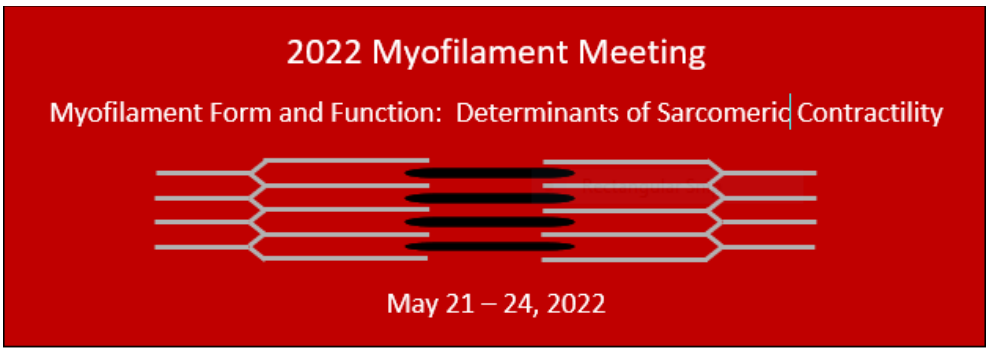

Myofilament Form and Function: Determinants of Sarcomeric Contractility

May 21-24, 2022

Monona Terrace Convention Center
Madison, Wisconsin

Sponsors:

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JGP Journal of General Physiology

MYOLOGICA

Welcome Participants!

It is our great pleasure to welcome you to Madison and to the 2022 Myofilament Meeting, "**Myofilament Form and Function: Determinants of Sarcomeric Contractility".** This is the seventh in a series of biennial meetings focused on myofibrillar and cytoskeletal proteins featuring both poster and platform sessions.

We are pleased to receive more than 160 registrants for this meeting and look forward to hearing about and discussing research of mutual interest and setting a framework for future directions in the field.

The following institutions and companies have provided generous sponsorship for our meeting, and we wish to thank them for their support:

The University of Wisconsin-Madison School of Medicine and Public Health

The University of Illinois at Chicago, Center for Cardiovascular Research

The University of Wisconsin-Madison Cardiovascular Research Center

Institute of CardioScience, Johns Hopkins University

Myologica LLC

Aurora Scientific

Rockefeller University Press, Journal of General Physiology

We hope you enjoy the meeting!

Organizers:

Richard L. Moss
University of Wisconsin-Madison

Brandon J. Biesiadecki
The Ohio State University

R. John Solaro
University of Illinois at Chicago

Anthony R. Cammarato
Johns Hopkins University

Pieter P. de Tombe
Loyola University Chicago

Coordinators:

Ladera Barnes
University of Wisconsin – Madison

Katie Randall
University of Wisconsin - Madison

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HELPFUL INFORMATION

Poster Presentations

All posters will be on display both Sunday and Monday, but poster presentations will occur on either Sunday or Monday according to subject-area categories:

Sunday Poster Session Categories

Determinants of Relaxation
Length Dependent Force Development
Models of Contraction/Regulation
Thick Filament Regulation in Striated Muscle
Thick/Thin Filament Structure

Monday Poster Session Categories

Cardiomyopathies
Elastic Proteins of Muscle
Regulation & Regulatory Signaling Networks
Myofilament Contribution To Human Diseases

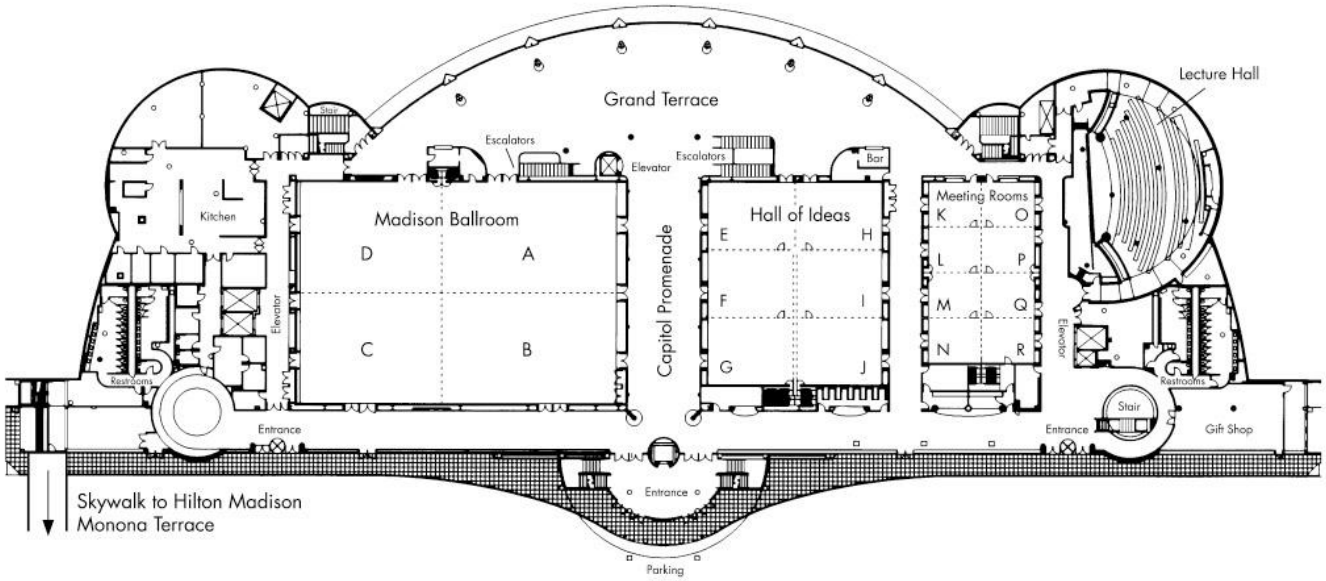
- An approximately equal number of poster presentations will take place Sunday and Monday.
- Presenters are responsible for being at or near their poster(s) during the poster presentation times indicated in the program.
- Awards will be made to the top 3 posters on Sunday and again on Monday. Awardees should be prepared to give a 2-slide talk during the Poster Awards session on Tuesday.

Internet Access at Monona Terrace Convention Center

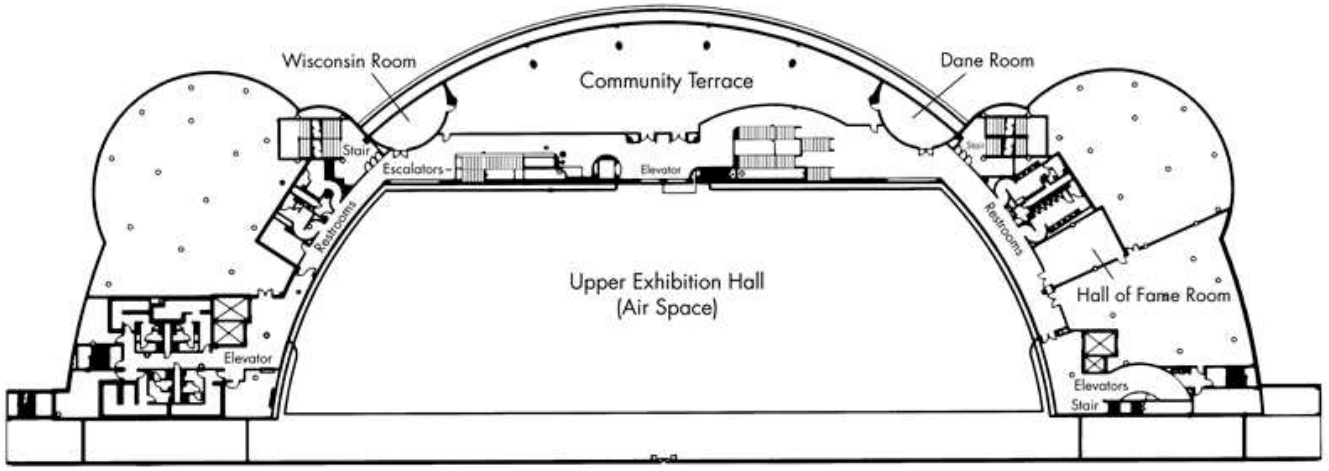
Wi-Fi is available at the conference site for all attendees free of charge.

Monona Terrace Convention Center

LEVEL 4 - MEETING ROOMS / GRAND TERRACE



LEVEL 2 - MEZZANINE



SATURDAY – MAY 21, 2022

9:00 am – 1:00 pm	Conference Registration	Monona Convention Center, Counter 4

Early Career Investigator Symposium

10:00 am – 5:30 pm
Monona Convention Center, Meeting Rooms Hall of Ideas E & F

Symposium Organizers:

Brett Colson
University of Arizona

Bertrand Tanner
Washington State University

Kathleen Woulfe
University of Arizona

David Barefield
Washington State University

10:10 am	Welcome
Trainee Group 1: Cardiomyopathies 10:15 am – 11:15 am (7-8 minute talks; with 2-3 minutes for questions & setup next speaker)	
10:15	***Prince Tiwari,*** University of Massachusetts Medical School
10:25	***Alison Vander Roest,*** Stanford University
10:35	***Mathew Childers,*** University of Washington
10:45	***Fiona Wong,*** University of Arizona
10:55	***Paulina Langa,*** University of Illinois at Chicago
11:05	***Alyssa Grogan,*** University of Maryland Baltimore
11:15 – 11:30am	Break
Trainee Group 2: Regulation of Contraction 11:30 am – 12:30 pm (7-8 minute talks, with 2-3 minutes for questions & setup next speaker)	
11:30	***Kyrah Turner,*** Washington State University
11:40	***Sarah Kosta,*** University of Kentucky

11:50 12:00 12:10 12:20	***Lorien Salyer,*** The Ohio State University ***Garrett Crosby,*** University of Arizona ***Henry Gong,*** Illinois Institute of Technology ***Cameron Hill,*** King's College London	
12:30 – 1:30 pm	Lunch (Ballroom C) – Gourmet Deli Buffer	
1:45 – 2:25 pm	Senior PI Plenary Presentation: ***Christine Cremo, PhD***, Professor of Pharmacology, U. of Nevada *"Tales of a Muscle Fossil"*	
Senior PI and Junior PI Q&A Career Advice Session 2:35– 3:10 pm		
	Christine Cremo, University of Nevada ***Kathleen Woulfe,*** University of Colorado ***Brandon Biesiadecki,*** The Ohio State University ***Jonathan Kirk,*** Loyola University Chicago ***Kaylyn Bell,*** Scientist, Bristol Myers Squibb	
Trainee Group 3: Modulation of contraction in health and disease 3:30– 4:30 pm (7-8 minute talks, with 2-3 minutes for questions & setup next speaker)		
3:30 3:40 3:50 4:00 4:10 4:20	***Joshua Smith,*** University of Nevada ***Claudia Crocini,*** University of Colorado ***Vivek Jani,*** Johns Hopkins University ***Axel Fenwick,*** Johns Hopkins University ***Kelly Araujo,*** Loyola University Chicago ***Hanzhong Feng,*** University of Illinois Chicago	
ECI Closing Discussion and Poster Judging Overview 4:30pm		
6:30 – 8:00 pm	***Welcome Reception***	Monona Convention Center Hall of Ideas Rooms H, I, J
	Poster set up for Sunday & Monday Poster Session	Hall of Ideas Rooms E-J
5:00 – 7:00pm	Conference Registration Continued	Monona Convention Center, Counter 4

SUNDAY, May 22, 2022

7:30 – 8:30 am	Conference Registration Continental Breakfast	Counter 4 Community Terrace

Welcome

8:00 am – 8:30 am
Monona Convention Center Lecture Hall

Oral Session I

Influence Of Cellular Microenvironment on Myofibrillar Function

8:30 am – 10:05 am
Monona Convention Center, Lecture Hall

Jolanda van der Velden
University of Amsterdam
Moderator

8:30 – 8:50 am (20 min including 5 min Q&A)	***Janice Raabe*** Studienstiftung des Deutschen Volkes *Protein kinase D-mediated regulation of myofilament function in cardiac health and disease*
8:50 – 9:10 am (20 min including 5 min Q&A)	***Robert Ross*** University of California San Diego *Junction proteins in myocardial function and rhythm*
9:10 – 9:30 am (20 min including 5 min Q&A)	***Farah Sheikh*** University of California San Diego *Policing Cell-Cell Connections: Uncovering Protein Degradation Pathways As Novel Regulators of Cardiac Desmosomal Biology and Disease*
9:30 – 9:50 am (20 min including 5 min Q&A)	***Jolanda van der Velden*** University of Amsterdam *Role of microtubules in disease development*
9:50 – 10:05 am (15 min including 5 min Q&A)	***Kalina Reese*** University of Wisconsin- Madison *Integrated Analysis of Patient-specific Human Induced Pluripotent Stem Cell-derived Engineered Cardiac Tissue Model of Hypertrophic Cardiomyopathy*

10:15 – 10:45	Break	Lecture Hall

Plenary Lecture

John Solaro

University of Illinois Chicago

Signaling in the Sarcomere Microenvironment: Perspectives on the Roles of the Hippo/Yap Cascade

10:45 am – 11:15 am

Monona Convention Center Lecture Hall

11:15 – 12:00	**Poster Session 1** ❖ Determinates of Relaxation ❖ Length Dependent Force Development ❖ Models of Contraction/Regulation ❖ Thick/Thin Filament Regulation in Striated Muscle ❖ Thick/Thin Filament Structure	Hall of Ideas Rooms E - J
12:00 – 1:00 pm	Lunch – Bucky's Tailgate Buffet	Monona Convention Center Community Terrace

Oral Session II

Thin Filament Linked Regulation of Contraction

1:15 pm – 3:00 pm

Monona Convention Center, Lecture Hall

Brandon Biesiadecki

The Ohio State University

Moderator

1:15 – 1:35 pm (20 min including 5 min Q&A)	***Neil Kad*** University of Kent *Single molecule imaging reveals the underlying mechanisms of thin filament regulation*
1:35 – 1:55 pm (20 min including 5 min Q&A)	***William Lehman*** Boston University *Phosphorylation of Cardiac Muscle Troponin-I Modulates Thin Filament Structure*
1:55 – 2:15 pm (20 min including 5 min Q&A)	***Jil Tardiff*** University of Arizona *The Role of the Cardiac Thin Filament in Myocardial Relaxation: Exploring Pathogenic Mechanisms that Determine Diastolic Dysfunction*

2:15 – 2:30 pm (15 min including 5 min Q&A) 2:30 – 2:45 pm (15 min including 5 min Q&A) 2:45 – 3:00 pm (15 min including 5 min Q&A)	***Brandon Biesiadecki*** The Ohio State University *Tyrosine phosphorylation of troponin I beneficially accelerates diastolic function* ***Vitold Galkin*** Eastern Virginia Medical School *Structural insights into cardiac thin filament regulation - what are we missing?* ***Farid Moussavi-Harami*** University of Washington *Danicamtiv reversed negative effects on cardiac myofilament function in the I61Q cTnC mouse model of dilated cardiomyopathy*	
3:00 – 5:30 pm	**Poster Session 1 (continued)**	Hall of Ideas Rooms E - J
6:00 – 9:00pm	Dinner on Rooftop Garden	Monona Terrace Convention Center

MONDAY, May 23, 2022

7:30 am – 8:30 am	Continental Breakfast	Monona Convention Center Community Terrace
Oral Session III **Thick Filament-Linked Regulation Contraction** 8:30 am – 10:10 am Monona Convention Center, Lecture Hall ***Malcolm Irving*** King's College London Moderator		
8:30 – 8:50 am (20 min including 5 min Q&A) 8:50 – 9:10 am (20 min including 5 min Q&A)	***Elisabetta Brunello*** King's College London *Load-dependence of the structural dynamics of the thick filament during contraction of intact cardiac trabeculae* ***Roger Craig*** University of Massachusetts *Switching off myosin II in filaments & molecules*	

9:10 – 9:30 am (20 min including 5 min Q&A)	***Thomas Kampourakis*** King's College London *Control of cardiac myosin motor function by the regulatory light chain*	
9:30 – 9:50 am (20 min including 5 min Q&A)	***Massimo Reconditi*** University of Florence *Thick filament regulation in cardiac muscle*	
9:50 – 10:10 am (20 min including 5 min Q&A)	***Dave Warshaw*** University of Vermont *Myosin-Binding Protein C: The many ways it regulates muscle contractility*	
10:15 – 10:45	Break	
Plenary Lecture ***Malcolm Irving*** Kings College London *Dual-filament Regulation in Striated Muscle* *Intrinsic Regulation of Contractility in the Heart* 10:45 am – 11:15 am Monona Convention Center, Lecture Hall		
11:15 – 12:15	**Poster Session 2** ❖ Cardiomyopathies ❖ Elastic Proteins of Muscle ❖ Regulation & Regulatory Signaling Networks ❖ Myofilament Contribution to Human Diseases	Hall of Ideas Rooms E-J
12:15 – 1:30 pm	Lunch – Mediterranean Buffet	Monona Convention Center Community Terrace
Plenary Lecture ***Leslie Leinwand*** University of Colorado *Myosin Modulation in Health and Disease* 1:30 pm – 2:00 pm Monona Convention Center, Lecture Hall		

Oral Session IV

Regulation Via Load-Dependent Signaling at M-Line & Z-Disc

2:00 pm – 2:55 pm
Monona Convention Center, Lecture Hall

Pieter de Tombe
University of Illinois
Moderator

2:00 – 2:20 pm (20 min including 5 min Q&A)	***Mathias Gautel*** King's College London *Constraints of Z-disk and M-band mechanosignalling from new structural insight and interactions*	
2:20 – 2:40 pm (20 min including 5 min Q&A)	***Henk Granzier*** University of Arizona *N2BA titin is vital for cardiac homeostasis and knock-down induces a DCM-like phenotype*	
2:40 – 3:00 pm (20 min including 5 min Q&A)	***Stuart Campbell*** Yale University *Effects of myosin modulators on length-dependent activation*	
3:00 – 3:15pm (15 min including 5 min Q & A)	***Weikang Ma*** *Illinois Institute of Technology* *Cardiac myosin filaments are directly regulated by calcium*	
3:30 – 5:30pm	**Poster Session 2 (continued)**	Hall of Ideas Rooms E-J
6:30pm	Evening of Networking	Park Hotel 22 S. Carroll St.

TUESDAY, May 24, 2022

7:00 am – 8:00 am	Continental Breakfast	Monona Convention Center Community Terrace

Oral Session V

Myofilament Mis-Regulation in Disease

8:00 am – 9:40 am
Monona Convention Center, Lecture Hall

	Samantha Harris University of Arizona Moderator	
8:00 – 8:20 am (20 min including 5 min Q&A)	***Samantha Harris*** University of Arizona *Mis-regulation of myosin binding protein-C in striated muscles: There may be a catch*	
8:20 – 8:40 am (20 min including 5 min Q&A)	***Katia Kontrogianni-Konstantopoulos*** University of Maryland *MYBPC1 associated myopathy with tremor: what we know, what we need to learn*	
8:40 – 9:00 am (20 min including 5 min Q&A)	***Kerry McDonald*** University of Missouri *Myofilament power in failing hearts*	
9:00 – 9:20 am (20 min including 5 min Q&A)	***Corrado Poggesi*** University of Florence *Impaired sarcomere energetics and E-C coupling remodeling are at the core of HCM caused by MyBP-C mutations: translational insight from a Tuscan founder population*	
9:20 – 9:40 am (20 min including 5 min Q&A)	***KC Woulfe*** University of Colorado *Functional consequences of acetylation of specific lysines on sarcomeric proteins*	
9:40 – 10:00	Break	
10:00 – 11:15 am	**Session VI** ***ECI Poster Award Session*** Monona Convention Center, Lecture Hall	
11:15 – 12:00 pm	Meeting Summary Panel Discussion – **The Field Today & Tomorrow**	Monona Convention Center, Lecture Hall
12:00 pm	Closing Remarks	
12:15 – 1:30 pm	Lunch & Meeting Closure – Heart of Italy Buffet	Monona Convention Center Community Terrace

Plenary Speaker Biographies

John Solaro, PhD, *University of Illinois Chicago*

R. John Solaro trained for the PhD in the Department of Physiology, at University of Pittsburgh School of Medicine and in 1971 immediately moved to a faculty position at the Medical College of Virginia. After moving on to Professor at University of Cincinnati, College of Medicine, he was appointed Head of the Department of Physiology and Biophysics at University of Illinois at Chicago (UIC) from 1988 to 2015. He was appointed Distinguished University Professor in the University of Illinois System in 1998. Solaro is founder and past director of the UIC Center for Cardiovascular Research. In 1975-76 he was a Fellow of the American and British Heart Associations and worked with S. V. Perry in Birmingham, England. In 1987 he was a Fogarty Fellow working with David Allen at University College London. Solaro's lab has focused on modifications in cardiac sarcomeres as significant elements in the regulatory cascades controlling cardiac dynamics, and more recently on sarcomeres as elements in mechano-signaling controlling cardiac long-term adaptations and maladaptations. In 1979, he explored the idea of developing drugs directly enhancing myofilament Ca-response. A 1982 paper in *Circulation Research* with Dr. Ruegg is a seminal paper supporting this idea.

Malcolm Irving, PhD, *King's College London*

Malcolm Irving is Professor of Biophysics at King's College London and Associate Director of Research at the Francis Crick Institute, London, where he is responsible for the Crick's partnership with Imperial College London, King's College London, and University College London (UCL). Following an undergraduate degree in Physics (Cambridge UK), Malcolm undertook postgraduate research training in physiology at UCL, followed by post-doctoral work at UCLA and Yale. His main research interest is in molecular mechanisms in the contraction and regulation of skeletal and cardiac muscle. Recent research has focused on the regulation of contraction by mechanisms involving structural changes in the myosin-containing filaments. Malcolm was elected a Fellow of the Royal Society in 2003 and a Fellow of the Academy of Medical Sciences in 2006.

Leslie Leinwand, PhD, *University of Colorado*

Leslie Leinwand, PhD is a Molecular, Cellular, and Developmental Biology (MCDB) Distinguished Professor and the Chief Scientific Officer of the BioFrontiers Institute at the University of Colorado Boulder. She received her Bachelor's degree from Cornell University, her PhD from Yale University and did post-doctoral training at Rockefeller University. She joined the faculty at Albert Einstein College of Medicine in New York in 1981 and remained there until moving to Colorado in 1995. She co-founded Myogen, Inc. which was sold to Gilead Pharmaceuticals. More recently, she was a co-founder of Hiberna, Inc, and of MyoKardia, Inc., a company founded to develop therapeutics for inherited cardiomyopathies. MyoKardia was acquired in 2020 by Bristol Myers Squibb. She is a Fellow of the AAAS, former MERIT Awardee of the NIH, Established Investigator of the American Heart Association and was recently elected to the

American Academy of Arts and Sciences and the National Academy for Inventors. She has been honored by the American Heart Association with its Distinguished Scientist Award and its Braunwald Academic Mentorship Award. The interests of Dr. Leinwand's laboratory are the genetics and molecular physiology of inherited diseases of the heart and particularly how biologic sex affects the heart in health and disease. The study of these diseases has required multidisciplinary approaches, involving molecular biology, mouse genetics, mouse cardiac physiology, and the analysis of human tissues. She has also studied the biology of the Burmese python to uncover cardio-protective mechanisms.

SUMMARY OF POSTER ABSTRACTS

(listed by Presenter's last name)

Last Name	First Name	Institution	Mentor	ECI Speaker	Poster No.	Abstract Category	Title
Alvarez-Arce	Alejandro	Loyola University Chicago	David Barefield		56	Cardiomyopathies	Premature Stop Mutations In MYBPHL Share The Common Pathomechanism Of Preventing Protein Incorporation Into The Cardiac Atrial Myofilament
Araujo	Kelly	Loyola University Chicago	David Barefield	X	1	Determinants of Relaxation	Myosin Binding Protein H-like Loss Of Function Mutations Alter Stoichiometry Of Myosin Binding Proteins And Cause Defects In Atrial Myofilament Relaxation
Awinda	Peter	Washington State Univeristy			24	Filament Regulation in Striated Muscle	MgADP Augments Passive Force & Ca2+-Sensitivity Of Contraction In Porcine Myocardial Strips
Badillo Lisakowski	Victor	Max Delbrück Center, Berlin, German	Michael Gotthardt		2	Determinants of Relaxation	RBM20 Inhibition With Antisense Oligonucleotides Improves Diastolic Function In The Titin N2B-KO & In Human Engineered Heart Tissue
Baker	Anthony	University of California - San Francisco			92	Myofilament Contribution To Human Diseases	Low MgATP In Heart Failure Causes Reduced Cardiac Muscle Power
Baker	Josh	University of Nevada			11	Models of Contraction/Regulation	A Binary Mechanical Model System:The Simple Physical Chemistry of Cardiac Pressure Volume Loops
Baldo	Anthony	University of Arizona	Steven D. Schwartz		25	Filament Regulation in Striated Muscle	Rare Event Simulations for Thin Filament and Myosin Systems
Barry	Meaghan	University of Massachusetts	Jeffrey Moore		26	Filament Regulation in Striated Muscle	Destabilizing Of Putative Tropomyosin-Troponin-I Interactions Increase Calcium Sensitivity
Batabyal	Anandi	Florida State University	P Bryant Chase		83	Regulation & Regulatory Signaling Networks	Effect Of Sex & Genotype On Gene Expression In Cardiac Hypertrophy (HCM)-Affected Mouse Cardiac Transcriptome
Bell	Kaylyn	Bristol Myers Squibb			12	Models of Contraction/Regulation	Sinusoidal Analysis As A Tool To Understand & Differentiate Small Molecule Myosin Modulators Developed For Cardiovascular Disease Treatment
Bennett	Maggie	Loyola University Chicago	Jordan Beach		13	Models of Contraction/Regulation	Smooth Muscle Myosin Monomer Pool is Dynamic

Cammarato	Anthony	John Hopkins University			57	Cardiomyopathies	The A331P Hypertrophic Cardiomyopathy Mutation In Cardiac Actin Enhances Basal Contractile Activity & Causes Resting Muscle Dysfunction
Campbell	Kenneth	University of Kentucky			93	Myofilament Contribution To Human Diseases	A Multiscale Model Of The Cardiovascular System That Incorporates Baroreflex Control Of Chronotropism, Cell-Level Contractility, & Vascular Tone
Campbell	Stuart	Yale University			8	Length Dependent Force Development	Effects of Myosin Modulators on Length-Dependent Activation
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Cleary	Sean	Loyola University Chicago	Seth L. Robia		84	Regulation & Regulatory Signaling Networks	Inhibitory and Stimulatory Micropeptides Preferentially Bind to Different Conformations of the Cardiac Calcium Pump
Childers	Mathew	University of Washington	Michael Regnier	X	58	Cardiomyopathies	MYH7 G256E & H251N Mutations Impair Relaxation By Altering The Stiffness Of The Transducer Region Of Myosin
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Hock	Marcus	University of California San Diego	Andrew McCulloch		49	Thick/Thin Filament Structure	Multiscale Simulations Of The Effects Of 2'-deoxy-ATP & Myosin Mutations On Actomyosin Interactions
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Iyer	Aishwarya	University of Maryland	Aikaterini Kontrogianni-Konstantopoulos		98	Myofilament Contribution To Human Diseases	Elucidating the Structural and Functional Consequences of a Novel Mutation in MYBPC1 Linked to Myopathy with Tremor
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Janssen	Paul	Ohio State University			18	Models of Contraction/Regulation	Contraction-Relaxation Coupling & Mechanical Dyssynchrony In End-Stage Human Heart Failure
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Premature stop mutations in *MYBPHL* share the common pathomechanism of preventing protein incorporation into the cardiac atrial myofilament

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Background. Cardiomyopathy and arrhythmia are diseases with significant genetic linkage. Cardiac myosin binding protein-C (cMyBP-C) is a myofilament associated protein with a C-terminal composed of repeating immunoglobulin (Ig) and fibronectin III (FnIII) domains that bind to the myosin thick filaments and titin. C-terminal truncating mutations in cMyBP-C result in improper myofilament incorporation and degradation of the truncated protein and are a major cause of hypertrophic cardiomyopathy. Myosin-binding protein-H like (MyBP-HL) is a protein that was recently identified that is structurally related to MyBP-C. MyBP-HL and cMyBP-C share high sequence homology in their C-terminal Ig and FnIII domains that bind to myosin. MyBP-HL is different in that it is primarily expressed in the atria where it that interacts with the myofilament. MyBP-HL premature stop mutations are associated with improper myofilament incorporation, like cMyBP-C. Prior investigation modeled the *MYBPHL* Arg255X mutation that is associated with ventricular conduction system abnormalities, atria enlargement, and dilated cardiomyopathy (DCM) in humans and this mutation resulted in no MyBP-HL localization into the myofilament. A null mouse model showed that loss of MyBP-HL resulted in arrhythmia and cardiomyopathy, like that observed in humans.
Method. The gnomAD database reports 9 stop-gain mutations for *MYBPHL* in humans including: Gln29, Trp54, Arg113, Tyr123, Trp158, Trp192, Lys250, Arg255, and Tyr307. We hypothesize that all MyBP-HL truncation mutations share a common mechanism by failing to produce MyBP-HL that contains the necessary myosin binding residues that required for integration into the sarcomere. We test this hypothesis by transfecting neonatal rat ventricular and atrial cardiomyocytes with mouse *Mybphl* constructs containing these human mutations.
Results. We tested MyBP-HL and MyBP-C overexpression, and both showed to be able to incorporate in a similar pattern to cMyBP-C C-zone doublets. We then tested Gln29, Trp54, Arg113, Tyr123, Trp158, Trp192, Lys250, and Arg255 MyBP-HL mutations, and observed that stop gained mutation disrupt normal localization as we expected.
Conclusions. Based on these data, we expect that truncating mutations in *MYBPHL* to act through a common loss of protein mechanism and may be functionally similar. This suggests that mechanistic insight into the pathogenicity of *MYBPHL* null alleles can be extrapolated to these nine known human *MYBPHL* truncating variants.

RBM20 inhibition with antisense oligonucleotides improves diastolic function in the titin N2B-KO and in human engineered heart tissue

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The elastic properties of the heart are largely determined by myocardial collagen deposition and isoform expression of the sarcomeric protein titin. Genetic deficiency of the titin splice regulator RBM20 leads to expression of more compliant titin isoforms and improves cardiac filling in diverse mouse models that replicate phenotypic features of heart failure with preserved ejection fraction (HFpEF). Here, we evaluated pharmacological RBM20 inhibition with antisense oligonucleotides (ASOs) in the titin N2B-KO with increased titin-based stiffness. Weekly injections of palmitoylated ASOs led to altered alternative splicing and restored cardiac filling as determined by echocardiography and conductance catheter. This approach was validated in naïve human engineered heart tissue (EHT), in which the ASOs were well tolerated and adapted contraction and relaxation kinetics without obvious adverse effects.

Strain-rate of low-amplitude stretches affect crossbridge distortion during relaxation in twitching, intact cardiac trabeculae

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Relaxation mechanisms include both calcium and myofilament kinetics. We have recently reported that Mechanical Control of Relaxation (MCR), or the acceleration of relaxation by fast stretch, exists in intact cardiac trabeculae, is likely due to crossbridge detachment that is accelerated by fast stretch (strain-rate).

This study was undertaken to further investigate strain-rate dependence of crossbridge kinetics after a ramp-stretch during the relaxation phase of a twitch in intact cardiac trabeculae. Rat cardiac trabeculae were allowed to twitch isometrically at Lo (0.5 Hz, 25C) except when experiments were performed. During data acquisition, twitch contractions were isotonically held at ~50% of the developed isometric stress by shortening the muscle. The muscle was then allowed to isometrically relax for 1 second, except when ramp-stretches were applied (following the load-clamp). Ramp-stretches (amplitude: 1% muscle length; strain rates: 25-1000 lengths/s) were performed at various times through the diastolic period, once per twitch. Twitches without ramp-stretches were used as control. Measurements of stress vs. time from control twitches were subtracted from the ramp-stretch twitches resulting in a 4-phase step response pattern. The stress response patterns were analyzed for stress and time parameters.

Results related to timing: Peak stress varied with the timing of stretch after the end of the load clamp, indicating that the number of bound crossbridges decreased as diastole progressed. The timing and magnitude of the transition between phase 2 (crossbridge detachment) and phase 3 (recovery) were reduced as time after the end of the clamp progressed.

Results related to strain rate: Peak stress also varied with the rate of stretch, suggesting increased distortion of bridges. The half-time of stress decay between the peak-to-minimum stress values were (stretch) strain-rate dependent, likely due to the increased distortion. The timing and magnitude of the end of the phase 2 and the timing of phase 3 were, at best, weakly dependent on strain rate.

Several computational models could not accurately fit all transients, especially at low strain rates. For example, the two exponential rates of relaxation and recruitment-distortion-instantaneous-elastance models did not consistently result in an accurate fit to the phase 2 and phase 3 components of the transient after stretch. This suggests limitations to assumptions in the current models. While the early (phase 1) portion of the maneuver can be explained by enhanced off-rate with lengthening, the recovery may require attributing a stretch-dependency to the on-rate of myosin crossbridges and/or the cooperative deactivation of the thin filament during relaxation.

In all, these data support that crossbridge dynamics are dependent on the strain-rate of stretch. However, further investigation into the precise mechanisms underlying the responses reported here would provide a more detailed account of myofilament kinetics during relaxation.

