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Elucidating the Functional Role of VDAC2 Mitochondrial Protein on Early Zebrafish Heart Development

Spring 2023 MCDB 198C

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Abstract

Voltage dependent anion channel 2 (VDAC2) is a channel protein that is widely expressed on the outer mitochondrial membrane (OMM). Despite the recent discovery of VDAC2's ability to suppress aberrant Ca^{2+}-induced cardiac arrhythmia, very little study has been done to elucidate the early functional role of VDAC2 and whether calcium trafficking is necessary for an early developing heart. Based on the knowledge we had on VDAC2's Ca^{2+} trafficking property to rescue *ncx1* mutant fish, we hypothesized that the Ca^{2+} trafficking property of VDAC2 is not only crucial for rescuing cardiac arrhythmia, but it is also crucial for the normal development and function of an early heart by maintaining calcium homeostasis early on.

We tested our hypothesis by first creating the VDAC2$^{-/-}$ zebrafish line. VDAC2$^{-/-}$ embryos exhibited cardiac arrhythmia as early as day 6 post fertilization. We transgenically induced the wildtype version of VDAC2 (*Tg:cmlcGal4EcR-EGFP-UAS-vdac2-FLAG*) and the loss-of-function mutant version of VDAC2 (*Tg:cmlcGal4EcR-EGFP-UAS-vdac2E73Q-FLAG*) that cannot traffick calcium into our VDAC2$^{-/-}$ zebrafish embryos. Rescue assessment showed that transgenic induction of wildtype VDAC2 could indeed rescue VDAC2$^{-/-}$ zebrafish embryos, while the mutant version of the protein that has a substitution of its 73rd amino acid to glutamine cannot rescue. On the other hand, transgenic induction of a gain-of-function VDAC3 (*Tg:cmlcGal4EcR-EGFP-UAS-vdac3Q73E-FLAG*) that can traffick calcium could indeed rescue VDAC2$^{-/-}$ embryos as well. This result underscores the importance of mitochondrial Ca^{2+} trafficking via VDAC2 with the SR in cardiomyocytes for proper functioning of an early normal heart.

Introduction

Mitochondria are small membrane bound organelles in almost every eukaryotic cell[1]. They are widely known to be involved in the bioenergetic process in cells; however, decades of research showed that mitochondria are also involved in a variety of cellular processes including apoptosis, cellular metabolism, and cellular Ca^{2+} signaling[2]. Recent studies highlighted the strong interplay between mitochondria and the sarcoplasmic reticulum (SR), where the highly-regulated cross talk is responsible for normal physiological processes[2]. In the heart, the interaction between mitochondria and the SR is especially important for maintaining calcium homeostasis for proper heart contraction[3]. This tight SR-mitochondria crosstalk is maintained through a tethering mechanism made up of a variety of SR, inner and outer mitochondrial membrane proteins at the specific subdomain of the SR membrane called mitochondria-associated membrane (MAM)[3]. One such membrane protein involved in SR-mitochondria crosstalk is the voltage-dependent anion channels (VDACs) found on the outer mitochondrial membrane[2].

There are three isoforms of VDACs: VDAC1, VDAC2, and VDAC3. They are known to transport an array of ions and metabolites in and out of the mitochondria, but despite their similarity in structures and sequences, they have been shown to have different biological functions[4]. In zebrafish cardiomyocytes, VDAC2 was shown to have Ca^{2+} trafficking property governed by its 73rd amino acid, where the overexpression of VDAC2 could suppress cardiac arrhythmia from Ca^{2+} overload in *tremblor/ncx1h* mutant zebrafish[2,5]. Despite the importance of VDAC2 in Ca^{2+} trafficking in adult zebrafish cardiomyocytes, little is known about the role of VDAC2 in early zebrafish cardiac function.

In our laboratory, we observed cardiac arrhythmia in VDAC2 knockout embryos as early as day 6 post fertilization (dpf). This suggests that VDAC2 may play an important role in developing normal cardiac function in early zebrafish. Our study seeks to elucidate the specific properties of VDAC2 that are crucial for the development of normal cardiac function. By combining the knowledge we had on VDAC2's calcium trafficking property to rescue *tremblor/ncx1h* mutant, **we believe that VDAC2, specifically its Ca^{2+} trafficking property, may be critical for normal zebrafish cardiac function by maintaining proper calcium homeostasis during early cardiac formation.** By expressing versions of the protein that either can or cannot traffic calcium, we will be able to assess the ability of each to rescue heart rhythm in the knockout animal.

Our experiment established a genetic platform to test different isoforms of the VDAC proteins. We transiently expressed the wildtype version of the VDAC2 protein and successfully restored cardiac rhythm in VDAC2 knockout ($VDAC2^{-/-}$) embryos at 6 dpf. We also transiently expressed the wildtype version of the VDAC3 protein, which is a VDAC isoform that lacks Ca^{2+} trafficking property and saw no rescue in $VDAC2^{-/-}$. This confirmed VDAC2 deficiency as the cause of the cardiac arrhythmia in $VDAC2^{-/-}$ embryos at 6 dpf and prompts further investigation on its property to control cardiac function in early zebrafish development. We also assessed the requirement of Ca^{2+} trafficking property in early cardiac function by conducting mutant experiments, where we found that transient expression of VDAC2 mutant protein that lacks Ca^{2+} trafficking property could no longer rescue $VDAC2^{-/-}$ cardiac arrhythmia, while transient expression of VDAC 3 mutant that has Ca^{2+} trafficking property could. This result confirmed our hypothesis that calcium trafficking is necessary for the early functioning of a zebrafish heart.

