The proprotein convertase furin regulates the development of thymic epithelial cells to ensure central immune tolerance
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SUMMARY
The generation of mature T cells and establishment of central tolerance is predominantly orchestrated by thymic epithelial cells (TECs). Proprotein convertases are responsible for the proteolysis of proproteins into their mature bioactive counterparts. Here, we found that Furin, a member of the subtilisin/kexin-like PCs family, is highly expressed in TECs compared with other members of this family. TEC-specific deletion of Furin caused severe thymic atrophy and predominantly reduced the number of medullary TECs and thymic tuft cells, and to a less degree, cortical TECs. Furin deletion attenuated the proliferation of TECs, impaired thymopoiesis, and led to autoimmune disorders in mice. Furin promotes the development of TECs via cleavage of proIGF1 receptor and pro-Insulin receptor and the activation of downstream ERK/MAPK and Akt signaling pathways. Thus, this study uncovered the role of furin in TEC development and function and highlighted the importance of post-translational modification of immature proproteins in TEC biology.

INTRODUCTION
The development of functionally diverse and self-tolerant T cells in the thymus is critical for the continuous generation of adaptive immune responses. Thymic epithelial cells (TECs) elegantly orchestrate the generation of functionally competent T cells by providing niches for the attraction, proliferation, selection, and survival of developing T cells (Kadouri et al., 2019). TECs can be roughly categorized into cortical TECs (cTECs) and medullary TECs (mTECs) based on their anatomical structure and molecular characteristics (Barthlott et al., 2021). cTECs and mTECs are derived from the common progenitors expressing CD205 or β5t (Baik et al., 2013; Ohigashi et al., 2013), while the mTEC-restricted progenitors are identified as SSEA-1+CD34+ TECs (Hamazaki et al., 2007; Sekai et al., 2014). The maturation of mTECs is defined by the concomitant upregulation of CD80, major histocompatibility complex class II (MHCII), autoimmune regulator (AIRE), and Fezf2 (Kadouri et al., 2019). Based on the expression of CD80 and MHCII, mTECs are subdivided into immature CD80loMHCIIlo mTEClo and mature CD80hiMHCIIhi mTEChi subsets. The expression of AIRE and Fezf2 is critical for the expression of tissue-restricted antigens (TRAs) in mTECs (Liang et al., 2018; Sansom et al., 2014; Takaba et al., 2015). In contrast to the high heterogeneity of mTECs, the developmental heterogeneity of cTECs is not well defined. It has been known that the maturation of cTECs is defined by the acquisition of CD40 and the high expression of MHCII (Anderson and Takahama, 2012; Shakib et al., 2009). Recently, a new subset within terminally differentiated mTECs was identified; these cells are called thymic tuft cells because they share a large similarity with small-intestinal tuft cells (Bornstein et al., 2018; Miller et al., 2018). The differentiation of thymic tuft cells is predominantly regulated by transcription factor Pou2f3 (Bornstein et al., 2018; Kadouri et al., 2019; Miller et al., 2018).

Proteolytic cleavage (PC) is one of the most important post-translational modifications of immature proproteins. The proteolytic cleavage of immature proproteins will generate functional mature proteins with critical roles in cell proliferation and survival, immunity and inflammation, and other important biological processes (Braun and Sauter, 2019). PCs are serine proteases that are responsible for the proteolytic cleavage of a variety of precursor proteins, including cytokines, extracellular matrix proteins, growth factors and their receptors, hormones, adhesion molecules, neuropeptides, and various proteins from pathogens (Brouwers et al., 2020; Seidah and Prat, 2012). Furin, also known as Pcsk3, was the first PC to be discovered...
(Thomas, 2002). Furin and six other PC members (Pcsk1, Pcsk2, Pcsk4, Pcsk5, Pcsk6, and Pcsk7) constitute the subtilisin/kexin-like PCs family that cleave proproteins at paired basic amino acids with the motif R/K-X_{n}-R/K (n = 0, 2, 4, or 6, and X is any aa, except Cys) (Al Rifai et al., 2017; He et al., 2020b; Susan-Resiga et al., 2011). Furin exhibits a strong preference for the cleavage of paired basic amino acids with the motif R-X-R/K-R (Braun and Sauter, 2019; Jaaks and Bernasconi, 2017). The proprotein substrates of furin in mammals include growth factors and their receptors, hormones, and adhesion molecules (Braun and Sauter, 2019). Germline knockout of Furin is embryonically lethal (Roebroeck et al., 1998), and furin is implicated in many biological processes, including ontogeny, tumor progression, cell-cell adhesion, pathogen infection, and inflammation (Bessonnard et al., 2015; Couture et al., 2015). Until now, the role of PCs in the development of TECs has been unknown. After finding that Furin is highly expressed in cTECs and mTECs compared to the other six members of the subtilisin/kexin-like PC family, we generated TEC-specific Furin ablation mice to study the role of furin in the development and differentiation of TECs. We showed here that TEC-specific ablation of Furin dramatically reduced the numbers of mTECs and, to a lesser degree, reduced the numbers of cTECs, which subsequently led to severe thymic atrophy. Moreover, TEC-specific ablation of Furin also substantially impaired the differentiation of thymic tuft cells. Importantly, TEC-specific ablation of Furin broke down the establishment of T cell central immune tolerance so that these mice displayed autoimmune disorders. Thus, our study elucidated a previously unrecognized role of proprotein convertase furin in TEC differentiation and the corresponding development of thymocytes in mice, supporting the importance of post-translational modification of proproteins, such as proteolytic cleavage, in regulating TEC development.

RESULTS
Furin deficiency remarkably impaired the development of thymic epithelial cells

To investigate the role of subtilisin/kexin-like PCs in TEC biology, we first assessed the expression of the seven subtilisin/kexin-like PCs in cTECs and mTECs according to the available RNA-seq data (Rodrigues et al., 2017). The results showed that the mRNA expression of Furin was much higher than the other six PC members in both cTECs and mTECs (Figure 1A). We thus focused our studies on the intrinsic role of Furin in TECs and generated mice with TEC-specific deletion of Furin by crossing mice with a loxP-flanked Furin allele to mice expressing the Cre recombinase under the control of the Foxn1 promoter. The genotypes of homozygous (Furin^{fl/fl}Foxn1^{Cre/+}) and heterozygous (Furin^{fl/+}Foxn1^{Cre/+}) TEC-specific Furin deletion mice were shown in Figure S1A, and the mRNA and protein expression of Furin in TECs of wild-type (WT), Furin^{fl/+}Foxn1^{Cre+}, and Furin^{fl/fl}Foxn1^{Cre+} mice were shown in Figures S1B and S1C.

We found that the heterozygous and homozygous knockout of Furin in TECs caused severe thymic atrophy and substantially reduced the ratio of thymus weight to body weight and the cellularity of thymocytes (Figures 1B–1D). Noticeably, the homozygous deletion of Furin in TECs caused obvious thymic atrophy and led to a significantly declined cell number of thymocytes and the ratio of thymus weight to body weight than heterozygous deletion of Furin in TECs did (Figures 1B–1D), indicating the potential dose-dependent role of Furin on the development of TECs in mice. The thymus is segregated anatomically into cortical and medullary regions that express its specific markers cytokeratin 8 (K8) and cytokeratin 5 (K5), respectively (Liang et al., 2021). Both H&E and immunofluorescence staining revealed that the thymic medulla of both Furin^{fl/+}Foxn1^{Cre+} and Furin^{fl/fl}Foxn1^{Cre+} mice dramatically declined compared with WT mice and that the cortical region of thymus in Furin^{fl/fl}Foxn1^{Cre+} mice also showed a decreased tendency (Figures 1E and 1F). Consistently, the frequency and absolute numbers of TECs in Furin^{fl/+}Foxn1^{Cre+} and Furin^{fl/fl}Foxn1^{Cre+} mice substantially decreased than Furin^{fl/+} control mice (Figures 1G and 1H). The percentage of mTECs was drastically reduced in Furin^{fl/+}Foxn1^{Cre+} mice and moderately reduced in Furin^{fl/+}Foxn1^{Cre+} mice compared with WT mice (Figure 1I). The homozygous or heterozygous Furin ablation in TECs considerably reduced the absolute numbers of mTECs compared with WT mice (Figure 1J). In contrast, the frequency of cTECs was substantially elevated in Furin^{fl/+}Foxn1^{Cre+} and Furin^{fl/+}Foxn1^{Cre+} mice compared with WT mice (Figure 1I), and homozygous or heterozygous Furin deletion only mildly diminished the numbers of cTECs (Figure 1J). These results indicated that furin regulated the development of mTECs likely in a dose-dependent manner and Furin deletion predominately impaired mTEC development and, to a lesser content, affected cTEC development.

