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Role of the *p63-FoxN1* regulatory axis in thymic epithelial cell homeostasis during aging

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The *p63* gene regulates thymic epithelial cell (TEC) proliferation, whereas *FoxN1* regulates their differentiation. However, their collaborative role in the regulation of TEC homeostasis during thymic aging is largely unknown. In murine models, the proportion of TAp63$^+$, but not ΔNp63$^+$, TECs was increased with age, which was associated with an age-related increase in senescent cell clusters, characterized by SA-β-Gal$^+$ and p21$^+$ cells. Intrathymic infusion of exogenous TAp63 cDNA into young wild-type (WT) mice led to an increase in senescent cell clusters. Blockade of TEC differentiation via conditional *FoxN1* gene knockout accelerated the appearance of this phenotype to early middle age, whereas intrathymic infusion of exogenous *FoxN1* cDNA into aged WT mice brought only a modest reduction in the proportion of TAp63$^+$ TECs, but an increase in ΔNp63$^+$ TECs in the partially rejuvenated thymus. Meanwhile, we found that the increased TAp63$^+$ population contained a high proportion of phosphorylated-p53 TECs, which may be involved in the induction of cellular senescence. Thus, TAp63 levels are positively correlated with TEC senescence but inversely correlated with expression of FoxN1 and FoxN1-regulated TEC differentiation. Thereby, the *p63-FoxN1* regulatory axis in regulation of postnatal TEC homeostasis has been revealed.

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Transcription factor *Trp63*, a homolog of the tumor suppressor *p53*, is pivotal in the development of stratified epithelial tissues, including the epidermis, breast, prostate, and thymus.[1] The *p63* gene encodes multiple products (isoforms). Specifically, its transcription, initiated from two different promoters, produces isoforms containing (TAp63) or lacking (ΔNp63) an N-terminal transactivation domain. Both transcripts undergo alternative splicing at the C-terminus leading to α, β, and γ isoforms of TAp63 and ΔNp63.[2] Thus, *p63* executes complex molecular functions to regulate various and sometimes paradoxical phenotypes. Although the exact roles of each *p63* isoform are still not clear, two fundamental functions have emerged: (i) tumor suppression through the induction of tumor cell senescence and apoptosis,[3–5] associated mainly with the TAp63 isoform and (ii) epithelial stem cell maintenance[1,6–8] through the regulation of self-renewal and proliferation, associated mainly with the ΔNp63 isoform.

The role of *p63* in thymic development is considered to be essential for the proliferation potential of thymic epithelial stem/progenitor cells, but it could be dispensable for lineage commitment and differentiation.[9,10] Generally, thymic development appears to be regulated by the ΔNp63 isoform rather than by the TAp63 isoform through the maintenance of epithelial progenitor 'stemness'. This was demonstrated *in vivo* by introducing the ΔNp63 or the TAp63 transgene into *p63*-knockout mice. The results show that ΔNp63, but not TAp63, could rescue defective thymus development in the *p63*-knockout mice.[9] However, the role of TAp63 in the thymus *in vivo* is largely unknown.

TAp63, has been shown to possess opposing functions—prevention of aging[11] and promotion of cellular senescence,[4] but studies of pan-*p63*'s roles in epithelial cell and organ aging show that reduction in *p63* expression caused cellular senescence and led to accelerated aging.[11,12] Similar paradoxical effects were observed in tumor studies as well. For example, *p63* was initially considered to be a tumor suppressor as it overlapped with *p53* in targeting genes.[2] Later, *p63* was found to function as a putative oncogene, as its expression was increased in early neoplasia.[13] This may be due to the molecular complexity of *p63*, which has both transactivating and transcriptional repressor activities that bind over 5800 target sites[14] to regulate a wide spectrum of target genes.[1] Thus, to determine whether TAp63 is associated with regulation of thymic epithelial cell (TEC) senescence[3–5] during thymic aging, both changes in expression and gain-of-function of TAp63 in the thymus under physiological aging conditions should be investigated.

Cellular replicative senescence was originally referred to as a proliferative end stage in cultured somatic cells (mostly fibroblasts).[15,16] It can be induced by telomere erosion, DNA damage, oxidative stress, and oncogene activation.[17,18] The concept of senescence *in vitro* may be applied to *in vivo* tissue

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Abbreviations: cTEC/mTEC, cortical/medullary thymic epithelial cells; fx, *loxP*-floxed-*FoxN1*; uCreER^T, ubiquitous promoter-driven Cre-recombinase and estrogen-receptor fusion protein; TM, tamoxifen; WT, wild-type
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homeostasis as it is related to natural aging, and could also have a role in organismal aging and age-related pathology.[19] For example, aged organs are considered to be sites of accumulated cellular senescence.[20,21] In the aged thymus, it is possible that there is an accumulation of senescent TECs as implied by senescence-associated β-galactosidase (SA-β-gal) staining activity[22] in a previously characterized premature aging mouse model.[23]

However, the mechanisms of age-related thymic involution are still unclear. In the postnatal thymus, both T-cell precursors (thymocytes) of hematopoietic origin and TECs of non-hematopoietic origin undergo age-related changes. Mounting empirical evidence has shown that the primary changes occur in TECs,[24,25] whereas in the postnatal thymus TECs undergo homeostasis supported by the continuous self-renewal and differentiation of epithelial stem/progenitor cells *in situ*. Thus, age-related thymic involution may potentially arise due to defective postnatal TEC homeostasis, which should be, at least in part, associated with *p63,* the regulator of epithelial progenitor proliferation,[9,10] and *FoxN1*, the regulator of epithelial progenitor differentiation.[26–28] Moreover, both these genes appear to function in the same TEC developmental and homeostatic pathway.

In this report, we demonstrate that TAp63$^+$ TECs colocalize with senescence marker p21 and correlate with an increase in senescent cell clusters in the naturally (wild-type, WT) aged murine thymi. Intrathymically infusing young WT mice with exogenous TAp63 cDNA induces TEC senescence, whereas blockade of TEC differentiation via conditional *FoxN1* gene knockout (a model of accelerated thymic aging[29]) accelerates the occurrence of this phenotype to early middle age. Therefore, dysfunction of the *p63-FoxN1* regulatory axis resulting in disrupted TEC homeostasis is a possible molecular mechanism of age-related thymic involution.

Results

Change in p63 expression, particularly TAp63, is positively correlated with thymic aging. Several studies have linked *p63* with organ aging and cell senescence, using strategies to reduce (loss-of-function)[11,12] or enhance (gain-of-function)[4] TAp63 (or pan-p63) expression to lead to accelerated aging or to promote cellular senescence, respectively. These findings may be pertinent to thymic aging. However, the functional characterization of *p63* expression in age-related thymic involution has not been performed yet. We therefore investigated age-related *p63* expression profile in WT murine thymi and found a dynamic change in the percentage of pan-p63$^+$ TECs with thymic age (Supplementary Figure S1). This change was observed as a V-shaped response curve (Supplementary Figure S1C), with higher proportions of pan-p63$^+$ TECs in both fetal (Supplementary Figure S1A) and aged (Supplementary Figure S1B, middle and bottom panels) thymi, but lower proportions in young thymi (Supplementary Figure S1B, top panel). These results imply that the changes in natural *p63* expression in the thymus are age-related.

