Chapter 10

Assessing the Roles of Potential Notch Signaling Components in Instructive and Permissive Pathways with Two *Drosophila* Pericardial Reporters

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Abstract

The highly conserved Notch signaling pathway brings about the transcriptional activation of target genes via either instructive or permissive mechanisms that depend on the identity of the specific target gene. As additional components of the Notch signaling pathway are identified, assessing whether each of these components are utilized exclusively by one of these mechanisms (and if so, which), or by both, becomes increasingly important. Using RNA interference-mediated knockdowns of the Notch component to be tested, reporters for two Notch-activated pericardial genes in *Drosophila melanogaster*, immunohistochemistry, and fluorescence microscopy, we describe a method to determine the type of signaling mechanism—instructive, permissive, or both—to which a particular Notch pathway component contributes.

Key words Notch signaling pathway, Notch permissive signaling mechanism, Notch instructive signaling mechanism, Gene regulation, Transcriptional regulation, Cardiac cell subtype-specific gene expression, *Drosophila* embryonic heart, Heart development, Enhancer–reporter constructs

1 Introduction

Cell–cell signaling via the Notch receptor is a highly conserved signaling pathway that regulates numerous metazoan developmental processes such as cell proliferation, differentiation, and apoptosis [1–6]. Notch signaling, initiated by ligand binding to Notch receptors, results in a proteolytic cleavage that releases the Notch intracellular domain (NICD) from the cell membrane. NICD then enters the nucleus and binds with the transcription factor CSL to activate the transcription of relevant target genes [2–6]. In the absence of Notch signaling, CSL associates with corepressors to form a repressor complex that binds dynamically and rapidly on and off the enhancers of target genes to prevent their transcription [4, 6–12]. During Notch signaling, the interaction of NICD with

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CSL forms an activator complex that competes dynamically with the CSL repressor complex for CSL binding motifs on the enhancers. NICD enhances both the recruitment of the NICD-CSL activator complex and its dwell time at the enhancer, thereby favoring its presence over the CSL repressor complex [12]. This allows for the transcription of target genes to occur by one of two mechanisms: (1) a Notch-permissive process in which alleviating repression through the competitive displacement of the CSL repressor complex is sufficient to initiate transcription due to the presence of other local activators that are then free to exert their positive effects, or (2) a Notch-instructive process, which necessitates the activating effect of the NICD-CSL activator complex that allows transcription initiation to occur [4, 6, 10–12]. Thus, as additional components of the Notch signaling pathway are identified, the assessment of whether these components are utilized exclusively by the Notch-permissive or the Notch-instructive mechanism, or by both, becomes important in describing Notch activity.

In the fruit fly *Drosophila melanogaster*, Notch signaling brings about cell type–specific gene expression in different cardiac cell types [13–20]. The *Drosophila* heart consists of two groups of cells: two paired inner rows of cardial cells (CCs) surrounded by pericardial cells (PCs) (Fig. 1). The CC nuclei express *Myocyte enhancer factor 2* (*Mef2*) while the PC nuclei express both *Zn finger homeodomain 1* (*zfh1*) and *Holes in muscle* (*Him*) [13, 20–24]. A subset of PCs, the Eve-PCs, also express *even-skipped* (*eve*) in addition to *zfh1* [25, 26]. The Delta ligand expressed by the CCs binds to the Notch receptor in the adjacent PCs (i.e., all PCs other than the Eve-PCs, henceforth referred to as core-PCs) to activate transcription of *Him* and *zfh1* in the latter, *Him* in a permissive manner by displacing the CSL repressor complex with the NICD-CSL complex on the *Him* enhancer (Fig. 2a), and *zfh1* in an instructive manner that requires the inductive activating effect of the NICD-CSL activator complex on the *zfh1* enhancer (Fig. 2d) [13, 14].

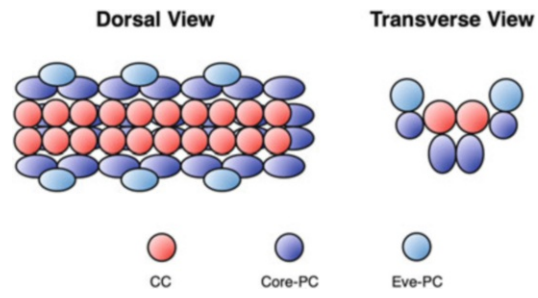

Fig. 1 Relative positions of the distinct cardiac cell nuclei in the *Drosophila* heart. The cardial cells (CCs) express *Mef2*, the core pericardial cells (Core-PCs) express both *zfh1* and *Him*, and the Eve pericardial cells (Eve-PCs) express *zfh1*, *Him*, and *eve*

