Study the role of patient-specific mutations by genetic disease modelling

From gene to function; A study to understand muscles

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I dedicate this thesis to Sophia and Embla, the brightest light bringers in my existence. They show not only the brilliance for themselves but bring out the best in the people around them; as said in Shakespeare's Hamlet from 1602: "*I am not only witty in myself, but the cause that wit is in other men.*"

I could not have hoped for better love and inspiration in my life.

"It is a profound and necessary truth that the deep things in science are not found because they are useful; they are found because it was possible to find them." - Julius Robert Oppenheimer, 1962

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ABSTRACT

Many genetic diseases inherited in a dominant fashion have a complex pathological pattern. *TOR1A* mediated Dystonia-1 (DYT1) is an example of incomplete penetrance, affecting only a third of the carriers. DYT1 is an early-onset neurological disease affecting dopamine release from substantia nigra to the striatum in the brain, causing muscle tremors in muscles. We have identified the first cases of homozygous *TOR1A* mutation together with a new *TOR1A* mutation all of them showing DYT1 symptoms from birth.

The main part of this thesis has gone to describing the skeletal myosin myopathies Laing early-onset myopathy (MPD1) and myosin storage myopathy (MSM). The diseases are known for causing slow progressive muscle atrophy with huge variations on progression rate. Individuals within the same family can exhibit wildly different speed of atrophy. We show with cell assays that various *MYH7*, which all leads to myosin storage myopathy, are caused by different mechanisms. We also show that *Drosophila melanogaster*, fruit flies, carrying MPD1 and MSM mutations becomes resilient when overexpressing the enzymatic ubiquitin E3-ligase TRIM32. The enzyme is a homolog to the human MuRF enzyme, known to mediate myosin breakdown. Lastly we have found a family where a mutation in the myosin folding chaperone UNC-45B drives the heart condition hypertrophic cardiomyopathy. UNC-45B have been shown to be important for embryonic heart development but never been found to be associated with any muscle disease before.

Keywords: Muscles, Myosin, MYH7, Myosin storage myopathy, Laing early-onset myopathy, Drosophila, TOR1A, DYT1, HCM, heart disease

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SVENSK SAMMANFATTNING

Många genetiska sjukdomar har en komplex sjukdomsprofil med många faktorer som spelar in. Dystonia-1 är ett bra exempel på detta. Den orsakas av mutationer i genen *TOR1A* som påverkar dopamin utsläpp från substantia nigra till striatum. Detta gör att patienten får muskelryckningar, kramper och får försämrad motorik, ungefär på samma sätt som Parkinsons sjukdom. Även om mutationerna i *TOR1A* är dominanta så är det bara en tredjedel av alla patienter som utvecklar dystonia-1. Vi rapporterar om de första fallen där patienter fått sjukdomsmutationen från båda sina föräldrar. Samtidigt har vi upptäckt en ny, tidigare okänd, *TOR1A* mutation som också leder till dystonia.

I min avhandling har jag mest fokuserat på myosin-muskelsjukdomar. Proteinet myosin är en av de viktigaste delarna för att muskler ska kontrahera. När muskeln får signal så binds myosinprotein till aktin och börjar vrida sig, vilket drar ihop muskelcellen till en kontraktion. Genen *MYH7* producerar myosin i hjärtat och i långsamma skelettmuskler som ska kunna jobba under längre perioder. Flera mutationer i *MYH7* orsakar inga hjärtproblem, men leder till en gradvis försvagning av skelettmuskler. Genom att mutera och odla muskelceller, har vår forskning visat att olika mutationer som leder till samma sjukdom drivs av olika mekanismer. Vi har sedan muterat bananflugor där vi lyckats visa att om flugorna överproducerar ett enzym, kallat TRIM32, får de ett molekulärt skydd emot *MYH7*-muskelsjukdomar. TRIM32 är en del av flugans ubiquitin-system som finns för att bryta ner gamla och slitna proteiner som behöver ersättas och förnyas. De här kunskaperna är viktiga för att förstå varför vissa patieter utvecklar sjukdomen mycket långsammare än andra för att inte tala om potentialen att utveckla terapeutiska läkemedel mot muskelsjkdomar.

Sist så har vi identifierat en ny gen som orsakar hjärtsjukdomen hypertrofisk kardiomyopati. Sjukdomen är den vanligaste orsaken till plötslig hjärtdöd hos unga atleter och drivs av att mutationer i olika muskelgener ökar tjockleken på hjärtats vänstra kammare. Detta blir ett ökat tryck på hjärtat som kan orsaka plötsligt hjärtstillestånd vid ansträngning. Vi har hittat och beskrivit en familj som bär på en mutation i genen *UNC45B*, som är viktig för att preparera myosin innan myosin sätts in i muskelcellens stora muskelkomplex. Man har i tidigare forskning visat att om *UNC45B* inte fungerar normalt så leder det till utvecklingsfel i hjärtat, men man har inte kunnat konstatera något fall där mutationen orsakar någon sjukdom hos patienter. Detta utvidgar vad vi innan visste om hypertrofisk kardiomyopati.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Halvarsson D.M.**, Pokrzywa M., Rauthan M., Pilon M., Tajsharghi H. Myosin Storage Myopathy in *C. elegans* and Human Cultured Muscle Cells.
PLoS One, 2017 Jan 26;12(1):e0170613.

II. Kariminejad A., **Halvarsson D. M.**, Ravenscroft G., Afroozan F., Goullee H., Davis R. M., Laing G. N., Tajsharghi H. Recessive TorsinA variants cause severe arthrogryposis developmental delay, strabismus and tremor. Journal of Brain, 2017 Sep 23; 140 (11): doi: 10.1093/brain/awx230

III. **Halvarsson D.M**., Olivé M., Pokrzywa M., Ejeskär K., Palmer R.H., Uv A.E., Tajsharghi H. *Drosophila* model of myosin myopathy rescued by overexpression of a TRIM-protein family member. PNAS, 2018 Jul 10;115(28) doi: 10.1073/pnas.1800727115

IV. **Halvarsson D.M**., Olivé M., Pokrzywa M., Norum M., Ejeskär K., Palmer R.H., Uv A.E., Tajsharghi H. *Drosophila* model of myosin storage myopathy rescued by overexpression of a TRIM-protein family member. Submitted manuscript, PNAS, 2019 Feb.

V. Emrahi L.*, **Halvarsson D.M**.*, Moselmi A.R., Hesse C, Goullée H., Laing G.N., Tajsharghi H. UNC45B, a co-chaperone required for proper folding and accumulation of myosin, as a novel gene associated with hypertrophic cardiomyopathy. Manuscript

* authors contributed equally to the study and share first authorship

CONTENT

ABBREVIATIONS

MyHC	Myosin heavy chain protein
ATP	Adenosine 5’ triphosphate
ADP	Adenosine 5’ diphosphate
MYH	Human myosin heavy chain gene
MyBPC	Myosin binding protein C
TRIM32	Tripartite Motif32
MuRF	Muscle RING finger protein
Hsp	Heat-shock protein
MPD1	Laing distal early-onset myopathy
DYT1	Early-onset Dystonia-1
HCM	Hypertrophic cardiomyopathy
GFP	Green fluorescent protein
C.elegans	*Caenorhabditis elegans* nematode worms
Drosophila	*Drosophila melanogaster* fruit flies
IFM	Indirect flight muscles
Mhc	Drosophila myosin heavy chain gene
UAS	Upstream activating sequence
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
PAM	Protospacer adjacent motif
TDT	Tergal depressor of trochanter (jump muscle)

DEFINITIONS IN SHORT

Sarcomere	A repetitive protein complex of muscle proteins. The complex becomes tighter when myosin begins to interact with actin, which is how a muscle contract.
In vitro and *In vivo*	"*In vitro*" means "in glass" and refers to biological experiments performed outside a living organism, unlike "*in vivo*" where experiments are done inside an organism. I place cellular assays into the *in vivo* category of experiments. This thesis will mainly describe and review *in vivo* experiments but will also compare and discuss results from *in vitro* protein-interaction assays.
Genes vs. proteins	The thesis follows the generally accepted standard of writing gene names with italic cursive style, while proteins are not (for example: The gene *UNC45B* produces the protein UNC-45B). Some established genetic *Drosophila* models (alleles) are written with small (superscript) letters (but with italic style), such as Mhc^{10}.
Genotype and phenotype	Common concepts in genetic science. Genotype means genetic makeup or a specific mutation (the $MYH7^{K1729\Delta}$ and normal *MYH7* are two different genotypes). Phenotype is visual traits that can be seen (such as how proteins can be seen to arrange themselves when stained through immunofluorescence).
MyHC-I and MyHC-II	MyHC refers to the protein myosin heavy chain, the number I and II indicates which type of skeletal muscle the MyHC isoform

	belongs to. MyHC-I is slow type I muscles and MyHC-II is a fast type II muscle.
Immunofluorescence staining	Immunofluorescence staining is a standard qualitative analytical technique, where cells or dissected samples are treated with antibodies that specific for a certain protein. The antibodies carry a "fluorochrome", a molecule that emits light of a certain colour/wavelength which can be seen in a light microscope. This means that e.g. muscle cells can be stained with antibodies where myosin is stained red, UNC-45B is stained green and titin is stained blue. Through this method, one can study the location or pattern of proteins in the sample.
-genesis	The word comes from Greek and mean "origin" and can in biology (and in this thesis) be put together with another word to describe a beginning of a process. Such as: Sarcomerogenesis – The beginning of sarcomere formation, Pathogenesis – the initiating process of a disease, Myofibrilogenesis – Early muscle fibre formation.
Myosin or Myosin heavy chain	When referring to "myosin heavy chain" (or MyHC) it is common to only say "myosin", but there is a risk of confusing it with the proteins "myosin essential light chain" and "myosin regulatory light chain". In this thesis, only the effects of myosin heavy chain are explored, so "myosin" and "myosin heavy chain" could be considered the same.

1 INTRODUCTION

1.1 Muscles – Function of the sarcomere

Striated muscle tissue is a highly specialised group of cell types consisting of skeletal and cardiac muscles. All striated muscle cells (myocytes) contain sarcomeres, which are repetitive muscle protein complexes. The sarcomere complex produces mechanical energy through structural changes in the constituent proteins, which shortens the sarcomere causing the muscle to contract.

Muscle research has been ongoing for hundreds of years as anatomy and pathology is one of the older studies within medicine. During the 19th century, scientist confirmed that some areas in the muscle filaments could be divided into darker and lighter areas (more or less protein-dense areas) when studied under light microscope (1). This led to a division of the muscle filament into separate zones; Z-discs, H-zone, M-, A-, and I-bands (Figure 1A). With time, different proteins have been found in the distinctive areas. The modern theory of how muscles contract started to take form back in the 1950s when thick filaments were observed to slide across thin filaments. This led to the understanding that proteins in the thick filaments (myosin) were pulling themselves across proteins in the thin filaments (actin) (2, 3). Myosin, or myosin heavy chain (MyHC), is attached to the centre of the sarcomere, in the M-band, and stretches through the H-zone and A-band. Actin on the other hand is fastened in the Z-disc and extends through I-band and a good way into the A-band. Both the M-band and the Z-disc consist of a number of proteins, and are connected with the cell membrane, anchoring the sarcomere structure in the cell.

The contraction is driven by MyHC binding and hydrolysing an adenosine 5' triphosphate (ATP) into an adenosine 5' diphosphate (ADP) (4). The "ATP-charged" MyHC attaches itself to actin, forming a cross bridge. When releasing the hydrolysed ADP and the phosphate, the MyHCs neck-hinge region performs a tilt motion. This conformational change pulls MyHC along actin, shortening the sarcomere. MyHC lets go of actin by binding to a new ATP. The process can then start over with MyHCs forming a new cross bridge but it starts with a shorter sarcomere. When the next cross bridge is formed, MyHC will drag the thick filament even further along the thin

filament with the ATP-driven force. This causes every myosin-actin interaction to shorten the sarcomere into a contraction. Not every MyHC finds an actin to form cross bridges at the same time; it is always a question of different amount of interactions. If only a few MyHCs can attach to actin, the thick filament slides down from the thin filament and the muscle relax, whereas several MyHCs interacts with actin causes the sarcomere to contract (5, 6). To regulate the cross bridge process, the protein complex tropomyosin-troponin blocks MyHC from interacting with actin by coiling around actin. To enable contraction, tropomyosin-troponin complex must slide across actin to expose it for MyHC. To initiate this process, the myocyte receives an electric signal from a cell, stimulating the membrane-bound sarcoplasmic reticulum to release calcium ions into the myocyte cytoplasm. The calcium binds to troponin, making the associated tropomyosin filament expose actin to MyHC and a cross bridge can be established (5, 6).

