PI: **Higuchi-Sanabria, Ryo**	Title: More than just a load control: cytoskeletal form and function during aging	
Received: 01/29/2019	FOA: PA19-130 Clinical Trial:Not Allowed	Council: 10/2019
Competition ID: FORMS-E	FOA Title: NIH Pathway to Independence Award (Parent K99/R00 - Independent Clinical Trial Not Allowed)	
1 K99 AG065200-01	Dual:	Accession Number: 4257698
IPF: 577502	Organization: UNIVERSITY OF CALIFORNIA BERKELEY	
Former Number:	Department: Molecular and Cell Biology	
IRG/SRG: NIA-B	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: 112,750 Year 2: 112,750 Year 3: 249,000 Year 4: 249,000 Year 5: 249,000	Animals: N Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: Early Stage Investigator:
Senior/Key Personnel:	*Organization:*	*Role Category:*
Ryo Higuchi-Sanabria	The Regents of the University of California	PD/PI
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Reference Letters

[redacted]

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Project Summary. Although the cytoskeleton has historically been understood as the structural framework of the cell, the proper function of actin is also required for a diverse array of cellular pathways. The collapse of these cellular processes manifests during aging and exposure to a myriad of stresses, which is in part due to the breakdown of the cytoskeleton under these conditions. Interestingly, the breakdown of the cytoskeleton throughout age has been adopted as common knowledge in the field of aging biology, despite the lack of clear and direct evidence. A major contributor to the lack of these essential studies is the lack of tools available for *in vivo*, live-cell imaging of the actin cytoskeleton in multi-cellular organisms. Early in my postdoctoral career, I developed a system for robust, tissue-specific, live-cell imaging of the cytoskeleton in the muscle, intestine, and hypodermis of *C. elegans*, utilizing LifeAct fused to a fluorescent molecule. LifeAct-mRuby reliably binds to F-actin, allowing visualization of functional, filamentous actin in the cells it is expressed. Using this system, I performed an exhaustive characterization of the decline of actin cytoskeletal integrity during aging.

This work laid the foundation of my currently ongoing work in identification of novel regulators of the actin cytoskeleton. Having set up a system to interrogate cytoskeletal quality, I can now interrogate novel genes in their potential role for actin regulation. Using this and other platforms, I performed a multi-pronged screening approach to identify novel genetic regulators of actin. These studies combined *in vivo* live cell imaging of actin filaments, synthetic lethality screening with known regulators of the actin cytoskeleton, and both transcriptome analysis and whole genome CRISPR-Cas9 screening of organisms experiencing actin stress. Cross-referencing these rich datasets has revealed two critical nodes of genes: 1) modifiers of chromatin state and their downstream transcriptional regulators and 2) genes involved in lipid storage and global lipid homeostasis.

In Aim 1, I propose to characterize these major molecular pathways and how they contribute to cytoskeletal integrity. I hypothesize that a general chromatin state exists to promote a healthy transcriptome for proper cytoskeletal form and function, and that this breaks down as a function of age. Moreover, a healthy metabolic state can work either upstream of – or independent of – chromatin remodeling to also promote cytoskeletal health. In Aim 2, I propose to study whether any of the identified processes can function in a cell non-autonomous manner, by answering two questions: 1) does non-autonomous *hsf-1* signaling occurs through changes in chromatin or metabolic state in peripheral tissue? and 2) does overexpression of major genes involved in chromatin remodeling or lipid homeostasis in neurons alone drive non-autonomous protection of the actin cytoskeleton in peripheral tissue, similar to HSF-1? Finally, Aim 3 uses a molecular approach to characterize protein interactors of actin important for its proper form and function. This study will open exciting avenues of research in understanding the role of cytoskeletal form and function on physiological aging.

Many cellular functions, such as autophagy, organelle dynamics, and endocytosis/exocytosis, as well as their dedicated quality control machineries, such as the ubiquitin-proteasome system and the heat-shock response, decline in efficiency and function during the aging process. The actin cytoskeleton is no exception, and exhibits marked decline in structural integrity and function at old age. I propose a multipronged approach to understand how the regulatory network involved in cytoskeletal maintenance deteriorates during aging, and how this contributes to the physiological consequences of aging.

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Candidate Background. I believe that I harbor a unique and eclectic skillset gained through years of devoted time both inside and outside of the laboratory. Countless hours in restaurant management have taught me invaluable lessons like the ability to husband resources and impeccable time management, while my expansive teaching experience has granted me effective and articulate communication as well as remarkable mentoring capacity and leadership. These worldly experiences combined with my scientific expertise on the actin cytoskeleton, cellular stress response, and aging, create an idiosyncratic profile, which I believe makes me a great candidate for the NIH Pathway to Independence award.

At Columbia, I worked vigorously in researching factors that contributed to aging using the budding yeast model, *S. cerevisiae*. Within my first year in the lab, I succeeded in putting together a manuscript that highlighted the importance of the actin cytoskeleton and its role in asymmetric segregation as a mitochondrial quality control machinery, *a work that was published in Current Biology and featured as an F1000 prime article.* My dedication has led to *seven first-author publications, and contributions to several other publications in the three years spent in the lab*. I have also pioneered independent research projects, including utilization of novel fluorophores to adapt 3-, 4-, and 5-color, live-cell, long-term imaging in yeast, a feat never accomplished before. My experiences have made me resilient and efficient, allowing me to *complete my PhD within four years with a 4.0 GPA*. In the Dillin lab, I am continuing my work on aging in the context of cellular stress response. I focused on two broad subjects: the heat-shock response and its role in cytoskeletal regulation, which my proposal aims to explore further, and the unfolded protein response of the endoplasmic reticulum (UPR^{ER}) and its impact on cellular metabolism and aging. Briefly, I have created tools to interrogate cytoskeletal quality during aging in an effort to answer open questions that have been unanswered for decades due to the lack of methods available to test the hypothesis of cytoskeletal aging. This work has been recently *published in the Molecular Biology of the Cell, along with a comprehensive review on cellular stress responses*. I have also built many fruitful and mutually beneficial collaborations both inside and outside of Berkeley, including collaborations with Dr. Barbara Meyer and Dr. Amy Herr at UCB and with Dr. Valerie Weaver at UCSF. All of the work performed in collaborations with these labs are currently at various stages of the publication cycle, and expected to be in press in early 2019.

In the ER field, I have worked with members of the Dillin lab to develop independent projects on ER quality control. First, I have found that ER stress can be communicated from neurons to activate UPR^{ER} signaling in distal tissue, partially through dopaminergic neurons. Moreover, this signal activates a unique and non-canonical arm of UPR^{ER} involved in ER remodeling and activation of lipophagy, which is distinct from canonical UPR^{ER} involving chaperones. Both arms are independently required for the beneficial effects of UPR^{ER}, including metabolic health and lifespan. I have also discovered a novel communication from the lysosome to the ER through amino acids, which is required for proper ER quality control through UPR^{ER} activation. Finally, I have discovered a novel UPR^{ER}-independent mechanism that promotes ER quality control and lifespan through p38/MAPK pathways. These works have evolved into *four different co-first author manuscripts over the course of my postdoc, one currently in revision at Nature, one submitted to Cell, and two in preparation for submission.*

I believe that my comprehensive work in multiple fields highlights my broad range of interests and ability to succeed in many different situations. My numerous collaborations show that I am capable of working well with others, building meaningful relationships, and multi-tasking. I am confident that my educational, worldly, and scientific experience illustrates my capacity to succeed through the proposed training to complete my postdoctoral work and transition into a tenure-track faculty position. With my work ethic, experience, and the knowledge of my committee, I have the tools to succeed in this ambitious, but highly important research proposal.

Career Goals and Objectives. My short term research goal is to continue pursuing my interests in interrogating the impact of actin cytoskeletal health on the physiological consequences of aging. I hope to establish an independent research group focusing on dissecting the complex map involved in cytoskeletal regulation. Obtaining the Pathway to Independence Award will give me the time to acquire new skills and knowledge, particularly in data science to dissect large sequencing and proteomic datasets with the help of Drs. Alice Ting, Brett Phinney, and Michael Eisen. These skillsets will be invaluable in my progression to a faculty position, where I will combine these tools with my expertise in cell biology and genetics to map cytoskeletal regulation and its breakdown during the aging process. Long term, I plan to expand my work to the cross communication of cytoskeletal stress response to the quality control and fitness of other organelles. My proposal touches on this briefly in an effort to study the communication between lipid homeostasis and cytoskeletal regulation, but in future proposals, I hope to interrogate the impact of cytoskeletal quality and health to other organelles, including ER and the mitochondria, both of which I have developed expertise in studying throughout my scientific career.

I hope to develop my research group in an academic environment with passion, dedication, and respect towards teaching and mentorship, such as in State University systems. I have taught courses at Columbia and Hunter college, have been an adjunct professor at the College of Mount Saint Vincent, and taught and mentored

many high school, undergraduate, masters, and graduate students in and out of the lab, which I believe make me proficient in teaching. I am also highly invested in increasing the presence of under-represented minority in STEM, and have been involved directly in programs focused in this cause including the NIH Bridges to Baccalaureate (B2B) program and the CHORI Summer Research program. I hope to evolve programs similar to these in my research institute to continue to expand science to a larger population of racial and socioeconomic minorities. I truly believe that an individual who can succeed in higher education while maintaining families and multiple part-time and full-time obligations is one who is sure to succeed in scientific research, and we cannot continue to lose these brilliant minds to other fields, simply due to lack of opportunities open to them. While the primary criticism of my interest in teaching may be to question my dedication for research, I combat this criticism by expressing my profound belief that teaching and education are the essence of research. To adequately teach and educate the next generation is to both preserve and expand the excellence of scientific research. It is through the scientists of tomorrow that we can make research an eternally successful creature.

