Foxn1-β5t transcriptional axis controls CD8⁺ T-cell production in the thymus

Muhammad Myn Uddin1,*, Izumi Ohigashi1,*, Ryo Motosugi2,*, Tomomi Nakayama2, Mie Sakata1, Jun Hamazaki2, Yasumasa Nishito3, Immanuel Rode4, Keiji Tanaka5, Tatsuya Takemoto6, Shigeo Murata2 & Yousuke Takahama1

The thymus is an organ that produces functionally competent T cells that protect us from pathogens and malignancies. Foxn1 is a transcription factor that is essential for thymus organogenesis; however, the direct target for Foxn1 to actuate thymic T-cell production is unknown. Here we show that a Foxn1-binding cis-regulatory element promotes the transcription of β5t, which has an essential role in cortical thymic epithelial cells to induce positive selection of functionally competent CD8⁺ T cells. A point mutation in this genome element results in a defect in β5t expression and CD8⁺ T-cell production in mice. The results reveal a Foxn1-β5t transcriptional axis that governs CD8⁺ T-cell production in the thymus.
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D8 + T cells have a central role in immune defense against viral infection, intracellular pathogens, and malignant tumours. CD8 + T cells are chiefly generated in the thymus through the process of positive selection. Positive selection of functionally competent CD8 + T cells is dependent on TCR engagement of immature thymocytes with self-peptides produced by the thymoproteasome, a thymus-specific form of the proteasome. The thymoproteasome is specifically expressed in cortical thymic epithelial cells (cTECs) because its unique catalytic subunit β5t or Psmb11 is exclusively transcribed in cTECs. However, how β5t is expressed specifically in cTECs is poorly understood. Previous studies showed that when the entire coding sequence of β5t in mouse genome is replaced with foreign sequences, including sequences encoding Venus fluorescence protein and Cre recombinase, the mouse retains cTEC-specific expression of Venus and Cre, respectively, suggesting that the genomic element that instructs the cTEC-specific expression of β5t is located mainly outside the β5t-coding sequence. Importantly, β5t expression in the embryonic thymus primordium is not detectable in Foxn1-deficient mice. Foxn1 is a transcription factor that governs the development of TECs, and thymus organogenesis is prematurely arrested in Foxn1-deficient mice and humans. However, whether Foxn1 directly controls the transcription of β5t or indirectly affects β5t by regulating molecules that are crucial for upstream TEC development has not been clarified.

Indeed, no direct targets of Foxn1 in its transcriptional regulation of gene expression have been identified in TECs, despite the importance of Foxn1 in TEC development. Like β5t, many molecules, including DLL4, CCL25 and PD-L1, expressed in TECs have markedly reduced expression in Foxn1-deficient mice. However, it is not established whether Foxn1 directly or indirectly affects the expression of any of those TEC-associated genes, including functionally relevant genes in the thymus.

Here we report the identification of a highly conserved Foxn1-binding sequence that is located proximal to the β5t-coding sequence in the genome. In vitro experiments show that Foxn1 protein binds to this sequence and promotes proximal gene transcription. In vivo experiments in mouse show that this cis-regulatory element is indeed essential for the optimal expression of β5t in cTECs and the optimal production of CD8 + T cells. Our results reveal a Foxn1-binding cis-regulatory element that is functionally relevant for the thymus to produce T cells.

Results

Foxn1-binding motifs adjacent to β5t-coding sequence. β5t-encoding gene in the mouse genome is encoded by a single exon located within the 14-kb region between β5t-coding and Cdhd24-encoding genes in chromosome 14 (ref. 6) (Fig. 1a). Within this 14-kb region, we searched for the 11-bp Foxn1-binding consensus motif, a/a g n g A G C G t a/t t, in which the middle trinucleotide in large letters represents the invariant core motif. Although there were no sites that perfectly matched this 11-bp consensus motif, we detected 18 sites that contained the 4-bp core motif (Fig. 1a,b). Among those 18 sites, site #13 located 80-bp upstream of β5t transcription initiation site best matched (2-bp mismatched) the 11-bp consensus motif (Fig. 1b). Among the four sites with second-best matched (3-bp mismatched) sequence, site #8 located 2.4-kb upstream of β5t transcription initiation site contained the longest (7-bp) region identical with the 11-bp consensus motif. The order and orientation of the neighbouring β5t-, β5t-, and Cdhd24-encoding genes were conserved in the genomes of various mammalian species, including human, chimpanzee, dog, rat, bat, elephant and horse (NCBI public database). Site #13 was well conserved among those species, whereas site #8 appeared less conserved (Supplementary Table 1).

