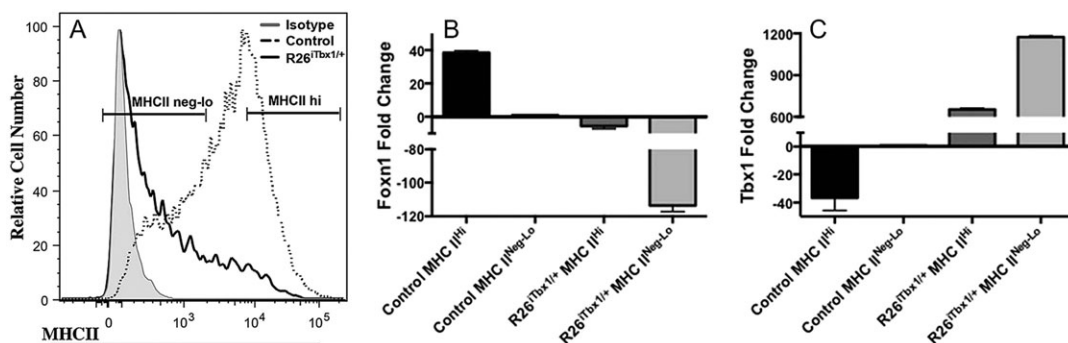


Fig. 5. Ectopic TBX1 blocks TEC differentiation and suppresses *Foxn1* expression. (A) Flow cytometric analysis of MHC class II expression on EpCAM⁺ CD45[−] control and *Foxn1*^{Cre};*R26*^{iThx1/+} TECs. The majority of control TECs are MHCII^{hi}, whereas most *Foxn1*^{Cre};*R26*^{iThx1/+} TECs are MHCII^{neg-lo}. (B,C) Representative bar graphs of qRT-PCR analysis showing *Foxn1* (B) or *Tbx1* (C) expression in E15.5 control and *Foxn1*^{Cre};*R26*^{iThx1/+} TECs. MHCII^{hi} and MHCII^{neg-lo} were obtained by FACS sorting using the indicated gates. Data are mean \pm s.d. for six control and six *Foxn1*^{Cre};*R26*^{iThx1/+} experiments.

