

From DEPARTMENT OF PHYSIOLOGY AND
PHARMACOLOGY
Karolinska Institutet, Stockholm, Sweden

FUNCTIONAL ROLE OF CYTOSKELETAL, CONTRACTILE AND REGULATORY PROTEINS IN MUSCLE DISEASE

MEI LI



**Karolinska
Institutet**

Stockholm 2015

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet.

Printed by Universitetsservice US-AB

© Mei Li, 2015

ISBN 978-91-7549-805-8

Functional role of cytoskeletal, contractile and regulatory proteins in muscle disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Mei Li

Principal Supervisor:

Prof. Anders Arner
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Genetic Physiology

Co-supervisor(s):

Prof. Thomas Sejersen
Karolinska Institutet
Department of Women's and Children's Health

Dr. Monika Andersson-Lendahl
Karolinska Institutet
Department of Cell and Molecular Biology

Opponent:

Prof. Carlo Reggiani
University of Padova
Department of Biomedical Science

Examination Board:

Prof. Jan Henriksson
Karolinska Institutet
Department of Physiology and Pharmacology

Prof. Madeleine Durbeej-Hjalt
Lund University
Department of Experimental Medical Science

Dr. Homa Tajshargi
University of Gothenburg
Institute of Biomedicine
Department of Pathology

To my beloved family
致我摯愛的家人

ABSTRACT

The function of skeletal muscle is an essential component of animal physiology and daily life. Hereditary muscle diseases are comparatively rare in humans, but often very severe. The disease causes are heterogeneous, defects in nerves, intracellular components, structural proteins and the contractile apparatus can be involved. Genetic linkage analyses have revealed associations between some genetic defects and muscle diseases. Causal relationships have been established in some animal models, but the exact function of several of the genes/proteins in the muscle and their roles in pathogenesis of the diseases have not been fully explored. The general aim of the thesis was to develop and analyze muscle disease models in the zebrafish larvae using a combined genetic and physiological approach, focusing on cytoskeletal/structural proteins and to explore different therapeutic options. In **Paper I**, desmin, which is a key intermediate filament protein, was knocked down in larval muscles by about 50 % using morpholino antisense oligonucleotide injection. This knockdown model had a significant impairment in muscle structure and decreased active force. X-ray diffraction analysis revealed swelling of the filament lattice after desmin knockdown, suggesting a role of desmin in the lateral anchoring of the contractile apparatus. Moreover, the vulnerability to eccentric contractions was lower after desmin knockdown suggesting that desmin is involved in lateral force transmission in the muscle cells. In **Paper II**, the zebrafish dystrophin null *Sapje* mutant, a model for Duchenne Muscular Dystrophy (DMD), was characterized. These mutant larvae had structural changes and compromised cell membranes, observed early during development (3 days post fertilization, dpf). Active force was significantly lower (about 50 % of that in the normal siblings). Two-day treatment with Ataluren, a compound causing read through of premature stop codons, partially restored the protein expression of dystrophin in the *Sapje* mutants. This was accompanied by a significant improvement of muscle structure and active force. The effect of Ataluren on active force revealed a bell-shaped dose dependency, similar to that suggested in initial clinical trials. The pathogenesis of DMD is complex, several factors can be involved. The contribution of the mechanical linkage provided by dystrophin/dystrophin-glycoprotein complex in the disease development of muscular dystrophin was examined in **Paper III**. *Sapje* mutants were fully immobilized by BTS (an actomyosin inhibitor) from 18 hours after fertilization until 4 dpf. The structural damage, as assayed by birefringence, was completely abolished by immobilization. To further validate the concept in another dystrophic model, *Candyfloss* mutants that were laminin- α 2 chain null, were treated using the same protocol. These mutants which had significantly impaired structure were also rescued by immobilization. Following washout of BTS and active swimming, structural damage developed supporting mechanical contractions as a primary factor in the development of structural changes in muscular dystrophy. In **Paper IV**, the role of a sarcomeric protein, myosin binding protein C (MyBPC) was examined in the skeletal muscle. The skeletal isoforms, were knocked down in larvae. Partial removal of the fast type (MyBPC-2) resulted in a severe form of skeletal myopathy with activated degeneration/regeneration processes. Significant alterations were observed in the sarcomeric structure suggesting that MyBPC-2 is required for normal sarcomere

assembly. The active force was significantly lower and the maximal shortening velocity was increased after MyBPC-2 knockdown, indicating that the close interaction between MyBPC-2 and the contractile filaments affects cross-bridge interaction. It is expected that a human myopathy associated with MyBPC-2 alterations, if present, would be severe with significant alterations in skeletal muscle structure and function. In general, the papers included in the thesis show that models for human muscle disease in the zebrafish can be analyzed with a clinically relevant functional read out, that novel therapeutic options can be examined in these models and that possible new muscle diseases associated with altered expression of structural muscle proteins can be identified.

LIST OF SCIENTIFIC PAPERS

- I. **Li M**, Andersson-Lendahl M, Sejersen T, Arner A. Knockdown of desmin in zebrafish larvae affects interfilament spacing and mechanical properties of skeletal muscle. *J Gen Physiol.* 2013;141:335-45.
- II. **Li M**, Andersson-Lendahl M, Sejersen T, Arner A. Muscle dysfunction and structural defects of dystrophin-null *sapje* mutant zebrafish larvae are rescued by ataluren treatment. *FASEB J.* 2014;28:1593-9.
- III. **Li M** and Arner A. Immobilization of dystrophin and laminin $\alpha 2$ -chain deficient zebrafish larvae in vivo prevents the development of muscular dystrophy. Manuscript.
- IV. **Li M**, Andersson-Lendahl M, Sejersen T, Arner A. Knockdown of fast skeletal myosin binding protein C in zebrafish results in a severe skeletal myopathy. Manuscript.

CONTENTS

1	INTRODUCTION	1
1.1	Skeletal muscles and skeletal muscle disease	1
1.2	Skeletal muscle diseases and related proteins	2
1.2.1	Myofibrillar myopathy and Desminopathy	2
1.2.2	Duchenne muscular dystrophy (DMD)	4
1.2.3	Merosin-deficient congenital muscular dystrophy (MDC1A)	7
1.2.4	Myosin binding protein C (MyBPC) and related myopathies	8
1.3	Zebrafish (<i>Danio rerio</i>)	8
1.3.1	Zebrafish, a model organism	8
1.3.2	Skeletal muscles in the zebrafish	9
1.3.3	Modeling muscle disease in zebrafish	10
1.3.4	Therapeutic screening in zebrafish larvae	12
2	AIM	13
3	MATERIAL AND METHODS	15
4	RESULTS AND DISCUSSION	25
4.1	Desmin - Desminopathy	25
4.1.1	Desmin knockdown in zebrafish larvae	26
4.1.2	Desmin deficiency results in structural alterations	27
4.1.3	Lowering of desmin content impairs muscle contraction, but prevents stretch-induced injury	28
4.2	Dystrophin - Duchenne Muscular Dystrophy	29
4.2.1	The Sapje/DMD zebrafish	30
4.2.2	Sapje/DMD zebrafish have impaired contractile function	31
4.2.3	Ataluren partially restores the protein expression, improves muscle structure and function in the Sapje/DMD model	31
4.2.4	Immobilization during early development prevents muscle damage in Sapje/DMD larvae	33
4.3	Laminin - Congenital muscular dystrophy	35
4.3.1	Laminin α 2-chain deficiency in Candyfloss zebrafish strain	35
4.3.2	Alterations in muscle structure and functional defects in Caf larvae	35
4.3.3	Contraction-induced injury plays a part in the pathogenesis of MDC1A	36
4.3.4	Strain differences in muscle activity affects development of muscle lesions	36
4.4	Myosin Binding Protein C - striated muscle myopathies	37
4.4.1	Partial loss of fast skeletal MyBPC-2 results in a severe muscle phenotype	37
4.4.2	Structural alterations and elevated degeneration/apoptotic signaling in MyBPC-2 morphants	38
4.4.3	X-ray diffraction studies of MyBPC-2 morphants	39

4.4.4	Contractile impairments in MyBPC-2 morphants.....	41
4.4.5	MyBPC-2 associated myopathy	42
5	CONCLUSIONS	44
6	ACKNOWLEDGEMENTS	45
7	REFERENCES	47

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BAG3	BAG family molecular chaperone regulator 3
BMD	Becker muscular dystrophy
BTS	N-benzyl-p-toluene sulphonamide
<i>Caf</i>	<i>Candyfloss</i> (zebrafish mutant)
CMD	Congenital muscular dystrophy
dCAPS	derived cleaved amplified polymorphic sequences
DGC	dystrophin-glycoprotein complex
DMD	Ducheene muscular dystrophy
DMSO	dimethyl sulfoxide
dpf	days post-fertilization
EDL	extensor digitorum longus
ENU	N-ethyl-N-nitrosourea
FHL1	four and a half LIM domains protein 1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
hpf	hours post-fertilization
L_{opt}	optimal length
LCB3	Microtubule associated protien 1 light chain 3
MDC1A	Merosin-deficient muscular dystrophy type 1A
MFM	myofibrillar myopathy
MO	Morpholino antisense oligonucleotide
MOPS	3-(N-morpholino)propansulfonic acid
MuRF	muscle RING finger protein
MyBPC	myosin binding protein C
PFA	paraformaldehyde
<i>Sap</i>	<i>Sapje</i> (zebrafish mutant)
SAXS	small angle X-ray scattering
SQSTM1	sequestosome-1

TU	Tübingen (wild type zebrafish strain)
V_{\max}	maximal shortening velocity
ZASP	Z-disk-associated protein

1 INTRODUCTION

1.1 SKELETAL MUSCLES AND SKELETAL MUSCLE DISEASE

Muscles are essential for the physiology of humans and other animals. The three main types, skeletal, cardiac and smooth muscles have key functions in the body. The cardiac muscle sustains the pumping activity of the heart and the smooth muscles maintain tone in the wall of hollow organs like blood vessels and intestines. The skeletal muscle supports locomotion, posture maintenance, respiratory ventilation and heat generation (Marieb, 1995). The skeletal muscle is cross-striated, when observed in the microscope, due to its sarcomeric organization, and the contraction is achieved by shortening of the sarcomeres caused by interaction between the contractile myosin and actin filaments, as described by “the sliding filament model” (Huxley and Niedergerk, 1954; Huxley and Hanson, 1954). When a muscle is activated via its motor neurons, myosin cross-bridges attach and exert power strokes to produce force and shortening. This process is regulated by intracellular Ca^{2+} binding to troponin on the thin filaments, which leads to activation of the actin-myosin interaction (Cooke, 1997). The contractile proteins, actin and myosin, constituting the thin and thick filaments, are anchored in a complex cytoskeleton which provides mechanical contacts in the cell, and also participates in cellular signaling.

Although skeletal muscle function is an important component in daily life, it can also be affected in several muscle diseases. In general, the causes for muscle disease are heterogeneous, from alterations in nerves, mitochondria, sarcoplasmic reticulum, to nuclei and other intracellular components (Goebel, 2011; Laing, 2012). The inheritable muscle diseases are relatively rare, but often devastating where patients become disabled often at an early age and can die prematurely. The disease prevalence is variable among different disease subtypes and the geographic locations, and a recent study on genetic muscle disease showed an overall prevalence of 37.0/100 000 in Northern England (Norwood et al., 2009). The cytoskeletal proteins, interacting or regulating the contractile apparatus, are often engaged in hereditary muscle disease, where mutations give rise to low protein content or aberrant protein function (Emery, 2002; Goebel, 2011). For the many forms of hereditary muscle disease hardly any effective treatments are available.

Today, clinical analysis of genetic mutations, with screening and identification of novel candidate genes for muscle disease, is rapidly developing. However, to seek and confirm causal relationships, between mutation and disease, mechanistic studies are needed (Laing, 2012). For understanding the role of the many muscle proteins (normal and mutated) involved in muscle disease, different animal models of muscle disease and new platforms for high throughput therapeutic screening have to be developed.

With this background, the thesis work was initiated to establish the zebrafish model for studying muscle protein function; and to examine pathological mechanisms and therapeutic

options in skeletal muscle diseases. In this introduction, I present a background on the skeletal muscle diseases addressed in the **Papers I-IV** included in the thesis.

Figure 1 depicts the contractile components, with a sarcomere, thick and thin filaments, in a muscle cell. The sarcomere and contractile filaments are connected via cytoskeletal proteins, some of which shown in the illustration. Desmin and the desminopathies introduced in Section 1.2.1 was the topic of **Paper I**. Dystrophin and Duchenne Muscular Dystrophy are introduced in Section 1.2.2 (**Papers II, III**), Laminin and Merosin-deficient congenital muscular dystrophy in Section 1.2.3 (**Papers II, III**) and Myosin Binding Protein C (MyBPC) in Section 1.2.4 (**Paper IV**).

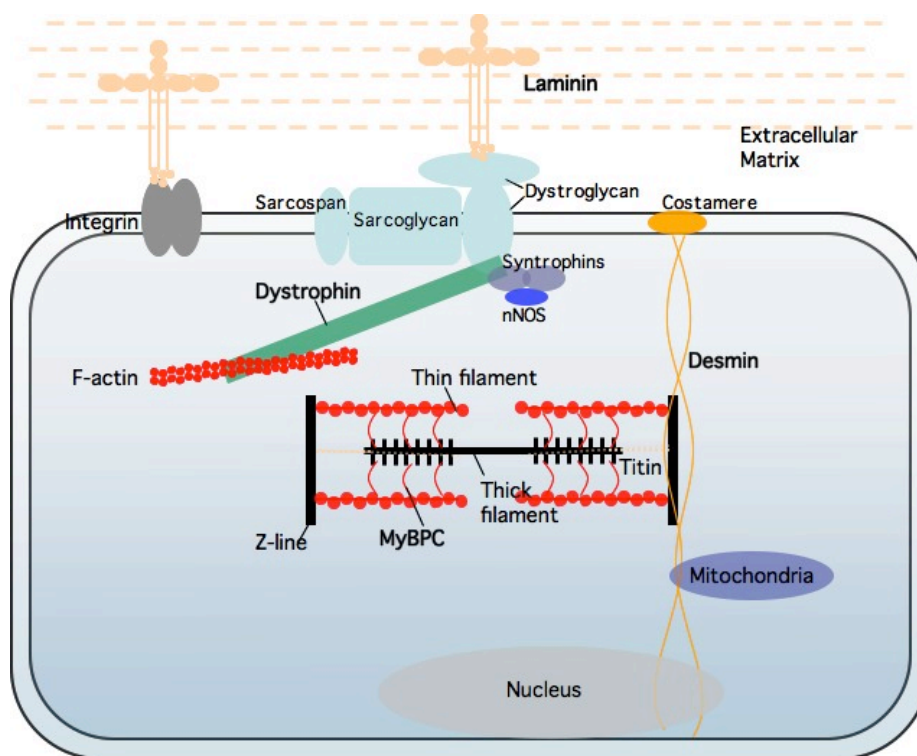


Figure 1. Schematic view of some key proteins and associated structures in the skeletal muscle cell.

1.2 SKELETAL MUSCLE DISEASES AND RELATED PROTEINS

1.2.1 Myofibrillar myopathy and Desminopathy

Myofibrillar myopathies (MFMs) are a large heterogeneous group of chronic neuromuscular disorders sharing similar pathological features, including degradation of the myofibrils, accumulation of degraded products as well as ectopic expression of several proteins (Selcen and Engel, 2011). These diseases are rare in humans, but often severe with cardiac and respiratory involvement. The onset in patients varies between infant to adulthood, and the clinical phenotype has a large spectrum. The inheritance has been reported as autosomal dominant or recessive in most cases, and causative mutations in multiple genes have been

identified. Genes and encoding proteins involved in MFMs mainly include desmin, α B-crystallin, myotilin, filamin C, ZASP, FHL1, BAG3, and myotilin (Schröder and Schoser, 2009).

Among the several protein candidates causing MFMs, desmin is the most commonly involved. Missense mutations have been identified in human desmin gene (*DES*), and being shown to be responsible for fragility of the myofibrils (Dalakas et al., 2000). Moreover, the locations of these mutations in *DES* are often related to the clinical phenotypes, e.g., mutations on 2B segment of *DES* primarily reveal a skeletal myopathy whereas the defects on 1B segment are closely related with cardiomyopathy (Goldfarb et al., 1998). The desmin-related myopathies constitute a distinct subgroup of MFMs, where the muscle weakness usually occurs in distal muscles and progresses to the proximal. Cardiomyopathy and respiratory impairment is often involved. Microscopic examination reveals general signs of degenerative myopathy, and, like in other MFMs, accumulation of protein deposits is a characteristic pathological finding in the desminopathy. The intracellular protein aggregates are often found along the Z-line, and formed by the mutated desmin proteins or other myofibrillar proteins (Goldfarb et al., 2008).

1.2.1.1 Desmin and Intermediate filaments

Desmin is a key member of the intermediate filament (IF) proteins, specifically expressed in muscle tissues forming the 10-nm filaments. In humans, the desmin protein is a 470-aa protein with molecular weight of ~50 kDa. The protein is highly conserved among different species. It is essential for the scaffolding structure around the Z-disk in striated muscles, thereby linking the contractile machinery to the cell membrane, having an important role in maintaining myofibrillar integrity and in transmitting forces in the muscle tissues (Lazarides, 1980). Moreover, the structural bridging by desmin IF also anchors several intracellular organelles and the nuclei in muscle cells, further providing integration in the whole cell (Tokuyasu et al., 1983).

1.2.1.2 Human and mouse studies on desminopathy

In human desminopathies, most of the cases are caused by dominant negative mutations in desmin or α B-crystallin and exhibit the pathological hallmark of the intra-sarcoplasmic protein accumulation (Goldfarb et al., 2008). The insoluble protein deposits are reported to be toxic to the muscle cells, leading to the progression of muscle degeneration. However, one patient case has been reported (Muñoz-Mármol et al., 1998), where a homozygous or hemizygous deletion in the *DES* gene resulted in a severe myopathy, thus suggesting that low desmin content also is a pathological mechanism.

Several studies on mouse models for desminopathies have been performed, based on the desmin knockout (*DES*^{-/-}) mouse created in 1996 (Li et al., 1996; Milner et al., 1996). The *DES*^{-/-} mice are viable and essentially normal without a severely impaired general phenotype. The complete loss of desmin did not affect the development/differentiation of the muscle cells, but resulted in a severe myopathy affecting all types of muscle tissues including

skeletal, cardiac and smooth muscles (Li et al., 1996). Specifically, the *DES*^{-/-} skeletal muscles had severely disrupted muscle architecture, widened lattice spacing, significantly reduced tension generation as well as altered vulnerability to eccentric contractions (Balogh et al., 2003; Milner et al., 1996; Sam et al., 2000; Wieneke et al., 2000). However, in the heterozygous animals (*DES*^{+/-}) where the protein content was significantly lower, no major pathology was detected in the muscles (Li et al., 1997; Milner et al., 1996). The difference between *DES*^{+/-} and *DES*^{-/-} mice thus suggests that a complete ablation of desmin protein is required for the pathogenesis of desminopathy in the mouse model. It should be noted that unlike the human desminopathy, the *DES*^{-/-} mice did not show any protein deposits. Transgenic mice carrying various *DES* mutations have also been investigated to understand the specific role of human mutations and encoding truncated proteins. It has been reported that over-expression of wild-type desmin did not show any alterations; whereas the introduction of a 7-amino acid deletion (R173 through E179) in desmin resulted in a severe cardiomyopathy with the appearance of aggregates within the muscle cells (Wang et al., 2001). Taken together, substantial knowledge on desminopathy has been obtained from human and mouse studies, but it still remains unclear if the alterations in desmin protein content would play a role in the disease or if the aggregates are the main mechanism. The comparatively mild phenotype in the mice models should also be taken into consideration, possibly suggesting that alternative animal models are needed.

1.2.2 Duchenne muscular dystrophy (DMD)

The Duchenne muscular dystrophy (DMD), first clinically described in mid-19th century, is the most common and severe myopathy in childhood. The clinical onset is early in life, between 2-4 years of age, then with progress leading to a severe disability with immobilization and need for wheelchair during puberty, and often death at 15-25 years of age. The muscle weakness affects mainly proximal muscles, and often includes a cardiac dysfunction (Jay and Vajsar, 2001). The disease is X-chromosome linked and affecting ~1/3500 boys. The muscle fiber size is often variable in DMD biopsies and cell necrosis, macrophage invasion and increased connective tissue are usually seen in the affected muscle tissues. Serum creatine kinase values are elevated, suggesting compromised cell membrane (Emery, 2002).

