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**Strategie terapeutyczne w dystrofii mięśniowej Duchenne’a:
badanie kompensacyjnej roli DP71 we wczesnych stadiach
rozwoju mięśni**

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**Therapeutic Approaches for Treatment of Duchenne Muscular
Dystrophy: Exploring the Compensatory Role of DP71 in the
Early Stages of Muscle Development**

Doctoral dissertation supervised by
dr hab. Patryk Konieczny, prof. UAM

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Streszczenie

Dystrofia mięśniowa Duchenne’a (DMD) to ciężka choroba prowadząca do zaniku mięśni, dotykająca 1 na 5000 chłopców i prowadząca do przedwczesnej śmierci około 30. roku życia. Choroba ta spowodowana jest mutacjami w genie *DMD*, prowadzącymi do utraty pełnej izoformy dystrofiny — DP427. U około 10% pacjentów brak jest także występującej w mioblastach izoformy DP71, co wiąże się z cięższym fenotypem choroby. Utrofina UP395, paralog dystrofiny DP427, której ekspresja dominuje w życiu płodowym, może częściowo kompensować brak DP427, natomiast funkcja DP71 w dojrzałej tkance mięśniowej wciąż nie została dokładnie poznana. Nasze badania koncentrowały się na wyjaśnieniu ograniczeń obecnych strategii terapeutycznych opartych na dystrofynie i utrofinie, określeniu ról DP427 i DP71 w proliferacji komórek oraz różnicujących włóknach mięśniowych, ocenie potencjału kompensacyjnego DP71 w mięśniach pozbawionych DP427, a także analizie terapeutycznego znaczenia różnych wariantów splicingowych DP71.

Zidentyfikowaliśmy regiony utrofiny ograniczające jej funkcję kompensacyjną oraz fragmenty dystrofiny, które mogą przyczyniać się do wywoływania odpowiedzi immunologicznej w terapiach DMD. Wykorzystując modele DMD, wykazaliśmy, że brak DP427 we włóknach mięśniowych oraz brak DP71 w proliferujących mioblastach prowadzą do podobnego fenotypu. Dodatkowo, nasze badania pokazały wzrost DP71 w komórkach pozbawionych DP427 oraz rolę kompensacyjną DP71 we wczesnym różnicowaniu mięśni poprzez poprawę przeżywalności komórek, redukcję ROS oraz napływu wapnia. Zbadaliśmy także wpływ różnych wariantów splicingowych DP71, ujawniając różnice w ich stabilności, lokalizacji wewnątrzkomórkowej i zdolności do przywracania funkcji komórkowych — niektóre z nich powodowały wzrost poziomu ROS w określonych przedziałach komórkowych.

Podsumowując, wykazaliśmy znaczenie specyficznych domen dystrofiny i utrofiny zarówno dla skuteczności terapii, jak i dla kontroli odpowiedzi immunologicznej. Nasze analizy podkreśliły podobieństwa oraz różnice funkcjonalne DP427 i DP71, dostarczając nowych informacji na temat potencjalnej roli DP71 jako czynnika terapeutycznego we wczesnych etapach różnicowania się mięśni pozbawionych dystrofiny.

Słowa klucz: dystrofina, utrofina, mięśnie, terapie DMD, warianty splicingowe

Summary

Duchenne muscular dystrophy (DMD) is a severe muscle-wasting disorder affecting 1 in 5,000 boys, leading to premature death around age 30. It is caused by mutations in the *DMD* gene, resulting in the loss of the full-length dystrophin isoform DP427. In about 10% of cases, a short DP71 isoform, synthesized ubiquitously in myoblasts, is also absent, and these patients tend to show more severe phenotype. The DP427 paralog, utrophin UP395, generated during fetal muscle development, can partially compensate for DP427 deficiency in patients. In contrast, the roles of DP71 in control and DMD muscle cells are not known. Our research focused on explaining limitations of current dystrophin- and utrophin-based therapeutic approaches in DMD, identifying the roles of DP427 and DP71 in cell proliferation and myofiber differentiation, determining compensatory potential of DP71 in DP427-deficient muscle fibers, and evaluating therapeutic roles of various DP71 splice variants.

We analyzed key dystrophin and utrophin domains for DMD treatment, identifying regions in utrophin that limit its compensatory function and potential immune triggers in dystrophin-based therapies. Next, using various DMD models, we found that loss of DP427 in myofibers and DP71 in proliferating myoblasts led to similar cellular defects and apoptosis. Further investigation showed that DP71 can compensate for DP427 loss during the early stage of myofiber differentiation, with its synthesis increasing in cells lacking DP427. This upregulation reduced ROS, calcium influx, and improved cell viability. We also explored the impact of different DP71 splice variants, revealing variations in their stability, localization, and ability to restore cellular functions, though some increased ROS levels in specific compartments.

In summary, we demonstrated the significance of specific dystrophin and utrophin domains in both treatment efficacy and immune response regulation. Our analysis also highlighted similar and distinct functions of DP427 and DP71, providing new insights into the therapeutic potential of DP71 during the early stages of muscle fibers lacking all dystrophins.

Keywords: dystrophin, utrophin, muscles, DMD therapies, splicing variants

List of abbreviations

ABD1/2	Actin-binding domain 1/2
CT	C-terminal end
CR	Cysteine-rich domain
BMD	Becker Muscular Dystrophy
DMD	Duchenne Muscular Dystrophy
<i>DMD</i>	Dystrophin gene
DGC	Dystrophin-Glycoprotein Complex
FDA	Food and Drug Administration
H1/3	Hinge 1, Hinge 3
MARK2	Microtubule Affinity Regulating Kinase 2
nNOS	neuronal Nitric Oxide Synthase
NT	N-terminal end
<i>UTRN</i>	Utrophin gene
UGC	Utrophin-Glycoprotein Complex
PAX7	Paired Box 7 (PAX7)
ROS	Reactive Oxygen Species
WT	Wild Type
μ Dys	Micro-Dystrophin
μ Utr	Micro-Utrophin

Chapter 1: Introduction and objectives of the study

Duchenne muscular dystrophy (DMD; OMIM 310200) is a severe and progressive muscle-wasting disease that primarily affects boys, occurring in approximately 1 in 5,000 live male births ¹⁻³. It is caused by mutations within the X-chromosome-linked *DMD* gene, which abolish the production of dystrophin proteins ^{4,5}. Apart from skeletal muscles, the disease affects the cardiac and smooth muscles, leading to cardiomyopathy and respiratory complications, which are major causes of morbidity and mortality in DMD patients ⁶. Additionally, some individuals may exhibit cognitive and behavioural impairments due to the absence of dystrophin in the central nervous system ^{7,8}. The first symptoms of DMD typically appear around the age of two when boys exhibit Gowers' sign, characterized by difficulty rising from the ground. An accumulation of fat tissue becomes evident, often seen as enlarged calves (pseudohypertrophy). By around the age of 10-12, affected individuals often require the use of a wheelchair. As the condition progresses, the heart and lungs are increasingly impacted, leading to respiratory and cardiac failure, which typically results in premature death between the ages of 20 and 40 ¹. While all DMD patients lack the full-length DP427 isoform, some may retain the expression of shorter dystrophins. However, some patients are also devoid of these shorter isoforms, which can influence disease severity, leading to more pronounced muscle degradation, neurological impairments, and cognitive deficits ^{9,10}.

Dystrophins are predominantly found in several tissues, exhibiting a tissue-specific and developmentally regulated expression pattern. The *DMD* gene, through its diverse promoters, produces several dystrophin isoforms. The longest isoforms DP427 are regulated by three promoters and are expressed mainly in skeletal muscle (DP427M), brain (DP427B), and Purkinje cells (DP427P). Other isoforms include DP260 (regulated by a promoter in intron 30) found in the retina, DP140 (promoter in intron 45) synthesized in the brain and kidney; DP116 (promoter in intron 56) present predominantly in Schwann cells, and also in the heart, and

DP71/DP40 (promoter in intron 63) generated ubiquitously across multiple tissues (Fig. 1)^{11,12}.

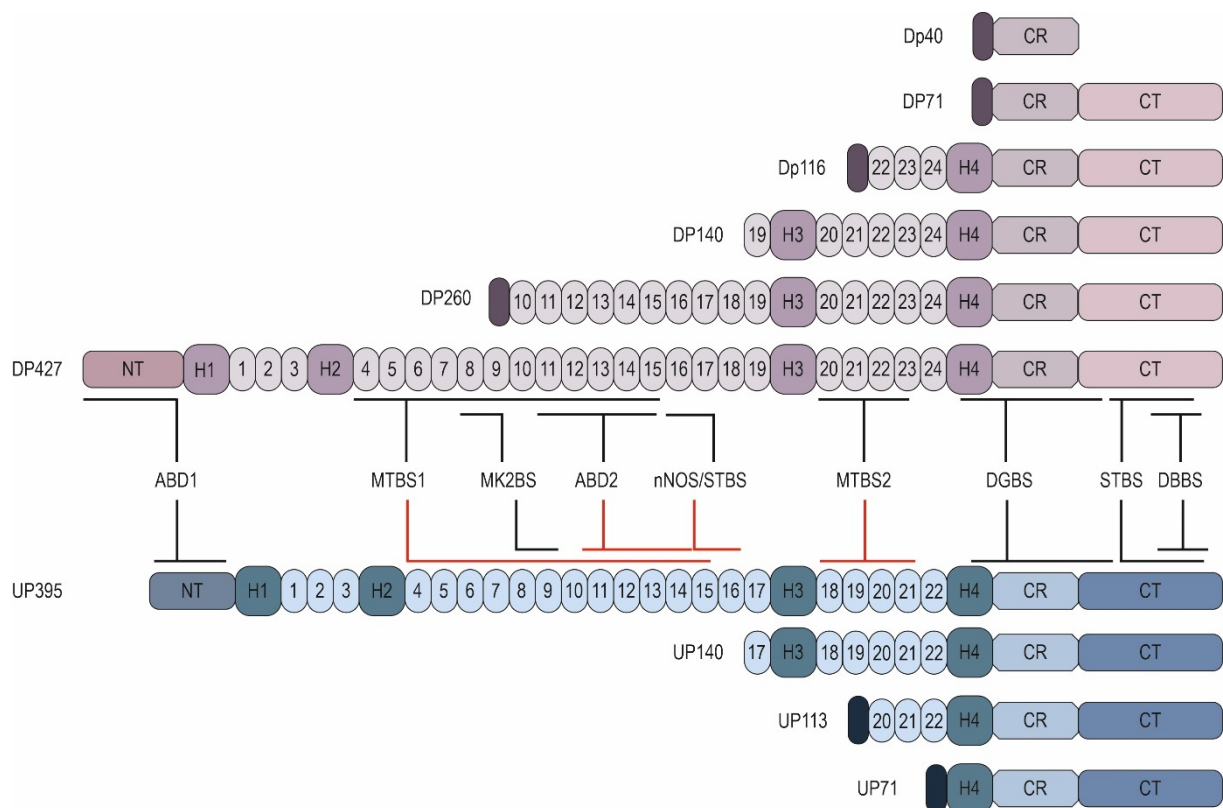


Figure 1 Schematic representation of dystrophin and utrophin with their major isoforms and domain organization

Both the full length DP427 dystrophin (pink) and UP395 utrophin (blue) have four main regions: the N-terminal domain (NT), the central rod domain composed of four hinges (H1–H4) and 24/22 spectrin repeats, the cysteine-rich domain (CR), and the C-terminal domain (CT). Red lines note that the binding properties of dystrophin are not retained in utrophin.

Most DMD cases are caused by deletions of one or more exons within the *DMD* gene, with two primary hotspot regions: 3–9 exons and 45–55 exons, with 7% and 47% of all *DMD* mutations, respectively^{1,13–15}. These mutations lead to loss of the longest isoforms, allowing for the expression of shorter ones. Reading frame–preserving mutations in the *DMD* gene typically produce truncated but partially functional dystrophin proteins, leading to the milder clinical presentation seen in Becker muscular dystrophy (BMD, OMIM 300376)¹⁶. BMD progresses more slowly, with later onset and less severe muscle weakness compared to DMD. Clinical observations reveal that patients lacking all dystrophin isoforms exhibit more severe

neurological symptoms and muscle pathology compared to those missing only the DP427^{17,18}. Patients lacking all dystrophin isoforms, including DP71, exhibit more severe cognitive impairments and lower IQ scores compared to those missing only DP427 or both DP427 and DP140^{19,20}. These findings underscore the crucial role of shorter isoforms in both muscle and brain function in DMD pathology. These results highlight the importance of shorter isoforms in DMD pathology.

The full-length 427 kDa dystrophin isoform, DP427, is a cytoskeletal protein that plays a critical role in maintaining muscle function⁵. It is a key component of the dystrophin-glycoprotein complex (DGC), which localizes to the muscle cell membrane, connecting the intracellular cytoskeleton to the extracellular matrix^{21,22}. This connection stabilizes the plasma membrane during muscle contraction, protecting it from contraction-induced damage. In addition to its structural role, the DGC also transmits extracellular signals regulating various signaling pathways²³. The loss of DP427 disrupts the assembly of the DGC complex, leading to a loss of cellular structural integrity, impaired contraction, and inhibition of several signaling pathways, ultimately resulting in muscle damage^{23,24}. In healthy muscle, damage triggers the activation of satellite cells, with the Paired Box 7 (PAX7), protein serving as a key marker of their activity²⁵. Research indicates that DP427 is involved not only in stabilizing the sarcolemma in muscle fibers but also in the activation, proliferation, and differentiation of satellite cells. These processes are crucial for muscle repair and regeneration^{23,26,27}. Over time, the extent of muscle damage surpasses the capacity of satellite cells to produce sufficient new myoblasts for muscle regeneration, leading to the replacement of muscle cells with fibrosis and fat tissue²². This suggests that the muscle phenotype in DMD results from the cumulative effects of two phenomena regulated by DP427: 1) the degeneration of muscle fibers due to the absence of the DGC complex on the sarcolemma, and 2) the lack of dystrophin in activated satellite cells, which leads to inefficient regeneration.

Data obtained from DMD patients, and the disease mouse models indicate that not only DP427 but also DP71 plays important roles in the function of skeletal muscles. Unlike the full-length DP427, DP71 lacks the major muscle-specific functions such as the ability to bind actin, microtubule, neuronal nitric oxide synthase (nNOS) and Microtubule Affinity Regulating Kinase 2 (MARK2) ⁴⁸. Despite it, DP71 can still play an important role in maintaining cellular integrity in other tissues. It is worth noting that there are several DP71 splicing variants, which leads to the generation of 14 distinct splice variants, which can be categorized into four main four distinct groups ^{28,29}. The first group, containing exon 78, comprises: DP71 (includes all exons), Dp71 Δ 71 (also known as DP71a), DP71 Δ 74, Dp71 Δ 71,74 (also known as DP71c), DP71 Δ 71,73-74 and DP71 Δ 71-74; the second, without exon 78 and an alternative reading frame of exon 79, resulting in a more hydrophobic C-terminal protein sequence, encompasses: DP71 Δ 78 (also known as DP71b), DP71 Δ 71 Δ 78 (also known as DP71ab), DP71 Δ 71 Δ 73 Δ 78, DP71 Δ 74 Δ 78 and DP71 Δ 71-74 Δ 78; the third group, with a retained part of intron 77 and no exons 78 and 79: DP71 Δ 71i77 and DP71 Δ 71-74i77, found in non-muscle rat tissues and cell lines; and the fourth, containing the splice variant DP71 Δ 68-76 Δ 78 Δ 79, detected in glioblastoma cells ^{28,30}. Recent studies suggest that DP71 regulates cell proliferation, particularly in the nervous system, where it contributes to neuronal stability and function ^{28,30}. The effects of DP71 on cell proliferation vary by isoform, with some variants involved in cell cycle progression and tissue repair. While DP71 interacts with other proteins to stabilize cellular structures and facilitate signaling in neuronal cells, its role in muscle regeneration and satellite cell function is less understood. In DMD, the absence of DP71 may impair regenerative capacity in both muscle and nervous tissues ^{31,32}.

Studies have shown that mice lacking all *Dmd* isoforms experience more severe abnormalities, including increased calcium salt deposition in muscle fibers and fibrosis, leading to an enhanced immune response characterized by macrophage infiltration, compared to those

missing only the DP427 isoform ³³. DMD patients are significantly shorter than individuals with BMD, with short stature being particularly prevalent in those with DP71 mutations ¹⁰. Additionally, DMD boys lack DP140, and those with combined DP140/DP71 deficiencies display worse motor function, highlighting the role of DP71 in muscles ³⁴.

An important aspect of DMD pathophysiology is the role of utrophin, an autosomal paralog of dystrophin ^{35,36}. Utrophin is a protein with structural and functional similarities to dystrophin, including its ability to assemble into a large transmembrane utrophin-glycoprotein complex (UGC) and bind actin filaments ^{37,38}. During fetal development and the early stages of tissue formation, the utrophin gene is predominantly expressed but is gradually replaced by dystrophin as tissue matures ^{39,40}. However, throughout life, utrophin synthesis persists in specific regions, such as neuromuscular and myotendinous junctions ⁴⁰. The utrophin gene (*UTRN*), similarly to the *DMD*, has several promoters that produce shorter isoforms: UP140, UP113 (also known as Utrophin-G), and UP71 ^{11,38} (Fig. 1). In the absence of dystrophin, utrophin is upregulated, partially replacing dystrophin's functionality ⁴¹ as shown in studies on DP427 and utrophin-deficient mice ⁴², and in experiments enhancing utrophin gene synthesis in dystrophin-deficient conditions ⁴³. However, despite the high structural similarity between dystrophin and utrophin, utrophin cannot fully compensate for the function of dystrophin. Some of these differences were indicated to result from the loss of utrophin's ability to interact with microtubules and proteins such as nNOS ³⁸. Currently, there is no highly effective therapy for Duchenne muscular dystrophy (DMD) that works universally for all patients and all types of mutations. The standard approach to managing DMD focuses on alleviating symptoms through

physiotherapy and the use of corticosteroids, such as Emflaza (deflazacort), a pro-drug approved by the Food and Drug Administration (FDA)^{38,44}.

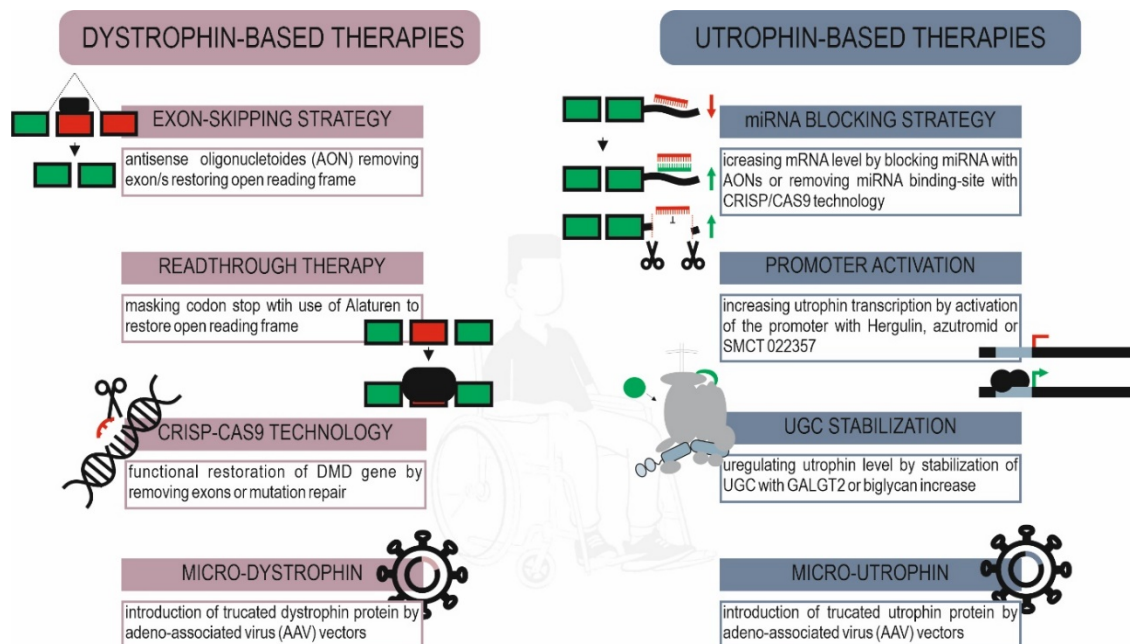


Figure 2 Schematic representation of dystrophin- and utrophin-based therapies

Dystrophin-based therapies focus on dystrophin restoration by exon skipping strategy, CRISP-Cas9 technology, and reading through therapy or by introducing new truncated, but functional micro-dystrophin. Utrophin-based therapies are designed based on mRNA stabilization by blocking microRNA (miRNA), increasing RNA transcripts by promoter activation, stabilizing protein through utrophin-glycoprotein complex (UGC) or introducing to the cell micro-utrophin protein.

Decades of research on different therapeutic approaches have resulted in the generation of several therapeutics for treating DMD (Fig. 2). These fall into two main categories, based on restoring dystrophin protein synthesis or utrophin upregulation. The first group leads to dystrophin restoration by correcting the reading frame using AONs⁴⁵, and introducing recombinant adeno-associated viral (rAAV) vectors carrying coding sequences encoding shorter but still functional micro-dystrophin (μ Dys) forms or using CRISPR-Cas9 technology to repair *DMD*⁴⁶. Although their efficacy is still questionable, clinical trials with improved chemistry (AONs) and sequences (AAVs) are currently ongoing⁴⁷. The second group focuses on increasing utrophin levels through promoter modulation, mRNA stabilization by blocking miRNA with AONs⁴⁸, or stabilizing UGC using biglycan⁴⁹ or GALGT2⁵⁰, and introducing

rAAV vectors encoding micro-utrophin (μ Utr) (Fig. 2)^{38,51}. Over the years some strategies have been dropped due to their limited efficacy. These include Ataluren/Tranlarna (PTC124), a molecule aimed at bypassing premature stop codons in the dystrophin gene to restore dystrophin protein expression, which was discontinued due to limited efficacy. Despite initial hope, later clinical trials failed to show significant improvements in key measures, such as walking ability, leading to the drug's withdrawal from DMD treatment options (EMA statement, January 2024)⁶³. Similarly in the case of utrophin-based approaches, were Ezotrumid (SMT C1100) was developed to increase the *UTRN* gene expression in DMD patients by targeting the utrophin gene's promoter region (Fig. 2). Although it showed some promise in preclinical studies, clinical trials did not demonstrate significant benefits in terms of muscle function or disease progression. The drug was discontinued due to its lack of sufficient efficacy and challenges in clinical outcomes^{52,53}.

The goal of this thesis was to expand our knowledge of the advantages and disadvantages of DMD therapies, with a focus on the comparative analysis of dystrophin and utrophin proteins. We extended our analysis to broaden our understanding of the functions of dystrophin isoforms DP427 and DP71. Since DP71 is one of the major isoforms in proliferating muscle cells and is present in most DMD cases, we directed our research toward understanding its compensatory properties in DMD models.

The following research hypotheses were addressed in this thesis:

1. The sequential and structural similarity between dystrophin and utrophin is critical for maintaining their functional roles and minimizing immune responses, which is essential for effective therapy management.
2. DP427 and DP71 are key regulators of muscle cell function, proliferation, and viability, primarily through their roles in maintaining the stability of the cell membrane, cytoskeleton, and mitochondria.
3. DP71 variants compensate for the lack of dystrophin and utrophin in DP427-deprived DMD cell models.

Chapter 2: Dystrophin- and Utrophin-Based Therapeutic Approaches for Treatment of Duchenne Muscular Dystrophy: A Comparative Review.

Szwec S, Kapłucha Z, Chamberlain JS, Konieczny P. *Dystrophin- and Utrophin-Based Therapeutic Approaches for Treatment of Duchenne Muscular Dystrophy: A Comparative Review.* BioDrugs. 2024 Jan;38(1):95-119. doi: 10.1007/s40259-023-00632-3. Epub 2023 Nov 2. Erratum in: BioDrugs. 2025 Jan;39(1):167. doi: 10.1007/s40259-024-00693-y. PMID: 37917377; PMCID: PMC10789850.

<https://link.springer.com/article/10.1007/s40259-023-00632-3>

Chapter 3: Dystrophin DP71, and DP427 isoforms determine cell viability during proliferation and myofiber differentiation

Szwec S, Durska A, Kościelniak-Wawro K, Chamberlain JS, Ostrovskyy O, Dominiak K, Konieczny P. *Dystrophin DP71, and DP427 isoforms determine cell viability during proliferation and myofiber differentiation* (manuscript submitted in 2025)

Dystrophins DP71 and DP427 determine cell viability during proliferation and myofiber differentiation

Running title: Dystrophins in cell proliferation and differentiation

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Key words: Duchenne muscular dystrophy, dystrophin DP71, dystrophin DP427, utrophin, muscle regeneration, muscle stem cells

ABSTRACT

Duchenne muscular dystrophy (DMD) is an X-linked disease caused by mutations in the dystrophin gene. DMD manifests with progressive skeletal muscle wasting, cardiac dysfunction, and cognitive and neuropsychiatric symptoms. As the disease progresses, affected boys lose their ability to walk and die prematurely. All DMD patients lack the full-length DP427 dystrophin, whereas approximately 10% lose all dystrophins, including DP71, synthesized in various cell types, including myoblasts. There is evidence that the cognitive symptoms of DMD patients vary depending on the location of the mutation site and the number of missing dystrophins, and there is some indication that this may also be the case in skeletal muscle. We therefore investigated the roles of DP427 and DP71 during cell proliferation and fiber differentiation. We included paralogous utrophins in our analyses, particularly the UP395 isoform, due to its presence in muscle and its partial ability to compensate for the lack of DP427. We demonstrate that DP71 and DP427 play important roles in cell viability during cell proliferation and fiber differentiation, respectively. Despite their different expression patterns, the absence of DP71 and DP427 resulted in a similar phenotype, including increased membrane permeability, mitochondrial aggregation, elevated ROS, and increased cyto- and genotoxicity. The phenotype occurred independently of myofiber contraction, dysfunction of neuromuscular or myotendinous junctions, or other cell types. We further show that utrophin UP395 can partially compensate not only for DP427 but also for DP71. These results explain the observed differences in disease severity among DMD patients and suggest that individuals deficient in both DP71 and DP427 may require a different therapeutic approach than patients deficient in only DP427.

INTRODUCTION

In the mid-19th century, the French neurologist Guillaume B.A. Duchenne de Boulogne identified a distinct type of muscular dystrophy that predominantly affected young boys. The disease named after him, Duchenne muscular dystrophy (DMD), affects approximately 1 in 5000 live male births and is not only one of the most severe forms of dystrophy but also the most common neuromuscular genetic disorder ¹. DMD manifests predominantly in progressive skeletal muscle wasting, respiratory difficulties, and cardiac dysfunction; however, it also affects smooth muscles, the nervous system and bones ². As the disease progresses, the affected boys lose their ambulation by the age of 13 and die prematurely in their 30s from heart or respiratory failure.

In the late 1980s, the dystrophin (*DMD*) gene was cloned, and DMD was characterized as a loss-of-function disease, in which the full-length 427 kDa dystrophin (DP427) protein was absent from muscle samples ³. At the same time, it became clear that mildly affected Becker muscular dystrophy (BMD) patients also suffer from mutations in the *DMD* gene, but unlike DMD patients, their biopsies retained some dystrophin reactivity. Indeed, consistent with the reading frame hypothesis, DMD patients typically have out-of-frame mutations that lead to premature stop codons and loss of dystrophin mRNA, whereas in BMD patients, in-frame mutations result in translation of shorter and partially functional dystrophins, a property utilized for developing drugs for boys with DMD ². Available treatments include antisense oligonucleotides and a gene therapy treatment designed to generate partially functional truncated/micro-dystrophins similar to BMD.

The phenotype of DMD patients varies depending on the site of mutation ². All DMD patients have no functional DP427; however, most of them lack one or more shorter dystrophins. For example, among the 181 patients tested by Yamamoto *et al.*, approximately 75% of individuals had mutations predicted to cancel the translation of DP260; 64% of DP260 and DP140; 12% of DP260, DP140 and DP116; and 7% of DP260, DP140, DP116 and DP71 ⁴. Dystrophin isoforms are synthesized from 7 promoters within the *DMD* gene in a tissue- and time-dependent manner ⁵, and there is also an alternative polyadenylation site, which results in the generation of the shortest dystrophin, DP40. Another level of dystrophin diversity is based on the alternative splicing of dystrophin transcripts, which can determine the cellular localization and function of the generated proteins ^{6,7}. One of the three full-length dystrophin proteins, DP427M, is translated in muscles, particularly in skeletal and cardiac

muscles, where it assembles the membrane-localized dystrophin-glycoprotein complex (DGC), which is crucial for transmitting forces during contraction and signal transduction. The remaining two long isoforms, DP427C and DP427P, are present in neurons of the cortex and cerebral Purkinje cells, respectively. The shorter dystrophin variant DP260 is present in the retina; DP140 and DP40 are typical of the brain; DP116 occurs in Schwann cells, striated muscle and fibroblasts; and DP71 is ubiquitously synthesized throughout the body ^{2, 5}. While DP427 can interact with many proteins, including actin, microtubules, nNOS, syntrophins or β -dystroglycan, shorter isoforms lose some of their binding capacity ⁸, but retain the ability to assemble the DGC ^{9, 10, 11}.

The dystrophin paralog utrophin has gained significant attention in the field of DMD therapy because of its increased synthesis in dystrophic myofibers and its potential to partially compensate for the lack of dystrophin ¹² without inducing an immune response ^{8, 13, 14}. Current results indicate that, similar to the *DMD* gene, the utrophin (*UTRN*) gene is also transcribed from various promoters, giving rise to full-length utrophin (UP395) as well as shorter UP140, UP113 and UP71 isoforms, the synthesis of which is often complementary to that of dystrophin. DP71, DP116, DP427 and UP395 are reportedly present at various stages of myofiber differentiation ^{4, 15, 16, 17, 18}. DP71 and UP395 are considered to be more fetal isoforms, whereas DP427 is regarded as the adult isoform, the absence of which leads to fiber degeneration ⁸. Nevertheless, the role of these dystrophin/utrophin isoforms at different stages of fiber differentiation is unclear and may be overshadowed by their mutual and compensatory functions.

Despite differences in mechanical properties and inability to bind proteins such as nNOS, UP395 interacts with actin filaments and assembles the utrophin-glycoprotein complex (UGC) ^{8, 19}. This dual activity is sufficient to partially prevent contraction-induced fiber damage and ameliorate muscular dystrophy in animal models of DMD, as also demonstrated in gene therapy treatments using recombinant AAV vectors carrying shortened dystrophin and utrophin sequences encoding micro-dystrophin and micro-utrophin proteins ^{13, 14, 20}. In contrast, DP71 synthesis in myofibers from a transgene in mice lacking DP427 (*mdx* mice) was shown not to be beneficial, even though the DGC was assembled ^{9, 10}. However, other data indicate that DP71 might have important functions during cell proliferation ^{6, 7}, whereas the absence of DP427 is thought to result in the myofiber death because of altered mechanical properties of the sarcolemma, increased reactive oxygen species generation, and overload of mitochondria with calcium ^{2, 21}. There are also data showing that the additional absence of

utrophin in *mdx* (*mdx/Utrn*^{-/-}; dko) mice worsens the mitochondrial phenotype in the muscle ²², possibly due to the lack of compensatory functions.

In the present work, we aimed to decipher the role of dystrophin and utrophin during cell proliferation and myofiber differentiation/regeneration. In particular, we aimed to 1) characterize the expression patterns of the *DMD* and *UTRN* genes at the RNA and protein levels, 2) assess compensatory expression of the *UTRN* gene in the absence of dystrophin, 3) evaluate the distinct and compensatory functions of various dystrophin and utrophin isoforms and 4) delineate the order in which pathologies occur in dystrophin and dystrophin/utrophin deficiency during myofiber differentiation.

METHODS

Mice

Age- and sex-matched wt (C57/Bl6) and *mdx4cv/Utrn^{-/-}* (dko ²³) 8-10-week-old mice were used throughout the study. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Washington.

Notexin (NTX) and BrdU injections

The EDL muscles were surgically exposed and injected with 100 μ l of 1.0 μ g/ml NTX as described previously ²⁴. A total of 750 μ g of BrdU (250 μ l; Sigma-Aldrich) was administered subcutaneously.

Cryosections and single-fiber cultures

Dissected muscles were frozen in cryomolds in liquid nitrogen-cooled isopentane and sectioned. Single muscle fibers were isolated from collagenase-treated EDL muscles, plated in the well of a Matrigel-coated chamber slide (NUNC, 177445) and incubated as previously described ²⁵. The media were supplemented with 1% penicillin/streptomycin solution.

Cell lines

The C2C12 and HeLa cell lines were obtained from ATCC, while the HSkM and HEK293 cell lines were obtained from Thermo Fisher. C2C12, HEK293, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Capricorn Scientific, DMEM-HA or Biowest, LO102-500) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich, Gibco) and 1% antibiotic antimycotic reagent (AA; Sigma Aldrich). HSkM cells were cultured in Nutrient Mixture F-10 Ham Medium supplemented with 20% FBS, 10 μ g/ μ l epidermal growth factor (EGF), 1 mg/ μ l dexamethasone, and 1% AA. For differentiation, C2C12 and HSkM cells were seeded to high confluence (> 80%) and then cultured in DMEM-based starvation medium (Capricorn Scientific, DMEM-HA) supplemented with 2% horse serum (Sigma-Aldrich) and 1% AA (Sigma Aldrich). C2C12 and HSkM cells were seeded on cell culture plates covered with 0.1% gelatin (Sigma Aldrich or Thermo Fisher). All the cells were grown at 37°C in a humidified incubator containing 5% CO₂.

siRNA transfection

Cell transfection with Silence Select siRNA (Thermo Fisher) was performed with Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher) according to the manufacturer's protocol. The final concentration of each siRNA was 25 nM (for double siRNA transfection, the final concentration of negative control siRNA was 50 nM). For the experiments involving proliferating cells, the material was collected 48 hours after transfection. For differentiation, siRNA was added simultaneously with the induction of differentiation. For C2C12 cells, siRNA transfection was performed twice, with an additional transfection on day 2. The siRNAs used in this study are listed in Table S1.

DMD ko cell lines

DMD ko cell lines were derived from HeLa and HEK293 cells. The cells were seeded at 30% confluency in 24-well plates and transfected twice, 24 and 48 hours after passage. For each transfection, 2.5 µl of Lipofectamine CRISPRMAX Cas9 Transfection Reagent (Thermo Fisher) was used per well, along with 7.5 pmol of TrueGuide sgRNA (Thermo Fisher) and 1.25 µg of TrueCut Cas9 Protein v2 (Thermo Fisher). The cells were collected 96 h after seeding, with half of the material used to evaluate knockout efficiency and the other half used for single-colony expansion. Single-cell clones for further validation and banking were obtained via the limiting dilution cloning method. Clones were validated by Sanger sequencing, RT-PCR, and Western blotting. The sgRNAs used in this study are listed in Supplementary Table S1.

Immunofluorescence and histochemical staining, and imaging

Tissue sections, single fibers and single fiber-derived cultures

Cultures derived from single fibers were immunostained according to the published protocol ²⁶. SDH, COX and costameric staining were performed as previously described ²⁷. To visualize BrdU and desmin, cryosections were fixed with acetone at -20°C for 10 minutes, denatured in 2.5 M HCl for 30 minutes and permeabilized with 0.25% Triton X-100 for 5 minutes before the MOM Basic Kit (Vector Laboratories) was used. For single PAX7 immunostaining, cryosections and freshly isolated fibers were subjected to 4% PFA fixation and antigen retrieval with citrate buffer at 100°C for 15 minutes. Cryosections were then permeabilized with 0.25% Triton X-100/PBS for 10 minutes, blocked and immunostained via the MOM kit. Fixed fibers were resuspended and blocked in 5% normal goat serum (NGS) and 0.2% Triton X-100/PBS for 1 hour and immunolabeled with the PAX7 antibody, which was

added directly to the blocking solution O/N at 4°C on a rotating mixer. For other single and double immunofluorescent labeling, cryosections were fixed with 2% PFA and processed without antigen retrieval. The samples were incubated with the secondary antibody for 45 minutes at RT. After the secondary antibody incubation, DAPI (Sigma Aldrich) was used to visualize the nuclei. Immunolabeled cell and tissue samples were mounted with Mowiol (Calbiochem). The antibodies used in this study are listed in Supplementary Table S2.

The images were captured with: Nikon Eclipse E1000 scope, QImaging QICAM Fast 1394 camera, Nikon Plan 10x/0.25 and Plan Apochromat 20x/0.75 objectives, QImaging QCapture software (version 2.95); Olympus ZX16 scope, DP71 camera, SDF PLAPO 2xPFC and 1xPF objectives, DP Controller software (version 3.1); Carl Zeiss 510 META confocal scope, Plan Apochromat 20x/0.75 and Plan Apochromat 63x/1.4 objectives, LSM510 software (version 4.2). Composite images were acquired via the Syncroscan stage attached to the Nikon Eclipse E1000 scope and Syncroscan software (4.05; Syncroscopy). Images were processed via the LSM Image Browser (generation of projections of confocal stacks, brightness and contrast adjustments; version 4.2; Carl Zeiss) and Photoshop CS3 (brightness and contrast adjustments, cropping, and splitting of color channels; Adobe). Fiber areas and the number of nuclei in fiber-derived cultures were assessed using ImageJ software.

Cell lines

The cells were plated on 0.1% gelatin-coated coverslips were fixed in 2% PFA for 15 minutes and then washed 3 times with PBS. The cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 minutes and then blocked with 5% normal donkey serum (NDS) and 0.2% Triton X-100 in PBS for 30 minutes. The samples were incubated with primary antibodies diluted in 2% NDS and 0.1% Triton X-100 in PBS for 2 hours at RT. The cells were then washed 3 times with PBS, incubated with secondary antibodies diluted in 2% NDS, 0.1% Triton X-100 in PBS for 1 hour at RT, and washed again 3 times with PBS. Coverslips were mounted on microscopic slides with ProLong™ Diamond Antifade Mountant (Thermo Fisher) and visualized on a Leica DM IL LED confocal system. The antibodies used in this study are listed in Supplementary Table S2.

Immunoblotting

Muscles

Muscle protein extracts were prepared as described elsewhere²⁷. Proteins were separated via NuPAGE 4-12% Bis-Tris gels (Invitrogen) and transferred onto PVDF membranes (GE Healthcare). Densitometric analysis was performed with ImageJ. Band intensities were normalized to the total protein content that was evaluated densitometrically from Coomassie–stained gels.

