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Title: FOXN1 in thymus organogenesis and development

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Abstract

Development of the primary T-cell repertoire takes place in the thymus. The linked processes of T-cell differentiation and T-cell repertoire selection each depend on interactions between thymocytes and thymic stromal cells; in particular, with the epithelial cells of the cortical and medullary thymic compartments (cortical and medullary thymic epithelial cells; cTECs and mTECs, respectively). The importance of the thymic epithelial cell lineage in these processes was revealed in part through analysis of *nude* (*nu/nu*) mice, which are congenitally hairless and athymic. The *nude* phenotype results from null mutation of the forkhead transcription factor FOXN1, which has emerged as a pivotal regulator both of thymus development and homeostasis. FOXN1 has been shown to play critical roles in thymus development, function, maintenance, and even regeneration, which positions it as a master regulator of thymic epithelial cell (TEC) differentiation. In this review, we discuss current understanding of the regulation and functions of FOXN1 throughout thymus ontogeny, from the earliest stages of organogenesis through homeostasis to age-related involution, contextualising its significance through reference to other members of the wider Forkhead family.

A) Thymus development, homeostasis and involution
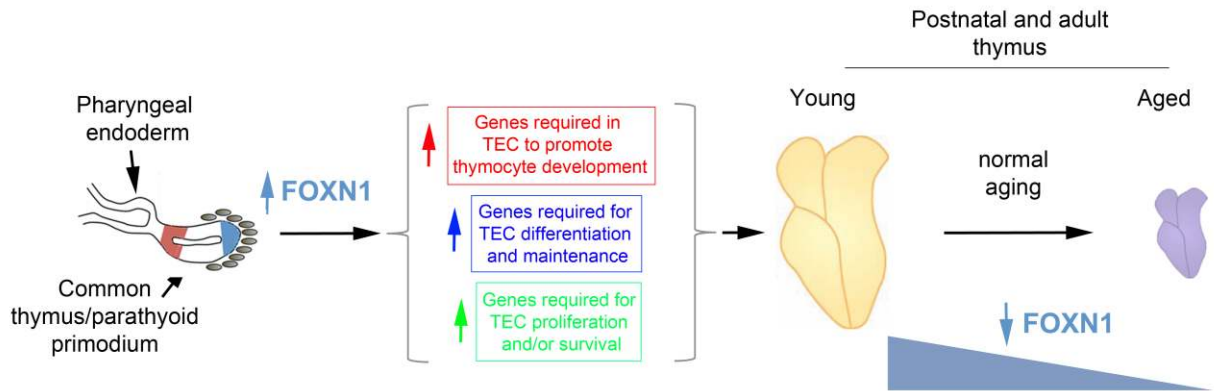
B) Regeneration of the aged thymus
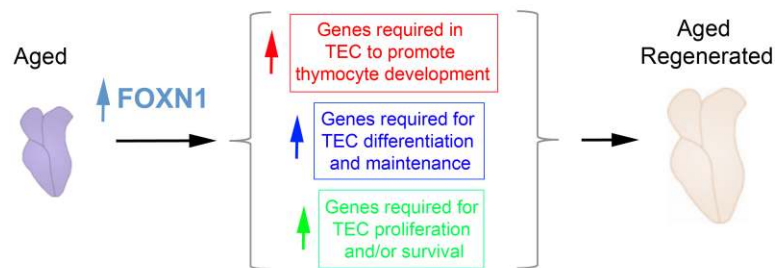
FOXN1 is a master regulator of thymus function, orchestrating thymic epithelial cell (TEC) differentiation and function in thymus development and homeostasis.

FOXN1 expression in TEC is downregulated with age.

TEC-specific restoration of high-level FOXN1 expression rejuvenates the fully involuted thymus

Expression of FOXN1 converts some other cell-types into functional TEC.

Introduction

The thymus is a central organ of the adaptive immune system due to its obligatory role in T-lymphocyte differentiation and repertoire selection [1]. These functions depend on the thymic stroma, which comprises a variety of cell types including mesenchymal cells, vascular endothelium, macrophages, dendritic cells and, importantly, a highly specialized epithelial compartment, which confers both structural and functional attributes to the organ. The thymus is divided into two broad regions, the cortex and the medulla (Figure 1). The epithelial cells in each of these compartments are functionally distinct, with cortical and medullary thymic epithelial cells (cTECs and mTECs, respectively) mediating different aspects of T-cell development [2-7]. T-cell development has been reviewed extensively elsewhere (see references [2-7]) and is not revisited in detail herein. Briefly, haematopoietic progenitors enter the thymus at the junction between cortex and medulla. Commitment to the T-cell lineage and differentiation as far as the CD4$^+$CD8$^+$ 'double positive' (DP) stage of T-cell development occurs in the cortex. As discussed in detail below, cTECs are required for commitment of haematopoietic cells to the T-cell lineage, and also mediate both the β selection and positive selection stages of T cell lineage development. Developing T cells (called thymocytes) that successfully undergo positive selection can then enter the medulla, the site of central tolerance induction, with tolerance induction being mediated by both mTECs and thymic dendritic cells (Figure 1). Not surprisingly, in view of their functional differences, cTECs and mTECs are also phenotypically distinct. These differences are discussed in further detail below but in brief, expression of Bp-1 (En-pep, the Ly-51 antigen) by cTECs, and binding of the lectin *Ulex europeaus* agglutinin 1 (UEA1) by mTECs identifies these TEC sub-lineages and permits their isolation and subsequent analysis. In the adult thymus, Ly-51$^+$ cTECs and UEA-1$^+$ mTECs each constitute heterogeneous populations that can be subdivided into a number of different subsets based on expression of additional surface markers, including MHC Class II [8-12].

