Contractile Systems, Mechanobiology & Regulation: Novel Concepts, Technologies & Applications

May 18-21, 2024

Monona Terrace Convention Center
Madison, Wisconsin

Sponsors:
Welcome Participants!

It is our great pleasure to welcome you to Madison and the 2024 Myofilament Meeting, “Contractile Systems, Mechanobiology & Regulation: Novel Concepts Technologies & Applications”. This is the eighth in a series of biennial meetings featuring both poster and platform sessions focused on myofibrillar and cytoskeletal proteins. We are pleased to have more than 180 registrants for the 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:

- Aurora Scientific
- Cytokinetics
- IonOptix
- Journal of General Physiology
- Journal of Molecular and Cellular Cardiology
- Myologica
- Sarver Heart Center | (heart.arizona.edu)
- Shanghai Model Organisms Center LLC
- University of Wisconsin-Madison Cardiovascular Research Center
- University of Wisconsin-Madison School of Medicine and Public Health
- UW Center for Translational Muscle Research (CTMR)

We hope you enjoy the meeting!

Scientific Organizing Committee:

Henk Granzier (Chair)  Aikaterini Kontrogianni-Konstantopoulos
University of Arizona  University of Maryland

Anthony R. Cammarato  Brandon J. Biesiadecki
Johns Hopkins University  The Ohio State University

Elisabetha Brunello  Jonathan Kirk
King’s College London  Loyola University Chicago

John Carter Ralphe  Jolanda van der Velden
University of Wisconsin-Madison  Amsterdam UMC

Michael Regnier  Richard L. Moss (Ex Officio)
University of Washington  University of Wisconsin-Madison
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**
- Thick/Thin Filament Structure
- Thick/Thin Filament Regulation in Striated Muscle
- Myofilament Contribution to Human Diseases

**Monday Poster Session Categories**
- Cardiomyopathies
- Length Dependent Force Development
- Regulation & Regulatory Signaling Networks
- Elastic Proteins in Muscle
- Models of Contraction/Regulation
- Determinants of Relaxation

- 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.

Internet Access at Monona Terrace Convention Center

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

1. The shuttle to the Tinsmith will be arriving at 5:15pm at the Monona Terrace - 1 John Nolen Drive, Madison, WI, 53703. Please be at this location before 5:15pm.

2. There will be assigned seating at the dinner.
   a. Your table number is located on the back of your name tag.
   b. Please bring your name tag to the dinner to ensure you receive the correct entrée.
   c. Dinner at Tinsmith will begin with a reception in the Tinsmith foyer. An announcement will be made when it is time for dinner – please find your assigned table and place your name tag on the seat of your choice (colored sticker facing up).

3. The shuttle to return to the Monona Terrace after dinner will arrive at The Tinsmith at 8:45pm.

If you wish to walk to or from The Tinsmith, please see the map below:

![Google Map of the area around The Tinsmith](image)
Things to do in Madison, WI!

With five lakes, multiple parks, museums, trails, and a world-renowned university, Madison has something for everyone. For more, see Destination Madison (https://www.visitmadison.com)

**Biking, Walking, Hiking**

Take a spin around our lakes or on the many bike paths using an ebike [https://madison.bcycle.com](https://madison.bcycle.com). Walk or run from the Memorial Union along the Temin Lakeshore Path. Or take an Uber to the UW-Madison Arboretum ([https://arboretum.wisc.edu](https://arboretum.wisc.edu)), where you can walk around and enjoy a brilliant curation of trees and wildlife.

**Paddling**


**Wet your Whistle**

Sample craft brews, cocktails, and mocktails at nearby restaurants like Merchant, Tornado Room, Nattspil, and the Robin Room. Or make a reservation at Fairchild, home of the 2023 James Beard’s Best Chef Midwest Award!

**Art**

Check out the Chazen Museum of Art on the UW-Madison campus or on State Street, closer to the meeting site, tour MMOCA, Madison’s modern art museum.
Thank you to our Myofilament Meeting Sponsors!

(Gold)

Cytokineti

(Silver)

ION OPTIX MYOLOGICA

(Bronze)

aurora SCIENTIFIC
(Other Meeting Sponsors)
Plenary Speaker Biographies

Elizabeth McNally, MD, PhD, Northwestern University

Elizabeth McNally directs the Center for Genetic Medicine at Northwestern University’s Feinberg School of Medicine in Chicago and is the Elizabeth J. Ward Professor of Genetic Medicine. She is a practicing cardiologist with expertise in cardiovascular genetics. She has a special interest in neuromuscular diseases like muscular dystrophy and their accompanying cardiovascular complications. In her research, her group has defined primary genetic causes and mechanisms causing myopathic processes, including understanding the mediators of sarcolemmal stability and membrane repair. Her group has pioneered methods for defining genetic modifiers of primary genetic mutations. Several of these genetic signals are now being used to drive the development of new treatments. She is a former Established Investigator of the AHA and is past Chair of the AHA’s Council on Basic Cardiovascular Sciences. She serves on the Board of Directors for the Muscular Dystrophy Association. She is a past president of the American Society for Clinical Investigation and the Association of American Physicians. In 2021, she was elected to the American Academy of Arts and Sciences and the National Academy of Medicine. She is the Editor-in-Chief of the Journal of Clinical Investigation.

R. John Solaro, PhD, University of Illinois at Chicago

R. John Solaro received a PhD in the Department of Physiology, University of Pittsburgh School of Medicine in 1971 and moved immediately 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 Solaro’s lab, has over 400 peer reviewed publications, has been continually funded by NIH/AHA for 50 years, and focuses 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. Seminal papers published in Nature established the critical roles of protein phosphorylation in control of the heartbeat. A 1982 paper in Circulation Research with Dr. Caspar Ruegg is a seminal paper supporting this idea of developing drugs directly enhancing myofilament Ca-response.
Raúl Padrón, PhD, University of Massachusetts

Raúl Padrón, a Professor at the University of Massachusetts Chan Medical School since 2018, is a structural biologist recognized for his work on the structure and function of myosin thick filaments of skeletal, cardiac, and smooth muscle. He is known particularly for his studies on the myosin interacting-head motif (IHM) structure and function and their implications on how the thick filaments of muscle relax, super-relax, and become activated, and its consequences on the molecular pathogenesis of human muscle diseases hypertrophic and dilated cardiomyopathy. Padrón was born and grew up in Caracas, Venezuela. He graduated from the Universidad Central de Venezuela with a degree in Electrical Engineering and from the Venezuelan Institute for Scientific Research with an M. Sc. in Biology and a summa cum laude Ph.D. in Biophysics and Physiology in 1979. He was a postdoctoral fellow in muscle structure and function at the MRC Laboratory of Molecular Biology (Cambridge, U.K.) in 1980. He joined the Venezuelan Institute for Scientific Research in 1983, where he founded the Center of Structural Biology, where he was an International Research Scholar at the Howard Hughes Medical Institute (HHMI) from 1997 until 2011. He was elected a member of the Latin American Academy of Sciences (ACAL) in 2002, a fellow of the World Academy of Sciences (TWAS) in 2004, and an international member of the U. S. National Academy of Sciences in 2018.

Malcolm Irving, PhD, FRS, King’s College London

Malcolm Irving is Professor of Biophysics at King’s College London, and is based in the Randall Centre for Cell and Molecular Biophysics and British Heart Foundation Centre of Research Excellence. Following an undergraduate degree in experimental physics, he did a PhD in physiology with a research project on muscle energetics. This triggered a career-long interest in muscle biophysics. His early research was focused on the force-generating working stroke in the myosin motor. He developed and applied synchrotron X-ray techniques with collaborators including Vincenzo Lombardi, Gabriella Piazzesi, Marco Linari, and Massimo Reconditi, and optical methods with David Trentham, John Corrie and Yale Goldman, that allowed the size and speed of the working stroke to be measured in an intact muscle fibre on the millisecond timescale. The results led to a description of the physiological performance of skeletal muscle in terms of changes in the conformation of the myosin motor in situ. Later, in collaboration with Brian Sykes, Yin-Biao Sun and others, he applied one of the optical methods, based on polarised fluorescence from bifunctional fluorophores crosslinking pairs of cysteines in a target protein domain, to determine the structural changes in troponin linked to calcium regulation of muscle
contraction. His more recent research, with Elisabetta Brunello, Luca Fusi, Thomas Kampourakis and others, applied the optical and X-ray methods to the study of thick- and thin-filament regulatory mechanisms in both skeletal and cardiac muscle.

Roger Craig, PhD, University of Massachusetts Chan Medical School

Roger Craig is Professor of Cell Biology and Imaging in the Department of Radiology at UMass Chan Medical School in Worcester, MA. He received his bachelor’s degree in zoology from the University of Sydney and PhD in biophysics from King’s College, London. Following postdoctoral training at Brandeis University, Oxford University, and MRC LMB (Cambridge), he joined the faculty at UMass Medical School. His lab is interested in the molecular structure/function of the thick and thin filaments of smooth, skeletal and cardiac muscle and of their constituent molecules. His research currently focuses on the structure, function and evolution of the interacting-heads motif (IHM) of myosin, from spiders to humans, using cryo-EM and single particle analysis. His lab’s most recent work revealed the organization of IHMs, cMyBP-C and titin in human cardiac muscle at high resolution, providing new insights into basic mechanisms of cardiac function and disease.

Stefan Raunser, PhD, Max Planck Institute of Molecular Physiology

Stefan Raunser is a structural biologist whose research focuses on understanding molecular mechanisms underlying cellular processes in the healthy and diseased organism. His pioneering structural studies have led to groundbreaking discoveries in the areas of muscle and cytoskeletal research as well as toxicology. Raunser is Managing Director of the Max Planck institute of Molecular Physiology, Adjunct Professor at Technical University Dortmund and Honorary Professor at University of Duisburg-Essen. With his research group, he develops and uses a multi-disciplinary approach, including biochemical reconstitutions, high-resolution electron cryomicroscopy (cryo-EM) and electron cryotomography (cryo-ET) primarily to investigate the structure of macromolecular complexes that play a crucial role in muscle contraction, the dynamics of the eukaryotic cytoskeleton and bacterial infections. A detailed understanding of these processes is of great importance as they ultimately serve to develop pharmaceutical measures to combat disease.

He has authored over 130 papers in the fields of structural and molecular biology and has given over 200 lectures and seminars around the world. He is a scientific member of the Max Planck Society, an elected member of the North Rhine Westphalian Academy of Sciences and Arts, the German National Academy of Sciences Leopoldina and EMBO.
Krishna Chinthalapudi, PhD, Ohio State University

Krishna Chinthalapudi, PhD, is a structural biologist and Assistant Professor in the Department of Physiology & Cell Biology at The Ohio State University College of Medicine. His academic journey includes a PhD from Hannover Medical School, Germany, and postdoctoral work at The Scripps Research Institute. Prior to his current role, he performed research on myosin motors at the National Heart, Lung, and Blood Institute in Bethesda, Maryland. Dr. Chinthalapudi’s research is pivotal in elucidating the structural mechanisms of several proteins with implications for human health. His work centers on the contractile forces of myosins and their impact on muscle and non-muscle cells, employing advanced techniques such as cryo-electron microscopy, biochemistry, and biophysics. Using these state-of-the-art techniques, he aims to unravel the dynamic intricacies of cardiac muscle function, driving scientific advancements that will inform novel rational therapeutics to improve patient outcomes. A prolific contributor to the scientific community, Dr. Chinthalapudi has published extensively in top-tier journals and is an active member of prestigious societies such as the Biophysical Society and the American Heart Association. He also serves as an ad hoc reviewer for major scientific journals and funding bodies including the NIH, NSF, and AHA.

Anne Houdusse, PhD, Curie Institute

Anne Houdusse, Ph.D. is a CNRS research director, head of the Structural Motility team of the Cell Biology and Cancer laboratory (CNRS/Institut Curie). Anne Houdusse was trained as a structural biologist and a chemist from ENS and Pasteur Institute. With HFSP and EMBO fellowships, she worked six years at Brandeis University, USA - as a post-doctoral fellow with Carolyn Cohen, Andrew Szent Gyorgyi and Hugh Huxley to provide high-resolution structures of muscle myosin. She initiated the study of structural biology at the Curie Institute in 1999 with the goal of answering critical questions in cell biology and developing new therapies against human diseases. The focus of her laboratory is on the study of nanomachines, these molecular motors that produce force within our cells, and which are essential for their dynamic organization, their ability to contract and migrate. From high-resolution structural knowledge, the challenge lies in identifying how allostery can tune these motors for precise yet distinct cellular roles and how their dysfunction leads to pathologies. By identifying cryptic pockets and understanding how modulators control activity, structural studies facilitate the discovery of new therapeutic solutions against cardiomyopathies, skeletal muscle diseases, malaria and cancer.
She received the CNRS Bronze and Silver Medals and she was elected as an EMBO member in 2013. Her contributions in the motor field have been recognized with the FEBS/EMBO Women in Science Award in 2009 and with the Prix Lecocq from the French Academy of Sciences in 2018. She has been elected as a member of the French Academy of Sciences in December 2019 and was nominated Knight in the National Order of Merit (2020).

Kenneth Taylor, PhD, Florida State University

Kenneth A. Taylor is the Donald L. D. Caspar Professor of Biological Science at Florida State University. His laboratories primary experimental tool is 3-D cryoelectron microscopy (cryoEM). He obtained his PhD in Biophysics from the University of California at Berkeley under the direction of Prof. Robert Glaeser where he was the first to demonstrate that molecular images could be obtained from specimens embedded in ice and that thin protein crystals were undamaged by freezing without cryoprotectants. He then spent almost four years at the MRC Laboratory of Molecular Biology in Cambridge, U.K. where he learned 3-D image reconstruction with the late Dr. L. A. Amos and began his career in muscle research with the late Dr. H. E. Huxley. His muscle research continued after taking his first academic position at Duke University Medical Center where he began a collaboration with the late Prof. Michael K. Reedy obtaining 3-D images using electron tomography of the highly ordered indirect flight muscles from the giant water bug Lethocerus indicus in various biochemical states including active contraction. His research program is possibly the only one to have imaged myosin head conformations within the muscle lattice during actual contraction using fast frozen, freeze substituted and sectioned muscle. In 1995 he moved to Florida State University where he developed and directed a modern cryoEM facility, continued working on Lethocerus flight muscle but also branched out into determining protein structures using lipid monolayers. One of these was the first 3-D image of the inhibited state of smooth muscle myosin, a conformation later shown nearly ubiquitous in multicellular organisms. Following the passing of Mike and Mary Reedy, his laboratory began work determining the high-resolution structure of striated muscle myosin filaments which resulted in the first subnanometer resolution 3-D image of a myosin filament from Lethocerus and later the first atomic model of the myosin tail in its native environment. These thick filament studies continue today producing subnanometer thick filament structures from the fruit fly, Drosophila melanogaster and the Asian bumble bee, Bombus ignitus. In the 40 years that he has been a Principal Investigator he has served on 81 study sections, on the NIGMS Council Advisory Committee for the PSI:Biology program in 2013, on the NIGMS Council Advisory Committee for the Structural Biology of AIDS Program from 2014-2018, is currently an Advisor to the NIH Common Fund cryoEM Centers, served on the External Advisory Committees of BioCAT beam line at Argonne National Lab, Dr. Tamir Gonin’s MEDIC P41 grant, has serve on the Editorial Board of the Journal of Structural Biology since its founding in 1990 and as Associate Editor since October 2018. He chaired a Gordon Research Conference on 3-D Electron Microscopy of
Macromolecules in 2003, when acting as chair, he initiated the change from a biennial conference to an annual one. The Microscopy Society of America named him a Fellow in 2016 and in 2022 the Distinguished Scientist in Biology.
# 2024 MYOFILAMENT MEETING

**Contractile Systems, Mechanobiology & Regulation: Novel Concepts Technologies & Applications**  
*May 18-21, 2024*

## SATURDAY – MAY 18, 2024

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>9:30 am–1:00 pm</td>
<td>Conference Registration</td>
<td>Monona Convention Center, Counter 4</td>
</tr>
</tbody>
</table>

### Early Career Investigator Symposium

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

*Symposium Organizers:*

- **Brett Colson**  
  University of Arizona
- **Kathleen Woulfe**  
  University of Colorado  
  Anschutz Medical Campus
- **Bertrand Tanner**  
  Washington State University
- **David Barefield**  
  Loyola University Chicago

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:10 am</td>
<td>Welcome</td>
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</tbody>
</table>

### Trainee Group 1: Myosin Structure-Function

10:15 am – 11:15 pm (8 minute talks, 2 minutes for questions & setup next speaker)

<table>
<thead>
<tr>
<th>Time</th>
<th>Speaker</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:15</td>
<td><strong>Alice Arcidiacono</strong>, King's College London</td>
</tr>
<tr>
<td>10:25</td>
<td><strong>Rama Goluguri</strong>, Stanford University</td>
</tr>
<tr>
<td>10:35</td>
<td><strong>Ruchi Sharma</strong>, University of Massachusetts</td>
</tr>
<tr>
<td>10:45</td>
<td><strong>Robbert van der Pijl</strong>, University of Arizona</td>
</tr>
<tr>
<td>10:55</td>
<td><strong>Michel Kuehn</strong>, University of Muenster</td>
</tr>
<tr>
<td>11:05</td>
<td><strong>Ilaria Morotti</strong>, University of Florence</td>
</tr>
</tbody>
</table>

11:15 – 11:30 am Break
### Trainee Group 2: Myofilament Regulation
11:30 am – 12:30 pm (8 minute talks, 2 minutes for questions & setup next speaker)

<table>
<thead>
<tr>
<th>Time</th>
<th>Speaker</th>
<th>Institution</th>
</tr>
</thead>
<tbody>
<tr>
<td>11:30</td>
<td>Catherine Hoover</td>
<td>University of Arizona</td>
</tr>
<tr>
<td>11:40</td>
<td>Taejeong Song</td>
<td>University of Cincinnati</td>
</tr>
<tr>
<td>11:50</td>
<td>Caterina Squarci</td>
<td>University of Kentucky</td>
</tr>
<tr>
<td>12:00</td>
<td>Chris Hoffer</td>
<td>University of Colorado Anschutz Medical Campus</td>
</tr>
<tr>
<td>12:10</td>
<td>Christine Delligatti</td>
<td>Loyola University Chicago</td>
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<tr>
<td>12:20</td>
<td>Nichlas Engels</td>
<td>University of Arizona</td>
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<tr>
<td>12:30 – 1:30 pm</td>
<td>Lunch (Ballroom C)</td>
<td></td>
</tr>
<tr>
<td>1:45 – 2:25 pm</td>
<td>Seeing is believing: focusing in on myofilament structure</td>
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<tr>
<td></td>
<td>Senior PI Plenary Presentation: Roger Craig, PhD</td>
<td>Division of Cell Biology and Imaging, Department of Radiology, University of Massachusetts Chan Medical School</td>
</tr>
</tbody>
</table>

### Trainee Career Development Breakout Session
2:35 – 3:40 pm 2x, 30 minute options (4 total topics), with 5 minute transition

- **Group A1) Academic Career Progression – Pre- to Post-doc**
- **Group A2) Academic Career Progression – Trainee to Faculty**
- **Group B) Leveraging Biophysical Training**
  - Outside the Faculty Track
- **Group C) Future of Muscle Biophysics in an AI World**
- **Group D) Intrinsically Disordered Discussions (not proteins)**

### Trainee Group 3: Myofilament Dysregulation in Disease
4:00 – 5:00 pm (8 minute talks, 2 minutes for questions & setup next speaker)

<table>
<thead>
<tr>
<th>Time</th>
<th>Speaker</th>
<th>Institution</th>
</tr>
</thead>
<tbody>
<tr>
<td>4:00</td>
<td>Ricardo Galli</td>
<td>Amsterdam UMC</td>
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<tr>
<td>4:10</td>
<td>Aishwarya Iyer</td>
<td>University of Maryland</td>
</tr>
<tr>
<td>5:20</td>
<td>Sonette Steczina</td>
<td>University of Washington</td>
</tr>
<tr>
<td>4:30</td>
<td>Christopher McAllister</td>
<td>University of Pennsylvania</td>
</tr>
<tr>
<td>4:40</td>
<td>Saffie Mohran</td>
<td>University of Washington</td>
</tr>
<tr>
<td>4:50</td>
<td>Ying-Hsi Lin</td>
<td>Duke-NUS (Singapore)</td>
</tr>
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</table>

### ECI Closing Discussion and Poster Judging Overview
5:00 pm

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:30 – 7:00 pm</td>
<td>Welcome Reception</td>
<td>Rooms H – J</td>
</tr>
</tbody>
</table>
Theme: Contractile Systems, Mechanobiology & Regulation: Novel Concepts, Technologies & Applications

Scientific Organizing Committee: B. Biesiadecki, E. Brunello, A. Cammarato, H. Granzier (Chair), J. Kirk, K. Kontrogianni-Konstantopoulos, J.C., Ralphe, M. Regnier, J. van der Velden, and R. Moss (ex officio)

ECI Committee: B. Tanner & B. Colson /K.C. Woulfe & D. Barefield. B. Biesiadecki and A. Cammarato are organizing committee liaisons to the ECI committee for Saturday, May 18. The program will feature scientific presentations by ECI investigators and career development activities with senior Keynote speaker, Roger Craig.

Local Organizing Committee: Carter Ralphe (Chair), Willem deLange, Ying Ge, Wei Guo, Katie Randall (Univ of Wisconsin), Cara Deery (Univ of Arizona)

The meeting comprises:

- Two plenary sessions plus panel discussion (75 minutes, 60 min, and 45 mins, resp.).
- Five oral sessions. Session I has 4 talks of 20 minutes each + one ECI talk of 10 minutes. Session II has three talks of 20 minutes each + one ECI talk of 10 minutes. Session III has 4 talks of 20 min, one talk of 10 min and 1 ECI talk of 10 min. Sessions IV and V each have four talks of 20 minutes each + one ECI talk of 10 minutes.
- Plenary lecture of 40 minutes.
- Two poster sessions.
- A closing session including Poster awards, ECI awards, discussion of current and future directions in the field

Each session will have an established investigator and an early career investigator (ECI) as co-moderators. The meeting organizing committee will work with the moderators to determine all but one speaker in each oral session. The remaining slot in each session was designated for an ECI scientist and has been filled in consultation with the ECI leadership.

<table>
<thead>
<tr>
<th>Saturday, May 18th</th>
<th>Sunday, May 19th</th>
<th>Monday, May 20th</th>
<th>Tuesday, May 21st</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECI Sessions (all day)</td>
<td>Welcome</td>
<td>Oral session III</td>
<td>Oral session V</td>
</tr>
<tr>
<td>Plenary session I</td>
<td>Plenary session II + panel discussion</td>
<td>Tissue slice workshop</td>
<td>Closing session</td>
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<tr>
<td>Oral session I</td>
<td>Oral session IV</td>
<td>Topical workshop I</td>
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<tr>
<td>Oral session II</td>
<td>Poster session II</td>
<td>Topical workshop II</td>
<td></td>
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<tr>
<td>Poster session I</td>
<td></td>
<td>Topical workshop III</td>
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<tr>
<td>Networking event</td>
<td>Meeting dinner</td>
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## Schedule of Events

### Saturday, May 18

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
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</thead>
</table>
| 10:00 – 5:30 | Hall of Ideas  
ECI Sessions (see website for program) |
| 5:30 – 7:30 | Welcome reception                                         |

### Sunday, May 19

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
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<tbody>
<tr>
<td>7:30 – 8:15</td>
<td>Continental Breakfast</td>
</tr>
<tr>
<td>8:15 – 8:30</td>
<td>Welcome</td>
</tr>
</tbody>
</table>
| 8:30 – 9:45 | Plenary Session I — High resolution myofilament structure  
(3x25; includes Q&A).  
Malcom Irving, moderator  
- Roger Craig, “Structure of the human cardiac thick filament C-zone at 6 Å resolution”  
- Stephan Raunser, “Unlocking the secrets of heart muscle structure”  
- Ken Taylor, “What can a pair of atomic resolution structures of Drosophila thick filaments tell us about how Vertebrate muscle works?” |
| 9:45 – 10:15 | Refreshment Break                                           |
| 10:15 – 11:15 | Plenary Session II—Implication of high-resolution myofilament structure  
(3x20; includes Q&A).  
John Solaro, moderator  
- Krishna Chinthalapudi, “Structural insights into thick filament regulation mechanisms”  
- Anne Houdusse “Essential insights into the regulation of thick filaments based on high-resolution structures and dynamic simulations”  
- Raul Padron, “HCM and DCM pathogenesis reside on different cardiac thick filament interfaces” |
<table>
<thead>
<tr>
<th>Time</th>
<th>Location</th>
<th>Event Description</th>
</tr>
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<tbody>
<tr>
<td>11:15 – 12:00</td>
<td>Lecture Hall</td>
<td>Panel discussion (plenary session speakers), Malcom Irving &amp; John Solaro (moderators)</td>
</tr>
<tr>
<td>12:00 – 1:00</td>
<td>Community Terrace</td>
<td>Lunch</td>
</tr>
<tr>
<td>3:40 – 5:30</td>
<td>Hall of Ideas</td>
<td>Poster session I (refreshments served)</td>
</tr>
<tr>
<td>6:30 – 8:00</td>
<td>Top of the Park</td>
<td>Evening event – Networking: standing dinner for all meeting participants (Best Western Park Hotel) 22 S. Carol St. Madison, WI</td>
</tr>
<tr>
<td>Time</td>
<td>Location</td>
<td>Session</td>
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<tr>
<td>7:30 – 8:15</td>
<td>Community Terrace</td>
<td>Breakfast</td>
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</tbody>
</table>
| 8:30 – 10:10 | Lecture Hall      | Oral Session III – MyBP-C, titin and other thick filament-associated proteins. (4 speakers x 20 min) + one speaker (10 min) + ECI speaker, (10 min). Sam Harris, moderator
Aishwarya Iyer, ECI moderator
- Brett Colson, “Roles of myosin-binding protein C in contractile function, disease and therapy”
- Michael Gotthardt, “From the chamber of proteins to the order of the filament: Following titin around the (cardio)myocyte”
- Coen Ottenheijm, “Diaphragm weakness in mechanically ventilated ICU patients: role for super relaxed myosins?”
- Danuta Szczesna-Cordary, “N-Terminus of the cardiac myosin essential light chain: Insights from cross-genotype mouse models”
- Pradeep Luther, “A new network cross-linking thick filaments near the edge of the bare region in vertebrate striated muscle may be due to titin and titin kinase”, 10 min
- ECI Speaker, Alejandro Alvarez-Arce. “The 62 amino terminal residues of MyBP-HL contain a unique domain that can regulate sarcomere function”
| 10:10 – 10:40| Lecture Hall      | Refreshment Break                 |
| 10:40 – 11:25| Lecture Hall      | Plenary Lecture – Elizabeth McNally. “Structural protein gene mutations and myocarditis” (40 min including 5 min for Q&A) |
| 11:30 – 1:00 | Community Terrace | Lunch + Tissue slice demo/workshop (Jolanda van der Velden, organizer) |

(continued on next page)
Monday, May 20

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| 1:00 – 2:45| Lecture Hall           | **Oral session IV – Filaments, membranes & enzymes influencing contractility,** (4 speakers x 20 min) + (ECI speaker 10 min). Farah Sheikh, moderator Steczina Sonette, ECI moderator  
  - Cecilia Ferrantini, “Electrical and mechanical impact of sealing cardiac t-tubules”  
  - Julia Gorelik, “Membrane nanodomains in cardiomyocytes”  
  - Jonathan Kirk, “An unexpected role for the sarcomere in Afib”  
  - James McNamara, “Exploring the function of Alpha Kinase 3: A novel component of the M-Band”  
  - ECI speaker. Garrett Hauck, “Calmodulin Kinase II is a mutation-specific driver of disease in hypertrophic cardiomyopathy” |
| 2:45 – 5:00| Hall of Ideas          | **Poster session 2** (refreshments served)                            |
| 5:15pm     |                        | **Shuttle to dinner at the Tinsmith**                                |
|            |                        | Shuttle Pickup: Monona Terrace - 1 John Nolen Drive, Madison, WI     |
| 5:30       | The Tinsmith           | **Dinner at the Tinsmith** – reception 5:30pm, seated dinner at 6:15pm |
**Tuesday, May 21**

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<tr>
<td>7:30 – 8:30</td>
<td>Community Terrace</td>
<td>Breakfast</td>
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<td>8:30 – 10:05</td>
<td>Lecture Hall</td>
<td>Oral Session V – Myofilament-based diseases</td>
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<td>(4 speakers x 20 min) + (ECI speaker 10 min) = 90 min</td>
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<td>Leslie Leinwand, moderator</td>
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<td>Vivek Jani, ECI moderator</td>
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<td>• Sharlene Day. “Genetic cardiomyopathies and the new therapeutic landscape”</td>
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<td>• Michael Regnier, “Mechanisms of altered cardiac myosin recruitment and chemo-mechanical crossbridge cycling with the MYH7 R403Q mutation”</td>
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<td>• Kricket Seidman. “Myofilament Protein Gene Mutations: Mechanistic Insights that Enable Therapies”</td>
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<td>• Miklos Kellermayer, “Titin in the DCM sarcomere”</td>
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<td>• KC Woulfe, ECI speaker, “Pathophysiologic modulation of sarcomeric acetylation contributes to disease”</td>
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<tr>
<td>10:05 – 10:45</td>
<td>Hall of Ideas</td>
<td>Refreshment Break</td>
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<td>11:30 – 1:00</td>
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<td>10:45 – 11:30</td>
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<td>Poster Awards (poster organizers)</td>
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<td>11:30 – 12:00</td>
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<td>ECI Awards (ECI leadership)</td>
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<td>12:00 – 12:45</td>
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<td>Summary panel (Katia Kontrogianni-Konstantopoulos, moderator; panel participants Malcolm Irving, Leslie Leinwand, Michael Regnier, Jil Tardiff)</td>
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<td>12:45 – 1:00</td>
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<td>Closing remarks (H. Granzier)</td>
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<td>1:00 – 1:45</td>
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<td>Box lunches in dining room or to take to topical workshops</td>
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<td>1:00 – 4:05 (optional)</td>
<td>Lecture Hall</td>
<td><strong>Topical Workshops:</strong></td>
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<td>• iPSC workshop, organized by Carter Ralphe and Mike Regnier.</td>
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<td>The goals of this workshop are to discuss the current state and value of hiPSC-derived muscle models, how subcellular to tissue level platforms allow to select ‘fit for purpose’ research and to brainstorm on areas of need and future growth of these model systems.</td>
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<td>• Computational approaches in myofilament research, organized by Mike Regnier. This workshop will introduce new models and analytical tools at two scales – the actomyosin complex and the</td>
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<td>Topical Workshops, continued</td>
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<td>Lecture Hall</td>
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sarcomere. The goal is to demonstrate the usefulness of these models and tools, then have a discussion of what additional development of modeling approaches would be most useful to experimentalists.

- **Low-angle X-ray diffraction**, organized by Weikang Ma and Tom Irving
  This workshop features discussions of new experimental opportunities for muscle diffraction studies of muscle at the BioCAT Facility in the Advanced Photon Source, Argonne National Laboratory:
  a. Introduction to the BioCAT resource, by Thomas Irving.
  b. Discussion of new kinds of experiments supported at BioCAT, by Weikang Ma.
  c. Describing how X-ray diffraction has been used to study how sarcomere level structural changes with obesity drives contractile dysfunction in human HFpEF, by Vivek Jani.

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Thank you for joining us at the 2024 Myofilament Meeting. We look forward to seeing you in 2026!
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<td>Thick/Thin Filament Regulation in Striated Muscle</td>
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<td>Molecular basis of length-dependent activation (LDA) in cardiac muscle</td>
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<td>Myosin-Modulating Compounds Result in Different Responses to Mechanical Control of Relaxation (Strain Rate dependent Tension) in Rat Cardiac Trabeculae</td>
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<td>Age-Related Post-Translational Modifications of Skeletal Muscle Myosin Heavy Chain Affect Muscle Function, Myofilibril Structure, and Myosin Biochemical Properties</td>
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<td>The Effects of Conditional Loss of Myosin Binding Protein H-Like on Cardiac Function</td>
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Low-angle X-ray fiber diffraction is a powerful technique for analyzing the molecular structure of the myofilaments of striated muscle *in situ* in different physiological states. It has made an important contribution to our understanding of the quasi-helical organization of myosin heads in thick filaments in the sarcomere of vertebrate skeletal and cardiac muscle in the relaxed state. Using X-ray diffraction, changes in filament structure occurring during contraction and with experimental perturbations can be detected on the Å length scale and millisecond time scale, leading to models of the structural basis of contraction and activation. As with all X-ray fiber diffraction studies, interpretation is based on modeling of the data, which is complicated by the contributions of multiple filament components to the X-ray reflections. We recently obtained an atomic model of the 430-Å repeat of the isolated, relaxed human cardiac thick filament C-zone (PDB 8g4l) based on a 6-Å-resolution cryo-EM reconstruction (Dutta et al., Nature 623, 853-862, 2023). Here, we have used this model to compute the contributions of heads, tails, titin, and cMyBP-C to the relaxed diffraction pattern by including/excluding these different components in the calculations. We confirm that the myosin heads are responsible for most of the intensity on the stronger myosin layer-lines (M1-M8), including the M3 meridional. Myosin tails contribute little to the layer lines, including the M6 meridional, consistent with their minimal mass variation along the filament length; this reflection arises mainly from other structures. The M11 layer line (39 Å spacing) arises mostly from the curved and kinked structure of titin, allowing eleven ~42-Å domains to fit into the 430 Å repeat. The M11 spacing can be used as a measure of strain in the myosin filament backbone as there is negligible head contribution. cMyBP-C domains C5-C10 in the model of the isolated filament extend longitudinally and contribute little to any layer line, except M10. In the sarcomere, where thin filaments overlap with thick filaments, cMyBP-C domains C0-C6 extend radially from the thick filament towards actin (Tamborrini et al., Nature 623, 863-871, 2023). This produces a 430-Å axial repeat of protein density that contributes substantially to “forbidden” meridional reflections on the M1, M2, M4, and some higher order layer lines that have not previously been well understood. Mobility of every third level (430 Å intervals) of myosin heads may also contribute to these meridionals. The insights gained in these studies may aid understanding the mode of action of each thick filament component in intact muscle in different conditions such as contraction and drug treatment. Supported by NIH HL164560 and AR081941.
Resolving the Disordered Cardiac Troponin T C-Terminal Tail and the Impact of cTnT Mutations D270N, K273E, and R278P

Garrett Crosby1, M.S., Bai Hei2, Melissa Lynn3, Ph.D., Steve Schwartz2, Ph.D., Jil Tardiff1,3,4, M.D./Ph.D.

