Insm1 regulates the development of mTECs and immune tolerance

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Abstract

The *Insm1* gene encodes a zinc finger protein with known functions in neuroendocrine cells and neurons. Here we characterized the expression and function of *Insm1* in medullary thymic epithelial cells (mTECs). *Insm1* is co-expressed with Aire in majority of Insm1- or Aire- positive cells, while a few Insm1-positive cells did not express Aire. Mutation of *Insm1* impair the expression of *Aire* and the generation of normal numbers of Aire-expressing mTECs during development. We detected downregulation of genes that expressed specifically in Aire-expressing mTEC and mimetic cells in *Insm1* mutant mTECs. Conversely, when *Insm1* was overexpressed in thymic epithelial cells *in vivo*, the size of the mTECs compartment was enlarged and the expression of *Aire* and genes expressed specifically in the neuroendocrine mimetic cells were increased. Mechanistically, Insm1 bound DNA in mTECs and the majority of the Insm1 binding sites were co-occupied by Aire. These Insm1 binding sites were enriched on super-enhancer regions and thus may contributed to remoted regulation. Both, mice with a thymus-specific mutation in *Insm1* or nude mice transplanted with *Insm1* mutant thymus, displayed autoimmune responses in multiple peripheral tissues. Together, our data demonstrate a role of Insm1 in development of mTECs and immune tolerance.

Key words

medullary thymic epithelial cells (mTEC), mimetic cells, tissue-restricted antigens (TRAs), Insulinoma-associated protein 1 (Insm1), autoimmunity, The autoimmune regulator (Aire)

Introduction

The thymus is a primary lymphoid organ where T cell progenitors undergo maturation and selection to become functional T cells. The medullary thymic epithelial cells (mTECs) control negative selection which eliminates self-antigen-recognizing T cells, and promote differentiation of Treg (Foxp3+CD25+) cells (1, 2). Expression of tissue-restricted antigens in mTECs, also called promiscuous gene expression, is believed to be a key for negative selection and the establishment of self-tolerance (3). The autoimmune regulator (Aire) is the essential transcription factor that directly regulate TRA expression and autoimmunity (4, 5). In the last two decades, studies showed that the Aire-expressing cells defined the functional mTEC populations and have provided key molecular insights into how the TRAs expression is regulated by Aire (6-12). Particularly, Aire was identified as a chromatin looping regulator in mTECs, where Aire binds to super-enhancers and promotes the interaction between enhancers and the TRA genes (13). Studying on Aire also revealed that correct TRAs expressing in the perinatal period but not at the adult stages is essential for the establishment of self-resistance (14, 15).

Recently, lineage specific transcription factor defined mTECs were believed appeared in post-Aire populations, which is called mimetic cells (16). Aire is partially and variably required for the development of mimetic cells and the expression of the mimetic cells expressing TRAs. For each lineage of mimetic cells, specific transcription factors were required for the driving of the lineage development and accumulation. Therefore, the Aire-dependent TRAs

and the mimetic cells expressing TRAs establishment the cellular and molecular bases of the central tolerance.

Herzig *et al*. screened potential regulators of Aire-expression, and identified Insulinoma-associated protein 1 (Insm1) as a transcription factor expressed in mature mTECs and a candidate regulator of Aire. However, no functional analysis of Insm1 was performed in mTECs (17). We present here a genetic and molecular analysis of Insm1 in the thymus, and show that Insm1 is a novel regulator in both Aire-expressing mTECs and mimetic neuroendocrine cells.

Results

Insm1 is expressed in Aire-expressing and post-Aire mTECs

To investigate the expression of *Insm1* in the thymus, we performed immunofluorescence analysis. For this we used *Insm1*$^{+/lacZ}$ and *Insm1*$^{lacZ/lacZ}$ mice in which one or two alleles of *Insm1* codon sequence were replaced by *lacZ* that encodes beta-galactosidase (β-gal) (18, 19). Therefore, the expression of *Insm1* can be monitored using β-gal. We compared signals obtained using antibodies against β-gal and Insm1, and observed that β-gal was expressed in the medulla of the thymus in both *Insm1*$^{+/lacZ}$ and *Insm1*$^{lacZ/lacZ}$ mice (Fig. S1A). Insm1 immunoreactivity overlapped with β-gal in the thymus of *Insm1*$^{+/lacZ}$ but was absent in *Insm1*$^{lacZ/lacZ}$ mice (Fig. 1A and Fig.S1A). Thus, the Insm1 antibody specific detected the endogenous Insm1 expression. Unlike the exclusive nuclear location in neuroendocrine cells (20, 21), Insm1 protein was detected in both, nuclei and cytoplasm of thymic cells (Fig 1B).

The thymic epithelial compartment is formed by cTECs and mTECs, and contains also lymphocytes and dendritic cells (22). To define the cell-type that expresses *Insm1*, we performed immunofluorescence using antibodies against Insm1 and cell type specific markers in both, the fetal and adult thymus. We did not observe co-expression of Insm1 with the lymphocyte specific marker CD45, or the dendritic cell marker Cd11b and Cd11c in thymuses of E18.5 and 6-week old mice (Fig. S1B,C). However, Insm1 was co-expressed with the mTECs marker keratin 5 (Krt5) but not the cTECs marker keratin 8 (Krt8) (Fig. 1C,D). Using Insm1/Aire double antibodies staining, we further observed that 70% of the Insm1-positive cells co-expressed Aire and vice versa (Fig. 1E). The similar co-expression pattern of Insm1 and Aire was also observed in thymuses of adult mice (Fig S1D). Moreover, the Aire-dependent TRA insulin and somatostatin can be detected co-expressing with Insm1 (Fig S1E and S2A). Using the published single cell RNAseq data (16), we investigated the sub-populations of mTEC that expressing *Insm1*. We identified that *Insm1* is expressed in Aire-expressing, transit-amplifying and neuroendocrine mTECs, while Aire is expressed mainly in Aire-expressing and transit-amplifying mTECs (Fig 1F,G and Fig S2B,C). This is consistent with the observation in immunofluorescence analysis that most of the of Insm1 expressing cells co-expressed Aire (Fig 1D). We further verified the co-expressing of Insm1 with neuroendocrine mimetic cell markers Foxa2 and chromogranin A in adult thymus (Fig 2A,B). In summary, *Insm1* is expressed in Aire-expressing mTECs and neuroendocrine mimetic cells.

Tissue morphology and cell populations in *Insm1* mutant thymus

There was no difference in the appearance of size and shapes of thymus at E18.5 in mutants (Fig S2D). The distribution and relative amounts of mTECs and cTECs identified by Krt5 and Krt8, respectively, were comparable between wildtype and *Insm1* mutant mice (Fig S2E,F). We used flow cytometry to further investigated subpopulation of mTECs. The number of EpCAM labeled thymic epithelial cells ($CD45^{-}EpCAM^{+}$) and UEA1 labeled mTECs ($CD45^{-}EpCAM^{+}UEA1^{+}$) were comparable in wildtype controls and *Insm1* mutants both at E18.5 and adults (Fig.2C,D). However, the proportions of Aire-positive cells were significantly decreased in populations of mTECs ($CD45^{-}EpCAM^{+}Ly51^{-}$) in *Insm1* mutants at E18.5 (Fig.2E). We did not observe significant alteration of Aire-positive cell proportions in mTECs in adults (Fig.2E). Thus, *Insm1* mutation affected the development of Aire-positive mTECs.

Using qRT-PCR, we examined the expression of genes that were identified specifically expressed in each of the mimetic cells(16) (Fig 2F upper panel). We detected genes that are specifically expressed in neuroendocrine, entero-hepatocyte and Ptf1a+pancreatic mimetic cells were downregulated in *Insm1* mutant adult mice (Fig 2F lower panel). Thus, the neuroendocrine, entero-hepatocyte and Ptf1a+pancreatic mimetic cells were affected by *Insm1* mutation.

Decreased expression of *Aire* and mTEC genes in *Insm1* mutants

As we observed a decreased number of Aire-positive cells in the *Insm1* mutant thymus, we investigated the expression levels of Aire. The immunofluorescence intensity which indicated the Aire protein level was

moderately but significantly decreased in mTECs of *Insm1* mutant mice (Fig.3A). Using real-time quantitative reverse transcription PCR (qRT-PCR), we detected decreased transcript levels of *Aire* in mTECs analyzing either in *Insm1* mutant E18.5 mice or thymic specific mutant adult mice (*Foxn1Cre;Insm1*$^{flox/flox}$, subsequently called *Thy-cKO*) (Fig. 2B,C). Thus, the *Insm1* mutation resulted in decreased expression of *Aire*.

To analyze the global gene expression changes in *Insm1* mutant thymus, we performed RNA-seq using mTECs isolated from both *Insm1* mutant E18.5 and *thy-cKO* adult mice. To our surprising, differential gene expression test by DESeq2 under cutoff of FDR<0.1 and fold change>1.5, identified 63 and 97 dysregulated genes in E18.5 and adult mice, respectively (Fig 3D,E and Table S1,S2). The numbers of the dysregulated genes were far less than that of Aire-regulated genes reported in previous study (23). Nevertheless, *Aire* expression was detected down-regulated in both E18.5 and adults in the RNAseq data (Fig 3D,E), which was consistent with the observation in qRT-PCR and immunofluorescence analysis (Fig 3A-C). Recent studies using single cell technology illustrated the sub-lineages of thymic epithelial cells (16, 24). To characterize the *Insm1* effects on different mimetic cell types, we analyzed the expression pattern of our dysregulated genes in the published thymus single-cell data (16). The down-regulated genes distributed both in Aire-expressing mTECs and in mimetic cells (Fig 3F upper and middle panels). However, the cTECs specific genes were up-regulated in mTECs of *Insm1* mutant adult mice (Fig 3F lower panel). Thus, although moderate numbers of the dysregulated genes were identified in *Insm1* mutants, Insm1 regulates the gene expression in Aire-expressing mTECs and mimetic cells.

Considering each of the TRA typically expressed in only 1-5% of mTECs (25), to clarify the TRA expression trends, we used a loose cutoff (p value <0.05 and fold change>1.5) (Fig 3D,E) and distinguished 78 and 81 downregulated TRA genes at E18.5 and adults, respectively (Fig S3A, Fig 3G and Table S3,S4). Using qRT-PCR, we verified some of the dysregulated TRAs genes that were identified with the this cutoff (Fig 3B,C). We compared the downregulated genes observed in *Insm1* mutant mTECs with the ones identified in *Aire* mutant mTECs (23). Among the dysregulated TRA genes, we found both, Aire-dependent and -independent TRAs to be downregulated (Fig 3G and Fig S3A). Among the 81 downregulated TRA genes in adults, tween-six was downregulated in both, *Aire* and *Insm1* mutants. However, fifty-five (68% of all TRAs) TRA genes were unique to *Insm1* mutant mTECs (Fig.3G). Thus, Insm1 regulates the expression of TRA genes in mTECs, and particularly both Aire-dependent and -independent TRAs.