L348P substitution in the M-Domain of Cardiac Myosin Binding Protein-C slows relaxation kinetics in murine cardiac single myofibrils

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Hypertrophic cardiomyopathy (HCM) is a severe human genetic cardiac disease with the prevalence of ~1:500 individuals which a big burden for healthcare systems. Mutations in gene encoding cardiac myosin-binding protein C (cMyBP-C) account for 20-25% of all HCM cases. The L348P substitution (L352P in humans) was identified as an HCM-associated missense mutation in the M-domain of cMyBP-C. Murine models developed in our laboratory demonstrated insufficient myocardial relaxation in these mice consistent with increased binding of cMyBP-C's M-domain to actin (Bezold et al., J Biol Chem, 2013), however the precise mechanisms by which cMyBP-C affects relaxation are still unclear. Here we performed single myofibril mechanics experiments to obtain data on relaxation kinetics using two approaches: myofibrils isolated from heterozygous L348P-CR mutant mice and myofibrils from homozygous SpyC3 mice where we used the cut-and-paste method to replace endogenous cMyBP-C with recombinant cMyBP-C with the L348P mutation. The latter method developed in our lab allows for specific and efficient proteolytic removal of endogenous wild type cMyBP-C N'-terminal domains and in situ replacement with recombinantly expressed mutant cMyBP-C via SpyCatcher-SpyTag covalent binding. Results showed that both approaches returned same result: the L348P mutation significantly reduced relaxation rates in both phases of relaxation that could be detected in single myofibrils: an initial linear phase as well as a fast exponential phase. In the linear phase, both slope of the relaxation and duration of phase were significantly impaired. We conclude that replacement of recombinant cMyBP-C with L348P substitution in SpyC3 cardiac tissue using the cut-and-paste approach has similar effects as seen in L348P-CR mice suggesting a direct effect of this mutation on relaxation kinetics in the sarcomere. Results also provide additional validation of the cut-and-paste method as useful experimental approach.
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Cardiac Myosin Binding Protein-C Provides Cooperative Coupling of Cross-bridge Cycling to Calcium Transient for Heart to Contract and Relax Effectively

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Background: We hypothesize that cooperative activation/de-activation by myosin-actin cross-bridges sustains force generation during calcium re-uptake and accelerates relaxation to provide effective coupling needed for normal heart function.
Methods: We conducted a study spanning survival, echocardiography, intact papillary muscle experiments, and protein expression analysis of calcium handling proteins on mouse models of cardiac myosin binding protein-C deletion (cMyBP(KO)) and its litter mates cMyBPC(tWT) with normal cMyBPC. Mutations of cMyBPC in patients have led to low expression levels to make cMyBPC(KO) to be clinically relevant.
Results: cMyBPC(KO) mice showed increased 18-months mortality. cMyBPC(KO) hearts exhibited depressed LVEF, diastolic dysfunction (slower e', higher E/e' ratio), and abbreviated systolic ejection duration on echocardiography. Simultaneously measuring intracellular calcium and force on intact papillary muscles showed that cMyBPC(KO) myocardium exhibited abbreviated force duration/calcium duration ratio and decreased ability to sustain force during calcium re-uptake, indicating decreased cross-bridge cooperative action. We then used ratio of dFR=(dF/dt min)/(dF/dt max) to measure myocardium's ability to accelerate relaxation with increasing pacing frequency. Unlike cMyBPC(tWT), cMyBPC(KO) myocardium showed unchanging dFR when pacing increased from 1 to 2 Hz to suggest inability to accelerate relaxation. Weibull analyses showed that presence of cMyBPC changed the shape of relaxation-time curve to suggest cooperative de-activation mediated acceleration of relaxation. Furthermore, cMyBPC(KO) hearts displayed increased expression of SERCA2a and RYR, which suggests a possible compensatory mechanism for increased calcium dependency.
Conclusion: cMyBPC mediates cross-bridge cooperative activation/de-activation to sustain systole and accelerates relaxation with increasing heart rate to provide normal diastolic function.

*** p<0.05 *vs.* tWT; # p<0.05 *vs.* 1 Hz**	**cMyBPC(tWT)**	**cMyBPC(KO)**
Survival at 18 months (%)	N=122, 87.1 ± 5.1	N=87, 37.6 ± 13.1*
Echocardiography	N=7	N=7
LVEF (%)	51 ± 3	32 ± 4 *
e' (mm/s)	28.2 ± 1.5	15.7 ± 1.1*
E/e'	23.8 ± 0.9	44.8 ± 3.3*
Systolic Ejection Duration (ms)	42.6 ± 1.6	26.1 ± 1.1*
Systolic Ejection Duration/(cardiac cycle duration) (%)	35.1 ± 2.1	22.0 ± 0.7*
Intact Papillary Muscle (Force & Intracellular Calcium)	N=8	N=8
Delay from calcium 0.5 rise to force 0.5 rise (ms) at 2Hz	44 ± 2	28 ± 2*
Force Duration/Calcium Duration at 2Hz	0.96 ± 0.09	0.62 ± 0.06*
Cross-bridge Cooperative Activation Hysteresis at 2Hz	0.45 ± 0.09	0.25 ± 0.02*
dFR=(dF/dt min)/(dF/dt max) at 1Hz	0.52 ± 0.02	0.52 ± 0.04
dFR=(dF/dt min)/(dF/dt max) at 2Hz	0.67 ± 0.03#	0.54 ± 0.04*
Weibull Shape (S) at 1Hz	1.76 ± 0.09	1.92 ± 0.05
Weibull Shape (S) at 2Hz	2.18 ± 0.06#	1.91 ± 0.06*
SERCA2a Expression (adj. volume/total actin)	N=6, 0.17 ± 0.05	N=6, 0.91 ± 0.26*
RYR Expression (adj. volume/total MyHC)	N=5, 0.70 ± 0.22	N=5, 2.00 ± 0.18*

Structural Basis of SRX and HRX States: Consequences of IHM Variants

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Super-relaxation (SRX) is a state of muscle thick filaments in which ATP turnover by myosin is much slower than that of myosin II in solution. This inhibited state, in equilibrium with a faster, disordered-relaxed state (DRX), is ubiquitous across muscle types and species. It is thought to be fundamental to muscle function, acting as a mechanism for switching off energy-consuming myosin motors when they are not being used. The structural basis of SRX is usually taken to be a motif formed by myosin in which the two heads interact with each other and with the proximal tail (subfragment 2, S2), forming an interacting-heads motif (IHM), which switches the heads off. However, recent studies show that even isolated myosin heads (~15%) can exhibit this slow rate, possibly due to a putative bent conformation that inhibits activity. We suggest that the high level of SRX in muscle (~75%) results from stabilization of the bent head structure through intramolecular interactions in the IHM, and of the IHM through intermolecular interactions occurring in the filament. A third, even more inhibited, state of myosin (a hyper-relaxed state, HRX) is found in certain invertebrate thick filaments and in vertebrate smooth and nonmuscle myosin molecules. It appears to result from additional interactions between the heads and the tail. We speculate on a relationship between animal lifestyle and the level of super- and hyper-relaxation in different species, and on the mechanism of formation of the IHM that produces the SRX and HRX states.

The simplest (canonical) IHM, described above, occurs in thick filaments of most species, where the interacting heads bend back on S2 and lie parallel to the filament axis. Two variants of this structure have been found. In one, occurring in insect indirect flight muscle, the interacting heads are perpendicular to the filament axis and don't interact with S2. In another, found in isolated smooth and nonmuscle myosin molecules in their switched-off (10S) conformation, S2 is shifted by 20 Å, and the rest of the tail folds twice and wraps around the interacting heads. The structures of the canonical IHM and the insect variant are known at low resolution, while the single molecule (10S) variant has been resolved in near-atomic detail. The differing structures of these IHMs appear to underlie key energetic, functional, and pathophysiological variations:

- The canonical IHM in vertebrate muscle is thought to produce the SRX state, while additional head-tail interactions in some invertebrate thick filaments and in the single molecule variant give rise to the more inhibited (HRX) state.
- The absence of S2-head interaction in the insect IHM may contribute to the relative instability of this IHM and be related to the high contraction frequency of indirect flight muscle.
- Analysis of the low-resolution canonical IHM structure in thick filaments has revealed that mutations causing hypertrophic cardiomyopathy are concentrated in regions of intramolecular interactions in the IHM; however, use of the near-atomic structure of the single molecule variant to obtain higher resolution is not appropriate, as the 20-Å shift in S2 precludes analyzing head-S2 interactions.
- The single molecule variant can be used to analyze the structural/functional impact of mutations in smooth and nonmuscle myosin.

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A Homozygous Missense Mutation in the M-Domain of Cardiac Myosin Binding Protein-C is Lethal in a CRISPR Gene Edited Mouse Model

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The L348P substitution (L352P in humans) is an HCM-associated missense mutation in cardiac myosin binding protein-C (cMyBP-C) that increases binding of cMyBP-C's M-domain to actin (Bezold et al., J Biol Chem, 2013). We previously developed a mouse model where transgenic expression of the L348P mutant protein was ~39 ± 4% of total cMyBP-C (van Dijk et al., J Mol Cell Cardiol, 2018). Here, we sought to increase the relative expression of the L348P mutant protein by developing a CRISPR gene edited mouse model that could be bred to homozygosity for the missense mutation. However, we found that homozygous expression is lethal prior to postnatal day 7, the time point at which mice were genotyped. All genotyped mice were wild type or heterozygous for the L348P-CR mutation. Western blots showed similar L348P expression in left ventricles of L348P-Tg mice compared to L348P-CR heterozygous mice. Also, consistent with the original transgenic model, echocardiography of L348P-CR mice showed diastolic dysfunction relative to wild type littermates. Permeabilized cardiomyocytes from L348P-CR heterozygous mice displayed increased viscosity, increased Ca^{2+} sensitivity of tension, and slowed relaxation rates as reported previously, supporting the hypothesis that the increased binding of cMyBP-C to actin inhibits normal cardiomyocyte relaxation. Due to the inability of the CRISPR gene edited model to measure the effects of L348P homozygosity, we also tested effects of the L348P mutation in our SpyC3 cut and paste mouse model, which allows for specific and efficient proteolytic removal of endogenous wild type cMyBP-C N'-terminal domains and *in situ* replacement with recombinantly expressed mutant cMyBP-C via SpyCatcher-SpyTag covalent binding. Preliminary data from permeabilized SpyC3 myocytes with L348P mutant replacement cMyBP-C N'-termini showed similar results, particularly with respect to an increase in Ca^{2+} sensitivity of tension. We conclude that the L348P missense mutation is a dominant negative mutation that slows relaxation rates and is incompatible with survival when inherited in the homozygous state. Supported by NIH HL080367 and HL140925.

Effects of Myosin Modulators on Length-Dependent Activation

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Recent years have seen the discovery of multiple small molecules capable of activating or inhibiting cardiac myosin. Studies of these so-called myotropes are progressing beyond simple measures of contractility to include assessment of their effects on length-dependent myofilament activation. In multiple experiments, we have studied the length-dependent effects of cardiac myotropes on functional performance of engineered heart tissues (EHTs) created from human induced pluripotent stem cell-derived cardiomyocytes. Several interesting observations have emerged from the aggregate results of our work. For instance, mavacamten, a cardiac-specific myosin inhibitor, depresses isometric twitch force but does so in a manner that can be reversed by stretching EHTs to longer lengths, in effect preserving a significant measure of recruitable contractile reserve. The myosin activators omecamtiv mecarbil and danicamtiv both enhanced isometric twitch force in EHTs, but with striking differences in their effect on length-dependent activation. Danicamtiv tended to proportionally increase twitch force at all EHT lengths, whereas omecamtiv mecarbil had the greatest contractile enhancement at short lengths. As EHTs were stretched, omecamtiv mecarbil had progressively smaller effects on peak isometric force, ultimately blunting the relative length sensitivity. A computational model of the myofilaments was subsequently used to provide plausible molecular explanations for the diverging length-dependent effects of omecamtiv mecarbil and danicamtiv. The diverse combination of functional effects manifest by these myotropic agents raises the possibility of matching specific drugs with specific disease states to achieve optimal therapy.

Removal of the N-terminal Extension of Cardiac Troponin I Enhances Frank-Starling Response of Mouse Heart by Increasing Myofilament Sensing of Resting Tension

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Differentiated from skeletal muscle troponin I (TnI), cardiac TnI (cTnI) of higher vertebrates has evolved with an N-terminal extension containing β-adrenergic dependent protein kinase A (PKA) phosphorylation sites. Deletion of the N-terminal extension via restrictive proteolysis occurs as a compensatory adaptation in chronic heart failure by increasing ventricular relaxation and stroke volume based on Frank-Starling mechanism. Here we first-time demonstrate a novel transgenic mouse model with 100% expressing N-terminal truncated cTnI (cTnI-ND) in the mouse hearts by crossing with cTnI knockout transgenic mice. The ex vivo working heart functional measurements discovered an extended Frank-Starling response to preload with reduced left ventricular end diastolic pressure. The enhanced Frank-Starling response effectively increases systolic ventricular pressure development and ventricular ejection time. A novel finding is that cTnI-ND increases left ventricular relaxation velocity and stroke volume without increasing the end diastolic volume. Consistently, resting sarcomere length (SL) in isolated cardiomyocytes and optimal SL for maximum force development in intact papillary muscle of cTnI-ND hearts were not different from WT controls. Despite the removal of PKA phosphorylation sites from cTnI, β-adrenergic stimulation remains effective on augmenting the enhanced Frank-Starling response of cTnI-ND hearts whereas titin isoforms and phosphorylation and MBP-C phosphorylation were not changed. Using skinned frozen sectioned strips of papillary muscle, force-pCa relationship study showed higher Ca^{2+} sensitivity in cTnI-ND vs WT groups in a resting tension-dependent manner at various SL. With fast step solution change to switch Ca^{2+} concentration among pCa 9, 7, 5.7 and 4.5, cTnI-ND papillary muscle strips showed shorter contractile and relaxation times than that of WT control at SL of 2.0 and 2.2 μm. The results demonstrate that restrictive N-terminal truncation of cTnI enhances Frank-Starling response via increasing myofilament sensing of resting tension other than directly depending on SL. This novel function of cTnI modification provides a myofilament mechanism and therapeutic target for enhancing Frank-Starling response to treat heart failure especially diastolic heart failure, i.e., heart failure with preserved ejection fraction.

Carbon Monoxide Release by oCOm-21 Enhances Ca^{2+} Sensitivity of the Cardiac Myofilament

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Introduction. Cardiac inotropic therapies increase contractile force by enhancing cytosolic Ca^{2+} cycling. However, elevated Ca^{2+} can adversely increase myocardial oxygen demand, inducing arrythmias and end-organ injury, prompting a demand for new treatments with little impact on intracellular Ca^{2+}cycling. Our lab has demonstrated that low carbon monoxide (CO) concentrations (1 - 10 μM) delivered by oCOm-21, a novel organic CO releasing molecule, evoke a concentration-dependent, positive inotropic and coronary vasodilatory effect within isolated Langendorff perfused hearts. Although the positive inotropic mechanism of CO remains unclear, CO can interact with myofilament proteins such as troponin C. While this action has not been examined in cardiomyocytes, we proposed that the positive inotropic effect of oCOm-21-derived CO may result from a CO-mediated increase in cardiac myofilament Ca^{2+}sensitivity.

Methods. Rat left ventricular cardiomyocytes were skinned, isolated, and attached to micromanipulators connected to a force transducer within a micro-organ bath at 15 °C. Force was measured as a function of pCa (-log [Ca^{2+}]) in the range of pCa 9.0-4.5 under 3 conditions: oCOm-21 at 3 and 10 μM, and the non-CO releasing drug control, BP-21 at 10 μM. Treatments were blinded, and randomly applied either before or after the control force-[Ca^{2+}] measurements, allowing both a control and treatment full force-[Ca^{2+}] sigmoidal curve to be generated within the same preparation. The Ca^{2+}concentration at which 50% of maximal force is produced (pCa_{50}), was then derived from each condition the preparation underwent, and was used to measure a change in Ca^{2+}sensitivity. Cardiomyocytes which declined in force by 15% or had a sarcomere length out of the range of 2.1-2.3 μm, were discarded from the analysis. Results were analysed using a paired Student's t-test where a significant result is considered when $P < 0.05$.

Results. Addition of oCOm-21 at 10 μM (pCa_{50} 5.37 – 5.419, oCOm-21 vs control; $P < 0.001$) but not 3 μM (pCa_{50} 5.375 – 5.398, oCOm-21 vs control; $P > 0.05$), increased myofilament pCa_{50} compared to the respective controls. Additionally, BP-21 at 10 μM did not significantly affect myofilament pCa_{50} compared to control (pCa_{50} 5.417 – 5.44, BP-21 vs control $P > 0.05$).

Discussion. These preliminary results support the hypothesis that CO release by oCOm-21 directly enhances Ca^{2+} sensitivity of the cardiac contractile filament. Importantly, BP-21 at 10 μM had no impact upon the pCa_{50}, indicating that CO release and not the drug structure, is responsible for this sensitizing effect. Interestingly, although oCOm-21 at 3 μM was shown to produce inotropy in the intact perfused rat hearts, we detected no change in pCa_{50} in the skinned cardiomyocytes. It is possible that oCOm-21 concentrations below 10 μM do not release sufficient CO to induce a Ca^{2+} sensitization effect.

A Binary Mechanical Model System: The Simple Physical Chemistry of Cardiac Pressure Volume Loops

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With no thermodynamic constraints and absent thermodynamic processes such as adiabatic and isothermal reactions, molecular mechanics provides the formal foundation for most models of force generation in biology. Here, I assume that thermodynamics applies to physiological systems and consider the simplest possible model of force generation by molecular motors: a binary mechanical system. This intriguing statistical mechanical problem remains unsolved even though molecular motors function as binary mechanical systems, i.e., mechanical switches equilibrated with a system force. Here, I develop the mechanics, energetics, and kinetics of this system and show that a simple binding reaction can occur through adiabatic, isothermal, and irreversible processes that together directly account for complex physiological behaviors such as the beating of the heart. The simple model unifies disparate models of molecular motor mechanochemistry, accounts for the mechanical performance of muscle in both transient and steady states, and provides new perspectives on biomechanics with a focus here on how muscle and molecular motor ensembles work.

Sinusoidal Analysis as a Tool to Understand and Differentiate Small Molecule Myosin Modulators Developed for Cardiovascular Disease Treatment

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Small molecule sarcomere modulators have been gaining attention as clinical candidates to treat heart failure and related disease. Two myosin binding molecules are currently awaiting FDA approval for treating HCM or HFrEF patients. As these and future therapeutics advance, it is imperative to fully understand their effects on myosin contractile kinetics and force production.

For years, sinusoidal analysis on skinned muscle fibers has been a useful tool to detect biochemical rates of the cross-bridge cycle in myofilaments under load. We designed and implemented a software program compatible with aurora scientific hardware and software to evaluate the effects of oscillatory length changes on porcine cardiac muscle force production, elastic and viscous modulus, and myosin kinetics using the conventional ABC model originally developed by Kawai and Brandt.

Skinned papillary fibers from healthy minipigs were set to sarcomere length 2.0 μm and incubated with one of the following small molecules: a myosin inhibitor, a myosin activator, a thin filament inhibitor, blebbistatin or a DMSO control. Under maximum [Ca2+] activation, each fiber underwent sinusoidal analysis (0.125% ML, 0.1-20 Hz) with both DMSO and a sarcomere modulator.

The myosin inhibitor and blebbistatin behaved similarly, showing decreases in both elastic and viscous moduli at frequencies above 3.5 Hz. Decreases in the B and C values were also observed suggesting an overall decrease in myosin head engagement, as well as slowed force producing kinetics with a reduction in $2\pi b$. In addition, $2\pi c$ decreased, but not significantly (9% and 12% for the inhibitor and blebbistatin, respectively). The thin filament inhibitor showed very little change in myosin kinetics, and showed small, but non-significant reductions in B and C values. This is likely due to the thin filament inhibitor predominantly decreasing calcium sensitivity, but not affecting myosin kinetics at maximum [Ca^{2+}] activation.

The most dramatic changes observed were with the myosin activator. The activator increased B and C values 3.5-fold, even though raw maximum tension only increased by 12%. The activator also slowed cross-bridge kinetics, reducing $2\pi b$ and $2\pi c$ by 59% and 77%, respectively. Total myosin kinetics were also slowed as shown by a 72% decrease in elastic modulus dip frequency. These kinetic changes were highlighted by leftward shifts in the elastic and viscous modulus traces.

This methodology highlighted the differences in mechanism between myosin and thin filament inhibitors and showed the potent effect a myosin activator had on myosin kinetics and head engagement. This is the first study to our knowledge performing sinusoidal analysis on porcine cardiac tissue. It is a valuable tool to investigate the mechanistic changes incurred by a sarcomere modulator in large animal models for advancing cardiac disease treatment.

Smooth Muscle Myosin Monomer Pool is Dynamic

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Vascular smooth muscle cells (SMCs) line blood vessels throughout the body, where they dynamically alter vessel diameter to regulate blood pressure, provide structural integrity, and absorb shock on a beat-to-beat timescale. Interactions between actin and the molecular motor smooth muscle myosin 2 (SM2) drive contractility within the cell and allow smooth muscle to perform these vital functions. As smooth muscle function fails, profound vascular disease can ensue with tragic results- even death. Integral to SM2's ability to function is the formation of SM2 filaments and the interaction with these filaments with actin. Although smooth muscle contraction is integral to health, there are many unknowns about SM2 filament assembly and dynamics and the role these dynamics play in SMC contraction. Our goals were first, to determine equilibrium kinetics between SM2 monomer and filaments, and second, to determine if this equilibrium is shifted during SMC activation to drive contraction. Fluorescence recovery after photobleaching (FRAP) of SM2 in A7R5 (rat aortic smooth muscle cells) shows SM2 in filaments are highly dynamic, with recovery halftimes on the order of 10s of seconds with SMC activation stabilizing filaments. The prevailing model in SMC contraction suggests that activation of existing filaments leads to cell wide contractile forces. However, our data and recent literature demonstrate a significant SM2 monomer pool (~25% of total SM2). By using an imaging-based triton permeabilization assay, the percent of filamentous and monomeric SM2 can be measured. Our results indicate the monomer pool decreases while filamentous myosin increases upon SMC activation. This indicates the monomer population is tunable, with additional monomers being recruited into filaments upon activation of SMCs by carbachol. Traction force microscopy in A7R5 cells transfected with a calcium sensor confirms that the increase in SM2 filament formation coincides with both intracellular Ca response to a stimulus and increase in force production. This supports a model of SMC contraction in which both existing filaments are activated and new filaments are formed to produce cell level contractile forces.

Molecular Study of the Effect of Omecamtiv Mecarbil on the Recovery Stroke and ATP Hydrolysis step of Human Cardiac Beta Myosin using Rare Event Methods.

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Omecamtiv Mecarbil (OM) is a cardiac myosin specific allosteric effector designed to enhance the contractile performance of the cardiac muscle. The drug directly binds to the motor domain of the myosin and is observed to affect the steps of the cross-bridge cycle central to the muscle regulation including the recovery stroke and ATP hydrolysis step. Experimental studies have shown that OM slows down the rate of the reverse recovery stroke and shifts the equilibrium towards the pre-powerstroke state as well as shifting the equilibrium of the ATP hydrolysis towards the ADP.P_i state, but little has been known at the molecular level. In this study, we sampled the transition of the OM-bound post-rigor to the OM-bound pre-powerstroke state using the method of Metadynamics and also generated unbiased thermodynamic ensemble of reactive trajectories for the ATP hydrolysis step utilizing Transition Path Sampling method to explore the effect of OM on the recovery stroke as well as the chemical step. Our results were able to predict the changes to dynamics of the recovery stroke caused due to OM as well as showed a similar trend to that of the experimental data. We were also able to observe the alterations in the active site as well as the pathway of the breakdown of ATP to ADP and HPO_4^{2-} and the transition states involved due to the presence of OM. Moreover, we have extended these methodologies to study mutant forms of myosin causing genetic cardiomyopathies: R712L, F764L and P710R to detect the variation in both the steps due to point mutations in presence of OM. This will help provide a molecular understanding of the effect of OM on the primary steps of the chemomechanical cycle of human cardiac beta myosin and hence, further motivate us to expand this approach to other key transitions affected by OM.

Mechanisms of postprandial cardiac growth in pythons

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Pythons are infrequent feeders that can ingest meals equal to their own body mass. The extreme metabolic response required to digest such large meals is associated with a dramatic increase in metabolism and the mass of most organs, including the heart. The functional effects of feeding have not been reported in python cardiomyocytes or myofibrils, limiting our understanding of the mechanisms responsible for the extreme post-prandial cardiac adaptation in reptiles. Here, we studied the cardiac transcriptome, ultrastructure, and function of fasted and 24h post-fed ball pythons (*Python regius*). After feeding, python hearts showed a ~28% increase in mass and a ~31% decreased in stiffness. Unexpectedly, electron microscopy revealed reduced cardiac myofibril area and RNAseq analysis showed downregulation of sarcomere-related gene transcripts. Conversely, maximal force generation was increased in myofibrils from 24h post-fed pythons as compared to pythons fasted for 28 days. Myofibrils also showed prolonged relaxation time and reduced passive tension. Proteomic analysis of myofibrils and titin separating gels indicated that myosin and titin isoforms were not different between 24h post-fed and fasted ball pythons, but post-translational modifications were different between fasted and post-fed hearts. Ca^{2+} transients of isolated cardiomyocytes from 24h post-fed pythons were prolonged, with increased time-to-peak and slower Ca^{2+} decay. Myofibril Ca^{2+} sensitivity and peripheral coupling area identified by electron microscopy were not different between 24h post-fed and fasted ball pythons. Compared to fasted, cardiomyocytes isolated from 24h post-fed pythons produced more ATP via oxidative phosphorylation accompanied by activation of AMP-dependent kinase and increased expression of fatty acid oxidation genes. Additionally, metabolomics analysis indicated increased aminoacyl-tRNA biosynthesis and amino acid metabolism in 24h post-fed python hearts. These results suggest that feeding promotes positive inotropy in python hearts by means of prolonged Ca^{2+} transients and increased myofilament force generation supported by increased energy production.

Multiscale Study of Differential Target Activation by Calmodulin Isoforms

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Calmodulin (CaM) serves as an important Ca2+ signaling hub that regulates many signaling pathways. Two such pathways are the calcineurin (CaN)/NFAT pathway that is associated with cardiac hypertrophy, and the myosin light chain kinase (MLCK) pathway that is associated with smooth muscle contraction. In this work, we aim to study the molecular interactions between CaM and its targets, CaN and MLCK, on multiple scales (molecular and cellular) to inform protein engineering strategies for the differential regulation of different targets. Previous studies revealed that CaM modulates CaN function through a primary and canonical CaM binding region, as well as a secondary interaction using the distal helix (DH) of CaN. Furthermore, the DH interaction is a key step for the activation of CaN by CaM. On the molecular level, our biochemical assay showed that soybean CaM isorform 4 (sCaM-4) exhibited reduced activation of CaN. Further, MD simulations indicated that sCaM-4 showed less favorable DH binding. On the cellular level, using a FRET-based sensor of CaN, we found that the kinetics of CaN activation by sCaM-4 is slow relative to mCaM. Using the cellular data, we built a systems model that couples the activation of CaN to the activation of NFAT followed by its translocation. We then introduced Ca2+ transients to simulate electrically-paced cardiac cells. This systems model allowed us to study how the molecular interactions between CaM and CaN are relayed to the downstream cellular signaling, and more importantly, how the differences on the molecular level influence cellular outcomes. Following a similar strategy, we investigated the CaM-MLCK system. We identified several interactions that contribute to CaM-MLCK binding through MD simulations. Furthermore, using a systems model, we showed that compromised MLCK activation leads to reduced contractility in smooth muscle cells.

Computer Simulations of Afterloaded Contraction Experiments with Fast Stretch

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Fast myocardial relaxation is important for cardiac function. Impaired relaxation compromises ventricular filling and leads to diastolic dysfunction. Prior work using intact trabeculae from mice, rats, and humans has shown that relaxation following an afterloaded contraction can be accelerated by a quick stretch. This is termed mechanical control of relaxation.

To investigate the molecular mechanisms underpinning molecular control of relaxation, additional experiments measured the peak stress response to ramp stretches imposed on rat trabeculae. The force responses scaled with the prevailing tension at the initiation of the stretch, and increased with both stretch velocity and stretch amplitude. Force measured after recovery from a stretch was greater than that measured in a paired trial performed without a perturbation. The increased force resulting from stretches imposed early in relaxation could exceed the increase from a late diastolic stretch, which is dominated by passive viscoelastic components. This suggests that the muscle exhibited stretch activation.

Simulations mimicking these experiments were performed using a Huxley-type distribution type model called MATMyoSim. Cross-bridges transitioned between a super-relaxed (SRX), a disordered relaxed (DRX), and a single attached state. The detachment rate of bound heads depended on the load and increased as the heads were pushed in the direction of shortening. This mimics experimental data recorded with single myosin heads via optical trapping.

The simulations mimicked most key aspects of the experimental data. In particular, the stretch responses increased with the prevailing tension and with stretch velocity and stretch amplitude. The model also exhibited stretch activation due to the quick stretches pulling bound heads to positions where they had a lower detachment rate. While this simulation did mimic key aspects of the experimental data, the fitting has not yet been performed.

These simulations suggest that cross-bridges can have a significant impact on diastolic function in the heart.

Contraction-Relaxation Coupling and Mechanical Dyssynchrony in End-Stage Human Heart Failure

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In twitch contractions of isolated cardiac muscle, we previously observed a strong coupling between the maximal speed of contraction and the maximal speed of relaxation when assessed under physiologically relevant conditions. In multicellular preparations of various murine models and of normal wild-type rats these maximal speeds of contraction and relaxation are very strongly coupled (R=0.99). When the twitch is modulated by length-dependent activation, frequency-dependent activation, or beta-adrenergic stimulation, or any combination thereof, this coupling persists. This coupling also extends to other kinetic parameters; time to peak tension correlates very strongly (R=0.99) with time from peak tension to 50% relaxation. In larger mammals, such as the rabbit and the dog, this coupling also exists, and is equally strong (R=0.99). Moreover, coupling appears to be unaffected by disease or exercise training. Dogs with a myocardial infarction, those trained on a treadmill, or a group receiving both interventions all showed strong coupling of relaxation and contraction.

Since most animal models are in-bred, and reflect a very narrow genetic variability, next we studied multicellular myocardial preparations from human origin. In total, data was collected from 91 end-stage failing human hearts, and from 85 non-failing human hearts over a span of 11 years. Over the range of different muscle lengths and frequencies (both spanning the normal in vivo range), as well as with beta-adrenergic stimulation, the coupling between contraction and relaxation was very strong (R values between 0.95 and 0.99, $P<0.0001$).

Next, we studied if these kinetic parameters were impacted by ventricular origin of the muscle. When stratified to LV and RV data, coupling was strongly present in both ventricles. Intra-ventricular coupling analysis of force and kinetics parameters was performed by correlation of contractile parameters of paired muscles that had been obtained from both the left and right ventricle of the same human heart. A comparison of RV to LV performance was done in 41 pairs of muscles from non-failing hearts, and 29 from failing hearts. In non-failing myocardium, the contractile kinetics of the muscle from the RV strongly correlated to those in the muscle from the LV of the same heart. Time to peak tension, time form peak tension to 50% relaxation, as well as dF/dt correlated strongly ($P<0.001$), indicating that the ventricles of these hearts were mechanically synchronized. In sharp contrast, in muscles from the RV and LV of the failing hearts did not correlate strongly, or even modestly; for the same contractile parameters, no significant correlation was found. This lack of correlation was found both in failing hearts of ischemic etiology, and of non-ischemic etiology. The data thus indicates that in end-stage heart failure the kinetics of both ventricles are not synchronized. This mechanical dyssynchrony may play a critical role in the poor overall cardiac performance in end-stage heart failure.

We conclude that contraction-relaxation coupling is a fundamental cardiac property, that is unaffected by disease, and that mechanical dyssynchrony of the RV and LV could be a fundamental pathological feature of end-stage failing myocardium.

A Computational Model Demonstrates How a Cardiac Myosin Binding Protein-C Mutation Impacts Calcium Sensitivity and Power Output in a Mutant Mice Model

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Cardiac myosin binding protein-C (cMyBP-C) is a sarcomeric protein that is essential for cardiac contraction regulation. Mutations in MyBP-C's cardiac-specific gene (MYBPC3) are a leading cause of hypertrophic cardiomyopathy (HCM). In particular, the L348P substitution (L352P in humans) is an HCM-associated missense mutation that increases binding of cMyBP-C's M-domain to actin. A CRISPR-gene edited mouse model was previously developed, with a ~40% expression of the L348P mutant protein. Permeabilized cardiomyocytes from these L348P-CR heterozygous mice (HET) showed increased Ca^{2+} sensitivity of tension and slowed shortening velocities compared to wild type (WT) mice.

Since cMyBP-C is exclusively located in the C-zone of the sarcomere and interacts with both the thick and thin filaments, spatially-explicit models can help gain insights about its role in cardiac contraction regulation. FiberSim (https://campbell-muscle-lab.github.io/FiberSim/) is a spatially-explicit model of myofilament-level contraction that simulates contractile properties. In this work, FiberSim was used to compare tension-pCa and force-velocity curves for three models, called WT, HET and HOM. For the WT model, cMyBP-C could either stabilize the super-relaxed state of myosin dimers, or bind to actin. For the HET model, 40% of the cMyBP-C had an increased attachment rate for actin compared to the WT model. Finally, a HOM model was generated where 100% of the cMyBP-C had an increased attachment rate for actin compared to the WT model.

The simulation results for WT and HET were in good agreement with experimental data obtained from L348P-CR mice. The HET model showed an increase in calcium sensitivity compared to WT. This can be explained by the increased number of bound cMyBP-Cs, which sustains thin filament activation and allows for more cross-bridges to bind to actin. The HET model also showed a decrease in shortening velocities compared to WT, suggesting that bound MyBP-Cs act like a drag that reduces maximal power output. The HOM model (for which no experimental data were available, as homozygosity is lethal prior to postnatal day 7) showed an even bigger increase in calcium sensitivity and a greater decrease in power output compared to WT. Finally, isometric twitch contractions showed decreased relaxation rates in the HET and HOM models compared to WT. This supports the hypothesis that increased binding of cardiac MyBP-C to actin inhibits normal cardiomyocyte relaxation, and suggests that the L348P missense mutation is incompatible with survival when inherited in the homozygous state.

