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Involvement of Vav2 in the EphA4 signaling pathway of *Xenopus laevis* as a possible Rho regulator

Abstract

The purpose of this research is to shed light on the involvement of the Vav2 guanine nucleotide exchange factor in the *Xenopus laevis* EphA4 signaling pathway. In the first phase of the project, the presence of Vav2 mRNA within seven different *Xenopus laevis* embryogenic stages was detected via embryogenic stage collection, retrotranscription, PCR, using forward and reverse Vav2 primers, agarose gel electrophoresis, and UV gel visualization. In the second phase of the project, Western Blotting techniques were used to determine the presence of Vav2 protein in *Xenopus laevis* embryogenic stages.

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**Involvement of Vav2 in the EphA4 Signaling Pathway of *Xenopus laevis* As A
Possible Rho Regulator**

by

Jessica Kopala

Abstract

The purpose of this research is to shed light on the involvement of the Vav2 guanine nucleotide exchange factor in the *Xenopus laevis* EphA4 signaling pathway. In the first phase of the project, the presence of Vav2 mRNA within seven different *Xenopus laevis* embryogenic stages was detected via embryogenic stage collection, retrotranscription, PCR, using forward and reverse Vav2 primers, agarose gel electrophoresis, and UV gel visualization. In the second phase of the project, Western Blotting techniques were used to determine the presence of Vav2 protein in *Xenopus laevis* embryogenic stages.

Introduction

The Eph family is the largest family of receptor tyrosine kinases, responsible for regulating cell and tissue interactions. In specific, EphA4 tyrosine kinase has been shown to disrupt cadherin-dependent cell adhesion, and is an active signaling pathway during *Xenopus laevis* embryogenesis (Dodelet and Pasquale, 2000). The target of EphA4 signaling in *Xenopus* embryos is the actin cytoskeleton, and Rho GTPases are also involved in the pathway by linking the receptor to the actin cytoskeleton. Previous studies have shown that inhibition of both Rho and Rac GTPase in *Xenopus* embryos leads to a loss of cell adhesion and blastocoel occlusion (Winning *et al.*, 2002). A link between the EphA4 signaling pathway and RhoA was also uncovered in more recent experiments, connecting the P59fyn tyrosine kinase to the EphA4 pathway. The activation of p59fyn leads to inhibition of RhoA, which in turn leads to blastocoel occlusion (Grabauskiene, 2003). The mechanism by which p59fyn affects Rho activity is currently unknown; however, we hypothesize that the Rho GEF Vav2 is involved. Vav2 is known to activate Rho from the inactive GDP Rho to the active GTP Rho (Grabauskiene, 2003). Active Rho inactivates the EphA4 pathway, so Vav2, which activates Rho, should be inactive during EphA4 signaling.

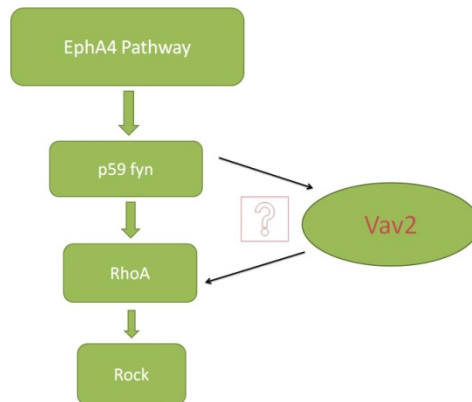


Figure 1. Hypothesized location of Vav2 oncogene in the *Xenopus laevis* EphA4 signaling pathway.

The Vav proto-oncogene family consists of three known subsets, Vav1, Vav2, and Vav3, which function as signal transduction molecules. Vav gene involvement in intracellular pathways has been documented for mitogenesis, cytoskeleton organization, and transcriptional dynamics as well. Specifically, they serve as Rho/Rac GTPase activators during cell signaling, primarily in hematopoietic signaling responses, specifically in the T-cell response (Sauzeau *et al.*, 2006). Vav2 and Vav3 genes are also expressed in non-hematopoietic tissue, as evidenced in Sauzeau and colleagues' experiment concerning Vav2 expression in mice. Disrupting Vav2 expression produced symptoms resembling cardiovascular disease, such as tachycardia, hypertension, and arterial wall defects. Kidney defects were also observed. The hypertensive conditions were believed to arise from chronic renin/angiotensin II and sympathetic nervous system stimulation after Vav2 disruption. Sauzeau and colleagues concluded Vav2 was responsible for maintaining cardiovascular homeostasis in mice (Sauzeau *et al.*, 2006). Inactivation of Vav2 genes in mice resulted in a phenotype resembling human essential hypertension, such as cardiovascular remodeling, and tissue fibrosis, suggesting Vav2 plays a similar role in humans (Sauzeau *et al.*, 2006).