Foxn1 can bind to a site proximal to β5t-coding sequence. We examined whether Foxn1 could actually bind to the candidate sites. To do so, we co-transfected HEK293T cells with a plasmid that expressed Foxn1 and a plasmid that contained a mouse genomic region proximal to β5t-encoding gene (Fig. 1c,d). Foxn1 protein was immunoprecipitated from the lysates of transfected cells, and co-precipitated DNA fragments were PCR-amplified for those candidate sites. We detected Foxn1-dependent immunoprecipitation and PCR amplification for site #13 and not the other sites including site #8 (Fig. 1d). The co-precipitation reflected specific binding to the β5t-proximal 3-kb genomic sequence because no signals were detected when the transfected plasmid did not contain this 3-kb fragment (Fig. 1e,f). A point mutation in the core sequence of site #13, which destroyed the capability of binding to Foxn1 protein, severely diminished the Foxn1-immunoprecipitated signals, whereas an equivalent mutation in the core sequence of site #8 did not affect the signals (Fig. 1e,f). These results indicate that Foxn1 protein can specifically bind to site #13 that is proximally located 80-bp upstream of β5t transcription initiation site.

Foxn1 can enhance transcription via β5t-proximal cis-element. We next examined whether Foxn1 binding to site #13 might indeed affect the transcription of proximal β5t-encoding gene. Towards this goal, we co-transfected HEK293T cells with a plasmid that co-expressed Foxn1 protein and tdTomato red fluorescence protein and a plasmid that contained the EGFP green fluorescence protein reporter sequence attached to the herpes simplex virus thymidine kinase gene promoter (HSV-tk) and a variety of the mouse genomic sequence 5′ to the β5t-encoding exon (Fig. 2a). In this reporter assay, the HSV-tk promoter was a weak promoter relative to the cytomegalovirus immediate early gene promoter (CMV-EGFP) (Supplementary Fig. 1) and thus was useful for the sensitive detection of transcriptional regulation mediated by a transfected molecule, such as Foxn1. Upon the plasmid transfection, we measured EGFP fluorescence reporter signals in tdTomato-expressing cells that did or did not co-express transduced Foxn1, in order to detect transcriptional regulation mediated by Foxn1 and cis-regulatory elements. We detected a Foxn1-dependent elevation of HSV-tk-driven EGFP reporter expression when the reporter plasmid contained the 3-kb genomic fragment (Fig. 2b and Supplementary Fig. 2a). The Foxn1-dependent elevation of the reporter expression was markedly abrogated by introducing a point mutation in the core sequence of site #13, which destroyed the Foxn1-binding capability (Fig. 1f), but not by introducing an equivalent mutation in site #8 (Fig. 2b). Foxn1 could elevate the HSV-tk-driven EGFP reporter expression when the reporter plasmid contained the 1-kb mouse genomic fragment, which contained site #13, but could not do so when the reporter plasmid contained no genomic fragment (Fig. 2b).

A point mutation in the core sequence of site #13 in the 1-kb genomic fragment was sufficient to abrogate the Foxn1-dependent elevation of the reporter expression (Fig. 2b). The mutant Foxn1 protein that lacked a DNA-binding domain (Fig. 2c) failed to elevate the reporter expression (Fig. 2d), reconfirming that the specific binding of Foxn1 protein to site #13 is responsible for the elevation of the reporter expression. These results indicate that Foxn1 binding to site #13 can elevate the transcription of a proximal reporter gene.
All statistical analyses were performed by student's t-test. ND, not detectable. (Figure 1) Foxn1 binds to β5t-proximal site #13. (a) Schematic diagram of the locations of 18 sites that contain the Foxn1-binding invariant core AGCG tetranucleotide within the 14-kb region proximal to β5t-encoding gene between two neighbouring genes in the mouse genome. Arrows indicate the orientation of the transcription. (b) Distances of the 18 sites from β5t translation initiation site are listed. The nucleotide sequences of those sites and their mismatches from the Foxn1-binding consensus 11-bp sequence previously reported21 are also listed. (c,d) HEK293T cells were transfected with a vector that expressed Foxn1 and a plasmid that contained mouse genomic DNA fragment proximal to β5t-encoding gene, as illustrated schematically (c). Forty-eight hours after the transfection, formaldehyde-fixed cell lysates that contained protein–DNA complexes were immunoprecipitated with either goat anti-Foxn1 antibody (filled bars) or control IgG (open bars) and PCR-amplified for the indicated candidate sites of the Foxn1-binding sequences. Graphs show the frequency of immunoprecipitated DNA in input DNA (mean ± s.e.m., n = 5), which was sonicated at mild or strong amplitude (d). *P < 0.05; NS, not significant; ND, not detectable. (e,f) A plasmid that contained the 3 kb DNA fragment upstream of β5t-encoding gene or its variants mutating at the indicated site was used for immunoprecipitation (e). A control plasmid that contained no genomic DNA fragment was used where indicated (mock). Graphs show the frequency of immunoprecipitated DNA in input DNA (mean ± s.e.m., n = 3) (f). *P < 0.05; NS, not significant; ND, not detectable. All statistical analyses were performed by student’s t-test.

