Protein homeostasis in muscle cells under mechanical stress conditions in health and disease

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The work described in this dissertation was conducted between September 2018 and January 2024 under supervision by Prof. Dr. Thorsten Hoppe at the CECAD Research Center, University of Cologne, Joseph-Stelzmann Straße 26, 50931 Cologne, Germany.

INHALTSVERZEICHNIS

ABSTRACT

Protein homeostasis (proteostasis) describes the balance of synthesis, folding, and degradation of proteins in a given cell or tissue, and is mediated by molecular chaperones and E3 ubiquitin ligases that are essential for the assembly, maintenance, and repair of complex protein machineries and for the response to stressful challenges. Especially in the sarcomeres of striated muscle, a tissue under constant stress and use, myosin motor proteins and actin filaments mediate contraction and the generated force leads to mechanical stress and misfolding of the intricate protein components. Conversely, inactivity and muscular diseases present stressful challenges that result in the increased demand for the degradation of damaged or excess proteins. The myosin chaperone UNC-45 is involved in the folding and assembly of myofilaments and has been implicated in the maintenance and response to mechanical stress. However, the exact roles of UNC-45 in human muscular disease, in muscle proteostasis, and particularly regarding the myosin motor protein under mechanical stress remain incompletely understood.

This work reports pathogenic variants in the *UNC45B* gene that cause progressive myopathy characterized by eccentric cores. Clinical observations, biochemical experiments, and transgenic rescue assays in *Caenorhabditis elegans* reveal that the disease mechanism involves alterations in *UNC45B* mRNA splicing and UNC-45B protein levels, as well as disruptions in UNC-45 localization, folding, and interaction with myosin. A ubiquitin fusion reporter strain in *C. elegans* body wall muscle allows to further explore proteostasis responses to UNC-45 mutation. In addition, a transgenic model for the OPTogenetic Induction of Mechanical MUscle Stress in *C. elegans* (OptIMMuS) in combination with sensitive proximity labeling assays identifies the small heat shock proteins of the HSP-16 family as well as the TRIM E3 ubiquitin ligase NHL-1 and its binding partner F40A3.6 as new interactors of UNC-45 and myosin. Genetic, behavioral, and co-immunoprecipitation experiments in myosin misfolding mutants and in a ligase-dead *nhl-1* mutant provide evidence that the ubiquitylation activity of this TRIM protein regulates myosin protein abundance and function under mechanical stress.

Together, this work establishes a role for UNC-45 in human muscular disease and in the molecular response to mechanically induced myosin misfolding in otherwise healthy muscle. These findings suggest that UNC-45 determines the fate of misfolded myosin molecules under mechanical stress and cooperates with a TRIM E3 ligase and the ubiquitin-proteasome system in myosin proteostasis in the muscle.

ZUSAMMENFASSUNG

Proteinhomöostase (Proteostase) beschreibt das Gleichgewicht von Synthese, Faltung und Abbau von Proteinen in Zellen oder Geweben und wird durch molekulare Faltungshelfer (Chaperone) und E3-Ubiquitin-Ligasen vermittelt, die essentiell sind für den Aufbau, die Wartung und die Reparatur komplexer Proteinmaschinen und in Reaktion auf Stress. Insbesondere in den Sarkomeren der quergestreiften Muskulatur, einem Gewebe, das ständiger Belastung und Nutzung ausgesetzt ist, ermöglichen Myosin-Motorproteine zusammen mit Aktinfilamenten die Kontraktion und erzeugen eine Kraft, die zu mechanischer Belastung und Fehlfaltung komplexer Proteinkomponenten führt. Hingegen stellen Nichtbeanspruchung und Muskelerkrankungen stressige Herausforderungen dar, die zu einem erhöhten Abbau beschädigter oder überschüssiger Proteine führen. Das Myosin-Chaperon UNC-45 ist sowohl an der Faltung und Assemblierung von Myofilamenten als auch an der Instandhaltung und der Antwort auf mechanische Belastung beteiligt. Welche genaue Rolle UNC-45 bei menschlichen Muskelerkrankungen, bei der Muskel-Proteostase und insbesondere im Hinblick auf das Myosin-Motorprotein unter mechanischer Belastung spielt, ist jedoch noch nicht vollständig geklärt.

Diese Arbeit berichtet von pathogene Varianten im *UNC45B*-Gen, die eine progressive Myopathie verursachen, gekennzeichnet durch exzentrische Cores. Klinische Beobachtungen, biochemische Experimente und transgene Rettungstests in *Caenorhabditis elegans* zeigen, dass der Krankheitsmechanismus mit Veränderungen im *UNC45B*-mRNA-Spleißen und im UNC-45B-Proteinspiegel sowie Störungen der UNC-45-Lokalisierung, -Faltung und -Interaktion mit Myosin einhergeht. Ein Ubiquitin-Fusionsreporterstamm im Muskel von *C. elegans* ermöglicht außerdem die Untersuchung von Proteostase-Antworten auf UNC-45-Mutationen. Darüber hinaus identifiziert ein transgenes Modell für die OPTogenetische Induktion von Mechanischem MUskelStress in *C. elegans* (OptIMMuS) in Kombination mit sensitiven Proximity-Labeling-Assays die kleinen Hitzeschockproteine der HSP-16-Familie sowie die TRIM E3-Ubiquitin-Ligase NHL-1 und den Bindungspartner F40A3.6 als neue Interaktoren von UNC-45 und Myosin. Genetische, Verhaltens- und Co-Immunpräzipitationsexperimente in Myosin-Fehlfaltungsmutanten und einer Ligase-toten *nhl-1*-Mutante liefern Hinweise darauf, dass die Ubiquitylierungsaktivität dieses TRIM-Proteins die Myosin-Proteinmenge und -Funktion unter mechanischem Stress reguliert.

Zusammengefasst belegt diese Arbeit, dass UNC-45 eine Rolle bei menschlichen Muskelerkrankungen und bei der molekularen Reaktion auf mechanisch induzierte Myosin-Fehlfaltungen im ansonsten gesunden Muskel spielt. Diese Ergebnisse legen nahe, dass UNC-45 das Schicksal fehlgefalteter Myosin-Moleküle unter mechanischer Belastung bestimmt und

mit einer TRIM-E3-Ligase und dem Ubiquitin-Proteasom-System bei der Myosin-Proteostase im Muskel zusammenarbeitet.

LIST OF PUBLICATIONS

The following publications form the basis of this thesis:

A Donkervoort, S.*, **Kutzner, C. E.***, Hu, Y., Lornage, X., Rendu, J., Stojkovic, T., Baets, J., Neuhaus, S. B., Tanboon, J., Maroofian, R., Bolduc, V., Mroczek, M., Conijn, S., Kuntz, N. L., Töpf, A., Monges, S., Lubieniecki, F., McCarty, R. M., Chao, K. R., Governali, S., Böhm, J., Boonyapisit, K., Malfatti, E., Sangruchi, T., Horkayne-Szakaly, I., Hedberg-Oldfors, C., Efthymiou, S., Noguchi, S., Djeddi, S., Iida, A., di Rosa, G., Fiorillo, C., Salpietro, V., Darin, N., Fauré, J., Houlden, H., Oldfors, A., Nishino, I., de Ridder, W., Straub, V., Pokrzywa, W., Laporte, J., Foley, A. R., Romero, N. B., Ottenheijm, C., Hoppe, T.**, Bönnemann, C. G.** (**2020**). Pathogenic Variants in the Myosin Chaperone UNC-45B Cause Progressive Myopathy with Eccentric Cores. *American Journal of Human Genetics*, *107*(6), 1078–1095. https://doi.org/10.1016/j.ajhg.2020.11.002
*These authors contributed equally. **These authors contributed equally.

B **Kutzner, C. E.**, Bauer, K. C., & Hoppe, T. (**2023**). A ubiquitin fusion reporter to monitor muscle proteostasis in *C. elegans*. *microPublication biology*, *2023*, https://doi.org/10.17912/micropub.biology.000824

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LIST OF FIGURES

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ARM	Armadillo repeat
ATP	adenosine triphosphate
CASA	chaperone-assisted selective autophagy
C. *elegans*	*Caenorhabditis elegans*
ChR2	channelrhodopsin-2 from *Chlamydomonas rheinhardtii*
CIM	critical illness myopathy
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
E4	ubiquitin chain-elongation factor
F-box	cyclin F domain
HECT	Homologous to E6-associated protein C-Terminus
HSP	heat shock protein
Ig	immunoglobulin
MHC B/A	myosin heavy chain B/A
OptIMMuS	OPTogenetic Induction of Mechanical MUscle Stress
PQC	protein quality control
RNAi	RNA interference
RBR	RING-Between-RING
RING	Really Interesting New Gene
sHSP	small heat shock protein
TPR	Tetratricopeptide repeat
TRIM	Tripartite motif
Ub	ubiquitin
U-box	UFD2-box
UFD	ubiquitin fusion degradation
UPS	ubiquitin-proteasome system

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Thank you all so very much!

ERKLÄRUNG ZUR DISSERTATION

„Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht."

Teilpublikationen:

A Donkervoort, S.*, **Kutzner, C. E.***, Hu, Y., Lornage, X., Rendu, J., Stojkovic, T., Baets, J., Neuhaus, S. B., Tanboon, J., Maroofian, R., Bolduc, V., Mroczek, M., Conijn, S., Kuntz, N. L., Töpf, A., Monges, S., Lubieniecki, F., McCarty, R. M., Chao, K. R., Governali, S., Böhm, J., Boonyapisit, K., Malfatti, E., Sangruchi, T., Horkayne-Szakaly, I., Hedberg-Oldfors, C., Efthymiou, S., Noguchi, S., Djeddi, S., Iida, A., di Rosa, G., Fiorillo, C., Salpietro, V., Darin, N., Fauré, J., Houlden, H., Oldfors, A., Nishino, I., de Ridder, W., Straub, V., Pokrzywa, W., Laporte, J., Foley, A. R., Romero, N. B., Ottenheijm, C., Hoppe, T.**, Bönnemann, C. G.** (**2020**). Pathogenic Variants in the Myosin Chaperone UNC-45B Cause Progressive Myopathy with Eccentric Cores. *American Journal of Human Genetics*, *107*(6), 1078–1095. https://doi.org/10.1016/j.ajhg.2020.11.002
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Köln, 13.08.2024, Carl Elias Kutzner

1 Introduction

The balance of synthesis, folding, and degradation of proteins in a given cell or tissue is termed protein homeostasis (proteostasis). Proteostasis factors, such as molecular chaperones and E3 ubiquitin ligases, are essential for the assembly, maintenance, and repair of complex protein machineries and in the response to stressful insults (Balch et al., 2008; Hipp et al., 2019; Pilla et al., 2017). Especially, tissues under constant stress and use, such as the muscle, require constant proteome surveillance and genetic dysfunction of tissue-specific proteostasis factors is associated with multiple progressive hereditary disorders (Carlisle et al., 2017; Collier and Benesch, 2020; Henning and Brundel, 2017; Sarparanta et al., 2020; Smith et al., 2014). In the sarcomere, the contractile unit of the muscle, myosin motor proteins align with actin filaments and spatiotemporally interact to perform contraction. Intense contractions can lead to mechanical stress and misfolding of intricate protein components that need to be precisely recognized. Conversely, disuse and muscular diseases present stressful stimuli creating the enhanced demand for the degradation of damaged or excess proteins (Bodine and Baehr, 2014; Höhfeld et al., 2021; Mukund and Subramaniam, 2020). However, how proteostasis in the muscle is maintained in general and, in particular, how the muscle motor protein myosin is regulated under mechanical stress remain incompletely understood.

Intriguingly, the myosin-directed chaperone UNC-45, which was first discovered in the nematode *C. elegans*, has been implicated not only in myofilament assembly but also in the response to mechanical stress (Hellerschmied and Clausen, 2014; Kim et al., 2008; Odunuga and Oberhauser, 2023; Pokrzywa and Hoppe, 2013). The following sections will introduce the concept of proteostasis, molecular chaperones, and E3 ubiquitin ligases, as well as describe striated muscle tissue, mechanical stress in muscle, and muscular diseases. The myosin chaperone UNC-45 will thereby be highlighted as a central proteostasis factor and *C. elegans* body wall muscle will be described as a model for mammalian striated muscle.

1.1 Protein homeostasis (proteostasis)

Proteins are fundamental macromolecules that are each built from a chain of amino acids and, in their three-dimensional, folded state, serve vital functions in the cell. The cellular proteome, encompassing all proteins within a cell at a given time, is constantly adjusted to maintain homeostasis in response to metabolic demands, environmental stimuli, and various types of stress. The proteostasis network thereby comprises protein quality control (PQC) factors that regulate protein conformation, interaction, and localization, and balance protein synthesis and degradation (Figure 1; Balch et al., 2008; Balchin et al., 2016; Hipp et al., 2019; Hoppe and Cohen, 2020; Kaushik and Cuervo, 2015; Labbadia and Morimoto, 2015; Pilla et al., 2017).

The life cycle of a protein begins during translation, when ribosomes catalyze the synthesis of polypeptide chains from individual amino acid building blocks in a unique sequence. For each protein, this sequence is encoded in the organism's DNA and is transcribed and delivered to the ribosome as mRNA. During and after translation, molecular chaperones ensure the proper three-dimensional folding of the newly synthesized polypeptide, prevent misfolding, and aid in correct localization (Balchin et al., 2016; Dahiya and Buchner, 2019; Hipp et al., 2019). In addition, chaperones can facilitate protein degradation when folding fails intrinsically or stressful insults to the cell enhance the accumulation of unfolded, misfolded, or damaged proteins (Kevei et al., 2017; Kriegenburg et al., 2012; McClellan et al., 2005). At the end of their lifetime, damaged or excess proteins are removed by two major regulated degradation machineries: the ubiquitin-proteasome system (UPS) recognizes and degrades individual damaged proteins, whereas larger protein aggregates and damaged organelles are removed by autophagy. In the process of macroautophagy, short autophagy, double-membrane structures called phagophores engulf damaged proteins or organelles into autophagosomes and fuse with lysosomes for degradation of the cargo by lysosomal hydrolases (Dikic, 2017; Li et al., 2022b; Pohl and Dikic, 2019; Wang and Le, 2019).

Under non-stress conditions, proteostasis mechanisms continuously adapt the proteome to physiological demands. In contrast, cellular stress caused by increased temperature, mechanical forces, high metabolic demands, or the generation of reactive oxygen species results in the accumulation of aberrant proteins (Dantuma and Lindsten, 2010; Höhfeld et al., 2021; Ravanelli et al., 2020; Vakkayil and Hoppe, 2022). Accordingly, accumulated aberrant proteins and toxic aggregates increase the strain on the proteostasis network and require a more pronounced activation of proteostasis mechanisms. Similarly, disease states and organismal aging contribute to a decline of proteostasis capacity and ultimately to the progression of age-related diseases such as neurodegenerative disorders, cardiovascular disease, sarcopenia, and cancer (Hipp et al., 2019; Kaushik and Cuervo, 2015; Kevei et al., 2017; Labbadia and Morimoto, 2015; López-Otín et al., 2023; Pilla et al., 2017; Vilchez et al., 2014). Therefore, understanding proteostasis mechanisms and their interplay is integral to healthy aging and to the successful treatment of many diseases.

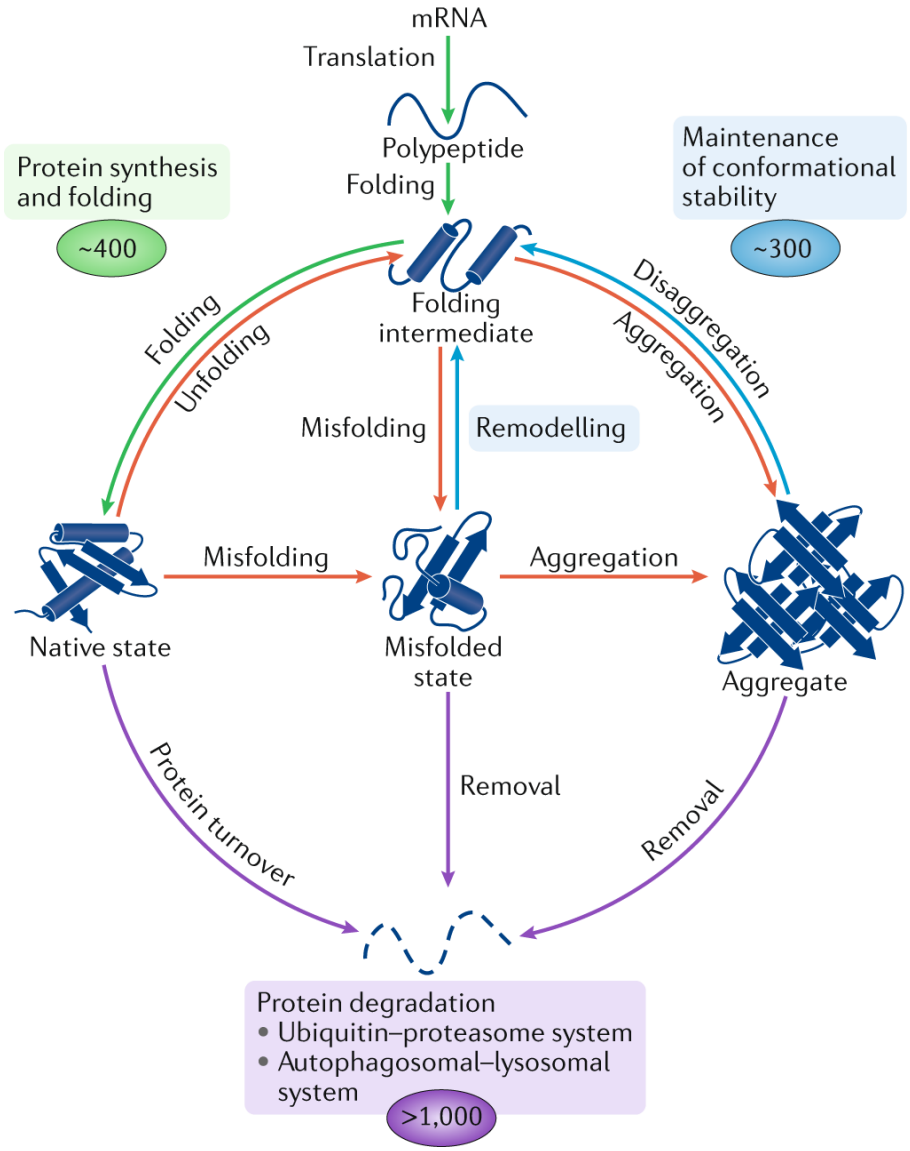

Figure 1 The cellular proteostasis network (cropped from Figure 1a in Hipp et al., 2019; reproduced with permission from Springer Nature; License Number 5718770857159).
The proteostasis network contains ~2000 factors that aid in protein synthesis and folding (green), maintain conformation (blue), and facilitate protein degradation (purple) preventing misfolding and harmful interactions (red). In humans, ~300 different molecular chaperones cooperate with the UPS and autophagy balancing protein synthesis, maintenance, and degradation (Brehme et al., 2014).

