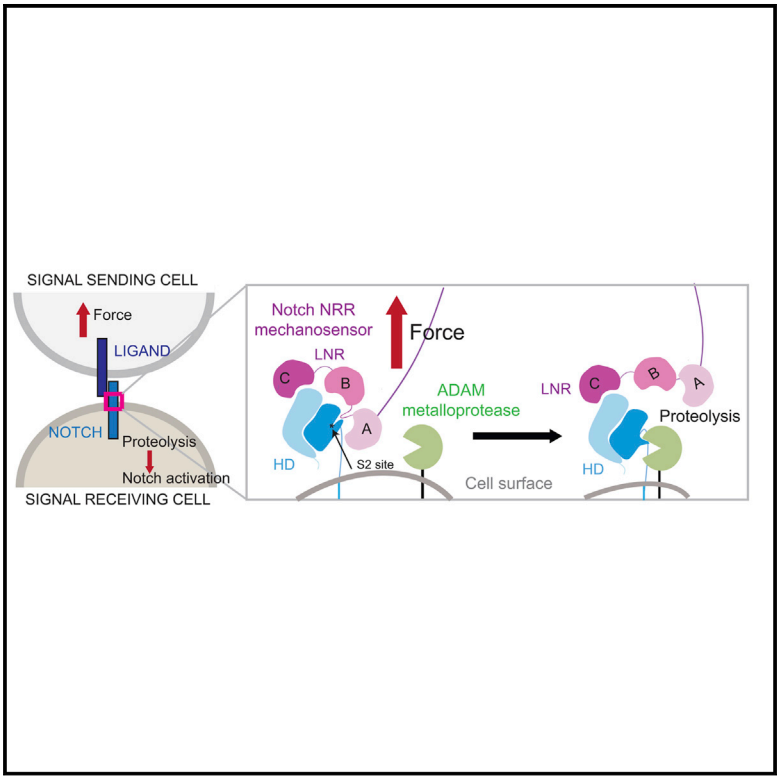
Short Article

Developmental Cell

Mechanical Allostery: Evidence for a Force Requirement in the Proteolytic Activation of Notch

Graphical Abstract



Highlights

- Development of single-molecule assays to monitor Notch proteolysis under force
- Exposure of the Notch1 masked proteolytic site occurs in a physiological force regime
- Dll4-induced activation of Notch1 requires the application of mechanical force
- Non-native tethering can substitute for receptor-ligand complexes in signaling

Authors

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In Brief

Gordon et al. develop and apply single-molecule and high-throughput magnetic tweezers assays to show that mechanical force is needed to unmask the metalloprotease site in the Notch "activation switch" and induce Notch signaling in cells. Synthetic signaling systems also show a ligand endocytosis requirement even without native ligand-receptor interactions.

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Mechanical Allostery: Evidence for a Force Requirement in the Proteolytic Activation of Notch

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SUMMARY

Ligands stimulate Notch receptors by inducing regulated intramembrane proteolysis (RIP) to produce a transcriptional effector. Notch activation requires unmasking of a metalloprotease cleavage site remote from the site of ligand binding, raising the question of how proteolytic sensitivity is achieved. Here, we show that application of physiologically relevant forces to the Notch1 regulatory switch results in sensitivity to metalloprotease cleavage, and bound ligands induce Notch signal transduction in cells only in the presence of applied mechanical force. Synthetic receptor-ligand systems that remove the native ligand-receptor interaction also activate Notch by inducing proteolysis of the regulatory switch. Together, these studies show that mechanical force exerted by signal-sending cells is required for ligand-induced Notch activation and establish that force-induced proteolysis can act as a mechanism of cellular mechanotransduction.

INTRODUCTION

Notch signaling conveys information between cells using a mechanism that is conserved in organisms ranging from flies to humans. These signals influence a wide range of cell fate decisions both during development and in adult tissue homeostasis. In addition, a number of human diseases are associated with mutations of Notch pathway components that result in loss or gain of function.

Notch signaling occurs when a transmembrane ligand of the Delta, Serrate, and Lag2 (DSL) family engages a transmembrane Notch receptor on a neighboring cell, inducing regulated intramembrane proteolysis (RIP) to produce a transcriptional effector (Kopan and Ilagan, 2009). During transport to the cell surface, Notch receptors are cleaved at site S1 by a furin-like protease, but are resistant to further proteolysis, because the activating cleavage site, called S2, is buried in an autoinhibited conformation within a negative regulatory region (NRR) consisting of three LNR modules and a juxtamembrane "heterodimerization domain" (HD) (Gordon et al., 2007, 2009). Ligand binding relieves autoinhibition by exposing S2 to ADAM metalloproteases (Brou et al., 2000; Mumm et al., 2000). Activating mutations of the Notch1 NRR that result in ligand-independent proteolysis are found frequently in human leukemias, highlighting the importance of tight control of metalloprotease access to the S2 site (Weng et al., 2004).

How ligand engagement relieves autoinhibition of Notch remains poorly understood. X-ray structures of the NRRs from Notch1 and Notch2 show that the S2 site near the C-terminal end of the HD is masked by the LNRs (Gordon et al., 2007, 2009), indicating that ligand binding must result in sufficient displacement of the LNRs to allow metalloprotease access to S2. Because the binding site for Notch ligands is centered on EGF repeats 11–12, more than 20 EGF modules away (Rebay et al., 1991), and because genetic and biochemical studies have established a requirement for endocytosis of ligand into signal sending cells (Nichols et al., 2007; Musse et al., 2012), it has long been speculated that endocytic internalization of Notch-bound ligands delivers a pulling force that relieves autoinhibition by exposing S2 (Musse et al., 2012). It remains unknown, however, whether S2 proteolysis can be induced in the physiologic force regime or whether force is even required to activate ligand-bound receptors on cells.

In the work reported here, we develop a single-molecule assay to determine the force required for NRR proteolysis in vitro, and using a cell-based magnetic tweezer assay we also developed, we show that force is required for relief of Notch autoinhibition in cells. We also designed two synthetic ligand-receptor systems, which both show that signal-sending cells supply sufficient force to induce metalloprotease sensitivity in the NRR in the absence of native ligand-receptor interactions, indicating that ligand binding does not need to exert an allosteric effect on the sensitivity of the NRR in order for activating proteolysis to occur. These results show that mechanical force generated by