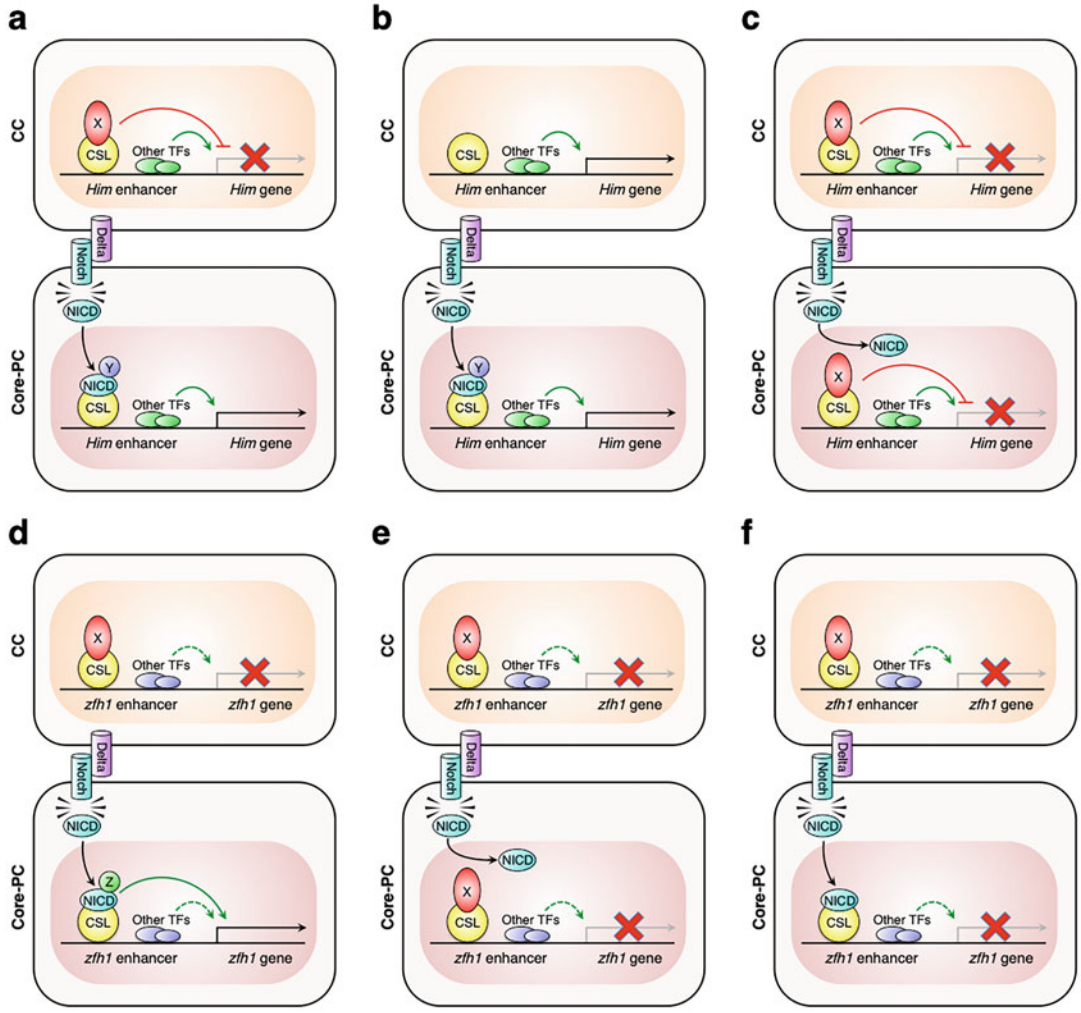

Fig. 2 Schematic of the Notch signaling pathway driving *Him* and *zfh1* expression in the *Drosophila* heart and the effect of knocking down components of the pathway. (**a**) *Him* is repressed in CCs by binding of the CSL repressor complex to its enhancer. In PCs, the competitive displacement of the CSL repressor complex by the NICD-CSL complex alleviates this repression and allows *Him* to be transcribed. (**b**) If the pathway component (X in the figure) being assayed is required for repression by the CSL repressor complex, for example, a corepressor, then its knockdown will result in *Him* being also ectopically expressed in CCs. (**c**) If, instead, the pathway component (Y in the figure) is required for the NICD-CSL complex to competitively displace the CSL repressor complex, for example, by stabilizing the binding of NICD to CSL, then its knockdown will result in *Him* expression being eliminated in core-PCs. (**d**) *zfh1* expression in core-PCs is achieved only through the instructive effect of the NICD-CSL activator complex binding to the *zfh1* enhancer. (**e**) Thus, knockdown of a component (Z in the figure) required to bring about this transcription by stabilizing the NICD-CSL activator complex would result in transcription being abrogated in core-PCs. (**f**) Alternatively, Z could be a component recruited or used by the NICD-CSL activator complex, such as a coactivator or member of a histone acetyltransferase complex, to promote transcription. In this case too, knockdown of Z would result in the elimination of *zfh1* expression in core-PCs

We have taken advantage of the distinct Notch signaling mechanisms for *Him* and *zfh1* to devise a simple RNA interference (RNAi) knockdown-based assay to examine whether newly discovered Notch components are utilized exclusively by the Notch-permissive or the Notch-instructive mechanism, or by both. Briefly, we have created transgenic fly lines with enhancer–reporter constructs for *Him* (*Him*^{WT}*-lacZ*) and *zfh1* (*zfh1*^{WT}*-lacZ*) that express the β-galactosidase reporter exclusively in PCs in embryos, that is, in the same expression pattern as the endogenous genes [13, 14]. The Notch signaling pathway component to be tested will be knocked down specifically in the cardiac mesoderm (the precursor of the heart) using the GAL4-UAS system by the *TinD-GAL4* driver which we have also included in these previously described fly lines [27, 28]. If the component functions in the permissive Notch signaling pathway solely by repressing the transcription of target genes in the absence of NICD, then its knockdown will result in the *Him*^{WT}*-lacZ* reporter being ectopically expressed in the CCs (Fig. 2b). If, instead, the component acts in the permissive Notch signaling pathway in concert with the NICD-CSL complex to bring about transcription of the target genes solely by displacing the CSL repressor complex, then its knockdown will result in the *Him*^{WT}*-lacZ* reporter expression disappearing in the core-PCs (Fig. 2c). Finally, if the component is required to activate transcription of target genes in the instructive Notch signaling pathway through the inductive effect of the NICD-CSL activator complex, then its knockdown will lead to the disappearance of *zfh1*^{WT}*-lacZ* reporter expression in the core-PCs (Fig. 2e, f). Additionally, knockdown of components that result in both *Him*^{WT}*-lacZ* reporter and *zfh1*^{WT}*-lacZ* reporter expression disappearing in core-PCs would indicate that those components are used in both the Notch permissive and Notch instructive pathways. Thus, examining the effect of the knockdown of individual Notch signaling pathway components on the cardiac expression of these reporters can serve to identify the specific category of Notch signaling mechanisms that they are involved in (summarized in Table 1).

The protocol for this assay involves acquiring the relevant fly lines (transgenic lines with the enhancer–reporter constructs and the *TinD-GAL4* driver from us as well as controls and transgenic lines with UAS-RNAi constructs for the pathway components to be tested), crossing these lines to obtain embryos of the desired genotype, dechorionating, fixing, and immunostaining these embryos with relevant primary and secondary antibodies, and then examining the embryos using fluorescent microscopy.

Table 1
Identification of the specific Notch signaling mechanism, permissive or instructive, a Notch signaling pathway component is involved in, based on the effect of its knockdown on the expression of *Him* and *zfh1* pericardial reporters

Effect of component knockdown	Interpretation
Ectopic expression of β-galactosidase driven by the *Him* enhancer in CCs	Component functions in the Notch-permissive signaling pathway to repress transcription of target genes in the absence of NICD
Elimination of *Him* enhancer-driven β-galactosidase expression in the core-PCs	Component functions in the Notch-permissive signaling pathway to enable the NICD-CSL complex to competitively displace the CSL repressor complex at the target gene enhancer
Elimination of *zfh1* enhancer-driven β-galactosidase expression in the core-PCs	Component functions in the Notch-instructive signaling pathway to activate transcription of target genes through the inductive effect of the NICD-CSL activator complex

2 Materials

Use distilled and deionized water.

