

Distribution Statement

Distribution A: Public Release.

The views presented here are those of the author and are not to be construed as official or reflecting the views of the Uniformed Services University of the Health Sciences, the Department of Defense or the U.S. Government.

Store Operated Calcium (Ca^{2+}) Entry is an essential regulator of cardiac physiology

By

Courtney Elizabeth Petersen

Dissertation submitted to the Faculty of the
Molecular and Cell Biology Graduate Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2021



UNIFORMED SERVICES UNIVERSITY, SCHOOL OF MEDICINE GRADUATE PROGRAMS
Graduate Education Office (A 1045), 4301 Jones Bridge Road, Bethesda, MD 20814



**FINAL EXAMINATION/PRIVATE DEFENSE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN
THE MOLECULAR & CELL BIOLOGY GRADUATE PROGRAM**

Name of Student: Courtney Petersen
Date of Examination: January 25, 2021
Time: 2:00 PM
Place: Virtual Meeting

DECISION OF EXAMINATION COMMITTEE MEMBERS:

	PASS	FAIL
Rachel Cox Digitally signed by Rachel Cox Date: 2021.01.28 13:43:34 -05'00'	<u>X</u>	_____
Dr. Rachel Cox DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY Committee Chair		

SMYTH.JEREMY.T.1180138249 Digitally signed by SMYTH.JEREMY.T.1180138249 Date: 2021.01.28 13:05:00 -05'00'	<u>X</u>	_____
Dr. Jeremy Smyth DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS Dissertation Advisor		

FLAGG.THOMAS.P.1376882930 Digitally signed by FLAGG.THOMAS.P.1376882930 Date: 2021.01.28 14:25:02 -05'00'	<u>X</u>	_____
Dr. Thomas Flagg DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS Committee Member		

KORTUM.ROBERT.1506017030 Digitally signed by KORTUM.ROBERT.1506017030 Date: 2021.01.28 14:44:07 -05'00'	<u>X</u>	_____
Dr. Robert Kortum DEPARTMENT OF PHARMACOLOGY AND MOLECULAR THERAPEUTICS Committee Member		

Indu S. Ambudkar -S Digitally signed by Indu S. Ambudkar -S Date: 2021.01.29 09:40:01 -05'00'	<u>X</u>	_____
Dr. Indu Ambudkar NATIONAL INSTITUTE OF DENTAL AND CRANIOFACIAL RESEARCH, NATIONAL INSTITUTES OF HEALTH Committee Member		



APPROVAL OF THE DOCTORAL DISSERTATION IN THE MOLECULAR & CELL BIOLOGY
GRADUATE PROGRAM

Title of Thesis: "Store-operated calcium (Ca^{2+}) entry is an essential regulator of cardiac physiology"

Name of Candidate: Courtney Petersen
Doctor of Philosophy
January 25, 2021

THESIS AND ABSTRACT APPROVED:

DATE:

Rachel Cox Digitally signed by Rachel Cox
Date: 2021.01.28 13:44:13 -05'00'

1/28/2021

Dr. Rachel Cox
DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
Committee Chair

SMYTH.JEREMY Digitally signed by
SMYTH.JEREMY.T.1180138249
Date: 2021.01.28 13:05:28
-05'00'

1/28/2021

Dr. Jeremy Smyth
DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS
Thesis Advisor

FLAGG.THOMA Digitally signed by
FLAGG.THOMAS.P.1376882930
Date: 2021.01.28 14:25:42 -05'00'

1/28/2021

Dr. Thomas Flagg
DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS
Committee Member

KORTUM.ROBERT. Digitally signed by
KORTUM.ROBERT.1506017030
Date: 2021.01.28 14:44:49 -05'00'

1/28/2021

Dr. Robert Kortum
DEPARTMENT OF PHARMACOLOGY AND MOLECULAR THERAPEUTICS
Committee Member

Indu S. Digitally signed by Indu S.
Ambudkar-S
Date: 2021.01.29 09:40:58
-05'00'

1/28/2021

Dr. Indu Ambudkar
NATIONAL INSTITUTE OF DENTAL AND CRANIOFACIAL RESEARCH, NATIONAL
INSTITUTES OF HEALTH
Committee Member

ACKNOWLEDGEMENTS

First, and foremost I must acknowledge my mentor, Dr. Jeremy Smyth. I am incredibly grateful for your mentorship. Thank you for all your time, patience, and consideration throughout this process. Thank you for encouraging my growth as a well-rounded, capable scientist. Thank you for learning with me and inciting my scientific curiosity.

Next, I would like to acknowledge my committee members, Dr. Rachel Cox, Dr. Thomas Flagg, Dr. Robert Kortum, and Dr. Indu Ambudkar. I know you all to be exceptional scientists, whom I greatly admire and respect. Your guidance has been fundamental to my success.

Thank you to everyone in the Smyth and Cox Lab, especially Darya Karabasheva, for training and supporting me.

Thank you to my fellow graduates, Erin Sheffels and Robert Geiger, who have stuck with me throughout this arduous journey. I am so proud of how far we have come, and I could not have asked for better colleges. We made it guys!

A special thank you to my partner, Michael Garrison, for all the reassurance, patience, kindness and love you have provided not only in this process but our life together.

Thank you to my friends and family for your love and encouragement.

I would also like to acknowledge the Collaborative Health Initiative Research Program (CHIRP) for supporting this research.

DEDICATION

I would like to dedicate this to my family, Christopher, Crystal, and Cameron Petersen, who have inspired and emboldened my personal and academic growth.

I would also like to dedicate this manuscript to the faculty, staff, and students at Uniformed Services University of the Health Sciences. It is here I found a sense of community that I am proud belong to, for the greatness of a community is attributable to the support and compassion and its members.

COPYRIGHT STATEMENT

The author hereby certified that the use of any copyrighted material in the dissertation manuscript entitled: Store Operated Calcium (Ca^{2+}) Entry is an essential regulator of cardiac physiology is appropriately acknowledged and, beyond brief excerpts, is with the permission of the copyright owners

[Signature]

A large black rectangular box redacting the signature of the author.

Courtney Elizabeth Petersen

Date: 1/11/2021

DISCLAIMER

The views presented here are those of the author and are not to be construed as official or reflecting the views of the Uniformed Services University of Health Sciences, the Department of Defense, or the U.S. Government

ABSTRACT

Calcium (Ca^{2+}) signaling serves as a nodal point in cardiac physiology through regulation of diverse cellular events like excitation-contraction coupling and transcriptional regulation. Accordingly, disrupted Ca^{2+} homeostasis is known to be a central cause of heart failure pathogenesis. Heart failure is a major global health burden that continues to increase in prevalence worldwide. Experimental therapeutics aimed at restoring proper Ca^{2+} dynamics may offer broad therapeutic potential but have yet to meet clinical expectations. To improve translational efficiency, we need to better understand how Ca^{2+} dependent processes specifically and selectively mediate maladaptive cardiac features. Recently, the store operated Ca^{2+} entry (SOCE) pathway, whereby Ca^{2+} influx is triggered upon depletion of endo/sarcoplasmic reticulum (ER/SR) stores, was found to be essential to the induction of pathological cardiac hypertrophy. SOCE is mediated by STIM proteins that function as luminal ER/SR Ca^{2+} sensors, and Orai Ca^{2+} influx channels in the plasma membrane. Despite accumulating evidence, the specific cardiac processes regulated by SOCE remain poorly understood.

Our results demonstrate SOCE is an essential regulator of *Drosophila* heart physiology. Heart specific SOCE suppression, by Stim and Orai RNAi, induced dilated cardiomyopathy with enhanced cardiac diameters and reduced contractile strength. Myofibrils in SOCE suppressed animals were highly disorganized and loosely packed. Conversely, heart specific SOCE upregulation by constitutively active Stim and Orai (Stim^{CA} and Orai^{CA}) expression induced cardiac hypertrophy and decreased cardiac dimensions, that was dependent on Ca^{2+} influx through SOCE specific, Orai channels.

SOCE suppressed and upregulated animals died significantly earlier than controls, suggesting pathologically impairment of cardiac function and heart failure. Cardiac defects caused by SOCE suppression and upregulation did not significantly affect heart rate or rhythmicity, which suggests SOCE signaling primarily regulates gene expression and maintains tissue homeostasis. Collectively, our results are consistent with previous reports and demonstrate an essential, heart-specific role for SOCE across species. Furthermore, we establish that the *Drosophila* model serves as an ideal platform to delineate the mechanism by which SOCE regulates heart function through the integration of tractable genetic tools with powerful experimental approaches to heart contractility and morphological analysis.

TABLE OF CONTENTS

LIST OF TABLES	xii
LIST OF VIDEOS	xiii
LIST OF FIGURES	xiv
CHAPTER 1: Introduction	1
Ca ²⁺ is an Essential Regulator of Cardiomyocyte Physiology	1
Ca ²⁺ influx in cardiomyocytes.....	1
Intracellular Ca ²⁺ release and cardiac contraction.....	3
Ca ²⁺ extrusion from cardiomyocytes.....	3
Nuclear Ca ²⁺ and regulation of gene expression	4
Altered Ca ²⁺ dynamics are detrimental to cardiac function	5
Dysregulated Ca ²⁺ Homeostasis is a Central Cause of Heart Failure	6
Heart failure onset and the neurohumoral response.....	6
Cardiac hypertrophy proceeds heart failure	7
Reduced global Ca ²⁺ transients	9
Reduced SR Ca ²⁺ store content	10
Triggered arrhythmias.....	11
Impaired mitochondrial function	12
Experimental therapeutics targeting abnormal Ca ²⁺ handling	13
Store Operated Ca ²⁺ Entry (SOCE) is an Important Regulator of SR Ca ²⁺	
Homeostasis	14
Discovery and upstream signaling in SOCE	14
SOCE I _{CRAC}	15
STIM.....	16
Orai	20
Physiological SOCE functions and genetic mutations.....	21
SOCE is an Essential Regulator of Physiological and Pathological Heart Function.....	23

SOCE in normal heart physiology	24
SOCE In pathological cardiac hypertrophy and arrhythmia.....	25
<i>Drosophila</i> in Cardiac Research	27
<i>Drosophila</i> heart morphology and function.....	28
<i>Drosophila</i> as a model for heart failure	30
SOCE in the <i>Drosophila</i> heart	31
Summary of Conclusions.....	32
CHAPTER 2: Suppression of store-operated calcium entry causes dilated cardiomyopathy of the <i>Drosophila</i> heart	34
Abstract	35
Introduction	36
Results	39
<i>Stim</i> and <i>Orai</i> suppression results in dilated cardiomyopathy.....	39
<i>Stim</i> and <i>Orai</i> suppression disrupts myofibril organization	42
Heart-specific <i>Stim</i> and <i>Orai</i> suppression reduces animal viability	43
Discussion	45
Materials and Methods	51
Fly stocks	51
Adult survival.....	51
Developmental timing.....	52
RNA isolation and RT-qPCR.....	52
Heart dissection, staining, and confocal imaging	53
Optical coherence tomography (OCT).....	54
Intravital fluorescence microscopy	55
Statistical analysis.....	56
Figures	57
Supplemental Figures	63
Supplemental Tables	66

Supplemental Videos.....	66
CHAPTER 3: A novel micro-computerized tomography (uCT) method reveals hypertrophy of the <i>Drosophila</i> heart caused by upregulation of store operated calcium entry	68
Abstract	69
Introduction	70
Results	74
MicroCT is a valuable tool to evaluate heart wall thickness in <i>Drosophila</i> adults and larva	73
SOCE upregulation results in pathological cardiac hypertrophy.....	77
SOCE upregulation impairs heart contractility.....	79
Stim ^{CA} -mediated hypertrophy and contractility impairment specifically requires Orai channels.....	80
Discussion	81
Materials and Methods	87
Fly Stocks.....	87
Micro-computerized Tomography (microCT).....	88
Adult Labeling	88
Larval Labeling.....	88
Sample Mounting and Scanning	89
Reconstruction	90
Heart Wall Thickness Measurements	90
Intravital Fluorescence Microscopy.....	90
Developmental Timing	91
Statistical Analyses	92
Figures.....	93
Supplemental Figures.....	100
CHAPTER 4: Discussion.....	101
SOCE is an Essential Component of Cardiomyocyte Ca ²⁺ Regulation in <i>Drosophila</i>	103

SOCE Ca ²⁺ Contributes to Cardiomyocyte Morphology and Function.....	106
Innovated New Techniques to Evaluate <i>Drosophila</i> Cardiac Structure and Function .	110
Intravital Fluorescent Imaging.....	110
Micro-computerized Tomography (MicroCT)	112
Concluding Remarks.....	115
References.....	117

LIST OF TABLES

Table 2-S1. Comparison of contractility parameters measured from OCT and CM-tdTom intravital imaging analysis of control animals.....	66
--	----

LIST OF VIDEOS

Video 2-S1. Full intravital imaging timelapse of the adult control heart shown in Figure 2-S3.....	66
Video 2-S2. Full intravital imaging timelapse of the adult <i>Stim</i> RNAi heart shown in Figure 2-S3.....	67
Video 2-S3. Full intravital imaging timelapse of the larval control heart shown in Figure 2-2A.....	67
Video 2-S3. Full intravital imaging timelapse of the larval <i>Stim</i> RNAi heart shown in Figure 2-2A.....	67

LIST OF FIGURES

Figure 1-1. Ca^{2+} Handling Mechanism in Cardiomyocytes.....	2
Figure 1-2. Ca^{2+} dependent regulation of prohypertrophic gene expression	9
Figure 1-3. SOCE Activation Mechanisms	18
Figure 2-1. Heart specific <i>Stim</i> and <i>Orai</i> suppression results in dilated cardiomyopathy in adults	57
Figure 2-2. Larval <i>Stim</i> and <i>Orai</i> suppressed heart are significantly dilated.....	58
Figure 2-3. <i>Stim</i> and <i>Orai</i> suppression results in myofibril disorganization in larval hearts	59
Figure 2-4. <i>Stim</i> and <i>Orai</i> suppression results in myofibril disorganization in adult hearts	60
Figure 2-5. Heart specific <i>Stim</i> and <i>Orai</i> suppression delays post-embryonic development.....	61
Figure 2-6. Heart specific <i>Stim</i> and <i>Orai</i> suppression results in early adult lethality	62
Figure 2-S1. <i>Stim</i> and <i>Orai</i> RNAi are efficient and specific	63
Figure 2-S2. Heart specific suppression of <i>Stim</i> and <i>Orai</i> does not affect adult or larval heart rate.....	64
Figure 2-S3. Intravital fluorescence imaging confirms dilated cardiomyopathy in adult <i>Stim</i> and <i>Orai</i> suppressed heart.....	65
Figure 3-1. MicroCT analysis of normal and hypertrophied hearts in adult <i>Drosophila</i>	93
Figure 3-2. MicroCT analysis of normal and hypertrophied hearts in third instar <i>Drosophila</i> larvae.....	94
Figure 3-3. SOCE upregulation results in hypertrophy of adult <i>Drosophila</i> hearts	95
Figure 3-4. SOCE upregulation results in hypertrophy of larval <i>Drosophila</i> hearts	96
Figure 3-5. SOCE upregulation results in altered heart contractility with reduced heart chamber dimensions.....	97
Figure 3-6. Cardiac hypertrophy caused by constitutively active <i>Stim</i> expression requires <i>Orai</i> channels.....	98
Figure 3-7. Altered heart contractility caused by constitutively active <i>Stim</i> expression requires <i>Orai</i> channels.....	99
Figure 3-S1. Heart specific expression of <i>Stim</i> ^{CA} results in significant larval and pupal lethality	100

CHAPTER 1: INTRODUCTION

Ca²⁺ is an Essential Regulator of Cardiomyocyte Physiology

Calcium (Ca²⁺) is a highly ubiquitous signaling factor found in all eukaryotic cell types where it regulates diverse cellular events that range from mitotic cell birth to apoptotic cell death (43; 103; 182). In the heart, Ca²⁺ is an important regulator of electrophysiology, excitation-contraction (E-C coupling), mitochondrial function, cell death, and transcriptional regulation (15; 55; 89). The most well characterized and critical functions of Ca²⁺ in the heart involve the regulation of electrical activity and cardiac contractility.

Ca²⁺ influx in cardiomyocytes

During the cardiac action potential, depolarization of the plasma membrane (sarcolemma) activates L-type or T-type voltage gated Ca²⁺ channels (VOCCs) to stimulate inward Ca²⁺ current (I_{CA}) (13; 15; 50; 58; 89). L-type VOCCs are primarily located in dyadic clefts, which are regions within the cell where specialized invaginations of the plasma membrane (T-tubules) come within close proximity to ryanodine receptors (RyR) on the sarcoplasmic reticulum (SR) (Figure 1-1). T-type VOCCs are not preferentially located at the dyadic cleft and produce a far weaker I_{CA} than L-type VOCCs (13; 15). Thus, T-type VOCCs are believed to have a negligible role in normal cardiac physiology. The accumulation of Ca²⁺ ions entering through L-type VOCCs during a single action potential is called a Ca²⁺ sparklet and increases Ca²⁺ concentration within the dyadic cleft from ~100nM to ~10μM (43; 55). During the action potential, I_{CA}

through L-type VOCCs is limited by Ca^{2+} -calmodulin (Ca^{2+} -CaM) dependent inactivation by phosphorylation on the cytosolic side. Ca^{2+} sparklets within the dyadic cleft induce opening of a cluster of ~6-20 RyRs on the SR to activate the release SR Ca^{2+} stores, in a process known as Ca^{2+} induced Ca^{2+} release (CICR). The release of SR Ca^{2+} stores by RyR is called a Ca^{2+} spark and further increases the concentration of Ca^{2+} within the cytosol to $>100\mu\text{M}$ in the dyadic cleft (43; 55; 89). Several thousand Ca^{2+} sparks produced at the dyadic junction within each cell are synchronized by the action potential, such that local rises in Ca^{2+} overlap spatially and temporally to produce a global Ca^{2+} increase from 500nM to $\sim 1\mu\text{M}$ (43; 50).

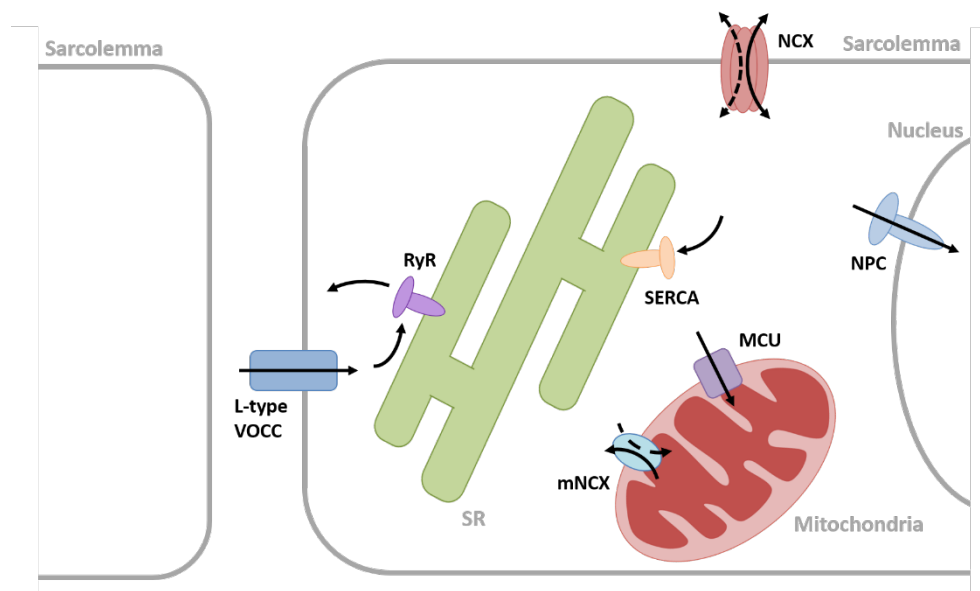


Figure 1-1. Ca^{2+} Handling Mechanisms in Cardiomyocytes. Schematic diagram of ion channels and transporters directly involved in maintaining cardiomyocyte Ca^{2+} homeostasis. Solid arrows indicate movement of Ca^{2+} and dashed arrows indicate movement of Na^+ . Ca^{2+} entry or efflux at the sarcolemma is mediated by L-type voltage gated channels (L-type VOCC) and $\text{Na}^+/\text{Ca}^{2+}$ exchangers (NCX). Ca^{2+} release or reuptake from internal organelles is regulated by ryanodine receptors (RyR) and sarcoendoplasmic reticulum Ca^{2+} ATPases (SERCA) on the sarcoplasmic reticulum (SR), or mitochondrial Ca^{2+} uniporter (MCU) and mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ antiporter (mNCX) on the mitochondria. Nuclear Ca^{2+} influx occurs via the Ca^{2+} nuclear pore complex (NPC).

Intracellular Ca^{2+} release and cardiac contraction

Ca^{2+} released from the SR diffuses out from the dyadic cleft to engage with contractile machinery, promoting cell shortening and generating contractile force. Ca^{2+} regulates cardiac contraction through interactions with the troponin-tropomyosin complex. Specifically, the troponin complex has 3 subunits, which include tropomyosin-binding (TnT), inhibitor (TnI), and Ca^{2+} sensor (TnC) (15; 50; 55; 58). TnT secures the troponin complex to tropomyosin. Under resting conditions, when intracellular Ca^{2+} levels are relatively low, TnI sterically blocks tropomyosin binding to actin through competition of a common actin binding site. Activation of the troponin complex occurs upon Ca^{2+} binding to TnC, which induces a conformational change that moves the troponin-tropomyosin complex deeper into the actin filament groove, exposing actin-tropomyosin binding sites. Myosin heads then attach to actin binding sites forming cross-bridges that induce ATP driven myofilament sliding resulting in cell shortening and contraction (50; 55; 58). In this way, Ca^{2+} couples the electrical excitation of the myocyte to the mechanical action of contraction.

Ca^{2+} extrusion from cardiomyocytes

For relaxation to occur, the concentration of Ca^{2+} within the cytosol must decline. E-C coupling events are short-lived and reach peak contraction within a few tens of milliseconds (55), thus relaxation requires rapid extrusion of Ca^{2+} from the cytosol. Under physiological circumstances, the amount of Ca^{2+} leaving the cell is equal to the amount entering (50; 110). The two primary mechanisms with which Ca^{2+} is extruded from the cytoplasm involve SR Ca^{2+} ATPase (SERCA) and sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchange (NCX) (Figure 1-1). The NCX is widely distributed within the sarcolemma

where it uses the electrochemical gradient of Na^+ to catalyze the reversible exchange of three Na^+ for one Ca^{2+} . Therefore, high intracellular $[\text{Ca}^{2+}]$ favor Ca^{2+} efflux, whereas elevated intracellular $[\text{Na}^+]$ induce Ca^{2+} influx (14; 15; 43; 50; 55; 110; 183). The relevance of Ca^{2+} influx by NCX during normal cardiac function remains controversial, though it has been suggested to contribute to Ca^{2+} influx early during action potential, thereby priming the dyadic cleft to enhance activation of L-type V_{OCC} I_{CA} and triggering SR Ca^{2+} release (181; 183). The second main mechanism of cytoplasmic Ca^{2+} removal is reuptake of Ca^{2+} into the SR by ATP driven SERCA pumps. SERCA pumps are typically located in SR regions separate from the dyadic junction and Ca^{2+} returns to dyadic junctions by moving through the SR network (14; 15; 43; 55). In addition to SERCA and NCX, a small amount of Ca^{2+} is removed by plasma membrane Ca^{2+} ATPases (PMCA) and the mitochondrial Ca^{2+} uniporter (MCU) (Figure 1-1). The relative contributions of these mechanisms vary between species with ~70% of cytosolic Ca^{2+} removed by SERCA, 28% by NCX, and 2% by MCU in humans, cats, dogs and rabbits. Whereas, in mice and rats SERCA removes ~92% of Ca^{2+} and NCX ~7% (15).

Nuclear Ca^{2+} and regulation of gene expression

Increased cytosolic Ca^{2+} transients during contraction are accompanied by an increase in nuclear Ca^{2+} (Figure 1-1). However, unlike cytosolic Ca^{2+} , nuclear Ca^{2+} transients are slower, with a lower peak, and take longer to return to baseline levels (43; 103). Recently, a process termed excitation-transcription coupling has been proposed to coordinate the molecular signals regulating E-C coupling with transcriptional activity. Although over 300 genes and ~30 transcription factors are known to be regulated by intracellular Ca^{2+} (45), only a fraction of Ca^{2+} -dependent gene regulatory pathways have

been delineated. In the heart, the most well characterized Ca^{2+} -dependent signaling pathways involve Ca^{2+} /calmodulin protein kinase II (CaMKII) and calcineurin (CN). Activation of CaMKII and CN signaling in the heart has been shown to regulate cardiac gene expression through MEF2 (myocyte-enhanced-factor 2) and NFAT (nuclear factor of activated T-Cells), respectively (43; 45; 153; 222) (Figure 1-2). However, the role of Ca^{2+} dependent regulatory networks in cardiac development and the distinct Ca^{2+} sources activating these pathways are poorly understood. Moreover, how Ca^{2+} transients regulating gene expression are distinct from contractile Ca^{2+} transients remain unknown.

Altered Ca^{2+} dynamics are detrimental to cardiac function

Overall, the plethora of Ca^{2+} -dependent processes in the heart involve a highly dynamic network of Ca^{2+} signaling pathways to regulate proper heart physiology. Acute dysfunctions in any Ca^{2+} -dependent mechanism can induce mechanical and/or electrical malfunctions and long-term maladaptations that severely impact heart function resulting in cardiac disease and heart failure. Importantly, altered intracellular Ca^{2+} homeostasis is known to be a central cause of heart failure pathogenesis (104; 106). Deciphering pivotal points of maladaptive Ca^{2+} dynamics may therefore prove essential to the development of novel treatment and prevention therapeutics. However, due to the complex nature of Ca^{2+} signaling in the heart, there is a need for better understanding of the distinct Ca^{2+} sources that regulate these processes, how specifically and selectively Ca^{2+} dependent pathways mediate maladaptive responses, and how Ca^{2+} signaling pathways are integrated to regulate cardiac function.

Dysregulated Ca^{2+} Homeostasis is a Central Cause of Heart Failure

Heart failure or the inability of the heart to properly pump blood, is a chronic progressive condition affecting nearly 20 million people worldwide and proposed to increase in prevalence by approximately 25% by 2030 (68; 116). Despite significant advances in treatment there continues to be no cure for heart failure and treatment options are limited to palliative pharmaceuticals and invasive therapies (117). This demonstrates a need for better understanding of the cellular and molecular mechanisms that regulate heart physiology and how dysregulation contributes to disease pathogenesis. Although hospitalization for heart failure has decreased recently, the cost of care remains high and if present care practices continue, US costs are expected to double by 2030 by approximately \$30 billion (68; 116). In accordance with this, heart failure thus represents a significant global health burden in terms of cost, quality of life, and longevity of treatment (104; 106). Importantly, dysregulated intracellular Ca^{2+} homeostasis is known to be a central cause of progression to heart failure and contributes to both contractile dysfunction and arrhythmia. Moreover, since Ca^{2+} serves as a signaling nexus regulating multiple aspects of heart function, targeting Ca^{2+} dependent pathways may offer broad therapeutic potential. (1; 43; 89; 98; 110; 191).

Heart Failure onset and the neurohumoral response

Heart failure is initiated by cardiac injury, which commonly results from inadequate myocardial flow (myocardial infarction), prolonged pressure overload (hypertension), and abnormal flow through heart valves (valvular stenosis) (104; 116). Regardless of the cause, to maintain cardiac output the heart attempts to compensate for injury through myocardial remodeling and neurohormonal activation (77; 116; 117; 191).

Over time however, these compensatory mechanisms ultimately result in adverse cardiac remodeling and electrical instability. Notably, each of these maladaptive events are associated with dysregulated Ca^{2+} handling. Following cardiac insult, decreased cardiac output leads to activation of the sympathetic nervous system and the renin-angiotensin-aldosterone (RAA) axis to maintain proper contractility and blood pressure, respectively (77; 116)). In particular, epinephrine and norepinephrine released in response to sympathetic stimulation bind β -adrenoreceptors (β -AR) on the plasma membrane activating several signaling pathways that ultimately increase intracellular Ca^{2+} (77). Furthermore, β -AR signaling regulates the expression and activity of essential Ca^{2+} handling proteins, including protein kinase A (PKA) via G coupled protein receptor activation of adenylate cyclase and CaMKII (104; 106).

Cardiac hypertrophy proceeds heart failure

The onset of heart failure is preceded by cardiac hypertrophy characterized by increased cardiac size and reactivation of developmental gene expression (69; 111). Under conditions of sustained stress, compensatory increases in cell size can become pathological and result in fibrotic replacement, myocyte loss and cardiac dysfunction (12; 111; 191). Several Ca^{2+} -dependent signaling pathways have been identified as essential to induction of pathological cardiac hypertrophy and contribute to the pathogenesis of heart failure. The best characterized of which involve Ca^{2+} /calmodulin regulated CaMKII and CN (Figure 1-2). Increased cytosolic Ca^{2+} initiates Ca^{2+} /calmodulin dependent activation of the serine threonine protein kinase, CaMKII. During cardiac hypertrophy, activated CaMKII phosphorylates transcription factors, epigenetic regulators, and histones to modulate gene expression (12; 43; 44; 52; 69). CaMKII is involved in chromatin

modification through phosphorylation of histones and histone deacetylases that increase the chromatin accessibility of prohypertrophic gene expression. In particular, CaMKII dependent phosphorylation of histone deacetylase 4 (HDAC4) has been found to block HDAC4 nuclear export, diminishing HDAC4-dependent myocyte enhanced factor 2 (MEF2) repression (8; 23; 43; 55; 127) (Figure 1-2). MEF2 activation has been shown to be important in cardiac remodeling, activation of fetal gene expression and inflammation (8; 85; 103; 154).

Ca²⁺/Calmodulin mediated activation of the serine-threonine phosphatase, calcineurin (CN), stimulates cardiomyocyte growth and cardiac remodeling through activation of NFAT (nuclear factor of activated T cells). Dephosphorylation of NFAT by CN induces NFAT nuclear translocation where, along with the cardiac restricted zinc finger transcription factor GATA4, NFAT stimulates the expression of cardiomyocyte growth genes (43; 126; 176) (Figure 1-2). In fact, activation of CN-NFAT has been found to be both necessary and sufficient to induce pathological cardiac hypertrophy in mammalian cardiomyocytes (126). Rodent models lacking CN have reduced heart mass and attenuate the hypertrophic response (39). Furthermore, MEF2 has also been suggested as a downstream effector of CN during hypertrophy (43), demonstrating the potential for CN-dependent regulation of multiple transcriptional networks during heart failure. Overall, we are only just beginning to understand how Ca²⁺-dependent gene regulation contributes to cardiac physiology and pathophysiology. Further research is needed to identify the specific Ca²⁺ sources regulating pathway activation.

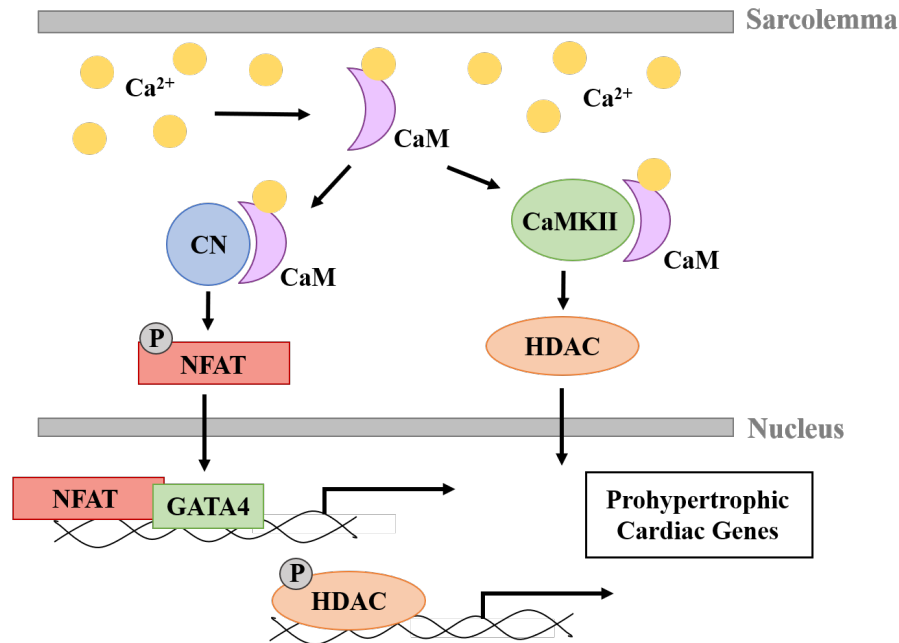


Figure 1-2. Ca^{2+} dependent regulation of prohypertrophic gene expression.

Intracellular Ca^{2+} binds and activates calmodulin (CaM). Ca^{2+} bound CaM mediates activation of calcineurin (CN) and Ca^{2+} /calmodulin protein kinase II (CaMKII). CN dependent dephosphorylation of nuclear factor of activated T-cells (NFAT) induces NFAT nuclear translocation. Inside the nucleus, NFAT recruits cardiac restricted zinc finger transcription factor (GATA4) to stimulate transcription of cardiac growth genes. Activated CaMKII phosphorylates histone deacetylases (HDAC) stimulating nuclear translocation and de-repression of prohypertrophic gene expression.

Reduced global Ca^{2+} transients

Failing cardiomyocytes exhibit abnormal E-C coupling characterized by reduced Ca^{2+} transients, enhanced diastolic SR Ca^{2+} leak, and decreased SR Ca^{2+} sequestration, which occur due to altered expression and activity of Ca^{2+} handling proteins (104; 106). Reduced Ca^{2+} transients observed in heart failure patients occur due to diminished capacity of I_{Ca} to trigger SR Ca^{2+} release and decreased SR Ca^{2+} stores. During heart failure, the dyadic junction undergoes significant reconstruction resulting in a loss of T-

tubules (50; 106). Defects in cytoskeletal and myofilament structure result in abnormal organization of ion channel and transporters at the plasma membrane. Moreover, dispersion of T-tubule structure can leave RyRs ‘orphaned’ from the dyadic cleft, thus impairing L-type VOCC coupling with RyRs and decreasing I_{CA} mediated SR Ca^{2+} release. Furthermore, disrupted T-tubule structure has been shown to cause asynchronous cellular Ca^{2+} release in failing human cardiomyocyte (104; 105), meaning SR Ca^{2+} release in some regions of the cardiomyocyte was delayed compared to other regions. The delayed Ca^{2+} response most likely results due to loss of L-type VOCC-RyR coupling and is suggested to contribute to early after depolarization arrhythmias.

Reduced SR Ca^{2+} store content

The global reduction of intracellular Ca^{2+} in failing cardiomyocytes, may also result from decreased SR Ca^{2+} store content. Reductions in SR Ca^{2+} stores may occur as a result of leaky RyR activity, reduced SR Ca^{2+} uptake via SERCA, or increased Ca^{2+} extrusion via NCX (16). Leaky RyR activity refers to the spontaneous or increased activation of RyR induced SR Ca^{2+} release. Overactivation of RyR during heart failure has been linked to RyR hyperphosphorylation by PKA and CaMKII (16; 62; 114). In particular, phosphorylation of RyR by PKA has been found to dissociate the FKBP12.6 regulatory subunit of RyR, increasing RyR open probability and SR Ca^{2+} leak in humans (106; 114). FKBP12.6 normally inhibits RyR coupled gating with VOCCs, and thus dissociation increases RyR activity, resulting in reduced Ca^{2+} spark amplitude. Conversely, in failing rabbit hearts, increased SR Ca^{2+} leak by RyR was found to be reduced by inhibition of CaMKII, but unaltered by PKA inhibition (2; 104). Thus, while significant evidence supports that altered RyR regulation contributes to abnormal Ca^{2+}

handling in the failing heart, further research is required to determine the significance and mechanism by which RyR alters SR Ca^{2+} transients.

