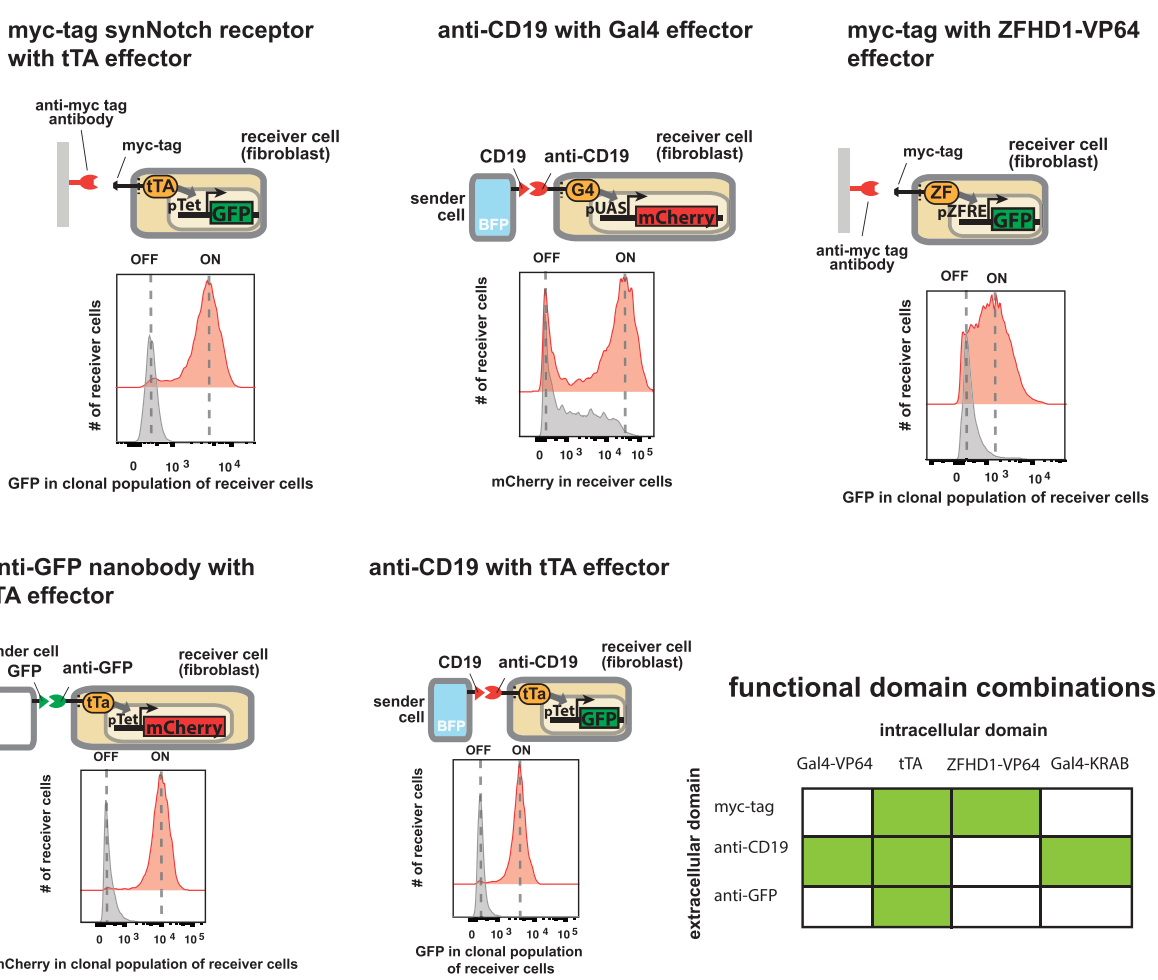
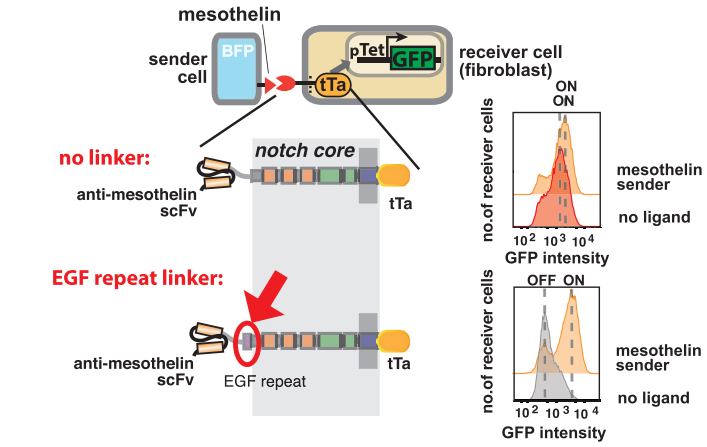
Cell