The epithelial component of the thymus arises from the endoderm of the pharyngeal pouches (PPs). These structures are bilateral outpocketings of the foregut endoderm. The number of PPs varies between species; in mouse and human it is the third PPs (3PPs) that generate the thymus, while other PPs also contribute in some species [13, 14]. In mice, the 3PPs form at around day 9 of embryonic development (E9.0). This initial budding is followed by outgrowth and patterning stages, such that each 3PP forms a shared primordium for two organs – the thymus and the parathyroid glands. These organ primordia can be distinguished on the basis of marker expression by E10.5 in mouse, when transcription factor Glial cells missing 2 (Gcm2) mRNA specifically delineates the parathyroid domain, and eventually separate from the pharyngeal endoderm and resolve into discrete organ primordia by about E12.5 [15]. In humans, the thymus domain within the 3PP is evident by week 6 of gestation [16]. The endodermal thymic rudiment within the 3PP is sufficient to direct thymus development, even after transplantation to an ectopic site [17], and appears to contain bipotent TEPCs that can generate both cortical and medullary TECs [18-21]. However, the normal process of thymus organogenesis involves interplay between a number of different cell types – including 3PP endoderm, neural crest-derived mesenchyme, endothelial progenitors and haematopoietic progenitors – all of which are components of the mature organ (reviewed in [22-26]) (Figure 2).

Some of the earliest insights into the function of the thymus came from studies on *nude* (*nu/nu*) mice, which carry an autosomal recessive mutation leading to congenital hairlessness and athymia [27, 28]. *Nu/nu* mice are correspondingly immunocompromised as they lack normal T-cell populations [27, 28]. The functional athymia in *nu/nu* mice results from a severe developmental block early in thymus organogenesis. The common thymus-parathyroid primordium forms normally and thymus organogenesis proceeds until E11.5-

E12.0. However, a maturational arrest in thymic epithelial progenitor cells occurs at around E12.0 [29], such that the *nude* thymic epithelium never becomes competent to support T-cell development. Indeed, the *nude* thymic rudiment is never colonized by haematopoietic or vascular progenitors; instead, these remain in the perithymic mesenchyme [30, 31]. Adult *nu/nu* mice retain a small, cystic, alymphoid thymic rudiment, which does not support T-cell development at any stage in ontogeny.

Identification of *Foxn1* as the nude gene

Foxn1 was originally identified as the gene mutated in *nu/nu* mice using genetic approaches [32, 33]. Following localization of *nu* to chromosome 11 in mice and subsequent fine-mapping, a member of the forkhead or winged helix superfamily, originally named winged helix nude (*whn;* later renamed *Foxn1*), was identified as the *nude* gene by positional cloning [32, 34]. Initial studies showed the *whn* transcript in *nu/nu* mice carried a single base pair deletion in its third exon, resulting in the absence of *Whn* mRNA due to nonsense-mediated decay [32]. RT-PCR analysis revealed that *Whn* was expressed in the developing mouse embryo from E9.5, and was restricted to skin and thymus in adult tissues [32]. Subsequently, a targeted null allele of *Whn* was generated by inserting a *lacZ M2-neo* cassette into exon 3 of *Whn*, close to the site of the spontaneous mutation in *nu*. Mice homozygous for this allele phenocopied *nu/nu* mice, confirming *Whn* as the *nude* gene [33].

The Forkhead family of transcription regulators

FOXN1 was one of the first members of the forkhead (FOX) superfamily of transcription factors (TFs) to be implicated in a specific developmental defect in vertebrates [35]. It is now known that this large family of TFs has important roles in the development, homeostasis,

function and aging of a variety of organs and tissues - including the immune system [36, 37]. As examples, FOXP3 is needed for the development and function of regulatory T cells (Treg cells) [38-40], FOXJ1 for suppression of T-cell activation [41], and FOXO3 for lymphocyte proliferation and apoptosis [42, 43], while FOXN1 itself is essential for production and maintenance of a functional thymus and is also required for hair production [29, 33, 44-48].

The FOX family is evolutionarily ancient. Its canonical member is the *Drosophila melanogaster* gene *fork head* (*fkh*) which, when mutated, causes a spiked head phenotype in adult flies [49]. Identification of the rat gene hepatocyte nuclear factor 3 alpha (*HNF3α*) in 1990 revealed an approximately 100 amino acid region of high homology between the HNF3α and fkh proteins, that was suggested to be a DNA binding domain (DBD) [35, 50]. TFs containing this 'winged helix' or 'forkhead' DBD (Structural Classification of Proteins (SCOP) classification n°46785) were subsequently identified in Eubacteria, Archaea, and Eukaryota, and classified as a new superfamily. More than 2000 FOX family members have now been identified in 108 species of animals and fungi, with numbers differing between species [36][51-53]. FOX proteins are currently classified based on phylogenetic analysis of their DBDs (forkhead domains), which are highly conserved and represent the only regions of peptide sequence that can be confidently aligned across all FOX proteins [34, 54]. Nineteen subclasses of FOX protein have now been identified - FOXA to FOXS - and of these, FOXQ, FOXR, and FOXS are vertebrate-specific [54].

The FOXN subfamily

The FOXN genes cluster separately from other FOX sub-classes, and the gene most closely related to *FOXN1* is its paralogue *FOXN4.* The evolutionary history of the FOXN subclass as currently understood is depicted in Table 1 [55, 56]. *Foxn4* first appeared in

cephalochordates (amphioxus) and is found in all higher organisms. Cephalochordates also contain a more ancient paralogue *Foxn4b*, which is present in Echinodermata and Cnidaria but absent from urochordates and all vertebrates. Jawless fish possess a gene very similar to *Foxn4*, termed *Foxn4-like* (*Foxn4L*). *Foxn1* is thought to be an orthologue of *Foxn4L*, based on protein sequence and short-range synteny relationships. The expression patterns of these genes further support this genealogy: *Foxn4* (or *Foxn4a*) being expressed in the pharyngeal endoderm and other sites in amphioxus; *FOXN4L*, in the epithelium lining the gill basket in lamprey; and *Foxn1* in the pharyngeal pouches giving rise to the thymus in cartilaginous fishes and all other jawed vertebrates [48]. The expression of *Foxn4* in the pharyngeal endoderm in amphioxus suggests that this gene contributed to the evolutionary emergence of thymopoiesis [48]. Indeed in evolution, the emergence of FOXN4L and thymus-like function preceded the pinching off of pharyngeal pouches into a distinct organ, as observed in the lamprey gill basket. Both *Foxn1* and *Foxn4* are expressed in the thymi of catshark, zebrafish, and medaka; however *Foxn4* is either not expressed or expressed at very low levels in higher order organisms [57]. Indeed, *Foxn1* appears to have a unique role in the thymus in jawed vertebrates, which cannot be completely substituted by *Foxn4* [57].

