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Notch Ligand Endocytosis Generates Mechanical Pulling Force Dependent on Dynamin, Epsins, and Actin

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SUMMARY

Notch signaling induced by cell surface ligands is critical to development and maintenance of many eukaryotic organisms. Notch and its ligands are integral membrane proteins that facilitate direct cell-cell interactions to activate Notch proteolysis and release the intracellular domain that directs Notch-specific cellular responses. Genetic studies suggest that Notch ligands require endocytosis, ubiquitylation, and epsin endocytic adaptors to activate signaling, but the exact role of ligand endocytosis remains unresolved. Here we characterize a molecularly distinct mode of clathrin-mediated endocytosis requiring ligand ubiquitylation, epsins, and actin for ligand cells to activate signaling in Notch cells. Using a cell-bead optical tweezers system, we obtained evidence for cell-mediated mechanical force dependent on this distinct mode of ligand endocytosis. We propose that the mechanical pulling force produced by endocytosis of Notch-bound ligand drives conformational changes in Notch that permit activating proteolysis.

INTRODUCTION

The Notch pathway is a highly conserved signaling system used extensively throughout embryonic development that continues to function in adult homeostasis. The integral membrane nature of Notch receptors and canonical ligands provides a mechanism for cells to directly interact and communicate with each other (Fortini, 2009). The ligand transmembrane structure also facilitates endocytosis, which is absolutely required for ligand cells to activate signaling in Notch cells (Weinmaster and Fischer, 2011). Despite extensive evidence implicating ligand endocytosis in Notch signaling, the basis of this requirement has remained poorly understood and controversial.

Sequential proteolysis of Notch regulates release of the Notch intracellular domain (NICD) that functions as the biologically active signal transducer (Kopan and Ilagan, 2009). Ligand binding induces a-disintegrin-and-metalloprotease (ADAM) cleavage in Notch that allows subsequent intramembrane $\gamma-$secretase proteolysis to generate the active NICD fragment, which moves to the nucleus to interact with the DNA-binding protein CSL (CBF1, Su(H), LAG-1) and activate Notch target genes. Although activating proteases have been identified, the molecular events required for ligand cells to trigger Notch proteolysis for downstream signaling are not well defined.

Consistent with a strict requirement for ligand endocytosis, proteolytic activation of Notch correlates with selective internalization of the Notch extracellular domain (NECD) by ligand cells referred to as transendocytosis (Nichols et al., 2007; Parks et al., 2000). Ligand endocytosis of Notch attached to an adjacent cell has been proposed to produce a molecular strain in Notch that allows NECD uptake by ligand cells. In the absence of ligand, a negative regulatory region in the Notch ectodomain masks the ADAM site to keep Notch in a protease-resistant state (Musse et al., 2012). These ideas form the basis of a pulling-force model proposing that mechanical force produced by ligand endocytosis physically pulls on Notch to expose the ADAM site, allowing activating proteolysis for downstream signaling. Although this model is consistent with a critical role for ligand endocytosis in Notch signaling, it is completely unknown if ligand cells produce mechanical force during NECD transendocytosis or if ligand-induced Notch signaling is force dependent.

To address the pulling-force model, we identified and characterized endocytic and cellular factors required for ligand cells to exert mechanical pulling force on Notch, internalize NECD, and activate signaling. Together, our findings identify a molecularly distinct mode of clathrin-mediated endocytosis (CME) requiring epsin endocytic adaptors and actin for ligand cells to pull on Notch and activate signaling.

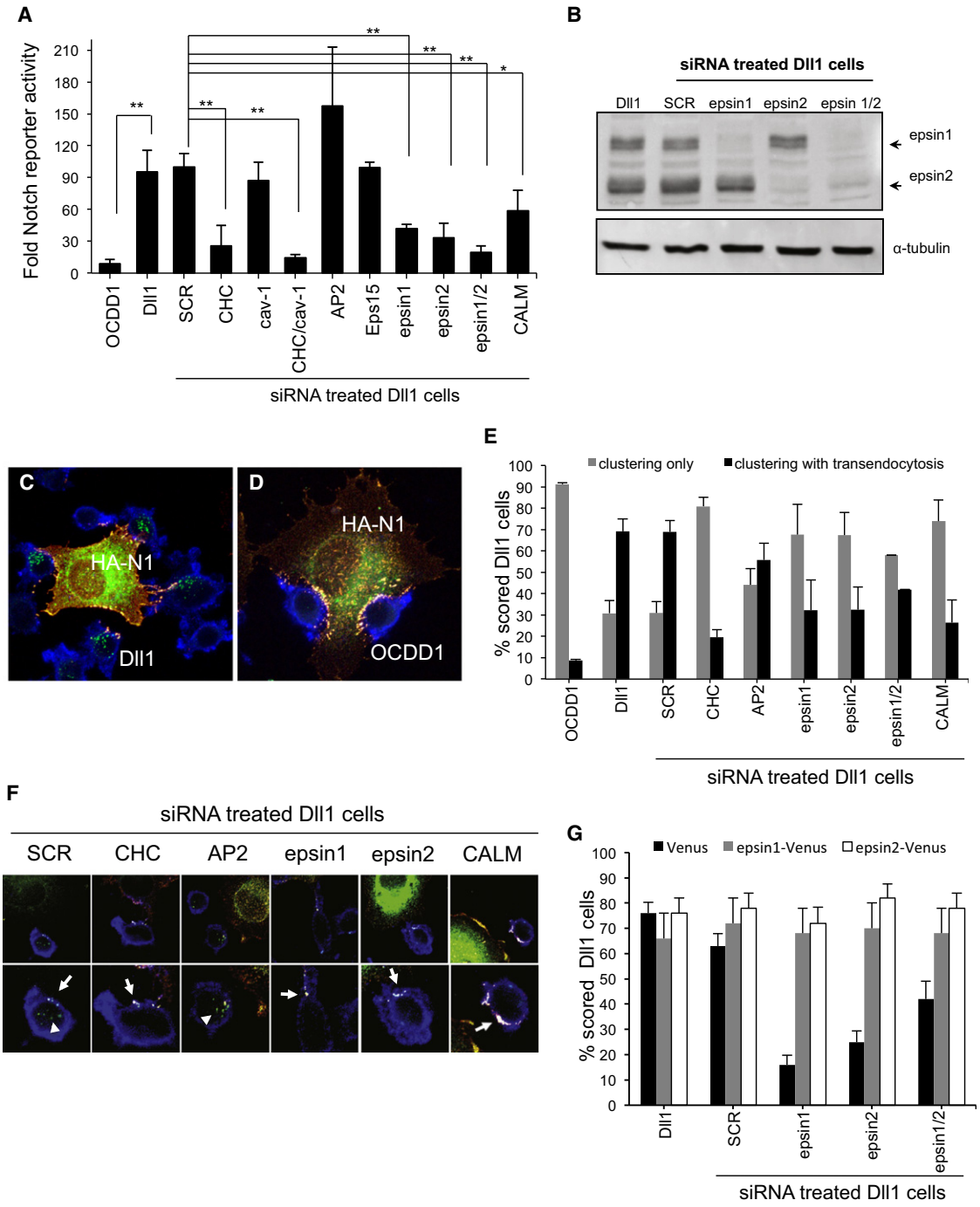

Figure 1. Dll1 Cells Use Epsin-Dependent CME to Activate Notch

(A) Notch reporter activity for HA-N1eGFP-expressing cells cocultured with cells expressing either the endocytic mutant OCDD1 or Dll1 treated with the indicated siRNAs. Values are mean of three independent experiments done in triplicates ± SEM and represent fold activation over cocultures with parental L cells. *$p < 0.05$ and **$p < 0.01$, Student's t test. SCR, scrambled; CHC, clathrin heavy chain; cav-1, caveolin 1.

(B) Western blot analysis of Dll1 cells treated with epsin1 and epsin2 siRNAs. α-tubulin indicates equal loading (bottom).

(C and D) Confocal images of coculture assays using HA-N1 cells with (C) Dll1 cells or (D) OCDD1 cells to detect and quantitate NECD transendocytosis. Surface Dll1 (blue), surface HA-N1 (red), postpermeabilizied HA-N1 signal (green), and pre- and postpermeabilization HA-N1 signal overlap (yellow).

