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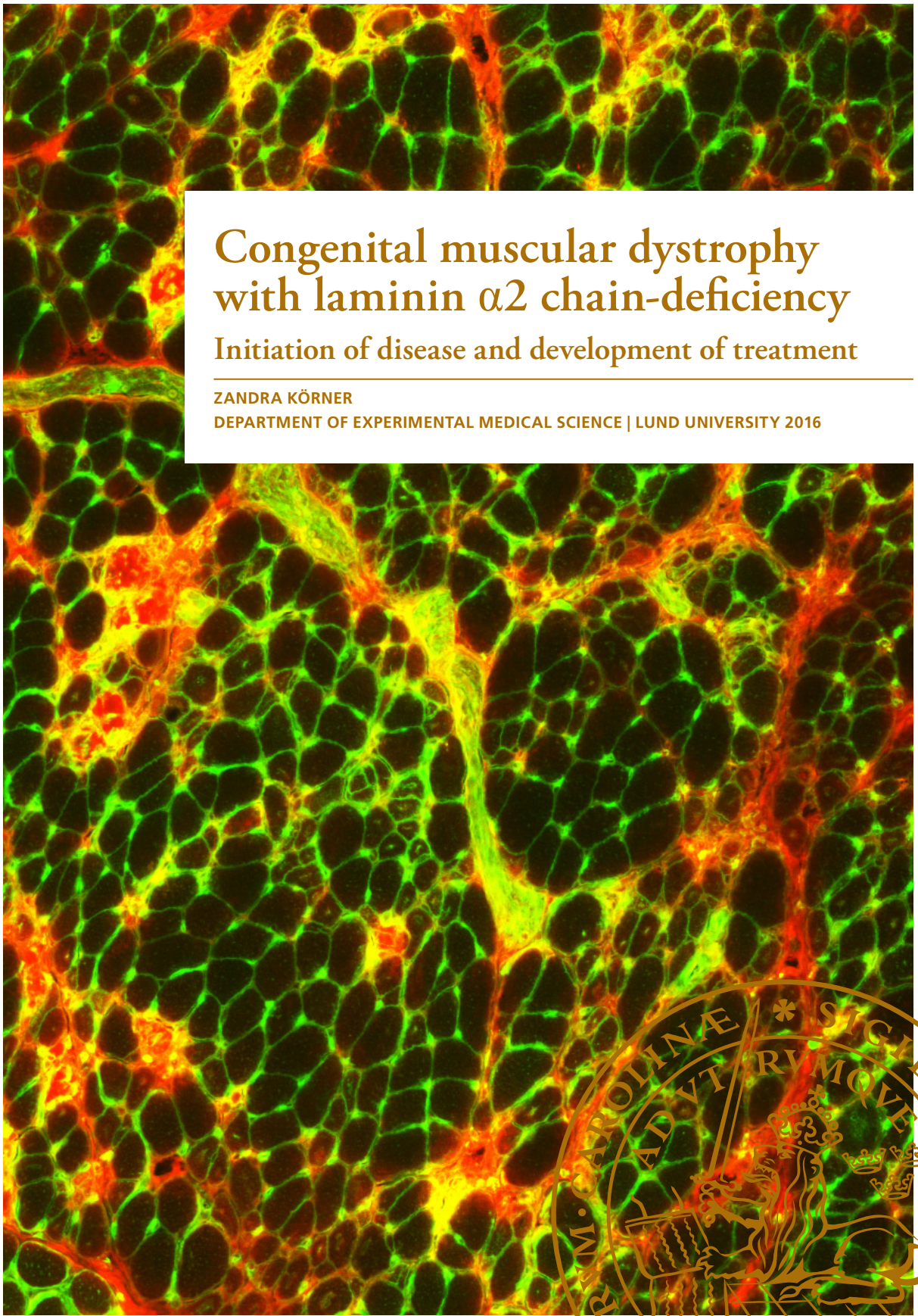

Congenital muscular dystrophy with laminin α2 chain-deficiency

Initiation of disease and development of treatment

ZANDRA KÖRNER
DEPARTMENT OF EXPERIMENTAL MEDICAL SCIENCE | LUND UNIVERSITY 2016

Congenital muscular dystrophy with laminin α2 chain-deficiency

Initiation of disease and development of treatment

Zandra Körner

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Abstract

Congenital muscle dystrophy type 1A (MDC1A) is a muscle disease caused by mutations in the LAMA2 gene, encoding the basement membrane protein laminin α2 chain. MDC1A patients exhibit neonatal onset of muscle weakness, progressive muscle wasting and hypotonia, joint contractures that mostly affect elbows, hips, knees and ankles along with scoliosis and delayed motor milestones. Currently, there is no cure for MDC1A and respiratory failure is the main cause of death. Patients with complete laminin α2 chain-deficiency have an early onset and also a more severe muscle phenotype whereas patients with partial loss usually have a milder disease course. The same genotype-phenotype correlations can be seen in the mouse models of MDC1A. The dy3K/dy3K knock-out model exhibits a much more severe phenotype than the dy2J/dy2J mouse model, which expresses a truncated laminin α2 chain. However, we have not before this thesis known how early the pathogenesis in the skeletal muscle starts. Here, we demonstrated that changes in skeletal muscle start with apoptosis already at day one after birth and inflammation at day four in dy3K/dy3K mice.

Previously, it was demonstrated that the ubiquitin-proteasome system is upregulated in the dy3K/dy3K mouse muscle. Moreover, by inhibiting the proteasome by using a lab-bench drug, dy3K/dy3K mice exhibited reduced muscular dystrophy. This led us to testing an approved FDA drug, bortezomib, which also inhibits the proteasome. By using bortezomib we could partially ameliorate the disease in the dy3K/dy3K mice with an increased lifespan and improved muscle function. However, this could not be recapitulated in the dy2J/dy2J mice. Furthermore, in this thesis we also showed that another pathway for cellular degradation, the autophagy-lysosome pathway, is upregulated in the dy3K/dy3K mouse muscle. By inhibiting the autophagy pathway, dy3K/dy3K mice exhibited improved muscle morphology and increased lifespan. In summary, I have shown that there is enhanced proteasome and autophagy activity in MDC1A muscle and that proteasome and autophagy inhibitors, respectively, can be used to reduce disease in mice. I hope that our studies can form the basis for the development of clinically relevant autophagy inhibitors. It may also be worth testing bortezomib as a possible supportive therapy for MDC1A. Furthermore, our data suggest that treatment should be initiated as early as possible given that we detected disease changes already one to four days after birth in mice.

Key words: Muscular dystrophy, laminin, MDC1A, autopahgy, proteasome

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For my family♡

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List of paper

This thesis is based on the following original articles:

I. Körner Z, Fontes-Oliveira CC, Holmberg J, Carmignac V, Durbeej M. **Bortezomib partially improves laminin α2 chain-deficient muscular dystrophy**, *Am J Pathol.* 2014 May;184(5):1518-28.

II. Körner Z, Durbeej M. **Bortezomib does not reduce muscular dystrophy in the *dy^{2J}/dy^{2J}* mouse model of laminin α2 chain-deficient muscular dystrophy**, *PLoS One.* 2016 Jan 5;11(1):e0146471.

III. Carmignac V, Svensson M, Körner Z, Elowsson L, Matsumura C, Gawlik KI, Allamand V, Durbeej M. **Autophagy is increased in laminin α2 chain-deficient muscle and its inhibition improves muscle morphology in a mouse model of MDC1A**, *Hum Mol Genet.* 2011 Dec 15;20(24):4891-902.

IV. Körner Z, Gawlik K, Durbeej M. **Early skeletal muscle pathology in the *dy^{3K}/dy^{3K}* mouse model of congenital muscular dystrophy with laminin α2 chain-deficiency**, *Manuscript*

Abstract

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Previously, it was demonstrated that the ubiquitin-proteasome system is upregulated in the dy^{3K}/dy^{3K} mouse muscle. Moreover, by inhibiting the proteasome by using a lab-bench drug, dy^{3K}/dy^{3K}mice exhibited reduced muscular dystrophy. This led us to testing an approved FDA drug, bortezomib, which also inhibits the proteasome. By using bortezomib we could partially ameliorate the disease in the dy^{3K}/dy^{3K}mice with an increased lifespan and improved muscle function. However, this could not be recapitulated in the dy^{2J}/dy^{2J} mice. Furthermore, in this thesis we also showed that another pathway for cellular degradation, the autophagy-lysosome pathway, is upregulated in the dy^{3K}/dy^{3K}mouse muscle. By inhibiting the autophagy pathway, dy^{3K}/dy^{3K} mice exhibited improved muscle morphology and increased lifespan. In summary, I have shown that there is enhanced proteasome and autophagy activity in MDC1A muscle and that proteasome and autophagy inhibitors, respectively, can be used to reduce disease in mice. I hope that our studies can form the basis for the development of clinically relevant autophagy inhibitors. It may also be worth testing bortezomib as a possible supportive therapy for MDC1A. Furthermore, our data suggest that treatment should be initiated as early as possible given that we detected disease changes already one to four days after birth in mice.

Abbrevations

BMD	Becker muscular dystrophy
cDNA	Complementary DNA
CK	Creatine kinase
CNS	Central nervous system
DGC	Dystrophin-glyoprotein complex
DMD	Duchenne muscular dystrophy
ECM	Extracellular matrix
Itga7	Integrin α7
KO	Knock-out
LGMD	Limb-girdle muscular dystrophy
MD	Muscular dystrophy
MDC1A	Congenital muscular dystrophy type 1A
MTJ	Myotendinous junction
qRT-PCR	Quantitative real-time PCR
Sgcb	β-sarcoglycan
UPS	Ubiquitin-proteasome system
WT	Wild-type

Background

Skeletal muscle

Skeletal muscle is a very important organ, present in all animals from small insects to big whales. It is the largest organ in the human body, filling up to 40% of the total body mass and is under voluntary control of the somatic nervous system.

Skeletal muscle is one of three major muscle types, the others being cardiac and smooth muscle. The main function of skeletal muscle is to generate mechanical force and motor activity in order to drive locomotion. The skeletal muscle is composed of bundles of muscle fibers that consist of multinucleated muscle cells. Every muscle fiber is enclosed by a plasma/cell membrane (sarcolemma) functioning as a barrier between intra- and extracellular compartments and every muscle fiber is surrounded by the endomysium. The muscle fibers are then organised in muscle bundles or fascicle surrounded by the perimysium. Finally, the whole muscle is encased by the epimysium (see Figure 1) [1-3].

To transmit the mechanical force between the inside and outside of the muscle cell, myotendinous junctions (MTJ) are needed to connect the skeletal muscle to the tendon [4]. These are formed by the perimysium and epimysium to increase the contact surface between these two tissues.