The primary cause for DMD is the defect in the dystrophin protein (Hoffman et al., 1987). The ultimate consequence of the genetic mutations in DMD is the complete absence of functional dystrophin, while the reduction or partial expression of normal dystrophin lead to a milder form as known as Becker Muscular Dystrophy (BMD). Recently, up to 4700 mutations have been reported in the Leiden DMD mutation database (Aartsma-Rus et al., 2006). The genetic alterations in the DMD gene are highly heterogeneous, including intragenic deletions (65%), duplications (5% to 15%), nonsense point mutations, frame-shift mutations (Muntoni et al., 2003).

1.2.2.1 Conventional treatment for DMD and Ataluren

Currently, there is no effective cure for DMD; most of the treatments are focused on the disease management. The standard clinical drug is glucocorticoids, used mainly to relieve the symptoms and slow down the disease progression, but also introducing secondary side effects. Novel therapeutics have shown some promise and undergone further investigations, including adeno-associated virus mediated gene therapy, exon-skipping agents and read-through compounds (Beytía et al., 2012).

Non-sense mutations, introducing premature stop codons occur in 10-15% of all DMD patients. It has been found that some aminoglycosides (e.g. gentamycin) promote read-through of pre-mature translation stop codons, and that this compound is effective in the dystrophic mdx mice carrying a point mutation in the dystrophin gene (Barton-Davis et al., 1999). The dystrophin levels were significantly increased after 14-day gentamycin treatment and were accompanied by recovery in force generation. The aminoglycosides have however side effects with nephrotoxicity and ototoxicity, preventing long term use in human dystrophy.

Ataluren, also known as PTC-124, is a small-molecular drug identified in chemical screening for suppressing the nonsense mutations and promoting the read-through of pre-mature termination in mRNA. The read-through effect of Ataluren is more prominent than that of the aminoglycosides, and with much less toxicity (Welch et al., 2007). It was reported to interact with the ribosome during protein translation, promoting the read-through of premature termination caused by nonsense mutations only, and not interfering with the normal stop codons. In addition to initial tests *in vitro*, it has also been introduced into several mouse models with nonsense mutations associated with human genetic disorders *in vivo*, including Duchenne muscular dystrophy (DMD), cystic fibrosis (CF) and haemophilia. For the treatment of DMD, an initial phase II study was unfortunately unsuccessful, possibly because of a small material and inclusion criteria. The drug showed no toxicity, but the results suggested a complex dose dependency with no effects of higher doses (Peltz et al., 2013). More research, including our **Paper II**, has addressed this problem and Ataluren has now entered in phase III clinical trials (Bushby et al., 2014).

1.2.2.2 Dystrophin and Dystrophin-glycoprotein complex (DGC)

DMD/dystrophin gene is the largest gene identified in the human genome, and the encoding dystrophin protein is about 427 kDa in weight (Kunkel et al., 1989). The expression is mainly in the striated muscle tissues. Dystrophin is a large cytoskeletal protein, located closely to the sarcoplasmic surface of the sarcolemma; together with trans-membrane proteins (e.g. sarcoglycans, dystroglycan) and other partners (e.g. dystrobrevin) to form a condensed complex, which provides attachment between the cytoskeleton and the cell membrane. At the extracellular matrix, the DGC also adheres to laminin via α -dystroglycan, and further stabilizes the integrity of the cells (Davies and Nowak, 2006). In addition to the mechanical role, dystrophin and its partners in the DGC are also suggested to play an important role in signaling relay/transduction between matrix and nucleus. It has been proposed that the

interacting signaling proteins included calmodulin, CaM kinase II, and mitogen-activated protein kinase kinase 2, nNOS etc. (Bhatnagar and Kumar, 2010). Considering the structural and functional importance of the dystrophin and DGC, the pathogenic mechanisms of DMD have been considered very complex, although failure of the important mechanical bridge provided by DGC has been proposed as the main cause for DMD (Brooks, 1998).

1.2.2.3 *Studies in mdx mouse*

The first animal model for human DMD was the mdx mutant mouse identified in the 1980s (Bulfield et al., 1984; Dangain and Vrbova, 1984; Tanabe et al., 1986). These mice carry a nonsense mutation in exon 23 of the dystrophin gene. The symptoms are also X-chromosome linked with similar features to human patients, but much milder. Unlike the severe disease in DMD patients, the mdx mice are comparable healthy, viable and fertile. The relative mild phenotype is probably due to compensation from utrophin (Matsumura et al., 1992) and a generally higher regenerative capacity in the *Mus* species (Megenny et al., 1996).

The structural alterations in mdx mouse muscles include fiber destruction, followed by regeneration processes. The muscle weakness and impairment of force generation was minimal (Tanabe et al., 1986). The severity of the phenotype also tends to vary with age of the animals (Coulton et al., 1988a; Coulton et al., 1988b; Williams et al., 1993). The mdx muscles have high susceptibility to contraction-induced injuries, possibly reflecting that dystrophin plays a protective role under mechanical stress (Dellorusso et al., 2001; Petrof et al., 1993).

1.2.2.4 *Zebrafish Sapje mutant*

The zebrafish *dmd* gene for dystrophin is located on chromosome 1, and it is an ortholog of the human dystrophin *DMD* gene. The dystrophin protein is fairly conserved, the structure of the key protein domains in the two species have strong similarities. The zebrafish *Sapje* mutant was first characterized in 2003 (Bassett et al., 2003). Similar to the mdx mice, the *Sapje* zebrafish also carries a point nonsense mutation in the dystrophin gene causing complete loss of functional dystrophin protein. The phenotype of zebrafish *Sapje*/DMD mutant is much more severe than in the mdx mice; the disease associated defects starts to reveal at 3 days post fertilization (dpf) in the larvae, and develop until 14-21 dpf when the mutant larvae die prematurely. It has been shown that the morphological alterations are severe. The cell membrane is often compromised in the mutant larvae, as illustrated by a microscopy picture of Evans Blue dye injected larvae from our laboratory (Figure 2) accompanied with muscle degeneration and regeneration processes. The pathological alterations mimic the structural phenotype observed in human DMD (Bassett and Currie, 2004; Berger et al., 2010), however, the defects in contractile performance of this model were not known.

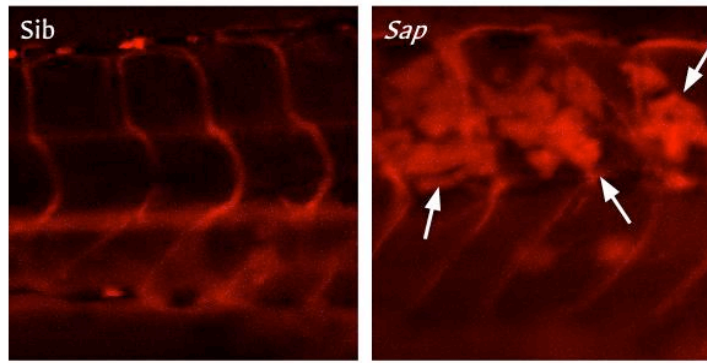


Figure 2. Evans blue dye accumulation in 4 dpf zebrafish larval muscles, indicating cell membrane ruptures (arrows) in the *Sapje* (*Sap*) mutant in contrast to the normal siblings (*Sib*), (Li, M. & Arner, A. unpublished)

1.2.3 Merosin-deficient congenital muscular dystrophy (MDC1A)

Merosin-deficient congenital muscular dystrophy (MDC1A) belongs to a heterogeneous group of congenital muscular dystrophies (CMD). The incidence of CMD is very low with geographic variations, and is estimated between 0.89-4.7/10 000 (Kirschner, 2013). The most frequent subtype is the collagen VI-related disorders or dystroglycanopathy. About ~10% of the CMD cases is MDC1A. The disease onset is early at birth or infancy, and progressively causing severe muscle weakness with delayed motor development, also spreading to other organs including heart and lung. The patients are usually never able to walk, and death occurs between 10-30 years of age due to cardiac or respiratory failure (Iannaccone and Castro, 2013). Currently, there is no curative treatment for this disease, and standard therapy is specifically targeting each individual symptom. To improve the quality of life in general, multidisciplinary care is considered, including physiotherapy, physical training as well as orthopedic surgeries. The MDC1A, also called *LAMA2*-related congenital myopathy, is primarily caused by the completely loss of normal laminin- α 2 chain (merosin) due to mutations in the *LAMA2* gene. The association between autosomal recessive mutations in *LAMA2* and MDC1A was first reported in 1995 (Helbling-Leclerc et al., 1995).

Animal models for studying MDC1A are mostly based on mutant (dy/dy , dy^{2J}/dy^{2J}) or knockout (dy^W/dy^W , dy^{3K}/dy^{3K}) mouse strains, which have varied phenotype with different genetic defects and protein contents (Gawlik and Durbeej, 2011). Nevertheless, many studies indicate that the most common pathogenesis of the disease lies in the structural integrity provided by laminin- α 2 chain between the cytoskeleton and the extracellular matrix (Brooks, 1998; Han et al., 2009; Petrof et al., 1993), although the exact role of mechanical contraction or intracellular laminin- α 2 signaling in the disease development remains unclear. The zebrafish MDC1A model (*Candyfloss* mutant) with a complete *LAMA2* deficiency was first presented in 2007 (Hall et al., 2007). These mutants revealed a severe phenotype affecting the muscle structure, movement as well as the animal life span. In the affected muscle regions, the muscle fibers appeared to detach from the myotendinous junctions with extensive degeneration and fibrosis. Inflammatory responses also occur. However, sarcolemmal rupture

was not primarily observed in this zebrafish model (Hall et al., 2007). The underlying mechanism and the precise role of mechanical stress in the disease development remain unresolved. Functional characterization is required to fully understand the *Candyfloss* mutant and to further explore the pathological mechanism in this model.

1.2.4 Myosin binding protein C (MyBPC) and related myopathies

Myosin binding protein C is a sarcomeric protein located at the M-zone of the striated muscle sarcomere. There are three types of this protein, cardiac, skeletal slow and skeletal fast. The cardiac type (MyBPC-3) is the most investigated. This work was initiated after the establishment of a causal relationship between mutations in this gene and human familiar cardiomyopathy (Watkins et al., 1995). The MyBPC-3 protein has 11 domains (C0-C10), where the N-terminus and C-terminus interacts with thin and thick filament/titin respectively (Winegrad, 1999). Phosphorylation/dephosphorylation of this protein is found to play an important regulatory role in positioning the myosin-head and affecting the cross-bridge kinetics as well as Ca^{2+} sensitivity (Harris et al., 2004; Kulikovskaya et al., 2003; Kunst et al., 2000; Weisberg and Winegrad, 1996). The structural tethering effect of MyBPC-3 was also reported in knockout mouse studies (Korte et al., 2003; Stelzer et al., 2006). Compared to the cardiac MyBPC-3, the skeletal types (MyBPC-1 and MyBPC-2) are less understood. Structurally, the key domains of three isoforms are fairly conserved, and it has been shown that the skeletal MyBPC binds to the sarcomere in a similar manner as the cardiac type, although the N-terminal C0 domain and several phosphorylation sites are absent (Luther et al., 2008; Oakley et al., 2004). Recently, mutations on MyBPC-1 gene have been associated with human distal atrogryposis type 1 myopathy (Gurnett et al., 2010) and congenital contractural syndrome type 4 (Markus et al., 2012), showing that the slow type of this protein is important in the skeletal muscle context.

The function of the skeletal form of MyBPC was examined in early work by Moss and colleagues (Hofmann et al., 1991a; Hofmann et al., 1991b). They used an extraction reconstitution strategy in skinned skeletal muscle fibers and showed that extraction of MyBPC caused an increase in maximal shortening velocity. It was suggested that the protein affected interaction between actin and myosin and thereby the filament sliding. Consistent with this model, knock out of the cardiac form in mice resulted in an increase in shortening velocity of cardiac muscle fibers (Harris et al., 2004; Kunst et al., 2000). To our knowledge, *in vivo* studies, where MyBPC have been removed in intact skeletal muscle are not available and no disease has been associated with alterations in the fast skeletal MyBPC-2.

1.3 ZEBRAFISH (*DANIO RERIO*)

1.3.1 Zebrafish, a model organism

The zebrafish is an emerging animal model for biomedical research. The rapid development, the optical transparency of embryos, the large number of off-springs, the less problematical ethical constraints and low economical costs of this species makes it advantageous as a model organisms (Ablain and Zon, 2013; Dooley and Zon, 2000). The animal offers powerful

possibilities to study the vertebrate gene function since genetic manipulations, with knockdown or over-expression, can be performed and mutagenesis screening and transgenic lines are accessible (Lieschke and Currie, 2007). Zebrafish have 25 chromosomes, the whole genome size is about half of that in humans (15 billions vs. 30 billions of base-pairs). Recent genome sequencing has shown that ~26,000 protein coding genes exist in zebrafish, and they share large conservative features with the mammalian genome. Specifically, ~70% of human genes have corresponding orthologs in zebrafish (Howe et al., 2013).

1.3.2 Skeletal muscles in the zebrafish

Skeletal muscles constitute a major part of the zebrafish trunk and tail, and are functional supporting animal motions already from ~ 18 hours post fertilization (hpf). The slow and fast muscle fibers are found in the trunk at separate locations; the slow fibers are found in the peripheral layer under the skin while the fast are more internal and more abundant (Devoto et al., 1996). General muscle cell structure, sarcomeres and anaerobic/aerobic functions are conserved in zebrafish muscles. But unlike the mammals, the zebrafish retains the structure of somites where the skeletal muscle fibers are arranged in parallel between two adjacent myosepta (arrows in Figure 3).

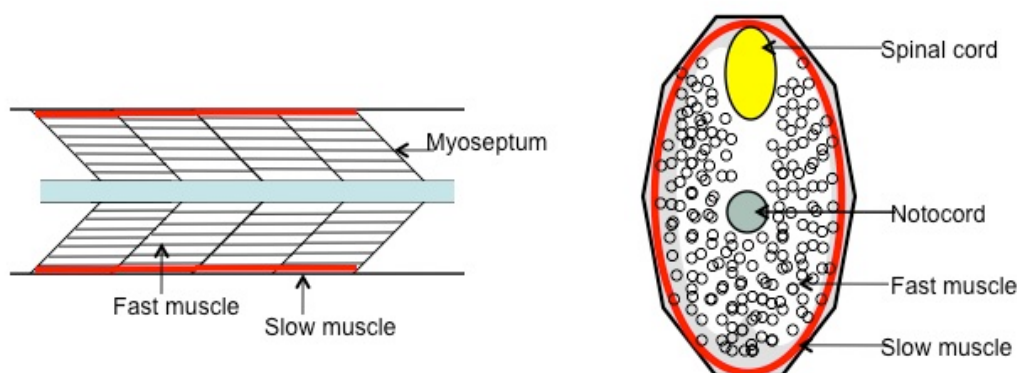


Figure 3. Schematic view of general muscle fiber organization and composition in zebrafish larval muscles (4 dpf). The slow fibers (indicated as red) form one single cell layer underneath skin, the majority of the trunk muscles are the fast type (white). Left: longitudinal section through the larval trunk region, muscle fibers attach between two adjacent myosepta. Right: transverse section at mid-trunk region.

1.3.3 Modeling muscle disease in zebrafish

For zebrafish, significant research interest has focused on the embryology and developmental biology due the “*ex-uterus*” development and optical transparency. As the knowledge and experience on this species is expanding, zebrafish have also been used to model human diseases. In 2003, the first zebrafish model for muscle disease, the *Sapje* mutant, was discovered and characterized (Bassett et al., 2003). In the field of muscle disease, several approaches have been applied in zebrafish to model human disorders, including forward/reverse genetic studies and transient genetic modifications (Wood and Currie, 2014).

1.3.3.1 Mutagenesis screening and transgenic strains

One of the oldest strategies, chemical mutagenesis by N-ethyl-N-nitrosourea (ENU), is powerful to introduce mutations on a large genome scale (Justice et al., 1997). Following the success in other organisms (mouse, fly, worms), ENU screening was established in zebrafish to study the forward genetics since the 1990s (Solnica-Krezel et al., 1994). However, the effect of ENU is highly nonspecific and random, the phenotypic screening requires large-scale tests. Furthermore, the subsequent genetic linkage mapping is needed to locate the mutation on the chromosome. Therefore, this method is suitable for large research centers with joint-efforts on maintaining of zebrafish lines, phenotyping and genetic mapping. Up to now, the zebrafish mutant lines with various genetic defects have been established in e.g. Tübingen, Germany and Boston, USA (Knapik, 2000). For several mutants, the genetic lesions have also been identified, e.g. *Sapje* (*dmd*), *Candyfloss* (*lama2*), *Softy* (*lamb2*). Transgenic zebrafish strains for human disorders have also been created using recombinases (Dong and Stuart, 2004). However, they are less studied due to the limited techniques for targeting the zebrafish embryonic cells (Fan et al., 2006). Recently, novel techniques have been developed to generate stable mutant and knockout lines, e.g., zinc-finger nucleases, transcription activator-like effector hybrid nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR's)/cas9 system (Doyon et al., 2008; Huang et al., 2011; Hwang et al., 2013).

1.3.3.2 Morpholino antisense-oligonucleotides (MOs)

“Morpholinos” are chemically modified oligonucleotide analogs. They inhibit the expression of selected genes through a base-pairing mechanism (Heasman, 2002). The inhibitory effects can target the splicing of premature RNAs (splicing blocking MOs) or the initiation of the protein translation (translational blocking MOs). Compared to small interfering RNAs (siRNA), the structural modifications of MOs enables a higher solubility, higher affinity for the target RNA sequence, longer stability in biological systems with significantly lower off-target effects (Summerton, 2007). Since the information on genetic sequencing is available in zebrafish, the design of MOs is fairly simple and available to users; different designs of MOs for one gene-of-interest could be obtained at low cost. For the knockdown in zebrafish, morpholinos are injected into the yolk of embryos at the 1-2 cell stage, and then taken-up into

the cells by the yolk stream. The effects of MOs are non-stable, transient during the larval stage, although the MOs are persistent and resistant to degradation *in vivo*. This approach has been popular in zebrafish field owing to the low cost and high efficiency.

There are, however, also some concerns with regard to the authenticity of the phenotype and the proper use of control MOs. The off-target effects are theoretically minimal in the use of MOs; however, a dose-associated toxicity and non-specific phenotype have been shown in some cases (Bill et al., 2009). Moreover, in some studies, the use of mismatched control MO, where several bases of the effect MO are mismatches, often interfere with endogenous RNA. The most commonly used control MO is the standard MO, which targets a human beta-globin intron mutation (designed by Gene Tools LLC). It has been shown to cause negligible phenotype effects in most test systems and can serve as a negative control. The accuracy and the specificity of MOs thus have to be validated with caution, it has been proposed that the phenotype caused by MOs should be verified in both blocking strategies (splicing blocking and translation/ATG blocking of mRNA). Phenotypic rescue of the corresponding mRNA should also be considered (Eisen and Smith, 2008).

1.3.3.3 Structural and functional evaluation of zebrafish skeletal muscles

The structural examination of zebrafish larval muscles has been well established. In early muscle developmental studies, several basic techniques, including tissue sectioning, whole-mount preparations, immunohistochemistry, hematoxylin and eosin stain, *in situ* hybridization, etc., have been well documented. In the first zebrafish model for muscle disorder (Bassett et al., 2003), birefringence microscopy was also applied and revealed characteristic features of dystrophic muscle fibers in the larval trunk. The measurement of muscle damage by birefringence quantification was further established (Berger et al., 2012) and also applied in other zebrafish mutants with muscle dystrophies, e.g., *Candyfloss* (Sztal et al., 2012). Specifically, Evans blue dye is also a useful tool to examine the sarcolemmal integrity, and revealed compromised cell membrane in zebrafish *Sapje* mutant (cf. Figure 2). Given the optic advantages of early zebrafish larvae, microscopic investigation has been an important approach. Ultra-structural examination was also available using electron microscopy. In addition, a classic structural evaluation method in muscle, small angle X-ray diffraction, has been applied in the larval muscles (Dou et al., 2008).

Compared to the structural investigations in zebrafish larvae, the functional characterization of muscle tissue was less established. The early studies have been focused on the swimming property and behavioral pattern of the larvae. The functional impairment observed in these studies could reflect more the general locomotor defects, but remain less specific for the muscle function *per se* (Saint-Amant and Drapeau, 1998). Moreover, in most cases, the subtle changes in the muscle force could be undetectable due to the limited resolutions of the assay. The first direct mechanical measurement of muscle force was reported in 2008 (Dou et al., 2008), where the active contraction could be recorded and length-tension relationship was characterized on the larval muscles. These functional techniques, measuring active force, are important for translation to the normal and diseased human muscle. More advanced

mechanical examinations on the contractile or elastic properties of the muscles can give further mechanistic information importance for understanding the function of normal and mutated muscle proteins.