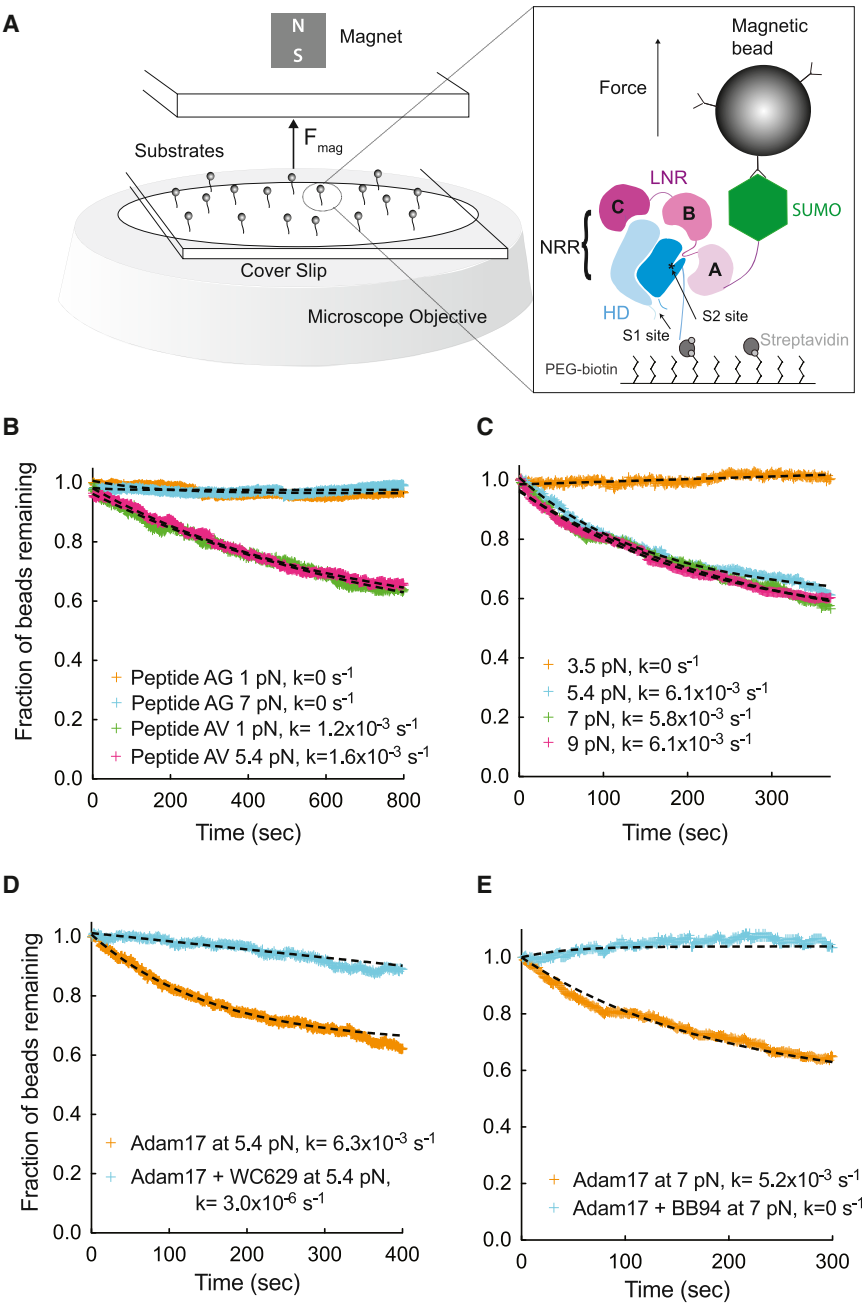

Figure 1. Single-Molecule Assay of Adam17-Mediated Proteolysis of the Notch1 NRR under Force

(A) Assay schematic and experimental design. Magnetic beads are tethered to proteins immobilized in a flow cell mounted on an inverted microscope. Force is applied to the beads by varying the distance between a magnet and the surface of the flow cell. Substrate proteolysis is monitored by determining the fraction of beads released over time. The expanded view in the right panel illustrates the Notch1 NRR, captured on the flow cell with streptavidin and tethered to the magnetic bead using anti-SUMO antibodies.

(B) Adam17-catalyzed proteolysis of biotinylated and SUMO tagged recombinant peptides, containing either the natural S2-cleavage site sequence (AV, green and pink), or a mutated sequence with a V1721G substitution (AG, orange and cyan) at the forces indicated.

(C) Adam17-catalyzed proteolysis of the Notch1 NRR, monitored as a function of time at different levels of applied force. Traces shown represent averages of two or three replicates.

(D and E) Effect of inhibitors on proteolysis of the Notch1 NRR in the single molecule cleavage assay. Traces shown represent a single experiment. (D) Effect of WC629, an anti-Notch1 inhibitory antibody that binds to the NRR, on the time course of Adam17-catalyzed NRR proteolysis. (E) Effect of BB94, an ADAM inhibitor, on the time course of Adam17-catalyzed NRR proteolysis. Additional control experiments are provided in Figure S1.

signal-sending cells is sufficient to unfold the NRR and sensitize Notch to proteolytic activation.

RESULTS

Physiologic Forces Sensitize the NRR to ADAM Cleavage

To test whether proteolytic cleavage of the activation switch occurs in a physiologic force regime, we developed a single-molecule, multiplexed magnetic tweezers assay to determine the proteolytic sensitivity of the isolated Notch1 NRR as a function of applied force (Figures 1 and S1). The Notch1 NRR, as well as control proteins intrinsically sensitive or resistant to Adam17 cleavage, was immobilized on the surface of a flow cell by streptavidin capture, tethered to magnetic beads coated with an anti-SUMO antibody, and subjected to Adam17 delivered by syringe pump. Enzymatic cleavage of tethered molecules was determined as a function of applied magnetic force, monitoring bead loss by dark field microscopy.

Proteolysis experiments using control peptides show that Adam17 cleaves a bead-tethered polypeptide that presents the native S2 processing site of Notch1 ("AV" peptide) when as little as 1 pN of force is applied. The kinetics of cleavage for the AV peptide are indistinguishable at 1 and 5.4 pN of applied force, already fully sensitive to Adam17 at 1 pN. In contrast, a control peptide with a mutated cleavage site (AG) is Adam17 resistant up to 7 pN of applied force (Figure 1B).

When the intact Notch1 NRR is examined in this assay, it resists Adam17 cleavage at a force of 3.5 pN, but undergoes proteolysis at forces $\geq$5.4 pN, indicating that the transition from resistance to sensitivity occurs in a physiologically accessible regime between 3.5 and 5.4 pN of force (Figures 1C and S1D). Both an NRR conformation-specific inhibitory antibody WC629 and the metalloprotease inhibitor BB94 prevent proteolysis by Adam17, confirming that bead release

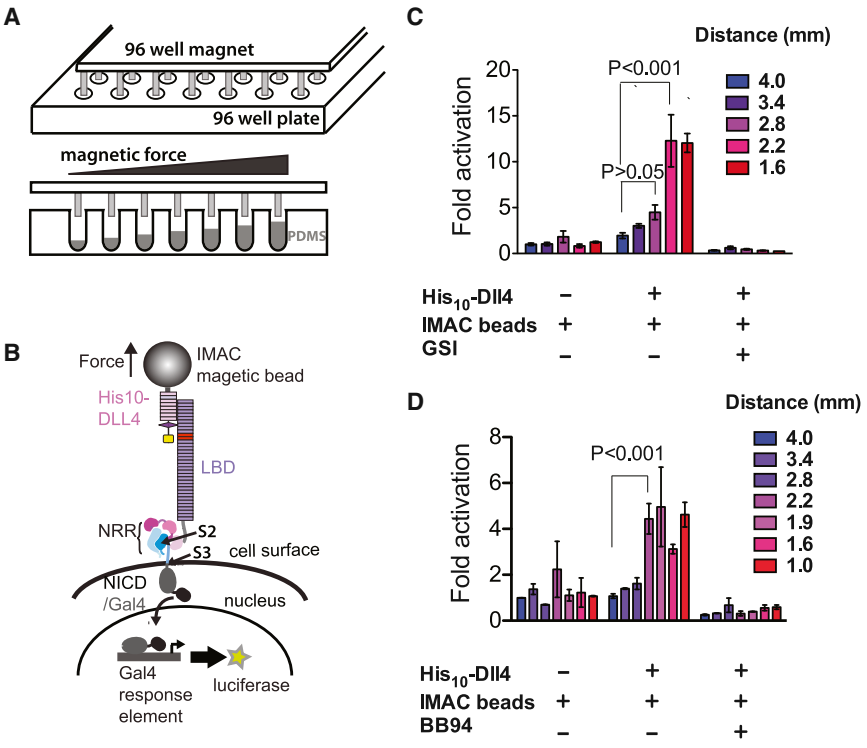

Figure 2. Response of Cell-Surface Notch Receptors to Applied Force using a Multiplexed Magnetic Tweezers Assay

(A) Experimental design. A plate containing 96 cylindrical magnets is positioned over a 96-well plate of cells in order to apply force to magnetic beads tethered to Notch molecules on the cell-surface. The distance between the cells and the magnet is varied by using the polymer PDMS to create terraces of different heights.

