Title

Exploring the role of Calcium Signaling via Calmodulin and Calcineurin and its activation on Nuclear Factor of Activated T-Cells in the Adult Drosophila Heart

Author

Paknoosh, Sean J

Publication Date

2018

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA SAN DIEGO

Exploring the role of Calcium Signaling via Calmodulin and Calcineurin and its activation on Nuclear Factor of Activated T-Cells in the Adult *Drosophila* Heart

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Sean J Paknoosh

Committee in charge:

Professor Gabriel Haddad, Chair
Professor Ethan Bier, Co-Chair
Professor Rolf Bodmer
Professor Karen Ocorr
Professor James Posakony

SIGNATURE PAGE

The Thesis of Sean J. Paknoosh as it is listed on UCSD Academic Records is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

Co-chair

_____

Chair

University of California San Diego

2018

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

CaM: Calmodulin

CaN: Calcineurin

CnA: Calcineurin A

CnB: Calcineurin B

CO: Cardiac Output

DD: Diastolic Diameter

DI: Diastolic Interval

dNFAT: *Drosophila* homolog of Nuclear Factor of Activated T-Cells

FS: Fractional Shortening

HP: Heart Period

HR: Heart Rate

NFAT: Nuclear Factor of Activated T-Cells

SD: Systolic Diameter

SI: Systolic Interval

SV: Stroke Volume

UAS: Upstream Activating Sequence

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

This work would not have been possible without the support and guidance of many people. Firstly, I would like to express my gratitude to my mentor, teacher, and friend Anjali Gupta. Thank you for your patience in answering all my questions and teaching me almost every laboratory technique I have used for this project. It truly has been a privilege to collaborate with you and I am eternally grateful for all your help. I would also like to especially thank Dr. Rolf Bodmer and Dr. Karen Ocorr for allowing me to work in their lab and participate in the Master's program at UCSD. Furthermore, I am appreciative for the support and great company of all present and past members of the Bodmer-Ocorr lab of whom I had the pleasure to meet during my time as an undergraduate and graduate student. Finally, I would like to thank my family and friends for their enduring support of all my efforts in pursuing my Master's degree and in all the other pursuits I have decided to undertake.

ABSTRACT OF THE THESIS

Exploring the role of Calcium Signaling via Calmodulin and Calcineurin and its activation on Nuclear Factor of Activated T-Cells in the Adult *Drosophila* Heart

by

Sean J Paknoosh

Master of Science in Biology

University of California San Diego, 2018

Professor Gabriel Haddad, Chair

Professor Ethan Bier, Co-chair

Heart disease has been the leading cause of death in the U.S. over the past century despite its decreasing rate over the last 50 years. Understanding the function of new genes and pathways in a simple model system may provide novel insights and lead to new therapeutic targets for heart disease in humans. In mammalian systems, calcineurin-NFAT signaling has been implicated in the regulation of the cardiac hypertrophic response. However, how this gene interacts with other molecular pathways in the heart is unknown. In order to better understand this signaling process, the singular function of calmodulin (CaM), calcineurin (Pp2B-14D), and

Nuclear Factor of Activated T-Cells (NFAT), in the heart was investigated, using the animal model system *Drosophila melanogaster*. Loss-of-function and gain-of-function studies were conducted in a tissue-specific manner using the GAL4/UAS system by means of RNAi and overexpression lines, respectively. The flies were dissected to expose the beating heart tube; hearts were filmed and analyzed using Semi-Automated Optical Heartbeat Analysis (SOHA) and key cardiac performance indicators were measured. Slightly significant differences between NFAT gene knockdown and respective controls using two RNAi lines were observed, yet there were no obvious changes observed in cardiac size. However, this may be due to insufficient KD or due to compensation by changes in expression of other genes. Overall, cardiac size was reduced in Pp2B-14D KD and increased in Pp2B-14D OE. Finally, CaM KD resulted in significant contractile defects when compared to their respective controls.

Introduction

A. Overview of Calmodulin, Calcineurin & NFAT

Calcium is one of the cell's most prevalent divalent ions and is often required in intra- and intercellular signaling. Resultantly, Ca^{2+} levels are tightly regulated and normally kept at low levels (50-100nM) in the cellular environment due to the messenger's role in many signaling pathways (Swulius and Waxham, 2008). One protein which often associates with calcium in order to further mediate secondary messenger effects is known as calmodulin (CaM) (Martin et al. 2004). Structurally, CaM is made up of 148 amino acids which are highly conserved between vertebrates and invertebrates. Humans contain three distinct CaM genes scattered throughout the genome which encode an identical protein whereas the model organism, *Drosophila melanogaster,* only contains a single gene with highly similar intron locations and fused exons (Friedberg et al. 2001). The protein has two domains that each contain a pair of EF-hand motifs consisting of a helix-loop-helix that are connected in close proximity by a flexible alpha helix linker. Each motif allows the binding of two calcium ions and the binding of CaM and Ca^{2+} to its targets modify their action in a calcium-dependent manner (Vetter et al. 2003). As a secondary messenger, CaM naturally has many targets with perhaps the most prominent of its interactions being with the myosin heavy chain (Tóth et al. 2005). However, CaM also acts on many transferases as well including the well-known phosphatase, calcineurin.

In mammalian systems, calcineurin (CaN) consists of a 60kDa catalytic subunit, calcineurin A (CnA), which is bound to its smaller regulatory subunit, calcineurin B (CnB), which is a Ca^{2+} binding protein. The *Drosophila* genome includes three genes (CanA1, Pp2B-14D, and CanA-14F) which make up the CnA subunit and two genes (CanB and CanB2) which makeup the CnB subunits; both of which are highly homologous to the vertebrate counterparts

(Miskei et al. 2011). CanA-14F and Pp2B-14D are both located sequentially on the X chromosome thus providing likely insight that they arose through a gene duplication event. Calcineurin is activated by its association with calmodulin at its catalytic subunit and the binding of increased levels of Ca^{2+} at its regulatory subunit. Once active, the phosphatase is able to dephosphorylate serine and threonine residues on the transcription factor known as NFAT (Nuclear Factor of Activated T-Cells). Once dephosphorylated NFAT is allowed to translocate into the nucleus and activate its target genes (**Figure 1**) (Luo et al. 1996). CaN's dephosphorylation activity on NFAT has only been confirmed in vertebrates, and its role on NFAT in an invertebrate model has yet to be shown. In addition, CaN can act in an inhibitory and activating manner on many targets such as MEF2 in both vertebrate and invertebrate systems; it is quite likely that there are additional unknown effectors.

There are five different genes of NFAT (NFATc1-c4, NFAT5) found in humans whereas the *Drosophila* genome only contains one NFAT gene (dNFAT). Originally found in vertebrate models, these transcription factors were initially found to activate a set of immune response genes like IL-2 when translocated into the nucleus; these genes then provide support to activate helper T-cells and other cytokines needed for a proper immune response (Clipstone and Crabtree, 1992). Once dephosphorylated, NFATc3 was later found to induce cardiac hypertrophy in transgenic mice through activation of CaN and its resultant dephosphorylation and translocation of NFATc3. These transgenic mice hearts were observed to possess extremely hypertrophic ventricular chambers with age as well as excessive deposits of collagen (Molkentin et al. 1998). Later it was found, that targeted inhibition of calcineurin via cyclosporine A and FK506 lead to a decrease in hypertrophy. These studies confirmed the necessity of calcineurin in the hypertrophic response (Windt et al. 2001). NFAT5 was later found to help express genes under osmotic stress

in humans by helping to regulate transmembrane proteins which transport osmolytes across the cell. (Miyakawa et al. 1999). **Unlike NFATc1-c4, NFAT5 was found to lack the transactivation domain required for calcineurin binding**; however, the entire vertebrate NFAT gene family contain highly conserved DNA binding domains known as the rel homology class.

In the fruit fly, dNFAT has been shown to significantly contribute to salt stress resistance (Keyser et al. 2007), yet a role in the fruit fly's cardiovascular system remains unexplored. NFAT5 is thought to be the most primordial gene out of the NFAT gene family as it is the most homologous NFAT gene expressed in *Drosophila melanogaster* (dNFAT). **Therefore, it remains unlikely that dNFAT is mediated through calcineurin activation due to the absence of a calcineurin binding site on NFAT5.** Additionally, it is now thought that NFATc1-c4 evolved in vertebrates for complex functions like the adaptive immune response and cardiovascular and skeletal muscle development likely through a gene duplication event involving NFAT5.

