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Optical Tweezers Studies on Notch: Single-molecule Interaction Strength is Independent of Ligand Endocytosis

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SUMMARY

Notch signaling controls diverse cellular processes critical to development and disease. Cell surface ligands bind Notch on neighboring cells yet require endocytosis to activate signaling. The role ligand endocytosis plays in Notch activation has not been established. Here we integrate optical tweezers with cell biological and biochemical methods to test the prevailing model that ligand endocytosis facilitates recycling to enhance ligand interactions with Notch necessary to trigger signaling. Specifically, single-molecule measurements indicate that interference of ligand endocytosis and/or recycling does not alter the force required to rupture bonds formed between cells expressing the Notch ligand Delta-like1 (Dll1) and laser-trapped Notch1-beads. Together, our analyses eliminate roles for ligand endocytosis and recycling in Dll1-Notch1 interactions, and indicate that recycling indirectly affects signaling by regulating the accumulation of cell-surface ligand. Importantly, our study demonstrates the utility of optical tweezers to test a role for ligand endocytosis in generating cell-mediated mechanical force.

INTRODUCTION

Activation of the evolutionarily conserved Notch signaling system requires cell-cell contact to facilitate interactions between Notch cell surface ligands and receptors. The transmembrane nature of the Notch ligands is consistent with the requirement for ligand endocytosis in activation of the Notch receptor (D'Souza et al., 2010; Fortini and Bilder, 2009). In the absence of endocytosis, ligands accumulate on the cell surface but fail to activate Notch signaling in neighboring cells (Itoh et al., 2003; Nichols et al., 2007a; Parks et al., 2000; Wang and Struhl, 2004), identifying a new paradigm for endocytosis in

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SUPPLEMENTAL INFORMATION

Supplemental information includes two figures, one table and Supplemental Experimental Procedures and can be found with this article online at

activation of a signaling pathway. The exact roles that ligand endocytosis serve in Notch signaling have remained poorly defined and controversial.

Studies in flies and mammalian cells suggest ligands undergo two distinct endocytic events to activate Notch signaling (D'Souza et al., 2010; Furthauer and Gonzalez-Gaitan, 2009). The first occurs prior to binding Notch and is proposed to facilitate recycling to generate an active ligand. Specifically, this recycling model proposes nascent ligand delivered to the cell surface cannot activate Notch. Instead, endocytosis and recycling back to the surface are necessary for ligand to effectively bind Notch and activate signaling (Heuss et al., 2008; Wang and Struhl, 2004). Ligand binding strength is critical for a model in which Notch ligands are proposed to generate mechanical force to pull on Notch (Nichols et al., 2007a; Parks et al., 2000). According to this pulling-force model, force produced through ligand endocytosis deforms bound Notch to permit activating proteolysis in the production of a cleaved Notch intracellular form that directly functions as the downstream signal transducer (D'Souza et al., 2010; Gordon et al., 2008; Nichols et al., 2007b). Additionally, studies in flies and mammalian cells suggest roles for endocytosis and recycling in the trafficking of ligand to specific cell surface microdomains to potentiate either affinity or availability for Notch (Benhra et al., 2010; Heuss et al., 2008; Rajan et al., 2009). Whether ligand endocytosis regulates recycling or mechanical force to activate Notch signaling is currently unknown. However, not all Notch-dependent developmental events require ligand recycling raising the possibility that recycling is not a general, core requirement for signaling activity (Banks et al., 2011; Jafar-Nejad et al., 2005; Windler and Bilder, 2010).

In this study, we directly tested whether ligand endocytosis functions to promote recycling to strengthen Notch binding. To this end, we developed a cell-bead optical tweezers assay to obtain rupture force measurements specific for cells expressing the Notch ligand Delta-like1 (Dll1) bound to laser-trapped Notch1 (N1) beads. Our biophysical experiments indicate that neither endocytosis nor recycling strengthens ligand binding to N1, eliminating a role for endocytosis in ligand affinity prior to interactions with N1. In the accompanying paper (Meloty-Kapella et al., 2012), we extend this biophysical approach to obtain support for a primary role for ligand endocytosis in generating mechanical pulling force downstream of Notch binding.

RESULTS AND DISCUSSION

Establishing Optical Tweezers to Measure Specific Dll1-N1 Binding Events

To determine if ligand endocytosis or recycling regulate ligand binding to Notch we used a cell-bead optical tweezers assay. Interactions between *Drosophila* and mammalian Notch ligands and receptors have been demonstrated using cell aggregation (Fehon et al., 1990; Parks et al., 2006; Rebay et al., 1991) and binding assays (Heuss et al., 2008; Hicks et al., 2002; Nichols et al., 2007a; Shimizu et al., 1999). Moreover, atomic force microscopy (AFM) measurement of rupture between *Drosophila* cells programmed to express Delta and Notch have reported force in the nanoNewton range (Ahimou et al., 2004). In contrast to these cell-based studies, the affinities reported for recombinant protein fragments containing Notch ligand and receptor binding domains are weak or undetectable (Cordle et al., 2008a; Cordle et al., 2008b). None of these studies, however, have directly tested requirements for ligand endocytosis and recycling in Notch ligand-receptor interactions. Towards this goal, we replaced the Notch cell with a bead functionalized with recombinant Notch1 (N1) protein containing the ligand-binding domain fused to human Immunoglobulin G (IgG)-Fc (N1Fc).

