Non-Canonical Notch Signaling During Early Heart Development

By
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A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science

Baltimore, Maryland
May 2017

Abstract:

The canonical Notch signaling pathway has been studied extensively and plays a key role during embryonic development. Recent evidence points to the existence of a non-canonical function of the Notch protein. Elucidation of a non-canonical Notch signaling pathway would significantly alter how the Notch protein is viewed in biological systems. Perhaps more importantly, the identification of downstream effectors could lead to discovery of novel gene networks functioning throughout development. One potential downstream effector of non-canonical Notch is Numb, which interacts with Notch during development. We provide evidence of the existence of a non-canonical pathway in both the embryonic stem cell and during early heart development. By developing two models that alter either the Notch protein or the canonical Notch signaling pathway, we studied non-canonical Notch signaling *in vitro* and *in vivo*. Upon overexpression of a tethered form of Notch, which cannot initiate canonical Notch signaling, we observed remarkable apoptosis in embryonic stem cells. Furthermore, Notch overexpression during early heart development led to decreased heart size due to decreased myocyte proliferation when the essential transcription factor to canonical Notch signaling, RBP-j, was knocked out. Similar phenotypes observed in a Numb knockout setting led us to hypothesize that an interaction between Numb and Notch is involved in heart development. Elucidation of a non-canonical Notch signaling pathway may lead to not only a better understanding of congenital heart disease, but also development of other organ systems, as well.

Reader: Dr. Chulan Kwon, Ph.D

Preface:

This is the culmination of my yearlong Masters research project. I have developed a deep interest for Notch (canonical and non-canonical) signaling pathways as a result. I hope that my passion for the topic will show through this thesis, and that the findings will one day contribute to the emerging field of non-canonical Notch signaling. I would like to thank my P.I Dr. Chulan Kwon and research advisor Dr. Peter Andersen for their contributions and support for this project.

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Introduction:

The development of the heart is a very complex process that involves multiple signaling pathways and tight spatial-temporal regulation. However, the potential for development to go awry still exists, leading to congenital heart defects, which affect thousands of individuals throughout the world (1). Thus, the study of the mechanisms that control heart development could prove invaluable in understanding the origin of these diseases and potential treatments.

Understanding the mechanisms responsible for cardiac development can also be useful in the emerging field of stem cell therapy. Patient derived pluripotent stem cells (PSCs) offer a promising treatment for many types of heart diseases. To be able to induce a patient's skin cells into a pluripotent state, and subsequently differentiate and mature them into transplantable cardiac tissue would significantly change how we view and approach heart disease. Better understanding of the mechanisms at work would aid in the development of methods to create nearly natural cardiac tissue that functions appropriately when transplanted back into a patient's heart.

A variety of signaling pathways are involved in the development of the heart. Some of the pathways that are involved in the development of the heart are the CXCR (2), Sonic Hedgehog (3), Wnt B-Cat (4), and Notch (5) signaling pathways. These pathways have been well characterized,

and evidence shows there is cross-talk between some of these pathways at different stages of development (6).

We have recently discovered a potentially new signaling pathway required for heart development. The endocytic adaptor protein Numb and its homologue Numb-like are required for the proper development of the heart in mouse embryos (7). When both Numb and Numb-like are knocked out in heart tissue during the stage of early heart development, the heart decreases significantly in size (7). However, when Numb and Numb-like are knocked out at the later stages of heart development, the heart *increases* in size compared to wild-type embryos (8). This phenomenon may be due to interactions between the Numb protein and a non-canonical Notch signaling pathway.

The canonical Notch signaling pathway is a well-established pathway that regulates development in many systems such as neural stem cells (9), cardiac valves (10), and angiogenesis (11), among a variety of other systems and applications. Initial work in *Drosophila* revealed a notch in the wings of affected flies (12), which led to the naming of the Notch receptor and pathway. The pathway has been extensively studied, leading to a widely-accepted consensus of the mechanism and many of its downstream effectors.

The canonical Notch signaling pathway requires a neighboring cell to initiate signaling. The cascade begins when an extracellular signal

from a neighboring cell, Delta, binds the extracellular receptor of the Notch transmembrane protein (13). Upon binding of the Notch receptor, a gamma-secretase complex cleaves the Notch receptor near the membrane, releasing the intracellular domain of the Notch receptor (NICD) from the membrane into the cytoplasm. The NICD translocates into the nucleus and interact with the transcription factor RBP-j and initiates transcription of target genes. The Notch signaling pathway is dependent on the signal from the neighboring cell, cleavage of the protein from the membrane, and transcription factors.

Growing evidence supports the existence of non-canonical Notch signaling activity. The non-canonical Notch pathway does not have a well-defined mechanism. In fact, its effects on gene expression and development are quite uncertain. A recent study demonstrated that Notch non-canonically regulates the levels of the Wnt signaling mediator active **β**-catenin (14). In line with this, other studies have shown how Wnt signaling is decreased upon the increase of non-canonical Notch signaling (15). Non-canonical Notch signaling is a broad characterization of a relatively unknown type of signaling. For our studies, we will define non-canonical Notch signaling to be signaling initiated by the Notch protein that does not initiate transcription through RBP-j in the nucleus. The uncleaved Notch or cytoplasmic NICD protein was previously thought to be biologically inactive. We developed two models to attempt to investigate if this is true. First, we used a model where a mutation

makes Notch resistant to cleavage. In other words, NICD is tethered to the plasma membrane. In doing so, the NICD is unable to initiate transcription as it cannot translocate to the nucleus. The Tethered-NICD will remain able to potentially interact with other molecules in the cytoplasm near the membrane. The second model we used was NICD overexpression in the cytoplasm while simultaneously knocking out the essential transcription factor RBP-j. This overexpresses the NICD while knocking out canonical Notch signaling. We used these two models to specifically study non-canonical Notch signaling. Our initial hypothesis was that Notch was interacting with Numb to cause a decrease in proliferation in early cardiac cells. Our observations provide evidence of such a relationship.

Materials and Methods:

Mice Crosses and Genetics:

Mesp-Cre; *RBP-f fl/fl or +*; *NICD Overexpression (OE)* mice were generated by crossing a *Mesp-Cre*; *RBP-J fl/+* male mouse with a *NICD OE/OE*; *RBP-J fl/fl* female. The *NICD OE* trait in a *Cre* positive mouse leads to overexpression of the *NICD*. *Cre* excises a stop site in front of the *NICD* overexpression coding region. We ensured that we were studying non-canonical Notch signaling through knockout of the essential transcription factor *RBP*-J through the same *Cre-lox* utilized to knockout the stop site preceding the *NICD* OE coding region. These mice were provided by Dr. Chulan Kwon of Johns Hopkins University, School of Medicine. To confirm the genotypes of the mice, 0.5 mm tail snips were obtained from 3-week-old mice. DNA was extracted using Phire Animal Tissue Direct PCR Kit (ThermoFisher Scientific). Polymerase Chain Reaction (PCR) was completed to amplify regions of interest. Promega Go-Taq Green Master Mix, primers designed by Dr. Hideki Uosaki, Jichi University, Japan, and supplied by Integrated DNA Technologies, and nuclease-free water were combined with the extracted DNA. After PCR was completed, samples were run on a 2.0% Agarose gel with ethidium bromide for 20 minutes at 200 volts. Gels were imaged using ultraviolet imaging.

