A

Actual Pathway Activity (simulated)

[Gal4]

Expected Fluorescent Signal

Total Citrine

Reconstructed Pathway Activity

$\frac{d[\text{Citrine}]}{dt}$

Time

B

4epi-Tc

Gal4-T2A-H2B-mCh   H2B-Citrine

H2B-Cit promoter activity ($\frac{dCit}{dt}$, A.U)

4epi-Tc concentration

- 0 ng/ml
- 50 ng/ml
- 100 ng/ml
- 200 ng/ml
- 1000 ng/ml

Gal4-T2A-H2B-mCh promoter activity ($\frac{dmCh}{dt}$, A.U)

C

Raw data

Dll1 (n = 262)
Dll4 (n = 200)

x10^4

Total Citrine (A.U)

Time (h)

D

Alignment only

Dll1
Dll4

Total Citrine (x10^4, A.U)

Time since activation (h)

Alignment + Normalization

Dll1
Dll4

Total Fluorescence (normalized)

Time since activation (h)

Dll1
Dll4

Promoter Activity (x10^2, A.U)

Time since activation (h)

Dll1
Dll4

Promoter Activity (normalized)

Time since activation (h)

E

Dll1

Fraction of cells

Dll4

Fraction of cells

Time of first activation (h)

F

x10^3

Dll1

Promoter Activity ($dCit/dt$, A.U)

Time (h)

Aligned at second activation

Dll1

Dll4

Promoter Activity (normalized)

n = 151 (35%)

Time after second activation (h)

G

Receiver cells on plate-bound Dll1 →

Promoter Activity (Normalized)

n = 120

Time after activation (h)

H

DAPT

[Gal4]

Notch inhibited

time (h)

Measured (n = 66 cells)
Fit (Gal4 t$_{1/2}$= 4h
Citrine t$_{1/2}$ = 3.4h)

Median Promoter Activity (normalized)

time (h)

I

t$_{rise}$ = 6 h
95% CI = [5.7, 7.1]

FWHM = 12.2h
95% CI = [12, 13.3]

Measured Promoter Activity (normalized)

Time since activation (h)

J

Simulated response to activation pulse

Simulated FWHM (h)

Simulated t$_{rise}$ (h)

Approx. measured values

Activation pulse duration

5 min   15 min   30 min   1h   2h

K

15 min activation pulse

Gal4 (protein)
Citrine (mRNA)

Simulated Promoter Activity (normalized)

Time since activation (h)

(legend on next page)

Figure S1. Characterization of Diverted Notch Reporter System and Dll1 Pulses, Related to Figure 1

(A) Simulation showing how the derivative of total fluorescence (d/dtCitrine = 'Promoter Activity') can recover underlying Notch activation dynamics. In response to a simulated pulse of Gal4 (orange trace), H2B-Citrine reporter fluorescence increases ('Total Citrine', green). The first derivative of total Citrine fluorescence (blue trace) provides a reconstructed estimate of the active Gal4 concentration (compare 'Reconstructed Pathway Activity' and 'Actual Pathway Activity').

(B) Correlation between Gal4 levels and promoter activity of the fluorescent reporter gene. (Inset) The cell line used for experiment expresses Gal4 (white, with a co-translationally expressed H2B-mCherry cassette, red) under control of a 4epi-Tetracycline ('4epi-Tc') inducible promoter. These cells also contain a fluorescent H2B-Citrine reporter gene (purple) that is responsive to Gal4 protein. (Main plot). Scatterplot of maximal (Gal4-T2A)-H2B-mCherry production rate (95th percentile value of promoter activity (d/dtmCherry) versus the maximal promoter activity of the H2B-Citrine reporter gene rate (95th percentile value of promoter activity d/dtCitrine), for different levels of 4epi-Tc induction. Each circle represents the response in a single cell. The line connects median values within equally-spaced bins of H2B-mCherry levels, and error bars indicate 25th to 75th percentile levels of the distribution of values within each bin. Cell-to-cell variability in induction of the reporter is partly due to extrinsic noise and can be observed in the promoter activity of a co-expressed constitutive H2B-Cerulean gene. Note that since the units of total fluorescence are arbitrary, the resulting derivatives are also in arbitrary units (A.U).

(C) Average of unprocessed total fluorescence traces (representing cumulative Notch activity) from the same set of activating cells plotted in Figures 1D and 1E. No alignment, normalization, or time derivative was applied to the data. A pulse of activity would lead to an initial rise in fluorescence followed by a plateau (as for Dll1, blue), while sustained activity would result in a continuous increase in total fluorescence (as for Dll4, red). Solid colored lines indicate median values, shaded regions indicate s.e.m. 'n' corresponds to number of traces included in the average.

(D) (Left) Median of total Citrine (top) and promoter activity (bottom) after alignment at the point of activation, defined as t = 0 (see STAR Methods). (Right) Corresponding plots after individual traces were not only aligned, but also individually normalized to the 90th percentile value within the averaging time window (STAR Methods). Note that these plots are the same as Figures 1D and 1E.

(E) Distribution of time points at which cells first activate during the Dll1 (top) or Dll4 (bottom) excess sender co-culture. Note that the time period prior to 15h was not considered in the analysis in order to eliminate contributions from transient effects on the reporter after transfer of cells to imaging conditions. Activation events in sender-receiver co-cultures are also delayed relative to the time of plating because cells are plated at sub-confluence (STAR Methods). Error bars indicate s.e.m (n = 2 experiments).

(F) (Left) Representative traces of cells displaying two pulses during co-culture with Dll1 (same dataset as in Figures 1D and 1E). Arrowheads indicate pulse peaks. See Movie S3. (Right) Median response profile of receiver cells activated by Dll1 that could not be classified as single pulses, aligned after a period corresponding to the first pulse (7.5h, see STAR Methods). Each trace is aligned at a point when the promoter activity subsequent to the initial phase of activation (0-7.5h) again reaches the peak activity of the initial phase (n = 151 traces, 35% of all traces, cf. Dll1 response in Figure 1D). Each trace is individually normalized to the 90th percentile of promoter activity value between the alignment point and the end of the trace. Solid line indicates the median of the normalized promoter activity, light shaded areas show s.e.m, and the light gray shaded area indicates the standard deviation. The dark gray box indicates time period prior to the alignment time point, and the dashed horizontal line indicates the median level of (normalized) promoter activity at the alignment point. The median response in Dll1 returns to this level after 25h, consistent with a systematic second pulse of activity. (Inset) The same procedure applied to the Dll4 data (from Figure 1D) does not reveal a second pulse.

