RESEARCH
Department of Health and Human Services
National Institutes of Health

NIH ⟩
National Institutes
of Health

NATIONAL INSTITUTE OF ARTHRITIS AND MUSCULOSKELETAL AND SKIN DISEASES

Grant Number: 1R01AR075836-01
FAIN: R01AR075836

Principal Investigator(s):
Clarissa A Henry, PHD

Project Title: Mechanisms of NAD+ action during muscle development and homeostasis in a zebrafish dystroglycanopathy model

Christopher Boynton
5735 Hitchner Hall
University of Maine
Orono, ME 044695717

Award e-mailed to: umgrants@maine.edu

Period Of Performance:
Budget Period: 08/02/2019 – 07/31/2020
Project Period: 08/02/2019 – 07/31/2024

Dear Business Official:

The National Institutes of Health hereby awards a grant in the amount of $321,200 (see "Award Calculation" in Section I and "Terms and Conditions" in Section III) to UNIVERSITY OF MAINE ORONO in support of the above referenced project. This award is pursuant to the authority of 42 USC 241 42 CFR 52 and is subject to the requirements of this statute and regulation and of other referenced, incorporated or attached terms and conditions.

Acceptance of this award including the "Terms and Conditions" is acknowledged by the grantee when funds are drawn down or otherwise obtained from the grant payment system.

Each publication, press release, or other document about research supported by an NIH award must include an acknowledgment of NIH award support and a disclaimer such as "Research reported in this publication was supported by the National Institute Of Arthritis And Musculoskeletal And Skin Diseases of the National Institutes of Health under Award Number R01AR075836. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health." Prior to issuing a press release concerning the outcome of this research, please notify the NIH awarding IC in advance to allow for coordination.

Award recipients must promote objectivity in research by establishing standards that provide a reasonable expectation that the design, conduct and reporting of research funded under NIH awards will be free from bias resulting from an Investigator's Financial Conflict of Interest (FCOI), in accordance with the 2011 revised regulation at 42 CFR Part 50 Subpart F. The Institution shall submit all FCOI reports to the NIH through the eRA Commons FCOI Module. The regulation does not apply to Phase I Small Business Innovative Research (SBIR) and Small Business Technology Transfer (STTR) awards. Consult the NIH website http://grants.nih.gov/grants/policy/coi/ for a link to the regulation and additional important information.

If you have any questions about this award, please contact the individual(s) referenced in Section IV.

Sincerely yours,

Andrew Jones
Grants Management Officer
NATIONAL INSTITUTE OF ARTHRITIS AND MUSCULOSKELETAL AND SKIN DISEASES

Additional information follows

SECTION I – AWARD DATA – 1R01AR075836-01

Award Calculation (U.S. Dollars)

Federal Direct Costs	$220,000
Federal F&A Costs	$101,200
Approved Budget	$321,200
Total Amount of Federal Funds Obligated (Federal Share)	$321,200
TOTAL FEDERAL AWARD AMOUNT	$321,200
AMOUNT OF THIS ACTION (FEDERAL SHARE)	$321,200

SUMMARY TOTALS FOR ALL YEARS		
YR	**THIS AWARD**	**CUMULATIVE TOTALS**
1	$321,200	$321,200
2	$321,200	$321,200
3	$321,200	$321,200
4	$321,200	$321,200
5	$321,200	$321,200

Recommended future year total cost support, subject to the availability of funds and satisfactory progress of the project
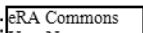
Fiscal Information:

CFDA Name:	Arthritis, Musculoskeletal and Skin Diseases Research
CFDA Number:	93.846
EIN:	1016000769A1
Document Number:	RAR075836A
PMS Account Type:	P (Subaccount)
Fiscal Year:	2019

IC	CAN	2019	2020	2021	2022	2023
AR	8472466	$321,200	$321,200	$321,200	$321,200	$321,200

Recommended future year total cost support, subject to the availability of funds and satisfactory progress of the project

NIH Administrative Data:
PCC: 2 B / **OC**: 414A / **Released:** eRA Commons 07/29/2019
Award Processed: 08/02/2019 12:04:49 AM

SECTION II – PAYMENT/HOTLINE INFORMATION – 1R01AR075836-01

For payment and HHS Office of Inspector General Hotline information, see the NIH Home Page at http://grants.nih.gov/grants/policy/awardconditions.htm

SECTION III – TERMS AND CONDITIONS – 1R01AR075836-01

This award is based on the application submitted to, and as approved by, NIH on the above-titled project and is subject to the terms and conditions incorporated either directly or by reference in the following:

a. The grant program legislation and program regulation cited in this Notice of Award.
b. Conditions on activities and expenditure of funds in other statutory requirements, such as those included in appropriations acts.
c. 45 CFR Part 75.
d. National Policy Requirements and all other requirements described in the NIH Grants Policy Statement, including addenda in effect as of the beginning date of the budget period.
e. Federal Award Performance Goals: As required by the periodic report in the RPPR or in the final progress report when applicable.
f. This award notice, INCLUDING THE TERMS AND CONDITIONS CITED BELOW.

(See NIH Home Page at http://grants.nih.gov/grants/policy/awardconditions.htm for certain references cited above.)

Research and Development (R&D): All awards issued by the National Institutes of Health (NIH) meet the definition of "Research and Development" at 45 CFR Part§ 75.2. As such, auditees should identify NIH awards as part of the R&D cluster on the Schedule of Expenditures of Federal Awards (SEFA). The auditor should test NIH awards for compliance as instructed in Part V, Clusters of Programs. NIH recognizes that some awards may have another classification for purposes of indirect costs. The auditor is not required to report the disconnect (i.e., the award is classified as R&D for Federal Audit Requirement purposes but non-research for indirect cost rate purposes), unless the auditee is charging indirect costs at a rate other than the rate(s) specified in the award document(s).

An unobligated balance may be carried over into the next budget period without Grants Management Officer prior approval.

This grant is subject to Streamlined Noncompeting Award Procedures (SNAP).

This award is subject to the requirements of 2 CFR Part 25 for institutions to receive a Dun & Bradstreet Universal Numbering System (DUNS) number and maintain an active registration in the System for Award Management (SAM). Should a consortium/subaward be issued under this award, a DUNS requirement must be included. See http://grants.nih.gov/grants/policy/awardconditions.htm for the full NIH award term implementing this requirement and other additional information.

This award has been assigned the Federal Award Identification Number (FAIN) R01AR075836. Recipients must document the assigned FAIN on each consortium/subaward issued under this award.

Based on the project period start date of this project, this award is likely subject to the Transparency Act subaward and executive compensation reporting requirement of 2 CFR Part 170. There are conditions that may exclude this award; see http://grants.nih.gov/grants/policy/awardconditions.htm for additional award applicability information.

In accordance with P.L. 110-161, compliance with the NIH Public Access Policy is now mandatory. For more information, see NOT-OD-08-033 and the Public Access website: http://publicaccess.nih.gov/.

In accordance with the regulatory requirements provided at 45 CFR 75.113 and Appendix XII to 45 CFR Part 75, recipients that have currently active Federal grants, cooperative agreements, and procurement contracts with cumulative total value greater than $10,000,000 must report and maintain information in the System for Award Management (SAM) about civil, criminal, and administrative proceedings in connection with the award or performance of a Federal award that reached final disposition within the most recent five-year period. The recipient must also make semiannual disclosures regarding such proceedings. Proceedings information will be made publicly available in the designated integrity and performance system (currently the Federal Awardee Performance and Integrity Information System (FAPIIS)). Full reporting requirements and procedures are found in Appendix XII to 45 CFR Part 75. This term does not apply to NIH fellowships.
Treatment of Program Income:
Additional Costs

SECTION IV – AR Special Terms and Conditions – 1R01AR075836-01

Clinical Trial Indicator: No

This award does not support any NIH-defined Clinical Trials. See the NIH Grants Policy Statement Section 1.2 for NIH definition of Clinical Trial.

COMPETING FUNDING LEVEL
This award includes funding adjustments in accordance with current NIAMS FY2019 policy.

SHORTENED FIRST YEAR BUDGET
This award includes funds for twelve months of support. The competing budget period is awarded for less than 12 months. Continuation of this award will cycle each year on August1st.

STAFF CONTACTS

The Grants Management Specialist is responsible for the negotiation, award and administration of this project and for interpretation of Grants Administration policies and provisions. The Program Official is responsible for the scientific, programmatic and technical aspects of this project. These individuals work together in overall project administration. Prior approval requests (signed by an Authorized Organizational Representative) should be submitted in writing to the Grants Management Specialist. Requests may be made via e-mail.

Grants Management Specialist: T. Erik Edgerton
Email: edgertont@mail.nih.gov **Phone**: 301-594-7760

Program Official: Thomas Cheever
Email: Thomas.Cheever@nih.gov **Phone**: (301) 594-5019 **Fax**: (301) 480-1284

SPREADSHEET SUMMARY
GRANT NUMBER: 1R01AR075836-01

INSTITUTION: UNIVERSITY OF MAINE ORONO

Budget	Year 1	Year 2	Year 3	Year 4	Year 5
TOTAL FEDERAL DC	$220,000	$220,000	$220,000	$220,000	$220,000
TOTAL FEDERAL F&A	$101,200	$101,200	$101,200	$101,200	$101,200
TOTAL COST	$321,200	$321,200	$321,200	$321,200	$321,200

Facilities and Administrative Costs	Year 1	Year 2	Year 3	Year 4	Year 5
F&A Cost Rate 1	46%	46%	46%	46%	46%
F&A Cost Base 1	$220,000	$220,000	$220,000	$220,000	$220,000
F&A Costs 1	$101,200	$101,200	$101,200	$101,200	$101,200

PI: **Henry, Clarissa A**	Title: Mechanisms of NAD+ action during muscle development and homeostasis in a zebrafish dystroglycanopathy model	
Received: 10/05/2018	FOA: PA18-484 Clinical Trial:Not Allowed	Council: 05/2019
Competition ID: FORMS-E	FOA Title: NIH Research Project Grant (Parent R01 Clinical Trial Not Allowed)	
1 R01 AR075836-01	Dual: HD	Accession Number: 4223362
IPF: 3833403	Organization: UNIVERSITY OF MAINE ORONO	
Former Number:	Department:	
IRG/SRG: SMEP	AIDS: N	Expedited: N
<u>Subtotal Direct Costs</u> <u>(excludes consortium</u> <u>F&A)</u> Year 1: 250,000 Year 2: 250,000 Year 3: 250,000 Year 4: 250,000 Year 5: 250,000	Animals: Y Humans: N Clinical Trial: N Current HS Code: Evaluative Info HESC: N	New Investigator: N Early Stage Investigator: N

Senior/Key Personnel:	Organization:	Role Category:
Clarissa Henry	University of Maine	PD/PI
Michelle Goody	University of Maine System acting through the Univ of Maine	Faculty
Benjamin King	University of Maine	Co-Investigator
Samuel Hess	University of Maine	Co-Investigator
Matthew Parent	University of Maine	Post Doctoral Associate

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	State Application Identifier

1. TYPE OF SUBMISSION*

○ Pre-application ● Application ○ Changed/Corrected Application

4.a. Federal Identifier

b. Agency Routing Number

2. DATE SUBMITTED	Application Identifier

c. Previous Grants.gov Tracking Number

5. APPLICANT INFORMATION

Organizational DUNS*: 1868757870000

Legal Name*: University of Maine System acting though the Univ. of Maine
Department:
Division:
Street1*: 5717 Corbett Hall
Street2: University of Maine
City*: Orono
County: ME
State*: ME: Maine
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 04469-5735

Person to be contacted on matters involving this application
Prefix: First Name*: Clarissa   Middle Name: Last Name*: Henry   Suffix: Ph.D.

Position/Title:
Street1*: 5735 Hitchner Hall
Street2: University of Maine
City*: Orono
County:
State*: ME: Maine
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 04469-5717
Phone Number*: 207-581-2816   Fax Number: 207-581-1479   Email: clarissa.henry@maine.edu

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)**   1016000769A1

7. TYPE OF APPLICANT*   H: Public/State Controlled Institution of Higher Education

Other (Specify):

Small Business Organization Type   ○ Women Owned   ○ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*

● New   ○ Resubmission
○ Renewal   ○ Continuation   ○ Revision

If Revision, mark appropriate box(es).
○ A. Increase Award   ○ B. Decrease Award   ○ C. Increase Duration
○ D. Decrease Duration   ○ E. Other *(specify)* :

Is this application being submitted to other agencies?* ○ Yes ● No   What other Agencies?

9. NAME OF FEDERAL AGENCY*
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Mechanisms of NAD+ action during muscle development and homeostasis in a zebrafish dystroglycanopathy model

12. PROPOSED PROJECT
Start Date*   Ending Date*
07/01/2019   06/30/2024

13. CONGRESSIONAL DISTRICTS OF APPLICANT
ME-002

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE   Page 2

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: Dr. First Name*: Clarissa Middle Name: A Last Name*: Henry Suffix:

Position/Title:

Organization Name*: University of Maine

Department:

Division:

Street1*: 5735 Hitchner Hall

Street2: University of Maine

City*: Orono

County: ME

State*: ME: Maine

Province:

Country*: USA: UNITED STATES

ZIP / Postal Code*: 04469-5735

Phone Number*: 207.581.2816   Fax Number: Email*: clarissa.henry@maine.edu

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested*	$1,825,000.00
b. Total Non-Federal Funds*	$0.00
c. Total Federal & Non-Federal Funds*	$1,825,000.00
d. Estimated Program Income*	$0.00

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ○ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR

○ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

*The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION   File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: First Name*: Christopher Middle Name: Last Name*: Boynton Suffix:

Position/Title*: Director, Office of Research Administration

Organization Name*: University of Maine

Department:

Division:

Street1*: 5717 Corbett Hall

Street2: University of Maine

City*: Orono

County: ME

State*: ME: Maine

Province:

Country*: USA: UNITED STATES

ZIP / Postal Code*: 04469-5717

Phone Number*: 207.581.1484   Fax Number: Email*: umgrants@maine.edu

Signature of Authorized Representative*	Date Signed*
Christopher Boynton	10/05/2018

20. PRE-APPLICATION   File Name:

21. COVER LETTER ATTACHMENT   File Name:

424 R&R and PHS-398 Specific
Table Of Contents

Project/Performance Site Location(s)

Project/Performance Site Primary Location

○ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name:	University of Maine System acting through the Univ. of Maine
Duns Number:	1868757870000
Street1*:	5735 Hitchner Hall
Street2:	University of Maine
City*:	Orono
County:	ME
State*:	ME: Maine
Province:	
Country*:	USA: UNITED STATES
Zip / Postal Code*:	04469-5735
Project/Performance Site Congressional District*:	ME-002

Additional Location(s)

File Name:

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ○ Yes ● No

1.a. If YES to Human Subjects

    Is the Project Exempt from Federal regulations? ○ Yes ○ No

        If YES, check appropriate exemption number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6 _ 7 _ 8

        If NO, is the IRB review Pending? ○ Yes ○ No

            IRB Approval Date:

            Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ● Yes ○ No

2.a. If YES to Vertebrate Animals

    Is the IACUC review Pending? ● Yes ○ No

    IACUC Approval Date:

    Animal Welfare Assurance Number   A3754-01

3. Is proprietary/privileged information included in the application?* ○ Yes ● No

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ○ Yes ● No

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an ○ Yes ○ No
environmental assessment (EA) or environmental impact statement (EIS) been performed?

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ○ Yes ● No

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ○ Yes ● No

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	1246-Abstract_v1.pdf
8. Project Narrative*	1247-Narrative.pdf
9. Bibliography & References Cited	1248-REFS.pdf
10. Facilities & Other Resources	1249-Facilities 10 01 18.pdf
11. Equipment	1250-Equipment 10 01 18.pdf

ABSTRACT

Congenital muscular dystrophies (CMDs) are progressive debilitating diseases without cures. Many CMDs disrupt the adhesion of muscle cells to their surrounding extracellular matrix (ECM). Muscle-ECM adhesion is critical for muscle development, homeostasis, regeneration, and resilience to stress. Mutations in genes that modulate muscle-ECM adhesion frequently lead to CMDs. For example, Dystroglycan (DG) and Integrin alpha7 (Itga7) are transmembrane ECM receptors that, when mutated, result in CMDs. Whether and/or how these transmembrane receptors interact during muscle development/homeostasis is not known. In addition, the roles that post-translational modification of DG plays in modulating both the ECM proper and muscle-ECM adhesion are not known. We previously found that exogenous NAD+ potentiates ECM deposition and that NAD+ improves dystrophic phenotypes in zebrafish lacking either DG or Itga7. The basic cell biological mechanisms that underlie NAD+-mediated improvement in muscle-ECM adhesion are not well understood. Our _long-term goal_ is to understand how signaling between muscle cells and their ECM mediates muscle health. Secondary Dystroglycanopathies are a subset of CMDs that result from mutations in genes that are necessary for glycosylation of DG, which is necessary for muscle-ECM adhesion. GDP-mannose, synthesized by GMPPB, is essential for glycosylation reactions. Mutations in _GMPPB_ result in GMPPB-associated Dystroglycanopathy. Preliminary data show that muscle development, homeostasis, and regeneration are disrupted in _gmppb_ mutants. In contrast to our previous data showing NAD+ improves ECM deposition in _dg_-deficient zebrafish, preliminary data show that NAD+ does not improve muscle structure in _gmppb_ mutants. In this grant, we will compare and contrast the mechanisms underlying the effects of DG glycosylation and NAD+ on muscle development, homeostasis, and regeneration. Our _central hypothesis_ is that both NAD+ and _gmppb_ regulate muscle cell adhesion by altering sarcolemma architecture and ECM organization. In Aim 1 we will test the hypothesis that NAD+ increases cell adhesion in DG mutant zebrafish by increasing Itga7 clustering; and that hypoglycosylated DG disrupts sarcolemma architecture and prevents NAD+-mediated Itga7 clustering and increased cell adhesion. We will do this with a combination of longitudinal light sheet microscopy studies and super-resolution microscopy. In Aim 2 we will identify new muscle cell adhesion regulators through comparative studies of dysregulated muscle development in three zebrafish models of muscular dystrophy. We will take an unbiased approach to identify ECM regulatory nodes by using network modeling and network resilience analysis of co-expressed coding and non-coding genes. Completion of this grant will provide new insight into how cell-ECM adhesion mediates muscle development and homeostasis in vertebrate models of CMDs. These basic _in vivo_ cell biological studies are crucial to provide a foundational understanding of the interplay between transmembrane receptors, ECM regulation, and cell adhesion.

We use the zebrafish as a genetically and embryologically accessible vertebrate model of congenital muscular dystrophies, in particular as a model of dystroglycanopathies. Adhesion of muscle cells to their surrounding extracellular matrix is disrupted in dystroglycanopathies. In this grant, we propose to focus on how NAD+ and GMPPB modulate organization of the muscle cell membrane and adhesion to the extracellular matrix.

Project Narrative

FACILITIES AND OTHER RESOURCES – UNIVERSITY OF MAINE

Environment
Henry Laboratory
The facilities and other resources needed to undertake and complete this project successfully are already in place and available to Dr. Henry and her research team at the University of Maine. The Henry lab is completely equipped for the experiments proposed in this project, and Dr. Henry has a track record of success in this environment. There are two other laboratories in the same building as Dr. Henry's lab that use zebrafish as a model system for human disease (innate immunity, *Candida* infection). The PIs and labs interact frequently through joint lab meetings and journal clubs. The Jackson Laboratory is only one hour away on Mount Desert Island. Dr. Henry and/or her lab travel to The Jackson Laboratory approximately once every other month to attend seminars, meet with seminar speakers, and attend graduate student committee meetings. Mount Desert Island Biological Laboratory, which is on the way to The Jackson Lab, also has three PIs using the zebrafish model. Dr. Henry's lab and these labs have periodic informal developmental biology fish club meetings. Gregory Cox is at The Jackson Laboratory. He has acted as an unofficial mentor to Dr. Henry for muscle biology and is an enthusiastic and critical colleague. Dr. Henry's lab attends the informal Connecticut Valley Zebrafish Meetings twice/year, with numerous labs from the Boston Area, UMass, and Yale. Dr. Henry's lab also attends the International Zebrafish Development and Genetics and North Atlantic Zebrafish Research Symposium meetings held every other year, as well as the Northeast Society for Developmental Biology meetings. Dr. Henry attends the Strategic Conference for Zebrafish Investigators held every other year. Dr. Henry and her lab members frequently attend the Frontiers in Myogenesis meeting (invited speaker 2019) and the Gordon conference on Myogenesis (co-vice chair 2019). The active participation of Dr. Henry and lab members in local, national, and international meetings provides a valuable opportunity for scientific exchange and new ideas.

King Laboratory
Resources and facilities required to successfully undertake and complete the proposed project are already available to Dr. King and his laboratory at the University of Maine (UMaine). Dr. King has extensive experience in conducting RNA-Seq studies and analyzing RNA-Seq data and molecular pathways. His laboratory is equipped for the research proposed in this project and has access to high-performance computing resources at UMaine. Dr. King' laboratory is on the same floor of Hitchner Hall as Dr. Henry's laboratory and this fosters interactions and collaboration between laboratories. Outside of UMaine, Dr. King travels at least once monthly to The Jackson Laboratory and/or Mount Desert Island Biological Laboratory in his role as the co-Director of the Bioinformatics Core for the Maine IDeA Network for Biomedical Research Excellence (ME-INBRE) program. The proximity of UMaine to both institutions and Dr. King's role in the statewide ME-INBRE program provides rich opportunities for collaboration across Maine. Dr. King also teaches three bioinformatics short courses. He is lead instructor for the Genome Access Course at Cold Spring Harbor Laboratory since 2005 that is offered 2-3 times a year (http://meetings.cshl.edu/courses.aspx?course=TGAC3&year=17). At Mount Desert Island Biological Laboratory, he co-organizes the annual Applied Bioinformatics (https://mdibl.org/course/applied-bioinformatics/) and Environmental Genomics (https://mdibl.org/course/environmental-genomics/) Courses. The Environmental Genomics Course is funded by an NIH Big Data to Knowledge (BD2K) R25 grant. Dr. King is also a faculty member of the Graduate School of Biomedical Sciences and Engineering at UMaine. Dr. King and lab members actively participate in scientific meetings and bioinformatics courses that provide a valuable opportunity to interact on a regional, national, and international scale.

Hess Laboratory
Resources and facilities required to successfully undertake and complete the proposed project are already available to Dr. Hess and his laboratory at the University of Maine.

Institutional Commitment

Dr. Henry
The environment at the University of Maine is incredibly supportive of Dr. Henry. The University of Maine provides full salary and benefits for Dr. Henry during the academic year on an ᴱᶠᶠᴼᴿᵀ research, ᴱᶠᶠᴼᴿᵀ teaching appointment that provides <u>protected time for research</u>. Dr. Henry teaches one class a year, rotating between: (1) a graduate level Professionalism in Biology class that teaches speaking, grant writing, reviewing,

Facilities & Other Resources

manuscript writing, and ethics, (2) the Graduate School of Biomedical Sciences and Engineering grant writing class, and (3) an undergraduate senior capstone class titled Morphogenesis in Development and Disease. The institutional commitment of the University of Maine to Dr. Henry is evident in their provision of retention funds: The University of Maine provided salary and benefits for Michelle Goody, a then post-doctoral fellow, for fiscal years 2016 and 2017.

Dr. King
The University of Maine is highly supportive of Dr. King. The University of Maine provides full salary and benefits for Dr. King during the academic year on a EFFO research, EFFORT teaching appointment that provides protected time for research. Dr. King teaches one class a year, an undergraduate bioinformatics course. Dr. King was hired in January 2017 and was provided a startup package from the Office of the Vice President for Research totaling $128,300 spread out over three years.

Dr. Hess
The University of Maine provides full salary and benefits for Dr. Hess during the academic year on a EFFORT research, EFFOR teaching appointment. Dr. Hess teaches one class per semester, rotating between upper-level undergraduate optics and electricity and magnetism.

