Intronic regulation of Aire expression by Jmjd6 for self-tolerance induction in the thymus

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The thymus has spatially distinct microenvironments, the cortex and the medulla, where the developing T-cells are selected to mature or die through the interaction with thymic stromal cells. To establish the immunological self in the thymus, medullary thymic epithelial cells (mTECs) express diverse sets of tissue-specific self-antigens (TSAs). This ectopic expression of TSAs largely depends on the transcriptional regulator Aire, yet the mechanism controlling Aire expression itself remains unknown. Here, we show that Jmjd6, a dioxygenase that catalyses lysyl hydroxylation of splicing regulatory proteins, is critical for Aire expression. Although Jmjd6 deficiency does not affect abundance of Aire transcript, the intron 2 of Aire gene is not effectively spliced out in the absence of Jmjd6, resulting in marked reduction of mature Aire protein in mTECs and spontaneous development of multi-organ autoimmunity in mice. These results highlight the importance of intronic regulation in controlling Aire protein expression.
The thymus has spatially distinct microenvironments, the cortex and the medulla, where developing T-cells are selected to mature or die through the interaction with thymic stromal cells. Cortical thymic epithelial cells (cTECs), a major stromal cell-type in the cortex, direct differentiation of CD4⁺CD8⁻ immature thymocytes that are capable of recognizing self-major histocompatibility complex (MHC) molecules. On the other hand, medullary thymic epithelial cells (mTECs) play an important role in self-tolerance induction by eliminating self-reactive T-cells. A unique property of mTECs is their expression of a diverse set of peripheral tissue-specific self-antigens (TSAs). This ectopic expression of TSAs largely depends on the transcriptional regulator Aire. 

The homeozymous mutations of human AIRE cause an autoimmune disease known as autoimmune-polyendocrinopathy-candidiasis ectodermal dystrophy. Similarly, Aire-deficient mice develop multi-organ autoimmunity with the failure to delete self-reactive T-cells. Despite an important role in self-tolerance induction, the mechanism controlling Aire expression itself is poorly understood.

Alternative splicing is a major cellular mechanism in metazoans for generating proteomic diversity. This is a posttranscriptional process in which premature transcripts are selectively cut and joined in more than one way to generate multiple mRNAs from a single gene. There are three forms of alternative splicing: exon skipping, alternative splice site usage and intron retention. Of these, intron retention is the least frequent alternative splicing form, which occurs when an intron, having been transcribed as a part of a pre-mRNA, is not spliced out. The sequence structure of most introns consists of a short 5' splice site boundary, a catalytic adenosine and a polypyrimidine tract. Mechanistically, intron retention is considered to be the result of weak splice site sequences that are not properly recognized by spliceosome. Intron retention often inserts an intron, having been transcribed as a part of a pre-mRNA, is not spliced out. The sequence structure of most introns consists of a short 5' splice site boundary, a catalytic adenosine and a polypyrimidine tract (PPT)

Results

Impaired tolerance induction by Jmjd6⁻/⁻ thymic stroma.

A previous study has shown that intrathymic T-cell development is partially impaired in Jmjd6-deficient (Jmjd6⁻/⁻) mTECs, and T-cells generated in such thymic microenvironments caused multi-organ autoimmunity in mice. Our findings indicate that Aire protein expression is tightly controlled through two discrete steps, intron retention and its relief, the latter of which involves the enzymatic activity of Jmjd6.

Defective Aire expression in Jmjd6⁻/⁻ mTECs.

To examine the function of Jmjd6 in TECs in more detail, we performed histological analyses. Haematoxylin and eosin staining of E15.5 embryos and spleens of Jmjd6⁻/⁻ mice revealed that thymic stromal cells in the spleen and peripheral lymph nodes (PLNs) 10 weeks after grafting (Fig. 1d). Jmjd6 deficiency in thymic stroma did not impair generation of regulatory T-cells (Fig. 1e). However, the frequency of CD4⁺ T-cells with an activated/memory phenotype (CD44⁺CD62L-lo) significantly increased in nu/nu/Jmjd6⁻/⁻ mice (Fig. 1f). Histological examination demonstrated inflammatory infiltrates in the stomach, salivary gland, pancreas and liver of nu/nu/Jmjd6⁻/⁻ mice (Fig. 1g). In addition, nu/nu/Jmjd6⁻/⁻ mice, generated autoantibodies against these tissues (Fig. 1h). Thus, Jmjd6 expression in thymic stroma is required for induction of self-tolerance in developing T-cells.