(E) Quantification of NECD transendocytosis by Dll1 cells treated with indicated siRNAs cocultured with HA-N1 cells. Values represent the percentage of Dll1 cells interacting with HA-N1 cells scored for "clustering only," indicated by yellow signals as in (D), or "clustering with transendocysosis," indicated by green signals as in (C) ± SEM of three independent experiments.

(F) Representative confocal images used for quantification in (E). Arrows indicate cell surface HA-N1 clustering; arrowheads indicate internal HA-N1. Bottom images are enlargements of the upper images.

RESULTS

Ligand Cells Require CME to Activate Notch Signaling

Genetic studies with *Drosophila shibire* first identified a requirement for the endocytic factor dynamin in Notch signaling (Seugnet et al., 1997). Studies in mammalian cells report that a dominant-negative dynamin2 (DynK44A) perturbs NECD transendocytosis and signaling induced by cells expressing the Notch ligand Delta-like 1 (Dll1) (Nichols et al., 2007). Dynamin functions in both clathrin-dependent and -independent endocytosis (Doherty and McMahon, 2009); thus, either or both pathways could function in ligand signaling activity.

To identify the specific endocytic pathway, Dll1 cells were treated with small interfering RNAs (siRNAs) to deplete endocytic factors prior to coculture with Notch1 (N1) cells expressing a Notch reporter (Bozkulak and Weinmaster, 2009; Nichols et al., 2007). Dll1 cells depleted of clathrin heavy chain (CHC) by more than 80% compared to control scrambled (SCR) siRNAs (Figures S1A and S1B available online) blocked CME as monitored by transferrin uptake (Figure S1C). The block was specific for CHC depletion (Figure S1D) and did not decrease Dll1 cell surface expression (Figure S1E). Despite this, Notch reporter activity was strongly reduced and similar to the endocytic mutant Dll1 (OCDD1) defective in Notch activation (Figure 1A) (Nichols et al., 2007), identifying CME as the major pathway for Dll1 signaling activity. In fact, Dll1 cells depleted of caveolin-1 (cav-1) (Figures S1A and S1B), which functions in clathrin-independent endocytosis (Hansen and Nichols, 2009) did not alter ligand activity (Figure 1A). Moreover, simultaneous knockdown of CHC and cav-1 did not further reduce reporter activity (Figure 1A), arguing against a role for caveolin-dependent endocytosis in Dll1 signaling activity.

Dll1 Signaling Activity Requires Alternative Clathrin Adaptors

The adaptor protein 2 (AP2) complex links clathrin to cargo and the membrane during CME of most proteins (Maldonado-Báez and Wendland, 2006). Despite losses in transferrin uptake (Figure S1C), Dll1 cells depleted of the μ2 AP2 subunit (Figures S1A and S1B) efficiently activate signaling (Figure 1A), suggesting that AP2 is not required for ligand signaling. In contrast, specific depletion of alternative clathrin adaptors epsin1 and/or epsin2 (Figure 1B), required for Notch signaling in flies, worms, and mice (Chen et al., 2009; Overstreet et al., 2003, 2004; Tian et al., 2004; Wang and Struhl, 2004), produced significant losses in reporter activity (Figure 1A). Surprisingly, Dll1 activity did not require the endocytic scaffold protein Eps15 known to interact with epsin during CME of other cargos (Huang et al., 2004; Kazazic et al., 2009). In addition to epsins, the alternative adaptor CALM (clathrin assembly lymphoid myeloid leukemia protein) was also required for reporter activity induced by Dll1 (Figure 1A; Figures S1A and S1B).

NECD Transendocytosis Is Epsin Dependent

To determine if endocytic factors identified for Dll1 cells to activate Notch signaling (Figure 1A) also function in NECD transendocytosis, we tested Dll1 cells depleted by siRNA for NECD uptake (Nichols et al., 2007). Dll1 cells cocultured with cells expressing N-terminal hemagglutinin (HA)-tagged N1 (HA-N1) produce a punctate HA signal (Figure 1C, yellow) at the interface between Dll1 (blue) and HA-N1 cells as well as display intracellular HA signals (Figure 1C, green) indicative of NECD transendocytosis. Although OCDD1 cells (Figure 1D, blue) accumulate strong HA signals at sites of HA-N1 cell contact (yellow), intracellular HA signals (green) are not detected. Thus, despite efficient ligand-receptor clustering, defects in Dll1 endocytosis severely compromise both NECD transendocytosis (Figure 1D) and Notch signaling (Figure 1A).

When siRNA-treated Dll1 cells depleted of CHC, AP2, epsin1, epsin2, or CALM were scored for "clustering only" and "clustering with transendocytosis," only cells depleted of AP2 were positive for both HA clustering and internal HA signals, similar to untreated and SCR-treated Dll1 cells (Figures 1C, 1E, and 1F). Therefore, NECD uptake by Dll1 cells requires CHC and the alternative adaptors, epsin1, epsin2, and CALM, rather than AP2 central to CME of most proteins. Moreover, loss of either epsin1 or epsin2 decreased NECD transendocytosis and Notch reporter activity (Figures 1A, 1E, and 1F), indicating that both isoforms function in Dll1 signaling activity. Since NECD transendocytosis defects for Dll1 cells depleted of epsin1, epsin2, or both are rescued by expression of either epsin1 or epsin2 siRNA-resistant constructs (Figure 1G), epsin concentration, rather than the specific isoform, appears critical for Dll1 CME to activate Notch. Identification of the same endocytic factors for Dll1 cell uptake of NECD and activation of Notch signaling strengthens the functional link between these events.

Epsin-Dependent and -Independent Dll1 CME

Notch ligand cells bind and internalize a recombinant, soluble form of N1 containing epidermal growth factor-like repeats 1–15 fused in frame with human immunoglobulin G-Fc sequences (N1Fc) (Hansson et al., 2010; Heuss et al., 2008; Nichols et al., 2007). To identify the endocytic pathway required for Dll1 cells to internalize soluble N1Fc, we compared the N1Fc endocytic values (Figure 2A) following siRNA knockdown. Although epsins are required for NECD transendocytosis (Figure 1E) and Notch signaling (Figure 1A), neither epsin1 nor epsin2, alone or in combination, were needed for soluble N1Fc uptake (Figure 2B). These findings suggested a specific requirement for epsins when Dll1 cells are bound to Notch attached to a neighboring cell.

We reasoned that attached N1 produced resistance to Dll1 endocytosis that required epsins, and thus, soluble N1Fc was attached to PrtA beads to produce resistance to N1Fc uptake. N1Fc was labeled with Cy5-anti-human Fc antibody prior to bead attachment (N1Fc-Cy5 beads) (see Figure S2A), and following incubation with Dll1 cells, an intracellular Cy5 signal

(G) Quantification by confocal microscopy of the rescue of NECD transendocytosis defects associated with epsin siRNA knockdown by expression of siRNA-resistant rat epsin1-Venus or rat epsin2-Venus constructs in Dll1 cells cocultured with HA-N1 cells. Values represents the percentage of Dll1 cells expressing Venus, epsin1-Venus, or epsin2-Venus scored for "clustering with transendocysosis" ± SEM of three independent experiments.
See also Figure S1.