1.1.1 Myosin heavy chain

The MyHC protein can be roughly divided into an ATP- and actin-binding head domain, a long tail domain used for stabilising and keeping the protein in place in the sarcomere, and a hinge/neck region in between to make the protein flexible (Figure 1B) (7, 8). In the head region, there is a series of secondary structures, consisting of 17 α-helices that surround seven β-sheets (8). Together they build up the actin-binding and ATPase functional domain that hydrolyses ATP. MyHCs are paired up two-and-two into a dimer by coiling their tail regions around each other, forming a structure often referred to as coiled-coil domain. The two MyHC monomers arrange themselves so that hydrophobic amino acids face the other monomer, because they are repelled by the surrounding liquid that make up the cell's cytosol, and hydrophilic faces outwards. This is possible due to the string of amino acids forming a repetitive pattern of twenty-eight residues (Table 1) (9, 10). The twenty-eight residues coil around its own axis eight times. This repetitious pattern is more commonly divided into four sets of seven amino acids, referred to as heptamers (Figure 2). This setup makes MyHC very stable as the hydrophobic agents are drawn together while the hydrophilic amino acid residues pull the MyHCs together.

There are several MyHC isoforms, produced by different *MYH* genes. Each isoform is specialised for different purposes and muscle tissues (11). A couple of the MyHCs are classified as non-muscular, also known as unconventional, MyHC (*MYH9, MYH10, MYH14*). They serve as vesicle transporters or mediate cell migration by pulling themselves across the cell skeleton (12). Other MyHC isoforms are considered mainly embryonic

(*MYH3* and *MYH8*) and are found primarily during foetal development and early childhood (11). The conventional muscular MyHC are mainly divided into fast type-II skeletal muscle myosin (*MYH1*, *MYH2*, and *MYH4*), slow type-I skeletal myosin fibres (*MYH7*), and cardiac myosin (*MYH6*, *MYH7*, and *MYH14*). The differences between the muscular isoforms are subtle and are mostly in the head- and hinge-regions to make each isoform more suited for its purpose. For example, there are differences between the force needed in the arms' fast muscle MyHC compared to the steady and continuous contraction of the heart muscle. Many types of muscles have a combination of different isoforms to be able to perform a variety of type of works.

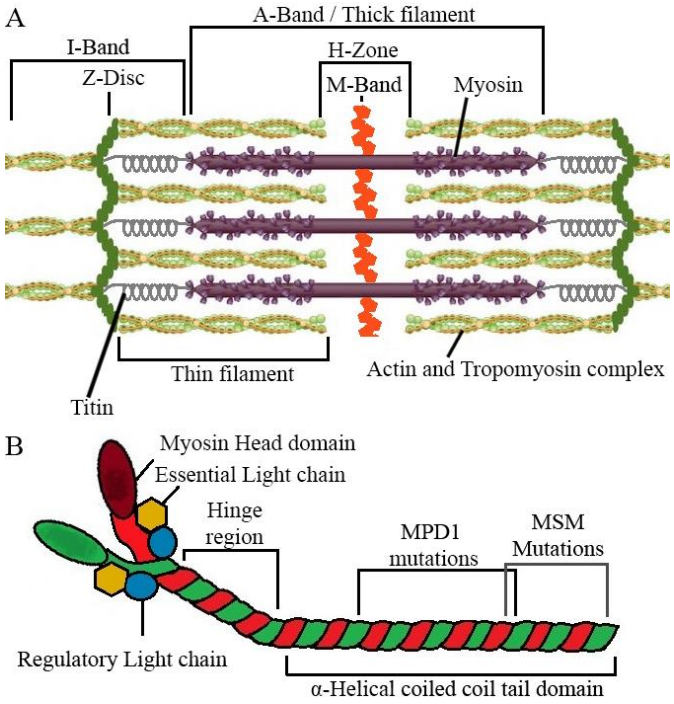

Figure 1 Schematics of the sarcomere complex and myosin (**A**) Sarcomeres are repetitive protein complexes divided into zones depending on what proteins they contain. Myosin is found in the thick filaments, called A-bands. (**B**) Two myosin proteins form a dimer by coiling their tail region together. Mutations in the end-part of the tail region cause myosin storage myopathy, while mutations in the middle part of the tail lead to Laing early-onset distal myopathy. Mutations in the head region give rise to different heart conditions.

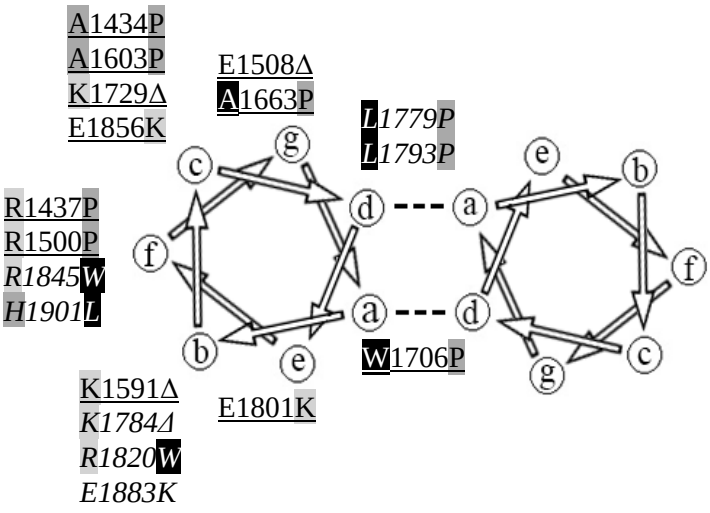

Figure 2: Amino acids in the myosin tail form four repetitive spirals of seven residues per spiral. The residues in myosin's tail coils around its axis. The positions of the amino acids are called a-b-c-d-e-f-g. Hydrophobic residues (dark) faces the other myosin monomer (position a and d). Hydrophilic amino acids face outwards (b, c, f, g, and e) consisting mainly of negative (white) and positive (light grey) residues but also an occasional neutral amino acid, not considered hydrophobic nor hydrophilic (dark grey). Amino acid substitutions cause either Laing distal myopathy or myosin storage myopathy depending on locus and nature of the mutation. Mutations associated with Laing distal myopathy are underlined and those linked to myosin storage myopathy are in italic. The conserved positions of each type of amino acid are presented in Table 1. General descriptions of amino acids' traits are presented in supplementary table 1.

1.1.2 Tripartite Motif proteins – TRIM proteins

To keep a cell healthy and alive it has to be able to break down and degrade old, damaged, or malformed proteins to give space to new fully functional ones. One way to do this is to ubiquitinate the bad protein, which means adding a ubiquitin-molecule to it. Ubiquitin is a signal to heat-shock proteins (Hsp) and other degrading units, telling them to break down the protein it is attached to (13). To make this system well functional there are hundreds of different ubiquitin-adding enzymes that are specialised in recognizing

Table 1: The amino acids in the myosin tail follow a conserved pattern of 28 residues.

Position in heptamer		a	b	c	d	e	f	g	a	b	c	d	e	f	g
Residue loci[a]	1-14	L	E	E	L	Q	E	Q	L	D	E	E	E	R	A
	15-28	L	A	K	L	E	K	Q	K	K	K	L	E	Q	E

a: Traits of amino acids are shown by colours. Dark - Hydrophobic; Dark grey – uncharged low hydrophobic; Light grey – Positive charged hydrophilic; White – Negative charged hydrophilic.

abnormal protein structures. These enzymes are called ubiquitin E3-ligases and each enzyme is highly specific to one specific kind of protein or structure (13-15). This specificity serves to minimise accidental targeting of normal, well-functioning proteins for breakdown or missing a damaged protein. In muscles, there is a set of E3-ligases specific for MyHC, called Tripartite Motif (TRIM) or, in humans, more commonly called Muscle RING-finger proteins (MuRFs). They scan MyHC, looking for changes in its structure, which makes them essential for long-term prosperity of the muscle cells. As such, MuRF is usually used as a marker to determine the levels of atrophy and muscle degradation (16). Rare variations of MuRFs have been shown to change the impact of mutations in *MYH7*, *MBPC3*, and *MYL2*, which are associated with the heart condition hypertrophic myopathy (17). The MuRF polymorphisms increase the pathological effect of the mutated genes, making the symptoms more prominent.

In article III and IV we explore how TRIM32, the *Drosophila* homologue of MuRFs, can influence the disease progression of skeletal myosin myopathies.

1.1.3 UNC-45B

After a protein has been produced it needs to be folded to gain its properties. Folding of proteins is performed by a series of Hsp and chaperons. One important folder of MyHC is UNC-45B, a chaperon that folds the secondary structure of the head domain. UNC-45B is composed of an N-terminal tetratricopeptide repeat domain that binds the co-chaperone Hsp90a, a

conserved central domain of armadillo repeats, and a C-terminal domain that interacts with the MyHCs motor domain (18, 19). UNC-45B binds to the MyHC head domain, recruits Hsp90a and, together with Hsp70, they fold MyHC into its proper form (20, 21). All striated muscle development and muscle regeneration is dependent on that these chaperons function properly, making UNC-45B a very important protein.

1.2 Muscle- and neurological diseases

Since the sarcomere consists of a large number of proteins, there are a lot of entities that has to cooperate for the whole unit to function. When one of the proteins has a decreased function or lacks ability to seat itself into the sarcomere it affects the whole muscle structure. Depending on which gene and protein that is mutated, the disease exhibits different mechanistic changes. This can lead to very distinct symptoms and characteristics that can be identified as a unique disease. In some cases where the mechanisms are known, the disease has been shown to be treatable (22, 23). The main symptoms observed in myopathies can be very similar, with muscle weakness, cramps, and muscle stiffness, but the nuances in pathology makes each disease distinct. This highlights the importance of researching what changes in protein interactions, are caused by various gain- or loss-of-function mutations.

1.2.1 Myosin storage myopathy – MSM

The gene *MYH7* is responsible for producing MyHC in slow skeletal muscles and in the heart's ventricles (11). The first genetic link to any striated muscle disease was a mutation in the head-domain of *MYH7* (R403Q), causing hypertrophic cardiomyopathy (24). In the year 2000, almost 10 years later, the first purely skeletal muscle disease was identified, caused by a mutation in the fast skeletal type-II muscle gene *MYH2* (E706K), which led to a high interest in myosin diseases (25). The first mutation causing myosin storage myopathy (MSM), $MYH7^{R1845W}$, was identified in 2003 due to new tools for genetics analyses (26). The disease had been clinically reported as early as in the 1970's, but back then no genetic analyses could be made (27). Over the years, several new MSM mutations (all of them dominant) have been found as the disease profile has slowly been revealed (11, 26, 28-31).

The most characteristic phenotype among patients is that MyHC-I aggregates to form clumps that are framed by other proteins, such as desmin, αβ-

crystalline, ubiquitin, and myosin binding protein C in slow muscle tissue. The aggregates, sometimes called hyaline bodies, have distinct vacuole-like structure and can be seen histologically when cross sectioning muscle tissue,. Patients show primarily skeletal muscle weakness and atrophy, with more prominent disease development in proximal parts of the limbs (thighs, pelvic-girdle, shoulder, biceps, and triceps). The disease is seldom connected with a shorter life expectancy, but the weakness often leads to problems with mundane tasks such as climbing stairs, reaching the arms above the head or raising oneself to sitting position after lying down. The loss of muscle strength gets progressively worse over time but the time points when patients begins to experience symptoms varies (this is called time-of-onset). The time-of-onset spans from clearly defined symptoms at birth to not having any prominent symptoms before the age of 30.

Even though *MYH7* is expressed in both skeletal and heart muscles, the position of the mutation decides whether the symptoms are going to be primarily skeletal or cardiac. Mutations in *MYH7* head domain causes heart diseases and mutations in the tail domain drives skeletal diseases (11, 32, 33). In a few cases, MSM patients have developed heart conditions, such as dilated cardiomyopathy, where the size of the muscle tissue on the heart's left ventricular side is increased (34). Why some MSM patients develop heart diseases while others have exclusively skeletal muscle symptoms is presently unclear. This shows that there are several unknown factors that dictate the pathological process. The loci of the MSM associated mutations are spread out among exon 37 to 40 which encode the end of the coiled-coil rod region (35). This is close to a 29 amino acid sequence (residues 1874 to 1902) known for assembly of the MyHC dimer and anchoring it to the M-band (36).

