Supporting Information

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SI Materials and Methods

Fetal Thymus Organ Culture. These cultures were performed as described previously (1). Briefly, E15 fetal thymic lobes were cultured on filters (pore size = 8 μm; Nuclepore) floating on complete RPMI medium containing 1.35 mM 2-deoxyguanosine (dGuo; Nacalai Tesque) for 7 d to deplete hematopoietic cells. Each dGuo-treated lobe was submerged in a well of a 96-well V-bottom plate. E15 fetal thymocytes were then inoculated into each dGuo-treated lobe at 10^3 cells/lobe and cultured in a plastic bag containing a gas mixture of 5% CO_2, 70% O_2, and 25% N_2.

Antibodies and Other Reagents. The following antibodies were used: anti-CD3 (2C11), anti-CD4 (GK1.5), anti-CD5 (53-7.3), anti-CD8 (53-6.7), anti-CD25 (PC61.5), anti-CD28 (37.51), anti-CD44 (IM7), anti-CD45 (30-F11), anti-CD69 (H1.2F3), anti–T-cell receptor β (TCRβ; H57-597), anti–Mac-1 (M1/70), anti–Gr-1 (RB6-8C5), anti-B220 (RA3-6B2), and anti–TER-119 (TER-119; all from BD Bioscience), rabbit polyclonal antibodies (pAbs) to claudin 1 (Cld1), Cld4, ZO-1, ZO-2, and ZO-3 (all from Invitrogen), anti–ERK-1(C-16), anti–ERK-2 (C-14), antiphosphor-ERK (E-4), anti-lck (3A5), anti-GFP (FL), anti-E2A (V-18X), and anti-HEB (A-20X; all from Santa Cruz Biotechnology), anti-H3K18ac (07-354) and anti-H3K4me3 (07-473; both from Millipore), anti-phosphor-ERK (197G2), anti-LAT, anti-phospho-LAT, and lck (all from Cell Signaling Technology), and anti-CD3ξ (G3; Abcam). Mouse anti–ZO-1mAb (T8-754) was a gift from Itoh (Dokkyo Medical University, Tochigi, Japan). Monoclonal antibody for the extracellular domain of mouse Cld4 (HKH-189) was generated by immunization with L cells transfected with mouse Cld4 cDNA (Cld4L). Preparation of the biotinylated C-terminal one-half fragment of *Clostridium perfringens enterotoxin* (C-CPE) was described previously (2).

Cells and Culture. The double-positive (DP) thymocyte cell line, SCID.adh (3), was provided by D. L. Wiest (Fox Chase Cancer Center, Philadelphia, PA). The OVA53 cell line was derived from a thymoma that developed spontaneously in a p53-deficient $RAG2^{-/-}$ OVA-specific TCR transgenic mouse. These cell lines were maintained in complete RPMI medium supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 100 μM nonessential amino acids, 1 mM sodium pyruvate, and antibiotics. The L cells expressing Cld1 and Cld4 were maintained in DMEM supplemented with 10% FCS and G418. Normal thymocytes and the OVA53 cell line were incubated with biotinylated or purified antibodies at 4 °C for 20 min and washed followed by the addition of cross-linkers, avidin or anti-IgG, at 37 °C. The OVA53/Cld4 cells were incubated with A20 cells that had been preincubated with OVA (1 μM) for 16 h as antigen presenting cells (APCs). For the cell dissociation assay (4), L cells at confluent culture were treated with 0.01% trypsin/1 mM EDTA in HBSS at 37 °C for 30 min, dissociated by pipetting 10 times, and examined under a photo microscope (Axiovert 40C; Carl Zeiss) equipped with an A-Plan (5/0.12 N.A; Carl Zeiss).