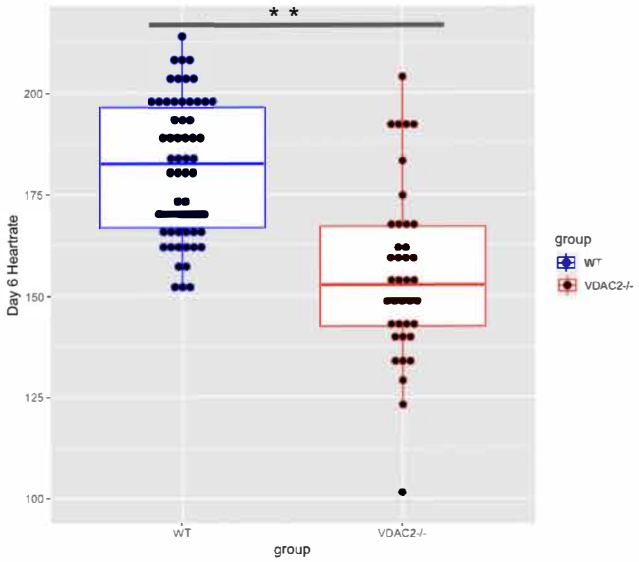
Results

Zebrafish Deficient in VDAC2 Results in Bradycardia with Incomplete Penetrance for Cardiac RArrhythmia at 6 Days Post Fertilization

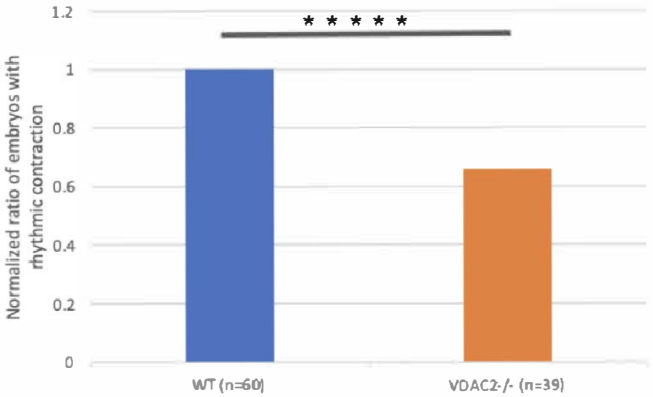
Homozygous knockout of VDAC2 protein resulted in embryos exhibiting bradycardia as early as 6 dpf (Figure 1A). The large standard deviation in the heart rate of those VDAC2$^{-/-}$ at day 6 prompted further investigation in the cardiac function of each embryo. Cardiac arrhythmia assessment showed that in a sample of 39 VDAC2$^{-/-}$ zebrafish, 14 exhibit significant arrhythmia (36%) at 6 dpf, while the rest of 64% maintained normal rhythmic cardiac contradiction (Figure 1B). The disproportion of embryos with quantifiable cardiac arrhythmia suggests an incomplete penetrance of the VDAC2$^{-/-}$ phenotype at 6 dpf. In order to conduct further analysis on the effect of VDAC2 deficiency, VDAC2$^{-/-}$ embryos were divided into two classes. VDAC2$^{-/-}$ embryos that exhibited quantifiable arrhythmia were grouped under "VDAC2$^{-/-}$ Class 1" and VDAC2$^{-/-}$ embryos that did not exhibit quantifiable cardiac arrhythmia were grouped under "VDAC2$^{-/-}$ Class 2". Heart trace diagrams of the wildtype and VDAC2$^{-/-}$ Class 2 appeared similar to one another with consistent intervals between each beat, however, the heart trace for VDAC2$^{-/-}$ Class 1 appeared to be drastically different with large intervals randomly throughout the trace (Figure 1C). Analysis showed that VDAC2$^{-/-}$ Class 1 had significant cardiac arrhythmia as determined by the standard deviation within the heart beat-to-beat interval. A cut off of 1.5 times the standard deviation of the wild type was used to determine the classification of each VDAC2$^{-/-}$ embryo into either Class 1 or Class 2. Previous observations made in our laboratory have suggested that this 1.5 threshold is sufficient to consistently identify 25-35% of embryos with more pronounced cardiac arrhythmia. No statistical difference was identified between the wildtype and VDAC2$^{-/-}$ Class 2. In a sample of 60 wild type zebrafish, the average standard

deviation of the interval between each heartbeat was 0.0223. VDAC2 Class 1 had a significantly increased standard deviation of 0.0630 and VDAC2 Class 2 with a standard deviation of 0.0217 ($p < 0.01$)(Figure 1D). Our laboratory also previously showed with EKG that by 3-months old, 100% of adult VDAC2$^{-/-}$ zebrafish exhibited cardiac arrhythmia. Deducing from the information we have thus far, VDAC2 deficiency has incomplete penetrance at 6 dpf but reaches full penetrace by the time of adulthood. We hypothesize that the phenotype of VDAC2 deficiency is a progressive event. At 6 dpf, Class 2 embryos have a slower progression of cardiac related issues than Class 1 for reasons not yet fully understood.