To further discuss the nature of MSM mutations, the traits and abilities of the amino acid residues in the MyHC tail are important to understand. As already mentioned, the MyHC tail arranges itself to make the hydrophobic amino acids face the other monomer-tail while the charged and/or hydrophilic amino acids lie outwards (Figure 2). A swap of a hydrophobic residue to a hydrophilic one, or vice-versa, would interfere enormously on the structure of MyHC dimers (Table 1). For example, *in vitro* studies show that mutations where a positively charged arginine is changed to an uncharged, hydrophobic tryptophan (such as the mutation R1845W) increase the binding force between the two MyHC molecules (37, 38). If this would cause an inability of MyHC to be broken down or to insert itself into the sarcomere is still not known. It is thus not certain if MSM-causing mutations make MyHC act in the same manner.

1.2.2 Laing early-onset myopathy – MPD1

While mutations in the far distal C-terminal end cause MSM, as mentioned above, mutations in the middle and semi-distal C-terminal end lead to Laing distal myopathy (MPD1) (11, 33, 39, 40). The loci of most mutations for MPD1 are found within exons 32 to 38 (11, 35). The general symptoms that patients experience are progressive atrophy and muscle weakness mainly in distal limbs rather than proximal limbs as in MSM. This causes weakness in the patients' hands, feet, wrists, and ankles. One characteristic that is highly specific for MPD1 and is considered a marker for the disease is "dropping-big-toe". This is caused by a diminishing of the muscles that operate the toes, making the patient unable to raise the big toes (11, 41). The disease does not exclusively affect muscles in distal libs, but in many cases shoulders and pelvis also suffers from muscle impairment. The level of atrophy and time-of-onset can vary even within the same family (42, 43).

Many patients develop pseudohypertrophy in the calves, were the muscle tissue becomes atrophic and deteriorate but the body builds up connective and fatty tissue that take the place of the muscles, making them look bigger (42, 44). The time-of-onset varies to the same levels as in MSM, between new born up to the age of 30. Cardiomyopathic involvement is rare but some cases include dilated cardiomyopathy (45). The two main features that set MPD1 apart from MSM are that MPD1 do not generally form hyaline body accumulations of MyHC and that MPD1 affects the distal limbs to a higher degree than MSM (11). The same principle of hydrophobicity-hydrophilicity switch in amino acid residues seen in MSM also applies to MPD1 (11). The affected amino acids are often those in the outer parts of the heptamer but they are not restricted to only outwards-facing amino acids. Compared to MSM, MPD1 is often caused by change of an amino acid to proline (46-48). Whether it is just that no MSM mutation featuring a proline has been found or if proline do not have traits that would lead to MSM is not known. But MPD1 is very diverse with reported mutations at all constellations in the heptate that can also overlap with the regions of mutations causing MSM. (11). This makes the mechanisms that drive *MYH7*-mediated diseases seem complex and shows the importance of further studies to understand and help patients suffering from these maladies.

1.2.3 Dystonia-1 – DYT1

Many genetic diseases have a multi-facetted profile with an incomplete penetrance making patients' symptoms appear different from case to case, such as the *MYH7* diseases already mentioned. Another example of incomplete penetrance is the neurological disease early-onset dystonia-1

(DYT1). The disease is often caused by an autosomal dominant mutation in the gene *TOR1A*. The disease is considered to be inherited in a dominant fashion but with an incomplete penetrance. Only between 30-40% of identified carriers show any sign of symptoms before 28 years of age, while those who do suffer from symptoms does so from infancy or early childhood. The most common mutation is an in frame three-base deletion of *TOR1A*, removing a glutamate residue close to the C-terminal end of the protein (E303Δ) (49). Less common mutations have been identified, such as a six amino acid deletion close by (F323Δ- Y328Δ) (50, 51). We present in article II a new mutation (G318S), found through NGS analysis of several patients that manifested the disease.

Symptoms associated with DYT1 are involuntary muscle tremors, and twitches. Infants also exhibit arthrogryposis, which is a high level of fibrosis in muscles, inhibiting them from extending and stretching the muscle due to its short length.

The *TOR1A* gene expresses TorsinA, an enzymatic protein with two ATP - interacting domains called α/β domains and a conserved string of 220 amino acids, which places it in the family of AAA+ adenosine triphosphatases family (family of ATPases with diverse functions and activities) (Figure 3) (52, 53).

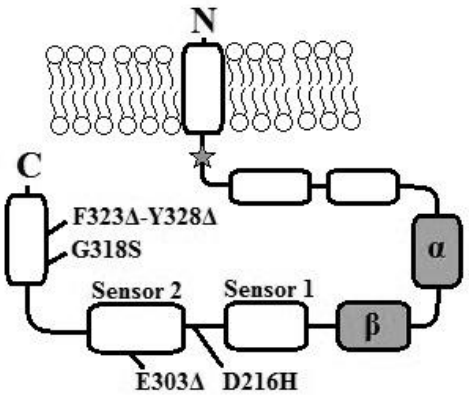

Figure 3: The TorsinA protein. TorsinA assists in dopamine release in the brain. Several known mutations in the end part of the protein have been found to cause dystonia-1 with involuntary muscle tremors. E303Δ is the most common mutation but others have been observed. Star shows where the enzyme is cut to start translocation into the endoplasmic reticulum lumen.

Downstream of the ATPase domains comes an α-helical domain called sensor 1, which is essential for performing ATP hydrolysis, as it mediates substrate interactions (54). Further downstream, close to the C-terminal end, is another α-helical domain called sensor 2, which assists in the transmission of the energy to the substrate by ATP-cleavage: It does so by forming a hexameric

pocket for ATP (55). The E303 amino acid is located in this sensor 2 domain, while G318 and the six amino acids F323-Y328 lie just downstream of the domain (54). The location of TorsinA proteins vary between cell types; In HEK cells, TorsinA is found enveloping the nucleus associated with nuclear envelop proteins, and inside the ER (56-58); In neurons, the presence of TorsinA is found in the synaptic end as well as in the ER lumen (59-61). TorsinA interacts with several proteins connected with vesicle transportat, such as synaptobrevin, VMAT transporter 2, and synaptotagmin I. When knocking down endogenous TorsinA in neurons, the cells begin to accumulate an increased amount of synaptogamin I along the synapse membrane, a phenomenon also detected in G303Δ (62-65). The protein is expressed to medium levels in several areas of the brain; cerebral cortex, striatum, hippocampus, midbrain, pons, and in motor neurons in the spinal cord. The most notable is that the dopaminergic neurons going from substantia nigra expresses high levels of TorsinA (55, 66). The neurons connect the substantia nigra to the striatum, which is responsible for both motor planning and sensorimotor processing (it is also the brain's reward system). The main mechanism that drives DYT1 is thought to be a loss of function in dopamine release in striatum, even though TorsinA is expressed in a lot of other areas of the brain (62, 67, 68). Patients have been observed to have developmental delays at early age but no degradation in any area has been found in patients.

An uncommon amino acid polymorphism in *TOR1A* has been found, where an aspartic acid is substituted to a histidine (D216H). This polymorphism has been determined to negate the effect of G303Δ (61).

Several forms of dystonia have been seen to be treatable with high doses of anticholinergic agents as early as in the 1980s, but since the drugs are non-selective muscarinic antagonists they cause a lot of side effects (69). Over the past two decades, we have seen an increase in research in potential treatments of dystonia diseases (70). Treatments for DYT1 today still consist of anticholinergic agents (such as trihexyphenidyl), botulinum toxin injections, and surgical therapies but evaluation of deep brain stimulation and new specialised drug treatments with fewer side effects have a high priority in the research field (70-73).

1.2.4 Hypertrophic Cardiomyopathy – HCM

Sudden cardiac arrest is a relatively common lethal condition among young athletes. Among the reported cases of sudden cardiac arrest, hypertrophic cardiomyopathy (HCM) is the most reoccurring. HCM is responsible for

approximately 40% of sudden cardiac arrest among athletes under the age of 35 and have been observed in all cultures (74-76). In the normal adult population the prevalence falls between 1:344 and 1:625 without much difference between countries (77-80). The general phenotype is an elevated thickness of the heart's left ventricle together with tissue fibrosis, decreasing the cardiac output (81). This puts the body's circulation and ventilation system on a strain. The lack of cardiac output can make the patient out of breath at low strain and can lead to heart attack or sudden cardiac arrest during heavy exercise (76, 77). The diagnostics includes reviewing of family history, ECG, echocardiography, cardiac MRI, and genetic screening (81). Some patients, especially among athletes, do not exhibit any symptoms at all before a sudden cardiac arrest event, making preventive diagnostics hard without prior knowledge of any case in the family (82). Heavy regular exercise in itself often causes a slight thickening of the left ventricle wall, which makes the disease even harder to diagnose. Genetically, HCM is a heterogeneous disease and can be caused by several mutations in different genes. The first mutation detected was a substitution mutation in the head region of *MYH7*, R403E (83, 84). *MYH7* have later been established as one of the main causes of HCM and together with the gene for myosin binding protein C-3 (*MYBPC3*) is responsible for half of all diagnosed HCM cases (32, 85-88). The mutations of *MYH7* associated with HCM are almost exclusively in the head region while, as mentioned in the sections on MSM and MPD1 diseases, mutations in the *MYH7* tail region causes skeletal myopathies, sometimes without any cardiomyopathic involvement. Mutations in several other genes have been shown to cause HCM, such as *ACTC1* (cardiac actin), *MYL2*, and *MYL3* (myosin light chain genes) (89, 90). We have found a family where a mutation in the *UNC45B* gene causes HCM. Since *MYH7* is one of the main causes of HCM, the chaperone UNC-45B, which helps fold MyHC, has been under investigation for any link to the disease (91). The chaperone normally attaches to the MyHC head and, together with Hsp70 and Hsp90, assists in assembling MyHC into the thick filament of the A-band (20, 21). We describe this novel *UNC45B*^{Q61L} genotype in article V.

1.3 The importance of models

In the process of studying any disease, the researcher has to apply some kind of model when setting up an assay. One way to perform experiments on muscle diseases is to use patient samples, such as biopsies and establishment of cell cultures from these. Patient samples can be seen as the first step to understand a malady since no genetic modifications are required as it already

carries the genetic defect causing the disease. Unfortunately, patient derived samples of uncommon diseases can sometimes be extremely scarce. To identify any mechanism of cardiac involvement, for example, could be considered impossible since obtaining biopsies from a living heart muscle is not possible with today's technology. Also, when studying cells, muscle fibres, and tissue from patient derived biopsies it can be hard to determine if any deviation is caused directly by the mutation. Any observed malformation or gain/change/loss-of-function could be a secondary feature that arises from the disease or from aging but is not typical among patients. An abnormal phenotype can also be caused by mechanical force when harvesting biopsies or cells. Finally, medical doctors and researchers around the world have different procedures to analyse the already very scarce material. All these factors make it very troublesome to determine exact mechanisms of the disease.

To identify key features and functional changes caused by specific mutations, it is therefore vital to consider alternative models and methods. Even though models are needed to get a deeper understanding of pathology and biological mechanisms, every model and assay has its own limitations and faults that must be considered. Simpler models might have fewer factors that distort the results, making the connections between genotype and phenotype more clear. But a simple model can be far from representative in showing how the disease takes form in a human. Add these two variables together and you get a spectrum that goes from simple but not representable on one extreme end and highly or fully representable but obscure and unable to identify the actual mechanisms on the other end. Consequently, balancing somewhere in the middle by compromising on one of the two factors and combining information from different models is a way to approach this dilemma. In this fashion one can set up models to complement each other, strengthening the weak points that each separate model possesses on its own.

2 AIMS

With this thesis, the general aim has been to investigate mechanisms that drive genetic diseases affecting muscle function. The diseases display an incomplete penetrance, showing high variation in progression rate and severity even between individuals within the same family. This means that unknown factors apart from the main causative mutation are involved in protecting the muscle tissue or driving the diseases. Many of the diseases had not earlier been explored to any detail, which prompted us to develop new models to investigate disease mechanisms and factors that influence the pathology.

Specific aims

Article I

In vitro experiments have shown that MSM-causing mutations promote different interaction traits in myosin monomers depending on the type of mutation. The aim was to determine if the different mechanisms cause different cellular phenotypes in an extrachromosomal assay.