The structures needed for contraction and force development, myofibrils, are a part of the muscle fibers. Myofibrils consist of several numbers of sarcomeres (composed of myosin and actin filaments and other proteins), that are the functional units [5]. The tightly packing of the sarcomeres is what enables the interaction between the filaments when the excitation signal comes from the central nervous system (CNS).

To sustain a proper muscle function and protection against contraction-induced damage, there are connections between the actin cytoskeleton of the muscle fiber and the basement membrane that is situated between the sarcolemma and the endomysium [6].

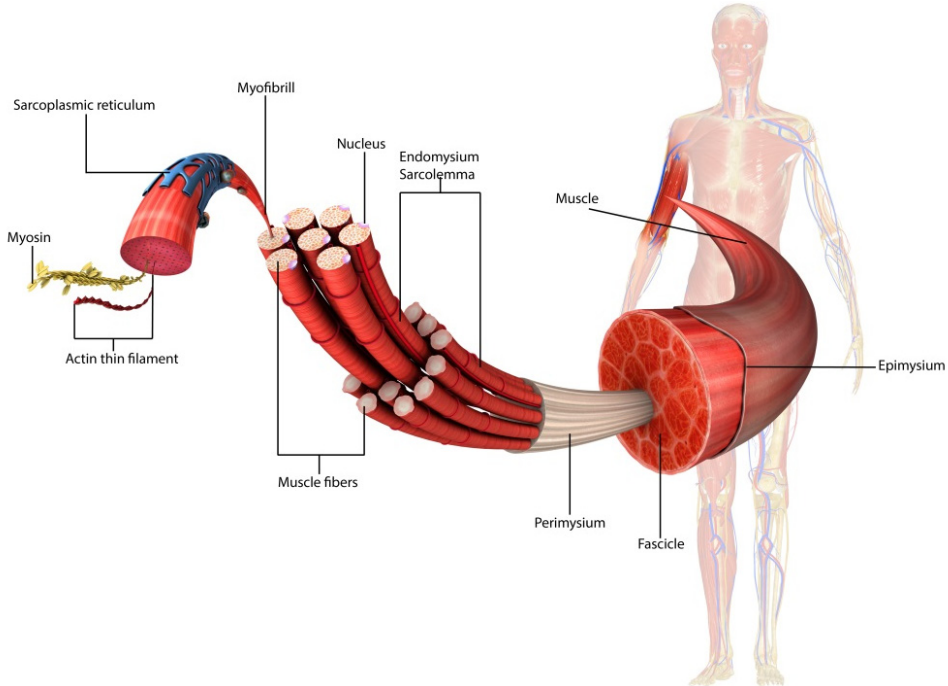

Figure 1. Overview of the organisation of the skeletal muscle.

Extracellular Matrix

All tissues are composed by cells and, by the cells produced extracellular matrix (ECM). The ECM is composed of different types of proteins (mostly collagens) and carbohydrates in a complex structure. The above mentioned endomysium, perimysium and epimysium are examples of such so called interstitial ECM. The basement membrane is a specialized ECM that surrounds muscle, fat, peripheral nerve cells and covers the basal side of endothelial and epithelial cells [6]. It also consists of many different proteins and one of the major constituents is laminin that together with type IV collagen, nidogen and perlecan form a tight network [7-10].

Laminins

Laminins are a family of cell adhesion proteins that are involved in various biological activities such as cell adhesion, growth, migration, angiogenesis and differentiation. They are heterotrimers consisting of one α, one β and one γ chain,

which assemble into a four-armed crucifix or T-shaped protein. To date, there are more than 16 different isoforms known *in vivo*, and they are named according to their chain composition [11-14]. The first isoform identified was laminin-111 and it was discovered by Rupert Timpl and colleagues almost 40 years ago [15].

The laminin assembly takes place inside the cell but some extracellular proteolytic processing of the chains may occur before the laminins reach their final form. The laminin is a mosaic protein with many critical domains for correct basement membrane assembly and function. Two of the most important domains on the laminins are the laminin N-terminal (LN) domain and the globular C-terminal (LG) domains that are expressed in a tandem manner. LN is the domain essential for self-assembly and therefore important for the assembly of the basement membrane [11-14]. The LG domains serve as binding sites for many different proteins, including cell surface receptors, to form a highly cross-linked basement membrane [12, 16]. The biological activity of the laminins is indeed mostly mediated by the binding to different cell surface receptors that link the laminin matrices to intracellular signaling pathways. The major surface receptors belong to the integrin family but laminins (laminin α1 and α2 chains in particular) also bind to the highly glycosylated α-dystroglycan, which is part of the dystrophin-glycoprotein complex (DGC) [11-13].

Laminins in skeletal muscle

Laminin-211 is the predominant isoform in the basement membrane of adult skeletal muscle and peripheral nerve [17-19] as well as cardiac muscle [19]. It consists of α2-, β1- and γ1-chains (see Figure 2). However, other laminin isoforms are expressed during myogenesis and also at the MTJ and neuromuscular junction. Laminin-111 is the predominant laminin isoform in embryonic skeletal muscle and may support normal development, however, this isoform is completely absent in adult muscle [17].

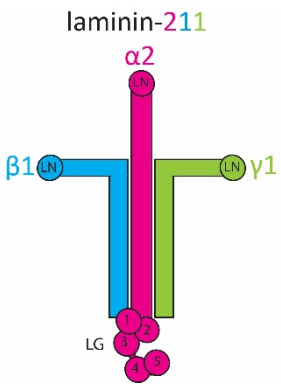

Figure 2. Schematic figure of laminin-211 and its most important domains. Laminin-211 consists of one α2 chain (pink), one β1 chain (blue) and one γ1 chain (green). Every arm contains N-terminal domains (LN) necessary for self-polymerization. Five laminin globular (LG) domains situated at the C-terminal end of the α-chain are involved in binding to cell-surfaces receptors (α-dystroglycan and integrin α7β1) and other ECM proteins such as heparin and perlecan.

Laminin-211 was originally isolated from placenta and initially called merosin [20]. The gene encoding laminin α2 chain (*LAMA2*) is located on chromosome 6q22-23 in humans and on chromosome 10 in mice [21-23]. Both laminin-211 and 221 are found in the basement membrane that surrounds the mature muscle cell [17, 24]. To provide the muscle cell with the needed support, the basement membrane needs to be tight and strongly cross-linked. This is accomplished by the cross-linkage between laminin-211/221 and other matrix proteins including nidogen (that in turn connects the laminin network to the collagen type IV network), perlecan, fibulins and agrin [25, 26] as well as between laminin-211/211 and cell surface receptors.

Laminin-211/221 receptors

The biological function of laminin-211/221 is triggered by the interaction between laminin and its transmembrane receptors, resulting in connection to the cytoskeleton and intracellular signaling pathways. The highly glycosylated protein α-dystroglycan and integrin α7β1 are the major receptors of laminin-211/221 in skeletal muscle. However, other cell-surface receptors, including syndecans and sulphated glycolipids, have also been identified in the neuromuscular system [13, 25].

The dystroglycan gene, *DAG1*, encodes a propeptide that is proteolytically cleaved into extracellular α-dystroglycan and transmembrane β-dystroglycan [27, 28]. To bind to the surrounding components in the ECM, α-dystroglycan must be heavily glycosylated [29-31] and the glycosylation is conferred by glycosyltransferases [30].

In skeletal muscle α-dystroglycan forms together with β-dystroglycan the backbone of the DGC that links laminin-211 to the intracellular components dystrophin and actin [32, 33]. The DGC is a large multisubunit complex of membrane-associated proteins and its primary function is thought to be protection of the muscle cells from contraction-induced damage [34-36]. The DGC consists of dystrophin, α-dystrobrevin, the syntrophins (α1, β1, β2), sarcospan, the sarcoglycans (α, β, γ and δ) and the dystroglycans (α and β) [33, 37, 38]. Dystrophin connects to cytoskeletal actin in the skeletal muscle fiber and to the transmembrane protein β-dystroglycan. The extracellular part of β-dystroglycan binds to the peripheral membrane protein α-dystroglycan, which through its glycostructures binds laminin-211 (and perlecan) in the basement membrane (see Figure 3) [22, 32, 39]. The binding is calcium dependent [32] and a phosphorylated O-mannosyl glycan on α-dystroglycan is required for binding laminin [29] whereas domains LG1-3 and LG4-5 on laminin α2 chain are crucial for α-dystroglycan binding [25, 40].

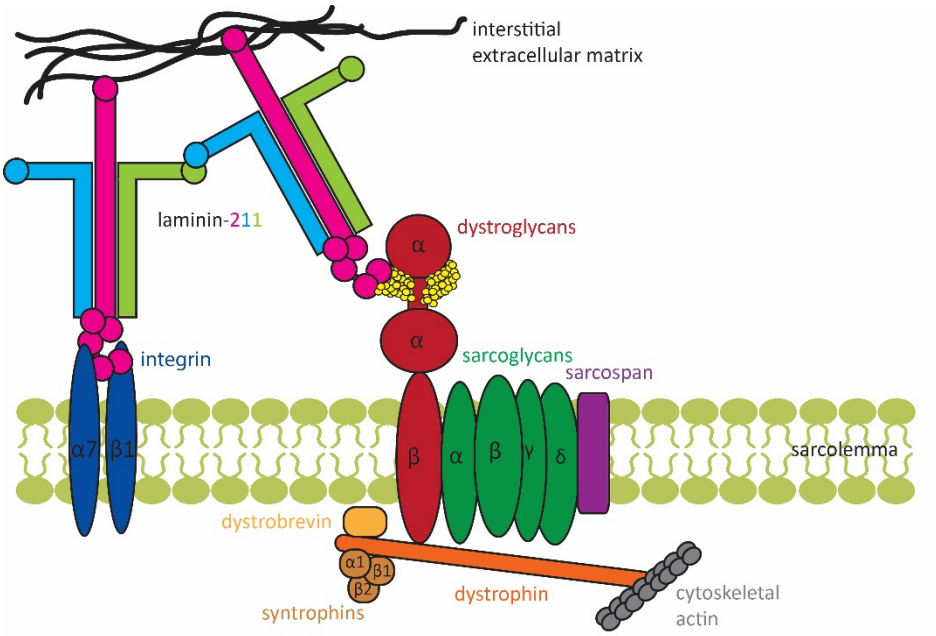

Figure 3. Laminin-211 receptors in skeletal muscle.
α-dystroglycan is a part of the DGC and together with integrin α7β1, it connects the basement membrane to the intracellular part of the muscle fiber.

