Supplemental Inventory

Figure S1, related to Figure 1

Figure S2, related to Figure 2

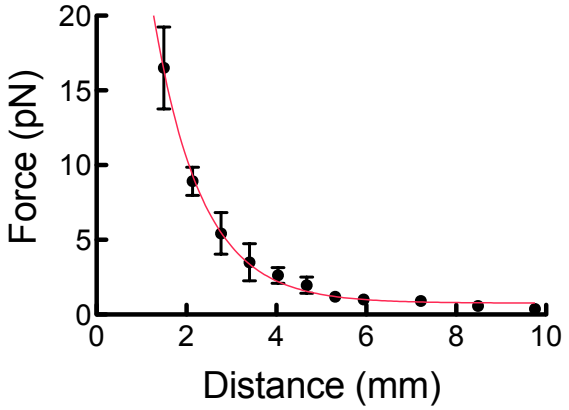
Figure S3, related to Figure 3

Figure S4, related to Figure 4

Supplemental Experimental Procedures

Supplemental Figure S1

A



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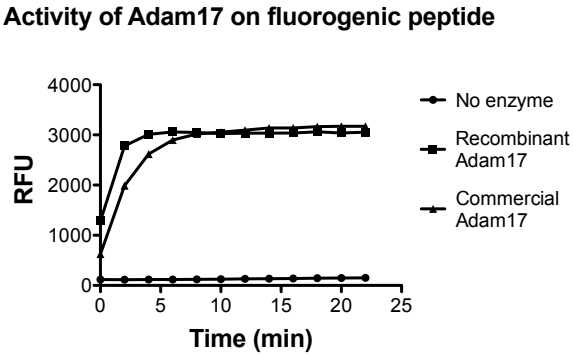

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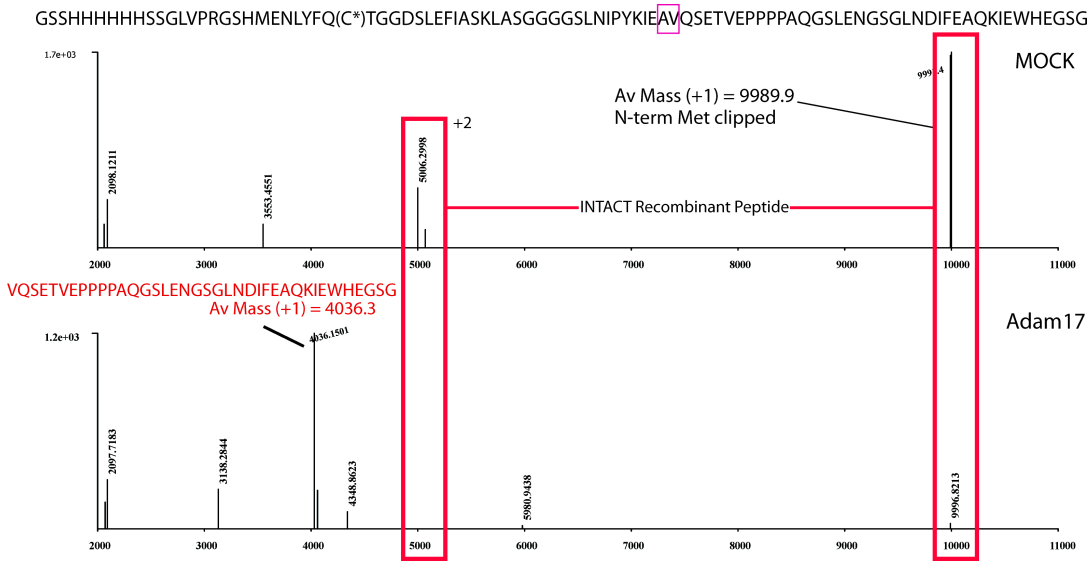