We further examined the effect of Furin ablation on the development of TECs at the embryonic stage. The results showed that the total thymocyte number decreased significantly in Furin^{fl/+}Foxn1^{Cre+} embryos compared with WT embryos (Figure S2A). The absolute cell number of TECs decreased obviously in...
TEC-specific Furin knockout mice, although the frequency of TECs was comparable between WT and *Furin*^fl/fl^ *Foxn1*^Cre^ embryos (Figures S2B and S2C). Similar to postnatal stages, *Furin* ablation in TECs reduced the cell number of mTECs dramatically and the cell number of cTECs mildly (Figures S2D and S2E). We next examined the effect of *Furin* deletion on the development of TEC progenitors. CD205^+^ TECs that could generate both cTECs and mTECs represent the common progenitors of cTECs and mTECs (Baik et al., 2013) and Cld3,4^hi^SSEA-1^+^ TECs were identified as mTEC progenitors (Sekai et al., 2014). We found that the proportions of both CD205^+^ TECs and Cld3,4^hi^SSEA-1^+^ TECs were similar between WT and *Furin*^fl/fl^ *Foxn1*^Cre^ mice (Figures S2B and S2C).
**Furin***/Foxn1*Cre embryos (Figures S2F and S2G), indicating Furin deletion did not alter the early development process of TECs. Combined with the results of 2-week-old mice, we concluded that Furin is indispensable for the development of TECs at the embryonic and postnatal stages.

To further assess the effect of Furin ablation on TEC development and heterogeneity, we detected the transcriptional profiles of TECs of 2-week-old WT and Furin*/Foxn1*Cre mice using single-cell RNA-seq (scRNA-seq) assays. Employing an unsupervised graph-based clustering strategy, 20 clusters of TECs and 5 clusters of non-TEC contaminants (clusters 8, 14, 19, 22, and 23) were identified (Figures S3A and S3B). Clusters 14 and 19, expressing CD4, CD8, and cTEC marker Psmb11, may represent thymic nurse cells, which envelop many viable thymocytes within their intracellular vesicles (Figure S3B). Clusters 8, 22, and 23 represent thymocytes, endothelial cells, and fibroblast contaminants, respectively (Figure S3B). Contaminated clusters were removed before further analysis, and all populations used were displayed in the Uni-form Manifold Approximation and Projection (UMAP) plots (Figure S4A). The cTECs and mTECs are obviously separated based on the cTEC-specific marker genes (Figures 1I, 2C, and 2F), but the expression levels of marker genes for mTEC I, II, and III were similar between WT and Foxn1Cre embryos (Figures S2F and S2G), indicating Furin deletion did not alter the early development process of TECs. Combined with the results of 2-week-old mice, we concluded that Furin is indispensable for the development of TECs at the embryonic and postnatal stages.

**Furin ablation had no obvious effect on the expression of maturation markers in thymic epithelial cells**

We further examined the maturation process of mTECs and cTECs in mutant mice. The proportion of mature mTECIII (CD80Hi-MHCIIHi) subpopulations was overtly normal in Furin**+/Foxn1*Cre and Furin**+/Foxn1*Cre mice compared with WT mice (Figure 2A). Similar results were observed for the mature AIRE**+ mTEC populations (Figure 2B). However, the number of cells of these mature mTEC subsets were dramatically reduced as a result of the reduction in total mTEC number (Figures S5A and S5B). Similarly, the percentage of CD40**+ cTECs were also unaltered by homozygous or heterozygous deletion of Furin (Figure 2C), but the cell numbers of CD40**+ cTECs were obviously decreased (Figure S5C).

mTECs are highly heterogeneous and were recently defined into four major populations by scRNA-seq analysis (Bornstein et al., 2018). Thus, we further analyzed the effect of Furin ablation on the differentiation and maturation of mTECs at the scRNA-seq level. We performed cluster analyses based on the expression of the specific marker genes of four major mTEC subsets (Bornstein et al., 2018; Cowan et al., 2019). The representative genes of each cluster are shown in Figures 2D and S6A. The scRNA-seq analysis showed that the proportions of mTEC I, II, and III were overtly unaltered between WT and Furin**+/Foxn1*Cre mice (Figures 2E and 2F) and the expression levels of marker genes for mTEC I, II, and III were similar between WT and Furin-deficient mTECs (Figure S6B), whereas the percentage of mTEC IV, a terminally differentiated mTEC subset called thymic tuft cells (Bornstein et al., 2018), was significantly reduced (about 2.5-fold) in Furin**+/Foxn1*Cre mice compared to WT mice (Figures 2E and 2F).

**Furin deletion resulted in a severe defect of thymic tuft cell differentiation**

Thymic tuft cells are similar to mucosal tuft cells and express the canonical taste transduction pathway genes and IL-25 (Bornstein et al., 2018; Miller et al., 2018). We further examined the expression level of some representative genes (Pou2f3, Gnat3, Il25, and L1cam) of thymic tuft cells, and the results showed that the expression level of Pou2f3, Il25, and L1cam had no obvious change in Furin-deficient thymic tuft cells compared with WT thymic tuft cells, and only Gnat3 exhibited reduced expression in thymic tuft cells after Furin ablation (Figures 3A and 3B), indicating Furin ablation mainly reduced the proportion of thymic tuft cells with little effect on the expression level of the representative genes of thymic tuft cells. Owing to the reduction in the proportion of thymic tuft cells, the results of bulk RNA-seq revealed that the expression of many genes that are highly expressed in thymic tuft cells (Table S1) (Bornstein et al., 2018) markedly decreased in mTECs obtained from Furin**+/Foxn1*Cre and Furin**+/Foxn1*Cre mice compared with those from WT mice (Figure 3C). Consistently, there was significant negative enrichment of genes in the taste transduction pathway (Kyoto Encyclopedia of Genes and Genomes [KEGG]: mmu04742) in Furin**+/Foxn1*Cre and Furin**+/Foxn1*Cre mTECs compared with WT mTECs (Figure 3D). These bioinformatic
information prompted us to further investigate the role of Furin in the differentiation of thymic tuft cells. It has been shown that thymic tuft cells exhibit high DCLK1 expression (Bornstein et al., 2018; Millere et al., 2018). Flow cytometry analysis showed that both the frequency and cell number of thymic tuft cells significantly decreased in Furin
fl/+Foxn1
Cre and Furin
fl/flFoxn1
Cre mice compared with WT mice (Figures 3E and 3F). Consistently, the reduction of DCLK1+ thymic tuft cells was confirmed by immunofluorescence staining of thymic sections (Figure 3G). To further examine whether Furin deletion favorably impaired the differentiation of thymic tuft cells, we subdivided mTECs into TAC-TEC, Ccl21a-high, Aire-positive, Late-Aire, and Tuft subsets according to another recent publication (Wells et al., 2020). The results showed that the proportion of Tuft subsets reduced obviously and the proportion of the other subsets had no detectable change in Furin
fl/flFoxn1
Cre mice compared with WT mice (Figures S6C and S6D). Collectively, these results indicated that TEC-specific deletion of Furin severely impaired the differentiation of thymic tuft cells.