In hematopoiesis, Vav2 serves an essential role in T-cell receptor signal transduction, as evidenced by Denkinger and colleagues (Denkinger *et al.*, 2000). In their experiment, "knockout" mice lacking Vav2 gene expression displayed decreased CD4+ or CD8+ single positive T-cells, and increased CD4+/CD8+ double positive T-cells. Data suggested T-cell receptor signal transmission loss resulted from Vav2 inactivation.

In this experiment, a possible role for the Vav2 gene of *Xenopus laevis* was utilized

in researching the EphA4 signaling pathway. The *Xenopus laevis* Vav2 gene consists of 96,509 nucleotides. Several regions of interest exist within the gene, the first being the “CH” region or the Calponin homology domain which functions in cytoskeleton and signal transduction proteins and is located at 2889 (Pubmed). The CH region serves as an actin-binding surface as well. The second region, known as “RhoGEF,” is a Rac/Rho guanine nucleotide exchange factor and has duplicate domains located at 29100 and 29135. Another region of interest is Protein Kinase C conserved region located at 28911. And, finally, a domain involved in the signal transduction and phosphorylated tyrosine kinase recognition is the Src homology 2 domain located at 29135.

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1 mtegwrqcgr wlidckvlpv dhrvwwpsav vfdlaqalrd gvllcqlhn lspgsidlkd
61 in frpqmsqf lclknirtfl kvchdkfqlr ncdlfdpfdl fdvrdfgkvi aalskisyhn
121 iaqtkgirpf psedttendd diyrsleela dehdlgendt ydvpceeddi yediikvenq
181 qpmkmgmted dkrnccllei retedryyrt lediknyyti plkqilsvqe itniflnlee
241 likvhfnflr tielsvmgg stigqvfldy keklliygey cshieysqkt ldqliatred
301 vrtkleecsl kvqegkfkqlq dllvipmqrv lkyhl11kel lshtadsper htlkealdam
361 qdlamyinev krdketlkki sefqnsienl qvnleefgrp kidgelkvrsv mnvqakqdry
421 lflfdkvviv ckrrgynyel keiellchk mnddpmmnkdk ikkwsygffl ihlqgkqgfq
481 ffcktedmkr kwmeqfemam snikpekata nhhnfqmftf dkttncack mflrgtffyqg
541 yfcslcvgvv hkecletiha ckiggstddv dalitvsgpk mvavqnyfgn pappgkpvl1t
601 fqkddviell rgdpnsqwwe grllltkksq yflsslvepc pveplvirsd yskypwfagn
661 verpqadnll kghvsgtyli rerpaeerf aisikfndev khikvvekn wihiteakkf
721 esllelveyy qmhs1kesfk qldttlkypy ksrtgrscss sprgpvftpr pigtaiaryn
781 faardmrels lregdvvkiy sriggdqgwv kgetngridw fpstyveeeg vq

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Figure 2. Vav2 *Xenopus laevis* protein domains. The calponin homology (CH) domain is shown in red, the RhoGEF domain is shown in blue, the pleckstrin homology (PH) domain is shown in green, the two Src homology 3 (SH3) domains are shown in orange and brown, and the Src homology 2 (SH2) domain is shown in purple.

Xenopus laevis is an excellent animal model for research, and has been utilized in developmental and molecular research for over 60 years (*Xenopus* Genetics, 2009). Several reasons contribute to the widespread use of *Xenopus laevis* as a research model; embryos develop very quickly and externally which allows researchers the ability to observe embryo development. *Xenopus laevis* can be induced to breed throughout the

year, and will produce hundreds to thousands of eggs in one clutch. Figure 3 identifies several *Xenopus laevis* embryonic stages of particular interest to this project.