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**Figure 1 | Jmjd6 expression in thymic stroma is required for T-cell tolerance induction.** (a) Real-time PCR analyses for Jmjd6 expression in mTECs, cTECs and MEFs. (b) Immunofluorescence staining showing nuclear localization of Jmjd6. UEA-1 was used to detect mTECs. Data are representative of two independent experiments. (c) Expression of CD4 and CD8 on thymocytes differentiated in the grafts. The number of total thymocytes was compared between nu/nu WT (n = 8) and nu/nu/Jmjd6⁻/⁻ mice (n = 7). (d) The numbers of CD3⁺ T-cells in the spleen and PLNs were compared among nu/nu WT (n = 8), nu/nu/Jmjd6⁻/⁻ (n = 7) and nude (n = 3) mice. (e) The numbers of CD25⁺Foxp3⁺ regulatory T-cells in the grafted thymi and PLNs were compared between nu/nu WT (n = 8) and nu/nu/Jmjd6⁻/⁻ mice (n = 7). (f) Expression of CD44 and CD26L on PLN CD4⁺ T-cells from nu/nu WT (n = 8) and nu/nu/Jmjd6⁻/⁻ mice (n = 7). (g) Haematoxylin and eosin staining of tissues from nu/nu WT and nu/nu/Jmjd6⁻/⁻ mice. Scale bars, 100 μM. The percentage of each organ with inflammatory infiltrates was compared between nu/nu WT (n = 8) and nu/nu/Jmjd6⁻/⁻ mice (n = 7). (h) Immunofluorescence staining showing the presence of autoantibodies (green) in nu/nu/Jmjd6⁻/⁻ mice. Scale bars, 100 μM. The percentage of mice with autoantibodies against each tissue was compared between nu/nu WT (n = 8) and nu/nu/Jmjd6⁻/⁻ mice (n = 7). Data are collected from four mice, six and three separate experiments and are expressed as mean ± s.d. *P < 0.05; **P < 0.01 (two-tailed Student’s t-test).
found between WT and Jmjd6−/− mice when thymic sections were stained for the cTEC marker keratin 8 and mTEC marker keratin 5 (ref. 29) (Fig. 2a). In addition, mTECs of both WT and Jmjd6−/− embryos were comparably stained with Ulex europaeus agglutinin-1 (UEA-1), a lectin that binds to mature mTECs29 (Fig. 2a), suggesting that Jmjd6 deficiency does not affect mTEC maturation. Consistent with this, the expression of another maturation marker CD80 was unchanged between WT and Jmjd6−/− mTECs4,6,30 (Fig. 2a). Surprisingly, however, the expression of Aire in mTECs of Jmjd6−/− embryos was

**nu/nu Jmjd6−/− nu/nu WT nu/nu Jmjd6−/− nu/nu WT**

**Stomach** **Salivary** **Pancreas** **Liver**

**nu/nu WT**

**nu/nu Jmjd6−/−**

**Inflammatory infiltrates (%)** **Autoantibody production (%)**

**nu/nu WT** **nu/nu Jmjd6−/−**

**Stomach** **Salivary** **Pancreas** **Liver**

**nu/nu WT**

**nu/nu Jmjd6−/−**

**Autoantibody production (%)**

**nu/nu WT** **nu/nu Jmjd6−/−**
Figure 2 | Defective Aire protein expression in Jmjd6−/− mTECs. (a) Thymic sections from E18.5 WT and Jmjd6−/− embryos were stained with UEA-1 and/or antibodies specific for keratin 5, keratin 8, CD80 and Aire. Scale bars, 50 μM. (b) Immunofluorescence analysis of thymi from WT and Jmjd6−/− embryos grafted under the kidney capsule of C57BL/6 mice. Six weeks after engraftment, thymic sections were stained with UEA-1 and/or antibodies specific for keratin 5, keratin 8, CD80 and Aire. Scale bars, 50 μM. (c) Flow cytometric analyses for Aire expression in mTECs prepared from E18.5 embryos. The percentages of UEA-1+ mTECs and Aire+ UEA-1+ mTECs were compared between WT (n = 3) and Jmjd6−/− mice (n = 5). (d) Flow cytometric analyses for expression of CD80 and MHC class II in mTECs prepared from E18.5 embryos. The percentages of CD80+ mTECs and MHC class IIhigh mTECs were compared between WT (n = 3) and Jmjd6−/− mice (n = 4). (e) Following stimulation with RANKL, anti-LtβR antibody and/or CD40L for 4 days, 2-DG-treated fetal thymic stroma from WT and Jmjd6−/− embryos were stained with UEA-1 and anti-Aire antibody. Scale bars, 50 μM. (f) Flow cytometric analyses for Aire expression in mTECs prepared from FTOC with or without RANKL stimulation. The percentages of UEA-1+ mTECs and Aire+ UEA-1+ mTECs were compared between RANKL-stimulated WT (n = 3) and Jmjd6−/− samples (n = 3). Data are collected from three (c,d,f) separate experiments and are expressed as mean ± s.d. **P<0.01 (two-tailed Student’s t-test).
markedly reduced at E18.5, as compared with that in WT controls (Fig. 2a and Supplementary Fig. 3a). This does not simply reflect delayed Aire expression in Jmjd6−/− thymi, because similar results were obtained when the 'artificial' thymi from Jmjd6−/− embryos were analysed 6 weeks after grafting (Fig. 2b and Supplementary Fig. 3b).