Furin deletion decreased cell proliferation of thymic epithelial cells

Next, we explored the molecular mechanisms that caused the reduction of mTECs and cTECs in Furin
fl/+Foxn1
Cre and Furin
fl/flFoxn1
Cre mice. We examined the altered biological processes in mTECs and cTECs of
Furin $^{fl/+}$ Foxn1 Cre and Furin $^{fl/fl}$ Foxn1 Cre mice compared to WT mice, using gene set variation analysis (GSVA). The RNA-seq count matrix (Table S2) was used for this analysis, and the results were listed in Table S3. The results revealed that there was no obvious expression change in the genes of the apoptosis pathway in mTECs and cTECs of Furin $^{fl/+}$ Foxn1 Cre and Furin $^{fl/fl}$ Foxn1 Cre mice compared with WT mice (Figure 4A). Consistent with the bioinformatic information, the apoptosis of mTECs and cTECs of Furin $^{fl/+}$ Foxn1 Cre and Furin $^{fl/fl}$ Foxn1 Cre mice was similar to that in the littermate controls as shown by cleaved caspase3 staining (Figures 4B and 4C). In contrast, the genes in cell proliferation-related biological processes gradually decreased in mTECs and cTECs of Furin $^{fl/+}$ Foxn1 Cre and Furin $^{fl/fl}$ Foxn1 Cre mice (Figure 4A). Importantly, the frequency of Ki67+ proliferating mTECs and cTECs dramatically decreased in Furin $^{fl/fl}$ Foxn1 Cre mice and decreased more moderately in Furin $^{fl/+}$ Foxn1 Cre mice compared with WT mice as detected by flow cytometry (Figures 4D and 4E). Similarly, the incorporation of bromodeoxyuridine (BrdU)
The proliferation of mTECs and cTECs decreased in Furin<sup>fl/+</sup>Foxn1<sup>Cre</sup> and Furin<sup>fl/fl</sup>Foxn1<sup>Cre</sup> mice (Figures 4F and 4G). Recently, Wells et al. showed that the highly proliferative mTECs are mainly restricted to TAC-TEC subset (Wells et al., 2020) and we further analyzed the expression of some representative proliferation-related genes in different TEC subsets according to this publication. The scRNA-seq results showed that the expression of some representative proliferation-related genes, including Ccnd1, Ccnd2, Cdk4, and Ranbp1, showed high expression in TAC-TEC and Ccl21a-high mTEC subpopulations and the expression of these genes decreased significantly in TAC-TEC and Ccl21a-high TEC subsets of Furin<sup>fl/fl</sup>Foxn1<sup>Cre</sup> mice compared with that of WT mice (Figure S7A). We further examined the expression Ki67 in CD80<sup>+</sup> and CD80<sup>−</sup>mTEC subsets and we found that the expression of Ki67 decreased in both CD80<sup>+</sup> and CD80<sup>−</sup>mTEC subsets of Furin-deleted mice (Figures S7B and S7C). To further prove the pro-proliferation role of furin in TECs, Furin was overexpressed in mTEC cell line 1C6, and the proliferation of Furin overexpressed cells was...
detected. The results revealed that Furin overexpression significantly increased the proliferation of 1C6 cells as assessed by 5-Ethynyl-2’-deoxyuridine (EdU) incorporation (Figures S7D and S7E). In summary, these results demonstrated that furin is essential for the proliferation of TECs.

Furin regulated thymic epithelial cell development via cleavage of proIGF1R and proIR

We further examined the signaling pathways that account for the impaired development and proliferation of TECs in Furin<sup>fl/fl</sup>Foxn1<sup>Cre</sup> and Furin<sup>fl/+</sup>Foxn1<sup>Cre</sup> mice. GSVA analysis revealed that the IGF1R and insulin pathways and their downstream ERK/MAPK and Akt pathways gradually declined in mTECs and cTECs of 2-week-old WT and Furin<sup>fl/+</sup>Foxn1<sup>Cre</sup> mice (Figure 5A). It has been shown that IGF1 and insulin promoted thymus development and TEC proliferation (Binz et al., 1990; Chu et al., 2008; de Mello Coelho et al., 2002). Moreover, proIGF1R and proIR are well-established Furin proteolytic substrates (Khatib et al., 2015).
et al., 2001; Roebroek et al., 2004). Thus, we postulated that the impaired development and proliferation of TECs in Furin<sup>fl/+< sup>Foxn<sup>Cre</sup>/mice and Furin<sup>fl/fl</sup>Foxn<sup>Cre</sup> mice may be caused by the proteolytic blockage of proIGF1R and proIR, which subsequently results in the attenuation of the ERK/MAPK and Akt pathways. The mRNA expression of IGF1R and Insr was comparable in mTECs and cTECs of WT and Furin<sup>fl/fl</sup>Foxn<sup>Cre</sup> mice (Figure 5B), indicating that the downregulation of the IGF1R and insulin pathways was not a result of the transcriptional alteration of IGF1R and Insr. Because of the scarcity of the freshly isolated TECs, we investigated the cleavage of proIGF1R and proIR using a TEC in vitro culture system (Liang et al., 2018) by culturing TECs of ER-Furin mice in which Furin was deleted by treatment with tamoxifen. We found that the proteolytic cleavage of proIGF1R and proIR was obviously blocked in the cultured Furin knockout TECs (Figure 5C). In contrast, proIGF1R and proIR were normally processed into mature IGF1R and IR in cultured WT TECs (Figure 5C). The binding of IGF1R and IR to their ligands results in the autophosphorylation of the β subunit of IGF1R and IR (Hakuno and Takahashi, 2018). Consistently, the autophosphorylation of IGF1R and IR was obviously reduced in cultured Furin knockout TECs (Figure 5D). Next, we examined the alteration of the downstream signaling pathways of IGF1R and IR and observed a strong reduction in ERK phosphorylation in Furin knockout TECs compared with WT TECs (Figure 5D), indicating the downregulation of the ERK/MAPK signaling pathway in Furin knockout TECs. Moreover, the phosphorylation of Akt at either Thr308 or Ser473 also substantially decreased in Furin knockout TECs compared with WT TECs, which indicated the downregulation of the Akt signaling pathway in Furin knockout TECs (Figure 5D).

To directly prove the processing blockage of proIGF1R and proIR caused by Furin deletion in vivo, we sorted mTECs from 2-week-old WT and Furin<sup>fl/+< sup>Foxn<sup>Cre</sup>/mice and examined the cleavage of proIGF1R and proIR by Simple Western. Coincident with the results obtained from the in vitro cultured TECs, we found that the cleavage of proIGF1R and proIR was also markedly blocked in mTECs of Furin<sup>fl/fl</sup>Foxn<sup>Cre</sup> mice compared with WT mice (Figure 5E). To directly assess the effect of the processing blockage of proIGF1R and proIR on the downstream signaling pathway, we stimulated cultured WT and Furin knockout TECs using IGF1 and insulin, respectively. As expected, the addition of exogenous IGF1 or insulin promoted the autophosphorylation of IGF1R and IR in WT TECs (Figure 5F). In contrast, Furin knockout TECs exhibited reduced phosphorylation of IGF1R and IR, as well as diminished phosphorylation of ERK and Akt, compared to WT TECs (Figure 5F). To further confirm the role of the ERK/MAPK and Akt signaling pathways in TEC development, we treated mice or FTOCs with ERK inhibitor (ERKi) and Akt inhibitor (AKTi), respectively. The results showed that administration of ERKi decreased the numbers of both mTECs and cTECs (Figure 5G) and that administration of AKTi significantly reduced the number of mTECs and had less effect on the number of cTECs (Figure 5H). Collectively, these results demonstrated that Furin ablation impaired the proteolytic cleavage of proIGF1R and proIR, which subsequently led to the downregulation of the ERK/MAPK and Akt signaling pathways.

**Furin expression in thymic epithelial cells was indispensable for multiple stages of thymopoiesis**

Next, we examined whether the impaired TEC microenvironment affected the development of thymocytes in 2-week-old Furin<sup>fl/+</sup>Foxn<sup>Cre</sup> and Furin<sup>fl/fl</sup>Foxn<sup>Cre</sup> mice. The frequency of early thymic precursors (ETPs), a population that is homing to thymus in response to chemokines CXCL12 and CCL25 expressed by cTECs (Abramson and Anderson, 2017; Liang et al., 2021; Zuklys et al., 2016), significantly decreased in Furin<sup>fl/+</sup>Foxn<sup>Cre</sup> and Furin<sup>fl/fl</sup>Foxn<sup>Cre</sup> mice relative to littermate controls (Figure 6A). The subsequent progression of DN1 through DN4 was overtly similar among these mice, except for an obvious diminution of the DN1 subset in Furin<sup>fl/+</sup>Foxn<sup>Cre</sup> and Furin<sup>fl/fl</sup>Foxn<sup>Cre</sup> mice compared with WT mice (Figures S8A and S8B). The frequency of CD4 single-positive (CD4SP) and CD8 single-positive (CD8SP) thymocytes significantly decreased in 2-week-old Furin<sup>fl/+</sup>Foxn<sup>Cre</sup> and Furin<sup>fl/fl</sup>Foxn<sup>Cre</sup> mice compared with age-matched WT mice (Figure 6B), and the frequency of CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) thymocytes was mildly increased (Figure 6B). The reduction of the CD4SP and CD8SP compartments correlated with impaired positive selection in Furin<sup>fl/+</sup>Foxn<sup>Cre</sup> and Furin<sup>fl/fl</sup>Foxn<sup>Cre</sup> mice, as the frequency of CD69<sup>+</sup>TCRβ<sup>int/thi</sup> thymocytes markedly decreased compared with controls (Figure 6C). We next evaluated the cortical and medullary negative selection by assessing the frequency of DP and CD4SP thymocytes that co-expressed Helios and PD-1, respectively (Daley et al., 2013; Rodrigues et al., 2017). The negative selection of DP thymocytes, as defined by the co-expression of Helios and PD-1 on CD25<sup>-</sup> DP thymocytes (Daley et al., 2013; Rodrigues et al., 2017), did not obviously change in Furin<sup>fl/+</sup>Foxn<sup>Cre</sup> and Furin<sup>fl/fl</sup>Foxn<sup>Cre</sup> mice in comparison with WT mice (Figures S8C and S8D). However, the negative selection of CD4SP thymocytes, as characterized by the
The co-expression of Helios and PD-1 on TCRβ+CD25−CD4SP thymocytes (Daley et al., 2013; Rodrigues et al., 2017), was significantly diminished in Furinfl/+Foxn1Cre and Furinfl/flFoxn1Cre mice compared with WT control mice (Figure 6D). On the other hand, in addition to support the conventional αβ T cell development, TECs also promote the development of γδ T cells (Roberts et al., 2012). Our results showed that Furin ablation had no obvious effect on the proportion of αβ T cells and γδ T cells (Figures S8E and S8F).