N1Fc specifically binds Dll1 expressing cells (Nichols et al., 2007a) and optical tweezers can present beads to live cells for accurate measurement of bond strength (Weisel et al., 2003). To determine if endocytosis or recycling enhance intrinsic ligand binding strength

independent of avidity (Weisel et al., 2003), it was imperative to minimize Dll1 clustering in cells following interactions with N1Fc-beads. In fact, the exceptionally strong AFM forces reported (Ahimou et al., 2004), likely reflect multivalent interactions between cells engineered to express high levels of Delta and Notch. To avoid N1-induced ligand clustering and facilitate single Dll1-N1 interaction measurements, ProteinA (PrtA) microbeads were coated with low N1Fc concentrations (Supplemental Experimental Procedures).

With optical tweezers, the position of the bead within the optical trap can be precisely monitored over time (sec) and used to calculate the force (pN) required to rupture interactions between N1Fc-beads and Dll1 cells (Figure 1A and Supplemental Experimental Procedures). Media containing 0.5ug N1Fc/ml produced functionalized beads that could be repeatedly bound and detached from Dll1 cells, which allowed serial measurements of rupture force between a single live cell and a laser-trapped bead. These modifications to a published method (Litvinov et al., 2002), allowed collection of large data sets for statistical analyses to accurately determine rupture force as a measure of bond strength.

Determining Rupture Force Measurements Specific for Dll1-N1 interactions

When N1Fc-beads interacted with Dll1 cells the majority of rupture events occurred between 0 and 40 pN (Figure S1A). To establish the specificity of these measurements, we tested conditions in which Dll1-N1 binding would not occur: Dll1 cells paired with Fc- or PrtA-beads (with and without BSA) and parental L cells paired with N1Fc-, Fc-, or PrtA/BSA-beads. All negative control spectra contain one large mode centered at a few pN (containing the maximum viscous drag force of ~0.6 pN estimated by Stokes flow and verified in cell-free experiments shown in Figure S1B) and a shorter shoulder bound by 12 pN, representing non-specific binding and rupture events (Figure 1B and insert). In support of this idea, spectra obtained for Dll1 cells bound to N1Fc-beads contained additional force modes with means of ~ 19 and 36 pN, suggestive of specific Dll1-N1 interactions. Further confirming the specificity of these measurements, L cells transiently expressing Dll1C284Y carrying the missense mutation reported to eliminate Delta binding to Notch in flies (Parks et al., 2006), did not display the two higher force modes detected for Dll1 cells (Figure 1C). Consistent with the inability to bind N1Fc, Dll1C284Y cells displayed reduced soluble N1Fc binding similar to that detected for L cell controls (Figure S1C, D).

Single-molecule interactions were promoted by limiting the bead-cell contact time to 20 msec. If multiple interactions formed within 20 msec, we would have expected to detect stepwise rupture as previously reported (Litvinov et al., 2002; Litvinov et al., 1994), rather than the observed steep rupture events (Figure 1A). Additionally, we predict that our laser tweezers cannot break more than four parallel bonds; assuming 19 pN to be the single-bond rupture force and considering that our maximum laser tweezers force is ~ 90 pN. In support of this, N1Fc-beads can be consistently pulled away from Dll1 cells at forces too weak to pull Dll1 out of the membrane (Shao and Hochmuth, 1999).

Poisson distribution statistics were used to determine if the 19 pN force mode corresponds to rupture of single Dll1-N1 interactions. Nonspecific rupture events account for 70% or more of our data, which corresponds to a rate of specific bond formation of 30% or lower, proposed to promote single molecule events (Panorchan et al., 2006). In our rupture force data for Dll1 cells bound to N1Fc-beads (Figure 1B), the rate of bond formation was 10.5% with 62% in the putative single bond mode and 38% in the double bond mode. Poisson distribution statistics (Tees et al., 2001), however, predict a higher rate of single (95%) and lower rate of double (5%) bonds than observed. Since Fc sequences dimerize N1Fc to produce two potential Dll1 binding sites, we wondered if dimer formation enhances the probability of double bond formation (Figure 1D). If this were the case, spectra should conform to Poisson distribution statistics when cells express low levels of Dll1 and can only

form single Dll1-N1 interactions. To directly test this idea, we measured rupture force for cells in which the level of Dll1 expression could be modulated using doxycycline (dox) induction (Sprinzak et al., 2010).

