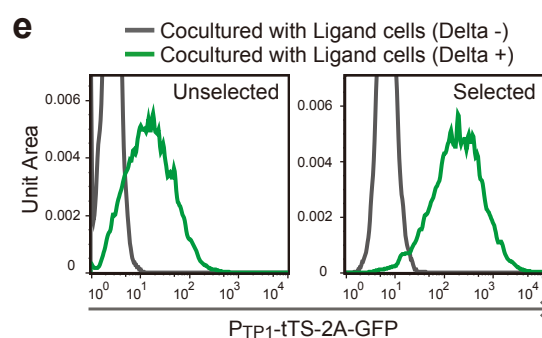
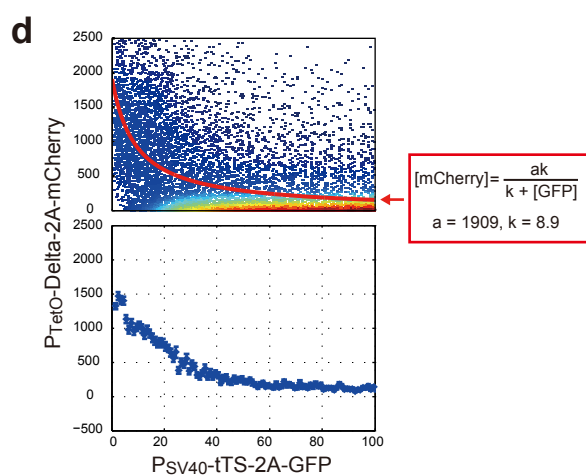
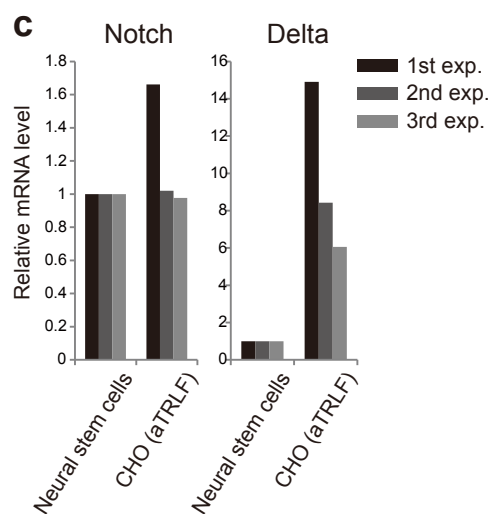
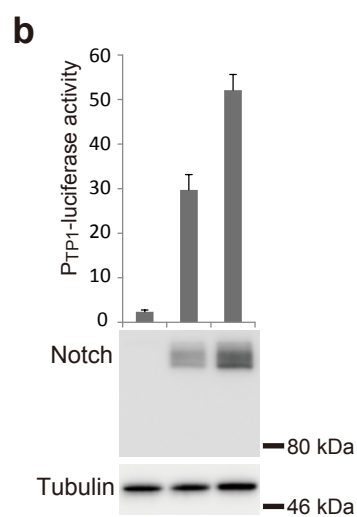
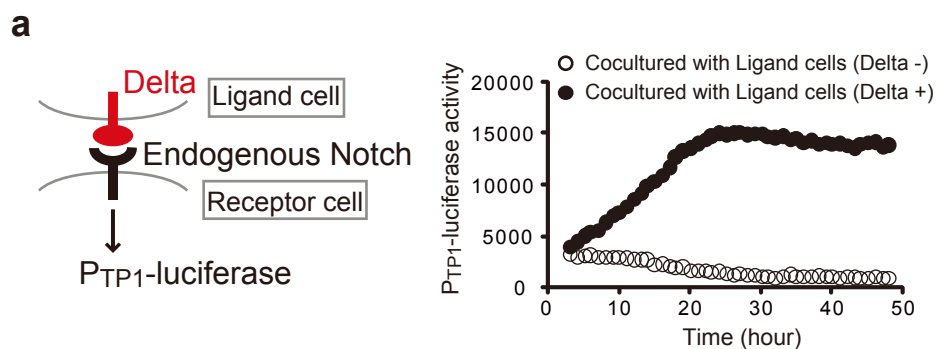