A




B




C

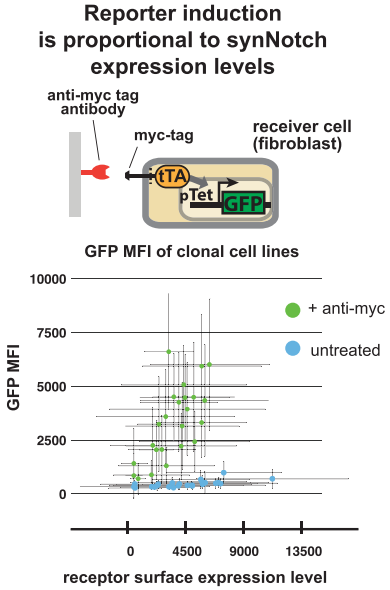



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**Figure S1. Modular Configuration of SynNotch Receptors, Related to Figure 1**

(A) Series of synNotch receiver cells with different extracellular and intracellular domains show activation upon stimulation with cognate ligand. Mouse fibroblasts (L929 line) or human epithelial cells (HEK293, for antiCD19/Gal4 synNotch) are engineered to express the indicated synNotch receptors and the corresponding reporter. The cells are stimulated either by plate-bound stimulus or with K562 sender cells expressing the ligand, as indicated in figure. Results show FACS data from representative experiments with clonal L929 cells or bulk HEK293, as indicated, from at least 10,000 cells per condition. Tested synNotch modular combinations are summarized in table.

(B) Addition of an EGF-repeat on the extracellular domain reduces basal activation of the anti-mesothelin synNotch. Mouse L929 fibroblasts are engineered to express anti-mesothelin synNotch receptors with or without an EGF repeat between the anti-mesothelin ScFv and the Notch core. The activation of these receptors activates a GFP reporter. Upper graph: without the EGF repeats, the induction of the reporter is constitutive even in the absence of the ligand; bottom graph: with the EGF repeats, the basal reporter activation is abolished (OFF line), and the induction brings the GFP intensity to the ON state. Data shown are FACS plots of at least 10,000 cells.

(C) SynNotch expression levels proportionally influence the intensity of reporter activation. Mouse L929 fibroblasts are engineered to express the myc-tag synNotch, and n = 24 clonal lines are obtained. The cell lines are then stimulated with plate-bound ligand (anti-myc antibody), and the graph shows reporter intensity against receptor expression levels as evaluated from Alexa647-anti-myc antibody staining for the 24 clones before and after stimulation. The blue dots are data from unstimulated cells; the green dots are data after stimulation. Data are mean and SD. A positive correlation between receptor expression and GFP intensity is appreciable.