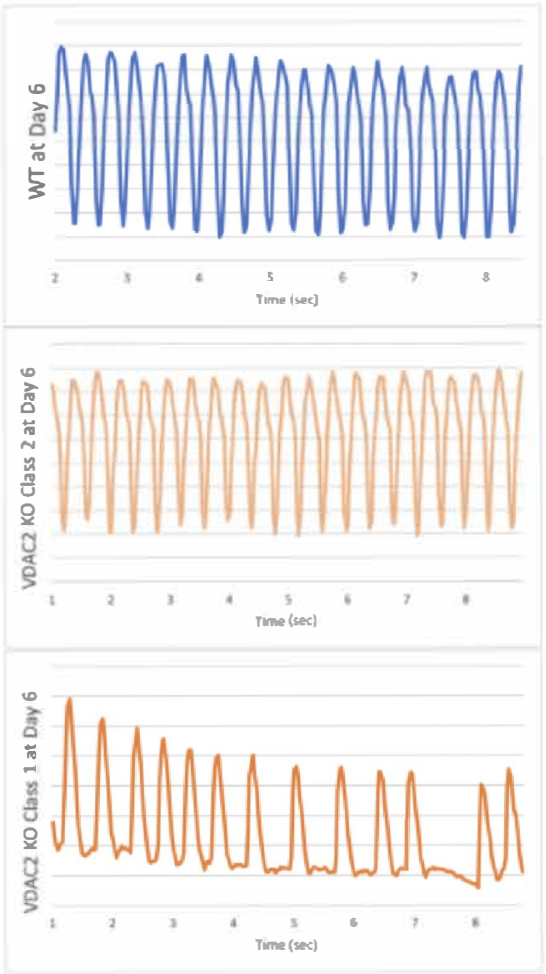
A


* *
Day 6 Heartrate
200
175
150
125
100
WT
VDAC2-/-
group
group
WT
VDAC2-/-

B


* * * * *
Normalized ratio of embryos with rhythmic contraction
1.2
1
0.8
0.6
0.4
0.2
0
WT (n=60)
VDAC2-/- (n=39)

C


WT at Day 6
Time (sec)
VDAC2 KO Class 2 at Day 6
Time (sec)
VDAC2 KO Class 1 at Day 6
Time (sec)

D

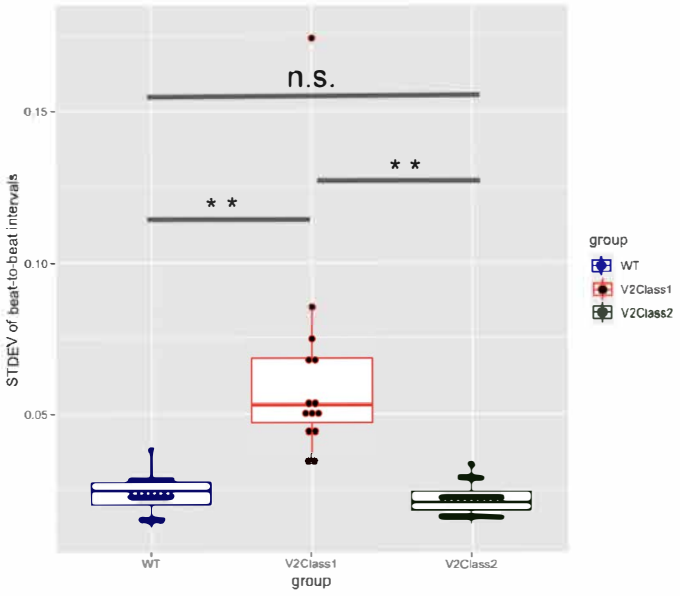
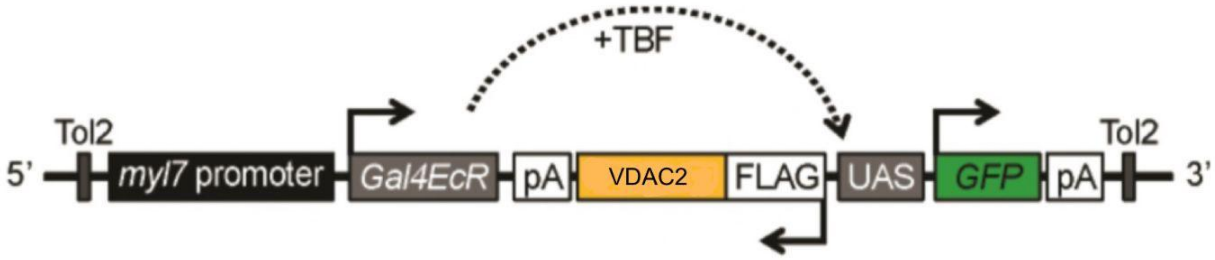

n.s.
* *
* *
STDEV of beat-to-beat intervals
0.15
0.10
0.05
WT
V2Class1
V2Class2
group
group
WT
V2Class1
V2Class2

