RESEARCH STRATEGY

Significance

Heart failure is the leading cause of hospitalization in the US and will affect 1 in 4 people during their lifetime[1,2]. The syndrome has different forms with non-ischemic cardiomyopathy (NICM) being the least understood. The most likely genetic cause of NICM is a truncation variant in the *TTN* gene (TTNtv), which encodes titin[3-9].

Titin, the largest known protein in humans, spans half sarcomeres and is essential for sarcomere assembly, passive tension, and force generation[7,9,10]. Myocyte quality control pathways must attempt to adapt to the atypical size of titin to rid the cell of its faulty transcripts and filaments. Nonsense-mediated and exosomal decay are two major surveillance mechanisms responsible for eliminating faulty transcripts, while the ubiquitin-proteasome system works similarly at the protein-level. The nonsense-mediated decay pathway, mediated by up-frameshift protein 1 (UPF1), recognizes and degrades truncated mRNA species before they are translated[11,12]. Similarly, the exosomal decay pathway, regulated in part by exosome complex 10 (EXOSC10), processes and degrades aberrant transcripts[13,14]. The ubiquitin-proteasome system uses ubiquitin to tag damaged proteins, typically via a K48-polyubiquitin linkage, for proteasomal degradation[15-17].

In NICM hearts, these pathways may be overloaded due to the burden of TTNtvs, which could lead to accumulation of cellular debris (lipofuscin) and/or incorporation of truncated titin into sarcomeres. The status of these mechanisms in TTNtv-associated NICM and their influence on disease progression is unknown.

This project will investigate how TTNtvs disrupt RNA and protein turnover, lipofuscin accumulation, sarcomeric titin integration, and myocardial mechanics (Fig. 1). By utilizing human tissue with matched clinical and multi-omics data, this study bridges molecular, cellular, and mechanical changes with direct translational implications.

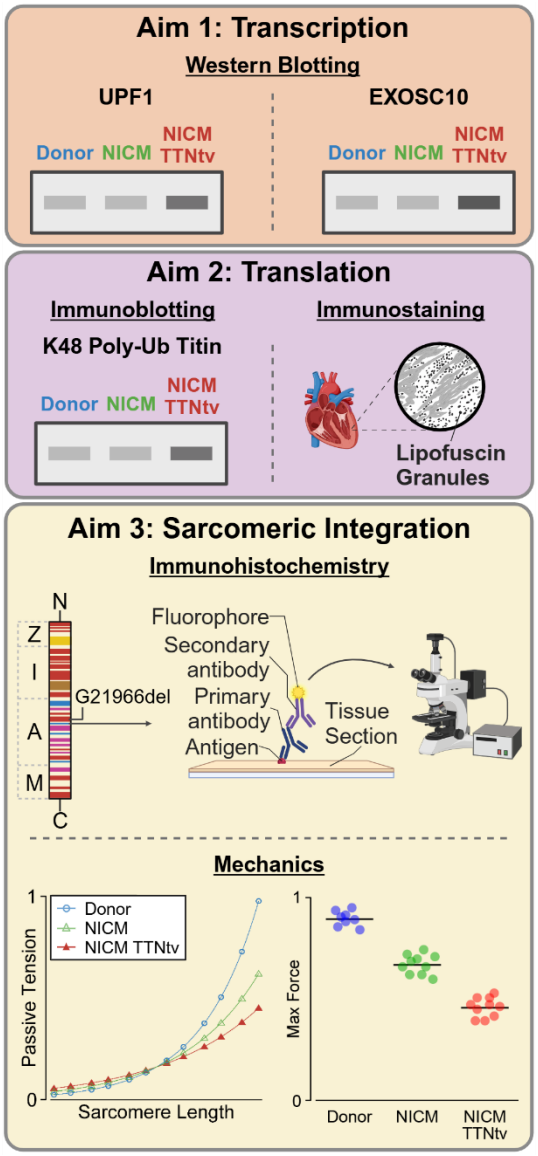

Figure 1. Project Overview

Innovation

This study integrates human myocardial tissue, multi-omics, and clinical data to examine disease mechanisms in TTNtvs. Unlike studies relying on animal models or in vitro systems, this approach provides a direct assessment of human myocardial function.

This research focuses on TTNtv-related inefficiencies in RNA and protein turnover, bridging nonsense-mediated and exosomal decay, proteasomal processing, and lipofuscinogenesis. While previous studies have emphasized titin's structural role, this project expands to explore protein quality control and degradation pathways.

By examining how truncated titin integrates into the sarcomere and affects intracellular tension and force generation, this work offers new insights into the mechanical consequences of TTNtvs. A newly developed image segmentation pipeline ensures accurate quantification of lipofuscin deposition, overcoming challenges posed by autofluorescence.