We characterized the expression patterns of downregulated TRAs in mimetic cells using the published thymus single-cell data (16). Although *Insm1* expression was restricted in neuroendocrine cells in mimetic cells (Fig 1F), the downregulated TRAs were not restricted in neuroendocrine cells, instead, the distribution of these TRAs was found in Aire-expressing mTECs and multiple mimetic cell types, i.e., enterocytes/hepatocytes and microfolds, both in E18.5 and in adult (Fig 3H). We investigated the tissue types where TRA genes are dominantly expressed using public microarray data (26). We noticed that Insm1-induced TRAs were expressed in all the tested peripheral tissues and with relative higher frequency in the gastrointestinal tract and neuroendocrine tissues (Fig.S3B).

Insm1 promotes *Aire* and TRA expression

We induced over-/ectopic-expression of *Insm1* (*Insm1OE*) *in vivo* using genetic tools (Fig S4), i.e., a mouse line that harbors a *loxp-STOP-loxp-Insm1* cassette in the *Rosa26* locus, and the F*oxn1Cre* transgenic allele that allows thymus specific expression. We observed increased numbers of Insm1-positive cells and enlarged Krt5-positive mTECs areas in the *Insm1OE* thymus (Fig 4A). Moreover, the Aire-positive cells were increased in the thymic sections, although the increase of such cells was less obvious as the one of Insm1-positive cells (Fig 4A). Increased expression levels of *Aire* were detected by qRT-PCR using RNA isolated from the whole thymus or from mTECs (Fig 4B,C). Interestingly, four of the nine TRA genes that were downregulated in *Insm1* mutant thymuses showed significantly increased expression upon *Insm1* overexpression (Fig 4B,C). We investigated the expression of mimetic cell marker genes that was downregulated in *Insm1* mutant mTEC. *Insm1* overexpressing promoted the neuroendocrine cells specific genes expression (Fig 4B,C). Thus, Insm1 promoted mTECs fate and the expression mTEC specific genes such as *Aire,* TRAs and neuroendocrine-specific markers.

Insm1 binds on promoters and super-enhancers in mTECs

Insm1 was identified as a transcriptional factor in endocrine cells (20). To investigate the mechanism of Insm1 function, we examined the genome wide DNA-binding of Insm1 using CUT & Tag analysis. We detected 5,206 Insm1 binding peaks (union of 3 repeats, q<1e-5) in mTEC of E18.5 and 1,458

(union of 2 repeats, q<1e-5) peaks in mTEC of adults (Fig 5A,B). Although a less numbers of Insm1 binding peaks were identified in adults, 1,382 (94.7%) peaks identified in adult mTEC were overlapped with the peaks detected in E18.5 mTEC, indicating a significant amount of consensus Insm1 binding on DNA in mTEC during development and in adults.

Although the majority of Insm1 binding sites were located on promoter regions in both E18.5 (50%) and adults (65.8%) (Fig 5A,B), only two (Fig 5C, E18.5) and three (Fig 5D, adult) of these Insm1 bound genes were dysregulated in Insm1 mutation (Fig 5C,D). Furthermore, we found the dysregulating genes, which were identified by a looser cutoff (p-value<0.05, FC>1.5) in the RNAseq data, were not enriched but depleted for Insm1 binding on the promoter (Fisher test, p<0.0001) (Fig 5E). These evidences suggest that Insm1 binds on promoters but did not contribute to the correlated gene expression.

Insm1 is co-expressed with Aire in mTECs (Fig1E, Fig S1D), we therefore compared the DNA binding of Insm1 with that of Aire identified in published data sets (23, 27). The majority of Insm1 binding sites (72-78%) were co-occupied by Aire (Fig S5A,B). Since Aire was reported binding on super-enhancers and performing regulatory roles in mTEC (13), we investigated the Insm1 binding sites and found significantly enriched binding of Insm1 on super-enhancers (Fig.5F-H). We further found that the dysregulated genes were significantly over-represented within 500kb of the Insm1-binding super-enhancers at E18.5 mTECs (Fig. 5I). These results imply that Insm1 could bind on super-enhancers and participate in the gene expression regulation in the developing mTECs.

Autoimmune responses in nude mice transplanted with *Insm1* mutant thymuses

We used thymus transplantation in nude mice to study autoimmune reactions. For this, T cells depleted thymuses that were isolated from fetal control (*Insm1*$^{+/lacZ}$) and mutant (*Insm1*$^{lacZ/lacZ}$) mice were transplanted into the renal capsule of six-week old nude mice (28) (Fig.6A,B). Eight weeks after transplantation, the size and structure of the transplanted thymuses were similar regardless whether they derived from control or *Insm1* mutant animals (Fig.6C and Fig.S6). Further, the numbers of CD4+ and CD8+ cells detected by flow cytometry were comparable in the transplanted control and mutant thymuses (Fig.6D). However, the spleens of nude mice transplanted with a mutant thymus (*KO/nu* mice) were increased in size and weight compared to the one of animals that received a control transplant (*Het/nu* mice) (Fig. 6E). Furthermore, the structure of the spleen was altered (Fig 6F), but the lymph nodes were unchanged (Fig S6). In particular, spleen follicles of *KO/nu* mice were smaller than that those of *Het/nu* mice (Fig. 6F). We investigated lymphocyte infiltration of various organs using hematoxylin eosin (H&E) staining, that detects the accumulation of lymphocyte nuclei. Increased infiltration was observed in pancreatic islets, lungs, kidneys and salivary glands of *KO/nu* mice (Fig.6G,H), but not in other investigated tissues (Fig.S6).

Next, we analyzed autoimmune reactions by the detection of autoimmune antibodies. Among the 13 investigated tissues, we observed autoimmune antibody reactions in pancreatic islets, kidney, and testis by staining these tissues with the serum obtained from the *KO/nu* mice (Fig 6I and Fig S7). In

particular, serum of *KO/nu* mice detected subsets of pancreatic polypeptide (Pp) positive cells, glucagon (Gcg) positive alpha cells, and somatostatin (Sst) positive delta cells, whereas insulin (Ins) positive beta cells were rarely detected (Fig 6J). Thus, autoimmune antibody against PP-, alpha- and delta-cells were present in *KO/nu* mice. To sum up, the transplantation of *Insm1* mutant thymuses into nude mice results in autoimmune responses in multiple tissues.

Thymus specific *Insm1* mutant mice show autoimmune responses

We employed thymus-specific *Insm1* mutant mice, *Foxn1Cre;Insm1*$^{flox/flox}$ (*Thy-cKO*), and littermates *Foxn1Cre* or *Insm1*$^{flox/flox}$ controls to further examine the autoimmune phenotype. The *Thy-cKO* mice were born healthy and displayed no obvious abnormalities in adulthood. We observed decreased expression levels of *Insm1, Aire* and the Aire-dependent and -independent TRAs in mTECs of *Thy-cKO* mice (Fig 3C), which is similarly to the changes observed in *Insm1* null mutant mTECs (Fig 3B). $CD4^+$ and $CD8^+$ T cells was properly generated in the thymus (Fig 7A) and developed in lymph nodes (Fig 7B) of *Thy-cKO* mice, as analyzed by flow cytometry. However, we detected significantly decreased numbers of Treg cells ($CD8^-$ $CD4^+Foxp3^+CD25^+$) in the thymus of *Thy-cKO* mice (Fig 7C), and we observed a trend towards a reduced number of Treg cells in lymph nodes that did not reach statistical significance (Fig 7D).

We investigated the infiltration of lymphocytes into various tissues at 6-week, 6-, 12- and 18-month-old *Thy-cKO* animals. Generally, we observed pronounced lymphocyte infiltration in multiple organs that increased in

frequency with the age of the animals. Infiltrated lymphocytes were observed in salivary glands of 6-week-old and in multiple organs of 1.5-year-old *Thy-cKO* mice, the infiltrated organs including the pancreas, the slavery gland, the liver, the lung and the brown fat (Table 1 and Fig 7E). Further, we detected autoimmune antibodies against multiple tissues including the pancreas, the salivary gland and the ovary when serum of *Thy-cKO* mice was used for immunostaining (Table 2 and Fig 7F). Thus, mutation of *Insm1* in the thymus resulted in autoimmune responses in periphery organs in a subset of the *thy-cKO* mice.

Discussion:

Insm1 was previously described as a factor functioning in neuroendocrine cells and neurons (19-21, 29-31). Herzig, Y *et al* reported the first evidence indicating Insm1 is a candidate transcriptional regulator of *Aire* (17). However, to our knowledge functional data on a role of Insm1 in thymus have not been systematic analyzed. We used *Insm1* null mutant mice, thymus-specific *Insm1* mutants and transplanted the thymus of *Insm1* mutants for an analysis of a role of Insm1 in autoimmunity.

We detected that *Insm1* is expressed in mTECs. Majority of Insm1 is expressed in Aire positive cells while a minor population of Insm1 positive cells did not express Aire. Using the published single cell RNAseq data (16), we found that *Insm1* is expressed in Aire-expressing and transit-amplifying TECs, which is similar as the expression pattern of *Aire*. In addition, *Insm1* but not *Aire* is expressed in neuroendocrine cells, a mimetic cell type in post-Aire population. In *Insm1* mutant mTECs, the expression of *Aire*, the Aire-

dependent and -independent TRAs was decreased. In consistent with the gene expression, *Insm1* mutation results in less Aire-positive cells in developmental mTECs. To identify the regulatory role of Insm1 in mimetic cells, we examined the mimetic cell-type-specific gene expression and found only those genes expressed in the neuroendocrine, enterohepatic, and pancreatic cells was downregulated in *Insm1* mutation. When *Insm1* overexpression was induced in the thymus, enlarged areas of Krt5 expression were observed, and the expression level of *Aire* was increased. Further, TRAs and neuroendocrine cell marker genes was induced. Currently, the molecular and cellular model of TRA expression showed that Aire-expressing mTECs, which developed from transit-amplifying TECs, express a selected set of TRAs that directedly regulated by Aire. Aire-expressing mTEC subsequently differentiate into diverse mimetic cell types, which produce mimetic cell specific TRAs. The maturing thymocytes then undergo negative or agonist selection against the full ranges of TRAs expressed by Aire-expressing mTECs and mimetic cells (24). In this model, Aire deficiency impaired the function of Aire-expressing mTECs and the development of mimetic cells, which results in diminished expression of numerous TRAs. In our study, we showed that *Insm1* is expressed in transit-amplifying TECs, Aire-expressing mTECs and neuroendocrine mTECs, indicating Insm1 exists in various developmental stages of mTECs. In addition, Insm1 regulates not only the expression of Aire and Aire-dependent TRAs but also neuroendocrine mimetic cell specific gene. Thus, Insm1 contributes to the development and function of Aire-expressing mTECs and post-Aire neuroendocrine mimetic cells.