To determine more precisely the expression level of Aire in mTECs, we performed flow cytometric analyses. E18.5 thymi from both WT and Jmjd6−/− mice contained comparable numbers of CD45−UEA-1+ mTECs (Fig. 2c). Although cell-surface expressions of CD80 and MHC class II were unchanged between WT and Jmjd6−/− mTECs (Fig. 2d), intracellular staining of Aire revealed that the proportion of Aire+ mTECs in Jmjd6−/− thymi was reduced to 25.1% of the WT level (Fig. 2c). Thus, Jmjd6 deficiency reduces Aire protein expression in mTECs without affecting mTEC maturation.
The Aire expression is induced in mTECs through interaction with cells such as lymphoid tissue inducers, positively selected TCRβ+ thymocytes, and TCR Vγ5+ dendritic epidermal T-cell progenitors30–33, which is mainly mediated by the signals through tumour necrosis factor receptor family members, such as receptor activator of NF-κB (RANK), CD40 and lymphotoxin β receptor (LTβR)34–36. When 2-DG-treated WT fetal thymic stroma was stimulated *in vitro* with RANK-ligand (RANKL), the Aire expression was markedly induced in a subset of UEA-1+ mTECs (Fig. 2e and Supplementary Fig. 3c). Although CD40-ligand (CD40L) or anti-LTβR antibody alone was not effective, they synergistically act with RANKL to augment Aire expression in WT mTECs (Fig. 2e and Supplementary Fig. 3c). However, the Aire expression was hardly detected in *Jmjd6*−/− mTECs even after stimulation with RANKL in combination with CD40L or anti-LTβR antibody (Fig. 2e and Supplementary Fig. 3c). This was again confirmed by flow cytometric analyses (Fig. 2f). These results indicate that *Jmjd6* is critical for Aire expression in mTECs.

**Intronic regulation of Aire gene by Jmjd6.** As *Jmjd6* is a nuclear protein that catalyses lysyl hydroxylation of multiple substrates22–25, it seemed likely that *Jmjd6* deficiency affects transcription of both upstream and downstream genes of *Aire*. To comprehensively identify genes controlled by *Jmjd6*, we prepared 2-DG-treated fetal thymic stroma with or without RANKL stimulation from WT and *Jmjd6*−/− mice and analysed their transcriptomes by RNA sequencing (RNA-seq). Although the gene expression of *Jmjd6* was unchanged between before and after stimulation (Supplementary Fig. 4), 1,850 genes were induced in response to RANKL stimulation, among which 1,020 genes were expressed in WT fetal thymic stroma at significantly higher levels than those in *Jmjd6*−/− samples (see top 200 genes in Supplementary Table 1). These included 23 genes encoding Aire-dependent TSAs such as insulin 2 and salivary protein 1 (refs 5,6,8) (Fig. 3a). This reduction of Aire-dependent TSAs was further confirmed by quantitative real-time PCR using samples from *Jmjd6*−/− fetal thymic stroma and *Jmjd6*−/− E18.5 thymi (Fig. 3b and Supplementary Fig. 5). On the other hand, *Jmjd6* deficiency did not affect gene expression of Aire-independent TSA, glutamate decarboxylase 67 (GAD67)5,6,8 (Fig. 3b and Supplementary Fig. 5). Similarly, gene expression of CD80 and CD40 were unaffected in the absence of *Jmjd6* (Fig. 3c).

To explore the underlying mechanism, we next analysed pre-mRNA splicing. Among 84,708 introns of detected genes, 1,051 introns were selected, because they were expressed at a relatively high frequency (intrinsic FPKM > 10). Bioinformatics analysis identified 57 introns preferentially expressed in *Jmjd6*−/− thymic stroma under RANKL-stimulated condition (Supplementary Table 2). These included the intron 2 of *Aire* (Fig. 3d). Unexpectedly, however, the abundance of *Aire* transcript was also comparable between RANKL-stimulated WT and *Jmjd6*−/− samples (Fig. 3b,c), suggesting that *Jmjd6* controls Aire expression through a posttranscriptional mechanism.