Flow Cytometric Analysis. The cells were stained intracellularly with anti-phospho-ERK antibody according to the manufacturer's protocol (Cell Signaling Technology) and analyzed with FACS Calibur (Beckton Dickinson). Thymocytes were loaded with 5 μM Fluo-4 AM (Dojindo Laboratories) in Tyrode's buffer (130 mM NaCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 5.6 mM glucose, 10 mM Hepes–NaOH, pH 7.3) containing 1% BSA at 37 °C for 45 min. The cells were incubated with biotinylated antibodies at 4 °C for 20 min, washed, and warmed to 37 °C, and the Ca^{2+} mobilization was scanned in Tyrode's buffer for 5-min period after the addition of avidin (25 μg/mL) with FACS Calibur. For K_i-67 staining, the thymocytes were incubated with biotinylated (b) anti-Cld4 (HKH-189) antibody and phycoerythrin-conjugated streptavidin, fixed in 70% ethanol for 2 h at −20 °C, and then stained with anti–K_i-67 (BD Pharmingen), anti-CD4, and anti-CD8 antibodies. Cells were analyzed with FACS Calibur (Beckton Dickinson). For BrdU uptake experiments, mice were injected intraperitoneally with 1 mg BrdU (BD Pharmingen); 2, 6, and 24 h later, the thymocytes were stained using FITC BrdU Flow Kit (BD Pharmingen) and analyzed with FACS Calibur (Beckton Dickinson).

Gene Transfection. OVA53 cells were infected with pMCs-Ires-EGFP retrovirus containing cDNA of *Cldn1*, *Cldn4*, or various *Cldn4* mutants as described before (5), and GFP^+ cells were sorted with the use of FACS Aria (BD Biosciences). For shRNA knockdown experiments, the double-stranded oligonucleotides were ligated into a BamHI and EcoRI site downstream of a U6 promoter in the pSIREN-RetroQ retroviral vector (Clontech) containing an *Ires-EGFP* fragment at the XhoI site. The primer sequences for *Cldn4* shRNA were as follows: sense, 5′-GATCCGTGGCAAGCATGCTGATTATTCAAGAGATAATCAGCATGCTTGCCACTTTTTG-3′; antisense, 5′-AATTCAAAAAGTGGCAAGCATGCTGATTATCTCTTGAATAATCAGCATGCTTGCCACG-3′. For the control, scrambled sequences were used as follows: sense, 5′-GATCCGGATCGACGTAGGTTCATATTCAAGAGATATGAACCTACGTCGATCCTTTTTG-3′; antisense, 5′-AATTCAAAAAGGATCGACGTAGGTTCATATCTCTTGAATATGAACCTACGTCGATCCG-3′. PLAT-E packaging cells were transfected with 5 μg pSIREN-RetroQ plasmids using Lipofectamine 2000 Reagent (Invitrogen). Fetal thymocytes were infected with the viral supernatants in the presence of 8 mg/mL polybrene (Wako) and 10 ng/mL IL-7 (Peprotech) at 32 °C for 60 min.

Immunoprecipitation and Immunoblotting. Cells were lysed in lysis buffer (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and a mixture of protease and phosphatase inhibitors) containing 1% Brij58. The lysates were incubated with 3 μg/mL biotinylated C-CPE or antibodies for 2 h at 4 °C followed by streptavidin Sepharose (Amersham) for 30 min at 4 °C. Immunoblotting was performed as before (5).

Immunostaining. Cells were fixed with 1% formaldehyde/PBS for 15 min at room temperature, permeabilized with 0.2% Triton-X-100/PBS for 5 min, washed, blocked with 1% BSA in PBS, and incubated with primary antibodies at RT for 1 h followed by the secondary antibodies for 30 min. The cells were examined under a fluorescence photo microscope (Axiovert 200M; Carl Zeiss) and a Plan Apochromat (10/0.45 NA, 20/0.80 NA, 40/0.95 NA, and 63/1.4 NA). Photographs were recorded with a cooled CCD camera (model AxioCam MRm) and Axiovision version 4.6 software.

