Video Article

Cell Aggregation Assays to Evaluate the Binding of the *Drosophila* Notch with *Trans*-Ligands and its Inhibition by *Cis*-Ligands

Ashutosh Pandey¹, Hamed Jafar-Nejad^{1,2}

¹Department of Molecular and Human Genetics, Baylor College of Medicine

²Program in Developmental Biology, Baylor College of Medicine

Correspondence to: Hamed Jafar-Nejad at hamedj@bcm.edu

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Abstract

Notch signaling is an evolutionarily conserved cell-cell communication system used broadly in animal development and adult maintenance. Interaction of the Notch receptor with ligands from neighboring cells induces activation of the signaling pathway (*trans*-activation), while interaction with ligands from the same cell inhibits signaling (*cis*-inhibition). Proper balance between *trans*-activation and *cis*-inhibition helps establish optimal levels of Notch signaling in some contexts during animal development. Because of the overlapping expression domains of Notch and its ligands in many cell types and the existence of feedback mechanisms, studying the effects of a given post-translational modification on *trans*- versus *cis*-interactions of Notch and its ligands *in vivo* is difficult. Here, we describe a protocol for using *Drosophila* S2 cells in cell-aggregation assays to assess the effects of knocking down a Notch pathway modifier on the binding of Notch to each ligand in *trans* and in *cis*. S2 cells stably or transiently transfected with a Notch-expressing vector are mixed with cells expressing each Notch ligand (S2-Delta or S2-Serrate). *Trans*-binding between the receptor and ligands results in the formation of heterotypic cell aggregates and is measured in terms of the number of aggregates per mL composed of >6 cells. To examine the inhibitory effect of *cis*-ligands, S2 cells co-expressing Notch and each ligand are mixed with S2-Delta or S2-Serrate cells and the number of aggregates is quantified as described above. The relative decrease in the number of aggregates due to the presence of *cis*-ligands provides a measure of *cis*-ligand-mediated inhibition of *trans*-binding. These straightforward assays can provide semi-quantitative data on the effects of genetic or pharmacological manipulations on the binding of Notch to its ligands, and can help deciphering the molecular mechanisms underlying the *in vivo* effects of such manipulations on Notch signaling.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56919/>

Introduction

Canonical Notch signaling is a short-range cell to cell communication mechanism that requires physical contact of neighboring cells to facilitate the interaction between Notch receptors and their ligands¹. Interaction of Notch receptor (present on the surface of signal-receiving cells) with ligands (present on the surface of signal-sending cells) initiates Notch signaling and is known as *trans*-activation². On the other hand, interaction between Notch and its ligands in the same cell leads to the inhibition of Notch pathway and is known as *cis*-inhibition³. The balance between *trans*- and *cis*-interactions is required to ensure optimum ligand-dependent Notch signaling⁴. *Drosophila* has one Notch receptor and two ligands (Delta and Serrate) as opposed to mammals, which have four Notch receptors and five ligands [jagged 1 (JAG1), JAG2, delta-like 1 (DLL1), DLL3 and DLL4]⁵. Having this simplicity, the *Drosophila* model offers the ease to dissect/study the effects of pathway modifiers on Notch-ligand interactions and subsequently on Notch signaling. In certain contexts during animal development (including wing development in *Drosophila*), both *cis*- and *trans*-interactions are involved to achieve proper Notch signaling and cell fate^{1,6}. It is important to distinguish the effects of Notch pathway modifiers in these contexts on *cis*- versus *trans*-interactions of Notch with its ligands.

Our group previously reported that addition of a carbohydrate residue called xylose to *Drosophila* Notch negatively regulates Notch signaling in certain contexts, including wing development⁷. Loss of *shams* (the enzyme that xylosylates Notch) leads to a "loss of wing vein" phenotype⁷. More recently, gene dosage experiments and clonal analysis were used to show that loss of *shams* enhances Delta-mediated Notch signaling. To distinguish whether the enhanced Notch signaling in *shams* mutants is a result of decreased *cis*-inhibition or increased *trans*-activation, ectopic overexpression studies of Notch ligands in larval wing imaginal discs using the *dpp-GAL4* driver were performed. These experiments provided evidence suggesting that Shams regulates *trans*-activation of Notch by Delta without affecting Notch *cis*-inhibition by ligands⁸. However, feedback regulations and effects of endogenous ligands might complicate the interpretation of ectopic overexpression studies^{1,6,9}.