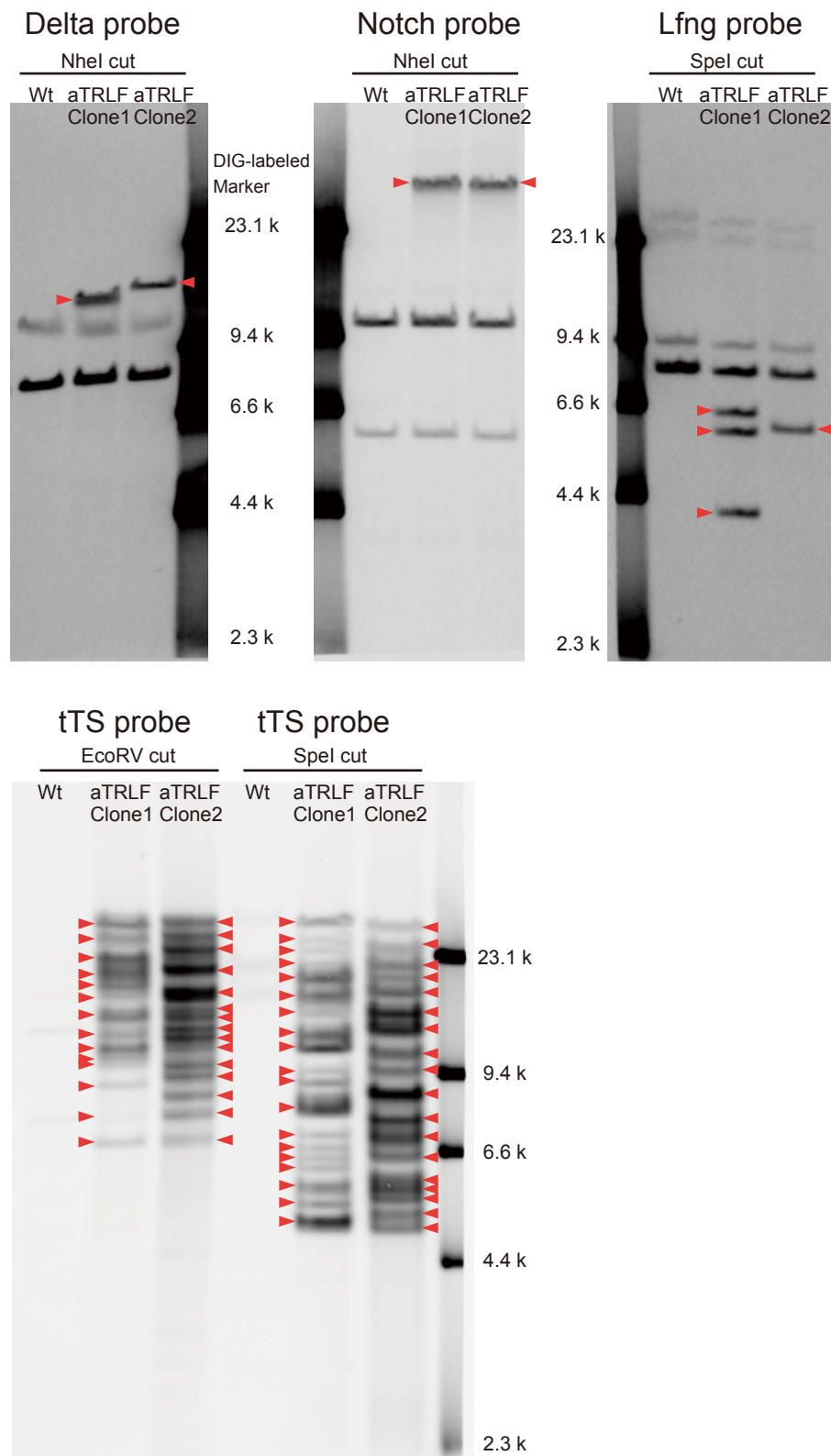
Supplementary Figure 1



Supplementary Figure 1. Construction of lateral inhibition circuits

(a) Activation of the P_{TP1} upon binding of Delta to Notch. CHO cells that have a P_{TP1} -luciferase construct, “Receptor cells” were cocultured with “Ligand cells” at a ratio of 1:9. The Ligand cells are either CHO cells that produce Delta under the control of a strong constitutive promoter, P_{CMV} (Delta-positive Ligand cells) or wild-type CHO cells (Delta-negative Ligand cells). The luciferase activities were monitored in real-time. (b) Enhancement of the P_{TP1} activity by overexpression of Notch. CHO cells that have P_{EF1a} -Notch and P_{TP1} -luciferase constructs were sorted into 3 fractions according to their expression levels of Notch by using a PE-conjugated anti-mouse Notch1 antibody (BioLegend; 1/1000). Cells from each fraction were cocultured with the Delta-positive Ligand cells. Forty hours later, the luciferase activity in each fraction was measured. The expression level of Notch in each fraction was assessed by immunoblotting. The control, Tubulin, was run on a different gel. Data are means + SEM ($n = 3$). (c) Comparison of expression levels of Notch and Delta in aTRLF cells with those in neural stem cells. The mRNA levels were measured by qRT-PCR. (d) Measurements of the tTS level required for repression of the P_{TetO} . CHO cells that have P_{SV40} -tTS-2A-GFP (continuous expression) and P_{TetO} -Delta-2A-mCherry constructs were analyzed by FACS for GFP and mCherry fluorescence. A hyperbolic function was fitted to the signal-response curve (upper). The mCherry intensity values were averaged over every 1 AFU of the GFP intensity (lower). Data are means $\pm$ SEM ($n = 150$). (e) Comparison of the tTS induction level in selected cells with that in unselected cells. CHO cells that have P_{EF1a} -Notch and P_{TP1} -tTS-2A-GFP constructs were cocultured with the Delta-positive Ligand cells. The cells that showed a higher induction level of tTS-2A-GFP were sorted by FACS. After the expansion of cells, the selected cells (right) and unselected cells (left) were again cocultured with the Delta-positive Ligand cells or Delta-negative Ligand cells and analyzed by FACS for GFP fluorescence.

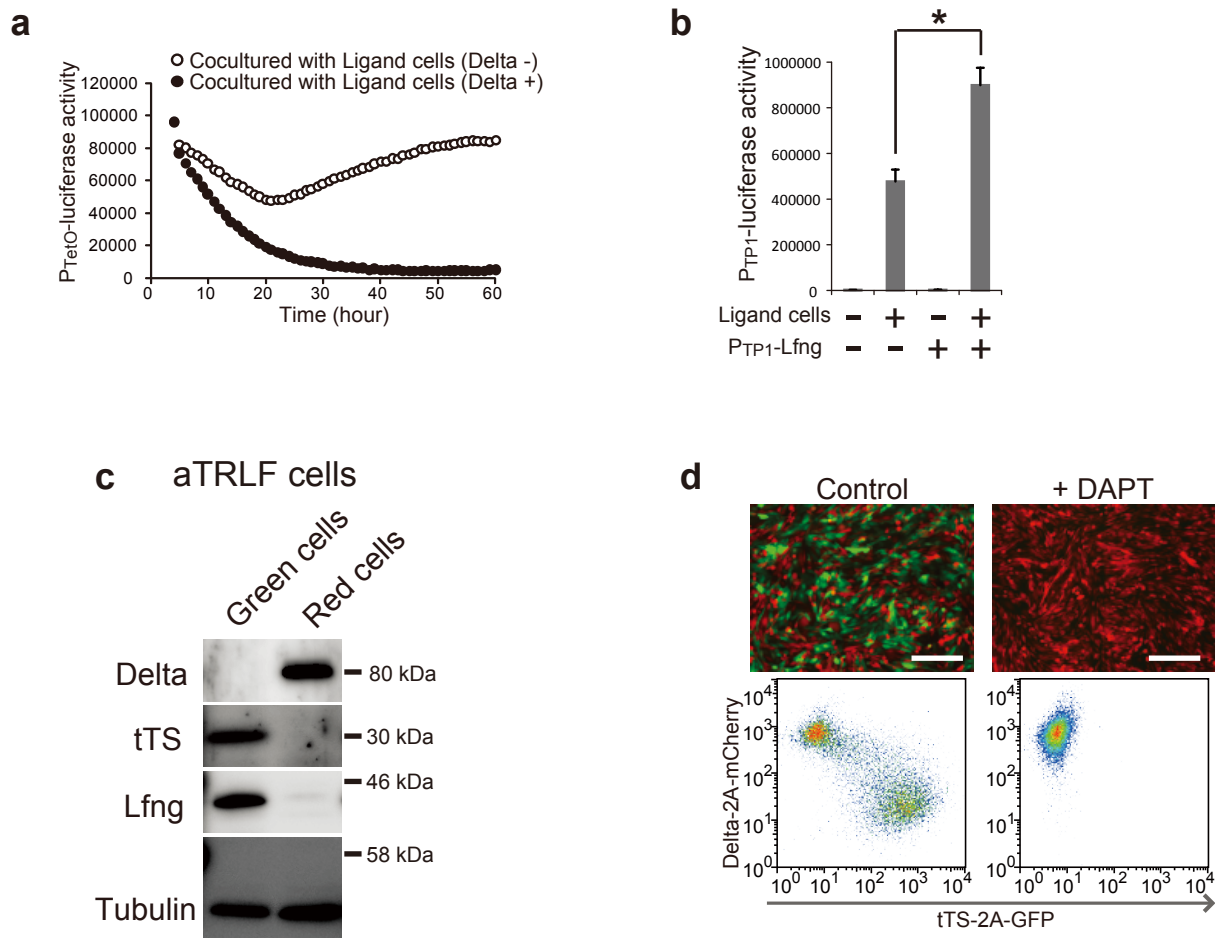
Supplementary Figure 2



Supplementary Figure 2. Copy numbers of genetic circuit components

Wild-type CHO cells (Wt), two clones of aTRLF cells were subjected to southern blotting, and the copy numbers of each genetic circuit component were evaluated. The tTS construct was introduced by a *piggyBac* vector, while other constructs were introduced by a lentiviral vector. The copy numbers were estimated as P_{TetO} -Delta-2A-mCherry = 1 copy, P_{EF1a} -Notch = 1 copy, P_{TP1} -Lfng = 1 and 3 copies, and P_{TP1} -tTS-2A-GFP = more than 10 copies. Because a Notch-expressing clone was first selected, and then other constructs were added to the clone, both aTRLF cell clones exhibit the same band patterns (i.e., same integration sites) for the Notch construct.