B. The Importance of the Calcineurin-NFAT Complex in Drosophila

Despite the established role of CaN-NFAT in the vertebrate immune (IL-2 activation) and cardiovascular systems (hypertrophic cardiomyopathy), a role in cardiovascular muscle development or cardiovascular function has not yet been determined in the fruit fly. Here, we investigated the effects of dNFAT through loss-of-function studies using RNAi on the adult *Drosophila* heart. **Given that the fruit fly has only one NFAT gene, and given that calcineurin activates multiple pathways, it is essential to understand the phenotypic effect of calcineurin-mediated NFAT activation in the fruit fly heart before other pathways involving calcineurin can be studied in this model system.** Therefore, it is also important to

understand the specific roles of upstream players, CaM and CaN, on the fruit fly heart. Since CaN mediated NFATc3 activation has been shown to lead to hypertrophic cardiomyopathy in mammalian systems, it is critical to understand if similar phenotypes can be induced through loss-of-function (LOF) and gain-of-function (GOF) through knockdown (KD) or overactivation (OE) of the gene of interest, respectively. Results from these studies will contribute to our foundational understanding of the role of CaM, CaN, and NFAT in the fruit fly heart and will further allow researchers to use the fruit fly as a model for studying heart disease.

C. Drosophila melanogaster as a Model Organism for Cardiovascular Disease

The versatility and commonality in genes with humans have made the fruit fly the ideal model organism for genetic and molecular studies over the past 100 years. *Drosophila* has only recently been uncovered as a great resource to study cardiovascular disease over the past few decades due to how genetically tractable the organism is across many generations. This has made the fruit fly a vital tool in the discovery of new genes as well as a great complement to mammalian models of heart development and disease (Vogler et al. 2009). Though the fruit fly heart is phenotypically very different than a human, both species have about the same heart rate. Additionally, the fruit fly contains three pairs of intake valves, or ostia, which creates a 4-chambered heart tube.

To investigate the effects of tissue-specific loss of function of CaM, CaN, and dNFAT, we utilized the UAS/GAL4 system in conjunction with gene-specific RNAi lines. RNAi has been a revolutionary method of knockdown in the fruit fly and has only contributed to the reason why the fruit fly is often used as a genetic model to study cardiac disease (Bier et al. 2004). Heart parameters such as systolic and diastolic diameters and intervals, heart rate, heart period, fractional shortening, stroke volume and cardiac output were then quantified and analyzed.

The GAL4/UAS system contains two parts: GAL4, which is a transcription activator in yeast, and UAS (Upstream Activating Sequence), which is an enhancer that GAL4 can bind to. Since GAL4 is not endogenous in fruit flies, the expression of GAL4 does not interfere with other processes in the cell (Duffy, 2002). GAL4 is typically placed under control by a native gene or driver using a P-element vector allowing researchers to express GAL4 only in tissues of interest. Without the presence of both the GAL4 downstream of a tissue-specific promoter and a UAS upstream of an RNAi element, knockdown will not occur. In this experiment, strong heart-specific promoters like Hand4.2-gal4 and TinCΔ4-gal4 were used. Hand4.2-gal4 is a driver line, which drives expression of GAL4 in the heart tube and the surrounding pericardial cells whereas TinCΔ4-gal4 drives expression of GAL4 only in the heart tube. In the fruit fly, pericardial cells serve as the intermediary in metabolism or excretion through pinocytosis, so it is possible that these cells are having a non-autonomous effect on the heart tube. The fat-body specific driver, LSP-gal4, was also used to test for cell non-autonomous effects on dNFAT KD in the heart tube. The fat bodies in insects play considerable roles in energy and metabolism, similar to liver function in mammals (Arrese et al. 2018).

The fruit fly only has four pairs of chromosomes and about 17,000 genes, comparable to the 21,000 found in humans. Additionally, balancer chromosomes are commonly used in *Drosophila* for screening populations as well as a genetic tool that prevents crossing over between homologous chromosomes during meiosis. Balancer chromosomes are also used as a tool to maintain deleterious mutations in a living stock. These chromosomes contain a recessive lethal mutation, an observable dominant phenotype as well as multiple overlapping inversions. The inversions which are built into each balancer are the reason why homologous recombination is unable to occur; while at the same time the recessive lethal mutation prevents the stock from

becoming homozygous for the balancer chromosome. This results in a net effect where lines carrying recessive mutations can be maintained in heterozygous form. The 4th pair of chromosomes are extremely small and do not undergo genetic recombination during meiosis. Resultantly, the fruit fly has become a perfect model to track genes between generations due to its simplicity.

Over the years two, different transgenic RNAi libraries were made at the Vienna Drosophila Resource Center. Both the GD and KK libraries contain 1000s of different RNAi lines but differ in the way the insertion is placed into the fly. The GD lines use a P-element to randomly insert the sequence anywhere on the 1st, 2nd or 3rd chromosome. However, the KK library uses a transgene whereby the insertion is always at a single defined insertion site on the 2nd chromosome. Interestingly, studies have shown that there are actually 2 landing sites, 40D3 and 30B3, on the 2nd chromosome which may contain the insertion (Green et al., 2014). To control for experiments involving these two libraries certain GAL4 responsive lines were made that contained no functional RNAi coding sequence. Therefore, the controls for the experiments involving these RNAi stocks involved using VDRC provided host strains which contained the respective landing sites from the KK and GD libraries. The creation of these libraries has greatly contributed to the resourcefulness of the fruit fly as a model organism for many different types of diseases.

Given that a role in *Drosophila* heart function has not been concluded for dNFAT and given that calcineurin activates a variety of transcription factors, we would like to independently understand the role of dNFAT and Pp2B-14D in the context of heart function in *Drosophila*. By conducting LOF and GOF studies on these genes, we will be able to understand if a similar hypertrophic pathway like the one seen in mammalian systems is inducible in the fruit fly and if

similar pathways are involved. Furthermore, comparing these studies with LOF studies on CaM will provide a baseline understanding of the role of each effector in this signaling pathway.

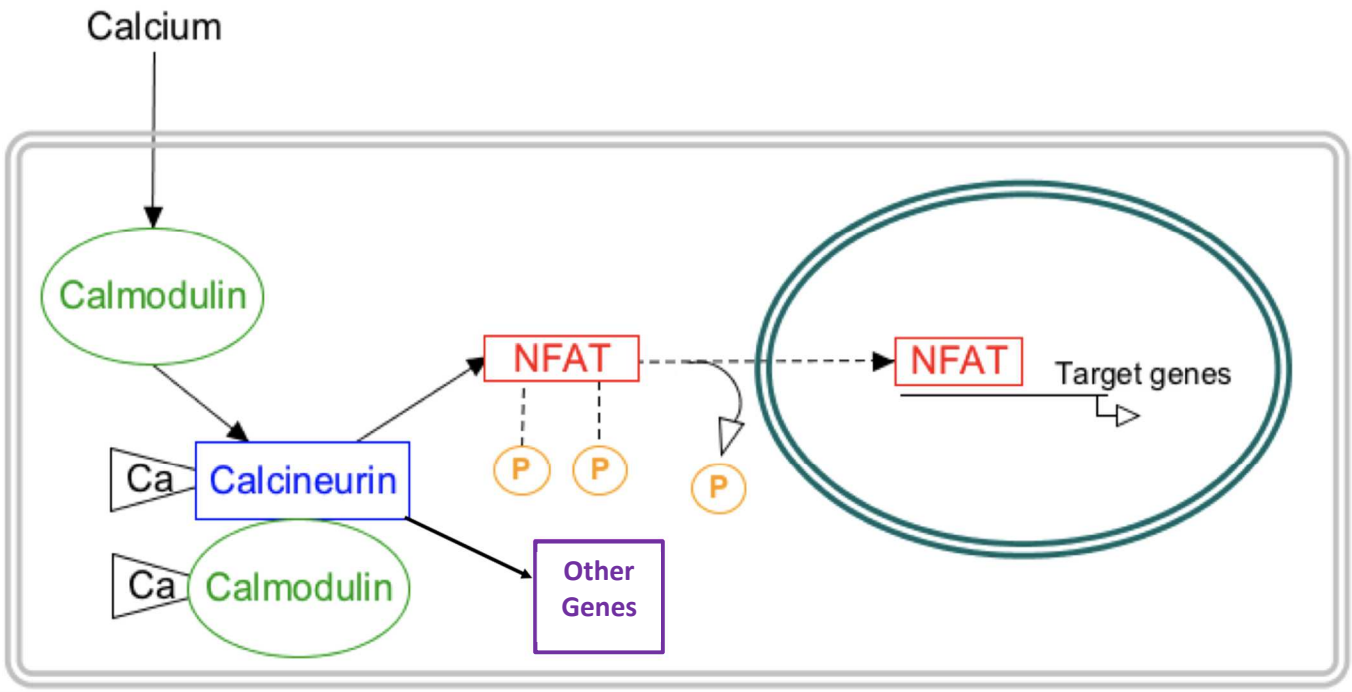

Figure 1: Schematic view of NFAT activation via the phosphatase, calcineurin. Intracellular Ca^{2+} enters the cell and binds to the EF-hand motifs on CaM. CaM is then able to activate the phosphatase, CaN, through its binding on its catalytic domain and the association of free Ca^{2+} ions on the regulatory domain of CaN. CaN has a myriad of targets including NFAT which will enter the nucleus once dephosphorylated to initiate transcription of target genes. Calcineurin's role on NFAT in a *Drosophila* heart model has yet to be shown.