Decreases in SR Ca^{2+} store content may also result from reduced Ca^{2+} sequestration by SERCA. Accordingly, many groups have reported reduced SERCA expression in failing cardiomyocytes that correlated with decreased SR Ca^{2+} uptake (13; 16; 51; 70). Decreased expression and activity of SERCA in heart failure may result from altered regulation of SERCA by phospholamban (PLN). SERCA is directly regulated by PLN, which inhibits SERCA activity when not phosphorylated. The phosphorylated, inactive state of PLN was found to be decreased in failing hearts, suggesting increased inhibition of SERCA leading to decreased SR Ca^{2+} store refilling (104; 173). PLN is phosphorylated by PKA and CaMKII and dephosphorylated by CN. In failing human hearts with dilated cardiomyopathy, studies have found increased dephosphorylation of PLN by CN (131). Knockout of PLN has been shown to significantly increase SR Ca^{2+} content and completely rescue dilated cardiomyopathy and heart failure in mouse models (123). Taken together, these findings demonstrate the importance of maintaining proper SR Ca^{2+} dynamics in cardiomyocytes and highlight how dysregulation of Ca^{2+} handling proteins deleteriously contributes to heart failure pathogenesis.

Triggered arrhythmias

Approximately 50% of HF patients die from progressive pump failure, the remainder die suddenly due to tachyarrhythmias often provoked by changes in Ca^{2+} handling (68; 104). Triggered arrhythmias can occur as early afterdepolarizations (EADs), delayed afterdepolarizations (DADs), or increased spontaneous depolarization events (15; 41; 50; 152). DADs occur after plasma membrane repolarization due to high

intracellular Ca^{2+} levels resulting from increased SR Ca^{2+} release or decreased SR Ca^{2+} sequestration (15). EADs are depolarization events that occur during the action potential plateau phase and result from reactivation of L type VOCCs or activation of the reverse mode of NCX. Phosphorylation of L-type VOCCs through PKA and CaMKII, enhance the probability of channel opening, leading to prolonged and frequent channel activation (104; 106; 152). NCX may also contribute to EADs through activation of the reverse mode. Decreased Ca^{2+} transients observed during heart failure result in elevated intracellular $[\text{Na}^+]$, which subsequently activates the reverse mode of NCX. Activation of the NCX reverse mode, increases Ca^{2+} influx into the cell inducing Ca^{2+} overload and triggering arrhythmias (181). Furthermore, during heart failure, CaMKII has been found to promote NCX reverse mode activation through phosphorylation of the cytoplasmic linker I-II region, inducing EADs and DADs (6).

Impaired Mitochondrial Function

Heart failure is associated with decreased energy production and impaired energy utilization. Loss of metabolic capacity during heart failure occurs due to the interdependence of Ca^{2+} handling and energy homeostasis (15; 106). Ca^{2+} communicates cellular ATP demands through Ca^{2+} dependent activation of key mitochondrial dehydrogenases. Under physiological conditions, beat-to-beat changes in mitochondrial Ca^{2+} are small, but cumulative due to the low uptake of Ca^{2+} through MCU (~1%) and slow Ca^{2+} extrusion via the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ antiporter (15; 214). However, when cytosolic Ca^{2+} transients are high, Ca^{2+} uptake by MCU dramatically increases. Although the increased mitochondrial Ca^{2+} influx initially acts as a protective mechanism against arrhythmias and hyper-contraction (15; 60; 93), excessive mitochondrial Ca^{2+} has

detrimental consequences. Mitochondrial Ca^{2+} overload induces opening of the mitochondrial permeability transition pore (mPTP), which dissipates membrane potential, releasing Ca^{2+} , apoptotic mediators and reactive oxygen species (ROS) that promote hyper-contraction and cell death (60; 106; 165). Despite significant evidence supporting a role for mitochondrial in heart failure etiology, few studies have evaluated whether or how mitochondrial Ca^{2+} uptake, transport, or homeostasis are altered during heart failure.

Experimental therapeutics targeting abnormal Ca^{2+} handling

In summary, heart failure is a highly complex and devastating disease that's progression centers around dysregulation of Ca^{2+} handling. Current pharmacological treatments, such as β -blockers, aldosterone, angiotensin receptor blockers, and angiotensin converting enzyme inhibitors, target suppressing neurohumoral overactivation and are shown to enhance cardiac function and improve clinical outcome (43; 77; 104). However, despite the success of neurohumoral blockade treatments, the mortality rate for heart failure remains high with 50% of Medicare beneficiaries not surviving 3 years after hospitalization (68). This demonstrates a need for better, more effective, treatment and prevention options against heart failure.

Aberrant Ca^{2+} handling serves as a nodal point in heart failure pathogenesis, and thus developing therapeutics aimed at restoring proper Ca^{2+} dynamics may offer broad therapeutic potential. Recently, a few promising experimental therapies have been created to target abnormal Ca^{2+} handling by improving RyR function and enhancing SERCA mediated SR Ca^{2+} reuptake. The small molecule, JTV519, was developed to reduce RyR Ca^{2+} leak by enhancing FKBP12.6 binding to RyR (104; 206). Chronic administration of JTV519 has been shown to prevent left ventricular remodeling and improve contractile

function in a canine model of heart failure (104; 216). Furthermore, methods to increase SERCA expression and PLN phosphorylation are being pursued to increase SR Ca^{2+} reuptake. Intracoronary injection of an adenovirus-associated virus packaged SERCA2c (human cardiac SERCA) expression vector has reached phase 2 clinical trials (67; 78; 108; 149). Encouragingly, 5 out of 9 patients had improved symptoms from baseline after two months (78). However, although these experimental therapeutics have shown promising results, they have yet to meet expectations in clinical trials. To improve our translational efficiency, we need a better understanding of how SR Ca^{2+} dynamics are regulated and integrated throughout heart failure progression.

Store-operated Ca^{2+} Entry is an Important Regulator of SR Ca^{2+} Homeostasis

Discovery and Upstream Signaling in SOCE

Due to the essential and highly integrated functions of Ca^{2+} within the cell, precise control of intracellular Ca^{2+} handling is required to maintain optimal function. Elevations in cytoplasmic Ca^{2+} occur primarily by extracellular Ca^{2+} influx across the plasma membrane or release from intracellular stores, such as the endoplasmic/sarcoplasmic reticulum (ER/SR). Store operated Ca^{2+} entry (SOCE) is a highly conserved Ca^{2+} signaling mechanism known to regulate ER/SR Ca^{2+} homeostasis in nearly all eukaryotic cell types (11; 82; 141; 155-158; 160; 182). The concept of store operated, or ‘capacitive’ Ca^{2+} entry was first proposed by Dr. Putney in 1986, describing a model whereby depletion of intracellular Ca^{2+} stores signaled Ca^{2+} channel opening at the plasma membrane that would subsequently refill intracellular stores (4; 156-158; 160). In non-

excitable cells, ER Ca^{2+} store depletion typically involves agonist binding to G coupled protein receptors or receptor tyrosine kinases at the plasma membrane, inducing phospholipase C to cleave phosphatidylinositol 4,5-bisphosphate (PIP_2) and produce inositol 1,4,5-trisphosphate (IP_3). IP_3 subsequently activates IP_3 receptors (IP_3R) on the ER to trigger the release of ER Ca^{2+} stores (11; 157; 161). Depletion of ER Ca^{2+} stores induce activation of the SOCE pathway. Notably, reduced ER/SR Ca^{2+} content, regardless of IP_3R induction, stimulates SOCE and the SOCE pathway has been found to be essential in excitable cells like neurons and striated muscle myocytes (7; 20; 22; 224). The close physical and functional association of store operated channels (SOC) to the ER/SR allows them to play a key homeostatic role in ER/SR Ca^{2+} refilling. Prior to intracellular store refilling, SOC mediated Ca^{2+} influx first enters the cytoplasm, thus SOCE Ca^{2+} has the potential to regulate broader signaling functions by elevating cytosolic $[\text{Ca}^{2+}]$ (155).

SOCE I_{CRAC}

The concept of SOCE was solidified upon discovery of an electrophysiological current activated by Ca^{2+} store depletion, by Hoth and Penner in 1992 (4; 156; 158). Markus Hoth and Reinhold Penner demonstrated that stimulated depletion of intracellular Ca^{2+} in mast cells activated a small inwardly rectifying current that was highly Ca^{2+} selective (72; 157). Due to the nature of current activation, it was termed I_{CRAC} for calcium-release-activated- Ca^{2+} current. Notably, I_{CRAC} differed from other Ca^{2+} currents in that the single channel conductance was very small, estimated to be at the order of 24fs though noise analysis by Zweifach and Lewis (232), which also suggested the molecular structure of SOC differed from currently known Ca^{2+} channels. Furthermore, SOC

mediated I_{CRAC} showed unusual regulation by Ca^{2+} , including that SOC can be inhibited by several intracellular Ca^{2+} mechanisms and potentiated by extracellular Ca^{2+} (160).

Although I_{CRAC} is the most extensively studied and best characterized SOC current, several publications have described potential store operated currents with electrophysiological properties that slightly differ from I_{CRAC} but findings have not been reproducible (141). However, a subgroup of the transient receptor potential (TRP) channel class, TRPC, has shown I_{CRAC} potential and was initially proposed to be SOCs. TRPs were first characterized as light-activated, non-selective, but Ca^{2+} permeable, cation channels in *Drosophila* photoreceptors (38; 63; 129). Since their characterization in *Drosophila*, seven mammalian isoforms TRPC1-7 (canonical TRP) have been identified. Electrophysiological characterization of TRPC channels confirmed that they are non-selective cation permeable channels and thus mediate currents that differ from I_{CRAC} (32; 141). Although TRPCs do not regulate I_{CRAC} , several studies have demonstrated the potential for TRPCs to form SOCs with properties that differ from I_{CRAC} . (32; 157) However, results between laboratories have been conflicting and TRPC activation independent of store depletion has been identified (40; 66; 157; 199). Thus, the possibility remains that TRPCs may interact with SOC specific channels, but further investigation is required.

STIM

In 2005-2006, the key components of the SOCE pathway were identified to include STIM (stromal interacting molecule) proteins which function as ER/SR Ca^{2+} sensors, and Orai proteins that act as SOC pore forming subunits. Activation of SOCE by depletion of ER/SR Ca^{2+} stores induce STIM oligomerization and repositioning to ER/SR

regions close to the plasma membrane where they activate Orai mediated Ca^{2+} influx (Figure 1-3). In mammals, two STIM homologs (STIM1 and STIM2) and three Orai (Orai1, 2, and 3) have been identified. The vast majority of research has focused on characterizing the function of Stim1 and Orai1 which show the widest tissue expression and highest degree of conservation across species (11; 82; 155). STIM proteins were initially characterized as integral membrane proteins that promote pre-B cell survival and proliferation (31; 155). The Ca^{2+} signaling role of STIM was later identified in an RNAi screen of *Drosophila* S2 cells for gene products required for SOCE activation. (4; 158; 182). In mammals, STIM1 and STIM2 share >74% sequence homology and show broad tissue and organ expression. In human tissues, the most robust STIM expression is found in the pancreas, brain, skeletal and cardiac muscle (155). Both STIM homologs are type 1 single-pass transmembrane proteins whose N-terminus is located in the ER/SR lumen and C-terminus in the cytoplasm. Several functional motifs have been identified in STIM, including the Ca^{2+} binding EF-hand (EF) domain and sterile α motif (SAM) on the luminal side, and a pair of coiled-coil domains, a serine/proline rich region and a polybasic domain on the cytoplasmic side (54; 135; 155; 184; 228; 230).

Under resting conditions, when Ca^{2+} stores are full, STIM proteins exist as mono or dimeric structures expressed uniformly across the ER/SR (184; 230) (Figure 1-3A). STIM1 proteins have also been found to be expressed in the plasma membrane, though their function remains unclear and several studies have concluded that only ER-localized STIM1 is required for SOCE activation (120; 212). Site directed mutagenesis and structural studies have shown the inactive state of STIM1 is maintained through Ca^{2+} binding to the EF-hand domain, which stabilizes the closely coupled SAM domain.

Therefore, upon depletion of ER/SR Ca^{2+} stores, Ca^{2+} dissociation from EF-hand promotes the open conformation of STIM1 allowing for oligomerization and subsequent translocation to ER-plasma membrane junctions (54; 135; 184). Fluorescence resonance energy transfer studies found that the initial oligomerization of STIM1 in the ER occurs rapidly within 4s (31; 102). Following initial oligomerization there is a bulk movement of STIM1 oligomers to ER/SR sites that closely appose the plasma membrane, forming punctate structures (Figure 1-3 B and C).

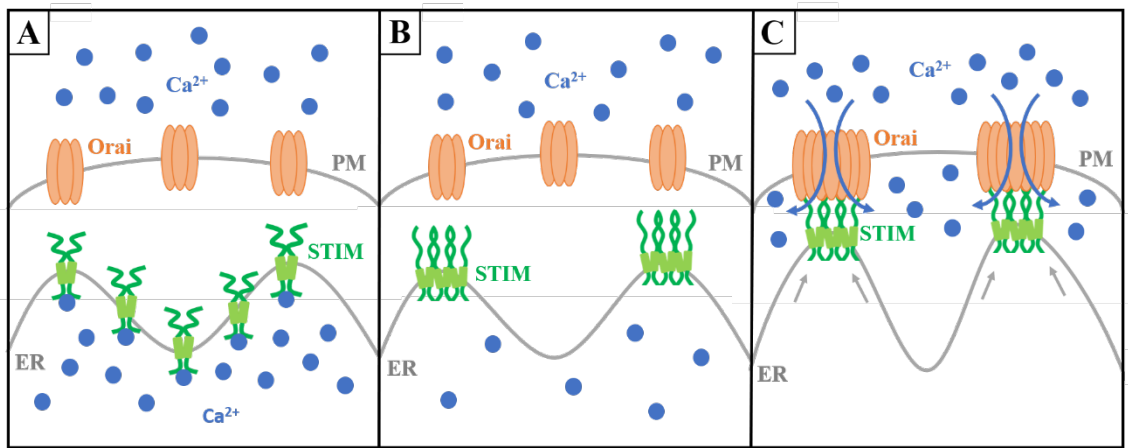


Figure 1-3: SOCE Activation Mechanisms. **A.** Under resting conditions, when endoplasmic reticulum (ER) Ca^{2+} (blue) stores are full, Ca^{2+} bound STIM proteins (green) are uniformly expressed across the ER membrane in stable mono- or dimeric structures and Orai (orange) pore forming subunits are located in the plasma membrane (PM). **B.** Depletion of ER Ca^{2+} stores activates the SOCE pathway. Ca^{2+} release from STIM promotes the STIM open configuration and subsequent oligomerization. **C.** After initial oligomerization, STIM oligomers move to ER-PM junctions and directly interact with Orai proteins stimulating Orai multimerization and channel activation. Pathway activation induces extracellular Ca^{2+} influx into the cytoplasm.

The redistribution of STIM1 oligomers to ER-plasma membrane (ER-PM) sites occurs through passive diffusion and relies on functional domains within the cytoplasmic C-terminus of STIM1. STIM1 oligomerization is believed to increase exposure of

positive charges in the C-terminal polybasic region. This enhances the binding ability of STIM1 to PIP2 lipids in the plasma membrane and retains STIM1 at ER-PM junctions (31; 87; 102; 212). Thus, the role of the STIM1 polybasic tail is believed to enhance STIM1 movement to ER-PM junctions. STIM1 oligomerization also induces a conformational change in the coiled-coil domain of STIM1 C-terminus region. Electron micrographs of Jurkat T cells suggest, opening of the long coiled-coil domain of STIM1 acts a bridge, aiding in movement of STIM1 oligomers to ER-PM sites (212). STIM1 targeting to ER-PM sites is not sufficient to induce Orai activation. Opening of the coiled-coil domain also exposes the STIM-Orai activating region (SOAR) and interaction of the STIM1 SOAR domain with Orai1 has been found to be sufficient for I_{CRAC} induction (142; 221).

Although STIM1 and STIM2 share high amino acid sequence homology and domain architecture, the physiological relevance of STIM2 remains poorly understood and controversial. In particular, STIM2 mediated activation of Orai1 has been shown to be slower and less efficient as compared to STIM1. Delayed unfolding and oligomerization of STIM2 may result from differences in the STIM2 N-terminal domain and increased stability of EF-SAM structures (185). For example, the Zhou et al group used chimeric replacement experiments to swap the N-terminal domains of STIM1 and STIM2 and found that when STIM1 has the N-terminal domain of STIM2, I_{CRAC} slows and SOCE is suppressed (229). STIM2 has also been found to have a lower Ca^{2+} binding affinity than STIM1, and thus requires a lower threshold of Ca^{2+} release for activation than STIM1 (18; 26). Overall, current evidence suggests that STIM2 is not a strong activator of SOCE but instead maintains basal cytosolic and ER Ca^{2+} levels (11; 26; 182).

Orai

A year after the discovery of STIM, whole genome siRNA screens in *Drosophila* S2 cells and human genetic linkage analysis identified Orai genes (named for the mythological gate keepers of heaven) to be required for I_{CRAC} (4; 155; 157; 160; 161; 182). Mammals have three Orai homologs, Orai 1, Orai 2 and Orai 3 that are broadly expressed across tissue types. Orai proteins have four α -helical transmembrane domains (TM1-4), two extracellular loops, and the N- and C-termini located in the cell cytoplasm (27; 29; 42; 217; 230). Although all three Orai homologs share 62% overall sequence identity and 92% homology of the transmembrane domains (94; 155), the physiological roles of endogenous Orai 2 and Orai 3 remain poorly understood. Under resting conditions, Orai proteins are uniformly expressed as dimeric or hexameric structures throughout the plasma membrane (Figure 1-3A and B). Direct interaction of Orai with the STIM SOAR domain induces Orai subunits to form multimeric channels, activating extracellular Ca²⁺ influx (142; 221; 228) (Figure 1-3C). The STIM SOAR domain is proposed to functionally interact with both the N- and C- termini of Orai1. The coiled-coil domain in the C-terminus of Orai1 critically regulates re-arrangement and colocalization of Orai1 with STIM1, and Orai1 N-terminal interactions with STIM1 are proposed to relieve inhibition of the STIM1 SOAR domain (27).

Orai channel structure was identified in 2013 by Long and colleagues using high resolution crystallography of the *Drosophila* Orai (dOrai) protein, which shares 73% primary sequence identity with human Orai (27). These results concluded that a single channel complex was found to contain six dOrai subunits arranged circularly around the central axis, with TM1 segments forming the inner most α -helix ring, TM2 segments the

middle ring, and TM3 the outermost ring (27; 29). The ion conduction pathway was found to have 3 distinct sections, including a cation binding region at the outer mouth of the channel, a short cylinder of hydrophobic residues for structural stability, and a section of positively charged residues lining the cytoplasmic end. With these findings, they proposed that STIM interactions with the Orai N-terminus results in dilation of the lower, positively charged section, allowing the electrochemical movement of Ca^{2+} ions from the external cation binding site through the dilated intracellular opening into the cytosol. Notably, the finding of hexameric Orai channel structures contradicted earlier reports which suggested Orai channels to exist as tetramers, using chemical cross linking of channel subunits, electrophysiological recordings, and single molecular fluorescence photobleaching techniques (29; 122; 148). Furthermore, it is also possible that different Orai subunits (Orai1-3) can form hetero-multimeric structures (120), but this has not been evaluated in depth. Overall, fundamental questions remain regarding some of the basic structural properties of Orai and their activation by STIM.

Physiological SOCE Functions and Genetic Mutations

The SOCE pathway is known to regulate a variety of biological processes, some of which include immune cell regulation, skeletal muscle function, smooth muscle migration and proliferation, inflammation, endothelial proliferation, and breast cancer cell metastasis (36; 157). However, the critical relevance of SOCE in physiology is best illustrated by patients with inherited loss and gain of function mutations in STIM1 and Orai1. Homozygous point mutations in *Stim1* and *Orai1* induce pathological phenotypes that begin early during infancy resulting from lack of functional STIM1 and Orai1 proteins. In contrast, heterozygous point mutant carriers do not present significant

physiological defects, suggesting the presence of a single wild-type *Stim1* or *Orai1* allele is sufficient to sustain function in humans, though SOCE is reduced (11; 94). To date, diseases caused by altered *STIM2*, *Orai2*, and *Orai3* have not been reported in humans.

Currently, ten different loss-of-function or null mutations in *Stim1* and *Orai1* have been identified and found to strongly abolish or impair SOCE activity. Individuals with loss-of-function mutations in *Stim1* and *Orai1* present with similar phenotypes and suffer from severe combined immunodeficiency (SCID), autoimmunity, congenital myopathy, chronic pulmonary disease, ectodermal dysplasia, and dental enamel calcification (11; 94; 161). The two most striking phenotypes are the severely compromised immune response and congenital myopathy. SCID-like symptoms in these individuals result from abrogated SOCE in peripheral T cells causing impaired T cell activation, reduced cytokine production, and absent proliferative responses. Impaired T cell response has been shown to result from decreased CN-dependent phosphorylation and nuclear translocation of NFAT (61). SCID patients also present with congenital non-progressive muscular hypotonia characterized by reduced muscle strength and endurance which manifests early in newborns (94). Reduced muscle function in SOCE deficient individuals is proposed to result from depletion of SR Ca^{2+} stores (91). Consistent with this, *STIM1* and *Orai1* deficient mice have been found to have reduced muscle mass and increased fatigue susceptibility after continuous stimulation (186; 207).

Comparatively, individuals with gain-of-function mutations in *Stim1* and *Orai1* have distinct yet overlapping disease phenotypes resulting from constitutive or increased activation of SOC channels. Clinically, these individuals present with non-syndromic tubular aggregate myopathy (TAM), York platelet syndrome and Stormorken syndrome,

and symptoms include skeletal muscle myopathy, bleeding disorders, and platelet dysfunction (11; 94). Skeletal myopathy is present in all three diseases and is characterized by progressive muscle weakness due to membranous tubular aggregation and atrophy. Notably, both gain- and loss-of function mutations in *Stim1* and *Orai1* result in skeletal muscle myopathy which demonstrates an important role for SOCE in regulating striated muscle Ca^{2+} homeostasis. This was a highly significant finding, since historically SOCE was not believed to contribute to striated muscle physiology due to a lack of evidence demonstrating a direct contribution of SOCE Ca^{2+} to E-C coupling (4; 7; 22). Although SOCE has subsequently been shown to be essential to skeletal muscle function, most likely through regulation of SR Ca^{2+} homeostasis and signaling pathways (121; 188), the mechanism by which SOCE contributes to pathogenesis is poorly understood. Furthermore, although these findings strongly support a conserved role for SOCE in striated muscle function, there are few studies evaluating the role of SOCE in cardiac muscle. Moreover, the effect of loss or gain-of function mutations in *Stim1* and *Orai1* on human heart physiology are unknown.

SOCE is an Essential Regulator of Physiological and Pathological Cardiac Function

A growing number of studies have shown the SOCE pathway components, STIM and Orai, are essential to both physiological and pathological cardiac function (7; 22; 36; 164). Initially, the functional relevance of SOCE in cardiomyocytes was overlooked because the large Ca^{2+} influxes that occur during each heartbeat were thought to make SOCE activity unnecessary (7; 22). However, as explained in the heart failure section, even modest changes in Ca^{2+} homeostatic mechanisms have the potential to profoundly

impact cardiac function. Consistent with a role for SOCE in cardiomyocyte function, embryonic, neonatal, and adult cardiomyocytes have been found to express STIM1 and Orai1 in rodent models (139; 140; 195; 202). However, expression alone does not constitute SOCE activity and reports of SOCE activity in cardiomyocytes have been conflicting. SOCE activity is typically measured by pharmacologically depleting intracellular Ca^{2+} stores, by blocking store refilling (thapsagargin) or stimulating SR Ca^{2+} store release (caffeine), in Ca^{2+} free media followed by re-addition of extracellular Ca^{2+} . Elevations in cytosolic Ca^{2+} upon extracellular Ca^{2+} re-addition result from SOCE activation (160). Using this method, several studies have shown that extracellular Ca^{2+} re-addition following store depletion in neonatal and adult cardiomyocytes increases cytosolic $[\text{Ca}^{2+}]$ (75; 76; 107; 198) and SR Ca^{2+} store refilling (76). However, other studies have demonstrated a variable response, with only ~50% of cardiomyocytes having increased cytosolic Ca^{2+} following store depletion and extracellular Ca^{2+} re-addition (22). These findings strongly support a role of SOCE in cardiomyocyte physiology, but the contribution of SOCE to normal heart function remains poorly understood and controversial.

SOCE in normal heart physiology

To date, there have been very few studies regarding the role of SOCE in normal cardiac function. STIM1 and Orai1 expression is shown to be developmentally dependent, with decreasing expression from embryonic to adult hearts, which suggests SOCE may be essential during cardiac development (35; 36; 164). In support of this, the Voelkers group demonstrated that suppression of *Stim1* and *Orai1* by shRNA significantly reduced neonatal ventricular rat myocyte (NVRM) size compared to

controls as detected by α -actinin immunostaining (202). *Stim1* and *Orai1* suppressed NVRMs were also found to have reduced mRNA expression of CaMKII and CN target genes, suggesting SOCE Ca^{2+} regulates cardiomyocyte size through these pathways. However, whether CN or other transcription factors are direct targets of SOCE in normal cardiac physiology has not been specifically evaluated. *In vivo* analysis from two independent studies found cardiomyocyte specific STIM1 deletion results in left ventricular dilatation and reduced ejection fraction in adult mice (35; 144). Reduced cardiac function was accompanied by expression of ER stress markers and altered mitochondrial morphology (35) as well as altered contractile Ca^{2+} transients and myofibril organization (144). These conclusions are further supported by Orai suppression in zebrafish embryos which reduced contractile function and heart failure (204). Although these results support the hypothesis that SOCE is required for normal heart function, the specific processes regulated by SOCE in cardiomyocytes remain unknown.

SOCE in pathological cardiac hypertrophy and arrhythmia

SOCE has recently been identified as an essential regulator of pathological cardiac hypertrophy (PCH). PCH results when initially compensatory increases in heart muscle mass induce detrimental cardiac remodeling, ultimately resulting in myocyte loss, fibrotic replacement, decreased cardiac output, and contractile dysfunction (12; 59; 163). PCH is a key risk factor in the development of heart failure and occurs in response to chronic increases in pressure or volume overload by stimuli such as hypertension, myocardial infarction, or genetic mutations (65; 69; 117).

Multiple studies have demonstrated the requirement for STIM1 and Orai1 to induction of PCH. For example, PCH induced by thoracic aortic constriction was found to upregulate STIM1 and Orai1 expression in mouse cardiac tissue, and cardiomyocyte specific suppression of STIM1 and Orai1 abrogated the hypertrophic response (10; 74; 107; 144). Similarly, endothelin-1 and phenylephrine induced cardiac hypertrophy was found to be attenuated by STIM1 and Orai1 suppression in rodent cardiomyocytes (74; 107; 202). Elevated adult expression of Stim1 and Orai1 in response to hypertrophic stimuli is also consistent with reactivation of developmental gene expression. Activation of fetal gene expression is a hallmark of PCH and known to play a causative role in heart failure pathogenesis (44). Enhanced SOCE in response to pathological stimuli likely activates the CN-NFAT signaling pathway to promote hypertrophic growth (74; 126; 176; 209). In support of this, multiple studies have found that transgenic overexpression of STIM1 enhances NFAT activity through luciferase reporter assays (37; 74; 107). However, whether NFAT nuclear translocation is directly the result of CN activation by SOCE Ca^{2+} has not been specifically shown.

In addition to transcriptional regulation, upregulated STIM1 and Orai1 is suggested to contribute to arrhythmias and altered contractile Ca^{2+} transients (37; 197; 202). Troupes, et al demonstrated that adenoviral transfer of human STIM1 in atrial feline myocytes induced diastolic Ca^{2+} sparks and spontaneous action potentials that were eliminated following BTP2 treatment (Orai channel inhibitor) or co-expression of a dominant negative Orai construct (197). Similarly, the Correll group, found transgenic overexpression of STIM1 in mice enhanced Ca^{2+} transients and Ca^{2+} spark frequencies that were attenuated by SKF-96365 treatment (SOCE specific inhibitor) and associated

with hypertrophy, sudden cardiac arrest, and heart failure (37). These results suggest SOCE is an important upstream source of Ca^{2+} contributing to abnormal Ca^{2+} handling in pathological cardiac hypertrophy and heart failure.

Despite significant evidence indicating an important role for STIM1 and Orai1 in cardiac physiology, the precise functions of SOCE in the heart are controversial. Analysis of SOCE signaling in mammalian cardiomyocytes is complicated by the presence of multiple STIM (STIM1 and STIM2) and Orai (Orai1-3) isoforms. In 2019, Orai3 was identified as a critical partner of STIM1 in voltage independent Ca^{2+} entry in hypertrophied cardiomyocytes. Researchers proposed this interaction may occur as a result of pre-existing STIM1 channels or from redistribution of Orai1 and Orai3 to form new channels (170). Moreover, studies in mammalian cardiomyocytes have also suggested Orai1-independent targets of STIM1, including TRPCs (140), L type VOCCs (143), T type VOCCs (133), and PLN (226). Thus, we have only just begun to understand how SOCE contributes to cardiac function, and further investigation is required regarding Stim and Orai in normal cardiac function and how dysregulation results in disease pathogenesis.

***Drosophila* in Cardiac Research**

The *Drosophila* heart has emerged as a powerful tool in cardiac research. *Drosophila* are the only invertebrate model with a working heart that shares developmental and functional homologies to vertebrates (17; 33; 151). Moreover, flies share strong gene conservation with humans, including the cardiac master genes *tinman*

(human *Nk2-5*) and *dHand* (human *Hand*) that direct heart development and regulate adult heart function, further extending conservation to include transcriptional and signaling networks (30; 80; 86; 99; 162; 189). Thus, the combination of strikingly well conserved structural and functional cardiac features with the advantages of invertebrate genetics and robust physiological measurements allows for meaningful comparison with vertebrate models.

Drosophila heart morphology and function

The *Drosophila* heart is a tubular structure that runs the length of the animal at the dorsal midline and pumps hemolymph, a lymph like fluid, throughout the body in an open circulatory system (17; 19; 167; 190). The two primary cell types of the *Drosophila* cardiac system include contractile cardiomyocytes and pericardial cells that aid in hemolymph filtration. In addition, specialized cardiomyocytes, ostia and intracardiac valves, regulate the inflow of hemolymph into the heart and promote unidirectional movement by preventing back flow, respectively (95; 167; 190). The main functions of the *Drosophila* heart are to provide nutrients and hormones to the body cavity by circulating and filtering hemolymph. In contrast to vertebrates, the *Drosophila* heart does not mediate tissue oxygenation. Instead, *Drosophila* have an elaborate tracheal system that supplies oxygen throughout the animal (19). The presence of a separate system for tissue oxygenation allows the animal to endure severe heart damage or malformation without death, permitting researchers to study aspects of heart differentiation, organogenesis, and performance that would be otherwise inaccessible in other animal models (151; 231).

Despite the later complexity of the vertebrate heart, the initial stages of vertebrate and *Drosophila* heart development are remarkably similar. Early during embryogenesis, *Drosophila* heart precursors derived from lateral mesoderm form bilateral symmetrical groups of cardiac mesoderm that assemble into the tube-like structure and form the luminal space (19; 190; 203). By the end of embryogenesis, the heart begins to contract and is divided into anterior and posterior regions, separated by an intracardiac valve (167; 189). Embryonic and larval heart contractions are myogenic and originate from the terminal region of the heart to induce anterograde movement of hemolymph from the posterior heart chamber up and out the anterior aortic region (19; 33; 95; 167; 190). The initial cardiac structure established during embryonic development is largely maintained through the larval lifecycle stage. However, during larval development the animal drastically grows, increasing in size, elongating the heart, and expanding the luminal diameter. Elongation of the heart occurs exclusively through increases in cell size (hypertrophy) without proliferation (hyperplasia) (19; 167; 190). Cardiac growth without cell proliferation similarly occurs during vertebrate heart development, which suggests larvae may serve as an important model for the study of physiological growth mechanisms, though this possibility has not been specifically evaluated. Furthermore, the transparent larval cuticle facilitates intact, *in vivo* optical measurements of heart rate and rhythm. (56; 151; 180).

During metamorphosis, the larval heart undergoes significant structural rearrangements to produce the adult heart structure. This includes the formation of additional ostia and intracardiac valves, remodeling of the terminal heart chamber, and extensive neuronal innervations (167; 190). Remodeling and simultaneous innervation of

the heart results in formation of a second pacemaker region in the conical chamber (47; 81). The conical chamber is located at the thorax to abdomen transition and functions as a collecting reservoir for hemolymph inflow from the anterior (aorta) and posterior regions of the heart. Heart rate is regulated by neurotransmitters and peptides acting on a collection of ion channels located in the pacemaker regions (81; 205). Notably, some ion channel genes essential to maintaining rhythmic contractions, such as KCNQ (136; 137), are well conserved functionally from *Drosophila* to humans. Interestingly, these same genes show no significant role in regulating the relatively fast mouse heart rate, which suggests that the fly heart model may be more informative in some aspects of cardiac function than mouse models (138).

Drosophila as a model for heart failure

Heart failure, the pinnacle of cardiac disease pathogenesis, is a profoundly complex disease that arises from a variety of initial cardiac insults and results in a deleterious cascade of declining heart function. Due to the convoluted nature of heart failure, neither the molecular mechanisms nor effective treatment and prevention strategies are fully understood. The *Drosophila* heart has proven to be a valuable tool in heart failure research, due to a combination of strong gene conservation, advanced genetic tools, and powerful methods to evaluate heart biology.

Drosophila genetic screens have been used to identify genes involved in congenital and adult human heart disease (17; 203; 210), including 46 genes required for muscle function that were not previously reported (231). Furthermore, combining genetic screens with high resolution methods to evaluate contractility and morphology, such as optical coherence tomography (OCT), intravital fluorescent imaging, and confocal

microscopy, allows for accurate high-throughput analysis of cardiac structure and function. For example, targeted genetic screens in *Drosophila* identified galactokinase as a novel regulator of calcineurin induced cardiomyopathy. Following identification, cardiac specific suppression of galactokinase by RNAi was found to suppress CN induced cardiomyopathy (97). In another study, heart specific activation of receptor tyrosine kinase signaling (RTK) components, *Ras* and *Raf*, in *Drosophila* resulted in cardiac hypertrophy characterized by reduced luminal diameter and increased cell size as shown by OCT and histological preparation (219). At the time, inhibition of RTK signaling was known to contribute to dilated cardiomyopathy, but the ability to regulate hypertrophic cardiomyopathy was unknown. *Drosophila* have also been used to evaluate arrhythmias, where heart specific overexpression of CaMKII has been shown to rescue age-dependent arrhythmias by increasing heart rate (171). Collectively, these studies provide compelling evidence for use of the *Drosophila* heart model to delineate fundamental mechanisms regulating heart failure. Moreover, insights gained from *Drosophila* cardiac research could be used to effectively direct translational studies in the more time consuming, costly, and technical challenging mammalian models.

SOCE in the Drosophila heart

Store Operated Ca^{2+} entry (SOCE) is suggested to be an essential regulator of physiological and pathological cardiac function, however the exact role by which SOCE contributes to cardiac physiology is poorly understood. The *Drosophila* heart may serve as an important model for uncovering the mechanism underlying SOCE cardiac function, yet the role of SOCE in *Drosophila* cardiac biology has not previously been explored. Research regarding SOCE function in mammalian models has been complicated by the

presence of multiple Stim and Orai isoforms. *Drosophila* have single isoforms of *Stim* and *Orai* that are most homologous to vertebrate *Stim1* and *Orai1*, which have the widest tissue distribution of SOCE isoforms in vertebrates (82; 155). This allows us to effectively target the SOCE pathway by genetically manipulating either Stim or Orai, without the possibility of functional redundancy or compensation by other isoforms. Furthermore, vertebrate studies have demonstrated the potential for Orai1-independent functions of STIM1, such as transient receptor potential (TRPC) channels (140). The *Drosophila* TrpC orthologs, *Trp* and *Trpl*, are only expressed in the visual system (63; 134), making them unlikely to function in *Drosophila* cardiac physiology. Thus, using the *Drosophila* model, we sought to evaluate the role of SOCE in physiological cardiac function and disease pathogenesis.

