Identification of deregulated AMPK and mTORC1 signalling in myotonic dystrophy type I and their potential as therapeutic targets

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Abstract

Myotonic Dystrophy type I (DM1) is a disabling multisystemic disease affecting skeletal muscle. The disease is caused by expanded (CTG)n repeats in the 3'UTR of the *DMPK* gene. (CUG)n-RNA-hairpins formed by the elongated transcripts lead to sequestration of splicing factors, and thereby to mis-splicing of various genes. Although different strategies have been tested to limit splicing defects, no causal treatment is available for this debilitating disease. To better understand the pathophysiology underlying the disease, we analysed whether DM1-associated muscle alterations may be related to a deregulation of central metabolic signalling and/or of the autophagy process in muscle. Although muscle atrophy in DM1 has been previously related to altered signalling and perturbation in catabolic processes, in-depth investigations into these areas are lacking. We showed that muscles from HSA^{LR} mice, a well-characterized mouse model for DM1, exhibit a defective response to energy restriction. Mutant muscles reveal blunted AMPK activation under staved conditions, which might be related to splicing-dependent CaMKII deficiency. Additionally, active mTORC1 signalling is maintained in muscle from starved mutant mice, while Akt is efficiently inhibited. We further observed that autophagy flux is impaired in HSA^{LR} muscle, which may arise from the deregulation of AMPK-mTORC1 signalling; autophagy is even more severely affected in human DM1 myotubes. Most importantly, normalization of these pathways with pharmacological or dietary approaches improved skeletal muscle strength and significantly reduced myotonia in HSA^{LR} mice. In particular, the AMPK agonist, AICAR, but not metformin, another drug known to induce the AMPK pathway, led to a marked amelioration of the relaxation time of mutant muscle, together with partial splicing correction of *CLCN1*. On the other hand, rapamycin, an mTORC1 inhibitor, and enduring low-protein diet, both reduced myotonia but not DM1-related mis-splicing. Furthermore, mTORC1 inhibition increases muscle force in HSA^{LR} mice. Taken together, this suggests that splicing-dependent as well as alternative, splicing-independent mechanisms can improve muscle function in DM1. These findings highlight the involvement of AMPK-mTORC1 imbalance in the disease and illustrate the importance of deregulated cellular processes contributing to DM1 muscle pathophysiology. At the same time this opens new avenues regarding therapeutic options for DM1, by modulating alternative processes aside from RNA toxicity.

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List of abbreviations

Ach:	Acetylcholine
AChE:	Acetylcholine esterase
AICAR:	5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside
AMP:	Adenosine monophosphate
AMPK:	AMP-activated protein kinase
AK:	Adenosine kinase
Akt/PKB:	Protein kinase B
AKT1S1/PRAS40:	AKT1 Substrate 1
AKT2:	V-Akt Murine Thymoma Viral Oncogene Homolog 2
ASO:	Antisense oligonucleotide
ATP:	Adenosine triphosphate
ATP2A1:	*ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 1*
CACNA1S:	Voltage-gated calcium channel subunit alpha Cav1.1
CaMKII:	Calcium/calmodulin-dependent protein kinase II
CaMKK2:	Calcium/calmodulin-dependent protein kinase kinase 2
CaV1.1:	Voltage-gated L-type calcium channel
CLCN1:	*Muscle specific chloride channel 1*
ClC-1:	Chloride voltage-gated channel 1
CUG-BP1:	CUG binding protein 1
HSA:	Human skeletal actin
DM:	Dystrophia myotonica/Myotonic Dystrophy
DMD:	Duchenne muscular dystrophy
DMWD:	*Dystrophia myotonica-containing WD repeat motif*
DMPK:	*Dystrophia myotonica protein kinase*
DM1:	Myotonic Dystrophy type I/Steinert´s disease
DM2:	Myotonic dystrophy type 2/proximal myotonic myopathy
E_{Cl}:	Cl^- equilibrium potential
EDMD:	Emery–Dreyfus muscular dystrophy
eIF4E:	Eukaryotic translation initiation factor 4E
ELAVL1/HuR:	ELAV-like protein 1
EMG:	Electromyogram
E_K:	K^+ equilibrium potential
FBXO32:	F-Box Protein 32
FKBP12:	FK506 binding protein
FoxO:	Forkhead box O
Fn14:	Fibroblast growth factor-inducible 14
$G_{Cl/K/M}$:	Cl^- / K^+ / Resting membrane conductance
GSK3β:	Glycogen synthase kinase 3β
IGF:	Insulin-like growth factors

HSA^{LR}:	Human skeletal actin - long repeat
INSR:	*Insulin receptor*
IR:	Insulin receptor
Kir2.1:	Inwardly rectifying potassium channel 2.1
LAMP-1:	Lysosomal-associated membrane protein 1
LC3/MAP1LC3:	Microtubule-associated protein 1A/1B-light chain 3
LDL:	Low-density lipoprotein
LGMD:	Limb-girdle muscular dystrophies
LKB1:	Serine/threonine liver kinase B1
LPD:	Low-protein diet
MBNL1:	Muscleblind-like protein 1
MDC1A:	Merosin-deficient congenital muscular dystrophy
MPC:	Mesodermal precursor cell
mRNA:	Messenger RNA
MuRF1:	Muscle RING-finger protein-1
mTORC1:	Mammalian target of rapamycin complex 1
nAChR:	Nicotinic acetylcholine receptor
NaV1.4:	Sodium channel protein skeletal muscle subunit alpha
NMD:	Neuromuscular disorder
Orai1:	Calcium Release-Activated Calcium Modulator 1
PI3K:	Phosphoinositide 3-kinase
PGC1α:	Peroxisome proliferator-activated receptor γ coactivator 1α
PKC:	Protein kinase C
PP2A:	Protein phosphatase 2a
p53:	Tumor protein p53
p62/SQSTM1:	Sequestosome 1
p70S6K:	p70 ribosomal S6 kinase
RBM3:	RNA Binding Motif Protein 3
RNA:	Ribonucleic acid
RNase H:	Ribonuclease H
RyR1:	Ryanodine receptor 1
SERCA1:	Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 1
siRNA:	Small interference RNA
SIRT1:	Sirtuin 1
SIX5:	*Sine Oculis Homeobox Homolog 5*
SR:	Sarcoplasmic reticulum
S6:	Ribosomal protein S6
TAK1:	Transforming growth factor β-activated kinase-1
TF:	Transcription factor
TNNT2:	Cardiac troponin T
TRIM63:	Tripartite Motif Containing 63
TSC2:	Tuberous sclerosis complex 2

Tweak:	Tumor necrosis factor-like weak inducer of apoptosis
ULK1:	Unc-51 like autophagy activating kinase 1
UPS:	Ubiquitin-proteasome system
UTR:	3´untranslated region
ZMP	5-aminoimidazole-4-carboxamide-1-D-ribofuranosyl-5'-monophosphate
ZNF9:	Zinc finger protein 9
4E-BP1:	Eukaryotic translation initiation factor 4E-binding protein 1

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Preface

The neuromuscular disorder Myotonic Dystrophy type I (DM1) is a multisystemic disease, which is the most common form of muscular dystrophy in adults. Even though, various approaches designed to treat this debilitating disease have been conducted, no cure is available to date and current therapies are quite limited in alleviating the symptoms associated to DM1. The lack of suitable therapeutic options may be linked to the complexity of the pathomechanism of the disease, which is still not fully understood.

The aim of my thesis was to shed light into the complex metabolic signalling pathways in DM1 muscle in order to identify novel therapeutic targets. This study delineates the discovery of altered cellular and metabolic processes that are involved in DM1 muscle pathophysiology, together with the identification of druggable targets. Furthermore this works provides different dietary and pharmaceutical approaches and discusses the therapeutic potential of the selected targets and treatments.

The project was conducted in the Neuromuscular Research Group of Prof. Michael Sinnreich, Department of Biomedicine, University of Basel under the supervision of Dr. Perrine Castets, Biozentrum, University of Basel. The thesis comprises a detailed introduction to this work, followed by the manuscript recently submitted to the *Journal of Clinical Investigation*, and lastly a detailed discussion with conclusion and outlooks.

1. Introduction

Muscle is the most abundant tissue in the human body, and is subdivided into skeletal, smooth and cardiac muscle. Skeletal muscle, a type of striated muscle, represents the largest organ in the body accounting for approximately 40% of the human body mass. It permits mechanical activity, allowing respiration, body movement and posture maintenance, thereby accomplishing essential physiological processes. Importantly, skeletal muscle triggers metabolic responses in consequence to contraction and further operates as secretory organ communicating with other organs, thus playing an important role on the whole body metabolism [1, 2].

Consequently, alterations affecting skeletal muscle can lead to various muscle diseases. These diseases can be of hereditary or acquired origin, and are embraced by the term of neuromuscular disorders (NMDs) [3]. NMDs commonly manifest with muscle weakness, sensory disturbances or reflex changes, and display anatomically and symptomatically divers patterns. Depending on the type of the disease, patients can present muscle pain, cramps, myotonia, severe muscle inflammation to abnormalities in the metabolic machinery and many other features that can, as the case may be, mildly affect the patients, lead to severe disability or in the worst case to premature death [4]. NMDs comprise neuropathies, which affect the peripheral nervous system, diseases of the neuromuscular junction (e.g. Myasthenia Gravis or the Eaton-Lambert syndrome), and diseases emerging directly from skeletal muscle including myopathies and muscular dystrophies [3, 5]. To this day, NMDs are mostly not effectively curable and may still be accompanied by major disabilities and life-threatening conditions [5].

As this work comprises a scientific study about the muscular dystrophy Myotonic Dystrophy type 1 (DM1), a summary of skeletal muscle organization and function together with selected metabolic processes and signalling pathways in muscle is given, followed by a detailed overview and description of DM1 disease and the underlying pathomechanism together with the current state of research in this field.

1.1 Skeletal muscle organization and function

1.1.1 Skeletal muscle architecture: an overview

Skeletal muscle is attached to the bones by tendons, and is composed of multiple bundles, which are ensheathed by connective tissue. Muscle bundles contain numerous long cylindrical multinucleated fibres, corresponding to syncytia originating from fusion of several myoblasts during myogenesis [6]. Myofibres are composed of aligned myofibrils, which consist of repeated units, named sarcomeres, which in turn comprise thin and thick filaments. Sarcomeres are the fundamental contractile units of skeletal muscle, forming visible bands, which are responsible for the striated appearance of the muscle fibres, visible under the microscope. The thin filaments are attached to the Z-lines, the borders of the sarcomere, and extend to the centre of the sarcomere. Between each layer of thin filament, a layer of thick filament is present in the centre of the sarcomere. Thick filaments are composed of bundles of myosin, while the thin filaments consist of three proteins called actin, tropomyosin and troponin. At rest tropomysin, that is controlled by troponin, blocks the myosin-binding sites on the actin molecules. Upon liberation of the myosin-binding sites, myosin acts as a motor molecule that interacts with the actin filaments, so that the filaments slide past each other in an energy-dependent fashion, ultimately leading to a shortening of the sarcomere and thereby effecting muscle contraction [6, 7].

1.1.2 Force generation and muscle contraction

Muscle contraction is initiated by single or consecutive action potentials, which are transmitted by the innervating motor neurons, through the neuromuscular junctions (NMJs) to the muscle fibres. The contraction resulting from one single action potential is designated as muscle twitch, while consecutive action potentials trigger a sustained muscle contraction termed tetanus. The arriving action potential causes Ca^{2+} influx through voltage-gated calcium channels at the presynaptic site, which triggers the release of the neurotransmitter acetylcholine (ACh) at the endings of the motor neuron into the synaptic cleft [8, 9]. ACh in turn binds to nicotinic acetylcholine receptors (nAChRs) at the sarcolemma of the muscle fibres and triggers the opening of these channels, ultimately leading to influx of Na^+ and efflux of K^+, thus generating a weak depolarization of the sarcolemma [8]. In response to the membrane depolarization,

voltage sensitive sodium channels $Na_V1.4$ (sodium channel protein skeletal muscle subunit alpha) open and allow large quantities of Na^+ to enter the muscle fibre, which greatly amplifies the depolarization [8]. In this manner an action potential is generated and propagates along the membrane of the fibre and into the T-tubules, which are invaginations of the sarcolemma [8]. The membrane depolarization induces a conformational change of voltage-dependent calcium channels $Ca_V1.1$/DHPR (voltage-gated L-type calcium channel), which further triggers the opening of the RyR1 (ryanodine receptor 1) calcium channels in the sarcoplasmic reticulum (SR) [6, 8, 10]. Ca^{2+} is released from the SR, binds to troponin and induces interaction of actin and myosin filaments, allowing muscle fibre contraction and force generation [6, 7, 11].

When the action potential ceases, ACh release is discontinued, the remaining ACh is reabsorbed into the ending of the motoneuron or is broken down by the acetylcholine esterase (AChE) [12]. Further, $Na_V1.4$ channels close, interrupting Na^+ influx [13]. Repolarization of the membrane is induced by the opening of potassium channels, allowing K^+ ions to exit the fibre, and is further enforced by the activation of the ClC-1 (chloride voltage-gated channel 1) chloride channels, which permit the entry of Cl^- ions into the cell [13]. Finally, the repolarization leads to inactivation of $Ca_V1.1$ and RyR1 [14]. Ca^{2+} ions are actively pumped back into the SR via SERCA1 (sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 1) calcium pumps, thus restoring the resting state of the cell [14]. As a consequence of the falling Ca^{2+} concentration in sarcoplasm, Ca^{2+} dissociates from troponin, the sarcomeres return into their resting length, and the muscle relaxes.

1.2 Processes and signalling pathways at play in muscle homeostasis

Skeletal muscle is a highly dynamic tissue that is exposed to divers physiological conditions, including variable nutrient availability, physical exercise or different types of hormones like anabolic hormones (e.g. growth hormone, Insulin-like growth factor 1) or stress hormones (e.g. glucagon, glucocorticoids) [15-17]. Adequate response and adjustments to these stimuli are crucial for maintaining a healthy and balanced cellular homeostasis for proper muscle performance. Deregulation of central metabolic signalling in muscle can be detrimental and alter muscle function.

1.2.1 Processes involved in proteostasis

In healthy skeletal muscle tissue, homeostasis relies on well-controlled proteostasis, which corresponds to the balance between protein synthesis and protein degradation. Rapid and efficient adjustment of required protein synthesis, as well as adequate protein breakdown, ensuring protein turnover and removal of toxic products, altered organelles, or aggregated proteins are indispensable for muscle function and maintenance of muscle mass [15]. Both, excessive or insufficient protein synthesis and inappropriate protein degradation in skeletal muscle may be very harmful for muscle tissue but can also greatly affect the whole body homeostasis [18]. Therefore, correct regulation of the anabolic and catabolic mechanisms is of major importance for muscle and body health.

The two major processes responsible for proteolysis are the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system [15]. Generally, the UPS selectively targets proteins, while the autophagy flux accounts for the degradation of large amounts of proteins and whole organelles [15, 19]. The UPS is implicated in controlling the amount of regular proteins and is particularly involved in clearing mis-folded and damaged proteins [19, 20]. A protein is selectively targeted for degradation by conjugation of a polyubiquitin chain, comprising a cascade of enzymatic factors, including the ubiquitin-activating enzymes (E1), the ubiquitin-conjugating enzymes (E2), and the ubiquitin–protein ligases (E3) [19]. The polyubiquitinated substrate is then transferred to the 26S proteasome complex that displays three main catalytic activities: the chymotrypsin-like, trypsin-like, and caspase-like activities [20]. The protein is degraded by the proteasome into short peptides, which can be recycled and used for synthesis of novel proteins [19]. It is known that muscle atrophy can be caused by increased activity of the UPS resulting in excessive protein degradation and rapid loss of muscle mass [21]. Hence, deregulation of UPS function has been found to be implicated in the pathogenesis of different diseases, including NMDs [20, 22, 23].

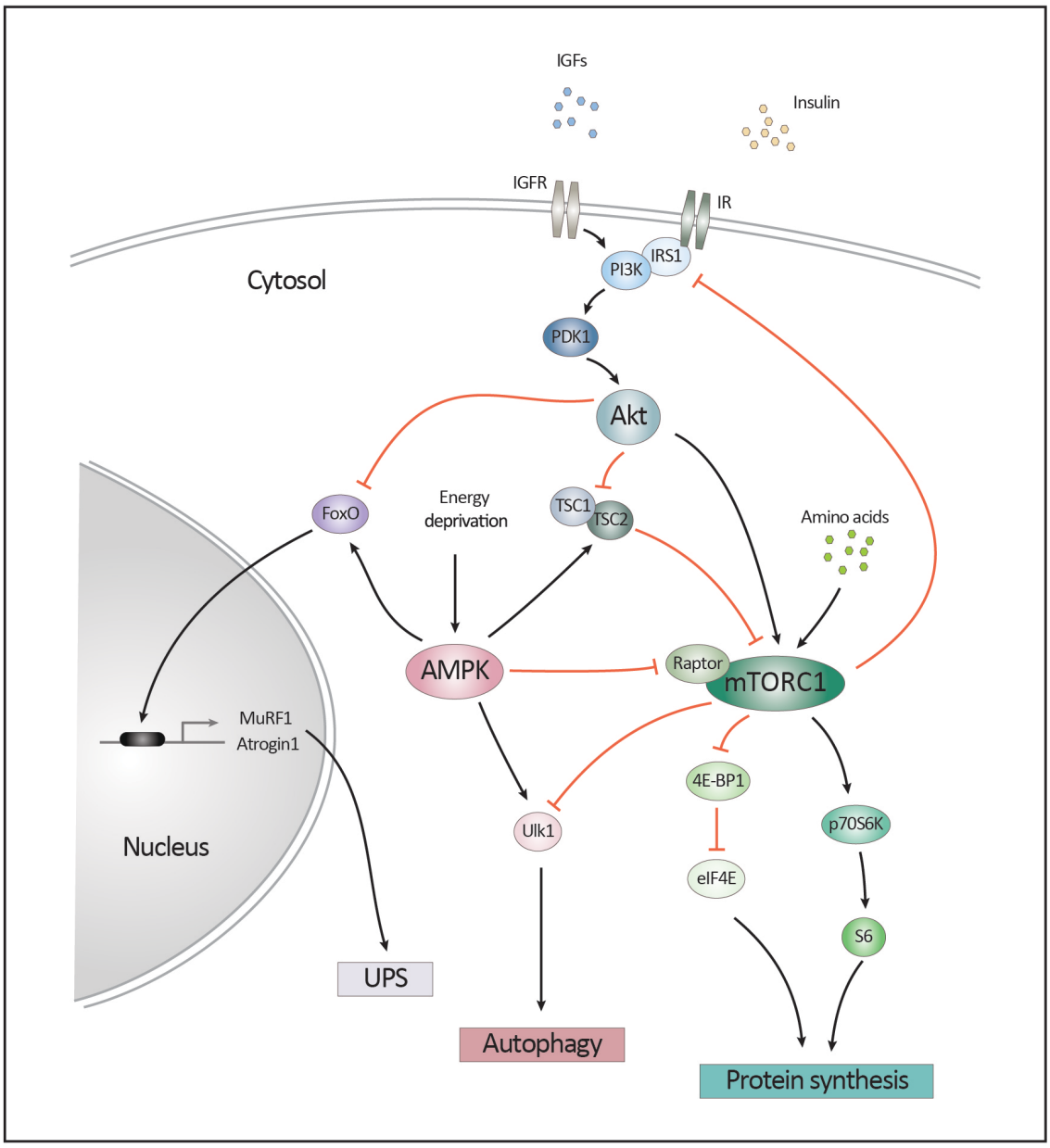
Autophagy is a complex process involving a dynamic flux of autophagic vesicles, being necessary for the basal turnover of cytosolic components and the removal of aggregated and toxic proteins [24]. Autophagy plays a major role in protein catabolism, whereby different stimuli like deprivation of nutrients, energy and hormones can elicit autophagy activity. For instance, nutrient restriction is a potent trigger of autophagy activity [24, 25]. Upon autophagy induction, double-membraned structures called phagophores are formed, further generating vesicles named

autophagosomes that enclose cytoplasmic proteins and organelles [24, 25]. The autophagosome then fuses with lysosomes, and the sequestered material is degraded into small peptides, serving as amino acid source for the cell [24, 25]. Detection of LC3 (MAP1LC3, microtubule-associated protein 1A/1B-light chain 3) has become a reliable method for monitoring autophagy flux. At autophagy induction, the cytosolic LC3I is lipidated to form LC3II, which is inserted into the autophagosomal membrane [26, 27]. At a later stage of autophagy flux, LC3II is degraded [25, 26]. The cargo receptor protein p62/SQSTM1 (sequestosome 1) is also an autophagy substrate and constitutes another marker commonly used to investigate autophagy; accumulation of p62 can be a sign of impaired autophagy [25, 28]. To date, it is known that an immoderate autophagy flux, resulting in excessive protein degradation provokes muscle wasting, while perseverative decreased autophagy flux triggers the accumulation of altered proteins and organelles, which can cause muscle myopathy with myofibre degeneration and muscle weakness [15, 21, 29, 30]. Hence, deregulated autophagy process has been found to be involved in the pathogenesis of various muscle diseases [30-32].

1.2.2 Signalling pathways regulating muscle homeostasis (Scheme 1)

Skeletal muscle homeostasis and proteostasis are controlled by key metabolic signalling, such as the pathways determined by mTORC1 (mammalian target of rapamycin complex 1) and AMPK (AMP-activated protein kinase) [33]. These kinases sense cellular conditions (i.e. energy and nutrient status) and are regulated by signals elicited by growth factors. They respond by switching to the energy and nutrient consuming anabolic processes, such as protein synthesis, or to energy and nutrient producing catabolic processes, such as protein degradation [33, 34].

Growth factors, including insulin and insulin-like growth factors (IGFs) act via the PI3K (phosphoinositide 3-kinase) pathway, in which Akt/PKB (protein kinase B) promotes mTORC1 signalling, amongst others, through inhibition of TSC2 (tuberous sclerosis complex 2) [35-37]. In parallel amino acids are able to activate mTORC1 via the Rag-Ragulator complex [35, 36]. Cell growth and proliferation are promoted by mTORC1-dependent induction of protein synthesis via different downstream effectors [38]. Activation of p70S6K (p70 ribosomal S6 kinase) and its downstream target S6



Scheme 1: The AMPK-mTORC1 signalling pathway. AMPK and mTORC1 activities are inversely related and integrate energy and nutrient (amino acids) signals and growth factors (insulin and IGFs) in order to determine if the cell will switch to anabolic (protein synthesis) or catabolic (autophagy, UPS) processes. In deprived conditions AMPK is activated, promoting autophagy and restricting protein synthesis by blocking mTORC1 via Raptor and TSC2. Ulk1 is responsible for autophagy induction and regulated by AMPK and mTORC1. FoxO transcription is induced by AMPK and further promotes genetic expression of the atrogens (MuRF1 and Atrogin1) that is required for the activation of UPS. Growth factors act via PI3K-Akt/PKB pathway, reducing FoxO-dependent transcription and inhibiting TSC1-2 complex activation. TSC1-2 inhibition and amino acids promote phosphorylation of mTORC1, thereby stimulating protein synthesis through activation of p70S6K-S6 and inhibition of 4EBP1-eIF4E. A negative feedback loop in which mTORC1 inhibits IRS1 (insulin receptor substrate 1) blunts PI3K-Akt/PKB signalling.

(ribosomal protein S6) as well as phosphorylation of 4E-BP1 (Eukaryotic translation initiation factor 4E-binding protein 1), preventing its binding to eIF4E, lead to protein synthesis by increasing gene transcription and translation [35]. A negative feedback loop from mTORC1-p70S6K that inhibits the insulin-IGF pathway further regulates this signalling process [39, 40]. On the other hand, mTORC1 is able to inhibit ULK1 (unc-51 like autophagy activating kinase 1), which leads to reduction of autophagy induction [15, 33].

Another important component regulated by the Akt/PKB pathway is FoxO (forkhead box O) transcription factor. In contrast to mTORC1, FoxO is inhibited by Akt/PKB and triggers catabolic signalling. Upon FoxO activation, transcription of autophagy-related genes as well as of atrogens, such as the muscle-specific ubiquitin ligases TRIM63/MuRF1 (Tripartite Motif Containing 63) and FBXO32/Atrogin1 (F-Box Protein 32) is induced [41, 42]. Consequently, FoxO triggers protein breakdown mediated by the autophagy-lysosome system and the UPS [11, 15, 41].

AMPK, which is activated upon energy deprivation, is an upstream activator of FoxO [11]. Further, AMPK is able to repress mTORC1 signalling through phosphorylation of TSC2 and raptor, a specific component of mTORC1 [11, 43]. AMPK also induces autophagy by interacting with and phosphorylating ULK1 [15, 33]. Hence, mTORC1 and AMPK activities are closely related and consist of the main regulators of autophagy and UPS.