Article II

TOR1A-derived dystonia is known for its incomplete penetrance, and only 1/3 of mutant carriers develop any symptoms. The aim of this project was to learn more about the profile of dystonia by identifying and describing the pathology of new TOR1A mutations that cause dystonia.

Article III

The disease MPD1 has not been studied to the same degree as for example MSM and there is not much known about the pathological progression. The aim of this project was to determine the deterioration of muscle fibres and a potential protecting effect of TRIM32 in MSM (caused by which mutation) by analysing the corresponding mutation (*Mhc*$^{K1728\Delta}$) in *Drosophila melanogaster*.

Article IV

The mutation *MYH7*^{R1845W} makes myosin dimers become extremely stable, resulting in the MSM-characteristic myosin aggregates. The aims were to investigate the pathogenesis and progression of MSM, as well as the impact of overexpressing TRIM32.

Article V

HCM is a heterogeneous disease, caused by several different mutations. Since *MYH7* is the most common gene that leads to HCM, UNC45B have been under investigation for heart diseases as its role is to fold myosin. The aims were to determine and describe the pathogenesis of a novel UNC45B mutation that causes HCM.

3 METHODS AND ASSAYS

3.1 Cell culturing

Growing and analysing cell cultures are a good way to study genetic diseases. Using human cells gives a good representation of what happens in humans on a cellular level but they lack the interaction that cells experiences when they grow inside an actual body. Cell cultures are usually grown on the bottom of plastic plates or flasks, although glassware can also be used (92). Cells do not like the surfaces of plastic or glass, which makes the process of growing them problematic. Research groups and companies have been experimenting with different coatings and surface treatments to come up with good products for cell culturing. A common culture plate treatment is to add synthetic polymers like poly-D-lysine to increase the cells' adhesion properties (93, 94). A diverse set of coatings with adhesion molecules such as gelatinous hydrogels, fibronectin, and other molecules can also be used (95-97). Different cell types behave differently on different plates. So when someone takes up cell culturing it is always a good idea to read up on reports and to learn about what works best for the cell in mind. The cell culture must also be covered by liquid medium, containing nurture, growth factors, insulin and other essential reagents important for cells to grow and survive. Labs usually have their own optimized protocols for their cells. A standard protocol usually consists of medium (like Dulbecco's Modified Eagle's Medium, DMEM), 10% bovine serum, and some amount of penicillin and streptomycin (to protect the cells from bacterial infections). There are also three main categories of cells when culturing: Cell lines, primary cell cultures, and secondary cell cultures. Cell lines are cells derived from a single cell and therefor have the exact same genetic background as each other. Cell lines have often been modified to be long-lived or immortal, making them easy to grow. Primary cells are derived directly from a piece of tissue, organ or biopsy (from patients or lab animals). When primary cells are subcultivated or frozen for cryopreservation they are considered secondary cultures.

3.1.1 Cellular gene transfection

Primary cell cultures from patients can carry a lot of other divergent changes. These mutations and changes can be without clinical importance but can change the cells' growth- or protein expression pattern (98).

A way to work around this problem is to use an established cell line or primary wild type cells and insert the mutated gene. Gene insertion can be done through several methods, such as lipofection, electroporation, and virus-mediated infection (99). Genes often have to be cloned into a DNA-ring, called plasmid, first. Today, plasmids are designed to be easily opened with enzymes to insert genes, using different gene-cloning kits. A tool that can be utilised is to use a plasmid that also contains a green fluorescent protein (GFP) –reporter gene. When the plasmid enters a cell and the endogenous gene expression system transcribes genes encoded in the plasmid, GFP is also expressed. The GFP signal thus shows in which cells the plasmids have entered. If the GFP protein-coding region is fused to that of the protein in interest, it can also be used to see where on the cells the proteins end up. With lipofection, one combines the plasmid with lipid drops and lipofection-mediating reagents that facilitates the process. The plasmids are taken up through the cells' own system of lipid endocytosis. Viruses have been shown to be a very effective gene-insertion method and can yield cells with stable transgene expression (100). Gene insertion through viruses possesses contamination- and health risks as viruses can accidently spread to other hosts (101). This makes lab security, efficient protocols and lab facilities a top issue when working with viruses. Electroporation is another way of introducing genetic material. The exact principle is unknown, but a short electrical charge causes some kind of disturbance in the cell membranes, making the cells take up the plasmids (102). The success rate of transfections is higher when relatively undifferentiated cells are used, compared with transfecting highly differentiated cells. This means that when working with muscle cells, the transfection efficiency is greater when using myoblasts than using myocytes. After the transfection, the cells can be differentiated to become fully differentiated myocytes with sarcomere complexes.

Lipofection-mediated gene insertion was used in articles I, II and V. In article I, myoblast cells were transfected with GFP-plasmids, containing either wildtype or mutated forms of *MYH7*. This made the cells express either wildtype MyHC-I or different MyHC-I mutations. The same protocol was used in article II where the hepatic HEK cells were exposed to *TOR1A* with the G318S mutation, causing DYT1. In manuscript V human myoblasts were transfected with wild type or mutated *UNC45B*, causing HCM.

3.2 Caenorhabditis elegans

Analysing cell cultures is a great way to deduce the effects of genetic defects but what one has to remember is that cell cultures do not mimic the environment of cells in an organism's tissue. To do so one must introduce the mutation into a living organism. Muscles and muscle proteins are very convenient to investigate in model animals because they are very conserved. This means they are highly alike when comparing muscle complexes between different species. The high level of conservation makes it possible to employ different animal models to study muscles, such as the nematode worm *Caenorhabditis elegans* (*C. elegans*). The worm is 1 mm in length and its skeletal muscles consist of 95 striated body wall muscles. The sarcomere structure has some divergence compared to vertebrates but is otherwise very comparable (103, 104).

C. elegans expresses four different MyHC isoforms, of which two are expressed in the body wall muscles. These are called MyHC-A and –B. The MyHC-A isoform, expressed by the gene *MYO3*, is gathered in the central part of the sarcomere and the MyHC-B isoform, produced by *UNC54*, forms the outer parts of the thick filaments (104-107). Apart from the two standard MyHC isoforms, *C. elegans* has a protein in the centre of the thick filament called paramyosin that functions as a regulator and mediator in thick filament assembly (107, 108). The paramyosin is homologous to the rod domain of myosin, but it lacks the ATPase and actin binding head region (107, 109). Both MyHC isoforms are vital for any muscle contraction to occur, but MyHC-A is especially important in the assembly of the thick filament while MyHC-B performs the majority of the force generation of the contraction. A *null-MYO3* mutant does not assemble any thick filaments while *null-UNC54* mutants have structurally normal thick filaments but lack the function to perform proper contractions (107, 110, 111). In Article I, plasmids carrying different *UNC54* mutant variants were inserted into *C. elegans* worms, making them produce myosin mutations associated with MSM. This was done in both a wild type background as well as in *null-UNC54* mutants. Since worms carrying *null-UNC54* still can assemble thick filaments, the experiment was focusing on detecting and measuring functional muscle differences.

3.3 Drosophila melanogaster

In the investigation of genetics and genetic diseases the fruit fly, *Drosophila melanogaster* (*Drosophila*), has been one of the frontier model organisms through the past 100 years (112, 113). The first transgenic fly, carrying a

gene from a foreign organism, was generated in the early 1980s (114). The whole *Drosophila* genome was mapped during year 2000 (115). After that, a multitude of different genetic- and molecular tools have been developed to open new and innovated ways to make good use of *Drosophila* in research (116-118). During early *Drosophila* embryogenesis the cell nuclei are not separated by membranes, but instead form a big syncytium, one huge cytosol with several nuclei (119). Therefore, if a plasmid or enzyme is injected into an early embryo it can access the nearby nuclei and cause mutations. Since some, but not all nuclei will implement the planned mutation, this will give rise to a mosaic individual with both wild type and mutated cells. One therefore crosses the mosaic fly to wild type flies in order to generate a fly that carries the mutation in all cells. Specific balancer chromosomes are used to keep the stock heterozygous without chromosomal recombination. A balancer chromosome has been genetically altered and is most often lethal or infertile in homozygous condition, but viable in heterozygous condition. So, if the mosaic fly carries a mutant germ cell, it can give rise to an individual whose all cells are heterozygous for the genetic change. The offspring of this fly can be analysed in a way to establish if it carries the mutation, e.g. through sequencing or by the presence of a simultaneously introduced alteration in the genome that causes a visible phenotype, for example change in eye colour (117).

When it comes to muscle research, *Drosophila* offers a great opportunity for scientists to discover fundamental knowledge in genetics, protein structures, and protein-protein interactions (120). To do so one must understand some of the organism's process of development. The structure of striated muscles in *Drosophila* varies between different developmental stages. The body wall muscles of the larvae, used for crawling, are much different from the adult indirect flight muscles (IFM), because they have to be able to perform different types of functions (Figure 4) (120).

3.3.1 Myosin Heavy Chain in *Drosophila*

The gene for myosin in *Drosophila* (*Mhc*) contains fundamentally the same domains found in human *MYH* genes. But instead of having several genes to produce specialised myosin isoforms, optimised for different tasks in the body, *Drosophila* employ alternative splicing to produce organ specific isoforms of MyHC (121). The tail region of *Mhc* lacks alternative exons, which makes mutations in the tail expressed in all striated muscles. The favourable aspect of this is that when introducing a mutation in tail-domain

of *Mhc*, all muscles will be affected no matter which isoform they express. In humans, other MyHC isoforms can be produced by another MYH gene to cover for the mutated MyHC but this is not possible in *Drosophila*. Although this makes the phenotype of the disease more distinct and clearer to follow, it is slightly different to what occurs in humans. It also follows that flies homozygous for a mutation in this region of *Mhc* might not be viable and dies at an early developmental stage, precluding analysis of adult muscles.

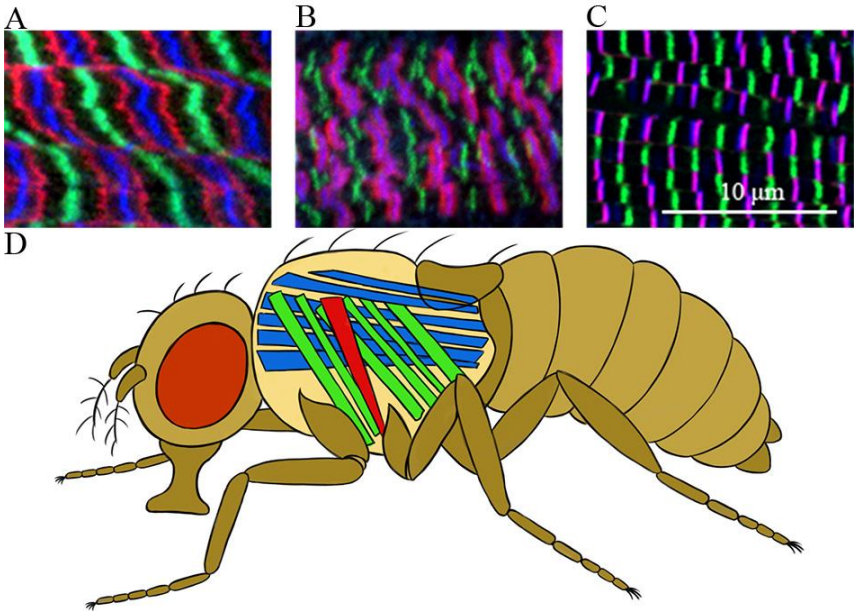

Figure 4: Muscles in *Drosophila melanogaster* (fruit flies) look different depending on muscle types. (**A-C**) Immunofluorescence stainings of different Drosophila muscles. Images were taken with confocal microscope x63 magnification. MyHC is stained red, titin/kettin is green (showing Z-discs), and obscurin is blue (showing the M-bands). Purple colour indicates overlap between MyHC and obscurin. (**A**) Immunofluorescence staining of larva body wall muscles showing broad sarcomeres. (**B**) Staining of larva heart muscle, showing a much tighter structure with only a slight space between each red myosin filament with a discrete blue obscurin stripe. (**C**) Staining of an adult fly's indirect flight muscles (IFM). Each filament is thinner and more compact in adult IFMs comparing with larval body wall muscles, with obscurin and MyHC fully overlapped. (**D**) A schematic, showing the placement of IFM (blue and green) and the jump muscle, TDT (red) in adult *Drosophila*.

One way around this is to make use of the *Mhc*^{10} allele, a null-mutation in an alternative exon of *Mhc* that is specific for IFM. Flies homozygous for *Mhc*^{10} do not produce any MyHC in those muscles (122), but are still viable. These flies can be crossed with flies carrying a mutation of interest in the *Mhc* gene to produce hemizygous offprings with only the mutated version of MyHC in their IFM.