Potential Path to Clinical Application

Better understanding the pathogenic mechanisms of TTNtvs has direct clinical implications. If truncated titin disrupts sarcomere integrity and reduces contractile function, therapies targeting protein quality control pathways—such as autophagy enhancement or proteasomal regulation—could be explored to mitigate pathogenic effects. Additionally, if TTNtv-associated mechanical dysfunction follows specific patterns, this knowledge could improve risk stratification, refine patient-specific interventions, and guide treatment decisions.

These techniques may distinguish high-risk TTNtv carriers for NICM onset and progression, supporting personalized treatments and advancing precision medicine for TTNtv-associated cardiomyopathies.

Preliminary Studies

The University of Kentucky performs ~1% of the world's heart transplants, and my lab has banked these myocardial specimens for over a decade, amassing over 20,000 samples from 650 patients. I coordinated the whole exome sequencing of 348 patients and analyzed the results to identify 24 patients who had NICM and a TTNtv (Table 1). I also have transcriptomic and (by May 2025) proteomic data for these individuals. I will use samples from age and sex-matched organ donors who did not have NICM or a TTNtv as physiological controls, along with patients with NICM (Table 1). To our knowledge, this is one of the largest datasets of TTNtv-related omics data available world-wide.

Table 1. Patient Characteristics

	Donor (n=24)	NICM (n=24)	NICM TTNtv (n=24)	*p-value*
Age, mean ± SD	39.7 ± 12.7	45.5 ± 15.9	45.6 ± 15.9	0.31
Male, %	66.7	66.7	66.7	
White, %	91.7	70.8	87.5	0.36
Diabetic, %	16.7	16.7	16.7	
BMI, mean ± SD	29.2 ± 6.4	28.3 ± 7.8	29.9 ± 6.0	0.70

Categorical and continuous variables were analyzed with chi-squared tests and one-way ANOVA.

Approach

Aim 1. Test the hypothesis that samples with a TTNtv have higher UPF1 and EXOSC10 abundance.

Rationale

Our transcriptomics data confirm prior studies showing that pathways regulating nonsense-mediated and exosomal decay are upregulated in patients who have TTNtvs[7,8,10,18]. Since nonsense-mediated and exosomal decay are central to clearing faulty transcripts, higher UPF1 and EXOSC10 activity in TTNtv samples is likely to manage the increased presence of aberrant RNAs.

UPF1 identifies and facilitates degradation of mRNAs with premature stop codons[8,10]. By comparison, EXOSC10 interacts with cofactors for transcript processing and serves as part of degradation machinery[14].

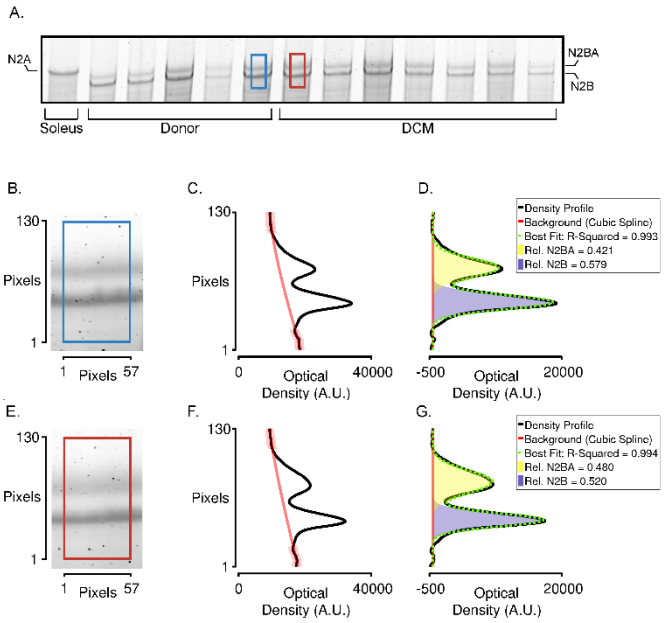

Figure 2. GelBox corrects for background variation and partially resolved bands to semi-automatically quantify gels.

Experimental Design

Left ventricular tissue from organ donors and NICM patients (with and without a TTNtvs) will be analyzed for UPF1 and EXOSC10 abundance via Western blot (n=24/group; Table 1).

Western Blotting

Samples will be resolved on 10% SDS-PAGE gels, transferred to PVDF membranes, and probed for UPF1 and EXOSC10. Total protein will be normalized using 2,2,2-trichloroethanol, and bands will be quantified via GelBox (Fig. 2)[19].