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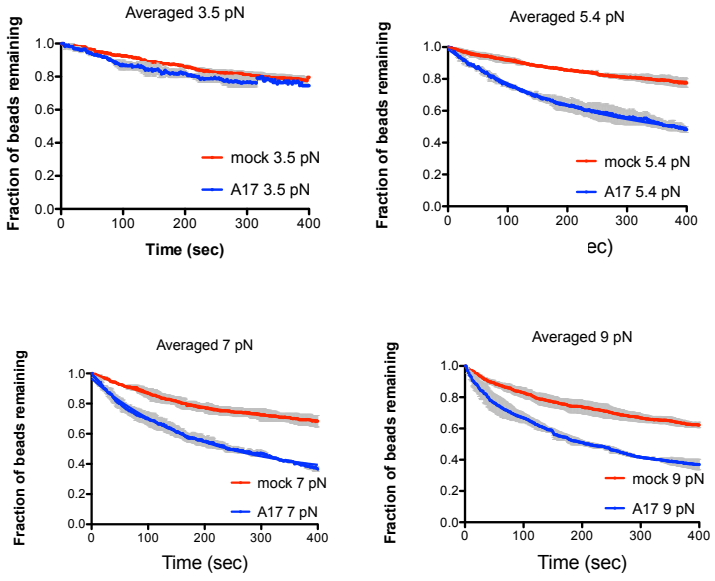
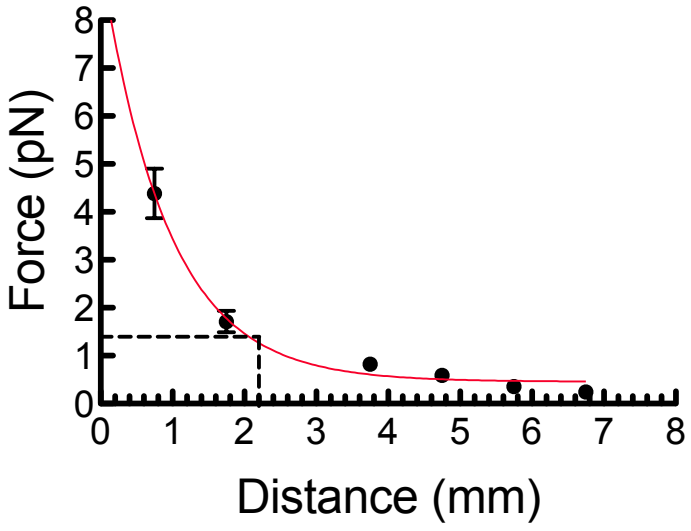