Laboratory facilities

Dr. Henry
Dr. Henry's laboratory space is 850 square feet. It is well equipped for embryo manipulation, microscopy, and molecular biology. The new light-sheet microscope is housed in a room at the back of Dr. Henry's lab. The laboratory houses four incubators (one for bacteria, and three at different temperatures to rear embryos), a shaking incubator, standard molecular biology equipment, and an electrode puller. They share a -80°C freezer, spectrophotometer, gel imaging system, protein gel imaging system, and a real-time qPCR system within the building. There is also a sequencing facility within the building.

Dr. King
Dr. King's laboratory space occupies a bay (approximately 300 square feet) within a large laboratory shared with three other PIs. The laboratory is located on the same floor as Dr. Henry's laboratory. Dr. King's laboratory includes a -80°C freezer and molecular biology equipment required for the proposed RNA extraction, qPCR, and Western blot validation studies. Nanodrop and real-time qPCR systems are located within the building. A sequencing facility equipped with an Applied Biosystems 3700 sequencer is located within the building.

Dr. Hess
Dr. Hess's laboratory space (2500 square feet) includes equipment for cell culture and molecular biology: Baker cell culture hood, CO_2 incubator; -80°C freezer, -20°C freezer; +4°C refrigerator; precision balances (x2); chemical safety hood; pH meter; pipettemen; Bright-line hemocytometer; National Standard Centrifuge; Eppendorf refrigerated ultracentrifuge; PCR; autoclave; gel electrophoresis; bacterial shaker/incubator; temperature-controlled dry incubator; refrigerated temperature-controlled water bath; Zeiss IM 35 inverted microscope with several objective lenses: 5X 0.1 NA, 10X 0.25 NA, 20X 0.4 NA, 40X 0.75 NA water, 32X 0.4 NA. Access to shared instrumentation (adjacent buildings) includes: Harvey Sterile Max Autoclave, Perkin Elmer High-Power (1500W) UV lamp; two -80 °C freezers.

Microscopy facilities

The Henry Laboratory has two Leica microscopes: a Leica TCS SP8 Digital Light Sheet Microscope (shared with two other labs) and a Leica MZ10F stereomicroscope with birefringence analyzer. There is an Olympus Bx45 for genetic mosaic experimentation and 5 dissecting microscopes with microinjection rigs. They also have access to a microscopy facility with a Zeiss VivaTome, Olympus FV-1000 confocal, SEM, and TEM that is staffed by an excellent full-time technician.

The Hess laboratory has three setups for single molecule localization-based super-resolution microscopy (FPALM) consisting of either an Olympus IX71 (x2) or Zeiss Axiovert stand, optics to use any of the above lasers for activation or imaging including TIRF, inclined, and widefield illumination; MadCity XYZ Piezo stage;

Thorlabs two-axis galvanometer; PIFOC objective collar; six CCD cameras: Hamamatsu sCMOS Flash 4.0, Andor iXon+ (x3), Cascade512B, Andor Luca; laser power meters; Tokai-Hit temperature and CO_2-regulated microscope stage-top incubator; three vibration isolation tables (5' x 8', 4' x 6', 3' x 5'); objective lenses (including 1.2NA 60X water, 1.35NA 60X oil, 1.45NA 60X TIRF); diode lasers at 405 nm (x3), 532 nm (x2), 555 nm (x2), 561 nm, 635 nm; gas lasers at 457 nm, 488 nm (x2), 496 nm, 514 nm, and 568 nm; Coherent Innova 300 water-cooled 6-watt Argon Ion laser; coherent Mira 900 Ti:Sapphire laser with control module, short, medium, and long mirror sets; Spectra-Physics MaiTaiHP femtosecond pulsed Ti:Sapphire laser tunable 680-1040 nm, Opal optical parametric oscillator (OPO) with doubling crystal, tunable from 390-520 nm and 550-2200 nm. Laser intensities can be modulated using a computer-controlled acousto-optic tunable filter (AOTF) and/or computer-controlled filter wheels. An additional shared instrument (adjacent building) includes a Spectra-Physics Tsunami multiphoton laser; a third FPALM microscope with IX71 microscope stand, 405 nm, 473 nm, and 642 nm lasers.

Confocal Microscopy: The Hess lab has its own confocal microscope (a Leica TCS-SP with KrAr (488 and 568 nm) and HeNe (633 nm) lasers and several objectives: 63X 1.2NA water, 20X 0.7NA, 63X 1.3NA oil); Access is also available to shared equipment on the UMaine campus including a Leica TCS-SP2 confocal microscope, including 458, 476, 488, 514, 543, and 633 nm laser lines; an Olympus FV-1000 confocal microscope with 405 nm, 458, 488 nm, 514, 543 nm, and 633 nm laser lines.

Spectroscopy: Ocean Optics USB2000-FL spectrometer; Ocean Optics Jaz USB spectrometer with cuvette adaptor, absorption, excitation, and emission spectrum capability; two auto- and cross-correlator PC cards for fluorescence correlation spectroscopy; multimode optical fibers; two fiber-coupled avalanche photodiodes (EG&G Perkin Elmer); three temperature-controlled water baths; vortexers; Beckman DU-65 spectrophotometer; Beckman Coulter DU 640 Spectrophotometer; Photon Technology International (PTI) Spectrofluorometer; Nanodrop spectrophotometer; Molecular biology facilities including DNA sequencing.

Mechanical: A machine shop and an electronics shop with professional staff are located in the same building as the Hess laboratory. The machine shop has facilities for custom metal, wood, plastic, and computer-controlled machining. The electronics shop has facilities for integrated circuit fabrication, and repair of electronic devices and computers.

Animal Facilities

The zebrafish facility is excellent. The facility is located in the same building as Dr. Henry's laboratory. The fish are incredibly healthy and spawn copious amounts of embryos. The facility is run by an experienced technician, Mark Nilan. Mark's degree is in aquaculture and he built the water filtration system incorporating knowledge from big fin fish facilities. The per-tank charge includes all aspects of zebrafish maintenance, including delivery of 1-cell embryos to the lab at any time of day. The Henry lab is also provided with assistance in maintaining mutant strains and growing new stocks. The Henry lab has 360 2.5L tanks in this facility.

Computational Infrastructure

The Advanced Computing Group (ACG) at the University of Maine provides high performance computing resources for faculty research programs. Among the ACG's high performance computing resources is a Linux cluster with 2,464 Intel Haswell/Broadwell processor cores running at 2.5/2.4 GHz with a performance rating of approximately 95.6 TFlops of compute performance. Cluster nodes have all required RNA-Seq analysis software already installed and are upgraded to new versions on a periodic basis. The cluster is connected over an InfiniBand network to three highly fault tolerant ZFS NAS systems with a total of 785 TB of usable disk storage. Data are backed up offsite to the University of Maine Portland Data Center using 40 Gbps Ethernet links.

Network Infrastructure

All offices are equipped with hard-wired and wireless high-speed internet connections. Internet access is provided by UNET (University of Maine System Internet provider) over an ATM circuit with a bandwidth of 3 Mbps (full-duplex). In association with UNET and other research laboratories in Maine, UMaine was recently granted associate membership to Internet 2. Internet 2 provides large amounts of bandwidth on direct connections between member institutions for live video conferencing and transfer of large data files. UNET

Facilities & Other Resources

provides a similar connection to Internet 2 for The Jackson Laboratory enabling easy transfer of large RNA-Seq data files between institutions.

Computer/Office

Dr. Henry
There are two offices located within the lab. One is occupied by the PI and research assistant professor, the other holds six desks for students, technicians, and post-docs. There are also two desks in the wet lab. There are four Mac Pros, one Mac G5, two Mac G4s, 4 Dells with Zeiss 3D reconstruction and quantification software, and a PC that runs the Leica TCS SP8 Digital Light Sheet Microscope.

Dr. King
Dr. King's office is 256 ft^2 and located on the 2nd floor of Hitchner Hall right near his laboratory. Dr. King has a MacBook Pro laptop.

Dr. Hess
The Hess laboratory contains ample office space for PI, students, and postdocs. Dr. Hess also manages an Image Processing Cluster with 32 multi-core PCs (2 x 2.6 GHz, 20 x 2.8-3.0 GHz, 10 x 3.4-4.0 GHz) which is directly linked to the primary microscope acquisition computer by a dedicated fiber optic network; Sixteen single-core PC desktops (2.0 Ghz Pentium 4 or faster), 1 dual core PC (3.2 GHz); direct internet access; data analysis software and programming languages; MATLAB site license including image processing, parallel processing, and statistics toolboxes); network attached storage terabyte servers (x2); three additional networked shared hard drives (3 TB networked storage in total); 40 TB storage on portable USB 2.0 and USB 3.0 hard drives.

Clinical Facilities
Not applicable.

EQUIPMENT – UNIVERSITY OF MAINE

All of the equipment needed for the proposed project is already available to Dr. Henry, Dr. King, Dr. Hess, and their staff.

Equipment in the Henry Lab:

The Henry lab houses the majority of the equipment needed and has access to remaining core equipment (below). Imaging equipment in the laboratory includes: a Leica TCS SP8 Digital Light Sheet Microscope (shared with two other labs), a Leica MZ10F stereomicroscope with birefringence analyzer, and an Olympus Bx45 for genetic mosaic experiments. Molecular biology equipment includes: -80°C freezer, -20°C freezer, 2 X 4°C refrigerators, Eppendorf and BioRad table-top centrifuges, 37°C shaking incubator, power supplies and chambers for SDS-PAGE and agarose gel electrophoresis, and water baths. Equipment used to rear/manipulate embryos includes: 5 dissecting microscopes equipped with rigs for microinjection, 4 incubators for rearing embryos at different temperatures, and an electrode puller.

Equipment in the King Lab:

The King lab is located in a bay within a larger laboratory shared by three other PIs that enables sharing of equipment among these laboratories in addition to core equipment (below). Molecular biology equipment includes: -80°C freezer, -20°C freezer, 4°C refrigerator, NanoDrop One spectrophotometer, Qubit 4 fluorometer, Eppendorf 5814R refrigerated centrifuge, BioRad C1000 Touch PCR thermocycler, power supply and chambers for SDS-PAGE and agarose gel electrophoresis, BioRad TransBlot Turbo Transfer System and heat block. Dissection microscopes, incubators, water baths, fume hoods and other equipment are readily available in the shared laboratory space.

Equipment in the Hess Lab:

Dr. Hess's laboratory space (2500 square feet) includes a Baker cell culture hood, CO_2 incubator, -80°C freezer, -20°C freezer; +4°C refrigerator, precision balances (x2), chemical safety hood, pH meter, pipettemen, Bright-line hemocytometer, National Standard Centrifuge, Eppendorf refrigerated ultracentrifuge, PCR, autoclave, gel electrophoresis, bacterial shaker/incubator, temperature-controlled dry incubator, refrigerated temperature-controlled water bath, a Zeiss IM 35 inverted microscope, three setups for single molecule localization-based super-resolution microscopy (FPALM) consisting of either an Olympus IX71 (x2) or Zeiss Axiovert stand, a confocal microscope (a Leica TCS-SP), and spectrometers.

Core Equipment: The Henry and King labs share access to centrifuges, gel imaging systems, RT-PCR, a Zeiss VivaTome, SEM, and TEM. The Henry, King, and Hess labs share access to NanoDrop spectrophotometers, an Olympus FV-1000 confocal, and DNA sequencing facilities. The Hess lab shares access to a Spectra-Physics Tsunami multiphoton laser, an additional FPALM microscope with IX71 microscope stand and lasers, a Leica TCS-SP2 confocal microscope, a Harvey Sterile Max Autoclave, Perkin Elmer High-Power (1500W) UV lamp, and two -80 °C freezers in adjacent buildings.

High-Performance Computing Infrastructure: The Advanced Computing Group (ACG) at the University of Maine provides high performance computing resources for faculty research programs. The King Lab will use one of the ACG's Linux cluster with 2,464 Intel Haswell/Broadwell processor cores running at 2.5/2.4 GHz with a performance rating of approximately 95.6 TFlops of compute performance. Cluster nodes have all required RNA-Seq analysis software already installed and are upgraded to new versions on a periodic basis. The cluster is connected over an InfiniBand network to three highly fault tolerant ZFS NAS systems with a total of 785 TB of usable disk storage. Data are backed up offsite to the University of Maine Portland Data Center using 40 Gbps Ethernet links.

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: Dr.	First Name*: Clarissa	Middle Name A	Last Name*: Henry	Suffix:

Position/Title*:
Organization Name*:   University of Maine
Department:
Division:
Street1*:   5735 Hitchner Hall
Street2:   University of Maine
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County: ME
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Country*:   USA: UNITED STATES
Zip / Postal Code*:   04469-5735

Phone Number*: 207.581.2816   Fax Number:

E-Mail*: clarissa.henry@maine.edu

Credential, e.g., agency login: eRA Commons

Project Role*: PD/PI   Other Project Role Category:

Degree Type: PhD   Degree Year: 2000

Attach Biographical Sketch*:   File Name: 1241-Henry_CV.pdf

Attach Current & Pending Support: File Name:

PROFILE - Senior/Key Person

Prefix: Dr.　First Name*: Michelle　　Middle Name　　　　　Last Name*: Goody　　　　Suffix:

Position/Title*:
Organization Name*:　　University of Maine System acting through the Univ of Maine
Department:
Division:
Street1*:　　　　5735 Hitchner Hall
Street2:
City*:　　　　Orono
County:
State*:　　　　ME: Maine
Province:
Country*:　　　USA: UNITED STATES
Zip / Postal Code*:　　04469-5735

Phone Number*: 207.581.2807　　　　Fax Number:

E-Mail*: michelle.goody@maine.edu

Credential, e.g., agency login: eRA Commons

Project Role*:　Faculty　　　　　Other Project Role Category:

Degree Type:　PHD　　　　　Degree Year:　2012

Attach Biographical Sketch*:　　File Name:　　1242-Goody_Biosketch.pdf

Attach Current & Pending Support:　File Name:

PROFILE - Senior/Key Person

Prefix: Dr.　First Name*: Benjamin　　Middle Name　　　　　Last Name*: King　　　　Suffix:

Position/Title*:　　Assistant Professor
Organization Name*:　　University of Maine
Department:　　Molecular Biomedical Sciences
Division:
Street1*:　　　　5735 Hitchner Hall
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City*:　　　　Orono
County:
State*:　　　　ME: Maine
Province:
Country*:　　　USA: UNITED STATES
Zip / Postal Code*:　　04469-5735

Phone Number*: 207-581-2803　　　　Fax Number:

E-Mail*: benjamin.l.king@maine.edu

Credential, e.g., agency login: eRA Commons

Project Role*:　Co-Investigator　　　　Other Project Role Category:

Degree Type:　PhD　　　　　Degree Year:　2016

Attach Biographical Sketch*:　　File Name:　　1243-King_Biosketch_R01_Oct_2018.pdf

Attach Current & Pending Support:　File Name:

PROFILE - Senior/Key Person		

Prefix: Dr.   First Name*: Samuel   Middle Name   Last Name*: Hess   Suffix:

Position/Title*:   Professor
Organization Name*:   University of Maine
Department: Physics
Division:
Street1*:   313 Bennet Hall
Street2:
City*:   Orono
County:
State*:   ME: Maine
Province:
Country*:   USA: UNITED STATES
Zip / Postal Code*:   04469-5735

Phone Number*: 207-581-1036   Fax Number:

E-Mail*: samuel.hess@maine.edu

Credential, e.g., agency login: eRA Commons User

Project Role*: Co-Investigator   Other Project Role Category:

Degree Type: PhD   Degree Year: 2002

Attach Biographical Sketch*:   File Name: 1244-HessNIHBiosketch06.12.18.pdf

Attach Current & Pending Support: File Name:

PROFILE - Senior/Key Person		

Prefix: Mr.   First Name*: Matthew   Middle Name   Last Name*: Parent   Suffix:

Position/Title*:   Graduate Student
Organization Name*:   University of Maine
Department: Physics
Division:
Street1*:   129A Bennet Hall
Street2:
City*:   Orono
County:
State*:   ME: Maine
Province:
Country*:   USA: UNITED STATES
Zip / Postal Code*:   04469-5735

Phone Number*: (207) 581-1009   Fax Number:

E-Mail*: matthew.t.parent@maine.edu

Credential, e.g., agency login:

Project Role*: Post Doctoral Associate   Other Project Role Category:

Degree Type: BS   Degree Year: 2011

Attach Biographical Sketch*:   File Name: 1245-MP_biosketch.pdf

Attach Current & Pending Support: File Name:

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. DO NOT EXCEED FIVE PAGES.

NAME: Henry, Clarissa

eRA COMMONS USER NAME (credential, e.g., agency login): [eRA Commons User Name]

POSITION TITLE: Associate Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	END DATE MM/YYYY	FIELD OF STUDY
University of Utah, Salt Lake City, UT	BS	05/1995	Biology
University of Washington, Seattle, WA	PHD	09/2000	Zoology
University of California Berkeley, Berkeley, CA	Postdoctoral Fellow	08/2004	Dev. Genetics

A. Personal Statement

The goal of my research program is to understand how dynamic interactions between muscle cells and their extracellular matrix mediate morphogenesis, and how disruption of cell-matrix interactions leads to disease states. Aberrant cell-matrix interactions cause or exacerbate many musculoskeletal disorders such as myopathies, tendonitis, traumatic muscle injuries, arthritis, and sarcopenia. My lab primarily focuses on how signaling between muscle cells and their extracellular matrix mediates musculoskeletal development and homeostasis with the goal of discovering new approaches for treatment of neuromuscular diseases.

Note on productivity gap 2013-2015: My lab did not publish primary research 2013-2015. The reason for this is that [Personal Info] I took the opportunity to reflect on what I found most interesting. I realized that I had essentially spent my entire career asking the question "how do embryos make structure when they are compromised?" As a graduate student I determined how somites could form in the absence of internal cells. As a post-doctoral fellow I asked how myotendinous junctions – which are derived from somite boundaries – form when somite boundaries do not form. As a PI we have addressed this question in nearly every manuscript my lab has published. It was during this period of reflection that I realized that cell adhesion complexes are likely a significant hub of compensatory responses in muscle (and proposed this in an invited review for Developmental Biology in 2015). I decided that the notably variable phenotype of Dystroglycanopathies, where cell adhesion is presumably disrupted due to failed glycosylation of Dystroglycan, would be the perfect system to combine my two favorite topics: compensation and cell adhesion. Unfortunately, it took my lab longer than predicted to use CRISPR/Cas9 to generate zebrafish models of Dystroglycanopathy. At the time it was not well known (or publicized) that CRISPR/Cas9 phenotypes in zebrafish are not reliable and fully penetrant until the third generation in most cases. Our zebrafish facility is excellent and the fish are incredibly fecund and long-lived. This comes at a price; rearing our zebrafish takes 4-6 months instead of 2 months. Due to this combination of factors, progress was slow. However, please note that we have been publishing yearly again beginning in 2016 (despite the absence of RO1-level funding). We have made significant progress on this project (see preliminary data) and have trained personnel who can accomplish the proposed experiments.

B. Positions and Honors

Positions and Employment

1992 - 1995	Undergraduate Research, University of Utah, Salt Lake City, UT
1995 - 2000	Graduate Student, University of Washington, Seattle, WA
2001 - 2004	Miller Postdoctoral fellow, University of California Berkeley, Berkeley, CA
2004 - 2010	Assistant Professor, University of Maine, Orono, ME
2011 -	Associate Professor, University of Maine, Orono, ME

Other Experience and Professional Memberships

2012 - 2012   Visiting faculty, Advanced Microscopy Course, Universidade Federal do Rio de Janeiro
2013 - 2013   Session Chair, "Muscle", Strategic Conference for Zebrafish Investigators
2013 - 2017   Member, Skeletal Muscle and Exercise Physiology Study Section
2014 - 2014   Co-Organizer, Northeast Society for Developmental Biology
2016 - 2019   Board Member, Zebrafish Disease Models Society
2019 -   Co-Vice Chair, Gordon Myogenesis Conference
2021 -   Co-Chair, Gordon Myogenesis Conference

Honors

1992   University of Utah Undergraduate Research Program Fellowship, University of Utah
1993   Howard Hughes Undergraduate Research Program Fellowship, University of Utah
1997   Molecular and Cellular Biology Training Grant, University of Washington
2001   Miller Fellow, University of California Berkeley

C. Contribution to Science

1. **Novel mechanisms of laminin polymerization:** Basement membranes (BMs) are specialized sheets of extracellular matrix. BMs are critical for animal development and homeostasis. In skeletal muscle, adhesion of muscle fibers to the BM is necessary for muscle development, regeneration, and homeostasis. This requirement for muscle-BM adhesion is highlighted by the genetic basis for congenital muscular dystrophies: these are progressive, debilitating diseases without cures. Many dystrophies, including Duchenne, Becker, and Merosin-deficient muscular dystrophies, result from mutations that disrupt adhesion of muscle fibers to their surrounding BM. Mechanisms that mediate BM assembly during muscle development and repair are not well understood. The protein laminin is a central component of BMs. We identified a novel Integrin signaling pathway that increases muscle-BM adhesion. This pathway involves Nicotinamide Riboside Kinase 2b (Nrk2b). Nrk2b is expressed specifically in skeletal muscle and generates NAD+. We showed that addition of either exogenous NAD+ or a vitamin precursor to NAD+ **is sufficient to correct dystrophic phenotypes in zebrafish lacking either Dystroglycan or Integrin alpha7.** This work was recommended by F1000, highlighted by a PLoS Biology Synopsis (http://www.plosbiology.org/article/info%3Adoi%2F10.1371%2Fjournal.pbio.1001410), highlighted in a PLoS Biologue (http://blogs.plos.org/biologue/), and highlighted in Science Daily. As mechanical failure in cell adhesion between muscle fibers and their surrounding BM underlies the etiology of many different dystrophies, the Nrk2b pathway may have therapeutic utility for multiple dystrophies.

   a. Goody MF, Kelly MW, Lessard KN, Khalil A, Henry CA. Nrk2b-mediated NAD+ production regulates cell adhesion and is required for muscle morphogenesis in vivo: Nrk2b and NAD+ in muscle morphogenesis. Dev Biol. 2010 Aug 15;344(2):809-26. PubMed PMID: 20566368; PubMed Central PMCID: PMC2917104.

   b. Goody MF, Kelly MW, Reynolds CJ, Khalil A, Crawford BD, Henry CA. NAD+ biosynthesis ameliorates a zebrafish model of muscular dystrophy. PLoS Biol. 2012;10(10):e1001409. PubMed PMID: 23109907; PubMed Central PMCID: PMC3479101.

2. **Fibronectin (Fn) in muscle development:** Remodeling of the ECM regulates adhesion and signaling between cells and their microenvironment. ECM remodeling is critical for embryonic development and regeneration. However, mechanisms underlying ECM remodeling in vivo remain elusive. We were the first to show that Fn is dynamically regulated at the myotendinous junction during muscle development: Fn is degraded as fast-twitch muscle fibers elongate and attach to the MTJ. Whether Fn played a role in muscle development was a central question. Answering this question was not trivial because Fn is required for formation of somites, which give rise to axial skeletal muscle. We used multiple morphometric assays and mutant analysis to show that Fn plays a critical role in MTJ maintenance. Next, we delved into molecular mechanisms of Fn regulation in vivo. We showed that normal laminin organization acts as a "checkpoint" for Fn downregulation. Furthermore, laminin signaling modulates both the expression of fn1b and localization of Matrix metalloproteinase 11 (Mmp11) to MTJs. We found that Mmp11 is both necessary and sufficient for Fn downregulation. Taken together, this study identified a new mechanism in the myomatrix

Biosketches

that regulates the remodeling process during morphogenesis in vivo, and that may be profitably targeted in the many pathological conditions in which Fn is dysregulated, to improve muscle tissue structure.

a. Jenkins MH, Alrowaished SS, Goody MF, Crawford BD, Henry CA. Laminin and Matrix metalloproteinase 11 regulate Fibronectin levels in the zebrafish myotendinous junction. Skelet Muscle. 2016;6:18. PubMed PMID: 27141287; PubMed Central PMCID: PMC4852425.

b. Snow CJ, Henry CA. Dynamic formation of microenvironments at the myotendinous junction correlates with muscle fiber morphogenesis in zebrafish. Gene Expr Patterns. 2009 Jan;9(1):37-42. PubMed PMID: 18783736; PubMed Central PMCID: PMC2655214.

c. Snow CJ, Peterson MT, Khalil A, Henry CA. Muscle development is disrupted in zebrafish embryos deficient for fibronectin. Dev Dyn. 2008 Sep;237(9):2542-53. PubMed PMID: 18729220; PubMed Central PMCID: PMC2572006.