To further characterize Foxn1-dependent cis-regulatory elements, we generated a series of luciferase reporter constructs that contained the mouse β5t 5’ genomic region and measured luciferase activity of cells expressing each construct in response to Foxn1 expression. Foxn1-mediated transcriptional activity was readily detectable with constructs that contained 5’-upstream to nucleotide position from −1 to −503, and its magnitude was comparable to that of construct containing longer sequences (Figure 2e), suggesting that the 503-bp β5t-upstream sequence containing sites #11, #12 and #13 is sufficient for the...
Foxn1-dependent promoter activity. We then deleted these three sites from the 503-bp region and examined luciferase activity. We found that the deletion of site #13 most profoundly decreased the Foxn1-dependent promoter activity (Fig. 2f). The deletion of site #12 less severely affected the activity, whereas the deletion of site #11 showed no significant effect on the promoter activity (Fig. 2f). These results reconfirm that site #13 is a potent cis-regulatory element for Foxn1-mediated reporter transcription, and further suggest the additional roles of other sites, including site #12.

**Induced mutation in proximal Foxn1-binding site in mouse.** Our results suggested the possibility that the binding of Foxn1 to the #13 cis-regulatory site would contribute to the expression of

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β5t in cTECs. Indeed, in vivo chromatin immunoprecipitation analysis showed Foxn1 binding to site #13 in the thymus but not the liver from fetal mice (Fig. 3a). Foxn1 binding to site #13 was readily detectable in isolated cTECs but not isolated medullary TECs (mTECs) (Fig. 3b). That Foxn1 binding to site #8 and site #18 was almost undetectable demonstrated the specificity of in vivo Foxn1 binding to site #13 (Fig. 3a,b). The detection of in vivo Foxn1 binding to site #13 specifically in cTECs but not mTECs, in which Foxn1 is expressed23,24, suggests the contribution of epigenetic mechanisms that allow in vivo Foxn1 binding to site #13 in cTECs but limit it in mTECs. In this regard, it is interesting to note that the expression levels of Foxn1 and β5t in freshly isolated cTECs are well correlated under different cell culture conditions (Fig. 3c). When cTECs from the thymus lobes were dispersed, β5t messenger RNA (mRNA) expression in cTECs was quickly lost within 12 h (Supplementary Fig. 3a). β5t mRNA expression remained low in two-dimensional (2D) flat dish culture for up to 72 h but partially recovered in reaggregation thymus organ culture (RTOC) after 48 h (Supplementary Fig. 3a). Microarray analysis of embryonic thymic stromal cells cultured in either 2D flat dish culture or RTOC for 72 h revealed that three transcription factors, Foxn1, Hey1 and Spatial, behaved similarly to β5t in these culture conditions (Fig. 3c). Among these three transcription factors, Foxn1 but not Hey1 or Spatial promoted the reporter expression in HEK293T cells through the β5t 5’ genomic region (Fig. 3d). These results reinforce the possibility that Foxn1 directly activates β5t expression in cTECs.

To directly examine whether Foxn1 binding to site #13 is functionally relevant in vivo, we introduced a point mutation into the site #13 sequence in the mouse genome and examined the phenotypes of those mutant mice. An improved CRISPR/Cas9-mediated genome editing technology25 was used to introduce the point mutation in the mouse genome. Three independent alleles of mutant mouse strains generated in this study identically contained the intended point mutation in site #13 (Fig. 3e), at the functionally relevant nucleotide in Foxn1-binding and Foxn1-dependent reporter transcription (Figs 1f and 2b). Chromatin immunoprecipitation analysis of cTECs isolated from site #13 homozygous mutant mice showed that Foxn1 binding to site #13 in cTECs in vivo was significantly (P<0.001; Student’s t-test) reduced by the introduced mutation in the genome (Fig. 3f). The non-specific cleavage of the off-target sequence is a possible risk of the CRISPR/Cas9-mediated genome editing. The cleavage efficiency is dependent on the number, position and distribution of mismatches26. We examined fifteen off-target genomic sequences that exhibited the highest homology to the RNA-guide sequence and the highest scores of the off-target likeliness, and found that all of them remained intact without mutations in all of the three mutant alleles (Supplementary Fig. 4), suggesting that the genome editing carried out in this study introduced no apparent off-target mutations in the mouse genome. The following phenotypes of the mutant mice reported in this study were reproduced in all the three independent alleles.