1.3.4 Therapeutic screening in zebrafish larvae

As discussed above, zebrafish develop rapidly and reproduce in large quantity; the small size and independency on cardiovascular or food support during early stage make them popular in high throughput screens. They have been used in multi-well system for chemical screening since 2000 (Peterson et al., 2000). The approach was simply carried out by adding the compound into the fish water and evaluating the phenotypes. For the zebrafish mutants with muscle disorders, the same approach could also be applied for therapeutic screening and evaluation of small molecules, where the effects on structural and functional improvement or toxicity could be assessed. It has been reported that therapeutic candidates for human DMD were tested in zebrafish *Sapje* mutant larvae, the phenotypic improvement was recorded and structural restorations were also evaluated (Kawahara et al., 2011). In addition to small molecule screening, other therapeutic strategy e.g. genetic modification with exon-skipping, has been tested using the zebrafish *Sapje* DMD model (Berger et al., 2011). So far most studies have focused in effects on structure. It should be noted that a main therapeutic goal in muscle disease is to improve muscle function in the patient, and it is therefore relevant to include analysis on the therapy effects on muscle force.

2 AIM

This thesis is aimed to answer the following research questions:

- Can physiological studies of zebrafish larvae be used for analysis of protein function in muscle?
- Can zebrafish models of muscle disease be used to explore pathological mechanisms relevant for the situation in humans and to examine effects of novel therapies?
- What are the structural and mechanical consequences of partial desmin removal in the zebrafish skeletal muscles?
- Do zebrafish models lacking dystrophin or laminin α 2-chain mimic clinical observations in Duchenne (DMD) and Congenital (CMD) Muscle Dystrophies?
- What are the effects and dose dependency of Ataluren, a read-through compound, on muscle function in the *Sapje*/DMD model?
- Can the muscle dystrophic development be influenced by muscle immobilization?
- What are the roles of Myosin Binding Protein C (MyBPC) in skeletal muscle development and function?

3 MATERIAL AND METHODS

3.1 Animals

The papers included in the thesis are based on studies of zebrafish models, either after gene knockdown with morpholino antisense oligonucleotides (cf. section 3.3) or using different mutated strains. The zebrafish wild-type strains (AB and TU, **Papers I and IV**), used in morpholino knockdown studies, were regular breeds at the CMB zebrafish facility, KI. The *dmd/sap*^{ta222a} (*Sapje*) mutant strain (**Paper II and III**) was imported from Tübingen, Germany and the *caf*^{eg15a} (*Candyfloss*) mutant strain (**Paper III**) was a gift from Monash University, Australia (Dr. P. Currie). For the mutants, the heterozygous animals carry a single missense mutation in the dystrophin (*Sapje* strain; Basset et al., 2003) or the laminin $\alpha 2$ (*Candyfloss* strain; Hall et al., 2007) gene on one allele. They have normal protein expression with healthy phenotype and normal life span; whereas the homozygote animals lack the corresponding protein and exhibit defects in musculature. The larvae were used within 6 days post fertilization (dpf). If not euthanized, the homozygous animals of each strain will die early, from about 10 dpf, most likely due to impaired food intake. The zebrafish larvae are easily anesthetized and immobilized using Tricaine (MS-222), and higher doses of the compound were used to euthanize the animals (Westerfield, 2000).

3.2 Genotyping (*Papers II and III*)

The homozygous mutant *Sapje* and *Candyfloss* strains were easily identified with birefringence (Section 3.5), but genotyping was also performed to identify heterozygous adult breeding pairs and homozygous larvae following treatment, when they did not exhibit structural changes (**Paper III**). Genomic DNA was extracted from the tip of tail-fin tissue (adult fish) or from the whole body (early larvae). The identification of the mutant allele was achieved using derived cleaved amplified polymorphic sequence (dCAPS) analysis (Neff et al., 1998). In principle, the gene fragment harboring the point mutation was flanked and amplified using standard three-step PCR, using primers resulting in a restriction site in the mutated samples. The PCR products were subsequently digested with the restriction enzyme, with a successful cut of the fragment from the mutant allele at the site of the point mutation. The corresponding PCR products from wild type allele remained intact after the digestion. Thus, the wild type (+/+), heterozygous (+/-) and homozygous (-/-) animals could be identified from the number of bands after restriction digestion. The primers and restriction enzymes used for each strain were described previously (Sztal et al., 2012).

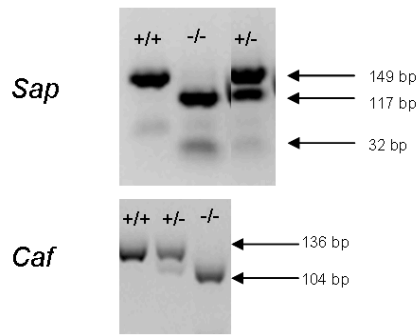


Figure 4: Genotyping of zebrafish mutants.

Following PCR amplification of genomic DNA, using primers flanking the region harboring the point mutation, the products were then digested with restriction enzyme that cuts at the mutation site, and separated on agarose gel. The corresponding bands from wild type (+/+), heterozygous (+/-) and homozygous (-/-) *Sapje* (*Sap*) and *Candyfloss* (*Caf*) could be identified.

3.3 Knockdown approach (Papers I and IV)

Morpholino antisense oligonucleotides (MO) are designed to target the splicing site (intron-exon or exon-intron junction) on the premature mRNA or the ATG site of mRNA, resulting in mis-spliced mRNA (splice blocking) or un-translated protein (translation blocking), respectively (Bill et al., 2009). The MOs were designed based on the DNA sequences, synthesized and obtained from Gene Tools LLC (Philomath, OR, USA). We used this technique to knock down desmin (**Paper I**) and myosin binding protein C (**Paper IV**). Since we identified several isoforms several MOs were used, either alone or in combination. The animals with gene/protein knockdown are denoted morphants. The morpholino oligonucleotides are small in size (25-bases), and can easily be delivered into the embryos by yolk-injection at the 1-2 cell stage. The amount of MO injected per larva was about 5 nL (~4 ng oligonucleotide). A standard control MO (5'-CCT CTT ACC TCA GTT ACA ATT TAT A 3') was used in both **Paper I** and **IV**. This sequence is supposed to have no target or biological activity in the zebrafish larvae (Gene Tools LLC), and was used to exclude unspecific effects of oligonucleotide injection. We did not in our studies observe any differences between wild type and the control MO injected larvae in the mechanical and structural experiments. The specific knockdown effects of designed morpholino oligonucleotides in the larvae was detected at the protein level using Western blotting, silver stained gels or immunohistochemistry; and, for the splice blocking MOs, also at the mRNA level using PCR.

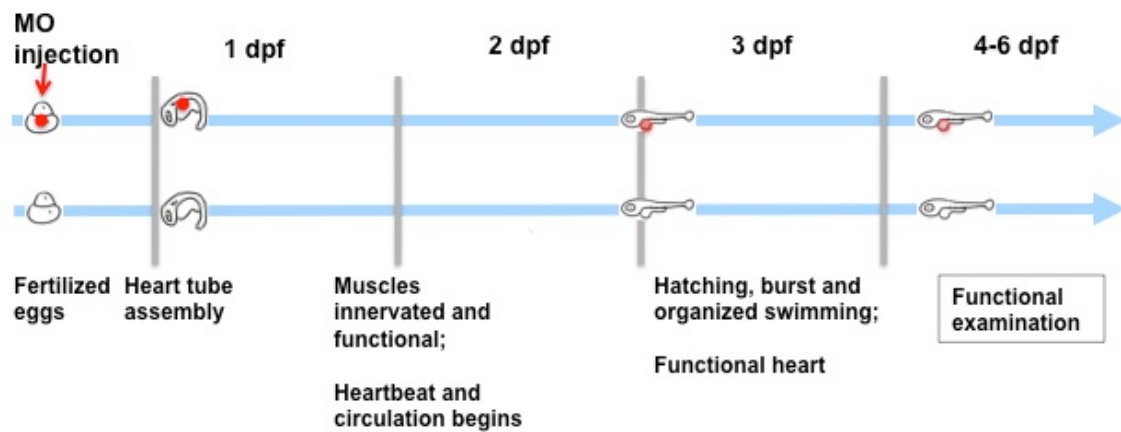


Figure 5: Schematic illustration of the early development of organ functions in zebrafish larvae and of the experiments using morpholino antisense oligonucleotide (MO) injection.

3.4 General morphology and immunohistochemistry

In all papers we examined the effects of MO injection or mutations on the larval morphology and muscle structure. The 4-6 dpf zebrafish larvae are small and transparent to light, which make it possible to examine the morphology of the whole body using microscopy. A general overview of the larvae (e.g. to identify large anatomical changes and measure length and width) was obtained using standard light microscopy of anesthetized or euthanized larvae. The birefringence technique used on living larvae is described below in Section 3.5. More specific information was obtained using fixed samples. The larvae were euthanized with Tricaine and then fixed in 4 % paraformaldehyde (PFA) at 4 °C overnight at defined degrees of stretch. For general examination of the sarcomeric structure, whole-mount preparations of larvae were stained with Rhodamine phalloidin, which stains F-actin filaments. This enabled studies of muscle fiber orientation and measurements of sarcomere length. In **Paper II**, the fixed preparations were stained with anti-dystrophin primary antibody (DSHB) and Alexa Fluor-488-conjugated secondary antibody to detect the protein content and location. To determine the relative content of fast and slow muscle myosins, the larvae were embedded in freeze section medium (O.C.T.), sectioned transversely (10 µm), fixed in acetone, and stained with S58 (mouse anti slow myosin) and F59 (mouse anti fast myosin) primary antibody and Alexa488 secondary antibodies. The collagen content was examined in cross-sections using Sirius Red staining. The images were recorded and analyzed using a confocal microscopy system (LSM 510; Carl Zeiss, Germany).

3.5 Birefringence assays (*Papers II and III*)

Skeletal muscle is birefringent, due to the optical properties of the sarcomere affecting the polarization of transmitted light. When the larval muscle is illuminated with polarized light and observed through a second polarizer at 90° angle, the background is dark and bright/light areas appear where the muscle is birefringent (i.e. with regular sarcomeric structure). This property has been explored to examine dystrophic changes in muscle structure of the zebrafish larvae (Berger et al., 2012). It has been shown that in zebrafish muscular dystrophy larvae (*Sapje* and *Candyfloss*), the affected muscle cells undergo necrosis/degeneration, indicated as dark patchy areas under birefringence microscopy. The homozygous mutants start to reveal these birefringence defects at 3 dpf, and allow the differentiation from their normal siblings. The polarized light intensity was quantified in **Paper III** as a measure of the muscle damage.

3.6 Small angle X-ray diffraction (*Papers I and IV*)

The regular arrangement of sarcomeres and contractile filaments give rise to light scattering, interference and diffraction. The length of the sarcomeres (in the μm dimension) can be estimated using light diffraction from a HeNe laser (wavelength 632.8 nm). However, for resolution of interfilament distances (20-50 nm) lower wavelength light has to be used (about 1 Å=0.1 nm). In principle the muscle is illuminated by focused X-ray light. Scattering and interference generates a diffraction pattern where periodic structures (e.g. distances between filaments) create reflections. The distance between these reflections is inversely related to the spacing of the diffracting structures. Since the distance between filaments is large compared to that in e.g. protein crystals the scattering angle is small requiring a comparatively long camera length (i.e. distance between sample and detector) to be resolved (i.e. Small Angle X-ray Scattering, SAXS). For example the spacing of the 1.1 and 1.0 reflections is reflecting lateral distances between the contractile actin and myosin filaments (Millman, 1998; Figure 6). The intense focused and high energy light with a broad wavelength spectrum generated by accelerated charged particles in synchrotron light facilities has been used in muscle research (Wray and Holmes, 1981). The experiments were done at the A2 beamline at the Hamburger Synchrotron- Strahlungslabor (HASYLAB)/Deutsches Elektronen-Synchrotron (DESY) synchrotron facility in Hamburg, Germany, and the I911-SAXS beamline at the MAX II ring of the MAX IV Laboratory in Lund, Sweden. In these beamlines, the X-ray beam is focused on the larvae (beamsize at Hamburg: $\sim 300 \times 2000 \mu\text{m}$; Lund: $100 \times 100 \mu\text{m}$). The 2-dimensional pattern of diffracted light is recorded using sensitive detectors (Mar CCD or Pilatus) at a distance of 3-4 meter. The position of the reflections was determined and the spacing between filaments could be calculated knowing wavelength and camera length or from calibrations using samples with known spacing (collagen or Silver behenate).

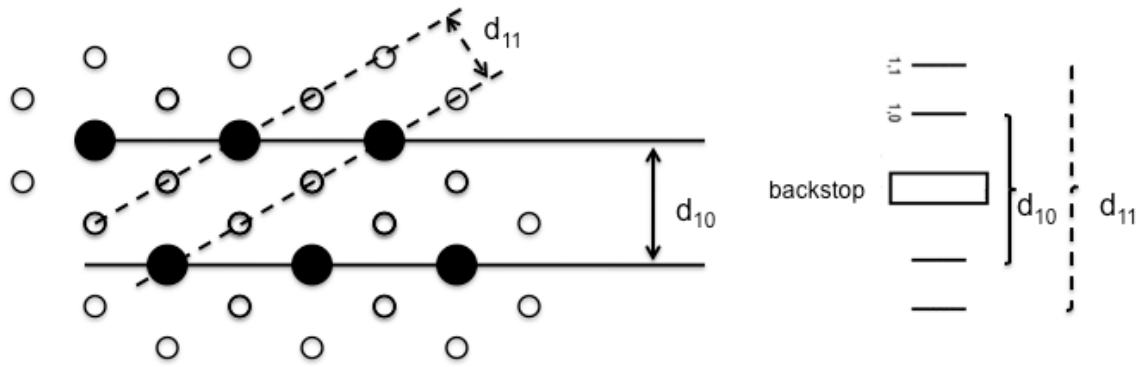


Figure 6. Left: transverse view of thick and thin filament lattice. Right: equatorial reflections in X-ray diffraction of skeletal muscles. Modified from Millman (1998).

Our group has previously demonstrated that X-ray diffraction patterns can be recorded from single zebrafish larvae (Dou et al., 2008) and the SAXS technique was used in **Paper I** and **IV** to determine the filament distances after knockdown of desmin and myosin binding protein C. The larval preparations were mounted horizontally in a Kapton-window cuvette with MOPS buffered physiological solution at room temperature. We exposed the muscles during 1-10 s and recorded the pattern. In these studies we focused on the equatorial patterns reflecting filament distances.

In **Paper I**, the larval preparations examined at varied stretch (i.e. sarcomere length) which enables estimation of sarcomere volume (Millman, 1998). We related the length changes in X-ray experiments to the mechanical data using the extent of stretch (muscle length relative to slack) or using the sarcomere length (determined with laser diffraction). To examine the influence of filament spacing in relation to active tension the osmolarity of the bathing solution was varied. The muscles were swelled (low NaCl) or compressed by adding sucrose in MOPS buffered solution. The structural data at different osmolarities was correlated with mechanical experiments as described below.

During contraction, myosin heads are considered to move out and attach to actin affecting the intensity of the equatorial 1.1 and 1.0 reflections (Haselgrove and Huxley, 1973). Outward movement towards actin weakens the 1.0 intensity and increases the 1.1 intensity. Since the SAXS studies can be performed in non-fixed, non-stained samples it is possible to record cross-bridge movement during contraction. In **Paper IV**, we established a procedure for the zebrafish larvae where the muscle was repeatedly (15 times) stimulated with short (200 ms) tetanic contractions. The detector was gated to be open in the relaxed state or during contraction, thus enabling us to record relaxed and contracted patterns. We also opened the X-ray shutter only when recording to minimize muscle exposure to the X-rays. To obtain a situation with maximal attachment of myosin, we recorded patterns in rigor conditions induced by incubation with 2 mM NaCN.

3.7 Protein expression analysis

To examine the knockdown effect of translation blocking MOs, as well as the splicing MOs on the protein level, protein expression of the control and MOs injected larvae was analyzed. Proteins were purified from early larval trunk muscles. The head, yolk, gut and other organs were removed as much as possible, to exclude possible contaminations from non-muscle tissue. The tissues were homogenized in an SDS containing solution (composition see **Paper I**), centrifuged and loaded on the gels.

Silver staining

In **Paper IV**, we estimated the content of Myosin Binding protein C using silver stained gels, since we could not obtain an antibody that could be used. The protein extracts were loaded and separated on 7% polyacrylamide gels. Extracts from one single larva were loaded on each lane. After the electrophoresis, the gel was washed briefly in water, subsequently stained and the proteins visualized using a SilverQuest™ Silver Staining Kit (Life technology). The intensity of the stained proteins bands was estimated using densitometry (Bio-Rad system, Bio-Rad Laboratories).

Western blotting

Extracts from 5-15 larvae were loaded on each lane of 10% polyacrylamide gels. Protein bands were transferred to nitrocellulose membranes and incubated with primary antibody (rabbit polyclonal anti-desmin in **Paper I** and mouse anti-dystrophin in **Paper III**). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was used in both studies as a control. ECL Western Blotting Detection Reagents, photographic film (Fuji), and optical detection (Bio-Rad system) were used to quantify the intensity of the protein bands.

3.8 Behavioral analysis

Optical analysis of larval swimming can be used as a semi-quantitative method to identify major motility problems (Granato et al., 1996). The zebrafish larvae were examined using an automated behavioral analysis system, ZebraLab V3 (ViewPoint Life Sciences Inc) at 4-6 dpf. The larvae were morphants, pre-injected with morpholinos (**Paper I**) or mutants, pre-selected under birefringence (**Paper III**). They were transferred to 24-well plate in E3 medium, 1 larva per well, and allowed to acclimatize for 30 min at 22 °C. The swimming pattern then was recorded for 1-2 min. Large and small movements of each larva were tracked and analyzed, the parameters included in the studies were: duration, distance covered and moving speed of each movement type.

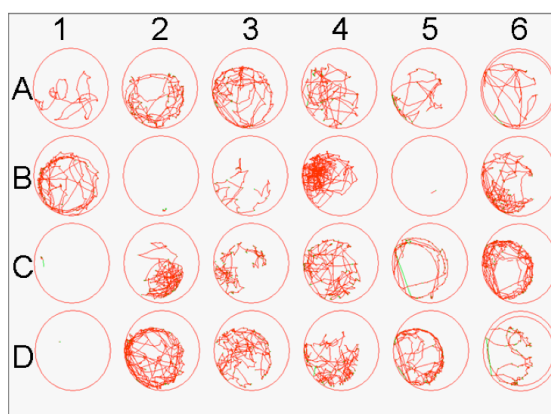


Figure 7. Representative recordings of 4 dpf TU larvae during 2 min swimming behavior analysis on 24-well plate, one larva per each well. Red traces indicate large movements.

3.9 Pharmacological treatment

Zebrafish larvae enable comparative high-throughput studies of the effects of pharmacological intervention. Using our mechanical approach, we provide a functional readout. The zebrafish larvae start to hatch from the chorion at 3 dpf and live on the yolk as nutrition source until ~ 6 dpf. They absorb oxygen and other chemicals (e.g. electrolytes and drugs) from the bathing E3 medium through skin or digestive track. Thus, the pharmacological treatments of the larvae can be applied directly to the bathing medium at the desired concentration. To ensure a proper absorption, the larvae were manually dechorionated before the treatment, and solutions were renewed every day. The animals were kept in 24-well plate during the treatment, one larva per well; the survival/mortality rate and touch-response were monitored through the treatment. The experiments were terminated and the muscle structure and/or function were analyzed after 2-4 days. For both Ataluren (**Paper II**, Section 3.9.1) and BTS (**Paper III**, Section 3.9.2) treatments, 0.1% DMSO was applied as a solvent control.

3.10 Ataluren treatment (Paper II)

In **Paper II**, we examined the functional and structural effects of a novel compound Ataluren (PTC 124) causing read-through of pre mature stop codon (Welch et al., 2007), in the *Sapje* dystrophy model. The compound was purchased from Selleck Chemicals (Houston, TX, USA), dissolved in DMSO to obtain two stock concentrations (5 mM and 35 mM), and then added to the E3 medium to reach the final dose (0, 0.1, 0.5, 1, 5 and 35 μ M). The treatment was initiated at 3 dpf, when the mutant larvae and siblings could be identified with birefringence microscopy. After the functional and structural analyses at 5 dpf, the larval preparations were kept and genotyped to confirm their genetic identities.