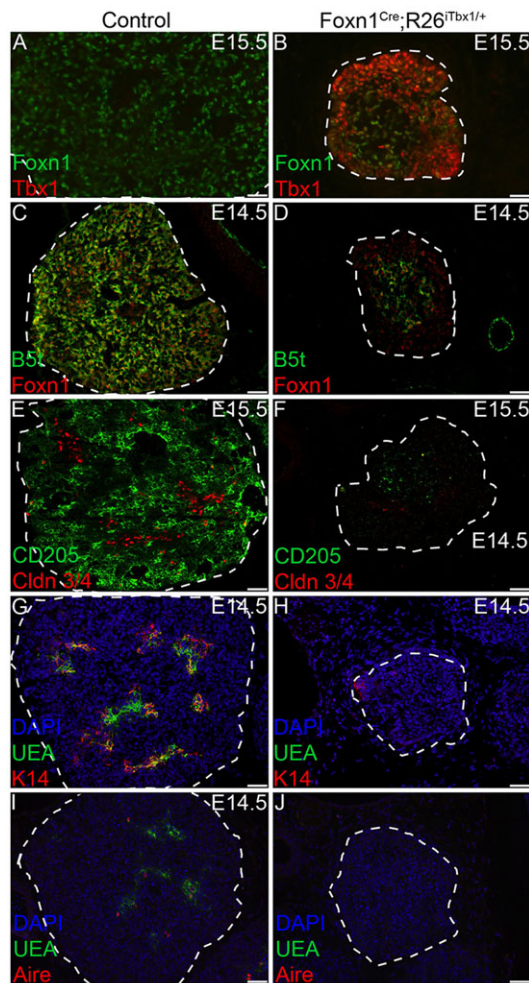


Fig. 6. A small number of FOXN1-positive TECs are present in the center of *Foxn1^{Cre};R26^{iTBx1/+}* fetal thymic lobes. (A–J) Representative immunohistochemical stains of transverse sections from control and *Foxn1^{Cre};R26^{iTBx1/+}* E14.5 or E15.5 thymic lobes show distinct patterns of TEC localization. (A) FOXN1-positive (green) TECs are dispersed throughout the control thymus and TBX1-positive cells are not found. (B) The small number of FOXN1-positive TECs (green) in *Foxn1^{Cre};R26^{iTBx1/+}* mutants localize to the center of each lobe and generally segregate away from the abundant TBX1-positive TECs (red) that are concentrated in the outer region of the each lobe. (C–F) Only a few of the mutant TECs are positive for β 5t and for CD205, which mark the cTEC lineage (green); these cells localize primarily in the center of each lobe where FOXN1-positive cells are found (see B). Scale bars: 50 μ m.

K14 expression and binding of the lectin *Ulex europaeus* agglutinin 1 (UEA1) are markers that distinguish mTEC subsets (Klug et al., 1998). Both K14-positive and UEA1-binding mTECs were present in the developing medullary regions of control thymus (Fig. 6G,I). However, only exceedingly rare cells expressed either of these mTEC markers in the *Foxn1^{Cre};R26^{iTBx1/+}* thymic lobes (Fig. 6H,J). Furthermore, the mutant TECs failed to express the nuclear protein AIRE (an autoimmune regulator), which regulates transcription of tissue restricted antigens and is a marker for mTEC maturation (Fig. 6J) (Gillard et al., 2007; Org et al., 2008; Gardner et al., 2009). Claudin (CLDN) 3 and CLDN4 are expressed on TEC precursors that are committed to the mTEC lineage (Hamazaki et al., 2007). There was a paucity of CLDN3/4-positive TECs in *Foxn1^{Cre};R26^{iTBx1/+}* thymi (Fig. 6F). Interestingly, the few CLDN3/4 positive precursors were positioned towards the outer region of the thymic lobes where TECs expressing higher levels of TBX1 and lower