1.1.1 Molecular chaperones

Molecular chaperones are essential proteostasis factors that assist proteins in obtaining their functional, three-dimensional structures. Upon synthesis, polypeptide chains can adopt various structural conformations before reaching the native state (Figure 2). While the native conformation of a protein primarily depends on the amino acid sequence, large proteins or protein complexes often require assistance in assembling correctly. Here, chaperone machineries stabilize the polypeptide chains, prevent unwanted interactions and aggregation, and help reach an energetically favorable conformation, while not being integrated into the

final structure (Balchin et al., 2016; Bukau et al., 2006; Dahiya and Buchner, 2019; Hartl et al., 2011; Hipp et al., 2019).

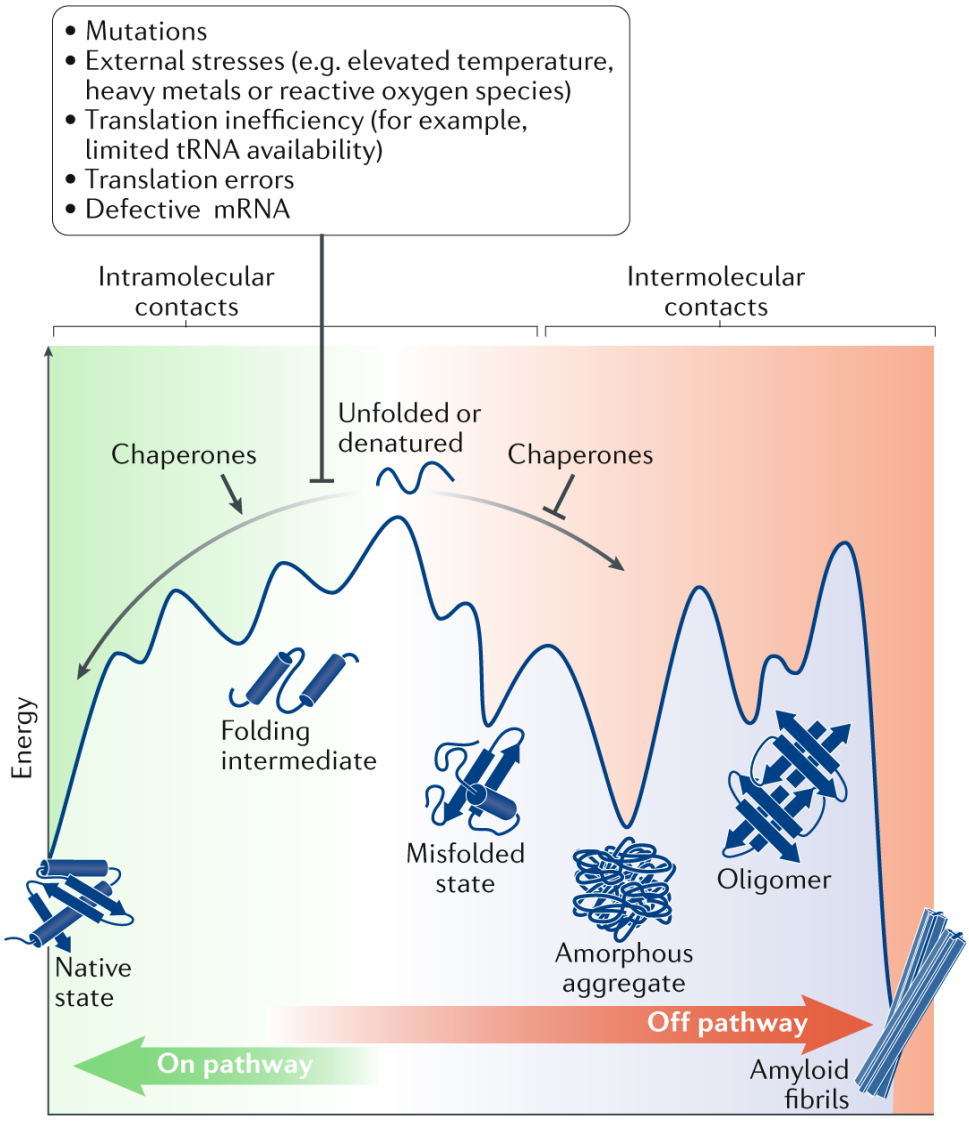

Figure 2 Protein folding regulation by molecular chaperones (cropped from Figure 1b in Hipp et al., 2019; reproduced with permission from Springer Nature; License Number 5718770857159).
To reach their thermodynamically stable native state, proteins must navigate a rugged thermodynamic energy landscape and form intramolecular contacts. During folding, proteins adopt diverse conformations, some resulting in misfolded states of low energy, effectively trapping the protein kinetically. Intermolecular contacts of proteins in theses misfolded states may lead to various aggregate species. Molecular chaperones support the on-pathway progression of folding and prevent off-pathway interactions. Various insults, e.g. mutations, stress, translation aberrations or mRNA defects, can shift the sensitive balance maintained by chaperones towards protein misfolding and aggregation.

Chaperones encompass several protein families classified by their molecular weight, including small heat shock proteins (sHSPs), HSP90, HSP70, HSP40 and HSP110 (HSP70 co-chaperones), HSP100 (AAA^+-ATPases), HSP60 (mitochondrial chaperonin), GimC/prefoldin and TRiC (Balchin et al., 2016; Hipp et al., 2019). Mechanistically, chaperones recognize exposed hydrophobic amino acid residues and promote folding through cycles of binding and release.

Foldases, adenosine triphosphate (ATP)-dependent chaperones like HSP90 and HSP70, thereby usually work in concert with co-chaperones that regulate ATPase function and nucleotide exchange, and mediate client specificity. Holdases, in contrast, are ATP-independent chaperones like the sHSPs and can form large heterogeneous oligomers that bind non-native states and buffer aggregation (Balchin et al., 2016; Dahiya and Buchner, 2019; de Graff et al., 2020; Hipp et al., 2019).

Particularly in specialized cell types, such as muscle cells, molecular chaperones are highly abundant and required for maintaining the functionality of the complex protein machineries in these cells (Carlisle et al., 2017; Kim et al., 2008; Smith et al., 2014; Wettstein et al., 2012). Accordingly, chaperone dysfunction is responsible for several hereditary disorders and muscular pathologies that will be introduced in Chapter 1.2.3 (Collier and Benesch, 2020; Henning and Brundel, 2017; Sarparanta et al., 2020; Unger et al., 2017; Weihl et al., 2018). Therefore, uncovering which and how tissue-specific chaperones prevent protein misfolding and aggregation during stress conditions will lead to a better understanding of these diseases.

1.1.2 E3 ubiquitin ligases and the UPS

The UPS is a vital cellular machinery for the targeted degradation of misfolded or excess proteins. It relies on the highly conserved 76-amino acid protein modifier ubiquitin (Ub), which is post-translationally attached to mark proteins for degradation by the 26S proteasome (Ciechanover, 2015; Pickart and Eddins, 2004; Rape, 2018).

Ubiquitin conjugation is orchestrated by three enzyme classes: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases (Figure 3). Canonically, these enzymes catalyze ATP-dependent reactions to couple the C-terminus of a Ub molecule to the ε-amino group of a lysine residue in the target protein (Hershko and Ciechanover, 1998; Komander and Rape, 2012; Müller et al., 2021b; Rape, 2018). With over 600 members in humans, E3 ligases represent the largest group of proteins within the UPS, providing substrate specificity for almost every protein in the cell (Balaji and Hoppe, 2020; Li et al., 2008; Yang et al., 2021; Zheng and Shabek, 2017). Based on their domain structure and ubiquitin-transferring mechanism, E3 ligases can be classified into Really Interesting New Gene (RING) finger, UFD2-box (U-box), Homologous to E6-associated protein C-Terminus (HECT), and RING Between RING (RBR) domain-containing E3 ligases. While RING and U-box E3 ligases act as scaffolds to facilitate the direct transfer of Ub from E2 enzymes to substrates, HECT and RBR E3 ligase active sites contain a cysteine residue that accepts Ub from an E2 enzyme followed by transfer onto the substrate (Komander and Rape, 2012; Pickart and Eddins, 2004; Rape, 2018; Sluimer and Distel, 2018). Commonly, E3 ligases attach several Ub molecules to their substrate and the

iterative ubiquitylation of Ub molecules at one of their seven lysine residues forms poly-ubiquitin chains, sometimes with the help of ubiquitin chain-elongation factors (E4; Hoppe et al., 2004; Koegl et al., 1999). These diverse linkages can serve either non-proteolytic functions like signaling complex assembly or subcellular localization (K63 linkages) or encourage proteasomal degradation (K48 linkages; Kwon and Ciechanover, 2017; Oh et al., 2018; Pohl and Dikic, 2019; Swatek and Komander, 2016; Yang et al., 2021).

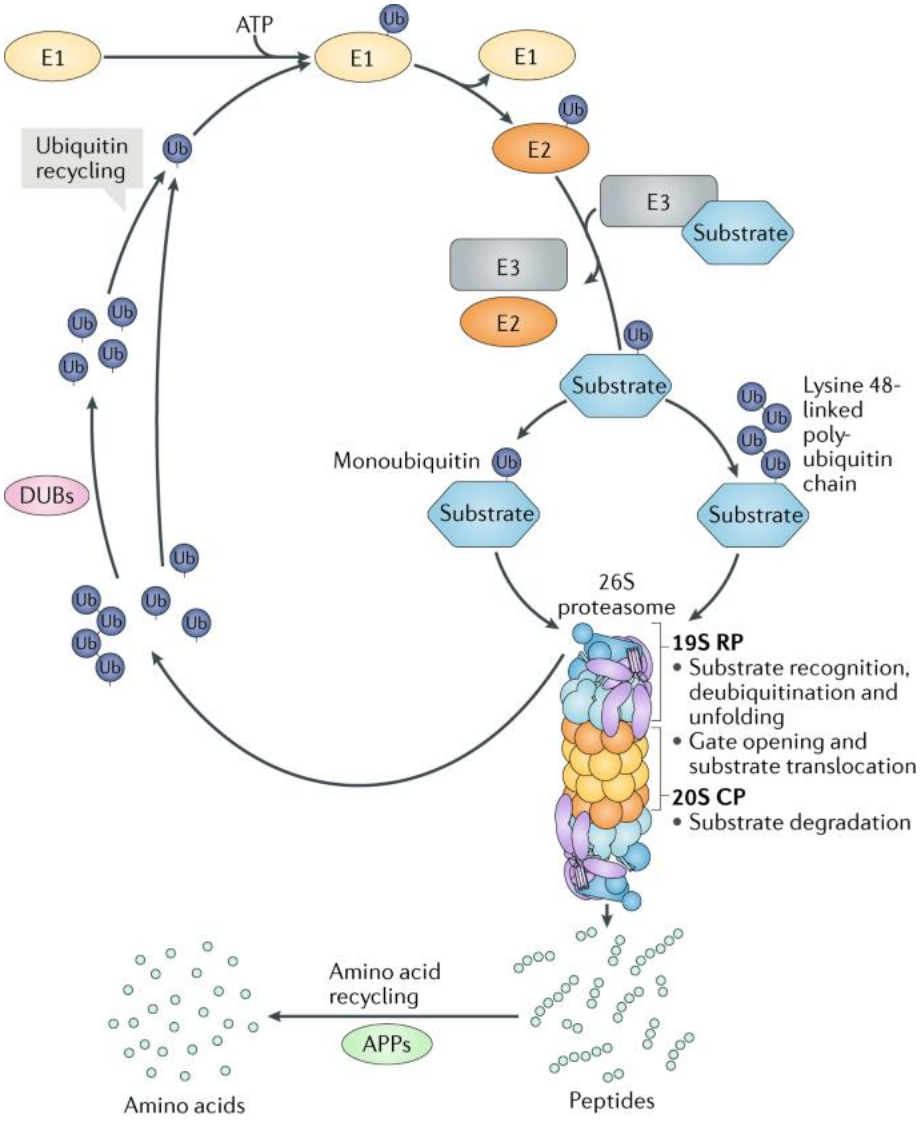

Figure 3 Ubiquitin conjugation and protein degradation via the UPS (Figure 1 in Rousseau and Bertolotti, 2018; reproduced with permission from Springer Nature; License Number 5718770496931).
First, using ATP, the ubiquitin-activating enzyme E1 forms an intermediate ubiquitin adenylate and links the C-terminal Gly residue of Ub to a Cys residue in a thioester bond. Next, activated Ub is transferred to an active site Cys residue of a ubiquitin-conjugating enzyme E2. In the last step of ubiquitin conjugation, an E3 ubiquitin ligase links Ub by its C-terminus to an ε-amino group of a Lys residue in the substrate in an amide isopeptide bond. Lys48-linked poly-ubiquitylated or in rare cases monoubiquitylated substrates can be degraded into small peptides by the 26S proteasome consisting of the 19S regulatory particle (RP) and the 20S core particle (CP) in a sequential cascade of recognition, deubiquitylation, unfolding, translocation, and degradation. The peptides resulting from substrate degradation are further metabolized by aminopeptidases (APPs) and Ub molecules that were removed prior to substrate degradation are recycled by deubiquitylating enzymes (DUBs).

The 26S proteasome, a multi-subunit complex in the cytoplasm and nucleus, degrades ubiquitin-marked proteins (Figure 3). The proteasome comprises a 19S regulatory cap and a 20S proteolytic core that sequentially recognize poly-ubiquitylated substrates, remove ubiquitin chains, unfold proteins, and catalyze proteolysis (Bard et al., 2018; Rousseau and Bertolotti, 2018; Vilchez et al., 2014). Cellular stress or chemical inhibitors such as bortezomib lead to the accumulation of aberrant proteins that can overload the proteasome and compromise proteasomal capacity (Dantuma and Lindsten, 2010; Pohl and Dikic, 2019; Qiu et al., 2022; Vilchez et al., 2014). To counteract this overload, protein synthesis is decreased and/or the abundance of PQC components is increased by multiple overlapping mechanisms (Pilla et al., 2017). For example, the Eukaryotic Initiation Factor 2α (eIF2α) that mediates the binding of initiator methionyl-tRNA to the ribosome is phosphorylated and thereby inactivated, resulting in translation attenuation (Sonenberg and Hinnebusch, 2009). Moreover, stress-responsive transcription factors such as Heat-Shock Factor 1 (HSF1) and Nuclear factor erythroid-derived 2–Related Factor 1 and 2 (NRF1 and NRF2) activate the expression of chaperone and proteasomal subunit genes, respectively (Dantuma and Lindsten, 2010; Lehrbach and Ruvkun, 2016; Pilla et al., 2017; Rousseau and Bertolotti, 2018).

Taken together, the proteostasis network is constantly challenged by stressful insults resulting in the accumulation of unfolded, misfolded, or damaged proteins; and UPS capacity therefore needs to be permanently adapted in order to maintain cellular and tissue functionality. Molecular chaperones and E3 ligases thereby cooperate closely to identify damaged proteins and remodel the cellular architecture (Carlisle et al., 2017; Kim et al., 2008; Pilla et al., 2017; Smith et al., 2014; Wettstein et al., 2012). Dissecting the close interplay of molecular chaperones, E3 ligases, and the proteasome will help in understanding how proteostasis is maintained, especially under stress conditions and in disease.

1.1.3 Protein triage

External or internal stresses as well as disease states can culminate in imbalances in proteostasis. The result is misfolding and aggregation of stress-sensitive protein components (Balch et al., 2008; Hipp et al., 2019; Pilla et al., 2017). Especially, when individual components of vital cellular machineries and the cytoarchitecture misfold, degradation of these particular damaged proteins needs to be precisely recognized and regulated for successful remodeling and sustained functionality of the cell (Carlisle et al., 2017; Johnson et al., 2007). However, how the proteostasis factors, chaperones and E3 ligases, recognize misfolded proteins and cooperatively decide on their fate has not been widely studied in multicellular organisms to date.