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Amplification of Aire cDNA with primers specific for the sequence of the exons 1 and 10 yielded four bands corresponding to the Aire transcript with or without retention of intron 2, intron 9 and intron 2 plus intron 9 (Fig. 3g). By measuring the intensity of each band, 41.5% of the Aire transcripts expressed in RANKL-stimulated WT fetal thymic stroma were found to be mature form, whereas this value decreased to 18.6% in Jmjd6−/− samples (Fig. 3g), because of increase in the frequency of retention of intron 2 and/or intron 9. Similar results were obtained when E18.5 thymi of WT and Jmjd6−/− embryos were analysed with this method (Fig. 3h).

It is known that Aire is also expressed in the reproductive organs and embryonic stem (ES) cells. To examine whether a similar mechanism operates in ES cells, we developed Jmjd6−/− ES cells and compared their Aire transcripts with those of WT ES cells. Jmjd6 deficiency in ES cells markedly increased the Aire transcript containing intron 2 (Fig. 4a), indicating that Aire expression in ES cells is also controlled by Jmjd6 through intron retention. Recent structural analysis of the catalytic domain of Jmjd6 indicated amino acid residues critical for binding to Fe(II), 2-oxoglutarate and substrate lysine. When five of these amino acid residues were mutated to alanine (designated 5 A mutant), the catalytic activity of Jmjd6 to hydroxylate lysine residues was completely lost (Fig. 4b). Although the transient expression of WT Jmjd6 in Jmjd6−/− ES cells significantly, albeit incompletely, improved the ratio of mature Aire transcript (without intron 2) to immature transcript (with intron 2), such improvement was not achieved by the 5 A mutant (Fig. 4c). These results suggest that Jmjd6 controls splicing events of Aire gene depending on its enzymatic activity.

The nature of immature Aire protein. The retention of intron 2 results in an appearance of a premature termination codon at the N-terminal portion of Aire (Fig. 5a and Supplementary Fig. 6), leaving only 103 amino acid residues presumably intact. To know the nature of this immature Aire protein generated by intron 2 retention, we first analysed its subcellular localization. As expected, the GFP-tagged mature Aire protein was localized to the nucleus when expressed alone in MEFs (Fig. 5a). On the other hand, the mCherry-tagged immature Aire protein preferentially accumulated in the cytoplasm, owing to the lack of the nuclear localization signal (Fig. 5a). Interestingly, localization of mature Aire protein was changed from the nucleus to the cytoplasm when immature Aire protein was co-expressed (Fig. 5a). Exactly the same results were obtained when GFP- and mCherry-tags were exchanged (Supplementary Fig. 7). This finding led us to
A cis-regulatory element for intron retention of Aire gene. To understand why the intron 2 of Aire gene is susceptible to intron retention, we compared the sequence structure between Aire intron 2 and other introns. Although the most introns have the PPT site immediately upstream of the 3′ terminal AG dinucleotide, Aire intron 2 encodes GAG instead of canonical pyrimidine-rich sequence at this position (Fig. 6a), resulting in ‘low’ 3′ splice site score, which is calculated based on the similarity to the consensus sequence (Fig. 6a). Comparison of 57 retained introns with 188,151 unretained ones suggested that the degree of intron retention is associated with 3′ splice site score, which is calculated based on the similarity to the consensus sequence (Fig. 6a). Comparison of 3′ splice site score among different species. Non-canonical sequences at PPT site are indicated in red.

Figure 6 | The 3′ splice site sequence is critical for intron 2 retention of Aire gene. (a) Comparison of 3′ splice site sequence of the Aire intron 2 with the consensus sequence. /, splice site. (b) Association between intron retention and 3′ splice site score revealed by comparing 57 retained introns with 188,151 unretained introns. (c) Effect of TTT mutation on intron 2 retention of minigene. Twenty-four hours after the transfection of WT and Δm6Δ.C0 MEFs with GAG- or TTT-type minigene, the ratio of mature to immature transcript was analysed by RT-PCR (n = 3). Data are collected from three separate experiments and are expressed as mean ± s.d. **P < 0.01 (two-tailed Student’s t-test). (d) Comparison of the 3′ splice site sequence among different species. Non-canonical sequences at PPT site are indicated in red.

examine whether the presence of immature Aire protein affects stability of mature Aire protein. For this purpose, we developed HEK293 cells that constitutively express mature Aire protein, but inducibly express immature Aire protein when exposed to doxycycline (Fig. 5b). Doxycycline itself did not affect stability of mature Aire protein (Fig. 5c). However, the amount of mature Aire protein decreased, as the expression of immature Aire protein increased in response to doxycycline treatment (Fig. 5c). However, the amount of mature Aire protein increased in response to doxycycline treatment (Fig. 5c). However, the amount of mature Aire protein increased in response to doxycycline treatment (Fig. 5c). However, the amount of mature Aire protein increased in response to doxycycline treatment (Fig. 5c). However, the amount of mature Aire protein increased in response to doxycycline treatment (Fig. 5c). However, the amount of mature Aire protein increased in response to doxycycline treatment (Fig. 5c). However, the amount of mature Aire protein increased in response to doxycycline treatment (Fig. 5c).
Cbr1 gene with low 3’ splice site score are preferentially retained in the absence of Jmjd6 (Supplementary Fig. 8).