Dll1 cell surface levels are low in the absence of dox, which likely accounts for the majority of rupture force measurements occurring within the non-specific range (<12pN) (Figure 1E). In contrast, cells cultured in 1 ng/ml dox showed an 8% rate of specific bond formation, of which 98% fall within the putative single bond mode (Poisson distribution statistics predicts 96%), while 2% fall within the double bond mode (Poisson distribution statistics predicts 4%). Therefore, at this low Dll1 cell surface level rupture force spectra are indeed consistent with a Poisson distribution model, indicating the 19 pN mode represents single bond ruptures. At 10 ng/ml dox, induced surface levels are similar to stable expressing Dll1 cells (see Figure 4), and the rate of bond formation was 30%, with 71% single (Poisson distribution statistics predicts 83%) and 29% double bond formation (Poisson distribution statistics predicts 15%). Thus, as ligand expression increases, the relative probabilities deviate from predicted, however, the 19 pN mode persists supporting our claim that it represents single-molecule Dll1-N1 rupture events.

To further demonstrate the 19 pN mode corresponds to single-bond ruptures, we measured rupture force spectra for Dll1 cells interacting with N1Fc-beads coated with conditioned media containing decreasing N1Fc and increasing Fc proteins (see Supplemental Experimental Procedures for details). As N1Fc concentration decreased from 0.5 μg/ml (Figure 1B, 1C) to 0.25 μg/ml and 0.1 μg/ml (Figure 1F), the probability of rupture events in the first specific mode increased (62%, 76%, and 84%, respectively), while those in the second decreased (38%, 24%, 16%, respectively), consistent with the 19 pN mode corresponding to single ligand-receptor interactions. At 0.005 μg/ml the rate of bond formation was 0.3% and neither mode was detected, underscoring the Dll1-N1 specificity of these rupture force measurements.

We next addressed the possibility that either of the two specific modes represents rupture of PrtA-Fc bonds (Figure 1D, x). The rupture force between rabbit IgG and PrtA has been reported to be ~ 30 pN at our typical loading rate of 250 pN/sec (Salomo et al., 2008). Laser tweezers analyses of IgG-PrtA rupture force for rabbit, mouse, bovine and goat IgG identified a median rupture force ranging from 25 to 44 pN (Stout, 2001), measured at loading rates ranging from 400 to 5300 pN/sec. Considering these findings, the second specific mode in our system could represent rupture of PrtA-Fc interactions. To test this, N1Fc attached to PrtA-beads was chemically cross-linked (see Supplemental Experimental Procedures). Rupture force analysis failed to show a change in the 19 pN rupture mode ($p > 0.05$) and only <1 pN change was detected for the second specific mode (Figure 1G). If the second specific mode represented PrtA-Fc bond rupture, it should be significantly reduced or eliminated following covalent cross-linking. Importantly, detection of the second specific mode further supports specificity of the Dll1-N1 interactions. Lastly, this analysis suggests that the density of N1Fc on the bead must be too low for the cross-linking to cluster N1Fc, further indicating that the first specific rupture mode represents measurement of single-molecule binding events.

Interference of Dll1 Endocytosis does not Decrease Dll1-N1 Interaction Strength

To directly test whether endocytosis influences Dll1 bond strength, cells expressing a Dll1 lacking intracellular domain sequences (OCDD1) that is consequently defective in endocytosis was assayed. Important for this analysis, OCDD1 binds N1 but is unable to activate signaling in co-culture assays (Nichols et al., 2007a). Rupture force spectra obtained for OCDD1 cells interacting with N1Fc-beads compared to control Fc-beads identified both specific modes (Figure 2A). Comparison of rupture force spectra for Dll1 and OCDD1 cells

did not identify significant reduction in the mean value of the first specific mode (Figure 2B, $p > 0.05$) as would be predicted if endocytosis is necessary for ligand binding to Notch. The taller peaks observed for OCDD1 likely reflect cell surface accumulation of this endocytic defective protein (Figure 4B and (Nichols et al., 2007a), which would increase the probability of forming interactions as observed with higher Dll1 expression (Figure 1E). In fact, concentration dependent increases in binding probability have been reported for fibrinogen-integrin binding with activated platelets (Litvinov et al., 2002).

Genetic mosaic analysis of Drosophila *shibire* indicates a requirement for the key endocytic factor dynamin by Delta cells in Notch activation (Seugnet et al., 1997). Dll1 cells expressing a dominant-negative dynaminK44A (Damke et al., 2001) are defective in endocytosis and activation of Notch signaling (Nichols et al., 2007a), and display reduced internalization of transferrin that requires dynamin (Figure S2B). The mean value of the single-molecule rupture force mode detected for Dll1 cells expressing dynaminK44A-eGFP or eGFP alone are statistically equivalent ($p > 0.05$; Figure 2C), indicating endocytosis is not a determinant of Dll1-N1 bond strength. A caveat of this interpretation is the possible induction of alternative modes of endocytosis by cells exposed to sustained perturbation in dynamin activity (Damke et al., 1995; Ferguson et al., 2009). Therefore, we induced an acute endocytic block with dynasore, a cell-permeable inhibitor of dynamin known to rapidly prevent dynamin-dependent vesicle formation (Macia et al., 2006). The mean rupture force value of the single molecule mode for Dll1 cells treated with dynasore was only 1 pN different from DMSO treated Dll1 cells (Figure 2D), and the leftward shift is likely due to cellular affects related to DMSO. Together, our findings indicate that neither short- nor long-term inhibition of dynamin activity decreased Dll1-N1 interaction strength, eliminating a role for dynamin-dependent ligand endocytosis in Notch ligand bond strength.