2.1 Drosophila *Lines*

1. *Him^{WT}-lacZ; TinD-GAL4 UAS-Dcr-2*: This fly line can be obtained from the authors (*see* **Note 1**). We also intend to submit this line to the Bloomington *Drosophila* Stock Center (https://bdsc.indiana.edu) for ease of access and dissemination.
2. *zfh1^{WT}-lacZ; TinD-GAL4 UAS-Dcr-2*: This fly line can also be obtained from us, and we intend to submit this line too to the Bloomington *Drosophila* Stock Center (*see* **Note 1**).
3. Canton-S or Oregon-R flies: These wild-type flies can be obtained from any *Drosophila* stock center or almost any research laboratory using *Drosophila*.
4. Transgenic fly lines containing UAS-RNAi constructs for Notch signaling pathway components to be tested. Appropriate RNAi lines can be obtained from the Bloomington *Drosophila* Stock Center, the Vienna *Drosophila* Resource Center (https://stockcenter.vdrc.at), the National Institute of Genetics—Japan (https://www.nig.ac.jp/nig), and individual investigators (*see* **Note 2**).

2.2 Fly Crosses, Egg Laying, and Embryo Collection

1. Basic apparatus for rearing, anesthetizing, and sexing flies: Plastic or polypropylene bottles with fly food for culturing *Drosophila*, an incubator capable of holding these bottles and adjustable to temperatures between 25 °C and 29 °C, apparatus for anesthetizing flies, and dissecting microscopes for sexing flies.

2. Small embryo collection cages: These can be purchased from commercial vendors or made in the laboratory (*see* **Note 3**).
3. Embryo collection plates: Heat 1 L of water until lukewarm in a pan or beaker on a stirring hotplate and gradually add in and stir 32 g of bacteriological agar such that it does not form clumps. Next, add in 189 g of molasses and heat the stirring mixture until it comes to a boil. Turn off the heat once it is boiling. Once the stirring mixture cools down to 60 °C, add 15 mL of tegosept solution (18 g of methyl 4-hydroxybenzoate in 750 mL of reagent alcohol), stir, and pour into 60 mm × 15 mm petri dishes until the bottom of the dishes are completely covered. Let the agar cool down until solidified, cover the petri dishes, and store at 4 °C.
4. Yeast paste: Mix dry yeast to a small quantity of water (approximately 1 g of yeast to 1.3 mL of water) until it has the consistency of cream cheese. Store at 4 °C.
5. Egg baskets: 100 μm cell strainers designed to fit 50 mL conical tubes.
6. Small fine-haired paint brushes.
7. Squirt bottle containing water.

2.3 Dechorionation

1. Petri dishes (60 mm × 15 mm).
2. 50% Bleach (Clorox) in water.
3. Squirt bottle containing water.
4. Squirt bottle containing 0.1% Triton X-100 in water.

2.4 Embryo Fixation

1. Scintillation vials.
2. Pasteur pipettes.
3. Small fine-haired paint brushes.
4. 5× PEM solution: 0.5 M PIPES, 10 mM $MgSO_4$, 5 mM EGTA. Adjust pH to 6.9 with KOH.
5. 16% EM grade formaldehyde.
6. Heptane.
7. Methanol.
8. Platform shaker.
9. 1.5 mL Microcentrifuge tubes.

2.5 Antibody Staining

1. 10× phosphate-buffered saline (PBS) stock solution: 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4 (*see* **Note 4**).
2. PBST solution: 1× PBS, 0.2% Tween 20. Make by diluting appropriate volumes of 10× PBS stock solution and 10% Tween 20 in water.

3. Normal goat serum (NGS).
4. Rabbit anti-Mef2 antibody (*see* **Note 5**).
5. Guinea pig anti-Zfh1 antibody (*see* **Note 6**).
6. Mouse monoclonal anti-β-galactosidase antibody (*see* **Note 7**).
7. Alexa Fluor 568 goat anti-rabbit IgG (H + L) cross-adsorbed secondary antibody (*see* **Note 8**).
8. Alexa Fluor 647 goat anti-guinea pig IgG (H + L) highly cross-adsorbed secondary antibody (*see* **Note 8**).
9. Alexa Fluor 488 goat anti-mouse IgG (H + L) cross-adsorbed secondary antibody (*see* **Note 8**).
10. Vectashield antifade mounting medium: available from Vector Laboratories.

2.6 Microscopy

1. Fluorescence microscope with filter sets capable of imaging Alexa Fluor 488, 568, and 647 antibodies: confocal microscopes or microscopes with apotomes capable of taking Z-stacks are preferred.
2. Secure Seal Imaging Spacers (0.12 mm depth, 20 mm diameter).
3. Slides and coverslips.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Fly Crosses and Egg Laying

1. Before setting up an embryo collection cage, put a small dollop of yeast paste on the molasses-agar surface at the center of each embryo collection plate (Fig. 3a).

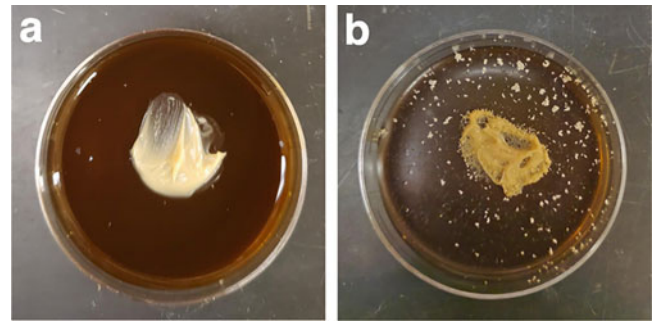

Fig. 3 Embryo collection plates before and after egg laying. (**a**) Plate immediately prior to being used in a cage. Note the small dollop of yeast paste at the center of the plate. (**b**) Plate after a 3 h egg laying period in the cage