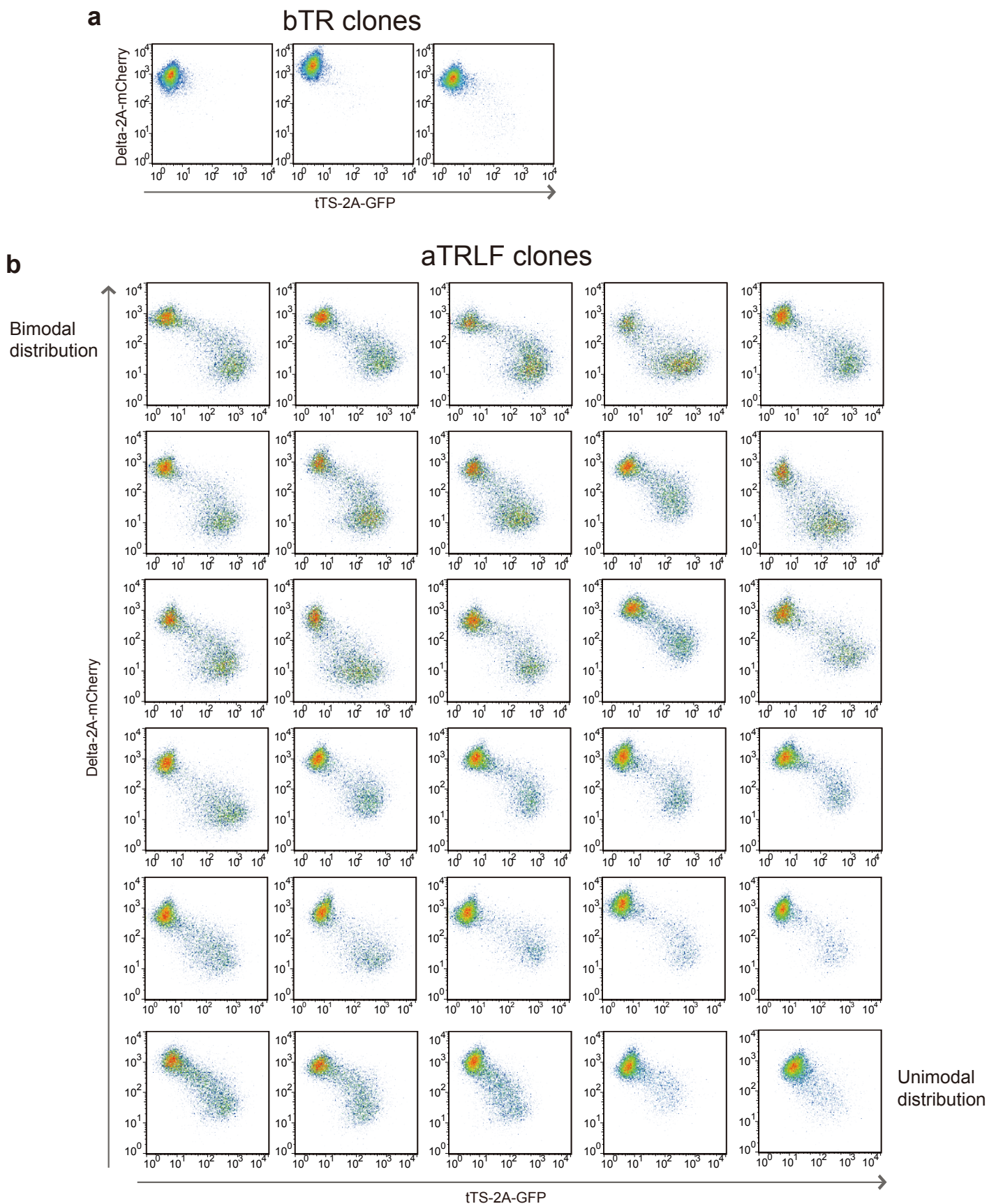
Supplementary Figure 3



Supplementary Figure 3. Characterization of aTRLF cells

(a) Repression of the P_{TetO} activity by the induction of tTS. CHO cells that have P_{EF1a}-Notch, P_{TP1}-tTS, and P_{TetO}-luciferase constructs were cocultured with the Delta-positive Ligand cells or Delta-negative Ligand cells, and the luciferase activities were monitored in real-time. (b) A positive effect of the Lfng-feedback on Delta-Notch signaling. CHO cells that have P_{EF1a}-Notch, P_{TP1}-luciferase, and P_{TP1}-Lfng constructs were cocultured with the Delta-positive Ligand cells or Delta-negative Ligand cells. The luciferase activities were measured in the absence or presence of the P_{TP1}-Lfng construct. Data are means $\pm$ SEM (n = 4). * p < 0.01 (paired t-test). (c) The red cells and green cells shown in Fig. 1f were sorted by FACS. The expression levels of Delta, tTS, and Lfng were measured in each fraction by immunoblotting. The control, Tubulin, was run on a different gel. (d) Pharmacological inhibition of Notch signaling. The aTRLF cells were seeded at a density of 8×10^5 cells/35 mm dish, and treated with 5 μ M DAPT, a γ -secretase inhibitor. Two days later, the cells were re-seeded and again treated with DAPT to make sure that the system reaches a steady state. Two more days later, the images were taken, and the cells were analyzed by FACS. Scale bar = 100 μ m.

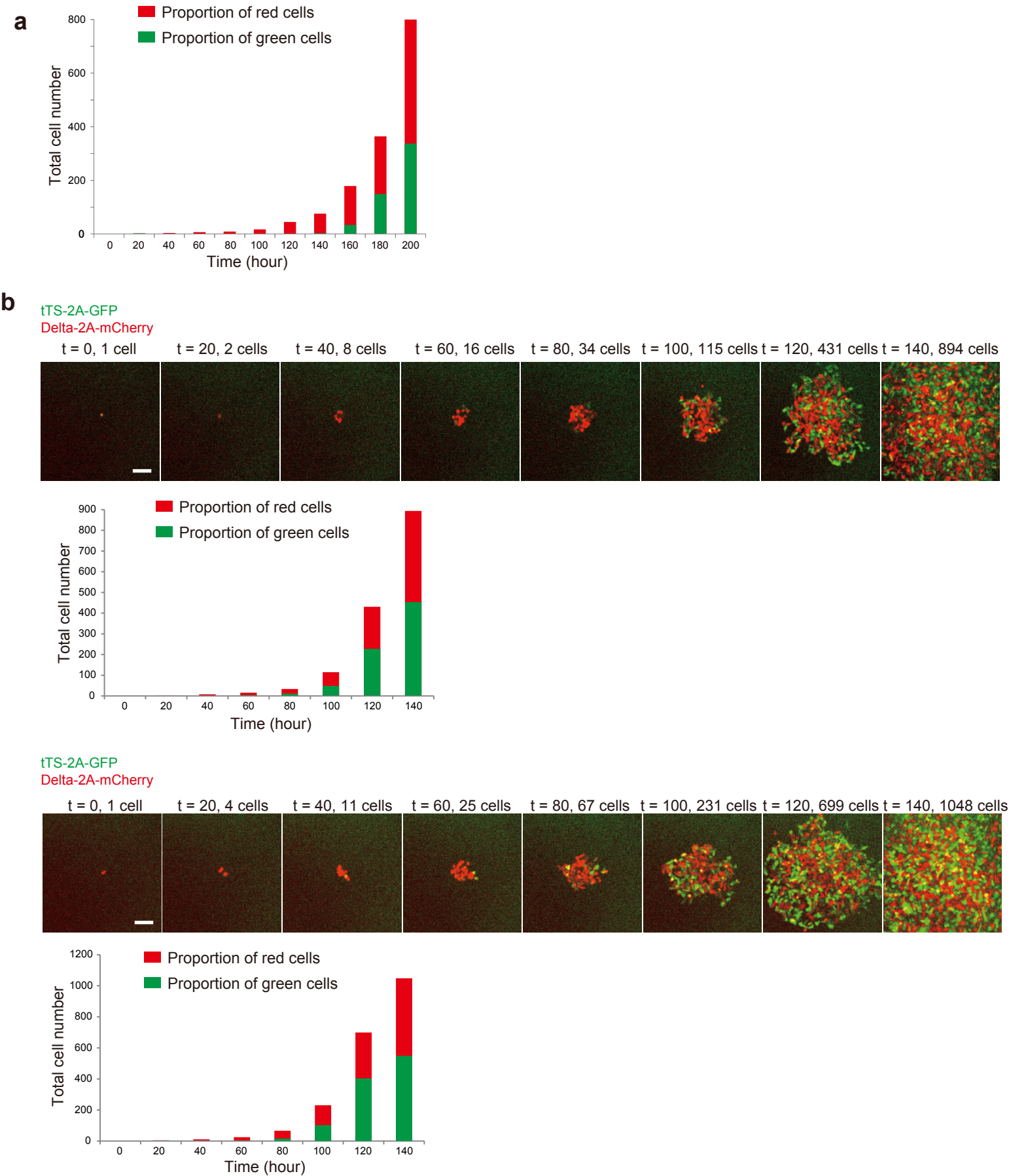
Supplementary Figure 4



Supplementary Figure 4. Comparison of aTRLF cells with bTR cells

(a) Repeated experiments for Fig. 1c using 3 other bTR cell clones. Because each genetic construct was introduced by a lentiviral vector or a *piggyBac* vector, the copy number of the construct and the genomic integration site differ among the clones. Each clone was analyzed by FACS. (b) Repeated experiments for Fig. 1f using 30 other aTRLF cell clones. More than 20 clones showed a clear bimodal distribution.

