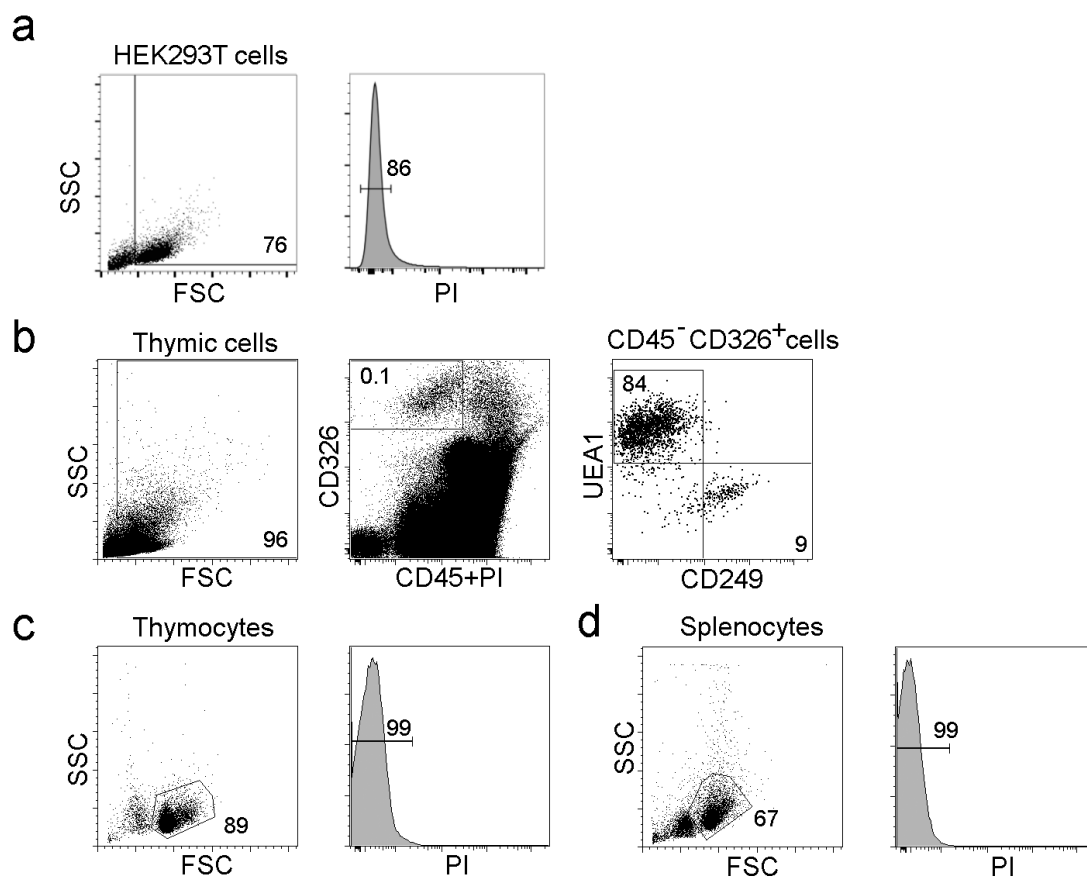