Summary of Conclusions

Our results clearly demonstrate that SOCE is an essential regulator of heart physiology, and that SOCE dysregulation contributes to the pathogenesis of heart failure. We show that heart specific suppression of *Stim* and *Orai* by RNAi in larvae and adults results in dilated cardiomyopathy, characterized by reduced heart dimensions and decreased contractile strength. Dilated cardiomyopathy in SOCE suppressed animals was associated with highly disorganized myofibril structure and decreased myofibril density. Cardiac dysfunction and abnormal structure significantly impaired animal survival resulting in early lethality, which suggests that cardiac defects caused by SOCE suppression were pathological. We also show that SOCE upregulation by heart specific expression of constitutively active Stim and Orai constructs pathologically increases

Drosophila heart wall thickness and decreases cardiac dimensions, consistent with pathological cardiac hypertrophy. Importantly, cardiac hypertrophy caused by constitutively active Stim was dependent on Orai channels, demonstrating a specific role for Ca^{2+} influx through the SOCE pathway in hypertrophic cardiomyocyte growth. These results are consistent with vertebrate models of Stim and Orai suppression (35; 71; 144; 197; 202; 204) and upregulation (37), which suggests a well conserved role for SOCE in cardiac physiology. Furthermore, we improved and expanded the *Drosophila* repertoire of heart physiological methods to include intravital fluorescence imaging for larval heart contractility, and micro-computerized tomography for analysis of cardiac hypertrophy. Taken together, these results will provide the foundation for future research regarding the mechanism by which SOCE regulates cardiac function.

CHAPTER 2: Suppression of store-operated calcium entry causes dilated cardiomyopathy of the *Drosophila* heart

Courtney E. Petersen¹, Matthew J. Wolf², and Jeremy T. Smyth^{3*}

¹Graduate Program in Molecular and Cellular Biology and ³Department of Anatomy, Physiology, and Genetics, Uniformed Services University of the Health Sciences, F. Edward Hébert School of Medicine, Bethesda, MD 20814

²Division of Cardiovascular Medicine, Department of Medicine, The University of Virginia School of Medicine, Charlottesville, VA 22908

Published in *Biology Open*, March 2020 (150)

ABSTRACT

Store-operated Ca^{2+} entry (SOCE) is an essential Ca^{2+} signaling mechanism present in most animal cells. SOCE refers to Ca^{2+} influx that is activated by depletion of sarco/endoplasmic reticulum (S/ER) Ca^{2+} stores. The main components of SOCE are STIM and Orai. STIM proteins function as S/ER Ca^{2+} sensors, and upon S/ER Ca^{2+} depletion STIM rearranges to S/ER-plasma membrane junctions and activates Orai Ca^{2+} influx channels. Studies have implicated SOCE in cardiac hypertrophy pathogenesis, but SOCE's role in normal heart physiology remains poorly understood. We therefore analyzed heart-specific SOCE function in *Drosophila*, a powerful animal model of cardiac physiology. We show that heart-specific suppression of *Stim* and *Orai* in larvae and adults resulted in reduced contractility consistent with dilated cardiomyopathy. Myofibers were also highly disorganized in *Stim* and *Orai* RNAi hearts, reflecting possible decompensation or upregulated stress signaling. Furthermore, we show that reduced heart function due to SOCE suppression adversely affected animal viability, as heart specific *Stim* and *Orai* RNAi animals exhibited significant delays in post-embryonic development and adults died earlier than controls. Collectively, our results demonstrate that SOCE is essential for physiological heart function, and establish *Drosophila* as an important model for understanding the role of SOCE in cardiac pathophysiology.

INTRODUCTION

Cardiomyopathies are a major cause of morbidity and mortality throughout the western world, with current treatment options limited to palliative pharmacological or invasive therapy (117). The discovery of curative treatments depends on thorough understanding of the molecular mechanisms that govern the onset and progression of cardiac pathophysiology. Significantly, irregularities in cardiomyocyte calcium (Ca^{2+}) homeostasis are a major contributing factor to cardiomyopathy and heart failure pathogenesis, and targeting Ca^{2+} signaling mechanisms may therefore be an important approach to novel therapeutic development (1; 88; 98; 110; 191).

The role of Ca^{2+} in the process of excitation-contraction (E-C) coupling, which drives cardiomyocyte contractility, is well established. In E-C coupling, membrane depolarization opens L-type voltage gated Ca^{2+} channels, generating localized Ca^{2+} elevations that activate ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR). Release of SR Ca^{2+} via RyRs results in a large cytoplasmic Ca^{2+} pulse that drives actomyosin contractility (13). In addition to E-C coupling, Ca^{2+} is also an important regulator of cardiomyocyte signaling pathways, such as those that control differentiation, cell growth, and pathological remodeling (200). Maintenance of Ca^{2+} homeostasis, including SR Ca^{2+} stores, is therefore essential to multiple aspects of cardiomyocyte physiology. Store-operated Ca^{2+} entry (SOCE) is a process that plays a major role in maintaining cellular Ca^{2+} homeostasis, as it couples the influx of extracellular Ca^{2+} to the depletion of sarco/endoplasmic (S/ER) Ca^{2+} stores. Ca^{2+} that enters the cell via SOCE can be pumped back into the S/ER to replenish depleted stores and restore S/ER Ca^{2+} homeostasis. Importantly, despite the prominent role for SR Ca^{2+} stores in cardiomyocyte physiology,

the functions of SOCE in cardiomyocytes and overall cardiac physiology are poorly understood.

The main components of the SOCE pathway are STIM (stromal interacting molecule) and Orai. STIM is a single-pass transmembrane protein that serves as an S/ER Ca^{2+} sensor via its N-terminal EF-hand domain, and Orai is a SOCE pore forming channel subunit in the plasma membrane (157; 159). In response to S/ER Ca^{2+} store depletion, STIM undergoes a large conformational change that results in oligomerization and exposure of a cytoplasmic Orai activating domain. Oligomerized STIM then translocates to S/ER-plasma membrane junctions where it interacts with and activates Orai to induce Ca^{2+} influx (159; 182). In mammals, there are two STIM isoforms, STIM1 and STIM2, and three Orai isoforms (Orai1-3), with STIM1 and Orai1 exhibiting the widest functional distribution across mammalian cell and tissue types.

Numerous studies strongly suggest that SOCE contributes to the pathogenesis of pathological cardiac hypertrophy, whereby heart muscle mass increases in response to stressors such as hypertension or valve dysfunctions. For example, induction of cardiac hypertrophy by pressure overload results in upregulation of STIM1 and Orai1 expression in mouse cardiac tissue, and cardiomyocyte-specific suppression of STIM1 and Orai1 attenuates the hypertrophic response (10; 74; 107; 144). Similarly, pharmacological induction of cardiac hypertrophy by phenylephrine and endothelin-1 is attenuated by suppression of STIM1 or Orai1 in rodent cardiomyocytes (74; 107; 202). Enhanced SOCE in response to pathological stimuli likely activates the calcineurin-nuclear factor of activated T-cells (NFAT) signaling axis, which is essential for reactivation of

developmental gene expression and promotion of cardiomyocyte growth (74; 126; 176; 209).

In light of this strong evidence that enhanced SOCE can drive pathological responses in cardiomyocytes, an important question remains: what is the role of SOCE in healthy cardiomyocytes and physiological heart function? To this end, two independent studies have shown that cardiomyocyte restricted STIM1 deletion in mice results in marked left ventricular dilation and reduced ejection fraction in adult hearts (35; 144). Decreased cardiac function was concomitant with indications of ER stress and changes in cardiomyocyte mitochondrial morphology (35), as well as altered contractile Ca^{2+} transients and myofibril organization (144). Additionally, Orai1 suppression in zebrafish embryos resulted in reduced fractional shortening and severe heart failure (204). These results support the conclusion that SOCE is essential for normal cardiac physiology. Importantly though, the specific cellular processes that are regulated by SOCE in cardiomyocytes remain unknown. It is also unclear whether these results reflect full suppression of SOCE activity, because functional contributions by other STIM and Orai isoforms cannot be ruled out in these vertebrate models. The goal of our current study was to address these important questions by testing the role of SOCE in *Drosophila melanogaster* heart function, a valuable animal model in which powerful genetic tools can be integrated with *in vivo* analyses of cardiomyocyte physiology and overall heart function.

The *Drosophila* heart is a muscular tube of cardiomyocytes that runs along the dorsal midline of the animal. Its primary function is to pump hemolymph, a plasma-like fluid, throughout the body in an open circulatory system (166). Importantly, the

contractile physiology of the *Drosophila* heart, including cardiomyocyte Ca^{2+} transport mechanisms and sarcomere composition, is highly conserved with mammals (101; 136), and the genetic and functional bases of many cardiomyopathies can be readily modeled and analyzed in flies (151). Simplified genetics is another important advantage of *Drosophila* over other animal models. In particular, *Drosophila* express single isoforms of *Stim* and *Orai*, thus precluding complications of functional overlap between multiple STIM and Orai isoforms encountered with vertebrate models. Taking advantage of the strengths of the *Drosophila* model, we demonstrate that animals with heart-specific suppression of the key SOCE pathway components, *Stim* and *Orai*, exhibit dilated cardiomyopathy characterized by enlarged end-diastolic and end-systolic dimensions and decreased fractional shortening. Myofibrils were highly disorganized and loosely spaced in *Stim* and *Orai* suppressed hearts, further consistent with disrupted contractile physiology. SOCE suppressed animals also exhibited significantly delayed post-embryonic development and died earlier than controls, suggesting pathological impairment of cardiac function. Our results, as well as those from other animal models, demonstrate that SOCE has highly conserved, essential roles in supporting physiological heart function, and lay the groundwork for future studies using *Drosophila* to mechanistically define SOCE functions in the heart.

RESULTS

***Stim* and *Orai* suppression results in dilated cardiomyopathy**

Drosophila Stim and *Orai* loss-of-function mutants fail to grow properly and die as second or third instar larvae (147), limiting their use in analysis of heart function. We therefore used inducible RNAi to suppress *Stim* and *Orai* expression specifically in the

heart and avoid the systemic effects of global SOCE suppression. Efficacy of the RNAi constructs was tested by expressing them ubiquitously in the whole animal and analyzing transcript levels and phenotypes. *Stim* and *Orai* RNAi driven by the ubiquitous *act-GAL4* driver suppressed *Stim* and *Orai* mRNA expression in whole first instar larvae by $72.67 \pm 3.18\%$ and $80 \pm 2.65\%$ (mean $\pm$ s.e.m.), respectively, compared to non-targeting RNAi controls, demonstrating potent knockdown of the targeted transcripts (Figure 2-S1A). Ubiquitous expression of *Stim* and *Orai* RNAi also resulted in reduced growth and larval lethality (Figure 2-S1B-F) similar to loss-of-function mutants (147), suggesting specific knockdown of the targeted gene products with few to no off-target effects. We also tested several other publicly available *Stim* and *Orai* RNAi *Drosophila* strains but found that they were ineffective at suppressing *Stim* and *Orai* transcript levels, respectively (data not shown).

We next evaluated heart contractility using optical coherence tomography (OCT) in fully intact, non-anesthetized animals with heart specific *Stim* and *Orai* suppression. Adult males with heart-specific *tinC-GAL4* driven *Stim* and *Orai* RNAi exhibited significantly increased end-diastolic dimensions (EDD) and a more pronounced increase in end-systolic dimensions (ESD) as compared to non-targeting RNAi controls (Figure 2-1A-H). Based on these measures, the calculated fractional shortening (FS), a direct measure of the contractile strength of the heart, was $88.73 \pm 2.04\%$ in non-targeting control hearts but was significantly reduced to $42.28 \pm 1.4\%$ and $49.21 \pm 3.11\%$ in *Stim* and *Orai* RNAi hearts, respectively (Figure 2-1I; mean $\pm$ s.e.m., $p < 0.001$). Importantly, enlarged ESD and EDD and reduced FS in *Stim* and *Orai* RNAi animals are consistent with dilated cardiomyopathy. Similar results were also observed using a second heart

specific driver, *4xhand-GAL4* (Figure 2-1J-R). Animals with *4xhand-GAL4* driven *Stim* and *Orai* RNAi again exhibited significantly larger EDD and ESD compared to nontargeting RNAi controls, and this resulted in significantly reduced FS in *Orai* RNAi animals. The decrease in FS was not significantly different in animals with *4xhand-GAL4* driven *Stim* RNAi, likely due to less efficient knockdown of *Stim* compared to *Orai* expression (see Figure 2-S1A). Neither *tinC-GAL4* nor *4xhand-GAL4* driven *Stim* and *Orai* RNAi significantly altered heart rates or caused notable arrhythmias compared to controls (Figure 2-S2A, B). Overall, heart-specific suppression of *Stim* and *Orai* driven by two independent heart-specific drivers resulted in dilated cardiomyopathy, suggesting an essential function for SOCE in adult *Drosophila* cardiac function.

Previous studies have demonstrated that cardiomyocyte-specific STIM1 suppression in mice results in age-dependent cardiomyopathy and heart failure in adults (35; 144), whereas severe heart failure was evident during embryogenesis in zebrafish with *Orai1* suppression (204). Therefore, we determined whether cardiomyopathy caused by SOCE suppression in *Drosophila* is also age-dependent by analyzing contractility in larvae, prior to the adult life cycle stage. OCT has previously been used to analyze larval heart contractility (25; 33; 208); however, we have found on our system that light scattering caused by the high fat content of larvae significantly interferes with OCT analysis. We therefore analyzed larval contractility using intravital fluorescence imaging of animals with cardiomyocyte-specific tdTomato (CM-tdTom) expression (83). Importantly, analysis of adult heart contractility by intravital CM-tdTom imaging yielded similar results to our OCT analysis, with significantly larger EDD and ESD and reduced

FS in *tinC-GAL4* driven *Stim* RNAi hearts compared to controls (Figure 2-S3, Table 2-S1, Video 2-1 and 2). Similar to adults, larvae with *tinC-GAL4* driven *Stim* and *Orai* RNAi exhibited significantly increased EDD and ESD compared to non-targeting RNAi controls (Figure 2-2A-H, Video 2-3 and 4), and this resulted in significantly decreased FS for *Orai*, though not *Stim* RNAi animals (Figure 2-2I). A small though significant decrease in heart rate was also observed in *Orai* but not *Stim* RNAi larvae (Figure 2-S2C). These results suggest that reduced cardiac output due to SOCE suppression is already evident in larval stages of *Drosophila* development, and thus is not exclusively an age-dependent effect in the adult heart. Larval heart contractility was also evaluated with *4xhand-GAL4* driven *Stim* and *Orai* RNAi and although this resulted in modest increases in ESD and EDD, FS was unchanged compared to controls (Figure 2-2J-R). It is possible that *4xhand-GAL4* does not drive RNAi expression as strongly in the larval heart as does *tinC-GAL4*, as has previously been noted (96), thus explaining the weaker effects of *4xhand-GAL4* driven *Stim* and *Orai* RNAi on larval heart contractility. Because of this, the *4xhand-GAL4* driver was not further used for larval heart analysis.

***Stim* and *Orai* suppression disrupts myofibril organization**

Disrupted myofibril organization is a common feature of dilated cardiomyopathy in mammalian (59; 187) and *Drosophila* (136; 138; 210) hearts. We therefore determined whether SOCE suppressed hearts similarly exhibit disorganized myofibrils. As shown in Figure 2-3A and B, hearts from *tinC-GAL4* driven control RNAi larvae exhibited evenly spaced myofibrils that were oriented circularly around the heart tube as revealed by actin staining and confocal microscopy. In contrast, myofibrils in *tinC-GAL4* driven *Stim* and *Orai* RNAi hearts were disorganized and more widely spaced compared to controls, as

indicated by a nearly 45% reduction in myofibril density in *Stim* and *Orai* RNAi versus control hearts (Figure 2-3C-E). In adult control animals, myofibrils again were densely packed and uniformly oriented circularly around the heart tube (Figure 2-4A, B, E). In marked contrast, however, myofibrils in adult *tinC-GAL4* driven *Stim* and *Orai* RNAi hearts were highly disorganized, with many myofibrils oriented parallel to the long axis of the heart (Figure 2-4C and D). Myofibril density was also significantly reduced in *Stim* and *Orai* RNAi compared to control adult hearts (Figure 2-4H). Similar results were also seen in adult hearts with *4xhand-GAL4* driven *Stim* and *Orai* RNAi, including myofibril disorganization and a significant decrease in myofibril density (Figure 2-4F, G, I). These results suggest that myofibril remodeling as a result of SOCE suppression may contribute to the aberrant contractility underlying dilated cardiomyopathy in these animals.

Heart specific *Stim* and *Orai* suppression reduces animal viability

We next determined whether the functional defects in *Stim* and *Orai* suppressed hearts affect animal health and viability. In support of this, larval development was significantly prolonged in animals with *tinC-GAL4* driven *Stim* and *Orai* RNAi, with nearly 86% fewer *Stim* and *Orai* RNAi animals having undergone pupariation by day five post-embryogenesis compared to controls (Figure 2-5A and B). Numbers of pupariated *Stim* and *Orai* RNAi animals were indistinguishable from controls by day six post-embryogenesis, indicating that the overall delay in pupariation was approximately 24 hours and that significant larval lethality was not observed. Eclosion, when adult animals emerge from their pupal cases, was also delayed by about 24 hours in *tinC-GAL4* driven *Stim* and *Orai* animals compared to controls, showing that development was not further

prolonged during pupal metamorphosis (Figure 2-5C and D). These results suggest that the contractile deficits caused by *Stim* and *Orai* suppression in the larval heart alter developmental physiology of the animal. Speculatively, reduced larval heart contractility may limit nutrient availability to developing tissues or reduce circulation of the hormone ecdysone that regulates larval developmental timing.

We then analyzed adult lifespan to determine whether heart dysfunction resulting from *Stim* and *Orai* suppression affects adult viability. Males with *tinC-GAL4* driven *Orai* RNAi died significantly earlier than those with non-targeting control RNAi, as indicated by a significant reduction of approximately 13 days in average median lifespan for *Orai* RNAi versus control animals (Figure 2-6A and B). Animals with *tinC-GAL4* driven *Stim* RNAi also exhibited an approximately 11 day reduction in average median lifespan compared to controls, though this difference was not statistically significant (Figure 2-6A and B). More profound effects on adult lifespan were observed with *4xhand-GAL4* driven *Stim* and *Orai* RNAi, as the average median lifespan of adult males was 11 ± 1.73 and 13 ± 2.65 days for *Stim* and *Orai* RNAi, respectively, compared to 43.64 ± 3.38 days for controls (Figure 6C-D; mean $\pm$ s.e.m.). Thus, whereas *Stim* and *Orai* suppression driven by *tinC-GAL4* resulted in greater deficiencies in heart contractility, suppression driven by *4xhand-GAL4* resulted in greater reductions in adult lifespan. This discrepancy may result from broader tissue distribution of *4xhand-GAL4* expression in adults compared to *tinC-GAL4*, and thus *4xhand-GAL4* driven RNAi may have effects on animal survival independent of heart function. Consistent with this, many *4xhand-GAL4* driven *Stim* and *Orai* RNAi adults exhibited severe wing damage, including blistering and tearing of large sections of the wings (Figure 2-6E). This is likely

due to *4xhand-GAL4* driven RNAi expression in wing hearts, which are muscles of cardiac origin that circulate hemolymph throughout the wings (192; 193). Interestingly, this suggests that SOCE is also required for proper wing heart function, though we did not investigate this further. However, we did consider the possibility that wing damage may have contributed to the early lethality of the *4xhand-GAL4* driven RNAi animals by causing them to become stuck in the food or on the vials. To test this and more clearly determine whether lethality is attributable to dysfunction of the primary heart, we repeated adult survival experiments with *4xhand-GAL4* driven RNAi animals whose wings were removed at the time of eclosion. As shown in Figure 2-6F and G, wingless *4xhand-GAL4* driven *Stim* and *Orai* RNAi animals indeed survived longer than those with wings (compare to Figure 2-6C and D), but these animals still died significantly earlier than corresponding wingless controls. Importantly, the approximately 12-day reduction in average median lifespan of wingless *4xhand-GAL4* driven *Stim* and *Orai* RNAi animals was similar to that of *tinC-GAL4* driven RNAi animals, demonstrating consistent early lethality due to abnormal heart function. Collectively, these results suggest that heart dysfunction caused by SOCE suppression pathologically impairs developmental physiology and longevity in *Drosophila*.

DISCUSSION

The critical importance of proper Ca^{2+} homeostasis and transport in cardiomyocytes is accentuated by the fact that nearly all cardiomyopathies involve Ca^{2+} dysregulation. Clear understanding of the cellular and molecular mechanisms that regulate cardiomyocyte Ca^{2+} handling is therefore fundamental to unraveling the complexities of cardiac pathophysiology. Our results demonstrate that SOCE is an

essential component of cardiomyocyte Ca^{2+} physiology in *Drosophila* because suppression of the key molecular components of the SOCE pathway, *Stim* and *Orai*, resulted in contractile deficiencies that are consistent with dilated cardiomyopathy. Concomitant with contractility defects, SOCE suppressed hearts also exhibited disrupted myofibril organization and density, a common feature of genetic cardiomyopathy models. Moreover, our results show that reduced functional output of *Stim* and *Orai* suppressed hearts significantly impaired overall animal physiology and health, as these animals developed significantly more slowly and died earlier than controls. Importantly, our findings are largely consistent with those from mice and zebrafish in which STIM1 or Orai1, respectively, were suppressed (35; 204), strongly suggesting that SOCE has essential, cardiomyocyte-specific functions that are highly conserved across species.

Our current study augments previous findings showing cardiomyocyte-specific functions of STIM and Orai proteins in new and important ways. Most notably, our results are the first to demonstrate that suppression of both *Stim* and *Orai* in the same animal model results in nearly identical cardiac phenotypes suggesting that these proteins are both required and function together to regulate SOCE in cardiomyocytes. This is significant because previous studies in mammalian cardiomyocytes have implicated Orai1-independent targets of STIM1 including canonical transient receptor potential (TrpC) channels (140) and L-type Ca^{2+} channels (37). While our results do not rule out the possibility of additional functional partners of STIM and Orai, particularly in vertebrate species, they do strongly suggest that Ca^{2+} influx mediated specifically by STIM-operated Orai channels is essential for proper cardiomyocyte physiology. This conclusion is further substantiated by the fact that *Drosophila* express single *Stim* and

Orai isoforms. Thus, we can effectively target the SOCE pathway by suppressing only *Stim* or *Orai*, without possible compensation by other isoforms such as STIM2, Orai2, or Orai3. Moving forward, utilizing these advantages of the *Drosophila* model will allow us to investigate the functional roles of SOCE in cardiomyocytes using genetic tools that are largely unattainable with vertebrate models, including powerful genetic screens.

Despite several recent studies demonstrating that SOCE is required for normal, healthy heart function, the specific cellular processes regulated by SOCE in cardiomyocytes have not been clearly defined. Maintenance of SR Ca^{2+} stores is a seemingly likely function of SOCE that would broadly impact cardiomyocyte physiology. For example, decreased SR Ca^{2+} content in the absence of SOCE may reduce RyR-dependent Ca^{2+} release that drives contraction, and would be consistent with the severe systolic dysfunction that we and others have observed in STIM and Orai suppressed hearts. In this case, the myofibril disorganization observed in SOCE suppressed hearts may be the result of decompensation in response to reduced contractile force generation (73). However, direct analyses of SR Ca^{2+} store content in STIM or Orai depleted cardiomyocytes have yielded conflicting results. For example, siRNA knockdown of STIM1 in freshly isolated neonatal rat ventricular myocytes led to a significant reduction of the caffeine-releasable SR Ca^{2+} pools (202), whereas SR Ca^{2+} store content was unchanged in adult cardiomyocytes isolated from STIM1 deleted mouse hearts compared to controls despite clear indication of dilated cardiomyopathy (144). This suggests that SR Ca^{2+} store depletion may not be a direct cause of heart failure when SOCE is suppressed. However, an independent analysis of STIM1 deficiency-associated dilated cardiomyopathy in mice demonstrated significantly

upregulated expression of ER stress response factors (35), possibly reflecting mild but chronic Ca^{2+} store depletion that may be difficult to measure in acutely isolated cells. Reorganization of cytoarchitecture is a common feature of the ER stress response mechanism, and therefore an important possibility in SOCE-suppressed cardiomyocytes is that ER stress leads to myofibril disorganization which contributes to impaired contractility and ultimately heart failure.

It is also possible that SOCE has specific signaling functions in cardiomyocytes that are required for heart development or tissue homeostasis. The best characterized signaling function of SOCE across cell and tissue types involves calcineurin-mediated dephosphorylation and activation of transcription factors such as NFAT (94).

Overwhelming evidence has established that upregulated calcineurin-NFAT signaling is an essential mechanism of cardiac hypertrophy pathogenesis (191), and SOCE upregulation also results in cardiac hypertrophy (10; 74; 144) and enhanced calcineurin activity (107). Collectively, these findings suggest that calcineurin is a key cardiomyocyte target of SOCE in the pathological, upregulated state. However, whether calcineurin or other signaling factors are essential targets of SOCE in normal heart physiology has not been determined. Arguing against a signaling role for SOCE specifically during heart development, STIM1 knockout mouse hearts do not exhibit altered phenotypes at early postnatal stages (35), and we also did not observe effects on *Drosophila* embryogenesis with heart-specific *Stim* or *Orai* suppression (data not shown). Additionally, cardiomyopathy in STIM1 knockout mouse hearts was progressive and age-dependent (35), further suggesting that any signaling functions of SOCE are likely to be homeostatic rather than developmental. Our findings in *Drosophila* indicate that

cardiomyopathy due to *Stim* and *Orai* suppression is already evident in larvae, early in the fly life cycle. This may also reflect post-developmental, homeostatic functions of SOCE that when suppressed, manifest earlier in flies due to their faster lifecycle compared to mice. Clearly, substantial work is still required to identify the specific functional roles of SOCE in physiological heart function, and genetic tools combined with *in vivo* analyses of heart physiology and Ca^{2+} dynamics in *Drosophila* will allow us to address this in novel and significant ways.

Our study is the first to demonstrate the use of intravital fluorescence imaging of heart contractility in *Drosophila* larvae, an important lifecycle stage that involves rapid animal growth and high nutritional demands. This is significant because larval heart contractility has been difficult to analyze using methods commonly employed for adults, such as OCT and direct imaging of dissected preparations. For example, the high fat content of larvae causes light scattering that can obscure OCT imaging, though some OCT analyses of larval heart function have been carried out successfully (25; 33; 208). In addition, partial dissections that faithfully preserve heart function can be challenging because the larval heart is extremely delicate and poorly attached to the body wall. Thus, intravital fluorescence imaging of larval hearts is a versatile and widely accessible method that is also amenable to longitudinal analysis. We validated our intravital imaging approach by confirming significantly increased ESD and EDD and decreased FS values in adult *Stim* RNAi versus control hearts, similar to our results from the more established OCT method. However, it was notable that even in controls, ESD and EDD values were substantially larger with intravital imaging compared to OCT measurements (Table 2-S1). This may be due in part to better spatial resolution of the heart walls with intravital

fluorescence imaging compared to OCT. Moreover, OCT measures the dorsal to ventral movement of the heart walls, whereas intravital fluorescence imaging measures lateral movement. This important distinction may also account for the differences we observed with the two methods. Importantly, our fluorescence imaging measurements of adult heart ESD, EDD, and FS were very similar to those from an earlier study that first described the CM-tdTom model for intravital fluorescence imaging (83), further validating the accuracy of our measurements.

Another notable finding of our study was that there were several key differences in the results of *Stim* and *Orai* RNAi driven with *tinC-GAL4* compared to *4xhand-GAL4*, both of which are commonly used as heart-specific *GAL4* drivers in *Drosophila* studies. Most strikingly, *4xhand-GAL4* but not *tinC-GAL4* driven RNAi resulted in significant effects on adult wing structure and integrity that were likely caused by defective wing heart function, given that *hand* but not *tinman* (the genes from which the respective *GAL4* drivers are derived) is expressed in wing heart myocytes (192; 193). Importantly, we found that the wing defects caused by *4xhand-GAL4* driven *Stim* and *Orai* RNAi significantly shortened animal lifespan, independent of effects on contractility of the primary adult heart. This illustrates that caution is warranted when attributing systemic phenotypes, such as premature lethality, to dysfunction of the primary *Drosophila* heart when using these *GAL4* drivers.

In conclusion, our results demonstrate that SOCE mediated by STIM and Orai is essential for proper function of the *Drosophila* heart, and add to a growing number of studies that collectively suggest that SOCE has highly conserved functional roles in cardiomyocytes across species. Paradoxically, it is still unknown whether loss of function

mutations in *Stim1* or *Orai1* result in heart defects or cardiomyopathies in humans. This is likely due to the fact that individuals homozygous for these mutations die from immunodeficiency in infancy or early childhood, before adverse heart phenotypes may fully manifest (164). Therefore, animal model studies, including powerful genetic screens and *in vivo* analyses in *Drosophila*, will continue to be vital to our understanding of how alterations to SOCE result in or contribute to devastating cardiomyopathies and heart failure.

MATERIALS AND METHODS

Fly Stocks

The following *Drosophila* stocks were obtained from the Bloomington *Drosophila* Stock Center: mCherry RNAi (35785; non-targeting control), GAL4 RNAi (35783; non-targeting control), *Stim* RNAi (27263), *Orai* RNAi (53333), and *act-GAL4* (3954). *tinC-GAL4* was obtained from Dr. Manfred Frausch (Friedrich Alexander University), and *4xhand-GAL4* was from Dr. Zhe Han (George Washington University School of Medicine). CM-tdTom flies were obtained from Dr. Rolf Bodmer (Sanford Burnham Prebys Institute). Flies were maintained on standard cornmeal agar food, and all crosses were carried out at 25°C.

Adult Survival

Virgin female *tinC-GAL4* or *4xhand-GAL4* flies were crossed with male RNAi animals, and progeny were raised to adulthood at 25°C. On the day of eclosion, adult progeny were collected and separated based on sex into vials containing up to ten flies per vial, with a total of 20-30 flies per group. Flies were maintained at 25°C throughout

the course of the experiments. Every three days, vials were checked for dead animals and surviving flies were transferred into new vials with fresh food. For wingless experiments, wings were removed immediately following eclosion on the day of adult collection. Kaplan-Meier survival curves and median lifespan were generated using GraphPad Prism.

Developmental Timing

Approximately 30-40 virgin female *tinC-GAL4* or *4xhand-GAL4* animals were mated with RNAi males for three days, at which time animals were transferred into egg laying chambers that consisted of a 100ml plastic beaker with holes for air exchange affixed over a petri dish containing grape juice agar (Genesee Scientific). A dollop of yeast paste (active dry yeast mixed with water) was placed in the center of each grape juice agar plate as food. Animals were acclimated in the chambers for 24 hours, and then transferred to new plates for 4 hours at 25°C for timed egg laying. After removing the adults, plates with eggs were incubated at 25°C for an additional 24 hours. Hatched larvae were then transferred to vials with standard fly food, with up to 30 larvae per vial, and maintained at 25°C over the course of the experiment. Vials were checked each day, and the numbers of newly formed pupae and eclosed adults were recorded.

RNA Isolation and RT-qPCR

Twenty to 30 first instar larvae were collected in 20µl cold Trizol and manually crushed with a pestle. Additional Trizol was then added to a total volume of 500µl, 100µl chloroform was added, and the aqueous layer containing extracted RNA was isolated.

Extracted RNA was further purified with the RNeasy kit (Qiagen) and converted to cDNA using an S1000 Thermo Cyclor (BioRad) with high capacity cDNA Reverse Transcription kit (ThermoFisher). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed on a StepOnePlus RT-qPCR machine (Applied Biosystems) with each reaction consisting of triplicate samples containing iTaq Universal Probes Supermix (BioRad), pre-validated 6-carboxyfluorescein (FAM)-labeled Taqman probes (Applied Biosystems) against *Stim*, *Orai*, and *RPL32* (housekeeping gene), and template cDNA diluted per manufacturer's instructions. For quantification, triplicate cycle threshold (Ct) values were averaged and normalized to the *RPL32* Ct value to calculate ΔCt . The $\Delta(\Delta\text{Ct})$ was determined by subtracting the control RNAi ΔCt value from the experimental ΔCt value, and fold changes expressed as $2^{-\Delta(\Delta\text{Ct})}$. Fold changes are expressed as a percentage of expression compared to non-targeting RNAi control.

Heart Dissection, Staining, and Confocal Imaging

Hearts from third instar larvae or five-day old adults were dissected and fixed as previously described (3). In brief, for adults the ventral abdomens and underlying tissues were removed to expose the contracting heart while bathed in oxygenated artificial *Drosophila* hemolymph (ADH; 108 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 8 mM MgCl₂, 1 mM NaH₂PO₄, 4 mM NaHCO₃, 10 mM sucrose, 5 mM trehalose, and 5 mM HEPES (pH 7.1)). Hearts were then fully relaxed by exchange with fresh ADH containing 10 mM EGTA. Larvae were pinned to Sylgard coated dishes at their anterior and posterior, and a slit was cut along the ventral midline in the presence of oxygenated ADH. Lateral cuts were then made along the sides of the animals, and the resulting cuticle flaps were pinned

to expose the internal organs. The gut was removed to expose the beating hearts, which were then relaxed with ADH containing 10 mM EGTA. Dissected adult and larval hearts were both fixed for 20 min at room temperature in PBS containing 4% paraformaldehyde. Following three 10 min washes in PBS containing 0.1% Triton X-100 (PBSTx) with gentle rotation, the samples were incubated in PBSTx containing 1.0 μ M Alexa Fluor 488 Phalloidin (ThermoFisher) for one hour at room temperature with gentle shaking. Samples were again washed three times in PBSTx at room temperature, and mounted on glass slides and coverslips with Vectashield (Vectashield Laboratories) as described (3). Samples were imaged with a Nikon A1R confocal microscope using 10X, 0.45 N.A. and 40X, 1.3 NA objectives. Phalloidin was excited with a 488 nm laser. Z-stacks at 1 μ m intervals were collected and images are presented as maximum intensity projections encompassing the whole heart for larvae or the dorsal half of the heart for adults to avoid the ventral layer of skeletal muscle. Myofibril density measurements on adults and third instar larval hearts were taken from the A2 segment of the heart. The number of myofibrils per 75 μ m in larvae and 50 μ m in adults was calculated manually.

Optical Coherence Tomography (OCT)

Adult heart contractility was analyzed using a custom-built OCT apparatus as previously described (210). In brief, five-day old males were briefly anesthetized with CO₂, embedded in a soft gel support, and allowed to fully awaken based on body movement. Animals were first imaged in B-mode in the longitudinal orientation to identify the A1 segment of the heart chamber. They were then imaged transversely in M-mode for 3 sec, and multiple M-modes were recorded for each fly. Animals were then re-

imaged in B-mode to ensure proper orientation of the heart chamber. M-modes were processed in ImageJ by referencing to a 150 μm standard. EDD, ESD, and heart rate were calculated directly from the processed M-mode traces. FS was calculated as $((\text{EDD}-\text{ESD}) / \text{EDD}) \times 100$.

Intravital Fluorescence Microscopy

Intravital fluorescence imaging of adult and third instar larval hearts was carried out using animals that express tdTomato under control of the cardiomyocyte-specific R94C02 enhancer element (83). Five-day old adult males were briefly anesthetized with CO_2 and were then adhered dorsal side down to a glass coverslip with Norland Optical Adhesive that was then cured with a 48-watt UV LED light source (LKE) for 60 seconds. Animals were allowed to recover for 10 mins prior to imaging. Third instar larvae were anesthetized by 1-minute cold exposure at 4°C and were adhered ventral side down to a glass slide with superglue. Larvae were flanked on the glass slide by double-sided tape that was then used to adhere a glass coverslip. The heart of larval and adult animals was imaged through the dorsal cuticle at a rate of 200 frames per second (fps) for ten seconds using an ORCA-Flash4.0 V3 sCMOS camera (Hamamatsu) on a Nikon Ti2 inverted microscope controlled with Nikon Elements software. Excitation light at 550 nm was provided by a Spectra-X illuminator (Lumencor) and emission was collected through a 555-635 band-pass filter. To generate M-modes, a 1 pixel wide line was drawn through the heart chamber in the A1 segment in adults and the A2 segment in larvae, and the fluorescence intensity along this line for the full time-course was plotted using ImageJ. EDD and ESD were calculated directly from the processed M-mode traces by manually

measuring the distance between the heart walls at full relaxation and full constriction, respectively. An average of five measurements of EDD and ESD was calculated from each trace. Heart rate was calculated by manually counting the number of systoles over 10 seconds. FS was calculated as $((\text{EDD}-\text{ESD}) / \text{EDD}) \times 100$.

Statistical Analyses

All statistical analyses were carried out using GraphPad Prism software. Contractility parameters and myofibril density measurements were analyzed by one-way ANOVA followed by Tukey's Multiple Comparisons Test. Differences were considered statistically significant at $p < 0.05$.