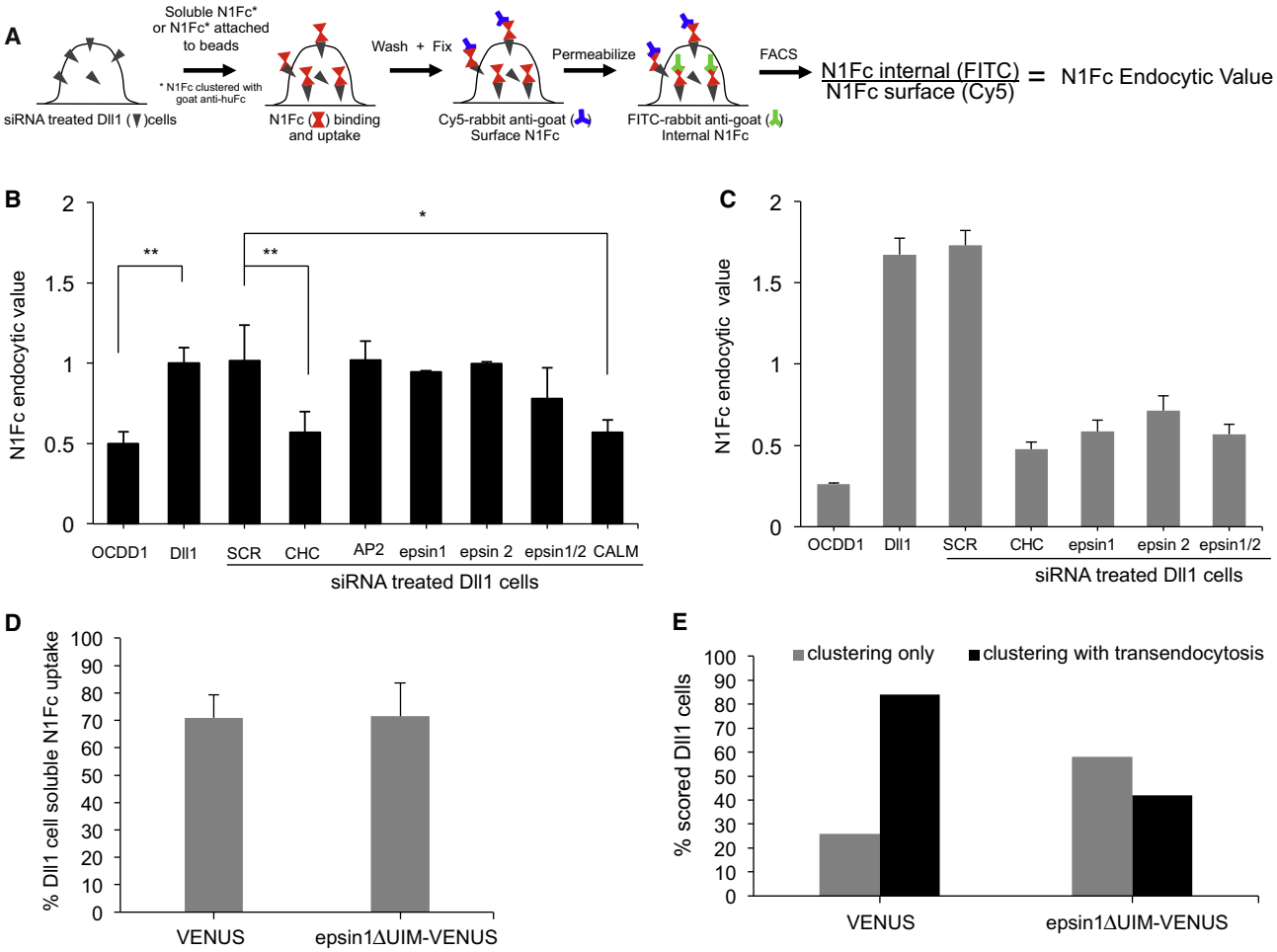

Figure 2. Dll1 Cell CME of Soluble or Attached N1Fc Requires Distinct Endocytic Adaptors

(A) Schematic of staining protocol to detect surface and internal N1Fc signals by FACS analysis to calculate N1Fc endocytic value (see Experimental Procedures for details).

(B and C) FACS analysis of uptake of (B) soluble N1Fc or (C) N1Fc attached to PrtA beads by OCDD1, Dll1, or Dll1 cells treated with the indicated siRNAs. Values represent mean of at least three independent experiments ± SEM. *$p < 0.05$. **$p < 0.01$.

(D) Quantification by confocal microscopy of soluble preclustered N1Fc uptake by Dll1 cells expressing Venus or dominant-negative epsin1ΔUIM-Venus. Values represent the percentage of Dll1 cells with internal N1Fc signal (n = 100) for three independent experiments ± SD.

(E) Quantification of NECD transendocytosis for Dll1 cells expressing Venus or epsin1ΔUIM-Venus cocultured with HA-N1 cells. Values represent the percentage of Dll1 cells expressing Venus or epsin1ΔUIM-Venus scored for "clustering only" or "clustering and transendocytosis."

See also Figure S2.

was detected (Figure S2B). Since crosslinking of N1Fc-Cy5 beads reduced Cy5 uptake (Figure S2B), we propose that Dll1 cells disrupt noncovalent Fc-PrtA bonds to internalize N1Fc-Cy5 but are unable to break covalent bonds produced by crosslinking.

Validating the N1Fc bead assay, Dll1 cells displayed considerably higher N1Fc endocytic values than OCDD1 cells (Figure 2C). N1Fc uptake was dependent on CHC and, like NECD transendocytosis, required epsins. Consistent with a specialized role for epsins in endocytosis of N1 attached to beads or cells, Dll1 cells expressing a dominant-negative epsin1 (epsin1ΔUIM-Venus) (Chen and Zhuang, 2008) internalized soluble N1Fc (Figure 2D) yet were defective in NECD transendocytosis (Figure 2E). Together, our different assays reveal two distinct modes of CME for Dll1 cells: (1) epsin-independent internalization of soluble N1Fc and (2) epsin-dependent internalization of N1 attached to beads or cells.

Dll1 Cells Require Actin Polymerization for CME of Attached Notch

Actin is absolutely required for CME in yeast; however, an obligatory role in mammalian cells is controversial (Aghamohammadzadeh and Ayscough, 2009; Robertson et al., 2009). To determine the actin requirements for Dll1 CME of soluble versus attached N1Fc, actin polymerization was inhibited by latrunculin B (LatB). Although LatB decreased F-actin staining with phalloidin (data not shown), it did not perturb soluble N1Fc uptake (Figure 3A), indicating that Dll1 cells do not require actin

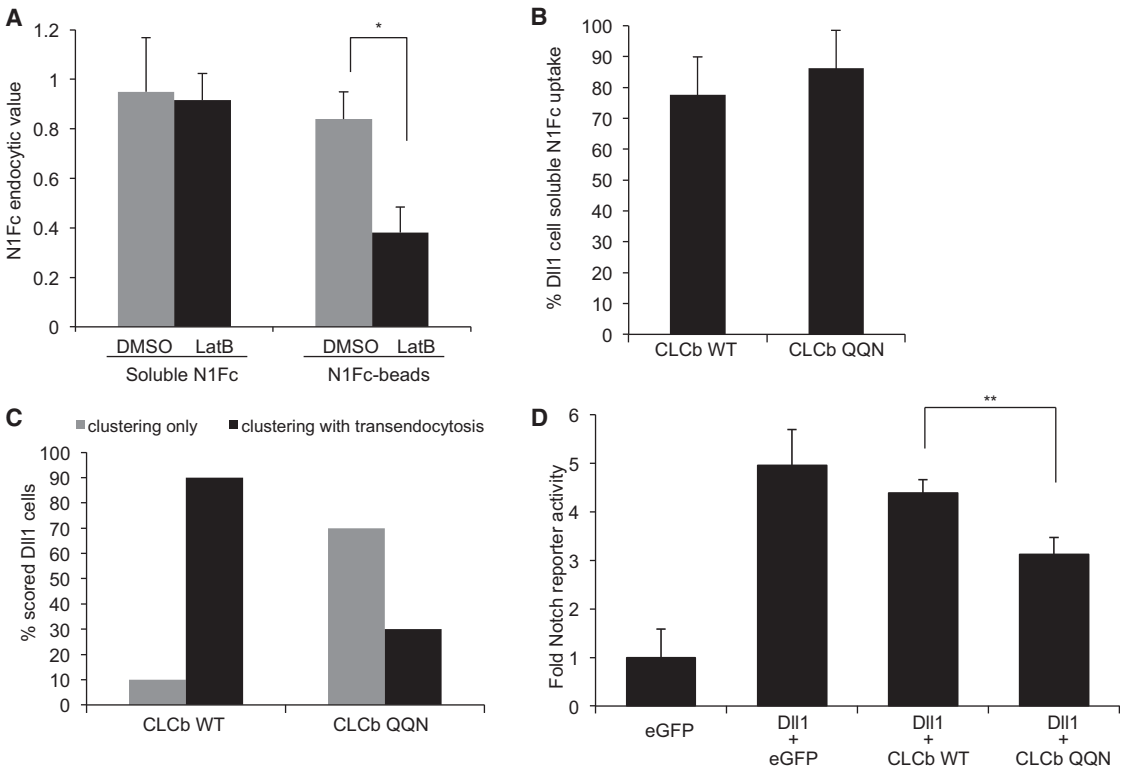

Figure 3. Distinct Requirements for Actin Polymerization in Dll1 Cell CME of Attached versus Soluble Notch

(A) FACS analysis of soluble N1Fc and N1Fc attached to beads in the presence of LatB. Values represent the mean of three independent experiments ± SEM. *$p < 0.05$.