Integrins are a large family of αβ heterodimeric transmembrane cell surface receptors that have functions in a wide variety of cellular events such as adhesion, migration and differentiation [41, 42]. Integrins consist of one long extracellular domain and a relatively short intracellular domain. In skeletal muscle the predominate integrin that binds laminin α2 chain is α7β1 [43, 44] and it has been suggested to take part in myogenesis and to be involved in the differentiation and migration processes [45, 46]. Integrin α7β1 binds to LG1-3 domains of laminin α2 chain [40].

Studies have shown that the two receptors and pathways work synergistically but also that they have separated functions [47-50]. For example, only dystroglycan is involved in the anchorage between the basement membrane and the sarcolemma [36]. Additionally, it seems that different muscles have different requirements of the dystroglycan-laminin interaction. It may be more crucial in limb muscle than in diaphragm [51]. The downstream actions from the laminin-211-dystroglycan/integrin α7β1 axis remain largely elusive and more research is clearly needed to elucidate them.

The importance of laminin-211 and its receptors to retain normal muscle function is emphasized by the fact that mutations in the genes encoding dystrophin, all four

sarcoglycans, laminin α2 chain and integrin α7 chain, respectively, are responsible for Duchenne/Becker muscular dystrophy (DMD/BMD); limb-girdle muscular dystrophy (LGMD) type 2C-F and congenital muscular dystrophy (CMD) [52-61].

Muscular dystrophy

More than 30 genes have been implicated to cause muscular dystrophy [37]. Muscular dystrophy is a general term that describes a very heterogeneous group of inherited and progressively attenuated myogenic disorders. Genetic mutations in the DGC cause many of the different muscular dystrophies, and they can be X-linked recessive as DMD/BMD, autosomal dominant as in LGMD type 1, or autosomal recessive as in LGMD type 2 and congenital muscular dystrophy type 1A (MDC1A) (see Table 1). DMD is the most common type of muscular dystrophy. It affects approximately 1 out of 3500 boys whereas the combined LGMD affect 1 out of 20,000. DMD patients have a childhood onset phenotype and due to either respiratory or cardiac failure they die in their early thirties, whereas patients with the milder form BMD have a moderate weakness in adulthood and a near normal lifespan. When it comes to the limb-girdle muscular dystrophies, they have a very variable time of onset and progression but they initially involve the shoulder and pelvic girdle muscle [62-65]. Integrin α7-deficiency is another extremely rare type of autosomal recessive congenital muscular dystrophy [59, 61]. Finally, laminin α2 chain-deficiency leads to yet another type of congenital muscular dystrophy, namely laminin α2 chain-deficient-deficient congenital muscular dystrophy type 1A (MDC1A). In this thesis, MDC1A is the main focus and will be discussed in detail below.

Table 1.
Summary table of muscular dystrophies discussed in this thesis.

Disease	Mode of inheritance	Gene product	Mouse model
X-linked MD			
Duchenne/Becker MD	XR	Dystrophin	*mdx*
Limb-girdle MD			
LGMD 2E	AR	β-sarcoglycan	*Sgcb*$^{-/-}$
Congenital MD			
α7 integrin CMD	AR	α7 integrin	*Itga7*$^{-/-}$
MDC1A	AR	Laminin α2	*dy*^{3K}/*dy*^{3K}, *dy*^{2J}/*dy*^{2J}

MD=muscular dystrophy, AR=autosomal recessive, XR=X-linked recessive

Laminin α2 chain-deficient congenital muscular dystrophy type 1A (MDC1A)

Mutations in the *LAMA2* gene, encoding the laminin α2 chain, result in laminin α2 chain-deficient congenital muscular dystrophy type 1 A (MDC1A) [66]. In Europe this type of congenital muscular dystrophy represents approximately 30-50% of the patients with classical CMD [67, 68] and the estimated prevalence is 7×10^{-6} and 2.5×10^{-5} based on samples from Italy and Sweden, respectively [69, 70]. In Sweden there are around eight to ten patients.

After the identification of the first causative mutation, we know now that there are more than 380 different missense, nonsense, splice sites and deletions mutations scattered over the 65 exons of the coding sequence of the *LAMA2* (www.dmd.nl, [71, 72]). The mutations can lead to complete or partial deficiency and complete deficiency causes a more severe phenotype than the partial, however, that is not true for all individual patients [72, 73]. Laminin α2 chain-deficiency leads to muscular dystrophy also in other species, including mice and dogs [74, 75] and nearly all MDC1A mechanistic studies have been performed in mice.

Clinical features of MDC1A

MDC1A patients exhibit neonatal onset of muscle weakness, progressive muscle wasting and hypotonia, joint contractures that mostly affect elbows, hips, knees and ankles along with scoliosis and delayed motor milestones. The patients also display increased creatine kinase (CK) levels at an early age [68, 76, 77]. Laminin α2 chain is also expressed in the central nervous system (CNS), peripheral nervous system (PNS) and heart [18] and therefore these tissues are also affected to various degrees in MDC1A. In most patients older than 1 year of age white-matter abnormalities are observed, however, this is not associated with any particular functional impairment. Nevertheless, some patients display structural brain changes that may be accompanied by epilepsy. MDC1A patients also display myelination defects resulting in decreased peripheral nerve conduction velocity. Heart problems are rare in MDC1A patients, but 30% of the patients display left ventricular dysfunction [68, 76-79]. The maximum motor ability that the patient can achieve is unsupported sitting. Often they can also stand with support but patients rarely learn to walk [78, 79]. Consequently, patients are confined to wheelchair, require assistance in ventilation, gastrostomy, and have a reduced life expectancy [76, 77, 80, 81]. Around 30% of the patients die within their first decade of life and the commonest cause of death is infections in the respiratory-tract [67, 81].

Non-neuromuscular effects have not been well characterized in the MDC1A patients. However, studies in laminin α2 chain-deficient animals resolve that apart from the neuromuscular disease, laminin α2 chain depletion also leads to hearing loss, aberrant thymocyte development, defective odontoblast differentiation and impaired spermatogenesis [71, 82-85]. Today, there is no effective treatment for MDC1A patients. This has led to the development of a wide range of mouse models to be able to test both the efficacy and safety of novel approaches as well as to study the disease progression.

Mouse models of laminin α2 chain-deficiency

Several different mouse models that exhibit laminin α2-deficiency have been produced and they all in some way adequately mirror the human disease (see Table 2). The first one to be identified was the *dy/dy* (dystrophia-muscularis) mouse that has a spontaneous, but yet unknown, mutation in the *lama2* gene [22, 23, 86]. Shortly after, the allelic mutant dy^{2J}/dy^{2J} was characterized [87]. Dy^{2J}/dy^{2J} mice display much milder characteristics of MDC1A compared to the other mouse models. They harbour a point mutation in the N-terminal domain of the laminin α2 chain gene, which leads to abnormal splicing of the gene and therefore expression of a truncated protein. Even though the dy^{2J}/dy^{2J} mice still express a truncated protein, muscles display the histopathological changes normally seen in muscular dystrophy; centrally located nuclei, variation in fiber size and fibrosis. They, however, display a better general health (compared to the other mouse models), have a near normal lifespan and are able to breed, but develop a pronounced hind-leg paralysis [87, 88].

Interestingly, neither of these two models (*dy/dy* or dy^{2J}/dy^{2J}) exhibits a complete deficiency even though both of them display muscular dystrophy. However, null mutant mouse models using homologous recombination have also been generated, dy^{W}/dy^{W} and dy^{3K}/dy^{3K}. These two models exhibit a much more severe muscular dystrophy due to the failure to form a laminin scaffold [89, 90], which is necessary for the structure of the basement membrane and for interactions with the DGC and integrins.

The dy^{3K}/dy^{3K} mouse model has been the main mouse model used in this thesis. They are outwardly indistinguishable from their wild-type (WT) littermates, until approximately 2 weeks of age when severe growth retardation and muscle wasting become apparent. They die shortly after the symptoms become more severe around week 3-4 after birth [89]. Even though this is a widely used mouse model, not much is known about when and what the disease progression starts with. Therefore we have studied the early pathogenesis in dy^{3K}/dy^{3K} mouse muscle (paper IV).

In summary, all mouse models exhibit pronounced transcended hind-leg lameness and to different extent histopathological signs of muscular dystrophy. The usage of mice as preclinical tools to model human disease can always be discussed. However, analyses of the various laminin α2 chain-deficient mouse models have resulted in significant improvement in the understanding of development of MDC1A. Even more importantly, they have helped to develop novel therapeutic concepts for laminin α2 chain-deficiency [76].

Table 2.
Summary table of MDC1A mouse models.

Mouse model	Mutation	Laminin	Phenotype	Lifespan	Reference
dy/dy	Unknown spontaneous mutation	Reduced levels of normal laminin α2	Moderate muscular dystrophy, peripheral neuropathy and CNS myelination defects	Lethal within 6 months of age	[22, 23, 91]
dy^{2J}/dy^{2J}	Splice site resulting in spontaneous mutation in LN domain	Expression of a truncated α2 chain devoid of LN domain	Mild muscular dystrophy, peripheral neuropathy	Normal lifespan	[87, 92]
dy^{W}/dy^{W}	Knock-out	Express small amount of truncated α2 chain devoid of LN domain	Severe muscular dystrophy, peripheral neuropathy	Lethal at 10-15 weeks of age	[90, 93, 94]
dy^{3K}/dy^{3K}	Knock-out	Complete deficiency	Very severe muscular dystrophy, peripheral neuropathy, impaired spermatogenesis	Lethal at 3-4 weeks of age	[82, 89, 95]

Other muscular dystrophy mouse models

In my studies I have also used other mouse models of muscular dystrophy and they are briefly described below.

Mdx

The mouse model of dystrophin-deficiency, *mdx*, is the best-characterized mouse model for muscular dystrophy. The loss of dystrophin is due to a point mutation in exon 23 of the dystrophin gene, which leads to a premature termination of the polypeptide chain [96]. The loss of dystrophin also affects the expression of several of the other DGC proteins [97]. Although this is the most widely used mouse model and has showed to be a valuable model for DMD, the mice exhibit a

much milder form of muscle wasting disease than the human patients [98], at least in certain fundamental muscles. The *mdx* mice exhibit signs of muscular dystrophy in many different muscles under their first six weeks of life but subsequently only show a little weakness and have a near-normal lifespan. This partial recovery can be due to two possible explanations. The first one is that the partial 'recovery' is due to the fact that the *mdx* mouse adapts to the muscle degeneration with an expansion of the satellite cell population and muscle hypertrophy. The other possible explanation is that the mice express the homologous protein utrophin, which in turn compensates for the lack of dystrophin. Despite this, the *mdx* mouse exhibits an elevated level of creatine kinase (CK) in serum, which is a well-known diagnostic criteria for muscular dystrophy [37, 98-106].