3.3.2 Upstream Activating Sequence – UAS

As researchers progressed in the knowledge of functional genetics during the 80's and 90's another implementation of gene expression was developed. A gene enhancer named Upstream Activating Sequence – UAS was found in yeast, *Saccharomyces cerevisiae* (123). It was deduced that the UAS-associated gene was activated and expressed in the presence of the transcription factor Gal4, which binds to UAS. This feature gave genetic researchers a new elaborate way to set up models. One *Drosophila* is genetically transformed to carry a gene (X) downstream of a UAS-cassette and another fly to carry a gene encoding the Gal4 transcription factor downstream of a promotor (Y). When the two are crossed, the offspring will carry both UAS-*X* and *Y-GAL4*. In cells where the Y promotor is activated, Gal4 will also be produced. Gal4 in turn, will activate transcription of gene X. In this way, the UAS*/GAL4* system makes it possible to insert and express a mutant version of a gene in a tissue of choice, by choosing the right GAL4-line. What is also convenient with this expressional system is that the production of Gal4 is temperature dependent, making the fly produce larger amounts at higher temperatures (29°C) than at lower temperatures (18°C). Thus, by incubating the UAS*/GAL4* flies at different temperature one can analyse how different levels of gene expressions causes phenotype variations. In Article IV UAS-*Mhc* with the MSM associated mutation is inserted into flies and the expression is driven by Mef2-Gal4, a line with GAL4 expression in muscle tissues, at different temperatures. Also, Articles III and IV make use of the UAS*/GAL4* system to overexpress the ubiquitin TRIM-protein TRIM32/Abba/MuRF and to determine its impact on flies that carried a mutation. UAS-*TRIM32* was driven by Mef2-Gal4.

3.3.3 Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR

Somewhere around 2013 a new method was presented based on a virus defence system in prokaryote (124). The bacteria were found to have the ability to identify when they have a viral attack and start up regulating antiviral genes. This was based on which viral DNA or RNA that is present. The system was named Clustered Regularly Interspaced Short Palindromic

Repeats (CRISPR) (125-127). With a lot of the genetic manipulating methods the goal is to insert a modified version of a gene and control its expression but with the CRISPR method one can make base-changes or deletion in the flies' own endogenous genome. This way fewer unfamiliar or modified genetic elements are inserted into the fly and the result becomes easier to interpret. The theory behind CRISPR is that the enzyme CRISPR-associated endonuclease, Cas, can perform a double-strand break of the DNA strand. Cas is guided by small RNA (or DNA) strands, almost in the same fashion as PCR primers guide a polymerase. The small RNA primers are often referred to as guiding-RNA or gRNA. To function, the gRNA needs to have three elements: A sequence of about 20 bases that are homologous to a locus where the cut will be done, a small section called Protospacer adjacent motif (PAM) that is important for Cas enzyme to bind, and lastly a sequence folded into a hairpin structure for the Cas enzyme to hitch onto (126). The gRNA identifies its homologous corresponding locus in the DNA and guides the Cas enzyme. Together with the hairpin structure, Cas forms a ribosome-like complex and cuts the double strands of DNA.

The first application of this method was to generate models with base deletions, making the Cas enzyme cut at two points to remove gene sections. When injecting fly embryos with enzyme and gRNA one can include a DNA template sequence that covers an area that is going to be deleted by Cas. The template could contain any base variation or mutation that one wants to study. The template should also include small silent base changes in the sections that PAM regions of the gRNA otherwise would recognise to stop Cas to cut the template. The cells' endogenous DNA repair system will use the template to patch together the DNA. There are *Drosophila* stock-flies that are expressing Cas enzymes, controlled by germ cell-specific transcription factors (128). If gRNA and templates are injected into these embryos, the CRISPR-Cas process only occurs in the germ cells. This reduces the number of species that has to be injected into the fly. In Article III CRISPR-Cas9 was used to produce *Drosophila* with the MPD1 myosin mutation $Mhc^{K1728\Delta}$ (corresponding to the human $MYH7^{K1729\Delta}$).

3.3.4 *Drosophila* dissections

When a model has been generated the next step is to prepare them for some form of data-collecting assay. There are several well-established assays and protocols used for studying *Drosophila* but there are always small variations in the setups or sample preparation processes between labs.

Drosophila has several developmental stages; all of them have their own value to investigate (119). First it goes through a ~24 hours long embryonic phase, which can be divided into 17 embryonic phases based on a series of developmental markers (119). The first muscle proteins and tissue can be detected around stage 16, roughly between 15 and 16 hours after being laid (at 25 °C). During the 17th stage the embryo further arranges the muscle tissue to resemble the larval body wall muscles before it hatches into a 1st instar larva. During roughly three to four days it goes through three different larval phases, called 1st-, 2nd-, and 3rd instar larval stage where it crawls around, eating a lot to grow several times its own size. At the end of 3rd instar larval stage it begins to creep upwards (on the wall of the fly vial or bottle) and proceeds to pupate into a pupa. During the pupation process, the larva degrades most of its muscles to reconstruct the tissue into the form of an adult fly. After seven to ten days the adult fly has matured and begins to break out of the pupa to begin the adult phase (breaching and leaving the pupa is called eclosion). An adult fly has a life expectancy of between five and up to eight weeks in an optimal environment at room temperature (129). This means one can follow the flies' development over several weeks to analyse vitality or disease progression after introducing a genetic variation or mutation.

Putting all this into context, it is important to decide what factors that are important for the experiment during the setup and relate it to which developmental stages or muscle types that will be analysed. For example, larval body wall muscles can be easier to analyse since they are more easy to prepare for immunofluorescence, but the larvae's movement and muscle function in a very slow pace, making it difficult to detect changes between different genotypes. The adult fly has several movement traits that can be studied but can be harder to analyse as the adults have more interactive muscle types. Also one can study adults over a longer period of time, determining muscle changes or deterioration over several weeks, which a larval assay is unable to be used for.

Larvae body wall dissection

Dissecting 3rd instar larvae (the later larvae stage) for immunofluorescence staining the body wall muscles is the easiest analysis to perform (Figure 5A). Start by grab the larva by the head or tail region with forceps and quickly dipping the larvae in 95 ºC to kill it (this procedure can be skipped if the analysed protein structure is very temperature sensitive). Place the larvae on a silicon plate and put a needle between the capillaries, straight through its back in the causal/rear end section to pin it down. Apply a few drops of PBS to cover the larvae. With a small scissor, cut of its head, insert the scissor into

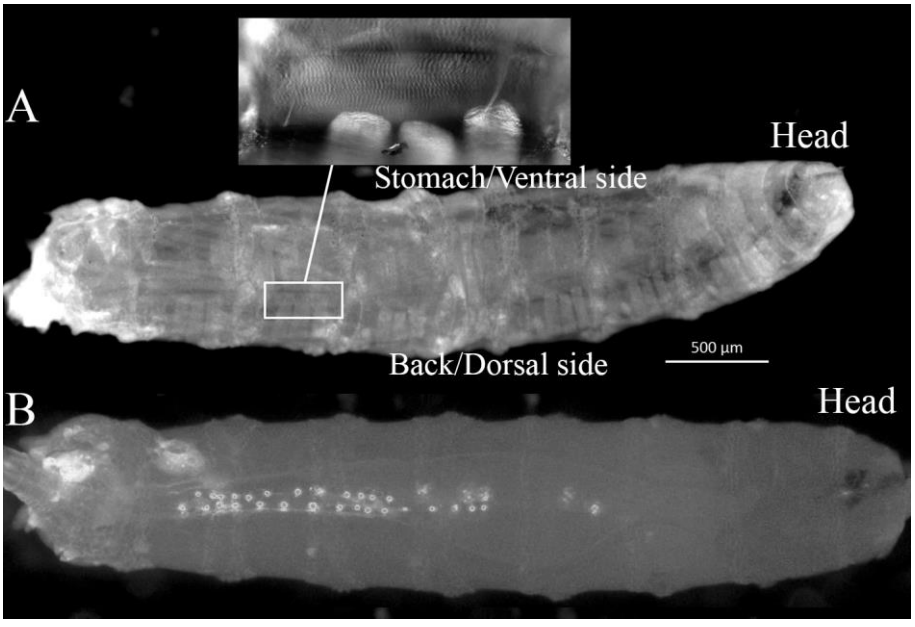

Figure 5: Drosophila larvae expressing fluorescent GFP-molecules in different tissues. (**A**) A larva, lying on the side, with its muscles visible by presence of SLS-GFP, making the larva produce titin/sallimus with GFP attached. The GFP signal is found close to the Z-disc in all muscles, which makes them look striped. (**B**) The protein HandC is expressed to a high degree in pericardial cells along the heart. This larva expresses GFP together with HandC, illuminating the heart along its back.

the opening and cut open the larvae's back all the way to the needle. Unfold the larvae by putting a needle on each side of its cranial end (where the head was removed) and cut of the caudal end of the larvae (remove the first needle) and pin down the caudal end with two pins, one on each side. Now with the larvae fully opened, work with scissor and forceps to remove as much of the gut, fat bodies, trachea, and brain lobes as possible or as needed. Change PBS regularly to remove tissue and fat. Much of the trachea can be kept intact to not damage the body wall muscles it is attached to. Remove the PBS and envelope the larvae in 3.7% - 4% formaldehyde and incubate for 10 minutes. Afterwards, remove the pins and transfer the sample to a small tube with PBS and proceed with a standard staining protocol. The staining can be performed with the dissected larva floating freely in the tube.

Larvae heart muscle dissection

The heart of a *Drosophila* is a tube of single muscle fibre that goes from head-to-tail of the animal, running on its posterior side. The heart is flanked by two rows of neuron centres that function as pacemakers (Figure 5B) (130, 131). When preparing a dissection sample of heart muscles in larvae it is these pacemaker nodes that can be seen while performing the dissection. The principle of dissection to expose heart muscles are the same as when exposing the body wall muscles except that instead of placing the larvae on its anterior side and cut open the back, the larvae must be placed on its back and the stomach/ventral side is opened. When the larva is opened, unfolded, and its interior is exposed, begin to slowly and carefully to remove gut, trachea and fat bodies. This process is more problematic and labour intensive to do than when the back is cut opened because the trachea, with its capillaries are now placed in the middle of the sample and binds together the organs and fat bodies in a bundle of trachea and tissues. The heart is positioned just beneath the dorsal skin, which means most of the organs must be carefully removed. The trachea poses the biggest challenge as it is also attached to the heart but cutting the major trachea tubes into smaller sections and carefully tugging it apart with forceps can remove it. The brain lobes can be left intact; as it is connected to the heart, keeping the brain will increase the chance that the heart is kept in place. When the organs have been removed, proceed as with larval body wall muscle preparation.

Adult fly indirect flight muscle dissection

The flight muscles of an adult *Drosophila* are excellent subjects to study cellular mechanisms of muscles. The indirect flight muscles (IFM) consist of a series of muscle fibres in the thorax (Figure 4D). The name "indirect" comes from that the muscles are not connected directly to the wings but to the cuticle around the thorax. When the fly contracts the horizontal IFMs, it pulls the cuticle ("skin") together, forcing the wings down. When relaxing them and contracting the diagonally IFMs, the wings go up again.

Anatomically, the diagonal IFM consists of six fibres on each side, running diagonally from the dorsal side down to the legs, while the horizontal IFMs goes horizontally from the head region towards the node between thorax and abdomen (Figure 4D; blue and green, respectively). Alongside the diagonally IFMs runs a muscle called jump muscle or tergal depressor of trochanter (TDT) (Figure 4D; red muscle group). It is attached to the middle legs' trochanter (the proximal knee joint) and is used when the fly jumps, which it does when it is about to start flying (122). The horizontally running IFMs, also specified as dorsal median IFM in literature, are the easiest muscles to expose in the adult *Drosophila*. Begin with putting the fly to sleep, using CO_2

and place it on a silicon plate on its side. Pin it down and kill it by putting a needle straight through its both eyes and then put a needle through the caudal abdomen/rear end. As only the end parts of the IFM-muscles are attached to the inside of the cuticle (contrary to the larval body wall muscles), the dissection has to be performed in 4% formaldehyde (or equivalent) to maintain stability. The cuticle of the *Drosophila* adult fly is highly hydrophobic, which causes it to be surrounded by air bubbles at first when covered with liquids. Cut away the wings and the legs and cut open a hole in the cuticle to expose the tissue inside to formaldehyde. Incubate for a few minutes to let formaldehyde enter the fly through the opening. This causes the fly to slowly loose its hydrophobic features. Cut away as much surrounding cuticle and tissue to make the sample as thin is possible. The first muscle fibres can be cut away to expose the muscles on the other side, to make the sample thinner. When the sample preparation has exposed sufficient amount of muscles, let it incubate for 10 minutes in formaldehyde to assure that all newly exposed tissue is also fixated, then proceed as with larvae.