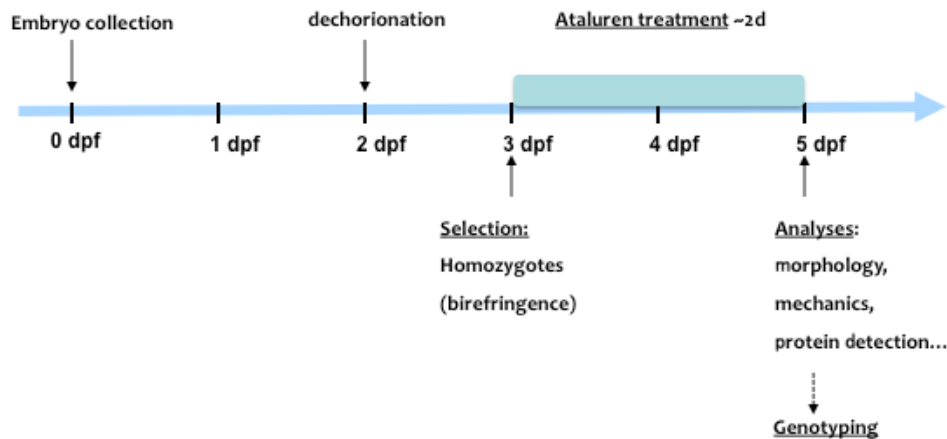


Figure 8: Schematic view of the experimental plan for Ataluren treatment.

3.11 BTS treatment (Paper III)

To examine the effect of immobilization on structural and mechanical dysfunction in *Sapje* and *Candyfloss* dystrophic larvae we applied N-benzyl-p-toluene sulphonamide (BTS), which is a compound that inhibits actin-activated myosin ATPase (Cheung et al., 2002) and contraction of mammalian (Pinniger et al., 2005) and zebrafish larval muscle (Dou et al., 2008). Since hatching involves active muscle movement the BTS treatment was initiated early at 18 hpf, when the homozygous mutants and siblings have the same phenotype. The larvae were dechorionated and then treated with 50 μ M BTS from 18 hpf to 4 dpf, a dose giving complete inhibition in mouse EDL (Fredsted et al., 2007), and then analyzed after 1 hr (at 4 dpf) and 1 day (5 dpf) washout in E3 medium, as illustrated in Figure 9 below. Since the treatment was initiated before the homozygous animals could be indentified, each sample was genotyped after analysis.

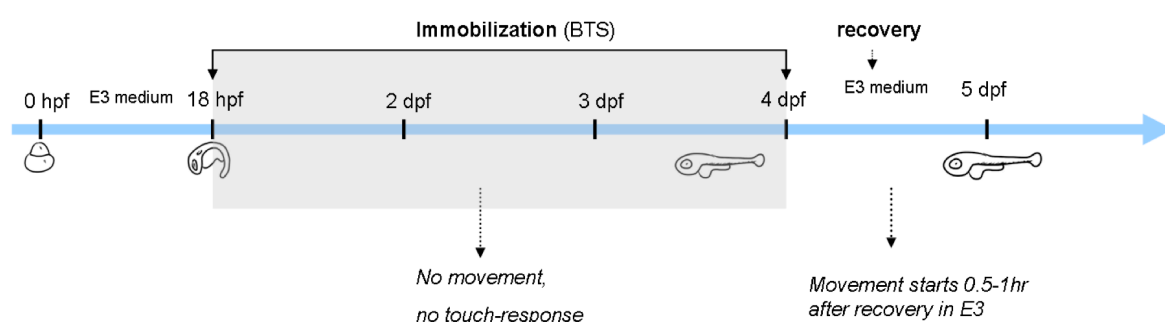


Figure 9. Schematic view of the experimental plan for larval immobilization using BTS.

3.12 Mechanical analysis

We analyzed muscle contraction as a key functional readout in the studies of morphants (**Paper I** and **IV**), in mutated strains and after treatment (**Paper II** and **III**). We used single twitch stimulation and determined maximal active force at optimal stretch (Section 3.12.1) In **Paper I** we also used tetanic stimulation and applied a stretch protocol to examine effects of

eccentric contractions (Section 3.12.2) The larvae were analyzed at 4-6 dpf and euthanized with Tricaine. Aluminum clips were wrapped around the larval head and the tip of the tail, and the preparation mounted horizontally in a cuvette between a force transducer attached to a micrometer screw and a hook attached to a motor enabling rapid length changes. The cuvette was perfused with a Krebs-Ringer bicarbonate buffered solution gassed with 95%/5% O₂/CO₂, or with MOPS buffered solution (composition of solutions is given in **Papers I and II**) at room temperature (22 °C). The muscles were stimulated via two platinum electrodes placed on the sides of the cuvette.

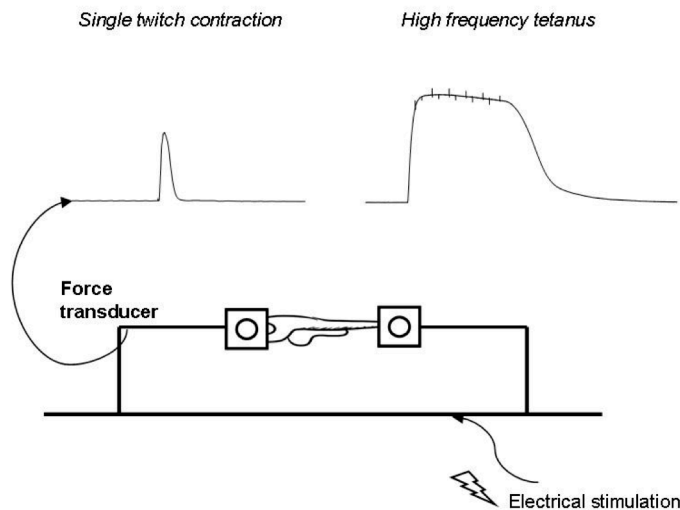


Figure 10: Mounting of larval preparation and force recording on 4-6 dpf larvae. Upper panels: original recording of force traces from a single twitch and high-frequency tetanus contraction. Lower: schematic view of the mounting strategy.

3.12.1 Single-twitch contractions and length-force relationships

It has previously been found that single twitch stimulation gives near maximal force in zebrafish larval muscle (Dou et al., 2008) and we therefore used this mode of stimulation in our analysis of contractile function. The larval preparations were stimulated with 0.5 ms electrical pulses (supramaximal voltage) at 2-min intervals. An initial contraction was recorded at slack length, and then the preparation length was increased step-wise between the contractions via the micrometer screw, until a length above the maximal for active force, to ensure that the optimal length (L_{opt}) for maximal active force was determined. At each step, length and the active and passive force were recorded. Force signals were recorded and analyzed using LabChart® (ADInstruments Ltd, Oxford, UK). The length-force relationship was determined by plotting the relative length of the preparation against the relative force generated at each length.

3.12.2 Tetanic stimulation and eccentric contraction (Paper I)

For tetanic contractions, larval muscles were mounted at L_{opt} , stimulated at 200 Hz for 200 ms, with 5 min intervals between the stimulations. The contractions fused at 60 ms after onset

of stimulation and exhibited a plateau during the tetanus. To examine stretch-induced damage on the larvae, a length ramp (10% stretch, at a rate of $2 L_{opt}/s$) was imposed on top of the tetanus after 60 ms using the length motor. The preparations were kept at the stretched length for 200 ms and returned to L_{opt} in the relaxed state after the contractions. The protocol is illustrated in Fig. 5 of **Paper I**. The eccentric contractions were applied for 10 times, the tetanus force during the first contraction, was used to normalize subsequent force responses. Control responses were recorded on muscle repeatedly activated.

3.12.3 Force-velocity relationship (Paper IV)

The force-velocity relationships of 4-5 dpf larvae were determined at lowered temperature (15 °C). A velocity ramp protocol was applied during single twitch contractions. To ensure a steady velocity, the shortening ramp was initiated 50 ms prior to the stimulation, and also the preparations were pre-stretched so that the actual length at peak of the twitch was the same. A series of different ramp speeds was applied at 2-min intervals, and the corresponding force was recorded. The isometric tension was also determined between each iso-velocity contraction. The technique is further discussed in relation to the results in Section 4.4.4.

4 RESULTS AND DISCUSSION

In the papers included in the thesis, we have focused on the contractile and structural functions of four key muscle proteins, Desmin, Dystrophin, Laminin and skeletal Myosin Binding Protein C (MyBPC). They locate at different sites in the muscle cells, and in many aspects, their functions are unknown or under-explored *in vivo*. They are all involved in human muscle disease. We applied and developed techniques for functional analysis in zebrafish larval muscle and examined the normal protein functions and their potential roles in disease, using knockdown strategies and mutated strains. The figure below illustrates the location of the proteins examined in the included papers.

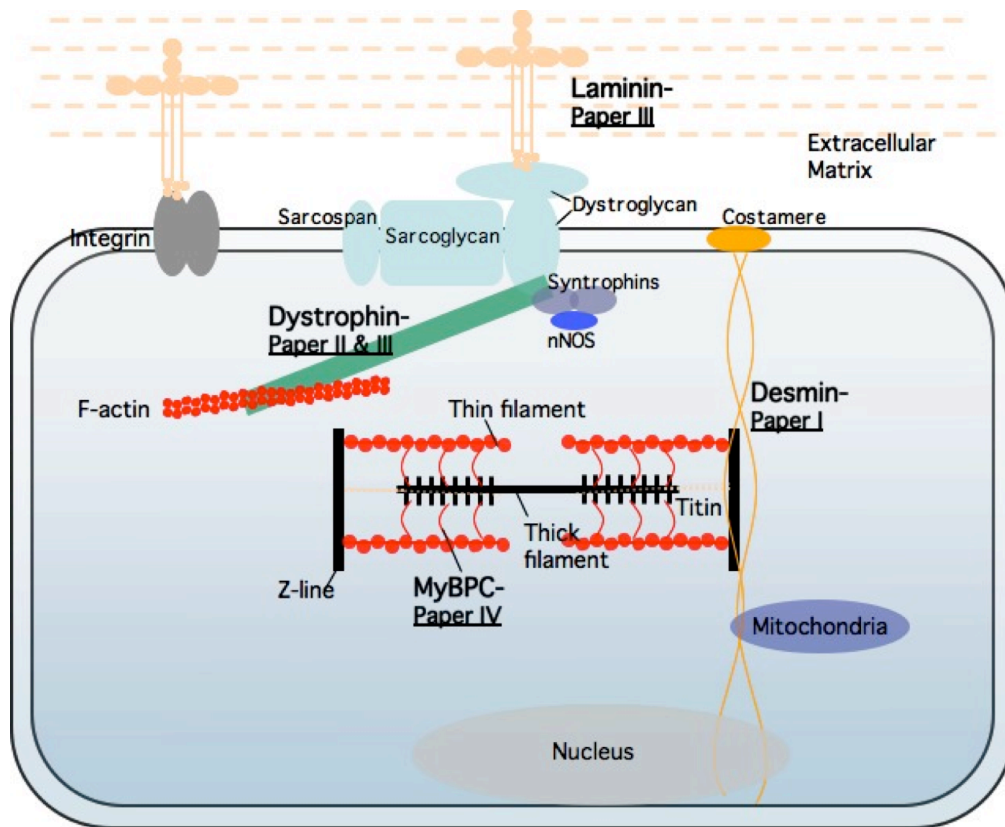


Figure 11. Schematic illustration of cytoskeletal proteins examined in the papers of the thesis.

4.1 DESMIN - DESMINOPATHY

Desmin is a major component of the intermediate filaments in muscle (Lazarides, 1980). Its structural role in anchoring the cellular components and maintaining the sarcomere arrangements in skeletal muscle has been well documented (Boriek et al., 2001; Capetanaki et al., 1997; Goldfarb et al., 1998). Several forms of human desminopathy caused by mutations in the desmin gene exist (Dalakas et al., 2000). As stated in the Introduction (Section 1.2.1), human desmin-related myopathies are manifested by loss of functional desmin, as well as by the appearance of intracellular protein aggregates. These protein deposits were suggested to be toxic (Sugawara et al., 2000). If the deposits are the main cause

or consequence of the disease, and whether toxic effects or a lack of functional protein are the pathological mechanisms remain open questions. Human desminopathy is a very heterogeneous disease and, most likely, the pathological mechanism might vary among individual forms. To investigate the exact mechanisms in human desminopathy, knock-out mouse models have been developed and extensively studied (Li et al., 1996; Milner et al., 1996). The links between desmin mutations, aggregates and disease phenotype have been investigated in the human studies and some mouse models (Carlsson et al., 2002; Muñoz-Mármol et al., 1998), showing that mutations in desmin can affect the formation and stability of the intermediate filaments, as well as the cellular localization of other cytoskeletal proteins. Studies on knockout (*DES*^{-/-}) mice, where desmin is completely absent, have revealed a comparatively mild phenotype with alterations in muscle structure and function, in the absence of protein aggregates (Balogh et al., 2005; Sam et al., 2000). However, the heterozygous (*DES*^{+/-}) mice, where desmin content is lower, did not show any disease phenotype (Li et al., 1996; Milner et al., 1996). Thus, the primary disease mechanisms in humans, where desmin is not completely absent, remain to be determined - whether it is the truncated protein aggregates or the lowered desmin that affect the muscle function. It is also relevant to mention, in this context, that most experimental models of desminopathy are developed in the mouse, and information from other species, perhaps better mimicking some aspects of human disease, could be of value. In **Paper I**, we used a morpholino antisense approach to lower desmin content in zebrafish larvae, and found that a partial decrease of desmin is sufficient to cause structural and functional damage in muscles. As discussed below we propose that this model, with partial desmin knockdown and absence of protein aggregates, mimics some aspects of human desminopathy. The desmin knockdown larval model also enabled us to explore more basic functions of desmin on the muscle contractile apparatus using a combined mechanical and structural approach.

4.1.1 Desmin knockdown in zebrafish larvae

Genes in zebrafish are often duplicated. For desmin, both genes (*desma* and *desmb*) were identified in the zebrafish using the NCBI (National Center for Biotechnology Information, USA) database. The desmin protein is fairly conserved when compared to human (71% identity of the sequence). In **Paper I**, we designed primers and demonstrated using RT-PCR that both genes were expressed in larval muscles at 4 dpf. Since the mRNAs of both desmin genes were present in zebrafish, we used a combination of morpholino antisense oligonucleotides (MOs) targeting both of the genes to achieve a significant knockdown. The amount of MOs was kept below the amount considered toxic and injection with the standard control MO did not have an effect on the measured parameters (e.g. larval body length/width, active force). Thus the control MO injected larvae were routinely used as control for comparisons with the animals injected with blocking MOs. Further, we used two different MO approaches: splicing and translation blocking MOs as discussed previously. The Figure 12 below shows mRNA expression in zebrafish larvae after injection with control and splicing blocking MOs, demonstrating a significant knockdown at the mRNA level by the blocking MOs.

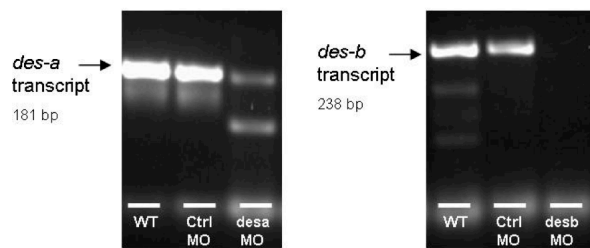


Figure 12. Gel separation of mRNA transcripts for desmin (des-a and des-b) in zebrafish larvae injected with control and splicing blocking MOs targeting each gene respectively.

It is also important to confirm that injection of blocking MOs is associated with lowering of protein content. With Western blotting, we detected a significantly lowered protein content by ~ 50% (Fig. 1 in **Paper I**), for both splicing and translational blocking MOs. Due to the nature of MOs and the delivery method used in the larvae (local injection into the yolk, MOs are taken-up into cells from yolk), the knockdown effect is transient (days to one week) and it is often impossible to achieve a complete knockout.

We examined the larvae at 4-5 dpf, and the desmin morphants showed a severe muscle phenotype. They also had shorter body length, cardiac edema and an impaired swimming activity compared to the control group (Table 1, Fig 2. of **Paper I**). These results show a prominent effect of knockdown on the skeletal muscle structure and function. Most likely the knockdown also affects cardiac and smooth muscles, since desmin is expressed in all muscle types (Lazarides, 1980). Defects in the heart or gut are however less likely to cause any secondary effects on the skeletal muscles, since cardiovascular/intestinal functions are not essential for zebrafish larvae as the oxygen is diffused through the skin and yolk stream supplies the nutrition (Lin, 2012).

4.1.2 Desmin deficiency results in structural alterations

We examined the structural effects of desmin knockdown in the zebrafish larvae in more detail using confocal microscopy and staining of F-actin with Rhodamine phalloidin. A picture is shown in Fig. 2 of **Paper I**. The desmin morphants had generally thinner and disarranged muscle fibers. We observed regions where the anchoring sites of the muscle fibers and the myosepta were compromised. No protein aggregates were observed in this knockdown model. These results thus show that loss of desmin significantly affects the muscle structure with effects on the regular arrangement of fibers and of their attachments sites. These results are similar to results from the desmin knockout mouse models (Li et al., 1997). However, the heterozygous desmin knockout mice do not have a clear pathological phenotype although protein content in muscles is lower (Li et al., 1997; Milner et al., 1996). Our results thus show that a partial loss of desmin is sufficient to cause structural alterations and a myopathy, which could be important for translation to the human desminopathy, where desmin is not completely absent.

Since desmin can be a major component in the lateral anchoring of the sarcomeres, binding between the Z-disks and the muscle costameres (Tidball, 1992), we further examined the myofilament structure using small angle X-ray scattering, addressing the hypothesis that the

desmin intermediate filaments affect the sarcomere structure. We obtained strong equatorial signals at the synchrotron facility and estimated the interfilament distances from the equatorial 1.0 and 1.1 reflections. The inner 1.0 reflection was strong and its spacing (d10) was primarily used in the analysis (Fig. 3 in **Paper I**). The d10 spacing was significantly wider in the desmin morphants. The difference appeared larger at shorter sarcomere length, showing, that when the sarcomeres are shortened the filaments move apart maintaining sarcomere volume (Millman, 1998), and that this behavior was altered in the desmin morphants. The mechanism for the wider filament spacing in desmin morphants thus seems to be related to the lack of lateral links of the sarcomeres and is similar to results from the desmin knock-out mouse (Balogh et al., 2005). Thus the desmin intermediate filaments affect the lateral compliance and have role in maintaining lattice spacing in the sarcomere.

As discussed below (Section 4.1.3) the active force was lower. It has been reported that moderate changes in fiber width and lateral spacing affect the contractile properties (Edman, 1999), and we examined if the spacing change in the desmin morphants was large enough to affect contraction. In the experiments, we therefore compressed the desmin morphants using sucrose in the bathing medium to the widths observed in controls; and expanded the lattice of the controls to a level of the morphants and examined contractions as discussed below (Section 4.1.3).

4.1.3 Lowering of desmin content impairs muscle contraction, but prevents stretch-induced injury

In general the zebrafish larvae have a low maximal active stress (about 20-50 mN/mm², **Paper I** and Dou et al., 2008), as estimated from muscle cross-sectional area and active force, compared to e.g. about 360 mN/mm² in single muscle fibers frog and mouse (Edman, 2005). For each muscle, we confirmed that the measurements were made at optimal stretch and with supra-optimal stimulation intensity. The tetanic contractions in the zebrafish are of about the same magnitude as the single twitch contractions (cf Dou et al., 2008), which show that we cannot obtain higher force with summation. One explanation for this is the very fast muscle type. It is likely that the fast zebrafish muscle fibers cannot maintain tension during the tetanus and that they fatigue during the sustained contraction and thus do not contribute with summation of tension. This is also supported by results showing that a myosin inhibitor (BTS) mainly inhibiting fast myosin, primarily attenuating the early phases of contraction (Dou et al., 2008). Another important contributing factor can be the predominant fast muscle type in the zebrafish (Devoto et al., 1996). A higher cross-bridge cycling of zebrafish muscle in general, since higher cycling rates will lead to shorter duty cycles (i.e. the time a cross-bridge is attached in a force generating state) and that give lower tension (Rome et al., 1999), which is also supported by the X-ray data discussed under Section 4.4.3.

The decrease in desmin content was ~50% in the morphant larvae (Section 4.1.2) and we observed that they generated a significantly lower active force at optimal stretch compared to the controls (Fig. 4 in **Paper I**). The length-force relationship was not affected by knockdown, suggesting that the altered filament spacing or the disrupted structural links did

not influence the length dependency of filament overlap. We estimated the active stress (i.e. the force per muscle cross sectional area) and conclude that a smaller muscle size was a contributing factor for the weaker contraction in the desmin morphants, but not the sole explanation (i.e. the reduction in force was larger than expected from the decrease in cross-sectional area). We also addressed the question if the wider filament spacing in the desmin morphants could explain the lower tension (Fig. 4 in **Paper I**), and showed that osmotic compression of the morphants to the spacing in controls did not rescue the impaired force and that swelling of controls to the level in the morphants did not lower tension. These findings thus support that the role of desmin in muscle contraction is mainly associated with the force transmission, by anchoring and integrating the sarcomeres as contracting units. Most likely lack of the desmin/intermediate filament attachments affect the force transmission between the muscle and myosepta, consistent with a proposed role in myotendinous junctions in mammalian muscle (Tidball, 1992). A further consequence of an affected Z-disk anchoring in the desmin morphants can be changes a lack of coordination of contractile unit lengths during contractions as discussed previously for smooth and striated mammalian muscle (Balogh et al., 2003; Sjuve et al., 1998). Previous work on the mechanical role of desmin has to a large extent been based on studies of desmin knock-out animals, as discussed above. An interesting aspect of the zebrafish morphants is that we show that ~50% reduction in desmin is sufficient to introduce both structural (Section 4.1.2) and contractile impairments.