To directly examine the effect of GAG sequence on the intron 2 retention, we created Aire minigene containing exons 1–5 surrounded by their intronic regulatory sequences with GAG or TTT at PPT site of the intron 2 (designated GAG-type or TTT-type) (Fig. 6c). When the GAG-type minigene was expressed in WT or Jmjd6–/– MEFs, Jmjd6 deficiency markedly increased the transcript containing intron 2 (Fig. 6c), which was consistent with the results on endogenous Aire gene expression in mTECs and ES cells (Figs 3g and 4a). However, TTT-type minigene yielded only mature transcript without intron 2 retention, irrespective of Jmjd6 expression (Fig. 6c). Thus, GAG sequence acts as a cis-regulatory element that causes intron 2 retention and inhibits Aire protein expression. Interestingly, this GAG sequence at PPT site of Aire intron 2 is highly conserved in mammals in the Euarchontoglires clade (Fig. 6d). Therefore, intron retention may have been evolved as a mechanism to prevent overexpression of Aire protein in these species.

Discussion
Intron retention is widely accepted as a consequence of mis-splicing, and its significance has been overlooked. However, recent evidence indicates that intron retention has a physiological role in some biological settings such as granulopoiesis. Here we have demonstrated that the expression of Aire is controlled by Jmjd6 through intron retention. Although Jmjd6 deficiency did not affect abundance of Aire transcript, the intron 2 of Aire gene was not effectively spliced out in the absence of Jmjd6, resulting in a marked reduction of mature Aire protein in mTECs. In both Jmjd6–/– E18.5 thymi and RANKL-stimulated Jmjd6–/– fetal thymic stroma, the reduction of Aire protein was more prominent than that of mature Aire transcript. The exact reason for this discrepancy remains unclear. However, reconstitution experiments revealed that immature Aire protein generated by intron 2 retention affects subcellular localization and stability of mature Aire protein. Therefore, the ratio of mature Aire protein to the immature form might be a critical factor that determines expression and function of Aire protein in the nucleus. Ectopic expression of peripheral TSAs by mTECs has been viewed as an essential mechanism for induction of central tolerance. Consistent with a reduction of mature Aire protein in mTECs, RNA-seq analyses revealed that the expressions of 23 Aire-dependent TSAs were markedly reduced in Jmjd6–/– thymic stroma. This was further confirmed by quantitative real-time PCR analyses. In addition, by grafting Jmjd6–/– thymic stroma into athymic C57BL/6 mice, we have shown that T-cells selected to mature in Jmjd6–/– thymic microenvironments caused multi-organ autoimmunity. As the number of thymocytes was significantly reduced in the grafted Jmjd6–/– thymus, disease manifestation in nu/nuJmjd6–/– mice might be exaggerated by homeostatic T-cell proliferation. However, it has been reported that, while lymphocytes from Aire-deficient mice cause autoimmune disease when transferred into recombinase-activating gene (Rag)-deficient recipients, adoptive transfer of WT lymphocytes fail to induce disease under the same conditions. Thus, it is likely that reduction of Aire-dependent TSAs underlies disease development in nu/nuJmjd6–/– mice.

Mechanistically, our results suggest that Aire expression is tightly controlled via two discrete steps. First, owing to the GAG sequence at PPT site, the intron 2 of Aire gene is highly susceptible to intron retention in Euarchontoglires, and as a result, Aire protein expression is expected to be kept at a low level. As Aire has been reported to act as a proapoptotic factor, this static regulation may be important to avoid a deleterious effect of Aire overexpression on the immune system or reproductive organs. The second important regulation is relief of intron retention. This is a dynamic process involving the enzymatic activity of Jmjd6, thereby raising the possibility that metabolic status and oxygen tension may influence intron retention. Although the direct substrate of Jmjd6 in this context is currently unknown, Jmjd6 interacts with multiple splicing regulatory proteins including U2 small nuclear ribonucleoprotein auxiliary factor 65 kDa (U2AF65) and other. Therefore, it seems likely that Jmjd6 could alter affinity of a given splicing factor to 3’ splice site of Aire gene through lysyl hydroxylation. Our findings thus define a previously unknown mechanism controlling expression of Aire protein critical for establishment of immunological self in the thymus.