Three major models for this recognition process of damaged proteins termed "protein triage" (Figure 4) have been proposed from experimental data generated in yeast, bacteria, or *in vitro* (Carlisle et al., 2017): First, the "kinetic model" describes chaperones and E3 ligases as competing entities that bind to and act on damaged proteins at random, driven by interaction kinetics. Second, the "co-factor model" suggests that the binding of co-chaperones that either promote refolding or degradation determines the fate of the substrate protein. Third, the "degradation complex model" proposes that the additional binding of an E3 ligase turns a chaperone/co-chaperone/substrate complex into a degradation complex. Candidates for chaperones, co-chaperones, and E3 ligases that cooperate in protein triage include, for example, HSP90, HSP70, and their co-chaperones as well as the chaperone-interacting E3 ligase CHIP. Overall, the best model of protein triage is likely a combination of all three, yet confirmatory evidence from *in vivo* studies in multicellular organisms is necessary.

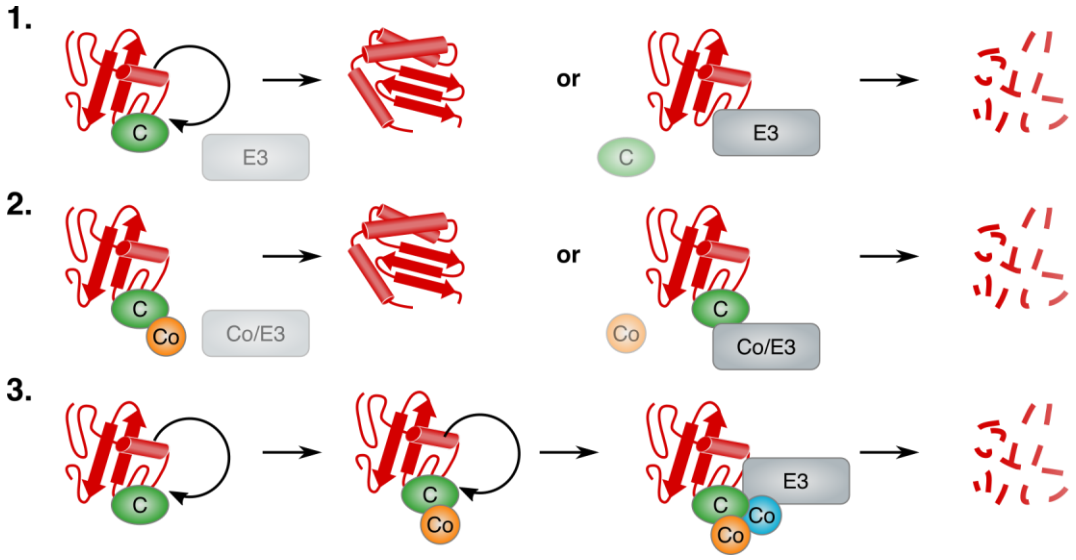

Figure 4 Models of chaperone and E3 ligase cooperation in protein triage (according to Carlisle et al., 2017; published under MDPI Open Access license).
1. In the "kinetic model" of protein triage, chaperones (C) either succeed or fail in refolding damaged proteins during multiple rounds of binding and release. Misfolded proteins may either rebind chaperones or interact with E3 ligases to be targeted for degradation. **2.** In the "co-factor model", chaperones bind damaged proteins and co-chaperones (Co) are recruited that either promote refolding or degradation of the substrate. **3.** In the "degradation complex model", chaperones bind damaged proteins and iteratively recruit co-factors that transform the chaperone complex into an E3 ligase complex targeting the substrate protein for degradation.

1.2 Striated Muscles and the Sarcomere

The striated muscles of the musculoskeletal system and the heart, also classified as skeletal and cardiac muscles, are highly active tissues under constant stress and use. While skeletal muscles consist of long, innervated, voluntary myofibers that exhibit fatigue, cardiac muscles comprise

individual, self-stimulating, non-fatiguing muscle cells that are in constant movement. In skeletal muscles, contractile cells are fused into multinucleated myofibers, whereas the mononucleated cardiomyocytes form a functional syncytium via connecting cell junctions at intercalated disks (Henning and Brundel, 2017; Mukund and Subramaniam, 2020).

Regardless of the muscle type, each muscle cell contains myofibrils, in which specialized structural and motor proteins are aligned in periodic arrays, the sarcomeres. In sarcomeres, myosin-containing thick filaments interdigitate with actin-containing thin filaments in a precise, semi-crystallin order (Clark et al., 2002; Geeves and Holmes, 2005; Mukund and Subramaniam, 2020; Sparrow and Schöck, 2009; Sweeney and Hammers, 2018). The striated appearance of skeletal and cardiac muscle results from alternating A-bands (thick filaments) and I-bands (thin filaments) in the sarcomeres, comprised of myosin motor proteins that are attached to M-lines in the center of A-bands and actin filaments that are attached to Z-disks in the center of I-bands (Figure 5a). In addition, a plethora of structural and regulatory proteins in the sarcomere cooperate to anchor the filaments (e.g. α-actinin, desmin, filamin C/γ-filamin), confer structure and elasticity (e.g., titin, nebulin), and regulate actin-myosin cross-bridge cycling (e.g. tropomyosin, troponins) (Figure 5b).

As a result, the formation of actin-myosin cross-bridges combined with filament gliding allows the generation of a directed force and thereby muscle contraction. Briefly, in the presence of ATP, myosin is energized by ATP hydrolysis to adenosine diphosphate (ADP) and inorganic phosphate. Upon arrival of an action potential, influx of calcium ions (Ca^{2+}) into the muscle cell changes the conformation of troponins, which releases tropomyosin from its blocking position on the actin thin filament, allowing the actin-myosin cross-bridge to form. Release of inorganic phosphate from myosin initiates the power stroke, in which rotation of the myosin head forces the actin filament past it, effectively shortening the sarcomere. The exchange of ADP with ATP releases myosin from actin to reinitiate the cycle (Mukund and Subramaniam, 2020; Sweeney and Hammers, 2018).

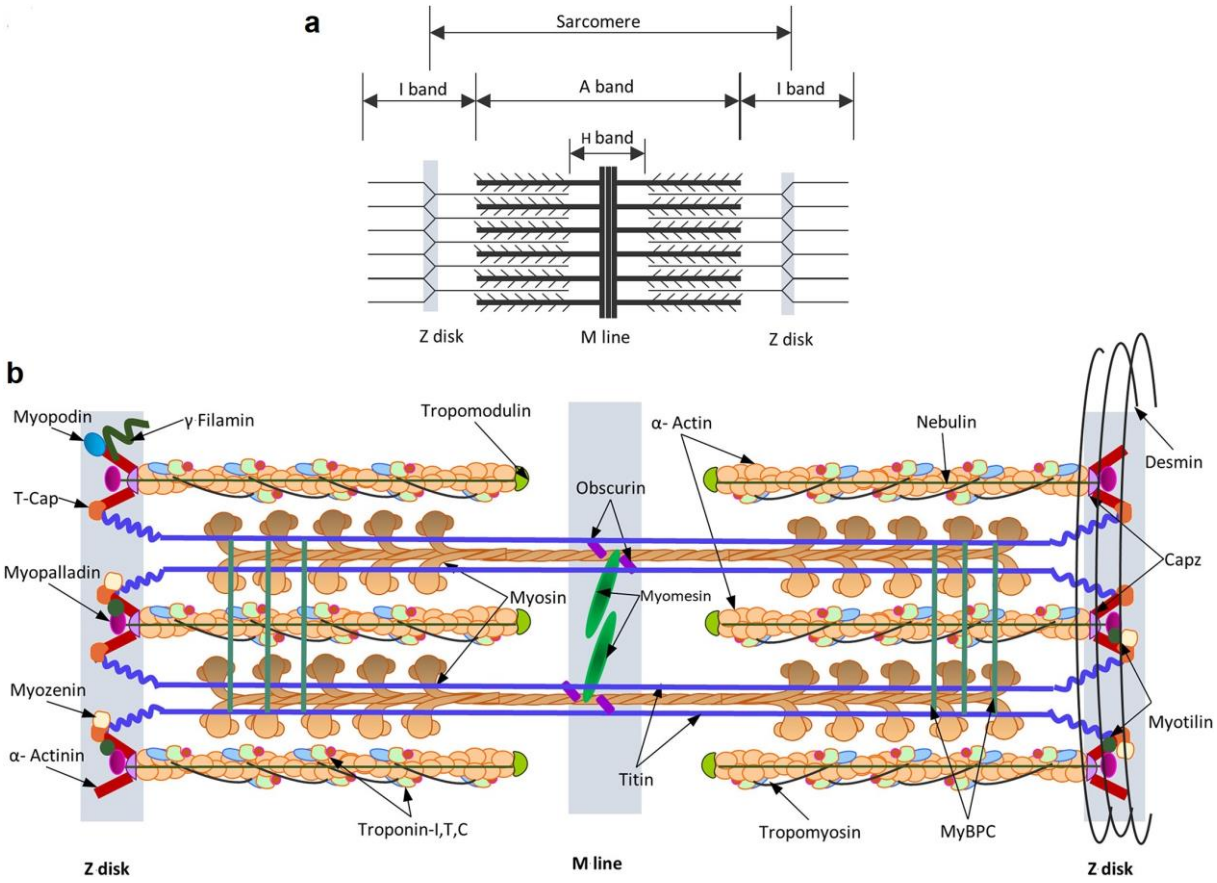

Figure 5 The muscle sarcomere (Figure 8 in Mukund and Subramaniam, 2020; licensed under CC BY 4.0; style of figure panel labels changed from original).
a The sarcomere is defined by the repetitive arrangement of myosin-containing thick filaments attached to M-lines in the A-bands and actin-containing thin filaments attached to Z-disks in the I-bands. **b** In molecular detail, a plethora of structural and regulatory proteins comprise the sarcomere and cooperate to allow actin-myosin cross-bridge cycling, filament gliding, and muscle contraction. The tropomyosin/troponin complex regulates actin-myosin binding in response to elevated cytosolic Ca^{2+} levels. Myosin heads interlock and successively glide along actin filaments, generating force and sarcomeric contraction. The giant protein titin is the backbone of the sarcomere and confers elasticity. Protein complexes at the filament anchor points maintain structural integrity, such as desmin intermediate filaments in the Z-disk that form transverse links between adjacent sarcomeres and myofibrils.

1.2.1 Myofibrillogenesis and proteostasis in the muscle

Throughout a lifetime, the muscle must continuously adapt to metabolic challenges, mechanical forces, or injury. For the regeneration of whole myofibers, skeletal muscle tissue retains unipotent stem cells, called satellite cells, that – after being activated from quiescence – proliferate and differentiate to replace damaged myofibers. In contrast, cardiomyocytes are terminally differentiated cells that are unable to clear aberrant proteins by cell division or apoptosis, and must cope with misfolded proteins internally (Henning and Brundel, 2017; Mukund and Subramaniam, 2020; Myhre and Pilgrim, 2012).

Both during differentiation and regeneration of skeletal and cardiac muscle cells, the assembly of functional myofibrils, the process of myofibrillogenesis, involves the correct folding,

the stoichiometric assembly, and the coordinated interaction of filament systems (Carlisle et al., 2017; Myhre and Pilgrim, 2012; Sanger et al., 2006). According to the "premyofibril model" of myofibrillogenesis, sarcomere assembly begins with integrins assembling attachment complexes at the muscle cell membrane that are called protocostameres. Recruitment of α-actinin and titin is followed by actin polymerisation into stress-fiber-like filaments. Non-muscle myosin II activity thereby aids in aligning the thin actin filaments to form mini-sarcomeres. Lastly, non-muscle myosin II is replaced by striated muscle myosin II and Z-disks develop, completing the maturation of the sarcomere. In this process, muscle cells conceivably rely on the proteostasis network and PQC factors to establish and maintain sarcomere integrity (Carlisle et al., 2017; Höhfeld et al., 2021; Kim et al., 2008; Myhre and Pilgrim, 2012; Smith et al., 2014; Sparrow and Schöck, 2009). Therefore, proteostasis mechanisms are essential for proper muscle development and function, especially during muscle repair and remodeling in response to stressful challenges like intense contractions and myofiber injury or in disease.

To maintain the functionality and optimal mechanical and metabolic output of the muscle in response to cellular stress and pathological states, the balance between protein synthesis and degradation must be precisely regulated to prevent the accumulation of aberrant proteins (Bodine and Baehr, 2014; Carlisle et al., 2017; Höhfeld et al., 2021; Phillips et al., 1997; Sartori et al., 2021). In otherwise healthy individuals, adequately intense muscle contraction and exercise result in protein synthesis dominating over degradation and enhanced muscle growth with an increased need for myofibrillogenesis. Conversely, upon inactivity, denervation, or disease, protein degradation is enhanced, resulting in a loss of muscle mass and atrophy. Upon intense contractions and mechanical stress, this balance is more intricately regulated. When individual protein components of the muscular cytoarchitecture misfold, these particular damaged proteins need to be precisely recognized and their targeted degradation needs to be tightly regulated for the successful remodeling and sustained functionality of the muscle during protein integration and exchange (Carlisle et al., 2017; Johnson et al., 2007).

1.2.2 Mechanical stress in muscle

Mechanical stress occurs in all muscles, both in those that are particularly stiff, e.g. skeletal muscles and muscles of the spinal column, and those that are continually moving, e.g. the cardiac muscle and the diaphragm. During muscle contraction, both compression and elongation of the tissue elicit pressure and stretch stress, respectively. The mechanical forces generated by myosin motor proteins are thereby transferred to other components of the sarcomere and intra-tissue contacts, which results in disruption of the sarcomeric structure

(Collier and Benesch, 2020; Höhfeld et al., 2021; Morita et al., 2013; Paulsen et al., 2009; Willis et al., 2008).

On the molecular level, the myosin motor domain itself undergoes significant conformational changes during contraction and is therefore prone to force- and heat-induced unfolding and misfolding (Bujalowski and Oberhauser, 2013; Johnson et al., 2007; Kaiser et al., 2012; Melkani et al., 2010; Nielsen, 2003; Rahmani et al., 2021; Reconditi et al., 2021). Other components of the sarcomere, especially immunoglobulin (Ig) domain containing proteins, are similarly unfolded in response to mechanical forces. To confer elasticity and generate passive force upon contraction, Ig domains in the sarcomere-spanning protein titin are elongated and unfolded, and intrinsically disordered regions are extended, which likely increases titin's vulnerability to ubiquitylation and degradation (Linke and Hamdani, 2014; Mártonfalvi et al., 2013; Müller et al., 2021a). Similarly, Ig domain unfolding of the actin-crosslinking protein filamin C exposes previously buried bindings sites in Ig domain 21 and allows for interactions that initiate signaling events and the degradation of unfolded proteins (Arndt et al., 2010; Klimek et al., 2017; Rognoni et al., 2014).

Accordingly, upon mechanical stress, chaperone and degradation machineries are engaged to recognize and remove damaged proteins. Here, interactions of general and specialized chaperones and sHSPs with cytoskeletal and sarcomeric proteins are intensified and influence muscle contraction and elasticity (Carlisle et al., 2017; Collier and Benesch, 2020; Höhfeld et al., 2021; Paulsen et al., 2009; Smith et al., 2014; Willis et al., 2008). For example, HSP90 and UNC-45 as myosin-directed co-chaperone were shown to maintain sarcomere integrity and to rapidly localize to the myosin-containing A-band upon myofiber damage in zebrafish (Etard et al., 2008; Lee et al., 2014). In mammalian muscle, the sHSP HSPB5/αB-crystallin is phosphorylated at serine-59 upon intense exercise increasing its chaperoning activity (Jacko et al., 2020; Peschek et al., 2013), and, together with HSPB1/HSP27, associates with mechano-sensitive elements of titin upon sarcomere stretch to prevent stiffening and aggregation (Kötter et al., 2014). Mechanical unfolding of filamin C recruits the co-chaperone BAG3 that coordinates the holding function of the sHSPs HSPB1/HSP27, HSPB5/αB-crystallin, HSPB6/HSP20, and HSPB8/HSP22 with the chaperoning function of HSP70 proteins (Rauch et al., 2017). Subsequent ubiquitylation of the client proteins by the HSP70-associated E3 ligase CHIP and recognition by the autophagic ubiquitin receptor SQSTM1/p62 target unfolded filamin C for degradation in the process of chaperone-assisted selective autophagy (CASA; Arndt et al., 2010; Gamerdinger et al., 2009; Haidar et al., 2019).

Interestingly, following intense mechanical stress, muscle protein degradation is increased and the expression of the E3 ligases Muscle RING Finger 1 (MuRF1)/TRIM63 and Muscle Atrophy F-box (MAFbx)/atrogin-1 is activated pointing to a role for these E3 ligases in

the degradation of misfolded muscle proteins and in tissue remodeling upon mechanical stress (Louis et al., 2007; Murton et al., 2008; Phillips et al., 1997; Yang et al., 2006). However, how these E3 ligases might recognize misfolded sarcomeric proteins remains elusive.