2. An individual cage is set up by placing anesthetized flies of the appropriate genotypes (about 200 virgin females and 100 recently eclosed males) in the cage, and sealing the cage opening with the yeast paste containing embryo collection plate. Invert the cage such that the embryo collection plate is on the bottom only after all the anesthetized flies have regained consciousness and have begun moving. For each fly line containing a UAS-RNAi construct for a candidate Notch signaling pathway component to be assessed, set up the four following simultaneous fly crosses in separate embryo cages in a 29 °C incubator (*see* **Notes 9–11**):
   (a) *Him*^{WT}*-lacZ; TinD-GAL4 UAS-Dcr-2* virgin females × wild-type males. This cross will produce embryos that allow the normal *Him* enhancer-driven transcription to be assessed as a baseline/control for the assay.
   (b) *Him*^{WT}*-lacZ; TinD-GAL4 UAS-Dcr-2* virgin females × males bearing the UAS-RNAi construct for the Notch pathway component to be tested. This cross will produce embryos that allow the effect of the RNAi-mediated knockdown on Notch-permissive *Him* enhancer-driven transcription to be assessed.
   (c) *zfh1*^{WT}*-lacZ; TinD-GAL4 UAS-Dcr-2* virgin females × wild-type males. This cross will produce embryos that allow the normal *zfh1* enhancer-driven transcription to be assessed as a baseline/control for the assay.
   (d) *zfh1*^{WT}*-lacZ; TinD-GAL4 UAS-Dcr-2* virgin females × males bearing the UAS-RNAi construct for the Notch pathway component to be tested. This cross will produce embryos that allow the effect of the RNAi-mediated knockdown on Notch-instructive *zfh1* enhancer-driven transcription to be assessed.
3. Change the embryo collection plate on each cage with a new plate (that has a fresh dab of yeast paste) every 8 h on the first day (Day 1). With a little practice, this is achieved by inverting the cage, tapping it on the bench to make the flies fall to the bottom, quickly replacing the old embryo collection plate with a new one before any flies can escape, and inverting it again such that the plate is on the bottom. Discard the old embryo collection plates.
4. From Day 2 onward, use the following procedure: first change the embryo collection plate, and discard the previous plate. After 3 h, change the plate again, but this time cover the removed plate and keep it at 29 °C in the incubator. This plate should now have many visible *Drosophila* eggs (white specks) laid on it during that 3 h period (Fig. 3b). After 12.5 h at 29 °C (which allows all the embryos to age to stage 16, a point at which the effect of the Notch pathway

component on the reporter expression in the heart can be conveniently assessed), begin the dechorionation and fixing process described in the following sections (*see* **Note 12**).

3.2 Fix Solution

1. While the embryos are maturing during the 12.5 h period in the incubator, prepare the fix solution by adding the following in the order they are listed to each scintillation vial.
   (a) 1.375 mL water.
   (b) 0.5 mL 5× PEM.
   (c) 0.625 mL 16% EM grade formaldehyde.
   (d) 2.5 mL heptane.

   Four (4) scintillation vials with fix solution are required for each fly line containing a UAS-RNAi construct for the candidate Notch signaling pathway component being assessed.
2. After adding the heptane, close the lids tightly and shake the vials vigorously by hand to saturate the heptane with formaldehyde.

3.3 Embryo Collection and Dechorionation

1. After 12.5 hours in the incubator, recover the embryo collection plates. The small white specks scattered on the surface of the plate are embryo-containing eggs. Add enough water to a plate to cover the eggs, and then use a paint brush to dissolve the remaining yeast paste (if any) and gently detach the eggs from the molasses-agar surface.
2. Once the yeast paste is dissolved, and most of the eggs have been detached from the molasses-agar surface, pour the mix of water, dissolved yeast paste, and eggs into a labeled egg basket. Repeat for the remaining genotypes (each in its separate embryo collection plate) into separate labeled egg baskets. The egg baskets serve as filtering devices to collect the embryo-containing eggs and remove dissolved yeast and water. Wash the eggs in the baskets with squirts of water until the water runs clear.
3. The following bleach treatment with vigorous washing removes the outer chorion layer of the embryo. Place the egg baskets in a petri dish partially filled with 50% bleach for 5 min such that the level of the bleach solution is just below the rim of the baskets. During these 5 min, use a pasteur pipette to repeatedly suck up bleach solution from the petri dish and expel it inside the baskets to continually rinse the embryos.
4. Immediately wash the embryos in the egg baskets, first by squirting with 0.1% Triton X-100, and then extensively with water to remove any traces of bleach or detergent.

3.4 Embryo Fixation

1. Blot the exterior of the egg baskets with a disposable laboratory tissue or a paper towel to remove excess moisture. Then use fine paint brushes to transfer the embryos from the egg baskets to the fix solutions in separate labeled scintillation vials. The embryos will easily come off the paint brushes and settle at the interface between the two phases. A single layer of embryos is desired at this interface. Perform this step quickly to prevent desiccation of the dechorionated embryos.
2. Close the lids tightly, secure the vials on their side on the platform shaker, and run the shaker at 125 rpm for 20 min.
3. After 20 min, gently release and remove the vials from the shaker. Moving the vials too vigorously at this point increases the risk of embryos getting stuck on the lids or the sides of the vials.
4. Draw off embryos from the interface using a pasteur pipette. Discard the lower aqueous phase in the pasteur pipette, and transfer the embryos to a labeled 1.5 mL microcentrifuge tube. Repeat for each genotype (each in its separate scintillation vial).
5. Wash embryos three times with heptane. Each wash is achieved by adding 1 mL of heptane gently to the embryos in the tube, then using a pasteur pipette to remove as much of the heptane as possible without drawing out the embryos.
6. Add 0.6 mL of heptane followed by 0.6 mL of methanol to the embryos in each tube. Hold tubes in a fist and shake vigorously for 60 s to facilitate devitellinization (removal of the opaque vitelline membrane) of the embryos.
7. Let the tubes stand vertically in a rack for 30 s. Devitellinized embryos will sink to the bottom of the tube.
8. Use a pasteur pipette to remove the top layer (heptane) and the interface (which will contain empty vitelline membranes and embryos which were not devitellinized).
9. Wash embryos five times with methanol. Each wash is achieved by adding 1 mL of methanol gently to the embryos in the tube, then using a pasteur pipette to remove as much of the methanol as possible without drawing out the embryos.
10. Add 1 mL of methanol to the embryos and store at −20 °C for antibody staining (*see* **Note 13**).