Table 2. Expected Results of Aim 1

Protein	Abundance compared to other experimental groups
UPF1	↑
EXOSC10	↑

Challenge and Potential Solution

Human cardiac tissue is difficult to procure and process consistently. This study circumvents this limitation by utilizing out biobank, which cryopreserves samples within 50 minutes post-explant, a method shown to preserve protein integrity and contractile function[20].

Outcomes

TTNtvs are expected to elevate UPF1 and EXOSC10 abundance, indicating increased activity of RNA degradation pathways in affected myocardium (Table 2).

Aim 2. Test the hypothesis that samples with a TTNtv have higher K48-linked polyubiquitinated titin and lipofuscinogenesis.

Rationale

Damaged proteins are targeted for degradation via K48-linked polyubiquitination, yet proteasomal efficiency declines with age, leading to lipofuscin accumulation[15-17]. These processes are accelerated in disease, compounding protein quality control deficits[15,21].

Titin's large size and abundance may overwhelm proteasomal degradation, promoting lipofuscin formation.

Experimental Design

Using the same myocardial samples as in Aim 1, K48-linked polyubiquitination of titin and lipofuscin granule accumulation will be measured via immunoblot/stain, respectively.

Aim 2.1 K48-Linked Polyubiquitination of Titin

As shown in Figure 2, Samples will be resolved on agarose-stabilized 1% gels, transferred to PVDF membranes, and probed for K48-linked polyubiquitin. Total protein will be normalized using Oriole fluorescent staining and quantified via GelBox[19].

Aim 2.2 Lipofuscin Granule Accumulation

10-µm cryosections will be immunohistochemically stained for alpha actinin and imaged with lasers to exploit lipofuscin's innate autofluorescence (~650 nm) and the attached fluorophore (~555 nm). I have written code to segment fluorescent images and normalize lipofuscin to total cardiomyocyte area (Fig. 3).

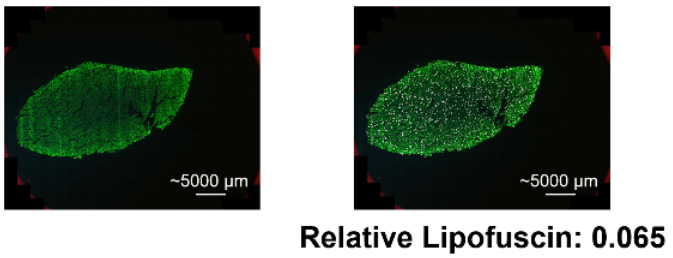

Figure 3. Customized image segmentation pipeline calculates relative lipofuscin from immunofluorescent images.

Challenge and Potential Solution

The size difference between titin (~3,000 kDa) and ubiquitin (~8.5 kDa) make it difficult to distinguish clear signals from noise in immunoblots. Moreover, variability in lipofuscin autofluorescence affects consistency of quantification. This investigation bypasses these hurdles by using highly sensitive and adaptive analysis tools to quantify gels and segment images (Figs. 2 & 3)[19].

Outcomes

TTNtvs are expected to increase titin polyubiquitination and lipofuscin accumulation, highlighting proteasomal inefficiency in NICM (Table 3).

Table 3. Expected Results of Aim 2

Target	Abundance compared to other experimental groups
Aim 2.1: Immunoblotting	
K48 Poly-Ub Titin	↑
Aim 2.2: Immunohistochemistry	
Lipofuscin	↑

Aim 3. Test the hypothesis that samples with a TTNtv incorporate truncated titin filaments into sarcomeres and exhibit lower intracellular passive and maximal isometric forces.

Rationale

Truncated titin is meant to be removed from the cell via quality control mechanisms, but prior studies have shown that TTNtv reduce sarcomere stability and alter kinase/phosphatase activity[9,11-13,15-17,22]. Cellular turnover pathways are modulated by kinase/phosphatase activity, suggesting that faulty titin from a TTNtv may overload turnover pathways, leading to sarcomeric integration of these filaments.

Experimental Design

I will use specimens from the same samples studies in Aims 1 and 2 to test how TTNtv impact muscle mechanics. I will also quantify if truncated titin filaments are incorporated into sarcomeres using immunohistochemistry.

Data will be collected from 3 cryosections or permeabilized cardiac fibers per patient (n=72). Power calculations (G*Power, Cohen's f = 0.17) indicate that this design can detect small-to-medium effect sizes. Statistical analyses will use linear mixed models to account for repeated measures, with a significance threshold of 5% ($p<0.05$).

Aim 3.1 Incorporation into Sarcomeres

10-µm cryosections will be immunohistochemically stained for a titin epitope near the sarcomeric M-line and alpha-actinin (Fig. 4). A custom image segmentation pipeline will quantify the relative proportion of titin by normalizing to total alpha-actinin content.