Defects in Rab11-dependent Recycling do not Alter Dll1-N1 Interactions

Our findings for Dll1 cells defective in endocytosis indirectly suggest that recycling is not required to strengthen the bond between Dll1 and N1, in contrast to that previous proposed (Heuss et al., 2008). Since this study did not directly test a role for Dll1 recycling in Dll1-N1 bond strength, we established methods to monitor and perturb Dll1 recycling to determine if trafficking through the recycling endosome enhanced ligand binding strength. To monitor Dll1 recycling, a cell surface labeling and stripping protocol (Heuss et al., 2008) that allows 90–100% removal of biotin from biotinylated cell surface Dll1 was employed (Figure 3A and 3B). Quantification of Dll1 cell biotinylation indicated that ~ 50% of internalized biotinylated Dll1 is recycled back to the cell surface after 30 min (Figure 3C) as previously reported (Heuss et al., 2008).

Since the small GTPase Rab11 that functions in the recycling endosome is indirectly associated with ligand signaling activity (Emery et al., 2005), we determined if Rab11 activity is required for Dll1 recycling. Simultaneous depletion of Rab11A and B using small interfering RNAs (siRNAs) decreased Dll1 recycling to ~ 1/3 detected for cells treated with scrambled (SCR) siRNAs (Figure 3B and 3C). Moreover, expression of the dominant-negative Rab11S25N-eGFP (Ullrich et al., 1996) produced a similar Dll1 recycling defect (Figure 3B and 3C). Given the incomplete block in Dll1 recycling and possible Rab-11-independent Dll1 recycling pathways, we estimate that this block detected at most 33% of Dll1 normally returned to the cell surface. If recycling strengthens ligand binding to Notch as proposed, then decreasing Dll1 recycling should produce a population displaying reduced rupture forces compared to cells that effectively recycle Dll1. Depending on the mean value of this putative population, it should either appear as a distinct mode between the nonspecific and single-bond modes, cause a leftward shift in the 19 pN mode, or fall completely in the nonspecific mode increasing its probability. Significantly, none of these predictions were supported by rupture force measurements of Dll1 cells blocked in recycling

through Rab11S25N-eGFP expression (Figure 3D and Figure S2C). Instead, as found for defects in Dll1 endocytosis, the single ligand-receptor rupture force mode was not statistically different from control eGFP cells ($p > 0.05$), indicating no change in Dll1-N1 interaction strength (Figure 3D).

Finally, that interference of either ligand endocytosis or recycling did not lead to significant increases in the frequency of single-molecule interactions, argues against a role for recycling to cluster ligand to produce high affinity Notch binding (Heuss et al., 2008). Rather, our data indicate that Dll1 recycling is not a determinant of Dll1-N1 bond strength, and are more consistent with genetic studies suggesting a context dependent requirement for ligand recycling in signaling activity (Banks et al., 2011; Windler and Bilder, 2010).

Epsins are not Required for Ligand Recycling or Dll1-N1 Interactions

Epsins are absolutely required for Notch ligands to activate signaling (Chen et al., 2009; Overstreet et al., 2003; Overstreet et al., 2004; Tian et al., 2004; Wang and Struhl, 2005), and it has been suggested that these endocytic adaptors function in ligand recycling to gain signaling activity (Wang and Struhl, 2004). However, Dll1 cells siRNA-depleted of epsin1 or expressing the dominant-negative epsin1ΔUIM-Venus (Chen and Zhuang, 2008) displayed similar levels of Dll1 recycling as control cells (Figure 3B and 3C). Moreover, the mean rupture forces for Dll1 cells expressing epsin1ΔUIM-Venus are similar (difference was less than 1 pN) to Dll1 cells expressing Venus alone (Figure 3E). Together, our biochemical and biophysical data exclude a role for epsins in Dll1 recycling to enhance Dll1-N1 bond strength. Since epsins are required for all Notch-dependent events, our findings suggest alternative roles for epsins in ligand signaling activity that do not include recycling.