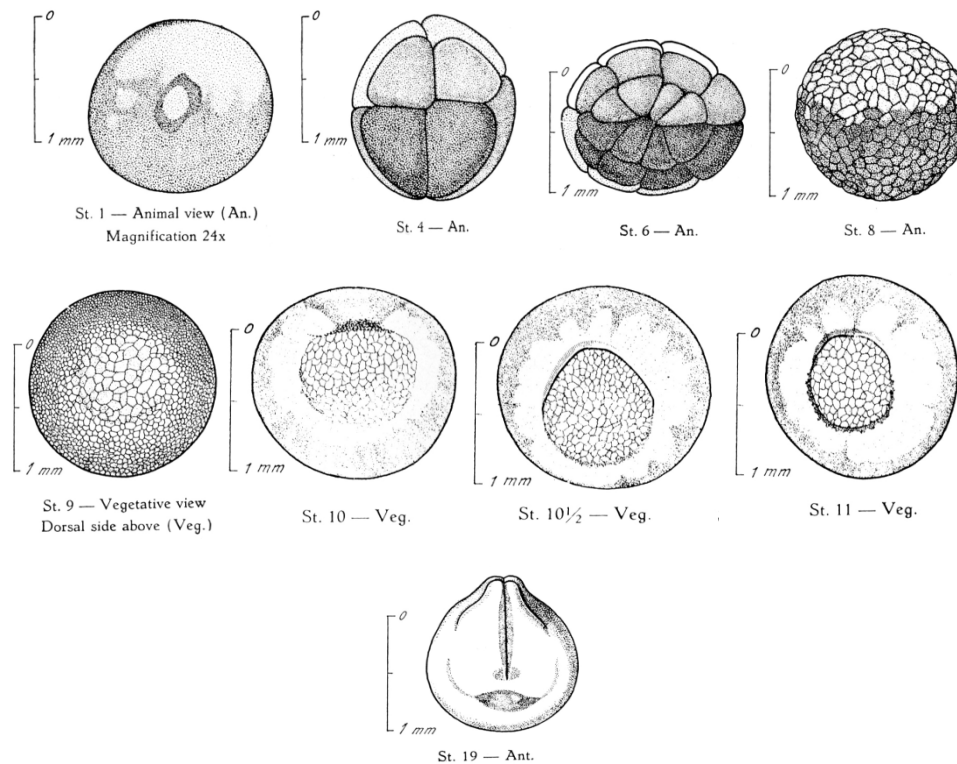


Figure 3. *Xenopus laevis* embryo stages (Cockerill, 1994). Copyright 1994 Pieter D. Nieuwkoop and J. Faber

We propose that the Vav2 signaling protein may be linked in the EphA4 pathway between p59fyn activation and inhibition of RhoA GTPase. However, for Vav2 to be involved in the EphA4 pathway, the protein must be present at stages when EphA4 is functioning. Furthermore, to test the above hypothesis using the same methodology that was used to test the involvement of RhoA (Winning *et al.*, 2003), the Vav2 protein must be present at blastula stage in *Xenopus laevis* embryos. This project will assess Vav2 expression at several embryonic stages using both RNA and protein to determine if the expression of Vav2 is consistent with a role for the protein in EphA4 signaling.

Materials and Methods

Embryo Collection

The first phase of the project consisted of *Xenopus laevis* embryo stage collection, followed by isolation of RNA or protein. Stages were collected over a span of two days from a Petri dish containing fertilized *Xenopus laevis* embryos following the embryo collection protocol (Appendix I). Stages 1, 6, 9, 11, 19, 27, and 33 were collected and stored at -20°C.

Embryo Homogenization and Extraction

Each embryo stage sample was homogenized or disrupted following the embryo homogenization and extraction protocol (Appendix II). Supernatants containing yolk proteins and other insoluble material were discarded to avoid having yolk protein overwhelm other protein bands during electrophoresis. The SDS sample buffer served as an anionic detergent, essentially combining with proteins and stabilizing protein size for SDS PAGE analysis, as well as maintaining a negative protein charge for cleaner results. High temperature denatured proteins, while DTT also denatured proteins by disrupting disulfide bonds.

Cell Disruption and RNA Purification

For RNA samples, staged embryo crude extracts underwent RNA purification, following Initial and Final RNA Purification protocol (Appendices III and IV). RNA purification was necessary in order to remove DNA, which can produce false-positive signals in RT-PCR experimentation, ultimately generating erroneous results.

UV Spectrophotometer Analysis

Following an initial Nucleic Acid Dilution protocol (Appendix V), RNA purified stage samples underwent UV spectrophotometer analysis (Appendix VI) to determine respective A_{260} values for later use in NMR (Appendix VI) calculations pertinent to Reverse Transcription.

Reverse Transcription

The Reverse Transcription protocol was followed using specific amounts of stage-specific RNA as calculated from A_{260} spectrophotometer values (Appendix VII). It is important to mention that the RNA constant value used in calculating stage RNA volumes for Reverse Transcription at 1 A_{260} is 40 μ g/ml, and the dilution factor is 100. RNA-dependent DNA polymerase essentially reverse transcribed single-stranded stage RNA into double stranded cDNA for use in Polymerase Chain Reaction (PCR) analysis, which requires DNA.

RT-PCR Analysis

RT-PCR analysis was used to measure Vav2 mRNA levels in each collected embryo. RNA was isolated from stages 4, 6.5, 8, 10, and 10.5, and underwent reverse transcription, utilizing the enzyme reverse transcriptase and the primer oligo-dT. Oligo-dT functions by annealing to the poly-A tail located at the 3' end of mRNA (Winning, 2010). Staged cDNA underwent PCR analysis in a MJ Designs mini-thermocycler using Vav2 Forward and Vav2 Reverse primers, measuring 19bp and 14bp respectively, which amplified cDNA to more quantifiable levels (Appendix VIII). PCR analysis amplified the DNA samples to measurable amounts able to be visualized by electrophoresis.

