

Supporting Material for:

Membrane Tethered Delta Activates Notch and Reveals a Role for Spatio-Mechanical Regulation of the Signaling Pathway

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Supporting Methods

DLL4 ligand labeling with Alexa Fluor dye

Deca-histidine tagged recombinant human DLL4 (R&D Systems, Minneapolis, MN) was reconstituted in PBS, pH 7.4 to 0.8 µg/ml. A sodium bicarbonate solution was added to the protein solution at a final concentration of 0.1 M. Twenty fold molar excess of Alexa Fluor 568 or 647 NHS ester (Life Technologies) was resuspended in DMSO and added dropwise to the pH adjusted protein solution. The reaction proceeded for a total of 1 h and was quenched with the addition of 1 M Tris buffer (final concentration, 10 mM).

Design and expression of DLL4-mCherry

The cDNA sequence of the human DLL4 ECD (corresponding to residues Ser27-Pro524) was cloned into the pcDNA3 mammalian expression vector (Life Technologies). The fluorescent protein mCherry, biotin acceptor peptide (GLNDIFEAQKIEWHE) and deca-histidine tag were fused in frame to the C-terminus of DLL4 ECD. The final construct, pcDNA3-DLL4-mCherry, was sequence verified. HEK 293T cells were cultured to 70% confluency (following ATCC conditions) in 10 cm dishes and transfected with 10 µg of pcDNA3-DLL4-mCherry using CaPO₄. After 16 h, the media was removed and indicator- and serum-free Eagle's minimum essential medium (EMEM) was added, and the cells were cultured for an additional 5 days. The conditioned media containing secreted DLL4-mCherry protein was collected and concentrated. The ligand was purified using Ni-NTA magnetic agarose beads (Qiagen, Valencia, CA), buffer exchanged into 10 mM Tris, 300 mM NaCl, pH 8.0 and stored at 4°C.

Biotin ligase modification of DLL4-mCherry

Purified DLL4-mCherry was concentrated and buffer exchanged into 10 mM Tris, 100 mM NaCl, pH 8.0. The substrate solution was combined with 10 mM ATP, 10 mM MgOAc, 50 µM D-biotin and 1.2 µg of biotin ligase in 50 mM bicine buffer, pH 8.3 (Genecopoeia, Rockville, MD). The reaction was incubated at 30° C for 5 h and purified by removing excess biotin using an Amicon Ultra 10k filter (Millipore, Billerica, MA) to yield DLL4-mCherry-biotin. The biotin ligase (BirA) site-specifically incorporates the biotin moiety at the lysine residue located in the biotin acceptor peptide sequence (1, 2).

Binding specificity of DLL4 functionalized membranes

A portion of the human Notch1 receptor, including residues Ala19-Gln526 of the extracellular domain, was fused to human IgG₁ (Pro100-Lys330) through a 6 amino acid linker (R&D Systems, Minneapolis, MN). This chimera is present in its soluble form as a disulfide-linked homodimer. The protein was labeled with Alexa 647 NHS ester as described above and purified using Bio-Rad P-4 gel (Bio-Rad, Hercules, CA) to yield NECD-647. DLL4 functionalized membranes were incubated with 33 nM of NECD-647 for 45 min, rinsed and imaged.

Immunostaining and analysis of NICD localization

Membrane surfaces with histidine-tagged DLL4 were prepared as described and 30,000 cells, which were either treated with 25 µM DAPT (Calbiochem, Billerica, MA) (negative control) or DMSO for 16 h, were added into each well. After 3 h with continued drug or vehicle treatment, the cells were rinsed with cold PBS, fixed with 4% paraformaldehyde for 12 min and permeabilized with 0.1% (v/v) Triton X-100 for 5 min. The samples were blocked with a 1%

(w/v) BSA in PBS solution overnight. The primary antibody for cleaved NICD (Cell Signaling Technology, Danvers, MA) was diluted 1:200 in 1% BSA and incubated for 1 h. After rinsing, the secondary antibody labeled with Alexa Fluor 647 (Life Technologies) was diluted 1:1000 and incubated for 30 min. Lastly, DAPI (Life Technologies) was added at a concentration of 300 nM for 5 min. To determine the intensity of the NICD within the nuclei, the DAPI signal was used to locate the nucleus and then the signal intensity from the secondary antibody within these areas was measured.