**Diminished β5t expression in cTECs in mutant mice.** Mice carrying either heterozygous or homozygous alleles for the site #13 mutation were born and fertile. No apparent abnormality in macroscopic appearance was noted. The thymus contained unaffected corticomedullary architectures and their weights were normal (Fig. 4a). Immunofluorescence analysis of the thymic sections and flow cytometric analysis of liberase-digested thymic cells showed that the cellularity of cTECs and mTECs in those mutant animals was undisturbed (Fig. 4b). Importantly, however, cTECs from mice carrying the homozygous mutation at site #13 had markedly reduced β5t expression (Fig. 4c). Flow cytometric analysis of isolated cTECs showed that the β5t expression was reduced significantly (P<0.001; Student’s t-test) and appeared homogeneous in the homozygous cTECs, whereas the β5t expression was reduced but still significantly (P<0.001; Student’s t-test) detectable when compared with the background signals detected in β5t-deficient cTECs (Fig. 5a). The expression levels of MHC class I and class II molecules in cTECs and mTECs were not reduced in those mutant mice (Fig. 5b,c). In contrast, β5t expression in cTECs of heterozygous mutant mice appeared unaffected (Fig. 5a). The amount of β5t mRNA detectable in isolated cTECs was well correlated with the amount of β5t proteins (Fig. 5d), suggesting that the reduction in β5t expression is due to reduced β5t transcription caused by the mutation in site #13. These results indicate that homozygous mutation in site #13 markedly diminishes β5t expression in cTECs in vivo.

**Defective CD8+ T-cell generation in mutant mice.** We finally examined whether and how the site #13 mutation would affect T-cell development in the thymus. The numbers of total thymocytes and CD4−CD8− TCRlow, CD4+CD8− TCRlow and CD4+CD8+ TCRhigh thymocytes were not affected in heterozygous and homozygous site #13 mutant mice (Fig. 6a). However, the cellularity of CD4−CD8− TCRhigh thymocytes was significantly (P<0.05; Student’s t-test) reduced in homozygous but not heterozygous mutant mice (Fig. 6a). The reduction in the number of CD4−CD8+ TCRhigh thymocytes in homozygous mutants was significant but less prominent than that in β5t-deficient mice (Fig. 6a). An essentially similar reduction in the number of CD4−CD8+ T cells was observed in the spleen of