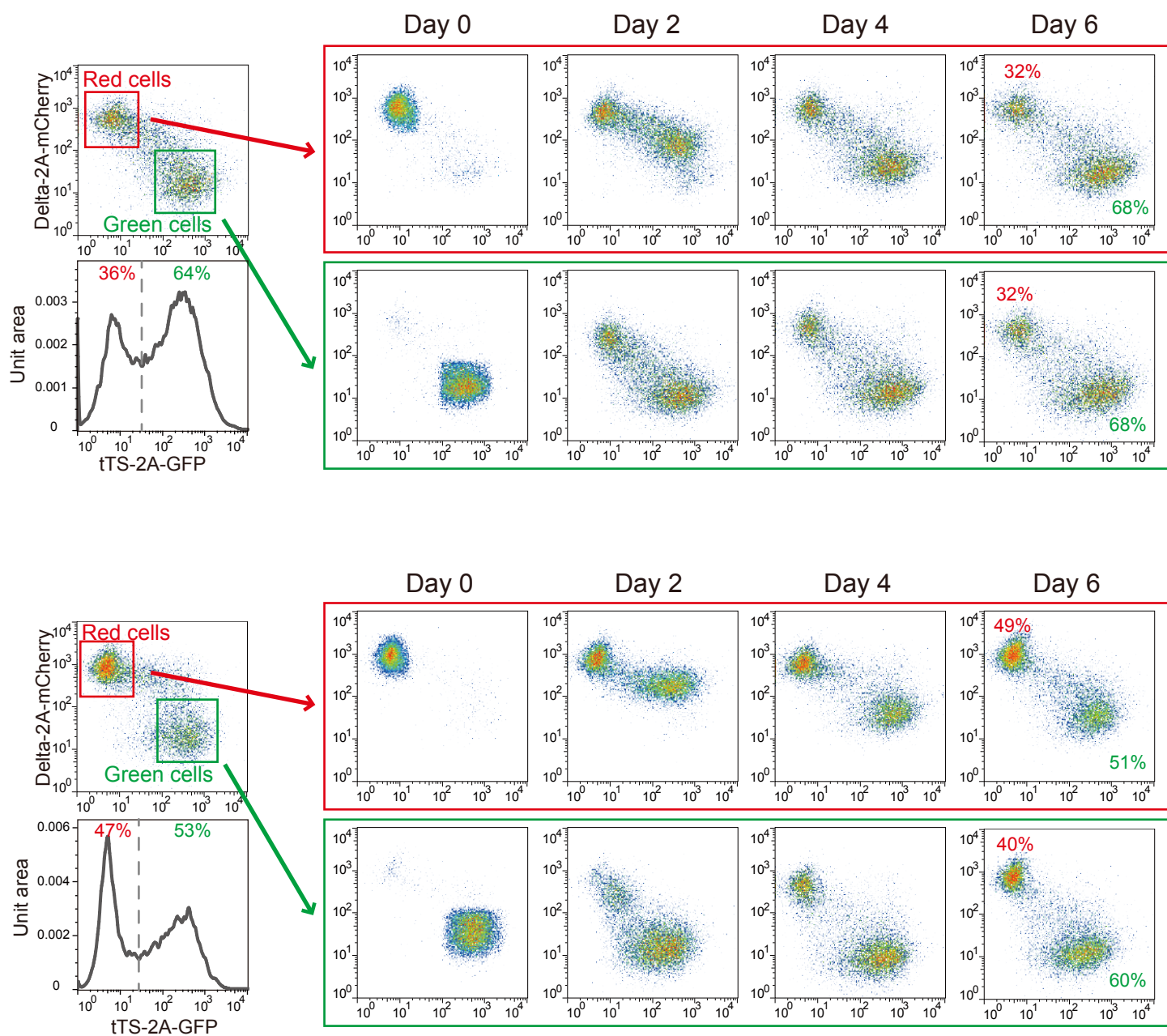
Supplementary Figure 5



Supplementary Figure 5. Time-lapse imaging of cell-type bifurcation in an aTRLF cell colony
(a) Quantification of Fig. 2a. (b) Repeated experiments for Fig. 2a using two other colonies. Scale bar = 100 μ m.

Supplementary Figure 6

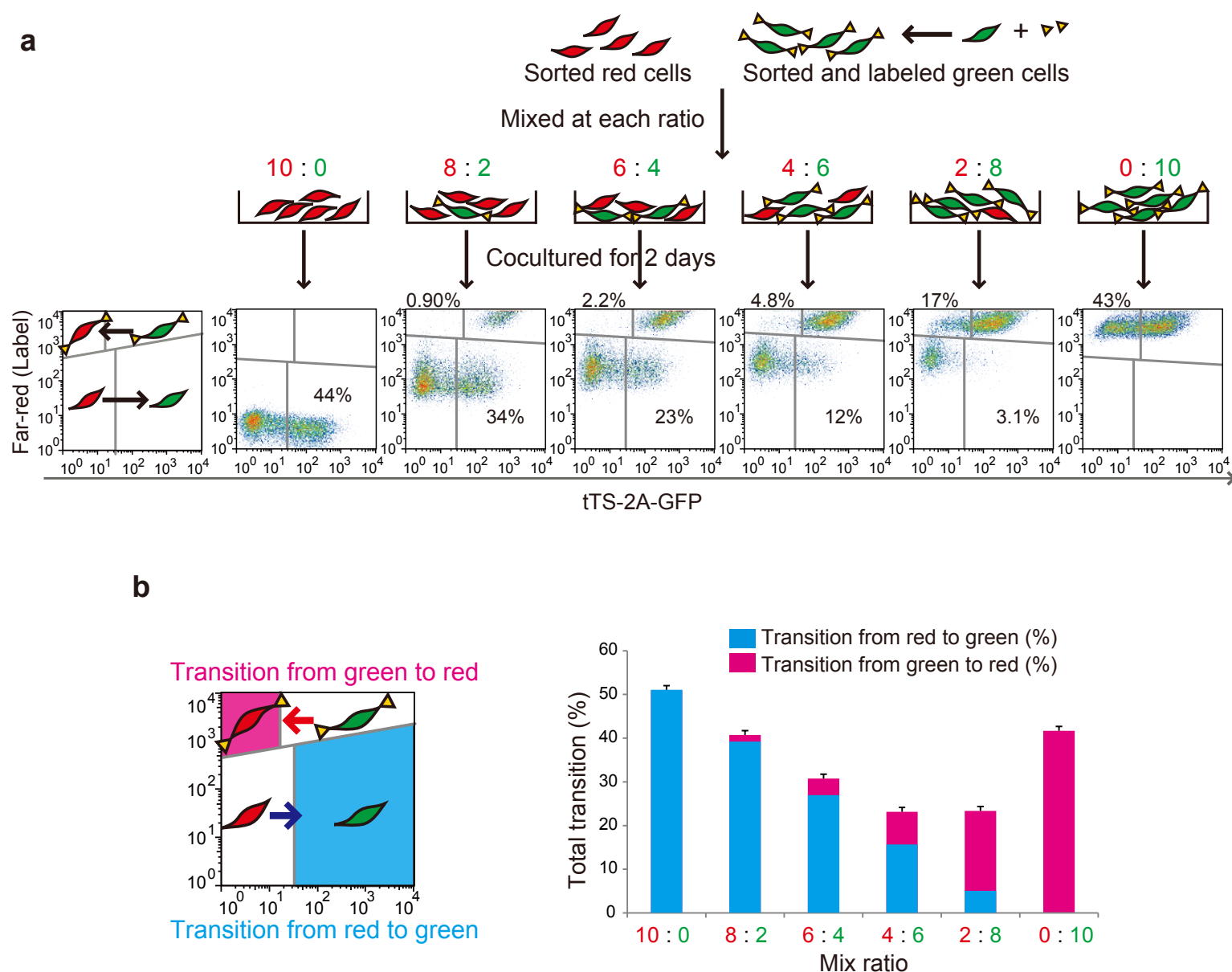
aTRLF clones



Supplementary Figure 6. Robustness of the cell-type ratio

Repeated experiments for Fig. 2c using 2 other aTRLF cell clones.