Supplemental Figure S2



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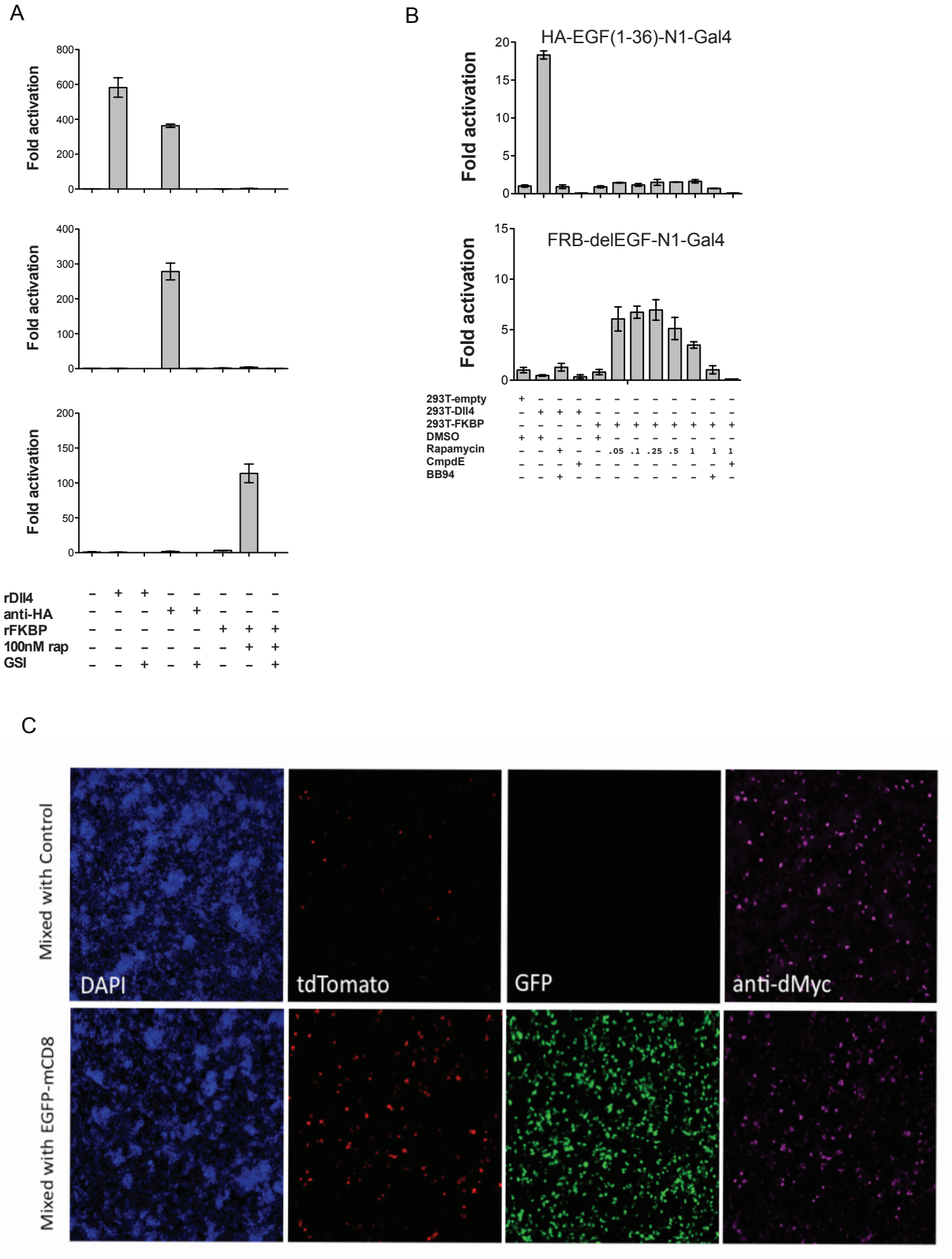
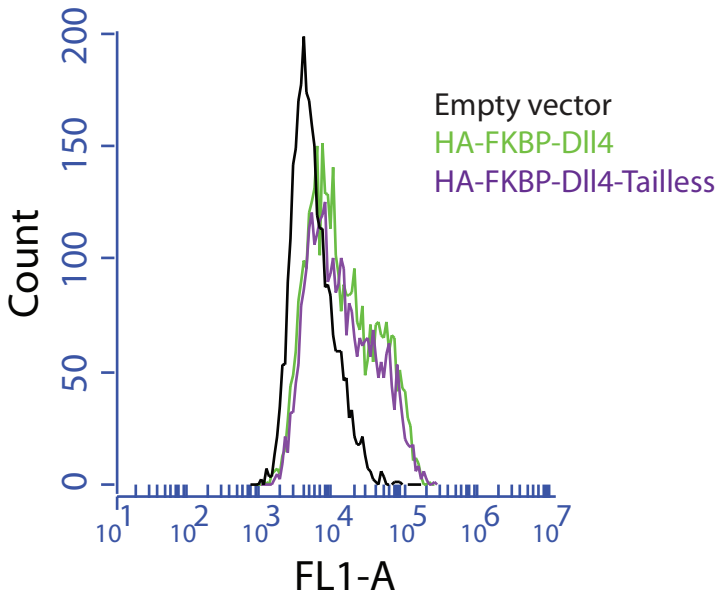


Supplemental Figure S4



Supplemental Figure Legends

Figure S1. Control experiments for single molecule magnetic tweezer assay, related to Figure 1. A. Calibration of the magnet for single molecule experiments. The magnet was calibrated as described in Methods using in-plane fluctuations of 2.8 μ paramagnetic beads. The force is plotted as a function of the distance of the magnet from the flowcell. Error bars represent standard error. The calibration was fit to a single exponential (red line). B. Analysis of the protease activity of recombinant Adam17. Proteolytic activity of recombinantly produced Adam17, compared with commercially available Adam17 (R&D Systems). The hydrolysis of a fluorogenic susbstrate was monitored as a function of time, using equimolar preparations of commercial or recombinant enzyme (see Methods). C. Cleavage products after incubation of a recombinant peptide substrate (top) with recombinant Adam17, analyzed by MALDI mass spectrometry. Mass spectra were acquired after a one-hour incubation of substrate in the absence (upper panel) or presence (lower panel) of enzyme. D. Raw data for NRR proteolysis as a function of time at different forces. Duplicate or triplicate curves were recorded in the presence (A17) or absence (mock) of Adam17 at four different forces. Data traces shown start at the time when force is applied. Number of beads was normalized to fraction of beads remaining by dividing by the total number of beads at the first time point in the experiment, which varied slightly from experiment to experiment, typically 100 to 300 beads. These curves were averaged and standard error bars shown in light grey. The

averaged mock curve for each force was subtracted from the averaged trace in the presence of enzyme.