It is well established that transient activation of the Akt-mTORC1 pathway is associated with muscle hypertrophy with an increase in the size of myofibres, whereas mTORC1 inactivation in skeletal muscle leads to atrophy and muscle degeneration [39, 44]. Activation of Akt/PKB-mTORC1 pathway can also counteract disuse-induced muscle wasting [44]. Inversely, its inhibition with the mTORC1 inhibitor rapamycin or by genetically induced repression prevents muscle hypertrophy [44]. However, it has also been shown that sustained activation of mTORC1 leads to a late-onset myopathy in mice with muscle atrophy and muscle weakness [29, 45]. In parallel, muscle hypertrophy has been described in AMPK-deficient mice and increased AMPK activity has been related to muscle atrophy [46, 47]. Hence correct and dynamic regulation of AMPK and mTORC1 signalling is essential for maintaining muscle homeostasis.

1.2.3 Deregulation of metabolic signalling pathways and NMDs

A proper balance between anabolic and catabolic processes is essential to maintain regular muscle function. Deregulation of Akt-mTORC1 and AMPK pathways, as well as abnormal autophagy and proteasomal activity have been related to the pathogenesis of different forms of myopathies and dystrophies, including Duchenne muscular dystrophy (DMD) or collagen VI-related myopathies [30, 48-50]. Constant activation of Akt-mTORC1 pathway in combination with defective autophagy has been observed in muscle of patients affected by DMD and *mdx* mice, a model for this disease [48, 50, 51]. Similarly, it has been demonstrated that impaired autophagy flux together with abnormal Akt and AMPK activity is involved in muscle pathophysiology of collagen VI-related myopathies [30]. Importantly myotonic dystrophy type I (DM1) is another disease that may be associated to deregulation of metabolic signalling. Indeed, different reports suggested that muscle atrophy and weakness in DM1 may be caused by an increased catabolic activity and related to abnormal Akt/PKB-mTORC1 signalling [21, 22, 31, 52, 53].

1.3 Myotonic dystrophy type I: from the genetic cause to therapeutic strategies

1.3.1 Clinical pattern of DM1

Myotonic dystrophy (Dystrophia myotonica, DM) is a dominantly inherited neuromuscular disorder that emerges as two different disease forms named myotonic dystrophy type 1 (DM1, Steinert´s disease, OMIM #160900) and myotonic dystrophy type 2 (DM2, proximal myotonic myopathy, OMIM #602668). DM1 and DM2 affect multiple organ systems, especially compromising skeletal muscle function, and are known to be the most common forms of inherited muscular dystrophies in adults [54]. DM1 and DM2 share clinical features and pathogenic mechanisms involving abnormal polynucleotide repeat expansion mutations. DM1 is more prevalent and generally more severe than DM2, and is caused by (CTG)n trinucleotide repeat expansion in the 3´untranslated region (UTR) of the *DMPK* (dystrophia myotonica protein kinase) gene located at the locus 19q13.3. DM2 is related to a (CCTG)n tetranucleotide expansion in the intronic region of the *ZNF9* (Zinc finger protein 9) gene located on chromosome 3 [55, 56].

DM1 presents with a decreased age of onset and increased severity in succeeding generations, called genetic anticipation. The severity of the disease roughly correlates with the augmentation of the length of the trinucleotide repeat expansion, and is increased in consecutive generations [57, 58]. Further the disease is associated with various phenotypes, ranging from severely affected new-borns afflicted by life threatening complications, to patients with adult onset showing no or only mild symptoms [59]. Accordingly DM1 is subdivided into four main clinical forms; congenital, childhood-onset, adult-onset and late-onset/asymptomatic, each manifesting distinct disease traits, requiring adequate care and therapy [59].

Myotonia is one of the major characteristics of DM1 being markedly present in most of the patients suffering from this disease. The affected individuals present a delayed relaxation of skeletal muscle, which can either occur spontaneously or be elicited by voluntary contraction or by mild stimulation, such as tapping on a muscle (percussion myotonia). Myotonia can manifest already at very early stages of the disease, resulting in muscle stiffness and difficulties in relaxing the grip but also causing problems when talking, chewing, or swallowing [60, 61]. The cause of myotonia in DM1 is generally attributed to reduced Cl^- conductance (G_{Cl}), due to deficiency of the chloride channel ClC-1, resulting in sarcolemmal hyperexcitability; the pathological origin of ClC-1 altered function will be discussed in a subsequent paragraph. Generally tetanic muscle stimulation is accompanied by extracellular K^+ accumulation and thus membrane depolarization at the T-tubules due to the shifted K^+ equilibrium potential (E_K) [62]. In healthy muscle ClC-1 is markedly activated after repetitive firing of action potentials, generating a Cl^- inward flux [62, 63]. Importantly, in addition to its small but significant contribution to repolarization of the sarcolemma, G_{Cl} is of major importance for re-establishing the resting membrane potential [62, 64]. As G_{Cl} accounts for approximately 80% of the resting membrane conductance (G_M), the accumulation of K^+ and thus the rise of E_K is quenched by the presence of the very high G_{Cl}, as the Cl^- equilibrium potential (E_{Cl}) stays relatively constant [65]. Additionally, the resting membrane potential being stabilized by E_{Cl} is more negative than E_K, resulting in enforced K^+ reuptake of the cell [66]. However, in DM1 muscle after tetanic stimulation the rise of E_K cannot be counteracted due to the important reduction of Cl^- flux and is sufficient to trigger muscle action potentials without incoming impulses from the nervous system [62, 67]. Moreover, K^+ ions persist longer in the T-tubule system due

the reduced driving force for K^+ reuptake. Thereby myotonia manifests after voluntary contraction in DM1 patients.

These spontaneous muscle fibre discharges due to membrane hyperexcitability can be monitored by a needle electromyogram (EMG). Insertion of the needle electrode into a myotonic muscle triggers consecutive discharges, that may last tens of seconds to minutes, whereas healthy muscle will only present a brief electronic signal lasting for a few msec [65, 68]. Interestingly, in a recent study the electrophysiological profiles of DM1 patients were investigated, by applying an EMG examination protocol that allows to distinguish ion-channel deficiencies. The electrophysiological profiles of all DM1 patients corresponded to chloride channel dysfunction [69]. However, 40% of the patients exhibited a certain variation from the typical chloride channel profile [69, 70]. It was hypothesized that these deviant profiles may be due to a simultaneous dysfunction of ClC-1 and other muscle channels like sodium channels, suggesting that the pathogenesis of myotonia in DM1 is more complex than initially suspected [69].

Besides myotonia, skeletal muscle alterations in DM1 patients include muscle wasting and weakness, which considerably affect the quality of life of the patients. The pattern of muscle involvement in DM1 comprises facial- and neck-muscle weakness but usually also the distal ends of the limbs, leading to weakness in feet and hands causing substantial disability; proximal weakness becomes symptomatic only late in the disease course [60]. Moreover, respiratory muscle weakness is common in DM1, eventually triggering respiratory failure, which is one of the prevailing causes of death together with heart conduction block. Another feature in DM1 is muscle pain that can be resistant to treatment, thus severely affecting the well-being of the individuals concerned [60]. A variety of gastrointestinal problems like gastroesophageal reflux, nausea, vomiting or incontinence rely as well on insufficient functioning of smooth muscles and of the muscles of the alimentary tract [60, 71]. Apart from skeletal and smooth muscle-related affection, DM1 patients usually show diverse symptoms like cataracts, cardiac conduction defects, neurological dysfunctions, endocrine abnormalities and metabolic derangements such as insulin resistance [58].

Formerly, muscle biopsies were frequently used for DM1 diagnosis, before being mostly replaced by genetic testing. Muscle abnormalities characteristic of DM1 include internalized nuclei, whose number correlates with the muscular involvement of the patient [72, 73]. Although centralized nuclei are usually associated to muscle

regeneration, the reasons for their accumulation in DM1 is still not fully understood and might have other reasons than regeneration [72-75]. Moreover, fibre size variation, disordered cytoarchitecture, deranged organization of myofibrils harbouring cellular aggregates, abnormal fibre type proportion as well as angulated, ring and atrophic fibres are also observed in DM1 muscle [72, 73, 75].

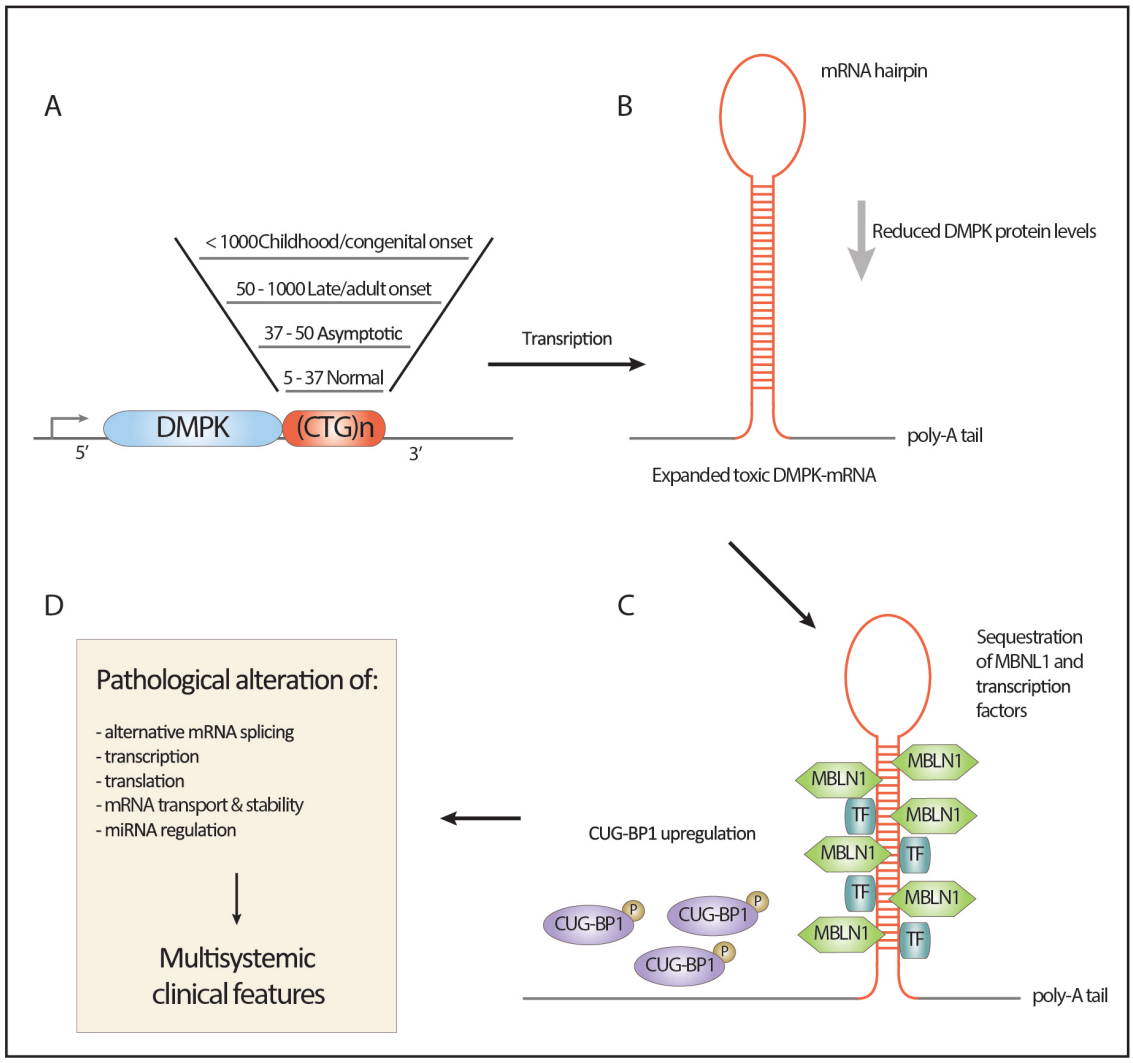
1.3.2 Pathogenic cause of DM1 (Scheme 2)

As aforementioned DM1 is caused by (CTG)n trinucleotide repeat expansion in the 3´untranslated region (UTR) of the *DMPK* gene. Currently, the commonly accepted hypothesis relates the primary cause of DM1 pathogenesis to a toxic gain-of-function of the expanded (CUG)n-RNA that is generated upon transcription of the genomic (CTG)n abnormal repeats [76].

Contribution of DM1 mouse models to the understanding of DM1 pathomechanism

Mutant *DMPK* transcripts are retained as foci within nuclei, which leads to reduced DMPK protein levels [77]. Initially, the reduced DMPK levels suggested a role for DMPK haploinsufficiency in the pathology. However, *Dmpk* knockout mice ($Dmpk^{-/-}$) that were generated to investigate this hypothesis, only exhibited a very mild phenotype and failed to reproduce the multisystemic DM1 pathology [78, 79]. Still, these mice exhibited a late onset progressive skeletal myopathy, some cardiac conduction abnormalities and altered calcium homeostasis, suggesting that DMPK deficiency may contribute in part to DM1 pathogenesis [78-81].

Later on, the transgenic HSA^{LR} mouse model, that expresses approximately 250 CTG repeats in the 3'UTR of the human skeletal actin gene (*HSA*), demonstrated the crucial role of toxic expanded RNA transcripts in DM1 muscle-pathology [82]. These mice reproduce the genetic pathological origin specifically in muscles and exhibit an distinctive phenotype, showing myopathy that aggravates during ageing. It has been established that this lineage reiterates the main features of the muscle pathophysiology described in DM1 patients, including myotonic discharges, and similar muscle histology with multiple central nuclei and variability of fibre size [82, 83]. This model also displays specific molecular characteristics described in patients such as MBNL1 sequestration by the (CUG)n-hairpins into nuclear foci, leading to reduced availability of MBLN1 [84, 85]. Analysis of the *Mbnl1* knockout mouse ($Mbnl1^{\Delta E3/\Delta E3}$), another DM1 mouse model, has been of major importance to understand that MBNL1 reduction



Scheme 2: Pathomechanism of DM1. A) The molecular mechanism of DM1 relies on a mutated (CTG)n repeat expansion in the 3'UTR of the *DMPK* gene. In healthy individuals the (CTG)n repeat fragment varies between 5 to 37 repeats. The severity of the disease correlates with the size of the repeat expansion and aggravates with the ascending amount of repeats. B) Upon transcription of the mutated *DMPK* gene a toxic gain-of-function mRNA is generated that forms double-stranded hairpin structures, leading to reduced DMPK protein levels. C) The toxic transcripts disturb the distribution and function of several RNA-binding proteins, leading to sequestration of the splicing factor MBNL1, leaching of different transcription factors (TFs) and stabilization of CUG-BP1. D) These processes trigger pre-mRNA mis-splicing, affect mRNA transport and stability, alter transcription and translation and influence miRNA regulation, which results ultimately in multiple disease symptoms.

is crucial for the pathogenesis of DM1. Depletion of MBNL1 in skeletal muscle leads to symptoms characteristic of DM1, like myotonia, altered muscle histology, cataracts as well as behavioural problems (e.g. increased anxiety or decreased motivation) [86, 87]. To date it is known that normal tissue development goes along with an enrichment of MBNL1 protein in the nucleoplasm. By contrast, in DM1 cells as well in HSA^{LR} mice the protein is sequestered by the (CUG)n-hairpins, leading to decreased MBNL1 levels [85]. MBNL1 has been reported to be a splicing factor that is crucial in regulating the transition from the neonatal to the adult splicing-program of its targets [85, 88]. Consequently, the reduction of MBNL1 leads to an abnormal adult-to-neonatal splicing-switch of various pre-RNAs, including *CLCN1* (*muscle specific chloride channel 1*) and *ATP2A1/SERCA1* (*ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+ Transporting 1*), in DM1 muscle as well as in $Mbnl1^{\Delta E3/\Delta E3}$ and HSA^{LR} mice [84-86]. Different studies have demonstrated a direct link between the development of myotonia and the persistence of neonatal ClC-1 isoform in muscle tissue of both mouse models, hence emphasizing the importance of expanded transcripts and MBNL1 sequestration in the development of DM1 pathogenesis [84-86].

Spliceopathy as a pathomechanism for DM1

Mis-splicing of several pre-mRNAs is thought to result in the multiple disease symptoms of DM1 (Scheme 2) [54, 89]. MBNL1 is not the only RNA-binding protein that is affected by the generation of toxic gain-of-function RNA. The mutant transcripts disturb the distribution and function of several RNA-binding proteins, amongst other of the splicing regulator CUG-BP1 (CUG binding protein 1), which has an antagonistic function to MBNL1 [90, 91]. Indeed, the elongated transcripts promote the activation of the PKC (protein kinase C) pathway, which in turn triggers phosphorylation and thus stabilization of CUG-BP1 [92]. In contrast to MBNL1, the steady-state levels of CUG-BP1 are increased in DM1 tissue, whereas a decrease is observed in healthy individuals upon embryonic-to-adult transition [90, 91].

Consequently, MBNL1 and CUG-BP1, which are both key splicing regulators, are suggested to strongly contribute to DM1 pathogenesis by triggering an inappropriate shift from postnatal to embryonic splicing patterns of more than 30 genes [85, 86, 93, 94]. *CLCN1*, *ATP2A1*, *INSR* (*insulin receptor*) and *TNNT2* (*cardiac troponin T*), all being mis-spliced in DM1 context, are well-known targets of one or both splicing factors [84, 93, 95-98]. Aberrant splicing of the *TNNT2* gene is associated with the

cardiac conduction defects detected in DM1 patients, while increased exon 11 skipping in the *INSR* pre-mRNA is responsible for the altered expression and signalling capacity of the insulin receptor (IR), and thereby for the insulin resistance observed in DM1 patients [89, 91, 95]. Myotonia has been related to mis-regulated splicing of the *CLCN1* gene, combining an inclusion of the exons 6B and/or 7A, and retention of intron 2, which induces a frameshift in *CLCN1* transcripts, generating premature termination codons [84, 99, 100]. Nonsense-mediated mRNA decay is responsible for the degradation of the expressed transcripts, while the spared transcripts produce a truncated protein, leading ultimately to decreased ClC-1 protein levels [99-101]. This gives rise to reduced resting chloride conductance contributing to myotonic discharges [99-101].

Other mis-splicing events affecting genes involved in muscle calcium-handling, like *RyR1* (*ryanodine receptor 1*) and *CACNA1S (voltage-gated calcium channel subunit alpha Cav1.1)* were linked to deregulated calcium homeostasis and excitation-contraction coupling, which may eventually participate in the pathological process leading to muscle weakness and atrophy and contribute to sarcolemmal hyperexcitability [102-104]. In particular, a recent report demonstrated functional differences between the adult (1a) and neonatal (1b) isoforms of SERCA1 protein, involved in intracellular calcium homeostasis in muscle. Therefore, increased expression of SERCA1b in the DM1 context may be part of the pathomechanism causing myotonia and other symptoms [105].

DM1, not only a spliceopathy?

Obviously, splicing alterations play a major role in the pathomechanism leading to DM1. Nevertheless, growing evidence suggests that mRNA toxicity has a broader spectrum of implications beyond mis-splicing and indicates that the molecular pathomechanism is more complex than previously expected [75, 106, 107].

Apart from their well-known function as splicing factors, MBNL1 and CUG-BP1 might affect mRNA transport and stability and may therefore have an impact on transcription, translation and thus on various cell signalling pathways [94, 106, 108]. It is suggested that reduced MBNL1 levels impact on the processing of certain miRNAs that are deregulated in DM1 heart and skeletal muscle tissue, hence contributing to disease pathology [109, 110]. Furthermore, a process known as RNA leaching corresponding to the interaction of transcription factors (TFs) with the expanded

(CUG)n-RNA occurs in DM1 cells [111]. Leaching of TFs supposedly relies on their ability to bind double-stranded DNA and double-stranded RNA, whereby the TFs are removed from the chromatin and displaced from their site of action [111]. In consequence, the leached TFs exhibit an abnormal activity, resulting in altered transcription of their target genes in DM1 tissue [111]. A well-known example is the modification of the levels of the myogenic factor MyoD, which contributes to the reduced differentiation and regeneration capacities of DM1 muscle cells [112].

Another process, independent of mis-splicing, has more recently been suggested to contribute to DM1 and corresponds to a cis-effect. The (CTG)n repeat expansion is supposed to alter the structure of the chromatin, thus influencing the expression of the *DMPK* gene itself and that of the adjacent genes [113, 114]. As mentioned before, DMPK deficiency may partially contribute to DM1 muscle pathology [78-81]. Moreover, in different mouse models the deletion of the *Six5* (*Sine Oculis Homeobox Homolog 5*) gene, which is situated next to *DMPK* in human, generated premature cataracts, cardiac conduction defects and testicular atrophy [115-118]. *DMWD* (*dystrophia myotonica-containing WD repeat motif*), another gene adjacent to *DMPK* was also shown to be decreased in human DM1 cells, substantiating the hypothesis that (CTG)n repeat expansion induces chromatin condensation and alters gene expression in DM1 context, thereby contributing to disease pathogenesis [114].

1.3.3 Pathomechanisms leading to muscle alterations in DM1

Despite the established hypothesis relating the primary cause of DM1 pathogenesis to a toxic gain-of-function RNA, the downstream processes by which muscle weakness, atrophy and histological alterations arise are still not well understood.

Altered cellular processes that may contribute to muscle atrophy

As mentioned before, correct functioning of autophagy is crucial for healthy muscle homeostasis and its deregulation can lead to muscle wasting and cell death. In myoblasts from DM1 patients, increased autophagy has been suggested, based on the presence of autophagic vacuoles and elevated expression of autophagy markers [52, 53]. Another recent report has related muscle atrophy in a DM1 *Drosophila* model to an increased autophagic flux [31]. At the same time it was shown that inhibition of autophagy had a beneficial effect on the muscle pathophysiology in the mutant flies, hence indicating that autophagy might play an important role in DM1 muscle pathology

[31]. Of note, down-regulation of the genes *AKT1S1/PRAS40* (*AKT1 Substrate 1*) and *AKT2* (*V-Akt Murine Thymoma Viral Oncogene Homolog 2*) negatively regulating autophagy could as well be detected in DM1 patient biopsies [31].

Another catabolic pathway being potentially altered in DM1 is the UPS. Increased activation of the UPS has been reported in a DM1 mouse model (*DM300-328*), suggesting a possible contribution to muscle wasting in these mice, and eventually being associated to muscle loss in DM1 patients [21, 22]. In addition, muscle wasting and weakness in DM1 has been linked to increased apoptosis, as human DM1 myotubes were shown to exhibit increased apoptotic activity [31, 52, 53, 119]. These results are consistent with the data obtained in the forecited DM1 *Drosophila* model, demonstrating that increased apoptosis goes along with decreased muscle area [31, 52, 53, 119].

To date, it is supposed that the process of myogenesis, which is responsible for generation and regeneration of muscle tissue, is impaired in DM1 disease [120]. For muscle regeneration, correct activation of myogenic progenitor cells, known as satellite cells, followed by proliferation of myoblasts and subsequent differentiation to myofibres is required [120]. Presumably, reduced regenerative capacities exhibited in DM1 muscle contribute to the muscle pathology in DM1 [53, 83, 121]. It has been speculated that reduced satellite cell activation, altered differentiation or a combination of these defects, contribute to impaired muscle regeneration and entail muscle weakness and atrophy in DM1 [52, 83, 122-124].

Perturbation of signalling pathways that may be involved in muscle alterations

Tweak-Fn14 (tumor necrosis factor-like weak inducer of apoptosis - fibroblast growth factor-inducible 14) signalling, being implicated in different processes including regeneration, autophagy and metabolism, proved to be of major importance concerning muscle pathology in an inducible DM1 mouse model (*DM5*), which overexpresses an eGFP-DMPK 3′ UTR $(CUG)_5$ mRNA [124, 125]. In fact, Fn14 was found to be induced in *DM5* mice but also in tissue from DM1 patients [124]. Blockage of Tweak-Fn14 signalling in mutant mice improved the deficient satellite cell response, as well as muscle histology and function, thereby pointing out its potential involvement in DM1 pathogenesis [124].

GSK3β (glycogen synthase kinase 3β) is another component being upregulated in DM1 context and has additionally been shown to be mis-regulated during

myogenesis in human DM1 cells [83]. Pharmacological treatment normalizing GSK3β levels in *HSA*^{LR} mice ameliorated satellite cell activation, muscle force, histology and decreased myotonia in the mutant mice [83].