3. **Visualization and quantification of morphogenesis in vivo:** In order to understand development it is absolutely essential to be able to visualize and quantify cell behaviors in vivo. I have been a pioneer of both of these aspects of morphogenetic analysis. As a graduate student, I did the first time-lapse analysis of organogenesis in a vertebrate (somite formation in zebrafish). As a post-doctoral fellow, I provided significant insight into somite patterning and interactions between slow- and fast-twitch muscle fibers (Recommended by Faculty of 1000, Highlighted in the Journal of Cell Biology (2004) 188, 11 "Research Roundup"). In my own laboratory, I have significantly contributed to our understanding of muscle morphogenesis by rigorously combining time-lapse analysis and computational methods to assay morphological change (Recommended by Faculty of 1000). We have also collaborated with Dr. Andre Khalil (University of Maine) to adopt image analysis algorithms from astrophysics. This allows quantification changes in cell and extracellular matrix organization in an unbiased manner, which is an essential step if morphological analyses are to be grounded in a more mechanistic cell biological understanding.

a. Henry CA, Hall LA, Burr Hille M, Solnica-Krezel L, Cooper MS. Somites in zebrafish doubly mutant for knypek and trilobite form without internal mesenchymal cells or compaction. Curr Biol. 2000 Sep 7;10(17):1063-6. PubMed PMID: 10996075.

b. Henry CA, Urban MK, Dill KK, Merlie JP, Page MF, Kimmel CB, Amacher SL. Two linked hairy/Enhancer of split-related zebrafish genes, her1 and her7, function together to refine alternating somite boundaries. Development. 2002 Aug;129(15):3693-704. PubMed PMID: 12117818.

c. Henry CA, Amacher SL. Zebrafish slow muscle cell migration induces a wave of fast muscle morphogenesis. Dev Cell. 2004 Dec;7(6):917-23. PubMed PMID: 15572133.

d. Snow CJ, Goody M, Kelly MW, Oster EC, Jones R, Khalil A, Henry CA. Time-lapse analysis and mathematical characterization elucidate novel mechanisms underlying muscle morphogenesis. PLoS Genet. 2008 Oct 3;4(10):e1000219. PubMed PMID: 18833302; PubMed Central PMCID: PMC2543113.

4. **Cell-extracellular matrix adhesion guides muscle development:** Although the impact of the extracellular matrix (ECM) on cell behavior has long been established in tissue culture, I have long been interested in understanding roles for cell-matrix adhesion in morphogenesis in vivo. As a graduate student I cloned two focal adhesion proteins, Fak and Paxillin (this was when cloning was done by screening phage libraries and sequencing involved pouring your own gels) and was the first to show subcellular localization of focal adhesion proteins during zebrafish development. In my own lab, we discovered new ECM microenvironments important for muscle morphogenesis, defined interactions between ECM proteins and Hedgehog signaling, and discovered new roles for the ECM proteins Fibronectin and laminin in myotendinous junction development.

a. Jenkins MH, Alrowaished SS, Goody MF, Crawford BD, Henry CA. Laminin and Matrix metalloproteinase 11 regulate Fibronectin levels in the zebrafish myotendinous junction. Skelet Muscle. 2016;6:18. PubMed PMID: 27141287; PubMed Central PMCID: PMC4852425.

b. Peterson MT, Henry CA. Hedgehog signaling and laminin play unique and synergistic roles in muscle development. Dev Dyn. 2010 Mar;239(3):905-13. PubMed PMID: 20063418; PubMed Central PMCID: PMC2954646.

c. Snow CJ, Peterson MT, Khalil A, Henry CA. Muscle development is disrupted in zebrafish embryos deficient for fibronectin. Dev Dyn. 2008 Sep;237(9):2542-53. PubMed PMID: 18729220; PubMed Central PMCID: PMC2572006.

d. Henry CA, Crawford BD, Yan YL, Postlethwait J, Cooper MS, Hille MB. Roles for zebrafish focal adhesion kinase in notochord and somite morphogenesis. Dev Biol. 2001 Dec 15;240(2):474-87. PubMed PMID: 11784077.

5. Interactions between muscle and the environment: One of the key unanswered questions regarding the variable age of onset and rate of progression of muscular dystrophies is whether the environment influences these factors. One roadblock has been that the basic biology of how environmental factors interact with muscle is unknown. We have been addressing this question in the zebrafish model. (1) Although muscle pain and weakness are symptoms of Influenza A and B viruses, the mechanisms of influenza-induced muscle damage were not known. We characterized muscle damage and assayed innate immune/inflammatory markers in response to IAV infection in vivo. We also showed that IAV infection greatly exacerbates the extent of fiber damage in zebrafish modeling Duchenne Muscular Dystrophy. Thus, these results showed an important gene-environment interaction between the pro-inflammatory innate immune response and the *DMD* gene in skeletal muscle. (2) Alcoholic myopathies are characterized by neuromusculoskeletal symptoms such as compromised movement and weakness. The effects of EtOH exposure on skeletal muscle during the growth period that follows primary muscle development are not well understood. We found that this period of development is highly sensitive to EtOH, which triggers a pro-inflammatory response. EtOH damaged muscle end attachments and caused membrane instability. EtOH also exacerbates muscle damage in the zebrafish model of Duchenne Muscular Dystrophy. Thus, our results suggest that EtOH exposure has pleiotropic deleterious effects on skeletal muscle.

a. Coffey EC, Pasquarella ME, Goody MF, Henry CA. Ethanol Exposure Causes Muscle Degeneration in Zebrafish. J Dev Biol. 2018 Mar 9;6(1)PubMed PMID: 29615556; PubMed Central PMCID: PMC5875561.

b. Goody M, Jurczyszak D, Kim C, Henry C. Influenza A Virus Infection Damages Zebrafish Skeletal Muscle and Exacerbates Disease in Zebrafish Modeling Duchenne Muscular Dystrophy. PLoS Curr. 2017 Oct 25;9PubMed PMID: 29188128; PubMed Central PMCID: PMC5693338.

For a complete list of publications in pubmed:
https://www.ncbi.nlm.nih.gov/sites/myncbi/clarissa.henry.1/bibliography/40509195/public/?sort=date&direction=ascending

D. Additional Information: Research Support and/or Scholastic Performance

Ongoing Research Support

This grant funds purchase of a light-sheet microscope and Imaris software. Henry, Clarissa (PI EFFORT). Co-Investigators Robert Wheeler, Andre Khalil, Leif Oxburgh, Kristy Townsend.
Role: PI

University of Maine System Undergraduate Research Reinvestment Fund (Ben King, PI)
05/01/18 – 04/30/19
Muscular Dystrophy Genomics Research Collaborative (35k)
The goal of this project is to characterize a novel zebrafish model for muscular dystrophy developed by Dr. Clarissa Henry's laboratory at the University of Maine where the GDP-mannose pyrophosphorylase B (*gmppb*) gene was knocked out using CRISPR-Cas9. A team of five undergraduates and one graduate student will identify use our preliminary RNA-seq data shown in the grant to understand impaired muscle function in the novel zebrafish mutant by computationally modeling how networks of genes are dysregulated.
Role: Collaborator

Completed Research Support

Private Source

    Henry, Clarissa (PI)
06/01/14-10/31/17
Mechanisms of NAD+-mediated Amelioration of Dystrophy in Zebrafish
Role: PI

R03 HD077545-02
    Henry, Clarissa A (PI)
07/05/13-06/30/16
Mechanisms underlying Paxillin-mediated amelioration of muscle degeneration
Role: PI

R01 HD052934-05
    Henry, Clarissa A (PI)
02/15/07-01/31/14
Analysis of Myotome Boundary Morphogenesis During Zebrafish Development
Role: PI

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. DO NOT EXCEED FIVE PAGES.

NAME: Goody, Michelle

eRA COMMONS USER NAME (credential, e.g., agency login): eRA Commons User Name

POSITION TITLE: Research Assistant Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	END DATE MM/YYYY	FIELD OF STUDY
University of Maine, Orono, ME	BS	05/2007	Biology
University of Maine, Orono, ME	PHD	08/2012	Biomedical Sciences
University of Maine, Orono, ME	Postdoctoral Fellow	08/2014	Kim Laboratory - Gene environment interactions in innate immunity
University of Maine, Orono, ME	Postdoctoral Fellow	05/2017	Henry Laboratory - Mechanisms of Integrin signaling in muscle development and disease

A. Personal Statement

I fell in love with developmental biology and morphogenesis in graduate school. The complexity, orchestration, and integration across biological size scales fascinates me. The unifying theme in the Henry Lab's research is elucidating how bidirectional cell-matrix signaling regulates neuromusculoskeletal development and disease. My graduate thesis research showed me that basic biological research can unlock potential therapeutics for diseases. In the Henry Lab, we used zebrafish to discover that the early developmental switch in muscle extracellular matrix from mainly Fibronectin to mainly laminin-111 involves NAD+ regulation of integrin-laminin interactions, that recruit Mmp11, which downregulates Fn. We also demonstrated that NAD+ supplementation improves laminin-111 organization in zebrafish models of congenital muscular dystrophies where laminin-111 is disrupted. This intervention prevents fiber detachment and prolongs mobility in these disease models. I have 10 years of experience working with the zebrafish model and expertise in molecular and in vivo cell biological techniques and muscle and innate immune tissues. For personal reasons I am geographically constrained, but am thrilled with the research, training, mentoring, and teaching opportunities that Dr. Henry and the University of Maine have afforded me. Dr. Henry and I work well and productively together and mutually respect one another. I hope to continue our collaboration long into the future.

1. Jenkins MH, Alrowaished SS, Goody MF, Crawford BD, Henry CA. Laminin and Matrix metalloproteinase 11 regulate Fibronectin levels in the zebrafish myotendinous junction. Skelet Muscle. 2016;6:18. PubMed PMID: 27141287; PubMed Central PMCID: PMC4852425.

2. Goody MF, Kelly MW, Reynolds CJ, Khalil A, Crawford BD, Henry CA. NAD+ biosynthesis ameliorates a zebrafish model of muscular dystrophy. PLoS Biol. 2012;10(10):e1001409. PubMed PMID: 23109907; PubMed Central PMCID: PMC3479101.

3. Goody MF, Kelly MW, Lessard KN, Khalil A, Henry CA. Nrk2b-mediated NAD+ production regulates cell adhesion and is required for muscle morphogenesis in vivo: Nrk2b and NAD+ in muscle morphogenesis. Dev Biol. 2010 Aug 15;344(2):809-26. PubMed PMID: 20566368; PubMed Central PMCID: PMC2917104.

4. Snow CJ, Goody M, Kelly MW, Oster EC, Jones R, Khalil A, Henry CA. Time-lapse analysis and mathematical characterization elucidate novel mechanisms underlying muscle morphogenesis. PLoS Genet. 2008 Oct 3;4(10):e1000219. PubMed PMID: 18833302; PubMed Central PMCID: PMC2543113.

B. Positions and Honors

Positions and Employment

2003 - 2006   Undergraduate researcher, University of Maine, Orono, ME
2007 - 2012   Graduate student, University of Maine, Orono, ME
2010 - 2010   Graduate teaching assistant, University of Maine, Orono, ME
2012 - 2017   Postdoctoral researcher, University of Maine, Orono, ME
2017 -   Lecturer and lab coordinator, University of Maine, Orono, ME
2017 -   Research Assistant Professor, University of Maine, Orono, ME

Other Experience and Professional Memberships

Honors

2009 - 2009   Travel Award to attend the ASCB/JSCB/RIKEN CDB Meeting on Building the Body Plan: How Cell Adhesion, Signaling, and Cytoskeletal Regulation Shape Morphogenesis, American Society for Cell Biology
2011 - 2011   College of Natural Sciences Forestry and Agriculture Graduate Research Excellence Award Scholarship, University of Maine

C. Contribution to Science

1. Mechanisms of muscle matrix remodeling during zebrafish development and disease: Remodeling of the ECM regulates adhesion and signaling between cells and their microenvironment. ECM remodeling is critical for embryonic development and regeneration. However, mechanisms underlying ECM remodeling in vivo remain elusive. In zebrafish and mouse muscle development, the myomatrix switches from mainly Fibronectin (Fn)-based somite boundaries to mainly laminin-based basement membranes. We identified a novel Integrin signaling pathway that increases muscle-laminin adhesion. This pathway involves Nicotinamide Riboside Kinase 2b (Nrk2b). Nrk2b is expressed specifically in skeletal muscle and generates NAD+. We showed that normal laminin organization then acts as a "checkpoint" for Fn downregulation. Furthermore, laminin signaling modulates both the expression of fn1b and localization of Matrix metalloproteinase 11 (Mmp11) to MTJs. We found that Mmp11 is both necessary and sufficient for Fn downregulation. Taken together, these studies identified a new mechanism in the myomatrix that regulates the remodeling process during morphogenesis in vivo.

   As many muscle diseases, including Duchenne, Becker, and Merosin-deficient muscular dystrophies, result from mutations that disrupt adhesion of muscle fibers to laminin and we found that Nrk2b-NAD+-Integrin-laminin signaling promotes muscle adhesion, we showed that addition of either exogenous NAD+ or a vitamin precursor to NAD+ is sufficient to correct dystrophic phenotypes in two zebrafish models of muscular dystrophies. This work was recommended by F1000, highlighted by a PLoS Biology Synopsis (http://www.plosbiology.org/article/info%3Adoi%2F10.1371%2Fjournal.pbio.1001410), highlighted in a PLoS Biologue (http://blogs.plos.org/biologue/), and highlighted in Science Daily. As mechanical failure in cell adhesion between muscle fibers and laminin underlies the etiology of many different dystrophies, the Nrk2b pathway may have therapeutic utility for multiple dystrophies.

   a. Jenkins MH, Alrowaished SS, Goody MF, Crawford BD, Henry CA. Laminin and Matrix metalloproteinase 11 regulate Fibronectin levels in the zebrafish myotendinous junction. Skelet Muscle. 2016;6:18. PubMed PMID: 27141287; PubMed Central PMCID: PMC4852425.
   b. Goody MF, Kelly MW, Reynolds CJ, Khalil A, Crawford BD, Henry CA. NAD+ biosynthesis ameliorates a zebrafish model of muscular dystrophy. PLoS Biol. 2012;10(10):e1001409. PubMed PMID: 23109907; PubMed Central PMCID: PMC3479101.
   c. Goody MF, Kelly MW, Lessard KN, Khalil A, Henry CA. Nrk2b-mediated NAD+ production regulates cell adhesion and is required for muscle morphogenesis in vivo: Nrk2b and NAD+ in muscle morphogenesis. Dev Biol. 2010 Aug 15;344(2):809-26. PubMed PMID: 20566368; PubMed Central PMCID: PMC2917104.

    d. Snow CJ, Goody M, Kelly MW, Oster EC, Jones R, Khalil A, Henry CA. Time-lapse analysis and mathematical characterization elucidate novel mechanisms underlying muscle morphogenesis. PLoS Genet. 2008 Oct 3;4(10):e1000219. PubMed PMID: 18833302; PubMed Central PMCID: PMC2543113.

2. Integration of development and disease across tissues and their matrices: Although the impact of the matrix on cell behavior has long been established in tissue culture, we and others are interested in understanding roles for cell-matrix adhesion in morphogenesis in vivo. The Henry lab discovered new ECM microenvironments important for muscle morphogenesis, defined interactions between ECM proteins and signaling pathways, and discovered new roles for the ECM proteins Fibronectin and laminin in myotendinous junction development. We've also been invited to synthesize and integrate the recent literature regarding in vivo cell-matrix interactions during development and disease, which has resulted in multiple publications.

    a. Goody MF, Henry CA. A need for NAD+ in muscle development, homeostasis, and aging. Skelet Muscle. 2018 Mar 7;8(1):9. PubMed PMID: 29514713; PubMed Central PMCID: PMC5840929.

    b. Goody MF, Carter EV, Kilroy EA, Maves L, Henry CA. "Muscling" Throughout Life: Integrating Studies of Muscle Development, Homeostasis, and Disease in Zebrafish. Curr Top Dev Biol. 2017;124:197-234. PubMed PMID: 28335860.

    c. Goody MF, Sher RB, Henry CA. Hanging on for the ride: adhesion to the extracellular matrix mediates cellular responses in skeletal muscle morphogenesis and disease. Dev Biol. 2015 May 1;401(1):75-91. PubMed PMID: 25592225; PubMed Central PMCID: PMC4402131.

    d. Goody MF, Henry CA. Dynamic interactions between cells and their extracellular matrix mediate embryonic development. Mol Reprod Dev. 2010 Jun;77(6):475-88. PubMed PMID: 20108219.

3. Assay and model development to study in vivo host-pathogen dynamics: Live, transparent zebrafish embryos and larvae are an ideal system in which to study infection dynamics using fluorescently-labeled pathogens and fluorescently-labeled host cell types. In a 2 year post-doc position in Dr. Carol Kim's laboratory at the University of Maine, I participated in projects that resulted in publishing a methods paper, a novel zebrafish model of human Influenza A virus infection, roles for SOD2 in bacterial infection, and a review of zebrafish models of human virus infections.

    a. Peterman EM, Sullivan C, Goody MF, Rodriguez-Nunez I, Yoder JA, Kim CH. Neutralization of mitochondrial superoxide by superoxide dismutase 2 promotes bacterial clearance and regulates phagocyte numbers in zebrafish. Infect Immun. 2015 Jan;83(1):430-40. PubMed PMID: 25385799; PubMed Central PMCID: PMC4288898.

    b. Gabor KA, Goody MF, Mowel WK, Breitbach ME, Gratacap RL, Witten PE, Kim CH. Influenza A virus infection in zebrafish recapitulates mammalian infection and sensitivity to anti-influenza drug treatment. Dis Model Mech. 2014 Nov;7(11):1227-37. PubMed PMID: 25190709; PubMed Central PMCID: PMC4213727.

    c. Goody MF, Sullivan C, Kim CH. Studying the immune response to human viral infections using zebrafish. Dev Comp Immunol. 2014 Sep;46(1):84-95. PubMed PMID: 24718256; PubMed Central PMCID: PMC4067600.

    d. Goody MF, Peterman E, Sullivan C, Kim CH. Quantification of the respiratory burst response as an indicator of innate immune health in zebrafish. J Vis Exp. 2013 Sep 12;PubMed PMID: 24056405; PubMed Central PMCID: PMC3871927.

4. Effects of environmental stressors on muscle homeostasis in wild-types and zebrafish modeling muscular dystrophy: The basic biology of how environmental stressors effect healthy and genetically compromised muscle is largely unknown. We used wild-types and zebrafish modeling Duchenne muscular dystrophy (DMD) to determine the effect of two environmental stressors (Influenza A infection (IAV) or alcohol exposure) on healthy or diseased muscle structure and function (after normal primary muscle development had concluded). We characterized muscle damage and assayed innate immune/inflammatory markers in response to IAV infection or EtOH exposure in vivo. We showed that IAV infection or EtOH exposure upregulated pro-inflammatory cell signaling cascades and disrupted cell-matrix adhesions as well as

sarcolemma stability in wild-type zebrafish; and either environmental stressor greatly exacerbated the extent of fiber damage in zebrafish modeling DMD. Thus, these results show important gene-environment interactions between stressors, the pro-inflammatory innate immune response, and the DMD gene in skeletal muscle.

a. Coffey EC, Pasquarella ME, Goody MF, Henry CA. Ethanol Exposure Causes Muscle Degeneration in Zebrafish. J Dev Biol. 2018 Mar 9;6(1)PubMed PMID: 29615556; PubMed Central PMCID: PMC5875561.

b. Goody M, Jurczyszak D, Kim C, Henry C. Influenza A Virus Infection Damages Zebrafish Skeletal Muscle and Exacerbates Disease in Zebrafish Modeling Duchenne Muscular Dystrophy. PLoS Curr. 2017 Oct 25;9PubMed PMID: 29188128; PubMed Central PMCID: PMC5693338.

Complete List of Published Work in My Bibliography:
https://www.ncbi.nlm.nih.gov/myncbi/michelle.goody.1/bibliography/52951851/public/

D. Additional Information: Research Support and/or Scholastic Performance

Biosketches

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: King, Benjamin L

eRA COMMONS USER NAME (credential, e.g., agency login): eRA Commons User Name

POSITION TITLE: Assistant Professor of Bioinformatics

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Boston University, College of Engineering, Boston, MA	B.S.	05/1994	Biomedical Engineering
Boston University, College of Engineering, Boston, MA	M.S.	09/1996	Biomedical Engineering
University of Maine, Graduate School of Biomedical Science and Engineering, Orono, ME	Ph.D.	05/2016	Biomedical Science

A. Personal Statement

My research program utilizes genomic and computational approaches to understand the mechanisms of stress responses with a specific focus is on non-coding RNAs, including microRNAs and long non-coding RNAs, that have important regulatory roles in heath and disease, including muscular dystrophies. I have specialized expertise in modeling mRNA and microRNA (miRNA) expression profiling data as integrated networks to understand complex biological processes. I have made significant contributions to many studies of mouse, zebrafish and other diverse animal models relevant to improving human health. For example, my recent research on miRNA-mRNA regulatory circuits for heart regeneration revealed a set of novel long non-coding RNA [Unpublished] [Unpublished] *in press*). This paper was co-authored by a University of Maine undergraduate from my lab. My research on appendage regeneration described a conserved gene regulatory network that described how miRNAs regulate genes required for blastema formation in three vertebrates that last shared a common ancestor over 420 million years ago (King & Yin, *PLoS ONE*, 2016). My other comparative studies include using high-throughput sequencing to characterize embryonic gene expression in three cartilaginous fishes together with the complete genome sequence of *Leucoraja erinacea* to study *Hox* clusters (King *et al*, *Science*, 2011). I was recruited to the University of Maine in January 2017 to establish my independent research program after directing bioinformatics core groups at The Jackson Laboratory and Mount Desert Island (MDI) Biological Laboratory for the past 12 years. Since 2002, I have organized and taught bioinformatics in several courses including the Environmental Genomics and Applied Bioinformatics Courses at MDI Biological Laboratory that provide intensive training on high-throughput RNA sequencing data analysis. Since 2005, I have been the organizer and lead instructor of The Genome Access Course at Cold Spring Harbor Laboratory, a two-and-a-half day introductory course in bioinformatics offered three times a year. As the Bioinformatics Core co-Director of the Maine Institutional Developmental Award Network of Biomedical Research Excellence (INBRE) Program, I coordinate collaborative data management and analysis services and training for faculty across 13 Maine institutions. In summary, my extensive experience in genomics and bioinformatics gives me the expertise necessary for the proposed research program.

1. King BL, Rosenstein MC, Smith AM, Dykeman CA, Smith GA, Yin VP. RegenDbase: a comparative database of noncoding RNA regulation of tissue regeneration circuits across multiple taxa. *NPJ Regen Med*. 2018. 3:10.

2. King BL, Yin VP. A conserved gene regulatory circuit is dynamically controlled during limb/appendage regeneration in three vertebrates. *PLoS ONE*. 2016.1(6):e0157106. PMCID: PMC4927183. Featured in *Science* Shot on June 29, 2016 DOI: 10.1126/science.aag0632.
3. King BL, Gillis JA, Carlisle HR, Dahn RD. (2011) A natural deletion of the *HoxC* cluster in elasmobranch fishes. *Science,* 334(6062):1517. PMCID: PMC3264428. Reviewed in Research Highlights *Nature* 480(22/29): 419, 2011.