The negatively selected thymocytes undergo further post-selection maturation in the medulla before they export as naive T cells to the periphery (Hauri-Hohl et al., 2014). The post-selection maturation of the thymocytes is marked by phenotypic and functional changes, including the upregulation of CD62L and the
The impaired development of NKT2 cells. The proportion of mature TCRβhiCD62LhiCD24hiCD45SP thymocytes was significantly reduced in Furinfl/+Foxn1Cre and Furinfl/flFoxn1Cre mice relative to WT control mice (Figure 6E), indicating a partial block in maturation. Similarly, CD8SP thymocytes in Furinfl/+Foxn1Cre and Furinfl/flFoxn1Cre mice exhibited a decreased downregulation of CD24 (Figure 6F), suggesting an impaired maturation of these cells. It has been well known that mTECs also promoted the development of tTreg cells by agonist selection (Abramson and Anderson, 2017). The mature CD25Foxp3+ tTreg cells in the thymus originate from CD25Foxp3− tTreg precursors (Josefowicz et al., 2012; Liang et al., 2018). The Furinfl/+Foxn1Cre and Furinfl/flFoxn1Cre mice showed a reduced frequency and cell number of tTreg cells than WT controls (Figure 6G). We further showed that the reduction of tTreg cells in mutant mice was at least partially caused by the developmental blockage of CD25Foxp3+ tTreg precursors into mature tTreg cells, as shown by the decreased ratio of CD25Foxp3+ mature tTreg to CD25Foxp3− tTreg precursors (Figures 6H and S8G). More recent studies showed the existence of another pool of immature CD25Foxp3− tTreg precursors (Cowan et al., 2016; Savage et al., 2020). We further examined the expression of CD25 versus Foxp3 in CD4+CD8− thymocytes. The results showed that the percentage of immature CD25Foxp3+ tTreg cells decreased in Furinfl/+Foxn1Cre and Furinfl/flFoxn1Cre mice compared with WT mice (Figures S8H and S8I), indicating Furin ablation in TECs impaired the development of immature CD25Foxp3+ tTreg cells. Collectively, these results indicated that homozygous or heterozygous deletion of Furin in TECs impaired TEC function in supporting thymocyte development marked by diminished ETPs, reduced positive and negative selection, partial blockage of post-selection maturation, and impaired tTreg generation.

Thymic tuft cells are critical for the development of type 2 invariant natural killer T (NKT2) cells, which further promote the generation of CD8+EOMES+SP thymocytes (Lee et al., 2013; Miller et al., 2018). Given the dramatic reduction of thymic tuft cells in Furinfl/+Foxn1Cre and Furinfl/flFoxn1Cre mice, we detected the development of NKT2 and CD8+EOMES+SP thymocytes (Figure S9C–S9E). Moreover, the percentage and cellularity of CD8+EOMES+SP thymocytes were obviously reduced in Furinfl/+Foxn1Cre and Furinfl/flFoxn1Cre mice compared with WT mice (Figures S9F and S9G). These results indicated that the defect of thymic tuft cells in Furinfl/+Foxn1Cre and Furinfl/flFoxn1Cre mice led to the impaired development of NKT2 cells.

These perturbations in thymopoiesis extended to the peripheral T cell compartment in 4-week-old Furinfl/+Foxn1Cre and Furinfl/flFoxn1Cre mice, as indicated by diminished CD4 and CD8 T cell counts in spleen (Figure S10A). Importantly, the percentage and number of recent thymic emigrants (RTEs), which are defined as CD3+CD4+CD62L+CD45RBhi (Bredenkamp et al., 2014) were significantly diminished in Furinfl/+Foxn1Cre and Furinfl/flFoxn1Cre mice in comparison with WT mice (Figure S10B). The CD4 and CD8 T cells in the spleen of Furinfl/+Foxn1Cre and Furinfl/flFoxn1Cre mice were decreased CD62L+CD44hi naive phenotype and increased CD44+CD62L− effector memory phenotype (Figure S11A). Although the percentage of Treg cells slightly increased, the number of Treg cells was drastically decreased in the spleen of 4-week-old Furinfl/+Foxn1Cre and Furinfl/flFoxn1Cre mice in comparison with age-matched WT mice (Figure S11B). These results demonstrated that the homeostasis of peripheral T cells is disturbed in Furinfl/+Foxn1Cre and Furinfl/flFoxn1Cre mice.

Thymic epithelial cell-expressed Furin was essential for the establishment of central immune tolerance

Mature mTECs express a wide spectrum of TRAs and present them directly, or indirectly through DCs, to developing thymocytes to impose T cell tolerance by eliminating self-reactive thymocytes from the aβTCR repertoire (Abramson and Anderson, 2017). A failure in this process leads to devastating autoimmunity. The impaired mTEC development in Furinfl/+Foxn1Cre and Furinfl/flFoxn1Cre mice prompted us to detect the establishment of central immune tolerance in these mice. We evaluated the expression of AIRE-dependent and AIRE-independent TRAs (Table S4) in mTECs of WT, Furinfl/+Foxn1Cre, and Furinfl/flFoxn1Cre mice using the RNA-seq data. The results showed that the expression of a large proportion of AIRE-dependent...
and AIRE-independent TRAs was substantially downregulated in mTECs of Furin\(^{fl/+}\)Foxn1\(^{Cre}\) mice compared with WT mice (Figure 7A). Many of the AIRE-dependent and AIRE-independent TRAs also moderately decreased in mTECs of Furin\(^{fl/+}\)Foxn1\(^{Cre}\) mice relative to WT mice (Figure 7A). We further examined the TRA expression level in individual mTEC cells of WT and Furin\(^{fl/+}\)Foxn1\(^{Cre}\) mice by constructing a TRA score according to previously published information (Cowan et al., 2019). The frequency of cells with a high AIRE-dependent TRA score significantly declined in the mTEC II and III subsets, and the frequency of cells with a high AIRE-independent TRA score was obviously reduced in the mTEC I, II, and III subsets (Figure 7B). The expression of some representative TRAs in WT and Furin-deficient mTECs was shown in the UMAP plots (Figure 7C). These results illustrated that homozygous or heterozygous deletion of Furin in TECs disturbed the expression of many AIRE-dependent and AIRE-independent TRAs, which implied that these mice might develop autoimmune diseases. Indeed, we observed obvious signs of autoimmunity in 6- to 8-month-old Furin\(^{fl/+}\)Foxn1\(^{Cre}\) mice relative to WT mice (Figure 7A). We further examined the TRA expression level in individual mTEC cells of WT and Furin\(^{fl/+}\)Foxn1\(^{Cre}\) mice by constructing a TRA score according to previously published information (Cowan et al., 2019). The frequency of cells with a high AIRE-dependent TRA score significantly declined in the mTEC II and III subsets, and the frequency of cells with a high AIRE-independent TRA score was obviously reduced in the mTEC I, II, and III subsets (Figure 7B). The expression of some representative TRAs in WT and Furin-deficient mTECs was shown in the UMAP plots (Figure 7C). These results illustrated that homozygous or heterozygous deletion of Furin in TECs disturbed the expression of many AIRE-dependent and AIRE-independent TRAs, which implied that these mice might develop autoimmune diseases. Indeed, we observed obvious signs of autoimmunity in 6- to 8-month-old Furin\(^{fl/+}\)Foxn1\(^{Cre}\) and Furin\(^{fl/fl}\)Foxn1\(^{Cre}\) mice, including weight loss, hunched posture, and hair slip, whereas the age-matched control mice were healthy (Figures S12A and S12B). Consistently, the 6- to 8-month-old Furin\(^{fl/+}\)Foxn1\(^{Cre}\) and Furin\(^{fl/fl}\)Foxn1\(^{Cre}\) mice developed marked lymphocytic infiltrations in multiple organs, such as liver, stomach, and salivary gland (Figures 7D and S12C). Moreover, these mice also displayed profound tissue cell-reactive autoantibodies against kidney, liver, prostate, and stomach in serum (Figure 7E and Table 1). In contrast, the age-matched control mice exhibited minimal lymphocytic infiltrations in these organs and showed no detectable autoantibody production in serum (Figures 7D, 7E, and S12C). Noticeably, the signs of autoimmunity in Furin\(^{fl/+}\)Foxn1\(^{Cre}\) mice were ameliorated compared with those in Furin\(^{fl/fl}\)Foxn1\(^{Cre}\) mice, which is coincident with the milder TEC impairment and TRAs dysregulation in
Furin-deficient Foxn1<sup>Cre</sup> mice than Furin<sup>fl/fl</sup> Foxn1<sup>Cre</sup> mice. These results demonstrated that homozygous or heterozygous deletion of Furin in TECs resulted in the breakdown of T cell central immune tolerance.

