

1 **Integrative analysis of scRNAs-seq and scATAC-seq revealed transit-amplifying thymic**
2 **epithelial cells expressing autoimmune regulator**

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34 **Summary**

35 Medullary thymic epithelial cells (mTECs) are critical for self-tolerance induction in T cells via
36 promiscuous expression of tissue-specific antigens (TSAs), which are controlled by
37 transcriptional regulator AIRE. Whereas AIRE-expressing (Aire⁺) mTECs undergo constant
38 turnover in the adult thymus, mechanisms underlying differentiation of postnatal mTECs remain
39 to be discovered. Integrative analysis of single-cell assays for transposase accessible chromatin
40 (scATAC-seq) and single-cell RNA sequencing (scRNA-seq) suggested the presence of
41 proliferating mTECs with a specific chromatin structure, which express high levels of Aire and
42 co-stimulatory molecules CD80 (Aire⁺CD80^{hi}). Proliferating Aire⁺CD80^{hi} mTECs detected by
43 using Fucci technology express a minimal level of Aire-dependent TSAs and are converted into
44 quiescent Aire⁺CD80^{hi} mTECs expressing high levels of TSAs after a transit amplification.
45 These data provide evidence for the existence of transit amplifying Aire⁺mTEC precursors
46 during Aire⁺mTEC differentiation process of the postnatal thymus.

47

48 **Keywords**

49 Medullary thymic epithelial cells, AIRE, Transit-amplifying cells, Self-tolerance,
50 Differentiation, Single cell RNA sequencing, Single cell ATAC sequencing

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55 Introduction

56 Medullary thymic epithelial cells (mTECs) are essential for induction of T cell self-tolerance in
57 the thymus^{1,2}. mTECs ectopically express thousands of tissue-specific antigens (TSAs), and
58 this expression is regulated by transcription factors, AIRE and FEZF2^{3,4}. TSAs are directly or
59 indirectly presented to developing T cells, and T cells that recognize TSAs with high affinity
60 undergo apoptosis or are converted into regulatory T cells, thereby suppressing the onset of
61 autoimmune diseases^{1,2}.

62
63 Several studies have suggested processes and underlying mechanisms of mTEC differentiation
64 during thymic organogenesis^{1, 2, 5, 6, 7, 8, 9, 10, 11}. In addition, some previous studies suggest that
65 mTEC turnover is homeostatic in the adult thymus, with a duration of approximately 2 weeks^{12,}
66 ^{13, 14}. Notably however, cellular mechanisms underlying maintenance of adult mTECs remain
67 unclear. mTEC subpopulations are largely classified based on their expression of cell surface
68 markers (mainly CD80 and MHC class II) and Aire in the adult thymus¹. CD80^{lo} and
69 Aire-negative (Aire⁻) mTECs (mTEC^{lo}) are thought to be immature, and they differentiate into
70 CD80^{hi} Aire-expressing (Aire⁺) mTECs that are reportedly post-mitotic¹³. Aire⁺ mTECs are
71 further converted into Aire-negative mTECs (post-Aire mTECs)^{15, 16, 17, 18, 19}. Moreover, a
72 previous study suggested that mTECs might be differentiated from stage-specific embryonic
73 antigen-1⁺ (SSEA-1) claudine3/4⁺ mTEC stem cells²⁰. These views are primarily based on fate
74 mapping studies involving transfer and re-aggregation of sorted cell populations with fetal
75 thymus^{5, 13, 20} and on experiments employing genetic marking^{15, 17}.

76
77 Single-cell RNA sequencing (scRNA-seq) technology has yielded new insights into cell
78 diversity and differentiation in various tissues. In TEC biology, previous scRNA-seq studies
79 revealed a stochastic nature of TSA expression in mTECs^{21, 22} and high heterogeneity of TECs
80 in mice^{23, 24, 25, 26}. Bornstein et al. showed that mTECs in the postnatal thymus are separated
81 into four subsets, mTEC I to IV²³. In addition to the classical mTEC^{lo} (mTEC I), Aire⁺ mTEC
82 (mTEC II), and post-Aire mTEC (mTEC III) types, a tuft-like mTEC subset (mTEC IV) was
83 identified^{23, 24}. Subsequent scRNA-seq studies suggested further heterogeneity of TECs, such as
84 cilium TECs²⁵, GP2⁺ TECs²⁵, intertypical TECs²⁶, neural TECs²⁶, and structural TECs²⁶,
85 according to specific gene expression profiles. However, it has not yet been clarified whether
86 this heterogeneity identified from gene expression profiles is correlated with differences in
87 chromatin structure.

88

89 In general, transit-amplifying cells (TACs) are a proliferating cell population linking stem cells
90 and differentiated cells²⁷. TACs are short-lived and undergo differentiation after a few cell
91 divisions. To date, the presence of TACs has been confirmed in some tissues such as intestines²⁸,
92 hair follicles²⁹, and neurons³⁰. Previous analyses of scRNA-seq data of murine adult TECs
93 revealed a cell cluster expressing an abundance of cell-cycle regulated genes, which implies the
94 presence of TACs for TECs (TA-TECs)^{25,31}. Computational trajectory analysis of scRNA-seq
95 data suggested that this population might give rise to Aire-expressing mTECs^{25,26}. Intriguingly,
96 another trajectory study predicted that this cell cluster might differentiate into Aire-expressing
97 mTECs and an mTEC population expressing CCL21a³¹. However, because the TA-TEC
98 candidate has not been isolated and specific marker genes of TA-TECs have not been reported,
99 exact properties of TA-TECs, in addition to their cellular fates, remain to be clarified.

100

101 In this study, droplet-based scRNA-seq and scATAC-seq of murine TECs were performed to
102 characterize TEC heterogeneity and differentiation dynamics. Integrative analysis of these data
103 showed that Aire⁺ mTECs are separated into at least 2 clusters with different gene expression
104 profiles and chromatin accessibilities. One of these Aire⁺ mTEC clusters exhibited high
105 expression of cell cycle-related genes, which accords with a previously proposed TAC
106 population of mTECs^{25,31}. By using the Fucci technology³², proliferating mTECs expressing
107 Aire and maturation marker CD80 were isolated as TA-TEC candidates. This proliferating Aire⁺
108 CD80^{hi} mTEC subpopulation showed minimal expression of TSAs regulated by AIRE, in
109 contrast to quiescent Aire⁺ CD80^{hi} mTECs. Moreover, *in vivo* BrdU pulse-labeling, and *in vitro*
110 reaggregated thymic organ culture suggested that proliferating Aire⁺ CD80^{hi} mTECs are
111 short-lived and that they differentiate into quiescent Aire⁺ CD80^{hi} mTECs, post-Aire mTECs,
112 and tuft-like mTECs. Consequently, these data strongly suggest that proliferating Aire⁺ CD80^{hi}
113 mTECs are TACs for mTECs expressing TSAs.

114

115 **Results**

116 *Droplet-based scATAC-seq reveals heterogeneity of TEC chromatin structure*

117 Given that chromatin structures can be changed during cell differentiation, scATAC-seq analysis
118 of TECs may offer some insights into TEC heterogeneity and differentiation dynamics.

119 Droplet-based scATAC-seq analysis was carried out with EpCAM⁺ CD45⁻ cells that were sorted
120 and pooled from thymi of 2 mice, 4 weeks of age. Unsupervised graph-based clustering and

121 dimensional reduction via uniform manifold approximation and production (UMAP) using the
122 Signac R package (<https://www.biorxiv.org/content/10.1101/2020.11.09.373613v1>) revealed 11
123 cell clusters from 8,413 cells (Figure 1A). We first analyzed chromatin accessibility of
124 previously known TEC marker genes. Clusters 0, 3, 4, 5, 8 and 9 contained relatively higher
125 numbers of cells having the open chromatin structure of the *Cd80* gene, a maturation marker of
126 TECs (Figure 1B and C). Among these clusters, the *cis*-regulatory element of the *Aire* gene³³
127 (about 3 kbp upstream of the transcriptional start site) is opened in clusters 0 and 3 (Figure 1D),
128 suggesting that these clusters (cluster 0 and 3) may be concordant with Aire-expressing mTECs
129 (*Aire*⁺ mTECs, also referred to as mTEC II²³). In contrast, the *cis* element of *Aire* genes is
130 closed in clusters 4, 5, 8, and 9 (Figure 1D), suggesting that these clusters may correspond to
131 post-Aire mTECs and other Aire-negative mature mTECs²³. Because the *Lrmp* gene region is
132 accessible in cluster 5 (Figure 1B and Figure 1—figure supplement 1A), this cluster may be
133 equivalent to tuft-like mTECs (mTEC IV)^{23,24}. CD80 and *Aire* gene regions in clusters 1, 2, and
134 6 are relatively closed, whereas the mTEC marker *Tnfrsf11a* is relatively accessible (Figure 1B
135 and C, and Figure 1—figure supplement 1A). Therefore, these clusters should be equivalent to
136 mTECs expressing low levels of CD80 and *Aire* (mTEC^{lo}). Cluster 7 should be cTECs, because
137 cTEC marker *Psmb11* gene region is opened (Figure 1B and Figure 1—figure supplement 1A).
138 Finally, cluster 10 was deemed thymocyte contamination because the *Rag1* gene was opened
139 (Figure 1—figure supplement 1A).

140
141 We next sought to correlate the scATAC data with TEC scRNA-seq data. Droplet-based
142 scRNA-seq analysis of 11,475 EpCAM⁺ CD45⁻ cells from age- and gender-matched mice
143 (4-week-old female mice) revealed 18 cell clusters (Figure 2A), and expression of TEC marker
144 genes in these clusters was analyzed (Figure 2B and Figure 2—figure supplement 1). Integrative
145 analysis with reported datasets suggests quality comparable to that of our dataset (Figure
146 2—figure supplement 2). Clusters R0, R1, R3, and R9 showed high expression of *Aire*,
147 suggesting that these clusters are equivalent to *Aire*⁺ mTECs (also referred to as mTECs II).
148 Clusters R2, R4 and R5 include cells showing relatively higher levels of *Iga6* and *Ccl21a*
149 expression and a very low level of *Aire* expression (Figure 2A and b), corresponding to mTEC I
150²³, CCL21-expressing mTECs³⁴, and possibly intertypical TECs²⁶. Cluster R6 expresses *Lrmp*
151 (Figure 2B), and should contain tuft-like mTECs (mTEC IV)²³. Clusters R7 and R10 were
152 marked with *Krt10* and *Pigr* genes, respectively (Figure 2B). Accordingly, these clusters should
153 be categorized as post-Aire mTECs (also referred as to mTECs III²³). Cluster R13 showed high

154 expression of chemokines, *Ccl6* and *Gp2* (Figure 2B and Figure 2—figure supplement 1A),
155 which should be concordant with Gp2⁺ TECs, as reported recently²⁵. Clusters R8 and R11
156 exhibited high expression of typical cTEC marker genes, *Psmb11* and *Dll4* (Figure 2B and
157 Figure 2—figure supplement 1A), and should be equivalent to cTECs. Given that thymocyte
158 genes are highly expressed, cluster R11 was most likely thymic nurse cells enclosing
159 thymocytes³⁵. Cluster R12 showed relatively high expression of *Pdpr* (Figure 2—figure
160 supplement 1A), which may comprise junctional TECs³⁶. Cluster R14 was considered
161 thymocyte contamination because thymocyte markers, but not TEC markers, were detected.
162 Cluster R15 apparently corresponds to structural TECs, reported recently, because of their
163 expression of *Car8* and *Cd177*²⁶ (Figure 2—figure supplement 1A). Cells in cluster R16 highly
164 express *Tppp3* and *Fam183b* (Figure 2—figure supplement 1A). Since these genes are
165 expressed in ciliated cells^{37,38}, this cluster may be equivalent to ciliated columnar TECs
166 associated with thymic cystic structure^{25,39,40}. We failed to assign cluster R17, which may be
167 contaminated with endothelial cells or macrophages, because they express *Ly6c1* and *Aqp1*, but
168 low levels of *Epcam* (Figure 2—figure supplement 1A). Overall, our data and assignments are
169 reasonably correlated with previous scRNA-seq data analyses^{23,25,26,31}.

170
171 We then bioinformatically integrated the scRNA-seq data with scATAC-seq data. Gene
172 expression, predicted from accessible chromatin regions of scATAC-seq data, was correlated
173 with scRNA-seq data using the Signac R package (Figure 3A and Figure 3—figure supplement
174 1). As described, clusters 0 and 3 in scATAC-seq analysis contain cells with the accessible
175 *cis*-regulatory element of the *Aire* gene (Figure 1D). Consistently, cluster 0 in scATAC-seq were
176 mostly transferred to cluster R0 (40.5 %) and R3 (26.3%) in scRNA-seq analysis (Figure 3B
177 and C, and Supplementary Table 1), which were assigned as Aire⁺ mTECs (Figure 2). Cells
178 transferred to R0 and R3 appear to be separately embedded in cluster 0 in the UMAP dimension,
179 implying that these two Aire⁺ mTEC subsets have slightly different chromatin structures.
180 Cluster 3 was mostly transferred to cluster R1 (88.2%) (Figure 3B and C), also designated as
181 Aire⁺ mTECs. Interestingly, cells transferred to cluster R9 are embedded around the junction
182 between cluster 0 and 3 (Figure 3B), suggesting that cluster R9 may be a transitional stage
183 between R1 and R0. Clusters 1, 2, and 6 are closely embedded in the UMAP dimension and
184 principally assigned to clusters R2, R4, and R5 (Figure 3B and C), suggesting that these clusters
185 are concordant with mTEC I or intertypical TECs assigned in the scRNA-seq data. Cluster 4
186 mainly contains cells transferred to cluster R7 (55.0%) and R10 (27.2%) (Figure 3B), which

187 were assigned as post-Aire mTECs (mTEC III). Cells assigned in R7 and R10 were embedded
188 in distinct regions of cluster 4, implying that post Aire⁺ mTECs consist of two cell types with
189 slightly different gene expression profiles and chromatin structures. As expected, cluster 5 with
190 an open *Lrmp* gene was transferred to cluster R6, a tuft-like mTEC subset (mTEC IV). Cluster 9
191 was assigned as cluster R13, which was Gp2⁺ TECs (Figure 3B and C). Cluster 7 was
192 transferred to cluster R8 and R12, assigned as cTECs and jTECs, respectively. Cluster 8
193 contains clusters R15 (64.7%) and R16 (34.2%), which express markers of structural TECs and
194 cilia TECs, respectively (Figure 3C and Figure 3—figure supplement 1). Finally, cluster 10 was
195 transferred to clusters R11 and R14, which are assigned as T cells and Nurse TECs (Figure 3C
196 and Figure 3—figure supplement 1). Although a few cells were transferred to R17 in
197 scRNA-seq, these cells did not form cluster in this analysis. Thus, TEC heterogeneity predicted
198 from scRNA-seq may be ascribed to differences in chromatin structure.