(G) Median promoter activity of receiver cells (same as Figure 1B) cultured on plate-bound Dll1ECD-Fc. Also shown are standard errors of the mean (light colored lines) and standard deviations (gray areas).

(H) (Top, schematic) Expected Gal4 response (orange trace) in cells allowed to activate for 7h, then inhibited (indicated by gray shading) by treatment with DAPT (t = 0h). (Bottom) Median promoter activity in receiver cells (blue line) after DAPT treatment. Red line shows simulated response using fitted parameters for Gal4 protein half life ('Gal4 t1/2' = 4h, 95% bootstrapped confidence interval [3.8h, 4h]) and H2B-Citrine mRNA half life ('Citrine t1/2' = 3.4h, 95% bootstrapped confidence interval [3.4h, 3.5h]). See STAR Methods for fitting procedure and Supplementary Information for model.

(I) Median (blue line) and standard error of the mean (lighter blue region) of normalized Dll1 pulse promoter activities (same as Figure 1E). 'trise' denotes time from 10% to 90% of the peak promoter activity, and FWHM denotes the full-width at half-maximum of the peak promoter value. '95% CI' indicates bootstrapped confidence intervals.

(J) Dependence of FWHM (blue circles) and trise (purple circles) values on duration of underlying Notch activation ('Activation pulse duration') based on a mathematical model for pulse-like activation, using values calculated in panel H (see Supplemental Information). The horizontal gray line indicates measured values for these quantities.

(K) Simulated time-course of Gal4 protein (orange, dashed) and H2B-Citrine mRNA (blue) for 15 min Notch activation, using values for half-lives calculated in panel H. Note similarity with measured pulse shape in panel I.
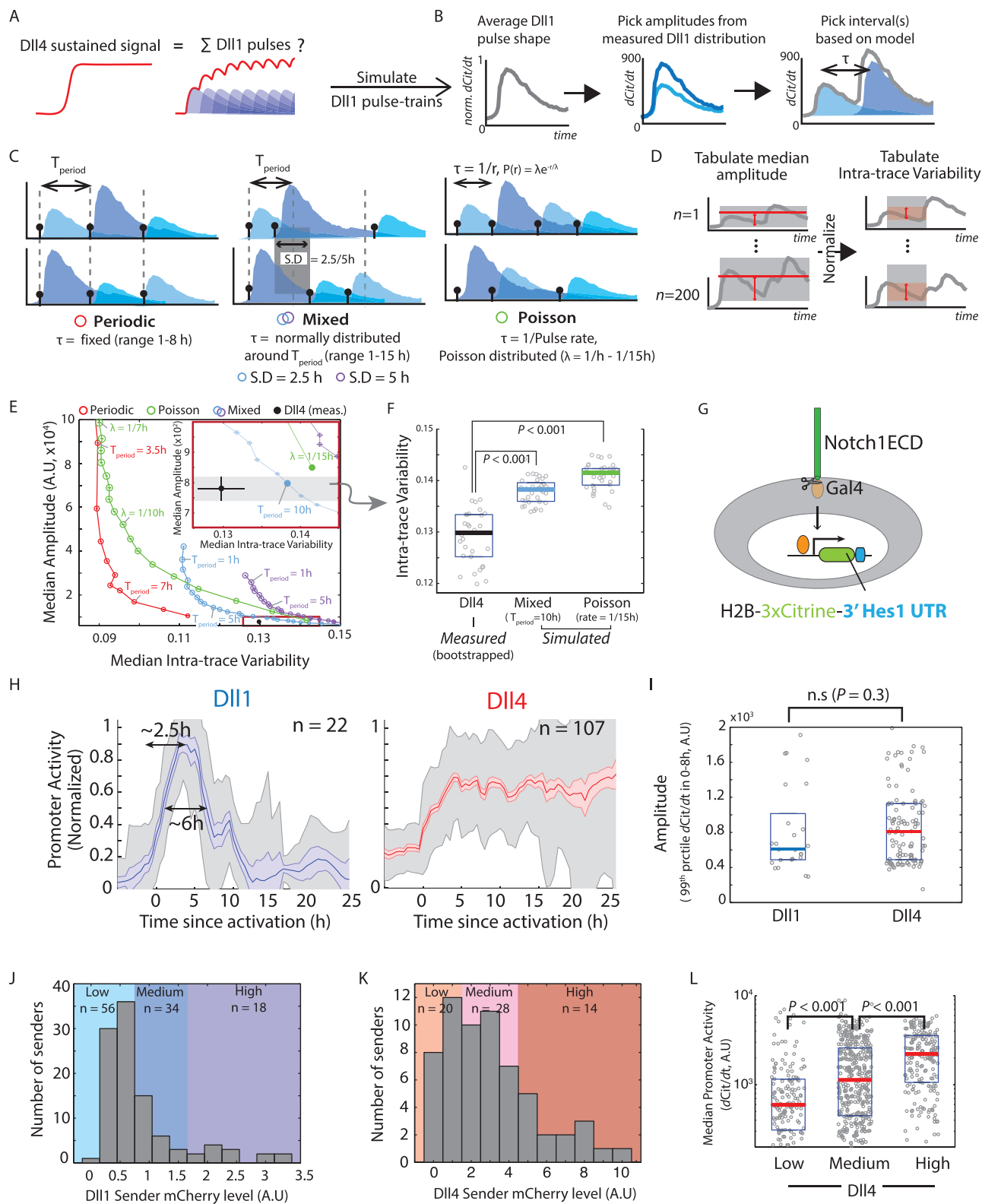
Figure S2. Pulse Train Model and Analysis of Increased Time-Resolution Reporter, Related to Figures 1 and 2

(A–F) Comparison of simulated Dll1 pulse trains to observed Dll4-induced responses.