Cell lines

Protein extraction from the cell lines was performed with CellLytic M (Sigma Aldrich) supplemented with cOmplete Mini Protease Inhibitor Cocktail (Sigma Aldrich). After the protein concentration was measured with a Pierce BCA protein assay kit (Thermo Fisher), the extracts were mixed with sample buffer (4x Sample Buffer: 0.8 g of SDS, 2 ml of 1 M Tris (pH=8.0), 4 ml of glycerol, 1 ml of 0.5 M EDTA (pH=8.0), 0.4 ml of β -mercaptoethanol, and 8 mg of Brilliant Blue) and then denatured for 5 minutes at 95°C. Western blotting was performed by loading 20 μ g of protein extracts per lane onto 6-12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Proteins were then transferred to methanol-activated PVDF membranes (0.45 μ m) (Thermo Fisher) for 16 hours at 25 V at 4°C via a wet blotting system (Bio-Rad). For large proteins such as DP427 and UP395, the transfer buffer was supplemented with 0.03% SDS. The membranes were blocked with 5% skim milk in TBS supplemented with 0.1% Tween (TTBS) for 1 hour at RT and incubated with primary antibody in blocking buffer O/N at 4°C or 2 hours at RT. After incubation with primary antibodies, the membranes were washed 3 times for 20 minutes with TTBS and then incubated with secondary antibodies in blocking buffer for 1 hour at RT, and the washing procedure was repeated. Protein detection with the Clarity™ Western ECL Substrate was performed on a ChemiDoc Imaging System (Bio-Rad). SimplyBlue SafeStain (Coomassie G-250; Thermo Fisher)-stained gels and Ponceau S Staining Solution (Thermo Fisher)-labeled membranes were used as loading controls. The antibodies used in this study are listed in Supplementary Table S2.

Real-time (RT) PCR

Total RNA was extracted with a Total RNA Zol-Out kit (A&A Biotechnology), and 1 μ g of total RNA was reverse transcribed with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). RT-PCR was

performed with Power SYBR™ Green PCR Master Mix (Thermo Fisher) with 10 µg of cDNA and primers at a final concentration of 1.5 µM. The primers used in this study are listed in Supplementary Table S1.

Cell viability and proliferation

Cell viability/proliferation was determined using the RealTime-Glo™ MT Cell Viability Assay (Promega). The cells were plated on a Nunc MicroWell 96-well plate (Thermo Fisher) at a density of 1000 for the HeLa and HEK293 cells and 5000 for the HSKM cells. Cell proliferation was monitored every 24 hours on a Tecan SPARK plate reader with luminescence.

Mitochondrial content and activity

The cells were plated on Greiner CELLSTAR® 96 well plates (Sigma-Aldrich), and the mitochondrial content was measured with MitoTracker Green FM (Thermo Fisher) on a Tecan SPARK plate reader. The Hoechst fluorescence signal was used to normalize the MitoTracker signal to the number of cells. Emission/excitation wavelengths were adjusted experimentally to avoid signal crossover. The following excitation/emission wavelengths were used: MitoTracker, 485/525; Hoechst, 350/461. Alternatively, the mitochondrial content was determined from the ratio of mitochondrial DNA (mtDNA, *ND1/Nd1* gene) to genomic DNA (gDNA, *SERPINA/Hk2* gene) via RT-PCR analysis. Image-iT TMRM Reagent (Thermo Fisher) was used to obtain mitochondrial membrane potential images.

Oxidative stress, cytotoxicity, genotoxicity, apoptosis, calcium levels, and plasma membrane staining

The cells were plated on Greiner CELLSTAR® 96 well plates (Sigma Aldrich), and readings were obtained via a Tecan SPARK plate reader. Oxidative stress was measured with CellROX Green Reagent and CellROX Deep Red Reagent (Thermo Fisher); cytotoxicity and genotoxicity were measured with an HCS DNA Damage Kit (Thermo Fisher); viability, cytotoxicity and apoptosis were measured with ApoTox-Glo Triplex Assay (Promega); and calcium levels were measured with Fluo-8, AM (AAT Bioquest), and plasma membrane staining with CellMask Plasma Membrane Stains (Thermo Fisher). The Hoechst fluorescence signal was used to normalize the fluorescence signal to the number of cells. Emission/excitation wavelengths were adjusted experimentally to avoid signal crossover. The following excitation/emission wavelengths were used: Cell Rox Deep Red, 640/690; CellMask Plasma Membrane

Stains, 649/666; genotoxicity (HCS Damage Kit), 540/590; Cell Rox Green Reagent, 485/550; cytotoxicity (HCS Damage Kit)/ Calcium, 485/530; cytotoxicity (ApoTox-Glo™) 480/525; viability (ApoTox-Glo™), 400/505; Hoechst, 350/461; and apoptosis (ApoTox-Glo™), luminescence.

RNA-sequencing

Sample preparation

HeLa and HSKM cells were transfected with siRNA and cultured in nine biological replicates for each siRNA. For RNA isolation, every three biological replicates were combined to create a single pooled replicate as one biological repeat. Total RNA was extracted via Total RNA Zol-Out™ (A&A Biotechnology) and RNA Clean & Concentrator-5 with DNase I (ZYMO Research). The quality of the total RNA was evaluated via a TapeStation 2200 with an RNA ScreenTape assay kit (Agilent). Only samples with an RNA integrity number (RIN) ≥ 8 were used for further analyses. Following rRNA depletion and cDNA library preparation, RNA-Seq libraries were sequenced on a NovaSeq platform (Illumina) to generate 150-bp paired-end reads (PE150).

rRNA depletion and cDNA library preparation

rRNA depletion was performed using the RiboCop rRNA Depletion Kit V2 (Lexogen) according to the manufacturer's protocol. The quality and quantity of depleted RNA were evaluated via the TapeStation 2200 with the High Sensitivity RNA ScreenTape assay kit (Agilent). cDNA libraries were prepared using the RNA-Seq V2 Library Prep Kit with UDIs (Lexogen) following the Long Insert Sizes (RTL) protocol. Libraries were amplified in 11 cycles. The concentration and size range of the cDNA libraries were determined using the TapeStation 2200 with the High Sensitivity DNA ScreenTape assay kit (Agilent). The resulting libraries were pooled, and the final concentration and size distribution were verified via the TapeStation 2200 (Agilent).

RNA-sequencing and sequencing data analysis

The RNA-Seq libraries were sequenced on a NovaSeq platform (Illumina) to generate 150-bp paired-end reads (PE150). Adapter removal was performed via Cutadapt (<https://cutadapt.readthedocs.io/en/stable/>) followed by alignment to the reference genome using Hisat2 (<https://daehwankimlab.github.io/hisat2/>)²⁸. The GRCh38.p14 reference genome was utilized

(https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000001405.40/). The number of paired reads mapped to individual genes was subsequently quantified via HTseq (https://htseq.readthedocs.io/en/release_0.10.0/index.html), with strand specificity considered (--stranded=reverse). Gene annotations were assigned on the basis of a gene description file available from the DRYAD database (<https://datadryad.org/stash>) containing InterProScan results ^{29, 30}.

The level of gene expression was calculated using the R package DESeq2. For gene expression analysis, a negative binomial distribution was applied, utilizing the gene-specific mean and dispersion parameters. The changes in transcript counts were tested via the Wald method, which allowed us to determine the statistical significance of the expression changes in the analyzed genes. The obtained p-values were corrected for multiple testing via the Benjamini-Hochberg method ($FDR \leq 0.05$). Only statistically significant genes with $FDR \leq 0.05$ were included in the comparative analyses of differentially expressed genes (DEGs). The results are presented as log2_Estimated_FoldChange (log2FC), which represents the logarithm base 2 of the fold change in transcript abundance between the experimental sample and the control sample.

KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis was performed by retrieving gene identifiers matching the KEGG database. For this purpose, BLASTP was used to search peptide sequences from the *Homo sapiens* genome that corresponded to genes in the KEGG database (<https://www.kegg.jp/kegg/kegg2.html>) ³¹. Only BLASTP results with identity scores above 90% were included in the KEGG analysis. The final results were processed in the R environment using the DESeq2 package ³². Biological pathways from the KEGG database for statistically significant genes with EntrezId identifiers in the analyzed comparisons were determined using the clusterProfiler package ^{33, 34}. This package has the advantage of using the latest version of the KEGG database. For KEGG analysis, only genes identified as significantly differentially expressed ($FDR \leq 0.05$) were included. Visualization of KEGG pathway data was performed via Pathview (<https://bioconductor.org/packages/release/bioc/html/pathview.html>) ³⁵.

RESULTS

Loss of dystrophin Dp427 and utrophin in skeletal muscles leads to proteomic changes and alterations in the organization of the mitochondrial network

To address the functions of DP427 in skeletal muscles, we used wild-type (wt) and severely affected DP427- and utrophin-deficient mice (*mdx/Utrn^{-/-}*, dko mice). Dko mice represent the muscle wasting phenotype of DMD patients much better than mildly affected *mdx* mice because they are much smaller, their movements are greatly restricted, and they live for only up to 20 weeks³⁶. This phenotypic severity is explained by the lack of compensatory synthesis of UP395, which is able to connect actin filaments to the extracellular matrix via the assembled UGC and partially prevents the contraction-induced degeneration of myofibers¹⁹.

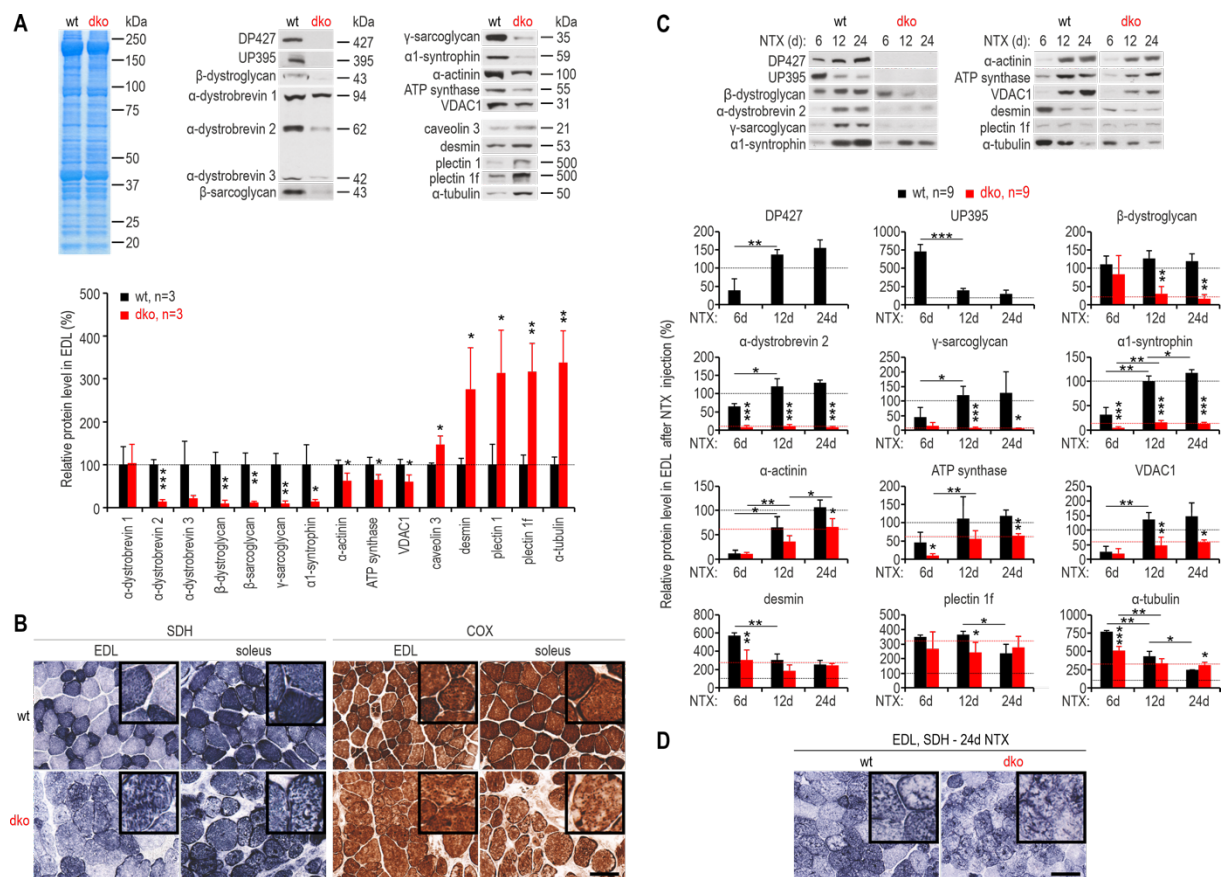


Figure 1: Loss of dystrophin DP427 and utrophin in skeletal muscle leads to proteomic changes and alterations in the organization of the mitochondrial network.

A, C Immunoblotting analysis of EDL muscles (**A**) and EDL muscles after NTX treatment (**C**) with densitometric evaluation of band intensities relative to wt levels. Coomassie-stained polyacrylamide gels were used as

normalizing controls. The black and red horizontal lines in **C** show protein levels in uninjected wt and dko EDL muscles. **B, D** Frozen sections of EDL and soleus muscles from wt and dko mice stained for the mitochondrial enzymes SDH and COX (**B**) and of EDLs from wt and dko mice isolated 24 days post NTX and stained for SDH (**D**). The insets show magnifications of representative section fragments. Bars, 50 μ m. The results in **A** and **C** are shown as the mean values + SD. n, the number of biological replicates (EDL muscles). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (t-test).

Our comparative immunofluorescence microscopy and Western blot analyses of wt and dko *extensor digitorum longus* (EDL) muscles confirmed markedly reduced DGC/UGC protein contents in dko tissues (Figs. 1a and S1). Specifically, we detected reduced amounts of α -dystrobrevin 2 and 3, β -dystroglycan, β - and γ -sarcoglycan, and α 1-syntrophin, along with the myofiber differentiation marker, α -actinin. In contrast, the level of a predominantly neuromuscular junction (NMJ)-associated α -dystrobrevin 1 was unaltered and elevated amounts of caveolin 3, and cytoskeletal proteins, such as desmin, plectin, α -tubulin and vimentin were observed ³⁷. An anti-vimentin antibody labeled cells exclusively outside of myofibers, revealing much greater numbers of non-myogenic cells in dko EDLs, and desmin was detected in myofibers, with a particularly high signal in small-diameter regenerating fibers of dko muscles (Fig. S1A). In turn, anti-pan plectin and anti- α -tubulin antibodies marked both non-myogenic cells and muscle fibers, with a particularly strong signal in the dko samples. In agreement with the staining pattern of the pan-plectin antibody, the plectin isoform 1f signal was observed on the sarcolemma, primarily in regenerating dko fibers, and plectin 1 was found in non-myogenic cells, and costameres and perinuclear regions of isolated fibers (Fig. S1A and B). Despite changes in protein content, the costameric localization of plectin and desmin remained unchanged (Fig. S1B).

In contrast to the intact organization of costameres in isolated EDL fibers, both the content and organization of the mitochondrial network were altered in dko muscles. In particular, the amounts of the mitochondrial marker proteins ATP synthase and VDAC1 were reduced in EDL protein lysates (Fig. 1A), and histochemical staining of succinate dehydrogenase (SDH) and cytochrome c oxidase (COX) in frozen EDL and soleus sections revealed decreased activity of mitochondria and disorganization of the mitochondrial network (Fig. 1B). Additionally, there was a clear distinction of fiber types in wt samples, whereas such a distinction was barely noticeable in dko muscles.

NTX-induced regeneration reveals a correlative increase in DGC and mitochondrial proteins during muscle regeneration

We next injected notexin (NTX) into wt and dko EDLs to degenerate muscle fibers and induce their synchronous regeneration from activated muscle stem cells (MuSCs) to confirm that the observed proteomic changes did not stem solely from the presence of immature fibers (Fig. S2). Analyses revealed the complementary synthesis of UP395 and DP427 during the regeneration of wt EDLs (Figs. 1C and S3A). Specifically, high amounts of utrophin were observed on the sarcolemma at 6 days post NTX treatment, whereas low amounts of utrophin were limited to NMJs in the later stages of regeneration (Fig. S3A). On the other hand, DP427 was present at low levels on the sarcolemma at the earliest differentiation time point, and its amount was significantly greater at 12 and 24 days after NTX treatment (Figs. 1C and S3A). Interestingly, all tested DGC components, including α -dystrobrevin-2, γ -sarcoglycan and α 1-syntrophin, but apart from β -dystroglycan, which was already high at the earliest time point tested, followed the dystrophin synthesis pattern (Figs. 1C and S3). During regeneration, the levels of the myofiber differentiation marker α -actinin, as well as mitochondrial marker proteins, also increased, whereas the amounts of desmin, plectin 1f and α -tubulin followed a reverse synthesis profile, in agreement with the results observed in the non-treated muscles (Figs. 1A and S1).

Analyses of dko muscles revealed β -dystroglycan levels comparable to those of the wt samples at 6 days post NTX and its decreasing amounts at later differentiation stages, whereas the other DGC components were present at very low quantities at the earliest analyzed time point (Figs. 1C and S3). Much lower levels of α -actinin and mitochondrial marker proteins were also detected in dko samples, especially on days 12 and 24 post NTX injection, whereas anti- α -tubulin, -desmin and -plectin 1f antibodies showed usually presented lower values in early regeneration stages and tended toward higher levels on the last analyzed day. Additionally, 24 days after NTX injection, the mitochondrial network in wt samples exhibited a uniform localization pattern (with rare exceptions, as shown in the inset), whereas in dko muscles, the staining was weaker and disorganized (Fig. 1D).

Dko EDL fibers show increased regeneration during early differentiation but reduced survival during later stages of differentiation

Proteomic analyses of NTX-injected EDLs indicated that the loss of DP427 and utrophin leads to faster generation of myofibers (significantly lower amounts of desmin and α -tubulin in dko samples than in

control samples 6 days post NTX injection) and either to their hindered regeneration in the later stages of differentiation or premature death and generation of new myofibers (significantly lower α -actinin and higher α -tubulin levels at 24 days post NTX injection) (Fig. 1C). To assess the time required to generate muscle fibers, we prepared frozen cryosections from muscles harvested 3, 6 and 12 days after surgery and double immunolabelled them for myosin and the fibroblast marker ER-TR7. Indeed, the dko sections presented larger myosin-positive fibers than the wt samples did at the earliest time point analyzed (Fig. 2A). To test how the lack of DP427 and utrophin affects fibers at later stages of differentiation, we first determined the timing of myoblast proliferation after NTX treatment. Immunofluorescence microscopy analysis revealed high numbers of BrdU-labeled nuclei when BrdU was injected 2 and 3 days after NTX treatment (Fig. 2B), indicating that cell proliferation is most robust at these time points. Wt and dko mice were then injected with BrdU twice, 2 and 3 days after NTX treatment, and the muscles were harvested on days 6 and 12 after NTX injection. While the number of nuclei in myofibers containing BrdU-labeled nuclei was comparable on day 6, approximately 50% fewer dko-labeled myofibers were observed 12 days after NTX treatment (Figs. 2C and S4), revealing their reduced survival.

We next examined the time points DP427 and utrophin begin to be synthesized following NTX injection and whether their synthesis affects myofiber regeneration from activated MuSCs. We did not observe DP427 in proliferating PAX7-positive cells, whereas utrophin was visible both in myoblasts and in newly formed myofibers (Fig. 2D). We then isolated single fibers from untreated and NTX-injected EDL muscles and immunolabeled them (Fig. 2E) to test whether the number of PAX7+ cells in the dko muscles could explain the increased myofiber formation in the dko EDLs. Consistent with our hypothesis, the analysis revealed a threefold greater number of PAX7+ cells on dko fibers than on wt fibers isolated from untreated muscles (Fig. 2E). Interestingly, the number of PAX7+ cells in wt EDLs increased markedly, remained elevated until 12 days post NTX injection, and then decreased to lower numbers (Figs. 2E and S5). In contrast, the number of these cells on the dko fibers remained approximately constant. PAX7+ cells on the dko muscles were able not only to generate new myofibers after NTX treatment but also to regenerate myofibers after subsequent degenerative processes, as the number of myofiber nuclei in the dko fibers increased to wt levels from days 12 to 24 after NTX treatment (Fig. 2F). Overall, these data indicate that the absence of DP427 and utrophin does not prevent the regeneration of myofibers from MuSCs.

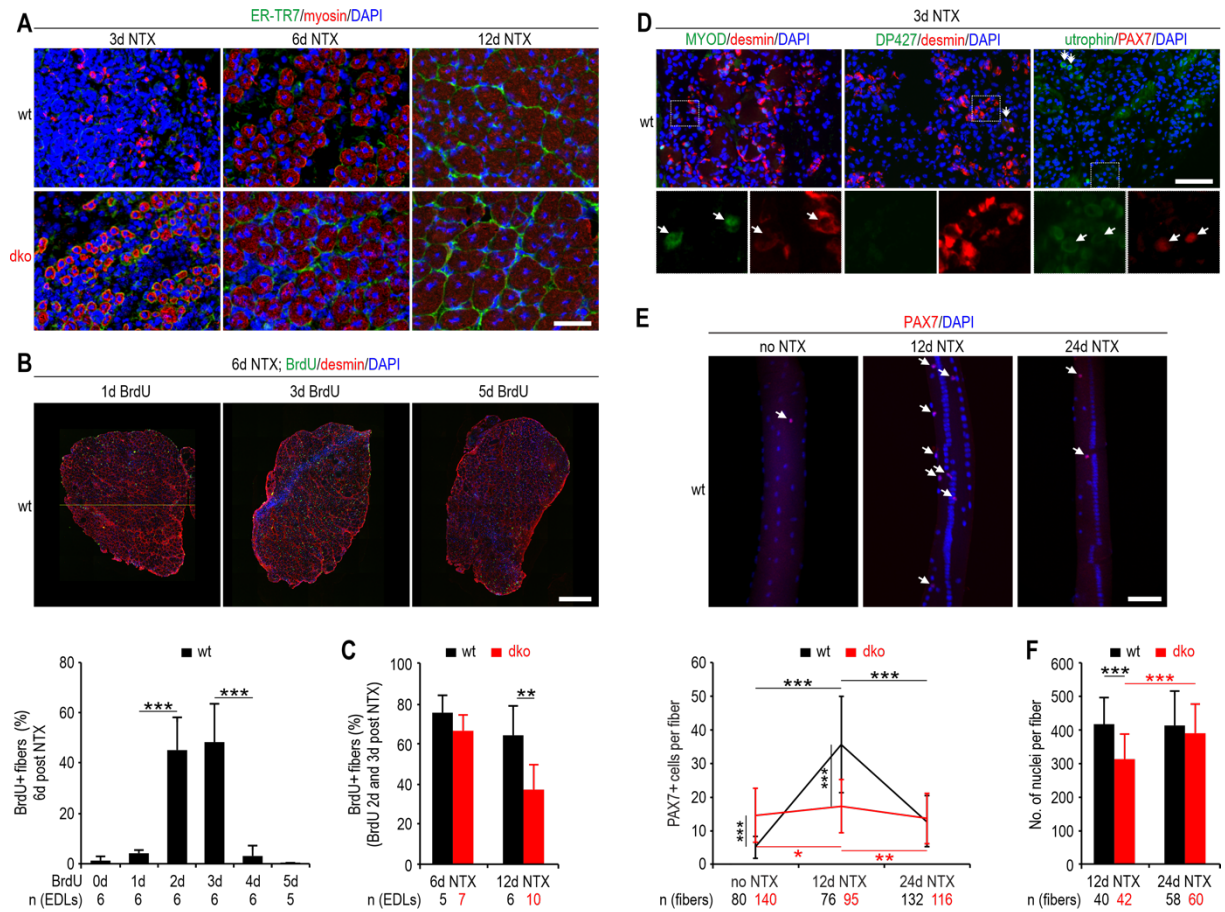


Figure 2: Dko EDL fibers show enhanced formation during early differentiation but high mortality rates in later differentiation stages.

A Frozen sections of EDL muscles from wt and dko mice 3, 6 and 12 days after NTX injection immunostained for ER-TR7 (fibroblast marker), myosin and nuclei (DAPI). **B** Quantification of the percentage of fibers containing BrdU+ nuclei 6 days after NTX injection. Composite images immunostained for BrdU, desmin and DAPI from wt EDL muscles that were either not injected with BrdU or were injected with BrdU 1, 2, 3, 4, and 5 days after NTX treatment were used for analysis. **C** Quantification of the percentage of wt and dko EDL fibers containing BrdU+ nuclei at 6 and 12 days post NTX injection. BrdU was injected twice, 2 and 3 days after NTX treatment. **D** Frozen sections of EDL muscles from wt mice 3 days post NTX injection immunostained for myoblast determination protein 1 (MYOD), DP427, utrophin, desmin, and nuclei (DAPI). The insets show a lack of DP427 in desmin+ myoblasts and the presence of utrophin in proliferating PAX7+ cells. **E** Quantification of PAX7+ nuclei in fibers isolated from uninjected or NTX-treated wt and dko EDL muscles. **F** Evaluation of the number of nuclei in wt and dko fibers collected from muscles injected with NTX. The results in **B**, **C** and **F** are presented as mean values + SD and those in **E** as mean values $\pm$ SD. n, the number of biological replicates (EDL muscles in **B**, **C** and fibers from EDL muscles in **E**, **F**). Bars, 50 μ m (**A**, **D**, **E**) and 300 μ m (**B**). * P <0.05, ** P <0.01, *** P <0.001 (t-test in **B**, **C** and z-test in **E**, **F**).

Fiber degeneration in dko muscles is caused by intrinsic cellular alterations independent of changes in NMJs and other cell types

Myofiber degeneration could be a consequence of intrinsic changes in myofibers, NMJs, or general alterations in the muscle tissue environment and impaired interactions with other cell types. To test this, we isolated single myofibers and allowed activated MuSCs to migrate from them, proliferate and generate new myotubes. As early as day 6 after plating, myosin-labeled dko cultures presented reduced areas (Fig. 3A). This difference was even more pronounced on day 12, when we also observed the death of dko myotubes. We also noted significantly greater numbers of nuclei in the dko cultures 3 days after plating, but on day 6, these numbers were similar in the wt and dko wells (Fig. 3B). After normalizing the number of nuclei to the number of PAX7+ cells on myofibers of untreated muscles, we observed lower numbers of nuclei in dko cultures (Fig. 3B), indicating their reduced ability to give rise to new nuclei.

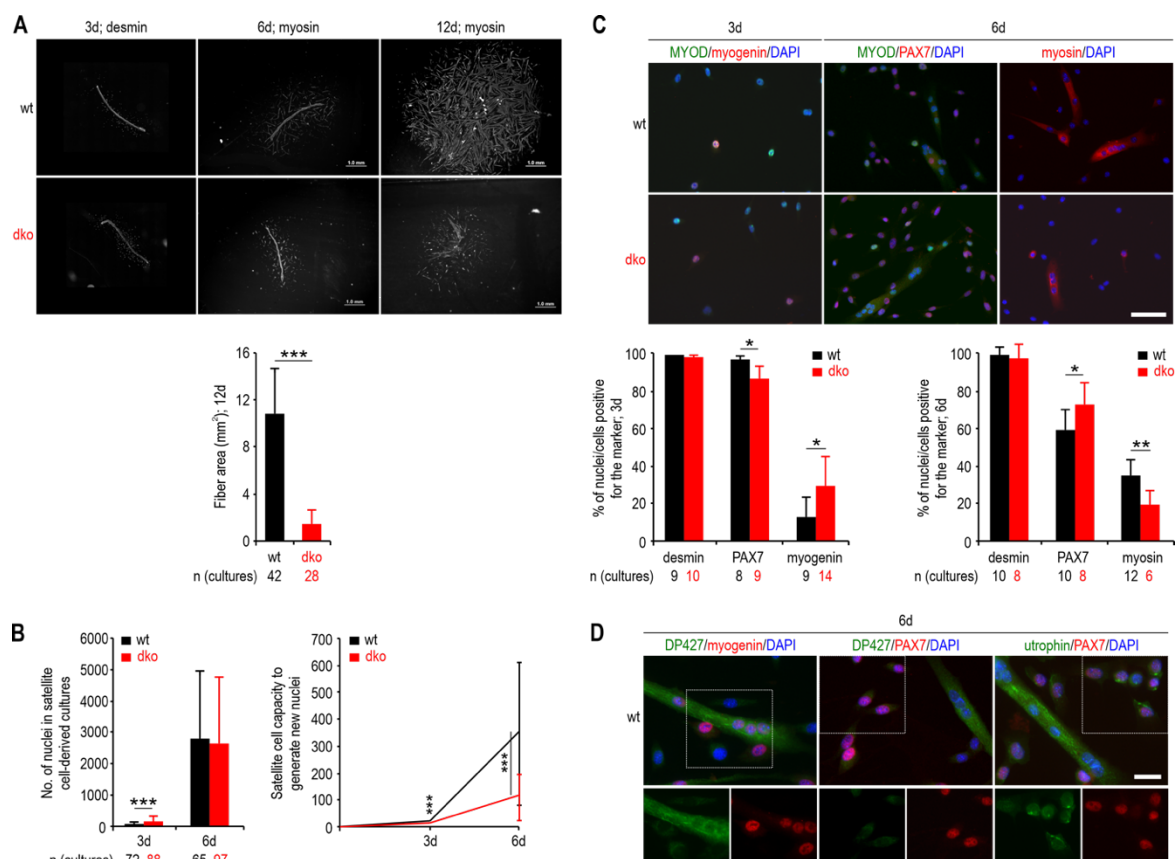


Figure 3: Dko fiber degeneration is caused by intrinsic cellular alterations independent of changes in NMJs and other cell types.

A Fiber-derived wt and dko cultures immunostained for either desmin (3 days post fiber plating) or myosin (6 and 12 days post fiber plating), and the quantification of myotube areas in wt and dko cultures 12 days post fiber plating. **B** Evaluation of the number of nuclei in fiber-derived cultures 3 and 6 days post fiber plating and normalization of the number of nuclei to the number of PAX7+ nuclei in uninjected EDL fibers (see Fig. 2E). **C** 3- and 6-day-old fiber-derived wt and dko cultures immunolabeled for differentiation markers and quantification of the percentage of nuclei/cells positive for desmin, PAX7, myogenin and myosin. **D** Fiber-derived wt cultures immunostained for DP427, myogenin, utrophin and PAX7 6 days post fiber plating. The results in **A**, **B** (left panel) and **C** are shown as the mean values + SD and those in **B** (right panel) as the mean values $\pm$ SD. n, the number of biological replicates (cultures from single EDL fibers). Bars, 50 μ m in **C** and 20 μ m in **D**. *P<0.05, **P<0.01, ***P<0.001 (z-test in **A**, **B** and t-test in **C**).

We then immunolabeled wt and dko cultures at days 3 and 6 after fiber plating. Consistent with the data showing enhanced dko fiber formation at the early stage of differentiation (Fig. 2A), dko cultures also presented a greater percentage of myogenin+ nuclei on day 3, whereas on day 6, the opposite pattern was observed, with a greater percentage of PAX7+ cells and a lower percentage of nuclei within myosin+ cells/myotubes (Fig. 3C). Analyses also revealed the presence of utrophin in proliferating PAX7+ cells and myotubes, whereas significant amounts of DP427 were present only in myotubes (Fig. 3D). Together, these results demonstrate that greater numbers of PAX7+ cells in dko fibers determine enhanced fiber formation; however, these fibers die at later differentiation stages. Moreover, the data indicate that premature death of dko fibers is not a consequence of damaged NMJs and defective interactions with neurons, or any other cell types.

siRNA-mediated downregulation of the dystrophin and utrophin genes leads to pathological abnormalities in myoblasts and muscle fibers

Because some of the observed changes in fiber-derived cultures could be due to prior alterations in the muscle tissue milieu, we decided to downregulate the *Dmd* and *Utn* genes in a commonly used murine C2C12 muscle cell line (Figs. 4, S6). Quantitative PCR analyses revealed very low dystrophin mRNA levels in proliferating cells (day 0) and their rapid increase during differentiation (from day 3 to day 12; Fig. 4A), along with the early and late differentiation markers myogenin (*MyoG*) and myosin (*Myh1*), respectively (Fig. S6A). On the other hand, utrophin mRNAs were at higher levels in the proliferation stage but increased to much lower levels during early differentiation and then declined on day 12.

Subsequent analysis revealed that *Dp71* mRNAs were the most abundant dystrophin transcripts in proliferating cells in contrast to myotubes, where *Dp427* was predominantly transcribed (Fig. 4A).

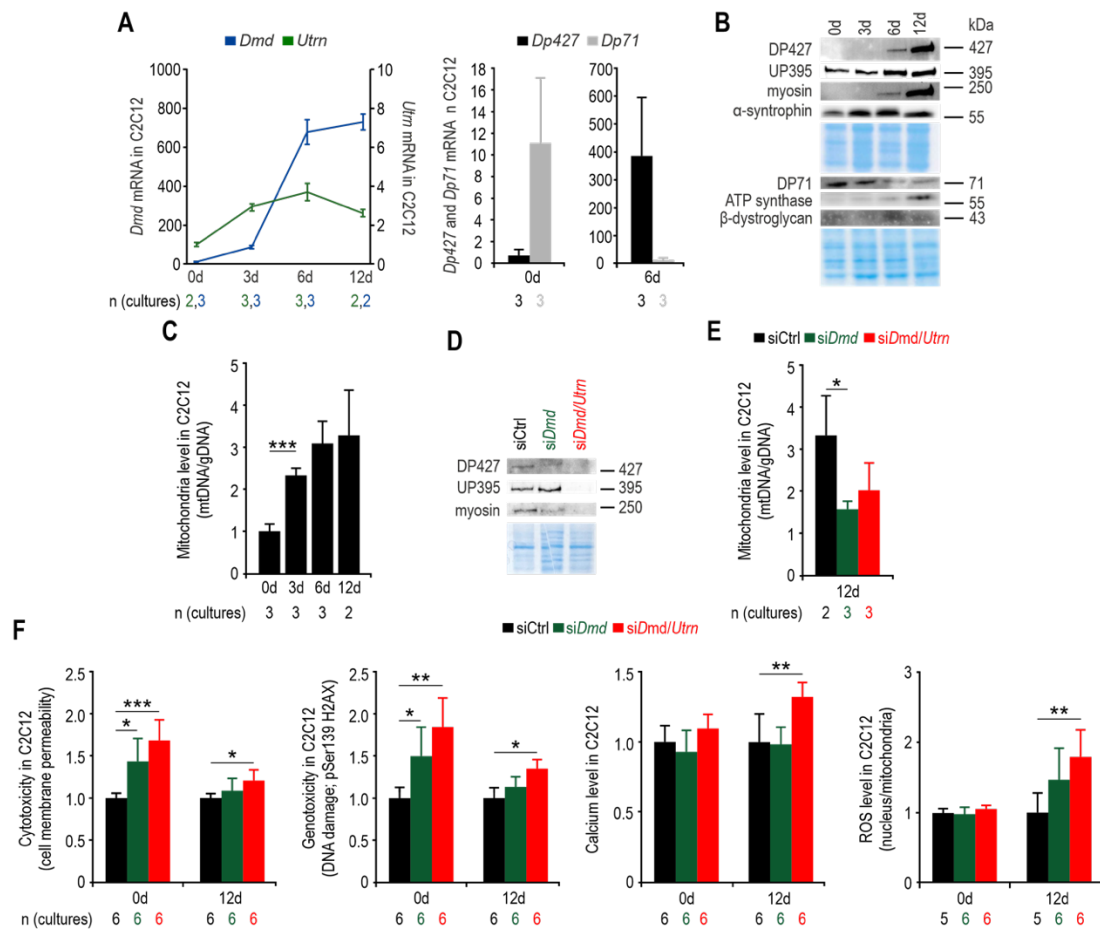


Figure 4: Proliferating and differentiated dystrophin and utrophin-deficient C2C12 cells show increased cyto- and genotoxicity.

A RT-PCR analyses of *Dmd*, *Utrn*, *Dp427* and *Dp71* mRNAs in the C2C12 cell line at different time points during differentiation. **B, D** Immunoblot analysis of C2C12 protein extracts from days 0, 3, 6 and 12 of differentiation (**B**) and from day 12 of differentiation after siRNA delivery (**D**). Coomassie-stained polyacrylamide gels were used to normalize the protein content. **C, E** Quantification of mitochondrial levels in C2C12 cells based on RT-PCR results of mitochondrial (*Nd1*) and nuclear (*Hk2*) DNA markers during differentiation (**C**) and in differentiated cells following RNA interference (**E**). **F** Cytotoxicity (cell membrane permeability) and genotoxicity (DNA damage), ROS production and calcium influx quantification in dystrophin- and dystrophin/ utrophin-deficient C2C12 cells. The results in **A** (left panel) are shown as the mean values $\pm$ SD and those in **A** (right panel), **C**, **E** and **F** as the mean values + SD. n, the number of biological replicates (cell cultures). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (t-test).

In agreement, the DP71 protein was the only dystrophin detected on day 0, but following differentiation initiation, this dystrophin was replaced by DP427 (Fig. 4B). Among the utrophin proteins, UP395 was detected at all stages of differentiation (Fig. 4B). In accordance with the proteomic data obtained after muscle degeneration with NTX (Fig. 1C), we also detected increasing levels of DGC components (α -syntrophin and β -dystroglycan; Fig. 4B) and mitochondrial markers (Fig. 4B, C). Immunofluorescence analysis with an antibody that does not distinguish dystrophin from utrophin (Fig. S7) also revealed the presence of dystrophin/utrophin at all stages of myotube formation (Fig. S6B).

After siRNA-mediated downregulation of the *Dmd* and *Utrn* genes in proliferating cells and myotubes (Figs. 4D, S6C), we observed several pathological changes (Fig. 4D-F). Proliferating dystrophin-deficient and dystrophin- and utrophin-deficient cells presented increased cyto- and genotoxicity (Fig. 4F). Similarly, differentiated myotubes lacking dystrophin and utrophin showed increased toxicity (Fig. 4F), as well as higher ROS and increased intracellular calcium levels (Figs. 4F, S6D, S8). Moreover, reduced myosin and mitochondrial levels were observed (Fig. 4D, E), indicating impaired differentiation of C2C12 cells treated with siRNAs targeting *Dmd*, and *Dmd* and *Utrn* transcripts.

To further assess whether the same phenotype could be observed in human myoblasts and myotubes, we delivered siRNAs to primary human skeletal muscle (HSkM) myoblasts and subjected them to proliferation or differentiation (Figs. 5 and S9A-C). SiRNA-mediated downregulation of the *DMD* and *UTRN* genes reduced the content of DGC/UGC components (Fig. S9D) and decreased the viability of HSkM myoblasts and myotubes (Fig. 5A-C). The culture plates containing myotubes lacking dystrophin and utrophin also presented reduced fiber areas, fewer nuclei, and increased nuclear areas (Figs. 5D, E and S10A). Mitochondrial marker analysis revealed that although mitochondrial levels remained unchanged (Fig. S10B), ATP synthase-positive aggregates could be observed in myotubes, particularly those treated with si*DMD/UTRN* (Fig. 5D). Furthermore, increased calcium and ROS levels were observed (Fig. 5F). These data indicate that contraction-induced changes in the sarcolemma are not a prerequisite for profound pathological alterations in myoblasts and myotubes lacking either dystrophin or dystrophin and utrophin.

