Regeneration of the aged thymus by a single transcription factor

Nicholas Bredenkamp*, Craig S. Nowell‡ and C. Clare Blackburn§

ABSTRACT

Thymic involution is central to the decline in immune system function that occurs with age. By regenerating the thymus, it may therefore be possible to improve the ability of the aged immune system to respond to novel antigens. Recently, diminished expression of the thymic epithelial cell (TEC)-specific transcription factor Forkhead box N1 (FOXN1) has been implicated as a component of the mechanism regulating age-related involution. The effects of upregulating FOXN1 function in the aged thymus are, however, unknown. Here, we show that forced, TEC-specific upregulation of FOXN1 in the fully involuted thymus of aged mice results in robust thymus regeneration characterized by increased thymopoiesis and increased naive T cell output. We demonstrate that the regenerated organ closely resembles the juvenile thymus in terms of architecture and gene expression profile, and further show that this FOXN1-mediated regeneration stems from an enlarged TEC compartment, rebuilt from progenitor TECs. Collectively, our data establish that upregulation of a single transcription factor can substantially reverse age-related thymic involution, identifying FOXN1 as a specific target for improving thymus function and, thus, immune competence in patients. More widely, they demonstrate that organ regeneration in an aged mammal can be directed by manipulation of a single transcription factor, providing a provocative paradigm that may be of broad impact for regenerative biology.

KEY WORDS: Thymic involution, FOXN1, Organ regeneration, Mouse

INTRODUCTION

The thymus, the obligate site of T lymphocyte development (Miller, 1961), is one of the first organs to degenerate in normal healthy individuals (Chinn et al., 2012). This process, termed 'age-related thymic involution', results in decreased production of naive T cells with age (Weng, 2006; Lynch et al., 2009; Chinn et al., 2012). This reduced output of naive T cells severely impairs the immune response to newly encountered antigens, making thymic involution a major cause of the age-related decline in immune system function. Thymic involution also results in an impaired capacity to recover adaptive immunity following immune depletion in patients (Holland and van den Brink, 2009; Lynch et al., 2009). The capacity to increase thymus function would therefore be beneficial

Medical Research Council Centre for Regenerative Medicine, Institute for Stem Cell Research, School of Biological Sciences, University of Edinburgh, SCRM Building, 5 Little France Drive, Edinburgh EH16 4UU, UK.
*Present address: Wellcome Trust - Medical Research Council Stem Cell Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK. ‡Present address: Ecole Polytechnique Fédérale de Lausanne, Swiss Institute for Experimental Cancer Research, Lausanne CH-1015, Switzerland.

§Author for correspondence (c.blackburn@ed.ac.uk)

in a wide variety of clinical settings, and consequently a number of strategies aimed at regenerating the aged thymus are currently under investigation (van den Brink et al., 2004; Holland and van den Brink, 2009; Markert et al., 2009).

The epithelial component of the thymic stroma is essential for intrathymic T cell development and undergoes a stereotypical age-related degeneration that is strongly implicated as a cause of age-related thymic involution (Mackall et al., 1998; Gray et al., 2006; Williams et al., 2008). The key transcription factor FOXN1 (Nehls et al., 1996) is crucially required throughout thymic epithelial cell (TEC) differentiation in the fetal and postnatal thymus (Chen et al., 2009; Nowell et al., 2011) and is downregulated with age in the thymic stroma (Ortman et al., 2002; Chen et al., 2009; Zook et al., 2011). Forced downregulation of *Foxn1* in the perinatal thymic epithelium results in loss of thymus homeostasis (Chen et al., 2009), while overexpression in young mice delays thymus degeneration (Zook et al., 2011). FOXN1 is thus implicated as one of the primary targets in age-related thymic involution.

Sex-steroid signaling is also thought to be an important regulator of involution. However, castration-induced thymic rebound was recently demonstrated to reflect enlargement of a thymus with an aged phenotype rather than restoration of the functionality and architecture of the young organ (Griffith et al., 2012). Therefore, the clinically important question of whether the effects of established age-related thymic involution can be reversed to drive rejuvenation of the fully involuted, aged thymus remains unanswered.

We have developed a novel transgenic model for conditional, inducible upregulation of FOXN1 function, and have used this to test the outcome of upregulating FOXN1 specifically in TECs in the fully involuted thymus. Our data establish that enhanced activity of this single transcription factor is sufficient to regenerate the fully involuted thymus such that its architecture, gene expression profile and function are restored to those characteristic of the juvenile organ. They further establish that this FOXN1-mediated thymic regeneration stems from proliferation of progenitor TECs.

RESULTS

A transgenic model for conditional inducible *Foxn1* expression

To test the hypothesis that upregulation of *Foxn1* in the aged, fully involuted thymus might reverse age-related thymic involution, we generated a transgenic mouse model that permits conditional, inducible overexpression of *Foxn1*, through production of a tamoxifen-inducible form of mouse FOXN1, FOXN1ER^{T2} (*ROSA26$^{CAG-STOP-Foxn1ERT2-IRES-GFP}$* mice, referred to as R26Foxn1ER; Fig. 1A; supplementary material Figs S1 and S2). When induced by tamoxifen, this FOXN1ER^{T2} protein showed equivalent activity to native FOXN1 in a minimal responsive element reporter assay (supplementary material Fig. S3A) previously utilized to validate the activity of another FOXN1ER protein (Janes et al., 2004), and could induce expression of known FOXN1 targets to levels equivalent to native FOXN1 (supplementary material Fig. S3B). To activate expression of this transgene specifically in TECs we crossed the R26Foxn1ER
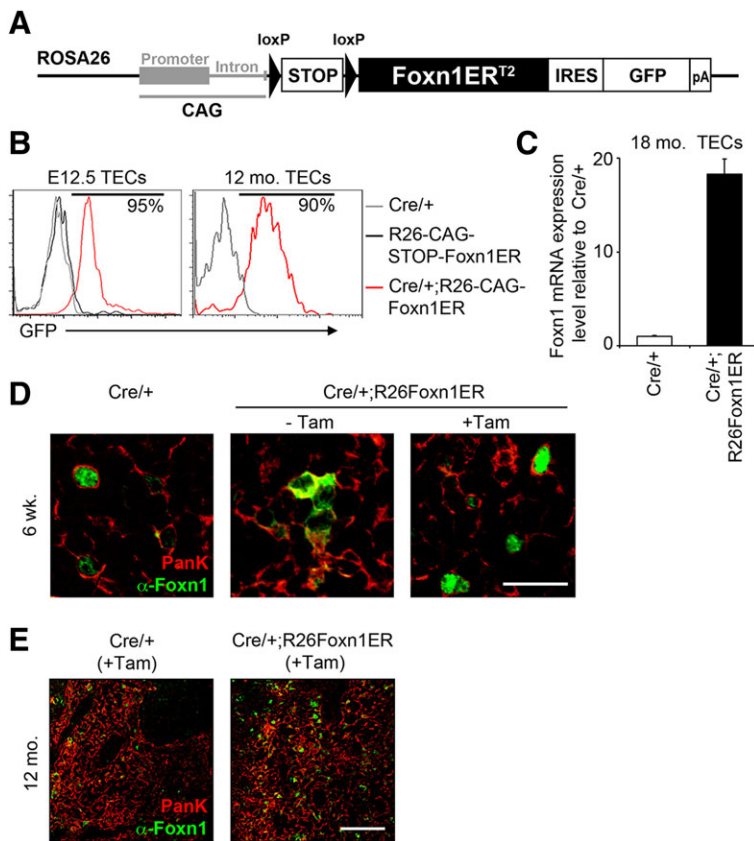
Fig. 1 . Generation of a regulatable FOXN1 mouse model.
(A) Schematic of *Foxn1ER^{T2}* transgene. (B) Plots show
GFP expression reporting *CAG-Foxn1ER* in Cre/+,
ROSA26$^{CAG-STOP-Foxn1ERT2-IRES-GFP/+}$ (STOP-Foxn1ER) and Cre/+;
R26Foxn1ER TECs at the ages shown. (C) *Foxn1* expression in
EpCAM$^+$ TECs in 18-month-old Cre/+ and Cre/+;R26Foxn1ER mice,
shown normalized to the geometric mean of three housekeeping
(3HK) genes and relative to Cre/+. (D) Images show thymi from
6-week-old mice injected with carrier-only or 3 mg tamoxifen, at
2 days post-injection, after staining with the markers shown. Scale
bar: 25 μm. (E) Images show thymi from tamoxifen-treated 12-month-
old mice of the genotypes shown, after staining with the markers
shown. Scale bar: 100 μm. Error bars, s.d. Mo, month. See also
supplementary material Fig. S1. $n \geq 3$ for all experiments.

line with *Foxn1$^{Cre/+}$* (Cre/+) mice (Gordon et al., 2007), generating
ROSA26$^{CAG-Foxn1ERT2-IRES-GFP/+}$; *Foxn1$^{Cre/+}$* (Cre/+;R26Foxn1ER)
mice. Most, if not all, TECs in both fetal and adult Cre/+;
R26Foxn1ER mice expressed *Foxn1ER* as reported by GFP
expression (Fig. 1B), with *Foxn1ER* mRNA highly overexpressed in
aged TECs (Fig. 1C). The tamoxifen inducibility of the FOXN1ER^{T2}
fusion protein in TECs was confirmed by immunohistochemical
(IHC) analysis (Fig. 1D,E) and immunoblotting (supplementary
material Fig. S3C,D). In the absence of tamoxifen, no differences
between control and Cre/+;R26Foxn1ER thymi were observed
at any stage of development (shown for 12-month-old mice in
supplementary material Fig. S4). Collectively, these data validated our
experimental model.

Increased FOXN1 function drives thymus regeneration in aged mice

To determine whether overexpression of FOXN1 in TECs could
reverse established thymus involution in aged mice, we treated 12- and
24-month-old Cre/+;R26Foxn1ER mice with tamoxifen for 1 month
to induce FOXN1ER^{T2} activity. Controls were provided by tamoxifen-
treated Cre/+, and untreated Cre/+;R26Foxn1ER and Cre/+ littermates.
These control groups revealed a mild and transient effect of tamoxifen
treatment on thymocyte number, but no effects on thymic architecture
or TEC subset distribution (supplementary material Fig. S4).
Tamoxifen-induced upregulation of FOXN1 activity at both 12 and
24 months of age in Cre/+;R26Foxn1ER mice resulted in an overt
increase in thymus size (Fig. 2A). Total thymocyte numbers increased
more than 2.5-fold (Fig. 2B), with a proportional increase in the major
thymocyte populations defined by expression of the CD4 and CD8
co-receptors (CD4$^+$CD8$^+$ double positive, CD4$^+$ single positive, CD8$^+$
single positive; Fig. 2C,D). The early thymic progenitor cell (ETP; lin$^-$
CD25$^-$ Kit$^+$) population (Allman et al., 2003), which contains the most

immature thymocytes, declines during age-related thymus involution
(Min et al., 2006) and among ETPs, the Flt3$^+$ fraction is considered to
contain canonical intrathymic T cell progenitors (Adolfsson et al.,
2005; Sambandam et al., 2005). This lin$^-$ CD25$^-$ Kit$^+$ Flt3$^+$ population
was also significantly increased (Fig. 2E-G) and the CD4$^-$CD8$^-$
double negative (DN) thymocyte populations defined by CD44 and
CD25 (also known as interleukin 2 receptor alpha chain, IL2ra)
expression were expanded by greater than 2.5-fold relative to controls
(Fig. 2H,I). Within the DN1 population (CD25$^-$CD44$^+$) the number of
B cells (Lin$^-$ B220$^+$; B220 is also known as PTPRC) was reduced by
half (Fig. 2J), suggesting increased commitment into the T cell lineage
(Koch et al., 2008). These data establish that upregulation of FOXN1
activity is sufficient to promote increased thymic function in aged
mice, as shown by expansion of all currently defined thymocyte
populations, including ETPs.

We note that the increase in thymus size reported here, of 2.7-fold
in 12-month-old mice, and 2.6-fold in 24-month-old mice after
1 month of tamoxifen treatment, corresponds well with those
reported for castration-induced or keratinocyte growth factor
(KGF)-induced rebound, which each typically result in a 2- to
3.5-fold increase in thymus size (Olsen et al., 2001; Alpdogan et al.,
2006; Min et al., 2007; Williams et al., 2008; Griffith et al., 2012).

FOXN1 upregulation restores the aged thymic epithelial microenvironment to a pre-involution phenotype

Age-related thymic involution is defined by a stereotypical
deterioration of the thymic epithelial compartment, characterized by
reduced distinction between the cortical and medullary regions;
reduction in the number of medullary islets per thymic lobe (Griffith
et al., 2012); a decline in total TEC number and in the rate of
TEC turnover (Gray et al., 2006); a specific loss of some TEC subsets
(Chen et al., 2009), including a proportional decrease in MHC Class II^{hi}
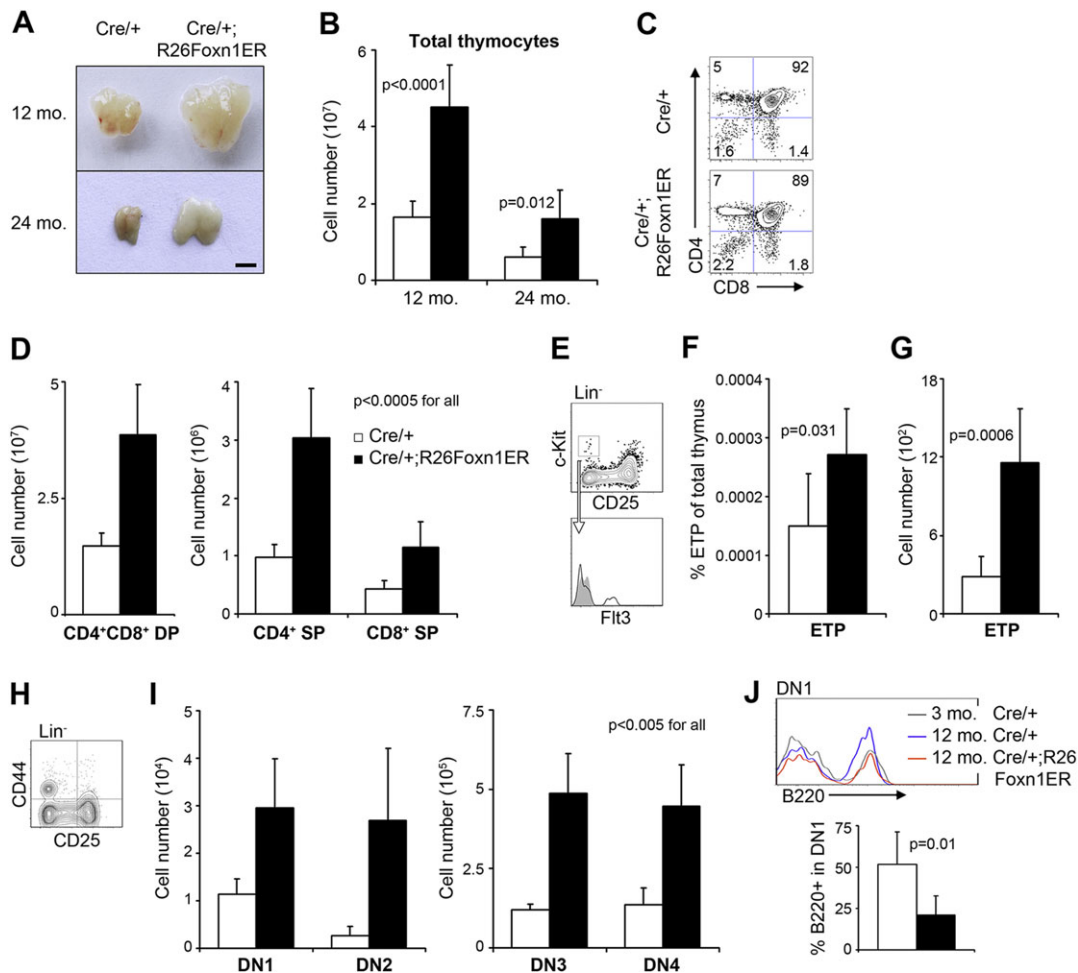
Fig. 2. Induction of FOXN1 drives thymus regeneration in aged mice. Cre/+;R26Foxn1ER and Cre/+ mice (12 and 24 months old) were treated with tamoxifen for 1 month as described. (A) Representative thymic lobes. Scale bar: 2 mm. (B) Total thymocyte number (12 months, n=12; 24 months, n=6). (C) Plots show flow cytometric analysis of CD4 and CD8 staining on thymic cell preparations from 12-month-old mice of the genotypes shown after gating on CD45^{+} cells. (D) Numbers of CD4^{+}CD8^{+} double positive (DP) and CD4^{+} and CD8^{+} single positive (SP) thymocytes in 12-month-old mice (n=10). Numerical fold change: DP, 2.6; CD4 SP, 3.1; CD8 SP, 2.6. (E) Gating strategy for ETPs (lin^{−} CD25^{−} Kit^{+} Flt3^{+}) in 12-month-old mice (lin: CD4, CD8, CD3e, TCRβ, TCRγδ, NK1.1, CD11c, CD11b, Gr-1, Ter119, B220). Filled gray area, isotype control. (F) Frequency of ETPs as a percentage of total thymus cells (n=6). (G) Number of ETPs (n=6). (H) Analysis of double negative (DN) lin^{−} thymocytes using CD44 and CD25. Numerical fold change: 4.1. (I) Enumeration of DN populations (DN1, CD25^{−}CD44^{+}; DN2, CD25^{+}CD44^{+}; DN3, CD25^{+}CD44^{−}; DN4, CD25^{−}CD44^{−}; n=6). Numerical fold change: DN1, 2.6; DN2, 9.9; DN3, 4.1; DN4, 3.3. (J) B cell proportions in the DN1 compartment. Top panel, B220 staining after gating on DN1 cells (lin^{−}CD25^{−}CD44^{+}, lin without B220). Bottom panel, average proportions of B220^{+} cells among DN1 (n=6). Black bars, Cre/+;R26Foxn1ER; white bars, Cre/+ controls, throughout. mo, month. Error bars, s.d.