B. Positions and Honors

Positions and Employment
1996-1997   Research Associate II, Research Computing Group, Genetics Institute, Inc., Cambridge, MA
1997-1999   Research Informatics Engineer, AstraZeneca R&D Boston, Cambridge, MA
1999-2004   Bioinformatics Analyst, The Jackson Laboratory, Bar Harbor, ME
2004-2009   Senior Manager, Computational Sciences, The Jackson Laboratory, Bar Harbor, ME
2009-2017   Senior Staff Scientist, MDI Biological Laboratory, Salisbury Cove, ME
2011-2017   ME-INBRE Bioinformatics Core Director, MDI Biological Laboratory, Salisbury Cove, ME
2017-Present Assistant Professor of Bioinformatics, University of Maine, Orono, ME
2011-Present ME-INBRE Bioinformatics Core co-Director, University of Maine, Orono, ME

Other Experience
2002-2005   Instructor, Genome Sequence Analysis: Theory and Practice, The Jackson Laboratory, Bar Harbor, ME (Topic: Sequence Similarity Searching)
2005-2010   Instructor, Short Course on Medical and Experimental Mammalian Genetics, The Jackson Laboratory, Bar Harbor, ME. (Topic: Advanced In Depth Bioinformatics Workshop and Clinic)
2005-Present Lead Instructor, The Genome Access Course, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (April, August and November 2005-2007,2011-present, April and November 2008-2010)
2006-2011   Instructor, Genomic and Proteomic Approaches to Complex Heart, Blood and Lung Diseases, The Jackson Laboratory, Bar Harbor, ME (Topic: Pathways Analysis and Genetic Candidate Analysis)
2010-2013   Instructor, Short Course on Systems Genetics, The Jackson Laboratory, Bar Harbor, ME (Topic: Transcriptional Profiling Using High-Throughput Sequencing)
2010-Present Instructor and co-organizer, Environmental Genomics course, Mount Desert Island Biological Laboratory, Salisbury Cove, ME (Topic: Microarray experimental design, statistical analysis of RNA-Seq data)
2012-Present Co-organizer and instructor, Applied Bioinformatics Course, Mount Desert Island Biological Laboratory, Salisbury Cove, ME (Topic: RNA-Seq data analysis)

Honors
1994   Member, *Tau Beta Pi* National Engineering Honor Society
2008   "Getting Connected With caBIG" Deployment Award given to Jackson Laboratory team, NCI Cancer Bioinformatics Grid National Meeting, June 24, 2008

C. Contributions to Science

1. **Applying genomics to characterize the structure and function of novel genomes**: Rapid development of inexpensive DNA sequencing technologies enabled transformative advances in our knowledge of genes and genomes and successful application of these technologies require bioinformatics. I was the lead bioinformatics expert for the first whole-genome comparison of two isolates of the same organism ever published (reference a). This work, cited over 2,006 times, compared the genomes of a recent clinical isolate of *Helicobacter pylori* with a lab strain. Our knowledge of mouse transcripts grew tremendously with the FANTOM projects where we discovered a large portion of the transcriptome was composed of non-coding RNAs (references b and c, cited 704 and 1,315 times, respectively). Using next-generation sequencing technologies, I compared *Hox* genes among three cartilaginous fishes by comparing embryonic transcriptomes and whole genome shotgun sequences from the skate, *Leucoraja erinacea* (reference d).
   a. Alm RA, Ling LS, Moir DT, King BL, Brown ED, Doig PC, Smith DR, Noonan B, Guild BC, deJonge BL, Carmel G, Tummino PJ, Caruso A, Uria-Nickelsen M, Mills DM, Ives C, Gibson R, Merberg D,

Mills SD, Jiang Q, Taylor DE, Vovis GF, Trust TJ. (1999). Genome-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature,* 397(6715),176-80.

b. The RIKEN Genome Exploration Research Group Phase II Team and the FANTOM Consortium. (2001). Functional annotation of a full-length mouse cDNA collection. *Nature,* 409, 685-690.

c. The FANTOM Consortium and RIKEN Genome Exploration Research Group Phase I & II Team. (2002). Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. *Nature*, 420, 563-73.

d. King BL, Gillis JA, Carlisle HR, Dahn RD. (2011). A natural deletion of the *HoxC* cluster in elasmobranch fishes. *Science*, 334(6062):1517. PMCID: PMC3264428. Reviewed in Research Highlights *Nature*, 480(22/29): 419, 2011.

2. **Building biomedical databases to integrate curated data on gene function**: Biomedical databases that feature curated information supported by peer-reviewed literature are essential to biomedical research. Model organism databases, such as Mouse Genome Informatics where I was a Bioinformatics Analyst for five years, provides an array of knowledge easily accessible through a web interface. My contributions to this project include integrating genome, transcript and protein sequence data into the Mouse Genome Database (references a and b below). I also redesigned the gene detail web page in 2004 to provide a high-level overview of all information about a gene, a design that is still used today. For six years, I was the biostatistician for the Comparative Toxicogenomics Database (CTD) that provides information about chemicals and their interactions with genes and how those chemicals are associated with diseases (reference c). A major contribution I made to CTD was developing a score to rank transitive inferences based on local network topology (reference d).

a. Baldarelli RM., Hill DP, Blake JA, Adachi J, Furuno M, Bradt D, Corbani LE, Cousins S, Frazer KS, Qi D, Yang L, Ramachandran S, Reed D, Zhu Y, Kasukawa T, Ringwald M, King BL, Maltais LJ, McKenzie LM, Schriml LM, Maglott D, Church DM, Pruitt K, Eppig JT, Richardson JE, Kadin JA, Bult CJ. (2003). Connecting sequence and biology in the laboratory mouse. *Genome Research*, 13(6B), 1505-19. PMCID: PMC403701.

b. Zhu Y, King BL, Parvizi B, Brunk BP, Stoeckert, Jr, CJ, Quackenbush J, Richardson J, Bult CJ. (2003). Integrating Computationally Assembled Mouse Transcript Sequences with the Mouse Genome (MGI) Database. *Genome Biology*, 4(2), R16. PMCID: PMC151306.

c. Davis AP, Grondin CJ, Johnson RJ, Sciaky D, King BL, McMorran R, Wiegers J, Wiegers TC, Mattingly CJ. The Comparative Toxicogenomics Database: update 2017. *Nucleic Acids Res.* 2017 45(D1):D972-D978. PMCID: PMC5210612.

d. King BL, Davis AP, Rosenstein MC, Wiegers TC, Mattingly CJ. (2012). Ranking transitive chemical-disease inferences using local network topology in the comparative toxicogenomics database. *PLoS One*. 7(11):e46524. PMCID: PMC3492369.

3. **Bioinformatics research training:** Training in bioinformatics is essential for biomedical researchers as large datasets have become common place and analysis of freely-accessible published datasets can inform hypotheses. Since 2002, I have taught bioinformatics as part of biomedical courses or organized and taught bioinformatics courses. Foremost of these is The Genome Access Course at Cold Spring Harbor Laboratory that I have organized and taught 33 times since 2005 to over 1,200 faculty, post doctoral fellows, graduate students and other researchers. The goal of this course and other trainings I provide an overview of major genomic and bioinformatics resources and tools and inspire students to learn more about these resources and tools by demonstrating their value. As part of the skate genome project, I co-organized a series of workshops that are described in the publication below.

a. Wang Q, Arighi CN, King BL, Polson SW, Vincent J, Chen C, Huang H, Kingham BF, Page ST, Farnum Rendino M, Thomas WK, Udwary DW, Wu CH; the North East Bioinformatics Collaborative Curation Team. (2012). Community annotation and bioinformatics workforce development in concert--Little Skate Genome Annotation Workshops and Jamborees. *Database (Oxford)*. 2012(0):bar064. PMCID: PMC3308154.

D. **Additional Information: Research Support and/or Scholastic Performance**

Ongoing Research Support

NSF OIA-1826777 (Kovach, PI)   08/15/18 – 07/31/22
Genomic Ecology of Coastal Organisms (GECO): A Systems-Based Research and Training Program in Genome-Phenome Relationships in the Wild.
This project investigates the genetic basis of organismal adaptation through the study of six different tidal marsh sparrow species across spatial and environmental gradients. The genomes of four sparrow species will be sequenced and assembled for the first time. I will also lead the characterize molecular pathways associated with kidney morphology and osmoregulation in species that adapted to tidal marsh environments compared to inland freshwater species with a graduate student. This award is a Track-2 Focused EPSCoR Collaboration (RII Track-2FEC).
**Role: Co-Principal Investigator** `EFFORT`

NIH/NIGMS P20 GM103423-17 (Coffman, PI)   05/01/17 – 04/30/19
Comparative Functional Genomics INBRE (IDeA Network in Biomedical Research Excellence) in Maine.
The goal of this program is to enhance a network for biomedical research training of faculty and students and for institutional development across 13 research and educational institutions in Maine.
Role: Bioinformatics Core Co-Director (Subaward; `EFFORT`

University of Maine System Research Reinvestment Fund (King and Henry, co-PIs) 05/01/18 – 04/30/19
Muscular Dystrophy Genomics Research Collaborative
The goal of this project is to characterize a novel zebrafish model for muscular dystrophy developed by Dr. Clarissa Henry's laboratory at the University of Maine where the GDP-mannose pyrophosphorylase B (*gmppb*) gene was knocked out using CRISPR-Cas9. A team of five undergraduates and one graduate student will identify molecular mechanisms that contribute to impaired muscle function in the novel zebrafish mutant by computationally modeling how networks of genes are dysregulated.
Role: Co-Principal Investigator

Support

NIH/NIBIB R25 EB022367-01 (Shaw, PI)   09/30/15 - 06/30/18
Establishing a Network of Skilled BD2K Practitioners: The Summer Workshop on Population-Scale Genomic Studies of Environmental Stress
The long-term goal of the proposed NIH Big Data to Knowledge (BD2K) training initiative in Environmental Genomics is to increase the number of BD2K practitioners and build a virtual network of big data scientists. We will couple three annual workshops that focus on population-scale genomics studies of environmental stress with networking tools aimed at keeping participants connected, trained and engaged in the application of modern sequencing technologies, computational approaches, and biostatistical methods.
Role: Instructor `EFFORT`
Recently Completed Research

Maine INBRE Research Resources Grant (PI)   10/30/17 – 4/30/18
Genome-wide Expression Profiling of Aberrant Neuromusculoskeletal Development in a Novel Model for GMPPB-Associated Dystroglycanopathy
The goal of this project was to characterized genome-wide gene expression in gmmbp zebrafish mutants using RNA-Seq to identify gene networks that are dysregulated in the mutants.
Role: Principal Investigator

NIH/NIGMS P20 GM103423-17 (Coffman, PI)   06/01/14 – 4/30/17
Comparative Functional Genomics INBRE (IDeA Network in Biomedical Research Excellence) in Maine.
The goal of this program is to enhance a network for biomedical research training of faculty and students and for institutional development across 13 research and educational institutions in Maine.
Role: Bioinformatics Core Director

NIH/NIGMS P20 GM104318-02 (Strange, PI)   09/01/13 – 12/30/16
Comparative Biology of Tissue Repair, Regeneration and Aging
The goal of this program was to create an innovative Center of Biomedical Research Excellence (COBRE) focused on the Comparative Biology of Tissue Repair, Regeneration and Aging.

Role: Comparative Functional Genomics Core Director

NIH/NIEHS R01 ES019604-05 (Mattingly, PI)   01/01/11 - 12/31/15
Generation of a Centralized and Integrated Resource for Exposure Data
This project curated and integrated chemical exposure data into the Comparative Toxicogenomics Database and enhance the capacity for prediction, analysis and interpretation of environment-disease networks by developing novel analysis and visualization tools that include exposure data.
Role: Biostatistician

Biosketches

BIOGRAPHICAL SKETCH
DO NOT EXCEED FIVE PAGES.

NAME: Hess, Samuel T.

eRA COMMONS USER NAME (credential, e.g., agency login): eRA Commons User Name

POSITION TITLE: Professor of Physics

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Yale University, New Haven, CT	B.S.	1995	Physics
Marine Biological Laboratory, Woods Hole, MA		1997-1997	Neurobiology
Cornell University, Ithaca, NY	M.S.	1998	Physics
Cornell University, Ithaca, NY	Ph.D.	2002	Physics
National Institutes of Health, Bethesda, MD		2002-2004	Membrane Biophysics

A. Personal Statement

I have the expertise, leadership, training, and motivation necessary to successfully carry out the proposed research project. In 2005, I invented the super-resolution microscopy technique called fluorescence photoactivation localization microscopy (FPALM; Hess et al. *Biophys. J.*, 2006), which was published in 2006 essentially simultaneously with photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM). **Super-resolution microscopy, including PALM, was awarded the Nobel Prize in Chemistry in 2014, and our invention of FPALM was cited within the Nobel Document "Super-Resolved Fluorescence Microscopy".** These methods were cited by *Science* magazine as one of the "Top Ten Scientific Breakthroughs" of 2006, and by *Nature Methods* as "Method of the Year" in 2008. My laboratory published the first localization microscopy of live cells in *PNAS* in 2007. We continue to pioneer new developments in localization microscopy, and have published three-dimensional (biplane) FPALM in *Nature Methods* (Juette et al., 2008), super-resolution anisotropy imaging in a second publication in *Nature Methods* in 2008, and multi-color FPALM (Gunewardene et al., *Biophys. J.* 2011). One of my most recent publications in *Biophysical Journal* specifically uses FPALM to image the actin cytoskeleton, and we have nearly a decade of experience using super-resolution microscopy to image a wide variety of subcellular structures in living and fixed samples. This expertise makes me very well qualified to carry out the super-resolution imaging portion of the proposed project.

In addition to super-resolution microscopy, I have been working in the field of membrane biophysics for over sixteen years, and have related publications in *Nature, Cell, Biophysical Journal*, and *PNAS* on the effects of lipids and the cytoskeleton on membrane organization. Our publications in *Biophysical Journal* (Gudheti et al., 2013) and (Gunewardene et al., 2011) specifically investigated the role of the cytoskeleton in organization of clustered membrane proteins. Thus, my experience in membrane biophysics also pertains to the proposed project.

In addition to expertise, my laboratory is well equipped for the proposed project. We operate and manage a super-resolution microscopy facility with all necessary technology, including a wide range of laser wavelengths, several EMCCD cameras capable of high frame rate single molecule imaging, a temperature and gas controlled incubator, stage autofocus, drift correction algorithms, and data analysis with a dedicated computer cluster. We already maintain several collaborations centered on the use of this FPALM facility. I also have experience running large collaborative grant projects; both active NIH grants funding my laboratory are based on interdisciplinary research with national and international collaborators.

Overall, I have maintained a high level of research productivity (61 publications in total including one in press; h-index = 28) and funding for my laboratory.

Hess, S.T; Girirajan, T; Mason, M.D. "Ultra-High Resolution Imaging by Fluorescence Photoactivation Localization Microscopy (FPALM)," *Biophysical Journal* 91: 4258-4272, 2006. PMCID: PMC1635685 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1635685/

Juette, M.F., Gould, T.J., Lessard, M.D., Mlodzianoski, M.J., Nagpure, B.S., Bennett, B.T., Hess, S.T., and Bewersdorf, J. "Three-Dimensional sub-100 nm Resolution Fluorescence Microscopy of Thick Samples," *Nature Methods* 5: 527-9, 2008. PMID: 18469823

Gunewardene M.S., Subach F.V., Gould T.J., Penoncello G.P., Gudheti M.V., Verkhusha V.V., and Hess S.T. "Superresolution Imaging of Multiple Fluorescent Proteins with Highly Overlapping Emission Spectra in Living Cells," *Biophysical Journal* 101: 1522-8, 2011. PMCID: PMC3177078
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3177078/

B. Positions and Honors
Positions and Employment
1995-1996	*Sage Graduate Fellow, Dept. of Physics, Cornell University, Ithaca, NY*
	Department Head: Douglas Fitchen, Ph.D., Professor of Physics, Cornell University
1996-1999	*Res. Fellow, Dept. of Physics, Cornell University*
	Advised by: W.W. Webb, Prof. of Applied Physics and S. B. Eckert Prof. in Engineering, Cornell University
1997-1997	*Student, Neurobiology Course, Marine Biological Laboratory, Woods Hole, MA*
1999-2002	*National Institutes of Health Molecular Biophysics Trainee, Cornell University*
	Advised by: W.W. Webb
2002-2004	*Intramural Research Training Award Fellow, National Institutes of Health, Bethesda, MD*
	Supervised by: Joshua Zimmerberg, M.D., Ph.D., Director, NASA/NIH Center for Three-Dimensional Tissue Culture, Chief of Laboratory of Cellular and Molecular Biophysics, National Institutes of Child Health and Human Development, National Institutes of Health
2004-2004	*Teaching Assist., Neurobiology Course, Marine Biological Laboratory, Woods Hole, MA*
2007-2007	*Teaching Assist., Neurobiology Course, Marine Biological Laboratory, Woods Hole, MA*
2004-2009	*Asst. Prof. of Physics, Dept. of Physics & Astronomy, University of Maine, Orono, ME*
2009-2014	*Assoc. Prof. of Physics, Dept. of Physics & Astronomy, University of Maine, Orono, ME*
2013-2015	*Faculty, Neurobiology Course, Marine Biological Laboratory, Woods Hole, MA*
2014-pres.	*Professor of Physics, Dept. of Physics & Astronomy, University of Maine, Orono, ME*

Academic and Professional Honors: Graduate Mentoring Award, University of Maine, Spring 2018. Cited in the Nobel Prize for Chemistry in the field of Super-Resolution Microscopy (2014). Early Tenure, University of Maine, Orono (2009). Nominated for a Blavatnik Award for Young Scientists. Nominated for a "Brilliant Ten" award (*Popular Science*). Work cited by *Science* as one of the "Top Ten Scientific Breakthroughs of the Year," (2006), as one of the "Methods to Watch" by *Nature Methods* (2007), and as "Method of the Year" by *Nature Methods* (2008), and as a "milestone in light microscopy," *Nature Milestones*, 2009. College of Engineering Early Career Teaching Excellence Award (2008). Cover images for *Nature*, *Biophysical Journal*, and *Journal of Physical Chemistry C*. National Science Foundation Graduate Research Fellow, Cornell University, Ithaca, NY (1996-1999); Phi Beta Kappa; Graduated *summa cum laude*, De Forest Prize in Physics, Yale University, New Haven, CT (1995). Eagle Scout (1991).

C. Contributions to Science
1. My interest in understanding the mechanisms of viral infection led to investigation of membrane lateral organization, first in lipidic systems, then in native cell membranes. The lack of optical resolution posed a significant limitation, motivating me to invent the super-resolution microscopy technique FPALM, which enabled imaging the membranes of living cells at the nanoscale. This capability led to the discovery that most of the published models of cell membrane organization, specifically relating to the clustering of hemagglutinin from influenza virus, had significant inconsistencies with our experimental results. We therefore were forced to develop a new model of membrane organization, invoking feedback between the actin cytoskeleton, membrane proteins, and cellular signaling pathways, as well as experimentally consistent aspects of other models. Interactions between the viral and host cell components are also closely tied to membrane lateral organization, not just in influenza virus, but also in a variety of other viruses, including snakehead rhabdovirus, components of which were imaged by FPALM (Gabor et al. 2013). By providing these new imaging capabilities, our understanding of viral infection and cell membrane organization has been advanced.

a. Baumgart, T., Hess, S.T., and Webb, W.W.*, "Imaging Coexisting Fluid Domains in Biomembrane Models Coupling Curvature and Line Tension," *Nature* 425: 821-824, 2003. PMID: 14574408

b. Hess, S.T.*, Gould, T.J., Gudheti, M.V., Maas, S.A., Mills, K.D., and Zimmerberg, J. "Dynamic Clustered Distribution of Hemagglutinin Resolved at 40 nm in Living Cell Membranes Discriminates Between Raft Theories," *PNAS* 104: 17370-5, 2007. PMCID: PMC2077263

c. Gudheti M., Curthoys N., Gould T., Kim D., Gabor K., Gunewardene M., Zimmerberg J., Gosse J., Kim C., Hess S.T.*, "Actin Mediates the Nanoscale Membrane Organization of the Clustered Membrane Protein Influenza Hemagglutinin," *Biophysical Journal* 104: 2182-92, 2013. PMCID: PMC3660645

d. Gabor K., Stevens C., Pietraszewski M., Gould T., Lam S., Gong Z., Hess S.T.*, Kim C.*, "Super Resolution Microscopy Reveals that Caveolin-1 is required for antiviral immune response," *PLoS One* 8: e68759, 2013. PMCID: PMC3706321 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3706321/
*corresponding author(s)

2. We have investigated mitochondrial membrane organization using super-resolution microscopy and related techniques, revealing nanoscale changes as a result of drug treatments with triclosan, an antimicrobial compound used in consumer products, which we showed acts as a proton ionophore. We also developed new methodologies for simultaneously imaging multiple molecular species within mitochondria in living cells.

a. Weatherly, L.M., Nelson, A.J., Shim J., Riitano, A.M., Gerson, E.D., Hart, A.J., de Juan-Sanz, J., Ryan, T.A., Sher, R., Hess, S.T., and Gosse, J.A. "Antimicrobial Agent Triclosan Disrupts Mitochondrial Structure, Revealed by Super-resolution Microscopy, and Inhibits Mast Cell Signaling via Calcium Modulation Toxicology and Applied Pharmacology," *Toxicology and Applied Pharmacology 349:39-54, 2018. PMID: 29630968*

b. Weatherly, L.M., Shim, J., Hashmi, H.N., Kennedy, R.H., Hess, S.T., and Gosse, J.A. "Antimicrobial Agent Triclosan is a Proton Ionophore Uncoupler of Mitochondria in Living Rat and Human Mast Cells and in Primary Human Keratinocytes," *Journal of Applied Toxicology Jul 23. doi: 10.1002/jat.3209,* 2015. PMID: 26204821

c. Wilmes, S., Staufenbiel, M., Liβe, D., Richter, C.P., Beutel, O., Busch, K.B., Hess, S.T., Piehler, J. "Live cell triple-color super-resolution imaging for probing submicroscopic receptor organization in the plasma membrane," *Angewante Chemie*, 51: 4868-4871, 2012. PMID: 22488831

d. Appelhans, T., Richter, C., Wilkens, V., Hess, S.T., Piehler, J., Busch, K.B. "Dynamic nanoscale organization of membrane protein complexes in mitochondrial micro-compartments of living cells," *Nano Letters* 12: 610-6, 2012. PMID: 22201267

3. In addition to the contributions described above, we have further pushed the development of novel instrumentation for the study of cellular organization. We were the first to publish live-cell localization microscopy images (Hess et al. *PNAS* 2007), and in vivo localization microscopy (K. Gabor et al., 2015). Our polarization-based FPALM method (Gould et al., *Nature Methods*, 2008) allows a super-resolution map of single-molecule anisotropies to be imaged, which can be used to extract information about molecular orientations in addition to positions at the nanoscale. A publication in press Unpublished
Unpublished
Unpublished   *in press*). A recently published spectrally-resolved version of FPALM (Mlodzianoski et al., *PLoS One*, 2016) allows the determination of the emission spectrum of every localized molecule, in addition to its position, making a large number of novel fluorescent sensor applications accessible to super-resolution localization microscopy, as well as multi-color imaging of up to 20 fluorescent species simultaneously.

Biosketches

a. Gould, T.J., Gunewardene, M.S., Gudheti, M.V., Verkhusha, V.V., Yin, S.R., Gosse, J.A., and Hess, S.T. "Nanoscale Imaging of Molecular Positions and Anisotropies," *Nature Methods* 5: 1027-30, 2008. PMCID: PMC2901392 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2901392/

b. Gabor K.A., Kim D., Kim C.H., Hess S.T. "Nanoscale imaging of caveolin-1 membrane domains *in vivo*." *PLoS One*. 2015 Feb 3;10(2):e0117225. doi: 10.1371/journal.pone.0117225. eCollection, 2015. PMID 25646724.

c. Mlodzianoski M., Gunewardene M.S., Hess S.T., "Spectral Fluorescence Photoactivation Localization Microscopy," *PLoS One* 11(3): e0147506, 2016. PMID: 27002724

d. Pennacchietti F., Gould T.J., Hess S.T., "The Role of Probe Photophysics in Localization Microscopy," *Biophysical Journal, 2017*. PMCID: PMC5686043

Complete List of Published Work:
https://scholar.google.com/citations?user=HDr0Ef8AAAAJ&hl=en

D. Additional Information: Research Support and/or Scholastic Performance
Ongoing Research Support

National Institute of General Medical Sciences (PI: Hess)   9/1/15-8/31/18
Mechanism of Association between Influenza Hemagglutinin and the Host Cell Actin Cytoskeleton R15-GM116002
This AREA award funded investigation of the interactions between influenza virus components, the host cell actin cytoskeleton, signaling pathways, and lipids. Super-resolution microscopy (FPALM) and other fluorescence spectroscopy methods are being used to quantify associations between viral and cellular components at nanometer length scales. *Role*: PI.