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The cardiac thin filament (CTF) is a key regulator of muscle contraction and relaxation. The complex structure contains multiple disordered regions that remain structurally unresolved. One such domain is the C-terminal tail of cardiac troponin T (cTnT), residues 270-288. Recent cryo-EM studies did not detect densities associated with this cTnT domain (the site of multiple HCM mutation hotspots). We employed Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) to investigate this domain using a fully reconstituted CTF. Measurements were obtained in absence of calcium (1mM EGTA), saturating calcium (pCa3), and with saturating calcium plus stoichiometric myosin S1 to resolve all three known states of thin filament activation. The open, M-state exhibited significant changes in distance and the full-width at half-maximum (FWHM), a measure of disorder, for the proximal tail at residue V274C compared to the distal tail at residue V283C, in relation to cardiac troponin C (cTnC), specifically residues T53C and G125C of the N- and C-terminal lobes respectively. TR-FRET distances were then coupled to molecular dynamics simulations (MDS) and used as constraints to generate average structures of the previously unresolved C-terminal tail. MDS predicted novel tropomyosin interactions thought to be crucial for thin filament regulation. As it is unknown how cardiomyopathic mutations in this domain of cTnT result in disease, we performed differential scanning calorimetry (DSC) to investigate CTF thermal stability. cTnT DCM mutation D270N, and HCM mutations K273E and R278P all decreased the melting temperature of tropomyosin-troponin on actin with K273E having the greatest effect, suggesting weaker interactions between tropomyosin and actin. This likely contributes to the observed reduction in myosin ATPase activity in the absence of calcium for cTnT K273E. To determine how these mutations affect CTF structure, we performed TR-FRET experiments on CTF’s with these mutations. In CTF’s containing cTnT K273E, the C-terminal tail is positioned to that of the myosin activated WT CTF, in all three states. As it is known the C-terminal tail inhibits thin filament activation, in the presence of cTnT K273E, the thin filament may be more easily activated due to the tail being placed in an activated position where it is less likely to interact with and hold tropomyosin in its inactivated state. Zhu et. al. 2023 demonstrated that negative charges close to the IT arm of cTnT stabilize the active state of actin using phosphomimetic cTnT substitutions, our FRET data with K273E cTnT demonstrate the same phenomena with a clinically severe HCM mutation which results in a charge swap. We are currently interrogating how the DCM D270N and HCM R278P affect CTF structure with high resolution TR-FRET. We would predict the hotspot at residue R278 may also severely affect C-terminal structure, specifically actin interactions, whereas the more proximal D270N mutation may alter the structure in which the C-terminal tail is closer to and more able to interact with tropomyosin, stabilizing the blocked state.
Cardiac muscle contraction occurs due to repetitive interactions between myosin thick and actin thin filaments (TF) regulated by Ca\textsuperscript{2+} levels, active cross-bridges, and cardiac myosin-binding protein C (cMyBP-C). The cardiac TF (cTF) has two nonequivalent strands, each comprised of actin, tropomyosin (Tm), and troponin (Tn). Tn shifts Tm away from myosin-binding sites on actin at elevated Ca\textsuperscript{2+} levels to allow formation of force-producing actomyosin cross-bridges. The Tn complex is comprised of three distinct polypeptides – Ca\textsuperscript{2+}-binding TnC, inhibitory TnI, and Tm-binding TnT. The molecular mechanism of their collective action is unresolved due to lack of comprehensive structural information on Tn region of cTF. C1 domain of cMyBP-C activates cTF in the absence of Ca\textsuperscript{2+} to the same extent as rigor myosin. Here we used cryo-EM of native cTFs to show that cTF Tn core adopts multiple structural conformations at high and low Ca\textsuperscript{2+} levels and that the two strands are structurally distinct. At high Ca\textsuperscript{2+} levels, cTF is not entirely activated by Ca\textsuperscript{2+} but exists in either partially or fully activated state. Complete dissociation of TnI C-terminus is required for full activation. In presence of cMyBP-C C1 domain, Tn core adopts a fully activated conformation, even in absence of Ca\textsuperscript{2+}. Our data provide a structural description for the requirement of myosin to fully activate cTFs and explain increased affinity of TnC to Ca\textsuperscript{2+} in presence of active cross-bridges. We suggest that allosteric coupling between Tn subunits and Tm is required to control actomyosin interactions.
Cardiac troponin T (cTnT) serves as the tropomyosin (Tm) binding subunit of the troponin complex and, when mutated, often leads to pathologic cardiac remodeling observed in patients with hypertrophic and dilated cardiomyopathies. Part of the N-terminus of cTnT is responsible for positioning Tm along the actin groove and it immediately flanks the Tm overlap domain. Understanding the molecular mechanisms underlying these observations has been hindered by the absence of a high-resolution structure in the highly flexible cTnT N-terminal domain. Here, we employ single-donor dual-acceptor Time-Resolved Foster Resonance Energy Transfer (TR-FRET) to probe the positioning of the cTnT N-terminal extension in fully reconstituted cardiac thin filaments (cTF). We hypothesize the N-terminal tail interacts with F-actin in the -Ca\(^{2+}\) condition aiding in stabilizing Tm in the blocked position. Using cTFs with IAEDANS-donor-labeled cysteine-substituted residues within the N-terminal domain (N100, N73, M60, A36, A20, and A2) and DABMI-acceptor-labeled F-actin 374C as TR-FRET probes, we were able to measure cTnT's position in relationship to F-actin in both ±Ca\(^{2+}\). Preliminary molecular dynamics was then performed, where the TR-FRET distances were used as constraints. These preliminary models of the blocked and closed states are the first structures of the cTF to include the cTnT N-terminal hypervariable domain. Residues A36 and A2 are seen to move closer to actin in presence of Ca\(^{2+}\) whereas residue A20 moves further from actin. These region-specific changes observed upon the addition of Ca\(^{2+}\) suggest that the hypervariable domain may be interacting with the troponin core of an adjacent thin filament functional unit, specifically the C-terminus of cardiac troponin I (cTnI). Continuing work aims to resolve the structure of the N-terminus of cTnT in the open state as well as explore the potential interactions of the N-terminal domain with the preceding functional unit, specifically by performing TR-FRET between the C-terminus of cTnI and the N-terminus of cTnT.
Comparison of the cardiac myosin head orientations determined by cryo-electron microscopy and in-situ polarized fluorescence

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In addition to the classical Ca\textsuperscript{2+}-dependent thin filament regulatory pathway, activation of the thick filaments themselves has emerged as a second regulatory step that controls myofilament contractile function. Similar to the thin filaments, thick filaments are believed to exist in both a diastolic OFF and systolic ON state, and the rate of transition between those states are likely rate-limiting for force development and mechanical relaxation.

The thick filament OFF state is structurally characterized by myosin heads sequestered onto the surface of the thick filaments in quasi-helical tracks, which is stabilized by both intra-molecular interactions between the two myosin heads of the dimeric myosin molecule in the so-called interacting-heads-motif (IHM) and interactions between myosin heads and their coiled-coil tail domains, and intermolecular interactions between myosin heads on adjacent crowns.

We compare the orientations of the regulatory light chain (RLC)-region of cardiac myosin determined by polarized fluorescence from relaxed ventricular trabeculae (Kampourakis et al., 2014) with recent cryo-electron microscopy (cryoEM) reconstructions of the thick filament C-zone in the Mavacamten-stabilized OFF state (Dutta et al., 2023, Tamborrini et al., 2023). We show that the orientations of the RLC-region determined by the two methods are in very good agreement within the experimental resolution. However, polarized fluorescence reports a dynamic equilibrium between distinct conformations of the myosin heads in diastolic heart muscle, in contrast to the single conformations reported by cryoEM. We also compare the orientations of myosin head domains in the pre- and post-power-stroke conformation docked onto recent cryoEM reconstructions of myosin S1-decorated actin filaments with the orientations determined by polarized fluorescence from both actively contracting and rigor-state trabeculae.

The results show that the RLC-region of cardiac myosin is in a dynamic equilibrium between well-defined ON and OFF conformations, which leads to a new hypothetical working model incorporating the OFF/IHM state into crossbridge cycle.
Myosin Folding Boosts Solubility in Cardiac Muscle Sarcomeres

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The polymerization of myosin molecules into thick filaments in muscle sarcomeres is essential for cardiac contractility, with the attenuation of interactions between the heads of myosin molecules within the filaments being proposed to result in hypercontractility, as observed in hypertrophic cardiomyopathy (HCM). However, experimental evidence demonstrates the structure of these giant macromolecular complexes is highly dynamic, with molecules exchanging between the filaments and a pool of soluble molecules on the minute timescale. Therefore, we sought to test the hypothesis that the enhancement of interactions between the heads of myosin molecules within thick filaments limits the mobility of myosin by taking advantage of mavacamten, a small molecule approved for the treatment of HCM. Myosin molecules were labeled in vivo with a green fluorescent protein (GFP) and imaged in intact hearts using multiphoton microscopy. Treatment of the intact hearts with mavacamten resulted in an unexpected >5-fold enhancement in GFP-myosin mobility within the sarcomere. In vitro biochemical assays suggested that mavacamten enhanced the mobility of GFP-myosin by increasing the solubility of myosin molecules, through the stabilization of a compact/folded conformation of the molecules, once disassociated from the thick filaments. These findings provide an alternative insight into the mechanisms by which molecules exchange into and out of thick filaments and have implications for how mavacamten may impact cardiac contractility.
Heart function depends on the cardiomyocyte contractile apparatus and proper sarcomere protein expression. Mutations in sarcomere genes cause inherited forms of cardiomyopathy and arrhythmias, including atrial fibrillation. Atrial fibrillation is the most common cardiac arrhythmia and is associated with ischemic stroke, heart failure, and substantial morbidity and mortality. A novel sarcomere component, myosin binding protein-H like (MyBP-HL), has recently been discovered and is mainly expressed in the cardiac atria. MyBP-HL is composed of two immunoglobulin (Ig) domains and one fibronectin (Fn) domain, with homology to last three C-terminal domains of cardiac myosin binding protein-C (cMyBP-C). MyBP-HL incorporates into the sarcomere localizing in C-zone doublets, competing with cMyBP-C for sarcomere incorporation. We identified a 62 amino acid sequence in the N-terminal of MyBP-HL (N62) that does not share homology with any known protein and has no predicted secondary structure. We aimed to evaluate the structural and physiological implications of the N62 domain for MyBP-HL sarcomere localization and contractile regulatory function. We hypothesized that the N62 domain can interact and regulate other sarcomere proteins.

We made a MyBP-HL construct with an N'-terminal orange fluorescent protein and a C'-terminal maroon fluorescent protein that work as fluorescence resonance energy transfer (FRET) pairs. We then measured FRET to determine if the N'- and C'-terminals can come close to each other. The Ig-Fn-Ig domains are approximately 12 nm long, too far for FRET to occur if the N62 region is densely packed. We were able to detect FRET using this construct, indicating that the N62 region can extend away from the thick filament binding domains and come close to the C'-terminal domain. Next, we wanted to determine whether N62 has any functional effects. We performed the mantATP assay to measure the population of myosin heads in the super-relaxed (SRX) and disordered-relaxed (DRX) states. We found that addition of synthesized N62 domain reduced the SRX percentage in atrial myocardium but not ventricular myocardium. We then measured the isometric calcium-force relationship in permeabilized ventricular cardiomyocytes treated with the N62 domain. We found that N62 significantly reduced the maximum tension without a change in the calcium sensitivity of force development.

Based on these data, we demonstrated for the first time that MyBP-HL N-terminal N62 domain can regulate myosin activity and force generation.
Molecular basis of length-dependent activation (LDA) in cardiac muscle

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Activation of the cardiac sarcomere is sensitive to changes in sarcomere length, so that increased length results in increased myofilament calcium (Ca\(^{2+}\)) sensitivity and, consequently, force of contraction. This phenomenon is known as length-dependent activation (LDA) and is thought to constitute the cellular equivalent of the Frank-Starling law of the heart, a key auto-regulatory mechanism for modulation of cardiac contractility on a beat-to-beat basis. Nevertheless, despite its importance in both cardiac physiology and pathology, the molecular mechanisms underlying LDA in the heart remain to be elucidated. Here, we used polarised fluorescence from bifunctional rhodamine probes in the N- and C-terminal lobes of cardiac troponin C (cTnC), exchanged into demembranated rat cardiac trabeculae, to investigate the effects of Ca\(^{2+}\) and stretch on the conformation of cTnC in the thin filament. These were characterised during activation by temperature jump at steady-state [Ca\(^{2+}\)], in conditions that preserve the OFF structure of the thick filament at diastolic [Ca\(^{2+}\)] (T=27°C, 3% Dextran T-500) (Ovejero et al.; J Gen Physiol, 2022). We determined the contribution of myosin to the Ca\(^{2+}\)- and length-dependent regulatory structural changes in cTnC using 25 \(\mu\)M of the myosin motors inhibitor Mavacamten. We show that at a sarcomere length of 2.0 \(\mu\)m Mavacamten did not affect the amplitude of the Ca\(^{2+}\)-dependent structural changes of the cTnC N-lobe at pCa 4.7, but it significantly reduced that of the cTnC C-lobe in the same conditions. Moreover, Mavacamten greatly decreased the Ca\(^{2+}\) sensitivity of the structural changes in the N- and C-terminal lobes of cTnC by \(\sim 0.5\) pCa units (\(\Delta pCa_{50}\)), indicating that myosin motors binding to actin strongly contribute to the sensitisation of both the N- and C-lobes of cTnC to Ca\(^{2+}\). In addition, increasing the sarcomere length from 2.0 \(\mu\)m to 2.3 \(\mu\)m in the presence of Mavacamten did not affect either the amplitude or the Ca\(^{2+}\) sensitivity of the structural changes in the N- and C-lobes of cTnC. Altogether, these results indicate that Ca\(^{2+}\) sensitisation by stretch requires myosin motors binding to actin, highlighting the importance of thick filament-based mechanisms for the regulation of LDA in the heart.

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Muscle beyond myosin: A cross-scale study of rodent jaw muscles

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Masticatory myosin (MHC-M) is a specialized isoform that is expressed in jaw muscles of some vertebrate species including Chondrichthyes (sharks), Reptiles (crocodiles) and mammals (cat, chimpanzee, squirrel, etc.). Previous studies on cat jaw muscles expressing MHC-M revealed a much higher force generation capacity at moderately fast contractile speeds as compared to muscles expressing conventional myosin (MHC-IIa, IIb, IIx, β). Accordingly, MHC-M expression has been associated with specific dietary and defensive requirements involving forceful or swift biting. However, jaw musculoskeletal systems are functionally complex, and jaw mechanics can be affected at multiple scales beyond myosin isoform expression, including skull morphology and muscle architecture. Therefore, more comprehensive studies are warranted to determine how expression patterns and functional properties of MHC-M correlates with its constituent jaw muscle function. We used SDS-PAGE to determine the expression pattern of MHC-M in functionally and developmentally diverse jaw muscles of several rodent species with different skull types. Furthermore, because the contractile kinetics of myosin motor proteins expressed in muscle is known to significantly contribute to whole muscle contraction mechanics, it has historically been assumed that differences in myosin motor proteins determine the difference between mechanical properties of muscles expressing MHC-M versus conventional myosins. Therefore, we also characterized MHC-M and MHC-II motor protein performance using the in vitro motility assay (IVMA) and measured contractile properties of intact muscles expressing either MHC-M or conventional MHC-II isoforms. Our myosin isoform analyses confirmed that MHC-M is not expressed in all rodents and revealed that its expression is restricted to muscles of 1st pharyngeal arch with a jaw closing function. For example, within sciuromorphs, only jaw closing muscles of gray squirrel that originate from 1st pharyngeal arch, including superficial masseter (SM-GS), express MHC-M while red squirrels only express MHC-II isoforms in the jaw muscles tested. Like red squirrels, rats (myomorphs) also only express MHC-II isoforms in jaw muscles, including superficial masseter (SM-Rt). Our in-situ testing showed that SM-GS achieves ~2-fold higher tension as compared to SM-Rt (SM-GS = 15.5 ± 5.2 N/g; SM-Rt = 7.9 ± 1.3 N/g) coupled with a ~2-fold lower peak shortening velocity (SM-GS = 3.8 ± 1.1 L₀/s; SM-Rt = 67 ± 0.9 L₀/s). By contrast to predictions based on whole muscle contractile parameters, myosin extracted from SM-GS propelled actin at a ~5-fold slower unloaded velocity (Vₜₐₜ₅ᵢ₅ᵲᵣ = 0.8 ± 0.1 µm/sec) as compared to myosin purified from rat SM (Vₜₐₜ₅ᵢ₅ᵲᵣ = 4.3 ± 1.4 µm/sec). Thus, our cross-scale data indicate a disconnect between molecular and muscle scale contractile properties of jaw muscle, matching our previous findings from fast and slow limb muscles of rats. Whereas myosins are the motors of muscle contraction, we have previously shown that several additional factors influence the contractile properties of intact muscle and may account for the disconnect across scales, as well as variation of myosin isoform in functionally similar muscles. Our future studies will investigate additional factors, including thin filament regulation, calcium handling, elastic energy storage, myosin ensemble kinetics and fiber architecture, along with direct in vitro measurements of MHC-M force generation.

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Loss of fast skeletal Myosin Binding Protein-C (fMyBP-C) increases crossbridge detachment rates following rapid stretch and shifts myosin heads to the ON state.

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Fast skeletal Myosin Binding Protein-C (fMyBP-C) is a regulatory protein of the sarcomere consisting of a series of immunoglobulin-like and fibronectin type 3 domains termed C1-C10. The C-terminus of fMyBP-C is anchored to the thick filament by C8-C10, while the N-terminal domains (C1-C7) extend away from the thick filament backbone and interact with both myosin heads and actin. Similar to cardiac myosin binding protein-C (cMyBP-C), we previously reported that fMyBP-C increases Ca²⁺-sensitivity and limits the rate of crossbridge redevelopment ($k_{tr}$). Additionally, in passive muscle, fMyBP-C maintains myosin head conformation in the OFF state. However, it is unclear whether fMyBP-C affects myosin head position in active muscle or if it has other effects on crossbridge mechanics. Here, we investigated the structural and functional effects of fMyBP-C in psoas fibers from SNOOPC2 mice that allow in situ removal of domains C1-C7 of fMyBP-C. We found that mechanical responses to a rapid stretch imposed in actively contracting fibers were dramatically altered following treatment of fMyBP-C with tobacco etch virus protease (TEVp) to remove C1-C7. In particular, the apparent rate of crossbridge detachment ($k_{rel}$) following a 2% stretch was significantly increased in TEVp treated fibers resulting in a response that resembled stretch activation in cardiac muscle. In X-ray diffraction experiments we also found that TEVp treatment of psoas muscle significantly increased M3 spacing in Ca²⁺ activated muscle when the fiber was stretched from sarcomere length (SL) 2.4 to 3.0 suggesting that more myosin heads are released from the thick filament backbone and shift to the ON state as the muscle is stretched. Taken together, these data suggest that loss of fMyBP-C influences rates of cross-bridge detachment and myosin head position in active muscle.
Effect of load and sarcomere length on the activation of myosin filaments in heart muscle cells.

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Contraction of striated muscle is regulated both at the level of the thin actin-containing filaments, via calcium, as well as at the level of the thick myosin-containing filaments. The latter involves multiple mechanisms including direct mechanical activation in skeletal muscle. Here we used time-resolved synchrotron small-angle X-ray diffraction from electrically paced intact trabeculae isolated from rat heart to elucidate the role of this mechano-sensing mechanism in the heart. We measured the kinetics of the structural changes in the cardiac thick filament during activation of intact trabeculae under conditions that either reduced filament stress to a very low level, resulting in sarcomere shortening, or increased filament stress at constant sarcomere length. The kinetics of some of the X-ray signals that report thick filament activation, the increase in the equatorial intensity ratio $I_{11}/I_{10}$ associated with the movement of myosin motors away from the surface of the thick filaments towards the thin filaments, and the increase in the spacing of the M3 and M6 reflections associated with a longer axial periodicity of the myosin motors and filament backbone, respectively, were strongly load-dependent, as expected from the mechano-sensing mechanism. However X-ray signals associated with the quasi-helical arrangement of OFF-myosin motors on the thick filament surface, such as the first myosin layer line and the M2H meridional reflection, decreased on activation in an almost load-independent manner. These results suggest that mechano-sensing is not the only mechanism of activation of the thick filaments in the heart. Other candidate mechanisms might include direct sensing of intracellular calcium or interfilament signalling.

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Cardiac myosin binding protein-C (cMyBP-C) is a regulatory protein composed of 11 folded domains numbered C0-C10. The functional effects of the N' and C'-termini of cMyBP-C have been well-studied, but the middle domains' (C3, C4, C5, C6 and C7) are relatively unknown. Unique to this region is a 10-11 amino acid flexible linker between domains C4 and C5 (C4-C5 Linker) and a 28 amino acid unstructured region within the C5 domain (C5 Loop). We therefore aimed to determine the functional effects of the C4-C5 Linker and C5 Loop on myofilament force using the SpyC3 mouse model that allows for the replacement of endogenous C0-C7 domains of cMyBP-C (cMyBP-C^{C0C7}) with recombinant domains at its precise location in the sarcomere. Homozygous SpyC3 mice have a 20 amino acid insertion between C7 and C8 that encodes a tobacco etch virus protease (TEVp) recognition site and a “SpyTag.” Detergent-permeabilized cardiomyocytes from SpyC3 mice were attached to a force transducer and exposed to TEVp to remove cMyBP-C^{C0C7}. The domains were then replaced with recombinant proteins encoding cMyBP-C domains and a SpyCatcher, which covalently ligates to the SpyTag of SpyC3 myocytes. Effects of recombinant proteins on myofilament calcium sensitivity (pCa_{50}) and crossbridge kinetics were then compared with or without the C4-C5 Linker (ΔLinker C0C7sc) and with or without the C5 Loop (ΔLoop C0C7sc) on force redevelopment and decay. Results showed that replacement of cMyBP-C^{C0C7} with ΔLinker C0C7sc led to a significant increase in the myofilament calcium sensitivity (ΔpCa_{50} 0.10 ± 0.03, mean ± S.D.), increased residual force after a release and re-stretch maneuver, and an altered response to a rapid 2% stretch (stretch activation). However, the ΔLoop C0C7sc had no discernable effects on force or crossbridge kinetics. Taken together, these data indicate that the C4-C5 Linker, but not the C5 Loop, contributes to the functional effects of cMyBP-C on force. This work was supported by NIH HL080367 (SPH), HL140925 (SPH), AHA Postdoctoral Fellowship (ACG), and a F32 HL170509-01 (ACG).
Comparing the in vivo cardiac effects of first and second generation myosin activators

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Danicamтив (DN) is a second generation small molecule myosin activator currently undergoing clinical trials to treat heart failure with reduced ejection fraction, characterized by left ventricular (LV) systolic deficiency. Initial clinical trials and in vivo rodent studies claim that DN improves upon the shortcomings of the first-generation myosin activator, omecamtiv mecarbil (OM), which greatly impaired myocardial relaxation at higher doses, limiting its therapeutic window. However, these studies do not directly compare the in vivo effects of OM and DN to appreciate their differential impact on LV function. To address this gap, we used LV pressure-volume (PV) loops and speckle-tracking echocardiography in healthy mice to comprehensively study DN and OM’s LV effects. Each drug was studied at two doses (DN 3 and 6 mg/kg, OM 0.75 and 1.5 mg/kg) one minute following bolus administration IV via the external jugular vein. At the low doses, DN and OM similarly increased LV stroke volume (DN: 4.4 ± 0.3 v. OM: 4.1 ± 0.4 μL, P > 0.05) and peak systolic radial strain (DN: 9.7 ± 1.4 v. OM: 6.4 ± 1.9 %, P > 0.05) compared to baseline. However, DN increased the time constant of pressure relaxation (DN: 0.10 ± 0.03 v. OM: 0.4 ± 0.1 ms, P < 0.05) and systolic duration (DN: 1.1 ± 0.2 v. OM: 2.8 ± 0.4 % cycle duration, P < 0.05) less than OM. At the high doses, relative effects of DN and OM on stroke volume (DN: 7.7 ± 0.8 v. OM: 7.5 ± 0.9 μL, P > 0.05), the time constant of pressure relaxation (DN: 1.4 ± 0.1 v. OM: 2.7 ± 0.2 ms, P < 0.01), and systolic duration (DN: 4.1 ± 0.7 v. OM: 7.3 ± 0.4 % cycle duration, P < 0.01) remained consistent with the low doses but with significantly larger changes from baseline. The high dose of DN increased peak systolic radial strain slightly more than the high dose of OM (DN: 18.4 ± 1.7 v. OM: 13.2 ± 1.2 %, P < 0.05). Our results support previous claims that DN reduces diastolic performance less than OM for similar systolic improvements. Therefore, DN may find more success in clinical trials than OM. However, higher doses of DN still impaired diastolic function. Thus, the same factors that limited OM’s therapeutic window likely apply to DN.
Calcium binding to troponin-C is required for activation of the thick filaments in rat cardiac trabeculae

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Contraction of cardiac muscle requires activation of both the thin and thick filaments. Whereas the mechanisms underlying thin filament activation by calcium binding to troponin are well characterised, those responsible for thick filament activation are less clear. Three generic mechanisms have been proposed: direct mechanical activation triggered by force generation by constitutively ON or sentinel myosins (Linari et al Nature 2015; Craig & Padron J Gen Physiol 2022), coupling to the regulatory state of the thin filament (Kampourakis et al PNAS 2014), and direct calcium activation of the thick filament (Ma et al J Gen Physiol 2022). We aimed to distinguish between these mechanisms by replacing the native calcium-binding subunit of troponin (TnC) in demembranated trabeculae from rat right ventricle by its E76A variant, which cannot bind calcium. Following overnight incubation at 4°C in a relaxing solution containing E76A TnC, active force following a temperature jump to 27°C at pCa 4.7 was abolished. Control trabeculae that had been incubated overnight in relaxing solution produced active force of about 60 kPa at maximal calcium concentration (pCa 4.7). The regulatory state of the thick filaments was determined by two independent structural techniques: polarised fluorescence from a bifunctional rhodamine probe cross-linking the B and C helices in the regulatory light chain of myosin (RLC-BC; Kampourakis et al 2014), and small-angle synchrotron X-ray diffraction (Ovejero et al J Gen Physiol 2022). The RLC-BC probe became progressively more parallel to the filament axis as calcium concentration was increased from pCa 9-7 to pCa 6 and 4.7 in control trabeculae, but these orientation changes were completely abolished in the E76A trabeculae. Similarly, the increase in the X-ray equatorial intensity ratio $I_{11}/I_{10}$ at pCa<7 that signals movement of myosin motors from the surface of the thick filaments towards the thin filaments was absent in the E76A trabeculae. The decrease in the intensity of the first myosin layer line signalling the loss of the helical order of the myosin motors at pCa<7, and the increases in the spacings of the M3 and M6 reflections associated with the longer thick filament periodicity in the ON state at pCa<7 were also absent in E76A trabeculae. Together these results provide strong evidence that activation of the thick filaments in rat trabeculae requires activation of the thin filament triggered by calcium binding to troponin. They are not consistent with direct activation of thick filaments by calcium in this preparation.

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Differences in thick filament activation in fast rodent skeletal muscle and slow porcine cardiac muscle

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There is a growing appreciation that regulation of muscle contraction requires both thin filament and thick filament activation in order to fully activate the sarcomere. The prevailing mechano-sensing model for thick filament activation was derived from experiments on fast-twitch muscle. We address the question whether, or to what extent, this mechanism is extrapolatable to the slow muscle in the hearts of large mammals, including humans. We investigated the similarities and differences in structural signatures of thick filament activation in porcine myocardium as compared to fast rat EDL skeletal muscle under relaxed conditions and sub-maximal contraction using small angle X-ray diffraction. Thick and thin filaments were found to adopt different structural configurations under relaxing conditions and myosin heads showed different changes in configuration upon sub-maximal activation when comparing the two muscle types. Titin was found to has an X-ray diffraction signature distinct from those of the overall thick filament backbone and its spacing change appeared to be positively correlated to the force exerted on the thick filament. Structural changes in fast EDL muscle were found to be consistent with the mechano-sensing model, in porcine myocardium, however, the structural basis of mechano-sensing is blunted suggesting the need for additional activation mechanism(s) in slow cardiac muscle. These differences in thick filament regulation can be related to their different physiological roles where fast muscle is optimized for rapid, burst-like, contractions and the slow cardiac muscle in large mammalian hearts adopts a more finely tuned, graded response to allow for their substantial functional reserve. Supported by NIH P30GM138395 and 1R01HL171657.
Spatially resolving how phosphorylation affects β-cardiac myosin activity in porcine myofibril sarcomeres with single molecule resolution.

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Cardiac muscle contraction is mediated by myosin binding from the thick filament of the sarcomere to the thin filament in an ATP powered reaction. This process is highly regulated on a beat-to-beat basis by calcium interactions with the thin filament, but more recent investigations have indicated that the availability of myosin heads is also regulated. A number of factors that lead to the release of heads from the thick filament include load, calcium and phosphorylation. The number of heads available to interact with the thin filament governs the contractile force that can be generated, and occurs at a cost of ATP, an explanation for why it is so well regulated. In this study, we have taken the approach recently developed of imaging the binding of fluorescent ATP to the myosins within a sarcomere to provide spatially explicit details of their activity. This enables the size and location of the cardiac reserve to be assessed across conditions. Studying porcine cardiac myofibrils provides a very close analogue to human tissue; we find three kinetic species when examining the myosin ATPase. The fastest is consistent with earlier reports of non-specific ATP binding to the surface of myosin. The slower two species are consistent with the previously identified DRX and SRX states. The former is thought to represent myosins in an ON state, ready to interact with the thin filament and the latter an OFF state with slowed ATPase that constitutes the cardiac reserve. We find that the cardiac reserve is ~50% in the sarcomere and this can be sub-divided into the P-, C- and D-zones, with the D-zone having the least population of OFF heads (~44%). Treatment with PKA to phosphorylate cardiac myosin binding protein-C (cMyBP-C), led to a ~16% reduction in reserve in the C-zone (where cMyBP-C is found), a ~10% reduction in the P-zone, and an unexpected ~8% increase in the D-zone. Alternatively, myosin regulatory light chain (RLC) phosphorylation with myosin light chain kinase (MLCK) resulted in a large general decrease of reserve myosins by ~24%, interestingly the least affected area of the sarcomere was the C-zone. Coupled with observations from other groups these latter data suggest the interaction between myosin’s RLC and cMyBP-C alters the behavior of the myosin; potentially suggesting that cMyBP-C acts as the master regulator of activity over RLC.
Searching for the SRX state of myosin in synthetic thick filaments

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The super-relaxed (SRX) state is a biochemically defined state of myosin characterized by low ATPase activity. In recent years, the SRX state has been studied heavily in the context of thick filament regulation during normal and pathological physiology. The mant-ATP displacement assay, commonly employed to assess the fraction of heads in the SRX state, is a model that assumes an equilibrium between disordered relaxed (DRX, a state of standard ATPase rate) and SRX states that can be perturbed by ionic strength, temperature, small molecules that bind to myosin (e.g. mavacamten, dATP), or mutations in myosin. However, the system cannot be at equilibrium on a timescale less than 100 sec if they generate 2 populations in the mant-ATP displacement experiment. Only a single population is expected. This presents a paradox as to how to interpret the data which has been used as evidence of two populations. Additional observations which challenge the two-population interpretation are:

1) The measured basal ATPase rate is often not compatible with estimates of the %SRX present.
2) Estimates of %SRX generally do not agree with estimates of the IHM or ordered thick filaments under similar conditions.

The interpretation of the assay results in a series of unresolved paradoxes, which means there is no self-consistent model for the relationship between the SRX and DRX states. Our work, published earlier this year, re-examined the simplest system that has been reported to show the presence of the SRX state, muscle myosin HMM isolated from porcine ventricle. We found no evidence of a distinct SRX state population in our assays¹.

Here we examine full-length myosin and synthetic thick filaments to estimate the population of SRX/DRX present under different conditions. We present mant-ATP displacement assays using full-length myosin from porcine ventricle in 0.5M KCl buffer (monomeric myosin) and 0.1M KCl buffer (synthetic thick filaments). In each case we see a single population of myosin heads turning over ATP at ~0.01 s⁻¹. Addition of mavacamten, a known promotor of the SRX state, inhibited the ATP turnover rate by ~75% or 90% (0.5 M or 0.1 M KCl respectively) of this single population for both conditions with inhibition constants of 0.16 µM at 0.5M KCl and 0.26 µM at 0.1M KCl. Conversely, substitution of mant-ATP with mant-dATP, a known activator of myosin, accelerated the single-population ATP turnover rate by 30%. Similarly, we see single populations in synthetic thick filaments derived from rabbit psoas and bovine masseter.

In summary, none of our data, from S1 to HMM to monomeric myosin and thick filaments, supports the presence of two populations of myosin heads with distinctly independent ATPases, e.g. SRX and DRX, that can be detected by the displacement assay. This work, however, does not challenge the structural data on the interacting-heads motif and the order-disorder equilibrium of myosin heads on a thick filament, but rather suggests that in the mant-ATP displacement assay the heads behave as a single population because the ordered/disordered heads are in equilibrium on a second time scale.

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Sequence Specialization is Critical for Myosin-Induced Tropomyosin Translocation on Cardiac Thin Filaments during Pre-Powerstroke to Post-Powerstroke / C- to M-State Transitions

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Cryo-EM reconstructions of S1-decorated cardiac muscle thin filaments have shown that Loop-4, at the tip of the myosin-motor head, extends toward tropomyosin on actin. It is the only domain of the myosin-head that is likely to interact with tropomyosin on thin filaments (Behrmann et al., 2012; von der Ecken et al., 2016; Doran et al., 2020, 2023; Doran and Lehman, 2021; Risi et al., 2021). It follows that during powerstroke-driven crossbridge movement, Loop-4 is responsible for tropomyosin azimuthal translation and/or rotation across actin filaments to its M-state position. As part of the process, Loop-4 displaces tropomyosin from its attractive C-state electrostatic interactions with residues Lys 326 and Lys 328 on actin (Doran et al., 2020, 2023).

We propose that myosin-based azimuthal movement of tropomyosin away from residues 326 and 328 involves charge repulsion between tropomyosin and strictly conserved Loop-4 residues Arg 369 and Glu 370 rather than attraction. In addition, we posit that Loop-4 – tropomyosin repulsion occurs as the myosin head transitions from its pre-powerstroke to its post-powerstroke mode, i.e. during myosin cleft closure and release of nucleotide hydrolytic products. However, current cryo-EM approaches have not captured structures of pre-powerstroke muscle myosin bound to C-state filaments or resolved transitions between pre- and post-powerstroke interactions; thus, myosin's corresponding impact on tropomyosin position is uncertain. Our strategy therefore has been to study these transitions computationally, using classical and targeted molecular dynamics.

Our molecular simulations show tropomyosin repositioning on thin filament actin as myosin transitions from the pre-powerstroke to post-powerstroke conformation. As proposed, the simulations illustrate charged sidechains of Arg 369 and Glu 370 extending from Loop-4 and encountering identically charged residues on tropomyosin. Indeed, the resulting charge-repulsion between polar residues appears to cause tropomyosin translocation across actin and to facilitate myosin movement along the thin filament. It follows that the distribution of polar residues along tropomyosin must be precisely ordered to enable effective Loop-4 – tropomyosin charge repulsion during the pre- to post-powerstroke transition of the myosin head on the thin filament.