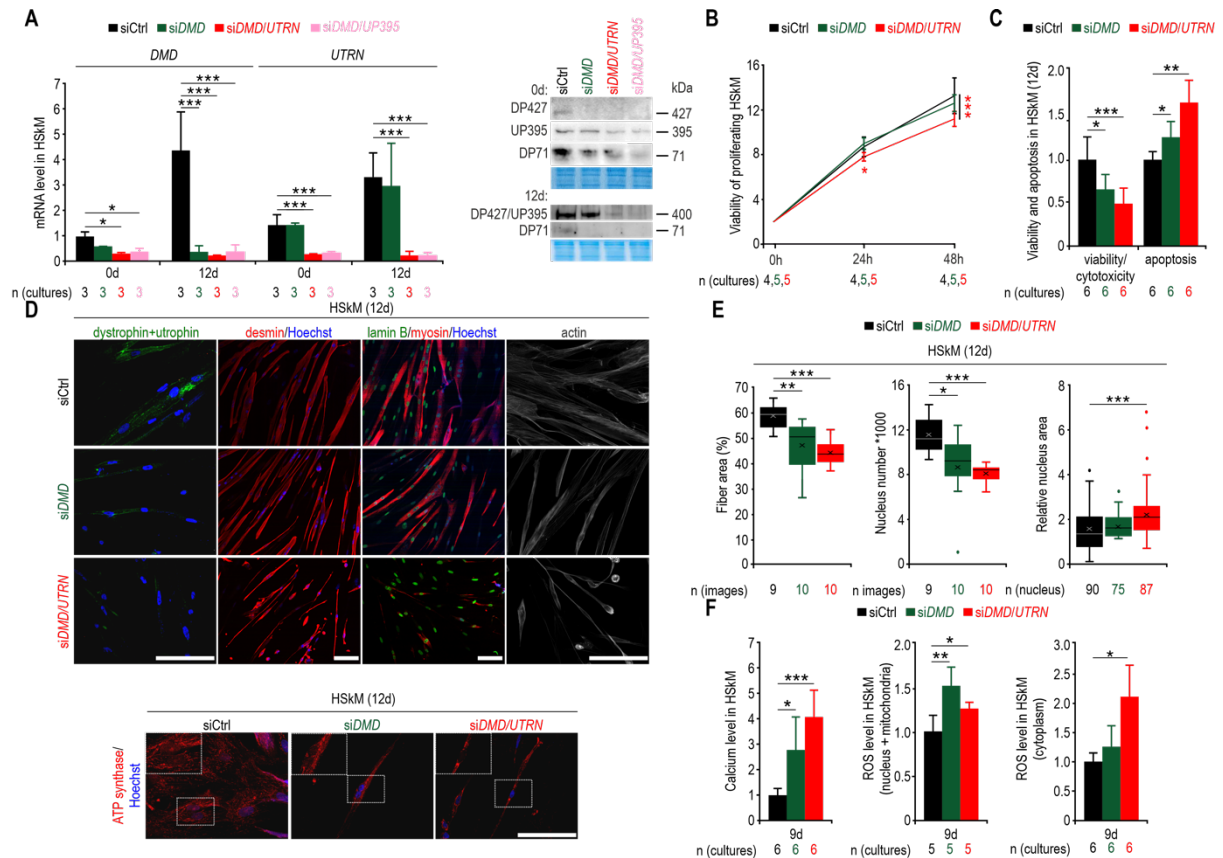


Figure 5: Downregulation of the dystrophin and dystrophin/utrophin genes in proliferating or differentiated HSKM cells leads to profound pathological changes.

A RT-PCR and immunoblotting analyses of proliferating (0d) and differentiated (12d) HSKM cells after siRNA administration. Coomassie-stained polyacrylamide gels were used to normalize the protein content. **B** Assessment of the viability of proliferating HSKM cells for 48 hours after downregulation of the *DMD* or *DMD/UTRN* genes. **C** Analysis of the viability and apoptosis of differentiated HSKM cells transfected with siRNA. **D** Immunostaining of HSKM cells on day 12 of differentiation for dystrophin/utrophin, desmin, lamin B, myosin, actin, ATP synthase and nuclei (blue channel, Hoechst). The insets show the separate red channel with ATP synthase labeling. Bars, 100 μ m. **E**, **F** Quantification of fiber plate coverage, nuclear density per targeted area, and relative nuclear area (**E**) and calcium influx and ROS production (**F**) in HSKM myotubes following RNA interference. In **A**, **C** and **F**, the data are shown as the mean values + SD and in **B** and **E** as the mean values $\pm$ SD. n, the number of biological replicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (t-test in **A**, **B**, **C**, **F** and z-test in **E**).

Utrophin UP395 partially compensates for the lack of DP71 in proliferating cells

Since DP71 and utrophin are ubiquitously synthesized in various cell types, we next generated HeLa and HEK293 dystrophin-deficient lines (Figs. S11 and S12) and subjected them to additional siRNA

delivery (Fig. 6). The wt HeLa and HEK293 lines lacked DP427 but presented some differences in DP71, UP395, DGC/UGC and mitochondrial protein contents (Fig. 6A).

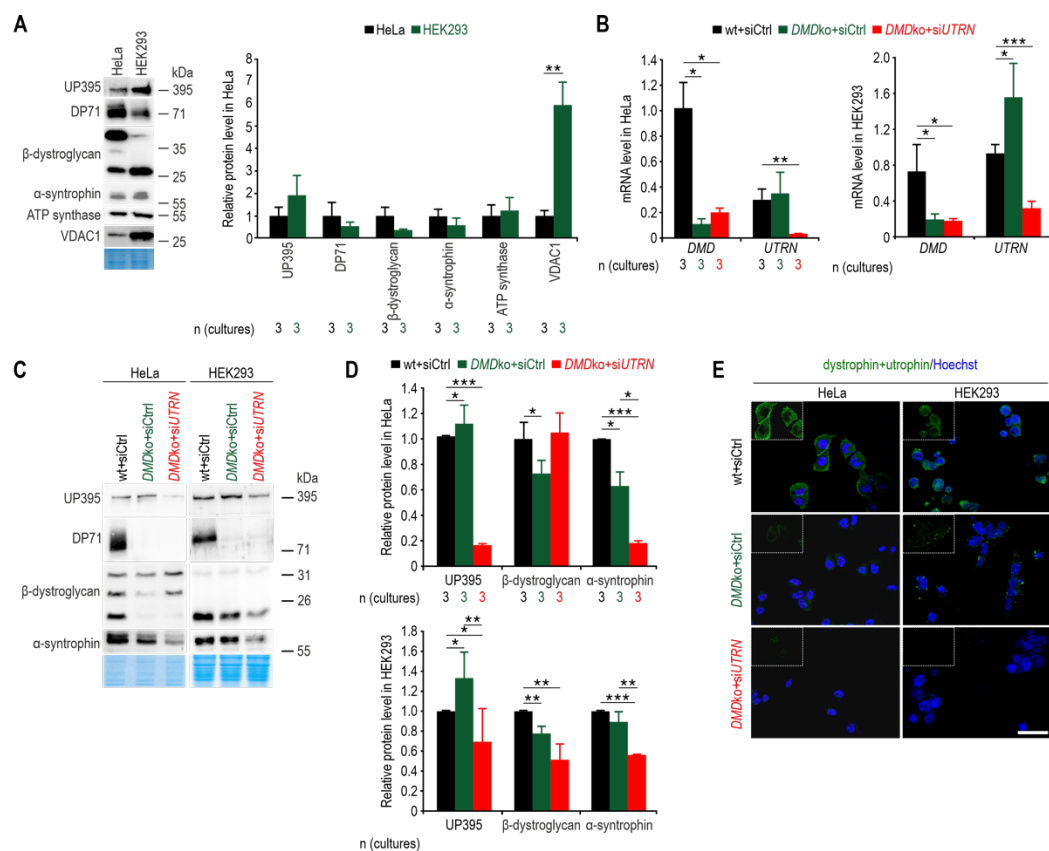


Figure 6: *DMD* ko HeLa and HEK293 cell lines show upregulation of the *UTRN* gene and a decrease in the level of DGC proteins.

A Immunoblot analysis of protein extracts from proliferating HeLa and HEK293 cells, with densitometric evaluation of band intensities normalized to HeLa signals. Coomassie-stained polyacrylamide gels were used for normalization. **B-E** Evaluation of dystrophin and utrophin levels in siRNA-treated wt and *DMD* ko HeLa and HEK293 cells via RT-PCR (**B**), immunoblotting analysis (**C**, **D**), and immunofluorescence microscopy (**E**). Immunoblotting was also used to assess β -dystroglycan and α -syntrophin levels (**C**, **D**). Coomassie-stained polyacrylamide gels were used for protein normalization. The insets in **E** show the separate green channel for dystrophin/utrophin labeling (without the Hoechst-labeled nuclei). Bar in **E**, 50 μ m. The results in **A**, **B** and **D** are shown as the mean values + SD. n, the number of biological replicates (cell cultures). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (t-test).

After CRISPR/Cas9-mediated *DMD* gene deletion, both cell types presented reduced DGC and increased UP395 protein levels (Fig. 6B-E), indicating a universal compensatory mechanism based on the upregulation of the *UTRN* gene in the absence of either DP427 or DP71. Indeed, DP71 deficiency

resulted in apoptosis and reduced the survival of HeLa and HEK293 cells, and an additional lack of UP395 markedly worsened the phenotype (Figs. 7A and S13).

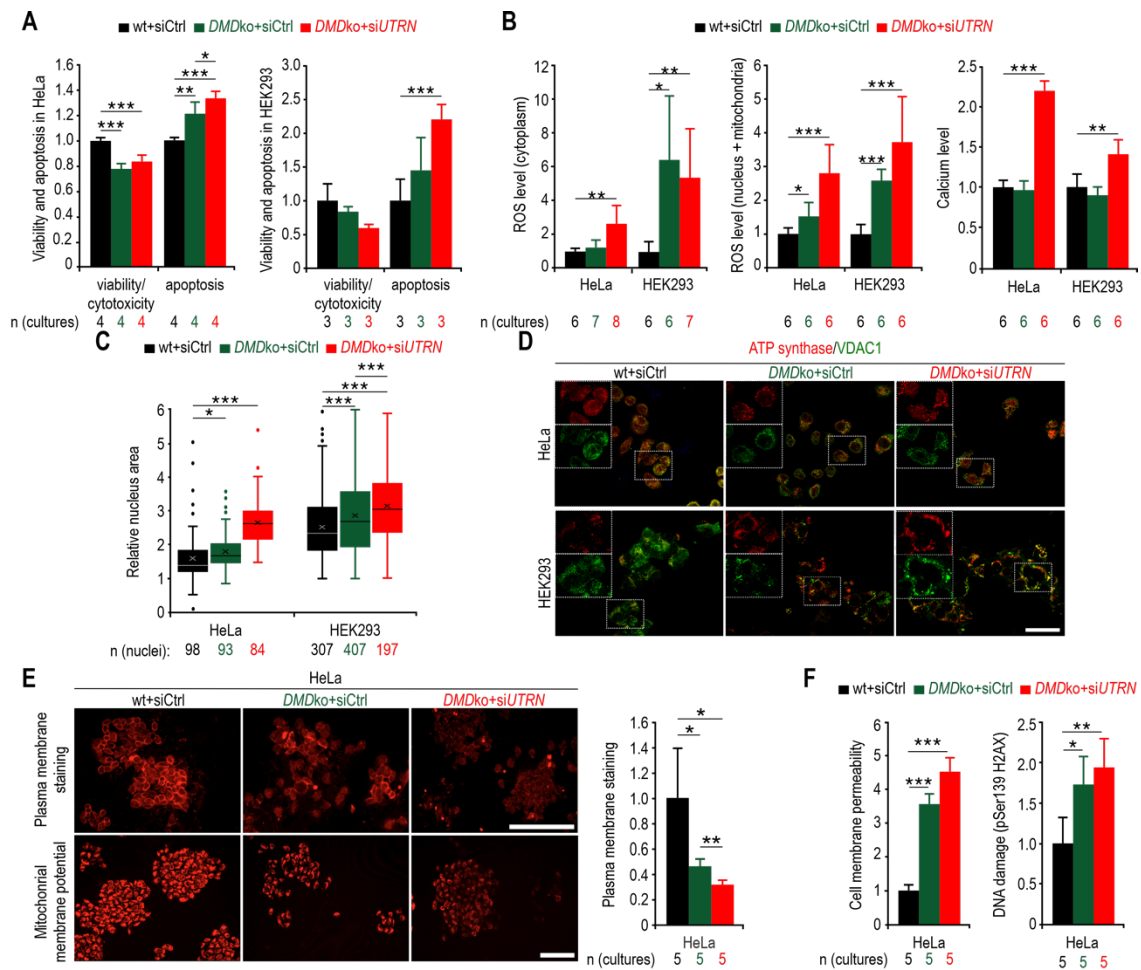


Figure 7: Dystrophin and utrophin deficiency in HeLa and HEK293 cell lines results in significant pathological alterations.

A-D Analysis of viability and apoptosis (**A**), calcium influx and ROS levels (**B**), relative nucleus area (**C**), and mitochondrial localization based on ATP synthase and VDAC1 immunolabeling (**D**) in siRNA-treated wt and *DMD* ko HeLa and HEK293 cells. The insets in **D** show the separate red channel with ATP synthase labeling and the green channel with VDAC labeling. **E** Images of dystrophin- and utrophin-deficient HeLa cells stained for the plasma membrane and mitochondrial membrane potential and quantification of the plasma membrane staining via a Tecan SPARK plate reader. **F** Cell membrane permeability and DNA damage in wt and *DMD* ko HeLa cells after siRNA treatment. In **A**, **B**, **E** and **F**, the data are shown as the mean values + SD and in **C** as the mean values $\pm$ SD. Bars, 50 μ m in **D** and 100 μ m in **E**. n, the number of biological replicates. * P <0.05, ** P <0.01, *** P <0.001 (t-test in **A**, **B**, **E**, **F** and z-test in **C**).

Furthermore, the DP71-deficient cell lines showed increased ROS levels and larger nuclear areas (Fig. 7B, C). Additionally, some phenotypic changes, such as increased intracellular calcium or decreased ATP synthase levels, were only visible in the absence of both DP71 and UP395 (Figs. 7B, S14). Like in HSkM dystrophin-deficient cells, overall mitochondrial levels remained unchanged in *DMD* ko HeLa and HEK293 cells (Fig. S15A), but their localization changed from dispersed to more aggregated (Fig. 7D). Further analyses in HeLa cells also revealed reduced cell membrane and mitochondrial membrane potential upon dystrophin or dystrophin and utrophin deficiency (Figs. 7E and S15B), as well as an increase in cell membrane permeability and DNA damage (Fig. 7F). Taken together, these data show that the lack of DP71 in proliferating cells can lead to profound pathological changes and apoptosis and that this phenotype can be partially compensated by UP395.

RNA sequencing of dystrophin- and utrophin-deficient HeLa cells and HSkM myotubes reveals profound transcriptomic changes related to phenotypic abnormalities

We then asked whether we could observe transcriptomic changes corresponding with the pathological phenotype in dystrophin- and utrophin-deficient HeLa cells and HSkM myotubes. Differential gene expression (DEG) analysis revealed 772 up- and 987 down-regulated genes in HeLa *DMD* ko cells following *siUTRN* treatment, whereas in HSkM cells treated with *siDMD/UTRN*, 295 up- and 282 down-regulated genes were identified relative to the control (Fig. 8, Dataset S1). Among these genes, those that were common to both cell types and exhibited either increased or decreased expression levels are highlighted in Dataset S2. Genes with significantly increased expression in dystrophin- and utrophin-deficient HeLa cells and HSkM myotubes were associated with oxidative stress (*AKR1B1*, *MSRB3*), mitochondria (*TOMM20*, *MCL1*, *EEF2*), apoptosis (*MCL1*, *EDN1*, *GDF15*, *TCIM*), muscle regeneration and cell growth (*MET*, *IGFN1*, *CDON*, *SH3PXD2A*), fibrosis and tissue remodeling (*EDN1*, *FKBP10*, *LAMA1*), metabolism (*AKR1B1* – also involved in ROS detoxification, *DGKD*, *SLC12A7*), signaling (*STAC3*, *CADM1/CADM2*, *CLCN5*, *PPM1L*, *ANKRD52*), as well as the immune response and cell adhesion (*NPTX1*), protein synthesis (*URB1*), cell proliferation (*AHCYL1*), gene transcription (*PBXIP1*), and cytoskeleton organization (*CCDC71L*). Similarly, genes with decreased expression in both cell lines were related to muscle regeneration and cell growth (*ITGA6*, *ITGB1*, *AXL*, *ETS1*), fibrosis and tissue remodeling (*FAP*, *ANXA1*, *CAV1*), metabolism (*SCD*, *MSMO1*, *ELOVL1*, *ELOVL5*), oxidative stress (*ALDH3B1*, *S100A10*, *S100A11*), mitochondria (*COX3*, *ATP2B1*), apoptosis (*MDM2*, *HSPA5*), cellular

function and signaling (*RAP1B*, *PIK3C2A*, *RRAS2*), transport and cytoskeleton organization (*VAMP3*, *TUBB6*, *TUBA1C*, *ACTN1*), cell-cell interactions (*CD151*), tight junction formation (*CLDN12*), heparin biosynthesis and cell signaling (*EXT2*), and protein degradation (*UBE2B*).

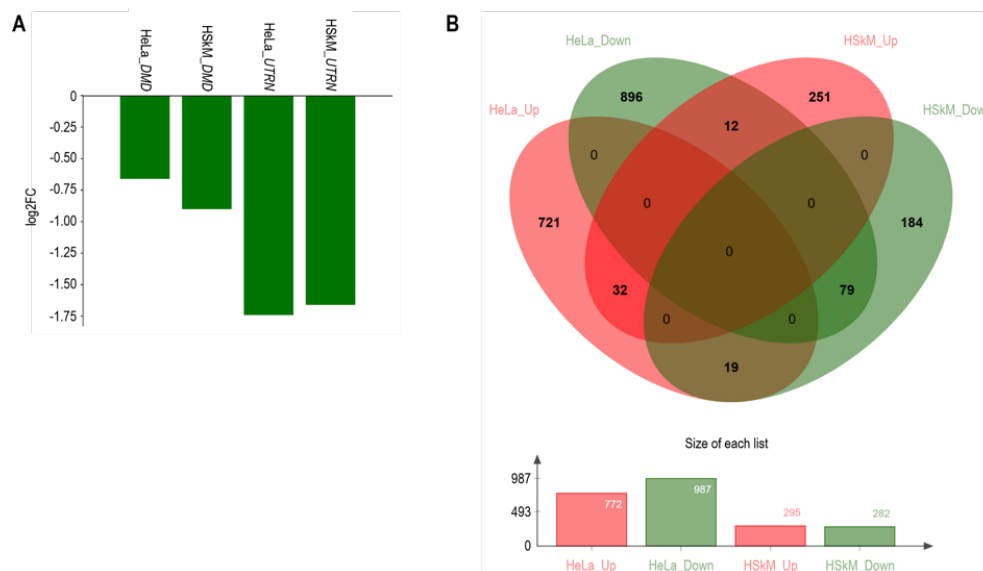


Figure 8: Dystrophin- and utrophin-deficient HeLa cells and HSkM myotubes show profound transcriptomic changes that correlate with phenotypic alterations.

A Expression levels of the *DMD* and *UTRN* genes in *DMD*ko+*siUTRN* HeLa cells and HSkM myotubes after *siDMD/UTRN* delivery compared to control samples, presented as log2FC values. **B** Venn diagram showing the number of up- and down-regulated genes in dystrophin- and utrophin-deficient HeLa cells and HSkM myotubes compared with control samples. Changes in expression fold were tested using the Wald test. The obtained p-values from the statistical test were adjusted for multiple testing using the Benjamini-Hochberg method ($FDR \leq 0.05$).

Analysis of KEGG pathways in *DMD*ko+*siUTRN* HeLa cells revealed significant changes in the expression of genes related to the cell cycle (Fig. S16), whereas in HSkM myotubes, the silencing of *DMD* and *UTRN* caused significant alterations in the expression of genes related to cytoskeleton organization, muscle contraction and motor proteins (Figs. S17-S19), receptor-extracellular matrix (ECM) and -cytokine interactions (Figs. S20 and S21), and in cardiomyopathy (Figs. S22-S24). Taken together, these transcriptomic changes correspond well to the phenotype observed in both dystrophin- and utrophin-deficient proliferating cells and myotubes.

DISCUSSION

To evaluate the role of different dystrophin isoforms and the compensatory role of utrophins in myoblasts and muscle fibers, we used the NTX-induced EDL muscle regeneration system, satellite cell-derived myofiber cultures, immortalized and primary cell lines, and RNA-sequencing analyses. We show that DP71 and DP427 play important roles in cell viability during proliferation and fiber differentiation, respectively. We further show that utrophin can partially compensate not only for DP427 but also for DP71. These results 1) provide an explanation for the observed differences in disease severity among patients who differ in the presence of DP71 and 2) indicate that patients deficient in both DP71 and DP427 in skeletal muscle might require a different therapeutic approach than patients deficient in only DP427.

Individuals with distal mutations in exons 63-79 of the *DMD* gene, which abolish translation of DP71 in addition to DP427, DP260, DP140 and DP116, constitute approximately 10% of all DMD patients^{4, 38}. Previous studies have shown that these patients have significantly lower stature and motor function than those with preserved DP71 translation^{38, 39}. Studies in mice have only partially confirmed these results. On the one hand, dystrophin-null mice, which lack all the dystrophin isoforms, presented significantly more severe muscle disease than did *mdx* mice because they showed decreased myofiber area, increased central nucleation, elevated fibrosis, fat accumulation, exacerbated calcification and altered infiltration patterns⁴⁰. On the other hand, the grip strength and running time of the mice lacking DP427 and DP140 were similar to those of the mice lacking DP71, indicating that the absence of DP140, an isoform synthesized specifically in the brain and kidney, could be responsible for the observed phenotype^{39, 41}. The discrepancies between the murine and human studies could be explained by the differences in the genetic background of the mouse strains, the age of the analyzed mice, or the species-dependent function of dystrophin in muscles and other tissues.

Because DP71 is ubiquitously synthesized throughout the body and constitutes the major isoform of dystrophin in the brain^{2, 5, 42, 43}, its absence in other tissues or cell types may indirectly affect muscle tissue performance. On the basis of experimental data, it has been speculated that the loss of DP71 or DP71/DP140 exacerbates muscle disease through alterations in the performance of lymphoblasts or brain cells, respectively^{39, 40}. Our data on HEK and HeLa cells are consistent with this view; however, we also show that the lack of DP71 has a direct effect on muscle cells, as they showed

increased cyto- and genotoxicity and apoptosis. Previous data also indicated a role for DP71 in cell proliferation ⁷, with specific splice variants having opposing functions in the process ⁶. While ectopic expression of DP71 in myotubes in *mdx* transgenic mice showed restored DGC, muscular dystrophy was not alleviated ^{9, 10}. It would be interesting to test whether the phenotype could be reduced by specific synthesis of DP71 in myoblasts/early myotubes deficient in all dystrophins.

PAX7 marks quiescent satellite cells, is maintained during proliferation, and is then lost during myogenic differentiation ⁴⁴. Our immunofluorescence microscopy analysis revealed threefold greater numbers of PAX7+ cells on DP427/utrophin-deficient myofibers than on wt myofibers; however, their myogenic potential was markedly lower than that of wt cells, as demonstrated in satellite cell-derived cultures. Their numbers also did not increase as significantly as those in wt muscles treated with NTX did. Similarly, previous studies reported increased numbers of PAX7+ cells in *mdx* mice and DMD patients and decreased myogenic potential of *mdx* and DMD myoblasts as well as CD133+ cells ^{45, 46, 47, 48, 49, 50}. Since we did not observe DP427 in proliferating PAX7+ cells, it could be concluded that this phenotype is due to a secondary effect of the disease, as previously indicated ⁴⁹. However, DP427 was shown to be present in activated satellite cells, where its absence leads to defective asymmetric divisions and increased symmetric expansion ^{30, 50}. Interestingly, we observed 7-fold and 3-fold greater numbers of PAX7+ cells on wt myofibers 12 and 24 days after NTX treatment, respectively. We hypothesize that this increase in PAX7+ cells after injury represents an intrinsic mechanism by which cells remain in an 'alert state' that allows for efficient and timely regeneration during the subsequent degenerative cycle. Since we observed an approximately 50% reduction in the number of BrdU+ nuclei in dko fibers 12 days after NTX treatment, we conclude that a large pool of PAX7+ cells in diseased muscles is in constant readiness to generate new fibers. Three days after NTX treatment, we observed faster dko fiber generation than wt fibers did, and a greater percentage of myogenin+ cells was observed in dko fiber-derived cultures at the earliest time point analyzed, which supports this hypothesis. We also speculate that as the disease progresses, this protective mechanism ceases to function because of telomere shortening, as previously shown by Sacco et al. ⁵¹.

Our data indicate that the increased cyto- and genotoxicity, and apoptosis of DP71-deficient myoblasts, HEK, and HeLa cells result from membrane damage, increased intracellular calcium levels, mitochondrial aggregation, changes in the nuclear area, and elevated ROS. This phenotype may be a direct consequence of reduced DGC levels, as in DP427-deficient myotubes. Indeed, previous

experimental data have shown that the absence of β -dystroglycan leads to an increased nuclear area in myoblasts^{52, 53}. Although the senescence of β -dystroglycan-deficient myoblasts is controversial^{52, 53}, it should be noted that mice with the conditionally deleted dystroglycan (*Dag1*) gene in myotubes had a relatively mild phenotype, unlike chimeric ko mice, indicating the crucial role of the DGC in the early stages of differentiation^{54, 55}. Interestingly, we observed that β -dystroglycan is less dependent on DP71/DP427 levels than other DGC components are, and on the basis of these results, we conclude that the complex is assembled starting with dystroglycans, then dystrophins, and then other complex components, including sarcoglycans, syntrophins, and dystrobrevins.

Genotoxicity and structural nucleus changes in the nucleus could be directly linked to the presence of DGCs (or DG-like complexes)^{53, 56}. However, we failed to detect significant levels of DP71 in this organelle. In contrast, we recorded its location in the cytoplasm and membrane. Indeed, previous results have shown that DP71 can form the DGC at the membrane and that it can be found in the cytoplasm^{9, 10, 56, 57}. The pathological phenotype was also much more pronounced in utrophin-deficient cells, indicating that, as in the case of DP427^{12, 19}, UP395 can compensate for its absence. Some previous results have indicated that dystrophin and utrophin exclude their presence at the sarcolemma⁵⁸. However, more recent data contradict this view^{12, 59}. Our results also indicate that DP71 and UP395 can coexist in myoblasts and at early stages of muscle fiber differentiation.

Previous data linked myofiber death in DMD to contraction-induced muscle damage^{60, 61}, calcium influx through mechanosensitive channels⁶², stretch-activated generation of ROS due to disorganized microtubules/NOX2^{62, 63}, changes in signal transduction influenced in part by loss and mislocalization of sarcolemmal nNOS^{2, 62, 64}, and a persistent chronic immune response^{65, 66}. Our results indicate that neither mechanical stress nor immune cell infiltration nor changes in NMJs, myotendinous junctions or other cell types are required for the pathological phenotype and the death of myofibers^{54, 67}. Furthermore, the observed increases in intracellular calcium, mitochondrial aggregation, and ROS production in proliferating cells and myotubes independent of dystrophin isoform loss may indicate that disease-related changes are only partially driven by nNOS- and microtubule-associated alterations because neither DP71 nor UP395 have nNOS binding sites, and DP427 and UP395 have different microtubule binding properties^{8, 68}. Direct comparisons of wt and dko muscles also revealed no obvious changes in the plectin/desmin cytoskeleton when the regenerative state of muscle fibers was considered¹⁷. Our data indicate that loss of DP71/DP427 induces increased membrane permeability, influx of

calcium ions, and mitochondrial aggregation and dysfunction, leading to increased cell death. Importantly, similar pathological changes were observed in non-muscle dystrophin-deficient HEK293 and HeLa cells as well as in brain tissue ⁶⁹.

In agreement with the observed phenotypic abnormalities, RNA-sequencing analyses revealed profound transcriptomic changes in both dystrophin- and utrophin-deficient HeLa cells and HSkM myotubes related to oxidative stress, mitochondrial function, structural and signaling alterations and apoptosis. The analysis showing both increased and decreased gene expression suggests that cells attempt to compensate for the loss of dystrophin and utrophin, but this is accompanied by a cellular imbalance. The increased expression of some genes (e.g., *STAC3*, *GDF15*, *MCL1*) may reflect attempts to compensate for damage, such as increasing protection against oxidative stress, inhibiting apoptosis, or supporting muscle regeneration. Conversely, decreased expression of other genes (e.g., *ITGA6*, *ITGB1*, *SCD*) may result from disruptions in cell signaling, metabolism, or regeneration. This cellular response indicates that cells are trying to counteract the loss of dystrophin and utrophin but are unable to fully compensate, resulting in progressive pathology. *DMDko*+*siUTRN* HeLa cells also revealed the down-regulation of *PLK1*, *SKP2*, *MDM2*, and *APC/C*, which might impair mitotic progression and activate cell cycle arrest at the G1/S and G2/M phases, whereas the up-regulation of *RAD21*, *PP2A*, *E2F*, and *MCM* may exacerbate replication stress and chromosomal instability. Consequently, these transcriptomic alterations might result in inhibited proliferation, accumulation of DNA damage, increased apoptosis, and errors in cell division. The loss of cytoskeletal proteins might also destabilize signaling between the cell membrane and the nucleus, further impairing repair and control mechanisms ^{70, 71}.

While the current therapeutic studies have focused on skeletal muscle therapy for DMD patients in the context of full-length dystrophin, it is becoming clear that DMD patients clearly require therapeutic intervention in other tissues such as the heart and brain ². Nervous tissue can be difficult to treat owing to the large number of dystrophin isoforms and their splicing variants, which are present in a cell- and developmental time-dependent manner. Here, we show that the treatment of skeletal muscle may be more complex than previously thought and that 10% of DMD patients who also lack DP71 in addition to DP427 may require a specifically tailored combination therapeutic approach.

CONFLICT OF INTEREST

JSC is one of the inventors on patents covering muscle-specific gene editing and patents covering muscle-specific gene regulatory cassettes, micro-dystrophins, and systemic delivery of AAV. JSC holds equity in and is a member of the scientific advisory board of Solid Biosciences. All the other authors declare that they have no competing interests.

AVAILABILITY OF DATA AND MATERIALS

RNA-seq data from HeLa cell lines are available at GEO accession GSE289357 at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE289357> by entering token yjqxmusclrovfgl into the box.

RNA-seq data from HSkM cell lines are available at GEO accession GSE303672 at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE303672> by entering token enkpkeuwutfenfkh into the box.

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AUTHOR CONTRIBUTION

PK, SS, AD, JSC, PKW designed the study. Data contribution: PK, figs. 1-3, S1-S5; SS, figs. 5, 6A, 7A-B, F, 8, S9-10, S13A, HeLa in figs. 6B-E, 7C-D, S14, S15A, HSkM in S8; AD, figs. 4, 7B, S6-S7, S11-12, S13B, C2C12 in S8; PKW, figs. 8, S16-S24, datasets S1-S2. OO, fig. 5D, HEK293 in 6B-E, 7C-D, S14; KD, figs. 7E, S15B. PK, SS, AD, PKW wrote the manuscript and prepared the figures.

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SUPPLEMENTARY INFORMATION

Dystrophins DP71 and DP427 determine cell viability during proliferation and myofiber differentiation

Running title: Dystrophins in cell proliferation and differentiation

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Table S1: A list of primers, sgRNA and siRNAs used in the study.

Target	Species	Forward	Reverse
<i>RPLP0</i>	Human	CGTCCTCGTGGAAGTGACAT	CTTGGAGCCCACATTGTCTG
<i>DMD</i>	Human	GCTTCCTTTGGGGGCAGTAA	CACTTGGCTTGATGCTTGGC
<i>UTRN</i>	Human	AAACTCCTCAGGCAGCACAA	GAGGCATCTGGATCAAGCGA
<i>Dp427</i>	Human	CTGCACTAGGCTGAATGGGAA	CTCCGCCAGGAATGTTTTTCAG
<i>Dp71</i>	Human	CTCGTACCCACACTCGACCG	TGCTGGTCCAAGGCATCACA
<i>PAX7</i>	Human	CACTGTGCCCTCAGGTTTAGT	GTTCCGACTCCACATCCGAG
<i>MYOD</i>	Human	CGCAACGCCATCCGCTAT	TGTAGTCCATCATGCCGTCG
<i>MYOG</i>	Human	TGCCATCCAGTACATCGAGC	CAGATGATCCCCTGGGTTGG
<i>MYH1</i>	Human	AGGTCGCATCTCTACGCCA	CGCTCCCTTTCAGACTTTTCG
<i>mtDNA_ ND1</i>	Human	TACGGGCTACTACAACCCTTC	ATGGTAGATGTGGCGGGTTT
<i>gDNA_ SERPINA</i>	Human	CAGTGAATAAATGAGGCGTACATCC	GACTGTTTCTCATGCCTCTGGAAAG
<i>CRISP_ DMD</i>	Human	GAGAGTGTGGTTCACGTTTGG	AGGGCCCTGTTGTAATGCTAA
<i>Rplp0</i>	Mouse	GCTTTGGGCATCACCACGAA	GTTGCGGACACCCTCCAGAA
<i>Dmd</i>	Mouse	GCTTCCTTTGGGGGCAGTAA	CACTTGGCTTGATGCTTGGC
<i>Utm</i>	Mouse	CTGACTCTGACTCCCGCATC	GTCCGTGCTAGTGTCGTGAG
<i>Dp427</i>	Mouse	CTCCACCCTCAGCACAAAGAG	TCATGCCAACATGCCCAAAC
<i>Dp71</i>	Mouse	ATGAGGGAACAGCTCAAAGG	TGCAGCTGACAGGCTCAAGA
<i>Pax7</i>	Mouse	CTGCTGAAGGACGGTCACTG	ATCCAGACGGTTCCTTTGT
<i>Myod</i>	Mouse	GCTACGACACCGCCTACTAC	GGTGGTGCATCTGCCAAAAG
<i>Myog</i>	Mouse	AATGCACTGGAGTTCGGTCC	CACCCAGCCTGACAGACAAT
<i>Myh1</i>	Mouse	TCCCTAAAGGCAGGCTCTCTC	AACACCGATGACTTGGCGT
<i>mtDNA_Nd 1</i>	Mouse	CTAGCAGAAACAAACCGGGC	CCGGCTGCGTATTCTACGTT
<i>gDNA_ Hk2</i>	Mouse	GCCAGCCTCTCCTGATTTTAGTGT	GGGAACACAAAAGACCTCTTCTGG

Name	Species	Sequence
sgRNA		
<i>DMD</i> ex65	Human	GACCACUAUUUUAUGACCGCC
siRNA		
<i>DMD/Dmd</i> (s501299)	Human, mouse	AAACAAAUUUCGAACCAAAtt
<i>UTRN</i> (s553059)	Human	GGAAUAUUGUAUACCUACAAtt
<i>Utn</i>	Mouse	CGAAUGAAGUUUUCAGCAAtt

Table S2: A list of antibodies used in the study.