Figure 1. VDAC2 Knockout Embryos Exhibit Cardiac Arrhythmia and Lower Heart Rate at 6 Days Post Fertilization (A) Bradycardia was observed in VDAC2$^{-/-}$ embryos with an average of 155.6 beats/min compared to the wildtype of 181.4 beats/min (B) Normalized graph comparing the ratio of embryos exhibiting normal rhythmic contraction between wild type and VDAC2$^{-/-}$ embryos (*****$p < 0.00001$). (C) Representative heart traces of the zebrafish embryos from WT, VDAC2$^{-/-}$ Class 1, and VDAC2$^{-/-}$ Class 2. (D) VDAC2$^{-/-}$ Class 1 exhibits significant cardiac arrhythmia as shown by higher STDEV of beat-to-beat interval than WT and VDAC2$^{-/-}$ Class 2 (**$p < 0.01$). There is no statistical difference between the cardiac rhythm of WT and VDAC2$^{-/-}$ Class 2 ($p > 0.05$).

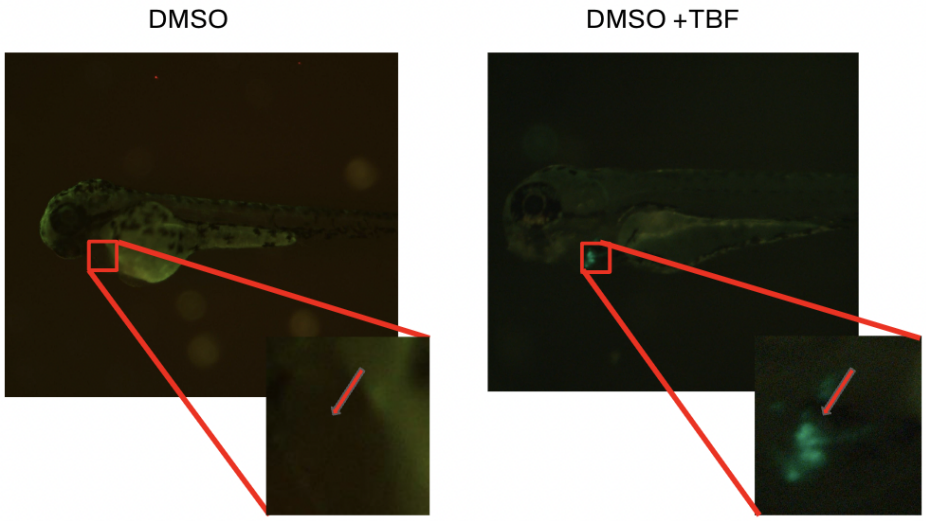
Expression of Transgenic VDAC2 Proteins Rescues VDAC2 Knockout Related Cardiac Arrhythmia

To gain temporal- and cell type-specific control on the induction of VDAC2 protein in VDAC2 deficient hearts, we created a transgenic construct as stated in Shimizu et al., 2021, where the expression of FLAG-tagged VDAC2 as well as a GFP reporter are regulated by the cardiomyocyte-specific promoter myl7 driven Gal4-ecdysone receptor fusion protein (Gal4-EcR; Figure 2A). VDAC2$^{-/-}$ embryos were injected with the transgenic VDAC2 plasmid and allowed to develop for 1 day. The embryos were then subjected to either vehicle (DMSO) or 1 uM tebufenozide (TBF) treatment. In the absence of TBF, Gal4-EcR is inactive and neither the VDAC2 transgene nor the GFP are expressed in the heart (Figure 2B). While the presence of TBF allowed for the activation of Gal4-EcR to act on the UAS to drive bidirectional transcription for VDAC2 and GFP (Figure 2B). Treatment of DMSO and TBF was done on VDAC2$^{-/-}$ embryos with no injection to ensure that the treatment of DMSO and TBF chemicals themselves do not affect the condition of VDAC2 deficiency-related cardiac arrhythmia (Figure 2C). As expected, the treatment of DMSO to our VDAC2$^{-/-}$ injection did not appear to rescue cardiac arrhythmia but the treatment with TBF resulted in a significant increase in the number of embryos exhibiting rhythmic contraction (Figure 2D).

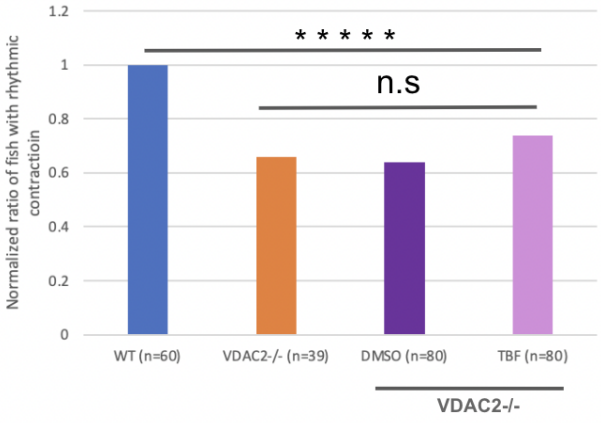
A


+TBF
Tol2
5'
myl7 promoter
Gal4EcR
pA
VDAC2
FLAG
UAS
GFP
pA
Tol2
3'