Materials and Methods

A. Drosophila Stocks and Culture

All RNAi stocks were obtained from the Vienna Drosophila Resource Center (VDRC) and the Pp2B-14D-OE stock was acquired through the Bloomington Drosophila Stock Center (BDSC) (**Table 1**). Other stocks used during these experiments, such as drivers, were obtained from the laboratory stocks. The fruit fly culture was made in the laboratory using a mixture of cornmeal, molasses, malt, yeast, agar, yeast and water. The stocks were maintained at room temperature when not in use and 25°C under experimental conditions.

Table 1: *Drosophila* stocks used for functional studies.

Simplified Genotype	Full Genotype	Library	CG #	Stock #	Stock Center
NFAT-RNAi	w1118; P{GD4379}v30566	GD	11172	30566	VDRC
NFAT-RNAi	P{KK102385}VIE-260B	KK	11172	107032	VDRC
CaM-RNAi	P{KK109037}VIE-260B	KK	8472	102004	VDRC
Pp2B-14D-RNAi	P{KK107714}VIE-260B	KK	9842	103144	VDRC
UAS-Pp2B14D-OE	w67c23 P{Mae-UAS.6.11}Pp2B-14DGG01028	--	9842	22025	BDSC

B. Imaging and Heart Function Analysis

Once aged to 3 weeks, the adult female fruit fly hearts were dissected to expose the beating heart tube and imaged using a high-speed camera (140 frames/second). The fruit flies were anesthetized for about 2 minutes using fly nap and laid out on a dish of vaseline in preparation for dissections. The heads are then cut off leaving only the thorax and abdomen on the dish. A solution of hemolymph, which contains certain sugars such as sucrose and trehalose, were then added in order to mimic conditions in the heart. The surrounding fat around the heart tube is removed using an ultra-fine glass suction micropipette and extra care is taken to avoid damaging the heart tube at this time. Once finished, the dissected flies were allowed to rest in O_2-rich hemolymph for approximately 20-30 minutes before filming. Thirty-second videos are

then taken of each heart tube using a high-speed camera and analyzed using software called Semiautomatic Optical Heartbeat Analysis (SOHA). Through this software, systolic and diastolic diameters were manually marked in the area surrounding the 2nd pair of ostia and a region of interest (ROI) is also marked to allow the program to analyze the intervals. The software quantifies key heart parameters such as diastolic/systolic diameters and diastolic/systolic intervals (Ocorr et al. 2014). R-Studio was used to calculate other heart parameters such as heart period, fractional shortening, stroke volume, heart rate and cardiac output. All statistical analysis was done using Prism 7 Statistical Software (GraphPad). Most of these studies were performed using one experimental condition and one or two controls. The controls for the knockdown data consisted of using the respective control stock from the correct VDRC library in order to test for issues with the UAS-RNAi and the driver being used (Hand4.2-gal4 or TinCΔ4-gal4). The controls for the Pp2B-14D OE data consisted of using another upstream activating sequence (UAS-GFP) in order to test for issues with the UAS-Pp2B-14D OE line and the driver being used. These data sets were analyzed using a t-test or 1-way ANOVA followed by Sidak's multiple comparisons post hoc tests, where appropriate. In all cases, $p<0.05$ was taken as significant.

C. Immunohistochemistry

In order to prepare for staining the flies were first fixed following dissections. Firstly, a calcium chelator, 0.5mM EGTA, was added to the dish containing the dissected flies. This stops the hearts from beating and prepares them for 3 consecutive washes in 1x phosphate buffered saline (PBS). Following this wash, the flies were allowed to sit in 4% paraformaldehyde for 20 minutes and further washed in 1x PBS 3 more times following the addition of paraformaldehyde. The flies were then prepared for staining by cutting off the remaining thorax leaving only the

intact abdomen. The abdomen was then trimmed to ensure that the heart sits flat when mounted. The respective genotypes were put in a 96-well plate and prepared for washing. Once added, the flies were washed in 0.05% PBTx, a buffer which contains Triton-X. The hearts were stained with phalloidin and anti-pericardin to visualize filamentous actin and pericardin, respectively (Alayeri et al., 2009). Following staining, the hearts were mounted on slides using Prolong-Gold as the mountant and allowed to dry at 4°C for a day before analyzed using confocal microscopy.

Results

A. Pp2B-14D Functional Studies

M-modes were generated through SOHA by analyzing changes over time in a single region of interest which were manually chosen and marked. This is done in order to visualize a mono-dimensional view of the heart and also to easily observe the diastolic/systolic intervals and diameters for each heart tube (**Figure 2**).

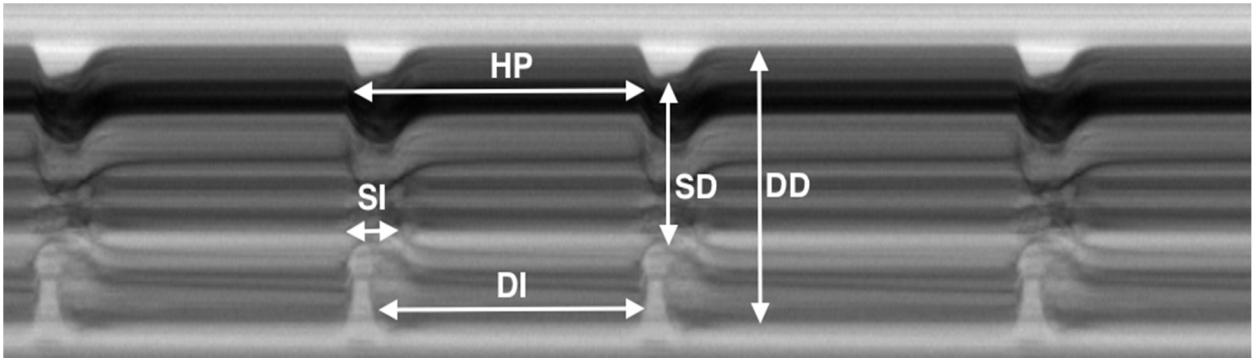

Figure 2: M-mode taken from a beating adult Drosophila heart tube. Diastolic/systolic diameters (DD & SD) intervals (DI & SI) are labeled as shown. SOHA is able to calculate these parameters through manual markings of heart films.

Firstly, the effects of Pp2B-14D knockdown (KD) and overexpression (OE) were tested in the fruit fly heart tube using the TinCΔ4-gal4 driver. Systolic and diastolic intervals were calculated during M-mode analysis. The diastolic interval (DI) is the amount of time it takes for the heart to fill with hemolymph; also known as the relaxation phase. The systolic interval (SI) is the time it takes for the heart to contract and pump out the hemolymph into the rest of the body. The completion of diastolic and systolic intervals in succession is known as the cardiac cycle. The length of time it takes to complete one cardiac cycle is known as the heart period. An increased systolic interval was observed for the Pp2B-14D KD whereas the diastolic interval was significantly decreased in the same KDs when compared to their respective controls. Ultimately, this led to a significantly shorter heart period in the Pp2B-14D KDs (**Figure 3**), indicating that knockdown of the CnA subunit results in an increase in the contraction phase and a decrease in

relaxation time. The Pp2B-14D OE showed opposite effects in comparison to the KD. Decreased systolic intervals were observed – meaning that less time was spent in the contraction phase when Pp2B-14D was over activated. A significant difference was not observed in diastolic intervals and heart periods.