Sgcb$^{-/-}$

The sarcoglycan complex consists of four transmembrane proteins (α-, β-, γ- and δ-sarcoglycan) that form a subcomplex together with sarcospan within the DGC [107]. The expression of the sarcoglycan-sarcospan complex is needed to stabilize α-dystroglycan at the membrane. It is well established that mutations in either of the sarcoglycans result in distinct forms of muscular dystrophy, collectively called sarcoglycanopathies [54-58, 108, 109]. LGMD type 2E is a sarcoglycanopathy caused by mutations in the β-sarcoglycan gene, which is expressed in skeletal, cardiac and smooth muscle tissues [55, 110, 111]. Consequently, mice deficient in β-sarcoglycan (Sgcb-null mice) develop a severe muscular dystrophy and cardiomyopathy but are both viable and fertile with a near-normal lifespan. They show typical pathological hallmarks of muscular dystrophy with extensive central nucleation, pronounced areas of focal necrosis, areas of fibrosis and a high degree of fatty infiltration and calcification. The mice also exhibit elevated CK levels in plasma. Additionally, vascular smooth muscle irregularities have been detected [109].

Itga7$^{-/-}$

As discussed before, integrin α7β1 is one of necessary cell-surface receptors for laminin-211 binding in muscle.

Mice lacking integrin α7 chain display a mild myopathy that mostly affects the MTJ. They are fertile and have a normal lifespan. The skeletal muscle develops normally, which shows that integrin α7 is not essential for myogenesis. They do, however, show typical symptoms of a progressive muscular dystrophy starting soon after birth, with variation in the different muscles. Integrin α7 is the only integrin α subunit in adult MTJ and therefore MTJ is very much affected in

Itga7$^{-/-}$ mice. [112]. In addition, there is an embryonic lethality in *Itga7*$^{-/-}$ offspring resulting in that heterozygous breeding does not follow the Mendelian ratio [113].

It is possible that the two receptors of laminin α2 chain, DGC and integrin α7 have overlapping functions. However, mice lacking both integrin α7 and dystrophin develop a severe dystrophy and die between 3-4 weeks of age [47]. The compensation reasoning seems even more compelling since the integrin α7 transcript is increased in the DMD patients and *mdx* mice [114, 115], which suggest that the integrin α7β1 may compensate for the absence of the DGC. Indeed, overexpression of integrin α7 subunit in mice lacking both dystrophin and utrophin has beneficial effects in skeletal muscle [116].

Pathogenesis of MDC1A

The absence of laminin α2 chain gives rise to a marked dystrophic pattern in skeletal muscle. The dystrophic muscles are characterized by the presence of centrally located nuclei, early inflammation, fiber-size variations, (atrophy dominate) massive degeneration and infiltration of fibrotic/connective tissue (see Figure 4) [76, 78]. Other histopathological changes are also detected for example upregulation of apoptosis [117, 118] and disrupted basement membranes [23, 119]. These are found both in laminin α2 chain-deficient mouse models and MDC1A patients.

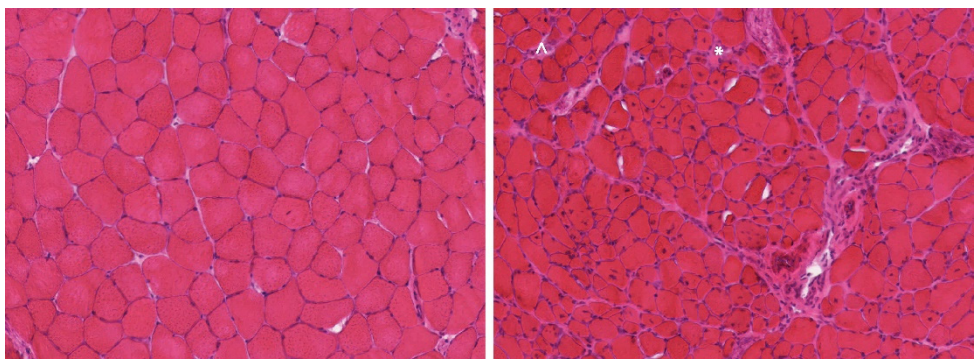

Figure 4. Cross-sections of WT and laminin α2 chain-deficient muscle, respectively.
Hematoxylin-eosin staining to visualize the histopathological changes in muscular dystrophy. Centrally located nuclei indicate an ongoing degeneration/regeneration. Connective tissue infiltration and fiber size variation are observed. *indicating necrotic/apoptotic fibers and ^indicating infiltration of mononuclear cells.

Even though the primary defect of MDC1A is loss of laminin α2 chain, it is the secondary effects and mechanisms that in the end lead to skeletal muscle degeneration. These secondary effects and mechanisms are less understood. For

example, only a few percentages of the muscle fibers in laminin α2 chain-deficient muscle are apoptotic [117]. Therefore, the excessive muscle wasting that is seen in MDC1A is probably a result from other deregulated mechanisms. It is well known that muscle atrophy is due to the imbalance between protein synthesis and protein degradation. The two major proteolytic systems in skeletal muscle are the ubiquitin-proteasome system and the autophagy-lysosome pathway [120]. These will be discussed later in this thesis.

Altered inflammation

Due to the muscle damage caused by the absence of the laminin α2 chain, there is an invasion of inflammatory cells in MDC1A muscle. This can definitely influence the outcome of fibrosis. It has been showed that inflammation is one of the first features of muscle pathology in both laminin α2 chain-deficient mice and patients [49, 119, 121, 122]. One prominent inflammatory cell type seen in laminin α2 chain-deficient muscle is the macrophage. However, the inflammatory response is transient and the phenotype evolves to a more fibrotic and degenerative one [49]. Inflammation has not been well studied in MDC1A but this acute inflammation may have a major role in the muscle pathogenesis of MDC1A [71].

Altered expression of ECM proteins

The absence of the laminin α2 chain also causes altered expression levels of other laminin chains and cell-surface receptors. Expression of the laminin β2 chain is drastically reduced from the sarcolemmal basement membrane [24] whereas the laminin α4, and also α5 chains are increased at the same site [17, 123]. However, since the laminin α4 chain cannot bind to α-dystroglycan the upregulation may not compensate for the loss of laminin α2 chain [11, 13, 16, 25, 124]. The upregulation of the laminin α4 chain is also seen in the extraocular muscle, where it is normally highly expressed. Here, it might have a protective role possibly due to the integrin α7β1 binding of laminin α4 chain [125, 126]. Regarding the receptors of laminin-211, there is a dramatic decrease of the integrin α7 subunit at the sarcolemma upon laminin α2 chain-deficiency [51, 114, 115, 127, 128]. In contrast, β-dystroglycan is upregulated at the sarcolemma [51, 129]. There have been some conflicting results when it comes to α-dystroglycan. The production has been showed to not be affected by the laminin α2 chain-deficiency [114, 127] or shown to be moderately increased [51]. On the other hand, the α-dystroglycan core protein has been showed to be severely reduced [129, 130]. Other ECM proteins, such as collagens, fibronectin, periostin, lumican and

vimentin, are also significantly increased in the laminin α2 chain-deficient muscles and constitute the source of the connective tissue infiltration of the muscles [49, 131, 132].

Transcriptomics and proteomics

A few transcriptomic and proteomic studies have been conducted of laminin α2 chain-deficient muscles. A microarray analysis of skeletal muscle from MDC1A patients showed that it is mainly ECM genes that are upregulated in diseased muscles [131]. The same trend was noted with a comparative proteomic analysis of dy^{3K}/dy^{3K} muscle [132]. Gene expression profiling of *dy/dy* and dy^{3K}/dy^{3K} muscle also showed that most of the upregulated genes encode ECM proteins as well as proteins that normally are expressed transiently during normal muscle development and regeneration [133, 134]. The downregulated genes are mainly involved in diverse metabolic processes and kinase activities [133]. The metabolic alterations were also seen in quantitative proteomic analysis together with calcium dysregulation [132]. The metabolic angle has never been explored in laminin α2 chain-deficiency and it is not the primary cause of disease but may play a significant role, and the same applies to the abnormal calcium handling. Finally, there are many different ways to regulate gene expression. One way is the use of microRNAs (miRNAs), which are small non-protein coding RNAs, which regulate the gene expression at the post-transcriptional level. Expression of selected miRNAs was recently evaluated and revealed that levels of the muscle specific miR-1, miR-133a and miR-206 are altered in skeletal muscle and plasma from laminin α2 chain-deficient mice. The study also indicated that miRNAs could function as biomarkers for non-invasive monitoring of the disease and also response to treatment [88].

Interventions to reduce MDC1A in mice

Genetic interventions

Many different genetic attempts to compensate for laminin α2 chain-deficiency have been explored during the past two decades. Most of the focus has been on ECM proteins due to the effects on the basement membrane in MDC1A muscle. Positive results have been seen by transgenic expression of laminin α1 and α2 chains, mini-agrin, integrin α7 chain and cytotoxic T cell Gal-Nac transferase [90, 129, 135-138]. An overexpression of linker molecules (such as mini-agrin and

agrin-perlecan fusion protein) in the dy^W/dy^W mouse model resulted in a prolonged lifespan and significantly improved muscle tissue. Mini-agrin has high-affinity binding sites for the laminins that are upregulated in the dy^W/dy^W mice (laminin α4 and α5 chains) and α-dystroglycan. Mini-agrin was also shown to be able to slowdown disease progression at every stage [129, 135, 139].
The overexpression of laminin α1 chain has also been extensively studied due to the fact that laminin α1 chain also binds to laminin α2 chain receptors α-dystroglycan and integrin α7β1. In the dy^{3K}/dy^{3K} mouse model overexpression of laminin α1 chain resulted in a near-normal lifespan and mice displayed significantly improved muscle morphology, heart and nerve morphology [136, 140, 141]. The overexpression also resulted in a reconstituted integrin α7 expression at the sarcolemma, which led to the reasoning that reduction of muscular dystrophy mainly involves the integrin α7β1. For that reason, a dy^{3K}/dy^{3K} mouse overexpressing laminin α1 chain lacking the dystroglycan binding site but still expressing the binding site for integrin α7β1 was created ($dy^{3K}\delta E3$). This mouse model showed increased lifespan and partially rescued dystrophic muscles, especially in the diaphragm [51]. Some minor defects were seen in older mice overexpressing full-length laminin α1 chain, which may be explained by the altered signaling cascades that laminin α1 may activate [136, 140].
Positive results have also been seen in transgenic dy^W/dy^W mice overexpressing integrin α7 subunit. These mice have a restored sarcolemmal localization of the α7β1 integrin, reduced muscle pathology and increased lifespan [138]. Despite these positive results, we need to keep in mind that these transgenic approaches are not feasible for clinical use. Therefore, adenoassociated virus-mediated gene transfer has been tested in the dy^W/dy^W and *dy/dy* mice. Systemic gene transfer of mini-agrin improved the overall phenotype and muscle function [142].
There have also been several attempts to target the secondary defects in MDC1A. There is some upregulation of apoptosis in MDC1A and therefore apoptosis has been embattled in two ways in the dy^W/dy^W mouse. Both inactivation of the pro-apoptotic protein Bax and overexpression of the anti-apoptosis protein Bcl-2 [143, 144] resulted in an improved health of the animals.