Methods
Mice. Jmjd6–/– mice have been described previously. Mice heterozygous for the fluorescent allele (Jmjd6+/-) were backcrossed onto a C57BL/6 background for more than 10 generations, and Jmjd6+/- mice were crossed to obtain Jmjd6–/– embryos. The morning of finding the vaginal plug was designated as E 0.5. B6.Cg-Foxn1nu > nu/Nrs (nude) female mice were purchased from Taconic or provided by RIKEN BRC through National Bio-Resource Project of the MEXT, Japan. P10 mice (F1) were obtained by the use of thymic grafts at the age of 6–8 weeks. Mice were kept under specific pathogen-free conditions in the animal facility of Kyushu University. The protocol of animal experiments was approved by the Committee of Ethics of Animal Experiments, Kyushu University.

Fetal liver chimera. Fetal livers were harvested from E14.5 embryos. A total of 1 × 10^6 fetal liver cells were injected in a volume of 300 μl phosphate-buffered saline (PBS) into the lateral tail vein of recipient (10 Gy) CD45.1 C57BL/6. Recipients were analyzed 30 days after reconstitution.

Fetal thymus organ culture (FTOC) and thymic graft. Thymic lobes were isolated from E15.5 WT and Jmjd6–/– embryos, and were cultured for 4 days on Nucleopore filters (Whatman) placed in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Nihirei Bioscience), 50 μM 2-mercaptoethanol (Nacalai tesque), 2 mM l-glutamine (Life Technologies), 100 U/mL penicillin (Life Technologies), 100 μg/mL streptomycin (Life Technologies), 1 mM sodium pyruvate (Life Technologies), MEM non-essential amino acids (Life Technologies) and 1.35 mM 2-DG (Sigma-Aldrich). After cultivation in complete RPMI medium without 2-DG for one more day, four pieces of WT and Jmjd6–/– fetal thymus were grafted under the renal capsule of athymic (nude) and WT C57BL/6 mice. Thymic chimera were analyzed 6–10 weeks after transplantation. In some experiments, TECs were stimulated in vitro with recombinant RANKL (1 μg/mL; Peprotech), agonistic anti-TLR2 antibody 3C8 (2 μg/mL; eBioscience) and/or recombinant CD40L (5 μg/mL; R&D systems) for 4 days before analyses.

Flow cytometry. The following antibodies and reagents were used at the indicated concentrations. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3e (145-2c11, 5 μg/mL), biotinylated anti-mouse CD4 (RM4-5, 5 μg/mL), phycoerythrin (PE)-conjugated anti-mouse CD45 (30-F11, 1 μg/mL), FITC-conjugated anti-mouse CD62L (MEL-14, 10 μg/mL), FITC-conjugated anti-mouse CD90.2 (30-H12, 2.5 μg/mL), FITC-conjugated anti-mouse Foxp3 (FJK-16s, 5 μg/mL), PE-conjugated anti-mouse MHC class II (I-A/I-E) (MS/114.15.2, 0.2 μg/mL) and PerCP-Cyanine5.5 conjugated streptavidin (0.4 μg/mL) were purchased from eBioscience. PE-conjugated anti-mouse CD8a (53–6, 7, 2 μg/mL), PE-conjugated anti-mouse CD25 (PC61, 2 μg/mL), PE-conjugated anti-mouse CD44 (IM7, 0.6 μg/mL), FITC-conjugated anti-mouse CD45 (30-F11, 10 μg/mL), FITC-conjugated CD45R/B220 (RA3-6B2, 10 μg/mL), PE-conjugated anti-mouse CD80 (16–10A1, 1 μg/mL), biotinylated anti-mouse CD45D2 (10.4, 5 μg/mL) and APC-conjugated streptavidin (0.4 μg/mL) were from BD Bioscience. Before staining with the antibodies, the cells were incubated for 10 min on ice with anti-Fc γ III/II receptor (2.4G2, 0.5 μg/mL; BD Bioscience) to block Fc receptors. For intracellular staining was performed with a staining kit (Biolegend) according to the manufacturer’s recommendations. For intracellular Aire staining of mTECs, cells were first stained with anti-CD45 antibody and UEA-1 (10 μg/mL; VECTOR Laboratories), followed by fixation in 4% (w/v) paraformaldehyde for 15 min at room temperature. After being permeabilized with 0.1% saponin (Sigma-Aldrich), cells were stained with Alexa Fluor 488-conjugated anti-mouse Aire (SH12, 5 μg/mL; eBioscience) for 30 min on ice. Flow cytometric analyses were done on FACs Calibur (BD Bioscience).