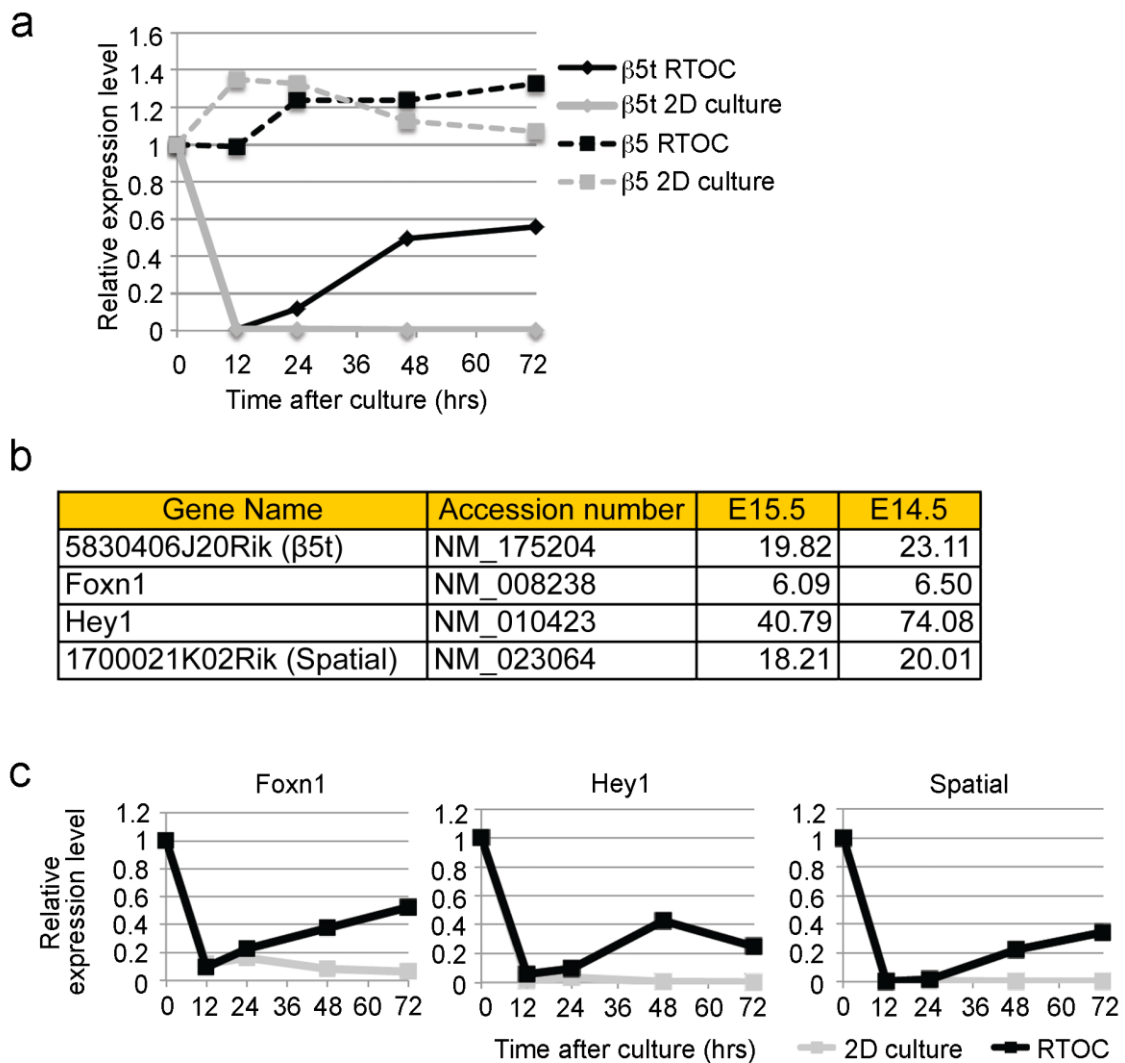