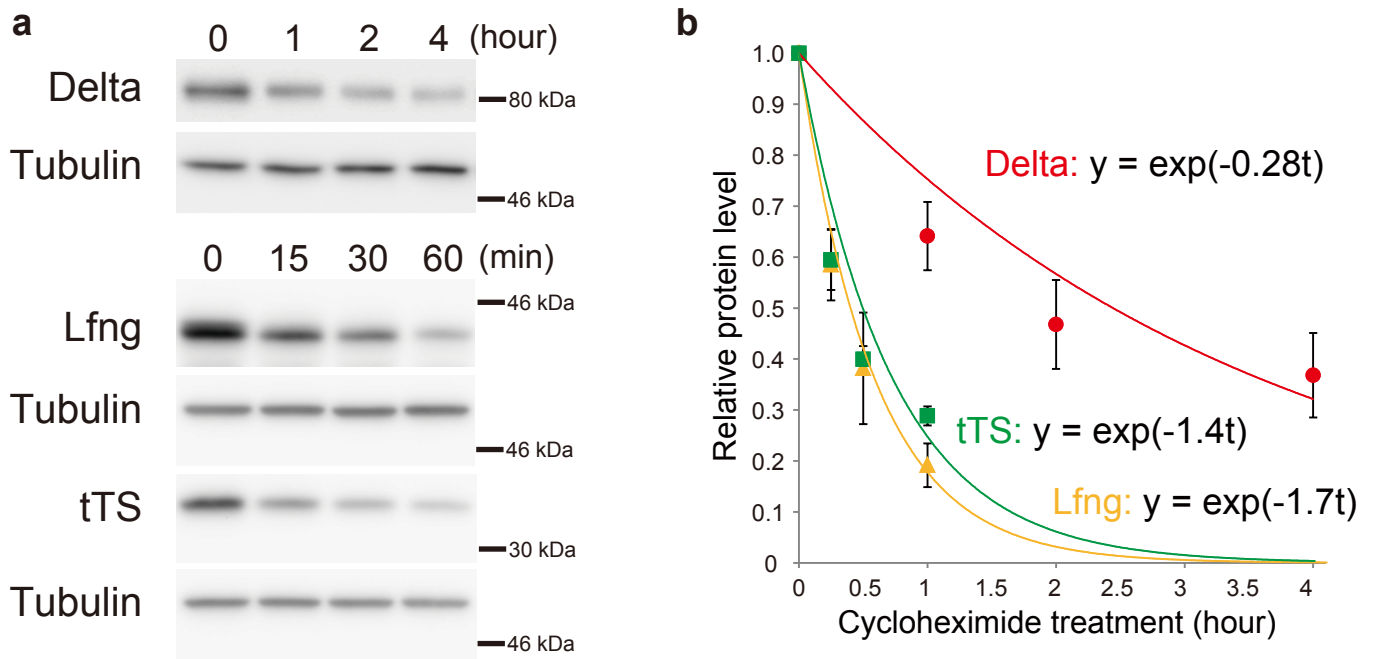
Supplementary Figure 7



Supplementary Figure 7. Static equilibrium of the cell-type ratio

(a) The aTRLF cells were sorted by their colors using FACS. The sorted green cells were labeled with a far-red fluorescent marker. Note that the sorted red cells were labeled in Fig. 3a. The unlabeled red cells and labeled green cells were mixed and cocultured at the indicated ratios. Two days later, the cells were analyzed by FACS for GFP and far-red fluorescence. (b) Quantification of the transition rates shown in (a). The labeled red cells indicate transitions from the green state to the red state, whereas the unlabeled green cells indicate transitions from the red state to the green state. Data are means + SEM (n = 3).

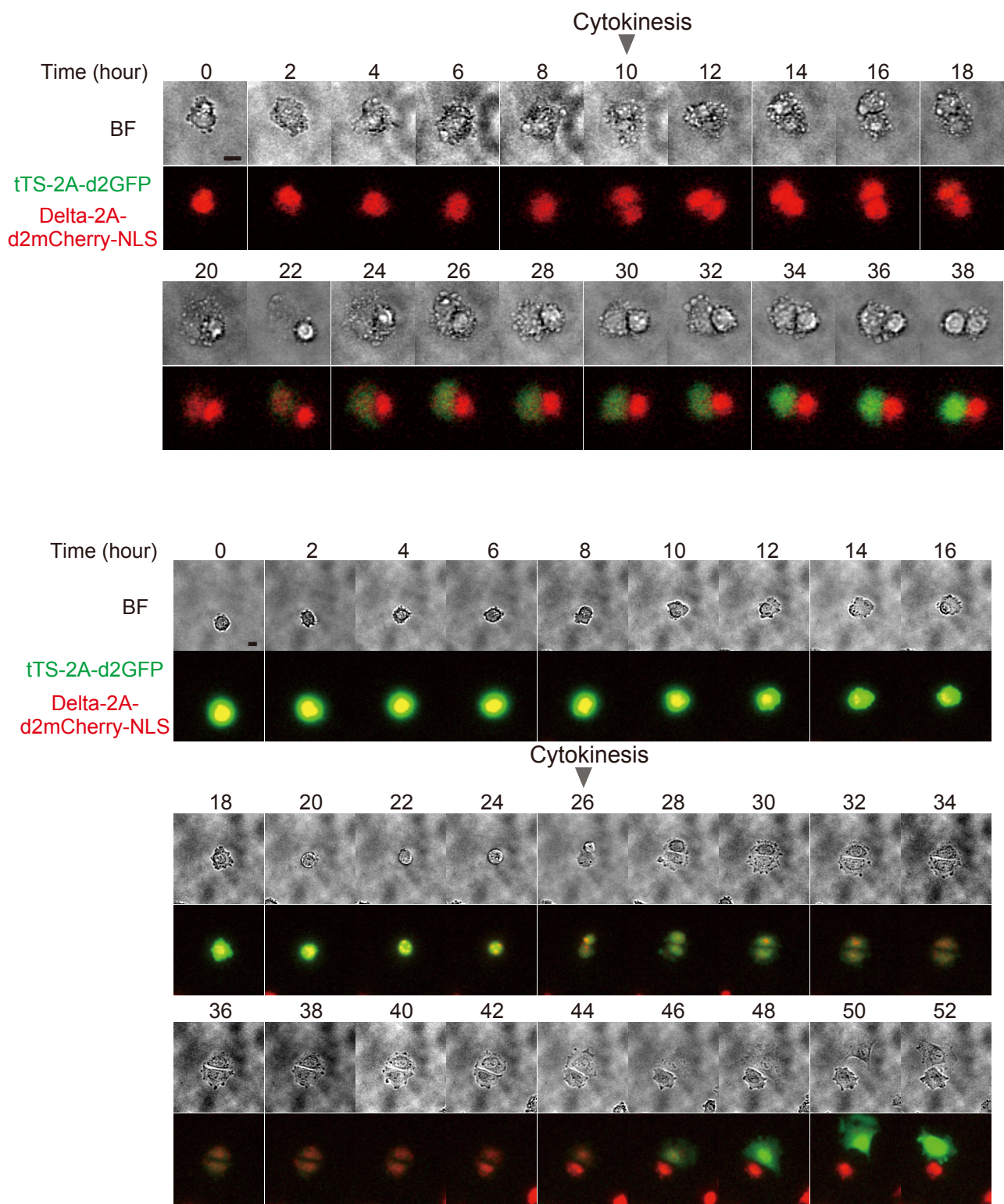
Supplementary Figure 8



Supplementary Figure 8. Measurements of the half-lives

(a) CHO cells that have a P_{CMV} -Delta, P_{CMV} -Lfng, or P_{EF1a} -tTS construct were treated with 20 $\mu\text{g/ml}$ cycloheximide. The expression levels of Delta, Lfng, tTS and Tubulin at each time point were measured by immunoblotting. The controls, Tubulin, were run on different gels. (b) Estimation of the decay rates. The exponential curves were fitted to the obtained data. Data are means $\pm$ SEM ($n = 3$).