3.5 Antibody Staining

1. Take out the microcentrifuge tubes of embryos that were fixed and stored at −20 °C. Place the tubes vertically in a rack such that the embryos settle to the bottom.
2. For each tube of fixed embryos, prepare 1 mL of 50% methanol in PBST.

3. Carefully aspirate the supernatant (methanol) from the tubes without disturbing or removing the fixed embryos, add 1 mL of 50% methanol in PBST to each tube, and rock the tubes at room temperature for 5 min to begin the process of rehydrating the embryos (*see* **Note 14**).
4. Place the microcentrifuge tubes vertically in a rack. After the embryos have settled to the bottom of the tube, aspirate the supernatant (50% methanol) without disturbing the embryos.
5. Add 1 mL of PBST to the microcentrifuge tubes and rock them at room temperature for 5 min. Then place them vertically in a rack to allow the embryos to settle to the bottom, and remove the supernatant (PBST) without disturbing the embryos.
6. Repeat **step 5** three (3) more times. These washes should remove all traces of methanol from the embryos.
7. While repeating **step 5**, prepare 2% NGS in PBST.
8. After the final aspiration of PBST from the tubes in **step 6**, add 1 mL of 2% NGS in PBST to each tube of embryos, and rock at room temperature for 1 h. The NGS in this and subsequent steps acts as a blocking agent to prevent nonspecific binding of antibodies to the embryo.
9. During **step 8**, prepare the primary antibody cocktail. Add all three primary antibodies (rabbit anti-Mef2 antibody, guinea pig anti-Zfh1 antibody, and mouse monoclonal anti-β-galactosidase antibody) to their prescribed dilutions in 2% NGS in PBST, and rock for at least 10 min to create the primary antibody cocktail (*see* **Note 15**).
10. After 1 h of incubation in the blocking solution (**step 8**), place the microcentrifuge tubes vertically in a rack and allow embryos to settle to the bottom of the tube. Aspirate the supernatant (2% NGS in PBST) without disturbing the embryos, add 0.5 mL of the primary antibody cocktail to each tube of embryos, and then rock the tubes at 4 °C overnight.
11. Prepare fresh 2% NGS in PBST the next day.
12. Place the tubes of embryos that had been rocking overnight at 4 °C in the primary antibody cocktail vertically in a rack such that the embryos settle to the bottom. Remove the supernatant (the primary antibody cocktail) (*see* **Note 16**).
13. Add 1 mL of 2% NGS in PBST to the microcentrifuge tubes and rock them at room temperature for 5 min. Then place the tubes vertically in a rack to allow the embryos to settle to the bottom, and aspirate the supernatant (2% NGS in PBST) without disturbing the embryos.

14. Repeat **step 13** seven (7) more times. These washes will remove all traces of the primary antibody cocktail from the tubes while continuing to prevent any nonspecific binding of the antibodies to the embryo.
15. During the wash process, that is, while repeating **step 13**, prepare the secondary antibody cocktail. Add all three secondary antibodies (Alexa Fluor 568 goat anti-rabbit IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 647 goat anti-guinea pig IgG (H + L) highly cross-adsorbed secondary antibody, and Alexa Fluor 488 goat anti-mouse IgG (H + L) cross-adsorbed secondary antibody) each to a 1:300 dilution in 2% NGS in PBST in a tube. Cover the tube completely with aluminum foil to prevent photobleaching of the antibodies and rock for at least 10 min.
16. After the final aspiration of 2% NGS in PBST from the tubes in **step 14**, add 0.5 mL of the secondary antibody cocktail to each tube of embryos. Immediately cover each tube completely with aluminum foil to prevent photobleaching.
17. Rock these covered tubes containing the embryos in the secondary antibody cocktail either at room temperature for 1 h or at 4 °C overnight.
18. Place these covered tubes that had been rocking in the secondary antibody cocktail vertically in a rack such that the embryos settle to the bottom. Aspirate the supernatant (the secondary antibody cocktail) without disturbing the embryos.
19. Add 1 mL of PBST to the microcentrifuge tubes and rock them at room temperature for 5 min. Then place them vertically in a rack to allow the embryos to settle to the bottom, and remove the supernatant (PBST).
20. Repeat **step 19** seven (7) more times. These washes will remove all traces of the secondary antibody cocktail from the tubes. Ensure that the tubes remain covered with aluminum foil at all times to prevent photobleaching.
21. After the final removal of PBST from the tubes in **step 20**, add 2–3 drops of Vectashield antifade mounting medium to each tube of embryos. Ensure that the tubes are still completely covered with aluminum foil and store them at 4 °C until microscopy.

3.6 Mounting and Microscopy

1. Peel and attach a Secure Seal Imaging Spacer on a clean microscope slide (Fig. 4a) (*see* **Note 17**).
2. Use a micropipette to transfer 10–15 μL of Vectashield with embryo suspension from the relevant microcentrifuge tube to the slide surface at the center of the hole in the spacer (Fig. 4b, c).

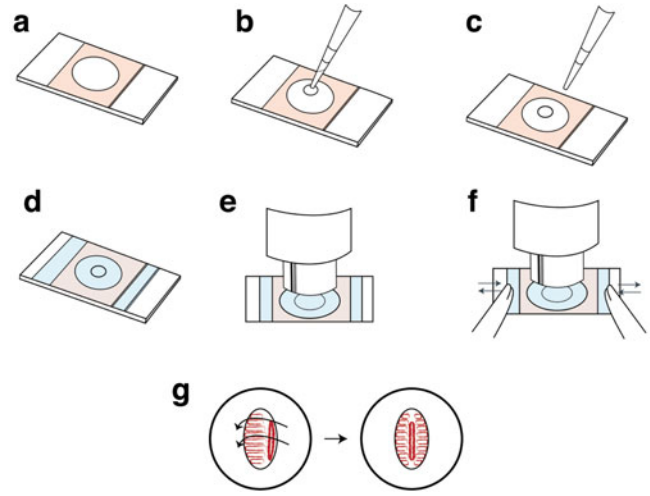

Fig. 4 Sequential steps in mounting and microscopy. (**a**) Attach a Secure Seal Imaging Spacer to the slide. (**b**, **c**) Transfer 10–15 μL of Vectashield with embryos suspended in it to the slide surface at the center of the hole in the spacer. (**d**) Gently place a coverslip on the spacer such that it also touches the drop of Vectashield. (**e**) Examine the embryos under low magnification. (**f**) Rotate embryos to bring them to the correct orientation by gently sliding the coverslip. (**g**) Schematic showing the rotation of embryos to the correct orientation by gentle sliding of the coverslip