TECs; and reduced expression of tissue-restricted antigens (Griffith et al., 2012). Therefore, we tested the effect of FOXN1 upregulation on these parameters. In striking contrast to aged-matched controls, the stromal compartment in 12- and 24-month-old tamoxifen-treated Cre/+;R26Foxn1ER thymi showed clearly defined cortical and medullary regions, establishing that upregulation of FOXN1 activity restored thymic epithelial architecture to be near indistinguishable from juvenile controls (Fig. 3A). Thymi from tamoxifen-treated 12-month-old Cre/+; R26Foxn1ER mice also exhibited increased numbers of medullary islets compared with age-matched controls, similar to juvenile thymi (Fig. 3B) (Griffith et al., 2012). The number of EpCAM[+] TECs (Fig. 3C) and both number and proportion of MHC Class II[hi] TEC were significantly increased (Fig. 3C-E). Furthermore, the ratio of medullary TECs (mTECs) to cortical TECs (cTECs), which decreases with age (Gray et al., 2006; Chen et al., 2009), was restored to near that of a young thymus (Fig. 3F,G).

We also examined TEC subset distribution. The *Ulex europaeus* agglutinin-1 (UEA-1)[hi] mTEC subpopulation, which is proposed to

regulate medullary organization (Naquet et al., 1999), declines with age and is also preferentially lost following premature downregulation of *Foxn1* expression in the postnatal thymus (Chen et al., 2009). This phenotype was rescued by upregulation of FOXN1 in aged thymi; the relative numbers of UEA-1^{hi} mTEC in tamoxifen-treated Cre/+;R26Foxn1ER mice were similar to those of young wild-type controls (Fig. 3A). The autoimmune regulator AIRE plays a crucial role in negative selection of thymocytes as it regulates expression in TECs of a set of genes that are otherwise expressed only in specific tissues or cell types (tissue-restricted antigens or TRAs) (Anderson et al., 2002; Mathis and Benoist, 2009). AIRE is expressed in an mTEC subpopulation that declines numerically with age and AIRE[+] mTEC number has been linked to *Foxn1* expression levels (Nowell et al., 2011). The number of AIRE[+] mTECs and the expression of *Aire* mRNA was significantly increased in aged, tamoxifen-treated Cre/+; R26Foxn1ER thymi versus controls (Fig. 3H-J; see supplementary material Fig. S5 for gating strategy for flow cytometric analysis and sorting of TEC subsets, and validation). The AIRE-dependent TRAs
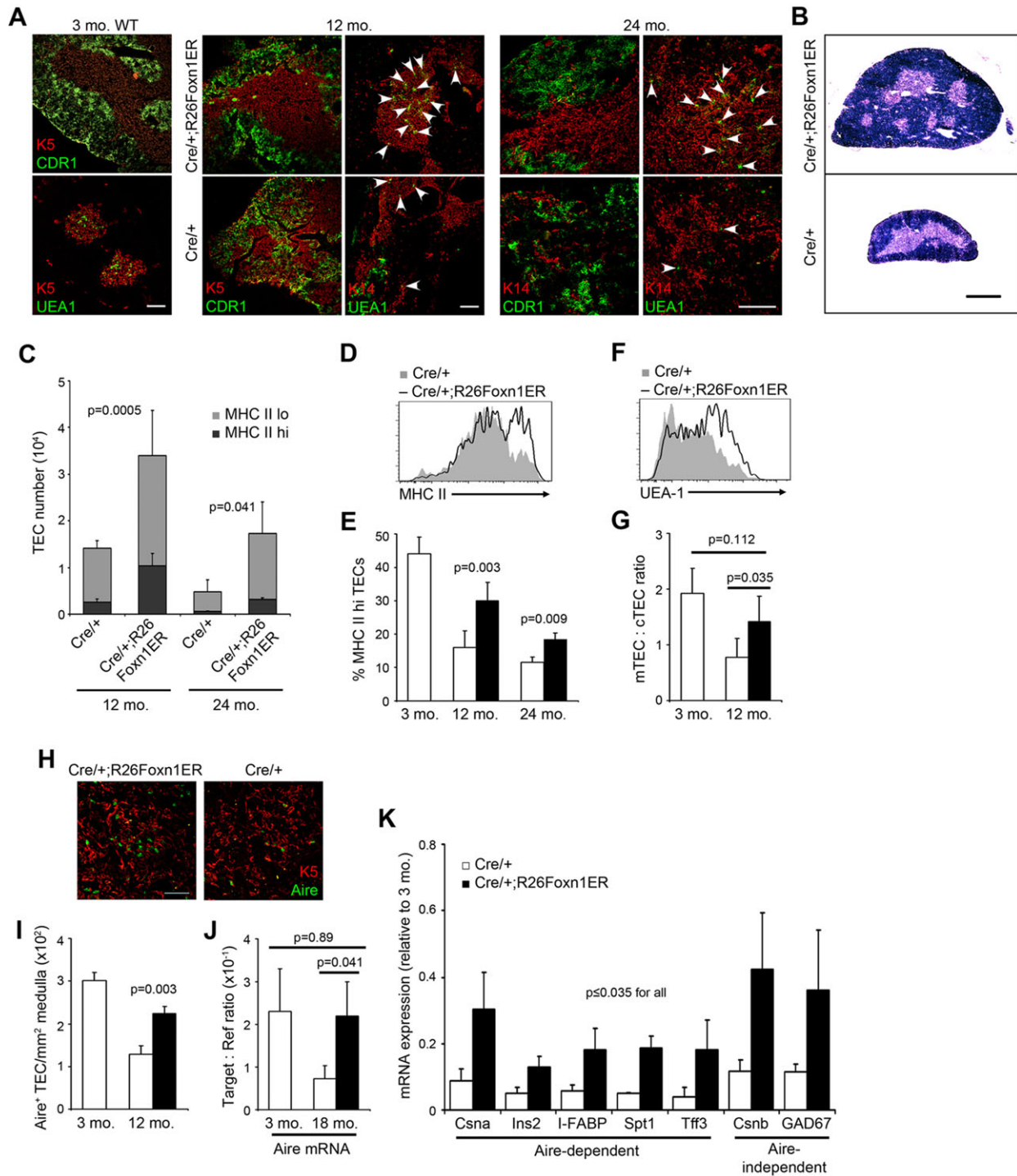
Fig. 3. Induction of FOXN1 restores the epithelial microenvironment in aged thymi. Cre/+;R26Foxn1ER and littermate Cre/+ mice (12 and 24 months old) were treated with tamoxifen for 1 month; thymi from these mice were then microdissected and processed for analysis. (A) IHC for the markers shown, revealing cortical and medullary epithelial regions [cortex, CDR1; medulla, Keratin (K) 5 or 14 and UEA-1]. Arrowheads indicate UEA-1$^+$ TEC clusters. Scale bars: 100 μm. (B) Hematoxylin and Eosin stained transverse sections from 12-month-old thymi. Number of medullary islets per lobe: Cre/+, 1.9; Cre/+; R26Foxn1ER, 3.6 (n=3, P=0.009). Scale bar: 1 mm. (C) TEC (CD45$^-$EpCAM$^+$) numbers, showing MHC Class II^{hi} (dark gray) and MHC Class II^{lo} (light gray) contributions (12 months, n=6; 24 months, n=3). Fold change in EpCAM$^+$ TECs: 12 months, 2.4; 24 months, 3.6. (D) MHC Class II profiles of TECs from tamoxifen-treated 12-month-old mice of the genotypes shown. (E) MHC Class II^{hi} TECs as a proportion of total TECs (12 months, n=6; 24 months, n=3). Fold change in MHC Class II^{hi} TECs: 12 months old, 4.1; 24 months old, 5.7. (F) UEA-1 profile of TECs from 12-month-old tamoxifen-treated mice of the genotypes shown (UEA-1$^+$ TECs are mTECs). (G) mTECs (UEA-1$^+$, Ly51$^-$) to cTECs (UEA-1$^-$, Ly51$^+$) ratio in 3- and 12-month-old mice, as determined from flow cytometric analysis (12 months, n=5). (H) IHC of tamoxifen-treated 12-month-old thymi of the genotypes shown, showing AIRE staining in mTECs (K5 positive). Scale bar: 50 μm. (I) Number of AIRE$^+$ cells per mm^2 of medullary area in tamoxifen-treated 3- and 12-month-old mice (12 months, n=3). (J) RT-qPCR analysis of *Aire* expression in mTECs from 3- and 18- to 24-month-old tamoxifen-treated mice, normalized to the geometric mean of three housekeeping genes (n=3). (K) TRA expression in total TECs in 12-month-old mice, shown relative to young TECs after normalization to the TEC housekeeping gene *Eva* (*Mpzl2*) (expression level in TECs at 3 months=1; n≥3 for all genes). mo, month; MHC II, MHC Class II. Error bars, s.d.

casein alpha (*Csna*; *Csn1s1* – Mouse Genome Informatics), insulin 2 (*Ins2*), intestinal fatty acid binding protein (*I-FABP*; *Fabp2* – Mouse Genome Informatics), salivary protein 1 (*Spt1*) and trefoil factor 3 (*Tff3*), which decrease during thymus involution (Anderson et al., 2002; Derbinski et al., 2005), were also upregulated upon induction of FOXN1 activity (Fig. 3K), establishing the functionality of the new AIRE[+] mTECs. The two AIRE-independent TRAs tested, casein beta (*Csnb*; *Csn2* – Mouse Genome Informatics) and glutamate decarboxylase 67 (*GAD67*; *Gad1* – Mouse Genome Informatics) (Anderson et al., 2002), were also upregulated. Collectively, these data indicate that thymus structure and function were regenerated to closely resemble their pre-involution phenotypes in the FOXN1-regenerated aged thymus.

FOXN1 upregulation restores the expression of genes required for TEC function

To probe further the effects of FOXN1 upregulation on the thymic microenvironment, we next tested the effects of normal ageing and of FOXN1 induction on a panel of genes required cell-autonomously in TECs for TEC development or for T cell differentiation and repertoire selection. Delta-like 4 (*Dll4*), chemokine (C-C motif) ligand 25 (*Ccl25*), kit ligand (*Kitl*) and chemokine (C-X-C motif) ligand 12 (*Cxcl12*) are putative transcriptional targets of FOXN1 that are expressed in cTECs and are required for the early stages of T cell development (Rodewald et al., 1997; Koch et al., 2008; Trampont et al., 2010; Zlotoff et al., 2010; Nowell et al., 2011; Calderon and Boehm, 2012); *Cd40*, *Cd80*, cathepsin L (*Ctsl*) and paired box 1 (*Pax1*) are expressed specifically in mTECs (*Cd80*), cTECs (*Ctsl*, *Pax1*) or both (*Cd40*), and are required in TECs to mediate normal T cell development (Reiser and Schneeberger, 1994; Van Den Berg et al., 1996; Wallin et al., 1996; Honey et al., 2002; Akiyama et al., 2008). Expression of all of these genes was substantially downregulated in aged versus young cTECs (Fig. 4A; see supplementary material Fig. S5 for gating strategy for flow cytometric analysis and sorting of TEC subsets, and validation), consistent with an age-related decline in TEC functionality. Fibroblast growth factor receptor 2 isoform IIIb (*FgfR2IIIb*), the receptor for fibroblast growth factors 7 and 10 (Revest et al., 2001), and the delta N isoform of transformation-related protein 63 (p63, also known as TRP63) ΔNp63 (Candi et al., 2007; Senoo et al., 2007) were also significantly downregulated in aged versus young TECs (Fig. 4A; note that *FgfR2IIIb* expression is restricted to cTECs), consistent with decreased TEC proliferation with age (Gray et al., 2006).

Tamoxifen-treated Cre/+;R26Foxn1ER mice exhibited increased expression of *Dll4* in TECs, such that the relative expression level was similar to that in TECs from 3-month-old mice (Fig. 4B,C; supplementary material Fig. S5 and Fig. S6A). Strikingly, *Dll4* was upregulated only in cTECs (Fig. 4C), where it was predominantly expressed in the MHC Class II[hi] subset (supplementary material Fig. S6A). Thus, although functional FOXN1 was upregulated in all TECs, FOXN1 targets maintained their expected TEC subset-specific expression. Of note is that the expression profile for DLL4 protein on TECs in the FOXN1-regenerated thymus is characteristic of the young rather than the aged thymus (Fiorini et al., 2008; Koch et al., 2008). *Ccl25*, *Kitl*, *Cxcl12*, *Cd40*, *Cd80*, *Ctsl* and *Pax1* were also upregulated close to levels found in 3-month-old TECs (Fig. 4B). Increased expression of these genes in the aged thymus in response to FOXN1 upregulation provides a mechanistic explanation for the specific impacts observed on the early thymocyte compartment, including the proportional drop in intrathymic B cells which probably results from increased DLL4 expression (Hozumi et al., 2008; Koch et al., 2008).

Among signaling pathways, the Wnt pathway is reportedly most affected in TECs by age-related thymic involution (Griffith et al.,

2012). Our analysis confirmed substantially downregulated expression of many Wnt pathway genes in aged versus young TECs, and extended previous findings to show that many of these genes are differentially regulated in c- and mTECs (Fig. 4D,D'). Strikingly, expression of most of the Wnt pathway genes analyzed were restored to young levels in tamoxifen-treated aged Cre/+; R26Foxn1ER TECs (Fig. 4D,D'). Again, these genes maintained their normal TEC subset-specific expression upon FOXN1 upregulation. Of note is that lymphotoxin receptor β (*Ltbr*), interleukin 7 (*Il7*), v-rel avian reticuloendotheliosis viral oncogene homolog B (*Relb*) and intracellular adhesion molecule 1 (*Icam1*) were expressed at equivalent levels in aged, young and tamoxifen-treated aged Cre/+;R26Foxn1ER TECs (supplementary material Fig. S7A).

Collectively, these data establish that reduced FOXN1 expression is the crucial factor limiting expression of a wide range of genes in aged TECs, and provide additional evidence that the FOXN1-regenerated thymus very closely resembles the young thymus.

FOXN1 mediates thymus regeneration by increasing proliferation of progenitor TEC

TEC proliferation is known to decrease with age, with an approximately threefold decrease in the proportion of proliferating TECs from 4 weeks to 10 months of age (Gray et al., 2006), and a link between FOXN1 and TEC proliferation has been established in fetal thymus (Itoi et al., 2001). To determine the cellular mechanism through which FOXN1 upregulation leads to thymus regeneration, we therefore tested whether the increase in TEC numbers resulted from increased TEC proliferation, using Ki67 staining. The proportion of proliferating cells in the total TEC population increased by twofold upon upregulation of FOXN1 function in 12-month-old mice (Fig. 4E; supplementary material Fig. S6B). Among cTECs, proliferation was significantly increased only in the MHC Class II[lo] compartment, which contains putative progenitor cTECs (Fig. 4F). The numbers of both MHC Class II[lo] and MHC Class II[hi] cTECs increased, however, indicating that in the regenerated thymi this MHC Class II[hi] cTEC compartment was rebuilt from a progenitor TEC pool (Fig. 4F,G). We found increased proliferation of both MHC Class II[lo] and MHC Class II[hi] mTECs (Fig. 4F); both of these mTEC compartments contain proliferating cells in young mice (Tykocinski et al., 2008).