As *p63* has multiple isoforms, we were curious as to which isoform(s) might be associated with thymic aging. We examined the percentages of ΔNp63$^+$ and TAp63$^+$ TECs in WT murine thymi of various ages using an immunofluorescence (IF) assay (Figures 1a–c). The expression of TAp63 and ΔNp63 in young and aged thymi was confirmed using real-time RT-PCR (Figure 1d). We found contrasting and dynamic changes in the percentages of ΔNp63$^+$ and TAp63$^+$ TECs in WT murine thymi at various ages (Figures 1a–c). Notably, the percentage of TAp63$^+$ TECs was very low or undetectable in the fetal thymus, but dramatically increased with age in the postnatal thymus (Figures 1b and c), whereas the percentage of ΔNp63$^+$ TECs was high in the fetal stage, but decreased in the postnatal thymus, showing no further reduction with advancing age (Figures 1a and c). The proportions of ΔNp63$^+$ and TAp63$^+$ TECs were equivalent in the thymi of 12-month-old animals (middle age) (Figure 1c). Real-time RT-PCR results also showed that the expression of TAp63 was significantly increased, whereas ΔNp63 was almost unchanged in the aged thymi *versus* the young ones (Figure 1d). This finding suggests that TAp63 is significantly associated with thymic aging, whereas the ΔNp63 isoform, as previously reported, is probably involved in the maintenance of TE progenitor 'stemness'.[9,30]

Increase in TAp63$^+$ TECs is positively correlated with the increase in senescence in naturally aged thymus, and TAp63 cDNA is able to promote senescence in the young thymus. As increased expression of TAp63 is proposed to be related to cellular senescence,[4,5] it is important to determine whether there is a continuous accumulation of senescent TECs in the thymus with age similar to senescence in other organs,[20,21] and whether increased TAp63$^+$ TECs are directly associated with the senescent cell clusters and co-localized with cellular senescence marker p21 in the aged thymus. We therefore analyzed SA-β-gal activity and co-localization of TAp63 and p21 in the thymi of WT mice at 2, 12, and 20–22 months of age. We found an age-related increase in the appearance of senescent cell clusters (Figure 2a) and clear co-localization of TAp63 and p21, characterized by yellow colored spots (Figure 2b, right panels) in the WT thymi. The results confirmed the continuous accumulation of senescent cells with age in the thymus, which is positively correlated with the increase in TAp63 expression (Figures 1b and 2b).

Although the increase in senescent cell clusters was correlated with the increase in TAp63$^+$ TECs with age, whether TAp63 can promote TEC senescence were largely unknown. We therefore tested the ability of TAp63 cDNAs to induce senescence in young thymi by intrathymically infusing TAp63γ or/and TAp63β cDNAs driven by the CMV promoter in pADTrack vector (Supplementary Figure S2A), using a polyethyleneimine (PEI) nonviral chemical delivery method.[29,31] We found that TAp63 was able to induce senescent cell clusters (Figure 3a) and increase p21$^+$ TECs (Figures 3b and c) in the young thymus compared with empty vector. The infusion of TAp63 also resulted in architectural cysts in the thymus (Supplementary Figure S3). Our *in vivo* results in the thymus confirmed the *in vitro* results in embryonic fibroblasts performed by Guo *et al.*,[4] in which the TAp63 isoforms were demonstrated to be robust mediators of the induction of senescence. In addition, we found that input of

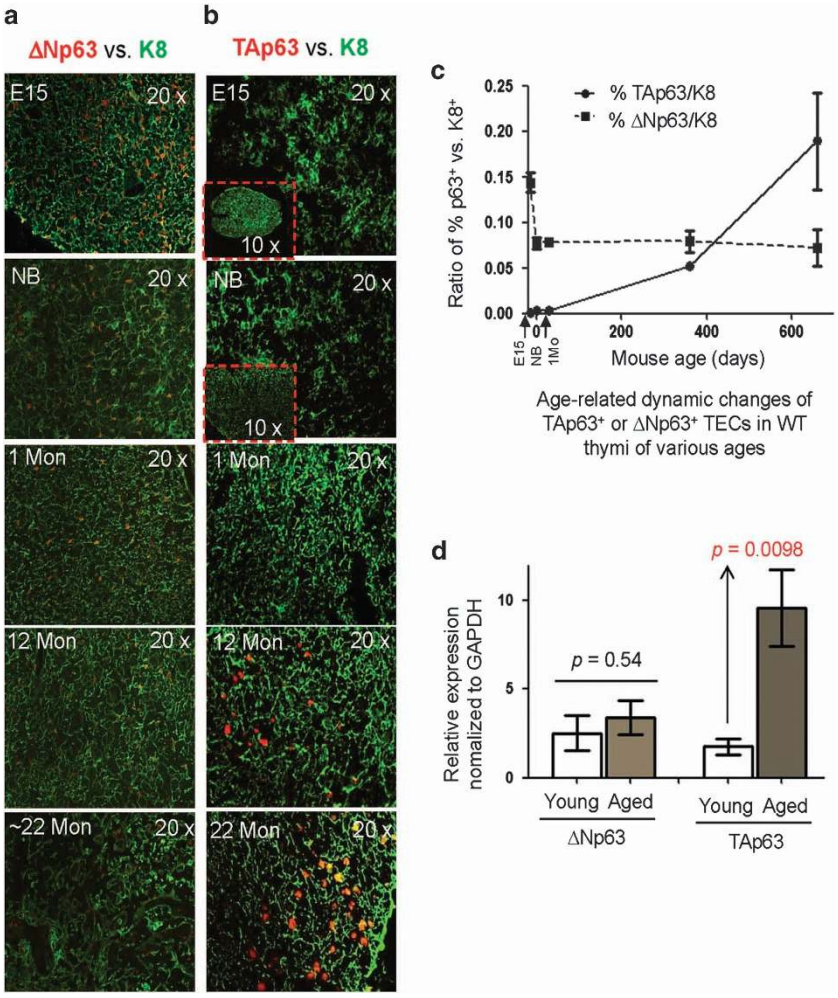

Figure 1 Age-related dynamic changes in the percentage of $\Delta Np63^+$ and $TAp63^+$ TECs, and ΔNp63 and TAp63 mRNA levels in WT murine thymi of various ages. (**a**) Representative IF staining of ΔNp63 (red) *versus* K8 (green) counterstaining, and (**b**) TAp63 (red) *versus* K8 (green) counterstaining in freshly isolated WT thymi of embryonic (E15 days) and postnatal mice at various ages. (**c**) Summary of ratios of % $\Delta Np63^+$ *versus* % $K8^+$ TECs (dotted line) and % $TAp63^+$ *versus* % $K8^+$ TECs (solid line) in WT murine thymi of various ages, analyzed by Image-J software. Each data point includes 2 to 3 animals. (**d**) Summary of real-time RT-PCR results showing relative expression of ΔNp63 and TAp63 from FACS-sorted $CD45^-MHC\text{-}II^+$ TECs of young (~2 months) and aged (≥18 months) WT mice. Each bar represents data (mean ± S.E.M.) from five animals with three real-time RT-PCR repeats

the TAp63 isoform cDNA neither affected FoxN1 expression nor induced thymic atrophy in the WT young thymus (data not shown). This suggests that FoxN1 is functionally independent of TAp63.