We note that charged residues on tropomyosin traversed by Arg 369 and Glu 370 on myosin Loop-4 in muscle sarcomeres are also strictly conserved including residues Arg 90, Arg 91, Glu 97, Glu 98, Asp 100, Arg 101, Glu 104, Arg 105, and Lys 112 on tropomyosin pseudo-repeat period 3, for example. We mutated tropomyosin Arg 91 and Arg 101 to glutamate and tested, in silico, whether the charge reversal affected the myosin-induced tropomyosin movement during the pre- to post-powerstroke transition. Strikingly, tropomyosin translated only incrementally while remaining in contact with actin residues 326 and 328 on actin. Such mutations in vivo would be expected to retard activation. Thus, our work highlights the view that myosin and tropomyosin sequence specialization defines thin filament regulatory dynamics. Our conclusions are supported by previous (Doran et al., 2023) and ongoing parallel examination of mutant myosin S1-decorated filaments in which polar residues on Loop-4 are replaced with glycine leading to comparable aberrant tropomyosin movement during the corresponding pre- to post-powerstroke transition.
Passive sarcomere stretch causes thin filament elongation, but the underlying mechanism is unclear. Here, we investigated the role of low-level cross-bridge formation in relaxed sarcomeres to elongate the thin filament during passive sarcomere stretch. We used small-angle X-ray diffraction to track sarcomeric protein structure and filament lengths in sarcomeres of skinned murine skeletal muscle before and after incubation with the myosin inhibitors, 2,3-butanedione monoxime (BDM) or mavacamten. Our preliminary results indicate that BDM or mavacamten treatment moves the myosin heads further to the "OFF"-conformation but does not affect elongation of thin filaments with increasing sarcomere length, suggesting myosins in the "ON"-states do not contribute much to the length-dependent effects on the thin filament. Apart from crossbridges, several other sarcomeric proteins such as titin and myosin-binding protein C (MyBP-C) could create thick-thin filament bridges resulting in thin filament elongation during passive stretch; indeed, our recent studies suggest that titin can explain some but not all thin filament elongation. We are currently investigating the role of MyBP-C thick-thin filament bridges in stretching thin filaments during passive sarcomere stretch. Overall, our study provides insights into the mechanism of thin filament elongation during passive sarcomere stretch and the role of sarcomeric proteins in this process. These are important considerations for developing accurate models and interpreting the effect of thick filament activator and suppressor drugs on the sarcomere.
Deleting one of titin’s A-band domains (A109) results in increased ATP turnover and calcium sensitivity

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Titin spans from the Z-disc to the M-band region of the sarcomere. Titin’s structure in the A-band is highly ordered with Ig- and Fn3-domains organized in super-repeats. It has been known for a long time that titin affects length-dependent activation; however, the exact mechanism by which titin activates the sarcomere is unknown. It has been hypothesized that the structural arrangement of titin along the thick filament, relative to myosin, plays an important role in shifting myosin from the OFF state (SRX) to the ON state. To test this, we genetically deleted a single titin domain (A109, a ~ 4nm long Ig domain) in the A-band (keeping the remaining 169 domains intact), causing a small misalignment with the thick filament backbone. The mouse model with the 109th A-band domain of titin deleted is referred to as TTN\textsuperscript{∆A109}. We also created a model there this domain is replaced with a sequence of unstructured amino acids (named TTN\textsuperscript{∆A109replace}), which will normalize the increased strain caused by the deleted A109 domain. Immuno-electron microscopy with antibodies against cardiac MyBP-C revealed 9 C-stripes at the expected positions in control papillary muscles, while muscles from TTN\textsuperscript{∆A109} mice showed misaligned C-stripes that on average were positioned further away from the M-band than in wildtype mice. TTN\textsuperscript{∆A109replace} mice were similar to controls. Single molecule turnover experiments using fluorescently-labelled ATP in cardiac and EDL myofibrils from TTN\textsuperscript{∆A109} mice showed binding events of shorter durations compared to wildtype mice. Survival plots of the event lifetimes were fitted with a double-exponential function, with the longer lifetime reflecting SRX myosin and the shorter lifetime DRX myosin. Results revealed a greatly decreased SRX state in TTN\textsuperscript{∆A109} mice. To investigate whether this was coupled to increased calcium sensitivity, we performed force-pCa experiments in single fibers from EDL and cardiac muscle bundles. Both skeletal and cardiac muscle preparations from TTN\textsuperscript{∆A109} mice showed larger forces at low levels of activation, which translated to higher pCa\textsubscript{50}-values. Taken together our data suggest that a small local deletion in the A-band portion of titin decreases the SRX/DRX ratio and increases force production at submaximal activation levels. These findings support that titin plays an important role in regulating thick filament activation.
How Phosphorylation of a Myofilament Protein Impacts Function: Actin-Binding LIM Protein 1

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As the body’s cardiac demands change, the heart adapts using a variety of mechanisms. Chemically modifying myofilament proteins responsible for cell contraction is a regular means to this end. Many of these chemical modifications, such as phosphorylation and ubiquitination, have been found to take place in the intrinsically-disordered regions (IDRs) of myofilament proteins, as opposed to more organized/structured regions. Though myofilament function shows a strong link to phosphorylation and other post-translational modifications (PTMs), determining how specific modifications of IDRs impact the function of the overall protein and of the myofilament has proven difficult. Clarifying the impact of these PTMs is important, as changes in PTM patterns have been associated with cardiac dysfunction. We hypothesized that for ABLIM1 protein, its phosphorylation at its IDRs impacts the protein’s conformation ensembles. Altering the conformations thereby toggles the ability for protein binding partners like titin to interact with the myofilament protein, thereby modifying its impact on myofilament function. To test this hypothesis, we created molecular dynamics models of ABLIM1’s N-terminus, a region containing LIM responsible for stress recognition, as well as an IDR between LIM domains 2 and 3, to determine the effects of phosphorylation. Our results show that phosphorylation of the IDR between domain 2 and domain 3 of ABLIM1 can impact the protein at both the local and global level. Local hydration levels differ around the motif in the IDR when phosphorylated, as do the conformations of both the LIM domain 2 and the whole protein. The differences found in the physicochemical properties of ABLIM1’s IDR when phosphorylated indicate a potential influence on its interactions with other myofilament targets via modulating its effective concentration. These results give insight into the potential mechanisms by which PTMs regulate myofilament proteins.
Thick filament regulation in intact porcine myocardium

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Contraction in heart muscle is highly regulated to ensure sufficient cardiac output for adequate end-organ perfusion to meet body’s various needs. We now realize that both classic thin filament and newly discovered thick filament mechanisms are required to fully activate striated muscle. Our limited understanding of the molecular mechanisms of thick filament regulation inside live myocardium is derived almost exclusively from studies of rodent myocardium. Whether findings from rodent myocardium can be directly translated to large mammalian hearts is an important open question. Here we used small-angle X-ray diffraction on twitching intact porcine myocardium to compare and contrast the X-ray diffraction signatures from porcine myocardium and from published studies on rodent myocardium. In contrast to rodent myocardium, the spacing and the intensity of the third-order meridional reflection decreases during twitch contractions in porcine myocardium. Unlike in the rodent heart where isoproterenol does not change the state of myosin motors in resting muscle, isoproterenol treatment in porcine myocardium releases the myosin heads from the ordered state on the surface of the thick filament towards the thin filament. These data provide evidence that thick filament regulation might act differently in hearts from different species in order to accommodate their different physiological roles. Fast rodent hearts work near their full capacity (< 30% cardiac reserve) and the slow cardiac muscle in large mammalian hearts adopts a more finely tuned, graded response to allow for their substantial functional reserve (5-to 10-fold reserve). We also observed the diffraction signatures of myosin head OFF to ON transitions during twitch and during zero-load shortening. During a normal twitch, myosin heads leave the vicinity of the thick filament and move towards the thin filament accompanied with a loss of the quasi-helical ordering of the myosin heads, i.e. an OFF to ON transition. The changes observed during zero load shortening were of similar magnitude. These data indicate that the degree of activation of myosin heads is comparable with and without active force at similar levels of calcium, consistent with a direct effect of calcium on the activation state of the thick filament.

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S-Glutathionylation Enhances Proteolysis of Myosin Light Chain 3 and Alpha Actinin 2 by Altering Their Structural Conformation

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Proteolytic degradation of cardiac sarcomeric proteins like myosin light chain 1 (MYL3) and alpha actinin 2 (ACTN2) by intracellular proteases during myocardial ischemia and reperfusion injury is well established. These proteins are crucial for sarcomeric function yet how they become targets for intracellular proteases like matrix metalloproteinase-2, calpain 1/2 and caspase-3 during ischemia and reperfusion events remain elusive. Despite their high α-helical content and well-folded structures, MYL3 and ACTN2 might unfold due to oxidative stress-induced modifications like S-glutathionylation, as both contain three and ten cysteine residues, respectively. We hypothesized that S-glutathionylation could render MYL3 and ACTN2 more prone to proteolytic attack.

Our aim was to elucidate the molecular link between the oxidative modification of cysteine residues in MYL3 and ACTN2 and their unfolded/partially unfolded state prior to proteolysis.

We explored the conditions leading to S-glutathionylation in MYL3 and ACTN2 using the thiol-reactive reagents such as S-nitroglutathione, GSSG, or GSH in combination with diamide. We also developed a recombinant MYL346-195 chimeric protein fused with its binding partner MYH7776-810, and a truncated form of recombinant ACTN222-266 to conduct unfolding studies. Using mass spectrometry, we confirmed glutathionylation in conserved cysteine residues of both proteins induced by S-nitroglutathione. Circular dichroism, intrinsic fluorescence, and nuclear magnetic resonance studies revealed that glutathionylation caused a disturbance in the α-helical structure and tertiary conformation of MYL3 and ACTN2. Moreover, two-dimensional NMR experiments demonstrated a partial unfolded state of MYL3 and a complete unfolding state of ACTN2 following glutathionylation. Notably, both proteins showed increased susceptibility to trypsin-induced proteolysis upon glutathionylation.

In summary, we provide evidence suggesting that glutathionylation likely induces a conformational shift in MYL3 and ACTN2 in spite of their inherent stability, potentially exposing the cleavage sites for intracellular proteases. Our research highlights the impact of S-glutathionylation in determining the susceptibility of MYL3 and ACTN2 to protease-mediated cleavage, providing valuable insights into their vulnerability under oxidative stress conditions.
LOADED SARCOMERE SHORTENING IN PERMEABILIZED CARDIAC MYOCYTES: ROLE OF CARDIAC MYOSIN BINDING PROTEIN-C (CMYBP-C)

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During the ejection phase of the cardiac cycle, left ventricular (LV) cardiac myocytes undergo loaded shortening and generate power. Our lab investigates factors that regulate loaded shortening and power in striated muscle cells. Few studies have measured sarcomere shortening during loaded contractions. For this study, we simultaneously monitored muscle length (ML) and sarcomere length (SL) during isotonic contractions in rat/mouse single permeabilized LV cardiac myocytes using the IonOptix SarcLen system. In rat permeabilized cardiac myocyte preparations, ML and SL traces were closely matched, as SL velocities were ~80% of ML velocities during half-maximal Ca2+ activation. This ML to SL relationship had an r² value of 0.98 (n = 4, 15 ± 1°C). SL traces were less closely matched to ML traces (i.e., SL velocities were ~70% of ML velocities, r² = 0.89) in mouse permeabilized LV cardiac myocyte preparations that were deficient in cMyBP-C (i.e., myocytes from cMyBP-C knock out (KO) mice). In addition, cMyBP-C KO cardiac myocyte preparations exhibited disproportionately high sarcomere shortening velocities at high loads (i.e., ~75% isometric force), a finding consistent with previously reports that tracked solely ML (Korte et al., Circ. Res. 2003; Hanft et al., J. Gen. Physiol. 2021). Interestingly, in cMyBP-C KO cardiac myocyte preparations during half-maximal Ca2+ activation, the small molecule myosin stabilizer, mavacamten, slowed SL loaded shortening across the force-velocity curve and normalized SL shortening velocity at high loads. Overall, these results suggest that cMyBP-C moderates sarcomere loaded shortening, especially at high loads, at least in part, by modulating cross-bridge availability.
Contribution of Ca\textsuperscript{2+} and Mechanosensing Regulation on Twitch Contractions

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Thin filament calcium regulation in muscle contraction is well established, and the most realistic model involves a continuous flexible tropomyosin-troponin chain mechanism (CFC). However, this mechanism alone is insufficient to explain the low resting tension and accelerated relaxation observed during twitch contractions in cardiac muscle. Recently, two mechanisms involving thick filament regulation via an inactive myosin heads state, known as the “parked state” (PS), have been proposed, namely by [Ca\textsuperscript{2+}] (1) and by force, called mechanosensing (2). To achieve the observed twitch contractions with the MUSICO simulations, it was necessary to incorporate both [Ca\textsuperscript{2+}] thin and thick filament regulation and mechanosensing into the computational model. Simulations showed that regulation of the thin filament by [Ca\textsuperscript{2+}] and either or both thick filament regulation mechanisms are necessary to achieve the observed tensions. However, the magnitude of these contributions can only be quantitatively evaluated through specifically designed experiments. In the absence of such experiments, the contributions of thick filament regulation mechanisms, together with thin filament regulation by [Ca\textsuperscript{2+}], on twitch contraction can only be assessed \textit{in silico}. The thick filament regulation model, where the transition rate from PS depends on [Ca\textsuperscript{2+}], as proposed by Mijailovich et al. (1), enabled matching the observed resting tension and accelerated tension relaxation during twitch contraction in human trabeculae (1, 3). In our previous work (3), simulations matched well the observed human tension transients. However, the regulation of the thick filament by [Ca\textsuperscript{2+}] was a dominant effect in reducing basal tension and twitch relaxation. Here we show how increasing the contribution of thin filament regulation can balance the two systems of regulation while maintaining a good match to force-pCa relations and twitch contractions in human trabeculae. Moreover, we also assessed the effect of thick filament activation by mechanosensing, as proposed by Linari at al. (2). MUSICO simulations showed that thick filament activation by mechanosensing can reduce resting tension but cannot achieve sufficient acceleration of twitch relaxation to match the observed tension transients in human trabeculae. Better fits were achieved when all three mechanisms are present, but the precise contribution of mechanosensing mechanism to the transition of myosin heads from inactive to active state needs to be more precisely quantified through specifically designed experiments.

Changes in affinity of leiomodin-2 for tropomyosin cause significant changes in thin filament length regulation and pointed end dynamics.

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Regulation of actin filament polymerization at thin filament pointed ends is controlled by structurally similar but functionally antagonistic proteins, leiomodin-2 (Lmod2) and tropomodulin-1 (Tmod1). In cardiomyocytes, Lmod2 has a higher affinity for tropomyosin-1.1 (Tpm1.1) within its Tpm-binding site (TpmBS1), compared with Tmod1. Lmod2 competes with Tmod1 for binding to the pointed end to enable controlled elongation of thin filaments. When the binding of Lmod2’s TpmBS1 to tropomyosin is abolished by a single point mutation, Lmod2 is unable to bind at the pointed end and therefore, is unable to displace (compete off) Tmod1 from the pointed end, resulting in shortening of the thin filaments. Since the TpmBS domain of Lmod3 (the skeletal muscle prominent isoform) has lower affinity for Tpm1.1, we changed Glu34 (E34) in Lmod2 TpmBS1 to Gln (Q), which is present at the corresponding position of Lmod3. We used circular dichroism to measure the thermally-induced denaturation of complexes between Lmod2 TpmBS1 (with and without the E34Q mutation) and a TpmBS1 αTM1-14Zip. The melting temperature of the Lmod2 TpmBS1 E34Q and αTM1-14Zip was reduced only by 2°C compared to the wild-type complex, indicating that despite some reduction in the affinity of Lmod2 TpmBS1 E34Q for Tpm1.1, Lmod2 retained high affinity for Tpm1.1 as compared to that of Lmod3. Interestingly, when we tested the function of the Lmod2 E34Q mutant in cardiomyocytes, we found that the modest change in affinity to Tpm1.1 affected the ability of Lmod2 to elongate thin filaments. The E34Q mutation also significantly reduced the ability of Lmod2 to displace Tmod1 at thin filament pointed ends. This investigation underlines the essential role of TpmBS1 in the dynamic equilibrium between Tmod1 and Lmod2 at the pointed ends of thin filaments in cardiac muscle sarcomeres.
Myosin Modulators Mavacemten and Aficamten Inhibit Cardiac Contractility Through Different Mechanistic Pathways

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Hypertrophic cardiomyopathy (HCM) is the most common form of genetic heart disease. HCM is characterized by left ventricular hypertrophy, hyperdynamic contraction, outflow tract obstruction, and impaired ventricular relaxation. Altered contractile function caused by mutations in sarcomere specific proteins is a significant contributing factor to the development of HCM. Recent FDA approval of Mavacemten (Mava | MYK-461) landmarked the first in-class myosin-specific small molecule therapeutic designed to regulate cardiac function at the sarcomeric level. In addition to Mava, a second small molecule myosin modulator, Aficamten (Afi | CK-274), has also been shown to reduce the hypercontractility associated with HCM. Here we present data assessing how Mava and Afi modulate the structural and mechanical function of the sarcomere to achieve reduced cardiac contractility in β-myosin expressing porcine cardiac tissue. Previous structural measurements utilizing X-ray diffraction showed that saturating Mava significantly decreased the intensity ratio (I1,1 / I1,0) as well as increased the myosin helical order of the thick filament (I MLL1 and IM3) [1]. These results suggest that Mava sequestered a significant number of myosin heads along the thick filament, reducing the pool of actively recruitable myosin heads during contraction. In contrast, at saturating Afi we find no significant difference compared to untreated controls (ND) in I1,1 / I1,0 (ND: 0.2812 ± 0.013 vs Afi: 0.278 ± 0.014) and, interestingly, a significant decrease in both IMLL1 (ND: 2.704 ± 0.207 vs Afi: 2.323 ± 0.219, p = 0.0025) and I M3 (ND: 2.219 ± 0.225 vs Afi: 1.986 ± 0.236, p = 0.0475). These results would suggest that, unlike Mava, Afi does not reduce the pool of actively recruitable myosin, and inversely, disorganizes the helical structure of the myosin crowns along the backbone. We next performed steady-state force and ktr measurements in demembranated tissue to assess contractility and crossbridge cycling kinetics. Compared to untreated controls, both Mava (1 µM) and Afi (1 µM) significantly inhibited max force generation (ND: 36.32 ± 2.58 vs Mava: 9.86 ± 1.28, p < 0.001, vs Afi: 13.21 ± 1.74, p < 0.001) and decreased the calcium sensitivity of force development (pCa50: ND: 5.53 ± 0.009 vs Mava: 5.34 ± 0.009, p < 0.001, vs Afi: 5.34 ± 0.009, p < 0.001) with no significant difference in max ktr. Further reductionistic experimentation utilizing the in-vitro motility assay (IVM) with purified HMM and unregulated thin filaments showed that only Mava inhibited the mean filament velocity at 1 µM (ND: 1.771 ± 0.08 vs Mava: 0.898 ± 0.05, p < 0.001, vs Afi: 1.549 ± 0.05, p = 0.87). Both Afi and Mava significantly reduced the fraction moving (ND: 0.854 ± 0.027 vs Mava: 0.381 ± 0.046, p < 0.001, vs Afi: 0.673 ± 0.034, p = 0.01). Preliminary investigate of contractile activation and relaxation kinetics through myofibril experimentation suggests that both Mava (0.5 µM) and Afi (1 µM) significantly inhibit force generation by ~50% while significantly accelerating both the fast (krel,fast) and slow (krel,slow) phases of relaxation. Future experiments utilizing the stop flow assay will investigate how each compound impacts actomyosin affinity and complex dissociation. These experiments are designed to better identify possible differences in the molecular mechanisms of force inhibition between the two myosin-targeting small molecules.
The dependence on the afterload of the degree of thick filament activation in the heart

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According to the Starling Law of the heart the dynamic equilibrium in mammalian circulatory system, driven by two pumps in series, is maintained by a mechanism that relates the work of the ventricle in systole to the degree of its diastolic filling. At the level of the sarcomere, the structural unit of the cardiac myocytes where myosin motors from the thick filaments pull the nearby thin filaments toward the sarcomere centre to generate force and shortening, this mechanism is classically attributed to the length-dependent activation that relates the systolic force to the end diastolic sarcomere length (SL). Here, the mechanism is tested using sarcomere level mechanics and small and ultrasmall angle X-ray diffraction from synchrotron light to measure the degree of myosin filament activation, the SL changes, and the power during afterloaded contractions of electrically paced intact trabeculae and papillary muscles of the rat ventricle. We find that, starting from the same end-diastolic SL, during the systole the fraction \( f_{ON} \) of myosin motors recruited from the OFF, ATP hydrolysis-unavailable state characteristic of the diastole, increases with the afterload \( T \), attaining almost saturation for \( T \geq \frac{1}{2} T_{p,max} \) (with \( T_{p,max} \), 110 kPa, the maximum force attained during an isometric twitch, Reconditi et al., 2017 PNAS, 114, 3240-3245). Accordingly, comparison of the mechanical output between afterloaded and isotonic-release contractions shows that, in the range \( 0<T<\frac{1}{2} T_{p,max} \), also the power during the systole depends on the afterload. At the organ level these results suggest that the aortic pressure, not the diastolic filling, determines the systolic performance, suggesting that the molecular basis of the Starling Law is the thick filament mechano-sensing, a downstream mechanism which allows, even at constant diastolic filling, the energetic cost of the systole to be rapidly tuned by the stress required to overcome the aortic pressure. Supported by MUR (Italy) and ESRF.
Myosin-nucleotide complex binds unregulated actin cooperatively

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We report the discovery of a new regulatory mechanism of the actomyosin system in muscle. We show that the weak binding of the myosin-nucleotide complex with unregulated F-actin is a cooperative process. Hundreds of myosin heads must work together for efficient force production in muscle, but the precise mechanism by which they coordinate remains elusive. It is known that myosin initially binds actin weakly, then transitions into a strongly bound state to produce force. Using the contiguous cooperative binding model, we interpreted our experimental results in terms of a cooperativity parameter defined as an increased probability for a myosin head to bind to the actin filament next to the already bound head. Considering the geometric organization of a sarcomere, we propose the formation of cross-bridge clusters composed of up to six myosin heads bound consecutively to actin. The cooperativity of weak actomyosin interaction may explain several challenging questions in muscle physiology, such as the role of myosin isoforms in mixed-isoform hybrid muscles, or the yield of supramaximal rate of force production in decorated skinned muscle fibers.
The binding of Ca\(^{2+}\) ions within the troponin core of the cardiac thin filament (CTF) regulates normal contraction and relaxation. Mutations within the troponin complexes are known to alter the normal functions and are thought to eventually result in the development of cardiomyopathy. However, despite the importance of the problem, detailed microscopic knowledge of the pathogenic potential of point mutations and their effects on the conformational free energy surface of CTF remains elusive. To explore the effect of point mutations on the conformational free energy barrier in the absence and presence of Ca\(^{2+}\), we have carried out metadynamics simulations for the WT and two mutants (cardiac troponin T R92L and R92W). To be more specific, we have investigated the Ca\(^{2+}\) ion binding process within the TnC core and the conformational modification of the Tm and Tn complexes during the calcium-binding process for both the WT and mutants. The free energy barriers for the Ca\(^{2+}\) binding process for the WT and mutants are consistent with our earlier work (Proc. Natl. Acad. Sci. U.S.A. 2016, 113, 3257–3262). A closer inspection of the data revealed a reduction in the free energy barriers for conformational transitions in the TnC and TnT complexes, whereas no significant effects on the free energy barriers were observed for Tm. Importantly, we found both mutations independently alter the free energy barrier of the cardiac TnI subunit. Such alteration in the free energy upon mutation of one protein in a complex affects the others through structural and dynamical changes to yield a pathogenic effect in the function of the thin filament.
The cardiac thin filament proteins troponin (Tn) and tropomyosin (Tpm) control actomyosin interaction, ATP hydrolysis and thus cardiac contractility. Calcium binding to Tn changes Tpm configuration along the actin thin filament from its B-state “blocking” position to the C-state “closed” position, thereby allowing myosin head binding to actin that is required for muscle contraction. Certain cardiomyopathies of genetic origin, including hypertrophic cardiomyopathy (HCM) and restrictive cardiomyopathy (RCM), caused by Tpm or Tn mutations, have been linked to perturbation of the tropomyosin regulatory positioning. Thus, understanding thin filament interactions in B- and C-states is critical to understand the molecular underpinnings for disease-linked alterations. A large number of disease-linked point-mutations are located within the C-terminal region of cardiac cTnI. This suggests that this region of TnI and possibly its binding partner tropomyosin represents a mutational “hot spot” prone to cause myopathies. For example, mutations R192H, G203S, and K206Q within the C-terminal domain of the cardiac cTnI are associated with HCM and RCM (reviewed in Galinska et al., 2010). Post-translational modification as well as limited proteolysis of TnI have also been implicated in modulation of TnI function. Here, the removal of C-terminal 17 amino acids from cTnI by Ca²⁺-dependent proteolysis is associated with myocardial stunning following brief ischaemia (Gao et al., 1997) and cTnI-Ser199 phosphorylation accompanies heart failure (Wijnker et al., 2015). Both increase calcium sensitivity measured in vitro. Similarly, corresponding studies have shown the importance of specific residues on tropomyosin which are linked to cTnI (Lehman et al., 2021; Lehman and Rynkiewicz, 2023). Indeed, we have highlighted that mutation of Glu139 of tropomyosin is critical for formation of Tpm-TnI interactions in the B-state (Barry et al., 2024).

In the current work, we have performed steered Molecular Dynamics simulations to estimate the energetics of the C- to B-state transition of tropomyosin in silico. We carried out these simulations in the presence and absence of WT and mutant troponin. We then measured the effects mutant and post-translationally modified TnI have on the energetics of Tpm movement. In our work, steering involved minimizing the root-mean-square deviation (rmsd) between a C-state Tpm starting structure and the target B-state Tpm, thus forming a graded pathway of ever-decreasing energy terms between the C-state tropomyosin and the B-state target. Each step along the C- to B-state pathway was extensively explored as a means of refining the transition (umbrella sampling). In turn, the method yielded free energy estimates for the transitions, and WT and mutant tropomyosin paths were compared. We show that in the presence of TnI the C- to B-state transition is an energetically favorable process as expected, presumably due to the extensive favorable Tpm-TnI interactions formed. However, the transition is less favorable for phosphorylated (S199⁹PhOS) TnI and in the presence of mutant Tpm (E139K), consistent with enhanced Ca²⁺-sensitivity. Currently, TnI mutants such as R192H and K206Q are being examined. We anticipate that our procedure will offer a means of assessing the severity of mutations in tropomyosin and TnI involved in switching muscles “on” and “off”.
Regional hierarchy of myosin motor recruitment from the thick filament in relation to heart performance

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The heart activity is regulated by mechanisms acting in both the thin, actin containing filament and the thick, myosin containing filament. In the thin filament, a Ca²⁺-dependent structural change in the regulatory proteins troponin and tropomyosin makes actin available for myosin motor attachment; in the thick filament, myosin motors must be released from their folded (OFF) state on the surface of the filament to be able to attach to actin and hydrolyze ATP. Switching ON of motors has been attributed to thick filament mechanosensing (Reconditi et al. 2017, PNAS 114, 3240-3245; Brunello et al. 2020, PNAS 117:8177-8186), a mechanism originally demonstrated in skeletal muscle (Linari et al. 2015, Nature 528:276-279), which adapts the number of motors switched ON to the load of the contraction. However, the molecular basis of mechanosensing as well as its hierarchical organization along the thick filament are not known. Here the regional contribution of the myosin filament to systolic force and its modulation are determined by high spatial resolution X-ray diffraction from electrically stimulated trabeculae and papillary muscles of rat ventricle (temperature 27°C, sarcomere length 1.9-2.2 µm). The force at the peak of the twitch (Tₚ) is varied in the range 5-100 kPa under different protocols able to vary the number of attached motors (Caremani et al. 2016, PNAS 113:3675-3680). The X-ray signals show that thick filament activation progresses starting from the periphery of the thick filament and spreading throughout the filament at relatively small systolic forces (≤1/2 the maximum force), at which motors attachment is limited to the C-zone. Higher systolic forces are associated to further motor attachment toward the periphery of the thick filament. These findings are explained in the light of the cryo-EM structure of the thick filament (Dutta et al. 2023, Nature 623:853-862; Tamborrini et al. 2023, Nature 623:863-871) and the cooperative thin filament activation by attached motors (McKillop and Geeves, 1993, Biophys J, 65:693–701; Desai et al. 2015, J. Biol. Chem. 290:1915–1925), operating at the sub-saturating level of intracellular Ca²⁺ of the cardiac twitch. Supported by MUR (PRIN2020, PNRR “Age-It”), Italy, and ESRF.
The Troponin Activator CK-136 Rescues Cellular Contractility in Human Heart Failure

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Direct sarcomere enhancers are a promising approach for treating heart failure with depressed systolic function, yet to date have shown little benefit in large-scale trials. In both heart failure with reduced ejection fraction (HFrEF) and preserved ejection fraction (HFpEF) with comorbid obesity, we have shown maximal calcium (Ca2+) activated tension (Tmax) is markedly reduced. Current thick filament activators, such as omecamtiv mecarbil, sensitize myofilaments to calcium but do not augment Tmax, potentially explaining their muted clinical effects. Here, we tested the hypothesis that a troponin activator CK-136 can both sensitize myofilaments to Ca2+ and also augment Tmax by indirect activation of the thick filament. Cardiomyocytes isolated from endocardial biopsies of 30 patients (5 non-failing controls, 25 HF) were studied. CK-136 both sensitized and increased Tmax in non-failing controls. In HF myocytes with Ca2+-tension relations that were already sensitized (left shifted), CK-136 increased Tmax but had no further impact on calcium sensitization. The rise in Tmax was surprising, since most troponin and thin filament activators sensitize but do not increase Tmax. To identify the mechanism by which this occurs, we performed small angle x-ray diffraction that showed CK-136 increases the spacing of the second and third troponin reflections but did not change the spacing of the sixth actin layer line. This suggests the increase in troponin reflection spacing was not due to thin filament strain. To our surprise, we also found an increase in equatorial intensity ratio (I1,1/I1,0), reduction in the intensity of myosin layer line 1 (MLL1) and the M3 and M6 reflections, and increased spacing of the M6 expression, all suggesting indirect thick filament activation. We next applied a high frequency strain perturbation to permeabilized myocytes, that showed an increase in stiffness after treatment with CK-136. Further interrogation of crossbridge kinetics showed an increase in ktr and myosin attachment rate indexed by 2πc. Taken together, these findings suggest that by increasing the proportion of weakly bound cross-bridges, CK-136 accelerates myosin attachment rate in the presence of calcium, thereby increasing the duty ratio and subsequently Tmax. In summary, because of its ability to augment Tmax, CK-136 may show promise for treatment of depressed contractility in human HFrEF and obese-HFpEF.
Leiomodin-2 Inhibits Actomyosin Interactions and Perturbs the Structure of the Thin Filament

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Muscle contraction depends on interactions between myosin thick and actin thin filaments coordinated by Ca²⁺ levels. Thin filament (TF) consists of actin, tropomyosin (Tm), and troponin (Tn) complex. Tn binds Ca²⁺ to move Tm strand away from myosin-binding sites on actin to permit actomyosin interactions required for force generation. The Tn complex has three subunits—Ca²⁺-binding TnC, inhibitory TnI, and Tm-binding TnT. TnT connects the two strands of the TF forming a so-called linker region. Leiomodin (Lmod2) protein controls the length of TFs in the sarcomere. Incorrect TF lengths are a source of fatal myopathies and altered contraction. Cardiac leiomodin-2 (Lmod2) binds to the pointed end of the TF to regulate its length. It has been proposed that Lmod2 acts as a “leaky cap” to modulate the actin polymerization at the pointed end of the TF. Recent data shows that Lmod2 also binds to the sides of the TFs, but the role of those interactions remains unknown. Here, we used cryo-EM to report the binding site of Lmod2 on the native cardiac TF (cTF). We show that Lmod2 binds to a specific region of the cTF between the junction region and the Tn core where it interferes with the myosin binding sites on the cTF. We used ATPase assay to confirm that Lmod2 inhibits actomyosin interactions at intermediate and high Ca2⁺ levels. Finally, we reveal that Lmod2 perturbs the structure of the TnT linker region which should affect the cross-talk between the two TF strands. We propose that the interaction of Lmod2 with the sides of the TF near its pointed end protects the terminal actin subunits and associated Tm molecules from the mechanical stress produced by actomyosin cross-bridges during the twitch.
Structural insights into the function of leiomodin-2 as a thin filament elongator

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Whether leiomodin-2 (Lmod2) in cells is an actin nucleator, a pointed end thin filament elongator, or both in cells has been a subject of debate for over a decade. We discovered that Lmod2 binds to the pointed end by solving the structure of the binding interface between tropomyosin (Tpm), and the only tropomyosin-binding site (TpmBS) in Lmod2. This structure is consistent with either of the proposed functions for Lmod2. To be an elongator, Lmod2 would need to bind to the pointed end, which our data show. In addition, the structure is also compatible with the hypothesis that thin filaments are formed by Lmod2 nucleating actin, polymerization proceeding from the barbed end, and Tpm being recruited, binding both actin and Lmod2. Thus, the question of whether Lmod2 is actually a pointed end thin filament elongator in cells was found by investigating the first actin-binding site (ABS1) within Lmod2.

Based on the structure of the TpmBS1/Tpm binding interface, we built a model of Lmod2 binding at the pointed end of the thin filament and proposed a mechanism of elongation. A single actin monomer binds to the ultimate protomer at the pointed end. This new actin protomer recruits another actin monomer which displaces ABS1 from the pointed end, after which polymerization can occur. Using NMR we determined the secondary structure and mapped the actin binding of ABS1. ABS1 has an N-terminal disordered half and a C-terminal \( \alpha \)-helical half, both of which interact with actin. The specific residues involved in interaction in both halves are separated by 18 residues of the disordered N-terminal half. Current experiments are focused on determining how ABS1 contributes to Lmod2's function as a pointed end elongator. We determined the structure of Lmod2 ABS1 in complex with actin by using transferred nuclear Overhauser effect NMR with small overlapping peptides representing Lmod2 ABS1. The molecular mechanism of elongation by Lmod2 and effects of mutations in ABS1 altering the elongation activity are explained by the structural data.
Unlocking the Role of sMyBP-C: A Key Player in Skeletal Muscle Development and Growth

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Myosin binding protein-C (MyBP-C) is a sarcomere regulatory protein that modulates contractility and calcium transient in striated muscles by interacting with actin and myosin. Among its three paralogs, slow myosin binding protein-C (sMyBP-C), encoded by the MYBPC1 gene, is globally expressed in all types of skeletal muscle fibers. A growing body of research suggests a potential link between MYBPC1 mutations and the development of congenital muscle diseases, such as distal arthrogryposis and lethal congenital contracture syndrome. However, comprehensive investigations into the role of sMyBP-C in skeletal muscle are lacking. In this study, we employed three novel mouse models: i) global sMyBP-C knockout (Mybpc1-/-), ii) MCK-Cre inducible constitutive sMyBP-C knockout (Mybpc1/MCKCre), and iii) HSA-Cre tamoxifen inducible conditional sMyBP-C knockout (Mybpc1/HSACre), to assess the structural and functional significance of sMyBP-C during embryonic, young, and adult stages of muscle development, respectively.

All Mybpc1/- pups exhibited severe tremors, respiratory distress, muscle atrophy, and functional deficits, leading to mortality within 24 hours after birth. RNAseq analysis revealed significantly increased expression of atrophy-related genes and decreased expression of muscle structural genes. Primary myoblasts isolated from Mybpc1/- pups demonstrated impaired myogenic development and reduced expression of myogenic markers and muscle structural genes during differentiation. Early sMyBP-C knockout (Mybpc1/MCKCre) resulted in reduced muscle growth and body mass, accompanied by decreased force generation, fatigue resistance, and calcium sensitivity measured in vivo, ex vivo, and in vitro. Histological examinations and X-ray diffraction studies showed severe muscle atrophy, fiber type switching from fast to slow, and disrupted sarcomere structure orientation following early sMyBP-C knockout. Although to a lesser extent than early postnatal knockout, sMyBP-C knockout in adult muscle (Mybpc1/HSACre) significantly impaired muscle function (exercise tolerance, grip strength, and force generation) and led to muscle atrophy with fiber type changes.

In conclusion, sMyBP-C plays a critical role in embryonic musculoskeletal development and is essential for postnatal muscle growth, homeostasis, and regulation of muscle function. Ablation of sMyBP-C results in severe muscle atrophy and functional loss in skeletal muscle.
Increased SRX myosin in hibernating bear hearts: a mechanism to conserve energy during hibernation.