Independence from Mentor: Dr. Dillin has an incredible reputation for being a supportive mentor and pushing his trainees to transition to independence. As evidenced by his previous postdoctoral trainees who have established their own labs, he maintains open communications, open availability of strains and resources, and complete transparency to avoid scientific overlap. Moreover, my work on cytoskeletal biology is unique in the Dillin lab, as this is not a primary focus of interest to Dr. Dillin or other members of the lab, so I do not anticipate heavy overlap. Dr. Dillin has also assured me that I am free to take any reagents and strains I need to establish my own lab, and that the scientific discoveries I make during the K99 phase will intellectually belong to me.

Plan for Career Development/Training Activities during Award Period. Throughout the award period, I will direct a majority of my time to the proposed research. My previous scientific training has been focused on understanding cell biology and genetics. This additional training will expand my experimental skillsets to data science, including understanding large transcriptome, ChIP-seq, ATAC-seq, and proteome datasets:

Learn more about mass spectrometry: my training in biochemistry is limited, and I plan to address this issue during the K99 award period by working closely with the Ting lab to develop an assay for proximity labeling of molecular interactors of actin. I will educate myself by reading instructional text, such as *Mass Spectrometry for Biotechnology* by Gary Suizdak, and I will also take instructional lessons at the Proteomic core at UC Davis.

Applying bioinformatics techniques: As a cell biologist and geneticist, my skillsets in bioinformatics are basic. Thus, I will spend a large amount of time with a post-doc in the lab, Dr. Raz Bar-Ziv, who is an expert in bioinformatics techniques using Galaxy and Matlab. I have already begun training with him in understanding how to analyze transcriptome datasets, and will continue to expand my knowledge by training with him and members of the Eisen lab to learn how to apply these techniques to other large datasets, including ChIP-seq and ATAC-seq. I also plan to take *Databases and tools of Bioinformatics* offered by the UC Berkeley learning center.

I will expand my experimental skills while establishing my fundamental work in cytoskeletal biology in the aging field. Moreover, I will gain new collaborations and career training from diverse experts to increase my scientific skills and my capacity to serve as a principal investigator. I have designed a scientific advisory panel including experts in diverse fields within the San Francisco Bay Area. I will work directly with the members of these labs, and have committee meetings every 6-9 months during the K99 phase. We will work on my scientific progress, career development, and future publication and grant applications.

Transcription expert: Michael Eisen, Ph.D. is a very well-known HHMI scientist whose lab applies a diverse array of biochemical, genetic, and computational approaches to answer complex questions about enhancers and general chromatin state. His expertise will be critical as I pursue complex techniques requiring both experimental and computational skills, such as ChIP-seq and ATAC-seq.

Cytoskeleton expert: David Drubin, Ph.D. is the co-chair of the MCB department at Berkeley and Ernette Comby Chair in Microbiology. He is a renowned expert on cytoskeletal biology, using both real-time microscopy of live cells and *in vitro* techniques to interrogate the cytoskeleton and its many functional roles.

Proximity labeling expert: Alice Ting, Ph.D. is a Chan Zuckerberg Biohub investigator and a professor at Stanford University. Her lab has truly revolutionized the field of proximity labeling and developed the miniTurbo method.

Career Development Training. In addition to my experimental work, I will engage in other forms of career training that will help increase my success as a principal investigator:

Teaching and mentoring: Within the Dillin lab, I will continue to mentor students on several levels: I currently have two graduate students, two undergraduate students, and one full-time technician that work in my team. This gives me an incredible opportunity to gain experience in running my own team in the safe and mentored environment of the Dillin lab. I will continue to train these and any additional students that I acquire. I will also take the time to continue working with Dr. Dillin in his molecular genetics lab in designing and carrying out a laboratory course. Throughout my graduate work, I have worked heavily in teaching lecture-type courses both

as a TA and an adjunct professor, but my work with Dr. Dillin will give me the experience and knowledge of how to successfully run a laboratory course. The training I will receive will allow me to not only firmly establish my scientific career, but also increase my capacity to be an independent researcher with teaching responsibilities.

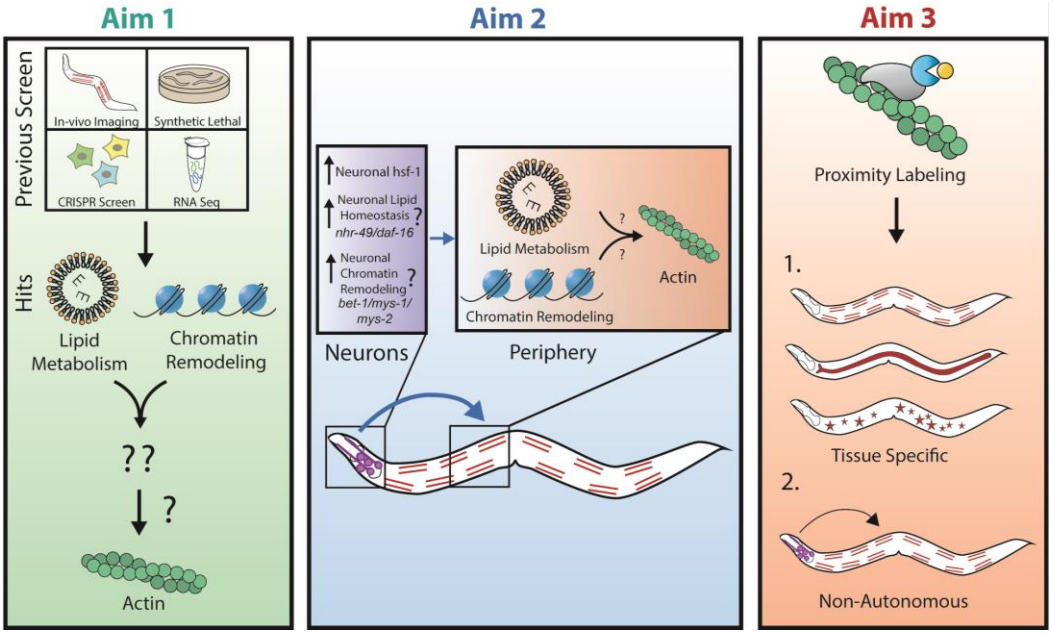
Increasing diversity in STEM: A large goal of mine is to increase the diversity – both of underrepresented minorities and underrepresented socioeconomic groups – in STEM. Currently, I am part of the B2B program at UC Berkeley, which is a mentorship program where students from underrepresented minority groups attending community colleges gain their first experience in a laboratory environment. This program involves direct mentoring of new students into a first lab experience, and includes lectures and courses on mentoring. I am also part of the CHORI/CIRM summer research program, which takes high school students from disadvantaged backgrounds (minority, low income, medical, etc.) and places them in a research environment. I am a mentor for this program, and recently have joined the selection committee. I will work with the heads of these programs to increase my involvement, but also to learn how to incorporate these types of programs into my future institute.

Lab management: I have a large amount of management experience both in restaurants and in retail. I had the privilege of translating these skillsets into laboratory management during my graduate work where I was serving as a secondary laboratory manager. Currently in the Dillin lab, I am expanding my expertise in lab management by working with our exceptional lab manager, Larry Joe, as he teaches me how to manage finances, personnel, and inventory of a lab. Moreover, he allows me to engage in direct negotiations with vendors.

Writing: I have had the fortune of being given the freedom to write my manuscripts independently in my graduate and postdoctoral labs. I will continue to write future manuscripts independently, with Dr. Dillin's input to refine and hone my writing skills. I have also written multiple grants both for myself, my students, and with Dr. Dillin, and write all progress reports for several grants, including my independent F32 and an R01 that I co-authored with Dr. Dillin. I will continue to expand my writing skills by co-authoring future R01 and R21-level grants.

Timelines for Career Benchmarks

Award Period	Timeline	Benchmark Goals
pre-award (Jan 2019-Nov 2019) Primary focus: -completion of post-doctoral work on ER quality control -laying ground-work for K99 research plan	Jan 2019	Submit papers on role of dopaminergic neurons in non-autonomous UPR^{ER} and TMEM2's role in ER stress resistance and longevity
	Mar 2019	Submit revisions for non-canonical UPR^{ER} paper
	Jun 2019	Submit revisions for 2 papers submitted in Jan 2019
	Jan 2019-Sep 2019	Continue project on characterizing the role of the lysosome on ER quality control
	Jan 2019-Sep 2019	Continue pilot experiments for K99 projects – characterizing role of chromatin modifiers on actin health (Aim 1.1) and start synthesis of strains for proximity-labelling system (Aim 2)
	Sep 2019-Nov 2019	Write and submit paper on characterizing the role of the lysosome on ER quality control
K99 year 1 (Dec 2019-Nov 2020) Primary focus: -complete chromatin modifier project -Begin proximity labelling project	Dec 2019-Feb 2020	Perform mass-spec on proximity-labeling (Aim 2)
	Feb 2020-Jun 2020	Continue work on characterization of chromatin modifiers on actin health (Aim 1.1)
	Jun 2020	Meet with advisory committee Submit revisions for lysosome on ER quality manuscript
	Jun 2020-Nov 2020	Finalize experiments for chromatin modifiers on actin health (Aim 1.1)
K99 year 2 (Dec 2021-Sep 2021) Primary focus: -chromatin modifier manuscript -acquire faculty position offer -complete proximity labeling	Dec 2020-Jan 2021	Write and submit paper for chromatin modifiers on actin health (Aim 1.1)
	Feb 2021-Apr 2021	Submit application packet for multiple faculty position
	Apr 2021-2022	Interview and negotiate for position and start-up
	Jun 2021	Meet with advisory committee
	Jun 2021	Submit revision for paper on chromatin modifiers on actin health (Aim 1.1
	Jun 2021-Sep 2021	Begin validation and secondary screening for hits identified in mass-spec (Aim 3)
R00 year 1 Sep 2021-Aug 2022 Primary Focus: -Lab set-up and secure staff -Manage teaching requirements -Begin thesis projects for students	Sep 2021-Dec 2021	Utilize start-up funds to purchase essential equipment and set up lab
	Sep 2021-Dec 2021	Recruit personnel: hire technician, recruit undergraduate and graduate students
	Sep 2021-Dec 2021	Fall semester lecture: molecular genetics or equivalent
	Jan 2022-Feb 2022	Write and submit methods/resource paper on proximity labeling (Aim 3)
	Feb 2022-May 2022	Spring semester lab class: molecular genetics or equivalent
	Feb 2022-Aug 2022	Recruit graduate students to begin work on lipid regulators on actin health (Aim 1.2) and non-autonomous HSF-1 signaling in actin health (Aim 2)
R00 year 2 Sep 2022-Aug 2023 Primary Focus: -Begin transition to new R01 work while completing R00 aims	Sep 2022-Dec 2022	Fall semester lecture: molecular genetics or equivalent
	Sep 2022-Dec 2022	Submit R01 on cross-communication between ER and cytoskeletal quality control & submit smaller grants to private institutions
	Sep 2022-Aug 2023	Continue to recruit students to begin work on ER and cytoskeletal cross talk.
	Feb 2023-May 2024	Spring semester lab class: molecular genetics or equivalent
R00 year 3 Sep 2023-Aug 2024 Primary Focus: -Secure R01 -Complete manuscripts for lipid regulation of actin and non-autonomous regulation of actin	Sep 2023	Re-submit R01
	Sep 2023-Dec 2023	Fall semester lecture: molecular genetics or equivalent
	Jan 2024-Feb 2024	Submit manuscripts on lipid regulators on actin health (Aim 1.2) and non-autonomous HSF-1 signaling in actin health (Aim 2)
	Feb 2024-May 2024	Spring semester lab class: molecular genetics or equivalent
	June 2024-Aug 2024	Submit revisions on manuscripts
	July 2024	Submit R21 on riskier project following up on other hits from K99 screens