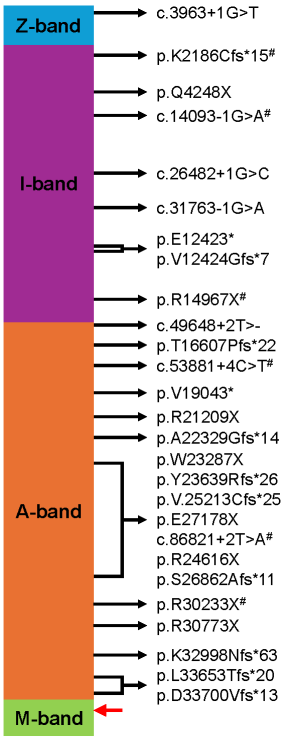

Figure 4. Sarcomeric locations of TTNtv. Red arrow indicates titin epitope. '#': frequency is >1 patient.

Aim 3.2 Biophysical Deviation

~100 mg of tissue per patient will be permeabilized to make ~200-µm x 600-µm cardiac fibers. These fibers will be anchored between a force transducer and length controller to generate force traces upon execution of stretch protocols[23]. Experiments will mimic physiological temperature (37°C) and sarcomere length (2.0 µm).

Fibers will be stretched before and after incubation with potassium chloride/iodide, which depolymerizes myofilaments, to separate intra/extracellular contributions to passive tension.

Fibers will be placed in calcium solutions of varying concentrations (pCa), and force traces will be fit to the Hill Equation, $F = F_0 + F_{max}([Ca^{2+}]^{nH}/([Ca^{2+}]^{nH} + [Ca_{50}^{2+}]^{nH}))$, to determine maximum isometric force (F_{max}; Fig 5).

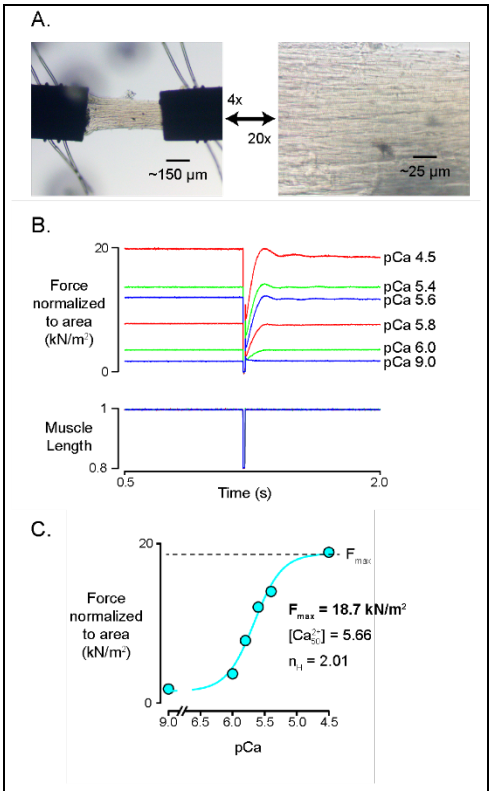

Figure 5. Muscle mechanics experiment I performed with tissue from a NICM patient with a TTNtv.

Challenge and Potential Solution

Detecting truncated titin may be difficult due to low expression and/or masking by full-length titin. This project utilizes a published, epitope-specific titin antibody to ensure adequate detection sensitivity (Myomedix TTN-M9).

Force measurements can be influenced by tissue heterogeneity, fiber quality, and/or uncontrolled sarcomere lengths. To maintain fiber quality and reproducible sarcomere length measurements, these experiments utilize a calibrated camera and exclude fibers with significant pre-experimental damage. Moreover, the design of triplicate measures per patient enhances statistical power and minimizes variability.

Outcomes

TTNtvs are expected to induce sarcomeric integration of truncated titin, leading to reduction of intracellular passive and maximal isometric forces (Table 4).

Conclusions

This study investigates the contribution of TTNtvs to NICM by integrating molecular, histological, and biomechanical analyses. By characterizing the burden of TTNtvs from transcript to protein and its impact on sarcomere function, I aim to clarify whether truncated titin filaments are incorporated into sarcomeres and how they affect myocardial mechanics.

Elucidating the status of myocyte quality control mechanisms in TTNtv-bearing NICM hearts will not only advance our understanding of NICM pathogenesis but also identify potential biomarkers and therapeutic targets for enhancing RNA and protein turnover.

Table 4. Expected Results of Aim 3

Metric	**Abundance compared to other experimental groups**
Aim 3.1: Immunohistochemistry	
Stain Intensity	↓
Aim 3.2: Muscle Mechanics	
Intracellular Passive Force	↓
Maximal Isometric Force	↓↓