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**Artificial restoration of the linkage between laminin and
dystroglycan ameliorates the disease progression of
MDC1A muscular dystrophy at all stages**

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TABLE OF CONTENTS

TABLE OF CONTENTS	1
FIGURES and TABLES	4
LIST OF ABBREVIATIONS	5
SUMMARY	6
I GENERAL INTRODUCTION	7
1 Adult Skeletal Muscle Characteristics	7
1.1 Structure and functional units of skeletal muscle	7
1.2 Sources of Energy	8
1.3 Molecular Basis of Force Transduction	8
1.3.1 The "actin-myosin sliding mechanism".....	8
1.3.2 From Activation to Contraction.....	8
1.3.3 Formation of the Neuromuscular Junction (NMJ)	9
2 Muscle Integrity: The Major Proteins in Skeletal Muscle	10
2.1 The Extracellular Matrix	10
2.1.1 The Basal Lamina.....	10
a. Laminins.....	11
b. Collagen.....	14
c. Nidogen	15
d. Heparan Sulfate Proteoglycans (HSPG).....	15
Agrin.....	15
Perlecan	16
2.2 Membrane-associated and Cytoplasmic Proteins	16
2.2.1 The Dystrophin-Glycoprotein Complex (DGC).....	16
a. Dystrophin.....	16
b. Dystroglycan	17
c. The Sarcoglycan-Complex and Sarcospan.....	17
d. Syntrophins and Dystrobrevin	18
2.2.2 Integrins.....	18
3 Laminin and its Receptors in the Peripheral Nervous System	19
4 Development of Skeletal Muscle: A Short Overview	20
5 Regeneration of Skeletal Muscle	21
5.1 The Regeneration Process	21
5.2 Satellite cells	22
5.2.1 Localization and Identification of Satellite Cells	22
5.2.2 Activation, Proliferation, Migration and Differentiation of Satellite Cells	22

5.2.3	The Satellite Cell Pool	23
6	Muscular Dystrophies	23
6.1	Congenital Muscular Dystrophies.....	25
6.1.1	Laminin α 2-deficient Congenital Muscular Dystrophy (MDC1A).....	26
a.	Molecular and Cellular Basis of MDC1A	26
b.	Mouse Models of MDC1A	27
c.	Clinical and Pathological Features of MDC1A	28
6.2	Strategies for Treatment of Muscular Dystrophies	28
6.2.1	Pharmacological Approaches	29
6.2.2	Gene Therapy.....	29
<i>In vivo</i>	Somatic Viral-based Gene therapy	30
II	THE TOPIC OF THE THESIS	32
1	Introduction.....	32
2	Aim of the Thesis.....	34
2.1	Evaluation of the potential of mini-agrin to slow down the disease progression of MDC1A muscular dystrophy when applied after onset of the disease.....	34
2.2	Evaluation of the Feasibility of Gene Therapy to Deliver Mini-agrin into the Diseased Muscles of MDC1A mice	35
2.3	Evaluation of the Potential of Full-length Muscle Agrin to Diminish the Disease Progression in MDC1A.....	35
III	MATERIALS AND METHODS.....	36
	Generation of the constructs	36
	Protein production	36
	Affinity binding assays.....	36
	Overlay assays.....	36
	Generation and genotyping of transgenic and dystrophic mice.....	37
	Breeding.....	37
	Transgene expression.....	37
	Regulation of the tet-off system.....	38
	Locomotion, muscle strength and creatine kinase assay	38
	Histology, immunohistochemistry and antibodies.....	38
	Quantification of immunostainings.....	39
	Muscle regeneration.....	39
	Intravenous injection of recombinant mini-agrin protein	40
	Generation of adenovirus (AdV).....	40
	In situ infection of triceps brachii	41
IV	RESULTS	42
1	Therapeutic Potential of Mini-agrin in a Mouse Model for MDC1A at All Stages of the Disease	42
a.	Cloning of the cDNA encoding the mouse mini-agrin-myc (m-mag)	42
b.	Testing of the function of the recombinant m-mag protein	44

c.	Generation of mice expressing m-mag under control of the inducible tetracycline-regulated "tet-off" expression system.....	44
d.	Tight spatial and temporal regulation of mouse mini-agrin-myc expression in $dy^{W/-}$ mice	47
e.	The disease progression is slowed down upon start of mini-agrin expression after birth.....	51
Physiological analysis	51	
Histological analysis	52	
Restoration of the regenerative capacity	54	
f.	Stability of mini-agrin at the muscle fiber membrane	56
g.	A preliminary attempt of a systemic mini-agrin protein application.....	58
2	Gene Therapy as a Means to deliver Mini-agrin into the diseased Muscles of MDC1A mice.....	59
a.	Generation of high titer stocks of adenoviral (AdV) vectors expressing mouse mini-agrin-myc (m-mag).....	59
b.	Infection of $dy^{W/-}$ muscle <i>in situ</i>	62
3	Full-length muscle agrin ameliorates the dystrophic phenotype in $dy^{W/-}$ mice	63
a.	Generation of $dy^{W/-}$ mice constitutively expressing chick full-length agrin in skeletal muscles	63
b.	Phenotype analysis of $dy^{W/-}/c$ -FLag mice	66
V	DISCUSSION AND PERSPECTIVES.....	67
1	Discussion.....	67
a.	Artificial restoration of the linkage between laminin and dystroglycan ameliorates the disease progression of MDC1A muscular dystrophy at all stages	67
Tight regulation of the "tet-off" system enables reliable evaluation	67	
Mini-agrin decreases the degeneration of intact muscle tissue of $dy^{W/-}$ mice	68	
Mini-agrin increases the regeneration capacity of intact muscle tissue in $dy^{W/-}$ mice	68	
Limitations of a mini-agrin-based treatment of MDC1A.....	69	
b.	Approaches to agrin-based application for the treatment of MDC1A	70
Advantages of mini-agrin for use in gene therapy of MDC1A.....	70	
Delivery of mini-agrin protein to the skeletal muscles.....	70	
Gene therapy as a means to introduce mini-agrin into the diseased skeletal muscles of $dy^{W/-}$ mice	71	
Upregulation of endogenous agrin expression	73	
2	Conclusion	74
3	Future Perspectives	75
VI	REFERENCES.....	76
VII	ACKNOWLEDGMENTS	85
VIII	APPENDIX I: Curriculum Vitae	86
IX	APPENDIX II: Publications	89

FIGURES and TABLES

I GENERAL INTRODUCTION

Figure	Description	Page
1	Structure and isoform-specific interactions mediated by laminins	12
2	Interactions of laminin-2	14
3	Overview of muscular dystrophies and their corresponding animal models	24
Table 1	Congenital muscular dystrophies and their mutated genes	25

II TOPIC OF THE THESIS

Figure	Description	Page
1	Innervated muscle fiber in wild-type-, MDC1A-, and MDC1A-mice treated with mini-agrin and potential mechanism involved in disease progression and treatment	33

IV RESULTS

Figure	Description	Page
1	Schematic representation of the mouse mini-agrin-myc construct and its orthologue chick mini-agrin	42
2	Affinity binding assays	43
3	The inducible tetracycline-regulated “tet-off” expression system	45
4	Analysis of m-mag expression activity in MCK-tTA ^{+/+} /tet-m-mag ^{+/+} mice	46
5	Spatial expression pattern of m-mag in the MCK-tTA ^{+/+} /tet-m-mag ^{+/+} mice	48
6	Temporal regulation of m-mag expression	49
7	Breeding strategy	50
8	Overall function of skeletal muscles in 4 and 6 week-old <i>dy</i> ^{W/-} mice starting mini-agrin expression 3, 14 and 28 days after birth	52
9	Phenotype analysis of <i>triceps brachii</i> cross-sections of 4 and 6 week-old <i>dy</i> ^{W/-} mice starting mini-agrin expression 3, 14 or 28 days after birth	53
10	Posttranslational stabilization of laminin- α 5 and α -dystroglycan and regeneration capacity after injury	55
11	Stability of the mini-agrin protein in skeletal muscle after temporal expression	57
12	Detection of m-mag in <i>triceps brachii</i> cross-sections 24 hours after intravenous injection of the recombinant protein.	58
13	Generation of adenoviral (AdV) vectors expressing mouse mini-agrin-myc (m-mag)	60
14	<i>In situ</i> infection of <i>triceps brachii</i> with AdV-m-mag	61
15	Schematic representation of the full-length agrin protein and construct	63
16	Evaluation of the expression levels in the transgenic mouse lines overexpressing chick full-length agrin (c-FLAG) in skeletal muscles	64
17	Phenotype analysis of 4 week-old <i>dy</i> ^{W/-} mice constitutively overexpressing chick full-length agrin (<i>dy</i> ^{W/-} /c-FLAG) in skeletal muscles	65

LIST OF ABBREVIATIONS

Repeatedly used abbreviations	
AAV	Adeno-associated virus
AdV	Adenovirus
AdV-MCK-m-mag	Recombinant adenoviral vectors carrying the mini-agrin under the control of the muscle-specific creatine kinase promoter
AgPerl	Fusion protein of the N-terminal laminin-binding part of agrin and the C-terminal α -dystroglycan-binding part of perlecan
c-FLag	Chick full-length muscle agrin
CK	Creatine kinase
c-mag	Chick mini-agrin
CMD	Congenital muscular dystrophy
CMV-promoter	Cytomegalovirus promoter
CNS	Central nervous system
DGC	Dystrophin-Glycoprotein complex
Dox	Doxycycline
Dox 1d / 2d ...	Re-application of doxycycline for <i>N</i> days
MCK-promoter	Muscle-specific creatine kinase promoter
MDC1A	Muscular dystrophy congenital type 1A (Laminin- α 2 deficient congenital muscular dystrophy; formerly: Merosin-deficient congenital muscular dystrophy)
m-mag	Mouse mini-agrin-myc
MTJ	Myotendinous junction
NMJ	Neuromuscular junction
PNS	Peripheral nervous system
tet-off system	Inducible tetracycline-regulated expression system, repressed in presence and activated in absence of Dox
tet-promoter	Tetracycline-dependent transcription activator (tta) -responsive promoter containing seven tta binding sites and a CMV-promoter localized upstream of the target gene
tTA	Tetracycline-dependent transcription activator
wd 3d / wd 6d	Withdrawal of doxycycline for 3 and 6 days, respectively

Genotypes of the mice	
$dy^{W-/-}$	Laminin- α 2 deficient mice, a mouse model for MDC1A exhibiting a severe phenotype
$dy^{W-/-}/c\text{-FLag}$	Laminin- α 2 deficient mice overexpressing chick full-length muscle agrin
$dy^{W-/-}/c\text{-mag}$	Laminin- α 2 deficient mice overexpressing chick mini-agrin
$dy^{W-/-}/MCK\text{-tTA}^{+/+}/tet\text{-m-mag}^{+/+}$	Laminin- α 2 deficient mice in which mouse mini-agrin expression in skeletal muscles can be temporally controlled
$dy^{W-/-}/m\text{-mag 3d, 14d or 28d}$	Laminin- α 2 deficient mice starting mouse mini-agrin expression at the age of 3, 14 or 28 days
$MCK\text{-tTA}^{+/+}/tet\text{-m-mag}^{+/+}$	Mice expressing mouse mini-agrin in skeletal muscles under control of the inducible "tet-off" system
$MCK\text{-tTA}^{+/+}$	Mice homozygously expressing the tTA activator transgene under control of the muscle-specific creatine kinase promoter
$tet\text{-m-mag}^{+/+}$	Mice mice containing m-mag under the control of the tet-responsive promoter

SUMMARY

Laminin- α 2 deficient congenital muscular dystrophy, classified as MDC1A, is a severe progressive muscle-wasting disease that leads to death in early childhood. MDC1A is caused by mutations in *lama2*, the gene encoding the laminin- α 2 chain being part of laminin-2, the main laminin isoform present in the extracellular matrix of muscles and peripheral nerves. Via self-polymerization, laminin-2 forms the primary laminin scaffold and binds with high affinity to α -dystroglycan on the cell surface, providing a connection to the cytoskeleton via the transmembranous protein β -dystroglycan. Deficiency in laminin- α 2 leads to absence of laminin-2 and to upregulation of laminin-8, a laminin isoform that cannot self-polymerize and does not bind to α -dystroglycan. Therefore, in laminin α 2-deficient muscle the chain of proteins linking the intracellular contractile apparatus via the plasma membrane to the extracellular matrix is interrupted. Consequently, muscle fibers lose their stability and degenerate what finally leads to a progressive muscle wasting.

In previous studies, we have shown that a miniaturized form of the extracellular matrix protein agrin, which is not related to the disease-causing *lama2* gene and was designed to contain high-affinity binding sites for the laminins and for α -dystroglycan, was sufficient to markedly improve muscle function and overall health in the *dy*^{W/-} mouse model of MDC1A. In a follow-up study we provided additional evidence that mini-agrin, both increases the tolerance to mechanical load but also improves the regeneration capacity of the dystrophic muscle.

We now report on our progress towards further testing the use of this approach for the treatment of MDC1A. To test whether mini-agrin application after onset of the disease would still ameliorate the dystrophic symptoms, we have established the inducible tetracycline-regulated “tet-off” expression system in *dy*^{W/-} mice to temporally control mini-agrin expression in skeletal muscles. We show that mini-agrin slows down the progression of the dystrophy when applied at birth or in advanced stages of the disease. However, the extent of the amelioration depends on the dystrophic condition of the muscle at the time of mini-agrin application. Thus, the earlier mini-agrin is applied, the higher is the profit of its beneficial properties.

In addition to gene therapeutical approaches, the increase of endogenous agrin expression levels in skeletal muscles by pharmacologically active compounds would be a safe and promising strategy for the treatment of MDC1A. To evaluate the potential and pave the way to further expand on the development of such a treatment, we determined whether full-length agrin ameliorates the dystrophic phenotype to a comparable extent as it was observed by application of mini-agrin. We provide evidence that constitutive overexpression of chick full-length agrin in *dy*^{W/-} muscle ameliorates the dystrophic phenotype, although not as pronounced as mini-agrin does.

In conclusion, our results are conceptual proof that linkage of laminin to the muscle fiber membrane is a means to treat MDC1A at any stage of the disease. Our findings definitely encourage to further expanding on this therapeutic concept, especially in combination with treatment using functionally different approaches. Moreover, these experiments set the basis for further developing clinically feasible and relevant application methods such as gene therapy⁴ and/or the screening of small molecules able to upregulate production of agrin in muscle.

I GENERAL INTRODUCTION

1 Adult Skeletal Muscle Characteristics

Three different kinds of muscles are found in vertebrate animals: cardiac muscle, smooth muscle and skeletal (or striated) muscle. The contraction of skeletal muscle is controlled by the nervous system and thus underlies voluntary control. In this respect, skeletal muscle differs from smooth and cardiac muscle, both able to contract without being stimulated by the nervous system.

1.1 Structure and functional units of skeletal muscle

A skeletal muscle consists of thousands of cylindrical muscle fibers, which are assembled by connective tissue and constitute the basic contractile units. The extensive connective tissue in skeletal muscles is responsible to combine the contractile myofibers into functional units (bundles or fascicles) and transform the contraction into movement. This force transduction is mediated via the myotendinous junctions (MTJ) at either end of the muscle, where myofibers attach to the skeleton by tendons.

Each muscle fiber comprises myofibrils that are aligned in parallel, contains many nuclei and mitochondria that both are located just beneath the plasma membrane and dispose of an extensive endoplasmic (sarcoplasmic) reticulum that extends between the myofibrils. Myofibrils consist of a chain of repeating units called sarcomeres, which involve two kinds of filaments arranged in parallel. The thick filaments with a diameter of ~15nm are composed of the protein myosin and the thin filaments having a diameter of ~5nm are composed of the protein actin along with smaller amounts of the proteins troponin and tropomyosin. Sarcomeres impose the striated appearance and are the force generators in skeletal muscles.

Individual adult skeletal muscles are composed of a mixture of myofibers with different physiological properties, Type I and Type II fibers, whereas a single motor unit always contains one type or the other, never both. The proportion of each fiber type within a muscle determines its overall contractile property. Type I fibers, also known as "slow-twitch" fibers, are resistant to fatigue and thus are dominant in muscles that depend on tonus, for instance those responsible for posture. They are loaded with mitochondria, depend on cellular respiration for ATP production and are rich in myoglobin and hence red in color. Type II fibers, known as "fast-twitch" fibers, fatigue easily and are dominant in muscles used for rapid movement. They contain few mitochondria, few myoglobin, but are rich in glycogen and depend on glycolysis for ATP production.

The number of muscle fibers is probably fixed early in life. In adults, muscle trophism is the balance between anabolic processes (the synthesis of contractile proteins and, to a minor extent, the proliferation and recruitment of progenitor cells) and catabolic processes (protein degradation due to calcium leakiness and the activation of proteases). Thus, increased strength and muscle mass is achieved by an increase in the thickness of the individual fibers as well as an increase of connective tissue.

1.2 Sources of Energy

The immediate source of energy for muscle contraction is ATP. Creatine phosphate is used as a storage of "high-energy" phosphate bonds and thus is produced when ATP levels are high. During exercise, creatine phosphate can donate the high-energy phosphate back to ADP to form and replenish ATP. This reversible reaction is catalyzed by the enzyme creatine kinase (CK). A limited source of ATP in skeletal muscle fibers is glycogen. Glycogen can degrade by glycogenolysis and is used to keep the muscle functioning in case it fails to receive sufficient oxygen to cover its ATP needs by respiration. Cellular respiration not only is required to cover the ATP needs of a muscle engaged in prolonged activity (thus causing more rapid and deeper breathing), but is also required afterwards to enable the body to re-synthesize glycogen from the lactic acid produced earlier.

1.3 Molecular Basis of Force Transduction

1.3.1 The "actin-myosin sliding mechanism"

Muscle contraction is produced by converting the chemical energy of ATP into mechanical work, which is executed by the sarcomeric units of the myofibrils. Shortening of the sarcomeres produces the shortening of the myofibril what in turn shortens the muscle fiber resulting in muscle contraction. Tightening of the sarcomere itself occurs by sliding of actin filaments against myosin filaments. Each molecule of myosin in the thick filaments contains a globular subunit, the myosin head, which contains binding sites for the actin molecules in the thin filaments and for ATP. Activation of the muscle fiber causes the myosin heads to bind to actin. An allosteric change occurs which draws the thin filament a short distance (~10nm) past the thick filament. Then the linkages break (for which ATP is needed) and reform farther along the thin filament to repeat the process. As a result, the filaments are pulled past each other.

1.3.2 From Activation to Contraction

Skeletal muscle cells are electrically excitable. Thus, the induction for contraction arrives as an electrical signal from the motor neuron contacting the muscle fiber at the highly specialized neuromuscular junction (NMJ). The terminals of motor axons contain thousands of vesicles filled with acetylcholine (ACh). When an action potential reaches the axon terminal, hundreds of these vesicles discharge their ACh onto the specialized area of the postsynaptic membrane containing

clusters of acetylcholine receptor channels (AChR). AChRs are 300kDa glycoproteins composed of five transmembranous polypeptides; α_2 , β , γ or ϵ , δ , together forming a cation channel. Binding of ACh molecules to the two α -subunits of each AChR, opens the channels and leads to a locally large Na^+ influx into the muscle fiber creating an end plate potential. If the end plate potential reaches the threshold voltage (~ -50 mV), sodium ions flow in with a rush and an action potential is created and propagates down the length of the fiber.

Immediately following the action potential, the enzyme acetylcholinesterase (AChE)⁵ breaks down the ACh in the synaptic cleft, the sodium channels close and the resting potential of the fiber is restored by an outflow of potassium ions. The muscle fiber is ready to receive and process a next nerve impulse.

Calcium ions (Ca^{2+}) link the action potentials in a muscle fiber to contraction. In resting muscle fibers, large quantities of Ca^{2+} are stored in the endoplasmic (sarcoplasmic) reticulum. Along the plasma membrane (sarcolemma) of the muscle fiber, invaginations of the membrane form tubules of the "T-system". These tubules plunge repeatedly into the interior of the fiber and terminate near the calcium-filled sarcoplasmic reticulum. Each action potential propagates quickly along the sarcolemma, hence is carried into the T-system and triggers the release of Ca^{2+} ions. The Ca^{2+} diffuses among the thick and thin filaments where it binds to troponin that is connected to tropomyosin located on the thin actin filaments. Ca^{2+} -binding to troponin allows the tropomyosin molecules to shift their position so that myosin heads can bind to the actin filament and the sarcomere can contract. When the process is over, the calcium is pumped back into the sarcoplasmic reticulum using a Ca^{2+} ATPase.

One muscle can be driven by many motor neurons, but each muscle fiber is innervated by a single axon terminal of a motor neuron that in turn can branch and innervate several muscle fibers.

The compound of muscle fibers innervated by the same motor neuron is called a motor unit. Although the response of a motor unit is all-or-none, the strength of the response of the entire muscle is determined by the number of motor units activated. A motor unit is small in muscles over which we have precise control. For example, a single motor neuron triggers fewer than 10 fibers in the muscles controlling eye movements, in contrast, a single motor unit for a muscle like the gastrocnemius muscle may include 1000–2000 fibers scattered uniformly through the muscle.

1.3.3 Formation of the Neuromuscular Junction (NMJ)

To ensure speed and reliability of transmission, the mature NMJ has to be highly specialized and organized. It is a morphologically complex structure formed by the presynaptic motor neuron, the Schwann cells, the interposed basal lamina and the postsynaptic muscle fiber, all tightly connected to each other. The postsynaptic muscle fiber membrane is depressed into shallow gutters, which in turn are invaginated into $1\mu\text{m}$ deep junctional folds, whose openings are directly opposite the presynaptic active zones enhancing transmission.

Formation of the neuromuscular junction depends on inductive interactions between the developing nerve and muscle^{6,7}. At the NMJ, several proteins are highly concentrated, specialized and organized compared to extrasynaptic regions. Underneath the neuromuscular synapse, aggregates of muscle nuclei selectively express mRNA encoding postsynaptic proteins such as rapsyn and AChRs^{8,9}. Moreover, highly influential postsynaptic organizers have the ability to aggregate and organize postsynaptic proteins. Nerve-derived agrin (7.4.8) turned out to play a key role in pre- and postsynaptic differentiations^{10,11-14,7,15}. During development, neural agrin (7.4.8) is synthesized by motoneurons, transported down axons and released by the motor axon terminal into the synaptic cleft. Its N-terminus binds with high affinity to all laminins present in the synaptic basal lamina^{16,17}. By interacting with a yet undefined receptor on the myotube surface, it initiates clustering of preexisting AChRs and other postsynaptic molecules under the nerve terminal. Thus, the uniformly distributed AChRs become highly concentrated in the postsynaptic membrane ($\sim 10,000/\mu\text{m}^2$ synaptically versus $<10/\mu\text{m}^2$ extrasynaptically). A cytoskeletal apparatus associated with the cytoplasmic domains of AChRs helps to generate and maintain this high AChR density at synaptic sites.

2 Muscle Integrity: The Major Proteins in Skeletal Muscle

2.1 The Extracellular Matrix

The intramuscular connective tissue (the interstitium) is extensively filled with blood vessels¹⁸⁻²⁰ what provides the contracting muscle with oxygen and substrate for energy production. It accounts for 1–10% of the skeletal muscle mass and varies quite substantially between different muscles^{21,22}. The interstitium is organized into three discrete but continuous sheets. The epimysium encircles the whole muscle, whereas the perimysium ensheats bundles of muscle fibers (fascicles) including blood vessels. The endomysium encloses each individual muscle fiber with a random arrangement of collagen fibrils and further harbors fibroblasts, mast cells and macrophages^{23,24}.

Beneath the endomysium there is a highly structured sheet, the so-called basement membrane, which considerably contributes to the structural integrity of the host tissue²⁵⁻²⁸. Basement membranes are composed of two layers. The external reticular lamina mainly accounts for muscle's elasticity based on its proteoglycan-rich ground substance embedding fibrillar collagens and elastin. The internal basal lamina (50-100nm) is directly linked to the plasma membrane and surrounds each muscle fiber, is fused to Schwann cells, passes the 50nm wide synaptic cleft and extends into junctional folds. It provides a structural and regulatory environment for the muscle fibers.

2.1.1 The Basal Lamina

Basal laminae are adhesive substrates, which provide mechanical support and harbor signalling components variously promoting proliferation, survival, differentiation and gene expression in

neighboring cells. A basal lamina is composed of several collagenous glycoproteins, non-collagenous glycoproteins, and proteoglycans. Its major constituents are collagen IV, laminins, nidogen, perlecan and fibronectin^{29,7,15}.

In the extrasynaptic basal lamina, the major structural component is the non-fibrillar collagen IV. Laminins constitute the major non-collagenous glycoproteins, while in adult muscle the predominant isoform is laminin-2 (α 2, β 1, γ 1)³⁰. Both, collagen and laminin self-assemble and form networks which are linked to each other by nidogen^{31,32,33}. This basic structure of the basal lamina provides the extracellular scaffold to which muscle fibers are tightly anchored through interactions with membrane-associated and transmembranous receptors, in turn interacting with the cytoskeleton. Most importantly, laminin-2 interacts with the dystrophin-glycoprotein complex (DGC) and both collagen IV and laminins interact with transmembranous integrins. Deficiency or dysfunction in one of the proteins implicated in the tight basal lamina-sarcolemma-cytoskeleton linkage, impairs the essential structural muscle integrity and leads to muscular dystrophy³⁴⁻³⁶.

Additionally, basal lamina components play active roles in myogenesis and synaptogenesis as well as in regenerative processes. Laminin and collagen IV provide binding sites for proteoglycans, in turn able to aggregate and present bioactive polypeptides critical for myogenesis such as fibroblast growth factors (FGF) and transforming growth factors (TGF)³⁷. Moreover, the basal lamina is supposed to orient the formation of new muscle during satellite cell-mediated regeneration. Indeed, muscles do regenerate in the absence of the basal lamina, but myotubes are not oriented in parallel what markedly affects the resulting stability and force generation.

At NMJ, the basal lamina is structurally and functionally highly specialized. Several protein isoforms are concentrated or specific for the synaptic basal lamina³⁸. It is supposed to markedly contribute to the tight adhesion at the neuromuscular junction³⁹.

a. Laminins

Laminins constitute large heterotrimeric glycoproteins of nearly 800kDa and ~100nm in length⁴⁰. At least 15 different heterotrimers are known to be formed out of 5 α , 3 β and 3 γ chains, each encoded by a different gene^{41-45,46}. Three chains, one of each subtype, bind together via their central coiled-coil domains and finally constitute a molecule with one long arm and up to three short arms⁴⁷. The α -chain of laminin extends beyond the long arm coiled-coil to form the G-domain, which typically consists of five globular modules (LG1-LG5)⁴⁸ important for interactions with cell surface binding partners.

Several laminin isoforms (laminin-1, -2, -3, -4, -10, -11, and -12) are able to self-assemble via their short arms and thus form a network to which other basal lamina constituents can bind⁴⁹⁻⁵². In contrast, laminin-6, -7, -8, -9, -14 and -15 fail to polymerize due to lack of most of the α -subunit

short arm. These truncated laminin isoforms might offer an advantage in a situation where large rearrangements in basement membrane are required as it is the case during development or regeneration processes⁵³.

Laminins are implicated in multiple interactions with other extracellular matrix or cell-surface proteins and thus play a crucial role in basal lamina integrity^{54,27,28}. The short-arm of the laminin γ 1 chain (the predominant γ -chain in muscle) is involved in nidogen binding and thus provides for the important connection of the laminin and collagen IV networks^{32,33}. Moreover, the basal lamina-proteoglycan agrin, binds strongly to the long arm of all laminin isoforms¹⁷. The G-domains of several laminin isoforms interact with β 1-integrins, heparin and heparan sulfates. Some laminin isoforms additionally bind to α -dystroglycan^{40,55,56,57}.

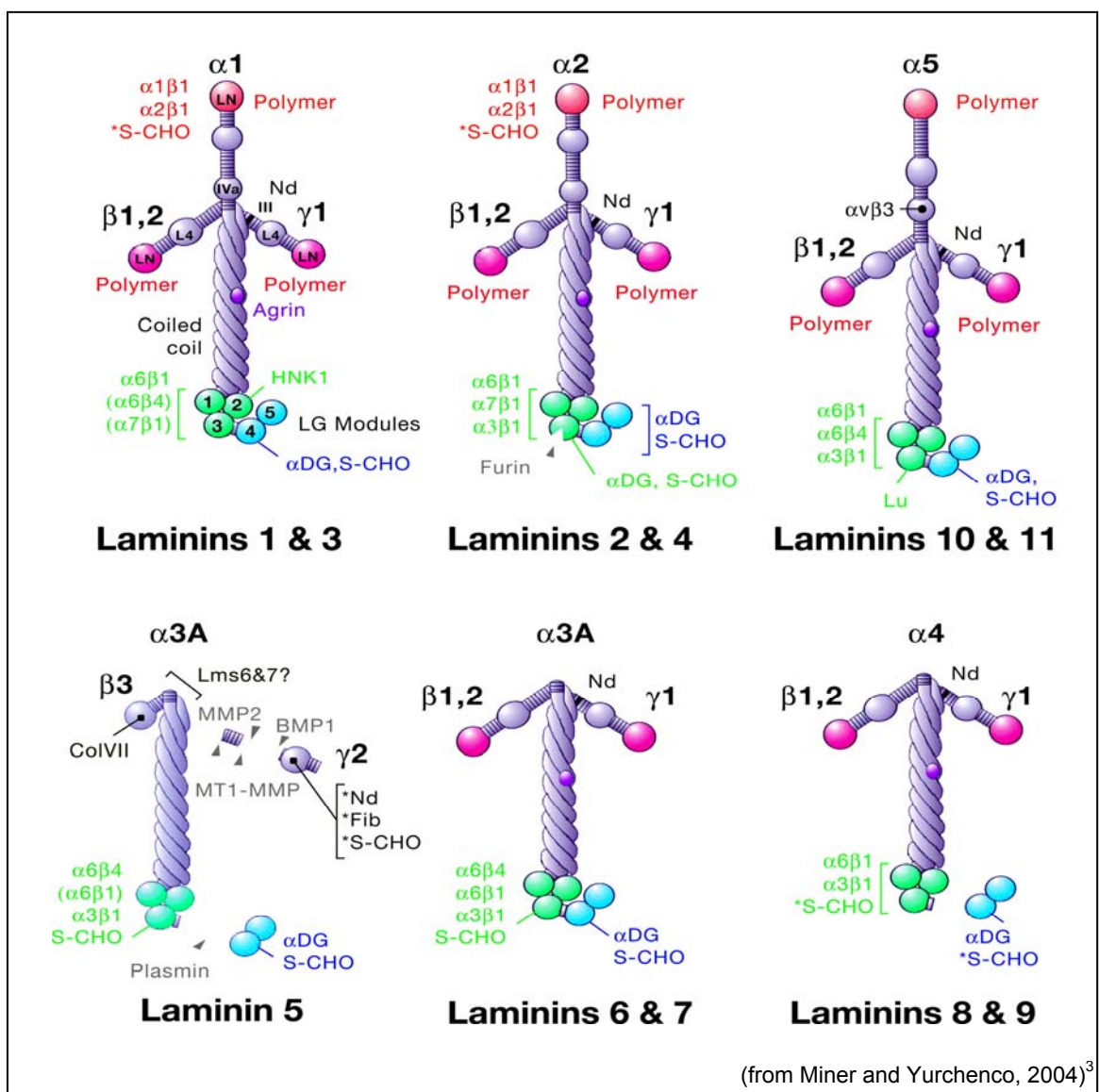


Figure 1: Structure and isoform-specific interactions mediated by laminins

Laminin isoforms have restricted distributions within the basal lamina, especially in regard to extrasynaptic and synaptic regions, what attributes highly specific functions to the different isoforms. Moreover, the expression of distinct laminin chains is tightly regulated during myogenesis, pointing to laminin's implication in myofiber formation and synaptogenesis⁵⁸⁻⁶⁰.

Initially, during embryonic development in mice, the laminin $\alpha 2$, $\alpha 4$ and $\alpha 5$ ⁶¹ chains are present in muscle basal lamina⁵³. These α -chains all assemble to heterotrimers together with the $\beta 1$ and $\gamma 1$ chains, giving rise to laminin-2 ($\alpha 2$, $\beta 1$, $\gamma 1$), laminin-8 ($\alpha 4$, $\beta 1$, $\gamma 1$) and laminin-10 ($\alpha 5$, $\beta 1$, $\gamma 1$). Postnatally, the laminin $\alpha 4$ and $\alpha 5$ chains disappear from the extrasynaptic but not from the synaptic region. Consequently, laminin-2 ($\alpha 2$, $\beta 1$, $\gamma 1$) becomes the predominant laminin isoform present in extrasynaptic basal lamina of the adult skeletal as well as in cardiac muscle and peripheral nerves^{57,62-64}.

Laminin-2 (Fig. 2) self-polymerizes to form a laminin network in the basal lamina. Moreover, the G-domain of laminin-2 (constituted by the laminin- $\alpha 2$ chain) binds to α -dystroglycan on the cell surface (LG4, LG5), as well as to the transmembranous integrin $\alpha 7 \beta 1$ (LG1-LG3), the principal integrin isoform in striated muscle⁶⁵⁻⁶⁸. Both of these laminin-2 receptors interact with the cytoskeleton and are thought to be critical to maintain normal muscle function since they control cellular activities such as adhesion or migration, differentiation and polarity, proliferation or apoptosis, and gene expression^{56,69-77}. Thus, Laminin-2 provides for the extracellular scaffold allowing the anchorage of muscle fibers to the basal lamina and at the same time serves as an extracellular ligand for membrane-associated and transmembranous receptors in turn interacting with the cytoskeleton. In conclusion, laminin-2 has an essential role in maintaining the structural integrity of the skeletal muscle.

Previously, laminin isoforms containing a laminin- $\alpha 2$ chain, namely laminin-2 ($\alpha 2$, $\beta 1$, $\gamma 1$), laminin-4 ($\alpha 2$, $\beta 2$, $\gamma 1$) and laminin-12 ($\alpha 2$, $\beta 1$, $\gamma 3$) were denoted as merosin. The importance of laminin- $\alpha 2$ subunits in the development and maintenance of muscle and nerve is demonstrated by mutations that affect this chain⁷⁸⁻⁸³. Laminin- $\alpha 2$ deficiency causes a severe muscular dystrophy, termed MDC1A.

The synaptic basal lamina is rich in $\beta 2$ -containing trimers, which were shown to stop motor axon growth and to initiate differentiation into nerve terminals *in vitro*^{84,85}. The presence of laminin $\alpha 2$, $\alpha 4$, and $\alpha 5$ chains in the synaptic basal lamina give rise to laminin-4 ($\alpha 2$, $\beta 2$, $\gamma 1$), laminin-9 ($\alpha 4$, $\beta 2$, $\gamma 1$) and laminin-11 ($\alpha 5$, $\beta 2$, $\gamma 1$), all of which might be involved in presynaptic differentiation. Laminin-11 promotes presynaptic differentiation and repels Schwann cell processes; laminin-9 promotes the precise alignment of pre- and postsynaptic specializations; and laminin-4 may be important for structural integrity^{60,86-88}.

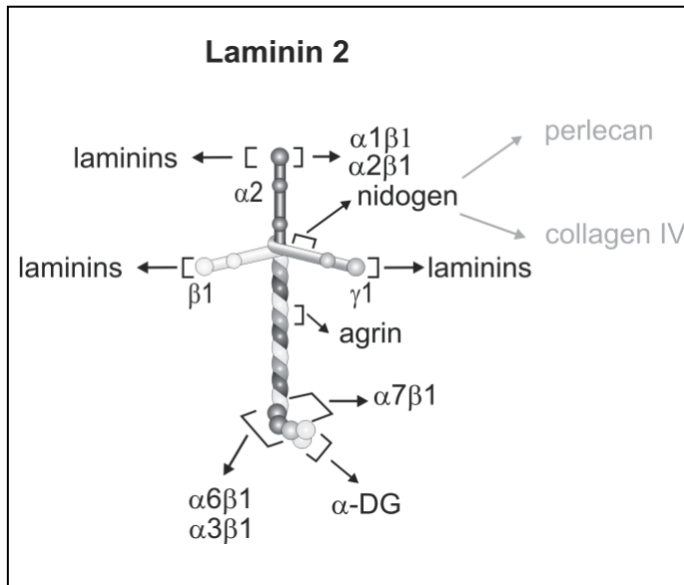


Figure 2: Interactions of laminin-2. Laminin-2 is the predominant laminin isoform present in the extracellular matrix of matured muscle and peripheral nerves.

b. Collagen

Proteins of the collagen family are the major structural components of the extracellular matrix^{24,89}. They are responsible for tissue strength and elasticity and also play dynamic roles in promoting cell growth and differentiation.

The structure of collagen constitutes a long and thin rod-like protein consisting of 3 coiled subunits. The triple helix gives collagen a rigid structure and renders it resistant to proteolysis. Collagen is principally produced by fibroblasts and their synthesis is regulated by complex interactions between growth factors, hormones and cytokines, whereas TGF β and PDGF play a stimulating and glucocorticoids an inhibiting role.

Several collagen types (up to 7) have been identified in intramuscular connective tissue⁹⁰⁻⁹². The fibrillar collagen types I, II, and III are major components of the epimysium and the perimysium, whereas the non-fibrillar type IV collagen constitutes the principal collagenous material in the myofiber basal lamina. In contrast to fibrillar collagens, the non-fibrillar type IV collagen has a more flexible structure. Hence, self-polymerization of collagen type IV does not form ordered fibrillar structures, rather than a flexible filamentous meshwork^{93,94} that is stably linked to the laminin network by interactions with nidogen and likely provides stability at sites or moments of mechanical stress^{27,50,95,96}.

Isoforms of collagen IV are differentially distributed with regard to the synaptic basal lamina, suggesting distinct isoforms to exhibit unique functions. Extrasynaptic portions of muscle contain the $\alpha 1(IV)$ and $\alpha 2(IV)$ chains, whereas synaptic portions primarily contain the $\alpha 3 - \alpha 5(IV)$ chains^{97,98}.

c. Nidogen

Nidogen, also known as entactin, is a ubiquitous component of basal laminas. Most importantly, nidogen provides the primary link between the laminin and collagen IV networks⁹⁹⁻¹⁰¹. It is a glycoprotein of about 150kDa composed of three globular domains (G1–3)^{102,103}. The G2 domain binds collagen IV, perlecan and fibronectin, whereas the G3 domain binds with high affinity to the laminin- γ 1 chain¹⁰⁴⁻¹⁰⁶. However, nidogen-deficient animals appear to develop normally and there is no evidence of structural deformity in the basement membrane¹⁰⁷, suggesting either that nidogen has no structural role in the basement membrane or more likely that its absence is compensated for by other proteins, such as nidogen-2¹⁰⁸.

d. Heparan Sulfate Proteoglycans (HSPG)

Proteoglycans are complex macromolecules consisting of a protein core to which one or more glycosaminoglycan (GAG) moieties are covalently attached. GAGs are linear polymers of repeating disaccharides. They constitute highly negatively charged, viscous molecules, whose low compressibility provides structural integrity to cells.

Heparan sulphates are the GAG chains of heparan sulphate proteoglycans (HSPG), which exist as integral membrane proteins or as secreted extracellular matrix proteins¹⁰⁹. The affinity for a large number of proteins has implicated HSPG in many cellular processes such as cell proliferation, cell differentiation, angiogenesis, metastasis, tissue repair, cell adhesion, sequestering of growth factors¹¹⁰⁻¹¹³, lining blood vessels¹¹⁴, and serving as a structural component of basal laminas¹¹⁵⁻¹¹⁹. In muscle, three main basement membrane HSPGs have been well characterized, agrin^{120,121}, perlecan¹²² and Collagen XVIII^{120,121}.

Agrin

Agrin, isolated by Nitkin et al. (1987)¹² is a HSPG with a protein core of ~225kDa in size. Attachment of GAG chains at two different sites elevate the molecular weight of agrin to 400 to 600kDa^{16,120,123,124}. It comprises nine follistatin-like domains resembling protease inhibitors, EGF-repeats and laminin-like globular domains^{125,126}.

Agrin is synthesized by different tissues such as kidney, lung, brain, spinal cord, muscles and neurons. As a large multidomain protein, agrin interacts with many basal lamina and cell surface proteins, including collagen type IV, laminin¹⁷, tenascin, neural cell adhesion molecules (N-CAM), α -dystroglycan¹²⁷ and integrins⁷ as well as with carbohydrates such as heparin, heparan sulphates and sialic acid¹²⁸.

The mRNA of agrin undergoes tissue-specific alternative splicing at several sites¹²⁹. Amino acid inserts at the carboxy-terminus (4.8) are termed A and B in chicken or y and z in rodents. The insert of eight amino acids at B/z site is responsible for agrin's ability to induce a signalling cascade resulting in AChR-aggregation as well as clustering of AChE and voltage-gated sodium channels at the NMJ^{130,131,10,132-134}. The insert of four amino acids at A/y site is required for agrin binding to heparin via the second laminin-like globular domain¹³². In contrast, non-neuronal agrin isoforms including muscle-derived agrin, lack amino acid inserts at the C-terminus and fail to

cluster AChRs^{131,135,127}. Instead, muscle-derived agrin binds with a 10 times higher affinity to α -dystroglycan than nerve-derived agrin¹³², suggesting an organizing and stabilizing function¹³⁶⁻¹³⁸.