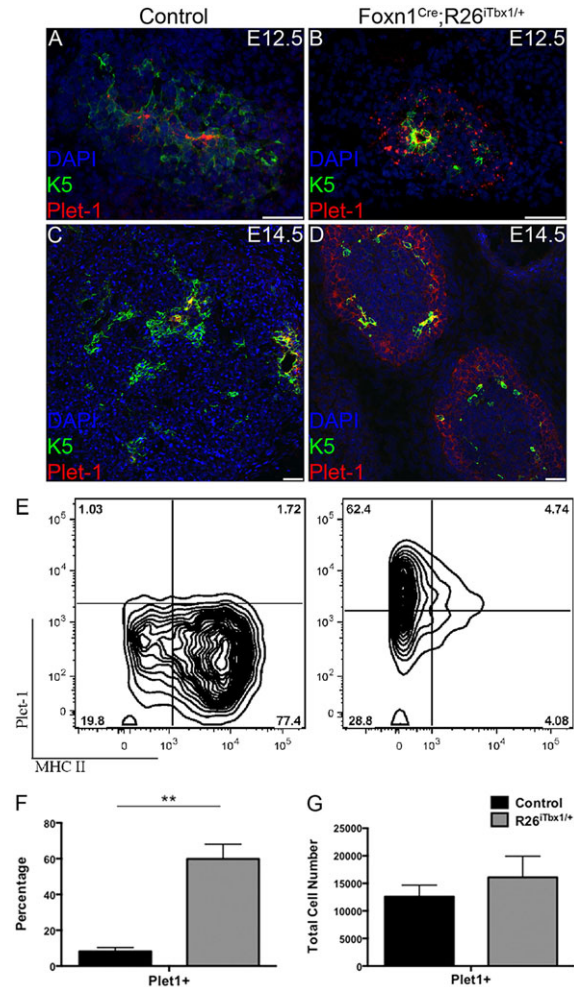


Fig. 7. *Foxn1^{Cre};R26^{iTBx1/+}* fetal thymi contain a high frequency of TEC progenitors. (A–D) Representative immunohistochemical stains of transverse sections from control and *Foxn1^{Cre};R26^{iTBx1/+}* E12.5 or E14.5 thymic lobes showing PLET1- (red) and K5- (green) positive cells. Note the high frequency of PLET1-positive TEC progenitors in the outer region of *Foxn1^{Cre};R26^{iTBx1/+}* thymic lobes (D). Scale bars: 50 μ m. (E) FACS plots showing MHC II and PLET1 expression on EpCAM+ CD45- control and *Foxn1^{Cre};R26^{iTBx1/+}* TECs. Numbers in each quadrant show percentage of cells. (F, G) The frequency (F) or number (G) of PLET1-positive cells in E14.5 control and *Foxn1^{Cre};R26^{iTBx1/+}* thymic lobes. Data are mean \pm s.d. for four control and four *Foxn1^{Cre};R26^{iTBx1/+}* thymic lobes. ** $P < 0.001$.

levels of FOXN1 are found. Taken together, these data suggest that ectopic TBX1 compromises differentiation of both cTEC and mTEC lineages.

Ectopic TBX1 results in an accumulation of early TEC progenitors

PLET1 is a cell-surface protein expressed by founder cells in the 3rd pp that give rise to both cTEC and mTEC lineages (Bennett et al., 2002; Gill et al., 2002; Bleul et al., 2006; Rossi et al., 2006; DePreter et al., 2008). A relatively high frequency of TECs in both *Foxn1^{Cre};R26^{iTBx1/+}* and control thymic rudiments express PLET1 at E12.5 (Fig. 7A,B), consistent with previously published data (Nowell et al., 2011). The frequency of PLET1-positive TECs decreases during ontogeny as TECs undergo differentiation and proliferation (Nowell et al., 2011). Thus, PLET1 expression is confined to a rare subset of TECs in control thymi by E14.5 (Fig. 7C). In striking

contrast, a higher frequency of PLET1-positive cells persists at E14.5 in the *Foxn1^{Cre};R26^{iTbx1/+}* thymi (Fig. 7D). Furthermore, the PLET1-positive cells in the mutant thymi are found in the outer region of the lobes, similar to the localization pattern observed for TBX1-expressing cells (Fig. 7D). These data are consistent with ectopic TBX1 expression blocking TEC differentiation at an early progenitor stage. Flow cytometric analysis confirmed an increased frequency of PLET1-positive cells in E15.5 *Foxn1^{Cre};R26^{iTbx1/+}* compared with control thymi (Fig. 7E). However, the absolute number of PLET1-positive cells was comparable in *Foxn1^{Cre};R26^{iTbx1/+}* and control thymi (Fig. 7F,G). The maintenance of the same number of PLET1-positive TECs in the hypoplastic *Foxn1^{Cre};R26^{iTbx1/+}* thymi at late stages of ontogeny is more consistent with TBX1 blocking differentiation of existing progenitors, rather than reverting differentiated TECs to a progenitor state.