Interestingly, the insulin receptor (IR), being altered in DM1, is upstream of the Akt/PKB pathway, which is involved in cell survival, growth and proliferation signalling. As quoted-above Akt/PKB is also a determinant coordinator of cell metabolism by regulating protein synthesis, autophagy and the UPS [126]. Akt/PKB was found to be inhibited in the heart of *Dmpk*$^{-/-}$ mice, but no data are available for the Akt/PKB status in skeletal muscle of these mice [127]. By contrast, one report suggested that human DM1 muscle biopsies show increased Akt/PKB levels, while the activation state of the Akt/PKB pathway in primary DM1 myoblasts is unclear [53, 128]. Hence, whether Akt/PKB deregulation could play a role in DM1 muscle pathophysiology is unknown. However, different reports identified a decreased activation of mTORC1, a downstream target of Akt/PKB [31, 52, 129]. One study relates abnormal activation of p53 to mTORC1 down-regulation and increased autophagy activation in human DM1 myoblasts [52]. The potential implication of mTORC1 deregulation in DM1 muscle pathology has further been emphasized in the DM1 *Drosophila* model. Overexpression of mTORC1 reduced autophagy and apoptosis and rescued muscle mass formation in the DM1 flies [31]. Overall this indicates that deregulated signalling pathways might play an important role in DM1 pathogenesis.

1.3.4 Current treatments and novel therapeutic strategies

At present, no treatment is available, which may halt the course of this debilitating disease. Patients are closely monitored and usually different treatments are combined in order to resolve or alleviate the symptoms. Diabetes is medicated by insulin or oral drugs like metformin, while methylphenidate is used occasionally to treat daytime sleepiness [58]. Severe health conditions might deserve surgical intervention, including pacemaker implantation, removal of cataracts or correction of major spine deformation in order to mitigate respiratory impairment [130]. Patients suffering from pain may benefit from anti-inflammatories or anti-myotonia therapy. Mexiletine has been reported to effectively improve myotonia in DM1. This drug does not seem to affect ClC-1 function, but reduction in myotonia probably results from nonspecific blockade of sodium channels [131, 132]. Further sodium channel blockers like tocainide,

procainamide and others, as well as calcium channel blockers such as nifedipine have also been reported to reduce myotonia and represent potential treatments for DM1 patients [131, 132]. However, the available therapeutic approaches remain insufficient, especially for the severe cases of DM1, hence pointing to the urgent necessity of exploring alternative, novel treatments for the disease.

With the progressing knowledge on the pathomechanism underlying DM1, new options have emerged for DM1-specific therapeutic strategies. Since the formation of toxic gain-of-function mRNA is central in DM1 pathology, targeting the elongated transcripts seems to be the most promising approach. This can be achieved in two different ways: either by liberating the RNA-binding proteins, making their binding sites unavailable, or by targeting the elongated transcripts for degradation. Two reports demonstrated efficient displacement of CUG-binding proteins from mRNA hairpins, using ASOs (antisense oligonucleotides) [133, 134]. ASOs were also applied in *in vivo* approaches, mediating degradation of the toxic RNA by the RNase H [135, 136]. These oligonucleotides either targeted directly the CUG repeat or alternatively targeted the 5'- or 3'-UTRs of the toxic transcripts in different DM1 mouse models [135, 136]. A major reduction of CUG-repeat transcript levels and nuclear foci was observed, as well as a partial improvement of splicing and myotonia [137]. Another auspicious study employed synthetic small interference RNA (siRNA) in order to target the expanded RNA in HSA^{LR} mice, leading to an almost complete abolition of mis-splicing and myotonia [137].

Based on the principle that MBNL1 displacement from the toxic RNA is sufficient to curtail DM1 disease, a vast range of potent small molecules has been tested and identified in DM1 cells and mouse models [133, 138]. At the moment small molecules represent another very attractive therapeutic alternative, showing a good efficacy, being cheap in production and simple to administrate. More screenings for hit compounds with suitable selectivity and low toxicity are ongoing, as well as investigations on structure and molecular composition of lead compounds in order to improve the efficacy of such strategies [138].

In a recent publication from Laustriat et al. (2015), metformin has been suggested as potential therapeutic treatment, irrespective of its anti-diabetic application [139]. Metformin has been shown to revert or alleviate altered splicing *in vitro*, such as *ATP2A1* mis-splicing in DM1 myoblasts or *CLCN1* mis-splicing in human DM1 MPCs

(mesodermal precursor cells) transfected with a *CLCN1* minigene [139]. It is suggested that the mechanism triggering splicing corrections involves metformin-induced AMPK activation and inhibition of *RBM3* (*RNA Binding Motif Protein 3*) gene expression [139]. This hypothesis is supported by the fact that another AMPK activator, named AICAR (5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside) was as well able to decrease *RBM3* expression and to restore mis-splicing of several gene transcripts in human DM1 myoblasts [139]. Another study (PCT publication no.: WO 2011121109 A1) did also suggest AMPK activators, like metformin or troglitazone, as potential therapy option in DM1 patients. Though, the correction of mis-splicing of *INSR*, *ATP2A1* and *TNNT2* transcripts due to AMPK activation has been linked to the modulation of the RNA-binding protein ELAVL1/HuR (ELAV-like protein 1) that exerts an opposed action to MBNL1, when situated in the cytoplasm. AMPK activators were shown to positively stimulate importin activity, which enhances ELAVL1 nuclear import and thus depletes the cytoplasmic levels of the protein. However, ELAVL1 does not co-localize and thereby act on foci level. Thus the positive effect upon treatment is rather linked to decreased cytoplasmic levels of ELAVL1, but the exact mechanism counteracting mis-splicing in DM1 cells due to ELAVL1 modulation remains unknown.

Together these studies provide quite promising results, although there are still unsolved issues, like delivery problems, toxicity and insufficient efficiency. Moreover, the complex pathophysiology associated with DM1 most likely requires targeting of multiple components, as single-agent or single-target therapeutic approaches are often limited in their efficacy [140]. While clinical trials are currently ongoing for better symptom management, broader investigations are required to further understand the mechanisms underlying DM1-associated muscle defects and identify solutions to circumvent functional muscle alterations.

2. Research aims and questions raised during the study

To date, most studies investigating the pathomechanism of DM1 have focused on the consequences of splicing defects generated by the toxic gain-of-function RNA. Hence, development of therapeutic strategies in order to abolish splicing abnormalities in DM1 context has been of major interest. In the last years, evidence emerged that the pathogenic cause of this disease is more complex than previously expected and that splicing-independent mechanisms or altered signalling processes that might be secondary to mis-splicing may considerably contribute to the multisystemic symptoms observed in DM1 patients. Thus, exploring the molecular mechanisms affecting skeletal muscle is crucial to understand the onset and progression of the DM1 disease, to be able to identify alternative therapeutic targets and to develop novel therapeutic approaches for this devastating disease.

In this study we focussed on the consequences triggered by the perturbation of certain metabolic pathways and processes. More specifically, we investigated the involvement of abnormal AMPK and Akt/PKB-mTORC1 signalling, as well as autophagy impairment in DM1 muscle pathology. Different studies reported a decreased activation of mTORC1 in DM1 context, that could eventually be responsible for the increased autophagic flux described in some DM1 cell lines [31, 52, 129]. Additionally, a potential deregulation of Akt/PKB was proposed, but the results remain contradictory regarding the status of the signalling in DM1 tissues and cells [53, 127, 128]. Considering that Akt/PKB-mTORC1 and AMPK signalling is central in regulating skeletal muscle homeostasis, examination of the state of these pathways is of major interest. Furthermore, it is paramount to clarify how or if the deregulation of Akt/PKB-mTORC1 and AMPK impacts on autophagy and if they contribute to muscle dysfunction in DM1 pathology. Moreover a recent report suggested that AMPK activation might be beneficial in DM1 cells, although the status of the signalling in the DM1 context was not investigated. Normalization of mTORC1, GSK3β, Fn14 and/or of abnormal autophagic flux, has recently been shown to be able to improve muscle pathophysiology in various neuromuscular diseases, including DM1, which opened new perspectives in terms of treatments [30, 31, 51, 83, 124]. For this reason, we addressed the question whether, pharmacological or dietary approaches would be suitable to normalize the identified deregulated processes, and be further able to resolve or improve splicing defects and muscle pathophysiology in DM1.

3. Results

3.1 Manuscript

The following manuscript refers to the version that was submitted to the *Journal of Clinical Investigation* in July 2016.

Targeting deregulated AMPK/mTORC1 pathways improves muscle function in myotonic dystrophy type I

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Conflict of interest
The authors have declared that no conflict of interest exists.

Abstract

Myotonic Dystrophy type I (DM1) is a disabling multisystemic disease affecting predominantly skeletal muscle. It is caused by expanded CTG repeats in the 3'UTR of the *DMPK* gene. RNA-hairpins formed by the elongated transcripts sequester RNA-binding proteins leading to mis-splicing of numerous pre-mRNAs. Here, we investigated whether DM1-associated muscle pathology may be related to a deregulation of central metabolic pathways, which may constitute novel therapeutic targets for the disease. We showed that activation of AMPK signaling is impaired under starved conditions in muscle from HSA^{LR} mice, a well-characterized mouse model for DM1, while mTORC1 remains active. As a consequence, autophagic flux was perturbed in HSA^{LR} muscle, as well as in cultured human DM1 myotubes. Pharmacological approaches targeting AMPK/mTORC1 signaling greatly ameliorated muscle function in DM1. AMPK activation by AICAR led to a strong reduction of myotonia, accompanied by partial correction of mis-regulated alternative splicing. Rapamycin, an mTORC1 inhibitor, improved muscle relaxation and increased muscle force in HSA^{LR} mice without affecting splicing. These findings highlight the involvement of AMPK/mTORC1 deregulation in DM1 muscle pathophysiology and may open new avenues for the treatment of the disease.

Introduction

Myotonic Dystrophy type I (DM1, #160900) is a multisystemic neuromuscular disorder, which represents the most common form of muscular dystrophy in adults [141]. In particular, DM1 patients suffer from muscle wasting, weakness, and myotonia. DM1 is an autosomal dominant disease caused by an expansion of unstable CTG repeats located within the 3' untranslated region of the dystrophia myotonica protein kinase (*DMPK*) gene. Toxic expanded transcripts containing RNA-hairpins formed by the triplet repeats accumulate as RNA foci in the nuclei of affected cells [142, 143]. These mutant transcripts are thought to sequester RNA-binding proteins, such as muscleblind-like 1 (MBNL1) and to increase CUG triplet repeat RNA-Binding Protein 1 (CUGBP1) levels. The resulting splicing defects are considered the primary cause of DM1 symptoms [82, 91, 144]. HSA^{LR} mice, which carry a CTG repeat expansion in the human skeletal actin (*HSA*) gene, constitute a well-characterized mouse model for DM1 [82]. These mice express (CUG)n-expanded transcripts specifically in skeletal muscle and reiterate the dystrophic phenotype and myotonic discharges observed in muscle of patients. HSA^{LR} mice also recapitulate DM1 molecular characteristics such as foci accumulation, MBNL1 sequestration, and splicing abnormalities [82, 145, 146]. Therapeutic strategies have mainly focused on targeting DM1-associated mis-splicing and mRNA toxicity [133, 147, 148], although a more complete understanding of pathogenic pathways would clearly be of interest for the development of alternative or additional therapeutic options.

Recently, deregulation of cellular processes and signaling pathways important for maintaining proper muscle homeostasis, has been reported in DM1. This includes abnormal activation of the ubiquitin-proteasome system and increased autophagic flux, which were both related to muscle atrophy and weakness in DM1 [149-151]. In parallel, perturbation in the PKB/Akt pathway may arise from altered expression of the insulin receptor, which correlates with glucose intolerance in DM1 patients [152]. Although PKB/Akt deregulation has been reported in *Dmpk*-deficient mice [127], in DM1 flies [150] and in DM1 human neural stem cells [153], contradictory results have been obtained in human muscle cells [128, 154].

To obtain further insight into the pathomechanisms associated with the disease, we investigated whether deregulated metabolic pathways may be involved in muscle

alterations in DM1. We uncovered that muscles from *HSA^{LR}* mice do not efficiently respond to fasting by displaying impaired activation of AMPK and delayed inhibition of the mTORC1 (mTOR complex 1) pathway. Moreover, we observed impaired autophagic flux in both *HSA^{LR}* muscle and myotubes from DM1 patients, which may arise from AMPK/mTORC1 deregulation. Importantly, we established that treatments normalizing these pathways improved skeletal muscle strength and strongly reduced the myotonia in *HSA^{LR}* mice. Our data provide evidence for the pathological role of metabolic pathways in DM1 and may open interesting avenues for alternative therapeutic strategies for the disease.

Results

AMPK and mTORC1 pathways are deregulated in HSA^{LR} muscle. To identify pathomechanisms involved in DM1-related muscle alterations, we examined the potential deregulation of metabolic pathways in *HSA^{LR}* mice (Supplemental Figure 1A). To this purpose, the activation state of key proteins was compared in muscle from 2-month-old mice analyzed in fed conditions or submitted to a physiological stimulus like fasting [155]. No major difference was observed in the activation state of AMPK, PKB/Akt and mTORC1 pathways in muscle from fed mutant and control mice, as reflected by the similar phosphorylation levels of AMPK (AMPK^{P172}), PKB/Akt (Akt^{P473}) and mTORC1 targets, ribosomal protein S6 kinase (p70S6K^{P389}) and S6 ribosomal protein (S6$^{P235/6}$) (Figure 1, A and B). After 24 h of starvation, *HSA^{LR}* mice showed impaired activation of the AMPK pathway, as revealed by the reduced levels of AMPK^{P172} in *tibialis anterior* (TA) mutant muscle (Figure 1A). Regardless of the nutritional status, protein expression of the known AMPK regulatory kinases, liver kinase B1 (LKB1) and transforming growth factor beta-activated kinase 1 (TAK1) was unchanged in *HSA^{LR}* muscle compared to control (Figure 1A). In contrast, mutant muscle displayed an altered expression profile for Ca^{2+}-calmodulin dependent kinase II (CaMKII) isoforms, with marked reduction in levels of the CaMKIIβM muscle-specific form and of its phosphorylated, active form (Figure 1A). Such deregulation was consistent with splicing defects in the *Camk2* genes previously described in tissues from DM1 patients and mouse models [148, 156-158]. We confirmed by quantitative PCR that splicing of *Camk2b* was altered in muscle from *HSA^{LR}* mice (exon 13 exclusion), while overall expression of *Camk2* transcripts was unchanged compared to controls (Supplemental Figure 1, B and C). As CaMKII regulates AMPK [159-161], these

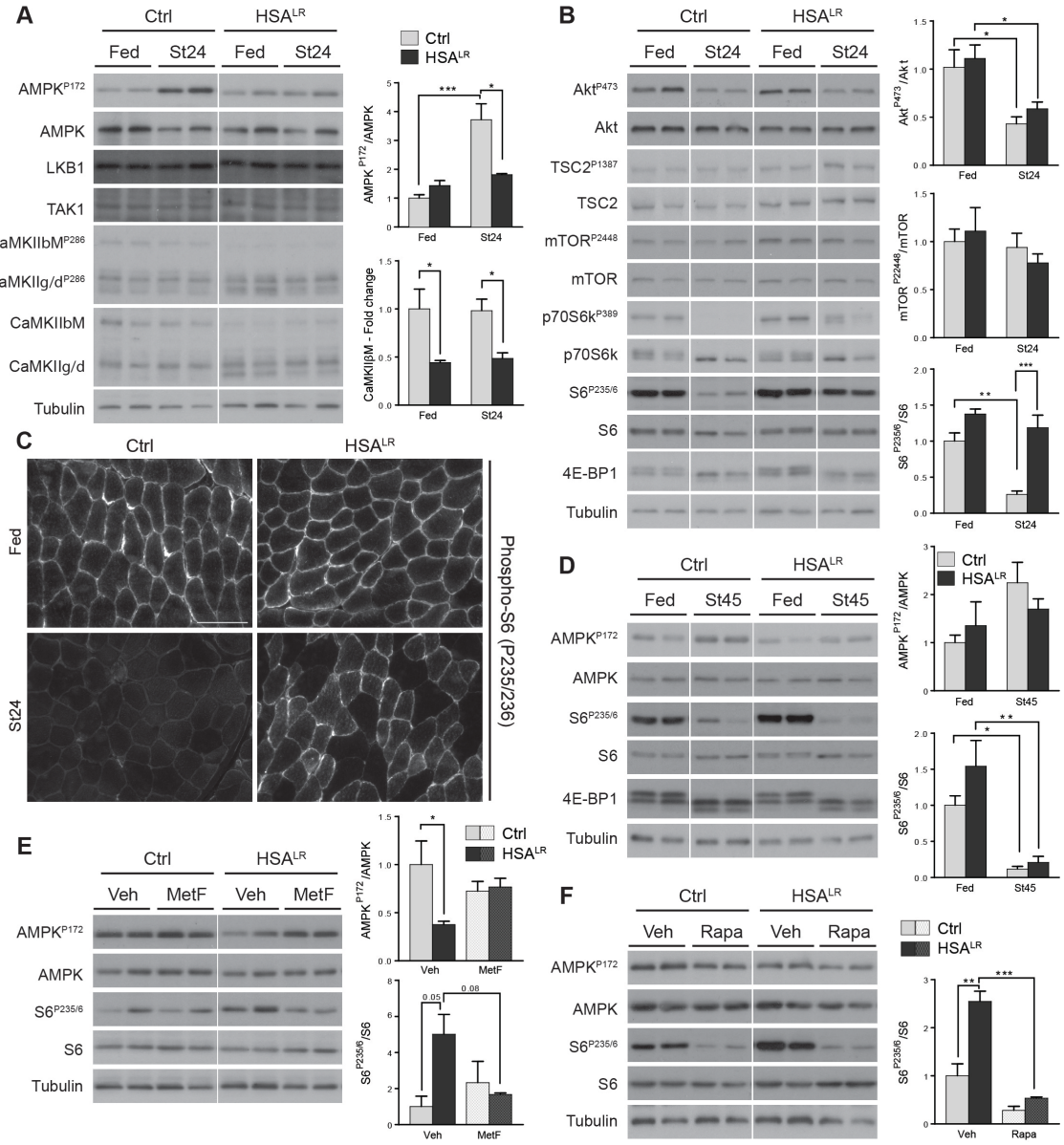

Figure 1: AMPK and mTORC1 pathways do not respond to starvation in *HSA^{LR}* muscle. (A, B) Two-month-old *HSA^{LR}* and control (Ctrl) mice were examined in fed conditions and after 24 h of starvation (St24). Immunoblots for phospho- (P) and total proteins of the AMPK (A, n=3-4 per group) and mTORC1 (B, n=3-7) pathways reveal reduced AMPK activation and increased phosphorylation of some mTORC1 targets upon starvation in mutant muscle. (C) Immunostaining on muscle cross-sections from fed and starved (St24) *HSA^{LR}* and control (Ctrl) mice shows high levels of phospho-S6 in mutant muscle upon starvation. Scale bar, 100 µm. (D - F) Immunoblots for phospho- (P) and total AMPK and S6 proteins reveal efficient inhibition of mTORC1 signaling upon 45 h of starvation (St45) (D, n=3-4) and with metformin (E, n=3-4) or rapamycin (F, n=3-4) treatment in muscle from *HSA^{LR}* mice. AMPK activation shows a trend towards increase in mutant muscle with metformin treatment (E). Data are relative to control fed (A, B, D) or vehicle-treated (E, F) mice and are mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, 2-way ANOVA with Tukey's multiple comparisons test correction.

results suggest that impaired AMPK activation in *HSA*^{LR} muscle may rely on mis-splicing-dependent CaMKII deficiency.

In parallel, higher phosphorylation of p70S6K and S6 was detected upon starvation in *HSA*^{LR} muscle compared to control muscle (Figure 1B). Accumulation of phosphorylated S6 in muscle from starved mutant mice was further confirmed by immunostaining (Figure 1C), suggesting an abnormal activation of the mTORC1 signaling in *HSA*^{LR} mice. Of note, no major change in the phosphorylation of mTOR was observed in mutant and control muscles from fed *vs*. starved mice (Figure 1B). Moreover, upon starvation, changes in 4E-BP1 levels were similar between *HSA*^{LR} and control muscles (Figure 1B), consistent with previous reports indicating differential regulation of mTORC1 targets [162]. Interestingly, mTORC1 deregulation was not related to abnormal activity of PKB/Akt since levels of the active phosphorylated form of PKB/Akt were efficiently decreased upon starvation in mutant mice (Figure 1B). Accordingly, we did not detect any changes in the splicing (exon 11, mis-spliced in DM1 patients) or expression of the gene encoding insulin receptor (*Insr*) in TA muscle from 2-month-old *HSA*^{LR} mice (Supplemental Figure 1D). Moreover, mTORC1 and AMPK activation state in non-muscle tissue, such as liver, was similar in control and mutant mice (Supplemental Figure 1E), indicating that deregulation of these pathways is confined to skeletal muscles, which specifically express (CUG)n-expanded transcripts.

In an attempt to normalize mTORC1/AMPK pathways, control and *HSA*^{LR} mice were subjected to starvation for 45 h. Mutant mice lost less weight than control mice after prolonged starvation (Supplemental Figure 1F). Moreover, upon 45 h of starvation, 4E-BP1 and S6$^{P235/6}$ levels were similar in mutant and control muscles, while AMPK phosphorylation showed only a trend toward increase in *HSA*^{LR} muscle (Figure 1D). We next addressed whether pharmacological treatments would be sufficient to modulate AMPK/mTORC1 pathways in *HSA*^{LR} mice. Control and mutant mice were treated for 5 days with metformin, a drug known to induce AMPK signaling. The treatment slightly activated AMPK in muscle from starved *HSA*^{LR} mice, which was accompanied by a decrease in S6$^{P235/6}$ levels (Figure 1E). Conversely, a single injection of rapamycin, a canonical inhibitor of mTORC1, strongly reduced S6$^{P235/6}$ levels in muscle from starved control and *HSA*^{LR} mice. Levels of AMPK^{P172} remained unchanged in rapamycin-treated mice (Figure 1F). Although no obvious change was detected in AMPK-dependent phosphorylation of TSC2 (TSC2^{P1387}), an upstream inhibitor of mTORC1

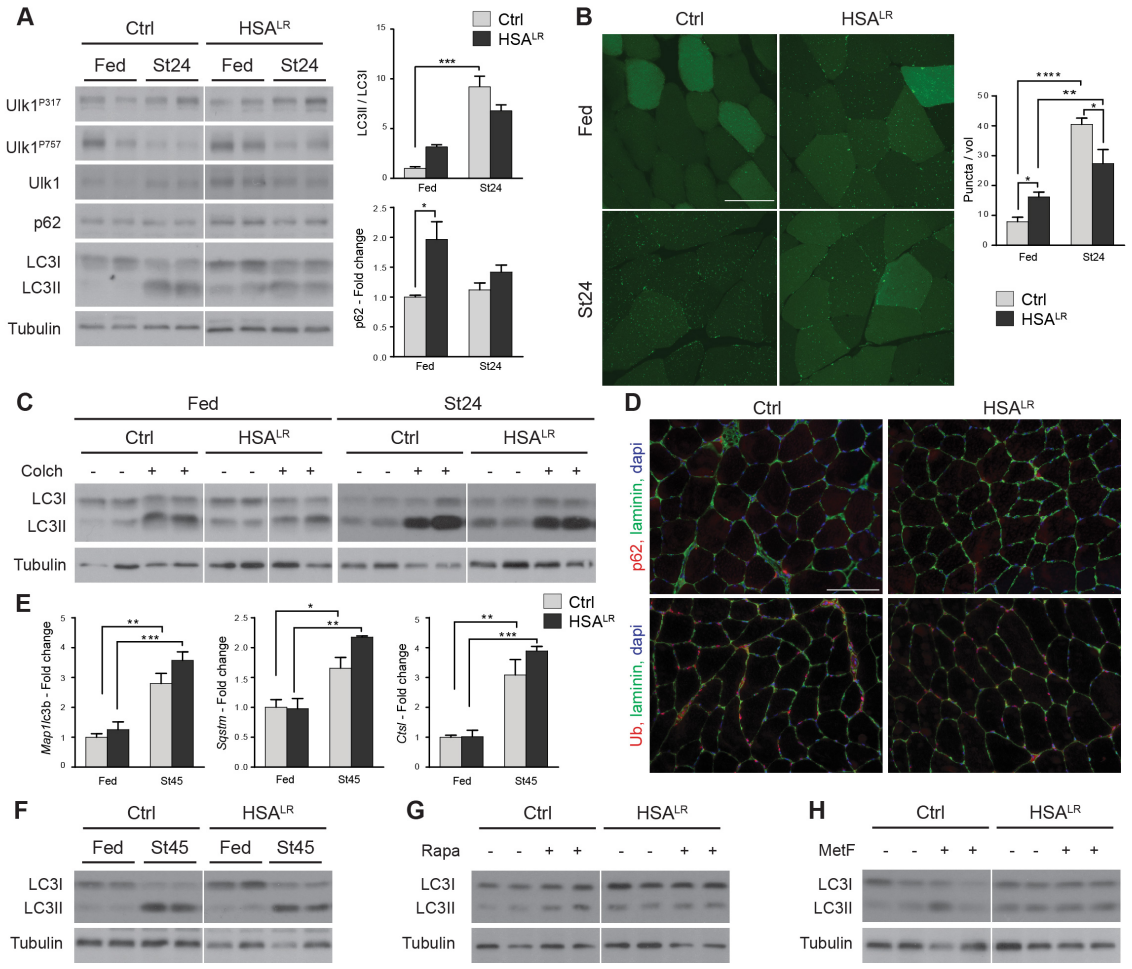

Figure 2: Autophagic flux is impaired in *HSA^{LR}* muscle. (A) Immunoblots for autophagy-related proteins show accumulation of autophagic substrates, i.e. LC3II and p62, in *HSA^{LR}* TA muscle in fed conditions (n=3 per group), while a reduced LC3I to LC3II switch is observed in mutant muscle upon 24 h of starvation (St24, n=4-7), compared to control (Ctrl) muscle. (B) *HSA^{LR}* mice expressing GFP-LC3 display increased number of GFP-positive puncta in TA muscle compared to control (Ctrl) in fed conditions (n=3-4), but reduced accumulation after 24 h of starvation (St24, n=3). Scale bar, 50 µm. A volume unit (Vol) corresponds to 2.8×10^3 µm^3. (C) Treatment with colchicine (Colch) leads to milder changes in LC3II levels in TA muscle from fed and starved (St24) *HSA^{LR}* mice, compared to control (Ctrl) mice. (D) Immunostaining of muscle sections from starved control (Ctrl) and *HSA^{LR}* mice reveals no major accumulation of p62 or ubiquitinated proteins in mutant muscle. Scale bar, 100 µm. (E) Expression of autophagy-related genes is efficiently up-regulated after 45 h of starvation (St45) in *HSA^{LR}* TA muscle. Data are normalized to *Actn* levels (n=3-4). (F-H) Immunoblots reveal limited or blunted switch from LC3I to LC3II in *HSA^{LR}* muscle upon 45 h of starvation (St45) (F), rapamycin (Rapa) (G) or metformin (MetF) (H) treatments, compared to controls (Ctrl). Data are relative to control fed mice and represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, 2-way ANOVA with Tukey's multiple comparisons test correction.