3. Gently place a coverslip on the spacer such that it also touches the drop of Vectashield containing the embryos (Fig. 4d). Try not to let air bubbles form in the Vectashield during this process.
4. Examine the fluorescent antibody-labeled embryos on the slide under low magnification (e.g., with a 10× objective) in a fluorescence microscope with filters set to detect Alexa Fluor 568 staining (Fig. 4e). The anti-Mef2 antibody-labeled nuclei of the somatic muscles and/or the CCs of the tubular heart should be clearly visible (Fig. 5). *Drosophila* Stage 16 embryos have a convex ventral surface, and a relatively flatter dorsal surface where the heart is located. Embryos may thus lie rotated along their long axis in a manner that makes viewing and imaging of the heart difficult (Fig. 5a). Gently slide the coverslip the tiniest degree along the spacer surface (Fig. 4f) while viewing an embryo through the eyepiece to rotate it such that the dorsal surface and the characteristic tubular heart now directly faces the objective (Figs. 4g and 5b, c).
5. Switch to higher magnification (20× or 40× objectives) and examine/take images of the heart with filters set to detect first Alexa Fluor 568 staining (for anti-Mef2-labeled CCs), then Alexa Fluor 647 staining (for anti-Zfh1-labeled PCs), and finally Alexa Fluor 488 (for β-galactosidase reporter

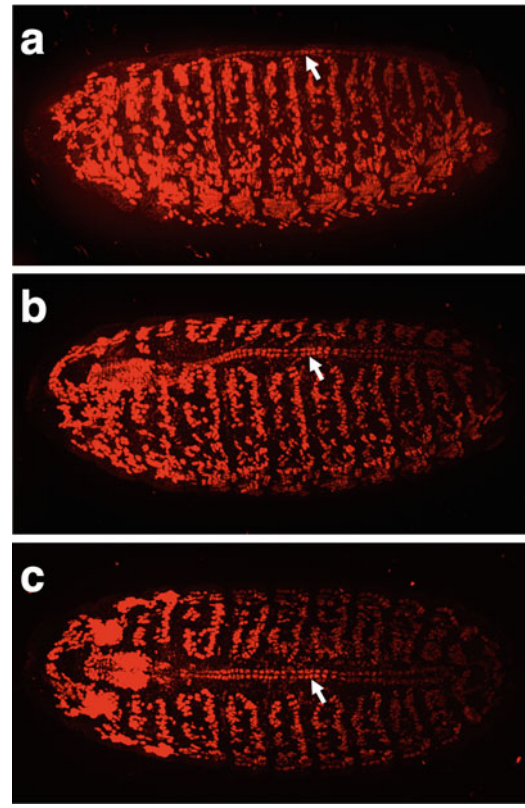

Fig. 5 Rotation of embryos to the correct orientation for imaging. Mef2-expressing nuclei (red) of the somatic muscles and heart in Stage 16 embryos observed under low magnification using a fluorescent microscope with filters set to detect Alexa Fluor 568 staining. (**a**) Embryo in an orientation that precludes clear imaging of the tubular heart (arrow). (**b**) Gentle sliding of the coverslip showing the gradual rotation of the same embryo. (**c**) The same embryo rotated to the proper orientation for detailed analysis of the heart at higher magnification and with all three fluorophores

expression). If the fluorescence microscope is a confocal microscope or equipped with an apotome, capture Z-stacks of sufficient depth that accommodate all the Mef2-labeled CCs and Zfh1-labeled PCs of the heart. Otherwise, take images along multiple different focal planes in all three filter set channels to ensure that every CC and PC is captured in focus. Examine 20–30 embryonic hearts for each genotype, paying particular attention to whether β-galactosidase reporter expression driven by the *Him* enhancer is ectopically present in CCs or missing in core-PCs in Notch pathway component knockdown embryos compared to controls. Similarly, note whether β-galactosidase reporter expression driven by the *zfh1* enhancer is missing in core-PCs in Notch pathway component knockdown embryos compared to controls.