199

200 *Aire-positive mTECs are divided into two subsets having distinct chromatin structures*

201 Previous scRNA-seq studies proposed the existence of a TEC population showing high
202 expression of cell-cycle-regulated genes^{25,26,31}. In our scRNA-seq data, cluster R1 (mTEC IIb)
203 appears equivalent to such a TEC subset, expressing cell-cycle-related genes (Figure 2—figure
204 supplement 1). Sub-clustering of cluster R1 showed its separation into 5 sub-clusters (R1A to
205 R1E in Figure 2—figure supplement 3A and B). Clusters R1A, R1B, R1C and R1D showed
206 expression of *Aire*. In contrast, *Ccl21a*, but not *Aire*, is highly expressed in cluster R1E (Figure
207 2—figure supplement 3C and D). This is largely consistent with a previous study. Thus, TECs
208 expressing cell-cycle-related genes defined in scRNA-seq analysis may be divided into
209 Aire-positive and Aire-negative *Ccl21a*^{high} subsets³¹.

210

211 Integrative analysis of scRNA-seq and scATAC-seq suggested that cells in cluster 3 in
212 scATAC-seq were transferred to cluster R1. Notably, although both clusters 0 and 3 have the
213 accessible enhancer element of the *Aire* gene (Figure 1), 327 genomic regions were significantly
214 opened, and 85 regions were closed in cluster 3, in contrast to cluster 0 (Supplementary Table 2
215 and Figure 2—figure supplement 1B). Thus, it is likely that the Aire⁺ mTECs subset expressing
216 cell cycle-related genes have a distinct chromatin structure relative to other TEC subsets.

217

218 Notably, some cells of clusters 1 (7%) and 0 were assigned as cluster R1 (Figure 3). This may
219 be consistent with the heterogeneity of R1, suggested from the subcluster analysis (Figure

220 2—figure supplement 3). Consistently, scATAC-seq analysis showed that chromatin
221 accessibility of a marker gene for cluster R1E (*Mgp*, Figure 2—figure supplement 3B) was low
222 in cluster 3 and relatively higher in cluster 1 (Figure 2—figure supplement 3E). Thus, this
223 analysis suggests that R1 includes a *Ccl21a*^{high} TEC subset having a chromatin structure
224 different from the *Aire*^{high} TEC subsets in cluster R1. Thus, it is possible that TECs expressing
225 cell-cycle-related genes, proposed by scRNA-seq analysis, contain at least two proliferating
226 TECs subsets having different chromatin accessibilities and gene expression profiles.

227
228 RNA velocity, which recapitulates differentiation dynamics by comparing unspliced and spliced
229 RNA in scRNA-seq data⁴¹, predicted that cluster R1 may differentiate into other *Aire*⁺ mTECs
230 (clusters R0, R3 and R9) (Figure 3—figure supplement 2A), which is consistent with analyses
231 of others²⁵. Moreover, trajectory analysis of scATAC-seq data using the Monocle3 package also
232 suggested a possible transition between cluster 3 and cluster 0 (Figure 3—figure supplement
233 2B). Thus, these trajectory analyses of scRNA-seq and scATAC-seq suggest that the *Aire*⁺
234 mTEC subset expressing cell-cycle-related genes may be precursors of other *Aire*⁺ mTECs.
235 Thus, integrative analysis of scATAC-seq and scRNA-seq data imply that cluster 3 (cluster R1)
236 may be equivalent to transiently amplifying cells (TA cells) with a distinct chromatin structure.

237
238 *A proliferative cell subset is present in Aire⁺ mTECs*

239 TA cells were defined as a proliferative, short-lived cell subset generated from progenitor or
240 stem cells and differentiating into mature quiescent cells^{27,42}. To search for evidence supporting
241 the presence of TA cells of mTECs (TA-TECs), we first sought to isolate the proliferating *Aire*⁺
242 CD80^{hi} mTEC subset as candidate TA-TECs. Fucci2a mice, in which cell cycle progression can
243 be monitored with mCherry (G1 and G0 phases) and Venus (G2, M, and S phases) fluorescence,
244 were used to isolate such proliferating cells (Figure 4A)^{32,43,44,45}, and were crossed with
245 *Aire*-GFP-reporter mice to facilitate detection of *Aire* expression⁴⁶. Flow cytometry analysis
246 indicated that Venus⁺ cells are present in mTECs expressing high levels of CD80 (mTEC^{hi}).
247 Moreover, these Venus⁺ mTECs^{hi} expressed *Aire*-GFP (Figure 4B). Thus, these data revealed the
248 presence of dividing cells in the *Aire*⁺ CD80^{hi} mTEC fraction. The fluorescence intensity of
249 *Aire*-GFP in Venus⁺ CD80^{hi} mTECs showed a broad peak and was slightly lower than that of
250 Venus⁻ mTEC^{hi} cells, which may be due to the relatively lower expression of *Aire* in Venus⁺
251 CD80^{hi} mTECs. However, the compensation between GFP and Venus proteins, which have very
252 close fluorescence spectra, hampered an exact comparison of *Aire* expression levels between

253 Venus⁺ mTEC^{hi} cells and Venus⁻ mTEC^{hi} cells. We next confirmed Aire protein expression in
254 proliferating mature mTECs. Immunostaining with an anti-Aire-antibody revealed the presence
255 of Aire protein localized in the nucleus of sorted Venus⁺ CD80^{hi} mTECs (Figure 4C). Moreover,
256 immunostaining of the thymic section from *Foxn1*-specific Fucci2a mice revealed that Venus⁺
257 cells are localized in the medulla, and some of the Aire⁺ mTECs were Venus⁺ (Figure 4D and
258 Figure 4—figure supplement 1). Taken together, these data confirm the presence of proliferating
259 Aire⁺ CD80^{hi} mTECs in the thymic medulla.

260

261 *Proliferating Aire⁺ mTECs express low levels of Aire-dependent TSAs*

262 We next addressed whether the proliferating Aire⁺CD80^{hi} mTECs subset has molecular
263 signature distinct from that of quiescent Aire⁺CD80^{hi} mTECs. RNA-seq analysis of sorted cells
264 from Fucci mice suggested that Venus⁺ CD80^{hi} mTECs and Venus⁻ CD80^{hi} mTECs subsets have
265 considerably different gene expression profiles (Figure 4E). As expected, gene ontology
266 analysis confirmed enrichment of cell cycle-related genes in Venus⁺ CD80^{hi} mTECs compared
267 with Venus⁻ CD80^{hi} mTECs (Supplementary Table 3). Notably, although expression levels of
268 Aire were comparable (Figure 4F), the Venus⁺ CD80^{hi} mTEC subset expressed lower levels of
269 Aire-dependent TSAs than the Venus⁻ CD80^{hi} mTECs subset (Figure 4F and G). However,
270 expression of Aire-independent TSAs was relatively comparable in the two subsets (Figure 4G).
271 These data suggested that proliferating Aire⁺CD80^{hi} mTECs are phenotypically immature,
272 compared to quiescent Aire⁺CD80^{hi} mTECs.

273

274 *Proliferating Aire⁺ mTECs are precursors of mature mTECs*

275 Because TA cells are defined as short-lived cells differentiating into mature cells²⁷, we next
276 addressed this issue regarding the proliferating Aire⁺ CD80^{hi} mTECs. *In vivo* pulse-labeling of
277 TECs with 5-bromo-2'-deoxyuridine (BrdU) was performed. Because mCherry^{hi} cells and
278 mCherry^{lo} were generally in G0 and G1 stages of the cell cycle, respectively⁴⁷, each fraction in
279 CD80^{hi} mTECs was sorted separately after *i.p* administration of BrdU, and thereafter stained
280 with anti-BrdU antibody (Figure 5A). This procedure was necessary because mCherry
281 fluorescence is lost after BrdU staining. Flow cytometric analysis showed that approximately
282 35% of mCherry^{lo} CD80^{hi} mTECs (hereafter referred as to mCherry^{lo}) were labeled at 12 h (Day
283 0.5) after the BrdU injection (Figure 5B). In contrast, about 3% of mCherry^{hi} CD80^{hi} mTECs
284 (referred as to mCherry^{hi}) were BrdU-positive (Figure 5B). Thus, as expected, cell cycle
285 progression of mCherry^{lo} is much faster than mCherry^{hi}. Importantly, cell number and the ratio

286 of BrdU-positive cells in the mCherry^{lo} fraction was significantly decreased 3.5 days after the
287 BrdU injection (Figure 5B and C). On the other hand, the frequency of BrdU-positive cells in
288 mCherry^{hi} was increased by day 3.5, and plateaued from day 3.5 to day 6.5 (Figure 5B and C).
289 Notably, fluorescence intensity (MFI) of BrdU staining in mCherry^{hi} at Day 3.5 was about 50%
290 lower than that in mCherry^{lo} at Day 0.5 (Figure 5D), suggesting that mCherry^{hi} at day 3.5 were
291 generated after cell division. Overall, these data suggest that mCherry^{lo} are transiently
292 proliferating, and after cell division, they are converted to mCherry^{hi} having low proliferative
293 activity.

294

295 To verify that mCherry^{lo} cells are precursors of mCherry^{hi}, we performed an *in vitro*
296 reaggregation thymic organ culture (RTOC) experiment (Figure 6A). The mCherry^{lo} fraction
297 (Figure 6—figure supplement 1) was reaggregated with wild type embryonic thymic cells. After
298 5-days of culture, mCherry^{hi} was detected in RTOC (Figure 6A). Because Venus⁺mCherry^{lo} cells
299 were practically absent in RTOC (Figure 6—figure supplement 1A), survived mCherry^{lo} cells
300 were mostly converted into mCherry^{hi} in RTOC. Interestingly, reaggregation with allogenic fetal
301 thymus (Balb/cA background) was not sufficient for the conversion to mCherry^{hi} (Figure
302 6—figure supplement 1B), implying that high affinity interaction between TCR and MHC
303 contributes to survival and maintenance of mCherry^{lo} TECs as described previously⁴⁸. Next, we
304 sorted mCherry^{hi} cells in the RTOC (referred as to mCherry^{hi}-RTOC) in addition to mCherry^{lo}
305 and mCherry^{hi} from the Fucci thymus, and analyzed gene expression by RNA-seq. As expected,
306 the mCherry^{lo} fraction expressed a lower level of Aire-dependent TSAs, compared to mCherry^{hi}
307 (Figure 6B), although Aire and Mki67 were highly expressed (Figure 6C). Importantly, in
308 comparison to the mCherry^{lo} fraction, the mCherry^{hi}-RTOC fraction showed higher levels of
309 Aire-dependent TSAs (Figure 6B). Moreover, beside cell-cycle-related genes, some genes were
310 highly expressed in all mCherry^{lo}, Venus⁺ cells, and cluster R1 cells (Figure 6—figure
311 supplement 2 and Supplementary Table 4). Notably, these gene set were down-regulated in
312 mCherry^{hi}-RTOC (Figure 6C and Figure 6—figure supplement 2). These data suggest that
313 mCherry^{lo} cells were converted into mCherry^{hi} in RTOC.

314

315 In order to detail phenotypes of mCherry^{hi}-RTOC, we next performed well-based scRNA-seq.
316 mCherry^{hi}-RTOC in addition to mCherry^{lo}CD80^{hi} and mCherry^{hi}CD80^{hi} mTECs from the
317 murine thymus were single-cell sorted by flow cytometry, and then gene expression in
318 individual cells was determined by random displacement amplification sequencing

319 (RamDA-seq) technology⁴⁹. After quality control of the data, gene expression matrix data of
320 single-cell RamDA-seq (scRamDA-seq) were integrated with the droplet-based scRNA-seq data
321 (Figure 6D). Although this integration slightly changed the UMAP dimension and clustering
322 compared to Figure 2, assignment of each cluster was successfully achieved in the practically
323 same fashion (Supplementary Figure 7E and F), except that cluster R15 (s-TEC) in Figure 3 was
324 incorporated into cluster R10 (mTEC IIIb) and one new cluster were separated from cluster R2
325 and R3.

326
327 Cells from the mCherry^{lo}CD80^{hi} mTEC fraction (total 36 cells) were assigned mainly to clusters
328 R1 (17 cells) and R9 (11 cells) (Figure 6E and F, and Supplementary Table 5). Some cells were
329 assigned to clusters R0 (3 cells) and R2 (2 cells). Although other cells were assigned to clusters
330 R4, R7 and R14, the embedded position was separated from each parent cluster, which may be
331 due to misclustering. In contrast, cells in the mCherry^{hi}CD80^{hi} mTEC fraction (total 35 cells)
332 were more heterogenous and consisted of cells assigned mainly to clusters R0 (7 cells), R3 (9
333 cells), R5 (4 cells), R7 (3 cells), R10/15 (5 cells), and R13 (2 cells) (Figure 6E and F, and
334 Supplementary Table 5). Except for cluster R5, these clusters were concordant with Aire⁺
335 mTECs, post-Aire mTECs, and GP2⁺ TECs. Notably, after the RTOC, heterogenous cell
336 populations including clusters R0 (18 cells), R3 (13 cells), R5 (5 cells), R6 (3 cells), R7 (8 cells)
337 and R10/15 (5 cells) were found in the mCherry^{hi}-RTOC population (total 65 cells). Its
338 composition was relatively similar to that of the mCherry^{hi}CD80^{hi} mTEC fraction (Figure 6F).
339 Moreover, these mCherry^{hi}-RTOC cells expressed high levels of TSAs (Figure 6G).
340 Interestingly, 5 cells in mCherry^{hi}-RTOC were assigned to cluster R5, which also reside in the
341 mCherry^{hi}CD80^{hi} mTEC fraction from the adult thymus. This finding is consistent with the idea
342 of an “intertypical” mTEC cluster, which reportedly contains both CD80^{hi} mTECs and
343 CD80^{lo} mTECs²⁶. Overall, these data suggest that mCherry^{lo}CD80^{hi} mTECs differentiate into
344 quiescent mature mTECs expressing high levels of TSAs, including Aire⁺ mTECs (mTEC II),
345 post-Aire mTECs (mTEC III), and tuft-like mTECs (mTEC IV).

346
347 *Proliferating Aire⁺ mTECs are present after puberty in mice*

348 We investigated whether proliferative Aire⁺ mTECs persisted in the thymi of older mice. TECs
349 were analyzed in 4-week-old, 8-week-old, and 19-week-old Fucci Aire-GFP mice. Flow
350 cytometric analysis showed that a Venus⁺ mTEC^{hi} subset was present in 19-week-old mice as
351 well as younger mice (Figure 7A). Moreover, Venus⁺ mTEC^{hi} cells expressed Aire genes (Figure

352 7A). These data strongly suggested that transit amplifying TECs persist in the adult thymus as a
353 source of mature mTECs.

354

355 Integrative computational analysis of our scRNA-seq data with a previously reported dataset of
356 fetal TECs (E12 to E18) showed considerably different cell embedding between adult TECs and
357 fetal TECs (Figure 7—figure supplement 1). A TEC-expressing subset was present in the fetal
358 thymus whereas Aire expression was low (Clusters F3 and F12, Figure 6—figure supplement 2).
359 This implies that fetal proliferating mTECs may have a different gene expression profile than
360 adult proliferating Aire⁺CD80^{hi} mTECs (Figure 6—figure supplement 2).

361

362 **Discussion**

363 With regard to mTEC differentiation in the adult thymus (Figure 7B), we hypothesize that Aire⁺
364 TA-TECs were generated from their Aire-negative progenitors. Aire⁺ TA-TECs (cluster 3)
365 undergo cell division and then differentiate into quiescent Aire⁺ mTECs (cluster 0) through a
366 transition stage, which corresponds to cluster R9 in scRNA-seq data. This differentiation
367 process is accompanied by a chromatin structure change. Post-mitotic Aire⁺ mTECs begin
368 high-level TSA expression, and further differentiate into post-Aire mTECs (R7, R10 and R13)
369 by closing the Aire-enhancer region. Differentiation of mTECs expressing TSAs may have to
370 coordinate differentiation with cell cycle regulation, as proposed in neural cells and muscle
371 differentiation⁵⁰.