In a previous study on the desmin knockout mouse (Sam et al., 2000), it was reported that these animals, although they had a lower active tension, were less affected by eccentric work (i.e. when muscles are stretched during contraction). We therefore established a protocol for eccentric contraction in the zebrafish larvae. Isometric tetanic contractions (i.e. without stretch) could be repeated 10 times with only moderate decrease in active force (Fig. 5 in **Paper I**). When we applied a lengthening step at the plateau of contraction, such repeated eccentric contractions resulted in a decrease in active tension of about 40-60% after 10 contractions in control larvae. In contrast, the desmin deficient morphants were less affected (~15-20% decrease) under the same conditions. The relative force increase during the stretch was similar in controls and morphants, showing that the challenge to the muscles was of the same magnitude. These findings suggest that the susceptibility of skeletal muscles to stretch is affected by the mechanical link inside cells provided by desmin. It has been shown that desmin is one of the proteins primarily degraded after eccentric work in some animal models (Barash et al., 2002), possibly reflecting that the protein is strained during eccentric stretch. It has been shown that swelling of muscle fibers lowers the resistance to stretch (Edman, 1999) and another interesting possibility is that the wider filament spacing in the desmin morphants might be protective against stretch induced injury, by allowing slipping of filaments in the sarcomeres and less injury during stretch.

4.2 DYSTROPHIN - DUCHENNE MUSCULAR DYSTROPHY

As presented in Introduction (1.2.2), Duchenne Muscular Dystrophy (DMD) is a severe muscle disorder of genetic origin. Mutations in the dystrophin gene have been identified in

DMD patients, leading lack of functional dystrophin expression (Aartsma-Rus et al., 2006). As illustrated in Figure 11 above, loss of dystrophin would compromise the dystrophin-glycoprotein complex, leading to an interruption of the structural link between membrane and contractile components in muscle. Significant experimental work has been invested into this field; human studies, as well as canine and transgenic mouse (mdx mouse) models have been extensively explored (Bieber and Hoffman, 1990; Dangain and Vrbova, 1984; Howell et al., 1997). Several pathological mechanisms, e.g. membrane injury, oxidative stress, cellular signaling (Bhatnagar and Kumar, 2010; Lynch et al., 2000; Whitehead et al., 2006) are implicated in the disease progression, but still not fully understood. No cure for the disease is currently available. In **Paper II** we examined the *Sapje* zebrafish model for DMD and examined the effects of a novel therapy based on a read-through compound and in **Paper III** we further examined the effects of active muscle contraction on disease development, also in comparison with a laminin deficient strain (Section 4.3).

4.2.1 The *Sapje*/DMD zebrafish

Like the mdx mouse, the *Sapje* (*dmd*^{ta222a}) zebrafish carries a point mutation in the dystrophin gene, resulting in a pre-mature stop codon (Bassett et al., 2003). This mimics the general situation in human DMD patients, where functional dystrophin is lacking and also specifically the case in ~15% of the human DMD patients where non-sense premature stop codon mutations are present (Tuffery et al., 1998). Interestingly, unlike the mildly affected mdx mice, the *Sapje* fish have generally more severe muscle phenotype and shortened lifespan, which may provide an alternative and possibly better model for human DMD.

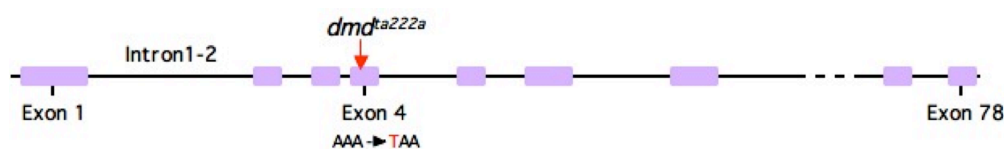


Figure 13. Schematic structure of the zebrafish dystrophin/dmd gene and the location of the point mutation in *Sapje* (*dmd*^{ta222a}). The nonsense mutation (A-to-T) is located in exon 4 (indicated with arrow), causing the pre-mature termination of protein translation.

As an indication of the muscular dystrophy in the *Sapje* zebrafish larvae, the mutants reveal patchy alterations under birefringence microscopy (Bassett et al., 2003; Fig. 1 in **Paper II**). These defects appear clearly at 3 dpf, providing a useful assay to differentiate the homozygous mutants from the siblings at this developmental stage. We confirmed using genotyping that the mutants identified with birefringence had the expected genotype. We also used Western blotting and immunostaining (Fig. 4 in **Paper II**) and showed that no dystrophin was expressed in the *Sapje* mutants.

To further investigate the structural alterations, especially the areas showing birefringence defects, we examined the general muscle morphology with F-actin staining (Fig. 4 in **Paper II**). The mutant larvae had well-preserved striations, but significantly altered muscle architecture; specifically, the muscle fibers appeared disarrayed with more space between the

bundles. The structural alterations seemed to be localized in certain somites, as shown by patchy damages under the birefringence assay. These morphological changes indicate that the dystrophin-null cells in the somites are damaged and undergo degeneration and necrosis. The structural damage started to develop from 3 dpf, when larvae start to hatch and swim, suggesting the progressiveness of the disease and its association with activity/movement (cf. Section 4.2.4). This is consistent with results from the mdx mice (Lynch et al., 2001) and possibly also human patients, showing that muscle activity partly contributes to disease progression. This aspect is further discussed in relation to the immobilization experiments.

4.2.2 Sapje/DMD zebrafish have impaired contractile function

In order to translate the zebrafish model to human disease, characterization of muscle function is essential. In **Paper II**, the active force generation was examined in 4-5 dpf *Sapje* larvae, to examine the muscle contractile performance. The homozygous mutated *Sapje* larvae were identified according to the birefringence defects and compared to the control siblings. To exclude any difference due to genetic background in the control group, we genotyped a group of control siblings after mechanical analysis, and found no difference in the function between wild type and heterozygous animals. The homozygous *Sapje* mutants generated significantly lower active force, with slower force transients during contraction and relaxation, compared to the siblings (Fig. 2 in **Paper II**). The reduction in active force can be partly related to a slightly smaller size, but the decay in force was larger than expected from the smaller muscle cross-section, and in view of the structural changes it is likely that muscle damage and loss of contractile tissue is a major contributing factor for the contractile impairment, as proposed for the mouse mdx model and humans (Lynch et al., 2000). In the mdx model, muscle damage is larger in active muscles and increases with age, suggesting activity-induced disease progression (Lynch et al., 2001). The mdx mice are less affected compared to the human patients, possibly related to compensatory mechanisms (Matsumura et al., 1992) or higher regeneration capacity (Megeney et al., 1996). In that aspect the *Sapje* larvae are more affected and thus possibly more similar to the human situation. Since the variations caused by age, muscle activity and fiber type composition are minimal in the *Sapje* zebrafish, these animals thus provide a well-defined model.

4.2.3 Ataluren partially restores the protein expression, improves muscle structure and function in the Sapje/DMD model

As discussed above, although significant efforts have been made, there is still no cure for the DMD patients. The zebrafish models make it possible to screen novel therapeutic regimens in a comparatively rapid and simple manner, with a clinically relevant functional readout. Ataluren (PTC124) is a small-molecular compound, which like some of the aminoglycoside antibiotics, has been proposed to enable the read-through of non-sense stop codon mutations (Barton-Davis et al., 1999; Pichavant et al., 2011). In the case of DMD, this type of mutations constitutes about 15%, which suggest that Ataluren can be an attractive treatment. It has been shown that Ataluren promotes the nonsense read-through *in vitro*, and improves mdx mouse muscles with low toxicity (Welch et al., 2007). The compound was examined in a first

clinical trial on a small number of DMD patients and, although no side effects were noted, the dose-dependency was complex, with no effect of a higher dose (Peltz et al., 2013). This temporarily halted further clinical trials and introduced a need for further examination of the dose-effect relationship.

In **Paper II**, we first examined the contractile function in the *Sapje*/DMD model (Section 4.2.2), and then examined the effects of Ataluren. The treatment started at 3 dpf, when the *Sapje* larvae hatch and start to swim freely. The *Sapje* mutants and their normal siblings were selected and grouped according to the birefringence assay. The treatment lasted from 3 to 5 dpf with a concentration range of 0-35 μ M Ataluren. 0.1% DMSO was applied as a solvent control. The animals were examined with force measurements and analysis of structure and dystrophin expression at 5dpf. During the treatment, the larvae were kept one per each well in a 24-well plate. The DMSO control and Ataluren did not have negative effects on animal behavior or contractile function, except for the highest dose of Ataluren (35 μ M), where both control and *Sapje* larvae groups had an increased mortality. The two-day treatment with an optimal dose of Ataluren (0.5 μ M) partially increased the dystrophin protein expression, as detected with Western blotting (Fig. 4, **Paper II**), showing that the read-through compound was effective in the zebrafish model. In addition, immunostaining on the treated larvae showed recovery of staining of dystrophin along the cell membrane in the somites in treated *Sapje* larvae. In contrast the non-treated *Sapje* completely lacked dystrophin expression.

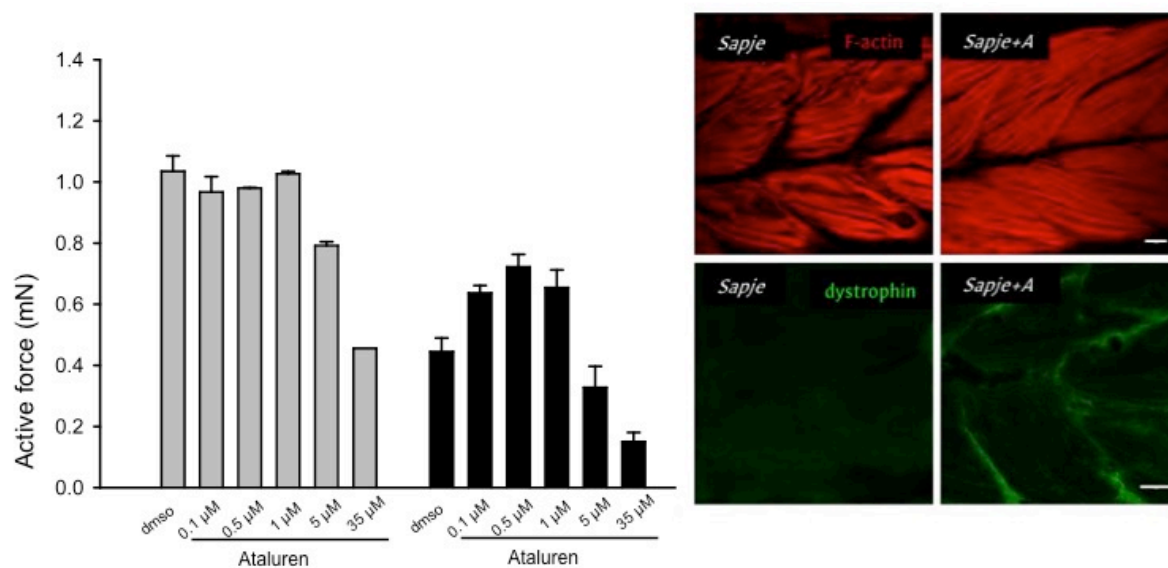


Figure 14. Summarized results of functional and structural rescue by Ataluren in *Sapje* mutant larvae. Left: dose-dependency of Ataluren (0-35 μ M) on the force generation; grey bars: healthy siblings; black bars: *Sapje* group. Right: structural recovery and protein restoration in the *Sapje* mutants with Ataluren (+A) at optimal dose. Modified from **Paper II**.

We showed that the muscle contractile function of the *Sapje* mutants was significantly improved after Ataluren treatment. The active force of *Sapje* muscles increased from ~40% of the control to ~70% at the optimal dose (0.5 μ M). However, the birefringence defects

remained in all *Sapje* groups even after treatment, suggesting that some muscle damage can occur already before 3 dpf or that a small decrease in dystrophin is sufficient to cause muscle injury. As discussed in relation to our immobilization experiments (**Paper III**, Section 4.2.4), active muscle contraction appears to be a major factor in the development of structural damage. Our data show that although Ataluren is effective in the *Sapje* model, the therapeutic concentration window of Ataluren is fairly narrow in the zebrafish larvae; beneficial effects appeared to be between 0.1 to 1 μM , while a higher dose (5 μM) lacked effects and even higher doses (35 μM) caused significant negative effects. This bell-shaped dose dependency demonstrated in the *in vivo* zebrafish model (**Paper II**) using a functional readout has also been seen in myotubes from patients and mdx mice (Finkel et al., 2013; Welch et al., 2007). This property might explain lack of effects in the first clinical trials and suggest a careful consideration of applied dosage in future clinical trials. Recently, the compound is in an active stage of development (Bushby et al., 2014; Ryan, 2014) and a new clinical phase III trial has been initiated (NCT01826487).

4.2.4 Immobilization during early development prevents muscle damage in *Sapje*/DMD larvae

The disease mechanisms in DMD are complex; several factors are proposed to play a role in the pathogenesis (Introduction section 1.2.2). The primary cause is the lack of dystrophin due to genetic mutations. Given the position of the dystrophin protein, and its associated dystroglycan complex (DGC, Figure 1), an impaired mechanical link of the intracellular components to the membrane has been proposed as a major mechanism (Brooks, 1998). The question: to what extent does the loss of the mechanical reinforcement by dystrophin contribute to the disease?, remains unresolved. Immobilization of muscles would be one approach to study this question. However, immobilization of hindlimb muscles *in vivo* using several different approaches have shown variable results in mdx mice (Hourd  et al., 2013; Mokhtarian et al., 1999). These previous studies on mice models have mainly focused on the later disease progression phase rather than the early onset, which raises another question - whether the early blocking of mechanical stress alone would abolish the pathological alterations in DMD.

We observed in **Paper II** that the structural damages were observed in the *Sapje* mutant larvae at 3 dpf. Since the embryos establish motor function and start to move at about 18 hpf, it is possible that two days of physical movement (18 hpf to 3dpf) is sufficient for introducing a structural damage. This property gives a time window when the early phases of disease development and the role of active contraction can be investigated in the DMD zebrafish model. The zebrafish also offers unique possibilities compared to mammals, since the animals can be completely immobilized without considering effects on respiration and cardiac function. Also early developmental stages can be affected since they develop “*ex utero*”. The specific role of mechanical stress in early development of DMD was investigated in **Paper III**. We used a pharmacological approach, where all active skeletal muscle contractions, as judged by larval movement, were abolished for 3 days using the small

molecule actomyosin inhibitor BTS (Pinniger et al., 2005). The treatment was started at 18 hpf before any physical movements occurred, and continued until 4 dpf when the muscle structure and function were analyzed. Since hatching of the larvae requires active contraction we manually hatched the larvae at 18 hpf.

During the BTS treatment period, the larvae developed essentially normally with regular heartbeats and blood circulation, although the larvae were non-responsive to touch. No edema was observed in the heart or intestines. The body length of these immobilized larvae was similar to the larvae treated with 0.1% DMSO solvent control (Table 1 in **Paper III**) showing that the growth rate was essentially normal. When the treatment was terminated at 4 dpf, the larvae were transferred to regular E3 medium. Under these conditions when BTS was removed they started to swim freely after ~0.5 hrs, which shows that the BTS immobilization effects are reversible. We observed that BTS treated *Sapje* larvae did not develop any structural damages under birefringence assay. To confirm that these *Sapje* larvae were of the correct genotype, all groups were genotyped at 4 dpf. A more detailed structural examination using Rhodamine phalloidin staining did not reveal any difference between the BTS treated *Sapje* larvae and their siblings; the characteristic muscle fiber disarrangement and distortion in the non-treated *Sapje* mutant larvae were absent (Fig.1 in **Paper III**). This shows that the structural damage in the *Sapje* larvae is caused by mechanical activity, most likely via rupture of membrane sites (Bassett and Currie, 2004).

We also observed that the general fiber alignment between the somites appeared slightly wavy in all BTS treated larvae, sibling controls and *Sapje*. This suggests that BTS treatment and immobilization has a general effect on muscle structure, indicating an association between mechanical silencing and altered-myofibrillogenesis, consistent with suggestions that cross-bridge cycling is crucial for the early myofibril development (Ramachandran et al., 2003). The results show that although BTS appears to have a general effect on muscle structure, the treatment abolishes the structural damage in the *Sapje* larvae. In addition to the structural rescue, the difference in muscle function between the *Sapje* mutants and their normal siblings was also absent after BTS immobilization (Fig. 4 in **Paper III**). The maximal active force measured after BTS washout at 4 dpf was similar in the *Sapje* mutants and their siblings. Nevertheless, there was a general muscle weakness caused by BTS immobilization in both groups compared to the DMSO controls. This shows that immobilization clearly affect contractile function possibly related to the impaired myofibrillogenesis as indicated by the wavy fiber arrangement observed after F-actin staining. We did not explore the nature of the lower active tension after BTS treatment, it might relate to changes in the myofibrillar arrangement or in establishment of the myofilaments. It is interesting to speculate of this mechanical and structural changes after immobilization, in some aspects resemble changes observed in human patients after long-term intensive care with pharmacological immobilization, the critical illness myopathy (Friedrich, 2006; Larsson, 2008). The general contractile impairment after BTS treatment was reversible since active movements reappeared after washout in E3 medium (cf. Section 4.3.4), although the extent of recovery and the time course in this model require further studies.

From a therapeutic perspective, the immobilization could be beneficial in DMD since the muscle cells might remain protected from membrane rupture, thus preventing the secondary damage responses, e.g., the $\text{Ca}^{2+}/\text{Na}^{+}$ influx, cell necrosis, inflammation, etc (Bhatnagar and Kumar, 2010). It should be noted that we used a complete immobilization not easily achieved and maintained in humans, and that the immobilization, also introduced an impaired muscle contractile function in controls with structural changes possibly by affecting activity-induced effects on myofibrillogenesis. Although full immobilization thus cannot be directly translated to a clinical setting in humans, the study indicates a mechanical effect in the pathogenesis of DMD, which might have an impact on the use of physical activity and exercise in DMD treatment.

In summary, we found that muscle contractions and free movements are responsible for the structural alterations observed in *Sapje* mutants. Early intervention by blocking contractions alone could abolish the onset of structural changes in the larvae.

4.3 LAMININ - CONGENITAL MUSCULAR DYSTROPHY

Congenital muscular dystrophy (CMD) is a heterogeneous group of neuromuscular disorders, with early onset and variable progression (Iannaccone and Castro, 2013). Like in DMD, genetic sequencing analyses have identified genetic mutations in several proteins. One common subgroup of CMD, the “merosin-deficient congenital muscular dystrophy type 1A” (MDC1A), is due to loss of the laminin $\alpha 2$ -chain (also known as merosin). Laminin is an extracellular component linking the dystrophin glycoprotein complex to the extracellular matrix (Figure 1). The molecular mechanisms underlying the pathological progression are however not clear yet. Different mouse models have been studied. Some strains contain low amounts of laminin, exhibiting a mild phenotype (dy/dy , $\text{dy}^{2j}/\text{dy}^{2j}$), and compensation from truncated laminin $\alpha 2$ -chain was observed in dy^w/dy^w mice (Guo et al., 2003); thus, the complete lack of laminin $\alpha 2$ -chain conditions observed in humans have not been fully resembled.

4.3.1 Laminin $\alpha 2$ -chain deficiency in Candyfloss zebrafish strain

The *Candyfloss* (*Caf*) zebrafish strain has recently been identified as a model for MDC1A. These mutants carry a non-sense mutation in *LAMA-2* gene, resulting in no expression of laminin- $\alpha 2$ via non-sense mRNA decay (Hall et al., 2007). The location of the mutation is a homologue to human exon 60, at the globular domain. The phenotype of the *Caf* mutants was severe in the musculature with fiber detachment and degeneration, and the animals die prematurely at between about 14-20 dpf.