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The super-relaxed (SRX) state of myosin serves as a pivotal regulatory mechanism in cardiac physiology, reducing myosin activity and lowering energy consumption. Hibernation, a state of energy conservation observed in mammals, induces reversible cardiovascular adaptations, potentially offering unique insights into the regulation of the thick filament of the heart. Our study investigates how hibernating grizzly bears (Ursus arctos horribilis) regulate thick filament activation at the biochemical and structural levels. We first focused on titin and myosin expression in a cohort of 6 active and 7 hibernating bears, investigating both atrial and ventricular cardiac tissue. Titin isoform ratios were affected in the left ventricle, favoring the stiffer N2B isoform over the compliant N2BA isoform in hibernating bears but myosin isoforms were unchanged. We then conducted both loaded and unloaded Mant-ATP chase experiments on bundles of isolated cardiomyocytes to measure resting ATP turnover and determined from this the population SRX and DRX myosin. Mant-ATP chase experiments at both SL 2.0 and 2.3 µm showed a significant increase in the population of SRX myosin. We subsequently determined mitochondrial activity by western blot for oxidative phosphorylation and found a general decrease in phosphorylation, supporting reduced production of ATP in left ventricle samples of hibernating bears. Similar experiments in the other cardiac chambers showed no difference in SRX/DRX. Preliminary x-ray diffraction experiments on left ventricular tissue indicate an increase in M3 intensity in hibernating animals, supporting a higher state of ordering of the myosin heads. Taken together, our findings highlight distinctive cardiac adaptations in hibernating bears, including reduced metabolic activity, and an elevated population of SRX myosin. Collectively, our findings underscore distinct cardiac adaptations in hibernating bears, characterized by reduced metabolic activity and an elevated population of SRX myosin. The shift toward SRX myosin during hibernation may represent a rapidly reversible mechanism for conserving energy and may be critical for survival.
Age-Related Post-Translational Modifications of Skeletal Muscle Myosin Heavy Chain Affect Muscle Function, Myofibril Structure, and Myosin Biochemical Properties

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We produced mutations in myosin’s globular head, its S2 hinge and its thick filament domain at amino acid residues that are post-translationally modified during human skeletal muscle aging (N81, R908 and N1168, respectively) (Li, et al., 2015). Our post-translational modification mimic mutations yielded defects in muscle function, myofibril structure and myosin biochemistry. Modifications in the homozygous state decreased jump ability by a third at three weeks of age and reduced flight ability to negligible levels in young flies, with severe effects on indirect flight muscle myofibril assembly and/or maintenance. Expression of mimic mutations in the heterozygous state or in a wild-type background yielded significant, but less severe, age-dependent effects upon flight muscle structure and function.

Mechanical analyses of indirect flight muscles expressing the post-translational modification mimics showed an ~80% decrease in maximum power production for skinned N81T/+ fibers and a ~70% decrease in maximum power for N1168D/+ fibers from 2-hour old flies. Sinusoidal analysis revealed that N81T/+ fibers had a slower rate of force production (lower 2πb) and a shorter average time that myosin spent attached to actin (higher 2πc), while N11168D/+ had an equivalent rate of force production and a shorter average time that myosin spent attached to actin (higher 2πc). In contrast, R908E homozygous fibers from 2-hour old flies displayed no reduction in maximal power or changes in myosin cross-bridge kinetics, even though flight ability of homozygous R908E flies was impaired, with a 30% reduction in wingbeat frequency.

At the biochemical level, the N81T mutation in the globular head disabled ATPase activity and in vitro actin filament motility, whereas the R908E S2 hinge mutation reduced actin-activated ATPase activity by 30%. The N1168D rod modification diminished filament formation in vitro. The latter mutation also reduced proteostasis, as demonstrated by enhanced accumulation of polyubiquitinated proteins. Interestingly, attempting to rescue flight function by overexpressing the proteostasis-enhancing transcription factor FOXO failed, yielding further reduced flight ability, suggesting that over-stimulating the FOXO pathway is incapable of alleviating such proteostasis defects.

Overall, we found that mutation of amino acids at sites that are chemically modified during human skeletal muscle aging can disrupt myosin ATPase, myosin filament formation and/or proteostasis, providing a mechanistic basis for the observed myofibrillar, muscle mechanical and locomotory defects. We conclude that aging-specific post-translational modifications present in human skeletal muscle are likely to act in a dominant fashion to affect muscle structure and function and may therefore be implicated in degeneration and dysfunction associated with sarcopenia.

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The Effects of Conditional Loss of Myosin Binding Protein H-Like on Cardiac Function

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Dilated cardiomyopathy (DCM) is a common cause of heart failure, with a strong hereditary component. Mutations in the recently discovered myosin-binding protein H-like (MYBPHL) gene are linked to hereditary DCM, atrial fibrillation, and atrioventricular arrhythmias. MyBP-HL is structurally similar to cardiac myosin-binding protein-C and is a sarcomeric protein essential for proper cardiac function. MyBP-HL protein expression is unique in that it is highly expressed throughout the atria with only scarce, distinct clusters of MyBP-HL positive cells found in the ventricles, mostly in or surrounding the ventricular conduction system (VCS). Constitutive knock-out of MyBP-HL in mice causes atrial dilation, ventricular dysfunction, arrhythmia, and DCM. What remains to be determined is whether MyBP-HL plays a developmental role, or if knock-down of the gene in adulthood will recapitulate a similar phenotype. Moreover, the significance of the MyBP-HL expressing cells in the VCS is currently unknown.

We hypothesize that MyBP-HL is essential for normal cardiac function, and conditional loss in adulthood will also create a diseased phenotype. To further elucidate the role of MyBP-HL, we have developed genetic knock-out mouse lines. The first is a ROSA-26-Cre-ERT loxP mouse that undergoes conditional Mybphl knock-down after tamoxifen treatment. Decrease of MyBP-HL expression is confirmed via qPCR and immunoblotting. Mice underwent functional testing following tamoxifen administration. Echocardiography was used to measure cardiac contractile function, and conscious telemetry allowed for monitoring of heart rhythm. The mice with conditional decrease of MyBP-HL in adulthood demonstrate a hypertrophic cardiomyopathy phenotype, along with atrial contractile changes.

To better study the role of MyBP-HL in the cardiac conduction system, we used a Contactin-2-Cre mouse that deletes Mybphl solely in the cardiac conduction system from birth. The expression pattern of MyBP-HL in specific puncta associated with the ventricular conduction system (VCS), including some Purkinje cells, is distinctive. The functional need for differential thick filament regulation in cells related to the VCS is unclear. We hypothesize that Contactin-2-Cre mice will have a higher rate of atrioventricular arrhythmias compared to wild-type mice. Conscious telemetry was used to evaluate arrhythmias and electrical signal conduction changes in response to physiological stressors. Echocardiography shows that deletion of Mybphl solely within the cardiac conduction system does not alter overt cardiac function.
Myofilament proteolysis may underlie contractile remodeling in atrial fibrillation

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Atrial fibrillation (AFib) is the most common cardiac rhythm disturbance. Treatment of AFib involves restoration of the atrial electrical rhythm. Following rhythm restoration, a period of depressed mechanical function, including decreased blood flow velocity and reduced atrial contractility, known as atrial stunning, occurs. This suggests that defects in contractility occur in AFib and are revealed upon restoration of rhythm. To assess contractile function, we used a canine atrial tachypacing model of induced AFib. Mass spectrometry analysis showed dysregulation of contractile proteins in samples from AFib compared to sinus rhythm atria. In atrial cardiomyocytes that were useable for skinned single cardiomyocyte force-calcium studies, we found reduced force of contraction. There were no significant differences in myosin heavy chain isoform expression. Resting tension is decreased in the AFib samples correlating with reduced full-length titin in the sarcomere. We measured degradation of other myofilament proteins including cMyBP-C, actinin, MHC, and cTnI, showing significant degradation in the AFib samples compared to sinus rhythm atria. Many of the protein degradation products appeared as discrete cleavage products that are generated by calpain proteolysis. We assessed calpain activity and found it to be significantly increased. Skinned cardiomyocytes from AFib atria showed increased calcium sensitivity that was consistent with increased cTnI degradation and decreased TnI phosphorylation. These results provide an understanding of the contractile remodeling that occurs in AFib.
Methylglyoxal Glycation Competes with Ubiquitination, Disrupting Sarcomere Function

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Methylglyoxal is a reactive carbonyl species that can non-enzymatically modify lysine and arginine residues via a process called glycation. Methylglyoxal is elevated in conditions that increase the risk of heart failure, such as diabetes and aging. Previously, we have shown sarcomere protein glycation induces contractile dysfunction. Glycation is irreversible and can only be removed by protein degradation. Here, we hypothesize methylglyoxal competes with and blocks protein ubiquitination on certain lysine residues, decreasing protein turnover and resulting in a feed-forward cycle of decreased protein turnover, increasing glycation, and worsening function. Indeed, mass spectrometry experiments in mouse and human cardiomyocytes have revealed methylglyoxal and ubiquitination share a subset of lysine targets on β-myosin heavy chain, myosin essential light chain, and actin.

We next determined whether methylglyoxal directly competes with and blocks ubiquitination. We found this to be the case, as C2C12 myoblasts and neonatal rat ventricular myocytes exposed to methylglyoxal (100μM C2C12s; 10μM NRVMs) exhibited reduced protein ubiquitination. We hypothesized that, due to reduced protein ubiquitination, there would be reduced protein turnover, and, as glycation is non-enzymatic, this would increase the likelihood of further glycation modifications accruing. To test this, we utilized BAG3 KO mice (heterozygous and homozygous), which have decreased sarcomere protein turnover. We found that glycation was increased on sarcomere proteins in BAG3 KO mice, indicating that glycation and ubiquitination do compete and suggest a dangerous, clinically relevant, feed-forward mechanism. Further, by mass spectrometry we found many of these residues with increased glycation in the BAG3 KO mice are also highly glycated in diabetic humans.

We hypothesized this may mean glycation is already impacting BAG3 KO myocyte function. To test this, we performed force-calcium experiments in skinned cardiomyocytes from BAG3 KO mice, comparing a baseline (pre-methylglyoxal exposure) measurement to a post incubation one. As anticipated, methylglyoxal incubation (100μM, 20m as we have previously used) of these myocytes resulted in a stark decrease of maximum calcium activated force (Fmax) in the wild-type group, but a reduced effect in the KO animals.

Lastly, we used western blot and mass spectrometry to show that 19-month-old rats exhibit increased glycation and ubiquitination of sarcomere proteins compared to young rats, suggesting this disease mechanism also occurs with aging. Overall, these results underscore the possibility that glycation/ubiquitination crosstalk impacts sarcomere function across multiple high-risk conditions. Therapeutics that break this loop may be efficacious in treating various diseases with increased glycation.
**Kbtbd13 knock-down restores muscle function in a mouse model of nemaline myopathy type 6**

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Nemaline myopathy type 6 is caused by variants in *KBTBD13*. Patients display muscle weakness and impaired muscle relaxation that significantly affects their daily life activities. Histopathologically, this disease is characterized by aberrant protein aggregation (nemaline rods) within the muscle cell, and a predominance of slow-contracting myofibers. Previous work suggested that *KBTBD13* is an actin binding protein and that variants cause stiffening of the actin-based thin filament, causing slow muscle relaxation. Most patients harbor the Dutch founder variant (*KBTBD13:p.Arg408Cys*). To date, no specific therapy is available.

Here, we used the patient-based *kbtbd13:p.Arg408Cys* knock-in mouse model. We aimed to characterize the natural history of disease to identify a therapeutic window. With a combination of whole muscle contractile measurements, single myofiber morphological and transcriptional assays, super-resolution imaging and nano-structural studies using x-ray diffraction, we established that the mouse model closely recapitulates the patient phenotype: contractile weakness and impaired relaxation kinetics were observed, in addition to the presence nemaline rods, and type I myofiber predominance, and thin filament stiffening. The pathological phenotype was absent at 1 month, developed between 1-3 months, and plateaued at 9 with little progression until 18 months.

Our treatment of choice was the knock-down of *Kbtbd13*. We have previously shown in a *Kbtbd13*-knockout model that *Kbtbd13*-deficiency is well tolerated. Knock-down was achieved by a intramuscular injection with an AAV9-containing a short-hairpin against *Kbtbd13*. We treated mice at 1-month (pre-phenotype) and at 3 or 7-months (overt phenotype). Our results show that at a pre-phenotype stage, *kbtbd13* knock-down was sufficient to fully prevent development of impaired relaxation kinetics, nemaline rod aggregation and slow-twitch myofiber predominance. At an overt-phenotype stage, treatment at 3-months as well as at 7-months was sufficient to significantly revert slow muscle relaxation kinetics, and, long-term *kbtbd13* knock-down completely reverted accumulation of nemaline bodies and type I myofiber predominance and, most importantly, it restored muscle force.

In conclusion, our results show the natural history of NEM6 in a patient-based mouse model that closely recapitulates patient phenotype. We further provide evidence that *Kbtbd13* interacts with the thin filament and the p.Arg408Cys variant impairs normal protein localization and function. And, most importantly, we identified a therapeutic window and highlighted the promise of knocking down *Kbtbd13* transcript as a therapeutic strategy.
Pathomechanisms of Monoallelic Variants in \textit{TTN} Causing Skeletal Muscle Disease

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Pathogenic variants in the titin gene (\textit{TTN}) are known to cause a wide range of cardiac and musculoskeletal disorders, with skeletal myopathy mostly attributed to biallelic variants. We identified monoallelic truncating variants (\textit{TTN}\textit{tv}), splice site or internal deletions in \textit{TTN} in probands with mild, progressive axial and proximal weakness, with dilated cardiomyopathy frequently developing with age. These variants segregated in an autosomal dominant pattern in 7 out of 8 studied families. We investigated the impact of these variants on mRNA, protein levels, and skeletal muscle structure and function. Results reveal that nonsense-mediated decay likely prevents accumulation of harmful truncated protein in skeletal muscle in patients with \textit{TTN}\textit{tv}s. Splice variants and an out-of-frame deletion induce aberrant exon skipping, while an in-frame deletion produces shortened titin with intact N- and C-termini, resulting in disrupted sarcomeric structure. All variant types were associated with genome-wide changes in splicing patterns, which represent a hallmark of disease progression. Lastly, RNA-seq studies revealed that GDF11, a member of the TGF-\(\beta\) superfamily, is upregulated in diseased tissue, indicating that it might be useful as a biomarker and/or therapeutic target in skeletal muscle titinopathies.
Contractile Function of Human Single Skeletal Muscle Fibers in End-Stage Heart Failure

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Heart failure (HF) is a chronic, multi-systemic condition that is typically treated through pharmacological and lifestyle intervention. However, the only cure for HF is heart transplantation. Heart failure is known to non-selectively decrease skeletal muscle mass and function, resulting in increased frailty. To be eligible for transplant, patients must have significantly decreased physical function, but interestingly, frailty is a contraindication for heart transplant, as increased frailty is associated with diminished post-surgical outcomes. Therefore, it is important to better understand the mechanisms underlying frailty associated with heart failure and to determine the role that skeletal muscle frailty plays in heart failure outcomes. Frailty associated with heart failure often exists with other conditions that further increase frailty, such as sarcopenia and sedentary behavior, complicating analysis of the specific role of heart failure. However, since these other co-morbidities tend to disproportionally affect load-bearing musculature, comparison of contractile function in single muscle fibers taken from two different muscle groups (load bearing and non-load bearing) promises to reveal cellular effects on muscle function specific to heart failure. Therefore, the aim of this study was to characterize contractile function of single skeletal muscle fibers (SMFs) from end-stage HF patients. We hypothesized that contractile function (force and power) would be reduced in fibers taken from load-bearing muscles (rectus abdominis [AB]) compared to non-load-bearing muscle (pectoralis major [PEC]). Skeletal muscle biopsies were collected from HF patients (n= 4) actively undergoing heart transplant or left ventricular assist device implantation. Bundles of muscle fibers were dissected from the biopsy samples, permeabilized, and SMFs were extracted. Loaded shortening velocity and peak isometric force ($P_0$) were measured using a force clamp technique and a force-velocity curve was generated for each fiber. Peak power output (PPO) and %Max Force at PPO were calculated from the force-power curve. Preliminary results show a significantly decreased mean cross-sectional area (CSA) in SMFs from PEC compared to AB ($p = 0.01$, n = 62 and 44 fibers respectively). There was no statistically significant difference in shortening velocity, specific force ($P_0$/CSA), or $P_0$ between SMFs from PEC and AB. However, there were trends for higher PPO and %Max Force at PPO for SMFs from AB compared to those from PEC ($p = 0.06$ and $0.0504$ respectively). Interestingly, these preliminary results oppose our current hypothesis that AB fibers would see a greater reduction in contractile function compared to PEC fibers. Moving forward, we plan to continue recruiting patients and begin to correlate changes in contractile function with patient post-operative outcomes with age and sex as covariates as our sample size increases.
Investigating the Molecular Pathogenesis of a Novel MYBPC1 Duplication Mutation Linked to Myopathy with Tremor (MYOTREM)

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Our group identified a novel dominant Leu266Lys267Arg268 (LKR) duplication mutation in the MYBPC1 gene, encoding the slow skeletal myosin binding protein-C (sMyBP-C), a critical sarcomeric protein that plays key structural and regulatory roles in skeletal muscle. Starting in infancy, the resulting clinical phenotype presents as Myotrem myopathy, manifesting as progressive generalized muscle weakness, hypotonia, skeletal deformities, dysmorphia, and tremor of likely myogenic origin. The molecular mechanisms underlying these pathological manifestations are elusive, and currently no therapeutics exist for this emerging sarcomeric myopathy. My work aims to characterize the LKR duplication on the atomic 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 responsible for dynamic interactions with myosin S2Δ and actin and thus the regulation of crossbridge cycling. I therefore hypothesize that the duplicated LKR residues alter the biochemical and/or structural properties of the M-motif, disrupting the sMyBP-C N-terminus functional activities. Our studies have yielded the following novel findings. First, in vitro motility and myosin enzymatic assays revealed the mutant M-motif to uniquely modulate crossbridge formation by dampening actin velocity and decreasing myosin ATPase activity, respectively, compared to wild-type. Second, 2D-nuclear magnetic resonance (NMR) spectroscopy titration experiments uncovered, for the first time, the specific amino acid residues involved in the M-motif – myosin S2Δ interaction, allowing identification of the minimal region necessary for binding. More importantly, our studies demonstrated that the LKR duplicated residues not only directly contribute to S2Δ binding, but also enhance the ability of distant and neighboring residues within the M-motif to interact with S2Δ and subsequently enhance the M-motif – S2Δ binding interaction. Third, 2D- and 3D-NMR chemical assignments indicated overall conservation of gross secondary structural elements in the M-motif, except for the terminal helix, which demonstrates increased helical propensity in the presence of the LKR duplication. Fourth, our current biophysical studies utilizing NMR relaxation methods suggest mutant-induced alterations to the M-motif tertiary structure. In summary, we characterized the first non-missense mutation in MYBPC1 associated with Myotrem myopathy on the atomic level, and uncovered biochemical and structural alterations underlying (at least in part) pathogenicity.
Myofilament Contractile and Relaxation Dysfunction in Human Heart Failure with Preserved Ejection Fraction

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Heart Failure with Preserved Ejection (HfP EF) affects over half of all heart failure patients, yet little is known about its underlying pathophysiology. This is further complicated by the shift in the presentation of the syndrome from one with predominant hypertension/hypertrophy (Ht/Hp) and diastolic dysfunction to one in which obesity and metabolic syndrome (Ob/Dm) predominate. HfP EF patients with an Ob/Dm phenotype present with systolic reserve limitations at the chamber level and depressed calcium-activated tension at the single cardiomyocyte level. This study aimed at elucidating mechanisms for this myocyte dysfunction, testing the hypothesis that myofilament contractile and relaxation dysfunction in HfP EF is mediated by alterations in acto-myosin crossbridge kinetics, thick filament inactivation, and ultrastructural changes. Studies were performed in >1500 isolated permeabilized myocytes obtained from 76 HfP EF patient endomyocardial biopsies, using small angle x-ray diffraction. Data were compared to results from 46 patients with dilated or hypertrophic cardiomyopathy (DCM, HCM). HfP EF Ob/Dm patients had depressed maximum power ($P_{max}$), reduced slope of the active length-tension relationship, and slowed myosin attachment ($2\pi b$). This is accompanied by reduced equatorial intensity ratio ($I_{1,1}/I_{1,0}$), a dilated lattice, increase in the proportion of super-relaxed (SRX) myosin and slower ATPase activity, and an inability to recruit SRX myosin upon sarcomere stretch. All these abnormalities are also observed in DCM despite huge differences in ejection fraction. By contrast, HfP EF Ht/Hp myocytes have accelerated myosin attachment ($2\pi b$), a compressed lattice, increased high frequency stiffness, and a normal proportion of SRX myosin, starkly different to human HCM myocytes, despite their apparent similarities at the chamber level. Dephosphorylation by active protein phosphatase 2A (PP2a) and phosphorylation by protein kinase G (PKG) increase myocyte contractility in HfP EF Ob/Dm but not HfP EF Ht/Hp, suggesting targeted perturbations in myofilament protein phosphorylation in both directions contribute to depressed myocyte contractility in HfP EF. By contrast, all HfP EF myocytes exhibited diastolic dysfunction, including reduced myosin detachment ($2\pi c$), increased calcium sensitivity, and increased titin stiffness after KCl/KI salt digestion, the former concordant with a slower linear phase of tension decline in myofibrils from the same patients. These diastolic abnormalities appear PKA-mediated, as incubation with the catalytically active PKA subunit rescued the phenotype. Thus, we identify a phenotype of myofilament dysfunction in HfP EF (both Ob/Dm and Ht/Hp) versus DCM or HCM. We also show one can identify specific subgroup-associated mechanisms for HfP EF myofilament dysfunction, and that such subgroup selection will likely be key to achieve maximal benefit.
Characterization of NEB pathogenic variants in patients reveals novel nemaline myopathy disease mechanisms and omecamtiv mecarbil force effects

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Nebulin, a critical protein of the skeletal muscle thin filament, plays important roles in physiological processes such as regulating thin filament length (TFL), cross-bridge cycling, and myofibril alignment. Pathogenic variants in the nebulin gene (NEB) cause NEB-based nemaline myopathy (NEM2), a genetically heterogeneous disorder characterized by hypotonia and muscle weakness, currently lacking curative therapies. In this study, we examined a cohort of ten NEM2 patients, each with unique pathogenic variants, aiming to understand their impact on mRNA, protein, and functional levels. Results show that truncation pathogenic variants affect NEB mRNA stability and lead to nonsense-mediated decay of the mutated transcript. Moreover, a high incidence of cryptic splice site activation was found in patients with splicing pathogenic variants that are expected to disrupt the actin-binding sites of nebulin. Determination of protein levels revealed patients with either relatively normal or markedly reduced nebulin. We observed a positive relation between the reduction in nebulin and a reduction in TFL, or reduction in tension (both maximal and submaximal tension). Interestingly, our study revealed a duplication pathogenic variant in nebulin that resulted in a four-copy gain in the triplicate region of NEB and a much larger nebulin protein and longer TFL. Additionally, we investigated the effect of Omecamtiv mecarbil (OM), a small-molecule activator of cardiac myosin, on force production of type 1 muscle fibers of NEM2 patients. OM treatment substantially increased submaximal tension across all NEM2 patients ranging from 87-318%, with the largest effects in patients with the lowest level of nebulin. In summary, this study indicates that post-transcriptional or post-translational mechanisms regulate nebulin expression. Moreover, we propose that the pathomechanism of NEM2 involves not only shortened but also elongated thin filaments, along with the disruption of actin-binding sites resulting from splicing pathogenic variants. Significantly, our findings highlight the potential of OM treatment to improve skeletal muscle function in NEM2 patients, especially those with large reductions in nebulin levels.
ADAPTIVE CHANGES IN THE A-BAND REGION OF THE GIANT PROTEIN TITIN IN DISEASED HUMAN CARDIAC SARCOMERE

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Titin, the largest protein known, spans the half-sarcomere, the contractile unit of skeletal and cardiac muscle through its Z-disk to M-line and interacts with thin and thick filaments in the I- and A-band of the muscle sarcomere, respectively. Titin’s A-band segment is not well understood but is shown to be orders of magnitude less extensible than the I-band region of the molecule. Heterozygous truncating mutations (TTNtv) affecting A-band titin are often associated with dilated cardiomyopathy (DCM). Marfan syndrome (MFS), a connective tissue disorder caused by mutations of the matrix glycoprotein fibrillin is also associated with impaired cardiac contractility but its exact pathomechanism is largely unknown. Here, we performed STED super-resolution microscopy on sections of stretched and fixed demembranated human cardiac myofibrils carrying heterozygous TTNtv mutations or on samples originated from MFS patients. Sequence-specific anti-titin antibodies included the 1) MIR, which labels titin at the ends of the thick filaments, 2) A170, that labels titin close to the M-line and its recognition motif includes the titin kinase (missing from the truncated titin isoform). Our experiments indicate that truncated titin is able to integrate into the cardiac sarcomere. Complex sarcomere length-dependent anti-titin epitope position-, shape- and intensity analysis pointed at structural defects in the I/A-junction and the M-line of the sarcomere. The distance of the A170 epitope measured from the M-line seems to depend from the locus of truncation, therefore from titin’s size but the A170 epitope distance does not simply linearly scale with the length of the truncated portion of titin suggesting anchoring hotspots across the A-band titin. We propose that the truncated titin cannot precisely register the ends of the thick filaments, and this can ultimately lead to the manifestation of DCM by disrupting the overlapping of thin and thick filaments. In MFS sarcomeres a pronounced, ~25 nm shift away from the M-line was found in the case of the A170 titin epitope suggesting that alterations in the M-band ultrastructure might be important contributors of the impaired cardiac performance observed in MFS patients.
**Age-related Changes in Human Single Muscle Fiber Contractile Properties**

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**BACKGROUND**: Sarcopenia is the age-related decline in muscle mass and function, including decreases in muscle force, velocity of shortening, and power output. Contractile function in single muscle fibers (SMF) is known to decline with age, but there is conflicting information about sarcopenic changes in certain specific single fiber contractile properties (i.e. Power). Some of this conflict may be due to the relatively few human aging studies that have examined single fiber contractile properties in both Type 1 and Type 2 fiber types. Therefore, the purpose of this study is to investigate the age-associated changes in SMF contractile properties in relation to their fiber types (as determined by their MHC isoform content) to elucidate potential cellular mechanisms of age-related muscle dysfunction. We hypothesize type 2 fibers will exhibit decreased Specific Force (SF; force normalized to cross-sectional area [CSA]) in the old compared to the young but maintained Normalized Power (NP; power normalized to CSA) with aging. We hypothesize that maximal velocity of shortening (Vmax) will not be significantly different between age groups in either fiber type. Additionally, we hypothesize a reduction in CSA in type 2 fibers with aging.

**METHODS**: Single muscle fibers were isolated from skeletal muscle biopsy samples obtained from the vastus lateralis of 7 young (4M:3F; 28.0 ± 1.7 years) and 5 old (2M:3F; 62.0 ± 3.1 years) participants to measure SF, NP, and CSA. SDS-PAGE and silver staining were used to determine fiber type. A total of 130 fibers in young and 76 fibers in old were analyzed with an average of ~6 Type 2 and ~12 Type 1 SMF’s per subject.

**RESULTS**: Peak isometric force (P₀) was not significantly different between the old and young in type 2 fibers (0.66 ± 0.24 vs. 0.99 ± 0.36 mN, p = 0.10), although the old type 1 fibers were significantly less (0.49 ± 0.17 vs. 0.78 ± 0.1 mN, p < 0.05). Like P₀, absolute power was not significantly different in the type 2 fibers (27.3 ± 14.1 vs. 46.8 ± 17.6 μN·FL/s, p = 0.07) but significantly different in the type 1 (9.6 ± 2.3 vs. 16.6 ± 4.0 μN·FL/s, p < 0.05). Specific force did not significantly differ between the old and young in type 2 (144.3 ± 43.7 vs. 173.1 ± 24.5 kN/m², p = 0.17) or type 1 fibers (107.3 ± 43.0 vs. 135.7 ± 20.0 kN/m², p = 0.15). Normalized power was significantly less in old type 2 fibers (5.86 ± 1.4 vs. 8.18 ± 1.1 W/L, p < 0.05), but not significantly different in the type 1 fibers (2.15 ± 0.97 vs. 2.85 ± 0.47 W/L, p = 0.13). CSA was not significantly different in the old type 2 (4680 ± 1600 vs. 6057 ± 2180 μm², p = 0.26) or type 1 fibers (4948 ± 1724 vs. 6008 ± 1422 μm², p = 0.27). Vmax showed no significant difference between old and young in type 2 (0.77 ± 0.3 vs. 0.70 ± 0.1 FL/s, p = 0.64) or type 1 fibers (0.44 ± 0.1 vs. 0.51 ± 0.1 FL/s, p = 0.06). CONCLUSION: Aging is known to be associated with type 2 fiber atrophy, but surprisingly our preliminary results show maintenance of CSA in both fiber types in old compared to the young. Interestingly, a significant decrease was found in the normalized power of the old type 2 fibers, while Vmax, specific force, and CSA were maintained. Absolute power was not statistically significantly decreased in the old type 2 fibers, although it was only ~60% of the young type 2 absolute power, which could account for the decreased normalized power. In summary, while SF and Vmax were not different between old and young fibers, the combination of force and velocity (i.e. Power) was significantly lower in the type 2 fibers of old compared to young. Power output is directly related to the fibers ability to perform work. This result suggests age-related cellular/molecular changes in contractile properties beyond changes in fiber size may contribute to age-related muscle dysfunction.
Sex-dependent progression of the MYBPC1 E248K Myotrem myopathy in response to aging

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Dominant missense mutations in MYBPC1, the gene encoding the essential sarcomeric slow myosin binding protein-C (sMyBP-C), have been associated with the development of a new, early-onset congenital myopathy termed “Myotrem”, characterized by muscle weakness, hypotonia, dysmorphia, skeletal deformities, and postural tremor of myogenic origin. Importantly, the clinical manifestation of Myotrem in mid and late adulthood remains unknown. Using the Myotrem MYBPC1 E248K murine model, we interrogated skeletal muscle organization and in vivo contractile performance in mid and late adulthood in both male and female mice. Our findings reveal a differential phenotypic manifestation of E248K Myotrem. At 12-months of age, both male and female E248K knock-in (KI) animals display comparable sarcomeric organization and contractile function to sex-matched wild type (WT) animals, although they present sex-specific (mal)adaptations. By 24-months of age, KI male muscles exhibit a sharp decline in force development and suppressed contractility kinetics compared to sex- and age-matched WT controls, secondary to sarcomeric disorganization. In contrast, 24-month-old female KI animals are not as severely affected, displaying contractile deficits only in certain muscles. Collectively, our studies reveal a sex-dependent and muscle-specific presentation of MYBPC1 E248K Myotrem in response to aging with sarcomeric disorganization being the primary driver of contractile dysfunction.
Scarcity of donor organs available for transplantation poses a challenge for the management of patients with end stage heart failure. As an alternative, some patients may receive a left ventricular assist device (LVAD) as either a bridge to transplant or destination therapy. These pumps mechanically unload the left ventricle to maintain adequate cardiac output independent of cardiac function. How the heart and cardiomyocytes response to this hemodynamic unloading is important for the management of these patients and development of new devices.

We have collected 31 pairs of left ventricular samples from patients with advanced heart failure. The first sample from each pair was acquired when the patient received an LVAD. The second sample was obtained sometime later when the patient received a heart transplant and their native heart was explanted. These samples provide an opportunity to investigate how mechanical unloading modulates myofilament biochemistry and function.

Gel electrophoresis was performed to determine the ratio of the N2B and N2BA isoforms of titin. Paired t-tests demonstrated that this ratio was not altered by LVAD support. However, prior to LVAD support, the N2BA/N2B ratio was correlated with both the patient’s cardiac output and their LV chamber diameter. Interestingly, these relationships did not persist after LVAD support. This may indicate a loss of mechano-sensitive regulation of titin isoform content. Consist with prior studies from our lab, the proportion of titin expressed as N2BA (the less stiff isoform) increased with extracellular collagen content. BAG3, a protein involved in autophagy, was decreased after mechanical unloading indicating that LVAD support does not rescue the decline of BAG3 often observed in heart failure.

Ongoing work is assessing how mechanical unloading alters the phosphorylation status of troponin I, regulatory light chain, and myosin binding protein-C. The paired samples provide a valuable opportunity to determine how mechanical signaling alters the biochemical status of myofilament proteins in human hearts.
HCM and DCM pathogenesis reside on different thick filament interfaces

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The human cardiac myosin filament C-zone atomic model (PDB 8G4L) reveals the protein-protein interfaces involved in cardiac muscle contractility, SRX state, LDA, and filament assembly1. We find five general interface classes involving: (1) the blocked head (BH) and free head (FH) of the interacting-heads motif (IHM)2,3, the BH and FH with subfragment 2 (S2), and the IHM with nearby tails, and S2 with titins; (2) BH/FH-ELCs with motor domains (MD) and lever arms, and the BH and FH RLCs with each other; (3) cMyBP-C domains with FHs and tails; (4) tails with titins; and (5) tails with tails. It has been a mystery how hypertrophic (HCM)4 and dilated (DCM) cardiomyopathy variants in sarcomeric genes can lead to profound abnormalities observed in cardiac contractility, relaxation, energy consumption, and filament assembly. The presence of multiple copies of myosin heavy chain (MYH7), ELC (MYL2), RLC (MYL3), cMyBP-C (MYBPC3), and titin (TTN) molecules makes the thick filament interactome very complex. We mapped HCM and DCM pathogenic/likely pathogenic (PLP) and benign/likely benign (BLB) variants in these proteins to disclose the structural impact of HCM and DCM variants curated from the ClinVar archive5 onto 8G4L. We found that PLP HCM variants cluster on many interfaces involving head-head, head-tail, MD/lever-ELC, RLC-RLC, tail-cMyBP-C, tail-tail, and tail-titin, while BLB variants are near-but not on- these PLP HCM sites. In contrast, PLP DCM variants cluster mainly in the MD, tail-tail, and tail-titin interfaces, consistent with the different prominent features of contractility, relaxation, energy consumption, and filament assembly between HCM and DCM. We suggest possible cardiac hypertrophy and dilatation mechanisms based on the dissimilar pathogenesis of HCM and DCM. The clustering of PLP variants in the numerous protein-protein interfaces of the myosin filament implies the critical role played by these interactions in normal cardiac structure and function.