1.2.3 Muscular pathologies (myopathies) and atrophy

Muscular disease can occur (1) as a result of inactivity as is the case with critical illness myopathy (CIM) upon extended intensive care stays; (2) during aging-associated diseases like cardiovascular disease, sarcopenia, and cancer, or (3) due to inherited genetic traits in the case of neuromuscular disorders and muscular dystrophies (Henning and Brundel, 2017; Larsson and Friedrich, 2016; Moreira-Pais et al., 2021; Rinaldi et al., 2016; Weihl et al., 2018). A hallmark of these pathological conditions is an imbalance in protein turnover in the muscle, with increased protein degradation due to reduced nerve input, inflammation, or protein misfolding among other causes, that leads to muscle wasting and atrophy.

In particular, a distinct subset of hereditary skeletal and cardiac myopathies, called chaperonopathies, arise from pathogenic variants in genes encoding chaperones and co-chaperones. The resulting chaperone dysfunction and client protein misfolding are accompanied by muscle weakness and muscular dystrophy (Collier and Benesch, 2020; Henning and Brundel, 2017; Lupo et al., 2016; Smith et al., 2014; Weihl et al., 2018). For example, a missense mutation in the sHSP HSPB5/αB-crystallin affects the folding of the muscle intermediate filament desmin and manifests clinically as αB-crystallinopathy (Vicart et al., 1998). Pathogenic variants in the HSP70 co-chaperone BAG3 impair protein clearance via CASA and manifest clinically as a progressive myofibrillar myopathy with cardiac and peripheral nerve pathologies (Selcen et al., 2009). Variants in the HSP40 family member DNAJB6 cause limb-girdle muscular dystrophy D1 (LGMD1D), which is characterized by progressive muscle weakness and accumulation of myofibrillar protein aggregates containing the Z-disk-associated intermediate filament desmin (Harms et al., 2012; Sarparanta et al., 2012). Chaperonopathies also include hereditary motor neuropathies with or without muscle involvement caused by gene defects in *HSPB1*, *HSPB3*, *HSPB8*, and *DNAJB2* (Lupo et al., 2016).

Enhanced catabolism and degradation of cytoskeletal and sarcomeric proteins in the muscle mark both genetic muscular pathologies and injury- or inactivity-induced atrophy conditions. Several E3 ubiquitin ligases have been implicated in PQC and degradation of myosin and other sarcomeric proteins: the tripartite motif (TRIM) proteins TRIM63/MuRF1, TRIM54/MuRF3, and TRIM32, as well as the cyclin F domain (F-box) proteins atrogin-1/MAFbx, MUSA1/Fbxo30, and Fbxo21, among others (Bodine and Baehr, 2014; Haberecht-Müller et al., 2021; Jeong et al., 2023; Sartori et al., 2021; Tocchini and Ciosk, 2015). MuRF1/TRIM63 and

MAFbx/atrogin-1 were the first E3 ligases found to be transcriptionally upregulated in skeletal muscle under atrophy-inducing conditions (Bodine and Baehr, 2014; Bodine et al., 2001) that, when knocked down, prevent the loss of thick filament proteins (Clarke et al., 2007; Fielitz et al., 2007). Mechanistically, these E3 ligases were shown to bind and ubiquitylate myosin and other myofibrillar proteins (Bodine et al., 2001; Clarke et al., 2007; Peris-Moreno et al., 2020). The E3 ligase TRIM32 is expressed in both brain and skeletal muscle and ubiquitylates the thin filament protein actin (Jeong et al., 2023; Kudryashova et al., 2005; Nicklas et al., 2012; Tocchini and Ciosk, 2015) as well as desmin filaments in the Z-disk (Aweida and Cohen, 2021, 2022; Cohen et al., 2012). Pathogenic variants in *TRIM32* cause limb girdle muscular dystrophy R8 (LGMD2H) (Jeong et al., 2023; Kudryashova et al., 2005; Nicklas et al., 2012; Tocchini and Ciosk, 2015), but unlike MuRF1 and atrogin-1, TRIM32 appears to play a more important role in skeletal muscle regrowth during recovery from atrophy than during atrophy itself (Kudryashova et al., 2012). Prior to ubiquitylation and degradation, the calcium-dependent, non-lysosomal cysteine protease calpain-1 is reportedly required to release sarcomeric proteins from the assembled myofibril and calpain activity is upregulated in muscle fiber atrophy (Potz et al., 2016; Willis et al., 2008).

Together, molecular chaperones, E3 ligases, proteases, and the degradation machineries UPS and autophagy collaborate to mediate protein turnover and tissue remodeling in the muscle upon stressful challenges. Accordingly, dysregulation of this delicate balance of collaboration or inherited pathogenic variants affecting proteostasis components can result in muscular diseases, weakness, and atrophy, and prevent the muscle from adequately performing its contractile function.

1.3 THE MYOSIN CHAPERONE UNC-45

The major motor proteins in the muscle, conventional type II myosins, are composed of two myosin heavy chains, each containing an identical myosin head domain, as well as four myosin light chains, one essential and one regulatory light chain per myosin head (Chantler et al., 2010; Lee et al., 2019; Thompson and Langford, 2002). Folding of the myosin head domain into a functional motor requires precise spatiotemporal control and is mediated by conserved UCS (UNC-45/CRO1/She4p) domain-containing proteins (Hellerschmied and Clausen, 2014; Hutagalung et al., 2002; Lee et al., 2014). As a founding member of the UCS family, UNC-45 was first identified in *C. elegans*, where temperature-sensitive missense mutations result in paralysis or an *uncoordinated* phenotype at the restrictive temperature above 22°C, along with disorganized myofilaments, reduced levels of the muscle myosin MHC B/UNC-54, and atrophy

(Barral et al., 1998; Epstein and Thomson, 1974; Moncrief et al., 2021). UNC-45 is highly conserved and several point mutations have been associated with impaired skeletal and cardiac muscle function in *Drosophila melanogaster*, *Xenopus tropicalis*, zebrafish, and mouse (Chen et al., 2012; Geach and Zimmerman, 2010; Lee et al., 2011; Melkani et al., 2011; Price et al., 2002; Wohlgemuth et al., 2007).

During evolution from invertebrates to vertebrates, gene duplication and functional divergence resulted in two UNC-45 isoforms in zebrafish and higher vertebrates (Figure 6a; Comyn and Pilgrim, 2012; Lee et al., 2014). The "general cell" isoform UNC-45A is expressed in proliferating cells, e.g. myoblast, epithelial, and cancer cells, and plays a role in cytokinesis by not only regulating the actomyosin cytoskeleton but also acting as a microtubule-destabilizing protein (Bazzaro et al., 2007; Clemente et al., 2021; Guo et al., 2011; Habicht et al., 2019, 2020; Mooneyham et al., 2019; Price et al., 2002). In contrast, the "striated muscle" isoform UNC-45B is expressed upon myogenic differentiation in skeletal and cardiac muscle and is crucial for myofibrillogenesis and contractility (Chen et al., 2012; Comyn and Pilgrim, 2012; Lu et al., 2023; Price et al., 2002; Wohlgemuth et al., 2007).

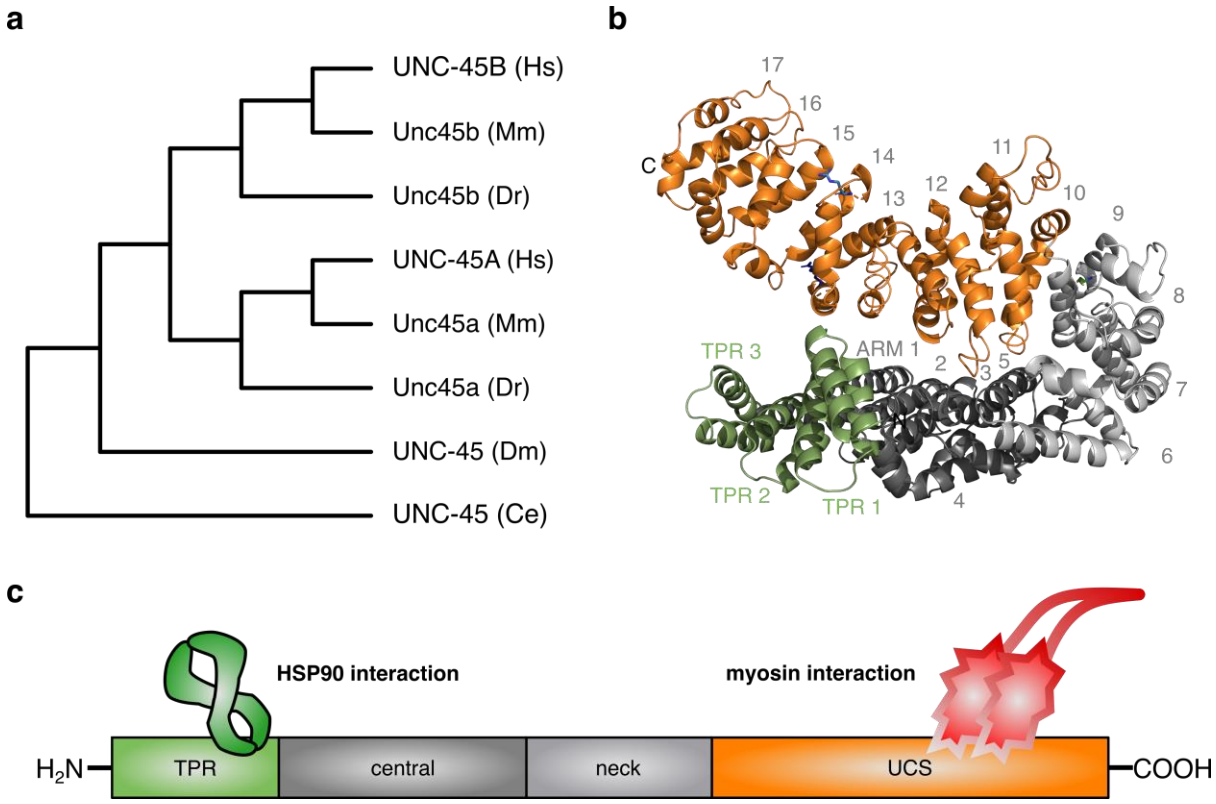

Figure 6 UNC-45 conservation and domain structure (according to Figure 1 in Comyn and Pilgrim, 2012; licensed under CC BY 4.0; and modified from Figures 1A and 5A in publication A, Donkervoort, Kutzner et al., 2020; style and colors modified from original).
a During evolution from *C. elegans* (Ce) to *Drosophila melanogaster* (Dm), zebrafish/*Danio rerio* (Dr), mouse/*Mus musculus* (Mm) and human/*Homo sapiens* (Hs), a gene duplication of the *UNC-45* gene occurred. **b** The three-dimensional protein structure of the human UNC-45B was modeled after the *C. elegans* crystal structure of UNC-45 (PDB: 412z) and is shown with numbered Tetratricopeptide (TPR) and

Armadillo (ARM) repeats. **c** The linear UNC-45 domain structure is shown with indicated binding sites for HSP90 and the myosin head domain.

The native UNC-45 protein has a size of approximately 107 kDa and contains four domains (Figure 6b-c; Gazda et al., 2013; Hellerschmied et al., 2019): an N-terminal Tetratricopeptide repeat (TPR) domain (TPR repeats 1–3), a central domain (Armadillo (ARM) repeats 4–5), a neck domain (ARM repeats 6–9), and a C-terminal UCS domain (ARM repeats 10–17). The N-terminal TPR domain is important for binding to the general chaperone HSP90, while the C-terminal UCS domain associates with the myosin head, together facilitating myosin folding and myofibrillogenesis (Barral et al., 2002).

Biochemical experiments have shown that UNC-45 exerts chaperone activity onto the myosin head by preventing its aggregation and facilitating refolding after mechanical unfolding (Barral et al., 2002; Kaiser et al., 2012; Melkani et al., 2010). In cooperation with the general chaperones HSP90 and HSP70, UNC-45 furthermore acts as a co-chaperone and enables *de novo* folding of the myosin motor domain (Liu et al., 2008).

1.3.1 UNC-45 role in myofibrillogenesis

In 2013, structural and biochemical analyses combined with complementary *C. elegans* rescue experiments discovered the mechanism of UNC-45-mediated myofilament assembly (Gazda et al., 2013). *In vitro*, the interaction of the TPR domain of one UNC-45 molecule with the neck domain of an adjacent UNC-45 molecule forms short UNC-45 filaments with the myosin-binding UCS domains protruding away from the filament axis. *In vivo*, mutations in this filament interface exert dominant negative effects on myofilament assembly and function. Together, the linear UNC-45 filament serves as a multisite-docking platform that regulates the precise cooperation with the general chaperones HSP90 and HSP70 to fold and incorporate myosin heads into the growing thick filament. The UCS domains protruding repetitively from the linear UNC-45 filament thereby serve as molecular ruler that defines the periodicity of staggered myosin motor domains (Gazda et al., 2013; Hellerschmied and Clausen, 2014; Pokrzywa and Hoppe, 2013). Further screens in *C. elegans* found that the co-chaperones Hop/Sti1, Aha1, and p23, which regulate the ATP-dependent client binding cycle of HSP90 and epistatically interact with UNC-45, are also involved in myofilament assembly (Frumkin et al., 2014). Thus, the myosin-directed chaperone UNC-45 provides substrate specificity for general chaperones during late stages of myofibrillogenesis (Figure 7).

It has been proposed that UNC-45 also plays a role in early stages of myofibrillogenesis (Myhre and Pilgrim, 2012). The "premyofibril model" describes the assembly of mini-sarcomeres composed of actin stress-fiber-like structures and non-muscle myosin II prior to

replacement of non-muscle myosin II by striated muscle myosin II (see Chapter 1.2). Non-muscle type II myosins, the force generating component of the actomysion cytoskeleton, are present in all eukaryotic cells and required for cell adhesion and migration. Interestingly, the contractile function of non-muscle myosin II was shown to depend on UNC-45 during cytokinesis of the early embryo in *C. elegans* and *D. melanogaster* and likely also in higher vertebrates (Kachur et al., 2004; Lee et al., 2011). Indeed, cardiac myofibrillogenesis has recently been reported to be mediated by UNC-45B already during protocostameres formation through interactions with the integrin-binding protein kindlin-2, non-muscle myosin II, and muscle myosin II (Lu et al., 2023). In addition, in skeletal muscle, UNC-45 is predicted to also influence myoblast aggregation and myotube fusion through the modulation of non-muscle myosin II motor function (Myhre and Pilgrim, 2012).

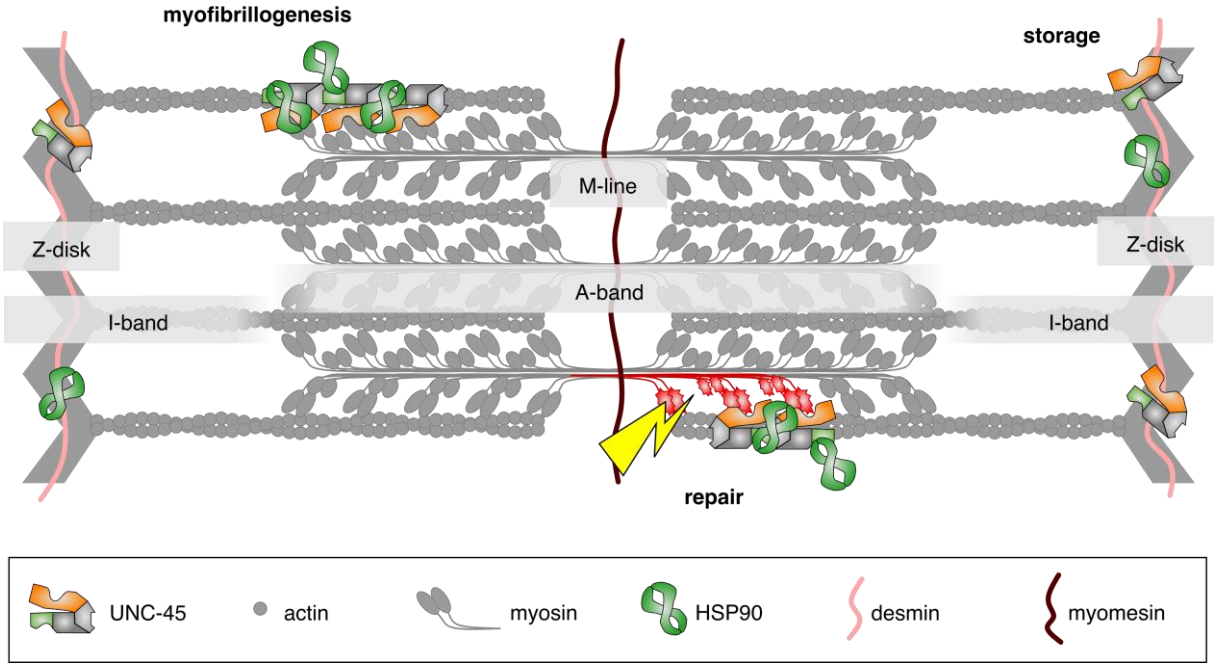

Figure 7 UNC-45 functions in the muscle sarcomere (modified from Figure 1B in publication **A**, Donkervoort, Kutzner et al., 2020; style and colors modified from original).
During myofibrillogenesis, UNC-45 forms short linear filaments that collaborate with HSP90 and its co-chaperones to fold and incorporate myosin into the thick filament. In *D. melanogaster*, zebrafish and higher vertebrates, UNC-45 is stored at the Z-disk. Upon myofiber damage in zebrafish (yellow lightning), Unc45b shuttles to the A-band to help refold damaged myosin (jagged red shapes) and repair the myofilament (Etard et al., 2008).