(B) Assay schematic. Cells expressing Notch1 receptors in which the ankyrin-repeat domain has been replaced by the Gal4 DNA-binding domain (Malecki et al., 2006) are stimulated by magnetic beads loaded with the ligand DLL4, followed by measurement of luciferase reporter gene activity.

(C and D) Luciferase reporter gene activity in response to various treatments as a function of the distance from the magnet. U2OS cells expressing Notch1-Gal4 receptors were incubated with magnetic beads alone or beads loaded with the ligand DLL4 in the absence or presence of a gamma secretase inhibitor (GSI) (C) or metalloprotease inhibitor BB94 (D). Luciferase reporter gene activity is reported relative to the response of cells to beads alone at a distance of 4 mm from the magnet. Error bars represent the SEM of triplicate measurements, and statistical significance was determined with a two-way ANOVA followed by a post hoc Bonferroni test. Magnet calibration (96-well) is provided in Figure S2.

results from metalloprotease cleavage at S2 (Figures 1D and 1E).

Force Induces Notch Activation in Cells

We next wished to determine whether force is required for the induction of a Notch signal when ligands bind to Notch receptors on cells. Because studies using genetically encoded or surface tethered force sensors have shown that signaling proteins such as integrins (Morimatsu et al., 2013; Wang and Ha, 2013), cadherins (Borghi et al., 2012), and vinculin (Grashoff et al., 2010) respond to applied force in the 1–40 pN range, we developed a high-throughput magnetic tweezers assay to apply a wide range of pN-scale forces to Notch receptors on the cell-surface. Our method uses magnetic tweezers in 96-well format and applies force to cell-surface receptor molecules bound to ligands on paramagnetic beads (Figure 2A). By controlling the distance between the cells and the magnet, it is possible to vary the force applied to cells as a function of their well position on the plate. In order to present the cells at different distances from the magnet, we dispensed different amounts of PDMS polymer into the culture chambers, creating a "terraced" configuration of wells of different depths across the plate. The range of forces sampled in a given experiment is specified simply by varying the heights of the terraces, the size of the beads and the characteristics of the magnet. For example, when 1 μm beads are used and the distance of the magnet from the cells ranges from 0.15 to 0.5 mm, the applied force estimated from force calibration using phage lambda DNA ranges from 0.5 to 2.5 pN (see Figure S2 for 96-well magnet calibration).

To probe the force dependence of Notch activation using this assay, we cultured cells expressing Notch1-Gal4 chimeric receptors (Malecki et al., 2006) in wells of different depths and treated the cells with paramagnetic beads loaded with the ligand DLL4. Force was applied to the beads by placing a 96-well magnet over the plate, and luciferase reporter-gene activity was measured 6 hr later (Figure 2B). When cells expressing the Notch1-derived receptors are incubated with DLL4-loaded magnetic beads, a statistically significant signal is induced only when the magnet is $\leq$2.2 mm from the beads and is suppressed in the presence of gamma-secretase or metalloprotease inhibitors (Figures 2C and 2D). (This magnet distance exerts a force of about 1.4 pN based on in vitro calibration with lambda DNA; Figure S2.) Given the many differences between the cell-based and in vitro proteolysis assays, it is not surprising that the amount of force sufficient to induce Notch proteolysis differs between the two experiments. In particular, the sustained delivery of force (over several hours) to receptors on cells combined with intrinsic protein dynamic motions promoting conformational opening likely results in irreversible capture of transiently open states by proteolysis at reduced forces and accounts for the lower force requirement in cells. Other factors, such as the influence of the membrane or its microenvironment, the ligand-binding domain of the receptor, or the clustering of receptors in response to bead-tethered ligand, may also contribute. Regardless, the key finding is that force must be applied to bead-tethered ligands in order to induce the canonical proteolytic steps responsible for Notch activation.

Robust Notch Signals in Synthetic Systems

To explore whether or not a signal-sending cell can directly deliver sufficient force to induce NRR proteolysis, we created "synthetic" ligand-receptor signaling systems that substitute