Perlecan

Perlecan is a large HSPG with a 270-467kDa core protein consisting of various domains^{122,139}. In muscle, perlecan is present in both extrasynaptic and synaptic basal lamina, but is concentrated at synaptic sites^{116,117,140}. Perlecan may promote the stability of basal lamina through a broad spectrum of protein interactions, including laminin, collagen IV, nidogen, fibronectin and itself¹⁴¹⁻¹⁴⁴. It is also involved in the adhesion of the basal lamina to the cell surface, since it binds to and co-localizes with α -dystroglycan on the muscle membrane¹⁴⁵. Moreover, perlecan may regulate the activity of growth factors¹⁴⁶, since its heparan sulphate chains bind fibroblast growth factors (FGF) and enhance their activity¹⁴⁷. In the synaptic basal lamina, perlecan mediates the anchoring of AChE¹⁴⁸.

2.2 Membrane-associated and Cytoplasmic Proteins

The muscle fiber plasma membrane (sarcolemma) is a highly specialized cellular structure. It constitutes the physical boundary to the cell and acts as the surface through which the cell interacts with its environment. The sarcolemma contains a very close and highly organized contact with the extracellular matrix.

2.2.1 The Dystrophin-Glycoprotein Complex (DGC)

The dystrophin-glycoprotein complex (DGC) is a specialization of cardiac and skeletal muscle membrane¹⁴⁹⁻¹⁵³. The DGC is composed of transmembranous, cytoplasmic, and extracellular proteins, including dystrophin, dystroglycan, sarcoglycans, sarcospan, syntrophins, dystrobrevins, caveolin-3, and neuronal nitric oxide synthase (nNOS). It provides for the tight link of the cytoskeleton via the sarcolemma to the basal lamina^{56,71,154}. This linkage is essential for muscle stability, since during muscle contraction, the contractile machinery inside the myofibers has to remain connected with the sarcolemma and the extracellular matrix to maintain muscle integrity. Without this association, proper movement would be hampered and muscle fibers would risk damage. Hence, mutations or lack in members of this complex lead to more or less severe muscular dystrophies^{155,156}. In addition to structural support, signal transduction properties are attributed to the DGC¹⁵⁷⁻¹⁵⁹. At the NMJ, the DGC is concentrated and specialized, implying its role for synaptic maturation as well as for maintenance of the NMJ¹⁶⁰.

a. Dystrophin

Dystrophin is a 427kDa cytoplasmic protein^{161,162} that serves as a molecular link between the contractile elements of muscle cytoskeleton and the muscle fiber membrane, which in turn is linked to the surrounding basal lamina by further components of the DGC¹⁵⁵. Dystrophin's N-terminus interacts with the filamentous γ -actin of the contractile apparatus of the muscle¹⁶³⁻¹⁶⁶,

whereas its C-terminus interacts with the intracellular portion of the transmembranous protein β -dystroglycan^{71,167-170}.

Genetic disruption of dystrophin leads to Duchenne muscular dystrophy (DMD) and the milder Becker muscular dystrophy (BMD)¹⁴⁹. In the absence of dystrophin, the transmembranous DGC elements as well as the sarcoglycan complex are unstable and are reduced at the sarcolemma. This renders the muscle abnormally susceptible to damage from contraction¹⁷¹.

At the NMJ, the dystrophin homologue utrophin is part of the DGC. Utrophin is localized with AChRs at the crests of postsynaptic junctional folds^{172,173} and is supposed to function in neuromuscular synapse maturation^{174-176,177}.

b. Dystroglycan

Dystroglycan acts as an essential core component of the DGC by connecting the cytoplasmic components via the sarcolemma to the extracellular matrix of the muscle fiber^{71,76,178,179}.

Dystroglycan is transcribed from a single gene and is posttranslationally processed into two subunits, namely α - and β -dystroglycan with a core molecular weight of 43kDa and 156kDa, respectively¹⁸⁰. Dystroglycan is heavily glycosylated, what determines much of its binding properties and hence gives rise to different isoforms conferring distinct functions.

β -dystroglycan is a single-pass transmembranous protein that intracellularly binds to dystrophin/utrophin, whereas the extracellular N-terminus is non-covalently anchored to its extracellular binding partner α -dystroglycan. Thus, β -dystroglycan tightly links the muscle membrane to its intra- and extracellular environment^{73,181,182}.

In turn, α -dystroglycan provides an important connection to the muscle fiber basal lamina scaffold by binding to different laminin isoforms. Moreover, α -dystroglycan interacts with agrin, perlecan, biglycan and neurexin^{134,160,183-188}. Glycosylation defects of α -dystroglycan disrupts the binding to extracellular ligands such as laminin and leads to muscular dystrophy¹⁸⁹⁻¹⁹⁶.

At the NMJ, dystroglycan is involved in the maturation and maintenance of the postsynaptic membrane in adult animals. β -dystroglycan interacts with rapsyn, a cytoplasmic protein required for acetylcholine receptor clustering at NMJ^{197,198}. Consequently the dystroglycan complex is necessary for stabilization of acetylcholine receptor clusters at NMJ¹⁹⁹ as well as the localization of perlecan and subsequently AChE^{200,201}. Moreover, β -dystroglycan binds to Grb2, what links the DGC to the Ras/MAPK signalling pathway²⁰².

c. The Sarcoglycan-Complex and Sarcospan

Sarcoglycans are glycosylated transmembranous proteins. In cardiac and skeletal muscle, they form a heterotetrameric complex composed of α -, β -, γ -, and δ -sarcoglycan, whereas the core is formed by β - and δ -sarcoglycan which are joined by the other subunits²⁰³. β - and δ -sarcoglycan

are also supposed to mediate the association of the sarcoglycan-complex to α -dystroglycan, thus fixing the complex to the rest of the DGC²⁰⁴. Moreover, the sarcoglycan complex binds to α -dystrobrevin²⁰⁵ and stabilizes sarcospan²⁰⁶ at the membrane. Nevertheless, there is no direct association with dystrophin.

The precise function of the sarcoglycan-complex has remained unclear. However, it stabilizes the dystrophin–dystroglycan interaction and may regulate the adhesion to laminin-2 in the extracellular matrix. The extracellular domains of α -sarcoglycan may be involved in signalling critical for muscle cell survival²⁰⁷. Mice lacking sarcoglycan genes effectively model human mutations leading to different types of limb girdle muscular dystrophy.

d. Syntrophins and Dystrobrevin

Three syntrophin isoforms exist in skeletal muscle, α -, β 1- and β 2-syntrophin²⁰⁸⁻²¹⁰. Syntrophins are cytoplasmic adaptor proteins thought to recruit signalling proteins to the muscle fiber membrane. The linkage to the DGC is mediated via binding to dystrophin/utrophin and α -dystrobrevin²¹¹. For this reason, syntrophins are secondarily abnormal in Duchenne muscular dystrophy^{212,213}. Moreover, α -syntrophin binds neuronal nitric oxide synthase^{214,215} (nNOS), sodium channels^{212,216,217}, aquaporin-4^{218,219}, kinases^{220,221}, the growth factor receptor bound adapter protein Grb2²²², and ErbB4, a signalling protein implicated in promoting transcription from sub-synaptic nuclei in skeletal muscle²²³.

In skeletal muscle, α -dystrobrevin-1, -2 and -3 are localized in the cytoplasm and, like syntrophins, are thought to serve as a scaffold for signalling proteins²²⁴⁻²²⁷. Moreover, α -dystrobrevin binds to syntrophins, the sarcoglycan–sarcospan complex and directly interacts with dystrophin^{228,229}.

2.2.2 Integrins

Integrins are integral receptor proteins which are of crucial importance for cell-extracellular matrix and cell-cell interactions^{230,231}. Functional integrins consist of a α and a β subunit, both transmembranous glycoproteins that are non-covalently linked to each other.

Integrin molecules are major structural components of adhesion complexes at the cell membrane^{232,233}. Extracellular matrix ligands for integrins are known to be collagens, fibronectin, tenascin and laminin, while the β -subunit cytoplasmic domain of integrin is interacting with the cytoskeleton^{234,235}. In this way, integrins establish a mechanical continuum along which forces can be transmitted from the outside to the inside of the cell, and vice versa²³⁶⁻²³⁹.

Integrin α 7 β 1 is the predominant integrin isoform found in skeletal and cardiac muscle. It is concentrated at MTJs but is also present along the sarcolemmal membrane and constitutes an

important receptor for laminin-1, -2 and -4. Integrin $\alpha7\beta1$ mediates indispensable interactions between the cytoskeleton and the extracellular matrix that are independent of the DGC-mediated connection^{240-243,244}.

3 Laminin and its Receptors in the Peripheral Nervous System

The speed and efficiency of propagation of action potentials along peripheral neurons are increased by insulating myelin sheaths formed by Schwann cells, which wrap around axons and form segments of about 1mm length. In between these segments, small regions of axon membrane remain bare, so-called nodes of Ranvier. Thus, the signal propagates along the axon by leaping from node to node. At site of muscle fiber contact, the axonal branch of a motor neuron loses its myelin sheath and makes a spray of fine boutons, which are capped by synapse-associated Schwann cells.

The peripheral nervous system (PNS) contains two distinct types of basement membranes. The perineurial basement membrane surrounds bundles of Schwann cell axon units and mainly contains laminin-9 ($\alpha4, \beta2, \gamma1$) and laminin-11 ($\alpha5, \beta2, \gamma1$)^{60,97,245}. The endoneurial basement membrane surrounds each individual Schwann cell and contains a complex system of laminin isoforms and receptors that are differentially expressed in a precise temporal and spatial distribution. Its major laminin isoforms during development are laminin-2 ($\alpha2, \beta1, \gamma1$) and laminin-8 ($\alpha4, \beta1, \gamma1$), whereas matured endoneurial basement membrane predominantly contains laminin-2, similar to muscle fiber basal lamina^{60,246}. Laminins in the endoneurial basement membrane are strictly required for Schwann cell survival, interaction with axons, myelination, myelin maintenance and nerve regeneration.

Aberrant laminin signalling induces pathogenesis such as for instance MDC1A and leprosy. For instance, mutations in the *lama2* gene, coding for the laminin- $\alpha2$ chain, cause both a muscular dystrophy and a peripheral neuropathy in human and mice (MDC1A). Null-mutants of laminin- $\alpha2$ chain showed presence of initial myelination. However, Schwann cells that lack laminin-2 ($\alpha2, \beta1, \gamma1$), cannot send processes within axonal bundles, proceed with radial sorting, nor achieve a 1:1 relationship with axons²⁴⁷. The sorting abnormalities are more severe in the proximal than in the distal PNS. Thus, the neuropathy derives from demyelination, mainly in the proximal part of the PNS, and results in a reduced nerve conduction velocity²⁴⁸.

Laminin receptors in the peripheral nervous system are collagens, integrins and dystroglycan. Schwann cell-specific inactivation of the $\beta1$ -integrin gene, prevented radial sorting of axons, similar to the phenotype observed in laminin- $\alpha2$ mutants. Hence, the defect may arise from disrupted signalling from laminin to the cytoskeleton due to lack of its receptor. This elevates the abundant $\alpha6\beta1$ -integrin to the putative laminin receptor involved in radial axonal sorting by

Schwann cells. Conclusively, signals from laminins allow Schwann cells to radially sort axons during development, by mediating the main regulatory events via β 1-integrin-mediated cytoskeletal rearrangements.

Glycosylation of α -dystroglycan is very important for its function as a laminin receptor. Indeed *Large^{myd}* mice, bearing a deletion of the LARGE glycosyltransferase leading to hypoglycosylation of dystroglycan^{193,249}, have both a muscular dystrophy and a peripheral neuropathy. Moreover, *Large^{myd}* neuropathy also includes unsorted naked axons suggesting that another substrate of LARGE may be β 1-integrin. In contrast, Schwann cell-specific inactivation of dystroglycan causes a late onset neuropathy, involving hypomyelination and abnormally folded myelin sheaths. However, there are no abnormalities in radial sorting.

Leprosy is one of the leading causes of non-traumatic peripheral neuropathy worldwide. The *Mycobacterium leprae* has a special tropism for Schwann cells because it binds laminin-2 in endoneurial basal lamina and uses laminin receptors, in particular α 6 β 4-integrin and dystroglycan, to enter Schwann cells. When the mycobacterium binds these receptors, it is able to cause Schwann cell de-differentiation, causing demyelination²⁵⁰.

4 Development of Skeletal Muscle: A Short Overview

All vertebrate skeletal muscles (apart from head muscles) derive from mesodermal precursor cells originating from the somites. During embryonic development, mesodermal precursor cells receive signals from surrounding tissues, which induce (Wnts, Sonic hedgehog, Noggin) or inhibit (BMP4) the expression of the primary myogenic regulatory factors (MRFs) Myf5 and MyoD. Myf5 and MyoD both are transcriptional activators and their upregulation specify the precursors to the myogenic lineage. Proliferative myogenic cells that are MyoD and/or Myf5 positive are termed myoblasts. Committed myoblasts migrate laterally to form the myotome, which eventually forms the skeletal musculature. Pax3 promotes myogenesis in the lateral myotome. To induce terminal differentiation of myoblasts into myocytes, proliferating myoblasts withdraw from the cell cycle by upregulation of the secondary MRFs, myogenin and MRF4. Subsequently, myocytes start to express muscle-specific genes such as myosin heavy chain (MHC) and muscle-specific creatine kinase (MCK). Finally, mononucleated myocytes specifically fuse to each other to form a multinucleated syncytium, the myofibers, which mature into contracting muscle fibers. After sexual maturity, skeletal muscle is a stable tissue characterized by multinucleated postmitotic muscle fibers with their postmitotic myonuclei located at the periphery.

A distinct subpopulation of myoblasts fails to differentiate, but remains associated with the surface of the developing myofiber as quiescent undifferentiated muscle satellite cells. Satellite cells are characterized by their high Pax7 expression, which is essential for the specification and

expansion of the satellite cell population. Satellite cell nuclei can be distinguished from myonuclei by their abundant heterochromatin reflecting their mitotic quiescence.

Only recently the muscle precursor cells accounting for muscle growth were identified²⁵¹. This newly identified population of muscle progenitor cells ingress the primary myotome directly from the central dermomyotome, express the transcription factors Pax3 and Pax7 and are maintained as a proliferating population in embryonic and fetal muscles throughout development. Subsequently, the Pax3/Pax7-positive cells become myogenic to form and contribute to the formation and growth of muscles during embryonic and fetal life. Thus, Pax3 and Pax7 have an important role in conferring myogenic potential on the muscle progenitor cells and thus assuring skeletal muscle formation²⁵². The same studies provide evidence that satellite cells derive from the same novel population of muscle progenitor cells^{251,252}.

5 Regeneration of Skeletal Muscle

Adult mammalian skeletal muscle is a stable tissue and elicits only a slow turnover of its constituent multinucleated muscle fibers (1–2 % of myonuclei are replaced every week). However, mammalian skeletal muscle has the ability of rapid and extensive regeneration in response to severe damage, resulting either from direct trauma such as extensive physical activity or from innate genetic defects.

Damage of muscle is generally accompanied by disruption of the muscle fiber integrity. It is believed that damage of the sarcolemma or the sarcoplasmic reticulum promotes increased calcium influx, resulting in a loss of calcium homeostasis and increased calcium-dependent proteolysis that drives tissue degeneration.

5.1 The Regeneration Process

The muscle regeneration process is characterized by an early degenerative followed by a regenerative phase. The initial degenerative phase upon muscle injury is characterized by necrosis and an increased number of non-muscle mononucleated cells within the damaged site. Factors released by the injured muscle activate inflammatory and mononucleated myogenic cells. Neutrophils are the first inflammatory cells to invade the injured muscle, followed by macrophages becoming predominant about 2 days postinjury²⁵³. Macrophages infiltrate the injured site to phagocytose cellular debris and to activate myogenic cells.

Muscle degeneration is followed by the activation of a muscle repair process. Myofiber regeneration is characterized by the activation of myogenic satellite cells to proliferate, differentiate, and fuse to necrotic fibers for repair or to each other for new fiber formation. Regenerating fibers are characterized by their small caliber and their centrally located myonuclei. Newly formed myofibers are often basophilic (reflecting high protein synthesis) and express embryonic/developmental forms of myosin heavy chain (MHC), reflecting de novo fiber

formation²⁵⁴. Once fusion of myogenic cells is completed, newly formed myofibers increase in size, and myonuclei move to the periphery of the muscle fiber. Under normal conditions, the regenerated muscle is morphologically and functionally indistinguishable from undamaged muscle.

5.2 Satellite cells

5.2.1 Localization and Identification of Satellite Cells

Muscle satellite cells were first described in 1961²⁵⁵. Recent studies suggest that satellite cells derive from the same Pax3/Pax7 expressing cell population as muscle progenitor cells^{251,252}. Satellite cells constitute a population of undifferentiated mononuclear myogenic cells that remain localized in the periphery of myofibers within the basal lamina, hence their name²⁵⁶. They are present in all skeletal muscles and are associated with all muscle fiber types, albeit unequally distributed. Within the same muscle, higher numbers of satellite cells are found associated with slow type I muscle fibers than with fast type II fibers. The satellite cell population varies also with age and decreases in density over time.

Pax7 is a transcription factor implicated in development of the skeletal muscle and is expressed only at low levels in adult muscle fibers. In contrast, it is a reliable marker of satellite cells, since it is specifically expressed in quiescent and activated muscle satellite cells up to myogenic differentiation when its expression is rapidly down-regulated^{257,258}. Mice deficient in Pax7 in skeletal muscles (Pax7^{-/-}), appear normal at birth but fail to grow postnatally. They show a decreased skeletal muscle mass resulting from a fiber size decrease rather than a decrease in fiber number. Pax7^{-/-} animals fail to thrive and usually die within 2 wk after birth^{258,259}. As expected, skeletal muscles have a striking absence of satellite cells and normal skeletal muscle regeneration is dramatically reduced²⁵⁸.

5.2.2 Activation, Proliferation, Migration and Differentiation of Satellite Cells

In the course of muscle regeneration, the myogenic program of satellite cells is activated. They exit their normal quiescent state and start to proliferate. Activation of satellite cells requires a temporally controlled upregulation of muscle transcription factors and muscle specific genes²⁶⁰⁻²⁶². This process is regulated through mechanisms involving cell-cell and cell-matrix interactions as well as extracellularly secreted factors. Muscle injuries have been shown to cause the release of biologically active molecules into the extracellular space. Such molecules include the hepatocyte growth factor (HGF), shown to be the primary muscle factor capable of inducing quiescent satellite cell activation, fibroblast growth factor (FGF)²⁶³, insulin growth factors (IGF) and transforming growth factors (TGF) of the TGF- β family, such as myostatin which inhibits both proliferation and differentiation.

Satellite cell activation is not restricted to the damaged site. Damage at one end of a muscle fiber will activate satellite cells all along this fiber leading to the proliferation and migration of the satellite cells to the regeneration site²⁶⁴. In a moderate injury, the satellite cells use the basement membranes of necrotic fibers as a scaffold to ensure a similar position of the new muscle fibers.

The process of satellite cell proliferation and differentiation during muscle regeneration is reminiscent of embryonic muscle development. In particular, the critical role of the MRFs is observed in both processes. At the molecular level, proliferation is characterized by the rapid upregulation of the two early MRFs, Myf5 and/or MyoD, followed by the co-expression of these two MRFs. Myf5 is supposed to promote satellite cell self-renewal, whereas MyoD promotes satellite cell progression to terminal differentiation²⁶⁵.

After several rounds of proliferation, the majority of the satellite cells differentiates and fuse to form new syncytial myofibers or to repair damaged one. This requires expression of the late MRF members Myogenin and MRF4, upregulated in cells beginning their terminal differentiation program^{263,266}. The differentiation program is then completed with the activation of muscle-specific proteins, such as myosin heavy chain (MHC), and the fusion to repair damaged muscle. Repaired or new myofibers grow to resemble original myofibers²⁶⁷.

5.2.3 The Satellite Cell Pool

Satellite cell self-renewal is a necessary process without which recurrent muscle regeneration would rapidly lead to the depletion of the satellite cell pool. Therefore a small proportion of satellite cells that has undergone proliferation returns to the quiescent state, thereby replenishing the satellite cell pool^{267,268}. The molecular mechanisms underlying the satellite cell self-renewal is unclear, although several lines of evidence suggest a role for Myf5 in facilitating satellite cell self-renewal.

Whatever the cellular mechanisms for satellite cell self-renewal are, they do not compensate for the chronic loss of myonuclei throughout a lifetime as reflected by the reduction in satellite cell number with aging, nor do they compensate for the depletion of the satellite cell pool resulting from continuous activation of muscle repair in dystrophic muscles^{269,270}. Exhaustion of the mitotic potential of satellite cells, or replicative senescence, may be responsible for the decrease in the satellite cell pool with age²⁷⁰.

6 Muscular Dystrophies

Muscular dystrophies are clinically and molecularly heterogeneous diseases of a genetic origin. They are characterized by a progressive wasting of skeletal muscle starting at any age from birth to middle years and resulting in significant disability. Frequently, respiratory and cardiac functions are affected, leading to respiratory failure and premature death. Most of the dystrophies have a

very poor prognosis. The underlying mutations often affect the proteins implicated in the tight physical linkage of the actin cytoskeleton and the extracellular matrix, namely members of the DGC and associated proteins such as laminin-2. The entire structure of proteins protects the integrity of the muscle tissue against physical damage during repeated rounds of contraction and relaxation. Disruption of this link by deficiency in one of its constituents leads to a decreased mechanical stability and an increased fragility of the sarcolemma, especially during intense contractile activity³⁴⁻³⁶. This, in turn, results in increased calcium entry (although the molecular mechanisms have not been elucidated in detail) and focal or diffuse damage to the fiber. Damaged or dead fibers can be repaired or replaced by satellite cells, responsible for muscle growth and regeneration in postnatal life. However, the satellite cells of patients with muscular dystrophy share the same molecular defect and produce fibers that are also prone to degeneration. With time, the population of satellite cells is exhausted and the muscle tissue is progressively replaced by non-muscle tissue.

It has proven very difficult to set up an accurate and clinically useful classification of the muscular dystrophies. Nevertheless, advances in the understanding of the molecular defects of these disorders have permitted a foundation for classification based on molecular biology.

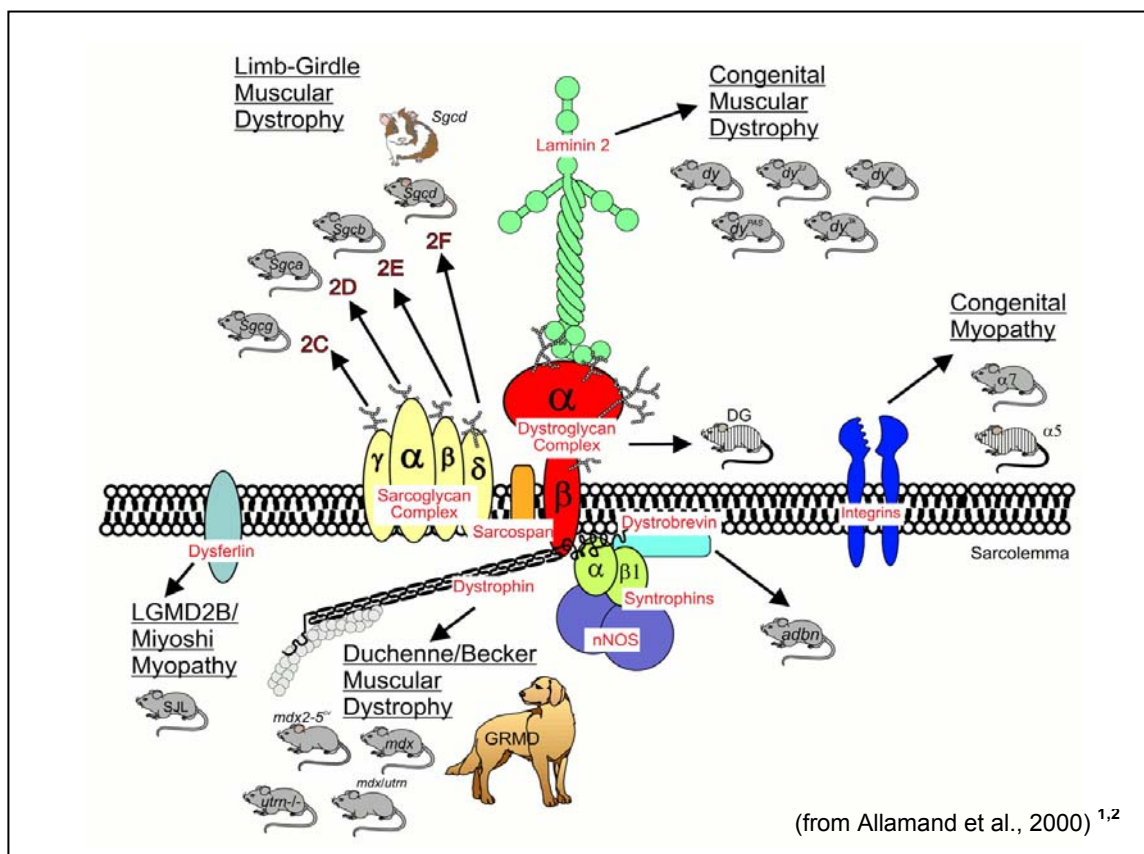


Figure 3: Overview of muscular dystrophies and their corresponding animal models.

Muscular dystrophies implicated in the alteration of proteins involved in the basal lamina-sarcolemma-cytoskeleton linkage are represented.

6.1 Congenital Muscular Dystrophies

Congenital muscular dystrophies (CMD) represent a clinically and molecularly heterogeneous group of autosomal recessively inherited neuromuscular disorders^{271,272}. The incidence of CMD is 4.65 in 100,000 and thus represents the most frequent autosomal recessive neuromuscular disease²⁷³. Various forms of congenital muscular dystrophies have been identified and attributed to mutations in several genes.

Disease	Gene	Product
MDC1A	lama2	Laminin- α 2
MDC1B	Unknown	Unknown
MDC1C	FKRP	Fukutin-related protein
MDC1D	LARGE	Glycosyltransferase-like protein LARGE
Walker-Warburg syndrome (WWS)	POMT1/2	Protein O-mannosyl-transferase 1
Muscle-eye-brain disease (MEB)	POMGnT1	O-mannoside N-acetylglucosaminyl transferase
Fukuyama CMD (FCMD)	FCMD	Fukutin
Congenital myopathy ²⁷⁴	ITGA7	Integrin α 7
CMD with early spine rigidity (RSS)	SEPN1	Selenoprotein N
Ullrich CMD	COL6A1/2	α 1 collagen VI + α 2 collagen VI
	COL6A3	α 3 collagen VI

Table 1: Congenital muscular dystrophies and their mutated genes

CMD is caused by mutations directly or indirectly interfering with the interaction of extracellular ligands and α -dystroglycan. These include cell surface receptors, such as integrins^{274,275} (congenital myopathy), basal lamina proteins, such as laminin-2 (MDC1A), and extracellular matrix proteins, such as collagen VI (Ullrich CMD)²⁷⁶. Recently, post translational modification of proteins has been defined as a new area of focus for congenital muscular dystrophy research by the identification of a group of disease genes that encode known or putative glycosylation enzymes^{190,194,277,278}. Walker-Warburg Syndrome (WWS²⁷⁹⁻²⁸⁴) and muscle-eye-brain disease (MEB^{280,285-287}) are caused by mutations in 2 genes involved in O-mannosylation, POMT1 and POMGnT1, respectively. Fukuyama muscular dystrophy (FCMD²⁸⁸) is due to mutations in fukutin, a phospholigand transferase. MDC1C and LGMD2I are allelic, due to mutations in fukutin-related protein (FKRP²⁸⁹⁻²⁹¹). Disruption of the glycosyltransferase-like protein LARGE is responsible for MDC1D¹⁹² caused by aberrant glycosylation of α -dystroglycan^{192,292}. A common feature of MDC1C, WWS, MEB and FCMD is hypoglycosylation of α -dystroglycan^{189,293}. Thus, a major feature in these pathologies is abnormal glycosylation of α -dystroglycan, impairing interactions

with its normal extracellular matrix partners and giving rise to progressive muscle degeneration and abnormal neuronal migration¹⁸⁹.

For most of the mutations causing CMD, corresponding animal models are available, effectively imitating human pathology and allowing for molecular investigations in regard to therapeutic interventions.

6.1.1 Laminin α 2-deficient Congenital Muscular Dystrophy (MDC1A)

The first report of a MDC1A patient derived from a French research group in 1994^{81,294}. In European populations, laminin- α 2- or merosin-deficient congenital muscular dystrophy (MDC1A) is one of the most frequent neuromuscular diseases in children, as it accounts for about 50% of all congenital muscular dystrophies^{78,80-82}.

a. Molecular and Cellular Basis of MDC1A

The predominant laminin isoform in the extracellular matrix of skeletal muscles and peripheral nerves is laminin-2 (α 2, β 1, γ 1). The three short arm N-terminals of laminin-2 allow self-polymerization providing for a supporting laminin network in the basal lamina. The C-terminal globular domains of the α 2 chain mediate the linkage to the sarcolemma mainly by interactions with the cell surface receptor α -dystroglycan which is part of the DGC^{56,69-77} and α 7 β 1 integrin. In MDC1A the laminin- α 2 chain is disrupted what consequently leads to absence of laminin-2 in the extracellular matrix of muscles and peripheral nerves. This leads to upregulation of the laminin- α 4 chain to yield laminin-8 (α 4, β 1, γ 1)^{246,295}. The laminin α 4 chain lacks the short N-terminal arm what impedes the ability to self-polymerize. In addition, laminin α 4 does not interact with the cell surface receptors α -dystroglycan and α 7 β 1 integrin. These properties are likely to be important for the integrity of the muscle. Hence, the tight basal lamina-sarcolemma-cytoskeleton linkage is disrupted, what decreases the mechanical stability of the muscle and evokes cycles of de- and regeneration³⁴⁻³⁶. With time, this leads to replacement of muscle with fibrous tissue and thus to the muscle waste typical for this disease.

MDC1A is caused by mutations in *lama2*, the gene encoding the laminin- α 2 chain. Its cDNA spans 9.5kb and harbors 64 exons²⁹⁶ giving rise to a polypeptide chain of 3110 amino-acid residues. In human, the *lama2* gene is located on 6q22–23. Most mutations in the *lama2* gene result in complete absence of laminin- α 2 protein; however, rare allelic mutations can result in partial protein reduction²⁹⁷. Analysis of the laminin- α 2 chain cDNA or the *lama2* gene itself showed that small deletions or insertions mostly localized in the N-terminal domain (exons 1–31), induce complete merosin deficiency and a severe phenotype²⁹⁸. Mutations in the last exons of the G domain (exons 58 to 64) result in the expression of partially functional laminin- α 2 chain, retaining at least some of its normal functions and thus are often associated with somewhat milder forms of CMD^{80,298,299}.

In addition to absence of laminin-2 ($\alpha 2$, $\beta 1$, $\gamma 1$), mutations in the gene for laminin $\alpha 2$ leads to lack of the laminin-4 ($\alpha 2$, $\beta 2$, $\gamma 1$) at the NMJ⁶⁰. Laminin-4-deficient synapses fail to develop proper postsynaptic folds^{300,301}. The mechanism by which laminin-4 regulates fold formation likely includes activation of $\alpha 7$ -integrins. Splice variants of integrin- $\alpha 7$ are concentrated at NMJs and MTJs, but absent from junctions in laminin-4 deficient mice.

In addition to the progressive muscular dystrophy, deficiency in laminin- $\alpha 2$ chain cause a peripheral neuropathy resulting in reduced nerve conduction velocity predominantly involving motor or sensory motor nerves. The neuropathy in MDC1A is of the demyelinating type. In large part, this abnormality is due to the inability of Schwann cells to send processes around axons as well as a decrease in number and proliferation of Schwann cells. The presence of naked axons is more evident in the proximal part of the PNS (especially roots) than in the distal part. This difference may result from compensation or redundancy by other laminin isoforms specific to the distal PNS. For example, laminin $\alpha 1$ is upregulated in distal nerves, but not roots, of dystrophic mice. Some fibers in the proximal PNS and most fibers in the distal PNS achieve the promyelinating state, and myelinate. But the dystrophic myelin is not normal: Thickness is reduced or increased, internodes are up to 50% shorter, nodes of Ranvier can be abnormally wide with nodal gaps up to 10 μ m and voltage-gated sodium channel clustering at nodes of Ranvier is reduced.

b. Mouse Models of MDC1A

Laminin-2 performs important functions in muscle^{53,302} and mouse models have been instrumental in elucidating these functions. As many as five mouse models for laminin $\alpha 2$ -deficiency are available³⁰³⁻³⁰⁷. Naturally occurring models include the $dy^{-/-}$, $dy^{2J^{-/-}}$ and $dy^{PAS^{-/-}}$ mice, while $dy^{3K^{-/-}}$ and $dy^{W^{-/-}}$ mice constitute genetically engineered mouse models of MDC1A.

The $dy^{-/-}$ mice express a laminin- $\alpha 2$ polypeptide of apparent normal size but in very small amounts, and the mice suffer from a very severe form of MDC1A. The mutation in the $dy^{-/-}$ mice has not yet been identified. The mutation in the *lama2* gene responsible for the $dy^{2J^{-/-}}$ mouse phenotype, is an in-frame deletion within the polymerizing N-terminal globular domain of the laminin- $\alpha 2$ chain^{304,305}. Although defective in self-assembly, the $\alpha 2$ -laminins remain within the basal lamina, probably tethered through nidogen, agrin, integrin, dystroglycan and other cell-surface binding molecules^{40,308,309}. The partial laminin- $\alpha 2$ deficiency in $dy^{2J^{-/-}}$ mice results in a mild phenotype. Both, the $dy^{PAS^{-/-}}$ and the $dy^{3K^{-/-}}$ mice completely lack laminin- $\alpha 2$ and represent a severe phenotype³¹⁰. For generation of $dy^{W^{-/-}}$ mice, a cassette containing the *LacZ* and *neo* genes was inserted shortly downstream the *lama2* gene ATG initiation site by homologous recombination in embryonic stem cells. The $dy^{W^{-/-}}$ mouse is not a null mutant, since a small amount of a truncated laminin- $\alpha 2$, which lacks domain VI but contains the G domain, is expressed. The severe phenotype in $dy^{W^{-/-}}$ mice may be related more to the low amounts of laminin- $\alpha 2$ than to the lack of domain VI in the protein.

The pathology in these mouse models resembles human MDC1A pathology. In all MDC1A mice, muscle development is seemingly normal, but a more or less severe muscular dystrophy is manifested postnatally^{303-305,311}. $\alpha7\beta1$ remains localized to the MTJs, whereas the sarcolemmal distribution of $\alpha7\beta1$ integrin is disturbed. Both at the synapse and in the extrasynaptic sarcolemmal basement membranes, lack of laminin- $\alpha2$ is compensated by expression of the functionally different laminin $\alpha4$ chain giving rise to laminin-8 isoform that is neither able to self-polymerize nor to interact effectively with α -dystroglycan. Similarly, the progressive lameness of hindlegs is characteristic of mice from all *dy* strains and is likely due to deficiency of laminin- $\alpha2$ in peripheral nerves³¹². Unlike in human MDC1A pathology, in none of the mouse models, changes of the white matter in the brain could be observed³⁰⁷.

c. Clinical and Pathological Features of MDC1A

The 'classical form' of MDC1A is associated with complete loss of laminin-2 and presents with early onset of a severe and progressive muscular dystrophy, hypotonia, peripheral neuropathy and characteristic white matter hypodensity on cerebral magnetic resonance imaging³¹³⁻³¹⁵. Children can sit without support but almost never achieve independent ambulation. Progressive joint contractures, rigidity and scoliosis of the spine are common, making ambulation more difficult. Respiratory failure followed by death in the first decade of life has been observed in 30% of patients with complete laminin- $\alpha2$ deficiency.

Dystrophic muscles are characterized by necrotic and regenerating fibers, centrally located myonuclei, increase in fiber size variation and non-muscle tissue as well as markedly elevated CK levels in the blood serum. Moreover, the muscle fiber regeneration capacity is markedly perturbed in MDC1A mice^{316,317}. Since Evans blue dye upon injection does not accumulate inside the muscle fibers of different mouse models, muscle membrane leakage seems not to be central to the pathogenesis of MDC1A³¹⁸. The neuropathy manifests in form of reduced nerve conduction velocity mainly in motor nerves³¹⁹.

6.2 Strategies for Treatment of Muscular Dystrophies

Muscular dystrophies are among the most difficult diseases to treat, even though most of the underlying molecular defects are now known. This is because skeletal muscle is the most abundant tissue of the body and is composed of large multinucleated fibers, the nuclei of which have permanently lost the ability to divide. Consequently, any cell or gene replacement must restore proper gene expression in hundreds of millions of postmitotic nuclei, which are embedded in a highly structured cytoplasm and surrounded by a thick basal lamina. Similarly, most pharmacological trials must overcome the complex as well as partly unknown biochemical mechanism of fiber degeneration that involves pathways, such as calcium fluxes and protease activity, for which inhibitors are associated with high systemic toxicity. Nevertheless, the results that have been accumulated during the last few years have opened new perspectives for different

treatment approaches. However, to date, there is no efficient treatment to prevent disability and death in muscular dystrophies. Treatments currently in use can only help to slow down the functional impairment of the disease.

6.2.1 Pharmacological Approaches

Several pharmacological strategies have been attempted to counteract the consequences of the dystrophic process, but only few of them have entered clinical trials.

Corticosteroids are anti-inflammatory molecules, which can delay the loss of independent ambulation in patients by 2 to 4 years, significantly reduce the risk of developing skeletal defects and delay the onset of respiratory and cardiac failure^{320,321}. However, their use is associated with significant side effects, such as weight gain and osteoporosis with the risk of bone fractures.

Other pharmacological strategies include the stimulation of anabolic processes using insulin growth factor-1 (IGF-1), a known muscle growth factor, compensation of the mutated protein by pharmacological upregulation of a functionally related protein as well as blocking of proteasomes to protect degradation of proteins in dystrophic muscles³²²⁻³²⁴.

6.2.2 Gene Therapy

There are two types of gene therapy. Germline gene therapy alters the reproductive cells of an individual's body and therefore is inheritable. Somatic gene therapy has no effect on future offspring because it only alters non-reproductive (somatic) cells. Currently, somatic cell gene therapy has been done on a larger scale than germline gene therapy because there are less ethical and social concerns associated with it. Somatic gene therapy can be performed in two ways: *ex vivo* (out of the body) and *in vivo* (in the body).

Ex vivo, somatic gene therapy involves two strategies. One is the use of cells obtained from a healthy donor, which express the normal copy of the mutated gene but induce an immune rejection unless the patient is permanently immune suppressed. The second one is the use of cells obtained from the patient, which do not require immune suppression but must be 'genetically corrected' *in vitro* to restore the expression of the mutated protein. The major problem still faced by this approach is the lack of dispersion of donor cells.

In vivo somatic gene therapy targets cells within the body to be genetically altered. It necessitates that the vectors are able to deliver the genes to the correct area of the body, deliver enough altered genetic material to the cells to ensure effectiveness, and remain otherwise undetected by the body's immune system. Disorders that arise from mutations in a single gene, as it is the case in muscular dystrophies, are the best candidates for gene therapy. The main strategies aim to replace the defective gene by introduction of a 'normal' copy or a compensatory gene by viral or non-viral delivery methods.

Moreover, current strategies also involve methods of gene modulation, such as antisense induced exon-skipping as well as endogenous correction of the defective gene using methods such as DNA–RNA chimeric oligonucleotides. However, these methods rely on exact mapping of the mutation in the individual patients.

***In vivo* Somatic Viral-based Gene therapy**

Viruses are naturally evolved vehicles, which efficiently transfer their genes into the host cells. For use in gene therapy, viruses are genetically modified to reduce expression of viral proteins, render them replication-deficient and to make space for insertion of the therapeutic transgene sequences. However, viruses still present a variety of problems to the patient, such as toxicity, immune responses, gene control and targeting issues as well as the potential recovery of the viruses to cause disease.

In order to develop valuable gene therapies, both methodological advances in viral vector design as well as the evaluation of effective genes to be introduced is necessary. For the latter purpose, transgenic analysis of putative therapeutic genes in animal models for muscular dystrophies^{325,326} is mandatory, enabling extensive pre-clinical studies on the safety and the functionality of various therapeutic approaches. Several transgenic approaches overexpressing the mutated gene or a structurally and functionally similar molecule in mouse models of muscular dystrophies resulted in a significant amelioration of the disease. However, replacing a mutated gene in all, or at least in a good proportion, of the post-mitotic nuclei of skeletal muscle is daunting. In addition, a major limitation of gene therapy is the large size of the genes to be compensated for in muscular dystrophies. An auspicious progress to overcome this problem, derive from approaches of transgenic overexpression of specifically designed molecules that are functionally but not structurally related to the disease-causing gene to be restored. Such rational design and the use of small mimetics of large proteins can be useful for gene therapy.

For effective treatment of muscular dystrophies, local intramuscular injection is definitively not feasible but a systemic delivery modus is required for a clinical setting. However, systemic delivery favors unwanted gene transfer to other tissues than muscles, what may pose safety concerns because of the potential to increase germline transmission, immunoreactivity and toxicity. Adeno-associated viral (AAV) vectors are among the most promising transfer vehicles and a lot of effort has been put on development and improvement of their delivery properties. AAV are non-pathogenic replication-deficient parvoviruses, characterized by low immunogenicity, able to integrate into host genome allowing for permanent genetic correction and able to infect multiple cell types, including non-replicating cells such as mature myofibers. The major disadvantage of AAV is the limited capacity for insert size (< 5kb). Recently, new AAV serotypes capable of disseminating systemically and having a high infectivity in muscle were discovered and made systemic muscle gene delivery possible³²⁷, pointing to a possibly viable therapeutic option particularly for childhood diseases that can be decreased with early intervention, as it is

the case for MDC1A. Very recently, the feasibility of systemic mini-agrin gene delivery without additional pharmacological intervention leading to long-term transduction of whole body skeletal and cardiac muscle in MDC1A mice and resulting in a substantial amelioration of the MDC1A phenotype was demonstrated³²⁸.