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Figure 2 | Foxn1 binding to site #13 enhances transcription of proximal gene. (a) HEK293T cells were transfected with a plasmid that co-expressed Foxn1 protein and tdTomato red fluorescence protein and a plasmid that contained the EGFP green fluorescence protein reporter sequence attached to the herpes simplex virus thymidine kinase gene promoter (HSV-tk) and a variety of the mouse genomic sequence 5’ to β5t-encoding gene as indicated. Site #13 cm, a point mutation in the core sequence of site #13; site #13 nm, a point mutation in the non-core sequence of site #13; site #13 cm, a mutation in both core and non-core sequence of site #13. (b) Dot plots show the expression of Tomato and EGFP in propidium iodide (PI)-negative viable HEK293T cells co-transfected with indicated EGFP reporter vectors and irres-tdTomato-expressing plasmid (upper profiles) or Foxn1-ires-tdTomato plasmid (lower profiles). Numbers in dot plots indicate mean fluorescence intensity (MFI) of EGFP expression in tdTomato+ PI- cells. Bar graphs show MFI (means ± s.e.m., n = 3) of EGFP expression in Tomato+ PI- cells. **P<0.01; ***P<0.001. See also Supplementary Fig. 2a. (c) Immunoblot analysis of Foxn1 protein or mutant Foxn1 protein without the DNA-binding domain (DBD). Calnexin was examined as the loading control. (d) HEK293T cells were co-transfected with indicated plasmids. EGFP reporter expression was measured as in b. ***P<0.005; n.s., not significant. (e) HEK293T cells were transfected with a series of luciferase reporter constructs that contained indicated lengths of the β5t 5’ genomic region, together with a Foxn1-encoding plasmid. Histograms represent relative luciferase activity, where the activity without genomic sequences was set as 1. Means ± s.d. (n = 3) are shown. **P<0.05. All statistical analyses were performed by student’s t-test.
Figure 3 | Generation of site #13 mutant mice. (a) Thymuses and livers isolated from E14.5 embryos were liberase-digested. Protein–DNA complexes were immunoprecipitated with goat anti-Foxn1 antibody (filled bars) or control IgG (open bars) and PCR-amplified for site #8 or site #13. Graph shows fold enrichment (means ± s.e.m., n = 9) of anti-Foxn1-precipitated signals normalized to the signals by control IgG. (b) CD45⁺ CD326⁺ UEA1⁺ CD249⁺ cTECs and CD45⁺ CD326⁺ UEA1⁺ CD249⁺ mTECs were isolated from 2-week-old C57BL/6 mice. Protein–DNA complexes were immunoprecipitated with anti-Foxn1 antibody (filled bars) or control IgG (open bars) and PCR-amplified for site #13 or site #18. Graph shows fold enrichment (means ± s.e.m., n = 3) of anti-Foxn1-precipitated signals normalized to the signals by control IgG. ***P < 0.001. (c) Comparison of gene expression profiles between RTOC and 2D culture of thymic stromal cells from E14.5 and E15.5 mice by microarray analysis. Grey dots represent ratios (log scale) of gene expression levels (33,749 transcripts) in the two culture conditions. Longitudinal and horizontal axes show the ratios in E14.5 and E15.5 thymic stromal cells, respectively. (d) A plasmid encoding Foxn1, Hey1 or Spatial was transfected into HEK293T cells together with a firefly luciferase that contained the 7-kb DC3' flanking region and a control plasmid encoding Renilla luciferase. Intensities of firefly and Renilla luciferase activities were measured 48 h after transfection. Histograms represent relative luciferase activity, where the activity in mock transfection was set as 1. All data are shown as means ± s.d. (n = 3). ***P < 0.005. (e) Mutant mice carrying the mutation in site #13 proximal to β5t-encoding gene were generated by the CRISPR/Cas9-mediated genome editing technology. The 2-bp mutation at site #13 (left) was confirmed in wild-type (wt), heterozygous, and homozygous mutant mice (right). (f) cTECs and mTECs were isolated from 2-week-old site #13 homozygous mutant mice. Graph shows fold enrichment (means ± s.e.m., n = 3) of mouse monoclonal anti-Foxn1-precipitated signals normalized to the signals by control IgG (filled bars) and control IgG (open bars) and comparison to the value in TECs isolated from C57BL/6 mice (open bars) as shown in b. ***P < 0.001; NS, not significant. All statistical analyses were performed by student’s t-test.

The results indicate that homozygous mutation in site #13 significantly diminishes the generation of CD8⁺ T cells in the thymus. A recent report described that CD4⁺ CD8⁺ thymocytes could be divided into three subpopulations on the basis of TCRβ and CD5 expression levels; TCRβlow CD5low DP1 thymocytes are enriched with pre-selection thymocytes; TCRβintermediate CD5high DP2 thymocytes are enriched with recently TCR-engaged thymocytes and contain both MHC class I- and MHC class II-restricted thymocytes; and TCRβhigh CD5intermediate DP3 thymocytes are enriched with MHC class I-engaged positively selected thymocytes that give rise to CD4⁺ CD8⁺ TCRβhigh thymocytes. We detected a significant (P < 0.05; Student’s t-test) reduction in DP3 thymocytes in homozygous mutant mice compared with heterozygous mutant mice, whereas the reduction in DP3 thymocytes was less severe than that in β5t-deficient mice (Supplementary Fig. 5). These results suggest that the homozygous mutation in site #13 disturbs positive selection of MHC class I-restricted CD4⁺ CD8⁺ thymocytes.

Discussion

The thymoproteasome component β5t has a pivotal role in the generation of functionally competent CD8⁺ T cells and is
wild-type cTECs. **

mRNA levels were normalized to those of CD45/C0 (grey shades) mice at 2 weeks old. Bar graphs show the relative fluorescence intensity (RFI, class II) expression normalized to the mean fluorescence intensity measured in wild-type cells. *

**5t expression in cTECs and diminishes the cellularity of mTECs. NS, not significant. Statistical analyses were performed by student's t-test. (c) Immunofluorescence analysis of **5t (green), Aire (red) and UEA-1-binding molecules (blue) in thymic sections from 2-week-old mice. Representative data from three independent experiments are shown. Scale bar, 2 mm.

**5t (green), Aire (red) and UEA-1 reactivity and CD249 expression in thymic sections from 2-week-old mice. Representative data from three independent experiments are shown. Scale bar, 75 μm.