Losses in Recycling Decrease Cell Surface Dll1 and Notch Signaling

Although Rab11-dependent Dll1 recycling does not strengthen Notch binding, depletion of Rab11A and B by more than 80% significantly reduced Notch reporter activity induced by Dll1 cells in coculture assays (Figure 4A). While Dll1 accumulates at the cell surface in the absence of endocytosis (Figure 4B and (Nichols et al., 2007a), Rab11A and B depletion produced significant reduction in cell surface Dll1 (Figure 4B). These data suggest that recycling regulates ligand cell surface expression, and thus, the amount of ligand available to activate Notch. Consistent with this idea, the level of cell surface Dll1 induced by dox (Figure 4C) directly correlated with the level of ligand-induced Notch signaling (Figure 4D). Together our optical tweezers and biochemical analyses suggest Dll1 recycling modulates Notch signaling by regulating the level of ligand at the cell surface, rather than enhancing Dll1-N1 bond strength as previously proposed (Heuss et al., 2008). Our findings also raise the possibility that the signaling defects previously reported for Delta-OP9 cells expressing Rab11S25N-eGFP (Emery et al., 2005), or the lysine-less Dll1 mutant defective in recycling (Heuss et al., 2008; Wang and Struhl, 2004), may actually reflect losses in cell surface ligand rather than changes in binding strength dependent on recycling.

CONCLUSIONS

Studies in *Drosophila* and mammalian cells have suggested that ligand signaling activity requires endocytosis and trafficking through the recycling endosome prior to engagement with Notch (Benhra et al., 2010; Emery et al., 2005; Heuss et al., 2008; Jafar-Nejad et al., 2005; Rajan et al., 2009; Wang and Struhl, 2004). More recent genetic studies in *Drosophila,* however, suggest the ligand recycling requirement is context dependent (Banks et al., 2011; Windler and Bilder, 2010). In fact, Rab11 and Rab5 (which directs access to the Rab11 recycling endosome) are not required for ligand signaling activity in the germline or

developing eye (Banks et al., 2011; Windler and Bilder, 2010). Based on these studies, ligand trafficking through the recycling pathway does not appear to be essential for all Notch-dependent developmental events. Our biophysical and biochemical studies also indicate recycling is not a general, core requirement for ligand signaling activity.

In summary, our biophysical approach determined rupture forces near 19 pN for single-molecule Dll1-N1 interactions, and eliminated requirements for ligand endocytosis and recycling in strengthening these binding events. The biophysical methodology outlined and developed here provides an exciting approach to detect and characterize mechanical force produced by Dll1 cells bound to laser trapped N1Fc-beads to test the pulling-force model (Meloty-Kapella et al., 2012).

EXPERIMENTAL PROCEDURES

Optical tweezers and force spectroscopy analyses

The cell lines used in this study have been previously published (Sprinzak et al., 2010) growth conditions, drug treatments, siRNA knockdown, plasmid transfection, cell surface biotinylation, recycling assay, FACS analysis, soluble N1Fc and transferrin uptake and Notch reporter assays are outlined in Supplemental Information. Analyses were carried out with custom-built optical tweezers (Kotlarchyk et al., 2011). Briefly, as external forces displace a bead from the center of the focused laser beam (Figure 1A, red hour glass), optical forces pull the bead back with equal force magnitude. The bead steers the laser beam as detected by a quadrature photodiode (QPD; 2903, Newport Corporation). Optical tweezers forces are computed as the product of optical trap stiffness and bead displacement. Multiple rupture force measurements were acquired for each cell-bead pairing (Table S1, Supplemental Information) as adopted from (Litvinov et al., 2002). Rupture Force spectra are calculated and analyzed in Matlab (Mathworks). Force spectra (eg. Figure 1B) are generated from force waveforms (e.g. Figure 1A) by tabulating the maximum positive force for each bead-cell interaction cycle. A two-mode Gaussian mixture model was fit to rupture force spectra using the expectation-maximization algorithm (McLachlan and Peel, 2000) for data ranging between 13 pN and 40 pN. P-values were calculated as described in Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Optical tweezers measure single-molecule Notch (N1)-ligand (Dll1) bond strength
- Blockade of dynamin activity does not alter the strength of Dll1-N1 interactions
- Rab11-mediated Dll1 recycling does not alter single-molecule Dll1-N1 bond strength
- Dll1 recycling influences signal intensity by regulating ligand accumulation