Understanding Conformational Transitions in the Cardiac Thin Filament Using Metadynamics

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Cardiac muscle contraction is a Ca^{2+} regulated system consisting of a complex cycle of conformational changes throughout the cardiac sarcomere. In a Ca^{2+}-unsaturated state, the cardiac troponin I (cTnI) c-terminal domain is strongly associated to actin, inhibiting contraction by blocking the myosin binding site on actin. Upon calcium binding to cardiac troponin C (cTnC), the troponin core undergoes a conformational rearrangement which exposes a hydrophobic pocket in cTnC to the surrounding solvent, which in turn increases the stabilization of cTnC-cTnI interactions. These rearrangements weaken the relative stability of the cTnI-actin interactions prompting the dissociation of the cTnI c-terminus from actin, ultimately revealing the myosin binding site. From here, myosin binds to actin resulting in contraction of the muscle. However, detailed molecular level understanding of these transitions is limited. Utilizing the rare event sampling method metadynamics, we calculated the free energy surface associated with each transition using our complete atomistic model of the cardiac thin filament. The free energy surface of cTnC conformational opening due to Ca^{2+} binding and cTnI releasing from actin, both in the Ca^{2+}-saturated and -unsaturated states were computed. Results revealed that for the cTnC opening transition, the Ca^{2+}-saturated state is favored compared to a Ca^{2+}-unsaturated state. Additionally, cTnI dissociating from actin is highly favorable when cTnC is in the open conformation compared to the closed conformation. We also identified several key residues for each transition.

PKD regulates myofibrillogenesis and intercalated disc composition

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Introduction:
The intercalated disc (ID) ensures electro-mechanical transduction for unperturbed function between neighbouring cardiomyocytes. Dysfunction of ID proteins has been linked to the development of cardiomyopathies, arrhythmia or early embryonic lethality in mouse models. An early ID protein that impacts myofibrillogenesis, the nebulin-related anchoring protein (NRAP), was found differentially expressed in cardiac disease.

Objectives:
Protein kinase D (PKD) plays a key role in the transcriptional regulation of pro-hypertrophic gene expression by phosphorylation of class II histone deacetylases (HDAC) and promotion of myocyte enhancer factor 2 (Mef2)-driven fetal gene expression, such as NRAP. Concomitantly, a yeast-two hybrid screen of a human cardiac library was performed with the catalytic domain of PKD1 as a bait and revealed NRAP as a putative novel interaction partner. We aimed to investigate the functional relationship between PKD and NRAP in the developing and diseased heart.

Materials and Methods:
Neonatal rat ventricular myocytes (NRVMs) were isolated. PKD activity was increased by exposure to the pro-hypertrophic stimulus phenylephrine (PE), adenoviral PKD overexpression or inhibited pharmacologically with BPKDi, a specific PKD inhibitor. NRVMs were analyzed by immunofluorescence and stable isotope labelling with amino acids in cell culture (SILAC) after modulation of PKD activity to investigate proteomic alterations. The functional consequences were assessed in NRVM-derived engineered heart tissues (EHTs).

Results:
Exposure of NRVMs to PE enhanced myofibrillar maturation and NRAP translocation towards the IDs. Conversely, pharmacological inhibition of PKD activity led to retained perinuclear accumulation of NRAP. Combined exposure to BPKDi and PE prevented ID localization and disturbed sarcomeric structure resembling a fetal phenotype. SILAC/proteomic analysis revealed lower NRAP expression and increased abundance of proteins involved in proteasomal, lysosomal and spliceosomal pathways after BPKDi treatment. Surprisingly, BPKDi exposure of EHTs led to improved force development compared to controls.

Conclusion:
PKD regulates NRAP expression, its translocation towards the IDs during myofibrillogenesis and negatively impacts cardiomyocyte force development. Further investigations aim to gain a better understanding of the molecular pathways goverend by PKD to regulate myofibrillar structure and function in cardiac health and disease.

2-deoxy-ATP Improves Systolic Ventricular Function in a Multiscale Computational Model of Heart Failure

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Introduction: 2-deoxy-ATP (dATP), a candidate therapeutic for heart failure, has been shown to enhance cardiac contractility and lusitropy by acting on myosin to increase both the rate of crossbridge binding and cycling and the rate of Ca^{2+} transient decay. However, the molecular mechanisms behind these effects and how they scale to improvements in cardiac function are not well understood. Furthermore, how cardiomyocyte and ventricular function are improved significantly even when dATP is only a few percent of total cytosolic ATP is not understood. It is also not known how these therapeutic mechanisms apply during heart failure when myocyte energy metabolism is impaired. Here, we used multiscale computational modeling to address these questions.

Methods: We conducted molecular dynamics (MD) and improved Brownian dynamics (BD) simulations to assess (d)ADP.Pi-myosin/actin association rates, overcoming limitations of previous simulations by assessing a broader range of possible myosin conformations. We then utilized these association rates to constrain a sarcomere mechanics model, and combined this with experimental Ca^{2+} transient data to simulate the effects of dATP at the myocyte level. We then utilized a model of rat biventricular mechanics, energetics, and circulation to predict the effects of elevated dATP at the tissue and ventricular scales. Heart failure was simulated by adjusting metabolite levels based on data from failing rats.

Results: BD simulations showed an increase in the myosin/actin association rate with dATP, which resulted in increased Ca^{2+} sensitivity and steady state force. Model predictions also suggested downstream effects of dATP on the crossbridge cycle. We found that even small fractions of dATP can cause large changes in force by disrupting the super-relaxed state of myosin. The integrative effects of dATP on improved Ca^{2+} handling and increased crossbridge binding and cycling augmented myocyte contractility and lusitropy, which resulted in a 15% improvement in ejection fraction with elevated dATP. Consistent with experimental observations, dATP decreased the creatine phosphate/ATP ratio in the model. In a model of failing heart energy metabolism, dATP increased myocardial oxygen consumption and ATPase rates, but not above normal, non-failing levels. dATP also increased energy efficiency (fraction of energy from ATP hydrolysis transferred to ventricular work) in the failing heart model but not in the healthy heart model, suggesting that dATP improves function to a greater extent in heart failure.

Conclusions: In this work, we developed a novel multiscale modeling framework extending from the atomic to full ventricle scale. Our model predictions suggest that the combined effects of dATP on myocyte Ca^{2+} handling and myosin crossbridge state transitions, including increased recruitment of myosin from the super-relaxed state, improve ventricular mechanics and energetics, especially under metabolically inhibited conditions of heart failure.

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The Curse of Dimensionality in Multifilament Modeling

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Computational models in biology often require setting a large number of free parameters using either experimental data for each parameter directly or by fitting simulations to experimental data. However, for many models of complicated systems, like muscle, determining large numbers of free parameters presents considerable challenges. For our recent half sarcomere model consisting of 6 myosin and 4 actin chemo-mechanical rate transitions, we attempted to fit six of the rate parameters to experimental data by comparing simulated force during twitches to experimental twitches taken from mouse cardiac muscle. Using Azure cloud computing, we simulated tens of thousands of combinations of rate parameters in order to estimate the manifold of constant model behavior corresponding to the experimental data – the 'wild type' isotwitch by identifying least-squares fit between simulation and measurements. We expected that the set of rate parameters that best reflect experimentally measured twitches would occupy a relatively small and sparse part of the possible parameter space. However, we instead found many combinations of parameters spanning the entire parameter space which yielded nearly indistinguishable twitches based on least squares comparisons between measured and predicted twitches. This is an example of the 'curse of dimensionality' in which relationships in high dimensional spaces become unintuitive and difficult to describe. Thus, we turn to dimensionality reduction techniques around the optimal parameter set and describe how model twitches change, but only in a local area. Our ultimate goal is to be able to describe 'isotwitch' manifolds in a high dimensional space of model outputs. This approach would allow us to identify ways in which adjustments in one aspect of the chemomechanical system can compensate for deficits in another.

MgADP Augments Passive Force and Ca^{2+}-sensitivity of Contraction in Porcine Myocardial Strips

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Myosin cross-bridges dissociate from actin following Mg^{2+}-adenosine triphosphate (MgATP) binding. Myosin hydrolyses MgATP into inorganic phosphate (P_i) and Mg^{2+}-adenosine diphosphate (ADP), and release of these hydrolysis products drives chemo-mechanical energy transitions within the cross-bridge cycle to power muscle contraction. Some forms of heart disease are associated with metabolic or enzymatic dysregulation of the MgATP-MgADP nucleotide pool, resulting in elevated cytosolic [MgADP] and impaired muscle relaxation. We investigated the mechanical and structural effects of increasing [MgADP] in permeabilized myocardial strips from porcine left ventricle samples. Sarcomere length was set to 2.0 μm at 28°C for all measurements, and all solutions used 3% dextran T-500 to reduce myofilament lattice spacing to near physiological values. Under relaxed conditions (pCa 8.0) passive tension increased as [MgADP] increased from 0-5 mM. Complementary small-angle x-ray diffraction measurements show that the $I_{1,1}/I_{1,0}$ intensity ratio also increased as [MgADP] increased from 0-5 mM, signifying myosin heads moving away from the thick-filament backbone towards the thin-filament concurrently with loss of helical ordering as indicated by decreasing intensity of myosin based layer lines. Ca^{2+}-activated force-pCa (= $-Log_{10}[Ca^{2+}]$) measurements show that Ca^{2+}-sensitivity of contraction increased with 5 mM [MgADP], compared to 0 mM [MgADP]. Altogether, these data suggest that MgADP augments passive tension and Ca^{2+}-sensitivity of contraction, which follow from MgADP destabilizing the myosin OFF state and shifting the population of cross-bridges towards the myosin ON state to enhance the probability of cross-bridge binding and increase cooperative cross-bridge contributions to dual-filament activation.

Rare Event Simulations for Thin Filament and Myosin Systems

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Both the thin and thick filaments of striated muscle have complex biochemical cycles that combine chemical potential to produce the observed mechanical action. However, due to the complexity of these systems as well as their integration with the remaining proteins of the sarcomere, there still remains gaps in the understanding of these complexities at the molecular scale. Computational studies utilizing molecular dynamics (MD) simulations have been performed to provide this molecular insight, however limitations of MD largely allows for the resolution of protein dynamics on the timescales of femtoseconds (*fs*) to nanoseconds (*ns*) and other processes that may occur on this timescale while having small thermodynamic barriers. To escape these constraints, enhanced sampling (ES) techniques can be applied in conjunction with MD simulations to further increase conformational and energetic sampling. To examine the effectiveness of ES methods, the blocked to myosin-state transition of tropomyosin (Tm) was sampled in an atomistic representation of the cardiac thin filament (cTF) with metadynamics, mimicking the initial myosin binding event. The free energy surface was determined along the transition and determined differences in the required work due to the influence of cardiac troponin T(cTnT). Within myosin, the mechanism of adenosine triphosphate (ATP) hydrolysis was determined computationally with another ES method, transition path sampling, within *Dictyostelium* myosin. Glutamate 459 was observed to play an active role in the chemical event that was missing in previous examinations with the remaining residues stabilizing a metaphosphate intermediate formed along the reaction coordinate. These studies and others allow for a clearer determination of native function and how it maybe perturbed with small drugs and/or mutations.

Destabilizing of putative tropomyosin-troponin-I interactions increase calcium sensitivity

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The cardiac thin filament, troponin (Tn), consisting of TnC, TnI, and TnT, and tropomyosin (Tpm) bound to filamentous actin, is the primary transducer that relates sarcoplasmic calcium levels to the contraction-relaxation cycles of the myocardium. Calcium binding to TnC propagates to induce a shift in Tpm, allowing for actomyosin interactions, which powers contraction with each heartbeat. While the general structure of the thin filament has been well characterized, vital regions of the communication pathway where Tn triggers Tpm movement remains unresolved. Specifically, helix 4 of the TnI C-terminus, thought to force Tpm in a position on actin to promote muscle relaxation, has only been determined at low resolution. Recent work from our collaborative team, using cryo-electron microscopy data coupled to computational chemistry, has provided insight to this region at molecular resolution which predicted a vital, electrostatic Tpm-TnI interaction at amino acid-residue level (Tpm-E139 with TnI-R170 and K174). By mutation Tpm-E139 to an opposite-charged lysine (E139K), we used mutation-induced alterations to determine Tpm-E139's role in maintaining the blocked-state during thin filament regulation. With *in vitro* motility assays, we examined the effects on thin filament activation and regulation with Tpm-E139K. Our results reveal an increased calcium sensitivity as the velocity of thin filaments shows a significant increase compared to the wild-type Tpm. Further, our data demonstrates the thin filament's inability to turn off completely at low Ca^{2+} in the presence of the E139K mutation. This suggests a potential disruption between a pivotal Tpm-TnI interaction that modulates cardiac thin filament regulation. Continued structural testing, molecular dynamic simulations and circular dichroism, is being performed in order to assess the structural changes introduced by the E139K mutation. Collectively, this study provides insight on the molecular mechanism in which Tpm and TnI interact to regulate cardiac muscle contraction at a residue level.

Identification and Characterization of a Novel Third Tropomyosin-Binding Site in the C-Terminal End Segment of Troponin T

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Troponin T (TnT) is the tropomyosin (Tm)-binding subunit of troponin. Previous studies have identified two Tm-binding sites in TnT, a T1 site in the middle region and a site at beginning of the T2 fragment (Jin & Chong, *Archives Biochem Biophys* 500:144-50, 2010). A Ca^{2+}-regulated Tm-binding site is recently found in the C-terminal end segment of troponin I (TnI) with inhibitory function (Wong et al, *JMCC* 136:42-52, 2019). Based on the common ancestry of TnT and TnI, a monoclonal antibody raised against the C-terminal end segment of TnI detected a similar epitope structure in the C-terminal end segment of TnT. Following this lead, we identified a third Tm-binding site in the C-terminal end segment of TnT. Using engineered TnT and peptide constructs, we first mapped the TnI-like structure to the C-terminal end 14 amino acids of cardiac TnT. Protein binding studies using mini-TnT constructs excluding the two previously known Tm-binding sites and C-terminal peptide fused with a carrier protein then demonstrated Tm-binding at submicromolar affinity. Function of the novel third Tm-binding site as a TnI C-terminal segment-like inhibitory structure is supported by a previous report that deletion of the C-terminal end segment of TnT resulted in a loss of troponin inhibitory regulation in reconstituted myofilament (Franklin et al, *Biophys J* 102:2536-44, 2012). Our finding also provides a molecular mechanism for the previous observation that restrictive N-terminal truncation of cardiac TnT in adaptation to acute ischemia or pressure overload reduces ventricular end systolic velocity to prolong ejection time and sustain stroke volume (Feng et al, *J Physiol* 586:3537-50, 2008) via conformational modulation that activates the TnI-like Tm-binding function. In contrast to the structural and coordination roles of the two constitutive Tm-binding sites 1 and 2, the third Tm-binding site of TnT represents a novel thin filament regulatory mechanism. Further studies are currently in progress to investigate the physiological function and Ca^{2+}-regulation of the TnT C-terminal peptide.

The cooperativity in thin filament activation of skeletal muscle is modulated by the force of the myosin motor

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Contraction of striated muscle is regulated by a dual mechanism involving both the thin, actin-containing filament and the thick, myosin-containing filament. Thin filaments are activated by Ca^{2+} binding to troponin leading to tropomyosin displacement. In this way actin sites are exposed for interaction with myosin motors extending from the neighbouring stress-activated thick filaments. Motor attachment to actin contributes to spreading activation along the thin filament, through a cooperative mechanism, still unclear, that determines the slope of the sigmoidal relation between isometric force and pCa (-log[Ca^{2+}]), estimated by Hill coefficient n_H. We use sarcomere-level mechanics in demembranated fibres isolated from rabbit soleus muscle activated by Ca^{2+} at different temperatures (12-35 °C) to show that, for a constant number of attached motors, n_H depends on the force of the attached motor (F_0). In the presence of the β/slow-myosin isoform effector omecamtiv mecarbil (OM), no-force generating OM-bound-motors inhibit cooperativity induced by force-generating motors. Extending the n_H -F_0 relation in control to higher F_0 values with data from fast skeletal muscle reveals that the cooperative mechanism that relates n_H to F_0 is unique for the two muscle types, and saturates at high values of F_0 according to a two-step process with a Michaelis-Menten kinetics.

Impaired Inhibition and Cooperative Ca^{2+}-Activation of Cardiac Thin Filament Sliding Suggests Altered Diastolic Function after Thrombin Removes Human cTnT N-terminus

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The most N-terminal "tail" portion of the troponin T (TnT) subunit has received less attention than the rest of troponin (Tn) and has not yet been resolved in X-ray structures of Tn or cryo-EM structures of the cardiac thin filament. Existing cryo-EM structures of cardiac thin filaments show, in diastole (low Ca^{2+}), the TnT tail extends one way from Tn core domain and crosses from one tropomyosin (Tm) strand to the other, while the C-terminus of troponin I (TnI) extends in the other direction along Tm toward the next Tn.[1,2] Functionally, the N-terminal tail peptide of either skeletal or cardiac TnT inhibits actomyosin in the absence of the rest of Tn. We therefore hypothesized that TnT's tail is required for full inhibition in diastole, and cooperativity of Ca^{2+}-activation would be altered when TnT's tail is absent. We tested these hypotheses by examining the functional role(s) of the first 68 amino acids of human cTnT. Human cardiac Tn subunits were co-expressed in *E. coli* and assembled Tn complexes (rhcTn) were purified. RP-HPLC and N-terminal sequencing demonstrated that the N-terminal 68 amino acids of human cTnT are removed by thrombin proteolysis of rhcTn at one site (rhcTnΔ68). Unlike rhcTn,[3] rhcTnΔ68 does not enhance myosin (HMM) MgATPase in solution in the absence of F-actin-Tm. rhcTnΔ68, like rhcTn, co-sediments with F-actin-Tm to reconstitute thin filaments (TF-rhcTnΔ68, TF-rhcTn). TF-rhcTnΔ68 sliding in motility assays was only partially inhibited at diastolic Ca^{2+}, unlike TF-rhcTn. Speed-pCa slope was less steep (n_{Hill}<1) for TF-rhcTnΔ68 compared to TF-rhcTn. Maximum sliding speed (high Ca^{2+}) was similarly faster for both TF-rhcTnΔ68 and TF-rhcTn compared with F-actin. We conclude the N-terminal tail of cTnT functions with cTnI's C-terminus to fully inhibit actomyosin interactions in diastole, while the full cTnT tail is necessary for cooperative activation of actomyosin interactions.

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Central and C-terminal Domains of Cardiac Myosin Binding Protein C Modulate Contractility in the Absence of N-terminal Domains

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Cardiac myosin binding protein c (cMyBPC) is a multi-domain sarcomeric protein that is integral to the regulation of cardiac function. The N-terminal portion of the protein consisting of domains C0 through C2 has been extensively investigated and is known to play a role in cMyBPC's modulation of cardiac muscle function. Recent studies have demonstrated that Adeno-Associated Virus (AAV9) mediated exogenous expression of C0-C2 in cMyBPC null (cMyBPC -/-) hearts is sufficient to ameliorate systolic and diastolic cardiac dysfunction by normalizing hypercontractile cross-bridge kinetics, thereby contributing to prolongation of ejection time, which is significantly abbreviated in the absence of cMyBPC. In contrast to the N-terminus of cMyBPC, relatively little is known about the functional roles of the central and C-terminal portions of cMyBPC. There is growing interest in domains beyond C0-C2 as there is evidence that other regions of the cMyBPC molecule have sites for post-translational modification that may alter structure and function. However, the functional relevance of these domains in vivo has yet to be fully explored. In this study, we examine the effects of N-terminal truncated cMyBPC on cardiac structure and function. Within 36 hours of birth, cMyBPC -/- mice were injected with saline or with AAV9 encoding either full length cMyBPC (FL) or N-terminal truncated cMyBPC domains C3 through C10 (C3C10). Six weeks post injection, in vivo cardiac function was assessed by transthoracic echocardiography. Presence and appropriate localization of exogenous cMyBPC in the sarcomere was confirmed by Western Blot and immunohistochemistry, respectively. Echocardiograms were analyzed to obtain conventional and speckle tracking-derived strain measurements indicative of systolic and diastolic function and morphology. Our results indicate that incorporation of cMyBPC domains C3C10 into the sarcomere yields improvements in systolic function, however, by a mechanism that is distinct from the reported effects of C0-C2. These findings support the existence of cMyBPC-mediated regulation that is independent of known N-terminal regulatory mechanisms.

Functional Implications of experimentally varying thin filament length in fast skeletal muscle of the mouse

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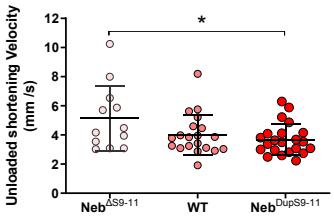
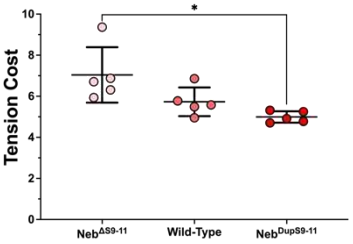
It is generally assumed that strict thin filament length control in skeletal muscle is functionally important, but if this is indeed the case and why is not well understood. Here we took advantage of genetic models that have different thin filament length (TFL) and studied the EDL muscle, a fast twitch muscle rich in type IIB fibers. We used the Neb$^{\Delta S9\text{-}11}$ model with 3 deleted nebulin super repeats and a TFL of 980 nm, and the Neb$^{DupS9\text{-}11}$ model with 3 super-repeats added and a TFL of 1193 nm and compared data with those of WT EDL muscle where the TFL is 1088 nm. We previously showed that these models have normal body weights, muscle weights and myosin isoform composition (Kiss et al., 2020).

Because of the differences in thin filament length, the optimal sarcomere lengths range for force development is predicted to be 1.81-2.11 µm in the Neb$^{\Delta S9\text{-}11}$ model, 2.03-2.3 µm in WT and 2.236-2.536 µm in the Neb$^{DupS9\text{-}11}$ model. Because of these differences, at the optimal length the number of sarcomeres per unit fiber length will vary: compared to WT. The Neb$^{\Delta S9\text{-}11}$ have 10.2% more sarcomeres in series per unit fiber length, while the Neb$^{DupS9\text{-}11}$ model had 8.3% fewer sarcomeres in series per unit fiber length. Because the shortening speed of sarcomere in series is additive, it is predicted that the shortening speed will be highest in fibers with the shortest TFL. We tested this prediction using maximally activated single fibers (type IIB EDL fibers) and the slack test (Edman, 1979). Results show that the maximal shortening speed is higher in Neb$^{\Delta S\ 9\text{-}11}$ mice than in Neb$^{DupS9\text{-}11}$ mice (see Figure 1).



Variation in the number of sarcomeres per unit length of fiber at the optimal sarcomere length, is also expected to alter the tension cost (ATP utilization/unit force) with the highest tension cost in the Neb$^{\Delta SR\ 9\text{-}11}$ model and lowest in the Neb$^{DupS9\text{-}11}$ model. (Forces of sarcomeres in series are not additive and more sarcomeres per unit length will therefore increase tension cost). To test this, we measured tension cost in muscle strips isolated from the EDL muscle. Using an enzyme coupled reaction where the regeneration of 1 ATP is coupled to the oxidation of 1 NADH to NAD^+ with the change in the concentration measured by the NADH absorbance at 340 nm. The rate of ATP consumption, and Tension development, is measured as a function of free calcium and the resulting linear relationship between Tension (X-axis) and ATPase (Y-axis) gives us the Tension Cost. Results show that the tension cost is significantly higher in Neb$^{\Delta S9\text{-}11}$ mice than in the Neb$^{DupS9\text{-}11}$ model (see Figure 2).



We conclude that variation in the thin filament length has functional implication at the level of the muscle fiber, with shorter than normal thin filaments resulting in higher shortening speeds and higher tension cost and conversely, longer than normal TFL resulting in lower shortening speeds and lower tension cost. Slow muscle fibers are known to have longer thin filaments than fast muscle (Gokhin, Kim et al. 2012) and the present findings show that this allows them to generate force at a lower tension cost. Mutations in sarcomeric proteins that alter thin filament length are thus expected to alter the shortening speed and energetics of skeletal muscle fibers which could trigger myopathy.

Exploring the Super-relaxed State of Myosin in Myofibrils from Fast-twitch, Slow-twitch and Cardiac Muscle

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Muscle myosin heads, in the absence of actin, have been shown to exist in two states, the relaxed (turnover ~0.05 s^{-1}) and super-relaxed states (SRX, ~0.005 s^{-1}) using a simple fluorescent ATP chase assay (Hooijman, P. et al (2011) *Biophys. J.* **100**, 1969–1976). Studies have normally used purified proteins, myosin filaments or muscle fibers. Here we use muscle myofibrils, which retain most of the ancillary proteins and 3-D architecture of muscle and can be used with rapid mixing methods. Recording time scales from 0.1 – 1000 sec provides a precise measure of the two populations of myosin heads present in relaxed myofibrils. We demonstrate that:
1.The population of SRX states is formed from rigor cross bridges within 0.2 sec of relaxing with fluorescently labelled ATP, We argue that the data are incompatible with a thermal equilibrium between myosin heads in the SRX and those in the DRX (disordered relaxed) states.

2. The population of SRX states is relatively constant over the temperature range of 5 °C – 30 °C. This result is incompatible with the temperature dependence of the order/disorder transition of myosin thick filaments in muscle which become disordered at low temperature.

3. The SRX population is enhanced in the presence of mavacamten. Slow-twitch and cardiac muscles myofibrils are more sensitive to mavacamten (K_d < 5 μM) than fast twitch myofibrils (30 μM), and mavacamten induced a larger increase in the population of the SRX state in slow twitch and cardiac myofibrils.

4. Mavacamten is much less effective at stabilizing the SRX state at physiological temperatures than at 5 °C. Suggesting that mavacamten may not affect the SRX in normal or wt myofibrils but reverse the effect of any SRX destabilizing mutation such as those associated with HCM.

5. The SRX population is reduced in the presence of deoxy-ATP consistent with the ability of deoxy-ATP to enhance crossbridge formation and the power output of cardiomyocytes.

6. These assays require small quantities of myofibrils (~ 10 μg of myosin in myofibrils), making them suitable for studies of model organism muscles, human biopsies, and human patient derived iPSCs.

Fast and slow-twitch skeletal muscle exhibit different structural dynamics during twitch and tetanic contraction

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Slow muscle and fast muscle are two different muscle types composed primarily of slow-twitch fibers and fast-twitch fibers, respectively. Structural dynamics studies of muscle have focused predominately on fast muscle or mixed-fiber muscle with slow muscle being much less studied. Here, we use time-resolved x-ray diffraction to investigate the dynamic behaviors of the myofilament proteins in relatively pure slow fiber rat soleus and pure fast fiber rat extensor digitorum longus (EDL) muscle during twitch and tetanic contraction at optimal length (L_o). During twitch contractions, the spacing of the third myosin-based meridional reflection (S_{M3}) increased 0.23% while the intensity of first myosin-based layer line (I_{MLL1}) decreased 26.6% indicate a transition of myosin heads from OFF to ON states. In soleus muscle, however, there was no significant change of S_{M3} (0.04%) and I_{MLL1} (3.3%) from rest to peak-tension. These data indicate that thick filament OFF to ON occur in EDL muscle but not in soleus muscle during twitches. To further investigate the structural basis of these different response to twitch stimulation in soleus as compared to EDL muscle, we also examined the structural responses during tetanic contraction. The spacing of the sixth myosin-based reflection (S_{M6}) which reports the axial periodicity of the thick filament increased 1.3% in EDL muscle but only increased 0.7% in soleus muscle comparing resting muscle to the plateau of tetanic contraction. The thin filament periodicity, however, increased by similar amount (~0.4%, p = 0.1) in both EDL and soleus muscle, indicating that the same amount of force was exerted on the myofilaments and that the difference of thick filament extensibility between soleus and EDL muscle is an intrinsic property of the thick filaments in the two muscles. The change in S_{M3} in response of active tension showed a sigmoidal shape in EDL muscle indicate a stepwise transition of myosin heads from the OFF to ON, while a linear relationship was observed in soleus muscle. These data indicate that while EDL exhibits the classic mechano-sensing mechanism where the stress imposed onto the thick filament is sufficient to recruit the myosin heads from OFF states to ON states during both twitch and tetanic contraction, thick filaments in soleus muscle appear to require a different mechanism.

Activation of Myosin Motors in Fast-Twitch Mouse Skeletal Muscle is Controlled by Mechano-sensing in the Myosin Filaments

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Myosin motors in resting muscle are inactivated by folding against the backbone of the myosin filament in an ordered helical array and must be released from that conformation to engage in force generation. Time-resolved X-ray diffraction from single fibres of amphibian muscle showed that myosin filament activation could be inhibited by imposing unloaded shortening at the start of stimulation, suggesting that filaments were activated by mechanical stress. Here, we improved the signal-to-noise of that approach using whole extensor digitorum longus (EDL) muscles of the mouse. Data were collected at the I22 beamline of the Diamond Light Source synchrotron facility where 2D X-ray diffraction patterns were collected on a Pilatus 2M detector in 5-ms frames at a camera length of 8.26m. EDL muscles were stimulated to contract tetanically at 28°C, with 10-ms unloaded shortening applied at the start of stimulation. Changes in X-ray signals associated with myosin filament activation, including the decrease in the first-order myosin layer line associated with the helical motor array, increase in the spacing of a myosin-based reflection associated with packing of myosin tails in the filament backbone and increase in the ratio of the 1,1 and 1,0 equatorial reflections associated with movement of motors away from the backbone, were delayed by imposing 10-ms unloaded shortening at the start of stimulation. These results show that myosin filaments are predominantly activated by filament stress, as in amphibian muscle. However, a small component of filament activation at zero load was detected, implying an independent mechanism of partial filament activation. X-ray interference measurements indicated a switch-like change in myosin motor conformation at the start of force development, accompanied by transient disordering of motors in the regions of the myosin filament near its midpoint, suggesting that filament zonal dynamics also play a role in its activation.

Potential Ca^{2+} Buffering Function of the Glu-rich N-terminal Segment of Troponin T in Cardiac Muscle

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The N-terminal hypervariable region of vertebrate troponin T (TnT) is rich in Glu residues especially in cardiac TnT and avian flight muscle TnT. Insect TnT also has a Glu-rich long C-terminal extension. Previous studies found that the N-terminal segment of avian flight muscle TnT binds Ca^{2+} with physiologically relevant affinity (Zhang et al., *Biochemistry* 43:2645-55, 2004). Deletion of the C-terminal extension of *Drosophila* TnT significantly altered muscle and heart functions (Cao et al., *JBC* 295:3794-807, 2020). Here we investigated the hypothesis that the Glu-rich segments of TnT serve as a myofilament Ca^{2+} buffer/reservoir to reduce the amount of Ca^{2+} cycled by Ca^{2+} pumps during muscle contraction and relaxation. A CRISP/Cas9 knock-in (KI) mouse model was constructed to replace the N-terminal variable region of cardiac TnT with 51 Glu residues derived from the C-terminal extension of bee TnT. Cardiac function is studied using ex vivo working hearts. At baseline, 51E-cTnT hearts showed lower systolic velocity than WT control. Raising [Ca^{2+}] in the perfusion buffer from 2.25 mM to 5 mM had similar positive effects on functions of 51E-cTnT hearts to with that of WT control but the difference remains. While 51E-cTnT and WT hearts both had positive responses to isoproterenol or Bay-K8644 treatment, 51E-cTnT hearts exhibited significantly more increases in ventricular peak systolic pressure and diastolic velocity than WT controls but stroke volume remained lower. 51E-cTnT hearts showed no change in LVEDV but LVESV is larger, resulting in lower ejection fractions in comparison to WT control. These phenotypes are likely from the added Ca^{2+} buffering effect on troponin response to intracellular calcium transient. Analysis of Ca^{2+} regulation of force in skinned papillary muscle sections showed decreased Ca^{2+} sensitivity in 51E-cTnT hearts especially at higher [Ca^{2+}]s. Isolated 51E-cTnT mouse cardiomyocytes showed significantly shorter resting sarcomere length, which is correctable in with BDM. The results suggest that the potential Ca^{2+} buffering capacity of TnT may play a role in contractile and relaxation kinetics, potentially more important in rhythm contractions of cardiac and flight muscles.

Differentiating the effects of age and exercise on calcium sensitivity of force and troponin-C conformational changes during isometric contraction in MHC I and II fibres from healthy young, healthy elderly cyclists and elderly hip fracture patients.

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Introduction: Ca^{2+} dependent structural changes in troponin are an essential step in controlling the interaction of myosin with actin during contraction in skeletal muscle. Whether ageing and/or exercise affects the Ca^{2+} sensitivity (pCa_{50}) of force production and troponin C (TnC) conformational changes ($<P_2>$) has not been previously investigated in humans. To test this, the pCa_{50} of force and $<P_2>$ during permeabilised fibre isometric contraction were compared between young adults (YAs, age 26), physically active master cyclists (MCs, age 75) and hip fracture patients (HFPs, age 75), and also between different muscle fibre types.

Methods: Bergström needle biopsy samples were taken from the vastus lateralis muscle of YA and MCs following local anaesthesia (2% lidocaine), and surgical biopsies from the HFPs. Single fibres were isolated and chemically skinned. pCa_{50} of force and $<P_2>$ were measured using polarized fluorescence from bifunctional rhodamine probes attached to the C helix of slow (ss) or fast (fs) TnC isoforms. SDS PAGE was then used to determine fibre type based on myosin heavy chain (MHC) isoform expression. Results presented are from MHC I fibres exchanged with ssTnC, and MHC II fibres with fsTnC, unless otherwise specified. MCs only exhibited MHC I fibres, so are not included in analyses involving MHC II fibres.