FIGURES

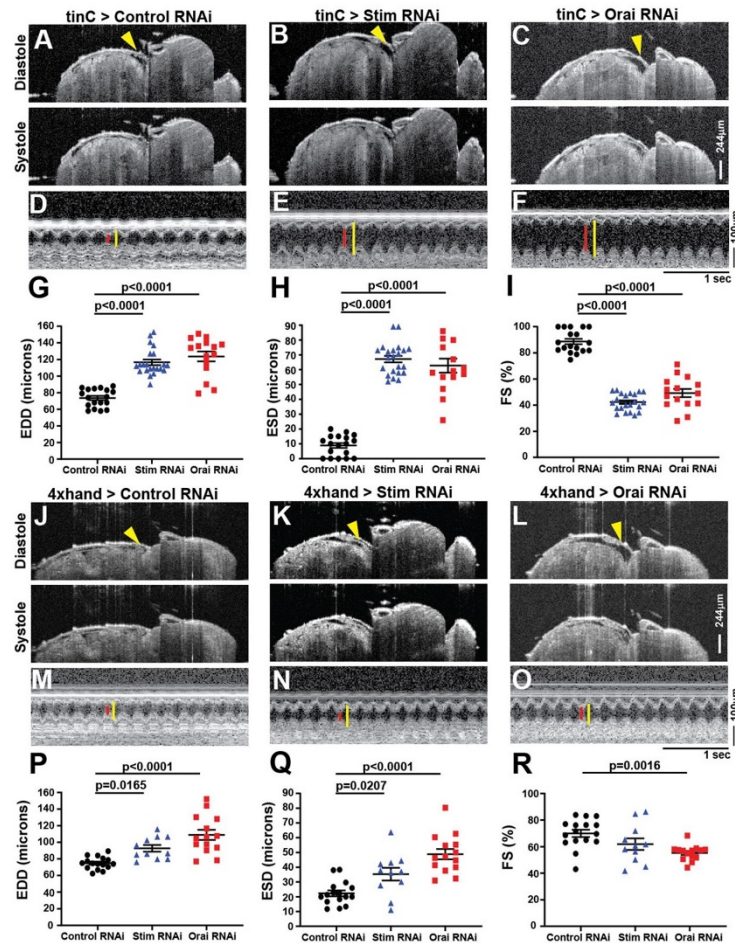


Figure 2-1. Heart specific *Stim* and *Orai* suppression results in dilated cardiomyopathy in adults.

A-C. Representative OCT longitudinal B-mode images of five-day old adult male flies with *tinC-GAL4* driven non-targeting control, *Stim*, and *Orai* RNAi during diastole (upper panels) and systole (lower panels). Arrowheads indicate the heart in diastole images. **D-F.** Representative M-mode recordings from *tinC-GAL4* driven non-targeting control, *Stim*, and *Orai* RNAi with red lines depicting systole and yellow lines diastole. EDD (**G**), ESD (**H**), and FS (**I**) were calculated from M-mode recordings of five-day old males, and each symbol represents a measurement from a single animal. Bars indicate mean $\pm$ s.e.m., and p-values were calculated from One-way ANOVA with Tukey's Multiple Comparison. **J-L.** Representative OCT longitudinal B-mode images of five-day old adult male flies with *4xhand-GAL4* driven non-targeting control, *Stim*, and *Orai* RNAi during diastole (upper panels) and systole (lower panels). Arrowheads indicate the heart in diastole images. **M-O.** Representative M-mode recordings from *4xhand-GAL4* driven non-targeting control, *Stim*, and *Orai* RNAi with red lines depicting systole and yellow lines diastole. EDD (**P**), ESD (**Q**), and FS (**R**) were calculated from M-mode recordings of five-day old males, and each symbol represents a measurement from a single animal. Bars indicate mean $\pm$ s.e.m., and p-values were calculated from One-way ANOVA with Tukey's Multiple Comparison.

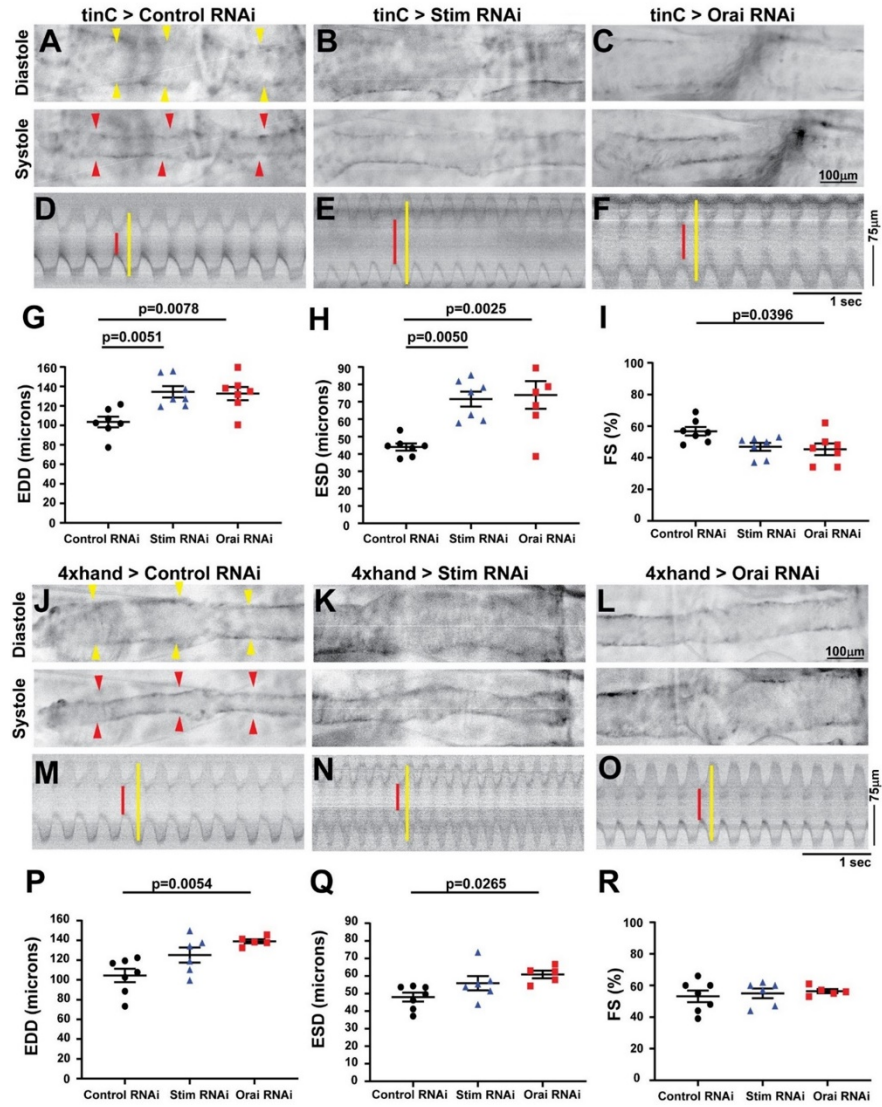


Figure 2-2. Larval *Stim* and *Orai* suppressed hearts are significantly dilated.

A-C Representative longitudinal images from intravital fluorescence imaging of R94C02-tdTom expressing third instar larval hearts with *tinC-GAL4* driven non-targeting control, *Stim*, and *Orai* RNAi during diastole (upper panels) and systole (lower panels). Images are presented in inverted grayscale for added clarity, due to low fluorescence intensity. Yellow arrowheads indicate heart in diastolic images and red arrowheads in systolic images. **D-F**. Representative M-mode images from *tinC-GAL4* driven non-targeting control, *Stim*, and *Orai* RNAi hearts. Red lines depict systole and yellow lines diastole. EDD (**G**), ESD (**H**), FS (**I**) were calculated from M-mode recordings of third instar larva, and each symbol represents a measurement from a single animal. Bars indicate mean $\pm$ s.e.m., and p-values were calculated from One-way ANOVA with Tukey's Multiple Comparison. **J-L**. Representative longitudinal images from intravital fluorescence imaging of R94C02-tdTom expressing third instar larval hearts with *4xhand-GAL4* driven non-targeting control, *Stim*, and *Orai* RNAi during diastole (upper

panels) and systole (lower panels). Yellow arrowheads indicate heart in diastolic images and red arrowheads in systolic images. **M-O**. Representative M-mode images from *4xhand-GAL4* driven non-targeting control, *Stim*, and *Orai* RNAi hearts. Red lines depict systole and yellow lines diastole. EDD (**P**), ESD (**Q**), FS (**R**) were calculated from M-mode recordings of third instar larvae, and each symbol represents a measurement from a single animal. Bars indicate mean $\pm$ s.e.m., and p-values were calculated from One-way ANOVA with Tukey's Multiple Comparison.

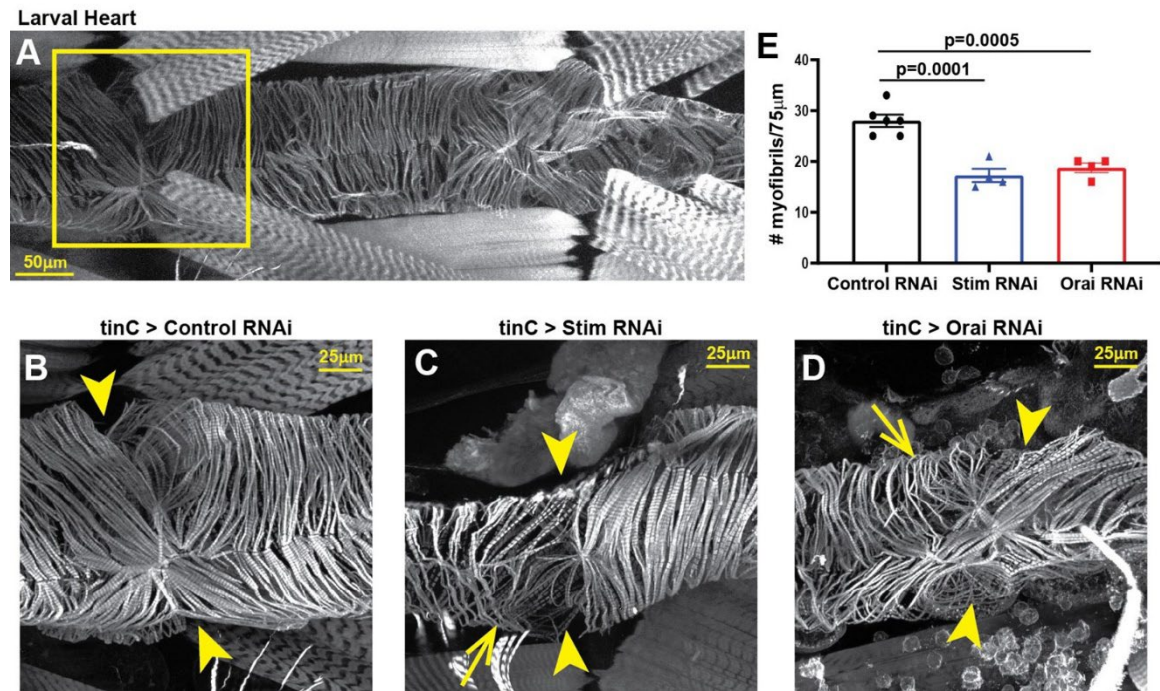


Figure 2-3. *Stim* and *Orai* suppression results in myofibril disorganization in larval hearts

A. Representative image of a control third instar larval heart stained with Phalloidin to visualize actin. The box denotes the region shown in high resolution images. **B-D.** Representative high resolution images of the region around the second ostium of third instar larval hearts with *tinC-GAL4* driven non-targeting control, *Stim*, and *Orai* RNAi. Arrowheads point to ostia, and arrows point to regions with disorganized myofibrils. **E.** Plot of the total number of myofibrils per 75 μm in larval hearts with *tinC-GAL4* driven non-targeting control, *Stim* and *Orai* RNAi. Each symbol represents a measurement from a single heart, and data are mean $\pm$ s.e.m. (p-values were calculated from One-way ANOVA with Tukey's Multiple Comparisons Test).

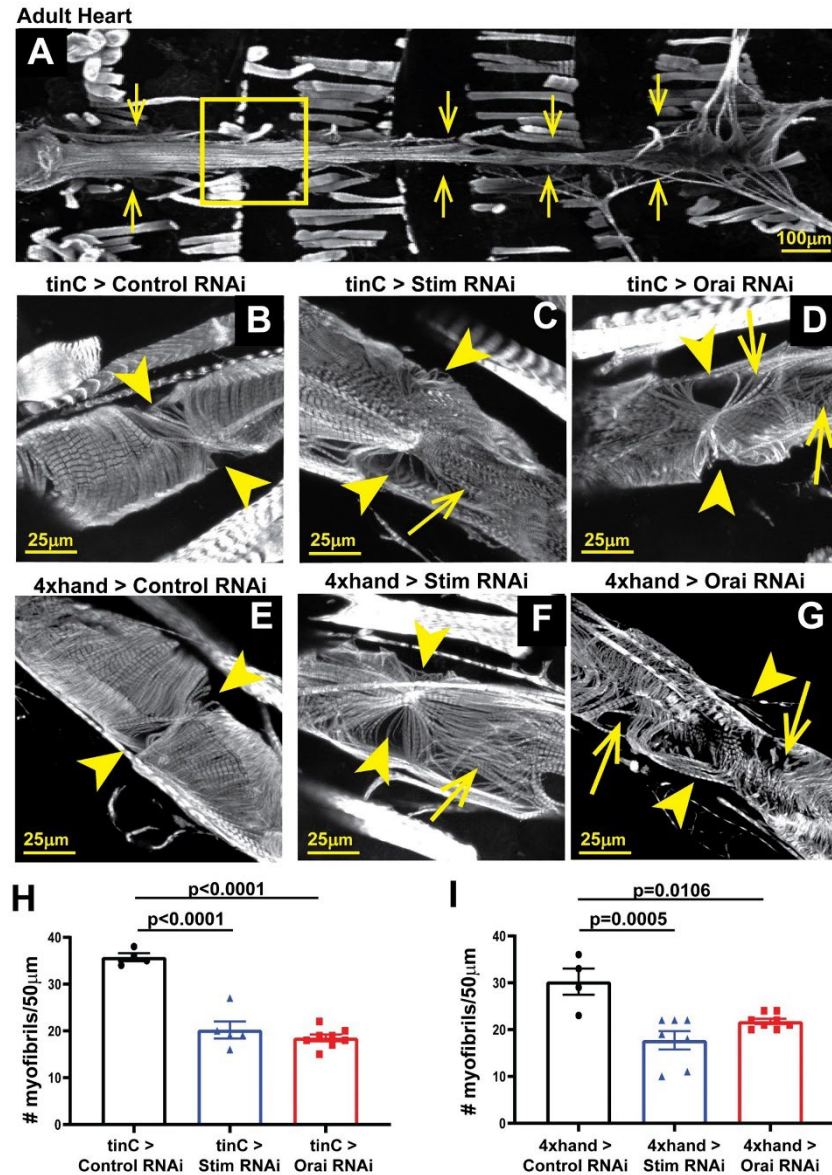


Figure 2-4. *Stim* and *Orai* suppression results in myofibril disorganization in adult hearts

A. Representative image of an adult control heart (indicated by arrows) stained with Phalloidin to visualize actin. The box denotes the region shown in the high-resolution images. **B-D.** Representative high resolution images of the region around the third ostium of five-day old adult male hearts with *tinC-GAL4* driven non-targeting control, *Stim*, and *Orai* RNAi. Arrowheads point to ostia, and arrows point to regions with disorganized myofibrils. **E-G.** Representative high-resolution images of the region around the third ostium of five-day old adult male hearts with *4xhand-GAL4* driven non-targeting control, *Stim*, and *Orai* RNAi. Arrowheads point to ostia, and arrows point to regions with disorganized myofibrils. **H.** Plot of the total number of myofibrils per 50 μ m in adult hearts with *tinC-GAL4* driven non-targeting control, *Stim* and *Orai* RNAi. Each symbol

represents a measurement from a single heart, and data are mean $\pm$ s.e.m. (p-values were calculated from One-way ANOVA with Tukey's Multiple Comparisons Test). **I.** Plot of the total number of myofibrils per 50 μ m in adult hearts with *4xhand-GAL4* driven non-targeting control, *Stim* and *Orai* RNAi. Each symbol represents a measurement from a single heart, and data are mean $\pm$ s.e.m. (p-values were calculated from One-way ANOVA with Tukey's Multiple Comparisons Test).

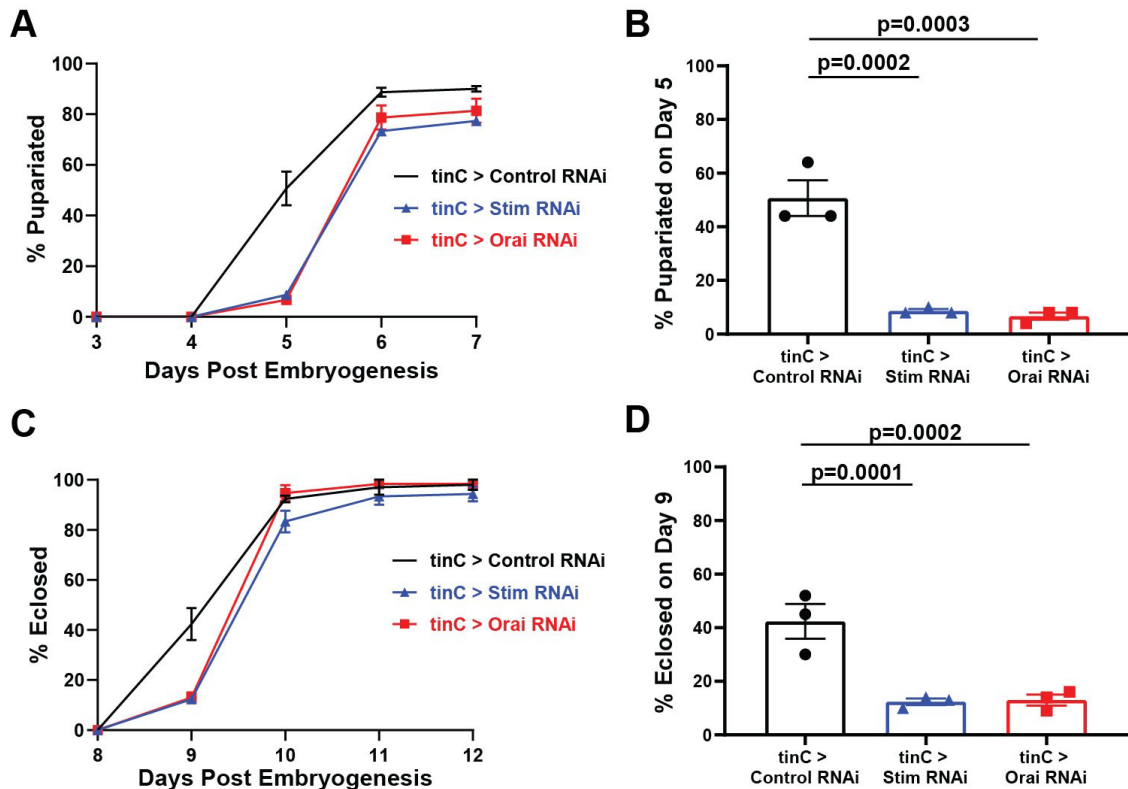


Figure 2-5. Heart specific *Stim* and *Orai* suppression delays post-embryonic animal development.

A. Plot of the percent of larvae that pupariated on each of the indicated days post-embryogenesis for *tinC-GAL4* driven *Stim*, *Orai*, and non-targeting control RNAi. Data are mean $\pm$ s.e.m. from three independent experiments with 25-50 animals per experimental group. **B.** Comparison of percent pupariated on day 5 post-embryogenesis for *tinC-GAL4* driven *Stim*, *Orai*, and non-targeting control RNAi from three independent replicates (p-values calculated from One-way ANOVA with Tukey's Multiple Comparisons Test). **C.** Plot of the percent of pupae that eclosed on each of the indicated days post-embryogenesis for *tinC-GAL4* driven *Stim*, *Orai*, and non-targeting control RNAi. Data are mean $\pm$ s.e.m. from three independent experiments with 25-50 animals per experimental group. **D.** Comparison of percent eclosed on day 9 post-embryogenesis for *tinC-GAL4* driven *Stim*, *Orai* and non-targeting control RNAi from three independent replicates (p-values calculated from One-way ANOVA with Tukey's Multiple Comparisons Test).

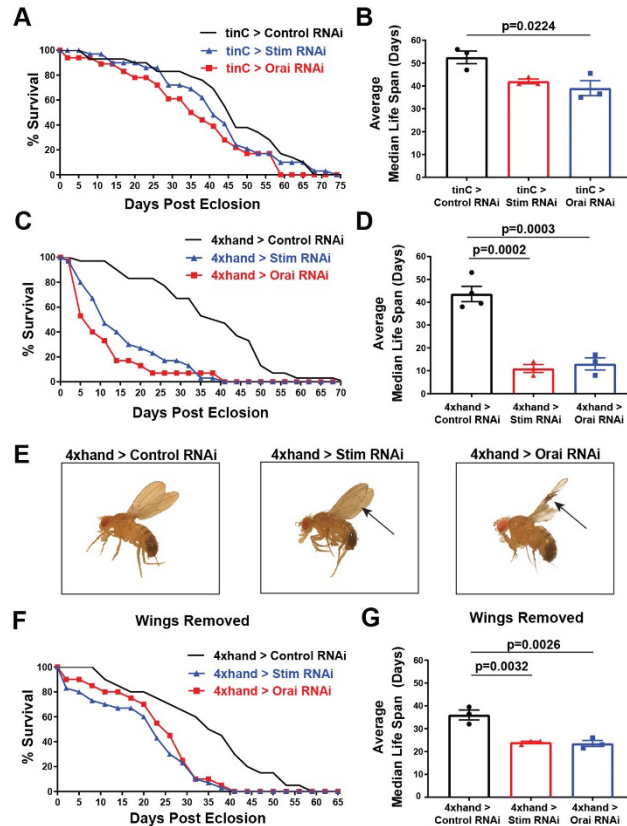


Figure 2-6. Heart specific *Stim* and *Orai* suppression results in early adult lethality
A. Representative survival curves in days post-eclosion for adult males with *tinC-GAL4* driven *Stim*, *Orai*, and non-targeting control RNAi (n = 30 animals per group). **B.** Plot of the average adult male median lifespan (MLS), calculated from three independent survival curves, for *tinC-GAL4* driven *Stim*, *Orai*, and non-targeting control RNAi. Error bars represent s.e.m., and p-values were calculated from One-way ANOVA with Tukey's Multiple Comparisons Test. **C.** Representative survival curves in days post-eclosion for adult males with *4xhand-GAL4* driven *Stim*, *Orai*, and non-targeting control RNAi (n = 30 animals per group). **D.** Plot of the average adult male median lifespan (MLS), calculated from three independent survival curves, for *4xhand-GAL4* driven *Stim*, *Orai*, and non-targeting control RNAi. Error bars represent s.e.m., and p-values were calculated from One-way ANOVA with Tukey's Multiple Comparisons Test. **E.** Representative images of adult males with *4xhand-GAL4* driven *Stim*, *Orai*, and non-targeting control RNAi on the day of eclosion. Note the blistered wing on the *Stim* RNAi animal and the severely damaged and frayed wings on the *Orai* RNAi animal (indicated by arrows). **F.** Representative survival curves in days post-eclosion for adult males with *4xhand-GAL4* driven *Stim*, *Orai*, and non-targeting control RNAi, with their wings removed on day of eclosion (n = 30 animals per group). **G.** Plot of the average adult male median lifespan (MLS), calculated from three independent survival curves, for *4xhand-GAL4* driven *Stim*, *Orai*, and non-targeting control RNAi animals with their wings removed on the day of eclosion. Error bars represent s.e.m., and p-values were calculated from One-way ANOVA with Tukey's Multiple Comparisons Test.

SUPPLEMENTAL FIGURES

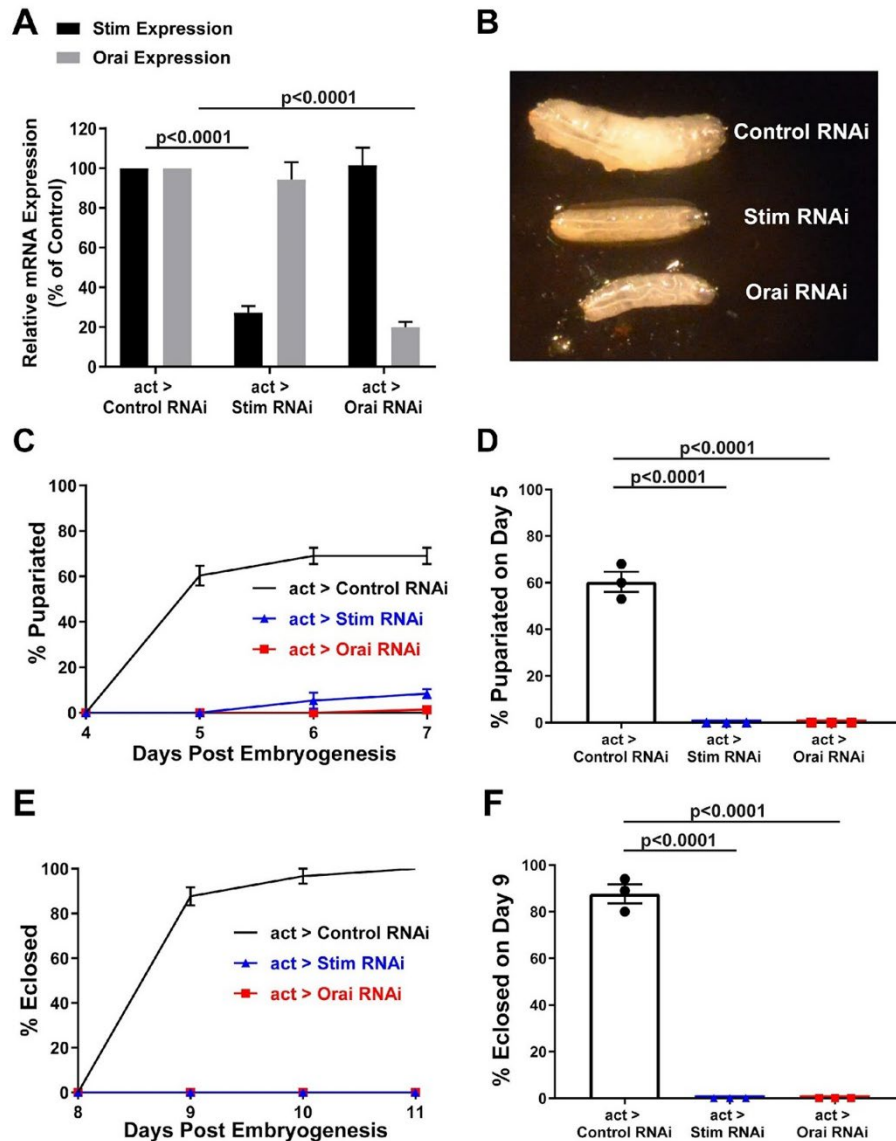


Figure 2-S1. *Stim* and *Orai* RNAi are efficient and specific

A. RT-qPCR analysis of relative *Stim* and *Orai* mRNA expression levels from first instar larvae with *act-GAL4* driven non-targeting control, *Stim*, and *Orai* RNAi. Data are represented as percent of non-targeting control RNAi (mean $\pm$ s.e.m. from three independent replicates; p-values were calculated from Two-way ANOVA with Tukey's Multiple Comparison). **B.** Representative images of third instar larvae with *act-GAL4* driven non-targeting control, *Stim*, and *Orai* RNAi. Note the significantly reduced size of *Stim* and *Orai* RNAi animals compared to controls. **C.** Plot of the percent of larvae that pupariated on each of the indicated days post-embryogenesis for *act-GAL4* driven *Stim*, *Orai*, and non-targeting control RNAi. Data are mean $\pm$ SEM from three independent experiments, with 25-50 animals per experimental group. **D.** Comparison of percent pupariated on day 5 post-embryogenesis for *act-GAL4* driven *Stim*, *Orai*, and non-targeting control RNAi from three independent experiments (p-values calculated from

One-way ANOVA with Tukey's Multiple Comparisons Test). **E.** Plot of the percent of pupae that eclosed on each of the indicated days post-embryogenesis for *act-GAL4* driven *Stim*, *Orai*, and non-targeting control RNAi. Data are mean $\pm$ SEM from three independent experiments, with 25-50 animals per experimental group. **F.** Comparison of percent eclosed on day 9 post-embryogenesis for *act-GAL4* driven *Stim*, *Orai* and non-targeting control RNAi from three independent experiments (p-values calculated from One-way ANOVA with Tukey's Multiple Comparisons Test).

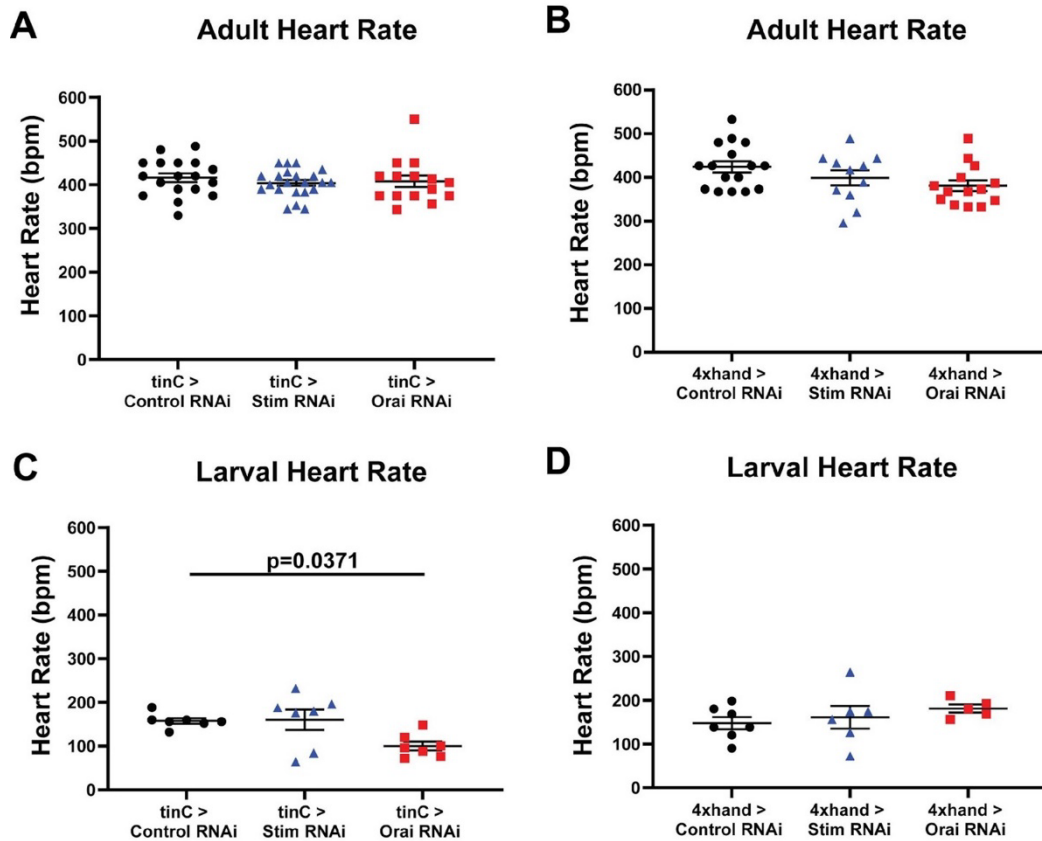


Figure 2-S2. Heart specific suppression of *Stim* and *Orai* does not affect adult or larval heart rate

Adult heart rate calculated as beats per min (bpm) from OCT imaging for *tinC-GAL4* (**A**) and *4xhand-GAL4* (**B**) driven non-targeting control, *Stim*, and *Orai* RNAi animals. Each symbol represents a single animal measurement; results were not significantly different (One-way ANOVA with Tukey's Multiple Comparisons Test). Third instar larval heart rate calculated as beats per min (bpm) from intravital fluorescence imaging for *tinC-GAL4* (**C**) and *4xhand-GAL4* (**D**) driven non-targeting control, *Stim* and *Orai* RNAi. Each symbol represents a single animal measurement; results were not significantly different except where indicated (p-value calculated from One-way ANOVA with Tukey's Multiple Comparison).

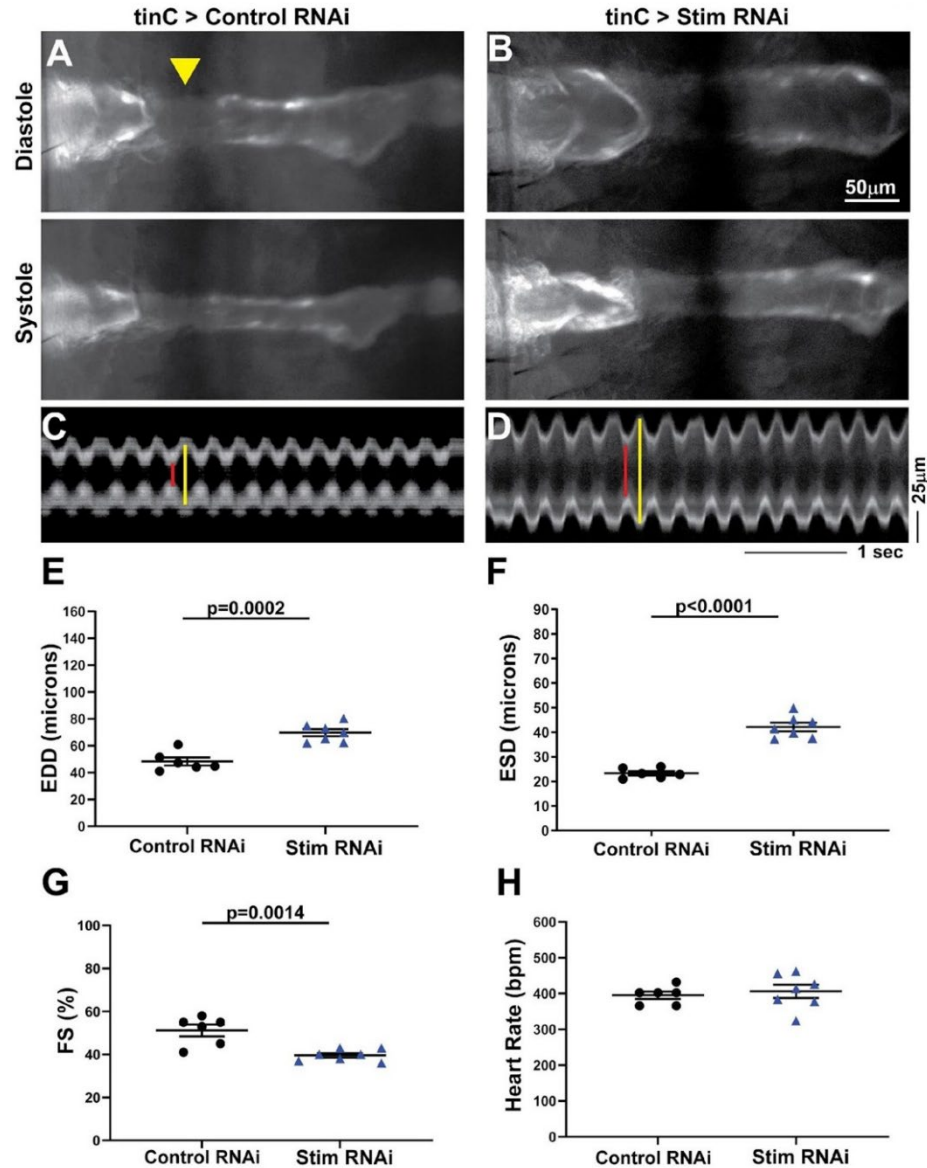


Figure 2-S3. Intravital fluorescence imaging confirms dilated cardiomyopathy in adult *Stim* and *Orai* suppressed hearts

A-B. Representative longitudinal B-mode images of intravital fluorescence imaging of R94C02-tdTom expressing five-day old adult male hearts with *tinC-GAL4* driven non-targeting control and *Stim* RNAi during diastole (upper panels) and systole (lower panels). Arrowhead points to region that is obscured by a dark abdominal stripe on the cuticle of the animal. **C-D.** Representative M-mode images from *tinC-GAL4* driven non-targeting control and *Stim* RNAi hearts, with red lines depicting systole and yellow lines diastole. EDD (E), ESD (F), FS (G), and heart rate (H) were calculated from M-mode recordings of five-day old males, and each symbol represents a measurement from a single animal. Bars indicate mean $\pm$ s.e.m., and p-values were calculated from unpaired t-tests.