Antibody	Species	Concentration		Manufacturer
		WB	IF	
α -Actinin	Mouse	1:1000		Sigma (A7811)
ATP synthase α (7H10)	Mouse	1:500 (muscle)		Invitrogen (A21350)
ATP Synthase β (3D5AB1)	Mouse	1:1000	1:200	Thermo Fisher (A21351)
BrdU (BU 1/75)	Rat		1:50	Novus Biologicals (NB500-169)
Caveolin 3	Rabbit	1:2000	1:500	Abcam, ab2912
Desmin (Clone D33)	Mouse	1:750	1:100	Agilent Dako (M076029)
Pan α -Dystrobrevin	Mouse	1:1000	1:100	BD Tranduction Laboratories (610766)
β -Dystroglycan (43DAG1/8D5)	Mouse	1:1000	1:10-1:50	Abcam (ab49515), Novocastra (NCL-b-DG)
Dystrophin (MANDRA1)	Mouse	1:2000	1:100	Sigma–Aldrich (ab7164); Santa Cruz Biotechnology (sc-73592)
Dystrophin	Rabbit	1:1000	1:50	Abcam (ab15277)
Dystrophin	Rabbit	1:2000	1:200	Proteintech (12715-1-AP)
Dytrophin 303-3 (N-terminus)	Rabbit		1:600	J. S. Chamberlain
ERTR7	Rat		1:300	Acris
Lamin B1	Rabbit		1:500	Abcam (ab16048)

MyoD (M-318)	Rabbit		1:100	Santa Cruz (sc-760)
Myogenin (F5D)	Mouse		1:20	DSHB
Myosin (B-5)	Mouse		1:50	Santa Cruz Biotechnology (sc-376157)
Myosin (MF-20)	Mouse		1:20	DSHB
Anti-Pax7 (concentrate or supernatant)	Mouse		1:10 (conc.) or undiluted (supern.)	DSHB
Phalloidin-iFluor 647 reagent			1:1000	Abcam (ab176759)
Plectin	Rabbit	1:1000		Abcam (ab83497)
Plectin #46	Rabbit		1:100	provided by G. Wiche
Plectin 1	Rabbit	1:2000	1:500	provided by G. Wiche
Plectin 1f	Rabbit	1:200	1:50	Provided by G. Wiche
β -Sarcoglycan (BSarc/5B1)	Mouse	1:200		Novocastra
γ -sarcoglycan (35DAG/21B5)	Mouse	1:500	1:100	Novocastra
α 1-syntrophin (1351)	Mouse		1:1000	provided by S. Froehner
α 1-syntrophin (SYN17)	Rabbit	1:2000		provided by S. Froehner
α -Tubulin	Mouse	1:500		Sigma (T6074)
α -Tubulin (DM1A)	Mouse	1:2000	1:500	Abcam (ab7291)

Utrophin (MANCHO3)	Mouse	1:200	1:100	Developmental Studies Hybridoma Bank (8A4)
Utrophin (MANCHO7)	Mouse	1:500	1:50	Santa Cruz Biotechnology (sc-81557)
Utrophin (UTR316)	Rabbit	1:1000	1:500	provided by S. Froehner
VDAC1/Porin	Rabbit	1:500 (muscle)		Abcam (ab15895)
VDAC1/Porin	Rabbit	1:1000	1:100	Sigma-Aldrich (SAB5700655)
Vimentin	Mouse		1:500	Abcam (ab20346)
Alexa 488 anti-rabbit	goat		1:1000	Molecular Probes
Alexa 488 anti-rabbit IgG H&L	donkey		1:400	Abcam (ab150073)
Alexa 488 anti-rat	goat		1:1000	Molecular Probes
Alexa 594 anti-mouse	goat		1:1000	Molecular Probes
Alexa 488, streptavidin			1:1000	Molecular Probes
Alexa 594, streptavidin			1:500	Molecular Probes
Cy5 anti-rabbit	goat		1:1000	Jackson Immuno Research
Rhodamine (TRITC) anti-mouse IgG (H+L)	donkey		1:400	Jackson Immuno Research
anti-mouse IgG (Fab specific)–peroxidase antibody	goat	1:10 000		Sigma–Aldrich (A9917)
anti-rabbit IgG	goat	1:10 000		Abcam (ab97051)

anti-mouse HRP-conjugated IgGs	donkey	1:20 000		Jackson Immuno Research
anti-rabbit HRP-conjugated IgGs	donkey	1:20 000		Jackson Immuno Research,

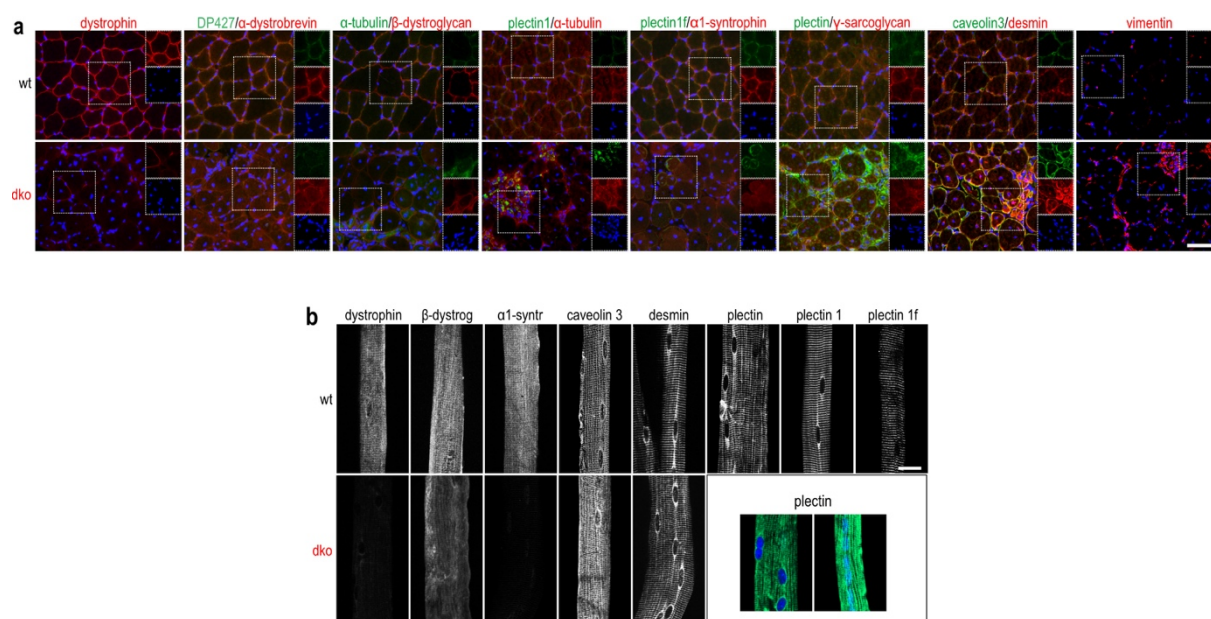


Figure S1: Loss of dystrophin DP427 and utrophin in skeletal muscle leads to proteomic changes.

A Frozen sections of EDL muscles from wt and dko mice immunostained for costameric and cytoskeletal proteins and nuclei (blue channel, DAPI). The insets show separate red, green and blue channels. Bar, 50 μ m. **B** Teased single fibers from wt and dko EDL muscles immunostained for costameric and cytoskeletal proteins. The inset shows two dko fibers differing in their regenerative state, immunolabeled with anti-plectin antibody and DAPI. Bars, 50 μ m (**A**) and 20 μ m (**B**).

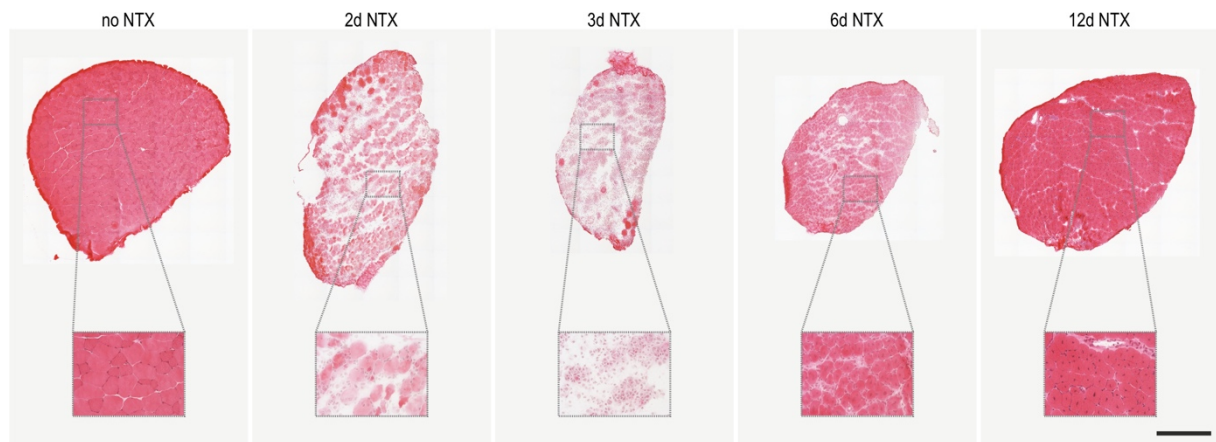


Figure S2: Notexin (NTX) induces effective degeneration of muscle fibers and their subsequent regeneration from activated muscle stem cells.

Composite images of sections from either uninjected (no NTX) or NTX-treated EDL muscles. Wt muscles were collected at various timepoints after NTX injection to assess efficiency of NTX-induced muscle damage and subsequent regeneration and maturation of myofibers. 2 days post NTX injection, massive myofiber death and infiltration of immune system cells was evident. By day 3, most of the dead fibers were removed and muscles were filled with mononuclear cells. 6 days post NTX *de novo* formed fibers were apparent, which then increased in size 12 days post NTX. Bar, 500 μ m.

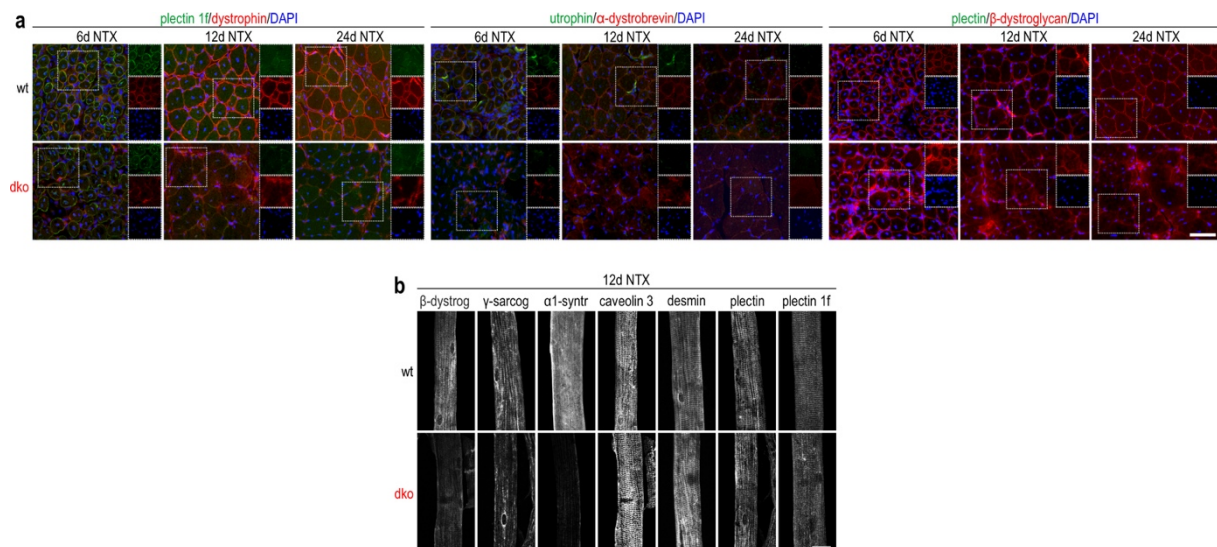


Figure S3: NTX-induced regeneration reveals complementary expression of *DMD* and *UTRN* genes and an increase in DGC proteins during fiber differentiation.

A Frozen sections of EDL muscles isolated 6, 12 and 24 days post NTX injection from wt and dko mice immunostained for costameric and cytoskeletal proteins and nuclei (DAPI). The insets show separate red, green and blue channels. **B** Teased single fibers from wt and dko EDL muscles harvested 12 days after NTX treatment, immunostained for costameric and cytoskeletal proteins. Bars, 50 μ m (**A**) and 20 μ m (**B**).

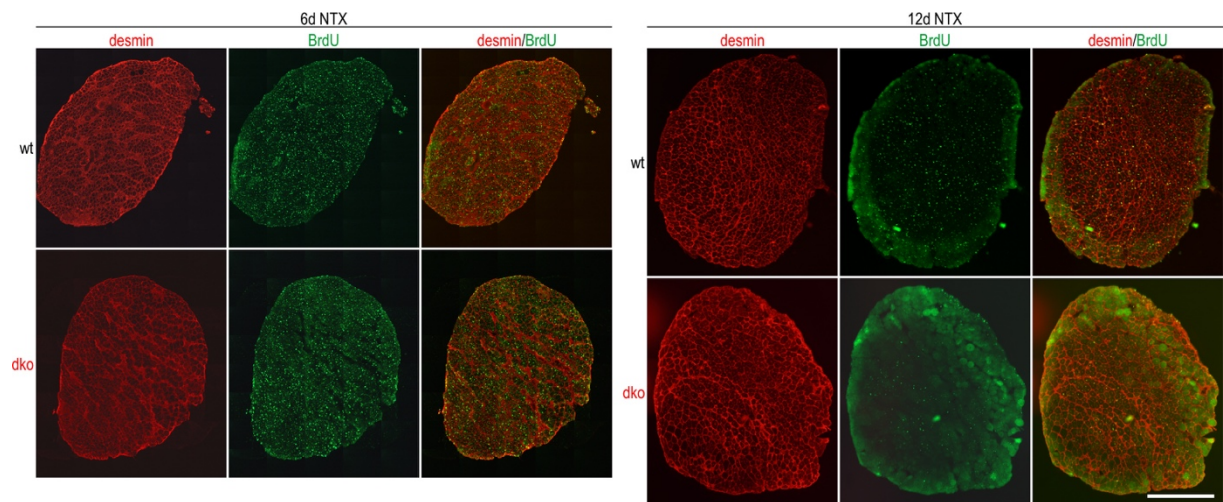


Figure S4: BrdU injections into wt and dko mice after NTX treatment reveal lower lifespans of dko myofibers.

BrdU was injected subcutaneously 2 and 3 days post EDL NTX injection. The muscles were isolated 6 and 12 days after NTX administration. Bar, 500 μm .

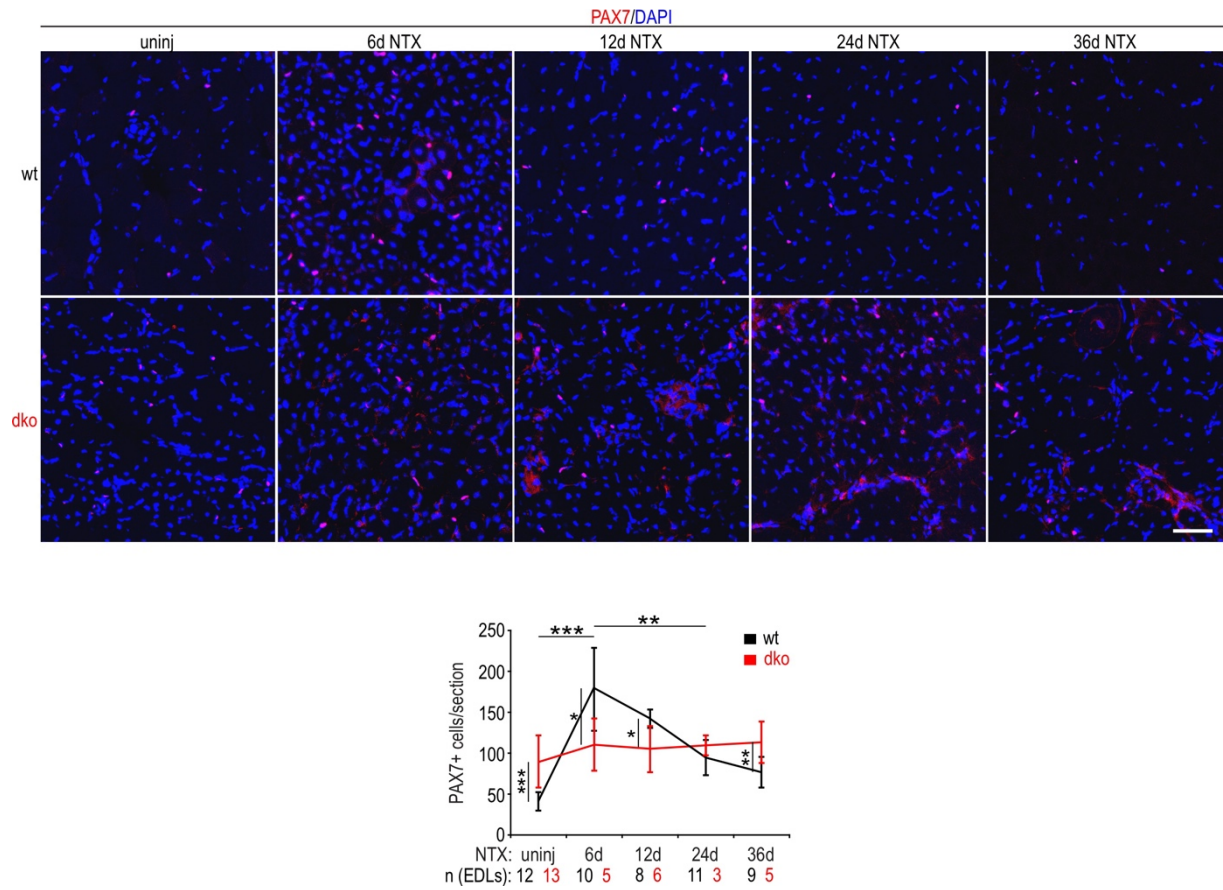


Figure S5: NTX treatment reveals high myogenic potential of wt EDL muscles.

Frozen sections from wt and dko EDL muscles immunolabeled for PAX7 and nuclei (DAPI) and quantification of the numbers of PAX7+ nuclei in uninjected and NTX-treated wt and dko EDL muscle sections. The results are shown as the mean values $\pm$ SD. n, number of biological replicates (EDL muscles). Bar, 50 μ m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (t-test).

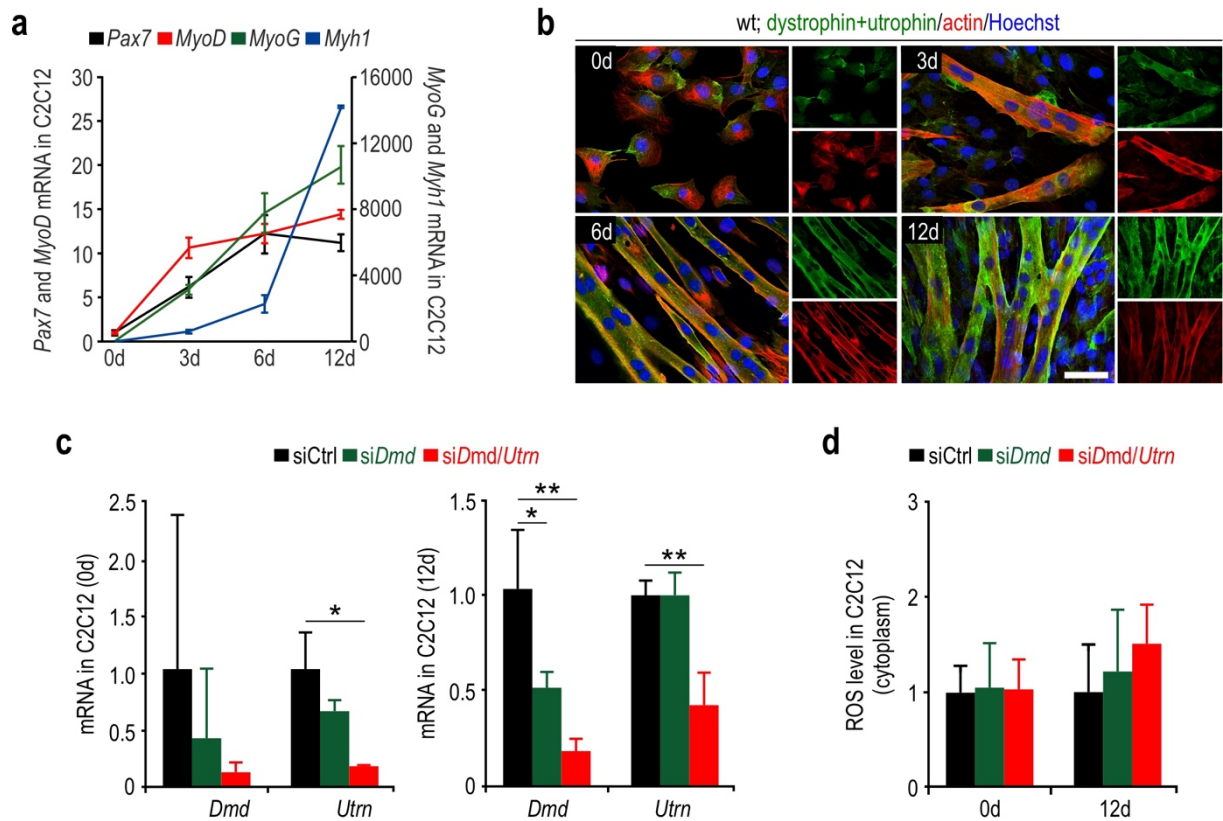


Figure S6: Dystrophin and utrophin are synthesized at various stages of myogenic differentiation.

A RT PCR analyses of myofiber differentiation markers (*Pax7*, *MyoD*, *MyoG*, *Myh1*) in the C2C12 cell line at different time points during differentiation. **B** Images of C2C12 myoblasts and myotubes immunolabelled for dystrophin and utrophin (Proteintech antibody, see also Fig. S7), actin and nuclei (Hoechst). Bar, 50 μ m. **C** RT PCR analysis of *Dmd* and *Utrn* knockdown efficiency using siRNA in proliferating (0d) and differentiated (12d) C2C12 cells. **D** ROS production in dystrophin- and dystrophin and utrophin-deficient C2C12 cells. The results in **A** are shown as the mean values $\pm$ SD and those in **C**, **D** as the mean values + SD. Bar in **B**, 50 μ m. * P <0.05, ** P <0.01 (t-test).

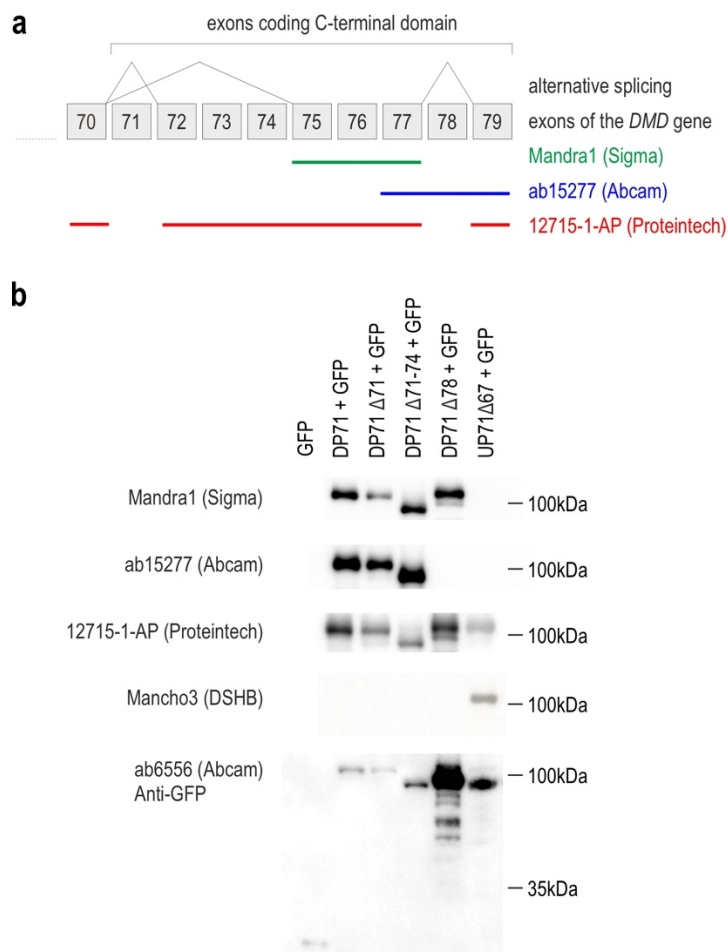


Figure S7: Comparison of the specificity of commercially available antibodies against dystrophin and utrophin.

A The dystrophin protein regions used as immunogens to obtain commercially available dystrophin antibodies. **B** A collection of vectors encoding GFP and GFP fusion proteins with different splicing variants of dystrophin isoform DP71 or utrophin UP71 were used to transfect *DMD* ko HEK293 cell line and prepare protein extracts for testing the specificity of various commercially available antibodies against dystrophin and utrophin by Western blot analysis. Note that whereas the dystrophin antibody 12715-1-AP (Proteintech) also recognizes utrophin, it detects all dystrophin isoforms and was most reliable in cell immunofluorescence and immunoblot studies.

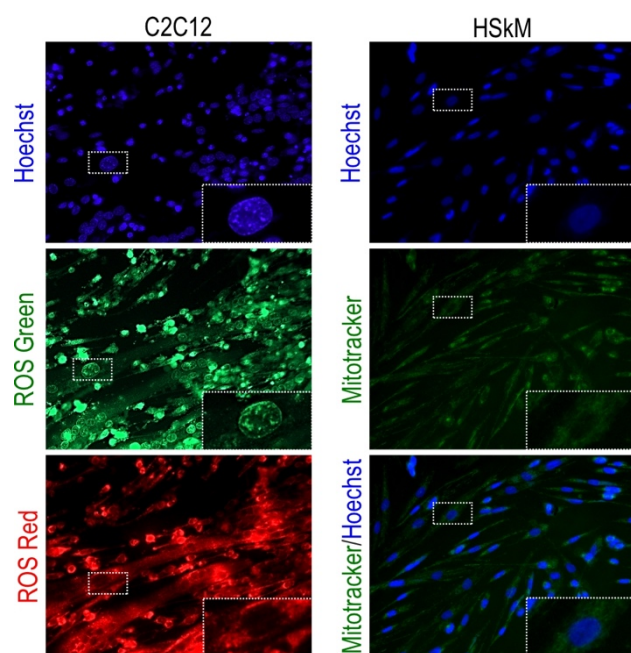


Figure S8: Microscopic validation of reagents for TECAN-based quantification of ROS production and mitochondrial content.

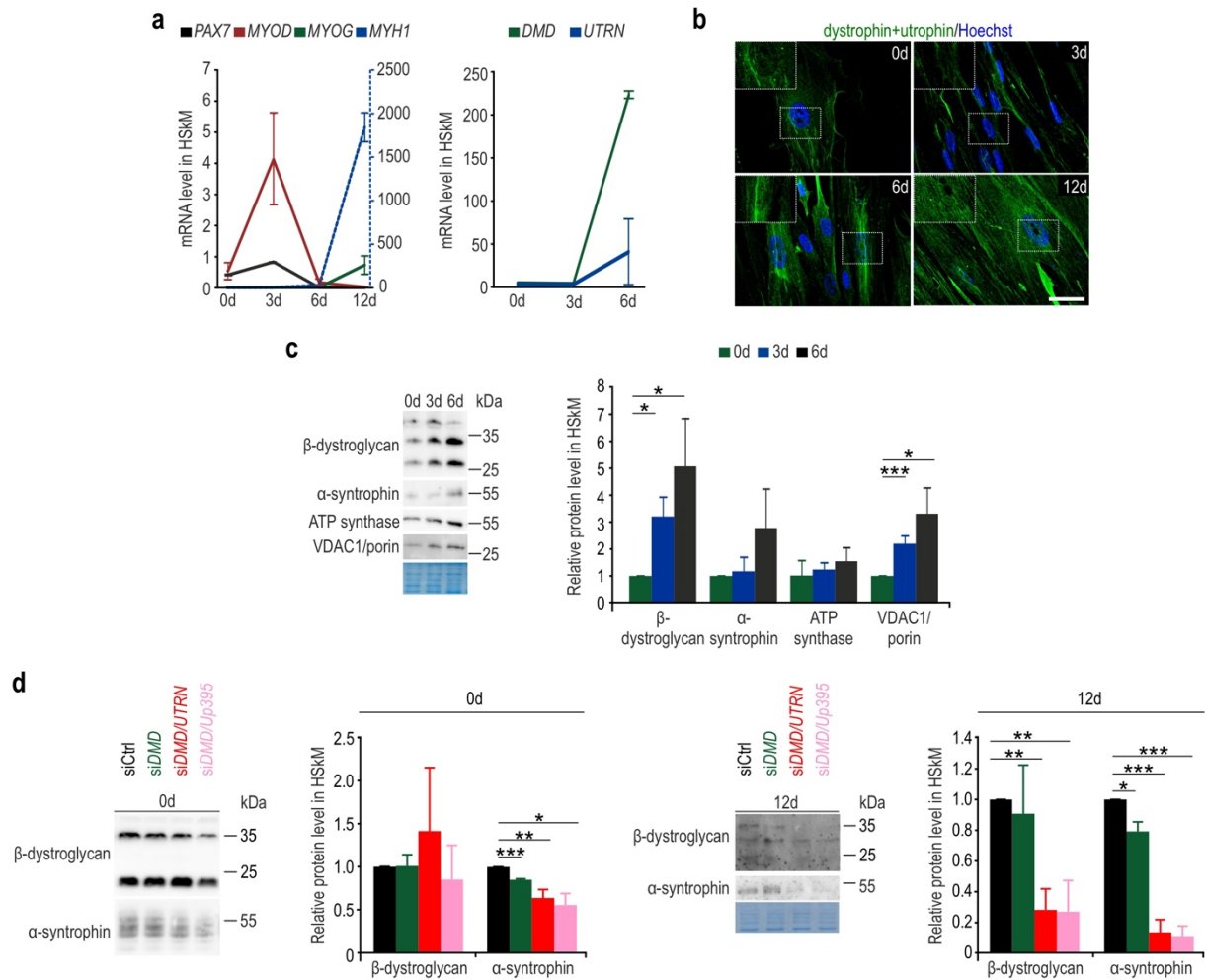


Figure S9: Downregulation of *DMD* and *UTRN* genes in proliferating and differentiated HSKM cells leads to reduction of DGC proteins.

A RT-PCR analysis of myofiber differentiation markers (*PAX7*, *MYOD*, *MYOG*, *MYH1*) along with the *DMD* and *UTRN* mRNA in the HSKM cell line at different time points during differentiation. **B** Immunostaining for dystrophin/utrophin (Proteintech antibody, see also Fig. S7), and nuclei (blue channel; Hoechst) in HSKM cells at 0, 3, 6 and 12 days of differentiation. The insets show the separate green channel for dystrophin/utrophin labeling. **C** Immunoblotting analysis of HSKM protein extracts from days 0, 3 and 6 of differentiation, with densitometric evaluation of band intensities relative to HSKM levels at day 0. Coomassie-stained polyacrylamide gels were used for normalization. **D** Immunoblot analysis of β-dystroglycan and α-syntrophin levels in HSKM myoblasts and myotubes after siRNA transfection, with densitometric evaluation of band intensities relative to wt levels. Coomassie-stained polyacrylamide gels (for d0, see Fig. 5a) were used for normalization. The results in **A** are shown as the mean values ± SD and those in **C**, **D** as mean values + SD. *P<0.05, **P<0.01, ***P<0.001 (t-test). Bar in **B**, 100 μm.

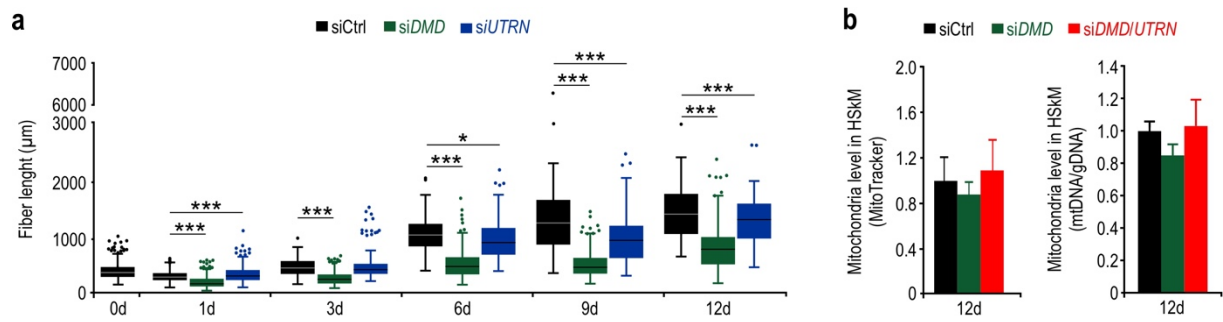


Figure S10: Dystrophin and utrophin deficiency affect fiber length in HSkM myotubes.

A Quantification of HSkM fiber lengths during differentiation after *DMD* and *UTRN* gene expression silencing. **B** Analysis of mitochondrial contents on day 12 of HSkM differentiation after siRNA transfection, based on the MitoTracker levels and mtDNA/gDNA ratio. The results in **A** are shown as mean values $\pm$ SD and in **B** as mean values $\pm$ SD. * $P < 0.05$, *** $P < 0.001$ (z-test in **A** and t-test in **B**).

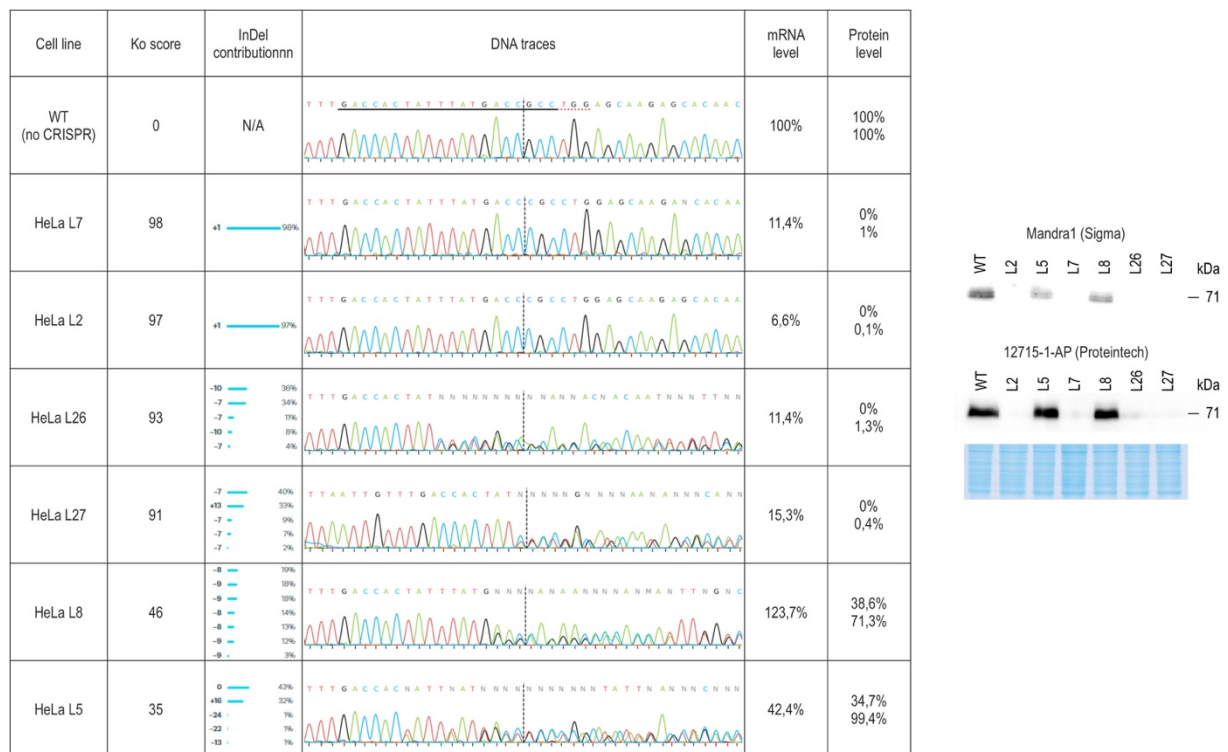


Figure S11: Characterization of CRISPR/Cas9 *DMD* ko HeLa cell lines.

Knockout (Ko) scores, InDel distribution and DNA traces were obtained after Sanger sequencing and data analyses with Synthego ICE Analysis Tool. Transcript (mRNA) levels were based on relative RT-PCRs. Protein levels were calculated by immunoblot analysis with Mandra 1 and 12715-1-AP Proteintech antibodies. Coomassie-stained polyacrylamide gel fragment shows equal sample loading. The order of *DMD* ko cell HeLa lines is based on the ko score.

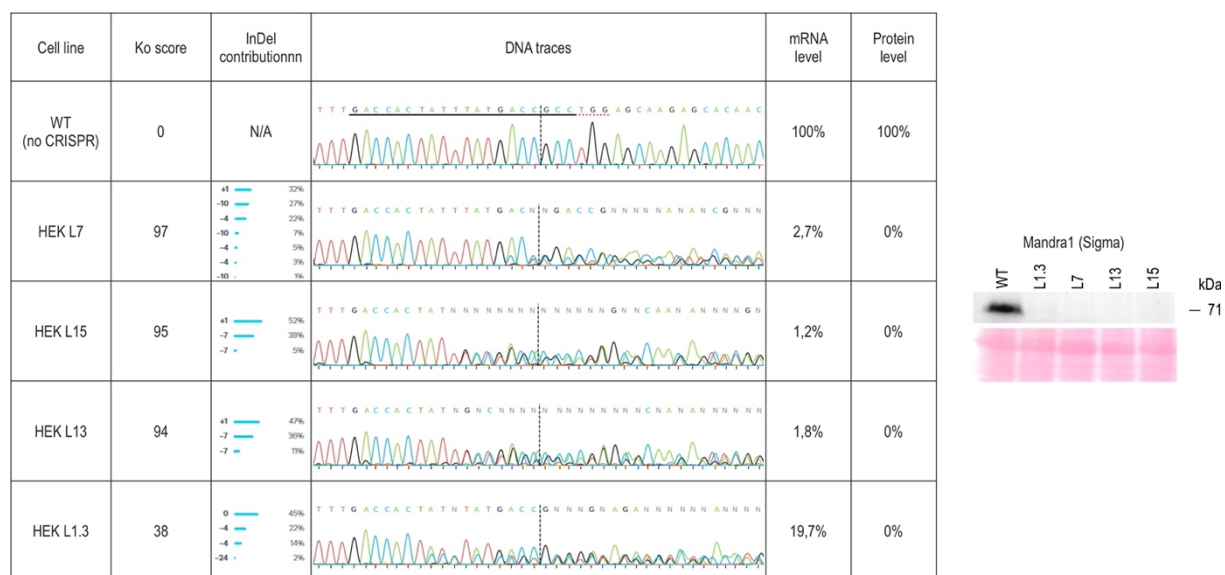


Figure S12: Characterization of CRISPR/Cas9 *DMD* ko HEK293 cell lines.

Ko scores, InDel distribution and DNA traces were obtained after Sanger sequencing and data analyses with Synthego ICE Analysis Tool. Transcript (mRNA) levels were based on relative RT PCRs. Protein levels were calculated by immunoblot analysis with Mandra 1 antibody. The Ponceau-labeled membrane fragment shows equal sample loading. The order of *DMD* ko cell HEK293 lines is based on the ko score.

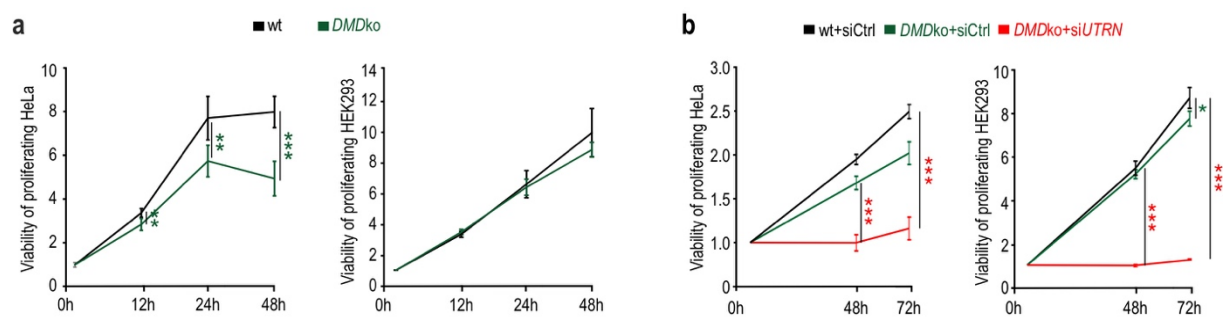


Figure S13: Utrophin deficiency in *DMD* ko HeLa and HEK293 cells suppresses cell proliferation.

A, B Assessment of viability of proliferating wt and *DMD* ko HeLa and HEK293 cells for 48 hours (**A**) and wt and *DMD* ko HeLa and HEK293 cells for 72 hours after siRNA treatment (**B**). The results are shown as mean values $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (t-test).

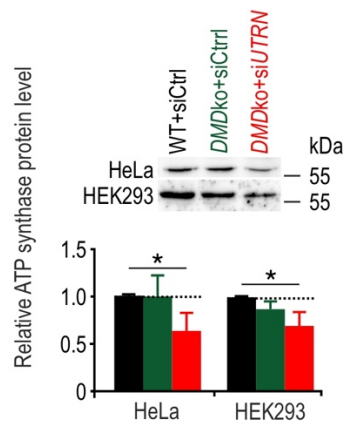


Figure S14: Utrophin deficiency in *DMD* ko HeLa and HEK293 cells suppresses ATP synthase production.

Immunoblot analysis of protein extracts from proliferating wt and *DMD* ko HeLa and HEK293 cells after siRNA transfection, with densitometric evaluation of band intensities normalized to wt levels. Coomassie-stained polyacrylamide gels from Fig. 6C were used for normalization. The results are shown as mean values + SD. *P<0.05 (t-test).