Biotin-functionalized glass for non-fluid DLL4 surfaces

All glass substrates (25 mm diameter, #2 coverslips, VWR, Radnor, PA) were sonicated in Nanopure water and piranha etched (H_2O_2 : H_2SO_4 ; 1:3; v:v) for 15 min. (Caution: piranha solution is an extremely strong oxidant and can become explosive if mixed with organics). After rinsing with copious amounts of water, the glass substrates were dried under nitrogen and immersed in toluene. The substrates were incubated for 1 h at room temperature in a 0.4% (v/v) solution of 3-aminopropyltriethoxysilane (Gelest, Morrisville, PA) in toluene. The substrates were rinsed with ethanol and dried under nitrogen. The amine-functionalized slides were reacted with 500 μl per slide of 0.05% (w/v) sulfo-NHS-LC-biotin (Pierce, Rockford, IL) and 5% (w/v) mPEG-NHS, MW 2000 (Nanocs, Boston, MA) in 0.1 M sodium bicarbonate overnight. The slides were rinsed with water, dried and placed into an Attofluor chamber (Life Technologies) for further functionalization (beginning with BSA blocking), see assembly of supported lipid membrane section for details.

Physisorbed DLL4 on glass

A solution of 12.5 $\mu\text{g}/\text{ml}$ DLL4/Fc (Sino Biological, China) or DLL4-mCherry and 5 $\mu\text{g}/\text{ml}$ fibronectin in PBS was incubated on a glass bottom 96 well plate for 1 h while shaking at 250 rpm. Excess fibronectin and ligand were rinsed away with 5 ml of PBS. The DLL4/Fc protein is composed of the extracellular domain of DLL4 (Ser27-Pro524) fused to the Fc region of human IgG₁ at the C-terminus. This recombinant human protein is present as a disulfide-linked homodimer.

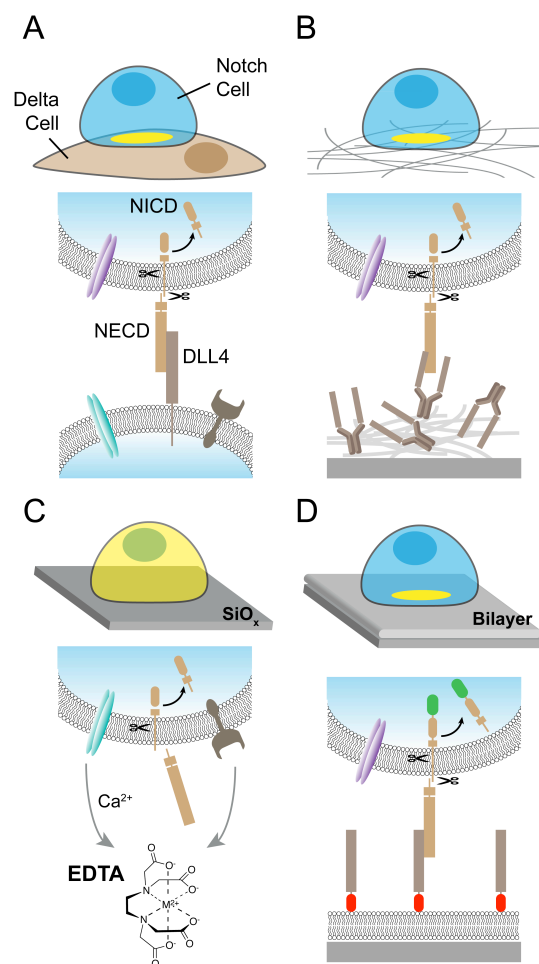


Figure S1. Summary of current methods to study Notch activation. This scheme illustrates the most commonly used strategies to activate Notch, which include: (A) cellular co-culture, (B) physisorbed ligand, (C) metal depletion, and (D) supported membrane tethered ligand. The yellow regions highlighted in the top diagrams are depicted in molecular detail below.