In the clinics, gene therapy has experienced only few modest successes but suffered from many failures, because of the death of a participant in a trial due to acute toxicity³²⁹ and the occurrence of leukemia in children who were treated with retrovirus-mediated gene transfer³³⁰.

These disappointing results from clinical trials imply the need of many more pre-clinical studies. In this respect, experiments on animal models will continue to provide crucial pieces of information in regard to issues such as the appropriate timing for intervention (the earlier the better seems to be a consensus), the risk–benefit ratio of current vectors and transgenes, and the assessment of functional benefit.

II THE TOPIC OF THE THESIS

1 Introduction

Laminin- α 2 deficient congenital muscular dystrophy, classified as MDC1A, is a severe progressive muscle-wasting disease that leads to death in early childhood³⁰⁷. MDC1A is caused by mutations in *lama2*, the gene encoding the laminin- α 2 chain being part of laminin-2 (α 2, β 1, γ 1), the main laminin isoform present in the extracellular matrix of muscles and peripheral nerves^{57,62-64}. The N-terminus of the laminin- α 2 chain accounts for the formation of the primary laminin scaffold by self-polymerization while C-terminal globular domains bind with high affinity to α -dystroglycan providing a connection to the cytoskeleton via the transmembranous protein β -dystroglycan. In laminin- α 2 deficient muscle, the laminin- α 4 chain is strongly upregulated to yield laminin-8 (α 4, β 1, γ 1)^{60,246}. Moreover, laminin-8 cannot self-polymerize and does not bind to α -dystroglycan³³¹, both properties that are likely to be important for the integrity of the muscle. Thus, in laminin α 2-deficient muscle the chain of proteins linking the actin cytoskeleton via the sarcolemma to the basement membrane is interrupted (Fig. 1). As a consequence, muscle fibers lose their stability and degenerate. In addition, the regenerative capacity of muscle is substantially lower^{30,317}. These deficiencies lead to the dystrophic phenotype characterized by high levels of creatine kinase in the blood, variations in fiber size and successive replacement of muscle by non-muscle tissue. As a mouse model, several laminin α 2-deficient mice are available. For our studies we used the *dy*^{W/-} MDC1A mouse model^{306,309}, which manifest a severe dystrophic phenotype becoming apparent at the age of two weeks.

In a previous study, we have shown that a rationally designed miniaturized form of the extracellular matrix protein agrin, which is not related to the disease-causing *lama2* gene, was sufficient to markedly improve muscle function and overall health of *dy*^{W/-} mice³¹⁶. In brief, in *dy*^{W/-} mice constitutively expressing chick mini-agrin in skeletal muscles were analyzed in regard to their dystrophic phenotype. Due to its laminin- and α -dystroglycan-binding properties^{17,127,332}, mini-agrin was supposed to reconnect the muscle fibers to the extracellular laminin network and thus restore the mechanical stability in MDC1A mice (Fig. 1). Indeed, such mice were clearly relieved from many of the pathological symptoms associated with the muscular dystrophy³¹⁶.

In a follow-up study we provided additional evidence that the mini-agrin-mediated amelioration is based on the direct linkage of muscle basal lamina with the sarcolemma, what in turn is important for proper muscle fiber regeneration after injury. Hence, mini-agrin both increases the tolerance to mechanical load but also improves the regeneration capacity of the dystrophic muscle. These findings suggest that treatment with mini-agrin might be beneficial over the entire spectrum of the MDC1A disease³¹⁷.

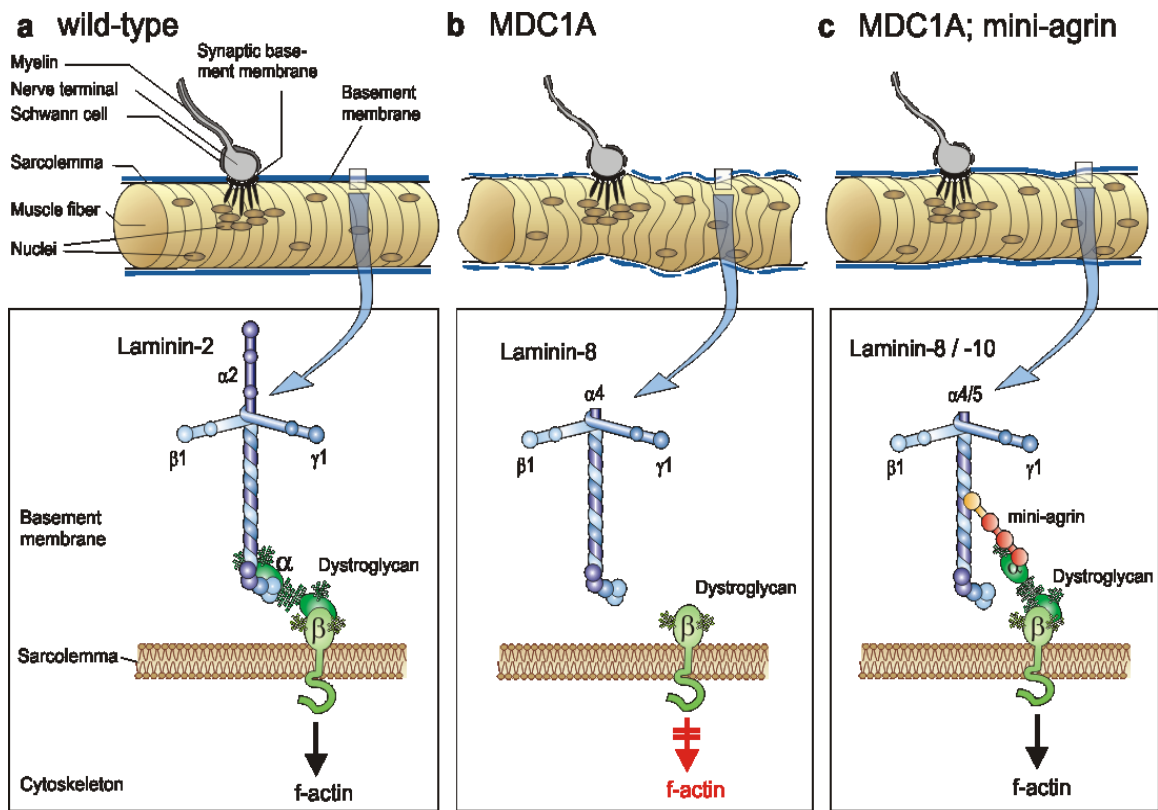


Figure 1: Innervated muscle fiber in wild-type- (a), MDC1A- (b), and MDC1A-mice treated with mini-agrin (c) and potential mechanism involved in disease progression and treatment (lower panels).

a. In wild-type mice, the peripheral nerve is well myelinated and the muscle fibers are healthy. This is based on the linkage of the basement membrane to the cytoskeleton (top panel). This is likely to be thanks to the tight connection of the basement membrane component laminin-2 with α -dystroglycan, which in turn is connected to the transmembranous component β -dystroglycan. β -Dystroglycan connects via linker molecules (not shown) to filamentous actin (f-actin). **b.** Dystrophic MDC1A muscle degenerates and peripheral nerve is demyelinated (top panel). Mutations in laminin- α 2 prevent synthesis of laminin-2. Instead, laminin- α 4 is synthesized in MDC1A muscle to form laminin-8. This isoform does not prevent the disease because it cannot link the basement membrane to α -dystroglycan and does not allow the formation of a proper basement membrane (symbolized by the interrupted line in the top panel). **c.** In MDC1A muscles treated with mini-agrin, both integrity of the muscle and the basement membrane is restored. In contrast, the peripheral nerve is still demyelinated (top panel). Mini-agrin binds via its amino-terminal region to laminin-8 and also stabilizes laminin-10 (α 5, β 1, γ 1). The carboxy-terminal part of mini-agrin connects to α -dystroglycan and restores the connection to the f-actin.

2 Aim of the Thesis

The underlying motivation of this study is to increase life quality and prolong life-span of MDC1A patients in the future. Hence, this investigation aims to contribute to the development of a feasible MDC1A treatment and additionally advance our knowledge of this disease.

2.1 Evaluation of the potential of mini-agrin to slow down the disease progression of MDC1A muscular dystrophy when applied after onset of the disease

This investigation further expands on the concept of the rationally designed mini-agrin able to ameliorate the dystrophic phenotype in mouse models of MDC1A substantially, and aims to make further steps in the evaluation of this strategy towards medical application.

The experiments conducted in the two underlying studies^{317,316}, all used the muscle-specific creatine kinase (MCK)-promoter to drive expression of mini-agrin. This promoter is activated during embryonic muscle development^{333,334} and consequently starts transgene expression before the onset of the disease. In respect to a future application of this approach in the treatment of human MDC1A patients, the main request of this thesis is to investigate the potential of mini-agrin to slow down the disease progression when applied at birth or in advanced stages of the disease in the $dy^{W/-}$ mouse model of MDC1A³⁰⁶.

In summary, we have established the inducible tetracycline-regulated tet-off expression system^{335,336} in $dy^{W/-}$ mice to temporally control mini-agrin expression in skeletal muscles. The tightly regulatable expression conditions enabled us to evaluate the amelioration of the dystrophic phenotype when mini-agrin was applied shortly after birth (at the age of 3 days), at the time point first dystrophic symptoms become apparent (at the age of 14 days) and in an advanced stage of the disease (at the age of 28 days).

We provide strong evidence that mini-agrin applied at birth as well as in advanced stages of the disease significantly diminishes the progress of the dystrophy in $dy^{W/-}$ mice. However, the dystrophic condition of the muscle at the time of mini-agrin application determines the extent of amelioration, since manifested dystrophic symptoms remain and can not be rescued by expression of mini-agrin. Moreover, start of mini-agrin expression stabilizes α -dystroglycan at the muscle membrane and hence profoundly increases the regeneration capacity of the remaining muscle fibers. Thus, the earlier mini-agrin is applied, the higher is the profit of its beneficial properties.

2.2 Evaluation of the Feasibility of Gene Therapy to Deliver Mini-agrin into the Diseased Muscles of MDC1A mice

We intended to critically test whether adenoviral (AdV) or adeno-associated viral (AAV) vector-mediated gene therapy could be used to introduce the mini-agrin into the skeletal muscles of $dy^{W/-}$ mice, in order to achieve a comparable amelioration of the dystrophic muscle pathology as in the transgenic approach.

In summary, adenoviral vectors expressing mini-agrin under control of the MCK-promoter, were amplified in 293 cells³³⁷ to produce high titer virus stocks and were used for *in situ* infection of muscles of $dy^{W/-}$ mice. The intramuscular injection presented reasonable transduction efficiencies but extensive necrosis at the site of injection.

Very recently, Qiao and colleagues⁴, demonstrated successful AAV-mediated systemic mini-agrin gene delivery leading to long-term transduction of whole body skeletal and cardiac muscle in MDC1A mice and resulting in a substantial amelioration of the dystrophic phenotype (see also Appendix II: News and Commentary). This rendered our experiments unnecessary.

2.3 Evaluation of the Potential of Full-length Muscle Agrin to Diminish the Disease Progression in MDC1A

Full-length muscle agrin is present at low levels in the extracellular matrix of adult skeletal muscles. The increase of endogenous agrin expression levels in skeletal muscles by pharmacologically active compounds would be a safe and promising strategy for the treatment of MDC1A. To this end, we aimed to investigate the efficacy of full-length muscle agrin to diminish the disease progression in $dy^{W/-}$ mice.

In summary, we show that constitutive overexpression of chick full-length agrin significantly ameliorates the dystrophic phenotype in $dy^{W/-}$ mice. However, the benefit of full-length agrin on the overall function of skeletal muscles, the slow-down of the disease progression, and the attenuation of ongoing degeneration was less pronounced compared to the effect attained by mini-agrin.

III MATERIALS AND METHODS

Generation of the constructs

The mouse mini-agrin cDNA was obtained by two independent RT-PCRs on mRNA isolated from mouse skeletal muscle. The 0.75kb 5' region encoding for the laminin binding and the 1.follistatin like domain was linked by *Nhe I* to the 2.2kb 3' part of agrin encoding the α -dystroglycan binding domains. A 5x myc-tag was inserted in-frame as a 0.25kb *Cla I - Sal I/SpeI* fragment to the 3' end of the construct. The mini-agrin-myc (m-mag) construct was sequenced and subcloned as a 3.2kb *EcoRV - Sal I* fragment downstream of the tet-responsive promoter of a) the uni-directional pTRE-2 vector (Clontech) and b) the bi-directional pBI-G vector (Clontech) co-expressing *LacZ*. The *Xho I* site in the pTRE-2 vector was replaced by a *Pac I* site what allowed to linearize the construct as a 4.9kb *Pac I - Ase I* fragment for pronuclear injection. The pBI-G vector was linearized as a 9.58kb *Ase I* fragment.

The chick full length agrin (c-FLag) construct was subcloned downstream the muscle-specific creatine kinase (MCK) promoter.

Protein production

The mouse mini-agrin and the perlecan-agrin constructs both were subcloned into pCEP-Pu vector and were transfected into HEK 293 EBNA cells constitutively expressing the EBNA-1 protein. Conditioned media was collected and tested by dot blots for its approximate content of protein. The supernatants were directly used for experiments.

Affinity binding assays

96 well plates were coated with either chick α -dystroglycan or laminin-1 (0.5 μ g / well), followed by incubation with chick mini-agrin (50nM as start conc.) and supernatant containing mouse m-mag protein (pure sup. as start conc.) at descending concentrations (dilution ratio 1:6). The proteins were detected by polyclonal antibodies against chick respectively mouse C-terminal agrin as well as by monoclonal antibody against the myc-tag. 1q5 minutes after activation of HRP, absorbances were measured at 405nm and then normalized. Dose-response curves are shown in half-logarithmic diagrams. Binding data were fitted by non-linear regression analysis, assuming a single class of equivalent binding sites.

Overlay assays

Protein extraction from wild-type chick and/or mouse skeletal muscles was separated on a 3-15% SDS gel. Proteins were blotted to nitrocellulose membrane and were incubated with supernatant containing mouse mini-agrin or perlecan-agrin protein. Protein bound to α -dystroglycan was detected by the monoclonal antibody against the myc-tag or the C-terminal part of agrin, respectively.

Generation and genotyping of transgenic and dystrophic mice

The tet-m-mag and the MCK-c-FLAG constructs were injected into male pronuclei. The chimeric offspring of all lines was tested for the content of the insert by southern blot and PCR of tail biopsies. Chick mini-agrin (c-mag) transgenic mice were created as described³¹⁶. MCK-tTA mice were obtained from Dr. Nina Raben³³⁸. *Dy*^{W/-} mice^{309,339} containing a *LacZ* insertion in the *lama2* gene served as the mouse model for MDC1A. Genotyping of heterozygous and homozygous *dy*^{W/-} mice was done by PCR with one primer within the *LacZ* insertion, (5'-GTCGACGACGACAGTATCGGCCTCAG-3') and two primers specific for the exons in the *lama2* gene (5'-ACTGCCCTTTTCTCACCCACCCTT-3' and 5'-GTTGATGCGCTTGGGAC-3'). Primers for genotyping PCR of tet-m-mag mice were designed to amplify a 683bp fragment including the linkage region of N25 and C95 part of the m-mag construct (5'-GCGGATCACTTTGCGGAACC-3' and 5'-TCGAACCTGAACTGTACATGACC-3'). C-FLAG as well as c-mag³¹⁶ mice were genotyped with primers amplifying a 591bp fragment in the C-terminal part of agrin (5'-ACCTGGATAAGCGTTTTGTT-3' and 5'-CTTCTGTTTTGATGCTCAGC-3'). Genotyping of AgPerl mice was performed on the chick agrin part of the construct (5'-GTCCCTTGCTGATGACCTTGA-3', 5'-ACCCAGCCCCTCAGTACATGT-3'). Distinction of hemi- from homozygous MCK-tTA mice was done by quantitative TaqMan PCR (TaqMan™ PCR core reagent kit, Applied Biosystems) on the tTA-transgene (5'-GCCTACATTGTATTGGCATGTAAAA-3', 5'-CAAAAGTGAGTATGGTGCCTATCTAACA-3', Probe 5' FAM-CTTTGCTCGACGCCTTAGCCATTGAG-TAMRA 3') normalized to β -actin (5'-CCACTGCCGCATCCTCTT-3', 5'-GCTCGTTGCCAATAGTGATGAC-3', Probe 5' FAM-CCCTGGAGAAGAGCTATGAGCTGCCTG-TAMRA 3').

Breeding

A mouse line heterozygous for the *lama2* mutation and hemizygous for the tet-m-mag transgene, and another mouse line heterozygous for the *lama2* mutation and homozygous for the MCK-tTA transgene were cross-bred to give rise to *dy*^{W/-} mice in which m-mag expression can be temporally regulated in skeletal muscles (*dy*^{W/-}/m-mag 3, 14, 28d). For the constitutively expressed transgenes (c-mag, c-FLAG) mice heterozygous for the *lama2* mutation and hemizygous for the c-FLAG or c-mag transgene were mated to mice heterozygous for the *lama2* mutation.

Transgene expression

Expression levels of the different transgenic lines were evaluated by Northern blot assays (Northern Max, Ambion), immunoblots and immunostainings. For Northern blots total RNA extracted from skeletal muscle was processed according to the Northern Max (Ambion) protocol. For immunoblots muscles were homogenized in 1ml extraction buffer (75mM Tris-HCl, pH 6.8, 3.8% SDS, 4 M urea, 20% glycerol and 5% β -mercaptoethanol). After protein denaturation (95 °C for 5min), non-dissolved protein was removed by centrifugation (14,000g for 5min). Equal amounts of protein were loaded onto each lane, separated on 3–12% SDS–polyacrylamide gel

electrophoresis (PAGE) and immunoblotted. For quantification, mRNA and protein signals of the transgenes were normalized to corresponding β -actin or tubulin signals and referred to expression in wild-type animals. Quantification of immunostainings and antibodies are described below.

For staining of the *LacZ* activity, fresh dissected muscles were fixed in 4% PFA/PBS. After washing (100mM sodium phosphate, 2mM $MgCl_2$, 0.01% Na-deoxycholate, - 0.02% Nonidet P40) whole muscles were stained for 24hrs at 37°C using a freshly prepared stain solution comprised of 5mM K-ferricyanide, 5mM K-ferrocyanide and 1mg/ml X-gal in rinse buffer. Stained muscles were postfixed in 10% formalin and then were transferred to 70% ethanol for long time storage or were mounted for analysis by light microscopy.

The inhibition of mini-agrin transcription after re-addition of doxycycline to mini-agrin expressing mice was tested by a quantitative TaqMan PCR (TaqMan™ PCR core reagent kit, Applied Biosystems) on the m-mag-transgene (5'- TGTGCCAATGTGACCGCTA -3', 5'- GCTGAAACCCTTGCCAGAA-3', Probe 5' FAM- CCCCCAAAGTCCTGTGATTCCC -TAMRA 3') normalized to β -actin (5'-CCACTGCCGCATCCTCTT-3', 5'-GCTCGTTGCCAATAGTGATGAC-3', Probe 5' FAM-CCCTGGAGAAGAGCTATGAGCTGCCTG-TAMRA 3').

Regulation of the tet-off system

Temporal regulation of m-mag expression under the tetracycline-regulated expression system³³⁵ was optimal by administration of 5ug doxycycline (Doxycycline hydrochloride, Sigma) per milliliter drinking water (enriched by 4% sucrose) in dimmed bottles. To repress the transgene after it was expressed, the concentration of doxycycline was increased to 50ug/mL.

Locomotion, muscle strength and creatine kinase assay

Locomotive behaviour in animals of different genotypes was determined as described elsewhere³¹⁶. In brief, mice were placed into a new cage and motor activity was measured for 10 minutes. All movements such as walking, digging, or righting up were included. Muscle strength was evaluated by a modified grid test³⁴⁰. In brief, animals of the different genotypes were placed on a vertical grid and the time until they fell down was measured. After 3 minutes, the test was finished. The procedure was repeated 3 times with each animal and values were referred to values obtained from wild-type animals. The creatine kinase levels were measured in the blood collected from the mouse-tail vein. 2 μ l of serum was applied using the creatine kinase CK-NAC Liqui-UV kit (Rolf Greiner Biochemica). In all tests, at least four animals of each genotype were analyzed.

Histology, immunohistochemistry and antibodies

Muscles were immersed in 7% gum Tragacanth (Sigma, St. Louis, MO, USA) and rapidly frozen in liquid nitrogen-cooled isopentane (-150°C). Cross sections of 12 μ m thickness were cut. General histology was performed using hematoxylin and eosin (H&E) (Merck, Rayway, NJ, USA). Membrane-bound and extracellular epitopes were visualized with Alexa-488-conjugated wheat

germ agglutinin (WGA; Molecular Probes, Eugene, OR, USA). Polyclonal rabbit anti-mouse laminin- α 5 (Ab 405) was a kind gift from Dr. L. Sorokin, Lund University. Polyclonal sheep anti-mouse α -dystroglycan was a kind gift from Dr. S. Kröger (University of Mainz). The remaining antibodies were produced in-house or purchased from commercial sources as follows: Monoclonal mouse anti-rat developmental myosin heavy chain (dMyHC; Novocastra), monoclonal rat anti-mouse laminin- γ 1 chain (Chemicon), polyclonal rabbit anti-chick agrin (Ab 3228¹⁰). Mouse monoclonal anti-Myc tag antibody (9E10) was produced and purified from hybridoma cell line 9E10. It was biotinylated (D-Biotinoyl-E-Aminocapronsäure N-Hydroxysuccinimidester, Roche) to prevent cross-reaction of the secondary antibody with the mouse tissue. Depending on the source of the primary antibody, appropriate Cy³-conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or Alexa-488-conjugated (Molecular probes) secondary antibodies were used. 4',6'-Diamidino-2-phenylindole hydrochloride (DAPI) was used to stain for nuclei.

Quantification of immunostainings

For quantification of the muscle fiber size distribution, pictures of WGAs were collected using a Leica DM5000B fluorescence microscope, a digital camera (F-View; Soft Imaging System, Lakewood, CO, USA), and analySIS® software (Soft Imaging System). The muscle fiber size distribution was determined using the minimum distance of parallel tangents at opposing particle borders (minimal "Feret's diameter") as described elsewhere³⁴¹. Measurement of minimal Feret's diameter of notexin-treated muscle was done on cross-sections stained for laminin- γ 1 and dMyHC, whereas only regenerating (dMyHC-positive) fibers were considered. Normalization of the number of fibers in each fiber feret class was based on the total number of muscle fibers on each picture. In all quantification experiments, a minimum of 4000 muscle fibers per mouse and at least three mice of each genotype were analyzed.

For quantification of the fibrosis, the fibrotic area of WGA-stained muscle cross-section was measured, normalized to the entire area of the cross-section and referred to wild-type animals. At least four cross-sections of *triceps brachii* were quantified for each genotype.

To determine transgene expression levels as well as laminin α 5 and α -dystroglycan protein expression in immunostainings, the primary antibodies were diluted such that the signal was not saturated. Images were collected and analyzed with a confocal microscope (Leica TCS-8P) and appropriate software. Specific intensity was calculated for each image as the signal intensity of the muscle circumference minus that of an adjacent, non-stained region³⁴². Five different pictures were taken using the same parameters on each section, and four different sections were used for each individual mouse.

Muscle regeneration

Skeletal muscle was injured by injection of the myotoxin notexin (Sigma) into the *tibialis anterior* of 35-day-old mice. *Dy*^{W/-}/m-mag 28d, *dy*^{W/-} and WT mice were anesthetized and a ~3mm-long incision into the skin was made at the proximal end of the muscle. A Hamilton micro syringe was

inserted and advanced to the distal end of the muscle. 15-20 microliters of notexin (50µg/mL) were injected slowly into the muscle while pulling the needle back to assure delivery of the myotoxin along the entire muscle. Mice were killed by CO₂ asphyxiation 6 or 14 days later; muscles were isolated and processed as described above.

Intravenous injection of recombinant mini-agrin protein

The mouse mini-agrin construct was subcloned into pCEP-Pu vector and was transfected into HEK 293 EBNA cells. Conditioned media was collected and the protein was purified by DEAE columns and concentrated to 1mg/ml. 0.1ml was slowly injected into the tail vein of a wild-type mouse. Muscles were collected 24 hours after injection.

Generation of adenovirus (AdV)

Recombinant adenoviral vectors carrying the mini-agrin under the control of the muscle-specific creatine kinase (MCK) -promoter, were produced using a shuttle cloning strategy based on the AdEasy™ XL system (Stratagene, La Jolla, CA). In short, the subcloning part is performed in the pShuttle plasmid, where expression cassettes consisting of promoter, gene of interest and poly-adenylation signal can be assembled prior to their transfer into an adenoviral backbone. In the transfer step one takes advantage of a homologous recombination in bacteria between the shuttle plasmid and the adenoviral backbone clone pAdEasy-1, which is initiated between 'left arm homologous (LAH)' and 'right arm homologous (RAH)' regions flanking the expression cassette and the corresponding LAH and RAH sequences in the adenoviral backbone. In a first step, a 0.12kb Sall-XhoI fragment containing the beta-Globin poly-adenylation signal (pA) sequence was subcloned into the multiple cloning site (MCS) of the pShuttle plasmid. The MCK1350, a 1.35kb BgIII - EcoRI fragment from the human muscle creatine kinase promoter was subcloned upstream of the pA sequence. The 3.2kb EcoRV -Sall fragment cDNA encoding mouse mini-agrin was put downstream of the respective promoter sequence. The following steps - linearization of the shuttle plasmid, transformation of each of the constructs into bacterial strain BJ5183-AD-1 (pre-transformed with pAdEasy-1 vector) and selection for KanR clones - resulted in recombinant adenoviral plasmids containing mini-agrin expression cassettes in an adenoviral backbone. After bacterial amplification and single cut restriction digest, the linearized plasmid was transfected into 293 cells to produce recombinant adenovirus. Positive clones have been selected on the basis of the cytotoxic effect on 293 cells after 48h, the absence of adenoviral E1 region in isolated adenoviral particles (as determined by PCR analysis with E1-specific primers) and the integrity of the genomic DNAs of the adenoviral constructs in isolated adenoviral particles (as determined by PCR and restriction digest analysis). Large-scale production of single constructs in 293 cells was done by repeated amplification (serial infection and lysis steps). Finally, 1-3 x 10E12 viral particles could be harvested from 10 l spinner cultures and were purified by CsCl step gradient centrifugation. In order to check the expression behavior of the respective mini-agrin constructs, we performed Western blot analyses on infected 293 cells using a myc-antibody confirmed proper mouse mini-agrin-myc expression.

In situ* infection of *triceps brachii

Two week-old wild-type and *dy*^{W/-} mice were anesthetized and the skin above the *triceps brachii* muscle was opened by minimal cut. A Hamilton micro syringe was introduced into the muscle and 15 μ l of either adenoviruses expressing mini-agrin (1×10^{11} pfu/ml) or PBS were slowly injected while pulling the needle back to assure delivery along the entire muscle. The wound was closed post-injectionally. Mice were killed by CO₂ asphyxiation 16 days later, injected muscles were isolated and processed as described above.

Statistical analysis

To compare the different genotypes, P-values were calculated using the unpaired two-sample *t*-tests, assuming equal variances.

IV RESULTS

1 Therapeutic Potential of Mini-agrin in a Mouse Model for MDC1A at All Stages of the Disease

Sarina Meinen and Markus A. Rüegg

a. Cloning of the cDNA encoding the mouse mini-agrin-myc (m-mag)

We aim to investigate the potential of mini-agrin to slow down the disease progression when it is applied at birth or in advanced stages of the disease in the $dy^{W/-}$ mouse model of MDC1A. This project is based on the striking amelioration observed in $dy^{W/-}$ mice constitutively overexpressing chick mini-agrin (c-mag) in skeletal muscles³¹⁶. In this underlying study, a chicken-derived agrin mini-gene was used, mainly in regard to facilitate its detection in the transgenic mice. It is well known that postnatal application of extraneous transgenes eventually evokes immune responses in the host. To minimize the immunological rejection of the postnatally expressed mini-agrin

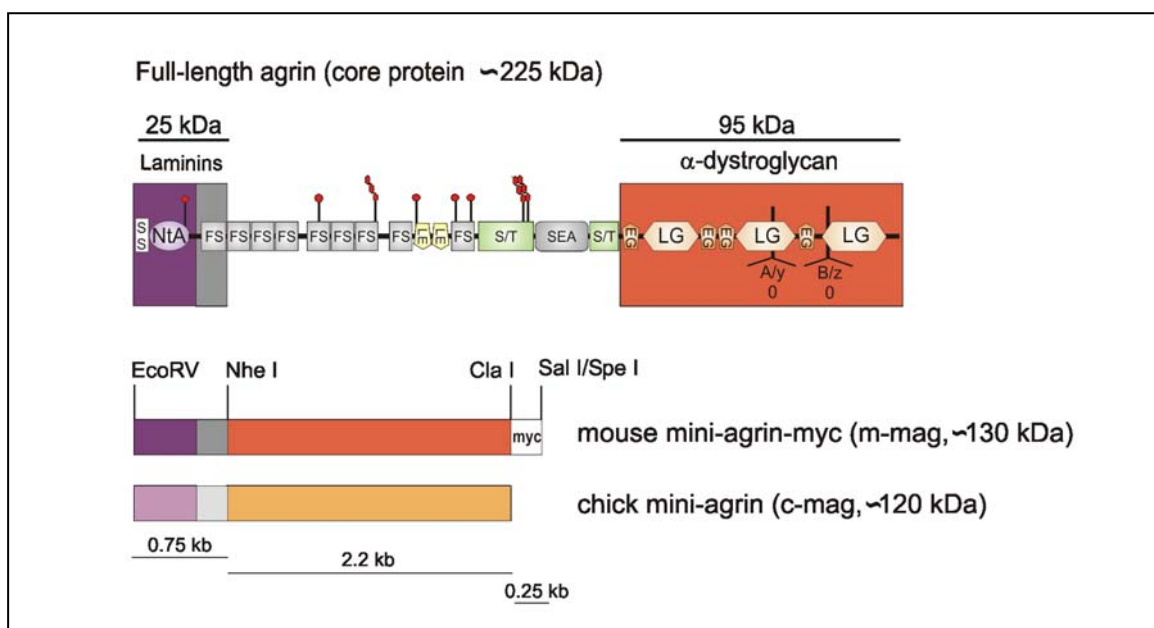


Figure 1: Schematic representation of the mouse mini-agrin-myc (m-mag) construct and its orthologue chick mini-agrin (c-mag) as described in Moll et al., 2001. Mini-agrin represents a fusion construct between the 0.75kb 5' region encoding the 25kDa N-terminal agrin (NtA) containing laminin-binding properties and the 1. follistatin-like (1.FS) domain, and the 2.2kb 3' region encoding the 95kDa C-terminus consisting of EGF-like and laminin G-like domains and containing α -dystroglycan binding properties. Mouse mini-agrin was generated out of mouse muscle-derived agrin cDNA. To allow specific detection in the transgenic mice, a 5x myc-tag was added to the 3' end of the mouse mini-agrin construct (m-mag). Restriction sites used to generate mouse mini-agrin are indicated.

we therefore decided to replace the chick mini-agrin by a mini-agrin deriving from the mouse muscle agrin isoform expressed at low levels in all skeletal muscles (Fig. 1).

Mini-agrin represents a fusion protein between agrin's laminin-binding N-terminus and its α -dystroglycan binding C-terminus. The corresponding encoding cDNAs were obtained by two independent RT-PCRs on mRNA isolated from mouse skeletal muscle. After subcloning and DNA sequencing, the two cDNAs were fused. To distinguish the mouse mini-agrin from the endogenously expressed mouse agrin in the transgenic mice, we added a five times repeated myc-tag in-frame to the 3' end of the mini-agrin construct giving rise to the 3.2kb mini-agrin-myc (m-mag) cDNA encoding a ~130kDa protein.

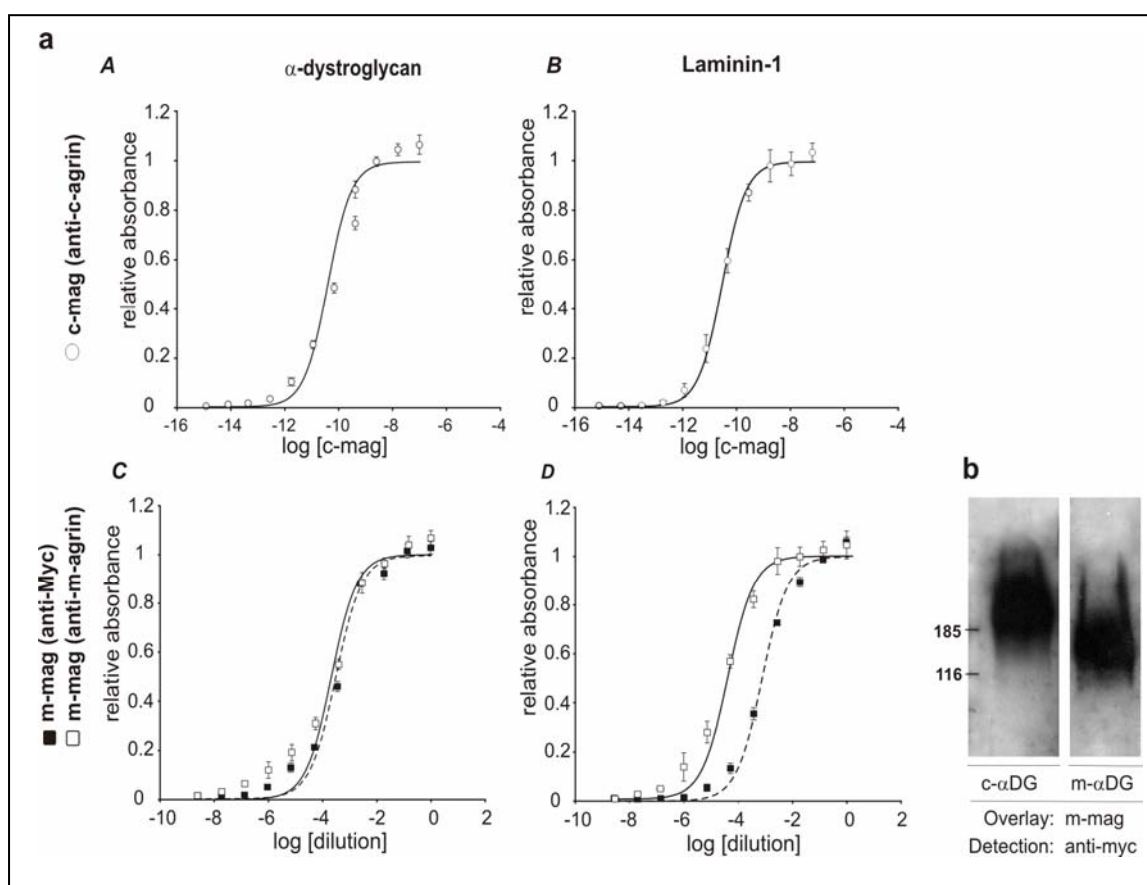


Figure 2: Binding of the transgenic mouse mini-agrin-myc (m-mag) to laminin and α -dystroglycan.

a. Solid-phase binding assays using 96-well plates coated with chick α -dystroglycan (A, C) or mouse laminin-1 (B, D). To detect chick mini-agrin, a polyclonal antiserum raised against chick agrin was used. Detection of mouse mini-agrin was done by using either polyclonal antibodies against C-terminal half of mouse agrin or the monoclonal antibody 9E10 against the myc-tag. Both c-mag and m-mag proteins bind to α -dystroglycan and laminin-1 in a dose-dependent, saturable manner. Each data point represents the mean \pm SD. (n=3). Binding data were fitted by non-linear regression analysis, assuming a single class of equivalent binding sites. **b.** Overlay binding assays using m-mag. Partially purified α -dystroglycan from chick (c- α DG) or mouse (m- α DG) skeletal muscle was separated by SDS-PAGE, transferred to nitrocellulose and incubated with supernatant from HEK 293 EBNA cells transfected with cDNA encoding m-mag. Bound protein was detected with 9E10 (anti-myc).

b. Testing of the function of the recombinant m-mag protein

The addition of the myc-tag to the C-terminal end of mouse mini-agrin eventually influences the binding properties of the mouse compared to the proved and non-tagged chick mini-agrin³¹⁶. To rule out major impairments we tested the binding of recombinant m-mag protein to laminin-1 and α -dystroglycan in affinity binding (Fig. 2 a) and overlay assays (Fig. 2 b)

For this purpose, recombinant mouse m-mag protein was produced in human embryonic kidney (HEK) cells constitutively expressing the EBNA-1 protein. Dot blot assays allowed to estimate an approximate concentration of mini-agrin-myc protein in the conditioned media (not shown), which was further used for dose-response curves to either chick α -dystroglycan or mouse laminin-1 as well as for overlay assays to α -dystroglycan. Results indicate that m-mag protein has similar binding properties as its chick orthologue and that the addition of the myc-tag to the C-terminal end of mouse mini-agrin does not impair this binding. Furthermore, the myc-tag is detectable when mini-agrin is bound to α -dystroglycan or laminin and therefore will allow the distinction of the transgenic mini-agrin from endogenous agrin in the transgenic mice (Fig. 2 a + b).

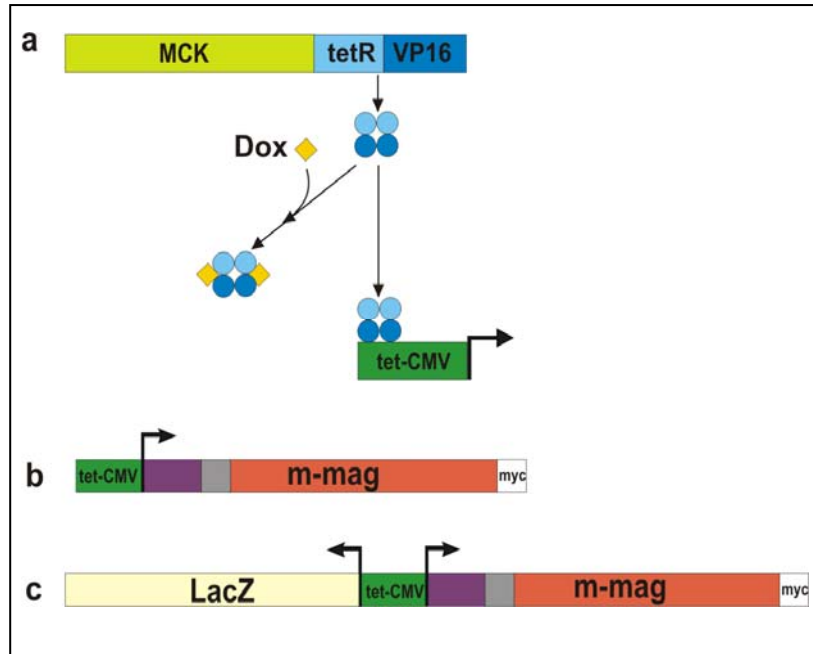
c. Generation of mice expressing m-mag under control of the inducible tetracycline-regulated "tet-off" expression system

Our aim to generate $dy^{W/-}$ mice in which mini-agrin expression in skeletal muscles can be temporally controlled, prompted us to establish the inducible tetracycline-regulated 'tet-off' expression system^{335,336} (Fig. 3 a). This system is designed to express the target transgene only upon binding of the tetracycline-dependent transcription activator (tTA) to the tet-responsive promoter (tet), containing seven tTA binding sites and a cytomegalovirus (CMV)-promoter localized upstream of the target gene. This binding is suppressed in presence of the antibiotic doxycycline (Dox). Doxycycline is known to pass placenta as well as to be transferred by nursing, implying the ability of the tet-off system to regulate transgene expression in the offspring.

In order to produce transgenic mice containing m-mag under the control of the tet-responsive promoter (tet-m-mag), we generated two independent constructs containing m-mag downstream of a uni-directional (Fig. 3 b) and a bi-directional (Fig. 3 c) tet-responsive promoter, the latter of which co-expresses nuclear localized *LacZ* as a reporter gene. The linearized constructs were injected into male pronucleus by the in-house "transgenic mouse core facility" to randomly insert into the genome. Male pronuclei later were implanted into pseudo-pregnant mice. Of the 69 and 35 mice born of the uni- and the bi-directional injection series respectively, tail biopsies were tested by PCR using primers designed to amplify a fragment including the linkage region between the 1. follistatin-like domain and the C-terminal region of the m-mag construct (data not shown). We detected eight chimeric founder mice (5 males; 3 female) carrying the uni-directional promoter and seven founders (6 males; 1 female) carrying the bi-directional promoter upstream m-mag. This result was verified by Southern blot analysis using a probe spanning the linkage region of the m-mag fusion construct (data not shown).