Transcriptional regulation by FOX proteins

The classical 'forkhead' DBD consists of three N-terminal α-helices (H1, H2, H3), three β-sheets (S1, S2, S3) and two C-terminal 'wing' regions/loops (W1, W2), arranged in the order H1-S1-H2-H3-S2-W1-S3-W2 [58]; an additional α-helix is present in some FOX proteins [59, 60]. The term 'winged helix' was coined to reflect the butterfly-like winged structure adopted by DNA-bound FOX proteins [59], which resembles structures formed during DNA interactions with linker histones such as H1 and H5 [59]. The DNA binding specificity of the forkhead domain depends on the variable region at the junction of the α-helices and wing

loops, which interact with bases in minor groove of DNA [61]. Whilst all FOX proteins share the forkhead domain, their specific functions are thought to reside in their transactivation or repression domains, which show almost no sequence homology between superfamily members [37]. Functional diversity is also determined by differences in interaction partners and spatio-temporal expression patterns such that, while FOX superfamily members have largely distinct functions, some functional overlap exists between members of the same sub-group [54].

Most FOX factors appear to bind to DNA as monomers [59], however cases of homodimers [62] and heterodimers [63] have also been documented. FOX proteins also interact with non-transcription factor proteins such as co-activators, co-repressors, and enzymes. Some FOX proteins also have posttranslational modifications - including phosphorylation, acetylation, methylation, and ubiquitination - which affect their binding affinity and specificity, nuclear localization, and stability [36]. Finally, FOX proteins act as effector molecules for several signaling pathways, coupling extra-cellular signals to changes in gene expression [36].

Recently, much interest has focused on the capacity of some TFs to act at the level of chromatin organization, opening regions of previously compacted chromatin thus enabling their transcription. TFs with this activity are called 'pioneers'. Pioneer factors are defined as being able to access their target sequence on nucleosomes and certain forms of compacted chromatin, to bind nucleosomes stably and before the binding of other TFs or initiation of the target gene expression, and to possess chromatin-opening capabilities [64]. Forkhead TFs – specifically the FOXA proteins FOXA1 and FOXA2 required for hepatic development [65] - were among the first to be identified as having 'pioneer' function. Detailed investigation of the interaction of the FOXA proteins with chromatin showed they could stably bind their

target sequences on *in vitro* assembled nucleosomes, and could open the local nucleosomal domain through the activity of their C-terminal domain [66, 67]. The similarities between the 'winged-helix' structure of the forkhead domain and structures of linker histones is thought to explain how these TFs can displace linker histones from compacted chromatin, even in the absence of the SWI-SNF chromatin remodeling complex. Whether FOXN1 has a similar pioneer activity within the TECs remains to be determined. Such activity could explain the broad range of functions regulated by FOXN1 in TECs (see below). However, while the FOXA factors are required for liver specification, FOXN1 does not appear to required for specification of the TEC lineage (see below), indicating the potential for different functional requirements from these two classes of FOX proteins.

FOXN1 in thymus development

Specification of the TEC lineage is independent of FOXN1

As discussed above, low-level *Foxn1* expression is evident in the pharyngeal endoderm as early as mouse E9.5 - the time of the initial outpocketing of the 3PP [32]. However, high-level expression is evident only from E11.25 [15]. This strong expression initiates in the most ventral tip of the 3PP and subsequently expands to encompass the entire thymus domain (see Figure 2). Histological analysis has established that FOXN1 is not required for formation of the 3PP or the thymic primordium itself [30, 68] and in keeping with this, several lines of evidence indicate that FOXN1 does not specify the thymic epithelial lineage [17, 29, 48, 68]. Following ectopic transplantation, the E9.0 3PPs (which do not yet express *Foxn1*) can generate an intact and functional thymus containing both cortical and medullary thymic epithelial compartments, indicating that 3PP cells are already committed to the TEC lineage [17]. Furthermore, both forkhead transcription factor g1 (*Foxg1*) and interleukin 7 (*Il7*) specifically mark the thymus domain of the 3PP and for each, this expression occurs

independently of FOXN1 [69, 70]. Additionally, analyses of revertible null or severely hypomorphic alleles of *Foxn1* have shown that thymic epithelial progenitor cells (TEPC) that lack *Foxn1* expression undergo a developmental arrest that can be reversed in neonatal and adult mice [21, 29, 71]. Thus, the fetal TEPC state appears to be extremely stable in vivo, strongly suggesting the presence of a stable transcriptional network upstream of *Foxn1* that confers TEPC identity and thus thymic epithelial lineage specification. Overall, these studies indicate that TEC lineage commitment does not depend solely on FOXN1, implicating an as yet unidentified genetic network in this process.

FOXN1 in TEC differentiation

As discussed above, the absence of functional FOXN1 arrests fetal TEPC in a bipotent progenitor cell state, and normal functioning in these developmentally arrested fetal TEPCs can be restored by permitting normal FOXN1 expression. This was initially demonstrated using a revertible *Foxn1* allele, *Foxn1*SA2 [21]. Using a Cre-ERT2 system that exhibited low-level activity in the absence of tamoxifen induction, reactivation of *Foxn1* in a single cell in the thymic rudiment of *Foxn1* null mice was shown to result in the generation of miniature thymi, each containing well-defined cortical and medullary areas [21]. How these findings relate to in vivo development is however still open to question, since $Foxn1^{-/-}$ thymi contain cytokeratin 5^{hi}, claudin 4^{hi} ($K5^{hi}Cldn4^{hi}$) and $K5^{-}Cldn4^{lo/-}$ regions. Since mTEC-restricted progenitors in the early fetal thymus are $Cldn4^{hi}$[72, 73], this suggests that the emergence of the mTEC sub-lineage may be *Foxn1*-independent and thus that the divergence of the cTEC and mTEC sub-lineages may occur earlier than implied by this particular genetic analysis [21, 48]. Current understanding is therefore that thymus organogenesis can be considered as two stages: early *Foxn1*-independent development which results in generation of the undifferentiated thymic primordium containing specified TEPCs, and later *Foxn1*-dependent

development in which FOXN1 expression in TEPCs results in their differentiation and the concomitant orchestrated development of the fully patterned and functional thymus [17, 21, 33, 70, 71].