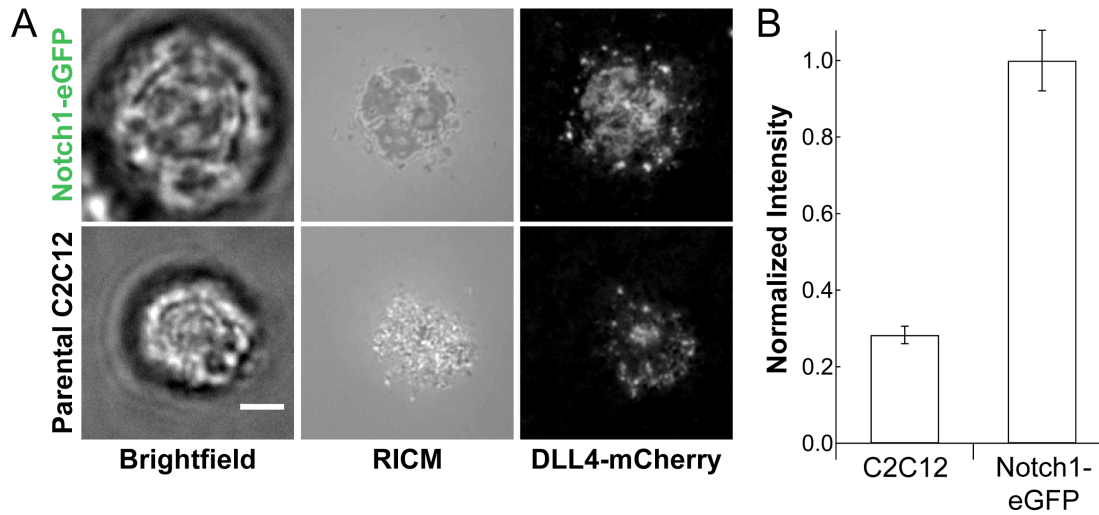


Figure S2. DLL4 binding of Notch receptors on parental C2C12 cells compared with Notch1-eGFP expressing cells. (A, Top panel) Representative images of Notch1-eGFP receptor binding to DLL4-mCherry on a supported membrane after 30 min reveal the formation of ligand-receptor clusters. (A, Bottom panel) Representative images of endogenous Notch receptors in parental C2C12 cells show binding to the DLL4-mCherry surfaces and formation of clusters that are smaller and less dense. Scale bar = 5 μ m. (B) Quantitative comparison of the amount of ligand-receptor binding for Notch1-eGFP and C2C12 cells, error bars represent SEM for $n = 60$ C2C12 cells and $n = 35$ Notch1-eGFP cells.

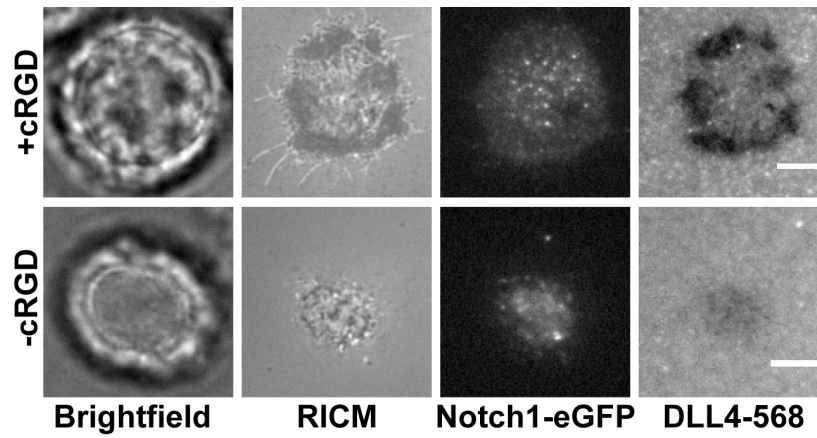


Figure S3. Specificity of DLL4 binding to Notch1. Notch1-eGFP cells were treated with soluble DLL4/Fc (185 nM) for 20 min before incubating on a DLL4-568 membrane surface. In order to facilitate imaging of cells, cRGD was added to the supported lipid membrane (top) to promote cell adhesion. Without cRGD-integrin binding, the Notch1-eGFP expressing cells very weakly associated with the supported lipid membrane (bottom).