Blockade of TEC differentiation via *FoxN1*^{flox} deletion accelerates the increase in $TAp63^+$ TECs and development of senescent cell clusters in the thymus. The transcription factor *FoxN1* acts in a cell-autonomous manner to regulate TEC differentiation, and its expression is decreased in the naturally aged thymus.[29,32] Conditional knockout of *FoxN1* via *FoxN1*^{flox} allele[28] in the postnatal thymus results in the acceleration of thymic aging.[29] As p63 and FoxN1 are thought to act in the same TEC developmental pathway, we wanted to see whether the profile of age-related dynamic changes in $p63^+$ TECs in the naturally aged thymus could also be observed in the conditional *FoxN1* deletion-induced accelerated-aged thymus. We analyzed the thymi of fx/fx-uCreER^T animals with spontaneous leaky uCreER^T expression-induced *FoxN1* deletion[29] in the early middle ages (1, 3, and 6 months of age), with IF staining and western blot, and found that the percentage of pan-$p63^+$ and $TAp63^+$ TECs (Figures 4a and b) and TAp63 mRNA expression in total thymic cells (Figure 4c) were increased with advancing age. We also observed accumulated senescent cell clusters in these thymi at 1, 3, and 8 months of age with SA-β-gal (Figure 4d) and IF (Figure 4e) staining, and found that the appearance of senescent cell clusters (Figure 4d) and $p21^+$ TECs accelerated in an age-related continuous manner in middle-aged thymi. These senescent cell clusters can even be seen in the newborn *FoxN1*^{flox}-uCreER^T thymus after tamoxifen (TM)-induced *FoxN1*^{flox} deletion (Supplementary Figure S4). SA-β-gal detection of cellular senescence in the thymi of *FoxN1*-deleted animals has been previously reported,[23] but here we show it as an age-related continuously advancing phenomenon that is tightly associated with the expression of senescence marker p21. The results indicate that the blockade of TEC

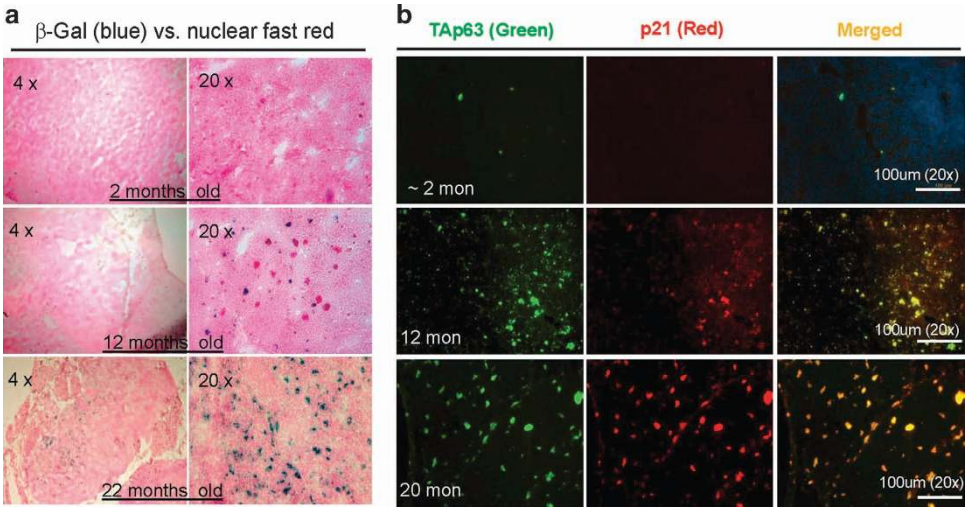

Figure 2 Increase in senescent cell clusters with advancing age in the thymi of WT mice. Cryosections from freshly isolated thymic tissues of WT mice at various ages (**a**) were stained with SA-β-gal and counterstained with nuclear fast red. The senescent cell clusters are shown as blue spots. Images were taken at $\times$ 4 (left panels) and $\times$ 20 (right panels) magnifications of the objective lens; (**b**) were stained with TAp63 (green) and p21 (red, a cellular senescence marker). Right-most panels show TAp63 and p21 co-localization. The experiment was repeated three times with three animals at each age producing consistent results

differentiation via *FoxN1*^{flox} deletion accelerates the process of TAp63 expression and the appearance of senescent cell clusters and p21$^+$ TECs.

Exogenous FoxN1 is unable to reduce TAp63$^+$ TECs, but induces an increase in ΔNp63$^+$ TECs in the naturally aged thymus. As reduced expression of FoxN1 induces TAp63$^+$ TECs (Figure 4), we wanted to determine whether this process can be reversed by supplying exogenous FoxN1 cDNA, which can partially, but significantly, rejuvenate aged thymic function.[29] Upon intrathymic injection of FoxN1 cDNA, as previously described,[29] into naturally aged mice, we found that this did not bring about a significant reduction in TAp63$^+$ TECs, although in some cases modest reduction of TAp63$^+$ TECs was seen (Figure 5a). The results suggest that, although the enhancement of FoxN1 expression can promote TEC progenitor differentiation, increased FoxN1 expression is not able to affect senescent TECs, which arise downstream of FoxN1's functional point during TEC homeostasis (discussed in Figure 7b). Meanwhile, we found that ΔNp63$^+$ TECs, which were not significantly changed with thymic aging (Figure 1d), were significantly increased in these rejuvenated thymi (Figure 5b). This is likely due to enhancement of TEC progenitor activation, resulting from feedback via FoxN1-enhanced TEC differentiation. Indeed, in the flow cytometry assay, we found that the ΔNp63$^+$ TEC population in the FoxN1-cDNA injected naturally aged thymus was the most proliferative population (Figure 5c).

***FoxN1*^{flox} deletion-induced p-p53$^+$ TECs were accumulated in TAp63$^+$ population in the thymus.** Activation of *p53* and increase in apoptotic TECs have been reported in the postnatal thymus of the *FoxN1*^{flox} conditional knockout animals.[28] However, *p53* is also paradoxically involved in the induction of cellular senescence.[33] As *FoxN1* deletion induced blockade of TEC differentiation accelerated the development of TAp63$^+$ and senescent TECs (Figure 4), it is worth knowing whether p53 is involved in these processes. We examined TECs expressing activated phosphorylated(p)-p53 in TAp63$^+$ and TAp63^{-neg} TEC populations in *FoxN1*-deleted mice, using flow cytometry. We found that the percentage of p-p53$^+$ TECs was significantly increased in the TAp63$^+$ TEC population but not in TAp63^{-neg} TEC populations (Figure 6). These results imply that in the *FoxN1*-deleted postnatal thymus (accelerated organ aging), the *p53* gene is likely involved in TAp63-induced TEC senescence.

