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Dystrophin Dp71: The Smallest but Multifunctional Product of the Duchenne Muscular Dystrophy Gene

Ramin Tadayoni · Alvaro Rendon · L. E. Soria-Jasso · Bulmaro Cisneros

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Abstract Dystrophin Dp71 is expressed in all tissues, with the exception of skeletal muscle, and is the main Duchenne muscular dystrophy (DMD) gene product in brain. As full-length dystrophin does in skeletal muscle, Dp71 associates with dystroglycans, sarcoglycans, dystrobrevins, syntrophins, and accessory proteins to form the dystrophin-associated protein complex (DAPC) in non-muscle tissues. Although it has been nearly 20 years since the discovery of Dp71, its study has become relevant only recently due to its direct involvement with the two main DMD non-muscular phenotypes: cognitive impairment and abnormal retinal physiology. In this review, we describe the historical background of Dp71 and the experimental models developed for its study. Additionally, we present and discuss the experimental evidence supporting the participation of Dp71 in different cellular processes, including cell adhesion, water homeostasis, cell division, and nuclear architecture. The functional diversity of Dp71 is attributed to the formation of Dp71-containing DAPC in numerous cell types and different subcellular compartments, including in plasma membrane and nucleus, as well as to the capability of Dp71-containing DAPC to work as the scaffold for proper clustering and anchoring of structural and signaling proteins to the plasma membrane and of nuclear envelope proteins to the inner nuclear membrane.

Keywords Duchenne muscular dystrophy · Dystrophin Dp71 · Retina · Nucleus · Mental retardation · Adhesion · Cell division · *mdx* mouse

Abbreviations

DMD	Duchenne muscular dystrophy
DAPC	Dystrophin-associated protein complex
PCR	Polymerase chain reaction
cDNA	Complementary DNA
Cre-loxP	Recombination system based on Cre site-specific DNA recombinase and loxP DNA sequences
Bgeo	β-Galactosidase–neomycin-resistant gene fusion
mdx	Mouse with X chromosome-linked muscular dystrophy
cAMP	Cyclic adenosine monophosphate
NGF	Nerve growth factor
Sp1	Specificity protein 1
Na^+	Sodium ion
K^+	Potassium ion
AP2α	Activating protein 2α
NLS	Nuclear localization signal
IQ	Intelligence quotient

Ramin Tadayoni and Alvaro Rendon contributed equally to this work.

R. Tadayoni
Ophthalmology Department, Hôpital Lariboisière, AP-HP,
Université Paris Diderot,
Paris, France

R. Tadayoni · A. Rendon
Institut de la Vision, INSERM UMR_S968, CNRS UMR_7210,
Université Pierre et Marie Curie Paris 06,
17 rue Moreau,
Paris 75012, France

L. E. Soria-Jasso
Área Académica de Medicina, I.C.Sa.,
Universidad Autónoma del Estado de Hidalgo,
Pachuca, Hidalgo, México

B. Cisneros (✉)
Departamento de Genética y Biología Molecular,
Centro de Investigación y de Estudios Avanzados—
Instituto Politécnico Nacional (CINVESTAV-IPN),
México Distrito Federal, México
e-mail: bcisnero@cinvestav.mx

LTP	Long-term potentiation
LTD	Long-term depression
ERG	Electroretinogram
AMPAr	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
NMDAr	*N*-methyl-D-aspartate receptor
OPL	Outer plexiform layer
Kir4.1	Inwardly rectifying potassium channels 4.1
AQP4	Water transport protein pore aquaporin-4
FAK	Focal adhesion kinase
BRB	Blood–retinal barrier

Introduction

Duchenne muscular dystrophy (DMD) is a common (one in 3,500 male births) and lethal X-linked inherited neuromuscular disorder characterized by progressive muscular degeneration and cognitive impairment [1–3]. Gene linkage and positional cloning analyses led to the identification of the genetic defect underlying DMD, which was mapped on the short arm of the X chromosome at band Xp21 [4, 5]. The DMD gene, the largest gene identified to date, spans >2.5 Mbp corresponding to 1.5% of the X chromosome and 0.1% of the whole human genome. The gene sequence comprises 79 exons, encoding for a 427-kDa cytoskeletal protein of 3,685 residues that is designated dystrophin [6].