Actin is arguably one of the most highly regulated proteins transcriptionally and translationally, popularizing it as a housekeeping gene and load control for a majority of qPCR and western blotting experiments alongside its cytoskeletal brother, tubulin. Despite this unequivocal regulation in expression, actin is one of the most dynamic proteins within the cell, consistently altering its structure, organization, and function in response to a wide range of cellular conditions. This dichotomy is explained in the plethora of actin-binding proteins involved in its fluid dynamics and regulation. The upstream network of genes responsible for regulating the expression of the many proteins responsible for cytoskeletal maintenance is poorly understood, likely owing to the complexity of cytoskeletal regulation. While our lab has identified HSF-1 function in regulation of actin during stress and aging, many actin-regulating genes are not bonafide targets of HSF-1, suggesting that other master regulators must exist, which function either independently or in parallel with HSF-1 to modulate the actin cytoskeleton. We further propose that similar to HSF-1 function, other master regulators of cytoskeletal form and function break down during the aging process, which is the key cause of cytoskeletal decline during aging. We thus propose an approach in which we dissect the genetic and molecular modulators both dependent and independent of HSF-1 in regulation of the actin cytoskeleton.

Aim 1: How does chromatin remodeling and lipid homeostasis regulate actin organization and function? We have performed a multi-pronged genetic screening approach to identify novel regulators of the actin cytoskeleton. These studies combined *in vivo* live cell imaging of actin, synthetic lethality screening with known regulators of the actin cytoskeleton, and both transcriptome analysis and whole genome CRISPR-Cas9 screening of organisms or cells experiencing actin stress. Cross-referencing these rich datasets has revealed two critical nodes of genes: 1) modifiers of chromatin state and their downstream transcriptional regulators and 2) genes involved in lipid storage and lipid homeostasis, which we believe either work in concert or independently to maintain a chromatin state and transcriptional program that allows efficient regulation of actin.

Aim 2: What is the nature of the neuronal communication to preserve the cytoskeleton in distal tissues via non-autonomous HSF-1? We have shown that overexpression of *hsf-1* in neurons is sufficient to protect the actin cytoskeleton in distal tissues during aging, including muscle, intestine, and hypodermis. We propose to dissect nature of the molecular and cellular changes occurring in distal tissue in response to non-autonomous HSF-1 signaling from neurons. We seek to determine whether regulation of the form and function of the actin cytoskeleton via chromatin remodeling, lipid homeostasis, or other large-scale transcriptional changes through candidate genes identified in Aim 1 are essential for the non-autonomous regulation of actin via *hsf-1*.

Aim 3: What is the molecular nature of the cytoskeleton resulting in major tissue-specific differences in cytoskeletal form and function? Recent work from our lab has shown direct evidence that the structure and integrity of the actin cytoskeleton breaks down during advanced aging in multiple tissue. Moreover, the nature and state of the actin cytoskeleton both at young age and throughout aging is significantly different between different tissue types. Therefore, we hypothesize that the actin cytoskeleton within each tissue is regulated by a unique set of molecular interactors, which break down at different rates and complexity during aging. To test this hypothesis, we propose to employ biochemical interrogation of cytoskeletal interactors by utilizing proximity labeling methods combined with LifeAct and miniTurbo technology. This study will accomplish several goals: 1) identification of novel molecular interactors of actin that are required for cytoskeletal maintenance, 2) characterization of differences in cytoskeletal regulators across tissues, and 3) characterization of the loss of cytoskeletal interactors that result in breakdown of the cytoskeleton during aging. Finally, we propose to determine whether the molecular interactome of actin significantly differ in peripheral tissue upon receiving non-autonomous signals from neuronal *hsf-1*.

Significance. The actin cytoskeleton, composed of a complex network of filaments held together by many actin-binding proteins, has historically been understood as the structural framework of the cell, with its primary function being ascribed to the sorting and transport of cellular cargo. However the proper function of actin is also required for a diverse array of cellular pathways, including autophagy, chaperone function, endocytosis, and exocytosis [1]–[5]. The breakdown of these cellular processes manifests during aging and exposure to stress, which is in part due to the breakdown of the cytoskeleton under these conditions [5]–[9]. The necessity of a properly maintained cytoskeleton in cellular homeostasis is evident in its requirement for a number of critical processes, yet the mechanisms involved in preservation of cytoskeletal form and function are not well understood.

One primary mechanism by which the cell protects its cytoskeleton during stress is through the heat shock response (HSR), mediated by the heat shock transcription factor, HSF-1. HSF-1 is activated under thermal stress and promotes protein homeostasis through upregulation of chaperones and other genes related to protein quality control [10]. Overexpression of HSF-1 is sufficient to confer thermal stress and increase lifespan in *C. elegans*, and alleviates the toxic effects associated with aging. Our lab has identified that HSF-1 also has the capacity to upregulate genes involved in maintenance of the actin cytoskeleton, including the troponin C/calmodulin homolog, *pat-10*, which is both sufficient and necessary for HSF-1-mediated thermotolerance and longevity [11]. However, many actin regulating genes are not bonafide targets of HSF-1, suggesting that other master regulators must exist, which function independently or in parallel with HSF-1 to modulate actin. Indeed, a closer look at cytoskeletal targets upregulated in HSF-1 overexpressers are primarily involved in formation of actin bundles, which are filaments of F-actin crosslinked into parallel bundles [11]. However, genes involved in forming actin networks, such as components of the ARP2/3 complex, are notably absent from our HSF-1 transcriptome datasets. It is clear that the regulation of actin and its many binding partners required for its function is extremely complex, and is through a concerted effort of multiple transcriptional regulators.

In this proposal, I will apply novel techniques to interrogate cytoskeletal quality during stress and aging in an effort to dissect the nature of cytoskeletal regulation. I employ multi-pronged genetic screening in an effort to identify critical transcriptional regulators involved in cytoskeletal regulation, and couple this with a biochemical approach to identify the molecular interactome for cytoskeletal regulation. Moreover, I propose to perform these experiments in a tissue-specific manner to understand the differences in actin regulatory proteins that allow a single protein to behave in massively different ways across tissue types. Finally, I will identify the extent to which HSF-1 coordinates changes in actin behavior and identify upstream and downstream requirements for HSF-1 signaling to preserve cytoskeletal quality. This work will not only increase the understanding of genes and proteins required for cytoskeletal preservation, but will increase the understanding of the requirement of actin filament structure and function in the collapse of homeostasis and health during organismal aging.

Innovation. Recently, interest has increased in the mechanistic breakdown of the actin cytoskeleton throughout aging. Indeed, there exists multiple studies that correlate cytoskeletal quality with aging: premature aging models in *S. cerevisiae* show declines in cytoskeletal integrity [6]; a study characterizing tissue-specific decline in cellular fitness in *C. elegans* as a function of age identified defects in actin organization in aged muscle cells [12]; loss of regulation of filamentous (F) actin in aged T cells prevents its activation in mammalian cells [13]; identification of cytoskeletal elements in proteome-wide analyses of age-associated changes in protein abundance in human fibroblasts [14]; and stabilization of the actin cytoskeleton results in increased lifespan in *C. elegans* [11]. *Through these indirect studies, it has been accepted as common knowledge that the actin cytoskeleton collapses as a function of age, although a direct and exhaustive characterization of actin cytoskeletal decline in multi-cellular organisms as a function of age has not been performed.* A major part of the absence of these essential studies is the lack of tools available to interrogate cytoskeletal integrity *in vivo*. I have developed a system whereby cytoskeletal integrity can be visualized specifically in multiple-tissue types in live *C. elegans*. Using these tools, *I obtained the first direct evidence that actin structure and integrity decline during late adulthood in multiple tissue types*, confirming the long-standing theory that actin faces an age-associated collapse.