Discussion

This study provides direct *in vivo* evidence linking *p63* with thymic epithelial senescence and thymic aging, which are associated with *FoxN1*-regulated TEC differentiation. Global function of *p63* in thymic development is essential for the proliferative capacity of thymic epithelial progenitor cells, which is maintained by the ΔNp63 isoform,[9,30] but it is dispensable for lineage commitment and differentiation in the thymus.[9,10] However, thymic epithelial senescence is hypothesized to be regulated by the TAp63 isoform as it was shown to induce senescence in fibroblasts.[4] TAp63 could also be a marker of delayed epithelial homeostasis because it is expressed in undifferentiated epithelium.[8] In this study, we focused on the role of TAp63 in the postnatal thymus. Although a dynamic change in the percentage of pan-p63$^+$ TECs with age was observed in a V-shaped response curve (Supplementary Figure S1C), thymic aging is accompanied by continuous upregulation of TAp63 expression (Figures 1b–d), which is associated with continuous accumulation of TEC senescent cell clusters and co-localized with senescence marker p21 (Figure 2). Furthermore, exogenous TAp63 cDNA

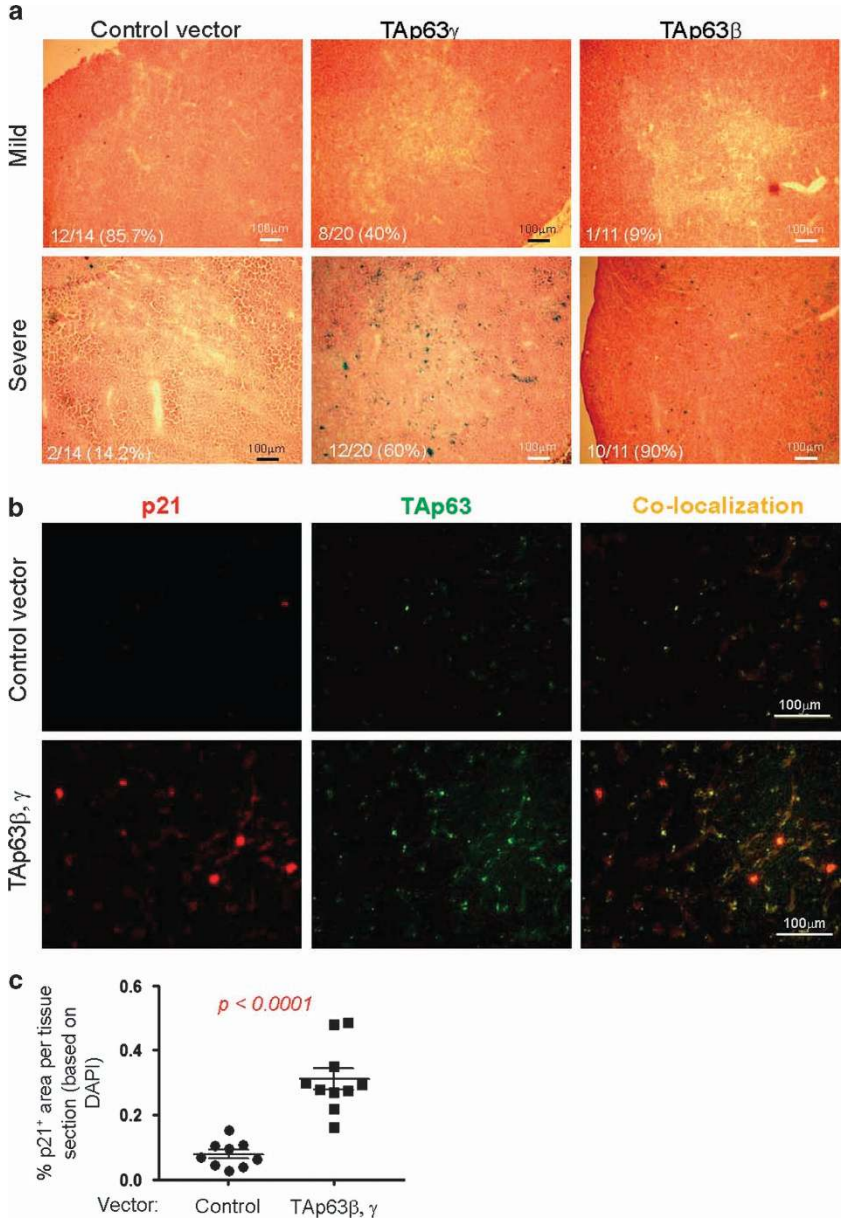

Figure 3 Increase in senescent cell clusters in TAp63 cDNA-infused young thymi. Young WT mice were intrathymically injected twice over a 2-week interval with PEI-treated empty vector (control vector), TAp63γ cDNA (see Supplementary Figure S2A), TAp63β cDNA, and TAp63$\beta\gamma$ (mixed cDNAs), respectively. Four weeks after the first injection freshly isolated thymi were cryosected. (**a**) The cryosections were stained with SA-β-gal and counterstained with nuclear fast red. On the basis of the amount of senescent cell clusters (blue spots), each group was divided into categories of mild and severe senescence. The numbers in the bottom-left of each panel indicate the tissue slide numbers in that category out of total observed tissue slides. (**b**) The cryosections were also stained with p21 and TAp63 (counterstained with DAPI) in order to semi-quantify senescent cells (p21) and input of TAp63 cDNA. (**c**) Summary of % p21$^+$ area per tissue section (based on DAPI staining) in control vector and TAp63$\beta\gamma$ cDNA-injected groups. Each data point represents a tissue slide observed. The experiment was repeated three times with three animals in each group producing consistent results

is able to induce these senescent cell clusters and p21$^+$ TECs in the young thymus (Figure 3). Although the PEI-mediated vector transformation approach with intrathymic injection *in vivo* is imperfect, it provides a clue for future study. Early high expression of pan-*p63* during the fetal stage is probably due to high ΔNp63 expression (Figure 1a). It is likely that TE stem/progenitor cells exist and are active in the aged thymus as the percentage of ΔNp63$^+$ TECs was not reduced (Figures 1c and d). When TEC differentiation was blocked by conditional deletion of the *FoxN1* gene, such phenotypes were accelerated in the thymi of early middle-aged *FoxN1*^{flox} animals[29] (Figure 4). As *p53* is paradoxically involved in the induction of cellular senescence,[33] we found a significantly increased percentage of p-p53$^+$ TECs in the TAp63$^+$ TEC population but not in the TAp63^{-neg} TEC population of the *FoxN1*-deleted thymi (Figure 6).