(B) Quantification of soluble N1Fc by confocal microscopy of Dll1 cells expressing eGFP-CLCb WT or eGFP-CLCb QQN. Values represent the percentage of Dll1 cells expressing eGFP-CLCb WT or eGFP-CLCb QQN with internal N1Fc signal (n = 100) for three independent experiments ± SD.

(C) Quantification of NECD transendocytosis of Dll1 cells expressing eGFP-CLCb WT or eGFP-CLCb QQN cocultured with HA-N1 cells. Values represent the percentage of Dll1 cells expressing eGFP-CLCb WT or eGFP-CLCb QQN scored for "clustering only" or "clustering and transendocytosis."

(D) Notch reporter activity for HA-N1eGFP cells cocultured with L cells transiently expressing eGFP, Dll1+eGFP, Dll1+ eGFP-CLCb WT, or Dll1+ eGFP-CLCb QQN. Values are the mean of one experiment done in triplicate ± SD. **$p < 0.01$.

See also Figure S3.

polymerization for uptake of soluble N1Fc. In contrast, LatB compromised Dll1 cell uptake of N1Fc attached to beads (Figure 3A), indicating a requirement for actin assembly. Furthermore, a dominant-interfering clathrin light chain b (CLCb QQN), known to uncouple actin dynamics during CME (Chen and Brodsky, 2005; Poupon et al., 2008), did not alter Dll1 cell uptake of either soluble N1Fc (Figure 3B) or transferrin (Figure S3). However, Dll1 cells expressing CLCb QQN were defective in NECD transendocytosis (Figure 3C) and reporter activity (Figure 3D). Together, our findings indicate that actin polymerization is important for Dll1 cell uptake of N1Fc attached to beads, NECD transendocytosis, and ligand signaling activity.

Notch Contact Induces Dll1 Ubiquitylation and Interactions with Epsin1

Genetic interactions between Notch ligands and epsins have been reported for Notch-dependent developmental events (Overstreet et al., 2003, 2004; Wang and Struhl, 2004, 2005). These findings may reflect physical interactions between ubiquitin (Ub) on the ligand intracellular domain and epsin ubiquitin-interacting motifs (UIMs), as reported for other epsin-specific cargos (Chen and Zhuang, 2008; Kazazic et al., 2009; Sugiyama et al., 2005; Wang et al., 2006). In fact, the E3 Ub ligase Mind bomb (Mib) binds and ubiquitylates Notch ligands and is necessary for signaling activity (Weinmaster and Fischer, 2011). Despite the absolute requirement for epsins in Notch signaling, evidence for ligands physically interacting with epsins in a Ub-dependent manner crtitcal to ligand activity has yet to be reported. We found that Dll1 cells require epsins to activate N1 signaling (Figure 1A) and that NECD transendocytosis requires the presence of epsin UIMs (Figure 2E), suggesting a functional interaction between Ub on Dll1 and epsin UIMs.

To directly address this idea, we asked if contact with attached N1 enhanced Dll1 ubiquitylation and complex formation with epsins. For these studies, L cells expressing Dll1, HA-Ub, or epsin1-Venus were grown on N1Fc- or Fc-coated plates. Immunoprecipitation (IP) of Dll1 from cell lysates followed by immunoblotting (IB) with HA antibodies revealed one major band ~130 KDa, as well as several minor higher molecular weight bands (Figure 4A), representing either ubiquitylated Dll1 and/or ubiquitylated Dll1-interacting proteins. Ubiquitylation of the 130 KDa HA-positive band was enhanced 1.9-fold when Dll1 cells were grown on N1Fc

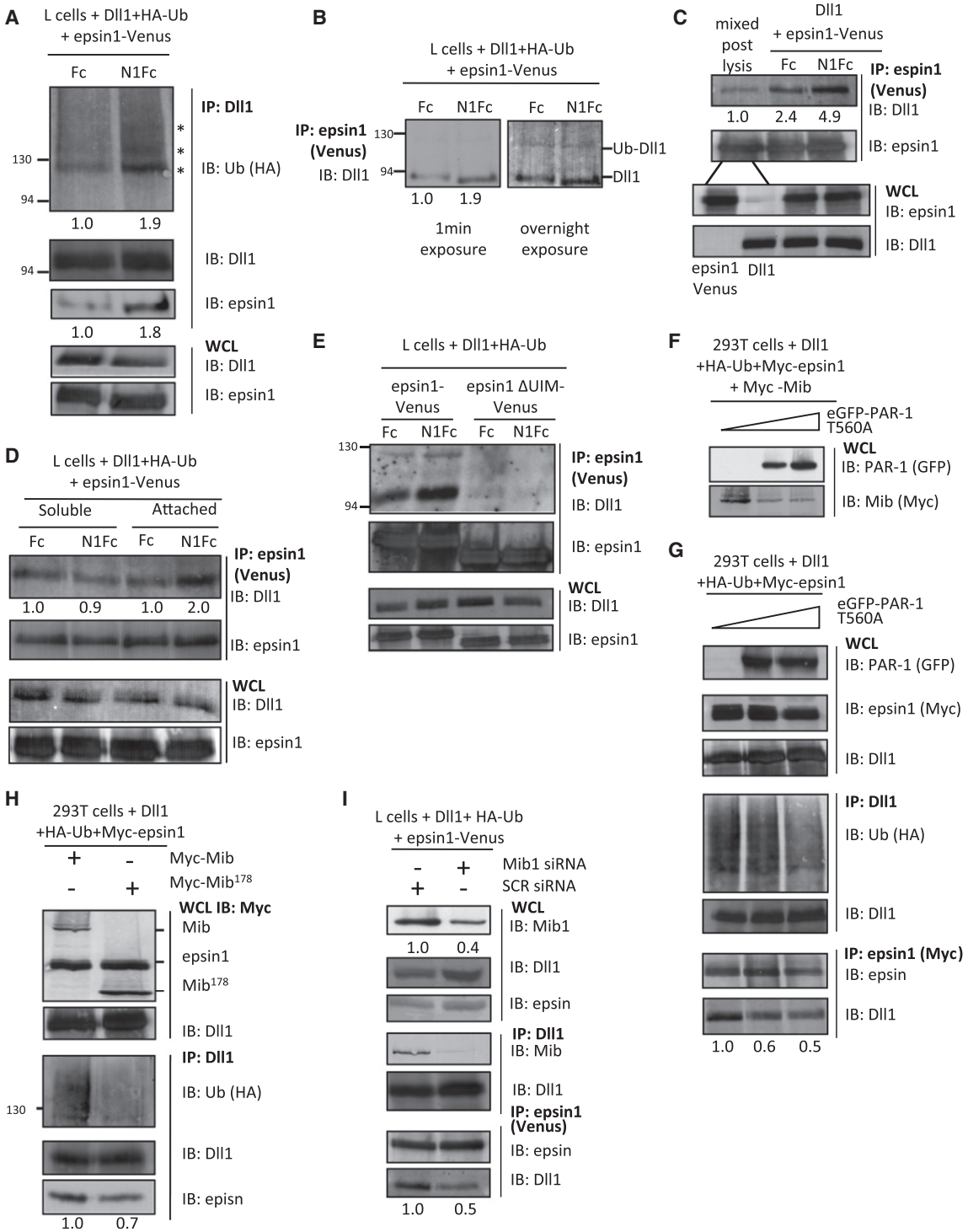

Figure 4. Notch Stimulates Dll1 Ubiquitylation and Complex Formation with Epsins

(A) Western blot analysis of lysates from L cells expressing Dll1, HA-Ub, and epsin1-Venus incubated on N1Fc- or Fc-coated dishes. Cell lysates were immunoprecipitated with anti-Dll1 ICD and immunoblotted with antibodies for HA (top), Dll1, and epsin1 (middle). Whole cell lysates (WCLs) were immunoblotted with antibodies for Dll1 and epsin1. Asterisks indicate different molecular weight forms of ubiquitylated Dll1.

(B) Short (left) and long (right) exposure of western blot analysis of lysates from L cells expressing Dll1, HA-Ub, and epsin1-Venus incubated on N1Fc- or Fc-coated dishes and immunoprecipitated with anti-GFP followed by IB with anti-Dll1.