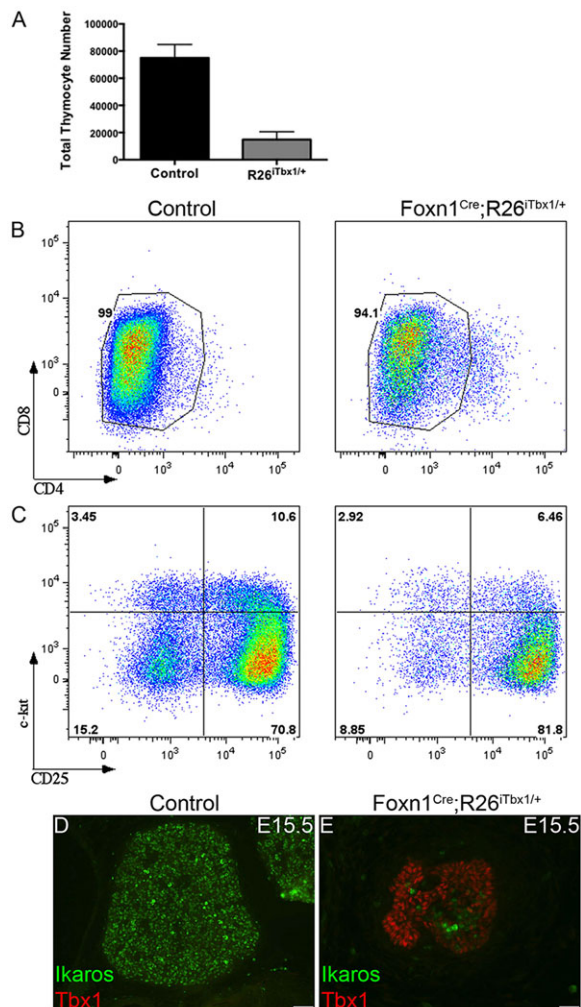


Fig. 8. Thymocyte localization, cellularity and development are aberrant in *Foxn1^{Cre};R26^{iTbx1/+}* fetal thymi. (A) The number \pm s.d. of thymocytes recovered from control ($n=2$) and *Foxn1^{Cre};R26^{iTbx1/+}* ($n=3$). (B) FACS plots showing that most E15.5 thymocytes are CD4 or CD8 double-negative (DN) cells. (C) FACS plots showing the distribution of DN subsets based on KIT and CD25 expression. There is a partial block at the DN3 (KIT⁺CD25⁺) to DN4 (KIT⁺CD25⁺) developmental transition in *Foxn1^{Cre};R26^{iTbx1/+}* thymocytes. (D,E) Representative immunohistochemical stains of transverse sections from control and *Foxn1^{Cre};R26^{iTbx1/+}* E15.5 thymic lobes show that thymocytes identified as Ikaros positive (green) are scattered through control thymic lobes, but are restricted to the central region of mutant lobes where FOXN1-positive cells are found (see Fig. 6B). Scale bars: 50 μ m.

Thymocyte development and localization is altered in the *Foxn1^{Cre};R26^{iTbx1/+}* thymic microenvironment

TECs are required for thymocyte proliferation, survival and differentiation (Ritter and Boyd, 1993; van Ewijk et al., 1994; Nitta et al., 2011; Anderson and Takahama, 2012). Given the profound arrest in TEC development resulting from ectopic *Tbx1* expression, we expected to find defects in thymocyte development. Total thymocyte cellularity was decreased by ~ 5 -fold in E15.5 *Foxn1^{Cre};R26^{iTbx1/+}* compared with control thymi (Fig. 8A). Flow cytometric analyses were performed to assess thymocyte differentiation. At this developmental stage, thymocytes have not yet differentiated beyond the immature CD4 and CD8 double-negative (DN) precursor subset (Fig. 8B). Expression of KIT and CD25 distinguishes DN subsets at sequential maturation stages, referred to as DN1–DN4. Flow cytometric analysis revealed a partial block at the DN3 to DN4 transition in *Foxn1^{Cre};R26^{iTbx1/+}* thymi (Fig. 8C). The reduction in thymocyte cellularity, together with the partial differentiation arrest at the DN3 stage, suggest that the aberrant TEC microenvironment in *Foxn1^{Cre};R26^{iTbx1/+}* thymi fails to provide adequate growth and differentiation signals to support normal thymocyte development. IHC analysis of E14.5 thymi revealed that thymocytes, identified by expression of the Ikaros (IKZF1) transcription factor (Georgopoulos et al., 1992), were distributed throughout the control thymic lobes (Fig. 8D). By contrast, the scarce thymocytes present in *Foxn1^{Cre};R26^{iTbx1/+}* fetal thymus lobes were concentrated in the central region in juxtaposition to TECs expressing higher levels of FOXN1 (compare Fig. 6B with Fig. 8E). Similar colocalization of thymocytes and TECs expressing low levels of FOXN1 was observed in the center of E17.5 mutant thymic lobes (supplementary material Fig. S3). This spatial relationship emphasizes the importance of TEC-thymocyte crosstalk in T-cell development.