How does FOXN1 effect these later stages of development, and what are its subsequent roles in thymus maintenance and function? Studies on FOXN1 function in early thymus development have revealed a role in TEC proliferation [31] and have also demonstrated its essential role in conferring competence to attract haematopoietic and endothelial progenitors upon TECs in the thymic rudiment [31, 74-76]. Furthermore, FOXN1 has been shown to regulate the maturation and migration of the neural crest cells that will form the thymic mesenchyme [48]. Interestingly, the expression of *Vegf-a* and *Pdgf-b* in TECs, thymic vasculature-associated mesenchyme, and endothelium, was severely reduced under conditions of low *Foxn1* expression, with the thymic rudiment showing fewer capillaries, leaky blood vessels, disrupted endothelium-perivascular cell interactions, endothelial cell vacuolization, and an overall failure of vascular organization at later stages of organ development [76, 77]. Thus, FOXN1 appears to regulate not only the initial colonization of the thymus with endothelial progenitors, but also normal vascularization of the organ [77].

Further insight into the role of *Foxn1* in TEC differentiation has been provided by studies in which *Foxn1* is either under- or over-expressed specifically in TECs. For example, mice homozygous for a hypomorphic *Foxn1* allele, *Foxn1*$^{\Delta}$, whose transcript lacks the N-terminal domain of FOXN1, develop severely hypoplastic and cystic thymi that lack distinct cortical and medullary regions [44]. T-cell development appears relatively normal in the fetal *Foxn1*$^{\Delta/\Delta}$ thymus. However, the adult *Foxn1*$^{\Delta/\Delta}$ thymus supports only aberrant thymopoeisis, characterized by the absence of CD25^{+} thymocytes (DN3 cells) and reduced expression of

TCRβ at the DP stage. Analysis of $Foxn1^{\Delta/\Delta}$ mice is consistent with TEC differentiation being initiated, but then blocked at an intermediate stage of development, such that the $Foxn1^{\Delta/\Delta}$ thymus has impaired functionality compared with that of the wild type [44]. A second severely hypomorphic $Foxn1$ allele, $Foxn1^{R}$, allowed investigation of FOXN1 function in TEC differentiation independently of its role in proliferation [48]. $Foxn1^{R}$ generates only around 15% of WT levels of normal $Foxn1$ transcripts, which results in development in $Foxn1^{R/R}$ mice of a hypoplastic thymus which can only sub-optimally support T-cell development such that fewer thymocytes are generated during differentiation. Analysis of an allelic series based on the $Foxn1^{R}$, $Foxn1^{-}$ and wild-type $Foxn1$ alleles revealed strong dose-dependent effects of $Foxn1$, in brief showing that increasing levels of $Foxn1$ expression are required for progression through multiple intermediate stages of TEC development – from exit from the earliest progenitor cell state(s) through to terminal differentiation, in both cTEC and mTEC sub-lineages in the fetal and adult thymus [48].

Despite 20 years having elapsed since confirmation of its identity as the *nude* gene product, the molecular functions of FOXN1 have not yet been determined in full, and indeed no direct targets have yet been verified in TECs by chromatin immunoprecipitation. However, FOXN1 has been shown to be required for the expression in TECs of proteins with essential roles in promoting thymocyte development, including Chemokine (C-C Motif) Ligand 25 (CCL25), C-X-C motif chemokine 12 (CXCL12; also known as stromal cell-derived factor 1 (SDF1), Delta-like ligand 4 (DLL4), Stem cell factor (SCF; also known as SCF, KIT-ligand, KL, or steel factor), Cathepsin L (CTSL), the 20S proteasome subunit beta-5t (β5t; also known as Psmb11) and MHC Class II [45, 48, 78]. CCL25 and CXCL12 are chemokines which are required for attracting thymic seeding cells into the developing thymic rudiment and the adult thymus [74, 79, 80]. DLL4 is a Notch ligand required for TECs for commitment of

haematopoietic progenitors to the T-cell lineage [81, 82], and SCF is required for thymocyte survival and proliferation [83]. Cathepsin L and β5t regulate the production of peptides required in TECs to effect optimal positive selection of CD4$^+$ and of CD8$^+$ thymocytes, respectively [84-87], while MHC Class II expression is critical for positive and negative selection of CD4$^+$ T cells. Notably, transgenic expression of *Dll4*, *Ccl25*, *Cxcl12* and *Scf* conferred some capacity to support production of CD4$^+$ and CD8$^+$ SP T cells to the *Foxn1*$^{-/-}$ thymic rudiment, although the TEPCs within the rudiment remained in an undifferentiated state [78]. Since the *Foxn1*$^{-/-}$ thymus expressing transgenic *Dll4*, *Ccl25*, *Cxcl12*, and *Scf* lacked functional TECs and normal thymus architecture, this study established that *Foxn1* must regulate additional genes that are required for TEC differentiation and function. In keeping with this observation, in addition to the genes discussed above, the genes encoding transformation related protein 63 (*Trp63*), Paired Box 1 (*Pax1*), Fibroblast growth factor receptor 2 isoform IIIB (*Fgfr2IIIb)*, Autoimmune regulator (*Aire*), Cluster of differentiation 40 (*CD40*), Cluster of differentiation 80 (*CD80*), and Cyclin D1 (*Ccnd1)*, which have known roles in TEC differentiation, proliferation, or function, and of several genes involved in Wnt signaling, are all FOXN1 responsive in TECs [48, 88]. Thus, although further insights, including delineation of which of these genes are direct FOXN1 targets, are undoubtedly required, the range of genes and breadth of functions known to be affected by FOXN1 expression already provides an indication of how this single TF can orchestrate thymus organogenesis and function.