Immunoelectron Microscopy. Immunoelectron microscopy using SDS-treated freeze–fracture replica was performed as described previously (6). Briefly, unfixed cell pellets were sandwiched between two aluminum plates and rapidly frozen by a HPM010 high-pressure freezer (Bal-Tec AG). The freeze–fracture replicas prepared at −120 °C were treated with 2.5% SDS/PBS and incubated with rabbit anti-Cld4 antibody followed by protein A conjugated with colloidal gold (5 nm diameter; University of

Utrecht). The specimens were observed with a JEOL 1400EX electron microscope operated at 100 kV, and electron micrographs were taken randomly.

ChIP Assay. ChIP assay was performed as described (7). Briefly, total thymocytes prepared from 4-wk-old mice were fixed with 1% formaldehyde for 20 min [for IgG, E2A, and HEB] or 5 min (for H3K18ac and H3K4me3) at room temperature. Soluble chromatin prepared from 3×10^6 fixed thymocytes was immunoprecipitated with rabbit IgG (Jackson Immunoresearch), anti-E2A (V-18X; Santa Cruz), or anti-HEB (A-20X; Santa Cruz) antibodies preadsorbed to Dynabeads Protein G (Dynal Biotech) or with anti-H3K18ac (07–354; Millipore) or anti-H3K4me3 (07–473; Millipore) antibodies followed by Protein G-Sepharose (GE Healthcare Life Sciences). DNA was purified from the bound and input fractions and quantified by real-time PCR using QuantiTect SYBR Green PCR mix (Qiagen) on a LightCycler 480 Real-Time PCR System (Roche).

The primer sets used for ChIP assay are as follows:

mTCRβ E 24325 F1 TCCTGGGACTTTTCGGTTCCT
mTCRβ E 24512 R1 TGAGTGAGTGGGGGAGCATTC
mCD4 E 169 F1 GAGCCCCACCCTAAGATGAAG
mCD4 E 319 R1 CCATGAGAAGACAGAGGTAGC
mMyoD ChIP YA F1 CGCCCTACTACACTCCTATTG
mMyoD ChIP YA R1 AAGGTTCTGTGGGTTGGAATG
m a-actin ChIP HS F AGAGTCAGAGCAGCAGGTAG
m a-actin ChIP HS R CAAGGCTCAATAGCTTTCTT
mCldn3 ChIP F7280 ATCAACTGCCCTTCGAAAACT
mCldn3 ChIP R7532 GACGGACCGGACTGGAC
mCldn4 ChIP F6599 GCCCAAGCTCAGGAATCCAGA
mCldn4 ChIP R6792 CGGTGAGCCTCAGGGACACA

1. Watanabe Y, Katsura Y (1993) Development of T cell receptor alpha beta-bearing T cells in the submersion organ culture of murine fetal thymus at high oxygen concentration. *Eur J Immunol* 23:200–205.
2. Hamazaki Y, et al. (2007) Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin. *Nat Immunol* 8:304–311.
3. Carleton M, et al. (1999) Signals transduced by CD3epsilon, but not by surface pre-TCR complexes, are able to induce maturation of an early thymic lymphoma in vitro. *J Immunol* 163:2576–2585.
4. Kubota K, et al. (1999) Ca(2+)-independent cell-adhesion activity of claudins, a family of integral membrane proteins localized at tight junctions. *Curr Biol* 9:1035–1038.
5. Shimatani K, Nakashima Y, Hattori M, Hamazaki Y, Minato N (2009) PD-1+ memory phenotype CD4+ T cells expressing C/EBPalpha underlie T cell immunodepression in senescence and leukemia. *Proc Natl Acad Sci USA* 106:15807–15812.
6. Fujita A, et al. (2007) Gangliosides GM1 and GM3 in the living cell membrane form clusters susceptible to cholesterol depletion and chilling. *Mol Biol Cell* 18:2112–2122.
7. Agata Y, et al. (2007) Regulation of T cell receptor beta gene rearrangements and allelic exclusion by the helix-loop-helix protein, E47. *Immunity* 27:871–884.