(A) (*Schematic*) Can sustained Dll4 signal (red) be composed of a series of Dll1-like pulses (blue)?

(B) Schematic diagram illustrating the pulse train simulation procedure (see also Supplementary Information). Each pulse train is constructed from a series of pulses with the average Dll1 pulse shape (*Left schematic,* median of averaged traces in Figure 1D), scaled by an amplitude randomly sampled from the empirically measured distribution of Dll1 pulse amplitudes (*Center schematic*, measured amplitude distribution shown in Figure 1F). Each pair of adjacent pulses (shades of blue) is temporally separated by an interval τ chosen based on one of the underlying pulse models shown in panel C, and combined to generate a particular pulse train (*Right schematic,* gray line).

(C) Schematics illustrating the underlying pulsing models. In each case two example traces are shown (top and bottom). (*Left*) In the Periodic model, the interval τ between adjacent pulses is fixed at a value T_{period}, that can range from 1h to 8h. Periods greater than 8h result in oscillating pulse trains in which the individual pulses can be clearly discerned. (*Right*) In the Poisson model, the interval between pulses i and $i+1$, τ_i, represents the inverse of a pulse rate, r_i, drawn from a Poisson distribution with parameter, λ, ranging from 1/h - 15/h. (*Center*) The mixed models interpolate between fixed and random intervals. In these models, the interval τ between adjacent pulses is drawn from a normal distribution with mean T_{period} (range 1- 15h) and standard deviation σ (= 2.5h or 5h). See Supplemental Information for further details.

(D) For each simulation, 200 pulse trains were simulated ('n' = 1 to 200). For each simulated trace, the median amplitude (*Left*) and the median temporal variability ('Intra-trace' variability, *Right*) were tabulated (see Supplemental Information).

(E) Relationship between median amplitude and median intra-trace variability for the different models. Each point corresponds to a different mean interval, and represents the median of 36 simulations. The black marker shows measured values of median amplitude and median intra-trace variability for Dll4 traces (using the data in Figures 1D and 1E). (*Inset*) Zoom into the region (red box) of the Dll4 data point (black). Error bars on Dll4 marker represent s.e.m. (n = 200 traces). The gray shaded area delineates region of the plot that has median amplitude within the measured error in the Dll4 median amplitude. Filled circles are points in the mixed (σ = 2.5h, T_{period} = 10h) and Poisson (λ = 1/15h) models closest to the experimental measurement.

(F) Boxplot comparison between median intra-trace variability in the simulations highlighted in panel E (inset) and the intra-trace variability measured for Dll4. For simulations, each point represents a single simulation (comprising 200 pulse trains). The colored horizontal lines (blue or green) represent the median value of these simulations (n = 36 simulations), while boxes delineate 25th – 75th percentile values. The black horizontal line represents a bootstrapped average of intra-trace variability values calculated from measured Dll4 data. See Supplemental Information for further details. *P*-value calculated by two-sided KS-test.

(G) (*Schematic*) Improved time-resolution reporter cell line, expressing the NotchECD (green)-Gal4 (orange) receptor and a Gal4-responsive H2B-3xCitrine (chartreuse) fused to 3′UTR (blue) derived from the mouse Hes1 gene (*cf.* Figure 1B, see STAR Methods).

(H) Median normalized promoter activity of reporter cells co-cultured with either Dll1- (blue) or Dll4- (red) expressing sender cells. Also shown are s.e.m. (light colored lines) and standard deviations (gray areas). See STAR Methods for alignment and normalization procedure. Note the decreased values of rise time ('t_{rise}') and full width at half max ('FWHM') of the Dll1 pulse compared to S1I, which shows corresponding values for the original reporter cell line.

(I) Boxplots of maximal promoter activities of reporter cells in response to Dll1 (blue) or Dll4 (red), calculated for the traces averaged in H. Colored lines represent median values, and boxes delimit 25th to 75th percentile values. *P value* calculated using two-sided KS-test.

(J), Distribution of mCherry levels in Dll1-T2A-H2B-mCherry sender cells used in Figures 2B and 2C, with 'Low', 'Medium', and 'High' fractions delineated. 'n' values correspond to the number of sender cells in the experiment (*cf.* Figure 2B).

(K) Distribution of mCherry levels in Dll4-T2A-H2B-mCherry sender cells used in Figures 2B and 2C, with 'Low', 'Medium', and 'High' fractions delineated. 'n' values correspond to the number of sender cells in the experiment (*cf.* Figure 2B).