Figure S2. Magnet calibration for 96-well magnetic tweezer experiments, related to Figure 2. The calibration was performed using in plane fluctuations of 1 μ paramagnetic beads (see Methods). Force is plotted as a function of distance of the magnet from the flowcell and error bars represent standard error. The calibration was fit to a single exponential (red line). The distance in which Notch activation was observed is shown with a dotted line, and corresponds to a force of 1.4 pN.

Figure S3. Additional control experiments for synthetic ligand-receptor system, related to Figure 3. (A) Cell-based reporter gene assay probing Notch or chimeric FRB-Notch activation by immobilized ligands. Luciferase reporter gene activity is reported as fold activation relative to the basal activity of cells cultured in the absence of any added ligand or small molecule. Error bars reflect the standard error of readings performed in triplicate. (B). Cell-based reporter gene assay probing activation of full-length human Notch and chimeric FRB-delEGF-Notch lacking the entire ligand binding domain (EGFs 1-36) in co-culture experiments with Dll4 or FKBP-Dll4. In this assay, 293T cells were used as signal-sending cells, and U2OS cells were used as signal-receiving cells. 293T cells were transfected with plasmids encoding wild-type Dll4, FKBP-Dll4, or empty vector (2 μg per well of a six-well dish), in the presence or absence of

rapamycin (0.05-1 μM), Compound E (GSI, 400 nM), or BB94 (20 μM). U2OS cells were transfected in 96-well format with plasmids encoding HA-Notch1-Gal4, or FRB-delEGFNotch1-Gal4 (lacking all 36 EGF-like repeats) along with a luciferase reporter plasmid containing the Gal4 response element and an internal control plasmid expressing Renilla luciferase. 24 hours after transfection, the 293T cells were added to the U2OS cells, and the luciferase reporter gene activity relative to the internal control was analyzed after cell lysis 24 hours later. Fold activation is reported relative to the activity of U2OS cells transfected with Notch molecules that were co-cultured with 293T cells transfected with empty vector. Error bars represent the standard error of triplicate measurements.

(C) Widefield view of data shown in Figure 3E. Cell mixing experiment was performed as previously described. The receptor positive cells and ligand positive (or control) cells were mixed in 1:5 ratio to maximize the induction efficiency. Notch receptor was stained by anti-Myc antibody and imaged in far red channel. The GBN-FlyNotch-QF receptor and EGFP-mCD8-Ser were used in the experiment.

Figure S4. Flow cytometry data associated with Figure 4. Flow cytometric analysis of the cell-surface levels of chimeric ligands in transfected cells, associated with Figure 4. HEK-293T cells were transfected with empty vector plasmid or a plasmid encoding either HA-FKBP-Dll4 or the tailless form of HA-FKBP-Dll4 (HA-FKBP-Dll4-tailless) in six-well dishes (2 μg plasmid per well). Cells were recovered 48 hours after transfection, and surface levels of the

chimeric ligands were analyzed using flow cytometry by detection of an HA epitope tag with an anti-HA antibody, followed by treatment with a FITC-conjugated anti-mouse secondary antibody. Plots show the fluorescence histogram.