To investigate the molecular basis of this increased proliferation, we analyzed expression of genes with roles in cell cycle progression and TEC proliferation. We observed an increase in the number of TECs expressing ΔNp63, which marks proliferative potential in epithelial cells including TECs (Candi et al., 2007; Senoo et al., 2007), in tamoxifen-treated aged Cre/+;R26Foxn1ER thymi versus age-matched controls (Fig. 4A,B,H); expression of the cell cycle regulator cyclin D1 and of *Fgf2RIIIb* was also upregulated (Fig. 4A,B). *Fgf2RIIIb* was previously been reported to be regulated by FOXN1 (Tsai et al., 2003). However, neither *Ccnd1* nor *p63* has previously been suggested to be a direct or indirect transcriptional target of FOXN1, and these data therefore expand the set of FOXN1-regulated targets to include these genes. As ΔNp63, cyclin D1 and *Fgf2RIIIb* have all previously been shown to regulate TEC proliferation (Robles et al., 1996; Revest et al., 2001), this provides a mechanistic link between increased FOXN1 expression and the expansion of TECs observed in these experiments.

Of note is that we could not detect interleukin 22 receptor (*Il22r*) expression (Dudakov et al., 2012) in tamoxifen-treated Cre/+; Foxn1ER TEC in aged mice (supplementary material Fig. S7B), indicating that FOXN1-mediated thymus regeneration acts via an IL22-independent mechanism.
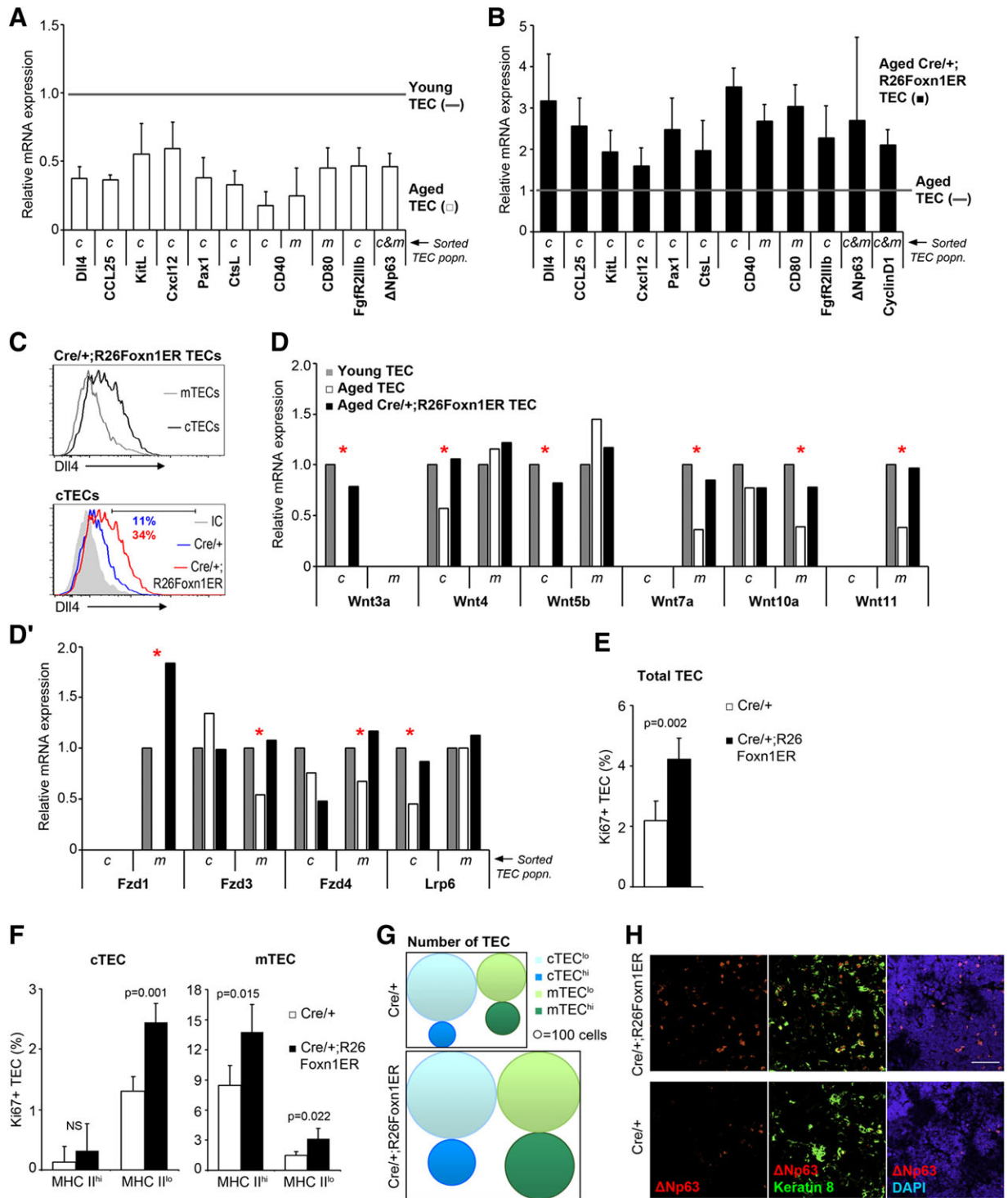
DEVELOPMENT

Fig. 4. Induction of FOXN1 elevates epithelial proliferation and gene expression. (A) mRNA expression levels of the genes shown in mTECs (m) and/or cTECs (c) from young (3-month-old) and aged (18-month-old) wild-type mice, shown as fold-change relative to young mice after normalization to the geometric mean of three housekeeping (3HK) genes (3 months, n=1; 18 months, n=3). (B) mRNA expression levels in TECs from tamoxifen-treated 18- to 24-month-old mice of the genotypes shown, shown as fold change relative to Cre/+ controls (gray line) after normalization to the geometric mean of 3HK genes (n=3). (C) Top: DLL4 expression on cTECs and mTECs from tamoxifen-treated 12-month-old Cre/+;R26Foxn1ER mice. Bottom: DLL4 expression on cTECs from tamoxifen-treated 12-month-old Cre/+ and Cre/+;R26Foxn1ER mice. (D,D′) mRNA expression levels of the genes shown in mTECs (m) and cTECs (c) from tamoxifen-treated young (3-month-old) and aged (12-month-old) mice, shown as fold-change relative to cTECs or mTECs from young mice after normalization to the geometric mean of 3HK. Data represent the expression levels in TECs pooled from four mice. Red asterisks indicate genes downregulated in involution and restored upon FOXN1 upregulation. (E,F) Proportion of Ki67^{+} TECs in total EpCam[+] TECs (E) and the major TEC subpopulations (F) (mTECs: UEA-1^{+}, Ly51^{−}; cTECs: UEA-1^{−}, Ly51^{+}) from tamoxifen-treated 12-month-old mice of the genotypes shown (n=4). (G) Schematic of TEC subpopulation numbers in tamoxifen-treated 12-month-old mice of the genotypes shown. hi and lo refer to MHC Class II surface expression levels. (H) Analysis of tamoxifen-treated 12-month-old thymi showing ΔNp63 staining. Scale bar: 50 μm. Black bars, Cre/+;R26Foxn1ER; white bars, Cre/+ controls, throughout. c, cTECs; m, mTECs; mo, month; MHC II, MHC Class II. Error bars, s.d.

FOXN1-mediated thymus regeneration impacts the peripheral immune system by increasing naive T cell numbers

A crucial question is whether these changes in thymocyte numbers translate into an altered peripheral lymphocyte pool. Age-related thymic involution leads to a numerical and proportional decrease in naive T cells in the peripheral pool, that is thought to be a major factor in the capacity of the immune system to respond to new antigenic challenge (Goronzy and Weyand, 2005). Both $CD4^+$ and $CD8^+$ splenocytes from aged, tamoxifen-treated Cre/+;R26Foxn1ER mice contained a significantly higher proportion of $CD3^+CD62L^+CD44^{lo}$ (CD62L is also known as SELL) naive T cells (Budd et al., 1987) than controls (Fig. 5A,B), demonstrating improved export of naive T cells from the thymus. To specifically identify recent thymic emigrants (RTEs), the CD45RB phenotype of $CD4^+$ splenocytes was analyzed (Boursalian et al., 2004). Upregulation of FOXN1-activity in 24-month-old Cre/+;R26Foxn1ER mice resulted in a higher proportion of $CD45RB^{lo}$ RTEs among naive splenic T cells (supplementary material Fig. S8A,B), and the proportion and numbers of RTEs in the total splenic $CD4^+$ compartment of 12- and 24-month-old tamoxifen-treated Cre/+;R26Foxn1ER mice were increased by twofold versus controls by 1 month after treatment commenced (Fig. 5C,D; supplementary material Fig. S8B). The increase in RTEs was also evidenced by a greater than twofold increase in T cell receptor excision (TREC) (Sempowski et al., 2002) number in both $CD4^+$ and $CD8^+$ $CD45RB^+$ naive splenocytes (Fig. 5E; supplementary material Fig. S8C). Thus, upregulation of FOXN1 in aged thymi resulted in an increase in the export of RTEs to the periphery with consequent increase in the numbers and proportions of naive T cells in the peripheral immune system.

DISCUSSION

We have shown that upregulation of a crucial regulator of TEC differentiation, FOXN1, is sufficient to drive regeneration of the aged thymus such that it closely resembles the juvenile, pre-involution thymus in terms of architecture, gene expression and functionality. This FOXN1-mediated thymus regeneration results in improved thymic output in aged mice, with increased numbers of naive T cells exported to peripheral lymphoid organs. Collectively, our data demonstrate that the major impacts of age-related involution on thymus structure and function can be substantially reversed by restoring FOXN1 function, establishing that loss of FOXN1 expression is the principal cause of these components of thymic involution. They further provide insight into the mechanisms by which FOXN1 mediates thymus regeneration.

Our findings contrast with the outcome of provoking thymus regeneration via sex-steroid ablation, which a recent study has shown results in transient generation of an enlarged, but phenotypically old thymus with no change in TRA expression or medullary complexity, and no upregulation of key regulatory and functional genes compared with aged controls (Griffith et al., 2012). In addition, most of the genes involved in the Wnt signaling pathway that were examined herein were downregulated during age-related thymic involution, in agreement with a previous study (Griffith et al., 2012). These genes were restored to young expression levels upon upregulation of FOXN1 activity in aged TECs, whereas their expression is not restored following sex steroid ablation-mediated rebound (Griffith et al., 2012). In vivo manipulation of FOXN1 expression, which not only enlarges the aged thymus but also restores its function, therefore presents a promising alternative strategy for clinical thymus
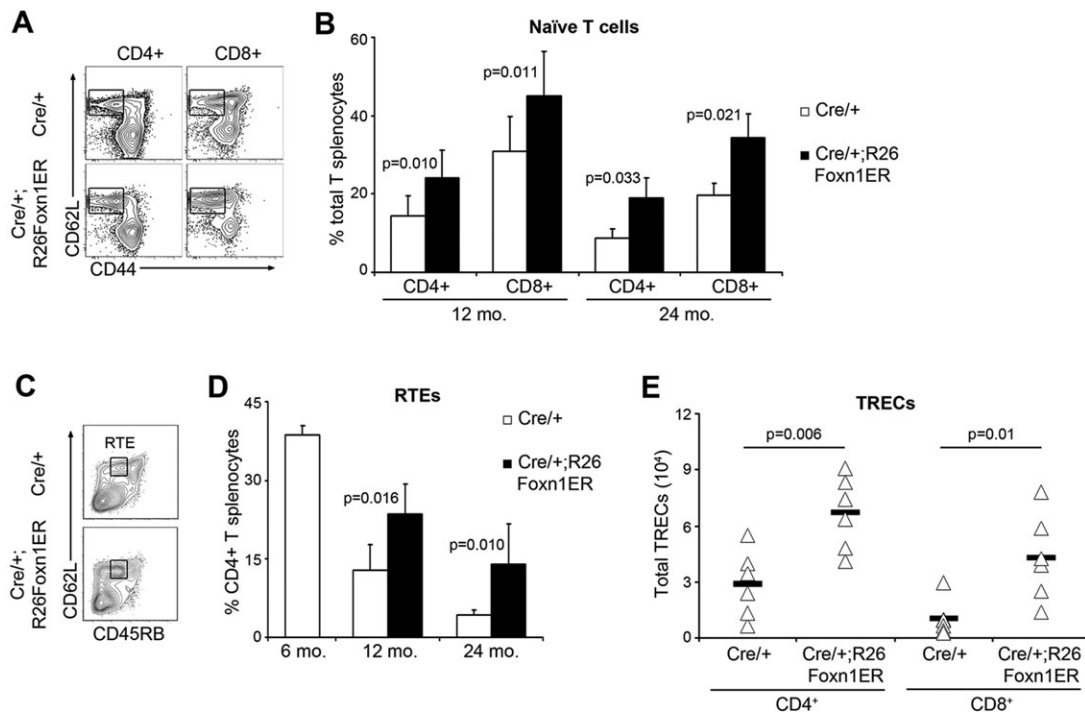

Fig. 5. Induction of FOXN1 improves thymic output in aged thymi. (A) Splenic cell preparations from tamoxifen-treated 12-month-old mice of the genotypes shown, after gating on $CD3^+$ cells. Naive T cells are $CD62L^+CD44^{lo}$. (B) Average proportions of $CD4^+$ and $CD8^+$ naive T cells in tamoxifen-treated 12- and 24-month-old mice of the genotypes shown (12 months, $n=6$; 24 months, $n=3$). (C) Splenic cell preparations from tamoxifen-treated 24-month-old mice of the genotypes shown, after gating on $CD3^+CD4^+$ cells. Recent thymic emigrants (RTEs) are $CD62L^+CD45RB^{int}$. (D) Average proportions of RTEs in tamoxifen-treated 12- and 24-month-old mice (12 months, Cre/+ $n=4$, Cre/+;R26Foxn1ER $n=6$; 24 months, Cre/+ $n=6$, Cre/+;R26Foxn1ER $n=8$). (E) Number of T cell receptor excision circles (TRECs) in naive splenic T cells ($CD62L^+CD45RB^+$) from tamoxifen-treated 24-month-old mice ($n=6$). Black bars, Cre/+;R26Foxn1ER; white bars, Cre/+ controls, throughout. mo, month. Error bars, s.d.

regeneration – for instance by small molecule manipulation of signaling pathways that regulate FOXN1 expression or directly augment FOXN1 function.

FOXN1 is a powerful regulator of TEC differentiation (Blackburn et al., 1996; Nehls et al., 1996; Chen et al., 2009; Nowell et al., 2011), is required throughout TEC development from exit from the earliest progenitor TEC state to terminal differentiation in both cTEC and mTEC sub-lineages (Nowell et al., 2011), and has also been implicated as a regulator of proliferation of fetal TECs (Itoi et al., 2001). In the context of our experimental model, however, upregulation of FOXN1 did not drive all TECs in the aged thymus to terminal differentiation, but rather caused a sustained increase in the size of both progenitor and terminally differentiated TEC compartments, driven by proliferation of progenitor cells. This increased proliferation was accompanied by upregulation of genes that promote cell cycle progression (cyclin D1, $\Delta Np63$, $FgfR2IIIb$) and that are required in TECs to promote specific aspects of T cell development ($Dll4$, $Kitl$, $Ccl25$, $Cxcl12$, $Cd40$, $Cd80$, $Ctsl$, $Pax1$). These data suggest a mechanistic basis for cell cycle promotion by FOXN1, and indicate that factors in addition to FOXN1 must limit the onset of terminal differentiation in progenitor TECs. They further establish, to our knowledge for the first time, that cyclin D1 and $\Delta Np63$ are direct or indirect targets of FOXN1. In conjunction with a recent report demonstrating that the retinoblastoma family of proteins can negatively regulate $Foxn1$ expression in TECs (Garfin et al., 2013), they thus suggest a complex interplay between FOXN1 expression and cell cycle regulation.