1.3.2 UNC-45 role in muscle stress response

Apart from its role in myofibrillogenesis, UNC-45 appears to also be required for sarcomere maintenance during adulthood and upon stress in the muscle (Figure 7). In adult *C. elegans* muscle, UNC-45 was observed to remain stably localized to myosin in mature myofilaments (Ao and Pilgrim, 2000; Gaiser et al., 2011). In *D. melanogaster*, muscle-expressed Unc45b was

shown to localize to the Z-disk and, in zebrafish, to shuttle from there to the myofilaments upon myofiber damage induced by cell membrane wounding, cold shock, or heat (Etard et al., 2008; Lee et al., 2011). In humans, the protein levels of cardiac UNC-45B are increased upon ischemic heart disease and heart failure, possibly to prevent myosin unfolding or to enable repair processes and incorporate newly synthesized myosin into the myofibrillar apparatus of damaged cardiomyocytes (Stanley et al., 2007).

Interestingly, *C. elegans* worms overexpressing UNC-45 display reduced myosin protein levels and diminished motility, which can both be partially restored by inhibiting the proteasome, indicating that UNC-45 can promote the degradation of misfolded or non-functional myosin by the UPS (Kachur and Pilgrim, 2008; Landsverk et al., 2007). However, how UNC-45 achieves myosin refolding and repair upon mechanical stress and what factors aid UNC-45 in degrading and exchanging misfolded myosin molecules remains elusive.

1.3.3 UNC-45 regulation

According to its central role as myosin-directed chaperone, UNC-45 protein levels need to be precisely regulated, since both UNC-45 deficiency and UNC-45 excess can interfere with myosin assembly (Kachur and Pilgrim, 2008; Landsverk et al., 2007). In *C. elegans*, UNC-45 is mainly expressed during larval development when *de novo* myofilament assembly occurs (Hellerschmied et al., 2018; Janiesch et al., 2007). Similarly, in C2C12 murine myoblasts and human embryo stem cell-derived cardiomyocytes, *UNC45B* expression is upregulated during myogenesis (Lu et al., 2023; Price et al., 2002).

UNC-45 protein degradation is mediated by the UPS coordinating myosin folding and assembly in both *C. elegans* and human myoblasts (Hoppe et al., 2004; Janiesch et al., 2007; Kachur and Pilgrim, 2008). *In vitro* ubiquitylation assays showed that the chaperone-associated E3 ligases CHN-1/CHIP and UFD-2 can individually ubiquitylate UNC-45 to a limited extent (Hellerschmied et al., 2018; Hoppe et al., 2004; Müller et al., 2021b). When incubated together, the two E3 ligases can however cooperate to form an E3/E4 ligase complex and poly-ubiquitylate UNC-45 (Hoppe et al., 2004; Koegl et al., 1999). The E3 ligases further collaborate with the ubiquitin-selective segregase CDC-48/p97/VCP to target UNC-45 for proteasomal degradation (Janiesch et al., 2007). Accordingly, mutations in the *chn-1*, *ufd-2*, or *cdc-48* genes can partially rescue the diminished motility of *unc-45* mutant worms *in vivo* (Hellerschmied et al., 2018; Janiesch et al., 2007). Conclusively, defective degradation of UNC-45B in patients with *VCP*-related inclusion-body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD) is linked to disorganized myofibrils and impaired sarcomeric

function (Janiesch et al., 2007). However, whether gene defects in *UNC45B* itself disturb muscle proteostasis and result in muscle pathologies has remained unknown until now.

1.4 THE BODY WALL MUSCLE IN C. *ELEGANS* AS A MODEL FOR MAMMALIAN STRIATED MUSCLE

For decades, the invertebrate nematode *C. elegans* has been used as a model organism to study the muscle sarcomere due to the high conservation of sarcomeric structures and protein complexes (Christian and Benian, 2020; Epstein and Benian, 2012). The major striated muscle in *C. elegans*, the body wall muscle, resembles both mammalian skeletal and cardiac muscle in that it is innervated and voluntary, but organized as individual, post-mitotic cells. The body wall muscle consists of 95 spindle-shaped mononuclear cells arranged in interlocking pairs in 4 opposing quadrants, whose alternating contraction and relaxation enables animal locomotion (Benian and Epstein, 2011; Gieseler et al., 2017). The striation in *C. elegans* muscle appear slightly oblique at an angle of 5.9° compared to the longitudinal axis of the muscle cell (Gaiser et al., 2011; Mackenzie and Epstein, 1980). In a body wall muscle cell, the sarcomeric lattice is restricted to a 1.5 μm zone at the outer side of the cell, where it attaches to the membrane and extracellular matrix via integrin adhesion sites in M-lines and in Z-disk-like "dense bodies" (Cox and Hardin, 2004). From there, the force of muscle contraction is transmitted via the hypodermis and the cuticle to the outside, allowing movement of the whole animal.

So far, almost 200 sarcomeric and other muscle proteins have been found to be conserved between *C. elegans* and human (Benian and Epstein, 2011). Many genes/proteins that result in various types of myopathies when mutated or that play a role in other muscular diseases and conditions have orthologues in *C. elegans* and are expressed in the body wall muscle. Notably, the first complete sequence of a myosin heavy chain from any organism was reported in *C. elegans* for the type II myosin heavy chain B (MHC B/UNC-54), which explained the parallel assembly of myosin rods and accurately predicted the conserved repetitive pattern of projecting myosin heads in the thick filament (McLachlan and Karn, 1982). In addition, the sole myosin-specific chaperone in striated muscle, UNC-45, was first discovered in a mutagenesis screen in *C. elegans* (Epstein and Thomson, 1974). Apart from the thick and thin filaments, the M-lines and dense bodies, the analogous membrane-extracellular matrix attachment structures in nematodes, also resemble costameres of vertebrate striated muscle and focal adhesions in non-muscle cells (Qadota and Benian, 2010; Samarel, 2005).

Therefore, significant genetic homologies and anatomic similarities in cytoskeletal structures between *C. elegans* and human allow the study of evolutionarily conserved

mechanisms of muscle function and disease in the nematode and facilitate the transferability of the findings (Benian and Epstein, 2011; Epstein and Benian, 2012; Henning and Brundel, 2017; Kim et al., 2008).

1.5 OBJECTIVES OF THIS THESIS

To date, proteostasis mechanisms responding to heat, oxidative, and general proteotoxic stress have been researched extensively. However, how the proteostasis network specifically in the muscle is rewired remains incompletely understood. In particular, how is PQC, refolding, and degradation of the muscle motor protein myosin regulated under mechanical stress? What role does the myosin-directed chaperone UNC-45 and associated proteostasis factors play in myosin folding, degradation, and exchange upon mechanical damage? What impact does UNC-45 have in human muscular disease? Therefore, to better understand how the proteostasis mechanisms contribute to the assembly, maintenance, and repair of myofilaments upon mechanical stress, I aimed to deeper characterize the role of the myosin chaperone UNC-45 and its interaction network.

Genetic experiments in the multicellular invertebrate *C. elegans* have already decisively shaped the functional understanding of protein components in the muscle, their organization and assembly, however, until now, a reliable *C. elegans in vivo* experimental model to systematically investigate molecular responses to mechanical stress in muscle is lacking. For this purpose, I developed the transgenic mechanical stress model OptIMMuS to identify and characterize new proteostasis regulators in the muscle that are critical under mechanical stress conditions and in disease.

Together, this thesis and the enclosed publications aim to address the following objectives:

(1) Determine the conserved role of UNC-45 in human muscular disease

(2) Explore the influence of UNC-45 dysfunction on muscle proteostasis

(3) Investigate the *in vivo* UNC-45 interaction network under mechanical stress conditions

2 RESULTS

2.1 PUBLICATION A: PATHOGENIC VARIANTS IN THE MYOSIN CHAPERONE UNC-45B CAUSE PROGRESSIVE MYOPATHY WITH ECCENTRIC CORES

2.1.1 Summary publication A

The myosin-directed chaperone UNC-45B is conserved from *C. elegans* to humans and essential for sarcomeric organization and muscle function. Yet, the pathological impact of UNC-45B in human muscle disease remained elusive. In this study, we reported ten individuals with four different bi-allelic variants in the *UNC45B* gene who present with childhood-onset progressive muscle weakness characterized by myofibrillar disarray and eccentric cores (Figure 8a-c). A common *UNC45B* variant was identified to act as a complex hypomorph splice variant and result in reduced UNC-45B protein levels. Immunofluorescence studies further demonstrated abnormal localization of mutant UNC-45B away from the A-band towards the Z-disk of the sarcomere. Structural modeling of UNC-45B domains affected by missense variants indicated potential disruption of myosin binding and protein stability. Biochemical analyses of purified UNC-45B mutant proteins showed changes in folding and solubility, indicating increased aggregation propensity. To determine the conserved role of pathogenic UNC-45 variants and investigate the physiological relevance of the clinical and biochemical observations, functional rescue experiments were performed by transgenic expression of UNC-45 missense variants in *C. elegans*. Three out of four UNC-45 variants failed to rescue the movement defect and muscle phenotype of the conditional loss-of-function *m94* allele, and one UNC-45 variant showed reduced myosin binding in co-immunoprecipitation experiments (Figure 8d-e). Together, these data demonstrated that UNC-45B impairment manifests as a chaperonopathy with progressive muscle pathology, underlining the conserved role of UNC-45B in myofibrillar organization and human muscular disease.

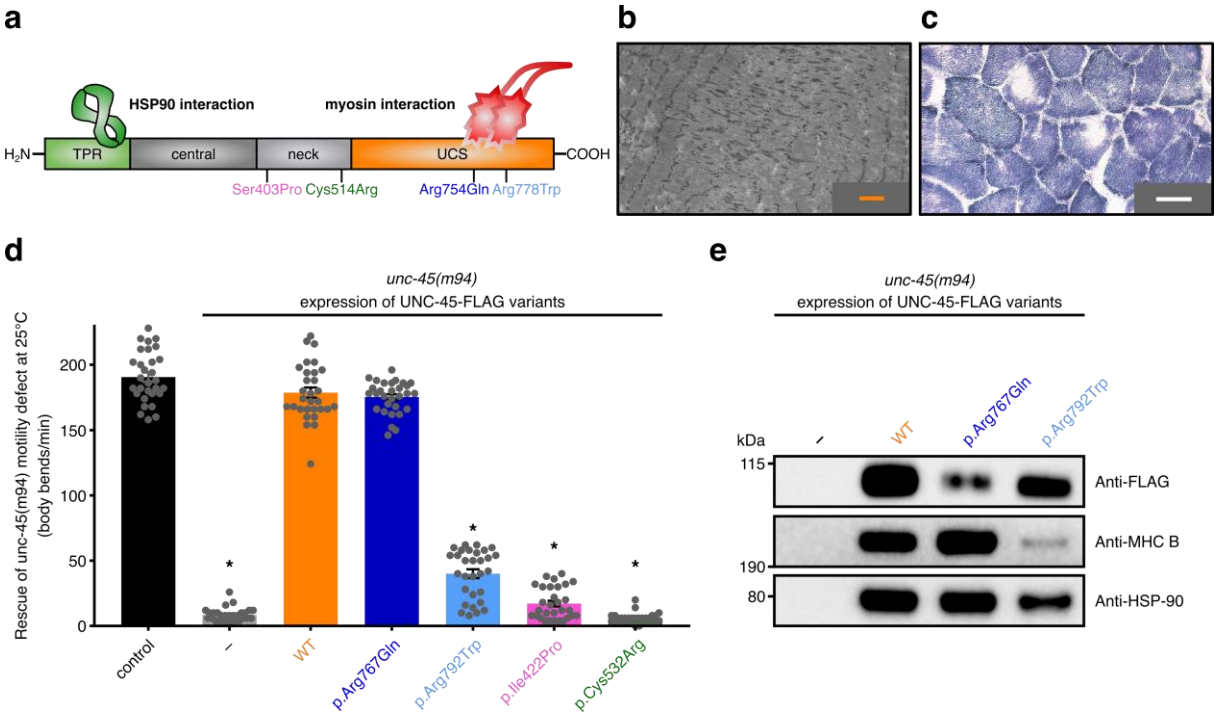

Figure 8 Pathogenic variants in the myosin chaperone UNC-45B cause progressive myopathy with eccentric cores (modified from Figures 1A, 2D, 2G, 6A, and 6F in publication **A**, Donkervoort, Kutzner et al., 2020; size, style, and colors modified from original).
a The recurring *UNC45B* hypomorph splice variant p.Arg754Gln (dark blue) and the p.Arg778Trp (light blue) variant affect conserved arginines in the myosin-binding UCS domain. The variants p.Ser403Pro (pink) and p.Cys514Arg (dark green) impact residues in the UNC-45B neck domain. **b** In electron microscopy of P3 biopsy, myofibrillar disarray were observed; orange scale bar represents 5 μm. **c** In histological analysis of P3 biopsy, core-like regions often located along the periphery of fibers were detected, consistent with eccentric cores on SDH staining; white scale bar represents 50 μm. **d** Transgenic UNC-45(p.Arg792Trp), UNC-45(p.Ser422Pro), and UNC-45(p.Cys532Arg) mutant proteins were unable to rescue the motility defect of *unc-45(m94)* worms grown at the restrictive temperature of 25°C, whereas UNC-45(p.Arg767Gln) rescued the *m94* motility defect in body bend measurements. **e** Co-immunoprecipitation of myosin heavy chain B (MHC B/UNC-54) and HSP-90 from cell lysates of *unc-45(m94)* mutant worms expressing the indicated FLAG-tagged UNC-45 variants grown at 25°C showed reduced binding of the UNC-45(p.Arg792Trp) variant to myosin.

2.1.2 My contribution to publication A

As co-first author of publication **A**, I designed and performed experiments and analyzed the data together with Sandra Donkervoort. I performed and analyzed all experiments concerning the recombinant expression of UNC-45B mutant proteins and subsequent biochemical assays. I generated transgenic *C. elegans* strains and performed the phenotypic analysis. I also performed *in silico* structural analyses. Sandra Donkervoort initiated the collaboration with clinicians and members of other research groups that are listed as co-authors who performed RNA sequencing, immunohistochemistry, and measurements of fiber mechanics in affected individuals' biopsies. Sandra Donkervoort also collected clinical and histological data on affected individuals from the co-authors. I helped with analysis and visualization of the data.

Sandra Donkervoort and I outlined and wrote the manuscript, and I designed the figures. As co-senior authors, Thorsten Hoppe and Carsten G. Bönnemann supervised experimental design, data interpretation, and manuscript writing.

2.2 PUBLICATION B: A UBIQUITIN FUSION REPORTER TO MONITOR MUSCLE PROTEOSTASIS IN C. *ELEGANS*

2.2.1 Summary publication B

Muscle is a highly dynamic tissue, in which folding and degradation processes work hand in hand to maintain proteostasis and functionality. Malfunction of the muscle-specific chaperone UNC-45 leads to misfolding of myosin, disorganization of myofilaments, and degradation of misfolded myosin molecules by the proteasome. To further investigate muscle proteostasis, the ubiquitin fusion degradation (UFD) model substrate Ub(G76V)-mCherry was expressed in body wall muscle of *C. elegans*. Under non-stress conditions, poly-ubiquitylation leads to proteasomal degradation of the substrate. Inhibiting proteasomal degradation with bortezomib or RNA interference (RNAi)-mediated depletion of UFD regulators stabilized the substrate. UNC-45 dysfunction induced by RNAi or temperature-sensitive mutants similarly resulted in substrate stabilization, indicating UPS overload likely due to myosin misfolding. This study further revealed that myosin misfolding in *unc-45* mutants activates the transcription factor SKN-1A, the NRF1/NRF2 orthologue in *C. elegans*, which adjusts proteasomal capacity by inducing the expression of the proteasomal subunit gene *rpt-3*. Finally, the muscle UFD substrate was used to study human disease pathology and showed that the dominant negative UNC-45 variant p.Ile422Pro can disrupt muscle proteostasis, potentially contributing to the progressive myopathy caused by pathogenic *UNC45B* variants.

2.2.2 My contribution to publication B

As first author of publication **B**, I conceived, designed, and performed experiments and analyzed the data. I generated and crossed transgenic *C. elegans* strains and performed Western blotting, microscopy, and motility experiments. Karen C. Bauer performed crossing and Western blotting of the *skn-1a(mg570) C. elegans* strain. I outlined and wrote the manuscript. Thorsten Hoppe supervised experimental design, data interpretation, and manuscript writing.