(L) Comparison of *median* promoter activities in activated receiver cells adjacent to sender cells expressing Low (light blue), Medium (navy blue), or High (dark blue) levels of Dll4 (same designations as used in Figure 2B). Grey circles represent individual responses, solid horizontal lines represent medians, while the boxes delineate 25th - 75th percentile values. *P*-values calculated by two-sided KS-test.
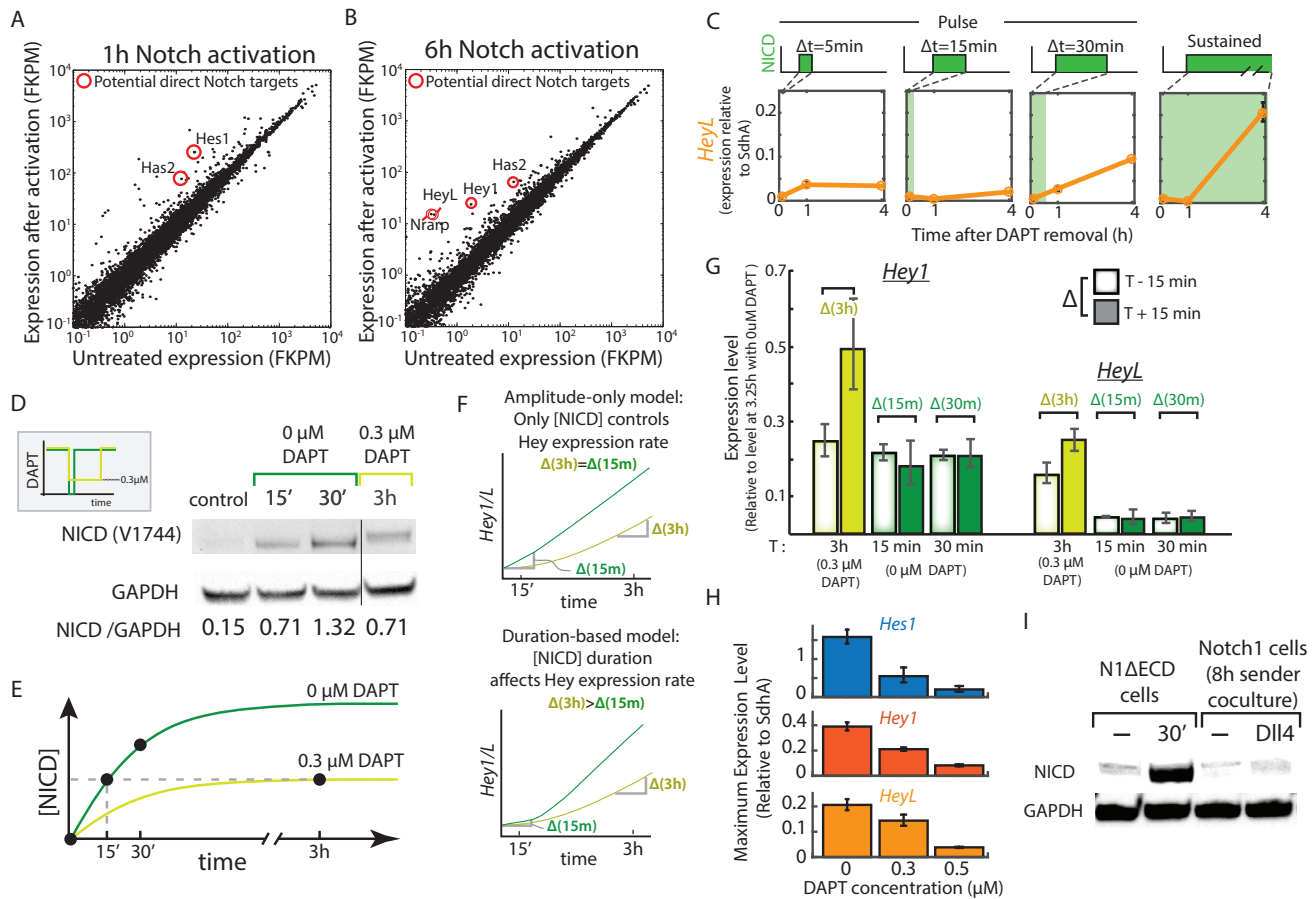
Figure S3. Duration Dependence of Gene Expression in C2C12 Cells, Related to Figure 3

(A) Transcript levels in C2C12-N1ΔECD cells in DMSO-treated cells versus cells activated for 1h by DAPT removal. Circled genes are putative direct Notch targets, upregulated by > 5-fold (Table S1).

(B) Transcript levels in C2C12-N1ΔECD cells in DMSO-treated cells versus cells activated for 6h by DAPT removal. Circled genes are putative direct Notch targets upregulated by > 5-fold (Table S1).

(C) Response of HeyL (orange) to complete DAPT removal for 5 min, 15 min, or 30 min followed by replenishment ('Pulse'), or removal without replenishment ('Sustained'). Error bars represent s.e.m. for duplicate experiments.

(D) (*Schematic*) For the experiment, DAPT is either washed out completely (final concentration 0 μM) for a brief period (15 min or 30 min, chartreuse line) or partially (final concentration 0.3 μM) for 3h (green line). (*Main panel*) Western blot analysis of cleaved NICD levels in cells after different DAPT washout treatments. NICD is detected using an antibody that detects the cleaved version (with N-terminal Val at amino acid position 1744, see STAR Methods). GAPDH levels represent the loading control. Note similar levels of NICD at 15' and 3h samples. The vertical line indicates splicing together of two parts of the same western blot (removing intervening lanes).

(E) (*Schematic*) Expected time evolution of NICD concentration within cells for complete (green line) or partial (chartreuse line) DAPT washout. Black markers indicate points at which NICD levels are measured in D.

(F) Predictions from Amplitude-only model and Duration-based models for control of Hey expression. (*Top*) In the amplitude-only model, the rate of Hey1/L expression is determined by the concentration of NICD. Therefore, the 15' complete DAPT washout (green line) and 3h partial DAPT washout (chartreuse line), which result in comparable NICD levels, should result in similar increases in Hey1/L expression levels (Δ) during a given time window at the end of treatment. (*Bottom*) In the duration based model, the rate of Hey1/L expression depends on the duration of NICD activation. Specifically, a short period of Notch activation (15') leads to a smaller increase in Hey1/L levels (Δ) during a given time window than a longer period of Notch activation (3h), despite similar NICD levels at the two time points.

(G) Median expression level of Hey1 and HeyL (each normalized to its expression level at 3.25h with complete DAPT removal) 15 min prior to ('T-15 min', empty bars) or 15 min after ('T+15 min', filled bars) a 15 min, 30 min, or 3h period of Notch activation with complete (dark green) or partial (chartreuse) DAPT removal. Error bars represent s.e.m. calculated from duplicate experiments.

(H) Maximum Hes1 (blue), Hey1 (burnt orange), and HeyL (orange) mRNA levels measured during the [0h, 1h, 4h] time-course, in response to sustained reduction of DAPT concentration to indicated levels (0, 0.3, or 0.5 μM). Error bars represent SEM calculated from duplicate experiments.