National Institute of Environmental Health Sciences (PI: Gosse)   9/1/14-8/31/17*
Mechanism of Triclosan Disruption of Mast Cell Function R15-ES024593
This AREA award has funded a study of the effects of the molecule triclosan on mast cell function, the actin cytoskeleton, intracellular Ca^{2+}, and organization of cell membranes. *Role:* Faculty
* a no cost extension was granted to allow the project to continue until 8/31/18.

National Aeronautics and Space Administration / Maine Space Grant Consortium (PI: Hess) 9/1/17-8/31/18
Imaging and Sensor Applications of Uninhabited Aerial Vehicles SG-18-10
This award funds research on Uninhabited aerial vehicles (UAVs), integration of aerospace content into physics classes at the University of Maine, undergraduate research projects, and outreach. *Role*: PI.

Completed Research Support

National Institute of Arthritis and Musculoskeletal and Skin Diseases (PI: Cox)   9/1/12-8/31/17
Genetic Control of Phospholipid Biosynthesis and Muscular Dystrophy R01-AR054170
This five-year project is investigating the role of mitochondrial morphology in the progression of muscular dystrophy in a mouse model using super-resolution microscopy (FPALM). Together with Dr. Cox (PI) of the Jackson Laboratory and Prof. Roger Sher (Dept. of Molecular and Biomedical Sciences at University of Maine), my laboratory is carrying out super-resolution imaging of muscle cells expressing mitochondrial and endoplasmic reticulum (ER) markers, comparing mitochondrial membrane organization including curvature and proximity to ER, in cells from mice with and without mutations leading to muscular-dystrophy-like symptoms. *Role:* Subcontract PI

National Institute for General Medical Sciences (PI: Hess)   9/1/10-8/31/14*
The Role of the Actin Cytoskeleton and Cholesterol in Influenza Virus Assembly R15-GM09471
This AREA award funded a super-resolution microscopy study of the effects of cholesterol, the actin cytoskeleton, and actin binding proteins on the lateral organization of cell membranes expressing

Biosketches

hemagglutinin, the fusion protein from influenza virus. This work led to discovery of an important role for actin and actin binding proteins in hemagglutinin membrane organization. *Role:* PI.
*a no-cost extension was approved to allow an end date of 8/31/15.

National Institute for Allergy and Infectious Diseases (PI: Hess)   9/1/05-7/31/12
The Role of Rafts in Virus-Induced Membrane Remodeling K25-AI065459
This Career Award funded a fluorescence spectroscopy and high-resolution microscopy study of the effects of cholesterol and lipid composition on the lateral organization of cell membranes expressing hemagglutinin, the fusion protein from influenza virus. *Role:* PI

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Parent, Matthew T

eRA COMMONS USER NAME (credential, e.g., agency login): eRA Commons User Name

POSITION TITLE: Graduate Research Assistant

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Maine, Orono	B.S.	05/2011	Physics
University of Maine, Orono	Ph.D.	Expected: Spring 2019	Physics

A. Personal Statement

I have been a graduate student and researcher under the advising of Samuel Hess for several years and I have the experience and knowledge to assist with the proposed work. I have extensive experience working with multicolor and 3D fluorescence photoactivation localization microscopy (FPALM), especially as it relates mitochondrial membrane imaging and nanoscale cell membrane organization. I also have several years of programming experience and expertise, including bleed-through correction, lateral and axial drift correction, colocalization analysis, and curvature analysis, and assisted in development of a novel bleed-through correction algorithm for multicolor single molecule localization methods (Kim et al, 2013).

Kim, D., Curthoys, N.M., Parent, M.T., and Hess, S.T. (2013). Bleed-through correction for rendering and correlation analysis in multi-colour localization microscopy. Journal of Optics.

B. Positions and Honors
Positions and Employment
2011-Current   Graduate Research Assistant, University of Maine

C. Contributions to Science

I've worked primarily on two areas of research during my time at the University of Maine:

1. In collaboration with Greg Cox (Jackson Laboratory), Roger Sher (University of Maine, Dept. of Molecular and Biomedical Sciences), and Samuel Hess, I contributed to a project to examine the role of mitochondrial morphology and their proximity to the endoplasmic reticulum as they relate to a rostrocaudal muscular dystrophy model in mice. Changes in mitochondrial membrane composition and curvature are hypothesized to lead to dysregulated mitochondrial fusion and development of megamitochondria, although the precise mechanism is not known. My role in this project was to provide super-resolution 3D FPALM imaging and analysis of fixed and living mouse muscle cells. I also developed investigative tools to identify mitochondria and measure membrane curvature from localized molecules.

Biosketches

2. Under the advising of Samuel Hess, I have contributed to the labs ongoing investigations of the clustering of influenza viral membrane protein hemagglutinin (HA). HA is the primary membrane protein component of influenza virions and clusters on the membrane of infected cells, even in the absence of other viral proteins. Generally accepted models of cell membrane organization have failed to adequately explain HA clustering and has led to a new proposed models involving feedback from the actin cytoskeleton and associated signaling pathways.

Curthoys, N.M., Parent, M.T., Mlodzianoski, M., Nelson, A.J., Lilieholm, J., Butler, M.B., Valles, M., and Hess, S.T. (2015). Dances with Membranes: Breakthroughs from Super-resolution Imaging

D. Additional Information: Research Support and/or Scholastic Performance

PHS 398 Cover Page Supplement

1. Vertebrate Animals Section

Are vertebrate animals euthanized?　　● Yes　　○ No

If "Yes" to euthanasia

Is the method consistent with American Veterinary Medical Association (AVMA) guidelines?

● Yes　　○ No

If "No" to AVMA guidelines, describe method and provide scientific justification

....

2. *Program Income Section

*Is program income anticipated during the periods for which the grant support is requested?

○ Yes　　● No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

　*Budget Period　　*Anticipated Amount ($)　　*Source(s)

PHS 398 Cover Page Supplement

3. Human Embryonic Stem Cells Section

*Does the proposed project involve human embryonic stem cells?   ○ Yes   ● No

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, check the box indicating that one from the registry will be used:

    Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s) (Example: 0004):

4. Inventions and Patents Section (Renewal applications)

*Inventions and Patents: ○ Yes   ○ No

If the answer is "Yes" then please answer the following:

*Previously Reported: ○ Yes   ○ No

5. Change of Investigator/Change of Institution Section

❏   Change of Project Director/Principal Investigator

Name of former Project Director/Principal Investigator

Prefix:

*First Name:

Middle Name:

*Last Name:

Suffix:

❏   Change of Grantee Institution

*Name of former institution:

PHS 398 Modular Budget

Budget Period: 1
Start Date: 07/01/2019   End Date: 06/30/2020

A. Direct Costs		Funds Requested ($)
	Direct Cost less Consortium Indirect (F&A)*	250,000.00
	Consortium Indirect (F&A)	
	Total Direct Costs*	250,000.00

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	Modified total direct costs	46.00	250,000.00	115,000.00
2.				
3.				
4.				

Cognizant Agency
(Agency Name, POC Name and Phone Number)

Indirect (F&A) Rate Agreement Date   Total Indirect (F&A) Costs   115,000.00

C. Total Direct and Indirect (F&A) Costs (A + B)	Funds Requested ($)	365,000.00

PHS 398 Modular Budget

Budget Period: 2			
Start Date: 07/01/2020 End Date: 06/30/2021			

A. Direct Costs			Funds Requested ($)
	Direct Cost less Consortium Indirect (F&A)*		250,000.00
	Consortium Indirect (F&A)		
	Total Direct Costs*		250,000.00

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	Modified total direct costs	46.00	250,000.00	115,000.00
2.				
3.				
4.				

Cognizant Agency
(Agency Name, POC Name and Phone Number)

Indirect (F&A) Rate Agreement Date	Total Indirect (F&A) Costs	115,000.00

C. Total Direct and Indirect (F&A) Costs (A + B)	Funds Requested ($)	365,000.00

PHS 398 Modular Budget

Budget Period: 3
Start Date: 07/01/2021   End Date: 06/30/2022

A. Direct Costs		Funds Requested ($)
	Direct Cost less Consortium Indirect (F&A)*	250,000.00
	Consortium Indirect (F&A)	
	Total Direct Costs*	250,000.00

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	Modified total direct costs	46.00	250,000.00	115,000.00
2.				
3.				
4.				

Cognizant Agency
(Agency Name, POC Name and Phone Number)

Indirect (F&A) Rate Agreement Date   Total Indirect (F&A) Costs   115,000.00

C. Total Direct and Indirect (F&A) Costs (A + B)	Funds Requested ($)	365,000.00

PHS 398 Modular Budget

Budget Period: 4
Start Date: 07/01/2022   End Date: 06/30/2023

A. Direct Costs		Funds Requested ($)
	Direct Cost less Consortium Indirect (F&A)*	250,000.00
	Consortium Indirect (F&A)	
	Total Direct Costs*	250,000.00

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	Modified total direct costs	46.00	250,000.00	115,000.00
2.				
3.				
4.				

Cognizant Agency
(Agency Name, POC Name and Phone Number)

Indirect (F&A) Rate Agreement Date	Total Indirect (F&A) Costs	115,000.00

C. Total Direct and Indirect (F&A) Costs (A + B)	Funds Requested ($)	365,000.00

PHS 398 Modular Budget

Budget Period: 5
Start Date: 07/01/2023   End Date: 06/30/2024

A. Direct Costs	Funds Requested ($)
Direct Cost less Consortium Indirect (F&A)*	250,000.00
Consortium Indirect (F&A)	
Total Direct Costs*	250,000.00

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	Modified total direct costs	46.00	250,000.00	115,000.00
2.				
3.				
4.				

Cognizant Agency
(Agency Name, POC Name and Phone Number)

Indirect (F&A) Rate Agreement Date	Total Indirect (F&A) Costs	115,000.00

C. Total Direct and Indirect (F&A) Costs (A + B)	Funds Requested ($)	365,000.00

PHS 398 Modular Budget

Cumulative Budget Information

1. Total Costs, Entire Project Period

Section A, Total Direct Cost less Consortium Indirect (F&A) for Entire Project Period ($)	1,250,000.00
Section A, Total Consortium Indirect (F&A) for Entire Project Period ($)	
Section A, Total Direct Costs for Entire Project Period ($)	1,250,000.00
Section B, Total Indirect (F&A) Costs for Entire Project Period ($)	575,000.00
Section C, Total Direct and Indirect (F&A) Costs (A+B) for Entire Project Period ($)	1,825,000.00

2. Budget Justifications

Personnel Justification	1234-Henry_UMaine_Personnel.pdf
Consortium Justification	
Additional Narrative Justification	

Personnel Justification

A. Senior/Key Personnel

Clarissa Henry, PhD, Principal Investigator, [EFFORT] effort [EFFORT] salary requested the first year, [EFFORT] salary, [EFFORT] person months, requested years 2-5). Dr. Henry has an [EFFORT] research [EFFORT] teaching appointment, thus she has [EFFORT] effort to commit to this project. Dr. Henry will oversee the project and coordinate monthly meetings of the research group. Dr. Henry is an expert on cell adhesion mechanisms underlying musculoskeletal development in the zebrafish model. Dr. Henry will meet with personnel in her lab weekly and personnel in the King and Hess labs every other week. Dr. Henry's lab will perform the bulk of the studies proposed, with exceptions noted below.

Benjamin King, PhD, Co-Investigator, [EFFORT] effort [EFFORT] salary requested the first year, [EFFORT] salary, [EFFORT] person months, requested years 2-5). Dr. King has a [EFFORT] research [EFFORT] teaching appointment and has the time to commit [EFFORT] effort to this project. Dr. King's lab will be responsible for characterizing the transcriptomes of the zebrafish dystroglycanopathy mutants to find genes and miRNAs to understand the molecular mechanisms of phenotypic variation and plasticity in these novel muscular dystrophy models and find candidate novel biomarkers for disease and disease progression. Dr. King's lab will prepare samples for RNA-Seq and miRNA-Seq, coordinate library preparation and sequencing, and analyze these data to find dysregulated candidate genes and conduct qPCR validation of candidates. Dr. King is an expert in genomics and bioinformatics with specialized expertise in the integrated analysis of miRNAs and mRNA expression data. Dr. King has extensive experience in genomics and bioinformatics as demonstrated by his 60+ publications. He led the bioinformatics analysis cores at The Jackson Laboratory for five years and Mount Desert Island Biological Laboratory for seven years prior to his appointment at University of Maine.

Sam Hess, PhD, Co-Investigator, [EFFORT] effort [EFFORT] salary requested the first year, [EFFORT] salary, [EFFORT] person months, requested years 2-5). Dr. Hess has significant experience with super-resolution microscopy: he invented the localization microscopy technique FPALM (S.T. Hess et al., *Biophysical Journal*, 2006), which is conceptually identical to PALM and STORM, and cited within the Scientific Background on the Nobel Prize in Chemistry 2014, "Super-Resolved Fluorescence Microscopy." Dr. Hess has also published the first live-cell localization microscopy in 2007, and the first *in vivo* localization microscopy in 2015, which was conducted in living zebrafish. He developed multicolor, three-dimensional, polarization, and spectral versions of localization microscopy, and has applied these methods to understand mitochondrial organization, membrane organization in general, and the role of host cell components on influenza virus infection. Dr. Hess will direct the super-resolution imaging portion of the project, providing all necessary resources and equipment, codes for data analysis, and co-advising the postdoc who will carry out the experiments.

B. Other Personnel

Michelle Goody, PhD, Research Assistant Professor, [EFFORT] effort Year 1 [EFFORT] months), [EFFORT] effort [EFFORT] months, years 2-5). Dr. Goody, along with a graduate student, will be primarily responsible for all of the experiments to be conducted in the Henry lab. Dr. Goody was the first person at UMaine to troubleshoot using CRISPR-Cas9 and generated the *gmppb* mutant and much of the preliminary data for this grant. She will be an invaluable and skilled contributor for this grant.

Mary Astumian, M.A., Technician, [EFFORT] effort ([EFFORT] person months, Year 1), [EFFORT] effort ([EFFORT] person months, Years 2-5). Ms. Astumian has been a technician in the laboratory for eight years. She will split her time between the Henry and King labs and will be responsible for prepping samples for RNA-seq Year 1. In Years 2-5 she will be responsible for assisting with qPCR validation of candidate genes identified in the mRNA-Seq and miRNA-Seq studies, as well as helping with all experiments.

Mat Parent, Post-doctoral fellow, [EFFORT] effort [EFFORT] person months, Years 1-5). Mr. Parent is currently finishing his PhD in the Hess lab and will graduate May 2015. Mat generated the preliminary super resolution data.

Personnel Justification

He will be responsible for the super- resolution studies proposed and will contribute to light-sheet microscopy studies in live zebrafish in the Henry lab.

Fringe Benefits
Fringe benefits for summer salary for the PI and co-Is are included at the University's federally negotiated rate of 7.8%. Fringe for regular employees is charged at 53.4%. Fringe is not charged on student salaries.

PHS 398 Research Plan

Introduction	
1. Introduction to Application	
(for Resubmission and Revision applications)	

Research Plan Section	
2. Specific Aims	1235-NAD.Aims.v8.pdf
3. Research Strategy*	1236-NAD approach_v17.pdf
4. Progress Report Publication List	

Other Research Plan Section	
5. Vertebrate Animals	1237-Vertebrate Animals 10 01 18.pdf
6. Select Agent Research	
7. Multiple PD/PI Leadership Plan	
8. Consortium/Contractual Arrangements	
9. Letters of Support	1238-Letters.pdf
10. Resource Sharing Plan(s)	1239-Resource Sharing Plan 10 01 18.pdf
11. Authentication of Key Biological and/or Chemical Resources	1240-Authentication Plan 09.14.18.pdf

Appendix	
12. Appendix	

SPECIFIC AIMS

Congenital muscular dystrophies (CMDs) are progressive debilitating diseases without cures. Many CMDs disrupt the adhesion of muscle cells to their surrounding extracellular matrix (ECM). Muscle-ECM adhesion is critical for muscle development, homeostasis, regeneration, and resilience to stress. Mutations in genes that modulate muscle-ECM adhesion frequently lead to CMDs. For example, Dystroglycan (DG) and Integrin alpha7 (Itga7) are transmembrane ECM receptors that, when mutated, result in CMDs. There are significant gaps in our understanding of basic DG and Itga7 biology. Whether and/or how these transmembrane receptors interact during muscle development/homeostasis is not known. In addition, the roles that post-translational modification of DG plays in modulating both the ECM proper and muscle-ECM adhesion are not known. We previously found that exogenous NAD+ potentiates ECM deposition[3]. NAD+ improves dystrophic phenotypes in zebrafish lacking either DG or Itga7. Although NAD+ is a well-known modulator of muscle health, most studies have focused on how NAD+ functions in the mitochondrial and nuclear compartments. Thus, the basic cell biological mechanisms that underlie NAD+-mediated improvement in muscle-ECM adhesion are not well understood. In this grant, we focus on the basic mechanisms, impacts, and limitations of NAD+-mediated potentiation of ECM deposition and muscle cell-ECM adhesion.

Our *long-term goal* is to understand how signaling between muscle cells and their ECM mediates muscle health. Secondary Dystroglycanopathies are a subset of CMDs that result from mutations in genes that are necessary for glycosylation of DG[16-18]. DG glycosylation is necessary for muscle-ECM adhesion[20]. GDP-mannose is essential for glycosylation reactions. GDP-mannose is synthesized by GMPPB. Mutations in *GMPPB* result in GMPPB-associated Dystroglycanopathy[26]. We used CRISPR-Cas9 to generate a zebrafish *gmppb* mutant. Preliminary data show that muscle development, homeostasis, and regeneration are disrupted in *gmppb* mutants. In contrast to our previous data showing NAD+ improves ECM deposition in *dg*-deficient zebrafish, preliminary data show that NAD+ does not improve muscle structure in *gmppb* mutants. In this grant, we will compare and contrast the mechanisms underlying the effects of DG glycosylation and NAD+ on muscle development, homeostasis, and regeneration. We will do this from the perspective of both the transmembrane ECM receptors and ECM proteins. Our *central hypothesis* is that both NAD+ and *gmppb* regulate muscle cell adhesion by altering sarcolemma architecture and ECM organization. We will test this hypothesis with two aims:

Aim 1: Test the hypothesis that NAD+ increases cell adhesion in DG mutant zebrafish by increasing Itga7 clustering; and that hypoglycosylated DG disrupts sarcolemma architecture and prevents NAD+-mediated Itga7 clustering and increased cell adhesion. Preliminary data show that NAD+ does not reduce muscle degeneration in *gmppb* mutants. We hypothesize that hypoglycoslated DG acts as a "dominant negative" and prevents clustering of the Itga7 receptor. We will test this hypothesis by: (1) generating *dg;gmppb* double mutants that we predict will be rescued with NAD+, and (2) using super-resolution microscopy to elucidate Itga7 and DG dynamics in the sarcolemma. Longitudinal light sheet microscopy studies will determine how NAD+ impacts cell adhesion, survival, and regeneration in *dg, gmppb,* and *dg;gmppb* double mutants. We will use super resolution microscopy to elucidate DG and Itga7 localization in normal and diseased muscle. We hypothesize that NAD+ increases ECM deposition in DG and Itga7 mutants by increasing clustering of the remaining receptor. Taken together, these studies will significantly contribute to our understanding of how NAD+ and DG glycosylation impact muscle-ECM adhesion.

Aim 2: Identify new muscle cell adhesion regulators through comparative studies of dysregulated muscle development in three zebrafish models of muscular dystrophy. Regulation of ECM composition is a critical aspect of cell adhesion. The mechanisms of ECM regulation during development and homeostasis are incompletely understood. We will take an unbiased approach to identify ECM regulatory nodes by using network modeling and network resilience analysis of co-expressed coding and non-coding genes. These experiments will determine how altered cell adhesion networks contribute to NAD+-mediated amelioration of muscle degeneration in DG and Itga7 mutants. We will investigate how these networks change when DG glycosylation is disrupted. Preliminary data indicate that 3 Elastase genes are significantly downregulated in *gmppb* mutant zebrafish. We hypothesize that the concomitant upregulation of Elastin, an ECM protein, modulates muscle development and degeneration and will test that hypothesis in this aim.

Completion of this grant will provide new insight into how cell-ECM adhesion mediates muscle development and homeostasis in vertebrate models of CMDs. These basic *in vivo* cell biological studies are crucial to provide a foundational understanding of the interplay between transmembrane receptors, ECM regulation, and cell adhesion.

A: SIGNIFICANCE

Muscle adhesion to the ECM is critical for muscle development, homeostasis, and regeneration: Cell-ECM adhesion complexes are hubs that integrate chemical and mechanical information that regulates muscle development and homeostasis[1]. The molecular mechanisms underlying muscle-ECM adhesion are not fully elucidated. Identification of mechanisms has been hindered by: (1) we don't know all the regulators of muscle cell adhesion, and (2) an inability to resolve the organization of transmembrane ECM receptors *in vivo*. We identified NAD+ biosynthesis as a novel regulator of muscle-ECM adhesion during muscle development [2,3]. The mechanisms by which NAD+ functions in muscle-ECM adhesion *in vivo* are not known. We propose to elucidate mechanisms of NAD+ action in muscle-ECM adhesion in dystroglycanopathies. The primary cause of muscle fiber death in *dg* and *gmppb* mutant zebrafish is disrupted cell-ECM adhesion. We hypothesize that NAD+ reduces muscle degeneration in zebrafish models of muscular dystrophies by improving cell-ECM
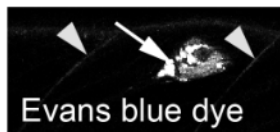
Fig. 1: <u>Fibers in *gmppb* mutants detach from the MTJ prior to membrane damage.</u> MTJs are denoted with yellow arrowheads. EBD is seen in detached muscle cells (white arrow).

adhesion. Detached muscle fibers in zebrafish muscle disease models can occur by two mechanisms. One is loss of sarcolemma integrity and subsequent fiber death. This mode occurs in *dmd* zebrafish models [4]. Alternatively, muscle fibers can detach from their surrounding ECM **prior** to loss of sarcolemma integrity and cell death. The latter mode occurs in *laminin a2 -/-* [5], *dg-/-*[6], and *gmppb-/-* (Fig. 1 and data not shown) zebrafish. Thus, failure of cell-ECM adhesion results in muscle degeneration, providing a strong premise for our focus on elucidating mechanisms of NAD+ action in cell-ECM adhesion and organization of transmembrane receptors within the sarcolemma.

Fig. 2: <u>Compartmentalization of NAD+ pools in skeletal muscle.</u> NAD+ is in mitochondrial, nuclear, cytosolic, and membrane proximal pools (shown) and vesicles (not shown). Enzymes that consume NAD+ are boxed. Integrin receptors and a membrane channel that transports NAD+ are labeled.

How does NAD+ impact cell adhesion? NAD+ function in skeletal muscle is thought to be compartmentalized to the nucleus, mitochondria, cytoplasm, and sarcoplasmic reticulum. We recently proposed the existence of a sarcolemma proximal compartment that plays a role in regulating muscle-ECM adhesion[7] (Fig. 2). Few groups study NAD+ function in this compartment[8–10]. We will identify mechanisms of NAD+ improvement of muscle and ECM organization in this grant.