My currently proposed studies are unique in that I use these novel tools to directly identify genes essential for cytoskeletal regulation. Moreover, I propose to adapt these tools even further for utility in biochemical assays to identify molecular interactors of actin in an effort to identify essential proteins for cytoskeletal regulation. While our methods are initially screen-heavy, I have already completed all the genetic screens and have even performed initial secondary and tertiary screening. To this end, I have the platforms for direct interrogation of the requirement of identified hits in regulation of actin under normal and aging physiologies. Finally, I ask direct and important questions to merge the fields of actin and aging: 1) to identify a critical chromatin and metabolic state that promotes transcriptomes characteristic of increased actin health, 2) characterization of molecular interactomes that allow for a virtually identical actin protein to function in extraordinarily different manners across

tissue, and 3) to dissect the node of cellular stress response involved in preserving actin under conditions of stress and aging.

Preliminary data. *I have created a system to interrogate cytoskeletal quality in vivo in C. elegans* in a tissue-specific manner by adapting the LifeAct system into *C. elegans*. LifeAct is a 17-amino acid peptide, which binds reliably to F-actin, and fusion of LifeAct to the fluorophore, mRuby, allows robust visualization of the actin cytoskeleton *in vivo* [15]. I created stable transgenic lines in which a single copy of LifeAct-mRuby is expressed under the control of a tissue-specific promoter in the muscle (*myo-3p*), intestine (*gyl-19p*), and the hypodermis (*col-19p*) (Fig. 1A). This peptide is sufficient to target mRuby to F-actin and exhibits none of the detrimental consequences caused by traditional visualization of actin, including actin fusions to fluorescent proteins or toxic dyes, like phalloidin [16], [17]. Using these newly synthesized strains, I performed the *first comprehensive analysis of changes to actin filaments during aging in C. elegans* [18]. I found that the actin cytoskeleton is structurally different across tissue at young age and throughout aging.

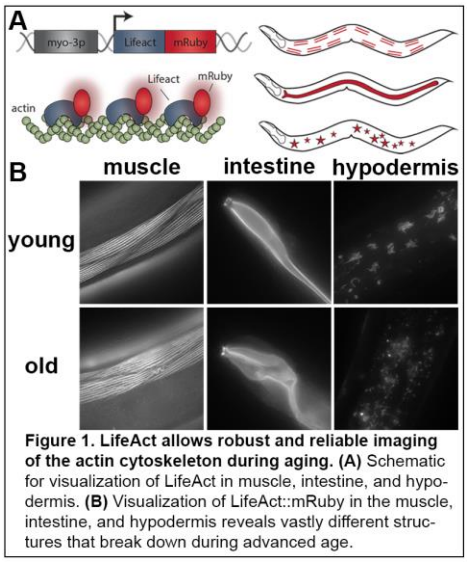


Figure 1. LifeAct allows robust and reliable imaging of the actin cytoskeleton during aging. (A) Schematic for visualization of LifeAct in muscle, intestine, and hypodermis. **(B)** Visualization of LifeAct::mRuby in the muscle, intestine, and hypodermis reveals vastly different structures that break down during advanced age.

The actin cytoskeleton in the muscle is visualized as linear striations of filamentous actin parallel to muscle fibers. Intestinal actin is visualized as two filaments along the intestinal lumen with more complex structures where it meets the pharynx and anus. The outer hypodermal layer under the cuticle of the worm has a large amount of actin throughout adulthood, but the inner hypodermal syncytium has punctate star-like cytoskeletal structures, which appear during early adulthood and resolve in late adulthood. All three tissue types show progressive decline of the cytoskeleton throughout aging, such that the structure and organization of actin is dramatically different between young and old worms (Fig. 1B).

I have found that HSF-1 plays a critical role in regulation of actin. Previous work from the lab has shown that HSF-1 can exert a beneficial effect on lifespan by transcriptional regulation of cytoskeletal genes, such as *pat-10* [11]. However, these studies lacked direct evidence that HSF-1 can modulate cytoskeletal integrity during aging, due to the lack of tools available. Using the LifeAct technology, *I have shown direct evidence that HSF-1 is essential for cytoskeletal maintenance.* Specifically, I showed that knockdown of *hsf-1* results in a premature decline in cytoskeletal integrity in muscle, intestine, and hypodermis during aging (Fig. 2A). Moreover, overexpression of *hsf-1* is sufficient to protect actin integrity, such that animals with *hsf-1* overexpression have more subtle defects in cytoskeletal integrity at late adulthood when wild-type animals exhibit major defects (Fig. 2B). Finally, I find that overexpression of *hsf-1* specifically in neurons is sufficient to protect cytoskeletal integrity in non-neuronal tissue (data not shown, but similar to Fig. 2B).

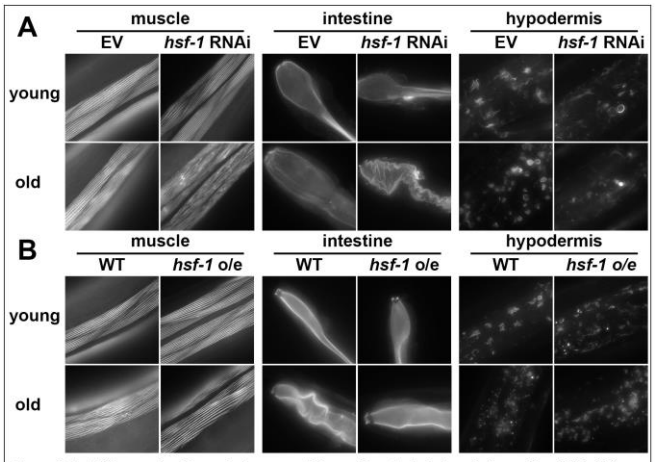


Figure 2. *hsf-1* is required for maintenance of the actin cytoskeleton during aging. (A) *hsf-1* RNAi results in premature breakdown of actin cytoskeletal integrity, such that at an age where empty vector (EV) control animals exhibit only mild cytoskeletal defects, *hsf-1* RNAi animals have grotesque defects in actin structure and organization. **(B)** Overexpression (o/e) of *hsf-1* in specific tissues results in protection of the actin cytoskeleton at old age where wild-type (WT) animals exhibit obvious abrogation of cytoskeletal integrity.

Research Plan. Aim 1: How does chromatin remodeling and lipid homeostasis regulate actin organization and function? *I hypothesize that a core machinery exists to preserve the actin cytoskeleton under conditions of stress and aging through broad, but specific changes to chromatin state.* The quantitative level of actin is under very tight regulation across various conditions, yet the proper function of the actin cytoskeleton is dependent on the exceptional dynamic properties of the protein. This dichotomy is explained by the large number of proteins involved in regulating actin dynamics, including actin-binding proteins and factors involved in its polymerization and depolymerization. I hypothesize that this large subset of genes fall under the control of master transcriptional regulators, allowing robust activation of a large subset of actin regulatory genes through modulation of a few transcription factors. HSF-1 functions as one of these transcription factors to preserve the cytoskeleton under conditions of heat-stress by promoting the expression of genes involved in increasing actin stability, including the troponin C homolog, *pat-10*, and the tropomyosin, *lev-11*.

Despite the identification of HSF-1 as a bonafide regulator of the actin cytoskeleton, a closer look at its downstream targets reveal that many critical components of actin maintenance are absent. In an effort to identify additional master regulators of the actin cytoskeleton, I performed a multi-pronged genetic screening approach (Fig. 3). I utilized 1) *in vivo* live-cell imaging of actin filaments to identify transcription factors required for cytoskeletal maintenance, 2) whole genome synthetic lethality screening to identify genetic interactors of *hsf-1* in *C. elegans*, 3) transcriptome analysis of *C. elegans* harboring a toxic, but sub-lethal dose of actin knockdown, and 4) whole genome CRISPR-Cas9 screening in human BJ fibroblasts to identify genes that are essential for survival under sub-lethal doses of the actin destabilizing agent, cytochalasin D. These screens have identified a number of candidate genes. Cross-referencing these rich datasets has revealed two critical nodes of genes: 1) modifiers of chromatin state and their downstream transcriptional regulators and 2) genes involved in lipid storage and global lipid homeostasis. The identification of various genes with overlapping functions across the screens provides a high level of confidence in these datasets.