Recently, Sun and colleagues reported that delivery of a FOXN1-expression plasmid to the aged thymus by intrathymic injection resulted in increased thymus size. However, as cellular composition, architecture and function of the thymus were not examined, that study was not informative regarding the effect of FOXN1 upregulation on hallmarks of thymic involution, including altered TEC phenotype and function (Sun et al., 2010). The importance of evaluating these parameters is highlighted by the findings of Griffiths and colleagues, discussed above, that the degenerative changes characteristic of age-associated thymic involution persist despite increased thymic size during castration-induced thymic rebound (Griffith et al., 2012). In a separate study, Zook and colleagues reported that overexpression of FOXN1 from the human keratin 14 (hK14; $KRT14$ – Human Gene Nomenclature Database) promoter, which can drive broad expression in fetal and postnatal TECs including cTECs (Laufer et al., 1996), delays but does not prevent the onset of age-related thymus involution (Zook et al., 2011). As thymic involution still occurred in the presence of FOXN1 overexpression in this model (Zook et al., 2011), these data suggested that upregulation of FOXN1 should not be sufficient to regenerate the age-related thymus. In our experiments, increased FOXN1 expression restored thymic architecture and function, despite the fact that involution was fully established before FOXN1 function was upregulated. Our data thus demonstrate unequivocally that FOXN1 upregulation is sufficient to reverse the crucial hallmarks of age-related thymic involution, based on all parameters commonly used to measure thymus structure and function. The reversibility of these hallmark changes indicates that the involuted state induced in the thymus by ageing is not locked, for instance by epigenetic or other mechanisms that result in permanent loss of key functions, even once fully established. Our data thus suggest that in the ageing and aged thymus, FOXN1 or its upstream regulators must be continuously repressed by an active mechanism in order to suppress FOXN1-mediated regeneration.

Since the discovery of induced pluripotent stem cells (Takahashi and Yamanaka, 2006), interest has increased in the capacity of a single or a few transcription factors to program/reprogram cell fate – for instance,

via direct conversion of cell fates or by directing differentiation of embryonic stem cells into defined cell types (Weintraub et al., 1989; Seguin et al., 2008; Zhou et al., 2008; Graf and Enver, 2009; Antonica et al., 2012). Our findings demonstrate that in the aged thymus FOXN1 is sufficient to promote proliferation of progenitor TECs and differentiation of these cells into fully functional thymic epithelium, extending this approach to the important goal of whole organ regeneration *in vivo*. As discussed, our data show that upregulating FOXN1 function leads to upregulation of key genes required for TEC proliferation and function. An important emerging question is therefore whether FOXN1 regulates this diverse set of targets via classical transactivation, or functions either additionally or alternatively as a 'pioneer factor', as recently demonstrated for FoxA proteins (Cirillo et al., 2002; Zaret and Carroll, 2011).

Collectively, the data presented herein have identified both the cell type and the gene required for thymus regeneration. These findings highlight FOXN1 as a specific target for strategies aiming to achieve thymus regeneration in patients, underlining the need to understand both its transcriptional regulation and downstream targets. Finally, by establishing that modulation of a single transcription factor is sufficient to instigate regeneration of an entire organ, our findings provide a provocative paradigm that may be of broad impact for regenerative biology strategies.

MATERIALS AND METHODS
Ethics statement
All animal work was conducted according to UK Home Office guidelines, as established in the Animals (Scientific Procedures) Act 1986.

Mice
$Rosa26^{CAG-STOP-Foxn1ERT2-IRES-GFP/+}$ (R26Foxn1ER) mice were backcrossed onto C57BL/6 for at least three generations then maintained via intercrossing. $Foxn1^{Cre/+}$ mice (Gordon et al., 2007) were maintained as homozygotes and crossed with $Rosa26^{CAG-STOP-Foxn1ERT2-IRES-GFP/+}$ mice as described to generate $Foxn1^{Cre/+}$;$Rosa26^{CAG-Foxn1ERT2-IRES-GFP/+}$ (Cre/+;R26Foxn1ER mice). Littermate controls were used in each experiment. For timed matings, noon of the day of the vaginal plug was taken as E0.5.

Generation of the Foxn1ER^{T2} targeting vector
A construct containing the targeting cassette (Fig. 1A; supplementary material Fig. S1A) was generated by standard molecular biology techniques (Nowell et al., 2011) and verified by sequencing. Conventional subcloning was used for all cloning steps.

Southern blotting
Genomic DNA was processed for Southern blotting as described (Morris et al., 2006; Nowell et al., 2011).

Gene targeting and blastocyst injection
Mouse sv129/ola ES cells (line E14tg2a) were electroporated with linearized targeting vector (supplementary material Fig. S1A) and grown under Geneticin selection. Correctly targeted clones were identified by Southern analysis (supplementary material Fig. S1B), expanded and injected into C57BL/6 blastocysts to generate chimeric mice. Germ-line transmission was confirmed for two independently targeted cell clones (supplementary material Fig. S1B); the resulting mouse line was designated $ROSA26^{CAG-STOP-Foxn1ERT2-IRES-GFP}$ (called R26Foxn1ER herein). The neomycin resistance cassette was removed by crossing of founder R26Foxn1ER mice with $Tg(CAG-FLPe)$ mice (Wallace et al., 2007) and confirmed by Southern analysis (supplementary material Fig. S1C).

Transient transfection
A luciferase reporter cell system (Janes et al., 2004) containing the wild-type and mutated minimal FOXN1 response elements (Schlake et al., 1997) was transiently transfected with empty, $CAG-Foxn1$ and $CAG-Foxn1ER^{T2}$

vectors using Lipofectamine 2000 (Invitrogen). Cells were cultured for 48 h with or without 4-hydroxytamoxifen (1 μM, Sigma), then assayed using a Luciferase Assay System (Promega) and analyzed on a Mediators PhL Luminometer (ImmTech).

Tamoxifen treatment
Mice were treated with a single intraperitoneal (IP) injection of tamoxifen (Sigma) prepared in ethanol and diluted appropriately in Cremophor (Sigma)/PBS. For regeneration experiments, mice were treated with a single 3 mg IP injection of tamoxifen and then with tamoxifen citrate salt (Sigma) in the drinking water (0.05 mg/ml) for one month.

Antibodies
The antibodies used for IHC and flow cytometry are listed in supplementary material Table S1.

Flow cytometry
Analysis
Mouse fetal TECs were processed for flow cytometric analysis as described (Bennett et al., 2002; Nowell et al., 2011); adult TECs were enzymatically digested to single cell suspensions as described (Gray et al., 2008) and processed for cytometric analysis without further enrichment. Naive T cells ($CD3^+CD62L^+CD44^{lo}$) (Budd et al., 1987) and RTEs ($CD3^+CD4^+CD62L^+CD45RB^{int}$) (Boursalian et al., 2004) were identified among splenocytes as described. Data were acquired using LSR Fortessa (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Sorting
For isolation of adult TECs, TECs were enzymatically digested to single cell suspensions and enriched as described (Gray et al., 2008). Total TECs ($EpCAM^+$) or cTECs ($EpCAM^+Ly51^+$) or mTECs ($EpCAM^+Ly51^-$) were purified after gating against $CD45^-Ter119^-$ cells. For isolation of splenocytes, mouse spleens were mechanically disrupted and red blood cells were lysed. $CD4^+$ and $CD8^+$ naive splenocytes were purified after gating on $CD3^+CD62L^+CD45RB^+$ cells. Cell sorting was performed using a FACSAriaII (BD Biosciences).

Immunohistochemistry
Adult thymi were processed for IHC as described (Nowell et al., 2011). Isotype controls (not shown) were included in all experiments. Staining was analyzed using a Leica AOBS confocal microscope (Leica Microsystems). The images presented are either single optical sections or projected focus stacks of serial optical sections. The number of $FOXN1^+$ or $AIRE^+$ TECs per area was established by analyzing three areas on each of two non-sequential sections from each of three individual mice. Statistical analysis was performed on the average number of $FOXN1^+$ or $AIRE^+$ cells per condition.

RNA isolation
RNA was prepared using an RNAeasy Mini Kit (Qiagen) according to the manufacturer's instructions. All samples were DNase treated.

RT-qPCR
cDNA was prepared using the Superscript II First Strand Synthesis Kit (Invitrogen) with Oligo-dT primers, according to the manufacturer's instructions. Relative expression levels were determined using the Roche Universal Probe Library on the Roche Lightcycler 480 after normalization to the geometric mean of three housekeeping genes (α-tubulin, HMBS and TBP) for all experiments except for TRAs, which were normalized to epithelial V-like antigen (EVA) (DeMonte et al., 2007). Technical triplicates were run for all samples and no reverse transcriptase and no template controls were included in all experiments. The primers used for RT-qPCR are listed in supplementary material Table S2.

TREC
Signal joint T cell receptor delta (TCRD) excision circle (sjTREC) quantification was based on a previously described method (Sempowski et al., 2002). Genomic

DNA was isolated from splenocytes by cell lysis (10,000 cells/μl) in 10 mM Tris with 0.05% Tween-20, 0.05% NP-40 and 100 μg/ml Proteinase K. TREC numbers were quantified on a Roche Lightcycler 480 using the following primer/probe set: forward primer, 5′-CATTGCCTTTGAAC-CAAGCTGA-3′; reverse primer, 5′-TTATGCACAGGGTGCAGGTG-3′; probe, FAM-GCAGGTTTTTGTAAAGGTGCTCACTTCT-BHQ (Sigma).

Samples were assayed at 25,000 cell equivalents per PCR reaction. A TREC standard was generated by PCR-cloning a 315-bp fragment, spanning the TCRD single joint site, from thymocyte genomic DNA into a TOPO-TA vector (Invitrogen) (primers: 5′-TAGGGAAGATGGGCCTCTCTG-3′, 5′-GTGTGTCCTCAGCCTTGATCCATC-3′). Serial dilutions of the TREC standard (10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 molecules per reaction) were used to generate a standard curve. To test for equivalent genomic DNA input, we separately amplified the α-tubulin reference gene.

Western blotting
Nuclear and cytoplasmic protein fractions were prepared using a nuclear extract kit (Active Motif) according to the manufacturer's instructions and processed for western blotting as described (Depreter et al., 2008; Nowell et al., 2011).

Statistical analysis
Statistical analysis was performed using the one-way ANOVA test (two tailed), as appropriate for normally distributed data (normal distribution was tested using χ^2 goodness of fit). The alpha level is taken as 0.05. Errors shown are standard deviations throughout. Sample sizes of at least $n=3$ were used for statistical analyses.

References
Adolfsson, J., Månsson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C. T., Bryder, D., Yang, L., Borge, O.-J., Thoren, L. A. M. et al. (2005). Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential. *Cell* **121**, 295-306.

Akiyama, T., Shimo, Y., Yanai, H., Qin, J., Ohshima, D., Maruyama, Y., Asaumi, Y., Kitazawa, J., Takayanagi, H., Penninger, J. M. et al. (2008). The tumor necrosis factor family receptors RANK and CD40 cooperatively establish the thymic medullary microenvironment and self-tolerance. *Immunity* **29**, 423-437.

Allman, D., Sambandam, A., Kim, S., Miller, J. P., Pagan, A., Well, D., Meraz, A. and Bhandoola, A. (2003). Thymopoiesis independent of common lymphoid progenitors. *Nat. Immunol.* **4**, 168-174.

Alpdogan, Ö., Hubbard, V. M., Smith, O. M., Patel, N., Lu, S., Goldberg, G. L., Gray, D. H., Feinman, J., Kochman, A. A., Eng, J. M. et al. (2006). Keratinocyte growth factor (KGF) is required for postnatal thymic regeneration. *Blood* **107**, 2453-2460.

Anderson, M. S., Venanzi, E. S., Klein, L., Chen, Z., Berzins, S. P., Turley, S. J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C. et al. (2002). Projection of an immunological self shadow within the thymus by the aire protein. *Science* **298**, 1395-1401.

Antonica, F., Kasprzyk, D. F., Opitz, R., Iacovino, M., Liao, X.-H., Dumitrescu, A. M., Refetoff, S., Peremans, K., Manto, M., Kyba, M. et al. (2012). Generation of functional thyroid from embryonic stem cells. *Nature* **491**, 66-71.

Bennett, A. R., Farley, A., Blair, N. F., Gordon, J., Sharp, L. and Blackburn, C. C. (2002). Identification and characterization of thymic epithelial progenitor cells. *Immunity* **16**, 803-814.

Blackburn, C. C., Augustine, C. L., Li, R., Harvey, R. P., Malin, M. A., Boyd, R. L., Miller, J. F. and Morahan, G. (1996). The nu gene acts cell-autonomously and is required for differentiation of thymic epithelial progenitors. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 5742-5746.

Boursalian, T. E., Golob, J., Soper, D. M., Cooper, C. J. and Fink, P. J. (2004). Continued maturation of thymic emigrants in the periphery. *Nat. Immunol.* **5**, 418-425.

Budd, R. C., Cerottini, J. C., Horvath, C., Bron, C., Pedrazzini, T., Howe, R. C. and MacDonald, H. R. (1987). Distinction of virgin and memory T lymphocytes. Stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. *J. Immunol.* **138**, 3120-3129.

Calderón, L. and Boehm, T. (2012). Synergistic, context-dependent, and hierarchical functions of epithelial components in thymic microenvironments. *Cell* **149**, 159-172.

Candi, E., Rufini, A., Terrinoni, A., Giamboi-Miraglia, A., Lena, A. M., Mantovani, R., Knight, R. and Melino, G. (2007). DeltaNp63 regulates thymic development through enhanced expression of FgfR2 and Jag2. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 11999-12004.

Chen, L., Xiao, S. and Manley, N. R. (2009). Foxn1 is required to maintain the postnatal thymic microenvironment in a dosage-sensitive manner. *Blood* **113**, 567-574.

Chinn, I. K., Blackburn, C. C., Manley, N. R. and Sempowski, G. D. (2012). Changes in primary lymphoid organs with aging. *Semin. Immunol.* **24**, 309-320.

Cirillo, L. A., Lin, F. R., Cuesta, I., Friedman, D., Jarnik, M. and Zaret, K. S. (2002). Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Mol. Cell* **9**, 279-289.

DeMonte, L., Porcellini, S., Tafi, E., Sheridan, J., Gordon, J., Depreter, M., Blair, N., Panigada, M., Sanvito, F., Merati, B. et al. (2007). EVA regulates thymic stromal organisation and early thymocyte development. *Biochem. Biophys. Res. Commun.* **356**, 334-340.

Depreter, M. G. L., Blair, N. F., Gaskell, T. L., Nowell, C. S., Davern, K., Pagliocca, A., Stenhouse, F. H., Farley, A. M., Fraser, A., Vrana, J. et al. (2008). Identification of Plet-1 as a specific marker of early thymic epithelial progenitor cells. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 961-966.

Derbinski, J., Gäbler, J., Brors, B., Tierling, S., Jonnakuty, S., Hergenhahn, M., Peltonen, L., Walter, J. and Kyewski, B. (2005). Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J. Exp. Med.* **202**, 33-45.

Dudakov, J. A., Hanash, A. M., Jenq, R. R., Young, L. F., Ghosh, A., Singer, N. V., West, M. L., Smith, O. M., Holland, A. M., Tsai, J. J. et al. (2012). Interleukin-22 drives endogenous thymic regeneration in mice. *Science* **336**, 91-95.

Fiorini, E., Ferrero, I., Merck, E., Favre, S., Pierres, M., Luther, S. A. and MacDonald, H. R. (2008). Cutting edge: thymic crosstalk regulates delta-like 4 expression on cortical epithelial cells. *J. Immunol.* **181**, 8199-8203.

Garfin, P. M., Min, D., Bryson, J. L., Serwold, T., Edris, B., Blackburn, C. C., Richie, E. R., Weinberg, K. I., Manley, N. R., Sage, J. et al. (2013). Inactivation of the RB family prevents thymus involution and promotes thymic function by direct control of Foxn1 expression. *J. Exp. Med.* **210**, 1087-1097.

Gordon, J., Xiao, S., Hughes, B., III, Su, D.-M., Navarre, S. P., Condie, B. G. and Manley, N. R. (2007). Specific expression of lacZ and cre recombinase in fetal thymic epithelial cells by multiplex gene targeting at the Foxn1 locus. *BMC Dev. Biol.* **7**, 69.

Goronzy, J. J. and Weyand, C. M. (2005). T cell development and receptor diversity during aging. *Curr. Opin. Immunol.* **17**, 468-475.