(I) Western blot analysis of cleaved NICD levels in N1ΔECD cells (see Figure 3A) and receiver cells expressing full-length human Notch1 (Figure S4D). N1ΔECD cells (co-cultured with CHO-K1 control cells to match total number of cells in co-culture) were maintained in DAPT (' – ') or DAPT was washed out completely for 30 min ('30 min'). Notch1 receiver cells were co-cultured with CHO-K1 control cells (' – ') or sender cells expressing maximal levels of Dll4 for 8h. NICD is detected using an antibody that detects the cleaved version (with N-terminal Val at amino acid position 1744, see STAR Methods). GAPDH levels represent the loading control.
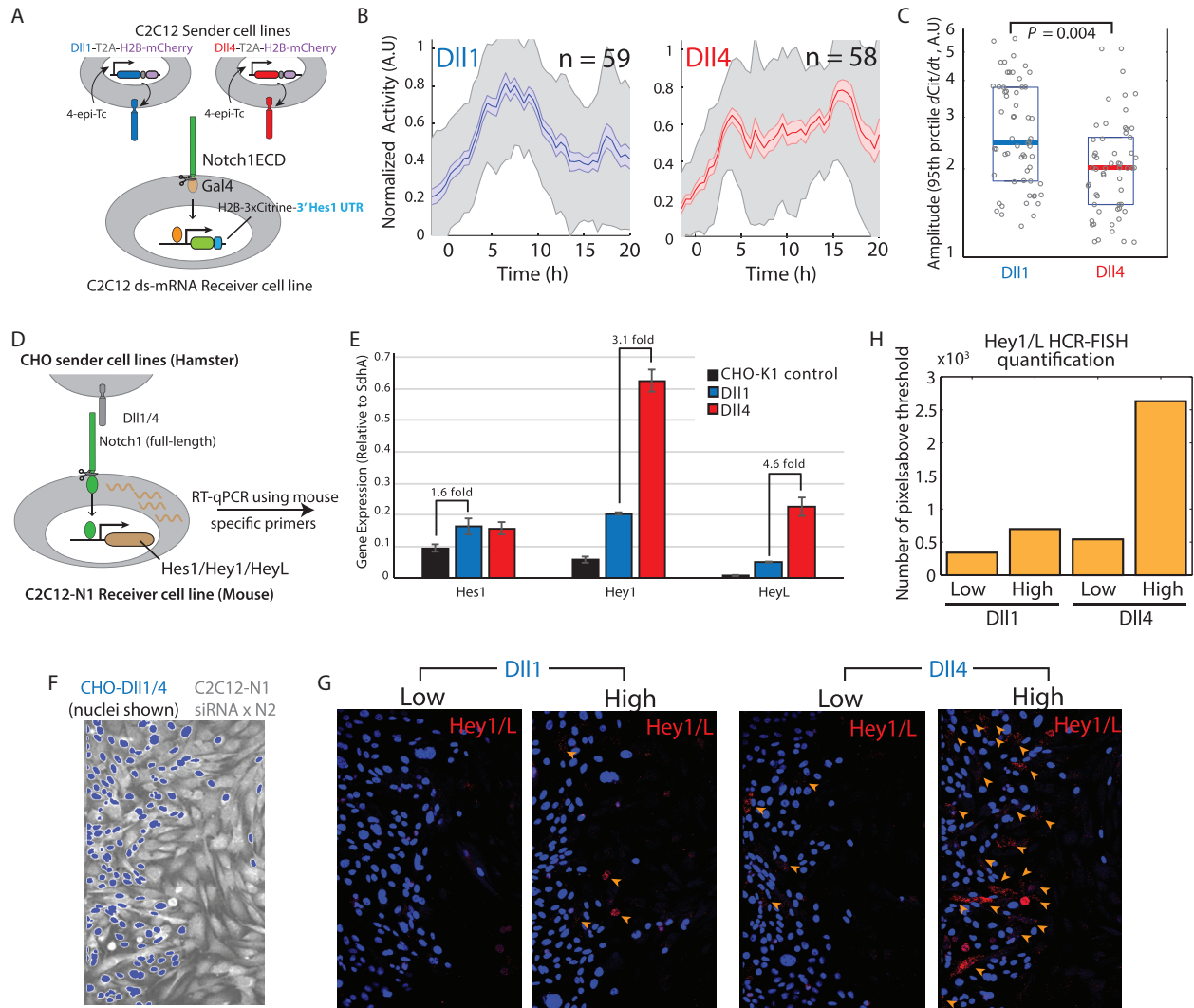
Figure S4. Dynamic Ligand Discrimination in C2C12 Cells, Related to Figure 3

(A–C) Dll1 and Dll4 activate Notch1 using pulsatile and sustained dynamics, respectively, in C2C12 cells.

(A) *(Top)* Engineered C2C12 sender cell lines contain stably integrated constructs expressing Dll1 (blue) or Dll4 (red), each with a co-translational (T2A, gray) H2B-mCh readout (purple). *(Bottom)* Receiver cell lines stably express a chimeric receptor combining the Notch1 extracellular domain (N1ECD, green) with a Gal4 transcription factor (orange) in place of the endogenous intracellular domain. Cells also contain a stably integrated H2B-3xCitrine fluorescent reporter (green), destabilized at the mRNA level by fusing it with a 3′UTR derived from the Hes1 cDNA (light blue).

(B) Median normalized promoter activities in receiver cells co-cultured with an excess of Dll4 (red) or Dll1 (blue) senders. Traces were aligned (see STAR Methods for special alignment procedure) and normalized to their 90th percentile value prior to averaging. Also shown are standard error of the mean (light colored lines), and standard deviations (gray areas).

(C) Boxplots of maximal promoter activities of the responses averaged in panel B. Each gray circle represents the response of a single cell to Dll1 (blue) or Dll4 (red). Colored lines represent the median, while boxes delimit 25th to 75th percentile values. *P value* calculated using two-sided *KS*-test.