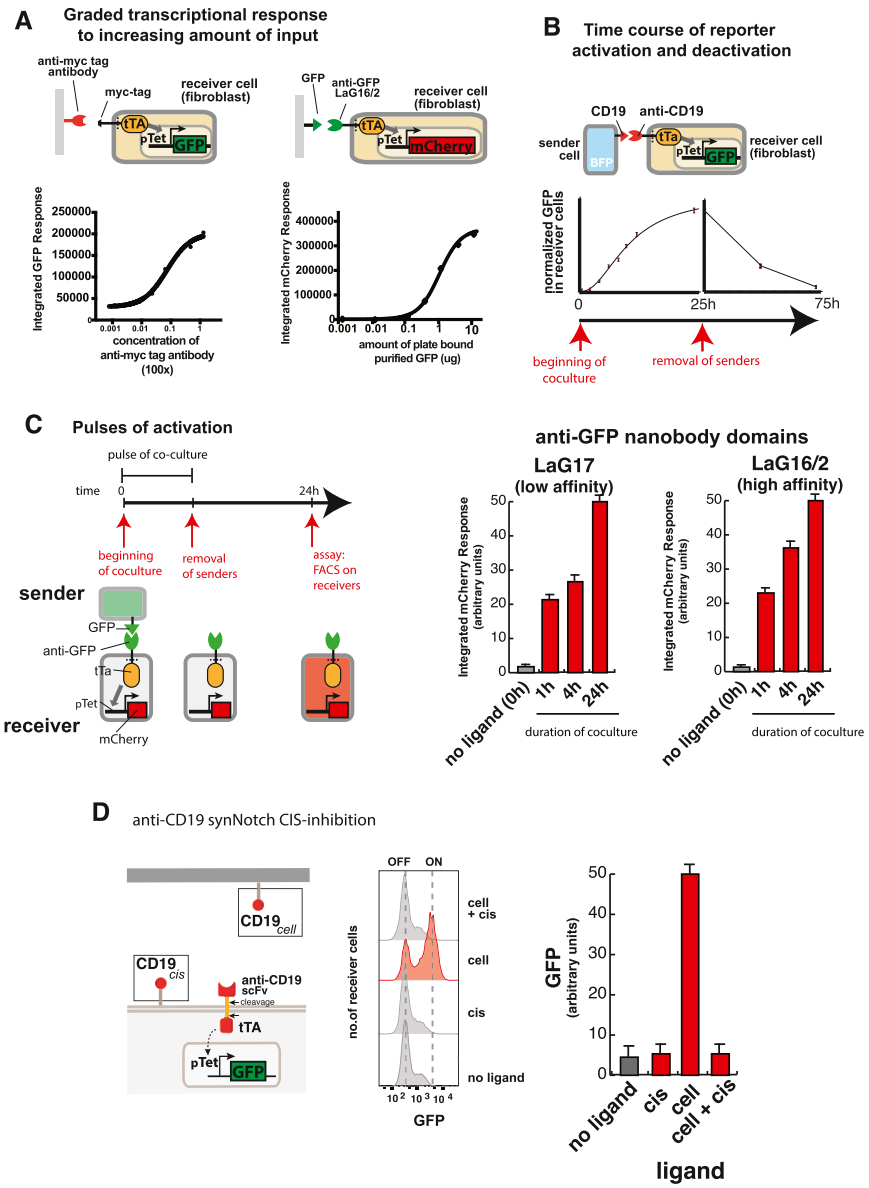


**Figure S2. SynNotch Receptors Can Be Used to Program Contact-Dependent Transcriptional Regulation, Related to Figure 2**

(A) Dose response of activation of receiver mouse fibroblast cells (L929) with different amount of plate-bound ligand. Myc-tag synNotch (left) and anti-GFP synNotch (right) receiver cells are exposed to increasing amount of cognate ligand. The myc-tag synNotch is stimulated with anti-myc antibody immobilized on A/G plates according to manufacturer instructions (Thermo Fisher Scientific); the anti-GFP synNotch is stimulated by GFP that is passively adsorbed on standard tissue culture plates for 24h at 4°C. The reporter integrated intensity at 24h is shown in the graphs, with overlaid sigmoid interpolation line.

(B) Time course of activation of anti-CD19 synNotch receiver cells upon sender cells addition (left); after full activation is reached, removal of sender cells induces inactivation of receiver cells (right). The mean and the SD of reporter integrated intensity after 24h of at least 10,000 cells is shown in the graphs, with overlaid sigmoid interpolation line for the activation.