DISCUSSION

These results clearly demonstrate that ectopic expression of *Tbx1* in the thymus-fated domain of the 3rd pp suppresses *Foxn1* expression and inhibits TEC proliferation and differentiation. These data directly contradict the prevailing model in which TBX1 is required for thymus development. Our data, together with the downregulation of *Tbx1* that occurs in the ventral domain of the wild-type 3rd pp (Vitelli et al., 2002; Manley et al., 2004; Liu et al., 2007), strongly support the conclusion that TBX1 is a negative regulator of thymus development.

Ectopic expression of TBX1 in ventral 3rd pp endoderm suppresses FOXN1

All of the defects in TEC maturation and proliferation observed in *Foxn1^{Cre};R26^{iTbx1/+}* thymi are consistent with the suppression of *Foxn1* expression, which is known to promote these processes. Downregulation of *Foxn1* occurred as early as E11.5, almost immediately upon ectopic expression of *Tbx1*. The inverse pattern of FOXN1 and TBX1 protein levels in E15.5 *Foxn1^{Cre};R26^{iTbx1/+}* TECs is further evidence that TBX1 negatively regulates *Foxn1* expression. TBX1 may shut down *Foxn1* expression by an indirect, rather than direct, mechanism, as there is not an obvious TBX-binding site in the *Foxn1* promoter (BLAT; UCSC Genome Bioinformatics). These findings are consistent with other work showing that ectopic activation of SHH signaling in 3rd pp endoderm both induces *Tbx1* expression and represses *Foxn1* (V.E.B. and N.R.M., unpublished).

The few FOXN1 positive TECs present in these mutants appear to be the progeny of 3rd pp progenitors that failed to effectively express ectopic TBX1, as these cells have very low or undetectable levels of TBX1. This failure could be due to inefficient or late

deletion of the stop codon in the $R26^{iThx1}$ allele, or due to secondary shutdown of the *Rosa26* locus. These FOXN1-positive cells tend to aggregate in the center of the lobe, forming a microenvironment that supports differentiation of a small number of thymocytes, with variable efficiency.

Ectopic TBX1 expression does not reverse thymus fate

By E11.5, most cells in the wild-type 3rd pp have acquired either a thymus or parathyroid fate, and express either *Foxn1* or *Gcm2* (Gordon et al., 2001). By contrast, the reduction of FOXN1-positive cells in the *Foxn1^{Cre};R26^{iThx/+}* 3rd pp, coupled with the lack of a compensatory expansion of *Gcm2* expression, results in a sizeable domain containing cells that fail to express either marker. However, as *Foxn1* is not required to establish thymus fate, these cells could still retain thymus fate specification. This appears to be the case, as these ventral cells do express *Il7* and FOXG1, both of which are expressed by thymus-fated cells independently of *Foxn1*-mediated regulation (Zamisch et al., 2005; Wei and Condie, 2011) (B. G. Condie, personal communication). These data also demonstrate that ectopic *Tbx1* cannot reverse thymus fate after it is established, as *Foxn1^{Cre}* is expressed in 3rd pp cells that are already committed to a thymus fate. The downregulation of *Tbx1* in the ventral domain during pouch outgrowth at E9–9.5, prior to expression of thymus-specific markers, raises the possibility that this downregulation is essential to establish thymus fate. Activation of $R26^{iThx1}$ in 3rd pp endoderm at an earlier developmental stage will be required to test whether TBX1 can block initial thymus fate commitment.