**5t expression is regulated was unknown. The present results show that the mouse genomic sequence termed site #13, located 80-bp upstream of **5t transcription initiation site in chromosome 14, is well conserved in the genomes of various mammalian species and functions as a Foxn1-dependent cis-regulatory element in the transcriptional promotion of **5t gene expression. CRISPR/Cas9-mediated editing of mouse genome reveals that a point mutation in site #13 reduces **5t expression in cTECs and diminishes the cellularity of CD8 T cells generated in vivo. Our chromatin immunoprecipitation analysis reconfirms the in vivo binding of Foxn1 to site #13 in cTECs and the reduction in the binding by the point mutation in site #13. Altogether, these results reveal a Foxn1-binding cis-regulatory element that has a pivotal role in thymic epithelial cell dependent positive selection of CD8 T cells. It has been suggested that Foxn1 regulates the expression of several genes, including DLL4, CCL25 and PD-L1 (refs 17–20), as well as **5t (refs 11,14), based on the reduced expression of those genes in the embryonic thymus primordium of Foxn1-deficient mice. In contrast, the present results unveil a cis-regulatory element that Foxn1 directly acts on to promote the transcription of a functionally relevant gene in TECs. Our results establish that Foxn1 directly enhances the transcription of
β5t, thereby directly controlling the thymus-dependent production of CD8+ T cells.

Interestingly, β5t is abundantly expressed in cTECs, but is not detectable in other cells including skin epithelial cells in which Foxn1 is expressed and important for hair follicle development. It is, therefore, reasonable to assume that Foxn1 does not always induce β5t expression regardless of cellular context. Instead, the expression of β5t may require an unknown cellular context that is unique in cTECs, in addition to the expression of Foxn1. In this regard, it is interesting to note that β5t is not detectable in the majority of mTECs, even though it is abundant in cTECs. Unlike β5t, however, Foxn1 is readily detectable in most mTECs and cTECs, and the development of mTECs as well as of cTECs is dependent on Foxn1. The disparity in expression between β5t and Foxn1 in mTECs further supports the possibility that β5t expression is additionally regulated by mechanisms other than the Foxn1-mediated transcriptional promotion. β5t is expressed in bipotent TEC progenitors that give rise to cTECs and mTECs and the vast majority of mTECs are derived from β5t-expressing progenitor cells. It is, therefore, possible that the additional mechanism regulating β5t expression may involve termination specifically in mTECs and/or maintenance specifically in cTECs. Alternatively, it is also possible that the difference in Foxn1 expression levels in cTECs and mTECs may account for the difference in β5t expression in those cells, because higher expression levels of Foxn1 are detectable in cTECs than mTECs (our unpublished results). In this context, it is interesting to note that our in vivo chromatin immunoprecipitation results demonstrated that Foxn1 binding to site #13 was clearly detectable in cTECs but not mTECs, suggesting that mTEC-specific epigenetic modification of the β5t-encoding genomic region may limit access of Foxn1 to site #13 in mTECs.

A recent report described that Foxn1 binds to many sites in the mouse genome, including the sites proximal to β5t gene, and that Foxn1 is capable of regulating β5t transcription in the in vitro reporter assay. The present results unveil a cis-regulatory element that Foxn1 directly acts on to promote β5t transcription in cTECs in vivo. We would like to reiterate that the direct target of Foxn1 in controlling the transcription of a functionally relevant gene in TECs in vivo has been revealed in this study.

Finally, our results showing that β5t expression in cTECs of site #13 homozygous mutant mice was ~50% of the normal expression levels in cTECs of control mice was in concordance with the reduction in the number of CD4−CD8+ TCRβhigh thymocytes to ~50% of normal cellularity in control mice. On the other hand, no significant reduction in β5t expression or CD4−CD8+ TCRβhigh thymocyte cellularity was detectable in site #13 heterozygous mutant mice. These results suggest that the cellularity of CD4−CD8+ thymocytes positively selected in the thymus is determined by the availability of β5t-dependent peptide-MHC complexes expressed by cTECs, a possibility that further suggests that the availability or avidity, in addition to the affinity, of peptide-MHC complexes contributes to inducing β5t-dependent positive selection of CD8+ T cells in the thymus. The novel Foxn1-β5t transcriptional axis presented in this study is expected to provide the basis for better understanding and future manipulation of the thymus-dependent generation and regeneration of functionally competent T cells.

**Methods**

**Genome sequencing and analysis.** Genome DNA was PCR-amplified and sequenced by using Big Dye Terminator V3.1 cycle sequencing kit (Applied Biosystems), and analysed by Genetic Analyser 3500 (Applied Biosystems). Genome sequences registered in the NCBI public database were analysed using Genetyx and mVista (DNAStar).