Figure 3: The inducible tetracycline-regulated “tet-off” expression system.

a. Functional elements of the inducible tetracycline-regulated ‘tet-off’ expression system. Function of the ‘tet-off’ system is composed of a team-work of two transgenes in response to the repressor agent doxycycline (Dox). The activator transgene, the tetracycline-dependent transcription activator tTA, is expressed under control of the muscle-specific creatine kinase (MCK-) promoter to target expression to skeletal muscles. It constitutes a fusion of the tet repressor of Transposon10 (tetR) and the transactivator domains of the



herpes simplex protein VP16. The second transgene is the tet-responsive promoter (tet) containing binding sites for tTA, followed by a CMV-promoter and the target gene m-mag. Binding of tTA to these sequences, activates the CMV-promoter and drives expression of m-mag. In the tet-off system, this binding is suppressed in presence of the antibiotic doxycycline (Dox), leading to inhibition of mini-agrin expression. **b.** - **c.** Constructs containing m-mag downstream the tet-responsive promoter. **b.** Uni-directional tet-responsive promoter (pTRE-2, Clontech), solely expressing m-mag. **c.** Bi-directional tet-responsive promoter (pBI-G, Clontech), co-expressing m-mag and *LacZ*.

All the chimeric founders were back-crossed to C57BL/6 mice and the F1 progeny was tested for the presence of the transgene. Four out of eight founders containing the uni-directional and all seven founders carrying the bi-directional tet-responsive promoter transmitted the transgene to the F1 progeny, giving rise to lines 1-4 for the uni-directional and 1-7 for the bi-directional tet-m-mag strain. To prevent later segregation and eventual subsequent loss of transgene expression levels due to multiple insertion sites of tet-m-mag in the genome, the transgenic F1 progeny was back-crossed to C57BL/6 for an additional generation and a 1:1 ratio of transgene transmission was confirmed in the F2 progeny.

Mice hemizygotously carrying the tet-m-mag transgene (tet-m-mag^{+/-}) were mated to mice homozygotously expressing the tTA activator transgene under control of the muscle-specific creatine kinase (MCK)-promoter^{338,343} (MCK-tTA^{+/+}). Skeletal muscles of offspring hemizygotously carrying both transgenes (MCK-tTA^{+/-}/ tet-m-mag^{+/-}) were then tested for their m-mag expression levels in skeletal muscles (Fig. 4). In the uni-directional tet-m-mag strain, mRNA transcripts in Northern blot (Fig. 4 a) and protein levels in immunostaining (Fig. 4 c) and Western blot analysis

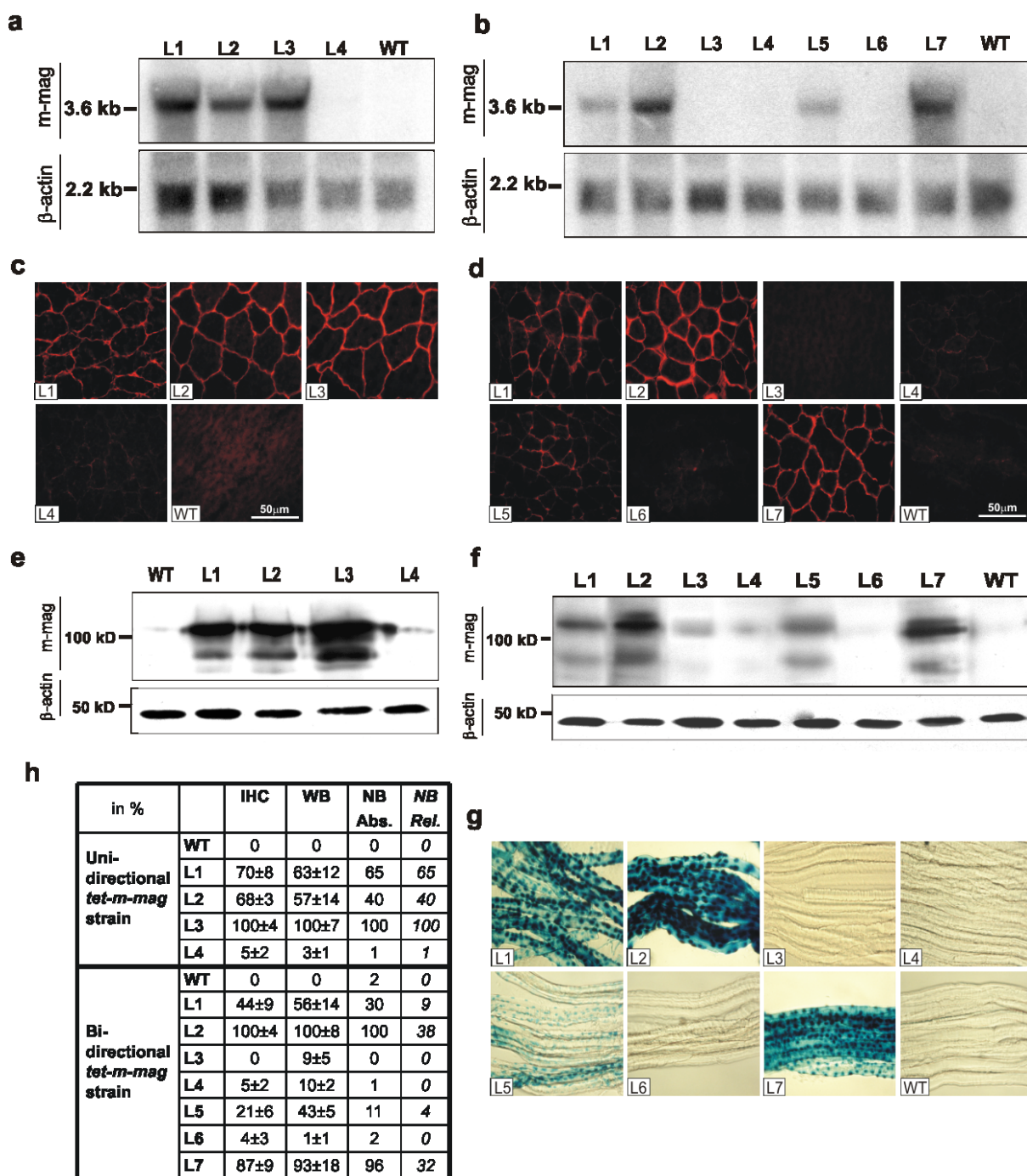


Figure 4: Analysis of m-mag expression activity in MCK-tTA^{+/+} tet-m-mag^{+/+} mice expressing m-mag driven by the uni- (a, c, e, h) and the bi-directional (b, d, f, g, h) tet-responsive promoter.

a. + b. Northern blot analysis of *quadriceps* muscle, **c. + d.** immunostaining of *triceps brachii* cross-sections and **e. + f.** Western blot analysis (**e + f**) of *triceps brachii* muscle. Analysis of m-mag mRNA transcription activity (**a + b**) and m-mag protein expression levels (**d - f**) both suggest mouse line 3 followed by line 1 and 2 of the uni- (**a, c, e, h**) and mouse line 2 and 7 followed by line 1 and 5 of the bi-directional (**b, d, f, h**) MCK-tTA^{+/+} tet-m-mag^{+/+} strain to express the transgene in a decreasing manner, respectively. **g.** Nuclear beta-galactosidase activity in the bi-directional strain reliably reflects m-mag transgene expression. **h.** Quantification of the m-mag expression levels in the different lines as determined by the analysis described in a-f. For quantification, mRNA levels in Northern blot and protein levels in Western blot analysis were normalized to corresponding β -actin signals. Based on these results we decided to continue our experiments with mouse line 3 of the uni- and with mouse line 2 of the bi-directional tet-m-mag^{+/+} strain.

(Fig. 4 e) suggest mouse line 3 followed by line 1 and 2 to have the highest m-mag expression activity. Nearly no m-mag expression was detected in line 4. In the bi-directional tet-m-mag strain, mRNA signals of m-mag in Northern blot analysis (Fig. 4 b) and protein levels visualized in immunostaining (Fig. 4 d) and Western blot analysis (Fig. 4 f), revealed highest transgene expression levels in line 2 and 7, followed by lines 1 and 5. No transcripts and proteins were detected in line 3, 4 and 6. In addition, m-mag expression activity in the bi-directional tet-m-mag strain correlated well with the nuclear expression of the reporter gene beta-galactosidase (Fig. 4 g). Moreover, muscles of mice only carrying the tet-m-mag transgene did not show any m-mag expression activity in none of the tests.

Based on these results, non-expressing lines were eliminated. Mouse line 7 of the bi-directional tet-m-mag strain represented health impairment and poor mating behavior (probably due to transgene incorporation into the genome at an important gene locus) and was eliminated as well. We decided to continue our experiments with mouse line 3 of the uni-directional and mouse line 2 of the bi-directional tet-m-mag strain, both showing a good overall health and mating behavior.

d. Tight spatial and temporal regulation of mouse mini-agrin-myc expression in $dy^{W/-}$ mice

Muscle-specific expression of the mini-agrin transgene relies on the expression of the tTA activator transgene driven by the muscle-specific creatine kinase promoter (MCK-tTA). To ensure the targeted expression of m-mag to muscles and thus prevent eventual side effects due to a widespread unspecific expression pattern, we evaluated the spatial regulation of tTA and consequent m-mag expression in the two MCK-tTA^{+/-}/tet-m-mag^{+/-} mouse lines shown to express high levels of m-mag in skeletal muscles (Fig.5 a - c). Western blot analysis of different tissues of the uni-directional line 3 (Fig. 5 a) and the bi-directional line 2 (Fig. 5 b) revealed high mini-agrin expression levels in skeletal and cardiac muscle. Only minimal amounts of m-mag were detected in liver and kidney, whereas no protein was present in lung and brain. In the bi-directional MCK-tTA^{+/-}/tet-m-mag^{+/-} line 2, this spatial expression pattern was confirmed by the exact conformance of *LacZ* activity in the corresponding tissues (Fig. 5c). In summary, these results suggest tTA expression, and thus subsequent m-mag expression, to be specifically targeted to skeletal and cardiac muscle where the MCK-promoter is known to be active.

In a next step, we optimized the temporal regulation of m-mag expression by administration of the repressor agent doxycycline. For this purpose, different concentrations of doxycycline were permanently administered to the drinking water of pregnant and gestating females (0.5mg/l; 5mg/l; 20mg/l; and 50mg/l). After 2 weeks of gestation, the antibiotic was withdrawn and the double transgenic offspring (MCK-tTA^{+/-}/tet-m-mag^{+/-}) was tested for induction of m-mag expression. In both of our double transgenic mouse strains, application of 5µg doxycycline per milli-liter drinking water to pregnant and gestating females, was found to completely inhibit mini-

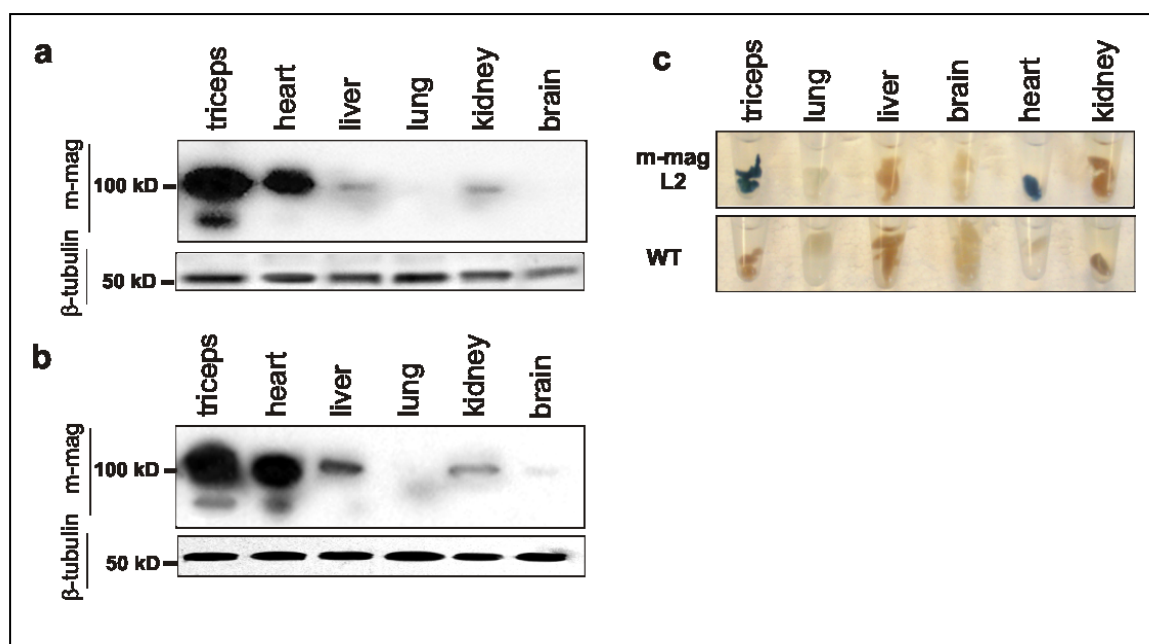


Figure 5: Spatial expression pattern of mini-agrin (m-mag) in the uni- (a) and the bi-directional (b, c) MCK-tTA^{+/}/tet-m-mag^{+/-} mice.

a. + b. Western blot analysis of various tissues (as indicated) suggest high m-mag protein expression in skeletal and cardiac muscle of both the uni- and the bi-directional line 3 (a) and 2 (b), respectively. Residual m-mag was detected in liver and kidney. No expression was detected in lung and brain. **c.** *LacZ* activity in the different tissues of the bi-directional line 2 exactly corresponds to the results obtained by Western blot (b). These results confirm tTA and subsequent mini-agrin expression to be targeted to skeletal and cardiac muscle as expected from tTA expression driven by the MCK-promoter.

agrin transcription and translation in the offspring and additionally allowed a rapid induction (Fig. 6). Northern blot analysis (Fig. 6 a + d) as well as immunostaining (Fig. 6 b + e), Western blot (Fig. 6 c) and *LacZ* activity (Fig. 6 f) all demonstrate a tight repression of m-mag expression in the offspring of gestating females permanently treated with doxycycline (Dox). As short as three days after withdrawal of the antibiotic (wd 3d) m-mag mRNA expression in the uni-directional MCK-tTA^{+/}/tet-m-mag^{+/-} strain was detected by Northern blot analysis (Fig. 6 a). At this time, in the bi-directional strain m-mag transcription has not yet started (Fig. 6 d). Correspondingly, the protein started to be translated in the uni- (Fig. 6 b + c) but not yet in the bi-directional (Fig. 6 e + f) line, as visualized by immunostaining (Fig. 6 b + e) and Western blot analysis (Fig. 6 c) or *LacZ* expression activity (Fig. 6 f). Six days after doxycycline withdrawal (wd 6d), in both strains m-mag expression activity reached levels as when no doxycycline has ever been applied (no Dox, Fig. 6 a - f). Thus, both double transgenic mouse lines expressing m-mag either under control of the uni- or the bi-directional tetracycline-regulated 'tet-off' expression system, allowed precise and efficient spatial and temporal regulation of m-mag expression, verified by quantification of the different analysis (Fig. 6 g).

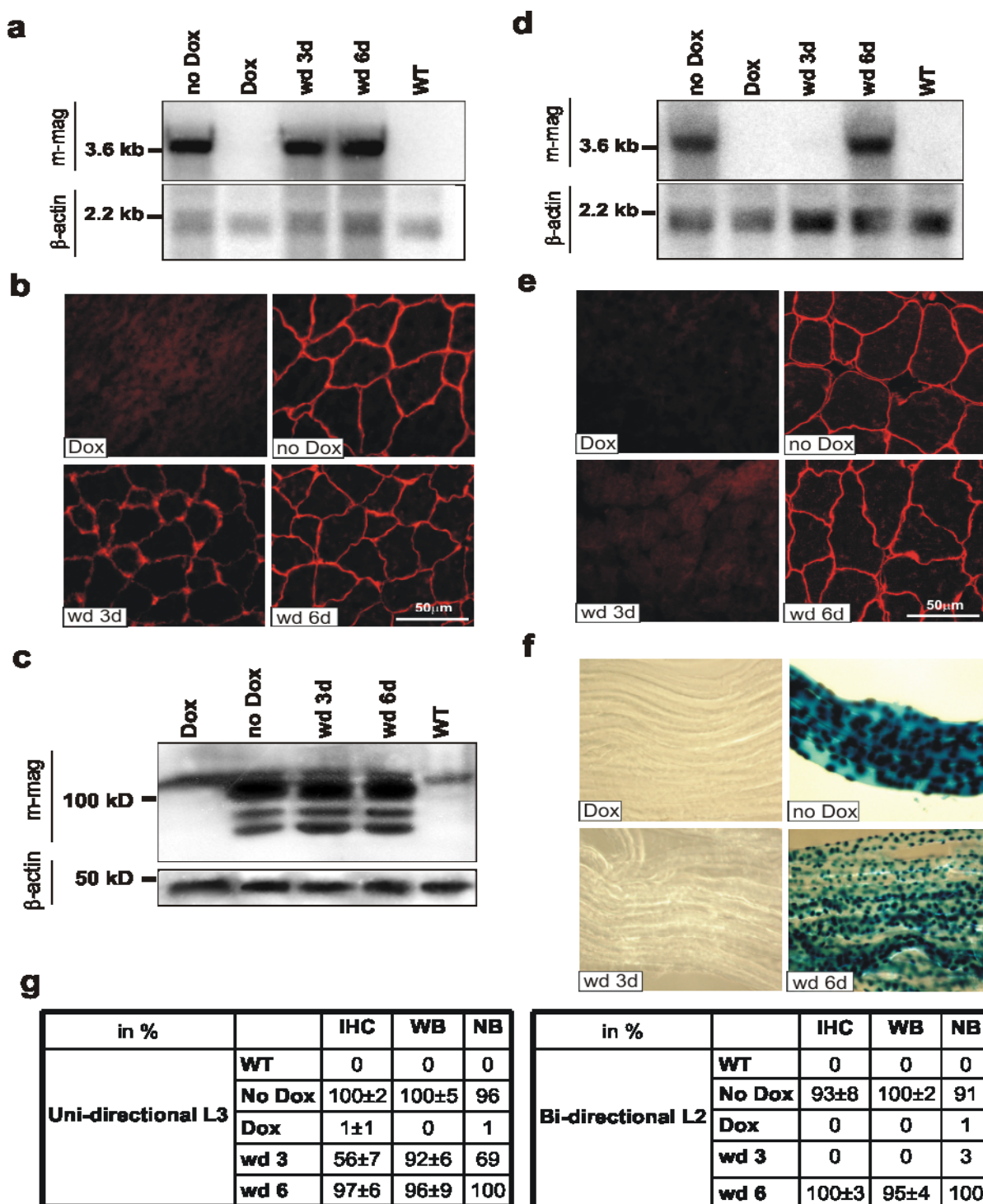


Figure 6: Temporal regulation of m-mag expression in the offspring from females permanently treated with doxycycline during pregnancy followed by withdrawal after 2 weeks of gestation.

a - c. Analysis of the uni-directional line 3 and **d - f.** of the bi-directional line 2 MCK-tTA^{+/+} tet-m-mag^{+/-} mouse strain. Northern blot analysis of *triceps brachii*, (**a + d**) immunostaining of *triceps brachii* cross-sections (**b + e**) as well as Western blot analysis of quadriceps muscle (**c**) and LacZ staining (**f**) of calf muscles all indicate that addition of 5 μg doxycycline (Dox) per milli-liter drinking water is capable to completely repress m-mag expression in both the uni- (**a - c**) and the bi-directional (**d - f**) MCK-tTA^{+/+} tet-m-mag^{+/-} strain. 3 days after withdrawal of the antibiotic (wd 3d), transcription and translation is induced in the uni-directional line 3 (**a - c**). 6 days after withdrawal (wd 6d) in both strains m-mag expression activity reaches equal levels as when no doxycycline (no Dox) was applied. **g.** For quantification, mRNA and protein levels of mini-agrin were normalized to corresponding β-actin signals. Analysis confirm the virtual results, suggesting a tight, rapid and reliable regulation of m-mag expression under control of the inducible tet-off expression system.

In order to generate $dy^{W-/-}$ mice in which mini-agrin expression in skeletal muscles can be temporally controlled, we first generated mouse lines heterozygous for the *lama2* mutation and hemizygous for either the uni- or the bi-directional tet-m-mag transgene ($dy^{W+/-}/tet\text{-}m\text{-}mag^{+/-}$). In parallel, another mouse line heterozygous for the *lama2* mutation plus homozygous for the MCK-tTA transgene was generated ($dy^{W+/-}/MCK\text{-}tTA^{+/+}$). Mating of these two mouse lines ($dy^{W+/-}/MCK\text{-}tTA^{+/+}$ X $dy^{W+/-}/tet\text{-}m\text{-}mag^{+/-}$) eventually give rise to $dy^{W-/-}$ mice in which m-mag expression can be temporally regulated in skeletal muscles (Fig. 7). Using this breeding strategy the probability to get $dy^{W-/-}/MCK\text{-}tTA^{+/-}/tet\text{-}m\text{-}mag^{+/-}$ mice, the genotype desired for further analysis, is one out of eight newborns.

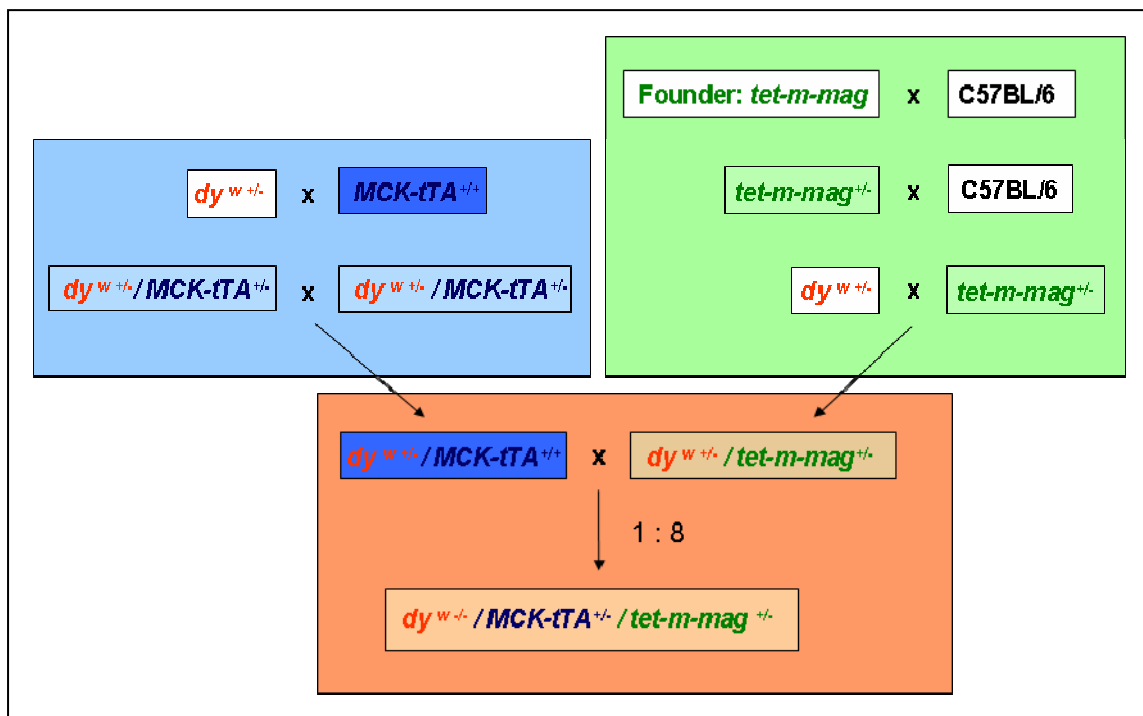


Figure 7: Breeding strategy.

Generation of $dy^{W-/-}$ mice in which mini-agrin expression in skeletal muscles can be temporally controlled.

In the course of producing $dy^{W-/-}/MCK\text{-}tTA^{+/-}/tet\text{-}m\text{-}mag^{+/-}$ mice, it became clear that the construct containing m-mag downstream the bi-directional tet-responsive promoter and giving rise to line 2, has inserted on the same chromosome as the *lama2* gene is located. This was concluded from the complete absence of the $dy^{W-/-}$ genotype containing the tet-m-mag transgene in the offspring. This fact rendered the bi-directional tet-m-mag line 2 useless for our further experiments. Thus, for all further investigations we focused on the uni-directional tet-m-mag line 3.

e. The disease progression is slowed down upon start of mini-agrin expression after birth

We decided to evaluate the amelioration of the dystrophy in 4 and 6 week-old $dy^{W/-}$ mice upon start of mini-agrin expression at different ages after birth. To this end, we induced mini-agrin expression by the removal of doxycycline at birth ($dy^{W/-}/m\text{-mag}$ 3d), at the age of 11 days ($dy^{W/-}/m\text{-mag}$ 14d) when first dystrophic symptoms become apparent, and at the age of 25 days ($dy^{W/-}/m\text{-mag}$ 28d) representing an advanced stage of the disease.

Physiological analysis

We first compared the physical shape of the different genotypes and in average noticed the body size of $dy^{W/-}$ mice to be larger the earlier their muscles started to express the mini-agrin transgene. However, physiological tests elucidate the overall function of skeletal muscles in the $dy^{W/-}$ mice starting mini-agrin expression at the different stages of the disease. The muscle strength of the different genotypes was evaluated by measuring the time the animals performed on a vertical grid before falling down (Fig. 8 a). The difference in the muscle strength of $dy^{W/-}$ mice starting m-mag expression at any age after birth compared to their $dy^{W/-}$ littermates was striking. However, in both 4 and 6 week-old animals, the duration of performance significantly and continuously increased the earlier mini-agrin started to be expressed.

Locomotive activity was estimated in an open-field walking test (Fig. 8 b) by placing the mice into a new cage and measuring the time over 10 minutes they spent exploring the unknown environment. As described³¹⁶, $dy^{W/-}$ mice constitutively overexpressing chick mini-agrin ($dy^{W/-}/c\text{-mag}$) presented near normal locomotion behavior, whereas the activity slightly decreased the later m-mag started to be expressed. However, in 4 week-old $dy^{W/-}$ animals starting m-mag expression at the age of 3 days, the time of activity was still doubled compared to $dy^{W/-}$ littermates.

In summary, both physiological tests indicate a slight decrease of the overall function of skeletal muscles the later mini-agrin was applied in the $dy^{W/-}$ mice. However, in all cases muscle strength and locomotory activity were significantly higher compared to age-matched $dy^{W/-}$ mice. Additionally, in 6 week-old animals inducing mini-agrin expression at the age of 4 weeks, muscle strength and locomotory behavior was significantly increased compared to 6 week-old but was maintained compared to 4 week-old $dy^{W/-}$ mice.

Creatine kinase (CK) is normally localized in the cytosol. It catalyzes the reversible exchange of high-energy phosphate bonds between creatine phosphate and ADP to provide a quick source of energy during contraction. In the course of MDC1A, muscles pass through degenerative processes, during which muscle cells break open, release their contents into the bloodstream and thus give rise to an increased amount of CK in the blood serum. Hence, elevated CK levels in the blood reliably indicate the extent of ongoing muscle fiber degeneration occurring at the time of measurement. Independent of the age, CK levels in $dy^{W/-}$ mice were 5-times elevated compared to wild-type littermates. In $dy^{W/-}$ mice constitutively expressing c-mag or starting m-mag expression at the age of 3, 14 or 28 days, CK levels were reduced to half of the amount

measured in $dy^{W/-}$ mice (Fig. 8 c). Thus, mini-agrin clearly decreases ongoing degeneration, independent of the damage that had occurred before mini-agrin expression has started.

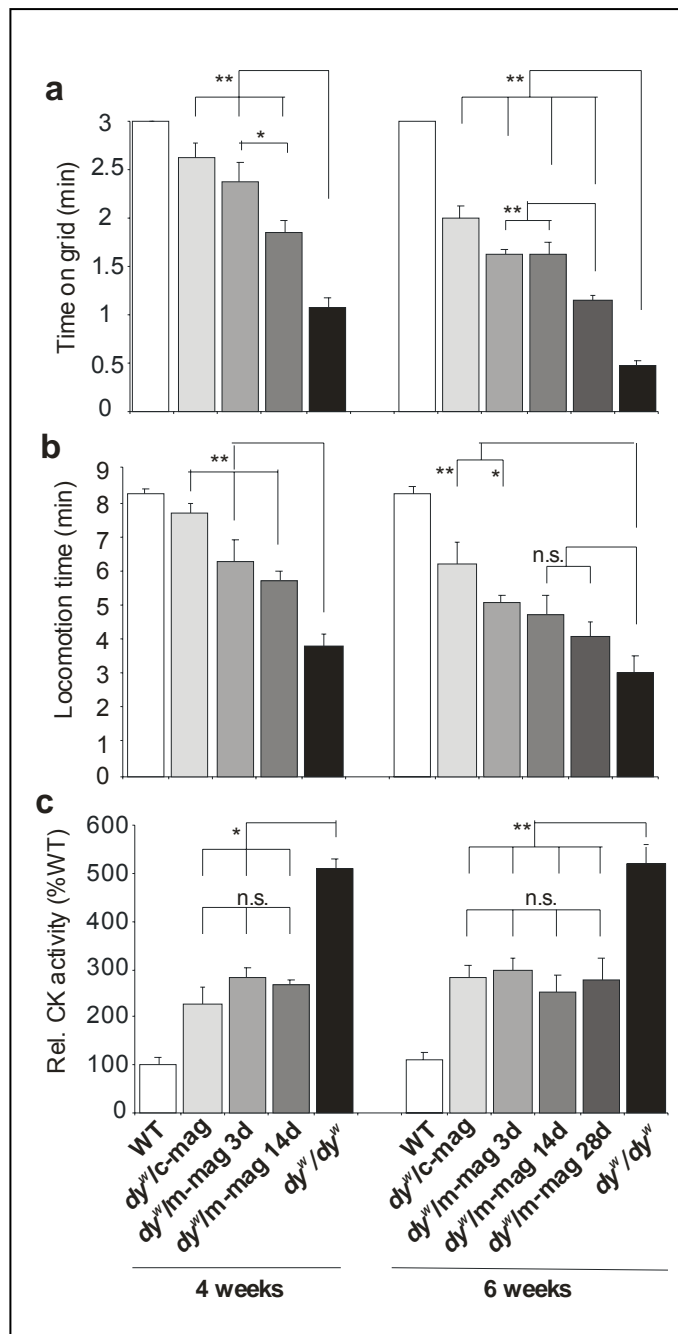


Figure 8: Overall function of skeletal muscles in 4 and 6 week-old $dy^{W/-}$ mice starting mini-agrin expression 3, 14 and 28 days after birth.

For each genotype at least 3 animals were analyzed. **a.** Muscle strength was evaluated by placing the mice on a vertical grid and measuring the time they were able to hold themselves. The earlier mini-agrin started to be expressed, the longer the mice could stay on the grid indicating increased muscle strength. Histogram represents average performance time \pm SD. **b.** Locomotive activity was estimated by an open-field walking test, measuring the time of activity over 10 minutes. In 4 and 6 week-old animals, $dy^{W/-}$ mice starting mini-agrin expression any time after birth showed a significant higher locomotive activity compared to $dy^{W/-}$ littermates. Histogram represents average moving time \pm SD. **c.** Creatine kinase (CK) levels detected in the blood of 4 and 6 week-old mice of the different genotypes. In all $dy^{W/-}$ mice starting mini-agrin expression at any age, we observed a similar decrease of the CK levels to less than half of the amount measured in $dy^{W/-}$ mice. This result indicates that presence of mini-agrin clearly decreases ongoing muscle degeneration. Histogram represents average CK levels \pm SEM. P-values (t test) are as follows: **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$.

Histological analysis

The severe dystrophic phenotype of $dy^{W/-}$ muscles is characterized by presence of many small and rounded fibers, replacement of muscle by fibrotic tissue, infiltration and central nucleation. Haematoxylin & Eosin (H&E) staining of *triceps brachii* deriving from 4 (Fig. 9 a) and 6 (Fig. 9 b) week-old mice, revealed that these pathological changes are continuously less pronounced the earlier mini-agrin expression was induced in the muscles of $dy^{W/-}$ mice. Quantification of this improvement was assessed by measuring the muscle fiber size distribution, reflecting muscle de-

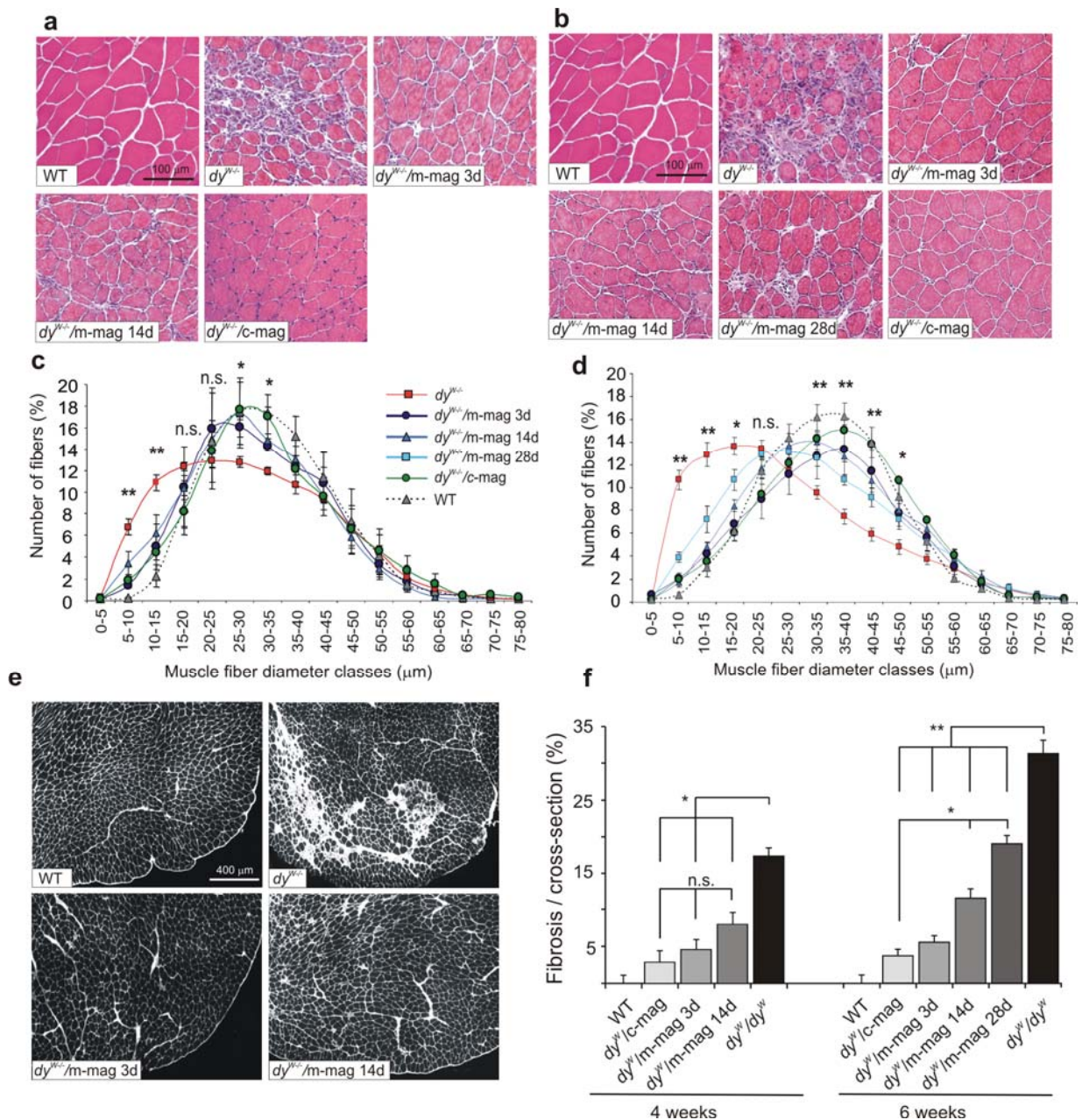


Figure 9: Phenotype analysis of *triceps brachii* cross-sections of 4 and 6 week-old $dy^{W/-}$ mice starting mini-agrin expression 3, 14 or 28 days after birth.

For each genotype at least 3 mice were analyzed. **a. - b.** Haematoxylin & Eosin (H&E) staining of 4 (**a**) and 6 (**b**) week-old mice revealed infiltration, central nucleation, many small fibers and extensive fibrosis in $dy^{W/-}$ muscle. The earlier mini-agrin was present in $dy^{W/-}$ muscle, the less pathological changes were observed. **c. - d.** The muscle fiber size distribution of 4 (**c**) and 6 (**d**) week-old animals was quantified using the minimal 'fiber feret's diameter'. Values were grouped into size classes of 5 μm per class and represent the average percentage \pm SEM. The different genotypes are represented as followed: $dy^{W/-}$ (red squares), $dy^{W/-}$ /m-mag 3d (dark blue circles), $dy^{W/-}$ /m-mag 14d (blue triangles), $dy^{W/-}$ /m-mag 28d (light blue squares), $dy^{W/-}$ /c-mag (green circles), WT (triangles, interrupted line). Muscles of $dy^{W/-}$ mice show a significant higher percentage of small fibers than age-matched muscles of $dy^{W/-}$ mice starting mini-agrin expression at any age. Note that the number of small fibers decreases the earlier mini-agrin expression was induced. **e. - f.** Fibrotic tissue was visualized (**e**) and quantified (**f**) from wheat germ agglutinin (WGA) staining, which detects membrane-bound and extracellular epitopes. (**e**) represents the condition of *triceps brachii* of the different genotypes at the age of 4 weeks. In $dy^{W/-}$ mice more than 25% of a muscle cross-section is replaced by fibrotic tissue (**f**), while the abundance of fibrotic areas significantly decreases the earlier mini-agrin starts to be expressed. P-values (*t* test) are as follows: **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$.

and regeneration. To this end, we evaluated the minimal fiber feret diameters in *triceps brachii* cross-sections of each genotype from 4 (Fig. 9 c) and 6 (Fig. 9 d) week-old mice. In a wild-type *triceps brachii* muscle cross-section the majority of the fibers represent a minimal diameter around 20-40 μ m or 30-50 μ m in 4 or 6 week-old mice respectively, whereas small fibers (< 15 μ m) represent a clear minority. Compared to wild-type, the fiber size distribution in $dy^{W/-}$ muscle was obviously shifted towards smaller fibers, since many of them did not exceed a minimal diameter of 15 μ m. The earlier mini-agrin expression has started, the more the fiber size distribution was re-shifted towards wild-type distribution.

Moreover, endomysial fibrosis is a general feature in MDC1A muscles and arises from replacement of muscle by non-muscle tissue. Fibrosis additionally limits the function of a muscle. Large parts of *triceps brachii* muscles from 4 and 6 week-old $dy^{W/-}$ mice revealed to be affected by fibrosis, whereas the abundance of fibrotic areas significantly decreased the earlier mini-agrin started to be expressed at the membrane (Fig. 9 e + f).

Restoration of the regenerative capacity

In contrast to $dy^{W/-}$ muscles, laminin- α 5 and α -dystroglycan protein but not mRNA levels are increased in $dy^{W/-}$ muscles constitutively overexpressing chick mini-agrin. We confirmed the posttranslational stabilization of these two proteins in the remaining intact muscle tissue of $dy^{W/-}$ mice starting mini-agrin expression 3, 14 and 28 days after birth, since we found the amounts of both proteins to be comparable in $dy^{W/-}$ mice constitutively overexpressing c-mag or starting m-mag expression at any age (Fig. 10 a + b).

In addition to the decreased mechanical stability in laminin α 2-deficient muscle, the capability to regenerate is markedly impaired^{317,339}. In muscle of $dy^{3K/-}$ mice, another mouse model for MDC1A, overexpression of mini-agrin successfully restored the regeneration capacity. This activity was attributed to the high affinity binding of mini-agrin to α -dystroglycan³¹⁷. Thus, we hypothesized that in co-incidence with the posttranslational stabilization of α -dystroglycan, the regenerative capacity in $dy^{W/-}$ muscle should be similarly restored upon mini-agrin expression after onset of the disease. To assess this question, we made use of the myotoxin notexin, a snake venom compound with phospholipase A2 activity that provokes transient necrosis of mature muscle followed by extensive myofiber regeneration. Inflammatory response and mononuclear cell proliferation is known to be most active within 1–4 days upon injection. Myogenic cell differentiation and new myotube formation is observed ~5–6 days post-injection. By 10 days post-injection, the overall architecture of the muscle is restored, although most regenerated myofibers are smaller and display central myonuclei. A morphologically and histochemically normal mature muscle is seen at ~3–4 week postinjection^{344,345}.