372

373 Generally, in other tissues, transit-amplifying cells constitute a link between stem cells and
374 mature cells⁴². An important question is what cells differentiate into proliferating Aire⁺ mTECs.
375 Previous studies have suggested that mTECs^{lo} expressing low levels of maturation markers (i.e.
376 CD80 or MHC II) are precursors^{1,13}. However, several recent studies have suggested that
377 mTEC^{lo} contains several subsets, including CCL21a-positive mTECs, tuft-like mTECs, and
378 others. One possible explanation for this is that a small number of mTEC stem cells or other
379 precursor cells may be present in the mTEC^{lo} subset¹³. Consistently RNA velocity analysis also
380 suggested that most mTEC^{lo} cells do not appear to differentiate into Aire-expressing mTECs.
381 Given that transit-amplifying mTECs are present, a small number of stem/precursor cells would
382 theoretically be sufficient for mTEC reconstitution. A previous study proposed that TECs
383 expressing claudin 3/4 and SSEA-1 had characteristic features of mTEC stem cells in
384 embryonic thymus²⁰. We failed to detect a corresponding cluster of mTEC stem cells as a

385 subset of adult scRNA clusters. This may be because corresponding mTEC stem cells in adult
386 thymus are included in the “intertypical” TEC cluster, which may be a mixture of various TECs
387 ²⁶. More detailed characterization of mTEC stem cells in the adult thymus is necessary to
388 illuminate the differentiation dynamics of mTECs.

389

390 Overall, the scRNA-seq analysis in the present study suggested the presence of a novel
391 differentiation process of TECs in the adult thymus. Disturbance of adult TEC homeostasis may
392 cause thymoma, autoimmunity, and other diseases. Further characterization of molecular
393 mechanisms underlying differentiation and maintenance processes in TECs will aid the
394 development of novel therapeutic strategies against these thymus-related diseases.

395

396 **Materials and Methods**

397 *Mice*

398 C57BL/6 mice were purchased from Clea Japan. Littermates or age-matched, wild-type mice
399 from the same colonies as the mutant mice were used as controls. *Aire-GFP* mice (CDB0479K,
400 http://www2.brc.riken.jp/lab/animal/detail.php?brc_no=03515) and
401 B6;129-Gt(ROSA)26Sor<tm1(Fucci2aR)Jkn> (RBRC06511) (Fucci2a) ³² were provided by the
402 RIKEN BRC through the National Bio-Resource Project of the MEXT, in Japan. CAG-Cre
403 transgenic mice were kindly provided by Dr. Jun-ichi Miyazaki ⁵¹. B6(Cg)-Foxn1tm3(cre)Nrm/J
404 are from Jackson Laboratory⁵². Fucci2a mice were crossed with CAG-Cre or Foxn1-Cre mice to
405 activate mCherry and Venus expression. Fucci mice crossed with CAG-Cre were used for all
406 experiments except for immunostaining experiments (Figure 4). All mice were maintained
407 under specific pathogen-free conditions and handled in accordance with Guidelines of the
408 Institutional Animal Care and Use Committee of RIKEN, Yokohama Branch (2018-075).
409 Almost all of available mutant and control mice were randomly used for experiments without
410 any selection.

411

412 *Preparation of TEC suspensions and flow cytometry analysis*

413 Murine thymi were minced using razor blades. Thymic fragments were then pipetted up and
414 down to remove lymphocytes. Then fragments were digested in RPMI 1640 medium containing
415 Liberase™ (Roche, 0.05 U/mL) plus DNase I (Sigma-Aldrich) via incubation at 37°C for 12 min
416 three times. Single-cell suspensions were stained with anti-mouse antibodies. Dead cells were
417 excluded via 7-aminoactinomycin D staining. Cells were sorted using a FACS Aria instrument

418 (BD). Post-sorted cell subsets were determined to contain > 95% of the relevant cell types. Data
419 were analyzed using Flowjo 10. No data points or mice were excluded from the study.
420 Randomization and blinding were not used.

421

422 *Droplet-based scRNA-seq analysis*

423 For scRNA-seq analysis, cell suspensions of thymi from 3 mice were prepared and pooled for
424 each individual scRNA-seq experiment. Two experiments were performed. Cellular suspensions
425 were loaded onto a Chromium instrument (10× Genomics) to generate a single-cell emulsion.
426 scRNA-seq libraries were prepared using Chromium Single Cell 3' Reagent Kits v2 Chemistry
427 and sequenced in multiplex on the Illumina HiSeq2000 platform (rapid mode). FASTQ files
428 were processed using Fastp⁵³. Reads were demultiplexed and mapped to the mm10 reference
429 genome using Cell Ranger (v3.0.0). Processing of data with the Cell Ranger pipeline was
430 performed using the HOKUSAI supercomputer at RIKEN and the NIG supercomputer at ROIS
431 National Institute of Genetics. Expression count matrices were prepared by counting unique
432 molecule identifiers. Downstream single-cell analyses (integration of two datasets, correction of
433 dataset-specific batch effects, UMAP dimension reduction, cell cluster identification, conserved
434 marker identification, and regressing out cell cycle genes) were performed using Seurat (v3.0)⁵⁴.
435 Briefly, cells that contained a percentage of mitochondrial transcripts > 15% were filtered out.
436 Genes that were expressed in more than 5 cells and cells expressing at least 200 genes were
437 selected for analysis. Two scRNA-seq datasets were integrated with a combination of Find
438 Integration Anchors and Integrate Data functions⁵⁵. To investigate the effects of regressing out
439 cell cycle genes on cell clustering, we compared three types of pre-processing; no regressing out,
440 regressing out the difference between the G2/M and S phase scores, and complete regressing out
441 of all cell cycle scores (Supplementary Fig. S3) after assigning cell cycle scores via the Cell
442 Cycle Scoring function. The murine cell cycle genes equivalent to human cell cycle genes listed
443 in Seurat were used for assigning cell cycle scores.

444

445 For comparison with a previously reported RNA sequence dataset obtained via a well-based
446 study²³, the expression matrix of unique molecule identifiers was used. Integration of the two
447 datasets was performed using the Seurat package as described above. RNA velocity analysis
448 was performed using velocity. Bam/sam files obtained from the Cell Ranger pipeline were
449 transformed to loom format on velocity.py. RNA velocity was estimated and visualized using
450 loom files by the velocity R package and pagoda2.

451

452

453 *Droplet-based scATAC-seq analysis*

454 In scRNA-seq analysis cell suspensions of thymi from 3 mice were prepared and pooled for
455 each individual scRNA-seq experiment. EpCAM⁺CD45⁻TER119⁻ fraction was collected by
456 using cell sorter (BD Aria). After washing with PBS containing 0.04% BSA, sorted cells were
457 suspended in lysis buffer containing 10mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂,
458 0.1% Tween-20, 0.1% NP-40, 0.01% Digitonin, and 1% BSA on ice for 3 min. Wash buffer
459 containing 10mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 0.1% Tween-20, and 1%
460 BSA was added to the lysed cells. After centrifuging the solution, a nuclear pellet was obtained
461 by removing the supernatant and the pellet was re-suspended in wash buffer. The concentration
462 of nuclei and their viability were determined by staining with acridine orange/propidium iodide,
463 and 10,000 nuclear suspensions were loaded onto a Chromium instrument (10× Genomics) to
464 generate a single-cell emulsion. scATAC-seq libraries were prepared using Chromium Next
465 GEM Single Cell ATAC Reagent Kits v1.1 and sequenced in multiplex on an Illumina HiSeq X
466 ten platform. Reads were demultiplexed and mapped to the mm10 reference genome with Cell
467 Ranger ATAC. Processing data with the Cell Ranger pipeline was performed using the NIG
468 supercomputer at ROIS National Institute of Genetics. Downstream single-cell analyses
469 (integration of two datasets, correction of dataset-specific batch effects, UMAP dimension
470 reduction, cell cluster identification, conserved marker identification, and regressing out cell
471 cycle genes) were performed using Seurat (v3.0)⁵⁴. Briefly, cells that contained a percentage of
472 mitochondrial transcripts > 15% were filtered out. Genes that were expressed in more than 5
473 cells and cells expressing at least 200 genes were selected for analysis. Two scRNA-seq datasets
474 were integrated with a combination of Find Integration Anchors and Integrate Data functions⁵⁵.
475 To investigate the effects of regressing out cell cycle genes on cell clustering, we compared
476 three types of pre-processing; no regressing out, regressing out the difference between the G2/M
477 and S phase scores, and complete regressing out of all cell cycle scores after assigning cell cycle
478 scores via the Cell Cycle Scoring function. The murine cell cycle genes equivalent to human
479 cell cycle genes listed in Seurat were used for assigning cell cycle scores.

480

481 *Well-based scRNA-seq analysis*

482 Single-cells were sorted into PCR tubes containing 1µl of cell lysis solution (1:10 Cell Lysis

483 buffer(Roche), 10U/ μ L Rnasin plus Ribonuclease inhibitor (Promega) using a cell sorter, shaken
484 at 1400 rpm for 1 min with a thermo mixer, and then stored at -80°C . Cell lysates were
485 denatured at 70°C for 90 s and held at 4°C until the next step. To eliminate genomic DNA
486 contamination, 1 μ L of genomic DNA digestion mix (0.5 \times PrimeScript Buffer, 0.2 U of DNase
487 I Amplification Grade, in RNase-free water) was added to 1 μ L of the denatured sample. The
488 mixtures were mixed by gentle tapping, incubated in a T100 thermal cycler at 30°C for 5 min
489 and held at 4°C until the next step. One microliter of RT-RamDA mix (2.5 \times PrimeScript Buffer,
490 0.6 pmol oligo(dT)18, 8 pmol 1st-NSRs, 100 ng of T4 gene 32 protein, and 3 \times PrimeScript
491 enzyme mix in RNase-free water) was added to 2 μ L of the digested lysates. The mixtures were
492 mixed with gentle tapping, and incubated at 25°C for 10 min, 30°C for 10 min, 37°C for
493 30min, 50°C for 5 min, and 94°C for 5 min. Then, the mixtures were held at 4°C until the next
494 step. After RT, the samples were added to 2 μ L of second-strand synthesis mix containing 2.25 \times
495 NEB buffer 2 (NEB), 0.625mM each dNTP Mixture (NEB), 40 pmol 2nd-NSRs, and 0.75 U of
496 Klenow Fragment (NEB) in RNase-free water. Mixtures were again mixed by gentle tapping,
497 and incubated at 16°C for 60 min, 70°C 10 min and then at 4°C until the next step. The
498 above-described double-stranded cDNA was purified using 15 μ L of AMPure XP SPRI beads
499 (Beckman Coulter) diluted 2-fold with Pooling buffer (20% PEG8000, 2.5 M NaCl, 10 mM
500 Tris-HCl pH8.0, 1 mM EDTA, 0.01% NP40) and Magna Stand (Nippon Genetics). Washed
501 AMPure XP beads attached to double-stranded cDNAs were directly eluted using 3.75 μ L of 1 \times
502 Tagment DNA Buffer (10 mM Tris-HCl pH8.5, 5 mM MgCl_2 , 10% DMF) and mixed well
503 using a vortex mixer and pipetting. Diluted Tn5-linker complex was added to the eluate and the
504 mixture was incubated at 55°C for 10 min, then 1.25 μ L of 0.2% SDS was added and incubated at
505 room temperature for 5 min. After PCR for adaptor ligation, sequencing library DNA was
506 purified using 1.0 \times the volume of AMPure XP beads and eluted into 24 μ L of 10 mM Tris-Cl,
507 pH 8.5.

508

509 *Standard RNA sequencing analysis*

510 Total RNA was prepared using TRIzol reagent (Thermo Fisher Scientific) in accordance with

511 the manufacturer's protocol. After rRNA was depleted using the NEBNext rRNA Depletion Kit,
512 the RNA sequence library was prepared using the NEBNext Ultra Directional RNA Library
513 Prep Kit (New England Biolabs). Paired-end sequencing was performed with NextSeq500
514 (Illumina). Sequence reads were quantified for annotated genes using CLC Genomics
515 Workbench (Version 7.5.1; Qiagen). Gene expression values were cut off at a normalization
516 expression threshold value of 3. Differential expression was assessed via empirical analysis with
517 the DGE (edgeR test) tool in CLC Main Workbench, in which the Exact Test of Robinson and
518 Smyth was used⁵⁶. An FDR-corrected *p* value was used for testing statistics for
519 RNA-sequencing analysis. Previously described lists of TSAs and Aire-dependent TSAs²¹ were
520 used for the analysis.

521

522 *RTOC and RNA-seq analysis*

523 mCherry¹⁰ cells ($4 \times 10^4 \sim 1 \times 10^5$) were sorted from Fucci mice and subsequently re-aggregated
524 with trypsin-digested thymic cells ($1 \sim 2 \times 10^6$) from E15.5 wild-type mice. RTOCs were
525 cultured on Nucleopore filters (Whatman) placed in R10 medium containing RPMI1640 (Wako)
526 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Wako), 1× nonessential
527 amino acids (NEAAs; Sigma-Aldrich), 0.1 pM cholera Toxin Solution (Wako 030-20621), 5
528 µg/ml Insulin solution from bovine pancreas (SIGMA I0516-5ML), 2 nM Triiodo-L-thyronine
529 (SIGMA T2877-100MG), 1000 units/ml LIF (nacalai NU0012-1), 0.4 µg/ml hydrocortisone, 10
530 ng/ml EGF (Gibco PMG8041), 1 µg/ml RANKL (Wako), penicillin-streptomycin mixed solution
531 (Nacalai Tesque), and 50 µM 2-mercaptoethanol (Nacalai Tesque) for 5 days. For RNA-seq of
532 RTOC experiments, random displacement amplification sequencing (RamDA-seq) were used⁴⁹,
533 which allows RNA-seq analysis of low numbers of cells. Briefly, sorted cells were lysed in TCL
534 buffer (Qiagen). After purification of nucleic acids by Agencourt RNA Clean XP (Beckman
535 Coulter) and subsequent treatment with DNase I, the RT-RamDA mixture containing 2.5x
536 PrimeScript Buffer (TAKARA), 0.6 µM oligo(dT)18 (Thermo), 10 µM 1st NSR primer mix, 100
537 µg/mL of T4 gene 32 protein, and 3× PrimeScript enzyme mix (TAKARA) were added to the
538 purified nucleic acids for reverse transcription. Samples were added to second-strand synthesis
539 mix containing 2× NEB buffer 2 (NEB), 625 nM dNTP Mixture (NEB), 25 µM 2nd NSR
540 primers, and 375 U/mL of Klenow Fragment (3'-5' exo-) (NEB). After cDNA synthesis and
541 subsequent purification by AMPure XP (Beckman Coulter), sequencing library DNA was
542 prepared using the Tn5 tagmentation-based method. Single-read sequencing was performed
543 using a HiSeq2500 (v4, high out mode). Sequence reads were quantified for annotated genes

544 using CLC Genomics Workbench (Version 7.5.1; Qiagen).