** Constructs and transfection.** Full-length Foxn1 complementary DNA was PCR-amplified from C57BL/6 mouse cTECs by PrimeSTAR DNA polymerase (Takara) and cloned into pcDNA-blunt vector (Invitrogen) and into CMV-promoter-driven bicistronic herpes simplex virus-1 thymidine kinase containing plasmid. Genomic fragments PCR-amplified from C57BL/6 mouse genomic DNA were cloned into HSV-tk-vector-driven EGFP reporter plasmid. Point mutations in the reporter plasmids were introduced with a PrimeSTAR mutagenesis kit (Takara). HEK293T cells were cultured in Dulbecco’s modified eagle medium supplemented with 10% fetal bovine serum and 100 U ml−1 penicillin streptomycin at 37°C and 5% CO2. Cells were transfected using X-tremeGENE nine DNA transfection reagent (Roche).
DNA and chromatin immunoprecipitation. Transfected cells or liberase-digested tissues isolated from E14.5 C57BL/6 mice were fixed in 1% formaldehyde for 10 min, neutralized with 125 mM glycine, and lysed with lysis buffer (1% NP-40, 1% Triton-X, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA) supplemented with the protease inhibitor cocktail (Sigma) for 20 min. Lysates were sonicated at 30% amplitude for five cycles (strong amplitude) or 20% amplitude for three cycles (mild amplitude) of 20 s on and 60 s off (Braunson Sonifier). DNA was pre-cleaned with 50% protein G-Sepharose (GE Healthcare) in salmon sperm DNA (Sigma) DNA–protein complex was immunoprecipitated with 2 μg of goat anti-Foxn1 polyclonal antibody (G-20, Santa Cruz) or control goat polyclonal IgG (Abcam), heated at 65 °C for 4 h, and treated with proteinase K and RNAse. Immunoprecipitated DNA was ethanol-extracted and quantitated by quantitative PCR.

Chromatin immunoprecipitation in thymic epithelial cells. Formaldehyde-fixed cTECs and mTECs isolated from 2-week-old mice were fixed with RIPPER buffer (0.1% SDS, 1% Triton X-100, 0.1% Na-DOC, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 140 mM NaCl) containing protease inhibitor cocktail. Lysates were sonicated in a Covaris S220 (Covaris). Immunoprecipitation was performed as previously described27. Briefly, DNA–protein complex was immunoprecipitated with 2 μg of mouse anti-Foxn1 or control mouse IgG, coupled to Dynabeads protein G (Vertis). DNA–protein complex was heated at 65 °C for 12 h. Immunoprecipitated DNA was purified with a Qiaquick PCR Purification Kit (Qiagen) and quantitated by quantitative nested PCR.

Culture of thymic stromal cells. To obtain thymic stromal cells, thymic lobes from E14.5 or E15.5 C57BL/6 mouse fetuses were cultured in the presence of 2 μg/ml monoclonal antibody 24, or control mouse IgG, coupled to Dynabeads protein G (Vertis). DNA–protein complex was heated at 65 °C for 12 h. Immunoprecipitated DNA was purified with a Qiaquick PCR Purification Kit (Qiagen) and quantitated by quantitative nested PCR.

In vitro transcription reporter assay. Forty-eight hours after the transfection, cell lysates were used for the expression of fluorescence proteins using FACSVerse (BD Biosciences). For luciferase reporter assay, transcriptional activity was measured using a dual luciferase reporter system (Promega). Genomic fragments were subcloned into pGL4.20 firefly luciferase vector and co-transfected into HEK293T cells along with pGL4.74 Renilla luciferase vector and a plasmid encoding a transcription factor using PEI MAX (Polysciences). Luciferase activity was measured according to the manufacturer’s instructions (Promega).

Immunoblot analysis. Cell lysates in lysis buffer (1% NP-40, 1% Triton-X, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA) supplemented with the protease inhibitor cocktail (Sigma) were subjected to SDS–polyacrylamide gel electrophoresis, transferred onto the polyvinylidene difluoride membranes (Millipore), and probed with either goat anti-Foxn1 antibody or rabbit anti-Calnexin antibody (Santa Cruz) and horseradish peroxidase conjugated secondary antibody. Signals were detected with ECL reagent (GE Healthcare) and detected with Image analysis system (Atto).