We injected notexin into the tibialis anterior muscles of 5 week-old wild-type, $dy^{W/-}$ and $dy^{W/-}$ mice who started mini-agrin expression at the age of 4 weeks ($dy^{W/-}$ /m-mag 28d) and analyzed the muscles 6 (Fig. 10 c) and 14 (Fig. 10 d) days post-injection (Fig. 10 c - f). 6 days after injury only few regenerating (dMyHC-positive) fibers were found in $dy^{W/-}$ muscle. In contrast, $dy^{W/-}$ /m-mag

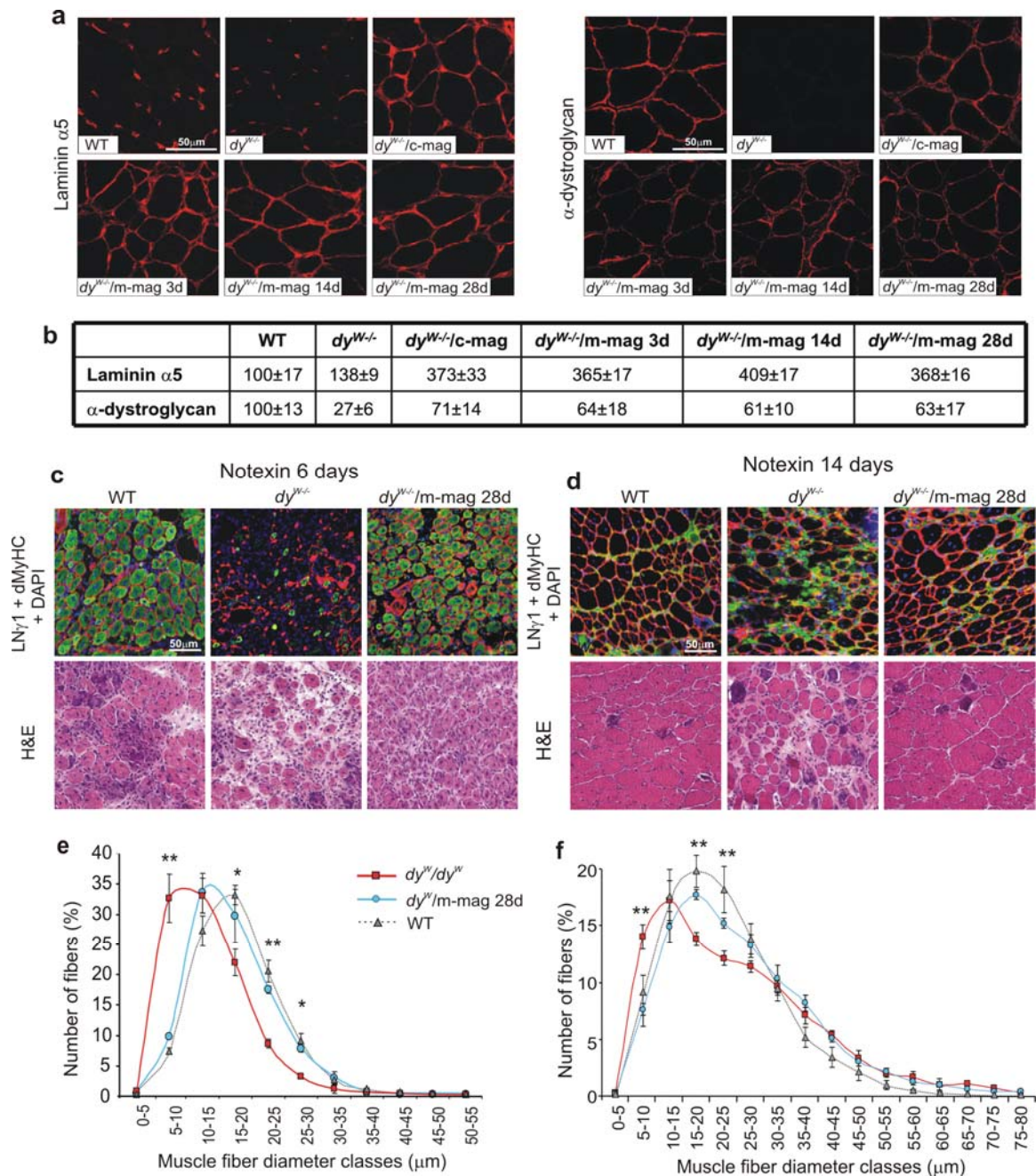


Figure 10: Stabilization of laminin- $\alpha 5$ and α -dystroglycan and regeneration capacity after injury.

a. - b. Posttranslational stabilization of laminin- $\alpha 5$ and α -dystroglycan at the membrane of *triceps brachii* of 6 week-old $dy^{W-/-}$ mice starting mini-agrin expression after birth. Amounts of both proteins were comparable in $dy^{W-/-}/c-mag$ and $dy^{W-/-}/m-mag$ mice starting expression at any age. **c. - d.** Recovery of *tibialis anterior* muscle analyzed 6 (**c**) and 14 (**d**) days after injury by injection of notexin. Upper panels represent staining of cross-sections with DAPI (blue), an antibody against developmental myosin heavy chain (dMyHC, green) which is re-expressed in regenerating muscle fibers and laminin $\gamma 1$ (red) to visualize the membranes. Lower panels reveal the muscle pathology by H&E staining. **c.** 6 days after notexin injection only a few very small regenerating (dMyHC-positive) fibers were found in $dy^{W-/-}$ muscle. In contrast, start of mini-agrin expression 7 days before injury ($dy^{W-/-}/m-mag$ 28d) could restore the regeneration capacity to a level indistinguishable from wild-type muscle as represented by the presence of many dMyHC-positive muscle fibers. **d.** 14 days after notexin injection, $dy^{W-/-}$ muscle is drastically affected by degenerative processes, while $dy^{W-/-}/m-mag$ 28d and WT muscles obviously are recovering from the injury. **e. - f.** Quantification of the muscle fiber size distribution of *tibialis anterior* 6 (**e**) and 14 (**f**) days after injury revealed the significantly enhanced regenerative capacity of muscle upon start of mini-agrin expression after onset of the disease. The different genotypes are indicated as followed: $dy^{W-/-}$ (red squares), $dy^{W-/-}/m-mag$ 28d (blue circles) and wild-type (grey triangles, interrupted line). P-values (*t* test) are as follows: **, $P < 0.01$; *, $P < 0.05$.

28d muscles showed a comparable amount of regenerating muscle fibers as injured wild-type muscles, represented by the presence of many dMyHC-positive muscle fibers. Thus, start of mini-agrin expression shortly before injury ($dy^{W-/-}/m\text{-mag}$ 28d) could restore the regeneration capacity to a level indistinguishable from wild-type mice. 14 days after muscle injury, $dy^{W-/-}$ muscle was fatally affected by degenerative processes whereas in $dy^{W-/-}/m\text{-mag}$ 28d and wild-type muscle the overall architecture was restored.

Quantification of the muscle fiber size distribution (Fig. 10 e + f) additionally revealed the significantly enhanced regenerative capacity of muscle upon start of mini-agrin expression after onset of the disease. 6 days after notexin injection the majority of regenerating (dMyHC-positive) $dy^{W-/-}$ muscle fibers were very small, while there was no statistically significant difference between the distribution of regenerating fibers of $dy^{W-/-}/m\text{-mag}$ 28d and wild-type muscle (Fig. 10 e). 14 days after injury (Fig. 10 f), the majority of the fibers in $dy^{W-/-}/m\text{-mag}$ 28d and wild-type muscles had a diameter of 10-20 μm , which is supposed to increase during the next few weeks. In contrast, in $dy^{W-/-}$ muscles fibers with a diameter of 10-20 μm were less abundant than fibers smaller than 10 μm or bigger than 20 μm , indicating insufficient regeneration as well as ongoing degeneration.

These results confirm our hypothesis, that start of mini-agrin expression stabilizes α -dystroglycan at the muscle membrane (Fig. 10 a + b) and hence profoundly increases the regeneration capacity of the remaining muscle fibers at all stages of the disease, independent of the condition of the rest of the muscle.

In summary, we show under tight controlled conditions (tet-off-system) that mini-agrin can slow down the progression of the disease in $dy^{W-/-}$ mice when it is applied at different stages of the disease. The earlier mini-agrin expression is induced, the more pronounced is the amelioration of the dystrophic phenotype. We report that the extent of amelioration depends on the condition of the muscle at the time of mini-agrin induction, since mini-agrin diminishes the disease progression by restoring the mechanical stability and the regenerative capacity of intact muscle tissue.

f. Stability of mini-agrin at the muscle fiber membrane

To further profit from the inducible tet-off system tightly regulating m-mag expression in skeletal muscles of our mice, we aimed to evaluate the stability of the m-mag protein bound to the basement membrane after a temporally limited expression (Fig. 11). To this end, we started m-mag expression in healthy animals ($dy^{W+/+}$) by withdrawal of doxycycline at the age of 3 weeks and repressed production at the age of 4 weeks by re-application of a 10-times increased amount of doxycycline (50 $\mu\text{g}/\text{ml}$). A complete repression was rapidly achieved as confirmed by real-time PCR quantification of m-mag mRNA transcripts in *triceps brachii* (Fig. 11 a). 24 hours (Dox 1d) after re-application of doxycycline m-mag transcription was reduced to 10% and after >48 hours (Dox 2d, 3d, 4d) was decreased to less than 1%. In order to evaluate the stability of m-mag at the

muscle fiber membrane, the protein was assessed by Western blot (Fig. 11 b) and by immunostaining (Fig. 11 c). Results indicate that upon repression, there is a continuous decrease of the m-mag protein bound to the basement membrane, which is halved within 5 days and has completely disappeared 9 days after re-application of the antibiotic.

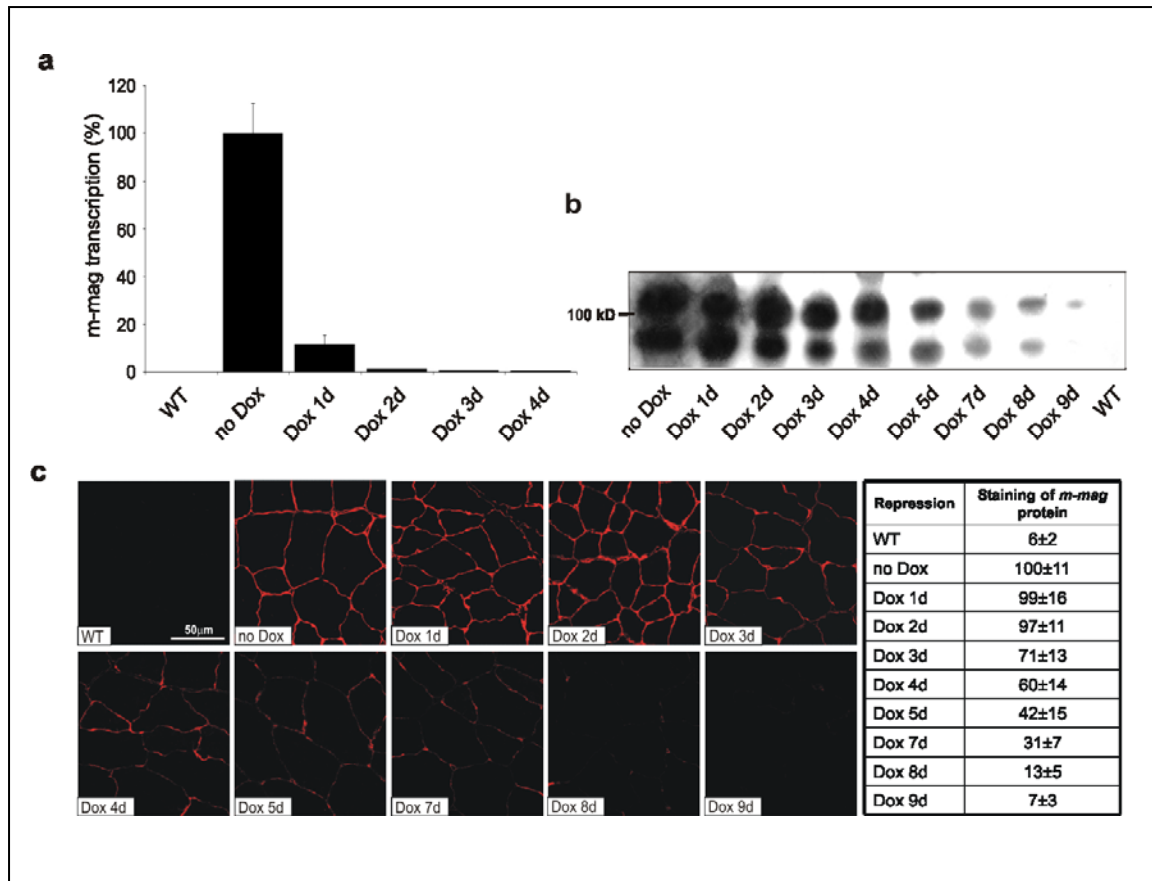


Figure 11: Stability of the mini-agrin protein in skeletal muscle after a temporal expression modus.
a. Real-time PCR quantification of mini-agrin mRNA transcripts in *triceps brachii* before repression (no Dox) and 1-4 days (Dox 1d, Dox 2d, Dox 3d, Dox 4d) after re-addition of doxycycline (50 μ g/ml). Values were processed according to the delta-Ct method and were referred to mRNA levels present in the muscle before repression has started (no Dox). Results suggest mRNA transcription to be reduced to 10% and less than 1%, within 24 hours (Dox 1d) and >48 hours (Dox 2d, 3d, 4d) after re-application of doxycycline, respectively. **b.** - **c.** Mini-agrin protein detected by Western blot using *quadriceps* (**b**) and by immunostaining using *triceps brachii* (**c**). Upon repression, the amount of mini-agrin protein bound to the basement membrane continuously decreased, was halved within 5 days and has completely disappeared after 9 days.

g. A preliminary attempt of a systemic mini-agrin protein application

Our studies proved mini-agrin to diminish the MDC1A disease progression when applied after birth and to remain stably bound to membrane for several days. Direct delivery of the mini-agrin protein to the skeletal muscles of diseased individuals would rule out several problems faced with viral-based gene therapy. Due to more than 600 muscles harbored in a human body, intramuscular injections are definitely ruled out as an application method. This prompted us to perform a preliminary approach of the most obvious way to systemically deliver m-mag to the extracellular matrix of skeletal muscles, namely intravenous injection of recombinant mini-agrin protein. 24 hours after protein application, mini-agrin was found co-localized with laminin- α 5 at the microvascular walls but did not exit the vascular system (Fig. 12), implying the requirement of further pharmacological intervention to succeed in this application modus.

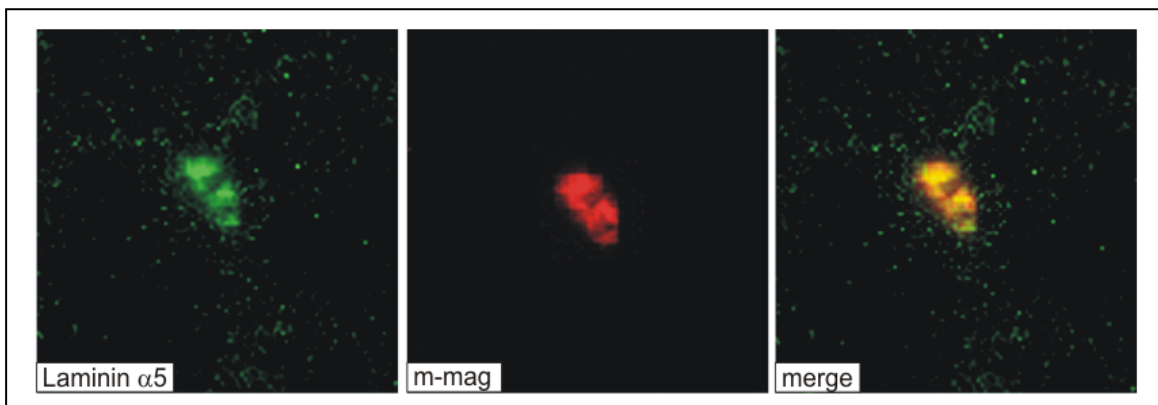


Figure 12: Detection of m-mag in *triceps brachii* cross-sections 24 hours after intravenous injection of the recombinant protein.

Mini-agrin co-localizes with laminin- α 5 at the microvascular walls but did not exit the vascular system.

2 Gene Therapy as a Means to deliver Mini-agrin into the diseased Muscles of MDC1A mice

Sarina Meinen, Rolf Stucka, Hanns Lochmüller and Markus A. Rüegg

In a parallel study to the conceptual approach of testing the mini-agrin for its effects after application in advanced stages of the disease, we aimed to critically test whether gene therapy could be used to introduce the mini-gene for agrin into skeletal muscles of diseased individuals. For this purpose, we intended to use adenoviral (AdV) and adeno-associated viral (AAV) vectors as delivery tools for mini-agrin.

Mini-agrin harbors several advantages for being successfully used in gene therapy. First, its cDNA is small enough to be incorporated into AAV vectors, the currently most promising viral-based delivery tool. Second, the notoriously low efficacy in the infection of muscle is not a problem since mini-agrin protein is secreted from infected muscle fibers and thus can also act on neighboring, non-infected fibers. Third, because MDC1A patients express agrin endogenously, the immunological rejection of the protein will be minimal³⁴⁶.

a. Generation of high titer stocks of adenoviral (AdV) vectors expressing mouse mini-agrin-myc (m-mag)

In a first step, our collaborators (Rolf Stucka and Hanns Lochmüller, Munich) subcloned the mouse mini-agrin-myc (m-mag) cDNA (see Fig. 1) into a 1. generation AdV vector. This virus can be easily produced to high titer concentrations, efficiently can infect replicating as well as differentiated cells and is safe for use in gene therapy since no malignancy is associated with it. However, AdV DNA does not integrate into the host genome and thus results only in a transient transgene expression and additionally may induce a host immune reaction to viral gene products³⁴⁷.

In order to generate recombinant AdV vectors carrying the mini-agrin under the control of the muscle-specific creatine kinase (MCK)-promoter (AdV-MCK-m-mag), a shuttle cloning strategy was followed, based on the AdEasy™ XL system (Stratagene, Fig 13 a). This system uses special competent cells that are pre-transformed with the AdEASY plasmid giving rise to the adenoviral backbone. The m-mag construct was subcloned downstream the MCK-promoter into a shuttle vector which was transformed to the AdEASY-pre-transformed cells. This allows homologous recombination in bacteria between the shuttle vector and the adenoviral genome. The linearized construct containing the MCK-promoter, m-mag and the adenoviral backbone (Fig. 13 b) then was transferred into 293 cells³³⁷, which complement for the E1 and E3 deficiency of the viral genome and thus allow amplification of the recombinant AdV.

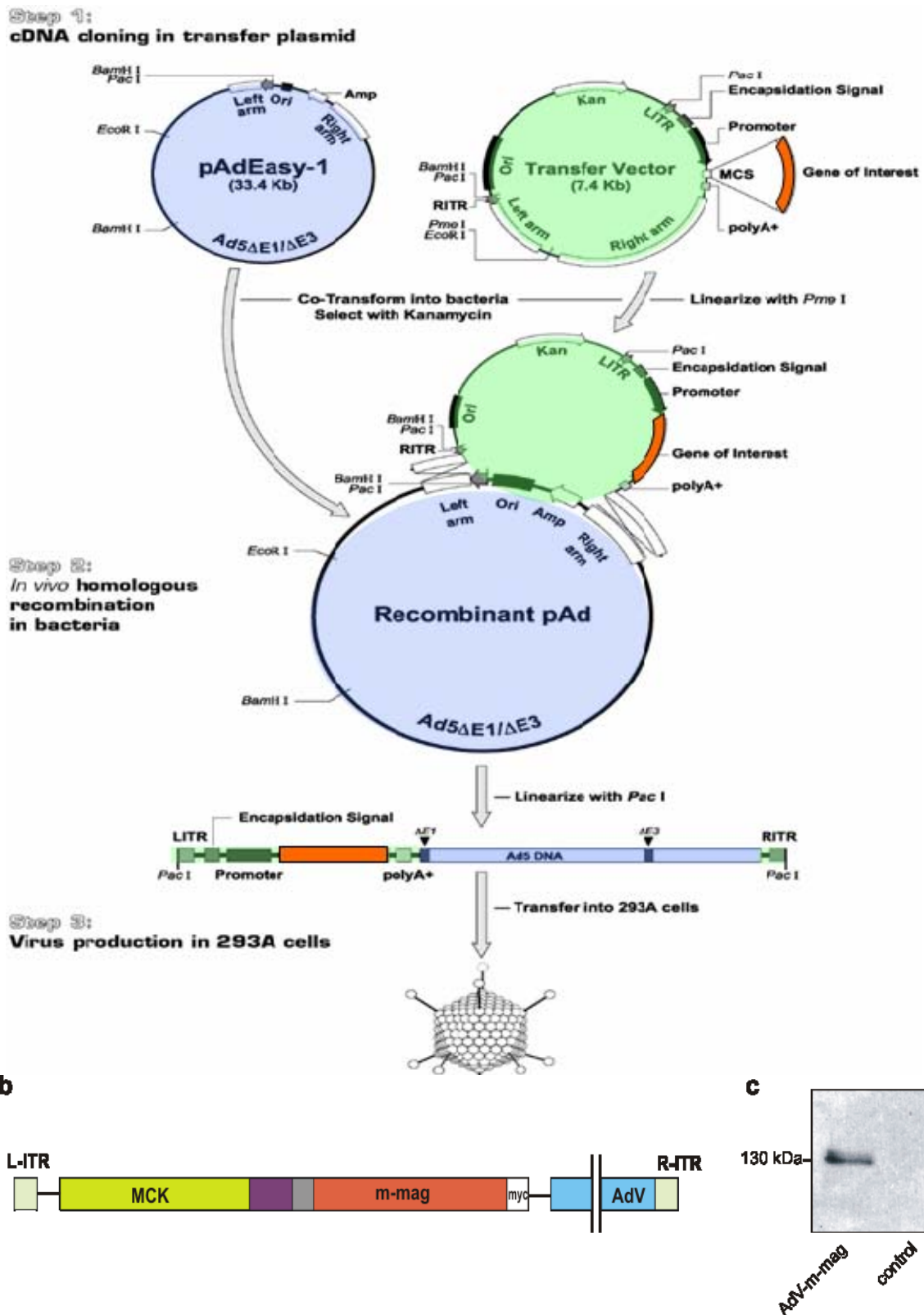


Figure 13: Generation of adenoviral (AdV) vectors expressing mouse mini-agrin-myc (m-mag).

a. The AdEasy™ XL system (Stratagene) was used to produce recombinant adenoviral vectors (AdV) carrying m-mag downstream the MCK-promoter. The AdEASY plasmid provides for the adenoviral backbone and was transformed to special competent cells. The m-mag construct was subcloned downstream the MCK-promoter into a shuttle vector, which then was transformed to the AdEASY-pre-transformed competent cells. This allows homologous recombination in bacteria between the shuttle vector and the adenoviral genome. **b.** The linearized construct containing the MCK-promoter, m-mag and the adenoviral backbone then was transferred into 293 cells, which complement for the E1 and E3 deficiency of the viral genome and thus allow amplification of the recombinant AdV. **c.** Western blot analysis revealed that infected cells express high amount of the m-mag protein.

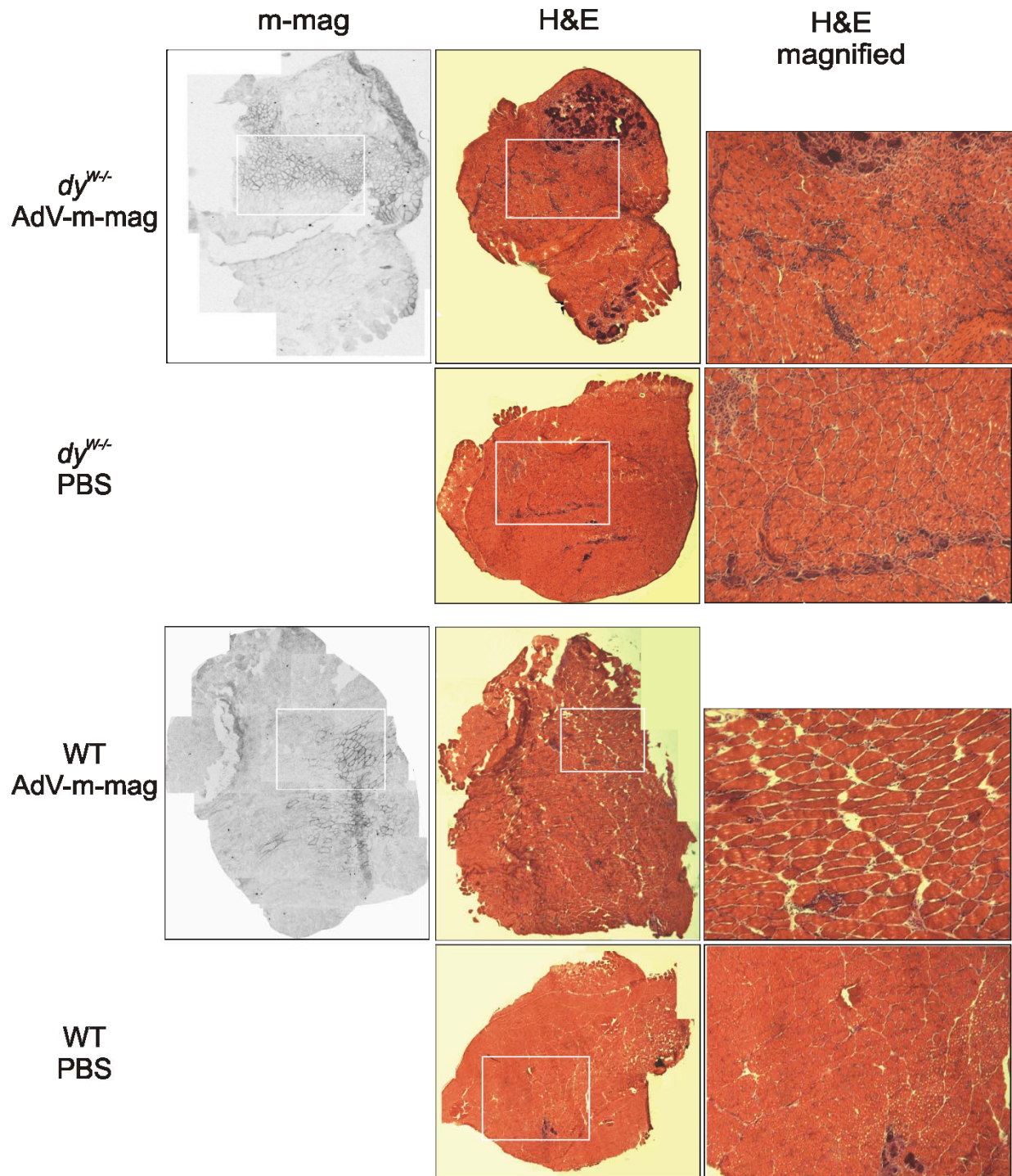


Figure 14: *In situ* infection of *triceps brachii* with AdV-m-mag.

AdV-m-mag-injected $dy^{W/-}$ muscle presented reasonable transduction efficiencies as detected by immunostaining of mini-agrin localized at the muscle fiber membranes using an antibody against the myc-tag (first panel, first column). In contrast, in WT muscle transduction of AdV-m-mag was much less efficient (third panel, first column). The site of injection site of AdV-m-mag-injected $dy^{W/-}$ muscle was inflicted by extensive necrosis (first panel, second and third column), whereas by far less necrosis was observed in AdV-m-mag-injected WT muscle (third panel, second and third column). PBS-injected muscles of both genotype (second and fourth panel for $dy^{W/-}$ and WT muscle, respectively) were only affected by minimal necrosis at the site of injection.

Large scale production of single constructs in 293 cells was done by repeated amplification, including serial infection and lysis steps. Finally, 1-3x10¹² viral particles could be harvested from 10 l spinner cultures. Using this system a high titer stock of AdV-MCK-m-mag was produced and purified by cesium chloride step gradient centrifugation. Finally, an infectious titer of 1x10¹¹ particles/ml was achieved. In order to check the correct expression behavior of m-mag, Western blot analyses of lysates of infected 293 cells was performed. The m-mag protein was visualized as a single band of ~130kDa, consistent with its calculated molecular mass (Fig. 13 c).

b. Infection of $dy^{W/-}$ muscle *in situ*

AdV harbors high transduction efficiencies upon local application and was previously shown to deliver mini-dystrophin to the diseased skeletal muscles of *mdx* mice where it could ameliorate the dystrophy³⁴⁸. Of course, local injections are definitely not feasible in the treatment of muscular dystrophies, since there is need to treat several hundred of muscles repeatedly. Nevertheless, in our approach local intramuscular injection can confirm the assumption that mini-agrin, which is secreted from infected muscle fibers and thus can also act on neighboring non-infected fibers, is able to efficiently transduce the muscle. Furthermore, we aimed to evaluate whether delivery of mini-agrin by means of adenoviral-based gene therapy efficiently could ameliorate the dystrophic phenotype of skeletal muscles in $dy^{W/-}$ mice.

For this purpose, we injected *triceps brachii* muscle of 2 week-old wild-type and $dy^{W/-}$ mice using AdV-MCK-m-mag. 16 days after virus injection, muscles were dissected for analysis (Fig. 14). The *in situ* infection of $dy^{W/-}$ muscle with AdV-MCK-m-mag represented reasonable transduction efficiencies (first panel, first column) as well as extensive necrosis at the site of injection (first panel, second and third column). Lower transduction efficiency and less necrosis were observed in virus-treated muscles of wild-type animals (third panel). Almost no necrosis was produced in PBS-injected muscles of both genotypes (second and fourth panel). Therefore, we attributed the extensive necrosis in AdV-MCK-m-mag -treated $dy^{W/-}$ muscle to an immune reaction to viral proteins. This problem could be overcome by administering immune-suppressants to the treated mice.

In parallel to our work, the feasibility of systemic mini-agrin gene delivery leading to long-term transduction of whole body skeletal and cardiac muscle has been demonstrated and was shown to result in a substantial amelioration of the disease in $dy^{W/-}$ mice³²⁸ (see Discussion and Appendix II: News and Commentary). Due to this impressive study³²⁸ we stopped further investigation following this approach and focused on the advantages provided by our conceptual approach using the tet-off system to regulate mini-agrin expression in $dy^{W/-}$ mice.

3 Full-length muscle agrin ameliorates the dystrophic phenotype in $dy^{W/-}$ mice

Sarina Meinen¹, Patrizia Barzagli¹, Shuo Lin and Markus A. Rüegg

¹ equal contribution

Another promising strategy for the treatment of MDC1A would be to screen for pharmacologically active compounds that increase the expression of endogenous agrin in skeletal muscles. Full-length muscle agrin is a highly glycosylated 400-600kDa heparan sulfate proteoglycan and served as a template for the generation of mini-agrin. It acts as an organizer and stabilizer of protein arrangements in the muscle basal lamina^{136,137,332} and is expressed at very low levels in matured muscle. However, before screening for such agents, it will be important to investigate the beneficial effects of full-length agrin on the dystrophic MDC1A phenotype.

a. Generation of $dy^{W/-}$ mice constitutively expressing chick full-length agrin in skeletal muscles

In a transgenic approach, we determined whether overexpression of full-length muscle agrin ameliorates the dystrophic symptoms in $dy^{W/-}$ mice as efficiently as constitutive expression of chick mini-agrin (c-mag) does. For this purpose, we generated mice constitutively overexpressing chick full-length muscle agrin (c-FLag) driven by the muscle-specific creatine kinase (MCK)-promoter (Fig. 15).

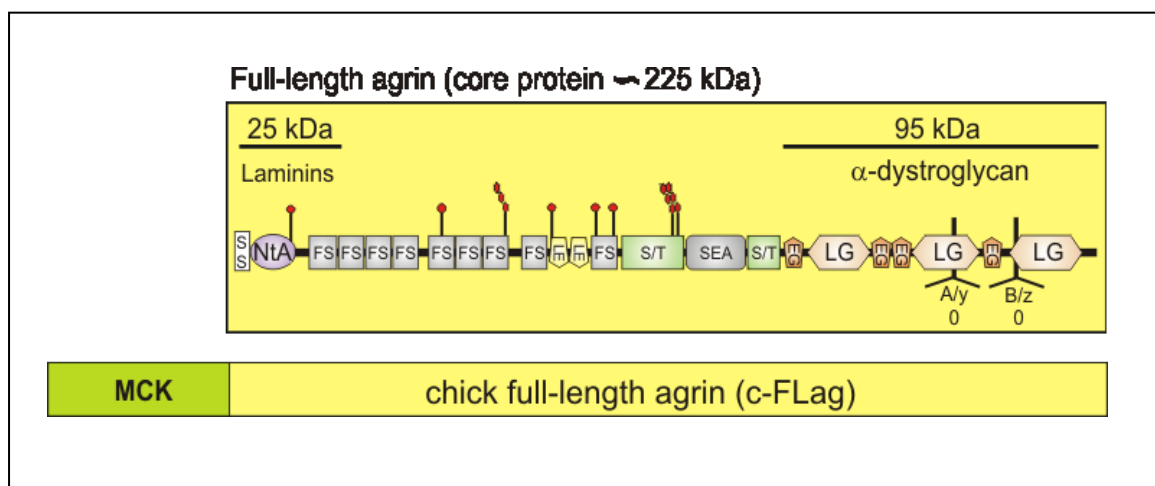


Figure 15: Schematic representation of the full-length agrin protein and construct.

The full length agrin protein (upper panel) contains laminin and α -dystroglycan binding sites at its N- and C-terminus, respectively. Full-length agrin is a heparan sulfate proteoglycan and thus is highly glycosylated. Its core protein represents ~225kDa, whereas glycosylations give rise to an apparent molecular weight of 400–600kDa. The ~6.3kb full-length agrin cDNA was gained out of muscle-specific chick full-length agrin and was subcloned downstream the MCK-promoter. This construct was used for generation of the transgenic mice overexpressing chick full-length agrin in skeletal muscles.

Evaluation of the transgene expression levels by Northern blot (Fig. 16 b), immunostaining (Fig. 16 a) and Western blot (not shown) analysis suggested three different mouse lines (L2, L4 and L9) to express the transgene at detectable levels. However, in all these mouse lines, expression levels of full-length agrin were substantially lower than those reached for c-mag using the same promoter (Fig. 16 a - c). Line 4, which was evaluated to achieve the highest expression activity on mRNA as well as on protein level (Fig. 16 c) and therefore was used to generate a mouse line heterozygous for the *lama2* mutation and hemizygous for chick full-length agrin ($dy^{W/-}/c\text{-FLag}$). An additional round of mating gave rise to laminin- $\alpha 2$ deficient mice overexpressing chick full-length agrin in skeletal muscles ($dy^{W/-}/c\text{-FLag}$). 4 week-old animals of this genotype were used for our comparative analysis of the dystrophic phenotype.

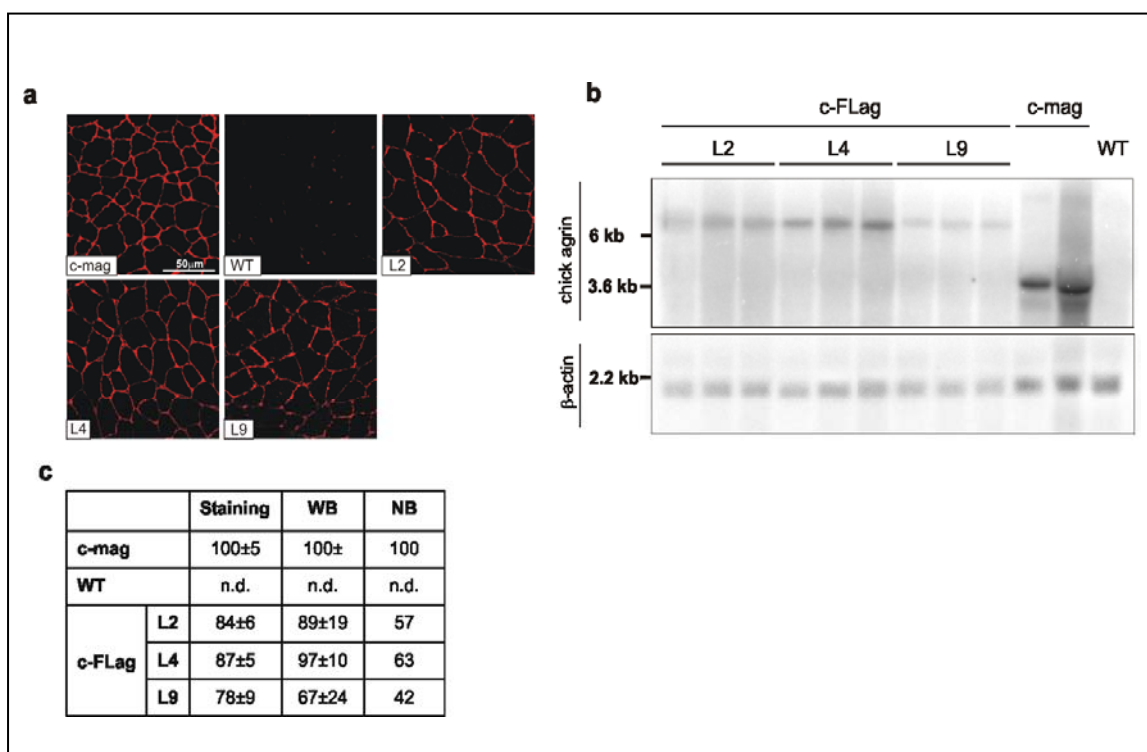


Figure 16: Evaluation of the expression levels in the transgenic mouse lines 2, 4 and 9 (L2, L4, L9) overexpressing chick full-length agrin (c-FLag) in skeletal muscles.

a. Immunostaining of c-FLag present at the membrane in the different transgenic mouse lines. **b.** Northern blot analysis of mRNA transcription levels of c-FLag in direct comparison to chick mini-agrin. **c.** Quantification of the mRNA (NB) and protein (IHC, WB) expression levels of the c-FLag in the different. c-FLag L4 was found to express the highest amount of the transgene, even though levels were substantially lower than in c-mag mice. Line 4 was used for further experiments.

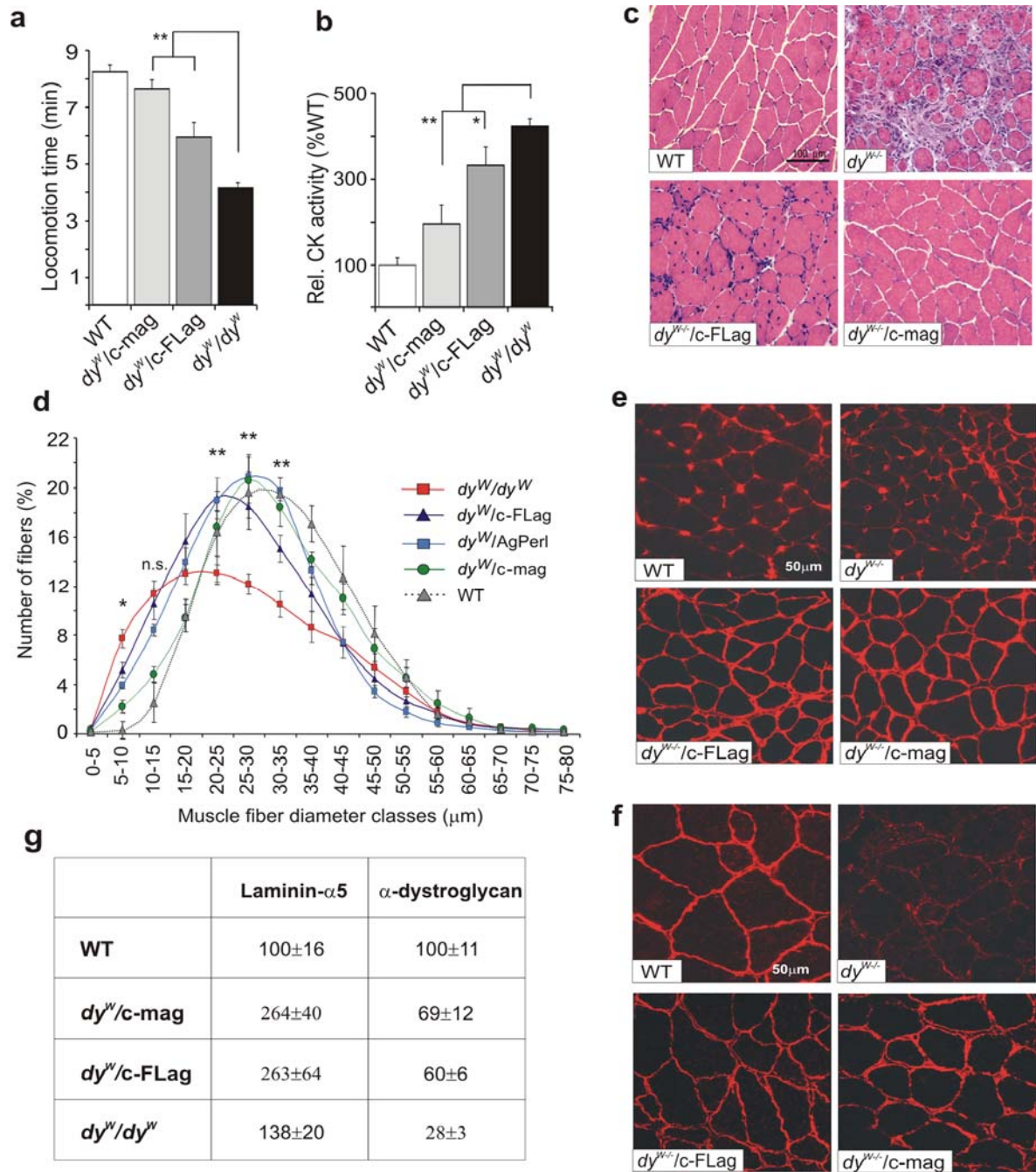


Figure 17: Phenotype analysis of 4 week-old $dy^{W/-}$ mice constitutively overexpressing chick full-length agrin ($dy^{W/-}/c\text{-FLag}$) in skeletal muscles.

a. Locomotion. Data represent average moving time \pm SD in an open-field walking test. $dy^{W/-}/c\text{-FLag}$ mice performed significantly longer than $dy^{W/-}$ mice. **b.** Evaluation of CK levels \pm SEM. $dy^{W/-}/c\text{-FLag}$ mice showed significantly decreased CK levels compared to $dy^{W/-}$ mice. However, c-FLag was not able to half the CK levels as it was the case using c-mag. **c.** Haematoxylin & Eosin staining of *triceps brachii* cross-sections visualized reduced pathological symptoms in $dy^{W/-}/c\text{-FLag}$ mice compared to $dy^{W/-}$ mice. The amelioration was not as prominent as observed in muscles of $dy^{W/-}/c\text{-mag}$ mice. **d.** Muscle fiber size distribution on cross-sections of *triceps brachii* of 4 week-old mice using the minimal 'fiber feret's diameter'. Values were grouped into size classes of 5 μm per class and represent the average percentage \pm SEM. The different genotypes are represented as follows: $dy^{W/-}$ (red squares), $dy^{W/-}/c\text{-FLag}$ (dark blue triangles), $dy^{W/-}/c\text{-mag}$ (green circles), WT (triangles, interrupted line). Muscles of $dy^{W/-}$ mice represent a significant higher percentage of small fibers than muscles of $dy^{W/-}/c\text{-FLag}$ mice. Results suggest a slightly increased de- and regeneration in $dy^{W/-}/c\text{-FLag}$ compared to $dy^{W/-}/c\text{-mag}$ muscle. **e.- f.** Posttranslational stabilization of laminin- α 5 (**e**) and α -dystroglycan (**f**) in *triceps brachii*. **g.** Quantification suggest an equal elevation of laminin- α 5 but a slightly decreased expression of α -dystroglycan in $dy^{W/-}/c\text{-FLag}$ muscles when compared to $dy^{W/-}/c\text{-mag}$ muscle. P-values (*t* test) are as follows: **, P < 0.01; *, P < 0.05; ns, P > 0.05.

b. Phenotype analysis of $dy^{W/-}/c$ -FLag mice

The general appearance, such as the shape, the size and hygienic maintenance of $dy^{W/-}$ mice overexpressing the chick full-length agrin was obviously improved when compared to $dy^{W/-}$ mice, but the amelioration was not as striking as in $dy^{W/-}/c$ -mag mice. The open-field walking test revealed $dy^{W/-}/c$ -FLag mice to be almost twice as active as age-matched $dy^{W/-}$ mice. However, $dy^{W/-}/c$ -FLag mice did not reach the near normal locomotion behavior presented by $dy^{W/-}$ mice overexpressing chick mini-agrin (Fig. 17 a).