Graf, T. and Enver, T. (2009). Forcing cells to change lineages. *Nature* **462**, 587-594.

Gray, D. H. D., Seach, N., Ueno, T., Milton, M. K., Liston, A., Lew, A. M., Goodnow, C. C. and Boyd, R. L. (2006). Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. *Blood* **108**, 3777-3785.

Gray, D. H. D., Fletcher, A. L., Hammett, M., Seach, N., Ueno, T., Young, L. F., Barbuto, J., Boyd, R. L. and Chidgey, A. P. (2008). Unbiased analysis, enrichment and purification of thymic stromal cells. *J. Immunol. Methods* **329**, 56-66.

Griffith, A. V., Fallahi, M., Venables, T. and Petrie, H. T. (2012). Persistent degenerative changes in thymic organ function revealed by an inducible model of organ regrowth. *Aging Cell* **11**, 169-177.

Holland, A. M. and van den Brink, M. R. M. (2009). Rejuvenation of the aging T cell compartment. *Curr. Opin. Immunol.* **21**, 454-459.

Honey, K., Nakagawa, T., Peters, C. and Rudensky, A. (2002). Cathepsin L regulates CD4+ T cell selection independently of its effect on invariant chain: a role in the generation of positively selecting peptide ligands. *J. Exp. Med.* **195**, 1349-1358.

Hozumi, K., Mailhos, C., Negishi, N., Hirano, K.-i., Yahata, T., Ando, K., Zuklys, S., Hollander, G. A., Shima, D. T. and Habu, S. (2008). Delta-like 4 is indispensable in thymic environment specific for T cell development. *J. Exp. Med.* **205**, 2507-2513.

Itoi, M., Kawamoto, H., Katsura, Y. and Amagai, T. (2001). Two distinct steps of immigration of haematopoietic progenitors into the early thymus anlage. *Int. Immunol.* **13**, 1203-1211.

Janes, S. M., Ofstad, T. A., Campbell, D. H., Watt, F. M. and Prowse, D. M. (2004). Transient activation of FOXN1 in keratinocytes induces a transcriptional programme that promotes terminal differentiation: contrasting roles of FOXN1 and Akt. *J. Cell Sci.* **117**, 4157-4168.

Koch, U., Fiorini, E., Benedito, R., Besseyrias, V., Schuster-Gossler, K., Pierres, M., Manley, N. R., Duarte, A., Macdonald, H. R. and Radtke, F. (2008). Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *J. Exp. Med.* **205**, 2515-2523.

Laufer, T. M., DeKoning, J., Markowitz, J. S., Lo, D. and Glimcher, L. H. (1996). Unopposed positive selection and autoreactivity in mice expressing class II MHC only on thymic cortex. *Nature* **383**, 81-85.

Lynch, H. E., Goldberg, G. L., Chidgey, A., Van den Brink, M. R. M., Boyd, R. and Sempowski, G. D. (2009). Thymic involution and immune reconstitution. *Trends Immunol.* **30**, 366-373.

Mackall, C. L., Punt, J. A., Morgan, P., Farr, A. G. and Gress, R. E. (1998). Thymic function in young/old chimeras: substantial thymic T cell regenerative capacity despite irreversible age-associated thymic involution. *Eur. J. Immunol.* **28**, 1886-1893.

Markert, M. L., Devlin, B. H., Chinn, I. K. and McCarthy, E. A. (2009). Thymus transplantation in complete DiGeorge anomaly. *Immunol. Res.* **44**, 61-70.

Mathis, D. and Benoist, C. (2009). Aire. *Annu. Rev. Immunol.* **27**, 287-312.

Miller, J. F. A. P. (1961). Immunological function of the thymus. *Lancet* **278**, 748-749.

Min, H., Montecino-Rodriguez, E. and Dorshkind, K. (2006). Reassessing the role of growth hormone and sex steroids in thymic involution. *Clin. Immunol.* **118**, 117-123.

Min, D., Panoskaltsis-Mortari, A., Kuro-o, M., Hollander, G. A., Blazar, B. R. and Weinberg, K. I. (2007). Sustained thymopoiesis and improvement in functional immunity induced by exogenous KGF administration in murine models of aging. *Blood* **109**, 2529-2537.

Morris, L., Gordon, J. and Blackburn, C. C. (2006). Identification of a tandem duplicated array in the Rhox alpha locus on mouse chromosome X. *Mamm. Genome* **17**, 178-187.

Naquet, P., Naspetti, M. and Boyd, R. (1999). Development, organization and function of the thymic medulla in normal, immunodeficient or autoimmune mice. *Semin. Immunol.* **11**, 47-55.

Nehls, M., Kyewski, B., Messerle, M., Waldschutz, R., Schuddekopf, K., Smith, A. J. H. and Boehm, T. (1996). Two genetically separable steps in the differentiation of thymic epithelium. *Science* **272**, 886-889.

Nowell, C. S., Bredenkamp, N., Tetélin, S., Jin, X., Tischner, C., Vaidya, H., Sheridan, J. M., Stenhouse, F. H., Heussen, R., Smith, A. J. H. et al. (2011). Foxn1 regulates lineage progression in cortical and medullary thymic epithelial cells but is dispensable for medullary sublineage divergence. *PLoS Genet.* **7**, e1002348.

Olsen, N. J., Olson, G., Viselli, S. M., Gu, X. and Kovacs, W. J. (2001). Androgen receptors in thymic epithelium modulate thymus size and thymocyte development. *Endocrinology* **142**, 1278-1283.

Ortman, C. L., Dittmar, K. A., Witte, P. L. and Le, P. T. (2002). Molecular characterization of the mouse involuted thymus: aberrations in expression of transcription regulators in thymocyte and epithelial compartments. *Int. Immunol.* **14**, 813-822.

Reiser, H. and Schneeberger, E. E. (1994). The costimulatory molecule B7 is expressed in the medullary region of the murine thymus. *Immunology* **81**, 532-537.

Revest, J. M., Suniara, R. K., Kerr, K., Owen, J. J. and Dickson, C. (2001). Development of the thymus requires signaling through the fibroblast growth factor receptor R2-IIIb. *J. Immunol.* **167**, 1954-1961.

Robles, A. I., Larcher, F., Whalin, R. B., Murillas, R., Richie, E. R., Gimenez-Conti, I. B., Jorcano, J. L. and Conti, C. J. (1996). Expression of cyclin D1 in epithelial tissues of transgenic mice results in epidermal hyperproliferation and severe thymic hyperplasia. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 7634-7638.

Rodewald, H.-R., Ogawa, M., Haller, C., Waskow, C. and DiSanto, J. P. (1997). Pro-thymocyte expansion by c-kit and the common cytokine receptor gamma chain is essential for repertoire formation. *Immunity* **6**, 265-272.

Sambandam, A., Maillard, I., Zediak, V. P., Xu, L., Gerstein, R. M., Aster, J. C., Pear, W. S. and Bhandoola, A. (2005). Notch signaling controls the generation and differentiation of early T lineage progenitors. *Nat. Immunol.* **6**, 663-670.

Schlake, T., Schorpp, M., Nehls, M. and Boehm, T. (1997). The nude gene encodes a sequence-specific DNA binding protein with homologs in organisms that lack an anticipatory immune system. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3842-3847.

Séguin, C. A., Draper, J. S., Nagy, A. and Rossant, J. (2008). Establishment of endoderm progenitors by SOX transcription factor expression in human embryonic stem cells. *Cell Stem Cell* **3**, 182-195.

Sempowski, G. D., Gooding, M. E., Liao, H. X., Le, P. T. and Haynes, B. F. (2002). T cell receptor excision circle assessment of thymopoiesis in aging mice. *Mol. Immunol.* **38**, 841-848.

Senoo, M., Pinto, F., Crum, C. P. and McKeon, F. (2007). p63 Is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* **129**, 523-536.

Sun, L., Guo, J., Brown, R., Amagai, T., Zhao, Y. and Su, D.-M. (2010). Declining expression of a single epithelial cell-autonomous gene accelerates age-related thymic involution. *Aging Cell* **9**, 347-357.

Takahashi, K. and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-676.

Trampont, P. C., Tosello-Trampont, A.-C., Shen, Y., Duley, A. K., Sutherland, A. E., Bender, T. P., Littman, D. R. and Ravichandran, K. S. (2010). CXCR4 acts as a costimulator during thymic beta-selection'. *Nat. Immunol.* **11**, 162-170.

Tsai, P. T., Lee, R. A. and Wu, H. (2003). BMP4 acts upstream of FGF in modulating thymic stroma and regulating thymopoiesis. *Blood* **102**, 3947-3953.

Tykocinski, L.-O., Sinemus, A. and Kyewski, B. (2008). The thymus medulla slowly yields its secrets. *Ann. N. Y. Acad. Sci.* **1143**, 105-122.

Van Den Berg, T. K., Hasbold, J., Renardel De Lavalette, C., Döpp, E. A., Dijkstra, C. D. and Klaus, G. G. B. (1996). Properties of mouse CD40: differential expression of CD40 epitopes on dendritic cells and epithelial cells. *Immunology* **88**, 294-300.

van den Brink, M. R. M., Alpdogan, Ö. and Boyd, R. L. (2004). Strategies to enhance T-cell reconstitution in immunocompromised patients. *Nat. Rev. Immunol.* **4**, 856-867.

Wallace, H. A. C., Marques-Kranc, F., Richardson, M., Luna-Crespo, F., Sharpe, J. A., Hughes, J., Wood, W. G., Higgs, D. R. and Smith, A. J. H. (2007). Manipulating the mouse genome to engineer precise functional syntenic replacements with human sequence. *Cell* **128**, 197-209.

Wallin, J., Eibel, H., Neubuser, A., Wilting, J., Koseki, H. and Balling, R. (1996). Pax1 is expressed during development of the thymus epithelium and is required for normal T-cell maturation. *Development* **122**, 23-30.

Weintraub, H., Tapscott, S. J., Davis, R. L., Thayer, M. J., Adam, M. A., Lassar, A. B. and Miller, A. D. (1989). Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5434-5438.

Weng, N.-P. (2006). Aging of the immune system: how much can the adaptive immune system adapt? *Immunity* **24**, 495-499.

Williams, K. M., Lucas, P. J., Bare, C. V., Wang, J., Chu, Y.-W., Tayler, E., Kapoor, V. and Gress, R. E. (2008). CCL25 increases thymopoiesis after androgen withdrawal. *Blood* **112**, 3255-3263.

Zaret, K. S. and Carroll, J. S. (2011). Pioneer transcription factors: establishing competence for gene expression. *Genes Dev.* **25**, 2227-2241.

Zhou, Q., Brown, J., Kanarek, A., Rajagopal, J. and Melton, D. A. (2008). In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. *Nature* **455**, 627-632.

Zlotoff, D. A., Sambandam, A., Logan, T. D., Bell, J. J., Schwarz, B. A. and Bhandoola, A. (2010). CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus. *Blood* **115**, 1897-1905.

Zook, E. C., Krishack, P. A., Zhang, S., Zeleznik-Le, N. J., Firulli, A. B., Witte, P. L. and Le, P. T. (2011). Overexpression of Foxn1 attenuates age-associated thymic involution and prevents the expansion of peripheral CD4 memory T cells. *Blood* **118**, 5723-5731.

DEVELOPMENT

Supplementary Figures
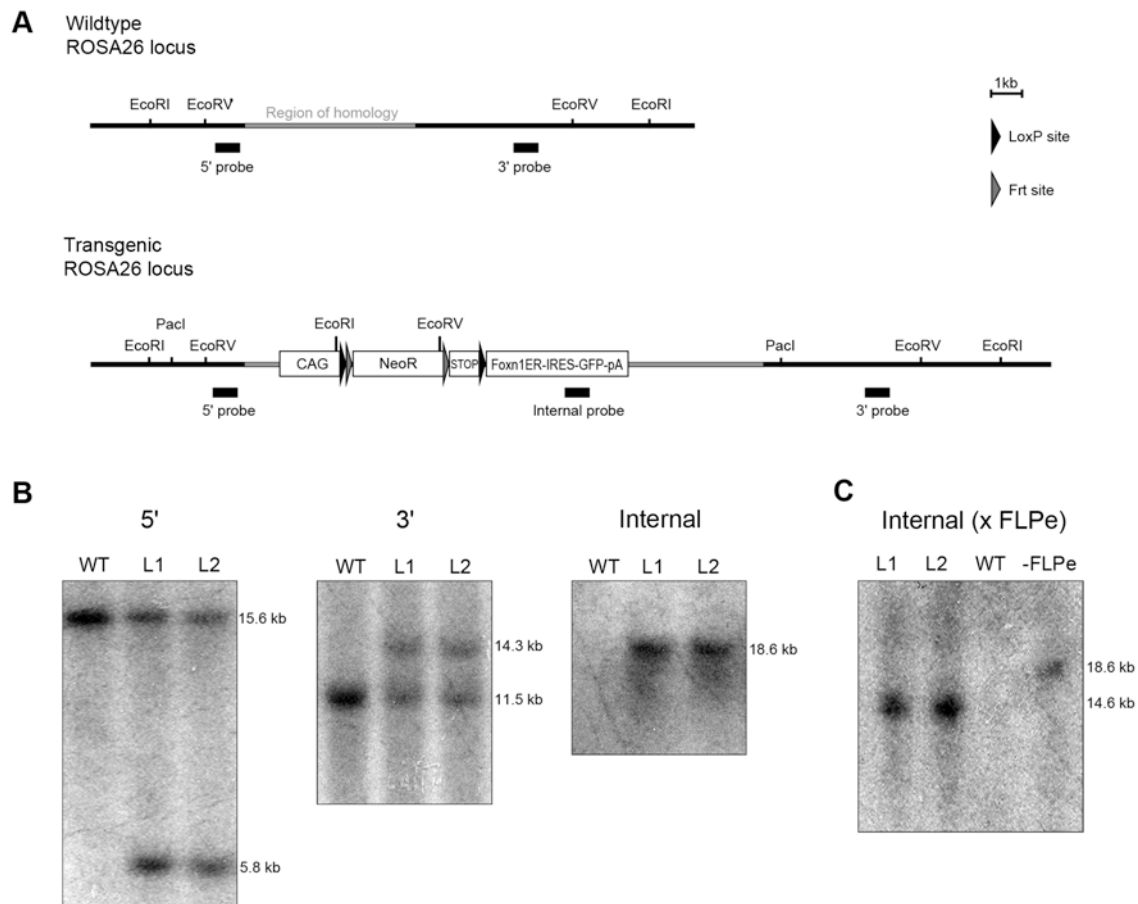
Figure S1. Generation of transgenic _Rosa26_^{_CAG-STOP-Foxn1ERT2-IRES-GFP_} mice. (A) The CAG-STOP-Foxn1ERT2 cassette shown in Figure 1 was introduced into the _Rosa26_ locus (Soriano, 1999) in mouse E14tg2a ES cells by homologous recombination using standard procedures, generating the _Rosa26_^{_CAG-NeoR-STOP-Foxn1ERT2-IRES-GFP_} allele. This cassette contained a cDNA encoding FOXN1ER^{T2}, under control of the CAG compound promoter and downstream of a LoxP-flanked CMAZ stop cassette (Ashfield et al., 1994), plus an IRES-GFP component to permit monitoring of _Foxn1ERT2_ expression. Neomycin-resistant colonies were picked and screened for targeted insertion by Southern blotting, using the strategy shown in (A) above. The position of restriction enzyme sites and Southern blot