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References


**Myofilament Mechanics of Human Epicardial Biopsies Related to Clinical Indicators of Diastolic Dysfunction**

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Heart failure due to diastolic dysfunction, also called heart failure with preserved ejection fraction (HFpEF), is diagnosed by the presence of several, but not necessarily all of the following factors: elevated left ventricular end-diastolic pressure (LVPed), reduced mitral valve ring tissue velocity (e'), high early trans-mitral blood velocity relative to e' (E/e'), increased LV mass and relative LV wall thickness (RWT), and elevated left atrial dimension (LAD) and volume (LAvol). This study focused on uncovering any relationships between these clinical indicators of HFpEF and cardiac myofilament mechanics. All patients had normal valvular function, wall motion, and ejection fraction (EF>50%) and had been scheduled for coronary bypass grafting. Those patients diagnosed with HFpEF were separated into two groups with and without diabetes mellitus (DM). Patients gave informed consent, and epicardial biopsies were obtained during open heart surgery. Biopsies were demembranated with detergent, sculpted to <150 um diameter, and placed between a force transducer and length motor. Relaxed force per cross-sectional area (Pₘᵟᵢₙ) at non-activating calcium concentration (pCa 7) was elevated in HFpEF+DM (5.99±0.55 mN.mm⁻², n=6, *p<0.05) compared to non-failing (NF) controls (3.95±0.38 mN.mm⁻², n=11) and HFpEF (4.78±0.48 mN.mm⁻², n=7) as determined by SNK post-hoc analysis. The number of myosin crossbridges per cross-sectional area attached at pCa 7 as detected by sinusoidal analysis (parameter C) was significantly higher in HFpEF+DM (119.64±21.09 mN.mm⁻², *p<0.05) compared to NF (72.07±7.40 mN.mm⁻²) and HFpEF (78.26±3.59 mN.mm⁻²). Passive stiffness, developed force per cross-sectional area, calcium sensitivity (pCa₅₀), and cooperativity (n-Hill) were not different among the groups. Myosin kinetics at sub-maximal activation (pCa 5.7) as detected by sinusoidal analysis (2πb and 2πc) and by quick stretch (k_rel and k_redev) were not different among the groups. Interestingly, there was a moderate yet significant correlation for n-Hill vs LVPed (r=0.642, p=0.013). There were other modest to strong correlations between mechanics at pCa 7 and clinical indicators of HFpEF: Pₘᵟᵢₙ vs LVPed (r=0.529*), Pₘᵟᵢₙ vs E/e' (r=0.530*), C vs LVPed (r=0.861**p<0.01), C vs LV mass (r=0.658**). These results for n-Hill, Pₘᵟᵢₙ and C collectively suggest that the formation of myosin crossbridges at non-activating calcium concentrations accompany and may underlie multiple indicators of diastolic dysfunction and was most prevalent in the HFpEF+DM patients. There were other moderate correlations between myosin kinetics at pCa 5.7 and HFpEF indicators: 2πb vs E/e' (r= -0.528*), 2πc vs LVPed (r= -0.640*), k_rel vs LVPed (r= -0.648**), and k_redev vs E/e' (r= -0.547*). These results further suggest that a prolonged lifetime of myosin crossbridges reflected in low 2πc and k_rel accompanies and may contribute to elevated LVPed, while a reduced myosin force redevelopment rate reflected in 2πb and k_redev is associated with changes to early diastolic filling.
Ablation of cardiac myosin binding protein-C induces cell signaling for fibrosis and hypertrophy prior to morphological remodeling of the myocardium

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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, clinical treatments for patients with HCM remain limited. Current therapies focus on decreasing cardiac stress rather than treating the underlying genetic condition. The heart comprises several myofilament proteins that work together to facilitate proper contraction and relaxation to pump blood throughout the body. Cardiac myosin binding protein-C (cMyBP-C) is a thick-filament regulatory protein and mutations in cMyBP-C are frequently linked with clinical cases of HCM. To further understand the role of cMyBP-C and its contribution to cardiac disease, we assessed the progressive development of morphological and molecular biomarkers associated with HCM in a transgenic mouse model lacking cMyBP-C. We assessed gene expression associated with sarcomeric proteins and HCM development via a custom cardiac gene panel using Nanostring nCounter analysis. Morphological alterations in cardiac tissue were evaluated using biochemical and histological assays. Our findings unveil significant dysregulation in genes associated with fibrosis and hypertrophy in cMyBP-C deficient mice as early as 21 days after birth, preceding observable morphological changes in the cardiac tissue. Alterations in expression of sarcomere specific proteins begin after notable cardiac remodeling. The early alterations to gene expression underscore the need for better understanding the mechanisms driving HCM development, which may offer potential avenues for therapeutic intervention before pathological remodeling occurs. Pharmaceutical interventions that target cardiac dysfunction are most viable prior to substantial cardiac remodeling, highlighting the potential utility for early screening and preventative strategies to manage genetic-based cardiomyopathies.
N2BA Isoform Expression, Collagen Content, and Tubulin Abundance Increased in Ischemic Heart Failure in Humans

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Myocardial stiffness, crucial for cardiac function, is regulated by factors that include the isoform and phosphorylation status of titin isoforms and the content of tubulin and collagen. This study investigated these factors in >175 organ donors and cardiac patients, encompassing various clinical sub-types of heart failure. The numbers of patients in each clinical sub-group were as follows: organ donors (21), dilated cardiomyopathy (29), ischemic heart failure (45), cardiac amyloidosis (5), titin truncation mutations (8), end-stage heart failure pre-Ventricular Assist Device (VAD) (35), and post-VAD (35).

Titin isoforms were separated using SDS-agarose gels, and phosphorylation levels were determined using agarose gel electrophoresis. Collagen content was assessed using a hydroxyproline assay, and tubulin abundance was measured through SDS-PAGE/Western blotting.

Data were analyzed using linear mixed models with clinical diagnosis as a main factor. One of many statistically significant results was the finding that the relative content of the N2BA isoform of titin was increased relative to that measured in organ donors and in patients with dilated cardiomyopathy. Titin phosphorylation effects were complex and difficult to interpret in isolation. Collagen content was higher in samples from patients with ischemic heart failure than in organ donors (p=0.0003). Alpha-tubulin abundance was also elevated compared in ischemic heart failure.

These findings suggest that ischemic heart failure leads to increased collagen and tubulin abundance, potentially prompting a compensatory shift toward N2BA expression to maintain myocardial passive stiffness.
Unveiling the Regulatory Mechanisms and Functional Significance of BAG3 Cleavage in Ischemic Cardiomyopathy

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Bcl2-associated athanogene-3 (BAG3) is a pro-autophagy co-chaperone that localizes to the cardiac sarcomere and is critical for proteostasis and maintenance of normal sarcomeric function. Loss of BAG3 in heart failure results in accumulation of ubiquitinated sarcomeric proteins, and depressed contractility. However, how BAG3 is regulated in the cell is not well understood, with uncertainty about its structure and proteoforms.

Analysis of various human heart samples showed BAG3 as a “doublet”, with one band at 74 kDa (BAG3-Z) and a second at 85 kDa (BAG3-FL). The BAG3-Z/BAG3-FL ratio was significantly increased in human heart samples with Ischemic Cardiomyopathy (ICM) compared to nonfailing samples. Previous studies hypothesized the 85 kDa band was due to phosphorylation. To test this, we treated human myocardial tissue with 10 units of alkaline phosphatase for 1 hour, which decreased troponin I phosphorylation by ~60%, but had no impact on the intensity of either BAG3 bands, suggesting these two bands are not due to phosphorylation. Subsequent PCR and mass spectrometry experiments confirmed that BAG3-FL is the full-length protein and BAG3-Z is a cleavage product at both the N- and C- termini, which has not been reported before.

To model ICM in-vitro, we incubated Neonatal Rat Ventricular Myocytes (NRVMs) in a low-glucose hypoxic environment for 6 hours and then allowed them to recover overnight in a normal environment. These hypoxia stress experiments caused a significant increase in BAG3 cleavage and a decrease in the level of BAG3-FL, similar to what we observed in human ICM. Our results were further supported using an Ischemia-Reperfusion (IR) mouse model, showing that the BAG3 cleavage is stress-induced. Mass spectrometry analysis and in-vitro cleavage assays using recombinant human BAG3 protein confirmed caspases 1, 8, and 9 as responsible for BAG3 cleavage and the formation of BAG3-Z. Furthermore, BAG3-Z appeared to be less stable than the full-length BAG3-FL as shown by a decrease in its half-life using a chase assay.

BAG3-Z has lost ~1/3 of its N-terminus WW domain. The WW domain of BAG3 has been shown to be critical for its role in protein delivery to, and formation of, the autophagosome, which fuses with the lysosome to degrade its cargo. Using high-resolution live-cell imaging, we found lysosomes, while highly dynamic, pause at individual z-disks before jumping to a neighboring z-disk, presumably to fuse with autophagosomes formed by BAG3. Interestingly, with a 50% knockdown of BAG3-FL, similar to that observed in ICM, lysosomes still localized to z-disks but got “stuck” there, exhibiting less dynamic behavior. This indicates a possible loss-of-function regarding autophagosome formation and regulation of the lysosomal degradation pathway associated with BAG3-FL cleavage. Additional experiments are being done to identify the interactome of BAG3-Z as well as its ability to localize to the myofilament.

Our findings provide novel mechanisms of regulation of the critical BAG3 protein which is necessary for developing more precise and efficient BAG3-based therapies for HF. It also provides better understanding of the autophagy pathway and protein quality control at the sarcomere of cardiomyocytes.
Mutations within the cardiac myosin binding protein-C gene (MYBPC3) are substantial contributors, accounting for 50-60% of hypertrophic cardiomyopathy (HCM) cases. Predominantly, truncations—encompassing nonsense mutations, frameshifts, and deletions—comprise 80% of MYBPC3 mutations. However, a notable subset of variants resides in intronic regions and non-canonical splice sites, which warrant deeper investigation as they could partially explain the reasons for negative genetic test results in HCM patients. Additionally, the molecular mechanisms underlying the pathogenicity of these intronic variants remain inadequately understood. To address this gap, we employed a multifaceted approach involving in silico prediction and in vitro experiments. Specifically, we focused on elucidating the significance of a previously described 25 bp deletion within intron 32 of MYBPC3 (MYBPC3Δ25bp), which has been associated with heightened HCM risk and severe myocardial infarction (MI). Utilizing in-silico predictors such as SpliceAI and Pangolin, we predicted multiple gains and losses of splice acceptor and donor sites across the region spanning exon 32 to exon 34 of MYBPC3. Experimental validation through exon trap assays in H9C2 cardiomyocytes confirmed both exon 33 cryptic splicing and exon 33 skipping events. Further support for these findings was derived from mini gene assays and RNA sequencing utilizing variant-specific hiPSC-derived cardiomyocytes, revealing the presence of cryptic splice products. Structural analysis using protein structure homology-modeling through SWISS-MODEL unveiled disruption in the IgG-like C2 type 7 structure of the C10 domain of MYBPC3, a consequence of the translated protein from the cryptic transcript and exon-skipped transcript. To ascertain the physiological relevance, transgenic mice expressing Mybpc3 with exon 33 omission were subjected to ischemia-reperfusion injury. Results demonstrated a significant increase in infarct size in transgenic mice compared to controls, indicating the deleterious impact of the MYBPC3Δ25bp on MI pathogenesis. Ongoing and future investigations will delve deeper into the molecular and functional aspects of the MYBPC3Δ25bp variant, shedding light on its role in both HCM and MI pathogenesis.
A thin filament targeting molecule reduces calcium sensitivity during muscle regulation

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Cardiomyopathy is a primary disease of heart muscle that is characterized by an abnormally functioning myocardium in the absence of any overt physiological cause (e.g., hypertension, valvular disease, etc). Upon diagnosis, many of these cases are found to result from inherited mutations in sarcomere proteins of the myocardium including the thin filament; consisting of actin, troponin (Tn), and tropomyosin (Tpm). As the thin filament response to myoplasmic calcium levels is key during the regulation of heart muscle contractions, it is not surprising that mutations in thin filament proteins have been shown to cause heart muscle dysfunction that can lead either hypotrophic cardiomyopathy (HCM) or dilated cardiomyopathy (DCM). The treatment options for patients over the past decade have improved in both effectiveness and precision. Until recently, patients either had lifestyle adjustments or a complete heart transplant. With improvements in genetic screening, recent work has shown that small molecules targeting myosin can modulate abnormal contractile behavior at the root cause of disease and are on the market as a therapeutic option for patients. To improve the precision and personalization of therapeutics for cases of thin filament disruption in disease, we have explored small molecule contractile modifiers that more specifically target the thin filament. In the computational screening, we used a library of about 8700 compounds obtained from the Center of Molecular Discovery at Boston University. First, potential small molecule binding sites were computationally predicted from the published blocked, low calcium B-state complex of the thin filament using the Schrodinger package with default parameters. Greater than 200 pockets were predicted and inspected manually to identify suitable pockets for docking of small molecule compounds. The molecular docking analysis was performed using Glide SP, with one compound, termed NCB1, showing a docking score of 9.318 kcal/mol, suggesting energetically favorable binding. Hence, the identified compound was taken further to evaluate experimentally. In vitro motility assays demonstrate that incubation of the compound with Tpm-Tn decreases calcium sensitivity of regulated thin filaments (DMSO control pCa₅₀: 6.35 mM ± 0.3, NCB1 pCa₅₀: 5.95 mM ± 0.6115; DMSO Hill Slope: 3.07 ± 1.20, NCB1 Hill Slope: 1.20 ± 1.4) without affecting acto-myosin motility or maximum velocity. Therefore, the data demonstrates that druggable sites, identified at the actin-Tpm interface, represent a potential therapeutic target that can modify contractility. This study highlights the potential for a thin filament targeted therapeutic to restore muscle contractility in cardiomyopathy cases where pathogenicity is hypercontractile.

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DILATED CARDIOMYOPATHY MUTATION IN BETA-CARDIAC MYOSIN ENHANCES ACTIN ACTIVATION OF THE POWER STROKE AND PHOSPHATE RELEASE

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Inherited mutations in human beta-cardiac myosin (M2β) can lead to severe forms of heart failure. The E525K mutation in M2β is associated with dilated cardiomyopathy (DCM) and was found to stabilize the interacting heads motif (IHM) and autoinhibited super-relaxed (SRX) state in dimeric heavy meromyosin (Rasicci et al. eLife, 2022, PMID 36422472). However, in monomeric M2β subfragment 1 (S1) we found that E525K enhances (3-fold) the maximum steady-state actin-activated ATPase activity ($k_{\text{cat}}$) and decreases (6-fold) the actin concentration at which ATPase is one-half maximal ($K_{\text{ATPase}}$). We also found a 3 to 4-fold increase in the actin-activated power stroke and phosphate release rate constants at 30 μM actin, which overall enhanced the duty ratio 3-fold. Loaded motility assays revealed that the enhanced intrinsic motor activity translates to increased ensemble force in M2β S1. The impact of free Mg$^{2+}$ on ADP-release kinetics was more pronounced in the E525K mutant compared to WT, suggesting Mg$^{2+}$ coordination may be altered by the mutation. Glutamate 525, located near the actin binding region in the so-called activation loop, is highly conserved and predicted to form a salt-bridge with another conserved residue (lysine 484) in the relay helix. Enhanced sampling molecular dynamics simulations predict that the charge reversal mutation disrupts the E525-K484 salt-bridge, inducing conformations with a more flexible relay helix and a wide phosphate release tunnel. Our results highlight a highly conserved allosteric pathway associated with actin activation of the power stroke and phosphate release and suggest an important feature of the autoinhibited IHM is to prevent this region of myosin from interacting with actin. We predict that the impact of the E525K mutation on stabilizing the IHM likely overrides the enhanced intrinsic motor properties, resulting in an overall reduction in cardiac muscle force and power and triggering DCM pathogenesis.
Two *Drosophila* Myosin Cardiac Isoforms of Myosin Assemble Normally in Indirect Flight and Jump Muscles but Differentially Affect Their Function

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To facilitate use of *Drosophila* as a model to study myosin-based human cardiomyopathies, we are investigating the structural, biophysical, and biochemical properties of its cardiac isoforms of myosin. Cardiomyocytes generate the force necessary for the pumping of hemolymph by the *Drosophila* cardiac tube, but isolating and characterizing cardiac myosins and directly measuring the contractile properties of the cardiac tube is not feasible. Therefore, we needed to determine which isoform(s) are present in the tube and express them in other muscles to enable isolation and to analyze the impact of said isoforms on biochemical and mechanical properties. Using RT-PCR and mass spectrometry, we identified two major myosin isoforms, referred to as “CardM1” and “CardM2.” Given that all *Drosophila* MHC isoforms are generated by alternative splicing events from a single gene, we know that the two isoforms differ at only the tip of the ATP-binding region, the relay domain, and the converter domain, suggesting they possess unique biochemical and/or biophysical properties. To enable myosin isolation as well as biochemical and mechanical analyses, we expressed each of these MHC isoforms in the indirect flight muscles (IFMs) and the tergal depressor of the trochanter (TDT or jump) muscles via transformation with the corresponding cardiac cDNA transgenes.

Each isoform successfully assembles normally into myofibrils in the IFM and TDT muscle as determined by transmission electron microscopy. Mechanical assays on isolated TDT muscle fibers show that CardM2 enables greater stretch activation, the delayed increase in force production following muscle stretch, than CardM1 and the endogenous TDT isoform. This suggests that significant stretch activation is occurring in *Drosophila* cardiac tubes, similar to what is observed for human hearts.

Relative to the fast IFM isoform, CardM1 and CardM2 exhibit significantly decreased actin-activated ATPase *V*\(_{\text{max}}\) values, and CardM1 shows significantly lower basal ATPase activity. CardM2 exhibits a lower actin-stimulated Michaelis-Menten constant, which suggests a higher affinity for actin. CardM1 shows significantly decreased *in vitro* actin filament sliding velocity compared to IFM myosin, which suggests it spends a longer time attached to actin and/or exhibits a decrease in step size of its power stroke. Preliminary data indicate that CardM2 shows a similar decrease of *in vitro* actin filament sliding velocity. Efforts to similarly characterize the biochemical properties of the TDT myosin isoform are ongoing. Flies expressing CardM1 or CardM2 yield reduced flight and jump abilities compared to control flies, with CardM2 flies displaying a greater loss of both.

Future work will include cryogenic electron microscopy (cryo-EM) of CardM1 and CardM2 bound to actin, to connect actomyosin structure with biophysical and biochemical properties. CRISPR-Cas9 mediated mutagenesis of the CardM1 and CardM2 cDNA constructs will reveal the biochemical, mechanical, and structural effects of two mutations linked with hereditary DCM, which have been shown to induce arrhythmia and increased diastolic and systolic diameters when expressed in the adult cardiac tube. Supported by NIH NIGMS 3R37GM032443, by the Rees-Steealy Research Foundation, and by the San Diego State University College of Graduate Studies.
A Novel TR-F based HTS to identify small molecules that mimic the effects of cTnl Ser23/24 phosphorylation

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Impaired relaxation is a hallmark of HCM and is commonly manifested in preclinical HCM cohorts. Ho et al identified that measurements of diastolic dysfunction via 2D echocardiography were highly specific in identifying preclinical mutation carriers. Specifically, a reduction in mitral annulus velocity (e') was commonly found prior to the development of left ventricular hypertrophy. Our R92L-cTnT HCM murine model phenocopied this preclinical population with a reduction in e' prior to changes in left ventricular wall thickness. The early onset of diastolic dysfunction in this model suggests a primary myofilament component driving the observed changes to diastolic function. At the level of the thin filament, the R92L-cTnT mutation significantly decreases the rate of calcium dissociation kinetics at baseline and shows a blunted response to beta-adrenergic activation as measured by a decrease in cTnI-Ser23/24 phosphorylation. Phosphorylation of these sites in the WT system significantly increases the rate of calcium dissociation and has been commonly shown to enhance diastolic function at the whole organ level. We postulated that the genetic incorporation of the phosphomimetic Ser23/24DD-cTnI (DD-cTnI) would recover diastolic function in this disease model. We generated cTnT-R92L/DD mouse lines with 50% expression of the R92L mutation and 95% expression of DD-cTnI. Incorporation of DD-cTnI prevented the development of diastolic dysfunction (measured by E/e') at 1mo and 5mo of age. This effect was accompanied by a reduction in hypertrophy and hyper systolic function as compared to the cTnT R92L model. Our echo data coupled with preclinical HCM patient data could suggest that early intervention with small molecules identified to mimic the effects of cTnl phosphorylation could be advantageous in the treatment of HCM. Therefore, we designed a time resolved fluorescence (TR-F) based high throughput drug screen optimized to identify these small molecules. Briefly, we conjugated an environmentally sensitive fluorescence probe to the N-terminus of cTnl and reconstituted it into our cardiac thin filament system. We then monitored the change in fluorescence lifetime in response to phosphorylation of cTnl. Our optimized screen yielded a Z' value of 0.86 indicating that this is an excellent assay for high throughput drug screening. We then tested our assay with the Selleck FDA approved drug library of 3057 compounds. Small molecules that altered the fluorescence lifetime by more than 5 times the standard deviation were selected for secondary screening. Primary screening yielded a 0.85% hit rate. We employed calcium dissociation kinetics experiments as our secondary screening assay. 75% of the selected small molecule hits altered calcium dissociation kinetics; however, only 3 accelerated the rate of calcium dissociation. These data indicate that our TR-F based screen was successful at identifying small molecules that alter the calcium handling properties of the thin filament. Future work will include additional screening of a 9000-compound diversity library and tertiary screening using myofibril ATPase to assess how these small molecules alter the calcium sensitivity of the myofilament.
Probing mutation-induced changes in post-powerstroke conformations of actomyosin via molecular dynamics simulations.

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Post-powerstroke conformations of the actomyosin complex bear force during the chemomechanical cycle and conformational transitions between such states determine relaxation kinetics in striated muscle. These structural transitions are driven by (1) release of ADP from the myosin nucleotide binding pocket, (2) binding of ATP to the myosin nucleotide binding pocket, and (3) detachment of ATP-bound myosin from actin filaments. Hypertrophic cardiomyopathy (HCM) associated mutations H251N, G256E, and R403Q in cardiac β-myosin have been linked with changes in relaxation kinetics (e.g. $k_{rel,\text{slow}}$, $k_{rel,\text{fast}}$, $t_{rel,\text{slow}}$) of myofibrils prepared from human induced pluripotent stem cell and porcine derived cardiomyocytes relative to wild type controls. Here, we employ molecular dynamics simulations of the ADP-bound and nucleotide actomyosin complex) to investigate the molecular mechanisms by which H251N, G256E, and R403Q modify muscle relaxation kinetics. Conventional MD simulations predict that the H251N and G256E disrupts myosin transducer structure and the conformation of ADP within the nucleotide binding pocket while the R403Q mutation alters the acto-myosin interface. Brownian Dynamics and enhanced sampling MD simulations of ADP release highlight the importance of E228 on nucleotide handling by myosin and predict that mutations altering nucleotide binding pocket structure impair the energetics and structural pathway of ADP release. These simulations collectively demonstrate that mutations can contribute to similar pathologies by adversely affecting multiple structural events in muscle relaxation.
Electrophysiological and mechanical characterization of human ventricular myocardium from patients with primary or secondary cardiac hypertrophy.

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Left-ventricular hypertrophy (LVH) is associated with clinical complications such as ventricular arrhythmias and diastolic dysfunction. The molecular and cellular mechanisms underlying such abnormalities in the human heart have been poorly investigated.

We collected myocardial samples from the upper interventricular septum of 132 patients with hypertrophic cardiomyopathy (HCM), 42 patients with aortic stenosis and severe LVH (AoS-LVH) and 12 non-failing non-hypertrophic patients with valve disease (NF-NH), who underwent myectomy operations at our cardiac surgery center. Samples were used to isolate single viable ventricular cardiomyocytes to perform patch-clamp electrophysiological studies and intracellular calcium measurements with fluorescent dyes. Intact trabeculae were also dissected to perform isometric force measurements of electrically-stimulated twitches.

Single-cell patch-clamp studies revealed a marked prolongation of action potential duration (APD) in HCM patient samples (N=82, mean APD at 90% repolarization=763±252ms at 0.5Hz) with respect to NF-NH (N=12, APD90%=447±88ms), while APD prolongation was less pronounced in AoS-LVH (N=22, APD90%=569±147ms). In both HCM and AoS-LVH cardiomyocytes, APD prolongation was associated with increased late-Na+ current and decreased delayed rectifier K+ currents with respect to NF-NH cells, while L-type Ca2+ current was enlarged only in HCM samples. Ca-fluorescence studies revealed markedly slower Ca-transient (CaT) kinetics in myocardial samples from HCM (N=38, mean CaT 50% decay time at 0.5 Hz = 658±179ms) and AoS-LVH patients (N=9, CaT50%= 668±187ms), when compared with NF-NH samples (N=8, CaT50%=283±59ms), paralleled by elevated diastolic [Ca2+] at high pacing rates. Twitch force measurements in intact trabeculae revealed prolonged isometric contractions in HCM (N=78, overall twitch duration at 0.5Hz= 730±147ms) and in AoS-LVH patient-samples (N=24, TwD=669±116ms), as compared with NF-NH (N=7, TwD=511±73ms). Force-frequency relationship was flat or negative in HCM and AoS-LVH samples, while NF-NH trabeculae showed a clear increase in twitch amplitude while increasing pacing rate to 2Hz. Interestingly, samples from the subgroup of HCM patients carrying sarcomeric mutations in MYH7 or MYBPC3 genes showed a shorter twitch duration with respect to HCM patients carrying no pathogenic mutations in sarcomeric genes.

All in all, our results suggest that the main features of functional cardiomyocyte remodeling (that is, changes in the expression and/or function of ion-channel and EC-coupling proteins) are qualitatively similar in primary vs. secondary LVH, albeit abnormalities are quantitatively more extensive in HCM samples. In line with our previous work (Pioner et al., Circulation 2023), we confirmed that the presence of sarcomeric thick filament mutations that accelerate cross-bridge kinetics reduce twitch duration in intact myocardium.

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Hypertrophic cardiomyopathy (HCM) is predominantly a disease of the sarcomere, with a majority of mutations found in cardiac myosin binding protein C (cMyBP-C) or beta myosin heavy chain (βMyHC). HCM-causing mutations in cMyBP-C can produce a dominant negative cMyBP-C protein or cause a failure in cMyBP-C protein production/incorporation, yet both insults result in the HCM phenotype. Our lab developed two HCM mouse models: a cMyBP-C null mouse (−/−) and a mouse with a cMyBP-C W792R mutation (792RR), which shows full incorporation of the protein into the sarcomere and functions through a dominant negative mechanism. In contrast to the −/− mice, the 792RR mice do not survive to weaning age. It is not clear whether these different primary contractile insults lead to HCM via converging or divergent cellular signaling mechanisms. We hypothesize that the −/− and 792RR HCM mouse models converge in their cellular signaling, albeit at different rates of progression, leading to the HCM phenotype.

We performed a comprehensive gene expression comparison of −/− and 792RR left ventricles (LVs) at postnatal day (PND) 10, prior to the drop in 792RR survival, using RNA Seq to compare global cell signaling. Within the large overlap of 1937 common genes found to be dysregulated relative to their respective, strain-matched controls, 167 of those genes were found to be dysregulated in opposite directions, with 103 of 106 genes belonging to the “cell cycle” GO category. All 103 cell cycling genes were upregulated in the −/− hearts and downregulated in the 792RR hearts. We questioned whether this result was indicative of a divergence in cell signaling or an offset in the timeline to disease progression. We thus harvested −/− and 792RR LVs at PND0, 2, 5, and 10 and performed gross morphological assessments at each timepoint and targeted qPCR at PND2,10. Heart weight/ body weight ratios (HW/BW) were significantly elevated in the −/− mice from PND2-10, while 792RR mice showed no elevation until PND10. Despite no early change in size, 792RR hearts displayed a markedly abnormal “apple-shaped” heart morphology by PND2, not mirrored by the −/− hearts. Targeted qPCR analysis suggests some metabolism-related gene dysregulation (e.g. Pdk4, Gck) at PND2 only in 792RR LVs. This precedes the cell cycling dysregulation (e.g. Ccna2, Anln, E2f1) in −/− and 792RR LVs, which appears by PND10, but not PND2. As in RNA Seq, cell cycling was upregulated in −/− and downregulated in 792RR. Experiments are continuing at PND0 and PND5 to give further detail to the timelines of cell signaling in −/− and 792RR hearts.

The −/− mouse, lacking cMyBP-C, and the 792RR mouse, which produces and incorporates a dominant negative cMyBP-C, both develop HCM. We show here dramatic, global dysregulation in opposite directions between −/− and 792RR LVs in cell cycling/ proliferation genes, which is preceded in the 792RR LV by altered expression of genes involved in metabolism. Striking differences in heart morphology between −/− and 792RR mice also point to either divergence in cell signaling or an altered timecourse of disease progression. Ongoing investigations aim to further define the extent of convergence/divergence in the cell signaling leading to HCM.
Impaired Myofibril Relaxation in Human HFpEF Biopsies Provides Insights into a Subphenotype's Diastolic Dysfunction

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Diastolic dysfunction is a hallmark of heart failure with preserved ejection fraction (HFpEF) and its subphenotypes, which include a ‘classic’ phenotype defined by hypertension/hypertrophy (Ht/Hp) as well as a phenotype that is observed comorbidly with obesity/diabetes (Ob/Dm). A prior cell mechanics study of HFpEF patient biopsies revealed reduced maximal contractile force ($F_{\text{max}}$) in Ob/Dm cardiomyocytes compared to non-failing samples, while Ht/Hp cardiomyocytes exhibited excessive force at diastolic calcium levels, though with relatively normal $F_{\text{max}}$. These findings suggest unique subcellular mechanisms that decrease force in Ob/Dm samples and elevate diastolic force in Ht/Hp samples, potentially impeding relaxation. To investigate these mechanisms, we assessed force production and activation/relaxation kinetics in myofibrils from Ht/Hp and Ob/Dm HFpEF patient biopsies. Measurements from a third cohort of patients who express a phenotype intermediate of the other two are ongoing. Myofibrils from both Ht/Hp and Ob/Dm patients showed a prolonged linear relaxation phase, as well as slower linear and exponential relaxation rates, indicating excessive actomyosin interactions following calcium removal. We also observed significantly decreased $F_{\text{max}}$ in Ob/Dm, but not Ht/Hp, myofibrils. X-ray diffraction was performed to determine whether anomalous structural relationships between myofilament proteins contribute to the Ob/Dm or Ht/Hp subphenotypes. Ob/Dm samples had increased lattice spacing and reduced equatorial intensity ratio, $I_{1,1}/I_{1,0}$, (indicative of decreased actomyosin interaction) compared to control, while neither property differed in Ht/Hp biopsies. These findings suggest altered sarcomere structure or thick filament activity as potential contributors to the Ob/Dm phenotype, while other mechanisms, such as aberrant thin-filament regulation, may explain impaired relaxation in Ht/Hp myofibrils and elevated diastolic force in cardiomyocytes. Finally, we assessed myofibril mechanics of three common HFpEF animal models: Göttingen minipigs, ZSF1-obese rats, and mice exposed to a high-fat diet and L-NAME. While all models showed organ-level diastolic dysfunction, only the rodent models exhibited impaired myofibril relaxation, most closely mimicking the human Ht/Hp phenotype, while minipig myofibrils exhibited decreased maximal force similar to the Ob/Dm phenotype, though with relatively normal relaxation.
R451G Desmoplakin Induces Contractile Deficits in Engineered Heart Tissue

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Arrhythmogenic cardiomyopathy (ACM) is a progressive disease characterized by mutations of desmosomal proteins which classically presents with arrhythmias originating from fibro-fatty infiltration of the right ventricle. Epidemiological studies have revealed that exercise can exacerbate the disease phenotype and in vitro work has implicated mechanical loading on disease progression. Here, we investigate the pathogenicity of R451G desmoplakin (DSP), a mutation previously shown to cause constitutive DSP proteolysis by calpain, with resultant disruption of Cx43 at the intercalated disk.

Using iPSC-derived engineered heart tissue, we demonstrate that DSP-R451G results in a phenotype that resembles dilated cardiomyopathy. DSP-R451G tissues produce less active force (456 μN vs 315 μN, n = 6) with faster systolic kinetics (TTP: 186 ms vs 156 ms, n = 6). DSP-R451G tissues also exhibit a faster spontaneous beating rate (55 bpm vs 66 bpm). Notably, our previous work, in which tissues were cultured under sub-physiological calcium levels (RPMI vs DMEM), did not show a hypo-contractile phenotype, potentially implicating calcium in the pathogenesis of R451G ACM. Next, to study tissue function under conditions of biomechanical stress, we applied 20% static stretch for 7 d after baseline mechanical characterization, then re-rested mechanical function. Preliminary data reveal a blunted response to mechanical stretch in DSP-R451G tissues compared to control (2.7-fold vs. 2.4-fold change in peak force, n = 4). Immunoblotting experiments show that the decrease in baseline force is not explained by differences in myosin heavy chain expression.

Though the pathogenesis of arrhythmogenic cardiomyopathy secondary to DSP R451G mutations is incompletely understood, our findings reveal the conditions under which calcium concentration and mechanical perturbations result in a hypocontractile phenotype. Future work will examine potential changes in calcium transients and sarcomere length in mediating this dilated cardiomyopathy-like phenotype.
Structural Origin of the Super Relaxed State of Human β-Cardiac Myosin

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In the sarcomere, force is produced by the sliding of myosin-containing thick filaments over actin-containing thin filaments. In the thick filaments, not all myosin heads are involved in force production, as some myosin molecules have their catalytic heads folded back onto their coiled coil tail in an inactive conformation referred to as the “folded back state” or the interacting heads motif (IHM). The IHM state plays a crucial role in determining cardiac contractility, and dysregulation of this structural state has been implicated to be the primary molecular effect of several different cardiomyopathy-causing mutations, based on recent studies. However, in most of these studies the IHM state was not directly measured using structural methods. Rather, the proportion of myosin in the IHM state was inferred from ATP turnover kinetics. Myosin can adopt an enzymatic state that turns over ATP 1000-fold slower than the active, actin-bound state. Although it is thought that the IHM myosin generally corresponds to this slow kinetic state, there are instances where this correlation is lost, and thus the slow kinetic state is not a reliable measure of the IHM state. Even structural biology methods such as electron microscopy (EM) or X-ray diffraction are not quantitative. We designed a FRET sensor using the atomic structure of the folded back state of human β-cardiac myosin, to quantify the IHM state in solution. We attached a donor fluorophore to the C-terminus of regulatory light chain of myosin and used Cy3-ATP bound at the nucleotide binding pocket in myosin head as an acceptor fluorophore. We tested the FRET sensor in different solution conditions and by using mutant variants of myosin that are known to stabilize/destabilize the IHM state. We observed a quantitative agreement between the biochemical SRX state and the structural IHM state quantified by the FRET sensor. This FRET sensor shows promise as a tool to determine the effects of cardiomyopathy-causing mutants on the IHM state and will be tested on other mutants and small-molecule myosin modulatory drugs.
Short duration space travel does not significantly affect cardiomyocyte sarcomere function

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Space exploration is important for scientific discovery, advancing technology, and survival of humanity. However, the impacts of microgravity and cosmic radiation during space travel on human physiology are still mostly unknown. While microgravity results in atrophy and loss of skeletal muscle function, the effect on cardiac muscle is not as clear. Here, we examine the effect of spaceflight on the myocardial contractile function in skinned cardiomyocytes from mice that went to space (spaceflight, \( N = 5 \)) and those that did not (ground control, \( N = 5 \); and vivarium, \( N = 3 \)). These experiments allow us to characterize the mechanical properties of the sarcomere, the fundamental unit of contraction. The spaceflight mice were housed in the Rodent Research Hardware System on the International Space Station (ISS) for five weeks. Meanwhile, the ground control group were housed on Earth in the same specialized cages and received the same specialized feed as the space mice. Vivarium control mice remained on Earth, in normal vivarium conditions. Upon return to Earth, the mice landed at NASA’s Kennedy Space Center in Florida and then transferred to Texas A&M University in College State Texas for examinations and preservation of samples (< 8 hours from splashdown to cryo-preservation).