Mechanisms that improve muscle-ECM adhesion: Clearly, adhesion of muscle cells to their ECM microenvironment is critical for development and homeostasis. The two major transmembrane receptors that anchor muscle cells to their ECM are DG and Itga7. We previously showed that NAD+ supplementation reduces muscle degeneration in zebrafish deficient for either DG or Itga7[3]. These two cell adhesion complexes display some degree of functional redundancy in both zebrafish and mouse models [3,11–13]. One therapeutic strategy involves promoting complementary, alternative mechanisms of cell-ECM adhesion. For example, transgenic expression of Itga7 rescues muscle in mouse models of Duchenne Muscular Dystrophy (DMD) or Merosin-deficient CMD type 1A [13,14]. While transgenic expression and/or protein therapy work in small mammals, there is concern that these methods won't be efficacious in larger mammals. One solution is that after compensatory cell adhesion mechanisms are identified, chemical screens can be conducted for small molecules that upregulate the desired transcript. For example, the small molecule SU9516 upregulates integrin alpha7beta1 in the mouse model of DMD and prednisone treatment upregulates itga7 and laminin alpha2 in GRMD dogs modeling DMD [12,15]. *These data suggest the hypothesis that innate, genetically encoded compensatory mechanisms of cell adhesion can be translated into potential therapies for CMDs.* The <u>roadblock</u> to this approach is that we do not yet know the suite of compensatory cell adhesion mechanisms that can be exploited. We are using *dg* and *gmppb* mutants as tools to elucidate compensatory cell adhesion mechanisms. Our preliminary data have already identified upregulated cell adhesion genes that may modulate the phenotype of *gmppb* mutants (Fig. 14). We will elucidate mechanisms and discover additional genes in Aim 2. Our focus on understanding <u>basic mechanisms of compensatory responses</u> is an <u>important first step</u> towards identifying future therapeutic targets.

Dystroglycanopathies result from mutations in genes responsible for glycosylation of α-DG[16–18] DG is a central component in the Dystrophin-glycoprotein complex (DGC)[22] that anchors muscle cells to their ECM.
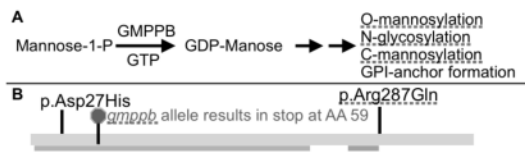
Research Strategy

Fig. 3: <u>GMPPB</u>. (A) GMPPB is required for synthesis of GDP-Mannose. (B) Diagram of GMPPB and the locations of the two most common, disease-causing point mutations. Blue and orange bars represent the nucleotidyl transferase domain and the Bacterial transferase hexapeptide domains, respectively. Adapted from Carss et al., 2013. The zebrafish *gmppb* mutant results in a premature stop at AA 59.

The DGC also links muscle to tendons at myotendinous junctions (MTJs)[23–25]. Glycosylation is necessary for normal DG-ECM binding[20,24,26]. There are no cures for dystroglycanopathies.

GMPPB-associated dystroglycanopathy: Mutations in *GMPPB* cause a dystroglycanopathy that varies in presentation from severe, infantile onset Walker-Warburg syndrome to mild, adult onset Limb Girdle Muscular Dystrophy[27–33]. GMPPB catalyzes GDP-mannose formation from mannose-1-phosphate and GTP (Fig. 3A). GDP-mannose is the major mannosyl donor for many glycosylation events, including C- and O-mannosylation, N-glycosylation, and GPI-anchor formation. We chose to focus on GMPPB in this grant for a couple of reasons: (1) the phenotypic spectrum of patients with *GMPPB* mutations is similar to the spectrum observed in other dystroglycanopathies, and (2) new individuals with mutations in *GMPPB* are identified frequently (41 already in 2018[31,34]). We used CRISPR-Cas9 to generate *gmppb* mutant zebrafish (Fig. 3B). This *gmppb* allele has a single nucleotide deletion in intron 2 that activates an alternative splice site, resulting in a truncated open reading frame with a premature stop codon at amino acid 59. Deep sequencing showed that 62 out of 151 *gmppb* reads were incorrectly spliced in *gmppb-/-* embryos. Thus, this hypomorphic allele is a **unique genetic resource.** There are no published mouse models of GMPPB-associated dystroglycanopathy. A zebrafish morphant was only superficially described as support that mutations in *GMPPB* cause dystroglycanopathy[26]. The morpholino caused a 29% reduction in DG glycosylation, which, together with our data, indicates that reduced GMPPB function has a dramatic phenotypic effect.
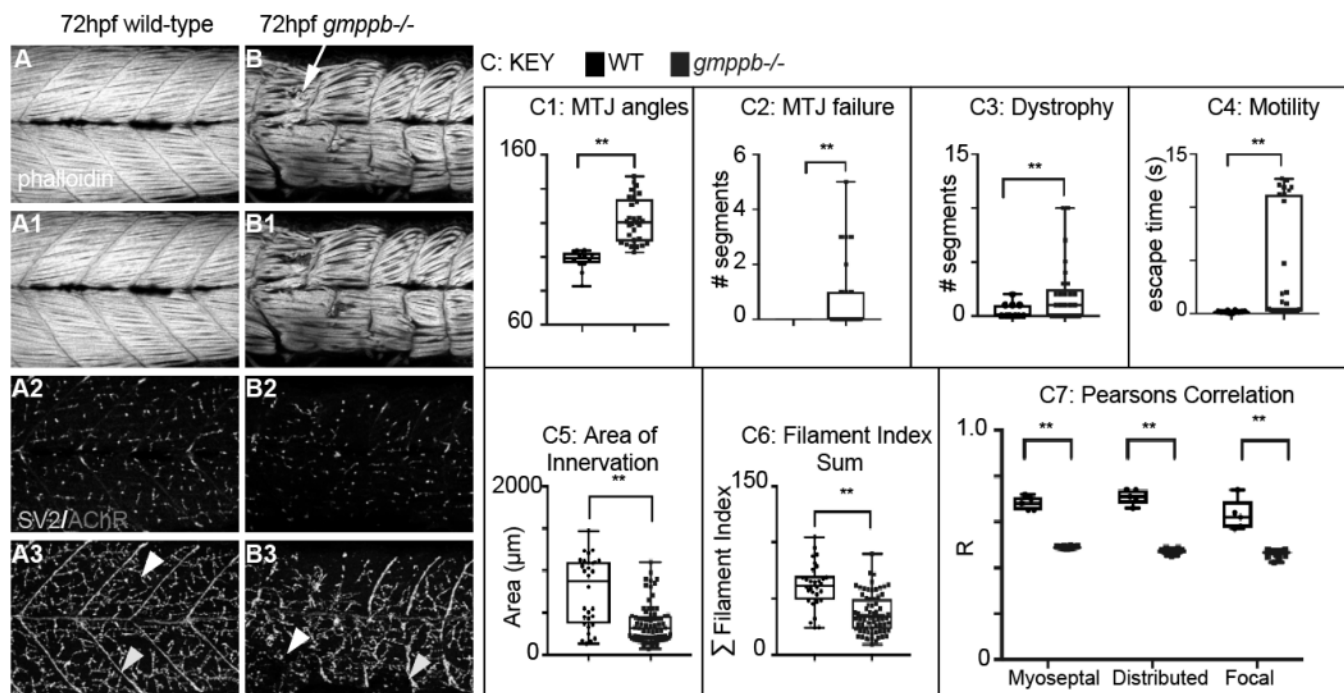
B: INNOVATION

Fig. 4: <u>Neuromuscular phenotype of *gmppb* mutants</u>. (A-B) Confocal micrographs, side view, anterior left, dorsal top. Phalloidin (white) denotes actin, SV2 antibodies (blue) mark pre-synaptic termini, and alpha-Bungarotoxin (fuchsia) labels post-synaptic termini. Note organized fibers in controls (A) and unbroken MTJs (pseudocolored yellow in A1). In contrast, MTJs are broken in *gmppb* mutants (yellow pseudocoloring, B1) and muscle fibers are detached (B, arrow, B1, red pseudocoloring). A2 and B2 are single focal planes showing innervation, A3 and B3 are projections. Yellow arrowheads point to myoseptal innervation of slow-twitch fibers. White arrowheads point to distributed innervation of fast-twitch fibers. (C) Color coded key. Box and whisker plots: whiskers display min to max. Note that *gmppb* mutants have significantly wider MTJ angles, higher incidences of MTJ failure and dystrophy, and worse motility. In addition, mutants have significantly less total NMJ occupancy area (C5), a lower filament index, and reduced Pearson's correlation coefficient. The filament index mathematically measures the spherical shape of an object: the higher the index, the less spherical the object is. These data suggest that both NMJ morphogenesis and synaptogenesis are disrupted ** *p < 0.01.*

Integration of muscle and MTJ development *in vivo:* Muscle and tendons function as an integrated unit to transduce force to the skeletal system and stabilize joints. Reciprocal interactions between muscle fibers and the myotendinous junction (MTJ) are critical for development in both zebrafish and mouse embryos (reviewed in[35]). Our preliminary data show for the first time that MTJ development is disrupted in a secondary dystroglycanopathy model (Fig. 4). A PubMed search for 'dystroglycanopathy and MTJ' yielded no results (8/12/2018).

Resilience of genetic networks in healthy and diseased tissues: How does one identify the most relevant mechanisms that regulate muscle cell adhesion? Conventional gene expression analyses rank candidate genes by gene expression changes. Although this approach is important, one pitfall is that changes in expression do not always correlate with function. Genes function together in networks to regulate complex biological processes that must sense and respond to a variety of inputs. Therefore, we are also taking the innovative approach of applying network science methods originally developed to study computer, physical and social networks over the last twenty years [36,37]. We hypothesize that genetic networks in the context of disease are less resilient to perturbations than in healthy tissues. We identify the most relevant genes in a network using a new computational approach that we developed to study the resilience of genetic networks to network attack. In this approach, individual genes with the highest degree of connectivity in the network are systematically removed until the network collapses. The rate at which the network sustains attack indicates its resilience. Fig. 5 shows application of this method to a microRNA co-expression network in normal and chemically treated zebrafish (this was done in the context of a different study that was not focused on muscle). In addition to characterizing the resilience of the network overall, these analyzes also identify individual genes that influence network resilience the most. These critical resilience genes are those that yield large changes in network topology. These are the labeled genes in Figure 4 that cause a dramatic decrease in network size. We will use this powerful analysis approach to: (1) study the resilience of gene networks and microRNA networks in dystrophic zebrafish with and without NAD+, (2) identify critical network regulators, and (3) identify candidates downstream of NAD+-mediated improvement.

Fig. 5: Network size of microRNA co-expression networks. Note the treated network collapses with fewer miRNAs removed. Numbers indicate breakpoints caused by removal of specific miRNAs.

Fig. 6: Isolated myofibers from 3 dpf larvae. Phalloidin (white), beta-DG (blue). White arrowhead in A1 points to normal beta-DG at terminal ends. Yellow arrowheads point to abnormal beta-DG accumulation (missing in B1, aggregated in C1) at fiber ends. White asterisk in B: abnormally frayed terminal end. Yellow arrow in C1: abnormal beta-DG. The area of myofibers (E) is not significantly different but their shape is: mutant myofibers have a significantly lower Filament Index (D). (F) MTJ angles are not significantly different in *gmppb* mutants with or without NAD+.

Hypothesis: Hypoglycosylated DG results in disorganized muscle cell membranes and blocks the NAD+-mediated increase in laminin organization: We previously identified a novel cell adhesion pathway required for laminin organization at the MTJ during zebrafish muscle development: Nrk2b-mediated NAD+ synthesis potentiates laminin organization at MTJs. We next showed that exogenous NAD+ is sufficient to *improve* laminin organization and *reduce* muscle degeneration in zebrafish deficient for DG or Itga7 [3]. To our knowledge, this was the first report identifying beneficial effects of NAD+ supplementation for muscular dystrophies. It has subsequently been shown that NAD+ plays a conserved beneficial role in muscle health, although most studies focus on how NAD+ levels increase mitochondria and muscle stem cell numbers and function (reviewed in [7]) rather than roles for NAD+ in cell-ECM adhesion.

As exogenous NAD+ ameliorates dystrophy in zebrafish deficient for DG, we hypothesized that NAD+ would be beneficial in zebrafish models of dystroglycanopathies. Surprisingly, NAD+ supplementation does not rescue *gmppb* mutant muscle: MTJ angles, muscle degeneration, and MTJ failure were not improved with NAD+ supplementation (Fig. 6F
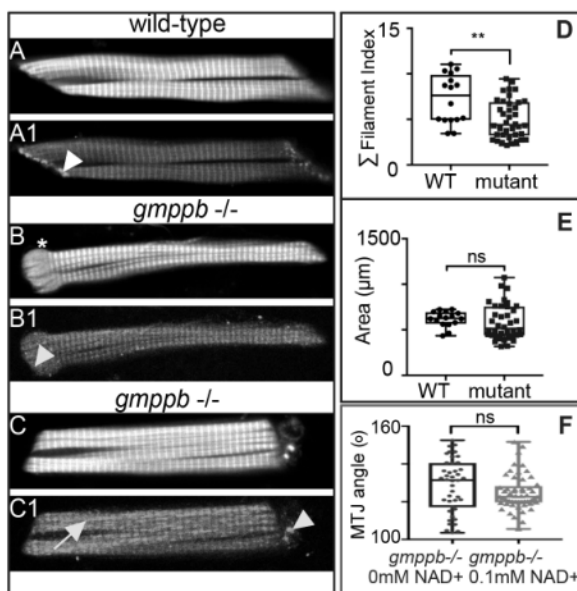
and data not shown). DG levels at the sarcolemma are variable in patients with GMPPB-associated dystroglycanopathy and sometimes aggregates of DG are observed[29,33,38,39]. In wild-type muscle fibers, DG concentrates at costameres and the terminal ends of muscle fibers (Fig. 6A1). In *gmppb* mutant fibers, DG is less frequently concentrated at terminal ends (Fig. 6B1) and is observed in large plaques (Fig. 6C1).

Given the above data, our new hypothesis is that <u>the presence of hypoglycosylated DG in the sarcolemma blocks the NAD+-mediated increase in laminin organization at the MTJ because Integrin clusters are unable to form and potentiate laminin organization (Fig. 7)</u>. We will first test this hypothesis by generating *gmppb;dg* double mutants. We predict that *gmppb;dg* double mutants will be rescued with NAD+. We will also test our hypothesis with super resolution microscopy. One **major gap** in our understanding of muscle cell membranes is whether/how the DGC and Integrin adhesion complexes interact. It was previously shown that these complexes can interact biochemically via sarcospan[40]. However, the frequency of these interactions and the spatial dynamics of these interactions are not known. This is a fundamental gap in knowledge that is important to understand because the DGC plays an important role in stabilizing sarcomeres in response to contraction[41]. Thus, these experiments will provide significant basic knowledge regarding muscle cell membranes.
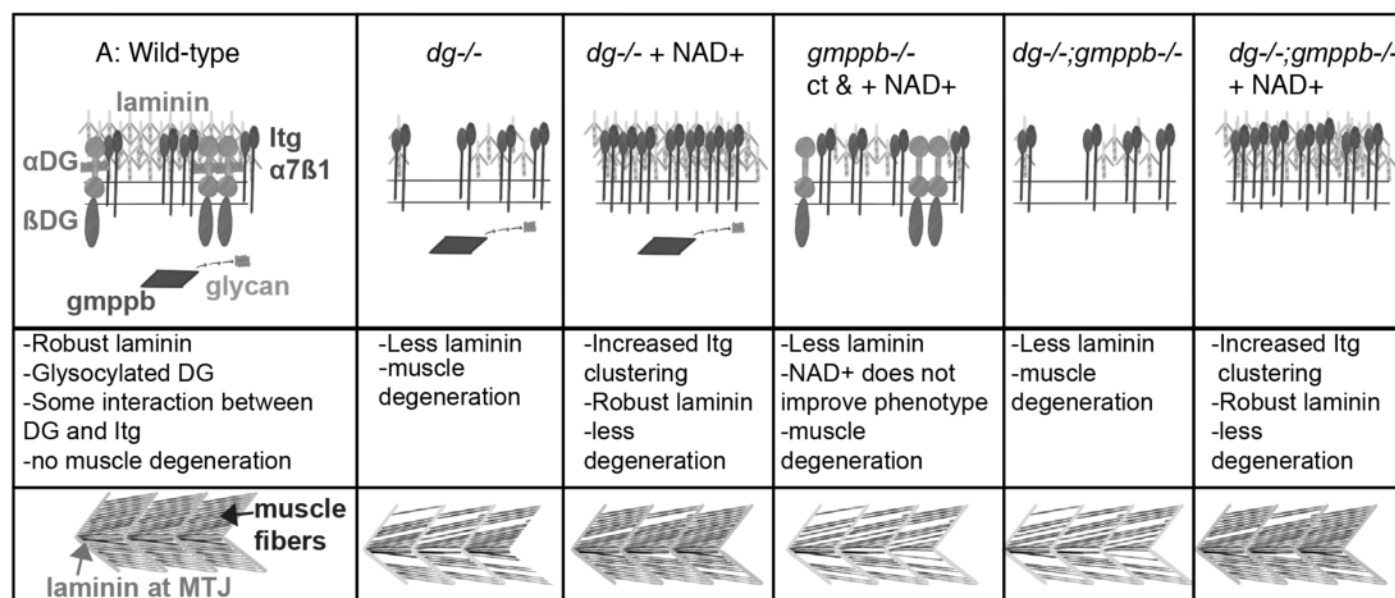

A: Wild-type	dg-/-	dg-/- + NAD+	gmppb-/- ct & + NAD+	dg-/-;gmppb-/-	dg-/-;gmppb-/- + NAD+
-Robust laminin -Glysocylated DG -Some interaction between DG and ltg -no muscle degeneration	-Less laminin -muscle degeneration	-Increased ltg clustering -Robust laminin -less degeneration	-Less laminin -NAD+ does not improve phenotype -muscle degeneration	-Less laminin -muscle degeneration	-Increased ltg clustering -Robust laminin -less degeneration

Fig. 7: <u>Cartoon of hypothesis</u>

C. APPROACH

The proposed experiments lay a critical foundation for understanding the basic biology underlying muscle-ECM adhesion. Our combined expertise in cell adhesion, quantitative phenotypic analyses, bioinformatics, and super-resolution microscopy make our groups uniquely positioned to address these Aims. Dr. Henry is an expert in zebrafish muscle development. Dr. Hess's invention of FPALM was cited within the Nobel document "Super-Resolved Fluorescence Microscopy". Dr. King has developed a new approach to identifying regulators in gene networks by incorporating the science of network resilience. Completion of this grant will provide new insight into how cell-ECM adhesion mediates musculoskeletal development and homeostasis.

<u>**Aim 1: Test the hypothesis that NAD+ increases cell adhesion in DG mutant zebrafish by increasing Itga7 clustering; and that hypoglycosylated DG disrupts sarcolemma architecture, preventing Itga7 clustering and increased cell adhesion with NAD+ supplementation.**</u>

Aim 1A: Test the hypothesis that NAD+ reduces muscle degeneration and increases muscle adhesion in *gmppb;dg* mutant zebrafish

Rationale: Preliminary data indicate that NAD+ is not sufficient to rescue *gmppb-/-* zebrafish (Fig. 6F). We hypothesize that hypoglycosylated DG prevents improved laminin organization because it physically interferes with Integrin receptor clustering needed to improve laminin organization. We will test our hypothesis by inhibiting both *dg* and *gmppb* and asking whether NAD+ is sufficient to reduce muscle degeneration and

increase cell adhesion. We propose a longitudinal study design that will ensure that, even if our hypothesis is wrong, completion of this aim will provide significant new insight into roles for NAD+ and GDP-mannose in muscle development, homeostasis, and regeneration. In addition, one aspect of dystroglycanopathies that is not well understood is what phenotypes are due to failure to glycosylate DG and what phenotypes are independent of DG. The classic approach to address this question is to generate double mutants/morphants as we propose to do here. Thus, whether or not our hypothesis is correct, completion of this experiment will result in new insight into requirements for *gmppb* versus *dg*.

Design: Rationale for increasing the timeline: We previously assessed the effects of NAD+ on *dg*-deficient embryos at 3 days post fertilization. Here we will determine if the beneficial effects of NAD+ perdure until fish feed independently (7dpf) and whether NAD+ increases survival. This longer experimental timeline will give us the opportunity to investigate muscle degeneration/regeneration cycles in *gmppb-/-* and *dg-/-* larvae. Given the requirement for dynamic ECM changes during regeneration[42,43], we will ask whether NAD+ improves regeneration in either model. **Genetics/pseudogenetics:** Unfortunately, *gmppb* and *dg* are both on Chromosome 22 with only 6 million base pairs separating them. Thus, it will be somewhat difficult to generate double mutants especially considering the suppression of recombination in zebrafish male meiosis[44]. The advantage is that once we recover double mutants the ratio of double mutants should be closer to 1/4 than 1/16. However, we also propose the complementary approach of combining morpholinos and mutants. We will inject *gmppb* morpholinos into *dg* mutants and do the reciprocal experiment of injecting *dg* morpholinos into *gmppb* mutants. We are experts in using combinations of mutants and morphants to conduct "pseudogenetic" epistasis analysis [2,3,45,46] and are confident that we can use this complementary approach. As a control we will also rescue the morpholino being injected with expression of the target gene that does not contain the morpholino targeting site. **Transgenic mutants being developed:** We crossed *gmppb* heterozygous carriers to *Tg(mylpfa:lyn-cyan, smych1:GFP, myog:H2B:RFP)* transgenics (Figs.9,10). The transgenics are on the *casper* background that does not have melanocytes or iridophores. We will cross *dg* heterozygous carriers with this transgenic line. These transgenic mutants will allow us to elucidate multiple aspects of muscle morphology through time (fiber length, cross-sectional area, nuclear positioning, etc.) (Figs.4,9). **Treatment:** Heterozygous carriers of *gmppb* or *dg* will be crossed and embryos will be split into two groups, a control group and a group that is injected with the reciprocal morpholino. These groups will then be split into two groups each: with and without NAD+. Every larva will be PCR identified at the end of the experiment (it is possible to recover embryos after staining and imaging).

Note: for the remainder of the grant we will refer to genotypes because that is much simpler than continuously writing "gmppb mutants injected with dg morpholinos and dg mutants injected with gmppb morpholinos", etc. All experiments will initially be conducted as described above (reciprocal mutant/morpholino combinations) and replicated with double mutants once they are generated.

Longitudinal study methods (see also Fig. 8): 1. Longitudinal birefringence imaging: Embryos are placed into 24 well plates. <u>Birefringence analysis:</u> Wild-type siblings will be imaged for birefringence daily.
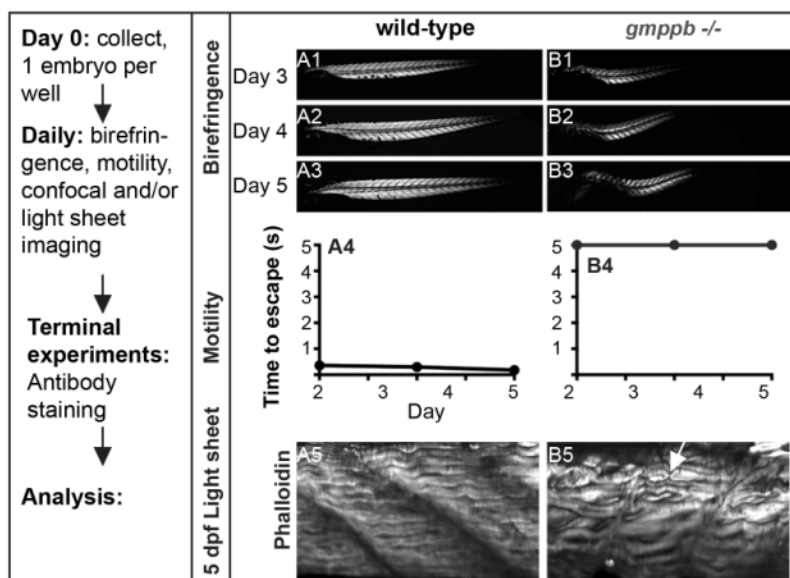

Fig. 8: Example of longitudinal experiments that interrogate musculoskeletal structure and function. (A) Wild-type. (B) *gmppb* mutant. (A1-A3, B1-B3) Birefringence through time, anterior left, dorsal top, side mounted live zebrafish. (A4, B4) Time in seconds to swim out of a 10 mm diameter circle after a touch stimulus. A score of 5 seconds was assigned to any embryo that didn't successfully exit the circle. Note that the *gmppb* mutant never exited the circle. (A5, B5) Side view, anterior left, dorsal top. Light-sheet imaging of the same embryos after they were fixed and stained with phalloidin to visualize actin. Note that myofibers in the *gmppb* mutant are disorganized and/or broken (arrow).

dmd   3 dpf   6 dpf   9 dpf
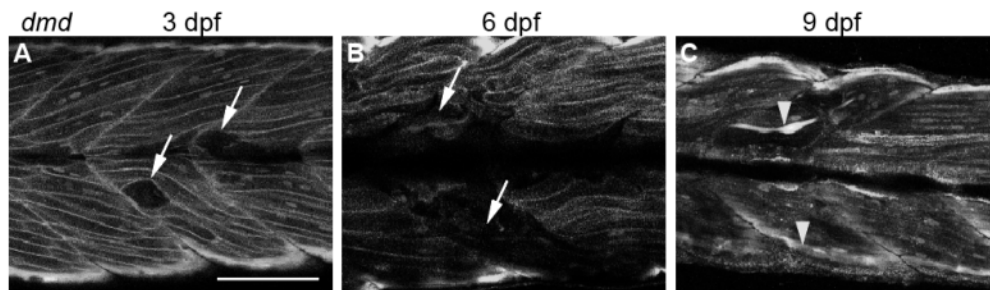
dmd;*Tg(mylpfa:lyn-cyan, smych1:GFP, myog:H2B:RFP)*

Fig. 9: Longitudinal confocal imaging of a *dmd* mutant with the transgenes that we will incorporate into *gmppb* heterozygotes. Note that damage begins at 3 days post-fertilization (dpf) (white arrows, A). By 6dpf there is significant degeneration. By 9dpf there has been some regeneration. Note some of the new fibers are slow-twitch fibers (yellow arrowheads). This particular *dmd* mutant regenerates comparatively well because it has undergone treatment that is not germane to this grant. Muscle cell membranes (green), myonuclei (red), and slow muscle fibers (cyan).