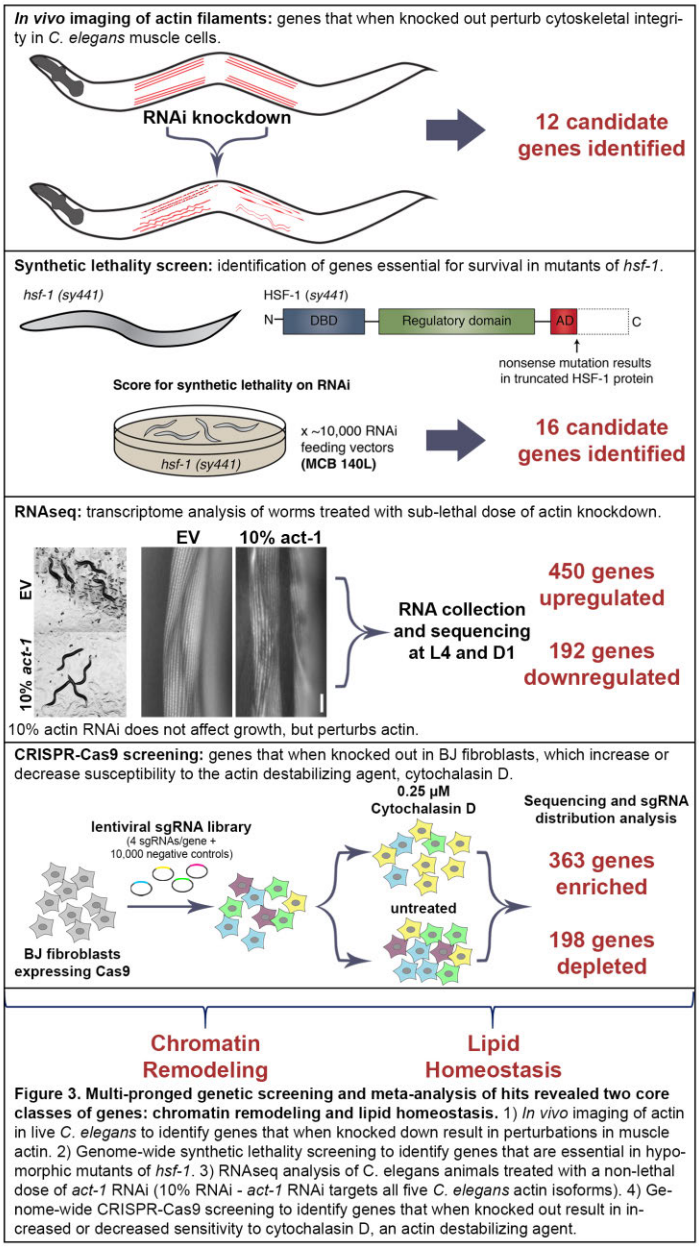


Figure 3. Multi-pronged genetic screening and meta-analysis of hits revealed two core classes of genes: chromatin remodeling and lipid homeostasis. 1) *In vivo* imaging of actin in live *C. elegans* to identify genes that when knocked down result in perturbations in muscle actin. 2) Genome-wide synthetic lethality screening to identify genes that are essential in hypomorphic mutants of *hsf-1*. 3) RNAseq analysis of C. elegans animals treated with a non-lethal dose of *act-1* RNAi (10% RNAi - *act-1* RNAi targets all five *C. elegans* actin isoforms). 4) Genome-wide CRISPR-Cas9 screening to identify genes that when knocked out result in increased or decreased sensitivity to cytochalasin D, an actin destabilizing agent.

<u>*Aim 1.1: How do chromatin modifiers alter actin form and function during aging?*</u> To dissect the complex architecture involved in transcriptional regulation of genes involved in cytoskeletal maintenance, I propose to test the requirement of the identified chromatin modifiers in regulation of the actin cytoskeleton. *I hypothesize that a specific chromatin state is maintained to preserve a transcriptional profile, which promotes a healthy actin cytoskeleton.* Moreover, aging results in breakdown of essential chromatin modifiers, which alters chromatin state, preventing efficient transcriptional regulation of the core cytoskeletal machinery, ultimately resulting in poor actin dynamics and the physiological consequences of aging. Our screens have revealed that major chromatin modifiers, such as the MYST histone acetyl transferases (HATs), are essential for maintaining a proper cytoskeletal state. I also identified, *bet-1*, a gene encoding a bromodomain protein, which exerts its transcriptional program by binding to acetylated histones that are maintained by MYST HATs [20].

<u>*Aim 1.1a (K99): Can BET-1 preserve actin filaments during the aging process?*</u> I have validated BET-1 as a bonafide cytoskeletal regulator, as knockdown or CRISPR-mediated knockout of *bet-1* results in increased sensitivity of actin to perturbation by heat (Fig. 4A). In addition, *bet-1* knockdown animals have a significant decrease in lifespan (Fig. 4B). Therefore, *I hypothesize that BET-1 function is essential for a healthy lifespan through its role in cytoskeletal maintenance, and that MYST HATs produce a chromatin state that allows BET-1 to exert its transcriptional program.* To further investigate the role of BET-1 on cytoskeletal health, I will quantitatively and qualitatively analyze tissue-specific patterns of actin filament structure and integrity using our LifeAct-mRuby lines. Filament structure and integrity will be qualitatively measured *in vivo* in muscle, intestine, and hypodermis of animals with knockdown or overexpression of *bet-1* throughout age at Day 1, D4, D7, D10, and D13. Moreover, quantitative analysis can be performed in a tissue-specific matter using measurements that have already been characterized in the lab to decline with age and be correlated with healthy actin ([18]; unpublished data): 1) thickness of muscle actin filaments measured as width of peaks at mid-height across a line profile (see similar quantifications in [21]); 2) integrated fluorescent intensity of hypodermal actin measured using a COPAS large particle biosorter (see similar quantifications in [22]); 3) indirect measurement of intestinal

filamentous actin by measuring the ratio of fluorescent intensity of LifeAct localized to filaments versus cytoplasmic signal; and 4) dynamics of actin measured using fluorescent recovery after photobleaching (FRAP) of LifeAct signal in star-like hypodermal actin structures. All knockdown experiments will be validated in null mutants synthesized by CRISPR-Cas9-mediated gene editing (experiments will first be performed in *bet-1* RNAi knockdown animals because RNAi knockdown has stronger phenotypes than *bet-1* loss of function [lof] animals, likely due to adaptation occurring upon chronic loss of this master transcriptional regulator across generations). I predict that across these measurements, *bet-1* knockdown will result in phenotypes associated with premature breakdown of the actin cytoskeleton, while overexpression of *bet-1* will protect the cytoskeleton in late age.

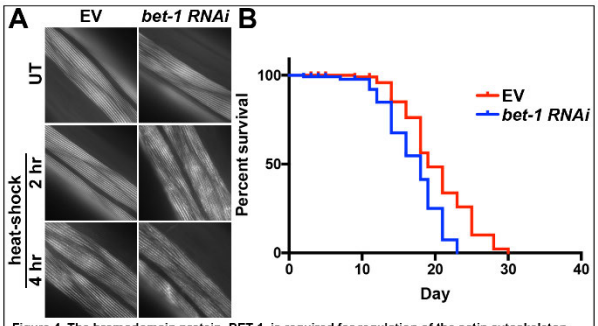


Figure 4. The bromodomain protein, BET-1, is required for regulation of the actin cytoskeleton and lifespan. RNAi-knockdown of *bet-1* results in increased sensitivity of the actin cytoskeleton to heat-induced stress **(A)** and decreased lifespan **(B)** compared to EV controls. Null mutants of *bet-1* created by CRISPR-Cas9 gene-editing yielded similar results.

To determine whether BET-1's role in cytoskeletal integrity alters organismal physiology, I will first determine whether overexpression of *bet-1* can extend lifespan and survival under thermal stress, since its knockdown decreases lifespan and increases sensitivity to thermal stress. If *bet-1* overexpression is sufficient to extend lifespan, I will determine whether BET-1-mediated lifespan extension is dependent on proper actin function to eliminate the possibility that BET-1 alters physiology through an alternative, actin-independent function. I will treat *bet-1* overexpressing strains with RNAi targeting actin and known actin-regulating genes, such as tropmyosins, the ARP2/3 complex, and formins, to determine whether loss of mRNA against broad and specific actin regulatory genes is sufficient to attenuate the lifespan extension found in *bet-1* overexpressing animals. *CAVEAT*: if *bet-1* overexpression does not extend lifespan, these proposed experiments can be done in *bet-1* knockdown or lof animals to determine whether their effects on lifespan or survival under thermal stress is additive (independent mechanisms) or not (similar mechanisms).

Aim 1.1b (K99): Is BET-1's role in cytoskeletal maintenance dependent on MYST HAT-mediated chromatin remodeling? Several MYST HATs were identified as genes essential for survival under actin destabilizing conditions in our screens. MYST HATs target H4 [23], [24] and their histone acetylation function have been ascribed to transcriptional activation in drosophila [25] and yeast [26]. In *C. elegans*, there are four primary MYST HATs, *mys-1, mys-2, mys-3, and mys-4*, and two putative MYST HATs, *C34B7.1* and *C34B7.2*. *mys-1* and *mys-2* are upstream of BET-1 and acetylate H4 at K5, K8, K12, and K16 [20]. These acetylation states allow BET-1 binding to H4 and subsequent transcriptional activation to promote cell-fate decisions.

To determine whether BET-1 function in cytoskeletal maintenance follows the same molecular mechanism as cell-fate decisions, I propose to interrogate cytoskeletal quality in animals with *mys-1* and *mys-2* knockdown using similar strategies proposed in Aim 1.1a. If BET-1 impacts actin health through its canonical role as a transcription factor, knockdown of either *mys-1* or *mys-2*, which prevents localization of BET-1 to DNA, should mimic defects in cytoskeletal health and lifespan, similar to *bet-1* knockdown. Moreover, knockdown of *bet-1* should not further abrogate cytoskeletal health or lifespan in *mys-1* or *mys-2* knockdown animals. Finally, if I see that *bet-1* overexpression improves cytoskeletal quality and lifespan in Aim 1.1a, I propose to extend these studies to determine whether *mys-1* or *mys-2* knockdown can suppress the beneficial effects found in *bet-1* overexpressing animals. As an extension to this aim, I will also test the role of the two putative MYST HATs, *C34B7.1* and *C34B7.2* in regulation of actin and lifespan as these genes also came up in our screens.

Aim 1.1c (K99): Does functional activity of BET-1 decline during aging? The functional activity of MYS-1 and MYS-2 is essential in maintaining BET-1 localization to the nucleus where it exerts its function as a transcription factor [20]. *It is possible that defects in actin during aging are due, at least in part, to functional decline of BET-1 during the aging process*. To test this hypothesis, I will determine whether nuclear localization of BET-1 declines during aging. A previous study has shown that BET-1::GFP fusion proteins maintain full function and its nuclear localization is a reliable marker for its transcriptional activity [20]. I will tag BET-1 with GFP at its genomic locus using CRISPR-Cas9 and will qualitatively and quantitatively assess BET-1::GFP localization in animals at D1, D4, D7, D10, and D13. To directly test BET-1 transcriptional activity, I propose to perform ChIP-seq (chromatin immunoprecipitation sequencing) of BET-1 (all ChIP-seq will be performed with the guidance of Dr. Michael Eisen – see letter of support). It is likely that during the aging process, BET-1 transcriptional activity is perturbed via decreased BET-1 recruitment to loci coding for actin-regulatory proteins, which results in the breakdown of the cytoskeleton with age. If this holds true, BET-1 function may decline due to the functional breakdown of MYS-1 and MYS-2 in maintaining a core chromatin state for BET-1 binding. Thus, I will test whether any measured breakdown of BET-1 function can be rescued by overexpression of *mys-1* or *mys-2*, and whether this can protect the actin cytoskeleton during aging to promote organismal lifespan.