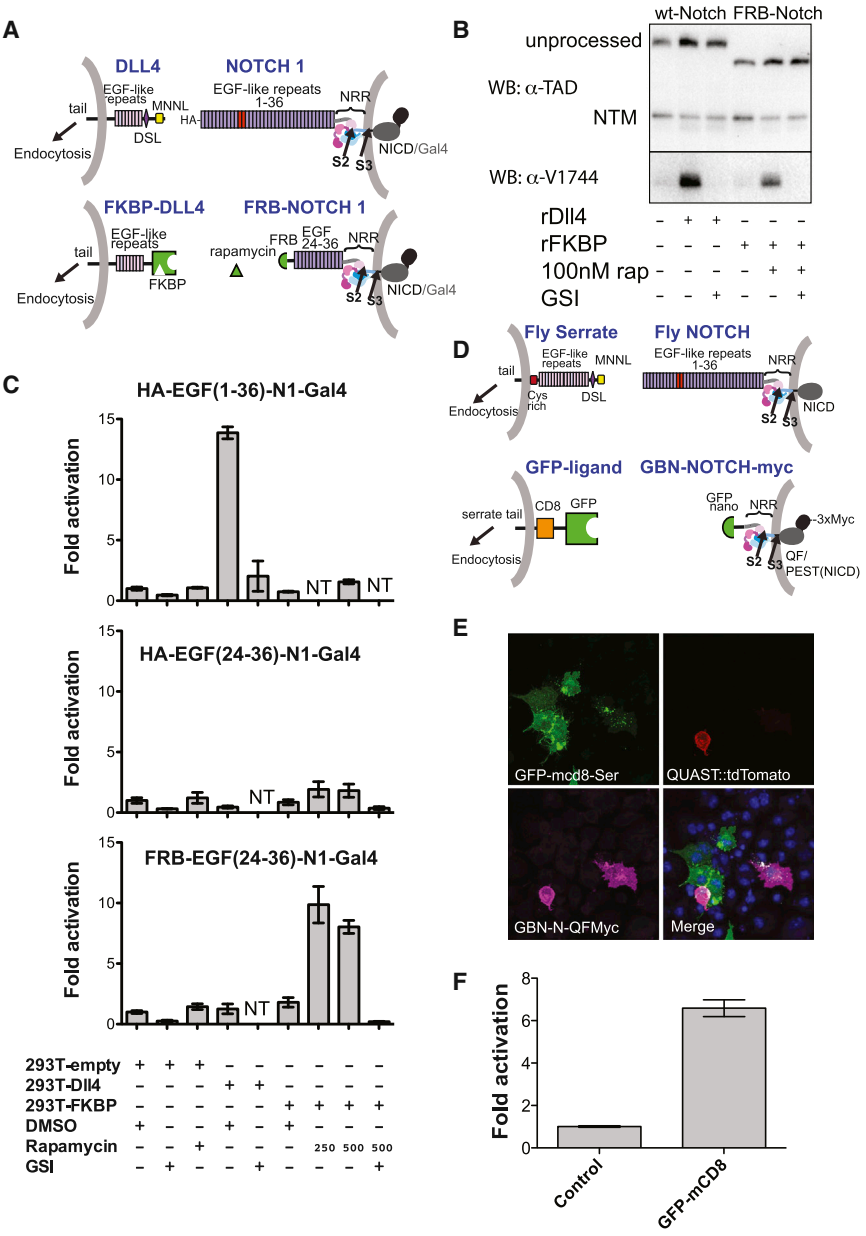

Figure 3. Development and Evaluation of Two Synthetic Notch Signaling Systems

(A) Schematic comparing the natural human Notch-ligand signaling system (top; EGF repeats 11–12 in red) to a synthetic signaling system placing NRR proteolysis under rapamycin-inducible control (bottom). Here, FKBP replaces the N-terminal portion of DLL4, and FRB replaces EGF-like repeats 1–23 of Notch1. The Notch1 ankyrin domain is also replaced with Gal4, as above (Malecki et al., 2006).

(B) Western blots monitoring receptor proteolysis. U2OS cells stably expressing wild-type or FRB-Notch1 were grown in the presence of the DLL4 ectodomain or FKBP immobilized on plastic tissue culture dishes in the absence or presence of rapamycin (100 nM) and/or a GSI (Compound E, 400 nM). Blots were probed with an antibody directed against an epitope of intracellular Notch1 (α-TAD) or the α-V1744 antibody to S3-cleaved Notch1 (Cell Signaling).

(C) Cell-based reporter gene assay. U2OS cells stably transfected with the indicated Notch variants were co-cultured with 293T cells transiently transfected with the indicated ligands. Luciferase activity for each U2OS line is reported relative to co-culture with 293T cells transfected with empty vector. Error bars reflect the SE of readings performed in triplicate. Additional control experiments are provided in Figure S3.

(D) Schematic illustrating design of a GFP-GFP-binding nanobody (GBN) synthetic ligand-receptor pair. Full-length fly Serrate and Notch are shown for reference. The artificial ligand consists of GFP, CD8, and the Serrate-derived tail. The ectodomain of the Notch-derived molecule consists of the GFP binding nanobody (GBN) and the NRR, and the intracellular domain contains the QF transcription factor, the Notch PEST domain, and a triple Myc tag.

(E) Co-culture assay. S2R+ cells expressing GFP-mcd8-Ser as ligand (green, upper left panel) were co-cultured with cells expressing GBN-Fly-Notch(NRR)-QF-3XMyc (GBN-N-QFMyc). Receptor is stained with anti-myc antibody (magenta, lower left panel). The tdTomato reporter signal is red (upper right panel). DNA was stained with DAPI (blue, lower right panel).

(F) QAS luciferase readout of cell-mixing experiment. Luciferase reporter gene activity for the GBN-Notch cell line is reported relative to co-culture with control cells. Error bars represent the SE of measurements performed in quadruplicate.

See also Figure S3.

the native binding interaction between Notch1 and DLL4 with non-native interacting pairs to tether signal-sending and receiving cells together. These systems dispense with native interaction domains and thus eliminate the possibility that formation of a native ligand-receptor complex allosterically lowers the barrier to proteolysis of the NRR.

In the first system, we tethered sending and receiving cells using the FRB domain of mTor and the FK506 binding protein (FKBP), which interact to form a stable complex only in the presence of rapamycin (Figure 3A). The chimeric DLL4 ligand molecules substitute FKBP in place of the Notch-binding MNNL and DSL domains, but retain the rest of their extracellular region, as well as the transmembrane region and cytoplasmic tail, which contains the sequences that target the ligand for endocytosis. The Notch-derived molecules substitute the FRB domain of mTor in place of EGF-like repeats 1–23 that encompass the ligand binding region, but retain repeats 24–36, the NRR, the transmembrane region, and the Notch1-Gal4 intracellular fusion for monitoring luciferase reporter activity with a Gal4 response element as above (Figure 3A).

We first tested the fidelity of this synthetic ligand-receptor pair and compared its signaling activity with normal ligand-receptor complexes using a well-established "plated ligand" assay, in which cells expressing receptors of interest are cultured in

dishes coated with a ligand ectodomain. Plated DLL4 (or plated anti-HA) stimulates proteolytic activation of the intact HA-Notch1-Gal4 fusion protein, but not the FRB-Notch1 chimera; in contrast, plated FKBP, when in the presence of rapamycin, induces proteolytic activation of the FRB-Notch1 chimera, but not the standard Notch1-Gal4 fusion (Figures 3B and S3A).

We next tested whether this synthetic system signals in a co-culture assay, in which ligand-expressing cells are used to stimulate a signal in receptor-expressing cells (Figure 3C). Control experiments confirm that DLL4 expressing cells induce a reporter response in the cells expressing full-length HA-tagged Notch1, but not in cells expressing a truncated HA-tagged receptor, or the chimeric FRB-Notch receptor. In contrast, cells expressing the FKBP chimeric ligand only activate signaling in cells expressing the FRB-Notch chimeric receptor in a rapamycin-dependent fashion (Figure 3C). This signaling activity is sensitive to a gamma secretase inhibitor and to the metalloprotease inhibitor BB94, indicating that activating proteolysis of the NRR at S2 and subsequent S3 cleavage can be triggered in the absence of native receptor-ligand interactions. Similar results are obtained when the experiment is performed with Notch molecules lacking all 36 EGF repeats (Figure S3B).

We also created a second chimeric signaling system derived from *Drosophila* proteins that pairs an anti-GFP nanobody and the QF transcriptional activator under Notch NRR control with a Serrate-derived protein that substitutes GFP (followed by CD8) in place of the normal Serrate ectodomain (Figure 3D). Thus, the entire ligand binding domain of Notch and the entire Notch-binding region of the ligand have been removed. Nevertheless, this system induces expression of tomato-GFP (under control of a QF-responsive element) only in nanobody-driven responder cells that are in direct contact with GFP-expressing ligand cells (Figures 3E and S3C) and signals in co-culture assays (Figure 3F). The robust signaling observed in two synthetic systems utilizing non-native modes of protein-protein interaction shows that a pair of interacting moieties sufficient to (1) bring signal sending and receiving cells into contact, and (2) withstand rupture under the force required to expose S2 is all that is needed to induce NRR proteolysis and transduce a signal. Although the native ligand-receptor interaction may alter the energy landscape associated with conformational exposure of the S2 site of the NRR, the synthetic systems show conclusively that an allosteric effect of ligand binding is not necessary for S2 cleavage to occur.