Interestingly, we observed that Insm1 binding on the super-enhancer regions in mTECs. These super-enhancers contribute to the expression of *Insm1*-dependent genes. In addition, the majority of Insm1 binding sites overlapped with those of Aire, pointing towards the functional importance of the set of cis-elements binding by both, Insm1 and Aire in mTEC. Although a relative high proportion of common Insm1 binding sites were observed in both developing and adult mTECs, the dysregulated genes were not conserved between E18.5 and adult in *Insm1* mutants. Rather, more up-regulated genes were identified in *Insm1* mutant adult mTECs than that of E18.5. These up-regulated genes are cTEC-specific genes, indicating a cTEC gene expression feature appeared in *Insm1* mutant adult mTECs. In addition, the Insm1-binding super-enhancers were significant correlated with the Insm1 dependent gene expression at E18.5 but not at adult stages, one explanation is that the dysregulated genes which are not directly regulated by Insm1 may have been accumulated in adults. As *Insm1* is expressed in several subpopulations of TECs, Further analysis on the mechanism of Insm1 function in the individual sub-population is needed, particularly in Aire-expressing mTECs and/or neuroendocrine mimetic cells.

Mutation of *Insm1* results in about 50% decrease of *Aire* expression. A dosage effect of Aire in TRAs regulation was discussed controversially. *Herzig* et al showed that 50% decreased Aire expression in *Aire*$^{+/-}$ mice did not alter the Aire-dependent TRAs expression (17). In contrast, others demonstrated an 80-90% reduction in the expression of a particular TRAs (*Ins2, Tff3, Mup1* and *Spt1*) in *Aire*$^{+/-}$ compared to wild-type littermate control mice (32). Additional studies also showed that TRAs levels were regulated by dosage of

Aire expression (33, 34). In the *Insm1* mutant thymus, we observed downregulated expression of Aire-dependent TRA genes, possibly due to the decreased Aire expression.

In nude mice transplanted with *Insm1* mutant thymuses or in thymus specific *Insm1* mutant mice, we observed lymphocyte infiltration and autoimmune antibodies. These autoimmune responses were directed against various tissues, among them are pancreatic islets, the salivary gland, the lung and the kidney. However, not all these were equally attacked by immune reactions. Whereas pancreatic islets and the salivary gland were affected frequently in both, nude mice transplanted with *Insm1* mutant thymuses or thymus specific *Insm1* mutant mice, immune reactions in other tissues were observed only in occasionally. In addition, autoimmune responses in brown adipose tissues were observed in *Insm1* mutants, and have not been reported for *Aire* mutant mice (4, 5, 9, 10, 12, 14). In conclusion, out data show that Insm1 is a novel regulator of mTEC development, gene expression and particularly of TRAs expression in mTECs. Mutation of *Insm1* can cause autoimmune reactions.

Materials and Methods:

Animals

Insm1$^{lacZ/lacZ}$ mice were described (18). E18.5 embryos were collected, genotyped and analyzed. A thymus specific mutation of *Insm1* was introduced by crossing *Insm1*$^{flox/flox}$ (20) and *Foxn1Cre* mice (JAX No. 018448). Tissues collected from *Rag1*$^{-/-}$ mice (GemPharmatech No.T004753) were used for autoimmune antibody test. The *Insm1KI*

mouse strain was generated by introducing Insm1 coding sequences preceded 5' by a stop cassette, that can be removed by Cre, into the *Rosa26* locus (Cyagen, Suzhou, China) (Fig S8). The littermate wildtype or heterozygous animals were used for control animals in the analysis. All animal experiments were approved by the institutional animal care and use committee of the Jinan University (IACUC-20211123-02).

Thymus transplantation

Thymuses were isolated from E18.5 *Insm1*$^{+/lacZ}$ and *Insm1*$^{lacZ/lacZ}$ mice and cultured for eight days in RPMI-1640 containing 10% FBS and 1.25 mM 2'-deoxyguanosine. One thymus was transplanted into renal capsule of a nude mouse (6-week-old, CD1 background) using the procedure described previously (28). Eight weeks after transplantation, the thymus and newly generated lymphocytes were collected from the nude mice, various tissues of the animals were also collected and analyzed.

Thymic cell dissociation and mTEC isolation

The fetal and adult thymic cells were dissociated using Liberase (35). In brief, the thymuses were separated and connective tissues and fat were removed. For E18.5 animals, 2-3 thymuses with of same genotype were pooled, and thymic lobes were cut into small pieces in RPMI to release the lymphocytes. Lymphocytes were collected in those cases when the entire thymic cell population was analyzed. Otherwise, the thymuses were digested in 100ul RPMI1640 containing 0.1mg/ml Liberase (Roche, Germany) and 20U/ml DNase I at 37°C for 4 minutes. The digestion procedure was repeated twice, and cells were collected after each round of digestion.

mTECs isolation was performed using a magnetic beads-based method as previously described (36). In brief, 1μl biotinylated UEA1 (Vector Laboratories, B-1065-2) was incubated with 25μl Biotin Binder Dynabeads (Thermo Fisher Scientific,11047) for 30 minutes at room temperature. Unbound UEA1 was removed by washing in 1ml FACS buffer (PBS with 0.02%BSA and 5mM EDTA). Thymuses from 2-3 E18.5 fetal or one adult mice were incubated with above prepared 25μl UEA1-coated beads at 4°C for 30 minutes. Unbound cells were removed by three washes with FACS buffer and by additional incubation with CD45 S-pluriBeads (pluriSelect Life Science, SKU#70-50010-11), before mTECs was collected for analysis. The isolated mTECs were used for RNAseq, Cut&Tag and qRT-PCR analysis.

Flow cytometry analysis

For analysis of lymphocytes in the transplanted thymuses, thymic lobes were gentle cut, released lymphocytes were collected and directly stained with CD4 and CD8 antibodies in FACS buffer for flow cytometric analysis. For analysis of thymic cells, stroma cells were dissociated using Liberase as described above. Staining with antibodies recognizing cell surface proteins were performed directly in FACS buffer, whereas staining with antibodies recognizing nuclear antigens was performed after fixation for 10 minutes in 4% PFA. Foxp3 staining was performed in Foxp3/Transcription Factor Staining Buffer (Invitrogen, 00-5523-00). Immunostaining procedures were performed as described (37). The antibodies used were listed in the supplementary table 5.

BD FACSCanto II or FACSAria II were used to analyze and collect

cells (Beckton Dickenson, Franklin Lakes, USA). Cell quantification was determined using FloJo 7.6.5 software.

Immunohistochemistry, western blot analysis and hematoxylin and eosin (H&E) staining

Immunohistochemistry and western blot analysis were performed as described (20). Fluorescence was imaged on a Zeiss LSM 700 confocal or a Leica DMi8 microscope, and the images were processed using Adobe Photoshop software. The antibodies used are listed in supplementary table 5. For the autoimmune antibody tests, serum of the *KO/nu* or *Thy-cKO* mice was used at the concentration of 1:25 on tissue sections obtained from *Rag1*$^{-/-}$ mice.

For H&E staining, the Lillie-Mayer method was used. In brief, 5% aluminum ammonium sulphate and 0.5% hematoxylin were used to stain the nuclei, followed by 0.3% acid alcohol incubation for the differentiation of nuclear staining. Acetified eosin was used for counterstain to reveal cellular detail.

***Insm1* mutants RNA-seq analysis**

For E18.5 mice, six to nine thymuses of each genotype (*Insm1* mutant and wildtype) were collected, and mTECs were isolated after pooling two to three thymuses of the same genotype. For 6-8 weeks mice, mTECs were isolated from each of the thymus for each genotype. Total RNAs were isolated using TRIZOL reagents. Three independent sequencing libraries for each genotype were generated using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA). Illumina NovaSeq 6000 was used for sequencing, and 150-nt paired-end reads were generated. Sequencing

data (6 Gb or more) with more than 94% of bases scoring above Q30 (accuracy rate 99.9%) were produced from each library sample. RNAseq fastq files were aligned to the mouse genome (mm10) using STAR (v2.5.2a) (38) with parameter --outFilterMismatchNmax 2. The proportions of uniquely mapped reads were 79.4%-85.9%. Gene read numbers were counted using HTSeq (v0.11.0) (39) based on mouse gene annotation Ensembl v79, and the value of fragment per kilobase of exon per million reads mapped (FPKM) was calculated. Next, we used DESeq2 (v1.34.0) (40) to detect dysregulated genes under cutoff FDR<0.1 and fold change>1.5. To analyze downregulated TRAs and dysregulating-trend genes for down-steam analysis, a looser cutoff (P value<0.05 and fold change>1.5) was performed. All the RNA-seq data are accessible on Gene Expression Omnibus (GSE193929).

For comparison of genes dysregulated after *Aire* mutation, published data were used (23). Gene read counts were downloaded from GEO (ID:GSE144877). Differential expressed genes were analyzed according to the method used in the referred paper (23).

GO enrichment analysis was performed by R package topGO (v2.38.1) (41) with “classic” algorithm and “Fisher exact test”, followed by the Benjamini-Hochberg multiple test correction using FDR=0.05 as a cutoff.

qRT-PCR analysis

Cells were lysed and total RNA was isolated using TRIZOL reagent (Invitrogen). For qRT-PCR analysis, RNA was isolated from thymuses or mTECs of E18.5 fetal mice. cDNA was synthetized using HiScript II Q RT

SuperMix for qPCR (+gDNA wiper) (R223-01, Vazyme, China) and analyzed using SYBR Green I based real-time quantitative PCR method on a CFX96 RT-PCR system (Bio-Rad). Expression levels were determined using the $2^{-\Delta\Delta Ct}$ method and *ActB* or *Krt5* as internal standards, and displayed as proportion of control. Primers used for quantitative analysis are listed in supplemental table S6.

Insm1 CUT&Tag-seq analysis

CUT&Tag (Cleavage Under Targets and Tagmentation) analysis was performed according to the instruction of the manufacturer (Novoprotein, N259-YH01) and previously published protocols (42). In brief, mTECs were isolated using UEA1 coupled Dynabeads from wildtype mice. 0.5μl of Insm1 antibody or 1μg IgG antibody was incubated overnight at 4°C with $5x10^4$ mTEC cells in a volume of 100μl first antibody buffer. Donkey anti-guinea pig (Jackson ImmunoResearch,706-005-148) secondary antibody was used and incubated with the mTEC for 45 minutes in 100μl secondary antibody buffer at room temperature. After washing to remove unbound antibodies, the ChiTag transposon was incubated with mTECs for one hour at room temperature. Fragmentation, DNA purification and library construction were performed according to the protocol of the manufacturer. At least three biological replicas were obtained.