hybridization probes are shown for the wild type and transgenic *Rosa26* locus. EcoRI, EcoRV and PacI restriction enzyme digests were used for 5′, 3′ and internal Southern blot analyses, respectively. **(B)** Correctly targeted colonies were identified (B) and used to generate chimeric mice via blastocyst injection. Germline transmission was confirmed from two independent ES cell lines (L1 and L2). **(C)** Founders from each of these *Rosa26*^{*CAG-NeoR-STOP-Foxn1ERT2-IRES-GFP*} lines were crossed with *Tg(CAG-FLPe)* mice (Wallace et al., 2007), in order to remove the neomycin resistance cassette (NeoR). Removal of this cassette was confirmed by Southern blotting**.** These lines were then backcrossed to C57BL/6 mice for three generations before analysis.

```
   1 - ATGGTGTCGCTACTCCCTCCGCAGTCTGACGTCACACTTCCAGGCTCCACCCGACTGGAGGGCGAACCCCAAGGGGACCTCATGCAGGCTCCGGGCCTCCCAGACTCCCCTGCCCCACAG -  120
   1 - M  V  S  L  L  P  P  Q  S  D  V  T  L  P  G  S  T  R  L  E  G  E  P  Q  G  D  L  M  Q  A  P  G  L  P  D  S  P  A  P  Q       -   40

 121 - AACAAGCATGCTAACTTCAGCTGCTCGTCGTTTGTGCCTGACGGCCCTCCAGAGAGGACACCCTCACTGCCCCCACACAGCCCCAGCATCGCATCTCCAGACCCAGAGCAGATCCAGGGC -  240
  41 - N  K  H  A  N  F  S  C  S  S  F  V  P  D  G  P  P  E  R  T  P  S  L  P  P  H  S  P  S  I  A  S  P  D  P  E  Q  I  Q  G       -   80

 241 - CACTGCACAGCCGGACCCGGCCCGGGCTCCTTCCGCCTTTCTCCTTCAGAAAAGTATCCTGGCTTTGGCTTTGAGGAGGGCCCAGCAGGCAGCCCAGGGCGCTTCCTCAAGGGCAACCAC -  360
  81 - H  C  T  A  G  P  G  P  G  S  F  R  L  S  P  S  E  K  Y  P  G  F  G  F  E  E  G  P  A  G  S  P  G  R  F  L  K  G  N  H       -  120

 361 - ATGCCTTTCCACCCCTACAAGAGGCACTTCCATGAGGACATCTTCTCTGAGGCCCAGACGGCCATGGCACTTGATGGACACTCCTTTAAGACTCAGGGGGCACTGGAAGCCTTTGAGGAG -  480
 121 - M  P  F  H  P  Y  K  R  H  F  H  E  D  I  F  S  E  A  Q  T  A  M  A  L  D  G  H  S  F  K  T  Q  G  A  L  E  A  F  E  E       -  160

 481 - ATCCCTGTGGACATGGGCGATGCTGAGGCCTTCCTGCCTAGCTTCCCAGCAGAGGCTTGGTGCAATAAACTCCCTTACCCCAGCCAGGAACACAACCAAATTCTGCAGGGGTCAGAGGTC -  600
 161 - I  P  V  D  M  G  D  A  E  A  F  L  P  S  F  P  A  E  A  W  C  N  K  L  P  Y  P  S  Q  E  H  N  Q  I  L  Q  G  S  E  V       -  200

 601 - AAGGTCAAGCCCCCAAGCTCTGGACAGTGGTCCTGGGATGTACTGCTACCAGCCTCCCTTGCAACATATGTACTGTTCTTCTCAGCCTGCCTTCCATCAGTACTCCCCGGGTGGAGGCAGC -  720
 201 - K  V  K  P  Q  A  L  D  S  G  P  G  M  Y  C  Y  Q  P  P  L  Q  H  M  Y  C  S  S  Q  P  A  F  H  Q  Y  S  P  G  G  G  S       -  240

 721 - TACCCTGTGCCCTACCTGGGCTCACCTCACTATCCCTATCAGAGGATTGCACCCCAGGCCAACGCCGAAGGTCACCAGCCACTCTTCCCAAAGCCCATCTACTCTTACAGCATCCTCATC -  840
 241 - Y  P  V  P  Y  L  G  S  P  H  Y  P  Y  Q  R  I  A  P  Q  A  N  A  E  G  H  Q  P  L  F  P  K  P  I  Y  S  Y  S  I  L  I       -  280

 841 - TTCATGGCCCTTAAGAACAGTAAGACCGGAAGCCTTCCAGTCAGTGAAATCTACAATTTCATGACGGAGCACTTCCCTTACTTCAAGACTGCTCCTGATGGCTGGAAGAATTCTGTTCGC -  960
 281 - F  M  A  L  K  N  S  K  T  G  S  L  P  V  S  E  I  Y  N  F  M  T  E  H  F  P  Y  F  K  T  A  P  D  G  W  K  N  S  V  R       -  320

 961 - CATAACCTGTCCCTCAACAAGTGCTTTGAGAAGGTGGAGAATAAATCCGGAAGTTCCTCTCGAAAGGGCTGTCTGTGGGCCCTCAATCCTTCCAAAATCGACAAGATGCAGGAAGAACTG - 1080
 321 - H  N  L  S  L  N  K  C  F  E  K  V  E  N  K  S  G  S  S  S  R  K  G  C  L  W  A  L  N  P  S  K  I  D  K  M  Q  E  E  L       -  360

1081 - CAGAAGTGGAAGAGGAAAGACCCCATTGCTGTGCGCAAAAGCATGGCCAAACCAGAAGAGCTGGACAGCCTCATTGGAGACAAAAGGGAAAAACTGGGCTCTCCGCTGCTGGGCGTGTCCA - 1200
 361 - Q  K  W  K  R  K  D  P  I  A  V  R  K  S  M  A  K  P  E  E  L  D  S  L  I  G  D  K  R  E  K  L  G  S  P  L  L  G  C  P       -  400

1201 - CCCCCTGGGCTGGCAGGCCCAGGTCCCATCCGGCCCATGGCACCATCAGCTGGTCTTTCCCAGCCTCTGCACCCAATGCATCCAGCTCCAGGCCCCATGCCTGGCAAGAACCCCCTGCAG - 1320
 401 - P  P  G  L  A  G  P  G  P  I  R  P  M  A  P  S  A  G  L  S  Q  P  L  H  P  M  H  P  A  P  G  P  M  P  G  K  N  P  L  Q       -  440

1321 - GACCTACTGGGTGGCCATGCTCCCTCCTGCTATGGGCAGACCTACCCACACCTTTCCCCCAGCCTGGCCCCTTCTGGACACCAGCAGCCATTGTTCCCACAGCCAGATGGGCATCTTGAG - 1440
 441 - D  L  L  G  G  H  A  P  S  C  Y  G  Q  T  Y  P  H  L  S  P  S  L  A  P  S  G  H  Q  Q  P  L  F  P  Q  P  D  G  H  L  E       -  480

1441 - CTGCAGGCCCAGCCAGGCACCCCCCAGGACTCACCTCTACCTGCCCCACACACCACCCAGCCACGGTGCCAAGCTGATGGCTGAGCCTTCCTCAGCCAGGACCATGCACGATACTCTACTG - 1560
 481 - L  Q  A  Q  P  G  T  P  Q  D  S  P  L  P  A  H  T  P  P  S  H  G  A  K  L  M  A  E  P  S  S  A  R  T  M  H  D  T  L  L       -  520

1561 - CCAGATGGAGACCTTGGGACTGACCTGGATGCTATCAACCCTTCTCTCACTGACTTCGACTTCCAGGGAAATCTGTGGGAGCAGCTGAAGGATGACAGCTTGGCCCTGGACCCCCTCGTA - 1680
 521 - P  D  G  D  L  G  T  D  L  D  A  I  N  P  S  L  T  D  F  D  F  Q  G  N  L  W  E  Q  L  K  D  D  S  L  A  L  D  P  L  V       -  560

1681 - TTGGTGACCTCGTCGCCGACGTCATCCTCCATGTTGCCACCCCCACCAGCAGCCCATTGCTTCCCCCCAGGGCCTTGTCTGGCAGAAACAGGCAATGAGGCAGGTGAACTGGCACCTCCA - 1800
 561 - L  V  T  S  S  P  T  S  S  S  M  L  P  P  P  P  A  A  H  C  F  P  P  G  P  C  L  A  E  T  G  N  E  A  G  E  L  A  P  P       -  600

1801 - GGCAGCGGCGGCTCCGGTGCTCTGGGAGACATGCACCTCAGCACTCTCTACTCCGCCTTTGTGGAACTGGAGTCCACGCCCTCCTCAGCAGCTGCCGGCCCTGCCGTGTACCTCAGTCCC - 1920
 601 - G  S  G  G  S  G  A  L  G  D  M  H  L  S  T  L  Y  S  A  F  V  E  L  E  S  T  P  S  S  A  A  A  G  P  A  V  Y  L  S  P       -  640

1921 - GGCTCAAAGCCATTGGCTCTGGCTGGAGGTGCTGGATCCGGAGACCCTTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACGCTCTAAGAAGAACAGC - 2040
 641 - G  S  K  P  L  A  L  A  G  G  A  G  S  G  D  P  S  A  G  D  M  R  A  A  N  L  W  P  S  L  M  I  K  R  S  K  K  N  S         -  680

2041 - CTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGTGCCTTGTTGGATGCTGAGCCCCCCATACTCTATTCCGAGTATGATCCTACCAGACCCTTCAGTGAAGCTTCGATGATGGGCTTA - 2160
 681 - L  A  L  S  L  T  A  D  Q  M  V  S  A  L  L  D  A  E  P  P  I  L  Y  S  E  Y  D  P  T  R  P  F  S  E  A  S  M  M  G  L       -  720

2161 - CTGACCAACCTGGCAGACAGGGAGCTGGTTCACATGATCAACTGGGCGAAGAGGGTGCCAGGCTTTGTGGATTTGACCCTCCATGATCAGGTCCACCTTCTAGAATGTGCCTGGCTAGAG - 2280
 721 - L  T  N  L  A  D  R  E  L  V  H  M  I  N  W  A  K  R  V  P  G  F  V  D  L  T  L  H  D  Q  V  H  L  L  E  C  A  W  L  E       -  760

2281 - ATCCTGATGATTGGTCTCGTCTGGCGCTCCATGGAGCACCCAGTGAAGCTACTGTTTGCTCCTAACTTGCTCTTGGACAGGAACCAGGGAAAATGTGTAGAGGGCATGGTGGAGATCTTC - 2400
 761 - I  L  M  I  G  L  V  W  R  S  M  E  H  P  V  K  L  L  F  A  P  N  L  L  L  D  R  N  Q  G  K  C  V  E  G  M  V  E  I  F       -  800

2401 - GACATGCTGCTGGCTACATCATCTCGGTTCCGCATGATGAATCTGCAGGGAGAGGAGTTTGTGTGCCTCAAATCTATTATTTTGCTTAATTCTGGAGTGTACACATTTCTGTCCAGCACC - 2520
 801 - D  M  L  L  A  T  S  S  R  F  R  M  M  N  L  Q  G  E  E  F  V  C  L  K  S  I  I  L  L  N  S  G  V  Y  T  F  L  S  S  T       -  840

2521 - CTGAAGTCTCTGGAAGAGAAGGACCATATCCACCGAGTCCTGGACAAGATCACAGACACTTTGATCCACCTGATGGCCAAGGCAGGCCTGACCCTGCAGCAGCAGCACCAGCGGCTGGCC - 2640
 841 - L  K  S  L  E  E  K  D  H  I  H  R  V  L  D  K  I  T  D  T  L  I  H  L  M  A  K  A  G  L  T  L  Q  Q  Q  H  Q  R  L  A       -  880

2641 - CAGCTCCTCCTCATCCTCTCCCACATCAGGCACATGAGTAACAAAGAATGGAGCATCGTACAGCATGAAGTGCAAGAACGTGGTGCCCCTCTATGACCTGCTGCTGGAGGCGGCGGAC - 2760
 881 - Q  L  L  L  I  L  S  H  I  R  H  M  S  N  K  R  M  E  H  L  Y  S  M  K  C  K  N  V  V  P  L  Y  D  L  L  E  A  A  D         -  920

2761 - GCCCACCGCCTACATGCGCCCACTAGCCGTGGAGGGGCATCCGTGGAGGAGACGGACCAAAGCCACTTGGCCACTGCGGGCTCTACTTCATCGCATTCCTTGCAAAAGTATTACATCACG - 2880
 921 - A  H  R  L  H  A  P  T  S  R  G  G  A  S  V  E  E  T  D  Q  S  H  L  A  T  A  G  S  T  S  S  H  S  L  Q  K  Y  Y  I  T       -  960

2881 - GGGGAGGCAGAGGGTTTCCCTGCCACAGCTTGA - 2913
 961 - G  E  A  E  G  F  P  A  T  A  *                                                                                             -  970
```

Figure S2. Sequence of the FOXN1ER^{T2} fusion protein.

The full length mouse *Foxn1* cDNA (red) was fused in-frame to cDNA encoding a mutated ligand binding domain of the human estrogen receptor (ER^{T2}, blue) (Feil et al., 1997). The fusion gene was constructed such that a glycine-rich linker (green) (Zeisig et al., 2004) separates the FOXN1 and ER^{T2} protein domains in the resulting FOXN1ER^{T2} fusion protein. This flexible linker was fused in frame to the sequence encoding the C-terminus of full-length FOXN1, without loss of any FOXN1 amino acids.
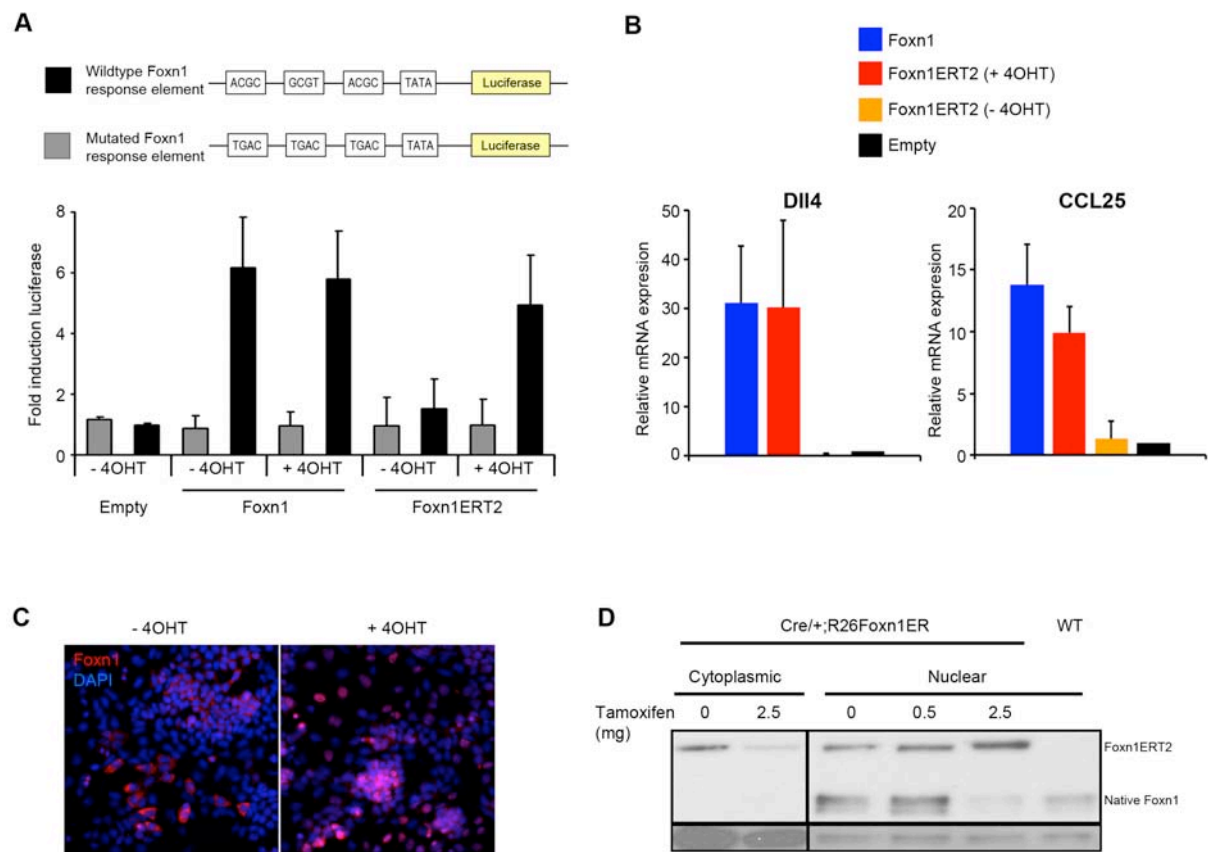
Figure S3. Verification of the transcriptional activity and tamoxifen inducibility of the Foxn1ERT2 fusion protein. (A) The FOXN1ER[T2] fusion protein is transcriptionally active. Wild-type and mutated FOXN1 transcriptional response elements (Schlake et al., 1997) upstream of a luciferase reporter (Janes et al., 2004) were stably transfected into COS-7 cells. Empty, Foxn1 or Foxn1ERT2 vectors (all driven by the CAG compound promoter (Chambers et al., 2003)) were transfected into these cells and luciferase expression assays were performed after 24 hours. In the presence of 4-hydroxytamoxifen (4OHT), FOXN1 and FOXN1ER[T2] induced luciferase at a similar level for the wild type response element (black), indicating a comparable transcription activity. FOXN1ER[T2] was also regulatable by 4OHT. Data represent 2 biological repeats. **(B)** *CAG-Foxn1* and *CAG-Foxn1ERT2* vectors were transiently transfected into E14Tg2A ES cells which were cultured in the presence or absence of tamoxifen, and RT-qPCR analysis for putative FOXN1 target genes, *Dll4* and *CCL25,* was