3.3.5 *Drosophila* functional muscle assays

Analysing immunofluorescence stainings to detect changes in protein patterns is a good way to draw conclusions on myofibril organization, but it often yields qualitative data that can have artefacts due to the dissection and staining procedures. To support any findings of abnormal muscle organization, one can set up functional assays to determine the flies' muscle strength or endurance as a quantitative complement to immunofluorescence analysis.

Larval motility analysis

When studying larval movement one can perform the analysis during any larval stage as long as all analysed individuals have reached the same stage. Analysis of locomotion in larvae can be very straightforward; put a larva on its back and measure the time it takes from its initial body twitches until it has fully turned around or let it crawl around on a plate containing agar and apple juice and measure how fast it crawls. An easy way to set up the latter would be to put the plate on a paper with a grid system printed on it and to film the larva for a set time span. The recorded data can later be analysed to determine how many portions of the grid system the larva passes and the number of squares divided by the time yields the traveling speed of the larva. One can also use different computer software to calculate the speed or to produce a graphic representation of the crawling pattern, which can prove to useful.

Larval Heart-rate analysis

To complement immunofluorescence data of larval hearts, one can determine the larval heart rate. Most setups consist of immobilizing the larva, place it under a microscope and record the larva during a certain timeframe (132). Because the heart is visual through the larva's cuticle, it is possible to analyse the recordings through different softwares to count the beats or to count them manually. It is also possible to make a visual representation, showing the heart rate as a single image. Other ways to quantify the heart rate is to employ a GFP-reporter line (130). As mentioned in the heart-dissection section, HandC is a protein expressed to a high degree in pericardial cells found along the heart in *Drosophila* (Figure 5B) (130, 131). If a mutant is crossed with a fly containing HandC-GFP the heart is clearly visible, making it possible to either count the beats manually, with a cell counter or to more easily make recordings through a microscope. If the microscope cannot make recordings or take pictures fast enough to reach a frame rate around 50-60 frames per second, all heartbeats would not be recorded, and it is better to directly count the heartbeats and eliminate this problem. A standard wild type 3rd instar larva has a heart rate around 220 beats per minute, making it a high risk of missing beats when collecting data without doing a video recording. Therefore, it is important to analyse every larva several times to yield a mean value for each individual before calculating the mean of the whole population.

Adult jumping analysis

Adult flies pose several locomotion traits that can be analysed. Their ability to jump is mainly facilitated by the TDT muscle that runs transversely through the thorax and is attached to the middle legs (Figure 4D). The MyHC expressed in TDT is the same isoform as that found in the IFM, which makes the *Mhc^{10}* a very useful tool to analyse *Mhc* mutations in a knockout background (133). The wings need to be removed at least a day before the analysis, because they use the TDT jump muscle as a mean to get altitude before taking flight. It is preferable to sedate the flies and cut of the wings at least one day before performing the data collection to make sure that the sedation does not disturbs their muscle performance. The flies can be placed one by one on a platform, between 7 cm to 10 cm high and be touched with a small brush to make them jump of the ledge (122). The horizontal distance of their jump can then be measured. It is often standard to measure the performance of the same fly 10 times and calculate the mean of its three longest jumps. Since the assay does not kill the flies, they can be analysed at different time points to determine any progressive phenotypes.

Adult climbing analysis

Much like the jump-muscle performance assay, the adult flies' ability to climb can be determined. Several studies on both neuromuscular- and muscular diseases employ climbing analysis (sometimes called rapid iterative negative geotaxis (RING) assay) (134, 135). Unlike the TDT muscles, the muscles in the legs express another isoform of MyHC that the IFM. This makes Mhc^{10} ineffective to use for studying climbing behaviour, as Mhc^{10} do not offer an *Mhc*-null background for climbing muscles. One simple way to set up a climbing assay is to place 10 flies in a plastic or glass vial with height measurements along its side. This could either be a plastic fly-food vial with markings added along it or a graduated cylinder otherwise used to measure liquids. A lamp should be placed above the vial or cylinder to attract the flies. The cylinder is then shaken in a non-rhythmic pattern to shake down all the flies and then their climbing patterns are recorded. What one looks for when measuring their climbing ability can vary between experimental setup. One can take a picture of the vial after 3-5 seconds and calculate the mean climbing speed for the flies. It is also possible to measure the time it takes for all flies to climb a certain distance or to reach the top of the cylinder. One can also film the cylinder to record all the movements of every fly during a set timeframe and analyse the climbing patterns of every single fly using imaging or tracing software. Another factor that can be looked at is how many times the flies lose their grip and fall from the cylinder's walls during a set timeframe. As in the case of jump analysis, the climbing assay can be performed at different time points to study the progression of a phenotype.

Flight analysis

As already mentioned, the muscles that the fly uses to beat its wings are called IFM and are found in the thorax. When it contracts and relaxes the different IFM in series, the changes in stiffness of the thorax makes the wings go up and down. The Mhc^{10} mutant flies that lack MyHC in the IFM have their wings facing straight up without the ability to pull them down, because they have no functioning IFM. The contraction-relaxation process in IFM needs to occur with an extremely high frequency to make the *Drosophila* take flight. The contraction-relaxation even occurs so fast in IFM that the normal Ca^{2+}-mediated activation and regulation of muscle contraction is not sufficient, since the release and removal of Ca^{2+} into and out of the sarcoplasmic reticulum is not rapid enough. Therefore, IFM has a feature called stretch-activation: The muscle cells maintain a Ca^{2+} concentration sufficient to keep them activated and when an antagonistic muscle contracts, the IFM is stretched and thereby activated. (136). The flight machinery is very delicate, and a slight muscle defect can easily inhibit flight ability, even if the flies display very little difference in other functionality-tests. The

ability to fly is often thought of as an on/off switch; either the flies can fly or they cannot. The simplest way to determine flight ability is to put flies in a vial and place a light source above it, in the same manner as when conducting a climbing analysis. The flies are observed during 20 minutes and are shaken down every five minutes. It is important to keep in mind that even when the flies are flightless, they can still jump or fall from the vials walls, making them appear to fly but through this assay one can usually tell if the flies are able to take flight. Other, more advanced setups can determine smaller variations in flight ability or measure the beating speed of the wings (137).

4 RESULTS AND DISCUSSION

4.1 PAPER I

Myosin Storage Myopathy in *C. elegans* and Human Cultured Muscle Cells. Halvarsson D.M., Pokrzywa M., Rauthan M., Pilon M., Tajsharghi H. (2017)

4.1.1 Background

The gene *MYH7* expresses the isoform of myosin heavy chain (MyHC) specific for slow/β-cardiac muscles. The *MYH7* mutations L1793P, R1845W, E1883K, and H1901L are dominant mutations that cause Myosin storage myopathy (MSM). The symptoms observed among patients are slow progressive muscle weakness and accumulation of MyHC aggregates, which overtime develops into muscle atrophy of slow skeletal muscles. The symptom severity and age-of-onset both display great variations, ranging from symptoms from birth up to symptom-free at the age of 30.

The four mutations are found in the coiled-coil tail domain of MyHC, which is important for anchoring the protein in the sarcomere. The amino acids in the tail-domain form an α-helix spiral with a repetitive amino acid sequence (Figure 2 and Table 1). L1793 faces inwards towards the MyHC's sister monomer (position *d*) while the other three amino acids; R1845, E1883, and H1901, all face outwards (position *f*, *b*, and *f* respectively), making them interact with other MyHC dimers and M-band proteins. The amino acid L (leucine) is hydrophobic while R, E and H (arginine, glutamic acid, and histidine) are all polar, hydrophilic residues. This is supposedly the key to what makes the mutation cause the progressive build-up of aggregated MyHC in the tissue. The actual mechanisms responsible for MyHC aggregation and what factors that makes an impact on the severity levels of the disease are poorly understood. It is possible to study MyHC and *MYH* mutations in other animals since the MyHC protein is widely conserved between species. The first aim of this paper was to identify malformations in sarcomere structure when transfecting human myoblasts with *MYH7* mutations. The second aim was to determine functional abnormalities when transfecting *C.elegans* nematodes with *UNC54* mutations (*UNC54* is the MyHC-B isoform that produces the most force during the contraction but only has a minor role in thick filament assembly).

Human myoblasts were cultivated on glass slides and transfected with EGFP-plasmids, carrying *MYH7* with mutations causing L1793P, R1845W, E1883K, or H1901L using a Lipofectamin2000™ kit. The myoblasts where then differentiated for either one or three days after the transfection. The cells were analysed through immunofluorescence staining for MyHC as well as either MyBPC or actin (phalloidin).

C.elegans worms with wild type- (Bristol N2) or *UNC54* knockout (CB190) background were transfected with *UNC54* carrying mutations that lead to R1854W, E1892K, or H1910L, which corresponds to the human R1845W, E1883K, and H1901L mutations, respectively. The one-day-old worms were placed on nematode growing plates where their positions were recorded every second over an hour to map their movements. The corresponding amino acid L1793 is not conserved in *C.elegans*, rendering it unsuited to test in the motility assay. L1793P was still included in the human cell culture assay because, together with R1845W, they are the most recurring MSM mutations found in patients. This made them the most important targets in the cell assay.

4.1.2 Results and Discussion

Through cell transfection assays, we showed that R1845W and H1901L form accumulations of MyHC as early as 1 day of differentiation into myocytes. After three days of differentiation, the mutant cells had still not began to form any distinct striated pattern of MyHC and MyBPC. This is a sign of delayed myofibrilogenesis and that muscle development is slower than in the control. The MyHC aggregates had increased in size after three days. Unlike R1845W and H1901L, the mutation L1793P never gave rise to any MyHC aggregations during the three days. Although all of the mutants showed a reduced ability to form sarcomeres, L1793P had begun to form striated sarcomeres after three days but to a much lower degree compared to the control.

The observation that L1793P causes reduced sarcomerogenesis rate has been reported *in vitro* before, where the self-assembly of MyHC into dimers where analysed (138). The group showed that L1793P, R1845W, and H1901L MyHC all dimerised to the same degree as the control, but that L1793P did it at a significantly reduced pace. Other *in vitro* experiments have shown that the mutations L1793P and E1883K have reduced MyHC dimer stability while R1845W and H1901L have a significantly increased stability (37, 38). Thus, it seems that mutations that lead to the same disease, with progressive muscle weakness and MyHC aggregations, are working through different

mechanisms. What sets the mutations apart is the nature of the amino acid that is changed. As mentioned earlier, the amino acid L1793 faces inwards in the coiled heptamer (placed at position *d*). The L1793P mutation makes the uncharged and hydrophobic leucine (L) to be replaced by the circular amino acid proline (P). Proline is known to have unique traits due to its circular aliphatic structure and is not normally found anywhere in the whole coiled-coil region of MyHC. An amino acid substitution where proline is involved has been shown to have more severe effects than many other substituted amino acids (47). All these *in vitro* experiments have shown that the mutation L1793P has a similar stability profile as E1883K. Unlike L1793P, E1883K faces outwards from the MyHC dimer, which would supposedly make the mutation behave as the two other mutations R1845W and H1901L, as all of them point outwards from the heptamer. E1883K is the only known MSM mutation found as homozygous genotype in patients, making it a mutation of high interest to study (139). What sets it apart from most other known MSM mutations is that it does not profoundly change the locus' hydrophilicity, since the glutamic acid (E) is an electronegative amino acid and lysine (K) is positively charged. The substitution still causes the locus to end up with a different trait (as the electronegativity is flipped), which disturbs the protein's stability. One can theorise that this is the reason why E1883K yields milder symptoms and can give rise to a viable homozygous genotype. E1883K has not only been linked to MSM but also to hypertrophic cardiomyopathy, which is very uncommon for mutations in the end part of the tail region (139). Other mutations of the same nature, such as the more proximal mutation E1356K, have only been found to causes hypertrophic cardiomyopathy with no skeletal myopathic involvement (38, 138, 140). Looking even further into similarities between mutations; E1856K, a mutation somewhat downstream from E1883K, has been reported to cause skeletal MPD1 combined with a range of dilated cardiomyopathies (141). While E1883 is found at *b*-constellation, both E1856 and E1356 are at *c*-position (all of them face outwards from the heptamer). These facts show that the disease mechanisms not only dependent on the amino acids' traits but may be more heavily dependent on the loci. Unfortunately, we did not have opportunity to transfect cells with $MYH7^{E1883K}$ to compare results with the other mutations. The R1845W and H1901L mutations have much in common; as already mentioned they are both located at *f*-position but they also have a hydrophilic positively charged amino acid substituted for an uncharged hydrophobic one (R1845W – arginine to tryptophan; H1901L – histidine to leucine). It is therefore understandable that phenotypes arising from these two could be very similar. Experiments where a positive arginine is replaced by a histidine, also positive, (R1559H) in Drosophila have been shown to cause progressive muscle atrophy (142). This shows that amino

acid substitutions must not necessary contain vast different traits to have a pathological phenotype in the organism. It is possible that the two residues have enough different physical steric attributes and is physically disturbing the the structure of the MyHC dimers.