To resolve this issue, *Drosophila* S2 cells¹⁰ were used, which provide a simple *in vitro* system for Notch-ligand interaction studies^{11,12}. S2 cells do not express endogenous Notch receptor and Delta ligand¹¹ and express a low level of Serrate¹³, which does not affect Notch-ligand aggregation experiments⁸. Therefore, S2 cells can be stably or transiently transfected by Notch and/or individual ligands (Delta or Serrate) to generate cells that exclusively express the Notch receptor or one of its ligands, or a combination of them. Mixing of Notch-expressing S2 cells with ligand-expressing S2 cells results in the formation of heterotypic aggregates mediated by receptor-ligand binding^{11,12,14}. Quantification of the aggregate

formation provides a measure of *trans*-binding between Notch and its ligands¹⁵ (Figure 1). Similarly, S2 cells can be co-transfected with Notch and Delta or Serrate ligands (*i.e.* *cis*-ligands). *Cis*-ligands in these Notch-expressing S2 cells abrogate the binding of Notch with *trans*-ligands and result in decreased aggregate formation^{8,12,14}. The relative decrease in aggregate formation caused by *cis*-ligands provides a measure of the inhibitory effect of *cis*-ligands on binding between Notch and *trans*-ligands (Figure 2). Accordingly, cell aggregation assays were utilized to examine the effect of loss of xylosylation on *trans*- and *cis*-interactions between Notch and its ligands.

Here, we present a detailed protocol for cell aggregation assays aimed to evaluate the binding of Notch with *trans*-ligands and its inhibition by *cis*-ligands using *Drosophila* S2 cells. As an example, we provide the data that allowed us to determine the effect of Notch xylosylation on binding between Notch and *trans*-Delta⁸. These straightforward assays provide a semi-quantitative assessment of Notch-ligand interactions *in vitro* and help determine the molecular mechanisms underlying the *in vivo* effects of Notch pathway modifiers.

Protocol

1. Preparation of Double Stranded RNA (dsRNA) for *Shams* Knockdown

1. PCR amplification of the products

1. Use wild-type *yellow white* (*y w*) genomic DNA and *pAc5.1-EGFP* as template and the following primer pairs to amplify the DNA fragments used in dsRNA synthesis. Use the following PCR thermal profile: Denaturation (95 °C, 30 s), Annealing (58 °C, 30 s) and Extension (72 °C, 1 min).

Enhanced Green Fluorescent Protein (EGFP) dsRNA primers (5'-3')-

Forward primer- GAAATTAATACGACTCACTATAGGGGGTGAGCAAGGGCGAGGAG

Reverse primer- GAAATTAATACGACTCACTATAGGGGGTCTTGCTCAGGGCGG

Shams dsRNA primers (5'-3')-

Forward primer- TTAATACGACTCACTATAGGGGAGATGCTGTATGTGGACACGGAT

Reverse primer- TTAATACGACTCACTATAGGGGAGATCCGTGGATAACCTTAACGA

NOTE: EGFP dsRNA is used as negative control.

2. Gel-purify the polymerase chain reaction (PCR) products using a commercial gel purification kit according to manufacturer's protocol.
3. Perform *in vitro* transcription using a commercial product capable of transcribing from a T7 promoter according to manufacturer's protocol.
4. Purify the dsRNA using an RNA purification kit according to manufacturer's protocol and store at -80 °C.
5. **Assess the efficiency of *shams* knockdown by using the following steps (1.5.1-1.5.5).**
 1. Count the cells manually using a hemocytometer and plate 5×10^5 S2 cells in each well of a 6 well plate in 1 mL/well of Schneider's medium supplemented with 10% Fetal Bovine Serum (FBS) and 100 U/mL Penicillin-Streptomycin.
 2. Add 7.5 µg of EGFP- or *shams* dsRNA to each well and incubate at 25 °C for 24 h.
 3. Harvest the control (EGFP dsRNA-treated) and *shams* dsRNA-treated S2 cells by gentle pipetting followed by pelleting down by centrifugation at 1,000 x g for 5 min at 25 °C and process for RNA isolation using an RNA extraction kit according to manufacturer's protocol.
 4. Quantify the RNA using a spectrophotometer and process 100 ng of RNA for 1-step qRT-PCR using commercial qPCR reagents and primer/probes and the following PCR thermal profile: Denaturation (95 °C, 15 s) and Annealing/Extension (60 °C, 1 min).
NOTE: Please see the **Table of Materials** for information on the primer/probe sets and the instrument used for *shams* and control qRT-PCR experiments in these studies.
 5. Calculate relative *shams* mRNA levels using the $2^{-\Delta\Delta CT}$ method¹⁶.