(D) *(Schematic)* RT-qPCR based measurement of Hes1/Hey1/HeyL response in C2C12-Notch1 + CHO-Dll1/4 co-cultures. C2C12-Notch1 receiver cells, expressing full length Notch1 (green) are co-cultured with CHO-K1 sender cells expressing either Dll1 or Dll4 (gray). The target genes (shown as brown gene target and corresponding brown mRNA) in C2C12-Notch1 receiver cells are specifically amplified using mouse-specific primers.

(E) RT-qPCR expression levels of Hes1, Hey1, and HeyL in C2C12-Notch1 receiver cells co-cultured with CHO-K1 control cells (black), Dll1- (blue), or Dll4-expressing (red) sender cells for 8h. Error bars represent standard error of the mean (n = 2 replicates). F-H, Characterization of *Hey* transcriptional response at the single-cell level using HCR-FISH.

(F) CHO sender cells (highlighted with blue nuclei) and C2C12-Notch1 receiver cells (gray cells) were co-cultured such that they interface along a line (see STAR Methods).

(G) HCR-FISH detection of Hey1+HeyL mRNA (red) in C2C12-Notch1 cells co-cultured with sender cells (blue nuclei shown) expressing low or high levels of Dll1 (left panels) or Dll4 (right panels). Arrowheads indicate cells showing clear HCR-FISH signal.

(H) Quantification of panel G images. Values indicate number of pixels in the left half of images that lie above a threshold intensity value.
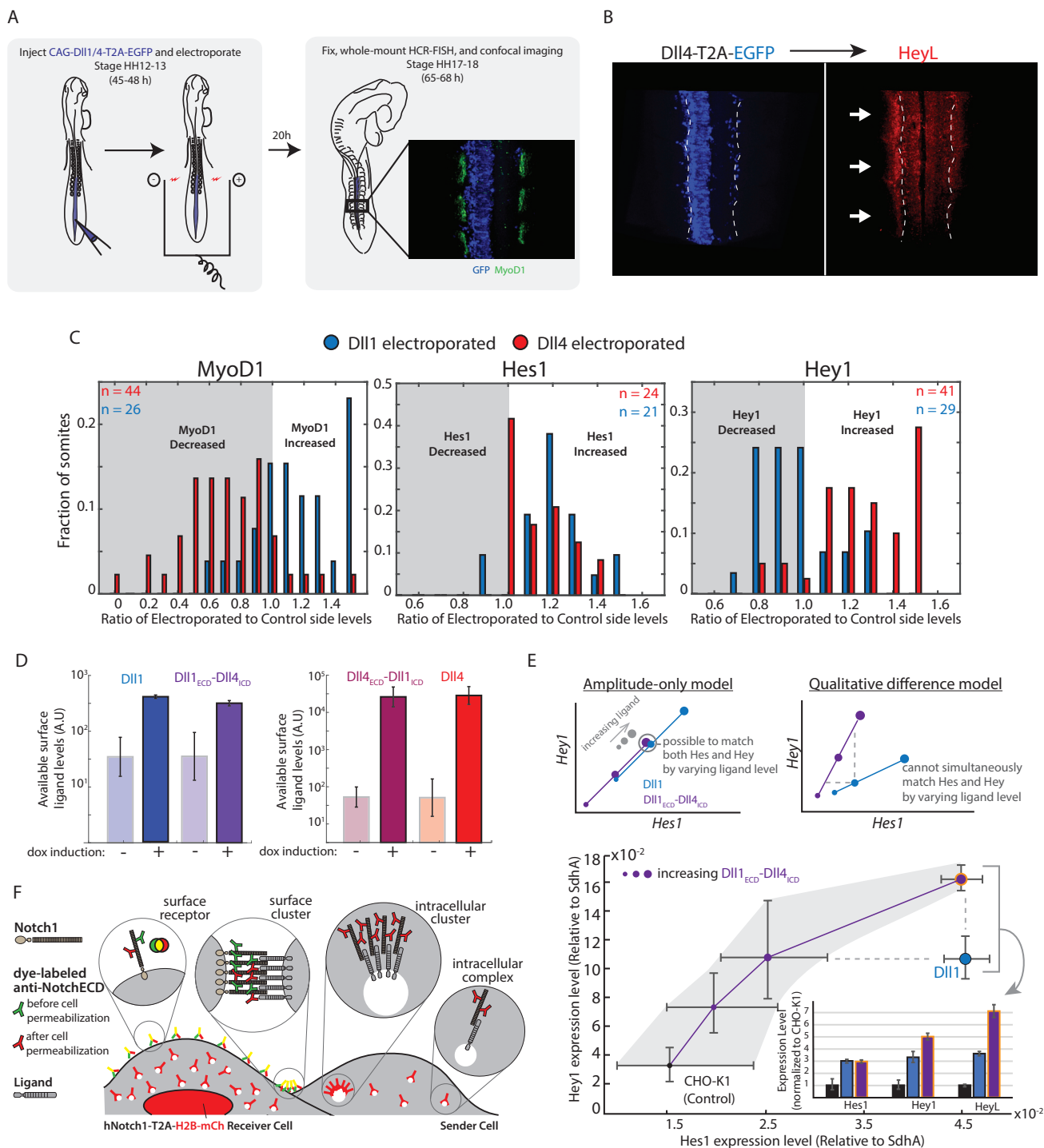
Figure S5. Quantitation of Effects on MyoD1, Hes1, and Hey1 *In Ovo* and Gene Expression Response to Dll1$_{ECD}$-Dll4$_{ICD}$ Ligand, Related to Figure 4 and Table 1

(A) Schematic for chick electroporation experiments. The neural tubes of HH stage 12-13 embryos were injected *in ovo* with CAG-Dll1/4-T2A-EGFP plasmid and a rightward current was applied at the level of the pre-somitic mesoderm. 20h later embryos were dissected, fixed, and subjected to 3-color whole-mount HCR-FISH to detect GFP (blue), MyoD1 (green), and Hes1/Hey1/HeyL (not shown). Inter-limb somites were then imaged using scanning laser confocal microscopy.