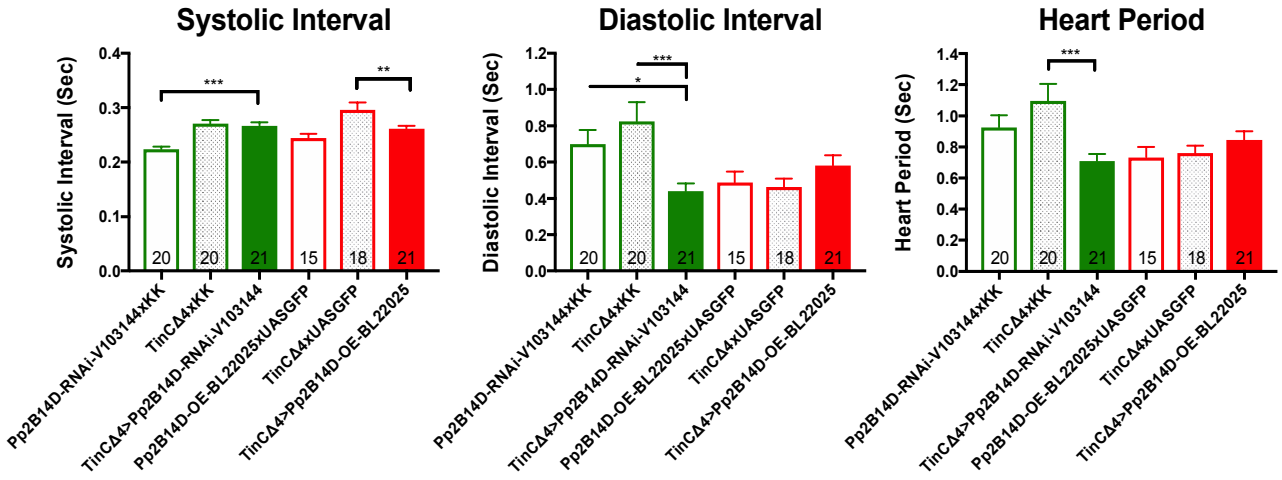

Figure 3: Diastolic and systolic interval responses to Pp2B-14D KD and OE. When compared to its controls the Pp2B-14D KD suffers an increased systolic interval while simultaneously undergoing decreased diastolic intervals. However, Pp2B-14D OE seems to have less of an effect on these parameters besides a significant decrease in its systolic interval when compared to one of its respective controls. *Data analysis: 1-way analysis of variance (ANOVA) and Sidak's multiple comparisons post-hoc test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Sample sizes are portrayed within each genotype and results are illustrated in Mean ± SEM.*

Previous studies have shown that calcineurin OE in transgenic mice have led to phenotypes such as an increase in heart size from the thickening of the ventricular walls that mimic human cardiomyopathies through hypertrophy. Similar phenotypes are seen when administering Pp2B-14D KDs and OEs in the *Drosophila* system. Both the systolic and diastolic diameters were reduced in the Pp2B-14D KDs (**Figure 4**). This directly correlates with studies done in mammalian systems where inhibition of calcineurin via cyclosporine A was seen to reduce the hypertrophic response. As expected, the Pp2B-14D OE resulted in larger systolic diameters whereas a firm conclusion cannot be made about the diastolic diameters in these fruit

flies. Fractional shortening is a measure of muscular contractility and is essentially the percent loss of diastole during systole. Since the systolic and diastolic diameters were altered in proportion to one another, no significant change in fractional shortening was observed. Fractional shortening can be calculated using the following equation:

$$\left(\frac{\mathrm{DD} - \mathrm{SD}}{\mathrm{DD}}\right) \times 100\% = \mathrm{FS}$$

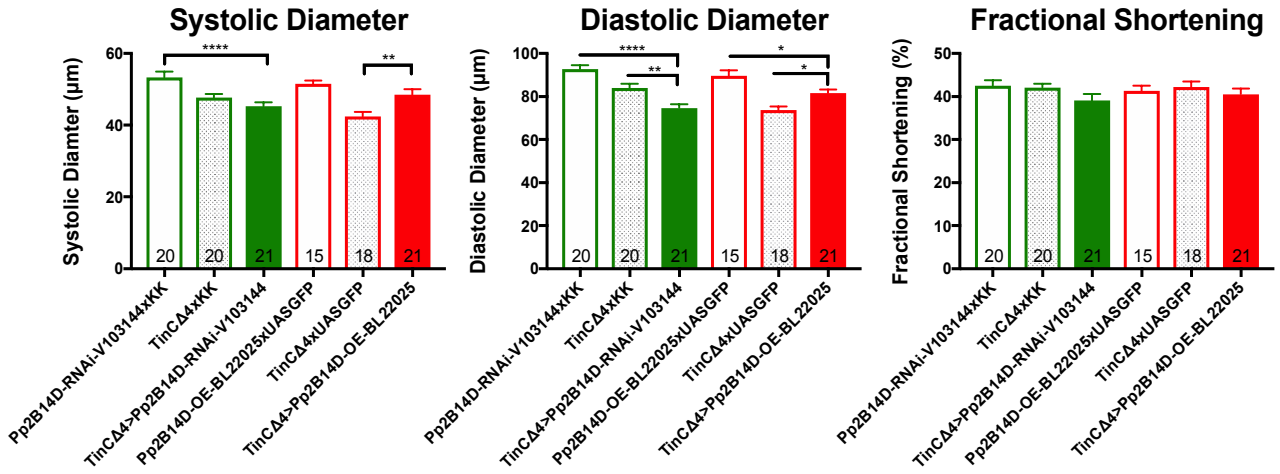

Figure 4: Diastolic and systolic diameter responses to Pp2B-14D KD and OE. Systolic and diastolic diameters were reduced in the Pp2B-14D KD while only systolic diameters are confirmed to be increased in the Pp2B-14D OE when compared to their respective controls. A firm conclusion on the effects of Pp2B-14D OE on diastolic diameter cannot be made. No significant change in fractional shortening was observed. *Data analysis: 1-way analysis of variance (ANOVA) and Sidak's multiple comparisons post-hoc test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Sample sizes are portrayed within each genotype and results are illustrated in Mean ± SEM.*

Lastly, heart rate and stroke volume were calculated and analyzed to determine cardiac output. The heart rate is essentially the average number of beats in a given time period—in our case this was 30 second. Stroke volume is a measure of the amount of hemolymph circulated during one heartbeat. The Pp2B-14D KD were found to experience significantly higher heart rates and lower stroke volumes in comparison to its respective controls. Since cardiac output is a function of heart rate and stroke volume, no significant difference was observed in this parameter

(**Figure 5**). The Pp2B-14D OE did not experience any significant change in heart rate however, a slightly significant decrease in stroke volume and cardiac output was found.

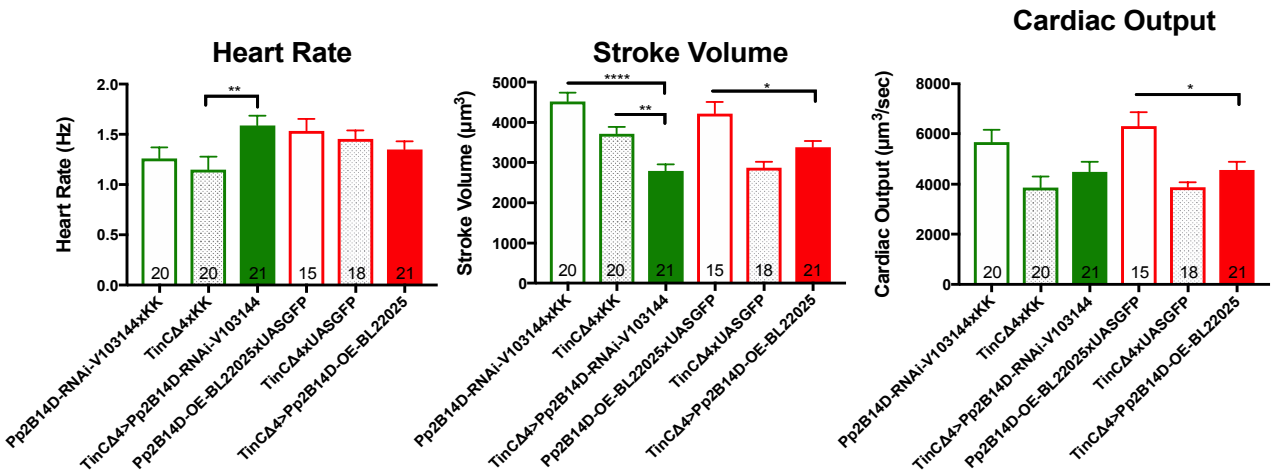

Figure 5: Heart rate, stroke volume, and cardiac output responses to Pp2B-14D KD and OE. Pp2B-14D KD were observed to have significantly higher heart rates while simultaneously experiencing significantly lowered stroke volumes. As a result, no significant change was detected in cardiac output. Pp2B-14D OE were found to experience similar heart rates while also experiencing smaller stroke volumes. Resultantly, a significant decrease in cardiac output was discovered. *Data analysis: 1-way analysis of variance (ANOVA) and Sidak's multiple comparisons post-hoc test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Sample sizes are portrayed within each genotype and results are illustrated in Mean ± SEM.*