Creatine kinase (CK) levels in the blood serum indicate ongoing degeneration in dystrophic muscles. $Dy^{W/-}/c$ -FLag mice showed significantly decreased CK levels compared to $dy^{W/-}$ mice, indicating the ability of full-length agrin to effectively decrease the susceptibility of the muscle. Nonetheless, ongoing degeneration was less extensively diminished than in $dy^{W/-}/c$ -mag mice (Fig. 17 b).

In line with these findings, the histological haematoxylin-eosin staining (Fig. 17 c) of *triceps brachii* cross-sections revealed that the dystrophic phenotype such as infiltration, increased number of small fibers and fibrosis in $dy^{W/-}/c$ -FLag muscle is less pronounced but more progressed in muscles of age-matched $dy^{W/-}$ and $dy^{W/-}/c$ -mag mice, respectively.

A hallmark of dystrophic muscle is the marked shift of the fiber size distribution towards smaller muscle fibers. We used the minimal 'fiber feret's diameter' for quantification of the muscle fiber size distribution on cross-sections of *triceps brachii* of 4 week-old mice of the different genotype (Fig. 17 d). A significant higher percentage of small fibers were determined in muscles of $dy^{W/-}$ mice in comparison to muscles deriving from $dy^{W/-}/c$ -FLag. Again, results suggest a slightly decreased amelioration of the dystrophy in muscles $dy^{W/-}/c$ -FLag compared to $dy^{W/-}/c$ -mag mice. Both, laminin- α 5 and α -dystroglycan were shown to be posttranslationally stabilized at the muscle fiber membranes of $dy^{W/-}/c$ -mag mice^{316,317}. In $dy^{W/-}/c$ -FLag mice, quantification of laminin- α 5 protein detected at the membrane of *triceps brachii* cross-sections (Fig. 17 e + g), suggested equal amounts of the protein as observed in $dy^{W/-}/c$ -mag muscles, whereas α -dystroglycan protein levels stabilized at the muscle fiber membranes were slightly but not significantly decreased compared to $dy^{W/-}/c$ -mag (Fig. 17 f + g).

In summary, overexpression of chick full-length agrin ameliorates the dystrophic phenotype in $dy^{W/-}$ mice, but the amelioration is less pronounced than in the $dy^{W/-}/c$ -mag mice. This effect might be due to the lower expression levels achieved for the chick full-length than for the chick mini-agrin. Moreover, full-length muscle agrin might provide a sterically less functional reconnection of α -dystroglycan to the laminin network.

V DISCUSSION AND PERSPECTIVES

1 Discussion

a. Artificial restoration of the linkage between laminin and dystroglycan ameliorates the disease progression of MDC1A muscular dystrophy at all stages

Seemingly, the embryonic development of muscle in MDC1A mice is normal, but a severe muscular dystrophy is manifested postnatally^{303-305,311}. In the *dy*^{W/-} mouse model, the disease starts to manifest shortly after birth and the animals stop gaining weight at the age of 2–3 weeks, when muscle degeneration becomes acute^{306,316}. This fact implies the possibility to diminish the disease progression by postnatal therapeutic intervention. Thus, we assumed application of mini-agrin at birth to be nearly as effective as when application was started during embryonic development, as it was the case in *dy*^{W/-} mice constitutively overexpressing mini-agrin³¹⁶.

Tight regulation of the "tet-off" system enables reliable evaluation

To reliably determine the potential of mini-agrin to slow down the disease progression when it is applied at birth or in advanced stages of the disease, we have established the tetracycline-regulated 'tet-off' expression system^{335,336} in the *dy*^{W/-} mice, to temporally control mini-agrin expression in skeletal muscles (Fig. 3 and 7). Previous studies using the tet-off system in conjunction with the MCK-tTA mice³³⁸ as its regulatory part, suggested an application of 50µg up to 1mg doxycycline per milli-liter drinking water to achieve a tight transgene repression^{343,349-352}. However, in our MCK-tTA^{+/-}/tet-m-mag^{+/-} mice application of 50µg doxycycline/ml drinking water tightly inhibited mini-agrin protein production, but took around 4 weeks to induce m-mag expression upon withdrawal of the antibiotic (data not shown), what definitively was not feasible for our approach. In purpose to achieve a reliable repression and a rapid induction of mini-agrin, we substantially decreased the amount of doxycycline. We provide evidence that administration of 5µg doxycycline per milli-liter drinking water to pregnant and gestating females completely inhibits mini-agrin transcription and translation in the offspring. Moreover, within less than one week upon withdrawal of the antibiotic, mini-agrin expression has recovered to levels indistinguishable from expression levels exhibited without administration of doxycycline (Fig. 6). Thus, we have adjusted the regulation of the tet-off system to be suitable for our approach and hence paved the way to reliably evaluate the amelioration of the dystrophic phenotype in *dy*^{W/-} mice when mini-agrin expression was induced after birth or after onset of the disease under tightly controlled temporal (Fig. 6) and spatial (Fig. 5) regulation.

Mini-agrin decreases the degeneration of intact muscle tissue of $dy^{W/-}$ mice

Indeed, we could demonstrate that mini-agrin significantly slows down the disease progression in $dy^{W/-}$ mice when applied shortly after birth (at the age of 3 days), at the time point first dystrophic symptoms become apparent (at the age of 14 days) and in an advanced stage of the disease (at the age of 28 days; Fig. 8 and 9). However, the improvement of the overall health, the increase in muscle strength (Fig. 8 a) and locomotion activity (Fig. 8 b) as well as the amelioration of the histological pathology of the skeletal muscles (Fig. 9) was more pronounced the earlier mini-agrin started to be expressed. This result indicates that the manifested dystrophic symptoms in the muscle at the time of mini-agrin application determine the extent of amelioration. This assertion was additionally confirmed by 6 week-old animals inducing mini-agrin expression at the age of 4 weeks ($dy^{W/-}/m\text{-mag}$ 28d), whose dystrophic phenotype was significantly alleviated compared to 6 week-old, but was maintained at the disease stage of 4 week-old $dy^{W/-}$ mice (Fig. 8 a + b and Fig. 9 e - f). We therefore suggest that the benefit exerted by mini-agrin depends on the amount of the remaining muscle fibers able to be accessed by the therapeutic mini-agrin protein. In such intact muscle tissue, mini-agrin exerts its beneficial effects by reducing the susceptibility of the muscle fibers to injury³¹⁶. This is underlined by the statistically equal decrease of the immediate ongoing muscle degeneration as represented by the blood serum creatine kinase levels measured in $dy^{W/-}$ mice starting mini-agrin expression at different time points (Fig. 8 c). In detail, $dy^{W/-}$ mice starting mini-agrin expression at any time, all represented an equal reduction of the creatine kinase levels to half of the amount observed in $dy^{W/-}$ mice. Hence, mini-agrin is able to decrease the muscle fiber degeneration in the remaining muscle tissue, independent of the damage that had occurred before mini-agrin expression has started.

In contrast to $dy^{W/-}$ muscles, laminin- α 5 and α -dystroglycan protein but not mRNA levels are increased in $dy^{W/-}$ muscles constitutively overexpressing chick mini-agrin^{316,317}. We confirmed the posttranslational stabilization of these two proteins in the remaining intact muscle tissue of $dy^{W/-}$ mice starting mini-agrin expression at later stages of the disease (Fig. 10 a + b). In the muscle basement membrane, laminin- α 5 chain assembles with the β 1 and the γ 1 chain to laminin-10, and is supposed to self-polymerize and form a laminin network. However, laminin-10 does not strongly bind to α -dystroglycan³¹⁷. Therefore, posttranslational stabilization of both laminin- α 5 and α -dystroglycan might be due to the high-affinity binding of mini-agrin. This connection of the muscle fiber to the laminin network is likely to be important for the mini-agrin-mediated amelioration of the dystrophic phenotype in MDC1A, since it maintains the binding and/or signalling to α -dystroglycan and stabilizes laminin-10 in the basement membrane.

In summary we showed that mini-agrin reduces the degeneration of intact $dy^{W/-}$ muscle tissue due to an increase of the tolerance of muscle fibers to mechanical demands and/or the recovery of ligands-mediated signalling³¹⁷ at all stages of the disease.

Mini-agrin increases the regeneration capacity of intact muscle tissue in $dy^{W/-}$ mice

In addition to the enhanced degenerative processes occurring in laminin α 2-deficient muscle, the initial capability to regenerate is markedly impaired^{306,317}. Secondly, in advanced stages of

muscle disease with abundant fibrosis, the basement membranes of necrotic muscle fibers are removed before they have a chance to act as scaffolds for generation of new muscle fibers^{353,354}. Hence, regeneration results in fibrotic muscle tissue with abnormal muscle fiber arrangement.

Another study arising from our laboratory, provided evidence that mini-agrin both increases the tolerance to mechanical load but also improves the regeneration capacity of the dystrophic muscle³¹⁷. The restoration of the regeneration capacity was attributed to the high affinity binding of mini-agrin to α -dystroglycan, whose protein but not mRNA levels were found to be increased by constitutive overexpression of chick mini-agrin in laminin α 2-deficient muscle^{316,317}. We showed that start of mini-agrin expression in $dy^{W/-}$ mice at different ages after birth, results in a comparable posttranslational stabilization of α -dystroglycan at the muscle fiber membranes (Fig. 10 a + b). Co-incidentally we provide evidence that the regenerative capacity of the remaining muscle fibers is restored to almost wild-type levels upon start of mini-agrin expression in $dy^{W/-}$ muscle (Fig. 10 c - f). Thus, in addition to the reduced degenerative processes, application of mini-agrin in $dy^{W/-}$ muscle at different stages of the disease allows for successful regeneration of the remaining muscle fibers.

In summary, we provide conceptual proof that the mini-agrin-mediated artificial restoration of the linkage between laminin and dystroglycan and/or the recovery of ligand-mediated signalling ameliorate the disease progression of MDC1A muscular dystrophy at all stages. We conclude that only intact laminin α 2-deficient muscle tissue profits from the beneficial activity of mini-agrin but that the benefit is exerted by the same mechanism and effectiveness independent of the dystrophic condition of the rest of the muscle. Consequently, the dystrophic manifestation in muscle at the time of mini-agrin application determines the extent of amelioration, since manifested dystrophic symptoms remain and can not be rescued by expression of mini-agrin.

Limitations of a mini-agrin-based treatment of MDC1A

MDC1A pathology involves other organs than skeletal muscles such as the peripheral nervous system (PNS) causing a severe neuropathy as well as the central nervous system (CNS) involving white matter changes. Hence, a holistic treatment would require an effective strategy targeting in particular the PNS in addition to the skeletal muscles. In our approach mini-agrin was specifically expressed in skeletal muscles (Fig. 1 and 5) what hindered the investigation of its effects on the progressive neuropathy caused by laminin- α 2 deficiency in the PNS. However, in a recent study³²⁸ expression of mini-agrin in peripheral nerves failed to prevent the neuropathology in the hind limbs of MDC1A mice. Hence, a mini-agrin-based treatment of MDC1A provides for a substantially diminished progression of some pathological manifestations in the muscle, but does not prevent or alleviate pre-existing symptoms, not to mention the severe neuropathy in MDC1A patients.

b. Approaches to agrin-based application for the treatment of MDC1A

Advantages of mini-agrin for use in gene therapy of MDC1A

Medicine currently offers no effective treatment for MDC1A patients. Therefore, high expectations are associated with the development of gene therapeutic applications of one of the transgenic approaches capable to diminish dystrophic symptoms in different mouse models of MDC1A^{306,316,317}. A general challenge in the treatment of muscular dystrophies is to deliver the therapeutic molecules by a feasible, safe and efficient application modus to the more than 600 skeletal muscles harbored in a human body.

However, the aim to re-insert the laminin- α 2 chain, which is missing in MDC1A patients, would face several problems. First, the large size (cDNA 9kb, protein 300kDa) of the protein complicates its delivery, second, the laminin- α 2 chain has to be incorporated properly into the laminin heterotrimer that gives rise to laminin-2 and last, the de novo presence of the laminin- α 2 chain in the muscle might trigger immune responses in the patient. As several domains of laminin- α 2 contribute to its functionality, it is also unfeasible to generate a miniaturized version without losing its function.

In contrast, part of these difficulties might be overcome by a treatment based on the functional but not structural related miniaturized form of the extracellular matrix molecule agrin. As outlined above, we provided strong evidence for the therapeutic efficacy of mini-agrin at all stages of the MDC1A muscular dystrophy (Fig. 8 - 10) and thus made a critical step towards medical application of mini-agrin in the treatment of MDC1A. Mini-agrin combines several advantages for application as a protein or in virus-based gene therapy. First, the notoriously low efficacy in the infection of muscle encountered in gene therapy trials is prevented because the mini-agrin protein is secreted from infected muscle fibers and thus could also act on neighboring, non-infected muscle fibers. Moreover, mini-agrin exerts its beneficial function by interacting with laminin and α -dystroglycan in the extracellular matrix, a fact that additionally would facilitate and enhance efficiency of application of the protein. Second, mini-agrin derives from species-specific endogenously expressed muscle agrin³⁴⁶, what minimizes its immunological rejection by the host. Third, mini-agrin is a relatively small molecule with a cDNA size of 3.2kb and a globular protein structure with an estimated length of approximately 20nm^{332,355,356}. Hence, the mini-agrin cDNA is small enough to allow the use of adeno-associated virus (AAV) vectors as a gene delivery tool. Moreover, the limited size of the mini-agrin protein raises the possibility of a protein delivery.

Delivery of mini-agrin protein to the skeletal muscles

Direct delivery of the mini-agrin protein to the skeletal muscles of diseased individuals would rule out several safety problems faced with viral-based gene therapy (see below). Before undertaking this endeavor, we evaluated the stability of mini-agrin bound to the basement membrane after a temporal expression of 3 days in our transgenic animal model (Fig.11 a - c). Five days after

repression of the transcription (Fig. 11 a) the amount of mini-agrin bound to the membrane has halved and after 9 days has completely disappeared (Fig. 11 b + c). To further expand on this strategy, it would be important to compare the efficiency of the amelioration in $dy^{W/-}$ mice that were discontinuously exposed to mini-agrin protein expression and $dy^{W/-}$ mice continuously expressing mini-agrin in skeletal muscles.

In a preliminary attempt, we tried to deliver recombinant mini-agrin protein by intravenous injection to the extracellular matrix of skeletal muscles. Capillaries running along the muscle fibers are characterized by low permeability³⁵⁷. This might account for the reason why mini-agrin despite its small structure (~20nm in diameter)^{332,355,356} was denied to exit the vascular system and instead remained localized to the capillary walls (Fig. 12). However, intravenous co-injection of mini-agrin protein and vascular endothelial growth factor (VEGF), known to transiently increase the microvascular permeability promptly after application^{348,358}, may allow mini-agrin to exit the capillaries and reach the extracellular matrix of skeletal muscles where it could exert its beneficial effects.

Gene therapy as a means to introduce mini-agrin into the diseased skeletal muscles of $dy^{W/-}$ mice

In a parallel set of experiments, we aimed to introduce the mini-agrin gene by adeno-associated viral (AAV) or adenoviral (AdV) vector-mediated gene therapy into the skeletal muscles of $dy^{W/-}$ mice. Since large-scale production of AAV is a time-consuming business, our collaborators in Munich first provided us with a 1.generation AdV vector serotype-2, expressing mini-agrin under control of the MCK-promoter (AdV-MCK-m-mag; Fig. 13). Adenoviral vectors can be produced to extremely high titer concentrations, are regarded to be safe for use in gene therapy since not malignant and are attributed to high transduction efficiencies in replication as well as differentiated cells. Despite deletion of the viral early genes E1 and E3, AdV may evoke a host immune reaction to viral proteins³⁴⁷. Moreover, adenoviral DNA does not integrate into the host genome, what on the one hand prevents eventual ablation of important genes due to virus insertion, but on the other hand causes only transient transgene expression and thus would involve the need for repetitive treatment. To date, no method is available allowing for efficient systemic delivery of AdV to muscles and local injections are definitely not the method of choice to treat muscular dystrophies, since several hundred of muscles would have been treated repeatedly. However, local application of AdV was previously shown to deliver mini-dystrophin to the diseased skeletal muscles of *mdx* mice where it was able to ameliorate the dystrophic phenotype³⁴⁸. In our preliminary approach, local intramuscular injection of AdV-MCK-m-mag was intended to confirm the ability of mini-agrin to achieve high transduction efficiency and to exert beneficial effects slowing down the progression of the dystrophy in the infected skeletal muscles of $dy^{W/-}$ mice.

Generally, transduction of immature, regenerating and dystrophic muscle fibers is favored compared to normal adult muscle fibers³⁵⁹, due by higher virus receptor expression and less tight extracellular matrix, allowing better adsorption and diffusion, respectively. Indeed, AdV-MCK-m-mag *in situ* infection of $dy^{W/-}$ muscle represented substantially higher transduction efficiencies

compared to infected wild-type muscle (Fig. 14). In contrast to wild-type, virus-infected $dy^{W/-}$ muscle represented extensive necrosis in a wide area surrounding the injection site, whereas intramuscular injection of PBS did not result in necrosis in both genotypes. Therefore, we attributed the pathological effect in AdV-MCK-m-mag-treated $dy^{W/-}$ muscle to an immune reaction to viral proteins. This may indicate the increased susceptibility of the dystrophic muscle to viral invasion³⁶⁰ and additionally may underline the impaired regeneration capacity. Mini-agrin was demonstrated to be present at the muscle fiber membranes in the intact part of the infected muscle, but as expected, was not present at sites of severe muscle damage (Fig. 14 first panel, first column), which hence can not profit from the benefit of mini-agrin. This corresponds to our findings in $dy^{W/-}$ mice starting mini-agrin expression at later stages of the disease, suggesting that mini-agrin only acts on intact laminin $\alpha 2$ -deficient muscle tissue and does not reverse manifested dystrophic symptoms (Fig. 8 - 10). Major immune responses to viral gene products might be suppressed by administration of immune-suppressants to the treated mice.

Although gene therapy has initially been met with great enthusiasm, clinical gene therapy trials suffered many set-backs. Many trials have failed to correct the disease being treated, some have caused other diseases such as leukemia³³⁰, and some have resulted in patient deaths due to acute toxicity³²⁹. These disappointing clinical experiences have left the general impression that gene therapy will never fulfill its initial promise. However, these clinical trials may have been conducted before the technology was sufficiently matured to make the technology therapeutically feasible.

Hitherto, most attempts of viral-based systemic gene delivery to muscles implicated complicated invasive surgical techniques or increasing of vessel leakiness by use of the inflammatory mediator histamine or vascular endothelial growth-factor (VEGF). Recently, VEGF-mediated delivery of an AAV-6 vector expressing "micro-dystrophin" was shown to result in body wide transduction of skeletal muscles in *mdx* mice³⁴⁸. Additionally, in a recent issue of PNAS, Qiao and colleagues⁴ presented the first study of a successful somatic gene therapy to treat laminin $\alpha 2$ -deficient congenital muscular dystrophy (MDC1A) in a mouse model. They delivered mini-agrin via a single intraperitoneal injection of AAV vectors serotype-1 (AAV1) into neonatal $dy^{W/-}$ mice. Their work impressively demonstrates the feasibility of systemic gene delivery without additional pharmacological intervention that leads to long-term transduction of whole body skeletal and cardiac muscle and that results in a substantial amelioration of the disease. This work may thus offer a new entry point to envisage gene therapeutic treatment of patients suffering from MDC1A. Moreover, parallel methodological advances indicate that AAV6 in conjunction with VEGF³⁴⁸ and AAV8³²⁷ might even be more efficient than AAV1.

All together, the potential of AAV-mediated, mini-agrin-based gene therapy of MDC1A is high but all the promising predictions must be carefully validated before this concept can be applied to human patients. Viruses still present a variety of problems to the patient, such as toxicity, immune

responses, gene control and targeting issues as well as the potential recovery of the viruses to cause disease. Importantly, much of our understanding of viral vectors is solely based on studies in mice, which tolerate treatment well. Humans might react differently and the efficacy of vector systems may be markedly different between the two species.

Upregulation of endogenous agrin expression

Since gene therapeutic approaches in the treatment of muscular dystrophy still present a variety of problems, an alternative and safe approach to achieve expression of a possibly functional therapeutic molecule in the diseased muscles of MDC1A patients would be the upregulation of endogenous expression of full-length muscle agrin by pharmacological agents. Full-length muscle agrin is a highly glycosylated 400-600kDa heparan sulfate proteoglycan and served as a template for generation of mini-agrin. It acts as an organizer and stabilizer of protein arrangements in the muscle basal lamina^{17,136,137} and its expression is markedly decreased but continued at low levels upon muscle maturation. Several indispensable investigations would be necessary to implement this basic approach, including the identification of the promoter driving endogenous full-length agrin expression in muscles, screening for pharmacologically active agents specifically enhancing the activity of this promoter and first of all, the evaluation of full-length agrin's benefit for the dystrophic MDC1A phenotype. To this end, we investigated the potential of the full-length muscle agrin to diminish the disease progression in *dy*^{W/-} mice and compared its benefit to the effects exerted by chick mini-agrin (c-mag).

Constitutive overexpression of full-length agrin significantly ameliorated the dystrophic phenotype in every respect when referred to *dy*^{W/-} mice (Fig. 17). But the benefit of full-length agrin on the overall function of skeletal muscles (Fig. 17 a), the deceleration of the disease progression (Fig. 17 c + d) and the attenuation of ongoing degeneration (Fig. 17 b) was less pronounced compared to the effect attained by mini-agrin. In contrast, posttranslational stabilization of laminin- α 5 and α -dystroglycan^{316,317} was similar in both genotypes (Fig. 17 e - g).

The decreased efficacy of full-length agrin might be attributed to the generally lower extent of overexpression when compared to chick mini-agrin, what might arise from the increased size and the posttranslational modifications attributed to the full-length agrin protein. The protein backbone of full-length agrin is $95\pm 15\text{nm}$ long³³² and contains a series of heparan sulphate glycosaminoglycan side chains that further enlarge the molecule. In contrast, mini-agrin protein misses the glycosylated domains and is supposed to fold into a globular protein structure with an estimated length of approximately 20nm ^{332,355,356}. However, we showed evidence that the mini-agrin protein remains stably bound at the muscle fiber membrane for several days (Fig. 11). Therefore, perpetual overexpression of chick full-length agrin is likely to saturate the muscle fiber membrane with time. Indeed, the difference of the full-length and mini-agrin protein levels is less pronounced than on the mRNA level (Fig. 16) and decreases more in older mice (data not shown). This saturation might account for the comparable amount of stabilized laminin- α 5 and α -

dystroglycan protein in $dy^{W/-}/c$ -FLag and $dy^{W/-}/c$ -mag mice (Fig. 17e - g). Thus, it is likely possible that the decreased efficacy of full-length agrin is due to its size and glycosylation, both providing for a sterically less effective reconnection of α -dystroglycan to the laminin network and promoting interactions with several other proteins *in vivo*.

Support for this assumption comes from a study that was performed in parallel in our laboratory (mainly by Patrizia Barzaghi). This additional approach aimed to elucidate the mechanism enabling mini-agrin to prevent muscle degeneration and to improve regeneration. Both processes are based on the mechanical re-establishment of the linkage of α -dystroglycan to muscle basement membrane and/or the recovery of ligand/mediated signalling³¹⁷. We generated a chimeric fusion protein consisting of the laminin-binding domain of agrin fused to the functionally but not structurally related α -dystroglycan binding domain of perlecan³³¹ (AgPerl, ~110kDa). Results showed that overexpression of AgPerl in $dy^{W/-}$ mice, exerts a comparable benefit as attained by mini-agrin. This provides strong evidence that the mechanical stabilization of the muscle fiber by reconnection of the sarcolemma to the extracellular matrix, via a transgene containing laminin and α -dystroglycan binding domains, is the major benefit exerted by mini-agrin and agrin-perlecan in $dy^{W/-}$ mice. These two miniaturized molecules are similar in size (~20nm in length) and thus lead to a similar sterical proximity of laminin and α -dystroglycan. This finding supports the assumption that full-length agrin due to its almost 5 times larger backbone (95 ± 15 nm), may provide only for a looser linkage of α -dystroglycan and laminin and thus a decreased mechanical stability what at least partly may account for the less pronounced amelioration in the $dy^{W/-}/c$ -FLag mice (Fig. 17).

2 Conclusion

All together, our findings constitute a critical step towards medical application of approaches that restore the tight linkage between laminin and dystroglycan in the treatment of MDC1A. We demonstrate the therapeutic efficacy of mini-agrin at all stages of the disease. Via its tight reconnection of the muscle fiber to the extracellular matrix, mini-agrin diminishes degeneration and improves the regeneration capacity of muscle fibers. Although both mechanisms are efficient and beneficial at all stages of the disease, successful restoration requires intact laminin $\alpha 2$ -deficient muscle tissue. Hence, the dystrophic condition of the muscle at the time of therapeutic intervention is critical for the extent of the amelioration, since manifested dystrophic symptoms can not be rescued. In conclusion, mini-agrin can substantially slow down the progression of MDC1A muscular dystrophy at all stages, but is not able to rescue the disease or to reverse manifested symptoms. Therefore, the earlier the linkage between laminin and dystroglycan is restored, the higher is the benefit for the diseased patient.

These data might help to improve the safety and efficacy of MDC1A treatment and definitely encourage to further expand on this therapeutic concept, especially in respect to combinational

treatment using functionally different approaches, such as preventing apoptosis³⁶¹⁻³⁶³ in diseased muscles and aiming to ameliorate the peripheral neuropathy. Moreover, these experiments set the basis for further developing clinically feasible and relevant application methods such as gene therapy⁴ and/or the screening of small molecules able to upregulate production of agrin in muscle.

3 Future Perspectives

As outlined above, laminin- α 2 deficiency disrupts the mechanical linkage of the muscle fiber to the extracellular matrix. We provided evidence that this pathological feature can be effectively treated at all stages of the disease, by reconnection of laminin and α -dystroglycan via mini-agrin. In addition, laminin- α 2 deficiency interrupts several signalling pathways, dependent on laminin binding to both α -dystroglycan and α 7 β 1 integrin on the muscle membrane³⁶¹. For instance, disruption of the interactions of α -dystroglycan with laminin- α 2 leads to disrupted PI3K/AKT signalling and apoptotic death of myotubes. Moreover, disruption of laminin-2/4 binding to α 7 β 1 integrin leads to apoptosis involving Src tyrosine kinase signalling (p60Fyn) and a p38 α SAPK-dependent pathway. In short, signalling from laminin- α 2 may provide a survival signal for muscle, and its absence in MDC1A is associated with particularly high levels of apoptosis^{30,361}. Therefore, prevention of apoptosis constitutes an additional promising strategy to ameliorate the muscular dystrophy in MDC1A. Indeed, overexpression of BCL-2, an inhibitor of apoptosis, in MDC1A mice was shown to substantially ameliorate the dystrophic phenotype³⁶¹⁻³⁶³. Taken together, a combination of these two functionally different approaches may provide an improved strategy for the treatment of the muscle pathology in MDC1A.

MDC1A pathology involves other organs than skeletal muscles, in particular the peripheral nervous system causing a severe neuropathy as well as the central nervous system involving white matter changes. Hence, a holistic MDC1A treatment would require a therapeutic strategy that is effective in several tissues. However, restoration of the muscle fiber membrane by mini-agrin as well as prevention of apoptosis by application of BCL-2, both are supposed to act solely on the muscle pathology but do not prevent the severe neuropathy caused by demyelination in the peripheral nervous system. Therefore, further understanding of the functions of laminin- α 2 in brain and peripheral nerve are indispensable to clarify the reason underlying the reduced nerve conduction velocity and the brain white matter changes and may help to develop a holistic treatment of MDC1A.

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VIII APPENDIX I

Curriculum Vitae (October 2005)

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Education

2002-2005	<p>Ph.D. student at the Biozentrum, University of Basel, Switzerland, Department of Neurobiology. Supervisor: Prof. Dr. Markus A. Rüegg</p> <p>Ph.D. thesis: "Artificial restoration of the linkage between laminin and dystroglycan ameliorates the disease progression of MDC1A muscular dystrophy at all stages"</p> <p>Referent of the exam committee: Prof. Dr. Markus A. Rüegg. Co-referent of the exam committee: PD Dr. Thomas Meier. Chairman of the exam committee: Prof. Dr. Tom Bickle</p>
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2000-2002 **1 ½ years diploma work including a 3 months training period at the Biozentrum, University of Basel, Switzerland, Department of Neurobiology.**

Supervisors: Prof. Dr. Markus A. Rüegg and Dr. Gabriela Bezakova.
Subject of the diploma work: "Proteolytic processing of agrin *in vivo*".
Received diploma in April 2002

1995-2000 **Five years of studies in Biology I (classical Biology) at the University of Basel.** Received certificate I in March 1997 and certificate II in March 1998

1987-1995 **Classical school education in the Gymnasium Bäumlhof in Basel, Switzerland.** Received certification in June 1995

1983-1987 **Primary school education at Wasserstelzen in Riehen, Basel, Switzerland**

Scientific trainings

2003 **LTK Modul 1:** Introductory course in laboratory animal science, in Roche, Basel, Switzerland

Oral presentations

2004 "Development of new approaches for the treatment of muscular dystrophies" at the 5th Swiss Meeting on Muscle Research, Magglingen, Switzerland

Poster presentations

2005	USGEB-SSN-SSBP Meeting, ETH Zürich, Switzerland
2004	5 th Swiss Meeting on Muscle Research, Magglingen, Switzerland
2004	Biozentrum Symposium, Chrischona, Basel, Switzerland
2004	Bench to bedside, Pharmazentrum, University of Basel, Switzerland
2003	Bench to bedside, Pharmazentrum, University of Basel, Switzerland
2002	4 th Swiss Meeting on Muscle Research, Magglingen, Switzerland

Publications

2006	Sarina Meinen and Markus A. Ruegg, 2006. <i>News and Commentary</i> . Congenital muscular dystrophy: Mini-agrin delivers in mice. <i>Gene Ther.</i> 13:869–870
2007	Sarina Meinen et al., 2007. Linker molecules between laminins and dystroglycan ameliorate laminin- α 2-deficient muscular dystrophy at all disease stages. <i>The Journal of Cell Biology (JCB)</i> , 2007. 176;7: 979-993

IX APPENDIX II

Publications

Year	Title	Authors	Journal
2006	<i>News and Commentary</i> Congenital muscular dystrophy: Mini-agrin delivers in mice	Sarina Meinen and Markus A. Ruegg	<i>Gene Therapy</i> , 2006. 13:869–870
2007	Linker molecules between laminins and dystroglycan ameliorate laminin- α 2-deficient muscular dystrophy at all disease stages	Sarina Meinen, Patrizia Barzaghi, Shuo Lin, Hanns Lochmüller and Markus A. Ruegg	<i>The Journal of Cell Biology (JCB)</i> , 2007. 176;7: 979-993

Congenital muscular dystrophy

Mini-agrin delivers in mice

S Meinen and MA Ruegg

Gene Therapy (2006) 13, 869–870. doi:10.1038/sj.gt.3302668;
published online 13 October 2005

In a recent issue of PNAS, Qiao *et al.*¹ present the first study of a successful somatic gene therapy to treat laminin $\alpha 2$ -deficient congenital muscular dystrophy (MDC1A) in a mouse model.

The researchers from the University of Pittsburgh used a state-of-the-art adeno-associated viral (AAV) vector to systemically deliver mini-agrin, whose efficacy for the treatment had

been demonstrated earlier in transgenic mice.^{2,3} Their work impressively demonstrates the feasibility of systemic gene delivery for long-term transduction of skeletal and cardiac muscle and subsequent substantial amelioration of the disease without any additional pharmacological intervention. This work points the way to a new approach to gene therapy for patients suffering from MDC1A.

MDC1A is an autosomal recessive muscle wasting disease that often leads to death in early childhood. It is caused by mutations in *LAMA2*, the gene encoding laminin $\alpha 2$, which assembles with the $\beta 1$ and the $\gamma 1$ chain to laminin-2, the main laminin isoform present in the basement membrane of muscle fibers and peripheral nerves (Figure 1a). Basement membranes are highly structured sheets of extracellular matrix molecules that surround many cells. Although other laminin isoforms are synthesized in the muscle of MDC1A patients, they do not form a proper basement membrane that is connected to the muscle sarcolemmal membrane (Figure 1b). Hence, the chain of proteins linking the actin cytoskeleton via the sarcolemma to the basement membrane is inter-

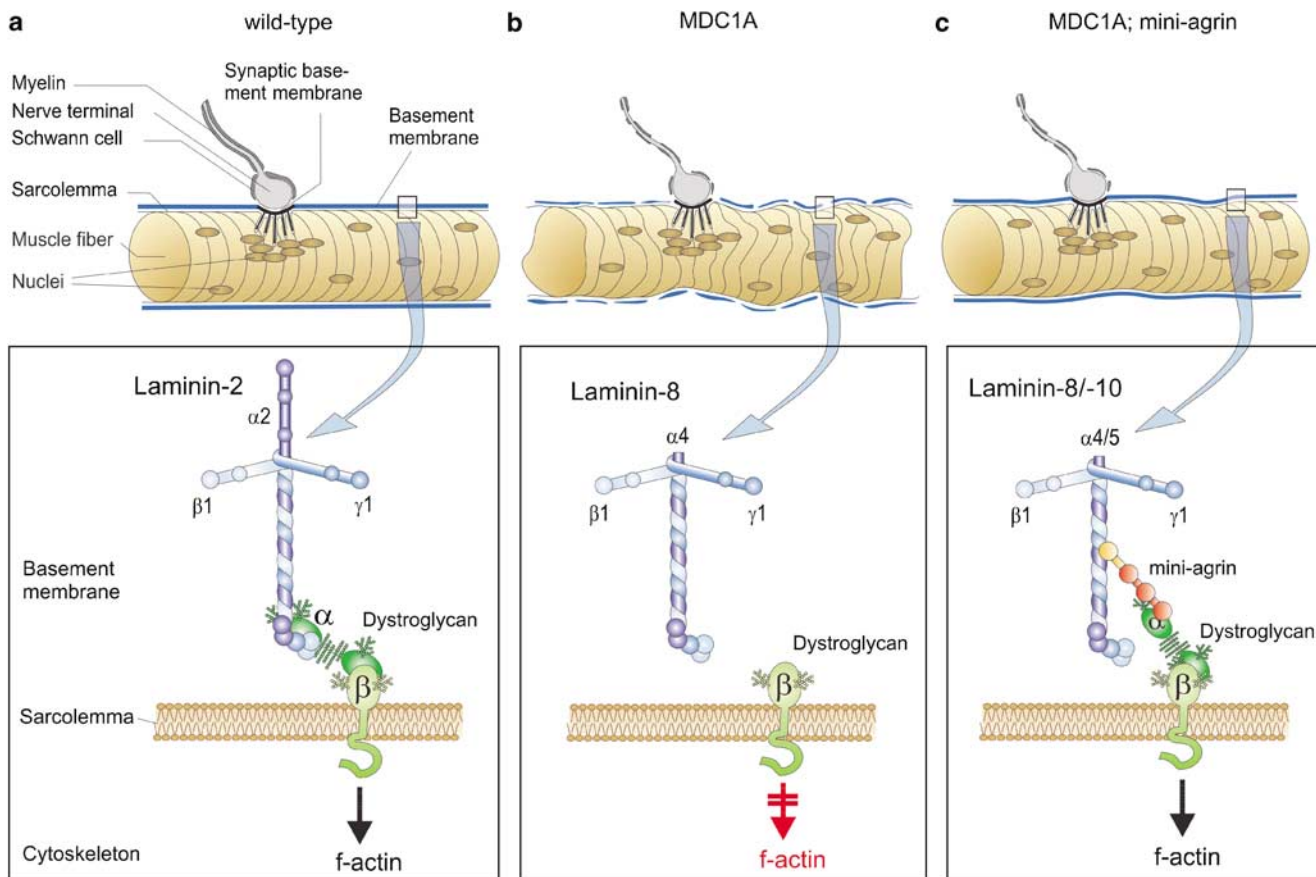


Figure 1 Innervated muscle fiber in (a) wild-type, (b) MDC1A, and (c) MDC1A mice treated with mini-agrin, and the potential mechanism involved in disease progression and treatment (lower panels). (a) In wild-type mice, the peripheral nerve is well myelinated and the muscle fibers are healthy. This is based on the linkage of the basement membrane to the cytoskeleton (top panel). This link is likely to be due to the tight connection of the basement membrane component laminin-2 with α -dystroglycan, which in turn is connected to the transmembrane component β -dystroglycan. β -Dystroglycan connects via linker molecules (not shown) to filamentous actin (f-actin). (b) Dystrophic MDC1A muscle degenerates and the peripheral nerve is demyelinated (top panel). Mutations in laminin $\alpha 2$ prevent synthesis of laminin-2. Instead, laminin $\alpha 4$ is synthesized in MDC1A muscle to form laminin-8. This isoform cannot link the basement membrane to α -dystroglycan and does not allow the formation of a proper basement membrane (symbolized by the interrupted line in the top panel). (c) In MDC1A muscles treated with mini-agrin, both integrity of the muscle and the basement membrane are restored. In contrast, the peripheral nerve is still demyelinated (top panel). Mini-agrin binds via its amino-terminal region to laminin-8 and also stabilizes laminin-10 ($\alpha 5$, $\beta 1$, $\gamma 1$). The carboxy-terminal part of mini-agrin connects to α -dystroglycan and restores the connection to the f-actin.

rupted. As a consequence, muscle fibers lose their stability and degenerate. In addition, the regenerative capacity of muscle is substantially lower.^{3,4}

Gene therapy has recently suffered from major setbacks because of the death of a participant in a trial due to acute toxicity⁵ and the occurrence of leukemia in children who were treated with retrovirus-mediated gene transfer.⁶ Gene therapies for muscle dystrophies are also hampered by the fact that more than 600 muscles must be reached to warrant optimal therapy. Thus, local intramuscular injection is definitively not a feasible strategy in a clinical setting. In this case the authors' major achievement came by using AAV1 vectors that allowed long-term (at least 4 months) expression of the transgene in all skeletal muscles examined and in the heart by a single intraperitoneal injection into neonatal mice. Recent methodological advances indicate that AAV6 in conjunction with vascular endothelial growth factor (VEGF)⁷ and AAV8⁸ might be even more efficient than AAV1.

The second important change was the use of mini-agrin instead of laminin $\alpha 2$. Re-insertion of laminin $\alpha 2$ would be extremely difficult because of the large size of the cDNA (9 kb), which prevents its packaging into AAV vectors. Moreover, laminin $\alpha 2$ must become incorporated into the laminin heterotrimer to be functional. As several domains of laminin $\alpha 2$ contribute to its functionality, it is also unfeasible to generate a miniaturized version without losing its function. Moreover, *de novo* expression of laminin $\alpha 2$ might trigger immune responses in patients. In contrast, the mini-agrin used by Qiao *et al.*¹ has several advantages. Firstly, its cDNA is small enough to be incorporated into AAV vectors. Secondly, because MDC1A patients express agrin endogenously, the immunological rejection of the protein will be minimal.

Agrin, well known for its role in the organization of the nerve-muscle synapse,⁹ shares with laminin $\alpha 2$ the ability to bind to α -dystroglycan, a protein that is involved in the linkage of basement membranes to the muscle sarcolemma (Figure 1). Moreover, an amino-terminal domain of agrin confers binding to all laminins. Transgenic overexpression of a mini-

agrin consisting solely of the laminin-binding and the α -dystroglycan-binding domain markedly improved the stability, function and regenerative capacity of muscle in mouse models for MDC1A.^{2,3} As a consequence, the mice had greatly prolonged lifespan and improved locomotion. As in the transgenic study, Qiao *et al.*¹ restored the structure of the muscle basement membrane, decreased dystrophy-related muscle fibrosis and significantly improved body growth, locomotor functions and lifespan. Mini-agrin expressed in nonmuscle tissue seemed to have no adverse effects during the time window examined. Although mini-agrin was present in the basement membrane of the peripheral nerve, it could not prevent demyelination.¹ This might arise either from insufficient levels of mini-agrin or from the different function of laminin $\alpha 2$ not using α -dystroglycan but integrins as a receptor in peripheral nerve.