Foxn1 in thymus homeostasis and involution

TECs in the adult thymus continue to express *Foxn1* [33], with cTECs expressing higher levels than mTECs, and MHC Class II^{hi} cells expressing higher levels of *Foxn1* than MHC Class II^{lo} TECs in each compartment [48, 89-91]. Several studies have demonstrated the

importance of FOXN1 in maintenance of the adult thymus [45-47]. Interestingly, down-regulation of *Foxn1* expression in the thymic stroma is one of the earliest events in the age-associated degeneration of the thymus [92], suggesting that FOXN1 could play an important role in postnatal thymus homeostasis and subsequent thymic involution. This hypothesis was supported by analysis of a *Foxn1* allele (*Foxn1*[lacZ]), which expresses normal levels of *Foxn1* in the fetal and newborn thymus, after which *Foxn1* expression declines to 20-30% of wild type levels by 5 weeks after birth [45]. *Foxn1*[lacZ/LacZ] mice exhibit a premature loss of thymus homeostasis, correlating with *Foxn1* downregulation, that phenocopies many of the hallmarks of age-related involution [45]. The TEC subsets most affected were those that normally express high levels of *Foxn1*, indicating their continued FOXN1 dosage sensitivity [45]. This study provided the first functional evidence linking FOXN1 down-regulation with age-related thymic involution. Consistent with this, ubiquitous deletion of *Foxn1* or of FOXN1^{+} cells in postnatal mice, resulted in rapid thymic atrophy, further supporting a role for FOXN1 in thymus homeostasis [46, 47, 93].

Foxn1 expression in the involuting thymus has recently been investigated at the single cell level. Using a new antibody generated against the C-terminus of FOXN1 protein and a tagged version of FOXN1, Rode and colleagues showed that in aging mice, *Foxn1* expression progressively decreases and there is an age-related accumulation of *Foxn1*^{-/low} TECs [90]. Lineage tracing of *Foxn1*-negative TECs has shown that these cells arise from Foxn1-positive precursors [47, 91, 94]. A second study used a *Foxn1*-eGFP reporter mouse line (in which eGFP was knocked into the *Foxn1* locus) to investigate transcriptional changes in *Foxn1* expression with age, and similarly showed that the emergence of *Foxn1*^{-} TECs correlates with the onset of age-related thymic involution [91]. This study suggested down-regulation of FOXN1 in a subset of cTECs as a primary event in age-related thymic

involution, and further showed that this FOXN1 downregulation correlated with diminished cTEC functionality based on decreased expression of genes required in cTECs to promote T-cell differentiation. Together, these analyses suggest that both the onset and progression of involution are the result of declining *Foxn1* expression.

The emerging evidence, discussed above, suggesting that down-regulation of FOXN1 might be a primary cause of age-related thymic involution has recently been tested in two studies, which have respectively determined the outcome of maintaining high-level FOXN1 expression throughout ontogeny [95], and of up-regulating FOXN1 function in the aged thymus [88]. The first used a strain of transgenic mice, hK14-Foxn1 (also known as Foxn1tg). In this strain the mouse *Foxn1* cDNA, under control of the human K14 promoter, was introduced into the genome by random insertion, resulting in twenty-fold over-expression of *Foxn1* in TECs due to multiple copies of the transgene [95]. These mice initially exhibited increased thymus size, with increased thymic output and numbers of early thymocyte progenitors (ETPs) [95]. However, although age-related thymic involution was delayed in this model, it was not prevented (Figure 3A,B) [95]. This suggested that FOXN1 is a target in age-related thymic involution, but that other targets might also exist.

The second study, from this laboratory, used a novel transgenic mouse strain, R26-*Foxn1ERT2*, which allows tissue-specific expression of a tamoxifen-regulatable form of FOXN1 (FOXN1ER) [88]. Using this model, we showed that increasing FOXN1 activity in TECs in 12- or 24- month old mice resulted in thymus regeneration, characterized by restoration of thymic architecture and functionality close to that found in young mice [88] (Figure 3C). This up-regulation of FOXN1 function led to increased expression of genes important for TEC biology and function, including *Dll4*, *Ccl25*, *Kitl*, *Cxcl12, Ctsl, Cd40, Cd80,*

Pax1, *Trp63*, *Fgfr2IIIb*, and *Aire*, to levels similar to those observed in the young thymus

(Figure 4A) [88]. It also led to increased proliferation in immature TEC subsets, strongly

suggesting that the observed thymus regeneration was instigated by a coordinated

proliferation and differentiation of TEC progenitors [88]. These data show that up-regulation

of FOXN1 function is sufficient to drive regeneration of the aged thymus, establishing

FOXN1 as the primary target of the mechanisms driving age-related thymic involution. Of

note is that uncontrolled differentiation of TEC progenitor/stem cells was not indicated in

either the K14-Foxn1 or R26-Foxn1ERT2 models [76, 82], suggesting that other factors must

interact with FOXN1 to regulate the balance between proliferation and differentiation.

FOXN1: a master transcriptional regulator of TECs

The evidence discussed above has established FOXN1 as a powerful mediator of TEC

differentiation and maintenance. Remarkably, recent work from this laboratory has shown

that overexpression of FOXN1 in an unrelated cell-type, mouse embryonic fibroblasts

(MEFs), is sufficient to reprogramme the MEFs into functional TECs [96] (Figure 3B). These

FOXN1-induced MEFs were shown to express genes indicative of TEC lineage identity,

including *Dll4*, *Ccl25*, and *Kitl* (Figure 3B), and to provide a permissive environment for the

maturation of ETPs to DP and SP thymocytes in vitro [96]. Furthermore, these 'induced'

TECs (iTECs), upon transplantation under the kidney capsule of 5-6 week old *nu/nu* or

syngeneic wild-type mice together with supporting thymic mesenchymal cells and immature

thymocytes, went on to generate a fully functional thymus, with characteristic cortical and

medullary architecture [96]. The iTECs were shown to express endogenous *Foxn1*,

consistent with the positive auto-regulation of *Foxn1* observed in the *K14-Foxn1* transgenic

mice, and iTECs recovered after transplantation expressed a range of genes required for

TEC differentiation, proliferation and function (Figure 4B) [95, 96]. This study thus extends

previous understanding to establish that FOXN1 functions as a master regulator of TEC differentiation, which is capable of initiating and maintaining the transcription factor network required to promote TEC identity (Figure 4).