Results and Discussion: Between groups, the Ca^{2+} sensitivity of MHC I fibre force was similar, whereas MHC II fibre force was more sensitive to Ca^{2+} in HFPs compared to YAs. The Ca^{2+} sensitivity of $<P_2>$ in MHC I and II fibres was also similar between groups, suggesting no age-related effect on the orientation of TnC during contraction. Interestingly, the standard deviation of $<P_2>$ in MHC I fibres was significantly greater in the HFPs, suggesting that the orientation of TnC during contraction was more variable compared to YAs, and that consistency may be preserved in MCs. Within groups, comparing force and $<P_2>$ revealed that force was more sensitive to Ca^{2+} than $<P_2>$ in MHC I fibres in all groups. In MHC II fibres, the same trend was present in HFPs, but not YAs where no difference was observed. Furthermore, the steepness (nH) of force-pCa curves from MHC I and II fibres was higher than for $<P_2>$, indicating that the cooperative mechanism of force generation requires more than Ca^{2+} binding alone, whereas the $<P_2>$ nH of ~2 was consistent with a structural change in TnC dependent on the binding of 2 Ca^{2+} ions. Between MHC I and II fibres, Ca^{2+} sensitivity of force was similar in YAs but higher in MHC II compared to MHC I in HFPs. The effect of TnC protein isoform on the calcium sensitivity of force was assessed by comparing data from MHC I and II fibres exchanged with either ssTnC or sfTnC. Switching TnC isoforms in YA fibres had no effect, likely due to the similar MHC I and II Ca^{2+} sensitivities, whereas in HFPs, both MHC I and II fibres exchanged with sfTnC exhibited higher Ca^{2+} sensitivity of force than fibres containing ssTnC. This supports that the Ca^{2+} sensitivity of force is at least partly regulated by the TnC isoform present.

Conclusions: Age and/or physical activity did not affect the pCa_{50} of force in MHC I fibres, or $<P_2>$ during contraction in MHC I and II fibres. $<P_2>$ was more variable during contraction in HFPs. An age-related effect on the pCa_{50} of force between MHC I and II fibres was observed in HFPs, although this may be influenced by variables such as their health history. The data also suggest that pCa_{50} of permeabilised fibre force is determined by TnC isoform.

In situ FRET-based localization of the N-terminus of myosin binding protein-C in heart muscle cells

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The coordinated activation and de-activation of both the actin-containing thin and myosin-containing thick filaments of the sarcomere, the basic contractile unit of the heart, is fundamental for normal heart muscle function. Cardiac myosin binding protein-C (cMyBP-C) is a thick filament-associated regulatory protein localised to a central region of the half-sarcomere, called the 'C-zone', via interactions of its C-terminal domains with titin and myosin tail domains. Recent *in vitro* experiments have highlighted the functional significance of the N-terminal region of cMyBP-C for heart muscle contraction, reporting its regulatory interactions with both thick and thin filaments. Moreover, pathogenic variants in the gene encoding for cMyBP-C (*MYBPC3*) are the second most common cause of heritable hypertrophic cardiomyopathy, further underlining the functional significance of cMyBP-C.

To better understand the interactions of the regulatory N-terminal domains of cMyBP-C in its native sarcomere environment, *in situ* FRET-FLIM binding assays were developed to determine the spatial relationship between the N-terminus of full-length cMyBP-C and the thick and thin filament in cardiomyocytes.

Control studies were performed using established biochemical and biophysical methods to determine the effects of introducing the fluorophores for FRET into sarcomeric proteins. The mTFP- and mVenus-labelled cMyBP-C, cRLC and cTnT had the same sarcomeric location as the endogenous proteins, to the C-zone, thick filament A-band and thin filament, respectively, when expressed in neonatal rat cardiomyocytes (NRCs) via adenoviral infection. *In vitro* studies showed that ligation of a genetically-encoded fluorophore to the N-terminus of cMyBP-C had no or little effect on its binding to thick and thin filament proteins, or on the ATPase activity of isolated cardiac myosin in the presence of native thin filaments.

After optimisation of the FRET-FLIM workflow, FRET was detected between mTFP on the N-terminus of cMyBP-C as the donor and Phalloidin-iFluor 514 labelling the actin filaments as the acceptor in skinned NRCs in both relaxing and rigor conditions. The measured FRET efficiency was intermediate between those observed when the donor was on the N-termini of cRLC and cTnT. A broad distribution of FRET efficiencies was observed between cMyBP-C and phalloidin, suggesting a broad distribution of distances between the N-terminal domains of cMyBP-C and the thin filament, particularly in relaxing conditions. These results are consistent with the co-existence of multiple populations of cMyBP-C, some with their N-terminal domains binding to the thin filament and others binding to the thick filament, supporting the hypothesis that the dynamic interchange between such populations may mediate inter-filament signalling in the regulation of contractility.

When the C-zone is Not a C-zone: Ultrafast Zebrafish Larval Tail Muscles Express Myosin-Binding Protein H in place of Myosin-Binding Protein C

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The “C-zone” of vertebrate sarcomeres is characterized primarily by the presence of MyosinBinding Protein C (MyBP-C), which binds myosin thick filaments via Ig/FN3 domains at its Cterminus (panel A). The N-terminal domains of MyBP-C, through its binding partner interactions (i.e., myosin head region and actin), are believed to modulate contractility in an isoform and phosphorylation-dependent manner. In addition to MyBP-C, fast-twitch skeletal muscle expresses Myosin-Binding Protein H (MyBP-H) to a limited extent, which localizes to the C-zone and is similar in structure to MyBP-C but lacks analogous regulatory N-terminal domains (panel B). The role of MyBP-H is still unknown. Interestingly, fast-twitch muscles of 5-day old zebrafish larvae are the fastest-shortening muscles known and express almost exclusively MyBP-H (panel C). To define the potential roles of MyBP-H, we sought to resolve its location within larval fast-twitch muscle sarcomeres and to generate mutant zebrafish that are null for MyBP-H to assess the impact of MyBP-H loss on muscle structure and function. MyBP-H and -C protein expression levels in 5-day-old zebrafish larval tails or adult fast-twitch muscle tissue were determined by Liquid Chromatography Mass Spectrometry (LCMS). At 5 days, MyBP-H was dominant, constituting ~95% of myosin-binding protein present, with the fastskeletal isoform MyBP-C2 the remaining ~5%. In the adult, the protein expression transitioned to ~60% MyBP-H and ~40% MyBP-C2, while total myosin-binding protein remained in a ~1:10 molar ratio with myosin heavy chain (MYH), in line with other vertebrate muscle types. To test whether both proteins localize to the C-zone, we injected 1-cell embryos with integrating DNA vectors containing inducible transgenes encoding either MyBP-H or MyBP-C2 fused with a C-terminal FLAG tag. At 5-days old, immunofluorescence confocal imaging of anti-FLAG stained larvae injected with either construct revealed doublets of fluorescence at the sarcomere center (panels D and E), characteristic of C-zone binding; further supporting the invariance of MyBP:MYH stoichiometry may be due to competition of MyBP-H and MyBP-C2 for limited binding sites within the C-zone. To define how the loss of MyBP-H impacts muscle structure and function, we used CRISPRCas9 to generate a deletional-null mutation in its gene. Heterozygotes developed normally to adulthood despite a ~40% reduction in MyBP-H content by larval day 5. Preliminary mechanical studies of fast-twitch muscles in homozygote (MyBP-H-null) larvae showed no change in twitch kinetics, tetanic force, or the Force:Velocity relationship. Furthermore, LCMS showed minimal replacement by MyBP-C2 in both heterozygotes and homozygotes at 5 days, indicating that Czone myosin-binding protein occupancy is significantly reduced in these fish. Despite its abundance, MyBP-H may not be essential to development of functioning larval muscle. However, subtle structural or contractile phenotypes may yet be detected. Additionally, MyBP-H’s dominance suggests ultrafast shortening speeds require the absence of MyBP-C analogous N-terminal regulatory domains. We plan to repopulate the C-zone using transgenes encoding MyBP genes with mutated, humanized, or novel N-terminal regions, to investigate the influence of C-zone MyBPs on muscle structure and function in normal and diseased states.

Myosin-Binding Protein C Stabilizes SRX Myosin in Striated Muscle

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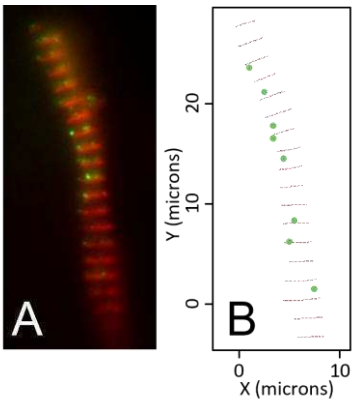
The myosin Super Relaxed (SRX) state plays a central role in the metabolic and functional regulation of striated muscle. SRX myosin demonstrate very low ATP consumption, 5-fold lower than other "relaxed," non-force generating myosin. Additionally in cardiac muscle, a population of SRX myosin that do not contribute to force production upon muscle activation may form a "reserve pool" that can be activated according to physiological needs. *To understand the mechanisms underlying the regulation of SRX myosin in various striated muscles, we sought to resolve the location of SRX myosin at the sub-sarcomeric level in different muscle fiber types.*



We prepared myofibrils from slow-twitch skeletal (rat soleus), fast-twitch skeletal (rat EDL), and mouse cardiac ventricular muscle. Isolated myofibrils were fluorescently stained using antibodies for myomesin, marking the center of the myosin thick filaments (red lines in panel A). Then, when imaged under relaxing conditions (zero calcium, 4mM ATP), along with 10nM BODIPY-ATP, discrete single-nucleotide binding events could be observed (green spots in panel A). Across all fiber types, distributions of observed event lifetimes were well fit as an admixture of two exponentially distributed populations with time constants (reciprocal rates) of ~25s and ~150s, in close agreement to literature values for myosin in the relaxed and SRX states, respectively. For both skeletal muscle types, longer-lived SRX events comprised ~28% of observed events, while cardiac muscle demonstrated a much greater fraction (59%) of SRX.

Utilizing super-resolution imaging methodologies, we were able to determine the sub-sarcomeric location of each event (panel B). In skeletal muscle, we found that the majority (80%) of SRX events were found between 180-530nm from the center of the thick filament, a region known as the "C-zone" due to the presence of Myosin Binding Protein C (MyBP-C), implicating this regulatory protein in the stabilization of the SRX state. Cardiac muscle demonstrated a similarly high incidence of SRX events within the C-zone (71%), but myosin outside the C-zone exhibited a 5-fold higher frequency of SRX events than in skeletal muscle. Additionally, we found that cardiac myofibrils from MyBP-C knockout mice demonstrated a 42% reduction in the overall proportion of SRX, indicating that myosin can inherently adopt the SRX state even in the absence of MyBP-C. Finally, although the central domains of MyBP-C may be implicated in stabilizing SRX myosin, we found that myofibrils from cardiac muscle expressing a truncated form of MyBP-C, lacking the N-terminal 30kDa, demonstrated an SRX content nearly identical to the complete MyBP-C knockout, suggesting a role for the N-terminus of MyBP-C in stabilizing SRX myosin.

In conclusion, we found that SRX myosin are predominantly found coincident with MyBP-C in the C-zone and that the N-terminal region of MyBP-C may play a role in stabilizing this configuration in cardiac muscle. This supports a model where myosins near the tip of the thick filament (devoid of MyBP-C) are the first to generate force upon activation, resulting in tension being transmitted through the thick filament towards the C-zone and thus "triggering" the release of myosin from the SRX state, where it predominantly exists.

Myosin conformational changes in *NEB*-related nemaline myopathy

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Nemaline myopathy (NM) is one of the most common congenital myopathies. NM is most frequently caused by mutations in the NEB gene. While exact disease mechanisms remain to be elucidated, histological analyses of NM patients' muscle biopsies reveal unexplained accumulation of glycogen and abnormally shaped mitochondria. Thus, the aim of the present work was to identify potential cause(s) of such changes in muscle energetics. Consequently, we applied Mant-ATP chase experiments on skeletal muscle fibres from patients diagnosed with NM and with NEB mutations. We observed a significant impairment in the myosin stabilizing conformational state, known as the super-relaxed state, which subsequently induced an increase in the energy (ATP) consumption of NM patients' muscle fibres at rest. Such alterations were related to a hyper-phosphorylation of myosin-binding protein C, a protein involved in linking myosin molecules to the thin filament. Hence, our results begin to uncover the contribution of myosin to some of the histopathological findings related to glycogen and mitochondria in NM.

Thick filament regulation in cardiac muscle

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In the cardiac muscle during diastole myosin motors are packed in helical tracks on the surface of the thick filament, folded toward the centre of the sarcomere and unable to bind actin and hydrolyse ATP (OFF state; Woodhead *et al*. 2005, *Nature* **436**:1195–1199; Stewart *et al*. 2010, *PNAS* **107**:430–435). The OFF state of both motors and thick filament is marked by characteristic signals in the X-ray diffraction pattern (Reconditi *et al*. 2017, *PNAS* **114**:3240–3245). In electrically paced trabeculae from rat ventricle, during diastole none of the signals related to the OFF state of the myosin motors are affected by inotropic interventions like increase in sarcomere length from 1.95 to 2.25 μm or addition of 10^{-7} M isoprenaline, which increase by twofold the peak force, indicating that the inotropic mechanism acts downstream with respect to Ca^{2+}-activation, likely modulating the gain of the mechanosensing-based thick filament activation (Caremani *et al*. 2019, *J. Gen. Physiol.* **151**:53–65). The effects on the thick filament structure in diastole of other interventions able to alter the OFF state of thick filament in cardiac demembranated trabeculae in relaxing solution, like lowering temperature below the physiological range (Xu *et al*. 2006, *Biophys. J.* **91**:3768–3775) or addition of the cardiac activator omecamtiv mecarbil (Kampourakis *et al*. 2018, *J. Physiol.* **596**:31-46), are defined here by collecting the X-ray diffraction pattern from intact trabeculae and small papillary muscles.

Troponin I Ser-150 Phosphorylation Increases Systolic Heart Function without Detrimental Effects

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Cardiovascular disease is a leading cause of death worldwide. Systolic heart failure results when the heart muscle is unable to pump enough blood to meet the metabolic needs of the body. Current heart failure therapies can alleviate some symptoms and prolong the progression of disease, but there are currently no approved therapies to improve the insufficient contractility at the root of systolic heart failure. Previously tested positive inotropes, or compounds used to increase the force of contraction, increased contractility by raising intracellular calcium. However, raising intracellular calcium resulted in detrimental effects and increased death so these therapies are not approved for long term treatment of heart failure. The purpose of this project is to establish a novel mechanism to increase contractility to improve systolic heart function for heart failure patients. One alternative to increase contractility without detrimentally increasing intracellular calcium is to increase the sensitivity of the myofilament regulatory proteins to calcium through post translational modifications. We previously demonstrated that phosphorylation of the key myofilament regulatory protein troponin I (TnI) at Ser-150 increases force development in muscle by increasing calcium sensitivity. We hypothesize that increasing TnI Ser-150 phosphorylation will increase contractility *in vivo* and improve cardiac function during pathological stress. To test this hypothesis, we have generated a phospho-mimetic mouse with Ser-150 mutated to Asp. Structural and functional measurements taken from echocardiography and pressure-volume hemodynamics demonstrate that increasing TnI Ser-150 phosphorylation results in increased *in vivo* systolic cardiac function and contractility without impairing diastolic function. Although increasing myofilament sensitivity to calcium has been associated with cardiac remodeling, we do not observe changes in left ventricle wall thicknesses or left ventricular mass. This suggests that the increase in cardiac function is not due to myocardial hypertrophy. Echocardiography measurements of these mice over 18 months demonstrate no adverse effects of long-term TnI Ser-150 phosphorylation on cardiac structure or function. Current experiments are focused on evaluating TnI Ser-150 phospho-mimetic mice following myocardial infarction to determine if increasing TnI Ser-150 phosphorylation improves heart failure outcomes. Overall, this data supports TnI Ser-150 phosphorylation as a novel signaling mechanism to increase systolic function without detrimental effects and is therefore a novel target for systolic heart failure therapies.

The effects of myosin regulatory light chain phosphorylation on Ca^{2+}-sensitivity of contraction are amplified in myocardial strips from cardiac myosin binding protein-C knockout mice

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Hypertrophic cardiomyopathy (HCM) is the leading genetic cause of heart disease. Although research has been focused on this condition for the last several decades, little progress has been made to improve treatment and outcomes for HCM patients. The heart comprises several proteins that work together to facilitate force production and pump blood throughout the body. Cardiac myosin binding protein-C (cMyBP-C) is a thick-filament protein and mutations in cMyBP-C are frequently linked with clinical cases of HCM. Within the sarcomere, the N-terminus of cMyBP-C interacts with myosin and is near the neck-region of myosin, where myosin regulatory light chain (RLC) is located. RLC is a subunit of myosin that modulates contractile dynamics via its phosphorylation state. Post-translational phosphorylation of RLC is thought to influence myosin head position along the thick-filament backbone, enabling more favorable binding with the thin-filament of actin to facilitate force production. However, it is unknown how cMyBP-C and RLC interact. We confirmed the cMyBP-C knockout (KO) mouse is a robust model for HCM as signs of cellular hypertrophy, disarray, and fibrosis were all present in transgenic mouse hearts. We tested the effects of RLC phosphorylation on Ca^{2+}-regulated contractility using biomechanical assays on skinned papillary muscle strips isolated from cMyBP-C KO mice and WT mice. RLC phosphorylation increased Ca^{2+}-sensitivity of contraction (i.e. pCa_{50}), from 5.80±0.02 to 5.94±0.03 (ΔpCa_{50} = 0.14; $p<0.001$) in the WT strips whereas RLC phosphorylation increased Ca^{2+}-sensitivity of contraction from 5.85±0.02 to 6.15±0.03 (ΔpCa_{50} = 0.30; $p<0.001$) in the cMyBP-C KO strips. These data suggest that the effects of RLC phosphorylation on Ca^{2+}-sensitivity of contraction are amplified when cMyBP-C is absent from the sarcomere. This implies that cMyBP-C and RLC act in concert to regulate contractility in healthy hearts, and mutations in the genes encoding these proteins that lead to HCM (or a loss of phosphorylation with disease progression) may disrupt important interactions between these thick-filament proteins.

Mechanistic study of mechanical stretch induced increase in Ca2+ sensitivity of troponin C

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The Frank-Starling Law of the heart is a direct consequence of myofilament length dependent activation (LDA) in the cardiac sarcomere, the phenomenon by which increased sarcomere length (SL) leads to both increased myocardial sensitivity to Ca^{2+} and maximum contractile force during the subsequent contraction. The molecular mechanisms underlying LDA, however, remain elusive. General consensus for LDA suggest SL-enhanced Tn sensitivity to Ca^{2+} is primarily caused by strong myosin-actin interactions via a crossbridge (XB) feedback mechanism, which can be further modulated by an allosteric interplay of other components of myofilament including myosin, titin, and myosin-binding protein C (MyBPC). It is known that LDA occurs immediately after mechanical stretch during diastolic filling when the myofilaments are still deactivated because of the low level of Ca^{2+}, and most myosin heads are at the off state preventing XB formation. Thus, key questions towards understanding LDA are how troponin-Ca^{2+} activation senses myocardial sarcomere stretch to promote myosin-actin interactions at the diastolic level of Ca^{2+} and how other regulatory components of myofilament play their roles in the mechanism. This study aims to explore a previously unappreciated mechanistic step that may play a role in initiating the LDA process in myocardium, i.e., mechanic stretching induces a direct troponin-myosin/N-domain of MyBPC interactions at diastole, leading to a partial activation of troponin through increasing sensitivity of cTnC to the diastolic level of Ca^{2+}, which is then able to initiate TF activation and release the inhibition of strong XB formation. Because multiple myosin heads can interact with each regulatory unit of the thin filament comprising 1 troponin, 1 Tm and 7 actins, a difficulty to study this mechanism within a sarcomere setting with such complex, interwoven regulatory components is how to distinguish the effect of the potential 1:1 myosin-Tn/N-MyBPC interaction from the effects generated by multiple dominant myosin-actin interactions. To address this issue, we implement a novel recombinant protein design and transgenic rat model to isolate the thin filament regulation process from the thick filament activity and use in situ FRET approach to examine troponin structural changes in response Ca2+, SL stretch and other regulatory components in a skinned muscle fiber setting. Briefly, we reconstituted skinned myocardial fibers from wilt-type rats and transgenic rats (in which C0-C2 of MyBPC is deleted) with Tn containing $cTnC(C35/51C)_{AEDENS\text{-}DDPM}$ and mutant cTnI (cTnI-ΔSp) in which the Sp of cTnI is replaced by a non-functional link sequence. FRET-based $cTnC(13C/51C)_{AEDANS\text{-}DDPM}$ was used to monitor Ca^{2+}-induced conformational change in N-cTnC. Our electrophoretic analysis showed >85% endogenous troponin was replaced by the mutants. Our force measurements showed that in the presence of cTnI-ΔSp, no Ca^{2+}-induced force development of the skinned myocardial fibers was observed. However, the Ca^{2+}-induced conformational change of troponin was observed (by monitoring fluorescence intensity change of AEDENS) in the presence of extrinsic peptide containing Sp residues 148-169 of cTnI. Normalized changes of pCa vs FRET fluorescence at different SLs shows Ca^{2+}-induced N-cTnC conformational change becomes more sensitive to Ca^{2+} as SL increases from 1.8 μm to 2.2 μm. Our results suggest that blocking strong XB had minimal effect on the SL-induced pCa_{50} shift, however, deletion of C0-C2 of MyBPC significantly attenuated, but not eliminate, the SL-induced pCa_{50} shift. These results provide key support for the hypothetical SL-induced troponin-myosin/N-MyBPC interactions, which enhances sensitivity of troponin to diastolic level of Ca^{2+} and initiate LDA process. It is likely that the hypothetical SL-induced troponin-myosin N-MyBPC interactions are caused by myocardial stretch induced reduction in interfilament spacing that promotes SRX to DRX transition and enables the myosin head in DRX state to interact with troponin in the diastolic state.

Cardiac Troponin T Mutations allosterically alter the N-terminus of Cardiac Troponin I

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The cardiac thin filament (cTF) is a crucial regulator of cardiac muscle contraction. The interface between the N-lobe of TnC and the N-terminus of cTnI (N-cTnI) is known to be a key modulator of calcium binding kinetics. Previous studies have also shown that Hypertrophic Cardiomyopathy causative mutations in cTnT, such as cTnT-R92L and cTnT-Δ160E, result in abnormal calcium binding kinetics. Both mutations listed exhibit significantly decreased calcium dissociation rates in the cTF system. We hypothesize that these mutations allosterically alter the structure of N-cTnI resulting in abnormal calcium dissociation rates. In order to investigate these structural changes, we performed time-resolved Forster resonance energy transfer (TR-FRET) experiments between cTnI-A28C to cTnC-84C, cTnI-A17C to cTnC-84C, and cTnI-A9C to cTnC-84C in fully reconstituted thin filaments to map N-cTnI with respect to cTnC across muscle biochemical conditions. We found that the more N-terminal residues of cTnI are more disordered in the absence of calcium. In the minus calcium state, we observed a full width half max (FWHM) of 18.4Å, 20.7Å, and 33.4Å with the TR-FRET pairs cTnI-A28C-cTnC-84C, cTnI-A17C-cTnC-84C, and cTnI-A9C-cTnC-84C, respectively. At saturating calcium levels, we observed a decrease in distance and increase in order between cTnI-A17C-cTnC-84C, and cTnI-A9C-cTnC-84C when compared to the minus calcium state. Both TR-FRET pairs displayed over a 6 Å decrease in distance upon the addition of calcium. The observed decrease in distance and increase in order upon the addition of calcium indicates that residues of the N-terminus of cTnI interact more with cTnC when calcium is bound. Probing of the cTnI-A17C-cTnC-84C TR-FRET pair in the presence of the cTnT-R92L and cTnT- Δ160E mutation resulted in an increase in order, with a FWHM of 11.9 Å and 14.7 Å, respectively. This increase in order observed for both mutations in saturating calcium levels may explain the decreased calcium dissociation rate. Conversely, TR-FRET between the cTnI-A28C-cTnC84C pair resulted in a decreased in FWHM for cTnT-R92L and not cTnT- Δ160E in the plus calcium condition. These results indicate that the cTnT-R92L and cTnT-Δ160E mutation differentially alter the structure of N-cTnI. This TR-FRET data along with future investigation into other HCM mutations will allow us to define the structural mechanism for changes in calcium dissociation observed in disease causing mutations.

The Interacting-Heads Motif Explains the X-ray Diffraction Pattern of Relaxed Vertebrate Skeletal Muscle

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Electron microscopy (EM) shows that myosin heads in thick filaments isolated from striated muscles interact with each other and with the myosin tail under relaxing conditions. This ‘‘interacting-heads motif’’ (IHM) is highly conserved across the animal kingdom and is thought to be the basis of the super-relaxed state. However, a recent X-ray modeling study concludes, contrary to expectation, that the IHM is not actually present in relaxed intact vertebrate skeletal muscle (Knupp *et al*., 2019, Biology (Basel), 8).
We propose that this conclusion results from modeling with a vertebrate thick filament 3D reconstruction in which the myosin heads have radially collapsed onto the filament backbone, not from absence of the IHM. Such radial collapse, by about 3-4 nm, is well established in EM studies of negatively stained invertebrate myosin filaments (Pinto *et al*., 2012, JSB, 180, 469; Woodhead *et al*., 2013, PNAS, 110,8561), and is therefore also likely to be present in the vertebrate reconstruction (Al-Khayat *et al.* 2013, PNAS 110, 318) on which the Knupp *et al.* study was based.

We have tested this idea by carrying out similar X-ray modeling, and determining the effect of the radial position of the heads on the goodness of fit to the X-ray pattern. We find that when the IHM is modeled into a thick filament at a radius 3-4 nm greater than that modeled by Knupp *et al*., there is good agreement with the X-ray pattern. When the original (collapsed) radial position is used, the fit is poor, in agreement with Knupp *et al.* We show that modeling of the low-angle region of the X-ray pattern is relatively insensitive to the conformation of the myosin heads but very sensitive to their radial distance from the filament axis.

We conclude that the IHM accounts well for the X-ray diffraction pattern of intact, relaxed vertebrate skeletal muscle when placed at the appropriate radius: there is no need to invoke a different head configuration. Our modeling supports previous studies showing IHMs in thick filaments and single myosin molecules from a wide variety of animal species, and removes the puzzling inconsistency between X-ray and EM suggested by Knupp *et al*. Our analysis is also consistent with the apparent ubiquity of the SRX state, thought to be based largely on the interactions of the IHM (Craig and Padrón, 2022, JGP, 154), and with studies of intact muscle using fluorescence polarization (Fusi *et al.* 2015, BJ, 109, 783). We conclude that the majority of myosin heads in intact muscle in the relaxed state are in the IHM conformation.

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The C-terminal Tail of Cardiac Troponin T Moves Closer to the N-lobe of Cardiac Troponin C upon Thin Filament Activation

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The cardiac thin filament is a multiprotein complex that contributes to the regulation of muscle contraction. Recent cryogenic electron microscopy studies have resolved much of the structure of the cardiac thin filament; however due to its inability to crystallize, the structure of the C-terminal tail of cardiac troponin T (C-cTnT) in the cardiac thin filament remains elusive. Functionally, C-cTnT has been shown to limit thin filament activation and is necessary for full contractile inhibition, and mutations in this region are known to cause cardiomyopathies. Elucidating the structure of the wild-type C-terminal tail of cTnT will clarify its regulatory function and the molecular mechanisms by which this region functions normally. In this study we have focused on structurally defining C-cTnT using bacterially expressed, human troponin subunits in reconstituted thin filaments. We hypothesize C-cTnT interacts with the Tn core, whereby it regulates thin filament activation. To test this, we used time-resolved fluorescence resonance energy transfer to determine the structural relationship between C-cTnT and the Tn core. Specifically, donor (IAEDANS) and acceptor (DABMI) probes were placed on C-cTnT and the N- and C- lobes of cTnC respectively. We found cTnT V274C is 45.2Å from cTnC T53C in low calcium (1mM EGTA) and this distance decreased to 44Å at saturating calcium (pCa 3) and further decreased to 30.5Å at pCa 3 +myosin S1 (S1). Additionally, the distribution of distances between these sites decreased upon activation with calcium and S1, indicating an increase in order. At low calcium the Full-Width at Half-Maximum (FWHM), which indicates the distance variability, between cTnT V274C and cTnC T53C decreased from about 33Å at pCa3 to 9.2Å at pCa 3 and +S1. The distance between cTnT V274C and cTnC G125C in 1mM EGTA was 52.8Å, this decreased to 47.1Å at pCa3, and further decreased to 45.8Å at pCa 3 +S1. The FWHM between these same sites were 31.3Å at pCa 3 and 11.1Å at pCa 3 +S1. Walking out towards the C-terminus of cTnT, we found V283C was 47.8Å from cTnC T53C at low calcium, 47.3Å at pCa 3, and 48Å at pCa 3 +S1. Relatively small changes in order were seen in these different biochemical conditions as demonstrated by a FWHM of 40Å at pCa 3 to 35.5 Å at pCa 3 +S1. Finally, we found that cTnT V283C was 58Å away from cTnC G125C in 1mM EGTA, 54Å at pCa 3 and 58.9Å at pCa 3 +S1. Our current probe pairs are not well suited to accurately measure the distance distributions between cTnT V283C and cTnC G125C as they may be too far apart for the necessary sensitivity. Taken together, these data suggest that the C-terminal tail of cTnT is dynamic and is closer to the N-lobe of cTnC than the C-lobe in all biochemical conditions tested. Interestingly, these data demonstrate that the C-terminal tail of cTnT, specifically V274C, becomes more ordered in relation to both the N-lobe (cTnC T53C site) and C-lobe (cTnC G125C site) upon addition of both saturating calcium and myosin S1; however, this same trend was not seen between V283C and T35C or G125C. This suggests that part of C-cTnT may be interacting with the N-lobe of the troponin core and myosin binding is responsible for this interaction.

Defining the Structure and Orientation of the N-Terminal Domain of Cardiac Troponin T in Relationship to Actin

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Cardiac troponin T (cTnT) is one of three proteins of the troponin core that is essential to calcium regulation in striated muscle. It serves as the tropomyosin binding subunit of the troponin complex and, when mutated, it often leads to pathologic cardiac remodeling observed in patients with hypertrophic (HCM) and dilated (DCM) cardiomyopathies. Part of the N-terminus of cTnT is specifically responsible for positioning tropomyosin along the actin groove and it immediately flanks the tropomyosin overlap domain. Understanding the molecular mechanisms underlying these observations has been hindered by the absence of a high-resolution structure in the highly flexible N-terminal domain. A recently published cryo-electron-microscopy structure shows interactions between the N-terminus of TnT, around amino acid 87, and the F-actin backbone; however, this interaction was not observed in calcium saturated conditions. Additionally, the remainder of the N-terminal tail remains unresolved due to its inherent flexibility. Here, we employ single-donor dual-acceptor time-resolved fluorescence resonance energy transfer (TR-FRET) to probe the positioning of the cTnT N-terminal extension in fully reconstituted cardiac thin filaments. We hypothesize the N-terminal tail interacts with F-actin in the absence of calcium and aides in stabilizing tropomyosin into its blocked position. In the presence of calcium condition, we hypothesize that the N-terminal tail is fly-casting and not interacting with F-actin. To test this, a donor probe (IAEDANS) was placed on either the Cys-substituted residues N73C or N100C of cTnT and an acceptor probe (DABMI) was placed on the endogenous Cys-374 of actin. Preliminary data revealed two measurable discrete distances for both residues, one short and one long, indicating that FRET occurred between the donor and two actin monomers. Initial data shows that N73C is 48.9Å and 27.4Å from actin during low calcium and moves to 51.3Å and 27.7Å during high calcium. N100C is 59.6Å and 26.7Å from actin during low calcium and moves to 46.4Å and 28.7Å during high calcium. These results suggest that the N-terminal tail is interacting with F-actin in high and low calcium conditions. Continuing work aims to probe additional sites on the N-terminus, specifically M60C and A20C. Resolving the structure and orientation of the N-terminal domain of cTnT is critical to understanding the regulatory mechanisms of the cardiac thin filament.

Multiscale Simulations of the Effects of 2'-deoxy-ATP and Myosin Mutations on Actomyosin Interactions

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Introduction: The actin-myosin cross-bridge cycle catalyzes the hydrolysis of ATP, leading to cardiomyocyte contraction. 2'-deoxy-ATP (dATP) is a myosin activator, which increases contractile force in cardiomyocytes even at low abundances in the adenine nucleotide pool. dATP has therefore been proposed as a potential therapeutic for heart failure with reduced ejection fraction. Previous Brownian dynamics (BD) simulations have demonstrated that, compared with ATP, dATP induces conformational and electrostatic changes in myosin that increase the association rate of myosin to actin. Here, we use a combined Markov-Brownian dynamics model to investigate changes in acto-myosin dynamics due to dATP compared with ATP, and the effects of dATP as a therapeutic for heart failure with reduced ejection fraction. We also simulate two known dilated cardiomyopathy mutations, β-myosin heavy chain myosin A223T and S532P, which have expected changes in crossbridge kinetics.

Methods: We hypothesized that using multiple structural conformations in Brownian dynamics (BD) simulations could provide greater insights into protein-protein association kinetics. We used molecular dynamics (MD) simulations to simulate pre-powerstroke myosin in four different conditions: Wild Type (WT), A223T, and S532P myosin in the present of ADP.Pi, as well as WT in the presence of dADP.Pi. The MD trajectories were clustered into Markov models of microstates, before being coarse grained into a reduced Markov model consisting of 15 meta-stable conformations, per each condition. The meta-stable myosin conformations were then used as input structures to BD simulations from which the association rate of actin and myosin is predicted.

Results: Markov modeling revealed that dADP.Pi reduces conformational variability within the pre-powerstroke myosin head, leading to slowed transition rates between metastable conformations when compared with ADP.Pi with WT myosin. Both the A223T and S532P mutation did not affect the rates of transitions between conformations. BD simulations also showed that conformational variability leads to significant differences in association rates of myosin to actin. The association rates computed by BD of different Markov state conformations from a single MD trajectory varied by a factor of up to 3, demonstrating that protein interactions depend significantly on small conformational changes. Furthermore, comparisons between simulation conditions demonstrate that dADP.Pi increases actin binding affinity of WT myosin by approximately 1.98x. The A223T + ADP.Pi condition showed a 0.56x decrease in association relative to WT myosin. Interestingly, the S532P + ADP.Pi condition increased actomyosin binding by approximately 3.0x relative to WT myosin.