Embryo Dissection:

Mice with *Mesp Cre; RBP-j fl/+* and *NICD OE/OE; RBP-j fl/fl* genotypes were mated to obtain the *Mesp-Cre*; *NICD OE*; *RBP-J fl/ fl or* + genotype. The following morning, mice were assessed for intercourse the previous night by vaginal plug inspection. If determined to be "plugged", that day is then referred to as embryonic day 0.5 (E.5). We were interested in looking at E7.5, E8.0, E8.5, and E9.0 embryos, and these dates were determined based on the date when the plug was observed. On dissection day, pregnant mothers were injected with 200 uL of 10 mM EDU, to mark proliferating cells. Mothers were dissected, and embryos harvested. Upon collection, embryos were fixed in 4% paraformaldehyde in PBS for 30 minutes, and transferred to 30% sucrose in PBS overnight. Images of the embryo were taken using an Axio-cam microscope camera, looking at the front, left, and right sides. Embryos were flash frozen in a bath of 2-methyl butanol and dry ice. Embryos were then sectioned and stained using Nkx 2.5 (Santa Cruz Biotechnology) with Alexa 488 anti-goat secondary antibody, Isl (Developmental Studies Hybridoma Bank) with Alexa 599 anti-mouse secondary antibody, cTnT (ThermoFisher Scientific) with Alexa 488 anti-mouse secondary antibody, pH3 (ThermoFisher Scientific) with Alexa 599 anti-rabbit secondary antibody, and Caspase (Santa Cruz Biotechnology) with Alexa 599 anti-rabbit secondary antibody.

Development of Cell Lines:

Stable cell lines were created by Dr. Peter Andersen, Johns Hopkins School of Medicine. A PiggyBac-Tethered Notch-GFP plasmid was transfected into an E14 embryonic stem cell (ESC) line. This plasmid overexpressed a doxycycline inducible form of the NICD that is unable to be cleaved from the plasma membrane. Transfection was completed using Lipofectamine 2000 (ThermoFisher Scientific). Doxycycline was added post-transfection, and GFP expressing cells were selected for using a Sony Cell Sorter, and were plated on a 10-cm gelatinized dish to ensure the transient transfection period had passed. Colonies that were still expressing GFP were picked and expanded into stable cell lines.

Cell Culture Experiments:

Embryonic stem cells (ESCs) were maintained in standard ESC culture medium, 2i medium. This medium is composed of GMEM, Leukemia Inducible Factor, Fetal Bovine Serum, Glutamax, Non-Essential Amino Acids, **β**-Mercaptoethanol, and two inhibitors PD and Chir. Cells were maintained in a gelatinized flask and were passaged every 3 days. Experiments were conducted using Lif media. Lif media and 2i media are similar, except that Lif does not have the 2 inhibitors, and it has extra Leukemia Inducible Factor.

Apoptosis Assay:

Apoptosis was measured in populations of ESCs. Cells were cultured for 60 hours in medium with and without doxycycline (Tethered Notch overexpressed and not overexpressed). Cells were dissociated with Trypl-e and washed with PBS, and apoptosis levels were assessed using FACS. As apoptosis occurs, the membrane phospholipid phosphatidylserine becomes exposed. The Annexin V antibody (BD Biosciences) binds to phosphatidylserine, which enables detection by FACS. A DAPI counter stain was used to measure the number of dead cells. Through use of a Sony Cell sorter, we could determine the proportion of dead, dying apoptotic, early apoptotic, and living cells in a sample.

qPCR:

Trizol RNA extraction and isolation methods were employed. Trizol was added to washed cells in cell culture dish. Bromochloropropanol, isopropanol, and ethanol were then added to extract and isolate RNA. The concentration of RNA was determined using a Nanodrop Spectrophotometer. Five hundred nanograms of cDNA was produced using RT-PCR kit (ThermoFisher Scientific). Taqman qPCR was completed. Gene expression was analyzed using RT-qPCR.

Western Blot:

Doxycycline was added to cells for periods of 24, 48, and 60 hours. Cells were lysed using RIPA buffer (Thermo Fisher Scientific), and stored with 1x protease and phosphatase inhibitor cocktail. Protein content was measured through BCA standard curve. Five micrograms of protein were added per well. Goat Anti-Numb (Abcam) primary antibody was used with a donkey anti-goat secondary antibody. Rabbit anti-GAPDH (Abcam) primary antibody was used with a mouse anti-rabbit secondary antibody.

Results:

Overexpression of RBP-j independent Notch intracellular domain at Mesp stage severely decreases heart size in embryonic day 8.0 embryos

To determine the effects of non-canonical Notch signaling on the heart during the early stages of heart development (Embryonic day 8-9), we generated mice that both overexpressed the Notch intracellular domain (*NICD*) while the essential transcription factor *RBP-J* was knocked out. This amplified *NICD*, while eliminating canonical Notch signaling; creating an excellent model for the focused study of non-canonical Notch signaling. We wanted to study this signaling pathway specifically in the early stages of heart development. We utilized a *Mesp* driven *Cre* to simultaneously initiate the overexpression of *NICD* and knock out of *RBP-j* in early heart tissue. *Mesp* is expressed in the mesoderm during gastrulation (16). By using this marker to drive *Cre* expression we could study non-canonical Notch signaling in the early stages of heart development. Overexpression of *NICD* was achieved by a *Cre* mediated knockout of a stop site in front of the *NICD* overexpression coding region. We achieved this by crossing a *Mesp*-Cre; *RBPj fl/+* male mouse with a *NICD OE/OE; RBPj fl/fl* female mouse, resulting in a mix of *Mesp-Cre; NICD OE; RBP-j fl/fl, Mesp-Cre; NICD OE; RBP-j fl/+*, and WT mice. We looked at the effects of *NICD* overexpression, with and without intact canonical Notch signaling. Wild type (WT) embryos showed normally developing hearts (Fig. 1A-C), while both *NICD OE; RBP-j fl/fl* and *RBP-j*

fl/+ embryos do not show a visible heart structure (Fig. 1D-F, G-I). However, the cardiac crescent is visible (n=5).