The force-calcium experiments showed that spaceflight mice (\( n = 13 \)) generated a maximum tension (\( T_{\text{Max}} \)) of 32.36 ± 2.65 mN/mm\(^2\), which was an insignificant increase compared to ground control (31.69 ± 2.49 mN/mm\(^2\), \( p = 0.9793, n = 11 \)) and vivarium (28.59 ± 2.08 mN/mm\(^2\), \( p = 0.5253, n = 11 \)). The spaceflight mice also exhibited a slight increase in calcium sensitivity (1.94 ± 0.1 \( \mu \)M) when compared to ground control (1.77 ± 0.1 \( \mu \)M, \( p = 0.3664 \)) and vivarium (1.67 ± 0.05 \( \mu \)M, \( p = 0.1019 \)). The kinetics of force-redevelopment (\( K_{\text{TR}} \)) experiments showed no notable differences among the groups at saturating calcium concentration (46 \( \mu \)M, 8.95 s\(^{-1}\) for spaceflight, 8.99 s\(^{-1}\) for ground control, and 8.73 s\(^{-1}\) for vivarium, \( p > 0.05 \)) and at calcium concentration where tension is half-maximal (1.7 \( \mu \)M, 3.12 s\(^{-1}\) for spaceflight, 3.35 s\(^{-1}\) for ground control, and 3.24 s\(^{-1}\) for vivarium, \( p > 0.05 \)). When stretching the sarcomere from 1.8 \( \mu \)m to 2.4 \( \mu \)m, there were no significant differences in passive tension (\( p > 0.05 \)) observed at any tested length between the groups. Gene ontology analysis of our mass spectrometry readout indicated that a few pathways were dysregulated in spaceflight mice such as granulocyte chemotaxis, the immune system, and others. However, dysregulations of pathways relating to the contractility of the sarcomere were not observed. Together, these results suggest that short-duration space travel does not substantially affect the intrinsic contractile state of the heart. Such insights are invaluable in preparing astronauts for the long-duration space expeditions in the near future, such as missions to Mars.
Multiomics reveals potential molecular correlates of phenotypic differences associated with disparate RBM20 variants

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Variants in RNA binding motif protein 20 (RBM20) are causative in a severe form of dilated cardiomyopathy (DCM) referred to as RBM20 cardiomyopathy. Studies have shown that pathogenic variants in RBM20 disrupt a nuclear localization signal (NLS) in the protein leading to mis-localization and accumulation in the cytoplasm. Emerging evidence has revealed phenotypic differences associated with different NLS variants. For example, our data show that mice carrying the S637A and S639G variants (analogous to S635A and S637G, respectively, in humans), exhibit significant differences in premature mortality with ~40% of Rbm20S639G mice dying within the first 200 days after birth versus ~12% of Rbm20S637A mice. Hence, it is critical to investigate the molecular bases for these differences. Recent studies have begun to unravel the mechanisms underlying RBM20 cardiomyopathy, however, analysis of gene expression has been restricted to the characterization of changes at the transcript level.

To address these knowledge gaps, we carried out the first analysis of gene expression changes at the protein level in mice carrying the pathogenic S637A and S639G variants in the NLS in RBM20. Consistent with the broadly similar phenotypes, both variants were associated with consistent changes in gene expression at the protein level, with characteristic up-regulation of well-established heart failure markers including NPPA and ANKRD1. Surprisingly, despite differences in premature mortality, only the protein dystonin was differentially expressed in the hearts of Rbm20S637A versus Rbm20S639G mice. Nevertheless, a comparison of protein expression in the hearts of Rbm20S637A and Rbm20S639G mice relative to WT yielded 197 and 400 proteins that were uniquely differentially expressed in the knock-in lines, respectively, suggesting that subtle differences in protein expression may explain phenotypic discrepancies. Gene ontology analysis indicated that uniquely differentially expressed proteins are involved in cardiac function, remodeling, and arrhythmia. Thus, subtle differences in the expression of proteins related to heart failure and arrhythmia likely underlie increased premature mortality in Rbm20S639G compared to Rbm20S637A mice.

Analysis of gene expression at the transcript level in Rbm20S637A versus Rbm20S639G mice allowed for the expression of approximately five times as many genes to be analyzed and identified several additional differences. These data further supported differences in the expression of genes related to pathological cardiac remodeling as a source of phenotypic disparities between mice with different RBM20 variants. Integration of the proteomics and transcriptomics data suggested that these variants differentially impact mitochondrial function leading to disparate outcomes.
Modulation of Cardiomyocyte Mechanics by HCM-Linked Mutation and Mavacamten: A Mant-ATP Study

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Background: Hypertrophic cardiomyopathy (HCM) is a prevalent genetic cardiac disorder where β-cardiac myosin mutations such as H251N contribute to its key phenotypes of hypercontractility, hypertrophy, and fibrosis, all of which are interconnected with altered cardiac mechanics. At the intersection of these phenotypes lies altered myosin-driven ATP turnover, pivotal for contractile force generation and cardiomyocyte energetics. The Mant-ATP assay serves as a vital tool for measuring ATP turnover rates, providing an index on the distribution of myosin heads in super relaxed (SRX) and disordered relaxed (DRX) states. Such measures have unveiled changes in tissue and protein studies but have scarcely been applied in stem cell-derived cardiomyocyte systems involving myosin mutations—a gap this study seeks to fill. By employing the Mant-ATP assay in such novel models, we aim to unearth molecular dynamics induced by HCM-causing mutations and dissect their systemic consequences.

Methods: We performed a Mant-ATP assay on permeabilized heterozygous mutant human-induced pluripotent stem cell (hiPSC) derived cardiomyocyte line and control cell line from H251N mutation to evaluate the fraction of myosin heads in the SRX and DRX states. Mavacamten's role as a modulator of these states was assessed by its effect on cell contractility, myofibril structural organization, and ATP fluorescence intensity decay, which follows a bi-exponential curve. Before conducting the Mant-ATP assay, we performed live-cell traction force microscopy (TFM) imaging to observe contractile dynamics; additionally, myofibril density and sarcomere shortening were captured by imaging GFP-tagged α-actinin using a 40x air objective before cell permeabilization. Post-experiment immunostaining for β-myosin provides insight into how expression and organization correlate with contraction and SRX rate (%).

Results: The H251N mutation led to an increased DRX state fraction, correlating with the hypercontractile behavior observed in TFM. Treatment with mavacamten recalibrated this balance toward the SRX state from 11% to 42% SRX rate, reducing the force exerted by these cells. Live cell TFM and post-experiment structural analysis of the cells by immunostaining provide mechanical correlation to the altered molecular kinetics induced by the HCM mutation. Mechanical and molecular events were both modulated by mavacamten.

Conclusion/Future Implications: Our findings reveal the intricate relationship between myosin ATPase activity altered by the H251N mutation and the resulting mechanical and structural changes in cardiomyocytes. Mavacamten's restoration of SRX and DRX states suggests a targeted therapeutic strategy for HCM. Moving forward, our research will extend to study the mechanobiology of cardiac fibroblast populations acknowledging the crucial role of altered cardiomyocyte mechanics in fibroblast activation and fibrotic phenotype development. Concurrently, organ-scale fibrosis modeling is being developed through the analysis of late gadolinium-enhanced (LGE)-MRI data from HCM patients, aiming to integrate cellular-level alterations with macroscopic fibrotic patterns in the heart. Correlating these findings will provide a more profound understanding of HCM pathology and open new avenues for therapies targeting the interconnection between ATP alterations, fibroblast activity, and cardiac fibrosis.
Calmodulin Kinase II is a Mutation-Specific Driver of Disease in Hypertrophic Cardiomyopathy

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In the heart, Ca/CaM Kinase II δ (CaMKIIδ) functions to regulate and maintain electromechanical and calcium homeostasis. Several post-translational modifications of CaMKIIδ, including autophosphorylation at Thr-287 and oxidation at Met-281/282, render its activity autonomous of Ca/CaM. In genetic hypertrophic cardiomyopathy (HCM), increased levels of autonomous CaMKIIδ have been linked to disease progression. We have shown previously that the pathogenic role of CaMKIIδ in HCM is specific to individual HCM-associated mutations. Specifically, autonomous CaMKIIδ is elevated in cardiac troponin T (cTnT)-R92W mice, but not in cTnT-R92L mice. These results pose a clinically relevant and critical question that remains unanswered: what is the specific trigger for CaMKIIδ dysregulation in HCM, such that it is only present in certain mutations? We hypothesize that CaMKIIδ dysregulation results from specific and distinct alterations in thin filament biophysical properties due to the presence of point mutations, and this variability contributes to the phenotypic variability observed in HCM patients. Specifically, cTnT-R92W thin filaments display accelerated calcium dissociation kinetics compared to wild type thin filaments, whereas cTnT-R92L thin filaments have slower dissociation kinetics, and as such we propose accelerated calcium dissociation kinetics as a mutation-specific trigger of CaMKIIδ dysregulation. To test this, we will use mass spectrometry following CaMKIIδ immunoprecipitation to assess the abundance of phosphorylation at Thr-287 and oxidation at Met-281/282 in transgenic mice expressing the cTnT-R94H, cTnT-I79N, or cTnT-R92W mutation, all of which are associated with HCM in humans, and which have differing calcium dissociation kinetics. Preliminary data demonstrates that CaMKIIδ oxidation can be readily detected in our immunoprecipitated samples. We will supplement these experiments with the use of the novel CaMKIIδ biosensor CaMKAR in cardiomyocytes isolated from these mice to evaluate CaMKIIδ activity. Our preliminary data demonstrates that CaMKAR expression in rat myocytes following adenoviral infection and that in our system, CaMKAR responds as expected to pacing, CaMKIIδ inhibition, and cytoplasmic calcium overload with caffeine and thapsigargin. In addition, we have previously shown that genetic inhibition of CaMKIIδ in cTnT-R92W mice improves diastolic function and atrial remodeling. We aim to extend this study and test the efficacy of the novel small molecule CaMKIIδ inhibitor ruxolitinib in cTnT-R92W mice. Two-week treatment of these mice with ruxolitinib improves both systolic and diastolic function, and has no effect in nontransgenic controls, implicating small molecule inhibition of CaMKIIδ as a potential therapeutic option in HCM. Future and ongoing work will explore changes in calcium handling in the three mouse models to further establish potential mechanistic pathways by which CaMKIIδ dysregulation occurs in HCM and will extend ruxolitinib treatment to several months to further evaluate its efficacy as an HCM therapeutic.
Regulation & Role of Obscurin Kin1 in cardiomyocytes

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Obscurins comprise a family of cytoskeletal proteins with known regulatory roles in calcium cycling, myofibrillogenesis and cellular adhesion. Obscurin-B (~870 kDa), the largest known isoform is a modular protein assembled from immunoglobulin-like (Ig) and fibronectin (Fn3) domains with two unique Ser/Thr kinase domains, Kin1 & Kin2, residing at the C-terminus. Recently, our group has elucidated a previously unknown signaling axis in cardiomyocytes involving Kin1’s phosphorylation of the adhesion molecule N-Cadherin, at Ser-788. Given Kin1’s essential role in cardiomyocyte coupling, an exhaustive investigation of the mechanisms that regulate Kin1’s activity is vital to delineating its (patho)physiological role in the heart. Sequence homology with members of the Myosin Light Chain Kinase (MLCK) family suggest regulation by Ca²⁺/Calmodulin (CaM) with in vitro studies supporting CaM binding. Additionally, obscurin-B’s modular architecture and subcellular localization suggests a potential role for mechanical force in its regulation. In accordance with this, preliminary in silico modeling and molecular dynamic simulations suggest the existence of a unique structural element within the regulatory domain of Kin1 whose unfolding allows Kin1 to adopt a semi-stable conformational state potentially capable of substrate binding. Current studies are focused on investigating Kin1’s mechanical properties via atomic force microscopy. Excitingly, pulldown assays have identified Ryanodine Receptor 2 (RyR2) as a binding partner of Obscurin Kin1 with preliminary in vitro kinase assays and mass spectrometry identifying Ser2457 as a novel phosphorylation site on the cardiac ryanodine receptor. This data is the first to suggest Obscurins as direct regulators of calcium cycling with current work focused on determining the in situ and in vivo relevance of this phosphorylation event.
Investigating the Effects of Two Thin Filament Mutations linked to Dilated Cardiomyopathy (DCM) on the Beta-Adrenergic Response

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The cardiac thin filament (CTF) is an essential regulatory unit in sarcomeric contraction and couples the availability of cytoplasmic calcium to force generation. It’s been established that thin filament mutations lead to symptomatic cardiomyopathies. The complexities of both the myocellular mechanism(s) involved and the clinical outcome(s) is an ongoing field of study to meaningfully link genotype to phenotype. Two DCM-causing thin filament point mutations (D230N-Tropomyosin and R173W-cardiac troponin T) have similar clinical presentations and were selected to investigate their disease trajectory. To investigate the effects of these mutations on systolic function, we conducted 2D-echocardiography on the transgenic mouse models and their non-transgenic (NTg) littermates at 2 and 4-month timepoints. At 2-months, the percent fractional shortening (%FS) in D230N-Tm mice was significantly reduced (21.79±1.19) compared to NTg (33.45±1.45). However, R173W-cTnT exhibited no reduction in %FS until 4 months of age (22.53±0.78). Thus, the 2D-echocardiography studies demonstrated that D230N-Tm presented an earlier onset of phenotype compared to R173W-cTnT. We then measured indices of sarcomeric contractility and relaxation in unloaded isolated cardiomyocytes from 4-month transgenic mice compared to their NTg littermates. At 4 months, both mutations displayed increases in rates of contraction (D230N-Tm 4.49±0.18 μm/sec, R173W-cTnT 5.83±0.27 μm/sec) and relaxation (D230N-Tm 3.14±0.19 μm/sec, R173W-cTnT 3.70 ±0.19 μm/sec) compared to NTg. To assess the effect of these mutations on the myocellular beta-adrenergic signaling pathway, we conducted an isoproterenol challenge on isolated D230N-Tm cardiomyocytes. D230N-Tm had elevated rates of contraction and relaxation at basal levels. Interestingly, upon isoproterenol treatment, there was no significant increase in rates of contraction or relaxation, suggesting a blunted beta-adrenergic response. We then sought to investigate potential underlying mechanism(s) in the myofilament linked to the blunted functional response. We isolated transgenic mouse hearts and perfused them with a calcium tyrode solution (no isoproterenol) or a 100 nM isoproterenol calcium solution. We prepared samples for western blots and probed for target proteins total phospholamban (PLB), phosphorylated PLB (pPLB-serine 16), cardiac troponin I (cTnI), and phosphorylated troponin I (pTnI-Ser23/Ser24). D230N-Tm had elevated basal levels of pTnI and pPLB, but following isoproterenol treatment, there were no significant changes in percent phosphorylation of cTnI or PLB. Based on the blunted PKA mediated-phosphorylation, we believe there is an underlying myofilament mechanism associated with the functional differences observed in D230N-Tm compared to their NTg littermates. We performed the same mechanistic assay for R173W-cTnT to collect a baseline of comparison. R173W-cTnT presented a significant increase in percent phosphorylation of both cTnI and PLB, which was consistent with our NTg controls, but deviated from the response of D230N-Tm. A significant beta-adrenergic response in R173W-cTnT suggests a possible differentiation in myocellular mechanisms and function between these mutations. Ongoing studies aim to further identify possible mechanism(s) by which these mutations express differences in phenotypic onset time and response to beta-adrenergic stimulation. Additionally, we will perform an isoproterenol challenge on isolated R173W-cTnT cardiomyocytes to compare their functional response to that of D230N-Tm and deduce if the blunted beta-adrenergic response in D230N-Tm is primarily due to elevated pTnI and pPLB levels.
Cardiac Troponin Activator CK-358 Increases Calcium Sensitivity and Cardiac Contractility in Two Mouse Models of Genetic Dilated Cardiomyopathy

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Genetic dilated cardiomyopathy (DCM) is a leading cause of heart failure, characterized by dilation of the left ventricle and impaired systolic function. Most therapeutics do not address the underlying contractile deficit causing the disease. However, over the last two decades there has been a push towards developing novel small molecules that directly target the sarcomere, the smallest contractile unit in muscle. Here we present data on the ability of the novel cardiac troponin activator, CK-3827358 (CK-358), to improve contractile function in two mouse models of DCM, one with the I61Q mutation in cardiac troponin C (cTnC I61Q) and a second with the D230N mutation in cardiac tropomyosin (cTm D230N). We measured steady state force and $k_{tr}$, a measure of crossbridge cycling kinetics, in demembranated ventricular tissue from control, cTnC I61Q, and cTm D230N mice in solutions with increasing concentrations of calcium, ranging from pCa 9 to pCa 4. Both DCM models showed a decrease in calcium sensitivity as previously published, and 0.5 μM CK-358 significantly increased calcium sensitivity in both models relative to control treatment (pCa50; I61Q: 6.05 ± 0.08 vs 5.50 ± 0.08, p = 0.005; D230N: 5.89 ± 0.03 vs 5.48 ± 0.02, p < 0.0001) without any change in maximum force. CK-358 also significantly increased $k_{tr}$ across all submaximal calcium concentrations in both models, suggesting activation of thin filaments consistent with the mechanism of action. We measured intact twitches in control and cTnC I61Q mice before and after incubation with 1 μM CK-358. cTnC I61Q mice showed decreased peak twitch. Both control and cTnC I61Q mice increased peak twitch vs control (Con: 66.8 ± 6.8 vs 56.4 ± 5.7 mN/mm², p = 0.003; cTnC I61Q: 39.1 ± 3.5 vs 30.9 ± 4.1 mN/mm², p = 0.005) as well as faster time to peak tension vs control (Con: 0.080 ± 0.005 vs 0.094 ± 0.005 sec, p < 0.0001; cTnC I61Q: 0.089 ± 0.005 vs 0.106 ± 0.005 sec, p < 0.0001) with no significant changes in relaxation kinetics. Lastly, steady state fluorescence spectroscopy was used to measure calcium affinity in isolated cTn complexes and decorated thin filaments (TF) fluorescently labeled with IANBD at C84 of cTnC. CK-358 did not affect calcium binding of cTn complexes. However, when incorporated into TF, CK-358 significantly increased pCa50 versus control for WT (6.86 ± 0.02 vs 6.73 ± 0.01, p = 0.001). A similar increase was seen in cTm D230N TF (6.82 ± 0.01 vs 6.67 ± 0.01, p < 0.0001); however, pCa50 of cTnC I61Q TF did not increase with CK-358 (5.77 ± 0.05 vs 5.75 ± 0.04, p = 0.995). Future directions aim to bridge the less pronounced effects of CK-358 on calcium binding and the more substantial effect on skinned and intact contractile function by looking at the transitions between blocked, closed, and open states of the thin filament as well as transitions to weak and strong crossbridge binding. Overall, we present data showing that the novel cardiac troponin activator, CK-358, improved both demembranated and intact cardiac function in two mouse models of DCM in part through increased binding of calcium to troponin C.
Desmoglein-2 is essential for age- and chamber-specific regulation of postnatal cardiac function

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Desmoglein-2 (DSG2) is an essential component of the cardiac desmosome – a junctional complex responsible for cell-cell integrity and electrical communication. Nearly 60% of arrhythmogenic cardiomyopathy (ACM) patient cases arise from pathogenic variants in desmosomal genes, including DSG2. However, the relationship between DSG2 and the arrangement and contractility of the cardiac sarcomere remains limited. Here, we hypothesized that DSG2 contributes to sarcomere composition, structure, and function; and thus, sarcomere disruption could be directly linked to reduced cardiac function in ACM. To study the role of DSG2 in cardiac muscle regulation, we utilized loss-of-function homozygous Dsg2-mutant mice (Dsg2mut/mut) that display severe arrhythmias, ventricular dysfunction, and myocardial fibrosis and inflammation by early adulthood (16-week-old); thus, replicating key ACM disease phenotypes. In left ventricular (LV) permeabilized cardiac muscle bundles from of postnatal (4-week-old) Dsg2mut/mut mice, we observed a significant reduction in cardiac force production and impaired cross-bridge kinetics vs. WT controls. A finding that was not observed in the right ventricle (RV). Importantly, alterations in muscle contraction preceded ECG repol depolarization anomalies and myocardial fibrosis. Interestingly, Western blot analysis of LVs from postnatal Dsg2mut/mut mice showed reduced levels of Z-disc and myofilament proteins; verified by electron microscopy studies which further revealed shorter sarcomeres with reduced Z-disc dimensions compared to WT controls. Additionally, postnatal Dsg2mut/mut LVs displayed an excess of myosin heads in the super-relaxed (SRX) state. Conversely, by adulthood, both ventricular chambers displayed mechanical and structural alterations. Overall, our findings show the absence of DSG2 is detrimental to sarcomere composition, structure, and function, as well as postnatal LV function. While postnatal RV function was preserved, RV dysfunction occurred over time (neonatal to adulthood). Collectively, this study underscores the critical contribution of DSG2 in ACM disease development. Understanding this impact is crucial for devising optimal and temporal therapeutic strategies to mitigate disease phenotypes in ACM, particularly those with RV- and LV-dominant involvement.
**Myosin Inhibition using mavacamten facilitates relaxation and improves compliance of the myofibrils from the HFpEF heart**

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**Background:** There are currently limited treatments for directly improving diastolic dysfunction in heart failure with preserved ejection fraction (HFpEF). Mavacamten, a small molecule inhibitor of myosin ATPase, has been developed as a treatment for hypertrophic cardiomyopathy (HCM), and it is currently being tested in HFpEF patients. Interestingly, emerging evidence suggests that mavacamten may not only modulate contractility but may also improve myocardial relaxation. The aim of this study was to investigate whether mavacamten directly modulates sarcomere mechanical properties to improve myocardial relaxation in a two-hit mouse HFpEF model.

**Methods:** Effects of mavacamten on the sarcomere function of the mouse hearts were first assessed using a myofibril mechanical system. To test whether mavacamten alleviates relaxation impairment of the diseased heart, adult C57Bl/6J mice were fed with (1) standard diet or (2) high-fat diet (HFD) + L-NAME to induce HFpEF (n=10 mice/group). Mouse myofibrils (n=3 per animals) were obtained and the contractile function of the sarcomere with or without the presence of mavacamten were investigated. Effects of mavacamten at the cellular level were determined in control adult mouse cardiomyocytes and human iPSC cardiomyocytes.

**Results:** Treatment of mavacamten facilitated the linear and exponential relaxation of the myofibrils isolated from control mouse hearts in a dose-dependent manner. Mechanical analysis showed myofibrils isolated from HFpEF mice had impaired relaxation when activated at sub-maximal [Ca²⁺], significantly higher stiffness and elevated Ca²⁺ sensitivity, compared with control animals. Ex vivo treatment of mavacamten alleviates relaxation impairment and completely normalised Ca²⁺ sensitivity and stiffness of the myofibrils from HFpEF animals. Furthermore, in vitro treatment of mavacamten shortened the relaxation time of both mouse and human cardiomyocytes, suggesting that the effects of mavacamten at the myofibril level could be translated to the cellular level.

**Conclusion:** This is the first study to characterise in detail the mechanical properties of myofibrils in the two-hit mouse HFpEF model. We demonstrated that myosin ATPase inhibition using mavacamten could normalise relaxation and stiffness abnormalities of the myofibrils in HFpEF. These findings position mavacamten to be a potential therapeutic intervention for improving diastolic function in patients with HFpEF.
Effects of MyBP-C Missense Variants on the Myofilament in Hypertrophic Cardiomyopathy

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Pathogenic variants in the sarcomeric gene Myosin-binding protein C3 (MYBPC3) are a primary cause of familial hypertrophic cardiomyopathy (HCM). MyBP-C (the protein encoded by MYBPC3) interacts with myosin and actin to regulate heart muscle contraction. Although both truncating and missense MYBPC3 variants are highly prevalent in HCM, only the effects of truncating variants on the myofilament have been well-defined. Truncating variants cause haploinsufficiency with a reduction in the total MyBP-C protein abundance. This protein reduction induces structural activation of myosin accompanied by diminished structural rigidity of the myofilament, shifts myosin from the super-relaxed state (SRX) to the disordered-relaxed state (DRX), and increases Ca2+ sensitivity with reduced force generation. Recent studies have shown that unlike truncating variants, missense MyBP-C properly incorporates into the sarcomere at the correct protein abundance. The consequences of the presence of missense MyBP-C in the myofilament on myosin have not been defined and could have major implications for the treatment of HCM patients with missense variants in MyBP-C.

We performed small-angle x-ray diffraction (X-rD) on human HCM myectomy tissue from patients with missense, truncating MyBP-C variants, and septum tissue from nonfailing donor controls. When nonfailing tissue was moved from resting (pCa 8) to activating (pCa 5) calcium conditions we observed an increase in the equatorial intensity ratio (I1,1/I1,0) indicating that myosin has moved away from the thick filament backbone and adopted an active conformation localizing along the thin filament. In accordance with what has been previously shown in mice truncating variants have a higher I1,1/I1,0 at resting calcium that increases further at activating calcium suggesting that at lower calcium levels truncating variants have a greater pool of myosin heads ready to engage the thin filament. However, in MyBP-C missense tissue I1,1/I1,0 is lower than nonfailing tissue at resting calcium and only increases moderately at activating calcium, indicating that even under activating conditions compared to nonfailing and truncating variants more is myosin remaining in the inactive conformation.

We next performed the Mant-ATP pulse-case assay to determine if the structural inactivation of myosin in missense hearts observed by X-rD is mirrored in the biochemical state. Previous studies have shown that in truncating MyBP-C and MYH7 HCM hearts there is a reduction in the amount of myosin in the slow energy-consuming biochemical state (SRX). However, we have observed that missense hearts do not undergo this reduction and have the same level of myosin in the slow ATP turnover state as nonfailing hearts. Treatment with the myosin modulator mavacamten increased the percentage of SRX myosin in both truncating and missense and MYH7 hearts.

Last, we performed contractile assays to determine how missense variants affect heart muscle contraction. Our tension-calcium experiments have revealed that both truncating and missense MyBP-C hearts have a reduced maximum force generation (Tmax) compared to nonfailing hearts, with this reduction being significantly lower in missense hearts. Missense hearts also exhibited a more severe increase in calcium sensitivity (EC50) relative to truncating and nonfailing hearts. Treatment with mavacamten caused a further reduction in Tmax and rescued the increased calcium sensitivity observed in missense and truncating hearts.
Reducing granule formation without splicing restoration is sufficient to alleviate cardiac dysfunction caused by Rbm20 genetic variants

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Background: RNA binding motif protein 20 (RBM20) cardiomyopathy has been characterized as a severe form of dilated cardiomyopathy (DCM). Genetic variants of Rbm20 hinder its nuclear import and promote the accumulation of pathogenic ribonucleotide protein (RNP) granules in the cytosol. We investigated whether reducing the formation of RNP granules by inhibiting Rbm20 expression could alleviate the DCM phenotype in an Rbm20 S639G knock-in (KI) mouse model (Rbm205639G).

Methods: We downregulated the Rbm20, utilizing antisense oligonucleotides (ASOs) that specifically inhibit Rbm20 expression. We employed this Rbm20-ASO in the Rbm205639G mice, the KI mouse model, which carries a serine-to-glycine substitution in the nuclear localization signal (NLS) region of Rbm20. The Rbm20-ASOs were administered subcutaneously at 25 mg/kg once a week, starting at 14 days of age and continuing for 8 weeks. The in-vivo cardiac function was assessed by echocardiography. RNP granules were identified through immunohistochemical staining, and the number and size of RNP granules were quantified using the Cell Profiler software. Alternative splicing of RBM20 target genes was determined by RT-PCR, and the titin isoform was analyzed by gel electrophoresis. Cardiomyocyte Ca2+ release-reuptake kinetics and mouse electrocardiographic (ECG) were also studied.

Results: The results revealed that reducing the level of RBM20 expression through the ASO treatment significantly decreased the number and size of RNP granules within the cardiomyocytes of Het Rbm205639G. The reduction of RNP granules led to improvement of cardiac function, including restoring ejection fraction and normalization of myocardial performance index. Furthermore, the decrease of RNP granules reduced the severity of LV chamber dilation, as evidenced by reduced LV internal diameter, and normalization of eccentricity. It was also effective in mitigating LV hypertrophic remodeling and improved ECG parameters observed as normalized P wave and QRS durations. These beneficial effects occur even without the restoration of mis-splicing of Rbm20 target genes, including the primary target gene titin and other genes such as CaMKIIδ, Ryr2, and Ank3.

Conclusions: The findings of this study demonstrated that RNP granules serve as the primary driver for RBM20 cardiomyopathy, and reduction of RNP granules through ASO treatment is a therapeutic option for RBM20 cardiomyopathy in patients carrying Rbm20 genetic variants in NLS region.
Genomic Characterization of Patients with Advanced Heart Failure at the University of Kentucky

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In collaboration with the clinical teams at UKHealthCare, the Campbell lab has created a cardiac biobank that now contains more than 15,000 myocardial specimens from 520 human hearts. Most of the samples were acquired from patients who received Ventricular Assist Devices or Heart Transplants. Genotyping has not been part of standard clinical care for this patient population, but cardiomyopathy panels were acquired for a subset of 33 patients (6%) who were considered at high risk of familial disease.

To further characterize our myocardial repository, DNA was extracted from 350 specimens and sent for whole exome sequencing. The average patient age is 51 years, ranging from 18 to 81 years, and over 90% reported non-Hispanic or Latino ethnic origin. There is a 7:1 male-to-female ratio, and the most common diagnoses were forms of non-ischemic heart failure (51%).

Sequencing revealed an excess of 100,000 variants per specimen, categorized as single nucleotide polymorphisms (SNPs) or insertions-deletions (INDELs). Tertiary genomic analysis involved filtering for 91 cardiomyopathic variants and non-benign ClinVar classifications. 3,749 variants fit these criteria, containing 2,287 and 1,462 SNPs and INDELs, respectively. Approximately 99% of these variants are considered of unknown significance or have conflicting classifications of pathogenicity, while 40 have ClinVar classifications of “Likely Pathogenic” or “Pathogenic”. These 40 variants encompass 17 cardiomyopathic genes, with 43% (17) residing in the gene encoding titin. Notable genes within this subset of variants include lamin A/C (4), troponin T (2), desmoplakin (2), phospholamban (1), dystrophin (1), plakophilin (1), myosin heavy chain 7 (1), and BCL2-associated athanogene 3 (1).

Further analysis of this data will provide a genetic atlas representative of heart transplant recipients in central Kentucky. Additional biochemical and biophysical studies are underway. Our team are happy to share deidentified samples and deidentified clinical data with research groups who can use them to help develop better therapies for patients who have heart failure.
One Drug does not Fit All - HCM Mutations Differentially Impact the Inhibitory Effect of Mavacamten and Aficamten

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Hypertrophic cardiomyopathy (HCM), affecting more than 1 in 500 people worldwide, is primarily caused by missense mutations in the proteins of the cardiac sarcomere, >70% of which affect β-cardiac myosin and myosin-binding protein C. Several small-molecule effectors directly binding to myosin and modulating its activity are now being developed to treat HCM and other heart diseases. Remarkably, Mavacamten, a first in class myosin inhibitor was recently approved for patients with symptomatic obstructive HCM by U.S. FDA. However, over 700 HCM mutations throughout the sequence of myosin have been reported, and disease heterogeneity makes it unlikely that a unique drug can be used to treat all cases of HCM as mutations can impair drug binding sites. I will discuss results from 8 different pathogenic myosin mutations whose effect on the inhibition by Mavacamten and Aficamten (currently in phase 3 clinical trials for the potential treatment of HCM) were compared using actin-activated ATPase assays. Five of the eight HCM mutations weakened the inhibitory effect of mavacamten- a higher IC₅₀ value was observed for these mutations compared to WT protein – while the inhibition by Aficamten was not affected by any of the mutations studied here. I will discuss these results in the light of what is known about the binding pockets of these drugs and how these are directly and/or allosterically modulated by HCM mutations. These results highlight the importance of continuing our efforts to develop a diverse set of small-molecule effectors targeting myosin to treat HCM. Development of new therapies depends on critical functional studies of different HCM mutations.
Atrial fibrillation (afib) is a cardiac arrhythmia characterized by disorganized and rapid electrical activity in the atria, resulting in an irregular and often rapid heartbeat, disrupting the normal synchronization of atrial contractions. Afib is the most common cardiac arrhythmia, affecting 1 in 4 individuals aged >40 years, and is strongly associated with an increased risk of morbidity, including stroke and heart failure. Therapeutic approaches to treating afib have largely focused on electrical modulation and structurally isolating the abnormal rhythm, but this approach does not address the underlying pathology of the atria, and unsurprisingly, many patients have recurrence due to further progression of atrial remodeling. There is growing evidence that a proportion of afib cases result from an atrial cardiomyopathic process in which the fundamental contractile function of the myocytes is altered. Broad genetic testing of a cohort of patients with very early-onset afib in the setting of a structurally normal heart and normal ventricular function revealed many more cardiomyopathy-causing gene mutations, suggesting the important link between atrial cardiomyocyte function and afib. In particular, multiple GWAS and familial linkage analysis studies of genetic afib and sick sinus syndrome (SSS) have implicated MYH6 (α-MHC) and MYL4 (ELCa) mutations. However, there is a lack of knowledge on how mutations in the atrial sarcomere proteins lead to atrial cardiomyopathy and, eventually, afib. Our overarching hypothesis is that contractile dysfunction at the sarcomere level leads to atrial arrhythmia in a manner similar to ventricular cardiomyopathy, where mutant sarcomeric proteins cause altered atrial cardiomyocyte function and contractility. To gain biochemical insights into the underlying myopathy in afib, we first cloned, expressed, purified, and characterized various constructs of recombinant human α-cardiac myosin and its MYH6 mutants. We selected two MYH6 mutants implied in SSS/afib (R721W and E933Δ) that are homologous to MYH7 (β-cardiac myosin) mutant residues (R719W and E931Δ) that cause ventricular hypertrophic cardiomyopathy (HCM). We hypothesize that MYH6 mutations result in hypercontractility due to releasing myosin heads from the sequestered state, resulting in a hypercontractile sarcomere similar to what we previously observed in the β-MHC R719W mutation. We prepared the wild-type (WT) recombinant human α-cardiac sS1, 8hep and 25hep HMM with the R721W mutation and the 25hep HMM with the E933Δ mutation in the 25hep HMM. Our preliminary biochemical data with WT human α-cardiac sS1, 8hep and 25hep HMM demonstrated a significantly higher actin-activated ATPase activity than the respective β-cardiac myosin constructs. The α-8hep and 25hep HMM with the R721W mutation and the 25hep HMM with the E933Δ mutation also showed significantly higher actin-activated ATPase activities than WT. The actin-activated ATPase curves of 8hep and 25hep R721W HMM did not show the large difference in activity seen for WT 8hep and 25hep HMM, suggesting that the R721W mutation disrupted the autoinhibition of 25hep HMM, resulting in higher activity. In the mant-ATP single turnover assay, the 25hep HMM with R721W or E933Δ demonstrated a significantly higher DRX fraction (76% and 77%, respectively) than WT (39-46%), and a faster ATP turnover rate. These findings suggest that R721W and E933Δ mutations destabilize the sequestered state of α-cardiac myosin, which may open more heads available to interact with actin and enhance the enzymatic function of the α-cardiac myosin. Our results support the hypothesis that these familial afib/SSS mutations result in hypercontractility of the atrial sarcomere, forming the basis of the atrial cardiomyopathic process. Our future work involves further analysis of other atrial myosin mutations (including MYL4) and establishing further mechanistic insights into the underlying pathogenesis of afib.
Structural Insights into Cardiac Dysfunction: Cryo-EM Study of Human Cardiac Myosin Exhibits Lever Arm Dynamics in Dilated Cardiomyopathy

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Cardiac myosin is the molecular motor that converts chemical energy from ATP hydrolysis into mechanical force, which powers muscle contraction in the heart. Dilated (DCM) and hypertrophic (HCM) cardiomyopathy are characterized by distinct cardiac muscle dysfunction. DCM exhibits reduced ejection fraction and contractility due to decreased actin- myosin interactions, while HCM essentially demonstrates the opposite (increased ejection fraction, contractility, and acto-myosin interactions) The MYH7 gene encodes β-cardiac myosin heavy chain (M2β), crucial for contraction. Here, we are reporting the cryo-EM structure of a human cardiac myosin construct (S1), which consists of a motor domain, a bendable lever arm containing ELC and RLC, and a C-terminal GFP tag. We have analyzed S1 constructs including both WT and a DCM mutant (E525K). Additionally, we investigated the impact of drugs on the structure of S1 E525K, including the cardiac inhibitor Mavacamten (Mava) and activator Omecamtiv Mecarbil (OM), which provided prominent structural insights into the movement of the lever arm, while density for the RLC and GFP was missing, possibly due to flexibility. The obtained EM maps exhibit resolutions of 3.3 Å (WT), 3.3 Å (E525K), 3.2 Å (E525K + OM), and 3.0 Å (E525K + Mava), and show ADP and Pi bound at the active site. Both Mava and OM bind at the same allosteric binding site (Auguin D et al. bioRxiv 2023.11.15.567213), inducing a pre-powerstroke (PPS) state, which traps the hydrolysis products with a primed lever arm. Notably, in the S1 E525K mutant, a small shift in the ELC position (bending towards the motor domain) is observed when compared with S1 E525K with Mava and OM. Key residues at the allosteric binding site of Mava and OM include Y164, T167, D168, H666, P710, N711, R712, I713, R721, Y722, D717, L770, and E774. These structural studies of S1 constructs of cardiac myosin in solution provide important insights to elucidate the effects of DCM mutations and drugs.