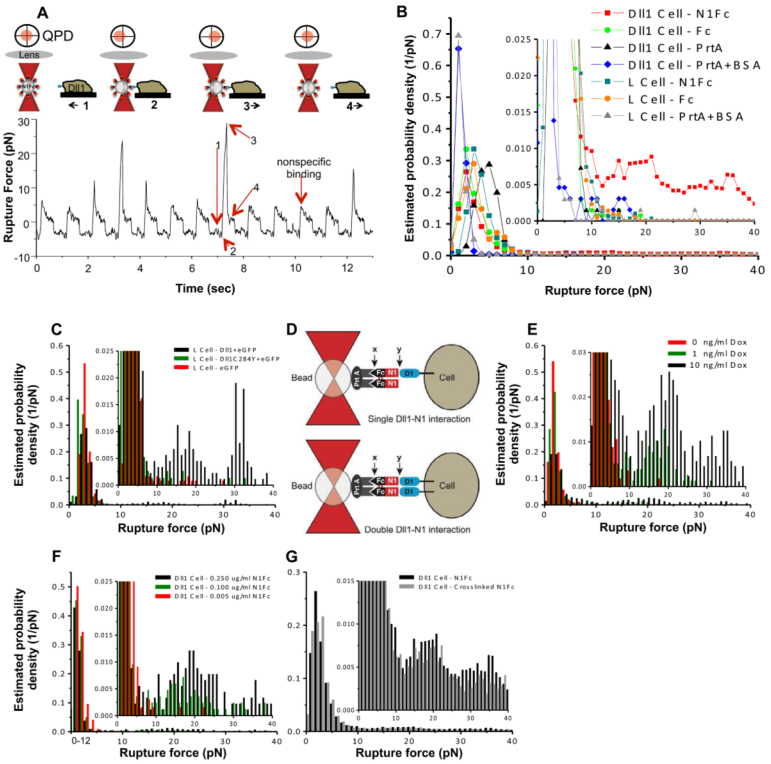

Figure 1. Optical tweezers to specifically measure the strength of Dll1-N1 interactions

(A) Schematic representation of a trapped bead presented to a live cell and a protoypical acquired force waveform. The optical tweezers beam is recollected by a lens and directed towards a quadrature photodiode (QPD) to monitor bead displacement. The experiment cycles through four stages: (1) cell moves towards the bead (small viscous forces push the bead slightly to the left); (2) cell is pushed against the bead to promote molecular interactions; (3) cell moves away from the bead until interactions rupture and (4) bead is pulled back into the trap and the cell continues to move rightward (viscous forces push the bead slightly to the right). Force is the product of trap stiffness and bead displacement. See Supplemental Information for details.

(B) Rupture force spectra obtained for N1Fc or control beads interacting with L and Dll1 cells. The tall peaks common to all spectra represent non-specific interactions. Insert represents enlargement of rupture force data.

(C) Rupture force spectra for cells expressing the Dll1 binding mutant Dll1C284Y compared to those obtained for Dll1 or eGFP controls .

(D) The N1Fc dimer presents two possible Dll1 binding sites for detection of single or double Dll1-N1 interactions. The two possible rupture sites for PrtA-Fc (X) or Dll1-N1 (Y) interactions are indicated.

(E) Rupture force spectra for N1Fc beads and cells treated with Dox to induce Dll1.

(F) Rupture force spectra for Dll1 cells interacting with beads functionalized with decreasing N1Fc and increasing Fc concentrations.

(G) Rupture force spectra for Dll1 cells with N1Fc beads following chemical crosslinking. See also Figure S1. Sample sizes for rupture force spectra can be found in Table S1.

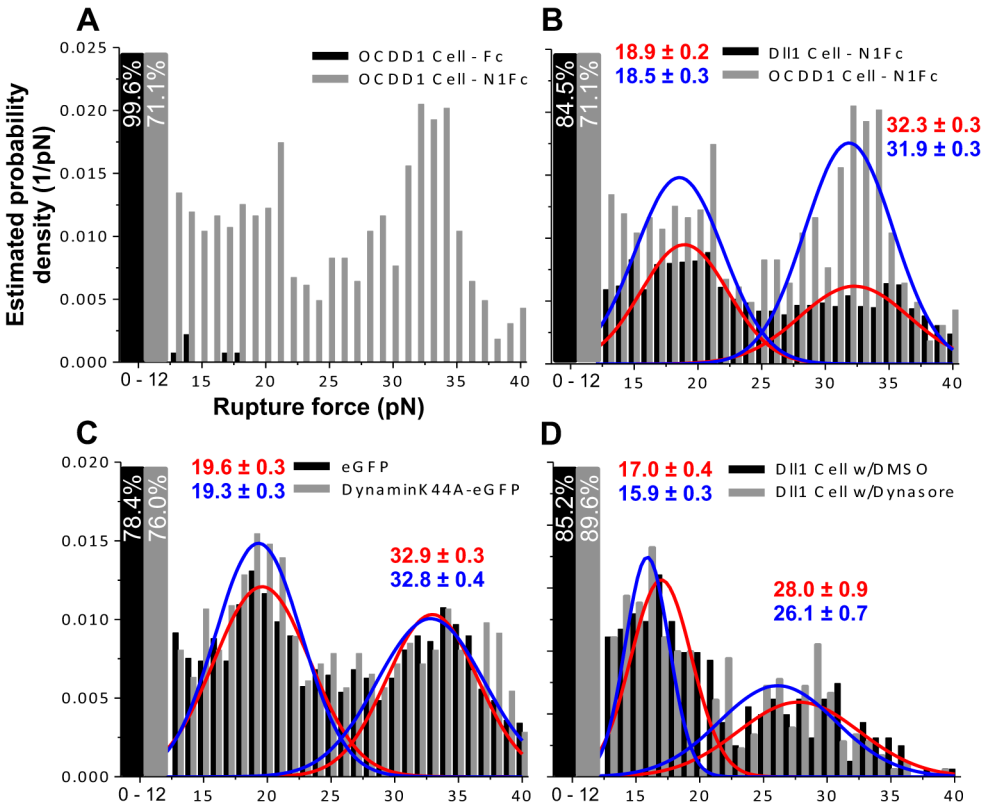

Figure 2. Dll1 endocytic defects do not alter Dll1-N1 interaction strength

(A) Rupture force spectra for Dll1 endocytic mutant (OCDD1) cells interacting with N1Fc or control Fc beads. The wide columns (0–12 pN) report the rate of non-specific binding events.