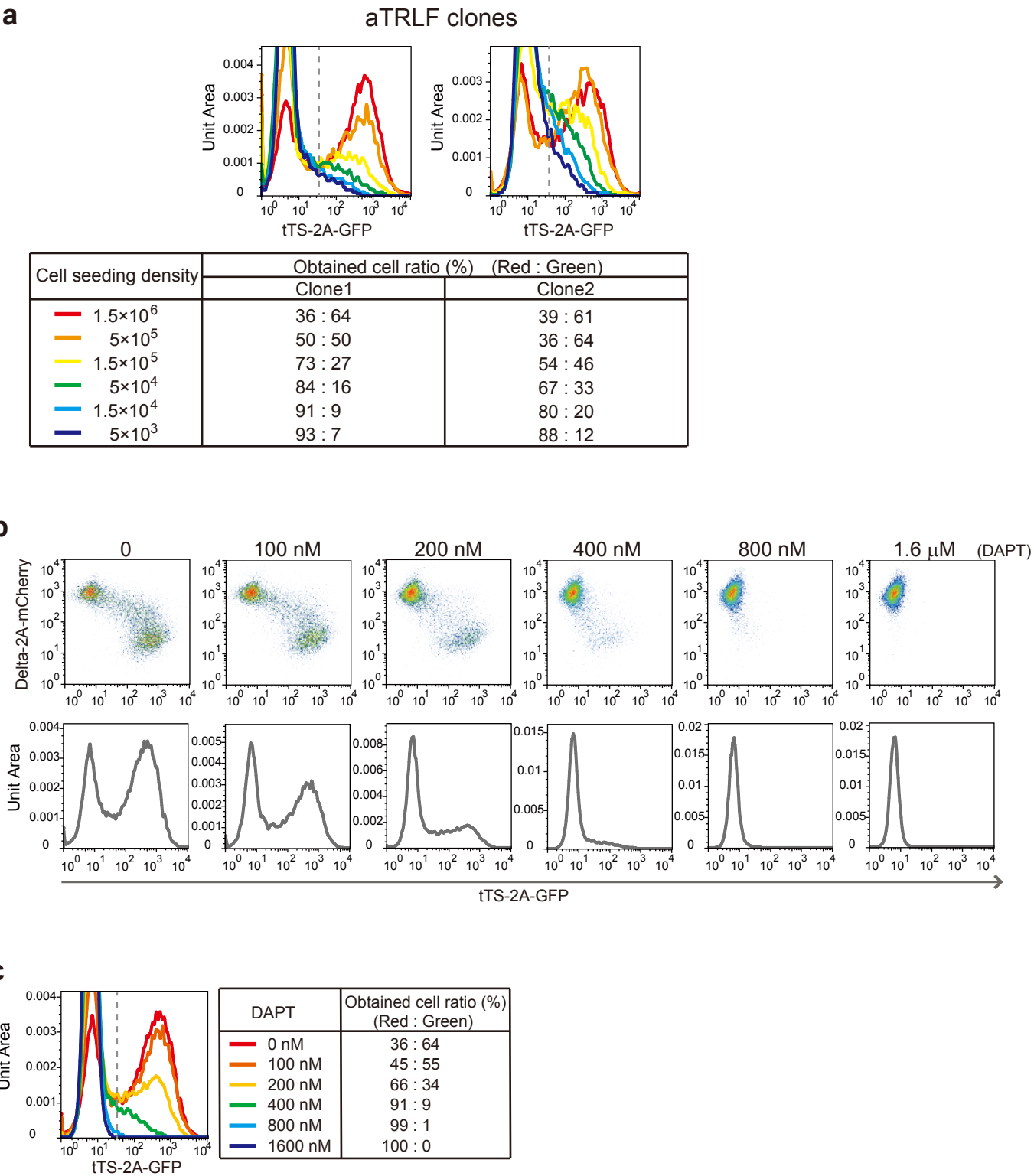
Supplementary Figure 9



Supplementary Figure 9. Cell-type bifurcation between two daughter cells

Repeated experiments for Fig. 4f in 2 other fields. Scale bar = 10 μ m. See Supplementary Movie 4.

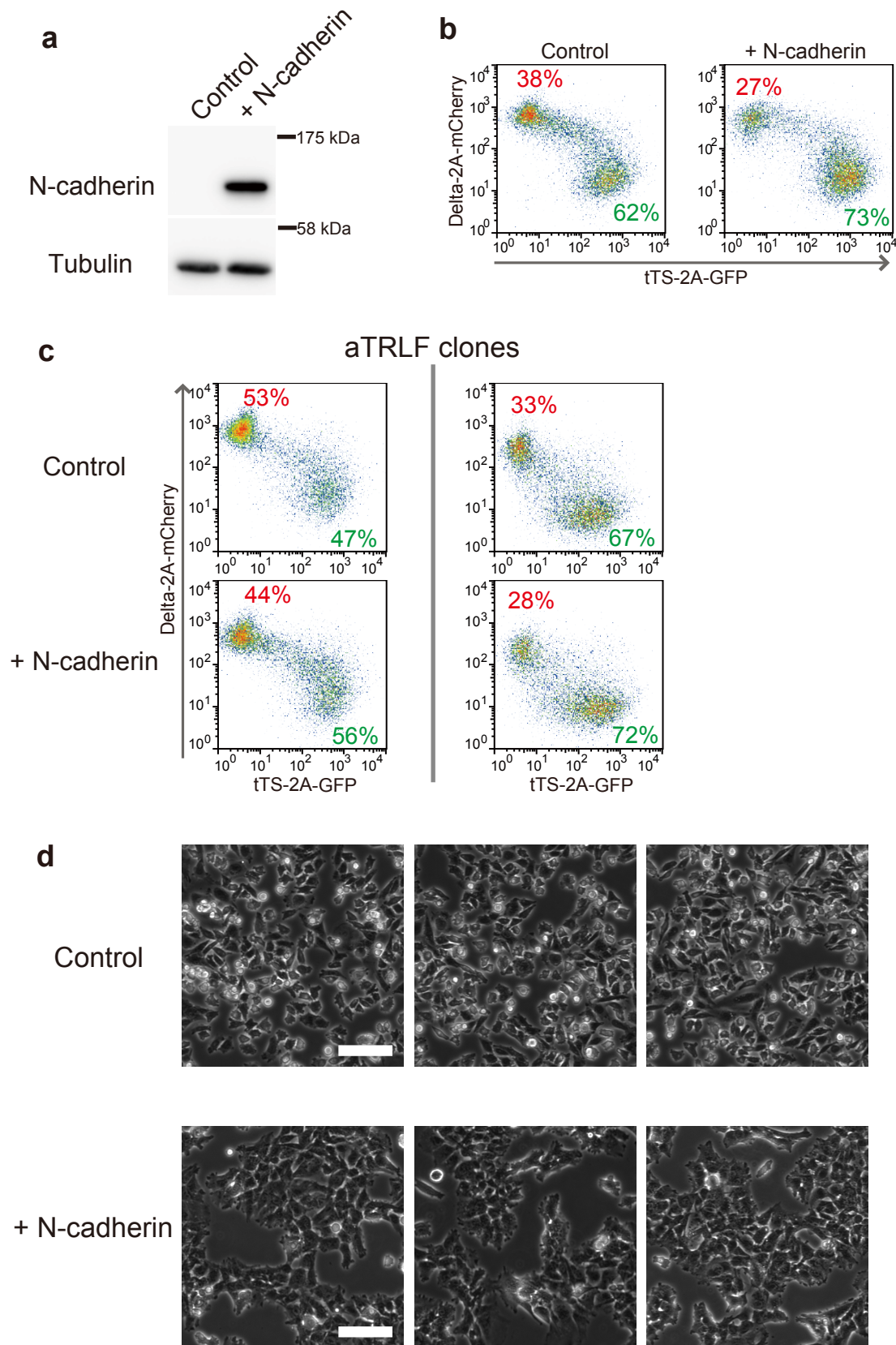
Supplementary Figure 10



Supplementary Figure 10. The cell-type ratio is adjustable by the degree of cell-cell attachment

(a) Repeated experiments for Fig. 5b using 2 other aTRLF cell clones. (b) The relationship between the concentration of DAPT and the ratio of two cell-types. The aTRLF cells were seeded at a density of 8×10^5 cells/35 mm dish, and treated with a γ -secretase inhibitor DAPT at the indicated doses. Two days later, the cells were re-seeded and again treated with DAPT to make sure that the system reaches a steady state. Two more days later, the cells were analyzed by FACS. (c) Quantification of the cell-type ratio of the FACS plots shown in (b).