Aim 1.1d (K99): Does there exist a core chromatin state that promotes a healthy actin transcriptome? I predict that MYST HATs function in maintaining a core chromatin state that promotes transcriptional activation of critical actin regulators through BET-1 and/or other transcription factors. To test this hypothesis, I propose to study chromatin state and transcriptomes of animals harboring deletion or overexpression of MYST HATs. I propose to perform ATAC-seq (assay for transposase-accessible chromatin using sequencing) on animals harboring knockdown of *mys-1* or *mys-2* to understand global changes in chromatin availability. ATAC-seq has recently been developed for adult *C. elegans*, and have been shown to provide robust and reproducible data and understanding of general chromatin accessibility throughout the developmental stages [27] (all ATAC-seq will be performed with the guidance of Dr. Michael Eisen). Studying global chromatin accessibility in animals with knockdown of *mys-1* and *mys-2* will give us an understanding of chromatin state under conditions when BET-1 is functionally inactive. I will also perform RNAseq in these animals to determine whether major transcriptional changes exist in cytoskeletal genes (all sequencing will be performed at the Vincent J. Coates Genomics Sequencing Laboratory and data analysis will be done using Galaxy and Matlab software with the help of the lab manager, Larry Joe, and postdoc, Raz Bar-Ziv, and members of the Eisen lab). This work may reveal putative transcription factor binding motifs or enhancers that exist that are shared amongst a subset of cytoskeletal genes.

Aim 1.2 (R00): How does lipid metabolism alter cytoskeletal integrity? Largely, aberrant metabolic state affects cytoskeletal regulation. For example, improper nutrient availability can perturb cytoskeletal dynamics through mTORC1 signaling [28]. This is unsurprising due to the high energy demands of maintaining proper cytoskeletal form and function. In our screens, I have found that several regulators of general metabolism – with an enrichment of genes involved in lipid metabolism – were essential for proper function of the actin cytoskeleton. Interestingly, I have found that RNAi knockdown of the *daf-16*/FOXO transcription factor, involved in insulin signaling, perturbs actin filament organization in the muscle. Our previous work has identified that *daf-16* is critical for the beneficial effects of non-autonomous HSF-1 signaling [29]. I have also identified *nhr-49*/PPARα, previously characterized for its role in β-oxidation and fatty-acid desaturation [30] (Fig. 5A).

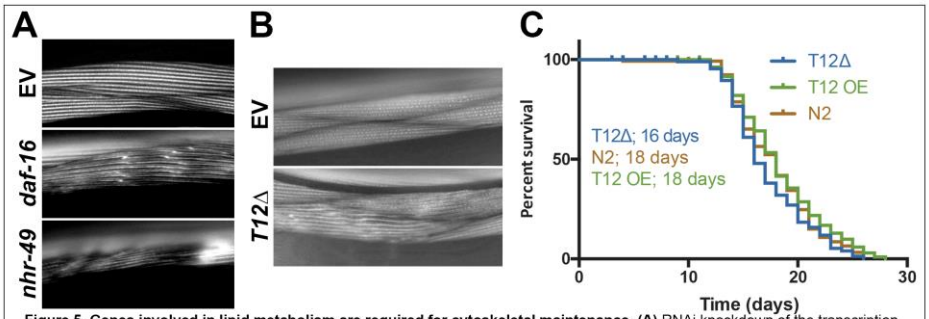


Figure 5. Genes involved in lipid metabolism are required for cytoskeletal maintenance. (A) RNAi knockdown of the transcription factors, *daf-16* and *nhr-49* result in perturbations of muscle actin. **(B)** lof mutants of *T12A2.1* (T12Δ) have defects in muscle actin structure. (C) lof mutants of *T12A2.1* (T12Δ) have decreased lifespan, but overexpression of *T12A2.1* (T12 OE) under its endogenous promoter does not affect lifespan.

Aim 1.2a (R00): Are master regulators of lipid homeostasis required for cytoskeletal health during aging? Our previous work identified a link between *hsf-1* and *daf-16*; however, this is the first time that a direct relationship between actin filaments and *daf-16* has been identified. Similarly, while energy deprivation and ATP availability have been correlated with actin function, no direct relationship between *nhr-49*/PPARα has been described. Importantly, overexpression of *daf-16* and *nhr-49* extends lifespan in *C. elegans* [30], [31]. Therefore, *I hypothesize that DAF-16 and NHR-49 impart a beneficial effect on lifespan through its role in regulation of actin.* To test this hypothesis, I will measure actin form and function during the aging process in animals with *daf-16* or *nhr-49* overexpression as described in Aim 1.1a. Moreover, I will determine whether *daf-16* and *nhr-49*-mediated lifespan extension is dependent on functional actin by determining whether RNAi knockdown of actin regulators suppress the lifespan effect of *daf-16* or *nhr-49* overexpression. In parallel, I will determine whether mutants of *daf-2*, the IGF-1/IIS receptor, also protect cytoskeletal health late into adulthood, as these mutants exhibit one of the highest recorded lifespans by activating DAF-16 [32].

I have also identified *dod-21*, a downstream target of DAF-16 to be highly upregulated in animals with dysfunctional actin. Although it has been shown that *dod-21* is massively upregulated in long-lived *daf-2* mutant animals, a functional role has not been ascribed to DOD-21. Therefore, it is possible that DAF-16 imparts its role in cytoskeletal function through DOD-21, either in concert with or independent of NHR-49. To test this hypothesis, I will determine whether DAF-16 and NHR-49-mediated lifespan extension is dependent on DOD-21. Moreover, I will determine whether *dod-21* overexpression is sufficient to protect cytoskeletal integrity and extend lifespan.

Aim 1.2b (R00): Does lipid homeostasis exert its effects on actin and aging through chromatin regulation? In my multi-pronged genetic screens utilizing highly variable and independent strategies, I found enrichment in two molecular pathways: chromatin remodeling and lipid homeostasis. There exist a growing body of evidence that lipids can indirectly impact chromatin states (reviewed in [33]), potentially linking these seemingly disparate classes of genes. For example, fatty acid oxidation acts as a source of acetyl-CoA, which can be utilized by HATs in histone acetylation [34]. Thus, *there is a possible linear mechanism by which NHR-49, DAF-16, etc.*

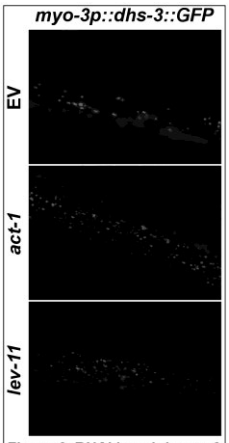


Figure 6. RNAi knockdown of *act-1* or the tropomyosin, *lev-11*, results in increased lipid droplet numbers in the muscle.

alter lipid metabolism to promote chromatin remodeling by MYS-1, MYS-2, etc., allowing BET-1 and other transcription factors to create a healthy actin transcriptome. To test this hypothesis, I propose both a directed and broad interrogation of chromatin state in animals with altered lipid homeostasis. First, I will test whether knockdown or overexpression of *nhr-49* alters H4 acetylation. As stated above, MYS-1 and MYS-2 exert their primary function by acetylating H4 at K5, K8, K12, and K16, and it is possible that NHR-49-mediated upregulation of beta-oxidation is a critical source of acetyl-CoA for acetylation of H4 by these HATs, such that knockdown of *nhr-49* will result in decreased H4 acetylation, and overexpression of *nhr-49* will result in increased H4 acetylation. H4 acetylation status will be tested by H4 immunoprecipitation followed by mass-spec [24]. I will extend these studies to animals with knockdown or overexpression of *daf-16* to determine whether DAF-16 can affect histone acetylation. In addition, I plan to perform BET-1 localization experiments and ChIP-seq of BET-1 as described in Aim 1.1c to determine whether NHR-49/DAF-16 modulates BET-1's transcriptional activity. *Caveat*: If changes in *nhr-49/daf-16* expression does not alter H4 acetylation or BET-1's function, it is still possible that *nhr-49/daf-16* alters larger, global chromatin states, which may affect cytoskeletal health independent of MYS-1/MYS-2/BET-1. Therefore, I propose to interrogate global changes in chromatin state by performing ATAC-seq in animals with knockdown or overexpression of *nhr-49*/*daf-16.* These studies may reveal a potential mechanism merging the functional roles of lipid homeostasis and chromatin remodeling on cytoskeletal health.