In summary, the potential of AAV-mediated, mini-agrin-based gene therapy of MDC1A is high, but all the promising results from the mouse studies must be carefully validated before they can be applied to human patients. Viruses still present a variety of problems for patients since much of our understanding of viral vectors is solely based on studies in mice, which tolerate treatment well. Humans might react differently and the efficacy of vector systems may be markedly different between the two species. Moreover, MDC1A pathology involves also organs other than skeletal and cardiac muscles, and a perfect treatment would also require infection of the peripheral and central nervous system. Since expression of mini-agrin in peripheral nerves failed to prevent neuropathology, it is unlikely that this treatment could alleviate all symptoms. Although the current study by Qiao *et al.*¹ applied mini-agrin at a later stage than the previous transgenic study,² it will be important to test the efficacy of mini-agrin that is applied when the symptoms of the dystrophy are apparent. Finally, more detailed information about the molecular mechanisms involved in the beneficial effect of mini-agrin might help to improve the safety and efficacy of MDC1A treatment, especially in combination with treatment

using functionally different approaches, such as the prevention of apoptosis.^{10,11} ■

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- 1 Qiao C, Li J, Zhu T, Draviam R, Watkins S, Ye X *et al.* Amelioration of laminin- $\alpha 2$ -deficient congenital muscular dystrophy by somatic gene transfer of miniagrin. *Proc Natl Acad Sci USA* 2005; **102**: 11999–12004.
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Linker molecules between laminins and dystroglycan ameliorate laminin- α 2-deficient muscular dystrophy at all disease stages

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Mutations in laminin- α 2 cause a severe congenital muscular dystrophy, called MDC1A. The two main receptors that interact with laminin- α 2 are dystroglycan and α 7 β 1 integrin. We have previously shown in mouse models for MDC1A that muscle-specific overexpression of a miniaturized form of agrin (mini-agrin), which binds to dystroglycan but not to α 7 β 1 integrin, substantially ameliorates the disease (Moll, J., P. Barzaghi, S. Lin, G. Bezakova, H. Lochmüller, E. Engvall, U. Müller, and M.A. Ruegg. 2001. *Nature*. 413:302–307; Bentzinger, C.F., P. Barzaghi, S. Lin, and M.A. Ruegg. 2005. *Matrix Biol.* 24:326–332.). Now we show that late-onset expression of mini-agrin still prolongs life

span and improves overall health, although not to the same extent as early expression. Furthermore, a chimeric protein containing the dystroglycan-binding domain of perlecan has the same activities as mini-agrin in ameliorating the disease. Finally, expression of full-length agrin also slows down the disease. These experiments are conceptual proof that linking the basement membrane to dystroglycan by specifically designed molecules or by endogenous ligands, could be a means to counteract MDC1A at a progressed stage of the disease, and thus opens new possibilities for the development of treatment options for this muscular dystrophy.

Introduction

Congenital muscular dystrophies (CMDs) represent a clinically and molecularly heterogeneous group of autosomal recessive neuromuscular disorders with a typical early onset of symptoms. Estimates in Italy suggest an incidence rate of 4.65×10^{-5} (Mostacciolo et al., 1996). Thus, after Duchenne muscular dystrophy (DMD), CMDs represent the second most frequent neuromuscular disorder. Laminin- α 2-deficient CMD, classified as MDC1A, accounts for ~30–40% of all CMD patients. MDC1A is a severe progressive muscle-wasting disease that leads to death in early childhood (Miyagoe-Suzuki et al., 2000; Muntoni and Voit, 2004; Ruegg, 2005). It shows a rather homogenous clinical picture, with severe neonatal hypotonia associated with joint contracture and inability to stand or walk.

Moreover, MDC1A is accompanied by a peripheral neuropathy that is caused by demyelination in the peripheral and central nervous system. However, no mental retardation is observed in most patients.

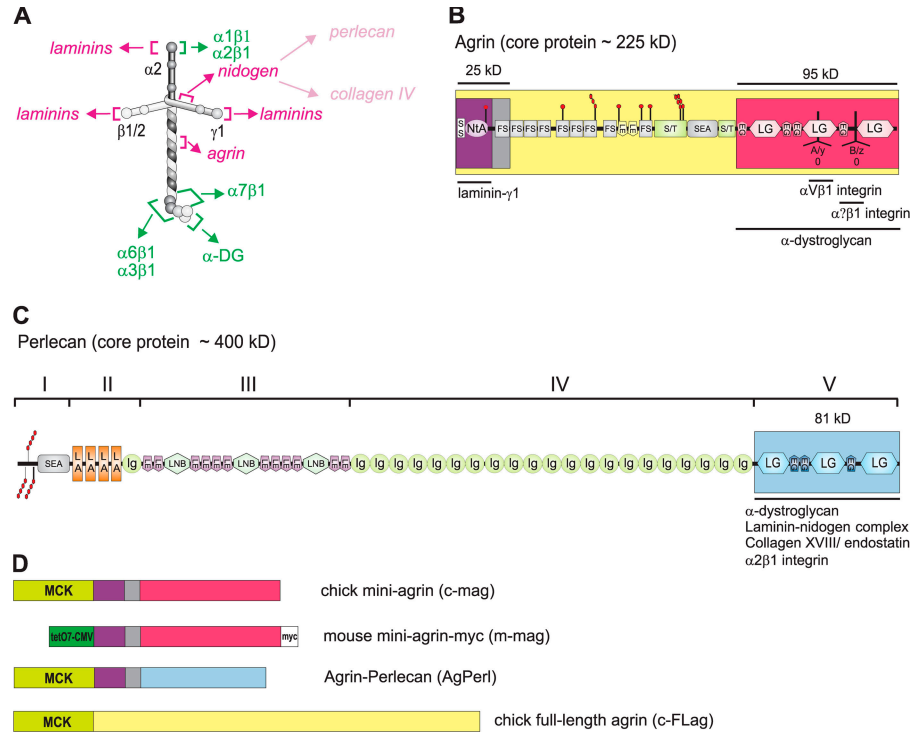
Laminins are cruciform-like molecules formed by α , β , and γ chains (Fig. 1 A). There are 5 α , 3 β , and 3 γ chains described so far that give rise to 15 isoforms (Aumailley et al., 2005). The central role of laminins can be explained by their dual function in organizing a structured basement membrane through interaction with other basement membrane proteins and connecting basement membranes to adjacent cells via cell surface receptors. Inactivation of different laminin chains in mice causes distinct phenotypes (for review see Miner and Yurchenco, 2004). The laminin- α 2 chain assembles to laminin-211 (LM-211; α 2, β 1, and γ 1) and LM-221. LM-211 is the main isoform in the basement membrane of muscle and peripheral nerve, whereas laminin-221 is restricted to neuromuscular junctions (Patton et al., 1997). In the basement membrane, LM-211 and -221 bind to other laminins, to nidogen (which in turn binds to collagen IV and perlecan), and to agrin (Fig. 1 A). The self-polymerization activity of LM-211 is thought to be particularly

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Abbreviations used in this paper: c-Flag, chick full-length muscle agrin; c-mag, chick mini-agrin; CK, creatine kinase; CMD, congenital muscular dystrophy; DGC, dystrophin-glycoprotein complex; DMD, Duchenne muscular dystrophy; dMyHC, developmental myosin heavy chain; HE, hematoxylin and eosin; MCK, muscle CK; m-mag, mouse mini-agrin; tTA, tetracycline-dependent transcription activator; WT, wild-type.

The online version of this article contains supplemental material.

Figure 1. Interactions of laminin-211 and scheme of constructs used in the study. (A) Structure and binding sites of LM-211. Laminins form by coiled-coil interactions of α , β , and γ chains. Interactions of LM-211 and -221 with extracellular matrix components are indicated in red and italics. The main receptors are indicated in green and include different integrins and α -dystroglycan. (B and C) Schematic representation of nonneuronal agrin (B) and perlecan (C). Domain structures and abbreviations are adopted from previous studies (Bezakova and Ruegg, 2003; Iozzo, 2005). The domains included in the constructs used in this study are color-coded, and relevant binding partners are indicated. (D) Schematic presentation of constructs used in the study. Promoters (green), domains (see color code in B and C), and tags are indicated for each construct. MCK represents the 1.3-kb fragment of the human MCK promoter. TetO7-CMV represents the tetracycline-responsive promoter (Fig. S1 A). Fig. S1 is available at <http://www.jcb.org/cgi/content/full/jcb.200611152/DC1>.



important for the formation of a proper muscle basement membrane. The main receptors for laminin- α 2 in adult muscle are dystroglycan and α 7 β 1 integrin (Fig. 1 A, green). Dystroglycan is cleaved into the peripheral α -dystroglycan and the transmembranous β -dystroglycan. In the membrane, dystroglycan associates with the sarcoglycans and sarcospan and intracellularly binds to dystrophin, which in turn links the complex to the f-actin cytoskeleton. The complex between LM-211, dystroglycan, sarcoglycans, and dystrophin, which is called the dystrophin-glycoprotein complex (DGC), has been shown to be of utmost importance for the maintenance of muscle integrity, as mutations in these components cause different types of muscular dystrophies (for review see Davies and Nowak, 2006). Similarly, mice or humans that are deficient of α 7 integrin display a mild muscular dystrophy (Mayer et al., 1997; Hayashi et al., 1998), and muscle-specific inactivation of β 1 integrins has a major impact on muscle development (Schwander et al., 2003). Thus, the evidence is strong that both receptor systems contribute to the linking of basement membrane to the f-actin cytoskeleton, and it is likely that the two systems act synergistically.

MDC1A is among the most severe muscle dystrophies, which may be based on the observation that the absence of laminin- α 2 leaves both receptor systems unoccupied by its ligand. As a compensatory mechanism, muscle fibers of MDC1A patients and laminin- α 2-deficient mice increase synthesis of laminin- α 4 (Patton et al., 1997; Ringelmann et al., 1999; Moll et al., 2001; Bentzinger et al., 2005). However, LM-411 is truncated at the N-terminal end, which prevents its self-polymerization, and it also does not bind to α -dystroglycan or α 7 β 1 integrin with high affinity (Kortessmaa et al., 2000; Talts et al., 2000). There is

also evidence that muscle fiber membranes of MDC1A patients, and mice models thereof, contain significantly lower levels of α 7 β 1 integrin (Vachon et al., 1997) and α -dystroglycan (Moll et al., 2001; Bentzinger et al., 2005). In addition, the ability of muscle to regenerate is greatly impaired (Kuang et al., 1999; Bentzinger et al., 2005). These deficiencies lead to the dystrophic phenotype characterized by high levels of creatine kinase (CK) in the blood, large variation in fiber size, successive replacement of muscle by fibrous tissue, and infiltration of adipose tissue. Good models for the disease are *dy^w/dy^w* mice generated by homologous recombination (Kuang et al., 1998). Like human patients, *dy^w/dy^w* mice have an early onset and severe dystrophic phenotype, which is often lethal between 6 and 16 wk. They grow at a slow rate, the histology of muscles is very similar to that of human patients, and they have a prominent peripheral neuropathy based on defective myelination of the peripheral nerve.

There is no curative treatment for MDC1A. However, a “replacement therapy” using a miniaturized form of the basement membrane component agrin (mini-agrin) was shown to markedly lower muscle degeneration and mortality in *dy^w/dy^w* mice (Moll et al., 2001). This is caused by both increasing the tolerance to mechanical load and improving the regenerative capability of the muscle (Bentzinger et al., 2005). These studies left several questions unanswered that were addressed in the current study. First, an efficacious treatment also needs to work after the onset of the disease. Second, a requisite to envisage pharmacological treatment options that aim at increasing synthesis of endogenous agrin is to show that full-length agrin can also have a beneficial effect. Finally, although it is highly suggestive that the beneficial effect of mini-agrin is based on the linking of the up-regulated LM-411 with α -dystroglycan

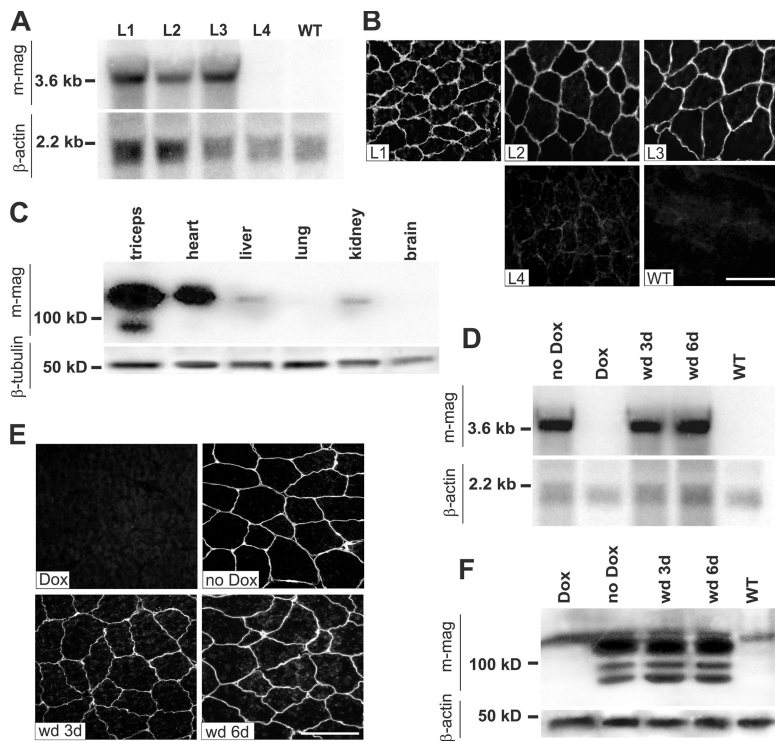


Figure 2. Expression of m-mag in transgenic lines. (A) Northern blot analysis of quadriceps and (B) immunostaining of triceps brachii cross sections from transgenic mouse lines 1–4 (L1–L4) and WT mice. Highest expression of m-mag mRNA (~3.6 kb) and protein was detected in L3. (C) Western blot analyses from different tissues of L3. High levels of m-mag (~130 kD) were detected in skeletal muscle (triceps brachii) and the heart. Very low levels of m-mag were observed in liver and kidney, but not in lung or brain. (D–F) Expression of m-mag in mouse line L3 is regulated by doxycycline. 5 mg/l doxycycline (Dox) in the drinking water suppresses the expression of m-mag at the transcriptional (D) and protein level (E and F). After withdrawal of doxycycline for 3 d (wd 3d), m-mag is detected, and levels similar to nontreated transgenic mice (no Dox) can be reached after 6 d (wd 6d). Note the lower molecular weight protein bands in (C and F), which are indicative of proteolytic degradation/processing. For normalization, probes for β -actin were used in Northern blot analyses (A and D) and antibodies against β -tubulin and α -actin in Western blots (C and F). For quantification see Table I. Bars, 50 μ m.

(Moll et al., 2001), other mechanisms (e.g., via integrins) may also contribute.

In an attempt to answer these open questions, we prepared a panel of constructs to generate different transgenic mouse lines (Fig. 1, B–D). First, we used the tet-off system (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200611152/DC1>) to generate dy^W/dy^W mice in which expression of mini-agrin can be temporally controlled (Gossen and Bujard, 1992). Second, we generated transgenic dy^W/dy^W mice that overexpress chick full-length muscle agrin (c-FLag) in muscle (Fig. 1, B and D). Third, we generated dy^W/dy^W mice that overexpress a fusion construct in which we replaced the α -dystroglycan binding region of chick mini-agrin (c-mag) with that of mouse perlecan (AgPerl; Fig. 1, C and D). Domain V of perlecan (also called endoreppelin; Iozzo, 2005) binds to α -dystroglycan (Talts et al., 1999), but not to integrins that are expressed in muscle. In this study, we show that mini-agrin can slow down the progression of MDC1A at any stage of the disease, full-length agrin is capable of improving muscle function, and the fusion construct between agrin and perlecan also counteracts the disease. In summary, our results are conceptual proof that linkage of laminin isoforms with α -dystroglycan is a means to treat MDC1A also at progressed stages of the disease.

Results

The most important questions for developing a treatment are to determine the efficacy of therapy at a progressed stage of the disease, to establish a molecular understanding of how the treatment interferes with disease progression, and to establish possible routes of applying the treatment. To this end, we generated a set of transgenic animals that overexpress artificially designed

proteins in skeletal muscle. All the constructs, including their promoters, are listed in Fig. 1 D.

Tight spatial and temporal regulation of mini-agrin expression

To test whether mini-agrin is also capable of ameliorating the disease when the phenotype is already apparent, we generated mice in which expression of mini-agrin can be controlled by removal of doxycycline (Gossen and Bujard, 1992). To minimize immune responses and for detection, we constructed mini-agrin from mouse cDNA and fused a c-myc tag to its C terminus (Fig. 1 D). Like c-mag, the tagged mouse mini-agrin (m-mag) bound to LM-111 and α -dystroglycan (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200611152/DC1>). Double transgenic mice in which expression of m-mag could be controlled by doxycycline (Gossen and Bujard, 1992; Ghersa et al., 1998) were generated (for details see Fig. 1 D, Fig. S1, and Materials and methods). When they were examined for the expression of m-mag, the highest levels on the mRNA and protein level were detected in line L3 (Fig. 2, A and B; Table I for quantification) which was used in all further experiments. Expression of m-mag was highest in skeletal muscle and heart, whereas only little or no m-mag was detected in liver, lung, kidney, or brain (Fig. 2 C). Next we determined the concentration of doxycycline needed to suppress expression of m-mag throughout embryonic development and to allow fast induction. We found that 5 μ g/ml doxycycline in the drinking water of pregnant and gestating females was sufficient to completely inhibit m-mag transcription (Fig. 2 D) and translation (Fig. 2, E and F). 3 d after withdrawal of doxycycline, m-mag was already expressed at high levels, and it reached a maximum after 6 d (Fig. 2, D–F; Table I for quantification).

Table 1. Quantification of mRNA and protein levels of m-mag in different transgenic lines, and induction by withdrawal of doxycycline

Line	Dox	NB	WB	IHC
L1		65	63 ± 12	70 ± 8
L2		40	57 ± 14	68 ± 3
L3		100	100 ± 7	100 ± 4
L4		1	3 ± 1	5 ± 2
L3	Dox	1	0	1 ± 1
L3	wd 3d	72	92 ± 6	56 ± 7
L3	wd 6d	102	96 ± 9	97 ± 6

Quantification of Northern blot analysis (NB), immunohistochemistry (IHC), and Western blot analysis (WB). Values show expression levels of m-mag in skeletal muscles of mouse lines L1–L4. Data of line L3 after suppression of expression by doxycycline (Dox) and subsequent withdrawal for 3 (wd 3d) or 6 d (wd 6d) are relative to levels measured in mouse line L3. Values represent the mean ± the SEM. $n \geq 3$.

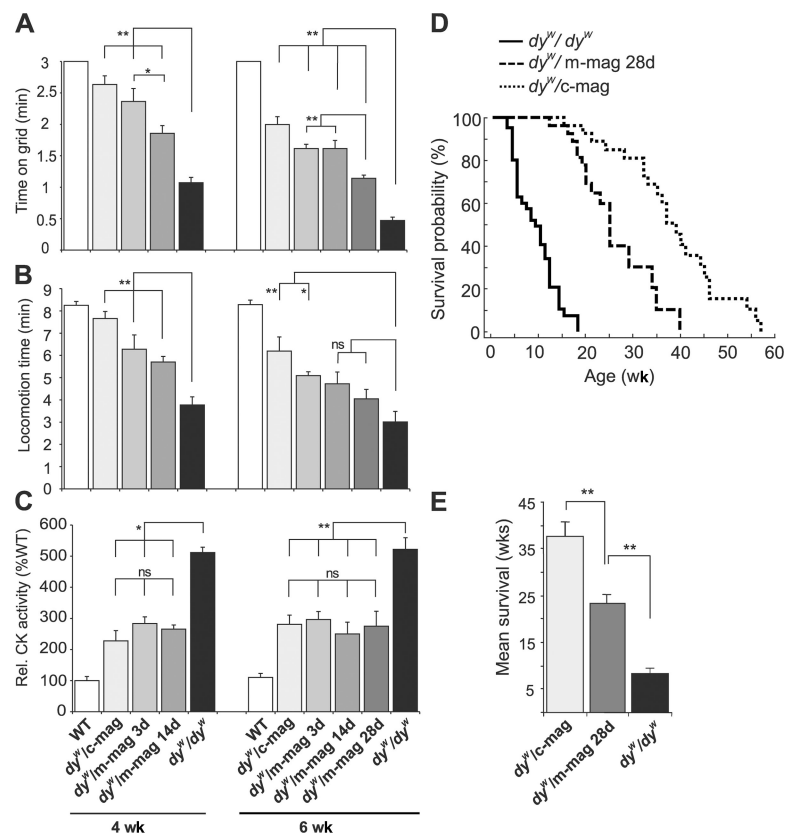
Late onset of mini-agrin expression ameliorates disease progression in dy^W/dy^W mice

To generate laminin- $\alpha 2$ -deficient mice that allowed controlling expression of m-mag, we mated line L3 with mice heterozygous for the laminin- $\alpha 2$ mutation. This breeding eventually resulted in dy^W/dy^W mice that contained all the necessary genetic elements (see Fig. S1 B for the breeding scheme). In such mice, which are called dy^W/m -mag, we removed doxycycline at birth, which resulted in expression of m-mag at postnatal day 3 (henceforth called dy^W/m -mag 3d), at day 11 (dy^W/m -mag 14d), or at day 25 (dy^W/m -mag 28d). Muscular dystrophy was always evaluated in 4- and 6-wk-old mice. In a grip test, dy^W/m -mag

mice always performed better than dy^W/dy^W controls, irrespective of when expression of m-mag was started (Fig. 3 A). In the 4- and 6-wk-old animals the improvement was less pronounced if m-mag expression was started late (dy^W/m -mag 28d). In an open field test, locomotory activity in 4-wk-old m-mag transgenic mice was significantly higher than in dy^W/dy^W mice, whereas in 6-wk-old mice, statistical significance could only be reached in mice expressing the transgene early (Fig. 3 B). To get a direct measure for ongoing muscle fiber damage, we measured the CK activity in the blood (Fig. 3 C). CK activity was ~ 5 times higher in dy^W/dy^W than in wild-type (WT) mice. Expression of m-mag lowered CK activity in dy^W/dy^W mice by approximately half (Fig. 3 C). In contrast to the behavioral tests, the lowering of the CK activity was not dependent on when mini-agrin expression started. Although the improvement in the capability of moving is certainly important to determine the benefit of a treatment, the strongest endpoint in such a severe disease is life expectancy. As shown in Fig. 3 D, treatment that started only after 4 wk substantially increased survival probability. In accordance with this, the mean lifespan of dy^W/m -mag 28d mice was approximately tripled compared with dy^W/dy^W mice (Fig. 3 E).

Another hallmark of the severe muscular dystrophy in dy^W/dy^W mice is the presence of many small fibers and of fibrotic tissue as visualized by hematoxylin and eosin (HE, Fig. 4 A) and Masson's Trichrome staining (Fig. 4 B) of cross sections from triceps brachii. In 6-wk-old dy^W/dy^W mice, muscle showed strong signs of degeneration and replacement of muscle with nonmuscle tissue (Fig. 4 A, top right). The nonmuscle cells

Figure 3. Mini-agrin improves overall performance, lowers muscle damage, and prolongs lifespan. Parameters were measured in 4- or 6-wk-old WT, laminin- $\alpha 2$ -deficient (dy^W/dy^W), and laminin- $\alpha 2$ -deficient mice expressing m-mag (dy^W/m -mag) or c-mag (dy^W/c -mag). (A) In grip strength, all mini-agrin-expressing mice show a significant improvement compared with dy^W/dy^W mice. The improvement is less in mice expressing mini-agrin late (dy^W/m -mag 14d or dy^W/m -mag 28d). (B) Locomotory activity within 10 min. In 4-wk-old mice, all mini-agrin-expressing mice show a significant improvement compared with dy^W/dy^W mice. In 6-wk-old mice, only early treatment (dy^W/c -mag and dy^W/m -mag 3d) is significant. (C) CK levels in the blood. All values are normalized to WT mice. CK activity is reduced to approximately half of that measured in dy^W/dy^W mice in all mini-agrin-expressing mice, irrespective of the onset of expression. (D and E) Survival curves of mice with different genotypes. Late start of mini-agrin expression (dy^W/m -mag 28d) increases the survival probability (D; $n \geq 29$) and the mean survival (E; $n \geq 16$) more than twice in comparison to dy^W/dy^W mice. Note that late expression of mini-agrin is significantly less effective than constitutive expression of c-mag (dy^W/c -mag mice). All values represent the mean ± the SEM. $n \geq 3$. P-values (t test) are as follows: **, $P < 0.01$; *, $P < 0.05$; ns (not significant), $P > 0.05$.



represented mainly fibrotic tissue, as suggested by the blue color in the Masson's Trichrome staining (Fig. 4 B). Moreover, muscle fibers in dy^W/dy^W mice often lost their characteristic polygonal shape, which is indicative of impaired nerve conduction. Although expression of the m-mag transgene prevented much of the fibrosis (Fig. 4, A and B), it did not affect the shape of the muscle fibers. The extent of fibrosis depended on the time point of the transgene expression. It was, however, compelling that a treatment of only 2 wk was still sufficient to improve the histological picture of the muscle. To measure these parameters more quantitatively, we determined the muscle fiber size distribution in 4-wk- (not depicted) and 6-wk-old mice (Fig. 4 C). Compared with WT mice, the fiber size distribution was obviously shifted toward smaller fibers in dy^W/dy^W mice, as many did not exceed a minimal diameter of 15 μm (Fig. 4 C). This shift was prevented by the expression of m-mag. To quantify fibrosis, we determined first the relative percentage of the area covered by nonmuscle tissue in a series of muscle cross sections. As shown in Fig. 4 D, expression of the m-mag transgene prevented the fibrotic phenotype of dy^W/dy^W muscle to a great extent. As an independent measure of fibrosis, we also determined the amount of hydroxylated proline in muscles of the different genotypes (Fig. 4 E). Hydroxyproline is a main constituent of collagens whose expression is high in fibrotic tissue. This quantification also showed the beneficial effect of m-mag. In contrast to the counting of nonmuscle tissue, the amount of hydroxyproline was at least twice as high in all mini-agrin trans-

genic dy^W/dy^W mice compared with WT controls, and this increase was independent of the time point of expression (Fig. 4 E).

Several lines of evidence strongly indicate that muscles of MDC1A patients, and animal models thereof, have a reduced capacity of regenerating upon damage (Miyagoe et al., 1997; Kuang et al., 1999). In dy^{3K}/dy^{3K} mice, another mouse model for MDC1A, this pathology is reversed by constitutive expression of mini-agrin (Bentzinger et al., 2005). To test whether the onset of expression of m-mag influences the outcome of the regeneration process, we induced degeneration by injection of notexin into the tibialis anterior muscle of $dy^W/m\text{-mag}$ 28d mice 1 wk after induction of the mini-agrin. Muscles were then examined 6, 14, and 28 d after injection and compared with WT and dy^W/dy^W mice. As shown in Fig. 5 A, 6 d after injection, many muscle fibers had already reformed in both WT and $dy^W/m\text{-mag}$ 28d mice. Indicative of ongoing regeneration, these muscle fibers expressed high levels of developmental myosin heavy chain (dMyHC; Fig. 5 B). In contrast, muscle of dy^W/dy^W mice contained mainly cells with a very small cytoplasmic surround and only a few dMyHC-positive fibers (Fig. 5, A and B). The difference between dy^W/dy^W and WT or $dy^W/m\text{-mag}$ 28d mice was highly significant in the quantitative assessment of the fiber size distribution (Fig. 5 C). 14 d after notexin injection, the muscle fiber size had further increased, and fibers no longer expressed dMyHC in both WT and $dy^W/m\text{-mag}$ 28d mice (Fig. 5 A). Myonuclei still had a central position, which was indicative of the recent regeneration (Fig. 5 A, arrows).

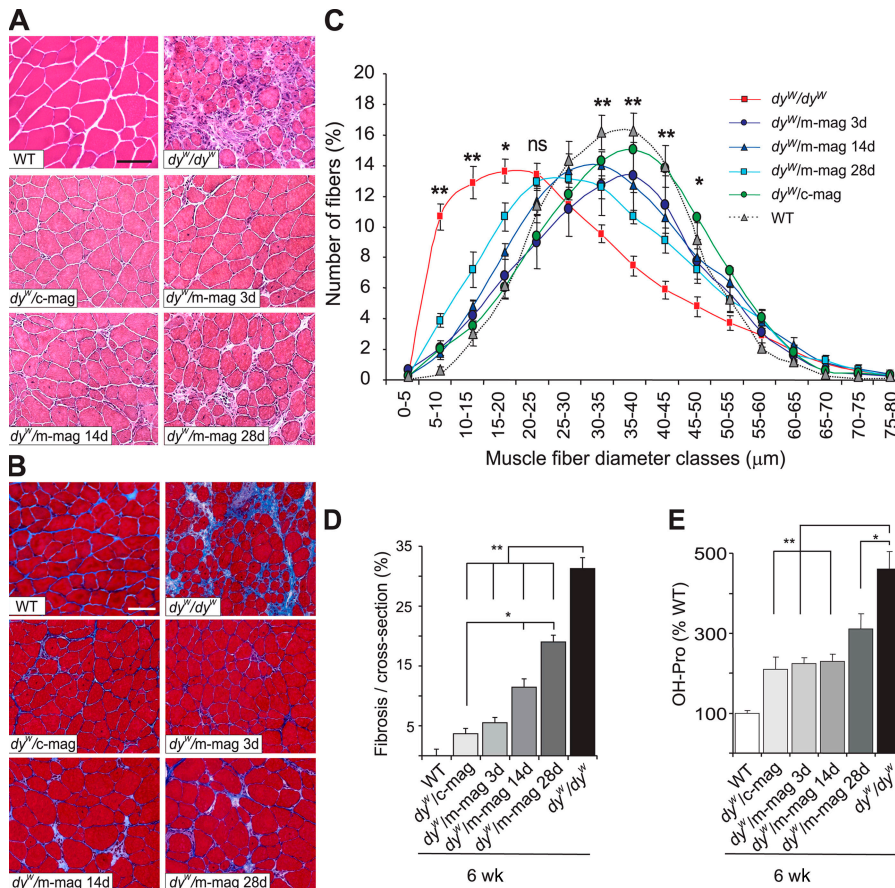


Figure 4. Phenotype analysis in triceps brachii muscle of 6-wk-old mice. HE (A) and Masson's Trichrome (B) staining of cross sections. Pathological changes in the muscle of dy^W/dy^W mice, i.e., fibrosis, variation in muscle fiber diameters, infiltration of nonmuscle tissue, and collagen-containing tissue (blue in B), are less pronounced in mice expressing mini-agrin, but are dependent on the time point of mini-agrin expression. Note that mini-agrin expression does not prevent appearance of polygonally shaped muscle fibers. (C) Muscle fiber size distribution. Values represent relative numbers of fibers in a given diameter class. Muscle fibers of dy^W/dy^W mice are significantly smaller than age-matched fibers of dy^W/dy^W mice expressing mini-agrin. (D) Relative contribution of fibrotic regions to the total area in cross sections. In 6-wk-old dy^W/dy^W mice, the fibrotic tissue represents >30% of the entire muscle. In all the mini-agrin transgenic dy^W/dy^W mice, the fibrosis is significantly reduced. (E) Relative amount of hydroxyproline (OH-Pro) in muscles of the different genotypes. The amount of OH-Pro is significantly reduced by mini-agrin ($dy^W/m\text{-mag}$ 3d and 14d, >50%; $dy^W/m\text{-mag}$ 28d, >30%). Values represent the mean \pm the SEM. $n = 3$. P-values (t-test) are as follows: **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$. Bar, 50 μm .

In contrast, cross sections from dy^W/dy^W mice contained large regions with mononucleated cells (Fig. 5 A, arrowheads), and the few muscle fibers did not express dMyHC (Fig. 5 B). This

deficiency in regenerative capacity of dy^W/dy^W mice was also eminent in the fiber size distribution (not depicted). 4 wk after notexin injection, muscles had almost completely recovered.

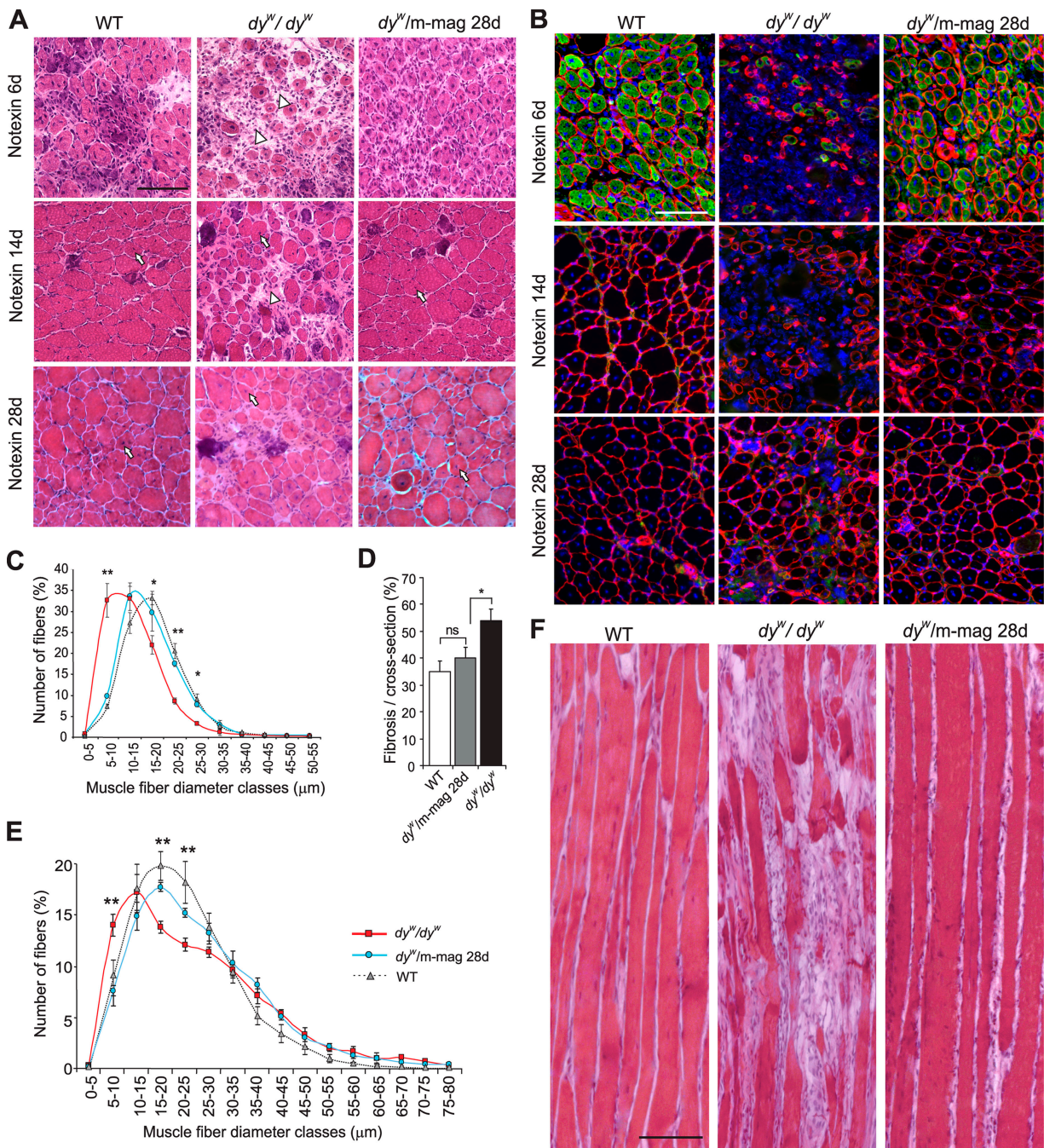


Figure 5. Expression of mini-agrin enhances regeneration of skeletal muscle after notexin-induced injury of tibialis anterior muscle. Notexin injection was performed in 5-wk-old mice in which mini-agrin expression had been started 1 wk before. Muscles were analyzed 6 (A and B, notexin 6d; C), 14 (A and B, notexin 14d), and 28 d (A and B, notexin 28d; D–F) after injection. (A) HE-stained cross sections. (B) Staining with the nuclear marker DAPI (blue), antibodies to dMyHC (green), and laminin- γ 1 (red) were used to determine the state of regeneration. 6 d after notexin injection, muscle fibers are regenerating in WT and $dy^W/m\text{-mag 28d}$ mice, while regeneration is poor in dy^W/dy^W mice; this is indicated by large regions containing mononucleated cells (arrowheads). 14 d after notexin injection, dy^W/dy^W muscle still contains many mononucleated cells (arrowheads) and little dMyHC is expressed. In WT and $dy^W/m\text{-mag 28d}$ mice, regeneration has progressed and most of the muscle fibers have lost expression of dMyHC. 28 d after notexin injection, muscle is restored in WT and $dy^W/m\text{-mag 28d}$, although centralized nuclei are still present. In dy^W/dy^W mice, large regions of the muscle fail to regenerate and are replaced by nonmuscle tissue. (C) Fiber size distribution 6 d after notexin injection. Quantification of fibrosis (D) and fiber size distribution 28 d after injection (E). There is a significant difference between dy^W/dy^W mice and the other two genotypes. (F) HE staining of longitudinal sections of muscles 28 d after notexin injection. dy^W/dy^W muscle is characterized by extensive fibrosis, and most of the remaining muscle fibers are smaller and thinner than in the other two genotypes. Values represent the mean \pm the SEM. $n \geq 3$. P-values (t test) are as follows: **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$. Bars, 50 μ m.

Table II. Quantification of laminin- α 5 and α -dystroglycan detected at the membrane of triceps brachii

Strain	Laminin- α 5	α -Dystroglycan
WT	100 \pm 17	100 \pm 13
dy^W/dy^W	138 \pm 9	27 \pm 6
$dy^W/c\text{-mag}$	373 \pm 33	71 \pm 14
$dy^W/m\text{-mag}$ 3d	365 \pm 17	64 \pm 18
$dy^W/m\text{-mag}$ 14d	409 \pm 17	61 \pm 10
$dy^W/m\text{-mag}$ 28d	368 \pm 16	63 \pm 17

Expression levels of laminin- α 5 and α -dystroglycan were comparable in $dy^W/c\text{-mag}$ and $dy^W/m\text{-mag}$ mice starting expression in advanced stages of the disease. Values are relative to WT mice and represent the mean \pm the SEM. $n \geq 3$.

Muscle of dy^W/dy^W mice still contained large regions that were reminiscent of fibrotic tissue (see Fig. 5 D for quantification), and the diameter of the muscle fibers was often $<15 \mu\text{m}$ (Fig. 5 E). To see whether the regenerated muscle fibers spanned the entire length of the muscle, we also examined longitudinal sections of tibialis anterior muscle. In contrast to WT mice, most of the regenerated muscle fibers were rather short and thin, and large parts of the muscle of dy^W/dy^W mice still contained mononucleated cells (Fig. 5 F). Muscle from $dy^W/m\text{-mag}$ 28d and WT mice showed a homogenous fiber size distribution and only little fibrosis. These experiments show that mini-agrin is sufficient to restore the regenerative capacity of muscle from dy^W/dy^W mice to almost WT levels. Importantly, 1 wk of m-mag expression is sufficient for this effect.

We have previously shown that constitutive overexpression of mini-agrin in dy^W/dy^W mice leads to increased levels of laminin- α 5 and α -dystroglycan (Moll et al., 2001), and that this is based on posttranscriptional effects (Bentzinger et al., 2005). The protein levels of laminin- α 5 were also increased in all $dy^W/m\text{-mag}$ mice, irrespective of the onset of m-mag expression

(Fig. S3 A, available at <http://www.jcb.org/cgi/content/full/jcb.200611152/DC1>; and Table II). Similarly, using antibodies directed to the core protein (Herrmann et al., 2000), we found increased levels of α -dystroglycan in all the transgenic mice (Fig. S3 A; Table II). In contrast, we could not detect any changes in the levels of α 7 integrin (not depicted), which is in agreement with earlier findings (Moll et al., 2001). Because recent experiments showed that transgenic expression of laminin- α 1 is highly beneficial in dy^{3K}/dy^{3K} mice (Gawlik et al., 2004), we also stained for this laminin chain. Basement membranes surrounding skeletal muscle did not contain detectable levels of laminin- α 1 (Fig. S3 B), which is consistent with published results (Patton et al., 1997). Our data thus show that an increase in the amount of laminin- α 1 is unlikely the mechanism of how mini-agrin ameliorates the disease in dy^W/dy^W mice.