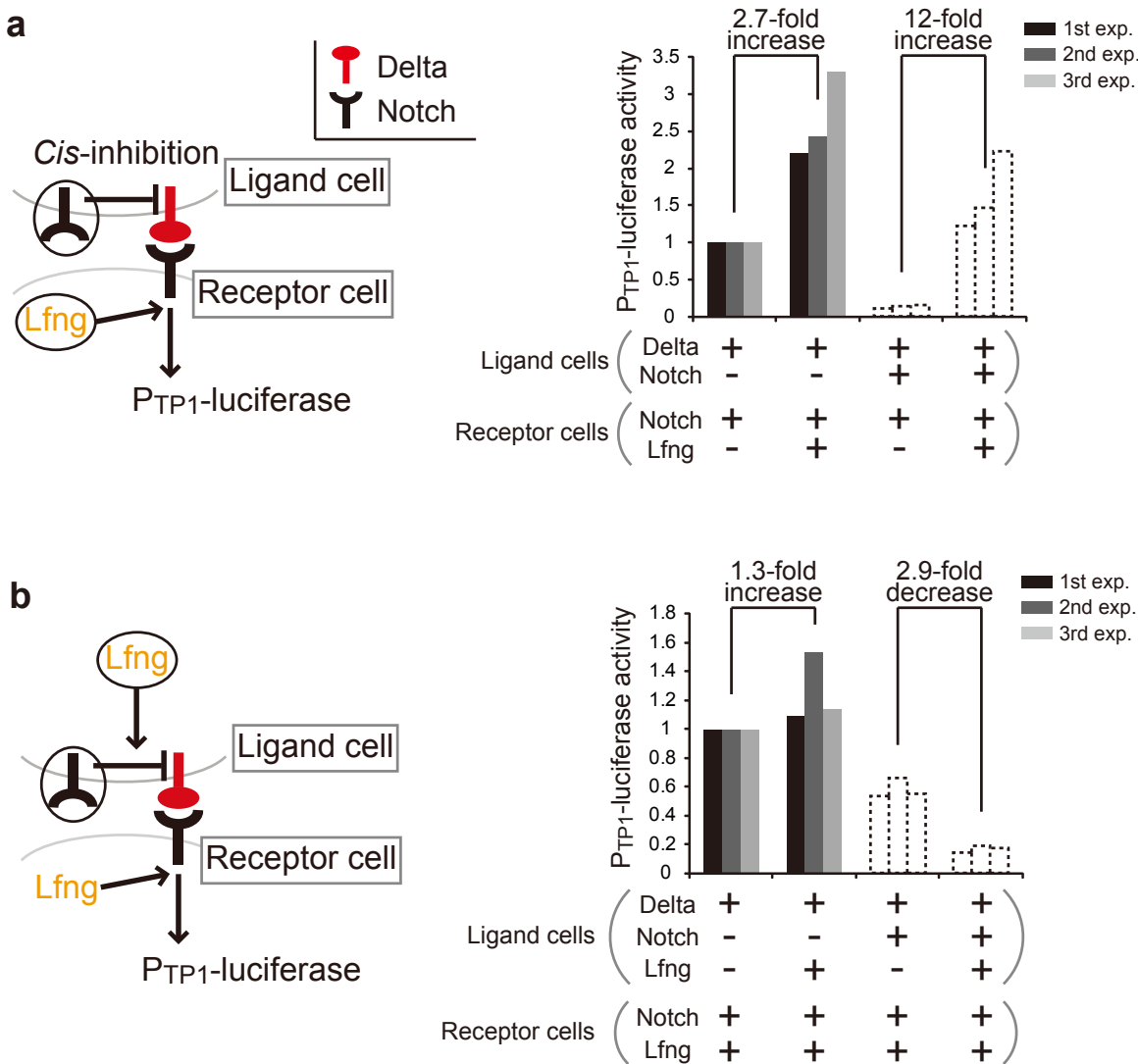
Supplementary Figure 11



Supplementary Figure 11. Effects of N-cadherin overexpression

(a) N-cadherin was introduced into the aTRLF cells. The expression level of N-cadherin was assessed by immunoblotting. (b) Original FACS plots for Fig. 5d. (c) Repeated experiments for (b) using 2 other aTRLF cell clones. (d) Clustering of N-cadherin-expressing cells. The aTRLF cells without or with N-cadherin overexpression were seeded at a density of 8×10^5 cells/35 mm dish. After 7.5 hours, the images were taken. Three different fields are shown. Scale bar = 100 μ m. See Supplementary Movie 5.

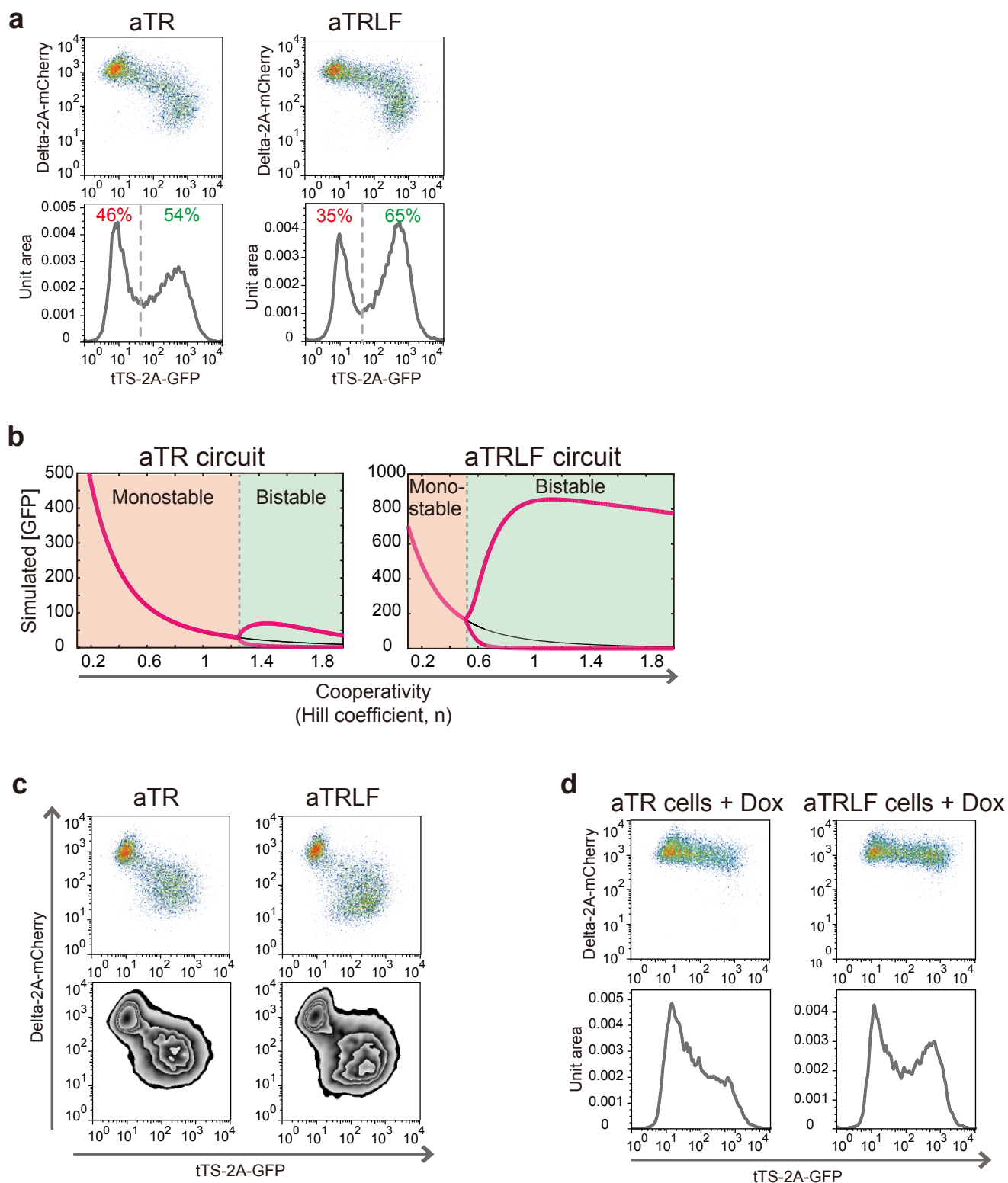
Supplementary Figure 12



Supplementary Figure 12. Dual roles of Lfng in Delta-Notch signaling

(a) A positive effect of Lfng on the Notch receptor. CHO cells that have P_{EF1a}-Notch, P_{TP1}-luciferase, and P_{CMV}-Lfng constructs (Receptor cells) were cocultured with CHO cells that have a P_{SV40}-Delta construct (Ligand cells) at a ratio of 1:9. The luciferase activities were measured in the absence or presence of Lfng in the Receptor cells. Lfng upregulated the luciferase activity only by 2.7 times in the absence of Notch in the Ligand cells. On the other hand, Lfng upregulated the luciferase activity by 12 times in the presence of Notch in Ligand cells (bars with broken lines), as shown in Fig. 6a. Thus, the positive effect of Lfng may be more prominent in the presence of *cis*-inhibition. See Discussion. (b) A negative effect of Lfng on the Delta ligand. CHO cells that have P_{EF1a}-Notch, P_{TP1}-luciferase, and P_{CMV}-Lfng constructs (Receptor cells) were cocultured with CHO cells that have P_{SV40}-Delta and P_{CMV}-Lfng constructs (Ligand cells). The luciferase activities were measured in the absence or presence of Lfng in the Ligand cells. Lfng did not change the luciferase activity significantly in the absence of Notch in the Ligand cells. On the other hand, Lfng downregulated the luciferase activity by 2.9 times in the presence of Notch in the Ligand cells (bars with broken lines), as shown in Fig. 6b. Thus, the negative effect of Lfng may be dependent on *cis*-inhibition. See Discussion.