**DISCUSSION**

We herein identified the critical role of proprotein convertase furin in the development of TECs. Ablation of Furin in TECs caused severe thymic atrophy and significantly reduced the cell number of TECs, and the number of mTECs and thymic tuft cells was decreased more dramatically than the cell number of cTECs after Furin inactivation in TECs. Notably, haploinsufficiency of Furin in TECs also caused obvious thymic atrophy and substantial numeric decline of TECs but less than full deletion of Furin. Thus, furin controls TEC development in a dose-dependent manner. In addition, Furin deletion also impaired the development of TECs at the embryonic stage. It has been shown that mTECs derived from progenitor cells expressing cTEC traits, including CD205, b5t, and so on (Baik et al., 2013; Ohigashi et al., 2013; Ribeiro et al., 2013) and Cld3,4hiSSEA-1<sup>+</sup> TECs was identified as mTEC progenitors (Sekai et al., 2014). Our results showed that Furin deletion had no obvious effect on the proportion of CD205<sup>+</sup> TECs and Cld3,4hiSSEA-1<sup>+</sup> TECs, indicating Furin deletion did not alter the early development process of TECs.

Our series of studies with bioinformatic, molecular, biochemical, and flow cytometry assays further showed that Furin deficiency predominately impaired cell proliferation but not cell death/survival of TECs. Furin inactivation reduced the cell proliferation of mTECs and cTECs, which is coincident with the pro-proliferation role of furin in β cells and some cell lines (Bassi et al., 2017; Brouwers et al., 2020). Although some studies demonstrated that Furin deficiency promoted cell apoptosis in some cancers (He et al., 2020a, 2020b), our results showed that Furin inactivation in TECs had no detectable effect on the apoptosis of mTECs and cTECs. This inconsistency may be a result of the difference in cell types and local microenvironments.

Furin deficiency in TECs resulted in severe processing blockage of proIGF1R and proIR to biofunctional counterparts. IGF1R has been shown to possess a high affinity for IGF1 and IGF2; IR has been shown to possess a high affinity for insulin and IGF2 (Hakuno and Takahashi, 2018; Kitamura et al., 2003). It has been shown that IGF1 or insulin administration could restore thymus atrophy in diabetic rats (Binz et al., 1990). IGF1 could promote the development and cell proliferation of TECs in vivo and in vitro (Chu et al., 2008; Timsit et al., 1992). Consistent with the defect of IGF1R and IR in Furin-deleted TECs, the activation of their intracellular downstream signal pathways was decreased. Furin deficiency led to attenuation of the ERK/MAPK and Akt signaling pathways in TECs as indicated by the decreased phosphorylation of ERK1/2 and Akt in Furin-deleted TECs in response to IGF1 and insulin. Inhibiting ERK and Akt activation decreased the cell number of TECs in vivo and in the FTOC system. Collectively, all these data indicated that deletion of Furin in TECs impaired the cleavage of proIGF1R and proIR to functionally competent receptors, which subsequently decreased the downstream ERK/MAPK and Akt signaling pathways to finally cause the defect of TEC development.

Thymic tuft cells represent a terminally differentiated mTEC subset that is characterized by the expression of the canonical taste transduction pathway (Bornstein et al., 2018; Miller et al., 2018). The scRNA-seq analysis revealed that Furin inactivation had no obvious effect on the proportion of mTEC I, II, and III subsets but specifically reduced the percentage of mTEC IV subpopulations (thymic tuft cells). In addition, when subdivided mTECs into TAC-TEC, Ccl21a-high, Aire-positive, Late-Aire, and Tuft subsets according to another recent publication (Wells et al., 2020), Furin ablation also only reduced the proportion of Tuft subsets. These data indicated that Furin deletion did not affect the early differentiation kinetics of mTECs but favorably blocked thymic tuft cell differentiation.

| Tissue | WT | Furin<sup>fl/+</sup>Foxn1<sup>Cre</sup> | Furin<sup>fl/fl</sup>Foxn1<sup>Cre</sup> |
|--------|----|-------------------------------|----------------------------------|
| Kidney | 0/4 | 2/4                           | 4/4                              |
| Liver  | 0/4 | 3/4                           | 3/4                              |
| Prostate | 0/3 | 2/3                           | 3/3                              |
| Stomach | 1/4 | 2/4                           | 3/4                              |

See Figure 7.
TECs are essential for the stepwise development and maturation of conventional αβ T cells and unconventional T cell lineages (Abramson and Anderson, 2017). Furin inactivation in TECs impaired multiple stages of thymopoiesis, including generation of ETPs, positive and negative selection of thymocytes, and development of nTreg cells. Although the numbers of cTECs only decreased slightly in mutant mice, some cTEC-dependent thymocyte development was also impaired obviously, which may be caused by the reduced expression of some genes that are critical for early thymopoiesis in Furin-deficient cTECs. Additionally, the development of NKT2 was also severely compromised, which is related to impaired thymic tuft cell differentiation. Moreover, the impaired thymopoiesis in Furinfl/+ Foxn1Cre and Furinfl/fl Foxn1Cre mice obviously disturbed peripheral T cell homeostasis as shown by the overrepresentation of activated/memory T cells and the reduction in the number of naive T and Treg cells. The appropriate expression of TRAs in mTECs is pivotal for T cells to establish central immune tolerance and to protect mice from suffering autoimmune disorders (Abramson and Anderson, 2017). Furin ablation remarkably interfered with the expression of many TRAs in mTECs as detected by RNA-seq and scRNA-seq assays. The reduced TRAs expression, impaired thymocyte negative selection and decreased Treg cell numbers in Furinfl/+ Foxn1Cre and Furinfl/fl Foxn1Cre mice collectively resulted in the development of autoimmune disease with obvious organ infiltration of lymphocytes and autoreactive antibody production. So Furin expression in TECs is essential for the establishment of central immune tolerance, at least in mice. It is reported that aberrant furin activity is associated with the progression of some cancers and the enzymatic activity of furin is exploited by many bacterial and viral pathogens, including the current global pandemic SARS-CoV-2 (Braun and Sauter, 2019; Peacock et al., 2021). Thus, inhibition of furin activity has been an attractive approach in the treatment of malignant and infectious diseases (Couture et al., 2015; Wu et al., 2020). Considering the critical role of furin in maintaining T cell central and peripheral immune tolerance as shown by other and our present studies (Pesu et al., 2008), the potential side effects of furin inhibitors on the immune system should be recognized and should cause our caution.

Limitations of the study
In this study, we uncovered the role of furin in the development of TECs and in the establishment of central immune tolerance. We also showed that Furin ablation caused the defective cleavage of proIGF1R and proILR, which subsequently led to the attenuation of the downstream ERK/MAPK and Akt signaling pathways by using the in vitro cultured TECs. However, owing to the scarcity of TECs, we could not get enough primary TECs to examine the changes in ERK/MAPK and Akt signaling pathways by Western blot. Although we found that Furin deletion favorably impaired thymic tuft cell differentiation, we haven’t fully uncovered the underlying mechanism. We should investigate these issues by integrating scRNA-seq, single cells transcriptomic assays, and spatially resolved proteomic analyses in the future.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Mice
  - In vitro culture of TECs
- METHOD DETAILS
  - Flow cytometry analysis
  - Immunofluorescence staining
  - Isolation of thymic stromal cells
  - BrdU labeling and staining
  - S-Ethynyl-2’-deoxyuridine (EdU) assay
  - Establishment of Furin overexpression cell line
  - Quantitative PCR
  - Bulk RNA-seq sample preparation and analysis
  - Single-cell RNA sequencing and analysis
  - Western blot
SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105233.