SUPPLEMENTAL TABLES

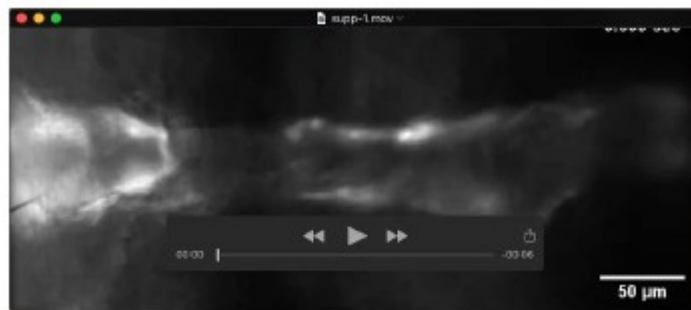
	Adult OCT	Adult CM-tdTom	Larval CM-tdTom
End Diastolic Dimensions	73.61 ± 2.54 μm	48.31 ± 2.89 μm	103 ± 5.41 μm
End Systolic Dimensions	8.89 ± 1.63 μm	23.32 ± 0.83 μm	43.92 ± 2.05 μm
Fractional Shortening	88.63 ± 2.04 %	51.17 ± 2.71 %	56.71 ± 2.76 %
Heart Rate	416.3 ± 9.87 bpm	395 ± 10.32 bpm	157.7 ± 6.23 bpm

Table 2-S1. Comparison of contractility parameters measured from OCT and CM-tdTom intravital imaging analyses for control animals.

Table indicates mean ± s.e.m. from OCT (n = 18) and CM-tdTom intravital imaging (n = 6) of *tinC-GAL4* driven nontargeting control RNAi adult hearts, as well as *tinC-GAL4* driven nontargeting control RNAi larval hearts measured by CM-tdTom intravital imaging (n = 7).

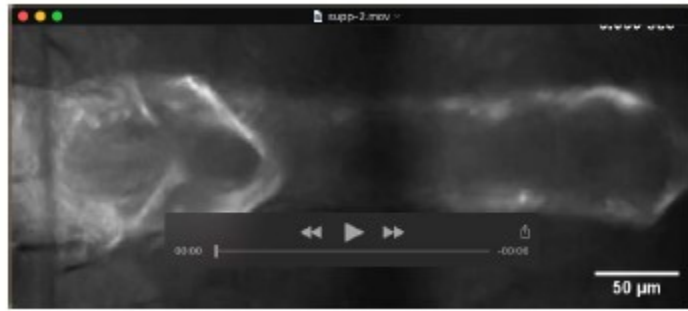
SUPPLEMENTAL VIDEOS

Supplemental videos can be found with supplemental material from publication in Biology Open (150)



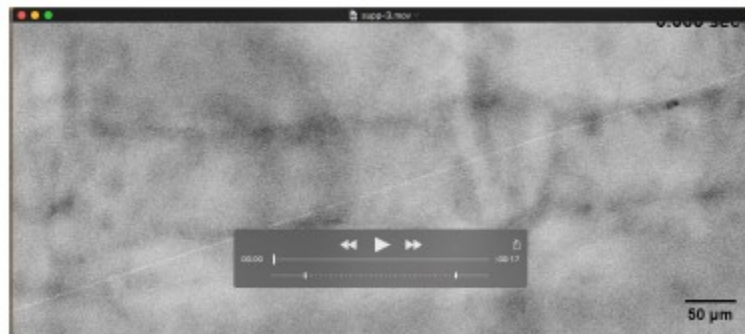
Video 2-S1. Full intravital imaging timelapse of the adult control heart shown in Figure 2-S3.

Images were acquired at a rate of 200 frames per second, and video playback is slowed to 100 frames per second to allow better visualization of contractions.



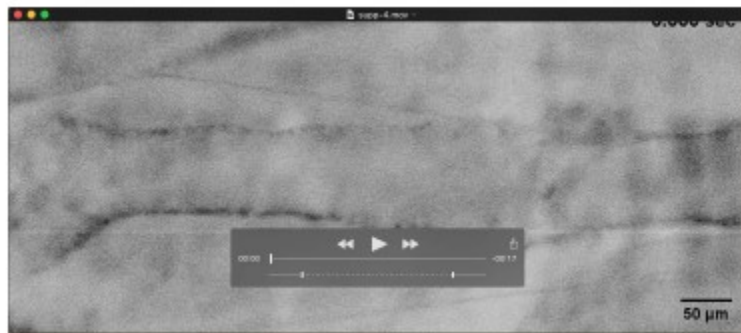
Video 2-S2. Full intravital imaging timelapse of the adult *Stim* RNAi heart shown in Figure 2-S3.

Images were acquired at a rate of 200 frames per second, and video playback is slowed to 100 frames per second to allow better visualization of contractions



Video 2-S3. Full intravital imaging timelapse of the larval control heart shown in Figure 2-A.

Images were acquired at a rate of 200 frames per second, and video playback is at full frame-rate.



Video 2-S4. Full intravital imaging timelapse of the larval *Stim* RNAi heart shown in Figure 2-B.

Images were acquired at a rate of 200 frames per second, and video playback is at full frame-rate.

**CHAPTER 3: A novel micro-computerized tomography method reveals
hypertrophy of the *Drosophila* heart caused by upregulated store
operated calcium entry**

Courtney Petersen^{1,2}, Todd A Schoborg³, and Jeremy T. Smyth^{1,2*}

¹Graduate Program in Molecular and Cellular Biology and ²Department of Anatomy,
Physiology, and Genetics, Uniformed Services University of the Health Sciences, F.
Edward Hébert School of Medicine, Bethesda, MD 20814

³Department of Molecular Biology, University of Wyoming, Laramie, WY 82071

Submitted to *BioRxiv*, January 2021

ABSTRACT

The onset of heart failure is often preceded by pathological cardiac hypertrophy, whereby thickening of the heart musculature is driven by complex gene regulatory and signaling processes that ultimately result in maladaptive cardiac remodeling and impaired contractility. The *Drosophila* heart has great potential as a genetic model for deciphering the mechanisms that underlie cardiac hypertrophy. However, current methods for evaluating hypertrophy of the *Drosophila* heart are laborious and difficult to reproducibly perform. In this study, we demonstrate that micro-computerized tomography (microCT) is an accessible, highly reproducible method for non-destructive, quantitative analysis of *Drosophila* heart morphology and size. To validate our microCT approach, we show that expression of constitutively active Ras (*Ras85D^{V12}*), previously shown to cause hypertrophy of the fly heart, results in significant thickening of adult and larval heart walls when measured from microCT images. We then show using microCT analysis that genetic upregulation of store-operated Ca^{2+} entry (SOCE), by expression of constitutively active Stim (Stim^{CA}) or Orai (Orai^{CA}) proteins, also results in significant hypertrophy of the *Drosophila* heart through a process that specifically depends on Ca^{2+} influx through Orai channels. Importantly, dysregulation of Ca^{2+} homeostasis in cardiomyocytes is a major driver of cardiac hypertrophy, but the underlying mechanisms are unclear. These results indicate that increased SOCE activity is an essential regulator of hypertrophic cardiomyocyte growth and demonstrate how microCT analysis combined with tractable genetic tools in *Drosophila* can be used to delineate the molecular mechanisms underlying cardiac hypertrophy and heart failure pathogenesis.

INTRODUCTION

Pathological cardiac hypertrophy occurs when the heart enlarges in response to conditions of chronic functional overload, such as hypertension or valve stenosis. This enlargement is initially compensatory to increase cardiac output in response to the underlying pathology. However, unmitigated hypertrophy eventually transitions to irreversible fibrosis of the heart musculature, stiffening of the ventricular walls, and decreased luminal diameter, resulting in impaired systolic function, and reduced cardiac output (12; 117; 191; 201). Pathological cardiac hypertrophy almost invariably progresses to heart failure, a major cause of death worldwide (68; 116). Despite significant advances in treatment, there is no cure for pathological cardiac hypertrophy or heart failure, demonstrating a critical need for better understanding of the cellular and molecular mechanisms that contribute to maladaptive cardiomyocyte growth.

A mechanistic hallmark of pathological cardiac hypertrophy is reactivation of fetal gene expression pathways in cardiomyocytes (44; 92; 111). Several key signaling processes are upregulated in cardiomyocytes from hypertrophic hearts, resulting in increased expression of cardiac contractility and growth genes including β -myosin heavy chain (β -MHC), myosin light chain 1/3 (MLC1/3), and atrial and brain natriuretic peptides (ANP and BNP) (84; 92). One of the most prominent of these signaling pathways is the Ca^{2+} – calcineurin – NFAT axis, whereby enhanced Ca^{2+} cycling in cardiomyocytes activates the NFAT transcription factor via the Ca^{2+} /calmodulin-regulated phosphatase calcineurin (28; 36; 126; 176; 200). Importantly, calcineurin activation is both necessary and sufficient for pathological cardiac hypertrophy in mammalian models (69; 126; 209), and calcineurin signaling has emerged as an attractive

therapeutic target for anti-hypertrophic drugs. However, the mechanisms that lead to enhanced Ca^{2+} cycling in hypertrophic cardiomyocytes are poorly understood. Moreover, signaling pathways involving phosphatidylinositol 4,5-bisphosphate kinase (PI3K), Ras and Raf small GTPases, nitric oxide, and Ca^{2+} /calmodulin-dependent protein kinases (CaMKs) are also essential mediators of hypertrophic cardiomyocyte growth (8; 52; 191), but integration of these signaling processes is highly complex and poorly understood. Resolution of this complexity will benefit greatly from molecular pathway dissection in a genetically tractable model like *Drosophila melanogaster*.

Drosophila have become firmly established as an important model organism for understanding the developmental genetics and signaling processes that regulate heart physiology. The *Drosophila* heart is a linear tube of cardiomyocytes that runs along the dorsal midline of the animal and pumps hemolymph, a lymph-like fluid, throughout the body in an open circulatory system (151; 167; 190). Importantly, *Drosophila* cardiomyocyte physiology is highly conserved with mammals, allowing for relevant comparison and analysis of the genetic and functional bases of cardiomyopathies (33; 101; 136; 138; 151). A major advantage of *Drosophila* over mammalian models in cardiac research is the ability to quickly and easily engineer multiple genetic alterations in the same animal for integrated signaling pathway analysis. These approaches have shown that both the Ras-Raf-MAPK and calcineurin-regulated signaling pathways drive cardiac hypertrophy in *Drosophila* (97; 218; 220), demonstrating that hypertrophic signaling in the fly heart is well conserved with mammals. However, the full potential of *Drosophila* for deciphering conserved mechanisms of cardiac hypertrophy has been

limited by a lack of readily accessible and reproducible methods for analyzing fly heart wall thickness.

Prior analyses of *Drosophila* cardiac hypertrophy have relied on histopathology (97; 215; 219; 220), which involves fixing and embedding whole flies, physical sectioning along the length of the heart tube, and microscopic imaging of individual sections to directly measure heart wall dimensions. This approach has several drawbacks that can limit throughput, reproducibility, and accuracy. First, it is very time-consuming and laborious, and this limits the feasibility of processing large numbers of samples required for genetic pathway analyses. Second, achieving consistent sectioning through the same regions of the heart and in the same orientation between samples is challenging, and this is critical for reproducibility of measurements. Third, physical sectioning through the heart yields relatively thick sections of 8-10 μ m, and this can limit the accuracy of individual measurements.

Several recent studies have described the use of micro-computerized tomography (microCT) for quantitative analysis of internal organs and structures in *Drosophila* (53; 64; 115; 124; 125; 175), and a major goal of our current study was to determine whether microCT is an effective tool for analysis of cardiac hypertrophy in flies. MicroCT is an x-ray based imaging platform in which the sample is incrementally rotated to generate a series of images that encompasses the full sample volume. Computerized algorithms are then used to generate three-dimensional (3D) reconstructions or tomograms of the entire sample. When applied to whole *Drosophila* larvae, pupae, or adults, microCT allows visualization of internal organs in any orientation and at any location within the specimen with unprecedented clarity and resolution (175). Importantly, microCT imaging is

isotropic, so measurements made in any dimension or orientation can be directly compared. Thus, with microCT, measurements can be reproducibly made from the same location within a specific organ or structure without reliance on imprecise physical sectioning. MicroCT also involves relatively simple fixation and staining protocols, with the ability to process multiple animals at a time. Reproducible measurements of *Drosophila* heart wall thickness have been made using microCT (175); however, whether microCT can be used to analyze pathological changes to heart morphology and architecture such as hypertrophy has not been determined. Importantly, integration of microCT analysis with combinatorial *Drosophila* genetic tools may provide powerful insight into molecular mechanisms of pathological heart remodeling.

Here we demonstrate that analytical microCT approaches can accurately and reproducibly report hypertrophy of the *Drosophila* heart. As proof-of-principle, we first show using microCT that expression of a constitutively active Ras mutant, previously shown to cause cardiac hypertrophy in *Drosophila* by histopathological analyses (218), results in significantly increased heart wall thickness in adult flies. We then apply additional genetic approaches and microCT analysis to demonstrate that upregulation of store-operated Ca^{2+} entry (SOCE) drives cardiac hypertrophy in *Drosophila*. SOCE is a highly conserved Ca^{2+} signaling mechanism that couples the influx of extracellular Ca^{2+} to the depletion of endo/sarcoplasmic reticulum (E/SR) Ca^{2+} stores. SOCE is mediated by Ca^{2+} sensing Stim proteins in the E/SR and Orai Ca^{2+} influx channels in the plasma membrane (11; 157; 182). Accumulating evidence suggests that SOCE upregulation in cardiomyocytes is necessary and sufficient for pressure-overload induced cardiac hypertrophy, likely due to direct SOCE activation of calcineurin signaling (37; 74; 202).

However, mechanisms that regulate SOCE in cardiomyocytes, and how SOCE signaling integrates with other hypertrophic signaling processes are largely unknown. Our study demonstrates that by combining microCT analysis with robust genetic tools in *Drosophila*, we can carry out highly reproducible and robust analyses of hypertrophic signaling mechanisms driven by upregulated SOCE. The microCT approach to analyzing cardiac hypertrophy is straightforward, accessible, and readily adaptable to the renowned genetic tools in *Drosophila*, creating a powerful experimental platform for delineating the complex cellular and genetic processes that drive pathological cardiac hypertrophy.

RESULTS

MicroCT is a valuable tool to evaluate heart wall thickness in Drosophila adults and larva

Direct measurements of heart wall thickness to analyze cardiac hypertrophy in *Drosophila* have previously been done using histopathological methods. These methods rely on mechanical tissue sectioning and microscopic imaging of individual tissue slices, making it difficult and laborious to acquire reproducible measurements from the same region of the heart between samples. Recent reports have demonstrated that microCT can non-invasively generate complete, 3-dimensional isometric image sets encompassing the entire *Drosophila* body in which all major internal organs, including the heart, can be clearly visualized. We therefore reasoned that microCT could be used to non-destructively image the entire *Drosophila* heart, allowing for reproducible heart wall measurements along the length of the heart tube. We began by analyzing hearts in adult

flies. Based on published protocols, whole intact adult flies were fixed in Bouin's solution and labeled with iodine to achieve sufficient X-ray attenuation of soft tissues. We then imaged adults by microCT at the highest resolution settings for which the entire animal still fits within the detector's field of view (1.15 μ m/pixel). Acquired tomograms produce a fully reconstructed animal (Figure 3-1A), and the entire heart tube, running anterior to posterior along the dorsal abdominal wall, is clearly visible in longitudinal views from high-resolution image stacks (Figure 3-1B). As previously described, these images have exceptional resolution that is sensitive enough to identify discrete structures such as ostia and intracardiac valves (175). To distinctly delineate the heart walls, we focused on the conical chamber at the anterior-most end of the heart where the heart chamber is largest, and the heart walls have the greatest separation. We then generated cross-sectional views from this region. As shown in Figure 3-1C and D, cross-sectional views from the conical chamber provided clear visualization of the heart walls around the complete circumference of the heart. Image sets from high-resolution scans provide superior visualization of heart structure and morphology. However, these scans require 4-6 hours to complete and are thus not practical to generate large datasets for quantitative analysis. Instead, scans of the entire adult fly at a lower resolution of 2.85 μ m/pixel can be completed in approximately 30 minutes, such that multiple animals can be scanned in several hours. As shown in Figure 3-1E and F, the heart walls can still be clearly delineated in low-resolution cross-sections of the conical chamber, though the margins are not as sharp. Importantly, low resolution images can be used to directly measure the thickness of the heart walls, and measurements from low-resolution scans of 22 *w¹¹¹⁸* control adults indicated a mean heart wall thickness in the conical chamber of $7.97 \pm$

0.13 μ m (mean $\pm$ SEM Figure 3-1G). Importantly, heart wall thickness measurements obtained from microCT scans were nearly identical to previously published measurements from histopathological preparations (97; 218; 220) . Moreover, the low variance of our measurements suggests the microCT-based methodology has a high degree of reproducibility.

We next determined whether our microCT-based approach can accurately detect hypertrophy of the adult *Drosophila* heart. We tested this by expressing a constitutively active mutant of Ras (Ras85D^{V12}) that has previously been shown to induce cardiac hypertrophy in flies (218). As shown in Figure 3-1C and D, heart-specific expression of Ras85D^{V12} using the *tinC-GAL4* driver resulted in noticeable thickening of the heart walls and reduced luminal area in high-resolution cross-section images of the conical chamber compared to *w¹¹¹⁸* controls. Direct measurements from low-resolution images (Figure 3-1E and F) revealed a significant increase in heart wall thickness of approximately 33% in Ras85D^{V12} compared to *w¹¹¹⁸* control hearts (Figure 3-1G). These results demonstrate that microCT imaging is a robust method for detection and analysis of hypertrophy of the *Drosophila* heart.

Drosophila heart development shares many similarities with the early stages of vertebrate heart development, and thus analysis of the *Drosophila* heart during developmental stages like larvae may prove invaluable for our understanding of human heart development and congenital heart defects (138; 167; 203). Additionally, the majority of cardiomyocyte growth in *Drosophila* occurs during the larval stage of development, which makes the larval heart a useful model for studying mechanisms that regulate both physiological and pathological heart growth (128; 151). We therefore

assessed the use of microCT to evaluate heart wall thickness in larva, and whether changes in thickness are detectable with this method. The heart spans approximately the posterior third of the third-instar larva and is situated just under the dorsal cuticle (Figures 3-2 A-D). Heart wall measurements made along the lateral sides of the heart tube in low-resolution cross-sectional images of *w¹¹¹⁸* controls revealed a thickness of $7.240 \pm 0.10\mu\text{m}$ (mean $\pm$ SEM Figure 3-2 E-G). Similar to adults, heart specific expression of Ras85D^{V12} resulted in a modest but highly reproducible and significant increase in larval heart wall thickness of approximately 26% compared to *w¹¹¹⁸* controls (Figure 3-2G). Collectively, these results demonstrate that microCT generates images of *Drosophila* larvae and adults with sufficient resolution and sensitivity to allow for accurate analysis of small changes in delicate tissues like the heart. Furthermore, we show that the combination of microCT with *Drosophila* genetic tools is a powerful analytical approach for studying mechanisms of cardiac hypertrophy.

SOCE upregulation results in pathological cardiac hypertrophy

Heart specific upregulation of SOCE is required for the induction pathological cardiac hypertrophy in vertebrate models (37; 74; 107; 197; 202). However, mechanisms that regulate SOCE in cardiomyocytes are still poorly understood. *Drosophila* may serve as an important model to elucidate how SOCE regulates cardiac hypertrophy, but whether SOCE upregulation results in hypertrophy of the *Drosophila* heart has yet to be determined. We therefore used microCT analysis to evaluate whether SOCE upregulation due to expression of constitutively active Stim and Orai mutants in *Drosophila* results in increased heart wall thickness. Constitutively active Stim (Stim^{CA}) contains two aspartic acid to alanine substitutions within the EF-hand domain, rendering Stim unable to bind

ER luminal Ca^{2+} (225). Constitutively active Orai (Orai^{CA}) has a glycine to methionine mutation in the hinge region of the channel, forcing the channel into an open conformation (227). As shown in Figure 3-3, heart specific expression of Stim^{CA} and Orai^{CA} resulted in significantly increased heart wall thickness compared to *w¹¹¹⁸* controls as well as animals expressing wildtype Stim and Orai constructs (Stim^{WT} and Orai^{WT}, respectively). Thickening of the heart walls in Stim^{CA} and Orai^{CA} expressing animals was comparable to increases observed with Ras85D^{V12} expression (Figure 3-3 C and D), demonstrating that our results with Stim^{CA} and Orai^{CA} are consistent with an established model of *Drosophila* cardiac hypertrophy.

Reactivation of signaling mechanisms that drive cardiomyocyte growth during heart development is a hallmark of pathological cardiac hypertrophy (44). In accordance with this, Stim1 and Orai1 are shown to have high expression levels during fetal development that drop precipitously into adulthood and are restored upon induction of pathological cardiac hypertrophy (36; 74; 107; 202). This suggests that SOCE signaling may be essential for developmental cardiomyocyte growth, however, little is known regarding the role of SOCE in cardiac development. We therefore determined whether SOCE upregulation results in increased heart wall thickness in larvae using microCT. Similar to adults, heart specific expression of Stim^{CA} resulted in significantly increased heart wall thickness in third-instar larvae compared to *w¹¹¹⁸* controls, as well as to larvae expressing Stim^{WT} (Figure 3-4). Increased larval heart wall thickness by Stim^{CA} expression was again similar to thickening caused by Ras85D^{V12} expression (Figure 3-4B).

SOCE upregulation impairs heart contractility

Cardiac hypertrophy impairs heart contractility and suppresses cardiac output due to reduced heart wall flexibility and chamber volume (12; 117; 201). Therefore, we next evaluated whether SOCE upregulation due to Stim^{CA} and Orai^{CA} expression affects heart contractility. Adult heart contractility was analyzed by intravital fluorescence imaging of animals with cardiomyocyte-specific tdTomato (CM-tdTom) expression (83; 150). As shown in Figure 5A-D, control hearts exhibited strong, consistent contractions with an average end diastolic dimension (EDD) of $65.17 \pm 1.33\mu\text{m}$, average end systolic dimension (ESD) of $32.15 \pm 0.83\mu\text{m}$, and average fractional shortening (FS) of $51 \pm 0.85\%$ (mean $\pm$ SEM). These parameters were not significantly altered by expression of Stim^{WT} or Orai^{WT}. In striking contrast, m-mode traces from Stim^{CA}, Orai^{CA}, and Ras85D^{V12} expressing hearts showed visibly narrowed diastolic dimensions (Figure 3-5A), and direct EDD measurements had significant reductions compared to controls of 26%, 32%, and 15% for Stim^{CA}, Orai^{CA}, and Ras85D^{V12}, respectively (Figure 3-5B). ESD was also significantly reduced in Stim^{CA}, Orai^{CA}, and Ras85D^{V12} expressing hearts (Figure 3-5C), whereas FS was unaltered in Stim^{CA} or Orai^{CA} and in fact modestly increased in Ras85D^{V12} expressing hearts (Figure 3-5D). Heart rate was reduced in Ras85D^{V12} hearts but was unchanged by Stim^{CA} and Orai^{CA} expression (Figure 3-5E). Importantly, reduced EDD is indicative of impaired relaxation while reduced ESD suggests decreased luminal volume, both of which are serious contractile impairments typically seen in hypertrophic hearts. Thus, these results demonstrate that SOCE upregulation results in contractile dysfunction consistent with cardiac hypertrophy observed in microCT analysis. Notably, the effects of SOCE upregulation on heart

structure and function were pathological, as the number of heart specific Stim^{CA} expressing animals reaching the pupal stage of development (pupariation) was reduced by approximately 70% compared to controls, and only about 20% of Stim^{CA} animals reached adulthood (Figure 3-S1).

Stim^{CA}-mediated hypertrophy and contractility impairment specifically requires Orai channels

It has been suggested that STIM proteins regulate Orai-independent targets in cardiomyocytes including L-type Ca²⁺ channels and phospholamban (143; 226). We therefore determined whether hypertrophy and impaired contractility caused by Stim^{CA} expression specifically required Orai channels. To evaluate this, we generated animals that co-express Stim^{CA} with Orai RNAi specifically in cardiomyocytes. As shown in Figure 3-6A and C, microCT analysis again showed significant thickening of heart walls in animals with heart specific Stim^{CA} expression alone. Strikingly however, the hypertrophic phenotype was completely suppressed by co-expression of Orai RNAi with Stim^{CA}, as heart wall thickness in these animals was similar to controls as well as animals with Orai RNAi alone. This strongly suggests that hypertrophy of Stim^{CA} expressing hearts results from upregulated Ca²⁺ influx through Orai channels. This conclusion is further supported by our finding that hypertrophy caused by Ras85D^{V12} expression was not suppressed by co-expression of Orai RNAi (Figure 3-6 B and D). Thus, the ability of Orai knockdown to suppress hypertrophy is specific to the effects of SOCE upregulation, as opposed to a generalized ability of Orai suppression to universally repress hypertrophic growth.

Additionally, we determined whether the effects of Stim^{CA} expression on heart contractility similarly depend on Orai channels. In support of this, co-expression of Orai RNAi with Stim^{CA} reverted the reductions in EDD, ESD, and heart rate seen with Stim^{CA} expression alone to control values (Figure 3-7A and C-F). The effect of Orai suppression was again specific to Stim^{CA}, as reductions in EDD, ESD, and heart rate caused by Ras85D^{V12} expression were unchanged by co-expression with Orai RNAi (Figure 3-7B and G-J) and in fact, EDD and ESD were further reduced compared to Ras85D^{V12} alone. These results collectively demonstrate that SOCE upregulation in *Drosophila* drives cardiac hypertrophy and pathologically impairs contractile heart function. Importantly, the effects of SOCE upregulation are specific and distinct from other mechanisms, such as upregulation of Ras signaling, that drive hypertrophic growth of the heart.

DISCUSSION

Cardiac growth and remodeling are essential for proper heart development and the ability to respond to physiological or pathological increases in functional demand, such as pregnancy and exercise or myocardial infarction and hypertension, respectively (12; 46; 119; 213). Thus, experimental approaches that combine molecular genetics with *in vivo* analysis of heart architecture and function are essential for understanding both physiological and pathological heart remodeling and hypertrophy. Here we have demonstrated that microCT is an accessible and powerful non-destructive imaging platform for analysis of *Drosophila* heart architecture that, when combined with genetic tools and functional heart analysis, can be used to decipher complex mechanisms of heart growth and pathological remodeling. Our introduction of microCT imaging to the

Drosophila heart analysis toolkit has the potential to significantly accelerate our understanding of the conserved genetic and signaling mechanisms that regulate heart development, and that are re-engaged during pathological hypertrophy and heart failure.

The *Drosophila* heart is a valuable model for understanding the molecular etiology of cardiomyopathies and heart failure. *Drosophila* have been widely used to study dilated cardiomyopathy, likely because dilated cardiomyopathy can be readily assessed based solely on relatively straightforward contractility analyses. Very few studies, on the other hand, have analyzed cardiac hypertrophy in flies. Notably, a finding of cardiac hypertrophy requires not only altered contractility, but also direct measurements indicating increased heart wall thickness. These measurements are challenging in flies due to the small size and delicate tissue structure of the fly heart. Accordingly, the most common approach, histopathological preparation, is time intensive and adversely affected by differences in tissue sectioning and mounting. We now show that microCT can yield heart size measurements in flies that are rapidly obtained, highly reproducible, and sensitive enough to detect changes in heart wall thickness indicative of hypertrophy. Another distinct advantage of microCT over histopathology is that microCT generates complete 3D reconstructions of the whole animal, such that the entire heart and other internal tissues and organs can be visualized in various orientations and perspectives. Thus, multiple landmarks external to the heart can be used to orient consistent measurements of the heart between animals. Furthermore, prolonged cardiac disease and heart failure have the potential to profoundly impact the physiology of other organs, such as skeletal muscle and renal function (77). Simultaneous analysis of multiple organs in the same animal with microCT therefore holds immense and relatively

unmatched potential for the study of multifactorial diseases that affect the heart in addition to other organs.

We first validated our microCT approach for detection of *Drosophila* cardiac hypertrophy by analyzing animals that express constitutively active Ras, an established model of cardiac hypertrophy in flies based on histopathological analysis (218). Importantly, our microCT measurements of heart wall thickness in adult controls were consistent with measurements made from histopathological preparations, indicating a normal thickness of about 6-8µm. MicroCT measurements from constitutively active *Ras85D^{V12}* animals resulted in a 3-4µm, or approximately 50%, increase compared to controls, indicating significant hypertrophy. Notably however, prior histopathological measurements showed an approximately 15µm increase in thickness with *Ras85D^{V12}* expression (218). A possible reason for this difference is that histopathological measurements were made from transverse heart sections that were 8µm thick, whereas our microCT measurements were made from 2.85µm optical slices. We expect that the smaller sections used for microCT measurements will result in more accurate, though potentially smaller measurements of heart wall thickness. It is also possible that our *Ras85D^{V12}* expression was not as penetrant as in previous studies. Of note, Yu et al. reported that heart-specific *Ras85D^{V12}* expression resulted in significantly reduced eclosion and poor survival of adult escapers, whereas we did not observe significant effects of *Ras85D^{V12}* expression on animal development or survival (not shown).

We have also demonstrated the utility of microCT for analysis of the larval *Drosophila* heart. This is significant, because the majority of physiological heart growth in *Drosophila* occurs during the larval stages of animal development (138; 151; 167).

Thus, application of microCT to larval heart analysis may be a valuable tool for delineating physiological mechanisms of developmental heart growth. And given that many of these developmental growth mechanisms are re-activated during pathological cardiac hypertrophy and heart failure, microCT analysis of the larval heart may be prove an essential tool for uncovering new mechanisms that drive heart failure as well (44; 69).

MicroCT has been largely underutilized for analysis of *Drosophila* anatomy and physiology, particularly when combined with other established genetic and imaging approaches. To demonstrate the power of microCT for analysis of signaling pathways that drive cardiac hypertrophy, we used this technique to investigate the role of SOCE signaling in hypertrophy of the *Drosophila* heart. Numerous studies have demonstrated that upregulated SOCE is essential for the induction of pathological cardiac hypertrophy (10; 74; 107; 144; 202). However, we still have much to learn about the precise roles of SOCE signaling in hypertrophic cardiomyocyte growth. Furthermore, individuals with gain-of-function mutations in STIM1 and Orai1 have not presented with cardiac phenotypes (94), further highlighting significant gaps in our understanding of SOCE function in the heart. Thus, a genetic model of upregulated SOCE activity may prove vital to understanding the mechanism by which SOCE regulates cardiac physiology and disease pathogenesis. Our microCT data clearly show that genetic upregulation of SOCE function in cardiomyocytes by expression of constitutively active Stim or Orai mutants results in significant thickening of the *Drosophila* heart, similar to heart wall thickening caused by *Ras85D^{V12}* expression. Importantly, we combined microCT measurements with direct, *in vivo* analysis of heart contractility by intravital imaging to determine the effect of hypertrophy caused by SOCE upregulation on heart function. Consistent with the

hypertrophic phenotype, which is known to restrict the inner dimensions of the heart chamber, Stim^{CA} and Orai^{CA} as well as *Ras85D^{V12}* expression resulted in significantly reduced diastolic and systolic dimensions. These results establish a new genetic model of SOCE-mediated cardiac hypertrophy that, when combined with other genetic analyses in *Drosophila*, may prove vital for delineation of the integrated role of SOCE signaling in cardiac disease pathogenesis. These results also demonstrate the power of integrating microCT analysis in flies with other measures of heart architecture and contractility for understanding the complex relationship between heart form and function.

The consistency of our microCT and contractility results between both Stim^{CA} and Orai^{CA} expression suggest a specific role for Stim/Orai mediated SOCE Ca²⁺ influx in cardiac hypertrophy, as opposed to SOCE-independent functions of Stim and Orai that have been previously demonstrated (48; 133; 169). This conclusion is further supported by our result showing that hypertrophy caused by Stim^{CA} expression specifically requires Orai channels, whereby Orai suppression reversed the hypertrophic effects of Stim^{CA} expression. Notably, this was not the case for *Ras85D^{V12}*-induced hypertrophy, as Orai suppression had no effect. Thus, Orai suppression does not have a generalized anti-hypertrophic effect. This result also suggests that Ras and Stim/Orai-mediated SOCE function in parallel hypertrophic signaling pathways, as opposed to a linear pathway in which Ras functions upstream of Stim and Orai.

Previous findings in mammalian models suggest that upregulation of wildtype Stim1 expression, as opposed to a constitutively active Stim1 mutant, is sufficient for induction of cardiac hypertrophy and heart failure (37). Our results, however, indicate that wildtype Stim expression alone is not sufficient for hypertrophy of the *Drosophila*

heart. One possible reason for this difference is that our expression levels of transgenic Stim in cardiomyocytes is not high enough to drive hypertrophy. It is also possible that the stoichiometry of Stim and Orai expression in *Drosophila* cardiomyocytes may differ from mammals. For example, if native Stim expression in *Drosophila* cardiomyocytes is already stoichiometrically matched to Orai expression, then increasing Stim expression may not significantly affect SOCE function without a compensatory increase in Orai expression. Furthermore, Orai1-independent targets of Stim1, including canonical transient receptor potential (TrpC) channels (49; 112; 140; 169), L-type Ca^{2+} channels (143), and phospholambans (226), may also contribute to Stim1-dependent cardiac hypertrophy in mammals, whereas our results suggest that Stim functions specifically through Orai channels to drive *Drosophila* cardiac hypertrophy. Thus, there may be differences between Stim and Orai function in mammalian versus *Drosophila* cardiomyocytes related to stoichiometry and/or functional targets. Importantly though, *Drosophila* offer a more simplified model in which we can focus our analyses specifically on Stim and Orai mediated SOCE function in cardiac hypertrophy.

Accumulating evidence suggests that SOCE-mediated Ca^{2+} influx acts through calcineurin signaling to drive hypertrophic cardiomyocyte growth and impaired heart contractility (10; 37; 74; 107). However, additional targets of upregulated SOCE in cardiomyocytes have also been reported. For example, increased STIM1 expression in hypertrophied mouse hearts is associated with enhanced CaMKII (37) activity and mTORC2/Akt (10) signaling that may also contribute to the hypertrophic phenotype (37; 197). Transgenic upregulation of STIM1 has also been shown to enhance Ca^{2+} spark frequencies and generate spontaneous action potentials in mammalian cardiomyocytes

(21; 37; 197; 202). Furthermore, defects in mitochondrial structure including increased fragmentation and loss of crista structure were also noted in transgenic STIM1 mouse hearts (37). Thus, consistent with the complex and multi-faceted etiology of pathological cardiac hypertrophy, the role of SOCE in this disease process likely involves multiple targets and regulatory processes. The advanced genetic tools coupled with microCT and contractility analysis in *Drosophila* will serve as a valuable experimental platform for deciphering the complex pathophysiology of pathological cardiac hypertrophy and heart failure.

MATERIALS AND METHODS

Fly Stocks

The following *Drosophila* stocks were obtained from the Bloomington *Drosophila* Stock Center: *w¹¹¹⁸* (3605), *UAS-Ras85D^{V12}* (64195), and *Orai RNAi* (53333). *tinC-GAL4* was obtained from Dr. Manfred Frausch (Friedrich Alexander University). CM-tdTom flies were obtained from Dr. Rolf Bodmer (Sanford Burnham Prebys Institute). Wildtype *Orai* (*Orai⁺*) and *Orai^{CA}* were obtained from Dr. Gaiti Hasan (National Center for Biological Sciences, Bangalore, India). *Orai^{CA}* has a glycine to methionine mutation at amino acid position 170 (227). To generate *UAS-Stim-GFP* flies, plasmid LD45776 containing the cDNA sequence of *Drosophila Stim* Isoform A was obtained from the *Drosophila* Genomics Resource Center. The *Stim* coding sequence was cloned by PCR and inserted into vector pPWG (Carnegie *Drosophila* Gateway Vector Collection), which introduces a C-terminal EGFP tag and upstream *UASp* promoter. This vector was then sent to Bestgene for embryo injection and selection of transgenic

animals. To generate *UAS-Stim^{CA}* animals, site-directed mutagenesis (Stratagene QuikChange XL Kit) was used to change aspartic acids at positions 155 and 157 to alanines using the *UAS-Stim-GFP* described above as template. Sequence confirmed plasmid was then sent to BestGene for embryo injection and selection of transformants.