2. Assessment of the Binding Between the Notch Receptor and *Trans*-ligands

1. Prepare signal-receiving cells (S2 cells expressing the Notch receptor).

1. Count the cells manually using a hemocytometer and plate 5×10^5 S2 and stable S2-Notch cells in each well of a 6-well plate in 1 mL/well of Schneider's medium supplemented with 10% FBS and 100 U/mL Penicillin-Streptomycin.
NOTE: For S2-Notch cells, which are available from *Drosophila* Genomics Resource Center (DGRC), add 200 nM methotrexate in media. To prepare a 0.5 mM methotrexate stock solution, first prepare a 20 mM methotrexate solution in 250 µL of 1 M NaOH. Next, dilute this solution to 0.5 mM in 1 M phosphate buffer saline and store at -20 °C. In S2-Notch cells, expression of the Notch protein is under the control of the *Drosophila* metallothionein promoter in the *pMT* vector.
2. Add 7.5 µg of dsRNA to each well and incubate at 25 °C for 24 h.
3. Add 0.7 mM CuSO₄ to induce the expression of Notch and incubate at 25 °C for 3 days.
NOTE: CuSO₄ is used to induce expression of proteins controlled by the metallothionein promoter.

2. Prepare signal-sending cells (S2 cell expressing Delta or Serrate ligands).

1. Count the cells manually using a hemocytometer and plate $\sim 5 \times 10^6$ stable S2-Delta or S2-Serrate^{Tom} cells in each well of a 6-well plate in 1 mL/well of Schneider's medium supplemented with 10% FBS and 100 U/mL Penicillin-Streptomycin.
NOTE: Add 200 nM methotrexate for S2-Delta cells and 100 µg/mL hygromycin for S2-Serrate^{Tom} cells in media. Expression of Delta and Serrate^{Tom}—a Tomato-tagged, functional version of Serrate¹²—is under *Drosophila* metallothionein promoter in the *pMT* vector.
2. Add 0.7 mM CuSO₄ to induce the expression of ligands and incubate at 25 °C for 3 h.

3. Perform aggregation between signal-sending and signal-receiving cells.

1. Harvest the dsRNA-treated S2 (control) and S2-Notch cells by gentle pipetting and plate 2.5×10^5 cells/well in a 24-well plate after manual counting by hemocytometer.

2. Add 5×10^5 stable S2-Delta or S2-Serrate^{Tom} cells in a total volume of 200 μ L of Schneider's medium (supplemented with 10% FBS and 100 U/mL Penicillin-Streptomycin).
NOTE: All S2 cells handling (including plating, induction and dsRNA treatment) was done under a biosafety cabinet.
 3. Place the plate on an orbital shaker at 150 rpm (942.48 rad/min).
 4. After 1 min, mix the contents of each well and take 20 μ L out for counting the number of aggregates.
 1. Simultaneously take a representative image under inverted compound microscope using 10x magnification (PLL 10/0.25 objective).
 2. Manually count the aggregates (>6 cells) using a hemocytometer.
 5. Place the plate on a shaker.
 6. Repeat the image acquisition and counting after 5 min and 15 min of aggregation.
4. **Perform quantification of *trans*-binding.**
1. Calculate the number of aggregates per mL between S2 cells and S2-Delta or S2-Serrate^{Tom} cells as a background control.
 2. Calculate the number of aggregates per mL between S2-Notch and S2-Delta or S2-Serrate^{Tom} cells (magnitude of *trans*-binding).