4.3.2 Alterations in muscle structure and functional defects in *Caf* larvae

The *Caf*/MDC1A mutants have several similarities with the *Sap*/DMD strain: 1) The general development of *Caf* mutant larvae is essentially normal, with no major abnormalities observed in heart or other organs. 2) They develop severe structural defects in muscle at about 3 dpf. The muscle cell differentiation of *Caf* mutants is unaltered, and the structural

lesions in skeletal muscles develop from 36 hpf with full penetration at 3 dpf. 3) The reduction of muscle birefringence is significant, with a patchy/uneven distribution. With Rhodamin-phalloidin staining, the general muscle fiber organization is distorted stochastically in the 4 dpf *Caf* larvae (**Paper III**). Overall, the structural alterations in the *Caf*/MDC1A model thus appear similar to the defects observed in *Sapje* DMD model, but with a larger magnitude as shown in the quantification of the birefringence intensity (Fig. 3 in **Paper III**). To understand the consequence of these observations in muscle function, we carried out a mechanical analysis on the *Caf* strain. The *Caf* mutants generated significantly lower active force compared to their siblings at 4 dpf and 5 dpf, showing that alterations in the extracellular laminin attachments leads to both structural and functional impairment. Interestingly, the normal *Caf* siblings were weaker compared to the *Sap* siblings at 4 dpf, but became equally strong at 5 dpf (Fig. 4 and 5 in **Paper III**). This might suggest a difference underlying the genetic background of the two strains as discussed below in 4.3.4.

4.3.3 Contraction-induced injury plays a part in the pathogenesis of MDC1A

The laminin- $\alpha 2$ chain is an essential ligand to α -dystroglycan of the DGC complex. As an extracellular part of the DGC integral system, laminin is shown to stabilize the cell membrane and protect the muscles from mechanical stress (Han et al., 2009). In addition to the mechanical strengthening role, laminin- $\alpha 2$ chain also binds to integrin-7, promoting the cell survival (Vachon et al., 1997). It has been proposed that the mechanical contraction-induced fiber detachment from the extracellular matrix is the initial cause for the MDC1A in the *Caf* zebrafish (Hall et al., 2007). In that study, a short-term immobilization (up to 3 dpf) of the *Caf* mutants with Tricaine was shown to prevent the disease phenotype. However, detailed analysis on the immobilized muscles was absent. In addition, the paralyzing effect from Tricaine is on the voltage-gated Na^+ channels, non-specific for the muscle contractions, and might include secondary effects in the muscles. To further investigate this, we applied the BTS immobilization protocol as described above (Section 4.2.4) to the *Caf* in comparison with the *Sap* larvae. BTS, specifically inhibit the cross-bridge cycling in skeletal muscles without non-selective effects on other cell types or intracellular organelles (Cheung et al., 2002; Pinniger et al., 2005). The treatment of *Caf* mutants was between 18 hpf to 4 dpf, and the larvae were kept fully immobilized during the period. The structural alterations were completely abolished, as shown with birefringence and Rhodamine phalloidin examination (Fig. 2 in **Paper III**). This shows that the mechanical injury via active contractions is a key pathological mechanism and that complete lack of laminin in *Caf* zebrafish mutant is not sufficient to cause muscle damage in the absence of mechanical stress.

4.3.4 Strain differences in muscle activity affects development of muscle lesions

Inter-strain difference is an important consideration in biomedical research on animal models. For the zebrafish, Tübingen (TU) is the main wild-type strain for the genome sequencing and phenotyping (Haffter et al., 1996; Haffter and Nüsslein-Volhard, 1996) and was used in **Paper I** and **IV**. For the mutated strains studied in **Paper II** and **III**, the normal siblings for

dystrophin and laminin were used as controls. We did not observe any differences between the heterozygotes and the wild type larvae. However, in **Paper III** we observed a generally more active swimming behavior in the *Caf* zebrafish control larvae compared to that of the *Sapje* controls. At 5 dpf, the total swimming duration was longer in the *Caf* controls (Table 2 in **Paper III**), which suggest that these animals were more mechanically active. The muscle activity might be involved in the growth in strength in *Caf* siblings from 4 to 5 dpf (Fig. 4 and 5 in **Paper III**), compared to the *Sapje* group, where the difference in active force between 4 to 5 dpf was minor. Interestingly this might relate to the appearance of structural injury after BTS immobilization. Both the *Sapje* and *Caf* strains were rescued, with regard to birefringence alterations, by the BTS immobilization with no structural damage observed at 4 dpf. However, the *Caf* developed birefringence lesions at 5 dpf after one-day recovery and activity. In contrast, the *Sapje* BTS group appeared essentially normal under birefringence at the same stage (5 dpf). This difference seems to relate to the difference in swimming activity. These data from recovery after immobilization further demonstrate that muscle contractions and swimming activity are associated with both DMD and MDC1A development in the zebrafish, and that the mechanical stress is an initial pathological factor.

4.4 MYOSIN BINDING PROTEIN C - STRIATED MUSCLE MYOPATHIES

The sarcomeric protein, Myosin Binding Protein C (MyBPC), has been extensively studied as a candidate for human familial cardiac hypertrophy (Watkins et al., 1995). Several mutations have been identified in the cardiac MyBPC (MyBPC-3) and the protein has several regulatory phosphorylation sites (Finley and Cuperman, 2014). In skeletal muscle, two isoforms of the protein are identified, slow (MyBPC-1) and fast (MyBPC-2). The structure of the different isoforms is fairly conserved (Oakley et al., 2004) and the cellular location and molecular interactions are similar to that of the cardiac MyBPC (Luther et al., 2008). The function of the skeletal isoforms is less well characterized. The slow MyBPC-1 has been linked to human muscle disease (Gurnett et al., 2010; Markus et al., 2012). The function of the fast skeletal isoform and a possible relation to muscle disease is less understood. In **Paper IV**, we applied the reverse-genetic approach as in **Paper I**, using morpholino antisense oligonucleotides in zebrafish early larvae, to knock down the skeletal MyBPC content, and investigate the consequences in vivo.

4.4.1 Partial loss of fast skeletal MyBPC-2 results in a severe muscle phenotype

We identified three genes for skeletal MyBPCs in the zebrafish, one slow (MyBPC-1) and two fast (MyBPC-2A; MyBPC-2B). Since pseudogenes often occur during gene duplication in the zebrafish we first examined the expression of all three genes. We determined the mRNA levels in the zebrafish larval trunk muscles using RT-PCR at different developmental stages of the larvae, including 1, 3 and 7 dpf (Fig.1 in **Paper IV**). The cardiac MyBPC-3 was not found, and the MyBPC-2A appeared non-transcribed at all times. The MyBPC-2B was the most dominant skeletal MyBPC isoform and was expressed already at 1 dpf. The mRNA for the slow type, MyBPC-1, was also present but at lower amounts.

To investigate the function of the MyBPC proteins, we applied a knockdown approach in early larvae as described in **Paper I** (Section 4.1.1). The morpholino antisense oligonucleotides (MO) were designed to target either the splicing or translation process of mRNA. In **Paper IV**, in addition to the standard control MO, the MO against MyBPC-2A also served as an internal control, since this isoform is not expressed. Similar to the knockdown effects achieved for desmin in **Paper I**, the MOs used in this study reduced the mRNA and protein content (for MyBPC-2 by about 50-70%, determined by RT-PCR and silver-stained protein gels (Fig. 2 in **Paper IV**). Knockdown of the fast MyBPC-2B gave a severe phenotype the larval muscles (Fig. 3 in **Paper IV**). We observed significant structural changes in the sarcomeres and filament arrangement as discussed below. The MyBPC-2B morphants also revealed signs of delayed development, cardiac edema, and had a high mortality rate compared to the controls. The contractile function was significantly impaired as discussed below (Section 4.4.4). The results thus show that knock down of the fast skeletal MyBPC-2 in the zebrafish results in a marked myopathy. To our knowledge, human skeletal myopathies associated with altered MyBPC-2 or animal models have not been described, and we can at present not make comparisons with any myopathies in other species. However, mutations in human MyBPC-1 gene have been associated with distal arthrogryposis (Gurnett et al., 2010) and congenital contractural syndrome type (Markus et al., 2012). Our study was focused on the fast MyBPC-2 form, but we also examined effects of knockdown of the slow MyBPC-1. Our analysis revealed that the MyBPC-1 morphants were essentially normal compared to the controls at 4dpf (Fig. 3 and 5, **Paper IV**). The minor effect of MyBPC-1 knock down was possibly due to the muscle type composition. In the zebrafish, slow muscle fibers only form one single layer underneath the skin; the rest of the myotome is essentially fast (Bryson-Richardson et al., 2005). Assuming that the fast and slow MyBPCs are fiber type specific, the decrease in MyBPC in the slow fibers might not have an impact. Slow fibers have been shown to be dominant at earlier developmental stages (Blagden et al., 1997) and our data further show that MyBPC-1 is not essential for the development of the trunk muscles. We did not observe any effects on contractile function after MyBPC-1. These results showing minor effects of MyBPC-1 knock down are different from a study showing negative effects on muscle structure following MyBPC-1 knock down in the zebrafish (Ha et al., 2013). We have no explanation for this difference, which might relate to the different design and amount of the MOs. We are, however, confident that we have a selective knockdown of MyBPC-1 by about 80% without effects on the MyBPC-2 expression or muscle structure.

4.4.2 Structural alterations and elevated degeneration/apoptotic signaling in MyBPC-2 morphants

Unlike in the desmin morphants (**Paper I**) or the muscular dystrophy mutants (**Paper II, III**), the structural alterations in the MyBPC-2 morphants involved marked changes in the sarcomere length distribution. As shown with Rhodamine-phalloidin staining, the MyBPC-2 morphants had clear striations indicating the presence of sarcomeres (Fig. 3 in **Paper IV**). However, the fiber arrangement was disordered and the sarcomere lengths were varied. The

quantification of the sarcomere length showed generally shorter sarcomeres with a wide distribution spectrum (Fig. 3 in **Paper IV**). This suggests that lack of MyBPC-2 results in a primary failure in sarcomere assembly.

The inter-fiber space was also widened, with gaps between muscle bundles, in the MyBPC-2B morphants compared with the control group (Fig. 3, **Paper IV**), suggesting muscle degeneration or apoptosis. Apoptotic changes were also higher in the MyBPC-2B morphant larvae as revealed by Acridine orange staining (Fig. 4, **Paper IV**). To further explore this possibility we examined expression of signaling components in degeneration using RT-PCR (Fig. 4, **Paper IV**). The transcription factors MuRF1-3, LCB3, SQSTM1 all associated with degeneration in the skeletal muscles (Cohen et al., 2009; McElhinny et al., 2004; Perera et al., 2011) were significantly upregulated. We also found that myogenin expression was two-fold upregulated compared to the control groups, suggesting activation of regeneration signaling (Onofre-Oliveira et al., 2012). These data thus show that lack of MyBPC-2 significantly affects muscle structure with activation of degeneration/apoptosis and regeneration, and with altered assembly of sarcomeres. At present we cannot provide data on the mechanism how lack of MyBPC-2 induces these alterations. Knockdown of MyBPC-2 results in lower active force in a similar manner as for desmin knockdown, but appears to have key functions also in signaling for sarcomere assembly. It should be noted that MyBPC has links to titin (Okagaki et al., 1993), which has signaling to the nucleus (Lange et al., 2005), and it is possible that MyBPC in some manner provides links between mechanical events in the sarcomere and signaling for sarcomere assembly or muscle degeneration/regeneration (Yang et al., 1998).

Despite the muscle damage early during sarcomere assembly, the structural failure in sarcomeres and large degenerative process were not accompanied by any increased fibrosis, since the Sirius red staining for collagen showed similar intensity in both groups (supplementary data, **Paper IV**). Fibrosis is often observed in muscle degeneration (Balogh et al., 2002; Ho et al., 2010; Stedman et al., 1991) and the findings in the MyBPC-2 morphants can reflect that we are studying comparatively early events in the degeneration process.

4.4.3 X-ray diffraction studies of MyBPC-2 morphants

To further characterize the structural alterations in the muscles with MyBPC-2 knockdown in **Paper IV**, we carried out X-ray diffraction experiments as described in **Paper I**. The X-ray patterns of the larval preparations were recorded in single larvae and we observed strong equatorial reflections (11, 10). At present the resolution of the meridional pattern was poor. This can be due to the orientation of the muscle fibers, which is essentially parallel with the larval long axis, but with some angular distribution. This can obscure the weaker meridional parts of the pattern and meridional layer lines. Our first experiments in **Papers I** and **IV** were performed at the A2 beamline HASY-lab, Hamburg that had comparatively larger focus (300 x 2000 μm), which gave strong signals but exposed a larger fraction of the larvae. Later experiments in **Papers II** and **IV** were done at the MaxIV laboratory, Lund which enabled a smaller focus (100 x 100 μm), thus illuminating a smaller part. This gave somewhat better

meridional patterns with the myosin 14.3 nm reflection visible in some samples, although not with a resolution enabling identification of the 44.2 nm MyBPC reflection (Rome et al., 1973) visible. We could thus not demonstrate a change in this reflection or in the myosin reflections following knock down of MyBPC-2. The meridional MyBPC reflection has been examined early in rabbit skeletal muscles (Rome et al., 1973), showing a periodicity at 44.2 nm. It is possible that further work on the zebrafish larval samples using other X-ray sources with smaller focus might resolve also other parts of the X-ray pattern.

Our main readout was the equatorial reflections (d_{10} , d_{11}) and their intensity ratio (I_{11}/I_{10}) reflecting the interfilament distances and the mass transfer between the thick and thin filaments, respectively (Haselgrove and Huxley, 1973; Millman, 1998). For interfilament spacing determination, the larvae were examined under different degrees of stretch and osmolarity (Fig. 7 in **Paper IV**). The muscles were first examined at an optimal stretch for active force. At low external osmolarity, the lattice spacing was wide and similar in both control and MyBPC-2 morphants suggesting that lack of the MyBPC does not affect the maximal lateral expansion of the lattice. However, when osmolarity was increased, to that in the standard Krebs-Ringer medium and above, the MyBPC-2 morphants were less compressed and the lattice was significantly wider. X-ray studies have been performed on cardiac muscle from MyBPC-3 knockout animals showing conflicting results (Colson et al., 2007; Palmer et al., 2004; Palmer et al., 2011). Our data suggest that absence of MyBPC affects the lateral coupling between the contractile filaments, so that lack of the protein removes a component keeping filaments together. This finding further supports the linkage role of MyBPC between actin and myosin filaments.

When the muscle was stretched the lattice was compressed in both controls and MyBPC-2B morphants (Fig. 7 in **Paper IV**) reflecting a constant volume behavior (Edman, 1999). In conclusion the structural and X-ray data suggest that the lack of MyBPC-2 results in shorter and expanded sarcomeres.

We used the intensity ratio of the equatorial reflections to examine the myosin head movements and positions. Cross-bridge movement out from the myosin filament towards actin is considered to increase the outer 1.1 reflection and decrease the inner 1.0 reflection giving an increased 1.1/10 intensity ratio (Haselgrove and Huxley, 1973). When a rigor state was induced by NaCN treatment a marked increase (about 4 times compared to the relaxed signal) was observed. We assume that all cross-bridges attach under these conditions and used the rigor value for normalization. We first examined the intensity ratio in the relaxed state and found that the intensity ratio, relative to the rigor value, was increased in the MyBPC-2B morphants. This can reflect that absence of MyBPC-2 results in an outward movement of the myosin heads in the skeletal muscle. This might resemble the effects observed in from cardiac muscle where Protein Kinase A-mediated phosphorylation of MyBPC-3 near the N-terminus weakens the interaction between MyBPC and myosin-S2 segment and results in outward movement of the myosin head (Colson et al., 2008).

To examine the motion of myosin heads during active contractions we developed a protocol where X-ray patterns were recorded in the relaxed state and during 200 ms tetani. Since about 10s recording was required to obtain a sufficiently strong signal, we accumulated the pattern during 15 successive contractions only recording (by gating the detector) during the contractions. We observed a clear increase in the intensity ratio during contraction consistent with a previous report (Dou et al., 2008), but, interestingly, the tetanic contractions did not result in a large increase of the attached cross-bridges in the larval muscles when compared to the relaxed state. The intensity ratio in the active muscle was ~30% of that in rigor (Fig. 7 in **Paper IV**). A corresponding value in frog muscle is ~45% (Haselgrove and Huxley, 1973). This suggests that a comparatively low number of cross-bridges are attached during active contractions in the zebrafish muscles. This can reflect a generally fast cycling rate in the zebrafish skeletal muscles during active contraction where the high rate results in a short time when the cross-bridges are attached to actin as discussed above (Section 4.1.3) in relation to active force.

In summary, the X-ray results suggest that skeletal MyBPC-2 affects the cross-bridge positions in the relaxed state, keeping them close to the thick filament. Lack of this structural constraint results in an outward movement. The MyBPC-2 morphant muscles also have an expanded lattice and shorter sarcomeres at optimal stretch. During active contractions cross-bridges move out towards actin, but the relative number of attached heads in the fast zebrafish muscle is low, and we could not observe any major differences in the MyBPC-2B morphants in this respect.

4.4.4 Contractile impairments in MyBPC-2 morphants

In **Paper IV**, we characterized the contractile performance of the MyBPC-2 morphants. We first found that the maximal active force at optimal stretch was significantly decreased (Fig. 5 in **Paper IV**) following the partial loss of MyBPC-2. A major contribution is the structural change with less contractile tissue most likely due to the degeneration processes described above. We also observed wider filament spacing (Section 4.4.3), but, as discussed in relation desmin and **Paper I** (Section 4.1.3), this change was not of a magnitude expected to influence the active force. Since we observed a major change in the sarcomeres towards shorter lengths and a wider length distribution (Section 4.4.2), we examined the length tension behavior (Fig. 5 in **Paper IV**) and found a less steep ascending limb. The ascending limb of the length tension curve is complex and includes not only changes in filament overlap but also other filament interactions and alteration in the degree of activation (Rüdel and Taylor, 1971). The wider sarcomere length distribution in the MyBPC-2 morphants is likely a major contributing factor to the less steep relationship, although other factors, e.g. a change in the length dependency of filament spacing and Ca^{2+} sensitivity can be involved. With regard to the latter factor, it has been shown the cardiac MyBPC-3 interacts with actin filaments and affects the thin filament regulation (Razumova et al., 2008) and our analysis of the single twitch force transients show that the rates of relaxation and contraction are altered in the MyBPC-2 morphants, possibly reflecting changes in activation/deactivation processes.

Early work on striated muscle MyBPC has suggested that the MyBPC is affecting the cross-bridge cycling rate. This was based on studies of skinned skeletal fibers where extraction of the MyBPC resulted in an increase in the maximal shortening velocity (V_{\max}), which was reversed upon re-addition of the protein (Hofmann et al., 1991a). Further work using *in vitro* motility applying MyBPC-3 assays support this view (Saber et al., 2008) and are interpreted that the MyBPC tethers the myosin head to thick filament backbone (Calaghan et al., 2000; Colson et al., 2008). Recent work has to a large extent focused on the cardiac MyBPC-3 based on knockout mouse models (Harris et al., 2002; Korte et al., 2003). In cardiac fibers from these animals it has been shown that the maximal shortening velocity is increased when the protein is absent or when it is phosphorylated (Colson et al., 2012; Harris et al., 2004; Kunst et al., 2000; Sadayappan et al., 2005). A corresponding regulation via phosphorylation has not been demonstrated in the skeletal MyBPC-2, which lacks two of the three phosphorylation sites, although other phosphorylation sites are present (Oakley et al., 2004). To our knowledge, knock-out mouse models for MyBPC-2 are not available and the knock-down MyBPC-2 morphant thus enables an initial approach to examine the mechanical function of MyBPC-2 in intact muscle fibers. We therefore also attempted to examine if the maximal shortening velocity was altered after MyBPC-2 knock-down in skeletal muscle. Since the later phases of tetanic contractions in the larval preparations might involve fatiguing of a fast muscle component (Dou et al., 2008), we used single twitch contractions. Since these are very rapid we applied a velocity ramp protocol, so that contractions could be initiated at defined shortening velocities. Using this approach we evaluated force responses at different velocities and constructed force velocity relationships. The data points at intermediate velocities/forces are affected by the non steady state situation since the contractile component will shorten and stretch the series elasticity while developing twitch force. We therefore only evaluated the extrapolated maximal velocity by extrapolating to zero loads. This value is most likely somewhat underestimated due to the less concave relationship. Using this approach we found a comparatively higher V_{\max} (~ 40% increased) in the MyBPC-2 morphants. A further complication in the analysis is the shorter sarcomere length (~18%) in the morphants, which would make the difference in velocity at the filament level smaller although still different (~ 14% higher in the morphants). With these reservations, we consider the velocity data to be consistent with a higher velocity upon removal of MyBPC-2 in skeletal muscle.

4.4.5 MyBPC-2 associated myopathy

Our data on MyBPC-2 knockdown in the zebrafish (**Paper IV**) show that partial removal of this protein results in a severe myopathy, characterized by muscle degeneration/regeneration and derangement of muscle fibers. At the level of the contractile apparatus, the sarcomeres are shorter and display a wider length distribution, and have a wider interfilament lattice spacing. These results can reflect a primary effect of MyBPC-2 on the regulation of filament assembly. The actin-myosin interaction is accelerated and the resulting force significantly lower. We are not aware of a human clinical condition associated with MyBPC-2 mutation. Since the phenotype following partial removal of MyBPC-2 in the zebrafish is comparatively

severe, it is possible that MyBPC-2 mutations are embryonic lethal, but the protein would still be an interesting candidate for further search in skeletal myopathies of unknown origin.