Overexpression of RBP-J independent NICD at Mesp stage results in smaller heart of embryonic day 9.0 embryos

After observing the little to no heart developed when *NICD* is overexpressed in the *Mesp* stage at E8.0, we wanted to see if the heart would develop further at a later embryonic day. Employing the same genetic crosses, we harvested embryos one day later, at E9.0, for imaging. Control embryos showed distinct heart formation (Fig. 2A-C). *NICD* overexpressing embryos showed a visible heart, but of a much smaller size (Fig. 2D-F). Mutant embryos also displayed slight developmental deformities, such as the uncurled tail and smaller total embryo size.

Embryo sectioning and staining shows decreased cardiac tissue in NICD overexpressing embryos

Embryonic day 9.0 embryos were sectioned and stained for Nkx2.5, Islet, and DAPI (Fig. 3A&B). Nkx staining outlines the developing heart and displays the smaller sized heart compared to control embryos.

pH3 and Caspase staining indicates decreased heart proliferation and increased apoptosis

We were interested in determining if the decreased heart size observed was due to differential proliferation, apoptosis, or a combination of both. pH3 staining was used to visualize proliferation in the developing heart (Fig. 4A&B). Arrows indicate regions of positive pH3 staining. Control embryos show pH3 expressing myocytes throughout the heart (Fig. 4A), while there was limited pH3 expression in the *NICD* overexpression embryos (Fig 4B). Differential pH3 expression points to decreased proliferation with *NICD* overexpression. Additionally, we were interested in investigating if apoptosis was occurring in the *NICD* overexpressing heart to lead to the smaller size. We stained for Caspase, an apoptosis indicator, and found that there was not apoptosis occurring in the heart compared to control embryos (Fig. 4C&D). We did, however, observe increased apoptosis near the neural tube of the embryo.

Development of Tethered Notch -GFP cell line

To investigate the mechanism of non-canonical Notch signaling further, we developed an *in vitro* model to study non-canonical Notch signaling, a cell line expressing Tethered notch (TN)-GFP (Fig. 5A). Tethered Notch is a mutated form of Notch protein with a mutation of Valine 1744, preventing cleavage of the *NICD* (Fig. 5B). A vector containing the TN coding region driven by a doxycycline inducible promoter was transfected

into an E14 embryonic stem cell (ESC) line. GFP expressing colonies were then sorted and plated using flow cytometry cell sorting, and picked, leading to the establishment of a TN-GFP cell line. We chose one cell line to use in these experiments based on the levels of GFP expression. We then sought to understand how this TN-GFP cell line worked by analyzing the expression levels of *Notch* through qPCR at different doxycycline concentrations. The doxycycline activated the TN-GFP (Fig. 5A). The GFP signal was most intense around the plasma membrane after 12 hours of doxycycline treatment, signaling that the TN was indeed unable to be cleaved from the membrane.

TN-GFP aggregates in cell membrane over time

In cells treated with doxycycline for 12-24 hours, an evenly distributed GFP signal was seen around the cell membrane (Fig. 6A). However, the signal was intensified on one side of the plasma membrane in cells treated with doxycycline for more than 36 hours (Fig. 6B).

Cell number decreases in TN overexpressing ESCs in Lif media

We grew TN-GFP cells in Lif-media for 60 hours with or without doxycycline treatment. TN-GFP expression was confirmed with fluorescence microscopy (Fig. 7B). Phase contrast microscopy showed fewer cells in TN-GFP overexpression samples compared to control (Fig. 7A&B). Cell counting analysis revealed an expected increase in cell

number in the control group over time (Fig. 7C). However, we observed fewer cells in the sample overexpressing TN after 60 hours (Fig 7C).

TN overexpression leads to apoptosis of ESCs in lif media

To determine the cause of the decrease in cell number we used a flow cytometry apoptosis assay to see if apoptosis was occurring. APC-Annexin 5 binds to receptors of cells undergoing apoptosis. We determined the ratio of live cells to dead and apoptotic cells by flow cytometry. TN overexpression led to significant apoptosis after 60 hours of doxycycline treatment (Fig. 8 A&B) when compared to control samples. In control samples, 99.2% of cells were alive (Fig. 8A), while only 56.9% were alive in TN over-expression, with 39% of this sample being apoptotic (Fig 8B).

2i media rescues TN overexpressing cells from apoptosis

We tested the effects of TN expression in 2i media. 2i media maintains ESCs long term in an undifferentiated state. 2i is short for 2 inhibitors, which are Chir and PD. Overexpression of TN led to slight apoptosis (Fig. 9D), and the cell morphology appeared similar in TN overexpression cells and control cells (Fig. 9A&B). The percentage of apoptotic cells decreased significantly to 3.4% apoptotic cells from 39% when the cells were in Lif media. These results suggested that factors related to the pluripotent state were rescuing the cells from apoptosis. qPCR was run for 10 genes

from cells in both media conditions to see if there was any correlation between genes involved in maintaining the ground state of embryonic stem cells and the apoptosis occurring in TN overexpressing cells (data not shown), but none was found.

TN overexpression up-regulates an isoform of Numb protein

Previous studies have shown that inhibition of Notch by Numb is required for certain cell fate decisions (16). This, combined with the similar *in vivo* phenotypes seen between *Numb* knock-out and *RBP-j* independent *NICD* over-expression points to a potential connection between the two proteins. We wanted to determine if *Numb* protein levels were altered following over-expression of *TN*. We cultured ESCs in Lif media for 8hr and 24 hr periods, and blotted for *Numb* (Fig. 10). We discovered two bands indicating Numb protein of smaller than expected size. The expected *Numb* bands were of similar intensity between control and TN over-expression samples; however, the extra bands are of interest for futures studies.

Discussion:

The results of the current study support the existence of a non-canonical Notch pathway. However, the different phenotypes observed when the pathway is activated at different times during development suggest that this pathway cannot be described simply. In embryonic stem cells, apoptosis was observed after overexpression of tethered-Notch, while decreased proliferation was seen when NICD was overexpressed in a RBP-j knockout setting. These different phenotypes observed during the different stages of development, lead to insights into how this non-canonical pathway functions.

We provide evidence for decreased heart proliferation and decreased heart size with RBP-j independent NICD overexpression. This result may explain why we see a small heart in E9.0 embryos but little to no heart in E8.0 embryos. However, the apoptotic cells surrounding the neural tube of the NICD over-expressing embryos cannot be explained as easily. Also of potential interest is the shape of the neural tube compared to control embryos (Fig. 4C&D). The neural tube is enclosed in WT embryos, while it is open in NICD over-expressing ones. We also observed that NICD over-expressing embryos cannot survive past ~E9.0. However, the apoptosis did not occur in cardiac cells, so the mechanism for how this apoptosis begins will need to be investigated further and is likely not related to the decreased heart size.