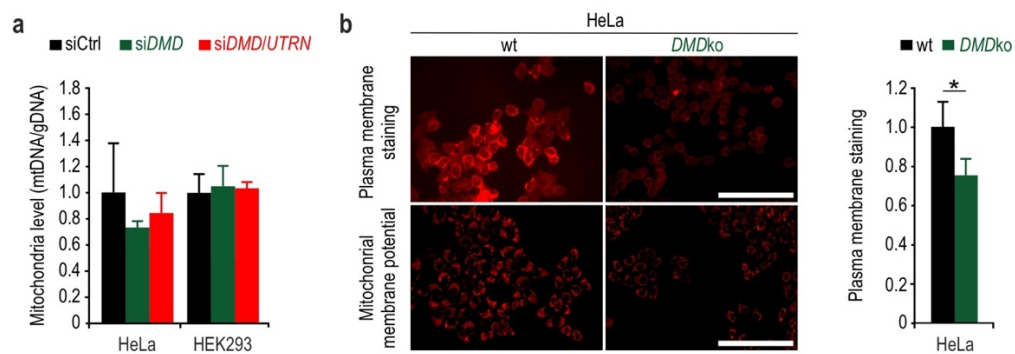


Figure S15: *DMD* ko HeLa cells show reduced plasma membrane and mitochondrial membrane potential.

A Mitochondria level analysis in wt HeLa and HEK293 cells after *DMD* or *DMD/UTRN* downregulation. **B** Images of wt and *DMD* ko HeLa cells stained for plasma membrane and mitochondrial membrane potential and quantification of the plasma membrane staining using Tecan SPARK plate reader. Bar, 100 μ m. The results are shown as mean values + SD. * $P < 0.05$ (t-test).

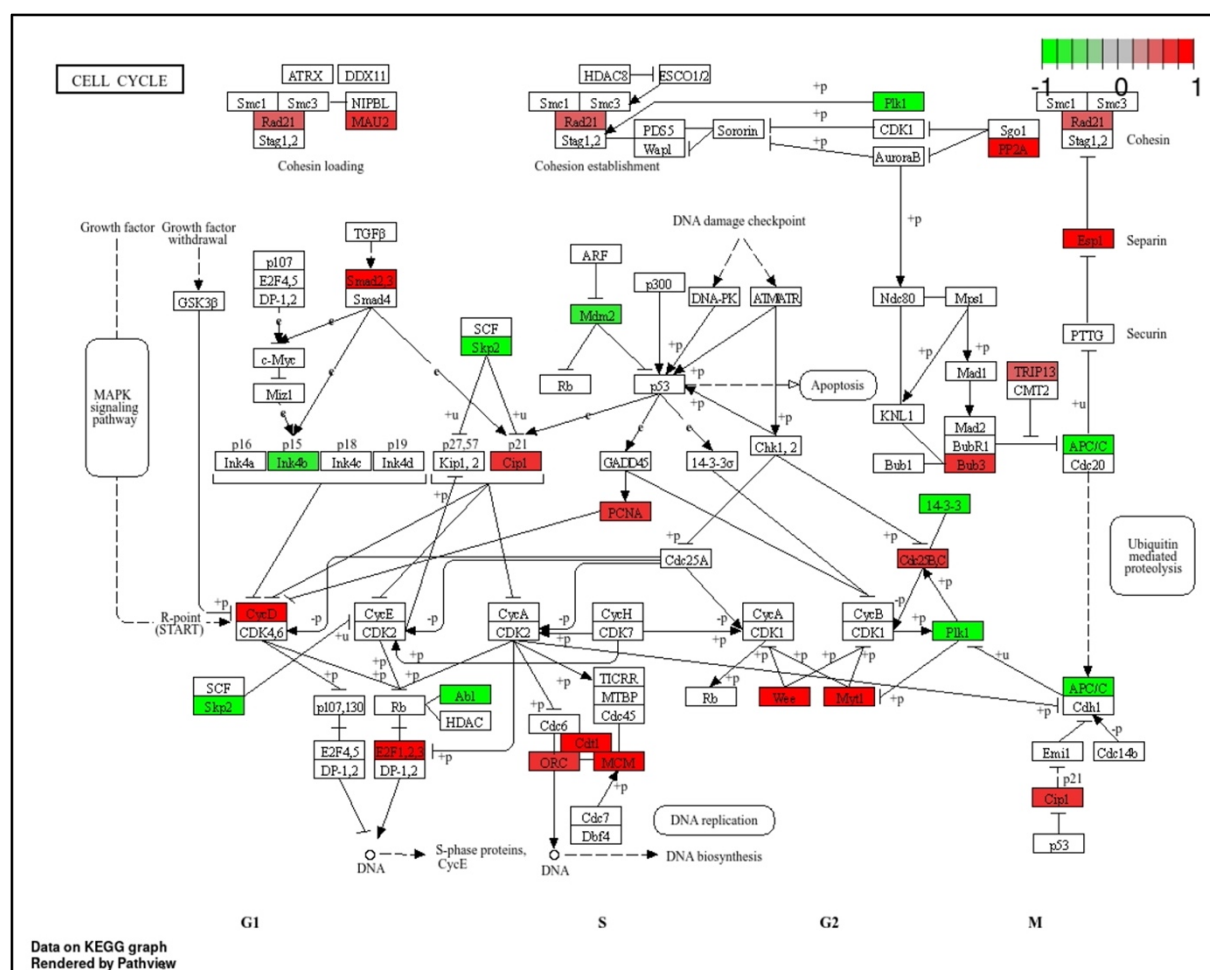


Figure S16: Schematic of the cell cycle metabolic pathway in dystrophin- and utrophin-deficient HeLa cells.

The image illustrates the cell cycle metabolic pathway identified in the KEGG analysis. The schematic highlights key stages of the cell cycle, including the G1, S, G2, and M phases, as well as major proteins and protein complexes involved in regulating these processes, such as cyclins (Cyc), cyclin-dependent kinases (CDK), checkpoint proteins, and DNA repair proteins. Green indicates genes with decreased expression, while red indicates genes with increased expression.

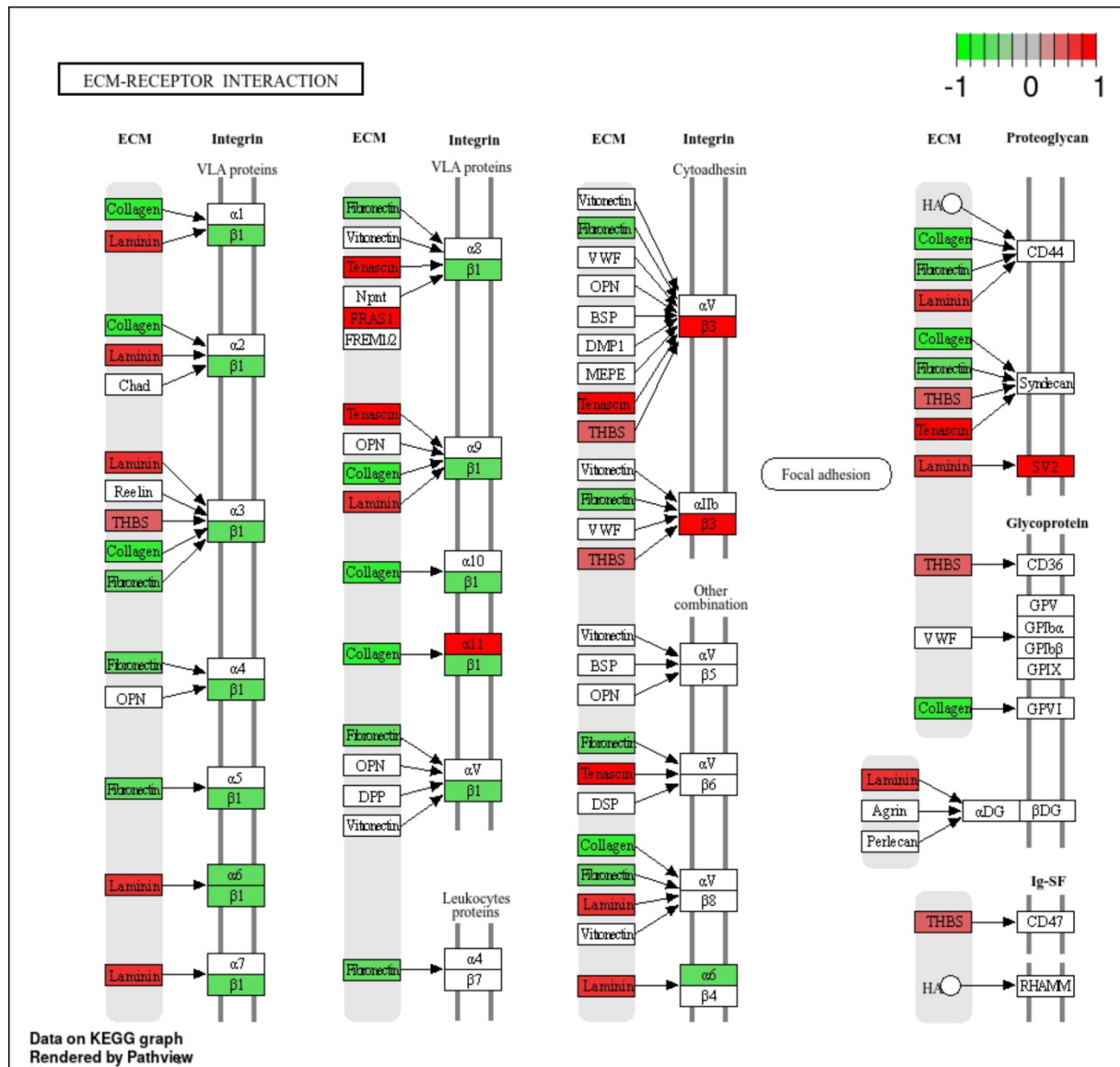


Figure S20: Schematic of the ECM-receptor interaction pathway in HSKM cells after silencing of the *DMD* and *UTRN* genes.

The image illustrates the ECM-receptor interaction pathway identified in the KEGG analysis (hsa04512). Key components such as integrins, collagens, laminins, and other extracellular matrix (ECM) proteins are highlighted. Green indicates genes with decreased expression, including collagen, *ITGB1*, *ITGA6*, and fibronectin, suggesting a disruption in ECM integrity and cell-matrix adhesion. Red indicates genes with increased expression, such as laminin, *THBS*, tenascin, integrin (*ITGA11*, *ITGB3*), and *SV2*, which may reflect compensatory mechanisms aimed at reinforcing ECM stability and enhancing cell adhesion and signaling pathways. Silencing of *DMD* and *UTRN* leads to decreased expression of key ECM and integrin genes, weakening the connection between the cytoskeleton and the ECM and increasing susceptibility to mechanical damage. Conversely, increased expression of certain genes may represent an adaptive response to stabilize the ECM and mitigate structural instability.

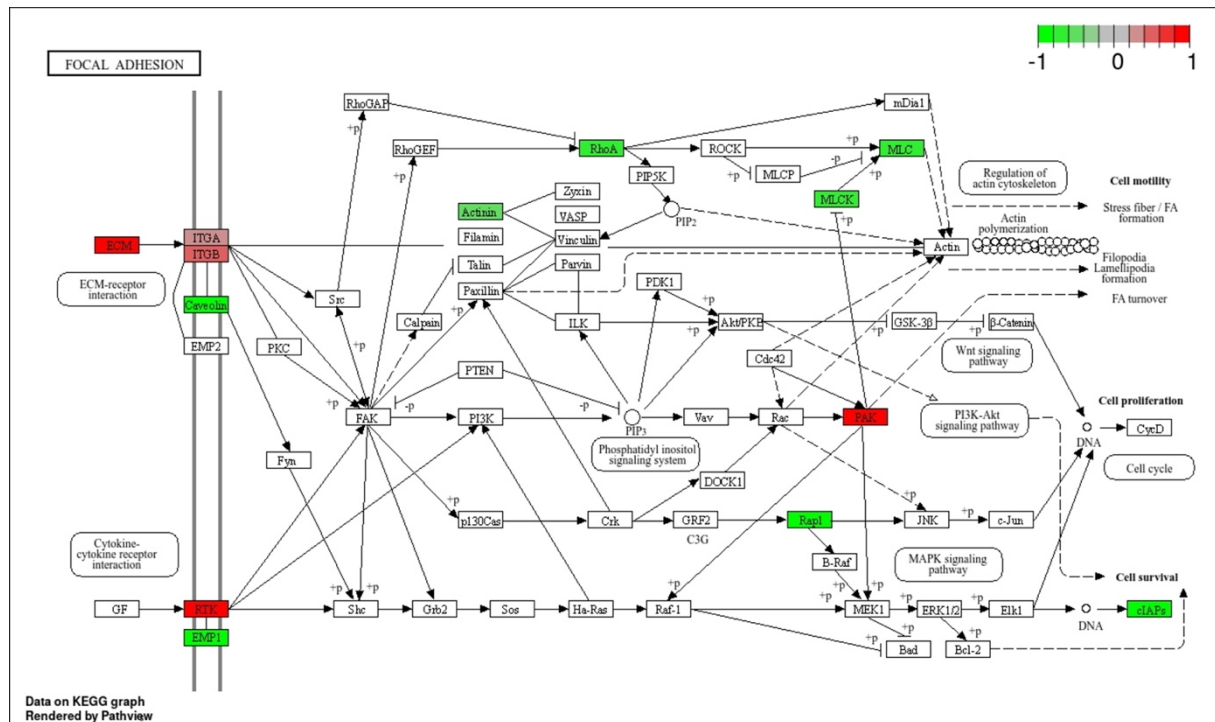


Figure S21: Schematic of the focal adhesion pathway in HSkM cells after silencing of the *DMD* and *UTRN* genes.

The image illustrates the focal adhesion pathway identified in the KEGG analysis (hsa04510). Key components such as integrins, cytoskeletal proteins, and signaling molecules are highlighted. Green indicates genes with decreased expression, including Caveolin, Actinin, *RhoA*, *MLC*, *MLCK*, *Rap1*, *EMP1*, and *cIAPs*, suggesting a disruption in cytoskeletal organization, cell contractility, and signal transduction. Red indicates genes with increased expression, such as ECM components, *ITGA*, *ITGB*, *RTK*, and *PAK*, which may reflect compensatory mechanisms aimed at enhancing cell-matrix adhesion, signal transduction, and cytoskeletal reorganization.

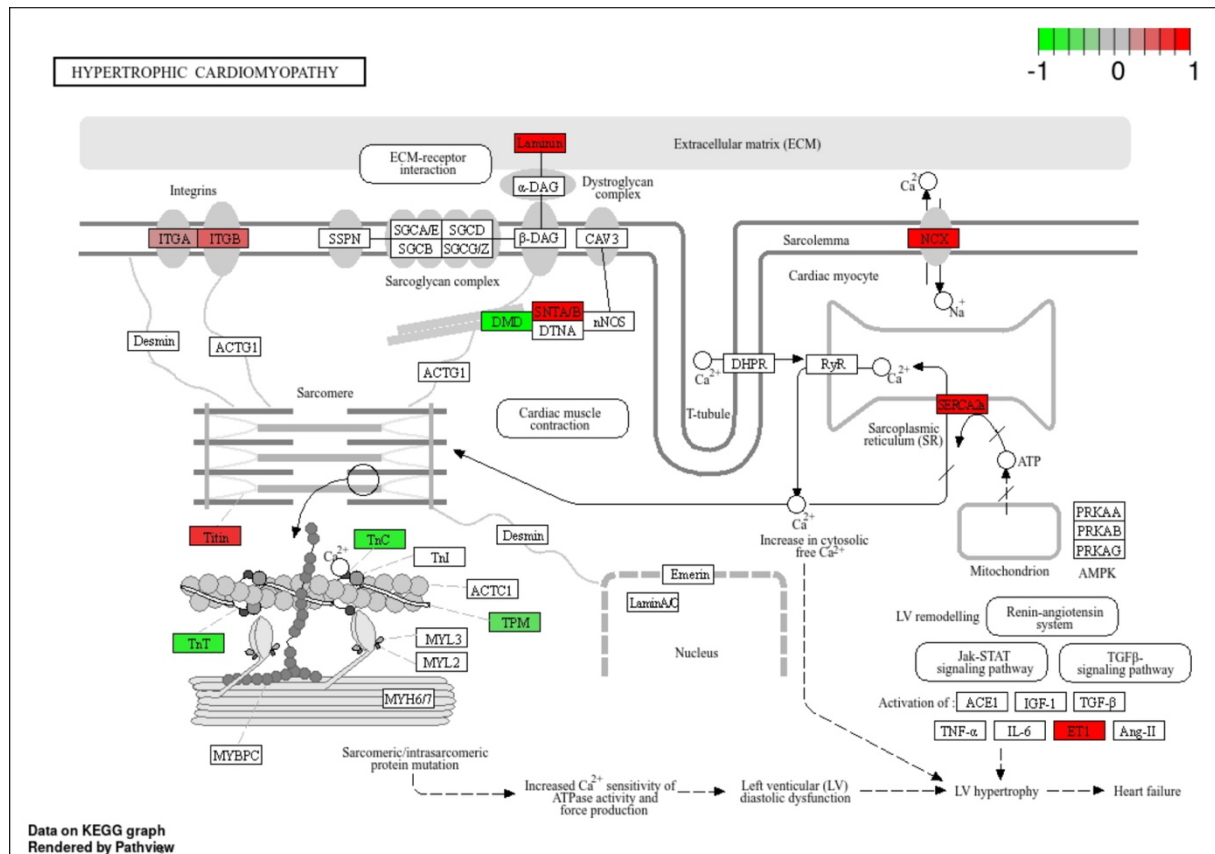


Figure S22: Schematic of the hypertrophic cardiomyopathy pathway in HSKM cells after silencing of the *DMD* and *UTRN* genes.

The image illustrates the hypertrophic cardiomyopathy pathway identified in the KEGG analysis (hsa05410). Green indicates genes with decreased expression, while red indicates genes with increased expression.

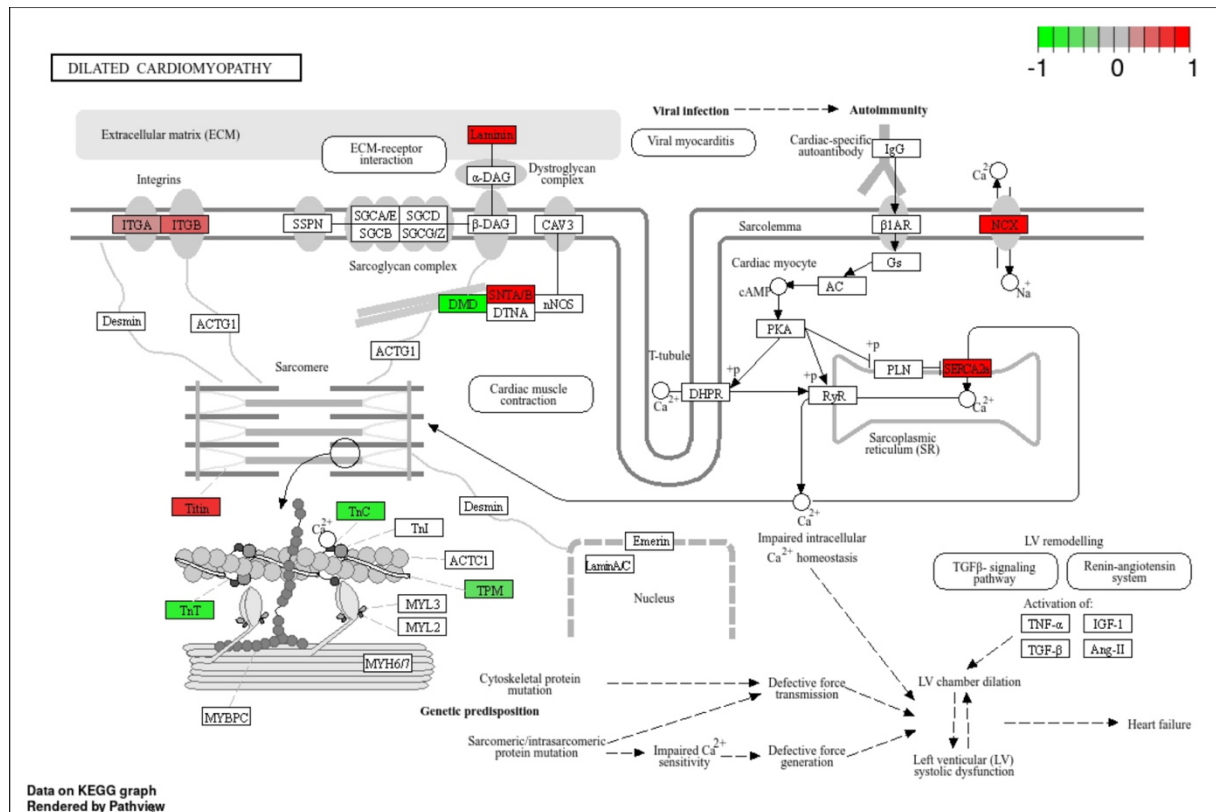


Figure S23: Schematic of the dilated cardiomyopathy pathway in HSKM cells after silencing of the *DMD* and *UTRN* genes.

The image illustrates the dilated cardiomyopathy pathway identified in the KEGG analysis (hsa05414). Green indicates genes with decreased expression, while red indicates genes with increased expression.

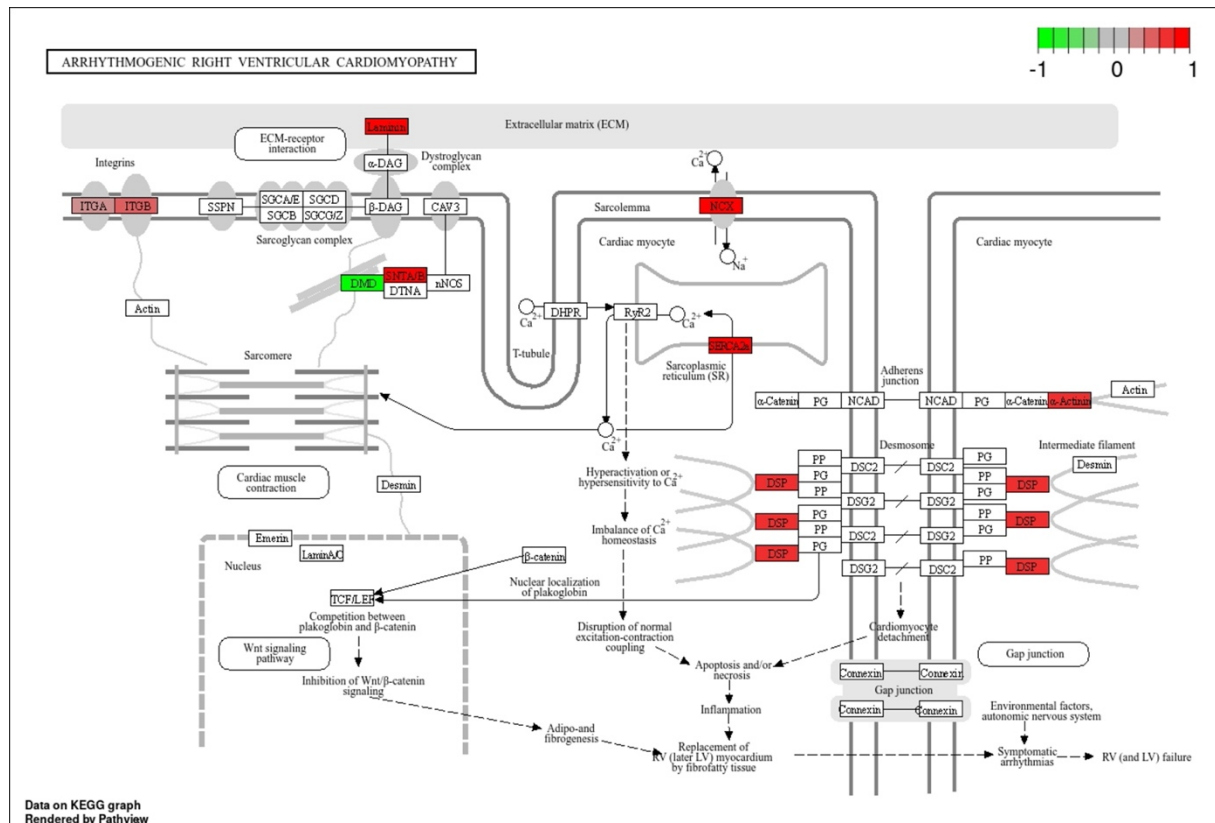


Figure S24: Schematic of the arrhythmogenic right ventricular cardiomyopathy pathway in HSKM cells after silencing of the *DMD* and *UTRN* genes.

The image illustrates the arrhythmogenic right ventricular cardiomyopathy pathway identified in the KEGG analysis (hsa05412). Green indicates genes with decreased expression, while red indicates genes with increased expression.

Chapter 4: DP71 compensates for full-length dystrophin DP427 in early stages of myofiber differentiation in cell models of Duchenne muscular dystrophy

Szwec S, Kościelniak-Wawro K, Durska A, Dominiak K, Konieczny P. *Compensatory properties of the short dystrophin isoform DP71 in cell models of Duchenne muscular dystrophy* (manuscript submitted in 2025)

DP71 compensates for full-length dystrophin DP427 in early stages of myofiber differentiation in cell models of Duchenne muscular dystrophy

Running title: DP71 compensates for DP427 in early DMD myofibers

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Key words: Duchenne muscular dystrophy, dystrophin, utrophin, DP71, DP427, splice variants

ABSTRACT

Duchenne muscular dystrophy (DMD) is an X-linked disease caused by mutations within the *DMD* gene and characterized by progressive muscle degeneration. All DMD patients lack the full-length 427 kDa dystrophin (DP427), with most individuals retaining the synthesis of the shorter DP71 isoform in various tissues, including muscle. In this study, we examined the putative compensatory role of DP71 and its various splice variants in cells deficient in DP427 and its paralogous utrophin protein UP395. Our results demonstrate that *DP71* expression is increased in human myofibers lacking DP427 and even more so in the absence of both DP427 and UP395, resulting in improved cell morphology, increased cell viability, reduced ROS production, and decreased calcium influx. Furthermore, our data indicate that the increase in *DP71* transcripts results from enhanced promoter activation rather than post-transcriptional mRNA stabilization. Subsequent forced expression studies revealed that DP71 splice variants differ in their stability, localization, ability to restore β -dystroglycan amounts, the cell plasma membrane function, mitochondrial transmembrane potential and calcium levels. Furthermore, some were more toxic than others, leading to increased ROS levels in specific cell compartments. These findings provide new insights into the role of DP71 in the pathophysiology of DMD and pave the way for the development of novel constructs for the treatment of DMD in myotubes and proliferating cells.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe, X-linked genetic disorder characterized by progressive muscle degeneration and weakness, cardiomyopathy, and cognitive and neuropsychiatric symptoms, affecting 1:5000 live male births ^{1, 2}. The disease arises from mutations in the dystrophin (*DMD*) gene, which encodes several dystrophin proteins ^{2, 3}. Most DMD cases are caused by deletion of one or more exons, with two hotspot regions: exons 3-9 and 45-55, with 7% and 47% of all mutations, respectively ^{4, 5, 6}. These genetic aberrations typically lead to a change in the reading of the coding sequence and the loss of the full-length 427 kDa dystrophin protein (DP427), and in some cases also DP260 and DP140, while allowing for the synthesis of shorter isoforms such as DP116 and DP71. Approximately 10% of individuals with distal mutations, spanning exons 63-79, lose all dystrophins, including the short DP71 isoform ^{5, 7}. Mutations that preserve the reading frame of the *DMD* gene generally result in truncated but partially functional dystrophin proteins. As a result, patients with such mutations often develop a milder form of the disease known as Becker muscular dystrophy (BMD) ⁸.

The production of dystrophins is regulated by seven promoters located within the *DMD* gene, three in the proximal region and four in the more distal part, leading to its cell-type and developmental stage-specific expression ⁹. *DP427B* (also called *DP427C*) is mainly transcribed in cerebral cortical neurons, *DP427M* in muscle fibers, *DP427P* in Purkinje cells, *DP260* in the retina, *DP140* in the brain and kidney, *DP116* in Schwann cells, and *DP71* ubiquitously in different cell types, including myoblasts. Patients lacking only DP427 generally do not exhibit strong cognitive deficits, in contrast to those lacking all dystrophin isoforms ^{10, 11, 12}. Clinical evaluations also indicated a significantly higher incidence of shorter stature and lower motor functions in DP71-missing DMD patients ¹³.

DP427 is a key muscular protein that stabilizes the sarcolemma during contraction and regulates activation of multiple signaling pathways via the assembled dystrophin-glycoprotein complex (DGC) and interaction with cytoskeletal proteins such as actin and microtubules ^{14, 15}. Others also indicated its role in the activation and proliferation of muscle stem cells (satellite cells), indicating that on one hand, its loss leads to myofiber degeneration and on the other, their inefficient regeneration. Conversely, *DP71* was indicated to be transcribed in proliferating myoblasts and to be downregulated during fiber differentiation ¹⁶. While its specific absence in mice did not lead to any obvious muscular phenotype ¹⁷, there are data showing its crucial role in cell proliferation and viability ^{18, 19, 20}. Specifically,

our recent data revealed that the loss of DP71 in various cell lines results in cyto- and genotoxicity, increased reactive oxygen species, and mitochondrial aggregation ²⁰.

14 splice variants of DP71 have been identified, which can be classified into four distinct groups ^{21, 22, 23}. The first group, containing exon 78, comprises: DP71 (includes all exons), Dp71 Δ 71 (also known as DP71a), DP71 Δ 74, Dp71 Δ 71,74 (also known as DP71c), DP71 Δ 71,73-74 and DP71 Δ 71-74; the second, without exon 78 and an alternative reading frame of exon 79, resulting in a more hydrophobic C-terminal protein sequence, encompasses: DP71 Δ 78 (also known as DP71b), DP71 Δ 71 Δ 78 (also known as DP71ab), DP71 Δ 71 Δ 73 Δ 78, DP71 Δ 74 Δ 78 and DP71 Δ 71-74 Δ 78; the third group, with a retained part of intron 77 and no exons 78 and 79: DP71 Δ 71i77 and DP71 Δ 71-74i77, found in non-muscle rat tissues and cell lines; and the fourth, containing the splice variant DP71 Δ 68-76 Δ 78 Δ 79, detected in glioblastoma cells ^{21, 24}. Current data indicate that each DP71 splice variant may exhibit a distinct synthesis pattern, localize to different cellular compartments, and have different functions, complicating the understanding of the role of DP71 ^{18, 23, 25, 26, 27, 28, 29, 30}. Some of these differences may stem from their varied ability to bind components of the DGC ^{31, 32}.

Paralogous to the *DMD* gene, the utrophin (*UTRN*) gene also encodes various isoforms, including UP395, UP140, UP113 (also known as utrophin G), and UP71 ³³. Similarly to DP71, the full-length UP395 product is synthesized in earlier myofiber differentiation stages than DP427 ^{16, 17, 33, 34}. However, while ectopic expression of *DP71* was shown not to be beneficial in mature fibers of DP427-deficient transgenic mice ^{32, 35}, UP395 was found to partially compensate for its absence, presumably due to its dual ability to assemble the UGC and bind actin filaments ^{36, 37}, the property also used to generate therapeutically relevant micro-dystrophin/utrophin proteins ³⁸.

While UP395 has been extensively studied as a therapeutic protein in DMD, the specific role of DP71 and its splice variants in control and diseased tissues lacking DP427 remains poorly understood ^{38, 39}. In this study, we aimed to 1) examine the expression patterns of the *DMD* and *UTRN* genes in different tissues and at various developmental stages, 2) assess the putative compensatory role of DP71 in muscle cell models deficient in DP427 and/or utrophin, 3) determine the localization and potential therapeutic roles of specific DP71 splice variants, and 4) characterize their binding partners.

MATERIALS AND METHODS

Mice

Wt (C57/Bl6) were used throughout the study. All experimental protocols were approved by the Polish Local Ethics Committee for Animal Research (LKE).

NTX injections

EDL muscles were surgically exposed and injected with 100 μ l of 1.0 μ g/ml NTX as described earlier⁵⁴ and harvested on days 3, 6, and 24 after NTX injection for further RNA isolation and RT-PCR analysis. Control EDLs were isolated from non-injected mice.

Cell lines

The C2C12 and HeLa cell lines were obtained from ATCC, whereas the HSkM, and HEK293 cell lines from Thermo Fisher, and the HL-1, and AC16 cell lines from Sigma Aldrich. The C2C12, HeLa, and HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Biowest, LO102-500) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich, Gibco) and 1x antibiotic-antimycotic reagents (AA). The HSkM cells were cultured in Nutrient Mixture F-10 Ham Medium supplemented with 20% FBS, 10 μ g/ μ l epidermal growth factor (EGF), 1mg/ μ l dexamethasone, and 1x AA. The HL-1 cells were cultured in Claycomb Basal Medium supplemented with 10% HL-1 qualified FBS (Sigma Aldrich) and 0.1 mM norepinephrine (Sigma Aldrich). The AC16 cells were cultured in DMEM/F12 medium supplemented with 12.5% FBS (Sigma Aldrich, Gibco). Both media for cardiomyocytes were enriched with 2 mM L-glutamine (Sigma Aldrich, Gibco) and 1x AA. Additionally, the HL-1 cells were cultured on wells covered with 0,01 % gelatin solution (Sigma Aldrich), and 5 μ g/mL fibronectin (Sigma Aldrich), and maintained in high confluency. The C2C12 and HSkM cells were cultured at low density to maintain an undifferentiated state. For experiments on differentiation toward myofibers, the C2C12 and HSkM cells were seeded on cell culture plates covered with 0,1% gelatin (Sigma Aldrich) to reach high confluence (>80%) and then cultured in a starving medium (DMEM (Capricorn Scientific, DMEM-HA) supplemented with 2% horse serum and 1x AA. All cells were grown at 37°C in a humidified incubator containing 5% CO₂.

siRNA transfection

The cell transfection with Silencer™ Select siRNA (Thermo Fisher) was performed with Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher) according to the manufacturer's protocol. The final concentration for each siRNA was 25 nM (in the case of double siRNA transfection, the final concentration of negative control siRNA was 50 nM). In experiments on proliferating cells, the siRNA was added 24h (HeLa, HEK293) or 4h (HSkM) from passage, and material was collected 48h after transfection. In the case of differentiation of HSkM, siRNA was added 4h after passage, and after another 48h of proliferation, the differentiation was induced. Two different *siDP427* were tested (Fig. S4A; *siDP427.1*, *siDP427.2*), and for further analysis, *siDP427.1* was chosen. The siRNA used in this study is listed in Supplementary Table S1.

Actinomycin D treatment

The HeLa cells transfected with control siRNA (siCtrl) or siRNA targeting *UTRN* transcripts (*siUTRN*) were cultured in 12-well plates. At 120 h post-transfection, actinomycin D (Sigma-Aldrich/Merck), prepared in DMSO, was added directly to the culture medium to a final concentration of 5 µg/mL to inhibit transcription. The cells were harvested at 0, 2, 4, 8, and 12 h following actinomycin D treatment for total RNA isolation. Reverse transcription and RT-PCR were then performed to quantify *DP71* mRNA levels in relations to *RPLP0* levels using *DP71/RPLP0*-specific primers (Supplementary Table S1).

DMD ko cell lines

The *DMD* knockout (ko) cell lines used in this study were obtained and characterized as described previously²⁰.

Plasmid preparation

Procedures for constructing the EF1α-GFP-FLAG-DP71 variants are illustrated in Supplementary Figure S5. Briefly insert was amplified using High-Fidelity Polymerase (Thermo Fisher) with primers containing additional nucleotides for restriction enzyme sites, following the manufacturer's protocol. Both the plasmid and the insert were digested overnight at 37°C with Fast Digest restriction enzymes (Thermo Fisher). After electrophoresis on a 1% agarose gel, the target band was isolated according to the manufacturer's protocol (GeneJET Gel Extraction Kit, Thermo Fisher), and both fragments were ligated

for 1h at room temperature using T4 DNA ligase (Thermo Fisher). The plasmid with the insert was then introduced into *E.coli* cells by the heat-shock method. The bacterial cells were first thawed on ice for about 20 minutes for the heat-shock procedure. Next, the ligation mixture was added to the bacterial cells, which were then incubated for 30 minutes on ice. After incubation, the cells were heat-shocked for 1 minute at 42°C, followed by a 2-minute incubation on ice. Then, 700 µl of LB medium preheated to 37°C was added to the cells (without antibiotics). The culture was shaken for 1h at 37°C and 300 rpm. Afterward, the cells were centrifuged for 5 minutes at 500 x g, and the pellet was resuspended in 50 µl of LB medium. Finally, the cells were spread onto a labeled LB agar plate (LB Agar, LabEmpire) containing the appropriate antibiotic (Carbenicillin disodium salt (as ampicillin substitution) or Kanamycin (Sigma). Such plasmids were tested by restriction enzymes and confirmed during electrophoresis, Sanger Sequencing, and Western Blot after transfection into the HeLa cell line. The antibodies used in this study are listed in Supplementary Table S2.

Plasmid transfection

The cell transfection with vectors was performed using FuGene HD or ViaFect (Promega) for the HeLa and HEK293 cell lines, and TransfeX (ATCC) for LHCN-M2 cells, maintaining a 2:1 transfection reagent to DNA ratio. For 6-well plates, 5.0 µL of transfection reagent was used; for 24-well plates, 2.5 µL; and for 96-well plates, 0.16 µL. Plasmid and the transfection reagent were added to Opti-MEM, mixed by pipetting, and incubated for 30 minutes (FuGene HD), 20 minutes (ViaFect), or 15 minutes (TransfeX). The DNA/transfection reagent mixture was then added to the cells. For proliferating cells, transfection was performed 24h after seeding. For the C2C12 cell differentiation, transfection was performed 24 hours after seeding, then after 48h the medium was replaced with starving medium, and cells were allowed to differentiate for 6 days.

Alternative splicing analysis

PCR was performed with GoTaq Polymerase according to the manufacturer's protocol. Electrophoresis was performed on a 2% agarose gel, while the results were detected on InGenius3 (SYNGENE). The analysis of the band was performed with the use of ImageJ software. The primers used in this study are listed in Supplementary Table S1.

Subcellular protein fractionation

Protein extraction was performed with a Subcellular Protein Fractionation Kit for cultured Cells (Thermo Fisher, 78840) according to the manufacturer's protocol. Subcellular fractions were confirmed by Western Blots by the presence of proteins specific to each targeted fraction. Antibodies used in this study are listed in Supplementary Table S2.

Imaging, immunoblotting, Real Time (RT) PCR, and cell assays

The experimental procedures were carried out as previously described ²⁰. Primers and antibodies used in this study are listed in Supplementary Tables S1 and S2.