Birefringence will be quantified as detailed in [47]. The mean gray value and birefringence area of the wild-type pool will be averaged and results for all individual embryos will be reported as % of average wild-type birefringence area and mean grey value. <u>Blinding and data analysis:</u> Blinding is done prior to analysis using a Perl script that renames files in a directory and provides an Excel sheet with the original and new file names. **2. Light-sheet and confocal imaging:** Embryo rearing and birefringence analyses will be conducted as above. For light-sheet or confocal imaging embryos/larvae will be mounted in low-melt agarose with MS-222 to inhibit movement, imaged, taken out of low-melt agarose, rinsed, and returned to their individual wells. Previous experiments in our laboratory have demonstrated that this approach does not disrupt viability (Figs. 9,10).

<u>*i*: Does NAD+ supplementation ameliorate degeneration in *gmppb-/-;dg-/-* double mutant zebrafish and do the beneficial effects of NAD+ supplementation perdure to the larval stage: improving muscle structure, motility, and lifespan?</u>

Rationale: The primary goal is to test the hypothesis that NAD+ supplementation will improve muscle structure and function in *gmppb;dg* double mutants. The secondary goals are to: (1) determine whether beneficial effects of NAD+ for *dg* mutants perdure past 3 dpf, and (2) quantify muscle development/ homeostasis in *gmppb* mutants. **Methods**: Longitudinal data will be obtained as described above. We are experts in mathematically characterizing muscle structure. We will quantify the following aspects of muscle structure: MTJ angles, fiber cross-sectional area, and % of segments with fiber degeneration. We will assess motility with the DanioVision system. To determine lifespan, we will use the Kaplan-Meier survival estimate. **Analysis**: A power analysis with alpha = 0.05, power = 0.9 and a medium effect size for a 7 (time point) X 12 (phenotype) ANOVA indicates that we will need 24 zebrafish per group. The 12 phenotypes are the following conditions with and without NAD+ supplementation (wt, *gmppb-/-, dg-/-, gmppb-/-;dg-/-, gmppb-/-;dgMOs, dg-/-;gmppb MOs*).
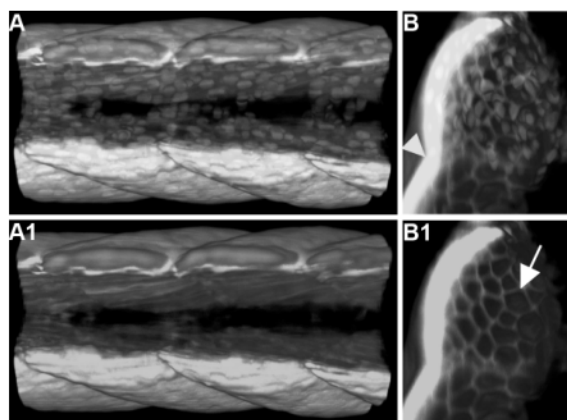

Fig. 10: Light-sheet imaging of live 7 day old *Tg(mylpfa:lyn-cyan, smych1:GFP, myog:H2B:RFP)* transgenics. Muscle cell membranes (green, white arrow in B1), myonuclei (RFP), and slow muscle fibers (cyan, yellow arrowhead in B). Dorsal view, anterior left. (B-B1) Transverse view (dorsal top, lateral left) of a partial myotome.
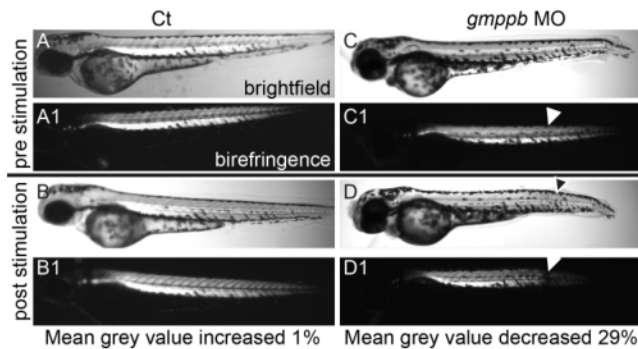
Potential outcomes and conclusions: The analysis of this experiment is straightforward. We predict that NAD+ supplementation will decrease MTJ angles, increase fiber cross-sectional area, and decrease the % of segments with fiber degeneration in *gmppb;dg* double mutants. We predict that beneficial effects of NAD+ in *gmppb;dg* double mutant zebrafish will be significant at 3 dpf. We hypothesize that the beneficial effects of NAD+ will continue past 3 dpf and the longitudinal study will allow us to test that hypothesis.

<u>*ii*: Does NAD+ supplementation increase cell adhesion?</u>
Rationale: We previously showed that NAD+ decreased muscle degeneration in DG-deficient zebrafish[3]. We proposed that the mechanism was increased cell adhesion but we did not have the tools to directly test whether cell adhesion was increased. Subsequently, Dr. Schilling and colleagues showed that muscle contraction induced by mild electrical stimulation can be used to quantitatively determine differences in muscle fiber-ECM attachment [48]. Our preliminary data show that 2 rounds of stimulation did not

Fig. 11: Electrostimulation causes fibers to detach in *gmppb* morphants but not in wild-type embryos. 2 dpf, anterior left, dorsal top. (A) Wild-type pre-stimulation and (B) post-stimulation. There is no loss in birefringence (mean grey value). (C) *gmppb* morphant. White arrowhead shows intact muscle. (D) Same *gmppb* morphant post-stimulation. White arrowhead points to detached muscle post stimulation. Birefringence decreased 29%.

cause muscle detachment in wild-type fish (Fig.11, 1 minute treatment, 8ms duration, 6ms delay, 30 Volts). In contrast, stimulation with the exact same parameters caused muscle detachments in *gmppb* morphants (Fig. 11D1, arrowhead). Thus, this approach can be used to compare relative strength of muscle fiber-ECM adhesion. **Design:** Testing the relative strength of muscle fiber-ECM attachments: We will test relative muscle fiber-ECM strength at 2,4,6, and 8 days. Larvae will be imaged with birefringence, electrostimulated, and imaged with birefringence immediately after stimulation as shown in our preliminary data (Fig. 11). Larvae will then be individually fixed, stained, and imaged to visualize muscle detachments (phalloidin) and MTJ structure (laminin, Collagen, Fibronectin) as we have done previously [2,3,45,46]. **Data Analysis:** We will analyze: (1) the change in birefringence with electrostimulation (see Fig. 11), (2) frequency of muscle fiber detachments, and (3) MTJ structure. We will use 24 fish per group per the power analysis in question 1. We will test whether any of these measures are altered using an ANOVA followed by Tukey's for post-hoc analysis. **Potential Outcomes and Conclusions:** We predict that NAD+ will increase muscle-ECM adhesion in *dg-/-* and *gmppb-/-;dg-/-* embryos. Increased cell adhesion would be indicated by significantly smaller reduction of birefringence in NAD+ supplemented *dg-/-* and *gmppb-/-;dg-/-* embryos compared to controls. We predict that NAD+ will not increase cell adhesion in *gmppb-/-* larvae. We predict this because NAD+ does not rescue *gmppb-/-* embryos (Fig. 6F). **Potential Pitfalls and Future Directions:** One pitfall is that the double mutant could be synthetically lethal prior to muscle development. This pitfall is unlikely but we could titrate morpholino doses if necessary. However, an alternate approach is to use genetic mosaic analysis to ask whether *dg;gmppb* double mutant cells are rescued in wild-type embryos treated with NAD+. We are experts in genetic mosaic analysis[3,49–51].
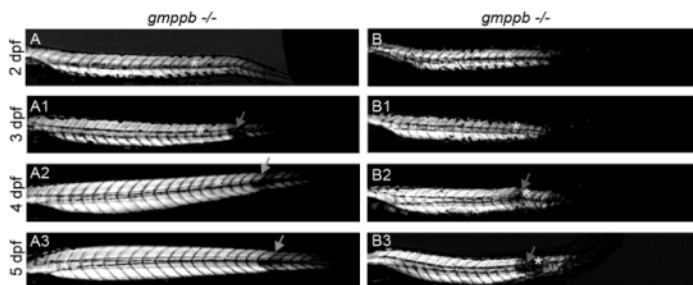

Fig. 12: Muscle degeneration and regeneration in *gmppb -/-* embryos. Anterior left, dorsal top, side mounted *gmppb -/-* embryos at 2dpf (A, B), 3dpf (A1, B1), 4dpf (A2, B2), and 5dpf (A3, B3). Yellow asterisks designate somite 20 in the embryos. Red arrows denote muscle degeneration, green arrows denote muscle regeneration. Note that muscle damage in the embryo shown in panel A slowly starts to regenerate, whereas muscle damage in the embryo shown in panel B2 worsens.

iii Does NAD+ supplementation promote regeneration? **Rationale:** Muscle degenerates in *gmppb* mutant embryos (Fig. 12). Interestingly, degeneration is not always accompanied by regeneration. Sometimes the patch of degeneration spreads (Fig. 12B3). Given that supplementation with a precursor to NAD+, NR, improves regenerative response after cardiotoxin-induced injury[52], we hypothesize that NAD+ supplementation may improve regeneration in zebrafish models of muscular dystrophy. **Methods:** We do not need to conduct an additional longitudinal study to answer this question because we will interrogate the longitudinal data obtained for question 1. **Analysis:** We will take multiple approaches to answer this question. First, we will visually screen birefringence data to identify larvae with large swaths of degenerating muscle. We

will measure overall birefringence and the area of degeneration and observe how these change over time. This will give us an overview of regeneration. Next, we will interrogate the light sheet and/or confocal data obtained to elucidate fast-twitch and slow-twitch muscle morphology during regeneration (or further degeneration). We will also determine how nuclei position and content changes through time with and without NAD+. **Outcomes:** We will determine whether NAD+: (1) improves regeneration in *dg-/-* and/or *gmppb-/-;dg-/-* zebrafish, (2) how NAD+ alters fiber specification after regeneration, and (3) whether NAD+ increases the probability of muscle regenerating in *gmppb-/-* mutants. These experiments will be the first large scale longitudinal analysis of muscle structure in a dystroglycanopathy model and will thus provide significant new insight into how NAD+ impacts degeneration/regeneration cycles in this context.

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Aim 1A: Future Directions Integrate NMJ and muscle morphogenesis, homeostasis, and regeneration. Our preliminary data clearly show that NMJ formation/homeostasis is altered in *gmppb-/-* mutants (Fig. 4). The NMJ plays a significant role in regulating muscle physiology[53]. It is therefore important to understand the cellular and molecular mechanisms that mediate adaptation of muscle and the NMJ. However, the interaction between muscle and the NMJ has been understudied in the context of dystroglycanopathies. In the future, we will determine the underlying mechanisms of NMJ disruption and how interactions between muscle and motoneurons affect development, homeostasis, and regeneration in *gmppb* mutants.

Aim 1B: Test the hypothesis that NAD+ does not rescue *gmppb-/-* zebrafish because hypoglycosylated DG blocks clustering of Itga7 receptors and concomitant increased laminin deposition
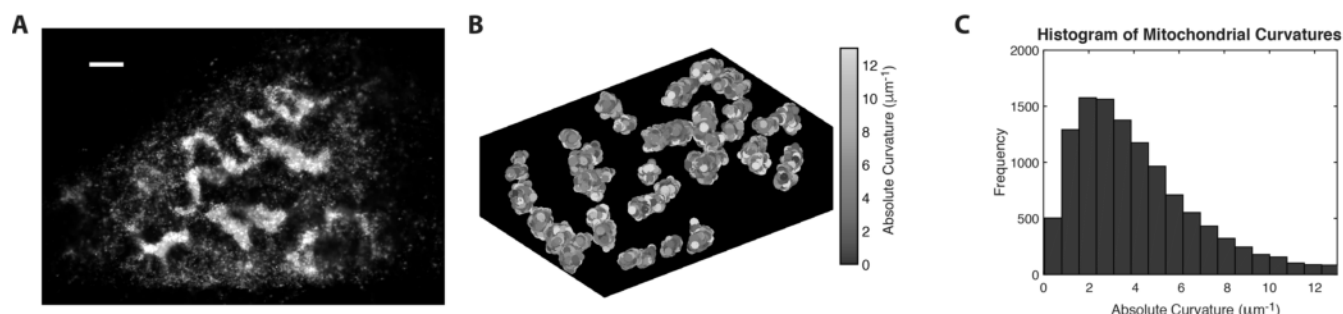

Figure 13. (A) Super-resolution (FPALM) image of mitochondria (green) and endoplasmic reticulum (magenta) in an NIH3T3 mouse fibroblast expressing both Tom20-Dendra2 (mitochondria) and Sec61b-PAmCherry (ER) plasmids. Scale bar is 3μm. (B) 3D point cloud representation of mitochondria from a live C2C12 differentiated mouse myotube expressing Tom20-Dendra2 and imaged using 3D FPALM. Individual mitochondria were identified using single linkage cluster analysis (SLCA) and boundary points were identified by determining an appropriate alpha shape for each mitochondrion. Localizations within a spherical region around each boundary point were fitted to a paraboloid to calculate the absolute local curvature at that point. Curvature scale is measured in μm-1. (C) Histogram of curvatures from the data shown in (B).

Rationale: Preliminary data indicate that NAD+ is not sufficient to rescue *gmppb-/-* zebrafish (Fig. 6F). We hypothesize that hypoglycosylated DG prevents improved laminin organization because it physically interferes with Integrin receptor clustering needed to improve laminin organization. We will test our hypothesis by using super-resolution microscopy (Fig. 13) to elucidate Integrin and DG dynamics in the sarcolemma. This Aim is significant because elucidating the dynamic interactions and behavior of transmembrane ECM receptors is critical for understanding basic muscle biology. This aim is innovative because nothing is known about DG and Integrin dynamics at the single molecule level. **Methods*: Transgenic mutants/morphants:** We will use the same mutants/morphants described above (wt, *gmppb-/-, dg-/-, gmppb-/-;dg-/-, gmppb-/-;dgMOs, dg-/-;gmppb MOs*) except that the transgenes will be different. The transgenes to be expressed include *dg;dendra2, itga7;PAmKate, and cav-1a;dendra2* under the myogenin promoter. We will compare DG and Itga7 localization in wild-type and *gmppb* mutants with/without NAD+. We cannot use DG in the *dg* mutants because that would defeat the purpose of having mutants. For those experiments we will use Caveolin-1a (cav-1a). We previously used FPALM to visualize caveolin-containing membrane domains in live zebrafish[54]. Very little appears to be known about Itga7 and Caveolin containing membrane domains (PubMed search for Itga7 and Caveolae returned 0 results 9/20/18). Interestingly, however, Itga6, a1, and a7 are found in caveolae-enriched membrane microdomains in cardiac myocytes[55]. We predict that Cav-1a will be an informative membrane domain marker and determining colocalization/anti-localization/spatial distribution of Itga7 and Cav-1a will provide significant insight into muscle sarcolemmal structure. We will use gateway cloning to generate these constructs and Tol2-mediated transgenesis to generate stable transgenics. All mutants/morphant groups will split into control and NAD+ treated populations. After muscle fiber isolation the remaining tissue will be used for PCR genotyping. **Approach:** *i) FPALM imaging of DG and Itga7 sarcolemmal localization in fixed zebrafish muscle fibers:* We will determine the baseline organization of DG and Itga7 in the sarcolemma. These data will provide the first nanoscale visualization of DG and Itga7 in muscle, revealing whether there is any co-clustering; and how NAD+ changes localization. **Analysis:** Colocalization will be quantified using a bleed-through corrected Pearson's coefficient[56,57] and relative positions of Itga7 and DG will be quantified with pair-correlation function (corrected for bleed-through)[56,57]. **Outcomes and conclusions:** There are obviously many potential outcomes because the organization of Itga7 and DG in muscle membranes is not known. Thus, we will describe the potential outcomes and conclusions that we would observe if our hypothesis is correct. Please note that even if our hypothesis is incorrect, these experiments will provide significant new insight into

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membrane organization in wild-type and dystroglycanopathy models in addition to insight into how NAD+ modulates membrane organization. **Potential outcomes and conclusions for Itga7 and DG colocalization:** The extent of nanoscale colocalization of Itga7 and DG will be indicted by the Pearson's correlation coefficient. A value of 1 would mean perfect nanoscale colocalization of Itga7 and DG and a value of -1 would mean that there is perfect anticorrelation. We predict that there will be some nanoscale colocalization of Itga7 and DG in wild-type (Fig. 14). We predict that Pearson's will decrease in *gmppb-/-* fibers and will not change with addition of NAD+. We would then conclude that the sarcolemma is indeed disorganized in *gmppb-/-* fibers and NAD+ is not sufficient to rescue this disorganization. **Potential outcomes and conclusions for Itga7 and DG pair-correlation:** This analysis quantifies the relative position of two entities and will elucidate if Itga7 and DG are

Fig. 14: <u>Potential outcomes</u> for Itga7 and DG and/or Itga7 and Cav-1a nanoscale colocalization.
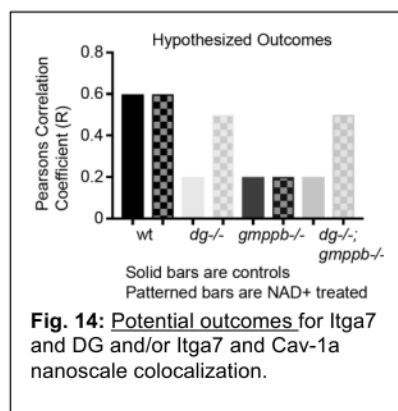
separate but consistently positioned with respect to each other. If we were to find that Itga7 is highly likely to be 1µm away from DG in wild-type embryos, then we would predict that this scenario would be significantly less likely in *gmppb-/-* embryos. **Potential outcomes and conclusions for Itga7 and Cav-1a:** We predict that organization (assessed by either colocalization or pair-correlation) will exist in wild-type myofibers. We predict that organization will be decreased in *gmppb-/-, dg-/-, and gmppb-/-;dg-/-* myofibers. We predict that NAD+ supplementation will increase organization in *dg-/-* and *gmppb-/-;dg-/-* myofibers but not in *gmppb-/-* myofibers. This result would support the hypotheses that disrupted muscle membrane organization is coupled to the *gmppb* phenotype, and that hypoglycosylated DG prevents NAD+-mediated increase in membrane organization. *Pitfalls and Alternatives:* If we observe background fluorescence from pigment, we will either use PTU to prevent

pigmentation or we will cross *gmppb;dg* to the homozygous Casper mutant (no melanocytes or iridophores)[59,60]. If needed, background fluorescence can also be mitigated using inclined illumination (functional setup available in the Hess lab)[56].

ii) FPALM imaging of DG and Itga7 sarcolemmal dynamics in live zebrafish muscle fibers: Next, two-color FPALM imaging will be performed in live cells. These data will allow quantification of the time-dependence of relationships established in fixed cells, such as colocalization, as well as the mobility of the individual molecules of DG and Itga7. Time-dependent colocalization could result from transient interactions between DG and Itga7, and the timescale of interaction could be quantified if it is within our time resolution (~0.1 seconds). Differences in the mobility or clustering of DG comparing WT and *gmppb* mutants would support the hypothesis that the *gmppb-/-* phenotype is correlated with altered muscle membrane organization.

iii) FPALM imaging of DG and Itga7 dynamics in live zebrafish: Finally, we will determine how ECM receptors are organized in the context of intact muscle. Dr. Hess's lab published the first FPALM study of membrane structure in live zebrafish embryos[61]. We will analyze Itga7 and DG organization in different sarcolemmal domains: at the MTJ, at NMJs, and throughout the myofiber. These experiments will provide the first insight into ECM receptor organization and interactions in different sarcolemmal domains in an intact vertebrate.

Future directions: We showed that NAD+ is also sufficient to improve muscle structure/function in Itga7 mutants and hypothesize that DG clustering is important for this effect. We generated *Itga7* mutants (not shown) and will use these to test this hypothesis. **Alternative approach:** Our hypothesis could be wrong. As one major impact of NAD+ is on mitochondria, we would then use constructs we have developed to visualize inner and outer mitochondrial membranes (Fig. 13) to ask how mitochondrial organization is altered in these different genotypes with and without NAD+. It is likely that NAD+ impacts *both* cell adhesion and mitochondrial function, thus elucidating mitochondrial structure is also a future direction.

Aim 2: Identify new muscle cell adhesion regulators through comparative studies of dysregulated muscle development in three zebrafish models of muscular dystrophy.

We aim to determine how NAD+ alters the ECM microenvironment and improves muscle structure/function in zebrafish muscular dystrophy models. Our rationale is that once these basic biological mechanisms are identified, this information can be used in the future to design chemical genetic screens or other approaches to enhance the ECM in a variety of muscle conditions. We fully expect to be able to identify novel mechanisms and networks for 2 reasons: (1) our preliminary data using *gmmpb* mutants at only one time point already

identified many ECM remodeling genes (Figs. 15, 16), and (2) ECM modifying genes were the second largest differentially expressed group in mouse *Large* mutants, *Dmd* mutants, and *Dmd;Large* double mutants [62].

We propose to characterize the underlying regulatory mechanisms of cell adhesion pathways through the

Fig. 15: MA plot of differentially expressed genes in preliminary RNA-Seq study of *gmppb* mutants compared to wild-type. The expression of each gene (dot) was plotted to visualize the relationship between fold change (y-axis) and the average level of expression across all samples (x-axis). Labeled gene candidates include ECM modifying proteins. Arrows point to genes of unknown function that may provide new insight into disease mechanisms.

Fig. 16: Heatmap of homologous genes differentially expressed in *gmppb* mutants and 21 day *Mdx* mice. A total of 21 homologous gene pairs were significantly differentially expressed in the two studies. These eleven gene pairs had a consistent fold change in mutants compared to wild-types and ten had conflicting fold changes (not shown).

analysis of networks that we will model from transcriptome data. We chose a transcriptome approach to determine regulatory factors that are upstream of post-translational events such as glycosylation. This approach is complementary to other high-throughput characterizations of muscular dystrophy models, such as genome chromatin state[63], proteome[64] and glycome[65]. Here we propose to acquire transcriptome data that is necessary to model co-expression networks, integrate regulatory non-coding RNA and analyze the resilience of these networks.

Aim 2A: Test the hypothesis that the topology and resilience of gene co-expression networks in *dg-/-, itga7-/-,* and *gmppb-/-* mutants in response to NAD+ correlate with phenotypes; and that specific gene sets contribute to the resilience of those networks.