Aim1.2c (R00): Is functional actin required for maintaining proper lipid homeostasis during aging? Lipid dysregulation and increased accumulation of lipid droplets is a bonafide marker of aging in multiple model systems. In our studies, I found that loss of genes involved in global lipid homeostasis, such as *nhr-49*, *daf-16*, and *T12A2.1*, result in defects in actin structural integrity (Fig. 5A-B). Previous reports have found that mutants of *nhr-49* have increased elevated fat content and decreased lifespan [35]; DAF-16 promotes longevity through transcriptional regulation of genes involved in both lipid synthesis and breakdown [36]; and mutants of *T12A2.1* have decreased lipid levels [37] and exhibit decreased lifespan (Fig. 5C). Aim 1.2a-b focus on studying the impact of lipid metabolism on actin health. Here, we propose to study the hypothesis that *a properly functional actin cytoskeleton is essential in regulation of lipid homeostasis, and that cytoskeletal breakdown can be a factor in lipid dysregulation during aging.*

A properly functional cytoskeleton is essential for many processes, such as mediating organelle dynamics of the mitochondria [6] and ER-mitochondria contacts [38], [39]. Moreover, F-actin/G-actin levels can alter signaling of the major transcriptional regulator, serum response factor (SRF), which can impact many different pathways including cell cycle, apoptosis, cell growth, and mitochondrial dynamics [40], [41]. Interestingly, I find that perturbations to the actin cytoskeleton via RNAi knockdown of actin itself or a tropomyosin, *lev-11*, results in increased lipid droplet numbers in the muscle (Fig. 6), which is a similar phenotype that I find in aged animals (data not shown). I propose to expand this study to measure organismal lipid levels in animals with defective actin cytoskeleton by using BODIPY and Nile Red staining using fluorescent microscopy and flow cytometry with a COPAS biosorter. Next, to determine whether protecting the cytoskeleton during aging can prevent an age-associated decline in lipid homeostasis, I propose to measure lipid droplet morphology and whole animal lipid levels in animals with a protected cytoskeleton during aging (using *hsf-1* overexpressing animals and any other overexpression line that I find in this study to protect the actin cytoskeleton into late adulthood). Finally, I will determine whether proper cytoskeletal function is required for NHR-49 and DAF-16-mediated effects on lipid homeostasis. In Aim 1.2a, I propose to test whether knockdown of essential cytoskeletal components could suppress the lifespan extension found in these animals. Here, I extend this study to determine whether knockdown of similar cytoskeletal components can perturb the lipid phenotypes found in these animals, including decreased fat content (measured using BODIPY and Nile Red staining) and increased β-oxidation and fatty acid desaturation (using qPCR against canonical targets upregulated by NHR-49 [30].

Aim 2: What is the nature of the neuronal communication to preserve the cytoskeleton in distal tissues via non-autonomous HSF-1? The nervous system is capable of sensing stress and initiating a protective transcriptional response in peripheral tissue. Moreover, it has been shown in many stress models that overexpression of a critical stress responsive transcription factor in the neurons, such as HSF-1, is sufficient to elicit a cytoprotective response within the entire animal to promote protein homeostasis and lifespan [19], [29], [42]. Recently, I have found that overexpression of *hsf-1* in neurons (heretofore referred to as neuronal *hsf-1*) is sufficient to protect the actin cytoskeleton in distal tissues during aging to the same extent as overexpression of *hsf-1* directly in the tissue (neuronal *hsf-1* showed virtually identical results to Fig. 2B). However, overexpression of *hsf-1* in peripheral tissues is not capable of signaling non-autonomously to other tissue to protect the

cytoskeleton (e.g. overexpressing *hsf-1* in muscle has no impact on intestinal or hypodermal actin) [18]. Therefore, in the case of HSF-1-mediated regulation of the cytoskeleton, only the neurons are capable of signaling distally to preserve cytoskeletal quality in peripheral tissues during aging. Here, I propose to dissect the molecular mechanism involved in non-autonomous HSF-1 signaling using the information gleaned in Aim 1.

Aim 2.1a (K99/R00): Does neuronal hsf-1 exert its effect on actin through chromatin remodeling? While I have shown that neuronal *hsf-1* is sufficient to protect cytoskeletal integrity in peripheral tissue, the mechanism underlying these phenotypic changes is still unknown. Here, I propose to test whether *neuronal hsf-1 exerts its functional role in cytoskeletal integrity by altering chromatin structure in peripheral tissue*, similar to the proposed function of MYS-1/MYS-2/BET-1-mediated cytoskeletal regulation. In plants, HSF-1 has been shown to cause chromatin remodeling via H3/H4 acetylation and H3 methylation to promote transcription initiation and elongation under conditions of heat stress [43]. Moreover, similar functions of HSF-1 in mediating chromatin opening to promote positive gene expression has been identified in mammalian models [44]. Here, I propose to test my hypothesis by determining whether major chromatin changes exist in peripheral tissue of neuronal *hsf-1* animals using a directed and broad approach: first, I will determine whether H4 acetylation status changes in these animals, then I will perform ATAC-seq to monitor global changes to chromatin state, with an initial focus on gene loci of cytoskeletal-related transcripts that I found upregulated in these animals (as described in Aim 1.1).

Aim 2.1b (K99/R00): Are bet-1 or mys-1/2 required for non-autonomous HSF-1 signaling to distal tissue? In addition to studying whether neuronal *hsf-1* can directly alter chromatin state in peripheral tissue, I propose to test *the requirement of the MYS-1/MYS-2/BET-1 paradigm in HSF-1-mediated cytoskeletal regulation.* Since HSF-1 has been shown to target H4 for acetylation similar to MYS-1/MYS-2, it is possible that neuronal *hsf-1* promotes BET-1 transcriptional activity in peripheral tissue. To test this, I will determine whether neuronal *hsf-1* can increase BET-1 nuclear localization, an H4-acetylation-dependent process that is a proxy for BET-1 activity. Moreover, I can perform BET-1 ChIP-seq similar to Aim 1.1c to test directly whether neuronal *hsf-1* affects BET-1 transcriptional activity. Finally, I propose to determine whether *bet-1*, *mys-1,* and *mys-2* are required for the beneficial effects of neuronal *hsf-1*. I will perform RNAi knockdown of *bet-1*, *mys-1*, and *mys-2*, and test whether this is sufficient to suppress the lifespan extension and actin preservation found in neuronal *hsf-1* animals.

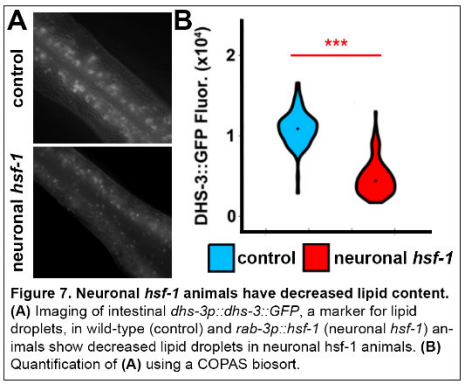


Figure 7. Neuronal *hsf-1* animals have decreased lipid content. **(A)** Imaging of intestinal *dhs-3p::dhs-3::GFP*, a marker for lipid droplets, in wild-type (control) and *rab-3p::hsf-1* (neuronal *hsf-1*) animals show decreased lipid droplets in neuronal hsf-1 animals. **(B)** Quantification of **(A)** using a COPAS biosort.

Aim 2.2 (R00): What is the epistatic relationship between nhr-49/daf-16 and non-autonomous HSF-1 signaling to distal tissue? In Aim 1, I describe preliminary results suggesting that NHR-49 and DAF-16 are each required for proper actin homeostasis. Moreover, previous work from our lab has shown that *daf-16* is required in peripheral tissue to mediate the beneficial response to HSF-1 signaling from neurons [29]. Finally, *nhr-49* is a direct target of DAF-16 [30], suggesting *a potential linear path of cytoskeletal regulation from HSF-1 to DAF-16 to NHR-49,* either directly or indirectly through its role in lipid regulation. Indeed, I find that neuronal *hsf-1* is sufficient to drive lipid depletion in peripheral tissue, similar to that found in *nhr-49* overexpression (Fig. 7). It is possible that this is mediated by DAF-16 and NHR-49 signaling downstream of HSF-1, and that this also drives cytoskeletal protection. To this end, I propose the following experiments: 1) *Is hsf-1 required for the effects of nhr-49 or daf-16 on actin homeostasis?* First, I will determine whether whole-animal overexpression of *hsf-1* is sufficient to mitigate the loss in actin filament integrity and shortened lifespan caused by *daf-16* and *nhr-49* RNAi. If I presume a linear relationship in which these transcription factors work downstream of HSF-1, the overexpression of *hsf-1* will be unable to rescue these deleterious phenotypes. 2) *Is neuronal hsf-1 signaling dependent on nhr-49 or daf-16 in the periphery?* Next, I will determine the impact of *nhr-49* and *daf-16* on non-autonomous *hsf-1* signaling. Previous work from our lab has shown that neuronal *hsf-1* drives upregulation of cytoskeletal targets, such as *pat-10* (data not shown). Using qPCR, I will validate whether actin regulatory targets of HSF-1 are differentially regulated by RNAi against these candidate transcription factors. Finally, I know from previous work that *daf-16* is required in peripheral tissues to respond to neuronal *hsf-1* signaling for chaperone induction [29]. Here, I will confirm whether a similar requirement is seen with *daf-16/nhr-49* for non-autonomous cytoskeletal regulation by testing the effect of *daf-16/nhr-49* knockdown on cytoskeletal protection and lifespan extension found in neuronal *hsf-1* animals.

Aim 2.3 (K99/R00): Are TFs required for actin homeostasis able to signal non-autonomously to distal tissue? As an extension to this aim, if overexpression of *nhr-49*, *daf-16,* or *bet-1* are sufficient to protect cytoskeletal integrity throughout aging, I propose additional studies where I will determine whether these genes can signal non-autonomously, similar to HSF-1. *I will overexpress nhr-49, daf-16, and bet-1 in neurons and determine whether this is sufficient to drive cytoskeletal protection in distal tissue and promote lifespan extension*.

In addition, I will test whether HSF-1 is required for the beneficial effects found in these animals. I will determine whether RNAi knockdown of *hsf-1* would abrogate the beneficial cytoskeletal or lifespan phenotypes found in both all-tissue and neuronal-specific overexpression of *nhr-49, daf-16,* and *bet-1*.

Aim 3: What is the molecular nature of the cytoskeleton resulting in major tissue-specific differences in cytoskeletal form and function? The nature and state of the actin cytoskeleton, both at young age and throughout aging, is significantly different between different tissue types, despite the actin protein being virtually identical. Therefore, I hypothesize that the actin cytoskeleton within each tissue is regulated by a unique set of molecular interactors, which break down at different rates and complexity during aging.