Cell therapy

Another treatment option for muscular dystrophy is cell therapy. Bone marrow transplantation of *dy/dy* animals resulted in improved lifespan, growth rate, muscle strength and respiratory function [145]. However, transplantation of myoblasts and CD90-positive cells to *dy/dy* and dy^{3K}/dy^{3K} mice, respectively, led to laminin α2 chain expression but no further improvement in either of the models was seen [146, 147].

Protein therapy

Protein therapy has also been evaluated for MDC1A. Intramuscular or intraperitoneal injections of laminin-111 in dy^W/dy^W mice resulted in reduced muscular dystrophy, improved muscle strength and also increased lifespan [148]. The same group has also demonstrated that laminin-111 treated muscles of dy^W/dy^W mice damaged with cardiotoxin display restored muscle regeneration, with increased number and size of myofibers [149].

Pharmacological intervention

Pharmacological treatment of MDC1A may be the most feasible option today. Treatment with anti-apoptotic substrates minocycline, doxycycline and omigapil, respectively, increased the lifespan of dy^W/dy^W mice and also reduced the muscle pathology [150, 151]. Omigapil also showed positive results in dy^{2J}/dy^{2J} mice [152]. These encouraging results have led to a clinical trial conducted by Santhera Pharmaceuticals, right now in phase 1. The trail will include 20 ambulatory and non-ambulatory CMD patients (with laminin α2 chain-deficiency and collagen VI-deficiency) aged 5 to 16 years old. The study will not just examine the drug safety but also the patients' respiratory function, muscle strength and motor function (http://musculardystrophynews.com).

The dy^W/dy^W mice also display swollen mitochondria, a typical feature of abnormal opening of the permeability transition pore caused by a strong increase of intercellular calcium. This can result in cell death due to mitochondria rapture because of the persistent opening. Treatment with cyclophilin-D, a permeability transition pore regulator, also resulted in reduced muscular dystrophy pathology [153].

Fibrosis is a typical feature of MDC1A pathogenesis and significantly contributes to muscle weakness in MDC1A. Therefore, fibrosis has also been a target for pharmacological treatment of MDC1A. Losartan is an angiotensin II type I receptor blocker that has showed positive results in dy^{2J}/dy^{2J} mice, with a reduction of fibrosis, apoptosis and also inhibition of the TGF-β signaling pathway [154, 155]. An analog derivative of losartan also showed encouraging results (but in the dy^W/dy^W mice) with a reduction of fibrosis and inflammation and also inhibition of TGF-β signaling pathway [156].

Pathways for protein degradation

In laminin α2 chain-deficient muscle wasting is pronounced. The excessive muscle wasting is probably not only due to the small upregulation of apoptosis, as previously discussed, but other pathways are likely to be affected too.

Muscle wasting may occur if there is an imbalance between protein synthesis and protein degradation. The latter is mediated by a higher activity in two highly conserved pathways; the ubiquitin-proteasome pathway and the autophagic/lysosomal pathway.

Proteasome

During protein degradation by the ubiquitin-proteasome system (UPS) proteins are conjugated to multiple ubiquitin molecules and are then tagged for degradation within the proteasome complex. This is done by three different groups of proteins, first the ubiquitin-activating enzyme (E1), then the ubiquitin-conjugating enzyme (E2) and last the ubiquitin ligase (E3) [157]. When the proteins are linked with ubiquitin they are recognized by the 26S proteasome that degrades them to small peptides. Two important proteins in skeletal muscle are the muscle specific-ubiquitin ligases, MAFbx/atrogin-1 and muscle ring finger protein 1 (MuRF1), which are confirmed markers for skeletal muscle atrophy as mice lacking the genes are very resistant against atrophy. During muscle atrophy there is an increased expression of both MAFbx/atrogin-1 and MuRF1 [157, 158]. There is still a debate on how these proteins are activated but it is thought that two different pathways are involved. The first pathway involves dephosphorylation of Akt, which removes its inhibitory effect on forkhead box O (FoxO) transcription factors making the FoxOs translocate to the nucleus from the cytosol [159, 160]. Akt is an important protein kinase that in muscles regulates proliferation, size, differentiation and survival. In the dy^{3K}/dy^{3K} mice, Akt phosphorylation is downregulated, which may result in the pathological changes of atrophy [161]. Stimulation of Akt, on the other hand, leads to hypertrophy of the skeletal muscle [162]. The other signaling pathway, which just seems to activate MuRF1, is the NF-κB pathway [163]. NF-κB is an important DNA transcription factor, and it is already present in cells in its inactive state, which means that it can act rapidly, without transcription. NF-κB consists of different subunits. The most distinct are subunits p50 and p65 that are inhibited by the IκB, which is sent to signal-induced degradation via the activation of IκB kinases.

It has been shown that the DGC interacts with phosphoinositide 3-kinase. This protein is one of the upstream activators of Akt and it was also shown that Akt

phosphorylation is dependent on laminin binding [164]. Akt phosphorylation is also associated with integrin α7β1 (Boppart, Burkin 2011). This lead to the idea that laminin α2 chain-deficiency might cause diminished Akt phosphorylation and consequently activation and upregulation of the UPS. The therapeutic potential of proteasome inhibition had also been evaluated pre-clinically for DMD [165-167]. Indeed, Carmignac et al. demonstrated that members of the UPS are upregulated in dy^{3K}/dy^{3K} muscle and that systemic treatment with the proteasome inhibitor MG-132 significantly improved the lifespan and muscle morphology in the dy^{3K}/dy^{3K} mouse [161]. The mice also exhibited normalized laminin α4 and β2 chain expression, with a restoration of β2 chain expression at the sarcolemma. Moreover, restored Akt phosphorylation was observed [161]. These positive results were obtained even though mice were only treated once or twice and mice treated with two injections did not show more beneficial effects than the ones treated once. Since MG-132 is just a lab-bench drug and not a specific inhibitor of just the proteasome, but also the calpain system, we wanted to analyse the effects of the specific proteasome inhibitor bortezomib in mouse models of laminin α2 chain-deficiency and also analyse if proteasome overactivation is seen in human MDC1A muscle cells (papers I and II).

Autophagy

The autophagic/lysosomal pathway is divided in different types of autophagy with different types of substrates; macroautophagy, microautophagy and chaperon-mediated autophagy. However, here I will focus on the macroautophagy (from here just called autophagy) process. Autophagy is involved in the degradation of damaged cellular organelles such as mitochondria, endoplasmic reticulum and ribosomes but also in the degradation of proteins aggregates, long-lived proteins, lipids and ribosomal RNA. It is a multi-step catabolic process that begins with sequestering of what should be degraded in autophagosomes [168-170]. Autophagosomes are double-membrane vesicles that are formed from small membrane structures called autophagosome precursors that are initiated by a class III phosphoinositide 3-kinase (Vps34) and autophagy-related protein-6 (Atg-6), also known as Beclin-1 [168, 171]. During the formation of autophagosomes two other systems are also involved. One system is composed of the microtubule-associated protein-1 light chain 3 B (LC3) and GABARAP, both homologues to ubiquitin-like protein Atg8 and Atg4 and the other is the Atg12-Atg5-Atg16 complex. LC3 is a protein that undergoes post-translation modifications during the activation of autophagy that converts it to a lower migrating form LC3II (first by conjugation by phosphatidylethanolamine and then the conversion by Atg4). Subsequently, the autophagolysosome is formed [168, 172]. This occurs by fusion of an autophagosome with a lysosome, which leads to the degradation of the

contents by lysosomal hydrolases. The lysosomes are filled with more than 50 acid hydrolases including several peptidases, phosphatases, nucleases, glycosidases, proteases and lipases. This function of this pathway is not to kill the cell but rather to maintain cellular homeostasis and differentiation by recycling. Lysosomal proteases are divided into three sub-groups depending on their active-site; cysteine, aspartyl and serine cathepsins. One of the most abundant lysosomal proteases is cathepsin L [168]. To be able to degrade cell organelles and especially the mitochondria and endoplasmic reticulum there is a need for the protein Bnip3 that interacts with LC3B [173]. This pathway is normally activated by deprivation of nutrients or growth factors but also endoplasmic reticulum stress to maintain cell survival. It can also be activated by the inhibition of Akt through the act of FoxO3 that can activate several autophagy-related genes (e.g. Bnip3, LC3, Vps34 and Gabarapl 1) [174, 175]. There is now also extensive evidence for the cross-talk between the UPS and the autophagic/lysosomal pathway [176]. P62 interacts with LC3 to deliver polyubiquitinated proteins for degradation. However, there is still the question if p62 is essential for the whole machinery of autophagy or just the delivery. Therefore, it is hard to pinpoint the activity for either of the pathways [168,173].

It has been shown that there is an impaired activation of autophagy in both patients and a mouse model of collagen VI-deficient muscular dystrophy and that the reactivation results in amelioration of the dystrophic phenotype [177]. However, much is still unknown when it comes to the role and regulation of autophagy and skeletal muscle, in particular since both upregulation and inhibition, respectively, results in atrophy [120, 178]. Since increased proteasome activity was noted in dy^{3K}/dy^{3K} muscle, we envisioned that autophagy might also be deregulated in laminin α2 chain-deficient mice. Hence, we functionally characterized autophagy in dy^{3K}/dy^{3K} muscle (paper III).

Main questions of the thesis

- Does the clinically relevant and FDA-approved drug bortezomib also reduce muscular dystrophy in the dy^{3K}/dy^{3K} mouse model, just like MG-132 did?
    - If so, does it also reduce muscular dystrophy in the dy^{2J}/dy^{2J} mouse, a model expressing a truncated laminin α2 chain?
- Is the autophagy/lysosomal pathway upregulated in the dy^{3K}/dy^{3K} knock-out mouse model for MDC1A?
    - If it is, can we by using an inhibitor of the pathway ameliorate the disease?
- When and what does the disease progression start with, in the dy^{3K}/dy^{3K} knock-out mouse model?