Illumina NovaSeq 6000 was used for sequencing, and 150-nt paired-end reads were generated. At least twelve Gigabyte pass filtering sequencing data was generated. Raw CUT&Tag sequencing data was trimmed to remove adaptor sequences using fastp (v0.12.0) (43) and aligned to mouse genome (mm10) using Bowtie2 (v2.2.9) (44) with the

parameters "--local --very-sensitive-local --no-unal --no-mixed --no-discordant --phred33 -X 700". Peaks were called using MACS2 (v2.2.6) (45) with the parameter "--keep-dup auto". IgG data were used as a control. Insm1-binding peaks identified in three replicates with q-value<10e-5 were merged for subsequent analysis.

To calculate the proportions of Insm1 binding on different genomic regions, peaks were assigned to different genomic regions based on their summit positions on the Ensembl transcriptome annotation according to the priority order of CDS, 5'-UTR, 3'-UTR, promoter, ncRNA, intron, and intergenic, where the promoter were defined as the regions of -2000 bp to +500 bp of transcription start site (TSS). The CUT&Tag sequencing data are accessible on Gene Expression Omnibus (GSE193929).

Single cell RNA-seq data analysis

Preprocessed transcript-by-cell matrix of scRNA-seq data of $Pdpn^{-}CD104^{-}$ $mTEC^{lo}$ from perinatal and adult mice was downloaded from GEO (ID:GSM5831744), and read by scanpy (46) for downstream analysis. First, the valid 8,236 mTECs used in the study (16) were selected according to the barcode list provided. The gene expression matrix was normalized to counts per 10,000 counts, followed by log-transfer and scaling to unit variance and zero mean. Next, the top 2,000 highly variable genes were identified and selected, and PCA was performed for dimensionality reduction. According to the elbow point of the PC contribution curve, the top 30 PCs were used for the neighborhood graph construction, and UMAP was created for visualization.

Cell clustering were preformed using the Leiden method (resolution=1.9). The scores of gene sets were calculated using the scanpy function tl.score_genes.

TRA gene analysis

the list of TRA genes were identified in an earlier study (47) and provided by the authors (Prof. Perreault C, Université de Montréal, personal communication). Aire-dependent TRAs were identified by the overlaps of downregulated genes from the public Aire mutant RNA-seq data (23). The TRA expression profiles of various mouse tissues were generated in pervious microarray studies (26) and downloaded from GEO (GSE10246). To visualize TRA expressions in different tissues (Figure S3), samples of each tissue were merged by mean expression values, followed by z-score normalization on each gene.

Super enhancer analysis

Super enhancer regions were identified in the published study (13) and were provided by the author via email (Prof. Diane Mathis, Harvard Medical School, personal communication). The control regions used in Fig 4B were the sequences with the same length as the super enhancer and with an offset of the distance of the super enhancer length plus 200kb.

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Author Contributions

W.T. designed the study and performed the molecular experiments. Y.W. and J.W. contributed to the animal experiments, tissues analysis and flow cytometry analysis. Y.W., Z.Y., W.Y. and G.Y. contributed to the molecular experiments, cells and tissues experiments. J.X. performed and managed the bioinformatic analysis and participated manuscript preparation. S.J. supervised the project, analyzed the data and wrote the manuscript. S.J. is the guarantor of this work and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure legends

Figure 1. *Insm1* is expressed in thymic epithelial cells

(A) Immunofluorescence analysis using antibodies against β-gal (red) and Insm1 (green); analyzed were thymuses of E18.5 *Insm1*$^{+/lacZ}$ and *Insm1*$^{lacZ/lacZ}$ mice. DAPI (48) was used as a counterstain. Scale bar = 40μm. (B) Immunofluorescence analysis of thymuses at E18.5 using antibodies against Insm1 (red), EpCAM (green) and DAPI (48). EpCAM is expressed in thymic epithelial cells and labels the cell membrane and cytoplasm. Yellow arrowheads indicated the cytoplasmic and nuclear location of Insm1, and the magenta arrowhead indicated a nucleus. Scale bar=10μm.

(C,D) Immunofluorescence analysis of thymuses of E18.5 (C) and 6 weeks old (D) mice using antibodies against Insm1 (red), Krt5 (green) and Krt8 (48). Magnifications are shown in the panels on the right. Scale bar = 100μm; and 20μm in magnified panels. (E) Immunofluorescence analysis detected Insm1 (green) and Aire (red) in thymuses of E18.5 mice. Insm1 is co-localized with Aire. Yellow arrowheads indicated the cell expressing only Insm1, magenta arrowheads indicated cells expressing only Aire.

Scale bar = 40μm. Quantification of single and double positive cells is shown on the right (animal number n=3, around 300 cells/animal/antibody were counted). (F) Insm1 expressing in subpopulations of Pdpn$^-$CD104$^-$ mTEC^{lo} cells analyzed using the published data (16). (G) Aire expressing in subpopulations of Pdpn$^-$CD104$^-$mTEC^{lo} cells analyzed using the published data (16).

Figure 2. Cellular phenotypes observed in the *Insm1* mutant thymus

(A,B) Immunofluorescence analysis of thymuses of 6-week-old mice using antibodies against (A) Foxa2 (green) and Insm1 (red), and (B) ChgA (green) and Insm1 (red). Scale bar = 25μm and 10μm in the main and magnified panels, respectively. (C) Flow cytometry analysis of CD45$^-$/EpCAM$^+$ epithelial cells (the upper panel) and CD45$^-$/EpCAM$^+$/UEA1$^+$ mTECs (the lower panel) in thymuses of *Insm1* mutant and wildtype mice (n=11). (D) Flow cytometry analysis of CD45$^-$/EpCAM$^+$ epithelial cells (the upper panel) and CD45$^-$/EpCAM$^+$/UEA1$^+$ mTECs (the lower panel) in thymuses of *Thy-cKO* and wildtype mice (n=6). (E) Flow cytometry analysis of Aire$^+$ cells in *Insm1* mutant and wildtype control animals at the age of E18.5 and adult (animal number n=4 for each genotype). Isotype control staining for Aire was showed on the left, quantifications were showed on the right of the panel. (F) Quantitative RT-PCR analysis of the expression of the mimetic cells specific genes. Upper panel showed the genes specific expressed in the mimetic cell types refereed to the published data (16). Red labeled the downregulated genes in *Insm1* mutants. Lower panel showed the qRT-PCR results. Significant downregulated genes were labeled red in the upper panel. Data in the

figure panel are presented as means ± SD, statistical significance was assessed by 2-tailed unpaired Student's t-test. Statistical significant was defined as $P < 0.05$.

Figure 3. Changes in gene expression in the *Insm1* mutant thymus

(A) Immunofluorescence analysis of the expression of β-galactosidase (β-gal, red) and Aire (green) in thymuses of E18.5 mice. DAPI (48) was used as a counterstain. Quantifications of the fluorescence intensity are shown on the right (animal number n=4 for each genotype, 80-100 Aire+/β-gal+ cells were counted). Scale bar=20μm. (B,C) Quantitative RT-PCR analysis of the expression of *Aire* and TRA genes in mTECs of E18.5 (B) and at adults (C) (n=4). Data in the figure panel are presented as means ± SD, statistical significance was assessed by 2-tailed unpaired Student's t-test. ns: $P>0.05$; *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. (D,E) Volcano blots of the RNAseq data in *Insm1* mutants versus wildtype controls at E18.5 (D) and adult stages (E). FDR $\leqq$ 0.1 or p-value $\leqq$ 0.05 combined with Fold change $\geqq$ 1.5 were used for identifying the dysregulated genes. The numbers of the dysregulated genes were showed in figures accordingly. (F) Expression distribution of the down- and up- regulated genes identified in the sub-populations of mTECs, presented by the violin plots of gene score for each cell. Upper panel, the down-regulated genes in *Insm1* mutants at E18.5; middle panel, down-regulated genes in *Insm1* mutants at adult; lower panel, up-regulated genes in *Insm1* mutants at adult. (G) Heatmap of dysregulated TRA genes expressed in mTEC of *cKO* mice. (H) Distribution of the gene scores for each cells showing the dysregulated TRA expression in the sub-populations of mTECs. The dysregulated TRAs

genes in *Insm1* mutants of E18.5 (the upper panel) and of the adult (the lower panel) were showed respectively.

Figure 4. Insm1 promotes thymic gene expression in the thymus

(A) Immunofluorescence analysis of the expression of Insm1 (green), and Krt5 (red, upper panels), Aire (red, lower panels) in *Insm1*-overexpression (*Insm1OE*) thymuses of postnatal 2-day animals. DAPI (48) was used as a counterstain. (B-C) Comparison of gene expression in *Insm1OE* and control thymuses using postnatal 2-day animals (animal number n=5-6) (B) and mTECs (animal number n=3-4) (C) using qRT-PCR. Data are presented as means ± SD, statistical significance was assessed by 2-tailed unpaired Student's t-test. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

Figure 5. Insm1 binds to super-enhancer loci

(A, B) Distribution of Insm1 binding sites in mTEC genome at the E18.5 stage (A) and at the adult stage (B). (C,D) the Insm1 binding traces on dysregulated genes at the E18.5 stage (C) and at the adult stage (D). (E) Proportions of genes with Insm1 binding sites in the promoter regions (-2000bp to +500bp of TSS) at the E18.5 stage (left) and adult stage (right). Insm1 binding sites are significantly depleted on the promoters of dysregulated genes (F) Density curves of detected loci Inms1 and Aire co-binding (48), Insm1-only binding (orange), and Aire-only binding (green), aligned to the center (0 position) of the super-enhancers. (G,H) Proportion of Insm1 binding peaks overlapping with super-enhancer and control loci at the E18.5 stage (G) and the adult stage (H). An identical length sequence located 200kb plus the length of super-enhancer away from each super-enhancer was selected as the control sequence. (I)

Proportions of super-enhancers located within ±500kb of genes that were dysregulated (p-value ≦ 0.05,FC ≧ 1.5) in *Insm1* mutant mTECs at the E18.5 stage. Stratified by the super-enhancers bound or unbound by Insm1. In (E), (G), (H) and (I), numerators and denominators of each proportion are given above the bars. The bars show 95% confidential intervals. The *p*-values of Fisher test are also given.