545

546 *Immunohistochemistry*

547 The thymus was fixed with 4% paraformaldehyde and frozen in OCT compound. After washing
548 cryosections (5 μ m) with PBS, sections were blocked with 10% normal goat serum. Keratin-5
549 was detected using a combination of a polyclonal rabbit anti-mouse keratin-5 antibody (1:500)
550 and AlexaFluor647-donkey-anti-rabbit IgG. Aire was detected using a labeled monoclonal
551 antibody (1:300). Confocal color images were obtained using a LAS X (Leica) microscope.

552

553 *Immunocytochemistry*

554 Thymic cell suspensions prepared via Liberase™ digestion were stained with anti-CD45-PE and
555 anti-TER119-PE. After depletion of labeled CD45⁺ and TER119⁺ cells via anti-PE microbeads
556 and a magnetic-activated cell sorting separator, negatively selected cells were stained with
557 anti-EpCAM (CD326), anti-CD80, anti-Ly51, and UEA-1. Venus⁺ CD80^{hi} mTECs were sorted
558 and spun down on glass slides using a Cytospin. The slides were then fixed with acetone and
559 stained with anti-Aire antibody and DAPI for nuclear staining. Confocal images were obtained
560 using an LAS X microscope.

561

562 *Statistical analysis*

563

564 Statistically significant differences between mean values were determined using Student's t-test
565 (**P < 0.001, **P < 0.01 and * P < 0.05). Principle component analysis was performed using
566 the prcomp function in R-project. The sample size was not predetermined by statistical methods
567 but based on common practice and previous studies^{6,57}. All replicates are biological replication.
568 All outliers were included in data.

569

570 **Data availability**

571 FASTQ data of RNA-Seq and ATAC-seq are deposited in DDBJ (DRA009125 DRA010209
572 DRA12308, DRA12309 and DRA012452). Any additional information required to reanalyze
573 the data reported in this paper was up-loaded as a zip file of Source_data.

574

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585

586 **Author contributions**

587 T.M, MM, TI, MY, HY, AI, and EO performed experiments and analyzed data. TK, TWT, SH,
588 KH, YT, and TS analyzed data. HI and NY established mutant mouse lines. ASS, AM, and AK
589 contributed to data analysis and interpretation. NA and TA designed the study, analyzed data,
590 and wrote the manuscript.

591

592 **Competing interests**

593 The authors declare no competing interests.

594

595

596 **Supplementary Table 1.** Integration of scRNA-seq cluster and scATAC-seq cluster

597 **Supplementary Table 2.** Open regions in cluster 3 as compared to cluster 0 in scATAC-seq.

598 **Supplementary Table 3.** GO analysis of genes differentially expressed in Venus+ cells

599 **Supplementary Table 4.** Possible marker gene candidates for transit amplifying TECs.

600 **Supplementary Table 5.** Summary for assignment of individual single cells in scRamDa-seq of
601 mCherry^{hi}, mCherry^{lo}, and mCherry^{hi}-RTOC

602

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795 **Figure Legends**

796 **Figure 1. Droplet-based scATAC-seq analysis of TECs in 4-week-old mice**

797 **A.** UMAP plot of scATAC-seq data from TEC cells (EpCAM⁺ CD45⁻ TER119⁻) from
798 4-week-old mice. Cell clusters are separated by colors and numbers in the plot. The graph on
799 the right shows percentages of each cluster in the total number of cells detected (8413 cells).
800 **B.** Chromatin accessibility of typical marker genes of TECs. Accessibility in each gene region is
801 represented in red.
802 **C.** Violin plot depicting chromatin accessibility in *Aire* and *Cd80* gene regions in each cluster.
803 **D.** Pseudo-bulk accessibility tracks of the *Aire* gene region in each cluster (upper panels)
804 and frequency of sequenced fragments within the *Aire* gene region of individual cells in cluster
805 0, 1 and 2 (lower panels)

806

807 **Figure 2. Droplet-based scRNA-seq analysis of TECs in 4-week-old mice**

808 **A.** UMAP plot of scRNA-seq data from TEC cells (EpCAM⁺ CD45⁻ TER119⁻) from
809 4-week-old mice. Cell clusters (R0 to R17) are indicated by colors and numbers in the plot. The
810 graph on the right shows the percentages of each cluster in the total number of cells detected
811 (11,792 cells).
812 **B.** Violin plots depicting expression level of typical TEC marker genes in each cluster.

813

814 **Figure 3. Integrative analysis of scATAC-seq data and scRNA-seq data of TECs**

815 **A.** Gene expression was predicted from scATAC-seq data using Signac. Individual cells in the
816 cluster from scATAC-data (cluster 0 to were assigned and transferred to the UMAP plot of
817 scRNA-seq cluster (R0 to R17).
818 **B.** Correlation between clusters derived from scATAC-seq and scRNA-seq datasets of TECs.
819 Cell types were annotated in scATAC-data set of TECs by transferring clusters from an
820 scRNA-seq dataset.
821 **C.** Ratio of cells assigned to each scRNA-seq cluster in each scATAC cluster.

822

823 **Figure 4. A highly proliferative subset of Aire⁺ CD80^{hi} mTECs**

824 **A.** Schematic depiction of cell cycles and Fucci fluorescence.
825 **B.** Flow cytometric analysis of TECs from Fucci2a mice crossed with Aire-GFP-reporter mice.
826 The gating strategy is shown. Intensities of GFP to monitor Aire expression in each subset
827 (Venus⁺ CD80^{hi} mTEC, Venus⁻ CD80^{hi} mTEC and CD80^{lo} mTEC^{lo}) are shown in the right

828 panels. Left, *Aire*^{gfp/+}::Fucci2a; right, control::Fucci2a. Typical figures of 3-independent
829 experiments are exhibited.

830 **C.** Immunostaining of a sorted Venus⁺ CD80^{hi} mTEC subset via anti-Aire antibody and DAPI
831 (nucleus staining). Typical panels of 3-independent experiments are exhibited. Scale bars, 10
832 μm

833 **D.** Immunostaining of thymic sections from Fucci2a mice with anti-Aire and anti-keratin-5
834 (Krt5) antibodies. Typical panels of 3-independent experiments are exhibited. Scale bars, 10 μm

835 **E** Scatter plots of RNA sequencing data from Venus⁺ CD80^{hi} mTEC and Venus⁻ CD80^{hi} mTEC
836 subsets. The left panel shows a plot of all detected genes and the right panel show TSA genes
837 detected. N = 3.

838 **F.** A typical RNA sequencing tracks of *Aire*, typical Aire-dependent TSA genes (*Ins* and *Sst*),
839 *Fezf2*, and *Top2a* (a marker of G2/M phase).

840 **G.** Scatter plots and volcano plots of RNA sequencing data from Venus⁺ CD80^{hi} mTEC and
841 Venus⁻ CD80^{hi} mTEC subsets. Upper panels show Aire-dependent TSAs, lower panels show
842 Aire-independent TSAs. Red dots in volcano plots indicate genes for which expression differed
843 significantly (2-fold change and FDR $P < 0.05$) in Venus⁺ and Venus⁻ CD80^{hi} mTEC subsets.
844 Numbers of differentially expressed genes are shown in the panels. N = 3. Y axis is log₁₀ of
845 FDR P-value.

846

847 **Figure 5. Fate mapping study by *in vivo* BrdU pulse-labeling of Fucci TECs**

848 **A.** Schematic procedure of *in vivo* BrdU pulse-labeling of Fucci mouse, and analysis of BrdU
849 staining in mCherry^{hi}CD80^{hi} and mCherry^{lo}CD80^{hi} mTECs by flow cytometric analysis. BrdU
850 staining was done after sorting each cell fraction.

851 **B.** Typical flow cytometric profile of BrdU staining in mCherry^{lo}CD80^{hi} mTECs (upper panels)
852 and mCherry^{hi}CD80^{hi} mTECs (lower panels) at Days 0.5, 3.5, and 6.5 after the BrdU injection.
853 Data for the ratio of BrdU⁺ cells in each mTEC fraction are summarized in right figures. N = 7
854 for 0.5 day after the BrdU injection, N = 3 for 3.5 day and 6.5 day after the injection. Two-tailed
855 Student's t-tests. ** $P < 0.01$ and * $P < 0.05$. NS, not significant ($P > 0.05$). $P = 1.5 \times 10^{-3}$ for the
856 upper figure and $P = 0.033$ for the lower figure.

857 **C.** Cell number of BrdU⁺mCherry^{lo}CD80^{hi} mTECs and BrdU⁺mCherry^{hi}CD80^{hi} mTECs at Days
858 0.5, 3.5, and 6.5 after the BrdU injection. Two-tailed Student's t-tests. ** $P < 0.01$. NS, not
859 significant ($P > 0.05$). $P = 4.3 \times 10^{-3}$ for the left figure and $P = 5.1 \times 10^{-3}$ for the right figure.

860 **D.** MFI of BrdU staining in mCherry^{lo}CD80^{hi} at Day 0.5 and mCherry^{hi}CD80^{hi} at Day 3.5 and

861 6.5. MFIs of other time points were difficult to evaluate because of very low cell numbers.
862 Two-tailed Student's t-tests. * P = 0.015 and ** P = 6.5×10^{-3} . NS, not significant (P > 0.05).
863
864 **Figure 6. Fate mapping study of proliferating Aire⁺ mTECs in *in vitro* reaggregated thymic**
865 **organ culture (RTOC)**
866 **A.** RTOC experiment to test the differentiation capacity of proliferating Aire⁺ mTECs.
867 Proliferating Aire⁺ mTECs (mCherry^{lo}) and E15.5 embryonic thymic cells were re-aggregated
868 and subsequently cultured for 5 days. Reaggregated thymic organ (RTO) was analyzed by flow
869 cytometer. Representative flow cytometric profiles of RTOC are shown. N = 5. The ratio of
870 mCherry^{hi} cells in TECs is summarized in right figure. *P < 0.05. P = 0.027 between CD80^{hi} and
871 CD80^{lo} in mCherry^{lo} and P = 0.024 between CD80^{hi} mCherry^{lo} and CD80^{hi} RTOC control.
872 **B.** Volcano plots of RNA-seq data from mCherry^{lo} CD80^{hi} mTECs (mCherry^{lo}), mCherry^{hi}
873 CD80^{hi} mTECs (mCherry^{hi}), and mCherry^{hi} CD80^{hi} mTECs in RTOC (mCherry^{hi} in RTOC).
874 Red dots in volcano plots indicate genes for which expression differed significantly between the
875 two subsets. Numbers of differentially expressed genes are shown in the panels. N = 3. Y axis is
876 log₁₀ of FDR P-value.
877 **C.** Expression levels of Aire and Mki67 in mCherry^{lo}, mCherry^{hi}, and mCherry^{hi} in RTOC.
878 **D.** Scatter plot of normalized expression values of TA-TEC marker candidates in mCherry^{lo} and
879 mCherry^{hi} in RTOC. TA-TEC marker candidate genes were selected from bulk RNA-seq data
880 and scRNA-seq data in Supplementary Figure 7.
881 **E.** Integration of well-based scRamDA-seq data (mCherry^{lo}, mCherry^{hi}, and mCherry^{hi} in
882 RTOC) with the droplet-based scRNA-seq data in Figure 2.
883 **F.** Frequency of each cell cluster in scRamDA-seq data of mCherry^{lo}, mCherry^{hi}, and mCherry^{hi}
884 -RTOC.
885 **G.** Volcano plot of TSA expression in each cell cluster in scRamDa-seq data of
886 mCherry^{hi}-RTOC as compared to mCherry^{lo}. Red dots indicate significantly changed TSA
887 genes.
888
889 **Figure 7. Proliferating Aire⁺ CD80^{hi} mTECs persist in older mice**
890 **A.** Flow cytometry analysis of CD80^{hi} mTEC subsets from Fucci2a mice aged 4, 8, and 19
891 weeks. Representative data are shown. Percentages of Venus⁺ cells in CD80^{hi} mTEC subsets are
892 summarized in the graph in the right panel. N = 4 each for Aire^{gfp/+}::Fucci2a (circle) and
893 control::Fucci2a (closed triangles)

894 **B.** Schematic depiction of the proposed process of Aire⁺ mTEC development in the adult
895 thymus. Transit-amplifying TSA^{lo} Aire⁺ TECs give rise to mature mTECs. Precursor cells to the
896 transit-amplifying TECs have not been determined yet. Cluster numbers in Figure 1 are shown
897 together with the model of mTEC subsets I to IV.
898

Figure 1

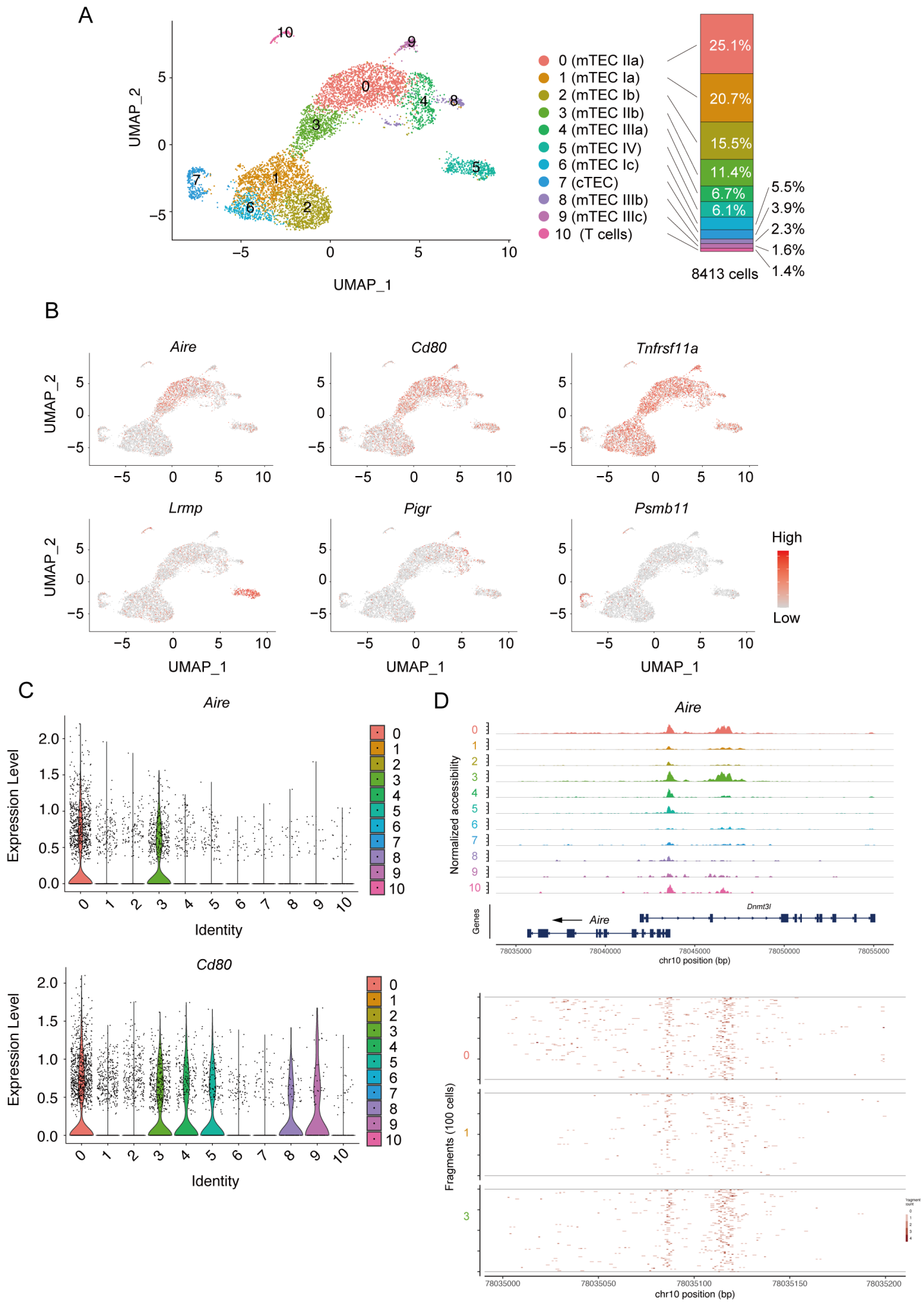
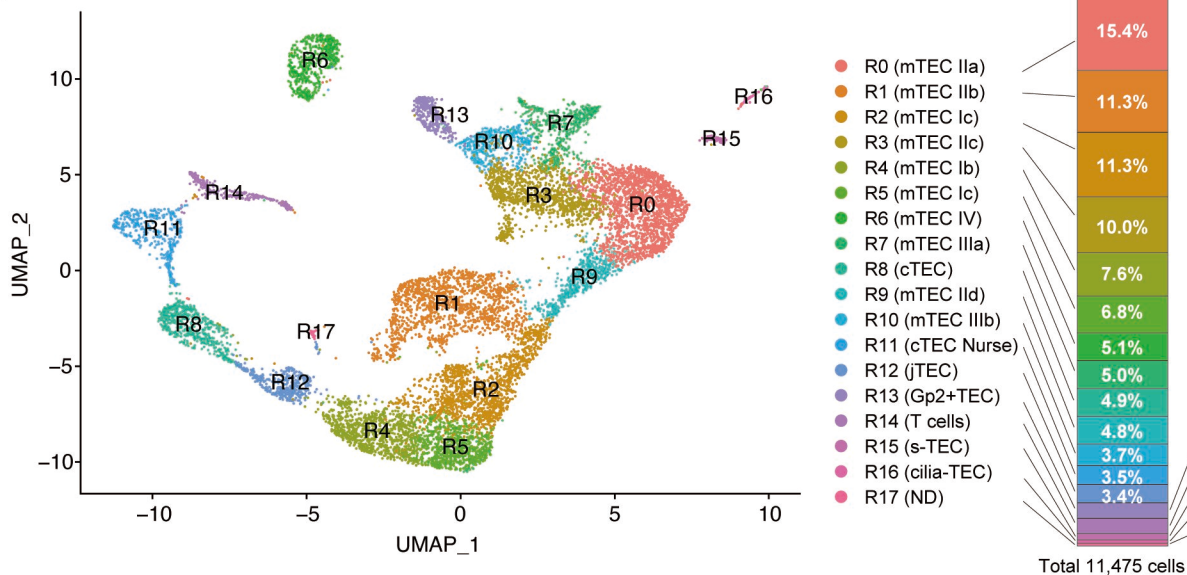