(B) Rupture force spectra for Dll1 cells compared to OCDD1 cells following interactions with N1Fc-beads are statistically equivalent ($p > 0.05$).

(C) Rupture force spectra for Dll1 cells compared to Dll1 cells blocked for dynamin activity. See also Figure S2B.

(D) Rupture force spectra for Dll1 cells treated with DMSO or dynasore to induced acute block of dynamin-dependent endocytosis. Red/blue numbers are the mean ± 95% confidence interval for two-mode Gaussian mixture models fit to the data (See Supplemental Experimental Procedures).

Sample sizes for rupture force spectra can be found in Table S1.

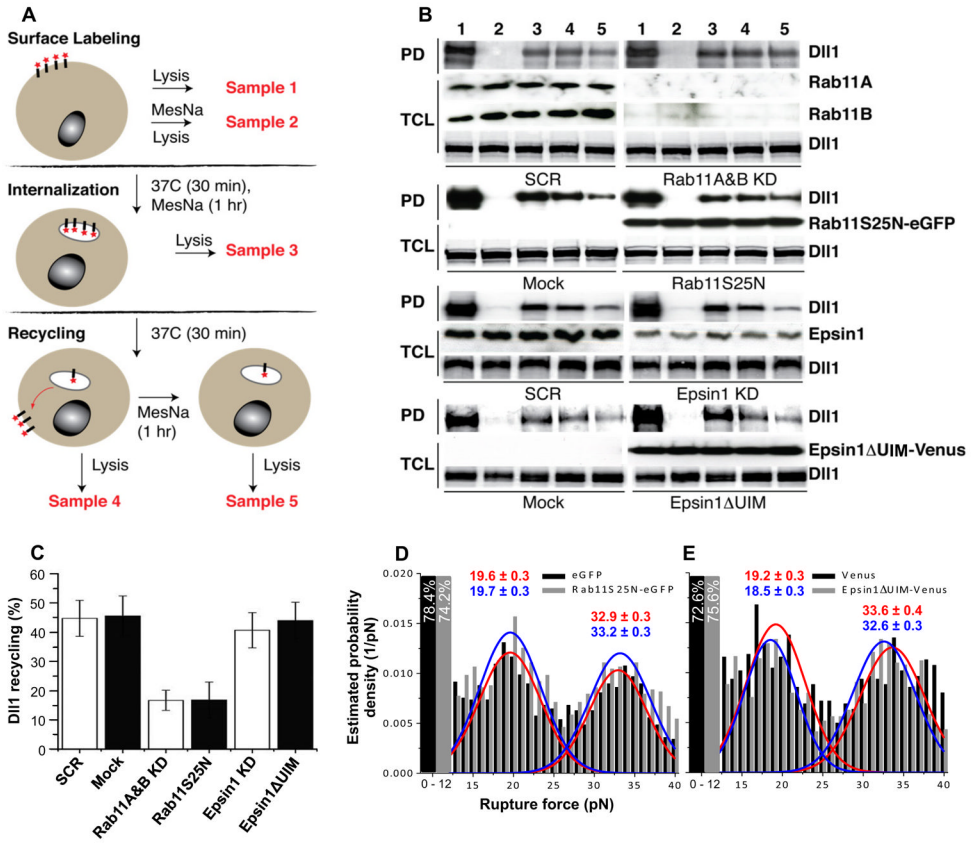

Figure 3. Perturbation of Dll1 recycling does not affect Dll1-N1 bond strength

(A) Schematic illustration of recycling assay. Sample numbers (1–5) are indicated as lanes in (B).

(B) Recycling of biotinylated Dll1 detected with NeutrAvidin pull-down (PD). The following lanes indicate surface biotinylation (1), efficiency of biotin stripping (2), Dll1 internalization (3), and Dll1 recycling (4 vs. 5). Immunoblots of total cell lysate (TCL) for Dll1, Rab11A, Rab11B, Rab11S25N-eGFP, Epsin1, and Epin1ΔUIM-Venus are shown.

(C) Quantification of recycled Dll1 (% difference between sample 4 and sample 5 in B). Error bars indicate standard deviation of the mean.

(D) Rupture force spectra for Dll1 cells compared to Dll1 cells blocked for Rab11-dependent recycling. See also Figure S2C.