3. Evaluation of Inhibition of Binding Between Notch and *Trans*-ligands by *Cis*-ligands

1. **Prepare signal-receiving cells.**
 1. Plate 5×10^5 S2 cells in each well of a 6-well plate in 1 mL/well of Schneider's medium supplemented with 10% FBS and 100 U/mL Penicillin-Streptomycin.
 2. Add 7.5 μ g of dsRNA to each well and incubate at 25 °C for 24 h.
 3. Co-transfect the dsRNA-treated cells with *pBluescript* and *pMT-Notch*¹¹ (DGRC) alone or with *pMT-Notch* and *pMT-Delta*¹¹ (DGRC) or *pMT-Serrate*¹⁷ for a total DNA concentration of 2 μ g/well using a commercial transfection reagent according to manufacturer's protocol.
NOTE: *pBluescript* is used as a control. The co-transfected cells will be called S2-Notch&Delta^{transient} or S2-Notch&Serrate^{transient} cells hereafter.
 4. Incubate the transiently-transfected S2 cells at 25 °C for 24 h.
 5. Add 0.7 mM CuSO₄ to induce the expression of Notch and the ligands and incubate at 25 °C for 3 days.
2. Prepare signal-sending cells as described in 2.2.
3. Perform aggregation between signal-sending and signal-receiving cells as described in section 2.3.
4. **Quantify inhibition of *trans*-binding by *cis*-ligands.**
 1. Calculate the number of aggregates per mL between S2 cells and S2-Delta or S2-Serrate cells as a background control.
 2. Quantify the magnitude of inhibition by *cis*-ligands as follows.
 1. Define A as Number of aggregates between S2-Notch^{transient} and S2-Delta cells.
 2. Define B as Number of aggregates between S2 cells transiently co-transfected with Notch and Delta (S2-Notch&Delta^{transient}) and S2-Delta cells.
 3. Calculate relative aggregation $C = (B \times 100)/A$
 4. Calculate magnitude of inhibition by *cis*-ligand (Delta or Serrate) as $100 - C$.
NOTE: As an example, if relative aggregation is 45%, then *cis*-ligands are considered to inhibit the *trans*-binding by 55%.

Representative Results

Our *in vivo* observations suggested that loss of the xylosyltransferase gene *shams* results in gain of Notch signaling due to increased Delta-mediated *trans*-activation of Notch without affecting the *cis*-inhibition of Notch by ligands⁸. To test this notion, *in vitro* cell aggregation assays were performed. First, *shams* expression in S2 cells was knocked down using *shams* dsRNA. *EGFP* dsRNA was used as control. Efficiency of knockdown (KD) was examined by real-time PCR and was shown to be greater than 80%⁸. Then, the binding of Notch receptor with *trans*-Delta upon *shams* KD was examined using aggregation assays. The aggregation between S2-Notch and S2-Delta cells was examined at various time points after mixing them. Aggregation between plain S2 cells and S2-Delta cells was taken as background control. **Figure 3** shows the graph indicating the number of aggregates formed at different time points (1, 5, 15 and 30 min) after mixing. A sharp increase was observed in the number of aggregates between 1 and 5 min. Afterwards, the number of aggregates continued to increase until 30 min at a slower rate. Therefore, aggregate formation was imaged at 1, 5 and 15 min and the number of aggregates was quantified after 5 min of mixing.

Figure 4A shows representative images of cell aggregation assays between stable S2-Notch and S2-Delta cells to assess the effects of *shams* KD on the binding between Notch receptor and *trans*-Delta. Images show the aggregation at three-time points (1, 5 and 15 min). For background control, plain S2 cells (treated with *shams* dsRNA) were mixed with S2-Delta cells. *EGFP* dsRNA-treated S2-Notch cells formed aggregates with S2-Delta cells, which increase in number with time. In contrast, *shams* dsRNA-treated S2-Notch cells formed aggregates faster, and the aggregates were bigger and greater in number than the control ones (**Figure 4B**). This indicated that decreasing Shams levels enhances the *trans*-binding of Notch with Delta⁹.