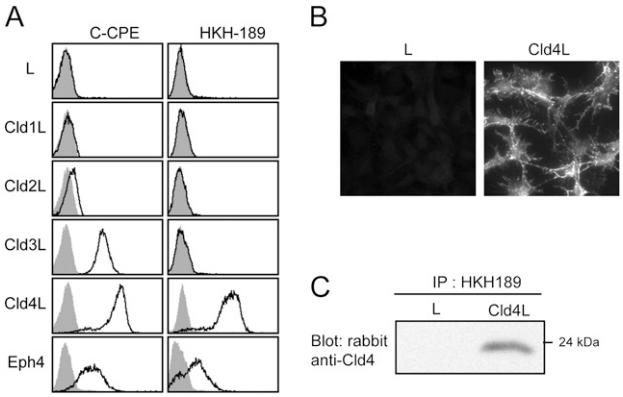

Fig. S1. A monoclonal anti-Cld4 antibody specific for the extracellular region. (*A*) L cells transfected with various *Cldns* (*Cldn1*, *-2*, *-3*, or *-4*), parental L cells, and Eph4 epithelial cell lines were stained with C-CPE or a newly developed monoclonal antibody (HKH-189) and analyzed with FACSCalibur. Shaded areas indicate staining with control IgG. (*B*) Parental and *Cldn4*-transduced L- (Cld4L) cells were fixed with 2% paraformaldehyde/PBS and stained with HKH-189. (*C*) L- and Cld4/L cells were lysed, immunoprecipitated with HKH-189, and immunoblotted with rabbit anti-Cld4 antibody specific for the intracellular region.

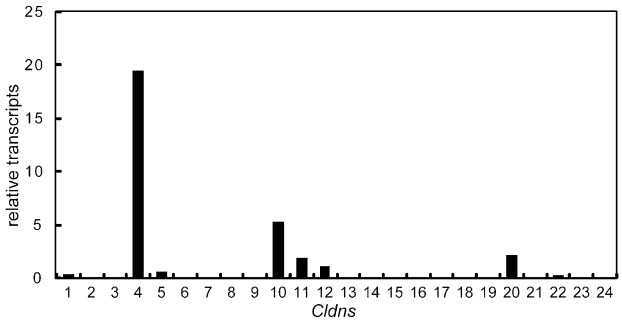

Fig. S2. Predominant expression of *Cldn4* among the Cld family members in normal thymocytes. RNA was extracted from the newborn thymocytes, and the transcripts of 24 Cld family members were assessed by quantitative RT-PCR. The transcript of each *Cldn* was normalized to the equivalent copy number of the corresponding *Cldn* plasmid. The primer sets are shown in Table S1.