(Figure 1B), these results suggest that AMPK deregulation may primarily be responsible for the defective response to starvation and for mTORC1 signaling perturbation in *HSA^{LR}* muscle.

Autophagic flux is impaired in HSA^{LR} muscle. It is well established that mTORC1 and AMPK are key regulators of autophagy and that perturbation of their activities can lead to severe tissue alterations, especially in skeletal muscle [163-165]. To determine whether the expression of the CUG repeats impairs the autophagy process, we assessed the ability of *HSA^{LR}* muscle to induce autophagy when the mice were subjected to starvation. First, we evaluated levels of the soluble (LC3I) and autophagosome-associated (LC3II) forms of the widely used LC3B (MAP1LC3 for microtubule-associated protein light chain 3) autophagy marker. The amount of LC3II correlates with the intracellular accumulation of autophagic vesicles [166]. Under fed conditions, LC3II levels were increased in mutant muscle, which reflects either an increased autophagy induction or a defect in the degradation steps (Figure 2A, and Supplemental Figure 2A). After 24 h of starvation, a clear switch from LC3I to LC3II occurred in control muscle, while *HSA^{LR}* muscle displayed reduced changes in LC3 levels and LC3II/LC3I ratio (Figure 2A, and Supplemental Figure 2A). To confirm these results, we starved *HSA^{LR}* and control mice expressing the GFP-LC3 fusion protein for 24 h. In control muscle, a striking increase in the number of GFP-LC3-positive puncta, representing autophagic vesicles, was observed upon starvation (Figure 2B). In *HSA^{LR}* mice, the number of puncta was higher under fed conditions, but was significantly less increased upon starvation as compared to control muscle (Figure 2B). These results confirmed that autophagy is perturbed in *HSA^{LR}* muscle, which may rely on impaired induction and/or degradation steps.

To assess the status of the autophagic flux, mice were treated for 2 days with colchicine, a drug preventing degradation of the autophagic content. Under both fed and starved conditions, colchicine induced a major switch from LC3I to LC3II in control muscle (Figure 2C). Comparing colchicine-treated and untreated mice, we observed that the fold change in the LC3II/LC3I ratio was less in *HSA^{LR}* muscle compared to controls, in both fed and starved conditions (Figure 2C, and Supplemental Figure 2B). This result ruled out that elevated LC3II levels in fed conditions were due to increased autophagy induction in *HSA^{LR}* muscle; accumulation of autophagic vesicles was therefor likely related to restricted degradation. Consistently, levels of the autophagosome cargo protein p62 were higher in muscle from fed mutant mice than in controls (Figure 2A).

Nonetheless, we detected neither p62 aggregates nor accumulation of ubiquitinated proteins in muscle from fed and starved HSA^{LR} mice (Figure 2D), suggesting that autophagy is not completely blocked. Similarly, distribution of lysosomal vesicles, visualized by Lamp1 immunostaining, was unchanged between 2-month-old mutant and control mice (Supplemental Figure 2C). Under fed conditions, increased amounts of p62 and LC3II were not due to abnormal transcript expression in HSA^{LR} muscle (Figure 2E). Moreover, expression of the *Map1lc3b*, *Sqstm* (encoding p62) and *Ctsl* (encoding Cathepsin L) genes was unchanged after 24 h of fasting, but we observed an efficient induction of the genes upon 45 h of starvation in both mutant and control muscles (Figure 2E, and Supplemental Figure 2D). It should be noted that following prolonged starvation, autophagy induction remained weaker in mutant muscle compared to control muscle (Figure 2F, and Supplemental Figure 2E). Together, these results suggest that autophagy deregulation in DM1 muscle results from impaired degradation in combination with a lowering of autophagy induction upon starvation.

To gain further insight into autophagy deregulation, we assessed the phosphorylation state of Unc-51-like kinase 1 (Ulk1), as mTORC1 and AMPK phosphorylate and thereby inhibit or activate Ulk1, respectively [163]. Upon starvation, levels of the inactive form of Ulk1 ($Ulk1^{P757}$) remained slightly higher in mutant muscle as compared to control muscle, while no major difference was observed for its active form ($Ulk1^{P317}$ - Figure 2A, and Supplemental Figure 2A). Interestingly, rapamycin and metformin treatments were both sufficient to increase LC3II levels in control muscle, but did not induce autophagy in HSA^{LR} muscle (Figure 2, G and H, and Supplemental Figure 2, F and G). Hence, mTORC1/AMPK deregulation in conjunction with mTORC1/AMPK-independent mechanisms perturbs the autophagic flux in HSA^{LR} muscle at both the induction and degradation steps.

Lastly, upon starvation, expression of the atrogenes, *Trim63* and *Fbxo32*, was similarly induced in HSA^{LR} and control muscles (Supplemental Figure 2H). However, caspase- and trypsin-like activities associated with the proteasome system were increased in muscle from fed and starved mutant mice, compared to control animals (Supplemental Figure 2I). This is consistent with a report showing higher proteasome activity in muscle from a DM1 mouse model expressing 550 CTG triplets [149].

Pathological involvement of autophagy impairment in DM1. To ascertain the relevance of the changes observed in *HSA*^{LR} mice for DM1 pathology, we evaluated the activation state of AMPK/mTORC1 signaling in muscle biopsies from three DM1 patients (P1-3) of 33, 34 and 49 years of age. There was no major difference in total and phosphorylated levels of PKB/Akt and AMPK proteins in muscle from DM1 patients compared to age-matched control individuals (C1/2 and C3-5 aged of 30 and 50 years, respectively) (Figure 3A). Notwithstanding, we noticed that levels of the active phosphorylated form of p70S6K and S6 were increased in muscle biopsies from DM1 patients compared to control individuals (Figure 3A). However, the nutritive status of the patients at the time of the biopsy could not be ascertained and may have influenced the results. For this reason, we next tested the ability of DM1 human muscle cells to modulate mTORC1/AMPK signaling in response to energy and nutrient deprivation. Fibroblasts of 3 DM1 patients (DM-L1-3) were transduced with MyoD and differentiated for 10 days into myotubes, before being subjected to growth medium or to amino acid- and glucose-deprived conditions (i.e. PBS) for 3 h. Upon deprivation, levels of the active phosphorylated form of PKB/Akt and S6 were strongly reduced in control muscle cells; there was no major activation of AMPK compared to enriched conditions (Figure 3B). A similar response was observed in DM1 muscle cells although they retained higher phosphorylation of S6 in deprived conditions compared to control cells (Figure 3B). In parallel, a major switch from LC3I to LC3II occurred in control muscle cells upon deprivation. LC3II levels were further increased in control cells treated with chloroquine, consistent with high autophagy induction in deprived cells (Figure 3C). In contrast, LC3II levels were only slightly changed when DM1 myotubes were submitted to deprivation, even in the presence of chloroquine, indicating that the autophagic flux is blocked at the induction steps (Figure 3C). Together, these data indicate that DM1 human muscle cells do not efficiently respond to nutrient/energy deprivation and display deregulation of the autophagy process**.**

To test the relevance of autophagy impairment in DM1, we looked for muscle alterations related to autophagy defects in muscle biopsies from DM1 patients and in muscle from aged *HSA*^{LR} mice. As previously reported [151, 167-169], vacuolated fibers were observed in muscle biopsy of one DM1 patient, out of the three examined (Figure 3D). Lysosome accumulation was also detected in affected fibers from DM1 muscle (Figure 3D). However, in contrast to biopsies from an IBM (Inclusion Body Myositis) patient, there was no accumulation of LC3, ubiquitinated proteins or p62 in

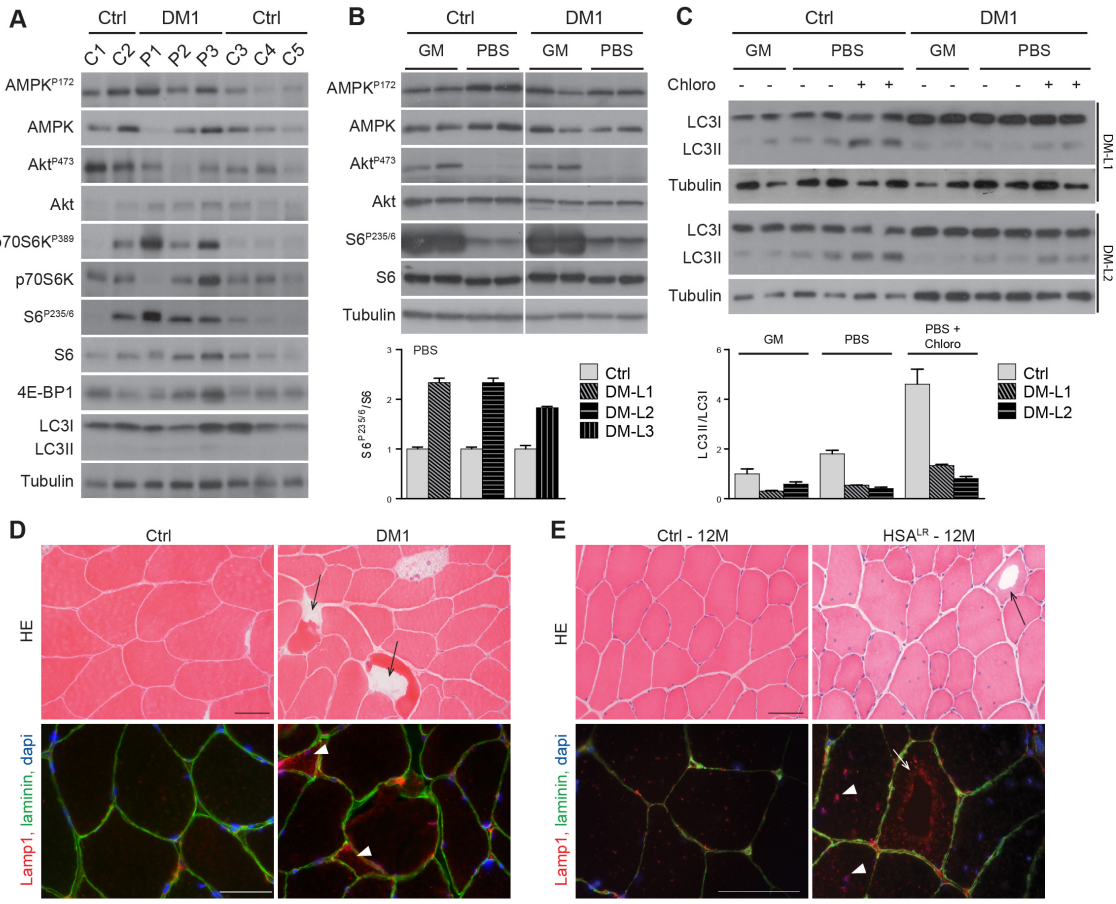

Figure 3: Autophagy perturbation contributes to muscle alterations in DM1. (A) Protein lysates from muscle biopsies of control individuals (C1-5) and DM1 patients (P1-3) were analyzed for phospho- (P) and total proteins of the AMPK and PKB/Akt-mTORC1 pathways. (B) MyoD transduced fibroblasts from controls (Ctrl) and DM1 patients were differentiated to myotubes and submitted to growth medium (GM) or deprived conditions (PBS) for 3 h. Immunoblots for phospho- (P) and total proteins reveal increased levels of $S6^{P235/6}$ upon deprivation in the three cell lines of DM1 patients (DM-L1-3), compared to controls. Quantification is given for deprived conditions; values are mean ± SEM of technical replicates. (C) Immunoblots for LC3 marker show defective accumulation of LC3II in DM1 myotubes upon energy and amino acid deprivation (PBS) as well as with deprived conditions and chloroquine treatment (Chloro), compared to control cells (Ctrl). Quantification of LC3II/LC3I ratio is shown for two DM1 cell lines (DM-L1/2) in enriched (GM) and deprived conditions; values are mean ± SEM of technical replicates. (D) H&E stain reveals the presence of vacuolated fibers (arrows) in muscle biopsy from one DM1 patient, together with lysosomal accumulation (arrowheads) observed by immunostaining in some affected muscle fibers (red, lower panel). Scale bar, 50 µm. (E) Vacuoles (arrows) are observed in muscle from aging *HSA^{LR}* mice; the periphery of the vacuoles is strongly reactive with anti-Lamp1 antibodies (red, lower panel) indicating accumulation of lysosomal structures in these regions. High density of lysosomes is also observed in non-vacuolated muscle fibers from 12-month-old (12M) mutant mice (arrowheads), compared to muscle from aged-matched control mice (Ctrl). Scale bar, 50 µm.

DM1 patient muscles (Supplemental Figure 3A). Consistently, LC3 levels detected by Western blot were similar in DM1 and control biopsies (Figure 3A). Interestingly, we frequently observed intracellular vacuoles in muscle from 12-month-old *HSA^{LR}* mice, as well as an accumulation of the lysosomal marker Lamp1 near the vacuolar structures and myonuclei (Figure 3E). Secondary antibodies alone did not react with the vacuoles and we did not observe such features in muscle from age-matched control mice (Figure 3E, and Supplemental Figure 3B). Electron microscopy confirmed the presence of vacuoles in mutant mouse muscle: they were surrounded by dense, disorganized areas of contractile elements and usually limited by a single, discontinuous membrane (Supplemental Figure 3C). Together with the lysosome staining, these features argue for the accumulation of autophagic vacuoles in *HSA^{LR}* muscle with age. These results suggest that autophagy perturbation may contribute to the progressive alteration of muscle tissue in DM1.

AMPK activation by AICAR abrogates myotonia in HSA^{LR} mice. In light of the deregulation of AMPK signaling in *HSA^{LR}* muscle, we investigated whether AMPK normalization would have a beneficial effect on muscle function in mutant mice. As readout of the disease, we evaluated myotonia by measuring the late relaxation time of skeletal muscle (i.e. time to reduce the maximal force from 50% to 10%) after *ex vivo* tetanic stimulation [146]. As reported previously, we observed no change in the late relaxation time of *soleus* muscle from *HSA^{LR}* mice compared to controls (Supplemental Figure 4A), whereas this parameter was strongly increased in *extensor digitorum longus* (EDL) mutant muscle (Figure 4A). Since AMPK activation by metformin has recently been shown to correct mis-splicing in human DM1 cells *in vitro* [170], 4- and 12-month-old control and *HSA^{LR}* mice were treated with metformin for 10 days. Despite using high doses of metformin, we observed only a limited and non-reproducible effect of the treatment on AMPK/S6 activation state in these groups of mice analyzed under basal nutritive conditions (Supplemental Figure 4B). Besides, the treatment failed to reduce the late relaxation time of EDL muscle in mutant mice (Figure 4B) and it did not modify the expression and splicing of genes affected in DM1, including *Clcn1* (encoding chloride channel protein 1, Clc-1 – exon 7a inclusion) and *Atp2a1* (encoding sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 1 – exclusion exon 22) (Figure 4, C and D, and Supplemental Figure 4C).

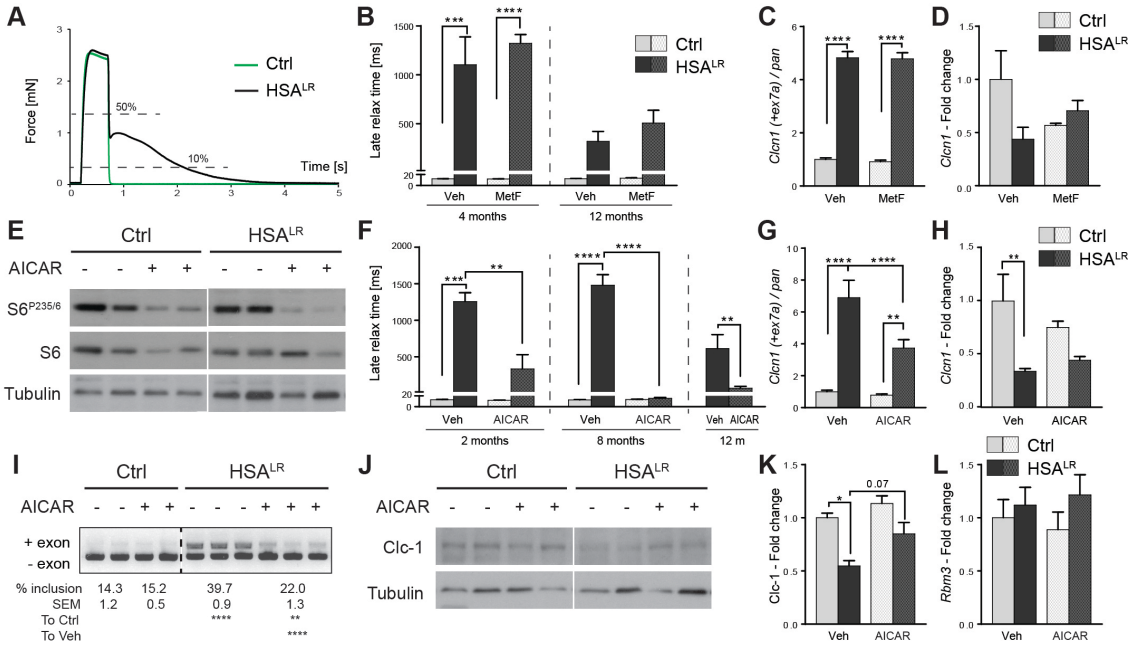

Figure 4: AICAR strikingly decreases myotonia in *HSA^{LR}* mice and reduces mis-splicing in mutant muscle. (A) *In vitro* tetanic stimulation (150 Hz) of EDL muscle leads to contraction of *HSA^{LR}* and control (Ctrl) muscles, with a strongly increased relaxation time in mutant muscle. (B) Metformin treatment does not reduce late relaxation time of EDL muscle from 4- and 12-month-old *HSA^{LR}* mice, as compared to age-matched vehicle-treated (Veh) mutant mice. n=3-5 control and 6-8 *HSA^{LR}* mice per group. (C, D) Inclusion of the exon 7a of the *Clcn1* gene and the overall expression of the gene are not changed in muscle from metformin (MetF)-treated *HSA^{LR}* mice, compared to vehicle (Veh)-treated mutant and control mice (n=3). (E) Immunoblots for phospho- and total S6 protein reveal efficient inhibition of AMPK-indirect target in muscle from control (Ctrl) and mutant mice treated with AICAR for 7 days. (F) Late relaxation time is significantly reduced in EDL muscle from 2- (n=3-4), 8- (n=3 Ctrl and 6-7 *HSA^{LR}*) and 12- (n=4-5) month (m)-old *HSA^{LR}* mice which were treated with AICAR for 7 days, as compared to age-matched vehicle (Veh)-treated mutant mice. (G-K) Quantitative PCR (G, H) and end-point PCR (I) reveal strong reduction in exon 7a inclusion of the *Clcn1* gene in muscle from *HSA^{LR}* mice treated with AICAR, compared to vehicle-treated (Veh) mutant mice (n=3-5). Protein levels of Clc-1 are also increased in mutant muscle from AICAR-treated mice (J, K – n=2 Ctrl and 5 *HSA^{LR}*). (L) Quantitative PCR shows similar transcript levels of *Rbm3* in muscle from AICAR-treated and untreated control and *HSA^{LR}* mice (n=3-4). Data are relative to vehicle-treated control mice (C, D, G, H, K, L) and represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, 2-way ANOVA with Tukey's multiple comparisons test correction (except 12M AICAR, unpaired two tailed Student's t-test).

As we did not detect any effect of metformin on *HSA*^{LR} muscle, we tested whether 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), an agonist of AMPK, may constitute an alternative strategy to target AMPK activation in muscle. Following a seven-day treatment with AICAR, control and mutant muscles showed a clear reduction in S6$^{P235/6}$ levels (Figure 4E). We further confirmed that AICAR increased phosphorylated levels of AMPK and those of its direct target acetyl-CoA carboxylase (ACC) shortly after the last injection (30 min), while inhibition of the AMPK indirect target, S6, was only detected after 2 h in control muscle (Supplemental Figure 4D). Importantly, following tetanic stimuli, a strong and significant reduction in the late relaxation time of EDL muscle was detected in 2- and 12-month-old AICAR-treated mutant mice, and myotonia was completely abrogated with AICAR in the group of 8-month-old *HSA*^{LR} mice (Figure 4F, and Supplemental Figure 4E). Normalization of the half relaxation time (i.e. time to reduce the maximal force from 100% to 50%) of mutant muscle was also observed upon treatment (Supplemental Figure 4F). As mis-splicing of the *Clcn1* gene is thought to be the primary cause of myotonia in DM1 [145, 171-173], we investigated whether the effect of AICAR was related to changes in *Clcn1* splicing. By quantitative PCR, a strong reduction in the expression of the mis-spliced *Clcn1* transcript (containing exon 7a) was detected in muscle from AICAR-treated *HSA*^{LR} mice, while overall transcript levels of *Clcn1* were unchanged compared to untreated mutant mice (Figure 4, G and H). We confirmed with end-point PCR that mis-regulated *Clcn1* splicing was significantly improved upon AICAR treatment (Figure 4I). Furthermore, AICAR led to a slight increase in Clc-1 protein levels in mutant muscle (Figure 4, J and K). It is worth to note that AICAR did not change splicing of the *Atp2a1* and *Camk2b* genes in *HSA*^{LR} muscle (Supplemental Figure 4, G and H). Moreover, we did not detect any reduction in transcript levels of *Rbm3*, encoding RNA binding-protein 3 (Figure 4L), previously suggested to mediate the effect of AMPK activation on splicing [170].