Ectopic TBX1 expression arrests TEC differentiation at an early progenitor stage

TEC differentiation is arrested at a PLET1-positive stage in *Foxn1*-null (nude) mutants (Blackburn et al., 1996; Depreter et al., 2008). *Plet1*-expressing TECs have been shown to contain a TEC progenitor activity in transplant experiments at both early and later stages of fetal thymus development (Bennett et al., 2002; Gill et al., 2002). These data strongly suggest that PLET1 likely marks a very early stage of committed thymus progenitor cells (reviewed by Manley and Condie, 2010). The loss of FOXN1 in *Foxn1^{+Cre};R26^{iThx1}* fetal thymi was associated with a marked increase in the frequency of PLET1-positive TECs. However, the number of PLET1-positive TECs was comparable in mutant and control fetal thymi. These data indicate that ectopic TBX1 results in arrested differentiation of PLET1-positive progenitors, rather than induction of their proliferation or reversal of a differentiated phenotype to a progenitor-like state.

Ectopic TBX1 expression uncouples FOXN1 and MHC Class II expression

Previous studies have suggested that *Foxn1* directly or (more likely) indirectly regulates expression of MHC Class II genes. MHC Class II levels are severely downregulated on TECs from *Foxn1^{ΔΔ}* mutants (Tennant et al., 1982; Chen et al., 2009). Furthermore, a direct relationship was observed between the frequency of MHC Class II-positive TECs and *Foxn1* levels in an allelic series of strains expressing progressively lower levels of *Foxn1* (Nowell et al., 2011). Therefore, we were surprised to find that the minor fraction of MHCII^{hi} *Foxn1^{Cre};R26^{iThx/+}* TECs expressed exceedingly low levels of *Foxn1* mRNA. The mechanism responsible for this finding is not clear, but this result does indicate that FOXN1 levels alone are not sufficient to determine the levels of MHC Class II genes. It is possible that ectopic *Tbx1* in *Foxn1^{Cre};R26^{iThx/+}* mutants disrupts the normal regulatory pathway linking *Foxn1* and MHC Class II

genes. Regardless of the mechanism, *Foxn1* and MHC Class II gene expression are uncoupled by ectopic *Tbx1* expression in TECs.

Ectopic TBX1 expression does not induce GCM2 expression

GCM2 is a transcription factor that marks the parathyroid-fated domain of the 3rd pp and is required for parathyroid survival and differentiation (Gunther et al., 2000; Gordon et al., 2001; Liu et al., 2007). *Tbx1* and *Gcm2* expression domains overlap in the dorsal 3rd pp, and previous reports have suggested that *Gcm2* may be a downstream target of *Tbx1*, based on microarray data in *Tbx1*-null mutants (Ivins et al., 2005) or on the persistence of *Tbx1* expression in *Gcm2*-null mutants (Liu et al., 2007). However, our data show that ectopic expression of TBX1 was not sufficient to induce an expansion of *Gcm2* expression into the ventral 3rd pp. This result is consistent with the finding that expression of a constitutively active allele of *Smo* throughout the 3rd pp expanded the *Tbx1* expression domain ventrally, but did not expand *Gcm2* expression (V.E.B. and N.R.M., unpublished). Thus, activation of additional positive regulators and/or suppression of negative regulators must be involved in regulating parathyroid fate specification and/or *Gcm2* expression during 3rd pp patterning.