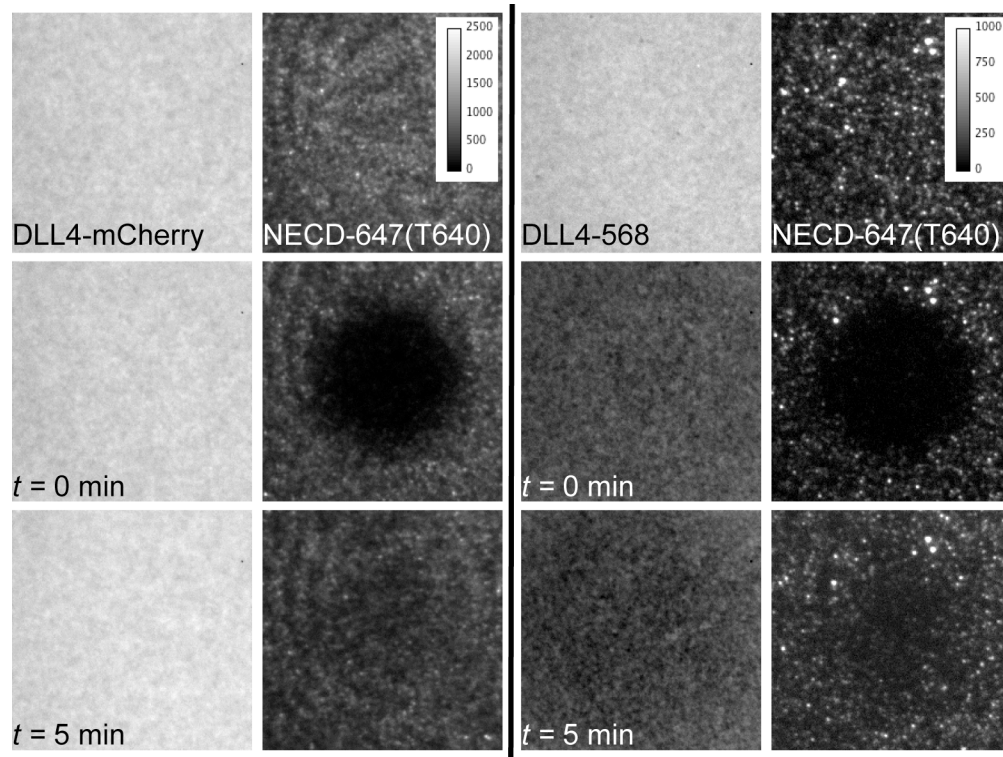


Figure S4. Clustering of DLL4 is Notch-cell driven. Binding of NECD-647 to DLL4 supported lipid membrane surface alone does not result in observable clustering. Note that the NECD-647 displays different affinity for the two types of ligand tested as indicated by the differences in intensity (calibration bars). FRAP assay shows that the NECD-647 is still laterally mobile when bound to DLL4.

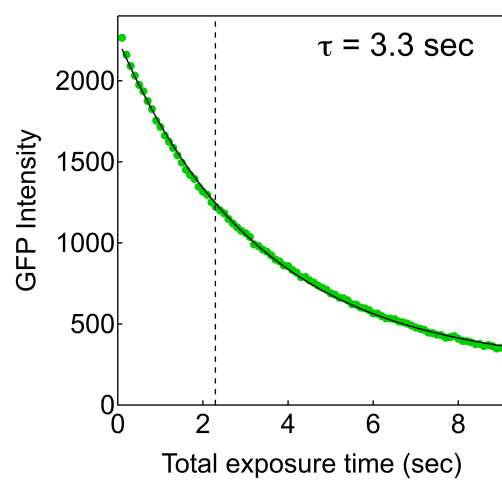


Figure S5. Photobleaching profile of eGFP physisorbed onto a glass surface. The experimentally measured values were fit to an exponential decay (solid line), and the half-life was calculated to be 2.3 sec or 23 frames taken at 100 msec exposure time (dashed line).

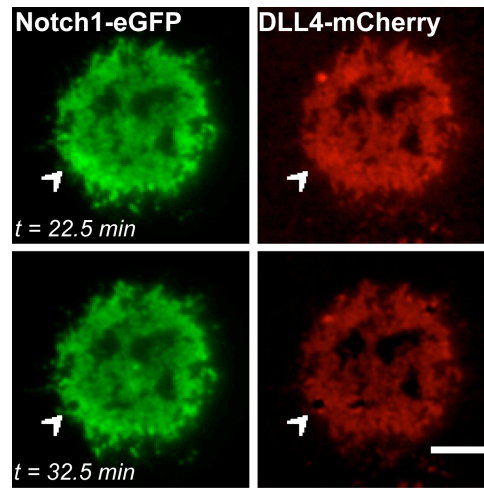


Figure S6. Time-lapse analysis of an individual DAPT treated cell. Arrow highlights an area where concerted loss of Notch1-eGFP and DLL4-mCherry was observed. Scale bar = 5 μm .