B. Effects of NFAT KD on Drosophila Heart Function

Our analysis on Pp2B-14D was also a proof of concept showing that there is a pathway which leads to a phenotype similar to hypertrophic cardiomyopathy in *Drosophila*. With this in mind, we decided that it was important to study the sole function of dNFAT in order to better understand its role in this pathway in fruit flies. In order to confirm reproducibility, two separate RNAi lines were used (**Table 1**). The effects of knockdown were first tested in the heart tube and surrounding pericardial cells using the Hand4.2-gal4 driver (**Figure 6**). dNFAT KD under the Hand4.2-gal4 driver showed no significant differences in both RNAi lines in systolic/diastolic diameters, heart rate, stroke volume, and cardiac output. However, when compared to its respective control dNFAT KD seemed to result in a slightly significant increase in systolic

interval as well as a slightly significant decrease in diastolic intervals in the 30566 RNAi line. Overall, the length of time in the cardiac cycle (heart period) remained unchanged. This phenotype is extremely similar to the results seen in the Pp2B-14D KD. However, no changes in systolic/diastolic diameters were observed in dNFAT KD. The controls are consistent with previous experiments further illustrating that these results are accurate and reliable.

Next, we decided to replicate this experiment in only the heart tube using the TinCΔ4-gal4 driver (**Figure 7**). In these experiments, similar outcomes were observed in significantly increased systolic intervals and consequently increased heart periods in the same 30566 RNAi line. Furthermore, a significant decrease was observed in heart rate and resultantly in the cardiac output of dNFAT KDs. In both of these tissue-directed NFAT KDs, the diameters remained unchanged and any effects seen were only observed in the 30566 RNAi line.

Lastly, we decided to test for cell non-autonomous effects of dNFAT in fat bodies using the LSP-gal4 driver and the same RNAi line that resulted in increases of systolic intervals in previous heart tube studies (**Figure 8**). Our results showed there were significant increases in systolic intervals when compared to respective controls. No other significant changes were found.

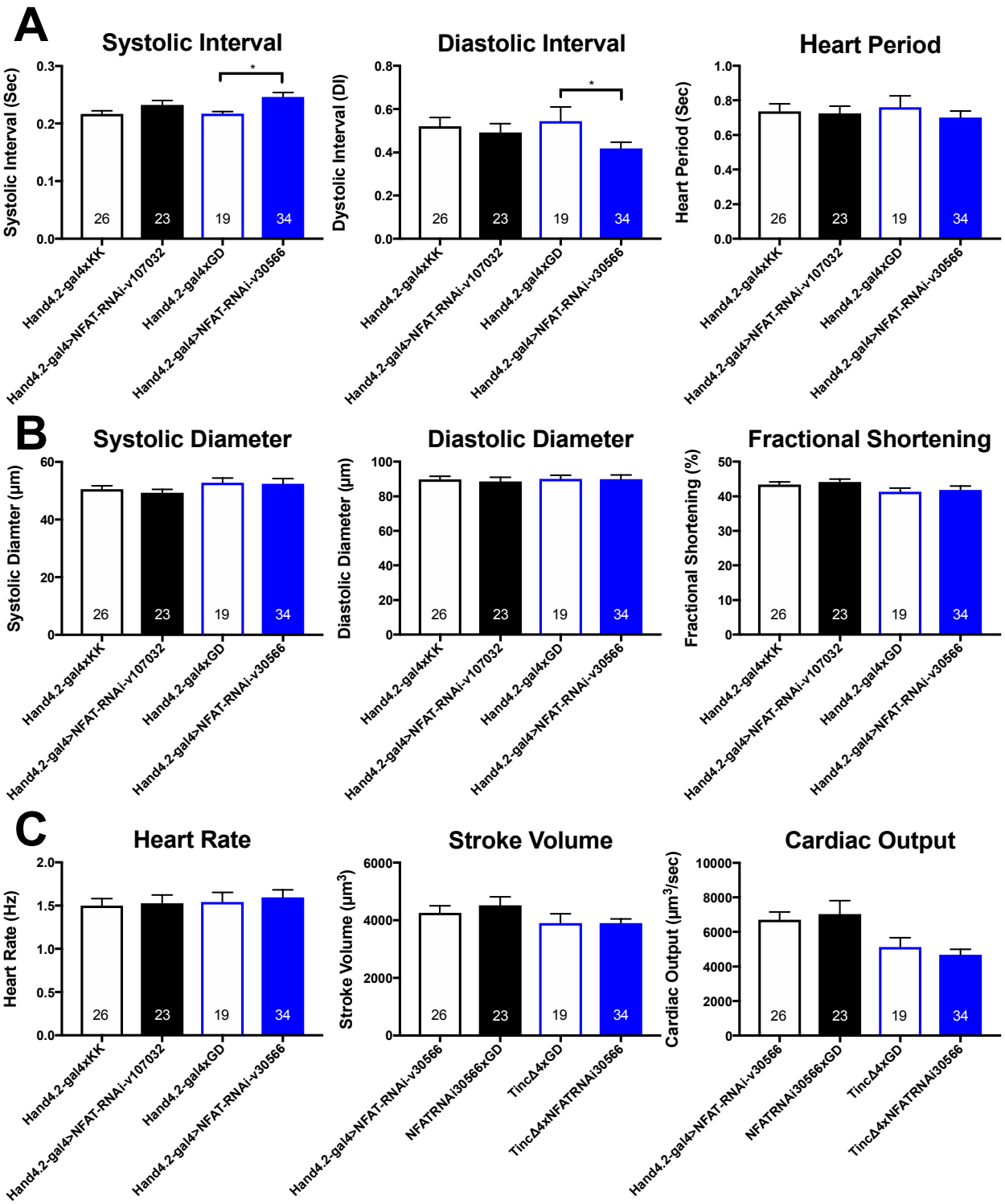

Figure 6: Hand4.2-gal4 directed NFAT KD responses in the *Drosophila* heart. A slightly significant increase in systolic intervals was observed in one of the two RNAi KD. *Data Analysis: Unpaired t-test assuming a Gaussian distribution; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Sample sizes are portrayed within each genotype and results are illustrated in Mean ± SEM.*