Histology and immunofluorescence. Tissues were fixed in 4% (w/v) paraformaldehyde and embedded in paraffin blocks. Sections (3 μm thick)
were stained with haematoxylin and eosin, and examined by light microscopy. For immunofluorescence analyses, tissues were embedded in OCT compound (Sakura Finetek U.S.A.) at −80°C. Cryosections were prepared by immersion for 10 min in ice-cold acetone and blocked with 10% horse serum (Sigma-Aldrich) for 1 h at room temperature. Sections were then incubated with rabbit anti-mouse keratin 5 (1 µg/ml; Covance), rat anti-mouse keratin 8 (TROMA-1, 1:1,000 dilution; Developmental Studies Hybridoima Bank), biotinylated UEA-1 (10 µg/ml; VICTOR Laboratories), Alexa Fluor 488-conjugated anti-mouse Aire (SH21, 2.5 µg/ml; 1; bioscience) and/or PE-conjugated anti-mouse CD80 (16-101A, 1 µg/ml; ebioscience) for 1 h at room temperature. After being washed with PBS, sections were incubated with appropriate second antibodies or reagents. For Jmjd6 staining, tissues were cultured on the poly-L-lysine coated glass bottom culture dishes (Matsunami), fixed with 4% (v/w) paraformaldehyde for 15 min, and permeabilized with 0.1% Triton X-100 for 1 h. After being blocked with 10% horse serum for 30 min at room temperature, cells were then stained with 4,6-diamidino-2-phenylindole (DAPL) (Dojindo Laboratories), Alexa Fluor 546-conjugated phallidin (Life Technologies) and mouse anti-Jmjd6 antibody (mAb328, 5 µg/ml; Synaptic Systems) for 1 h at room temperature, followed by incubation with Alexa Fluor 488-conjugated donkey anti-mouse IgG (Fab fragment, 1.5 µg/ml; Jackson Immunoresearch). To detect autotandishes, acetone-fixed cyrossections of C57BL/6 nude mouse tissues were incubated with diluted serum (1:40) obtained from transfected mice, followed by staining with Alexa Fluor 488-conjugated anti-mouse IgG (4 µg/ml; Life Technologies). All images were obtained with a laser scanning confocal microscope (LSM510 META; Carl Zeiss).

Cell preparation and culture. To enrich TECs, thymic lobes were prepared from E18.5 or 6–8-week-old C57BL/6 mice, cut into small pieces, and dispersed further with pipetting to remove the majority of thymocytes. The resulting thymic fragments were digested with 0.12% (w/v) collagenase D/disprase type II, and 0.1% (w/v) DNase I (Roche) in RPMI1640 medium for 1 h at 37°C. The supernatants containing dissociated TECs were centrifuged and were washed with PBS. Before cell sorting, TECs were further enriched by depleting CD45+ haematopoietic cells using CD45 MicroBeads (Milteny Biotech), and stained with the relevant antibodies and reagents. TECs and mTECs were further sorted into CD45- MHC class II+ UEA-1- cells, CD45- MHC class II+ UEA-1+ cells, respectively.

RNA-Seq analysis and 3' splice site scoring. Total RNA was extracted from WT and Jmjd6+/− FTOC samples with (two samples for each category) or without (one sample for each category) RANKL stimulation. One microgram of total RNA was used for library construction with TruSeq RNA Sample Prep kit v2 (Illumina) according to the manufacturer’s protocol. Briefly, poly-A-containing mRNAs were recovered from isolated poly(A)+ RNA using a magnetic bead-based isolation procedure. Pooled RNA samples were fragmented into 200–500 bp fragments with divalent cations under elevated temperatures and then converted to dsDNA by two rounds of dsDNA synthesis using reverse transcriptase and DNA polymerase I. After an end repair process, DNA fragments were ligated with adaptor oligos. The ligated products were amplified by eight cycles of PCR to generate RNA-seq library. Library integrity was verified by Bioanalyzer DNA1000 assay (Agilent Technologies). Sequencing was performed in 101-bp paired-end mode using an Illumina HiSeq (Illumina). A total of 177,060,020 reads were obtained for six samples. Filtered reads were mapped to the UCSC mm10 using the TopHat program (v2.0.10)14 with the default parameters. The Cufflinks program (v2.1.1)15 was then used to assemble 22,448 transcripts and to calculate the fragment per kilobase per million reads (FPKM) values, which are normalized measurement of gene expression levels, with the non-default option, and the counts are converted into the FPKM values for each intron (intron FPKM). There are 1,051 introns with intronic FPKM more than 10 for at least one of the six samples, and the degree of intron retention (IR value) was calculated by dividing intronic FPKM by conventional FPKM value for each gene. By filtering IR value of 0.5, we finally selected 57 introns that are preferentially expressed in Jmjd6+/− samples under RANKL stimulation. The 3' splice site score was calculated by ‘Splice-Site analyser tool’ (http://ibs.tau.ac.il/sat/SliceSiteFrame.htm)38. Shortly, the score expresses to what extent the splice-site sequences match the following consensus sequence: TTTTTTTTCTAG/G (7 indicate the intron/exon junction).