Micro-computerized Tomography (microCT)

The following microCT methods were adapted from protocols published by Schoborg et al (174; 175)

Adult Labeling

5-20 adult flies were anesthetized with CO₂ and transferred to a 1.5mL Eppendorf tube containing 1ml of phosphate buffered saline + 0.5% Triton-X 100 (0.5% PBST). Tubes were capped and gently inverted then incubated for 5 min at room temperature to remove the wax cuticle. Flies were then transferred to tubes containing 1ml Bouin's fixative solution (5% acetic acid, 9% formaldehyde, 0.9% picric acid; Sigma) for 24 hours. Samples were washed 3x30 min on a shaker in 1 ml μ CT Wash Buffer (0.1M Na₂HPO₄/NaH₂PO₄ + 1.8% Sucrose, pH 7.0), followed by staining with 1ml of 0.1N iodine-iodide solution (Lugol's solution) for 48hrs. Flies were then washed with two changes of ultrapure water and stored at room temperature for up to one month prior to scanning.

Larval Labeling

Third instar, wandering larvae were collected and placed in 1.5 ml Eppendorf tube with 1 ml 0.5% PBST. Tubes were then placed on a 100°C heat block for 20 seconds, followed by cooling at room temperature for 5 mins. Larvae were then

fixed in Bouin's solution for 24 hours and washed as described for adults. Prior to labeling with Lugol's solution, larval cuticles were punctured at their anterior and posterior ends with a microdissection needle, avoiding areas with underlying soft tissue, to allow penetration of the labeling solution. Punctured larvae were then incubated in Lugol's solution for 48 hrs, washed with two changes of ultrapure water, and stored at room temperature for up to one month prior to scanning.

Sample Mounting and Scanning

Individual adult and larval samples were placed head-down in heat sealed 10 μ l micropipette tips containing ultrapure water, and a dulled 20-gauge needle was used to gently lower the animals until they fit snugly in the taper of the pipette tip. Parafilm was wrapped around base of micropipette tip to prevent water leakage. Mounted samples were secured to the stage of the microCT scanner, pipette base down, using mounting putty. Samples were scanned with a Bruker SkyScan 1172 desktop scanner controlled by Skyscan software (Bruker) operated on a Dell workstation computer. The following X-ray source voltage and current settings were used: 40kV, 110 μ A, and 4 W. A Hamamatsu 10 Mp camera with 11.54 μ m pixel size coupled to a scintillator was used to collect X-rays and convert to photons. Fast scans utilized a medium camera resolution setting of 2.85 μ m, and slow, high-resolution scans used a small camera resolution setting of 1.15 μ m with 360 degrees of sample rotations. Frame averaging was set to four for all scans.

Reconstruction

Tomograms were generated using NRecon software (Bruker MicroCT, v1.7.0.4). The built-in shift correction function of NRecon, which uses reference scans to compensate for sample movement during scanning (e.g., thermal fluctuations or slowly varying sample movements), was used for image alignment and reconstruction. Any remaining misalignment was manually fine-tuned using the misalignment compensation function. Ring artifact correction was set to max (50) and beam hardening was set to 0%.

Heart Wall Thickness Measurements

Fully reconstructed microCT tomogram image series were imported into ImageJ (NIH) and viewed using the Orthogonal Views function to locate and orient the heart in XY, XZ, and YZ orientations. The conical chamber of the heart is located in the anterior-most region of the abdomen and can be easily identified in images in which the last segments of the thoracic muscles are visible. Using images that showed the largest opening of the conical chamber in cross section, lines were drawn through the thickest sections of the lateral heart walls and lengths of the lines recorded in μm . An average of the ten measurements from five slices was then used to represent each animal.

Intravital Fluorescence Microscopy

Intravital fluorescence imaging of adult hearts was carried out using animals that express tdTomato under control of the cardiomyocyte-specific R94C02 (CM-tdTom) enhancer element (83) as previously described (150). Five-day old adult females were

briefly anesthetized with CO₂ and adhered dorsal side down to a glass coverslip with Norland Optical Adhesive cured with a 48-watt UV LED light source (LKE) for 60 seconds. Animals were allowed to recover for 10 mins prior to imaging. The heart was imaged through the dorsal cuticle at a rate of 200 frames per second (fps) for twenty seconds using an ORCA-Flash4.0 V3 sCMOS camera (Hamamatsu) on a Nikon Ti2 inverted microscope controlled with Nikon Elements software. Excitation light at 550 nm was provided by a Spectra-X illuminator (Lumencor) and emission was collected through a 555-635 band-pass filter. To generate M-modes, a 1-pixel wide line was drawn through the heart chamber in the A2 segment, and the fluorescence intensity along this line for the full time-course was plotted using ImageJ. EDD and ESD were calculated directly from the processed M-mode traces by manually measuring the distance between the heart walls at full relaxation and full constriction, respectively. An average of five measurements of EDD and ESD was calculated from each trace. Heart rate was calculated by manually counting the number of systoles over 20 seconds. FS was calculated as $((\text{EDD}-\text{ESD}) / \text{EDD}) \times 100$.

Developmental Timing

Approximately 30-40 virgin female *tinC-GAL4* or *w¹¹¹⁸* animals were mated with males carrying specified transgenes under *UAS* control for three days, at which time animals were transferred into egg laying chambers that consisted of a 100 ml plastic beaker with holes for air exchange affixed over a petri dish containing grape juice agar (Genesee Scientific). A dollop of yeast paste (active dry yeast mixed with water) was placed in the center of each grape juice agar plate as food. Animals were acclimated in

the chambers for 24 hours, and then transferred to new plates for 4 hours at 25°C for timed egg laying. After removing adults, plates with eggs were incubated at 25°C for an additional 24 hours. Hatched larvae were then transferred to vials with standard fly food, with up to 30 larvae per vial, and maintained at 25°C over the course of the experiment. Vials were checked each day, and the numbers of newly formed pupae and eclosed adults were recorded.

Statistical Analyses

All statistical analyses were carried out using GraphPad Prism software. Contractility parameters and heart wall measurements were analyzed by One-way ANOVA followed by Tukey's Multiple Comparisons Test. Differences were considered statistically significant at $p < 0.05$.

FIGURES

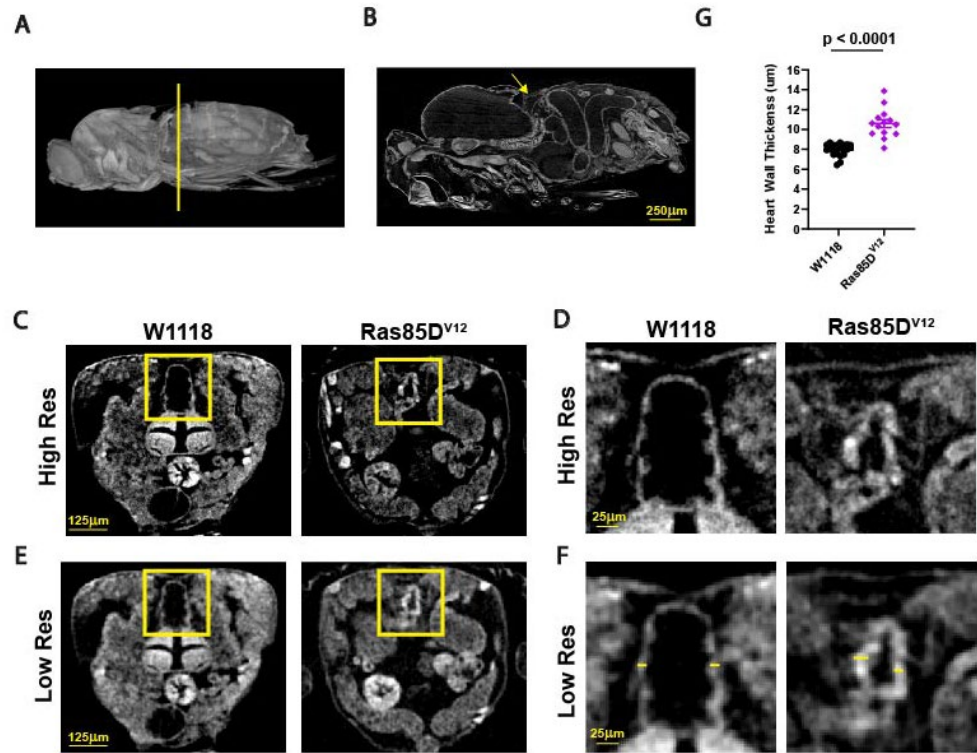


Figure 3-1. MicroCT analysis of normal and hypertrophied hearts in adult *Drosophila*

A. Maximum intensity projection of the full, high-resolution microCT image series of a 7-day old *w¹¹¹⁸* adult female fly, providing a 3D representation of the whole animal. The yellow line indicates the location of the cross-sectional images used for heart wall measurements. **B.** Single longitudinal microCT image through the center of a 7-day old *w¹¹¹⁸* adult female fly imaged at high resolution. Yellow arrow indicates the conical chamber of heart. **C.** Single cross-section high-resolution microCT images at the thorax to abdomen transition and bisecting the conical chamber, from 7-day old *w¹¹¹⁸* (left) and *tinC-GAL4* driven *Ras85D^{V12}* adult female flies. Yellow boxes denote the conical chambers of the hearts. **D.** Expanded images of the regions within the yellow boxes in C, showing magnification of the conical chambers. **E.** Single cross-section low-resolution microCT images at the thorax to abdomen transition and bisecting the conical chamber, from the same 7-day old *w¹¹¹⁸* (left) and *tinC-GAL4* driven *Ras85D^{V12}* adult female flies shown in C. Yellow boxes denote the conical chambers of the hearts. **F.** Expanded images of the regions within the yellow boxes in E, showing magnification of the conical chambers. Yellow lines on heart walls indicate areas where heart wall measurements were made. **G.** Plot of heart wall measurements taken from low-resolution microCT scans of *w¹¹¹⁸* and *tinC-GAL4* driven *Ras85D^{V12}* 7-day old adult females. Each symbol represents an average of ten measurements from a single animal. Bars indicate mean $\pm$

s.e.m., and p-values calculated from One-way ANOVA with Tukey's Multiple Comparison.

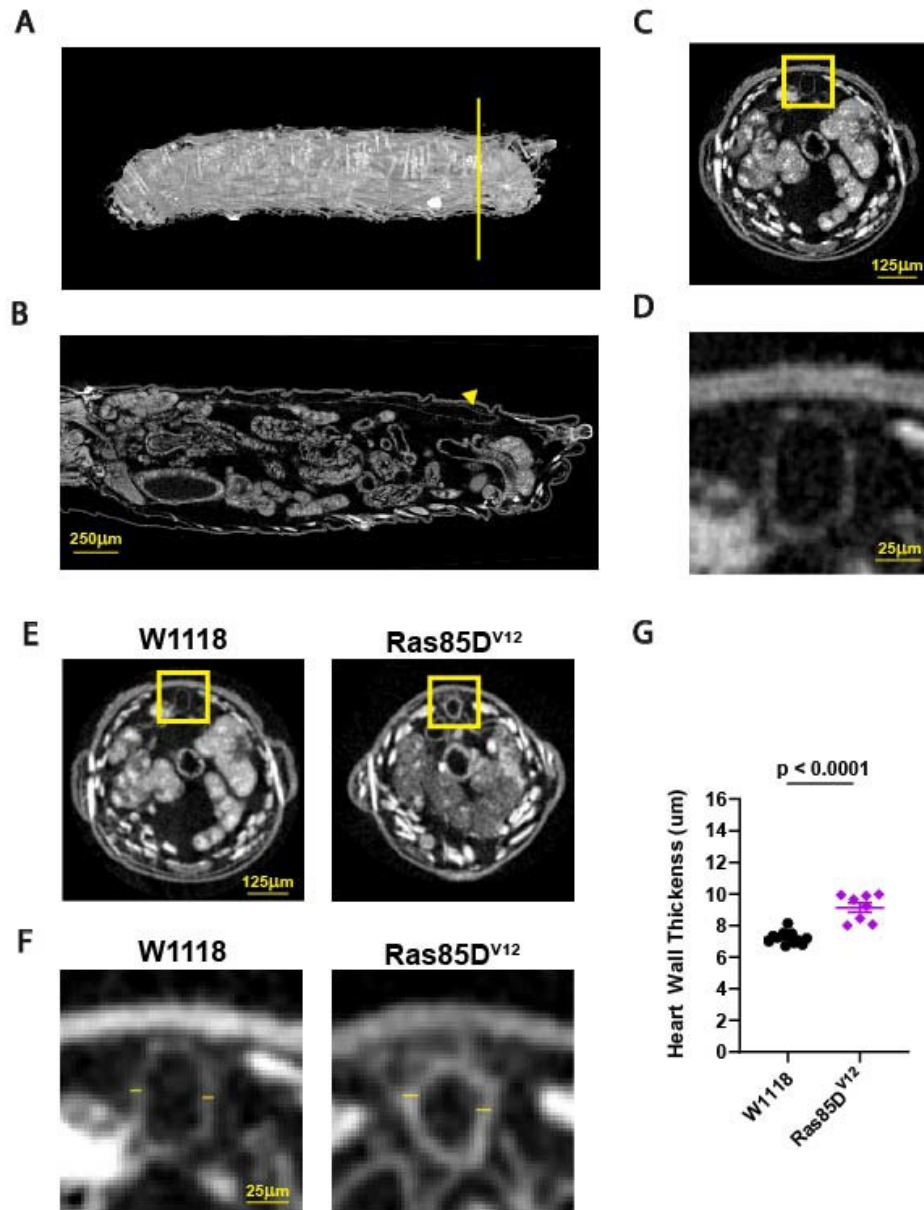


Figure 3-2. MicroCT analysis of normal and hypertrophied hearts in third instar *Drosophila* larvae

A. Maximum intensity projection of the full, high-resolution microCT image series of a 3rd instar larva, providing a 3D representation of the whole animal. The yellow line indicates the location of the cross-sectional images used for heart wall measurements. **B.** Single longitudinal microCT image through the center of a 3rd instar larva imaged at high resolution. Yellow arrowhead indicates the heart. **C.** Single cross-section high-resolution microCT image bisecting the heart from the high-resolution microCT scans represented

in B. The yellow box surrounds the heart and denotes the region expanded in D. to show the heart at higher magnification. E. Single cross-section low-resolution microCT images from the posterior of w^{1118} (left) and $tinC-GAL4$ driven $Ras85D^{V12}$ 3rd instar larvae. The yellow box surrounds the heart and denote the regions expanded in F. to show the hearts at higher magnification. Yellow lines in F indicate areas where heart wall measurements were made. G. Plot of heart wall measurements taken from low-resolution microCT scans of w^{1118} and $tinC-GAL4$ driven $Ras85D^{V12}$ 3rd instar larvae. Each symbol represents an average of ten measurements from a single animal. Bars indicate mean $\pm$ s.e.m., and p-values calculated from One-way ANOVA with Tukey's Multiple Comparison.

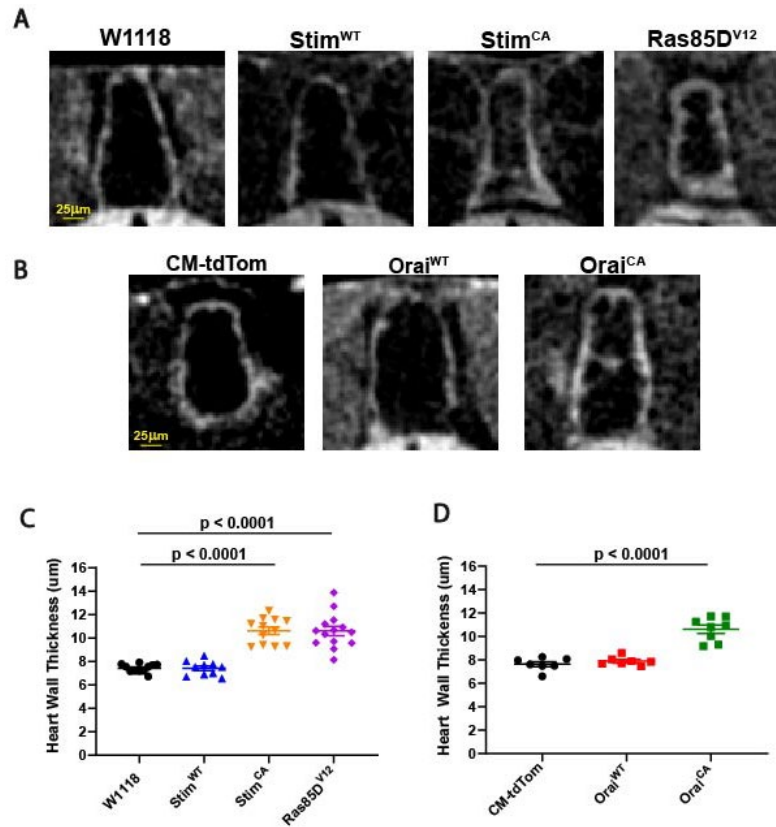


Figure 3-3. SOCE upregulation results in hypertrophy of adult *Drosophila* hearts.

A-B. Representative cross section low-resolution microCT images of the abdomen to thorax transition, cropped to show conical chambers of 7-day old adult females with $tinC-GAL4$ and w^{1118} (control), and $tinC-GAL4$ driven $Stim^{WT}$, $Stim^{CA}$, and $Ras85D^{V12}$ (A), and with $tinC-GAL4$ and $CM-tdTom$ (control), and $tinC-GAL4$ $Orai^{WT}$, and $Orai^{CA}$ (B). **C-D.** Plots of heart wall measurements taken from low-resolution microCT scans of 7-day old adult females with the genotypes shown in A and B, respectively. Each symbol represents an average of ten measurements from a single animal. Bars indicate mean $\pm$ s.e.m., and p-values calculated from One-way ANOVA with Tukey's Multiple Comparison.

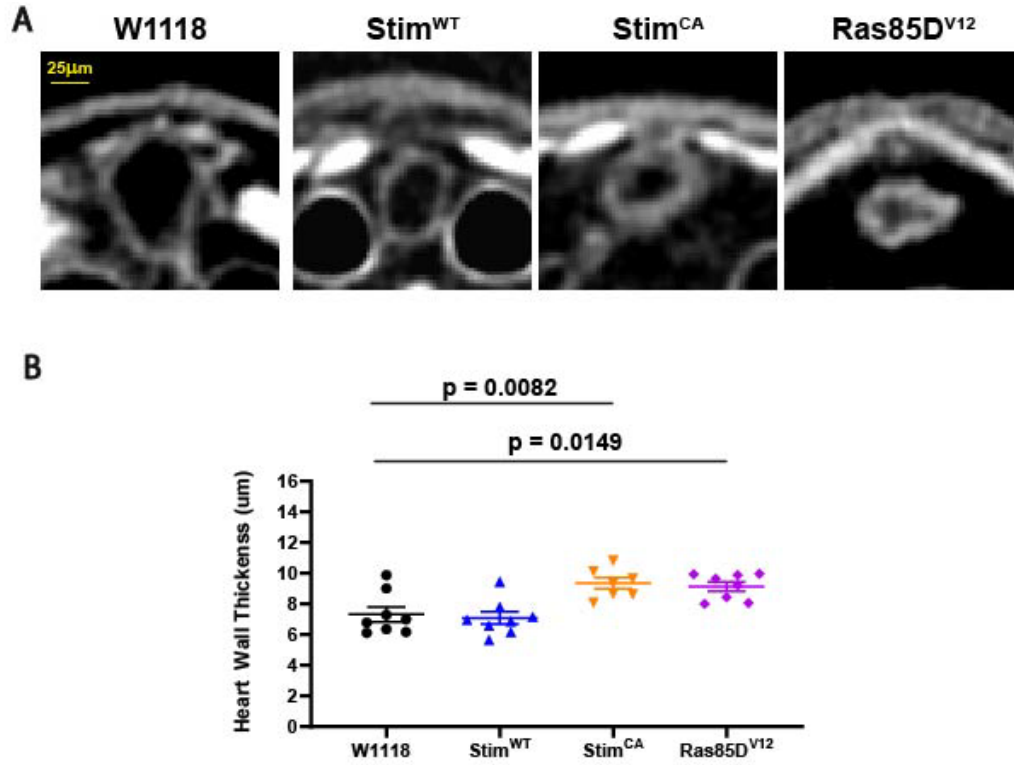


Figure 3-4. SOCE upregulation results in hypertrophy of larval *Drosophila* hearts.

A. Representative cross section low-resolution microCT images from the posterior of 3rd instar larvae, cropped to show the hearts from animals with *tinC-GAL4* and *w¹¹¹⁸* (control), and *tinC-GAL4* driven Stim^{WT}, Stim^{CA}, and Ras85D^{V12}. **B.** Plot of heart wall measurements taken from low-resolution microCT scans of 3rd instar larvae with the genotypes shown in A. Each symbol represents an average of ten measurements from a single animal. Bars indicate mean ± s.e.m., and p-values calculated from One-way ANOVA with Tukey's Multiple Comparison.

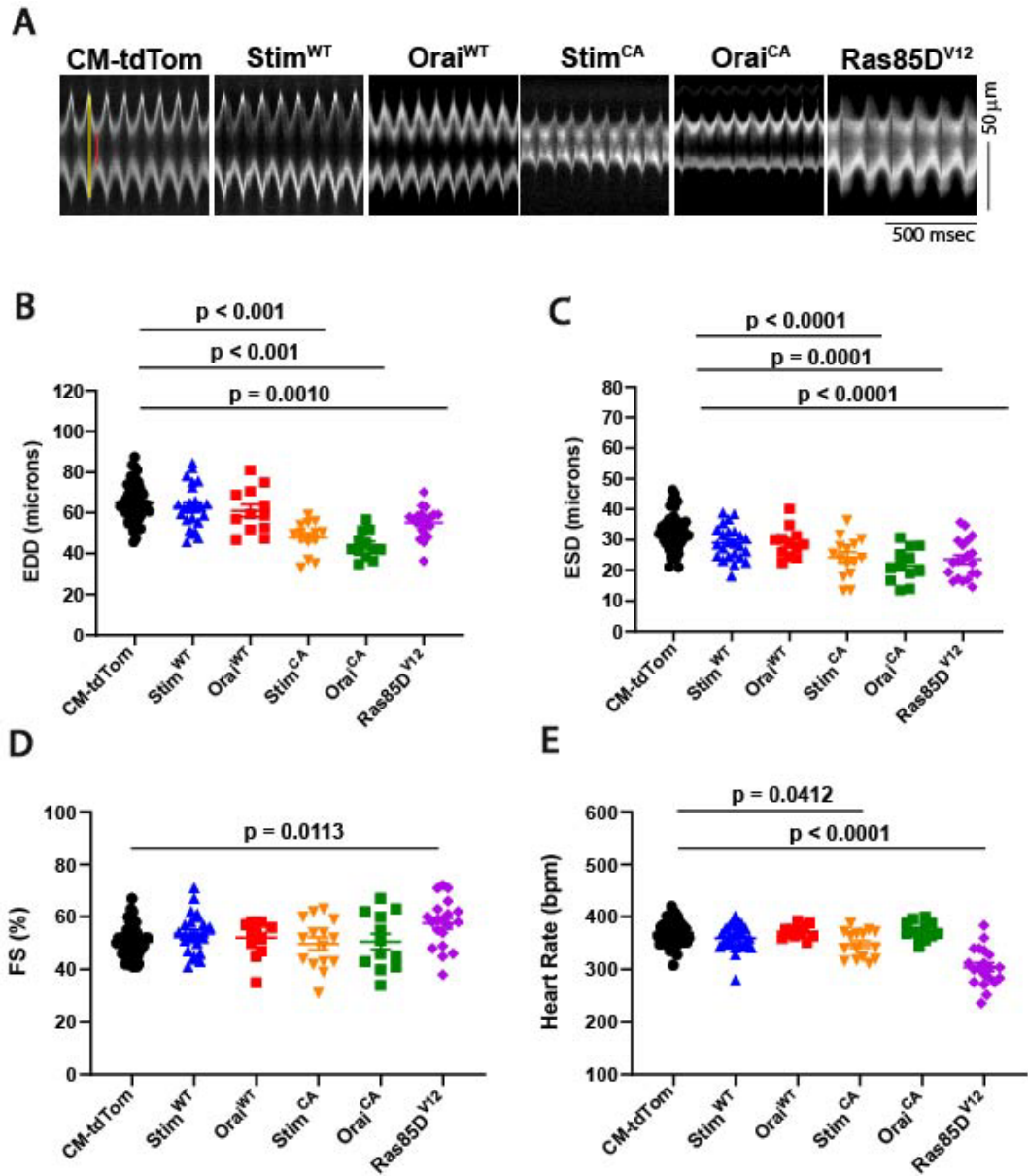


Figure 3-5. SOCE upregulation results in altered heart contractility with reduced heart chamber dimensions.

A. Heart contractility was analyzed by intravital imaging of 5-day old adult females expressing CM-tdTom. Shown are representative M-mode traces from animals with *tinC-GAL4* and CM-tdTom (control), and *tinC-GAL4* driven Stim^{WT}, Orai^{WT}, Stim^{CA}, Orai^{CA}, and Ras85D^{V12}. The red line in the CM-tdTom trace depicts systole and the yellow line depicts diastole. **B-E.** Plots of EDD (**B**), ESD (**C**), FS (**D**), and HR (**E**) calculated from M-mode traces of the genotypes shown in **A**. Each symbol represents an average of five measurements from a single animal. Bars indicate mean $\pm$ s.e.m., and p-values were calculated from One-way ANOVA with Tukey's Multiple Comparison.

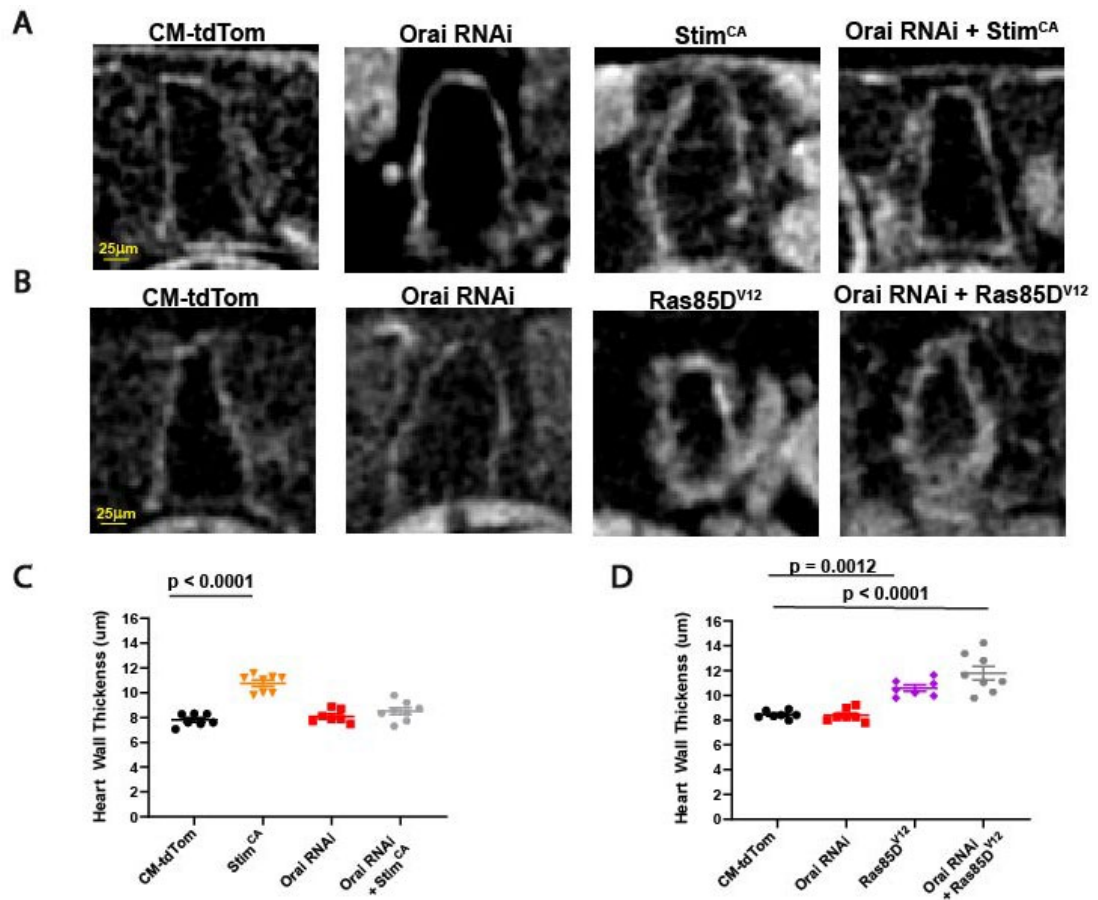


Figure 3-6. Cardiac hypertrophy caused by constitutively active Stim expression requires Orai channels.

A-B. Representative cross section low-resolution microCT images of the abdomen to thorax transition, cropped to show conical chambers of 7-day old adult females with *tinC-GAL4* and CM-tdTom (control), and *tinC-GAL4* driven Orai RNAi, Stim^{CA}, and Orai RNAi plus Stim^{CA} (**A**), and *tinC-GAL4* and CM-tdTom (control), and *tinC-GAL4* driven Orai RNAi, Ras85D^{V12}, and Orai RNAi plus Ras85D^{V12} (**B**). **C-D.** Plots of heart wall measurements taken from low-resolution microCT scans of 7-day old adult females with the genotypes shown in A and B, respectively. Each symbol represents an average of ten measurements from a single animal. Bars indicate mean $\pm$ s.e.m., and p-values calculated from One-way ANOVA with Tukey's Multiple Comparison.

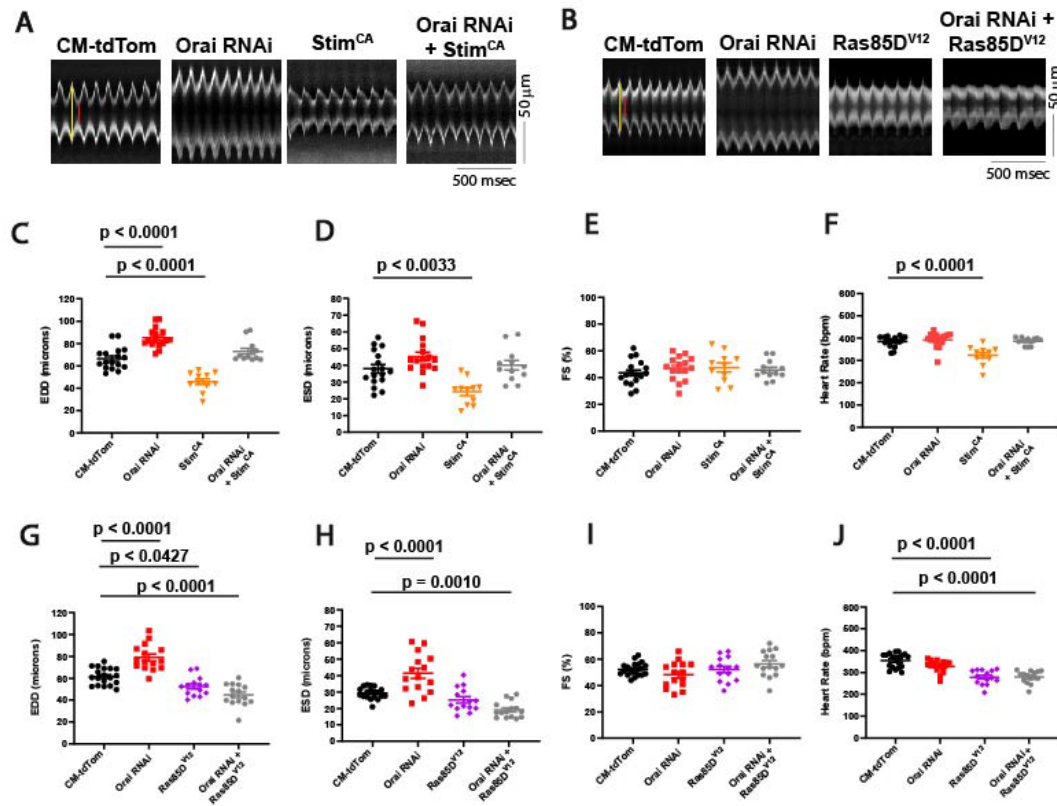


Figure 3-7. Altered heart contractility caused by constitutively active Stim expression requires Orai channels.

A-B. Representative M-mode traces from intravital imaging of 7-day old adult females with *tinC-GAL4* and CM-tdTom (control), and *tinC-GAL4* driven Orai RNAi, Stim^{CA}, and Orai RNAi plus Stim^{CA} (**A**), and *tinC-GAL4* and CM-tdTom (control), and *tinC-GAL4* driven Orai RNAi, Ras85D^{V12}, and Orai RNAi plus Ras85D^{V12} (**B**). **C-J.** Plots of EDD (**C,G**), ESD (**D,H**), FS (**E,I**), and HR (**F,J**) calculated from M-mode traces of the genotypes shown in A and B. Each symbol represents an average of five measurements from a single animal. Bars indicate mean $\pm$ s.e.m., and p-values were calculated from One-way ANOVA with Tukey's Multiple Comparison.

SUPPLEMENTAL FIGURES

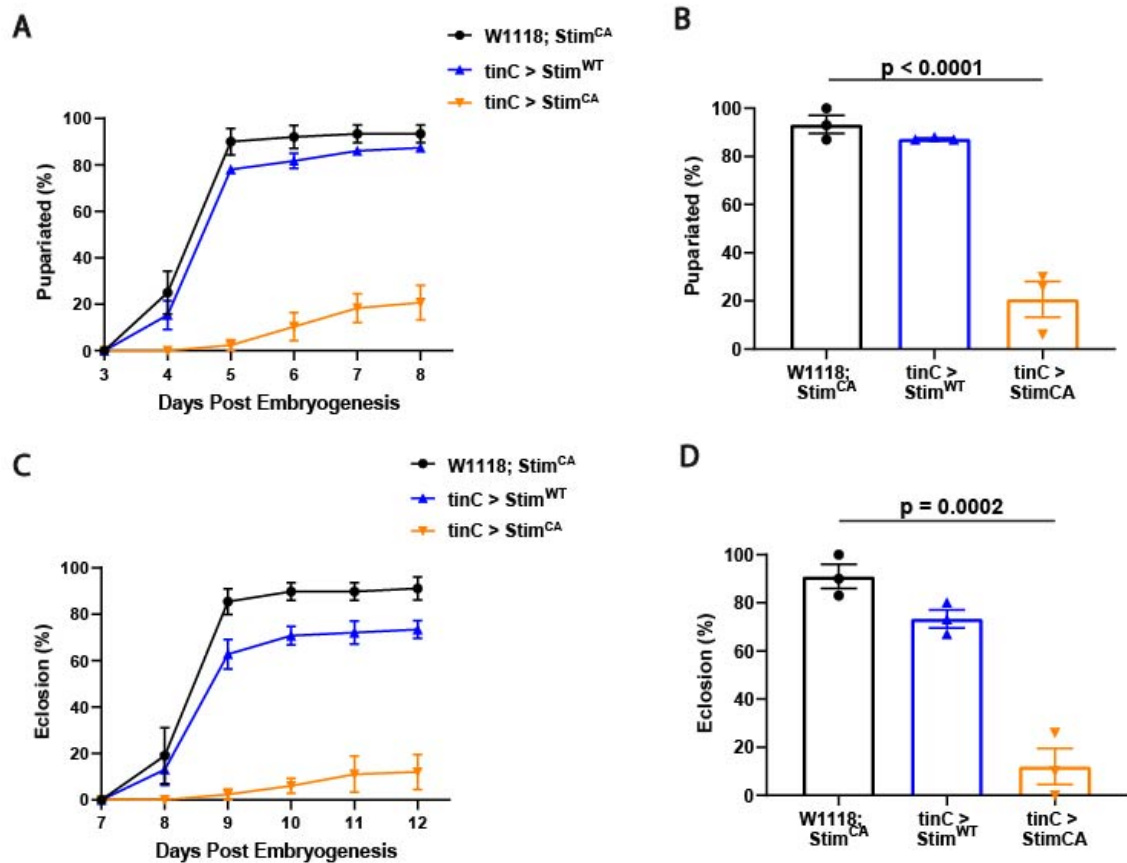


Figure 3-S1. Heart specific expression of *Stim^{CA}* results in significant larval and pupal lethality.