(C) Pulsed activation. To stimulate anti-GFP synNotch receiver cells for a short amount of time, receiver cells (L929) are seeded on the plate for 24h and then incubated with suspension sender cells (K562s expressing transmembrane GFP) for 1h or 4h; after that, suspension sender cells are washed away, and at t = 24h from the first addition of sender cells the fluorescence in receiver cells is measured by FACS. Bar graphs are integrated fluorescence response of at least 10,000 cells for each condition. Data are average and SE. Data are for anti-GFP LaG17 and LaG16/2 extracellular domains as indicated.

(D) Stimulation of mouse fibroblasts expressing anti-CD19 synNotch with ligands in different formats. anti-CD19 synNotch receiver cells are simulated with K562 sender cells expressing CD19 (cell); the receiver cells in the condition denoted "*cis*" express the CD19 ligand as well. The receiver cells show activation only when the ligand is present on an opposing surface (cell); the expression of the ligand in the receiver cells render the receiver cells unresponsive to surface stimulation (*cis*+cell). The FACS plots are recorded at 24h after the beginning of stimulation. Histograms include at least 10,000 cells for each condition. Data are mean and SE.

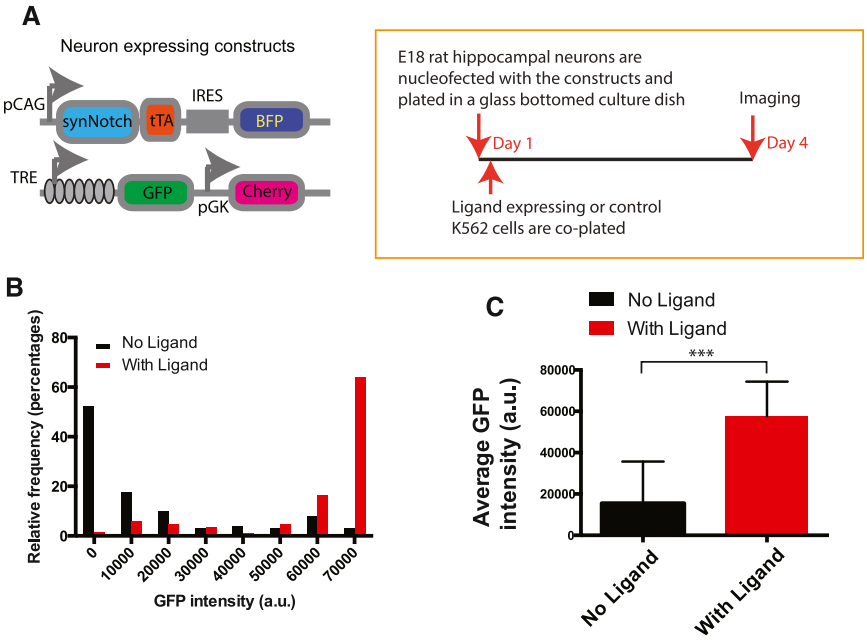


**Figure S3. SynNotch Receptors Work in Primary Neurons, Related to Figure 3**

(A) Demonstration of the experiment and constructs that are expressed in neurons in Figure 3. Primary hippocampal neurons are disassociated from E18 rat embryos and are nucleofected with constructs that express the synNotch receptor as well as the TetO-GFP reporter. Neurons are plated on glass-bottom 35mm culture dish coated with poly-D-lysine and laminin. 2 hr after neuron plating, K562 sender cells are added to the culture to form co-culture system. Images are taken from live cells at day 4 after plating.

(B) Distribution of the GFP fluorescent intensity in 100 neurons for each treatment. GFP intensity is calculated from the fluorescence confocal images.

(C) Quantification of the average GFP fluorescent intensity from about 100 neurons for each treatment. Data are mean and SE.