Endocytosis Is Required for S2 Site Exposure

To address whether or not endocytosis of the ligand is required for proteolytic activation of the receptor, we blocked ligand endocytosis in the mammalian and rapamycin-dependent co-culture assays using two different approaches: (1) deletion of the cytoplasmic tail of the ligand (which is required for endocytosis-dependent activation), and (2) treatment of ligand-expressing cells with hydroxydynasore, a small-molecule endocytosis inhibitor (McCluskey et al., 2013). The response to both interventions in the rapamycin-based synthetic signaling system mirrors that of Notch1 responding cells to DLL4-expressing signal-sending cells. Tailless ligands, which are transported to the cell surface as well as ligands with intact cytoplasmic tails (Figure S4), attenuate production of the gamma-secretase cleaved product (Figure 4A) and reporter gene expression (Figure 4B). Similarly, treatment of ligand cells with hydroxydynasore suppresses accumulation of the S3-cleaved product in both wild-type Notch-DLL4 and synthetic signaling systems (Figure 4C). Production of the S3-cleaved product is comparably attenuated in both systems by treatment with a gamma-secretase inhibitor, the metalloprotease inhibitor BB94, or the anti-Notch1 inhibitory antibody WC75, which binds specifically to the Notch1 NRR and stabilizes the autoinhibited conformation. Similar decreases in signaling activity occur upon hydroxydynasore treatment in the *Drosophila* GFP nanobody-GFP synthetic system (Figure 4D). Together, these data show that endocytosis of ligands artificially tethered to receptor molecules promotes proteolytic activation of Notch signaling in a fashion that remains dependent on a conformational change in the NRR permissive for S2 and S3 cleavages. Importantly, tethering alone without ligand endocytosis is insufficient for activation. We conclude that a step dependent on ligand endocytosis is required for signal-sending cells to deliver sufficient mechanical force to the receptor to induce the proteolytic cascade responsible for receptor activation and downstream signaling events.

DISCUSSION

The goal of these studies was to gain insight into the still elusive mechanism of ligand-induced proteolysis of Notch receptors. Previous X-ray structures of the Notch NRR "activation switch" show that a major conformational change must occur in order to unmask the solvent inaccessible S2 processing site for metalloprotease cleavage. A leading model (Parks et al., 2000) proposes that endocytosis of ligands applies a pulling force to bound Notch receptors, thereby exposing the S2 proteolytic site. In this model, the NRR would then be a mechanosensitive switch responding to this pulling force. A number of indirect lines of evidence are consistent with the mechanotransduction model, and the requirement for a specialized pathway for endocytosis of ligands in the signal-sending cells is well established (Musse et al., 2012).

As appealing as a mechanotransduction model might be, however, it has remained unclear (1) whether or not the force required to induce proteolytic sensitivity in vitro or in vivo lies in a physiologically accessible force regime, (2) whether allostery is required to lower the barrier to proteolysis, and (3) whether or not the force is delivered by ligand endocytosis. AFM studies in which the Notch2 NRR was pulled under high loading rates showed that multiple unfolding transitions occur in the 100 pN range, but how these findings relate to a physiological context is unclear (Stephenson and Avis, 2012). Studies using plated ligands conjugated to tension-gated tethers (TGT), which sense forces imposed on cellular receptors based on rupturing short DNA duplexes, led to the conclusion that Notch activation occurs at forces under 12 pN, but could not establish whether or not applied force was needed at all because of the limits of DNA duplexes as force sensors (Wang and Ha, 2013). The use of plate-bound ligands as activators also does not address whether or not signal-sending cells are capable of supplying an activating force.

Here, we developed assays to determine how the proteolytic sensitivity of site S2 varies as a function of applied force both

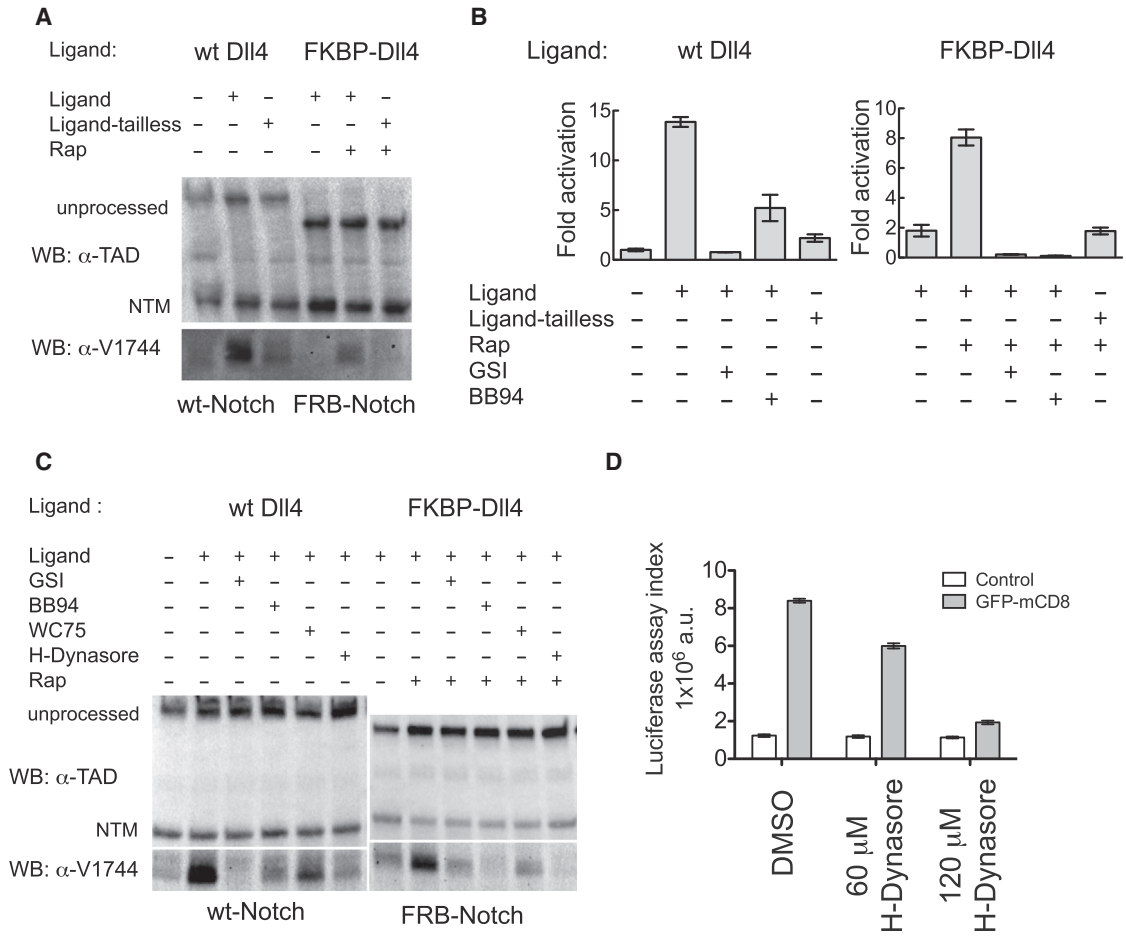

Figure 4. Analysis of Natural and Synthetic Notch Receptor Signaling in Co-culture Assays

(A) Effect of ligand-tail deletion on signaling. Wild-type and synthetic Notch receptors were co-cultured with full-length or tail-deleted cognate ligands (Ligand-tailless) and in the absence or presence of rapamycin (250 nM), as indicated. Blots were probed with an antibody directed against an epitope of intracellular Notch1 (α-TAD), or the α-V1744 antibody to S3-cleaved Notch1 (Cell Signaling).