Table 1. Thermocycler conditions for PCR Analysis. DNA was denatured to single strands at 95°C, Vav2 primers annealed to complementary regions on DNA at 50°C, and DNA Taq polymerase synthesized complementary DNA strands at 72°C. The process underwent 30 cycles and rested at 4°C until removed from the thermocycler.

Temperature (°C)	Time (minutes)	Cycles
(Denature) 95	1	30
(Anneal) 50	1	30
(Extend) 72	1	30
4	∞	Hold

Agarose Gel Electrophoresis and UV Visualization

PCR-amplified DNA samples underwent Agarose Gel Electrophoresis as defined in Appendix IX. Synthegel was used as a synthetic substitute for Agarose to accommodate small sample DNA fragment sizes. Amplified DNA samples were loaded into gel wells after combining 1/10 volume of 10X gel loading buffer, and underwent electrophoresis. During electrophoresis, the negatively charged DNA fragments traveled through gel pores where they separated depending on individual molecular weight. Gels were stained in ethidium bromide (EtBr) for 15 minutes, followed by destaining in distilled water for 15 minutes. The gel was visualized on a UV lightbox after treatment with ethidium bromide.

Western Blot Analysis

Western Blot Analysis was used to determine the Vav2 protein levels in embryos at stages 4, 6.5, 8, 10, and 10.5.

Table 2. Novel PCR conditions for Forward Vav2 and C-terminal Vav2 primers.

Step	Temperature (°C)	Time (minutes)
1	94	10
2	94	1
3	50	1
4	72	3
5	Go to step 2	39 cycles
6	72	10
7	4	∞

Crude extracts were isolated from embryos using freon to remove yolk proteins that would interfere with Vav2 protein identification. Extracts were then combined with dithiothreitol and protein sample buffer, and heated for 5 minutes at 100°C in order to denature the proteins. Proteins were subsequently separated using SDS-polyacrylamide gel electrophoresis, transferred to a hybond filter, exposed to a primary Vav2 antibody (Vav2 H200), then exposed to a secondary Vav2 antibody (Secondary goat anti-rabbit IgG – AP). Enhanced chemifluorescent substrate (ECF; Amersham) was added, and cleaved by alkaline phosphatase (covalently linked to the secondary antibody) resulting in a fluorescent product able to be detected on a Fuji FLA 3000 fluorimeter.

Results and Discussion

UV Spectroscopy

Resulting UV spectroscopy values for each stage are listed in Table 3. A_{260}/A_{280} ratios around 1.9 indicate that the RNA isolated was of acceptable purity.

Table 3. Nucleic acid analysis values at 260 λ and 280 λ for each stage via UV Spectrophotometer.

Stage #	260 λ (nm)	280 λ (nm)	Ratio (260 λ /280 λ)
1	.259	.137	1.895
6	.131	.068	1.917
9	.226	.120	1.877
11	.226	.120	1.891
19	.080	.042	1.897
27	.176	.095	1.860
33	.100	.054	1.848

PCR Analysis

Specific RNA concentrations were calculated from 260 λ UV spectrophotometer values and used to determine RNA volumes containing 2 μ g to be used for reverse transcription.

$$\text{concentration}(\mu\text{g} / \text{ml}) = (A_{260}) \times (40 \mu\text{g} / \text{ml}) \times 100$$

$$\text{st.11} = (0.226) \times (40 \mu\text{g} / \text{ml}) \times 100$$

$$\text{st.11} = 904 \mu\text{g} / \text{ml}$$

$$\text{vol.RNA} = \frac{2}{904 \mu\text{g} / \text{ml}}$$

$$\text{vol.RNA} = 2.21 \mu\text{l}$$

Figure 4. Equations for calculating stage concentrations (used to determine RNA volumes), followed by RNA volume calculations used for reverse transcription . The calculated RNA volumes are listed in Table 4, along with respective distilled water volumes.

Table 4. Specific distilled water and purified stage RNA volumes used in Reverse Transcription.

Stage #	Vol. RNA (μl)	Vol. distilled water (μl)
1	.450	9.55
6	.383	9.62
9	.617	9.38
11	.900	9.10
19	1.450	8.55
27	1.558	8.44
33	1.240	8.76

Agarose Gel Electrophoresis

Based on the resulting gel image (Fig. 5), Vav2 mRNA exists in all embryonic stages of *Xenopus laevis*. In addition to the specific bands expected from amplification, several other bands appeared on the gel. Three different sets of bands are present for each stage, as seen in Fig. 5. The first set of bands occurs at ~400bp, which is believed to signify Vav2 expression in all embryonic stages. The second set occurs at ~450bp and

bands appear to increase in thickness and clarity as the stages increase from stage 1 to stage 33. The third set occurs at ~500bp, and the bands appear uniform as the stages increase. The two sets of bands aside from the 400bp bands could be due to the presence of dimers, trimers, tetramers, etc. which formed during the RT-PCR annealing process. The dimers and trimers can confound data by hindering proper BP and forming new bands on the gel image. Annealing temperature can be increased in an attempt to prevent astringency between primers that could then form dimers or trimers. The presence of dimers and trimers could also be due to increased C/G content.