(C) Lysates from L cells expressing either Dll1 or epsin1 mixed postlysis (left lane), or from L cells transfected with both epsin1-Venus and Dll1 incubated on either Fc- (middle lane) or N1Fc-dishes (right lane) IP with anti-GFP followed by IB with anti-Dll1 and anti-epsin. Bottom: WCLs corresponding to cells in the above panel immunoblotted with anti-epsin1 and anti-Dll1.

compared to Fc plates. The increased ubiquitylation correlated with a 1.8-fold increase in epsin1-Venus coimmunoprecipitated with Dll1 (Figure 4A), and ectopic epsin2 and endogenous epsin1 also coimmunoprecipitated (data not shown). These data indicate that Dll1 physically interacts with epsin1 and identify a role for N1 in promoting Dll1 ubiquitylation and epsin interactions.

In a reciprocal approach, IB of epsin1-Venus IPs with Dll1 antibodies detected a 1.9-fold increase in the 94 KDa unmodified Dll1 (Figure 4B). Longer exposure revealed a 130 KDa band (Figure 4B) similar in size to the major ubiquitylated form with Dll1 IPs (Figure 4A), indicating that ubiquitylated Dll1 (130 KDa) and unmodified Dll1 (94 KDa) coimmunoprecipitated with epsin1 (Figure 4B). Although a low level of Dll1 coimmunoprecipitated with epsin1 from mixed postlysates (Figure 4C), the amount of Dll1 coimmunoprecipitated with epsin1 increased 2.4-fold when epsin1 and Dll1 were coexpressed and 4.9-fold when cells were grown on N1Fc-coated plates (Figure 4C), suggesting that contact of Dll1 cells with attached N1Fc stimulates and/or stabilizes Dll1-epsin1 interactions. Intriguingly, exposure of Dll1 cells to soluble N1Fc did not increase Dll1-epsin1 complex formation (Figure 4D). Even though the majority of Dll1 coimmunoprecipitated with epsin1 appeared unmodified (Figure 4B), detection of both forms required epsin1 UIMs (Figure 4E), demonstrating the dependence of epsin1-Dll1 complexes on epsin UIMs and indirectly implicating Dll1 ubiquitylation.

To investigate this, we perturbed Mib-mediated Dll1 ubiquitylation. First, expression of the polarity regulator PAR-1 (PAR-1T560A-eGFP), reported to target Mib for proteosome-mediated degradation and reduce Dll1 ubiquitylation and Notch signaling (Ossipova et al., 2009), induced a dose-dependent decrease in Mib (Figure 4F) that correlated with losses in Dll1 ubiquitylation and a 2-fold decrease in Dll1 coimmunoprecipitated with epsin1 (Figure 4G). Second, a dominant-negative Mib (Mib^{178}) that antagonizes Mib E3 ligase activity (Itoh et al., 2003) decreased Dll1 ubiquitylation and epsin1 interactions (Figure 4H). Third, siRNA-treated Dll1 cells depleted of Mib1 exhibited a 2-fold decrease in Dll1 interactions with epsin1 (Figure 4I). Together, these findings indicate that Dll1 ubiquitylation is a prerequisite for epsin1 interactions, corroborating the UIM requirement for epsin1 to interact with Dll1 (Figure 4E).

Optical Tweezers Detect Dll1 Cell-Mediated Force Specific for Binding N1

Requirements for epsins and actin in Dll1 CME unique to removal of N1Fc from beads or NECD from cells, but not soluble N1Fc uptake, are in line with the pulling force model. To directly determine if ligand cells produce mechanical force following N1 binding, we developed a cell-bead optical tweezers assay to detect and quantify force produced by Dll1 cells bound to laser-trapped N1Fc beads. Briefly, Dll1 cells placed in contact with trapped N1Fc beads promote Dll1-N1 interactions (Figure 5A). Bead displacements monitored by the quadrature photodiode measure positive cell-mediated forces that pull the bead from the center of the laser trap and negative cell-mediated forces that push on the bead.

Dll1 cells bind and pull trapped N1Fc beads, compared to control PrtA or Fc-coated beads (Table 1). A prototypic force tracing for Dll1 cells bound to N1Fc beads indicates sustained cell-mediated force over a 60 s period (Figure 5B). Specifically, this Dll1 cell exerted ~10 pN of pulling force on the N1Fc bead (Figure 5B), while considerably weaker force values were measured for PrtA (Figure 5C) or Fc beads (Figure 5D). Additionally, the average pulling force obtained for Dll1 cells with N1Fc beads was significantly stronger than that measured for PrtA or Fc beads ($p < 0.05$; Figure 5E; Table 1), and Dll1 cells pull more than push N1Fc beads. In general, Dll1 cell force traces for N1Fc beads (Figure 5B) have weaker fluctuations compared to PrtA (Figure 5C) or Fc beads (Figure 5D), reflecting restricted Brownian motion due to specific Dll1-N1 binding. In fact, Brownian motion for a trapped N1Fc bead in media (Figure S4) is similar to that presented in Figures 5C and 5D. Together, our data suggest that only N1Fc beads allow strong Dll1 cell-mediated pulling force, providing direct evidence for mechanical force dependent on N1 binding. Whether the measured Dll1 cell force magnitudes reflect that produced during physiological Notch signaling remains to be determined; however, detection of force specific for Dll1-N1 interactions allowed molecular analyses of Dll1 cell-mediated force.

Dll1 Cell-Mediated Pulling Force Requires Endocytosis Dependent on Dynamin, Epsins, and Actin

To determine if Dll1 cell pulling force requires endocytosis, we first tested OCDD1 cells defective in endocytosis and Notch signaling (Figures 1 and 2) (Nichols et al., 2007). Even though intrinsic cell movement is expected to exert force on trapped beads, OCDD1 cells did not produce positive pulling forces with N1Fc (Figure 5F) or Fc beads (Figure 5G). Rather, OCDD1 cells mostly push both bead types (Figure 5H) in contrast to the positive forces measured for Dll1 cells ($p < 0.05$; Figure 5I;

(D) Lysates from L cells transfected with Dll1, HA-Ub, and epsin1-Venus incubated with either soluble Fc or N1Fc or cultured on Fc- or N1Fc-coated dishes were immunoprecipitated with anti-GFP and immunoblotted with anti-Dll1 and anti-epsin1. WCLs were immunoblotted with anti-Dll1 and anti-epsin1 (bottom panels).
(E) Lysates from L cells expressing Dll1, HA-Ub, and either epsin1-Venus or epsin1ΔUIM-Venus incubated on Fc- or N1Fc-coated dishes were immunoprecipitated with anti-GFP and immunoblotted with anti-Dll1 and anti-epsin1. WCLs were immunoblotted with anti-Dll1 and anti-epsin1 (bottom panels).
(F) Lysates from 293T cells transfected with Dll1, HA-Ub, Myc-epsin1, Myc-Mib, and increasing amounts of eGFP-PAR-1-T560A were immunoblotted with anti-GFP and anti-Myc to detect Mib1 protein.
(G) Lysates from 293T cells expressing Dll1, HA-Ub, Myc-epsin1, and increasing amounts of eGFP-PAR-1-T560A were immunoprecipitated with anti-Dll1 and immunoblotted with anti-Ub and anti-Dll1 (middle panels) or immunoprecipitated with anti-Myc and immunoblotted with anti-epsin1 and anti-Dll1 (bottom panels). WCLs were immunoblotted with anti-GFP, anti-Myc, and anti-Dll1 (top panels).
(H) Lysates from 293T expressing Dll1, HA-Ub, and either Mib or dominant-negative Mib^{178} were immunoprecipitated with Dll1 and immunoblotted with anti-HA, anti-Dll1, and anti-epsin1 (bottom panels). WCLs were immunoblotted with anti-Myc and anti-Dll1 (top panels).
(I) Lysates from L cells expressing Dll1, HA-Ub, and epsin1-Venus treated with Mib1 or SCR siRNAs were immunoprecipitated with anti-Dll1 and immunoblotted with anti-Mib1 and anti-Dll1 (middle panels) or immunoprecipitated with anti-GFP and immunoblotted with anti-epsin and anti-Dll1 (bottom panels). WCLs were immunoblotted with anti-Mib1, anti-Dll1, and anti-epsin1 (top panels).