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AUTHOR CONTRIBUTIONS

Conceptualization, Z.F.L.; Methodology, Z.F.L. and Q.Z.; Software, Z.Q.Z.; Investigation, Z.F.L., Q.Z., X.D., T.L. and X.F.Y.; Resources, J.W. M.C; Visualization, Q.Z.; Writing, Z.F.L.; Supervision, Y.Z. and B.J.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: May 12, 2022

Revised: August 29, 2022

Accepted: September 25, 2022

Published: October 21, 2022
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## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-Helios-APC     | Biolegend | Cat#137221; RRID:AB_10662535 |
| Anti-Ki-67-PE       | BD Biosciences | Cat#556027; RRID:AB_2266296 |
| Anti-RORγt-BV421    | Thermo Fisher Scientific | Cat#562894; RRID:AB_2687545 |
| Anti-PLZF-AF488     | Thermo Fisher Scientific | Cat#53-9320-80; RRID:AB_2574444 |
| DCAMKL1 (DCLK1)     | Abcam | Cat#ab31704; RRID:AB_873537 |
| AF647-conjugated donkey anti-rabbit IgG (H+L) | Jackson ImmunoResearch Labs | Cat#711-605-152; RRID:AB_2492288 |
| Anti-CD45-PerCP/Cy5.5 | BioLegend | Cat#103132; RRID:AB_893340 |
| Anti-EpCAM-PE/Cy7   | BioLegend | Cat#118215; RRID:AB_1236477 |
| Anti-Ly51-AF647     | BioLegend | Cat#108312; RRID:AB_2099613 |
| Anti-i-A/I-BV421    | BioLegend | Cat#107632; RRID:AB_2650896 |
| Anti-CD40-PE        | BioLegend | Cat#124610; RRID:AB_1134075 |
| Anti-CD4-FITC       | BioLegend | Cat#100406; RRID:AB_312691 |
| Anti-CD4-APC        | BioLegend | Cat#100412; RRID:AB_312697 |
| Anti-CD4-APC/Cy7    | BioLegend | Cat#100414; RRID:AB_312699 |
| Anti-CD8a-BV421     | BioLegend | Cat#100738; RRID:AB_11204079 |
| Anti-CD44-PE        | BioLegend | Cat#103055; RRID:AB_2564043 |
| Anti-TCRβ-Pe/Cy7    | BioLegend | Cat#109222; RRID:AB_893625 |
| Anti-Ter19-FITC     | BioLegend | Cat#135216; RRID:AB_10689635 |
| Anti-CCR7-PE        | BioLegend | Cat#116206; RRID:AB_313707 |
| Anti-CD80-PE        | BioLegend | Cat#120106; RRID:AB_389358 |
| Anti-CD117-PE/Cy7   | Thermo Fisher Scientific | Cat#12-0801-82; RRID:AB_465752 |
| Anti-CD24-FITC      | Thermo Fisher Scientific | Cat#25-1171-81; RRID:AB_469643 |
| Anti-CD62L-PE       | Thermo Fisher Scientific | Cat#11-0242-82; RRID:AB_464988 |
| Anti-CD25-PE        | Thermo Fisher Scientific | Cat#12-0621-82; RRID:AB_465721 |
| Anti-CD45RB-FITC    | BioLegend | Cat#11-0455-82; RRID:AB_465064 |
| Anti-CD11b-FITC     | Thermo Fisher Scientific | Cat#11-0112-85; RRID:AB_464936 |
| Anti-CD1d tetramer   | ProlImmune | Cat#D001-2X |
| rabbit anti-K5      | Covance | Cat#PRB-160P-100; RRID:AB_10063444 |
| rat anti-K8         | DSHB | Cat#TROMA-I; RRID:AB_531826 |
| chicken anti-K5     | Biolegend | Cat#905904; RRID:AB_2721743 |
| anti-rabbit-HRP     | KPL | Cat#070-1506 |
| anti-mouse-HRP      | KPL | Cat#074-1806 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| β-Actin             | Sigma-Aldrich | Cat#A5441; RRID:AB_476744 |
| IGF-I receptor β    | Cell Signaling Technology | Cat#9750; RRID:AB_10950969 |
| Insulin receptor β  | Cell Signaling Technology | Cat#23413 |
| p-IGF1R/IR          | Cell Signaling Technology | Cat#3024; RRID:AB_331253 |
| ERK1/2              | R and D Systems | Cat#AF1576; RRID:AB_354872 |
| p-ERK1/2            | R and D Systems | Cat#MAB1018; RRID:AB_2140122 |
| AKT                 | R and D Systems | Cat#MAB2055; RRID:AB_2224581 |
| p-AKT473            | R and D Systems | Cat#MAB887; RRID:AB_10973140 |
| p-AKT308            | Cell Signaling Technology | Cat#4056; RRID:AB_331163 |
| IGF1R antibody      | Novus Biologicals | Cat#NBP1-77679; RRID:AB_11011902 |
| Insulin receptor β  | Cell Signaling Technology | Cat#3025 |
| AF 488 AffiniPure donkey anti-rabbit IgG (H+L) | Jackson ImmunoResearch Labs | Cat#715-545-150; RRID:AB_2340846 |

### Chemicals, peptides, and recombinant proteins

| Chemical, peptides, and recombinant proteins | SOURCE | IDENTIFIER |
|-----------------------------------------------|--------|------------|
| Fixation/Permeabilization Concentrate         | Thermo Fisher Scientific | Cat#00-5123-43 |
| Fixation/Permeabilization Diluent             | Thermo Fisher Scientific | Cat#00-5223-56 |
| Cytofix/Cytoperm solution                      | BD Biosciences | Cat#554722 |
| Perm/Wash the solution                         | BD Biosciences | Cat#554723 |
| collagenase/dispase                            | Sigma-Aldrich | Cat#11097113001 |
| DNAse I                                        | Sigma-Aldrich | Cat#D5025 |
| SYBR Premix Ex Taq™                            | TaKaRa | Cat#RR420 |
| CD45 MicroBeads                                | Miltenyi Biotec | Cat#130-052-301; RRID:AB_2877061 |
| TRizol reagent                                  | Thermo Fisher | Cat#15596018 |
| IGF1                                           | Abcam | Cat#ab198569 |
| Insulin                                        | Beyotime | Cat#P3376-100IU |
| RIPA                                           | Beyotime | Cat#P0013B |
| PMSF                                           | Beyotime | Cat#ST506 |
| PhosSTOP phosphatase inhibitor                 | Roche | Cat#04906845001 |
| nonfat dried milk                              | OXOID | Cat#LP0031 |
| SCH772984 (ERK inhibitor)                      | Selleck | Cat#S7101 |
| MK-2206 2HCl (AKT inhibitor)                   | Selleck | Cat#S1078 |
| PEG300                                         | Selleck | Cat#S6704 |

### Critical commercial assays

| Critical commercial assays | SOURCE | IDENTIFIER |
|----------------------------|--------|------------|
| PE Active Caspase-3 Apoptosis Kit | BD Biosciences | Cat#550914; RRID:AB_393957 |
| APC BrdU Flow Kit           | BD Biosciences | Cat#552598; RRID:AB_2861367 |
| BeyoClick™ EdU Cell Proliferation Kit | Beyotime | Cat#C00785 |
| MicroElute Total RNA Kit    | Omega Bio-tek | Cat#R683 |
| SuperScript III Reverse Transcriptase Kit | Invitrogen | Cat#18080-093 |
| Enhanced BCA Protein Assay Kit | Beyotime | Cat#P0010S |
| 12-230 kDa Separation Module | ProteinSimple | SM-W002-1 |

### Deposited data

| Deposited data | SOURCE | IDENTIFIER |
|----------------|--------|------------|
| Bulk RNA-seq data | This paper | GEO: PRJNA794306 |
| scRNA-seq data   | This paper | GEO: GSE193456 |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yong Zhao (zhaoy@ioz.ac.cn).