Figure 2

A



B

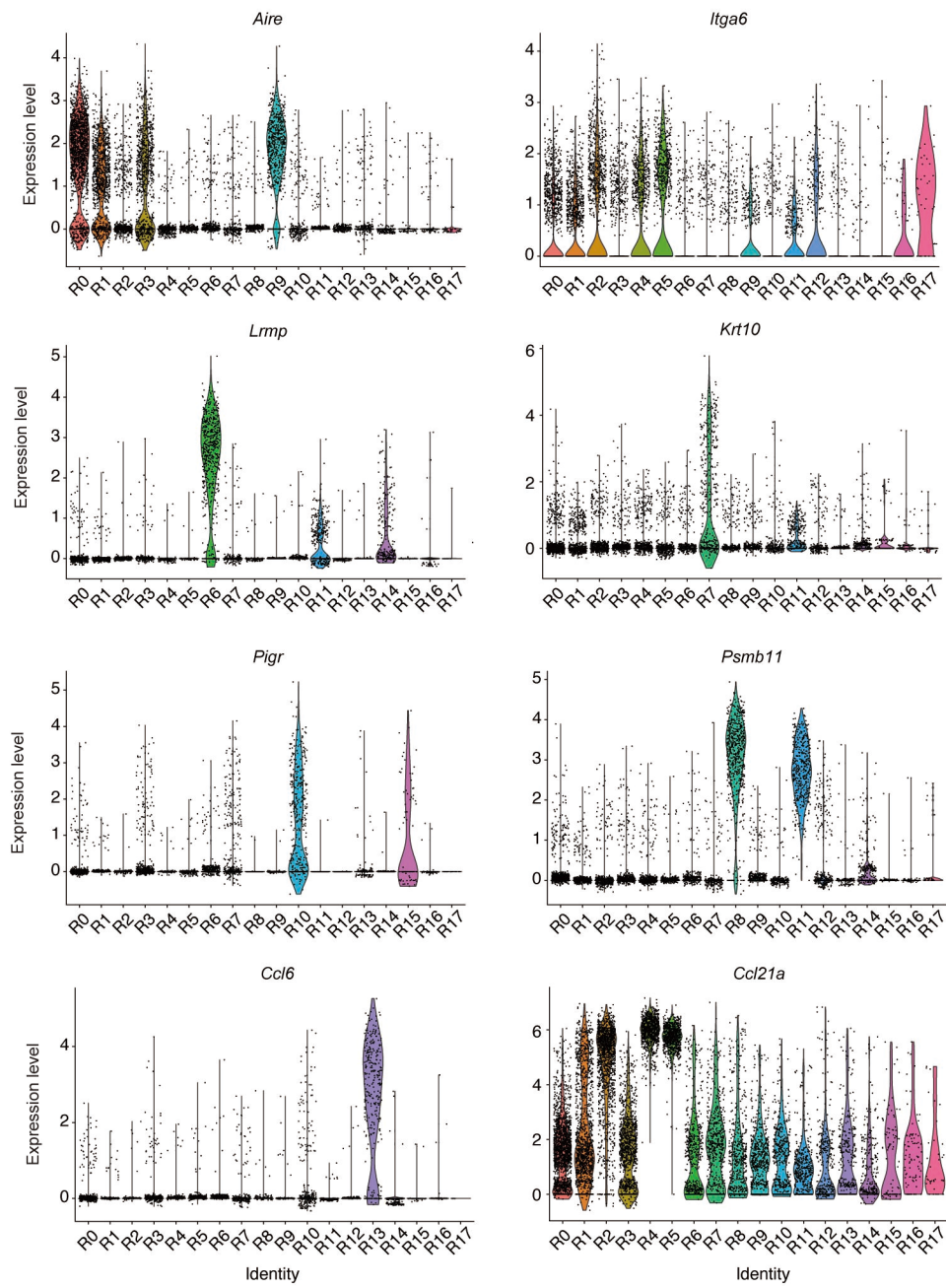


Figure 3

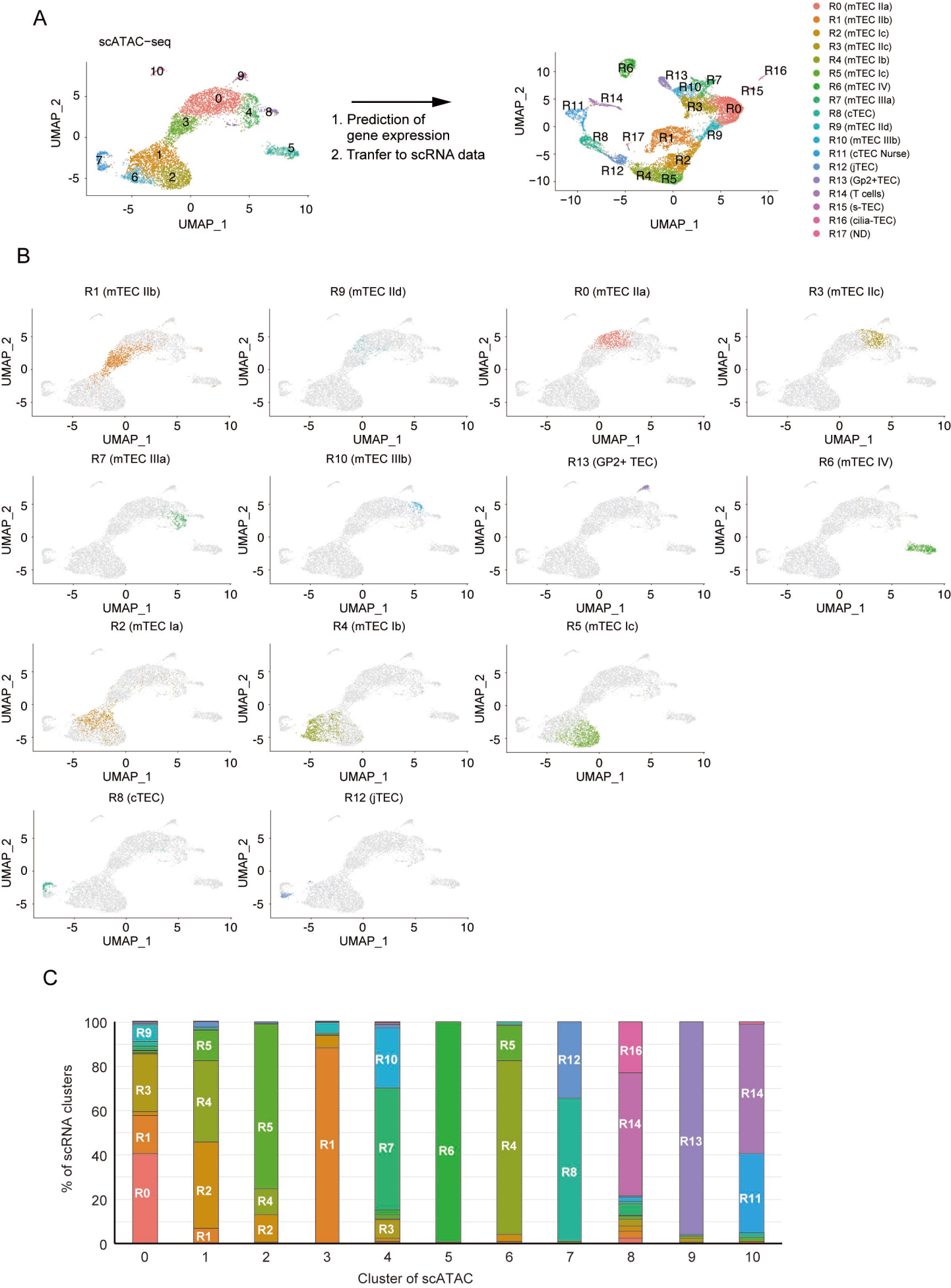


Figure 4

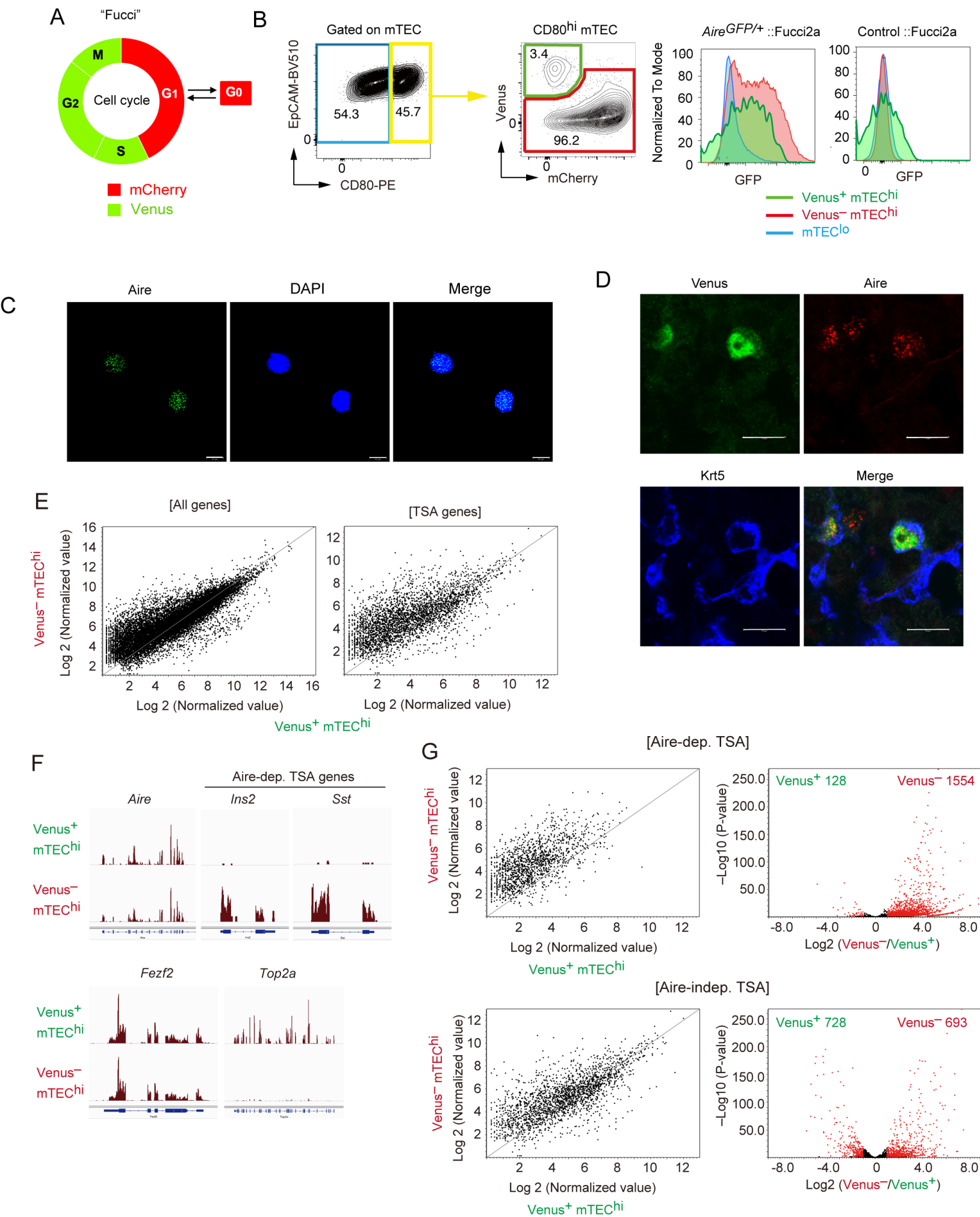
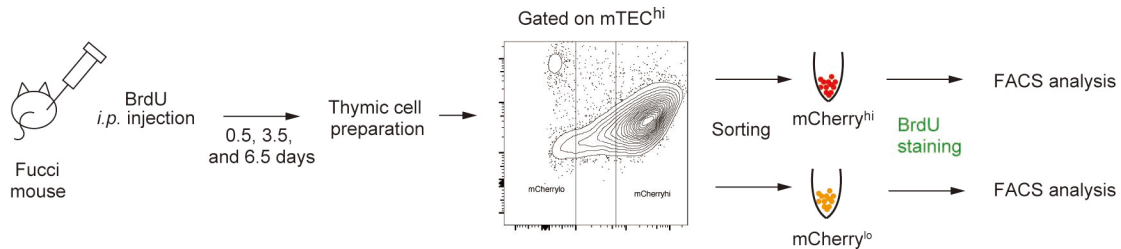
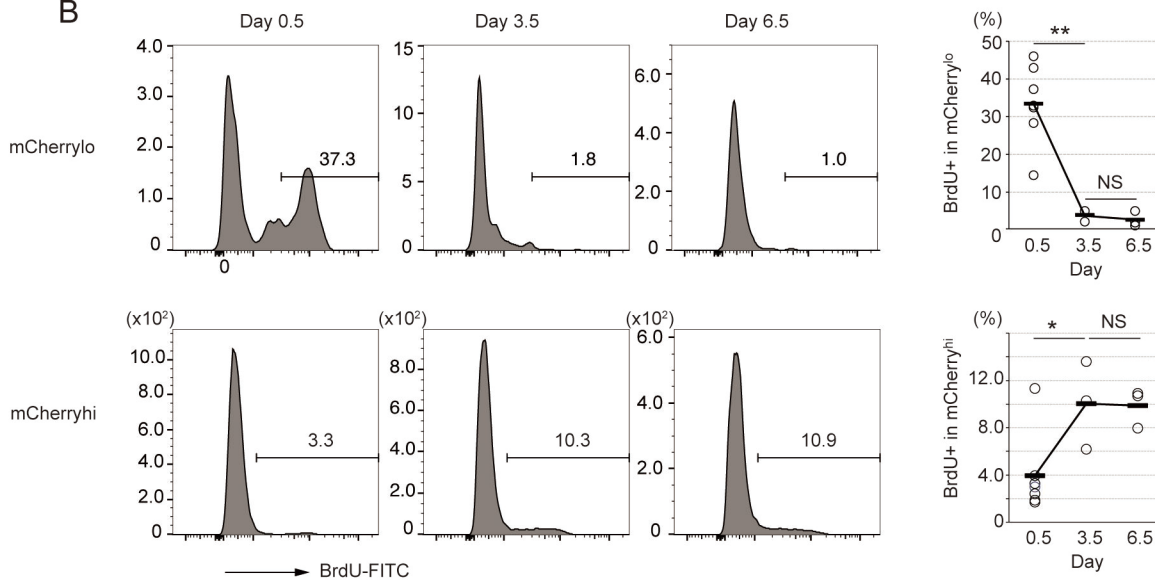