Supplementary Figure 1. EGFP expression by two different promoters.

Representative profiles ($n = 3$) of EGFP expression in HEK293T cells transfected with EGFP-expressing plasmid driven by herpes simplex virus thymidine kinase gene promoter (tk-EGFP, left panel) or cytomegalovirus immediate early gene promoter (CMV-EGFP, right panel). Shaded histograms indicate EGFP expression in control cells.



Supplementary Figure 2. Flow cytometry strategies. Dot plots and histograms show the representative flow cytometry profiles for the gating for (a) viable HEK293T cells, (b) liberase-digested viable CD249⁺UEA1⁻CD45⁻CD326⁺ cortical thymic epithelial cells and CD249⁻UEA1⁺CD45⁻CD326⁺ medullary thymic epithelial cells, (c) viable thymocytes, and (d) viable splenocytes. FSC, forward scatter; SSC, side scatter; PI, propidium iodide.

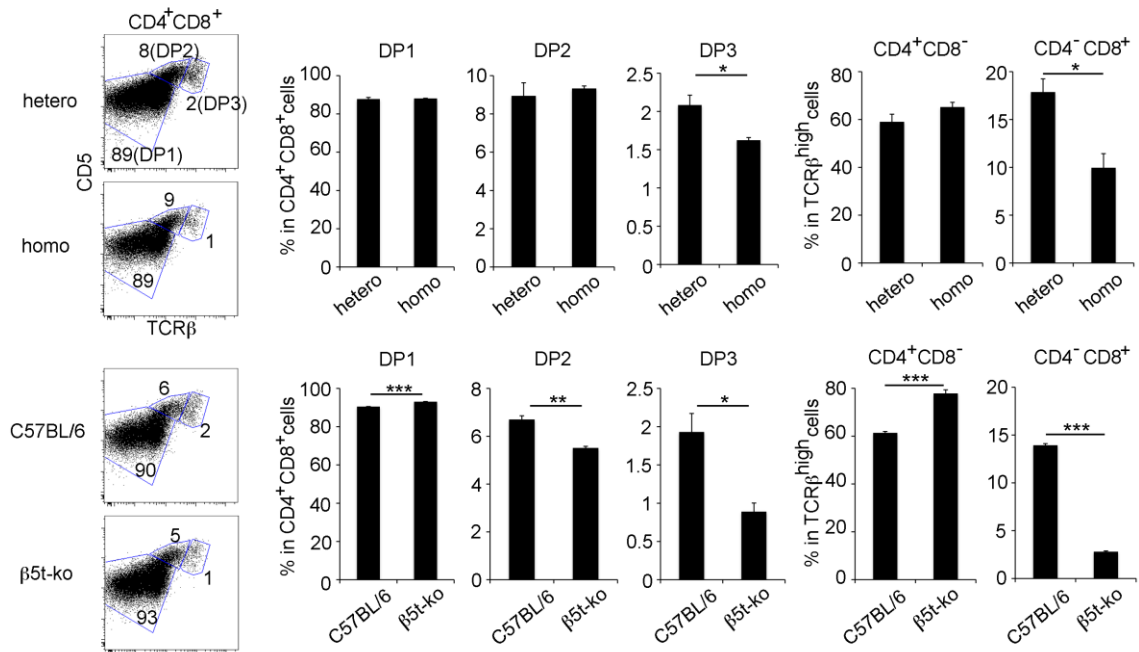


Supplementary Figure 3. Kinetics of gene expression in RTOC and 2D culture. (a) Relative mRNA expression of $\beta 5t$ (solid lines) and $\beta 5$ (dashed lines) in thymic stromal cells during RTOC (black lines) and 2D culture (gray lines). The expression level at 0 hr (just after disperse treatment) was set as 1. (b) Ratio of $\beta 5t$, *Foxn1*, *Hey1*, and *Spatial* mRNA expression in RTOC versus 2D culture in microarray analysis of thymic stromal cells. (c) Relative mRNA expression of *Foxn1*, *Hey1*, and *Spatial* in thymic stromal cells during RTOC (black lines) and 2D culture (gray lines).

	sequence	location	homology to on-target	score
on-target	<u>GGAGGACGCTGTGGAGAAGCTGG</u>	chr14, β 5t, 5'genomic region	100%	
off-target1	<u>AGAGGAAGCTGTGGAGAAGCAGG</u>	chr14, Sacs, intron2	90%	4.6
off-target2	<u>GGGGGAAGCTGTGGAGAAGCTGG</u>	chr2, Ryr3, intron73	90%	4.0
off-target3	<u>GGGGGACCCAGTGGAGAAGCTGG</u>	chr18, Zfp608, 3'genomic region	85%	2.4
off-target4	<u>AGAGGACGGAGTGGAGAAGCAAG</u>	chr4, Padi4, intron10	85%	1.5
off-target5	<u>AGTGGTCGCTGTGGAGAAGCAGG</u>	chr17, Cyp1b1, intron1	85%	1.5
off-target6	<u>GGTGAACGCTGAGGAGAAGCAGG</u>	chr4, Runx3, 5'genomic region	85%	1.3
off-target7	<u>GCAGGACACTGTGAGCAGCAGG</u>	chr8, Agpat5, 5'genomic region	85%	1.3
off-target8	<u>GGAGAACACTGGGGAGAAGCCAG</u>	chr5, 2900026A02Rik, exon10	85%	1.3
off-target9	<u>GGAGGACCCAGAGGAGAAGCTGG</u>	chr12, Nr3c1, 5'genomic region	85%	1.1
off-target10	<u>TGAGGTGCTGTGGAGAAGCAGG</u>	chr1, Esrrg, 5'genomic region	85%	1.1
off-target11	<u>GGAGAACCCTGTGGAGAACCCTGG</u>	chr7, 9030624J02Rik, intron9	85%	1.0
off-target12	<u>GGCGGAAGCTGTGGAGAAGGAGG</u>	chr8, Maf, 5'genomic region	85%	1.0
off-target13	<u>GGGGCACACAGTGGAGAAGCCAG</u>	chr15, Prlr, 5'genomic region	80%	1.3
off-target14	<u>TGAGAACCAGTGGAGAAGCAGG</u>	chr2, Ssfa2, 3'genimoc region	80%	1.3
off-target15	<u>TCAAGAGGCTGTGGAGAAGCAAG</u>	chr1, Gaptc2, 3'genomic region	80%	0.9