B


DMSO
DMSO +TBF

C


Normalized ratio of fish with rhythmic contractioin
1.2
1
0.8
0.6
0.4
0.2
0
* * * * *
n.s
WT (n=60)
VDAC2-/- (n=39)
DMSO (n=80)
TBF (n=80)
VDAC2-/-

D

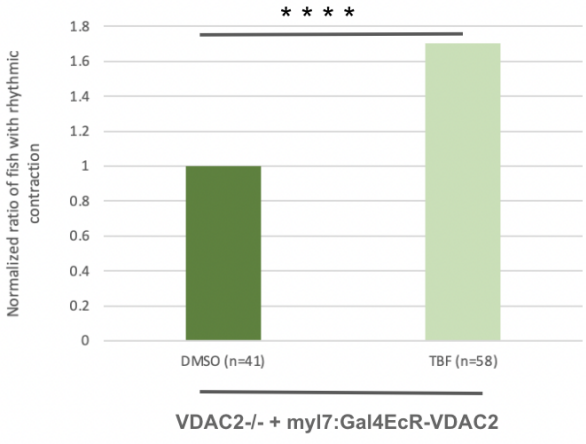

Normalized ratio of fish with rhythmic contraction
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
* * * *
DMSO (n=41)
TBF (n=58)
VDAC2-/- + myl7:Gal4EcR-VDAC2

Figure 2. Induction of Mosaic VDAC2 Expression Restores Rhythmic Cardiac Contraction but Shows Sign of Tachycardia in VDAC2$^{-/-}$ Embryos (A) Schematic diagram of the VDAC2 transgenic construct. The cardiomyocytes-specific promoter myl7 drives Gal4-ecdysone receptor fusion protein (Gal4EcR), which becomes transcriptionally activated in response to tebufenozide (TBF), an ecdysone receptor agonist and binds to the upstream activating sequence (UAS), resulting in the simultaneous expression of both FLAG-tagged VDAC2 and GFP reporter. (B) Fluorescent imaging for GFP in no fluorescent background demonstrated VDAC2 expression was successfully induced in the heart upon TBF treatment in VDAC2$^{-/-}$ embryos. Embryos were treated with either DMSO or TBF+DMSO from 24 hpf until they were imaged at day 6 post fertilization. (C) Controlled experiment showed no significant effect of DMSO and TBF chemicals alone on cardiac function. (D) Normalizing against the DMSO group, the TBF treated injected embryos exhibited close to 1.7 fold increase (****$p < 0.0001$) in the number of embryos with rhythmic cardiac contraction.

Expression of Transgenic VDAC3 Proteins Unable to Rescue VDAC2 Knockout Related Cardiac Arrhythmia

To examine whether the successful cardiac arrhythmia rescue was due to the Ca^{2+} trafficking property of VDAC2 or other intrinsic factor of the protein, we expressed transgenic VDAC3 proteins, a VDAC isoform that specifically lacks Ca^{2+} trafficking property, in VDAC2$^{-/-}$ embryos following the same procedure as described above (Figure 3A). Interestingly, both DMSO and TBF treated groups exhibited comparable levels of cardiac arrhythmia to each other (Figure 3B). This showed that the transgenic expression of VDAC3 was unable to rescue cardiac arrhythmia in VDAC2$^{-/-}$ embryos. The differences in the ability to rescue VDAC2$^{-/-}$ embryos between the transgenic induction of VDAC2 and VDAC3 proteins suggested functional differences between the two proteins in early zebrafish heart function. The rescue pattern of VDAC2$^{-/-}$ was very similar to our laboratory's previous research done on *ncx1* mutant, where overexpressing VDAC2 but not VDAC3 could suppress aberrant Ca^{2+}-induced cardiac arrhythmia in *ncx1* mutant zebrafish[2]. The similarity led us to believe that the trafficking of Ca^{2+} may explain the discrepancy of VDAC2$^{-/-}$ rescue between VDAC2 and VDAC3 expression and further suggested the important necessary step of Ca^{2+} trafficking between mito-SR cross-talk in normal heart development and function.