Besides its effect on myotonia, we wondered whether AICAR treatment would change muscle force in mutant mice. As initially reported [82], we detected neither muscle wasting nor reduction in total twitch (Pt) and tetanic (Po) muscle forces in *HSA*^{LR} mice compared to controls (Table 1, and Supplemental Table 1). Nonetheless, as cross sectional area (CSA: mass/(density*length*correction factor)) of EDL muscle was increased in mutant mice, specific muscle forces (sPt and sPo), representative of the contractile capacity of the myofibers, were reduced in *HSA*^{LR} mice compared to control

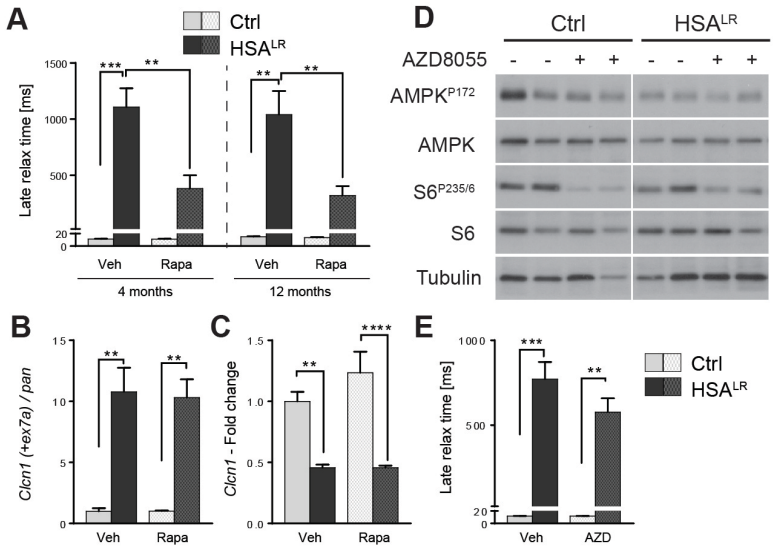

Figure 5: Rapamycin improves muscle function in *HSA^{LR}* mice via splicing-independent mechanisms. (A) Rapamycin (Rapa) treatment significantly reduces late relaxation time of muscle from 4- (n=4 Ctrl and 8-10 *HSA^{LR}* per group) and 12- (n=3 Ctrl and 5-6 *HSA^{LR}*) month-old *HSA^{LR}* mice, as compared to age-matched, vehicle (Veh)-treated mutant mice. (B, C) Splicing (B) and overall transcript expression (C) of the *Clcn1* gene are not modified in muscle from rapamycin (Rapa)-treated *HSA^{LR}* mice, compared to vehicle (Veh)-treated mutant mice. Values are relative to vehicle-treated control mice. (D) Treatment with AZD8055 (AZD) for 10 days efficiently reduces phosphorylation of mTORC1 target, S6, in control (Ctrl) and *HSA^{LR}* muscle, but does not change AMPK activation. (E) AZD8055 (AZD) does not reduce late relaxation time of EDL mutant muscle, compared to vehicle (Veh)-treated mutant mice. n=3 Ctrl and 5-8 *HSA^{LR}* mice. Data represent mean ± SEM. **p<0.01, ***p<0.001, 2-way ANOVA with Tukey's multiple comparisons test correction.

animals (Table 1, and Supplemental Table 1). Upon AICAR treatment, we observed that both total and specific forces of EDL muscle were increased in 2-month-old *HSA*^{LR} and control mice, but not in older mice (Table 1, and Supplemental Table 1). Altogether, these results indicate that targeting AMPK activation by AICAR improves muscle function in *HSA*^{LR} mice by reducing myotonia and potentially increasing muscle force, at least in part, through splicing correction.

Rapamycin treatment improves muscle function in HSA^{LR} *mice*. Based on the abnormal activation of mTORC1 signaling detected in *HSA*^{LR} muscle, we wondered whether indirect mTORC1 inhibition was part of the effect of AICAR and whether direct mTORC1 inhibition would improve muscle function in mutant mice. To this purpose, we subjected 4- and 12-month-old mice to rapamycin treatment for 7 and 10 days, respectively. Rapamycin treatment efficiently inhibited mTORC1 signaling, as shown by reduced S6$^{P235/6}$ levels in muscle from control and *HSA*^{LR} mice (Supplemental Figure 5A). Rapamycin did not affect muscle half relaxation time (Supplemental Figure 5B), but significantly reduced the late relaxation time of EDL muscle from 4- and 12-month-old *HSA*^{LR} mice (Figure 5A, and Supplemental Figure 5C). Moreover, we detected a significant increase in total and specific muscle forces in young rapamycin-treated mutant mice compared to vehicle-treated animals. Forces remained unchanged upon treatment in older mice (Table 1, and Supplemental Table S1).

To test whether the effect of rapamycin on muscle function relied on splicing improvement, we assessed, by quantitative PCR, *Clcn1* mis-splicing (i.e. exon 7a inclusion). Interestingly, rapamycin affected neither *Clcn1* splicing (Figure 5B) nor the overall transcript expression of the gene (Figure 5C) in mutant muscle. Consistently, splicing of the *Atp2a1* gene was also not restored in rapamycin-treated *HSA*^{LR} mice (Supplemental Figure 5D). Together, these data suggest that mTORC1 inhibition by rapamycin is sufficient to improve muscle function in *HSA*^{LR} mice likely through splicing-independent mechanisms.

Since rapamycin has been shown to impact on channel function (e.g. ryanodine receptor 1, RyR1) via its binding to FKBP12, we wondered whether mTORC1 inhibition or the drug itself mediated the effect of the treatment on myotonia. Hence, control and *HSA*^{LR} mice were treated for 10 days with AZD8055, an ATP-competitive

inhibitor of mTORC1. We confirmed that AZD8055 strongly decreased levels of $S6^{P235/6.}$ in control and mutant muscle, while the activation state of AMPK remained

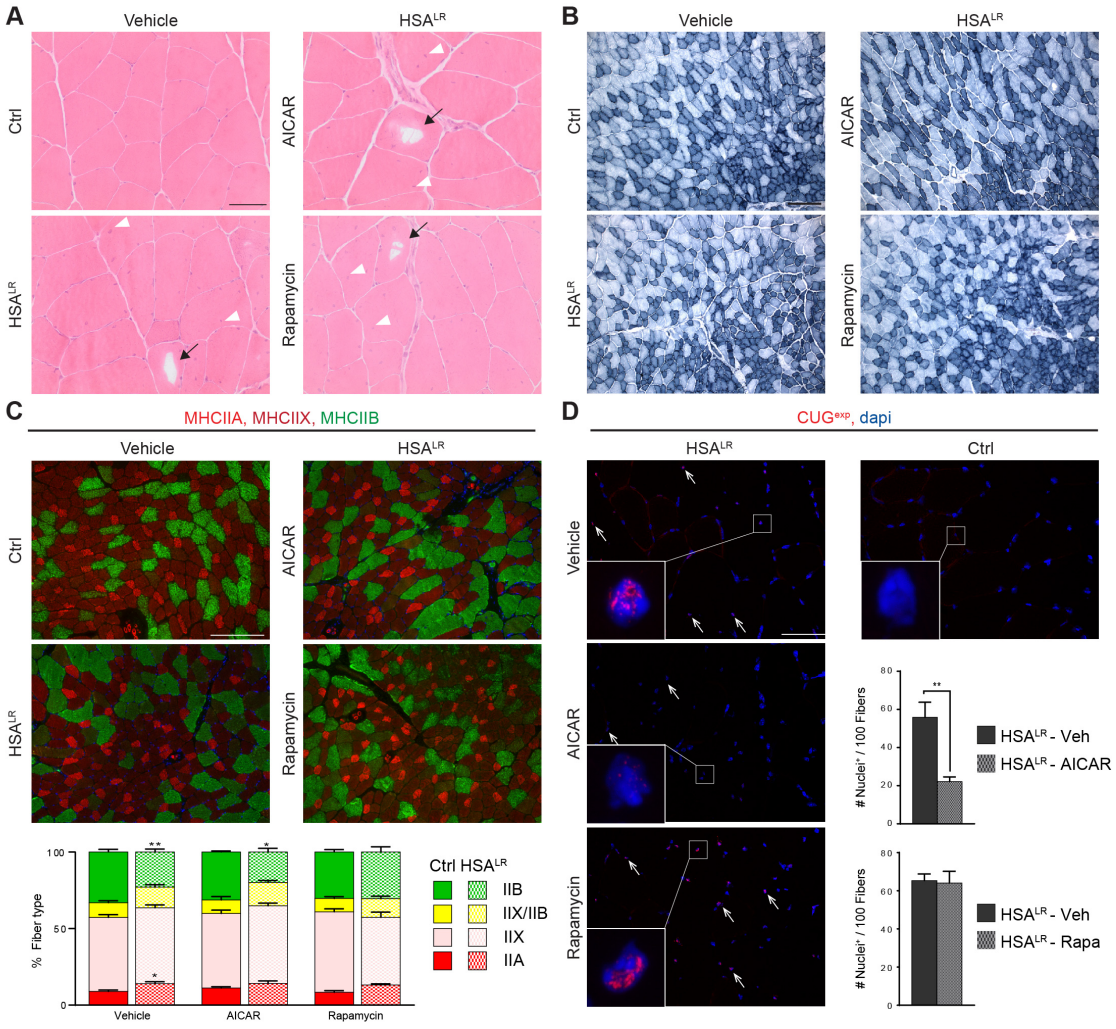

Figure 6: AMPK activation by AICAR leads to nuclear foci dispersion in *HSA^{LR}* muscle. (A, B) H&E (A) and NADH (B) stains reveal no major change in muscle histopathology and oxidative capacity upon AICAR or rapamycin treatment in *HSA^{LR}* mice. Arrowheads and arrows show internalized nuclei and vacuoles, respectively. Scale bar, 50 µm (A), 200 µm (B). (C) Immunostaining for type IIA (bright red), IIX (dark red) and IIB (green) myosin heavy chains (MHC) reveals no change in the respective proportion of fiber types in TA mutant muscle upon AICAR (n=3-4 per genotype) or rapamycin (n=3-4) treatment, compared to vehicle-treated *HSA^{LR}* mice (n=6-7). (D) Fluorescence *in situ* hybridization on TA muscle sections with a Cy3-CAG_{10} DNA probe shows accumulation of nuclear foci in *HSA^{LR}* muscle (arrows). The number of stained nuclei is significantly decreased upon AICAR treatment (n=4), but not with rapamycin (Rapa, n=3), compared to vehicle (Veh)-treated mutant mice. Foci are not detected in control (Ctrl) muscle. Scale bar, 50 µm. Data represent mean ± SEM. *p<0.05, **p<0.01, 2-way ANOVA with Tukey's multiple comparisons test correction.

unchanged (Figure 5D). In contrast to rapamycin, AZD8055 had no effect on late relaxation time of mutant muscle (Figure 5E). Nonetheless, total and specific forces of EDL muscle were increased in AZD8055-treated mutant mice, as observed with rapamycin (Table 1, and Supplemental Table 1). Together, these results indicate that mTORC1 inhibition may ameliorate the contractile capacity of muscle in HSA^{LR} mice, while muscle relaxation improvement upon rapamycin and AICAR treatments is likely independent of mTORC1.

AICAR, but not rapamycin, leads to nuclear foci dispersion in HSA^{LR} muscle. To further understand the beneficial effect of AICAR and rapamycin in HSA^{LR} mice, we investigated whether the treatments improved muscle function by affecting theproperties of the diseased muscle. First, we did not observe major changes in the histopathology of HSA^{LR} muscle upon 7-day AICAR or 10-day rapamycin treatment, compared to untreated conditions (Figure 6A). Of note, vacuoles remained present in muscle fibers from 12-month-old, AICAR- or rapamycin-treated HSA^{LR} mice (Figure 6A), indicating that the treatments were not sufficient to reverse muscle alterations related to impaired autophagy in aging mice. As no myotonia was detected in the slow, *soleus* muscle of HSA^{LR} mice and as AICAR and rapamycin were previously shown to alter muscle fiber types [174, 175], we tested whether changes in muscle function upon treatments were related to modification of muscle metabolic and contractile capacity. By nicotinamide adenine dinucleotide (NADH) staining, we first observed that the overall oxidative property of TA muscle was unchanged in AICAR- and rapamycin-treated mutant mice, compared to untreated animals (Figure 6B). Immunostaining against type I, IIA/X and IIB myosin heavy chains (MHC) was then conducted in TA muscle from HSA^{LR} and control mice to identify changes in muscle contractile properties upon treatment. Only few type I fibers were present in all the muscles analyzed (data not shown). Mutant muscle displayed a switch to slower fibers (i.e. increased and reduced proportion of IIA and IIB fibers, respectively) compared to control muscle (Figure 6C). However, upon AICAR or rapamycin, there was no significant change in the proportion of the different fiber types in comparison to vehicle-treated mice (Figure 6C).

Since aggregation of (CUG)n-expanded RNA in nuclear foci is one histological hallmark in DM1 diseased muscle, we next wondered whether the treatments affected their accumulation in HSA^{LR} muscle. To this purpose, we performed fluorescence *in*

Table 1: Changes in muscle cross section area and tetanic forces upon treatments in *HSA^{LR}* and control mice.

	Ctrl		*HSA^{LR}*	
	Vehicle	AICAR	Vehicle	AICAR
CSA EDL (mm^2)				
2M	1.38 ± 0.02	1.31 ± 0.03	1.65 ± 0.05$^{###}$	1.62 ± 0.01$^{###}$
8M	1.52 ± 0.00	1.70 ± 0.2	1.86 ± 0.07	1.70 ± 0.04
12M	-	-	2.46 ± 0.07	2.32 ± 0.02
Po (mN)				
2M	185.20 ± 9.01	255.79 ± 9.09*	197.91 ± 13.96	279.97 ± 17.25**
8M	285.37 ± 33.67	273.71 ± 36.71	288.31 ± 15.15	274.39 ± 12.27
12M	-	-	260.90 ± 26.53	339.42 ± 24.22$^{0.07}$
sPo (mN/mm^2)				
2M	134.58 ± 4.84	194.73 ± 2.67*	121.23 ± 12.16	173.60 ± 11.05*
8M	187.58 ± 22.01	168.80 ± 34.37	156.89 ± 11.95	161.94 ± 7.34
12M	-	-	107.33 ± 13.02	146.46 ± 10.51$^{0.06}$

	Vehicle	Rapamycin	Vehicle	Rapamycin
CSA EDL (mm^2)				
4M	1.69 ± 0.12	1.61 ± 0.07	2.04 ± 0.07$^{##}$	1.96 ± 0.02$^{##}$
12M	1.67 ± 0.09	1.50 ± 0.10	1.90 ± 0.06	1.77 ± 0.09
Po (mN)				
4M	180.94 ± 26.37	170.07 ± 3.91	193.80 ± 13.01	274.48 ± 16.11$^{**##}$
12M	226.14 ± 28.30	245.67 ± 16.07	209.59 ± 30.94	240.28 ± 18.95
sPo (mN/mm^2)				
4M	114.98 ± 21.53	111.32 ± 8.79	99.41 ± 7.64	144.37 ± 8.32**
12M	137.66 ± 23.49	168.87 ± 20.50	109.78 ± 15.01	137.12 ± 12.41

	Vehicle	AZD8055	Vehicle	AZD8055
CSA EDL (mm^2)	1.24 ± 0.42	1.67 ± 0.03	2.09 ± 0.09$^{#}$	2.06 ± 0.04
Po (mN)	185.19 ± 76.17	241.13 ± 80.46	257.96 ± 28.02	353.06 ± 8.90
sPo (mN/mm^2)	190.13 ± 23.13	192.38 ± 6.94	124.30 ± 13.88$^{#}$	171.96 ± 5.22*

CSA, cross sectional area; Po, tetanic muscle force. CSA = weight/(1.06*length*0.44), where 1.06 corresponds to the density of the muscle and 0.44 the correction factor for EDL muscle. AICAR, 2M (n=3-4), 8M (n=3 Ctrl and 6-7 *HSA^{LR}*), 12M (n=4-5); Rapamycin, 4M (n=4 Ctrl and 8-10 *HSA^{LR}*), 12M (n=3 Ctrl and 5-6 *HSA^{LR}*); AZD8055 (n=3 Ctrl and 5-8 *HSA^{LR}*). Values are mean ± SEM. # p< 0.05, ## p<0.01 compared to control mice with same treatment; * p<0.05, ** p<0.01, *** p<0.001 compared to same genotype treated with vehicle, 2-way ANOVA with Tukey's multiple comparisons test correction – For 12M AICAR, unpaired two tailed Student's t-test.

situ hybridization using a CAG_{10} DNA probe on TA muscle sections from mutant untreated- and treated-mice. Numerous foci were observed in HSA^{LR} muscle while none were detected in control muscle (Figure 6D). Interestingly, the number of nuclei showing foci was significantly decreased in muscle from AICAR-treated mutant mice, while no change was observed with rapamycin (Figure 6D). Moreover, foci appeared more diffuse in positive nuclei from mutant muscle upon AICAR treatment, compared to untreated conditions (Figure 6D). Altogether, these results indicate that changes in muscle metabolic and contractile properties do not account for the beneficial effect of the short treatments applied to HSA^{LR} mice, whilst reduced muscle pathology upon AICAR-mediated acute AMPK activation likely involves nuclear foci dispersion in the mutant muscle.

Discussion

The pathogenic mechanisms underlying DM1 disease are still not well understood and most investigations so far have focused on splicing defects caused by mRNA toxicity. In this study, we uncovered that in DM1, the AMPK and the mTORC1 pathways are deregulated and that the autophagic flux is perturbed in skeletal muscle. Most importantly, we established that pharmacological interference with AMPK/mTORC1 signaling by AICAR or rapamycin ameliorates DM1 muscle function.

AMPK and PKB/Akt-mTORC1 signaling are central metabolic pathways in muscle cells, and their deregulation has been related to muscle alterations and disease [164, 176, 177]. We found that DM1 muscle shows an altered response to energy/nutrient-deprived conditions, with impaired AMPK activation and abnormal activation of mTORC1 signaling. Although we have not studied the upstream mechanisms involved in this deregulation, mis-splicing-dependent CaMKII deficiency could well account for the limited AMPK activation in DM1 muscle [156, 158-161]. Interestingly, Jones et al. recently reported increased GSK3β levels and activity in HSA^{LR} muscle [178], which may also contribute to the perturbation of AMPK in DM1 muscle [179]. In parallel, AMPK constitutes an upstream regulator of mTORC1 [180] and its deregulation could thus be responsible for abnormal mTORC1 activation in DM1 muscle cells. Nonetheless, as we did not detect changes in 4E-BP1 levels or mTOR phosphorylation in mutant muscle, evidence that the state of mTORC1 is modified in DM1 muscle and not only the activation of its downstream axis p70S6K/S6

is missing. Previous reports suggested that mTORC1 is inhibited in DM1 human neural and muscle cells [151, 153] although results were only obtained *in vitro* and the underlying mechanisms have not been investigated. Based on the abnormal splicing and protein trafficking of the insulin receptor in metabolic tissues in DM1 [127, 152], it has also been hypothesized that PKB/Akt-mTORC1 may be less responsive to insulin. However, results regarding the activation state of PKB/Akt signaling in DM1 human muscle biopsies or cells are conflicting [128, 154]. In our experiments, we did not detect changes in PKB/Akt activation or in the expression of *Insr* in HSA^{LR} muscle, suggesting that AMPK/mTORC1 deregulation is independent of insulin receptor deficiency.

Autophagy as a major catabolic process essential for proteostasis has also been suggested to contribute to muscle alterations in DM1 [181]. The involvement of autophagy in DM1 was largely deduced from the presence of autophagic vesicles and/or accumulation of autophagic markers in DM1 cells, but usually without dynamic measurement of the autophagic flux [150, 151, 153, 154, 168, 169, 182]. In our study, we combined several methods and established that changes in LC3, increased levels of p62 and fiber vacuolization in muscle from older HSA^{LR} mice are caused by restricted autophagic flux at the degradation steps. Further, we showed that even prolonged starvation did not fully induce autophagy in mutant muscle. We hypothesize that AMPK- and mTORC1-independent mechanisms contribute to this autophagy defect, as rapamycin and metformin were not sufficient to normalize the flux in HSA^{LR} mice. Although autophagy impairment may contribute to muscle atrophy in DM1, it is unlikely to be the main pathogenic event, as autophagic features were scarce in DM1 muscle biopsies, compared to diseases primarily related to autophagy defects, such as vacuolar myopathies.

Importantly, we identified that AMPK/mTORC1 deregulation likely contributes to alteration of muscle function in DM1. Myotonia, which is due to membrane hyperexcitability, is thought to be caused primarily by mis-splicing and thereby deficiency in the chloride channel 1 [82, 145, 171, 173]. In contrast to a recent report studying cultured human DM1 cells [170], we did not find any effect of metformin on the mis-splicing of DM1-affected genes or on the severe myotonia observed in HSA^{LR} mice. While we cannot rule out that changes in dosage and administration may lead to different results, much higher concentrations may be required to efficiently stimulate AMPK in rodent muscle tissue. In contrast to metformin, we found a profound effect of AICAR, a more potent AMPK agonist. AICAR led to a strong reduction of myotonia in

HSA^{LR} mice, which correlated with improved splicing of the *Clcn1* gene and increased protein levels of the channel. While AMPK activation was related to repression of the RNA-binding protein *Rbm3 in vitro*, *Rbm3* expression was unchanged in *HSA*^{LR} muscle upon AICAR treatment. As we observed dispersion of the nuclear foci formed by the (CUG)n-expanded RNA aggregation after AICAR treatment, the effect of AMPK activation on splicing may be mediated by its interaction with other RNA-binding proteins, such as hnRNP H, which were implicated in foci stability in DM1 [183, 184]. Hence, one may argue that AMPK deregulation likely contributes to pathogenesis in DM1 muscle by perturbing RNA-binding proteins and thereby accentuating foci stability and mis-splicing events [185-187]. Of note, we cannot rule out that amelioration of muscle relaxation also relies on splicing-independent mechanisms. In particular, changes in sodium and calcium-activated potassium channels or in Ca^{2+} homeostasis, which have also been suggested to contribute to myotonia in DM1 [188-193], may mediate some of the observed effect. Consistently, AMPK has been shown to modulate chloride and potassium channels in several cell types, including cardiomyocytes [194, 195]. Such mechanisms may also contribute to the beneficial effect of rapamycin, as it occurs in the absence of splicing changes. Rapamycin could influence intracellular calcium mobilization by dissociating FKBPs (FK506-binding protein) from RyR1, thereby modifying channel activity [196-198].

While muscle weakness is observed in DM1 patients, it was initially not reported in *HSA*^{LR} mice [82]. Consistently, in our study, total muscle force was not affected in mutant mice but we observed reduced specific muscle strength in *HSA*^{LR} mice. Both specific and total muscle forces were increased upon AICAR, rapamycin and AZD8055 treatments in young mutant mice, which may be mediated by mTORC1 inhibition as the signaling was shown to modulate Ca^{2+} homeostasis and excitation-contraction coupling in skeletal muscle [199]. Myotonia reduction and increase in muscle force were not caused by modified metabolic and contractile properties of the mutant muscle upon the applied short-term treatments. However, one can hypothesize that the changes expected upon long-term administration of these drugs (i.e. switch toward slower fibers) may further positively affect DM1 muscle function.

In conclusion, we identified that deregulation of AMPK/mTORC1 signaling, together with autophagy impairment contribute to DM1-associated muscle alterations. We showed that treatments targeting the AMPK/mTORC1 imbalance are beneficial for muscle function, though to varying degrees. Whether alternative AMPK/mTORC1-

targeting compounds, as well as changes in the dosage, administration mode or treatment duration may further improve muscle function remains to be investigated. As the drugs used in our study can target the pathways body wide, they may also be beneficial in other tissues and thus may represent new treatment options for DM1.

Materials and methods

Mice

Homozygous mice of the mouse line LR20b carrying around 250 (CTG) repeats within the *HSA* transgene (HSA^{LR}) were obtained from Thornton et al. [82]. Mice of the corresponding background strain (FVB/N) were used as control. *GFP-LC3:HSA*^{LR} and GFP-LC3:FVB/N mice were obtained by crossing GFP-LC3 expressing mice [155] with *HSA*^{LR} or FVB/N mice. Mice were genotyped for *HSA*^{LR} transgenes by quantifying *ACTA1* levels normalized to endogenous actin (mouse *Acta1*) in genomic DNA. Mice were maintained in a conventional SPF facility with a fixed light cycle (23°C, 12 h dark-light cycle). Mice were intraperitoneally injected with colchicine (Sigma, 0.4 mg/kg) for 2 days, rapamycin (LC Laboratories) for 1 day (4 mg/kg), 7 or 10 days (2 mg/kg), AICAR (Toronto Research Chemicals, 500 mg/kg) for 7 days, or AZD8055 (LC Laboratories, 10 mg/kg) for 10 days. Mice were treated with metformin (Sigma-Aldrich, 300 mg/kg) by gavage for 5 or 10 days. For starvation experiments, mice were sacrificed after 12 h food deprivation followed by 4 h free access to food (fed), or after 24 h or 45 h food deprivation but free access to water (starved). *In vitro* force measurement of EDL and *soleus* muscles was conducted as previously described [164]. Half- and late-relaxation times were calculated according to Moyer et al. [146].

Human muscle cells and biopsy samples

Muscle biopsies frozen in nitrogen-cooled isopentane from 3 DM1 patients, aged 33, 34 and 49 years and from an IBM patient were analyzed and compared to 5 control muscle samples from age-matched individuals showing no clinical signs of DM1 and normal muscle histology. MyoD-transduced fibroblasts from control individuals and patients were cultured in growth medium (DMEM, 10% FBS, 50 μg/mL gentamicin) at 37°C under 5% CO2. At confluency, transduction into myoblasts was induced by differentiation medium (DMEM, 50 μg/mL gentamycin, 3 μg/mL doxycycline hyclate,

10 µg/mL human recombinant insulin) [200]. Myotubes obtained after 10 days were incubated for 3 h in growth medium (refed), PBS (starved), or PBS supplemented with chloroquine (100 µM).

Western Blotting

Cell pellets and muscles powdered in liquid nitrogen were lysed in cold RIPA^{+} buffer (50 mM Tris HCl pH8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton-X, 10% glycerol, phosphatase and protease inhibitors). Following dosage (BCA Protein Assay, Sigma-Aldrich), proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membrane. Blots were blocked in TBS, 3% BSA, 0.1% Tween-20, incubated overnight at 4°C with primary antibodies, and then for 2 h with HRP-labelled secondary antibodies. Immunoreactivity was detected using the ECL western blot detection reagent LumiGLO (KPL) and exposed to Super RX-N films (Fujifilm). Protein expression was normalized to α-actinin, α-tubulin or the total protein of the corresponding phosphorylated form. Antibodies used are listed in Supplemental Material.