2.3 PUBLICATION C: OPTOGENETIC INDUCTION OF MECHANICAL MUSCLE STRESS IDENTIFIES MYOSIN REGULATORY UBIQUITIN LIGASE NHL-1 IN C. *ELEGANS*

2.3.1 Summary publication C

Mechanical stress during muscle contraction is a constant threat to proteome integrity. However, versatile experimental models to identify critical proteostasis regulators under mechanical stress conditions are scarce. This study introduced the transgenic *C. elegans* model OptIMMuS (OPTogenetic Induction of Mechanical MUscle Stress) to investigate changes in the proteostasis network associated with mechanical forces. Repeated blue light exposure of the muscle-expressed *Chlamydomonas rheinhardii* channelrhodopsin-2 variant ChR2(C128S;H134R) triggered sustained muscle contraction and resulted in mechanical stress phenotypes as seen in imaging, behavioral, and omics analyses (Figure 9a-c). *In vivo* proximity labeling and mass spectrometry experiments localized the chaperone UNC-45 to the myosin head complex in muscle cells together with sHSPs of the HSP-16 family already under non-stress conditions. Under OptIMMuS-induced mechanical stress conditions, the TRIM E3 ubiquitin ligase NHL-1 and its binding partner F40A3.6 were identified as new interactors of UNC-45 that cooperate with the chaperone in the regulation of muscle myosin proteostasis. Yeast two-hybrid, co-immunoprecipitation, and genetic epistasis experiments in myosin misfolding mutant strains confirmed the interaction of NHL-1 and F40A3.6 with UNC-45 and muscle myosin (Figure 9d). Experiments using RNAi-mediated knockdown and the ligase-dead RING domain mutant strain *nhl-1(syb8175)* provided evidence that the ubiquitylation activity of NHL-1 regulates myosin protein levels and function under mechanical stress (Figure 9e-f). In the future, OptIMMuS will help to identify additional muscle-specific proteostasis regulators of therapeutic relevance.

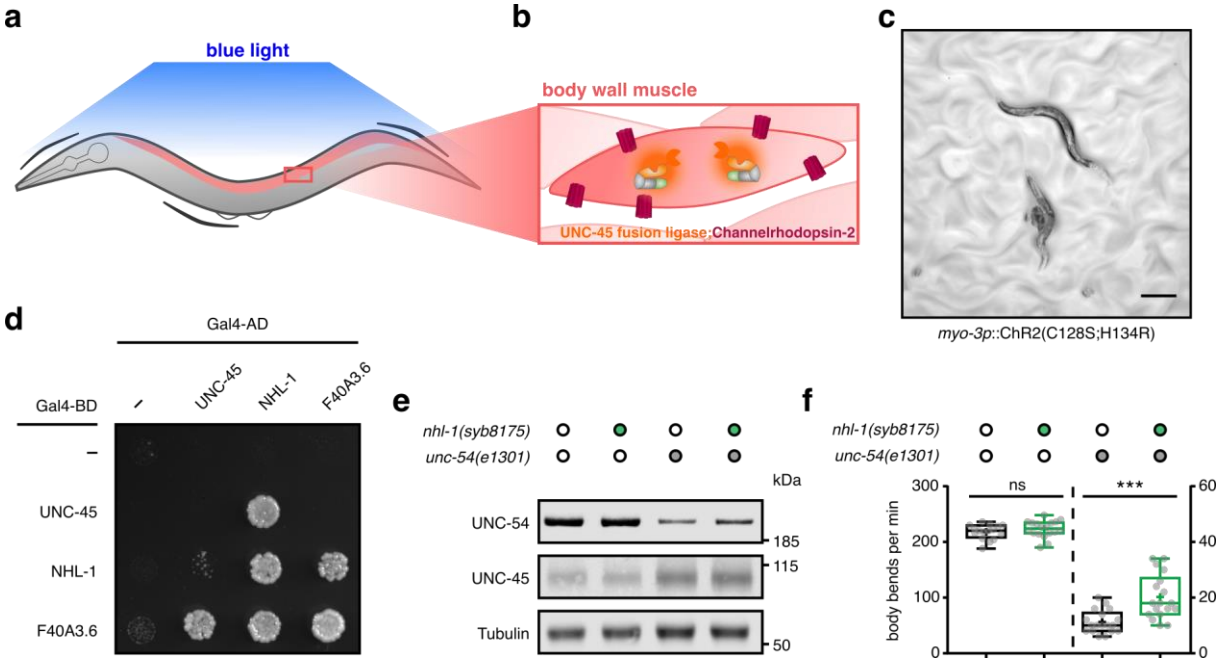

Figure 9 Optogenetic induction of mechanical muscle stress (OptIMMuS) identifies myosin regulatory E3 ubiquitin ligase NHL-1 in *C. elegans* (cropped and modified from Figures 1a, 4b, 6b, and 6c in publication **C,** Kutzner et al., 2024; size, style, and colors modified from original).
a Transgenic worms express *Chlamydomonas rheinhardii* channelrhodopsin-2 (ChR2, dark red barrel) in body wall muscle cells enabling muscle contraction upon blue light exposure. **b** Additional expression of a UNC-45 fusion biotin ligase (UNC-45-miniTurbo, orange shape) allowed the identification of the E3 ligase NHL-1 and its binding partner F40A3.6 in close proximity to UNC-45 under mechanical stress conditions. **c** Bright-field light microscopy of ChR2(C128S;H134R)-expressing worms after a single 5-s blue light pulse showed contraction, paralysis, and rupture; scale bar represents 500 µm. **d** Yeast two-hybrid assay showed growth and binding of NHL-1, UNC-45, and F40A3.6. **e** Western blot of UNC-54/myosin, UNC-45, and the housekeeping protein tubulin in lysates from the indicated mutant worms showed an increase in myosin/UNC-54 protein levels upon presence of the *nhl-1(syb8175)* RING domain mutant in myosin misfolding mutant backgrounds compared to control without *nhl1(syb8175)*. **f** Body bend counts per minute in liquid of indicated mutant worms showed partial rescue of the motility defect of *unc-54(e1301)* myosin misfolding worms by the *nhl-1(syb8175)* RING domain mutation at the restrictive temperature of 25°C.

2.3.2 My contribution to publication C

As first author of publication **C**, I conceived, designed, and performed the majority of experiments and analyzed the data. Thorsten Hoppe presented me with the idea for optogenetic induction of mechanical stress in muscle, and I generated transgenic *C. elegans* strains and established the experimental techniques for the OptIMMuS model. The OptIMMuS incubator was configured by the Biozentrum Zentralwerkstatt, namely Leo Leson and Christopher Kier. Karen C. Bauer helped with crossing transgenic OptIMMuS strains and performed and analyzed fluorescence and confocal microscopy experiments. Anwesha Sarkar performed and analyzed WormLab experiments. I performed transmission electron microscopy experiments with help from Karen C. Bauer and Felix Gaedke from the CECAD Imaging Facility. I performed ARENA population motility measurements with help from Karen C. Bauer. I designed and performed

RNA sequencing and mass spectrometry experiments and analyzed the data with help from the Cologne Center for Genomics, Jan-Wilm Lackmann from the CECAD Proteomics Facility, and Richard J. Acton from the CECAD Bioinformatics Facility. Karen C. Bauer collected samples for UNC-45 proximity labeling mass spectrometry under mechanical stress during her Master thesis. I performed yeast two-hybrid experiments with help from Karen C. Bauer. I further designed, performed, and analyzed experiments regarding the characterization of the new UNC-45 interactors NHL-1 and F40A3.6: generation of transgenic strains and crossing, fluorescence microscopy, co-immunoprecipitation, RNA extraction and qRT-PCR, RNAi experiments, motility measurements, and Western blotting. The *nhl-1(syb8175)* RING domain mutant strain was ordered from Suny Biotech. Wojciech Pokrzywa performed and analyzed UNC-45 co-immunoprecipitation in *unc-45* mutants. I outlined and wrote the manuscript and designed the figures with help from Karen C. Bauer and generous comments from Angela Andersen. As senior author, Thorsten Hoppe supervised experimental design, data interpretation, and manuscript writing.

3 Discussion

Proteostasis in tissues under constant mechanical stress requires precise regulation and collaboration of different factors of the proteostasis network (Carlisle et al., 2017; Collier and Benesch, 2020; Höhfeld et al., 2021; Morita et al., 2013). Until now, proteostasis and PQC mechanisms targeting the motor protein myosin in muscle cells under mechanical stress as well as the role of the myosin-chaperone UNC-45 in mechanical stress and disease had remained poorly understood. The following sections will discuss the findings of this work that aimed to determine how dysfunction of the conserved chaperone UNC-45 causes human muscular disease and to investigate how this chaperone plays a role in myosin proteostasis under mechanical stress. Furthermore, the interaction network of UNC-45 will be summarized, which allows comparing this chaperone network to other proteostasis networks that target sarcomeric proteins in the muscle. Last, by using *C. elegans* as model organism for human muscular disease and for the consequences of high intensity muscle contraction, this work encourages a discourse regarding the use of invertebrate models for medical and muscle exercise research.

3.1 UNC-45 FUNCTION IN HUMAN DISEASE

Although the importance of UNC-45 for muscle proteostasis and myosin folding has long been recognized, pathogenic variants in the human muscle-specific paralog *UNC45B* that cause muscular disease phenotypes remained elusive. Previous work in various model organisms, including *C. elegans*, *D. melanogaster*, zebrafish, and mice, had shown that loss of UNC-45/UNC-45B is embryonically lethal (Barral et al., 1998; Chen et al., 2012; Etard et al., 2007; Lee et al., 2011). Accordingly, no human individuals with bi-allelic null variants and no homozygous null variants have ever been identified, suggesting that the complete loss of UNC-45B may likely not be tolerated. So far, only one missense variant in the myosin-binding UCS domain of UNC-45B had been reported to result in autosomal dominant juvenile cataracts in humans and impaired lens development in zebrafish, yet without effects on muscle function (Hansen et al., 2014). Now, after over a decade of search, this work reports individuals with bi-allelic variants in the *UNC45B* gene that are causally associated with muscular pathology (publication **A**). The affected individuals present with childhood-onset progressive muscle weakness characterized by myofibrillar disarray and histologic evidence for eccentric cores (Donkervoort, Kutzner et al., 2020). Functional characterization of the here identified *UNC45B* variants revealed that the disease mechanism not only involves altered mRNA splicing accompanied by decreased UNC-45B protein levels, but also impaired protein folding, localization, and molecular interactions of UNC-45B (publication **A**), as well as disturbed proteostasis balance in muscle (publication **B**). Prior to publication, a single myopathy case was

reported of an individual carrying the common splice variant also found here, yet without further characterization of the disease mechanism (Dafsari et al., 2019). Later in 2022, a case was published of a newborn with bi-allelic *UNC45B* mutation that only survived for 23 days after birth due to myofibrillar disarray of the diaphragm and heart defects (Shi et al., 2022). Together, as of now, a total of seven different human pathogenic variants in *UNC45B* were found, of which six result in diverse muscular phenotypes likely caused by multiple developmental as well as progressive disease mechanisms.

Apart from the correct folding and adequate functionality of UNC-45B, a balanced protein abundance is crucial for maintaining a functional sarcomere, as proposed previously (Kachur and Pilgrim, 2008; Kim et al., 2008; Landsverk et al., 2007). Accordingly, experimental setups that reduced UNC-45B expression in various model organisms revealed myofibrillogenesis impairment, myofibrillar disorganization, and loss of contractile function (Chen et al., 2012; Etard et al., 2007; Frumkin et al., 2014; Gaiser et al., 2011; Melkani et al., 2011). Conversely, pathogenic variants in the ubiquitin segregase CDC-48/p97/VCP, which is involved in UNC-45B degradation via the UPS, resulted in elevated protein levels of UNC-45B that also led to disorganized myofibrils in both humans and *C. elegans* (Janiesch et al., 2007). In addition, in nonhereditary muscular disease, UNC-45B protein levels appear to be similarly regulated, either by increased transcription or attenuated protein degradation. In individuals suffering from ischemic heart disease and heart failure, proteomic analysis detected increased protein levels of UNC-45B (Stanley et al., 2007). In immobilized and mechanically ventilated individuals suffering from CIM due to extensive stays in the intensive care unit, increased mRNA expression of *UNC45B* and *HSP90* was detected in *tibialis anterior* skeletal muscle, along with the expression of atrophy markers such as *FBXO30*, *TRIM32*, *TRIM63*/MuRF1, and *FBXO32*/atrogin-1 (Llano-Diez et al., 2019). Therefore, combined data from this work and the literature highlight that the availability of UNC-45B together with other chaperones and E3 ligases in the muscle plays a role in the coordinated assembly and disassembly of myofibrils both in response to stress and as adaptation during critical illness and inactivity.

The second human paralog, *UNC45A*, has been associated with ovarian and breast cancers, in which increased levels of this chaperone were detected and shown to enhance cell proliferation and migration, and possibly metastasis (Bazzaro et al., 2007; Guo et al., 2011). Depletion of *UNC45A* in these cancer cells suppressed cell proliferation and migration through modulating myosin II-actin interactions. Interestingly, osteosarcoma cells depleted for *UNC45A* conversely displayed increased migration velocities yet impaired adhesion and tail retraction of the migrating cells (Lehtimäki et al., 2017). Recently, compound heterozygous variants in *UNC45A* have been reported to cause a syndrome associating cholestasis, diarrhea, impaired hearing, and bone fragility (Esteve et al., 2018). In subsequent studies, the disease mechanism

was demonstrated to involve the UNC-45A-mediated regulation of extracellular matrix adhesion, intestinal epithelial barrier function, and microvilli development (Lechuga et al., 2022; Li et al., 2022a). Chaperone clients of UNC-45A in this context are also myosins: non-muscle myosin IIA (NM-IIA/MYH9) and B (NM-IIB/MYH10) and unconventional myosin VB (MYO5B). Therefore, in addition to the function of UNC-45B in muscle cells, its paralog UNC-45A regulates multiple cellular adhesion- and migration-related processes in non-muscle cells by also chaperoning myosin proteins and UNC-45A dysfunction results in diverse disease phenotypes.

Since the two human paralogs UNC-45A and UNC-45B are expressed in different tissues but appear to exert similar functions on myosin motor proteins, it could be speculated that they partially compensate for defects in the respective other under certain stress conditions. Indeed, UNC-45B can compensate for loss of UNC-45A and colocalizes with NM-II in zebrafish embryos and in human osteosarcoma cells (Comyn and Pilgrim, 2012; Lehtimäki et al., 2017; Myhre et al., 2014). Regarding muscle development and myofibrillogenesis, however, compensation of UNC-45B function is not possible as shown in zebrafish and mouse (Chen et al., 2012; Comyn and Pilgrim, 2012; Price et al., 2002). Together, this work and work from others underline the importance and complex regulatory functions of the myosin-directed chaperone UNC-45 in health and disease in different cells and tissues.

3.2 UNC-45 ROLE IN MECHANICAL STRESS RESPONSE

As described above, in zebrafish, Unc45b was shown to localize to the Z-disk and shuttle to myofilaments after myofiber damage induced by cell membrane wounding, cold shock, or heat shock (Etard et al., 2008). Interestingly, in human individuals with pathogenic variants in *UNC45B*, the aberrant UNC-45B protein is mislocalized away from the M-line and the surrounding A-band and accumulates at the Z-disk (publication **A**). Likely, not only the abundance but also the dynamic shuttling of the chaperone is impaired, and the residual protein is prevented from fulfilling its function in maintaining and repairing the myosin heads in the A-band. Under conditions of constant mechanical stress and use, the lack of proper UNC-45B localization and function could result in the disruption and disarray of the myofilaments as seen in histologic and ultrastructural analyses of the affected individuals, which supports a role for this chaperone in the response to mechanical stress.

To further investigate the role of UNC-45 under mechanical stress, this work established an *in vivo* transgenic model able to induce mechanical stress in the body wall muscle of *C. elegans* called OptIMMuS (publication **C,** Kutzner et al., 2024). Interestingly, temperature-

sensitive mutations in *unc-45* prevented effective muscle contraction in OptIMMuS and blue light treatment of these mutants resulted in paralysis already at the permissive temperature, likely due to defects in myosin folding and repair. Although the experimental analyses in this context were unable to recapitulate shuttling of UNC-45 in the sarcomere observed in vertebrates, since UNC-45 remains stably attached to the thick filament in *C. elegans* throughout adulthood, OptIMMuS allowed to follow the chaperone's interaction network to investigate the proteostasis of the chaperone's substrate myosin upon mechanical stress. Indeed, *in situ* proximity labeling revealed increased association of UNC-45 and myosin with sHSPs of the HSP-16 family and with the E3 ligase NHL-1 and its binding partner F40A3.6 under mechanical stress (Figure 10).

C. elegans NHL-1 shares structural and functional similarities with the mammalian TRIM E3 ligases TRIM63 and TRIM32, among others. In zebrafish, *trim63* expression was reported to be upregulated in *unc45b* mutants, possibly in an Hsf1-dependent manner, since it possesses a predicted transcription factor binding site (Etard et al., 2015). Upon muscle pathological conditions, both *UNC45B* and *TRIM63* were transcriptionally upregulated in parallel as described above (Llano-Diez et al., 2019). Apart from the transcriptional co-regulation of chaperone and E3 ligase, this work now demonstrated the physical interaction of UNC-45 and the orthologous TRIM protein NHL-1 in mechanically stressed muscle. Here, NHL-1 was characterized to similarly target the UNC-45 substrate myosin (MHC B/UNC-54) for ubiquitylation and subsequent degradation via the UPS and thereby influence muscle contraction and recovery from mechanical stress (publication **C**).