MATERIALS AND METHODS

Mice

We generated a conditional *Tbx1* knock-in strain by targeting a *Tbx1* inducible expression cassette to the endogenous *Rosa26* locus to generate a $R26^{iThx1}$ allele. In this *Tbx1* inducible cassette, a full-length mouse *Tbx1* cDNA and an IRES-GFP were inserted downstream of a floxed stop cassette (vector provided by Dr A. McMahon). Expression of the $R26^{iThx1}$ allele was activated in TECs by crossing $R26^{iThx1}$ females with *Foxn1^{Cre}* males (Gordon et al., 2007). $R26^{iThx1/+}$ and $R26^{+/+}$ littermate controls were maintained on a mixed C57Bl/6 genetic background at the MD Anderson Cancer Center Science Park in accordance with the guidelines set forth by the Association for the Accreditation of Laboratory Animal Care. Timed pregnancies were set up in the evening and monitored daily thereafter. The day of vaginal plug was considered embryonic day (E) 0.5. Morphological cues were used to determine embryonic stage. Yolk sac DNA was genotyped using *Rosa26* forward 5'-TTGCAATACCTTTCTGGGAGTT-3', *Rosa26* reverse 5'-AACCCAGATGACTCCTATCCT-3' and β-galactosidase reverse 5'-GACAGTATCGGCCTCAGGAAG-3'. All experiments were performed in accordance with the MD Anderson Animal Care and Use Committee.

Histochemistry and immunohistochemistry

Embryos harvested for frozen sections were fixed in 4% PFA for 15 min (E11.5) or 30 min E13.5–15.5, washed in PBS, dehydrated in sucrose and embedded in OCT. Serial sections (7 μm) were air dried and washed in PBS. Tissue was incubated overnight at 4°C in 120 μl containing primary antibody, 5% donkey serum and 0.05% Triton X-100 in PBS. Slides were washed in PBS and incubated with 120 μl of secondary antibody and 0.05% Triton X-100 in PBS for 1 h at room temperature, in the dark. Slides were washed in PBS, incubated with 4',6-diamidino-2-phenylindole (DAPI) for 3 min at room temperature, and washed in PBS. Slides were mounted with ProLong Gold anti-fade reagent (Life Technologies) and coverslipped for imaging.

Embryos collected for paraffin embedding were fixed for 1 h in 4% PFA, washed in PBS, dehydrated in an ethanol gradient, permeabilized using xylenes and embedded in paraffin. Serial sections were cut at 7 μm on a Leica ThermoShandon Finesse 325 microtome. Tissue was washed in xylene and rehydrated through a methanol gradient to tap water. Immunostaining for paraffin-embedded tissue required antigen retrieval. Paraffin-embedded tissue was incubated in AR buffer [10 mM sodium citrate (pH 6), 0.05% Tween 20] at 95°C for 30 min. Slides were allowed to cool to room temperature. Paraffin-embedded tissue was incubated in primary and secondary antibodies as described above. Paraffin-embedded tissues collected for Hematoxylin and Eosin (H&E) staining were processed according to standard techniques.

Primary antibodies used were goat anti-Foxn1 (1:200, Santa Cruz, G-20), rabbit anti-Tbx1 (1:100, Abcam, ab18530), rabbit anti-Gcm2 (1:200, Abcam, ab64723), rabbit anti-Foxg1 (1:50, Abcam, ab18259), rat anti-BrdU (1:10, Serotec, OBT0030S), goat anti- β 5t (1:200, MBL, PD021), rat anti-Ly75 (1:100, Abcam, ab51820), K14 (1:400, Covance, PRB-155P-100), biotinylated UEA1 (1:200, Vector, B-1065), rabbit anti-Aire-1 (1:50, Santa Cruz, M-300), rabbit anti-claudin 3 and anti-claudin 4 (1:200, Invitrogen, 341700 and 364800), K5 (1:400, Covance, PRB-160P-100), PLET1 (kindly provided by Dr Clare Blackburn, University of Edinburgh), and Ikaros (1:100, Santa Cruz, M-20). All secondary antibodies were conjugated with DyLight 488 (705-546-147), Dylight 594 (711-586-152), Cy3 (711-166-152) or Cy5 (712-176-153) (Jackson ImmunoResearch, 1:400).