(E) Rupture force spectra for Dll1 cells compared to Dll1 cells defective in epsin activity. Red/blue numbers and curve fits are calculated as in Figure 2. Sample sizes for rupture force spectra can be found in Table S1.

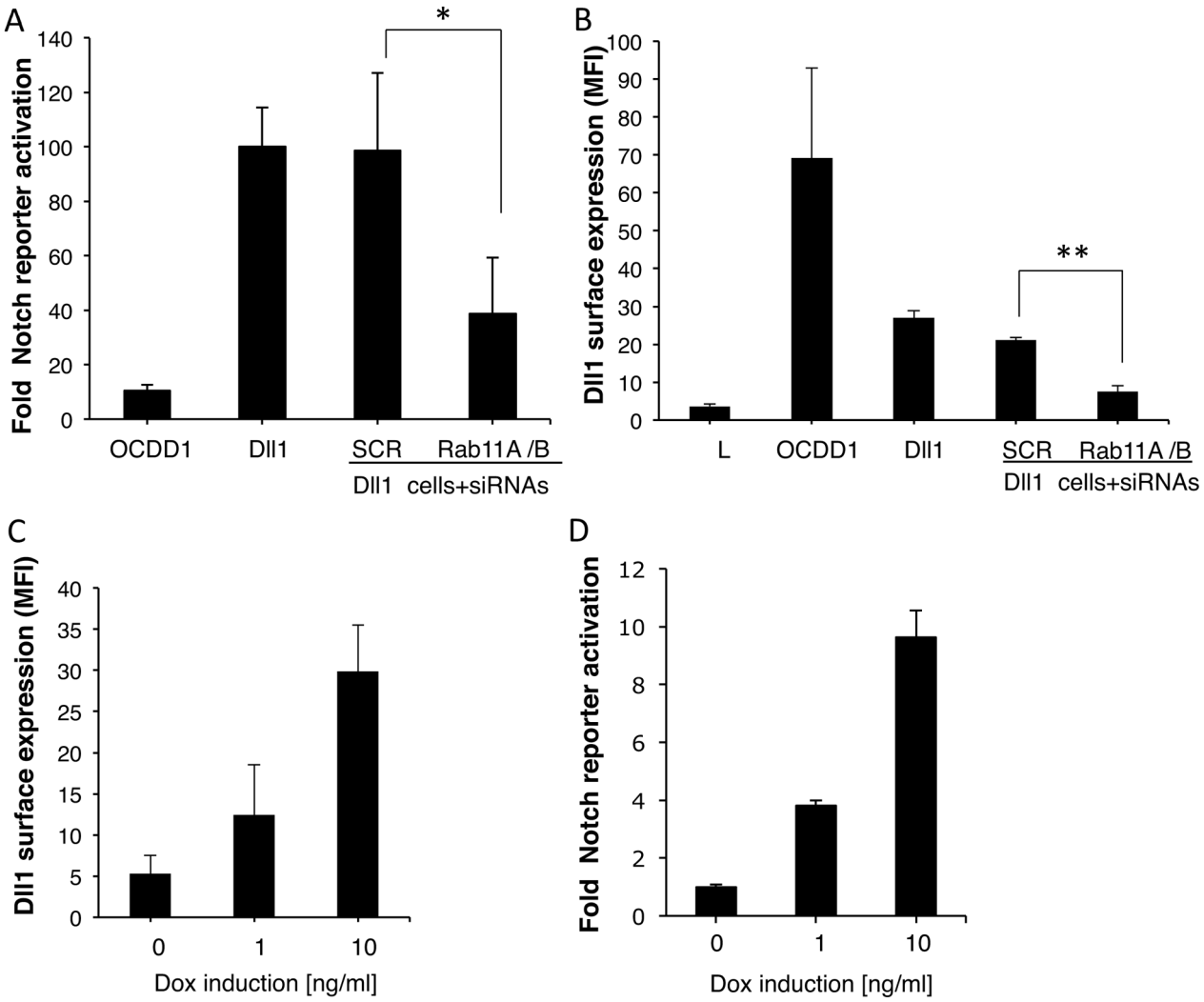

Figure 4. Recycling determines ligand cell surface level and signaling intensity

(A) Ligand signaling quantified by reporter activity obtained for OCDD1, and siRNA treated Dll1 or Dll1 cells co-cultured with N1 cells expressing a Notch reporter (*p= 0.02).

(B) Cell surface levels of ligand quantified by FACS following staining of L cells, OCDD1, and siRNA treated Dll1 or untreated Dll1 cells with extracellular Dll1 antibodies (**p= 0.007).

(C) Dll1 cell surface expression induced following dox treatment quantified as in (B).

(D) Notch reporter activity for Dll1 cells induced with dox before co-culturing with N1 cells as in (A). Values represent fold-induction over co-cultures with uninduced Dll1 cells. Error bars in (A–D) indicate the standard deviation of the mean, * is $p < 0.05$ and ** is $p < 0.01$.