Dystrophin consists of an N-terminal actin-binding domain, a central rod-like domain comprising 24 spectrin-like triple helical coiled coils, and a cysteine-rich C terminus that allows dystrophin to associate with a group of extracellular (α-dystroglycan), integral membrane (sarcoglycans and β-dystroglycan), and cytoplasmic (syntrophins and dystrobrevins) proteins to form the dystrophin-associated protein complex (DAPC) [7–9]. Specifically, dystrophin binds to both actin and β-dystroglycan; the extracellular domain of β-dystroglycan binds to α-dystroglycan, which in turns binds to laminin in the extracellular matrix [10]. In this manner, the DAPC serves as a bridge to connect the extracellular matrix to the cytoskeleton, providing structural stability to the sarcolemma during muscle contraction and modulating the cell signaling that takes place across the plasma membrane [11]. Lack of dystrophin, due to DMD gene mutations, promotes DAPC disintegration and consequently the development of muscular dystrophy [12]. Likewise, gene mutations in other components of the DAPC, such as sarcoglycans, give rise to other forms of muscular dystrophy (limb-girdle and congenital muscular dystrophies) [13], illustrating the vital role of this complex in the maintenance of muscle integrity.

Further analysis revealed the generation of short C-terminal DMD products through the alternative usage of internal promoters. DMD gene products differ among themselves in structure and expression patterns and are named according to their molecular mass as Dp260, Dp140, Dp116, and Dp71 [14–17]. Dp71 is expressed in a wide variety of non-muscle tissues and is the major DMD gene product in the nervous system [14, 18, 19]. Furthermore, Dp71 is expresses in pluripotent embryonic stem cells, being the first product of the gene detectable during development [20].

Recently, the study of Dp71 has acquired relevance because the deficit of this protein has been involved in at least two main clinical features of DMD, which are mental retardation and retinal dysfunction, and because its participation in different cellular processes has been recently revealed. In this review, we present an updated description of the study of Dp71, including the historical background that led to its discovery, the development and characterization of animal and cellular models for Dp71, as well as the recapitulation and discussion of all of the experimental data showing the functional diversity of this protein.

Identification of Dp71 and its Splice Variants

After identification and characterization of dystrophin in muscle tissue, research on this field was focused on the analysis of DMD gene expression in non-muscle tissues. By using Northern blot and RNase protection assays, several laboratories concurred with the identification of a 4.8- to 6.5-kb mRNA in a wide variety of tissues, including brain, liver, testis, lung, stomach, and kidney, but not skeletal muscle [14, 18, 19]. Further characterization by complementary DNA (cDNA) cloning and sequencing [14, 18, 19] demonstrated that this transcript is generated from a putative promoter located to the distal part of the DMD gene, between exons 62 and 63. It contains a unique first exon with an ATG start site in frame with the consecutive dystrophin cDNA sequence, but with both exons 71 and 78 spliced out of the transcript [18, 21]. *In vitro* transcription and translation of the novel coding sequence produced a 70 75-kDa protein [21]. In concordance, a protein band of similar molecular mass (70–80 kDa) was revealed by Western blot analyses with anti-dystrophin antibodies in non-human muscle and murine tissues [18, 19, 21]. Taken together, these results established a correspondence between the 4.8–6.5-kb transcript and the 70–75-kDa protein; which was named apodystrphin-1, and later, Dp71.