(B) Representative image showing an increase in HeyL (red, *right*) in the DML of somites when the neural tube and crest were electroporated with Dll4-T2A-EGFP (blue, *left*). Arrows indicate the electroporated side.

(C) Fold-changes in expression of MyoD1, Hes1, and Hey1 within somites (see STAR Methods), in response to electroporation of Dll1 (blue bars) or Dll4 (red bars), calculated relative to the control side. 'n' values correspond to number of somites analyzed.

(D) Cell surface levels of natural and chimeric ligands, detected using soluble Notch1ECD-Fc (see STAR Methods). (*Left*) Dll1 (blue bars) and $Dll1_{ECD}$-$Dll4_{ECD}$ (purple bars) are detected at similar surface levels before ('-', light shading) and after ('+', dark shading) maximal induction with doxycycline ('dox induction'). (*Right*) $Dll4_{ECD}$-$Dll1_{ICD}$ (magenta bars) and Dll4 (red bars) are detected at similar surface levels before (light shading) and after (dark shading) maximal induction. Error bars indicate standard deviation. Note that Notch1ECD has a higher affinity for the Dll4ECD than the Dll1ECD (Andrawes et al., 2013); hence ligand levels detected after induction in the *left* plot are higher than in the *right* plot.

(E) (*Top, schematics*) Expected dose-response behavior of Hes1 and Hey1 gene expression to varying Dll1 (blue) or $Dll1_{ECD}$-$Dll4_{ICD}$ (purple) ligand levels if (*Left*) ligands varied only in the amplitude of signaling ('Amplitude-only' model) and if (*Right*) ligands produced qualitatively different gene expression ('Qualitative-difference' model). In an amplitude-only model, it is possible to simultaneously match the Hes1 and Hey1 gene expression levels produced by one ligand by varying levels of the other. On the other hand, if ligands produced qualitatively different Hes1/Hey1 expression patterns, there exist Hes1/Hey1 expression levels induced by one ligand (for e.g., Dll1) that cannot be matched by varying the levels of the other ligand. (*Bottom*) Hes1 and Hey1 expression levels in N1 receiver cells co-cultured with CHO-K1 cells (control, black marker), Dll1 senders (blue marker), or sender cells expressing three different levels of $Dll1_{ECD}$-$Dll4_{ICD}$ (purple marker, increasing marker sizes indicate increase levels of level induction). Error bars represent SEM from duplicate experiments. (*Inset*) Comparison of fold-changes in Hes1, Hey1, and HeyL expression levels (relative to CHO-K1 control co-culture) in receiver cells co-cultured with Dll1 senders (blue bars, same experiment as indicated by blue marker) or $Dll1_{ECD}$-$Dll4_{ICD}$ senders (purple bars with orange outlines, same experiment as indicated by purple marker with orange outline).

(F) Schematic of types of staining observed in co-culture NotchECD immunostaining assay and the molecular species they are expected to represent. Intracellular Notch1 will only be labeled only after cell-permeabilization and should only be detected in a single channel (shown here as red). White circles within cells represent endosomes. All cell-surface Notch1, including unbound and ligand-bound forms, will be labeled both before and after cell-permeabilization; it should thus be detected in two channels (shown here as red + green = yellow). Receiver cells can be distinguished from sender cells based on expression of nuclear H2B-mCherry, which will also be detected in the red channel. Ligand-NotchECD complexes within the sender cell could exhibit two types of staining: large, bright puncta, corresponding to endocytosed ligand-receptor clusters, and low intensity staining, corresponding to individual or few endocytosed ligand-receptor complexes.
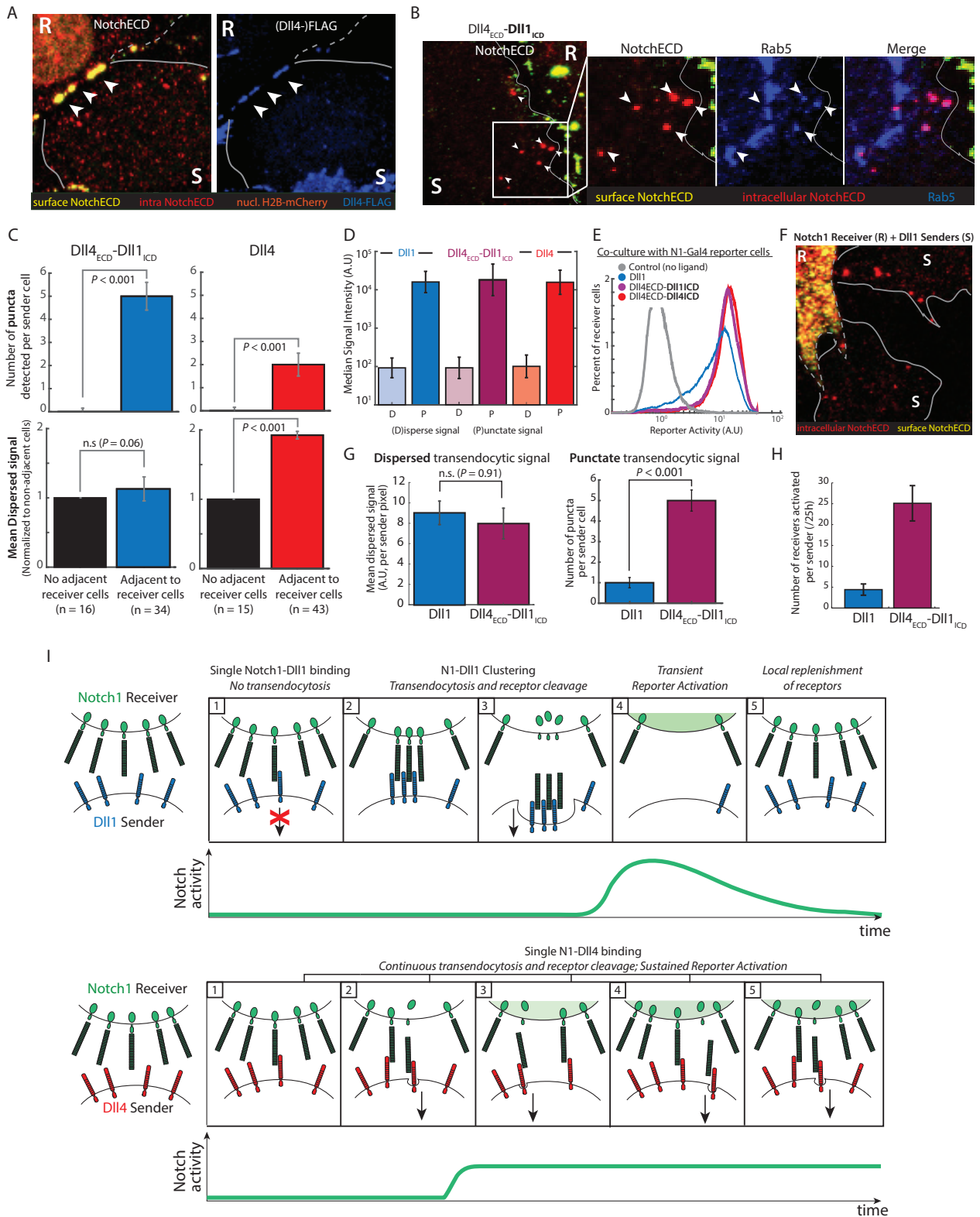
Figure S6. Role of Ligand Intracellular Domains in Receptor Transendocytosis, Related to Figure 5