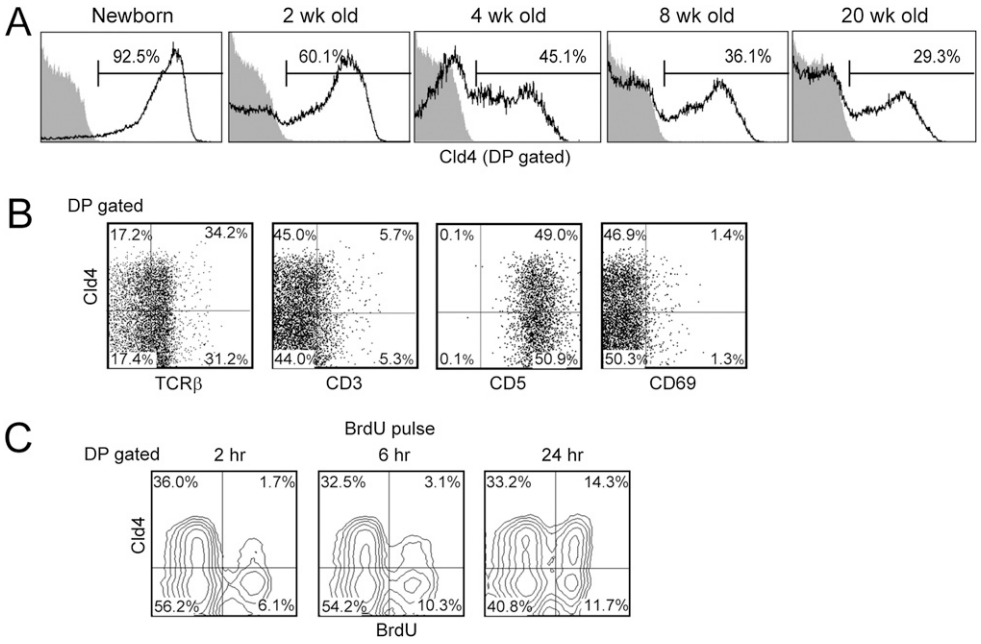

Fig. S3. Age-dependence, maturation profiles, and proliferation status of Cld4^{+} DP thymocytes. (*A*) The thymocytes derived from newborn, 2-, 4-, 8-, and 20-wk-old normal B6 mice were three-color stained with anti-Cld4, anti-CD4, and anti-CD8 antibodies. The proportions of Cld4^{+} cells in the DP cell gate are indicated. Shaded areas indicate the control IgG staining. Data are the representative of at least two independent experiments. (*B*) Adult thymocytes were stained with anti-Cld4, anti-CD4, and anti-CD8 antibodies together with anti-TCRβ, anti-CD3, anti-CD5, or anti-CD69 antibodies. The FACS profiles of Cld4 vs. indicated markers in the DP cell gate are shown. (*C*) Eight-week-old B6 mice were injected with 1 mg BrdU, and 2, 6, and 24 h later, the thymocytes were four-color stained with anti-Cld4, anti-CD4, anti-CD8, and anti-BrdU antibodies. The FACS profiles of Cld4 vs. BrdU in the DP cell gate are indicated. Data are representative of two independent experiments.