Microarray analysis. Fetal thymic stromal cells were cultured in RTMC or 2D culture for 72 h. Total RNA was extracted using RNeasy Mini Kit (QIAGEN). Microarray analysis was measured using a dual luciferase reporter system (Promega). Genomic fragments were subcloned into pGL4.20 firefly luciferase vector and co-transfected into HEK293T cells along with pGL4.74 Renilla luciferase vector and a plasmid encoding a transcription factor using PEI MAX (Polysciences). Luciferase activity was measured according to the manufacturer’s instructions (Promega).

Mice. β5-deficient mice were described previously3. All mouse experiments were performed with consent from the Animal Experimentation Committee of the University of Tokushima (#13116). Mutant mice were generated as previously described27. Briefly, zygotes from (C57BL/6xDBA/2)F1 mice were electroporated with 400 ng g−1 Cas9 mRNA, 200 ng g−1 sgRNA-β5 and 400 ng g−1 single-strand oligodeoxynucleotide for base substitution (ssODN). Electroporated zygotes were transferred into the oviduct of pseudopregnant female mice, and the mutant mice were born on E19. Cas9 mRNA was synthesized using Sall linearized pSp64TL-bCas9 and an in vitro RNA transcription kit (mMESSAGE mMACHINE SP6 Transcription Kit, Ambion), according to the manufacturer’s instructions. A pair of oligo-DNAs targeting β5 (5'-AAACGGCTTCCACACAGGCTTC-3' and 5'-TGGGAGAGGTGCTGGAAGG-3') was annealed and inserted into the Bsal site of pDR72 (Addgene) to produce pDR75-β5. sgRNA-β5 was synthesized using the Dral linearized pDR72-β5 as template and the MEGAshortscript T7 Transcription Kit (Ambion). Synthesized Cas9 mRNA and sgRNA were purified by phenol–chloroform–isoamyl alcohol extraction and isopropanol precipitation. The precipitated DNA was dissolved in Opti-MEM I (Life Technologies) at 2–4 μg ml−1, and stored at −20 °C until use. ssODN (5'-CATTGGAGGCGCCGTTTCAGAGGAGGAGGACAC TATGGAGAAGCTGGGTCAGCCAGAAACCCCAAGAGTGGGAGG-3') was purchased from Sigma.

Thymus section analysis. Frozen thymuses embedded in OCT compound (Sakura Finetek) were sliced into 5-μm-thick sections. Thymic sections stained with haematoxylin and eosin were examined under a light microscope. For immunofluorescence analysis, the thymuses were fixed in 4% (v/v) paraformaldehyde and embedded in OCT compound. Frozen thymuses were sliced into 5-μm-thick sections and stained with anti-β5 antibody and UEA-1, followed by AlexaFluor488-conjugated anti-rabbit IgG antibody. For the analysis of thymocytes and splenocytes, cells were stained for the expression of CD4, CD8 and TCRβ (BioLegend). Multicolor flow cytometry and cell sorting were performed on FACSAriva1 (BD Biosciences).

Quantitative reverse transcription–PCR analysis. Total cellular RNA was reverse-transcribed with oligo-dT primers and SuperScript III reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa) and the StepOnePlus Real-Time PCR System (Applied Biosystems) and LightCycler Probes Master in a LightCycler 480 (Roche). The amplified products were confirmed to be single bands by gel electrophoresis and normalized to the amounts of Gapdh amplification products. The primers used were as follows: β5, 5'-CTCTGGTGGCTGGACACCTC-3' and 5'-TCCGCTTCCCGAGGCTGGTGCGG-3'; Foxn1, 5'-CTGGTGTTTGTGCCGTCAGAC-3' and 5'-TGCCGTTGTGAGGCGCAGCAGC-3'; HIC1, 5'-CAAGATGTTAACGACCACCC-3' and 5'-CCCAGATAGCAGCTTGTCG-3'; HIC2, 5'-TTGACGCACTGTACCTTCAC-3' and 5'-GGCGCCGACCC-3'. To obtain thymic stromal cells, thymic lobes from E14.5 or E15.5 C57BL/6 mouse fetuses were cultured in the presence of 2 μg/ml monoclonal antibody 24, or control mouse IgG, coupled to Dynabeads protein G (Vertis). DNA–protein complex was heated at 65 °C for 12 h. Immunoprecipitated DNA was purified with a Qiaquick PCR Purification Kit (Qiagen) and quantitated by quantitative nested PCR.

Statistical analysis. Statistical comparison was performed with the two-tailed Student’s t-test using Prism 6 software (GraphPad).

Data availability. Microarray data that support the findings of this study have been deposited in GEO with the primary accession code GSE4222. Genomic sequence data of β5 locus referenced in this study are available in NCBI with the submission code NC_000806.0. The data presented in this study are available from the authors upon reasonable request.

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**Author contributions**


**Additional information**

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