Figure 6. Transplantation of *Insm1* mutant thymuses in nude mice

(A) Schematic outline for transplantation experiments. Thymuses were isolated, depleted for lymphocytes by 8-day culture in 2-deoxygranosine, transplanted into nude mice, and the mice were analyzed 8 weeks after transplantation. (B) Transplantation of the thymus under the kidney capsule of the nude mouse. Enlarged picture shows the kidney with the transplanted thymus. (C) Isolated kidneys showing the thymuses under the kidney capsule 8-week after transplantation. *Het/nu*: the thymus was isolated from an *Insm1*$^{+/lacZ}$ mouse and transplanted into a nude mouse; *KO/nu*: the thymus was isolated from an *Insm1*$^{lacZ/lacZ}$ mouse and transplanted into a nude mouse. (D) CD4 and CD8α staining and flow cytometry analysis of thymocytes isolated from the transplanted thymuses of *Het/nu* and *KO/nu* mice (n=3). (E) Appearance (upper panel) and quantification of the weight (lower panel) of spleens isolated from *Het/nu* and *KO/nu* mice (n=5-6). (F) H&E staining shows the structures of spleens isolated from *Het/nu* and *KO/nu* mice. (G) Summary of lymphocytes infiltration in multiple tissues of *Het/nu* and *KO/nu* mice (n=6). (H) H&E staining of the pancreas, the lung, the kidney and the salivary gland isolated from *Het/nu* and *KO/nu* mice. 6-8 sections from the non-serial

sections were used for H&E staining for each tissue. Red arrows indicate sites of lymphocytes infiltration. (I) Immunostaining using serum (green) isolated from *Het/nu* and *KO/nu* mice on sections that were prepared from *Rag1*$^{-/-}$ mice. 3-4 sections collected from the non-serial sections of two *Rag1*$^{-/-}$ mice were used for the serum staining for each tissue. (J) Shown is the co-staining of the *KO/nu* serum (green) with pancreatic islet specific hormones PP, Gcg, Sst and Insulin (red, as indicated in the panels). Statistical data are presented as means ± SD, significance was assessed by 2-tailed unpaired Student's t-test. *: $p<0.05$.

Figure 7. Autoimmune phenotype in thymus specific *Insm1* mutant mice

(A) CD4 and CD8α staining and flow cytometry analysis of thymocytes from thymuses of *Thy-cKO* and wildtype mice (mean and SD showed in figures, n=10-12). (B) CD4 and CD8α staining and flow cytometry analysis of thymocytes from axillary lymph nodes of T*hy-cKO* and wildtype mice (mean and SD showed in figures,n=9). (C, D) Flow cytometry analysis of $CD4^+/CD25^+/Foxp3^+$ Treg cells from the thymus (C, n=8) and the axillary lymph node (D, n=9) of *Thy-cKO* and wildtype mice. (E) H&E staining of brown fat, pancreas, salivary gland, liver and lung in *Thy-cKO* and *Insm1*$^{f/f}$ or *Foxn1Cre* control mice. 6-8 sections from the non-serial sections were used for H&E staining for each tissue. Animal numbers were list in Table 1. (F) Immunostaining using serum isolated from *Thy-Cko* and *Insm1*$^{f/f}$ or *Foxn1Cre* mice on sections of pancreas, salivary gland and ovary prepared from *Rag1*$^{-/-}$ mice. 3-4 sections collected from the non-serial sections of two *Rag1*$^{-/-}$ mice were used for the serum staining for each

tissue. The animals that the serum was collected were list in Table 2. Statistical data are presented as means ± SD, significance was assessed by 2-tailed unpaired Student's t-test. $p>0.05$ in figure A and B, p values showed in figures C and D.

Table 1. Lymphocytes infiltration in tissues

	17-month									6-month								6-week							
Genotypes	*thy-cKO*					*Ctl*				*thy-cKO*				*Ctl*				*thy-cKO*				*Ctl*			
Mice	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Gender	F	F	F	M	M	F	F	M	M	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
Pancreas	+	-	-	+	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salivary gland	++	+/-	-	++	++	+/-	+/-	+/-	+/-	+/-	+/ -	+	+	nt	+/-	+/-	-	+	-	+	-	-	-	-	-
Liver	++	+/-	+/-	-	nt	+/-	+/-	nt	-	+/-	++	++	+/-	-	+/-	+/-	-	+	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spleen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Brain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ovary	-	-	-			-	-			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Intestine	-	-	-	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nt	-	-	-
Colon	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stomach	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eye	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ganglia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Heart	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lung	++	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Skeletal muscle	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nt	-	nt	-	-	nt	-	-
Brown fat	-	+	+-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Testis				-	-			-	-																

Severe infiltration: ++; frequently observed infiltration: +; occasionally observed infiltration: +/-; Negtive: -; nt: not test. F: female; M: male.

Table 2. Autoimmune antibody reaction in tissues

	17-month									12-month						6-month											
Genotypes	*thy-cKO*					*Ctl*				*thy-cKO*			*Ctl*			*thy-cKO*				*Ctl*				*thy-cKO*		*Ctl*	
Mice	1	2	3	4	5	6	7	8	9	26	27	28	29	30	31	10	11	12	13	14	15	16	17	32	33	34	35
Gender	F	F	F	M	M	F	F	M	M	F	F	M	F	F	M	F	F	F	F	F	F	F	F	M	M	M	M
Pancreas	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
Salivary gland	-	-	-	++	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Brain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ovary	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-
Intestine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Colon	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stomach	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eye	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ganglia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nerve fiber	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Heart	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Skeletal muscle	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Brown fat	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Testis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Severe positive: ++; Positive: +; Negtive: -. F: female; M: male.

886

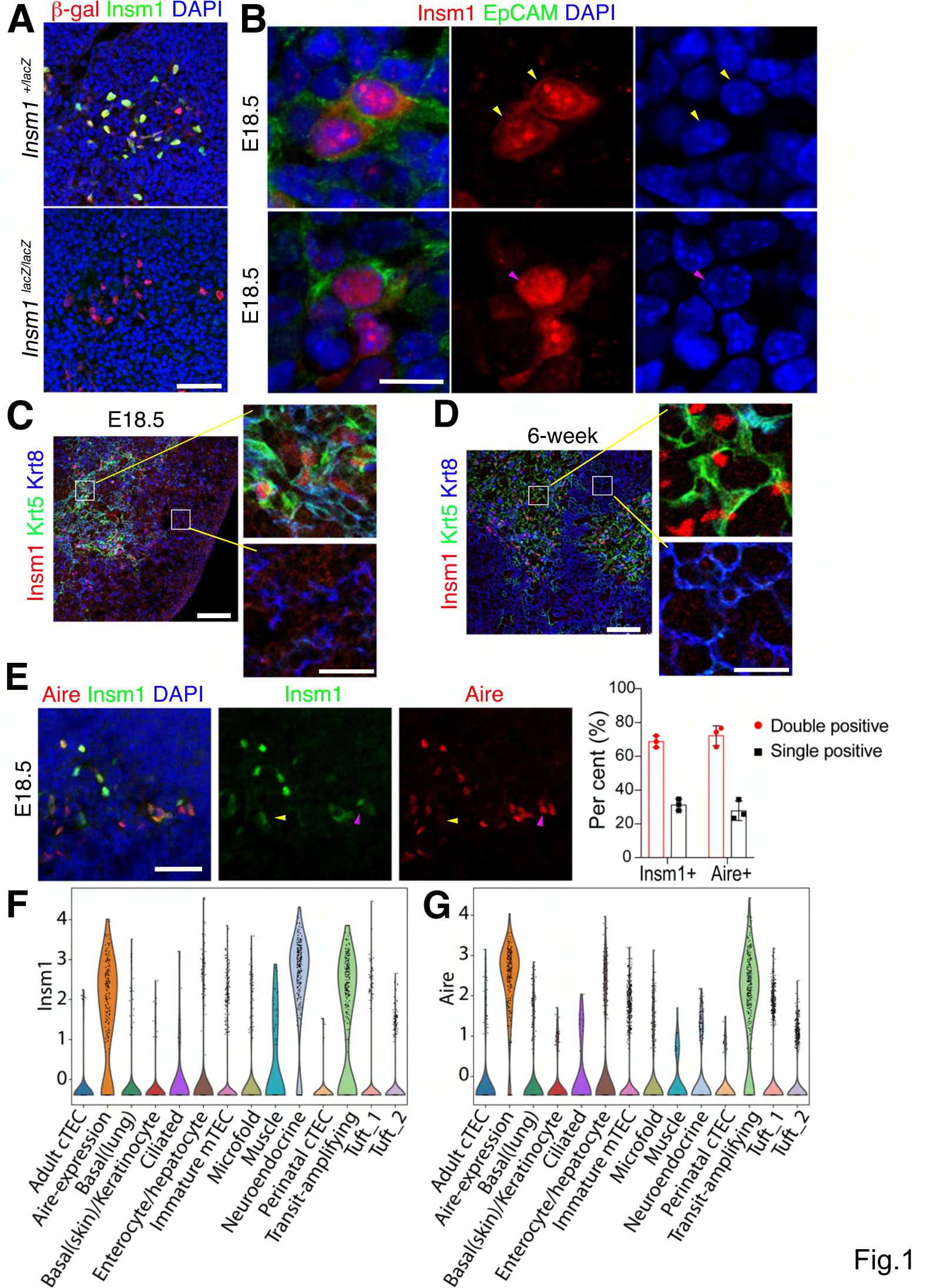
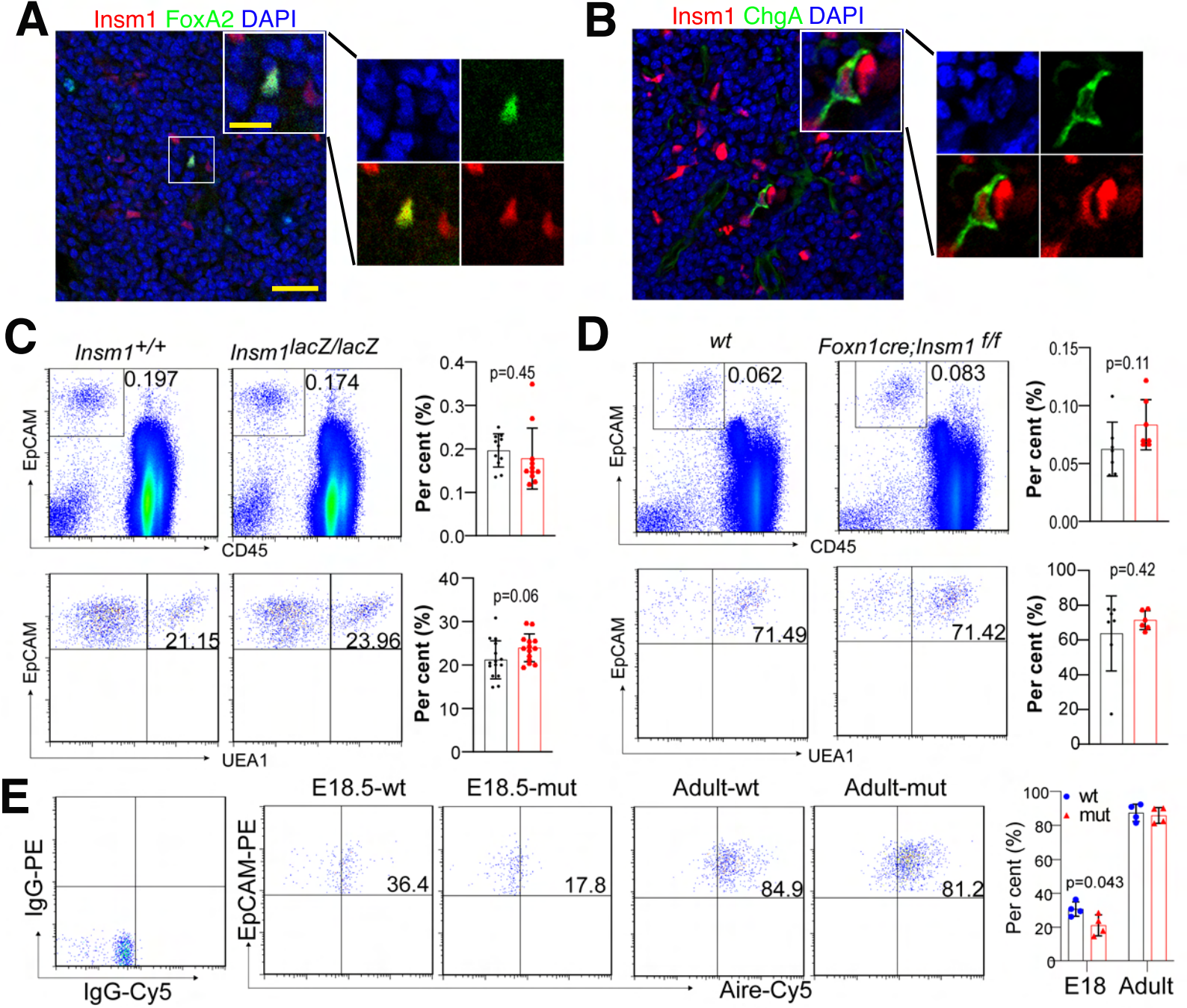