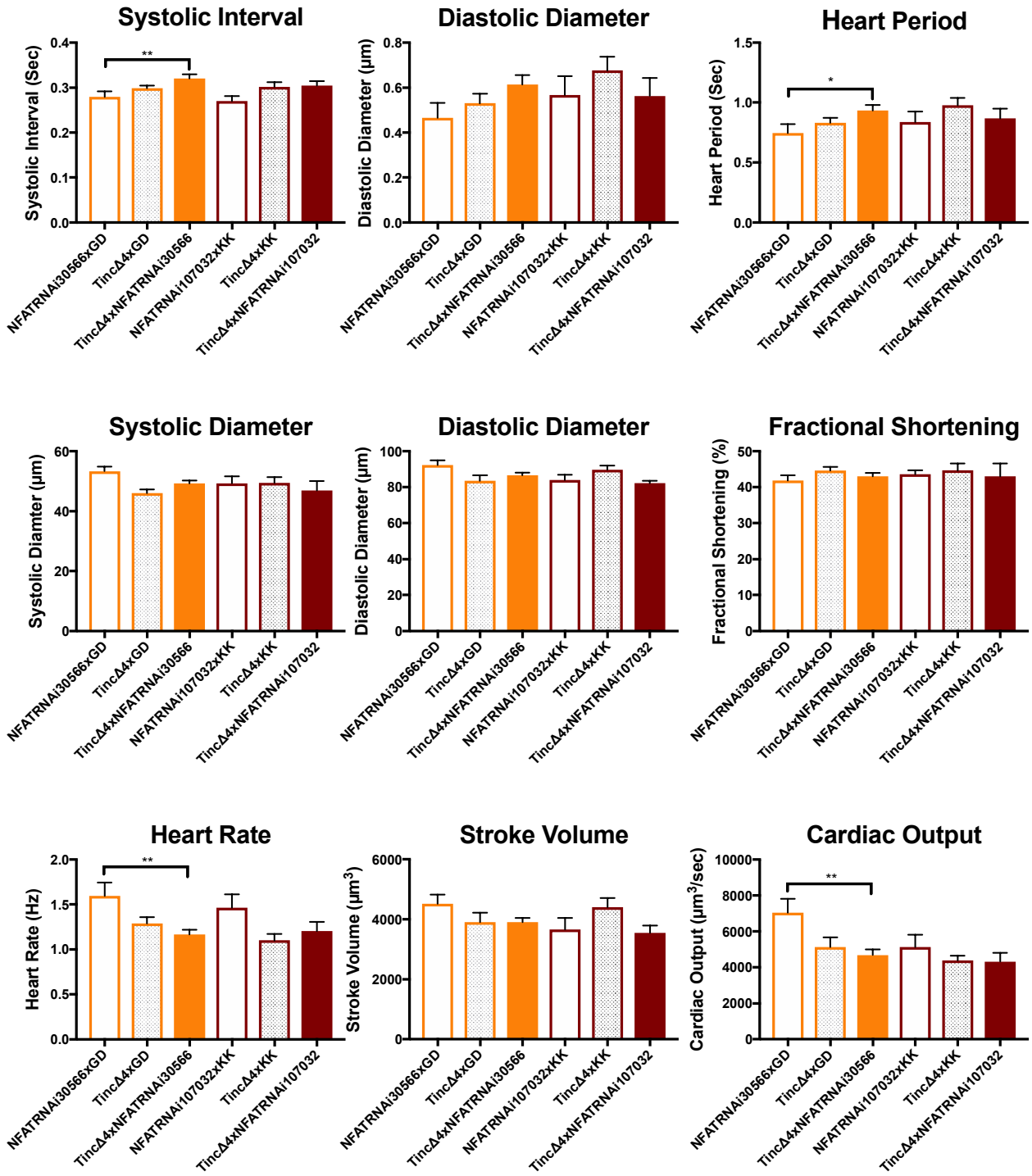

Figure 7: TinCΔ4-gal4 directed NFAT KD responses in the *Drosophila* heart. A slightly significant increase in systolic intervals was observed in one of the two RNAi KD lines. This resulted in slightly increased heart periods. Heart rate and cardiac output were also significantly decreased in these KDs. *Data analysis: 1-way analysis of variance (ANOVA) and Sidak's multiple comparisons post-hoc test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Results are illustrated in Mean ± SEM.*

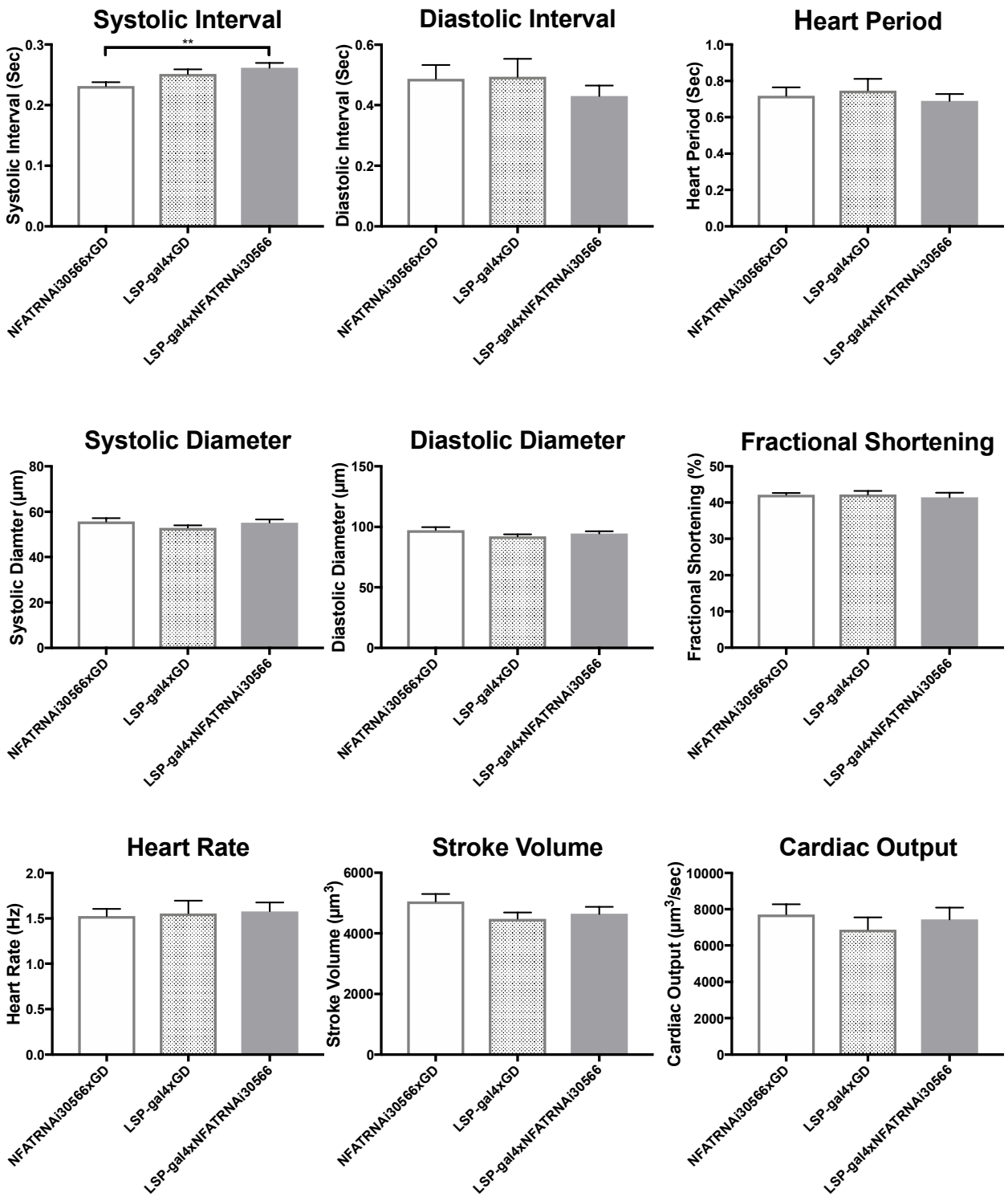

Figure 8: LSP-gal4 directed NFAT KD responses in the *Drosophila* heart. A slightly significant increase was seen in the systolic intervals when compared to its respective controls. No other significant differences were seen in any heart parameter. *Data analysis: 1-way analysis of variance (ANOVA) and Sidak's multiple comparisons post-hoc test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Results are illustrated in Mean ± SEM.*

C. Effects of Calmodulin (CaM) KD on Drosophila Heart Function

After observing the effects of dNFAT and Pp2B-14D on heart function we decided to look at the effects of calmodulin (CaM) on heart function using the TinCΔ4-gal4 driver. The phenotypes observed in the CaM-RNAi KD resulted in more than half of the hearts to be non-contractile (**Figure 9**). CaM-RNAi KD likely plays many roles in the *Drosophila* heart besides the activation of the hypertrophic pathway. The contractile defects which were seen were visually characterized and were broad in range from completely to partially non-contractile.