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Experimental models: Cell lines** |
| 1C6-mock           | This paper | N/A        |
| 1C6-Furin          | This paper | N/A        |
| BOSC 23 cells      | ATCC    | Cat# CRL-11270 |
| **Experimental models: Organisms/strains** |
| Furin<sup>fl/fl</sup> mice | Roebroek et al., 2004 | N/A        |
| Foxn1-Cre mice     | Liang et al., 2018 | N/A        |
| ER-Cre mice        | Zhao et al., 2018 | N/A        |
| **Oligonucleotides** |
| Furin-forward      | Roebroek et al., 2004 | N/A        |
| Furin-reverse      | Roebroek et al., 2004 | N/A        |
| Foxn1-Cre-forward  | Liang et al., 2018 | N/A        |
| Foxn1-Cre-reverse  | Liang et al., 2018 | N/A        |
| ER-Cre-primer1     | Zhao et al., 2018 | N/A        |
| ER-Cre-primer2     | Zhao et al., 2018 | N/A        |
| ER-Cre-primer3     | Zhao et al., 2018 | N/A        |
| **Recombinant DNA** |
| Plasmid: MSCV-PGK-IRES-GFP empty vector | This paper | N/A        |
| Plasmid: MSCV-PGK-Furin-IRES-GFP | This paper | N/A        |
| Plasmid: pCL-Eco    | Addgene | Cat# 12371; RRID:Addgene_12371 |
| **Software and algorithms** |
| GraphPad Prism Version 8.0 | GraphPad | https://www.graphpad.com |
| FlowJo v10         | FlowJo, LLC | https://www.flowjo.com/ |
| Compass for SW     | ProteinSimple | https://www.bio-techne.com/cn/brands/proteinsimple |
| cellranger-3.1.0   | Zheng et al., 2017 | https://support.10xgenomics.com/single-cell-gene-expression/software/overview/welcome |
| Seurat             | Butler et al., 2018 | https://satijalab.org/seurat/articles/install.html |
| ggplot2            | Wickham, 2016 | https://ggplot2.tidyverse.org/ |
| deseq2             | Love et al., 2014 | https://bioconductor.org/packages/release/bioc/html/DESeq2.html |
Materials availability
Plasmids generated in this study are available upon request from the lead contact, Yong Zhao (zhaoy@ioz.ac.cn).

Data and code availability
The bulk RNA-seq data and scRNA-seq data for this study have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. All data reported in this paper will be shared by the lead contact upon request.

This study did not generate original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS
Mice
All animal experiments were approved by the Animal Ethics Committee of the Institute of Zoology, Beijing, China. Male C57BL/6 mice (aged six weeks) were purchased from the Beijing Laboratory Animal Research Center (Beijing, China). TEC-conditional two-allele or one-allele Furin deletion mice (male and female) were generated by crossing Furin<sup>loxP/loxP</sup> mice (Roebroek et al., 2004) with Foxn1-Cre mice (Liang et al., 2018). Tamoxifen-induced Furin knockout mice (male and female) were obtained by crossing Furin<sup>loxP/loxP</sup> mice with ER-Cre mice (Zhao et al., 2018). Furin<sup>loxP/loxP</sup> mice were the generous gifts of Dr. John W. M. Creemers from the Department of Human Genetics, KU Leuven, Leuven, Belgium. All mice used in the study were bred under specific pathogen-free conditions.

In vitro culture of TECs
For in vitro TEC culture, thymi from ER-Cre-Furin<sup>loxP/loxP</sup> mice neonatal mice were digested as described below. Small thymic fragments from each step were collected and pooled. Fragments were allowed to settle and washed twice with CnT07 medium (CELLnTEC, CnT-BM.4). The remaining thymic explants were plated in 24-well plates with CnT07 medium and cultured at 37°C with 5% CO2 for 5 days.

METHOD DETAILS
Flow cytometry analysis
Single-cell suspensions were prepared and stained with anti-CD16/32 antibodies to block Fc receptors. For cell surface staining, single-cell suspensions were stained for 30 min at 4°C in FACS buffer (PBS containing 0.1% BSA, 0.02% NaN3). For intracellular staining, cells were fixed and permeabilized with fixation buffer (eBioscience, 00-5123-43 and 00-5223-56) and permeabilization buffer or the Cytofix/Cytoperm (BD Biosciences, 554722) and Perm/Wash (BD Biosciences, 554723) solutions according to the manufacturer’s instruction. The staining of CCR7 was performed at 37°C. Stained samples were acquired on LSRFortessa X-20 Cell Analyzer (BD Biosciences) or Gallios (Beckman Coulter) Flow Cytometer. The detailed information of the fluorochrome-conjugated antibodies used for flow cytometry were listed in key resources table.

Immunofluorescence staining
Tissues were embedded into optimum cutting temperature compounds. For the staining of K5 and K8, thymus was cut into 6 μm sections, and for the staining of DCLK1, the thymus tissue was cut into 25 μm sections. Sections were fixed with 4% paraformaldehyde and blocked with donkey serum, followed by overnight incubation with primary antibodies. After washing with PBS-0.05% Tween, sections were incubated with secondary antibodies for 1 hour at room temperature. Color images were made on the N-SIM Super-Resolution Confocal Microscope (Nikon, Tokyo, Japan). The following antibodies were used for staining: rabbit anti-K5 (Covance, clone AF138), rat anti-K8 (DSHB, Troma-I), chicken anti-K5 (Biolegend, 905904), and rabbit anti-DCAMKL1 (DCLK1) (Abcam, ab31704).

Isolation of thymic stromal cells
Mouse thymi were isolated, cleaned of fat, and cut into pieces with a dissection scissors. Tissue pieces were resuspended with DMEM containing 2% FBS, then moved into 15 mL tubes and pipetted up and down
several times. Fragments were allowed to settle before removing the medium and replacing it with 2 mL of digestion medium containing 1 mg/mL collagenase/dispase (Sigma-Aldrich, 11097113001) with 20 U/mL DNase I (Sigma-Aldrich, D5025) in DMEM. Tubes were then incubated at 37°C for 45 min. At the end of the digestion, cell suspensions were gently agitated; then, 5 mL PBS containing 1% FBS and 5 mM EDTA were added to neutralize the digestion. Finally, the cells were centrifuged and resuspended in DMEM (containing 2% FBS), then counted.

**BrdU labeling and staining**
Two-week-old mice were intraperitoneally injected with BrdU (1 mg per mouse). Twenty-four hours later, the mice were sacrificed, and thymic stromal cells were prepared as described above. After cell surface staining, the BrdU staining was performed using the APC BrdU Flow Kit (BD Biosciences, 552598) according to the manufacturer’s instructions.

**5-Ethynyl-2’-deoxyuridine (EdU) assay**
The mouse Furin cDNA was constructed into MSCV-PGK lentiviral vector. The mTEC cell line 1C6 was transfected with MSCV-PGK-Furin (1C6-Furin) or empty vector (1C6-mock), and stable transfected clones were selected and amplified. For the EdU assay, 1C6-mock and 1C6-Furin cell lines seeded in 12-well plates were cultured to 70% confluence and then treated with EdU (10 μM) for 2 hours. The EdU incorporation was detected using BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 594 (Beyotime, C0078S) according to the manufacturer’s instructions.

**Establishment of Furin overexpression cell line**
The full length of Furin cDNA was subcloned into retroviral vector MSCV-PGK followed by IRES and GFP (MSCV-PGK-Furin-IRES-GFP). For retrovirus production, 2.4 μg of MSCV-PGK-Furin-IRES-GFP or empty vector in combination with 0.3 μg of pCL-Eco retrovirus packaging vector was transfected into BOSC 23 cells using jetPRIME transfection reagent (Polyplus). The culture supernatant containing retrovirus was collected after 48 and 72 hours through 0.45-μm filters. 1C6 cells was transduced by retrovirus carrying MSCV-PGK-Furin-IRES-GPF (1C6-Furin) or empty vector (1C6-mock). The single transduced cells were sorted using a Fusion cell sorter according to the expression of GFP, the the sorted cells was amplified and passaged to establish stable cell line.

**Quantitative PCR**
Single-cell thymic stromal suspensions were isolated and stained with appropriate antibodies, and mTECs were then sorted using an FACS BD fusion (BD Biosciences). RNA was isolated using the MicroElute Total RNA Kit (Omega Bio-tek, R6831) and reverse transcribed using the SuperScript III Reverse Transcriptase Kit (Invitrogen, 18080-093). The resulting cDNA was used as templates for quantitative PCR with SYBR Premix Ex Taq™ (TaKaRa, RR420) on a CFX96 apparatus (Bio-Rad Laboratories).