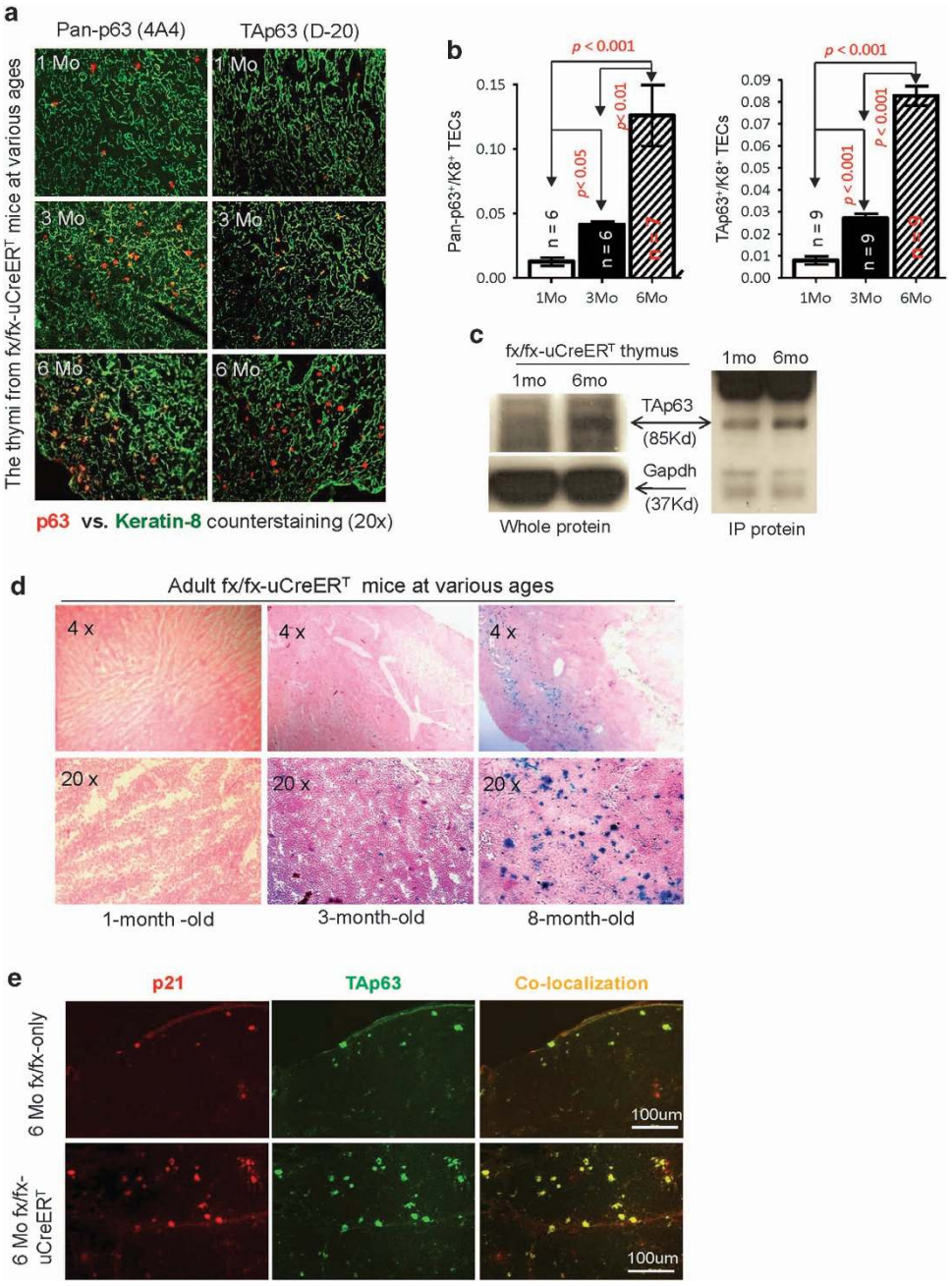

Figure 4 Blockade of TEC differentiation via a low level of spontaneous postnatal *FoxN1*^{flox} deletion in the thymi of fx/fx-uCreER^T mice accelerated the increase in TAp63$^+$ TECs, TAp63 expression, and senescent cell clusters. (**a**) Representative IF staining of pan-p63 (left panels) (red) and TAp63 (right panels) (red) *versus* K8 (green) counterstaining in freshly isolated thymic tissues of fx/fx-uCreER^T mice at 1, 3, and 6 months of age. (**b**) Summary of ratios of % pan-p63 (left bar graph) and % TAp63 (right bar graph) *versus* % K8 counterstaining in freshly isolated fx/fx-uCreER^T thymi of various ages, analyzed by Image-J software. (**c**) Western blot results show TAp63 expression in the thymi of 1-month-old and 6-month-old fx/fx-uCreER^T mice using whole thymic protein (left panel) or protein IP with pan-p63 conjugated protein A/G PLUS-agarose (right panel). This experiment was repeated three times producing consistent results. (**d**) Increase in senescent cell clusters in early middle-aged spontaneous postnatal *FoxN1*^{flox}-deleted murine thymi. The experiment was performed as in Figure 3, with three repeats using three animals at each age producing consistent results. (**e**) Representative IF staining of p21 (left panels) (red), TAp63 (middle panels) (green), and p21 *versus* TAp63 co-localization (yellow) in freshly isolated thymic tissues of fx/fx-uCreER^T and littermate control fx/fx-only mice at 6 months of age, with three animals at each group producing consistent results

Temporal studies of TAp63 and ΔNp63 expression profiles under physiological conditions can indicate their roles associated with physiological events at specific time periods. Their expression profiles have been reported in ectodermally derived skin epithelial cells during development[8,30] and tumor cells during tumorigenesis.[34,35] However, virtually no study has determined the expression profile of TAp63 and ΔNp63 in endodermally derived TECs, which could facilitate our understanding of the impact of the *p63* gene on the regulation of thymic development and aging. During skin embryogenesis, TAp63 is expressed first to initiate stratification of the epithelium and prevent terminal differentiation.