During the embryonic stem cell stage, overexpression of Tethered Notch caused a significant increase in apoptosis compared to control cells in Lif media. Lif media is typically used to maintain embryonic stem cells in a somewhat pluripotent state. However, when cells were maintained in 2i media, designed to keep cells in as pluripotent a state as possible, apoptosis was minimal compared to control cells. This is an interesting phenomenon that we hope to explain further *in vivo* in future experiments. Embryonic stem cells *in vitro* most closely resemble cells in the inner cell mass. Oct-4 marks the ICM starting at the 4-cell stage (17). By using Oct-4 to drive Cre function at this stage, we can over-express NICD and knock-out RBP-j specifically in these ESC-like cells. We will then be able to examine if and when apoptosis begins *in vivo*, potentially confirming the observed *in vitro* results.

Our initial hypothesis was that the non-canonical Notch signaling pathway signals through the protein Numb, and an endocytic adaptor, because knock-out of Numb leads to similar phenotypes as overexpression of NICD in the setting of canonical Notch signaling knockout, during the early and late stages of heart development (18). This hypothesis was rooted in the observation of similar phenotypes at the early and late heart development stages upon overexpression of NICD and knock-out of Numb. As seen in the experiments, however, at the embryonic stem cell stage, different phenotypes were seen *in vitro*. We expected to see Numb knockout ESCs exhibit the same apoptosis that

occurred when TN was overexpressed, but this was not observed. There was a slight decrease in proliferation, but this could be explained by other factors. First, the knockout of Numb may not lead to as drastic of a phenotype because rather than overexpressing a protein, in a knockout, the endogenous protein is just removed. However, significant TN overexpression might lead to a more severe phenotype. Regardless, future studies into this potential relationship are necessary. Preliminary western blots have shown up-regulation of a smaller sized Numb in TN overexpressing cells, suggesting some sort of interaction. A potential mechanism is that in the presence of Numb, Notch in the cytoplasm or the plasma membrane can sequester ß-catenin, which would decrease proliferation.

The decreased heart size observed when NICD is overexpressed during early heart development is similar to the phenotype seen when Numb was knocked out at the same time during development. This evidence of decreased proliferation seen in the hearts of these embryos, and not increased apoptosis, suggests that the non-canonical Notch signaling mechanism may be different depending on the stage in which it is activated. Our results provide evidence for the existence of a complex non-canonical Notch signaling pathway that, with future studies, can be elucidated more fully and examined in other developmental systems.

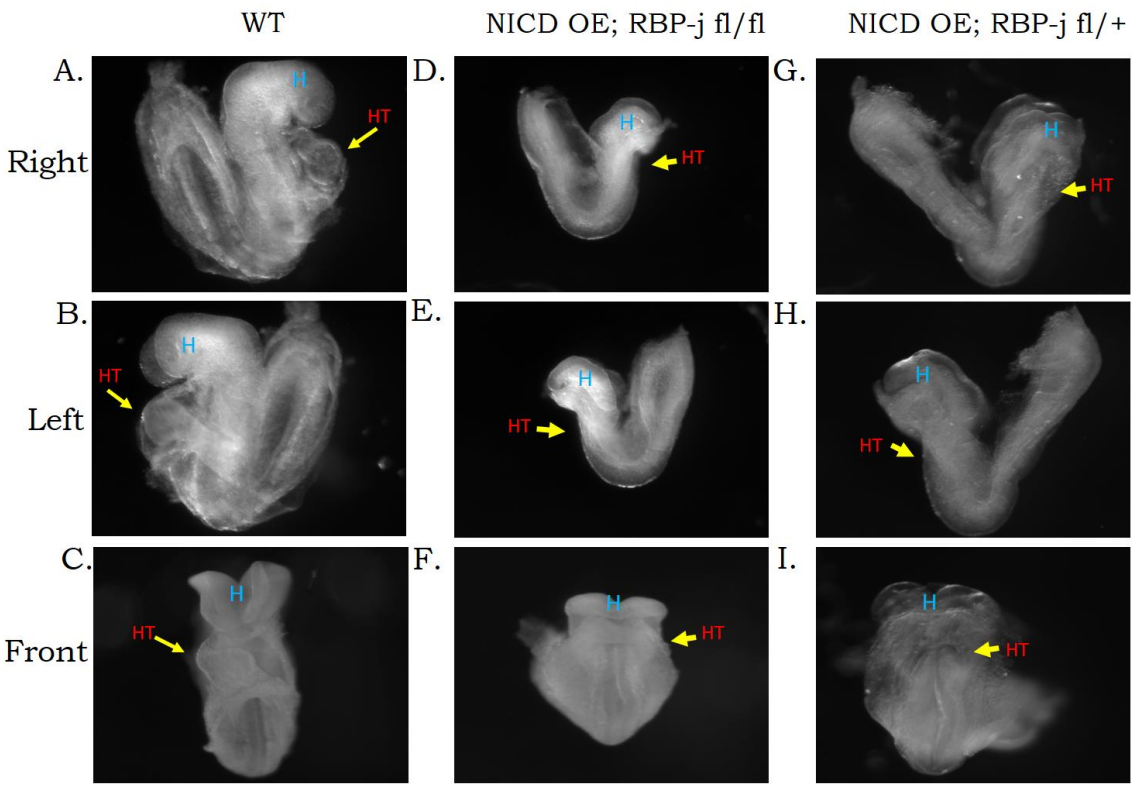

Figure 1: RBP-j independent Notch overexpression eliminates heart in embryonic day 8.0 mice

Orientation of embryo indicated by location of head (H) and heart (HT). Right, left, frontal view of E8.0 mouse control (A-C), Mesp-Cre; NICD OE; RBP-j fl/fl (D-F), and Mesp-Cre; NICD OE; RBP-j fl/+ (G-I) embryos. Arrows point to developing heart and cardiac crescent. n=5.