Regulation of *Foxn1* expression in TECs

Given the importance of FOXN1 in thymus biology, there has been considerable interest in its upstream regulation. However, information regarding transcriptional regulators of *Foxn1* is surprisingly scarce. Concrete evidence supports positive autoregulation of *Foxn1* in TECs [95, 96]. However, whether this is direct or indirect remains to be determined. Recently, some members of the E2F family of transcription factors (E2Fs; specifically E2F3 and E2F4), which mediate cell cycle progression among other functions and are negatively regulated by Retinoblastoma tumour suppressor (Rb) proteins, have been shown to be able to bind to their consensus binding site in the presumptive *Foxn1* promoter in vitro [97]. Additionally, increased activity of E2F3 in vivo was shown to correlate with increased expression of *Foxn1* in TECs [97]. This link between E2F3 activity and *Foxn1* expression was revealed by analysis of compound transgenic mice that lack the Rb family genes Rb and p103, and carry only a single copy of the third Rb family member p107 (Mx1-Cre;Rb$^{lox/lox}$; p130$^{lox/lox}$; p107$^{+/-}$: called *Mx1-Cre p107-single* mice). By 9 months old, these mice exhibit severe thymus hyperplasia, characterized by increased cellularity and increased *Foxn1* expression levels in TECs. Further genetic analysis showed that reduced levels of *Foxn1* expression (achieved by breeding the *Foxn1^{LacZ}* allele onto the Mx1-Cre p107-single background) were sufficient to reverse this hyperplastic phenotype, implicating RB and hence E2Fs in *Foxn1* regulation [97]. Another candidate transcriptional regulator of *Foxn1* in TECs is the T box transcription factor TBX1, mutation of which is thought to cause DiGeorge Syndrome [98, 99]: induced expression of TBX1 in *Foxn1* expressing cells of the E11.5 3PP resulted in down-regulation

of *Foxn1* expression, indicating that TBX1 can repress *Foxn1* transcription in TECs [100].

Consistent with its expected effect on *Foxn1* repression, the forced TBX1 expression in

Foxn1[Cre];*R26-iTbx1* thymi appeared to block TEC differentiation in an early progenitor state,

evidenced by the accumulation of progenitor-phenotype cells (characterized by expression

of Placenta expressed transcript 1; PLET1) and the absence of differentiated TECs in the

fetal thymus [100]. However, it remains to be determined whether TBX1 regulates *Foxn1*

directly or indirectly, and in this light TBX binding sites have not yet been identified in the

putative *Foxn1* promoter regions [100]. Transcriptional regulation of *Foxn1* in the hair follicle

and skin may also provide some clues as to its regulation in TECs, and notably, a homeobox

family member, *Hoxc13*, has been suggested to regulate *Foxn1* in skin and hair follicle.

However whether *Hoxc13* is also involved in regulation of *Foxn1* expression in TECs

remains to be determined [101]. Finally, the BMP and WNT-signaling pathways have been

implicated in regulating *Foxn1* expression in TECs [102-111], although again, the molecular

details have not been reported.

Surprisingly, the regulatory regions governing *Foxn1* expression in TEPCs or TECs are also

still only poorly characterized. Several studies have investigated whether a minimal genomic

region surrounding the *Foxn1* gene on mouse chromosome 11 can reproduce the wild-type

Foxn1 expression pattern in skin and thymus. The largest region tested was 110kb,

containing the entire *Foxn1* locus with an additional 74kb of 5`-flanking sequence and 12kb

of 3`-flanking sequence; this region rescued the *nude* phenotype in vivo, indicating that it

contains all the regulatory elements required for normal expression of *Foxn1* [112]. A 26kb

region of genomic DNA encompassing the coding exons of *Foxn1* plus 8.5kb of 5`-flanking

sequence and 3kb of 3`-flanking sequence could rescue the hairless but not the athymic

phenotype of *nude* mice, showing that it lacked at least some of the regulatory regions

required for *Foxn1* expression in TECs [113]. However, a 30kb fragment containing the entire upstream sequence between the first coding exon of *Foxn1* (exon-2) and the upstream gene *Slc13a2* can recapitulate *Foxn1* expression pattern in the developing thymus [114], although definitive characterization of its capacity to drive normal *Foxn1* expression in the postnatal and adult thymus has not been provided. Within these regions, the promoter and enhancers governing the expression of *Foxn1* in TEPCs and TECs remain to be definitely identified and similarly the identity of the tissue-restricted transcription factors important for its expression remains elusive.

Conclusions

FOXN1 plays a critical role in thymus biology, functioning as a master regulator of TEC differentiation, function and maintenance in the fetal and adult thymus and displaying remarkable potency as a regeneration and reprogramming factor. Further investigation of FOXN1 function will thus illuminate TEC biology during development, homeostasis and ageing, and contribute to a broader understanding of how master regulator TFs function to regulate and coordinate gene expression programs. Elucidation of the transcription factor networks responsible for regulating the initiation and maintenance of *Foxn1* in different TEC subsets will also be crucial. This presents a major challenge, but should now become tractable in light of recent technological advances allowing interrogation of global gene expression and TF binding in single cells/small cell populations. In this regard, the recent identification of TBX1 and E2F as transcriptional regulators of *Foxn1* should provide a tangible starting point for deciphering the molecular details of *Foxn1* transcriptional regulation.

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Conflict of interest statement

CCB and ABL declare no financial or commercial conflict of interest. HJV is a consultant for a privately held company.

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Phylum	Subphylum	Superclass or Class	Foxn4b	Foxn4	Foxn4L	Foxn1
Chordata	Vertebrata	Gnathostomata (jawed vertebrates)		▇		▇
		Agnatha (jawless fish)		▇	▇	▇
	Urochordata (Tunicates)	Ascidiacea (sea squirts)		▇		
	Cephalochordata (Lancelets)		▇	▇		
Echinodermata			▇			

|			
Cnidaria			

Table 1: *Foxn1* **and its orthologues and paralogues through evolution** (adapted from [46]). Table indicates the presence or absence of *Foxn1, Foxn4, Foxn4b and Foxn4l* in different subphyla or superclasses. Based on sequence and synteny homology, *Foxn1* and *Foxn4* are thought to have arisen from a common ancestor gene, *Foxn4b.* Green denotes the presence of the gene in the genome.