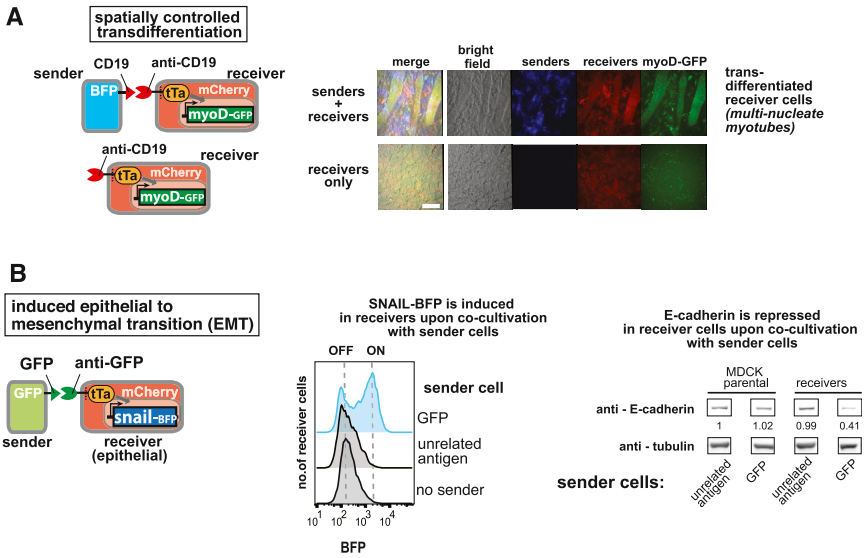


**Figure S4. SynNotch Receptors Yield Spatial Control of Diverse Cellular Behaviors, Related to Figure 4**

(A) High magnification of two fields of view of the endpoint of the experiment shown in Figure 4B. C3H mouse fibroblasts are engineered as follows: sender cells express extracellular CD19 linked to a transmembrane domain, plus a tagBFP marker; receiver cells express the anti-CD19 synNotch with tTA intracellular domain, along with a TRE→myoD effector construct and a constitutive mCherry marker. Sender fibroblasts are plated first in a limited region of the plate; after 1h, the sender cells are attached to the plate, and the receiver cells are plated to cover all the glass plate. Representative fields where receiver cells are next to sender cells (upper) or receiver cells that are not in contact with sender cells (bottom) are shown. Scale bar, 50um.

(B) Epithelial cells (MDCKs) are engineered as follows: receiver cells express the anti-GFP synNotch with LaG17 anti-GFP nanobody as extracellular domain, and tTA as intracellular domain, alongside a TRE→Snail-ires-BFP effector construct. Sender cells are GFP-expressing K562s. FACS plots of receiver cells BFP signal is shown in presence of no sender cells, of sender cells expressing an unrelated ligand, and of sender cells expressing the cognate ligand GFP.

On the right, protein quantification via western Blot reports the E-cadherin expression levels in the receiver cells or in parental cells in the indicated experimental conditions. E-cadherin levels are downregulated in receiver cells only in the presence of the cognate antigen GFP-expressing sender cells (last column).