To examine whether Shams regulates the inhibitory effect of *cis*-ligands on binding between Notch and *trans*-Delta, we first established that co-expression of Notch and Delta in the signal-receiving cell can decrease the cell aggregation mediated by Notch and *trans*-Delta. **Figure 5A** shows the graph indicating the number of aggregates formed between S2-Delta and S2-Notch&Delta^{transient} cells with different amounts of *cis*-Delta. Transfecting equal amounts of Notch- and Delta-expression constructs into S2 cells dramatically decreases the number of aggregates formed between these cells and S2-Delta cells. Moreover, decreasing the amount of *cis*-Delta results in an increase in the number of aggregates, indicating that in the range of Notch/*cis*-Delta ratios used in these assays, *cis*-Delta can inhibit the binding between Notch and *trans*-Delta in a dosage-dependent manner. Since the S2-Notch&Delta^{transient} cells express both Notch and Delta, they might form homotypic

aggregates independently of the S2-Delta cells. To examine if the formation of homotypic aggregates among S2-Notch&Delta^{transient} cells can be a confounding factor in the *cis*-inhibition assays shown in **Figure 5A**, we mixed these cells with S2 cells and quantified the aggregates. **Figure 5B** shows the number of aggregates formed after mixing S2 cells with S2-Notch&Delta^{transient} cells expressing various Notch/*cis*-Delta ratios. A rather small number of homotypic aggregates compared to heterotypic aggregates as shown in **Figure 5A** was observed. We conclude that these aggregation assays faithfully measure the effect of *cis*-Delta on binding between Notch and *trans*-Delta.

We next examined the effect of *shams* KD on the inhibitory ability of *cis*-Delta. To this end, aggregation between S2-Delta and S2-Notch&Delta^{transient} cells was examined. **Figure 6A** shows representative images of aggregation between S2-Delta cells and S2-Notch^{transient} (control) or S2-Notch&Delta^{transient} cells. S2-Delta and *EGFP* dsRNA-treated S2-Notch^{transient} cells formed aggregates, which increase in number with time, similar to the aggregation between S2-Delta and stable S2-Notch cells (**Figure 4**). Notably, mixing S2-Delta and S2-Notch&Delta^{transient} cells co-transfected with equal amounts of Notch- and Delta-expression plasmids formed less aggregates upon both *EGFP* KD and *shams* KD, suggesting that decreasing the level of Shams does not affect the ability of *cis*-Delta to block Notch/*trans*-Delta binding. To better assess the effects of *shams* KD on the activity of *cis*-Delta, the magnitude of inhibition by *cis*-Delta was measured by calculating the relative aggregation percentage over a range of Notch/*cis*-Delta ratios as explained in Section 3.4.2. Quantification of relative aggregation showed a comparable inhibition of aggregation upon *EGFP* and *shams* dsRNA treatment (**Figure 6B**). Together, these observations strongly suggest that loss of xylosylation promotes *trans*-binding without affecting *cis*-inhibition⁸, and provide a molecular mechanism for the wing vein loss phenotype observed in *shams* mutants.

Figure 1: Schematic Representation of Cell Aggregation Assay for *Trans*-binding. Diagram showing induction of receptor (S2-Notch) and ligand (S2-Delta or S2-Serrate) expressing S2 cells by 0.7 mM CuSO₄. Induction of both S2 cells is followed by mixing, which tends to make aggregates. These aggregates (>6 cells) are imaged and quantified. [Please click here to view a larger version of this figure.](#)

Figure 2: Schematic Representation of Cell Aggregation Assay to Assess *Cis*-inhibition. S2 cells were transiently transfected with *pMT-Notch* (S2-N^{transient}), *pMT-Notch* and *pMT-Delta* (S2-Notch&Delta^{transient}), or *pMT-Notch* and *pMT-Serrate* (S2-Notch&Serrate^{transient}). After induction by 0.7 mM CuSO₄ and mixing with S2-Delta cells, aggregates (>6 cells) are quantified. Inhibition by *cis*-ligands is measured in terms of relative aggregation percentage in the presence and absence of each *cis*-ligand. The effects of *cis*-ligands on binding between Notch and *trans*-Serrate can be determined by mixing the transiently-transfected cells with S2-Serrate^{Tom} cells (not shown). [Please click here to view a larger version of this figure.](#)

Figure 3: Aggregation between S2-Notch and S2 or S2-Delta Cells at Different Time Points. Graph shows the number of aggregates per mL at different time points (1, 5, 15 and 30 min) between the indicated types of cells. Error bars indicate standard error of the mean (SEM) of three replicates. [Please click here to view a larger version of this figure.](#)