**Bulk RNA-seq sample preparation and analysis**
Single-cell epithelial suspensions were isolated and enriched using anti-mouse CD45 microbeads (Miltenyi Biotec, 130052301), and cTECs and mTECs were then sorted using a Fusion cell sorter (BD Biosciences). The total RNA of the sorted cTECs and mTECs was extracted using TRIzol reagent (Thermo Fisher, 15596018), and amplified cDNA was prepared using the Smart-Seq2 method according to the manufacturer’s instructions. Amplified cDNA quality was assessed by the Agilent 2100 High Sensitivity DNA Assay Kit (Agilent Technologies), and cDNA library construction was prepared using the Bioruptor Sonication System and CWBIO Gel Extraction Kit. After the library construction, the insertion size was assessed by the Agilent Bioanalyzer 2100 system, and the accurate insertion size was quantified by the TaqMan fluorescence probe of the AB Step One Plus Real-time PCR system (library valid concentration >10 nM). The libraries were then sequenced by an Illumina Hiseq platform with a 150 bp paired end. The raw data were assessed by FastQC, and the adaptor sequence was filtered by Trimgalore. We used the mapping software HISAT2 to map the reads to the mm10 reference genome and StringTie, to construct transcripts independently for each cell. The differentially expressed genes (q-value <0.05, |logfc| >0) were identified using DEGseq by comparing mTECs and cTECs from *Furin<sup>fl/fl</sup>*Foxn1<sup>cre</sup> and *Furin<sup>fl/fl</sup>*Foxn1<sup>cre</sup> mice to those in WT mice, respectively. The differentially expressed genes were subjected to GSVA to calculate individual gene set enrichment scores by searching the c2.all.v7.4 symbols database according to published methods (Cowan et al., 2019; Hanzelmann et al., 2013). Visualization of GSVA results was obtained using R3.6.0. We used the
DAVID Bioinformatics Resources 6.8 online search tool (https://david.ncifcrf.gov/) and the Kobas online search tool (http://kobas.cbi.pku.edu.cn/) to search for gene ontology (GO) functional annotation and KEGG pathway analysis using all differential genes. All analyses were selected with $p < 0.05$ as the cutoff criterion. The bulk RNA sequencing data sets for this study can be found in Gene Expression Omnibus (GEO): PRJNA794306.

**Single-cell RNA sequencing and analysis**

The TECs from 2-week-old WT and *Furin*<sup>fl/fl</sup> Foxn1<sup>Cre</sup> mice were sorted using a Fusion cell sorter (BD Biosciences). Barcoded single cells were captured using the 10X Chromium microfluidics system (10X Genomics). Single-cell RNA-seq libraries were constructed using the Single Cell 3’ Library and Gel Bead Kit V3.1 (10X Genomics, 1000075) according to the manufacturer’s instructions. Finally, the libraries were sequenced using an Illumina Novaseq 6000 sequencer with a sequencing depth of at least 100,000 reads per cell with pair-end 150 bp (PE150) reading strategy (performed by CapitalBio Technology, Beijing). The Cell Ranger pipeline (10X Genomics, version 3.0.2) was used to demultiplex cellular barcodes.

Sequences from scRNA-seq were processed using cellranger-3.0.2 software and the sequences were processed using the cellranger-mm10-3.0.0 genome and gff file. Raw data generated by Cellranger were then read into the Seurat v3.2.3 R package with at least 200 genes per cell and at least 3 cells. Cells with <5% mitochondrial gene count and expressing 200–5,000 detected genes were retained for a total of 12,979 cells (5,431 for WT and 7,547 for *Furin*<sup>fl/fl</sup> Foxn1<sup>Cre</sup> mice). The remaining cells and genes were used for downstream analysis. The data were normalized by using ‘LogNormalize’ method and data scaled with ‘scale.factor=10000’ from Seurat. For each sample, variable genes were found by using ‘FindVariableGenes’ with the following options mean.function = ExpMean, dispersion.function = LogVMR, x.low.cutoff = 0.1, x.high.cutoff = 8, y.cutoff = 1. Then we use IntegrateData to create an ‘integrated’ data assay. For the initial analysis the returned variable genes were used as the gene list given to the RunPCA function. Clusters were determined using the ‘FindClusters’ function with the following options reduction.type = ‘pca’, dims.use = 1:20, resolution = 1. Then remove T cells\endothelial cells\immune cells according to CD3e\CD34\CD45 to get TECs. Further TEC dimensionality reduction was performed by using RunUMAP using the options reduction.type = ‘pca’, dims.use = 1:20, resolution = 0.5. Markers of each cluster were found using the ‘FindMarkers’ command and highly similar clusters were merged. mTECs dimensionality reduction was performed by using RunUMAP using the options reduction.type = ‘pca’, dims.use = 1:20, resolution = 1 and also merged highly similar clusters and mTECs were clustered into four major populations, according to a previous report (Bornstein et al., 2018). Differential gene expression was performed by running FindMarkers on all pair-wise clusters (so that differentially expressed genes could be shared between clusters). The threshold of differentially expressed genes is logfc.threshold = 0.25, test.use = “wilcox”, min.pct = 0.1. To account for the TRA heterogeneity in the mTEC subsets, we used the function “AddModuleScore” from Seurat (Tirosh et al., 2016) and the list of AIRE-dependent and AIRE-independent TRA-associated genes (Sansom et al., 2014) to calculate AIRE-dependent and AIRE-independent scores for each cell; this score was then regressed out. The scRNA-seq sets for this study can be found in GEO: GSE193456.

**Western blot**

Tamoxifen (0.125 μM) and vehicles were administrated to the in vitro cultured TECs for 2 days. When needed, cultured TECs were starved overnight and stimulated with 100 ng/mL IGF1 (Abcam, ab198569) and 200 ng/mL Insulin (Beyotime, P3376-100IU) for 20 min. The in vitro cultured TECs were lysed with radio-immunoprecipitation assay (RIPA) (Beyotime, P0013B) containing PMSF (Beyotime, ST506) and PhosSTOP phosphatase inhibitors (Roche, 04906845001). Protein concentration was determined using the Enhanced BCA Protein Assay Kit (Beyotime, P00105). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membrane (Merck Millipore, IPFL00010). The PVDF membrane was blocked with 5% nonfat dried milk (OXOID, LP0031) for 1–2 hours, then incubated overnight with primary antibodies on a shaker at 4°C. After washing three times with PBST, the PVDF membrane was incubated with HPR-coupled secondary antibodies, and the concentration of proteins was detected through chemiluminescence (Merck Millipore, WBKLS0500). The detailed information of primary and secondary antibodies used for western blot were listed in key resources table.

**Protein expression analysis by Simple Western**

The sorted mTECs were lysed with RIPA as described above. Simple Western was performed using 12–230 kDa Separation Module (ProteinSimple, SM-W002-1) according to the manufacturer’s instructions.
The results were analyzed using Compass for SW (ProteinSimple). The primary antibodies used for Simple Western are as follows: β-Actin (Sigma-Aldrich, A5441), IGF1R antibody (Novus Biologicals, NBP1-77679), and Insulin receptor β (CST, 3025).

**ERK inhibitor and Akt inhibitor treatment**

The ERK inhibitor, SCH772984 (Selleck, S7101), was dissolved in 5% DMSO+30% PEG300 (Selleck, S6704) +65% deionized water. Four-week-old C57BL/6 mice were treated intraperitoneally with either SCH772984 (25 mg/kg) or vehicle once a day for 7 days. The Akt inhibitor, MK-2206 2HCl (Selleck, S1078), was dissolved in DMSO. The thymic lobes of FTOC (Liang et al., 2018) were treated with either MK-2206 2HCl (1 mM) or vehicle for 4 days and then digested for the examination of mTECs and cTECs by flow cytometry.

**Detection of autoantibodies**

For the detection of autoantibodies, serum was collected from 6- to 8-month-old WT, Furin^{fl/+} Foxn1^{Cre}, and Furin^{fl/fl} Foxn1^{Cre} mice. Various tissue sections from Rag2^{-/-} mice were then stained with the serum at 1:50 dilution, followed by incubation with AF488 donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, 715-545-150). Images were acquired with a laser scanning N-SIM Super-Resolution Confocal Microscope (Nikon, Tokyo, Japan).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All data are presented as the means ± SD. The Student’s unpaired t-test for comparison of means was used to compare groups. A p-value of <0.05 was considered statistically significant.