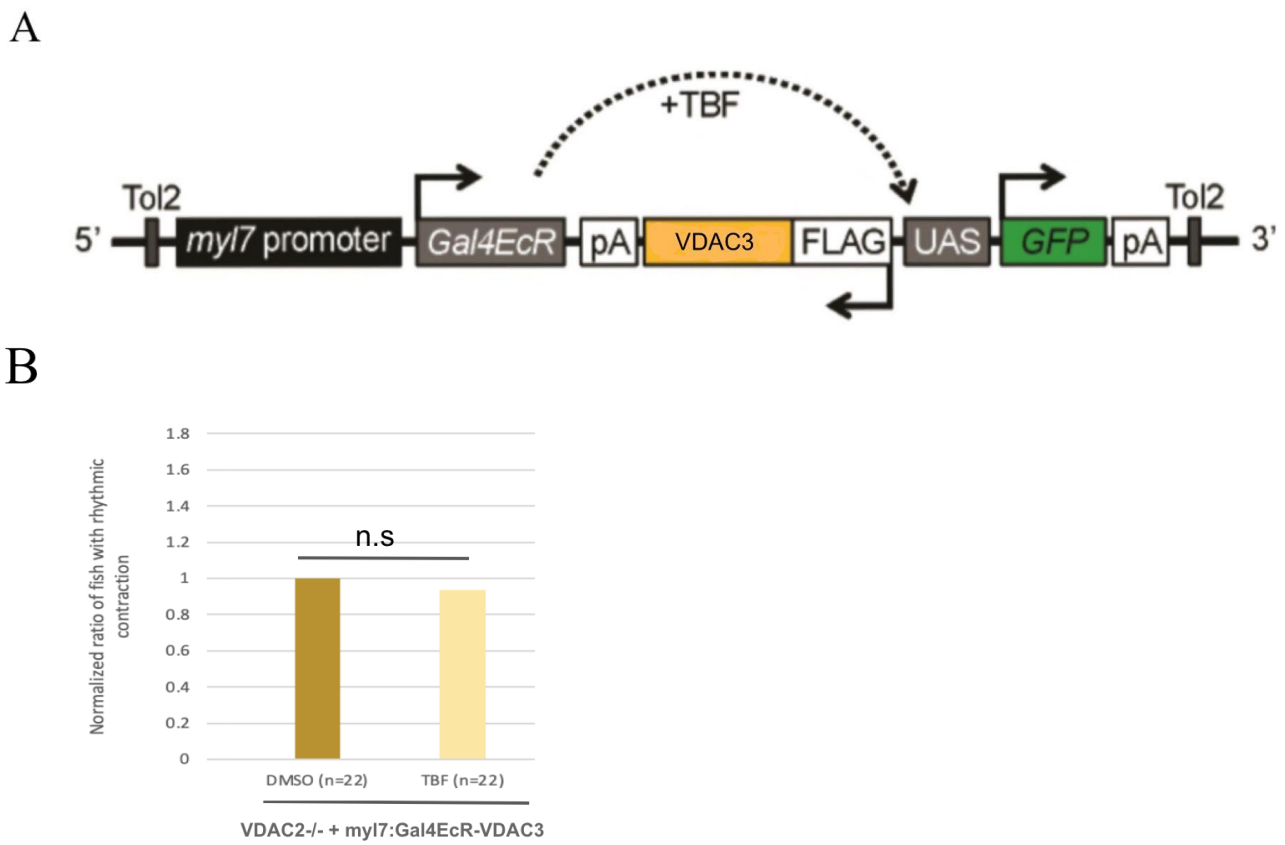
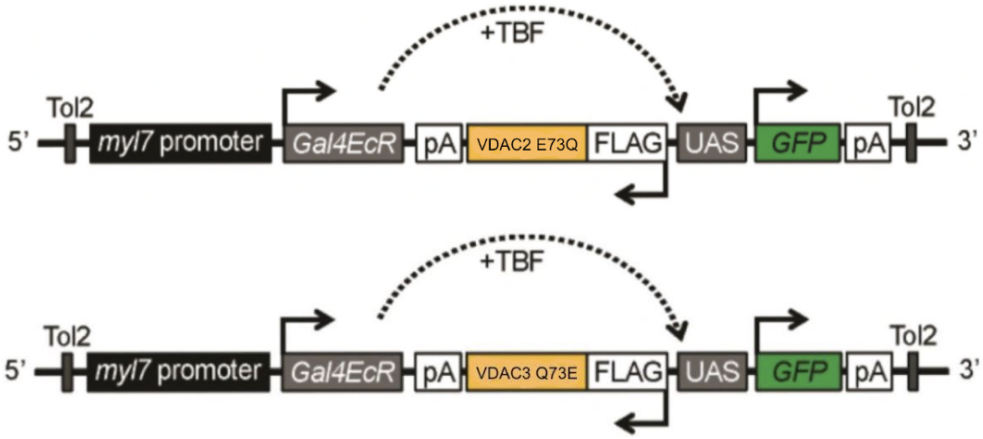


Figure 3: Induction of Mosaic VDAC3 Expression Unable to Restore Rhythmic Cardiac Contraction or Heart Rate (A) Schematic diagram of the VDAC3 transgenic construct. The cardiomyocytes-specific promoter myl7 drives Gal4EcR, which becomes transcriptionally activated in response to TBF, an ecdysone receptor agonist and binds to the UAS, resulting in the simultaneous expression of both FLAG-tagged VDAC3 and GFP reporter. (B) Normalizing against the DMSO group, the TBF treated injected embryos did not result in significant fold change in the number of embryos exhibiting rhythmic contraction.