(B) Cell-based reporter gene assay probing Notch activation in co-culture experiments. 293T cells were signal-sending cells, and U2OS cells were signal-receiving cells. 293T cells were transfected with plasmids encoding wild-type Dll4, tailless Dll4, FKBP-Dll4, FKBP-Dll4-tailless, or empty vector in the presence or absence of rapamycin (250 nM), Compound E (GSI, 400nM), or BB94 (20 μM). U2OS cells were transfected in 96-well format with plasmids encoding HA-Notch1-Gal4 (left), or FRB-Notch1-Gal4 (right) along with a luciferase reporter plasmid containing the Gal4 response element and an internal control plasmid expressing Renilla luciferase. Twenty-four hours after transfection, the 293T cells were added to the U2OS cells. Luciferase activity relative to the Renilla control was determined 24 hr later. Fold activation is relative to U2OS cells transfected with HA-Notch1-Gal4 and co-cultured with empty-vector transfected 293T cells. Error bars represent the SE of triplicate measurements.

(C) Effect of various drug or antibody treatments on signaling by wild-type or synthetic receptors when co-cultured with cognate ligands. Blots were probed with α-TAD or the α-V1744 antibody as in (A).

(D) Effect of hydroxydynasore in the fly synthetic signaling assay. Ligand expressing cells and untransfected control cells were first treated with the indicated concentration of H-Dynasore for 30 min. Receptor and ligand (or control) cells were then mixed together in a 1:5 ratio. Fresh drug was added to maintain the desired concentration, and luciferase activity was determined 6 hr later. Trypan blue staining after 10 hr of co-culture showed no difference in viability between DMSO and drug treatment (not shown).

See also Figure S4.

in vitro and in cells under physiologically relevant conditions. The in vitro magnetic tweezers assay revealed that the isolated activation switch undergoes a transition from protease resistance to sensitivity between 3.5 and 5.4 pN of force. Typical rate constants of cleavage were $\sim 6 \times 10^3\ M^{-1}s^{-1}$, in line with reported catalytic efficiencies for the isolated metalloprotease domain of Adam17 (Caescu et al., 2009). Cell-surface Notch1 receptors also exhibit mechanosensitivity upon application of force via ligand tethered magnetic beads, as the presence of ligand-coated beads alone is not sufficient to induce Notch activation.

The low forces required to relieve Notch autoinhibition show that the NRR is a highly mechanosensitive switch. How do these forces compare to the forces required for unfolding of other mechanosensors, or for other biological force-dependent events? The A2 domain of von Willebrand factor, a mechanosensor that undergoes proteolysis in response to shear stress, unfolds with a transition at 8 pN of force (Zhang et al., 2009). Similarly,

the binding of vinculin to talin relies on unfolding in the talin R3 domain over a force range of ~2–5 pN (del Rio et al., 2009; Yao et al., 2014). In addition, the force required for S2 exposure is comparable to the 3–4 pN force generated by a myosin motor taking a step on actin (Finer et al., 1994) and stall forces measured for kinesin (4–6 pN) and dynein (1 pN) (Blehm et al., 2013). Importantly, the force required to relieve autoinhibition of the activation switch is lower than the forces measured by optical tweezers to rupture ligand-receptor interactions and in line with the measured stall force generated by endocytosis of DLL1 (Meloty-Kapella et al., 2012; Shergill et al., 2012) and with the force experienced by EGFR during endocytosis (Stabley et al., 2012). Our data using synthetic signaling systems now show that allostery is not required to render the NRR sensitive to proteolytic activation. Moreover, these experiments directly link ligand-receptor engagement to proteolytic site exposure of the NRR in a step that depends on ligand endocytosis, though whether or not endocytosis itself supplies the pulling force remains to be determined.

Our studies investigating the responsiveness of the Notch1 NRR to force also raise a number of new questions about the mechanosensitive behavior of Notch receptors. What degree of domain movement is required to relieve autoinhibition? Is the barrier to mechanical exposure of the metalloprotease site in Notch1 influenced by the EGF-repeat region, or is the mechanosensitive property of the entire receptor completely encoded within the NRR? How do lateral interactions among Notch receptors in the membrane affect receptor mechanosensitivity? And how does the intrinsic sensitivity to force vary among the various Notch receptors, both in isolation, in response to disease-associated mutations, different ligands, or mechanical forces generated in the cellular microenvironment (e.g., by blood flow or muscle contraction)?

The methods developed here to investigate the role of Notch signaling should have wide utility for exploring the consequences of Notch signal transduction under precise chemical and temporal control and for investigation of other mechanosensitive processes in biology. The synthetic GFP-nanobody and rapamycin-dependent signaling systems open up new possibilities for controlling and reporting on Notch activation in a defined cellular context. The approaches can be used to investigate the kinetics of metalloprotease recruitment, receptor proteolysis, as well as events downstream of receptor cleavage. The assays can also report on whether or not two cells contact each other in vivo. Finally, the cell-based magnetic tweezers assay should facilitate new studies of other biological processes that may rely on mechanical force for the induction of signaling, such as ephrin-ephrin receptor signaling (Salaita et al., 2010), atypical cadherin complexes of the inner ear (Sotomayor et al., 2012), and other transmembrane signaling events.

EXPERIMENTAL PROCEDURES

Materials

A complete description of constructs, recombinant proteins, and cell lines are provided in the Supplemental Experimental Procedures.

Single-Molecule Magnetic Tweezer Experiments

Briefly (see Supplemental Experimental Procedures), single-molecule experiments were performed using custom microfluidic flow cells with glass coverslips as described previously (Tanner and van Oijen, 2010). Two stacked 6-mm cube magnets were attached to a mount containing a micrometer in order to control the distance from the magnet to the flow cell. Biotinylated NRRs or peptides are delivered into the flowcell with a syringe pump and captured in the flowcell with streptavidin. After delivery of magnetic beads followed by extensive washing, buffer with Adam17 (1 μM), and $ZnCl_2$ (4 μM) was added. Movies were recorded using Metavue or MicroManager in 1-s increments for up to 30 min. The total number of beads in each frame (10× objective) was counted using a built-in algorithm in ImageJ. 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. The magnet calibration is described in Supplemental Experimental Procedures.

96-Well Magnetic Tweezer Assays

PDMS Components A and B (Sylgard 184, Dow Corning) were added to a 50 ml falcon tube in a ratio of 10:1 and were mixed by slow rotation over 30 min. The mixture was centrifuged at 4,000 × *g* for 5 min and then dispensed with an Eppendorf digital repeat pipette using the slowest setting to ensure reproducible dispensing. The PDMS was dispensed into 96-well TC-coated plates in volume "steps" from 40–120 μl and was cured overnight at 37°C. Before cells were plated, the wells were bathed in 70 μl of fibronectin (10 μg/ml in PBS; Sigma) for 1 hr at 37°C. U2OS cell lines stably expressing Flag-Notch1-Gal4 were then reverse-transfected with luciferase reporter plasmids as above, treated with 1 μM doxycycline to induce protein expression, and plated onto the PDMS-modified wells. After 24–48 hr, cells were incubated in DMEM with or without 500 nM recombinant Dll4 ectodomain (R&D Systems). After 20 min, an excess of 1 μm IMAC magnetic beads in DMEM (Dynal) was added, and a plate with the 96-well configuration of magnets was placed over the cells (Alpaqua). The level of luciferase reporter activity was determined 6 hr later using a Promega Dual Luciferase kit.