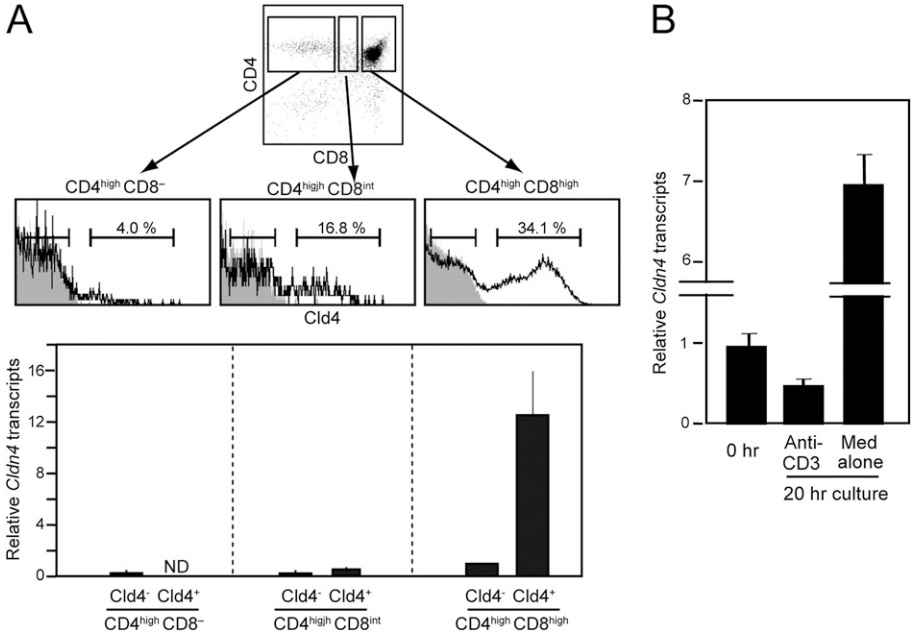

Fig. S4. Cld4 expression in DP cells is rapidly repressed during the transition to the SP cells in vivo and by the stimulation with anti-CD3 antibody in vitro. (*A*) The thymocytes of 8-wk-old B6 mice were stained with anti-Cld4, anti-CD4, and anti-CD8 antibodies, and the expression of Cld4 in the CD4^{high} CD8^{high}, CD4^{high}CD8^{int}, and CD4^{high} CD8$^{low/-}$ gates is shown. The Cld4^{-} and Cld4^{+} populations in each gate (bowed) were sorted, RNA was extracted, and relative *Cldn4* transcripts were assessed by quantitative RT-PCR. Means and ranges of duplicate analysis are indicated. ND, not determined. (*B*) The thymocytes of 8-wk-old B6 mice were incubated in the absence or presence of solid-phase anti-CD3 antibody for 20 h, and proportions of Cld4^{+} DP cells were analyzed. RNA was extracted from the aliquots of these cultured cells and precultured thymocytes, and the relative *Cldn4* transcripts were assessed by quantitative RT-PCR. Means and ranges of duplicate analysis are indicated.

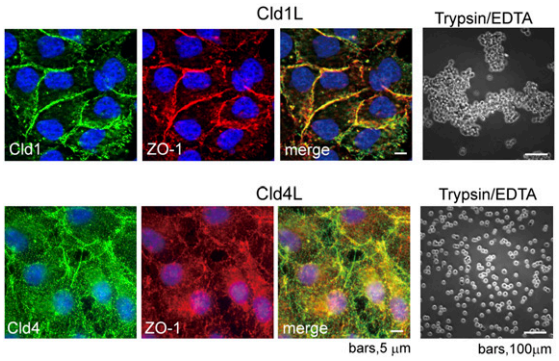

Fig. S5. Cld4 shows negligible homotypic adhesion activity in L-fibroblast. L-fibroblastic cells stably transfected with *Cldn1* or *Cldn4* (Cld1L and Cld4L) were two-color stained with the indicated antibodies. Merged images are also shown. Confluent cultures of Cld1L and Cld4L were treated with 0.01% trypsin/1 mM EDTA followed by gentle pipettings (far right column).

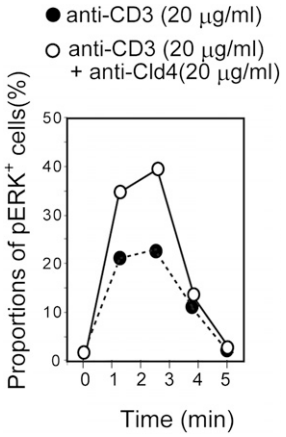

Fig. S6. The kinetics of ERK activation are unaffected by cross-linking of CD3 with Cld4. The thymocytes treated with 20 μg/mL b–anti-CD3 and 20 μg/mL b-IgG or b–anti-Cld4 antibody were cross-linked with avidin and analyzed with anti-pERK antibody after indicated times. Data are representative of two independent experiments.

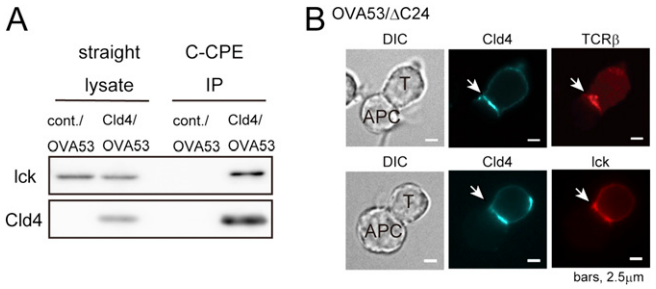

Fig. S7. Immunoprecipitation of lck by C-CPE is dependent on Cld4, and the ΔC24 mutant of Cld4 is efficiently recruited to the immunological synapse. (*A*) The OVA53 transduced with *Cldn4* (Cld4/OVA53) and parental Cld4^{-} OVA53 (cont/OVA53) cells was lysed and immunoprecipitated with b–C-CPE and avidin beads followed by immunoblotting with the indicated antibodies. Total straight lysates were also immunoblotted. (*B*) OVA53/ΔC24 cells were incubated for 6 h with APCs (A20 cells) preloaded with OVA (1 μM), fixed, and immunostained with the indicated antibodies.