Discussion: This newly developed computational approach emphasizes the importance of including dynamical protein information in BD methods and provides parameters that can be used in larger scale coarse-grained muscle models. The reduced transition rates in the dADP.P simulation suggests that dADP.Pi has a stabilizing effect on the myosin head. Further, the highly elevated binding affinity seen due to dADP.Pi helps to explain the significant change in contractile force seen even with minimal dATP, as seen in the literature. Further, the diverging trends due to the A223T mutation and S532P mutation show that additional steps in the cross-bridge cycle must be modeled to fully understand the mechanism leading to DCM, and different mechanisms may require different targeted approaches.

Myosin is on the Move within the Confines of a Single Sarcomere

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The heart contracts continually throughout one's lifetime through the antiparallel sliding of actin-based thin filaments along myosin-based thick filaments, organized within muscle sarcomeres. A recent biochemical study from our lab demonstrated that the half-life of the thick filament proteins is ~10 days in adult mouse hearts, suggesting that these large macromolecular complexes are continually remodeled to maintain structural and functional fidelity. The mechanism of thick filament protein replacement was stochastic, with newly synthesized molecules being randomly incorporated into preexisting thick filaments. Based on these findings, we hypothesize that the replacement of myosin molecules involves a dynamic equilibrium between single molecules and the thick filament *in vivo*. To test this hypothesis, we generated an adeno-associated virus (AAV) that replaces the regulatory light chain (RLC) on myosin molecules, with RLC that has a fluorescent GFP-tag fused to its C-terminus. Injection of mice with the AAV resulted in 27±7% replacement of the endogenous RLC with GFP-RLC within 12±2 days, which had no impact on actin filament sliding velocities in an *in vitro* motility assay. We then visualized the incorporation of these molecules in cardiomyocytes within intact hearts using two-photon microscopy. At high magnification, individual sarcomeres were observed within myofibrils, with the GFP-RLC being localized to the thick filaments. Next, we measured the movement of fluorescently labeled myosin molecules within the heart by irreversibly photobleaching limited areas of the cells and quantifying the fluorescence recovery after photobleaching (FRAP), over a ~30-minute period. No fluorescence recovery was observed after photobleaching regions encompassing entire sarcomeres. This was expected because only a minimal amount of myosin was synthesized on this timescale. Alternatively, rapid FRAP occurred when photobleaching partial sarcomeres, because the fluorescence signal moved from the unbleached portion of the sarcomere. These changes in fluorescence, were restricted to the boundaries of single sarcomeres. These data demonstrated that myosin molecules freely exchange between thick filaments, but they cannot exchange between adjacent sarcomeres or myofibrils. The kinetics of recovery within the sarcomere were fastest near the tips of the thick filaments, suggesting enhanced exchange of myosin molecules in this region of the filament, where the molecules are packed less densely. These data collectively demonstrate that thick filament structure is dynamic *in vivo*. The thick filaments, being large macromolecular complexes, appear to be designed in such a way that allows for the rapid incorporation and release of myosin molecules, while maintaining the structural integrity that is essential to support muscle contractility.

Cryo-electron tomography of intact cardiac muscle reveals myosin binding protein-C linking myosin and actin filaments

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Myosin binding protein C (MyBP-C) is an accessory protein of the thick filament in vertebrate cardiac muscle arranged over 9 stripes of intervals of 430Å in each half of the A-band in the region called the C-zone. Mutations in cardiac MyBP-C are a leading cause of hypertrophic cardiomyopathy the mechanism of which is unknown. It is a linear protein composed of 10 or 11 immunoglobulin- or fibronectin-like domains labelled C0 to C10 which binds to the thick filament via its C-terminal region. MyBP-C regulates contraction in a phosphorylation dependent fashion that may be through binding of its N-terminal domains with myosin or actin. Understanding the 3D organisation of MyBP-C in the sarcomere environment may provide new light on its function. We report here the fine structure of MyBP-C in relaxed rat cardiac muscle by cryo-electron tomography and subtomogram averaging of refrozen Tokuyasu cryosections. We find that on average MyBP-C connects via its N-terminal domains myosin to actin across a disc perpendicular to the thick filament. The path of MyBP-C suggests that the central domains may interact with myosin heads. Surprisingly MyBP-C at Stripe 4 is different; it may run axially and interact with myosin S2 or head on an adjacent crown. Given that the same feature at Stripe 4 can also be found in several mammalian cardiac muscles and in some skeletal muscles, our finding may have broader implication and significance. In the D-zone, we show the first demonstration of myosin crowns arranged on a uniform 143 Å repeat.

High Sensitivity Top-Down Proteomics Reveals Heterogeneous Proteoform Profiles Amongst Various Single Skeletal Muscle Fibers

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Single muscle fibers (SMFs) are multinucleated single cells that are classified as fast- and slow-twitch fibers. Post-translational modifications (PTMs) and isoforms of myofilament proteins within SMFs have profound effects on fiber functional properties. To better understand how PTMs and isoforms affect fiber function, it is important to analyze myofilament proteins within SMFs instead of their constituent muscles, which contain a heterogeneous mixture of fast/slow-twitch SMFs, connective tissue, and blood vessels. Top-down mass spectrometry (MS)-based proteomics is a premier technology for the analysis of proteoforms – protein products that arise from single genes due to events such as genetic mutations, alternative splicing, and PTMs. Due to the inherent challenges associated with top-down proteomics, conventional methods extract proteoforms from large pieces of muscle, which convolutes the relationship between proteoforms and SMF function. Therefore, we have developed a highly sensitive top-down proteomics strategy for the analysis of SMF proteoforms, which revealed distinct fiber-to-fiber proteoform profiles. In brief, SMFs were dissected from rat vastus lateralis (VL), plantaris (PLN), and soleus (SOL) hindlimb muscle in relaxation buffer containing protease and phosphatase inhibitors. To minimize sample losses, a one-vessel extraction method was used to extract myofilament proteoforms from SMFs (n=6 per tissue) using MS-compatible solvents and a freeze-thaw lysis. Online liquid chromatography-MS was performed using a capillary reversed phase column with a Newomics microflow-nanoelectrospray ionization source housing a 10 µm inner diameter multinozzle emitter. MS data were acquired using a Bruker maXis II quadrupole time-of-flight mass spectrometer. The MS data was analyzed using Bruker DataAnalysis and MASH Explorer. Our novel top-down proteomics method is highly sensitive which enabled successfully extraction and characterization of contractile proteoforms within single muscle cells. The data revealed distinct differences in PTMs and isoform expression in SMFs across different muscles. There were several instances of proteoform heterogeneity in sarcomeric proteins α/βtropomyosin, cypher 2s/4s, the fast and slow isoforms of myosin light chain 2 (MLC-2), among others. For example, the total level of phosphorylation (Ptotal) of the fast isoform of MLC-2 displayed Ptotal values ranging from 0.21 to 0.39 demonstrating nearly two-fold changes in phosphorylation from fiber-to-fiber. In addition, proteoforms extracted from VL, PLN, and SOL contained both different composition and abundance of myofilament isoforms corresponding to the function of the muscle. VL and PLN SMFs expressed myofilament proteoforms in their fast isoforms, whereas SOL SMFs contained myofilament proteoforms in their slow isoforms. We observed remarkable fiber-to-fiber and fiber-to-tissue difference in troponin T isoforms which demonstrated the diversity of isoform expression. Importantly, we were able to detect fiberspecific isoforms of myosin heavy chain (223 kDa)enabled by this highly sensitive platform for the first time from SMFs. Performing top-down proteomics at the single fiber level provides a more accurate assessment of fiber-specific proteoforms compared to bulk tissue analysis, providing new molecular insights at the single fiber level. In the future, we plan to implement this high sensitivity top-down proteomics method to measure proteoforms from single stem cell derivedcardiomyocytes for the study of maturation during the differentiation process.

Modulation of Cardiac Muscle Thin Filament Structure by Tyrosine Phosphorylated Troponin-I Analyzed by Molecular Dynamics Simulation.

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At low-Ca^{2+}, C-terminal domains of troponin subunit-I (TnI) trap tropomyosin in a position on thin filaments that interferes with myosin-binding on actin, thus causing muscle relaxation. This steric inhibition is reversed at high-Ca^{2+} when TnI releases from F-actin-tropomyosin as Ca^{2+} and the TnI switch-peptide bind to the N-lobe of troponin subunit-C (TnC). In the heart, TnI contains a well-known cardiac-specific, ~30 residue-long N-terminal peptide that is the target of kinases that alter the relaxation rate of cardiac muscle upon phosphorylation[1]. A well-characterized example is PKA-dependent phosphorylation of serine residues 23 and 24 (Ser23/24) which modulate Ca^{2+} and switch-peptide binding to TnC, causing more rapid relaxation (lusitropy)[1]. More recent studies indicate that tyrosine 26 (Tyr26) on the same cardiac specific N-terminal domain of TnI also may be phosphorylated by an as-yet uncharacterized tyrosine kinase[2,3]. Tyrosine phosphorylation may therefore augment or complement the PKA modulation of cardiac function[2,3]. Interestingly, both Ser23/24 and Tyr26 phosphorylation are decreased during heart failure[2,3], while cardiotoxicity has been associated tyrosine kinase inhibitors in humans[4]. Here, we used protein-protein docking and molecular dynamics simulation-based protocols to build a troponin model that was guided initially by the seminal Yamada structure of Ca^{2+}-activated thin filaments[5]. We find that phosphorylated TnI Tyr26 when present on thin filaments forms electrostatic interactions with an adjacent Lysine 161 tropomyosin residue. Interaction energy measurements indicate that the salt bridge formed is as favorable as ones produced by TnI phospho-Ser23/24 – tropomyosin linkages[6]. We anticipate that such interactions would likely bias tropomyosin to an off-state positioning on actin. In situ, any enhanced relaxation kinetics associated with tropomyosin repositioning would promote cardiac lusitropy.

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Myosin Subfragment-2 Binding is Cooperatively Related to Myofibril Contractility

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Myosin subfragment-2 (S2) is a coiled coil that is reported to contain unstable hinges, binding sites for myosin binding protein C (MyBPC), and a docking site for the interacting heads motif (IHM). Monoclonal antibodies, site-specific polyclonal antibodies, and rationally-designed fluorescent peptides have been developed to bind specific sites of vertebrate striated muscle myosin S2 and also light meromyosin (LMM) as a control. The location of their binding to S2 has the potential to interfere with the binding of the IHM and/or MyBPC to S2 as well as destabilizing the S2 coiled coil. In addition to affinity measurements, the impact of their binding to S2 was assessed using a myofibril contractility assay that measures the extent of sarcomere shortening in response to added ATP. It was found that the apparent Kd for binding to myosin S2 is greater than the EC50 for enhancing myofibril contractility by 2-4 times. The binding isotherms display positive cooperativity as expected for the predicted binding mechanism, yet the dose-response of myofibril contractility to the reagents appears to be hyperbolic. These differences suggest that S2 binding by the reagents may block a more complex underlying process that is saturated by substoichiometric binding of the reagent. One hypothesis is that MyBPC may be stabilizing the OFF states of several IHM and that the binding of one anti-S2 reagent inactivates that stabilization by MyBPC. To test the hypothesis that MyBPC competes with the anti-S2 reagents, the uniformity of A band labeling by the anti-S2 reagents was analyzed using super-resolution expansion microscopy of myofibrils. Each of the reagents demonstrates a noticeable depression in binding to the MyBPC containing C zone of the thick filament when compared to the D zone which lacks MyBPC. A monoclonal antibody against LMM used as a control has a greater C zone to D zone fluorescence intensity ratio than a monoclonal antibody against S2 suggesting that it may have less competition with MyBPC. This anti-LMM antibody also has no effect on myofibril contractility while the anti-S2 reagents all enhance the contractility. To further test whether the anti-LMM antibody was susceptible to competitive binding in the C zone, dual- and triple-labeling experiments were performed with the anti-LMM monoclonal antibody, the anti-S2 monoclonal antibody, and the anti-S2 site-specific polyclonal antibody. All three antibodies have different antigenic sites on the myosin tail, but their close proximity in the thick filament structure could lead to steric clashes. As the number of antibodies added increases, the fluorescent intensity ratio of the C zone to the D zone decreases in each case, but to a much greater extent for the anti-LMM monoclonal antibody suggesting that it has less competitive binding with the endogenous sarcomeric proteins. These observations are consistent with competitive binding between the anti-S2 reagents and MyBPC. Further evidence supporting this hypothesis is that the anti-S2 monoclonal antibody inhibits myofibril contractility where MyBPC is present in the myofibrils; however, in vitro motility assays of actin sliding over purified synthetic myosin filaments in the absence of MyBPC, the anti-S2 monoclonal antibody has no detectable effect. These results are consistent with a hypothesis that the anti-S2 reagents that may compete with MyBPC binding and prevent MyBPC from stabilizing several IHM's that act as OFF states for myofibril contractility.

Leiomodin Assembly at the Pointed End of Thin Filaments

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Leiomodin (Lmod) is an important muscle protein with a role in regulating thin filament formation. Malfunction of Lmod is associated with nemaline myopathy and dilated cardiomyopathy, both life-threatening diseases. The mechanism of Lmod function is heavily debated. Our current hypothesis suggests that Lmod allows thin filament elongation through direct competition with a thin filament capping protein tropomodulin (Tmod) at the pointed end. That competition implies the ability of the actin-binding site 1 (ABS1) of Lmod to compete with the ABS1 of Tmod while allowing for actin filament elongation that is not observed when Tmod caps the pointed end. This ability might be conferred by the specifics of interactions between actin and the Lmod2 ABS1 region comprised of an amphipathic helix and the preceding intrinsically disordered sequence. Using NMR, we mapped interactions of the ABS1 with actin. We found that two distinct parts of the region interacting with actin are separated by a stretch of noninteracting residues. Based on these results we propose a refined model for Lmod binding at the pointed end.

Premature stop mutations in *MYBPHL* share the common pathomechanism of preventing protein incorporation into the cardiac atrial myofilament

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Background. Cardiomyopathy and arrhythmia are diseases with significant genetic linkage. Cardiac myosin binding protein-C (cMyBP-C) is a myofilament associated protein with a C-terminal composed of repeating immunoglobulin (Ig) and fibronectin III (FnIII) domains that bind to the myosin thick filaments and titin. C-terminal truncating mutations in cMyBP-C result in improper myofilament incorporation and degradation of the truncated protein and are a major cause of hypertrophic cardiomyopathy. Myosin-binding protein-H like (MyBP-HL) is a protein that was recently identified that is structurally related to MyBP-C. MyBP-HL and cMyBP-C share high sequence homology in their C-terminal Ig and FnIII domains that bind to myosin. MyBP-HL is different in that it is primarily expressed in the atria where it that interacts with the myofilament. MyBP-HL premature stop mutations are associated with improper myofilament incorporation, like cMyBP-C. Prior investigation modeled the *MYBPHL* Arg255X mutation that is associated with ventricular conduction system abnormalities, atria enlargement, and dilated cardiomyopathy (DCM) in humans and this mutation resulted in no MyBP-HL localization into the myofilament. A null mouse model showed that loss of MyBP-HL resulted in arrhythmia and cardiomyopathy, like that observed in humans.
Method. The gnomAD database reports 9 stop-gain mutations for *MYBPHL* in humans including: Gln29, Trp54, Arg113, Tyr123, Trp158, Trp192, Lys250, Arg255, and Tyr307. We hypothesize that all MyBP-HL truncation mutations share a common mechanism by failing to produce MyBP-HL that contains the necessary myosin binding residues that required for integration into the sarcomere. We test this hypothesis by transfecting neonatal rat ventricular and atrial cardiomyocytes with mouse *Mybphl* constructs containing these human mutations.

Results. We tested MyBP-HL and MyBP-C overexpression, and both showed to be able to incorporate in a similar pattern to cMyBP-C C-zone doublets. We then tested Gln29, Trp54, Arg113, Tyr123, Trp158, Trp192, Lys250, and Arg255 MyBP-HL mutations, and observed that stop gained mutation disrupt normal localization as we expected.

Conclusions. Based on these data, we expect that truncating mutations in *MYBPHL* to act through a common loss of protein mechanism and may be functionally similar. This suggests that mechanistic insight into the pathogenicity of *MYBPHL* null alleles can be extrapolated to these nine known human *MYBPHL* truncating variants.

The A331P Hypertrophic Cardiomyopathy Mutation in Cardiac Actin Enhances Basal Contractile Activity and Causes Resting Muscle Dysfunction

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Pathogenic mutations that increase the duration and/or magnitude of tension during cardiac twitches strongly correlate with concentric myocardial hypertrophy. The ACTC A331P hypertrophic cardiomyopathy (HCM) mutation is located on a subdomain 3, surface strand of cardiac actin that is vital for troponin-tropomyosin-based regulation of contraction. Previous studies found A331P ACTC mouse models were indistinguishable from control, and data generated with reconstituted bovine trabeculae suggested that the variant reduced calcium sensitivity and tension, results at odds with those found for other HCM mutations. Here we employed a multidisciplinary approach to further examine the effects of the mutation *in vivo*, *in vitro*, and *in silico* to better understand disease pathogenesis. Our *Drosophila* models, benefitting from reduced genetic redundancy and complexity, helped to resolve genotype-phenotype associations and showed A331P actin reduced cardiac output by elevating diastolic tension and restricting filling. The variant likewise caused indirect flight muscle hypercontraction and destruction, hallmarks of impaired thin filament-based inhibition. Regulated *in vitro* motility assays using recombinant human cardiac actin plus bovine cardiac troponin-tropomyosin showed no change in calcium-sensitivity of A331P thin filament sliding or maximum velocity vs. control. However, A331P filaments displayed significantly higher sliding speeds under low calcium. 3.6 Å cryo-EM-based reconstructions of human wild-type and A331P filamentous actins (F-actins) showed no overt structural differences. When the F-actin cryo-EM models were used in MD simulations, we discovered that the 331-containing surface strand (i.e. residues 320-334) was more mobile in wild-type compared to mutant protomers. As suggested previously, actin flexibility may be critical for inhibitory tropomyosin positioning. Hence, MD simulations of wild-type and A331P F-actins, in the presence of tropomyosin and the regulatory components TnT1 and the C-terminus of TnI, were subsequently performed in order to test the dynamics of B- and C-state interactions. We found that the 320-334 strand along the mutant filament biases tropomyosin to the C-state configuration, which may impede its ability to properly return to or occupy the B-state, and prohibit thin filaments from fully turning off under relaxing conditions. Therefore, elevated diastolic/resting tension as observed in our fly models, potentially even in the face of reduced active tension as seen in reconstituted fibers, may be sufficient to drive pathological hypertrophy.

MYH7 G256E and H251N mutations impair relaxation by altering the stiffness of the transducer region of myosin

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Reported associations of genetic mutations in sarcomeric proteins associated with familial cardiomyopathies are myriad. However, detailed structural and functional studies are required to describe the mechanistic relationship between a mutation and its pathology. To accelerate such studies, we are developing a combined computational and experimental platform to probe how mutations to b-myosin heavy chain lead to familial cardiomyopathies. Experimentally, we utilize CRISPR edited human induced pluripotent stem cell derived cardiomyocytes to examine the effects of MYH7 mutations on the mechanical and biochemical properties of the contractile machinery. Computationally, we utilize molecular dynamics simulations to probe the structural changes that mutations induce in multiple chemomechanical states of myosin. Here, we report preliminary data from our studies on two hypertrophic cardiomyopathy-associated MYH7 mutations, H251N and G256E. These mutations reside in the transducer region of the myosin motor domain, which integrates and transmits structural information between the nucleotide binding pocket and actin binding regions. The specific force generation and activation rate of mutant G256E myofibrils was greater than isogenic control myofibrils. The initial slow phase relaxation kinetics of G256E myosin were reduced relative to isogenic controls, indicating a slower cross-bridge detachment rate. These data suggest a hypercontractile phenotype with impaired early relaxation for G256E myosin. Molecular dynamics simulations of the rigor (nucleotide-free actin-myosin complex) and myosin.ATP chemomechanical states showed that both H251N and G256E lead to distinct disruptions of native structure within the transducer region. G256E disrupted main chain hydrogen bonding patterns within the transducer whereas H251N disrupted main chain and side chain hydrogen bonding networks within the transducer and between the transducer and the upper 50 kDa domain. Comparison of the rigor state and myosin.ATP state simulations suggest that these structural changes would impede the structural transition associated with ATP binding, cleft opening, and actomyosin dissociation. Based on our combined computational and experimental results, we propose a putative structural mechanism by which the H251N and G256E mutations lead to hypercontractile phenotypes. Modulation of the transducer structure alters communication between the nucleotide binding pocket and the actin binding surface during steps in the chemo-mechanical cycle that involve transducer motion such as nucleotide release-induced strong actin binding and ATP binding-induced release from actin. Other effects on myosin structure and its activation during contraction are underway. This work was supported by NIH R01HL128368, P30AR074990 and RM1GM131981 to M.R. and T32 HL007828 to M.C.C. This work used the XSEDE resource COMET through allocation TG-MCB200100 to M.C.C. and M.R.

Transcriptional Analysis of Human iPSC-Engineered Cardiac Tissue Constructs Lacking Cardiac Myosin Binding Protein C Identifies Early Dysregulation of Sarcomeric, Metabolic and Ca^{2+}-Handling Genes

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Truncation mutations in *MYBPC3*, encoding cardiac myosin binding protein C (cMyBP-C), are common causes of hypertrophic cardiomyopathy (HCM). Heterozygous *MYBPC3* truncation mutation carriers present with classical HCM with disease presentation potentially slightly later in life than patients carrying mutations in other sarcomeric protein-encoding genes. Though rare, instances of homozygous *MYBPC3* truncation mutation carriers present with early onset HCM that rapidly progress to left-ventricular dilation and heart failure within the first year of life. In order to study the pathogenesis of cMyBP-C truncation mutations we used CRISPR-Cas9 to introduce heterozygous (+/-) and homozygous (-/-) frame-shift mutations into *MYBPC3* in the DF19-9-11T.H human iPSC line. Cardiomyocytes and cardiac fibroblasts derived from these isogenic lines were used to generate engineered cardiac tissue constructs (ECTs) that were subsequently characterized for contractile function and Ca^{2+}-handling. We previously showed that while contractile function was similar between the three genotypes after two weeks in ECT, there were subtle differences in Ca^{2+}-handling, of which slow Ca^{2+}-release was the most prominent. Following an additional four weeks in ECT culture, the Ca^{2+}-handling abnormalities became more pronounced in both +/- and -/-, and force production became severely depressed in -/- ECTs.
In order to gain insights into the molecular pathways that lead to the observed phenotype, we performed RNAseq analysis on two-week-old +/+, +/- and -/- ECTs.

Overall expression levels of differentially expressed genes were often positively or negatively correlated with cMyBP-C levels with 1,360 genes differentially expressed when comparing +/+ vs. +/- ECTs, 1,127 genes when comparing +/- vs. -/- ECTs and 4,102 genes when comparing +/+ vs. -/- ECTs. Gene ontology analysis identified specific enrichment of differentially expressed genes involved in hypertrophic remodeling and those encoding sarcomeric proteins (62/245 +/+ vs. +/- and 127/245 +/+ vs. -/-), genes involved in Ca^{2+}-handling and Ca^{2+}-response genes (31/123 +/+ vs. +/- and 62/123 +/+ vs. -/-) as well as genes involved in glucose metabolism and fatty acid oxidation (17/146+/+ vs. +/- and 52/146 +/+ vs. -/-).

Our data suggest that while cMyBP-C haploinsufficiency or ablation may primarily affect myosin crossbridge orientation, the observed contractile phenotype is Ca^{2+}-mediated. RNAseq data presented here, shows an enrichment in differentially expressed genes that are involved in cardiac metabolism, Ca^{2+}-handling and hypertrophic signaling that precedes the development of an overt contractile phenotype. Early differentially expressed genes and pathways identified in this study may be attractive therapeutic targets for treating HCM.

Determining the Effects of cTnT Linker Mutations on the Flexible Cardiac Troponin T Linker Region

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Contraction of the heart is driven by cyclic interactions between the thick and thin filament proteins, mediated by Ca^{2+} level fluctuations. Recent advances in electron microscopy (EM) and molecular dynamics studies have provided structural understanding of the cardiac thin filament (cTF). However, for certain cTF domains the structure and precise nature of the inter- and intra-protein interactions remain unknown. One such region is the extended cardiac troponin t (cTnT) linker between TNT1 and TNT2, which remains structurally undefined due to its inherent flexibility. It is comprised of a hinge region (residues 158–166) and a flexible region (residues 166–203), which modulates the cTn-Tm complex's position on actin, affecting the crossbridge cycling efficiency. The cTnT linker contains two mutational hotspots which cause severe and highly penetrant hypertrophic (HCM) and dilated (DCM) cardiomyopathies. Thus cTnT linker structural alterations could affect both inter- and intra-protein interactions and alter myofilament activation. Previous work in our lab coupled an atomistic computational cTF model with time-resolved fluorescence resonance energy transfer (TR-FRET) measurements in both $\pm Ca^{2+}$ conditions utilizing fully reconstituted cTFs to refine a structural model of the cTF inclusive of the cTnT linker. We have continued this coupled approach to determine how cTnT linker point mutations linked to cardiomyopathies altered the wildtype linker structure, HCM-causative cTnT Δ160E; and DCM-causative R173W and R173Q. For FRET, cTnT linker residues A168, A177, A192, and S198 were sequentially cysteine-substituted and IAEDANS-donor-labeled and the 5-IAF-acceptor was attached to actin 374Cys for cTnT linker to actin distance measurements. The computational cTF model was then constrained to reflect the measured TR-FRET distances and processed to an equilibrated state. The constraints were then removed and the computational cTF model reprocessed to develop refined structures of each cTnT linker containing a cTnT linker mutation. Using this coupled approach, we developed a cTF high resolution structure that included the refined cTnT linker structure and looked at mutational effects; providing mechanistic insight into the cTnT linker structural dynamics in both myofilament activation and disease.

Dystrophic Cardiomyopathy: Role of the Myofilaments

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Duchenne Muscular Dystrophy (DMD) is an inherited muscle wasting disease with a world-wide incidence of 1 in 3,500 live births. DMD is caused by the absence of dystrophin, a protein that connects the cytoskeleton of muscle cells to the extracellular matrix. Clinically, DMD-associated muscle weakness presents early in life and progresses rapidly, leading to premature death. In children, subclinical signs of cardiac disease present and usually progress to dilated cardiomyopathy in late stage DMD patients. With advances in skeletal muscle treatment, a cardiac component of DMD has been unmasked with heart failure becoming a leading cause of death in patients with DMD. Currently, it is unknown how dystrophin deficiency causes dystrophic cardiomyopathy and heart failure. This study addressed the effects of dystrophin deficiency on cardiac myofilament function. Single permeabilized left ventricular cardiac myocyte preparations from dystrophin-deficient $DMD^{mdx\text{-}4CV}$ and wildtype littermate mice were attached to a force transducer and motor and contractile properties were measured. Myocyte preparations developed similar tension and rates of force development during maximal Ca^{2+} activation between wildtype and $DMD^{mdx\text{-}4CV}$ groups. Interestingly, sarcomere length dependence of loaded shortening and power was greater in $DMD^{mdx\text{-}4CV}$ myocyte preparations. These results suggest that dystrophin mitigates length dependence of muscle activation and augmented sarcomere length dependence of myocyte contractility may accelerate progression of ventricular myocyte damage in dystrophic hearts.

Essential role of Obscurin Kinase-1 in cardiomyocyte coupling via N-Cadherin phosphorylation

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Obscurins comprise a family of giant cytoskeletal proteins with structural and regulatory roles in striated muscles. Obscurin-B (-870 kDa), the largest known isoform, contains two enzymatically active Ser/Thr kinase domains, kin1 and kin2, that belong to the myosin light chain kinase family. Our previous work established that kin1 binds and phosphorylates N-cadherin, a major component of the intercalated disc (ICD), the unique sarcolemmal microdomain that mediates the mechanochemical coupling of neighboring cardiomyocytes. Herein we report that obscurin-B containing kin1 co-localizes with N-cadherin at cell junctions in embryonic rat ventricular myocytes (ERVM) and that this co-distribution is regulated by Ca^{2+}. Phosphoproteonomics analysis revealed that obscurin-kin1 phosphorylates N-cadherin at Ser-788 located within the juxtamembrane domain of its cytoplasmic tail with an apparent Kcat of 0.29 sec^{-1}. Overexpression of constitutively active obscurin-kin1 or phosphomimic-Ser-788-Glu N-cadherin in ERVM markedly increases cell adhesion and chemical coupling. Importantly, phosphomimic-Ser-788-Glu N-cadherin exhibits significantly reduced binding to p120-catenin, while overexpression of phosphoablated-Ser-788-Ala N-cadherin results in increased RhoA activity. Consistent with a critical role of the obscurin-kin1/N-cadherin axis in cardiomyocyte coupling, our findings further show that it is deregulated in end-stage human heart failure biopsies. Taken together, these findings illuminate a novel signaling axis at the ICD with pathophysiological relevance to cardiac structure/function.

Disruption of the nuclear localization signal (NLS) in the RS domain of RBM20 is causative in dilated cardiomyopathy

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Background: RNA binding motif 20 (RBM20) is a muscle-specific splicing factor that belongs to the serine/arginine-rich (SR) protein family. SR proteins contain a C-terminal arginine/serine-rich (RS) domain, as well as one or more RNA recognition motifs (RRM) that bind RNAs. The RS domain in SR proteins is critical for spliceosome assembly and nuclear localization.

Objective: To elucidate the function of the RBM20 RS domain in cardiomyopathy.

Methods: To determine the function of the RS domain in RBM20, we generated mice with RBM20 lacking this domain ($Rbm20^{\Delta RS}$) using CRISPR-Cas9 genome editing. Cardiac structure and function in male and female homozygous $Rbm20^{\Delta RS}$ were assessed by gross anatomy, histology and echocardiography. RBM20 expression and localization in the hearts of $Rbm20^{\Delta RS}$ mice were assessed using Western blot, qPCR, and immunohistochemical staining. Titin splicing in $Rbm20^{\Delta RS}$ mice was examined by agarose gel electrophoresis. *In silico* analysis was carried out to establish putative sequence elements responsible for RBM20 nuclear localization. To identify the RBM20 NLS, mutant RBM20 constructs missing predicted NLS (termed D1 and D2) or surrounding sequences were expressed in H9c2 cells and localization was established with immunocytochemistry. Furthermore, to establish the role of RS domain phosphorylation in RBM20 nuclear localization, we developed RBM20 constructs with single or multiple amino acids mutated to un-phosphorylatable (Ala/Gly) or phosphomimetic (Asp) residues in the NLS sequences for expression in H9c2 cells.

Results: Histological examination and echocardiographic analysis of hearts from homozygous $Rbm20^{\Delta RS}$ mice revealed that $Rbm20^{\Delta RS}$ mice developed a dilated cardiomyopathy (DCM)-like phenotype with ventricular dilation and systolic dysfunction. Immunohistochemical staining revealed that RBM20 was mis-localized to the sarcoplasm in the hearts of $Rbm20^{\Delta RS}$ mice. In accordance, titin size was increased in $Rbm20^{\Delta RS}$ mouse hearts. *In vitro* expression of RBM20 constructs lacking one or both of the D1 and D2 sequence elements confirmed that D1 alone is essential for RBM20 nuclear localization. Our forthcoming data with Ser mutagenesis will provide new information regarding the role of RS domain phosphorylation in RBM20 nuclear localization, and RNAseq data will yield further mechanistic insights regarding the function of the RS domain.

Conclusions: Our current data reveal that the D1 sequence element constitutes the NLS for RBM20 and disruption of nuclear localization results in DCM. Our study also provides insight into the molecular mechanism(s) underlying RBM20 cardiomyopathy.

Molecular basis for atrial fibrillation and remodeling in mice lacking obscurin Ig58/59

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Obscurin (720-870 kDa) is a giant, modular, cytoskeletal protein that regulates striated muscle structure and function. Immunoglobulin domains 58/59 (Ig58/59) of obscurin interact with diverse proteins including titin variants and phospholamban. Mutations within Ig58/59 that alter these binding interactions have been linked to the development of myopathy in humans, underscoring the pathophysiological significance of this module. We previously generated a constitutive deletion mouse model, *Obscn-ΔIg58/59*, that expresses obscurin lacking Ig58/59 and comprehensively characterized the effects of this deletion on cardiac morphology and function through aging. Our findings demonstrated that *Obscn-ΔIg58/59* male animals develop severe arrhythmia characterized by spontaneous atrial fibrillation and junctional escape by 6-months of age, with atrial enlargement, ventricular remodeling, and contractile impairment manifesting by 12-months. Together, these findings demonstrated that the obscurin Ig58/59 module is essential for normal muscle function and that elucidating its impact has important implications for cardiac health and disease.

To understand the mechanistic basis for the severe atrial fibrillation and remodeling present in aging *Obscn-ΔIg58/59* males, we systematically evaluated Ca^{2+} cycling dynamics in isolated atrial cardiomyocytes. Strikingly, *Obscn-ΔIg58/59* atrial cardiomyocytes exhibited increased Ca^{2+} spark frequency and age-specific alterations in Ca^{2+} cycling kinetics and sarcoplasmic reticulum Ca^{2+} content that preceded those previously observed in *Obscn-ΔIg58/59* ventricular cardiomyocytes. Moreover, ultrastructural evaluation of aging male *Obscn-ΔIg58/59* atria revealed prominent Z-disk streaming and misalignment. To comprehensively characterize the molecular defects responsible for these Ca^{2+} cycling abnormalities and structural deficits, we performed proteomic and phospho-proteomic analyses in aging *Obscn-ΔIg58/59* atria. These studies revealed extensive and novel alterations in the expression and phosphorylation profile of major cytoskeletal proteins, Ca^{2+} regulators, and Z-disk associated protein complexes in *Obscn-ΔIg58/59* atria through aging that likely underlie the observed atrial pathologies. Notably, we detected altered expression and phosphorylation levels of T-cap, a titin-binding protein that links T-tubules to the Z-disk, in *Obscn-ΔIg58/59* atria in an age-dependent manner.