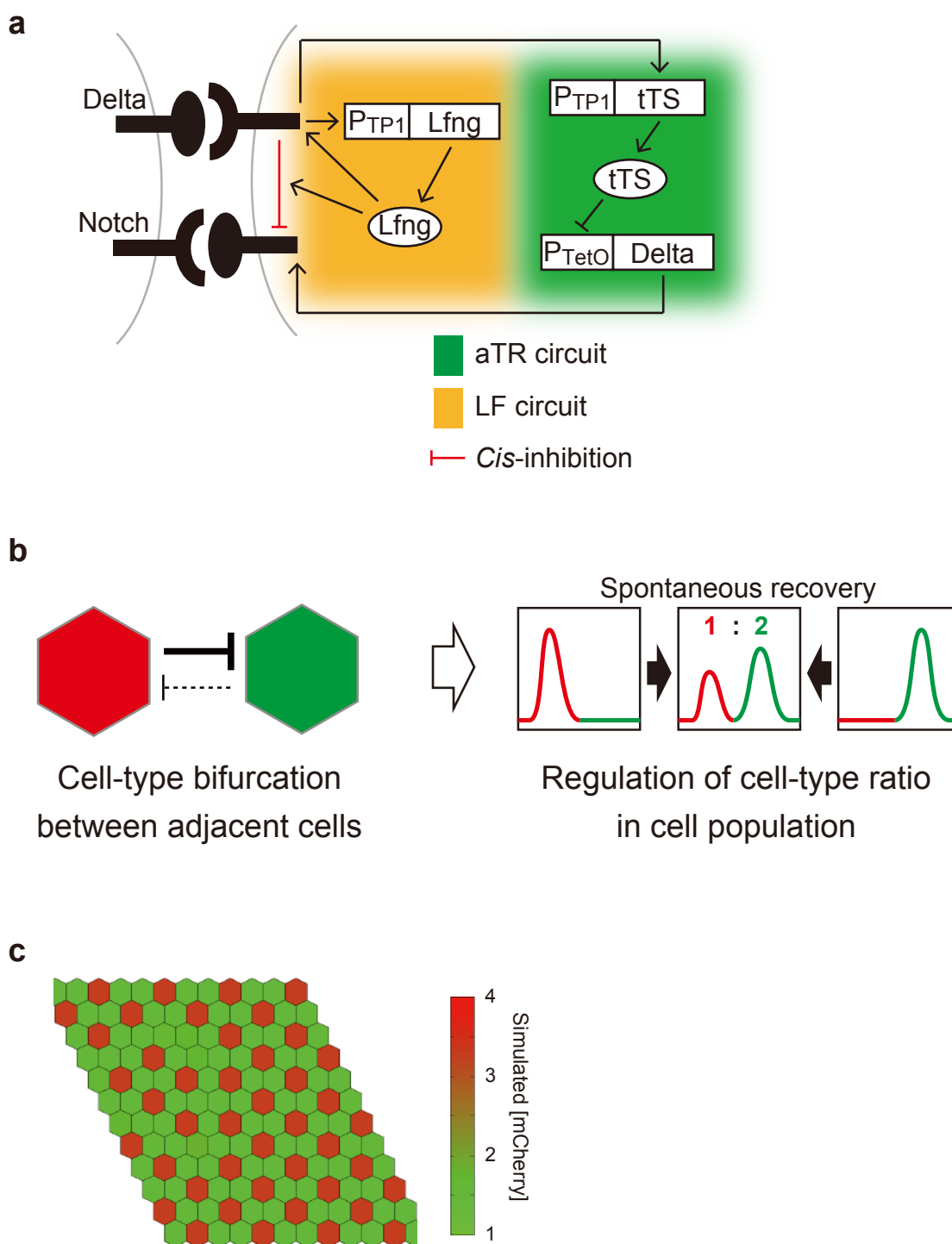
Supplementary Figure 13



Supplementary Figure 13. Comparison of aTR cells with aTRLF cells

(a) Repeated experiment for Fig. 6f using another set of aTR and aTRLF cell clones. (b) Bifurcation diagrams of the mathematical models for the aTR circuit and aTRLF circuit. (c) An aTR cell clone and the corresponding aTRLF cell clone, a different set of cell clones from Fig. 6f, were analyzed by FACS (upper). Contour plots are also shown (lower). The aTRLF cells showed clearer separation of two cell populations in the bimodal distribution. (d) Repeated experiment for Fig. 7c using another set of aTR and aTRLF cell clones.

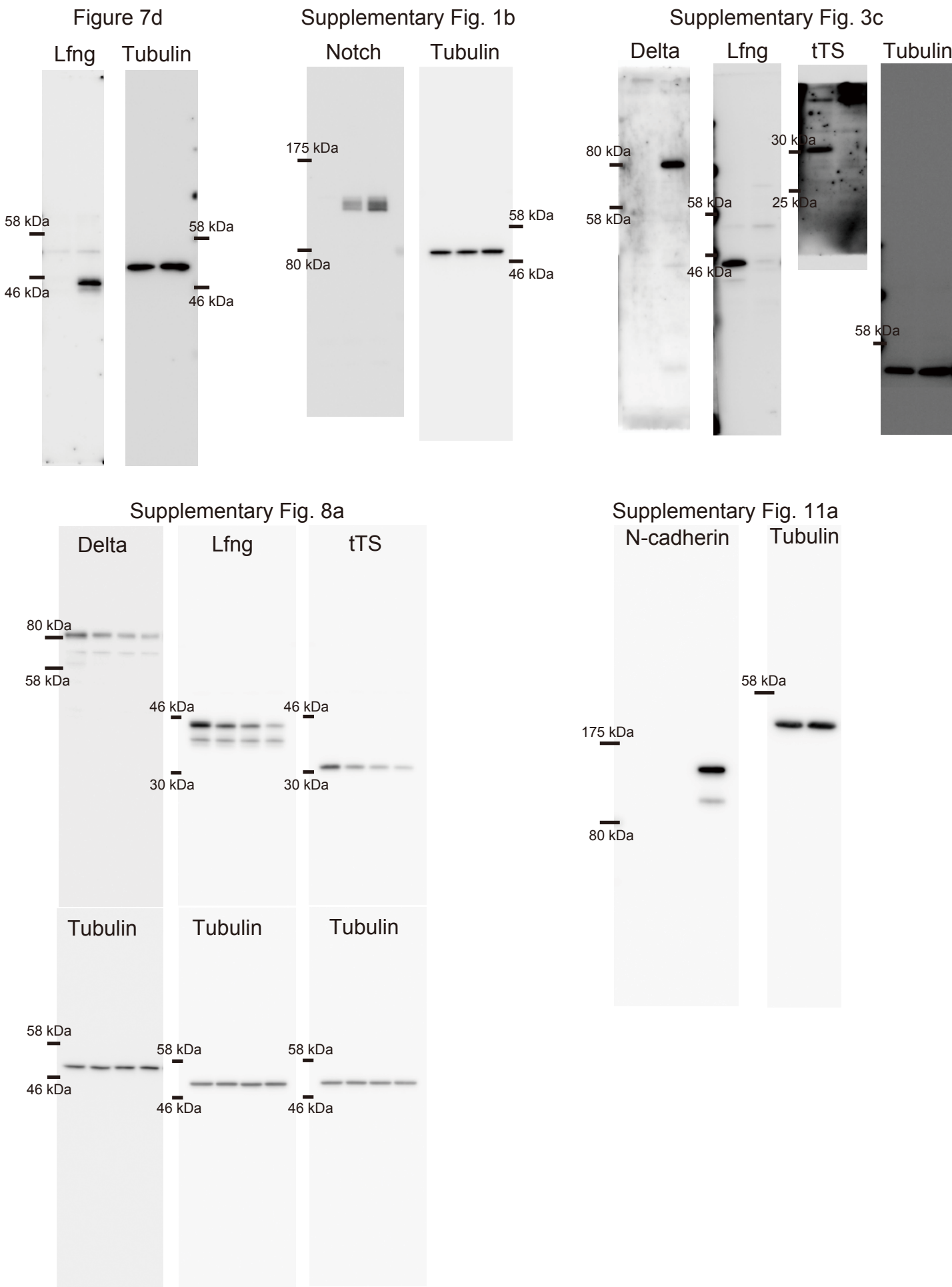
Supplementary Figure 14



Supplementary Figure 14. Lateral inhibition and cell population homeostasis

(a) A revised scheme for the aTRLF circuit, which comprises the aTR circuit and LF circuit. We found that Lfng has dual effects: a positive effect on Notch and a negative effect on Delta. We hypothesize that the negative effect of Lfng may be mediated by *cis*-inhibition. (b) A summary of this study. The lateral inhibition mechanism between adjacent cells not only causes spontaneous cell-type bifurcation but also regulates the cell-type ratio robustly in the cell population. (c) A numerical simulation of the mathematical model for the aTRLF cells with 12×12 cells and a hexagonal cell arrangement. The six immediate neighbors were considered as the adjacent cells. Hill coefficient $n = 2$. A regular salt-and-pepper pattern was formed.

Supplementary Figure 15



Supplementary Figure 15. Original scans of immunoblots