A. Plot of the percent of larvae that pupariated on each of the indicated days post-embryogenesis for animal with *w¹¹¹⁸* and *Stim^{CA}* (control), and *tinC-GAL4* driven *Stim^{WT}* and *Stim^{CA}*. Data are mean $\pm$ s.e.m. from three independent experiments with 20-30 animals per experimental group. **B.** Comparison of the percentage of animals that pupariated on day 8 post-embryogenesis for the genotypes shown in A. Bars indicate mean $\pm$ s.e.m. and p-values were calculated from One-way ANOVA with Tukey's Multiple Comparison. **C.** Plot of the percent of animals that eclosed on each of the indicated days post-embryogenesis for animal with *w¹¹¹⁸* and *Stim^{CA}* (control), and *tinC-GAL4* driven *Stim^{WT}* and *Stim^{CA}*. Data are mean $\pm$ s.e.m. from three independent experiments with 20-30 animals per experimental group. **D.** Comparison of the percentage of animals that eclosed on day 12 post-embryogenesis for the genotypes shown in C. Bars indicate mean $\pm$ s.e.m. and p-values were calculated from One-way ANOVA with Tukey's Multiple Comparison.

CHPATER 4: DISCUSSION

The importance of maintaining proper Ca^{2+} homeostasis in cardiomyocytes is well established. Ca^{2+} signaling serves as a nodal point in cardiac physiology through the regulation of diverse cellular events such as E-C coupling and gene transcription (7; 22; 36). Accordingly, dysregulation of intracellular Ca^{2+} homeostasis is known to be a central cause of heart failure pathogenesis (104; 106; 196). Experimental therapeutics aimed at restoring Ca^{2+} dynamics have had promising results but ultimately fall short of meeting clinical expectations (67; 78; 108; 110; 149; 178; 206; 216). This demonstrates a need for better understanding of the molecular and cellular mechanisms regulating Ca^{2+} -dependent processes in the heart and how dysregulation contributes to disease. However, due to the complex and dynamic nature of cardiomyocyte Ca^{2+} handling, several key aspects remain poorly understood, including (1) identifying distinct upstream Ca^{2+} sources, (2) determining what pathological processes are regulated by Ca^{2+} dependent pathways, (3) how specifically and selectively Ca^{2+} mediates maladaptive features, and (4) resolving how Ca^{2+} dependent pathways are integrated (13; 15; 41; 43; 50; 55; 89; 113; 172).

Recently, the store operated Ca^{2+} entry (SOCE) pathway has been suggested to be an essential upstream Ca^{2+} source in physiological and pathological cardiac function. Numerous studies have shown the main SOCE pathway components, Stim and Orai, are present in embryonic, neonatal, and adult cardiomyocytes (36; 74; 76; 139; 140; 195; 202). Despite the presence of Stim and Orai, the physiological relevance and specific processes regulated by SOCE in cardiomyocytes remains controversial. Moreover,

STIM1 has been shown to be a crucial mediator in physiological (35; 144; 204) and pathological (10; 37; 74; 107; 133; 139; 140; 197; 202; 223) heart function, but the role of Orai is less clear (36; 170; 177; 179; 204). To begin addressing these discrepancies, we analyzed the role of SOCE in *Drosophila* heart physiology. The strong functional and genetic conservation as well as simplified genetics of the *Drosophila* heart make it an ideal model to characterize the role of SOCE in cardiomyocytes (19; 33; 151; 167; 189; 203; 210). Furthermore, the combination of advanced genetic tools with *in vivo* contractility measurements and high-resolution morphological analysis make *Drosophila* an optimal platform to efficiently direct SOCE research in vertebrate models (17; 136; 151; 180; 210; 231).

Our results demonstrate SOCE mediated by Stim and Orai is an essential regulator of *Drosophila* heart function. *In vivo* analysis of heart function determined that suppression of Stim and Orai by RNAi induced dilated cardiomyopathy characterized by enlarged heart dimensions and reduced contractile strength. Dilated cardiomyopathy was concomitant with myofibril disorganization and decreased myofibril density. Defects in cardiac function and morphology upon SOCE suppression were pathological with animals developing slower and dying sooner than controls. In contrast, heart specific upregulation of SOCE activity by expression of constitutively active Stim and Orai mutants (Stim^{CA} and Orai^{CA}) induced pathological cardiac hypertrophy and reduced cardiac dimensions. Morphological analysis with the novel, micro-computerized tomography (microCT) method, revealed Stim^{CA} and Orai^{CA} in the *Drosophila* heart induced significant increases in heart wall thickness as compared to controls. Consistent with cardiac hypertrophy, SOCE upregulated animals had significantly decreased cardiac

dimensions. Importantly, cardiac hypertrophy and reduced cardiac dimensions in Stim^{CA} animals were rescued upon co-expression with Orai RNAi, demonstrating the defects in heart physiology specifically result from elevated Ca²⁺ influx through SOCE specific, Orai channels. Collectively, these results clearly demonstrate a well conserved requirement for properly regulated SOCE in cardiac physiology and establish *Drosophila* as a valuable model to delineate the mechanism by which SOCE regulates cardiomyocyte function.

SOCE is an Essential Component of Cardiomyocyte Ca²⁺ Regulation in *Drosophila*

Our results are the first to demonstrate that genetic manipulation of Stim and Orai induces comparable cardiac defects in the same animal model. We show that suppression and upregulation of either Stim or Orai induce nearly identical cardiac defects, including dilated cardiomyopathy and cardiac hypertrophy, respectively. Suppression of Stim or Orai by RNAi with two independent heart specific drivers, resulted in similarly significant increases in diastolic and systolic heart dimensions, decreased fractional shortening, and reduced myofibril density. Stim and Orai RNAi constructs were highly efficient and specific as shown by qRT-PCR in animals with ubiquitous RNAi expression. These results strongly suggest that both proteins function together to regulate SOCE activity in *Drosophila* cardiomyocytes. Correspondingly, upregulated SOCE activity by heart specific expression of Stim^{CA} or Orai^{CA} also induced comparable heart defects. Stim^{CA} or Orai^{CA} expression pathologically increased heart wall thickness and reduced systolic and diastolic heart dimensions. Importantly, enlarged heart walls and reduced heart dimensions in Stim^{CA} animals were rescued by co-expression of Orai

RNAi, which strongly suggests Ca^{2+} influx through Orai channels induced hypertrophic cardiac defects. The ability of Orai suppression to rescue hypertrophy was shown to be specific to Stim^{CA} induced defects, as opposed to a general ability to repress hypertrophic growth, since co-expression of Orai RNAi was unable to restore heart wall parameters in an independent, established model of *Drosophila* cardiac hypertrophy (Ras85D^{V12}) (218). Overall, the strikingly similar results of Stim and Orai suppression and upregulation, strongly suggest Stim and Orai function together to regulate *Drosophila* heart structure and function.

These findings are highly significant as previous reports have produced conflicting results regarding the physiological relevance of SOCE in the heart (22; 75; 76; 104; 198). Moreover, it is unclear whether the cellular actions of Stim and Orai are due to SOCE dependent Ca^{2+} influx or through SOCE independent mechanisms (7; 9; 22; 36; 48; 74; 107). Analysis of SOCE in vertebrate heart models is complicated by the presence of multiple Stim (STIM1 and STIM2) and Orai (Orai1-3) homologues. Although the specific functions of STIM2, Orai2, and Orai3 are poorly understood, they are believed capable of compensating for altered STIM1 or Orai1 function during stressful or pathological conditions (11; 26; 27; 31; 54; 82; 122; 146; 148; 155). In support of this, recent reports have demonstrated the potential for Orai3 and Orai1 homologues to form heteromultimeric channels and mediate store independent Ca^{2+} entry in adult cardiomyocytes (168; 170; 211). Using the *Drosophila* model, we effectively eliminate the potential for functional overlap or compensation by other Stim and Orai isoforms because *Drosophila* have single Stim and Orai isoforms that are most homologous to Stim1 and Orai1 (82; 155). This allows us to efficiently target the SOCE

pathway with genetic manipulation of a single pathway component and supports the conclusion that cardiac defects in Stim and Orai upregulated or suppressed animals result from dysfunction of SOCE mediated Ca^{2+} influx. However, further studies are needed to definitively demonstrate that cardiac defects in Stim and Orai suppressed or upregulated flies results from reduced or elevated Ca^{2+} influx, respectively.

Several vertebrate studies have also identified potential Orai1-independent targets of STIM1 in the heart, including TRPCs (48; 49; 112; 140; 169), L-type VOCCs (143), T-type VOCCS (133), and phospholamban (226). Of these potential STIM1 functional partners, TRPCs have been the most extensively studied in cardiac tissue. However, whether TRPC channels contribute to store operated Ca^{2+} (SOC) influx or work in parallel to Stim/Orai mediated SOCE in striated muscle function remains highly controversial (32; 40; 48). In neonatal rat ventricular cardiomyocytes, elevated SOCE due to chronic aldosterone stimulation was found to be attenuated by expression of dominant negative constructs of multiple TRPC isoforms, as well as SOCE specific channel blockers (169). Another group demonstrated knockdown of STIM1 inhibited TRPC1 upregulation and the hypertrophic response induced by pharmacological agents (140). Conversely, other groups have found STIM1 incapable of functioning with TRPCs and thus concluded TRPC verses Stim-Orai mediated Ca^{2+} entry mechanisms are distinct (40). For our research, the *Drosophila* heart provides a unique advantage to specifically evaluate Stim/Orai mediated Ca^{2+} influx, since the *Drosophila* Trp orthologs, *Trp* and *Trpl*, are exclusively expressed in the visual system and unlikely to function in the heart (57; 63; 129; 134). Although our results do not fully eliminate the possibility of other functional partners for Stim, the consistency of our results strongly suggests Stim and

Orai function together to induce the observed cardiac defects. Moreover, these findings illustrate the difficulty of studying Ca^{2+} dynamics in vertebrate hearts and highlight how the *Drosophila* model can aid in delineating the role of SOCE in physiological and pathological heart function.

SOCE Ca^{2+} Contributes to Cardiomyocyte Morphology and Function

Despite numerous studies demonstrating a role for SOCE in cardiac physiology, the specific processes regulated by SOCE remain poorly understood. SOCE is thought to primarily mediate local Ca^{2+} signaling and transcriptional networks to regulate cardiomyocyte gene expression, architecture, and survival (7; 22; 36; 163; 164). In support of this, multiple studies have shown upregulated SOCE activity contributes to pathological cardiac hypertrophy through regulation of CN-NFAT (37; 74; 107; 140; 202) and CaMKII (37; 79) mediated gene expression. Furthermore, decreased cardiac function in mice with cardiac restricted STIM1 deletion, was found to be accompanied by myofibril disorganization (144), abnormal mitochondrial structure (35), and increased expression of ER stress markers (34), suggesting homeostatic functions for SOCE.

The requirement for SOCE in cardiomyocyte SR Ca^{2+} store refilling or to augment contraction by contributing to contractile Ca^{2+} transients was originally believed unlikely, as the large Ca^{2+} influxes that occur during E-C coupling were believed to make SOCE Ca^{2+} influx unnecessary. However, newly emerging evidence suggests otherwise. Several groups have reported transgenic upregulation of STIM1 enhanced Ca^{2+} spark frequencies and generated spontaneous action potentials (21; 37; 132; 197; 202). These

findings indicate the potential for SOCE Ca^{2+} to contribute to contractile Ca^{2+} transients as well as serve homeostatic and gene regulatory functions. Maintenance of SR Ca^{2+} stores by SOCE would broadly impact cardiomyocyte physiology and may explain reported defects in both contractility and morphology. For instance, reduced SR Ca^{2+} store refilling in SOCE suppressed animals could reduce the quantal Ca^{2+} signal that drives contractility during E-C coupling resulting in decreased contractile force, and subsequently decompensation and myofibril disorganization. However, direct analyses of SR Ca^{2+} stores have yielded conflicting results. STIM1 knockdown in isolated neonatal rat ventricular cardiomyocytes was found to significantly reduce releasable SR Ca^{2+} pools (202), whereas cardiomyocyte-specific STIM1 deletion in mouse hearts resulted in dilated cardiomyopathy but SR Ca^{2+} store content was unchanged (144). It is possible that altered SR Ca^{2+} store homeostasis resulting from abnormal SOCE signaling does not directly cause heart failure. Instead, these inconsistencies may indicate that altered SOCE signaling results in small, but cumulative consequences to SR Ca^{2+} homeostasis, and that these modest changes in Ca^{2+} store content may be difficult to measure in acutely isolated cells. The power of invertebrate genetics may prove highly advantageous for this evaluation, through the use of genetically encoded Ca^{2+} indicators that allow for *in vivo* analysis of Ca^{2+} dynamics (100; 231).

The lack of rhythmic defects paired with abnormal morphology and altered cardiac dimensions in our SOCE suppressed and upregulated animals, suggests SOCE may have specific cardiomyocyte signaling functions required for regulating gene expression or maintaining tissue homeostasis. Our results show, SOCE suppression by heart specific Stim and Orai RNAi, induced myofibril disorganization and significantly

decreased myofibril density. Conversely, heart specific expression of Stim^{CA} and Orai^{CA} significantly increased *Drosophila* heart wall thickness as shown by micro-computerized tomography. One of the most well characterized signaling functions of SOCE is CN-dependent dephosphorylation of NFAT, and CN-NFAT signaling is known to be an essential regulator of genes involved in developmental and pathological cardiomyocyte growth. Multiple groups have shown, upregulated SOCE activity in response to hypertrophic stimuli is associated with increased NFAT nuclear translocation (37; 74; 107; 140; 202). However, whether CN is specifically a target of SOCE Ca²⁺ during heart development or disease pathogenesis has not been determined.

Recent studies have also found Ca²⁺/calmodulin-dependent protein kinases (CaMKs) participate in the expression of hypertrophic cardiac genes and have been shown to have upregulated activity during pathological cardiac hypertrophy (8; 69; 79; 90). CaMKs modulate transcriptional activity through phosphorylation of histone deacetylases which relieve repression of heart specific transcription factors. Moreover, elevated STIM1 expression during pathological cardiac hypertrophy has been linked to elevated CaMK activity (37; 79). However, the ability of SOCE Ca²⁺ to specifically activate CaMKs, or other NFAT-independent CN transcriptional networks, has not been assessed. Although we have yet to extensively evaluate the downstream transcriptional consequences of altered SOCE activity, the advantages of *Drosophila* genetics may prove highly beneficial for this research. In particular, the single *Drosophila* NFAT isoform is not activated by CN, which provides a unique opportunity to study NFAT-independent CN transcriptional networks, like myocyte enhanced factor 2 (Mef2). Mef2 is a well conserved transcription factor that is activated by CN dephosphorylation and known to be

essential to physiological and pathological cardiac growth (5; 23; 24; 118). Furthermore, several studies have shown that dominant-negative Mef2 expression in mice attenuates postnatal cardiomyocyte growth in response to CN induced hypertrophy (44; 85). Thus, our studies in *Drosophila* will serve as a platform for further research regarding SOCE regulated cardiomyocyte transcriptional networks.

Abnormal cardiac morphology and contractility defects in SOCE suppressed and upregulated animals was present as early as the larval stage of development, which suggests SOCE Ca^{2+} may be essential for proper heart development. Previous studies have shown SOCE activity and component expression is highest in embryonic and neonatal cardiomyocytes and decreases into adulthood (22; 35; 36; 163; 164; 202). However, the role of SOCE in normal cardiac physiology and development is poorly understood. In support of a role for SOCE in the developing heart, SOCE suppressed animals had significantly prolonged development, specifically during the larval lifecycle stage. This demonstrates SOCE mediated Ca^{2+} influx may particularly be important to larval heart development, which predominantly involves cardiomyocyte growth with minimal remodeling of the embryonic heart (138; 167; 180; 190). Both SOCE upregulated and suppressed animals died significantly earlier than controls. However, lethality of SOCE upregulated animals began early during larval development with only 70% of animals reaching the pupal stage of development and about 20% eclosed to adulthood. Arguing against a role for SOCE in cardiac development, cardiomyopathy in STIM1 knockout mouse hearts was found to be progressive and age dependent with no notable changes during early postnatal stages (35). We also did not observe changes to *Drosophila* embryogenesis upon SOCE suppression or upregulation (data not shown).

This suggests SOCE may have post-developmental, homeostatic functions and cardiac defects may manifest earlier in flies due to their faster lifecycle compared to vertebrate models. Taken together these findings demonstrate a need for further information regarding the role of SOCE in physiological cardiac function.

Innovative New Techniques to Evaluate *Drosophila* Cardiac Structure and Function

The *Drosophila* model has proven to be a powerful tool in cardiac research due to the integration of advanced genetic tools with accurate analysis of cardiac physiology. Notably, our studies expand current experimental approaches to analyzing *Drosophila* heart contractility and morphology in novel and significant ways. Our studies have optimized several new technological methods which include intravital fluorescent imaging for the *in vivo* analysis of heart function and micro-computerized tomography (microCT) for analysis of cardiac hypertrophy.

Intravital Imaging

Intravital fluorescent imaging is a relatively new technique that utilizes cardiomyocyte specific expression of the red fluorescent protein, tdTomato (CM-tdTom), (83) to evaluate heart contractility in the non-anesthetized, fully intact fly. Animals expressing CM-tdTom are attached by the dorsal abdomen to a coverslip and the heart is imaged directly through the dorsal cuticle at 200 frames per second on an inverted microscope. This approach provides remarkable resolution with clear delineation of heart walls and internal cardiac structures such as the ostia and intracardiac valves. Movement traces (M-modes) generated from captured image sequences can be used to evaluate

various aspects of heart physiology, including heart dimensions, contractile strength, rate, and rhythmicity (83). We validated the intravital imaging approach by confirming the dilated cardiomyopathy phenotype of SOCE suppressed adults was comparable to our results using the more established optical coherence tomography (OCT) method. OCT is a nondestructive, *in vivo* light imaging technique that captures three dimensional images from optical scattering media (109; 210). Of note, intravital imaging of control animals yielded substantially larger heart diameters compared to OCT. This difference may result from better spatial resolution of heart walls. Although both techniques provide high resolution image sequences, intravital imaging is significantly more sensitive, with a resolution of approximately 0.65 μ m/pixel compared to about 10 μ m/pixel with OCT (210) Furthermore, one of the main disadvantages of OCT is that it requires highly specialized equipment, unlike intravital fluorescence imaging, thereby limiting the techniques general application.

We are the first to demonstrate the use of intravital imaging for analysis of larval *Drosophila* heart contractility. Larvae are an important stage in *Drosophila* development that involves rapid animal growth and high nutritional demands (17; 19; 33; 151). Previously, analysis of larval heart contractility has proven challenging using methods commonly employed for the adults, such as imaging of partially dissected preparations and OCT. Partial dissections that faithfully preserve heart function can be difficult and laborious, as larval heart tissue is extremely delicate and predominantly free floating in the body cavity (167; 190). Evaluating larval heart contractility by OCT can be complicated by the high fat content of larval which may obscure OCT imaging and interfere with reconstruction of optical scattering media. Overall, intravital fluorescent

imaging is a powerful technique that allows for rapid, high resolution analysis of heart physiology at multiple stages of the *Drosophila* lifecycle. Moving forward, the development of software aimed to specifically analyze intravital imaging sequences will revolutionize the analysis of *Drosophila* heart function.

Micro-computerized Tomography (MicroCT)

Our results showcase the use of a powerful new imaging technique for the analysis of *Drosophila* cardiac hypertrophy, known as micro-computerized tomography (microCT). Previously, analysis of cardiac hypertrophy in *Drosophila* has been limited to histopathological preparations (97; 215; 218; 220), which involve fixing and embedding whole flies followed by mechanical sectioning and imaging of individual tissue sections to directly measure heart wall thickness. This is a highly laborious and challenging process that severely restricts throughput, reproducibility, and accuracy. Histopathological analysis is heavily reliant on proper sample mounting and preparation which limit the feasibility of processing large sample sets and complicate acquisition of reproducible measurements from the same region of the heart between samples.

Recent reports have demonstrated the use of microCT for the quantitative analysis of internal organs and structures in whole *Drosophila* larvae, pupae, or adults (53; 64; 115; 124; 125; 174; 175). MicroCT is a nondestructive, high resolution, x-ray imaging technique that relies on the attenuation of X-rays to generate image contrast in acquired radiographs encompassing the entire specimen (130; 175). Acquired radiograph images are then run through computerized algorithms to generate three-dimensional (3D) reconstructions of the whole animal, called tomograms. Notably, microCT imaging is isotropic which allows for the direct comparison of measurements made in any dimension

or orientation. MicroCT measurements can therefore be reproducibly acquired from the same region of an organ or tissue structure without reliance on imprecise physical sectioning. Furthermore, microCT sample fixation and staining are relatively quick and simple procedures allowing for high throughput sample evaluation. Despite these advantages, the use of microCT to evaluate *Drosophila* biology or in conjunction with other experimental techniques like light microscopy and molecular genetics has been largely underutilized.

We are the first to demonstrate the use of microCT imaging for the quantitative analysis of pathological changes in *Drosophila* cardiac morphology. To validate our approach, we analyzed heart wall thickness in a previously characterized model of *Drosophila* cardiac hypertrophy, *Ras85D^{V12}*. Heart specific expression of the constitutively activate Ras mutant, *Ras85D^{V12}*, was shown to induce significant increases in adult heart wall thickness by histological analysis (218). Our microCT measurements similarly found expression of *Ras85D^{V12}* in the adult heart significantly enhanced heart wall thickness compared to controls, and control heart wall values were nearly identical to previous reports (218). However, our *Ras85D^{V12}* heart wall measurements were smaller than previously reported, which may result from the increased sensitivity of microCT imaging. Mechanical sectioning for histopathological analysis yields relatively thick tissues sections of 8-10µm, which can limit the accuracy of individual measurements. Importantly, the variance of our measurements was extremely low which suggests a high degree of reproducibility using the microCT-based methodology.

We then applied the microCT approach to analyze the effects of SOCE upregulation on *Drosophila* heart morphology. Accumulating evidence suggests

increased SOCE in cardiomyocytes is necessary and sufficient for pressure overload induced cardiac hypertrophy. However, the mechanism by which SOCE regulates cardiomyocyte hypertrophy is unknown. *Drosophila* may serve as an important model to answer this question, but whether enhanced SOCE results in *Drosophila* cardiac hypertrophy had not previously been evaluated. Using microCT we show that, consistent with previous reports and similar to *Ras85D^{V12}* animals, heart specific upregulation of SOCE activity by Stim^{CA} and Orai^{CA} significantly increased heart wall thickness in *Drosophila* adult hearts. Importantly, co-expression of Orai RNAi completely restored heart wall thickness in Stim^{CA} animals to control values, which strongly suggests elevated Ca²⁺ influx via the SOCE specific, Orai channel is responsible for Stim^{CA} induced cardiac hypertrophy.

In addition to adult cardiac hypertrophy, we also demonstrate the use of microCT to evaluate larval heart wall thickness. Heart development in *Drosophila* shares many similarities with vertebrate heart development, and thus can be used for better understanding of human heart development and congenital heart defects (138; 151; 203). Additionally, a hallmark of adult-onset heart disease is reactivation of developmental gene expression (44; 69; 92; 119). Thus, insights gained from studying larval cardiomyocyte growth mechanisms may contribute to our understanding of the role of gene regulatory networks in adult-onset cardiac disease. Similar to adult hearts, morphological analysis in larvae is largely limited to histopathological preparations. Intact larval heart analysis with light microscopy has been challenging due to the light scattering effects of the larval cuticle, and partial dissections only faithfully preserve a small subset of tissue (145; 175). Our microCT analysis of larval heart wall thickness

found, similar to adults, heart specific expression of *Ras85D^{VI2}*, *Stim^{CA}*, and *Orai^{CA}*, induced modest yet highly reproducible increases in heart wall thickness compared to controls. Thus, our results collectively demonstrate that microCT imaging of *Drosophila* larva and adults is a robust method for detection and analysis of even small changes in delicate tissues, like the heart.

Concluding Remarks

Dysregulated Ca^{2+} homeostasis is known to be a central cause of cardiac disease and heart failure pathogenesis (104; 106; 196). Despite significant advances in treatment, the incidence and prevalence of heart failure continues to increase worldwide (68; 116; 194). Heart failure therefore represents a significant global health burden in terms of cost and quality of life. Due to the highly complex nature of cardiomyocyte Ca^{2+} handling, more research is needed to delineate Ca^{2+} -dependent processes and how specifically or selectively these processes mediate heart dysfunction. Better understanding of how Ca^{2+} -dependent pathways are regulated and integrated in cardiomyocyte physiology will provide the foundation for development of novel treatment and prevention strategies.

Collectively, our findings demonstrate a highly conserved requirement for the SOCE pathway in cardiac function. We identify SOCE as an important upstream source of Ca^{2+} in cardiomyocytes that regulates processes involved in cardiac remodeling and contractile function. Our results are largely consistent with previous reports and demonstrate the potential for SOCE Ca^{2+} to serve as a pivotal regulator of cardiomyopathy pathogenesis, where pathway suppression induces dilated

cardiomyopathy and upregulation results in cardiac hypertrophy. Importantly, we establish the *Drosophila* model as ideal experimental platform to evaluate the mechanism by which SOCE regulates cardiomyocyte function and determine how SOCE Ca^{2+} dependent pathways are integrated with global cardiomyocyte Ca^{2+} dynamics. The combination of renowned *Drosophila* genetic tools with powerful experimental approaches, like intravital fluorescence imaging and microCT, will allow us to efficiently direct translational research to more complex models. Deciphering the function of SOCE in cardiomyocyte physiology will greatly advance our understanding of cardiac Ca^{2+} dynamics and may prove vital for novel therapeutic development against heart failure.

REFERENCES

1. Abraham DM, Wolf MJ. 2013. Disruption of sarcoendoplasmic reticulum calcium ATPase function in *Drosophila* leads to cardiac dysfunction. *PloS one* 8:e77785
2. Ai X, Curran JW, Shannon TR, Bers DM, Pogwizd SM. 2005. Ca²⁺/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca²⁺ leak in heart failure. *Circulation research* 97:1314-22
3. Alayari NN, Vogler G, Taghli-Lamalle O, Ocorr K, Bodmer R, Cammarato A. 2009. Fluorescent labeling of *Drosophila* heart structures. *Journal of visualized experiments : JoVE*
4. Albarran L, Lopez JJ, Salido GM, Rosado JA. 2016. Historical Overview of Store-Operated Ca(2+) Entry. *Advances in experimental medicine and biology* 898:3-24
5. Altarejos JY, Montminy M. 2011. CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. *Nature reviews. Molecular cell biology* 12:141-51
6. Ashpole NM, Herren AW, Ginsburg KS, Brogan JD, Johnson DE, et al. 2012. Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) regulates cardiac sodium channel NaV1.5 gating by multiple phosphorylation sites. *The Journal of biological chemistry* 287:19856-69
7. Avila-Medina J, Mayoral-Gonzalez I, Dominguez-Rodriguez A, Gallardo-Castillo I, Ribas J, et al. 2018. The Complex Role of Store Operated Calcium Entry Pathways and Related Proteins in the Function of Cardiac, Skeletal and Vascular Smooth Muscle Cells. *Frontiers in physiology* 9:257
8. Backs J, Song K, Bezprozvannaya S, Chang S, Olson EN. 2006. CaM kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. *The Journal of clinical investigation* 116:1853-64
9. Bartoli F, Sabourin J. 2017. Cardiac Remodeling and Disease: Current Understanding of STIM1/Orai1-Mediated Store-Operated Ca(2+) Entry in Cardiac Function and Pathology. *Advances in experimental medicine and biology* 993:523-34
10. Benard L, Oh JG, Cacheux M, Lee A, Nonnenmacher M, et al. 2016. Cardiac Stim1 Silencing Impairs Adaptive Hypertrophy and Promotes Heart Failure Through Inactivation of mTORC2/Akt Signaling. *Circulation* 133:1458-71; discussion 71
11. Berna-Erro A, Woodard GE, Rosado JA. 2012. Orais and STIMs: physiological mechanisms and disease. *Journal of cellular and molecular medicine* 16:407-24
12. Bernardo BC, Weeks KL, Pretorius L, McMullen JR. 2010. Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. *Pharmacology & therapeutics* 128:191-227
13. Bers DM. 2002. Cardiac excitation-contraction coupling. *Nature* 415:198-205
14. Bers DM. 2002. Cardiac excitation-contraction coupling. *Nature* 415:198-205
15. Bers DM. 2008. Calcium cycling and signaling in cardiac myocytes. *Annual review of physiology* 70:23-49
16. Bers DM, Eisner DA, Valdivia HH. 2003. Sarcoplasmic reticulum Ca²⁺ and heart failure: roles of diastolic leak and Ca²⁺ transport. *Circulation research* 93:487-90
17. Bier E, Bodmer R. 2004. *Drosophila*, an emerging model for cardiac disease. *Gene* 342:1-11
18. Bird GS, Hwang SY, Smyth JT, Fukushima M, Boyles RR, Putney JW, Jr. 2009. STIM1 is a calcium sensor specialized for digital signaling. *Current biology : CB* 19:1724-9
19. Bodmer R. 1995. Heart development in *Drosophila* and its relationship to vertebrates. *Trends in cardiovascular medicine* 5:21-8

38. Cosens DJ, Manning A. 1969. Abnormal Electroretinogram from a *Drosophila* Mutant. *Nature* 224:285-7
39. De Windt LJ, Lim HW, Bueno OF, Liang Q, Delling U, et al. 2001. Targeted inhibition of calcineurin attenuates cardiac hypertrophy in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 98:3322-7
40. DeHaven WI, Jones BF, Petranka JG, Smyth JT, Tomita T, et al. 2009. TRPC channels function independently of STIM1 and Orai1. *J Physiol* 587:2275-98
41. Deo M, Weinberg SH, Boyle PM. 2017. Calcium Dynamics and Cardiac Arrhythmia. *Clinical Medicine Insights. Cardiology* 11:1179546817739523
42. Derler I, Schindl R, Fritsch R, Heftberger P, Riedl MC, et al. 2013. The action of selective CRAC channel blockers is affected by the Orai pore geometry. *Cell calcium* 53:139-51
43. Dewenter M, von der Lieth A, Katus HA, Backs J. 2017. Calcium Signaling and Transcriptional Regulation in Cardiomyocytes. *Circulation research* 121:1000-20
44. Dirx E, da Costa Martins PA, De Windt LJ. 2013. Regulation of fetal gene expression in heart failure. *Biochimica et biophysica acta* 1832:2414-24
45. Dolmetsch R. 2003. Excitation-Transcription Coupling: Signaling by Ion Channels to the Nucleus. *Science's STKE* 2003:pe4
46. Dorn GW, 2nd. 2007. The fuzzy logic of physiological cardiac hypertrophy. *Hypertension (Dallas, Tex. : 1979)* 49:962-70
47. Dulcis D, Levine RB. 2003. Innervation of the heart of the adult fruit fly, *Drosophila melanogaster*. *The Journal of comparative neurology* 465:560-78
48. Eder P. 2017. Cardiac Remodeling and Disease: SOCE and TRPC Signaling in Cardiac Pathology. *Advances in experimental medicine and biology* 993:505-21
49. Eder P, Molkentin JD. 2011. TRPC channels as effectors of cardiac hypertrophy. *Circulation research* 108:265-72
50. Eisner DA, Caldwell JL, Kistamas K, Trafford AW. 2017. Calcium and Excitation-Contraction Coupling in the Heart. *Circulation research* 121:181-95
51. Eisner DA, Caldwell JL, Trafford AW, Hutchings DC. 2020. The Control of Diastolic Calcium in the Heart: Basic Mechanisms and Functional Implications. *Circulation research* 126:395-412
52. Erickson JR. 2014. Mechanisms of CaMKII Activation in the Heart. *Frontiers in pharmacology* 5:59
53. Fabian B, Schneeberg K, Beutel RG. 2016. Comparative thoracic anatomy of the wild type and wingless (*wg(1)cn(1)*) mutant of *Drosophila melanogaster* (Diptera). *Arthropod structure & development* 45:611-36
54. Fahrner M, Schindl R, Muik M, Derler I, Romanin C. 2017. The STIM-Orai Pathway: The Interactions Between STIM and Orai. *Advances in experimental medicine and biology* 993:59-81
55. Fearnley CJ, Roderick HL, Bootman MD. 2011. Calcium signaling in cardiac myocytes. *Cold Spring Harbor perspectives in biology* 3:a004242
56. Fink M, Callol-Massot C, Chu A, Ruiz-Lozano P, Izpisua Belmonte JC, et al. 2009. A new method for detection and quantification of heartbeat parameters in *Drosophila*, zebrafish, and embryonic mouse hearts. *BioTechniques* 46:101-13
57. Fowler MA, Montell C. 2013. *Drosophila* TRP channels and animal behavior. *Life sciences* 92:394-403
58. Gambardella J, Trimarco B, Iaccarino G, Santulli G. 2018. New Insights in Cardiac Calcium Handling and Excitation-Contraction Coupling. *Advances in experimental medicine and biology* 1067:373-85
59. Garfinkel AC, Seidman JG, Seidman CE. 2018. Genetic Pathogenesis of Hypertrophic and Dilated Cardiomyopathy. *Heart failure clinics* 14:139-46

60. Griffiths EJ, Balaska D, Cheng WH. 2010. The ups and downs of mitochondrial calcium signalling in the heart. *Biochimica et biophysica acta* 1797:856-64
61. Gwack Y, Feske S, Srikanth S, Hogan PG, Rao A. 2007. Signalling to transcription: store-operated Ca^{2+} entry and NFAT activation in lymphocytes. *Cell calcium* 42:145-56
62. Gyorke S, Terentyev D. 2008. Modulation of ryanodine receptor by luminal calcium and accessory proteins in health and cardiac disease. *Cardiovascular research* 77:245-55
63. Hardie RC, Minke B. 1992. The trp gene is essential for a light-activated Ca^{2+} channel in *Drosophila* photoreceptors. *Neuron* 8:643-51
64. Harrison JF, Waters JS, Biddulph TA, Kovacevic A, Klok CJ, Socha JJ. 2018. Developmental plasticity and stability in the tracheal networks supplying *Drosophila* flight muscle in response to rearing oxygen level. *Journal of insect physiology* 106:189-98
65. Harvey PA, Leinwand LA. 2011. The cell biology of disease: cellular mechanisms of cardiomyopathy. *The Journal of cell biology* 194:355-65
66. Hassock SR, Zhu MX, Trost C, Flockerzi V, Authi KS. 2002. Expression and role of TRPC proteins in human platelets: evidence that TRPC6 forms the store-independent calcium entry channel. *Blood* 100:2801-11
67. Hayward C, Banner NR, Morley-Smith A, Lyon AR, Harding SE. 2015. The Current and Future Landscape of SERCA Gene Therapy for Heart Failure: A Clinical Perspective. *Human gene therapy* 26:293-304
68. Heidenreich PA, Albert NM, Allen LA, Bluemke DA, Butler J, et al. 2013. Forecasting the impact of heart failure in the United States: a policy statement from the American Heart Association. *Circulation. Heart failure* 6:606-19
69. Heineke J, Molkentin JD. 2006. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nature reviews. Molecular cell biology* 7:589-600
70. Hobai IA, O'Rourke B. 2001. Decreased sarcoplasmic reticulum calcium content is responsible for defective excitation-contraction coupling in canine heart failure. *Circulation* 103:1577-84
71. Horton JS, Buckley CL, Alvarez EM, Schorlemmer A, Stokes AJ. 2014. The calcium release-activated calcium channel Orai1 represents a crucial component in hypertrophic compensation and the development of dilated cardiomyopathy. *Channels (Austin, Tex.)* 8:35-48
72. Hoth M, Penner R. 1992. Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* 355:353-6
73. Houser SR, Margulies KB. 2003. Is depressed myocyte contractility centrally involved in heart failure? *Circulation research* 92:350-8
74. Hulot JS, Fauconnier J, Ramanujam D, Chanaan A, Aubart F, et al. 2011. Critical role for stromal interaction molecule 1 in cardiac hypertrophy. *Circulation* 124:796-805
75. Hunton DL, Lucchesi PA, Pang Y, Cheng X, Dell'Italia LJ, Marchase RB. 2002. Capacitative calcium entry contributes to nuclear factor of activated T-cells nuclear translocation and hypertrophy in cardiomyocytes. *The Journal of biological chemistry* 277:14266-73
76. Hunton DL, Zou L, Pang Y, Marchase RB. 2004. Adult rat cardiomyocytes exhibit capacitative calcium entry. *American journal of physiology. Heart and circulatory physiology* 286:H1124-32
77. Jackson G, Gibbs CR, Davies MK, Lip GY. 2000. ABC of heart failure. Pathophysiology. *BMJ* 320:167-70
78. Jaski BE, Jessup ML, Mancini DM, Cappola TP, Pauly DF, et al. 2009. Calcium upregulation by percutaneous administration of gene therapy in cardiac disease (CUPID Trial), a first-in-human phase 1/2 clinical trial. *Journal of cardiac failure* 15:171-81