Rationale: The fact that NAD+ improves muscle structure/function in *dg-/-* and *itga7-/-* but not *gmppb* mutants provides a valuable set of comparisons needed to identify gene sets that mediate the beneficial effects of NAD+. Characterization of the three mutants are necessary to find those genes that are commonly differentially expressed with NAD+ treatment in *dg-/-* and *itga7-/-* but not in *gmppb* mutants. To our knowledge, transcriptome and non-coding RNA analyses have not been conducted on dystroglycanopathy models. *Design:* We will identify molecular mechanisms by conventional bioinformatics methods, as we have done previously[61,62], and also apply novel network topology/attack analyses to interrogate the resilience of gene co-expression networks of *dg-/-, itga7-/-* and *gmppb* mutants compared to wild-types. Integration of these complementary approaches will prioritize genes and pathways for validation studies. MiRNA and mRNA gene co-expression networks will be constructed from high-throughput RNA sequencing (RNA-Seq) of 6 biological replicates of *wt, dg-/-, itga7-/-, and gmppb-/-* genotypes with and without NAD+ supplementation. Barcoded small RNA and strand-specific ribosomal RNA-depleted mRNA libraries will be pooled and sequenced to generate a minimum of 30 million 50bp paired-end reads for each sample using the Illumina platform. **Analysis Methods:** A five-part analysis workflow will be used (Fig. 17). 1: mRNA-Seq data will be used to determine genes that are differentially expressed and have a significant interaction between genotype and NAD+ supplementation factors using
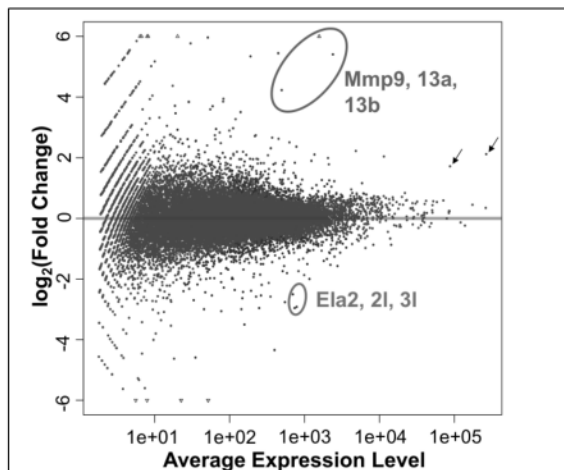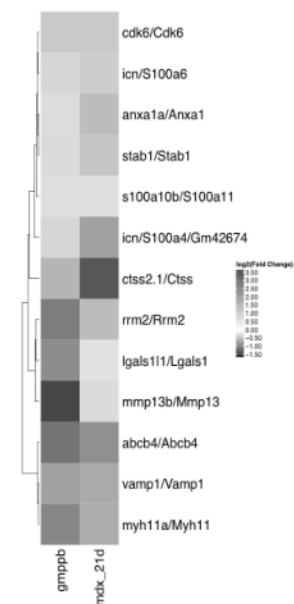
best practices of the conventional RNA-Seq analysis workflow. Briefly, the workflow consists of mapping adapter clipped reads to the current zebrafish transcriptome annotated by Ensembl to estimate read counts per gene in each sample using RSEM[68]. Differentially expressed genes will be determined using R/DESeq2[69]. Our preliminary study of *gmppb* mutants provided a list of 365 differentially expressed genes (Fig. 15 and data not shown). Lists of differentially expressed genes will be further studied to determine what pathways and/or GO biological process, molecular function and cellular component annotations are enriched[70,71]. We will compare the lists of differentially expressed genes with recently published transcriptome data from mouse models[62] (a subset is shown in Fig. 16). We will also examine the mRNA-Seq data for candidate biomarkers for disease and disease progression in these novel dystroglycanopathy models. 2: Small RNA-Seq reads will be analyzed

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to determine the expression of mature miRNA products annotated in MiRGeneDB[68] that Dr. King co-developed using miRExpress[73]. R/DESeq2 will be used to find differentially expressed miRNAs with a significant interaction between genotype and NAD+ supplementation factors. 3: miRNA and mRNA expression data will be integrated to find putative differentially expressed mRNA targets of differentially expressed miRNA using Dr. King's previously published methods to integrate miRNA and mRNA expression data[61,62]. 4: The topologies of gene co-expression networks for each mutant will be compared in order to rank mRNAs and miRNAs with the largest number of retained, lost, and gained co-expression relationships. Undirected mRNA and miRNA co-expression networks will be constructed using an adjacency matrix computed from pairwise Spearman correlations of normalized read counts using NetworkX[74]. mRNAs and miRNAs will be ranked by graph edit distance (the number of retained, lost and gained edges between networks)[75]. 5: The resiliency of networks for each mutant to network attack will be compared to find the mRNAs and miRNAs that result in large changes in network topology. Network attack is where nodes (mRNAs or miRNAs) or edges are systematically removed until the network collapses. Nodes are ranked by degree (number of edges for a node) in descending order and then sequentially removed from the network in that order. Differences in rates of which networks collapse are indicative of differences in the resilience of the networks. In addition to characterizing the resilience of the network overall, these analyses identify critical resilience nodes that are most important to changes in network topology. *These resiliency analyses will test our hypothesis that network resilience will correlate with NAD+ rescue and also identify mRNAs and miRNAs that contribute the most to network resilience. **Candidates for future studies will be prioritized by considering the rank order from the resiliency, topological, and conventional RNA-Seq analysis using rank product.***

Fig. 17: Cartoon of Workflow

Pitfalls: Skeletal muscle comprises the vast majority of zebrafish tissue at this age, thus we expect that most identified genes will be differentially expressed in skeletal muscle. One alternate approach is to cut off the heads prior to RNA extraction. ***Expected Outcomes***: We will identify networks that regulate musculoskeletal development, how those networks are dysregulated in dystroglycanopathies, and how networks are modified with NAD+ supplementation. This network-based analysis of miRNA and mRNA expression will provide prioritized lists of candidate pathways and specific miRNA and mRNA candidates for further experiments. This list also provides the basis of our **future directions** where we will leverage our expertise in muscle development and homeostasis to identify new regulators of muscle cell adhesion and their mechanisms.
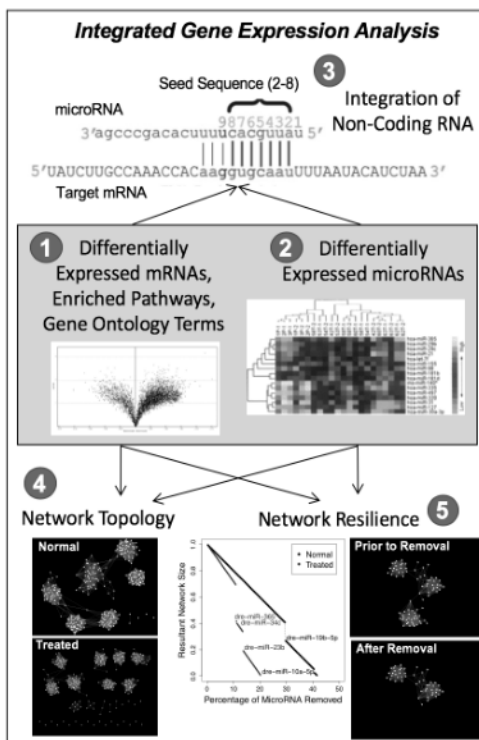
Aim 2B: Test whether downregulation of three elastase genes is a compensatory response that increases elastin content in the MTJ and improves MTJ development and homeostasis in *gmppb-/- larvae.*

Rationale: *Despite the importance of tightly regulated ECM remodeling for normal muscle development and function, mechanisms underlying ECM remodeling in vivo remain elusive.* We and others have shown that the zebrafish muscle and MTJ ECM undergoes a stereotypical sequence of remodeling events where the primary component shifts from Fibronectin (Fn), to laminin-111, to laminin-211 over developmental time[11,45,76,77]. These muscle ECM changes are conserved: they are also observed during mouse muscle development[78,79]. We subsequently showed that laminin polymerization indirectly promotes Fn downregulation via a matrix metalloproteinase 11 (Mmp11)-dependent mechanism. Thus, our lab has been a major contributor to elucidating the muscle/MTJ ECM regulatory network [35]. Our goal is to expand the ECM regulatory network by incorporating elastases that were identified as differentially expressed in our preliminary RNA-seq experiment. Three elastase genes (*ela3, ela2l,* and *ela3l*) are significantly downregulated in *gmppb* mutants (Fig. 15). Elastases degrade elastins. Elastins are a component of the tendon ECM and are enhanced within fascicles

and in the interfascicular matrix[80,81]. *However, the exact role that elastins play in tendon development, homeostasis, and repair is not well understood.* Aging results in decreased and disorganized Elastin in energy storing tendons[82]. Some tendons in elastin haploinsufficient mice show increased stiffness[83]. Elastin immunostaining is increased in torn human rotator cuffs and thought to play a role in healing[84]. The above results suggest a beneficial role for elastin in musculoskeletal health. However, increased elastin also promotes inflammation: wrapping rabbit tendon repairs with a collagen-elastin matrix increases inflammation and reduces healing[85]. We hypothesize that reduced expression of three elastase genes in *gmppb* mutants has a physiological impact on musculoskeletal development; but whether that impact is beneficial or deleterious is not clear. **Approach:** *i) validation of ela3, ela2l, ela3l:* We will use an antibody against elastin that has been used in zebrafish (Santa Cruz, sc-374638) to first determine elastin levels at MTJs during normal musculoskeletal development, and then ask how elastin levels change in *gmppb* mutants. Preliminary data indicate that Elastin is upregulated at 2dpf in *gmppb* mutants (not shown). *ii) Test the hypothesis that upregulated elastin (the hypothesized consequence of reduced elastase expression) is beneficial for musculoskeletal structure in gmppb mutants:* We will generate *elastin;gmppb* double mutants. There are multiple alleles for both elastin genes (that result in premature stops or affect splicing) that are available for the price of shipping from ZIRC (Zebrafish International Resource Center). We will use the methods/analyses described in Aim 1 to determine if loss of elastin function exacerbates the *gmpp* mutant phenotype, and elucidate mechanisms. *iii): Test the hypothesis that reintroduction of elastases will worsen the gmppb-/- phenotype:* We will conduct the reverse experiment and use the heat shock promoter/tissue specific promoters to determine where/when increased elastases impact the *gmppb-/-* phenotype. **Outcomes:** We predict that these experiments will elucidate new roles for elastins and elastases in MTJ development, homeostasis, and disease. **Pitfalls:** It is possible that changes in elastase expression are either spurious or have no impact on musculoskeletal development. We would move on to other candidates identified if this is the case. **Future Directions:** We will use the information gleaned in Aim 2A to test roles for putative significant cell adhesion modifying genes in response to NAD+ and use techniques we have published to place these genes in the muscle ECM regulatory network. For example, preliminary data show that *mmp13* is upregulated in macrophages at regenerating muscle in *gmppb* mutants (not shown). Thus, this is an excellent future direction.

SUMMARY The zebrafish is a fantastic animal model for elucidating basic mechanisms of musculoskeletal development and homeostasis. This proposal takes a multifaceted approach to elucidate new mechanisms underlying musculoskeletal development and homeostasis. This basic knowledge could, in the future, inform efforts to improve muscle health.

Experiments	Year 1	Year 2	Year 3	Year 4	Year 5
Generate *gmppb;dg* double mutants	■				
Aim 1A: Longitudinal studies with mutant/morphant combinations, repeat with double mutants, analysis	■	■			
Aim 1B: Generate transgenic mutants for FPALM	■	■			
Aim 1B: Super-resolution experiments in isolated muscle cells		■	■		
Aim 1B: Super-resolution experiments in live zebrafish				■	■
Aim 2A: Conduct RNA &miRNA -seq experiments	■				
Aim 2A: Analysis, validation	■	■			
Aim 2A: Functional analysis of the most impactful genes			■	■	■
Aim 2B: Elucidate roles for Elastases and Elastin in musculoskeletal development	■	■	■		

TIMELINE

STANDARDS FOR RIGOR, RESEARCH DESIGN, DATA ANALYSIS, AND DATA REPORTING

In order to reduce bias, facilitate the transparency and reproducibility of our results, and optimize the impact of this project, this proposal will adhere to a core set of research design and data reporting standards[86]. All data produced by this project will be stored on multiple redundant disk arrays housed in a secure, climate-controlled data center with backup power. We have begun to publish raw data and full statistical analyses in supplemental information and figshare and will continue to do so.

The genetics of sex determination in zebrafish is not yet understood and is likely polygenic [44,87–89]. As our proposed analyses are prior to sexual maturity, we cannot account for numbers of animals used from each sex. However, animals of a particular sex will not be intentionally excluded from our studies.

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PHS Human Subjects and Clinical Trials Information

Are Human Subjects Involved	○ Yes	● No
Is the Project Exempt from Federal regulations?	○ Yes	○ No
Exemption Number	❏ 1 ❏ 2 ❏ 3 ❏ 4 ❏ 5 ❏ 6 ❏ 7 ❏ 8	
Does the proposed research involve human specimens and/or data	○ Yes	● No
Other Requested information		

VERTEBRATE ANIMALS

Aims 1-2 of this proposal will use live zebrafish *(Danio rerio)* as a model for dystroglycanopathies.

1. Description of Procedures

Work involving zebrafish described in this proposal will be performed at the University of Maine. As this proposal uses zebrafish as a genetic system, male and female zebrafish will be spawned and raised in the University of Maine zebrafish facility. This facility is under the supervision of the campus veterinarian Dr. James Webber. For the proposed project, wild-type, mutant, double mutant, and transgenic mutant strains will be used. The wild-type strain will be ABC. For this project we are keeping 3 genetic stocks: *gmppb-/-, dg-/-,* and *gmppb;dg-/-*; and are also keeping 6 transgenic lines for live imaging, including lines with photoactivatable fluorescent proteins for super-resolution microscopy, that are being crossed with the *gmppb* mutants. We will maintain 4 tanks per stock so that we can always have breeding pairs. 72 tanks with 20 fish/tank so that we can always have breeding pairs. We predict we will generate about 1440 adult fish/year for a total of 7200 zebrafish.

Fish are kept until the end of their fertile lives, about 1.5 years, unless they are euthanized earlier. Zebrafish embryos will be collected from these strains and will be used for the observation and manipulation of neuromusculoskeletal phenotypes. The proposed procedures to be performed include tail fin biopsies for animal genotyping, chemical treatments of larval zebrafish, larval motility assays, microscopic observations, harvesting of larval zebrafish for gene and protein expression analyses, assessment of contractile strength, and assessment of neural transmission.

2. Justification

An animal model is necessary to address the Aims of this proposal because neuromusculoskeletal phenotypes are too complex to completely model in cell culture or in any *in vitro* system. In the past 15 years, the zebrafish has emerged as an important model organism for muscular dystrophy. As a vertebrate, its early development, including skeletal muscle development, is much like that of mammals, and thus it is appropriate to consider it a model for human muscle disease. Zebrafish embryos are transparent and develop externally, allowing observation of cells over time in wild-type and mutant fish. Equally important for genetic analysis is that, for a vertebrate, the zebrafish has a relatively short generation time of about 11 weeks, and fertile adults are highly fecund: a single female can give several hundred eggs on an ongoing weekly basis. The numbers of animals to be used are the minimal numbers necessary to generate the data as based on previous experimental designs and to achieve adequate statistical power.

3. Minimization of Pain and Distress

Most of the proposed experiments will be carried out with zebrafish embryos that are at stages before central nervous system pain centers have developed (which occurs around 8 days post-fertilization). After the stages when neurologic activity is made evident by muscular contractions (17 hours post-fertilization), embryos will be anesthetized in tricaine prior to live imaging to prevent any possible distress. If any procedures appear to cause severe discomfort for larval zebrafish, such as abnormally rapid twitching, we will cease the treatment and euthanize the animals. Adult fish are gently handled by netting and are anesthetized with tricaine for routine procedures such as genotyping. Anesthetized fish recover consciousness within minutes of being returned to fresh water and thereafter behave normally with respect to eating and breeding.

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October 1, 2018

Clarissa Henry, Ph.D.
School of Biology and Ecology
University of Maine
Orono, ME 04669

Dear Clarissa,

I am delighted to collaborate with you on this project. I am eager to apply my experience in super-resolution microscopy to provide innovative methods for visualization and analysis of muscle cell organization and physiology. My laboratory's role in our proposed project is to perform super-resolution microscopy of living and fixed muscle fibers, analyze the resulting data, and test for spatial relationships between Dystroglycan and integrins.
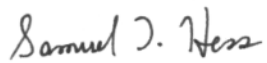
As you know, my laboratory invented Fluorescence Photoactivation Localization Microscopy [FPALM; S.T. Hess et al., *Biophysical Journal*, 2006], which allows for resolution of a few tens of nanometers, in living cells [Hess et al. *PNAS* 2007], fixed cells and tissue, or even *in vivo* [Gabor et al., *PLoS One* 2015]. I have nearly 13 years of experience with super-resolution microscopy, including 47 related publications (out of 61 total), mention in the "Method of the Year" award by *Nature Methods* in 2009, in the "Top Ten Scientific Breakthroughs of 2006" in *Science*, and a citation in the "Scientific Background on the Nobel Prize in Chemistry 2014" for Super-Resolved Fluorescence Microscopy, written by the Nobel Committee.

Furthermore, we have all necessary equipment for the proposed super-resolution microscopy, including multiple FPALM (localization) microscopes capable of multicolor [Gunewardene et al. *Biophysical Journal* 2011], live-cell [Hess et al. *PNAS* 2007], three-dimensional [Juette et al. *Nature Methods* 2008] and spectrally-resolved [Mlodzianoski et al. *PLoS One* 2016] super-resolution microscopy, pulsed and CW lasers spanning the visible and near-IR wavelengths, multiple EMCCD and sCMOS cameras, stage incubators, autofocus, and a dedicated computer cluster (20 PCs) with GPUs for real-time data analysis.

I am very happy that we were able to recruit a senior graduate student from my laboratory, Matthew Parent, to also work on this project. Mat already has extensive experience using FPALM in various forms (multi-color, three dimensional, live and fixed cell), including imaging of muscle cells, mitochondria, and membrane protein complexes. He is also very familiar with the kinds of data analysis we will need for this project. Based on our experience with FPALM and your extensive experience in the field of muscle physiology and muscular dystrophy, I expect that this will be a very successful project.

My laboratory is eager to collaborate, and we will do everything possible to ensure the success of this project. I look forward to working with you.

With best wishes for a successful application,

Letters of Support

Samuel T. Hess, Ph.D.
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October 1, 2018

Clarissa Henry, Ph.D.
School of Biology and Ecology
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Dear Clarissa,

I am delighted to collaborate with you on this project. This letter affirms my commitment to collaboratively investigate the molecular mechanisms of phenotypic plasticity in the novel zebrafish models for dystroglycanopathies you have developed. We both bring complementary expertise to this project and I am eager to apply my genomics and computational expertise to advance our knowledge of these mechanisms. My laboratory's role in our proposed project is to prepare samples for the RNA-Seq studies, coordinate library preparation and sequences, analyze the resulting data and validate candidate genes. Importantly, my lab will model networks of co-expressed coding and non-coding genes that will provide new insight into biological functions may contribute to disease phenotypes.

My laboratory studies the systems biology of stress responses and utilizes genomic and computational approaches to understand disease mechanisms. We apply network analysis methods to study how genes function together with multiple layers of genetic regulation that include both protein coding and non-coding genes. A specific focus of my laboratory is on non-coding RNA, including microRNA (miRNA) and long non-coding RNA, that have important regulatory roles in health and disease. I have specialized expertise in the analysis of high-throughput sequence data and miRNA that can be used to understand complex biological processes. Most recently, my recent research on miRNA-mRNA regulatory circuits for heart regeneration revealed a set of novel long non-coding RNA (King *et al, NPJ Regen Med,* 2018). Our proposed project requires the same overall approach of integrating miRNA and mRNA expression data to advance our knowledge of neuromusculoskeletal development, homeostasis, and plasticity.

My laboratory is eager to collaborate, and we will do everything possible to ensure the success of this project. We are equipped with the necessary technical capacities and experience to carry out our portion of the project. I look forward to working with you.

With best wishes for a successful application,

Benjamin L. King, Ph.D.
Assistant Professor of Bioinformatics

Letters of Support

RESOURCE SHARING PLAN

1. Data Sharing Plan

Not Applicable (<$500,000 in direct costs per year) but we will share RNA-Seq, miRNA-Seq, and phenotypic data after publishing.

RNA-Seq data will be deposited and made freely accessible in NCBI's Gene Expression Omnibus and Short Read Archive. Dr. King has deposited 8 previous RNA-Seq studies to these databases.

Phenotypic data – It is our hope that phenotypic data obtained will have an impact beyond this project and that making this data freely available will allow other researchers to analyze the data for their tissue/structure of interest. Unlike other fields, such as genomics, there are not well-established protocols/publication requirements/standards for sharing this type of image data. Thus, we aim to establish a best practices methodology that will be reviewed yearly and modified if needed. All original data will be archived and basic metadata regarding sample species, genotype, age, and markers/stains/antibodies used will be maintained in a spreadsheet. A spreadsheet containing the information above for experiments used in a particular study will be included as supplemental information when the data is published. This will allow other researchers to request access to the original data.

The approach that we are taking is to utilize The Advanced Computing Group at the University of Maine (ACG@UMaine) for long term archiving because the ACG@UMaine services include training and support for all technical aspects of the data produced during the course of this project necessary to maintain security, dissemination, and preservation. The ACG@UMaine primary data repository is the Maine Dataverse Network (MDVN). Those who generate data as a result of this project are responsible for adding that data to the MDVN repository immediately after publication. It is the responsibility of the individual PIs to NOT add data to the repository that violates privacy, confidentiality, security, or intellectual property concerns. The data will be retained indefinitely. Adherence checks will include review of the MDVN content, number of studies released, availability for each study of subsettable/preservation-friendly data formats (possibly embargoed, but listed), availability of documentation (public), and correctness of data citation (including an integrity check).

2. Sharing Model Organisms

As for our plan to share materials and our management of intellectual property, we will adhere to the NIH Grant Policy on Sharing of Unique Research Resources, and in particular the NIH Policy on Sharing of Model Organisms for Biomedical Research. Model organisms that may be generated by this project include new mutant and/or transgenic zebrafish strains. All model organisms generated by this project will be distributed freely or deposited into a repository, such as the Zebrafish International Resource Center (Eugene, OR), making them available to the broader research community, either before or immediately after publication. We have previously demonstrated our commitment to sharing by providing published mutant and/or transgenic zebrafish to the research community.

The Henry lab will assume responsibility for distributing the newly generated model organisms and will fill requests in a timely fashion. In addition, we will provide relevant protocols, published DNA clones, and published genetic and phenotypic data upon request. Material transfers will be made with no more restrictive terms than in the Simple Letter Agreement (SLA) or the Uniform Biological Materials Transfer Agreement (UBMTA) and without reach through requirements. Should any intellectual property arise which requires a patent, we will ensure that the technology (materials and data) remains widely available to the research community in accordance with the NIH Principles and Guidelines document.

3. Genome-Wide Association Studies

Not Applicable.

Authentication of Key Resources:

(1) <u>The *gmppb^{c125delA}* mutant zebrafish strain.</u> We generated this mutant strain using CRISPR-Cas9. We will use multiple approaches to ensure the specificity and proper maintenance of this stock. **Stock propagation:** Stocks for this allele are maintained separately and have been generated by outcrossing one *gmppb^{c125delA}* carrier fish with one ABC wild-type fish every generation. Currently, we are spawning the F3 generation and experiments are being conducted on the F4 generation. We will continue outcrossing and sequencing each successive generation to ensure that no allelic contamination has occurred. <u>Multiple alleles for each dystroglycanopathy</u>: We are currently generating multiple alleles for *gmppb* to validate our results. <u>Genotype:Phenotype correlation:</u> We have specific PCR probes that will detect *gmppb* mutant zebrafish. Thus, we will PCR genotype every larva when experimentation is complete.

(2) Antibodies. All of the antibodies/stains we propose to use are commonly used and commercially available. Every time we receive a new batch, we run an experiment with positive (wild-type fish with very well characterized staining pattern) and negative (no primary antibody and, if available, the appropriate mutant fish) controls. We have never had any issues with the antibodies/stains that we propose to use in this proposal.

Authentication of Key Biological and/or Chemical Resources