A
β-gal Insm1 DAPI
Insm1 +/lacZ
Insm1 lacZ/lacZ
B
Insm1 EpCAM DAPI
E18.5
E18.5
C
E18.5
Insm1 Krt5 Krt8
D
6-week
Insm1 Krt5 Krt8
E
Aire Insm1 DAPI
Insm1
Aire
E18.5
Per cent (%)
100
80
60
40
20
0
Double positive
Single positive
Insm1+
Aire+
F
Insm1
4
3
2
1
0
Adult cTEC
Aire-expression
Basal(lung)
Basal(skin)/Keratinocyte
Ciliated
Enterocyte/hepatocyte
Immature mTEC
Microfold
Muscle
Neuroendocrine
Perinatal cTEC
Transit-amplifying
Tuft_1
Tuft_2
G
Aire
4
3
2
1
0
Adult cTEC
Aire-expression
Basal(lung)
Basal(skin)/Keratinocyte
Ciliated
Enterocyte/hepatocyte
Immature mTEC
Microfold
Muscle
Neuroendocrine
Perinatal cTEC
Transit-amplifying
Tuft_1
Tuft_2

Fig.1



F

Neuroendocrine	EnteroHepato	Ptf1a+ pancreatic	Muscle	transit-amplifying	Microfold	Skin/keratinized	lung/basal	Ionocyte	Goblet	Ciliated	Tuft1	Tuft2	Immature
Clca3b	Reg3b	Ctrb1	Acta1	Mki67	Ccl20	Flg	Gm2a	Atp6v1g3	Ltf	Ly6c1	Fyb	Gnat3	Col6a1
Cd177	**Aldob**	**Reg1**	Myog	Ccna2	Prg2	Klk7	Serpinb2	Cxcl17	Lcn2	Ccdc153	Hck	Slit2	Mgp
Car8	**Reg1**		Myl1						Trf	Fam183b			Myl9
Resp18													
Chgb													
Cacna2d1													

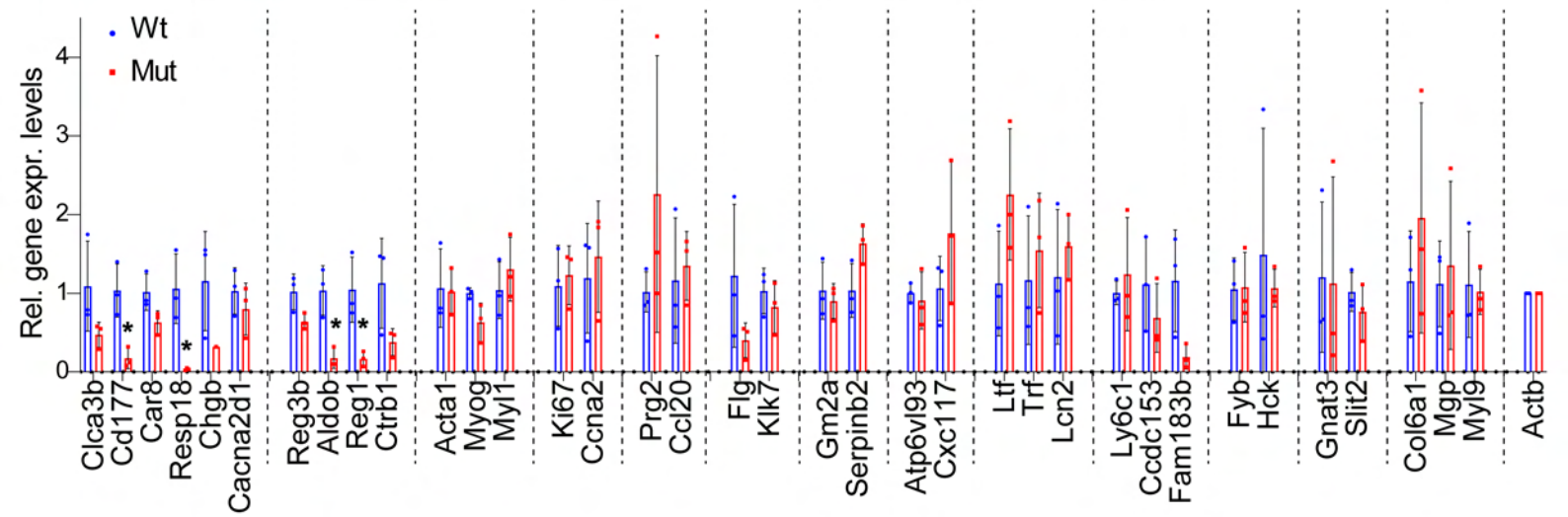


Fig. 2

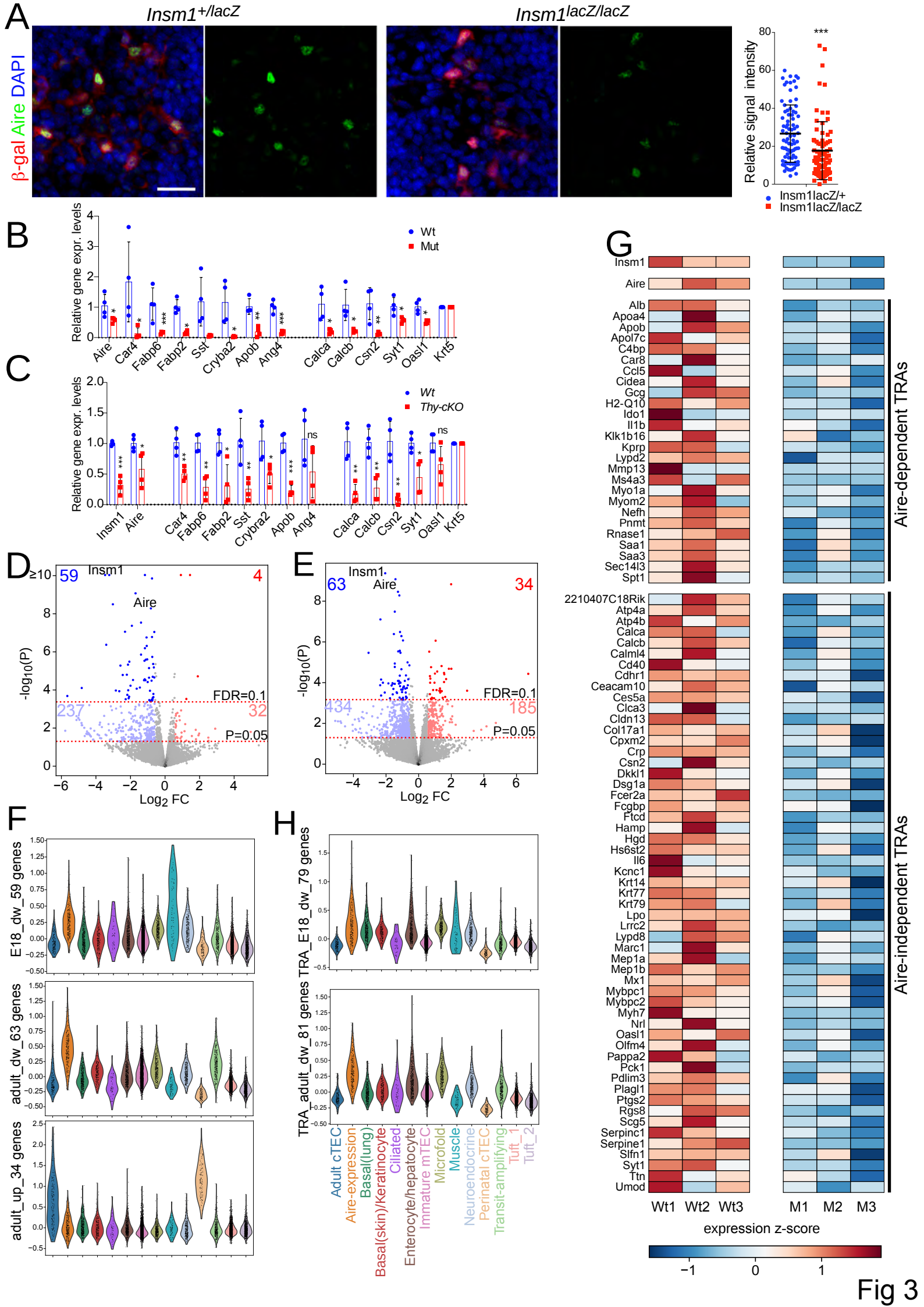

A
Insm1+/lacZ
Insm1lacZ/lacZ
β-gal Aire DAPI
Relative signal intensity
Insm1lacZ/+
Insm1lacZ/lacZ
B
Relative gene expr. levels
Wt
Mut
Aire
Car4
Fabp6
Fabp2
Sst
Crybα2
Apob
Ang4
Calca
Calcb
Csn2
Syt1
Oasl1
Krt5
C
Relative gene expr. levels
Wt
Thy-cKO
Insm1
Aire
D
Insm1
Aire
FDR=0.1
P=0.05
-log10(P)
Log2 FC
E
Insm1
Aire
FDR=0.1
P=0.05
-log10(P)
Log2 FC
F
E18_dw_59 genes
adult_dw_63 genes
adult_up_34 genes
H
TRA_E18_dw_79 genes
TRA_adult_dw_81 genes
Adult cTEC
Aire-expression
Basal(lung)
Basal(skin)/Keratinocyte
Ciliated
Enterocyte/hepatocyte
Immature mTEC
Microfold
Muscle
Neuroendocrine
Perinatal cTEC
Transit-amplifying
Tuft_1
Tuft_2
G
Insm1
Aire
Aire-dependent TRAs
Aire-independent TRAs
Wt1
Wt2
Wt3
M1
M2
M3
expression z-score