Together, these findings suggest that, apart from its role in *de novo* myosin folding and myofilament assembly, the chaperone UNC-45 can also adopt a specific function in myosin protein triage upon misfolding due to mechanical stress (Figure 10). UNC-45 thereby likely determines whether an unfolded myosin molecule can be refolded with the help of general chaperones or whether it should be degraded and replaced by a newly synthesized myosin molecule as had been proposed previously and described above (Chapters 1.1.3 and 1.3.2; Figure 4; Carlisle et al., 2017; Kachur and Pilgrim, 2008). The observations in publication **C** allow several interpretations: First, increased availability of NHL-1 in proximity of UNC-45 and myosin could indicate that UNC-45 and NHL-1 compete with each other for binding to misfolded myosin ("kinetic model of protein triage"). Second, UNC-45 binding to NHL-1 instead of HSP90 could promote degradation of misfolded myosin ("co-factor model") although no significant change in HSP90 abundance in UNC-45 proximity was detected upon mechanical stress. Third, the detection of a protein complex including myosin, UNC-45, and NHL-1 in *in vivo* co-immunoprecipitation experiments could hint to the notion that additional NHL-1 binding to the UNC-45-myosin chaperone-substrate complex is required for the degradation of misfolded myosin ("degradation complex model"). Likely, the interactions and kinetics are more

complex than it was possible to dissect in this work. Nevertheless, the identification of this new substrate-chaperone-E3 ligase complex in C. *elegans* body wall muscle combined with further analyses of the developed transgenic models and biochemical studies will enable the mechanistic characterization of UNC-45-mediated triage of myosin in the future.

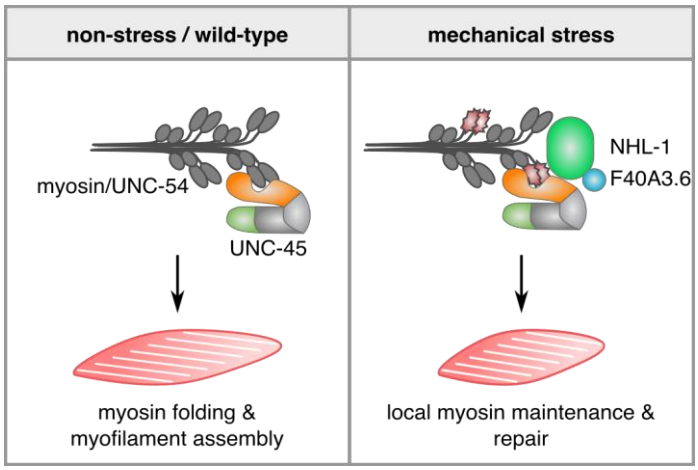

Figure 10 Protein triage of misfolded myosin by UNC-45, NHL-1, and F40A3.6 upon mechanical stress in the muscle (cropped and modified from Figure 7 in publication **C,** Kutzner et al., 2024; size, style, text, and colors modified from original).
During development and under non-stress conditions (left panel), the chaperone UNC-45 (orange/gray/green shape) folds and assembles myosin (gray shapes) into growing myofilaments (parallel white stripes) in the muscle, likely with the help of general chaperones HSP90, HSP70, and co-chaperones. Upon mechanical stress (right panel), the E3 ubiquitin ligase NHL-1 (green rectangle with rounded corners) and its binding partner F40A3.6 (blue circle) accumulate in proximity of UNC-45 and myosin and determine, whether a misfolded myosin molecule (jagged red shapes) requires refolding or degradation and replacement.

3.3 UNC-45 GENETIC AND PHYSICAL INTERACTIONS

Since shortly after its discovery, UNC-45 has been known to genetically and physically interact with different types of its substrate myosin (Ao and Pilgrim, 2000; Barral et al., 1998; Epstein and Thomson, 1974). This interaction was first demonstrated in C. *elegans* for the main muscle myosins MHC B/UNC-54 and MHC A/MYO-3 (Barral et al., 2002; Kaiser et al., 2012; Ni et al., 2011). This work corroborates not only the direct binding of UNC-45 to MHC B/UNC-54, which is influenced by a pathogenic variant in the UNC-45 UCS domain (publication **A**), but also the positioning of UNC-45 near the myosin head. In an unbiased proximity labeling assay, the type II myosins UNC-54 and MYO-3 as well as the myosin essential light chain MLC-3 were found to be significantly enriched near UNC-45 (publication **C**). Genetic and physical interactions with both conventional muscle and non-muscle type II myosins have been reported in *D. melanogaster*, zebrafish, mice, and humans (Chen et al., 2012; Etard et al., 2008; Guo et al., 2011; Kachur et al., 2004; Lehtimäki et al., 2017; Melkani et al., 2011). A very recent

study demonstrated that UNC-45B mediates protocostamere formation and cardiac myofibrillogenesis in human embryonic stem cell-derived cardiomyocytes by colocalizing and interacting spatiotemporally with the non-muscle myosin II NM-IIB/MYH10, the muscle myosin II MYH6, and the focal adhesion protein kindlin-2 (Lu et al., 2023). In addition, UNC-45A was found to mediate the stability and function of unconventional myosins: type I MYO1C in osteosarcoma cells and type V MYO5B in intestinal cells (Lehtimäki et al., 2017; Li et al., 2022a). Similarly, next to muscle myosins, the *C. elegans* non-muscle myosin II NMY-2 and the unconventional myosin V HUM-2 have been found to interact with UNC-45 in yeast two-hybrid assays (Kachur et al., 2004; Lee et al., 2014), but were not found to be significantly enriched in UNC-45 proximity in the muscle in this work (publication **C**).

First identified as an HSP90 co-chaperone, UNC-45 closely interacts and collaborates with other general chaperones, co-chaperones, and sHSPs. Apart from HSP90 and HSP70, whose interaction and collaboration with UNC-45 have been proven plenty of times *in vitro* and *in vivo* (Barral et al., 2002; Gaiser et al., 2011; Gazda et al., 2013; Liu et al., 2008; Lu et al., 2023), also other HSP90 co-chaperones epistatically interact with UNC-45. In a candidate knockdown screen in *C. elegans*, the client recruiter Hop/Sti1, the ATPase activator Aha1, and the maturation co-chaperone p23, which regulate the ATP-dependent client binding cycle of HSP90, were identified to aggravate the motility and myofilament organization phenotype of *unc-45* conditional loss-of-function worms (Frumkin et al., 2014). This work now adds the transient physical interaction with sHSPs of the HSP-16 family in *C. elegans* to the list (publication **C**). Previously, the HSP-16 small HSPs have only been reported to be transcriptionally upregulated in response to heat stress and to *unc-45* or *hsp-90* depletion (Schmauder and Richter, 2021), and to be altered in their conformation in *unc-54* conditional myosin misfolding worms (Sui et al., 2022). *C. elegans* HSP-16 family sHSPs show sequence similarities to several mammalian muscle-expressed sHSPs including HSPB1/HSP27, HSPB2/MKBP, HSPB3/HSPL27, HSPB4/αA-crystallin, HSPB5/αB-crystallin, HSPB6/HSP20 and HSPB8/HSP22 and similarly exist in multimeric complexes exhibiting chaperone activity (Collier and Benesch, 2020; Peter and Candido, 2002). One could speculate that unlike the foldases HSP90 and HSP70 that play an active role in the folding and assembly of myosin, sHSPs may aid in stabilizing myosin upon proteotoxic insults or mechanical stress. Indeed, αB-crystallin was shown to prevent myosin aggregation *in vitro* and preserve its ATPase activity (Melkani et al., 2006; Smith et al., 2014).

E3 ubiquitin ligases were first implicated in the regulation of UNC-45 protein levels by ubiquitylation and degradation via the UPS. Yeast two-hybrid, co-immunoprecipitation, and *in vitro* ubiquitylation assays identified the E3 ligases CHN-1/CHIP and UFD-2/UBE4B that are able to ubiquitylate UNC-45 and, when knocked down, rescue UNC-45 protein levels and the motility defect of *unc-45* mutant worms (Hoppe et al., 2004). Subsequent studies found that

CHN-1 and UFD-2 work together with the ubiquitin-selective segregase CDC-48/p97/VCP to target UNC-45 for proteasomal degradation in both *C. elegans* and humans (Janiesch et al., 2007). In addition, it has been reported that, *in vitro*, UFD-2 ubiquitylates heat-denatured myosin/UNC-54 presented by UNC-45 (Hellerschmied et al., 2018). This work now identifies the muscle-expressed E3 ligase NHL-1 as a physical interactor of UNC-45 *in vivo* in mechanically stressed muscle (publication **C**). Unlike previously identified E3 ligases, NHL-1 does not target UNC-45 itself but rather its substrate, the muscle myosin II MHC B/UNC-54. Notably, in *unc-54(e1301)* conditional loss-of-function mutants, only depletion or mutation of *nhl-1* but not depletion of *ufd-2* was able to rescue the mutants' motility defect (Janiesch et al., 2007) and increased the mutants' diminished myosin/UNC-54 protein levels (publication **C**). These findings suggest that UFD-2 regulates the myosin-chaperone UNC-45, however, that NHL-1 is the E3 ligase that directly targets myosin *in vivo* during mechanical stress, myosin misfolding, and atrophy. Nevertheless, considering the diverse number of existing E3 ligases, it is likely that there are multiple E3 ligases that influence UNC-45 and myosin function and protein levels under different physiological and stress conditions. Since NHL-1 shares similarities with the mammalian TRIM E3 ligases TRIM63 and TRIM32, it could be speculated that UNC-45 similarly interacts with TRIM63/MuRF1, TRIM32, or other orthologous E3 ligases in human muscle upon mechanical stress and regulates the function and levels of myosin or other sarcomeric proteins in this context. Corroboration of this hypothesis would present opportunities for research into new roads for therapeutic intervention and pharmacological support during recovery from muscle injury by targeting the interaction of UNC-45 and TRIM E3 ligases.

Finally, this work adds weight to the notion that UNC-45 is a central factor of the proteostasis network in the muscle not only mediating folding but also degradation of myosin via the UPS. Previously, UNC-45 has been shown to promote the degradation of misfolded or non-functional myosin via the UPS (Kachur and Pilgrim, 2008; Landsverk et al., 2007). *C. elegans* overexpressing UNC-45 have reduced myosin protein levels and diminished motility, which can both be partially restored by inhibiting the proteasome (Landsverk et al., 2007). Conclusively, missense mutations in the *C. elegans* myosin gene *unc-54* that result in intrinsic myosin misfolding achieve proteasomal induction via the stress-responsive NRF1/NRF2-orthologous transcription factor SKN-1A that activates the expression of proteasomal subunits, e.g. the ATPase subunit *rpt-3*. This work now demonstrates that conditional loss-of-function mutations in *unc-45* similarly engage the UPS via *skn-1a* and *rpt-3*, likely due to the resulting accumulation of misfolded myosin (publication **B,** Kutzner et al., 2023), and that myosin degradation in *C. elegans* is mediated by the E3 ligase NHL-1 (publication **C**).

Taken together, the manifold genetic and physical interactions of UNC-45 with sarcomeric components and proteostasis factors form a network that regulates the folding,

assembly, quality control, and degradation of the motor protein myosin, thereby enabling precise control of the contractility of the muscle and the motility of the organism (Figure 11).

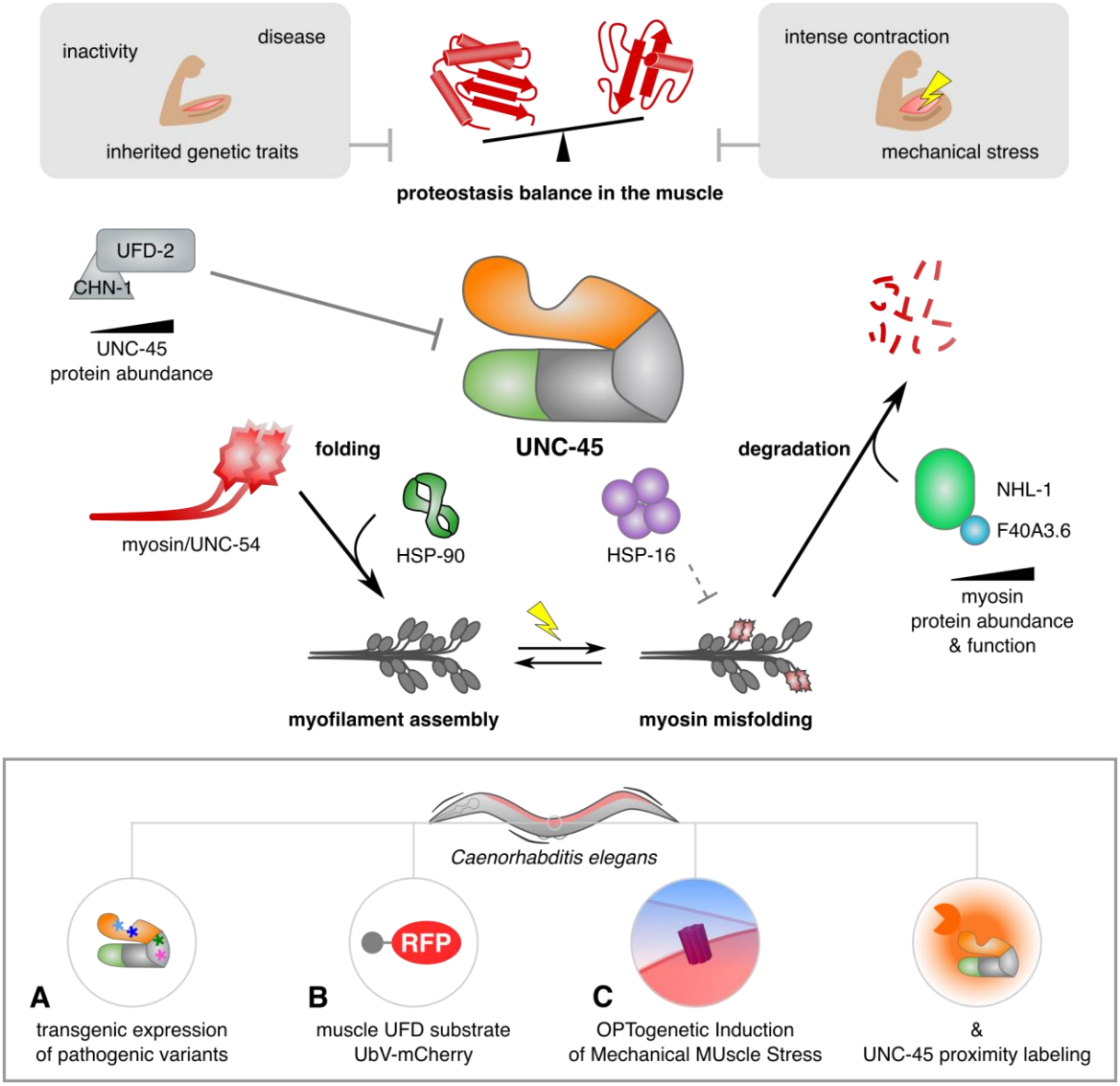

Figure 11 The myosin-directed UNC-45 proteostasis network in the muscle (according to literature and this work).
Proteostasis in the muscle, the balance of protein synthesis, folding, and degradation, is challenged by pathological states, such as inactivity, muscular disease, and inherited genetic traits, or intense contraction and the resulting mechanical stress (gray boxes with rounded corners at the top). The myosin-directed chaperone UNC-45 is at the heart of a proteostasis network regulating the muscle motor protein myosin. A complex of the E3 ligases CHN-1 and UFD-2 regulates UNC-45 protein abundance (blunted gray arrow; Hoppe et al., 2004; Janiesch et al., 2007). Together with general chaperones HSP90 and HSP70, UNC-45 folds and assembles myosin into myofilaments (Barral et al., 2002; Gazda et al., 2013). Upon mechanical stress (yellow lightning) and myosin misfolding, UNC-45 together with the E3 ubiquitin ligase NHL-1 and its binding partner F40A3.6 target myosin for proteasomal degradation, thereby regulating myosin protein abundance and function (black arrows). Small HSPs of the HSP-16 family could prevent myosin misfolding and aggregation (blunted gray arrow with interrupted line). In publications **A**, **B**, and **C**, which formed the basis of this work (gray bordered box at the bottom), transgenic models and reporters in the body wall muscle of the nematode C. *elegans* were generated and used to identify and characterize pathogenic variants in UNC-45 and new regulators of myosin proteostasis in the muscle upon mechanical stress (in gray circles).

3.4 THE UNC-45 INTERACTION NETWORK COMPARED TO OTHER PROTEOSTASIS NETWORKS IN THE MUSCLE

This work described a variety of genetic and physical interactions of UNC-45 with general chaperones HSP90 and HSP70, with sHSPs of the HSP-16 family (publication **C**), with E3 ubiquitin ligases CHN-1/CHIP, UFD-2, and NHL-1 and its binding partner F40A3.6 (publication **C**), and with the transcription factor SKN-1A and the UPS (publication **B**) that cooperate in muscle proteostasis during myosin folding, misfolding, mechanical stress, and disease (Figure 11).