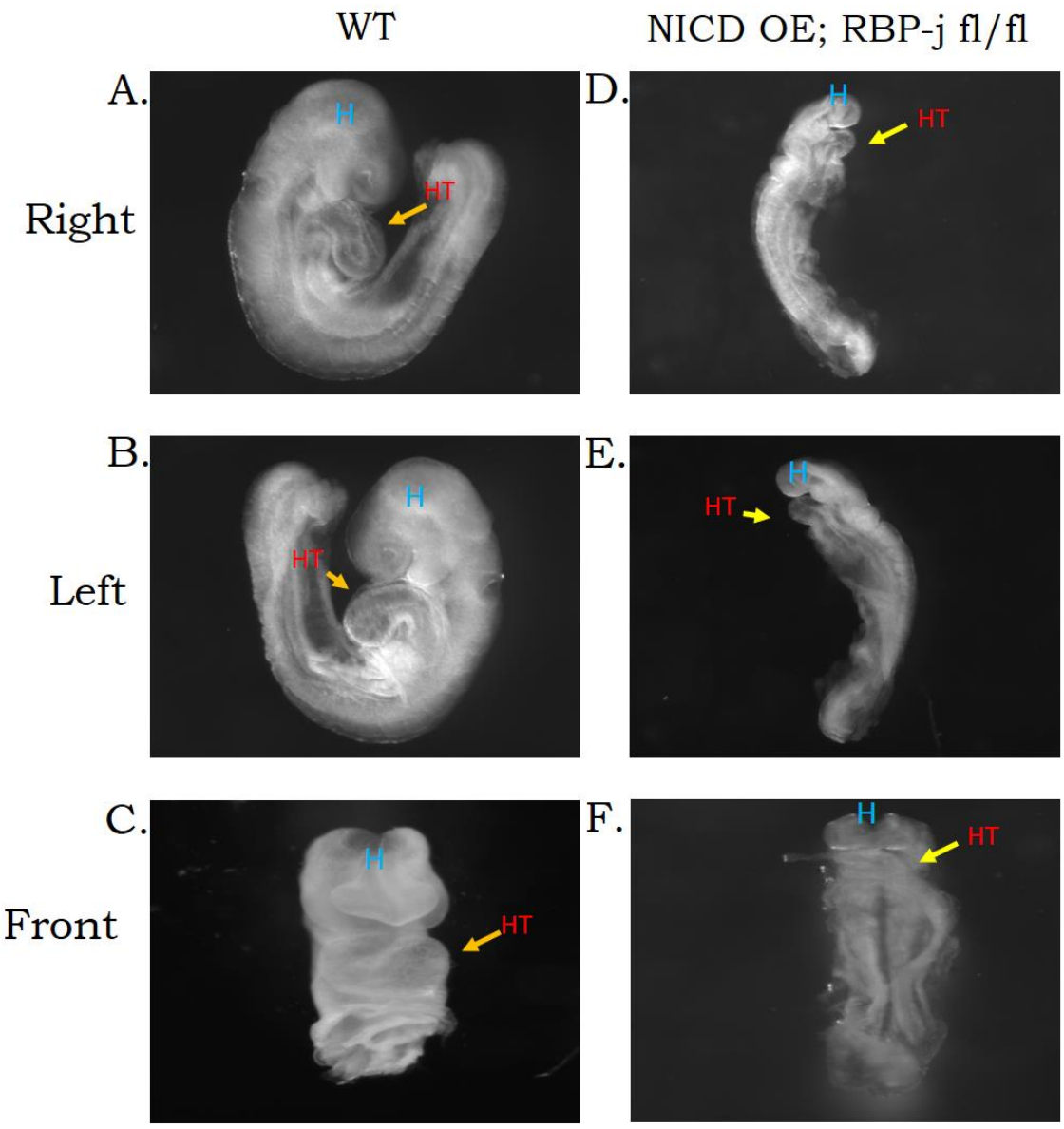

Figure 2: RBP-j independent Notch overexpression leads to decreased heart size in embryonic day 9.0 mice

Orientation of embryo indicated by location of head (H) and heart (HT). Right, left, frontal view of E9.0 mouse control (A-C), Mesp-Cre; NICD OE; RBP-j fl/fl (D-F) embryos. Arrows point to developing heart. n=2.

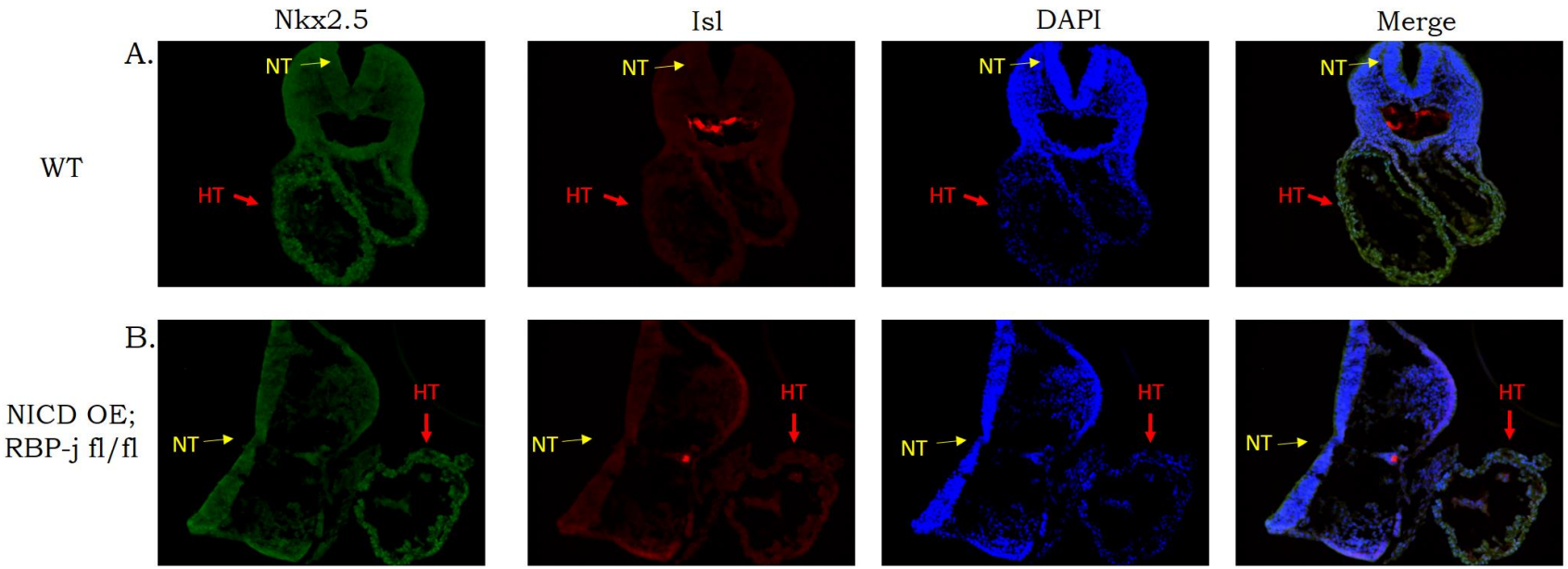

Figure 3: Cross sectioning of E9.0 embryo shows decreased heart size

Cross sections of WT (A) and Mesp-Cre; NICD OE; RBP-j fl/fl (B) E9.0 embryos stained for Nkx2.5(Green), Islet(Red), and DAPI(Blue). Yellow arrows point to location of heart within cross section. Red arrows point to location of neural tube within cross section.