RT-PCR and Southern blot analysis. After treatment with RNase-free DNase I (Life Technologies), RNA samples were reverse-transcribed with oligo(dT) primers (Life Technologies) and SuperScript III reverse transcriptase (Life Technologies) for amplification by PCR. The following PCR primers were used for RT-PCR and/or Southern blot analysis. Aire ( exon 1 – exon 10); 5′-AGTGGGGGATGGAAATATGCCTGCCT-3′ and 5′-TACAGATGCTTCTCTCCGTCTC-3′; Cbr1 (exon 1 – exon 3); 5′-TAACTACACGCTTTTCGGCTGAC-3′ and 5′-GGGACGCTCTTGGACAGTTG-3′; Actb (exon 1 – exon 10); 5′-CTCTCGATCACTGCCTAATG-3′ and 5′-ACCAACAGAGGAACTTGGAAGTGC-3′; Gapdh (exon 1 – exon 3); 5′-TACCACCTGCTGCCTGCACATG-3′ and 5′-AACTGGTGATGAGACCGGAGGA-3′. RT-PCR and Southern blot analysis. Aire I-Suc II fragment of Aire cDNA (506 bp fragment corresponding to exons 3–8) was used as a probe. Quantification of the intensity was performed with BAS-2500 bio-imaging analyser and the Image Gauge 4.0 software (Fujiﬁlm Co.). Information about all deterred bands was conﬁrmed by sequencing.

Plasmids and transfection. For expression of N-terminally-tagged GFP- or mCherry-fusion proteins in mammalian cells, expression vector was created by subcloning a cDNA encoding EGFP or mCherry into pCI (Promega). The genes encoding the full-length Aire protein (mature Aire) and the truncated Aire protein (imature Aire) containing only exon 1 and exon 2 owing to intron 2 retention were subcloned into these vectors to analyse their subcellular localisation in MEFs. Transfection was performed with Lipofectamine 2000 reagent (Life Technologies). The EGFP signals were used as a reporter to monitor transfectant. Immunoblotting. Cells were lysed on ice in 20 mM Tris-HCl buffer (pH 7.5) containing 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, and protease inhibitors. After centrifugation, the supernatants were mixed with sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, and protease inhibitors. After centrifugation, the supernatants were mixed with sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, and protease inhibitors.
Hydroxylation assays. Hydroxylation assays were performed as previously described. Briefly, the assays consisted of the substrate mixture: LUC7-like peptide (LUC7L267-276, 100 µM), 2-oxoglutarate (500 µM), ascorbate (100 µM) in Tris (50 mM, pH 7.5) and the enzyme mixture: Fe(II)/L-α-aminolevulinic acid (100 µM) and recombinant JmjC domain (100 µM) in Tris (50 mM, pH 7.5). The reaction was initiated by mixing the substrate and enzyme mixtures in a final volume of 20 µl and by incubating at 37 °C for 1 h. The reaction was quenched by adding 10 µl of 10% (w/v) CF3CO2H and 2% acetonitrile, 1 µl of which was directly spotted onto 0.5 µl of µ-cyano-4-hydroxycinnamic acid matrix on the MALDI target plate and allowed to dry. The mass corresponding to LUC7L267-276 and LUC7L267-276 + 16, equivalent to one hydroxylation, were identified.

Statistical analysis. For statistical analysis, P values were calculated with a two-tailed unpaired Student’s t-test. P values <0.05 were considered significant.

Error bars denote ± s.d.

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Author contribution

T.Y., F.S., Y.H., M.W., Y.W. and Y.T. performed functional and histological analyses; T.Y., M.M. and N. Shida performed RNA sequencing data. T.S. and M.S. analysed these data; T.U. and X.D. performed biochemical analyses; T.Y. and T.T. contributed to development of Jmdj6–/– ES cells; T.Y., Y.T., Y.N. contributed to writing the manuscript; Y.F. conceived the project, interpreted the data and wrote the manuscript.

Supplementary information

The supplemental information is available at Nature Communications online.
Additional information
Accession codes: RNA-seq data have been deposited in the gene expression omnibus (GEO) database under accession code GSE61444.

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