Main methods

Mouse models and drug administration

Many different mouse models were used for my studies, including dy^{3K}/dy^{3K} and dy^{2J}/dy^{2J} mice. We also used tissues from the following muscular dystrophy mouse models: *mdx*, $Sgcb^{-/-}$, $Itga7^{-/-}$ and $dy^{3K}\delta E3$.

The administration of the drugs in paper I, II and III, respectively, was made by different types of injection routes. In paper I, the systemic injections of bortezomib were done intravenously (i.v.). In paper II the first three injections were i.v. while the other three were sub-cutaneous (s.c.). 3-methyladenine (3-MA) in paper III was injected intraperitoneally (i.p.) while MG-132 was injected i.v. [179-181].

Murine tissue and histology

Skeletal muscle was either snap-frozen (for quantitative real-time PCR) or embedded in OCT medium for cryosectioning.

The muscle was sectioned (8 μm thick) using a cryostat. The sections were then processed differently. For evaluation of overall histology and centrally located nuclei the sections were stained with hematoxylin and eosin. Masson Trichrome staining was used to analyze the fibrosis and especially the collagen content. Otherwise, the majority of the sections were analyzed by immunofluorescence using different antibodies and also specific protocols. In this way, apoptosis, inflammation and fibrosis were analyzed (see Table 3). Fiber size was evaluated by incubating sections with biotinylated wheat germ agglutinin (WGA), which is a lectin that binds to glycoproteins in the cell membrane.

Table 3.
Summary table of staining protocols.

Histology	
Analysis	*Staining protocol*
Central nuclei	Hematoxylin and eosin (H&E)
Fibrosis	Masson Trichrome
Immunofluorescence	
Analysis	*Protein of reaction*
Fiber size	Biotinylated WGA
Fibrosis	Collagen III, Tenascin-C, Fibronectin
Inflammation	CD11b
Apoptosis	Caspase-3

Quantitative real-time PCR

To analyse involvement of different protein degradation pathways, we conducted quantitative real-time PCR (qRT-PCR). In Paper I we evaluated the expression of proteasome-related genes and in paper III the expression of autophagy-related genes. RNA was extracted from quadriceps muscle from the different mouse models and their WT littermates. Thereafter, complementary DNA (cDNA) was synthesized. The qRT-PCR amplifications were performed in a LightCycler. All the primers were previously described; NF-κB-p65, FoxO1, MAFbx/atrogin-1, MuRF1 and ubiquitin for the proteasome system [161, 174, 182, 183] and Bnip, Bnip3l, p62, LC3B, Gabarapl1, Atg4b, Vps34, Beclin, Cathepsin L and Lamp2a for the autophagy pathway [175]. GAPDH and RPLP0 were used as control genes. Human cells were also analysed using qRT-PCR, (in paper I and III9. Moreover, in paper I and II miRNA levels were analysed. In paper I we analysed the levels of two muscle specific miRNAs in quadriceps muscle; miR-133a and miR-1. As internal controls let-7a and U6 snRNA were used. In paper II the miRNA levels were analysed in plasma and the control was miR-122.

Cell culture

In paper I and III we cultured cells *in vitro*. Primary myoblasts (passage 2 and 3) were obtained from a control fetus (12 weeks of gestation) and a MDC1A fetus (15 weeks of gestation), presenting a homozygous nonsense mutation in exon 31 of the *LAMA2* gene [184]. To obtain myotubes the cells were incubated with fusion medium. In paper I both myotubes and myoblasts were used for qRT-PCR,

measuring the expression levels of the proteasom–related genes, using primers against NF-κB-p65, 20S core particle subunit α2, USP19 and ubiquitin. In paper III the same was done but with primers for the autophagy pathway; LC3BII, Vps34, Cathepsin L and Beclin. GAPDH was used as a control. In addition a proteasome activity test was conducted (paper I) in myoblasts, myotubes and also in fibroblasts from a MDC1A patient and a control subject. In short, the 20S proteasome activity was determined using a fluorometry-based microplate assay that detects chymotrypsin-like activity, and results were obtained using a fluorescence plate-reader.

Results of the present studies

Paper I

Carmignac et al. had previously showed an upregulation of proteasome-related genes and proteins in dy^{3K}/dy^{3K} mouse muscle and that the dystrophic phenotype was significantly improved when using the proteasome inhibitor MG-132 [161]. However, MG-132 is just a lab bench drug and not used clinically. Moreover, it is not an inhibitor of just the proteasome system but also the calpain pathway.

Therefore, I wanted to test the effects of a clinically relevant proteasome inhibitor. Bortezomib was identified in 1995 by Myogenics Company and is the first proteasome inhibitor towards malignant disease approved by the FDA. Today it is used alone or in combination against relapsed/refractory multiple myeloma and mantle cell lymphoma. In cancer treatment it works as a proapoptotic drug resulting in apoptotic cell death in malignant cells [185]. When we started the study we administrated mice using the same dosage that was used in a study on *mdx* mice with good results [186]. However, the dy^{3K}/dy^{3K} mice died when receiving bortezomib at 0.8 mg/kg. Therefore, we systemically delivered bortezomib at 0.4 mg/kg instead (two times). Mice were analysed at 5.5 weeks of age, a time-point when dy^{3K}/dy^{3K} mice should be dead. Quadriceps muscles from treated dy^{3K}/dy^{3K} mice exhibited increased fiber-cross sectional area with a shift to WT levels and a reduction of apoptosis and fibrosis. Some improvement was also seen in diaphragm. The mice also exhibited a better overall health status with increased locomotional activity and survival. However, bortezomib had no effect on the peripheral neuropathy. We also analysed the expression of two muscle specific miRNAs; miR-1 and miR-133a, which was shown to be downregulated in both quadriceps muscles from dy^{3K}/dy^{3K} mice and in myoblasts from the MDC1A patient. Importantly, the miRNA levels were partially normalized in bortezomib-injected dy^{3K}/dy^{3K} mice.

Cell culture analyses showed that there is an upregulation of ubiquitin-proteasome-related genes and proteasome activity also in human MDC1A myoblasts and myotubes. After bortezomib administration there was a reduction of the proteasome activity in myogenic cells.

We also analysed the expression of ubiquitin-proteasome related genes in quadriceps muscle from other mouse models than the dy^{3K}/dy^{3K}. The results showed, as previously described, that there is an upregulation of proteasome-related genes in 3.5-week-old dy^{3K}/dy^{3K} quadriceps muscle. We also noted a similar increase in the milder mouse model dy^{2J}/dy^{2J}. The results also showed that the upregulation seems just to be a feature of laminin α2 chain-deficiency, since no upregulation was seen in *mdx*, $Sgcb^{-/-}$ or $Itga7^{-/-}$ quadriceps muscle.

Results obtained in paper I confirm the beneficial effects of a proteasome inhibitor in the dy^{3K}/dy^{3K}mouse model. We not only show beneficial effects on the muscular dystrophy hallmarks but also an increased lifespan and locomotional activity. Furthermore, we also demonstrated that the upregulation of ubiquitin-proteasome related genes is a feature of laminin α2 chain-deficiency only.

Paper II

After we obtained beneficial results with bortezomib in the dy^{3K}/dy^{3K} mice, we wanted to see if the same results could be obtained in dy^{2J}/dy^{2J} animals. Furthermore, in paper I we also showed that there is an upregulation of proteasome-related genes in the dy^{2J}/dy^{2J} muscle, indicating that proteasome inhibition could also be advantageous in the milder mouse model. First, we treated dy^{2J}/dy^{2J} mice twice with the same dosage as for dy^{3K}/dy^{3K} mice. However, there were no beneficial results in dy^{2J}/dy^{2J} mice after this treatment. Therefore, we decided to prolong treatment and treating mice six times instead (one week apart). When doing the treatment with six injections the bortezomib concentration was gradually decreased in order to avoid potential adverse effects. Yet, this strategy had shown positive results in mice with collagen-induced arthritis (another musculoskeletal disorder) [187].

However, no improvement of the histological hallmarks of the disease (such as fiber size distribution and fibrosis infiltration) was seen in quadriceps or triceps muscles of dy^{2J}/dy^{2J} mice after six injections. No increased locomotive activity or muscle strength were noted either. Instead, we detected an adverse effect of bortezomib in male dy^{2J}/dy^{2J} mice as they exhibited a weight loss of about 10% compared to WT mice.

The expression of two muscle specific miRNAs in plasma was shown to be upregulated in dy^{2J}/dy^{2J} as well as in dy^{3K}/dy^{3K} mice. Notably, bortezomib administration downregulated plasma expression of miR-1 and miR-133a in dy^{3K}/dy^{3K} mice [88]. However, bortezomib had no effect on plasma levels of miR-1 and miR-133a in dy^{2J}/dy^{2J}mice.

Paper I and II show that the beneficial effects of proteasome inhibitor bortezomib are only seen in a mouse model of complete laminin α2 chain-deficiency but not in a mouse model with partial deficiency.

Paper III

In paper III, we tested the hypothesis that the autophagy/lysosomal pathway is upregulated in dy^{3K}/dy^{3K} muscle, as we previously had shown that the proteasome system is enhanced [161]. Indeed, we showed that the autophagy/lysosomal pathway was not only upregulated in the dy^{3K}/dy^{3K} muscle but also in myotubes from a MDC1A fetus. Interestingly, there was no upregulation in quadriceps muscle from $dy^{3K}\delta E3$ or *mdx* mice. These results indicate that the dystroglycan connection may not be involved in the downstream autophagic machinery.

Thereafter, we showed that the disease in dy^{3K}/dy^{3K} mice can be ameliorated using an autophagy inhibitor, 3-methyladenine (3-MA). The small molecule 3-MA inhibits Vps34 activity (by binding to the ATP-binding pocket of Vps34) and autophagosome formation [188]. The mice were again analyzed at 5.5 weeks of age, a time-point when dy^{3K}/dy^{3K} mice should be dead. The results included: restored expression of autophagy-related genes to the basal level of WT, reduction of fibrosis and apoptosis, increased average fiber diameter and improved regenerative capacity (all in quadriceps muscle). The mice also exhibited a better overall health status with increased locomotion and enhanced survival. However, the 3-MA injections did not improve the peripheral neuropathy.

Finally, we tested a combinational treatment with 3-MA and MG-132 in order to inhibit the autophagy/lysosomal pathway and the proteasome system at the same time, hoping to see even better results. However, this was not the case. There was a reduction of tenascin-C but no additional reduction in fibrosis compared with therapy with either drug alone. These results indicated that both pathways intersect in the dy^{3K}/dy^{3K} muscle.

With paper III we show that the dy^{3K}/dy^{3K} mouse muscle exhibits an upregulation of the autophagy/lysosomal pathway and that the inhibition of this pathway results in amelioration of the disease. In addition, we also demonstrate that the autophagy/lysosomal pathway and the proteasome system intersect in the dy^{3K}/dy^{3K} muscle.