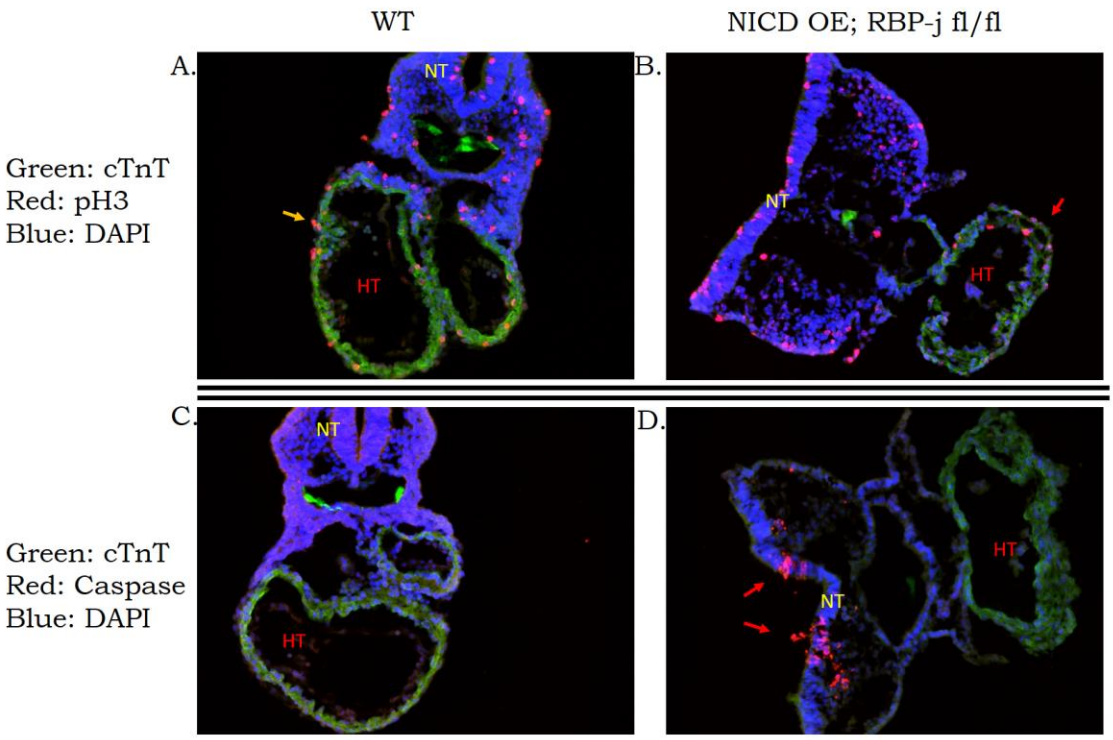

Figure 4: Cross sectioning of E9.0 embryo shows decreased heart proliferation and increased apoptosis

Cross sections of WT (A) and Mesp-Cre; NICD OE; RBP-j fl/fl (B) E9.0 embryos stained for cTnT (Green), pH3(Red), and DAPI(Blue). Arrows point to areas of pH3 positive staining. Cross sections of WT (C) and Mesp-Cre; NICD; RBP-j fl/fl (D) E9.0 embryos stained for cTnT (Green), Caspase (Red), and DAPI (Blue). Arrows point to areas of Caspase positive staining.

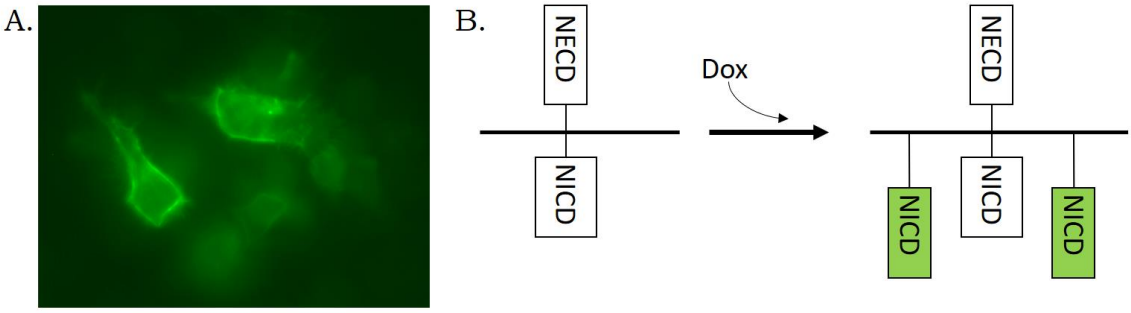

Figure 5: Tethered-Notch-GFP localizes in plasma membrane

A. Fluorescence microscopy image at 40x magnification of Tethered-Notch expressing cells. B. Experimental model used in *in vitro* experiments

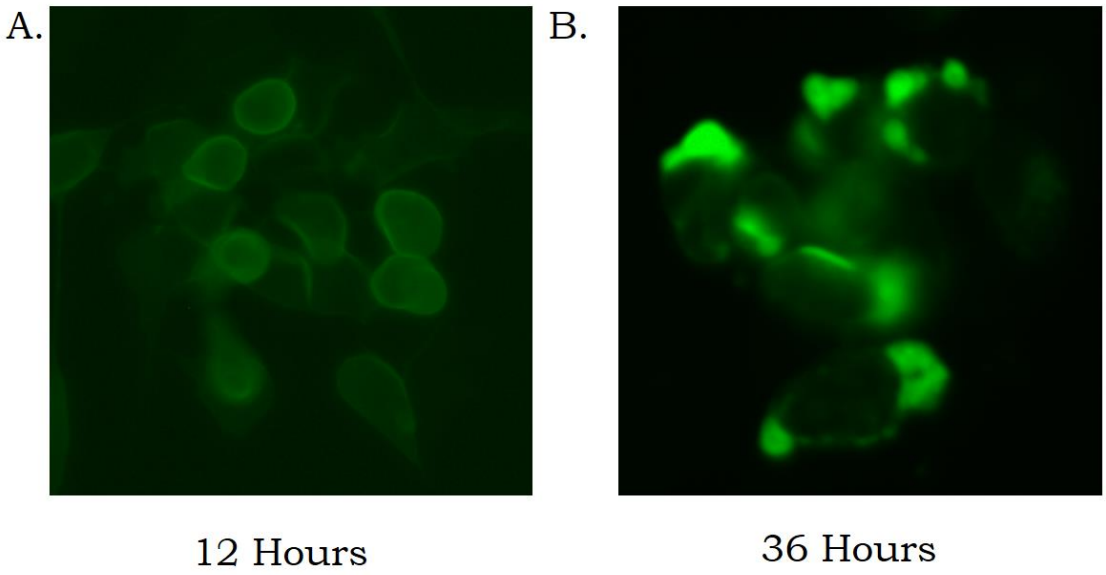

Figure 6: Tethered-Notch-GFP localizes asymmetrically in plasma membrane

Representative images of cells treated for 12 hours (A) or 36 hours (B) with doxycycline.