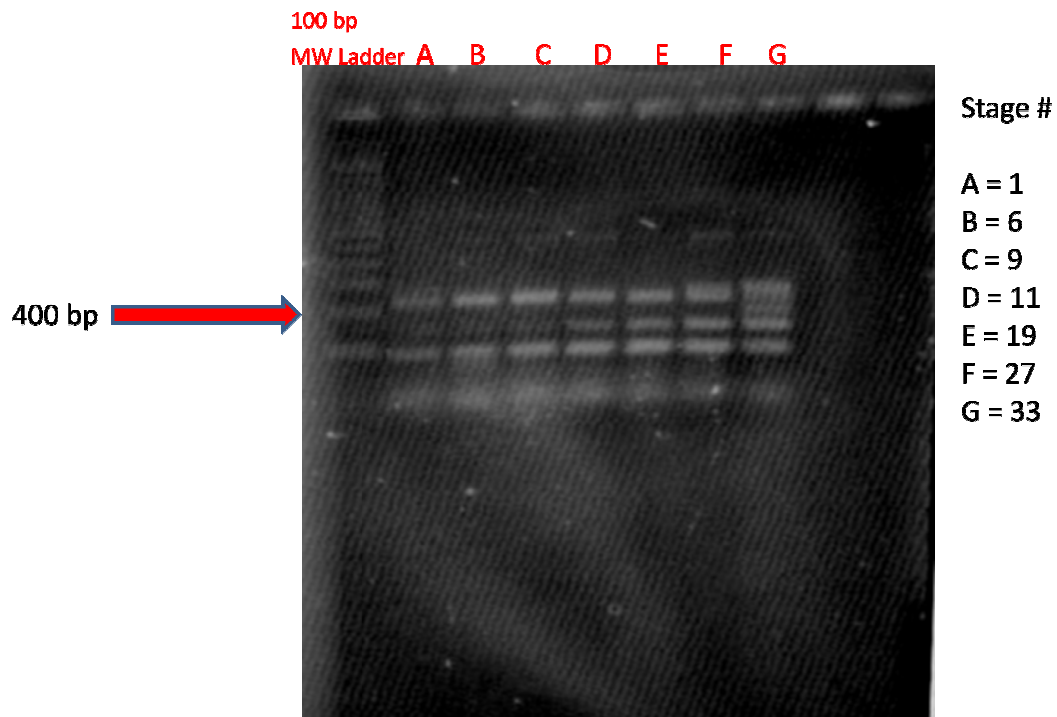


Figure 5. UV Visualized agarose gel image for stages 1, 6, 9, 11, 19, 27, and 33. All stages express bands at 400bp, indicating Vav2 expression.

Western Blot Analysis

Subsequent Western Blot analysis using Vav2 H200 primary antibody and Vav2 goat anti-rabbit IgG-AP secondary antibody was performed on protein extracts from stages 4.5, 6, 8, 10, and 10.5. The resulting immunoblot indicated the presence of the Vav2

protein at 64 kDa (Fig 6).