(A) Representative image showing Dll4 co-localization with Notch in clusters at the cell interface between the Notch1 receiver cell ('R') and a Dll4-FLAG expressing sender cell ('S') shown in Figure 5B. Cells are immunostained with antibodies for Notch1ECD (red, green, *left panels*, same as Figure 5B) and FLAG (blue,

(legend continued on next page)

right panels). Cell-surface receptors appear as yellow because they are labeled in two channels (red + green). White arrowheads indicated co-clustered ligands and receptors at the interface between the sender and receiver cell.

(B) Representative image showing co-localization of transendocytosed NotchECD puncta with the early endocytic marker Rab5 within a $Dll4_{ECD}$-$Dll1_{ICD}$ -expressing sender cell ('S') next to a Notch1 receiver cell ('R'). Cells are immunostained for Notch1ECD (red, green) and the early endocytic marker Rab5 (blue). Cell-surface receptors appear as yellow because they are labeled in two channels (red + green).

(C) Transendocytosis staining patterns in $Dll4_{ECD}$-$Dll1_{ICD}$ senders (*Left*) or Dll4 senders (*Right*) adjacent to or not adjacent to receiver cells within the same co-culture. (*Top Left*) Median values of the number of puncta detected (see STAR Methods) in $Dll4_{ECD}$-$Dll1_{ICD}$ sender cells that are adjacent to (magenta bar) or not adjacent to (black bar) receiver cells. (*Top Right*) Corresponding median values of number of puncta detected in Dll4 cells. (*Bottom Left*) Median values of the mean pixel intensity of dispersed signal in $Dll4_{ECD}$-$Dll1_{ICD}$ sender cells that are adjacent to (magenta bar) or not adjacent to (black bar) receiver cells. (*Bottom Right*) Corresponding median values of dispersed signal in Dll4 cells. Error bars represent standard error of the mean. *P value* calculated using the two-sided KS-test. 'n' indicates number of sender cells considered in the analysis.

(D) Median values for intensities of pixels categorized as 'disperse' staining (light shading) and for cumulative intensities of 'puncta' (dark shading) in immunostained co-cultures of receiver cells with Dll1 (blue bars), $Dll4_{ECD}$-$Dll1_{ICD}$ (magenta bars), or Dll4 (red bars) sender cells. Error bars represent standard deviations from the mean.

(E) Flow cytometry histograms of activation levels in a Notch-Gal4 receiver cell line (same as used in Figures 1, 2, and 5A; background activity indicated by gray histogram) co-cultured with Dll1 (blue), $Dll4_{ECD}$-$Dll1_{ICD}$ (magenta) or Dll4 (red) cells expressing ligand levels similar to that used in Figures 5C and 5D.

(F) Representative image of co-culture of Notch1 receiver cells ('R') and Dll1 sender cells ('S'), immunostained for NotchECD. The red channel shows staining for intracellular receptors. Cell-surface receptors appear as yellow because they are labeled in two channels (red + green).

(G) (*Left*) Median values of the background subtracted mean pixel intensity of dispersed signal in Dll1 (blue) or $Dll4_{ECD}$-$Dll1_{ICD}$ (magenta) sender cells that abut receiver cells (see STAR Methods). (*Right*) Median values of the number of puncta detected (see STAR Methods) in Dll1 (blue) or $Dll4_{ECD}$-$Dll1_{ICD}$ (magenta) sender cells that abut receiver cells. Error bars represent standard error of the mean. *P value* calculated using the two-sided KS-test.

(H) Median values of the rate at which receiver cells are activated by $Dll4_{ECD}$-$Dll1_{ICD}$ (magenta) sender cells or Dll1 (blue) sender cells in excess receiver conditions. Error bars indicate standard error of the mean.

(I) (*Schematic*) Molecular basis of pulsatile and sustained signaling. *Top*, (1) Dll1 ligands (blue) do not activate Notch1 receptors (green) efficiently in single ligand-receptor complexes, but (2) ligand- receptor complexes can assemble into larger clusters, leading to (3) efficient transendocytosis and coordinated activation of constituent receptors in the receiver cell. (4) This releases a burst of NICD (green ovals) which is reflected in a strong pulse of downstream response in receiver cell (green shading, green trace in plot). (5) The system resets (locally) and awaits another clustering event. *Bottom*, Dll4 ligands (red) transendocytose Notch1 (green) receptors efficiently even when bound in single ligand-receptor complexes (1, 2), or in small clusters (not shown). (3-5) This leads to a steady rate of NICD cleavage (green ovals), and a sustained downstream response in the receiver cell (green shading, green trace in plot).