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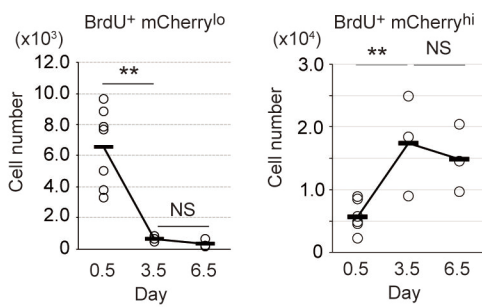
A



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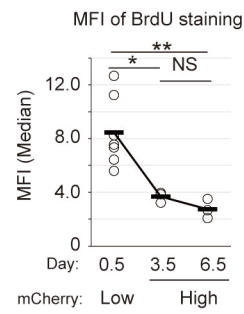


Figure 6

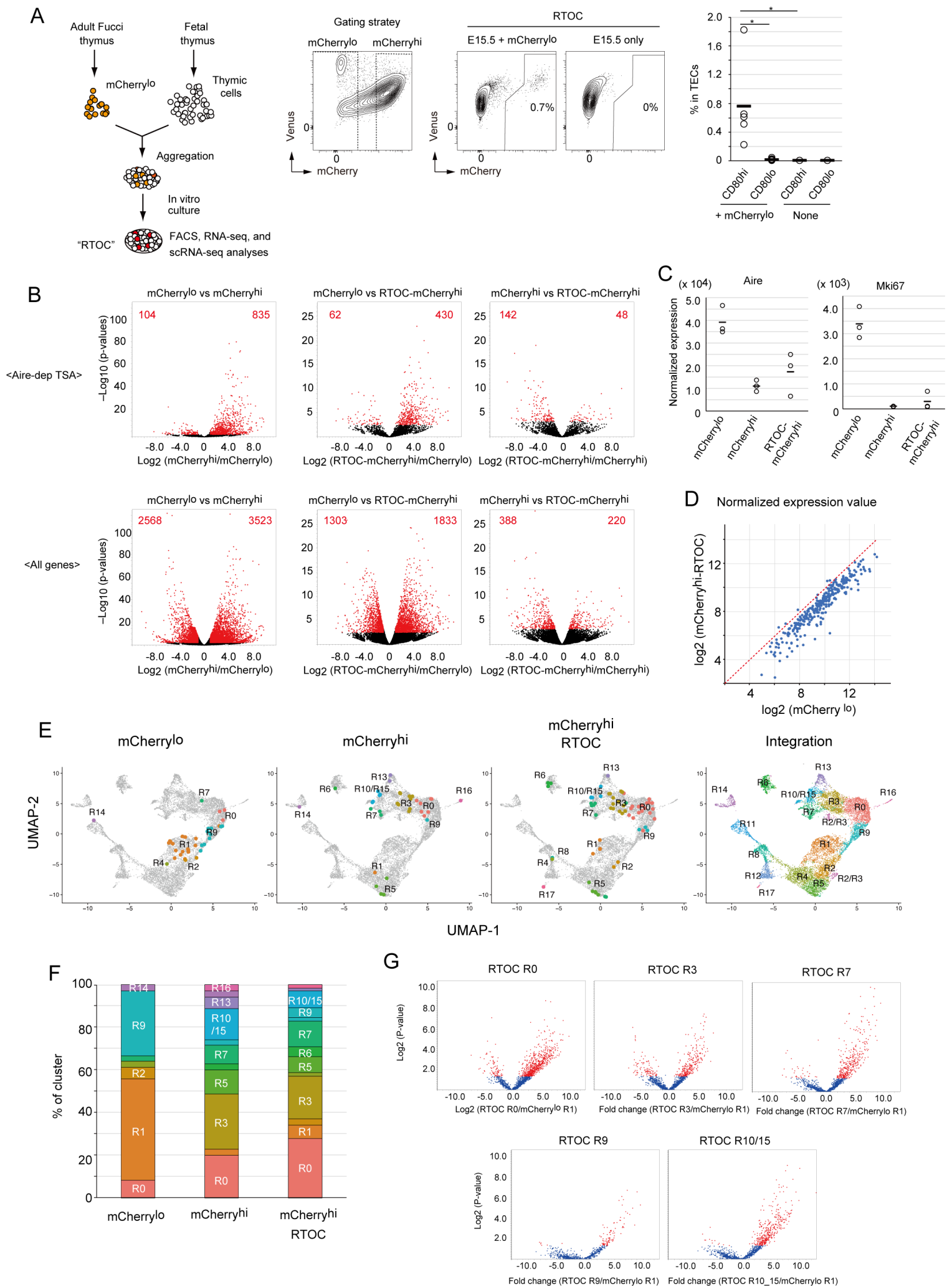


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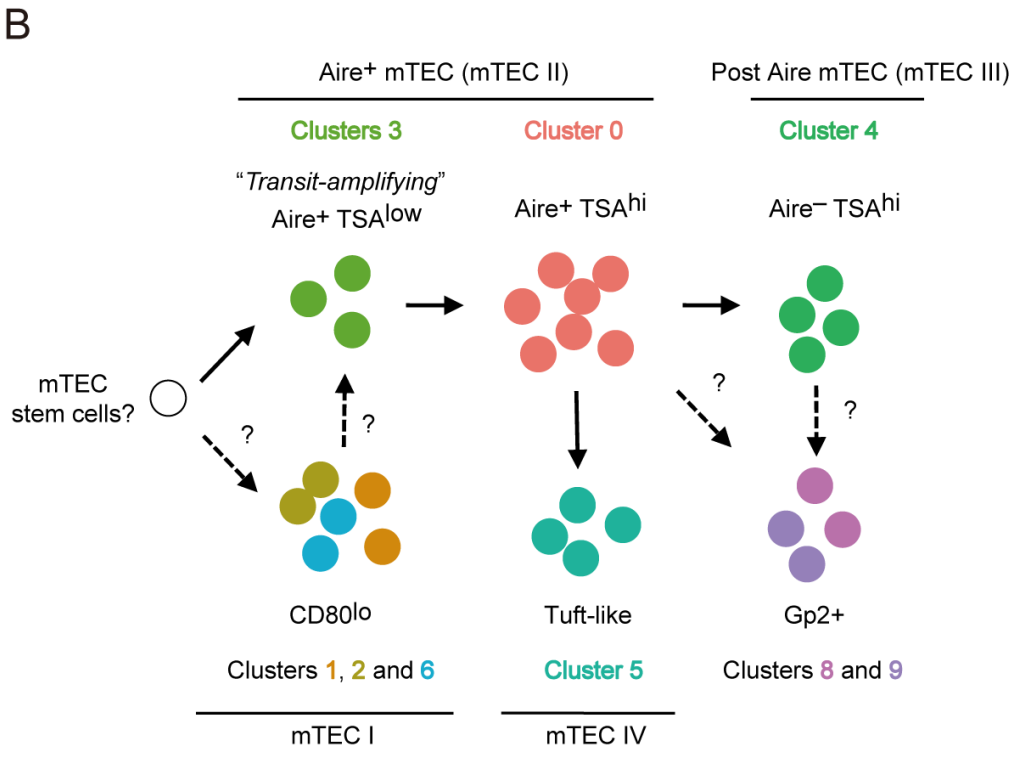
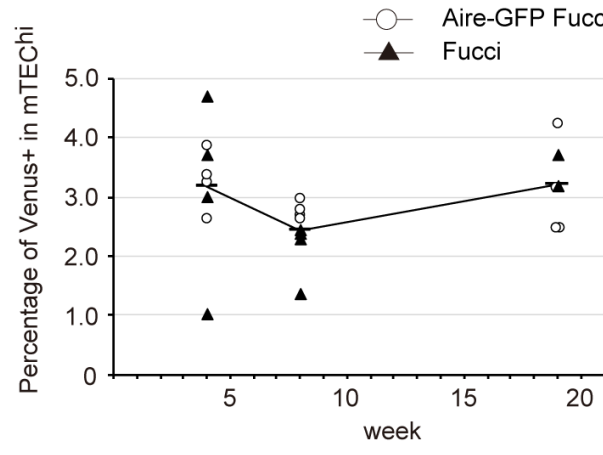
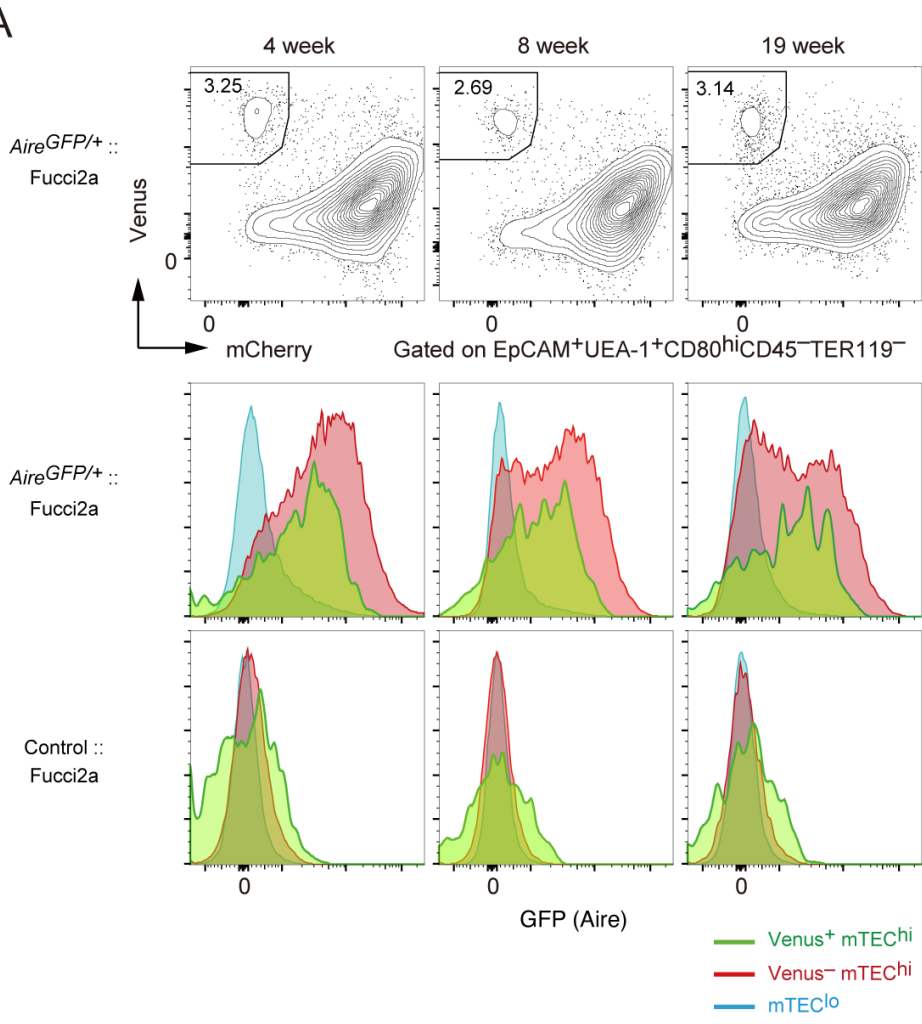
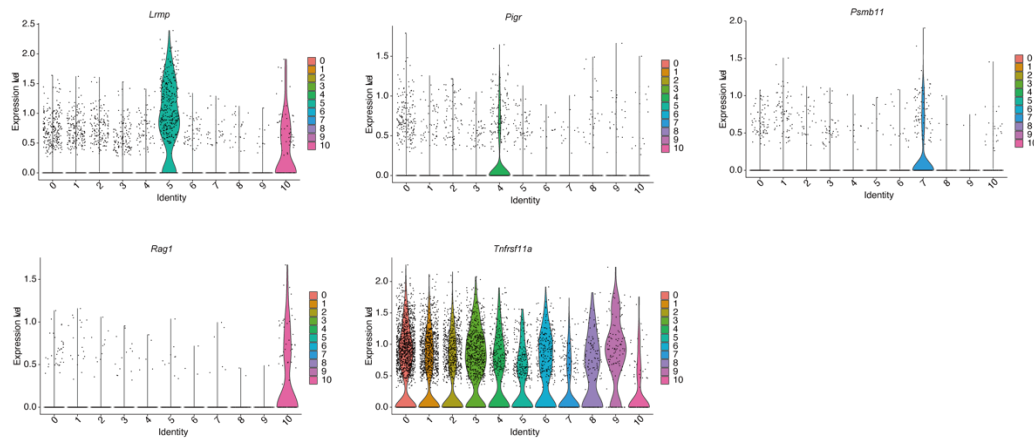


Figure 1-figure supplement 1

A



B

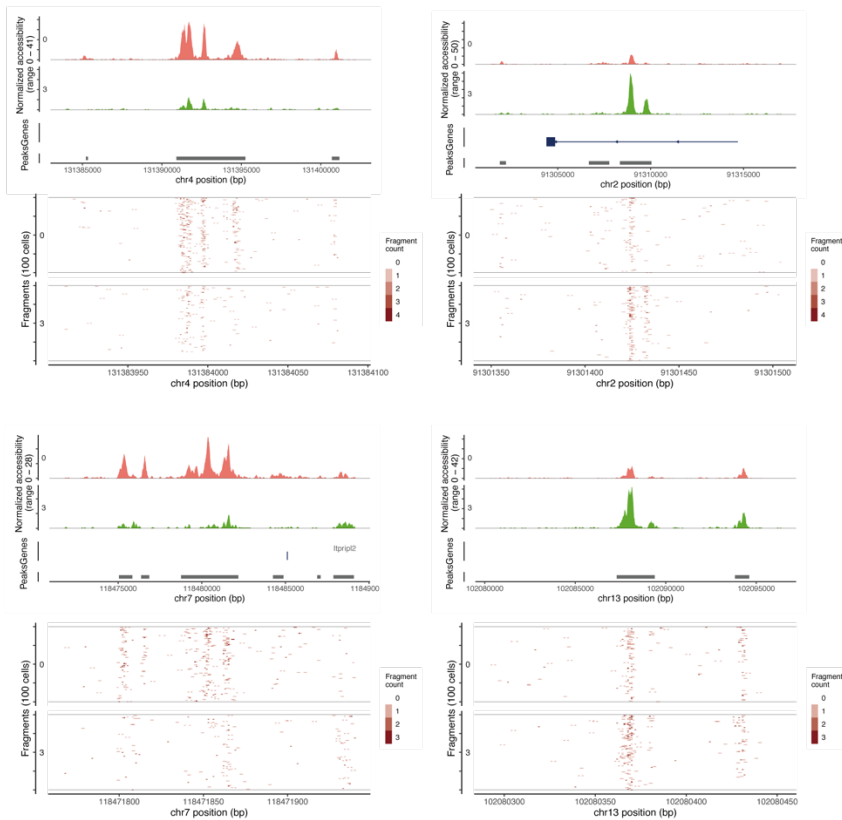


Figure 1-figure supplement 1.