Cell viability and proliferation

The cell viability/proliferation was determined using the RealTime-Glo™ MT Cell Viability Assay (Promega). The cells were plated on a Nunc™ MicroWell™ 96-well plate (Thermo Fisher) at a density of 7000 cells. The cell proliferation was monitored every 12h on a Tecan SPARK plate reader with luminescence.

Mitochondrial content, oxidative stress, apoptosis, calcium levels, and plasma membrane staining

The experimental procedures were carried out as previously described ²⁰.

RNA sequencing

The HSkM cells were transfected with siRNA and cultured in nine biological replicates for each siRNA. For RNA isolation, every three biological replicates were combined to create a single pooled replicate as one biological repeat. Total RNA from cells was extracted using the Total RNA Zol-Out™ (A&A Biotechnology) kit and RNA Clean & Concentrator-5 with DNase I (ZYMO Research). The quality of the total RNA was evaluated using TapeStation 2200 with the RNA Screen Tape assay kit (Agilent). Only samples with RNA integrity number (RIN) ≥ 8 were used for future analyses. The HSkM silencing was then confirmed with RT-PCR analysis. Detailed description of sample preparation and sequencing data analysis followed the procedures described in ²⁰. Expression analysis of the DP71 isoform of the DMD

gene at the exon level was performed using the DEXSeq package (version 1.52.0) in the R environment (version 4.4.2). The input data for the analysis were BAM files generated from RNA-seq analysis ⁴⁰.

RESULTS

DP71 and DP427 expression patterns vary by cell and tissue type, and the developmental stage

We first compared *DMD* and *UTRN* gene expression patterns in various human tissues, human and murine cell lines, and in notexin (NTX)-treated extensor digitorum longus (EDL) muscles (Figs. 1 and S1). *DP71* and *UP395* were the most abundant isoforms across different human tissues, except skeletal muscle, heart, and brain, where greater amounts of *DP427* were also observed (Fig. S1). Relatively high levels of *UP113* were noted in the adult brain and fetal kidney, while *UP140* was seen in a wide variety of tissues but at lower levels. Significant amounts of *DP40* were also detected in skeletal muscle, heart, brain, fetal kidney, and lung, as well as in the adult liver. *DP140*, a less abundant isoform, was identified only in the brain and kidney, with reduced levels in adult tissues compared to fetal. *DP116* showed notable expression in the fetal skeletal muscle, heart, and brain, but its levels decreased in adult tissues (Fig. S1A).

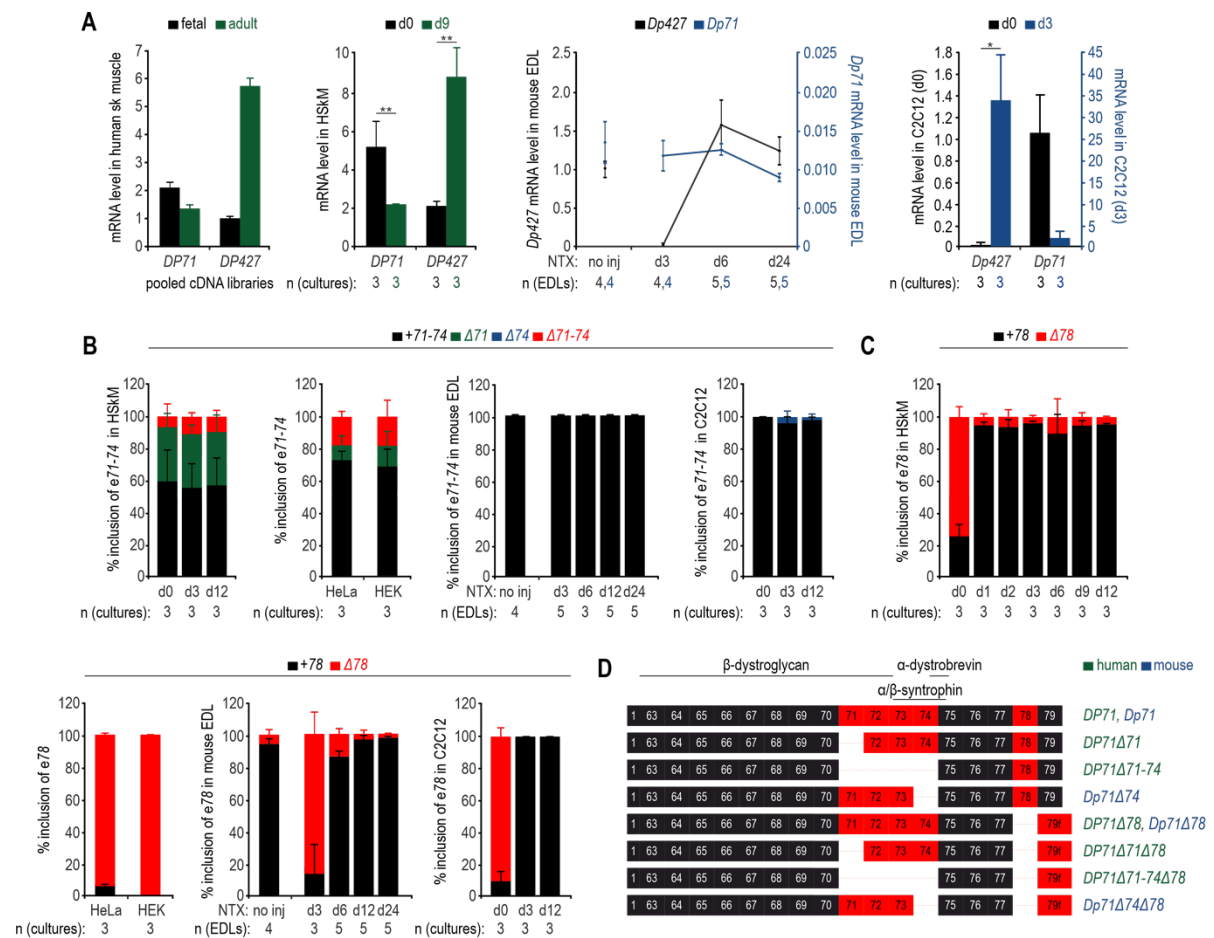


Figure 1: *DP71* and *DP427* exhibit distinct expression patterns depending on tissue type, developmental stage, and origin.

A Real-time (RT)-PCRs of *DP427* and *DP71* mRNA of Clontech human cDNA libraries from fetal and adult skeletal muscle, proliferating (d0) and differentiated (d9) HSkM cells, EDLs non-injected or at different time points following NTX-injection, and proliferating (d0) and differentiating (d3) C2C12 cells. **B, C** Quantitative analysis of exon 71-74 (**B**) and 78 (**C**) inclusion in human cell lines (HSkM, HeLa and HEK293), non-injected and NTX-treated EDLs, and C2C12 cell line. **D** Graphic representation of *DP71* mRNA splice variants detected in human and mouse samples. The results in **A** (middle panel) are shown as mean values $\pm$ SD and those in **A** (left and right panels), **B** and **C** as the means + SD. n, the number of biological replicates. *, $p < 0.05$; **, $p < 0.01$ (t-test).

Results of RT-PCR analyses of mouse-derived HL-1 cardiomyocytes, human-derived AC16 cardiomyocytes, and the human primary myoblast cell line HSkM coincided with the *DMD* and *UTRN* expression patterns in human skeletal muscles and hearts (Figs. S1, 1A). Some differences, such as low levels of *DP116* and no or only some *DP427* transcripts in cardiomyocyte cell lines and relatively high levels of *UP395* mRNA in HSkM myotubes (Fig. S1B), are likely due to their early differentiation state. Importantly, in all tested models, *DP71* exhibited an expression pattern specific to proliferating cells, while *DP427* expression was more characteristic of mature cells (Figs. 1A, S1A). We also observed species-specific differences, namely, in human cells, *DP71* accounted for a higher proportion of total *DMD/UTRN* transcripts compared to NTX-treated EDL muscles and C2C12 and HL-1 cell lines (Figs. 1A, S1B).

Multiple *DP71* variants are generated in human fetal and adult tissues

Since some studies indicated different localization and function of various *DP71* proteins, we next examined alternative splicing of *DP71* transcripts in human tissues (Fig. S2) and total dystrophin mRNAs in muscle and non-muscle human cell lines (HSkM, HeLa, and HEK293), NTX-treated EDL muscles, and C2C12 myoblasts/myotubes (Fig. 1B-D). We focused on major splice variants, i.e., containing various combinations of exons 71-74 and with or without exon 78. PCR analyses of human tissues revealed the presence of multiple *DP71* splice variants with differential expression between fetal and adult stages (Fig. S2). Amplification of the region spanning exons 71-74 revealed that the +71-74 and Δ 71 variants were most ubiquitous in fetal skeletal muscle, spleen, and thymus, and adult liver, pancreas, and placenta, and +71-74 was almost the only isoform in the adult skeletal muscle. Δ 71-74 transcripts were predominant in the brain, heart, and kidney, and lower amounts were detected in other tissues, such as fetal skeletal muscle, lung, liver, and adult pancreas, while the less abundant variants

were restricted to the fetal brain and lung tissues ($\Delta 71,73,74$), fetal kidney ($\Delta 71,74$), and adult brain and skeletal muscle ($\Delta 74$). Amplification of the second tested region revealed that the $\Delta 78$ transcript was the major mRNA variant regardless of tissue type, except for adult skeletal muscle, where only transcripts with exon 78 were observed.

Analyses of human tissues indicated that the presence of multiple *DMD* splice variants coincides with the expression of *DP71* mRNAs, while in differentiated muscle cells, where predominantly *DP427* synthesis is observed, only a splice variant containing all exons is primarily generated. Splicing analyses in cell lines and regenerating EDL muscles confirmed these results, as we identified several *DP71* splice variants in human and murine proliferating cells, the main one being $+71-74\Delta 78$ (Fig. 1B-D). Although this variant was almost exclusively transcribed in murine cells, variants lacking exons 71 ($\Delta 71$) and 71-74 ($\Delta 71-74$) were also consistently observed in human cell lines. Altogether, our findings demonstrate that *DP71* is ubiquitously expressed across different tissues and is the major isoform in proliferating cells and fetal tissues. As differentiation progresses, *DP71* is gradually replaced by *DP427*, especially in skeletal muscle and heart. Additionally, we observed that *DP71* transcripts lacking exon 78 are strongly associated with the proliferation phase. Notably, human-derived cells displayed higher levels of *DP71*, and a greater diversity of splice variants compared to mouse models.

Loss of *DP427* and *UP395* results in increased expression of *DP71*

Because previous results indicated differences in the severity of the phenotype between patients deficient in only *DP427* and lacking in addition to the full-length isoform also shorter variants, and our results indicated that *DP71* is the major dystrophin in non-differentiated muscle cells and tissues, we decided to investigate the role of *DP71* in muscle cells deprived of *DP427*. Considering the differences in the expression of *DP71* in mouse and human cells (Figs. 1A, S1B) and similar patterns of transcription in the human-derived HSkM cell line and human skeletal muscles (Fig. 1A, Fig. S2), we selected HSkM cells grown on gelatin coating as the primary model for our investigations (Fig. S3) and complemented these studies by using human HeLa and HEK293 cell models.

Our RT-PCR and microscopic analyses revealed that the absence of *DP427* in differentiated HSkM cells results in increased *DP71* expression (Figs. 2A, S4A). Because *UP395* can partially compensate for the loss of *DP427* and *DP71*, we also silenced all utrophin isoforms, which further enhanced the increase in *DP71*, leading to comparable amounts of total *DMD* transcripts to those from

wt cells (Figs. 2A, S4A). Importantly, downregulation of utrophin transcripts alone in either HSKM cells or a HeLa cell line, which naturally lacks DP427, also increased the *DMD* gene expression (Fig. 2B-C).

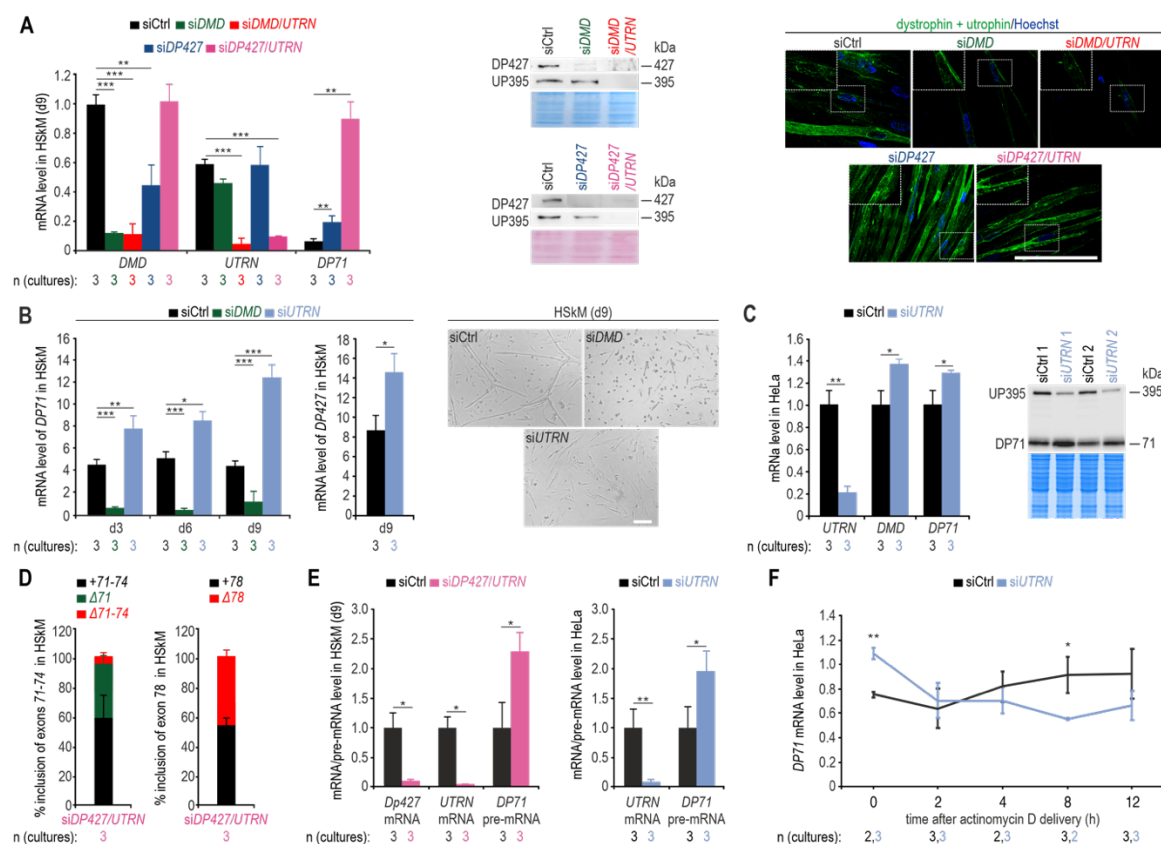


Figure 2: DP71 levels increase in cells deficient in DP427 and/or UP395.

A RT-PCR, immunoblotting, and immunofluorescence analyses assessing dystrophin and utrophin mRNA and protein levels in differentiated (d9) HSKM cells after siRNA administration. Coomassie-stained polyacrylamide gels or ponceau-stained membranes were used to normalize protein content and immunostaining. The images were generated from six separate pictures, taken at various z-positions using a microscope with the same parameters for each image. **B** RT-PCR analysis of *DP71* and *DP427* in HSKM cells during differentiation after *UTRN* or *DMD* silencing, with live-cell images representing HSKM phenotype at day 9 of differentiation. **C** RT-PCR and immunoblotting analyses of dystrophin and utrophin mRNA and protein levels in HeLa cells after siRNA delivery. HeLa *DMD* ko lysate was used as a negative control for dystrophin detection. Coomassie-stained polyacrylamide gels were used to normalize protein content. **D** Quantitative analysis of exon 78 and 71-74 inclusion in differentiated HSKM cells deprived of DP427 and utrophins. **E** RT-PCR analysis of *UTRN* mRNA and *DP71* pre-mRNA levels in HeLa cells after *UTRN* silencing. **F** Time-course of *DP71* mRNA decay (relative to *RPLP0* levels) in HeLa cells after transcriptional inhibition with actinomycin D, comparing siCtrl and siUTRN conditions. The results in **A**, **B**, **C**, **D** and **E** are shown as mean values + SD and those in **F** as the means $\pm$ SD. Bars in **A** and **B**: 100 μ m. n, the number of biological replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (t-test).

Transcript analyses in HSkM cells revealed that most of newly generated *DP71* mRNAs in myofibers lacking both *DP427* and *UTRN* had a splicing pattern characteristic for proliferating cells, with an approximately equal ratio of +78 and Δ 78 variants, while no change was observed in the alternative splicing of exons 71-74 (Fig. 2D). Based on these results, we hypothesized that the *DP71* increases might be due to higher stability of transcripts generated during the proliferation stage. However, RT-PCRs did not confirm this, because we also observed higher levels of *DP71* pre-mRNA in the absence of *DP427* and utrophins (Fig. 2E), and actinomycin D treatment showed rather decreased stability of *DP71* mRNAs following si*UTRN* delivery (Fig. 2F). These results demonstrate that loss of *DP427* results in increased *DP71* expression, and that this phenotype is enhanced by the concomitant absence of UP395. Furthermore, our data indicate that the increase in *DP71* transcripts is due to enhanced promoter activation rather than post-transcriptional mRNA stabilization.

DP71 compensates for DP427 during the early differentiation stage of muscle cells

To assess whether the increase in *DP71* levels can impact the functionality of myotubes deficient in *DP427* or *DP427* and utrophins, we examined morphology, viability, and mitochondrial functionality of HSkM cells following siRNA delivery. Nine days after differentiation initiation, myotubes lacking *DP427* or *DP427* and utrophins exhibited proper arrangement of actin, α -tubulin, and myosin, whereas cells lacking *DMD* or *DMD/UTRN* showed protein rearrangements, with significant aggregation of α -tubulin (Fig. 3A). A mitochondrial protein marker, ATP synthase, also showed a more compact distribution in *DMD*- and *DMD/UTRN*-deprived cells compared to the wt (Fig. 3A). Increase in the *DP71* expression in *DP427*-deficient myotubes inhibited this phenotype, with ATP synthase arrangement similar to that observed in wt cells (Fig. 3A).

Statistical analyses confirmed that the fibril area was significantly higher in cells deprived of *DP427* compared to those lacking all dystrophin isoforms (Fig. 3B), and the number of nuclei in si*DP427*-treated cells, unlike in cells deficient in all dystrophin isoforms, was not significantly lower than in wt cells. In agreement, additional silencing of *DP71* in si*DP427/UP395*-treated myotubes resulted in a more severe phenotype (Fig. S4B). Further analysis also confirmed the ameliorated phenotype of *DP427*-lacking myotubes when compared to the samples deficient in all dystrophins, as they revealed reduced reactive oxygen species (ROS) production and calcium influx, as well as increased viability (Fig. 3B, C). Surprisingly, the *DP427*-deficient myofibers showed elevated levels of the apoptosis marker, indicating

that cell degradation might eventually happen but later during differentiation (Fig. 3B). Altogether, our data indicate that DP71 compensates for the lack of DP427 at early stages of myofiber differentiation, delaying cellular degradation, and maintaining proper mitochondrial distribution and functions.

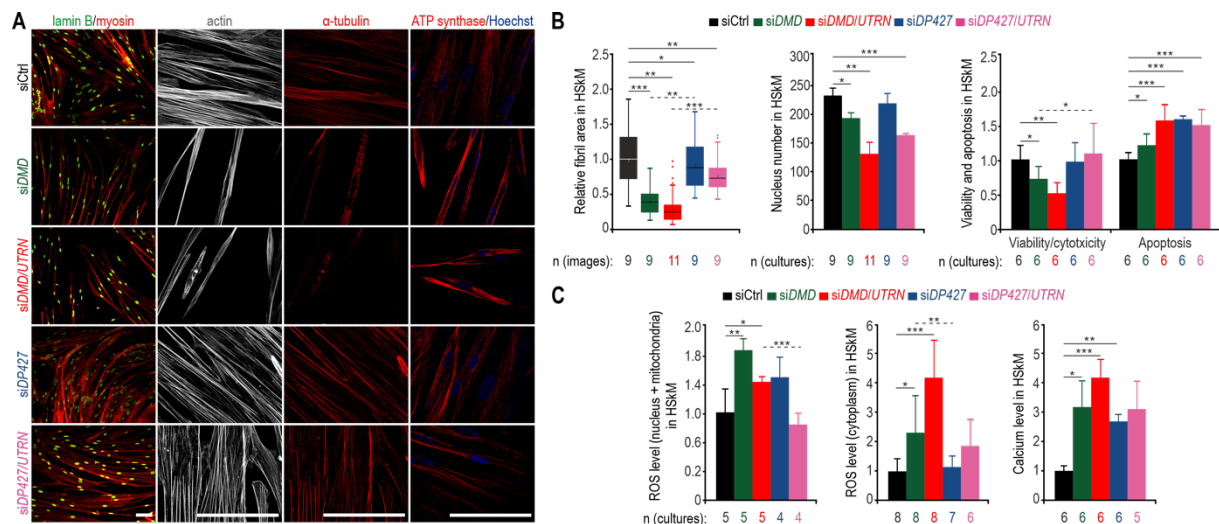


Figure 3: DP71 compensates for the loss of DP427 in muscle cells.

A Microscopic analysis of differentiated HSKM cells (d9) deficient in all dystrophins or DP427 and utrophins immunostained for lamin B, myosin, actin, tubulin, ATP synthase, and nuclei (blue channel, Hoechst). The images were generated from six separate pictures, taken at various z-positions using a microscope with the same parameters for each image. **B** Quantification of relative myofiber areas, nucleus numbers (d9), and viability and apoptosis (d12) in siRNA-treated HSKM (d9) cells. **C** ROS and calcium influx levels quantification in differentiated HSKM cells (d9) after siRNA treatment. Bars in **A**, 100 μ m. The results in **B** (left panel) are shown as mean values $\pm$ SD and those in **B** (middle and right panels) and **C** as the means + SD. n, the number of biological replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (t-test).

Exclusion of exons 71-74 reduces DP71 stability and impairs its ability to bind α -syntrophin

DMD knock-out (ko) HeLa and HEK293 cells demonstrated mitochondrial dysfunction, elevated ROS production, and calcium levels, enhanced cell membrane permeability, DNA damage, and apoptosis²⁰, indicative of important DP71 functions in the cytoplasm and at the cell membrane. Indeed, immunofluorescence microscopy analysis of wt HeLa cells revealed DP71 and utrophin localization to the cytoplasm and the membrane (Fig. 4A). In accordance, protein fractionation and immunoblotting analysis showed predominant presence of DP71, along with β -dystroglycan and UP395, in these fractions (Fig. 4B). However, while β -dystroglycan and UP395 were largely absent from the cytoskeletal

compartment, DP71 variants lacking exon 78 were particularly abundant in this fraction. On the contrary, the +78 variants occurred almost exclusively in the cytoplasm.

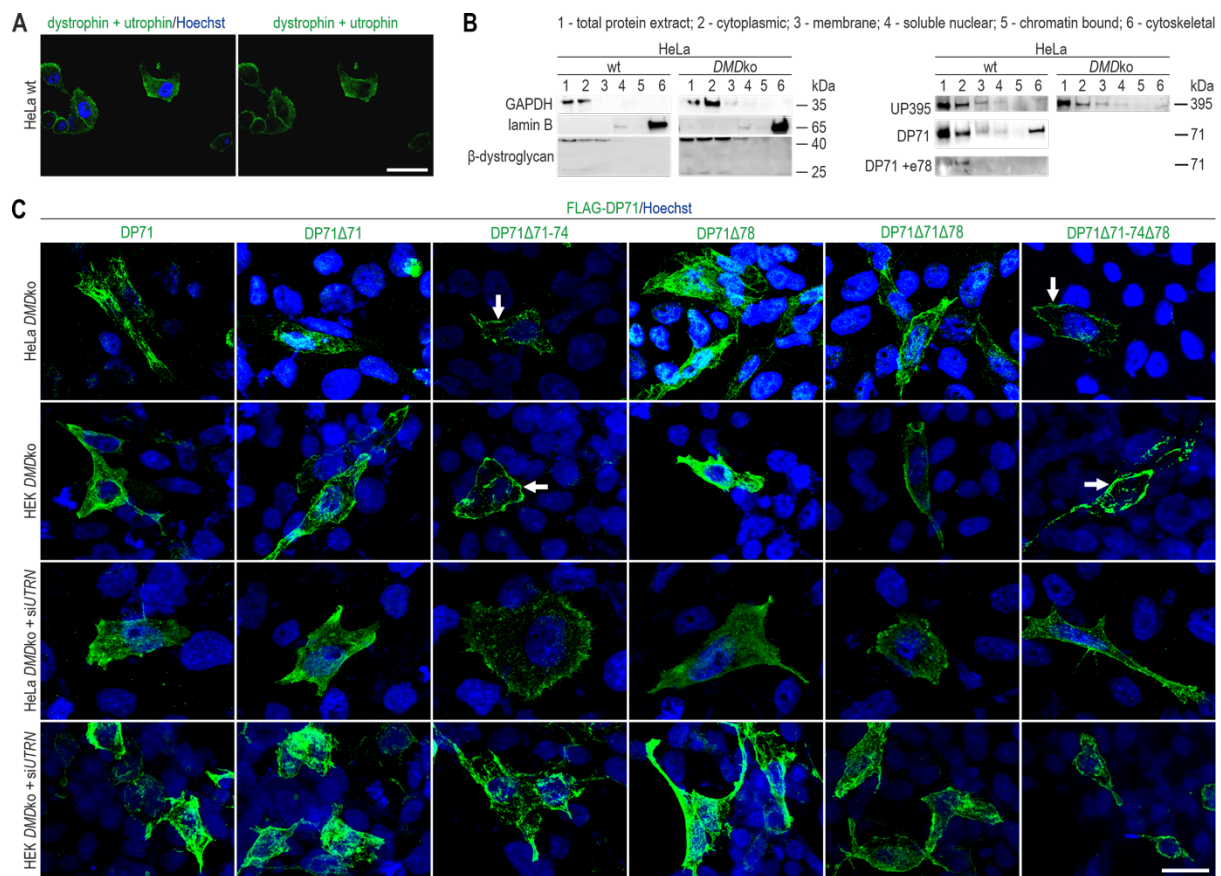


Figure 4: DP71 exhibits distinct cellular localization depending on the inclusion of alternative exons and the presence of utrophin.

A Microscopic analysis of wt HeLa cells immunolabelled for dystrophin and utrophin. **B** Immunoblotting analysis of protein fractions from wt HeLa cells. **C** Immunostaining of *DMD* ko HeLa and HEK293 cells for ectopic FLAG-DP71 proteins and nuclei with an antibody against FLAG and Hoechst following transfection with FLAG-DP71 splice variant-specific plasmids, either alone or in combination with siUTRN. Different gain parameters were applied to prevent excessive signals in the most effective plasmids and to avoid undetectable signals in less stable proteins. In **A** and **C**, the images were generated from six separate pictures, taken at different z-positions using a fluorescence microscope. Bars in **A** and **C**, 50 μm.

To test the localization and therapeutic potential of various DP71 variants, we generated vectors encoding flag-tagged proteins under the EF1α promoter (Fig. S5), which were then delivered to *DMD* ko HeLa and HEK293 cells (Figs. 4C and 5A). Immunoblotting analyses revealed proper molecular mass of DP71 variants that ranged from 58 to 72 kDa; however, isoforms lacking exons 71-74

(DP71 Δ 71-74, DP71 Δ 71-74 Δ 78) were present at much lower levels in both cell types (Fig. 5A). Immunofluorescence analyses showed DP71 cytoplasmic and membrane localization, with Δ 71-74 variants found mainly at the membrane (arrows, Fig. 4C). Interestingly, upon siRNA-mediated silencing of *UTRN* transcripts, DP71 Δ 71-74 and DP71 Δ 71-74 Δ 78 proteins partially relocated to the cytoplasm, the fraction in wt cells most abundant in UP395 (Fig. 4C).

DP71 variants show different capacity to restore the cell plasma membrane, mitochondrial transmembrane potential, calcium levels, and proliferation

We first estimated the therapeutic potential of different DP71 variants in dystrophin-deficient HeLa cells by evaluating their ability to lower the UP395 levels and restore the synthesis of DGC components, β -dystroglycan and α -syntrophin (Fig. 5A, B).

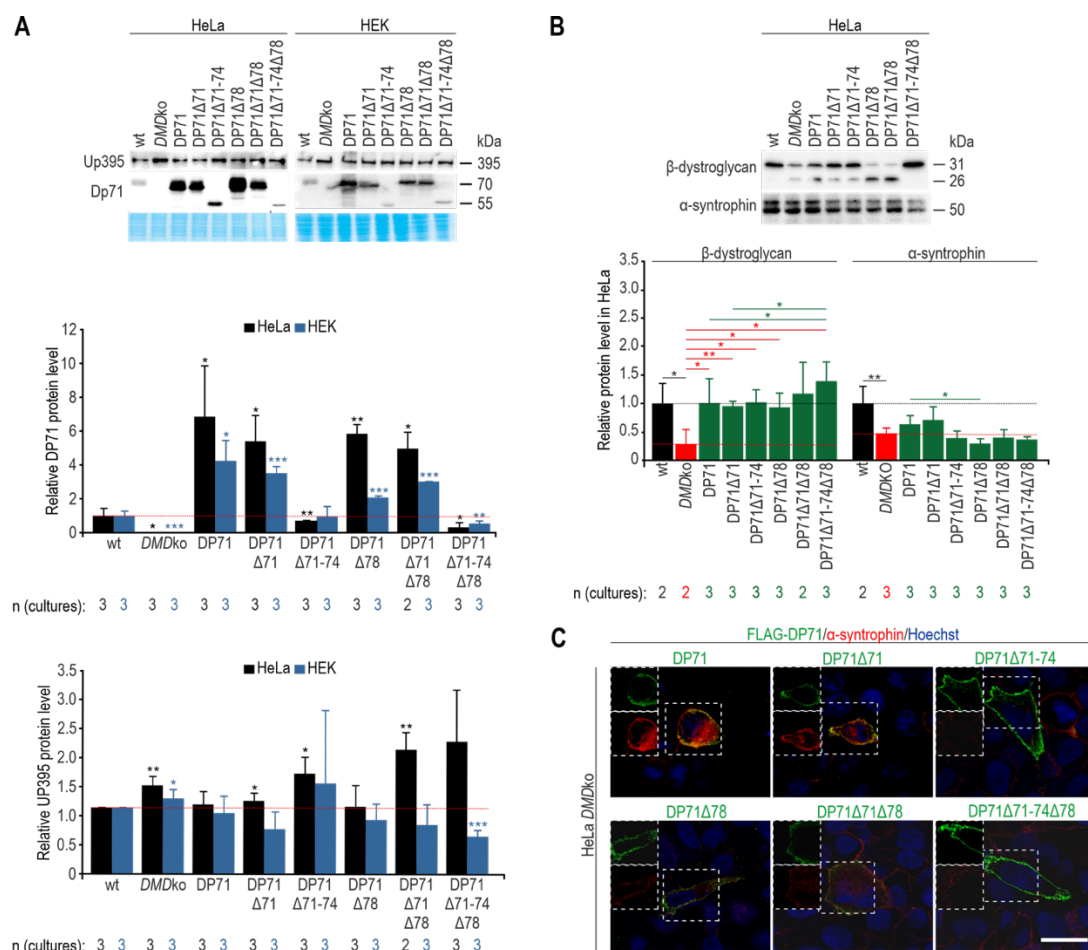


Figure 5: Exons 71-74 are crucial for the stability of DP71 and its interaction with α -syntrophin.

A and **B** Immunoblotting analysis of protein levels in *DMD* ko HeLa and HEK293 (A) or HeLa (B) cells following transfection with plasmids encoding FLAG-tagged DP71 splice variants. As controls, wt and *DMD* ko cells with

administered vector encoding FLAG-tag were used. Densitometric evaluation of band intensities was performed relative to wt samples with FLAG-tag. **C** Immunostaining for FLAG-DP71, α -syntrophin, and nuclei (Hoechst) in *DMD* ko HeLa cell line after administration of various DP71 vectors. For each image, the microscope z-position was chosen based on α -syntrophin localization, and the same parameters were set for all images. The results in **A** and **B** are shown as means + SD. Bar in **C**, 50 μ m. n, the number of biological replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (t-test).

While we observed varied ability of ectopic DP71 proteins to influence the UP395 levels, all variants could restore the β -dystroglycan amounts, with cells containing DP71 Δ 71-74 Δ 78 showing the highest β -dystroglycan contents (Fig. 5B). In contrast, the α -syntrophin levels were mostly unaltered, with only DP71 and DP71 Δ 71 showing capacity for restoring its levels. Immunofluorescence analysis confirmed these data, as the latter variants revealed the strongest colocalization with α -syntrophin (Fig. 5C). In contrast, the Δ 71-74 variants were the only DP71 proteins that did not exhibit any overlapping signals.

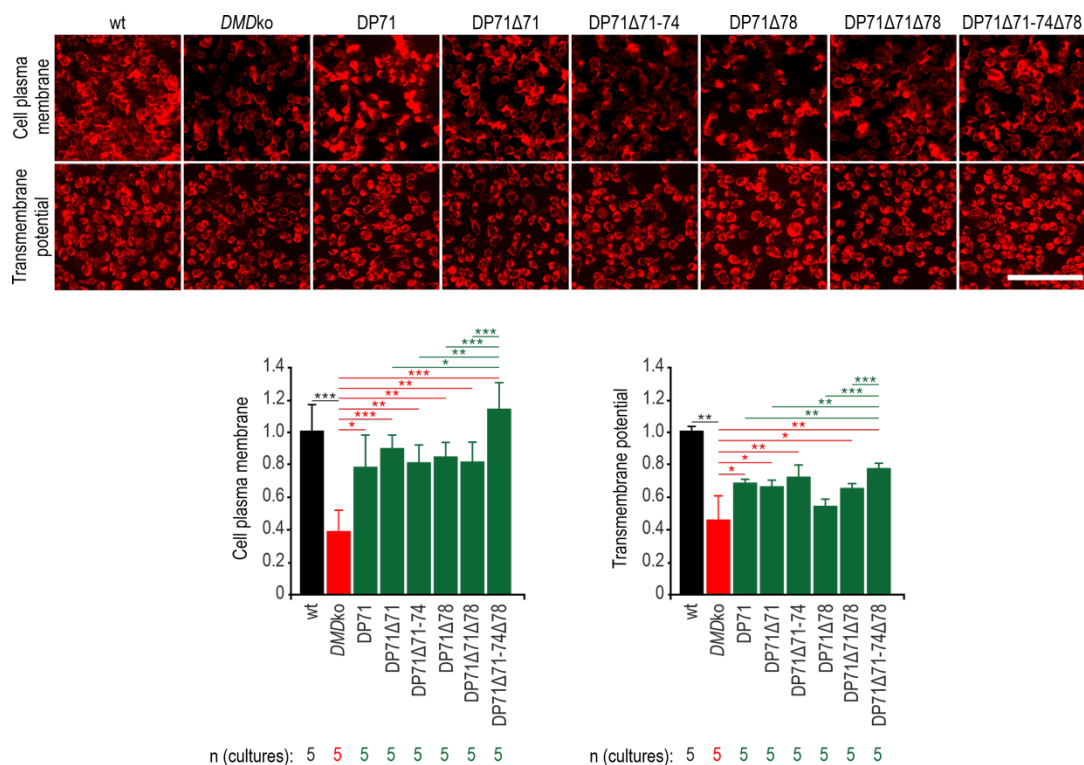


Figure 6: DP71 variants exhibit different capacities to restore plasma membrane integrity and mitochondrial transmembrane potential.

Assessment of cell plasma membrane and mitochondrial transmembrane potential in *DMD* ko HeLa cells after transfections with different *FLAG-DP71* vectors. As controls, wt and *DMD* ko cells with vector encoding FLAG-tag were used. Quantifications were obtained based on Tecan fluorescence values of cell plasma membrane and

mitochondrial transmembrane potential normalized to Hoechst signals. Bar, 100 μ m. The results in lower panel are shown as means + SD. n, the number of biological replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (t-test).

Next, we assessed whether DP71 proteins have the capacity to restore the cell plasma membrane and the mitochondrial transmembrane potential (Fig. 6). Importantly, all variants partially restored both functions in *DMD* ko HeLa cells, with the highest potential observed for DP71 Δ 71-74 Δ 78 that most markedly completely restored the cell plasma membrane. We then evaluated the impact of different DP71 variants on active mitochondria, ROS production, calcium influx, and cell proliferation (Fig. 7). None of the constructs restored the ROS production to the wt levels, with the synthesis of Δ 78 variants leading to an even higher content of mitochondria and associated ROS, with the highest values for the nuclear and mitochondrial ROS observed for DP71 Δ 71 Δ 78 and the cytoplasmic ROS and mitochondria for DP71 Δ 71-74 Δ 78, respectively (Fig. 7A, B). Elevated cellular calcium levels were also observed for Δ 78 variants, while these contents were either partially or completely restored to the wt levels by DP71 proteins containing exon 78 (Fig. 7B). *DMD* ko HeLa cells containing the Δ 78 variants also showed lower numbers in cell proliferation assays, below the *DMD* ko values, with only the DP71 containing all exons showing a significantly positive effect (Fig. 7C).

Altogether, this data shows a varied capacity of DP71 proteins to impact the function of different cellular compartments. Membrane-bound DP71 Δ 71-74 Δ 78 most significantly restored β -dystroglycan levels, the cell plasma membrane, and mitochondrial transmembrane potential, presumably by increasing the content of active mitochondria. The number of mitochondria was also significantly elevated in cells containing other exon 78-lacking DP71s, paralleled by an elevated ROS and calcium levels that presumably negatively affected cell numbers. Particularly, the DP71 Δ 71 Δ 78 protein increased most significantly nuclear/mitochondrial ROS levels and markedly lowered cell numbers. In contrast, variants with exon 78 tended to lower intracellular calcium levels, with DP71 and DP71 Δ 71 showing preferential localization with α -syntrophin in the cytoplasm and DP71 having a positive effect on restoring cell numbers.