Summary of Image Analysis

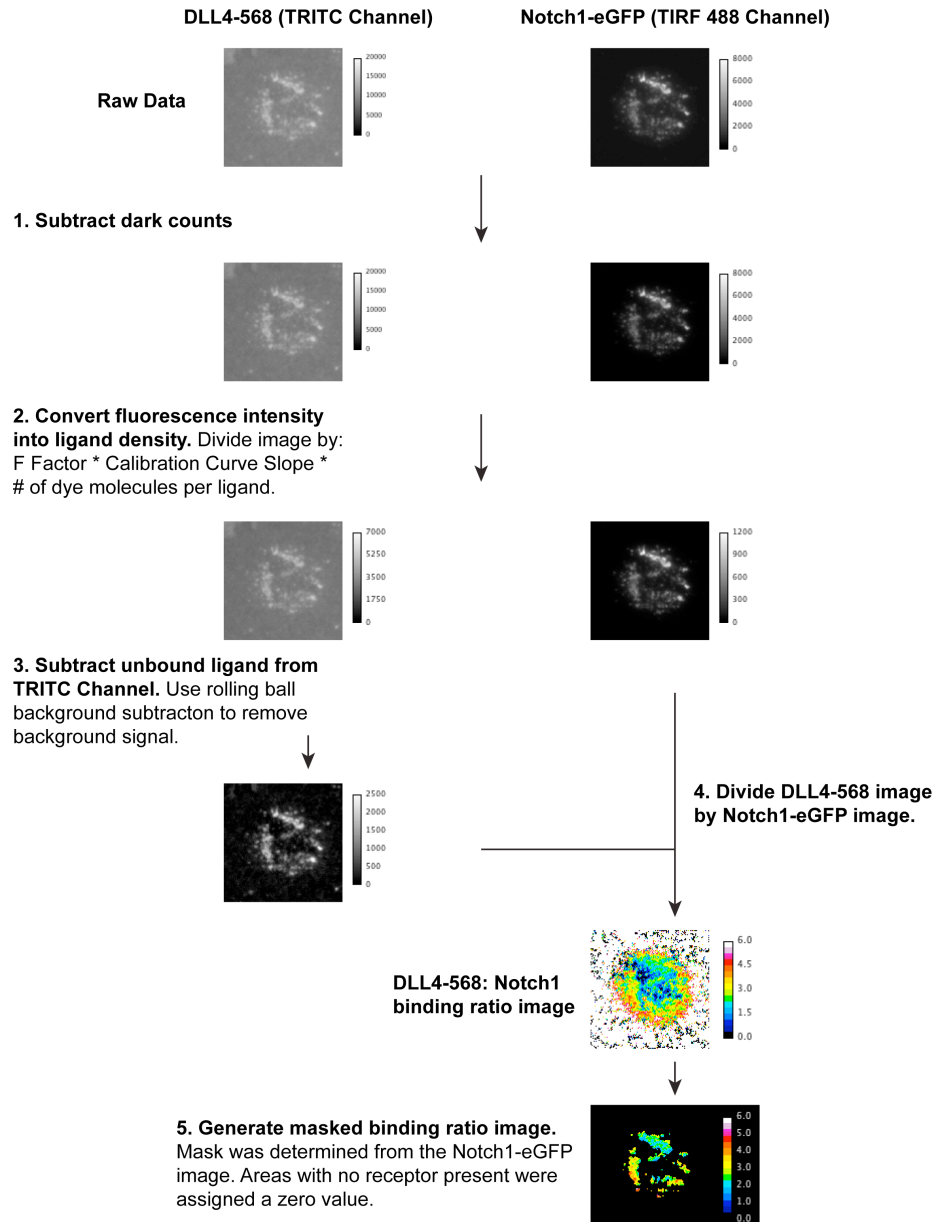


Figure S7. Summary of image analysis to determine the binding ratio between DLL4 and Notch1.

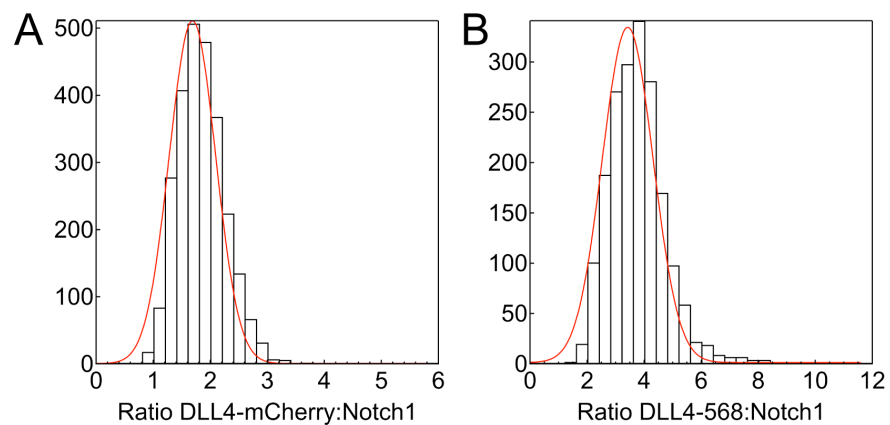


Figure S8. Representative histograms of ratio values fit with a Gaussian for binding of (A) DLL4-mCherry to Notch1-eGFP or (B) DLL4-568 to Notch1-eGFP.