These studies are the first to evaluate the role of obscurin in atria and to reveal chamber-specific molecular alterations due to the Ig58/59 deletion. Moreover, our findings provide new molecular insights into a genetic model of spontaneous atrial fibrillation and remodeling where atrial dysfunction precedes ventricular maladaptation.

Kinetic characterization of Super relaxed state of human β-Cardiac myosin

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Muscle protein myosin brings about muscle contraction by converting chemical energy of ATP hydrolysis into force generation by movement of its lever arm. Super relaxed state (SRX) of myosin is an inactive, energy sparing, evolutionary conserved state of myosin. SRX state hydrolysis ATP 10-fold slower than the active DRX (disordered relaxed) state of myosin. Regulation of cardiac output by modulating SRX state of myosin is a unifying mechanism of action several mutations of sarcomeric proteins that cause hypertrophic cardiomyopathies (HCM). Despite the important role of SRX state of myosin in physiology and pathophysiology of muscle there are several fundamental questions that needs to be addressed regarding the SRX↔DRX equilibrium. Structurally SRX state is known to have the lever arm bent towards the motor head and occludes the nucleotide binding pocket preventing the exit of hydrolyzed products of ATP hydrolysis. We designed a FRET sensor to characterize the SRX state of β-cardiac myosin. The donor label is attached to the C-terminus of the Regulatory light chain (RLC) bound to the lever arm the fluorescent labeled ATP bound at the nucleotide binding pocket is used as an acceptor. We expect to see more FRET in the SRX state since the lever arm moves close to the nucleotide binding pocket. Using stopped flow transient kinetics and kinetic modelling we aim to quantify rate constants and equilibrium constants of SRX↔ DRX interconversion.

Speckle Tracking Echocardiography Details Progression of Left Ventricular Mechanical Dysfunction in Genetic Models of Heart Failure

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Mouse models of heart failure (HF) offer important platforms to study how specific elements of cardiovascular pathophysiology may contribute to the overall disease. While studies of these models at the cellular and subcellular levels excel at describing possible molecular mechanisms, translating results to the whole organism and humans remains a challenge given the intricate architecture of the intact heart and the presence of various multiorgan regulatory mechanisms. Thus, gold-standard assessments of cardiac hemodynamic function in vivo via pressure-volume (PV) loops and mechanical function with cardiac magnetic resonance imaging (cMRI) in mice remain invaluable translational tools. However, these techniques also come with limitations such as the non-survivability of the PV-loop procedure and long acquisition time of cMRI. Furthermore, human PV-loop and cMRI data are less available for translational comparisons versus alternative techniques like echocardiography. Here, we demonstrate how the experimental flexibility of echocardiography using modern high-resolution transducers and speckle tracking (ST) analysis can offer novel functional insights into cardiac function and mechanics. We performed longitudinal studies of two phenotypically distinct genetic models of HF, the cardiac myosin binding protein-c knockout mouse ($MYBPC3^{-/-}$) and the muscle lim protein knockout mouse ($MLP^{-/-}$), and their wild-type controls at three, six, and ten weeks of age. At each time point, images were collected to obtain conventional echocardiographic measures of left ventricular (LV) morphology, systolic and diastolic function, ST derived strain, and torsional mechanics at basal conditions and following β-adrenergic (β-AR) stimulation. Our results illustrate how LV function and its response to β-AR stimulation differentially evolve with age in $MYBPC3^{-/-}$ and $MLP^{-/-}$ models of HF. Additionally, the enhanced mechanical granularity afforded by ST analysis allows for updated comparisons of mechanistic findings in $MYBPC3^{-/-}$ and $MLP^{-/-}$ cellular/subcellular systems to whole heart function. Ultimately, our results highlight modern echocardiography's utility to basic HF research and clinical translation.

Altered actin-binding, stability, and folding of MyBP-C N-terminal domains with hypertrophic cardiomyopathy-causing mutations

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Mutations in the gene encoding cardiac myosin-binding protein C (MyBP-C) represent a leading cause of hypertrophic cardiomyopathy (HCM). To better understand how different missense mutations in MyBP-C cause HCM, we examined biochemical and biophysical properties of MyBP-C N-terminal domains C0-C2 due to P161S, Y237S, or P371R. We evaluated human recombinant protein folding and stability expressed in bacteria using temperature-based solubility tests and differential scanning calorimetry (DSC), and actin-binding using a fluorescence lifetime assay. At physiological temperature (37ºC), P161S and Y237S were insoluble and protein yields at optimal growth temperature (23ºC) were reduced compared to wild-type. Both mutations reside in the hydrophobic core of C1, suggesting that these non-surface exposed residues are important for proper folding of C1 in expression of C0-C2. For P371R, solubility and yield were similar to wild-type, but shifts in its DSC thermal stability plot suggested effects of P371R on C0-C2 stability. Interestingly, when C0-C2 was bound to fluorescently-labeled F-actin, P371R caused a greater change in the lifetime of 1μM actin compared to wild-type over a range of concentrations (0-20 μM C0-C2), indicating increased binding and/or an altered mode of binding. P371R phosphorylated by PKA also changed actin lifetime more than phosphorylated wild-type. Introduction of a positive charge by the proline-to-arginine substitution may contribute to enhanced binding with a net negatively charged actin surface. P371R is surface exposed and although folding resulted in soluble C0-C2, its stability measured by DSC was modified likely due to changes in C2 structure. Our findings are consistent with the idea that mutations leading to reduced MyBP-C incorporation or MyBP-C with higher affinity for actin contribute to hypercontractility in cardiac muscle, commonly observed in HCM patients. We conclude that protein-level binning of HCM-causing mutations in MyBP-C are useful to provide insight for understanding and treating HCM.

Mutation-specific Changes to Thin Filament Calcium Exchange in Hypertrophic Cardiomyopathy

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Cardiac troponin T (cTnT) is a protein of the cardiac thin filament (CTF) and assists in conferring calcium regulation to muscle contraction. Mutations in cTnT often cause hypertrophic cardiomyopathy (HCM), a disease affecting ~1/500 people worldwide. This study focuses on six HCM-causing, highly penetrant mutations located within the cTnT N-terminus (R94H/C, R92L/W/Q, and I79N) which are each associated with distinct phenotypes and severities in human patients. The first goal of this study was to determine the effects of HCM-causing mutations in cTnT on the calcium-based regulation of muscle activation. Using fluorescently labeled, bacterially expressed, recombinant human protein, we measured the sensitivity of CTF complexes to calcium via spectrofluorimetry as well as the kinetics of calcium exchange with cTnC via stopped-flow. All disease-causing HCM mutations in this cohort significantly sensitized CTF complexes to calcium compared to WT controls. Although all mutations significantly increased calcium sensitivity of CTFs, four mutations (R92L/Q and R94H/C) significantly decreased the rate of calcium dissociation (1.2-1.5 fold), whereas two mutations significantly accelerated calcium dissociation (1.1-1.4 fold). Three mutations significantly accelerated calcium association (R92W, I79N, and R94C) 2.8-4.5 fold while a fourth trended with a slight, albeit functionally significant acceleration (R94H) at 2.0 fold. Thus, the calcium sensitization reported here for each mutation is accomplished via mutation-specific changes to the kinetics of calcium exchange with cTnC. Furthermore, the mutations in this cohort can be broken into three distinct groups (bins) based on their changes to the kinetics of calcium exchange with cTnC. Bin 1 mutations decelerated calcium dissociation with no change in calcium association. Bin 2 mutations accelerated both calcium association and dissociation. Bin 3 mutations decelerated calcium dissociation while accelerating calcium association. The next goal of this study was to provide a mechanistic rationale for the differential effects of mutations on the kinetics of calcium exchange with cTnC. To do this, we utilized a four-state mathematical model of cTnC activation originally published by Siddiqui et al. 2016. This model incorporates calcium and cTnI switch peptide binding to cTnC in order to simulate steady-state and kinetic experimental data. We found that by changing the input calcium association rate to reflect the experimental data, we could accurately simulate the steady-state and calcium dissociation experimental data by modulating factors associated with cTnI availability. For bin 1 mutations, increasing cTnI availability and slowing cTnI dissociation was sufficient to simulate experimental data. For bin 2 mutations, accelerating calcium association, decreasing cTnI availability, and accelerating cTnI dissociation was sufficient to simulate experimental data. Lastly, for bin 3 mutations, successful simulations resulted from accelerated calcium association together with increasing cTnI availability and decelerated cTnI dissociation. Thus, the results of the simulation studies suggest that in addition to changing the apparent calcium association rate, the mutations in this cohort may also alter cTnI availability in accordance with their binned group. Taken together, results from these studies demonstrate that HCM-causing mutations can alter calcium sensitivity of CTF activation through specific combinations of changes to the kinetics of calcium exchange with cTnC. Furthermore, these changes to calcium exchange kinetics can be explained by mutation-specific changes to cTnI availability.

Postnatal Hypertrophic Cardiomyopathy is Associated with Alterations in Coronary Flow, YAP Signaling and Angiogenesis

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Introduction: Hypertrophic cardiomyopathy (HCM) is a genetic cardiovascular disease caused by mutations in the sarcomeric proteins. Despite a significant body of research, critical gaps still exist as to how maladaptive biophysical signals within the sarcomere are transduced to other cellular compartments within the heart, and their contribute to disease progression.
Objectives: To investigate the sequalae of early HCM disease progression we focused on the onset of myofilament dysfunction during neonatal development with alterations in cardiac dynamics, coronary structure and function, vascular architecture, and mechano-transduction signaling in mice harboring a thin-filament HCM mutation.
Materials & methods: We studied HCM disease during postnatal days 7-28 (P7-P28) in transgenic (TG) TG-cTnT-R92Q and non-transgenic (NTG) mice using skinned fiber mechanics, echocardiography, biochemistry, histology, and immunohistochemistry. Statistical analysis was done using 2-way Anova followed by Fisher's LSD test or t-test, $P<0.05$.
Results: At P7, skinned myofiber bundles demonstrated increased Ca^{2+}-sensitivity (pCa_{50} TG:5.97±0.04, NTG: 5.84±0.01, p=0.002) associated with cTnT-R92Q expression on the background of fetal troponin I and myosin heavy chain isoforms expression. Despite the transition to adult isoform expression between P7-P14, the increased Ca^{2+}-sensitivity persisted through P28 in TG mice. During this period no significant changes in cardiac gross morphology were evident but significant diastolic dysfunction accompanied by coronary flow perturbation (mean diastolic velocity, TG:222.5±18.81, NTG: 338.7±28.07mm/s, p=0.001) was apparent as early as P7, increased phosphorylation of phospholamban (PLN), indicative of abnormalities in Ca^{2+} homeostasis and localized fibrosis (percent area, TG:4.36%±0.44, NTG:2.53%±0.47, p=0.034). By P14 there was a notable decline in small arteriolar cross-sectional area and an expansion of fibrosis (percent area, TG: 9.72%±0.73, NTG:2.72%±0.2, p<0.001). Evidence of changes in mechano-transduction signaling in the arterioles was found as the levels of endothelial YAP expression increased, a decrease in ratio of nuclear to cytosolic YAP at P14 which was reversed at P28. Despite transient increases in eNOS (P7) and NOX2 (P14) expression, we found no clear evidence of oxidative stress in the TG hearts during early stage of HCM.
Conclusion: Our studies reveal that early in neonatal HCM disease development the diastolic dysfunction resulting from increased myofilament Ca-sensitivity, also impacts coronary flow, normal arteriogenesis and fibrotic processes which presage the onset of cardiac hypertrophy. Our data also stress the importance of cross talk between cardiac myocytes and other compartments and suggest that the vasculature could be a new therapeutic target for HCM.

Danicamtiv reversed negative effects on cardiac myofilament function in the I61Q cTnC mouse model of dilated cardiomyopathy

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Dilated cardiomyopathy (DCM), which affects approximately 1 in 250 people, is characterized by enlarged ventricles, thinned walls and reduced systolic function. Genetic causes are responsible for 25-50% of cases, including mutations in proteins involved in sarcomere contraction. Here, we study the effects of the positive myotrope, danicamtiv (dani), on contractile function in the cardiac troponin C (cTnC) I61Q mouse model of DCM. At baseline, cTnC I61Q mice show a contractile deficit in demembranated ventricular tissue mounted between a piezo motor and force transducer, including reduced calcium sensitivity and maximal calcium-activated force in comparison to littermate controls. After incubation in 1 μM dani, calcium sensitivity significantly increases while maximal force does not change. The most potent effects of dani occur in submaximal calcium concentrations, which leads to a flattening of the force-calcium relationship, suggesting a decrease in cooperativity. Interestingly, maximal rates of tension redevelopment are decreased by approximately 60% in the presence of dani. In intact cardiac trabecula paced at 1 Hz, cTnC I61Q also shows an impairment in comparison to controls at baseline, with decreased peak twitch tension. This leads to a negative tension index, a predictive metric that uses the area under the cardiac twitch to indicate hypo- or hypercontractility in the whole organ. Preliminary data with increasing concentrations of dani (0.5, 1, and 3 μM) show progressive increases in peak twitch tension as well as longer times to peak, 50% relaxation, and 90% relaxation, resulting in an increase in the tension index. Effects in both demembranated and intact tissue appear comparable across genotypes. To further elucidate the mechanism by which dani alters function in cardiac muscle, we will isolate cardiac myofibrils from control and cTnC I61Q mice and measure force, the rate of activation, and the rates of slow and fast relaxation. We will also use these myofibrils to measure the proportion of cardiac myosin in the super-relaxed (SRX) and disordered-relaxed (DRX) states using stopped flow spectroscopy. Together, these data will provide a detailed view of the mechanism by which dani improves cardiac function in the cTnC I61Q model of DCM.

Allosteric Destabilization of the Super-Relaxed State of Cardiac Myosin by Hypertrophic Cardiomyopathy-Causing Mutations

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Hypertrophic cardiomyopathy (HCM), a leading cause of heart failure and sudden death, results from mutations in sarcomeric proteins. ~35% of causative mutations affect β-cardiac myosin. It is now recognized that a large fraction of these may be affecting the conformational dynamics of myosin, and not its catalytic activity. Studies over the past decade have shifted the focus of HCM research to the evolutionarily conserved 'super-relaxed' (SRX) state of myosin, which is an important modulator of cardiac contractile output and energy consumption. HCM mutations are predicted to weaken the intramolecular head-tail interactions that allow myosin to adopt the auto-inhibited SRX state where the catalytic heads fold back onto their tails and no longer engage with actin to produce force. Destabilization of the SRX state releases active heads, which can cause a pathological increase in the net force produced by the heart muscle. The clinical relevance of the SRX state can't be overstated, yet we know very little about it. Recent in-silico studies indicate that destabilization of the SRX state may be a more pervasive effect than currently imagined because residues lying outside the predicted head-tail interface may also allosterically modulate the stability of this state. Such residues may account for as many as 1/3rd of all myosin HCM mutations, yet this class of HCM mutations is poorly characterized. HCM mutations in this class are expected to show diverse effects on the motor function of myosin because they are localized to structurally important regions of myosin.

Here, we focus on two such pathogenic HCM mutations: Y115H (in the transducer) and E497D (near the end of the relay helix). I will present our results from single-molecule and ensemble measurements that reveal the effect of these mutations on the biomechanical properties of myosin and the stability of the SRX state. We believe that such allosterically acting mutations affect the stability of the SRX state by modulating the stability of the pre-power stroke state (PPS) of myosin, which is an 'on pathway intermediate' for the SRX state; I will talk about results from molecular dynamics simulations that support this hypothesis. I will also discuss that a few HCM mutations, including Y115H and E497D, affect the ability of mavacamten to inhibit myosin. I will discuss these to highlight the importance of continuing our efforts to develop a diverse set of small-molecule effectors targeting myosin to treat HCM. The presence of hundreds of HCM mutations in myosin implies that we can't pin our hopes on a single drug molecule to treat this clinically heterogenous disease.

Harnessing the Human Cardiac Troponin C-Troponin I Interaction to Develop Fluorescence Polarization-based High-Throughput Drug Screening Strategies.

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Cardiomyopathies and heart failure are the leading causes of death worldwide. Majority of the current treatment strategies are dependent on either modulation of the intracellular calcium levels or modulation of the neuro-humoral signalling within the muscle cells to regulate contractility. However, these strategies show variable efficacy and are coupled with multiple side effects interfering with other cellular processes. In contrast, recently the focus of biomedical research has shifted towards sarcomeric proteins as promising new drug targets.

Activation of cardiac myofilament contraction is regulated by calcium binding to troponin in the actin-containing thin filaments. Within the troponin complex, troponin C (TnC) makes contact with Troponin I (the inhibitory component of the Troponin complex) in a calcium dependent manner. The N-lobe of Troponin C has a low affinity calcium binding site which is occupied after an increase in intra-cellular calcium levels. This results in opening of the hydrophobic pocket and subsequent binding of the switch region of Troponin I. This calcium dependent interaction results in moving away of the inhibitory region of TnI from the thin filament surface causing an azimuthal shift of Tropomyosin molecule which in turn exposes the myosin binding site on actin and the onset of contraction. In contrast, the C-terminal domain of TnC makes a strong structural interaction with the anchoring region of Troponin I, thus anchoring TnC in the troponin complex.

Utilising this information, we have developed two fluorescence polarisation based high-throughput drug screening assay systems. Fluorescent peptide probes were synthesised using the switch (FAM-SP) and anchoring region (FAM-AR) sequences of Troponin I, and their binding to human cardiac Troponin C characterized by fluorescence polarization measurements. Both assays showed excellent assay windows, signal-to-noise ratios and stability towards organic solvents, and we routinely achieved Z' factors >0.8.

Using these assays, we screened three different FDA approved small molecule libraries, and identified small molecules that could act as both inhibitors as well as enhancers of TnC-TnI_{SP} and TnC-TnI_{AR} interactions. We characterised hits from the primary screens for their functional effects on cardiac myofilament function using a wide range of biophysical and biochemical techniques. We further characterized compound binding to TnC and associated structural effects using a wide array of biophysical techniques including MST, ITC and NMR.

Going forward, we aim to screen more small molecule libraries using our high throughput assays and lead optimise the identified hit compounds to enhance their efficacy and tissue specificity.

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UNDERSTANDING THE MOLECULAR BASIS OF HCM-CAUSING MUTATIONS IN β-CARDIAC MYOSIN

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Hypertrophic cardiomyopathy (HCM), a heritable cardiovascular disease marked by hypercontractility and abnormal thickening of heart muscle, affects upto 1 in 200 individuals. Mutations in sarcomere proteins result in HCM, but the mechanism by which these mutant proteins lead to hypercontractility and finally heart failure remains poorly understood, limiting clinical interventions for the same. We are currently focussing on two sarcomere proteins that together comprise ~70% of all known HCM mutations: β-cardiac myosin and cardiac myosin binding protein-C, cMyBPC (a regulatory protein in the thick filament). Functionally, cardiac myosin in the thick filament can sample two states: an 'open' state where the heads are available to interact with actin, and a 'closed' state where the heads fold back onto their tails and can no longer interact with actin. Increasing the proportion of myosins in 'open' state (Na) can increase force production by the sarcomere, resulting in hypercontractility. We have previously shown that several HCM mutations in myosin lead to hypercontractility by weakening its folded-back state, thereby increasing active heads (Na). We have also recently shown that cMyBPC can sequester myosin heads (decrease Na) upon binding to myosin. Several HCM mutations in cMyBPC likely result in hypercontractility by affecting its ability to bind myosin and rendering them temporarily inactive. I will present our preliminary results we obtained with HCM mutations in myosin's proximal S2 (R869H, E894G, D906G and L908V), which include biochemical characterisation of ATPase rates, single nucleotide turnovers and binding of wild-type cMyBPC to mutant β-cardiac myosin. Using purified recombinant human proteins, we show that cMyBPC binds β-cardiac myosin, sequestering myosin heads, whereas some of the HCM mutations tend to weaken this binding likely leading to more 'open' myosin heads (Na). I will also be presenting data on biochemical characterization of HCM mutations in the motor domain of β-cardiac myosin. We plan to look at several HCM mutations in the β-cardiac myosin to comprehensively understand the molecular basis of hypercontractility.

MAVACAMTEN DEPRESSES HUMAN ATRIAL CONTRACTILE TENSION IN THE SAME EC50 RANGE AS FOUND IN HUMAN VENTRICULAR MYOCARDIUM

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In obstructive Hypertrophic CardioMyopathy (HCM) patients, recent phase III placebo-controlled randomized EXPLORERHCM clinical trial demonstrated the efficacy and safety of Mavacamten in reducing left ventricular (LV) outflow tract gradient and ameliorating exercise capacity (Olivotto et al. 2021). Both effects are attributable to a local reduction of LV septal contractility. Although very promising, this class of small molecules can globally depress cardiac muscle contractility with potential detrimental consequences on the overall cardiac output. Here we characterized the effect of Mavacamten on the contractile properties of human atrial myocardium (from surgical samples), expressing different levels of α and β myosin isoforms. In human atrial myofibrils expressing >95% α myosin (15°C), Mavacamten (0.5-5.0 μM) had a fast, fully reversible, and dose-dependent negative effect on Ca^{2+}-activated isometric force, with an EC50 close to that of human ventricular myofibrils expressing close to 100% β-myosin (Scellini et al. 2021). At variance with what reported in ventricular myofibrils, Mavacamten depressed the kinetics of force generation of human α-myosin atrial myofibrils similarly to what had been observed in fast skeletal muscle myofibrils. In intact trabeculae Mavacamten (5 μM) reduced the amplitude of atrial twitches with variable effects on twitch duration, that were partly related to different α:β myosin ratios and to the different impact of the drug on the fast and slow myosin isoforms.

Disease and aging may significantly increase the relative amount of the slow β-myosin expressed in human atrial myocardium (from 5–25% to close to 50%) potentially modifying the impact of Mavacamten on atrial contraction kinetics. In addition, it should also be considered that in obstructive HCM ventricular filling strongly relies on atrial contraction and that depressing atrial contractility with Mavacamten could detrimentally reduce ventricular systolic function.

Deletion of cardiac Filamin C disrupts myocyte force development and mechanosensitive gene regulation

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Dilated cardiomyopathy (DCM) is a common and deadly form of heart disease that is typically characterized by progressive thinning of ventricular walls, chamber dilation, and systolic dysfunction. DCM is often associated with mutations in genes encoding sarcomere or cytoskeleton proteins that dysregulate cardiac contractility. At the cellular level, cardiomyocytes from DCM hearts often exhibit eccentric hypertrophy (elongation) and subcellular remodeling characterized by sarcomere and Z-disk disarray. The cytoskeletal actin-binding protein Filamin C (FLNC) interacts with many proteins in the Z-disk and the costamere, suggesting that it contributes to the stability of Z-disks and maintains mechanical force transmission in cardiomyocytes. Moreover, mutations in the gene that encodes FLNC have recently been associated with all major forms of human cardiomyopathies, with many unique FLNC-truncating mutations found in patients with DCM. However, the underlying mechanisms that lead to DCM in patients with FLNC variants are poorly understood. The goal of our study was to elucidate mechanisms by which FLNC regulates systolic force transmission in the heart and how a loss of functional FLNC affects cardiomyocyte structure and function during the progression of DCM. To determine how FLNC regulates systolic force transmission and DCM remodeling, we used an inducible, cardiac-specific FLNC-knockout (FLNC-KO) model to produce a rapid onset of DCM in adult mice. Loss of FLNC reduced systolic force development in single cardiomyocytes and isolated papillary muscles but did not affect twitch kinetics or calcium transients. Electron and immunofluorescence microscopy showed significant defects in Z-disk alignment in FLNC-KO mice and altered myofilament lattice geometry. Moreover, a loss of FLNC induces a softening myocyte cortex and structural adaptations at the subcellular level that contribute to disrupted longitudinal force production during contraction. Spatially explicit computational models showed that these structural defects could be explained by a loss of inter-myofibril elastic coupling at the Z-disk. Finally, preliminary *in-vitro* studies suggest that FLNC is required for regulating the genetic programs that are triggered by directional-specific myocyte stretch. Thus, our work identifies FLNC as a key regulator of the multiscale ultrastructure and mechanical force transmission in adult mouse cardiomyocytes, the dysfunction of which may be central to systolic impairment and mechanosensitive gene reprogramming that may be involved in driving progressive DCM. Our work provides new insights into the pathological mechanisms by which dysfunctional FLNC causes systolic abnormalities and myocyte remodeling in patients FLNC-associated cardiomyopathies.

Integrated Analysis of Patient-specific Human Induced Pluripotent Stem Cell-derived Engineered Cardiac Tissue Model of Hypertrophic Cardiomyopathy

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Background: Hypertrophic cardiomyopathy (HCM) is the leading cause of sudden cardiac death in young adults. Coined "disease of the sarcomere," nearly 1,500 HCM-related mutations in sarcomeric genes have been identified, but the molecular events leading to the disease phenotype remain largely unknown. While previous studies have focused on studying HCM pathogenesis in 2D cell cultures, hiPSC-CM model systems remain underdeveloped. 3D engineered cardiac tissue (ECT) constructs created from hiPSC-CMs can better approximate the complexity of the human heart than 2D cell cultures. Thus, ECTs have emerged as superior disease model systems to model complex cardiac diseases. Herein, we have leveraged the power of tissue engineering to create ECT models with HCM patient lines and studied them using integrated functional assessments and mass spectrometry (MS)-based proteomics to understand molecular events underlying disease pathogenesis.

Methods: ECTs were generated from family members with no mutation (control, n=5) and the β-myosin heavy chain (MHC) R663H mutation (HCM, n=5) were assessed using an integrated method that permits sequential analysis of functional properties and top-down mass spectrometry (MS)-based proteomics. Isometric twitch force measurements were performed to characterize contractile performance between the control and HCM ECTs. Using the exact same ECT construct, sarcomere proteins were analyzed using top-down MS-based proteomics to detect and quantify sarcomeric protein products arising from isoforms and post-translational modifications, termed proteoforms. Global bottom-up proteomics was performed on a separate group of control and HCM ECTs to quantify protein expression differences.

Results: We have successfully created patient-specific 3D ECT constructs incorporating HCM-associated genetic mutation of β-MHC. We employed integrated functional and proteomics assessments to measure twitch force kinetics and sarcomeric proteoform landscape on the same ECT construct. Using this methodology, we compared ECTs constructed using HCM-associated genetic mutation of ß-MHC to the control. Isometric twitch force measurements showed HCM ECTs displayed a prolonged time to maximum contraction compared to the control group ($p<0.05$). The time from maximum contraction to maximum relaxation was also significantly longer in the HCM ECTs ($p<0.05$). The twitch force magnitude was similar between the two groups ($p>0.4$). Proteomics measurements detected a significant decrease in total phosphorylation for contractile proteins α-tropomyosin and cardiac troponin T in the HCM samples ($p<0.05$). Global bottom-up proteomics data determined that extracellular matrix (ECM) proteins such as collagen I were downregulated in the disease models. Along with key hypertrophic factors, proteins involved in glucose metabolism were upregulated in the HCM samples.

Our results revealed altered twitch kinetics and sarcomere proteoform landscape of HCM ECTs compared to control ECTs. Our integrated methodology suggests a group-dependent correlation between the measured contractile parameters and sarcomeric proteoforms. Indeed, sequential evaluation of functional and molecular parameters within the same construct provides mechanistic insight into the progression of cardiac disease.

Structural Basis of Cardiac Myosin ADP Release

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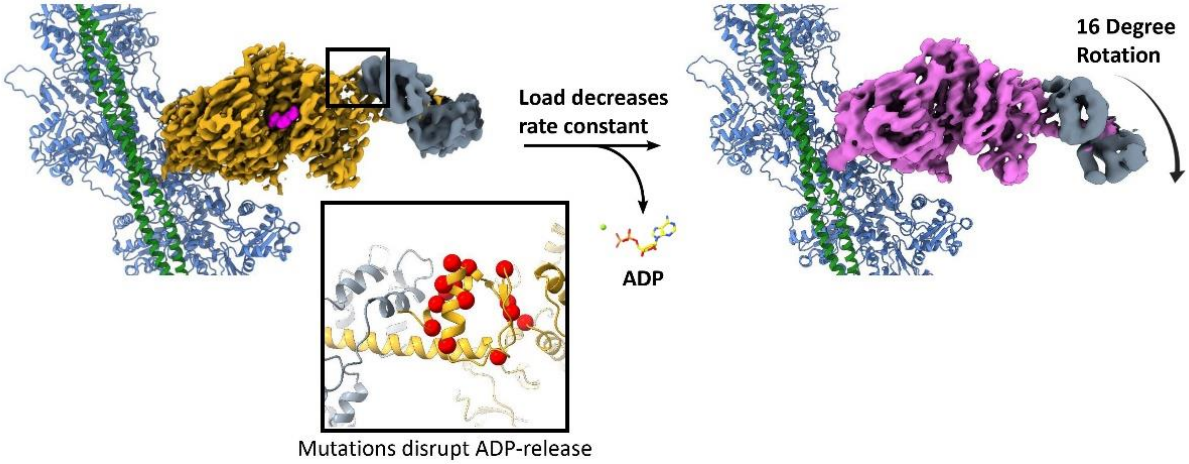
Myosins are members of a large superfamily of ATP-driven motor proteins that bind to and generate force on F-actin tracks. The binding of myosin to actin activates the myosin ATPase that is coupled to force generation through a stepwise series of interactions between myosin and actin. These steps, in turn, catalyze sequential release of Pi and ADP products of ATP hydrolysis from the motor. All myosin isoforms follow the same kinetic scheme during ATP hydrolysis and the accompanying actomyosin structural cycle; however, the conformational rearrangements during mechanochemical transduction are isoform-specific. These differences most likely modify actin-myosin affinity, the actomyosin ATPase, and the structural mechanics of the actin-myosin cycle, including the sensitivity of the various myosin motors to load. For example, the myosin load-dependency of actomyosin detachment in intact cardiac muscle allows the ventricular muscle to generate enhanced power to match the changing cardiac afterload during contraction. In the current study, we have focused on the structural interaction of expressed human β-cardiac myosin II (the major motor protein responsible for cardiac muscle contraction) with actin filaments. Our ultimate aim is to assess the structural effect of pathogenic mutations that disrupt motor function and lead to hypertrophic or dilated cardiomyopathy. To that end, we solved cryo-EM structures of human cardiac actin-myosin complexes at 3.3-3.8 Å resolution in both its rigor state and saturated with MgADP, to mimic the strong-binding ADP state. A composite strategy was employed to improve the resolution of the electron density of the actin-myosin interface (helical reconstruction) and the lever arm/light chain regions (single-particle reconstruction). The structures reveal large-scale changes in the myosin relay helix and converter domain as well as a lever arm displacement of 16 degrees associated with ADP release. These structures represent the start and end states of the rate-limiting step of the actomyosin attachment. Using our structures as starting points for molecular dynamics simulations and allosteric network analysis derived from that data, we provide a model to identify cardiomyopathy mutations that would affect the ADP release steps of the catalytic cycle.