Chimeric Notch/Ligand Experiments

Co-culture experiments, human cell lines. On day 1, Notch1-Gal4 fusion constructs and reporter plasmids were reverse transfected into U2OS cells in 96-well format as above. Ligand molecules were reverse transfected separately into 293T cells in 6-well plates (2 μg ligand/well) using Lipofectamine 2000. On day 2, ligand-transfected cells were resuspended in fresh DMEM with 10% FBS, drugs were added as indicated, and the 293T cells were plated on top of the Notch-expressing cells. On day 3, the luciferase reporter activity was determined as above.

Co-culture experiments, *Drosophila* cell lines. On day 1, S2R+ cells were transfected in 6-well dishes with 400 ng total DNA/well using Effectene Transfection Reagent (QIAGEN). Receptor positive cells were generated by transfection of 396 ng of QUAT::tdTomato and 4 ng ubi::GBN-fly-Notch(NRR)-QF-3XMyc DNA. Ligand-positive cells were generated by transfection of 100 ng Actin::Gal4 together with 300 ng UAST::GFP-mcd8-Ser or UAS-GFP-mcd8-Dl. On day 3, receptor and ligand positive cells were each washed in fresh culture medium to remove transfection reagents and dislodged from dishes by pipetting. Half of the receptor positive cells were mixed with ligand positive cells, while the other half were mixed with the same number of untransfected S2R+ cells (control). The cell mixture was transferred into a 1.5 ml Eppendorf tube and slowly rotated at room temperature for 1 hr to allow the ligand and receptor positive cells to bind to each other. Then the cell mixture was plated back into a new 6-well dish and cultured for one additional day before assay. For immunofluorescence imaging, the cell mixture was plated on coverglass bottom chamber slides (Lab-Tek) coated with Concanavalin-A. Cells were fixed in 4% formaldehyde, stained with mouse anti-Myc antibody (1:400, 9E10, Santa Cruz Biotech) followed by Alexa 647 goat anti-mouse IgG (1:500, Invitrogen), and observed after mounting in a Zeiss LSM 780 confocal microscope using a 63×/N.A. 1.4 oil objective. Western blot methods are provided in Supplemental Experimental Procedures.

Statistical analysis of reporter assays. Error bars in reporter assays represent SEM of triplicate or quadruplicate measurement. Statistical analysis to assess significance (p values) was performed with GraphPad Prism software using two-way ANOVA followed by a Bonferroni post hoc test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.05.004.

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Developmental Cell

Supplemental Information

Mechanical Allostery:

Evidence for a Force Requirement in the Proteolytic Activation of Notch

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Supplemental Inventory

Figure S1, related to Figure 1

Figure S2, related to Figure 2

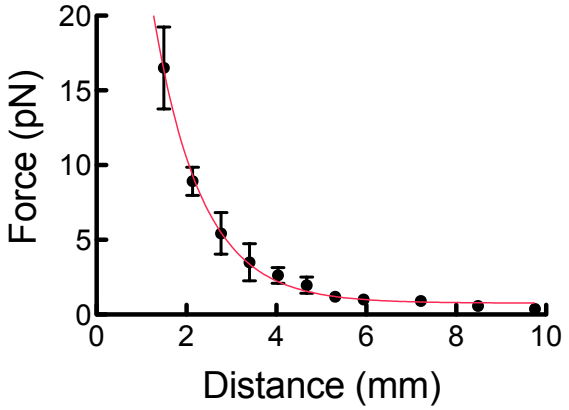
Figure S3, related to Figure 3

Figure S4, related to Figure 4

Supplemental Experimental Procedures

Supplemental Figure S1

A



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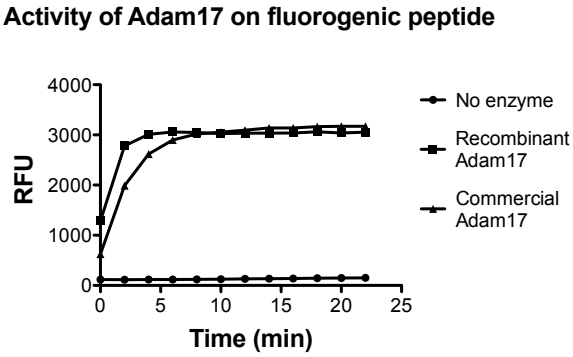

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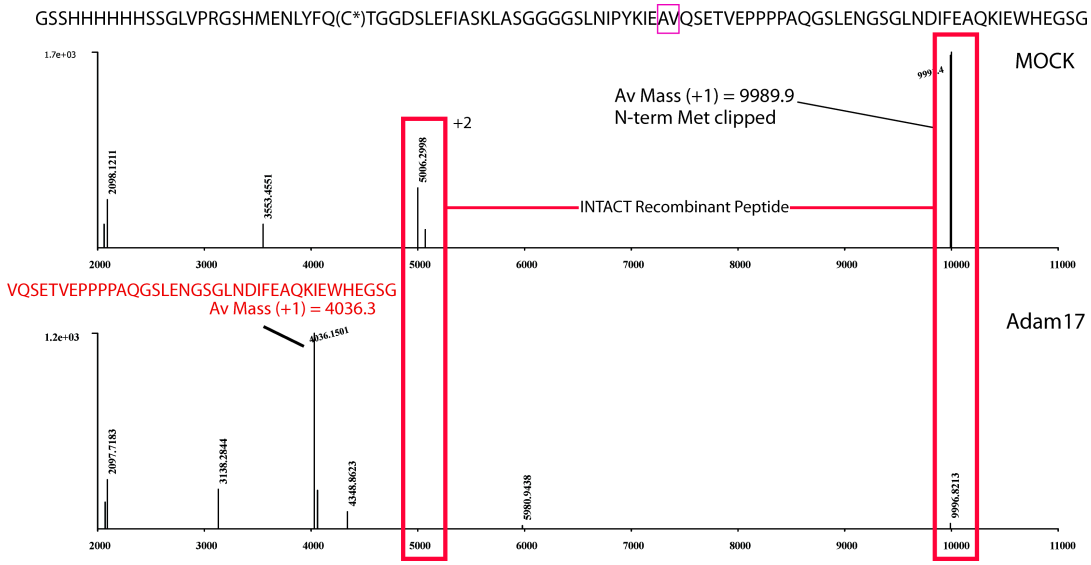