Polymerase chain Reaction (PCR)

Total RNAs were extracted with the RNeasy Mini Kit (Qiagen), reverse transcribed with the SuperScript III First-Strand Synthesis System (Invitrogen) and amplified with the Power Sybr Green Master Mix (Applied Biosystems) or the Hot FirePol EvaGreen qPCR Mix (Solis BioDyne). Expression of specific spliced or pan transcripts was analyzed by end-point PCR and electrophoresis, or by quantitative PCR with the Step One software and normalization to *Actn* expression. Primers are listed in Supplemental Material, Table S2.

Histology and Immunofluorescence

Muscles were frozen in liquid nitrogen-cooled isopentane. 8 μm muscle sections were stained with Hematoxylin/Eosin (H&E) or NADH, and observed with an upright microscope (DMR, Leica). For immunostaining, sections were unfixed or fixed with 4% paraformaldehyde (PFA), cold acetone or methanol; for some, microwave antigen

retrieval was used. Sections were then blocked in PBS, 3% BSA, incubated sequentially with primary and appropriate secondary fluorescent antibodies (Invitrogen), mounted with Vectashield medium (Vector) and observed with a Leica fluorescent microscope.

GFP-LC3 puncta analysis

For GFP-LC3 detection, mice were perfused with 4% PFA and muscles were incubated in 30% sucrose overnight. Cryosections were washed and mounted. Images were recorded using a Leica confocal microscope with 63x objective. The number of GFP-LC3 puncta was counted on the 3D reconstructed images with Imaris (version 8.1.2) software. Seven to twelve image stacks were quantified for each muscle and the average number of GFP-LC3 puncta per volume unit defined within a single fiber (20.8 x 20.8 x 6.5 μm^3) was used for statistical analyses. All GFP quantifications were done in a blinded way.

Fluorescence *in situ* hybridization (FISH)

FISH was conducted on muscle cryosections as previously described by Batra et al. [201], using a Cy3-CAG_{10} DNA probe. Nuclear foci were observed with a Leica confocal microscope with 40 and 100x objectives.

Statistics

Quantitative data are displayed as mean ± SEM of independent samples, with n (number of individual experiments) ≥ 3. Statistical analysis of values was performed using Student's t-test or two-way ANOVA test with Tukey's multiple comparisons test correction, with a 0.05 level of confidence accepted for statistical significance.

Study approval

Muscle biopsies from DM1 patients were obtained from the Neuromuscular Tissue Bank (Department of Neurosciences, University of Padova, Padova, Italy) through the Telethon Network of Genetic Biobanks and the EuroBioBank, in accordance with

European recommendation and Italian legislation on ethics. Control and IBM human muscle biopsies were from the Department of Pathology, University Hospital of Basel (Basel, Switzerland); their use was approved by the Ethical Committee of the University Hospital of Basel. Human fibroblast cell lines were obtained from the platform for immortalization of human cells at the Institut de Myologie (Paris, France). Fibroblasts derived from skin biopsies were obtained from the MyoBank-AFM bank of tissues for research at the Institut de Myologie, a partner in the EU network EuroBioBank, in accordance with European recommendation and French legislation on ethics. All animal studies were performed in accordance with the European Union guidelines for animal care and approved by the Veterinary Office of the Canton of Basel city (application number 2601).

Author contributions

MB and PC performed most of the experiments, analyzed the data and wrote the paper with input from all authors. NR, KC, TW, CE and BE conducted qPCR and Western blot analyses, GFP-LC3 quantification, muscle dissection, cryosections and inorganic staining, and electron microscopy analyses, respectively. SF and CA provided human muscle biopsies. DF developed and provided transduced fibroblasts from DM1 patients. MR and MS helped conceiving the project and edited the manuscript; MS secured funding. PC designed and directed the research project.

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the Swiss Foundation for Research on Muscle Diseases (MAR, MS) and the Swiss National Science Foundation (PC, MS).

Supplemental Material

Targeting deregulated AMPK and mTORC1 pathways improves muscle function in myotonic dystrophy type I

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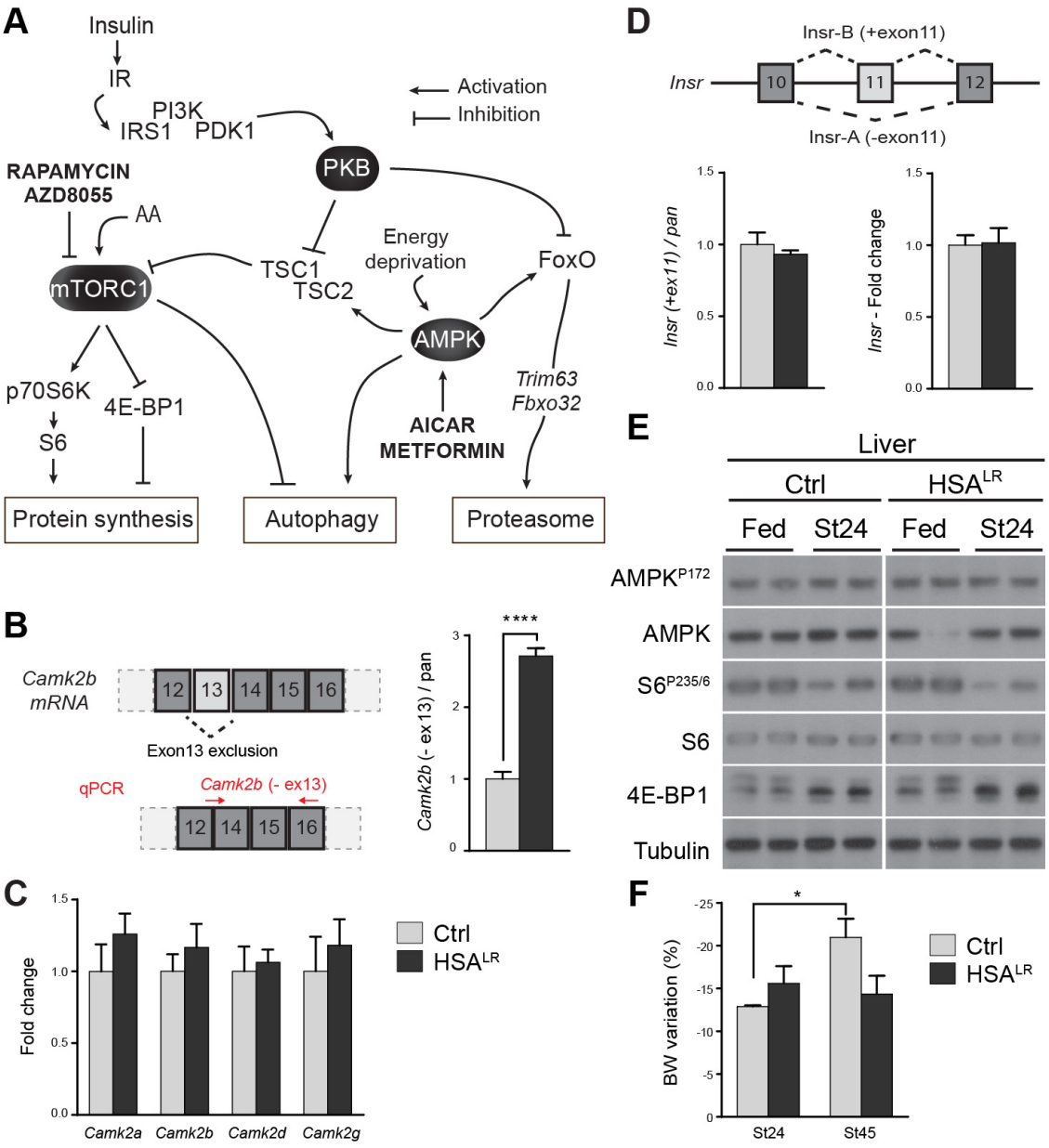
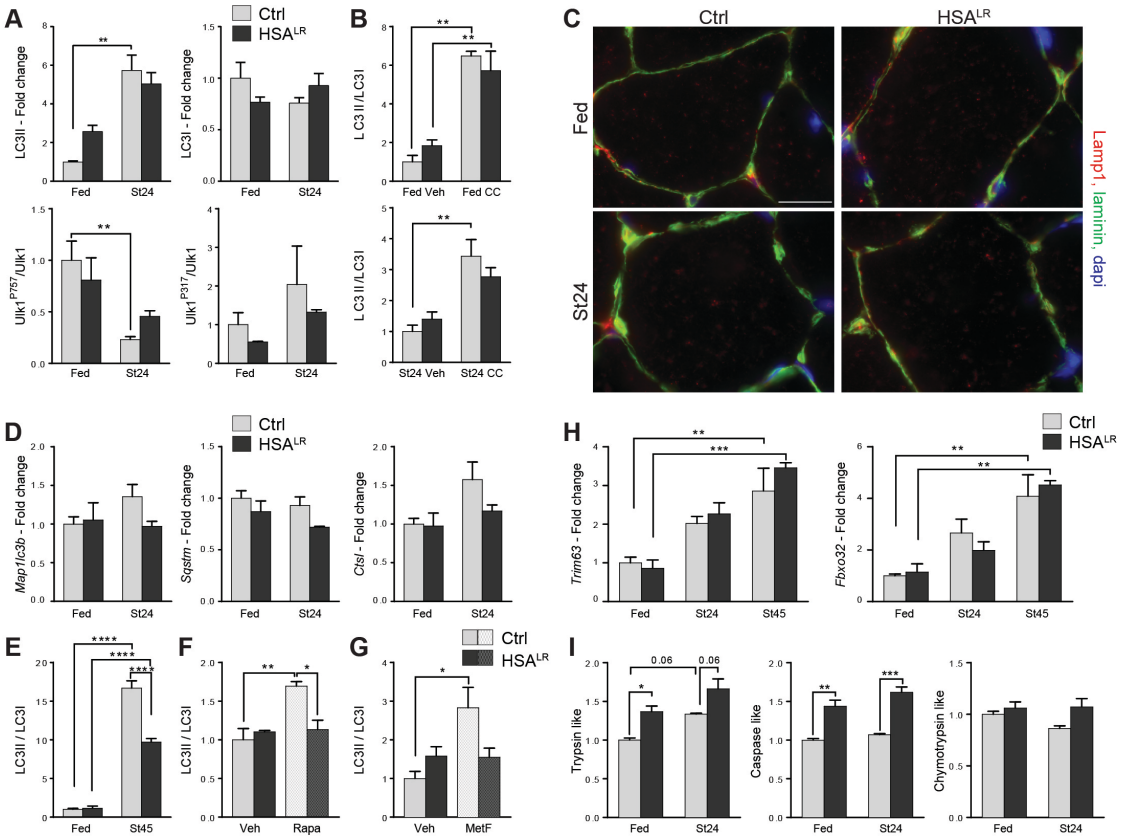
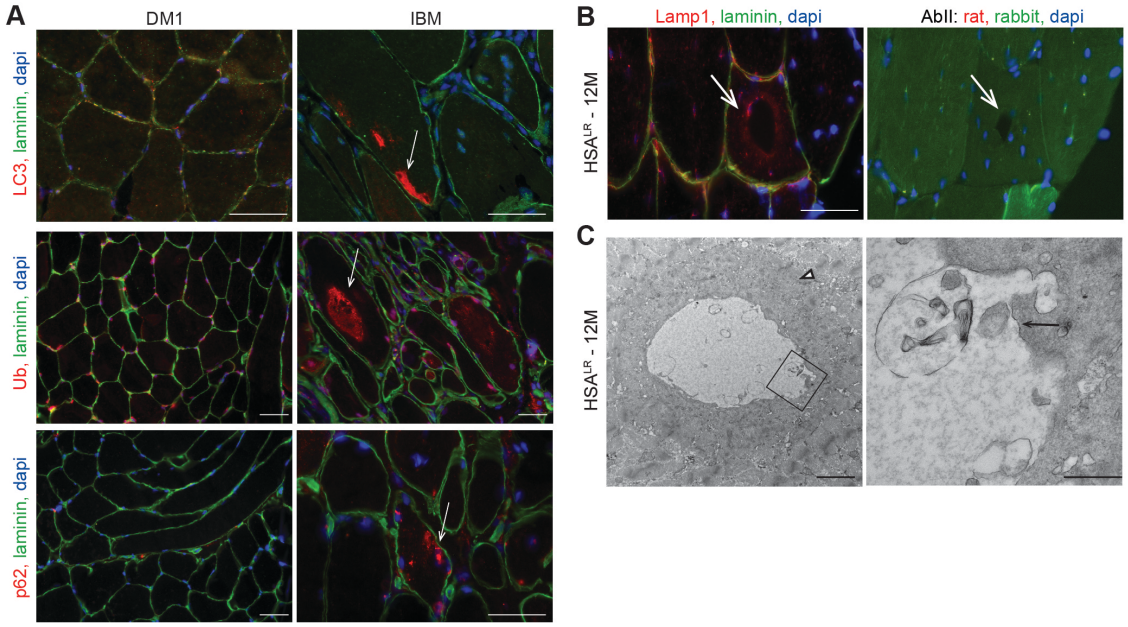
Supplemental material includes: 5 supplemental figures, 2 supplemental tables and supplemental methods



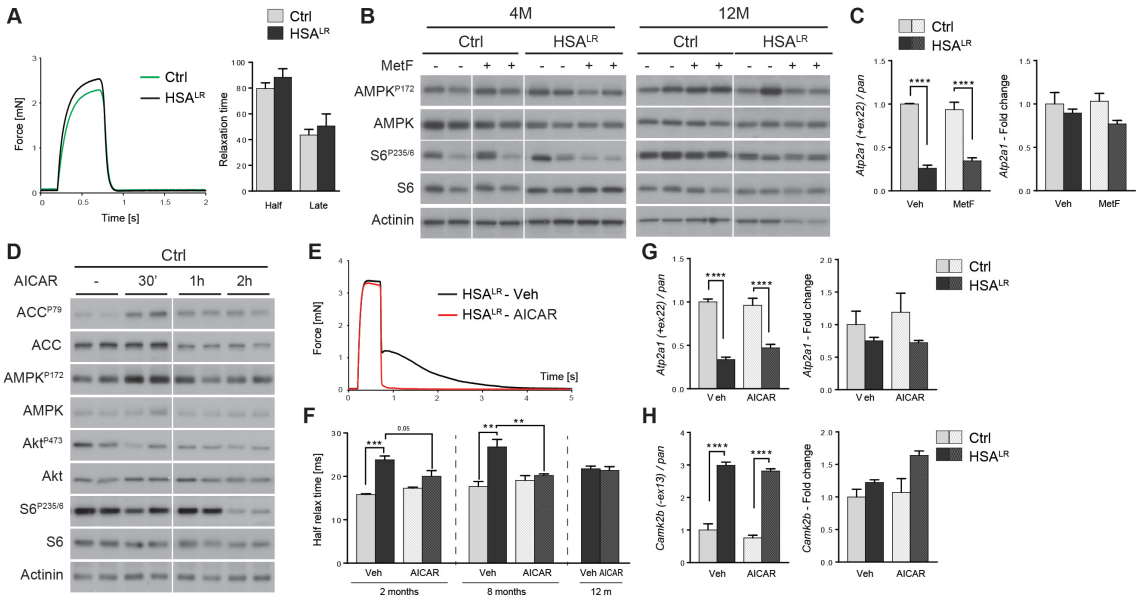
Supplemental Figure 1: Imbalance in AMPK / mTORC1 pathways is not related to insulin receptor (IR)-PKB/Akt deregulation and is limited to muscle tissue in *HSA^{LR}* mice. (A) Simplified scheme of the metabolic signaling involved in proteostasis in skeletal muscle. (B) Representation of the known alternative splicing of *Camk2b* mRNA in DM1 (exon 13 exclusion) and of the primers used to quantify its expression. Quantitative PCR shows increased levels of *Camk2b* transcript with exon 13 exclusion (-ex13) in *HSA^{LR}* muscle. n=4 per group. (C) Transcript levels of *Camk2a*, *2b, 2d* and *2g* are not affected in *HSA^{LR}* muscle (n=4). Expression is normalized to *Actn*. (D) Splicing of the *Insr* gene, corresponding to the mis-splicing event in DM1 patients, is not altered in muscle from 2 month-old *HSA^{LR}* mice (n=4). (E) Imbalance in AMPK and mTORC1 signaling is not observed in liver from fed and starved (St24) *HSA^{LR}* mice compared to control (Ctrl) animals. (F) Loss of body weight increases from 24 to 45 h of starvation in control (Ctrl) mice but not in *HSA^{LR}* mice. Loss is expressed as a percentage of the initial body weight. n=3-4. Data are relative to control (B-D) and represent mean ± SEM. ****p<0.0001, unpaired two tailed Student's t-test (B); *p<0.05, 2-way ANOVA with Tukey's multiple comparisons test correction (F).



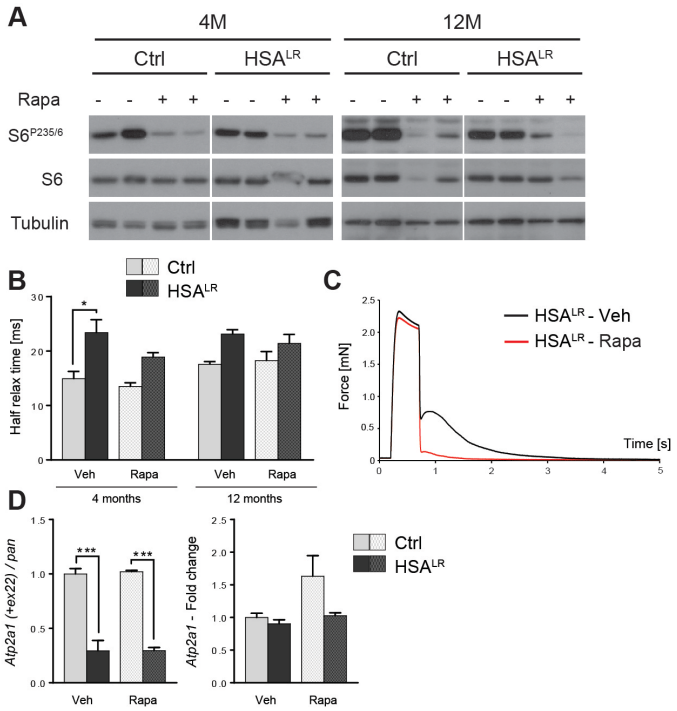
Supplemental Figure 2: *HSA^{LR}* muscles display impaired autophagic flux and increased proteasome activity. (A, B) Quantification of immunoblots for autophagy-related proteins in TA muscle from fed and starved (St24) control (Ctrl) and *HSA^{LR}* mice. Mice were treated with colchicine (CC) to analyze the autophagic flux. Protein expression is normalized to α-actinin (A, n=3-7 per group; B, n=3-4). (C) Immunostaining for Lamp1 protein (red) reveals similar lysosomal distribution in muscle from fed and starved (St24) *HSA^{LR}* and control (Ctrl) mice. Scale bar, 20 µm. (D) Expression of autophagy-related genes remains unchanged in control (Ctrl) and *HSA^{LR}* muscle after 24 h of starvation (St24). n=3-4. (E-G) After 45 h of starvation (St45, E), rapamycin (Rapa, F) or metformin (MetF, G) treatment, changes in LC3II to LC3I ratio are reduced in muscle from *HSA^{LR}* mice as compared to control (Ctrl) mice (n=3-4). (H) Expression of atrogenes is efficiently induced in *HSA^{LR}* muscle upon 45 h of starvation (St45). Expression is normalized to *Actn* (n=3-4). (I) Trypsin- and caspase-like activities are increased in muscle from fed and starved (St24) *HSA^{LR}* mice, compared to control (Ctrl) mice in the same nutritive condition (n=3). Data are relative to control mice in fed conditions (A, D, E, H, I) or to vehicle-treated control mice (B, F, G). Values represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, 2-way ANOVA with Tukey's multiple comparisons test correction.



Supplemental Figure 3: Few autophagic features are observed in muscle from DM1 patients and *HSA^{LR}* mice. (A) Muscle biopsies from DM1 patients display no accumulation of LC3 (red, upper panel), ubiquitinated proteins (red, middle panel) or p62 (red, lower panel) by immunostaining, contrasting with muscle from an IBM patient (arrow). Scale bar, 50 µm. (B) Immunostaining on serial muscle sections from 12-month (M)-old *HSA^{LR}* mice shows high density of lysosomes, reacting with anti-Lamp1 antibodies (red), at the periphery of a vacuole; no positive staining is observed in the negative control with only secondary antibodies (red, anti-rat) in the region of the same vacuole. Scale bar, 50 µm. (C) Electron microcopy confirms the presence of vacuolar structures in muscle from 12-month (M)-old *HSA^{LR}* mice. Vacuoles are limited by discontinuous membrane structures (arrow) and surrounded by disorganized regions of contractile materials (arrowhead). Scale bar, 2 µm for left panel; 0.5 µm for right panel.



Supplemental Figure 4: AICAR and metformin treatments have distinct effects on DM1 muscle function and splicing. (A) *In vitro* tetanic stimulation (120 Hz) of the *soleus* muscle leads to contraction of *HSA*^{LR} and control muscles; half and late relaxation times are not increased in mutant *soleus* muscle. (B) A ten-day oral treatment with metformin does not lead to reproducible decreased S6 phosphorylation in control (Ctrl) and mutant muscles. (C) Splicing and transcript expression of the *Atp2a1* gene are not changed in muscle from metformin (MetF)-treated *HSA*^{LR} mice, compared to vehicle (Veh)-treated mutant mice (n=3 per group). (D) Immunoblots for phospho- and total ACC, AMPK, PKB/Akt and S6 proteins show efficient phosphorylation of AMPK and ACC, 30 min after AICAR injection in control (Ctrl) muscle. Phosphorylation of S6, an AMPK-indirect target, is strongly reduced 2 h after AICAR injection, while no major change in PKB/Akt activity is detected. (E) AICAR treatment normalizes the time to relax of *HSA*^{LR} muscle upon tetanic stimulation (150 Hz), compared to muscle from vehicle-treated mutant mice (Veh). (F) Half relaxation time is reduced in muscle from 2 and 8 month (m)-old *HSA*^{LR} mice treated with AICAR, as compared with untreated (Veh) mutant mice (n=3-4 Ctrl and 4-7 *HSA*^{LR}). (G, H) Mis-splicing and transcript expression of *Atp2a1* (G) and *Camk2b* (H) genes are not changed in muscle from AICAR-treated *HSA*^{LR} mice, compared to vehicle (Veh)-treated mutant mice (n=3-4). Values represent mean ± SEM. **p<0.01, ***p<0.001, ****p<0.0001, 2-way ANOVA with Tukey's multiple comparisons test correction.



Supplemental Figure 5: Rapamycin treatment efficiently inhibits mTORC1 signaling but impacts neither on half relaxation time nor on splicing. (A) Immunoblots for phospho- and total S6 proteins reveal efficient mTORC1 inhibition in 4- and 12- month(M)-old, rapamycin (Rapa)-treated control (Ctrl) and *HSA*^{LR} mice. (B) Half relaxation time remains unchanged upon rapamycin (Rapa) treatment of *HSA*^{LR} mice, as compared to vehicle (Veh)-treated mutant mice (n=3-4 Ctrl and 5-10 *HSA*^{LR} per group). (C) Rapamycin treatment strongly reduces the time to relax of *HSA*^{LR} muscle upon tetanic stimulation (150 Hz), compared to muscle from vehicle (Veh)-treated mutant mice. (D) Rapamycin treatment does not change splicing and expression of the *Atp2a1* gene in mutant mice (n=3-4). Values represent mean ± SEM. *p<0.05, ***p<0.001, 2-way ANOVA with Tukey's multiple comparisons test correction.

Supplemental Table 1: Changes in EDL muscle mass and twitch forces upon treatments in *HSA^{LR}* and control mice.