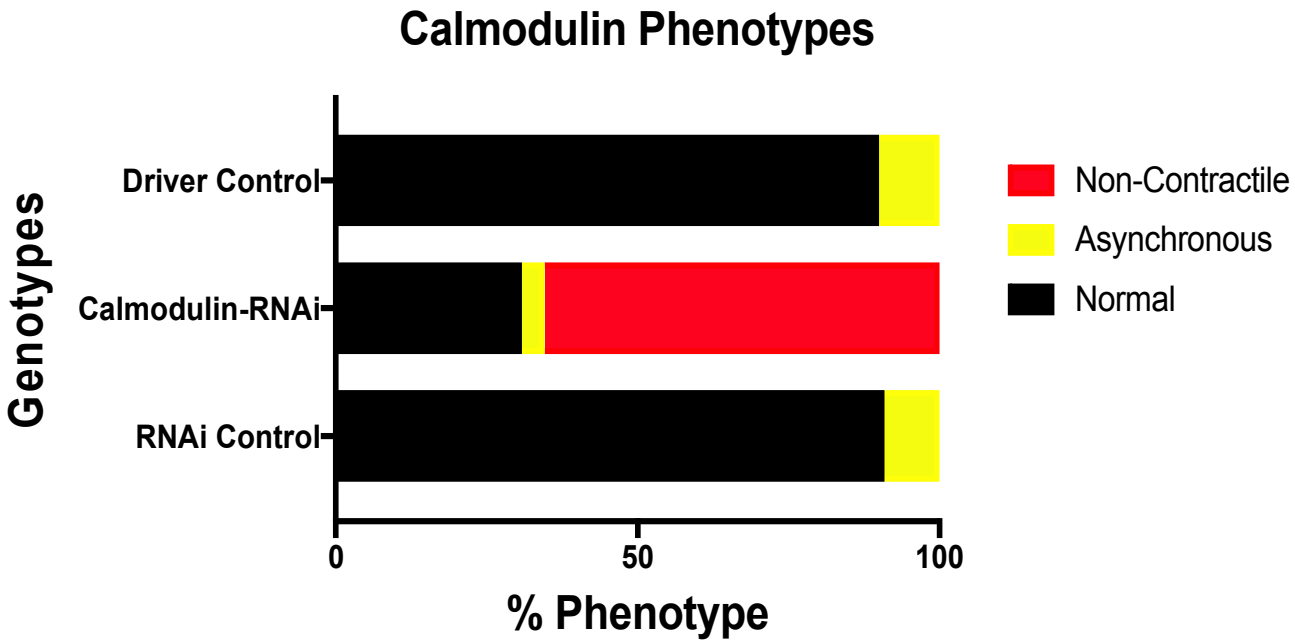

Figure 9:Phenotypes observed after TinCΔ4-gal4 directed CaM KD on *Drosophila* heart function. The majority of phenotypes observed in the CaM KD were non-contractile. *Data analysis: Chi-square test (p=0.0002)*

D. Immunohistochemical Analysis

In order to determine if there were any structural changes, the hearts were stained and analyzed using confocal microscopy. An anti-pericardin antibody was used in order to visualize pericardin, a *Drosophila* specific type IV collagen-like protein often found in the extracellular matrix. Filamentous actin was visualized using fluorescent phalloidin. The heart structure between dNFAT gene knockdown and relevant controls were very similar and no obvious differences were observed in terms of structural changes (**Figure 10**). Both conditions portrayed highly organized hearts and were correctly stained. The alary muscles were not stained during this procedure however, they were still observed during dissections. These muscles are essential in supporting the heart tube and serve as an attachment between the heart tube and abdominal walls. Furthermore, the ventral longitudinal muscle layers stained very well during this procedure whereas the circular cardiomyocytes do not.

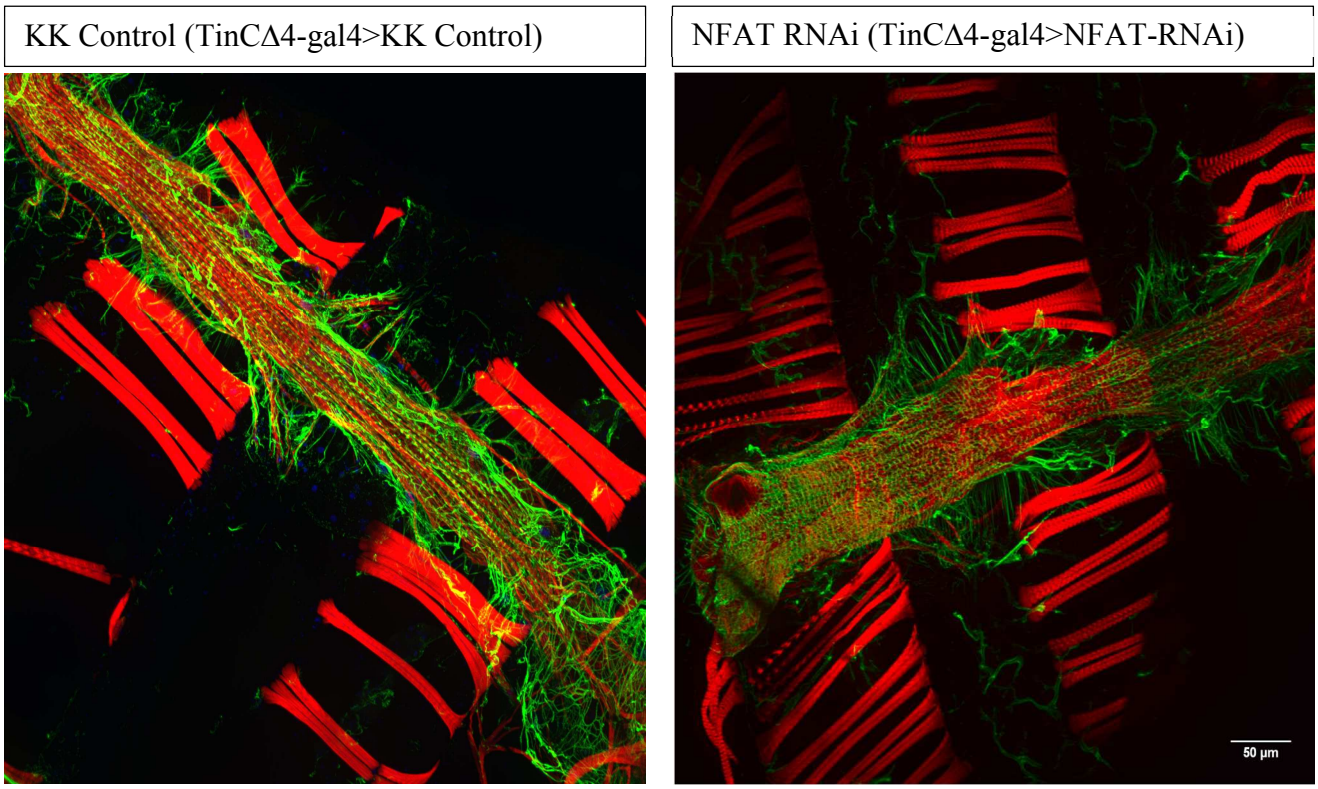

Figure 10: Structural changes were not observed under dNFAT KD. Visual analysis revealed organized myofibrils and no excessive deposits of collagen around the heart tube. (Collagen-IV Actin)

Discussion

A. Functional Analysis

The experimentation conducted throughout this study was done in hopes of understanding the independent role of Pp2B-14D and dNFAT on heart function in our model organism, *Drosophila melanogaster*. The loss and gain of function studies performed on one on of the 3 genes which make up the CnA subunit, Pp2B-14D, firstly showed that the fruit fly model is capable of undergoing phenotypes similar to what is seen in cardiac hypertrophy in mammalian systems. Secondly, heart specific knockdown of Pp2B-14D resulted in an overall decrease in cardiac size (SD &DD) whereas the overexpression of this gene resulted in an increase in cardiac size (**Figure 4**). Accordingly, fractional shortening, a measure of contractility, remained unchanged. Special attention was given while investigating the systolic and diastolic diameters as these parameters are especially important in investigating hypertrophy in the fruit fly. During hypertrophy heart muscles become thicker leading to a decrease in the actual chamber size. Therefore, changes in heart diameter are a good indication that a hypertrophic pathway was activated. In humans, ventricular hypertrophy often manifests as diastolic dysfunction and impaired ventricular filling. This goes hand in hand with what is seen when observing the systolic/diastolic intervals the experiments involving Pp2B-14D (**Figure 3**). However, it is important to note that it is unclear whether the increase in cardiac size was due to dilation or thickening of the heart muscle (cardiac hypertrophy).

Since the hypertrophy pathway was found to be activated by calcineurin and its subsequent activation of NFAT through dephosphorylation in vertebrate systems, the independent effects of heart specific knockdown of dNFAT was also studied. The two RNAi lines used in this experiment were purposefully chosen to explore the possibility regarding

whether different RNAi libraries will produce any difference in knockdown between one another (**Table 2**). Though the 107032/KK line contained 1 off-target effect while its GD counterpart contained none, more drastic effects were observed when the GD library was used rather than the KK. Despite the differences, both lines produced very similar results. Additionally, testing both RNAi lines confirmed that no technical errors were made. By testing for reproducibility, we can confirm the notion that these results are accurate. At most, dNFAT knockdown in the cardiomyocytes and pericardial cells using the Hand4.2-gal4 driver were observed to portray higher systolic intervals in comparison to controls. Since no changes in diameter were seen it is highly unlikely that the functional changes that are seen through this knockdown are calcineurin-mediated. Additionally, no changes were seen in dNFAT knockdown in the cardiomyocytes using the TinCΔ4-gal4 driver. Therefore, it is probable that the changes seen in intervals are due from the surrounding pericardial cells near the heart tube. However, pericardial specific drivers, such as SNS-gal4, must be used to confirm this notion. Hand4.2-gal4 is also regarded as a stronger driver than TinCΔ4-gal4; therefore, the effects seen in intervals can be due to stronger knockdown.