Dp71 lacks the actin-binding N-terminal domain and the entire large region of spectrin-like repeats found in full-length dystrophin, but does contain the C-terminal and cysteine-rich domains, common to all DMD-gene proteins, flanked by a unique seven amino acid N terminus and COOH-terminal sequences. The unique N terminus possesses a novel actin-binding motif composed of six amino acid residues [22] (Fig. 1a). The specific C terminus domain of Dp71 arises from excision of dystrophin exon

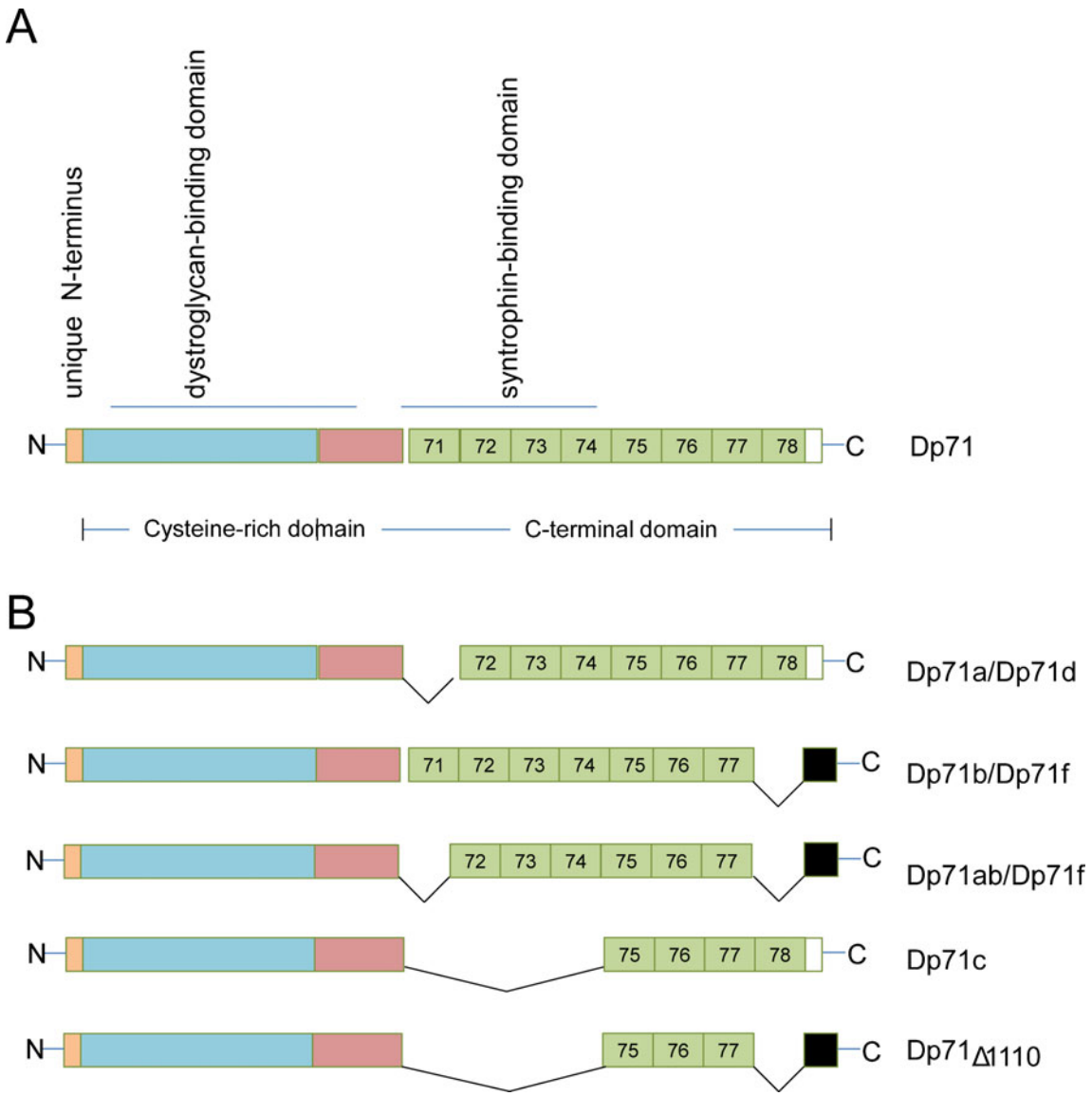

Fig. 1 Dp71and its splice isoforms. **a** Schematic representation of Dp71 protein. The unique seven amino acid N terminus and the cysteine-rich and C-terminal domains are shown. Dystroglycan and syntrophin binding sites on Dp71 are denoted at the top of the scheme. **b** Schematic representation of Dp71 splice variants. The spliced exons are denoted in each isoform. Loss of exon 78 causes a change in the reading frame with the last 13 amino acids of Dp71 (*open box* at the end of exon 78) replaced by 31 novel amino acids (founder sequence, denoted by *black box*) in Dp71b and Dp71ab proteins. Dp71a is also known as Dp71d, whereas Dp71b and Dp71ab are known as Dp71f variants too. Loss of exons 71–74 causes an in-frame deletion of 330 bp encoding the 110 amino acid syntrophin-binding domain in the Dp71c protein. $Dp71\Delta_{110}$ variant lacks exons 71–74 and 78

78, resulting in a shift of the reading frame that replaces the last 13 amino acids in the protein, forming a hydrophilic C terminus with 31 new residues, in turn forming a hydrophobic C terminus (denominated the Dp71 founder sequence) (Fig. 1b).

Alternative splicing is a mechanism commonly employed for increasing the coding diversity of eukaryotic genes. In many cases, it gives rise to protein isoforms that share extensive regions of identity and that vary only in specific domains, thus allowing fine modulation of protein function. Feener and collaborators [23] provided the first evidence showing that the dystrophin transcript can be alternatively spliced at its 3′ end to create numerous protein isoforms that differ from each other at their carboxyl domains. These authors screened the different regions of human dystrophin mRNA by polymerase chain reaction (PCR) amplification of reverse-transcribed RNA and further sequencing of the amplified products. Later, Bies and collaborators [24] utilized a similar PCR-based strategy to show that alternative splicing occurring at the 3′ end of dystrophin mRNA is common to both human and mouse species and, more importantly, that the pattern of alternatively spliced transcripts is characteristic of both tissue type and developmental stage.