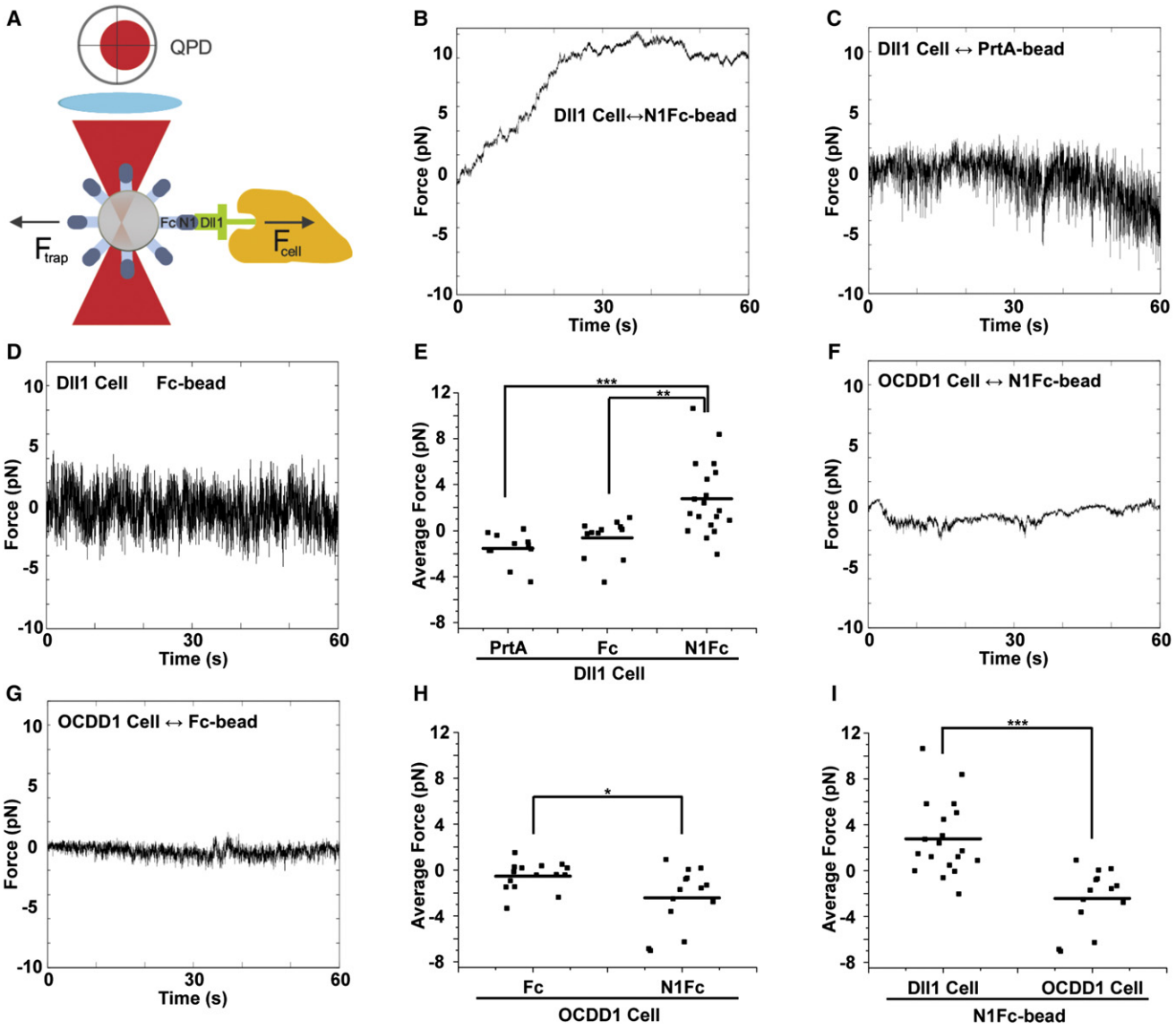

Figure 5. Laser Tweezers Detect Mechanical Forces Exerted by Ligand Cells on Trapped N1Fc Beads

(A) Schematic of optical tweezers system used to measure Dll1 cell-mediated forces exerted on trapped N1Fc beads.

(B) Prototypic force tracing for Dll1 cells bound to laser trapped N1Fc beads.

(C) Prototypic force tracing for Dll1 cells interacting with uncoated PrtA beads.

(D) Prototypic force tracing for Dll1 cells interacting with Fc beads. See also Figure S4.

(E) Average of the average force measurement for Dll1 cells interacting with PrtA, Fc, or N1Fc beads. $^{**}p < 0.01$. $^{***}p < 0.001$. See also Table 1.

(F and G) Prototypic force tracings for OCDD1 cells interacting with (F) N1Fc or (G) Fc beads.

(H) Average of the average force measurement for OCDD1 cells interacting with Fc or N1Fc beads. $^{*}p < 0.05$. See also Table 1.

(I) Average of the average force measurement for Dll1 cells and OCDD1 cells interacting with N1Fc beads. $^{***}p < 0.001$.

See also Table 1 and Figure S4.

Table 1), establishing a correlation between ligand endocytosis and pulling force.

To directly test endocytosis in Dll1 cell-mediated pulling force, DynK44A-eGFP was used to block dynamin-dependent endocytosis (confirmed by reduced transferrin uptake; Figure S3). Compared to eGFP (Figure 6A), DynK44A-eGFP severely compromised Dll1 cell pulling on N1Fc beads (Figure 6B), and multiple bead-cell pairings identified significant differences in force ($p < 0.05$; Figure 6C; Table 1). In contrast, losses in Dll1 recycling induced by Rab11S25N-eGFP (Shergill et al., 2012) did not diminish Dll1 cell-mediated pulling force ($p > 0.05$; Figure 6C; Table 1). Brownian motion for N1Fc beads bound to either Dll1 cells expressing DynK44A-eGFP (Figure 6B) or OCDD1 cells (Figure 5F) was reduced compared to Dll1 cells with Fc beads (Figure 5D), indicating specific Dll1-N1 binding. Nonetheless, the ligand endocytic defects resulted in losses in positive force

Table 1. Bead Displacement and Force Data

Cell Type	Bead Type	n	$\overline{D^{max}_{pulling}}$ (um)	$\overline{D^{max}_{pushing}}$ (um)	$\overline{F_{pulling}}$ (pN)
Dll1	N1Fc	19	0.27	0.08	2.8
	PrtA	11	0.11	0.23	−1.5
	Fc	12	0.10	0.15	−0.6
OCDD1	N1Fc	14	0.09	0.26	−2.4
	Fc	15	0.07	0.10	−0.5
eGFP	N1Fc	18	0.26	0.07	2.8
DynaminK44A-eGFP	N1Fc	16	0.07	0.20	−1.7
Rab11S25N-eGFP	N1Fc	21	0.21	0.12	1.2
eGFP-PAR-1	N1Fc	16	0.10	0.09	0.1
eGFP-PAR-1T560A	N1Fc	15	0.15	0.23	−1.1
CLCb WT-eGFP	N1Fc	23	0.22	0.09	1.7
CLCb QQN-eGFP	N1Fc	23	0.08	0.17	−1.4
Venus	N1Fc	17	0.16	0.03	1.6
epsin1ΔUIM-Venus	N1Fc	18	0.12	0.24	−1.7
SCR	N1Fc	14	0.84	0.17	1.5
epsin1 siRNA	N1Fc	31	0.56	0.57	−0.4
epsin2 siRNA	N1Fc	23	0.31	1.00	−1.1
epsin1/2 siRNA	N1Fc	20	0.89	0.67	−0.9
SCR	N1Fc	21	0.39	0.15	2.5
AP2	N1Fc	26	0.45	0.17	2.9
DMSO	N1Fc	15	0.23	0.16	1.7
Dynasore	N1Fc	21	0.14	0.12	0.3
D1D3	N1Fc	11	0.13	0.30	−1.3

n, number of samples; D, bead displacement in μm; F, force in pN; $\overline{\mathbf{max}}_{\mathbf{pulling}}$, average of maximum pull by cell type; $\overline{\mathbf{max}}_{\mathbf{pushing}}$, average of maximum push by cell type; $\overline{\mathbf{pulling}}$ average of pulling by cell type, (−) indicates pushing.

(Table 1), indicating that the cell-mediated pull on N1Fc beads is dependent on endocytosis.