5 CONCLUSIONS

- The zebrafish larva is a suitable model for mechanistic studies of functional consequences of contractile and structural protein alterations in skeletal muscle.
- The mechanical readout, in combination with structural data, provides a clinical-relevant parameter that can be measured with high resolution, enabling structure-function analyses in muscle diseases and screening of novel therapeutic approaches.
- Partial loss of desmin in the skeletal muscle affects the lateral anchoring of contractile filaments and protects the muscle from force decay during eccentric contractions.
- Loss of dystrophin in the *Sapje* zebrafish model of Duchenne Muscular Dystrophy (DMD) results in a significant muscle weakness, showing that interference with the membrane anchoring of the muscle cytoskeleton impairs muscle structure/function.
- Ataluren, a read-through compound, partially rescues the contractile function in the *Sapje*/DMD larvae by restoring dystrophin protein expression and exhibited a biphasic dose dependency relating to findings in clinical studies.
- Loss of laminin $\alpha 2$ chain in the *Candyfloss* zebrafish model results in a significant muscle weakness.
- Both *Sapje* and *Candyfloss* mutant models are protected from structural injury by early immobilization, indicating that mechanical stress plays a primary role in disease development in muscular dystrophy.
- Fast skeletal Myosin Binding Protein C (MyBPC-2) affects development, structure and cross-bridge interaction of the muscle sarcomere. Lack of this protein results in a severe form of skeletal myopathy.

6 ACKNOWLEDGEMENTS

I would like to thank all the colleagues and friends who have helped and supported me during my PhD study here at Karolinska Institutet. It is not a short journey from home, but truly a pleasant and rewarding one, because of your company.

Firstly, I would like to thank my main supervisor, **Anders Arner**, for giving me the opportunity of pursuing my PhD degree. I could not tell how lucky I am to have you as my supervisor. Your broad knowledge, excellent guidance and supports have helped me tremendously all along. But above all, you truly showed me what it takes to be a scientist.

Thomas Sejersen, thank you for being my co-supervisor. The scientific discussions and meetings with you have always been motivating. I truly appreciate your suggestions and ideas on the projects, and it was very nice of you for sharing the clinical experience.

Monika Andersson-Lendahl, my co-supervisor, for sharing your broad knowledge and experience on zebrafish with me. It has been an honor to work together with you at the CMB zebrafish facility. And the zebrafish course that you organized was excellent, I was very grateful to be involved and learn the basics about zebrafish. My PhD would have been a lot harder without your great help.

Prof. Paul Edman. I am much honored to have worked with the fantastic research equipment for muscle physiology established by you. And I am very grateful for the opportunity to meet and discuss with you in Lund, your constructive and encouraging comments are always appreciated very much.

Kent Ivasen, for welcoming me in the fish house and training on the zebrafish handling to keep the fish happy and healthy; and former colleagues at the zebrafish facility, CMB, **Gayathri Chandrasekar**, **Jing Lu** and **Tilo Wünsch**, for the friendly discussions and suggestions on zebrafish work.

Dr. Sérgio S. Funari at DESY/HASY LAB, Hamburg, and **Dr. Ana Labrador** at MAX Laboratory, Lund, for the competent help with the X-ray experiments and all helpful suggestions, patience and nice discussions.

Prof. Peter Currie and the group members, at Monash University, Australia, for kindly receiving me as a guest in your laboratory. The research visit was short, but I really enjoyed and learned so much about zebrafish biology because of your generous help. The collaborative work has been rewarding. Special thanks to **Dr. Joachim Berger**, for valuable suggestions and advice on everything; and **Silke Berger** for preparing and shipping the mutant lines from the other half of the world.

Prof. Hans Hertz and the group members at KTH, **Daniel Larsson** and **William Vågberg**, for providing a stimulating research collaboration. The collaborative work with you is so special, looking forward to more fantastic zebrafish images.

Dr. Rachel Ashworth and **Lise Mazelet**, at Queen Mary, University of London, for the zebrafish collaboration and stimulating discussions. It was a great experience to work with you.

Per Arlock, for sharing your knowledge and experience in electrophysiology and the work on zebrafish heart.

All members at Arner lab: **Cecilia Lövdahl**, **Ferenc Szekeres** and **Per Wikström**, it is so delightful to share the same office with you, our daily chatting really makes my work and life lighter, and I am very lucky to be sitting in the room which is always filled with smile and laughter; special thanks to **Ferenc**, you have truly helped me to get the tough work done, and your suggestions are always valued. **Sae-Il Murtada**, your kind encouragement and career advice are very much appreciated. And former members, **Awahan Rehemani**, **Benjamin Davis**, **Johan Sällström**, **Lilian Sundberg**, I really enjoyed working with you, and your interests and advice on my work had been so helpful; and **Katharina Jenniches**, for the critical comments and discussions on research projects.

I also owe my sincere gratitude to everyone at **FyFa** for the kind assistance during the course of my study.

All the friends in Stockholm, for the lovely gathering and fun time; also, **Angela Zhang**, **Li Chen**, **Yang Yang**, **Shogo Wada**, **Lei Gong** for the sweet visits from all over the world.

Most importantly, my family, for the unconditional love and care. My parents, **Chaolai** and **Xiujuan**, for believing in me and supporting me through all these years. Mom, I would never have started my PhD without your encouragement, you taught me how to lay the burdens down and travel light. Thank you for visiting me in Stockholm, it meant a lot to me.

我親愛的家人，我愛你們。

7 REFERENCES

- Aartsma-Rus, A., Van Deutekom, J.C., Fokkema, I.F., Van Ommen, G.J., and Den Dunnen, J.T. (2006). Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule. *Muscle Nerve* 34, 135-144.
- Ablain, J., and Zon, L.I. (2013). Of fish and men: using zebrafish to fight human diseases. *Trends Cell Biol* 23, 584-586.
- Balogh, J., Li, Z., Paulin, D., and Arner, A. (2003). Lower active force generation and improved fatigue resistance in skeletal muscle from desmin deficient mice. *J Muscle Res Cell Motil* 24, 453-459.
- Balogh, J., Li, Z., Paulin, D., and Arner, A. (2005). Desmin filaments influence myofilament spacing and lateral compliance of slow skeletal muscle fibers. *Biophys J* 88, 1156-1165.
- Balogh, J., Merisckay, M., Li, Z., Paulin, D., and Arner, A. (2002). Hearts from mice lacking desmin have a myopathy with impaired active force generation and unaltered wall compliance. *Cardiovasc Res* 53, 439-450.
- Barash, I.A., Peters, D., Fridén, J., Lutz, G.J., and Lieber, R.L. (2002). Desmin cytoskeletal modifications after a bout of eccentric exercise in the rat. *Am J Physiol Regul Integr Comp Physiol* 283, R958-963.
- Barton-Davis, E.R., Cordier, L., Shoturma, D.I., Leland, S.E., and Sweeney, H.L. (1999). Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of mdx mice. *J Clin Invest* 104, 375-381.
- Bassett, D., and Currie, P.D. (2004). Identification of a zebrafish model of muscular dystrophy. *Clin Exp Pharmacol Physiol* 31, 537-540.
- Bassett, D.I., Bryson-Richardson, R.J., Daggett, D.F., Gautier, P., Keenan, D.G., and Currie, P.D. (2003). Dystrophin is required for the formation of stable muscle attachments in the zebrafish embryo. *Development* 130, 5851-5860.
- Berger, J., Berger, S., Hall, T.E., Lieschke, G.J., and Currie, P.D. (2010). Dystrophin-deficient zebrafish feature aspects of the Duchenne muscular dystrophy pathology. *Neuromuscul Disord* 20, 826-832.
- Berger, J., Berger, S., Jacoby, A.S., Wilton, S.D., and Currie, P.D. (2011). Evaluation of exon-skipping strategies for Duchenne muscular dystrophy utilizing dystrophin-deficient zebrafish. *J Cell Mol Med* 15, 2643-2651.
- Berger, J., Sztal, T., and Currie, P.D. (2012). Quantification of birefringence readily measures the level of muscle damage in zebrafish. *Biochem Biophys Res Commun* 423, 785-788.
- Beytía, M.e.L., Vry, J., and Kirschner, J. (2012). Drug treatment of Duchenne muscular dystrophy: available evidence and perspectives. *Acta Myol* 31, 4-8.
- Bhatnagar, S., and Kumar, A. (2010). Therapeutic targeting of signaling pathways in muscular dystrophy. *J Mol Med (Berl)* 88, 155-166.
- Bieber, F.R., and Hoffman, E.P. (1990). Duchenne and Becker muscular dystrophies: genetics, prenatal diagnosis, and future prospects. *Clin Perinatol* 17, 845-865.
- Bill, B.R., Petzold, A.M., Clark, K.J., Schimmenti, L.A., and Ekker, S.C. (2009). A primer for morpholino use in zebrafish. *Zebrafish* 6, 69-77.

- Blagden, C.S., Currie, P.D., Ingham, P.W., and Hughes, S.M. (1997). Notochord induction of zebrafish slow muscle mediated by Sonic hedgehog. *Genes Dev* 11, 2163-2175.
- Boriek, A.M., Capetanaki, Y., Hwang, W., Officer, T., Badshah, M., Rodarte, J., and Tidball, J.G. (2001). Desmin integrates the three-dimensional mechanical properties of muscles. *Am J Physiol Cell Physiol* 280, C46-52.
- Brooks, S.V. (1998). Rapid recovery following contraction-induced injury to in situ skeletal muscles in mdx mice. *J Muscle Res Cell Motil* 19, 179-187.
- Bryson-Richardson, R.J., Daggett, D.F., Cortes, F., Neyt, C., Keenan, D.G., and Currie, P.D. (2005). Myosin heavy chain expression in zebrafish and slow muscle composition. *Dev Dyn* 233, 1018-1022.
- Bulfield, G., Siller, W.G., Wight, P.A., and Moore, K.J. (1984). X chromosome-linked muscular dystrophy (mdx) in the mouse. *Proc Natl Acad Sci U S A* 81, 1189-1192.
- Bushby, K., Finkel, R., Wong, B., Barohn, R., Campbell, C., Comi, G.P., Connolly, A.M., Day, J.W., Flanigan, K.M., Goemans, N., *et al.* (2014). Ataluren treatment of patients with nonsense mutation dystrophinopathy. *Muscle Nerve* 50, 477-487.
- Calaghan, S.C., Trinick, J., Knight, P.J., and White, E. (2000). A role for C-protein in the regulation of contraction and intracellular Ca²⁺ in intact rat ventricular myocytes. *J Physiol* 528 Pt 1, 151-156.
- Capetanaki, Y., Milner, D.J., and Weitzer, G. (1997). Desmin in muscle formation and maintenance: knockouts and consequences. *Cell Struct Funct* 22, 103-116.
- Carlsson, L., Fischer, C., Sjöberg, G., Robson, R.M., Sejersen, T., and Thornell, L.E. (2002). Cytoskeletal derangements in hereditary myopathy with a desmin L345P mutation. *Acta Neuropathol* 104, 493-504.
- Cheung, A., Dantzig, J.A., Hollingworth, S., Baylor, S.M., Goldman, Y.E., Mitchison, T.J., and Straight, A.F. (2002). A small-molecule inhibitor of skeletal muscle myosin II. *Nat Cell Biol* 4, 83-88.
- Cohen, S., Brault, J.J., Gygi, S.P., Glass, D.J., Valenzuela, D.M., Gartner, C., Latres, E., and Goldberg, A.L. (2009). During muscle atrophy, thick, but not thin, filament components are degraded by MuRF1-dependent ubiquitylation. *J Cell Biol* 185, 1083-1095.
- Colson, B.A., Bekyarova, T., Fitzsimons, D.P., Irving, T.C., and Moss, R.L. (2007). Radial displacement of myosin cross-bridges in mouse myocardium due to ablation of myosin binding protein-C. *J Mol Biol* 367, 36-41.
- Colson, B.A., Bekyarova, T., Locher, M.R., Fitzsimons, D.P., Irving, T.C., and Moss, R.L. (2008). Protein kinase A-mediated phosphorylation of cMyBP-C increases proximity of myosin heads to actin in resting myocardium. *Circ Res* 103, 244-251.
- Colson, B.A., Rybakova, I.N., Prochniewicz, E., Moss, R.L., and Thomas, D.D. (2012). Cardiac myosin binding protein-C restricts intrafilament torsional dynamics of actin in a phosphorylation-dependent manner. *Proc Natl Acad Sci U S A* 109, 20437-20442.
- Cooke, R. (1997). Actomyosin interaction in striated muscle. *Physiol Rev* 77, 671-697.
- Coulton, G.R., Curtin, N.A., Morgan, J.E., and Partridge, T.A. (1988a). The mdx mouse skeletal muscle myopathy: II. Contractile properties. *Neuropathol Appl Neurobiol* 14, 299-314.

- Coulton, G.R., Morgan, J.E., Partridge, T.A., and Sloper, J.C. (1988b). The mdx mouse skeletal muscle myopathy: I. A histological, morphometric and biochemical investigation. *Neuropathol Appl Neurobiol* 14, 53-70.
- Dalakas, M.C., Park, K.Y., Semino-Mora, C., Lee, H.S., Sivakumar, K., and Goldfarb, L.G. (2000). Desmin myopathy, a skeletal myopathy with cardiomyopathy caused by mutations in the desmin gene. *N Engl J Med* 342, 770-780.
- Dangain, J., and Vrbova, G. (1984). Muscle development in mdx mutant mice. *Muscle Nerve* 7, 700-704.
- Davies, K.E., and Nowak, K.J. (2006). Molecular mechanisms of muscular dystrophies: old and new players. *Nat Rev Mol Cell Biol* 7, 762-773.
- Dellorusso, C., Crawford, R.W., Chamberlain, J.S., and Brooks, S.V. (2001). Tibialis anterior muscles in mdx mice are highly susceptible to contraction-induced injury. *J Muscle Res Cell Motil* 22, 467-475.
- Devoto, S.H., Melançon, E., Eisen, J.S., and Westerfield, M. (1996). Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. *Development* 122, 3371-3380.
- Dong, J., and Stuart, G.W. (2004). Transgene manipulation in zebrafish by using recombinases. *Methods Cell Biol* 77, 363-379.
- Dooley, K., and Zon, L.I. (2000). Zebrafish: a model system for the study of human disease. *Curr Opin Genet Dev* 10, 252-256.
- Dou, Y., Andersson-Lendahl, M., and Arner, A. (2008). Structure and function of skeletal muscle in zebrafish early larvae. *J Gen Physiol* 131, 445-453.
- Doyon, Y., McCammon, J.M., Miller, J.C., Faraji, F., Ngo, C., Katibah, G.E., Amora, R., Hocking, T.D., Zhang, L., Rebar, E.J., *et al.* (2008). Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat Biotechnol* 26, 702-708.
- Edman, K.A. (1999). The force bearing capacity of frog muscle fibres during stretch: its relation to sarcomere length and fibre width. *J Physiol* 519 Pt 2, 515-526.
- Edman, K.A. (2005). Contractile properties of mouse single muscle fibers, a comparison with amphibian muscle fibers. *J Exp Biol* 208, 1905-1913.
- Eisen, J.S., and Smith, J.C. (2008). Controlling morpholino experiments: don't stop making antisense. *Development* 135, 1735-1743.
- Emery, A.E. (2002). The muscular dystrophies. *Lancet* 359, 687-695.
- Fan, L., Moon, J., Crodian, J., and Collodi, P. (2006). Homologous recombination in zebrafish ES cells. *Transgenic Res* 15, 21-30.
- Finkel, R.S., Flanigan, K.M., Wong, B., Bönnemann, C., Sampson, J., Sweeney, H.L., Reha, A., Northcutt, V.J., Elfring, G., Barth, J., *et al.* (2013). Phase 2a study of ataluren-mediated dystrophin production in patients with nonsense mutation Duchenne muscular dystrophy. *PLoS One* 8, e81302.
- Finley, N.L., and Cuperman, T.I. (2014). Cardiac myosin binding protein-C: a structurally dynamic regulator of myocardial contractility. *Pflugers Arch* 466, 433-438.

- Fredsted, A., Gissel, H., Madsen, K., and Clausen, T. (2007). Causes of excitation-induced muscle cell damage in isometric contractions: mechanical stress or calcium overload? *Am J Physiol Regul Integr Comp Physiol* 292, R2249-2258.
- Friedrich, O. (2006). Critical illness myopathy: what is happening? *Curr Opin Clin Nutr Metab Care* 9, 403-409.
- Gawlik, K.I., and Durbeej, M. (2011). Skeletal muscle laminin and MDC1A: pathogenesis and treatment strategies. *Skelet Muscle* 1, 9.
- Goebel, H.H. (2011). Congenital myopathies. Introduction. *Semin Pediatr Neurol* 18, 213-215.
- Goldfarb, L.G., Olivé, M., Vicart, P., and Goebel, H.H. (2008). Intermediate filament diseases: desminopathy. *Adv Exp Med Biol* 642, 131-164.
- Goldfarb, L.G., Park, K.Y., Cervenáková, L., Gorokhova, S., Lee, H.S., Vasconcelos, O., Nagle, J.W., Semino-Mora, C., Sivakumar, K., and Dalakas, M.C. (1998). Missense mutations in desmin associated with familial cardiac and skeletal myopathy. *Nat Genet* 19, 402-403.
- Granato, M., van Eeden, F.J., Schach, U., Trowe, T., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., *et al.* (1996). Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. *Development* 123, 399-413.
- Guo, L.T., Zhang, X.U., Kuang, W., Xu, H., Liu, L.A., Vilquin, J.T., Miyagoe-Suzuki, Y., Takeda, S., Ruegg, M.A., Wewer, U.M., *et al.* (2003). Laminin alpha2 deficiency and muscular dystrophy; genotype-phenotype correlation in mutant mice. *Neuromuscul Disord* 13, 207-215.
- Gurnett, C.A., Desruisseau, D.M., McCall, K., Choi, R., Meyer, Z.I., Talerico, M., Miller, S.E., Ju, J.S., Pestronk, A., Connolly, A.M., *et al.* (2010). Myosin binding protein C1: a novel gene for autosomal dominant distal arthrogryposis type 1. *Hum Mol Genet* 19, 1165-1173.
- Ha, K., Buchan, J.G., Alvarado, D.M., McCall, K., Vydyanath, A., Luther, P.K., Goldsmith, M.I., Dobbs, M.B., and Gurnett, C.A. (2013). MYBPC1 mutations impair skeletal muscle function in zebrafish models of arthrogryposis. *Hum Mol Genet* 22, 4967-4977.
- Haffter, P., Granato, M., Brand, M., Mullins, M.C., Hammerschmidt, M., Kane, D.A., Odenthal, J., van Eeden, F.J., Jiang, Y.J., Heisenberg, C.P., *et al.* (1996). The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* 123, 1-36.
- Haffter, P., and Nüsslein-Volhard, C. (1996). Large scale genetics in a small vertebrate, the zebrafish. *Int J Dev Biol* 40, 221-227.
- Hall, T.E., Bryson-Richardson, R.J., Berger, S., Jacoby, A.S., Cole, N.J., Hollway, G.E., Berger, J., and Currie, P.D. (2007). The zebrafish candyfloss mutant implicates extracellular matrix adhesion failure in laminin alpha2-deficient congenital muscular dystrophy. *Proc Natl Acad Sci U S A* 104, 7092-7097.
- Han, R., Kanagawa, M., Yoshida-Moriguchi, T., Rader, E.P., Ng, R.A., Michele, D.E., Muirhead, D.E., Kunz, S., Moore, S.A., Iannaccone, S.T., *et al.* (2009). Basal lamina strengthens cell membrane integrity via the laminin G domain-binding motif of alpha-dystroglycan. *Proc Natl Acad Sci U S A* 106, 12573-12579.