Figure 4: Cell Aggregation Mediated by Notch and *trans*-Delta. (A) Images show aggregation at different time points (1, 5 and 15 min). Aggregation between *shams* dsRNA-treated plain S2 cells and S2-Delta cells was taken as background control. *Shams*-dsRNA treated S2-Notch cells show an increase in the number and size of aggregates compared to *EGFP* dsRNA-treated (control) S2-Notch cells after mixing with S2-Delta cells. Scale bar = 100 μ m. (B) Quantification of number of cell aggregates greater than 6 cells after 5 min of aggregation. Error bars indicate SEM. * $P < 0.01$ (One-way Analysis of Variance (ANOVA)). Three replicates with three independent repeats were performed. [Please click here to view a larger version of this figure.](#)

Figure 5: *Cis*-Delta Inhibits the Cell Aggregate Formation Mediated by Notch and *trans*-Delta in a Dosage-dependent Manner. (A) Graph shows the aggregation (in terms of number of aggregates/mL) between S2-Delta cells and S2 cells transiently transfected with different ratios of expression vectors for Notch and *cis*-Delta (1:0, 1:1, 1:0.5, 1:0.2 and 1:0.1). Error bars indicate SEM. * $P < 0.01$ (One-way ANOVA). Three replicates with three independent repeats were performed. Note that decreasing the level of *cis*-Delta results in an increase in aggregation, likely due to reduced *cis*-inhibition. (B) Graph showing homotypic aggregation (in terms of number of aggregates per mL) in S2 cells transiently transfected with different ratios of Notch and Delta (1:0, 1:1, 1:0.5, 1:0.2 and 1:0.1) and mixed with plain S2 cells. Error bars indicate SEM. Number of aggregates is much smaller than the number of heterotypic aggregates formed between S2-Delta and S2-Notch&Delta^{transient} cells. [Please click here to view a larger version of this figure.](#)

Figure 6: Knockdown of *shams* does not Affect the Inhibitory Activity of *cis*-Delta. (A) Shown are representative images of the effect of *shams* KD on *cis*-Delta-mediated inhibition of cell aggregation mediated by Notch/*trans*-Delta binding. Images show aggregation at different time points (1, 5 and 15 min). Aggregation between *shams* dsRNA-treated plain S2 cells and S2-Delta cells was taken as background control. Similar to stably transfected S2-Notch cells, *shams* KD increased the number of aggregates between S2-Delta and S2-Notch^{transient} cells as compared to control (EGFP dsRNA-treated). Co-expression of *cis*-Delta with Notch in a 1:1 ratio resulted in a comparable decrease in the number of aggregates in control (EGFP dsRNA-treated) as well as in *shams* dsRNA treated cells. Scale bar = 100 μm. (B) Graph shows relative aggregation between S2-Delta cells and S2 cells transiently co-transfected with indicated ratios (1:0, 1:1, 1:0.5, 1:0.2 and 1:0.1) of Notch and *cis*-Delta. EGFP and *shams* dsRNA treatment show a comparable decrease in aggregation at each Notch/*cis*-Delta ratio. Data presented here are representative of three independent experiments. [Please click here to view a larger version of this figure.](#)

Discussion

Canonical Notch signaling depends on the interactions between the Notch receptor and its ligands⁵. Although most studies on the Notch pathway primarily consider the binding of Notch and ligands in neighboring cells (*trans*), Notch and same-cell ligands do interact, and these so-called *cis*-interactions can play an inhibitory role in Notch signaling^{3,4}. Accordingly, to decipher the mechanisms underlying the effects of a modifier on canonical notch signaling, it is often pertinent to examine both types of Notch-ligand interactions (*trans* and *cis*). However, in many contexts during animal development, involvement of both of these interactions and their feed-back regulation complicates the *in vivo* studies^{1,6}. In the present protocol, we provide a detailed methodology for *in vitro* cell aggregation assays using *Drosophila* S2 cells to evaluate the binding of Notch receptor with *trans*-ligands and its inhibition by *cis*-ligands. We have presented the utilization of this methodology to assess the effect of a genetic modifier of Notch signaling (the xylosyltransferase Shams^{7,8}) on receptor-ligand binding.