DynK44A-eGFP imposes a sustained block in dynamin-dependent endocytosis, which is compensated by other endocytic pathways (Damke et al., 1995; Ferguson et al., 2009). Therefore, we determined whether acute dynamin blockade with dynasore, a potent dynamin inhibitor that induces immediate effects when added directly to cells (Macia et al., 2006), also reduced Dll1 cell pulling force. Although the average pulling forces for cells treated with dimethyl sulfoxide (DMSO) (Figure 6D) are not significantly different from untreated cells ($p > 0.05$; Figure 6F; Table 1), addition of 80 uM dynasore for less than 20 min destroyed Dll1 cell pulling force (Figure 6E). Average pulling forces for dynasore-treated Dll1 cells were significantly different from both untreated ($p < 0.05$) and DMSO-treated cells ($p < 0.05$; Figure 6F). These studies identify a requirement for dynamin in pulling force that is specific for Dll1 cells bound to N1Fc beads. Finally, the loss in pulling force is specific to AP2-independent endocytosis, since Dll1 cells depleted of AP2 produce sustained pulling force (Figure S5A).

Epsins are required for Dll1 cells to internalize attached but not soluble N1Fc (Figure 2). Consistent with this, Dll1 cells expressing epsin1DUIM-Venus bound N1Fc beads and displayed lower pulling force (Figure 6G) compared to Venus (Figure 6H). Moreover, analysis of multiple bead-cell pairings identified significantly different average force measurements ($p < 0.05$; Figure 6I and Table 1). Furthermore, epsin1 and epsin2 siRNAs, alone or together, reduced Dll1 cell pulling (Figure S5B). Together, these findings correlate with the absolute requirements for epsins and ligand ubiquitylation in Notch signaling and establish a link between Dll1 cell pulling force and signaling activity.

Supporting that the epsin requirement in pulling force is related to Dll1 ubiquitylation, PAR-1, which degrades Mib and reduces Dll1 ubiquitylation (Figures 4F and 4G) (Ossipova et al., 2009), reduced pulling force. Specifically, either wild-type (WT) PAR-1 (Figure 6J) or the stabilized, active PAR-1T560A (Figure 6K) reduced pulling force relative to eGFP (Figure 6A). The average force for PAR-1 was significantly reduced compared to eGFP, and an even greater decrease was measured for PAR-1T560A ($p < 0.05$; Figure 6L), which is more active than WT PAR-1 (Ossipova et al., 2007). These biophysical data complement our epsin-Dll1 interaction study (Figure 4) and provide support for Dll1 ubiquitylation and epsin complex formation in cell-mediated pulling force generation. In fact, cells expressing the D1D3 chimeric protein lacking intracellular lysines and defective in signaling (Geffers et al., 2007; Heuss et al., 2008; G.W., unpublished data), produced lower magnitude forces than Dll1 cells (Figure S5C). Moreover, the negative force measured for cells expressing D1D3 or OCDD1 are not statistically different ($p > 0.05$), supporting a requirement for Dll1 ubiquitylation in cell-mediated pulling force.

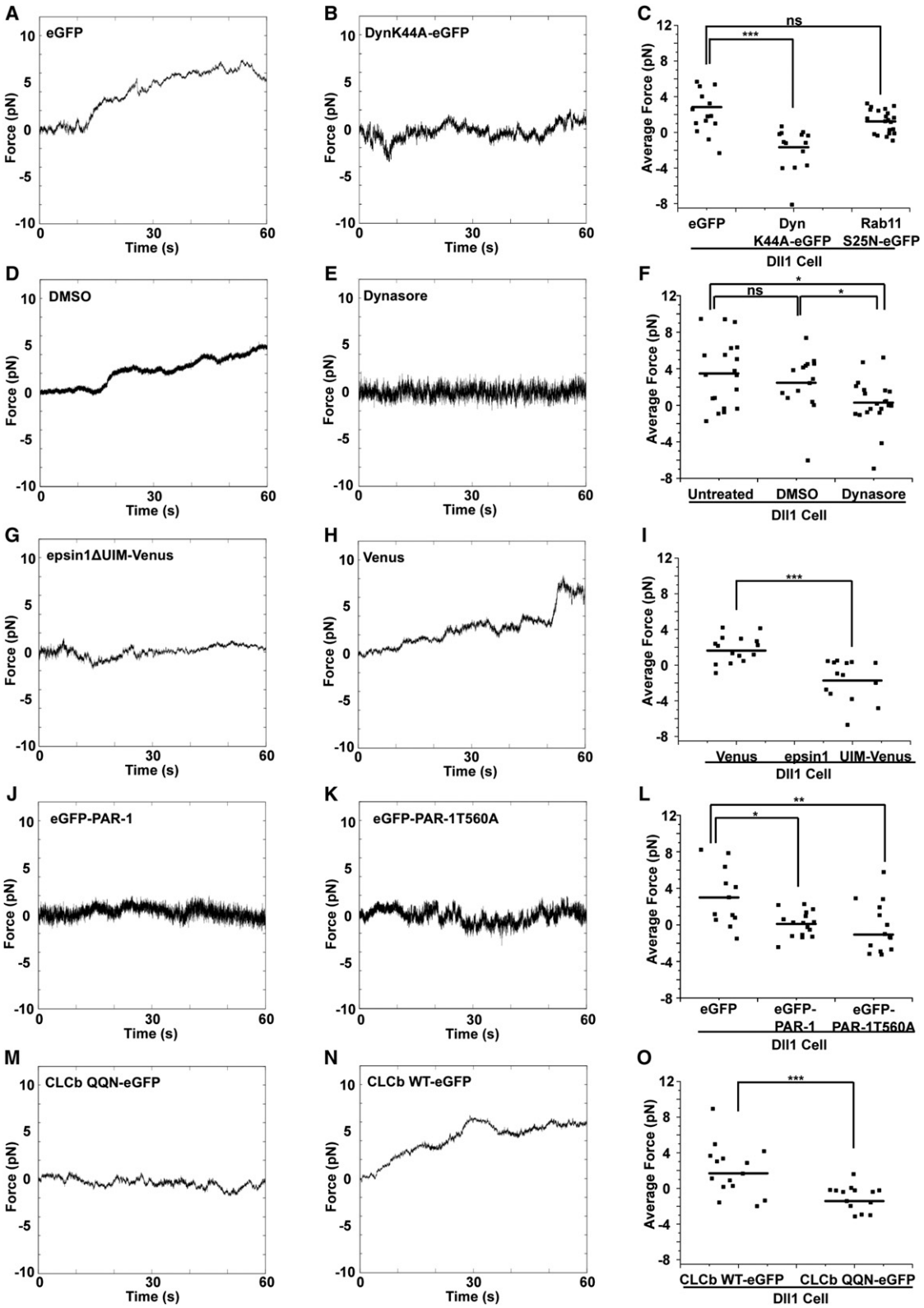

Figure 6. Dll1 Cells Pull on Laser-Trapped N1Fc Beads with Sustained Force Requiring Endocytosis Dependent on Dynamin, Epsins, and Actin

(A and B, D and E, G and H, J and K, and M and N) Prototypic force tracings for Dll1 cells expressing (A) eGFP or (B) DynK44A-eGFP, (G) epsin1ΔUIM-Venus or (H) Venus, (J) eGFP-PAR-1 or (K) eGFP-PAR-1T560A, (M) CLCb QQN-eGFP or (N) CLCb WT-eGFP, or (D) treated with DMSO or (E) Dynasore when bound to trapped N1Fc beads.

Actin polymerization is associated with mechanical force to drive membrane invagination during endocytosis (Liu et al., 2010; McMahon and Gallop, 2005). Implicating actin regulation in pulling force, CLCb QQN-eGFP, which is known to compromise actin dynamics during CME (Chen and Brodsky, 2005; Poupon et al., 2008), reduced Dll1 cell pulling force (Figure 6M), and multiple bead-cell pairings identified reduced average force measurements for CLCb QQN-eGFP ($p < 0.05$; Figure 6O and Table 1). Since Dll1 cells expressing CLCb QQN are defective in NECD transendocytosis and reporter activity (Figures 3C and 3D), these findings provide further support for Dll1 cell pulling force in signaling activity.

DISCUSSION

Structural studies suggest that Notch receptors are locked down in a protease-resistant state and that force-induced conformational changes are required to expose the ADAM site for activating proteolysis (Musse et al., 2012). Our biochemical, cellular, and biophysical findings suggest that interactions between Notch ligand and receptor cells produce resistance to ligand endocytosis that stimulates ligand ubiquitylation and recruitment of epsins. Together with actin, ligand cells form a specialized mode of CME associated with mechanical force to pull on Notch, which we propose, induces Notch conformational changes that permit activating proteolysis for downstream signaling.