Fig 3

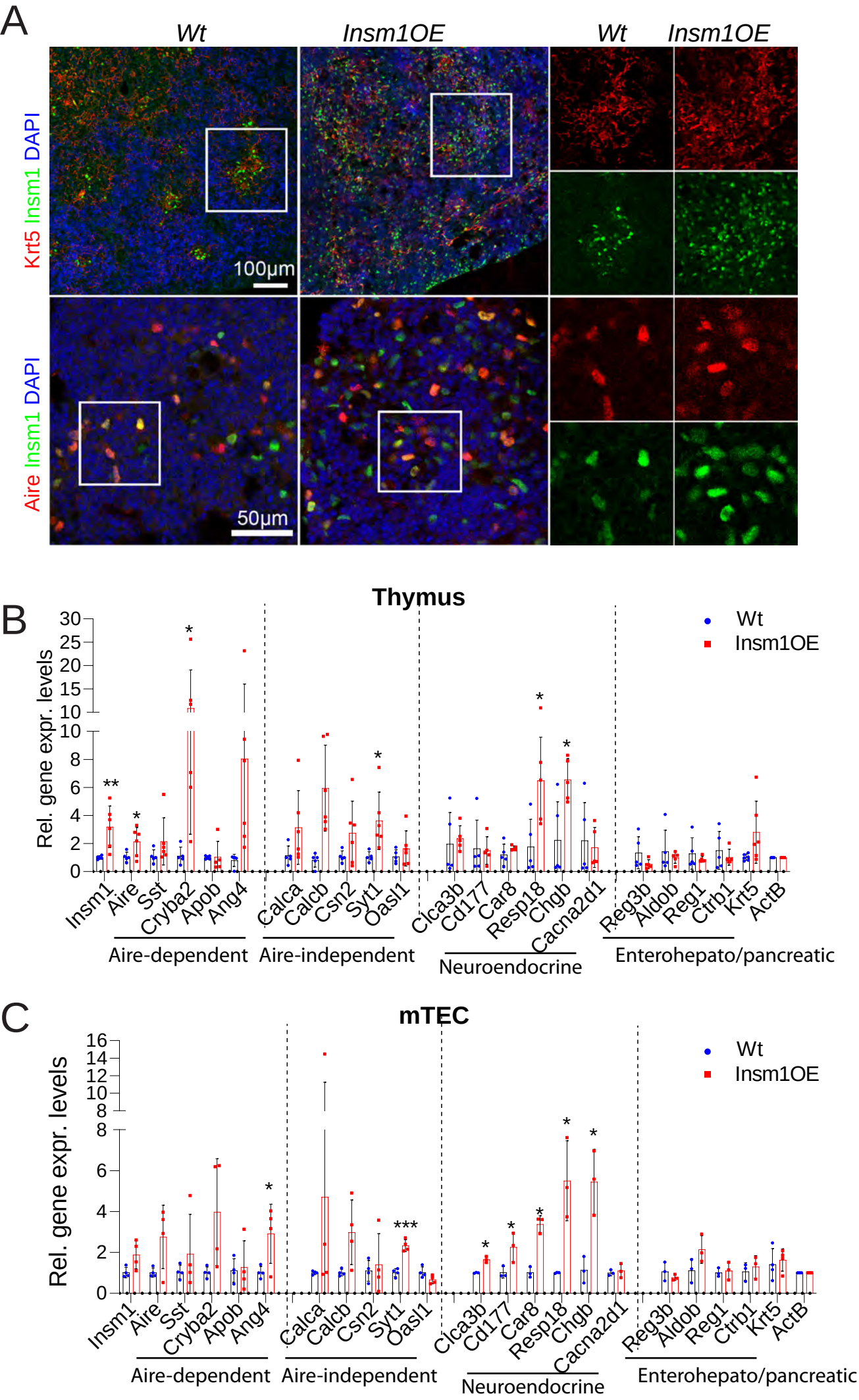

A
Wt
Insm1OE
Wt
Insm1OE
Krt5 Insm1 DAPI
Aire Insm1 DAPI
100µm
50µm
B
Thymus
Rel. gene expr. levels
Wt
Insm1OE
Insm1
Aire
Sst
Cryba2
Apob
Ang4
Calca
Calcb
Csn2
Syt1
Oasl1
Clca3b
Cd177
Car8
Resp18
Chgb
Cacna2d1
Reg3b
Aldob
Reg1
Ctrb1
Krt5
ActB
Aire-dependent
Aire-independent
Neuroendocrine
Enterohepato/pancreatic
C
mTEC
Rel. gene expr. levels
Wt
Insm1OE
Insm1
Aire
Sst
Cryba2
Apob
Ang4
Calca
Calcb
Csn2
Syt1
Oasl1
Clca3b
Cd177
Car8
Resp18
Chgb
Cacna2d1
Reg3b
Aldob
Reg1
Ctrb1
Krt5
ActB
Aire-dependent
Aire-independent
Neuroendocrine
Enterohepato/pancreatic

Fig. 5

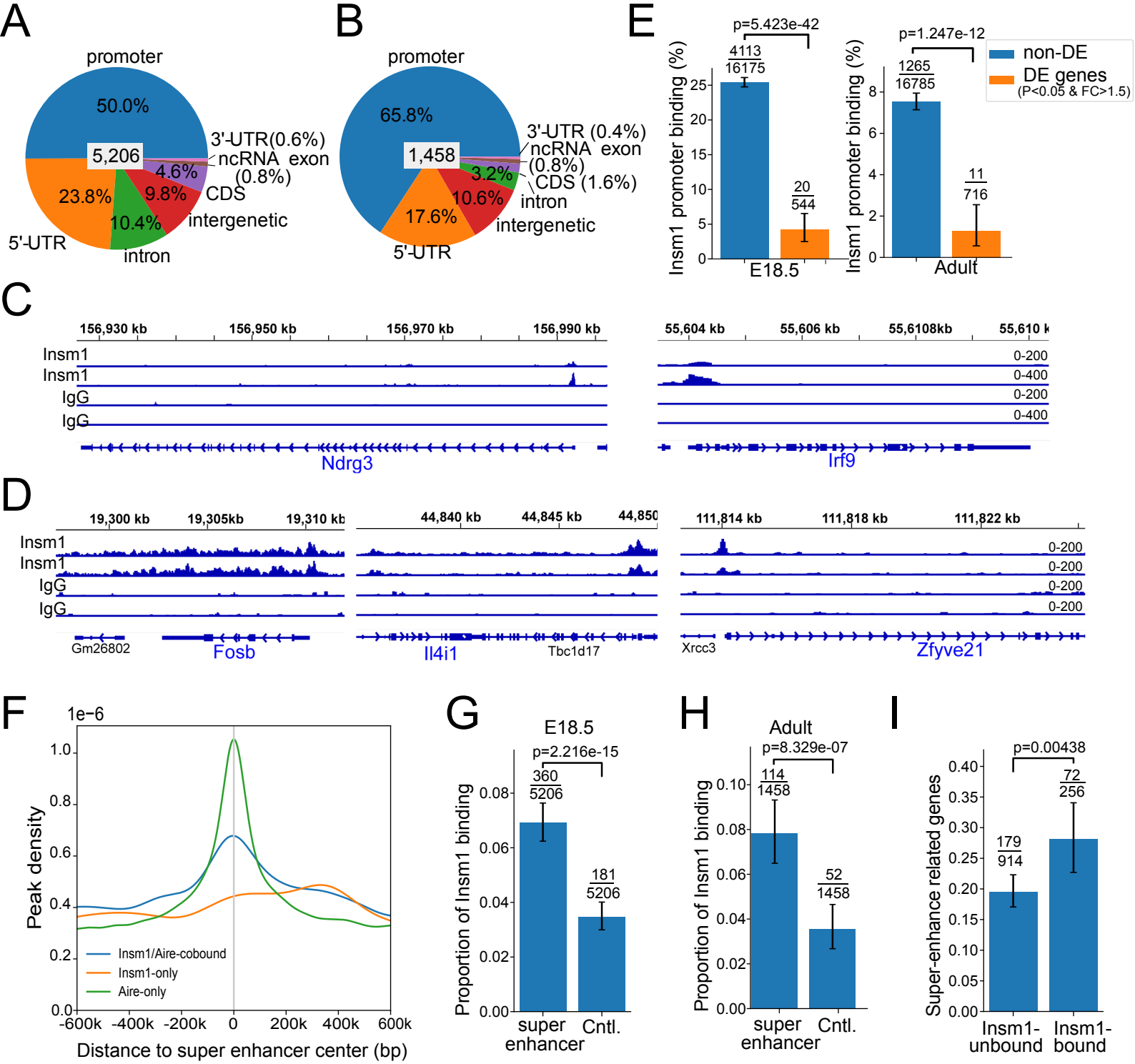

A
promoter
50.0%
5,206
3'-UTR(0.6%)
ncRNA exon
(0.8%)
4.6%
9.8%
CDS
23.8%
10.4%
intergenetic
5'-UTR
intron
B
promoter
65.8%
1,458
3'-UTR (0.4%)
ncRNA exon
(0.8%)
3.2%
CDS (1.6%)
10.6%
intron
17.6%
intergenetic
5'-UTR
E
p=5.423e-42
4113
16175
20
544
Insm1 promoter binding (%)
E18.5
p=1.247e-12
1265
16785
11
716
Adult
non-DE
DE genes
(P<0.05 & FC>1.5)
C
156,930 kb
156,950 kb
156,970 kb
156,990 kb
Insm1
Insm1
IgG
IgG
Ndrg3
55,604 kb
55,606 kb
55,6108kb
0-200
0-400
0-200
0-400
Irf9
D
19,300 kb
19,305kb
19,310 kb
Insm1
Insm1
IgG
IgG
Gm26802
Fosb
44,840 kb
44,845 kb
Il4i1
Tbc1d17
111,814 kb
111,818 kb
111,822 kb
0-200
0-200
0-200
0-200
Xrcc3
Zfyve21
F
1e−6
Peak density
Insm1/Aire-cobound
Insm1-only
Aire-only
-600k -400k -200k 0 200k 400k 600k
Distance to super enhancer center (bp)
G
E18.5
p=2.216e-15
360
5206
181
5206
Proportion of Insm1 binding
super enhancer
Cntl.
H
Adult
p=8.329e-07
114
1458
52
1458
Proportion of Insm1 binding
super enhancer
Cntl.
I
p=0.00438
179
914
72
256
Super-enhance related genes
Insm1-unbound
Insm1-bound