CHRONIC EXPOSURE TO MERCURY AGGRAVATES THE EFFECTS OF ACUTE MYOCARDIAL INFARCTION IN RATS

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Background: Environmental contamination has exposed humans to metal agents, including mercury (Hg). Studies suggest that chronic exposure to Hg can affect the cardiovascular system. However, the present study evaluated whether chronic exposure to Hg can increase mortality due to arrythmias in rats underwent Myocardial Infarction (MI).
Methods: Male rats (12 weeks old) were divided into four groups: Sham+Sal, Sham+Hg, MI+Sal, and MI+Hg. Animals received *i.m* injections of $HgCl_2$ (1st dose 4.6µg/kg, subsequent dose 0.07µg/kg/day to cover daily loss) or vehicle-saline (Sal) for three weeks. At the end of the third week, the animals were submitted to infarction surgery through of ligation of the anterior descending left coronary artery. Shams were submitted the same procedure except to coronary ligation. Electrocardiographic (ECG) recordings were performed 5 minutes before and 20 minutes after surgeries. Number of ventricular extra systoles (VES); duration of ventricular tachycardia (VT) and atrioventricular blocks (AVB) were analyzed. One week after MI, hemodynamic measurements were performed and ponderal data were analyzed. Also, levels of the reactive oxygen species (ROS) superoxide anion (O_2^-) and nitric oxide (NO) in the cardiac muscle of the animals were performed through fluorescence analyses using Dihydroethidium (DHE) and Diaminofluorescein (DAF), respectively. The protocol was approved by CEUA (20/2018 and 24/2020).
Results: The scar size was not different between MI groups. The mortality rate in MI+Hg was 31.82% while MI+Sal was 21.43%. ECG recordings showed an increase in AVB in IM groups (min: Sham+Sal=0.00±0.00%; Sham+Hg=0.81±0.67%; MI+Sal=4.35±0.96%*#; MI+Hg=3.64±0.88%*#; *p<0.05vsSham+Sal, #p<0.05vsSham+Hg). Basckó coefficient showed that arrhythmias after MI were aggravated by exposure to Hg (Sham+Sal=0.24±0.19; Sham+Hg=0.75±0.35; MI+Sal=2.97±0.30*#; MI+Hg=4.00±0.21*#+; *p<0.05vsSham+Sal, #p<0.05vsSham+Hg, +p<0.05vsMI+Sal). Also, there was strong correlation between mortality and AVB (r=0.7379), VES (r=0.9487), VT (r=0.9487) and Basckó coefficient (r=0,9487). Hemodynamic assessment showed that Sham+Hg group has increased of the systolic blood pressure, however these values were decreased in IM groups (mmHg: Sham+Sal=105±2.98; Sham+Hg=115±3.71*; MI+Sal=95.47±3.58*#; MI+Hg=90.01±2.96*#; *p<0.05vsSham+Sal, #p<0.05vsSham+Hg). Left Ventricle end Diastolic Pressure was increased in MI+Sal group (Sham+Sal=7.31±1.25; Sham+Hg=5.38±1.44; MI+Sal=15.95±2.84*#; MI+Hg=9.56±1.38+; *p<0.05vsSham+Sal, #p<0.05vsSham+Hg, +p<0.05vsMI+Sal). dP/dt+ and dP/dt- values, were decreased in IM groups (dP/dt+ in mmHg:Sham+Sal=5515±543; Sham+Hg=5779±728; MI+Sal=3623±315*#; MI+Hg=2782±322*#; dP/dt- in mmHg: Sham+Sal=-3500±403; Sham+Hg=-3180±354; MI+Sal=-2248±122*#; MI+Hg=-1833±125*#; *p<0.05vsSham+Sal, #p<0.05vsSham+Hg). The heart weight and body weight ratio was increased in IM groups (Sham+Sal=2,82±0,04; Sham+Hg=2,87±0,12; MI+Sal=3,83±0,26*#; MI+Hg=3,73±0,22*#; *p<0,05vsSham+Sal, #p<0.05vsSham+Hg) as well as the lung weight and body weight ratio (Sham+Sal=5,11±0,19; Sham+Hg=4,69±0,17; MI+Sal= 8,97±0,79*#; MI+Hg=7,50±0,67*#+; *p<0,05vsSham+Sal, #p<0.05vsSham+Hg, +p<0,05vsMI+Sal). ROS levels were higher in groups that associated exposed to Hg and IM (O_2^- in a.u:Sham+Sal=1.79±1.79; Sham+Hg=58.88±6.01; MI+Sal=171±7.26*#; MI+Hg=221±12.14*#; NO in a.u: Sham+Sal=57,27±8.05; Sham+Hg=106±10.97; MI+Sal=207±8.87*#; MI+Hg=246±4.54*#+; *p<0.05vsSham+Sal, #p<0.05vsSham+Hg, +p<0.05vsMI+Sal).
Conclusion: Mercury intoxication caused more arrhythmias in infarcted animals, increased ROS and changed pressure parameters. These results suggest a worsening of cardiac events triggered by MI, as well as increased mortality after the injury.
Keywords: Mercury; Myocardial Infarction; Electrocardiogram.
Financial Support: CAPES, CNPq, UFES, FAPES

Structure, Function, Formation, and Mutation of the Interacting-Heads Motif

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The interacting-heads motif (IHM) is a configuration of myosin in thick filaments and isolated molecules in which myosin's two heads (blocked and free) interact with each other and with sub-fragment 2 (S2). It is ubiquitous across muscle types and species, where it functions to conserve energy by contributing to the super-relaxed (SRX) state. The structure of the IHM in tarantula myosin filaments determined by cryo-EM at 20 Å resolution (Woodhead et al., 2005) has been used to map disease-causing mutations in myosin in cardiac muscle. Most mutations are concentrated in regions of intramolecular (head-head and head-S2) interaction in the IHM, suggesting that the motif is critical to normal cardiac function (Alamo et al., 2017, Nag et al., 2017). A high resolution (~4 Å) structure of the IHM has been reported in the switched-off form of smooth muscle myosin, in which the heads interact with each other, and the tail folds on itself and wraps around the heads forming a compact ("10S") structure. It has been suggested that the high resolution of the 10S structure can be used to map more precisely mutations occurring in cardiac myosin (Heissler et al., 2021, Scarff et al., 2020). However, this will not work for interactions between the heads and S2 because S2 in the 10S structure is shifted 20 Å compared with the filament IHM. Thus, a high-resolution structure of the cardiac IHM is urgently needed. The 10S structure can be used to map mutations in smooth and nonmuscle myosin, where they tend to occur in regions of head-tail and tail-tail interaction, suggesting that the 10S conformation plays a key role in normal function.

The cardiac IHM is quite labile but in heavy meromyosin is found to be stabilized by the DCM mutation E525K in the myosin motor domain, simultaneously enhancing SRX; this correlation supports the hypothesis that the IHM underlies the SRX. Based on the low-resolution (20 Å) tarantula thick filament IHM reconstruction, E525 (on the blocked head mesa) interacts with a negative patch on S2. Stabilization of the IHM by the E to K charge-reversal supports the validity of the S2 position based on the low-resolution map. S2 thus occupies different positions in different IHMs: running over the mesa in HMM and the thick filament, but 20 Å closer to the edge of the blocked head in 10S myosin.

The IHM structures observed by EM reveal key inhibitory interactions that stabilize the off-state, but not how the IHM forms in solution. We have studied this by single molecule EM of smooth muscle myosin under conditions favoring the extended (6S) or folded (10S) structure, revealing dynamics of the molecule in solution. Under high salt (6S) conditions, myosin showed a gently curving tail, with two hinge points, while at low salt in the presence of ATP, the folded (10S) structure was formed (as in past studies). When we repeated the experiments in the presence of crosslinker, the 10S conformation formed in both low and high salt, with or without ATP. Our results imply that myosin II is highly dynamic in solution, forming transient 10S-like structures at high and low salt, in the presence and absence of ATP. Crosslinking captures these conformations, which are otherwise lost during specimen preparation. In vivo, similar dynamics may occur, captured by intramolecular ionic interactions favored by the intracellular milieu. We propose that the IHM of both filaments and molecules is formed by transient folding of one head back onto the tail, where it is captured by interaction with S2. The second head folds back and is captured by binding to the first. In the case of 10S myosin, the tail then folds at two hinges and displaces S2 20 Å towards the tip of the head.

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Visualizing myofibril contractile dynamics after myosin perturbations in patterned human induced pluripotent stem cell derived cardiomyocytes

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Approximately one third of known hypertrophic cardiomyopathy mutations are found in betacardiac myosin, the motor protein responsible for contraction in human ventricles. Given the known hypercontractile phenotype of HCM in patients, our biochemical studies have investigated how these mutations alter kinetic rates of myosin function and myosin regulation. We have previously reported profound differences in the effects of different mutations on molecular metrics of myosin function, but our ongoing work aims to clarify how these mutations affect contraction in a cellular context. Omecamtiv Mercabil (OM) is a myosin activator that can potently affect myosin function and muscle contractility in vitro, but its effects in patients experiencing heart failure were less clear. A pediatric onset HCM mutation, H251N, was edited onto a healthy background line with a GFP-tagged alpha actinin protein to allow for live cell tracking of cardiac differentiation, cell size, and sarcomeric contraction. These cells were differentiated into cardiomyocytes following established protocols and matured for 30 to 60 days. H251N cells were replated onto glass dishes patterned with lines of matrigel at day 30 and the remainder replated onto unpatterned coverslips at day 60 and fixed and stained for cardiac troponin after 48 hours. H251N cells plated on patterned glass were imaged before, immediately after and 24 hours after treatment with 300 nM OM, and patterns of sarcomere lengths during contraction were visualized with kymograph plots generated and quantified in ImageJ. Initial sarcomere kymographs showed more coordinated motion and more distinct diastolic rest periods in control cells compared to H251N. Immediately after the addition of OM, both cell types experienced longer duration contractions with limited relaxed phase and instances of overextended sarcomeres at sporadic points along the myofibril, especially in the H251N cells. After 24 hours, the magnitude of sarcomere movement along the myofibrils were significantly reduced in both cell lines, and we observed examples of cytoskeletal and myofibril reorganization. Quantification of the average sarcomere lengths revealed a significant increase in % shortening of sarcomere lengths in control cells after acute treatment with OM, and more variable sarcomere lengths in the H251N cells. We further confirmed beta myosin expression in these cells via immunostaining and measured the spread area of both patterned and unpatterned cells. In contrast to our previous measurements of cells with the P710R mutation, cells with the H251N mutation had significantly lower baseline cell spread area at day 62, but both patterned and unpatterned H251N cells treated with OM for 24 hours were not significantly different from control cells. Analysis of myofibril and sarcomere dynamics in control and H251N cells before and after perturbing their homeostasis with OM treatment revealed differences in patterns of myofibril contraction that were exacerbated by acutely increased force. These differences may correspond to the myofibril disarray and adverse remodeling observed clinically in patients with hypertrophic cardiomyopathy. Future experiments will determine if inhibiting myosin activity with mavacamten in the H251N cells rescues sarcomeric organization and cell spreading, and will characterize cellular responses with different mutations.

Hypertrophic cardiomyopathy mutations in MyBP-C detected to enhance myosin binding

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Mutations in myosin-binding protein C (MyBP-C) account for about half of the instances of hypertrophic cardiomyopathy (HCM), and in cases caused by another sarcomeric protein mutation, MyBP-C phosphorylation is frequently altered. MyBP-C regulates normal cardiac contractility through its interactions with myosin and actin, and this regulation is dependent upon phosphorylation. Efforts to elucidate the details of MyBP-C interactions with myosin and actin have been limited, in part, due to low-throughput and labor-intensive approaches. To resolve this, we have established new assays using Time-Resolved Fluorescence Energy Transfer (TR-FRET) that report binding of N-terminal MyBP-C (domains C0-C2) to myosin (in synthetic thick filaments) and F-actin. To establish assays for C0-C2 binding to myosin and actin by intermolecular TR-FRET, a fluorescent donor probe was placed on myosin's regulatory light chain (RLC) at V105C or actin at C374 and combined with an acceptor probe attached to the C1 domain of C0-C2. These assays detected binding of C0-C2 to both myosin and actin and were validated as binding measured by TR-FRET was reduced when C0-C2 was phosphorylated. To test the hypothesis that *MyBP-C HCM mutations impact N-terminal MyBP-C binding to myosin and/or actin*, we investigated 4 MyBP-C HCM mutations (± phosphorylation) that are likely pathogenic and positioned to modulate its complex with myosin (or actin): R282W, E334K, L349R, and L352P. Changes in FRET Efficiency showed that with 1 μM of myosin and 10 μM of C0-C2, R282W did not differ from wild type, whereas E334K, L349R, and L352P significantly increased FRET by 30.2%, 35.5% and 15.2%, respectively. These findings suggest increased binding of these mutations to cardiac myosin. Using 2 μM of actin and 2.5 μM of C0-C2, our assay confirmed increased actin binding of L352P, as expected. When L352P was phosphorylated by PKA, FRET was increased by 26.0% compared to WT, and thereby reduced the phosphorylation-dependent effect on actin binding. We conclude that the TR-FRET assays are useful to detect unique binding and to bin effects of HCM mutations on interactions with myosin versus actin. Future studies will elucidate how enhanced myosin-MyBP-C interactions could lead to hypercontractility and HCM pathogenesis.

N2BA titin is vital for cardiac homeostasis and knock-down induces a DCM-like phenotype

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Abstract

Titin is the main source of passive tension in the cardiac myocyte. Changes in titin can lead to cardiac dysfunction and titin is one of the main genes linked to dilated cardiomyopathy (DCM). DCM often coincides with an increase in N2BA titin, the less abundant and more compliant isoform, compared to the stiff N2B isoform. How N2BA titin contributes to DCM is poorly understood. Here we present a mouse model, *$Ttn^{\Delta ex112\text{-}158}$, which specifically shortens the spring region the cardiac N2BA isoform and leaves N2B isoform unaffected. Splicing studies with RNA sequencing and SDS-AGE showed a reduction in both the spring region of N2BA as well as a general reduction in N2BA isoform levels. This N2BA knock-down coincided with only modest increases in passive tension. However, mice presented with progressive systolic dysfunction with increased left ventricular filling as confirmed by echocardiography and pressure-volume analysis. Western blotting revealed that several titin-associated proteins, such as: MLP, FHL1/2, CRYAB and MARP1 are downregulated during the DCM-phase, while gene expressions studies indicate that pre-DCM there is a disruption in metabolic pathways and kinase activity. Thus, N2BA titin is vital for maintaining cardiac health and a N2BA signaling network might contribute to DCM development.*

Effect of sex and genotype on gene expression in cardiac hypertrophy (HCM)-affected mouse cardiac transcriptome

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Abstract
Cardiac ailments including cardiomyopathies continue to be among the leading causes of sudden cardiac death (SCD) across the developed world. Although genetic predisposition is a key factor contributing to one or more types of cardiomyopathies, biological sex has been recognized as an important modifier of cardiac condition.[1] As a result of ongoing research to understand the functional effects of troponin protein mutants in context of cardiac health, the cardiac troponin protein cTnC-A8V mutant has attracted much attention. Shi et al. reported that cardiac health in alpha-1B-glycoprotein (*A1BG*) female knockout mice is more severely affected compared to *A1BG*-KO male mice.[2] Female *A1BG*-KO mice displayed severely compromised cardiac function. Our study explores global gene expression by analysis of RNA-sequencing (RNA-seq) data in cardiac left ventricular tissue samples extracted from 3-month old homozygous cTnC-A8V male and female mice affected by hypertrophic cardiomyopathy (HCM) and from wild type (WT) littermates.[3] Our findings indicate that expression of *A1BG* transcripts is controlled by genotype and sex in an additive manner and we hypothesize that this differential gene expression occurs in a sex-dependent manner. This is in accordance with other results that show *A1BG* expression is upregulated in female mice hearts compared to male mice hearts.[2] STRING analysis indicates interaction networks between *A1BG* and other genes which show significantly higher expression levels in our RNA-seq dataset. Statistical analyses further revealed that specific genes within this interaction network are differentially regulated by genotype, sex and/or a combination of both. ANOVA analysis of linear models considering genotype and sex for *AKT1*, *PIK3R1* and *GAB1* suggested that the expression of these genes is controlled by an interaction between genotype and sex. On the other hand, *PIK3R1* and *HCK* show a predominantly genotype-based expression regulation wherein sex and genotype play an additive role. Similarly *AHSG* expression also follows an additive model of regulation by sex and genotype, with sex playing the dominant role. These findings suggest that there is a molecular interaction that takes place between *AKT1*, *PIK3R1*, *GAB1* and *HCK*, probably through protein kinase activity where posttranslational modification by phosphorylation can alter both enzymatic activity and/or subcellular localization of the proteins. Increase in cell proliferation leads to thickening of cardiac ventricular wall, which is a major marker of a hypertrophic heart. These results are in agreement with Dieseldorff Jones et al., who demonstrated a sex-based difference in cardiac phenotype in HCM-affected mouse hearts, measured by echocardiography.[3] The data showed that the HCM-affected female mice hearts exhibited distinct thickening of ventricular wall compared to male mice hearts. *AHSG*, a glycoprotein and a promoter of endocytosis, probably acts in the same pathway to enhance wall thickening of cardiac ventricles. Further studies in this direction might help understand signaling pathways between key molecular players involved in mediating HCM pathogenesis.

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Inhibitory and Stimulatory Micropeptides Preferentially Bind to Different Conformations of the Cardiac Calcium Pump

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Abstract:
The ATP-dependent ion pump SERCA sequesters Ca^{2+} in the endoplasmic reticulum to establish a reservoir for cell signaling. Because of its central importance in physiology, this transporter is tightly controlled by physical interactions with tissue-specific regulatory micropeptides that tune SERCA function to match changing physiological conditions. In the heart, phospholamban (PLB) inhibits SERCA, while dwarf open reading frame (DWORF) stimulates SERCA. These competing interactions determine cardiac performance by modulating the amplitude of Ca^{2+} signals that drive the contraction/relaxation cycle. The distinct functions of these peptides may relate to their reciprocal preferences for SERCA binding. While SERCA binds PLB more avidly at low cytoplasmic Ca^{2+}, it binds DWORF better at high Ca^{2+}. In the present study, we determined that this opposing Ca^{2+} sensitivity is due to preferential binding of DWORF and PLB to different intermediate states that the pump samples during the Ca^{2+} transport cycle. PLB binds best to the SERCA E1-ATP state, which prevails at low Ca^{2+}. In contrast, DWORF binds most avidly to E1P and E2P states that are more populated when Ca^{2+} is elevated. The results suggest a mechanistic basis for inhibitory versus stimulatory micropeptide function. Moreover, FRET microscopy revealed dynamic shifts in SERCA-micropeptide binding equilibria during cellular Ca^{2+} elevations. In a computational model of these regulatory interactions under different heart rates, we found DWORF exaggerates the changes in PLB-SERCA binding during the cardiac cycle. The data suggest a new role for DWORF as a modulator of dynamic oscillations of the PLB-SERCA regulatory interaction.

MYOMEGALIN, A 250 kDa PHOSPHODIESTERASE INTERACTING PROTEIN (PDE4DIP), SHOWS A NOVEL PROTEIN INTERACTION WITH CARDIAC MYOSIN BINDING PROTEIN C

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Cardiac myosin binding protein C (cMyBP-C) is a sarcomeric protein critical for regulating the strength and kinetics of cardiac contraction. The N- and C-terminals of cMyBP-C bind to well-established protein binding partners that include myosin heavy chain, actin, and titin. However, the cMYBP-C central domains lack known binding partners, as well as a clear physiological relevance to the overall function of the protein. Interestingly, central domain C3 has been shown to contain a positively charged surface that could serve as a putative binding interface for potential protein partners. However, to date a binding partner to this region has not yet been identified. If present, a novel binding partner to cMyBP-C may establish new importance and potential functional relevance to the central domain.
In an effort to uncover a potential binding partner, we generated a GST-tagged cMyBP-C fragment of central domains C2 through C4, containing the putative binding region, to perform a pull-down using neonatal mouse cardiac homogenate. Mass spectrometry on the bound proteins revealed, among other expected proteins, a 250 kDa phosphodiesterase-4D-interacting protein (PDE4DIP), also known as myomegalin. To further investigate a potential interaction between cMyBP-C and this large isoform of myomegalin, we assessed through a Duolink proximity ligase assay (PLA) on cultured and fixed neonatal mouse cardiomyocytes whether these proteins are close enough to physically interact *in situ*. Cardiomyocytes from cMyBP-C$^{+/+}$ mice showed a positive PLA signal while those from control cMyBP-C$^{-/-}$ mice showed no signal, suggesting these proteins reside <40nm apart in the native setting within the cell. To provide additional evidence for a physical interaction between cMyBP-C and myomegalin, we performed co-immunoprecipitation using an anti-cMyBP-C antibody or anti-mouse IgG (control) in lysate from cultured neonatal cardiomyocytes. Western blot analysis for myomegalin revealed isoforms of ~140 kDa, 180kDa, and >250 kDa that co-immunoprecipitated with cMyBP-C in the presence of the anti-cMyBP-C antibody but not with the mouse IgG antibody control. Isoforms of myomegalin of approximate corresponding molecular weights are known to exist and have been reported in the literature.

These results from protein pull-down, *in vitro* proximity assay, and co-immunoprecipitation provide strong evidence of a novel and physiologically relevant interaction of the central domains of cMyBP-C with myomegalin, a protein purported to serve as scaffolding to localize components of cAMP signaling, by compartmentalizing AKAPs, PKA, phosphatases, and phosphodiesterases, specifically PDE4D, to the sarcomere. We hypothesize that localization of a cAMP signalosome to cMyBP-C through binding to myomegalin, could provide the focal, rapid changes in phosphorylation of cMyBP-C to quickly modulate cardiac contraction and kinetics. Furthermore, we postulate that mutations in cMyBP-C that could disrupt the positively charged binding interface in the C3 domain, such as R495Q and R502W, known hypertrophic cardiomyopathy (HCM) – causing mutations, may alter the interaction of cMyBP-C with myomegalin and the cAMP signalosome, eliciting perturbations in phosphorylation-dependent regulation of cMyBP-C. Further investigation into the possible role that myomegalin-cMyBP-C binding has on anchoring a cAMP signalosome to the sarcomere, and the consequences of disrupting this interaction are warranted.

Sweet and umami receptors are expressed in the heart and regulate contractility

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The tongue can distinguish between five different tastes via the taste receptors, which are G-protein coupled receptors (GPCRs). There are two classes of taste receptors, the TAS1 (T1) and TAS2 (T2) families, and the TAS1R1-TAS1R3 dimer senses the umami taste and T1R2-T1R3 senses the sweet taste. Recently, the taste receptors have also been found in the brain, lungs, intestine and pancreas, where they sense changes in the nutrient environment and respond through GPCR signalling. Given the importance of glucose and amino acid metabolism in the heart, we hypothesized that the sweet and umami taste receptors have an important function in the heart. Using a variety of technologies and disease states, we have identified that TAS1R1, TAS1R2 and TAS1R3 are expressed in the heart. Mass spectrometry of a dog model has shown the presence of TAS1R1, TAS1R3 and TAS1R2. RNA seq of human patients who received a Left Ventricular Assist device and those who did not also revealed the presence of TAS1R1 and TAS1R3. The expression of these proteins was also confirmed using Western blot. We further showed TAS1R2 and TAS1R3 protein is localized in the plasma membrane of the cardiomyocytes by immunofluorescence (colocalized with Na/K ATPase) and plasma membrane enrichment. We further found that TAS1R2 was overexpressed in Dilated Cardiomyopathy (DCM), showing that taste receptors may be important in nutrient sensing in disease. Furthermore, when neonatal rat ventricular myocytes were treated with sweet and umami agonists they had increased calcium transients shown by an increase in peak calcium. Engineered heart tissue constructs showed increased contractility upon agonist treatment, as shown by an increase in peak force. We tested the phosphorylation of several PKC targets that would explain the positive ionotropic response, since PKC is activated as part of the GPCR signaling pathway. We found that upon agonists stimulation, phosphorylation of phospholamban increased, however phosphorylation of troponin I and MyBPC remain unchanged, signifying that the increased ionotropic response from the taste receptor agonists was due to calcium released from the SR through phospholamban phosphorylation. We hypothesize that in the heart, sweet and umami receptors induce positive inotropy upon a change in nutrient environment. To test this hypothesis, we are constructing CRISPR constructs to knockout TAS1R3 in iPSC- derived cardiomyocytes.

Titin assists myosin filament mechanosensing during muscle contraction

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The giant protein titin spans the whole half-sarcomere, through the I-band, connecting the Z-line at the end of the sarcomere to the tip of the myosin filament, and through the A-band, associated to myosin filament, up to M-line at the centre of the sarcomere. Consequently, the I-band region of titin transmits the stress also when no myosin motors are attached and is responsible for the passive force developed when a skeletal muscle fibre is stretched at rest. However, the underlying passive stiffness in the range of physiological sarcomere lengths (SL, < 2.7μm) is too low to account for the role of I-band titin as an efficient mechanical link between the Z-line and the myosin filament, able to sustain mechanosensing-based myosin filament activation (Linari et al., *Nature*, 528,276-279, 2015) and to prevent development of sarcomere length inhomogeneity during contraction. Here we define titin mechanics in intact frog muscle fibers using 20μM para-nitro-blebbistatin (PNB, which induces 100% inhibition of *in vitro* actin-activated myosin ATPase) to suppress any force response borne by myosin motors. Under these conditions, with combined sarcomere-level mechanics and X-ray diffraction at the European Synchrotron ESRF we demonstrate that upon tetanic stimulation (*i*) I-band titin switches from an entropic spring with large SL-dependent extensibility to a mechanical rectifier that opposes stretching with a stiffness one order of magnitude larger and independent of SL; (*ii*) A-band titin interactions with the myosin filament are responsible of a hierarchically organized series of X-ray signals marking the activation of the myosin filament, starting from a load dependent reduction in the helical symmetry of motor disposition accompanied by filament extension. Supported by ECR, University of Florence (Italy) and MIUR (Italy) under the European Joint Programme on Rare Diseases (EJP-RD).

Evaluation of 4-hydroxycoumarin Derivatives as Inhibitors of Class II Myosin ATPase Activity

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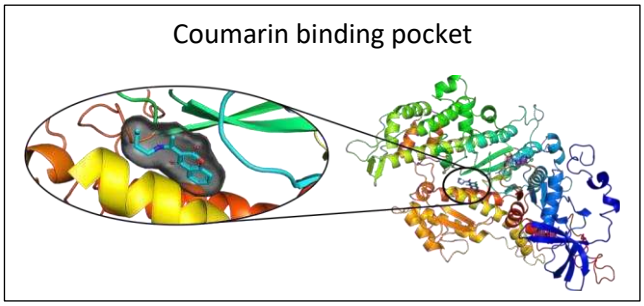
We are synthesizing novel small molecules and screening for their potential to treat conditions that could be improved by promoting relaxation of muscles from various organs. Our published study of structure-activity relationships for derivatives of 4-hydroxycoumarin imines (Brawley et al, J Med Chem 2020, 63, 11131-11148) suggests that this scaffold has promise for developing therapeutics that inhibit the ATPase activity of class II myosins. Competition and molecular docking results suggest that selected coumarin derivatives and blebbistatin bind to the same site on myosin and similarly inhibit phosphate release from the acto-myosin ADP-Pi state. They share a contact residue on Switch II adjacent to the active site which appears to be important for binding and inhibition.



Here we present new results about the selectivity of these coumarin derivatives for various myosin II isoforms, including cardiac (CMM), smooth (SMM), skeletal (SKM), and non-muscle myosin (NM IIb). Compounds were evaluated for their potency to inhibit SKM, CMM, and SMM acto-myosin ATPase using purified proteins. Cytotoxicity and cytokinesis inhibition, a proxy for NM IIb activity, was evaluated using COS7 cells. Previous work has shown that blebbistatin inhibits cytokinesis by targeting NM IIb. Interestingly, none of the 48 coumarin derivatives synthesized inhibited cytokinesis at 40 μM, suggesting little to no activity toward NM IIb. Cytotoxicity of the 4-hydroxycoumarin imine scaffold was low as no derivatives showed an increase in COS7 cell death above control levels. None of the derivatives inhibited SMM ATPase activity. These results suggest that the coumarin scaffold is highly selective for CMM and SKM. In general, the length of the derivative R group increased potency while the longest substituents were selective for SKM over CMM. Branched-chain aliphatic groups decreased potency while aromatic groups increased potency with the exception of ortho-substitutions on the aromatic ring. Para-substitutions caused selectivity for SKM while meta-substitutions were selective for CMM.

Whole-cell mechanical loading and unloading triggers more post-translational modifications in α-actinin than myosin activators and inhibitors

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The hypothesis tested is that unloading of mechanical forces affects acetylation, ubiquitination, and phosphorylation of the actin-binding protein α-actinin located in the Z-disc. We applied targeted proteomics to interrogate the post-translational modification changes during loading and unloading. Cell morphology and post-translational modifications were determined in a mechanical intervention consisting of 1 Hz cyclic strain for 24 hr (loaded) followed by 6 hr rest (unloaded) of cultured neonatal rat ventricular myocytes (NRVMs). This was compared to a chemical intervention consisting of treating NRVMs with the myosin inhibitor Mavacamten (1 μM, 6hr) or activator Omecamtiv Mecarbil (0.5 μM, 6hr). Quantitative immunofluorescence showed both chemical and mechanical loading increased α-actinin content while Mavacamten decreased the α-actinin content. Mass spectrometry analysis of affinity-purified α-actinin revealed that the mechanical intervention led to increased levels of post-translational modifications compared with the chemical interventions. Specifically, α-actinin ubiquitination increased with mechanical loading-unloading; acetylation decreased with mechanical loading-unloading and increased with mechanical loading; and phosphorylation remained unchanged with mechanical loading-unloading but increased with mechanical loading. Fluorescence recovery after photobleaching (FRAP) experiments demonstrated that Mavacamten increased the dynamics of overexpressed YFP-tagged α-actinin and a GFP-tagged CapZ in NRVMs when compared to Omecamtiv Mecarbil treatments and controls. Overall, the results suggest a link between sarcomere homeostasis and mechanical forces via mechanisms involving acetylation, phosphorylation and ubiquitination of α-actinin and a second Z-disc protein, CapZ. These findings could have consequences for cardiac heart disease with abnormal sarcomeric proteostasis. Funded by NIH grants HL151825 (CS) and HL62426 (RJS, BR, and CMW).

Longitudinal muscle hypertrophy following stretch is under the control of titin mechanosensing and mTOR signaling.

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ABSTRACT
Titin-based mechanosensing is considered a potent source of trophicity signaling in striated muscle. Although the connection between titin “sensing” and remodeling of striated muscle is well known, the mechanisms underlying the signaling cascades remain poorly understood. To study the downstream signaling of titin mechanosensing, we used unilateral diaphragm denervation (UDD), a surgical model for muscle stretch and titin-stiffness dependent cross-sectional and longitudinal hypertrophy. We applied UDD to wildtype mice and characterized the transient hypertrophy, and we focused on longitudinal hypertrophy. Functionality of hypertrophy was established using intact muscle mechanics and electron microscopy, showing structural abnormalities that resulted in a ~75% reduction in maximum tetanic tension. Increases in passive tension led us to examine titin splicing, using RNA sequencing and SDS-AGE, which showed increased titin splicing that explained the increased passive tension. To discriminate between signaling activated by stretch versus denervation, we performed global transcriptomics and proteomics after UDD and bilateral diaphragm denervation (BDD) in rats. Results indicate activity of the titin-associated proteins in UDD. We subsequently confirmed these proteins to be active in mice after UDD. We targeted several strongly upregulated titin-associated proteins and performed UDD on mouse models for Fhl1, MuRF1, Capn3 and MARPs (Ankrd1, 2 and 23), revealing the MARPs control the hypertrophy response to UDD. To investigate downstream signaling involved in longitudinal hypertrophy; we used pharmacological inhibition in mice with rapamycin and cyclosporin B. Rapamycin inhibited longitudinal hypertrophy following UDD, indicating that the mTOR pathway is responsible for longitudinal muscle growth. Thus, longitudinal hypertrophy following titin-mechanosensing is regulated through mTOR signaling and is dependent on titin-associated MARP family of proteins.

Co-translational Profiling in the Cardiac Endothelium During the Development of Sepsis

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The production of functional proteins is a multistep process whereby newly synthesized polypeptides are enzymatically processed, folded, and assembled into oligomeric complexes. These key maturation processes occur co-translationally by coupling nascent chain maturation and mRNA translation on the ribosomes, assisted by a network of regulatory proteins. Limited data exist regarding co-translational regulation in intact functioning mammalian systems and cells. To confront this limitation, we exploited translating ribosome affinity purification (TRAP) of the HA-tagged ribosomal protein L22 reporter mouse (RiboTag) crossed with an inducible endothelial-specific *Cdh5creERT2* mouse (*RiboTag*$_{EC}$). This model allowed for simultaneous immunoprecipitation (IP) of mRNA and nascent proteins for RNAseq and proteomic analysis from the endothelium and whole heart homogenates. Mice were injected with E. *coli* lipopolysaccharide (LPS) (6mg/Kg, i.p. 12 hours) to induce sepsis and perturb endothelial cell function. Hearts were homogenized, a small amount (~10%) was used for RNA-Seq and proteomics input controls (IN) and the remainder was used to IP ribosomal bound polyA+ mRNA and nascent proteins. Principal components analysis of the RNAseq data showed clear separation of the IP (endothelial cells) and IN (whole heart) transcriptomes between control and LPS groups. Transcripts characteristic of endothelial cells were enriched, whereas markers characteristic of cardiomyocytes, fibroblasts, and smooth muscle cells were depleted. Eluted proteins were digested, labeled with isobaric tags for quantification, and analyzed by synchronous precursor selection MS3 with a Thermo Scientific Fusion Lumos Orbitrap. Overall, >7000 proteins were identified, and initial analysis showed similar depletion and enrichment of proteins in the control and LPS hearts. Alignment of transcript and protein datasets was used for bioinformatic analysis. A hierarchical view of one of the top networks in which concordant expression of both EC transcripts and proteins identified interferon-g (IFNG) as an upstream signal with LPS treatment. These data highlight the power of our "functional translatome" discovery approach to provide a deeper predictive analysis of disease pathways and interventions.

Low MgATP in heart failure causes reduced cardiac muscle power

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Introduction: Cellular MgATP concentrations in the myocardium of heart failure (HF) patients can be reduced to levels one half of that observed in healthy controls. This marked reduction (from approximately 8-10 mM MgATP in healthy controls to as low as 3-4 mM in HF) has been suggested to contribute to impaired myocardial contraction, and to the decreased pump function that is characteristic of heart failure. However, *in vitro* measures of maximum myofilament force generation, maximum shortening velocity, and the actomyosin ATPase activity show an effective K_m of less than 250 μM, *well below the intracellular MgATP level in heart failure*. Thus, it is not clear that the fall of myocardial MgATP observed in heart failure is sufficient to appreciably impair the function of the contractile proteins.

Goal: Determine if the low MgATP level that is typically found in heart failure causes impaired contraction of cardiac myofilaments.

Methods: Demembranated mouse cardiac muscle preparations were attached to a muscle test system (Aurora Scientific) to measure force and shortening velocity. Muscles were maximally activated by using solutions containing MgATP levels typical of the range found in non-failing (8 mM) and failing hearts (4 mM). The relationships between muscle force, velocity and mechanical power were determined. Cross-bridge cycling kinetics were assessed from the rate constant of force redevelopment (k_{tr}) after mechanical disruption of cross-bridges.

Results: Consistent with previous studies, we found that a 50% reduction in MgATP level (from 8 mM to 4 mM) did not reduce maximum force generation or maximum velocity of shortening. However, we found that a 50% reduction in MgATP level caused: i) 20-25% reduction in maximal power generation measured during muscle shortening against a load; and ii) a 20% slowing of cross-bridge cycling kinetics.

Conclusions:

1. These results suggest the decreased cellular MgATP level occurring in heart failure contributes to the impaired pump function of the failing heart.

2. Since the ATP-myosin ATPase dissociation constant is estimated to be sub-millimolar, these findings also suggest that ATP concentration affects cross-bridge dynamics through a mechanism that is more complex than through the direct dependence of MgATP concentration on myosin ATPase activity. This is currently being investigated using computational models.

3. Finally, these studies suggest that therapies targeted to increase adenine nucleotide pool levels in cardiomyocytes might be beneficial for treating heart failure.

A multiscale model of the cardiovascular system that incorporates baroreflex control of chronotropism, cell-level contractility, and vascular tone

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Multiscale models of the cardiovascular system can provide new insights into physiological and pathological processes. Models that incorporate molecular-level effects may be particularly useful for clinical applications because they can predict the functional consequences of pharmaceuticals that modulate the properties of molecules and/or the rate at which they undergo reactions. PyMyoVent is a computer model that bridges from molecular to organ-level function and simulates a left ventricle pumping blood through the systemic circulation. Initial work with PyMyoVent focused on the End Systolic Pressure Volume Relationship and ranked potential therapeutic strategies by their impact on contractility. This work extends PyMyoVent by adding baroreflex control of arterial pressure. This new capability may be useful for future work focused on growth and biological remodeling in response to changing hemodynamic loads. The reflex algorithm is inspired by the underlying biology and uses an afferent signal derived from arterial pressure to drive a kinetic model that mimics the net result of neural processing in the medulla and cell-level responses to autonomic drive. The kinetic model outputs control signals that are constrained between limits that represent maximum parasympathetic and maximum sympathetic drive and which modulate heart rate, intracellular Ca^{2+} dynamics, the molecular-level function of both the thick and the thin myofilaments, and vascular tone. Simulations show that the algorithm can regulate mean arterial pressure at set-points ranging from ~30 to ~150 mmHg as well as maintaining arterial pressure when challenged by rapid changes in blood volume or sudden increases in aortic resistance.