The mutations R1845W, E1883K, and H1901L were also inserted into *C.elegans* through plasmid injections (with the corresponding *UNC54* mutations: R1854W, E1892K, or H1910L). The worms' movement was recorded for one hour to determine if they experienced any decrease in functionality or muscle strength. All mutants exhibited about the same crawling speed. Furthermore, injection of mutant genes rescued the crawling phenotype when performed in *UNC54*-double knockouts (which has no MyHC-B of themselves) just as much as those injected with $UNC54^{control}$. Since *UNC54* expresses the MyHC-B isoform, which is more important for performing the contraction than sarcomere formation, we drew the conclusion that the mutations affect the stability and structure of the sarcomere more than its function. These results must be viewed in the light that it is a plasmid-mediated extrachromosomal assay. The injected plasmid causes an overexpression in the worms, which might override much of the animals' problems with thick filament assembly that it otherwise would have experienced. Further analysis with *C.elegans* could include comparison of *UNC54* and *MYO3* mutations to determine if the mutations would cause a disruption in thick filament assembly.

4.2 PAPER II

Recessive TorsinA variants cause severe arthrogryposis developmental delay, strabismus and tremor. Kariminejad A., Halvarsson D.M., Ravenscroft G., Afroozan F., Goullee H., Davis R.M., Laing G.N., Tajsharghi H. (2017)

4.2.1 Background

The malady dystonia-1 or sometimes called early-onset generalized limb-onset dystonia (DYT1) is a neurological disease where patients suffer from both sustained involuntary muscle contractions and fibrosis in the muscles. This causes patients to experience muscle twitches and develop abnormal postures, often from birth. DYT1 is linked to mutations in the gene *TOR1A* that expresses the ATPase TorsinA, a vital factor in vesicle transportation and release of dopamine in the stratum in the brain. The most common *TOR1A* mutation is E303Δ, an in-frame 3-base deletion in a domain that assists the protein in transferring the energy from cleaved ATP onto an associated protein. Through a screening of twenty-five patients with congenital contractures in several body parts using NGS (as well as physical examinations and review of the patients' medical history) four cases of DYT1 were found. Two individuals, who were siblings, carried the previously unknown mutation $TOR1A^{G318S}$; one of them with as homozygous genotype while the other was heterozygous (Family 1: Case 1 – Heterozygous $TOR1A^{G318S}$, Case 2 – Homozygous $TOR1A^{G318S}$). Two cases of $TOR1A^{E303\Delta}$ were identified in two separate families; One with a homozygous genotype while the other was heterozygote (Family 2: Case 3 – Homozygous $TOR1A^{E303\Delta}$; Family 3: Case 4 – Heterozygous $TOR1A^{E303\Delta}$). The parents from all three families displayed no symptoms yet all were heterozygotes for the separate variants.

4.2.2 Results and Discussion

Since the $TOR1A^{G318S}$ mutation has not previously been identified, a cell culture assay was performed to determine if this mutation causes the same cellular phenotype as the more common variant $TOR1A^{E303\Delta}$. Cells from the human embryonic kidney cell-line HEK293 were transfected with EGFP plasmids carrying either wild type *TOR1A* or $TOR1A^{G318S}$ using lipofection. Confocal images showed intense accumulation of GFP-tagged proteins around cell nuclei transfected with $TOR1A^{G318S}$, while those transfected with the wild type version displayed a normal spread of GFP-positive TorsinA with only ever so slightly more intensity close to the nuclei.

HEK293 cells have often served to analyse *TOR1A* mutations to understand some of their fundamental cellular traits. When transfected with mutated *TOR1A* they display the abnormal phenotype of accumulating TorsinA around the nuclear envelope, which is also seen in patient-derived fibroblasts carrying $TOR1A^{E303\Delta}$ or $TOR1A^{F205I}$ (143, 144). The TorsinA-inclusions are not associated with all *TOR1A* mutations though; the six amino acid deletion $TOR1A^{F323\text{-}Y328\Delta}$ for example, has been shown to not cause any nuclear envelope aggregation even if the patients display the same symptoms (145). This concludes that $TOR1A^{G318S}$ and $TOR1A^{E303\Delta}$ affect cells in a very similar fashion. Studies of homozygous *TOR1A* knock-out and $TOR1A^{E303\Delta}$ knock-in mice have previously been done but have shown that the animals' brain development has been so severely affected that none of them survived 48 hours (63, 146). The finding of a homozygous patient shows that there are complexities regarding the disease in different species that is yet not understood. All the patients did exhibit a reduced cognitive ability and delayed development, such as not being able to communicate verbally (also in the heterozygous children). None of the parents displayed any symptoms nor was there any prior documented history of DYT1 in any of the families, which again shows that there is an incomplete penetrance of the mutation.

As mentioned earlier in the introduction, TorsinA exhibits a somewhat different localisation pattern in neurons, where it is not only found in the endoplasmic reticulum and nuclear envelope. TorsinA in neurons is closely associated with the dopamine transporter DAT in the synaptic ends as well as the microtubule subunit kinesin light chain 1 in the ganglion (or axons?) (54, 59-61). To really understand how the novel $TOR1A^{G318S}$ functions it is essential to study the localisation pattern in neurons when conducting cell assays. To employ animal models is also vital to further understand DYT1. The rise of CRISPR-Cas techniques could be one of the key assets in generating disease models. For example, one could generate cell lines of neuron-derived cells and introduce $TOR1A^{G318S}$ using the CRISPR-Cas method to investigate how the mutation acts when not expressed extra-chromosomally (as with plasmid mediated lipofection). It would also be possible to introduce the penetrance-reducing polymorphism $TOR1A^{D216H}$ together with $TOR1A^{G318S}$ to determine the effect the polymorphism would have on the mutation. Several mouse experiments have already been conducted with great results, but it would be possible to study the gene in other animals as well since the gene is highly conserved between species. *Drosophila melanogaster* carries a gene called *TORP4A*, while nematodes have *OOC5*, which both are equivalent to *TOR1A*.

4.3 PAPER III

***Drosophila* model of myosin myopathy rescued by overexpression of a TRIM-protein family member.** Halvarsson D.M., Olivé M., Pokrzywa M., Ejeskär K., Palmer R.H., Uv A.E., Tajsharghi H. (2018)

4.3.1 Background

One of the most recurring mutations known to cause Laing early-onset distal myopathy (MPD1) is an in-frame three base pair deletion in *MYH7*, removing a lysine (*MYH7*$^{K1729\Delta}$). The lysine is localised at position *c* in the MyHC rod heptamer, facing outwards from the dimer. Compared to amino acid substitution mutations, it is harder to predict what effect a deletion would have on the protein structure. *MYH7*$^{K1729\Delta}$ is a dominant mutation that causes the general symptoms associated with MPD1 with progressive muscle atrophy in slow skeletal muscles. The muscles affected are mostly the distal limbs; calves, forearms, feet, and hands. One of the characteristics of MPD1 that sets it apart from e.g. myosin storage myopathy is a reduced tension in ankle tendons resulting in reduced strength in the feet. This symptom is often recognised as "dropping-big-toe". As *MYH7* is not only expressed in the slow skeletal muscle but also in the heart ventricles, some patients have been found to exhibit dilated cardiomyopathy. Even though patients would express the mutated *MYH7* in the heart ventricles, the vast majority of them do not show any heart conditions at all.

Drosophila flies expresses all their MyHC isoforms from a single gene, called *Mhc*. The *MYH7*^{K1729} amino acid is located in the highly conservative α-helical tail-domain and it corresponds to the *Drosophila* residue *Mhc*^{K1728}. Because *Drosophila* only has a single myosin gene, it produces specialised MyHC isoforms through alternative splicing, yielding MyHC with different exons (121). The *Mhc*^{K1728} amino acid is located in exon 16, which is not an alternative exon. This means that when *Mhc*$^{K1728\Delta}$ is introduced into the fly, the mutated MyHC will be expressed in all of its skeletal and cardio muscles.

Using the CRISPR-Cas9 method, we generated a heterozygous fly line carrying *Mhc*$^{K1728\Delta}$. Homozygous *Mhc*$^{K1728\Delta}$ larvae were viable but suffered from severe movement and motility reduction. The homozygous individuals had a 100% mortality rate during pupation, which means that no homozygotes survived to adult stage. To investigate how the mutation affects muscles in a pseudo-homozygous genotype (this is called hemizygous), they were crossed with flies carrying *Mhc*^{10}, which does not produce MyHC in indirect flight muscles (IFM) and jump muscle (TDT) (described in Material & Method (3.3.1)). Thus, *Mhc*$^{K1728\Delta}$*/Mhc*^{10} flies express a mutant and wild

type version of MyHC in all its muscles, except for in those specific muscles where $Mhc^{K1728\Delta}$ is the sole MyHC produced. This fly survives to adult stage but its flight and jump muscles could be analysed as if they were homozygous. We also crossed the mutants with flies that overexpress the ubiquitin E3-ligase TRIM32/Thin (homolog to the human MuRF enzyme) (147, 148). As the TRIM32 protein's role is to assist in breaking down and clear away malformed MyHC, the aim was to determine how the disease would behave when increasing the myosin degradation system.

Flies were tested through immunofluorescence staining, survivability, and motility/functionality assays to determine protein pattern, formation, and the general disease progression.

4.3.2 Results and Discussion

The phenotype of $Mhc^{K1728\Delta}$ adult flies showed a close relation to how MPD1 patients progress. Larvae muscle staining of heterozygotes showed little deviation in morphology from the wild type situation, but the larvae had reduced crawling speed. The adult flies had significantly reduced strength in their jump muscles and reduced climbing speed from an early age. The muscle strength was further reduced with age. At an early age, the adult mutants formed aggregation-like accumulations of MyHC. After two weeks the flies started to develop areas of disorganisation in their myofibres. These areas further developed into big lesions of disorganised not only MyHC but also of Z-disc- and M-band proteins. Similar lesions of disorganised proteins are often found in patients. When overexpressing TRIM in these flies, a significant resilience to the $Mhc^{K1728\Delta}$ mutation was seen with muscle strength at the same level as control animals.

These findings show that TRIM-proteins can cause a variation in an individual's disease progression depending on how active the ubiquitination system is. Human polymorphisms of TRIMs that worsen the pathogenesis of hypertrophic cardiomyopathy have been found, but it is not yet known if any variation leads to a resilience among patients (17). The MPD1 disease mechanisms are problematic to target because muscles are tangled with a multitude of proteins and factors that facilitate protein complex formation and function. Our results that TRIM-protein expression can "hold off" the disease can also be seen as a possible target for therapeutic treatment. The problem would be that the disease is systemic; it is manifested in several skeletal muscles, and sometimes in the heart. This would mean that a treatment might have to be to systemically drive the production of TRIM in the patient, which could cause serious side effects over time.

What exact defects the mutation causes is still not clear through our experiments. Other mutations, where an amino acid has been replaced by another in the MyHC rod-domain, are easier to theorise on. But when the mutation causes an amino acid deletion it becomes much harder. Comparing the immunofluorescence pictures of heterozygous flies from day four and two weeks shows an interesting shift in phenotypes. The small aggregates seen in early flies seem to have disappeared after two weeks but instead, big but few areas of disorganisation are present.