	Ctrl		*HSA^{LR}*	
	Vehicle	AICAR	Vehicle	AICAR
EDL mass/BW (‰)				
2M	0.44 ± 0.01	0.42 ± 0.01	0.51 ± 0.03$^{\#}$	0.48 ± 0.01
8M	0.40 ± 0.02	0.42 ± 0.03	0.45 ± 0.03	0.36 ± 0.02
12M	-	-	0.40 ± 0.01	0.38 ± 0.02
Pt (mN)				
2M	15.40 ± 1.45	22.04 ± 0.84**	12.22 ± 0.50	25.14 ± 1.16***
8M	30.68 ± 5.08	29.93 ± 3.21	24.02 ± 1.35	25.85 ± 1.51
12M	-	-	29.27 ± 2.86	37.50 ± 1.48$^{0.06}$
sPt (mN/mm^2)				
2M	11.18 ± 0.92	16.84 ±1.03**	10.51 ± 0.60	15.58 ± 0.72**
8M	20.16 ± 3.33	18.06 ± 2.52	13.08 ± 1.02$^{\#}$	15.24 ± 0.86
12M	-	-	12.01 ± 1.39	16.18 ± 0.64*

	Vehicle	Rapamycin	Vehicle	Rapamycin
EDL mass/BW (‰)				
4M	0.38 ± 0.02	0.37 ± 0.02	0.42 ± 0.02	0.43 ± 0.01
12M	0.36 ± 0.03	0.33 ± 0.02	0.38 ± 0.02	0.32 ± 0.03
Pt (mN)				
4M	20.58 ± 1.69	17.77 ± 0.80	19.61 ± 1.55	29.70 ± 2.23$^{**\#\#}$
12M	22.40 ± 4.12	19.17 ± 0.35	17.69 ± 3.92	20.51 ± 2.18
sPt (mN/mm^2)				
4M	12.50 ± 1.71	11.08 ± 0.54	9.71 ± 0.89	15.23 ± 1.18**
12M	13.72 ± 3.08	13.93 ± 1.25	9.26 ± 1.97	11.77 ± 1.46

	Vehicle	AZD8055	Vehicle	AZD8055
EDL mass/BW (‰)	0.37 ± 0.02	0.34 ± 0.02	0.42 ± 0.02	0.41 ± 0.01
Pt (mN)	32.01 ± 3.43	31.53 ± 0.54	26.58 ± 2.68	35.55 ± 0.64**
sPt (mN/mm^2)	19.85 ± 3.42	18.84 ± 0.07	10.60 ± 2.31$^{\#}$	17.32 ± 0.44*

BW, body weight; Pt, twitch force. AICAR, 2M (n=3-4), 8M (n=3 Ctrl and 6-7 *HSA^{LR}*), 12M (n=4-5); Rapamycin, 4M (n=4 Ctrl and 8-10 *HSA^{LR}*), 12M (n=3 Ctrl and 5-6 *HSA^{LR}*); AZD8055 (n=3 Ctrl and 5-8 *HSA^{LR}*). Values are mean ± SEM. # p< 0.05, ## p<0.01 compared to control mice with same treatment; * p<0.05, ** p<0.01, *** p<0.001 compared to same genotype treated with vehicle, 2-way ANOVA with Tukey's multiple comparisons test correction – For 12M AICAR: * p<0.05 compared to same genotype treated with vehicle, unpaired two tailed Student's t-test.

Supplemental Table 2: List of primers.

Gene	Forward primer	Reverse primer
ACTA1 (human)	5'-CGAGACCACCTACAACAGCA-3'	5'-GGCATACAGGTCCTTCCTGA-3'
Acta1 (mouse)	5'-CCGGAAAGAAATCTCAACCA-3'	5'-CCAAGTCCTGCAAGTGAACA-3'
Actn	5'-CTGGTCTTCGACAACAAGCA-3'	5'-TTGTCAGGATCTGGGTCTCC-3'
Atp2a1 +ex22	5'- GCCCTGGACTTTACCCAGTG-3'	5'-ACGGTTCAAAGACATGGAGGA-3'
Atp2a1 pan	5'- GCCCTGGACTTTACCCAGTG-3'	5'-CCTCCAGATAGTTCCGAGCA-3'
Camk2a	5'-TTTGCCCTCTTCAGGCTTTA-3'	5'-GTGGACAGGGGCATGTTAG-3'
Camk2b -ex13	5'-TTTCTCAGCAGCCAAGAGTTT-3'	5'-TTCCTTAATCCCGTCCACTG-3'
Camk2b pan	5'-GCACGTCATTGGCGAGGA-3'	5'-ACGGGTCTCTTCGGACTGG-3'
Camk2d	5'-CTGGCACACCTGGGTATCTT-3'	5'-ATCCCAGAAGGGTGGGTATC-3'
Camk2g	5'-ACCGACGACTACCAGCTTTTC-3'	5'-GCAGCATATTCCTGCGTAGATG-3'
Ctsl	5'-GTGGACTGTTCTCACGCTCA-3'	5'-TCCGTCCTTCGCTTCATAGG-3'
Clcn1 +ex7a	5'-GGGCGTGGGATGCTACTTTG-3'	5'- AGGACACGGAACACAAAGGC-3'
Clcn1 pan	5'-CTGACATCCTGACAGTGGGC-3'	5'- AGGACACGGAACACAAAGGC-3'
Clcn1 (end-point)	5'-GGAATACCTCACACTCAAGGCC-3'	5'-CACGGAACACAAAGGCACTGAATGT-3'
Fbxo32	5'-CTCTGTACCATGCCGTTCCT-3'	5'-GGCTGCTGAACAGATTCTCC-3'
GFP (multiplex)	5'-ATAACTTGCTGGCCTTTCCACT-3'	5'-CGGGCCATTTACCGTAAGTTAT-3'
		5'-GCAGCTCATTGCTGTTCCTCAA-3'
Insr +ex11	5'-TATGACGACTCGGCCAGTGA-3'	5-ACCATTGCCTGAAGAGGTTT-3'
Insr pan	5'-ATGGGCTTCGGGAGAGGAT-3'	5'-GGATGTCCATACCAGGGCAC-3'
Map1lc3b	5'-CACTGCTCTGTCTTGTGTAGGTTG-3'	5'-TCGTTGTGCCTTTATTAGTGCATC-3'
Rbm3	5'-CTTCTGCCATGTCGTCTGAA-3'	5'-TGGGTTTGTGAAGGTGATGA-3'
Sqstm	5'-GCTCAGGAGGAGACGATGAC-3'	5'-AGAAACCCATGGACAGCATC-3'
Trim63	5'-ACCTGCTGGTGGAAAACATC-3'	5'-AGGAGCAAGTAGGCACCTCA-3'

Supplemental Methods

Antibodies

The following antibodies were used for immunoblotting or immunoflurescence: PKB/Akt (#9272), Phospho-Akt^{Ser473} (#4058), S6 (#2217), Phospho-S6$^{Ser235/236}$ (#2211), LC3B (#2775), AMPKα (#2532), Phospho-AMPKα^{Thr172} (#2531), Ulk1 (#8054), Phospho-Ulk1^{Ser757} (#6888), Phospho-Ulk1^{Ser317} (#6887), mTOR (#2972), Phospho-mTOR^{Ser2448} (#2971), 4E-BP1 (#9452), Tuberin/TSC2 (#3635), Phospho-Tuberin/TSC2^{Ser1387} (#5584), Phospho-CaMKII^{Thr286} (#12716), LKB1 (#3047), TAK1 (#5206), p70S6K (#9202), Phospho-p70S6K^{Thr389} (#9209) from Cell Signaling; α-Actinin (A5044) from Sigma; p62 (GP62C) from Progen; α-Tubulin (ab15246), Laminin (ab11575 and ab11576), Lamp1 (AD4B) from Abcam; CaMKII (C-20) from Santa Cruz; MHCIIA (A4.74) and IIB (BF-F3) from DSHB. Clc-1 antibody was a generous gift from Prof. Thomas Cooper (Baylor College of Medicine, USA) [173]. HRP-tagged and fluorescent secondary antibodies were from Abcam and Jackson Immunoresearch.

Proteasome activity assay

Protein extracts and acquisition of proteasome activity using the Proteasome-Glo 3-Substrate System (Promega) was described before by Strucksberg et al. [202].

Transmission electronic microscopy (EM)

EM analysis was conducted on ultra-thin (70 nm) sections of gastrocnemius muscle, as previously described [203]. Sections were stained with uranyl acetate and lead citrate, and observed on electron microscope (Philips CM100).

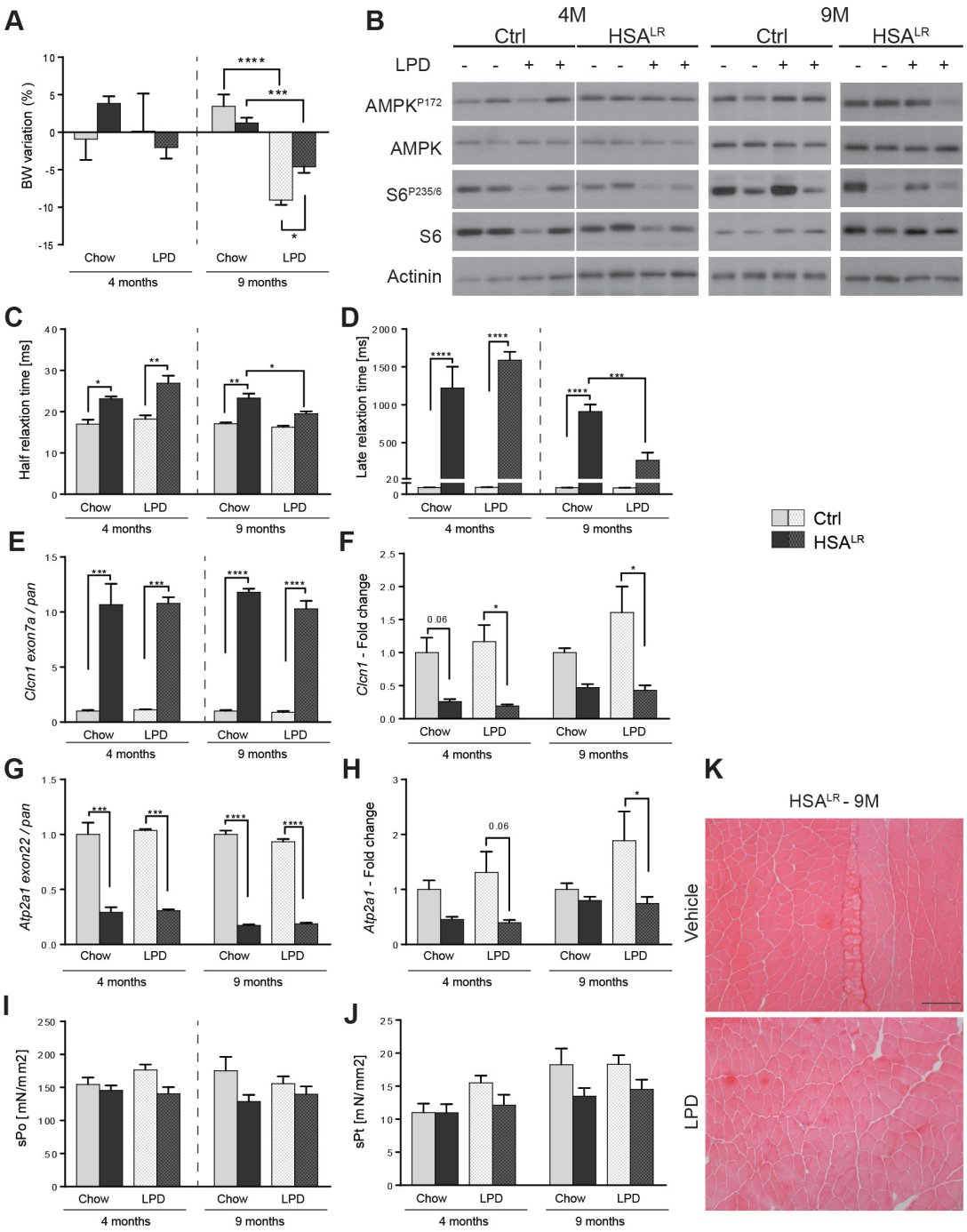


Figure 7: Low-protein diet improves muscle function in aging, but not young *HSA^{LR}* mice. (A) Significant loss of body weight is observed at the end of the 4 weeks low-protein diet (LPD) in 9 month-old *HSA^{LR}* and control mice, but not in young mice, as compared to age-matched mice fed with chow diet. Values are mean ± SEM; *p<0.05, ***p<0.001, ****p<0.0001. (B) Four weeks LPD administration does not lead to major changes in AMPK and mTORC1 signalling in muscle from 4 and 9 month (M)-old control (Ctrl) and *HSA^{LR}* mice. (C - D) LPD reduces half (C) and late (D) relaxation times of EDL muscle from 9 month-old *HSA^{LR}* mice compared to mutant mice fed with chow diet. (E - H) Protein restriction does not reduce mis-splicing (E) and transcript expression (F) of the *Clcn1* gene nor does it change alternative splicing (G) and expression (H) of the *Atp2a1* gene. (I - J) LPD does not improve specific tetanic force (sPo) (I) and specific twitch force (sPt) (J) of muscle from 4 and 9 month-old *HSA^{LR}* mice. Values are mean ± SEM; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (K) General histology observed with H&E stain is not changed in muscle from 9 month-old *HSA^{LR}* mice fed for 4 weeks with LPD. Scale bar, 200 µm. (D) Specific twitch force (sPt) is unchanged in EDL muscle from 4 and 9 month-old *HSA^{LR}* mice fed with LPD, as compared with mutant mice fed with chow diet. Data are mean ± SEM.

3.2 Complementary results

3.2.1 Low-protein diet improves myotonia in older *HSA^{LR}* mice

AMPK and mTORC1 are major sensors of the nutrient status of the cell: nutrient deprivation and more specifically protein restriction induce AMPK signalling and inhibit mTORC1. Given that drugs targeting AMPK activation or mTORC1 inhibition improved muscle function in *HSA^{LR}* mice, we investigated whether protein restriction would also have a beneficial effect on muscle function. To this end, we fed 4- and 9-month-old mice during 4 weeks with a low-protein diet (LPD), containing only 5% proteins; reference groups were fed with standard chow diet with 20% proteins. LPD led to a significant loss of body mass only in the older control and mutant mice, while body weight was not affected in young animals (Figure 7A). Interestingly, as observed with prolonged starvation, aged *HSA^{LR}* mice were more resistant to mass loss with LPD than controls (Figure 7A). Of note, no reliable change in the activation state of AMPK and mTORC1 signalling could be detected in muscle from LPD-fed mice compared to chow-fed animals, at the time of sacrifice (i.e. at daytime - Figure 7B). Importantly, while LPD did not modify muscle relaxation in 4 month-old mutant mice, it significantly reduced half and late relaxation times of EDL muscle from aging *HSA^{LR}* mice, compared to age-matched mutant mice on normal chow diet (Figure 7C, D). This differential effect of the dietary treatment was consistent with the stronger response of 9-month-old mice to protein restriction compared to young animals. Despite the effect of LPD on myotonia in aging mutant mice, we found no change in the expression or the splicing of the *Clcn1* and *Atp2a1* genes (Figure 7E - H). Moreover, LPD had no effect on specific muscle force (Figure 7I - J) and muscle histology (Figure 7K) in control and mutant mice. Thus, these results underline that splicing-independent mechanisms targeted by dietary approaches may have beneficial effects on muscle function in DM1 disease.

3.2.2 FoxO signalling is altered in *HSA^{LR}* muscle

FoxO transcriptions factors are known to be negatively regulated by Akt, while AMPK promotes their activity. Furthermore, FoxO induces the expression of different autophagy genes as well as atrogens, which are required for sustained autophagy flux and UPS activation. In light of the obtained results regarding blunted AMPK signalling,

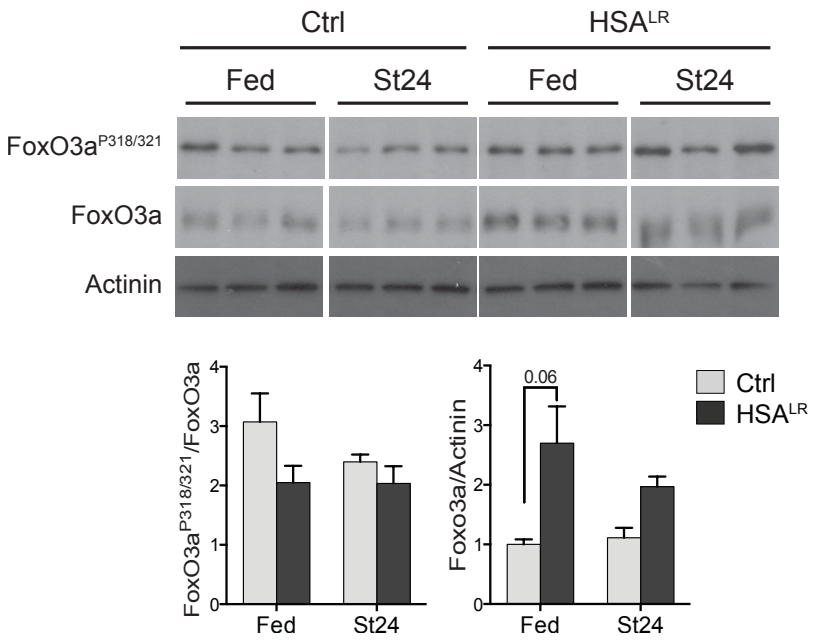


Figure 8: Foxo3a is deregulated in *HSA^{LR}* muscle. Foxo3a total protein expression and Foxo3a$^{P318/321}$ levels are potentially increased in fed and starved mutant muscle.

altered autophagic flux and increased proteasomal activity in *HSA*^{LR} muscle, we suspected that FoxO might as well be deregulated in mutant muscle. Preliminary western blot analysis revealed that protein levels of FoxO3a are increased in *HSA*^{LR} muscle, in both fed and starved conditions (Figure 8). In control muscle, the Akt-dependent inhibitory phosphorylation of FoxO3a (sites Ser318/321) showed a decreasing trend upon starvation. In contrast, levels of Foxo3a$^{P318/321}$ were increased in mutant muscle compared to controls and did not show reduction upon fasting. As the ratio between Foxo3a$^{P318/321}$ and total FoxO3a was not changed comparing mutant to control muscle, one can hypothesize that accumulation of the inactive form of FoxO3 is related to its increased protein expression, rather than excessive inhibition by Akt (Figure 8). This would be consistent with the effective inhibition of Akt detected in muscle from starved *HSA*^{LR} mice (Figure 1). Hence, FoxO signalling might as well be altered in DM1 and may contribute to the deregulation of catabolic processes in the diseased muscle. Further investigations remain to be conducted in order to clarify the activation state of FoxO signalling and the mechanism leading to its potential deregulation. In the short term, we will analyze the localization of FoxO by immunohistochemical analysis on muscle sections as FoxO nuclear import parallels its activation. qPCR will also be performed in order to detect potential changes in FoxO transcriptional expression. Lastly, examination of other phosphorylation sites (e.g. Foxo3a$^{P413)}$), which are phosphorylated by AMPK and/or trigger FoxO activity, may furnish more information about FoxO status in *HSA*^{LR} muscle.

4. Discussion and Outlook

To date, the detailed mechanism underlying the multisystemic DM1 pathology is still not well understood and most investigations and therapeutic developments have focused on splicing defects caused by mRNA toxicity. In the course of my PhD studies, I investigated whether deregulation of key metabolic components may contribute to DM1 pathogenesis and may constitute novel potential therapeutic targets. I identified that DM1 pathology is associated with a perturbation of AMPK and mTORC1 signalling in skeletal muscle as well as with an impairment of the autophagic flux. Normalization of AMPK and/or mTORC1 pathway through pharmacological or dietary approaches resulted in improved muscle performance outcomes and in markedly decreased myotonia in HSA^{LR} mice. Of note, activation of AMPK signalling by the AMPK-agonist AICAR in HSA^{LR} muscle, led to a substantial reduction of myotonia, together with partial correction of *Clcn1* mis-splicing. These findings nicely illustrate the involvement of abnormal AMPK-mTORC1 signalling in DM1 muscle pathophysiology and point to AMPK-mTORC1 modulation as potential therapeutic strategy in the DM1 context.

4.1 Deregulation of central metabolic pathways in DM1 muscle

The proper interplay between the two protein kinases AMPK and mTORC1, that sense the energy and nutrient status of the cell, determines the balance between anabolic and catabolic processes and therefore plays a major role in order to maintain regular muscle function [33, 204]. The fact that deregulation of Akt/PKB-mTORC1 and AMPK pathways has been related to the pathogenesis of several muscle diseases together with different reports suggesting a potential alteration of Akt/PKB-mTORC1 signalling in human DM1 cells, patient biopsies as well as in DM1 mouse models, prompted us to investigate the signalling characteristics of these central metabolic actors in DM1 muscle [21, 22, 30, 31, 48-50, 52, 53]

4.1.1 Abnormal AMPK-mTORC1 status in HSA^{LR} skeletal muscle

I identified an impaired activation of AMPK in HSA^{LR} muscle upon energy deprivation. Even after prolonged starvation AMPK activity was still reduced in mutant mice

compared to control mice, suggesting that the response to energy deprivation might be deteriorated or delayed in *HSA*^{LR} muscle. We further analyzed the activity of mTORC1, an indirect downstream target of AMPK. The increased phosphorylation and cellular accumulation of mTORC1 downstream targets, including p70S6k and S6, strongly suggests augmented mTORC1 activity in *HSA*^{LR} skeletal muscle. However, the phosphorylation status of mTOR was unchanged. Moreover, 4E-BP1 levels showed no difference between mutant and control mice, which could be due to the diverging regulation of mTORC1 targets [205]. AMPK-dependent TSC2 phosphorylation was neither altered in *HSA*^{LR} muscle. Though, further experiments are required, such as analysis of raptor phosphorylation status, in order to determine whether mTORC1 is effectively deregulated.

Since DM1 patients display mis-splicing and abnormal protein trafficking of IR, an upstream regulator of the Akt/PKB-mTORC1 pathway, we suspected a connection between deregulated IR signalling and altered AMPK-mTORC1 activity in *HSA*^{LR} muscle [95, 127, 206]. However, an involvement of IR deregulation seems to be unlikely, as IR mis-splicing is not reproduced in *HSA*^{LR} mice. Previously, slight changes in the splicing of *Insr* were only reported in *soleus* muscle from aging mice of another DM1 mouse model [206]. Moreover, no major change in the levels and activation of Akt/PKB was detected comparing control and mutant mice, thus indicating that mTORC1 deregulation in *HSA*^{LR} mice is independent of the proposed Akt/PKB alteration [53, 127, 128].

DM1 patients commonly exhibit metabolic disturbances including high serum triglycerides and LDL (low-density lipoprotein) cholesterol levels, elevated body fat content and insulin resistance [207]. However, these global metabolic perturbations are most likely not arising in the muscle specific *HSA*^{LR} mouse model and are probably not involved in altering the central metabolic signalling in *HSA*^{LR} muscle tissue. Nevertheless, we cannot exclude that the muscle specific expression the (CTG)n repeat expansion in in *HSA*^{LR} mice triggers metabolic alterations that may contribute to whole body metabolic deregulation. Therefore further experiments remain to be conducted in order to investigate the contribution of diseased muscle to body-wide metabolic changes observed in DM1 patients.

4.1.2 Mechanisms involved in AMPK and mTORC1 deregulation

Dietary and pharmacological treatments normalize abnormal AMPK-mTORC1 signalling in HSA^{LR} mice

I tested prolonged starvation and different pharmacological compounds in order to assess their ability to normalize abnormal AMPK-mTORC1 signalling in HSA^{LR} muscle. Long-lasting food restriction was able to normalize mTORC1 but not AMPK signalling, suggesting that impaired AMPK activation is more affected than mTORC1 signalling in HSA^{LR} mice. In addition, mutant mice were protected from extensive weight loss compared to controls, which might be linked to the deregulation of metabolic signalling in HSA^{LR} muscle. Metformin, an anti-diabetic drug known to stimulate AMPK activity [208], tended to normalize AMPK status in muscle from starved HSA^{LR} mice, and further slightly ameliorated deviant mTORC1 activation in mutant muscle. AICAR, another AMPK activator, proved to be even more effective in muscle, with a high but transient phosphorylation of AMPK followed by a strong inhibition of mTORC1 activity. Rapamycin was as well sufficient to reverse mTORC1 activation, but did not restore AMPK status. Since AMPK was shown to be an upstream regulator of mTORC1 [209], one can hypothesize that AMPK deregulation is a primary event in HSA^{LR} mice and is responsible for the abnormal activation of mTORC1.

Potential mechanisms leading to primary AMPK deregulation in DM1 muscle

Protein levels of AMPK-activating kinases LKB1 (serine/threonine liver kinase B1) and TAK1 (transforming growth factor β-activated kinase-1) [210, 211] were unchanged in HSA^{LR} muscle. However, I could not yet determine whether levels of the active phosphorylated form of these kinases were altered in mutant muscle. Therefore we cannot exclude their potential involvement in the deregulation of AMPK. In order to further investigate the cause for impaired AMPK signalling, we will evaluate the activity status of the AMPK-upstream kinases LBK1, TAK1 and CAMKK2 (calcium/calmodulin-dependent protein kinase kinase 2) [212]. Moreover, measurement of AMP:ATP levels in muscle from control and mutant mice in fed and starved conditions will allow us to judge whether altered energetic status in HSA^{LR} muscle is the cause for blunted AMPK activation [213].