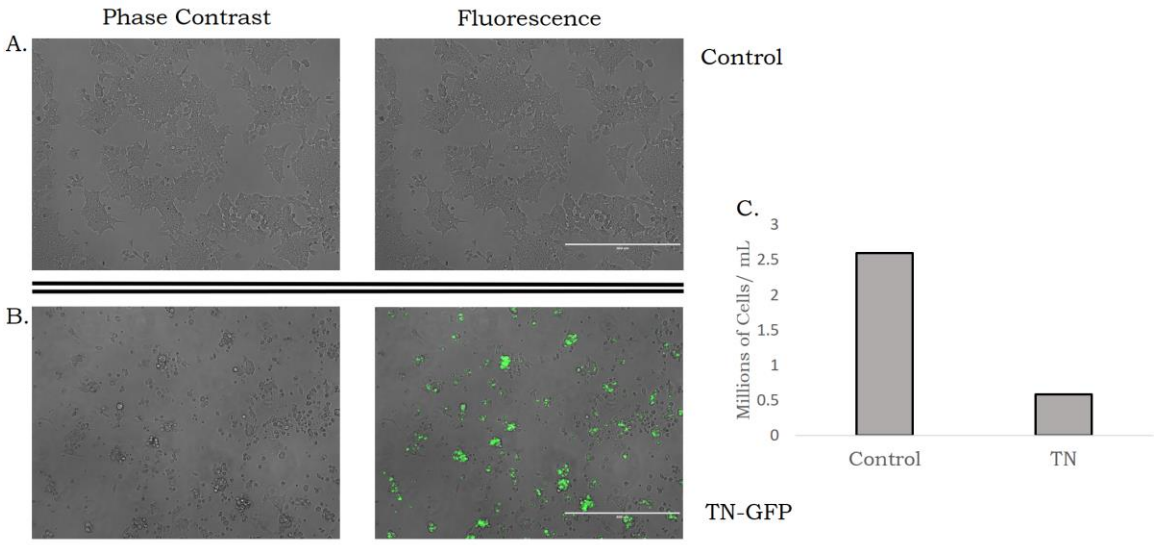

Figure 7: Cell number decreases in Tethered-Notch overexpressing cells.

Representative phase and phase/fluorescence overlay images of (A) control and (B) TN-GFP overexpressing cells after 60 hours in Lif media. C. Cell number quantification of both control and experimental group.

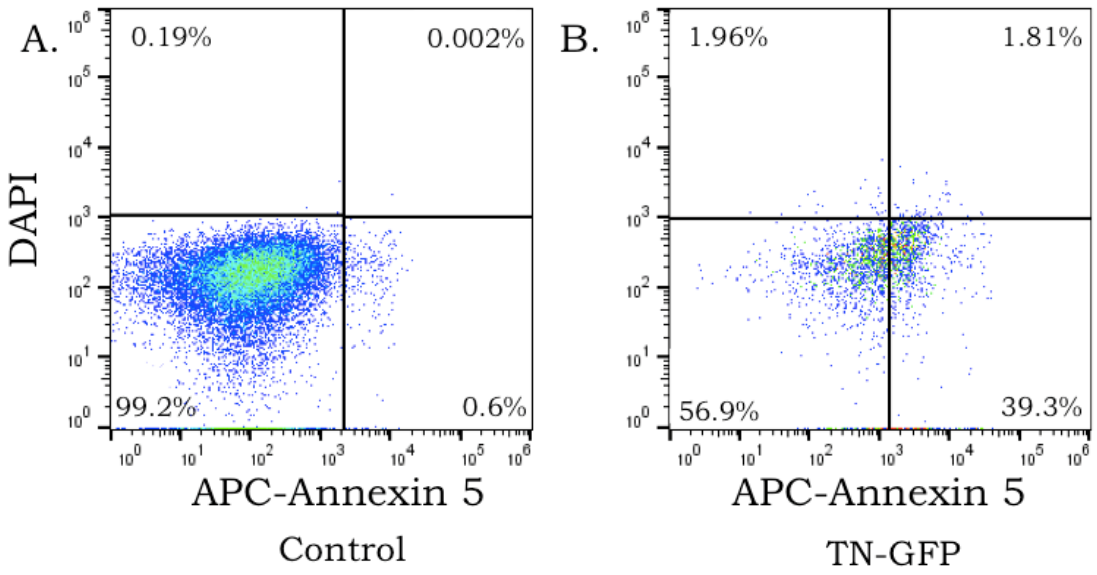

Figure 8: Tethered-Notch overexpression induces apoptosis in Lif media

Flow cytometric analysis depicting apoptosis levels in control (A) and TN overexpressing cells (B). X-axis measured apoptosis using APC-Annexin V antibody and Y-axis measured cell death using DAPI stain.