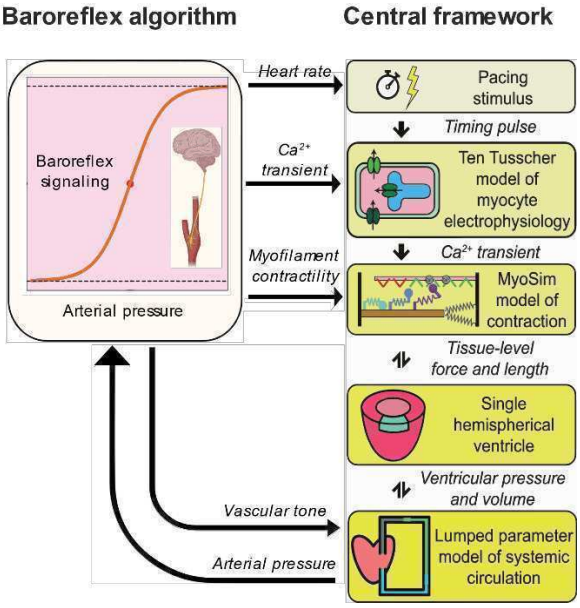

Figure 1. Overview of the model framework

Atrial cardiomyocytes from a canine model of atrial fibrillation demonstrate reduced specific force and sensitization of calcium sensitivity of force development

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Background. Atrial fibrillation is the most common cardiac rhythm disturbance and is associated with worse outcomes in many cardiovascular diseases. Although it is common, most studies surrounding atrial fibrillation focus on the electrophysiology of the disease and there is little known about the role of atrial contractility in atrial fibrillation. In this study, we performed cardiomyocyte force-calcium measures on atrial cardiomyocytes from a dog model of atrial fibrillation.
Methods. A dog model was developed to induce atrial fibrillation by atrial tachypacing for 8 weeks. These dogs were studied via echocardiogram to analyze whole heart function. The hearts were harvested and frozen. Atrial cardiomyocytes were isolated from the frozen tissue, permeabilized with detergent, and analyzed via force calcium experiments. Skinned atrial cardiomyocytes were attached to two needles and stretched to sarcomere length of 2.1 μm. The samples were perfused with buffers containing ascending calcium concentrations and then placed in a relaxing solution between each measurement. N = 3 – 4 atrial cardiomyocytes from 4 atrial fibrillation hearts, 3 – 4 atrial cardiomyocytes from 5 healthy hearts.

Results. In the atrial fibrillation cardiomyocytes, we saw a significant reduction in the maximum specific force of contraction compared to healthy controls. When normalized to maximum force, we saw a significant increase in calcium sensitivity of force development in the atrial fibrillation cardiomyocytes. We also saw a reduction in the Hill Coefficient in the atrial fibrillation model.

Conclusions. Both the reduction in maximum specific force and the increase in calcium sensitivity have important implications for the contractile function in atrial fibrillation. Increased calcium sensitivity may translate to prolonged contraction with slower relaxation *in vivo*. Prolonged contraction along with reduced force of each contraction could support the development of clots in the atrial chamber. Discerning the role of contractility in the pathology of atrial fibrillation could provide novel avenues into how to treat the disease.

Crosstalk Between Acetylation and Methylglyoxal Modifications on Myofilament Proteins Affects Sarcomere Function

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Diabetes doubles the risk of developing heart failure, independent of coronary artery disease and hypertension. The mechanisms connecting these two diseases are poorly understood, although we have previously shown one connection is likely due to methylglyoxal (MGO), a highly reactive glycolysis byproduct that can irreversibly modify lysine (K) and arginine (R) residues in a process called glycation. We found MGO glycation of the myofilament decreased calcium sensitivity and maximum calcium activated force in diabetes. Here we hypothesized MGO may further impact function by competing for lysine residues that would otherwise be targets for acetylation, which has been shown to impact cardiac function. To assess this hypothesis, non-failing human left ventricular (LV) myocardial tissue was exposed to MGO (100 μM) or vehicle and then the treated with acetic anhydride in acetonitrile (300 μM, Ac_2O-ACN) overnight. Ac_2O-ACN increased myofilament protein acetylation in both groups, but this effect was greatly reduced in samples pre-treated with MGO, suggesting these two PTMs do compete for a subset of lysine residues. To discover the specific sites of this acetylation-glycation crosstalk, we compared mass spectrometry data sets from diabetic humans, MGO treated mouse myocytes, and acetylated myocytes, which revealed sites of crosstalk on actin, myosin, and myosin light chains – proteins essential for normal contractile function. Importantly, these glycation-acetylation crosstalk sites were found in human samples, and the data suggest the balance of these may change in disease states. To explore the functional consequence(s) of this crosstalk, we incubated human LV isolated skinned myocytes with Ac_2O-ACN (300 μM, overnight), which enhanced function by significantly increasing calcium sensitivity. However, pre-treatment with MGO (100 μM) completely blocked this functional effect of acetylation. Together, these data demonstrate that glycation and acetylation compete for certain myofilament lysine residues, and that glycation can block the beneficial functional effects of acetylation. These results provide insight into the molecular mechanisms behind diabetic cardiomyopathy and the utility of targeting acetylation clinically.

X-ray Diffraction and Myofibril Mechanics Analysis Reveal Disparities in Myofilament Structure and Function between Distinct HFpEF Subphenotypes

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Heart Failure with preserved Ejection Fraction (HFpEF) is a complex, multi-organ disease primarily defined by cardiac aberrations including diastolic dysfunction with normal resting systolic function and depressed systolic reserve, hypertrophy, and impaired relaxation. However, this classification has been complicated by recently recognized phenotypical variations that occur co-morbidly with obesity and diabetes (Ob/Dm), distinguishing it from the 'classic' phenotype defined by hypertension and hypertrophy (Ht/Hp). Despite widespread and ongoing efforts, our understanding of the mechanistic deficiencies at the cellular and molecular levels related to HFpEF and its distinct subphenotypes remain largely unknown. Recent mechanics studies, using single cardiomyocytes from HFpEF patient biopsies, showed that maximal contractile force was significantly reduced in the Ob/Dm group compared to non-failing controls, but not in cells from the Ht/Hp cohort. Conversely, force under low-Ca^{2+} conditions (~pCa 6) was significantly increased in Ht/Hp compared to non-failing controls, but not in Ob/Dm cardiomyocytes. These findings suggest subcellular differences in Ht/Hp HFpEF patients that elevate force during diastole, inhibit relaxation, and preserve systolic force compared to non-failing samples. To determine whether anomalous structural relationships between myofilament proteins could help elucidate these mechanisms and potential phenotypical differences, we acquired X-ray diffraction patterns from flash-frozen, biopsied human cardiac tissue. We found that under relaxing conditions (pCa 8), Ht/Hp samples had significantly reduced myofilament lattice spacing ($d_{1,0}$) compared to non-failing controls, while Ob/Dm samples did not. Therefore, reduced lattice spacing in Ht/Hp HFpEF may serve as a compensatory mechanism which increases potential actomyosin interactions, restoring normal myofilament force during systole at the expense of elevated force during diastole (diastolic dysfunction). To test whether myofilament relaxation is also impaired in HFpEF, we have begun to examine the mechanical properties of single myofibrils isolated from the Göttingen minipig HFpEF model. This model recapitulates the spectrum of multiorgan pathophysiology characteristic of human HFpEF, including severe left ventricular diastolic dysfunction, myocardial hypertrophy, fibrosis, and pulmonary and systemic hypertension. Our preliminary data indicate that HFpEF minipig myofibrils produce significantly less maximal force compared to controls, with no change in relaxation kinetics. These data suggest that the Göttingen minipig model may more closely resemble the Ob/Dm vs. the Ht/Hp human subphenotype, at least in terms of expected myofibril mechanics. Further experiments will utilize myofibrils isolated from human biopsies to compare activation and relaxation kinetics between the various HFpEF subphenotypes and non-failing controls. We will also repeat our X-ray diffraction analysis with minipig tissue to assess lattice spacing in the model. In summary, we show that biopsies from HFpEF patients classified as Ht/Hp have reduced lattice spacing, which likely contributes to elevated diastolic force. Furthermore, we have begun to classify mechanical properties of cardiac myofibrils from the Göttingen minipig model and show that force and relaxation kinetics may be more similar to those expected for the human Ob/Dm subphenotype.

Histone deacetylase 8 regulates myofibril relaxation

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Background: Eighty-five percent of women older than 65 years of age who develop heart failure, develop a particular type of heart failure called heart failure with preserved ejection fraction (HFpEF). The underlying mechanisms that contribute to this sex difference in prevalence of HFpEF are not well defined. Since HFpEF is characterized by impaired relaxation, our primary objective is to define age-induced changes in sarcomeric relaxation that may increase the likelihood for women to develop diastolic dysfunction. We hypothesize that specific post-translational modifications of sarcomeric proteins are uniquely altered in female hearts with age and these modifications prolong sarcomeric relaxation.

Methods: Myofibrils were isolated from left ventricles of male and female non-failing donors 20-40 years of age and over 60 years of age and sarcomere mechanics measured. Acetylation of protein residues was measured using mass spectrometry. Regulators of acetylation that may change with age were evaluated in transcriptomic data of non-failing donor hearts. Based on identifying upregulation of histone deacetylase 8 (HDAC8) in aging female hearts, HDAC8 was inhibited or overexpressed in primary cultured rat cardiomyocytes and myofibril mechanics were measured.

Results: Myofibrils from female non-failing donors over 60 years of age relax more slowly and are less acetylated than myofibrils from female non-failing donors 20-40 years of age. In contrast, relaxation duration and acetylation are not different between males as they age. HDAC8 expression is higher in aging female hearts and HDAC8 is enriched in the myofilament protein fraction. Primary cultured cardiomyocytes overexpressing HDAC8 relax more slowly whereas cardiomyocytes treated with HDAC8 inhibitor relax faster.

Conclusions: Differential regulation of myofibril acetylation observed in females and males as they age may elucidate mechanisms that increase propensity to develop diastolic dysfunction.

Elucidating the Structural and Functional Consequences of a Novel Mutation in *MYBPC1* Linked to Myopathy with Tremor

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Myosin binding protein-C (MyBP-C) encompasses a family of sarcomeric proteins that contribute to thick filament stabilization and actomyosin crossbridge regulation in striated muscles. MyBP-C's regulatory role is governed by the ability of its N-terminus to dynamically interact with myosin and actin. Our group has identified a novel Leu266Lys267Arg268 (LKR) duplication in the *MYBPC1* gene encoding the slow skeletal isoform (sMyBP-C), that is associated with generalized muscle weakness, skeletal deformities, and a unique postural tremor of likely myogenic origin. The underlying molecular mechanism behind these pathological manifestations is elusive, and currently no therapeutics exist for this sarcomeric myopathy. The focus of my project is to characterize the LKR duplication on a molecular level and delineate the structural and functional alterations that it elicits. Interestingly, the LKR duplication localizes to the sMyBP-C N-terminus, specifically the highly conserved M-motif region. **I therefore hypothesize that the duplicated LKR residues alter the biochemical properties of the M-motif, disrupting the sMyBP-C N-terminus structure and function.** Molecular dynamics simulations, validated by circular dichroism experiments, suggest that the LKR duplication stabilizes the terminal α-helix present in the M-motif. Additionally, isothermal titration calorimetry experiments demonstrate the mutant M-motif to interact strongly with myosin with low nanomolar binding affinity, while the wildtype M-motif exhibits a weaker binding affinity in the micromolar range. These findings suggest that the LKR duplication alters the M-motif's structure and binding interactions. Future assays will assist in elucidating the molecular and functional basis of this novel myopathy, ultimately aiding in the development of targeted therapeutics that will improve the quality of life for these patients.

Role of Melanocortin-4 Receptor (MC4R) in Cross-bridge Function in Cardiac Muscle Fibers

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We examined the dynamics of the cross-bridge function of MC4R-/-, which is known to cause dilated cardiomyopathy (DCM) in humans and mice. We used skinned cardiac muscle strips (fibers), activated in the presence of Ca^{2+} and ATP, and changed the length in sinusoidal wave forms (15 frequencies ranging 1 and 187 Hz) at a small amplitude (0.2% L_0). We studied tension transients, and correlated them to the kinetic constants of the cross-bridge cycle by studying the ATP and Pi effects. We conclude that the cross-bridge functions that include the actin-myosin interaction are not severely altered by MC4R protein deficiency, except that we saw some effects possibly from sarcomere disorganization. At the same time, we observed significant differences between males and females. In wildtype (WT) animals, tension and stiffness during full activation and rigor were ~50% larger in males than in females (see Figure); and the equilibrium constant of the force generation step (K_4) and the Pi association constant (K_5) were ~120% larger in males than in females. In contrast, in the MC4R-/- models, the forward rate constant (r_4) and backward rate constant (r_{-4}) of the force generation step were 50-150% larger in females than in males. However, these differences did not cause a significant difference in cross-bridge distributions.

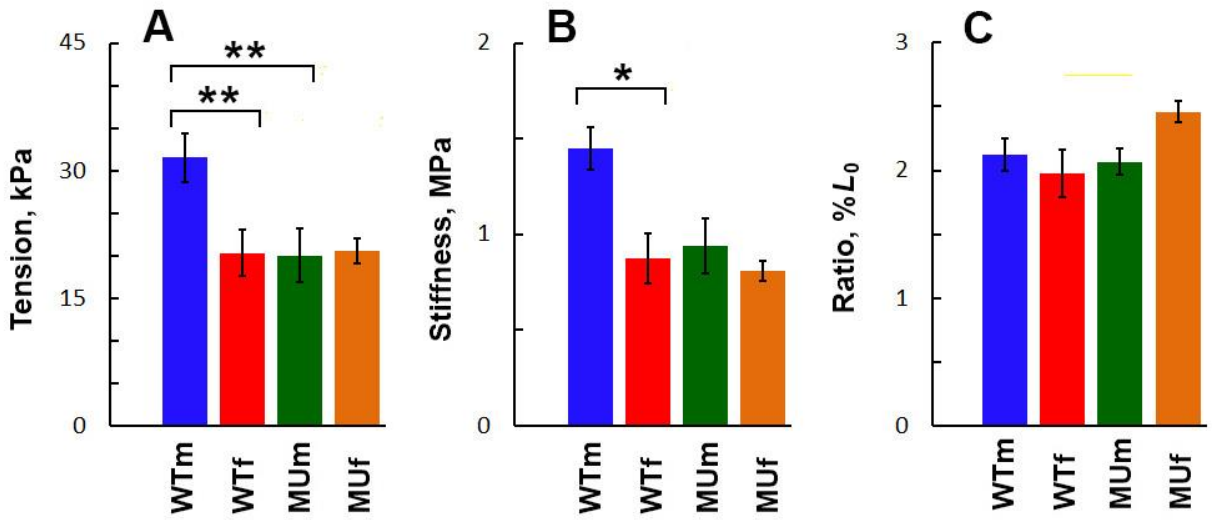

Figure. Results from standard activation. A: isometric tension, B: stiffness (Y_∞), and C: ratio (=Tension/Y_∞)L_0. The mean and SEM are shown. **p≤0.01 and *0.01<p≤0.05 by one-way ANOVA. WTm=Wildtype male (N=35), WTf=WT female (N=31), MUm=Mutant male (N=36), and MUf=Mutant female (N=33). Mutants are homozygote $MC4R^{-/-}$.

Dissecting the mechanisms by which a pathogenic variant in cardiac troponin T tail domain leads to disturbed myofilament performance

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Missense variant Ile79Asn in human cardiac troponin T (HcTnT-I79N) has been associated with familial hypertrophic cardiomyopathy (HCM), arrhythmia, and sudden cardiac death. Little is known about effects of this pathogenic variant on cardiac myofilament function and structure. To fill this gap, left ventricular papillary muscle bundles were harvested from non-transgenic (NTg) control mice and transgenic mice bearing HcTnT-I79N. The samples were permeabilized and mounted for mechanical measurements. Sarcomere length (SL 1.9, 2.1, or 2.3 μm) was set at pCa 8 using HeNe laser diffraction, followed by Ca^{2+}-dependence of isometric force, sinusoidal stiffness (SS, 0.2% PTP length oscillation), and rate of tension redevelopment (k_{TR}) measurements. We found that HcTnT-I79N tissue exhibited increased Ca^{2+}-sensitivity of force and SS, slower k_{TR} at all levels of Ca^{2+}-activation, and less length-dependent activation (LDA). Small-angle X-ray diffraction revealed that HcTnT-I79N permeabilized cardiac muscles exhibit smaller myofilament lattice spacing at longer SLs (2.1 μm and 2.3 μm) compared to NTg. Moreover, Tg-I79N exhibited a statistically significant reduction in I_{M3} at longer SLs (2.1 μm and 2.3 μm). Next, analysis of the angular spread (angle σ) and the width σ for the 1,0-equatorial reflection indicated that both NTg and HcTg-I79N exhibited better myofibrils alignment with the longitudinal axis upon cardiac muscle stretch. In addition, our data showed that the HcTg-I79N myocardium does not suffer from myofibrillar disorientation compared NTg. Using 3% Dextran T500 to osmotically compress the myofilament lattice (at SL 2.1 μm), HcTnT-I79N showed no change in myofilament lattice spacing and little response in contractile parameters associated with osmotic compression. Interestingly, in the presence of Dextran, HcTnT-I79N displayed a decrease in disordered relaxed state (DRX, ON state) of myosin and an increase in super-relaxed state (SRX, OFF state) of myosin compared to HcTnT-I79N with no dextran. We conclude that altered muscle mechanics, lack of responsiveness to osmotic compression, and reduced LDA observed with HcTnT-I79N are partially due to a combination of altered the relative ordering of myosin heads and disturbed ON and OFF states of myosin.

Pharmacological inhibition of myostatin improves muscle function and size in a mouse model of typical nemaline myopathy

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Nemaline myopathy is one of the most common non-dystrophic congenital myopathies. Patients with nemaline myopathy have muscle weakness and atrophic muscles that often require supportive interventions such as wheelchairs and/or respiratory support. Around half of the patients have compound heterozygous nebulin mutations that usually cause the typical form of the disease. There are no approved treatments for the disease as of today. Myostatin is a negative regulator of muscle mass, and we hypothesized that myostatin inhibition counteracts the muscle atrophy associated with the disease. To investigate this proposition, we used our mouse model with compound heterozygous nebulin mutations that phenocopies the typical form of the disease and treated them with mRK35*, a myostatin antibody. We performed weekly intraperitoneal injections of 10mg/kg mRK35 starting at two weeks of life until the mice were euthanatized, and tissue collected at four months of life. The treatment increased body weight and improved grip strength in both healthy and diseased mice. Running capacity was unchanged. *In vitro* mechanical experiment on intact extensor digitorum longus muscle revealed an increased physiological cross-sectional area and absolute force production in both wildtype and compound heterozygous mice. Specific force was unaltered by the treatment. The weight of most muscles was increased by ~20%. Cross-sectional area analysis of muscle fibers and signaling pathways involved in muscle hypertrophy are ongoing. Taken together, these data indicate that inhibition of myostatin counteracts the muscle deficits in typical nemaline myopathy and could represent a much-needed therapeutic option.
**mRK35 was kindly provided by Pfizer, Inc.*

MYBPC1 E248K Myopathy Structural and Functional Development from Early through Late Adulthood

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Myosin-binding protein C (MyBP-C) comprises a family of accessory proteins expressed in striated muscles that function in sarcomeric organization and actomyosin cross-bridge regulation. Mutations in *MYBPC1*, the gene encoding the slow skeletal isoform (sMyBP-C), have been linked to a novel myopathy characterized by muscle weakness, hypotonia, dysmorphia, skeletal rigidity and most distinctly, a high-frequency low-amplitude tremor. The absence of neuropathy in these patients as well as the restricted expression of sMyBP-C to skeletal muscle, suggest that the tremor represents a new entity originating at the level of the sarcomere. Patients harboring the *MYBPC1* E248K mutation display generalized myopathy and tremor more prominently in the upper and lower extremities, which evolves from an intense tremor at birth to a tremor accentuated only with posture in late adolescence and young to mid adulthood. However, the clinical presentation of the disease as well as its pathogenesis into late adulthood remain unknown.

To study this myopathy, our lab has generated a knock-in (KI) murine model expressing the E248K mutation that recapitulates the clinical pathology and disease progression seen in humans. Using this mouse model, we propose that disease progression of an affected individual is as follows: a high-intensity tremor and reduced force production are present and persist from birth through adolescence, followed by a period of stabilization where there is no further deterioration in muscle structure/function. This period of stabilization expires in late adulthood where muscle structure/function rapidly decline. We aim to extensively characterize the disease mechanisms at young-, mid-, and late-adulthood. We show that mid-adult KI mice (i.e., 12-months of age) exhibit severe deficits in sarcomeric and internal membrane structure accompanied by a reduction in expression of thick and thin filament-associated proteins, an expanded and misaligned t-tubule system, and an increased mitochondrial count. Further studies are underway aiming to decipher the structural and functional alterations taking place during the lifespan of the E248K model at the molecular, cellular, organ, and animal level.

Viral Vectors for Myofilament Research

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Our objective is to provide investigators access to vectors for translational studies and other basic research applications. We construct, purify and titer recombinant viral vectors for in vitro and in vivo use. rAdenovirus (rAd) and rAdeno-Associated Virus (rAAV) are the major vectors in demand, however, we also construct and purify rLentivirus. We utilize serotype 5 human adenovirus which is replication defective by deletion of the E1 and E3 genes. For in vitro myocyte studies, these vectors are well suited with fast expression profile, high titers, high capacity and easily renewable stocks. However they are problematic for in vivo use with significant immune response, higher safety profile, and transient expression. Thus, for in vivo use rAAV are the preferred mode. We currently have AAV serotypes 1, 2, 4, 6, 8, 8i, 9, 9.45 and PHP. We have ready-made reporter gene vectors for many of these in our inventory. We also can assist with design, cloning, mutagenesis and plasmid production.

In spite of incredible advances in the development of transgenesis models with tissue restriction, targeting and CRISPR, it is still primarily limited to murine models. Viral vectors can be utilized to manipulate the “genetics” of larger species and for targeted expression. Adeno-associated viruses (AAVs) have rapidly become versatile tools for these aims. Due to their dependant nature, decreased immune reactivity, and lack of disease association, recombinant AAVs also have lower safety concerns than most recombinant viruses. In addition, specific AAV serotypes have been suggested to have organ and cell type tropism for infection, particularly in rodent models, and especially with targeted promoters. Several examples of AAV use in myofilament and other relevant models are displayed in the poster. These delivery vehicles offer additional options for the study of human disease pathology in various experimental models.

pCa_{50} Correlates with Stroke Volume and Left Ventricular End Diastolic Volume in Heart Failure Patients

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Clinical work-ups for patients with heart failure often use echocardiography to measure dimensions of the heart and assess cardiac function. Commonly reported parameters include ejection fraction, left end diastolic volume, and stroke volume. Basic scientists can also use permeabilized myocardial preparations to measure cellular-level contraction. These experiments provide insight into the molecular mechanisms that regulate myocardial contraction and can be impaired in disease. Few studies have compared echo-based measurements with myofilament-level properties measured using cells from the same patients. We are currently mining previously-published contractile data to explore potential relationships between echo and cell-based measurements. Initial results suggest that the Ca^{2+}-sensitivity of myofilament level contraction (pCa_{50}) is positively correlated with left ventricle end diastolic volume and stroke volume. That is, high pCa_{50} values (high calcium sensitivity) are associated with high stroke volume and LVEDV. In contrast, there is no obvious relationship between ejection fraction and Ca^{2+}-sensitivity in patients with heart failure (n=27) who have ischemic or non-ischemic heart failure. We are continuing to explore these and other potential relationships in additional datasets. Our goal is to investigate how cell-level contraction translates to organ-level function.

Embryonic myosin mutations T178I and R672C result in mechanical and structural dysfunction in hiPSC derived skeletal myotubes

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Distal arthrogryposis (DA) is a skeletal muscle disorder characterized by joint contractures predominantly localized in the distal extremities. DA is associated with syndromes like Freeman-Sheldon (FSS), induced by mutations in the sarcomeric protein-encoding gene MYH3. This gene is responsible for generating embryonic skeletal muscle myosin during advancement. We have previously reported demembranated myofibrils from affected individuals expressing the R672C mutation in MYH3 showed reduced specific force and longer relaxation kinetics (Racca et al., 2015). To study the mutation in developing muscle, we generated three human induced pluripotent stem cell (hiPSC) skeletal myotube lines that express T178I (patient derived) or R672C (CRISPR generated heterozygous and homozygous lines) *MYH3* gene mutations associated with FSS. Preliminary myofibril measurements from T178I myotubes generated significantly greater ($p<0.05$) specific force (201±32 mN/mm^2) compared to control myofibrils (127±20 mN/mm^2). Diseased myofibrils also expressed significantly ($p<0.05$) slower rates of relaxation ($k_{REL,fast}$, 3.9±0.3 s^{-1}) and prolonged thin filament deactivation ($t_{REL,slow}$, 54.7±4.5 ms) compared to control myofibrils (6.9±1.7s^{-1} and 39.0±2.2 ms). In addition to myofibril mechanics, myofibrils were utilized for stop flow ATP binding experiments. Preliminary results from homozygous expressing R672C myofibrils showed faster ATP binding rates (0.7127 uM^{-1}s^{-1}) compared to heterozygous (0.377 uM^{-1}s^{-1}) and control (0.3503 uM^{-1}s^{-1}) preparations. To understand the structural dysfunction produced by these mutated embryonic myosin expressing cells, we have also used molecular dynamics simulations to investigate the structural basis for altered relaxation in T178I and R672C. The two mutant residues neighbor one another in the central β-sheet of myosin, positioned between the nucleotide binding pocket and a converter-connected α-helix. In post rigor state simulations, R672C and T178I mutations produced greater distances between the B-sheet and SH-Helix (10.1 Å and 9.5 Å respectably) when compared to control (9.1 Å). Simulations also showed that mutations disrupted local salt bridges and hydrogen bond formation between several different interacting amino acid residues that help stabilize the nucleotide binding pocket and converter-connected helix. These simulations suggest impaired relaxation due to interrupted structural communication between the nucleotide binding pocket and surrounding functional regions. To further investigate the role of actin-myosin binding and crossbridge cycling kinetics, ongoing studies will utilize myofibril kinetics, *in*-vitro motility (IVM) assay and stop flow to assess the specific chemo-mechanical dysfunctions that occur with mutated MYH3. Supported by RM1GM131981, HD48895, GM131891, EB001650.

Do aging-related post-transcriptional myosin modifications contribute to sarcopenia?

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Diminished skeletal muscle function with age contributes to many health problems and accrues economic burdens. While a decline in muscle function is normal with age, excessively reduced skeletal muscle mass, power, and endurance associated with age is known as sarcopenia. Muscle function decline with age is thought to stem from a decline in muscle regeneration capacities, reduced protein synthesis, and enhanced protein degradation [1]. Recently, mass spectrometry revealed specific, age-related post-translational modifications (PTMs) to the myosin motor protein [2]. While corelative, there is no experimental evidence to show these PTMs directly contribute to impaired muscle function. We are testing causation by creating mutations in *Drosophila* myosin that mimic known human myosin PTMs and determining how they impact myosin and muscle properties. In this study, we focused on carbonylation of arginine at amino acid 908 in the S2 linker domain (R908E) and hydroxylation of asparagine at amino acid 81 (N81T) in the catalytic domain. We took advantage of the single *Drosophila* muscle myosin heavy chain gene to engineer transgenic fly lines expressing these mutations in their flight and jump muscles to determine if these PTMs directly cause alterations to muscle structure, locomotion, and myosin and muscle function.

EM images of indirect flight muscle from 2-hour-old homozygous R908E flies exhibited no differences in the structural integrity of their sarcomeres compared to control flies, while 2-dayold flies showed slight disruptions which progressed with age. Flight ability of 2-day-old flies was slightly decreased, with average wing beat frequency (WBF) of the R908E homozygous flies slowed about 12%. Maximum actin activated ATPase rate of myosin isolated from homozygous R908E flies decreased approximately 40%. Surprisingly, IFM fibers isolated from 2-hour-old flies did not show any changes in muscle mechanical parameters such as power, work, or frequency at which maximum power occurred (f_{max}) compared to control flies.

In contrast, N81T homozygous flies showed more severe disruption of sarcomere structure, already by two hours of age, and could not fly. Myosin isolated from these flies showed no actin-activated ATPase activity. The more severe structural disruption precluded muscle mechanics experiments, therefore, we investigated heterozygous flies which had much better sarcomere structure. 3-day old N81T heterozygotes could fly with an ~14% decrease in wing beat frequency. Preliminary muscle fibers results from heterozygous flies suggest less power production, >50%, than control fibers and lower f_{max}. These results imply that while the N81T mutation severely impacts muscle function, its effects are not fully dominant over wild type myosin.

Overall, we found that both mutations negatively impact muscle structure and some functional properties, supporting the hypothesis that myosin PTMs contribute to sarcopenia. The lack of alteration to muscle mechanical function for R908E suggests that stability of thick filaments or other sarcomere structure likely has more of a negative impact on locomotion than the observed decrease in enzymatic activity (ATPase rate). The more severe functional changes from N81T and its location in the myosin head suggest its impact is more directly associated with altered muscle function and that the structural disruptions may be a secondary effect.

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[2] Li M et al. (2015) *Aging Cell*, **14**: 228-235.

Recruiting Super-Relaxed Myosin to Restore Contractility in Human Right Heart Failure

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Right heart failure worsens outcomes in patients with Heart Failure with Reduced Ejection Fraction (HFrEF), yet the precise mechanisms behind depressed right ventricular (RV) function in this population remain unknown. We have reported depressed maximal calcium activated tension (T_{max}) in HFrEF patients with RV dysfunction, identifying one likely cause. Here, we tested the hypothesis that reduced T_{max} is associated with a greater proportion of myosin in a super-relaxed (SRX) state of cardiac myosin that lacks force-producing capability and have low ATP turnover rates. To test this, we examined explanted RV tissue from 25 HFrEF patients and 9 non-failing controls and quantified mechanics from permeabilized RV myocytes and small angle X-ray diffraction patterns from the same tissue. Compared to non-failing controls, we found a 50% decline in T_{max}, elevated calcium sensitivity, a 40-50% depression in maximum power, and elevated viscoelasticity in RV HFrEF samples. We then trained a Gaussian Mixture Model, an unsupervised machine learning approach that fits high-dimensional multivariate Gaussians, to measured myocyte mechanical properties, and found that T_{max} underlies the majority of observed variance in both mechanics and clinical RV hemodynamics. Importantly, the latter was not used in training the model. As T_{max} explained most of the heterogeneity in myocyte mechanics and clinical hemodynamics, we sought underlying abnormalities that could explain T_{max} depression, in particular structural changes in the sarcomere and the proportion of SRX myosin. To quantify structural changes, X-ray diffraction patterns were obtained of isolated skinned fibers from the previously frozen myocardial tissues. We found HFrEF with RV failure (e.g., low T_{max}) had reduced equatorial intensity ratio ($I_{1,1}/I_{1,0}$) and decreased spacing of the M3 reflection (S_{M3}) when compared to HFrEF patients with normal range T_{max} or to non-failing controls. These findings support increased SRX myosin in low RV-T_{max} HFrEF. We further confirmed this biochemically by quantifying ATP turnover of myosin in relaxing conditions. We then pharmacologically manipulated the ratio of SRX myosin, confirmed with X-ray diffraction and biochemically: we employed mavacamten (to increase SRX) and deoxy-ATP and EMD-57033 (to decrease SRX). We could selectively augment T_{max} with both deoxy-ATP and EMD-57033 in HFrEF RV myocytes with a low T_{max}, while depressing it with mavacemten in RV HFrEF myocytes with normal T_{max} or in non-failing controls. In summary, we find depressed myocyte mechanics and increased SRX myosin contribute to RV failure and heterogeneous RV function in HFrEF. Small molecules that recruit SRX myosin may have therapeutic benefit in the former group.

Prior freezing has minimal impact on the contractile properties of permeabilized human myocardium

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Abstract

Background:

Experiments measuring the contractile properties of human myocardium are important for translational research but complicated by the logistical difficulties of acquiring specimens. Accordingly, many groups perform contractile assays using samples that are acquired from patients at one institution and shipped to another institution for experiments. This necessitates freezing the samples and performing subsequent assays using chemically permeabilized preparations. It is unknown how prior freezing impacts the contractile function of these preparations.

Methods and Results:

To examine the effects of freezing we measured the contractile function of never frozen and previously frozen myocardial samples. Samples of left ventricular tissue were obtained from 7 patients who were having a ventricular assist device implanted. Half of each sample was chemically permeabilized and used immediately for contractile assays. The other half of the sample was snap frozen in liquid nitrogen and maintained at –180 °C for at least 6 months before being thawed and tested in a second series of experiments. Maximum isometric force measured in pCa 4.5 solution, passive force measured in pCa 9.0 solution, and Hill coefficients were not influenced by prior freezing ($p = 0.07$, $p = 0.14$, and 0.27 respectively). pCa_{50} in never frozen samples (6.11 ± 0.04) was statistically greater ($p < 0.001$) than that measured after prior freezing (5.99 ± 0.04) but the magnitude of the effect was only ~0.1 pCa units.

Conclusions:

We conclude that prior freezing has minimal impact on the contractile properties that can be measured using chemically permeabilized human myocardium.

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