Figure legends

Figure 1. Thymus structure and development. Schematic representation of a human thymus. Left panel shows location of the thymus, at the midline above the heart. Middle panel shows representation of a section through a young thymus, indicating the thymic cortex (c) and medulla (m). Right panel shows detail of stromal cells (thymic epithelial cells, TECs; dendritic cells, macrophages and blood vessels.) Note that mesenchymal cells and the vascular network are omitted for clarity, although the mesenchymal capsule bounding the thymus is shown. Haematopoietic progenitors enter the thymus at the junction between cortex and medulla. Commitment to the T-cell lineage and differentiation as far as the $CD4^+CD8^+$ 'double positive' (DP) stage of development occurs in the cortex. Thymocytes that successfully undergo positive selection can then enter the medulla, which is the site of central tolerance induction. $CD4^+$ and $CD8^+$ single positive (SP) T cells exit the thymus from the medulla (see references [2-7]). DN, $CD4^-CD8^-$ 'double negative' thymocytes.
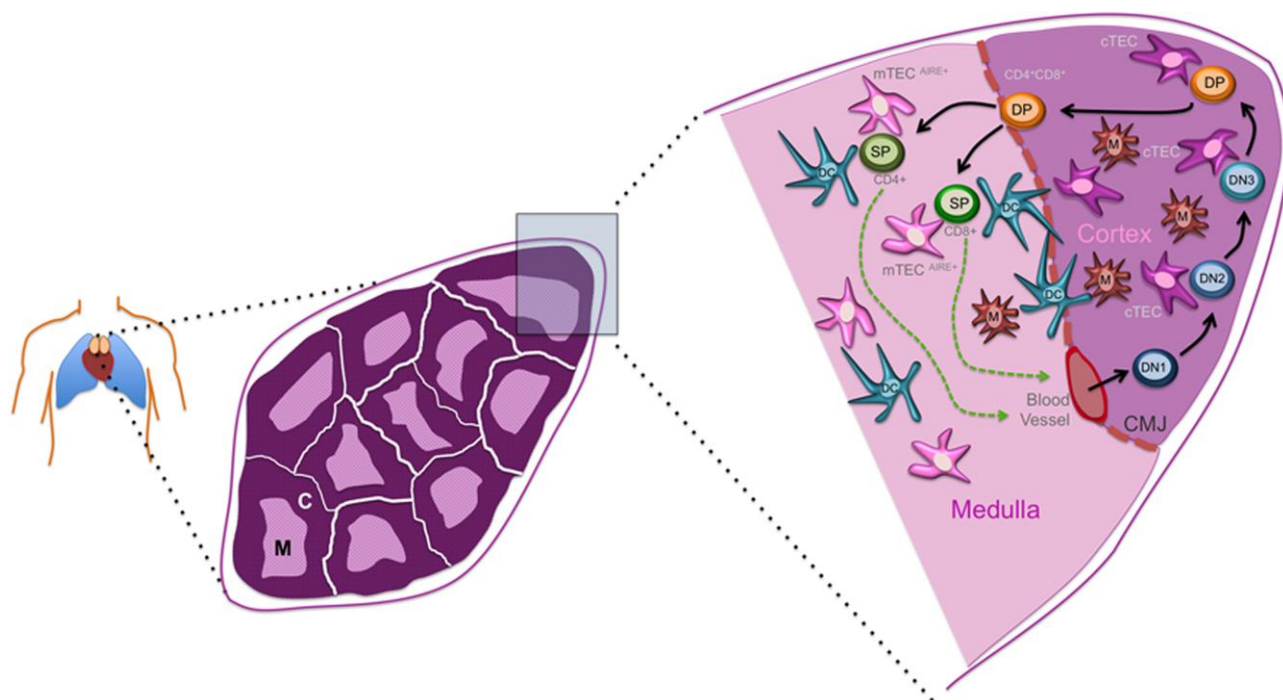
Figure 2. Early events in thymus development. Schematic representation of early thymus development in the mouse. 3PP, third pharyngeal pouch. Grey ovals represent neural crest-derived mesenchymal cells. Red denotes region of GCM2 expression, marking the parathyroid primordium, blue denotes region of FOXN1 expression, indicating the thymus primordium. E10.5, E11.5 and E12.5 denote day 10.5, 11.5 and 12.5 of embryonic development, respectively.
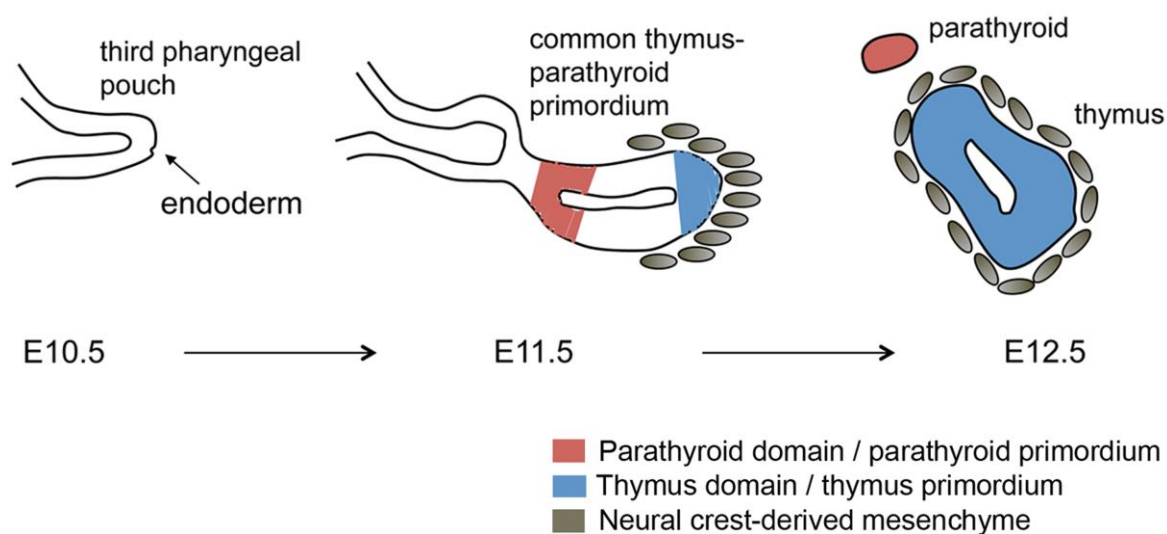
Figure 3. Foxn1 expression levels and thymic involution. The level of expression of