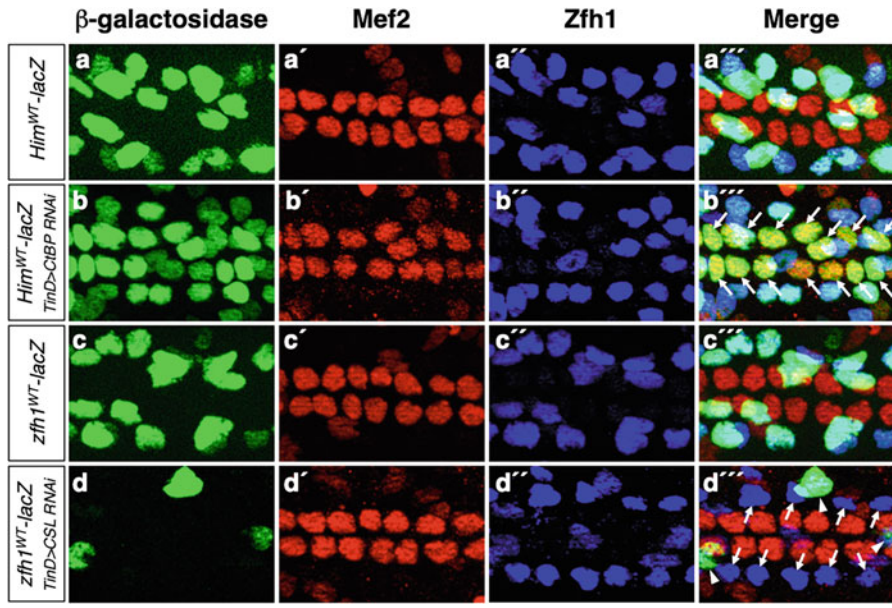

Fig. 6 Examples of RNAi-mediated knockdown of Notch signaling pathway components on reporter expression driven by the Notch-permissive *Him* and the Notch-instructive *zfh1* enhancers. (**a**–**d**‴) *lacZ* reporter activity (β-galactosidase, green) driven by wild-type *Him* or *zfh1* enhancers in Stage 16 embryos. All CCs express Mef2 (red) while PCs are marked by Zfh1 (blue). (**a-a**‴) In an otherwise wild-type embryo, β-galactosidase, driven by the *Him* enhancer is present only in PCs. (**b-b**‴) Knockdown of a component required for repressing the transcription of target genes in the absence of NICD, in this case CtBP, a corepressor that forms the repressor complex with CSL, results in derepression in the CCs (arrows) of β-galactosidase driven by the *Him* enhancer. (**c-c**‴) β-galactosidase, driven by the *zfh1* enhancer is also present only in PCs in otherwise wild-type embryos. (**d-d**‴) Knockdown of a component required for instructive transcriptional activation by the NICD-CSL activator complex, in this case CSL itself, eliminates *zfh1* enhancer-driven β-galactosidase expression in the core-PCs (arrows). β-galactosidase expression will still be detected in the Eve-PCs (arrowheads). Note that a very similar elimination of *Him* enhancer-driven β-galactosidase expression in the core-PCs would also be seen if the knocked down component functioned in the Notch-permissive signaling pathway to enable the NICD-CSL complex to competitively displace the CSL repressor complex at the target gene enhancer

3.7 Analysis and Interpretation

1. Examine the hearts of embryos obtained from the cross: *Him^{WT}-lacZ; TinD-GAL4 UAS-Dcr-2* virgin females × wild-type males. These are the controls for the *Him* enhancer–reporter construct. Ensure that β-galactosidase is expressed in each and every Zfh1-labeled PC but not in a single Mef2-labeled CC as in Fig. 6a-a‴.
2. Examine the hearts of embryos obtained from the cross: *Him^{WT}-lacZ; TinD-GAL4 UAS-Dcr-2* virgin females × males bearing the UAS-RNAi construct for the Notch pathway component to be tested. First assess whether β-galactosidase is expressed in one or more Mef2-labeled CCs in addition to all Zfh1-labeled PCs. If ectopic expression of β-galactosidase is seen in some CCs in addition to all PCs as in Fig. 6b-b‴, then

the component knocked down by RNAi functions in the Notch-permissive signaling pathway to repress transcription of target genes in the absence of NICD (Table 1) (*see* **Note 18**). If no ectopic expression of β-galactosidase is detected in CCs, assess whether β-galactosidase is expressed in each and every Zfh1-labeled PC, as in the control in **step 1**. If β-galactosidase reporter expression is missing in some or all PCs, similar to what is seen in Fig. 6d-d‴, then the component knocked down by RNAi functions in the Notch-permissive signaling pathway to enable the NICD-CSL complex to competitively displace the CSL repressor complex at the target gene enhancer (Table 1).

3. Examine the hearts of embryos obtained from the cross: *zfh1*^{WT}*-lacZ; TinD-GAL4 UAS-Dcr-2* virgin females × wild-type males. These are the controls for the *zfh1* enhancer–reporter construct. Ensure that β-galactosidase is expressed in each and every Zfh1-labeled PC but not in a single Mef2-labeled CC as in Fig. 6c-c‴.
4. Examine the hearts of embryos obtained from the cross: *zfh1*^{WT}*-lacZ; TinD-GAL4 UAS-Dcr-2* virgin females × males bearing the UAS-RNAi construct for the Notch pathway component to be tested. Assess whether β-galactosidase is expressed in each and every Zfh1-labeled PC, as in the control in **step 3**, or whether β-galactosidase expression is missing in some PCs. If β-galactosidase reporter expression is missing in some or all PCs, as in Fig. 6d-d‴, then the component knocked down by RNAi functions in the Notch-instructive signaling pathway to activate transcription of target genes through the inductive effect of the NICD-CSL activator complex (Table 1) (*see* **Note 19**).
5. Based on the expectations described above and in Table 1 and the effect of the Notch pathway component knockdown on reporter expression driven by the *Him* and *zfh1* enhancers, determine whether the component is utilized by the Notch-permissive and/or the Notch-instructive mechanism.

4 Notes

1. In addition to the enhancer reporter constructs (*Him*^{WT}*-lacZ* or *zfh1*^{WT}*-lacZ*) and the cardiac mesoderm-specific driver (*TinD-GAL4*), these lines also contain the transgenic construct *UAS-Dcr-2*. Inclusion of this *UAS-Dcr-2* construct ensures that the long dsRNAs used in the earlier generation of UAS-RNAi constructs also provide efficient knockdowns. The cardiac mesoderm-specific driver, *TinD-GAL4*, ensures that

the Notch signaling pathway component being assessed is not knocked down elsewhere in the embryo where it may be essential.

2. Use the RSVP tool (https://fgr.hms.harvard.edu/rsvp) tool to identify and obtain existing UAS-RNAi stocks for the Notch signaling pathway component to be knocked down from the Bloomington Drosophila Stock Center, the Vienna Drosophila Resource Center, or the National Institute of Genetics—Japan [29]. We recommend acquiring all available UAS-RNAi stocks for a particular component and performing separate series of crosses with them, since they will differ in knockdown efficiency. We also recommend performing and comparing reverse transcription quantitative real-time PCR (RT-qPCR) on total RNA collected from Stage 13–16 control and knockdown embryos (9–15.5 h after egg laying) from these series of crosses to determine RNAi-mediated knockdown efficiency. If the Notch signaling pathway component was identified in a system other than flies, the DIOPT tool (https://www.flyrnai.org/diopt) can be used to identify the *Drosophila* ortholog of the component in order to secure the relevant UAS-RNAi stocks [30]. Finally, FlyBase (http://flybase.org) can be used to identify UAS-RNAi fly lines constructed by and available from individual investigators that may not be present in the previously mentioned stock centers.
3. Small embryo collection cages can also be made from 100 mL polypropylene beakers whose open ends fit snugly over 60 mm × 15 mm petri dishes. Heat the tip of a syringe needle in the flame of a Bunsen burner and use it to make a number of air holes in the side of the beaker. Make certain that the holes are too small to prevent the flies from escaping. Prepare cages by dropping anesthetized flies into such a beaker, closing the opening with an embryo collection plate, using rubber bands or adhesive tape to keep the embryo collection plate in position, and inverting the cage such that the plate is at the bottom when the flies begin to stir (Fig. 7). We find making cages in this manner considerably more economical than purchasing commercial cages.
4. While 10× PBS pH 7.4 is relatively easy to prepare in the laboratory, it is also available from many vendors.
5. We use and recommend the rabbit anti-Mef2 antibodies that we requested and obtained as gifts from either Dr. Roger Jacobs in the Department of Biology at McMaster University or Dr. Bruce Paterson at the National Cancer Institute, NIH [31, 32]. Use at a 1:1000 dilution.
6. We use and recommend the guinea pig anti-Zfh1 antibody that we requested and obtained as a gift from Dr. James Skeath in