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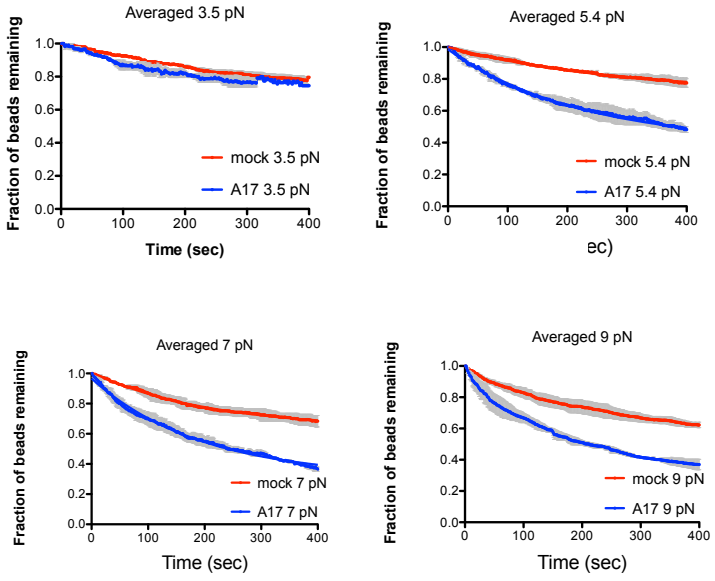
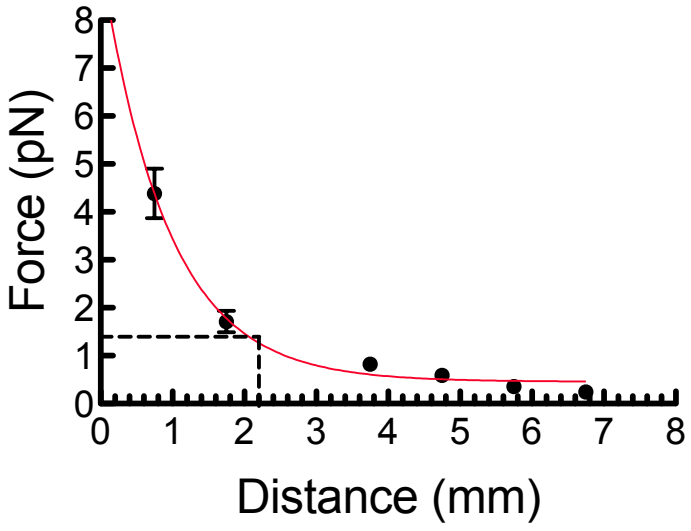
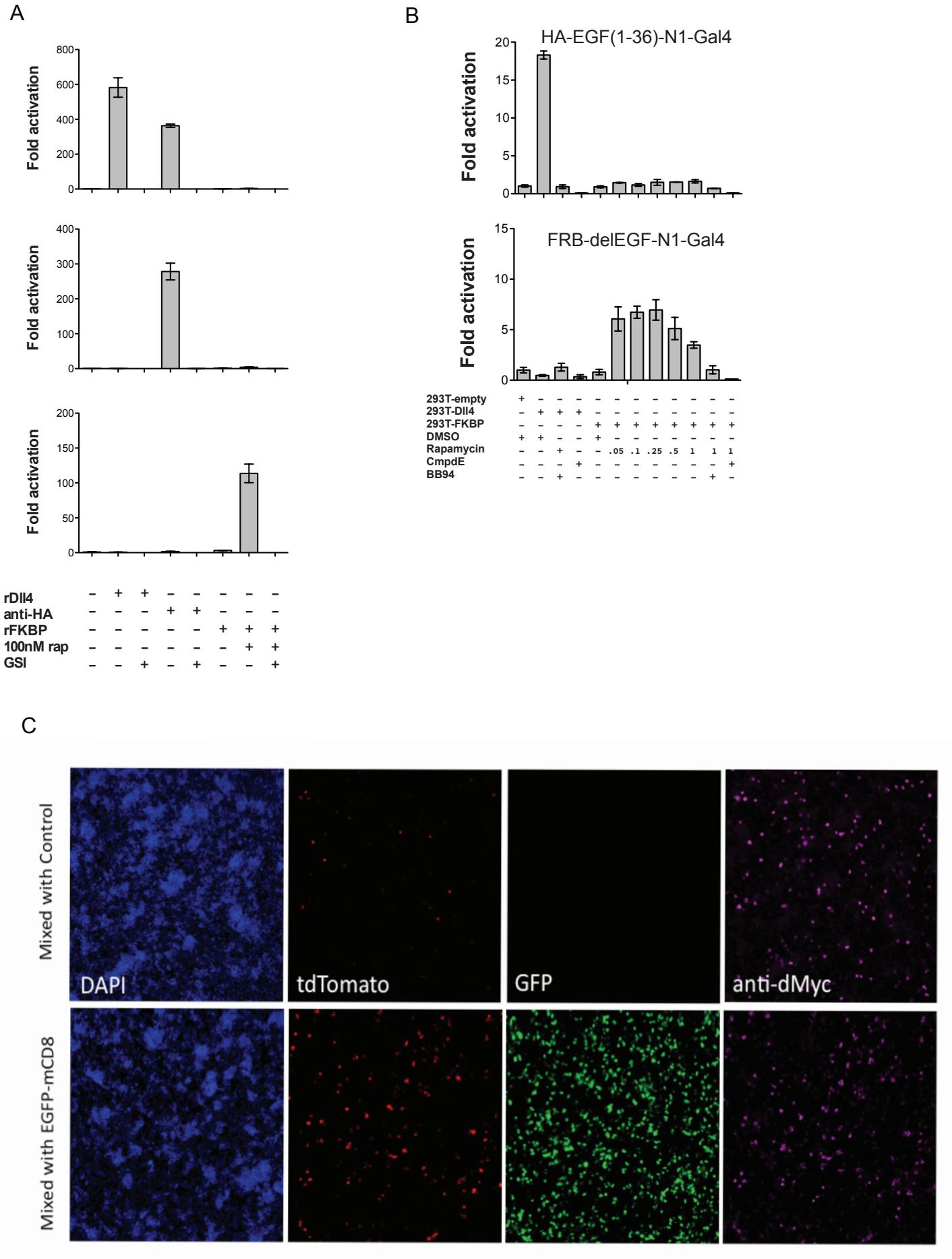


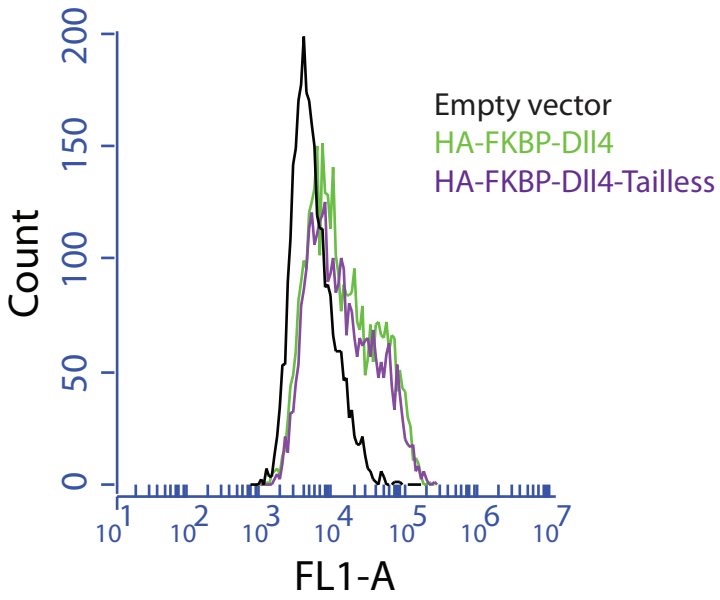
Supplemental Figure S2



Supplemental Figure S3



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.

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.