subsequently performed. FOXN1ER^{T2} and FOXN1 exhibited comparable abilities to regulate putative target genes, *Delta-like 4 (Dll4)* and *Chemokine (C-C motif) ligand 25 (CCL25)* (Bajoghli et al., 2009; Nowell et al., 2011) upon transfection *in vitro*. **(C)** FOXN1ER^{T2} expressed from the *Rosa26$^{CAG-STOP-Foxn1ERT2-IRES-GFP}$* allele is responsive to tamoxifen *in vitro*. *Rosa26$^{CAG-STOP-Foxn1ERT2-IRES-GFP}$* ES cells were transfected with a plasmid expressing Cre recombinase in order to excise the Stop cassette, then cultured in the absence and presence of 1μM 4-hydroxytamoxifen (4OHT) and subjected to immunohistochemical analysis of FOXN1 expression using an α-FOXN1 antibody. Panels show cytoplasmic and nuclear Foxn1 expression (red) in the absence (left image) and presence (right image) of 4OHT, respectively. DAPI, blue. **(D)** FOXN1ER^{T2} expressed from the *Rosa26$^{CAG-STOP-Foxn1ERT2-IRES-GFP}$* allele is responsive to tamoxifen *in vivo*. Image shows immunoblots of sub-cellular protein extracts (Nowell et al., 2011) from E14.5 TECs, following a single tamoxifen (Tam) injection of the amount shown at E13.5, after probing with α-FOXN1 or α-alpha-tubulin; top band is FOXN1ER^{T2}, bottom band is endogenous (native) FOXN1. Bottom panel, loading control (alpha-tubulin). ImageJ quantification of nuclear FOXN1ER^{T2} [arbitrary units] - 0mg tamoxifen, 1U; 0.5mg, 1.5U; 2.5mg, 3.75U. These data demonstrate increased nuclear FOXN1ER^{T2} protein following tamoxifen treatment, with a corresponding decrease in cytoplasmic FOXN1ER^{T2}. Of note is that endogenous FOXN1 protein levels diminished upon translocation of FOXN1ER^{T2} into the nucleus, suggesting the existence of a negative autoregulatory mechanism in fetal TEC.
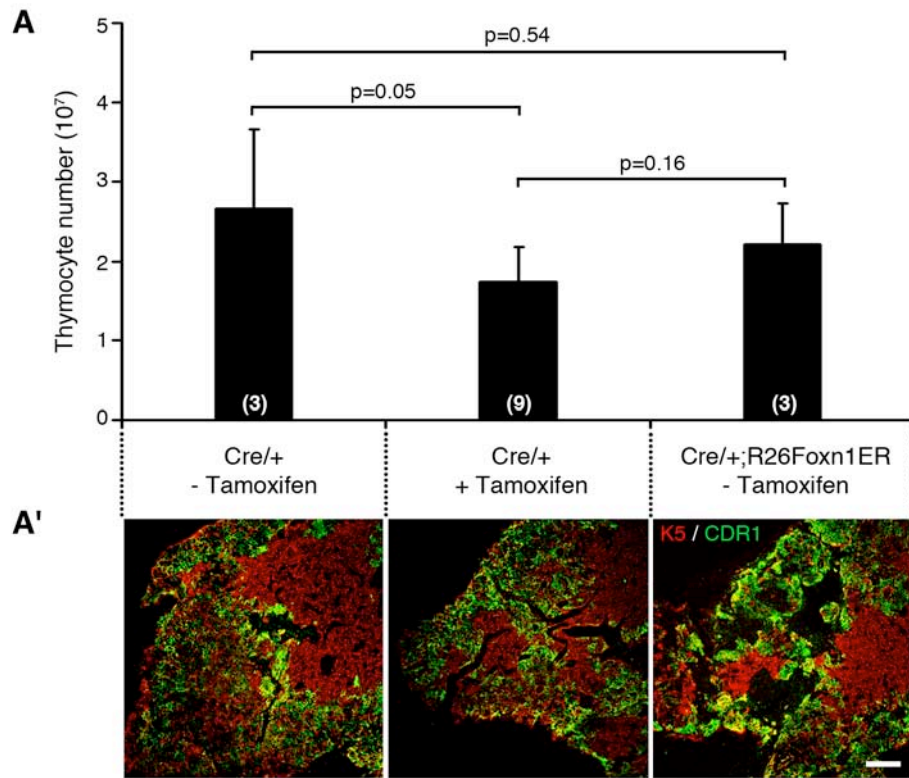
Figure S4. Analysis of thymi from control mice. (A) Total thymocyte number in thymi from 12 month old Cre/+ mice in the presence and absence of tamoxifen treatment, and Cre/+;R26Foxn1ER mice in the absence of tamoxifen treatment. (**A′**) Representative epithelial architecture for thymi from each of these conditions. Cytokeratin 5, mTEC; CDR1, cTEC. No difference in thymic architecture was observed between the different conditions. Scale bar, 100μm.
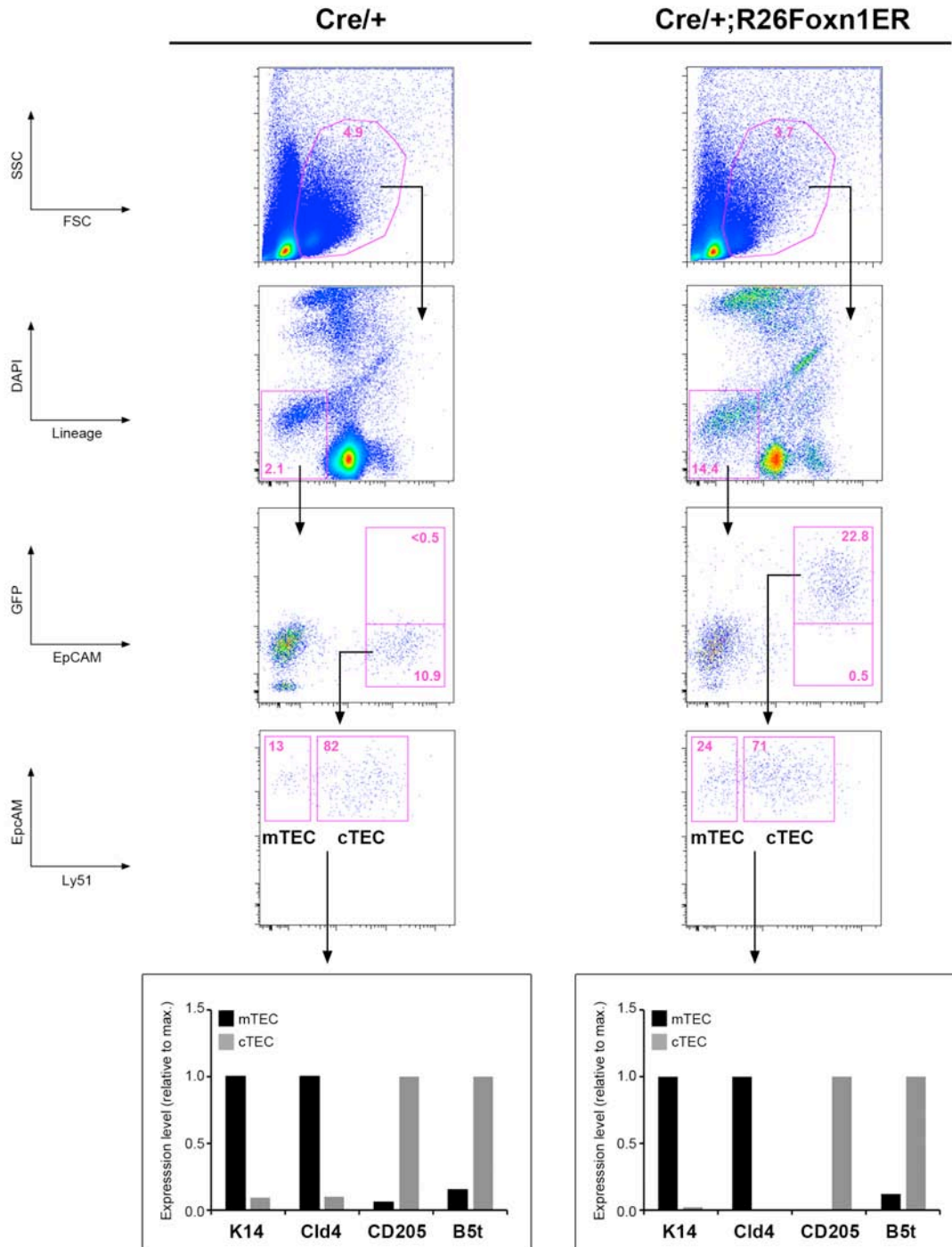
Figure S5. Strategy for flow cytometric isolation of mTEC and cTEC populations.

Representative plots show the gating strategy for isolation of mTEC and cTEC populations

from aged Cre/+ and Cre/+;R26Foxn1ER thymi (n > 10). Note that Cre/+;R26Foxn1ER TEC

are GFP$^+$ (expressed from the *Foxn1ER-IRES-GFP* biscistronic mRNA). The flow cytometric

isolation of TEC subsets was verified by gene expression analysis of mTEC and cTEC specific genes in the isolated populations (mTEC, K14 and Cld4; cTEC, CD205 and β5t).
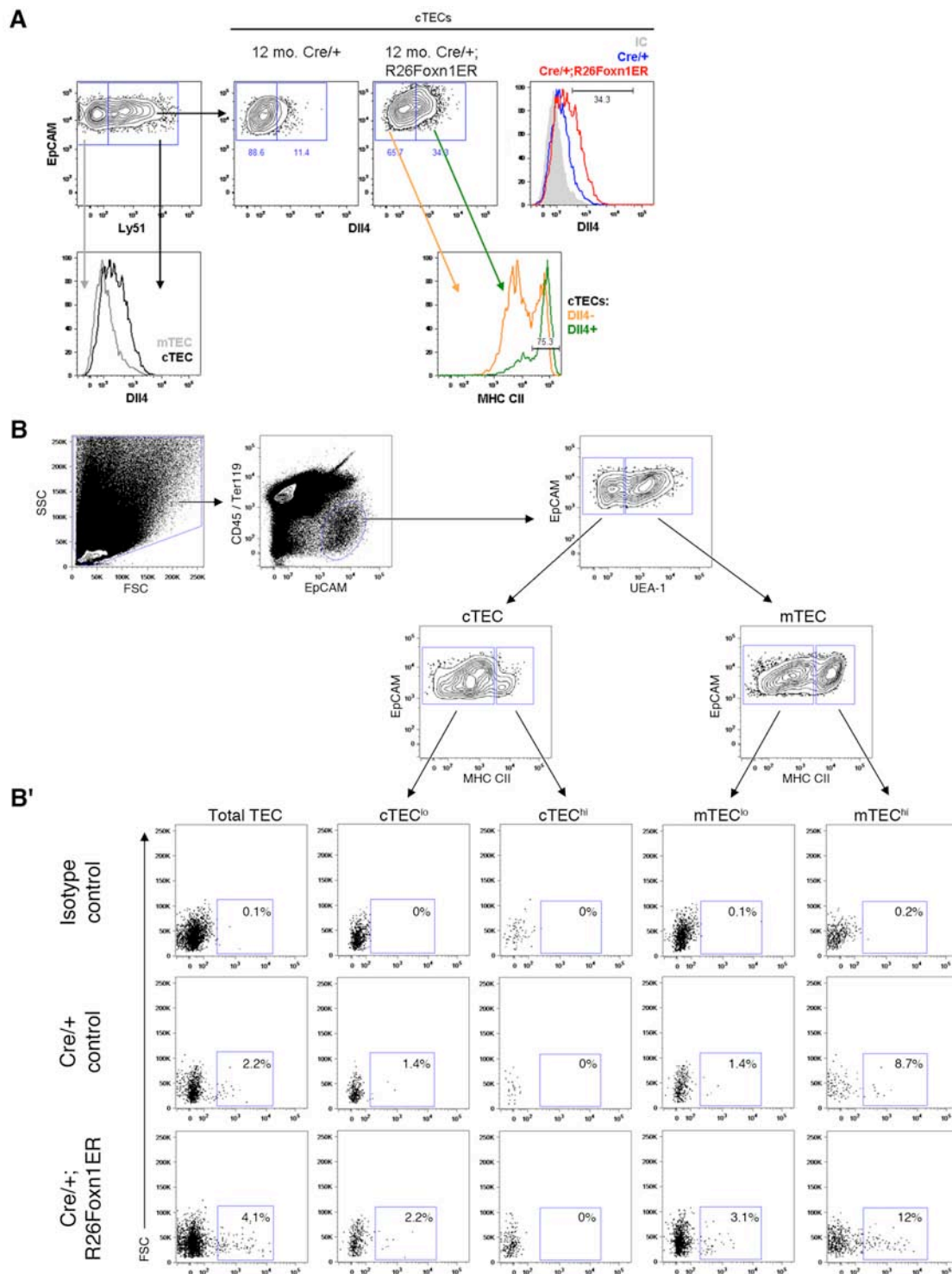
Figure S6. Analysis of Dll4 expression and proliferation in tamoxifen-treated R26Foxn1ER and control mice. (A) Flow cytometric analysis of Dll4 expression following induction of FOXN1 expression in aged thymi. Dll4 protein was detected only in cTEC and was predominantly present in MHC Class II^{hi} cells. IC, isotype control. **(B)** Effect of FOXN1