Because the Dp71 transcript shares exons encoding the cysteine-rich and COOH-terminal domains with dystrophin, it was suspected that some of the alternatively spliced mRNAs assumed at first to correspond exclusively to dystrophin might belong to Dp71. Kramarcy and collaborators [25] confirmed this hypothesis, revealing the existence of two Dp71 subpopulations containing either the 17-aa hydrophilic or the 31-aa hydrophobic C terminus. These findings encouraged researchers in the laboratory of Dr. Peter Ray to identify Dp71 alternatively spliced transcripts without doubt. These authors cloned and sequenced different Dp71 cDNA from cultured human amniocytes and revealed the existence of splicing variants with the presence or absence of exon 71 and/or exon 78 within the transcript [26]. In addition, they produced in *Escherichia coli* the recombinant proteins that correspond to the alternatively spliced cDNA of Dp71, and identified the C-terminal sequences that either in- or excluded exon 78, using antibodies directed specifically against the 17-aa C terminus or the founder sequence, respectively. Later, an additional Dp71 isoform spliced out of exons 71–74 and 78 was found in human brain tissue and different human embryonic neuronal tissues, including telen-, mesen-, and myelencephalon, and spinal cord [27]. The loss of exons 71–74 (330 bp) does not affect the reading frame, but the protein would lack the binding site to syntrophin, a DAPC component, and has a predicted molecular weight of 58–60 kDa (Fig. 1b). The expected protein product, later designated as $Dp71_{\Delta 110}$, was recognized by immunoblot analysis utilizing dystrophin-specific C-terminal antibodies against epitopes in either exon 77 or 78 [26]; furthermore, failure to detect $Dp71_{\Delta 110}$ with a monoclonal antibody directed against epitopes within exons 73–74 provided definite evidence that this isoform is derived from Dp71 transcripts deleted for the syntrophin-binding domain. Finally, a 2.2-kb transcript named apo-dystrophin-3 was recognized by screening a rat Schwannoma cDNA library [28]. The apo-dystrophin-3 open reading frame predicts a 341 amino acid protein with an estimated molecular weight of 40 kDa, containing the first seven amino acid N terminus and the cysteine-rich domain of Dp71. Because apo-dystrophin-3 possesses an identical 5′ first exon sequence to that of Dp71 and a similar range of tissue expression, it may be also transcribed from the Dp71 promoter. However, no protein of this size has been detected with anti-dystrophin antibodies, probably due to the lack of antibodies that recognize epitopes mapping to the cysteine-rich domain of which apo-dystrophin-3 is nearly exclusively encoded.