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Cardiomyopathy Induced by Reduction of the Mitochondrial Protein MTCH2 Shifts Myofilament Protein Composition

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BACKGROUND: MTCH2 is an outer mitochondrial membrane protein that regulates protein insertion into the membrane. MTCH2 is reduced in human end-stage failing hearts, and we found Mtch2 to be reduced in a mouse model of heart failure. We therefore hypothesized that having less MTCH2 contributes to impaired heart function.

METHODS/RESULTS: To study the role of MTCH2 in cardiac function, we created cardiomyocyte-specific Mtch2 KO mice (cMtch2 KO). cMtch2 KO mice developed systolic dysfunction with age, which was accompanied by increased fibrosis and elevation of heart failure markers. Male cMtch2 KO mice developed reduced systolic function earlier than female cMtch2 KO mice and also exhibited altered body composition with less body fat than controls. We sought to identify any changes to the myofilament in young mice that might be predictive of future systolic dysfunction. At 12 weeks, male cMtch2 KO mice had increased transcript levels of MYBPC3, but significantly reduced cardiac myosin binding protein-C (cMyBP-C). Male cMtch2 KO mice also had increased levels of the regulatory phosphorylation sites pS272/S282/S302 cMyBP-C. As cMyBP-C negatively regulates myosin, we propose these molecular shifts compensate to maintain cardiac function, and that this compensation comes at high energetic cost. Consistent with Mybpc3 KO models, cells from male cMtch2 KO mice have a decreased resting sarcomere length. We next evaluated mitochondrial dysfunction in cMtch2 KO mice prior to the development of systolic dysfunction. Isolated cMtch2 KO cardiomyocytes from 12 week mice loaded with Mitotracker green and TMRE had reduced mitochondrial membrane potential compared to control. In the presence of glucose, oxygen consumption rates were reduced in cMtch2 KO heart tissue, and targeted metabolomics of cMtch2 KO ventricle exhibited an accumulation of palmitate, indicating cMtch2 KO hearts do not efficiently utilize glucose and fatty acids. Consistent with the functional assays, mass spectrometry of mitochondria revealed a downregulation of proteins associated with fatty-acid beta oxidation and pyruvate metabolism in the cMtch2 KO mitochondria. Finally, we found that young, pre-cardiomyopathic cMtch2 KO mice were sensitized to stress. When young cMtch2 KO mice were challenged with acute isoproterenol, compared to controls, hearts from cMtch2 KO mice failed to respond with physiological hypertrophy and animals could not maintain body mass.

CONCLUSIONS: MTCH2 is vital for maintaining normal mitochondrial function. Reduction of MTCH2 impairs mitochondrial function, sensitizing the heart to stress, and ultimately reducing global heart function with effects on body composition. To compensate, Mtch2-deficient hearts reduce cMyBP-C and increases its phosphorylation consistent with mitochondrial sarcomere crosstalk.
Mechanisms of Contractile Dysfunction Due to the β-MHC-R403Q Mutation in Both Porcine Ventricular Tissue and Human Induced Pluripotent Stem Cell Derived Cardiomyocytes

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The β-myosin heavy chain (β-MHC) R403Q mutation is one of the most extensively studied genetic mutations linked to Hypertrophic Cardiomyopathy (HCM). However, conflicting contractile kinetics between human myectomy samples and transgenic rabbits has motivated us to study the R403Q mutation in a novel porcine model as well as a human CRISPR/Cas9 engineered R403Q induced pluripotent stem cell (hiPSC) line differentiated to cardiomyocytes (hiPSC-CM). Isolated myofibril contractile kinetics from porcine tissue demonstrated the mutation results in slower force development ($k_{ACT}$, R403Q = 1.07s$^{-1}$ ± 0.06 vs WT = 1.43s$^{-1}$ ± 0.08) and early phase relaxation ($k_{REL,slow}$, R403Q = 0.31s$^{-1}$ ± 0.02 vs WT = 0.58s$^{-1}$ ± 0.05); indicating a reduced rate of cross-bridge detachment. Isolated myofibrils from day 45 hiPSC-CMs exhibited similarly altered contractile kinetics during force development ($k_{ACT}$, R403Q = 0.71s$^{-1}$ ± 0.07 vs WT = 0.78s$^{-1}$ ± 0.13) and the early phase relaxation ($k_{REL,slow}$, R403Q = 0.16s$^{-1}$ ± 0.03 vs WT = 0.24s$^{-1}$ ± 0.03). Myosin recruitment during maximal force generation was ~22.5% lower in both the porcine and hiPSC-CM myofibrils for the R403Q mutation compared to controls. The agreement in myofibril contraction and relaxation kinetics between porcine and hiPSC-CM myofibrils are in conflict with myofibril results collected from human myectomy tissue with the R403Q mutation (Belus et al., 2008, J Physiol). This suggests that the above myofibril kinetics are likely the early-stage developmental phenotype induced by the R403Q mutation, and that the myofibril level phenotype changes with disease progression. Interestingly, R403Q porcine tissue exhibited an elevated tension cost similar to previous reports using R403Q human myectomy tissue (Witjas-Paalberends et al., 2014, J Physiol) (36% and 65% increase, respectively), indicative of energetic inefficiency due to the mutation. Using single molecule tracking of fluorescent ATP, we were able to track individual ATP binding events with both spatial and temporal resolution within the sarcomere of relaxed porcine myofibrils. These results provide rates of ATP turnover attributed to myosins in the disordered-relaxed state (DRX) versus super-relaxed state (SRX) occurring within the P-, C-, and D-zones of the thick filament. Results from porcine myofibrils, suggest that the R403Q mutation pulls myosin heads out of the SRX state from the thick filament P- (%SRX, R403Q = 38.9% ± 0.59 vs WT = 63.8% ± 0.071) and D-zones (%SRX, R403Q = 36.7% ± 0.20 vs WT = 65.9% ± 0.025), but not in the C-zone (%SRX, R403Q = 48.4% ± 0.064 vs WT = 40.1% ± 0.058), which contains cardiac myosin binding protein C. In the presence of a myosin activator, dATP, ATP turnover was accelerated across all zones equally in WT porcine myofibrils, however, the impact of dATP was blunted in the R403Q porcine myofibrils, suggestive that the R403Q tissue is already in a state of accelerated ATP turnover at baseline. These biochemical measurements agree with structural X-ray diffraction results exhibiting greater myosin disorder in resting R403Q porcine cardiac tissue (pCa 9.0) with decreased intensities in the myosin-based reflections ($I_{MLL1} = 6.30$ vs 0.69, $I_M3 = 9.35$ vs 3.56). Using the porcine tissue model of this mutation, we are further investigating through stopped-flow biochemistry how decreases of both myosin recruitment and cross-bridge cycling rate results in elevated force at sub-maximal calcium levels (greater calcium sensitivity), energetic inefficiency, and biochemical activation of myosin ATPase rates. With the R403Q hiPSC-CM line, we aim to further validate our early-stage human disease model by probing the metabolic profile (Seahorse assay) and twitch force and kinetics (hiPSC-CMs cast into engineered heart tissue (EHT) constructs). Complete validation of this human R403Q hiPSC-CM disease model will allow us to probe therapeutic interventions that specifically target the mutation specific mechanisms described above.
Hypertrophic cardiomyopathy (HCM) is a genetic cardiac disorder frequently caused by mutations in sarcomeric genes that are responsible for contractility. It is the leading cause of sudden cardiac death in young adults, with an estimated prevalence of 0.2% (1 in 500 adults). HCM is typically characterized by cardiomyocyte hypertrophy, hypercontractility, and diastolic dysfunction. Over 1000 mutations have been found, with a third of the mutations being identified in β-myosin, the dominant myosin isoform in the mature human heart. The condition presents heterogeneously, both genetically and clinically, making it challenging to diagnose and therefore treat. Further, the altered mechanics of the heart’s mechanosensing machinery in HCM are poorly understood.

Costameres, which are rib-like structures aligned predominantly with z disks in cardiac muscle, are made up of a complex network of mechanosensitive proteins that are crucial for lateral force transmission and coordinated contraction of the heart muscle. One of these proteins is vinculin, which has historically been used as a distinguishing marker of costameres. It is a ubiquitously expressed protein that binds to talin and α-actinin, and functions as a linker between the extracellular matrix and the cytoskeleton. While mutations related to vinculin rarely cause hypertrophic cardiomyopathy, it has been found to be implicated in a missense mutation in obstructive hypertrophic cardiomyopathy. Its expression levels have also been found to be elevated in diseased myocardium tissue. These key findings suggest the involvement of vinculin in cardiomyopathies either directly through mutations, or indirectly as a compensatory mechanism for cardiac failure.

Here, we aim to understand how a point mutation from proline to arginine at the P710 residue (P710R) in β-cardiac myosin impacts force sensitivity by leveraging the structure and molecular dynamics of vinculin, along with Forster Resonance Energy Transfer (FRET). The P710 residue is located at the proximal edge of the converter domain, which is crucial for communication between the catalytic motor and the lever arm in myosins, and thus myosin’s force-producing power stroke. We investigate how this P710R mutation alters force dynamics at cell-cell and cell-matrix junctions and how pharmacologics affect these dynamics by studying vinculin’s force-sensitive responses. Structurally, vinculin has a globular head linked to a flexible tail by a proline-rich hinge region. It has been described as a “clutch” system, such that when engaged, the head binds to talin, while the tail binds to α-actinin, causing it to be in an open conformation. Here, we use a tunable vinculin tension-sensing probe that is flanked with two fluorophores such that when in a closed conformation, the fluorophores are close enough for energy transfer to occur, thus enabling a read-out of force dynamics. P710R hiPSC-CMs were micropatterned on polyacrylamide gel at 10 kPa, which closely mimics the physiological stiffness, to allow for the alignment of myofibrils. Our image-splitting optical system allows for high-speed ratio metric imaging for quantification of sensitive, dynamically changing protein interactions. Our FRET metrics suggest that when P710R cells are treated with mavacamten, a novel drug used to treat obstructive hypertrophic cardiomyopathy, force is reduced. This suggests that altered force generation in live micropatterned P710R hiPSC-CMs affects the tension-sensing dynamics of vinculin in the myocardium. Taken together, our findings suggest that vinculin is crucial for force stabilization in HCM, and understanding its interactions with cardiac myofilament as well as other costameric proteins could elucidate its role in HCM.
Familial dilated cardiomyopathy (DCM) is a genetic heart disorder characterized by enlargement of one or both ventricles, rendering the heart unable to pump blood efficiently. Mutations in cardiac thin filament (CTF) regulatory proteins have been associated with well characterized clinical manifestations of DCM. Genetic segregation identified a mutation in α-Tropomyosin (Tm) at residue 230 (D230N-Tm) in two unrelated, multigenerational families linked to severe, early onset DCM. To meaningfully link genotype to phenotype our group sought to investigate the effects of D230N-Tm on the biophysical structure of Tm filament. We showed that D230N-Tm caused a compaction of the cardiac Troponin T (cTnT)–Tm overlap region accompanied by a decreased in flexibility of the Tm filament. Given the altered biophysical structure we investigated whether we could target this decrease in flexibility via small molecule intervention and improve function. Computational modeling and biophysical experiments identified small molecule Z06 that was shown to improve compaction of the cTnT-Tm overlap and restore flexibility of the Tm filament. We hypothesized that the decrease in flexibility of Tm impairs its ability to shift through its different conformational states, leading to impaired crossbridge formation. To investigate whether Z06 improves myofilament function, we performed an NADH-coupled ATPase assay using isolated ventricular myofibrils from our D230N-Tm transgenic mice and compared them to Non-Transgenic (NTg) littermates. ATPase activity was measured at saturating calcium (100uM) in the presence (250uM) and absence (0uM) of Z06. In the absence of Z06, D230N-Tm exhibited a decrease in ATPase activity (1.793±0.203 umol/min) compared to NTg littermates (5.420±0.562 umol/min), a common trend in DCM models. In the presence of 250uM Z06, D230N-Tm ATPase rates improve to levels comparable to NTg mice (4.816±0.544 umol/min). Moving forward, we will perform calcium titrations, allowing us to investigate if Z06 can restore cooperativity of myofilament activation. Additionally, we will test if the restorative effects of Z06 are present at the myocellular level, and if so, we will optimize delivery to test in vivo effects of Z06 in our D230N-Tm DCM mouse model.
Screening Single Nucleotide Changes to Tropomyosin and Troponin-I to Identify Novel Cardiomyopathy Mutants

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Hypertrophic and dilated cardiomyopathy (HCM and DCM) are conditions characterized by pathological remodeling of the heart; in HCM the ventricle becomes thickened whereas in DCM the ventricle becomes enlarged with thin walls. In many affected patients, mutations in their genetic sequence can be found in sarcomeric proteins such as tropomyosin, troponin, or myosin, all proteins involved in the molecular mechanism and regulation of contraction in cardiac muscle. These mutation-induced alterations can lead to either hypercontraction or hypocontraction. While the heart can compensate over the short term for these alterations, over time HCM or DCM remodeling results. The pathways connecting the initial mutational insult to the contractile properties of the heart to the eventual remodeling are not clear. Understanding these pathways may lead to the development of therapies targeting the early stage of cardiomyopathy that will avoid later complications in patients; therefore, there is a need for good model systems to study. Here, we develop a method for quickly screening mutations in tropomyosin and troponin-I using energy minimization calculations to find mutants predicted to have the large effects on regulation. The screened list of mutants includes not only clinically relevant HCM and DCM mutations from the ClinVar database, but also hypothetical mutants that could arise from single nucleotide sequence alterations to the tropomyosin or troponin-I genes. These mutations are incorporated into thin filament models containing tropomyosin, troponin, myosin, and actin in three regulatory states – the blocked, B-state associated with relaxed muscle, the closed, calcium-bound C-state associated with activated muscle, and the myosin-bound open, M-state associated with fully active thin filaments generating force. After minimization, the impact of the mutation on contractile activity is predicted based on its effect on the interaction energies between tropomyosin, troponin-I, actin, and myosin. The mutations that have the largest energetic effects are then further investigated using molecular dynamics simulations to quantify any alterations to thin filament structure. Using this methodology, we have discovered a number of novel mutants in tropomyosin and troponin-I that are predicted to cause large changes in contractile regulation likely leading to hypercontraction or hypocontraction and represent good candidates for analysis in in vitro motility assays and engineered heart tissues to examine the early phases of HCM and DCM, while the structural impact of the mutants will be determined by cryoelectron microscopy. This work is supported by National Institutes of Health grants R01HL036153 (to W. Lehman) and R01HL136590 (to S. Campbell).
MYBPC3 CHD-Linked Mutation Manifests as Hypercontractile Phenotype with Faster Relaxation Kinetics in Human Engineered Heart Tissues

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Congenital Heart Disease (CHD) refers to a variety of cardiac structural abnormalities and is the most common birth defect in humans. A subset of CHD, single ventricle defects (SVD) cause heart failure in 30% of surviving adults. Although mutations to sarcomeric proteins are often associated with adult-onset cardiomyopathies, there is increasing evidence of their ability to cause CHD. Among them, a homozygous missense variant of the MYBPC3 gene, MYBPC3\textsuperscript{F1183L}, was recently identified in SVD patients through exome screening. Despite clues this and other mutations could offer about CHD mechanisms, no study has yet examined their phenotypic effects on muscle contraction.

In this work, we leverage human induced-pluripotent stem cells (hiPSC), CRISPR/Cas9, and tissue engineering to explore the mechanobiology of the MYBPC3\textsuperscript{F1183L} CHD-linked mutation. An isogenic (WT) control hiPSC line and one carrying the MYBPC3\textsuperscript{F1183L} homozygous genotype are differentiated into cardiomyocytes and seeded onto decellularized porcine myocardium scaffolds, forming anisotropic engineered heart tissues (EHTs). After three weeks of isometric culture, EHTs are tested on a custom setup, where each tissue is connected to a force transducer and micromanipulators inside a temperature-controlled Tyrode’s bath with electrical pacing. Tissue contractile behavior is measured at various pacing frequencies and tissue lengths.

For MYBPC3\textsuperscript{F1183L}, results show increased force production both at culture length and during progressive stretching compared to WT, displaying a length-dependent activation (LDA) typical of the hypercontractile phenotype observed in tissues carrying adult-onset hypertrophic cardiomyopathy genes. Specifically, the LDA slope is steeper in the case of mutant tissues. Similarly, we detect a higher passive stiffness indicating a potential elevated resistance to diastolic filling. Surprisingly, the twitch relaxation time is shortened, while the contraction time (from stimulus to peak) does not show any significant difference from WT. Western blots show the cardiac myosin binding protein C is under expressed in F1183L samples compared to WT. Taken together, these results suggest a potential mechanistic link between MYBPC3\textsuperscript{F1183L} and the pathogenesis of SVD, emphasizing the role of sarcomeric mutations in altered contractility, stiffness, and relaxation dynamics in CHD.
Protein Tyrosine Phosphatase 1B mediates Length Dependent Activation via Glycogen Synthase Kinase 3β

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Phosphorylation of glycogen synthase kinase 3β (GSK-3β) at Y216 drives it to the sarcomere z-disc where it alters myofilament function. Sarcomere-localized GSK-3β is suppressed in human heart failure and mouse models of myocardial infarction, resulting in loss of Length-Dependent Activation. Thus, identifying the mechanisms that regulate phosphorylation of Y216 and GSK-3β localization are attractive therapeutic targets. To better understand the function of pY216, we utilized CRISPR-Cas9 to generate a phospho-null Y216F mouse model of altered sarcomeric GSK-3β localization with preserved expression. However, mice with the heterozygous mutation exhibited ~40% reduction in GSK-3β levels. Furthermore, the homozygous mutation is embryonic lethal, similar to a constitutive GSK-3β KO, suggesting Y216 phosphorylation is essential for either protein translation or stability. To test whether Y216 phosphorylation impacted stability, neonatal rat ventricular myocytes (NRVMs) transduced with Myc-tagged WT or Y216F GSK-3β adenovirus, treated with cycloheximide, and measured decay of exogenous GSK-3β via western blot. The Y216F mutant was as stable as WT GSK-3β. Likewise, RT-qPCR confirmed no changes in transcription. This, in tandem with other evidence that Y216 is auto-phosphorylated cotranslationally, suggests that post-translationally, pY216 is regulated by a phosphatase. While alkaline phosphatases are unable to dephosphorylate Y216, incubation of homogenized mouse left ventricle with recombinant active protein tyrosine phosphatase 1B (PTP1B) showed specific de-phosphorylation of Y216, pinpointing it as a potential candidate. Furthermore, evidence that PTP1B is elevated under stress is supported by RNA-sequencing data from human heart failure samples, demonstrating an upregulation of PTP1B in a pattern that would drive GSK-3β away from the sarcomere. Additionally, constitutive cardiomyocyte-specific PTP1B knockout mice exhibit altered Y216 phosphorylation and sarcomere localization of GSK-3β. Together, these results establish new evidence for potential mechanisms of sarcomeric GSK-3β localization, providing potential therapeutic strategies for heart failure.
Titin underpins the history-dependent properties of residual force enhancement, residual force depression, and the stretch-shortening cycling effect.

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Skeletal muscle active tension is dependent on its history. When activated isometrically, tension can be deduced based on the sarcomeric tension-length relationship, where force is proportional to the overlap of the myosin-containing thick filaments and actin-containing thin filaments. However, when muscle is activated and then subject to a length change, the forces no longer follow the tension-length relationship. Contrary to expectations, an active stretch-hold leads to increased tension (residual force enhancement), active shorten-hold leads to less tension (residual force depression), and an active stretch-shorten-hold leads to less tension but not at the level of a pure shortening-hold (stretch-shortening cycle effect). The I-band titin spring is thought to be the primary driver of these history-dependent effects, hypothesized to be through a change in titin-based stiffness upon activation, although the mechanism remains elusive. Using small angle X-ray diffraction in combination with a novel mouse model that specifically cleaves 50% of I-band titin, we evaluated the relationship between titin-based force, history-dependent properties, and sarcomere protein order and orientation. Our results demonstrate that cleaving 50% of I-band titin reduces the history-dependent tension effects towards those expected under purely isometric conditions. Furthermore, each of the isometric or length-change conditions had unique structural signatures that help to explain differences in force, which seem to be more related to changes in titin-based tension on the sarcomere and force transmission, and less dependent on changes to cross bridge kinetics. Furthermore, our results demonstrate that an activation-dependent change in titin-based stiffness is likely but the mechanism that accounts for this remains to be deduced.
Stretch Activation Combats Decreased Muscle Force Production from Fatigue 
In Mouse Type II Fibers

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Stretch activation (SA) is a delayed increase in muscle force following a rapid stretch and
improves muscle performance during repetitive cyclical contractions in insect flight and cardiac
muscles. Although historically considered too low to be physiologically relevant in skeletal muscle,
our recent work showed that higher phosphate concentrations ([Pi]) increased SA 4-fold in mouse
soleus muscles fibers (Straight, et al., 2019). These results suggested SA has a role combating
muscle fatigue, which increases [Pi], lowers pH and reduces activating calcium concentration
([Ca²⁺]). To test this new idea, we measured SA during Control (pCa 4.5, pH 7.0, 5 mM Pi), High
[Ca²⁺] Fatigue (pCa 4.5, pH 6.2, 30 mM Pi) and Low [Ca²⁺] Fatigue (pCa 5.1, pH 6.2, 30 mM Pi)
conditions by inducing the SA response in myosin heavy chain (MHC) type I and IIA fibers from
mouse soleus muscles and IIX and IIB fibers from EDL muscles. SA force (FSA) in myosin heavy
chain type IIA fibers from mouse soleus muscles increased 46% under High [Ca²⁺] Fatigue
conditions and was unchanged under Low [Ca²⁺] Fatigue conditions compared to Control
conditions. FSA of Type IIX and IIB fibers from EDL were unchanged under both fatigue conditions
compared to Control conditions. We found that calcium-activated isometric force (F₀) decreased
going from Active to High [Ca²⁺] Fatigue and further decreased from High [Ca²⁺] Fatigue to Low
[Ca²⁺] Fatigue in all type II fibers. Combined with our FSA measurements, this means that stretch
activation’s percent contribution to total muscle force production (FSA/(F₀ + FSA)) is much greater
under fatigue than control conditions. FSA’s contribution to total force production under
fatiguing conditions ranged from a 58% increase to a 114% increase depending on the
MHC II isoform and fatigue conditions. Interestingly, FSA from soleus type I fibers was 80%
lower than solus type II fibers under control conditions and the SA peak (phase 3 peak) was not
visibly apparent under either fatigue condition. Comparing the timing of the stretch activation
response of Type II fibers with in vivo mouse soleus and EDL muscle length change and activation
patterns suggests the delayed SA force increase would occur during in vivo shortening thus
increasing work and power. These results show SA improves force production under
fatiguing conditions in MHC type II fibers. This could play an important role in increasing
muscle endurance for muscles that are lengthened prior to shortening by supplementing
calcium-activated force production.

Further analysis of fibers from our previous stretch activation experiments with mouse soleus
fibers’ FSA values at different [Pi]s (Straight, et al., 2019) revealed that only MHC type IIA fibers’
FSA increased with Pi while type I FSA was unchanged. Type IIB EDL fiber FSA values decreased
with increasing [Pi]. To explain the [Pi] and the fatigue experimental results, and our previous
Drosophila myosin versus [Pi] findings (Zhao, et al., 2013), we propose two myosin-based
mechanisms, the main difference being that for some myosin isoforms and conditions,
stretch causes a reversal of the myosin power-stroke allowing for a subsequent “second”
power-stroke, while in other cases, stretch forcibly detaches myosin from actin in a post-power
stroke state.

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Influence of hERG channel structure on its function and membrane trafficking: Insights from molecular dynamics simulations

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The human Ether-à-go-go-Related Gene (hERG) channel is a voltage gated potassium channel whose function is crucial for cardiac repolarization. Many instances of Long QT syndrome 2 (LQT2), which is associated with arrhythmias, are caused by hERG mutations. 88% of LQTS-causing mutations have a trafficking-deficient phenotypes, but there are a few mutations identified with trafficking-correcting properties. To uncover the mechanistic basis of this differential behavior, we developed atomistic models of the wild-type hERG, along with six single mutants and five double mutants, each involving either a trafficking-deficient mutation, a trafficking-competent one, a trafficking-correcting one, or a combination of two different types of mutations. The Molecular Dynamics simulations performed on our models shed light on the main structural differences in the pore domain of the trafficking competent and the trafficking deficient hERG mutants. Based on these data, we propose that improper folding of the pore domain induced by non-trafficking variants interferes with normal trafficking. These findings may provide a mechanistic basis for assessing the trafficking phenotype of novel hERG mutants.
Mechanisms of BAG3 Regulation in Ischemia-Reperfusion Injury

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The co-chaperone BAG3 is critical for protein quality control at the cardiac sarcomere. BAG3 binds to Hsp70 and coordinates the assembly of the CASA (chaperone-assisted selective autophagy) complex, thus supporting protein homeostasis and cardiomyocyte contractility. Decreased BAG3 levels are associated with heart disease, whereas BAG3 overexpression rescues ventricular function in animal models of heart failure (HF). Despite BAG3's potential as a therapeutic target, the mechanisms underlying BAG3 regulation are largely unresolved. Here, we investigate the mechanisms of BAG3 downregulation after stress. We found that BAG3 protein is reduced in dilated cardiomyopathy (DCM) compared to non-failing hearts, yet there is an increase in bag3 mRNA transcript, suggesting BAG3's downregulation in heart disease may be controlled post-transcriptionally. To identify these post-transcriptional pathways, we subjected neonatal rat ventricular myocytes (NRVMs) to prolonged hypoxia-reoxygenation (H/R) stress, which recapitulated the decrease in BAG3 levels observed in human heart disease. Notably, disrupting Hsp70 binding to BAG3 in NRVMs via the drug JG-98 decreased BAG3's half-life by ~90%, suggesting that Hsp70 protects BAG3 from degradation. Loss of Hsp70-mediated protection could contribute to declining BAG3 levels, so we quantified Hsp70 abundance after H/R stress in NRVMs, finding no significant change. We also found that overexpressing inducible Hsp70 did not rescue BAG3 levels. This data suggests that BAG3 downregulation in H/R stress is not caused by loss of Hsp70 binding/protection. To examine BAG3 regulation in vivo, we subjected wildtype mice to ischemia-reperfusion injury. After 24 hours, we observed a decrease in BAG3 levels in the left ventricle and no significant change in Hsp70 abundance. Interestingly, the decline in BAG3 was accompanied by an increase in BAG3 cleavage, which will be explored as a potential mechanism of BAG3 loss in the future. Such mechanisms will provide insight into the maintenance of BAG3 levels, and thus cardiac function, during chronic stress.
**Ca2+ increases the viscous component of the passive response of cardiac muscle to stretch**  
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Recent studies reported that electrical stimulation of skeletal muscle resulted in an increase of muscle stiffness that was independent of active contraction.

The goal of this study was to determine if Ca2+-activation of cardiac muscle increased passive muscle stiffness, independent of active contraction. The passive mechanical response of mouse demembranated cardiac trabeculae to stretch was probed at different calcium levels by inhibiting active contraction using the myosin ATPase inhibitor para-nitroblebbistatin (PNB). Myocardial stiffness was assessed by muscle stretches (> 20% initial length) using stretch velocities varying over three orders of magnitude.

In response to stretch, muscle force rose to a peak and then relaxed toward a lower steadystate level, consistent with the viscoelastic nature of cardiac muscle. Peak force was higher with faster stretch velocity, but the steady-state force was independent of stretch velocity, consistent with the presence of both apparent viscous and elastic components of the stretch response. The major finding of this study was that with active contraction inhibited by PNB, Ca2+ increased the viscous force response to stretch by > 3-fold compared to the response measured under relaxed (low Ca2+) conditions. Moreover, there was a sigmoidal relationship between increased viscous force versus Ca2+ level, consistent with a regulated response.

To comprehensively analyze these mechanical phenomena, we developed an ensemble model of titin elastic domain mechanics that accounts for the dynamic unfolding of globular domains along the titin chain. This model simulates the observed effects of stretch on cardiac muscle stress: i) stress relaxation; ii) sensitivity to stretch velocity; and iii) sensitivity to Ca2+. For cardiac muscle, Ca2+-activation of a viscous mechanical phenomenon may be an important determinant of both systolic and diastolic properties.
Titin’s P-zone segment has muscle-specific roles in thick filament alignment and force generation

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Titin is a giant, filamentous protein that spans half of the sarcomere, the smallest contractile unit of striated muscle. Titin’s A-band segment is part of the myosin-based thick filament and is essential to sarcomere function through roles in thick filament length regulation, myosin activation, and mechanosensitive signaling. The A-band segment contains multiple sub-segments; herein we focus on dissecting the structural and mechanical properties of the poorly understood P-zone (domains A164-170). The P-zone is located near the edge of the bare zone of the thick filament and links titin’s M-band segment to its C-zone segment, where it may support alignment of the remainder of titin’s A-band with myosin and myosin binding protein-C (MyBP-C). We probed the contribution of titin’s P-zone to A-band regulatory function by deleting its first four domains, A164-167, in a mouse model (Ttn\(^{\Delta A164-167}\)). SDS-AGE indicates that total titin levels are unchanged in Ttn\(^{\Delta A164-167}\) mice, but homozygous mice express a second N2A titin in skeletal muscle. We found that although skeletal muscle weights are unaffected by the P-zone segment deletion, cardiac muscle weights were significantly increased, indicating hypertrophy. Echocardiography studies indicated mild diastolic dysfunction. Front-limb grip strength assays indicate that homozygous Ttn\(^{\Delta A164-167}\) mice are weaker than littermates, and whole-muscle mechanics assays show decreased force generation and altered contraction kinetics of EDL, but not soleus muscles. We followed these functional experiments with immuno-electron microscopy and immunofluorescence with super-resolution microscopy (SIM) to correlate structural changes to functional findings. We measured thick filament length and location of MyBP-C stripes to determine if deleting domains A164-167 has consequences on the alignment of the titin-myosin-MyBP-C complex. We propose that titin’s P-zone facilitates alignment between myosin and MyBP-C and thereby plays a role in muscle contraction.
Deletion of titin’s Z-repeats maintains sarcomere structure but increases eccentric contraction induced muscle damage.

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Titin contains 7 Z-repeats (Zr) encoded by exons 8-14 that exist within the Z-disc region of the sarcomere. All striated muscle types express Zr 1-3 and 7, while 4, 5, and 6 are differentially expressed in cardiac and slow skeletal muscle, with only adult hearts expressing all 7. The previously reported correlation between the number of expressed Zr and the thickness of the Z-disc in different muscle types has given rise to the view that titin serves as a ruler for determining Z-disc width. To test this, a mouse model was created with the complete deletion of the Zr region (Ttn^ΔZr1-7). Ttn^ΔZr1-7 mice are born in mendelian ratios, survive to adulthood and are visually indistinguishable from WT littermates. The ultrastructure of the sarcomere appears normal in both cardiac and skeletal muscle of Hom Ttn^ΔZr1-7 mice, with only a modest ~10 nm reduction in Z-disc width in the soleus, a predominantly slow muscle. With an estimated Zr length of 12nm, the reduction in Z-disc width is far smaller than anticipated. Despite normal appearance, the soleus produces less maximal isometric force compared to WT and experiments with permeabilized single fibers indicate that this attenuation is limited to Type 1 fibers. Furthermore, both fast (EDL) and slow (soleus) skeletal muscles are more susceptible to force loss from repeated eccentric contractions, with the effect being far more severe in the soleus, which is accompanied by substantial Z-disc and I-band disruption. Our findings suggest that titin’s Zr play a minimal role in Z-disc width regulation, but when mechanical stresses are high, Zr are critically important for providing integrity to the sarcomere.
**Transition kinetics between OFF and ON states of titin upon stimulation of skeletal muscle depends on temperature as expected from the Ca$^{2+}$ transient**

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A transition of I-band titin from an OFF state characterized by a large SL-dependent extensibility to an ON state characterized by a high, SL-independent stiffness ($\geq 3$ pN/nm per half-thick filament, htf) has been demonstrated in single fibers of frog skeletal muscle stimulated in the presence of 20 $\mu$M of the myosin inhibitor paranitroblebbistatin (PNB) to suppress contraction (Squarci et al. 2023, PNAS, 120:e2219346120). The transition has been attributed to a Ca$^{2+}$-dependent process that, by promoting titin-actin interaction, removes the contribution of the proximal tandem Ig segment to the compliance of I-band titin, making it an efficient coupler for stress transmission to A-band titin and myosin motors also at physiological SL. Titin structural dynamics associated to stimulation could play a role in the transition of myosin motors from the helically ordered, folded (OFF) state to the disordered (ON) state available for actin attachment (Dutta et al. 2023, Nature, 623:853-862; Tamborrini et al. 2023, Nature, 623:863-871). In this view, it is compelling to establish the kinetics of titin switching ON following the start of electrical stimulation. To this end we exploited the PNB-single fiber approach to extend the I-band titin stiffness analysis to the high frequencies domain by imposing small 4 kHz length oscillations both at rest and during stimulation. The distortion of the elastic response present at this frequency due to the propagation time of the perturbation along the fiber was minimized by using the striation follower to record the half-sarcomere length changes in a population of sarcomeres close to the force transducer end. The elastic modulus gained from the 4kHz oscillations analysis, an estimate of the quasi-instantaneous stiffness ($e_1$), increases following the start of stimulation attaining a plateau value ($\sim 13$ pN/nm per htf) which is independent of temperature (range 4-14 °C). The time course of $e_1$ increase is characterized by a latency following the start of stimulation ($t_{L,ON}$) that is 9.3 ms at 4 °C and threefold shorter at 14 °C. $t_{L,ON}$ and its temperature dependence are similar to the time taken by Ca$^{2+}$ to rise to its maximum value following the start of stimulation (Miledi et al. 1982, J Physiol, 333:655-679; Caputo et al. 1994, J Physiol, 478:137-148; Sun et al. 1996, Exp Physiol, 81:711-724). We conclude that switching ON of titin is a Ca$^{2+}$-dependent process and is fast enough to drive thick filament activation. Supported by Ente CRF and MUR (PNRR-PRIN2022), Italy.
Titin N2B deficient human engineered heart tissue as a tool to dissect titin mechanics and mechanotransduction

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Titin, a giant sarcomere protein and plays a central role in regulating the passive stiffness of muscle. Its cardiac isoform specific N2B region acts as a molecular spring. It interacts with various partners in a stretch-dependent manner and links to hypertrophy signaling via FHL2. The exact mechanism by which the N2B region responds to cyclic stretch to adapt cardiac mechanics and growth is still largely unknown. Studies with N2B knock-out (N2B KO) mice have shown that these mice have stiffer, atrophic hearts. We are currently investigating titin N2B deficiency in a 3D human iPSC-derived model and analyze molecular, structural and functional similarities and differences between species. To this end, we used CRISPR/Cas9 gene editing to generate N2B-deficient hiPSC and derived cardiomyocytes (hiPSC-CMs) and confirmed their normal growth, differentiation, and morphology. To measure the contractility of mature N2B KO hiPSC-CMs, we generated human engineered heart tissue (EHTs). Interestingly, the N2B KO EHTs generated more force than the isogenic control EHTs. Both relaxation and contraction velocity were increased in N2B KO EHTs compared to isogenic controls. To investigate N2B based mechanotransduction, we use afterload enhancement in the N2B KO EHT, which recapitulates pressure overload after transaortic constriction. Strikingly, N2B KO EHTs produced robust contractions during intervention and generated significant force compared to wild type. Transcriptomic data of N2B KO EHTs indicate significant regulation of extracellular matrix and molecular transducer activity as the mechanistic basis for the higher force production and increased tensile strength. Our work should provide valuable insights into the role of the human titin-N2B region in cardiac mechanics and growth and potentially pave the way for new therapeutic strategies in both systolic and diastolic dysfunction.
Hearts may grow eccentrically and concentrically to stabilize titin-based stress and ATP concentration respectively

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Cardiac growth is fundamental to clinical care. Cardiologists use physical exams and non-invasive imaging including, for example, echocardiography, to assess the shape, size, and function of a patient's heart and to check for the progression of disease. Doctors know that specific conditions produce distinct patterns of eccentric (chamber dilation / constriction) and concentric (wall thickening / thinning) growth but there is no single quantitative explanation as to why different perturbations cause hearts to evolve in distinct ways in common clinical conditions.