79. Ji Y, Guo X, Zhang Z, Huang Z, Zhu J, et al. 2017. CaMKII δ mediates phenylephrine induced cardiomyocyte hypertrophy through store-operated Ca(2+) entry. *Cardiovascular pathology : the official journal of the Society for Cardiovascular Pathology* 27:9-17
80. Jin H, Stojnic R, Adryan B, Ozdemir A, Stathopoulos A, Frasch M. 2013. Genome-wide screens for in vivo Tinman binding sites identify cardiac enhancers with diverse functional architectures. *PLoS genetics* 9:e1003195
81. Johnson E, Sherry T, Ringo J, Dowse H. 2002. Modulation of the cardiac pacemaker of *Drosophila*: cellular mechanisms. *Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology* 172:227-36
82. Kar P, Parekh A. 2013. STIM proteins, Orai1 and gene expression. *Channels (Austin, Tex.)* 7:374-8
83. Klassen MP, Peters CJ, Zhou S, Williams HH, Jan LY, Jan YN. 2017. Age-dependent diastolic heart failure in an in vivo *Drosophila* model. *eLife* 6
84. Kohli S, Ahuja S, Rani V. 2011. Transcription factors in heart: promising therapeutic targets in cardiac hypertrophy. *Current cardiology reviews* 7:262-71
85. Kolodziejczyk SM, Wang L, Balazsi K, DeRepentigny Y, Kothary R, Megeney LA. 1999. MEF2 is upregulated during cardiac hypertrophy and is required for normal post-natal growth of the myocardium. *Current biology : CB* 9:1203-6
86. Kolsch V, Paululat A. 2002. The highly conserved cardiogenic bHLH factor Hand is specifically expressed in circular visceral muscle progenitor cells and in all cell types of the dorsal vessel during *Drosophila* embryogenesis. *Development genes and evolution* 212:473-85
87. Korzeniowski MK, Popovic MA, Szentpetery Z, Varnai P, Stojilkovic SS, Balla T. 2009. Dependence of STIM1/Orai1-mediated calcium entry on plasma membrane phosphoinositides. *The Journal of biological chemistry* 284:21027-35
88. Kranias E, Bers D. 2007. *Calcium and Cardiomyopathies*. 523-37 pp.
89. Kranias EG, Bers DM. 2007. Calcium and cardiomyopathies. *Sub-cellular biochemistry* 45:523-37
90. Kreusser MM, Backs J. 2014. Integrated mechanisms of CaMKII-dependent ventricular remodeling. *Frontiers in pharmacology* 5:36
91. Kurebayashi N, Ogawa Y. 2001. Depletion of Ca²⁺ in the sarcoplasmic reticulum stimulates Ca²⁺ entry into mouse skeletal muscle fibres. *J Physiol* 533:185-99
92. Kuwahara K, Nishikimi T, Nakao K. 2012. Transcriptional regulation of the fetal cardiac gene program. *Journal of pharmacological sciences* 119:198-203
93. Kwong JQ, Lu X, Correll RN, Schwanekamp JA, Vagnozzi RJ, et al. 2015. The Mitochondrial Calcium Uniporter Selectively Matches Metabolic Output to Acute Contractile Stress in the Heart. *Cell reports* 12:15-22
94. Lacruz RS, Feske S. 2015. Diseases caused by mutations in ORAI1 and STIM1. *Annals of the New York Academy of Sciences* 1356:45-79
95. Lammers K, Abeln B, Husken M, Lehmacher C, Psathaki OE, et al. 2017. Formation and function of intracardiac valve cells in the *Drosophila* heart. *The Journal of experimental biology* 220:1852-63
96. Lee S, Bao H, Ishikawa Z, Wang W, Lim HY. 2017. Cardiomyocyte Regulation of Systemic Lipid Metabolism by the Apolipoprotein B-Containing Lipoproteins in *Drosophila*. *PLoS genetics* 13:e1006555
97. Lee TE, Yu L, Wolf MJ, Rockman HA. 2014. Galactokinase is a novel modifier of calcineurin-induced cardiomyopathy in *Drosophila*. *Genetics* 198:591-603
98. Limas CJ, Olivari MT, Goldenberg IF, Levine TB, Benditt DG, Simon A. 1987. Calcium uptake by cardiac sarcoplasmic reticulum in human dilated cardiomyopathy. *Cardiovascular research* 21:601-5

99. Limpitikul WB, Viswanathan MC, O'Rourke B, Yue DT, Cammarato A. 2018. Conservation of cardiac L-type Ca^{2+} channels and their regulation in *Drosophila*: A novel genetically-pliable channelopathic model. *Journal of molecular and cellular cardiology* 119:64-74
100. Lin N, Badie N, Yu L, Abraham D, Cheng H, et al. 2011. A method to measure myocardial calcium handling in adult *Drosophila*. *Circulation research* 108:1306-15
101. Lin N, Badie N, Yu L, Abraham D, Cheng H, et al. 2011. A method to measure myocardial calcium handling in adult *Drosophila*. *Circulation research* 108:1306-15
102. Liou J, Fivaz M, Inoue T, Meyer T. 2007. Live-cell imaging reveals sequential oligomerization and local plasma membrane targeting of stromal interaction molecule 1 after Ca^{2+} store depletion. *Proceedings of the National Academy of Sciences of the United States of America* 104:9301-6
103. Ljubojevic S, Bers DM. 2015. Nuclear calcium in cardiac myocytes. *Journal of cardiovascular pharmacology* 65:211-7
104. Lou Q, Janardhan A, Efimov IR. 2012. Remodeling of calcium handling in human heart failure. *Advances in experimental medicine and biology* 740:1145-74
105. Louch WE, Bito V, Heinzel FR, Macianskiene R, Vanhaecke J, et al. 2004. Reduced synchrony of Ca^{2+} release with loss of T-tubules-a comparison to Ca^{2+} release in human failing cardiomyocytes. *Cardiovascular research* 62:63-73
106. Luo M, Anderson ME. 2013. Mechanisms of altered Ca^{2+} handling in heart failure. *Circulation research* 113:690-708
107. Luo X, Hojaye B, Jiang N, Wang ZV, Tandan S, et al. 2012. STIM1-dependent store-operated Ca^{2+} entry is required for pathological cardiac hypertrophy. *Journal of molecular and cellular cardiology* 52:136-47
108. Lyon AR, Babalis D, Morley-Smith AC, Hedger M, Suarez Barrientos A, et al. 2020. Investigation of the safety and feasibility of AAV1/SERCA2a gene transfer in patients with chronic heart failure supported with a left ventricular assist device - the SERCA-LVAD TRIAL. *Gene therapy* 27:579-90
109. Ma L, Bradu A, Podoleanu AG, Bloor JW. 2010. Arrhythmia caused by a *Drosophila* tropomyosin mutation is revealed using a novel optical coherence tomography instrument. *PloS one* 5:e14348
110. MacLeod KT. 2016. Recent advances in understanding cardiac contractility in health and disease. *F1000Research* 5
111. Maillet M, van Berlo JH, Molkentin JD. 2013. Molecular basis of physiological heart growth: fundamental concepts and new players. *Nature reviews. Molecular cell biology* 14:38-48
112. Makarewich CA, Zhang H, Davis J, Correll RN, Trappanese DM, et al. 2014. Transient receptor potential channels contribute to pathological structural and functional remodeling after myocardial infarction. *Circulation research* 115:567-80
113. Marks AR. 2003. Calcium and the heart: a question of life and death. *The Journal of clinical investigation* 111:597-600
114. Marx SO, Reiken S, Hisamatsu Y, Jayaraman T, Burkhoff D, et al. 2000. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* 101:365-76
115. Mattei AL, Riccio ML, Avila FW, Wolfner MF. 2015. Integrated 3D view of postmating responses by the *Drosophila melanogaster* female reproductive tract, obtained by micro-computed tomography scanning. *Proceedings of the National Academy of Sciences of the United States of America* 112:8475-80
116. Mazurek JA, Jessup M. 2017. Understanding Heart Failure. *Heart failure clinics* 13:1-19
117. McKenna WJ, Maron BJ, Thiene G. 2017. Classification, Epidemiology, and Global Burden of Cardiomyopathies. *Circulation research* 121:722-30

118. McKinsey TA, Zhang CL, Olson EN. 2002. MEF2: a calcium-dependent regulator of cell division, differentiation and death. *Trends in biochemical sciences* 27:40-7
119. McMullen JR, Jennings GL. 2007. Differences between pathological and physiological cardiac hypertrophy: novel therapeutic strategies to treat heart failure. *Clinical and experimental pharmacology & physiology* 34:255-62
120. Mercer JC, Dehaven WI, Smyth JT, Wedel B, Boyles RR, et al. 2006. Large store-operated calcium selective currents due to co-expression of Orai1 or Orai2 with the intracellular calcium sensor, Stim1. *The Journal of biological chemistry* 281:24979-90
121. Michelucci A, García-Castañeda M, Boncompagni S, Dirksen RT. 2018. Role of STIM1/ORAI1-mediated store-operated Ca(2+) entry in skeletal muscle physiology and disease. *Cell calcium* 76:101-15
122. Mignen O, Thompson JL, Shuttleworth TJ. 2008. Orai1 subunit stoichiometry of the mammalian CRAC channel pore. *J Physiol* 586:419-25
123. Minamisawa S, Hoshijima M, Chu G, Ward CA, Frank K, et al. 1999. Chronic phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. *Cell* 99:313-22
124. Mizutani R, Saiga R, Takeuchi A, Uesugi K, Suzuki Y. 2013. Three-dimensional network of Drosophila brain hemisphere. *Journal of structural biology* 184:271-9
125. Mizutani R, Takeuchi A, Akamatsu G, Uesugi K, Suzuki Y. 2008. Element-specific microtomographic imaging of Drosophila brain stained with high-Z probes. *Journal of synchrotron radiation* 15:374-7
126. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, et al. 1998. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* 93:215-28
127. Mollova MY, Katus HA, Backs J. 2015. Regulation of CaMKII signaling in cardiovascular disease. *Frontiers in pharmacology* 6:178
128. Monier B, Astier M, Sémériva M, Perrin L. 2005. Steroid-dependent modification of Hox function drives myocyte reprogramming in the *Drosophila* heart. *Development (Cambridge, England)* 132:5283
129. Montell C, Rubin GM. 1989. Molecular characterization of the Drosophila trp locus: a putative integral membrane protein required for phototransduction. *Neuron* 2:1313-23
130. Morton EJ, Webb S, Bateman JE, Clarke LJ, Shelton CG. 1990. Three-dimensional x-ray microtomography for medical and biological applications. *Physics in medicine and biology* 35:805-20
131. Münch G, Bölc B, Karczewski P, Schwinger RH. 2002. Evidence for calcineurin-mediated regulation of SERCA 2a activity in human myocardium. *Journal of molecular and cellular cardiology* 34:321-34
132. Neely GG, Kuba K, Cammarato A, Isobe K, Amann S, et al. 2010. A global in vivo Drosophila RNAi screen identifies NOT3 as a conserved regulator of heart function. *Cell* 141:142-53
133. Nguyen N, Biet M, Simard É, Béliveau É, Francoeur N, et al. 2013. STIM1 participates in the contractile rhythmicity of HL-1 cells by moderating T-type Ca²⁺ channel activity. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1833:1294-303
134. Niemeyer BA, Suzuki E, Scott K, Jalink K, Zuker CS. 1996. The Drosophila light-activated conductance is composed of the two channels TRP and TRPL. *Cell* 85:651-9
135. Nwokonko RM, Cai X, Loktionova NA, Wang Y, Zhou Y, Gill DL. 2017. The STIM-Orai Pathway: Conformational Coupling Between STIM and Orai in the Activation of Store-Operated Ca(2+) Entry. *Advances in experimental medicine and biology* 993:83-98
136. Ocorr K, Perrin L, Lim HY, Qian L, Wu X, Bodmer R. 2007. Genetic control of heart function and aging in Drosophila. *Trends in cardiovascular medicine* 17:177-82
137. Ocorr K, Reeves NL, Wessells RJ, Fink M, Chen HSV, et al. 2007. KCNQ potassium channel mutations cause cardiac arrhythmias in Drosophila that mimic the effects of

- aging. *Proceedings of the National Academy of Sciences of the United States of America* 104:3943-8
138. Ocorr K, Vogler G, Bodmer R. 2014. Methods to assess *Drosophila* heart development, function and aging. *Methods (San Diego, Calif.)* 68:265-72
139. Ohba T, Watanabe H, Murakami M, Iino K, Adachi T, et al. 2017. Stromal interaction molecule 1 haploinsufficiency causes maladaptive response to pressure overload. *PloS one* 12:e0187950
140. Ohba T, Watanabe H, Murakami M, Sato T, Ono K, Ito H. 2009. Essential role of STIM1 in the development of cardiomyocyte hypertrophy. *Biochemical and biophysical research communications* 389:172-6
141. Parekh AB, Putney JW, Jr. 2005. Store-operated calcium channels. *Physiological reviews* 85:757-810
142. Park CY, Hoover PJ, Mullins FM, Bachhawat P, Covington ED, et al. 2009. STIM1 clusters and activates CRAC channels via direct binding of a cytosolic domain to Orai1. *Cell* 136:876-90
143. Park CY, Shcheglovitov A, Dolmetsch R. 2010. The CRAC channel activator STIM1 binds and inhibits L-type voltage-gated calcium channels. *Science (New York, N.Y.)* 330:101-5
144. Parks C, Alam MA, Sullivan R, Mancarella S. 2016. STIM1-dependent Ca(2+) microdomains are required for myofilament remodeling and signaling in the heart. *Scientific reports* 6:25372
145. Parton RM, Vallés AM, Dobbie IM, Davis I. 2010. *Drosophila* larval fillet preparation and imaging of neurons. *Cold Spring Harbor protocols* 2010:pdb.prot5405
146. Parvez S, Beck A, Peinelt C, Soboloff J, Lis A, et al. 2008. STIM2 protein mediates distinct store-dependent and store-independent modes of CRAC channel activation. *FASEB J* 22:752-61
147. Pathak T, Trivedi D, Hasan G. 2017. CRISPR-Cas-Induced Mutants Identify a Requirement for dSTIM in Larval Dopaminergic Cells of *Drosophila melanogaster*. *G3 (Bethesda, Md.)* 7:923-33
148. Penna A, Demuro A, Yeromin AV, Zhang SL, Safrina O, et al. 2008. The CRAC channel consists of a tetramer formed by Stim-induced dimerization of Orai dimers. *Nature* 456:116-20
149. Penny WF, Hammond HK. 2017. Randomized Clinical Trials of Gene Transfer for Heart Failure with Reduced Ejection Fraction. *Human gene therapy* 28:378-84
150. Petersen CE, Wolf MJ, Smyth JT. 2020. Suppression of store-operated calcium entry causes dilated cardiomyopathy of the *Drosophila* heart. *Biol Open* 9
151. Piazza N, Wessells RJ. 2011. *Drosophila* models of cardiac disease. *Progress in molecular biology and translational science* 100:155-210
152. Pogwizd SM, Bers DM. 2004. Cellular Basis of Triggered Arrhythmias in Heart Failure. *Trends in cardiovascular medicine* 14:61-6
153. Porter GA, Jr., Makuck RF, Rivkees SA. 2003. Intracellular calcium plays an essential role in cardiac development. *Developmental dynamics : an official publication of the American Association of Anatomists* 227:280-90
154. Potthoff MJ, Olson EN. 2007. MEF2: a central regulator of diverse developmental programs. *Development (Cambridge, England)* 134:4131
155. Prakriya M, Lewis RS. 2015. Store-Operated Calcium Channels. *Physiological reviews* 95:1383-436
156. Putney JW. 2011. Origins of the concept of store-operated calcium entry. *Front Biosci (Schol Ed)* 3:980-4
157. Putney JW. 2011. The physiological function of store-operated calcium entry. *Neurochemical research* 36:1157-65

20. Bollimuntha S, Pani B, Singh BB. 2017. Neurological and Motor Disorders: Neuronal Store-Operated Ca(2+) Signaling: An Overview and Its Function. *Advances in experimental medicine and biology* 993:535-56
21. Bonilla IM, Belevych AE, Baine S, Stepanov A, Mezache L, et al. 2019. Enhancement of Cardiac Store Operated Calcium Entry (SOCE) within Novel Intercalated Disk Microdomains in Arrhythmic Disease. *Scientific reports* 9:10179
22. Bootman MD, Rietdorf K. 2017. Tissue Specificity: Store-Operated Ca(2+) Entry in Cardiac Myocytes. *Advances in experimental medicine and biology* 993:363-87
23. Borghi S, Molinari S, Razzini G, Parise F, Battini R, Ferrari S. 2001. The nuclear localization domain of the MEF2 family of transcription factors shows member-specific features and mediates the nuclear import of histone deacetylase 4. *Journal of cell science* 114:4477-83
24. Bour BA, O'Brien MA, Lockwood WL, Goldstein ES, Bodmer R, et al. 1995. Drosophila MEF2, a transcription factor that is essential for myogenesis. *Genes & development* 9:730-41
25. Bradu A, Ma L, Bloor JW, Podoleanu A. 2009. Dual optical coherence tomography/fluorescence microscopy for monitoring of Drosophila melanogaster larval heart. *Journal of biophotonics* 2:380-8
26. Brandman O, Liou J, Park WS, Meyer T. 2007. STIM2 is a feedback regulator that stabilizes basal cytosolic and endoplasmic reticulum Ca²⁺ levels. *Cell* 131:1327-39
27. Braun AP. 2013. CRACKing the structure of Orai: new work reveals the intramolecular properties of a store-operated, calcium influx channel. *Channels (Austin, Tex.)* 7:71-3
28. Bueno OF, Wilkins BJ, Tymitz KM, Glascock BJ, Kimball TF, et al. 2002. Impaired cardiac hypertrophic response in Calcineurin Abeta -deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* 99:4586-91
29. Cai X, Zhou Y, Nwokonko RM, Loktionova NA, Wang X, et al. 2016. The Orai1 Store-operated Calcium Channel Functions as a Hexamer. *The Journal of biological chemistry* 291:25764-75
30. Cannon L, Zambon AC, Cammarato A, Zhang Z, Vogler G, et al. 2017. Expression patterns of cardiac aging in Drosophila. *Aging cell* 16:82-92
31. Carrasco S, Meyer T. 2011. STIM proteins and the endoplasmic reticulum-plasma membrane junctions. *Annual review of biochemistry* 80:973-1000
32. Chen X, Sooch G, Demaree IS, White FA, Obukhov AG. 2020. Transient Receptor Potential Canonical (TRPC) Channels: Then and Now. *Cells* 9
33. Choma MA, Suter MJ, Vakoc BJ, Bouma BE, Tearney GJ. 2011. Physiological homology between Drosophila melanogaster and vertebrate cardiovascular systems. *Disease models & mechanisms* 4:411-20
34. Collins HE, He L, Zou L, Qu J, Zhou L, et al. 2014. Stromal interaction molecule 1 is essential for normal cardiac homeostasis through modulation of ER and mitochondrial function. *American journal of physiology. Heart and circulatory physiology* 306:H1231-H9
35. Collins HE, He L, Zou L, Qu J, Zhou L, et al. 2014. Stromal interaction molecule 1 is essential for normal cardiac homeostasis through modulation of ER and mitochondrial function. *American journal of physiology. Heart and circulatory physiology* 306:H1231-9
36. Collins HE, Zhu-Mauldin X, Marchase RB, Chatham JC. 2013. STIM1/Orai1-mediated SOCE: current perspectives and potential roles in cardiac function and pathology. *American journal of physiology. Heart and circulatory physiology* 305:H446-58
37. Correll RN, Goonasekera SA, van Berlo JH, Burr AR, Accornero F, et al. 2015. STIM1 elevation in the heart results in aberrant Ca(2+)(+) handling and cardiomyopathy. *Journal of molecular and cellular cardiology* 87:38-47

158. Putney JW. 2017. Store-Operated Calcium Entry: An Historical Overview. *Advances in experimental medicine and biology* 981:205-14
159. Putney JW. 2018. Forms and functions of store-operated calcium entry mediators, STIM and Orai. *Adv Biol Regul* 68:88-96
160. Putney JW, Jr. 2007. Recent breakthroughs in the molecular mechanism of capacitative calcium entry (with thoughts on how we got here). *Cell calcium* 42:103-10
161. Putney JW, Steinckwich-Besançon N, Numaga-Tomita T, Davis FM, Desai PN, et al. 2017. The functions of store-operated calcium channels. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1864:900-6
162. Qian L, Wythe JD, Liu J, Cartry J, Vogler G, et al. 2011. Tinman/Nkx2-5 acts via miR-1 and upstream of Cdc42 to regulate heart function across species. *The Journal of cell biology* 193:1181-96
163. Rosenberg P. 2011. Socking It to cardiac hypertrophy: STIM1-mediated Ca²⁺ entry in the cardiomyocyte. *Circulation* 124:766-8
164. Rosenberg P, Katz D, Bryson V. 2019. SOCE and STIM1 signaling in the heart: Timing and location matter. *Cell calcium* 77:20-8
165. Rossi A, Pizzo P, Filadi R. 2019. Calcium, mitochondria and cell metabolism: A functional triangle in bioenergetics. *Biochimica et biophysica acta. Molecular cell research* 1866:1068-78
166. Rotstein B, Paululat A. 2016. On the Morphology of the Drosophila Heart. *Journal of Cardiovascular Development and Disease* 3:15
167. Rotstein B, Paululat A. 2016. On the Morphology of the Drosophila Heart. *Journal of cardiovascular development and disease* 3
168. Ruhle B, Trebak M. 2013. Emerging roles for native Orai Ca²⁺ channels in cardiovascular disease. *Current topics in membranes* 71:209-35
169. Sabourin J, Bartoli F, Antigny F, Gomez AM, Benitah JP. 2016. Transient Receptor Potential Canonical (TRPC)/Orai1-dependent Store-operated Ca²⁺ Channels: NEW TARGETS OF ALDOSTERONE IN CARDIOMYOCYTES. *The Journal of biological chemistry* 291:13394-409
170. Saliba Y, Keck M, Marchand A, Atassi F, Ouillé A, et al. 2014. Emergence of Orai3 activity during cardiac hypertrophy. *Cardiovascular research* 105:248-59
171. Santalla M, Valverde CA, Harnichar E, Lacunza E, Aguilar-Fuentes J, et al. 2014. Aging and CaMKII alter intracellular Ca²⁺ transients and heart rhythm in Drosophila melanogaster. *PloS one* 9:e101871
172. Santulli G, Lewis DR, Marks AR. 2017. Physiology and pathophysiology of excitation-contraction coupling: the functional role of ryanodine receptor. *Journal of muscle research and cell motility* 38:37-45
173. Schmidt U, Hajjar RJ, Kim CS, Lebeche D, Doye AA, Gwathmey JK. 1999. Human heart failure: cAMP stimulation of SR Ca(2+)-ATPase activity and phosphorylation level of phospholamban. *The American journal of physiology* 277:H474-80
174. Schoborg TA. 2020. Whole Animal Imaging of Drosophila melanogaster using Microcomputed Tomography. *Journal of visualized experiments : JoVE*
175. Schoborg TA, Smith SL, Smith LN, Morris HD, Rusan NM. 2019. Micro-computed tomography as a platform for exploring Drosophila development. *Development (Cambridge, England)* 146
176. Schulz RA, Yutzey KE. 2004. Calcineurin signaling and NFAT activation in cardiovascular and skeletal muscle development. *Developmental biology* 266:1-16
177. Segin S, Berlin M, Richter C, Flockerzi RMV, Worley P, et al. 2020. Cardiomyocyte-Specific Deletion of Orai1 Reveals Its Protective Role in Angiotensin-II-Induced Pathological Cardiac Remodeling. *Cells* 9

178. Seidler T, Hasenfuss G, Maier LS. 2007. Targeting altered calcium physiology in the heart: translational approaches to excitation, contraction, and transcription. *Physiology (Bethesda, Md.)* 22:328-34
179. Selvaraj S, Singh B, Bollensdorff C, Suwaidi JA, Yacoub M. 2015. A CRITICAL ROLE OF TRPC1-ORAI1-STIM1 MEDIATED STORE OPERATED CALCIUM ENTRY IN CARDIAC HYPERTROPHY. *Journal of the American College of Cardiology* 65:A902
180. Shahrestani P, Burke MK, Birse R, Kezos JN, Ocorr K, et al. 2017. Experimental Evolution and Heart Function in *Drosophila*. *Physiol Biochem Zool* 90:281-93
181. Shattock MJ, Ottolia M, Bers DM, Blaustein MP, Boguslavskyi A, et al. 2015. Na⁺/Ca²⁺ exchange and Na⁺/K⁺-ATPase in the heart. *J Physiol* 593:1361-82
182. Smyth JT, Hwang SY, Tomita T, DeHaven WI, Mercer JC, Putney JW. 2010. Activation and regulation of store-operated calcium entry. *Journal of cellular and molecular medicine* 14:2337-49
183. Sobie EA, Cannell MB, Bridge JH. 2008. Allosteric activation of Na⁺-Ca²⁺ exchange by L-type Ca²⁺ current augments the trigger flux for SR Ca²⁺ release in ventricular myocytes. *Biophysical journal* 94:L54-6
184. Stathopulos PB, Ikura M. 2013. Structure and function of endoplasmic reticulum STIM calcium sensors. *Current topics in membranes* 71:59-93
185. Stathopulos PB, Zheng L, Ikura M. 2009. Stromal interaction molecule (STIM) 1 and STIM2 calcium sensing regions exhibit distinct unfolding and oligomerization kinetics. *The Journal of biological chemistry* 284:728-32
186. Stiber J, Hawkins A, Zhang ZS, Wang S, Burch J, et al. 2008. STIM1 signalling controls store-operated calcium entry required for development and contractile function in skeletal muscle. *Nature cell biology* 10:688-97
187. Sussman MA, Welch S, Cambon N, Klevitsky R, Hewett TE, et al. 1998. Myofibril degeneration caused by tropomodulin overexpression leads to dilated cardiomyopathy in juvenile mice. *The Journal of clinical investigation* 101:51-61
188. Sztretye M, Geyer N, Vincze J, Al-Gaadi D, Oláh T, et al. 2017. SOCE Is Important for Maintaining Sarcoplasmic Calcium Content and Release in Skeletal Muscle Fibers. *Biophysical journal* 113:2496-507
189. Taghli-Lamalle O, Plantié E, Jagla K. 2016. *Drosophila* in the Heart of Understanding Cardiac Diseases: Modeling Channelopathies and Cardiomyopathies in the Fruitfly. *Journal of cardiovascular development and disease* 3:7
190. Tao Y, Schulz RA. 2007. Heart development in *Drosophila*. *Seminars in cell & developmental biology* 18:3-15
191. Tham YK, Bernardo BC, Ooi JY, Weeks KL, McMullen JR. 2015. Pathophysiology of cardiac hypertrophy and heart failure: signaling pathways and novel therapeutic targets. *Archives of toxicology* 89:1401-38
192. Togel M, Meyer H, Lehmacher C, Heinisch JJ, Pass G, Paululat A. 2013. The bHLH transcription factor hand is required for proper wing heart formation in *Drosophila*. *Developmental biology* 381:446-59
193. Togel M, Pass G, Paululat A. 2008. The *Drosophila* wing hearts originate from pericardial cells and are essential for wing maturation. *Developmental biology* 318:29-37
194. Tomasoni D, Adamo M, Lombardi CM, Metra M. 2019. Highlights in heart failure. *ESC heart failure* 6:1105-27
195. Touchberry CD, Elmore CJ, Nguyen TM, Andresen JJ, Zhao X, et al. 2011. Store-operated calcium entry is present in HL-1 cardiomyocytes and contributes to resting calcium. *Biochemical and biophysical research communications* 416:45-50
196. Towbin JA, Bowles NE. 2002. The failing heart. *Nature* 415:227-33

197. Troupes CD, Wallner M, Borghetti G, Zhang C, Mohsin S, et al. 2017. Role of STIM1 (Stromal Interaction Molecule 1) in Hypertrophy-Related Contractile Dysfunction. *Circulation research* 121:125-36
198. Uehara A, Yasukochi M, Imanaga I, Nishi M, Takeshima H. 2002. Store-operated Ca^{2+} entry uncoupled with ryanodine receptor and junctional membrane complex in heart muscle cells. *Cell calcium* 31:89-96
199. Varga-Szabo D, Authi KS, Braun A, Bender M, Ambily A, et al. 2008. Store-operated Ca^{2+} entry in platelets occurs independently of transient receptor potential (TRP) C1. *Pflugers Archiv : European journal of physiology* 457:377-87
200. Vega RB, Bassel-Duby R, Olson EN. 2003. Control of cardiac growth and function by calcineurin signaling. *The Journal of biological chemistry* 278:36981-4
201. Vikhorev PG, Vikhoreva NN. 2018. Cardiomyopathies and Related Changes in Contractility of Human Heart Muscle. *International journal of molecular sciences* 19
202. Voelkers M, Salz M, Herzog N, Frank D, Dolatabadi N, et al. 2010. Orai1 and Stim1 regulate normal and hypertrophic growth in cardiomyocytes. *Journal of molecular and cellular cardiology* 48:1329-34
203. Vogler G, Bodmer R, Akasaka T. 2009. A Drosophila model for congenital heart disease. *Drug Discovery Today: Disease Models* 6:47-54
204. Volkers M, Dolatabadi N, Gude N, Most P, Sussman MA, Hassel D. 2012. Orai1 deficiency leads to heart failure and skeletal myopathy in zebrafish. *Journal of cell science* 125:287-94
205. Wasserthal LT. 2007. Drosophila flies combine periodic heartbeat reversal with a circulation in the anterior body mediated by a newly discovered anterior pair of ostial valves and 'venous' channels. *The Journal of experimental biology* 210:3707-19
206. Wehrens XH, Lehnart SE, Reiken SR, Deng SX, Vest JA, et al. 2004. Protection from cardiac arrhythmia through ryanodine receptor-stabilizing protein calstabin2. *Science (New York, N.Y.)* 304:292-6
207. Wei-Lapierre L, Carrell EM, Boncompagni S, Protasi F, Dirksen RT. 2013. Orai1-dependent calcium entry promotes skeletal muscle growth and limits fatigue. *Nature communications* 4:2805
208. Weismann CG, Blice-Baum A, Tong T, Li J, Huang BK, et al. 2019. Multi-modal and multiscale imaging approaches reveal novel cardiovascular pathophysiology in Drosophila melanogaster. *Biol Open* 8
209. Wilkins BJ, Dai YS, Bueno OF, Parsons SA, Xu J, et al. 2004. Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. *Circulation research* 94:110-8
210. Wolf MJ, Amrein H, Izatt JA, Choma MA, Reedy MC, Rockman HA. 2006. Drosophila as a model for the identification of genes causing adult human heart disease. *Proceedings of the National Academy of Sciences of the United States of America* 103:1394-9
211. Wolkowicz PE, Huang J, Umeda PK, Sharifov OF, Tabengwa E, et al. 2011. Pharmacological evidence for Orai channel activation as a source of cardiac abnormal automaticity. *European journal of pharmacology* 668:208-16
212. Wu MM, Buchanan J, Luik RM, Lewis RS. 2006. Ca^{2+} store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane. *The Journal of cell biology* 174:803-13
213. Wu QQ, Xiao Y, Yuan Y, Ma ZG, Liao HH, et al. 2017. Mechanisms contributing to cardiac remodelling. *Clinical science (London, England : 1979)* 131:2319-45
214. Xu HX, Cui SM, Zhang YM, Ren J. 2020. Mitochondrial Ca^{2+} regulation in the etiology of heart failure: physiological and pathophysiological implications. *Acta pharmacologica Sinica* 41:1301-9

215. Xu P, Damschroder D, Zhang M, Ryall KA, Adler PN, et al. 2019. Atg2, Atg9 and Atg18 in mitochondrial integrity, cardiac function and healthspan in *Drosophila*. *Journal of molecular and cellular cardiology* 127:116-24
216. Yano M, Kobayashi S, Kohno M, Doi M, Tokuhisa T, et al. 2003. FKBP12.6-mediated stabilization of calcium-release channel (ryanodine receptor) as a novel therapeutic strategy against heart failure. *Circulation* 107:477-84
217. Yeromin AV, Zhang SL, Jiang W, Yu Y, Safrina O, Cahalan MD. 2006. Molecular identification of the CRAC channel by altered ion selectivity in a mutant of Orai. *Nature* 443:226-9
218. Yu L, Daniels J, Glaser AE, Wolf MJ. 2013. Raf-mediated cardiac hypertrophy in adult *Drosophila*. *Disease models & mechanisms* 6:964-76
219. Yu L, Daniels J, Glaser AE, Wolf MJ. 2013. Raf-mediated cardiac hypertrophy in adult *Drosophila*. *Disease models & mechanisms* 6:964-76
220. Yu L, Daniels JP, Wu H, Wolf MJ. 2015. Cardiac hypertrophy induced by active Raf depends on Yorkie-mediated transcription. *Science signaling* 8:ra13
221. Yuan JP, Zeng W, Dorwart MR, Choi YJ, Worley PF, Muallem S. 2009. SOAR and the polybasic STIM1 domains gate and regulate Orai channels. *Nature cell biology* 11:337-43
222. Zarain-Herzberg A, Fragoso-Medina J, Estrada-Avilés R. 2011. Calcium-regulated transcriptional pathways in the normal and pathologic heart. *IUBMB life* 63:847-55
223. Zhang B, Jiang J, Yue Z, Liu S, Ma Y, et al. 2016. Store-Operated Ca(2+) Entry (SOCE) contributes to angiotensin II-induced cardiac fibrosis in cardiac fibroblasts. *Journal of pharmacological sciences* 132:171-80
224. Zhang I, Hu H. 2020. Store-Operated Calcium Channels in Physiological and Pathological States of the Nervous System. *Frontiers in cellular neuroscience* 14:600758
225. Zhang SL, Yu Y, Roos J, Kozak JA, Deerinck TJ, et al. 2005. STIM1 is a Ca²⁺ sensor that activates CRAC channels and migrates from the Ca²⁺ store to the plasma membrane. *Nature* 437:902-5
226. Zhao G, Li T, Brochet DX, Rosenberg PB, Lederer WJ. 2015. STIM1 enhances SR Ca²⁺ content through binding phospholamban in rat ventricular myocytes. *Proceedings of the National Academy of Sciences of the United States of America* 112:E4792-801
227. Zheng H, Zhou M-H, Hu C, Kuo E, Peng X, et al. 2013. Differential roles of the C and N termini of Orai1 protein in interacting with stromal interaction molecule 1 (STIM1) for Ca²⁺ release-activated Ca²⁺ (CRAC) channel activation. *The Journal of biological chemistry* 288:11263-72
228. Zhou Y, Cai X, Nwokonko RM, Loktionova NA, Wang Y, Gill DL. 2017. The STIM-Orai coupling interface and gating of the Orai1 channel. *Cell calcium* 63:8-13
229. Zhou Y, Mancarella S, Wang Y, Yue C, Ritchie M, et al. 2009. The short N-terminal domains of STIM1 and STIM2 control the activation kinetics of Orai1 channels. *The Journal of biological chemistry* 284:19164-8
230. Zhu J, Feng Q, Stathopulos PB. 2017. The STIM-Orai Pathway: STIM-Orai Structures: Isolated and in Complex. *Advances in experimental medicine and biology* 993:15-38
231. Zhu S, Han Z, Luo Y, Chen Y, Zeng Q, et al. 2017. Molecular mechanisms of heart failure: insights from *Drosophila*. *Heart Failure Reviews* 22:91-8
232. Zweifach A, Lewis RS. 1993. Mitogen-regulated Ca²⁺ current of T lymphocytes is activated by depletion of intracellular Ca²⁺ stores. *Proceedings of the National Academy of Sciences of the United States of America* 90:6295-9