Table 2: Key comparisons of two NFAT RNAi lines used throughout KD experiments.

VDRC 30566/GD	VDRC 107032/KK
Chromosome 2 insertion	Chromosome 2 insertion
No Balancer	CyO Balancer
0 off-target effects	1 off-target effect

Since there were no changes in cardiac size in the fruit fly heart due to dNFAT knockdown, there are likely other calcineurin-mediated pathways which lead to cardiac hypertrophy. Previous studies have shown that calcineurin and its subsequent activation of NFAT resulted in cardiac hypertrophy in mammalian systems. In fruit flies, only the over-

activation of the calcineurin homolog, Pp2B-14D, were observed to result in increases in cardiac size. Calcineurin acts on a variety of genes in both vertebrates and invertebrates; it is probable that there are other unexplored transcription factors in the fruit fly that result in cardiac hypertrophy similar to the calcineurin-NFAT mediated pathway in mammals (**Figure 1**). These results also support the hypothesis that dNFAT is the most primordial gene in the NFAT gene family. dNFAT lacks a calcineurin binding site and is also the most structurally similar to NFAT5, which was found to not play a role in hypertrophy, in vertebrates. Therefore, the slightly significant increase in systolic intervals observed in dNFAT KD is likely unrelated to hypertrophy. Furthermore, there may be possible compensation changes made by other genes to account for the dNFAT knockdown thus producing a net effect that is similar in phenotype to the controls. It would be valuable to study the effect of NFAT KD in sensitized backgrounds, such as NFAT heterozygotes, and measure mRNA levels via qPCR, to help narrow down the all the potentials.

The fact that mammalian systems contain three distinct CaM genes which encode identical proteins may provide clues to its importance in calcium signaling. This genetic redundancy serves as a lifeline in case of a deleterious mutation because there would be little to no change in the biological phenotype. Since the *Drosophila melanogaster* model only contains a single CaM gene, it would make evolutionary sense that knockdown of this gene will lead to a drastic change in phenotype (**Figure 9**). Based on the results, it is apparent that CaM is playing multiple roles in the heart likely through muscle contraction and other activity in ion pumps. The inability for Ca^{2+} to interact with the myosin heavy chain in CaM KD lines up very well with the non-contractile phenotype observed in these flies. Furthermore, this set of experiments serves as

a great example in the power of using the model organism, *Drosophila melanogaster*, in heart function studies.

Besides the aforementioned possibilities, it is possible that there was insufficient knockdown of NFAT in the fruit fly heart. It is likely that relatively low amounts of the transcription factor, NFAT, are needed to induce expression of target genes. Therefore, even if the knockdown did produce a significant decrease in NFAT mRNA levels it is still quite possible that there will be enough available to have an effect on the cell. To confirm this is not the case, future experiments should include conducting qPCR or RNA *in situ* hybridization on isolated hearts and comparing the levels of NFAT mRNA transcript between the controls and knockdown hearts. Additionally, a double knockdown line can be created by crossing two different RNAi lines which contain insertions on different chromosomes. This would be useful in observing the effects of Pp2B-14D KD and dNFAT KD in the same fruit fly. Next, it would be useful to create a Pp2B-14D mutant to truly understand the roles this gene plays in cardiovascular muscle development and remodeling. Finally, to study the relationship between NFAT and Pp2B-14D, mutants can be created to inhibit binding of calcineurin to NFAT.

B. Structural Analysis

The dNFAT KD heart structures analyzed during the staining procedure produced no obvious differences between the controls. Moreover, all the hearts contained a high amount of contractile muscles near the conical chamber. Thus, confirming that there was no disorganization in pericardin surrounding the heart tube. Since heart function was not affected by NFAT gene knockdown, the absence of structural changes in the heart provides further indication that the knockdown had no effect. However, it is unclear whether this is due to insufficient knockdown. Further immunohistochemical analysis and structural studies are needed to confirm that the

functional changes seen in Pp2B-14D knockdown and overexpression are due to hypertrophy rather than dilation. These studies will also provide details on how heart structure changes during cardiac remodeling if the hypertrophic pathway was indeed activated. Excessive deposits of collagen-IV are also expected to be seen if hypertrophy was active in the fruit fly heart.

References

Alayari, Nakissa N, Georg Vogler, Ouarda Taghli-Lamallem, Karen Ocorr, Rolf Bodmer, and Anthony Cammarato. “Fluorescent labeling of Drosophila heart structures.” Journal of visualized experiments: JoVE no. 32 (2009).

Arrese, Estela L., and Jose L. Soulages. “INSECT FAT BODY: ENERGY, METABOLISM, AND REGULATION.” *Annual review of entomology* 55 (2010): 207–225. *PMC*. Web. 17 May 2018.

Bier, Ethan, and Rolf Bodmer. “Drosophila, an emerging model for cardiac disease.” *Gene* 342, no. 1 (2004): 1–11.

Clipstone, Neil A., and Gerald R. Crabtree. “Identification of Calcineurin as a Key Signalling Enzyme in T-Lymphocyte Activation.” Nature, vol. 357, no. 6380, 1992, pp. 695–697., doi:10.1038/357695a0.

Duffy J.B. GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis, 34 (1–2) (2002), pp. 1–15

Friedberg, F, and Rhoads - AR life. “Evolutionary aspects of calmodulin.” *IUBMB life* (2001). Available: http://onlinelibrary.wiley.com/doi/10.1080/1521654

Green, Edward W., Giorgio Fedele, Flaviano Giorgini, and Charalambos P. Kyriacou. "A Drosophila RNAi collection is subject to dominant phenotypic effects." *Nature Methods* 11.3 (2014): 222-23. Web.

Keyser P, Borge-Renberg K, Hultmark D. The *Drosophila* NFAT homolog is involved in salt stress tolerance. Insect Biochem Mol Biol. 2007;37:356–362.

Luo, C, K T Shaw, A Raghavan, J Aramburu, F Garcia-Cozar, B A Perrino, P G Hogan, and A Rao. “Interaction of calcineurin with a domain of the transcription factor NFAT1 that controls nuclear import.” Proceedings of the National Academy of Sciences of the United States of America 93, no. 17 (August 2, 1996): 8907–12.

Martin, Stephen R, and Peter M Bayley. “Calmodulin bridging of IQ motifs in myosin V.” *FEBS letters*567.2-3 (2004): 166–170.

Miskei M, Ádám C, Kovács L, Karányi Z, Dombrádi V. Molecular evolution of phosphoprotein phosphatases in Drosophila. PLoS One. 2011;6:e22218. doi: 10.1371/journal.pone.0022218.

Miyakawa, H, SK Woo, and Dahl - SC of the …. “Tonicity-Responsive Enhancer Binding Protein, a Rel-like Protein That Stimulates Transcription in Response to Hypertonicity.” Proceedings of the … (1999). doi:10.1073/pnas.96.5.2538.

Molkentin, J D, J R Lu, C L Antos, B Markham, J Richardson, J Robbins, S R Grant, and E N Olson. “A calcineurin-dependent transcriptional pathway for cardiac hypertrophy.” Cell 93, no. 2 (April 5, 1998): 215–28.

Ocorr K, Vogler G, Bodmer R. Methods to assess Drosophila heart development, function and aging. Methods. 2014;68:265–272. doi: 10.1016/j.ymeth.2014.03.031.

Swulius, M. T., and M. N. Waxham. “Ca2 /Calmodulin-Dependent Protein Kinases.”*Cellular and Molecular Life Sciences*, vol. 65, no. 17, 2008, pp. 2637–2657.

Tóth, Judit, Mihály Kovács, Fei Wang, László Nyitray, and James Sellers. “Myosin V from Drosophila Reveals Diversity of Motor Mechanisms within the Myosin V Family.” Journal of Biological Chemistry 280, no. 34 (2005): 30594–30603.

Vetter, S.W. and Leclerc, E. (2003): Novel aspects of calmodulin target recognition and activation. Eur. J. Biochem. 270, pp. 404-414.

Vogler G, Bodmer R, Akasaka T. A Drosophila model for congenital heart disease. Drug Discovery Today: Disease Models. 2009;6:47–54. 10.1016/j.ddmod.2009.11.002.

Windt, De LJ, HW Lim, and Bueno - OF of the …. 2001. “Targeted Inhibition of Calcineurin Attenuates Cardiac Hypertrophy in Vivo.” Proceedings of the …. doi:10.1073/pnas.031371998. http://www.pnas.org/content/98/6/3322.short.