A. Violin plot of chromatin accessibility in TEC marker gene regions in each cluster.

B. Pseudo-bulk accessibility tracks and frequency of sequenced fragments. Typical differentially accessible regions between clusters 0 and 3 are depicted from Supplementary Table 1.

Figure 2-figure supplement 1

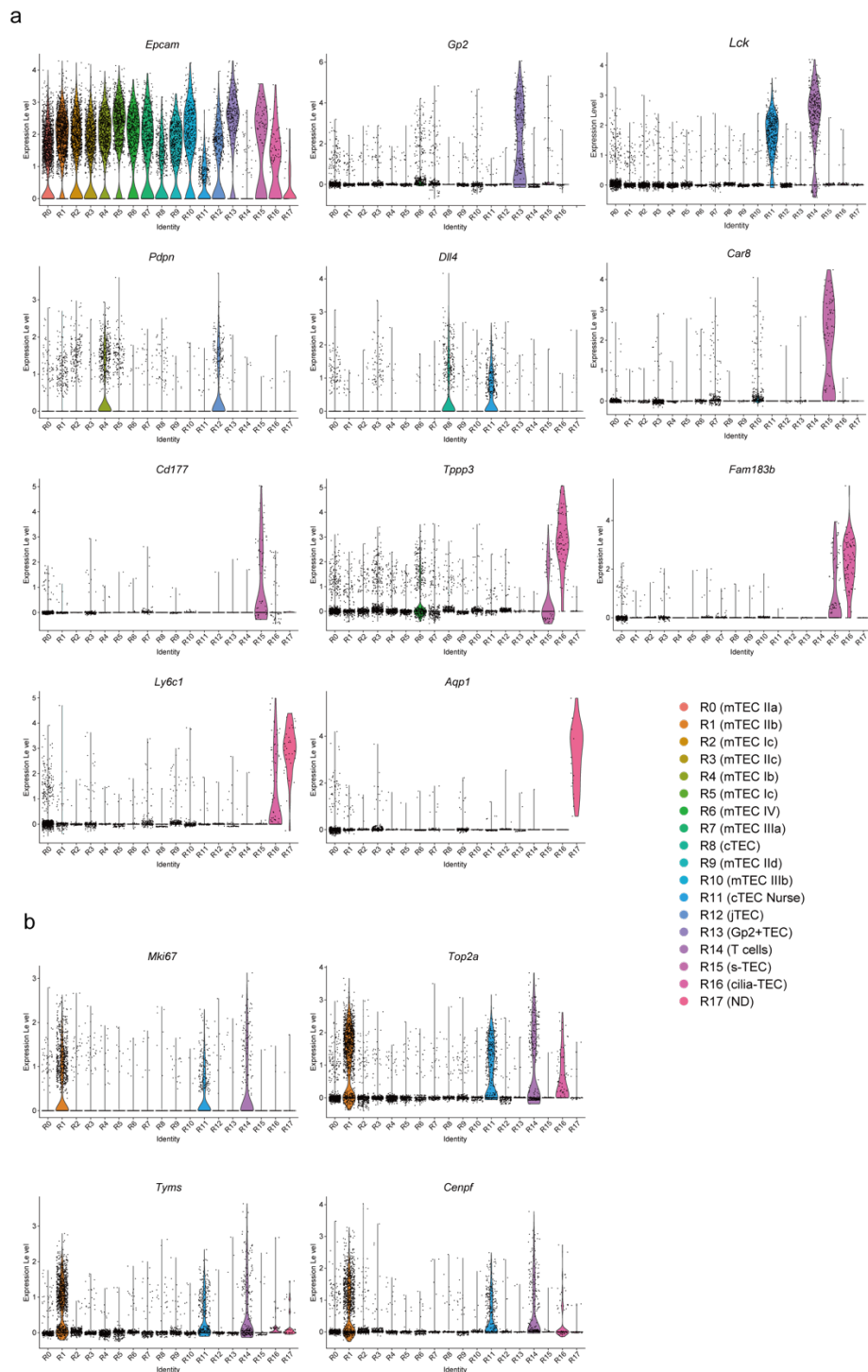


Figure 2-figure supplement 1.

A. Violin plots for expression level of typical TEC marker genes in scRNA-seq analysis of TECs

B. Violin plots for expression level of cell-cycle-related genes in scRNA-seq analysis of TECs

Figure 2-figure supplement 2

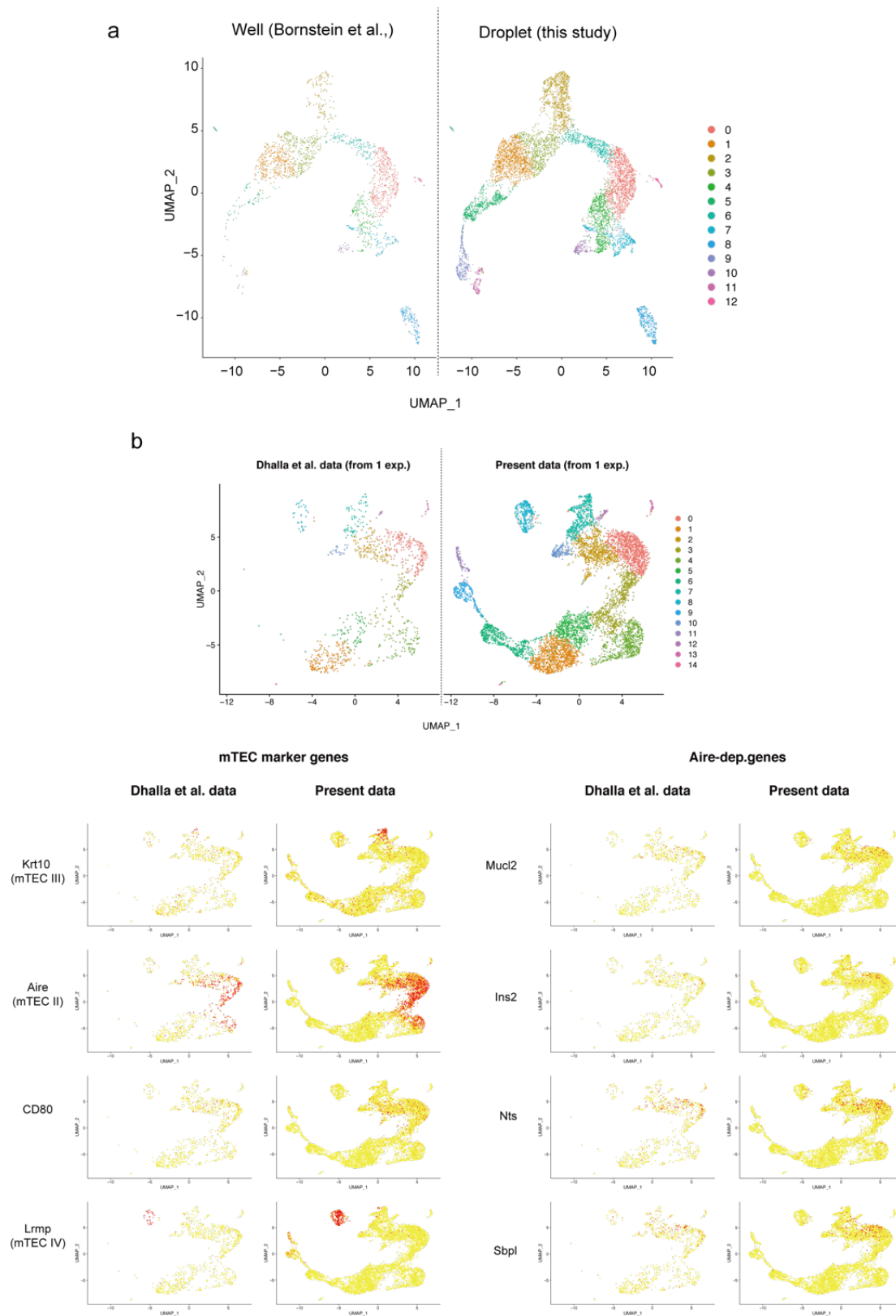


Figure 2-figure supplement 2

A. Integration of scRNA-seq data derived from a previously reported well-based study¹ and scRNA-seq data derived from the present droplet-based study. scRNA-seq data from the two studies were integrated

B. UMAP projections of the two scRNA-seq datasets are shown. Data from a previous study² were re-analyzed and integrated with data from the present study. Expression of typical marker genes in each data.

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Figure 2-figure supplement 3

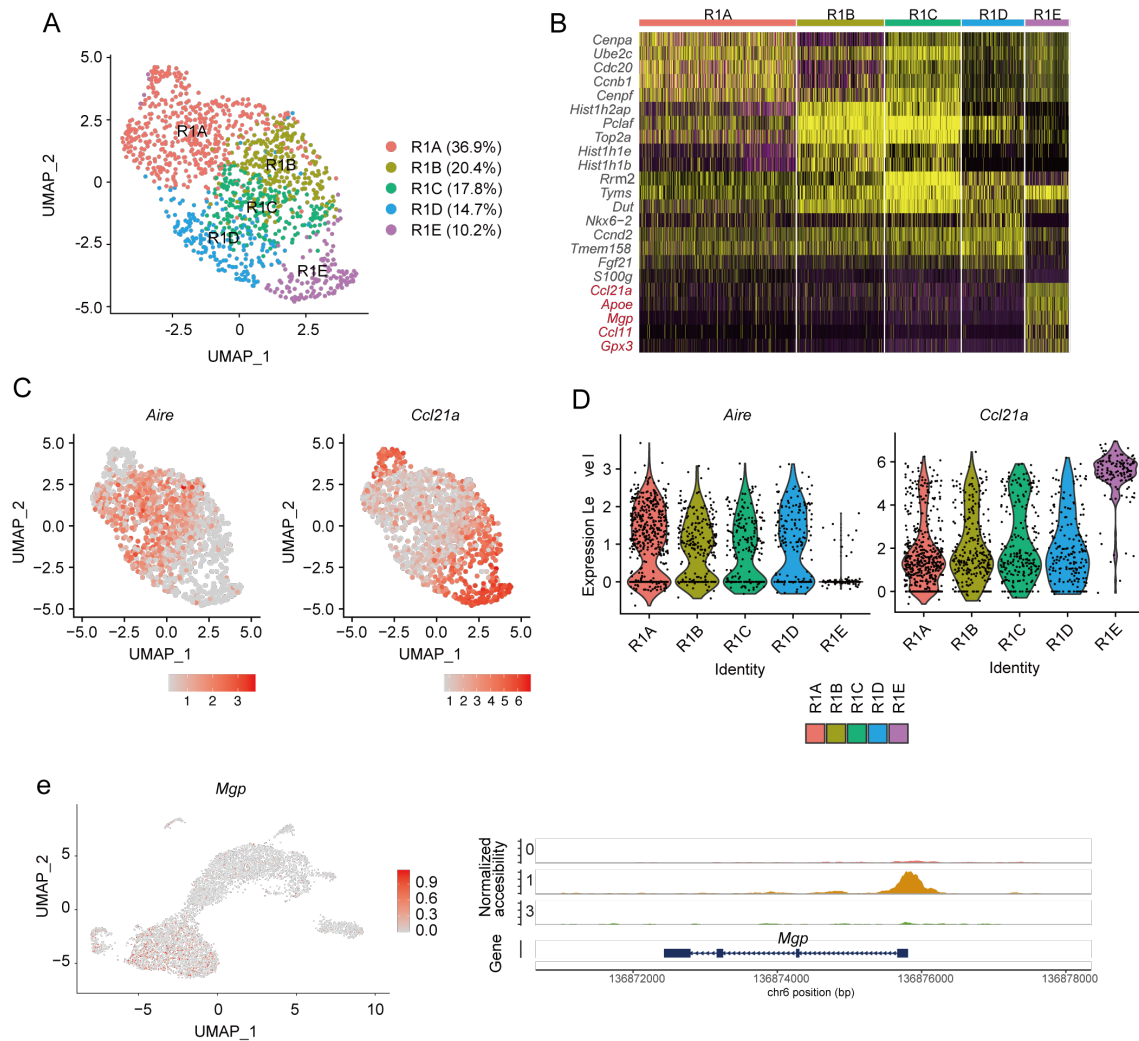


Figure 2-figure supplement 3

Sub-cluster analysis of the TEC subset expressing a high level of cell-cycle-related genes.