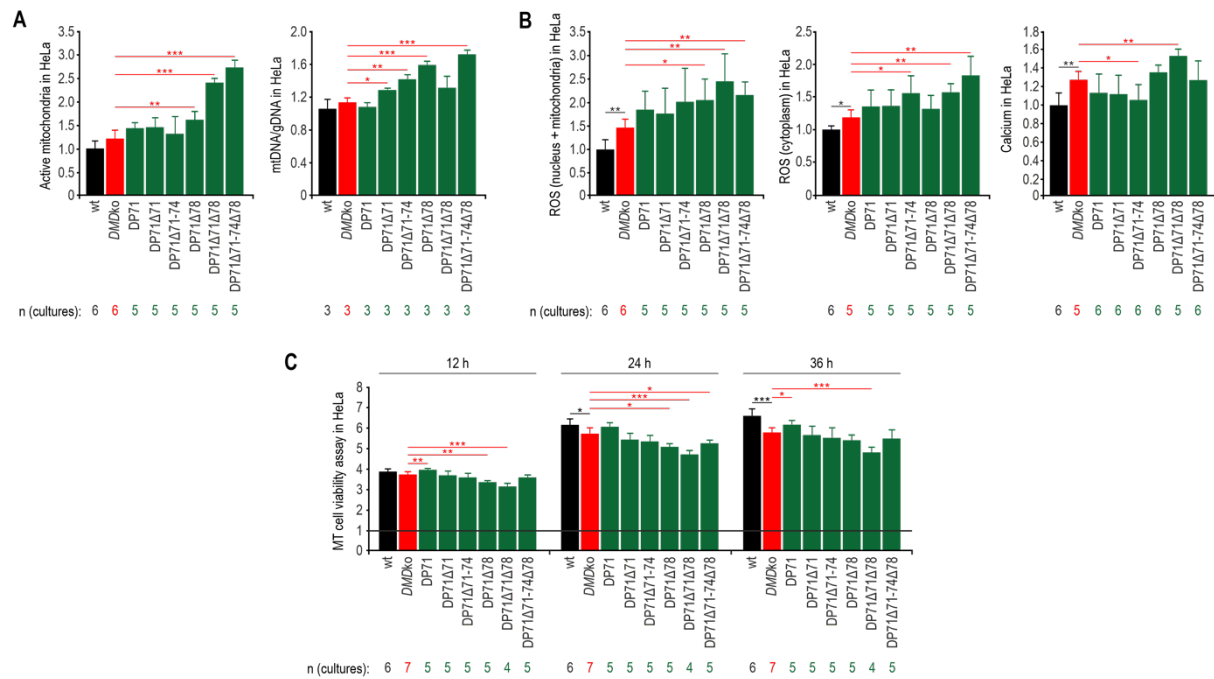


Figure 7: DP71 splice variants affect active mitochondria level, ROS production, calcium influx, and viability of DMD ko HeLa cells.

A and **B** Quantification of active mitochondria (MitoTracker) and mtDNA (**A**), ROS production, calcium influx (**B**), and viability/proliferation (**C**) in DMD ko HeLa cells after delivery of vectors encoding FLAG-tagged DP71 variants. As controls, wt and DMD ko cells with a vector encoding FLAG-tag were used. Quantifications were obtained based on Tecan values normalized to Hoechst signals. The results **A**, **B** and **C** are shown as means + SD. n, the number of biological replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (t-test).

DP427- and DP71/DP427-deficient HSkM myotubes show distinct changes in the expression of genes involved in the membrane integrity and cytoskeletal functions

To further investigate the differences between DP427- and DP71/DP427-deficient cells, we performed RNA-seq of HSkM myotubes treated with siRNAs targeting corresponding regions in DMD mRNAs, with and without downregulation of the UTRN transcripts. In agreement with the RT-qPCR data (Fig. 2A and S6A), we observed efficient downregulation of DMD transcripts (exons 63-79) in samples deficient in either DP71/DP427 (log2FC of -2.92) or DP71/DP427 and utrophins (log2FC of -3.76), while a substantial increase of DP71 transcripts was observed in samples lacking only DP427 (log2FC of 10.32, comparison to siDMD samples) or DP427 and utrophins (log2FC = 11.21, comparison to siDMD/UTRN samples) (Fig. S6B).

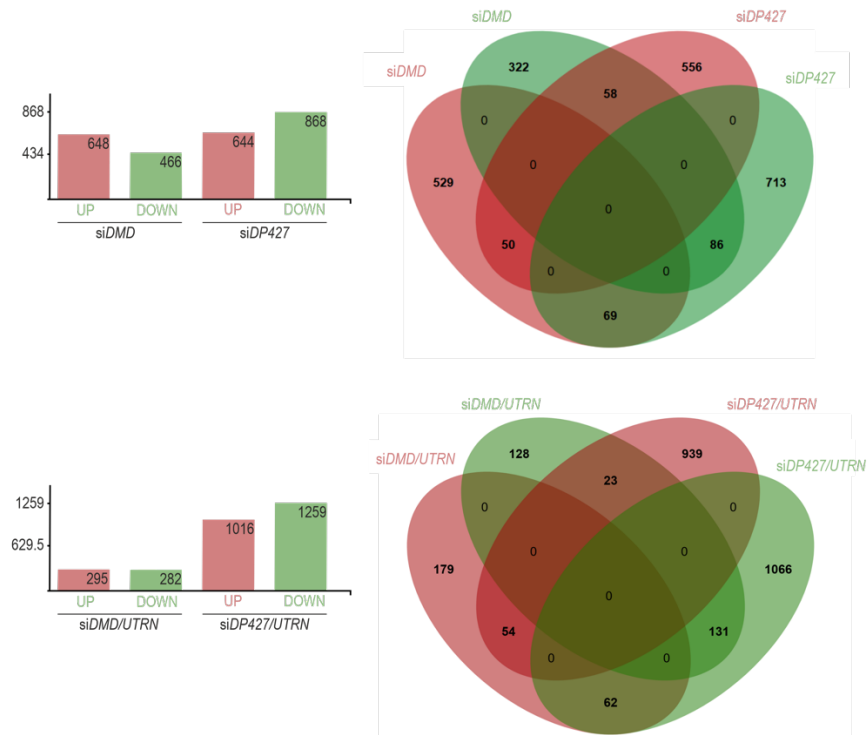


Figure 8: DP427-deficient cells exhibit distinct transcriptomic changes compared to DMD-deficient cells.

Summary of the number of upregulated (UP, red) and downregulated (DOWN, green) genes in *DMD/UTRN*- and *DP427/UTRN*-deficient HSkM cells at day 12 of differentiation. The Venn diagram illustrates the overlap between differentially expressed genes in both models, highlighting shared and unique transcriptional changes. Differential expression analysis was conducted using the Wald test, and p-values were adjusted for multiple comparisons using the Benjamini-Hochberg method (FDR ≤ 0.05).

The analysis of differentially expressed genes (DEGs) in comparison to control cells identified 295 upregulated and 282 downregulated genes in HSkM cells following *siDMD/UTRN* treatment and 1,016 upregulated and 1,259 downregulated genes in HSkM cells after *siDP427/UTRN* delivery. Some interesting differences in gene expression were observed between tested samples. Specifically, 62 genes were upregulated in *siDMD/UTRN* but downregulated in *siDP427/UTRN* (Fig. 8). These genes were associated with muscle contraction regulation (*TNNC2*, *ACTA1*), myofibril stabilization (*NEB*, *UNC45B*), mitochondrial function (*BCKDHB*, *ACTN2*, *TTN*), oxidative stress (*ADARB1*, *DUSP4*, *EGR1*), apoptosis (*HDAC4*, *MEGF10*, *TRIO*), muscle regeneration and differentiation (*MYOD1*, *MYOG*, *LMOD2*, *TNNC2*), fibrosis and tissue remodeling (*COL14A1*, *OLFML2B*), and cellular signaling (*RASSF4*, *SETBP1*) (Dataset S1). Conversely, 23 genes displayed the opposite trend, being downregulated in *siDMD/UTRN*, but upregulated in *siDP427/UTRN* (Fig. 8). These genes were linked to mitochondrial function (*ISCU*, *PGK1*, *FTL*), oxidative stress (*GCLM*, *GPX8*, *TRAM1*), apoptosis (*BIRC3*, *FSTL1*),

muscle regeneration and differentiation (*ADAM12*, *INHBA*, *FBN1*), fibrosis and tissue remodeling (*FSTL1*, *FBN1*), cellular signaling (*FRMD6*, *ITGB1*, *UBC*), cytoskeleton organization and cellular structure (*CAPZA1*, *TAGLN*, *ARPC2*, *CNN3*), nicotinamide metabolism (*NNMT*), metal transport such as zinc and iron (*SLC39A14*), and lipid metabolism (*UGCG*) (Dataset S1).

We also identified similarities between these models, with 54 genes upregulated in *siDMD/UTRN* and *siDP427/UTRN* samples, while 131 genes were downregulated in both conditions, indicating shared transcriptional permission and repression (Fig. 8). Upregulated genes were associated with mitochondrial function (*TOMM20*, *MSRB3*), oxidative stress (*AKR1B1*, *GDF15*), apoptosis (*MCL1*, *FKBP10*), muscle regeneration and differentiation (*MET*, *IGFBP2*, *IGFBP5*), fibrosis and tissue remodeling (*CCN2*, *FBLN5*, *THBS2*), cellular signaling (*CXCL12*, *PIEZO2*, *PRICKLE1*), cytoskeleton organization and cellular structure (*MYH10*, *DSP*), metabolism (*SLC16A6*, *CDS2*), phospholipid biosynthesis (*CDS2*), tissue elasticity (*ELN*), and regulation of cell proliferation and differentiation (*SPOCK1*). The downregulated genes were associated with mitochondrial function (*NCEH1*, *ATP2B1*, *SOD2*), oxidative stress (*ALDH3B1*, *TXNIP*, *SOD2*), apoptosis (*HSPA5*, *PARP14*, *PARP9*), muscle regeneration and differentiation (*DMD*, *TNNT2*, *TPM3*), fibrosis and tissue remodeling (*COL1A1*, *COL3A1*, *FN1*), cellular signaling (*CXCL1*, *CXCL3*, *ETS1*), cytoskeleton organization and cellular structure (*VIM*, *TUBB6*, *PLEC*), metabolism (*SCD*, *ELOVL5*, *HMGCS1*), and immune and inflammatory responses (*ISG15*, *MX1*, *IFI44*) (Dataset S1).

Next, we tested which pathways are significantly affected in myotubes treated with *siDP427* and *siDMD* (Dataset S1). KEGG pathway analyses revealed significant alterations in samples with and without concomitant downregulation of the *UTRN* gene in cytoskeletal organization, metabolism, motor proteins, and ECM-receptor interactions (Fig. S6-8). In contrast, these models were similarly affected in regard to the regulation of the actin cytoskeleton and the focal adhesion pathway (Fig. S9-10), probably due to the fact that, unlike DP427, DP71 does not possess an actin binding site (Dataset S2). Our analysis highlights distinct and overlapping regulatory responses associated with the presence of DP71 in DMD myotubes. Specifically, we show that DP71 positively affects muscle fibers during the early stage of myofiber differentiation due to its influence on expression of genes involved in muscle contractility regulation, myofibril stabilization, cytoskeletal organization, mitochondrial function, oxidative stress, cellular signaling and apoptosis. Similarly, the KEGG pathway analyses indicated convergence

of multiple gene products in skeletal muscle cytoskeleton, motor proteins, and ECM-receptor interactions pathways.

DISCUSSION

To shed more light on the function of different dystrophin isoforms, we examined expression patterns of the *DMD* and *UTRN* genes in various cell types and tissues at different developmental stages. The results showed that among all isoforms, *DP71* mRNA was the most abundant in most samples, with its elevated presence in proliferating cells and at early developmental stages, which aligns with previous studies^{9, 41}. In highly specialized differentiated tissues, such as skeletal muscle, heart, and brain we noticed relatively high levels of *DP427*, consistent with the fact that these tissues are most affected in DMD patients. As recent results showed that approximately 10% of affected individuals lose all dystrophin isoforms^{7, 13}, and that there are some phenotypic differences between them and patients missing just *DP427*, not only in cognitive performance but also in growth^{9, 13}, we hypothesized that this may be directly related to the presence and function of *DP71* in skeletal muscle. Surprisingly, we noticed significantly higher levels of *DP71* in *DP427*-deficient myotubes, and this increase was even greater when utrophins were missing.

Dystrophin and dystrophin/utrophin deficiency resulted in myofiber degradation, characterized by significantly shorter fibers and disruption of cytoskeletal proteins such as tubulin, myosin, and actin. The increase in *DP71* was accompanied by restored β -dystroglycan amounts in force expression studies and a decrease in DGC-related mRNAs in RNA-seq experiments, suggesting sufficient DGC proteins levels, eliminating the need for compensatory transcriptional activation. Furthermore, we observed striking improvements in myofiber morphology and function, and restoration of the proper arrangement of cytoskeletal proteins. These results seem to contradict previous results showing that *DP71* can assemble the DGC in muscles of transgenic *mdx* mice (lacking only *Dp427*), but without any therapeutic improvement^{32, 35}. However, in these mice, only one variant was generated, and its synthesis was limited to mature fibers. Importantly, we also observed an increase in apoptosis marker at later stages of differentiation in *DP427*-deficient fibers, suggesting that the *DP71*-induced therapeutic effects are indeed limited to the early stage of differentiation and that its increase cannot fully prevent myofiber degradation but rather delays its progression. This also confirms the current state of knowledge, indicating a differential role of *DP71* and *DP427* during myogenesis³⁴ and the crucial role of the actin-binding N-terminal part of dystrophin in mature myofibers⁴².

It is also important to note species-dependent differences in the synthesis of DP71 variants, as our data indicate much higher and more heterogeneous expression of the *DP71* gene in humans, with multiple splice variants present in proliferating cells of different origin and at the early stage of myofiber development. Our data also point to transcriptional activation of the *DP71* promoter in the absence of DP427 rather than the post-transcriptional increase in the stability of DP71 transcripts. This observation agrees with previous findings, where Sp1, Sp3, and YY1 transcription factors were identified as key regulators of the Dp71/Dp427 myogenesis feedback loop ^{16, 43}, suggesting that the absence of DP427 prevents the transcriptional shut down of the *DP71* promoter. Silencing of *UTRN* transcripts either in HSkM or HeLa cell models also increased the expression of the *DMD* gene, indicating that the loop involves not only *DP71* and *DP427* but also the *UP395* gene, and may include also other utrophin genes in other tissues ⁴⁴.

The lack of either DP71 in proliferating cells or DP427 in myofibers resulted in a plethora of phenotypic aberrations including affected membrane permeability, increased ROS production, mitochondria dysfunction and aggregation, and cyto- and genotoxicity ²⁰. All these processes are intertwined affecting overall cell performance, i.e. affected membrane function can lead to ROS overproduction and calcium influx, leading to mitochondria damage and apoptosis. On the other hand, overproduction of ROS can disturb calcium homeostasis and as a result to membrane permeability, mitochondria dysfunction, further leading to DNA damage and apoptosis ^{45, 46, 47}.

We show that a compensatory increase in DP71 reduced ROS production, calcium influx, and increased viability of DP427-deficient myofibers. As we observed several DP71 variants in either proliferating cells or in myofibers lacking DP427, we addressed their putative different functions by performing forced expression studies in *DMD* ko HeLa and HEK293 cells. These studies showed that DP71 splice variants differ in their stability, localization, ability to restore β -dystroglycan amounts, the cell plasma membrane function, mitochondrial transmembrane potential and calcium levels. Although some authors reported DP71 proteins in the nucleus ²⁴, our results point to their predominant cytoplasmic and membrane localization. Membrane-bound DP71 Δ 71-74 Δ 78 most significantly restored β -dystroglycan levels, plasma membrane and mitochondrial transmembrane potential, presumably by increasing the content of active mitochondria. In contrast, exon 78 variants had a positive impact on lowering calcium levels, with DP71 and DP71 Δ 71 showing preferential interaction with α -syntrophin, and the DP71 variant partially restoring cell numbers. Full dystrophin- β -dystroglycan and - α -syntrophin

binding sites were previously mapped to exons 62-73 and 73-75, respectively^{38, 48, 49}. Here we show that neither exons 71-74 nor 78 are required for β -dystroglycan binding, while α -syntrophin interacts preferentially with the DP71 variants containing either all exons or lacking only exon 71. Presumably the lack of exons 71-74 and 78 causes that DP71 is not binding to cytoskeletal proteins and then it is free to interact with binds then preferentially β -dystroglycan. Our results also indicate that β -dystroglycan and α -syntrophin localize to different cell compartments and do not participate in the formation of a DGC-like complex in proliferating cells.

Given the multiple *DP71* transcript splice variants in the tested cell models, as well as the different localization and exerted effects of DP71 proteins, the less beneficial outcome in the *DMD* ko HeLa cell line than in DMD muscle fibers may be due to uncontrolled synthesis of a single variant. Our results indicate that excessive DP71 proteins increase ROS levels and that their highest nuclear/mitochondrial levels correlate with the lowest cell numbers upon *DP71 Δ 71 Δ 78* delivery, possibly due to accumulated DNA damage. Others indicated that in muscle fibers, excessive DP71 may displace DP427 from its interaction partners⁵⁰ and, given the similar binding properties of dystrophins and utrophins, DP71 may exert its dominant negative effect on UP395. Skipping exon 78 and delivery of micro-dystrophin without exon 78 was shown to induce muscle fiber abnormalities⁵¹. Our results show that also in proliferating cells, where exon 78 variants predominate, DP71 proteins lacking exon 78 may be more toxic when synthesized in an uncontrolled manner.

RNA-seq analyses indicated that the DP71-mediated improvement in DP427-deficient muscle fibers may be due to functional restoration of gene products involved in cytoskeletal assembly, membrane stability maintenance, mitochondrial numbers, oxidative stress, cellular signaling, and apoptosis. In contrast, the lack of significant changes in actin cytoskeleton regulation and focal adhesion pathways between models lacking all dystrophins and those deprived of only DP427 confirm that DP71 cannot restore proper actin binding and the downstream signaling³⁸, presumably leading ultimately to myofiber death. Graham et al. previously suggested that DP71 may play a role in focal adhesion⁵². However, our data indicate that an increase in DP71 alters the focal adhesion pathway compared to wt cells and there are no significant differences between the loss of all dystrophin/utrophin isoforms and the loss of only DP427 and utrophins. Furthermore, integrins, which form focal adhesions, are upregulated in myofibers treated with *siDP427* and *siDP427/UTRN* compared to *siDMD* and

siDMD/UTRN, suggesting that a high increase in DP71 may further disrupt focal adhesion dynamics, leading to the activation of compensatory mechanisms in integrin synthesis.

Current DMD therapies primarily focus on dystrophin restoration and upregulation of the *UTRN* gene, both of which have shown promising results. However, each strategy has its limitations. Utrophin alone cannot fully compensate for the loss of dystrophin, micro-dystrophin therapies may provoke immune responses, and dystrophin restoration using CRISPR/Cas9 or AONs is limited to DMD patients with specific mutations, inducing dystrophin expression in only a relatively small number of muscle fibers³⁸. What is important, Guiraud et al. demonstrated that combined dystrophin and utrophin therapies could improve therapeutic outcomes⁵³. Our findings show that utrophin and DP71 can coexist within a single cell, suggesting that modulating both utrophin and DP71 could be explored as an alternative therapeutic strategy, especially considering the immune response that is related to the N-terminal and rod domain parts of dystrophin^{38, 54}. Promoter modulators or factors that stabilize mRNA are significantly smaller than micro-dystrophin, which facilitates their delivery into cells. Furthermore, targeting specific DP71 promoter isoforms could allow cells to regulate isoform expression based on their functional needs, preventing uncontrolled expression of a single variant, as observed in HeLa cells. This approach could offer a more physiological solution by enabling cells to produce the necessary isoforms in a controlled and context-dependent manner.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AVAILABILITY OF DATA AND MATERIALS

RNA-seq data from HeLa cell lines are available at GEO accession GSE289357 at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE289357> by entering token yjqxmusclrovfgl into the box.

RNA-seq data from HSkM cell lines are available at GEO accession GSE303672 at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE303672> by entering token enkpnewutfenfkh into the box.

AUTHOR CONTRIBUTION

PK and SS designed the study. Data contribution: SS, figs 1-5,7-8, S1, S3-6; PKW, figs 2C, 8, S6B, S7-11; KD, figs. 2E-F and 6, 7A; AD, C2C12 cells in fig 1B, C, S2 and contribution in plasmid preparation in fig S5. SS, PK and PKW wrote the manuscript. SS, PK, PKW, KD and AD prepared the figures.

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SUPPLEMENTARY INFORMATION

DP71 compensates for full-length dystrophin DP427 in early stages of myofiber differentiation in cell models of Duchenne muscular dystrophy

Running title: DP71 compensates for DP427 in early DMD myofibers

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Table S1: A list of primers and siRNAs used in the study.

Target	Species	Forward	Reverse
RT-PCR			
<i>RPLP0</i>	Human	CGTCCTCGTGGAAGTGACAT	CTTGGAGCCCACATTGTCTG
<i>DMD</i>	Human	GGAGGAGATGGCAGTGGAGAC	CACTTGGCTTGATGCTTGGC
<i>Dp427</i>	Human	CTGCACTAGGCTGAATGGGAA	CTCCGCCAGGAATGTTTTTCAG
<i>DP140</i>	Human	TGGGGACCGAAAGAGGTTTT	CAGGCTTCCCAATTTTTCCTG
<i>DP116</i>	Human	TCAGTGCTGTAGTGCCCGGT	CAGGACTGCATCATCGGAACCT
<i>DP71</i>	Human	CTCGTACCCACACTCGACCG	TGCTGGTCCAAGGCATCACA
<i>UTRN</i>	Human	AAACTCCTCAGGCAGCACAA	GAGGCATCTGGATCAAGCGA
<i>UP395</i>	Human	GTTCCAGAGCTCAACACACTGC	TGCCCTGTCAAAACAACCTTC
<i>UP140</i>	Human	CACAATACTTGGGTGGATTCAATGC	TGCCTGGGAGAGTTTCTGCAC
<i>UP113</i>	Human	ACTTAAATGATGGCTGTGGTGAGA	TCTGCCTGTTCCGTCAATGCT
<i>UP71</i>	Human	GCATTGGGTTTCTGAATGGTTGA	CCAAGGGATTGAAAGAGTTCGG
<i>MYOD</i>	Human	CGCAACGCCATCCGCTAT	TGTAGTCCATCATGCCGTCG
<i>MYOG</i>	Human	TGCCATCCAGTACATCGAGC	CAGATGATCCCCTGGGTTGG
<i>MYH1</i>	Human	AGGTGCGATCTCTACGCCA	CGCTCCCTTTCAGACTTTCG
<i>Rplp0</i>	Mouse	GCTTTGGGCATCACCACGAA	GTTGCGGACACCCTCCAGAA
<i>Dmd</i>	Mouse	CTCCACCCTCAGCACAAAGAG	TCATGCCAACATGCCCAAAC
<i>Dp427</i>	Mouse	ATGAGGGAACAGCTCAAAGG	TGCAGCTGACAGGCTCAAGA
<i>Dp260</i>	Mouse	ACAACAGATTGCAACAAGTGA	AATTCCTGGGCAGACTGGAT
<i>Dp140</i>	Mouse	GCATTGCTGACTGTTCTGAGC	CCCAGTTGCATTCAGTGTTCTG
<i>Dp116</i>	Mouse	TTTCTTAGGTTTGCTATGCAACAGG	CAGGGGTGCTTCATCCGAAC
<i>Dp71</i>	Mouse	ATGAGGGAACAGCTCAAAGG	TGCAGCTGACAGGCTCAAGA
<i>Utrn</i>	Mouse	AGAGCACTATGACCCCTCCC	ATCCTCCACGCTTCCTGTTG
<i>Up395</i>	Mouse	CCTGACAATGGGCAGAACGA	GCTGGTGGTGAAGTTGAGG
<i>Up140</i>	Mouse	AGGCCCTCTTCCAGTTCCTA	AAGACATTATAGCCCTACCCCG
<i>Up113</i>	Mouse	TCATCCAGTCGATGCTGAAG	CAAAGGATGGCAGTTTCCTC
<i>Up71</i>	Mouse	AAGAGCTCATTTTAGGATGAT	CTAGATTTCTGAGCGATCC
PCR			
<i>DP71</i> (PCR1)	Human	AAAACCATGCGGGAATCAGG	AAAACCATGCGGGAATCAGG

<i>DMD ex70,76</i> (PCR2)	Human	TTGCGAAGCATCCCCGAAT	ACTGCTGTCTGGACCTCTGTA
<i>DMD ex76,79</i> (PCR3)	Human	GGTTGGCAGTCAAACCTTCGG	TCCATCGCTCTGCCCAAATC
<i>Dmd ex69,75</i> (PCR2)	Mouse	CATGGTAGAGTATTGCACTCCG	GGAGGAGATGGCAGTGGAGAC
<i>Dmd ex77,79</i> (PCR3)	Mouse	TCTTCTGAGTCCTCCCCAGG	CTCCATCGCTCTGCCCAAAT

Name	Species	Sequence
siRNA		
<i>DMD/Dmd</i> (s501299)	Human, mouse	AAACAAAUUUCGAACCAAAtt
<i>DP427.1</i>	Human	GGAUAAACCCAAAUCAGAUUtt
<i>DP427.2</i>	Human	GGAGGGAACUACAUGAAGAtt
<i>DP71</i>	Human	GCUCACUCCUCCACUCGUAtt
<i>UTRN</i> (s553059)	Human	GGAAUAUUGUAUACCUACAtt

Table S2: A list of antibodies used in the study.

Antibody	Species	Concentration		Manufacturer
		WB	IF	
Dystrophin (+78)	Rabbit	1:1000		Abcam (ab15277)
Dystrophin	Rabbit	1:2000	1:200	Proteintech (12715-1-AP)
Lamin B1	Rabbit	1:1000	1:500	Abcam (ab16048)
Myosin (B-5)	Mouse		1:50	Santa Cruz Biotechnology (sc-376157)
FLAG	Rabbit	1:2000	1:100	Abcam (ab1162)
α -Tubulin (DM1A)	Mouse	1:2000	1:500	Abcam (ab7291)
GAPDH	Mouse	1:1000		Proteintech (60004-1-Ig)
β -Dystroglycan (43DAG1/8D5)	Mouse	1:1000	1:50	Abcam (ab49515)
α -syntrophin	Mouse	1:2000	1:20	Abcam (ab11425)
anti-mouse IgG (Fab specific)–peroxidase antibody	goat	1:10 000		Sigma–Aldrich (A9917)
anti-rabbit IgG	goat	1:10 000		Abcam (ab97051)
Rhodamine (TRITC) anti-mouse	Donkey		1:1000	Jackson Immunoresearch (715-025-150)
Alexa 488 anti-rabbit IgG H&L	Donkey		1:400	Abcam (ab150073)
Alexa 594, streptavidin			1:500	Molecular Probes

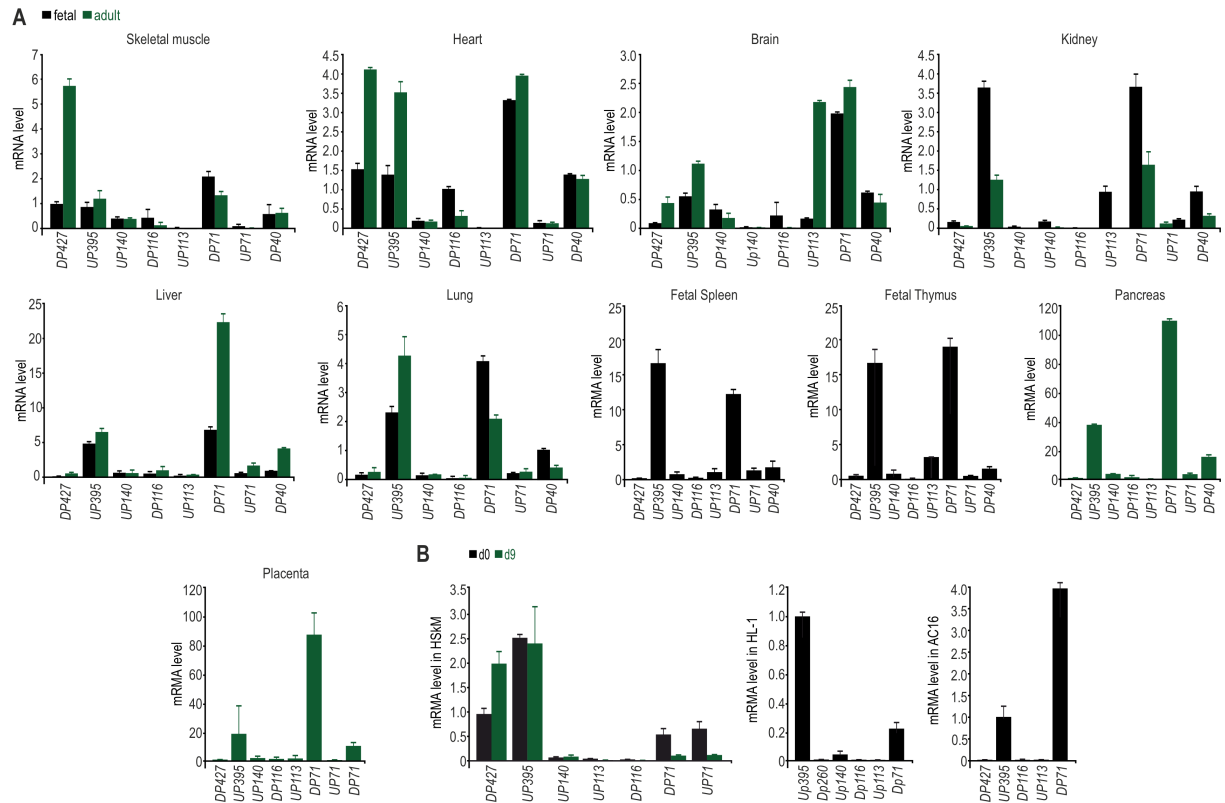


Figure S1: The *DMD* and *UTRN* genes exhibit varying expression patterns depending on tissue type and developmental stage.

A RT-PCR analyses of *DMD* and *UTRN* gene expression in fetal and adult tissues (skeletal muscle, heart, brain, kidney, liver, and lung), fetal tissues (spleen and thymus), and adult samples (pancreas, placenta). The mRNA level was normalized to the *DP427* level in fetal skeletal muscle tissue. **B** RT-PCR analyses of *DMD/UTRN* expression in proliferating (d0) and differentiated (d9) human skeletal muscle cells (HSkM) and proliferating human (AC16) and mouse-derived (HL-1) heart muscle cells. The results in **A** and **B** are shown as mean values + SD.

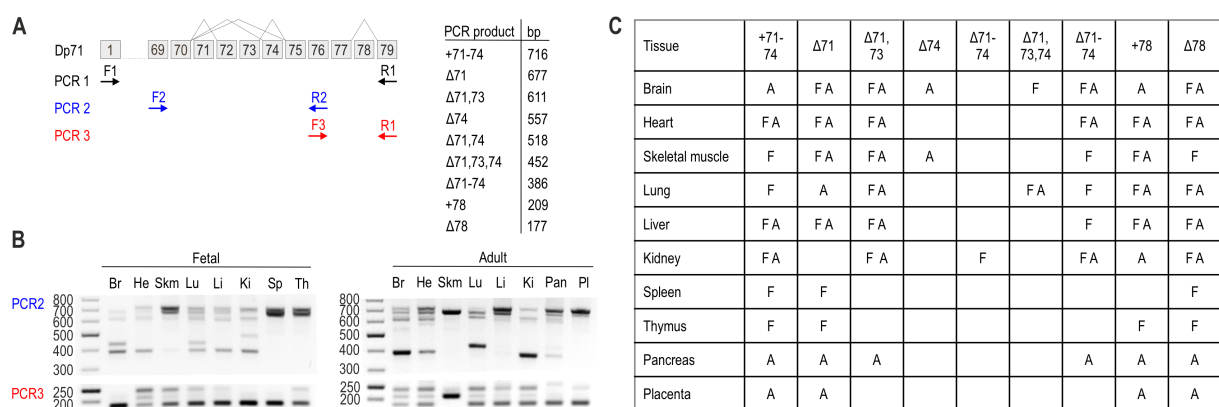


Figure S2: Stage- and tissue-dependent splicing diversity of *DP71* transcripts.

A Schematic illustration of PCR primers used for whole *DP71* isolation (F1/R1) and detection of splice variants targeting exon 71-74 (F2/R2) and exon 78 (F3/R1) exclusion, with a corresponding table of product sizes. **B** The corresponding PCR results display the amplified products in fetal and adults tissues: Brain, Br; Heart, Hr; Skeletal muscle, Skm; Lung, Lu; Liver, Li; Kidney, Ki; Spleen, Sp, Thymus, Th; Pancreas, Pa; Placenta, Pl. Hybrid duplex is a result of the hybridization of two products, +78 and $\Delta 78$. **C** Summary of *DP71* isoform transcript levels across different tissues with their distribution at both fetal (F) and adult (A) stages.

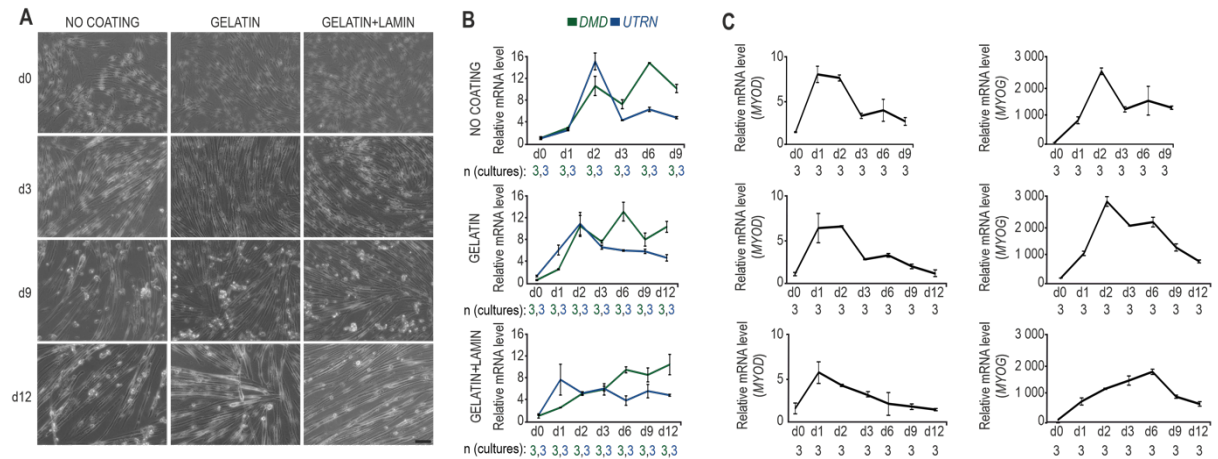


Figure S3: The HSkM cell line exhibits a similar differentiation pattern regardless of coating type.

A Live-cell images of HSkM cells at different myogenesis time points, cultured on various substrate coatings (no coating, gelatin alone or gelatin and laminin). **B** and **C** RT-PCR analyses of the *DMD/UTRN* (**B**) as well as differentiation markers (*PAX7*, *MYOD*, *MYOG*, *MYH1*; **C**) genes in the HSkM cell line after differentiation induction on plates without coating or coated with gelatin or gelatin/laminin. Bar in **A**, 50 μ m. The results in **B** and **C** are shown as mean values $\pm$ SD. n, the number of biological replicates.

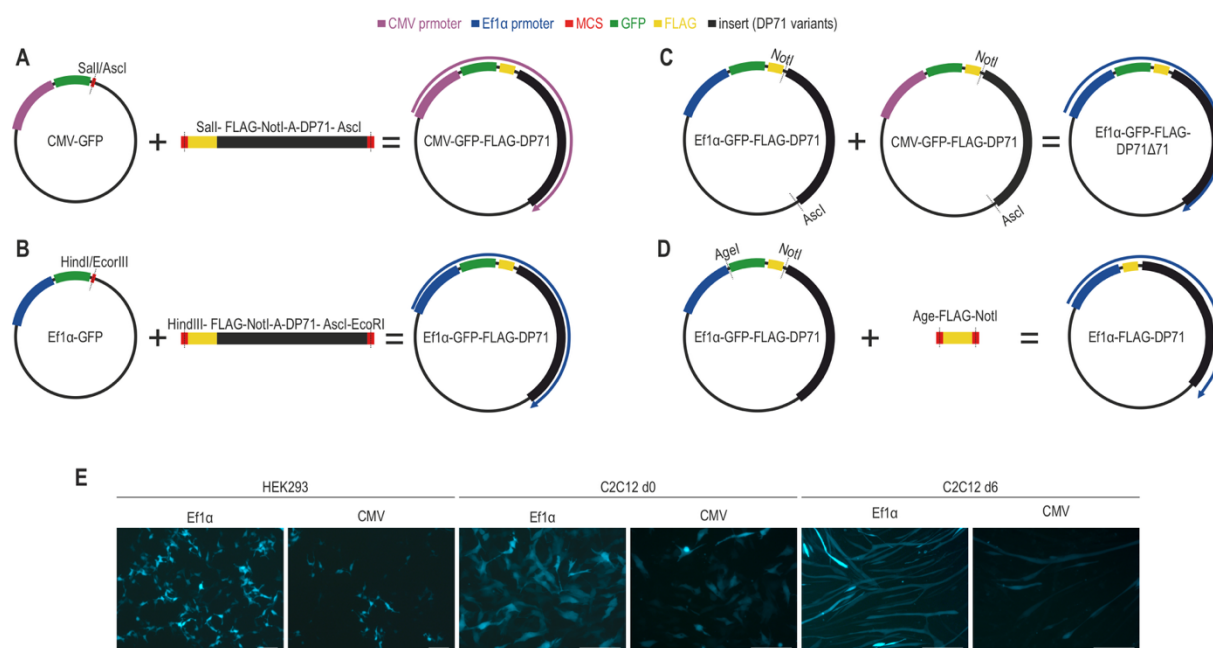


Figure S5: Preparation of CMV and EF1α plasmids.

A Graphic representation of cloning strategies and preparation of CMV/Ef1α-FLAG-DP71 vectors w/o GFP. **B** Comparison of CMV and αEF1 promoter activities in HEK293 and C2C12 cells following delivery of CMV-GFP-FLAG and αEF1-GFP-FLAG vectors. Bar in **E**, 50 μm.

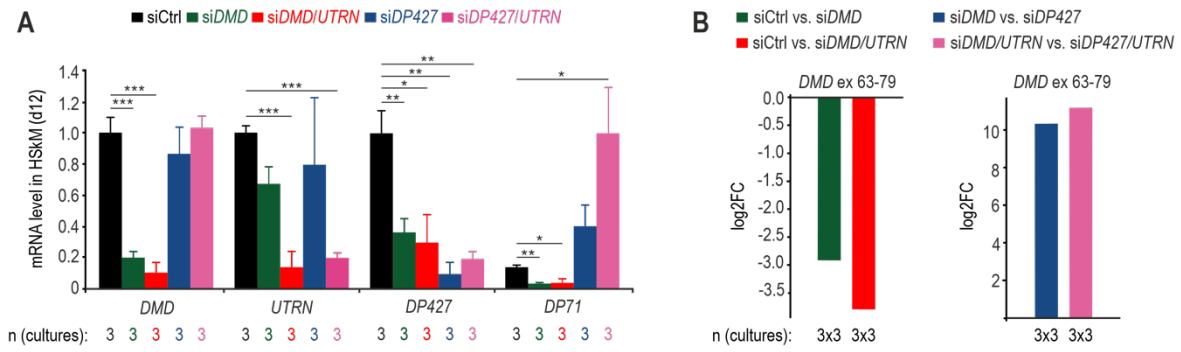


Figure S6: Validation of siRNA knockdown efficiency prior and after RNA Sequencing.