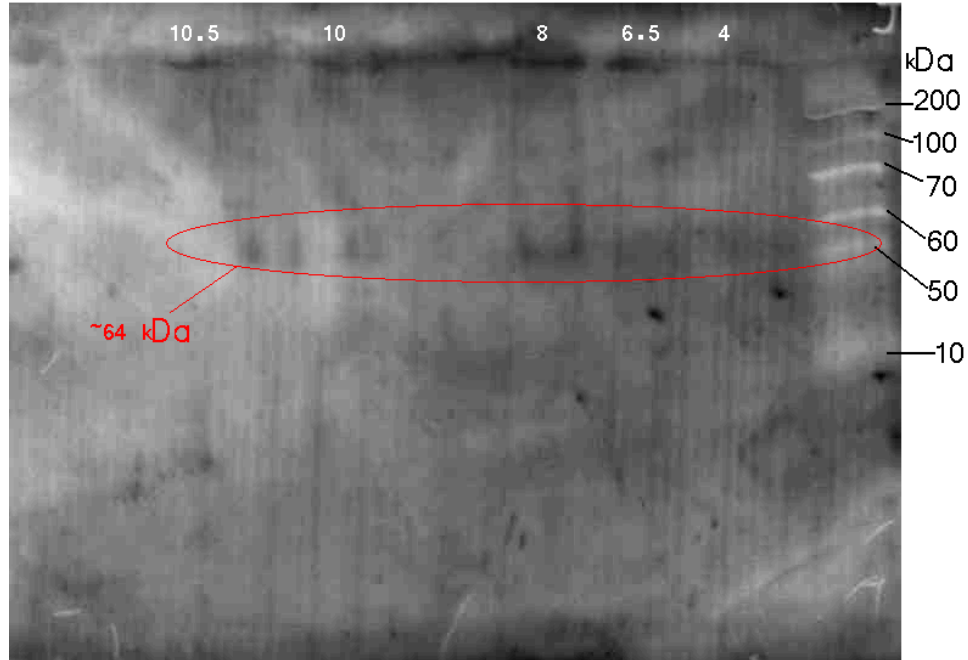


Figure 6. Vav2 Western Blot. Stages 4, 6.5, 8, 10, and 10.5 all exhibit bands at approximately 64 kDa.

Conclusion

The goal of this project was to determine the expression pattern of the Vav2 protein in the embryonic stages of *Xenopus laevis*. Although the role of Vav2 in the EphA4 signaling pathway of *Xenopus laevis* was not directly assessed, its presence was detected in embryonic stages, particularly during gastrulation (stages 10 and above) when the EphA4 signaling pathway is active, consistent with a role for Vav2 in the EphA4 signaling pathway. Further protein-level analysis is necessary to validate these results.

Future experimentation should consist of generating constitutively active and dominant inhibitory forms of the Vav2 protein for incorporation into the EphA4 signaling pathway, followed by subsequent monitoring of embryonic development via light and scanning electron microscopy with a focus on possible dissociation and blastocoel occlusion.

Subsequent observation of Vav2 behavior in EphA4 signaling may establish a greater understanding of Vav2 function as a proto-oncogene in various cancers and cardiovascular health. Further experimentation may identify Vav2 as a candidate for therapeutic treatment of cancers and cardiovascular ailments, perhaps generating a less toxic alternative to current chemotherapeutic agents with low selective toxicity.

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Appendix (Detailed Materials and Protocols)

I. Embryo Collection

1. Label one sterile centrifuge tube with embryonic stage number and the date.
2. Transfer ~20 embryos to the tube using a pipette with a wide mouth.
3. Remove excess liquid from the tube.
4. Repeat steps 1-4 for successive stages.
5. Store stages in freezer at 4°C for future use.

II. Embryo Homogenization and Extraction

1. Make homogenization solution:
 - a. 75µL HEPES
 - b. 225µL NaCl
 - c. 15µL EDTA
 - d. 15µL EGTA
 - e. 15µL NP-40
 - f. 1155µL distilled water (from EASYpure RF compact ultrapure water system)
2. Add 134µL homogenization solution to each tube containing embryo stages.
3. Use metal rod as a pestal and “smash” embryos with the homogenization solution.
4. Extract samples with 134µL 1,1,2 trichloro-trifluoro-ethane.
5. Vortex for 5-10 seconds.
6. Centrifuge samples for 15 min. at 4°C at 14,000 rpm.
7. Transfer supernatant to fresh centrifuge tubes and label.
8. Add 67µL NEB buffer to each sample.
9. Add 6.8µL 30X DTT to each sample.

10. Vortex samples for 3-5 seconds.
11. Boil samples for 5 minutes at 100°C.
12. Store samples at -20°C.

III. Cell Disruption and Initial RNA Purification

1. Homogenize collected embryo stages with 1mL TRI reagent using 1000µL pipette, and mix by pipetting.
2. Incubate tubes for 5 minutes at room temperature.
3. Add 200 µL of chloroform to each centrifuge tube.
4. Vortex tubes for 15 seconds at full speed.
5. Incubate tubes for 5 minutes at room temperature.
6. Centrifuge samples at 12,000 X g for 10 minutes at 4°C .
7. Transfer 400µL aqueous phase to new 1.5mL micro centrifuge tube.

IV. Final RNA Purification

1. Add 200µL 100% ethanol to each sample from the initial RNA purification and vortex for 5 seconds.
2. Pass sample through filter cartridge.
3. Centrifuge at 12,000 X g for 30 seconds at room temperature, remove flow through, and replace the filter.
4. Wash filter with 500µL wash solution and centrifuge for 30 seconds at room temperature.
5. After removing flow through, add another 500µL wash solution to each tube and centrifuge for 30 seconds at room temperature.
5. Remove flow through and centrifuge once more under the same conditions to remove

any excess solution.