Supplemental Experimental Procedures

Constructs: The sequence of the human Notch1 NRR (amino acid residues 1426-1733) was first amplified using primers that also introduced a 5' Nhe1 site, and a 3' Bsu36i site followed by an Avi tag and a stop codon, and then inserted into the pM-Mammalian Secretory SUMOstar vector (Lifesensors) using InFusion cloning (Clontech). Plasmids for expression of the positive (AV) and negative (AG) control peptides (NIPYKIEAVQS and NIPYKIEAGQS, respectively) were assembled by inserting the sequences encoding the peptides between the Nhe1 and Bsu36i sites. The catalytic domain of murine Adam17 (residues 1-477) was amplified by PCR using primers that incorporated a C-terminal His_8 tag, and subcloned into pcDNA3 The sequence encoding BirA was amplified from a commercial BirA vector (Avidity) using primers that introduced a C-terminal KDEL sequence followed by a stop codon, and inserted into pcDNA3.1 behind an N-terminal Notch1 signal sequence. Various Notch1-Gal4 cDNAs were assembled by derivatizing previously described Notch1-Gal4 expression constructs (Malecki et al., 2006). An Avi tag was also incorporated between the HA tag and the first EGF repeat of the Notch1 coding sequence. FRB-Notch1-Gal4 was prepared by substituting a fragment encoding the FRB domain (the FRB template was a kind

gift from Tom Muir) for EGF repeats 1-23 in the Notch1-Gal4 cDNA. Full-length human DLL4 was subcloned into pcDNA3.1. The chimeric FKBP-DLL4 construct was assembled by replacing the signal sequence, MNNL, and DSL domains of DLL4 with an immunoglobulin kappa signal sequence and a DNA sequence encoding FKBP. The tailless versions of DLL4 and FKBP-DLL4 were constructed by introducing a stop codon after amino acid residue 559. For expression of the isolated FKBP protein, the FKBP coding sequence was subcloned into pET15b behind an N-terminal His_6 tag.

To generate GBN-FlyNotch(NRR)-QF-3XMyc, full length QF2.0 (Potter et al., 2010) (a gift from Christopher J. Potter) was first cloned into pDONR221 vector through Gateway BP recombination (BP Clonase II, Invitrogen). Next, a signaling peptide (1-32 amino acid residues from mouse lymphocyte marker mCD8), a GFP binding nanobody (GBN) (Rothbauer et al., 2006), and fly Notch NRR domain together with transmembrane domain (1473-1960) were sequentially inserted before the first codon of QF using InFusion Cloning. A 3XMyc tag was then inserted at the C-terminal of QF. Finally, the whole GBN-flyNotch(NRR)-QF-3XMyc construct was cloned into a fly Gateway expression vector with Ubiquitin promoter (Ubi) (LR Clonase II, Invitrogen). The GFP-mcd8-X (X=Dl, or Ser) ligand was constructed by insertion of EGFP together with a C-terminal 17 amino acid linker (GGGASGGGSGGGGSGGG) after the signal peptide (residue 35) of mCD8 without the cytosol domain (1-222) using InFusion Cloning. To generate proper pulling force, the cytoplasmic domain of the *Drosophila* Notch ligand Delta

(Dl, 719-833) and Serrate (Ser, 1246-1407) were inserted after the transmembrane domain of mCD8, respectively. The GFP ligands were next cloned into the pUAST vector (Brand and Perrimon, 1993). Gal4 under the control of the actin promoter (Actin::Gal4) was used to drive expression of UAST-GFP ligands in fly cells. The QUAST-tdTomato-3XHA reporter for QF activity was a gift from Christopher J. Potter.

Recombinant proteins. The Notch1 NRR, as well as the positive and negative control peptides, were co-transfected with a plasmid for BirA expression into 293T cells. Cells were grown in a mixture of optimem and serum-free DMEM media supplemented with biotin (25 μM). The protein was collected from the conditioned media 2-3 days after transfection and purified by Ni-NTA affinity chromatography. Bound proteins were eluted in 250 mM imidazole, concentrated, and either used directly in single molecule experiments or after further purification by size exclusion chromatography on an S200 column. The Adam17 catalytic domain was secreted into the conditioned media of 293T cells and purified using Ni-NTA affinity chromatography. Eluted protein was passed through a concentrator with a MW cutoff of 100,000 Daltons, which was washed with Tris buffer (50 mM, pH 8) containing 150 mM NaCl, and 5 μM $ZnCl_2$. The flowthrough and wash were combined and concentrated using a concentrator with a MW cutoff of 10,000 Daltons and further purified by size exclusion using an S200 column. Activity assays of recombinant and commercially supplied (R&D Systems) Adam17 (Figure S1) were performed using the recommended fluorogenic peptide (R&D Systems), and fluorescence was recorded using a

SpectraMax M5 Microplate Reader (Molecular Devices). Recombinant FKBP was expressed in Rosetta pLysS cells using IPTG induction, and purified from the soluble fraction using Ni-NTA affinity chromatography. The elutate was buffer exchanged into Tris-buffered saline, concentrated, and stored at -80 C.