up-regulation on TEC proliferation. Flow cytometric analysis of Ki67 following induction of Foxn1 expression in 12 month-old thymi. Representative plots in (B) show the flow cytometric gating strategy used to resolve the major TEC subpopulations. Total TECs were identified in unenriched thymic cell preparations by gating against CD45$^+$ and Ter119$^+$ and for EpCAM$^+$ cells. Next, four major TEC subpopulations were identified: cTEC^{lo} (UEA1$^-$ MHC CII^{lo}), cTEC^{hi} (UEA1$^-$ MHC CII^{hi}), mTEC^{lo} (UEA1$^+$ MHC CII^{lo}) and mTEC^{hi} (UEA1$^+$ MHC CII^{hi}). (B′) Representative plots show Ki67 expression in these TEC subpopulations. All sample gates based on subpopulation-specific isotype control gates, as indicated.
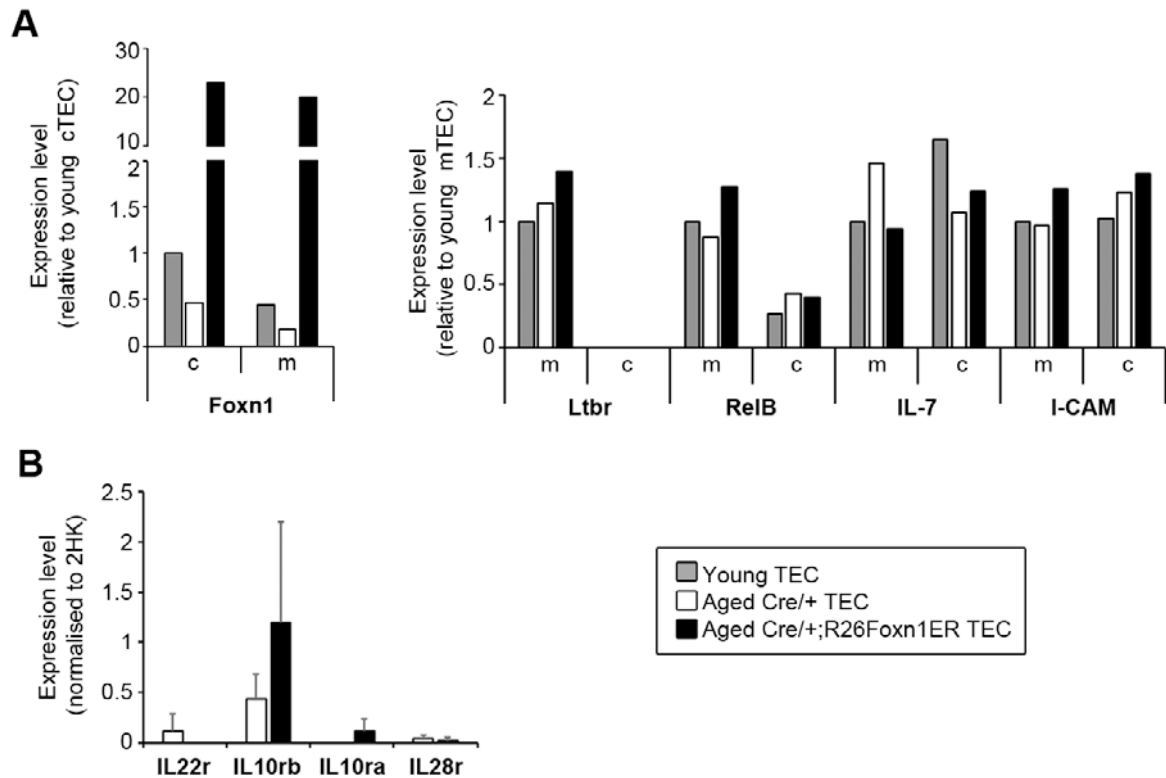
Figure S7. Analysis of gene expression in tamoxifen-treated R26Foxn1ER and control mice. (A) RT-qPCR analysis for genes in TEC isolated from tamoxifen-treated young (3 month old) Cre/+ mice and tamoxifen-treated aged (12-18 month old) Cre/+ and Cre/+;R26Foxn1ER mice. **(B)** RT-qPCR analysis of receptors for the interleukin 10 family of cytokines (Trivella et al., 2010; Wolk et al., 2010) in TEC isolated from 18-24 month old tamoxifen-treated Cre/+ and Cre/+;R26Foxn1ER mice. IL22R and IL10Rb form the receptor for IL20, IL22 and IL24. IL10Rb together with IL10Ra forms the receptor for IL10, and IL10Rb with IL28R forms the receptor for IL28A, IL28B, and IL29. mRNA expression is shown relative to the geometric mean of three housekeeper genes. We could not detect *IL22r* expression in tamoxifen-treated Cre/+;R26Foxn1ER TEC in aged mice indicating that IL22R signaling is not required for Foxn1-mediated thymus regeneration (n=3). c, cTEC; m, mTEC.
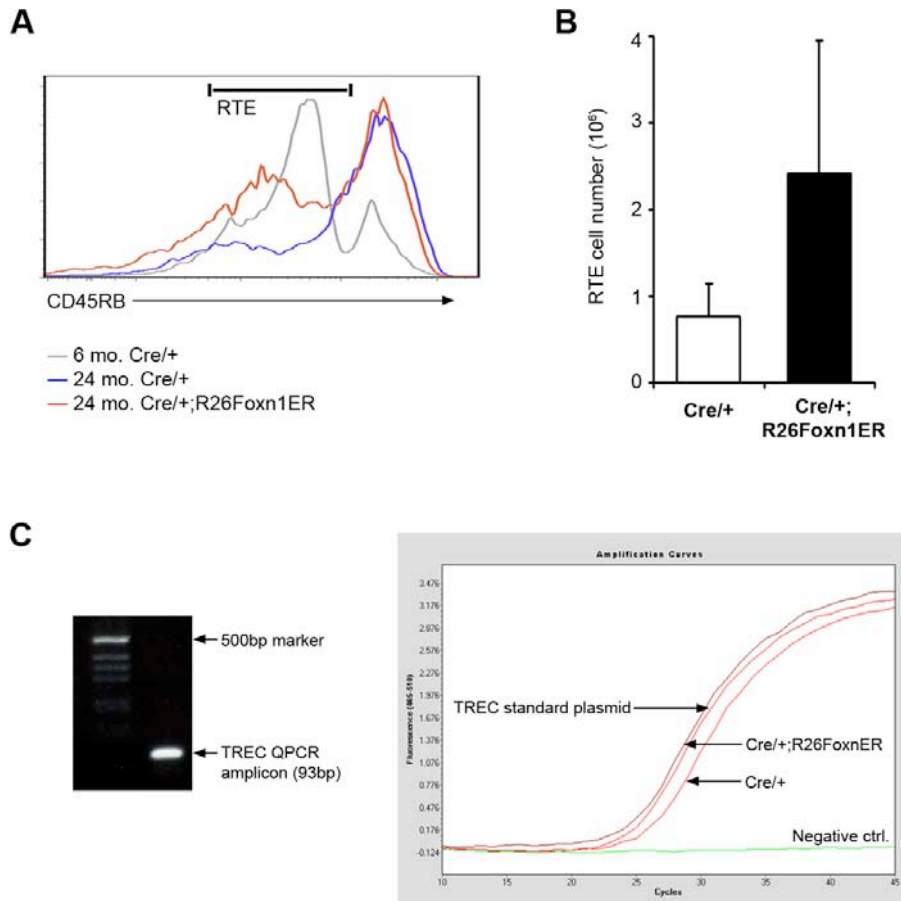
Figure S8. Analysis of recent thymic emigrants in tamoxifen-treated R26Foxn1ER and control mice. (A) Up-regulation of FOXN1 in the aged thymus results in increased numbers of peripheral naïve T cells. Histogram in shows CD45RB profile of CD4$^+$ splenocytes after gating on CD3$^+$CD4$^+$CD62L$^+$ cells. RTE are CD45RB^{lo}. The proportion of RTE was increased following tamoxifen-treatment of Cre/+;R26Foxn1ER mice (red line). **(B)** Graph shows numbers of RTE as defined in (A) in tamoxifen-treated Cre/+;R26Foxn1ER mice and tamoxifen-treated Cre/+ controls. n=4 for Cre/+ and n=5 for Cre/+;R26Foxn1ER, P=0.075. **(C)** Image shows PCR products from TREC analysis after separation on a 2% agarose gel, and representative graph of amplification curves from qPCR TREC analysis.

Supplementary Tables

Table S1. Antibodies used for IHC and flow cytometry.

Antibody	Clone	Supplier	Staining
Aire (D-17)	Polyclonal	SCBT	IHC
B220 PE-Cy7	RA3-6B2	BioLegend	FC – TN/Intrathymic B cell analysis
CD11b FITC	M1/70	E-Bioscience	FC – TN analysis
CD11c FITC	N418	E-Bioscience	FC – TN analysis
CD11c PerCP-Cy5.5	N418	E-Bioscience	FC – TEC isolation
CD25 PerCP-Cy5.5	PC61	BioLegend	FC – TN analysis
CD31 PerCP-Cy5.5	390	BioLegend	FC – TEC isolation
CD3ε FITC	145-2C11	E-Bioscience	FC – TN analysis
CD3ε PE	145-2C11	BioLegend	FC – Splenocyte analysis/isolation
CD4 FITC	RM4-5	BD Biosciences	FC – TN/CD4 v. CD8 analysis, Splenocyte analysis/isolation
CD4 PerCP-Cy5.5	RM4-5	E-Bioscience	FC – TEC isolation
CD44 APC	IM7	E-Bioscience	FC – TN analysis
CD44 APC-eFluor780	IM7	E-Bioscience	FC – Splenocyte analysis/isolation
CD45 APC	30-F11	E-Bioscience	FC – CD4 v. CD8 analysis
CD45 PerCP-Cy5.5	30-F11	E-Biosciences	FC – TEC analysis/isolation
CD45RB APC	C363.16A	E-Bioscience	FC – Splenocyte analysis/isolation

CD62L PE-Cy7	MEL-14	E-Bioscience	FC – Splenocyte analysis/isolation
CD8 PerCP-Cy5.5	53-6.7	E-Bioscience	FC – Splenocyte analysis/isolation
CD8α FITC	53-6.7	E-Bioscience	FC – TN analysis
CD8α PE	53-6.7	BD Biosciences	FC – CD4 v. CD8 analysis
CD8α PerCP-Cy5.5	53-6.7	E-Bioscience	FC – TEC isolation
CDR1	CDR1	Gift from B Kyewski	IHC
c-kit APC-eFluor780	2B8	E-Bioscience	FC – TN analysis
Cytokeratin 14 (AF 64)	Polyclonal	Covance	IHC
Cytokeratin 5 (AF 138)	Polyclonal	Covance	IHC
Cytokeratin 8	Troma1	DSHB	IHC
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)		Invitrogen	Viability dye
EpCAM APC	G8.8	BioLegend	FC – TEC analysis/isolation
Flt3 PE	A2F10	BioLegend	FC – TN/ETP analysis
Foxn1 (G-20)	Polyclonal	SCBT	IHC, Immunoblotting
Gr-1 FITC	RB6-8C5	E-Bioscience	FC – TN analysis
Ki-67 Isotype control (Mouse IgG1,k PE)	MOPC-21	BD Biosciences	FC – TEC analysis

Ki-67 PE	B56	BD Biosciences	FC – TEC analysis
Ly51 PE	6C3	BioLegend	FC – TEC analysis/isolation
MHC Class II PE-Cy7	M5/114.15.2	BioLegend	FC – TEC analysis/isolation
NK1.1 FITC	PK136	BD Biosciences	FC – TN analysis
Pan-Cytokeratin	Polyclonal	DAKO	IHC
TCRβ FITC	H57-597	BD Biosciences	FC – TN analysis
TCRβ PerCP-Cy5.5	H57-597	BioLegend	FC – TEC isolation
TCRγδ FITC	GL3	BioLegend	FC – TN analysis
Ter119 FITC	Ter119	E-Bioscience	FC – TN analysis
Ter119 PerCP-Cy5.5	Ter119	E-Bioscience	FC – TEC analysis/isolation
UEA-1 biotin	Lectin	Vector Labs	FC – TEC analysis/isolation
ΔNp63 (N-16)	Polyclonal	SCBT	IHC

Table S2. Sequences of primers for RT-qPCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Aire	GGTTCCTCCCCTTCCATC	GGCACACTCATCCTCGTTCT
Alpha-tubulin	CGGACCACTTCAAGGACTAAA	ATTGCCGATCTGGACACC
CCL25	GAGTGCCACCCTAGGTCATC	CCAGCTGGTGCTTACTCTGA
CD40	GAGTCAGACTAATGTCATCTGTGGTT	ACCCCGAAAATGGTGATG
CD80	TCGTCTTTCACAAGTGTCTTCAG	TTGCCAGTAGATTCGGTCTTC
Csna	GACATCTCTCAGGAACTCCACA	TCCATAGAATGAATAGAGAGACATGAG
Csnb	GGTGAATCTCATGGGACAGC	TGACTGGATGCTGGAGTGAA
CtsL	CAAATAAGAATAAATATTGGCTTGTCA	TCGTCTTTCACAAGTGTCTTCAG
Cxcl12	GGTTCTTCGAGAGCCACATC	TGTTCTTCAGCCGTGCAA
Cyclin D1	GAGATTGTGCCATCCATGC	CTCCTCTTCGCACTTCTGCT
Dll4	AGGTGCCACTTCGGTTACAC	GGGAGAGCAAATGGCTGATA
EVA	TGTGCTTCCACTTCTCCTGA	TCCACAGCTTCTGTAGGACAAA
FgfR2IIIb	CGGGGTGTTGGAGTTCAT	CCTGCGGAGACAGGTAACA
Foxn1	TGACGGAGCACTTCCCTTAC	GACAGGTTATGGCGAACAGAA
Fzd1	ATCTGGTCCGGCAAGACA	GCTGTTGGTAAGCCTCGTGT
Fzd3	GCAGCCTCCACAGGTCAC	ACATGCTGCCGTGAGGTAG
Fzd4	AACTTTCACGCCGCTCAT	CCGAACAAAGGAAGAACTGC
GAD67	ATACAACCTTTGGCTGCATGT	TTCCGGGACATGAGCAGT
HMBS	TCCCTGAAGGATGTGCCTAC	ACAAGGGTTTTCCCGTTTG
I-FABP	ACGGAACGGAGCTCACTG	TTACCAGAAACCTCTCGGACA
Ins2	GAAGTGGAGGACCCACAAGT	AGTGCCAAGGTCTGAAGGTC
Kit L	TCAACATTAGGTCCCGAGAAA	ACTGCTACTGCTGTCATTCCTAAG

Lrp6	TCCTCGAGCTCTGGCACT	CCTCCCCACTCAGTCCAATA
Pax1	CTCCGCACATTCAGTCAGC	TCTTCCATCTTGGGGGAGTA
Spt1	TGAAACTTCTGGAACTGCTGA	TTTTGATCAGTACTATCTGCCTGAG
TBP	GGGGAGCTGTGATGTGAAGT	CCAGGAAATAATTCTGGCTCA
Tff3	CTGGGATAGCTGCAGATTACG	CATTTGCCGGCACCATAC
Wnt10a	GGCGCTCCTGTTCTTCCTA	GTCGTTGGGTGCTGACCT
Wnt11	CAGGATCCCAAGCCAATAAA	TCCAGGGAGGCACGTAGA
Wnt4	ACTGGACTCCCTCCCTGTCT	TGCCCTTGTCACTGCAAA
Wnt5b	AGCACCGTGGACAACACAT	AAGGCAGTCTCTCGGCTACC
Wnt7a	CGCTGGGAGAGCGTACTG	CGATAATCGCATAGGTGAAGG
Wnta3a	CTTAGTGCTCTGCAGCCTGA	GAGTGCTCAGAGAGGAGTACTGG

References

Ashfield, R., Patel, A. J., Bossone, S. A., Brown, H., Campbell, R. D., Marcu, K. B. and Proudfoot, N. J. (1994) 'MAZ-dependent termination between closely spaced human complement genes', *EMBO J* 13(23): 5656-67.

Bajoghli, B., Aghaallaei, N., Hess, I., Rode, I., Netuschil, N., Tay, B. H., Venkatesh, B., Yu, J. K., Kaltenbach, S. L., Holland, N. D. et al. (2009) 'Evolution of genetic networks underlying the emergence of thymopoiesis in vertebrates', *Cell* 138(1): 186-97.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S. and Smith, A. (2003) 'Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells', *Cell* 113(5): 643-55.

Feil, R., Wagner, J., Metzger, D. and Chambon, P. (1997) 'Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains', *Biochem Biophys Res Commun* 237(3): 752-7.

Janes, S. M., Ofstad, T. A., Campbell, D. H., Watt, F. M. and Prowse, D. M. (2004) 'Transient activation of FOXN1 in keratinocytes induces a transcriptional programme that promotes terminal differentiation: contrasting roles of FOXN1 and Akt', *J Cell Sci* 117(Pt 18): 4157-68.

Nowell, C. S., Bredenkamp, N., Tetelin, S., Jin, X., Tischner, C., Vaidya, H., Sheridan, J. M., Stenhouse, F. H., Heussen, R., Smith, A. J. et al. (2011) 'Foxn1 regulates lineage progression in cortical and medullary thymic epithelial cells but is dispensable for medullary sublineage divergence', *PLoS Genet* 7(11): e1002348.

Schlake, T., Schorpp, M., Nehls, M. and Boehm, T. (1997) 'The nude gene encodes a sequence-specific DNA binding protein with homologs in organisms that lack an anticipatory immune system', *Proc Natl Acad Sci U S A* 94(8): 3842-7.

Soriano, P. (1999) 'Generalized lacZ expression with the ROSA26 Cre reporter strain', *Nat Genet* 21(1): 70-1.

Trivella, D. B., Ferreira-Junior, J. R., Dumoutier, L., Renauld, J. C. and Polikarpov, I. (2010) 'Structure and function of interleukin-22 and other members of the interleukin-10 family', *Cell Mol Life Sci* 67(17): 2909-35.

Wallace, H. A., Marques-Kranc, F., Richardson, M., Luna-Crespo, F., Sharpe, J. A., Hughes, J., Wood, W. G., Higgs, D. R. and Smith, A. J. (2007) 'Manipulating the mouse genome to engineer precise functional syntenic replacements with human sequence', *Cell* 128(1): 197-209.

Wolk, K., Witte, E., Witte, K., Warszawska, K. and Sabat, R. (2010) 'Biology of interleukin-22', *Semin Immunopathol* 32(1): 17-31.

Zeisig, B. B., Milne, T., Garcia-Cuellar, M. P., Schreiner, S., Martin, M. E., Fuchs, U., Borkhardt, A., Chanda, S. K., Walker, J., Soden, R. et al. (2004) 'Hoxa9 and Meis1 are key targets for MLL-ENL-mediated cellular immortalization', *Mol Cell Biol* 24(2): 617-28.