BrdU (300 μ l of 10 mg/ml) was injected by the intraperitoneal (IP) route into pregnant females and embryos were harvested after a 90 min chase. Cells from serial sections were counted based on expression of FOXN1, GCM2, TBX1 and/or BrdU using Cell Profiler (Broad Institute) or Imaris (Bitplane).

Flow cytometry

For TEC analysis, E15.5 thymi were digested with 0.125% collagenase (Sigma) and 0.1% DNase (Roche) as previously described (Gray et al., 2002). For analysis of thymocyte subsets, E15.5 thymi were pressed through a 70 μ m strainer (Fisher). Cells were stained with fluorochrome-conjugated antibodies in FACS buffer [PBS (pH 7.2), 0.005 M EDTA, 2% FBS] for 20 min on ice and washed with FACS buffer. Propidium iodide (Invitrogen) was added (0.5 μ g/ml) to each sample prior to analysis to exclude dead cells. Anti-CD326 (clone G8.8) conjugated to PE/Cy7; anti-I-A/I-E (clone M5/114.15.2) and anti-CD25 (clone PC61) conjugated to Pacific Blue; anti-CD326 (clone G8.8) conjugated to allophycocyanin; and anti-CD117 (clone 2 B8) conjugated to allophycocyanin-Cy7 were purchased from BioLegend. Anti-CD44 (clone IM7) conjugated to allophycocyanin; anti-CD8 α (clone 53-6.7) conjugated to PE-Cy7; and anti-CD45 (clone 30-F11) conjugated to PerCP-Cy5.5 were purchased from eBioscience. Anti-CD4 (clone RM4-5) conjugated to Qdot 605 was purchased from Invitrogen. To exclude erythrocytes, granulocytes, dendritic cells, macrophages and NK cells from the thymocyte analyses, the following antibodies, all conjugated to PE-Cy5 were purchased from eBioscience: TER-119, CD11c (clone N418, 0.17 μ g/ μ l), CD11b (clone M1/70, 0.17 μ g/ μ l), NK-1.1 (clone PK136, 0.17 μ g/ μ l) and Ly-6G (clone RB6-8C5, 0.17 μ g/ μ l). Cells were analyzed or sorted on a FACS Aria II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

RNA isolation, cDNA synthesis and real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

FACS-sorted R26^{Tbx1} and control TECs from a given litter were pooled and lysed in Trizol (Ambion). RNA was isolated using chloroform and linear acrylamide (Ambion) and RNA was resuspended in DEPC water until ready for cDNA synthesis. First-strand cDNA was synthesized using a SuperScript III First-Strand cDNA synthesis kit (Invitrogen). For quantitative reverse transcriptase PCR (qRT-PCR) analysis of *Foxn1*, *Tbx1* and α -tubulin (endogenous control), we used TaqMan Gene Expression Master Mix (Applied Biosystems) and TaqMan primers (Applied Biosystems) in a LightCycler 480 (Roche). In each experiment, comparators were set to a value of 1. All experiments were repeated three times and analyzed using the $\Delta\Delta C_t$ method.

Statistics

Prism 6.0 (GraphPad version 6.0b) and Microsoft Excel were used for statistical analyses. We used a Student's *t*-test to evaluate differences between controls and mutants regarding the number of cells expressing FOXN1, GCM2, TBX1 and/or BrdU. *P*<0.05 was considered statistically significant.

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Competing interests

The authors declare no competing financial interests.

Author contributions

K.A.G.R. and K.T.C. designed and performed the experiments and analyzed the data under the supervision of E.R.R.; V.E.B. confirmed results and contributed images to Figs 2 and 6; Z.L. and M.L. assisted in making the construct and generating and testing the knock-in mice; E.R.R. and N.R.M. conceived the project; K.A.G.R., E.R.R. and N.R.M. wrote and edited the manuscript.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.111641/-/DC1>

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