Cell lines. 293T and U2OS cells were used for transient transfections. Stable U2OS cell lines expressing Flag-Notch1-Gal4 and FRB-Notch1-Gal4 were prepared using the Invitrogen Flp-In system, as previously described (Malecki et al., 2006). *Drosophila* S2R+ cell lines were used to test the activation of the GBN-FlyNotch(NRR)-QF-3XMyc receptor by GFP-mcd8-Serrate ligand.

Single molecule magnetic tweezers experiments.

Glass coverslips were functionalized with 0.5% biotinylated PEG succinimidyl valerate and 99.5% methyl-PEG succinimidyl valerate (Laysan Bio) in 0.1 M $NaHCO_3$ (pH 8.2). Dried coverslips, stored under vacuum, were stable for several months. Recombinant SUMO-X-biotin (X= AV peptide, AG peptide, or Notch1 NRR) was captured onto the flowcell at the biotinylated end using streptavidin, and was bound to anti-SUMO antibody- (Lifesensors, Inc.) coated magnetic beads at the other end (tosyl-activated, 2.8-μm diameter; Dynal). Before an experiment, the biotin-PEG functionalized coverslip surface was incubated with 0.2 mg ml^{-1} streptavidin (Sigma) in PBS for 30 min and then washed with working buffer (20 mM Hepes, pH 7.4, 20 mM NaCl, 0.2 mg/mL BSA, 0.005% Tween 20). Anti-SUMO coated beads were flowed in to ensure that the surface was properly blocked- generally only 1-2 beads stuck non-specifically. The channel was washed again with working buffer. SUMO-X-biotin

was added to 500 µL working buffer containing 1 mM $CaCl_2$ at a concentration of about 5-100 pM and drawn into the flow cell at 0.025 ml min^{-1} with a syringe pump (Harvard Apparatus 11 Plus), allowing binding of SUMO-X-biotin to immobilized streptavidin sites. To minimize the probability of multiple tethers to a given bead, the optimal concentration of SUMO-X-biotin was determined for each protein prep by first flowing in 5 pM of protein, followed by beads, and increasing the concentration until the optimal field of about 200 beads at 10x (~1mm^2 field of view) was achieved. A stock of α-SUMO antibody–functionalized polystyrene beads (tosyl-activated, 2.8-µm diameter; Dynal) was prepared as previously described (Tanner and van Oijen, 2010). A 2 µL bead stock was diluted with 500 µL HBS-P buffer containing Surfactant P20 (GE Healthcare) and drawn into the flow cell at 0.015 ml min^{-1} to specifically bind the SUMO-labelled substrates. Excess beads were removed from the flow cell by washing with 0.5 mL working buffer plus calcium at 0.025 ml min^{-1}.

When the volume of buffer was reduced to 30 µl, the flow was stopped and 30 µl of working buffer plus calcium containing Adam17 (1 µM), and $ZnCl_2$ (4 µM) was added. For peptide experiments, the magnet was lowered to a distance corresponding to 1 or 5.4 pN of force. The solution containing Adam17 was loaded at a rate of 0.008 ml min^{-1} and flow stopped after the enzyme was loaded (~ 7 minutes). Movies were recorded using Metavue or MicroManager in one-second increments for 15-30 minutes. The total number of beads in each frame (10x objective) was counted using a built-in algorithm in ImageJ. The resulting trajectories of bead loss were corrected for nonspecific bead loss by taking the