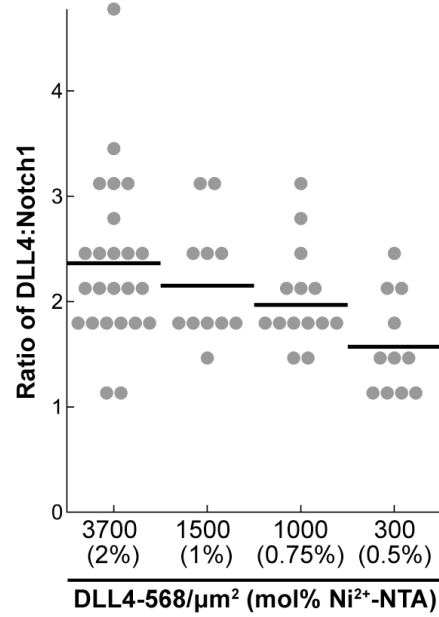


Figure S9. Determining the stoichiometry of DLL4 to Notch1 binding at live cell-supported lipid membrane junctions. The stoichiometry of binding was plotted as a function of ligand density. In these experiments, mole percent of Ni²⁺-NTA lipid was reduced from 2% to 0.5% and resulted in a lower binding ratio between DLL4-568 and Notch1. Each circle represents binding between a single cell and the membrane containing tethered ligand, and the mean ratio is indicated with a horizontal line.

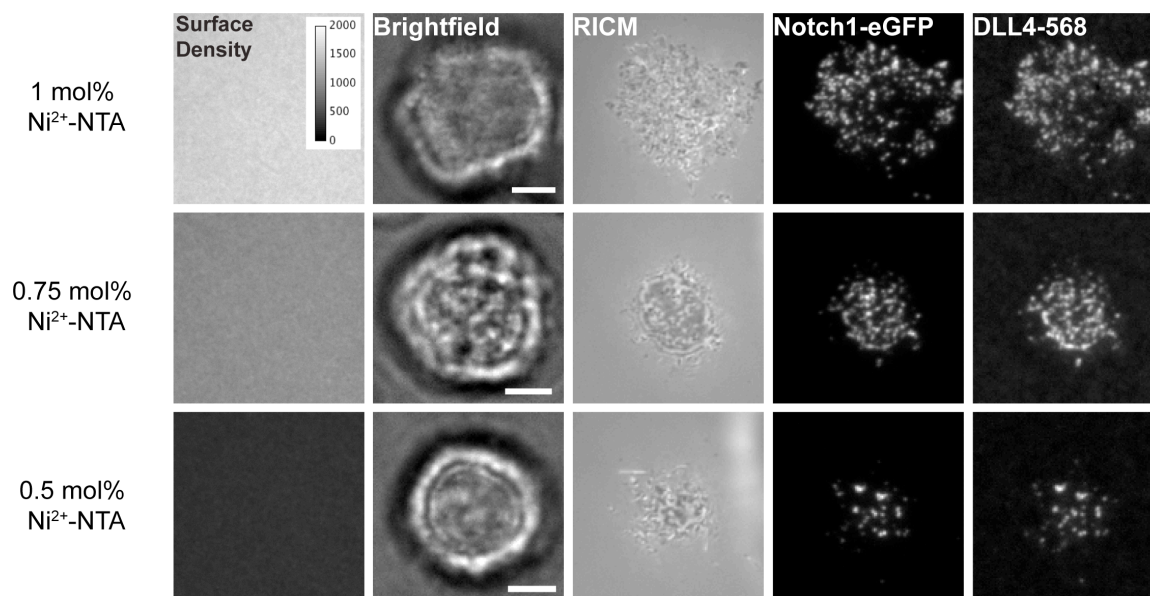


Figure S10. Effect of DLL4-568 density on Notch1 binding. Surfaces with varying densities of DLL4-568 were produced (left panel) and Notch1-eGFP expressing cells were incubated for 30 min prior to imaging. The strength of cell contact is seen in RICM and diminishes as the amount of available surface ligand is reduced. Ni^{2+} -NTA lipid concentrations below 0.5 mol% resulted in no cell attachment (data not shown). Scale bar = 5 μm .