Foxn1 is correlated with and influences the age-related thymic involution. (A) During normal healthy aging, FOXN1 expression levels decrease concomitant with the decrease in size, organization and TEC functionality that characterizes age-related thymic involution. (B) Enforced high-level FOXN1 expression in TECs, as with the *Foxn1*tg transgene, delays but does not prevent age-related involution. (C) Induction of increased levels of functional FOXN1 in TECs in the fully involuted, aged thymus, leads to true thymus regeneration evidenced by increased thymus size, increased thymopoiesis and output of naïve T cells, and restoration of thymus architecture and TEC phenotype to close to those of the young thymus.
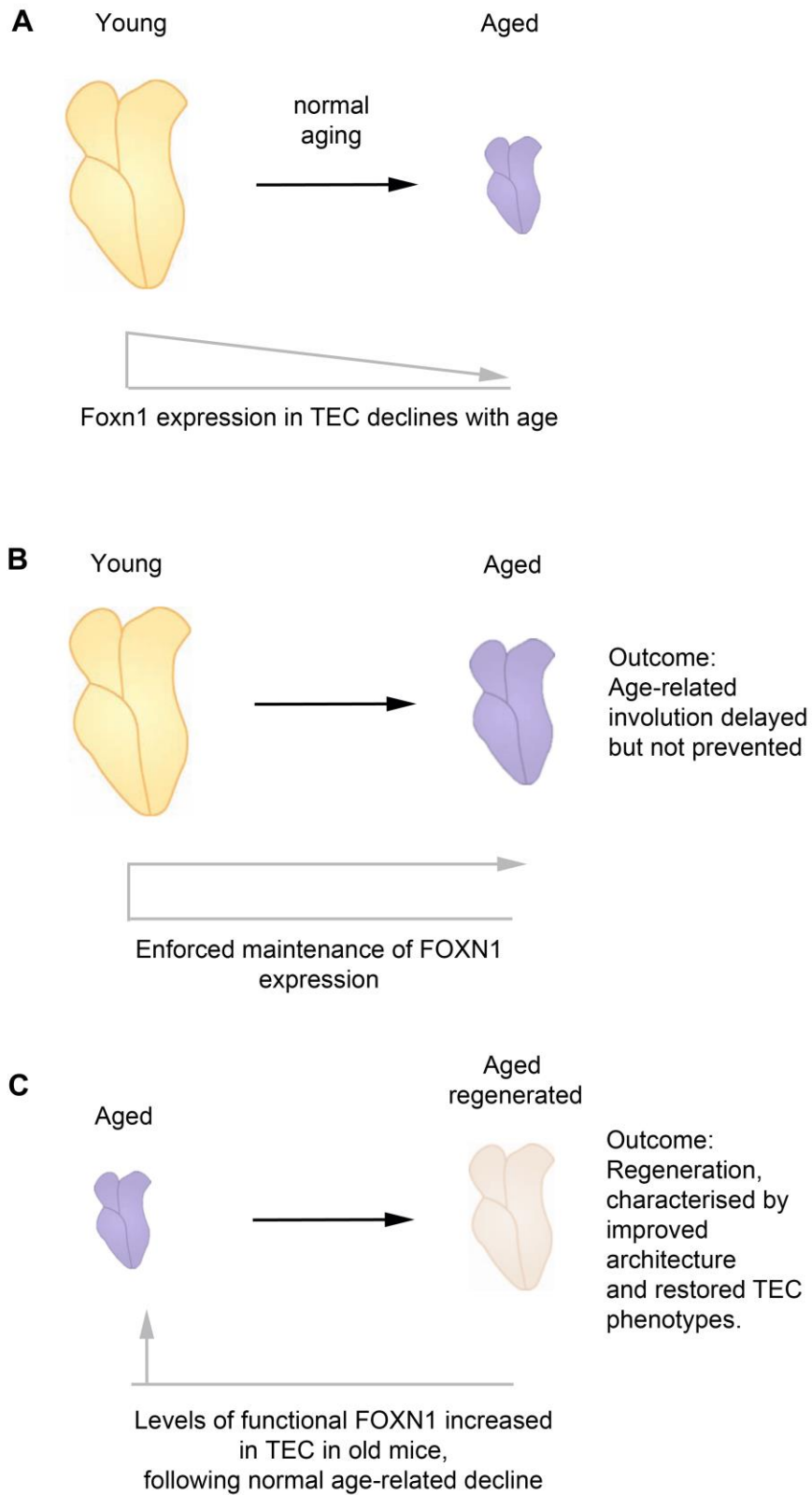
Figure 4. FOXN1 – a master transcriptional regulator of TEC gene expression. FOXN1 acts as a master transcription factor in TECs. It directly or indirectly regulates the expression of a number of genes in TECs during *Foxn1*-induced thymus regeneration (A) and transdifferentiation of MEFs to iTECs (B). The genes regulated by FOXN1 are responsible for a variety of functions in TECs, including differentiation, maintenance, and function, as well as including those with as yet unknown functions. Lower panel in (A) shows genes whose expression in TECs is down-regulated with age, and restored to close to juvenile expression levels when FOXN1 function is increased in aged TECs, grouped according to their known functions in TECs. Lower panel in (B), TEC identity and function is evidenced by expression of the genes shown. * denotes genes whose expression was demonstrated in MEF-derived iTECs recovered after transplantation but which were not present or not tested in iTECs prior to transplantation. Note that the cohort of genes regulated by FOXN1 in (A) and (B) is likely to be broader than depicted here.