slope of the initial 400 sec of data before enzyme reached the flow cell, and subtracting from the observed bead-loss curve after arrival of enzyme. For NRR experiments, Adam17 was loaded into the flow cell at ~1 pN force, and the magnet subsequently lowered to the appropriate distance corresponding to the desired applied force. Movies were recorded in Metavue at one frame per second for 15-30 minutes. Beads were then counted as described above. For each force, data was collected in the presence and absence of Adam17. Duplicate or triplicate traces for each mock and enzyme treated experiment were averaged after normalizing from the number of beads to the fraction of beads remaining. The averaged mock curve was then subtracted from the enzyme treated curve. The cleavage kinetics we observe are single-exponential under all conditions, strongly supporting the conclusion that only one NRR or peptide tethers each magnetic bead to the surface. BB94 experiment: Experiments were performed as above for the Notch1 NRR. BB94 was added to a final concentration of 0.67 mM into the enzyme solution prior to loading into the flow cell. Experiments were conducted under 7 pN of force. WC629 blocking antibody: WC629 (20 μg/mL) was loaded into the flow cell for 10 minutes prior to adding Adam17. The same concentration of WC629 was added to the Adam17 solution that was loaded into the flow cell. 5.4 pN of force was applied in this experiment. Details of magnet calibration can be found in supplemental experimental procedures.

Magnet calibration. Calibration of the magnets for both the single molecule force experiments and the 96 well magnetic tweezers experiments was

performed by measuring the in-plane fluctuations at 40x magnification of a magnetic bead attached to immobilized lambda DNA as a function of distance of the magnet from the flow cell. Bead positions were tracked using Diatrak, and force calculated as previously described (Strick et al., 1998).

Plated ligand assays. On Day 1, recombinant human Dll4 ectodomain (R&D Systems), recombinant FKBP ligand, or "non-native" ligand to epitope tags (in this case, anti-HA.11 antibody; Covance) was added to individual wells of a 96-well tissue-culture plate at 10 μg/ml, and incubated in PBS overnight at room temperature. On Day 2, Notch/Gal4 fusion constructs and reporter plasmids were reverse transfected with Lipofectamine 2000 into U2OS cells in Optimem media (Life Sciences). Transfected DNA amounts per well were 0.5-1 ng for Notch/Gal4 chimeras, 40 ng for Gal4-firefly luciferase, 0.8 ng pRTLK Renilla luciferase. The plate was washed once with PBS, and then 70 μl of cell-liposome suspension was transferred to each well. Three hours after transfection, 70 μl of media (DMEM+10% FBS; including Compound E (400 nM final concentration) and rapamycin (100-500 nM final concentration) as indicated) were added. On day 3, the luciferase reporter activity was determined using a Promega Dual Luciferase kit.

Western blots.

Plated ligand assays. On Day 1, U2OS cells stably expressing full-length Notch1/Gal4 or FRB-Notch1/Gal4 were plated into Uplift dishes (Thermo) in order to avoid the use of trypsinization during transfer onto ligand-coated wells. Doxycycline was added to a final concentration of 1 μm. Ligand or non-native

ligand (300 μL at 10 μg/ml in PBS buffer) was immobilized on 12 well plates overnight at room temperature. On day 2, Notch-expressing cell lines were allowed to "uplift" from Thermo plates and cells were transferred to the plates with immobilized ligand (in the presence of 400 nM Compound E and 100-500 nM rapamycin as indicated). Three hours later, RIPAA buffer minus SDS plus protease inhibitors was added, and the plates were kept at 4 C for 20 minutes before the lysates were subjected to SDS-PAGE and Western blotting. The anti-V1744 antibody (Cell Signaling) was used at 1:1000 dilution and the anti-Notch1-TAD antibody (Wang et al., 2011) was used at 1:5000.

Co-culture assays. On Day 1, U2OS cells stably expressing full-length Notch1/Gal4 or FRB-Notch1/Gal4 were plated into 12 well plates and doxycycline was added at a final concentration of 1 μm. Ligands were reverse transfected into 293T cells in 12 well plates (1mg/well). On day 3, ligand-expressing cells were resuspended in fresh media, and co-cultured with the Notch cells. The plate was centrifuged briefly at 500 *g* for 3 minutes after adding ligand cells. In experiments using the endocytois inhibitor, hydroxydynasore, co-culture of all ligand-expressing cells was performed in serum-free DMEM. Hydroxydynasore was used at a concentration of 30 μM. Three hours after the initiation of co-culture, cells were lysed with RIPAA buffer and Western blotting was performed as above.