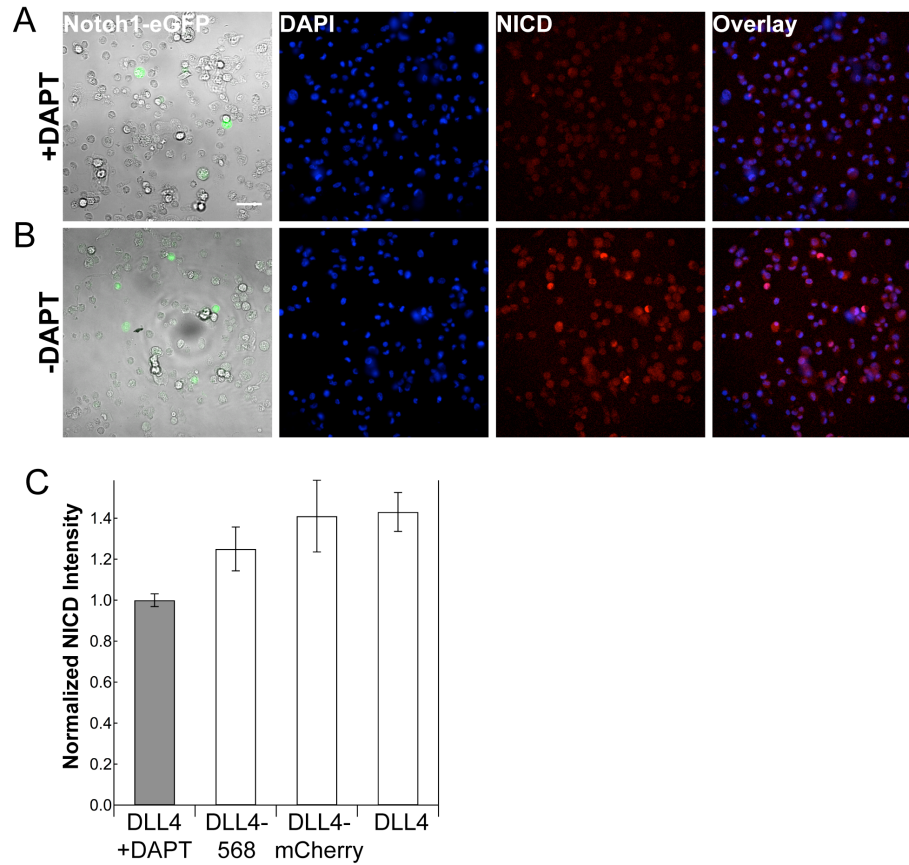


Figure S11. Activation of the Notch signaling pathway using the supported membrane platform. (A) DAPT treated or (B) untreated Notch1-eGFP cells were engaged to DLL4-functionalized membranes for 3 h and fixed and stained prior to imaging. Fluorescence images of Notch1-eGFP (green) overlayed with the brightfield view, nuclear stain with DAPI (blue), cleaved NICD (Val1744) (red), and an overlay of the DAPI and NICD channels showing translocation of the NICD into the nucleus. Scale bar = 50 μ m. (C) Quantitative analysis of average nuclear localization of the NICD as visualized through antibody staining, which is a measure of Notch activation. As a negative control, cells were treated with 25 μ M DAPT during the course of the experiment (shaded bar). Error bars represent the standard error of the mean (SEM) for experiments performed in triplicate.

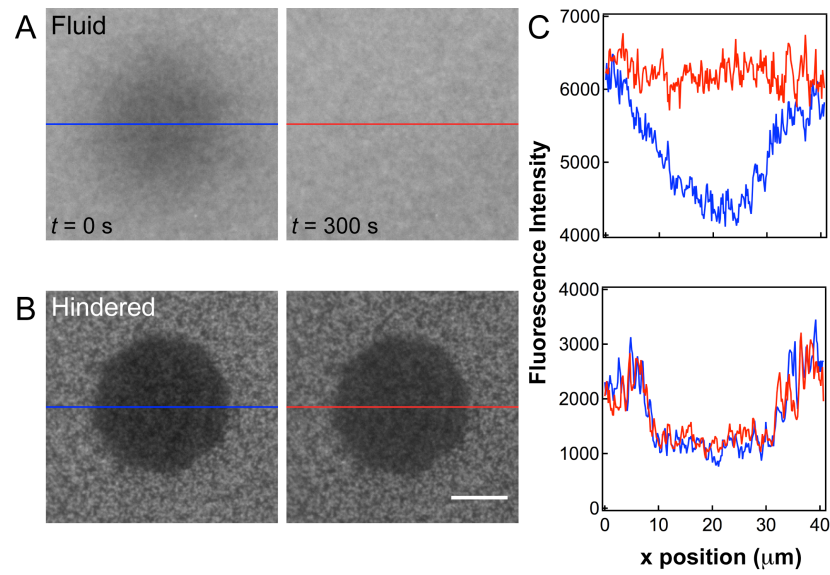


Figure S12. Lateral mobility of supported membranes with tethered DLL4 ligand. FRAP assay of (A) fluid and (B) hindered DLL4-mCherry-biotin functionalized lipid membranes that were photobleached and allowed to recover for 5 min. Scale bar = 10 μm . (C) Lines scans of fluorescence intensity over the indicated areas of images (A) and (B).