Fig. 5

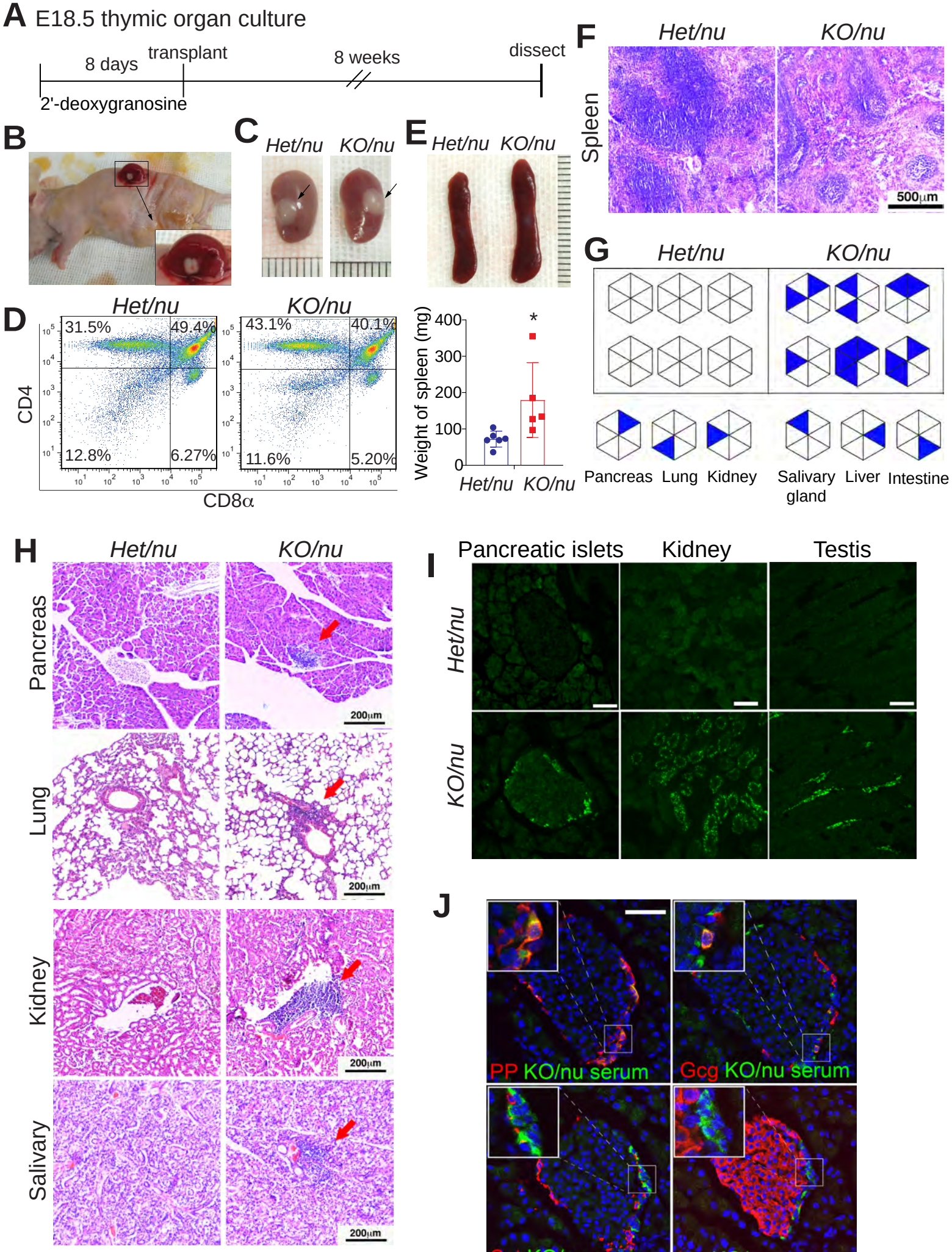

A
E18.5 thymic organ culture
8 days
transplant
8 weeks
dissect
2'-deoxygranosine
B
C
Het/nu
KO/nu
E
Het/nu
KO/nu
F
Het/nu
KO/nu
Spleen
500μm
D
Het/nu
KO/nu
31.5%
49.4%
12.8%
6.27%
43.1%
40.1%
11.6%
5.20%
CD4
CD8α
Weight of spleen (mg)
400
300
200
100
0
*
Het/nu
KO/nu
G
Het/nu
KO/nu
Pancreas
Lung
Kidney
Salivary gland
Liver
Intestine
H
Het/nu
KO/nu
Pancreas
Lung
Kidney
Salivary
200μm
I
Pancreatic islets
Kidney
Testis
Het/nu
KO/nu
J
PP KO/nu serum
Gcg KO/nu serum
Sst KO/nu serum
Ins KO/nu serum

Fig. 6

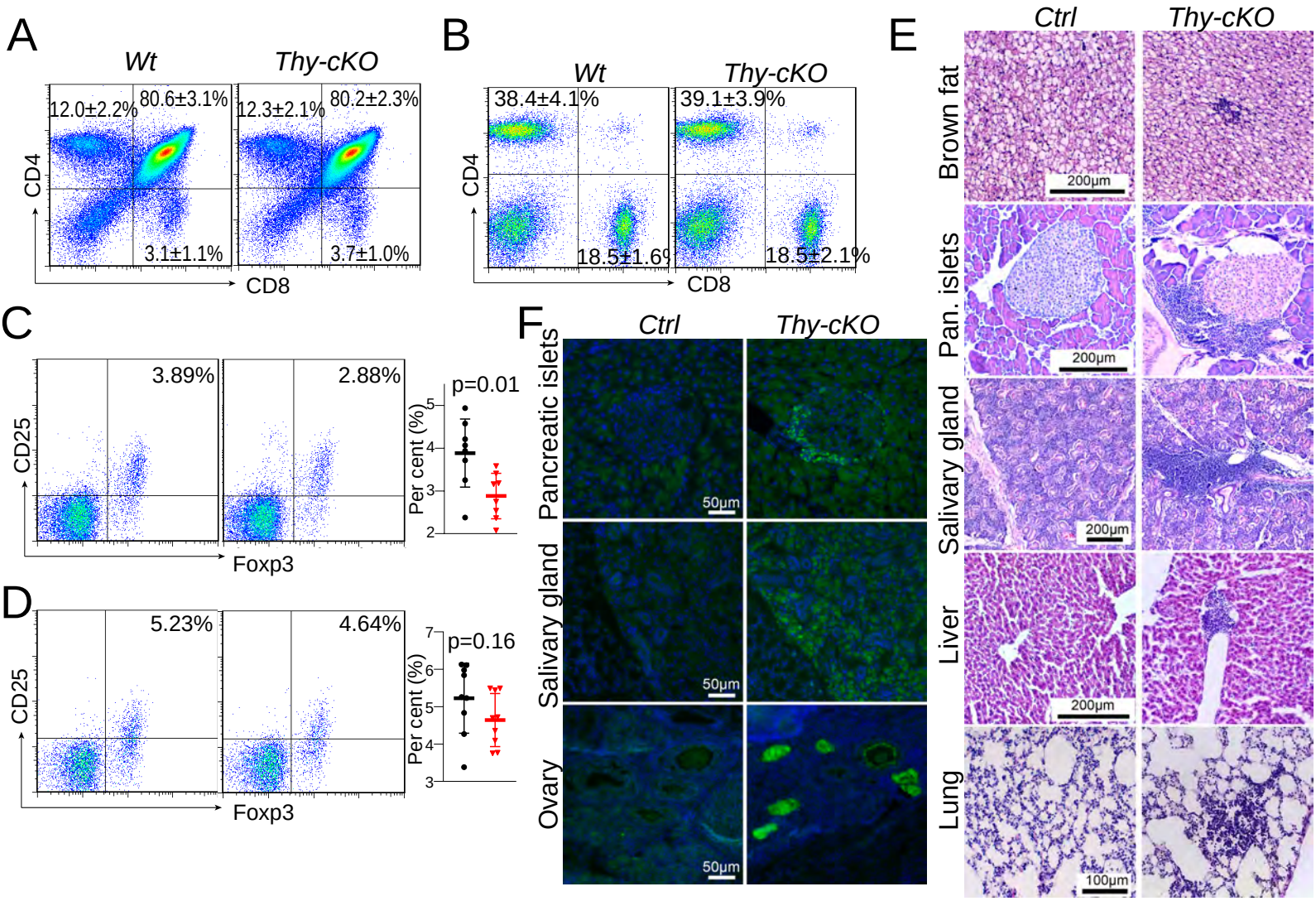

A
Wt
Thy-cKO
12.0±2.2%
80.6±3.1%
3.1±1.1%
12.3±2.1%
80.2±2.3%
3.7±1.0%
CD4
CD8
B
Wt
Thy-cKO
38.4±4.1%
18.5±1.6%
39.1±3.9%
18.5±2.1%
CD4
CD8
C
3.89%
2.88%
CD25
Foxp3
p=0.01
Per cent (%)
D
5.23%
4.64%
CD25
Foxp3
p=0.16
Per cent (%)
F
Ctrl
Thy-cKO
Pancreatic islets
Salivary gland
Ovary
50μm
E
Ctrl
Thy-cKO
Brown fat
Pan. islets
Salivary gland
Liver
Lung
200μm
100μm

Fig. 7