6. Remove flow through and place filter into RNase-sterile centrifuge tubes and label the caps with the stage number.

7. Elute RNA with 100 μ L Elution buffer and incubate for two minutes at room temperature.

8. Centrifuge for 30 seconds at 12,000 X g.

9. Remove filter, cap tubes, and store in freezer at -70°C

V. Dilution for Nucleic Acid Analysis for UV Spectrophotometry

1. Prepare 90 μ L of distilled water to 1 μ L of each sample from the final RNA purification and place into separate test tubes.

VI. UV Spectrophotometer Analysis

1. Set the spectrophotometer to read 260 λ :280 λ setting.

2. Insert the blank the cuvette containing distilled water only and blank the spectrophotometer, then insert the cuvette containing 100 μ L sample, pipetting to mix before placing in the spectrophotometer.

3. Repeat step 2 for every sample, making sure to use kimwipes to clean the cuvettes before placing them into the spectrophotometer.

4. Return samples to freezer for storage.

VII. Reverse Transcription

1. Add the following components to separate micro-centrifuge tubes for each RNA sample:

- a. 1-2 μ g sample RNA
- b. 2 μ L oligo(dT)
- c. 12 μ L Nuclease-free water

2. Mix the samples using a pipette, vortex the samples for 5 seconds, and heat for three minutes at 70-85°C.
3. Place the tubes on ice, vortex for another 5 seconds, and place on ice again.
4. Add the following components to each RNA sample:
 - a. 4µL dNTP mix
 - b. 1µL RNase Inhibitor
 - c. 1µL MMLV-RT+
5. Mix the contents of each tube with a pipette, vortex for 5 seconds, and incubate at 42-44°C for 1 hour.
6. Incubate at 92°C for 10 minutes.
7. Store samples at -20°C for future use.

VIII. PCR Analysis

1. Create PCR Mastermix:
 - a. 20µL 10X PCR Buffer -Mg
 - b. 4µL 10mM dNTP
 - c. 6µL 50mM MgCl₂
 - d. 10µL Forward-Vav2 Primer
 - e. 10µL Reverse -Vav2 Primer
 - f. 1µL template DNA
 - g. 1µL Taq DNA Polymerase
 - h. 141µL distilled water
2. Add components into a 0.5mL microcentrifuge tube on ice.

3. Cap tubes and centrifuge for 5 seconds.
4. Incubate samples in a thermocycler at 94°C for 3 minutes to denature the template.
5. Perform 30 cycles of PCR amplification under the following conditions in the minithermocycler:
 - a. Denature at 95°C for 1 minute
 - b. Anneal at 50°C for 1 minute
 - c. Extend at 72°C for 1 minute
6. Incubate at 72°C for five minutes and maintain reaction at 4°C .
7. Store samples at -20°C until ready for use.

IX. Agarose Gel Electrophoresis and UV Visualization

1. Weigh out 0.33g Synthegel into 125mL flask
2. Add 1.35mL ethanol with a pipette, add to flask and swirl to mix.
3. Add 0.7g Agarose.
4. Add 50 mL 1XTAE
5. Microwave at 1 minute increments until gel becomes clear and ethanol evaporates (~2 minutes).
6. Cool flask by swirling the base under cool water taking care to avoid pouring water into the flask.
7. Pour gel into the electrophoresis chamber and wait for gel to set (~ 1 hour).
8. Add 5µL loading dye to each sample and mix by pipetting.
9. Load 20µL of each sample to separate gel wells.
10. Load 20µL of the 100bp molecular weight ladder into a separate gel.
11. Run electrophoresis chamber at ~87 Volts for 1 hour.

12. Incubate gel in ethidium bromide for ~ 5minutes making sure to use gloves and avoid contact with the ethidium bromide.

13. Incubate the gel in distilled water destaining solution for ~5 minutes.

14. Visual the gel image using UV visualization.

X. Western Blot

A. Prepare the plates:

1. Clean both plates with windex and isopropanol
2. Align the plates in the loading chamber.

B. Assembling Separating Gel:

1. Add the following components to clean test tube:
 - a. 2.0mL distilled water
 - b. 1.25mL 1.5M TRIS HCl pH 8.8
 - c. 1.67mL Acrylamide/Bis (30%)
 - d. 50 μ L 10% SDS
 - e. 25 μ L 10% APS
 - f. 10 μ L TEMED
2. Add the separating gel mixture to the Western Blot plates using a Pasteur pipette and top off the gel with a thin layer of butanol.
3. Allow the separating gel to set for ~20 minutes.
4. Drain the butanol layer from the separating gel and rinse with distilled water.
5. Assemble the stacking gel components in a clean test tube:
 - a. 1.2 mL distilled water
 - b. 0.5 mL 0.5M TRIS HCl pH 6.8

c. 0.26 mL Acrylamide/Bis

d. 20 μ L 10% SDS Stock

e. 10 μ L 10% APS

f. 4 μ L TEMED

6. Invert the stacking gel mixture GENTLY ~5 times to mix

7. Add the stacking gel solution using a fresh Pasteur pipette until the gel reaches the top of the smaller glass plate.

8. Place the gel comb in at an angle to avoid air bubbles under the teeth.

9. Store the gel in a Tupperware container in a fridge overnight with several damp paper towels and wrap the plates in parafilm to avoid drying out.