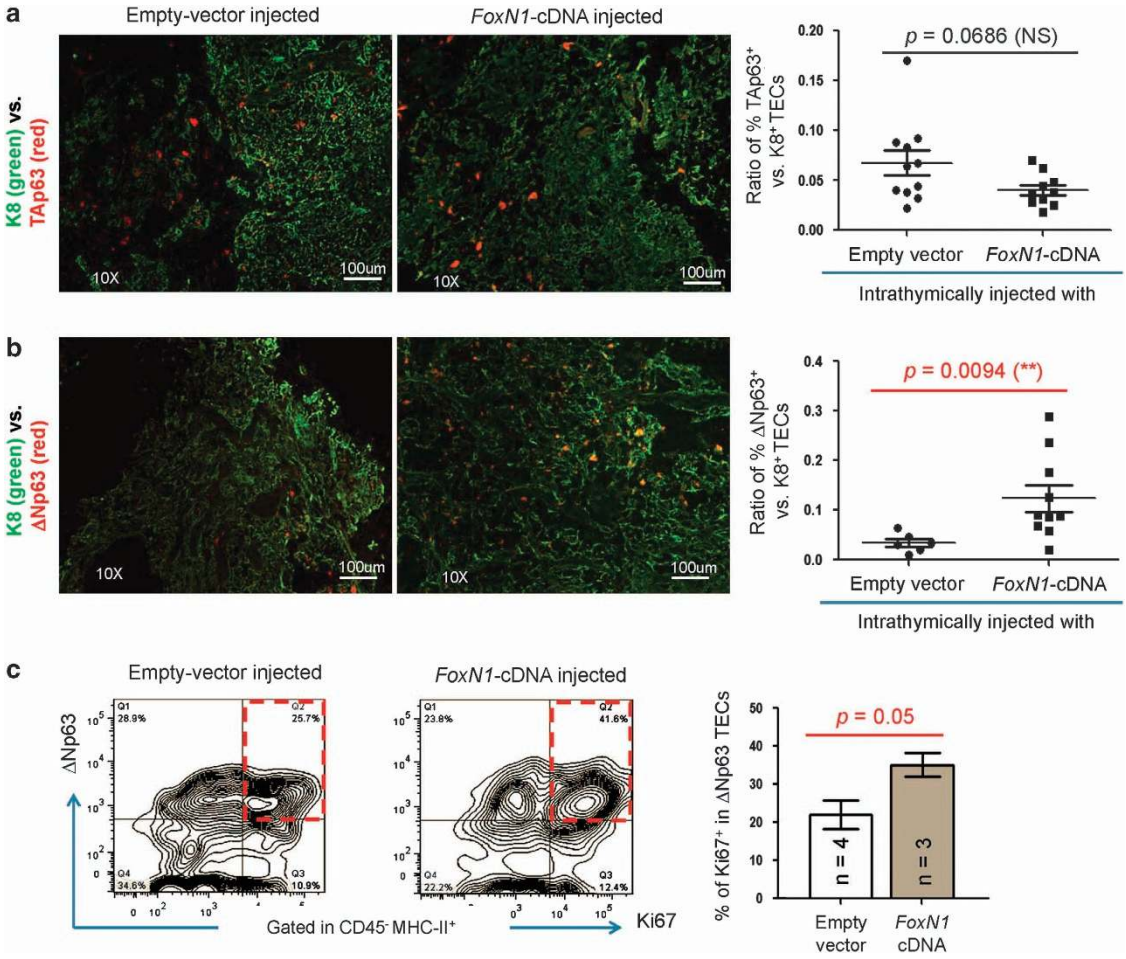

Figure 5 Exogenous *FoxN1* cDNA is unable to reduce TAp63$^+$ TECs, but induces an increase in ΔNp63$^+$ TECs in the naturally aged thymus. Naturally aged WT mice ($\geq$ 18 months old) were intrathymically injected twice over a 2-week interval with PEI-treated empty vector (10 mice) or vector-FoxN1 cDNA (10 mice). Four weeks after the first injection, the thymi were isolated for cryosectioning and IF staining of K8 *versus* TAp63 or ΔNp63. (**a**) Images show representative results of K8 *versus* TAp63 staining; right dot plot shows summarized results of ratio of % TAp63$^+$ *versus* % K8$^+$ TECs. (**b**) Images show representative results of K8 *versus* ΔNp63 staining; right dot plot shows summarized results of ratio of % ΔNp63$^+$ *versus* % K8$^+$ TECs. Each symbol represents one tissue slide, with 1–2 tissue slides per mouse being photographed. (**c**) Left flow cytometric plots show gates of Ki67$^+$ΔNp63$^+$; right bar graph shows summarized results of % Ki67$^+$ TECs in ΔNp63$^+$ TEC population in each group (n = mouse numbers)

Once stratification is initiated, the level of ΔNp63 increases to allow keratinocytes to respond to signals that are required for the maturation of the epidermis and commitment to terminal differentiation.[8] Expression of ΔNp63 *versus* TAp63 is thought to be counterbalanced or reciprocated not only in the development of epithelium but also in tumorigenesis. Malignant tumors with high ΔNp63 expression are characterized as aggressive tumors, whereas benign tumors with high TAp63 expression were significantly smaller and less aggressive.[35] This suggests that the role of TAp63 involves senescence-induced suppression of tumorigenesis.[4] Our results also show a relative reciprocity in the expression of the ΔNp63 and TAp63 isoforms in young and aged thymi, with a particularly noticeable increase in TAp63 in the aged thymi. The levels of ΔNp63 and TAp63 expression intercept at around 12 months of age (middle age) (Figure 1c). The expression profiles of *p63* isoforms in several tissues are summarized in Table 1.

Loss of TAp63 at the germline level in the prenatal stage was reported to induce premature aging in the epidermis and senescence in the hair follicle stem cells,[11] likely owing to hyperproliferation-induced epithelial stem cell exhaustion or accumulation of DNA damage leading to senescence.[36,37] As the undifferentiated ectoderm predominantly expresses TAp63 to mediate initiation of the stratification program,[8] it can be inferred that the knockout of TAp63 in the prenatal stage leads to developmental failure-associated premature aging.[11] However, elevated expression of TAp63 at the postnatal level is probably a sign of stress[37] or it could directly induce cellular senescence[4] associated with natural organ aging.

As increased TAp63 expression is associated with increased incidence of senescent cell clusters and senescence marker p21 in the aged thymus, and TAp63 cDNA is able to induce an increase in senescent cell clusters and p21$^+$ TECs in the young thymus, it should be appreciated that the role of TAp63 in the thymus is potentially related to TEC senescence *in vivo*, in a manner similar to the induction of senescence in cultured cells *in vitro*.[3–5] Cellular senescence characteristics *in vitro* generally include an irreversible proliferation arrest, enhanced SA-β-gal activity, increased

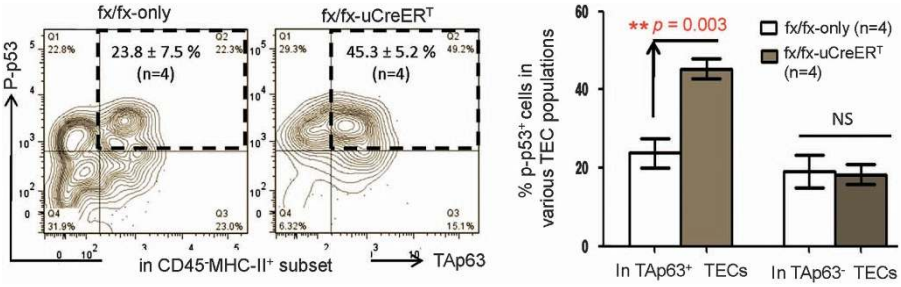

Figure 6 Increased p-p53$^+$ TECs in the TAp63$^+$ population in the conditional *FoxN1*^{flox}-knockout thymi. Left contour plots show representative flow cytometric gates of p-p53$^+$ TECs in TAp63^{-neg} TEC subpopulation (upper left quadrant of the plots) and TAp63$^+$ TEC subpopulation (upper right quadrant of the plots). The accompanying bar graphs on the right summarize the % p-p53$^+$ TECs in the TAp63$^+$ TEC subpopulation (two bars on the left) and TAp63^{-neg} TEC subpopulation (two bars on the right) in the *FoxN1*^{flox}-knockout (filled bars, fx/fx-uCreER^T mice, treated with TM, TM x4) and littermate control (open bars, fx/fx-only mice, which are equivalent to WT mice, treated with TM x4). ** = statistically very significant; NS = not significant