on-target	GGAGGAC <u>GCTGTGGAGAAGCTGG</u>	off-target6	GGTGAACGCTGAGGAGAAGCAGG	off-target12	GGCGGAAGCTGTGGAGAAGGAGG
mouse	***** * *	mouse	*****	mouse	*****
genome	GGAGGAC <u>ACTATGGAGAAGCTGG</u>	genome	GGTGAACGCTGAGGAGAAGCAGG	genome	GGCGGAAGCTGTGGAGAAGGAGG
off-target1	AGAGGAAGCTGTGGAGAAGCAGG	off-target7	GCAGGACACTGTGGAGCAGCAGG	off-target13	GGGGCACACAGTGGAGAAGCCAG
mouse	*****	mouse	*****	mouse	*****
genome	AGAGGAAGCTGTGGAGAAGCAGG	genome	GCAGGACACTGTGGAGCAGCAGG	genome	GGGGCACACAGTGGAGAAGCCAG
off-target2	GGGGGAAGCTGTGGAGAAGCTGG	off-target8	GGAGAACACTGGGGAGAAGCCAG	off-target14	TGAGAACCAGTGGAGAAGCAGG
mouse	*****	mouse	*****	mouse	*****
genome	GGGGGAAGCTGTGGAGAAGCTGG	genome	GGAGAACACTGGGGAGAAGCCAG	genome	TGAGAACCAGTGGAGAAGCAGG
off-target3	GGGGGACCCAGTGGAGAAGCTGG	off-target9	GGAGGACCCAGAGGAGAAGCTGG	off-target15	TCAAGAGGCTGTGGAGAAGCAAG
mouse	*****	mouse	*****	mouse	*****
genome	GGGGGACCCAGTGGAGAAGCTGG	genome	GGAGGACCCAGAGGAGAAGCTGG	genome	TCAAGAGGCTGTGGAGAAGCAAG
off-target4	AGAGGACGGAGTGGAGAAGCAAG	off-target10	TGAGGTGCTGTGGAGAAGCAGG		
mouse	*****	mouse	*****		
genome	AGAGGACGGAGTGGAGAAGCAAG	genome	TGAGGTGCTGTGGAGAAGCAGG		
off-target5	AGTGGTCGCTGTGGAGAAGCAGG	off-target11	GGAGAACCCTGTGGAGAACCCTGG		
mouse	*****	mouse	*****		
genome	AGTGGTCGCTGTGGAGAAGCAGG	genome	GGAGAACCCTGTGGAGAACCCTGG		

Supplementary Figure 4. No detection of off-target mutations in site #13 mutant mice. On-target site #13 and fifteen off-target sites with the high sequence match (80% to 90% of the similarity excluding the protospacer adjacent motif) to the on-target site (top) were sequenced in the genome of mutant mice (bottom). Underlines indicate the protospacer adjacent motif sequence. No off-target mutations were detected in our site #13 mutant mice.



Supplementary Figure 5. Diminished DP3 thymocytes in site #13 mutant mice.

Flow cytometric analysis of thymocytes from 5-week-old mice. Shown are dot plots for TCRβ and CD5 expression in CD4⁺CD8⁺ cells. Boxes indicate DP1 (TCR^{low} CD5^{low}), DP2 (TCR^{intermediate} CD5^{high}) and DP3 (TCR^{high} CD5^{intermediate}) subpopulations. Numbers in dot plots indicate frequency of cells within indicated area. Bar graphs show the frequency (means and s.e.m., n = 3) of indicated thymocyte populations. *, P<0.05; **, P<0.01; ***, P<0.001. Statistic analyses were performed by student's *t*-test.

Supplementary Table 1. Nucleotide sequences of site #8 and site #13 in different species. Letters in red indicate the Foxn1-binding invariant core ACGC tetranucleotide.

	site #8	site #13
Mouse	GCTT ACGC TTT	GAGG ACGC TGT
Human	TAAC ACTC ACC	AGGG ACGC TAC
Chimpanzee	GCTG AGGC AGG	AGGG ACGC TAC
Dog	GTAT ATCAT CT	AGAG ACGC TAA
Rat	GCAT ACTC TTT	GAGG ACGC TGT
Bat	TATC ACTT ACC	AAGG ACGC TAA
Elephant	CCTACTCATAA	AGGG ACGC TAG
Horse	GGTC A AAGGGT	AGGG ACGC TAA