Paper IV

In Paper IV, we demonstrated that the disease progression in the dy^{3K}/dy^{3K}mouse model starts already at postnatal day 1. In 18-day-old embryonic muscles, we could not detect any histological alterations or signs of inflammation and apoptosis. At day 1 after birth an increase of apoptosis and a diffuse increase of inflammation were observed but not any histological changes of muscle fibers. In 4-day-old dy^{3K}/dy^{3K} muscle, we could observe clear signs of apoptosis and inflammation. The histological alterations, such as centrally located nuclei and fiber-size variation, were not seen until day 7. One-week-old dy^{3K}/dy^{3K} muscle also exhibited an increase of apoptosis, inflammation and an increased deposition of ECM proteins meaning increased fibrosis in skeletal muscle. At day 14 and 21 we could see large groups of muscle fibers with centrally located nuclei, fiber size variability, increased apoptosis, inflammation and fibrosis. In WT muscle there was a gradual decrease of the analysed ECM proteins, fibronectin and collagen III. There was almost no obvious staining of fibronectin in WT muscle at day 21. However, there was some staining of collagen III in the perimysium at this age.

One other feature of the disease in dy^{3K}/dy^{3K} mice is the pronounced decrease of weight. In paper IV we observed that the dy^{3K}/dy^{3K} mice start to be significantly smaller at 1 week of age, which is around one week before we can distinguish the KO mice from their WT littermates just by the eye.

These results imply that the pre-clinical treatment of dy^{3K}/dy^{3K} mice should start as early as possible, preferably within the first week of life.

Discussion and future perspectives

The main focus of this thesis has been MDC1A and finding a treatment to give to patients suffering from this disease.

The MG-132 study in dy^{3K}/dy^{3K} mice formed the basis for my thesis. MG-132 is just a lab-bench drug and not suitable as a therapeutic compound in humans and it also inhibits the calpain system [189]. Therefore, we wanted to test a more suitable proteasome inhibitor and we chose bortezomib, an already FDA approved drug used for patients with relapsed multiple myeloma and mantle cell lymphoma. Bortezomib had also already showed positive results in the *mdx* mice and in cultured biopsies from DMD patients with increased membrane localization of DGC components [186, 190]. It also showed positive results in *GRMD* dogs, a dog model for Duchenne muscular dystrophy, even after already apparent clinical signs of muscular dystrophy [191]. Taken into account, all these positive results of bortezomib administration in *mdx*, *GRMD* and DMD patient cells and adding our results of the increased gene expression of the proteasome-related genes, we hypothesized that bortezomib treatment of laminin α2 chain-deficient mice would be beneficial. Certainly, bortezomib partially reduced laminin α2 chain-deficient muscular dystrophy. Moreover, bortezomib has also been shown to restore the biological function of a mutated dysferlin in human limb girdle muscular dystrophy 2B muscle cells [192, 193]. Thus, bortezomib treatment may be beneficial in many types of muscular dystrophy, although the underlying reason for its positive effects may be different in the different muscular dystrophies.

The gene expression analysis in *mdx* muscle, revealed no upregulation of major proteasome-related genes (paper I). It has, however, been shown before that there is an upregulation NF-κB-p65 and that reduction of the NF-κB signaling improves muscle pathology in *mdx* mice [194]. One of bortezomib's functions is to suppress the NF-κB signaling pathway [185], which probably partly explains the positive results in the *mdx*, *GRMD* and DMD patient cells. Elevated levels of NF-κB signaling have also been demonstrated in dy^{W}/dy^{W} mice [131] but whether bortezomib reduced NF-κB in dy^{3K}/dy^{3K} muscle remains to be elucidated.

The results obtained in paper I show that bortezomib partly ameliorated muscular dystrophy in dy^{3K}/dy^{3K}mice, at least in quadriceps muscle. The same positive results were not observed in diaphragm muscle. This should be taken into account

since respiratory failure is the common cause of death of MDC1A patients [67, 81].

Proteasome inhibition is not expected to cure MDC1A, but may work as a supportive treatment. I would like to stress that bortezomib did not cause any adverse side effects in treated mice, at least not after two injections. Bortezomib is for cancer treatment used as a proapoptotic drug [185], but at the low dosage and few injections that we administrated in mice it probably only works as a proteasome inhibitor, since we did not see an upregulation of apoptosis in the treated mice. In contrast, we actually found reduced apoptosis.

One of the main questions is still: why does proteasome inhibition work in dy^{3K}/dy^{3K}mice and what proteins are prevented from degradation in muscle? Laminin α2 chain-deficiency leads to dysregulated expression of ECM proteins and laminin-211 receptors. For example, the expression of laminin β2 chain was reconstituted in the basement membrane after proteasome inhibition and therefore it is possible that laminin β2 chain is degraded in the proteasome when laminin α2 chain is absent [49, 51, 114, 115, 127-132, 161]. However, a majority of the proteins that are destroyed by the proteasome in laminin α2 chain-deficient muscle cells are most likely part of the common atrophy program and probably include actin and myosin heavy chain [195-197]. Still, the substrates of the UPS in laminin α2 chain-deficient muscle remain to be identified.

Despite the positive results we obtained in dy^{3K}/dy^{3K} mice, we did not see any beneficial effects of bortezomib in dy^{2J}/dy^{2J} mice. This can depend on that the muscle atrophy is not as pronounced in the dy^{2J}/dy^{2J} mice since the body weight is only slightly decreased, which is in sharp contrast to dy^{3K}/dy^{3K} mice. Also, the expression of proteasome-related genes is only increased 1.5-2-fold in the dy^{2J}/dy^{2J} muscle, while it is more than 10-fold enhanced in the dy^{3K}/dy^{3K} muscle. Another possibility is that the dy^{2J}/dy^{2J} mice may require a different dosing of bortezomib. We started with a concentration of 0.8 kg/mg for both $dy^{3K}/dy^{3K}$and dy^{2J}/dy^{2J} mice since this was the concentration previously administered to *mdx* mice [186]. However, this concentration was devastating for both $dy^{3K}/dy^{3K}$and dy^{2J}/dy^{2J} mice resulting in the death of sick mice shortly after injections at 2.5 weeks of age. Normally, dy^{2J}/dy^{2J} mice have a near normal lifespan. Therefore, we injected the same concentration that we gave the dy^{3K}/dy^{3K}mice in paper I; 0.4 mg/kg, followed by gradually lower doses. This leads to the thought that maybe we would have found beneficial effects between 0.5–0.7 mg/kg for the dy^{2J}/dy^{2J} mice but this seems somehow unlikely. In addition, we did observe an adverse effect in injected dy^{2J}/dy^{2J} WT male mice with a 10% decrease of weight after six doses.

Since we found an upregulation of UPS in the dy^{3K}/dy^{3K} model, we reasoned that also the other highly conserved protein degradation pathway, the autophagic/lysosomal pathway, might be dysregulated in laminin α2 chain-

deficient muscle. Indeed, the results in paper III showed that autophagy is also upregulated in both dy^{3K}/dy^{3K} muscle and myogenic cells from MDC1A patients. As a proof of concept, we tested inhibition of the pathway with 3-MA, which reduced several of the pathological symptoms of dystrophic mice. 3-MA is class III PI3K inhibitor that inhibits Vps34 activity and autophagosome formation [188]. It can probably not be used clinically and therefore new autophagy inhibitors must be developed for future therapeutic use.

Laminin α2 chain-deficiency results in upregulation of three degradation pathways; the apoptotic, the UPS and the autophagic/lysosomal pathway. To analyse the cross-talk between the UPS and the autophagic/lysosomal pathway, we did a combinational treatment with MG-132 and 3-MA in paper III. The results showed that the combinational therapy was not more beneficial for the dy^{3K}/dy^{3K} mice than either drug alone. It has been suggested that there is a crosstalk between the UPS and autophagic-lysosomal pathway, since ubiquitinated proteins can be delivered to the autophagosomes [198-200] and the results in paper III indicate that the two systems intersect, at least in dy^{3K}/dy^{3K} muscle.

How can loss of one ECM protein result in such UPS and autophagy dysregulation? The laminin α2 chain does not only provide support and anchorage between the ECM and the muscle cell but it probably also works as a signal transducer through both DGC and integrin α7β1. Not much is known about the laminin α2 chain-induced signaling pathways in skeletal muscle [76], especially the downstream targets from dystroglycan and integrin α7β1 remain elusive. Both UPS and autophagy are suppressed by Akt activation and Akt phosphorylation was normalised with 3-MA or MG-132 administration [161] (paper I). So which is the crucial cell surface receptor involved in the proteasomal breakdown and autophagy in laminin α2 chain-deficient muscle? The absence of laminin α2 chain causes a secondary loss of integrin α7 chain [128] and maybe this leads to the reduction of Akt phosphorylation and activation of the degradation pathways. Indeed, it has been demonstrated that integrin α7 is activated in response to ECM attachment, resulting in phosphorylation of Akt in skeletal muscle [201]. Also, disruption of dystroglycan-laminin interactions, *in vitro*, resulted in decreased phosphorylation of Akt [202], indicating that dystroglycan may also be involved. However, the expression of proteasome-related genes was not altered in integrin α7-deficient mouse muscle or in $dy^{3K}\delta E3$ muscle (expressing a laminin chain without the dystroglycan binding site), suggesting that neither integrin α7 nor dystroglycan is involved in the downstream proteasomal breakdown (paper I). Similarly, the expression of autophagy-related genes was not altered in $dy^{3K}\delta E3$ muscle (paper III). Maybe there is a third, yet unidentified, laminin-211 receptor that without laminin-211 binding either gets degraded directly in the proteasome or upon loss of laminin-211 binding starts a signaling cascade that leads to upregulation of the proteasome. The third unknown receptor may also work as a

stabiliser of the cell membrane and without proper binding the cell membrane gets permeable and causes an overactivated proteolytic enzymatic cascade.

Altogether, since laminin α2 chain-deficiency seems to cause many different secondary effects, combinational therapy is probably what will be more relevant as MDC1A treatment. There have been two combinational therapies tested with additive positive results in the dy^W/dy^W mouse model. The first one was overexpression of mini-agrin combined with apoptosis inhibition. The apoptosis was either inhibited by the overexpression of the anti-apoptotic protein Bcl-2 or by administration of the apoptosis inhibitor omigapil. Notably, the combination resulted in additive benefits of the disease progression in the dy^W/dy^W mice [203]. The second approach was knocking-out the proapoptotic protein Bax combined with an activation of regeneration. The regeneration was activated either by overexpression of the insulin-like growth factor-1 (mIGF-1) under a muscle specific promoter or by systemic delivery of recombinant human IGF-1. This also resulted in an additive benefit in the dy^W/dy^W mice [204].