First, aggregation assays were performed between stable S2-Delta and EGFP or *shams* dsRNA-treated stable S2-Notch cells to examine the effect of *shams* KD on *trans*-binding. Aggregation of S2-Delta cells with *shams* dsRNA-treated plain S2 cells was taken as background control. *Shams* KD enhanced the aggregation between S2-Notch and S2-Delta cells compared to control. It is important to perform background

experimental control aggregations between plain S2 cells and ligand-carrying S2 cells in parallel to the *trans*-binding experiments. Although *Drosophila* S2 cells do not express endogenous Notch receptor and Delta ligand¹¹, expression of low levels of Serrate is reported in S2 cells¹³. Considering this, plain S2 cells were used in cell aggregation assays as background control; these cells did not show any aggregation (Figure 5B). Aggregates are counted manually using a hemocytometer. To ensure consistency in aggregate quantification, 10–20 μ L of mixed cell population can be pipetted out from at least three different areas per well for counting. These assays require healthy growing S2 cells. Considering the semi-adherent nature of S2 cells, gentle pipetting while collecting the cells and constant shaking during the aggregation assay are important.

Previously, we and others have used well-established, soluble ligand-receptor binding assays for a quantitative assessment of receptor-ligand binding in *trans*^{17–20}. Since aggregation assays are semi-quantitative, soluble ligand binding assays were also performed to examine the effect of *shams* KD on Notch-ligand *trans*-binding. The observed results were similar in trend with those obtained from aggregation assays⁸. This attested the faithful evaluation of binding of Notch with *trans*-ligands using cell aggregation assays. Although the aggregate formation is a readout of receptor-ligand binding, it can be intricate if the surface expression of the Notch receptor or its ligands is affected by the given modifiers. This highlights the limitation of the cell aggregation assays, as it cannot be distinguished if the change in aggregate formation is because of altered receptor-ligand binding or altered surface expression. Therefore, parallel to aggregation assays, the effects of each modifier on surface expression of Notch and ligands needs to be tested in S2 cells. In the context of our studies, surface levels of Notch and Delta were not affected in *shams* dsRNA-treated S2 cells⁸.

To examine the inhibition of *trans*-binding by addition of *cis*-ligands, aggregation assays were performed between S2-Delta cells and *EGFP* or *shams* dsRNA-treated S2-Notch&Delta^{transient} cells. The number of aggregates formed between these cells was compared with the number of aggregates formed between S2-Delta cells and S2-Notch^{transient} cells. The relative decrease in the number of aggregates was calculated and used as an indicator for the magnitude of inhibition by *cis*-Delta. The control experiments showed that *cis*-ligands show a robust and dosage-dependent inhibition of Notch/*trans*-Delta binding over a rather wide range of Notch to *cis*-ligand ratios (1:1 to 1:0.1) (Figure 5A)⁸. A 1:1 ratio resulted in the strongest degree of *cis*-inhibition, and decreasing the relative level of *cis*-Delta weakened *cis*-inhibition. Accordingly, the KD experiments over this range of ratios was performed. Of note, the amount of Notch receptor and the total amount of transfected DNA was kept constant in all experiments. This will ensure that aggregate formation is primarily affected by the activity of *cis*-ligands and not by altered levels of Notch expression by the cells.

It was previously shown that co-expression of Notch and *cis*-ligands in S2 cells can result in the formation of homotypic aggregations¹⁴. To examine whether similar homotypic aggregations among S2-Notch&Delta^{transient} or S2-Notch&Serrate^{transient} cells can compromise our interpretations of the aggregate quantifications in *cis*-ligand studies, a control aggregation assay was performed between S2-Notch&Delta^{transient} and plain S2 cells. The number of S2 cells used in these experiments was the same as S2-Delta cells used in *cis*-Delta experiments. Some homotypic aggregation was observed, but quantification showed that the resultant aggregates contribute a low fraction of the total aggregates compared with the heterotypic aggregates between S2-Notch&Delta^{transient} and S2-Delta cells (Figure 5B). We conclude that the changes observed in the number of aggregates in *cis*-ligand assays are primarily due to the inhibitory effect of *cis*-ligands on binding between Notch and *trans*-Delta.

In summary, the aggregation assays presented in this protocol provide an easy and straightforward methodology for semi-quantitative assessment of the *trans*- and *cis*-interactions of the Notch receptor and its ligands. These assays can be used to examine the effects of genetic or pharmacological Notch pathway modifiers on Notch-ligand binding *in vitro* and therefore can help elucidate the mechanisms underlying the *in vivo* effects of these modifiers on Notch signaling.

Disclosures

The authors have no conflict of interest.

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