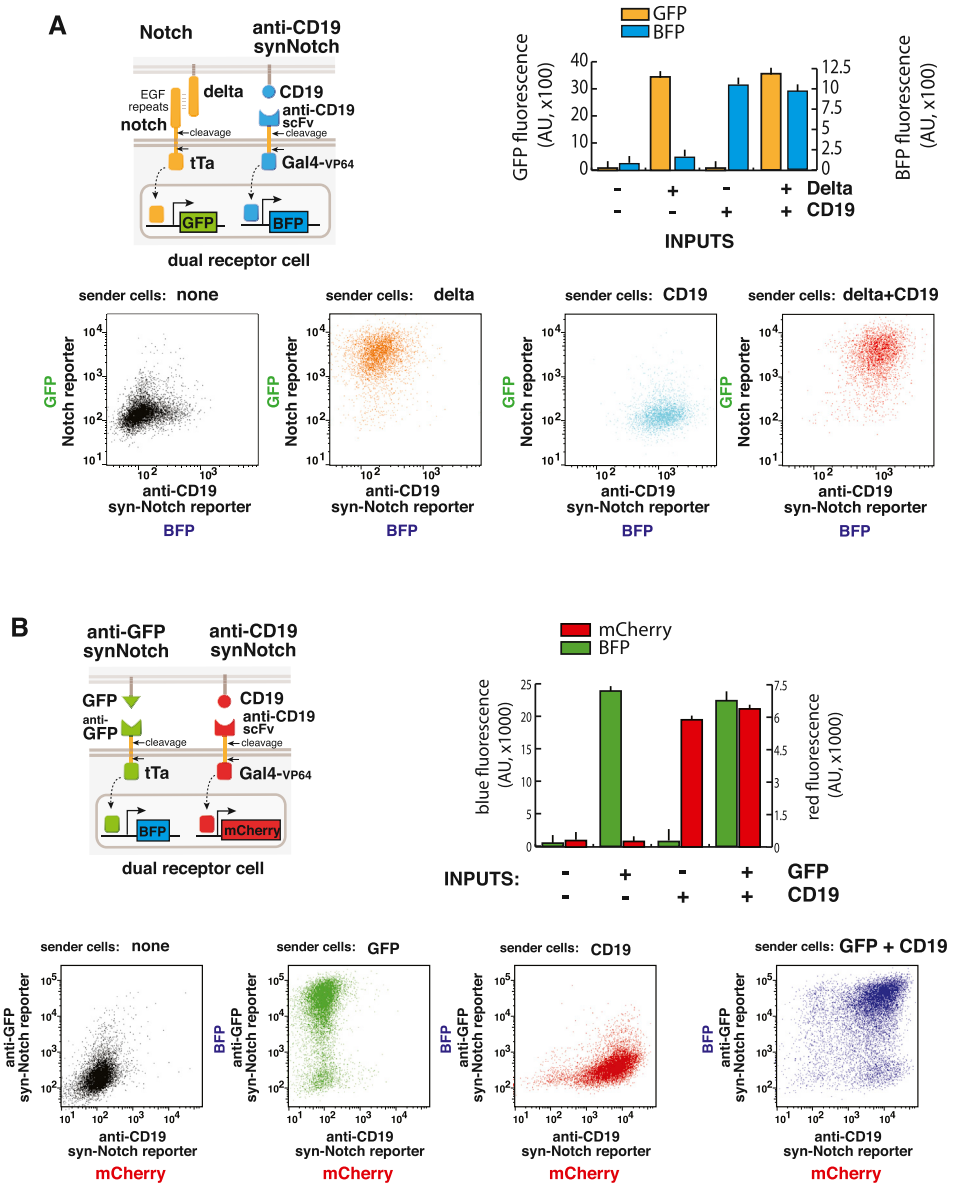


**Figure S5. SynNotch Receptors Are Orthogonal to One Another, Related to Figure 5**

(A) SynNotch and wild-type Notch activate orthogonal signaling pathways. L929 mouse fibroblasts receivers are engineered to express (i) the wild-type Notch receptor with a tTA intracellular domain and a TRE→GFP reporter, and (ii) a synNotch receptor with anti-CD19 extracellular domain and Gal4-VP64 intracellular domain, and a UAS→tagBFP reporter. The graphs on the right show the receiver cell fluorescence signal for the BFP and the GFP reporters in the indicated conditions. Sender cells are mouse L929 fibroblasts. Data are median and coefficient of variation of at least 10,000 cells per condition. On the bottom, single population two-dimensional FACS plots are shown from Figure 5A.

(B) Multiple synNotch receptors are orthogonal to one another. L929 mouse fibroblast receiver cells are engineered to express (i) the anti-CD19 synNotch receptor with a tTA intracellular domain and a TRE→BFP reporter; and also (ii) the synNotch receptor with anti-CD19 extracellular domain and Gal4-VP64 intracellular domain, and a UAS→mCherry reporter. The graphs on the right show the receiver cell fluorescence signal for the BFP and the GFP reporters in the indicated conditions. Sender cells are K562 cells. Data are median and coefficient of variation of at least 10,000 cells per condition. On the bottom, single population two-dimensional FACS plots are shown from Figure 5B.