Full-length agrin or an agrin-perlecan fusion protein ameliorate disease progression

Another treatment option for MDC1A patients is the up-regulation of the expression of endogenous agrin in muscle, similar to what has been proposed for utrophin in DMD patients (for review see Miura and Jasmin, 2006). Because full-length agrin is a large, highly glycosylated protein, its efficacy in ameliorating the disease might differ from mini-agrin. To test this, we generated transgenic mice that overexpress c-FLag in muscle (Fig. 1, B and D). In another set of experiments, we wanted to test our initial hypothesis that the beneficial effect of mini-agrin is based on the linking of the up-regulated laminin isoforms containing laminin- α 4 to α -dystroglycan (Moll et al., 2001) and not to the integrins. To this end, we generated a fusion construct in which we replaced the 95-kD, C-terminal half of agrin with domain V/ endorepellin of mouse perlecan (Fig. 1, C and D). Like mini-agrin, this fusion protein (AgPerl) bound to α -dystroglycan (Fig. S2 C). These data are consistent with the finding that

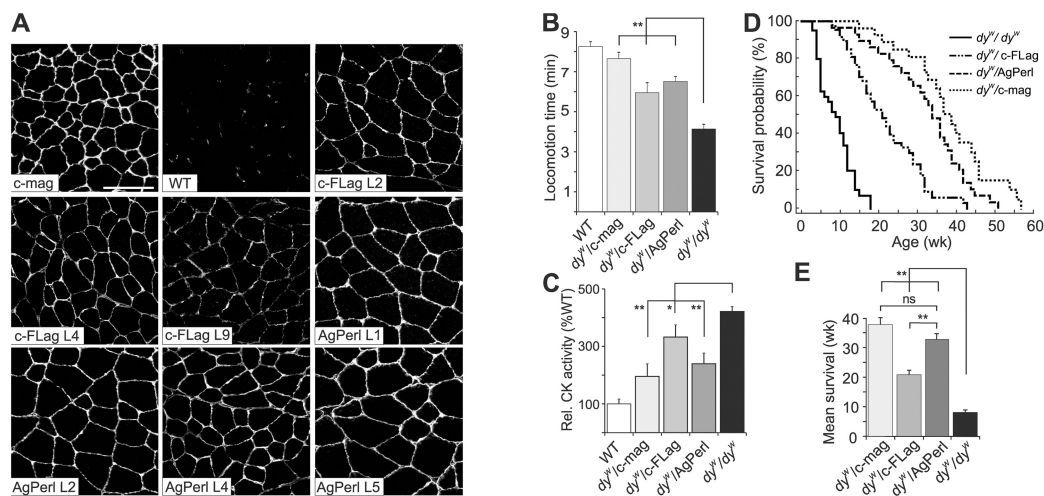


Figure 6. Transgenic expression of c-Flag and an AgPerl fusion protein improves muscle function and lifespan in dy^W/dy^W mice. (A) Immunostaining of transgenes in triceps brachii muscle of transgenic mouse lines for c-Flag (L2, L4, and L9) or AgPerl (L1, L2, L4, and L5). L4 (c-Flag L4) and L1 (AgPerl L1) express the highest levels for c-Flag and AgPerl fusion construct, respectively. For quantification see Table III. Improvement in locomotion (B) or CK values in the blood (C) is evident for all the transgenic mice at an age of 4 wk. (D and E) Life expectancy. The survival probability (D; $n \geq 29$) and the mean survival (E; $n \geq 23$) are more than doubled in the transgenic compared with the dy^W/dy^W mice. Note that in most of the parameters measured there is a trend that the amelioration is less pronounced in $dy^W/c\text{-FLag}$ mice than in $dy^W/c\text{-mag}$ or dy^W/AgPerl mice. Values in all quantifications represent the mean \pm the SEM. $n \geq 3$. P-values (t test) are as follows: **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$. Bar, 50 μm .

Table III. Quantification of mRNA levels by Northern blot analysis (NB) and protein levels by immunohistochemistry (IHC) or Western blot analysis (WB) in different transgenic lines expressing c-FLAG or AgPerl

Strain	Line	NB	WB	IHC
c-mag		100	100 ± 6	100 ± 5
c-FLAG	L2	57	89 ± 19	84 ± 6
	L4	63	97 ± 10	87 ± 5
	L9	42	67 ± 24	78 ± 9
AgPerl	L1	210	79 ± 2	84 ± 5
	L2	72	70 ± 5	78 ± 6
	L4	42	69 ± 1	87 ± 3
	L5	50	60 ± 9	85 ± 6

Values show the expression in skeletal muscles of c-FLAG in mouse lines L2, L4, and L9 and of AgPerl in mouse lines L1, L2, L4, and L5. Data are relative to levels measured in mice transgenic for c-mag (Moll et al., 2001). Values represent the mean ± the SEM. $n \geq 3$.

domain V/endorepellin binds to α -dystroglycan with similar affinity as laminin- α 2 (Talts et al., 1999) or agrin (Gesemann et al., 1998).

Between the different mouse lines, the mRNA levels of the transgenes varied substantially, whereas the amount of protein detected in muscle was similar (Fig. 6 A; Table III). To assess the capability of the transgenes to ameliorate the disease in dy^w/dy^w mice, the mouse lines with the highest expression levels were crossbred and analyzed (i.e., c-FLAG L4 and AgPerl L1). In the locomotory test, all the transgenic lines showed a highly significant improvement compared with dy^w/dy^w mice (Fig. 6 B). Moreover, CK levels in the blood were significantly lower (Fig. 6 C). Most importantly, the survival probability and the mean survival of the transgenic mice were higher than in dy^w/dy^w mice (Fig. 6, D and E).

Muscle histology was substantially improved as shown by HE staining of triceps brachii from 4-wk-old mice (Fig. 7 A), and the size distribution of the muscle fibers was shifted to larger fibers (Fig. 7 B). Consistent with the hypothesis that the mechanism of amelioration by the transgenes is the same as in mini-agrin transgenic mice, protein levels for both laminin- α 5 and α -dystroglycan were elevated (Fig. 7 C; Table IV for quantification). Our experiments thus show that full-length agrin and a fusion protein of agrin and perlecan ameliorate the disease phenotype in dy^w/dy^w mice. In most measurements, mice expressing the AgPerl transgene showed a better improvement than those expressing c-FLAG.

If human patients were to be treated, an appropriate route of application must be defined. Such routes for mini-agrin could be viral vectors (Qiao et al., 2005), but also injection of recombinant protein, as done for other muscle diseases (Bogdanovich et al., 2002; Raben et al., 2003). To test the feasibility of protein application, we determined the turnover rate of mini-agrin in our mouse model. To this end, mice were raised in the presence of doxycycline (i.e., m-mag not expressed), followed by 1 wk without doxycycline (m-mag expressed). Thereafter, doxycycline was reapplied and m-mag expression was followed on the mRNA and the protein level over time. 1 d after readdition of doxycycline, the mRNA encoding mini-agrin had already dropped to \sim 10%, and it could not be detected anymore after 2 d (Fig. 8 A). Concomitantly, with the repression of transcription, m-mag protein steadily declined, as determined by Western blot analysis (Fig. 8 B) and immunohistochemistry (Fig. 8 C).

Quantification of the amount of mini-agrin after suppression of its transcription indicates a half-life of 4.5 d (Fig. 8 D).

To get an estimate of how high the levels of agrin must be to achieve an improvement, we compared the levels of endogenous mouse agrin found in other tissues to the levels of the transgenes expressed in muscles of our mice. We first compared the levels of the transgenic protein for mini- and full-length agrin using antibodies that recognize chick, but not mouse, agrin (Fig. 8 E, left column). The amount of c-mag was \sim 20% higher than c-FLAG (Fig. 8 F; left column). We then compared staining intensity of the transgenic m-mag in muscle with that for endogenous agrin in kidneys using antibodies directed to mouse agrin (Fig. 8 E, right column). Expression levels of the transgenic m-mag were 13% lower than the levels of endogenous agrin detected in kidney (Fig. 8 F, right columns). To compare levels of endogenous agrin in kidney and the amount of c-FLAG in muscle, we assumed that the amount of c- and m-mag were the same. This assumption is based on the fact that the overall improvement in the phenotype is the same in dy^w/c -mag and dy^w/m -mag mice. Expression levels of c- and m-mag transgenes were therefore set as being equal. Based on this, the protein level of the transgenic c-FLAG (81% of c-mag) is substantially lower than the amount of endogenous agrin found in kidney (113% of m-mag). Thus, expression levels of endogenous agrin in kidney are even substantially higher than the levels of the transgenic c-FLAG in the muscle. Thus, agents that increase the amount of agrin in muscle to the amount in kidney are sufficient to be of benefit for dy^w/dy^w mice.

Discussion

In our previous work (Moll et al., 2001; Bentzinger et al., 2005), we provided in vivo evidence that mini-agrin could be a means to prevent muscular dystrophy in MDC1A patients. The work described in this study approaches both therapeutic and mechanistic aspects of how mini-agrin ameliorates the phenotype in dy^w/dy^w mice. It provides strong evidence that mini-agrin also decelerates disease progression when applied at late stages, and it shows that full-length agrin, if expressed at a level similar to that in kidney, is capable of ameliorating the disease. Finally, our evidence that the fusion construct between the laminin-binding domain of agrin and the α -dystroglycan-binding domain of perlecan has the same ameliorating activity in dy^w/dy^w

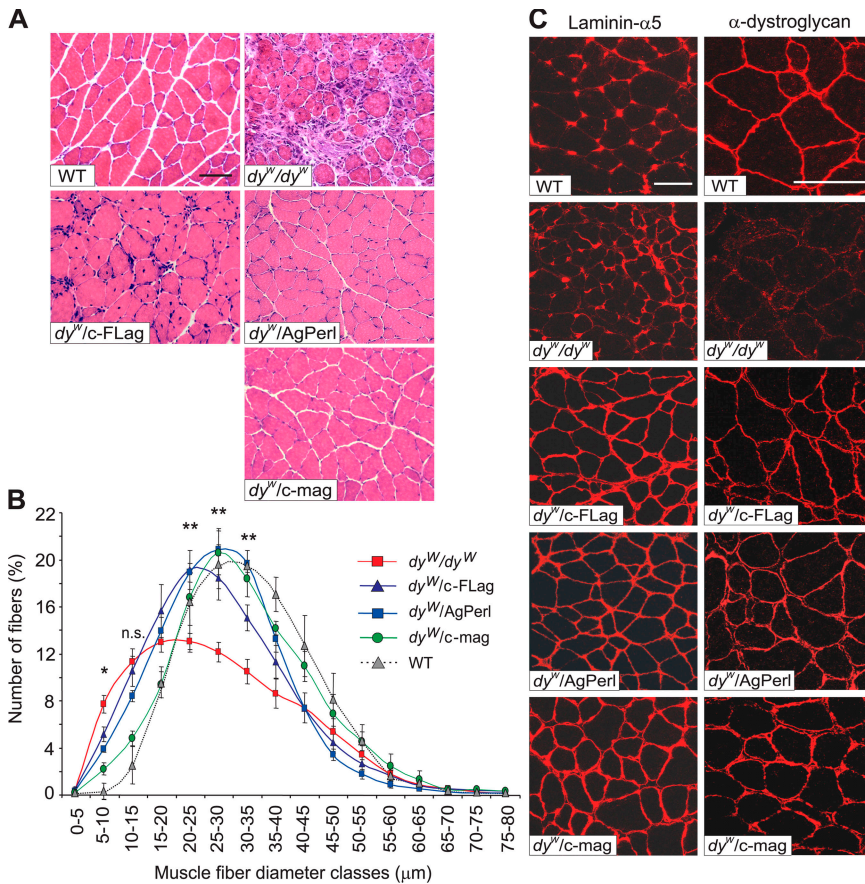


Figure 7. Characterization of muscles from mice transgenic for c-Flag or the AgPerl fusion protein. Animals were analyzed at 4 wk of age. (A) HE staining of triceps brachii cross sections. (B) Muscle fiber size distribution. Values are given as relative number of fibers in each diameter class. Muscles of *dy^W/dy^W* mice contain a significantly higher percentage of small fibers. (C) Levels of laminin- α 5 (left column) and α -dystroglycan (right column) are increased in muscles of *dy^W/c-Flag* or *dy^W/AgPerl* mice relative to *dy^W/dy^W* mice. See Table IV for quantification. Values in all quantifications represent the mean \pm the SEM. $n \geq 3$. P-values (*t* test) are as follows: **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$. Bars, 50 μ m.

mice as mini-agrin clearly indicates that the amelioration is based on the linking of muscle basement membrane to the DGC, and not to integrins.

Mini-agrin slows down MDC1A disease progression

In MDC1A patients, the disease is often diagnosed in the first year of life because of the floppy appearance of the infants. However, muscular dystrophy has already started to manifest at the time of diagnosis, and treatment of infants faces difficulties. Therefore, it is important to evaluate the potential of mini-agrin treatment at progressed stages of the disease. To test this, we generated *dy^W/dy^W* mice that allow the temporal control of the expression of mini-agrin in muscle using the tet-off system (Gossen and Bujard, 1992; Ghersa et al., 1998). We show that mini-agrin is of clear, but attenuated, benefit when applied at progressed disease stages. Importantly, expression of mini-agrin after 4 wk, when the disease is already far progressed, still tripled the mean survival. Our evidence indicates that mini-agrin mainly acts on the tissue that has not yet been destroyed in the course of the disease. This is best manifested by the finding that late expression of mini-agrin seems not to affect already existing fibrosis (Fig. 4), and that early treatment is superior in the behavioral tests (Fig. 3, A and B). There is also evidence that the time point of transgene expression is not relevant for parameters that measure acute responses, such as CK activity in the blood (Fig. 3 C) or the regeneration upon injury (Fig. 5). Our experiments are thus evi-

dence that even late application of mini-agrin is highly beneficial, but that treatment is most successful if initiated early.

Mini-agrin combines several advantages for a feasible treatment of MDC1A patients

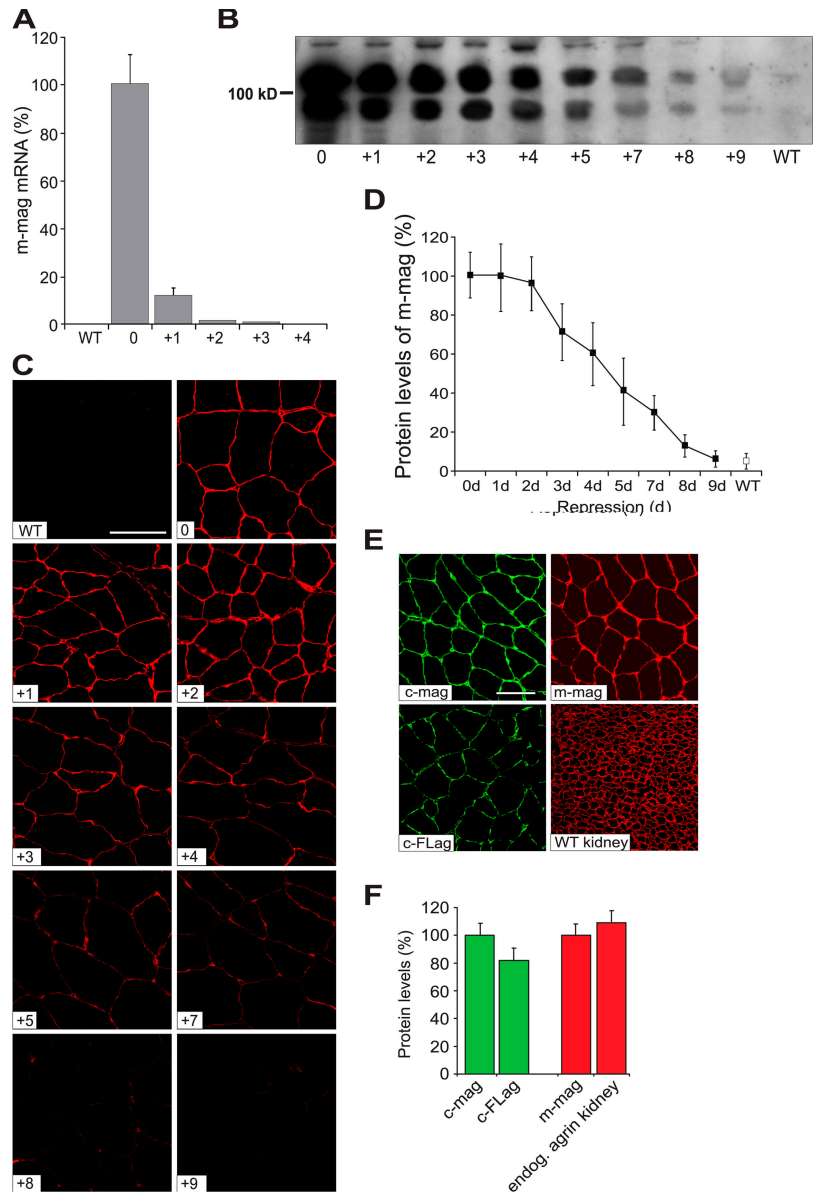
Our proof-of-concept experiment using transgenic mice is a crucial step toward devising ways of treating MDC1A patients. Similar to mini-agrin, transgenic expression of laminin- α 1 also improves muscle function (Gawlik et al., 2004). Although this approach may be interesting for therapy, the use of laminin- α 1 seems less feasible than that of mini-agrin. First, the size of its cDNA (>9 kb) prevents its packaging into AAV vectors. Second, laminin- α 1 must also become incorporated into the laminin heterotrimer to be functional, which makes it difficult to generate a miniaturized version of laminin- α 1 because

Table IV. Quantification of laminin- α 5 and α -dystroglycan in triceps brachii of *dy^W/dy^W* mice expressing *dy^W/c-Flag* or *dy^W/AgPerl*

Strain	Laminin- α 5	α -Dystroglycan
WT	100 \pm 16	100 \pm 11
<i>dy^W/dy^W</i>	138 \pm 20	28 \pm 3
<i>dy^W/c-Flag</i>	263 \pm 64	60 \pm 6
<i>dy^W/AgPerl</i>	266 \pm 39	61 \pm 6
<i>dy^W/c-mag</i>	264 \pm 40	69 \pm 12

Both transgenes increase the concentration of laminin- α 5 and α -dystroglycan in *dy^W/dy^W* mice. Similar values were observed in *dy^W/c-mag* mice. Values are relative to WT mice and represent the mean \pm the SEM. $n \geq 3$.

Figure 8. Feasibility for the use of mini-agrin or up-regulation of endogenous agrin expression as a treatment option. (A–D) Stability of mini-agrin in skeletal muscle of 4-wk-old transgenic mice. (A) Time course of m-mag transcripts in triceps brachii after repression by 50 mg/liter doxycycline in the drinking water, as determined by quantitative real-time PCR. After 2 d, m-mag transcripts cannot be detected anymore. Concomitantly, mini-agrin protein is lost from the muscle basement, as determined by Western blot analysis from quadriceps (B) and quantitative immunostaining of cross sections from triceps brachii muscle (C). (D) Quantitative immunohistochemistry indicates a half-life of mini-agrin protein of ~ 4.5 d. Values are expressed as percentages of staining relative to values before suppression by doxycycline. (E) Staining of transgenic c-mag and c-FLag (left) in triceps brachii muscle using antibodies recognizing chick, but not mouse agrin. Staining using antibodies against mouse agrin in triceps brachii muscle of mice transgenic for m-mag or in kidneys of WT mice (WT kidney; right). Note that staining in green (left) detects only the transgenes, whereas staining in red (right) can detect both the m-mag and endogenous agrin. (F). Quantification of staining intensity. Protein expression levels of c-FLag reach 81% of c-mag (left). Endogenous agrin expression in kidney is 13% higher than the levels of transgenic m-mag in muscle (right). For details see the text and the Materials and methods. Values represent the mean \pm the SEM. $n \geq 3$. Bar, 50 μm .



several domains contribute to its functionality. In contrast, mini-agrin combines several advantages, and thus might be a promising strategy for the treatment of MDC1A patients. As the next step will be the defining of a route of application, we determined the half-life of mini-agrin in the transgenic mice. We found that it was ~ 4.5 d, which is substantially less than what has been estimated for full-length agrin when injected into rat muscle (Bezakova et al., 2001). One of the reasons for this difference could be the lack of any *O*-glycosylation in mini-agrin. Moreover, mini-agrin also seems to be targeted by proteases of unknown identity, as the protein displays distinct bands in Western blots (Moll et al., 2001; this study). Nevertheless, the good stability of mini-agrin, in combination with the fact that it acts extracellularly, makes it a valuable candidate gene for gene therapy. Indeed, recent experiments in dy^w/dy^w mice showed that transduction of skeletal and heart muscle by recombinant adeno-associated virus that express mini-agrin restored muscle function (Qiao et al., 2005; Meinen and Ruegg, 2006).

An alternative way of treating patients might also be the use of recombinant protein and its targeting to the affected tissue. Examples for the successful targeting of recombinant enzymes and antibodies to muscle are the treatment of lysosomal storage diseases (Desnick, 2004) and muscle wasting (Bogdanovich et al., 2002), respectively. The major obstacle for mini-agrin's reaching muscle is, however, its laminin-binding, as laminins line the endothelial wall of blood vessels (Hallmann et al., 2005). Thus, it will be important to reduce the size of the injected protein (e.g., the binding of agrin to α -dystroglycan requires only two laminin G-like domains) and to apply enhancers of endothelial permeability, such as VEGF or histamine.

Up-regulation of endogenous agrin provides an alternative treatment option

An alternative treatment option is the use of molecules that increase the expression of the endogenous agrin protein in

MDC1A patients. In *mdx* mice, which are mouse models of DMD, up-regulation of the endogenous utrophin, which is the autosomal homologue of dystrophin, has been shown to ameliorate the dystrophic phenotype (Miura and Jasmin, 2006). Recently, intraperitoneal injection of a peptide derived from heregulin was shown to increase expression of utrophin and thereby ameliorate the disease in *mdx* mice (Krag et al., 2004). We show that c-FLag, indeed, ameliorates the disease and prolongs lifespan in *dy^w/dy^w* mice. This is experimental proof that the aforementioned strategy might be promising for the treatment of MDC1A patients. We found that the improvement with c-FLag is less effective than with mini-agrin. This difference might arise from a distinct orientation of the domains important for laminin and α -dystroglycan binding caused by the size difference or differences in glycosylation. Full-length agrin is \sim 95 nm long (Denzer et al., 1998), whereas mini-agrin folds into a globular structure with an estimated length of 20 nm. Moreover, the high carbohydrate content of full-length agrin may impose a different orientation of the two functional domains, and the presence of heparan sulfate glycosaminoglycan side chains may lower its apparent binding affinity to α -dystroglycan, as binding of agrin to α -dystroglycan is inhibited by heparin (Gee et al., 1994; Gesemann et al., 1996). Finally, the amount of full-length agrin expressed in our transgenic mice is lower than that of mini-agrin. However, the difference in the protein concentration between the two transgenes became smaller the older the mice were (unpublished data). Thus, it is likely that the slow turnover rate of full-length agrin in the basement membrane allows it to accumulate over time and to eventually reach saturation, despite being less strongly expressed. Accumulation of agrin would be highly desirable in a pharmacological approach because even a moderate increase in agrin transcripts would then result in high concentrations of the protein over time. Moreover, the levels of transgenic agrin necessary for its ameliorative effect are even lower than those found in kidney. Thus, such an approach may indeed be feasible.

A mechanistic explanation of agrin's activity

We also provide conceptual proof that mini-agrin's beneficial effect on the disease progression in *dy^w/dy^w* mice arises from the reconnection of muscle basement membrane with the cytoskeleton via the DGC, as a chimeric fusion protein between AgPerl has the same efficacy in ameliorating the disease as mini-agrin. Both mini-agrin and AgPerl bind to laminins and α -dystroglycan, and they compete for the same binding sites (unpublished data). Our experiments therefore indicate that integrins do not contribute to the ameliorating activity because the C-terminal proportions of AgPerl bind to different integrins (Brown et al., 1997; Burgess et al., 2002). In addition, the α 2 β 1 integrin receptor of domain V/endorepellin is not even expressed in muscle. Our model that reconnection of laminins and α -dystroglycan is the underlying mechanism for the beneficial effect of mini-agrin and AgPerl is also corroborated by the fact that CMDs with phenotypes similar to those of MDC1A are based on mutations in glycosyltransferases that have α -dystroglycan as their main substrates (Muntoni and Voit, 2004).

One of the most striking findings is that mini-agrin, irrespective of the onset of its expression, increases the regenerative capacity of muscle fibers in *dy^w/dy^w* mice. After notexin-induced muscle damage, many fibers in the WT and the *dy^w/m*-mag mice regenerate within the first week, as indicated by the expression of dMyHC. In contrast, in *dy^w/dy^w* mice, dMyHC was expressed only marginally in the early phase, but was also not up-regulated later. Moreover, muscle fibers that had formed in *dy^w/dy^w* mice were shorter, and the muscle contained large regions with mononucleated cells (Fig. 5). This is evidence that muscle regeneration in *dy^w/dy^w* mice is not simply delayed, but that some of the crucial steps cannot be accomplished. The mechanism behind how LM-211 influences regeneration is not known. For example, expression of LM-211 in satellite cells themselves may improve proliferation or survival. Alternatively, satellite cells may depend on LM-211 bound to muscle basement membrane for adhesion and/or survival, which, in turn, would allow their fusion. These events may even be interdependent, as muscle fibers are known to undergo detachment-induced apoptosis, which is termed anoikis, during regeneration (Kuang et al., 1999). The fact that only 1 wk of mini-agrin expression can restore muscle regeneration to levels indistinguishable from WT mice suggests that its binding to α -dystroglycan may activate pathways that prevent anoikis. Indeed, disruption of the binding of laminin with α -dystroglycan induces cell death in cultured muscle cells because of the perturbation of the phosphoinositide 3-kinase–protein kinase B pathway. Thus, we favor a mechanism in which mini-agrin bound to muscle basement membranes allows the survival of satellite cells and early myotubes.

We also find that the level of laminin- α 5, but not laminin- α 1, is increased in all mice that express a transgene. The increase in laminin- α 5 is not based on changes in transcription (Bentzinger et al., 2005), but may be based on its immobilization in the muscle basement membrane. As laminin- α 5 does not bind to α -dystroglycan (Ido et al., 2004), it probably does not contribute to linking α -dystroglycan to basement membrane. However, laminin- α 5 is not truncated at the N-terminal end, a site important for the formation of the primary laminin scaffold. Thus, the increased concentration of LM-511 in the transgenic mice may be important for the restoration of muscle basement membrane. We also observed a restoration of the amount of α -dystroglycan in mice that express the transgene (Fig. 7 and Fig. S3). This change was very striking when we used an affinity-purified antiserum directed against the protein backbone of α -dystroglycan (Herrmann et al., 2000), but was not seen with the antibody IIIH6 directed to the carbohydrate moiety (Gawlik et al., 2004; unpublished data). It may be possible that alterations in the proteolytic processing of dystroglycan, may lead to the loss of the epitope recognized by the antipeptide antibody, whereas glycosylation is not affected.

Future directions in the development of a MDC1A treatment

We and others (Qiao et al., 2005) noticed that mini-agrin does not remove all of the symptoms. Laminin- α 2 deficiency in nonmuscle tissue, particularly in the peripheral nerve, clearly

contributes to the pathology in dy^W/dy^W mice, and because our transgenes are only expressed in muscle, the pathology in non-muscle cells is not reversed. Interestingly, some symptoms are still present in dy^W/dy^W mice that express human laminin- $\alpha 2$ in skeletal muscle (Kuang et al., 1998), whereas amelioration is more complete in mice that express laminin- $\alpha 1$ under the control of the ubiquitously expressed chicken β -actin promoter (Gawlik et al., 2004). Thus, the exclusive expression of all our transgenes in muscle does contribute to the incompleteness of the amelioration. In addition, it is also probable that mini-agrin cannot substitute all of the functions of laminin- $\alpha 2$. For example, mini-agrin is not known to bind to those integrins that are expressed in muscle, and thus, any function mediated by the binding of laminin- $\alpha 2$ to integrins cannot be compensated for. Several lines of evidence strongly suggest that binding of laminin- $\alpha 2$ to $\alpha 7\beta 1$ integrin is important to prevent anoikis (Vachon et al., 1996, 1997). As recent findings indicate that prevention of apoptosis by genetic manipulation is also beneficial for dy^W/dy^W mice (Girgenrath et al., 2004; Dominov et al., 2005), it might be possible that antiapoptotic agents act synergistically with mini-agrin. Several apoptosis inhibitors are used in clinical development. Thus, the combination of antiapoptotic drugs with the expression of mini-agrin in muscle, and/or the up-regulation of endogenous agrin, might be a promising approach to help MDC1A patients. Future experiments will be aimed at critically testing such a strategy.

Materials and methods

Generation of the constructs and transgenic mice

The m-mag cDNA was obtained by two independent RT-PCRs on mRNA isolated from mouse skeletal muscle. The 0.75-kb cDNA encoding the 25-kD N-terminal agrin and the 2.2-kb cDNA encoding the 95-kD C-terminal half were ligated, and a $5 \times$ myc-tag (0.25 kb) was added to the 3' end to yield the m-mag-myc (m-mag) construct. The 3.2-kb m-mag construct was sequenced and subcloned downstream of the uni-directional pTRE2 tet-responsive promoter (tetO7-CMV; BD Biosciences; pTRE2, 3.8 kb). A PacI site was inserted into the pTRE2 vector to allow linearization of the construct as a 4.9-kb PacI-Asel fragment for injection into mouse oocytes. All transgenic mouse lines in which the cDNA was stably inserted into the genome were mated with transgenic mice expressing the tetracycline-dependent transcription activator (tTA) under the control of a 3.3-kb fragment of the human muscle CK (MCK) promoter (Fig. S1). MCK-tTA mice were obtained from N. Raben (National Institutes of Health, Bethesda, MD; Ghera et al., 1998) and were shown to drive expression of transgenes in skeletal and heart muscle (Ghera et al., 1998; Raben et al., 2001). The AgPerl fusion protein was created by fusing the cDNA encoding the 0.75-kb 5' region of chick agrin (Mascarenhas et al., 2003) with a cDNA coding for domain V of mouse perlecan (full-length cDNA encoding mouse perlecan was a gift from T. Sasaki, Max-Planck-Institut für Biochemie, Martinsried, Germany). Both the 6.2-kb c-Flag and the 3.1-kb AgPerl were subcloned downstream of the 1.3-kb MCK promoter (Fig. S1, B–D). Constructs were linearized and injected into male pronuclei. C-mag transgenic mice (c-mag) were created as previously described (Moll et al., 2001). dy^W/dy^W mice (Kuang et al., 1998) containing a LacZ insertion in the LAMA2 gene served as the mouse model for MDC1A.

Genotyping

Genotyping of heterozygous and homozygous dy^W/dy^W mice was done as previously described (Kuang et al., 1998). M-mag mice were genotyped by primers designed to amplify a 683-bp-long fragment, including the linker region of the N- and C-terminal parts of the m-mag construct (5'-GCGGATCACTTTGCGGAACC-3' and 5'-TCGAACCTGAAGTACATGACC-3'). Both c-Flag and c-mag mice were genotyped with primers amplifying a 591-bp-long fragment coding for the C-terminal part of agrin (5'-ACCTGGATAAGCGTTTGT-3' and 5'-CTCTGTTTTGATGCTCAGC-3').

Genotyping of AgPerl transgenic mice was performed on the chick agrin portion of the construct (5'-GTCCCTTGCTGATGACCTTGA-3', 5'-ACCCAGCCCTCAGTACATG-3'). To distinguish hemi- from homozygous MCK-tTA mice, we performed quantitative TaqMan PCR (TaqMan PCR core reagent kit; Applied Biosystems) on the genomic DNA. The following primers were used: tTA-transgene, 5'-GCCTACATTGATTGGCATGTA-3', 5'-CAAAAGTGAGTATGGTGCCTATCTAACA-3', and Probe 5'-FAM-CTTGTCTCGACGCCTTAGCCATTGAG-TAMRA 3'. For normalization of copy number, the following probes for β -actin were used: 5'-CCACTGCCGCATCCTCT-3', 5'-GCTCGTTGCCAATAGTGATGAC-3', and Probe 5'-FAM-CCCTGGAGAAGAGCTATGAGCTGCCTG-TAMRA-3'.

Regulation of the tet-off system

For temporal regulation of m-mag expression under the tetracycline-regulated tet-off expression system (Gossen and Bujard, 1992), 5 μ g doxycycline (doxycycline hydrochloride; Sigma-Aldrich) per milliliter of drinking water (enriched by 4% sucrose) was administered in dimmed bottles. For repression after transgene expression, 50 μ g of doxycycline per milliliter of drinking water was applied.

Protein production

The cDNAs encoding m-mag or AgPerl were subcloned into the pCEP-Pu vector (Kohfeldt et al., 1997) and transfected into HEK 293 EBNA cells. Conditioned medium was collected, and the relative amount of the protein was determined by dot blot assays. Such supernatants were directly used for experiments.

Solid-phase binding assays

96-well plates were coated with either chick α -dystroglycan enriched from skeletal muscle as previously described (Gesemann et al., 1998) or with laminin-111 (0.5 μ g/well), which was a gift from J. Engel (Biozentrum, University of Basel, Switzerland). Proteins were coated in 50 mM sodium carbonate buffer, pH 9.6, and incubated overnight at 4°C. After blocking with PBS containing 0.05% Tween-20, 1 mM CaCl_2 , 1 mM MgCl_2 , and 3% BSA (blocking buffer), wells were incubated with a dilution series (1:6) of supernatant containing m-mag (pure supernatant as the starting concentration) or of purified c-mag (50 nM as the starting concentration). The wells were washed with blocking buffer. Bound protein was detected with polyclonal antibodies raised against the C-terminal, 95-kD part of chick or mouse agrin. Alternatively, the monoclonal antibody 9E10 (Evan et al., 1985) directed against the myc-tag was used. For detection, appropriate horse radish peroxidase-conjugated antibodies, followed by McEvans solution, ABTS, and H_2O_2 , were used. The absorbance was measured on an ELISA reader at 405 nm after 15 min.

Overlay assays

Lysates enriched for α -dystroglycan were obtained from chick or mouse skeletal muscles, as previously described (Gesemann et al., 1998). Proteins were separated on a 3–15% SDS gel and blotted to nitrocellulose membrane. Blots were blocked for 2 h with PBS containing 0.05% Tween-20, 1 mM CaCl_2 , 1 mM MgCl_2 , and 5% dry milk powder (blocking buffer). Supernatants containing recombinant proteins were added and incubated overnight at 4°C. After several washes with blocking buffer, bound m-mag was detected with the anti-myc antibody 9E10, whereas detection of AgPerl was done using a polyclonal antiserum raised against the N-terminal part of agrin. For detection, appropriate horse radish peroxidase-conjugated antibodies were used, and immunoreactivity was visualized by the ECL detection method (Pierce Chemical Co.).

Immunoblots

Tissues were homogenized in protein extraction buffer (75 mM Tris-HCl, pH 6.8, 3.8% SDS, 4 M urea, 20% glycerol, and 5% β -mercaptoethanol). Equal amounts of protein were separated on a 3–12% SDS-PAGE and immunoblotted. Protein signals were normalized to β -actin (Santa Cruz Biotechnology; sc-8432) or β -tubulin (BD Bioscience).

Northern blot analysis and quantitative TaqMan PCR

Northern blot assays were performed on total RNA extracted from skeletal muscles using Northern Max Kit (Ambion). Signals were normalized to corresponding β -actin signals. Quantitative TaqMan PCR was performed on the m-mag transgene (5'-TGTGCAATGTGACCGCTA-3', 5'-GCTGAAACCCTTGCCAGAA-3', and Probe 5'-FAM-CCCCAAAGTC-CTGTGATTCCC-TAMRA 3') and was normalized to β -actin (5'-CCACTGCCGCATCCTCT-3', 5'-GCTCGTTGCCAATAGTGATGAC-3', and Probe 5'-FAM-CCCTGGAGAAGAGCTATGAGCTGCCTG-TAMRA-3').

Locomotion, muscle strength, and CK assay

Locomotive behavior was determined as previously described (Moll et al., 2001). In brief, mice were placed into a new cage and motor activity (walking, digging, and standing upright) was measured for 10 min. Grip strength was evaluated by placing the animals onto a vertical grid and measuring the time until they fell down. The cut-off time was 3 min. Blood for CK assays was collected from the tail vein. 2 μ l of serum was applied using the CK CK-NAC Liqui-UV kit (Rolf Greiner Biochemica). In all tests, at least three animals of each genotype were analyzed, and values were normalized to values obtained from WT animals.

Histology, immunohistochemistry, and antibodies

Muscles were immersed in 7% gum tragacanth (Sigma-Aldrich) and rapidly frozen in liquid nitrogen-cooled isopentane (-150°C). 12- μm -thick cross sections or longitudinal sections were cut and collected on SuperFrost Plus slides (Menzel-Glaser). In the case of longitudinal sections, the slides were pretreated with 3% aqueous EDTA. General histology was performed using HE (Merck). Masson's Trichrome staining (Luna, 1968) was used to visualize collagenous tissue. Membrane-bound and extracellular epitopes were visualized with Alexa Fluor 488-conjugated WGA (Invitrogen). Polyclonal rabbit anti-mouse laminin- $\alpha 5$ (Ab 405) and monoclonal rat anti-mouse laminin- $\alpha 1$ (Ab 198; Sorokin et al., 1992) were a gift from L. Sorokin (Lund University, Lund, Sweden). Polyclonal sheep anti-mouse α -dystroglycan was a gift from S. Kröger (University of Mainz, Mainz, Germany). The remaining antibodies were produced in-house or obtained as follows: monoclonal mouse anti-rat dMyHC (Novocastra), monoclonal rat anti-mouse laminin- $\gamma 1$ chain (CHEMICON International, Inc.), polyclonal rabbit anti-chick (produced in-house; Gesemann et al., 1995), and anti-mouse agrin (produced in-house). Mouse monoclonal anti-myc antibody (9E10) was produced and purified from hybridoma cell line 9E10 and was biotinylated (D-Biotinoyl-E-aminocaproic acid-N-hydroxysuccinimidester; Roche). Depending on the source of the primary antibody, appropriate Cy3-conjugated (Jackson ImmunoResearch Laboratories) Alexa Fluor 488-conjugated secondary antibodies (Invitrogen) or TRITC-labeled streptavidin were used for visualization. DAPI was used to stain nuclei.

Quantification of immunostainings

The muscle fiber size was quantified using the minimum distance of parallel tangents at opposing particle borders (minimal "Ferret's diameter"), as previously described (Briguet et al., 2004). Pictures of WGA-stained cross sections were collected using a fluorescence microscope (DM5000B; Leica), a digital camera (F-View; Soft Imaging System), and analysis software (Soft Imaging System). Measurement of minimal Ferret's diameter of notexin-treated muscle was done on cross sections stained for laminin- $\gamma 1$ and dMyHC. Normalization of the number of fibers in each fiber Ferret class of 5 μm was based on the total number of muscle fibers in each picture. Fibrosis was quantified by measuring the fibrotic area of WGA-stained muscle cross section and normalizing it to the entire area of the cross section. For quantification of immunostainings of m-mag, c-mag, c-Flag, laminin- $\alpha 5$, or α -dystroglycan, images were collected and analyzed by a confocal microscope (TCS-SP; Leica) and appropriate software. InSpeck Microscope Image Intensity Calibration kit (Invitrogen) was used to determine the linear range of the laser. Specific intensity was calculated for each image as the signal intensity of the muscle circumference minus that of an adjacent, unstained region (Turney et al., 1996). Five different pictures were taken using the same parameters on each section, and four different sections were used for each individual mouse. In all quantification experiments, at least three mice of each genotype were analyzed.

Evaluation of full-length agrin expression

Transgenic c-mag and c-Flag were detected by the polyclonal rabbit anti-chick agrin (Gesemann et al., 1995). For comparison of the transgenic m-mag and the endogenous agrin, an antiserum recognizing the 95-kD, C-terminal half of mouse agrin was used. Chick and mouse agrin immunostainings were quantified separately, as described in the previous section. Under the premise that c- and m-mag ameliorate the disease phenotype to the same extent, the relative expression levels of both were set to 100%. This clearly shows that levels of endogenous agrin expressed in kidney (expression level, 113% of m-mag) are sufficient to at least produce the ameliorating effect of c-Flag (expression level, 81% of c-mag).

Notexin-induced muscle damage

Tibialis anterior of 5-wk-old mice was injured by injection of 15–20 μl notexin (50 $\mu\text{g}/\text{ml}$; Sigma-Aldrich), as previously described (Bentzinger

et al., 2005). Mice were killed 6, 14, or 28 d after injection, and muscles were isolated and processed as described in Quantification of immunostainings.

Hydroxyproline assay

Fibrosis in triceps brachii muscles was measured by assaying for the exclusive collagen-specific modified amino acid hydroxyproline (Woessner, 1961; Edwards and O'Brien, 1980). Tendons were carefully removed before muscles were vacuum-speed dried and sent to Analytical Research Services (Bern, Switzerland) for amino acid analysis. There, each muscle was hydrolyzed under vacuum in 50 μl of 6 N HCl for 22 h at 115°C . Hydrolysates were evaporated to dryness and resuspended in 0.1% trifluoroacetic acid. Aliquots were diluted 1:100 for determination of amino acids by a routine method (Cohen et al., 1986), including derivatization with phenylisothiocyanate, followed by HPLC, identifying, and quantifying the collagen-related amino acid hydroxyproline. Relative hydroxyproline amount was assessed in reference to the total amount of amino acids.

Statistical analysis

To compare the different genotypes, p-values were calculated using the unpaired two-sample t-tests, assuming equal variances.

Online supplemental material

Fig. S1 represents the regulation of expression by the inducible tetracycline-regulated tet-off expression system (Gossen and Bujard, 1992) and the breeding scheme to obtain dy^w/dy^w mice with a tight spatial and temporal regulation of mini-agrin expression. Fig. S2 shows the binding of the transgenic m-mag and the fusion protein AgPerl to laminin and α -dystroglycan in both solid-phase and overlay binding assays. In Fig. S3, immunohistochemical staining of cross sections visualizes the regulation of different agrin-binding proteins, including laminin- $\alpha 5$, α -dystroglycan, and laminin- $\alpha 1$ in dy^w/m -mag mice. The online version of this article is available at <http://www.jcb.org/cgi/content/full/jcb.200611152/DC1>.

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