Of note, CaMKII that was reported to regulate AMPK activity [214-216], is composed of different isoforms, including CamkIIα CamkIIβ, CamkIIδ and CamkIIγ that are known to be abnormally spliced in DM1 context [156, 217, 218]. In fact, we

could confirm the decreased inclusion of exon 13 of *Camk2b* transcript in HSA^{LR} muscle [137, 217]. Additionally, CaMKII isoforms exhibited a severely altered pattern of expression and phosphorylation, suggesting potential alteration of the other isoforms. It has alternatively been suggested that CaMKII activates and inhibits AMPK, but the exact mechanism underlying the regulation of AMPK by CaMKII in skeletal muscle remains unknown [219-222]. Therefore, altered AMPK signalling in HSA^{LR} muscle may be related to abnormal CaMKII expression, although additional investigations need to be conducted. In particular, potentially altered splicing events in the different *Camk2* pre-mRNAs will be examined. Moreover, analyses of transfected DM1 cells expressing constitutively active isoforms of CAMKII as well as electroporation of HSA^{LR} muscle introducing active CAMKII isoforms, may allow us to evaluate their effect on AMPK signalling in HSA^{LR} muscle.

Previously, increased GSK3β levels were detected in HSA^{LR} mice, which could provide an alternative explanation for the altered AMPK-mTORC1 signalling [83]. GSK3β is implicated in the regulation of these pathways [223, 224], however it is not clear whether its deregulation could constitute the primary event for AMPK/mTORC1 imbalance, as GSK3β was shown to inhibit AMPK but also mTORC1 [223-226]. Moreover, AMPK and mTORC1 were also shown to regulate GSK3β activation making it difficult to render a statement about GSK3β impact on the altered signalling [227-229].

4.1.3 Augmented mTORC1 activity in human DM1 muscle?

In conformity with the results obtained in mice, muscle biopsies and MyoD-transduced fibroblasts from DM1 patients presented elevated activation of some mTORC1 targets. Nonetheless the *in vitro* energy deprived condition did not allow us to detect AMPK activation in control cells. Therefore, it is not feasible to make a meaningful statement about AMPK signalling in DM1 cells. Further adjustment of the protocols for *in vitro* experiments may help to conclude on the status of AMPK in human DM1 muscle cells. Moreover, analysis of additional biopsies with standardized nutritive status of the patients at the moment of the biopsy may also bring further insight on the involvement of AMPK-mTORC1 deregulation in the pathology. It is known that the HSA^{LR} mouse model does not reproduce all aspects of DM1 pathology, e.g. insulin receptor mis-

splicing, which may further impact on the state of these signalling in DM1 human muscle.

4.2 Abnormal activity of cellular degradation systems in DM1 context

Reduced Akt/PKB-mTORC1 signalling has been linked previously to abnormal autophagic flux in DM1 [52, 53, 129]. However, the dynamics of the autophagic flux in DM1 context have never been precisely investigated and generally increased autophagy was spuriously suggested from increased levels of different autophagy markers [31, 52, 53, 129]. At the same time, some reports have presumed that elevated autophagic flux contributes to muscle atrophy in DM1 [31, 52, 53].

4.2.1 Abnormal autophagic flux and proteasomal activity in HSA^{LR} muscle

Consistent with the observed AMPK-mTORC1 deregulation, being key regulators of autophagy, we detected impaired autophagic flux in HSA^{LR} muscle. By combining several approaches, we observed that both autophagy induction and the degradation steps are deregulated in mutant muscle. Fed HSA^{LR} mice displayed increased LC3II levels, pointing to limited degradations steps, however only in a moderate extent as there were no obvious aggregates of the autophagy and lysosomal markers p62 and LAMP-1 in muscle from young mutant mice [25, 230]. Upon energy deprivation, autophagy was not efficiently induced as the LC3I to LC3II switch was generally less pronounced in HSA^{LR} mice compared to controls. Colchicine treatment induced a major switch of LC3I to LC3II in fed and starved control mice. LC3II also accumulated in mutant mice after colchicine treatment, however the fold change of the LC3II/LC3I ratio was less pronounced in HSA^{LR} mice compared to control mice, further underlining the degradation defect in fed conditions as well as the induction defect in deprived conditions.

Although deregulation of AMPK and mTORC1 is likely to be involved in such autophagy impairment, alternative mechanisms may also contribute to these defects since the mTORC1-AMPK modulating agents, rapamycin and metformin, were not sufficient to normalize the flux in mutant muscle. In aged HSA^{LR} mice, histological alterations related to autophagy impairment became obvious, as vacuoles were

frequently observed in mutant muscle fibres. The abnormal lysosomal distribution present in 12 month-old but not in young mice supports the hypothesis that these histological features arise in aged animals in consequence of deregulated autophagy process.

In agreement with a previous study [22], we also detected an increase in some proteasomal enzymatic activities in HSA^{LR} muscle. It is unlikely that AMPK deregulation in mutant muscle contributes to elevated proteasomal activity as blunted AMPK would rather reduce the activity of FoxO transcription factors, which are central regulators of the proteasome [23]. Preliminary results suggest that FoxO signalling may be altered in mutant muscle, although further investigations, as mentioned in the part 3.2.2, are required to confirm this hypothesis. Since we did not detect major changes in the expression of atrogens and autophagy-related genes, one may argue however the signalling may not be responsible for the deregulation of the catabolic processes. Therefore, additional processes might as well participate in the deregulation of proteasome activity in mutant muscle. Examination of other signalling pathways involved in proteasome activity regulation may help to shed light into the mechanisms linked to proteasome activation in HSA^{LR} muscle. Moreover energy imbalance may impact on ATP-dependent proteasome activity, thus measurement of AMP:ATP levels in muscle of mutant and control mice may furnish supplemental information.

Together these data show that HSA^{LR} muscle exhibit a moderate deregulation of the protein degradation system. This includes a defective or delayed response to energy deprivation with impaired autophagy induction, restricted autophagy-related degradation and increased proteasomal degradation, which are altogether most likely contributing to the progressive muscle alterations seen in mutant muscle.

4.2.2 Deregulated autophagy in DM1 human muscle

In conformity with the results obtained in HSA^{LR} mice, we observed an abnormally regulated autophagy flux in DM1 human muscle cells. Energy restriction and blockage of the degradation steps by chloroquine treatment uncovered that there is a fundamental difference in autophagy flux comparing control and DM1 cells. These treatments were not able to induce any increase in LC3II levels in DM1 human muscle cells, illustrating a major defect in autophagy induction in response to energy deprivation. There was no indication for diminished autophagic degradation steps in DM1 cells, as the flux was

strongly blocked at the induction. Similar to *HSA*^{LR} muscle, few vacuolated fibres were observed in the muscle biopsy from one DM1 patient. Nevertheless, accumulation of p62, LC3 and ubiquitinated proteins, commonly observed in autophagy related muscle diseases, was absent in DM1 muscle indicating that deregulated autophagic flux might be involved but not central to DM1 muscle pathophysiology.

In summary, autophagy flux is likely perturbed in DM1 at the induction and degradation steps, although they seem to be altered at different degree *in vitro* in DM1 human muscle cells and *in vivo* in DM1 mice. It is difficult to judge whether the difference obtained between *HSA*^{LR} muscle and DM1 cells is due to *in vitro* vs. *in vivo* conditions, due to differential regulation of the autophagy flux in human *vs*. rodent, or due to specific disease-related deregulations, such as IR mis-splicing, which are not reproduced in the *HSA*^{LR} model and that may impact on autophagy.

4.3 Effect of pharmacological and dietary treatments targeting AMPK and mTORC1 signalling on DM1 muscle function

Pursuing the hypothesis that deregulated AMPK-mTORC1 signalling is of major importance for muscle pathogenesis in DM1, we assumed that normalization of these pathways might lead to an improvement of muscle function in *HSA*^{LR} mice. One of the most important results of this study is the identification of the deregulation of AMPK and mTORC1 activity, which may be of paramount importance in the alteration of muscle function in DM1. We demonstrate that normalization of AMPK and mTORC1 signalling is sufficient to improve muscle function via splicing-dependent and -independent mechanisms, hence opening new avenues regarding therapeutic options for the disease.

4.3.1 AICAR improves muscle performance in *HSA*^{LR} mice through splicing-dependent mechanisms

AICAR but not metformin significantly reduces myotonia through corrected Clcn1 splicing

The AMPK-activating drug metformin was our first choice in order to assess its effect on *HSA*^{LR} muscle function, as it is known to be a well-tolerated drug that is commonly used to treat type 2 diabetes patients [231]. However, metformin treatment had no

positive effect on muscle function in *HSA^{LR}* mice and did not correct DM1-associated mis-splicing in mutant muscle. By contrast, AICAR, the second AMPK-stimulating drug we tested, markedly reduced late relaxation time in young and old *HSA^{LR}* mice and completely abrogated myotonia in 8 month-old animals. At the same time we detected increased ClC-1 protein levels together with improved splicing of *Clcn1* mRNA. This is most likely the reason for the strong reduction of the late-relaxation time, as myotonia in DM1 is thought to be primarily caused by the mis-splicing and thereby deficiency of ClC-1 [82, 84, 100, 101]. Surprisingly, AICAR treatment had no corrective effect on other mis-spliced genes associated to DM1, like *Atp2a1* and *Camk2b* [85, 217]. Hence, it can be hypothesized, that blunted AMPK signalling might secondarily contribute to mis-splicing in DM1, but may impact specifically on some affected genes.

What are the mechanisms underlying AMPK-dependent correction of mis-splicing?

Considering the mechanism responsible for partial splicing correction in *HSA^{LR}* muscle, different options should be considered. First, AICAR itself might interact with the toxic mRNA, which may promote the release of RNA-binding proteins, such as MBNL1. Alternatively, AICAR and/or AMPK may influence actors that are involved in splicing or gene expression regulation. It was previously suggested that AMPK signalling is implicated in alternative splicing regulation [139, 232]. Several AMPK activating agents, such as berberine, curcumine and resveratrol have been reported to modulate splicing events [233-235]. It remains unclear by which mechanisms these compounds affect mRNA splicing and only a few splicing modulating factors were shown to be directly or indirectly regulated by AMPK [139, 232, 236].

In a study from Laustriat et al. (2015), AMPK activation was shown to correct the alternative splicing of DM1-affected genes *in vitro* [139]. AICAR-treated human DM1 myoblasts displayed restored mis-splicing of several gene transcripts, including *ATP2A1*. Metformin did as well revert splicing alterations of *ATP2A1* and corrected *CLCN1* mis-splicing in DM1 cells [139]. The authors further suggested that the beneficial effect of AMPK on mis-splicing was mediated by the RNA-binding protein RBM3 [139]. Both AICAR and metformin induced a reduction of *RBM3* gene expression, and silencing of *RBM3* expression entailed changes in alternative splicing of several gene transcripts [139]. In our study, we did not detect any changes in the transcriptional levels of *RBM3* in muscle from AICAR-treated *HSA^{LR}* mice. Hence, another mechanism that remains to be elucidated may be responsible for the splicing

correction of *Clcn1* transcripts triggered by AICAR and/or AMPK activation. Another study (PCT publication no.: WO 2011121109 A1) linked the corrective effect on splicing of AMPK activators, including metformin, to the modulation of the RNA-binding protein ELAVL1/HuR. AMPK activators may target importins, which enhance ELAVL1 nuclear import, going along with a depletion of ELAVL1 cytosolic levels and thereby trigger the restoration or amelioration of mis-splicing of different transcripts. At the same time ELAV1 overexpression aggravated DM1-associated splicing defects. However, we still need to verify this hypothesis by assessing ELAV1 localization and expression in mutant muscle after AICAR treatment.

Recent analysis of the nuclear foci formed by the aggregation of (CUG)n-expanded transcripts brought further insight on the mechanism of action of AICAR regarding the amelioration of *Clcn1* splicing. Indeed, by conducting fluorescence *in situ* hybridization with a fluorescent $(CAG)_{10}$ DNA probe on muscle sections, we observed a drastic decrease in the proportion of nuclei containing foci in mutant muscle from AICAR-treated mice, compared to untreated HSA^{LR} mice. This suggests that AMPK signalling may improve splicing by promoting the dissociation of the nuclear foci formed in DM1 muscle. Destabilization of the foci by perturbing RNA-binding proteins, such as hnRNP H, which is known to influence foci stability in DM1 and interacts with AMPK, may favour the release of MBNL1 and restore splicing [237, 238]. Although it is unlikely that AICAR restores MBNL1 distribution, as MBNL1-dependent *Camk2b* and *Atp2a1* mis-splicing [96, 137, 217] is not improved in muscle from AICAR-treated mutant mice, we will investigate if the levels and subcellular localization of MBNL1 are affected upon AICAR treatment. Moreover, as other alternative splicing events are likely corrected upon AICAR treatment, further splicing analyses in muscle from AICAR-treated mice may unveil potential candidates mediating the effect of AMPK on alternative splicing. Lastly, we will need to test another AMPK-activating compound, with a different mechanism of action compared to AICAR. This will allow us to judge whether the corrected splicing defect of *Clcn1* gene and the reduction of myotonia can be attributed to the stimulation of AMPK activity or if AICAR itself mediates muscle function amelioration.

AICAR versus metformin action in skeletal muscle

Regarding the incongruity between the results obtained in metformin- and AICAR-treated mice, it has to be mentioned that the mechanism of action of AICAR and

metformin is very different. After entering the cell, the adenosine kinase (AK) converts AICAR to ZMP (5-aminoimidazole-4-carboxamide-1-D-ribofuranosyl-5'-monophosphate), which acts as an AMP (adenosine monophosphate) analog, thus allosterically activating AMPK [239]. Metformin inhibits mitochondrial complex I, resulting in reduced ATP levels and finally in an accumulation of AMP, thereby activating AMPK [240]. Furthermore a wide spectrum of mechanistic effects of metformin has been recorded and the underlying molecular mechanisms are still incompletely understood. Metformin is toxic at high doses, severely impairs skeletal muscle oxidative capacity and is known to cause side effects like muscle pain and cramps and sometimes weakness in patients [241-244]. Additionally, high concentrations of metformin are required to acutely stimulate AMPK signalling in rodent muscle tissue, which may explain the absence of beneficial effect of the drug in our study [245].

The diverging molecular mechanisms of metformin and AICAR might be a reason for the differences comparing our results and those of Laustriat et al. (2015) [139]. AICAR and metformin action may vary between species and tissues, but these compounds could also be more efficient *in vitro* than *in vivo* [246]. This might explain why AICAR-treated human DM1 myoblasts displayed restored mis-splicing of *ATP2A1* and a down-regulation of *RBM3*, while we could not detect any of these changes in muscle from AICAR-treated HSA^{LR} mice [139]. Regarding the results for metformin, we assume that its impact was simply not strong enough to have an effect on splicing in muscle tissue as the activation state of AMPK and mTORC1 targets was not strongly and reproducibly changed in muscle from metformin-treated mice.

Importantly, the therapeutic potential of metformin is currently being studied in a clinical trial with DM1 patients (EudraCT number: 2013-001732-21). In view of our results and of its forecited side-effects in muscle, one may hypothesize that metformin might not be suitable as a therapeutic option, at least referring to muscle pathology in DM1. In contrast, AICAR could be very beneficial regarding muscle function in DM1 patients but it is also known to have serious side effects triggering injurious health risks, such as alteration of heart metabolism and function, thus minimizing the possibility that this compound may be applied in clinics [247]. Hence, dissecting the exact mechanism of action by which AICAR induces the substantial improvement of muscle function is of major importance and might open new avenues for developing pharmacological compounds to treat DM1 patients.

4.3.2 Rapamycin and low-protein diet reduce myotonia in *HSA^{LR}* mice

The last-mentioned results suggest a potential implication of AMPK deregulation in DM1 muscle dysfunction. However, during the course of the study, we had to consider that indirect mTORC1 inhibition by AICAR, mediates the effect of the drug on DM1 muscle function. Hence, we investigated whether direct modulation of mTORC1 activity has as well a positive effect in *HSA^{LR}* mice.

Rapamycin treatment reduces myotonia independently of mTORC1 inhibition and Clcn1 mis-splicing in HSA^{LR} mice

Rapamycin treatment resulted in a strong inhibition of mTORC1 and significantly reduced myotonia in *HSA^{LR}* mice. Of note, rapamycin had no effect on the mis-splicing of *Clcn1*, indicating that reduction of myotonia occurs independently of the restoration of ClC-1 activity. Therefore, alternative compensatory effects may contribute to the normalization of such pathophysiological defect. We had to consider that rapamycin additionally influences intracellular Ca^{2+} mobilization, and consequently muscle contraction and relaxation via its interaction with FKBP12 (FK506 binding protein) and the subsequent perturbation of RyR1 activity [175, 197, 248-250]. Therefore, we assessed myotonia in mice treated with the ATP-competitive mTOR inhibitor AZD8055 [251] and showed that, despite the efficient inhibition of mTORC1, the treatment did not decrease the late relaxation time of *HSA^{LR}* muscle. Thus, we conclude that the reduction of myotonia following rapamycin injections is caused by the drug itself and potentially by its effect on FKBP12, rather than due to the modulation of mTORC1 signalling.

Protein restriction diminishes myotonia through splicing-independent mechanisms in HSA^{LR} mice

We further demonstrate that submitting aging *HSA^{LR}* mice to low-protein diet (LPD) induces a significant reduction of DM1-associated myotonia. LPD led to body weight loss and corrected myotonia in 10 month-old animals, while it had no effect in young mice. It is well-known that protein restriction is more effective in older animals, as protein synthesis is blunted in their muscle when the quantity of protein-intake undercuts a certain limit, while in young mice a more drastic reduction of proteins is required to abate protein synthesis [252-254]. As the above-quoted results most likely exclude an involvement of mTORC1 deregulation in triggering myotonia, we suspect

that AMPK activation upon LPD may be responsible for the reduced relaxation time of mutant muscle. However, in contrast to AICAR, LPD did not correct the mis-regulated alternative splicing of *Clcn1* in HSA^{LR} muscle, indicating that reduction in myotonia through protein restriction occurs independently from restoration of ClC-1 activity. One can hypothesize that LPD is not sufficient to strongly induce activation of AMPK signalling, and the associated changes in *Clcn1* alternative splicing. Hence, alternative mechanisms may contribute to myotonia reduction upon LPD and may rely on other signalling targeted by protein restriction.

4.3.3 Alternative splicing-independent mechanisms involved in myotonia improvement

Regarding the effects of AICAR treatment and LPD, we have to consider that additional mechanisms apart from the correction of *Clcn1* mis-splicing promote the reduction of myotonia in HSA^{LR} mice. In particular, AMPK is involved in the regulation of various ion-channels and can influence the function of muscle-specific ion channels and pumps, like the potassium channel Kir2.1, the sodium channel $Na_V1.4$, or the calcium channel Orai1 (Calcium Release-Activated Calcium Modulator 1) [255-258]. These components are involved in the control of the resting membrane potential, the initiation and propagation of the action potential, as well as in membrane repolarization [255-258]. Modulation of the channels can thus affect the contraction and the relaxation of muscle fibres [255-258]. In accordance, different drugs such as sodium and calcium channel blockers exert an anti-myotonic effect in DM1 patients, without altering abnormal splicing [131, 132]. Hence, we can hypothesize that AMPK activation may regulate muscle relaxation in HSA^{LR} mice through its impact on various ion channels in skeletal muscle [255-258]. The effect of AMPK on myotonia may also be indirect and related to AMPK-dependent regulation of other signalling, such as GSK3β, whose modulation was proven to be beneficial for muscle function in HSA^{LR} mice [83, 227-229]. However the underlying mechanisms linking these signalling pathways to myotonia have, not yet been identified. Lastly, one can hypothesize that AMPK activation by AICAR indirectly improves muscle function in HSA^{LR} mice by modifying the contractile properties of the mutant muscle. In particular, reduction in myotonia may be achieved by a fast-to-slow fibre type transition, as this might be indicated by the normal relaxation time of soleus muscle in HSA^{LR} mice. Ljubicic et al. (2011) demonstrated that chronic AICAR-mediated AMPK activation triggers a fast-to-slow fibre type switch in muscle from *mdx*

mice [259]. It is well known that AMPK signalling stimulates SIRT1 (Sirtuin 1), ultimately leading to increased PGC1α (peroxisome proliferator-activated receptor γ coactivator 1α) expression, which has been shown to promote the slow myogenic program [260, 261]. To test this hypothesis, we recently conducted nicotinamide adenine dinucleotide (NADH) staining and immunostaining against the different types of myosin in order to identify potential changes in the metabolic and contractile properties of treated mutant muscle. However, after 7 days of AICAR treatment as well as after 10 days of rapamycin treatment, HSA^{LR} muscles did not display modifications of their oxidative capacity nor could we detect alterations in the proportion of the different fibre types. Similar experiments remain to be conducted for mutant mice submitted to LPD, to determine whether this treatment induced changes in the properties of the muscle and thus mediated reduction of myotonia.

4.3.4 Inhibition of mTORC1 activity partially enhances muscle force in HSA^{LR} mice

Concordant with the results obtained with AICAR and rapamycin treatments, we observed a significant increase in muscle strength in the animals that received AZD8055. Hence, it can be hypothesized that normalization of altered mTORC1 signalling induces force gain in DM1 muscle. The augmentation in muscle strength after AICAR treatment is consistent with the results obtained in a previous study showing force gain in *mdx* mice, following AICAR injections [262]. Decreased muscle degeneration, as shown in AICAR-treated *mdx* mice and in HSA^{LR} mice treated with different agents modulating GSK3β levels might account for improved muscle strength [83, 262]. However, we have to consider that these treatments were applied for 4 and 2 weeks respectively, whereas the AICAR-treatment we applied lasted only for 7 days. Therefore, it is questionable whether in this short time frame muscle regeneration is sufficiently advanced, so that it has an impact on muscle strength. mTORC1 may alternatively influence muscle force generation by increasing the half-time of Ca^{2+} decay, affecting RyR1 and the synthesis and/or the stability of $Ca_V1.1$, further influencing the frequency and amount of calcium releasing processes [248, 263, 264]. Notably, it has been reported that the expression of the embryonic $Ca_V1.1$ isoform, causing increased and harmful calcium influx, correlates with the degree of muscle weakness in DM1 patients and may contribute to DM1 muscle pathophysiology [104, 265, 266]. Hence one can speculate that inhibition of mTORC1 may improve muscle

function, by triggering a decrease in $Ca_V1.1$ protein levels subsequently modulating altered Ca^{2+} homeostasis in *HSA*^{LR} muscle [263].

In the short term, further investigations will be conducted to judge whether AICAR, rapamycin and AZD8055 augment muscle strength by promoting muscle regeneration and/or by inducing changes in Ca^{2+} handling in *HSA*^{LR} muscle. Moreover, the treatment duration might not have been long enough to obtain visible results in muscle, as we could not observe amelioration in muscle histology. Longer treatment periods will also be tested to evaluate the long-term potential of these drugs on DM1 muscle function.

5. Conclusion

During my PhD, I identified that deregulation of AMPK-mTORC1 signalling, in association with an impairment of the autophagic flux, may constitute key pathogenic mechanisms in DM1 disease. Autophagy seems to be moderately deregulated, but may contribute to muscle alterations in DM1. Approaches targeting AMPK and mTORC1 signalling considerably improved muscle function in a DM1 mouse model, hence underlining the implication of these central metabolic pathways in the disease. Blunted AMPK activity may worsen the myotonic phenotype through splicing-dependent and -independent mechanisms, and deregulated mTORC1 signalling may accentuate muscle weakness in DM1.

This study raises further questions and paves the way for future investigations. As skeletal muscle consists of the biggest organ in human it should be considered that the identified defective metabolic signalling may influence the whole body homeostasis and could greatly affect different organ function. In addition, non-muscle tissues might also be affected by similar signalling deregulation in DM1 patients, which may contribute to the multisystemic symptoms observed in the disease. Another interesting, open question may concern the involvement of mitochondrial defect, and thus energy management in DM1 disease, in conjunction with impaired AMPK activity and its potential impact on PGC1α [267]. The effect of CaMKII deficiency, beside its potential involvement in metabolic signalling imbalance on DM1 muscle, also constitutes an interesting research topic. Indeed, with regard to the role of CaMKII in the maintenance and the plasticity of NMJs, its deregulation in DM1 muscle might perturb NMJ function and thereby may contribute to muscle alterations in the disease [268].

In conclusion, the results obtained during my PhD may help to further understand the mechanisms underlying the multisystemic DM1 pathology. This work illustrates that deregulated cellular processes and signalling pathways are of major importance for the DM1-related muscle disease. Hence, we bring new insight to an aspect of DM1 pathogenesis, which did not attract too much interest in the past, but may account for central disease features, like myotonia and muscle weakness. At the same time, this work indicates the participation of novel unknown mechanisms in DM1 pathology and opens new research prospects. Lastly, we furnish alternative approaches in addition to the targeting of toxic transcripts that may provide a basis to develop novel therapeutic strategies to treat muscle pathophysiology in DM1 patients.

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