A. UMAP plot of scRNA-seq data and the percentage of each cluster (R1A to R1E) in R1.

B. Heatmap of the top 5 genes selectively expressed in each cluster. Yellow color indicates high expression.

C. Expression levels of *Aire* and *Ccl21a* in the sub-cluster is shown in dot plots.

D. Expression levels of *Aire* and *Ccl21a* in the sub-cluster is exhibited as violin plots.

E. Chromatin accessibility of a typical marker gene for sub-cluster R1E (*Mgp*). Accessibility in *Mgp* gene regions is represented in red (left). Pseudo-bulk accessibility tracks for *Mgp* in cluster 0, 1 and 3 is exhibited (bottom)

Figure 3-figure supplement 1

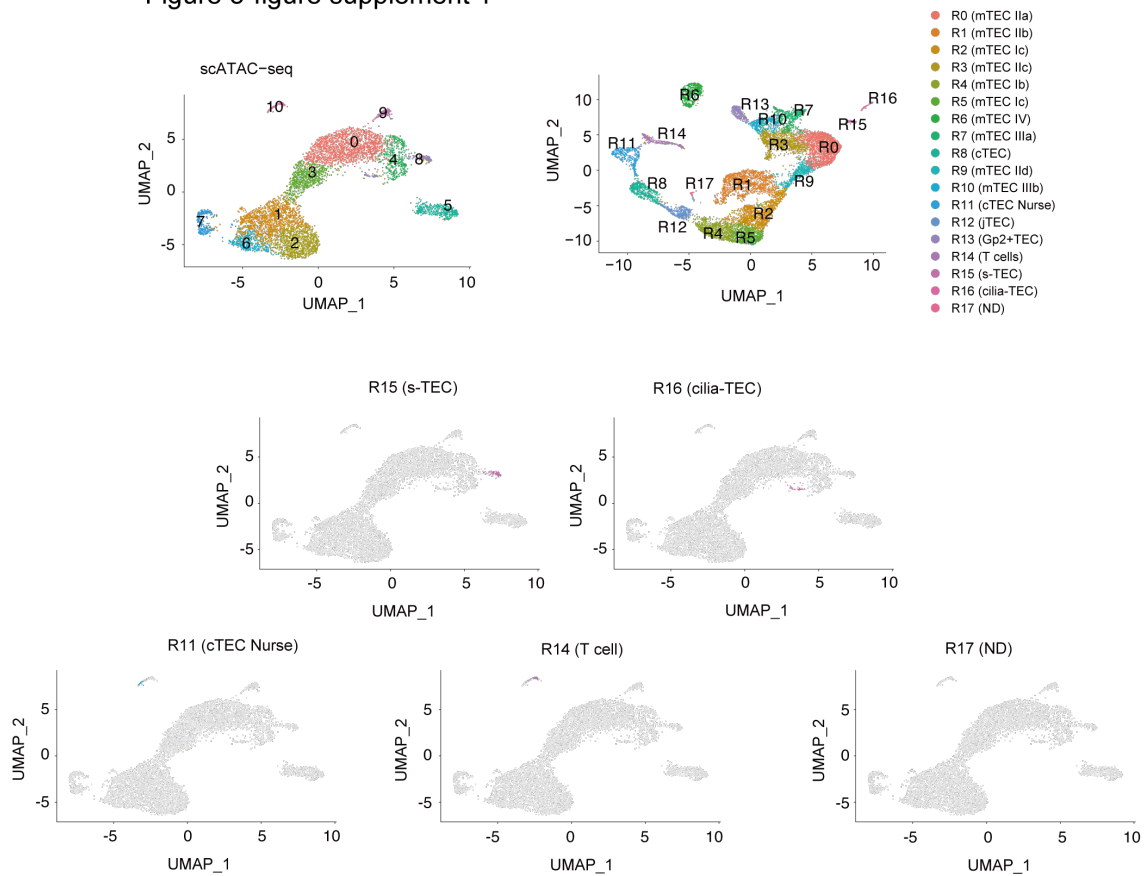
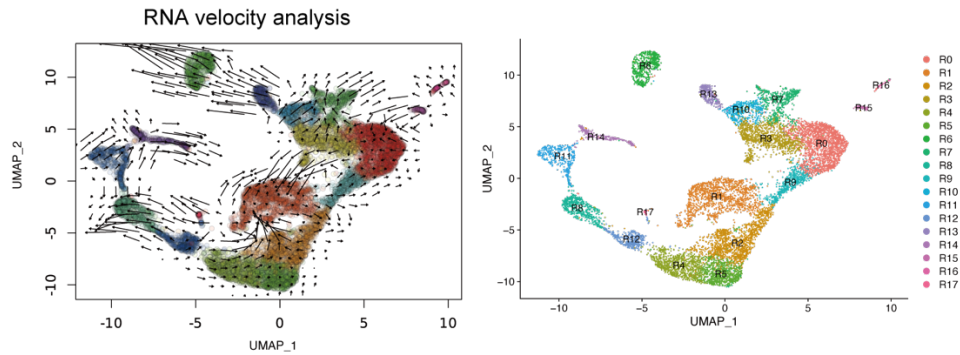


Figure 3-figure supplement 1

Integrative analysis of scATAC-seq data and scRNA-seq data of TECs. Gene expression was predicted from individual cells in scATAC-seq data (clusters 9 and 10). Individual cells in the scATAC-data were assigned to a scRNA-seq cluster (R0 to R17).

Figure 3-figure supplement 2

A



B

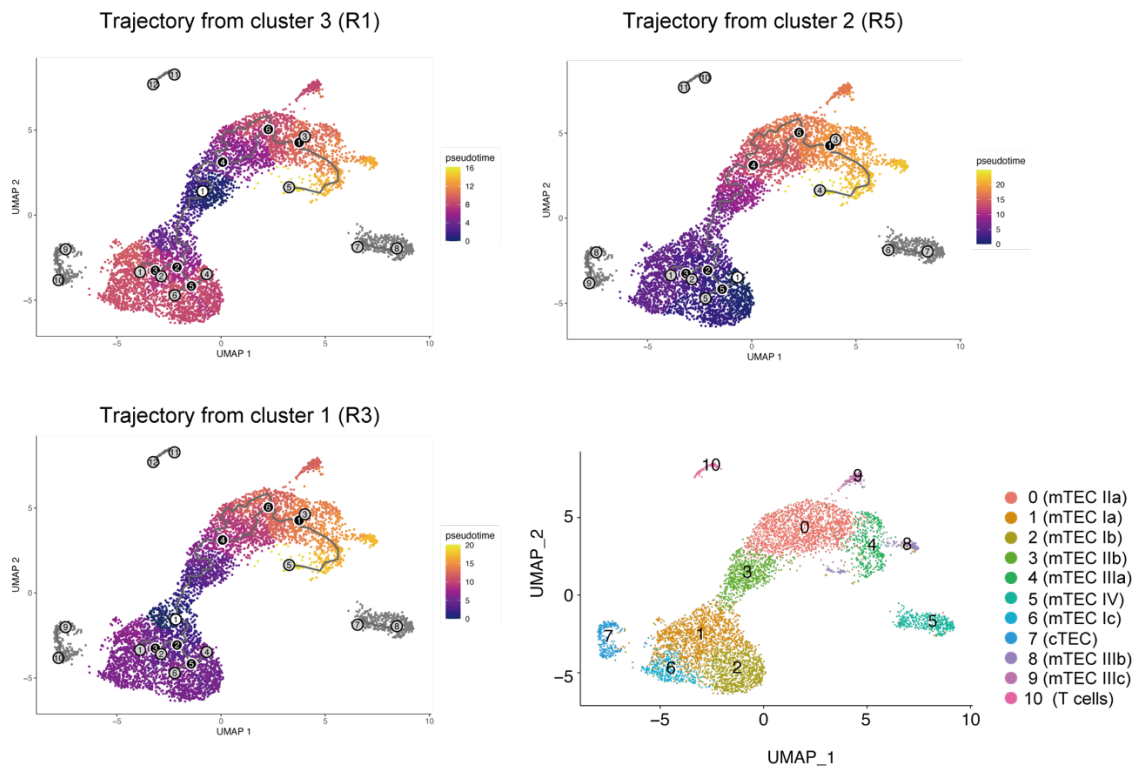


Figure 3-figure supplement 2

A. RNA velocity analysis of scRNA-seq data.

B. Monocle 3 trajectory analysis of scATAC-seq data. The trajectory was manually started from cluster 3, 2 or 1.

Figure 4-figure supplement 1

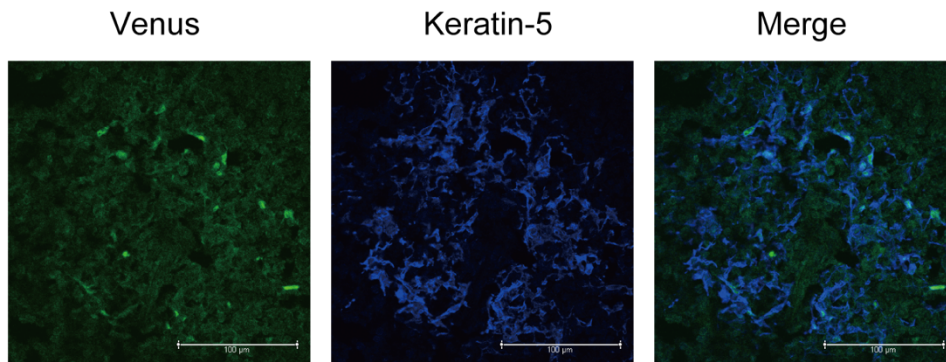


Figure 4-figure supplement 1

Immunostaining of thymic sections from Fucci2a mice with anti-GFP (for Venus staining, green) and anti-keratin-5 (Krt5, blue) antibodies. Typical panels of 3-independent experiments are exhibited. Scale bars, 100 µm.

Figure_6-figure supplement 1

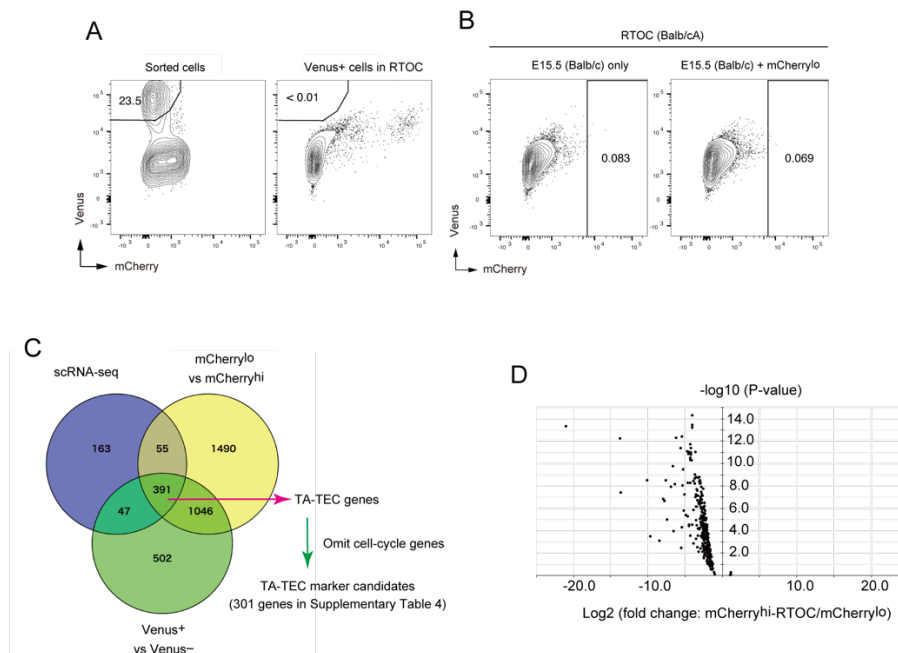


Figure 6-figure supplement 1

A. Ratio of Venus⁺ cells in sorted mCherry^{lo} and RTOC

B. Flow cytometric analysis of RTOC using allogenic fetal thymus (Balb/cA)

C. Venn diagram of gene lists expressed in proliferating TECs at higher level in the 3 different RNA-seq datasets (mCherry^{lo} vs mCherry^{hi} in Fig. 6, Venus+ vs Venus- in Fig. 4 and cluster R1 in Fig. 2). TA-TEC gene candidates were selected from the Venn diagram. TA-TEC marker gene candidates were selected by omitting cell cycle-related genes (GO:0007049 and Tirosh et al.³) from the TA-TEC gene candidates. The list of genes is summarized in Supplementary Table 4.

D. Volcano plot for TA-TEC marker candidate expression in mCherry^{lo} and mCherry^{hi} in RTOC.

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Figure_6-figure supplement 2

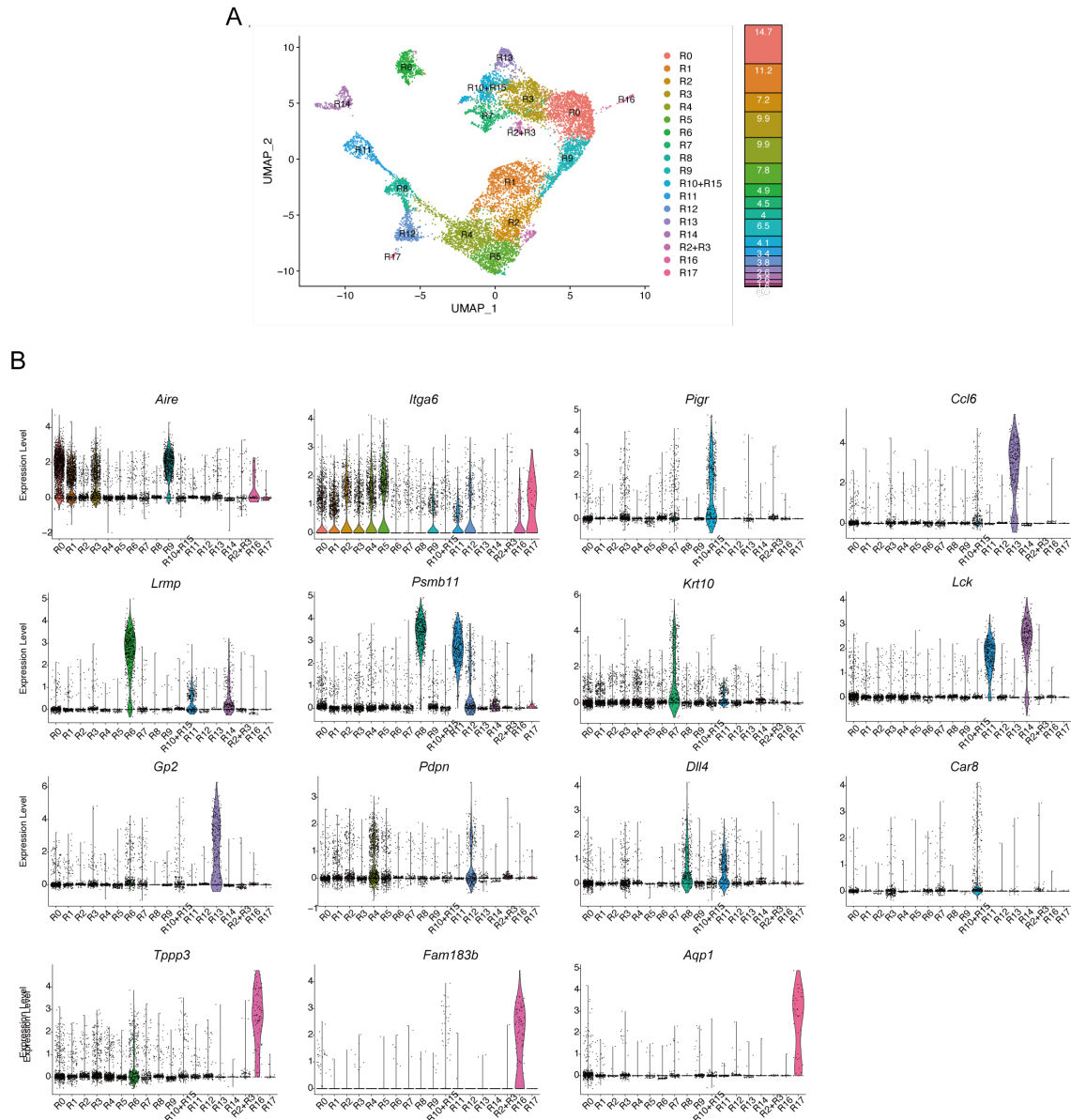


Figure 6-figure supplement 2

A. UMAP plot of droplet-based scRNA-seq and well-based scRamDA-seq data after their integration. Cell clusters are separated by colors and numbers in the plot. The graph on the right shows the percentages of each cluster in the total number of cells detected. Each cluster was assigned based on gene expression profile and corresponded with clusters in Fig. 2.

B. Violin plots depicting expression level of typical TEC marker genes in each cluster.

Figure_7-figure supplement 1