Table 1 Expression profiles of *p63* isoforms in various tissues

Tissues	TAp63 (role or implication)	ΔNp63 (role or implication)	Ref.
Skin (during embryogenesis)	Early developmental stages (undifferentiated epithelium) (expressed first to initiate stratification of the epithelium and prevent terminal differentiation)	After initiation of stratification (allows keratinocytes to respond to signals that are required for the maturation of the epidermis and commitment to terminal differentiation)	Koster *et al.*[8] Candi *et al.*[42]
Epithelial tumors (during tumorigenesis and in response to therapy)	Benign, smaller and less aggressive tumors (mediates suppression of tumorigenesis through the induction of senescence)	Malignant and aggressive tumors (mediates tumorigenesis, probably associated with the existence of a large number of tumor stem cells)	Guo *et al.*[4] Mitani *et al.*[35] Candi *et al.*[42]
Thymus	Aged thymus (expression increases in correlation to thymic epithelial cell senescence, due to blockade of TEC differentiation)	Fetal and young thymus (associated with activated thymic epithelial progenitor cells)	Candi *et al.*[9] and this report

Abbreviation: TEC, thymic epithelial cell.

p21 expression, and activation of p53. Although it is still unclear whether organ aging is the result of an accumulation of senescent cells and whether senescent cells in aged organ have the same characteristics as that *in vitro*, increased senescent cells have been observed in some aged organs,[22,38,39] and we, in this manuscript, found increased senescent cell clusters in the aged thymi with increased SA-β-gal activity and p21 expression. Therefore, thymic aging, at least in part, is probably due to an accumulation of senescent cells. This should serve as further validation of a link between cellular senescence and organismal aging, which has been discussed in-depth in a prior review.[19] The cells that undergo senescence in the thymus are likely to be TECs rather than thymocytes of lymphoid origin because TAp63 (expressed in TECs rather than in lymphocytes) was co-localized with p21 (senescence marker). In addition, thymocytes are mobile with a short resident time in the thymus. Furthermore, T cells in aged individuals never become fully senescent because IL-2 cytokine can restore their function.[40]

Although *p63* is expressed in TE progenitor cells of *FoxN1*^{null} mice,[10] these p63$^+$ cells cannot develop into mature TECs as nude (*FoxN1*^{null}) mice do not have mature TECs. Furthermore, TECs in *p63*^{null} mice express FoxN1, but these TECs suffer proliferation defects and cannot develop a normal-sized thymus.[10] In the thymus, the *p63* gene is the primary regulator of proliferation and regeneration of epithelial stem/progenitor cells.[9,10] *FoxN1*, on the other hand, is required for the differentiation of TE stem/progenitor cells into functional cortical and medullary TECs during organogenesis,[26,27] and for homeostatic control of postnatal TECs.[23,28] Therefore, these two genes should function collaboratively and in tandem in the same TEC developmental pathway.

A conceptual model of the mechanism by which *p63* and *FoxN1* co-regulate TEC homeostasis and are involved in the activation of *p53* expression is proposed in Figure 7. In normal homeostasis, the pool of TE stem/progenitor cells undergo expansion via proliferation and self-renewal, which are regulated by *p63*, predominantly the ΔNp63 isoform. The TE progenitors should then undergo differentiation, regulated by *FoxN1*, to develop into mature cortical and medullary TECs (cTECs and mTECs). When the expression of *FoxN1* is reduced by aging or as a result of conditional gene knockout in the postnatal thymus,[28] the differentiation pathway is blocked, causing an increased turnover of immature TECs (i.e., putative transit-amplifying (TA) or intermediate precursor cells)[10,41] in order to replenish the loss of mature TECs. The feedback signaling increases *p63* expression—most likely the ΔNp63 isoform as it was not reduced in the aged thymus (Figures 1c and d) and was in fact enhanced when exogenous FoxN1 was supplied to the naturally aged thymus—to accelerate TE stem/progenitor cell self-renewal and proliferation. However, the proliferative TA cells still cannot differentiate into mature cTECs and mTECs due to an insufficient differentiation signal from *FoxN1*. The increased turnover coupled with blockade of differentiation may result in

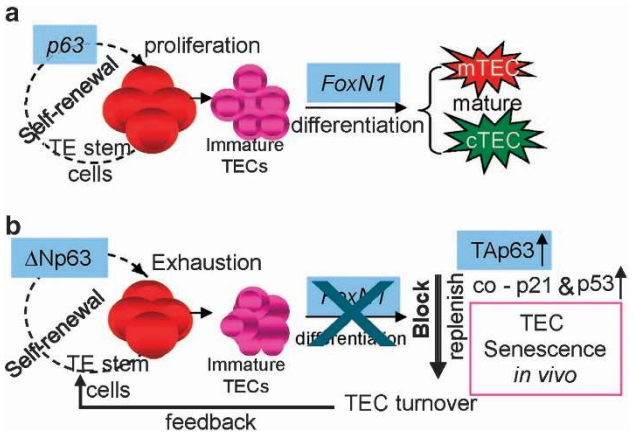

Figure 7 A conceptual model of the potential mechanism by which TEC homeostasis is co-regulated via the *p63-FoxN1* regulatory axis. (**a**) Flow diagram of normal postnatal TEC homeostasis, in which proliferation and self-renewal of TE stem/progenitor cells are regulated by *p63*, and differentiation into mature cortical and medullary TECs (mTECs and cTECs) is regulated by *FoxN1*. (**b**) Flow diagram of blockade of postnatal TEC homeostasis in the scenario of *FoxN1* being reduced (natural aging) or deleted (*FoxN1* conditional knockout). In order to replenish the loss of mature TECs, signals feedback to TE stem cells to direct stem/progenitor cells to accelerate self-renewal and proliferation, characterized by a relative increase (not reduced with age) of ΔNp63 expression and Ki67$^+$ TECs in ΔNp63$^+$ population when exogenous FoxN1 is provided in the naturally aged thymus. However, if FoxN1 is still insufficient, the immature or called intermediate precursor TECs cannot sufficiently differentiate into mature cTECs and mTECs. The continuous increase in turnover accompanied by blockade of differentiation of immature TECs will eventually result in exhaustion of the TE stem/progenitor cell pool and increase mature TEC senescence associated with increase in TAp63, co-localized with p21, as well as activation of p53. These senescent cells will ultimately be depleted via apoptosis, and the thymus undergoes involution (shrinking)

exhaustion of TE progenitors and increased TEC senescence associated with an increase in TAp63, p21, and p53.[4,33] These senescent cells should eventually be depleted via apoptosis *in vivo* due to cell cycle arrest. Moreover, TAp63α, an isoform of TAp63, probably elicits apoptosis by triggering signaling via the death receptor and mitochondria.[42]

In general, adult organ size is governed by the tissue-specific stem cell pool.[43,44] There are two types of tissue-specific pools: infinite and restricted pools. Some organs, such as the liver, have a high capacity for expansion of their tissue-specific stem cell pool following injury. However, such compensation does not take place in the injured pancreas, because it has a restricted and finite epithelial progenitor pool.[44] The thymic progenitor pool is similar to that of the pancreas and being restricted and finite cannot expand too much.[44] Consequently in the event of a blockade of differentiation and exhaustion of the TE progenitor pool, the thymus can only undergo involution (shrinking) resulting in a decline in function. In summary, postnatal TE progenitor pool is associated with TEC homeostasis, which is primarily controlled by the *p63-FoxN1* regulatory axis.