A RT-PCR analyses of *DMD/UTRN* and *DP427/DP71* isoforms in HSKM cell line at day 12 of differentiation after siRNA treatment. **B** RNA-seq analysis of *DMD* exons 63-79 levels in HSKM cell line at day 9 of differentiation following siRNA treatment. Note downregulation of *DP71/DP427* mRNAs after si*DMD* delivery and upregulation of *DP71* levels after si*DP427* administration. The results in **A** are shown as mean values + SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (t-test).

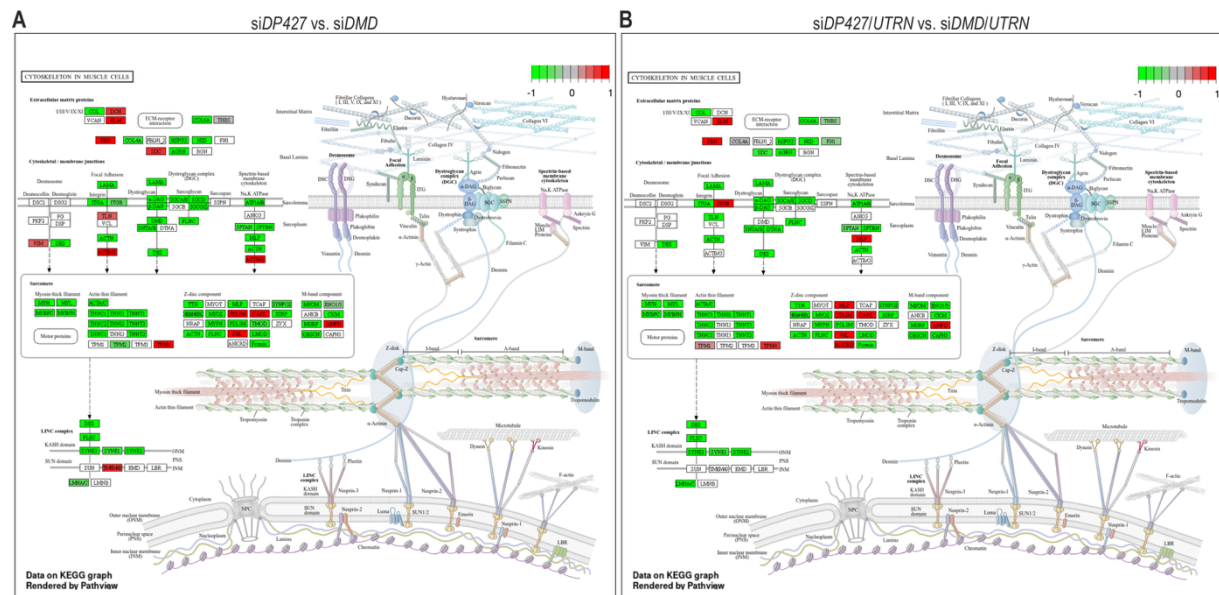


Figure S7: Differential expression of genes involved in the cytoskeleton-related metabolic pathway in DP427- and dystrophin-deficient HSkM myotubes.

A and **B** Comparison of cytoskeleton-related metabolic pathway alterations in myotubes lacking all *DMD* isoforms versus those lacking only *DP427* isoform (**A**) and cells deprived of both *DMD* and *UTRN* versus those lacking *DP427* and *UTRN* (**B**). The image illustrates the cytoskeleton-related metabolic pathway identified in the KEGG analysis (hsa04820). Green indicates genes with decreased expression, red indicates genes with increased expression.

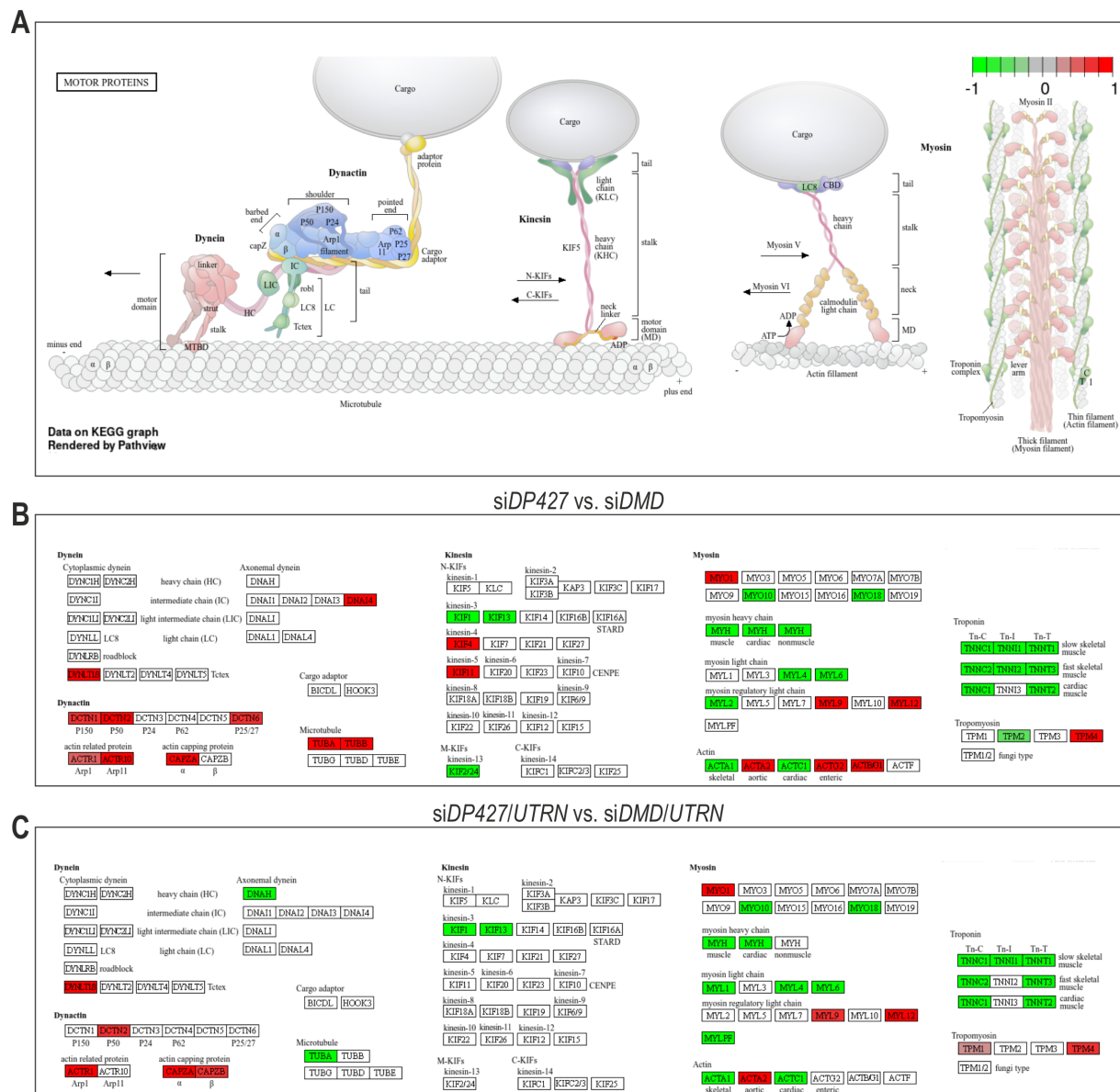


Figure S8: Differential expression of genes involved in the motor proteins pathway in DP427- and dystrophin-deficient HSKM myotubes.

A Graphic representation of motor proteins. **B** and **C** Comparison of motor proteins pathway alterations in myotubes lacking all *DMD* isoforms versus those lacking only *DP427* isoform (**B**) and cells deprived of both *DMD* and *UTRN* versus those lacking *DP427* and *UTRN* (**C**). The image illustrates the motor proteins pathway identified in the KEGG analysis (hsa04814). Green indicates genes with decreased expression, red indicates genes with increased expression.

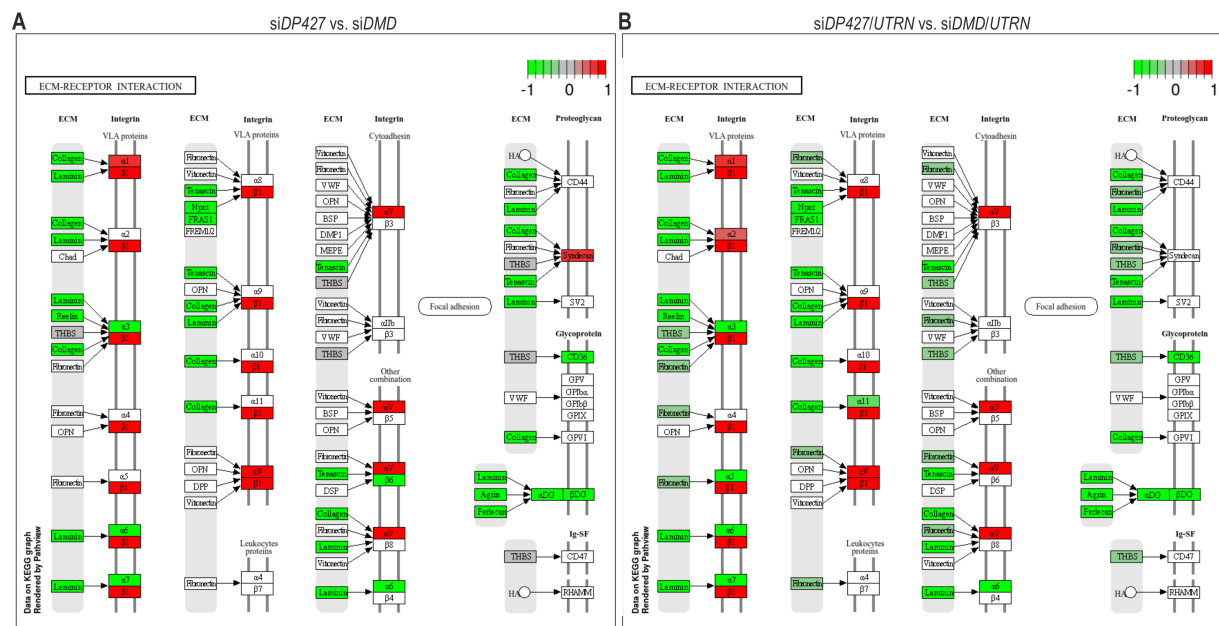


Figure S9: Differential expression of genes involved in the ECM-receptor interaction pathway in DP427- and dystrophin-deficient HSkM myotubes.

A and **B** Comparison of the ECM-receptor interaction pathway alterations in cells lacking all *DMD* isoforms versus those lacking only *DP427* isoform (**A**) and cells deprived of both *DMD* and *UTRN* versus those lacking *DP427* and *UTRN* (**B**). The image illustrates the ECM-receptor interaction pathway identified in the KEGG analysis (hsa04512). Green indicates genes with decreased expression, red indicates genes with increased expression.

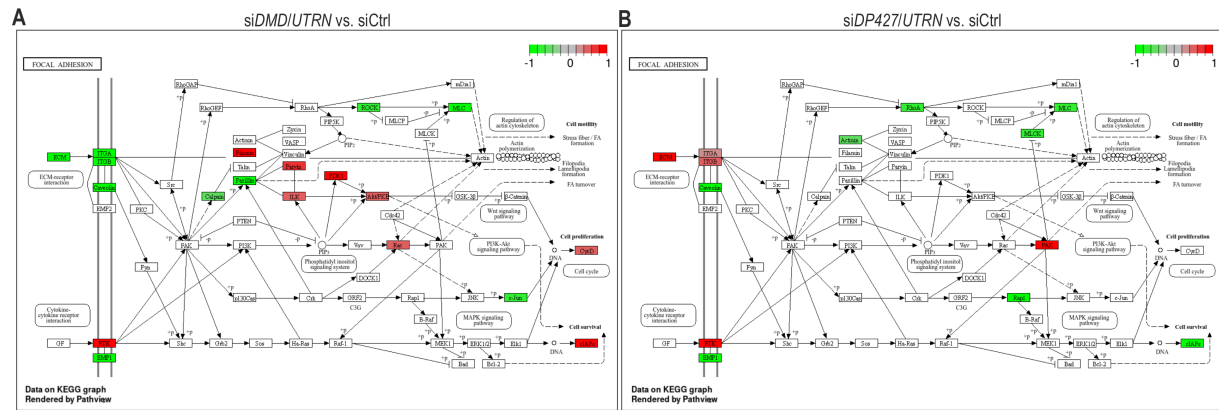


Figure S11: Similarly affected expression of genes involved in the focal adhesion pathway in DP427- and dystrophin-deficient HSkM myotubes.

A and **B** Comparison of the regulation of focal adhesion alterations in siCtrl-treated cells versus those lacking *DMD* and *UTRN* (**A**) or *DP427* and *UTRN*. The images illustrate the focal adhesion alteration pathway identified in the KEGG analysis (hsa04810). Green indicates genes with decreased expression, red indicates genes with increased expression.

Chapter 5: Summary and perspectives

Dystrophin- and Utrophin-Based Therapeutic Approaches for Treatment of Duchenne Muscular Dystrophy: A Comparative Review

Extensive research on the molecular mechanisms of DMD has led to the development of genetic therapies designed to restore the dystrophin reading frame or enhance the production of truncated dystrophin proteins using viral vectors^{54–58}. Despite these advances, recent studies suggest that therapeutic dystrophins may be immunogenic in patients, raising the possibility that utrophin, a dystrophin paralog, could serve as a more effective therapeutic alternative^{48,59,60}. In this study, we compared the amino acid sequences and structures of dystrophin and utrophin, integrating existing data with our expanded in-silico analyses. Additionally, we extended our research to in silico comparisons of various μ Dys proteins that are currently tested in clinical studies, taking into consideration beneficial outcomes, amino acid composition, and protein folding similarity to wild type (wt) dystrophin.

Sequence analysis revealed that the full-length dystrophin synthesized in muscle (DP427m) and utrophin isoform UP395A share 86.37% nucleotide and 41.61% amino acid identity, with higher similarity observed in domains responsible for binding β -dystroglycan, F-actin, α -dystrobrevin, and MARK2. Conversely, regions such as the nNOS binding site and the secondary actin-binding domain (ABD2) exhibit lower homology, accounting for functional differences between DP427 and UP395. In silico folding analyses confirmed a high degree of structural similarity in domains with significant amino acid conservation. Using the TM-align algorithm, we observed strong structural alignments in regions such as ABD1, the MARK2 binding site, and β -dystroglycan-binding domains, while the syntrophin-binding site displayed notable structural divergence.

Furthermore, in-silico analysis of μ Dys constructs in clinical trials revealed that while μ Dys–SB (manufactured and tested by Solid Biosciences)⁶¹ and μ Dys–ST/G (manufactured

and tested by Sarepta Therapeutics or Genethon in collaboration with Sarepta Therapeutics)⁶² maintain a rod domain structure similar to dystrophin, μ Dys–P (manufactured and tested by Pfizer)⁶³ and μ Dys–Y (designed by Yuasa et al.)⁶⁴ showed a more condensed structure with potential folding issues. rAAV-based therapies, however, face challenges such as pre-existing antibodies, and vector loss during cell division, with the immune system potentially recognizing dystrophin as foreign, thus diminishing therapy effectiveness. We identified that hinge 1 (H1) and 3 (H3) regions that have been commonly used in μ Dys share no sequence homology to the corresponding regions in utrophin that is naturally present in dystrophic tissues, and thus could, theoretically, more easily induce the immune response in treated DMD boys. In contrast, μ Utr circumvents immune reactions more efficiently, displaying lower immunogenicity and enhanced therapeutic potential, as evidenced by recent studies in dogs and mice, which showed μ Utr induces fewer adverse immune responses compared to μ Dys, positioning it as a more viable therapeutic option⁶⁵.

Our data demonstrated the potential of using in-silico amino acid sequence and protein structure analysis to determine the functional properties of new dystrophin- and utrophin-based therapies, enhancing their effectiveness and minimizing their limitations.

The ability of utrophin and dystrophin to co-localize opens the potential for combined therapeutic strategies. Guiraud et al. demonstrated that partial restoration of dystrophin (30% via AON) coupled with utrophin overexpression results in greater therapeutic outcomes than either approach alone⁶⁶. This evidence supports the idea that integrating multiple therapies could enhance efficacy and address the shortcomings of single-treatment approaches.

Dystrophin DP71 and DP427 isoforms determine cell viability during proliferation and myofiber differentiation

All DMD patients lack the full-length dystrophin DP427, and approximately 10% also lose all dystrophin isoforms, including DP71, which is widely expressed in various cell types, including myoblasts^{1,13–15}. This study aimed to determine the roles of dystrophin isoforms DP427 and DP71, as well as utrophin, in cell proliferation and myofiber differentiation. To address these research questions, we used both immortalized and primary cell lines to examine their functional roles.

We showed that loss of dystrophins solely or together with utrophins leads to dysfunction of HeLa and HEK293 cell lines, and proliferating and differentiated human primary skeletal muscle (HSkM) cells. We demonstrated that DP71 has an essential role in cell viability during proliferation, while DP427 is critical for proper muscle fiber differentiation. Despite their different synthesis profiles, loss of either DP71 or DP427 resulted in a similar pathological phenotype, including increased membrane permeability, elevated intracellular calcium, mitochondrial aggregation, ROS accumulation, and enhanced cyto- and genotoxicity leading to apoptosis. Additionally, CRISPR-mediated *DMD* knockout (ko) in HeLa and HEK293 cells highlighted utrophin's compensatory role, where UP395 partially mitigated the detrimental effects of the DP71 loss. Similar compensatory functions of UP395 were previously shown in cells deficient in DP427. Overall, our data indicate that DP71 and DP427 deficiencies impair cells during the proliferation stage and in myofiber differentiation, respectively, with utrophin offering partial compensation.

Altogether, these results explain the variability in disease severity among patients with differing in the presence of DP71 and suggest that individuals lacking both DP71 and DP427 in skeletal muscle may require distinct therapeutic approaches compared to those deficient only in DP427.

Dystrophin DP71, and DP427 isoforms determine cell viability during proliferation and myofiber differentiation

As DP71 is one of the isoforms synthesized in most DMD cases and we showed its important functions in proliferating cells, we aimed to investigate its potential compensatory role in DP427-deficient cell models. First, we conducted an extensive analysis of all dystrophins and utrophins variants across different tissues at fetal and adult stages, during the muscle regeneration process, and in different cell types. Next, we developed DP71/DP427- and DP427-deficient cell models. Finally, using plasmids encoding various DP71 splice variants, we explored their roles and therapeutic potential in dystrophin-deficient cell lines.

Our results highlighted the diversity of dystrophin and utrophin isoforms across different tissue types, developmental stages, and cell origins. We demonstrated that *UP395* and *DP71* are the most ubiquitously expressed isoforms, with *DP71* being particularly abundant during fetal stages. In contrast, *DP427* showed high expression as the predominant isoform in differentiated cells and adult tissues compared to fetal tissues, as well as in differentiated/regenerating cells compared to proliferating ones. Additionally, we found that DP71 has a higher level in human cells than in mouse cells, and this finding was confirmed in both skeletal and cardiac muscle cells.

Splicing analysis revealed that exon 78 exclusion is specific to proliferating cells, while its inclusion increases over time. This analysis also showed that human cells exhibit greater diversity in dystrophin variants than mouse cells and tissues.

We utilized an HSkM cell model with siRNA-mediated silencing of *DMD* or *DP427*. Given previous findings that utrophin can compensate for dystrophin deficiency, we also generated models with additional *UTRN* silencing. The data revealed an increase in DP71 synthesis following *DP427* silencing, which was further enhanced by the *UTRN* gene downregulation. Importantly, an increase in DP71 showed beneficial effects, including

improved cell differentiation, restoration of proper cytoskeletal protein arrangement, increased cell viability, and enhanced mitochondrial functionality. However, an increase in an apoptosis marker was still detected, suggesting that long-term cell viability may be compromised even in DP427- and DP427/utrophin-depleted cells. We also used RNA-seq analysis to compare signaling pathways between wt cells, cells lacking all dystrophin isoforms, and cells lacking only DP427 with additional silencing of all utrophin isoforms. Our data revealed that the expression of *DP71* in muscle cells, compared to all dystrophin/utrophin-deprived cells, alters the expression of factors regulating membrane integrity and cytoskeletal functions, potentially leading to an improvement in the phenotype. However, due to the absence of an actin-binding site in DP71, we did not observe a significant difference in actin cytoskeleton regulation, confirming that DP71 is not capable of maintaining actin functionality. Possibly, the lack of this function might lead to the activation of apoptosis in DP427-deficient cells.

To determine if a single DP71 isoform could mitigate the DMD phenotype, we engineered plasmids encoding different DP71 splice variants and introduced them into HeLa and HEK293 *DMD* ko cell models. Our analyses revealed that these isoforms differ in stability, subcellular localization, ability to restore β -dystroglycan levels, plasma membrane integrity, mitochondrial membrane potential, and intracellular calcium homeostasis. Notably, exons 71–74 determined DP71 stability, α -syntrophin interaction, and cytoplasmic localization in the presence of UP395. The membrane-associated DP71 Δ 71-74 Δ 78 isoform most effectively restored β -dystroglycan levels, plasma membrane integrity, and mitochondrial membrane potential, likely by enhancing the levels of active mitochondria. In contrast, isoforms containing exon 78 were more effective at reducing intracellular calcium levels, while DP71 Δ 71 Δ 78 notably elevated nuclear and mitochondrial ROS levels and markedly lowered cell numbers. Given the presence of multiple DP71 transcript variants in proliferating wt cells and DP427-

deficient myofibers, the limited rescue observed in *DMD* ko HeLa cells may be attributed to the uncontrolled synthesis of a single DP71 variant.

In summary, our data highlights the critical differences between murine and human DMD models. We demonstrated that DP71 can play a compensatory role; however, the internal regulation of splicing isoform synthesis within cells appears to be essential for proper DP71 functionality. Additionally, we emphasize the importance of exons 71–74 in maintaining dystrophin stability, modulating utrophin-dystrophin competition, and α -syntrophin binding. These findings should be carefully considered when designing therapeutic approaches for DMD. Furthermore, our findings show that utrophin and DP71 can coexist within a single cell, suggesting that modulating both utrophin and DP71 could be explored as an alternative therapeutic strategy.

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Additional research

During my PhD, I focused on optimizing research methodologies in biotechnology, along with other techniques discussed in this work. Additionally, I obtained the necessary certifications for conducting in vivo experiments, attended several conferences, and supervised both master's and bachelor's degree projects in the laboratory.

1. Plasmid design and evaluation

I designed and constructed multiple plasmids, which were tested across various cell lines. Our data revealed that the addition of a GFP tag at the C-terminus, combined with the insertion of DP116, exhibited cytotoxic effects (Fig. 3C). In contrast, a plasmid containing the same promoter but with the GFP tag positioned at the N-terminus remained fully functional (Fig. 3D). These findings highlight the importance of GFP localization in proper plasmid design. I also constructed plasmids with the EF1 α promoter (Fig. 3E). All plasmids were engineered for flexibility, allowing easy subcloning between constructs containing the CMV and EF1 α promoters (Fig. 3F). Additionally, the design permitted the removal of the GFP tag while retaining the FLAG tag (Fig. 3F).

Subsequently, I evaluated different promoters to identify the most efficient one for muscle cell lines and optimized transfection conditions by testing various transfection reagents. As described in the thesis, we demonstrated that EF1 α is the most effective promoter, and it was selected for future experiments.

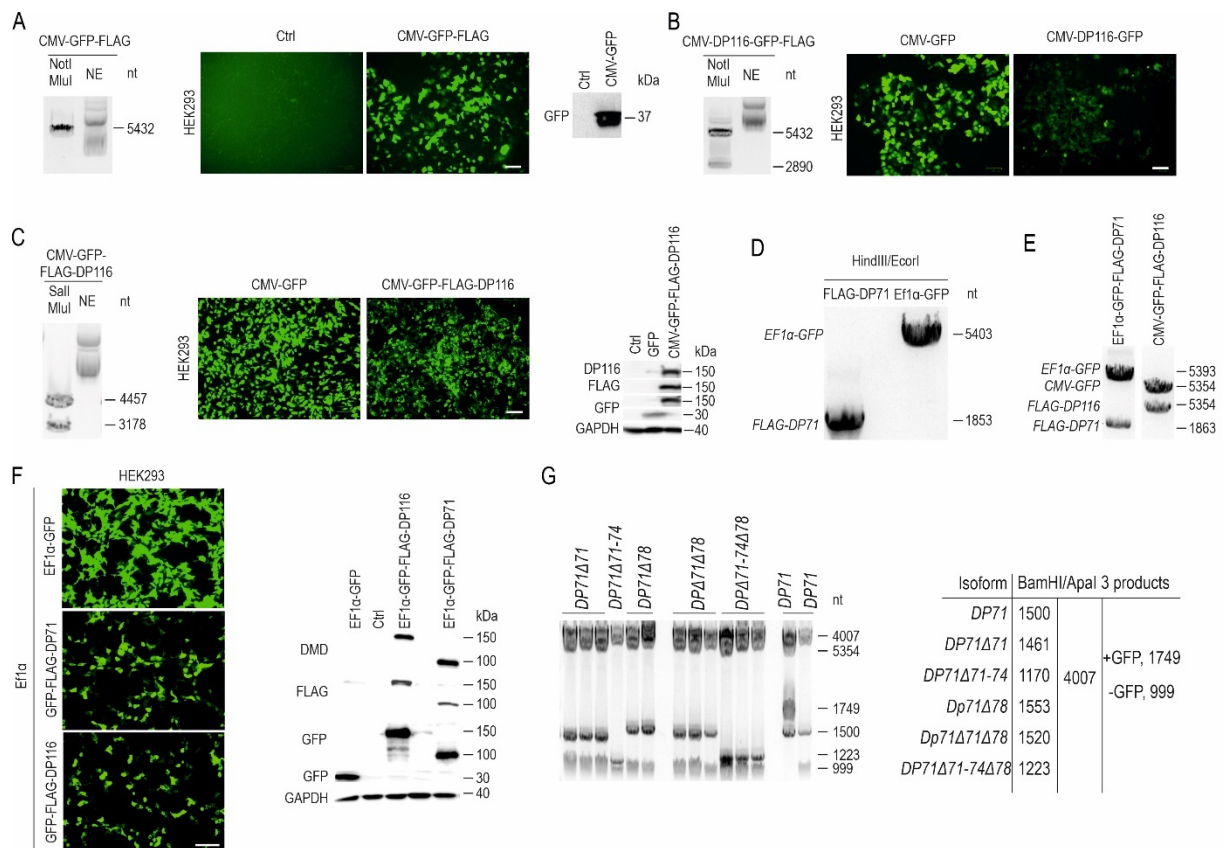


Figure 3 Validation of Plasmid Construction and Protein Expression

A Agarose gel electrophoresis confirming digestion of GFP and C-terminal pFLAG-C1 (CMV-FLAG) plasmid with BamHI and SalI restriction enzymes and confirmation of GFP insertion by immunofluorescence and immunoblotting; lipofectamine-transfected cells serve as a control, Ctrl. **B** Confirmation of *DP116* insertion into CMV-GFP-FLAG using NotI and MluI digestion, with non-digested plasmid (NE) as a control, verified by GFP fluorescence in live-cell imaging. **C** Cloning of *DP116* into pEGFP plasmid (N-terminal GFP fusion) confirmed by SalI and MluI digestion, GFP fluorescence in live-cell imaging, and immunoblotting. **D** Preparation of *DP71* with an additional FLAG sequence in EF1α plasmid using HindIII and EcoRI digestion. **E** Agarose gel electrophoresis showing transfer of *DP116* from CMV-GFP-FLAG-*DP116* to EF1α-GFP-FLAG-*DP71*. **F** Confirmation of *DP116* and *DP71* insertion into EF1α via GFP fluorescence in live-cell imaging and immunoblotting. **G** Electrophoresis confirming preparation of EF1α-FLAG-*DP71* variants without GFP, with expected restriction digestion patterns from BamHI and ApaI. *DP71* variant was used as a control of with without GFP plasmids.

2. Cardiomyocyte cell lines evaluation

I conducted an extensive analysis of available cardiomyocyte cell lines, specifically the human-derived AC16 and the murine HL-1 cell line. AC16 is a human cardiomyocyte cell line derived from ventricular heart tissue. It is commonly used for studying cardiac biology, metabolism, and disease mechanisms, including cardiotoxicity and heart failure research. Previously, researchers demonstrated that AC16 cardiomyoblasts were capable of differentiating into cardiomyocytes. We evaluated two different culture media (Fig. 3A). Initially, the cells were maintained in a proliferative medium, which was replaced with a differentiation medium containing 2% horse serum, 1× ITS, and 1× Penicillin-Streptomycin solution using either DMEM/F12 (Medium 1) or DMEM/F12 with HEPES (Medium 2). While the differentiation of AC16 cells into cardiomyocytes depends on the inactivation of SV40, we silenced SV40 after one day to initiate the differentiation process. Due to the higher viability of cells cultured in Medium 1, we selected this condition for further experiments (Fig. 4A). AC16 cells were cultured for six days, and we assessed SV40 levels along with differentiation markers known to indicate cardiomyoblast progression.

Although we successfully reduced SV40 expression initially, its levels began to increase after day 1. While still lower than in wt cells, this increase could potentially affect the differentiation process. We analyzed expressions of differential markers such as; *FATC4* and *GATA4*, which, based on previous studies, were expected to be significantly downregulated. However, our data revealed an increase in both genes, suggesting an unsuccessful differentiation process.

Additionally, we examined DP427 and UP395 levels, anticipating an increase in DP427 and a decrease in UP395. Instead, we observed the opposite trend, further supporting the hypothesis that AC16 cells did not undergo proper differentiation. We suspected that siRNA-mediated silencing was insufficient to induce differentiation. However, we were able to confirm

the ability to silence all dystrophin, utrophin, and single *UP395* isoform in this model. Due to the low level of *DP427*, the decrease was insignificant (Fig. 4C).

For future experiments, we plan to implement a CRISPR/Cas9-based approach or optimize siRNA silencing by transfecting at multiple time points to enhance the efficiency of SV40 inactivation and promote successful cardiomyocyte differentiation.

We also characterized dystrophin and utrophin isoforms in HL-1 cells. Our findings confirmed those presented in the thesis, demonstrating that *UP395* and *DP71* are the predominant isoforms (Fig. 4D-E).

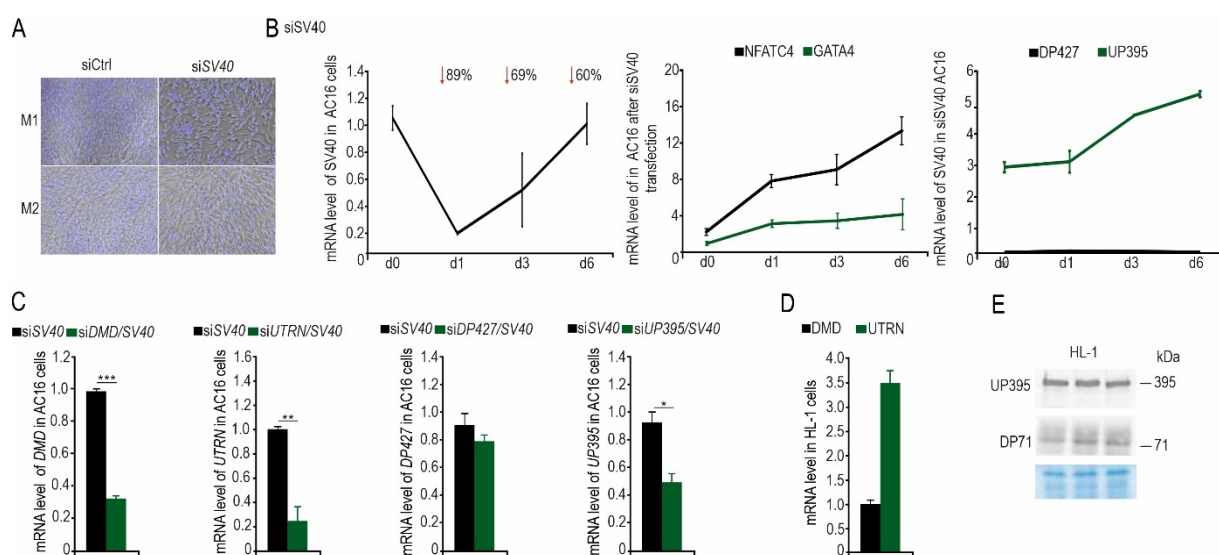


Figure 4 Validation of AC16 and HL-1 Cardiomyocyte Cell Lines

A Live-cell imaging of AC16 cells stained with Hoechst (blue), cultured in different media (Medium 1, M1; Medium 2, M2) following SV40 silencing, assessed five days post-differentiation induction. **B** RT-PCR analysis of SV40, differentiation markers (*NFATC4*, *GATA4*), and dystrophin-associated transcripts (*DP427*, *UP395*) after siSV40 transfection. Red arrows indicate *SV40* decrease in comparison to siCtrl-transfected cells. **C** RT-PCR analysis of *DMD*, *UTRN*, *DP427*, and *UP395* following gene silencing with siRNA. **D** RT-PCR analysis of *DMD* and *UTRN* expression in HL-1 cells. **E** Immunoblotting of HL-1 cells during proliferation, with Coomassie-stained polyacrylamide gels used for normalization.

3. Overall cell work

My research spanned several cell lines, including skeletal muscle (HskM, C2C12, LHCN-M2), cardiomyocytes (HL-1, AC16), neuronal cells (SHY-5Y, CAD), HeLa, and HEK293. I successfully performed CRISPR/Cas9 on the LHCN-M2 cell line and established a single-cell-derived cell line.

4. Mouse work

I obtained certifications from the Polish Society of Laboratory Animal Sciences (PolLASA) to perform and plan animal experiments, including:

- Level 1: Participation in experimental procedures and animal euthanasia
- Level 2: Conducting procedures and animal euthanasia
- Level 3: Planning procedures, conducting experiments, and animal euthanasia

I worked on muscle regeneration models using notexin and muscle-freezing techniques. Additionally, I isolated organs, including the brain, heart, muscles, and nerves, as well as collected blood samples and administered analgesic medications. I also conducted preliminary tests on siRNA induction in mouse cells.

5. Additional activities

I participated in several conferences, presenting my research on Duchenne Muscular Dystrophy (DMD) and related topics:

- "The short Dp71 dystrophin isoform has compensatory roles in cell models of Duchenne Muscular Dystrophy " – 8th International Congress, Myology 2024 Paris, France, 2024
- "Dp71 Compensatory Properties in Cell Models of Duchenne Muscular Dystrophy (DMD)" – 50th European Muscle Conference (EMC), Florence, Italy, 2023

- "Structural Properties of Micro-Dystrophins and Utrophins for Therapeutic Treatment of DMD" – 49th EMC, Prague, Czech Republic, 2022
- "Dystrophin/Utrophin Hybrid Proteins in DMD Therapy: Sequential, Structural, and Functional Comparisons" – Doctoral Forum 2022, Adam Mickiewicz University, Poznań, Poland
- "Cell Matrix and Muscle Cell Survival, Morphology, and Differentiation" – Doctoral Forum 2021, Adam Mickiewicz University, Poznań, Poland
- "Tissue-Specific Regulation of Dystrophin and Utrophin" – Doctoral Forum 2020, Adam Mickiewicz University, Poznań, Poland

6. List of publications not included in the doctoral dissertation

Urbanek-Trzeciak MO, Galka-Marciniak P, Nawrocka PM, Kowal E, **Szwec S**, Gieffing M, Kozłowski P. *Pan-cancer analysis of somatic mutations in miRNA genes*. EBioMedicine. 2020 Nov;61:103051. doi: 10.1016/j.ebiom.2020.103051. Epub 2020 Oct 7. PMID: 33038763; PMCID: PMC7648123.

Author contribution statements of publication and manuscripts included in chapters 2-4

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Author contribution statement

I declare that I am a co-author of the article “Dystrophin- and Utrophin-Based Therapeutic Approaches for Treatment of Duchenne Muscular Dystrophy: A Comparative Review” by Sylwia Szvec, Zuzanna Kapłucha, Jeffrey S. Chamberlain, and Patryk R. Konieczny published in BioDrugs in 2024, and I am aware that this publication is a part of Sylwia Szvec PhD dissertation. The author's contribution is as follows:

Sylwia Szvec – designed the study, wrote the original draft, and conducted the bioinformatics analysis. Sylwia Szvec created the original figures and tables and contributed to the manuscript revision.

Zuzanna Kapłucha - designed the study and wrote the original draft.

Jeffrey S. Chamberlain – revised the manuscript.

Patryk Konieczny – designed the study and wrote and revised the original manuscript. Patryk Konieczny revised and updated the figures and tables, secured funding, and managed correspondence with the journal and reviewers.

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April 2, 2025

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Sept. 24, 2025

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Author contribution statement

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Author contribution statement

I declare that I am a co-author of the manuscript “Compensatory properties of the short dystrophin isoform DP71 in cell models of Duchenne muscular dystrophy” by Sylwia Szvec, Paulina Kościelniak-Wawro, Alicja Durska, Karolina Dominiak and Patryk Konieczny, and I am aware that this manuscript is included as a chapter in Sylwia Szvec PhD dissertation. The author's contribution is as follows:

Sylwia Szvec – designed the study and performed experiments from figures (figs) 1 (with exclusion of C2C12), 2a-b, c (RT-PCR analysis), d, 3-5, 7a (Active mitochondria analysis), b-c; 8, S1, S3-5, S6a performed analysis for C2C12 cells in figs 1b-c, prepare HSkM material for cDNA libraries preparation (figs 8 and S6b, S7-S11), designed original plasmids, optimized its preparation and prepare CMV-GFP-FLAG, EF1 α -GFP-FLAG (DP71 variant), EF1 α -FLAG (all DP71 variants) plasmids (fig. S5). Sylwia Szvec wrote the original draft of manuscript, prepared original figures (figs 1-5, 7-8 and S1, S3-5) and revised final manuscript and figures versions.

Paulina Kościelniak-Wawro - prepared cDNA libraries and performed RNA-sequencing analyses (figs 8 and S6b, S7-S11). Paulina Kościelniak prepared the data summary and dataset for original draft of the results (Section: *DP427- and DP71/DP427-deficient HSkM myotubes show distinct changes in the expression of genes involved in the membrane integrity and cytoskeletal functions*), performed experiments from 2c (Western Blot analysis), prepared original figures (figs 8 and S7-S10) and contributed to figure legends (figs 8 and S6b, S7-S11).

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