We have created a multiscale computer model called MyoVent that spans from molecular to organ-level function, that predicts realistic Wiggers diagrams and pressure-volume loops, and can regulate arterial pressure via baroreflex control. Early versions of the model simulated sarcomere-level contraction using a Huxley-type distribution-based model called MyoVent. This has been upgraded in the current work to a spatially-explicit model called FiberSim. One important advantage of the new approach is a simpler way of simulating the different potential functions of myosin binding protein-C.

The working hypotheses are that myocytes grow (1) eccentrically (adding / removing sarcomeres in series) to normalize passive forces transmitted through titin to the Z-disc, and (2) concentrically (adding / removing myofibrils in parallel) to stabilize the intracellular concentration of ATP.

Pilot simulations demonstrate that, working synergistically, these growth mechanisms reproduce structural responses that mimic diverse conditions including, but not restricted, to hypertension, aortic valve stenosis, mitral valve insufficiency, and mitochondrial dysfunction.

Additional calculations show that the heart thickens if the myosin OFF / super-relaxed / interacting heads motif state becomes destabilized. This might reflect mutations to myosin binding protein-C. Thick hearts return to a normal size if the suppressed myosin state is repopulated as may happen in patients treated with the myotrope mavacamten.

These simulations help to extend myofilament-level mechanisms beyond the sarcomeres and might represent a useful step towards using computer models to help optimize therapies for patients who have heart failure.
Role of mitochondria-ER Ca\textsuperscript{2+} crosstalk in cell contraction

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As an energy production compartment, mitochondria sustain numerous cellular processes and use Ca\textsuperscript{2+} signaling as a regulatory mechanism. The main energy source of mitochondria Ca\textsuperscript{2+} is ER. While the importance of mitochondria-ER Ca\textsuperscript{2+} crosstalk is well known, its regulatory role in cell contraction remains understudied. We hypothesize that the mitochondria Ca\textsuperscript{2+} handling with ER allows localized Ca\textsuperscript{2+} signaling that activates calmodulin (CaM) and myosin light chain kinase (MLCK) that subsequently results in cell contraction. In this work, we performed a hybrid experimental/computational study of the role of mitochondria-ER Ca\textsuperscript{2+} crosstalk in regulating cell contraction. Experimentally, we leveraged Ca\textsuperscript{2+} imaging and traction force microscopy to understand the kinetics of mitochondria-ER Ca\textsuperscript{2+} communication and how this communication is related to cell contraction. Computationally, we use the experimental data to inform our systems model that models the Ca\textsuperscript{2+}-CaM-MLCK-contraction pathway. Interestingly we found that the onset of mitochondria Ca\textsuperscript{2+} uptake in response to extracellular ATP lags the corresponding ER Ca\textsuperscript{2+} release. This is consistent with the thresholding and cooperativity mechanism of the mitochondria Ca\textsuperscript{2+} uniporter (MCU). We therefore incorporated this kinetic aspect into our systems model. We also investigated the Ca\textsuperscript{2+} dependence through looking at effects of CaM variants that influence MLCK activation. These results provide insight into future therapy development for disease associated with cell contraction such as pulmonary arterial hypertension.
Troponin I serine 150 phosphorylation as a novel cardiac inotrope without detrimental effects

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Heart failure with systolic dysfunction is characterized by reduced contractility, resulting in an insufficient volume of blood pumped to the body. Current guidelines of care for heart failure treat symptoms, however there are currently no approved therapies to increase contractility and directly improve the ability of the heart to pump blood. Previously tested positive inotropes increased heart function through mechanisms that also increased intracellular calcium. Unfortunately, these early inotropes were associated with detrimental effects and worsened outcomes and therefore are not approved for long-term use. There remains a need for an alternative mechanism to increase contractility without increasing intracellular calcium. We previously demonstrated that phosphorylation of the inhibitory subunit of the troponin complex, troponin I (TnI) at serine residue 150 (S150) increases force development in ex vivo muscle by increasing calcium sensitivity. Increasing the sensitivity of the myofilament to calcium is an alternative mechanism to increase contractility without increasing intracellular calcium. We therefore hypothesize that increasing TnI-S150 phosphorylation in vivo would improve systolic function without harmful effects. To determine the effects of TnI-S150 phosphorylation in vivo, we generated a phosphorylation-mimetic mouse with TnI-S150 mutated to aspartic acid (TnI-pS150). Structural and functional measurements derived from echocardiography and hemodynamics demonstrate that TnI-pS150 mice have increased cardiac systolic function and contractility in vivo. We confirm that the mechanism for increasing in vivo function is through increased myofilament calcium sensitivity. Detrimental effects commonly observed with the use of inotropes (e.g. hypertrophy, hypertension, severe diastolic dysfunction, exercise intolerance, increased arrhythmia susceptibility, increased mortality) were not observed in TnI-pS150 mice. Additionally, we did not observe any adverse long-term detrimental effects on cardiac structure and function in aged TnI-pS150 mice. These results support the phosphorylation of TnI-S150 as a novel signaling mechanism to increase in vivo systolic function without detrimental effects and is therefore a novel target for systolic heart failure therapies.
Spatially-Explicit Simulations Predict How Different Modes of MyBP-C Function Modulate Isometric Twitches

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Multiple groups are trying to develop sarcomere-based therapies for Heart Failure with reduced Ejection Fraction. Trials that attempted to activate myosin with omecamtiv mecarbil showed minimal benefit, in part because the increased contraction compromised diastole. Cardiac Myosin Binding Protein-C (cMyBP-C) regulates both contraction and relaxation under physiological conditions and could be a more effective therapeutic target. However, cMyBP-C’s complex function makes it difficult to study in biological experiments. Here we used FiberSim, a spatially-explicit model of half-sarcomeres (https://campbell-muscle-lab.github.io/FiberSim/) to investigate how different potential modes of cMyBP-C function modulate contractile properties. In the model, cMyBP-C molecules are restricted to 9 stripes in the C-zone of the half-sarcomere where they have the appropriate stoichiometry (3 cMyBP-C molecules to 18 myosin molecular per 43 nm thick filament repeat). The cMyBP-C molecules can transition between a null state (no effect) and two states that respectively stabilize myosin in its suppressed super-relaxed / interacting heads motif / OFF configuration or bind to available sites on the thin filament. cMyBP-C molecules that are bound to actin increase thin filament activation via cooperative effects.

As shown in Fig 1, the time-course of isometric twitch contractions is prolonged when cMyBP-C molecules bind to the thin filament. Peak force is reduced when cMyBP-C stabilizes the SRX state. Simulations in which some cMyBP-C molecules bind actin and some stabilize myosin in the suppressed state have smaller slower twitches.

Ongoing work is testing how cMyBP-C modulates afterloaded twitches that may be more representative of myocardial function in vivo.

Categorie: Models of Contraction/Regulation

Figure 1: FiberSim-based simulations of isometric Twitches.
Identifying underlying mechanisms and therapeutic targets in muscle disease using Bayesian parameter estimation with conditional variational autoencoders

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Both genetic and acquired muscle disorders can interrupt the complex chemo-mechanical events of contractions. Computational muscle models can enable researchers to connect estimates of state transition rates to observed mechanical behaviors of both healthy and abnormal muscle contraction. These approaches generally fall into the category of forward models -- that is they generate predictions of muscle contractile behavior based on the best possible estimates of the kinetics of state transitions as well as the mechanics and geometry of the contractile lattice. However, it can be difficult to know which interventions in abnormal muscle may be useful to restore healthy function. This is an example of an inverse problem, one in which we have a target -- healthy muscle behavior -- and want to know what and want to know which state's rate functions to modify to restore force. Inverse problems are difficult due to noise inherent in stochastic models, and the under-determined nature of high dimensional problems. Advances in machine learning techniques can assist us in quantifying our uncertainty in the relationship between parameters and data when fitting using Bayesian techniques. We chose 9 rate parameters in our previously published spatially explicit sarcomere model which can be associated with small molecules or mutations causing cardiomyopathies. We then randomly varied these rate parameters and simulated an isometric twitch for each combination to generate a large training dataset. We used this training dataset to train a Conditional Variational Autoencoder (CVAE), a technique used in Bayesian parameter estimation. Given a simulated or experimental isometric twitch, this machine learning model can accurately estimate the probability distribution over rate space which corresponds to a particular isometric twitch. We then predict the set of rate parameters associated with both control and the cardiac Troponin C (cTnC) I61Q variant in mouse trabeculae, a hypocontractile variant known to cause dilated cardiomyopathy. We found that rate constants for the calcium thin filament on and off rates are among the most divergent between the two twitches, which is consistent with the known experimental data of the I61Q cTnT variant. This technique may help in estimating therapeutic targets and hypothesis generation of mechanisms stemming from disease-causing mutations and the myofilament level.
Characterization of the Cardiac Troponin I S150D mouse model

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Abstract: Cardiac function can be modulated by post translational modification of the myofilament proteins. Troponin I (TnI) is a key myofilament regulatory protein that can be phosphorylated to modulate cardiac function. Within TnI, Serine 150 (S150) is located in the inhibitory peptide that contributes to inhibition of the actin-myosin interaction and has been demonstrated to be phosphorylated. Previous work from our lab demonstrated exchanging troponin containing S150D pseudo-phosphorylated TnI into skinned cardiac muscle increased calcium sensitive force development. To study the effects of this phosphorylation site in vivo, we generated TnI S150D pseudo-phosphorylation (serine 150 mutated to an aspartic acid), and a TnI S150A phospho-null (serine 150 mutated to an alanine) mouse models.

Objective: The objective of the present work is to determine the effects of TnI S150 phosphorylation or ablation on papillary force generation and cardiomyocyte shortening of these mouse models.

Hypothesis: TnI S150D will increase calcium sensitive force production and increase cardiomyocyte shortening.

Methods: Calcium dependent force production was measured following exposure to varied calcium concentrations in skinned left ventricular papillary bundles from wild-type (WT), S150D, and S150A mice. Cardiomyocyte shortening and relengthening parameters were measured in freshly isolated cells from WT, S150D, and S150A mice.

Results: Papillary bundles from TnI S150D mice had greater calcium sensitive force production as demonstrated by an increased pCa50 compared to those from WT and S150A mice. The S150D mouse bundles also had a decreased Hill Coefficient compared to WT and S150A mice. There was no difference in maximal force production of bundles from any of the mice. Cardiomyocytes isolated from S150D mice exhibited similar % shortening, time to peak shortening, and time to 70% baseline to that of cardiomyocytes from WT and S150A mice. Interestingly, S150D myocyte diastolic sarcomere distance was shorter, and myocytes were shorter in length compared to WT and S150A myocytes, however cell width and length/width ratio were not different between groups.

Conclusions: Myocytes from TnI S150D mice have increased calcium sensitivity without altered shortening at shorter diastolic sarcomere distances. These results demonstrate cardiac tissue from the TnI S150D mouse heart has increased calcium sensitivity, and this mouse model can therefore be used to investigate the sensitizing effects of TnI Ser150 phosphorylation on in vivo cardiac function.
Myosin-Modulating Compounds Result in Different Responses to Mechanical Control of Relaxation (Strain Rate dependent Tension) in Rat Cardiac Trabeculae

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Introduction: Myosin-modulating compounds are now being used to treat cardiac diseases. Recent work from our lab has investigated the impact of Omecamtiv Mecarbil on Mechanical Control of Relaxation, or the strain rate dependent decline in tension after a contraction in rat intact cardiac trabeculae. The studies showed no increase in developed tension, a prolonged contraction, and a doubling of Mechanical Control of Relaxation when developed tension declined by 50%. This study sought to investigate the effect of additional myosin-modulating compounds on Mechanical Control of Relaxation.

Methods: Cardiac trabeculae were obtained from adult rats (female Sprague Dawley or male and female WT and Heterozygous RBM20 mutant). Trabeculae were mounted between a length motor and force transducer (322 and 403A, Aurora Scientific, Aurora ON Canada) and superfused in an experimental chamber (802 1900A, Aurora Scientific) using a HEPES buffered solution. Trabeculae were paced at 0.5 Hz and stretched to optimal length (Lo) manually, where length and cross-sectional area were measured. Mechanical Control of Relaxation was acquired and determined as previously reported, at a fixed isotonic afterload of ~50% of the developed force. The afterload was held via length control to varying conditions, leading to a range on lengthening strain rates using a custom control system (SLControl). Increasing doses of Danicamtiv, Mavacamten (trade name Camzyos), and Aficamten were added to the solution and allowed to equilibrate for at least 20 minutes. Mechanical Control of Relaxation was again acquired in the new condition. Tension was calculated by dividing the force by cross sectional area, and tensions, relaxation rates, and strain rates were determined. Mechanical Control of Relaxation was calculated as the slope of the relaxation rate to strain rate relationship.

Preliminary Results: Danicamtiv did not produce an increase in developed tension, but did result in increased contraction duration. Mechanical Control of Relaxation increased only after developed tension was reduced by 50%. While Danicamtiv shows similar contractile responses to Omecamtiv Mecarbil, the increase in Mechanical Control of Relaxation is attenuated in Danicamtiv by comparison. Mavacamten produced a dose-dependent decrease in developed tension but no change in Mechanical Control of Relaxation even at high doses. Aficamten produced a dose-dependent decrease in developed tension. In contrast to Mavacamten, Aficamten resulted in a 50% increase in Mechanical Control of Relaxation when developed force decreased by 50%.

Summary: Myosin-modulating drugs are currently reflecting two use cases: enhanced contractility (Omecamtiv Mecarbil and Danicamtiv) and suppressed contractility (Mavacamten and Aficamten). Differing responses to Mechanical Control of Relaxation, especially for the contraction-suppressing compounds, suggest that relaxation may be differentially modified when contractility is altered. The mechanism(s) of this differential response is unclear. While the response to these compounds is to enhance Mechanical Control of Relaxation, the relaxation phase of the cardiac cycle may merit further study for contraction-modifying compounds.
Effects of cMyBP-C phosphorylation on relaxation rates in myofibrils

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Cardiac myosin binding protein-C (cMyBP-C) is a thick filament accessory protein that modulates cardiac contractility and contributes to regulation of the On/Off states of myofilaments. Mutations in MYBPC3, the gene encoding cMyBP-C, are a leading cause of hypertrophic cardiomyopathy (HCM). We previously demonstrated that the L348P mutation in the tri-helix bundle (THB) of the M-domain that increases binding affinity of cMyBP-C for actin, activates the thin filament and forms long-lasting interfilament mechanical C-links. Consistent with this, we have reported that L348P significantly prolonged relaxation in various models. Recently we obtained evidence that C-links contribute to slowing the fast exponential phase of relaxation in single myofibrils. Because phosphorylation of cMyBP-C by protein kinase A (PKA) decreases binding affinity of its N-terminal domains for both actin and myosin and accelerates activation and relaxation in cardiac muscle, we investigated effects of cMyBP-C constructs not treated and treated with PKA in single myofibrils using the SpyC “cut and paste” delivery system. We show that while presence of L348P mutation in THB slowed relaxation rates and hyper-activated myofilaments, phosphorylated cMyBP-C had the opposite effects, accelerating relaxation and reducing activation compared to non-phosphorylated protein.
Cardiac diastolic dysfunction, characterized by prolonged relaxation, is associated with higher mortality. Moreover, subclinical diastolic dysfunction increases with age wherein over 30% of individuals aged 65 and older demonstrated impairment of diastolic function. Prolonged relaxation in diastolic function can be attributed to multiple mechanisms, with altered sarcomere function emerging as a significant contributor. We have found that myofibrils isolated from the hearts of women over 60 years of age relax significantly slower than myofibrils from the hearts of women 20-40 years of age.

Myofibril enriched proteins isolated from left ventricles of male and female non-failing (NF) donors were analyzed by mass spectrometry to identify differentially expressed proteins. One of the most significantly down-regulated proteins in the hearts of females over 60 years of age was short-chain enoyl-CoA hydratase (ECHS1). Since down regulation of ECHS1 leads to increased crotonyl-CoA, we hypothesize that age-associated decrease of ECHS1 in female hearts increased myofilament crotonylation. Assessment by Western blot revealed myofilament-enriched proteins isolated from the hearts of female NF donors over 60 years of age have higher crotonylation than myofilament-enriched proteins isolated from the hearts of adult female NF donors. To determine the functional role of increased crotonylation, primary cultured female adult rat ventricular myocytes (ARVMs) were treated with sodium-crotonate and cell dynamics were measured by Ionoptix. Crotonylation was higher in proteins isolated from ARVMs treated with sodium-crotonate. Moreover, these cells displayed slower kinetics of cell shortening and re-lengthening. To assess direct myofilament crotonylation, myofibrils isolated from adult female rats were treated ex-vivo with crotonyl-CoA for 1 hour at 37°C. Myofibrils treated with crotonyl-CoA demonstrated increased crotonylation of myofilament proteins coupled with slower activation and relaxation kinetics.

Our preliminary results reveal, for the first time, that aging induces alterations in myofilament crotonylation, consequently resulting in prolonged myofibril relaxation kinetics. This highlights the significance of myofilament crotonylation as a novel modulator of sarcomere dynamics.
Histone deacetylase 8 modulates myofibril relaxation

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Background: Diastolic dysfunction, characterized by prolonged relaxation, has proven difficult to pharmacologically target due to multiple contributing factors such as increased fibrosis, calcium handling, and sarcomere dysfunction. Since post-translational modifications (PTMs) of sarcomeric proteins can dynamically and reversibly regulate myofilament relaxation, targeting key PTMs on sarcomeric proteins has the potential to improve diastolic function. Previous studies have shown that increased acetylation of sarcomeric proteins accelerates relaxation; however, the enzymes that regulate this process are unknown. We hypothesize that inhibiting histone deacetylase (HDAC) isoforms increases acetylation on specific sarcomeric protein residues and leads to faster relaxation.

Methods: Adult rat ventricular myocytes (ARVMs) were isolated and treated with HDAC inhibitors of different specificities and cellular dynamics and myofilament acetylation were measured. Myofibrils were isolated from ARVMs treated with HDAC inhibitors and sarcomeric kinetics were measured.

Results: ARVMs treated with PCI-34051, an inhibitor that specifically inhibits HDAC8, have increased myofilament acetylation and faster cellular relaxation. In contrast, inhibiting HDACs 1,2 and 3 with MGCD-0103, inhibiting HDACs 4, 5, 7 and 9 with TMP-195, and inhibiting HDAC11 with FT895 do NOT modify cellular relaxation and inhibiting HDACs 6 and 10 with tubastatin A, slowed relaxation. Myofibrils isolated from ARVMs treated with PCI-34051 have faster relaxation kinetics than in ARVMs treated with vehicle. In contrast, treating myofibrils with recombinant HDAC8 decreases myofilament acetylation, and prolongs relaxation kinetics.

Conclusions: This study shows for the first time that HDAC8 can modulate myofibril relaxation through the deacetylation of sarcomeric proteins. The identification of HDAC8 as a novel modulator of relaxation provides a new pathway for the development of therapeutics to treat patients with diastolic dysfunction.
Tropomyosin Snapback on Actin after Myosin Detachment from Cardiac Thin Filaments

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Dynamic interactions between the myosin motor-head on thick filaments and the actin molecular track on thin filaments drive the myosin-crossbridge cycle that powers muscle contraction. The process is initiated by Ca\(^{2+}\) and the opening of troponin-tropomyosin-blocked myosin-binding sites on actin. This is followed by the recruitment of pre-powerstroke myosin-heads from thick filaments, accompanied by their attachment and transformation to post-powerstroke conformations on actin, thereby producing the force required for contraction. Concentrating on cardiac thin filament regulatory mechanisms, we previously compared cryo-EM-based atomic models of troponin–tropomyosin present on inhibited and Ca\(^{2+}\)-activated cardiac thin filaments. This confirmed that tropomyosin occupies three configurations on actin during regulation. We characterized two of these, showing tropomyosin pivoting on actin away from its TnI-imposed B-state (myosin-blocking) position to a C-state position allowing weak myosin binding to thin filaments (Lehman & Rynkiewicz, 2023). This reorganization of the thin filament only partially exposes myosin-binding sites. Thus, the released troponin-tropomyosin-imposed steric inhibition remains incomplete following Ca\(^{2+}\) activation. More recently, we have begun to elucidate the effect of myosin-binding on further tropomyosin translocation from the C-state to the M-state during thin filament activation. Here, we performed classical and targeted molecular dynamics simulations of the transition between myosin’s pre- and post-powerstroke conformations on actin in the presence of cardiac troponin-tropomyosin. The simulations indicate a stepwise translocation of tropomyosin on actin as myosin reconfigures from its modeled pre-powerstroke to its post-powerstroke conformation. The simulations show Arg 369 and Glu 370 on the tip myosin Loop-4 encountering identically charged residues on tropomyosin. The charge-repulsion between polar residues causes tropomyosin translocation across actin and is expected to facilitate myosin movement along the thin filament. In the heart, this behavior could add to positive cooperativity accompanying force development. We note that a sole myosin head bound to a single actin subunit along thin filaments causes M-state tropomyosin movement at the site of interaction as well as on neighboring actin subunits. However, once myosin is detached from the post-powerstroke modeled thin filaments and then molecular dynamics is launched, the tropomyosin moves back toward its C-state position. We suggest that myosin head binding to actin and concomitant Loop-4 – tropomyosin repulsion drives tropomyosin to an energetically unfavorable M-state position on actin during myosin binding. At the same time, the structural mechanics of the semi-rigid tropomyosin coiled coil are likely to be affected during its C- to M-state azimuthal movement on actin that accompanies the pre- to post-powerstroke myosin transition. Indeed, tropomyosin undergoes a local counterclockwise twisting motion as it transitions to its M-state configuration, which is likely to add to its super-helical strain (cf. Doran et al., 2023). We suggest that release of the torque imposed on M-state tropomyosin following ATP-induced dissociation of myosin from actin will cause tropomyosin to snapback to its default C-state. Then, during low-Ca\(^{2+}\) muscle relaxation, tropomyosin will be attracted to the C-terminal domain of Tnl producing the inhibitory B-state configuration. In terms of cardiac contractile function, any strain-induced snapback of tropomyosin from the M-state could be part of a negative cooperativity mechanism that helps make way for thin filament shut down and thereby facilitates rapid relaxation at the end of cardiac systole.
A Rapid Automatic System for Negative Staining EM

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Negative staining is a long-established protocol for observing macromolecules and their assemblies by transmission electron microscopy. It is widely used to optimize specimens for cryo-EM. The stain, typically a uranyl salt, surrounds the structure, providing an outline view at about 20 Å resolution. Most macromolecules are relatively stable and rigid, and negative stain images provide a reasonable facsimile of their structure. However, others are labile or flexible, and their structure or assembly state can be altered by binding to the carbon substrate of the grid before the specimen is fixed by the stain. In these cases, the negatively stained appearance does not faithfully represent the structure in solution. An example from our lab is the folded-back structure of the heads in the interacting-heads motif (IHM) of myosin II, which is rapidly disrupted by binding to the glow-discharged carbon film. We find that this problem is significantly alleviated, though not solved, when samples are incubated on the carbon surface for shorter times (5 sec) rather than typical times (30-60 sec) before staining. To further shorten incubation time, and thus minimize specimen disruption prior to staining/fixing, we have developed an automated, rapid negative staining device. Our system utilizes 3D-printed components to secure the EM grid, a stepper motor for precisely timed movements, and an Arduino-controlled interface to execute commands. This system can produce consistent, reproducible results at sample incubation times as short as 10 msec. Using this system to study a short-tailed (15-heptad) construct of cardiac myosin under solution conditions that promote the IHM, more than 50% of molecules show the IHM appearance with 10 msec incubation. With conventional incubation times (5-30 sec), few molecules show the IHM. We are using this approach to improve the reliability of studies of the IHM in myosin constructs containing disease mutations or treated with drugs, and to improve preservation of the labile helical array of myosin heads in native thick filaments. Our innovation promises to improve the reliability, precision and speed of negative staining procedures and, most importantly, to enhance the structural preservation of any labile protein. It will also be possible to include additional steps, such as timed incubation with enzyme substrate before staining.

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Exploring the formation of the Myosin Interacting-Heads Motif by EM: Effect of dilated cardiomyopathy E525K mutation and mavacamten on myosin constructs

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Cardiac myosin, a molecular motor, powers contraction in the heart, by converting chemical energy into force. Myosin heads (S1) comprise a motor domain that hydrolyses ATP and a lever arm. In relaxation, myosin’s two heads interact with each other, folding back onto the tail (subfragment-2, S2), forming the self-inhibited interacting-heads motif (IHM). IHM is an important conformation of the two heads switching both heads off to regulate activity. To decipher the structural basis of IHM formation and its association with SRX and the impact of a Dilated Cardiomyopathy (DCM) mutation and the drug (Mavacamten) on this process, we used negative staining EM to study cardiac myosin constructs of single heads (S1) and of 2-headed molecules with 2-, 15-, and 25-heptads of S2. 2D class averages of WT S1 molecules showed a ~90° bend between the MD and the lever arm, present in <10% of WT molecules and WT with mavacamten, 25% with the E525K mutant, and 70% with the mutant plus mavacamten. This bent structure was conserved in the IHM which may contribute to the SRX state formation. In the WT 15-heptad construct, ~25% of the heads were bent and folded, forming IHMs. This increased to ~38% with mavacamten, to ~40% with the E525K mutation, and to ~65% with both. We observed a similar trend with the 25-heptad construct. In the WT 2-heptad construct, with S2 too short to form the IHM, the heads were also bent, and head-head interactions were rare. Thus, the longer S2 may be important for IHM formation and stability. These interactions increased to ~11% in WT with mavacamten, to ~14% in E525K, and to ~30% in E525K with mavacamten. We conclude that the bent S1 structure and a minimum S2 length are essential for IHM formation, which the E525K mutation and mavacamten can further enhance. Further structural insights into these conformations and IHM formation require high-resolution cryo-EM.

Supported by NIH HL164560 and AR081941.
Assessing the microtubule contribution to diastolic performance in ZSF1 rat myocardial slices

Emmaleigh N. Hancock, Alexa R. Price, Shaina L Weingart, Raihan Kabir, Brad M Palmer, Matthew A Caporizzo

Heart failure with preserved ejection fraction (HFpEF) arises from increased ventricular stiffening and impaired relaxation and cardiac microtubules have been implicated in mechanical dysfunction during heart failure. However, systemic intolerance and non-cardiac effects of microtubule-depolymerizing compounds have made it challenging to precisely determine the effect of microtubules on working myocardial performance. Herein we leverage recent advancements in living myocardial slices to develop a preparation that recapitulates the complexity of diastole and exhibits long-term stability. To determine the effect of cardiac microtubule depolymerization on diastolic performance, slices were perfused with media optimized to maintain isometric twitch force and force-length work loops were collected before and after 90 minutes of treatment with either DMSO (vehicle) or colchicine (to depolymerize microtubules). A trapezoidal stretch was added to the work loop to mimic late-stage-diastolic filling driven by atrial systole. Force-length work loops were obtained at fixed preload and afterload and velocity traces were obtained as an analog to trans-mitral Doppler. Consistent with the HFpEF etiology, myocardium from ZSF1 obese rats was found to exhibit prolonged contractile transients with normal force production, preserved stroke work. Isovolumic relaxation time was not reduced in the ZSF1 obese myocardium but characteristics of early filling were compromised, specifically the slope of early deceleration. Colchicine was found to increase work output by increasing stroke length and accelerating relaxation and contraction with an improvement in the early filling characteristics of ZSF1 obese and lean myocardium. Taken together, these results indicate that the microtubule destabilizer colchicine can improve the performance of HFpEF myocardium.
Autophagy activates sarcomere remodeling in cardiomyocytes.

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1University of California Santa Barbara, Santa Barbara, CA, Mechanical Department
2University of Kentucky, College of Medicine, Department of physiology

Sarcomere remodeling in the heart is a dynamic process influenced by several factors, including mutations associated with hypertrophic cardiomyopathy (HCM) and heart failure. In response to these conditions, sarcomeres can undergo structural alterations, leading to changes in myocardial function. However, our comprehension of the impact of naturally occurring compounds, such as polyamines, on sarcomere remodeling remains limited. Here, we present evidence suggesting that the autophagy activator spermidine can play a significant role in stimulating sarcomere remodeling in human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). Here, we exposed hiPSC-CMs to 50 µM and 100 µM Spermidine to enhance autophagy and promote cell remodeling, cell enlargement what also happens in HCM conditions. Sarcomere structures were monitored before adding the drug, at 48 hours, and at 72 hours on a single-cell pattern (7:1 ratio, 1500 µm^2) hydrogel platform (10 kPa mimicking physiological conditions). A control sample without Spermidine was included for the three time points. We quantified the length of z-discs and the number of sarcomere structures using engineered cell lines with alpha-actinin tagged with green fluorescent protein and Labkit segmentation by Fiji. Preliminary results showed an enlargement of z-disc length with 100 µM Spermidine by 17% at 48 hours and 100% at 72 hours respect to the control at that time point. Samples treated with 50 µM Spermidine exhibited a 48% increase at 48 hours and a 140% increase at 72 hours respect to the control at that time point. Additionally, we observed a 6% increase in the number of sarcomeres with 100 µM Spermidine at 48 hours and a 7% increase at 72 hours. Samples treated with 50 µM Spermidine showed an 11% increase at 48 hours and a 20% decrease in the number of sarcomeres at 72 hours. These results suggest that z-disc enlargement, indicative of parallel addition of sarcomeres, is more predominant than sarcomere addition in series for Spermidine treatment. Further research is needed to validate these findings, and we have developed a platform that can be used to explore other perturbation effects on sarcomeres in hiPSC-CMs. This finding sheds light on a potential mechanism through which sarcomere remodeling can be modulated, offering new avenues for therapeutic intervention in cardiac pathologies characterized by aberrant sarcomere function.
Atomistic-level mechanisms of cardiac myosin powerstroke and activation

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Allosteric communications between distal domains in myosin play essential roles in its mechanochemical cycle. Understanding how small changes in myosin affect these communications is crucial for elucidating mechanisms underlying cardiomyopathy. Molecular dynamics (MD) simulations, though capable of providing atomistic details, are limited in studying the motor functional cycle owing to the slow timescale and rugged energy landscape. Here, by integrating Rosetta and AlphaFold with enhanced sampling MD simulations, we characterize the conformational changes of myosin in two key processes of its functional cycle. Firstly, despite the absence of key experimental intermediate structures, we are able to model the cardiac actomyosin conformational ensembles, which show that myosin tight binding to actin is coupled with the motor core transitions and products release from the active site. Additionally, we find the allosteric transition is facilitated by a cardiomyopathy-associated mutant. Furthermore, we demonstrate how myosin can be released from the inhibited state, in which the two heads interact with each other and fold back onto the tail. Our results uncover the principles by which myosin conformational distributions and dynamics encode its allosteric communications.
### Recommended Madison Restaurants: Walking Distance from Myofiliment Meeting

<table>
<thead>
<tr>
<th>Name</th>
<th>Price</th>
<th>Type</th>
<th>Location</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eno Vino Downtown</td>
<td>$50–100</td>
<td>Tapas</td>
<td>1 N Webster St</td>
<td>Modern tapas, rooftop views, great EtOH, feels like NYC</td>
</tr>
<tr>
<td>Graze Restaurant</td>
<td>$30–50</td>
<td>American</td>
<td>1 S Pinckney St</td>
<td>Lively gastropub with Capitol views</td>
</tr>
<tr>
<td>Merchant Madison</td>
<td>$$</td>
<td>American</td>
<td>121 S Pinckney St</td>
<td>Farm-to-table New American: Hip spot, great cocktails</td>
</tr>
<tr>
<td>The Old Fashioned</td>
<td>$10–20</td>
<td>Restaurant</td>
<td>23 N Pinckney St #1</td>
<td>Retro tavern for beers, brats &amp; cheese: You are in Wisconsin</td>
</tr>
<tr>
<td>Cento</td>
<td>$30–50</td>
<td>Italian</td>
<td>122 W Mifflin St</td>
<td>Stylish Italian eatery with lots of good wine</td>
</tr>
<tr>
<td>Lucille</td>
<td>$20–30</td>
<td>Pizza</td>
<td>101 King St</td>
<td>Gourmet pizza in stylish digs, also great cocktails</td>
</tr>
<tr>
<td>Osteria Papavero</td>
<td>$30–50</td>
<td>Italian</td>
<td>128 E Wilson St</td>
<td>Cozy spot for seasonal Italian fare</td>
</tr>
<tr>
<td>Sardine</td>
<td>$30–50</td>
<td>Bistro</td>
<td>617 Williamson St</td>
<td>Authentic French Bistro Great food, zinc bar.</td>
</tr>
<tr>
<td>Settle Down Tavern</td>
<td>$$</td>
<td>American</td>
<td>117 S Pinckney St</td>
<td>Relaxed hangout for comfort fare, outdoor seating</td>
</tr>
<tr>
<td>The Coopers Tavern</td>
<td>$20–30</td>
<td>Restaurant</td>
<td>20 W Mifflin St</td>
<td>Contemporary, upscale Irish tavern, great tap beer selection. Outdoor seating</td>
</tr>
<tr>
<td>Tornado Steak House</td>
<td>$50–100</td>
<td>Meat n’ potatoes</td>
<td>116 S Hamilton St</td>
<td>Classic Wisco retro steak house- go big or go home</td>
</tr>
<tr>
<td>Canteen</td>
<td>$10–20</td>
<td>Street tacos and more</td>
<td>111 S Hamilton St</td>
<td>Mexican kitchen with plenty of tequilas</td>
</tr>
<tr>
<td>Marigold Kitchen</td>
<td>$10–20</td>
<td>Cafe</td>
<td>118 S Pinckney St</td>
<td>Awesome breakfast just off the square.</td>
</tr>
<tr>
<td>RED</td>
<td>$50–100</td>
<td>Sushi</td>
<td>316 W Washington Ave #100</td>
<td>Great sushi (yes, it is)</td>
</tr>
</tbody>
</table>

Not responsible for disappointment... JCR