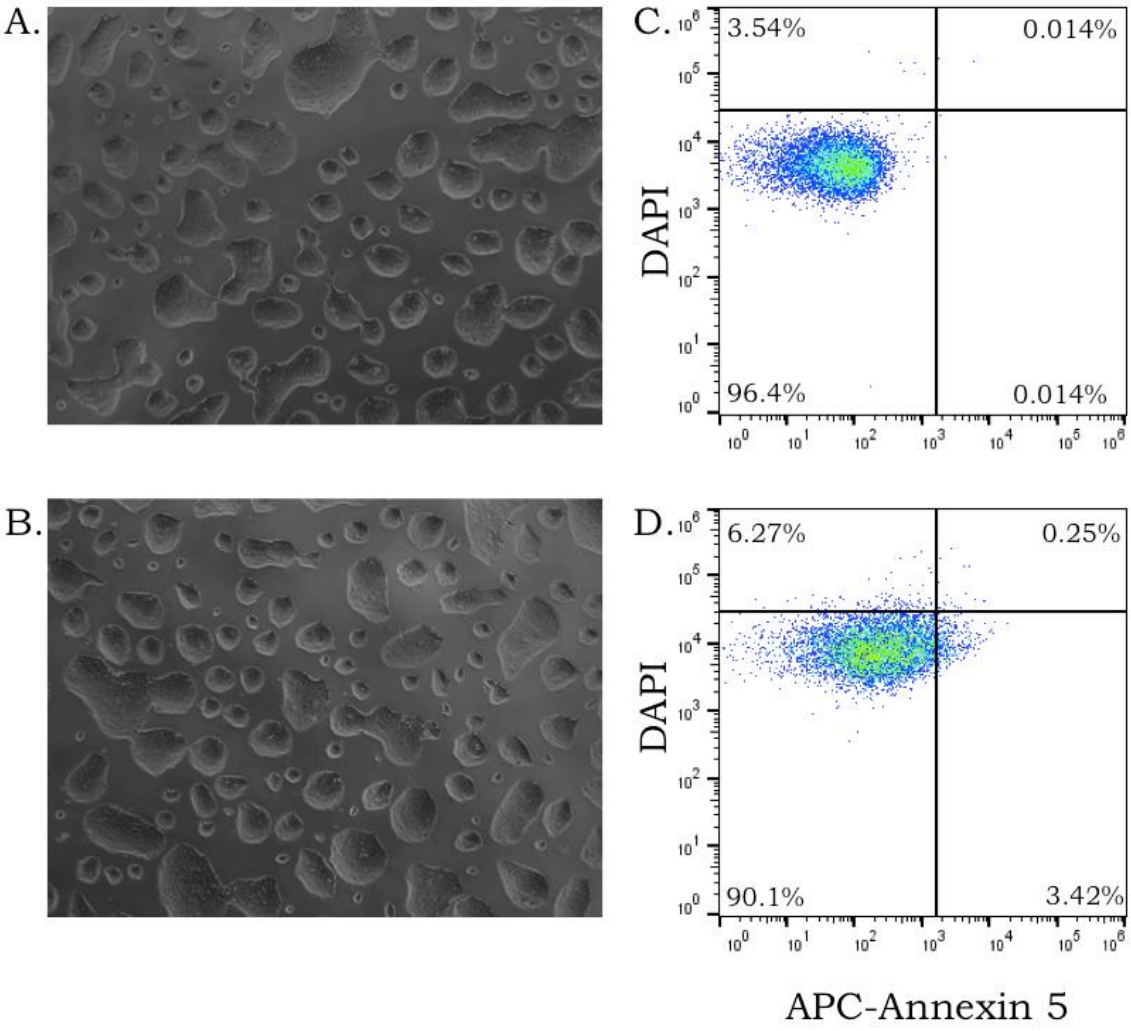

Figure 9: 2i media decreases apoptosis of Tethered-Notch overexpressing cells.

Representative phase contrast images of control (A) and TN over-expressing cells (B). Flow cytometric analysis depicting apoptosis levels in control (C)and TN overexpressing cells (D).

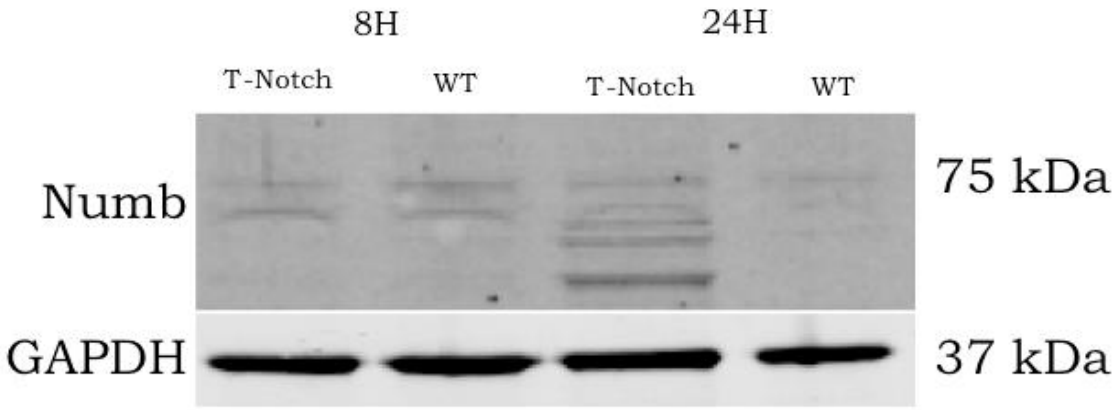

Figure 10: Western Blot of Tethered-Notch overexpressing cells anti-Numb

Western Blot depicting Numb protein levels after 8 and 24 hours of doxycycline treatment.

References:

1. Heery, Emily, Aisling Sheehan, Alison While, and Imelda Coyne. "Experiences and Outcomes of Transition from Pediatric to Adult Health Care Services for Young People with Congenital Heart Disease: A Systematic Review." *Congenital Heart Disease*, 9 Feb. 2015.

2. Escot, S, C Blavet, S Hartle, JL Duban, and C Fournier-Thibault. "Misregulation of SDF1-CXCR4 signaling impairs early cardiac neural crest cell migration leading to conotruncal defects." *Circ Res*, 16 Aug. 2013.

3. Hami, D, AC Grimes, HJ Tsai, and ML Kirby. "Zebrafish cardiac development requires a conserved secondary heart field." *Development*, June 2011.

4. Buikema, JW, AS Mady, NV Mittal, A Atmanli, and IJ Domian. "Wnt/β-catenin signaling directs the regional expansion of first and second heart field-derived ventricular cardiomyocytes." *Development*, Oct. 2013.

5. Luxan, G, G D'amato, D MacGrogan, and JL de la Pompa. "Endocardial Notch Signaling in Cardiac Development and Disease." *Circ Res*, 8 Jan. 2016.

6. Garside, VC, AC Chang, A Karsan, and PA Hoodless. "Co-ordinating Notch, BMP, and TGF-β signaling during heart valve development." *Cell Mol Life Sci*, Aug. 2013.

7. Shenje, LT, P Andersen, H Uosaki, L Fernandez, and C Kwon. "Precardiac deletion of Numb and Numblike reveals renewal of cardiac progenitors." *Elife*, 24 Apr. 2014.

8. Sulistio, E Unpublished 2017

9. Gaiano, N, and G Fishell. "The role of notch in promoting glial and neural stem cell fates." *Annu Rev Neurosci*, 25 Mar. 2002.

10. MacGrogan, D, L Luna-Zurita, and JL de la Pompa. "Notch signaling in cardiac valve development and disease." *Circ Res*, 8 Jan. 2016.

11. Liu, ZJ, T Shirakawa, Y Li, A Soma, and M Herlyn. "Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis." *Mol Cell Biol*, 23 Jan. 2003.

12. Morgan, TH. "The Theory of the gene." *The American Naturalist*, 1917.

13. Lieber, T, S Kidd, E Alcamo, V Corbin, and MW Young. "Antineurogenic phenotypes induced by truncated
Notch porteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei." *Genes & Development*, Oct. 1993.

14. Acosta H, et al. “Notch destabilizes maternal beta-catenin and restricts dorsal-anterior development in Xenopus.” *Development*, 2011

15. Brennan K, et al. “Wingless modulates the effects of dominant negative notch molecules in the developing wing of Drosophila.” *Dev Biol*, 1999.

16. Liang, Q, C Xu, X Chen, X Li, and N Sun. "The roles of Mesp family proteins: functional diversity and redundancy in differentiation of pluripotent stem cells and mammalian mesodermal development." *Protein Cell*, Aug. 2015.

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Publications:

Uosaki, Cahan, Lee, Wang, **Miyamoto**, et al. "Transcriptional Landscape of Cardiomyocyte Maturation" *Cell Reports* Nov 15, 2012.