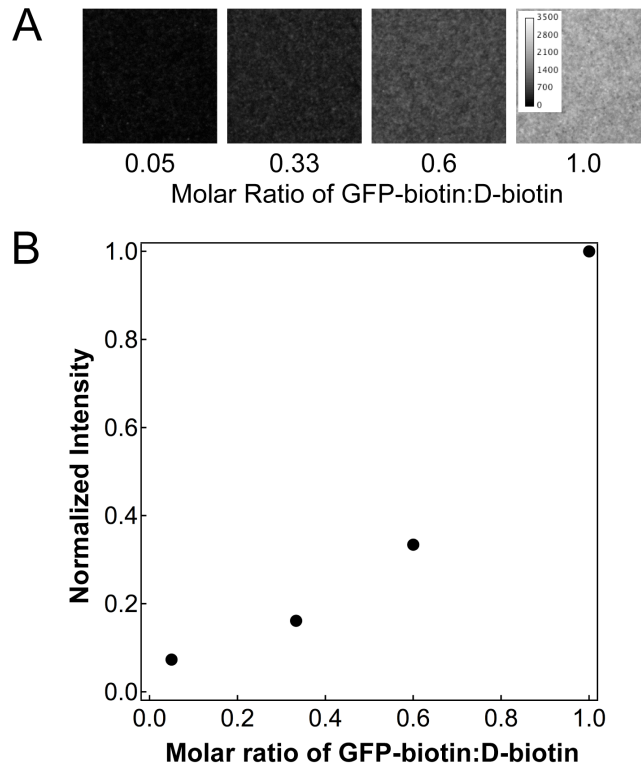


Figure S13. Competitive binding experiment between GFP-biotin and D-biotin on a lipid membrane. In this experiment, GFP-biotin was selected as a model ligand in place of DLL4-mCherry-biotin. The goal was to titrate different molar ratios of biotinylated (fluorescent) ligand to D-biotin on a membrane surface and to observe the effect on fluorescence intensity. First, eGFP α -thioester (expressed and purified using the IMPACT kit from New England Biolabs, Ipswich, MA) was site specifically modified with cysteine-biotin (Carbosynth, San Diego, CA) using expressed protein ligation (3). The resulting GFP-biotin was incubated on a streptavidin functionalized 0.1 mol% biotin-DPPE membrane at a series of molar ratios of GFP-biotin to D-biotin. The total biotin concentration was maintained at 250 nM. (A) Representative epifluorescence images of biotin-DPPE membranes incubated with different molar ratios of GFP-biotin to D-biotin. (B) Plot illustrating the fluorescence intensity as a function of the molar ratio of GFP-biotin to D-biotin. This plot was used to make an initial estimate for the amount of free D-biotin needed to produce a 4 mol% biotin-DPPE bilayer with the same ligand density (fluorescence intensity) as a 0.1 mol% biotin-DPPE surface.

Ligand	Ratio DLL4:Notch1	Average receptor- bound DLL4 density	Average Notch1 density	N	mol% Ni ²⁺ - DOGS-NTA	Initial surface density (ligand/ μm^2)
DLL4- mCherry	1.3 ± 0.5	490	420	85	2	1400
DLL4- mCherry + DAPT	1.1 ± 0.4	520	500	41	2	1100
DLL4-568	2.4 ± 0.8	820	390	24	2	3700
DLL4-568	2.2 ± 0.5	970	480	11	1	1500
DLL4-568	2.0 ± 0.5	1000	560	14	0.75	1000
DLL4-568	1.6 ± 0.4	700	510	11	0.5	300

Table S1. DLL4-Notch1 ratio determined from quantitative fluorescence analysis. Average density values for DLL4 and Notch1 refers to the average pixel value for all clusters of a single cell. See Methods for details on data analysis.

Supporting References

1. Beckett, D., E. Kovaleva, and P. J. Schatz. 1999. A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. *Protein Sci.* 8:921-929.
2. Chen, I., M. Howarth, W. Lin, and A. Y. Ting. 2005. Site-specific labeling of cell surface proteins with biophysical probes using biotin ligase. *Nat. Methods* 2:99-104.
3. Muralidharan, V., and T. W. Muir. 2006. Protein ligation: an enabling technology for the biophysical analysis of proteins. *Nat. Methods* 3:429-438.