Expression of Transgenic Mutant VDAC3 but not Mutant VDAC2 Proteins Rescues VDAC2 Knockout Related Cardiac Arrhythmia

The difference in rescue level between transgenic expression of VDAC2 and VDAC3 proteins suggested an important functional difference between the two isoforms. To investigate whether this Ca^{2+} trafficking property played a role in the rescue of cardiac arrhythmia, we created mutant VDAC2 ($VDAC2^{E73Q}$) and mutant VDAC3 ($VDAC3^{Q73E}$) proteins. In $VDAC2^{E73Q}$, the 73^{rd} amino acid position of VDAC2 was substituted from glutamate to glutamine resulting in a loss-of-function mutation of VDAC2 that could not traffic calcium[2]. On the other hand, in $VDAC3^{Q73E}$, the 73^{rd} amino acid position of VDAC3 was substituted from glutamine to glutamate, resulting in a gain-of-function mutation of VDAC3 that could traffic calcium[2]. Injection and rescue assessment of $VDAC2^{E73Q}$ and $VDAC3^{Q73E}$ was done in the same way as previously described (Figure 4A). Result showed that the transgenic expression of $VDAC2^{E73Q}$ with TBF did not have significant effect on the fold change of embryos exhibiting rhythmic cardiac contraction. However, the transgenic expression of $VDAC3^{Q73E}$ with TBF resulted in over 1.6 fold increase in the number of embryos with rhythmic cardiac contraction compared to the DMSO group (Figure 4B). All the data combined showed that both the transgenic expression of VDAC2 and $VDAC3^{Q73E}$ could rescue cardiac arrhythmia, underscoring the importance of Ca^{2+} trafficking property in regulating cardiac rhythm in early zebrafish development (Figure 4C).

A


+TBF
Tol2
5'
myl7 promoter
Gal4EcR
pA
VDAC2 E73Q
FLAG
UAS
GFP
pA
Tol2
3'
+TBF
Tol2
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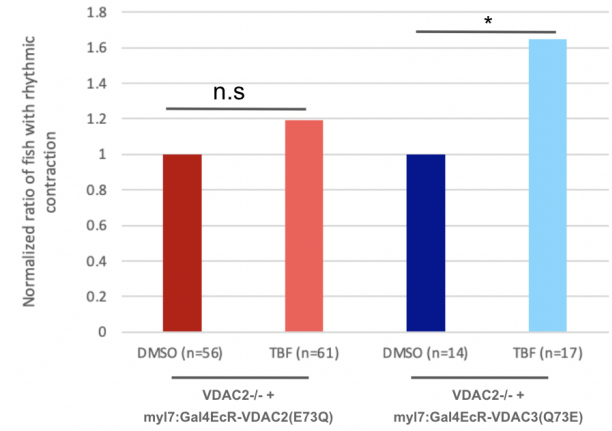
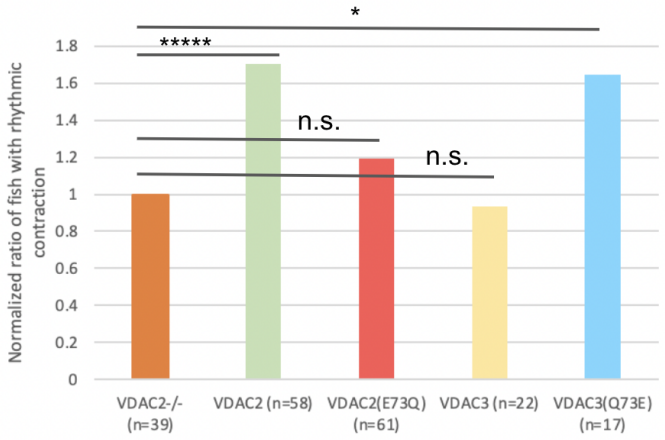
Gal4EcR
pA
VDAC3 Q73E
FLAG
UAS
GFP
pA
Tol2
3'

B


Normalized ratio of fish with rhythmic contraction
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
n.s
*
DMSO (n=56)
TBF (n=61)
DMSO (n=14)
TBF (n=17)
VDAC2-/- + myl7:Gal4EcR-VDAC2(E73Q)
VDAC2-/- + myl7:Gal4EcR-VDAC3(Q73E)

C


Normalized ratio of fish with rhythmic contraction
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
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*
*****
n.s.
n.s.
VDAC2-/- (n=39)
VDAC2 (n=58)
VDAC2(E73Q) (n=61)
VDAC3 (n=22)
VDAC3(Q73E) (n=17)

Figure 4: Induction of Mosaic VDAC3 Q73E Expression Restores Rhythmic Cardiac Contraction but not Heart Rate (A) Schematic diagram of the VDAC2^{E73Q} and VDAC3^{Q73E} transgenic construct. (B) Transgenic induction of VDAC2^{E73Q} with TBF result in little fold change in the number of embryos exhibiting rhythmic cardiac contraction ($p > 0.05$), while transgenic induction of VDAC3^{Q73E} yield over 1.6 fold change in the number of embryos showing rhythmic cardiac contraction ($*p \leq 0.05$). (C) Combined data showed that both VDAC2 and VDAC3^{Q73E} expression could significantly restore cardiac rhythm, but not VDAC3 or VDAC2^{E73Q}.