- Harris, S.P., Bartley, C.R., Hacker, T.A., McDonald, K.S., Douglas, P.S., Greaser, M.L., Powers, P.A., and Moss, R.L. (2002). Hypertrophic cardiomyopathy in cardiac myosin binding protein-C knockout mice. *Circ Res* 90, 594-601.
- Harris, S.P., Rostkova, E., Gautel, M., and Moss, R.L. (2004). Binding of myosin binding protein-C to myosin subfragment S2 affects contractility independent of a tether mechanism. *Circ Res* 95, 930-936.
- Haselgrove, J.C., and Huxley, H.E. (1973). X-ray evidence for radial cross-bridge movement and for the sliding filament model in actively contracting skeletal muscle. *J Mol Biol* 77, 549-568.
- Heasman, J. (2002). Morpholino oligos: making sense of antisense? *Dev Biol* 243, 209-214.
- Helbling-Leclerc, A., Zhang, X., Topaloglu, H., Cruaud, C., Tesson, F., Weissenbach, J., Tomé, F.M., Schwartz, K., Fardeau, M., and Tryggvason, K. (1995). Mutations in the laminin alpha 2-chain gene (LAMA2) cause merosin-deficient congenital muscular dystrophy. *Nat Genet* 11, 216-218.
- Ho, C.Y., López, B., Coelho-Filho, O.R., Lakdawala, N.K., Cirino, A.L., Jarolim, P., Kwong, R., González, A., Colan, S.D., Seidman, J.G., *et al.* (2010). Myocardial fibrosis as an early manifestation of hypertrophic cardiomyopathy. *N Engl J Med* 363, 552-563.
- Hoffman, E.P., Brown, R.H., and Kunkel, L.M. (1987). Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* 51, 919-928.
- Hofmann, P.A., Greaser, M.L., and Moss, R.L. (1991a). C-protein limits shortening velocity of rabbit skeletal muscle fibres at low levels of Ca²⁺ activation. *J Physiol* 439, 701-715.
- Hofmann, P.A., Hartzell, H.C., and Moss, R.L. (1991b). Alterations in Ca²⁺ sensitive tension due to partial extraction of C-protein from rat skinned cardiac myocytes and rabbit skeletal muscle fibers. *J Gen Physiol* 97, 1141-1163.
- Hourdé, C., Joanne, P., Medja, F., Mougnot, N., Jacquet, A., Mouisel, E., Pannerec, A., Hatem, S., Butler-Browne, G., Agbulut, O., *et al.* (2013). Voluntary physical activity protects from susceptibility to skeletal muscle contraction-induced injury but worsens heart function in mdx mice. *Am J Pathol* 182, 1509-1518.
- Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., *et al.* (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496, 498-503.
- Howell, J.M., Fletcher, S., Kakulas, B.A., O'Hara, M., Lochmuller, H., and Karpati, G. (1997). Use of the dog model for Duchenne muscular dystrophy in gene therapy trials. *Neuromuscul Disord* 7, 325-328.
- Huang, P., Xiao, A., Zhou, M., Zhu, Z., Lin, S., and Zhang, B. (2011). Heritable gene targeting in zebrafish using customized TALENs. *Nat Biotechnol* 29, 699-700.
- Huxley, A.F., and Niedergerk, R. (1954). Structural changes in muscle during contraction; interference microscopy of living muscle fibres. *Nature* 173, 971-973.
- Huxley, H., and Hanson, J. (1954). Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. *Nature* 173, 973-976.
- Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Kaini, P., Sander, J.D., Joung, J.K., Peterson, R.T., and Yeh, J.R. (2013). Heritable and precise zebrafish genome editing using a CRISPR-Cas system. *PLoS One* 8, e68708.

- Iannaccone, S.T., and Castro, D. (2013). Congenital muscular dystrophies and congenital myopathies. *Continuum (Minneapolis)* 19, 1509-1534.
- Jay, V., and Vajsar, J. (2001). The dystrophy of Duchenne. *Lancet* 357, 550-552.
- Justice, M.J., Zheng, B., Woychik, R.P., and Bradley, A. (1997). Using targeted large deletions and high-efficiency N-ethyl-N-nitrosourea mutagenesis for functional analyses of the mammalian genome. *Methods* 13, 423-436.
- Kawahara, G., Karpf, J.A., Myers, J.A., Alexander, M.S., Guyon, J.R., and Kunkel, L.M. (2011). Drug screening in a zebrafish model of Duchenne muscular dystrophy. *Proc Natl Acad Sci U S A* 108, 5331-5336.
- Kirschner, J. (2013). Chapter 143 - Congenital muscular dystrophies, Vol 113 (Elsevier).
- Knapik, E.W. (2000). ENU mutagenesis in zebrafish--from genes to complex diseases. *Mamm Genome* 11, 511-519.
- Korte, F.S., McDonald, K.S., Harris, S.P., and Moss, R.L. (2003). Loaded shortening, power output, and rate of force redevelopment are increased with knockout of cardiac myosin binding protein-C. *Circ Res* 93, 752-758.
- Kulikovskaya, I., McClellan, G., Levine, R., and Winegrad, S. (2003). Effect of extraction of myosin binding protein C on contractility of rat heart. *Am J Physiol Heart Circ Physiol* 285, H857-865.
- Kunkel, L.M., Beggs, A.H., and Hoffman, E.P. (1989). Molecular genetics of Duchenne and Becker muscular dystrophy: emphasis on improved diagnosis. *Clin Chem* 35, B21-24.
- Kunst, G., Kress, K.R., Gruen, M., Uttenweiler, D., Gautel, M., and Fink, R.H. (2000). Myosin binding protein C, a phosphorylation-dependent force regulator in muscle that controls the attachment of myosin heads by its interaction with myosin S2. *Circ Res* 86, 51-58.
- Laing, N.G. (2012). Genetics of neuromuscular disorders. *Crit Rev Clin Lab Sci* 49, 33-48.
- Lange, S., Xiang, F., Yakovenko, A., Vihola, A., Hackman, P., Rostkova, E., Kristensen, J., Brandmeier, B., Franzen, G., Hedberg, B., *et al.* (2005). The kinase domain of titin controls muscle gene expression and protein turnover. *Science* 308, 1599-1603.
- Larsson, L. (2008). Acute quadriplegic myopathy: an acquired "myosinopathy". *Adv Exp Med Biol* 642, 92-98.
- Lazarides, E. (1980). Desmin and intermediate filaments in muscle cells. *Results Probl Cell Differ* 11, 124-131.
- Li, Z., Colucci-Guyon, E., Pinçon-Raymond, M., Mericskay, M., Pournin, S., Paulin, D., and Babinet, C. (1996). Cardiovascular lesions and skeletal myopathy in mice lacking desmin. *Dev Biol* 175, 362-366.
- Li, Z., Mericskay, M., Agbulut, O., Butler-Browne, G., Carlsson, L., Thornell, L.E., Babinet, C., and Paulin, D. (1997). Desmin is essential for the tensile strength and integrity of myofibrils but not for myogenic commitment, differentiation, and fusion of skeletal muscle. *J Cell Biol* 139, 129-144.
- Lieschke, G.J., and Currie, P.D. (2007). Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* 8, 353-367.
- Lin, Y.Y. (2012). Muscle diseases in the zebrafish. *Neuromuscul Disord* 22, 673-684.

- Luther, P.K., Bennett, P.M., Knupp, C., Craig, R., Padrón, R., Harris, S.P., Patel, J., and Moss, R.L. (2008). Understanding the organisation and role of myosin binding protein C in normal striated muscle by comparison with MyBP-C knockout cardiac muscle. *J Mol Biol* 384, 60-72.
- Lynch, G.S., Hinkle, R.T., Chamberlain, J.S., Brooks, S.V., and Faulkner, J.A. (2001). Force and power output of fast and slow skeletal muscles from mdx mice 6-28 months old. *J Physiol* 535, 591-600.
- Lynch, G.S., Rafael, J.A., Chamberlain, J.S., and Faulkner, J.A. (2000). Contraction-induced injury to single permeabilized muscle fibers from mdx, transgenic mdx, and control mice. *Am J Physiol Cell Physiol* 279, C1290-1294.
- Marieb, E.N. (1995). Human anatomy and physiology, 3. edn (Redwood City, Calif.: Benjamin/Cummings).
- Markus, B., Narkis, G., Landau, D., Birk, R.Z., Cohen, I., and Birk, O.S. (2012). Autosomal recessive lethal congenital contractural syndrome type 4 (LCCS4) caused by a mutation in MYBPC1. *Hum Mutat* 33, 1435-1438.
- Matsumura, K., Ervasti, J.M., Ohlendieck, K., Kahl, S.D., and Campbell, K.P. (1992). Association of dystrophin-related protein with dystrophin-associated proteins in mdx mouse muscle. *Nature* 360, 588-591.
- McElhinny, A.S., Perry, C.N., Witt, C.C., Labeit, S., and Gregorio, C.C. (2004). Muscle-specific RING finger-2 (MURF-2) is important for microtubule, intermediate filament and sarcomeric M-line maintenance in striated muscle development. *J Cell Sci* 117, 3175-3188.
- Megeney, L.A., Kablar, B., Garrett, K., Anderson, J.E., and Rudnicki, M.A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev* 10, 1173-1183.
- Millman, B.M. (1998). The filament lattice of striated muscle. *Physiol Rev* 78, 359-391.
- Milner, D.J., Weitzer, G., Tran, D., Bradley, A., and Capetanaki, Y. (1996). Disruption of muscle architecture and myocardial degeneration in mice lacking desmin. *J Cell Biol* 134, 1255-1270.
- Mokhtarian, A., Lefaucheur, J.P., Even, P.C., and Seville, A. (1999). Hindlimb immobilization applied to 21-day-old mdx mice prevents the occurrence of muscle degeneration. *J Appl Physiol* (1985) 86, 924-931.
- Muntoni, F., Torelli, S., and Ferlini, A. (2003). Dystrophin and mutations: one gene, several proteins, multiple phenotypes. *Lancet Neurol* 2, 731-740.
- Muñoz-Mármol, A.M., Strasser, G., Isamat, M., Coulombe, P.A., Yang, Y., Roca, X., Vela, E., Mate, J.L., Coll, J., Fernández-Figueras, M.T., *et al.* (1998). A dysfunctional desmin mutation in a patient with severe generalized myopathy. *Proc Natl Acad Sci U S A* 95, 11312-11317.
- Neff, M.M., Neff, J.D., Chory, J., and Pepper, A.E. (1998). dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. *Plant J* 14, 387-392.
- Norwood, F.L., Harling, C., Chinnery, P.F., Eagle, M., Bushby, K., and Straub, V. (2009). Prevalence of genetic muscle disease in Northern England: in-depth analysis of a muscle clinic population. *Brain* 132, 3175-3186.
- Oakley, C.E., Hambly, B.D., Curmi, P.M., and Brown, L.J. (2004). Myosin binding protein C: structural abnormalities in familial hypertrophic cardiomyopathy. *Cell Res* 14, 95-110.

- Okagaki, T., Weber, F.E., Fischman, D.A., Vaughan, K.T., Mikawa, T., and Reinach, F.C. (1993). The major myosin-binding domain of skeletal muscle MyBP-C (C protein) resides in the COOH-terminal, immunoglobulin C2 motif. *J Cell Biol* 123, 619-626.
- Onofre-Oliveira, P.C., Santos, A.L., Martins, P.M., Ayub-Guerrieri, D., and Vainzof, M. (2012). Differential expression of genes involved in the degeneration and regeneration pathways in mouse models for muscular dystrophies. *Neuromolecular Med* 14, 74-83.
- Palmer, B.M., McConnell, B.K., Li, G.H., Seidman, C.E., Seidman, J.G., Irving, T.C., Alpert, N.R., and Maughan, D.W. (2004). Reduced cross-bridge dependent stiffness of skinned myocardium from mice lacking cardiac myosin binding protein-C. *Mol Cell Biochem* 263, 73-80.
- Palmer, B.M., Sadayappan, S., Wang, Y., Weith, A.E., Previs, M.J., Bekyarova, T., Irving, T.C., Robbins, J., and Maughan, D.W. (2011). Roles for cardiac MyBP-C in maintaining myofilament lattice rigidity and prolonging myosin cross-bridge lifetime. *Biophys J* 101, 1661-1669.
- Peltz, S.W., Morsy, M., Welch, E.M., and Jacobson, A. (2013). Ataluren as an agent for therapeutic nonsense suppression. *Annu Rev Med* 64, 407-425.
- Perera, S., Holt, M.R., Mankoo, B.S., and Gautel, M. (2011). Developmental regulation of MURF ubiquitin ligases and autophagy proteins nbr1, p62/SQSTM1 and LC3 during cardiac myofibril assembly and turnover. *Dev Biol* 351, 46-61.
- Peterson, R.T., Link, B.A., Dowling, J.E., and Schreiber, S.L. (2000). Small molecule developmental screens reveal the logic and timing of vertebrate development. *Proc Natl Acad Sci U S A* 97, 12965-12969.
- Petrof, B.J., Shrager, J.B., Stedman, H.H., Kelly, A.M., and Sweeney, H.L. (1993). Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc Natl Acad Sci U S A* 90, 3710-3714.
- Pichavant, C., Aartsma-Rus, A., Clemens, P.R., Davies, K.E., Dickson, G., Takeda, S., Wilton, S.D., Wolff, J.A., Wooddell, C.I., Xiao, X., *et al.* (2011). Current status of pharmaceutical and genetic therapeutic approaches to treat DMD. *Mol Ther* 19, 830-840.
- Pinniger, G.J., Bruton, J.D., Westerblad, H., and Ranatunga, K.W. (2005). Effects of a myosin-II inhibitor (N-benzyl-p-toluene sulphonamide, BTS) on contractile characteristics of intact fast-twitch mammalian muscle fibres. *J Muscle Res Cell Motil* 26, 135-141.
- Ramachandran, I., Terry, M., and Ferrari, M.B. (2003). Skeletal muscle myosin cross-bridge cycling is necessary for myofibrillogenesis. *Cell Motil Cytoskeleton* 55, 61-72.
- Razumova, M.V., Bezold, K.L., Tu, A.Y., Regnier, M., and Harris, S.P. (2008). Contribution of the myosin binding protein C motif to functional effects in permeabilized rat trabeculae. *J Gen Physiol* 132, 575-585.
- Rome, E., Offer, G., and Pepe, F.A. (1973). X-ray diffraction of muscle labelled with antibody to C-protein. *Nat New Biol* 244, 152-154.
- Rome, L.C., Cook, C., Syme, D.A., Connaughton, M.A., Ashley-Ross, M., Klimov, A., Tikunov, B., and Goldman, Y.E. (1999). Trading force for speed: why superfast crossbridge kinetics leads to superlow forces. *Proc Natl Acad Sci U S A* 96, 5826-5831.
- Ryan, N.J. (2014). Ataluren: first global approval. *Drugs* 74, 1709-1714.
- Rüdel, R., and Taylor, S.R. (1971). Striated muscle fibers: facilitation of contraction at short lengths by caffeine. *Science* 172, 387-389.

- Saber, W., Begin, K.J., Warshaw, D.M., and VanBuren, P. (2008). Cardiac myosin binding protein-C modulates actomyosin binding and kinetics in the in vitro motility assay. *J Mol Cell Cardiol* 44, 1053-1061.
- Sadayappan, S., Gulick, J., Osinska, H., Martin, L.A., Hahn, H.S., Dorn, G.W., Klevitsky, R., Seidman, C.E., Seidman, J.G., and Robbins, J. (2005). Cardiac myosin-binding protein-C phosphorylation and cardiac function. *Circ Res* 97, 1156-1163.
- Saint-Amant, L., and Drapeau, P. (1998). Time course of the development of motor behaviors in the zebrafish embryo. *J Neurobiol* 37, 622-632.
- Sam, M., Shah, S., Fridén, J., Milner, D.J., Capetanaki, Y., and Lieber, R.L. (2000). Desmin knockout muscles generate lower stress and are less vulnerable to injury compared with wild-type muscles. *Am J Physiol Cell Physiol* 279, C1116-1122.
- Schröder, R., and Schoser, B. (2009). Myofibrillar myopathies: a clinical and myopathological guide. *Brain Pathol* 19, 483-492.
- Selcen, D., and Engel, A.G. (2011). Myofibrillar myopathies. *Handb Clin Neurol* 101, 143-154.
- Sjuve, R., Arner, A., Li, Z., Mies, B., Paulin, D., Schmittner, M., and Small, J.V. (1998). Mechanical alterations in smooth muscle from mice lacking desmin. *J Muscle Res Cell Motil* 19, 415-429.
- Solnica-Krezel, L., Schier, A.F., and Driever, W. (1994). Efficient recovery of ENU-induced mutations from the zebrafish germline. *Genetics* 136, 1401-1420.
- Stedman, H.H., Sweeney, H.L., Shrager, J.B., Maguire, H.C., Panettieri, R.A., Petrof, B., Narusawa, M., Leferovich, J.M., Sladky, J.T., and Kelly, A.M. (1991). The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. *Nature* 352, 536-539.
- Stelzer, J.E., Fitzsimons, D.P., and Moss, R.L. (2006). Ablation of myosin-binding protein-C accelerates force development in mouse myocardium. *Biophys J* 90, 4119-4127.
- Sugawara, M., Kato, K., Komatsu, M., Wada, C., Kawamura, K., Shindo, P.S., Yoshioka, P.N., Tanaka, K., Watanabe, S., and Toyoshima, I. (2000). A novel de novo mutation in the desmin gene causes desmin myopathy with toxic aggregates. *Neurology* 55, 986-990.
- Summerton, J.E. (2007). Morpholino, siRNA, and S-DNA compared: impact of structure and mechanism of action on off-target effects and sequence specificity. *Curr Top Med Chem* 7, 651-660.
- Sztaf, T.E., Sonntag, C., Hall, T.E., and Currie, P.D. (2012). Epistatic dissection of laminin-receptor interactions in dystrophic zebrafish muscle. *Hum Mol Genet* 21, 4718-4731.
- Tanabe, Y., Esaki, K., and Nomura, T. (1986). Skeletal muscle pathology in X chromosome-linked muscular dystrophy (mdx) mouse. *Acta Neuropathol* 69, 91-95.
- Tidball, J.G. (1992). Desmin at myotendinous junctions. *Exp Cell Res* 199, 206-212.
- Tokuyasu, K.T., Dutton, A.H., and Singer, S.J. (1983). Immunoelectron microscopic studies of desmin (skeleton) localization and intermediate filament organization in chicken skeletal muscle. *J Cell Biol* 96, 1727-1735.
- Tuffery, S., Chambert, S., Bareil, C., Sarda, P., Coubes, C., Echenne, B., Demaille, J., and Claustres, M. (1998). Mutation analysis of the dystrophin gene in Southern French DMD or BMD families: from Southern blot to protein truncation test. *Hum Genet* 102, 334-342.

- Vachon, P.H., Xu, H., Liu, L., Loechel, F., Hayashi, Y., Arahata, K., Reed, J.C., Wewer, U.M., and Engvall, E. (1997). Integrins ($\alpha 7\beta 1$) in muscle function and survival. Disrupted expression in merosin-deficient congenital muscular dystrophy. *J Clin Invest* 100, 1870-1881.
- Wang, X., Osinska, H., Dorn, G.W., Nieman, M., Lorenz, J.N., Gerdes, A.M., Witt, S., Kimball, T., Gulick, J., and Robbins, J. (2001). Mouse model of desmin-related cardiomyopathy. *Circulation* 103, 2402-2407.
- Watkins, H., Conner, D., Thierfelder, L., Jarcho, J.A., MacRae, C., McKenna, W.J., Maron, B.J., Seidman, J.G., and Seidman, C.E. (1995). Mutations in the cardiac myosin binding protein-C gene on chromosome 11 cause familial hypertrophic cardiomyopathy. *Nat Genet* 11, 434-437.
- Weisberg, A., and Winegrad, S. (1996). Alteration of myosin cross bridges by phosphorylation of myosin-binding protein C in cardiac muscle. *Proc Natl Acad Sci U S A* 93, 8999-9003.
- Welch, E.M., Barton, E.R., Zhuo, J., Tomizawa, Y., Friesen, W.J., Trifillis, P., Paushkin, S., Patel, M., Trotta, C.R., Hwang, S., *et al.* (2007). PTC124 targets genetic disorders caused by nonsense mutations. *Nature* 447, 87-91.
- Westerfield, M. (2000). The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*). , 4th ed. edn (Univ. of Oregon Press, Eugene.).
- Whitehead, N.P., Yeung, E.W., and Allen, D.G. (2006). Muscle damage in mdx (dystrophic) mice: role of calcium and reactive oxygen species. *Clin Exp Pharmacol Physiol* 33, 657-662.
- Wieneke, S., Stehle, R., Li, Z., and Jockusch, H. (2000). Generation of tension by skinned fibers and intact skeletal muscles from desmin-deficient mice. *Biochem Biophys Res Commun* 278, 419-425.
- Williams, D.A., Head, S.I., Lynch, G.S., and Stephenson, D.G. (1993). Contractile properties of skinned muscle fibres from young and adult normal and dystrophic (mdx) mice. *J Physiol* 460, 51-67.
- Winegrad, S. (1999). Cardiac myosin binding protein C. *Circ Res* 84, 1117-1126.
- Wood, A.J., and Currie, P.D. (2014). Analysing regenerative potential in zebrafish models of congenital muscular dystrophy. *Int J Biochem Cell Biol* 56C, 30-37.
- Wray, J.S., and Holmes, K.C. (1981). x-ray diffraction studies of muscle. *Annu Rev Physiol* 43, 553-565.
- Yang, Q., Sanbe, A., Osinska, H., Hewett, T.E., Klevitsky, R., and Robbins, J. (1998). A mouse model of myosin binding protein C human familial hypertrophic cardiomyopathy. *J Clin Invest* 102, 1292-1300.