D. Running the Western Blot Gel:

1. Measure 350mL of 1X Protein Running Buffer.

2. Boil purified RNA samples and pre-stained fluorescent 1kB molecular weight ladder at 100°C for 5 minutes in a Fisher Scientific Dry Bath Incubator.

3. Assemble the Western Blot chamber and add 125mL 1X Protein Running Buffer (up to the level of the lower glass plate).

4. Pour the remaining 1X Protein Running Buffer outside of the chamber.

4. Load 10 μ L of each sample into separate gel wells.

5. Load 10 μ L of the fluorescent 1kb MW ladder into a separate well.

6. Run the blot at 200V for ~45 minutes or until the samples reach the very bottom of the gel.

E. Electrophoretic Transfer:

1. Assemble the Transfer Buffer:
 - a. 3.03g TRIS
 - b. 14.4g Glycine
 - c. 200mL MetOH
 - d. Distilled water to IL total volume
2. Cut a piece of Hybond-P to ~60 X 84 mm and label one corner with a pencil making sure to handle the Hybond-P with gloves.
3. Soak Hybond-P in methanol for 10 minutes.
4. Soak Hybond-P in distilled water for 10 minutes.
5. Soak Hybond-P in Transfer buffer until ready for use.
6. Pour Transfer buffer into Electrophoresis Transfer chamber until it reaches half the chamber volume.
7. Assemble the transfer “sandwich” on the black end of plastic tray:
 - a. Soak Fiber pad in Transfer buffer and lay onto tray.
 - b. Soak filter paper in Transfer buffer and lay onto fiber pad.
 - c. Soak gloved fingertips in Transfer Buffer and place Western Blot gel facing up on the filter paper, gently smoothing out any air bubble.
 - d. Place Hybond-P face-down onto the gel, soaking gloved fingertips in Transfer buffer and gently smoothing out any air bubbles.
 - e. Soak second filter pad in Transfer buffer and place on Hybond-P.
 - f. Soak a second Fiber pad in Transfer buffer and place onto

Hybond-P.

g. Fold the clear plastic side of the tray over the “sandwich” and lock it together.

8. Place the “sandwich” into the electrophoresis chamber making sure the place the black side of the plastic tray towards the black side of the chamber.

9. Add the ice pack and pour Transfer buffer to maximum capacity into the chamber.

10. Run the transfer at 150mA for 1 hour.

F. Immunoblotting:

1. Assemble 1L PBS:

a. 100mL 10X PBS

b. 900mL distilled water

2. Add 500 μ L Tween-20 wash to the 1X PBS and invert to mix.

3. Measure out 20mL PBS-Tween solution into a plastic tray and add 1g Albumin and mix.

4. Place the Hybond-P into the PBS-Tween Albumin solution and incubate in the fridge until next lab period.

5. Rinse the Hybond-P first with distilled water, then twice in PBS-Tween (PBST) for 15 minute increments.

6. Rinse the Hybond-P twice more in PBST for 5 minute increments.

7. Dilute the Vav2 H200 primary antibody with PBST:

a. 100 μ L Vav2 H200 Antibody

b. 10mL PBST

8. Incubate the blot in the primary antibody solution for 1 hour at room temperature on a DAIGGER orbital shaker.

9. Repeat steps 1-6 and dilute the secondary Vav2 antibody:

a. 5 μ L Vav2 goat anti - rabbit IgG-AP

b. 10mL PBST

10. Incubate the Hybond-P in the secondary Vav2 antibody solution at room temperature on a DAIGGER orbital shaker for 1 hour.

11. Repeat steps 1-6 and store the Hybond-P in PBST until next lab.

G. Western Blot Visualization:

1. Place blot protein-side up on plastic wrap

2. Add 1.2mL ECF reagent onto the blot surface, distributing the ECF reagent as evenly as possible over the blot surface.

3. Incubate blot in ECF solution for 30 minutes.

4. Drain blot and lay on Whatman Filter Paper (#1 Qualitative) for 30 minutes.

5. Visualize the Western Blot using the FUJIFILM FLA-3000 infrared Microarray Scanner and FUJIFILM Image Gauge program under the following conditions:

a. Sampling Area: Free

b. Gradation: 65536 (16 bit)

c. Resolution: 50

d. Sensitivity: F1