We identified two distinct modes of endocytosis for Notch ligands, yet only CME involving alternative endocytic adaptors and actin functions in ligand-induced Notch signaling. Genetic studies in flies have identified clathrin-dependent (Banks et al., 2011; Eun et al., 2007, 2008; Hagedorn et al., 2006; Kandachar et al., 2008) and -independent (Banks et al., 2011; Windler and Bilder, 2010) endocytosis required for ligand signaling activity, indicating context-dependent endocytic requirements. While most CME requires AP2, losses in AP2 activity in Dll1 cells consistently increased rather than decreased Notch signaling. This enhancement in ligand signaling activity may reflect increased availability of endocytic factors such as epsins when AP2 complex formation is disrupted (Mettlen et al., 2009). Consistent with a high demand for endocytic components, requirements for the alternative clathrin adaptors epsin1, epsin2, and CALM are not functionally redundant for Dll1 signaling activity.

Genetic studies also indicate an absolute requirement for the E3 ligase Mib in ligand signaling activity and further suggest that ligand ubiquitylation reflects the need for epsin-dependent ligand endocytosis and/or trafficking to obtain signaling activity (Weinmaster and Fischer, 2011). Here we show that interactions with Notch promote Dll1 ubiquitylation as reported for Jagged1 (Hansson et al., 2010). Additionally, our experiments provide evidence that this modification promotes recruitment of epsins that depend on UIMs, as reported for other epsin-specific cargos (Chen and Zhuang, 2008; Kazazic et al., 2009; Sugiyama et al., 2005; Wang et al., 2006). Although direct interactions are possible, the majority of Dll1 captured by epsin1 did not appear to contain Ub. Whether this finding reflects Dll1 deubiquitylation during the analysis or has more mechanistic implications involving cell surface clustering of ubiquitylated with unmodified Dll1 remains to be determined. In this regard, homotypic interactions have been reported for Notch ligands (Fehon et al., 1990; Sakamoto et al., 2002; Wright et al., 2011), and heterotypic interactions between zebrafish DeltaD and DeltaC ligands have been shown to regulate surface expression and proposed to regulate signaling (Wright et al., 2011). Alternatively, Ub-independent interactions of Dll1 with the endocytic machinery or indirect interactions via ubiquitylated adaptors (Hislop and von Zastrow, 2011) may promote Dll1-epsin interactions.

Deformation of the endocytic membrane is expected to require mechanical force (Liu et al., 2009; Liu et al., 2010), and mechanical forces have been recently linked to endocytosis using a fluorescent sensor (Stabley et al., 2011). Our findings indicate that Dll1 cell-mediated pulling force requires dynamin-dependent endocytosis, which could reflect GTPase activity intrinsic to dynamin associated with mechanical twisting (Roux et al., 2006) proposed to drive membrane deformation during endocytosis (Liu et al., 2009; Liu et al., 2010). Dynamin is also known to regulate actin polymerization, and a close interplay between dynamin and actin dynamics regulates endocytic membrane shape (Ferguson et al., 2009; Itoh and De Camilli, 2006; Itoh et al., 2005). Actin polymerization is also proposed to generate mechanical force to bend the membrane for invagination during endocytosis (Itoh and De Camilli, 2006; Itoh et al., 2005; Liu et al., 2009, 2010). Consistent with this idea, we find that Dll1 cells expressing the CLCb mutant that disrupts Hip1R-regulated actin dynamics (Chen and Brodsky, 2005; Poupon et al., 2008) are defective in pulling force generation.

Along with dynamin and the actin cytoskeleton, epsins also are implicated in membrane bending during invagination (Liu et al., 2010; McMahon and Gallop, 2005). Epsins contain an ENTH domain that functions directly in membrane curvature (Horvath et al., 2007) and influences actin dynamics (Aguilar et al., 2006; Brady et al., 2010). Our tweezer studies show that epsins are required for Dll1 cell pulling force, which is consistent with a role for epsin in force-dependent membrane bending. Interestingly, cells lacking both epsin1 and epsin2 are competent for general CME (Chen et al., 2009), suggesting additional proteins implicated in membrane curvature must compensate for the loss of epsins. Nonetheless, mouse embryos lacking both epsin1 and epsin2 display classic Notch mutant phenotypes, likely reflecting a role for epsins in ligand signaling activity and underscoring the absolute requirement for epsins in Notch-dependent events. Furthermore, since Dll1 ubiquitylation and epsin UIMs are required for Dll1-epsin complex formation, Dll1 cell-mediated force, and Delta signaling activity in flies (Xie et al., 2012), we hypothesize that recruitment of epsins by ubiquitylated ligands is critical for endocytic force to activate Notch.

We conclude that the primary role of ligand endocytosis is to generate mechanical force to activate Notch signaling. Future studies to quantify the force required to dissociate Notch, as

(C, F, I, L, and O) Average of the average force measurement for Dll1 cells expressing (C) eGFP, DynK44A-eGFP or Rab11S25N-eGFP, (I) Venus or epsin1ΔUIM-Venus, (L) eGFP, eGFP-PAR-1 or eGFP-PAR-1T560A, (O) CLCb QQN-eGFP or CLCb WT-eGFP, or (F) for Dll1 cells untreated or treated with DMSO or dynasore. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.
See also Table 1 and Figure S5.

well as directly demonstrate mechanical force applied to Notch activates signaling, will extend our findings and further test the pulling-force model. Our characterization of ligand cell endocytic pulling force induced by Notch identifies a role for endocytosis in receptor activation and intercellular signaling.

EXPERIMENTAL PROCEDURES

Mammalian Expression Constructs, Cell Lines, and siRNA Treatment

Cell lines used here have been previously described elsewhere (Nichols, 2007) and the growth, experimental conditions, and constructs are described in detail in the Supplemental Experimental Procedures. For siRNA knockdown, Dll1 cells were sequentially transfected with Lipofectamine RNA interference MAX reagent (Invitrogen) with 50 nM siRNA duplexes targeting specific sequences for the indicated mouse proteins (see Supplemental Experimental Procedures for specific nucleotide sequences). Cells were assayed 72 hr post-transfection for Notch reporter activity, NECD transendocytosis, and N1Fc uptake as described previously (Bozkulak and Weinmaster, 2009; Nichols et al., 2007). In parallel, WCLs were immunoblotted and quantified to monitor knockdown efficiency of targeted proteins. For rescue experiments, Dll1 cells were transfected with siRNA-resistant rat epsin1-Venus or rat epsin2-Venus constructs prior to coculture with HA-N1 cells.

N1Fc Uptake Assays

For soluble N1Fc uptake assays, N1Fc-conditioned media (5 μg/ml) was preclustered overnight at 40°C with goat anti-human Fc (1:500, Jackson Laboratories) and incubated with cells for 1 hr at 40°C and then at 370°C for 25 min. Subsequent staining and analysis by flow cytometry were as described (Nichols et al., 2007). For N1Fc bead uptake assays, preclustered N1Fc was incubated with PrtA-agarose beads for 30 min at room temperature to generate N1Fc beads that were incubated with cells for 2 hr at 370°C, followed by staining and flow cytometry analysis (see Supplemental Experimental Procedures for details). To monitor uptake of soluble N1Fc or N1Fc attached to beads in the presence of 10 μM Lat B (CALBIOCHEM), cells were pretreated with drug or DMSO (Sigma) for 30 min at 370°C prior to addition of soluble N1Fc or N1Fc beads and analyzed as above.

Notch Ligand and Epsin Interaction Analysis

L cells were reverse transfected with Lipofectamine 2000 in Optimem (Invitrogen) according to manufacturer's instruction and plated 36 hr posttransfection on coated dishes for 45 min at 37°C (see Supplemental Experimental Procedures). 293T cells were transfected by calcium phosphate. Equal amounts of total protein were incubated with anti-GFP or anti-Dll1 ICD (1:200) and 10 μM NEM followed by incubation with PrtA agarose (Roche). Western blot analysis was performed as previously described (Bozkulak and Weinmaster, 2009).

Statistical Analysis

Statistical significance was calculated by Student's t test for two-tailed distribution with equal variances using Microsoft Excel software (Microsoft). Error bars indicate the mean ± SD.

Optical Tweezers and Analysis

Optical tweezers experiments were conducted using a custom-built instrument (Kotlarchyk et al., 2011) and the experimental details are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2012.04.005.

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