The early aggregates could originate from decreased function of the MyHC-degradation system. Since the larva breaks down its skeletal muscles to rebuild them during the pupal stage, a malfunctioning degradation would cause a build-up of mutated MyHC. Our further experiments with TRIM-enzymes showed that mutated MyHC could be degraded by overexpressing ubiquitin, which either disproves a “decreased degradation” hypothesis or shows that the level of enzymes need to exceed a certain threshold to work more efficiently. Another hypothesis is that the mutation makes MyHC less stable and have a harder time to assemble itself into dimers or into the sarcomere, which after two weeks are gathered into the bigger lesions.

What could be done further is to study assembly and dimerization speed *in vitro* as well as the stability of the dimer to understand what drives the disease. To make similar experiments with a mouse or a rat model might prove to be very informative.

4.4 PAPER IV

***Drosophila* model of myosin storage myopathy rescued by overexpression of a TRIM-protein family member.** Halvarsson D.M., Olivé M., Pokrzywa M., Norum M., Ejeskär K., Palmer R.H., Uv A.E., Tajsharghi H. (Submitted manuscript, PNAS 2019 Feb)

4.4.1 Background

To investigate the condition myosin storage myopathy (MSM) deeper, we generated a *Drosophila* model that carried the most recurrent MSM mutation, Mhc^{R1844W} (corresponding to the human $MYH7^{R1845W}$). Expression of the allele was driven by the *UAS-GAL4* system, Thus, *UAS-*Mhc^{R1844W} flies were crossed with flies carrying *MEF2-GAL4*, producing offsprings with both *UAS-*Mhc^{R1844W} and *MEF2-GAL4*. Mef2, or Myocyte-specific enhancer factor 2, is an important transcription factor expressed to a high degree in muscle cells when muscle proteins are starting to be expressed. Therefore, when the flies normally produce muscle proteins, the *UAS* is activated and the mutated MyHC is also produced. As a control, a wild type version of *UAS-Mhc* was also developed.

To briefly describe the background of the two inserted *Mhc* genes; The genes were cDNA constructs of *Mhc*. This means that the genes do not contain any non-coding introns but only the exons. The gene used was an embryonic *Mhc* isoform to ensure that the flies would survive during their embryonic stages into larval stage. When the *UAS-Mhc* was injected into the embryo, the construct does not incorporate itself in the endogenous *Mhc* gene but is inserted at an *attP* site that had previously been inserted.

Since the *UAS-*Mhc^{R1844W} construct does not remove the fly's normal *Mhc* gene, this could interfere with the results, making the fly produce much more normal MyHC than the mutated $MyHC^{R1844W}$. Therefore, we wanted to knock-out the endogenous *Mhc* gene, which was positioned on the same chromosome as *UAS-*Mhc^{R1844W}. To do so, the mutants were crossed with a fly stock carrying a heterozygous MyHC knock-out mutation, called Mhc^{1}. Mhc^{1} has a stop-codon in an early exon, which hinders any functional MyHC to be produced. The offspring were crossed and screened to detect whether sexual recombination had occurred to yield Mhc^{1} and *UAS-Mhc* on the same chromosome (w^{-}; Mhc^{1}, *UAS-*Mhc^{R1844W}*/CyO, Dfd>GFP*). The same procedure was done with the wild type *UAS-Mhc* insert.

The *UAS-GAL4* system is temperature dependent, as already have been descried in the Materials and Method section (3.3.2). Animals were therefore

kept in incubators at either 25 °C or 29 °C, which causes different amount of MyHC to be expressed. *UAS-Mhc^{R1844W}* and *UAS-Mhc* were also expressed together with *UAS-TRIM*.

The *Drosophila* were tested through immunofluorescence staining, survivability, and motility/functionality assays to determine protein pattern, sarcomere formation, and the general disease progression.

4.4.2 Results and Discussion

The perk of using *UAS-GAL4* is that we can study different levels of expression to determine the lethality or pathological impact a mutation or gene has on the *Drosophila*. When incubated at 25 °C, the mutant flies exhibit similar pathological markers as seen in patients, with accumulation of MyHC and other proteins, which increased with time. At 29 °C the muscles deteriorated at a much faster rate. The muscles had filamentous accumulations of MyHC and other sarcomere proteins between their muscle fibres. Over time, the muscles lost most of their integrity, displaying whole filaments with disorganised proteins. Mutants kept at 29 °C also showed major developmental anomalies in the heart muscles while no major defect was detected at 25 °C.

Patients suffering from *MYH7^{R1845W}* develop aggregations of MyHC in slow-type 1 muscles that are gathered close to the cell membrane. We showed in article I that MyHC accumulation occurs at an early stage of myofibrilogenesis when *MYH7^{R1845W}* is present. It has also been established, through *in vitro* experiments, that there is a link between *MYH7^{R1845W}* and irregularly high stability of MyHC dimers (37, 38). Compared to our analysis of *UAS-Mhc^{R1844W}* and *Crispr-Mhc$^{K1728\Delta}$* in article III, one sees that *MHC$^{K1728\Delta}$* causes MyHC to be disorganised within the fibre while *Mhc^{R1844W}* makes MyHC accumulate in dense structures between the fibres. This indicates that the mutation behaves in the same fashion in the *UAS-Mhc^{R1844W}* model as in human patients.

A study of several MSM mutations in *Drosophila* were recently published, studying hemizygous *Mhc*-mutants (one of them was *Mhc^{R1844W}*) crossed with *Mhc^{10}* (37). They showed that when the mutations were in trans to *Mhc^{10}*, the flies were unable to form proper sarcomeres in IFMs at one day of age in adult stage and that no striations of mutated MyHC was present. They combine this data with *in vitro* experiments showing that the *Drosophila* isoform of *Mhc^{R1844W}* becomes super stable when dimerised, just like in the humane *MYH7^{R1845W}* isoform.

This agrees with our data, where *UAS-Mhc*^{R1844W} crossed with *Mhc*^{10} does not form any proper filaments, with MyHC accumulations and overall protein disruptions all over the fibres. The article only explores the MyHC formation up to day one in adult stage. What happens overtime in the model can be seen as vital for further understanding the disease, since the disease is progressive. What we saw in the heterozygous mutants was that MyHCs display disarrays in their pattern at four days of age, while after two weeks the aggregation of proteins can be seen between the fibres.

MYH7^{R1845W} does seldom cause any cardiomyopathy in patients, even though the *MYH7* isoform is expressed in the heart's left ventricle. It is therefore very interesting that the heart of mutant larvae showed major anomalies with fragmented Z-discs at 29 °C while they at 25 °C showed no major change on sarcomere level but had filamentous MyHC formations between cardiomyofibres.

When overviewing the skeletal and cardiac pathological profile of *UAS-Mhc*^{R1844W} we conclude that flies incubated in 25 °C was highly parable with MSM patients while 29 °C gives us information on how the mutation behaves when overexpressed.

When TRIM32/Thin is overexpressed in *Mhc*^{R1844W} flies, they become significantly stronger, showing resilience to the mutation at both temperatures, much like the results seen with Crispr-*Mhc*$^{K1728\Delta}$. What is interesting is that immunofluorescence stainings of mutants with overexpression of TRIM32 reared at 25 °C showed a lot of areas of disarray, which were not seen when the same cross was done at 29 °C. These two phenotypes give an indication that the two *UAS*-constructs; *Mhc* and *TRIM32*, might be competing for Gal4 activation and therefore TRIM32 does not pass an expression threshold to have enough therapeutic effect. What also could be interfering is that flies kept at a higher temperature have an increased movement activity and different metabolism than those kept at lower temperature (149). The flies kept at 29 °C would therefore not only have a higher level of TRIM-enzymes but would also move around and get more exercise, affecting the muscles' maintenance and wellbeing.

The next step of take to understand more about MSM and *Mhc*^{R1844W} would be to study the effects of exercise on flies. This would firstly give us quantitative data on how physical activity influence the pathogenesis of MSM and would also determine if this had any significant interference with the TRIM32 experiments.

Establishing a *Drosophila* Mhc^{R1844W} model using CRISPR-Cas technique would also be important to learn more about the disease. This would make the inserted gene independent of Gal4 for expression and can be analysed with less invasive factors. Introducing the mutation into a mammal, such as mouse or rat, would be a major step towards understanding the mechanisms of MSM.

4.5 PAPER V

UNC45B, a co-chaperone required for proper folding and accumulation of myosin, as a novel gene associated with hypertrophic cardiomyopathy. Manuscript Emrahi L.*, Halvarsson D.M.*, Moselmi A.R., Hesse C, Goullée H., Laing G.N., Tajsharghi H. (Manuscript)

* authors contributed equally to the study and share first authorship

4.5.1 Background

Hypertrophic cardiomyopathy (HCM) is a relatively common heart condition. In many cases it goes undiagnosed but can trigger sudden heart failure, especially among young athletes. About half of the cases of HCM are linked to mutations in the head region of *MYH7* or to myosin binding protein C but there is a large number of HCM cases and sudden heart failures where no genetic connections have been established. What we present in this study are recent findings where a family have been diagnosed with HCM and carries a mutation in the gene *UNC45B*. The gene expresses the myosin-specific chaperone UNC-45B that folds the myosin head. UNC-45B has been established as especially important during embryonic heart development and muscle regeneration due to its myosin-folding feature but have not before been identified in any muscle disease.

Three siblings in the same Iranian family carried an in-frame substitution mutation in the *UNC45B* gene, causing a glutamine to be replaced by a leucine (Q61L). The mutation was found during whole exome sequencing screening process of HCM patients while targeting several known HCM associated genes. To determine that the mutation would cause a pathological effect on myocytes, a cell assay was set up where GFP-plasmids, carrying $UNC45B^{Q61L}$ or $UNC45B^{Control}$, were inserted into myoblasts by lipofection. The myoblasts were then differentiated for five days, after which they were stained with immunofluorescent antibodies to determine how UNC-45B and MyHC behaved under the different conditions.

4.5.2 Results and Discussion

Out of eight siblings, three were identified as carriers of the mutation $UNC45B^{Q61L}$. No other mutation or unknown polymorphism was found in any other known HCM associated gene. After the three siblings were diagnosed, other relatives to the family were found to have a developed HCM. All patients had a heterozygous genotype. The pattern of heritage shows clear signs that the mutation is dominant.

Immunofluorescence staining of myocytes transfected with wild type *UNC45B* showed normal formation of MyHC after five days of differentiation. Proper striation into thick filaments were observed, which overlapped with presence of UNC45-B. Myoblasts transfected with *UNC45B*^{Q61L} showed a significantly reduced tendency to form sarcomere striation. GFP-positive UNC45-B was seen in some instances to form dense clusters together with MyHC inside myofibres. This indicates that UNC-45B^{Q61L} has a changed function and not simply lost its properties completely.

As mentioned earlier, the gene has been studied in *unc45b* knockdown models (where the activity of the gene is silenced by RNAi) and null mutations in zebra fish. These experiments showed that inhibiting *unc45* causes cardiomyopathy and embryonic heart malformations (91). Although no muscular disease has directly been associated with UNC45-B in humans before, a case of juvenile eye cataract has been found (20). It was determined to be caused by an amino acid change from an arginine to a tryptophan (R805W), disturbing the development of eye lens maturation.

We conclude through these findings that *UNC45B*^{Q61L} is a dominant mutation that causes HCM.

As this is a completely new mutation associated with HCM, it is important to start analysing what the mutation does to UNC-45B and its functionality. The previous zebra fish studies have given us information about what happens when knocking the gene out completely, but now there is a more specific mutation to study.

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APPENDIX

Table S1: Short description of amino acids commonly found in myosin tail-region or highly associated with skeletal diseases.

Abbreviation	Amino acid	Traits
L	Leucine	Very hydrophobic
A	Alanine	Uncharged, low hydrophobic
Q	Glutamine	Uncharged, hydrophilic
E	Glutamic acid	Negatively charged, hydrophilic
D	Aspartic acid	Negatively charged, hydrophilic
R	Arginine	Positively charged, hydrophilic
K	Lysine	Positively charged, hydrophilic
W	Tryptophan	Nonpolar, cyclic, hydrophobic
P	Proline	Nonpolar, cyclic, low hydrophobic
H	Histidine	Positively charged, hydrophilic

Figure 1A was obtained from the homepage https://biology.stackexchange.com/questions/10945/isotropy-of-sarcomere-bands-in-skeletal-muscle-cells (2019-01-15), where authors have stated that other individuals are allowed to use and edit their picture (last edited 2017-04-13).

Figure 3 was made by Martin Dahl Halvarsson with figure from article as template (65).

Figure 4D was made by Martin Dahl Halvarsson and Emma Dahl.

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