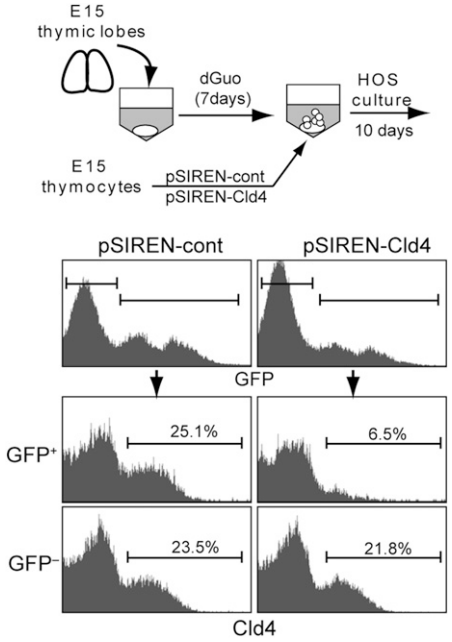

Fig. S8. Cld4 is down-regulated by infection of pSIREN retrovirus vector containing *Cldn4* shRNA in FTOC. Schematic representation of the FTOC procedure (*Upper*). pSIREN retrovirus vectors containing *Cldn4* shRNA (pSIREN-Cld4) or scrambled oligonucleotides (pSIREN-cont) were introduced into the E15 thymocytes and cultured with the E15 thymic lobes that had been treated with dGuo. Seven days later, the expression of Cld4 was analyzed in the GFP^+ and GFP^- gates (*Lower*).

Table S1. Primer sets used in Fig. S2 (means of duplicate analysis are shown)

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
Cldn1	actccttgctgaatctgaacagt	ggacacaaagattgcgatcag
Cldn2	gtagccggagtcatcctttg	ggcctggtagccatcatagt
Cldn3	tgggagctgggttgtacg	caggagcaacacagcaagg
Cldn4	ggcgtctatgggactacagg	gagcgcacaactcaggatg
Cldn5	acgggaggagcgctttac	gttggcgaaccagcagag
Cldn6	ggtggctgatgctcaaaag	tccacccagcagcaaaag
Cldn7	gacgcccatgaacgttaagta	ttgctttcactgcctggac
Cldn8	aagccctctacataggctgga	acaacaaaacacacaacagaacag
Cldn9	cccccgtcacactttgag	aacgggaagggatggagtag
Cldn10	tgatcctctctatatggagcaaaag	agaagctcctgcccatcc
Cldn11	gcctggagtggccaagta	agatggtggcgacaatgg
Cldn12	gctagctcaggggtctgtt	gcatgcacaataccaaacga
Cldn13	cagctccctgctgctgtt	ctaggggtctcaatgcaacg
Cldn14	accctgctctgcttatcctg	ggtagccgtggtggtagc
Cldn15	gtctccaactgctgggactt	agaaagaggcccaggaagc
Cldn16	ccatatatgcagaacatcccttg	ggtaggaacttcacacagtcca
Cldn17	tttctacgacccaaccgttc	aggaagagtgctcctccaagt
Cldn18	ccggccatacttcaccat	catcagggctcgtacagctt
Cldn19	ggtgcaatgcaaactctacg	cgtgctgactggatatgacc
Cldn20	attccggaaagccacaaata	atcgccgaaataaacccaat
Cldn21	gggatgggttctagccattat	ccatctcgttcagctccaa
Cldn22	cctcgagtcactatgcagtgg	gatttctggattggcttgct
Cldn23	aagacagccacctcccaag	cacagggcaacgaattttg
Cldn24	tcttcctggatggctgatg	tggaaggaaccatcaaattctt