Materials and Methods

Mice, age groups, genotyping, and animal care. All mice used in this study were from the C57BL/6 genetic background (age in months indicated in each figure). Aged (≥18 months old) WT mice were purchased from the National Institute on Aging (Bethesda, MD, USA). The *FoxN1*^{flox} (fx) mice carrying a TM-inducible ubiquitous CreER (uCreER^T), termed fx/fx-uCreER^T were generated and genotyped as described previously[28] and are available from Jackson Laboratories (Bar Harbor, ME, USA) (no. 012941). Mouse age groups are young (±2-month), late young (>3 months), early middle-aged (6∼9-month), middle-aged (±12-month), and aged (≥18-month), based on WT mouse thymic size in our previous experiments.[24,45] All animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center at Fort Worth, in accordance with guidelines from the National Institutes on Aging, Bethesda, MD, USA.

Intrathymic transformation of TAp63-cDNA or FoxN1-cDNA. TAp63γ and TAp63β cDNAs (kindly provided by Dr. Mills[4]) were sub-cloned into the CMV promoter-driven pADTrack vector (Supplementary Figure S2). *FoxN1*-cDNA placed in the CMV promoter-driven pADTrack vector was kindly provided by Dr. Brissette.[46] The control vector was an empty pADTrack plasmid. The cDNA plasmid was delivered *in vivo* by a nonviral PEI-mediated method, as described previously.[29] A mixture of plasmid and PEI (VWR, no. 201-20G) at ionic balance N/P ratio = 8, in ∼25 μl volume was intrathymically injected into young (TAp63γ TAp63β or TAp63βγ mixed cDNAs, as the TAp63βγ isoforms were shown to be the most robust senescence inducers[4]) and aged (*FoxN1*-cDNA) mice under anesthesia using suprasternal notch surgery.[29] Each mouse was injected twice over a 2-week interval, and 4 weeks after the first injection the thymi were isolated for analyses.

Senescence-associated β-galactosidase assay. Cryosections of differently aged mouse thymus tissues (16 μm thick) were analyzed for SA-β-gal activity using a Senescence β-Galactosidase Staining Kit according to the manufacturer's protocol (Cell Signaling Technology, Inc., Danvers, MA, USA, no. 9860), and counterstained with nuclear fast red (RICCA Chemical no. R5463200) solution.

IF staining. Cryosections (6 μm thick) were fixed in cold acetone, blocked with 10% donkey serum in Tris-buffered saline (TBS), and stained with optimized dilutions of dual primary antibodies, followed by optimized dilutions of fluorochrome-conjugated dual secondary antibodies. The primary antibodies used were Pan-p63 (4A4) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA, sc-8431), ΔNp63 (BioLegend, San Diego, CA, USA, no. 619001),[11] TAp63 (D-20) (Santa Cruz, sc-8608), rabbit anti-mouse claudin-3,4 (Invitrogen, Grand Island, NY, USA, no. 34–1700 and no. 36–4800), p21 (Santa Cruz, F-5, sc-6346), and Keratin-8 (Troma-1 supernatant). The secondary antibodies used were Cy3-conjugated donkey anti-mouse, -goat, or -rabbit IgG (Jackson ImmunoResearch Lab), or Alexa-Fluor-488-conjugated donkey anti-rat IgG (Invitrogen). IF labeled samples were mounted using anti-fade aqueous mounting medium, which usually contains 4',6-diamidino-2-phenylindole (DAPI). The positively stained areas were quantified by NIH Image-J software. The magnification in the figures indicates the objective lens of a Nikon Eclipse Ti-U fluorescence microscope.

Real-time RT-PCR. Total RNA from fluorescence-activated cell sorting (FACS)-sorted TECs (gate CD45$^-$MHC-II$^+$ population) was prepared and reverse transcribed with the SuperScriptIII cDNA kit (Invitrogen). Real-time RT-PCR was performed in a Step-One-Plus thermal cycler system (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) with SYBR-green reagents. The sequences of ΔNp63 and TAp63 primers were as previously published.[4] The relative expression levels of TAp63 and ΔNp63 mRNAs from aged animals were compared with those from young animals. The average $\Delta\Delta C_T$ value from multiple young animals was always arbitrarily set as 1.0 in each real-time PCR reaction. Samples were also internally normalized to GAPDH controls.

Western blot analysis and immunoprecipitation (IP). The whole thymus was subjected to homogenization and protein extraction in RIPA lysis buffer (Sigma, St Louis, MO, USA, #R0278). Protein, ∼25 μg/lane, was loaded under reducing condition for direct western blot assay with TAp63 antibody (D-20, Santa Cruz, sc-8608), and GAPDH was used as an internal loading control. Alternatively, the protein was precipitated with Pan-p63 antibody (4A4) using protein A/G PLUS-agarose (Santa Cruz, sc-2003) at 4 °C overnight and subjected to western blot analysis with TAp63 antibody.

Flow cytometry assays. *FoxN1*^{flox} mice were injected intraperitoneally with TM (2 mg/mouse/day) for 4 successive days.[28] On the fourth day after the last TM injection, the mice were killed for flow cytometry assay of p-p53 in TAp63$^+$ population. The thymi were torn apart in PBS to release thymocytes, and dissociated by

incubation through three enzyme cycles (Collagenase-V/DNase-I) to enrich TECs.[25,47] The single cell suspension of thymic cells was stained with combinations of fluorochrome-conjugated antibodies against cell surface markers: anti-mouse-PE/Cy5-CD45 and PE-MHC-II (M5/114) (BioLegend). Cells were then fixed with 2% PFA/PBS, permeabilized with 0.1% TritonX-100, and intracellularly stained for TAp63 with D-20 antibody (goat), followed by incubation with APC-anti-goat IgG, and then further intracellularly stained with p-p53 antibody (Ser-15, rabbit, Cell Signaling Technology Inc., Cat no. 12571). FoxN1 cDNA vector-injected aged thymi were also subjected to flow cytometry assay to analyze proliferation using intracellular staining of ΔNp63 (BioLegend, no. 619001) and Ki67 (BioLegend, clone 16A8). Data were acquired using a BD LSRII Flow Cytometer (BD Bioscience, San Jose, CA, USA) and analyzed using FlowJo software (FlowJo Home: Tree Star, Inc., Ashland, OR, USA).

Statistics. Statistical significance was analyzed by unpaired Student's *t*-test. Differences were considered statistically significant at values of $P < 0.05$.

Conflict of Interest

The authors declare no conflict of interest.

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