Similarly, in the BAG3-centric CASA network, general chaperones of the HSP70 family, the co-chaperone BAG3, and sHSPs such as HSPB6/HSP20 and HSPB8/HSP22 among others, interact with an E3 ligase, CHIP, to coordinate the sequestration and degradation of unfolded or misfolded substrate proteins (Arndt et al., 2010; Höhfeld et al., 2021; Rauch et al., 2017). During contraction stress, BAG3 and associated sHSPs act on the actin-crosslinking protein filamin C in the Z-disk, identify mechanically damaged forms of this protein, and initiate filamin C degradation by autophagy (Arndt et al., 2010; Klimek et al., 2017; Ulbricht et al., 2013). In contrast, UNC-45 and NHL-1 target misfolded myosin in the thick filament and initiate its degradation by the UPS. Whether and how UNC-45-mediated targeting of unfolded myosin can result in the degradation of sarcomeric proteins by autophagy remains to be investigated. Insults to the muscle that induce catabolic atrophy conditions activate both the UPS and autophagy in parallel, as seen in CIM occurring during intensive care stays. In this situation, not only chaperones and E3 ligases are increasingly expressed, but also the expression of lysosomal proteases (cathepsins), the microtubule-associated protein 1 light chain 3β *MAP1LC3B*, and the substrate receptor *SQSTM1/p62* is increased (Llano-Diez et al., 2019). Furthermore, structural similarities of NHL-1 to mammalian MuRF1/TRIM63 could indicate an involvement of this E3 ligase also in autophagic targeting, since MuRF1 was reported to target its substrates to both the UPS and autophagy as in the case of titin (Kötter and Krüger, 2022; Müller et al., 2021a).

The finding that UNC-45 and NHL-1 interact with myosin both during mechanical stress and during genetically induced atrophy suggests a resemblance between these two insults. In both cases, molecular misfolding appears to be the trigger for the increased interaction of chaperone and E3 ligase. It remains to be investigated whether NHL-1 is similarly upregulated in muscle atrophy conditions induced by inactivity or disease, as is the case for MuRF1/TRIM63 and TRIM32. Both have been implicated in the coordinated disassembly of filamentous myofibrils in atrophic conditions such as fasting, denervation, and aging, and in various

diseases, e.g. cancer, cardiac failure, diabetes, and sepsis (Aweida and Cohen, 2021; Bodine and Baehr, 2014; Tocchini and Ciosk, 2015).

In particular, TRIM32 plays a role in the initial degradation of structural desmin filaments in the Z-disk, thereby destabilizing the myofibril and allowing for the ubiquitylation and extraction of actin and myosin from the tightly packed thin and thick filaments (Aweida and Cohen, 2021; Cohen et al., 2012). In this case, phosphorylation of desmin filaments by the glycogen synthase kinase-3β (GSK3β) precedes and promotes the ubiquitylation of desmin by the E3 ligase TRIM32 (Aweida et al., 2018). In addition, the AAA-ATPases ATAD1 and p97/VCP and the protease calpain-1 aid in extraction and pre-digestion of desmin from filaments before degradation via the UPS (Aweida and Cohen, 2022). At least in *C. elegans*, it seems possible that UNC-45 initiates the extraction of individual misfolded myosin molecules from the sarcomere without prior disintegration of the myofibril. Evidence for this notion comes from several reported findings: First, as of now, no orthologue of desmin has been identified in *C. elegans* (Benian and Epstein, 2011). Second, in *C. elegans*, UNC-45 remains stably attached to the myosin head complex throughout adulthood and during contraction (Ao and Pilgrim, 2000; Gaiser et al., 2011). And third, *in vitro*, UNC-45B has been shown to be able to halt myosin-actin cross-bridge cycling (Nicholls et al., 2014), which would allow for the extraction of a single myosin molecule from the contracting thick filament. Similarly, the finding that depletion of *F40A3.6* significantly increased the motility of *unc-54(e1301)* and *unc-45(m94)* mutant worms (publication **C**) allows for the speculation that upon misfolding, F40A3.6 stops the molecular movement of the myosin motor to enable UNC-45 and NHL-1 to extract the misfolded myosin molecule for ubiquitylation. Nevertheless, it remains to be investigated how the C-terminal tail of the myosin molecule is extracted from the filament, since UNC-45 has only been shown to interact with the myosin head domain. It is conceivable that AAA-ATPases such as p97/VCP are involved in the uncoiling of the myosin tail and extraction of ubiquitylated proteins from myofibrils or that proteases such as calpains cleave the myosin head before extraction and degradation (Aweida and Cohen, 2021; Piccirillo and Goldberg, 2012).

Together, the findings of this work broaden the UNC-45 interaction network and its role in myosin proteostasis in the muscle. Next to UNC-45 as myosin chaperone, the E3 ubiquitin ligase NHL-1 emerges as central executive factor of myosin protein turnover in *C. elegans*. A similar role was demonstrated for TRIM63/MuRF1 in mammals, yet the identified collaboration of a TRIM E3 ligase with UNC-45 as mediator of client specificity implies the existence of more intricate mechanisms of myosin protein quality control in muscle upon mechanical stress.

3.5 C. *ELEGANS* AS A MODEL ORGANISM FOR HUMAN MUSCULAR PATHOLOGIES AND MECHANICAL STRESS RESPONSES

Appropriate experimental models are necessary to identify and characterize proteostasis mechanisms that respond to mechanical stress and myosin misfolding in muscle. Since UNC-45 was initially identified in *C. elegans*, this work used this nematode as the main model organism to investigate the role of UNC-45 in human muscular disease and in response to contraction-induced stress (Figure 11). In publication **A**, I employed a previously established experimental approach to investigate the impact of single point mutations on the functionality of the myosin chaperone. The transgenic expression of variant proteins of UNC-45 in the background of an *unc-45* conditional loss-of-function mutation had already proven successful and correlated well with *in vitro* properties of the variant proteins (Gazda et al., 2013). In publication **B**, I developed the muscle-specific UFD substrate strain *myo-3p::UbV-mCherry* on the basis of a ubiquitously expressed UFD substrate strain (Liu et al., 2011; Segref et al., 2011). The muscle-specific UFD substrate reports the proteostatic state of the body wall muscle and complements the established transcriptional reporter strain *rpt-3p::GFP* that indicates the upregulation of proteasomal subunits upon misfolding in the muscle (Lehrbach and Ruvkun, 2016, 2019). In publication **C**, I established the transgenic *C. elegans* model OptIMMuS to repeatedly trigger sustained contractions and thereby induce mechanical stress in muscle and used imaging, behavioral, and proteomic read-outs to study changes in the proteostasis network associated with mechanical forces and myosin misfolding.

Muscle and mechanical stress research is advancing thanks to models ranging from cell culture, *D. melanogaster*, zebrafish, and rodent models to human subjects. Two- and three-dimensional cell culture models using electrical pulse stimulation (EPS) can provide reproducible data on protein interactions and cellular responses to highly defined treatments and conditions (Hofemeier et al., 2021; Orfanos et al., 2016; Reimann et al., 2020). Nevertheless, cell culture lacks the complexity to account for tissue- and organism-wide adaptations to mechanical stress. Accordingly, muscle tissue can be analyzed *ex vivo* from *D. melanogaster* or rodent dissections (Akimoto et al., 2013; Ducomps et al., 2003), or human biopsies, or *in vivo* using exercise regimens followed by biopsies or non-invasive imaging (Bilston et al., 2019; Gehlert et al., 2016). Disadvantages of vertebrate studies are high variability and low versatility due to limited sample availability and method accessibility, respectively. Notably, *C. elegans* combines the advantages of both cell culture and *in vivo* models, including reproducibility, methodological versatility, and organismal complexity (Freires et al., 2023). The nematode model offers a wide variety of behavioral, microscopic, and genetic methods and reporters for the analysis of cellular

and organismal responses (Bukhari and Müller, 2019; Praitis and Maduro, 2011). In addition, significant anatomical and genetic similarities between *C. elegans* body wall muscle and human skeletal and cardiac muscle facilitate the transferability of findings (see Chapter 1.4; Benian and Epstein, 2011; Epstein and Benian, 2012; Kim et al., 2008).

When designing an experimental system for the characterization of cellular responses to mechanical muscle stress, three key aspects have to be considered: The system should be (1) sufficiently complex, (2) methodologically versatile, and (3) highly reproducible. First, other experimental systems either employ uniform cell cultures or heterogeneous tissue samples. In contrast, using *C. elegans* and in particular OptIMMuS, not only cellular responses in muscle cells but also organismal responses and phenotypes can be analyzed *in vivo*. Combined with sensitive proximity labeling and proteomics assays, a wide range of effects can be characterized, from population motility changes to cellular architecture rearrangements and transient protein-protein interactions. Second, in *C. elegans*, a wide variety of methods can be applied, such as behavioral assays, imaging approaches, whole worm and subcellular omics, and genetic experiments. These methods enable the analysis of single cells, tissues, individuals or large populations of a multicellular organism. Third, unlike human or rodent models that are limited to low sample numbers, are labor-intensive, and require ethical oversight, *C. elegans* reproduces quickly and in large quantities increasing sample availability, and can be easily manipulated genetically. Despite cell culture being versatile and highly reproducible, *C. elegans* similarly offers manifold cellular reporter assays and additionally the option to identify inter-tissue and behavioral complexities. Collectively, *C. elegans* as a model organism for muscle and mechanical stress research merges the complexity of *in vivo* models with the reproducibility and versatility of cells.

Together, as this work demonstrates, *C. elegans* is suited to quickly and efficiently analyze human pathological variants in conserved muscle proteins and to identify as yet unknown factors that regulate proteostasis on an organism- and tissue-wide scale. The newly developed transgenic models add to the available models of mechanical stress and open diverse opportunities for *in vivo* research on proteostasis mechanisms in an inexpensive and accessible organism.

3.6 CONCLUSION AND OUTLOOK

In summary, this work characterized pathogenic variants in the myosin chaperone UNC-45B that cause a progressive myopathy in humans (publication **A**), reinforced the role of UNC-45 in muscle proteostasis (publication **B**), and identified new regulators of myosin proteostasis in response to mechanical stress in *C. elegans* muscle (publication **C**). This work thereby established a role for UNC-45 in human muscular disease and in the molecular response to mechanical stress in otherwise healthy muscle tissue.

Follow-up experiments in *C. elegans* should further elucidate the collaboration of UNC-45 and NHL-1 as chaperone-E3 ligase complex in myosin protein triage in order to determine interaction kinetics, sequence of events, and relevance in different stress scenarios (Carlisle et al., 2017). In the process, additional sarcomeric substrates of NHL-1 and other collaborators in muscle proteostasis, such as AAA-ATPases and cellular proteases, will likely be identified. In addition, the role of HSP-16 family sHSPs in myosin folding, PQC, and degradation, and in mechanical stress requires detailed investigation, since related mammalian sHSPs execute diverse cellular functions and appear to be heavily involved in mechanical stress protection (Collier and Benesch, 2020). UNC-45 and other proteostasis factors are possibly also involved in the global transcriptional regulation of muscle proteostasis. Indeed, UNC-45B has been reported to genetically and physically interact with transcription factors in zebrafish embryos and murine heart, Hsf1 and GATA4, respectively, and thereby coordinate the transcriptional output during muscle development (Chen et al., 2012; Etard et al., 2015).

Conceivably, the here developed transgenic model, OptIMMuS, will support the examination of the roles of other proteostasis factors in mechanically stressed muscle, e.g. E3 ubiquitin ligases, sHSPs, and transcription factors. Pathogenic variants in UNC-45 or other muscle proteins could thereby serve as sensitized backgrounds or dominant negative disturbances. Using proximity labeling, the *in vivo* interactome of relevant E3 ligases (e.g. NHL-1, CHN-1, or UFD-2) upon mechanical stress could be analyzed to identify as yet unknown context-specific substrates. Refinement of the proximity labeling method by performing the trypsin digest prior to the streptavidin pull-down and subsequent mass spectrometry of biotinylated peptides could offer site-specific insights into interactors of UNC-45 or other proteostasis factors. In addition, the development of advanced bioinformatic approaches will enable the analysis of published and yet-to-generate datasets on tissue-wide transcriptomic, proteomic, and metabolomic responses in the muscle upon mechanical stress. To elucidate the role of autophagy in the response to mechanical stress in *C. elegans* muscle, further experiments using transgenic reporters and inhibitor-based assays will be necessary. Eventually, by using other ChR2 variants, the OptIMMuS model could be refined to allow for the investigation of the

potentially beneficial effects of light mechanical stress or exercise-equivalent contractions as observed in mammalian exercise regimes.

To transfer the findings presented in this work to humans, the corresponding functional interaction of the chaperone UNC-45B and MuRF1/TRIM63 or other TRIM E3 ligases should be corroborated and the impact of pathogenic variants in UNC-45B on this interaction should be examined. Interesting follow-up questions would be the influence of MuRF1/TRIM63 on UNC-45B localization and shuttling or vice versa, as well as the identification and characterization of an orthologue of F40A3.6, whose concrete function remains elusive. In the future, the here reported findings could present research opportunities into new roads for therapeutic intervention, such as the development of protein-protein interaction-mediating pharmaceuticals that will help balance muscle proteostasis during recovery from muscle injury and atrophy, thereby supporting healthy aging and the successful treatment of muscular diseases.

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RESEARCH EXPERIENCE

Research Fellow, PhD Candidate | supervisor: Thorsten Hoppe 2018 – present

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- Research area: UNC-45 and muscle proteostasis in development, aging, and disease

Master Student Intern | supervisor: Assaf Rudich 2016 – 2017

National Institute for Biotechnology in the Negev, Ben-Gurion University, Beer Sheva, Israel

- Research area: Autophagy in white adipose tissue and liver of mice on high fat diet

Research Fellow, Master and Bachelor Student | supervisor: Ralph Burkhardt 2015 – 2018

Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, Faculty of Medicine, Leipzig University, Leipzig, Germany

- Research area: TRIB1-dependent macrophage polarization and interactions in adipose tissue

UNIVERSITY EDUCATION

MSc Biochemistry (Biomedicine) | Final grade: very good (1.1, A) | Leipzig University 2015 – 2017

BSc Biochemistry | Final grade: very good (1.5, approximately A-) | Leipzig University 2012 – 2015

PEER-REVIEWED PUBLICATIONS

Kutzner, C. E., Bauer, K. C., Lackmann, J.-W., Acton, R. J., Sarkar, A., Pokrzywa, W., Hoppe, T. Optogenetic induction of mechanical muscle stress identifies myosin regulatory ubiquitin ligase NHL-1 in *C. elegans*. *Nat Commun*. **2024** *15*(1), 6879. doi: 10.1038/s41467-024-51069-3

Kutzner CE, Bauer KC, Hoppe T. A ubiquitin fusion reporter to monitor muscle proteostasis in *C. elegans*. *microPublication biology*. **2023** doi: 10.17912/micropub.biology.000824

Donkervoort S*, **Kutzner CE***, Hu Y, Lornage X, …, Pokrzywa W, …, Hoppe T**, Bönnemann CG**. Pathogenic Variants in the Myosin Chaperone UNC-45B Cause Progressive Myopathy with Eccentric Cores. *Am J Hum Genet*. **2020** Dec 3;107(6):1078-1095. doi: 10.1016/j.ajhg.2020.11.002 *Co-first author

Müller L, **Kutzner CE**, Balaji V, Hoppe T. In Vitro Analysis of E3 Ubiquitin Ligase Function. *J Vis Exp*. **2021** May 14;(171). doi: 10.3791/62393

Arndt L, Dokas J, Gericke M, **Kutzner CE**, Müller S, Jeromin F, Thiery J, Burkhardt R. Trib1 deficiency modulates function and polarization of bone marrow derived macrophages. *J Biol Chem*. **2018** Jul 20;293(29):11527-11536. doi: 10.1074/jbc.RA117.000703

Wolf J, Haendel N, Remmler J, **Kutzner CE**, Kaiser T, Mothes T. Hemolysis and IgA-antibodies against tissue transglutaminase: When are antibody test results no longer reliable? *J Clin Lab Anal*. **2018**;32:e22360. doi: 10.1002/jcla.22360

APPENDIX

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Donkervoort, S.*, **Kutzner, C. E.***, Hu, Y., Lornage, X., Rendu, J., Stojkovic, T., Baets, J., Neuhaus, S. B., Tanboon, J., Maroofian, R., Bolduc, V., Mroczek, M., Conijn, S., Kuntz, N. L., Töpf, A., Monges, S., Lubieniecki, F., McCarty, R. M., Chao, K. R., Governali, S., Böhm, J., Boonyapisit, K., Malfatti, E., Sangruchi, T., Horkayne-Szakaly, I., Hedberg-Oldfors, C., Efthymiou, S., Noguchi, S., Djeddi, S., Iida, A., di Rosa, G., Fiorillo, C., Salpietro, V., Darin, N., Fauré, J., Houlden, H., Oldfors, A., Nishino, I., de Ridder, W., Straub, V., Pokrzywa, W., Laporte, J., Foley, A. R., Romero, N. B., Ottenheijm, C., Hoppe, T.**, Bönnemann, C. G.** (**2020**). Pathogenic Variants in the Myosin Chaperone UNC-45B Cause Progressive Myopathy with Eccentric Cores. *American Journal of Human Genetics*, *107*(6), 1078–1095. https://doi.org/10.1016/j.ajhg.2020.11.002

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Publication B

Kutzner, C. E., Bauer, K. C., & Hoppe, T. (**2023**). A ubiquitin fusion reporter to monitor muscle proteostasis in *C. elegans*. *microPublication biology*, *2023*, https://doi.org/10.17912/micropub.biology.000824

Publication C

Kutzner, C. E., Bauer, K. C., Lackmann, J.-W., Acton, R. J., Sarkar, A., Pokrzywa, W., Hoppe, T. (**2024**). Optogenetic induction of mechanical muscle stress identifies myosin regulatory ubiquitin ligase NHL-1 in *C. elegans*. *Nature Communications, 15*(1), 6879. https://doi.org/10.1038/s41467-024-51069-3

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