Discussion

In this experiment, we demonstrated that transgenic induction of VDAC2 and VDAC3^{Q73E} could rescue cardiac arrhythmia in VDAC2$^{-/-}$ at 6 dpf, while the transgenic induction of VDAC3 and VDAC2^{E73Q} could not. This strongly indicates the functional difference between the two VDAC protein isoforms and that glutamate at the amino acid position 73rd of VDAC2 proteins plays an important role in facilitating proper heart function in early zebrafish development. However, it's crucial to note that the transgenic expression in this experiment results in mosaic expression in the heart as seen in the non-uniformed fluorescence of the heart in Figure 2B. This might be a problem if the level of cardiac arrhythmia rescue is dependent on protein abundance. Further experiments should aim to investigate whether uniform VDAC2 expression could improve rescue and whether there is a threshold requirement for VDAC2 expression for rescue. This could be done by conducting rescue assessment using the F1 embryos of founder fish that have incorporated out injected constructs into the germline. Nevertheless, in our mosaic expression, significant rescue was observed, suggesting a cell

autonomous role for VDAC2. Our mutant experiments further confirmed the Ca^{2+} trafficking property hypothesis in facilitating proper cardiac rhythm during early mito-SR cross-talk.

It is interesting to also note that even though $VDAC3^{Q73E}$ expression resulted in a much more significant fold change in the amount of embryos exhibiting rhythmic cardiac contraction than that of $VDAC2^{E73Q}$ expression, $VDAC2^{E73Q}$ still resulted in a 1.2 fold change that could suggest additional functional roles of VDAC2 in early cardiomyocytes to slightly rescue VDAC2 deficiency-related cardiac arrhythmia. This result prompts further experiments to fully elucidate the functional role of VDAC2 in early zebrafish cardiomyocytes.

Materials and Methods

Zebrafish Husbandry, Generation of Transgenic Fish Line, and Chemical Induction

Zebrafish used in this experiment are all bred and maintained under standard laboratory fish room conditions. The $VDAC2^{-/-}$ fish line was generated previously with the Zinc Finger Nuclease (ZFN) gene targeting approach, where the ZFN mRNA was injected into the wild type embryo resulting in 34-bp deletion at exon3 of the VDAC2 gene. The deletion resulted in a premature stop codon and successfully prevented the translation of a wild type VDAC2 gene.

To induce transgenic VDAC2 into the injected embryos, 1uM of tebufenozide (TBF) was added into the fish media after 1 day post fertilization (dpf).

Plasmid Construction

Plasmid contains a full length wild type VDAC2 cDNA. This was copied from our myc tag vector via PCR and ligated onto our 3xFlag tag vector. VDAC2 along with the 3xflag tag

sequence were then PCR out and ligated into the pEnter vector containing the recognition sites for the generation of a gateway entry plasmid with the Tol2Kit. Using the kit, the 5' entry consists of cmlc and Gal4EcR, the middle entry consists of the VDAC2-flag UAS, and the 3' entry plasmid consisting of GFP reporter and poly-A tail are ligated together to create the full inducible expression construct. Same procedures were performed to create VDAC2^{E73Q}, VDAC3 and VDAC3^{Q73E} plasmid constructs by changing the middle entry of the Tol2Kit and ligated them with the same 5' and 3' entries

Imaging

Cardiac phenotype of the embryos was imaged at 6 dpf with 5.3x magnification using *Zeiss SteREO Discovery. V8 microscope*. The embryos at 6 dpf were first treated with tricain and placed ventral side up in 3.25% methylcellulose. Videos were captured with a standard iPhone 11 with 3x zoom mounted to the eyepiece of the *Zeiss SteREO Discovery. V8 microscope*.

Data Analysis

Videos of the embryos' hearts were uploaded to a software developed by an undergraduate student in the laboratory in 2014 to obtain heart traces[5]. The fish used in this experiment were cmlc GFP positive. The software tracks the fluctuation of the fluorescence intensity in one specific region of the heart frame by frame as the heart beats and outputs the sinusoidal graphs mimicking the heart beat pattern. The data points outputting the sinusoidal graphs were then uploaded to Excel for T-test statistical analysis.

Injection

H buffer was prepared on the day of injection with 7g of NaCl, 0.4g of NaHCO3, 0.24g of CaCl2*2H2O, 0.1g of KCl mixing in two liters of ddH2O and adjust the pH to ~7 with HCl. VDAC2$^{-/-}$ embryos were dechorionated with a filtered pronase mix (1mL of 20mg/mL pronase and 14mL of H buffer) in a 6cm glass dish. After placing the embryos in the filtered pronase mix, look for the very first embryo to become dechorionated and begin subsequent washing with a waterfall method with around 500mL of H buffer. Dechorionated embryos were moved to a new 10cm glass dish containing H buffer with flamed smooth glass pipette and can be loaded onto the injection ramp for injection. Roughly 5 hours after injection, the embryos were cleaned and transferred to a new 10cm glass dish containing E3. Embryos were collected from VDAC2$^{-/-}$ lines and were injected at one cell stage with 35 ng/ul of the transgenic constructs and 140 ng/ul of transposase.

Acknowledgements

I would like to give special thanks to Dr. Lauren Crisman and Dr. Jau-Nian Chen for being amazing mentors throughout this project. Their constant support and assistance played major roles in ensuring the progression of this project. I would also like to thank Adam Langenbacher and Zhiyu Tian for their insights throughout my microinjection process. This project has been a fantastic learning experience, exposing me to the field of cardiac research and development. As I continue to pursue a career related to cardiac health, I'm forever grateful for my family and everyone in the Chen Lab for their incessant guidance and mentorship.

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