Nonetheless, gene therapy is still not applicable in human patients. Therefore, other combinational therapies should be analysed. One very interesting approach would be the combination of apoptotic inhibition and either autophagy or UPS inhibition. It has already been shown that apoptosis and autophagy share many regulatory proteins (e.g. Atg5, BCL-2 proteins and mTOR) and that they can act in a coordinated way to induce cell death and autophagy can both counteract or facilitate apoptosis [205].

To this date there has not been a pharmacological focus on peripheral neuropathy, so future therapies should take peripheral nerve into consideration. Improvement of peripheral neuropathy in a mouse model was first demonstrated by transgenic expression of laminin α1 chain [141] and later Girgenrath and co-workers demonstrated that doxycycline treated mice displayed delayed onset of hindlimb paralysis [150].

Conclusions

The main findings of my thesis are as follows:

- The UPS is upregulated in mouse skeletal muscle with complete and partial laminin α2 chain-deficiency, but not in muscles from other muscular dystrophy models.
- Proteasome activity is enhanced in human myogenic cells.
- The proteasome inhibitor bortezomib ameliorates the disease in mice with complete laminin α2 chain-deficiency, but not in mice with partial deficiency.
- Laminin α2 chain-deficient muscle also exhibits an upregulation of genes coupled to autophagy. In addition, autophagy inhibition reduces muscular dystrophy symptoms in dy^{3K}/dy^{3K} mice.
- The upregulation of UPS and autophagy may not depend on any of the two laminin-211 receptors dystroglycan and integrin α7β1.
- Combinatorial therapy with proteasome and autophagy inhibition does not work better than single inhibition, probably because the pathways intersect in laminin α2 chain-deficient mouse muscle.
- The complete laminin α2 chain-deficient mouse model exhibits disease hallmarks already at day one, implying that pre-clinical treatment should start as early as possible.

In summary, the present studies have been focused on finding ways to pharmacologically treat MDC1A and also finding the correct time point for starting treatment. We show that both the autophagy and the proteasome protein degradation systems are upregulated in laminin α2 chain-deficient muscle and that pharmacological inhibition of both systems reduces muscular dystrophy in mice. Even though these interventions do not target the primary genetic cause and cannot in that aspect completely cure MDC1A, they did counteract some of the major pathological features. Hence, it would be worth testing bortezomib as a supportive therapy in MDC1A patients completely lacking laminin α2 chain and perhaps to combine it with the apoptosis inhibitor omigapil. Moreover, new clinically relevant autophagy inhibitors should be developed. Finally, pre-clinical interventions in mice aimed at reducing the dystrophic pathology should start as early as possible.

Sammanfattning på svenska

Kongenital muskeldystrofi med lamininbrist: Sjukdomsinitiering och utveckling av behandling

Skelettmuskler, extracellulär matrix och lamininer

Skelettmuskler är ett av våra viktigaste organ och finns hos alla arter av djur från små insekter till stora valar. Det är kroppens största organ och utgör upp till 40 % av människans totala kroppsvikt. Våra muskler är uppbyggda av en massa muskelbuntar där varje muskelbunt innehåller ett stort antal muskelceller, även kallade muskelfibrer. Alla våra vävnader, inklusive muskler, består av celler samt en extracellulär matrix, som består av ett nätverk av specialiserade proteiner. Varje muskelfiber omges av en extracellulär matrix som kallas basalmembran (BM). Detta membran innehåller flera olika proteiner som är viktiga för normal muskelfunktion. Defekter i något av basalmembranets proteiner resulterar i olika muskelsjukdomar. Detta visar hur viktigt det är med ett fungerade BM. Lamininer är en av de viktigaste familjerna av proteiner i BM. Än så länge har man upptäckt 16 olika lamininer. De är alla heterotrimerer vilket betyder att de sammansatta av 3 enheter, en α, en β och en γ kedja. Lamininerna förankrar inte bara muskelcellerna utan de sänder även signaler in i muskelcellen. Signalerna hjälper cellen att veta vad den ska göra eller vad den ska bli - om den ska dela sig, utveckla sig, migrera eller dö. I muskler är det framför allt lamininer med en α2 kedja som uttrycks. Mutationer i genen som kodar för laminin α2 kedjan resulterar i kongenital (medfödd) muskeldystrofi typ 1A (MDC1A).

Muskeldystrofi, MDC1A

Muskeldystrofi är ett samlingsnamn för en grupp nedärvda sjukdomar som främst drabbar musklerna. Musklerna förtvinar som ett resultat av att muskelfibrerna förstörs på olika sätt. Det finns flera typer av muskeldystrofi och många orsakas av mutationer i olika gener som oftast kodar för proteiner som finns i eller är associerade med BM. Än så länge har man identifierat mer än 30 olika sådana

gener. De olika mutationerna resulterar i olika typer av muskeldystrofi och har alla varierande symptom. Många av dessa sjukdomar leder till döden och än så länge finns inget botemedel för muskeldystrofi.

MDC1A än en väldigt ovanlig men allvarlig muskeldystrofi som drabbar nyfödda barn och som blir gradvis värre med åldern. Patienternas symptom karakteriseras av muskelsvaghet, muskelslapphet, nedbrytning av muskelvävnad samt problem med de perifera nervsignalerna mellan nerver i centrala nervsystemet och muskler. Många patienter utvecklar även defekter i hjärnan. Det flesta barn med MDC1A lär sig aldrig att gå och får andningssvårigheter. Cirka 30 % av de drabbade överlever aldrig tonåren.

Det är än så länge inte helt förstått varför avsaknaden av laminin α2 kedjan leder till muskeldystrofi. En förklararing kan vara att muskelcellerna dör då förbindelsen mellan den extracellulära matrixen och muskelcellen försvinner när laminin α2 kedjan saknas. För att studera laminin α2 avsaknad så används mest musmodeller. Det finns många olika musmodeller som alla speglar olika nivåer av laminin α2 avsaknad. De går från att helt sakna laminin α2 kedjan till att producera en felaktig laminin α2 kedja. När det gäller musmodellerna så vet man inte riktigt när och vilka sjukdomsprocesser som aktiveras i tidigt stadium. Detta hade varit bra att känna till för att vi ska veta vilka sjukdomsmekanismer vi ska rikta vår forskning och utveckling av behandling emot.

Syfte

Just nu finns det ingen behandling att ge patienter med MDC1A. Det har gjorts en del lovande studier i de olika musmodellerna som visar att sjukdomen kan lindras i möss. Bland annat har man lyckats återställa förbindelsen mellan ECM och muskelcellen. Denna metod är dock svårt att tillämpa kliniskt. Därför har jag i mitt avhandlingsarbete valt att fokusera på läkemedelsbehandling.

Vår forskningsgrupp har i en studie tidigare visat att det sker en oönskad nedskrotning av proteiner i muskelcellen då laminin α2 kedjan saknas. Detta äger rum i proteasomen, som är en slags destruktionsanläggning i cellen. Om man ger möss som saknar laminin α2 kedjan ett preparat som förhindrar den oönskade proteinnedbrytningen så förbättras mössens muskler avsevärt. Detta preparat kan dock inte användas på människor. Därför ville jag testa ett godkänt läkemedel, bortezomib, på möss. Bortezomib förhindrar också proteindestruktion i proteasomen och används idag för behandling av blodcancer. Jag har även studerat autofagi i MDC1A. Detta är en annan process som innebär nedbrytning av cellkomponenter. Om både proteasomnedbrytning och autofagi är uppreglerade leder detta till muskelatrofi och MDC1A karakteriseras just av förlust av muskelmassa.

Resultat

Vi har utfört studier på olika musmodeller och speciellt på den som helt saknar laminin α2 kedjan.

Behandling med bortezomib ledde till väldigt positiva resultat i möss som helt saknar laminin α2 kedjan. Behandlade möss uppvisar förbättrad muskulatur, de rör sig mer och har även ökad livslängd. Bortezomib visar även positiva resultat i muskelceller från MDC1A patienter. Däremot såg vi inte samma goda effekt i en musmodell som fortfarande uttrycker lite laminin α2 kedja.

Vi har visat att många autofagigener är uppreglerade i mössen vid 3,5 veckors ålder. Vi har även demonstrerat att autofagiinhibering leder till att mössen får förbättrad muskulatur, rör sig mer och dessutom lever längre jämfört med sjuka möss som inte blivit behandlade. Däremot ser vi ingen ökad positiv effekt om man förhindrar både proteasom- och autofagiprocesserna samtidigt i mössen.

I musmodellen som helt saknar laminin α2 kedjan fann vi att sjukdomsförändringarna startar i skelettmusklerna redan en dag efter födelsen med en uppreglering av apoptos (programmerad celldöd). Vid dag fyra såg vi en uppreglering av inflammation. Detta visar att muskler som saknar α2 kedjan är påverkade i ett mycket tidigt skede.

Slutsats

Det finns idag ingen bot för MDC1A patienter. Mina studier visar att separata behandlingar med bortezomib och en autofagi-inhibitor har goda effekter i möss. Därför föreslår jag att användning av preparat som förhindrar antingen proteasomnedbrytning eller autofagiprocessen skulle kunna hjälpa MDC1A patienter som helt saknar laminin α2 kedjan. Jag hoppas att våra studier kan ligga till grund för utveckling av kliniskt relevanta autofagi-inhibitorer och att kliniska prövningar med bortezomib startas. Vidare tyder våra data på att behandlingar bör sättas in så tidigt som möjligt med tanke på att vi detekterade sjukdomsförändringar redan en till fyra dagar efter födseln i möss.

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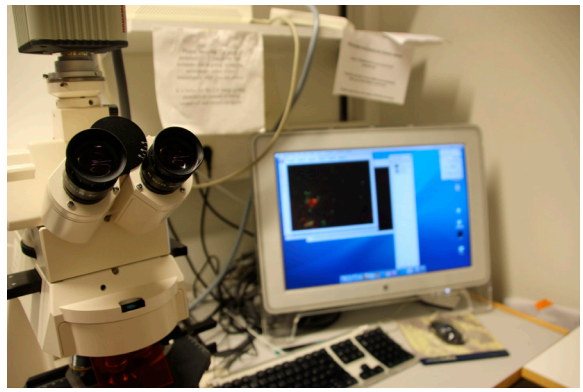

Partners in crime

Without these two there would not be any thesis or any research for that matter. The upper picture shows the cryostat where all the tissues were sectioned before staining procedures. The lower picture shows the fluorescence microscope where the sections stained with antibodies were analyzed.

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