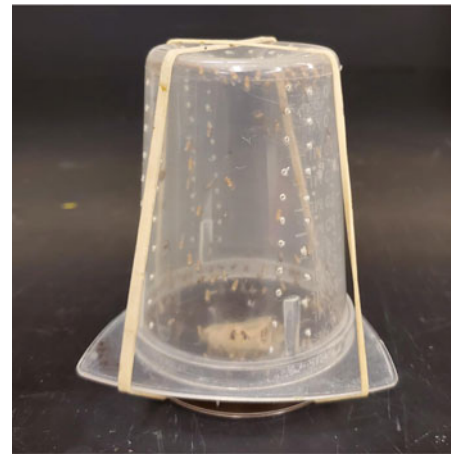

Fig. 7 Small embryo collection cage prepared as described in **Note 3**

the Department of Genetics at Washington University School of Medicine in St. Louis [33]. Use at a 1:1000 dilution.

7. We use and recommend the mouse monoclonal anti--β-galactosidase antibody available from Promega (catalog no. Z3783). Use at a 1:500 dilution.
8. The Alexa Fluor conjugated secondary antibodies from ThermoFisher Scientific that we suggest are effective at distinguishing Mef2, Zfh1, and β-galactosidase staining with the filter sets we use. Investigators are welcome to use different sets of fluorescent secondary antibodies that work best with their specific microscopy setups. We do recommend using a fluorescent secondary antibody for the anti-Mef2 primary antibody that is easily visible through the eyepiece (i.e., not Alexa Fluor 647 or any fluorophore in the "far-red" range) since we use the Mef2 staining for proper rotation and orientation of the embryos.
9. We recommend performing the crosses at 29 °C since the GAL4-UAS system is most efficient at that temperature. However, for certain genotypes, this temperature can lead to very few or no embryos surviving to Stage 16. In such cases, lower the temperature to a level that allows sufficient embryos to survive.
10. While the reciprocal crosses can be performed, we have empirically found that we obtain more efficient knockdowns by crossing males with the UAS-RNAi construct to virgin females bearing the *TinD-GAL4* driver, the *UAS-Dcr-2* construct, and the enhancer–reporter construct.
11. The crosses described in this method assume that the male flies bearing the UAS-RNAi constructs are homozygous viable. However, in some instances, the insertion of the UAS-RNAi

construct into the chromosome may be lethal when homozygous, resulting in the construct being carried over a balancer. In such cases, first put the UAS-RNAi construct over an embryonic *lacZ*-expressing balancer such as *CyO, ftz-lacZ* or *TM3, ftz-lacZ* and then perform the crosses with males bearing the construct over these marked balancers. Identify the knockdown embryos as those lacking the marked balancer since they fail to express β-galactosidase in the same pattern as the balancer.

12. Note that multiple embryo plate collections can be obtained from the same cage in a day. After the first 3 h collection, simply perform another 3 h collection on the second plate, allow it to age for 12.5 h before dechorionation and fixation, and then repeat again if necessary. Also note that the 12.5 h of aging is specific for embryos being raised at 29 °C. If, due to reduced embryo survival, the crosses and aging of the embryos are being performed at 25 °C, age the embryos for 13 h to reach Stage 16.
13. While the embryos can be used immediately for antibody staining, we have found that storing them overnight at −20 °C improves immunostaining.
14. For this and many of the subsequent wash steps, we find it easiest to simply aspirate the supernatant with a micropipette tip at the end of a pasteur pipette attached to a vacuum line (with appropriate safety trap containers and filters). However, a micropipette or even a pasteur pipette with a rubber bulb can also be used to remove the supernatant.
15. The primary antibody cocktail consists of 1:1000 dilutions of both the rabbit anti-Mef2 and guinea pig anti-Zfh1, and 1:500 dilutions of the Promega mouse anti-β-galactosidase we recommended. Please determine the appropriate dilutions empirically if the primary antibodies are obtained from other sources.
16. Since we have limited amounts of some of the primary antibodies, we remove the primary antibody cocktail with a micropipette, store it at 4 °C, and reuse it for subsequent immunostainings.
17. The Secure Seal Imaging Spacer both prevents the coverslip from flattening the embryos and allows adjustment of the orientation of the embryos for imaging by slight sliding of the coverslip on the spacer. Temporary spacers can also be prepared in the laboratory by placing three strips of Scotch Magic Tape on top of one another on a glass sheet. Cut off small pieces of this triple layered tape strip with a razor blade and place on both sides of the Vectashield drop on the slide. Use these tape pieces as bridge supports on which to place the coverslip.

18. Ectopic β-galactosidase need not be present in each and every CC of the heart since the *TinD-GAL4* driver does not function with identical efficiency in each and every heart cell. Reproducible β-galactosidase expression that is detected in even some CCs of the embryos with candidate component knockdowns but never in the CCs of the controls is sufficient to conclude that the knocked down component functions in the Notch-permissive signaling pathway to repress transcription of target genes in the absence of NICD.
19. Since disruption of the Notch-instructive signaling pathway does not affect *zfh1* enhancer-driven expression in the Eve-PCs, absence of β-galactosidase expression in the core-PCs is sufficient to conclude that the knocked down component functions in the Notch-instructive signaling pathway to activate transcription of target genes through the inductive effect of the NICD-CSL activator complex, even if β-galactosidase continues to be expressed in the Eve-PCs. This result is generally detected as the disappearance of β-galactosidase expression in the majority of the Zfh1-labeled PCs. However, if more specificity is required in the analysis, the Eve-PCs can be distinguished from the core-PCs as being the most dorsally positioned cells among the Zfh1-labeled PCs. Alternatively, staining with antibodies against the Eve protein can also serve to distinguish the Eve-PCs which express Eve from the core-PCs which do not.

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