Aim 3.1: *Development of proximity-dependent protein labeling of actin (K99).* Proximity labeling using BirA and other similar biotin-tagging enzymes have been used to robustly map proteomes of specific cells, organelles, and subdomains without a need for purification or fractionation methodology [45], [46]. Recently, the Ting lab has performed directed evolution to produce miniTurbo, a mutant of biotin ligase with much greater efficiency in proximity labeling [47]. miniTurbo allows robust biotinylation of proteins in close proximity to the enzyme, with much less background and higher specificity than similar constructs. Therefore, I propose to employ a *biochemical interrogation of cytoskeletal interactors by utilizing miniTurbo in combination with LifeAct technology* (with the guidance of Dr. Alice Ting – see letter of support).

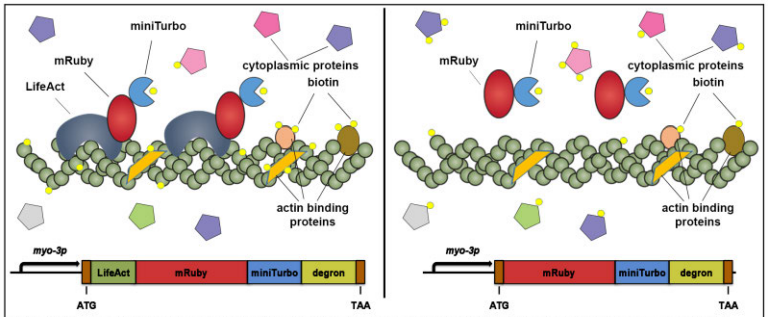


Figure 8. Proximity labeling of actin-binding proteins using LifeAct-miniTurbo system. LifeAct::mRuby::miniTurbo is expressed using tissue-specific promoters, such as myo-3p visualized here for the muscle. The construct will be degraded when animals are supplemented with auxin, and animals will be released from auxin to allow temporal resolution of proximity labeling. Cytoplasmic noise will be erased by subtracting proteins enriched in animals expressing mRuby::miniTurbo without LifeAct.

I have already established LifeAct in *C. elegans* as a robust and reliable method to visualize F-actin in a tissue-specific manner in live worms by fusion to a fluorescent molecule. I propose to adapt this technique by fusing LifeAct to miniTurbo (Fig. 8). I employ tissue-specific promoters as I did for our original LifeAct constructs for spatial control. For temporal control, I have attached a degron tag to the construct, which allows robust degradation of the protein when animals are supplemented with auxin [48]. Animals will be removed from auxin 24 hours prior to collection of protein to allow for synthesis of our construct and robust, yet temporally controlled, biotinylation of proteins. Currently, I have created transgenic lines carrying a single-copy integration of LifeAct::mRuby::miniTurbo::degron in the muscle, intestine, and hypodermis, and see that this larger construct still reliably localizes to the actin cytoskeleton without perturbations in cytoskeletal structure (data not shown). Currently, I am creating identical lines with LifeAct removed. This cytoplasmic mRuby::miniTurbo::degron system will allow us to identify cytoplasmic proteins, which can be removed from our dataset.

After confirming spatial and temporal control and efficacy of our miniTurbo constructs, I will purify biotinylated proteins to identify actin interacting partners using tandem mass tag (TMT) labeling coupled to quantitative MS/MS analyses. TMT labeling allows multiplexing of up to 10 samples in a single MS/MS run [49]–[51]. I will first test the ability to pulldown proteins that are in close proximity to our LifeAct construct by probing pulldowns with anti-actin antibodies, which I have verified to work robustly in the lab (data not shown). My LifeAct pulldowns should have massive enrichment for actin compared to the cytoplasmic pulldowns. Once I am confident in the pulldowns, I will subject the samples to TMT labeling and send them to UC Davis for MS/MS analysis. The proteomic analysis workflow will be done in full collaboration with Brett Phinney, head of the mass spectrometry core facility at UC Davis, who will perform the mass spectrometry and analysis using X! Tandem, Scaffold Q, and SafeQuant softwares (see attached letter of support). I will also work closely with the Ting lab to learn how to analyze mass spectrometry data independently as more data is collected.

Aim 3.2: What are the molecular interactomes that result in tissue-specific form and function of actin? I hypothesize that cross-comparison of molecular interactors of actin between different tissue types will reveal the critical interactome of actin required to exhibit tissue-specific form and function of immensely variable dynamics of actin. Indeed, I find that utilization of actin destabilizing drugs that target different forms of actin do not affect actin integrity equally in all tissues. For example, I find that the muscle actin is highly insensitive to CK-666, an arp2/3 inhibitor, and is only affected by long-term treatment with cytochalasin D, a drug that binds F-actin preventing polymerization of monomeric actin onto F-actin ends, and latrunculin A, a drug that sequesters g-actin to prevent further polymerization (data not shown). These data suggest that *actin in the muscle is less branched and more linear in nature, and is highly stable*. In contrast, hypodermal actin is highly sensitive to every actin destabilizing drug tested (CK-666, cytochalasin D, latrunculin A, and SMIFH2, a formin inhibitor), while intestinal actin is highly insensitive to these drugs. I also confirmed these results using genetic perturbations

of cytoskeletal regulators by RNAi. This suggests that *hypodermal actin is very dynamic and contains both branched and linear actin filaments, while intestinal actin is a unique and hyper-stable form of actin.*

To test these hypotheses, I will use the method developed in Aim 3.1 to identify molecular interactors of the actin cytoskeleton in a tissue-specific manner and across various time points in aging. I will start with the muscle, intestine, and hypodermis, which I have already verified have robust labeling of actin using LifeAct. I will perform pulldowns and mass-spec at young and old adult animals at various stages where I have already characterized a healthy and dysfunctional cytoskeletal network (see Fig. 1B for an example, and [18] for comprehensive analysis). For muscle and intestine, I will collect samples at D1 and D10, and for the hypodermis I will collect samples at D3 and D7. As a positive control, I will collect samples of young-age animals treated with a condition, which I have already validated perturbs cytoskeletal integrity to a similar extend to aging (tropomyosin knockdown for muscle, arp-2/3 inhibition for hypodermis, and actin knockdown for intestine).

To make these large datasets more accessible, our secondary analyses of our hits will be prioritized to genes that have known functions in actin regulation, but show significant differences across tissues. I will perform RNAi knockdown and tissue-specific overexpression of these candidate genes and measure their impact on cytoskeletal health during aging across the muscle, intestine, and hypodermis. For those genes that protect cytoskeletal integrity in at least one tissue when overexpressed, I will measure its impact on longevity.

Caveat: If no known actin regulatory proteins identified in this screen show tissue-specific differences, I will expand my studies to all proteins identified in the mass-spec analyses. While I expect these lists to be expansive, my lab has expertise in massive, large-scale screens to interrogate cytoskeletal quality as described in Aim 1. I can very quickly and efficiently perform RNAi screens to identify genes, that when knocked down, perturb cytoskeletal integrity and perform synthetic lethality screening with mutants of cytoskeletal regulators.

Aim 3.3: Do major differences exist in the molecular interactome involved in autonomous and non-autonomous protection of actin via HSF-1 signaling? As an extension to this aim, I propose to interrogate non-autonomous HSF-1 signaling and its impact on molecular interactors of actin. I have already shown that neuron-specific overexpression of HSF-1 is sufficient to protect cytoskeletal integrity through late age in multiple non-neuronal tissue, including muscle, intestine, and hypodermis. Therefore, I propose to perform *proximity labeling in animals with neuronal hsf-1 overexpression to determine what changes occur in the molecular interactome of actin in peripheral tissues of these long-lived animals.* Moreover, I question whether changes in molecular interactome differ across tissue types, despite an identical HSF-1 stress signal being received in each tissue.

Summary. Regulation of the actin cytoskeleton is a complex and multi-faceted process. This proposal uses an in depth approach to dissect the process of cytoskeletal regulation during aging. I will determine the role of chromatin remodeling and lipid homeostasis on cytoskeletal regulation and map the molecular interactome of the cytoskeleton in a tissue-specific manner. I will perform these studies under regular aging conditions, and long-lived paradigms of non-autonomous communication of *hsf-1* and other cytoskeletal regulators.

Rigor of experimental design. Across all experiments, rigorous testing methods will be used to ensure the most valid results possible. All experiments requiring qualitative measurements will be double-blinded, including lifespans and microscopy. All animals and samples will be assigned at random. All replicates and sample sizes will be provided for all analyses to provide transparency. All questions will be interrogated using multiple independent approaches to ensure against biases of a single method.

Timeline for Research Goals

Timeline	Aim	K99 Year 1	K99 Year 2	R00 Year 1	R00 Year 2	R00 Year 3
BET-1's role in actin homeostasis and lifespan	1.1	x				
MYS-1/2's role in actin homeostasis and lifespan regulation	1.1	x				
ChIP-seq of BET-1	1.1	x				
ATAC-seq on mutants of *mys-1* and *mys-2*	1.1	x	x			
Characterizing HSF-1/NHR-49/DAF-16 signaling modality	1.2			x	x	
DAF-16/DOD-21 signaling on actin health and lifespan regulation	1.2			x	x	
Epistatic relationship of TFs on actin homeostasis	1.2				x	x
Functional role of *hsf-1* in chromatin remodeling	2.1				x	x
Epistatic relationship between *hsf-1* and *nhr-49/daf-16*	2.2				x	x
Non-autonomous signaling of *bet-1/nhr-49/daf-16*	2.3		x		x	x
Generation and validation of turboID strains	3.1	x				
Tissue-specific mass-spec of actin using turboID	3.2	x	x			
Non-autonomous effects on actin using turboID	3.3		x	x		