Based on GenBank nomenclature, Dp71 isoforms are termed Dp71 (with exons 71 and 78), Dp71a (without exon 71; AY326947), Dp71b (without exon 78), Dp71ab (without exons 71 and 78), and Dp71c (without exons 71–74; AY326949) (Fig. 1a, b). Dp71a is more commonly known in the literature as the Dp71d isoform, and Dp71b and Dp71ab, as Dp71f (f, founder sequence) variants (Fig. 1a, b). Generation of Dp71 isoform-specific antibodies has permitted discrimination between Dp71f and Dp71d splice isoforms. Then, when identification of a particular Dp71 splice isoform was conducted in a particular study, it is indicated in the text.

Regulation of Dp71 Expression

As previously mentioned, expression of Dp71 is ubiquitous with the exception of skeletal muscle. Nevertheless, its levels vary greatly not only between different tissues and cell types but also during differentiation. Early studies showed that Dp71 mRNA and protein levels increase during dibutyryl–cyclic adenosine monophosphate (cAMP)-induced differentiation of rat brain astrocytes [29] and nerve growth factor (NGF)-induced differentiation of PC12 cells [30, 31], clearly indicating that Dp71 expression is mainly modulated at the transcriptional level.

The Dp71 promoter was mapped in the intron between exons 62 and 63 of the DMD gene, it is located more than 2,000 kb 3′ to the mouse and brain type dystrophin promoters, and only 150 kb from the 3′ end of the gene [21]. Characterization of the Dp71 promoter by anchored PCR primer extension and functional analysis of reporter gene constructs revealed the structure of a typical housekeeping promoter [21]; it has a GC-rich region with several specificity protein 1 (Sp1)-binding sites, but lacks TATA and CAAT motifs. The presence of GC-rich regions is a distinctive characteristic of TATA-less promoters. Binding of Sp1 to GC-rich regions stabilizes the transcriptional machinery and defines the transcription start site in TATA-less promoters. An elegant strategy to monitor Dp71 promoter activity during development was designed at the same time by two different laboratories [32, 33]. These authors specifically inactivated Dp71 expression in embryonic stem cells by replacing its first and unique exon with a sequence encoding a *βgeo* fusion protein in order to generate chimeric mice and to evaluate the endogenous Dp71 promoter during embryonic development, employing a sensitive assay based on the β-galactosidase reporter gene. Relatively high Dp71 promoter activity was found associated with morphogenic events and terminal differentiation of several mouse tissues and organs, including the central and peripheral nervous systems, limb buds, lungs, blood vessels, eyes, inner ear, and nasal organs [33]. Interestingly, the forebrain area, which gives rise to the hippocampus, was intensively stained. Consistent with this study, Dp71 mRNA was found highly expressed in the mouse hippocampal dentate gyrus and, to a lesser extent, in the cerebral cortex, olfactory bulb, and the hippocampal CA-3 region of adult mouse [34].

To delineate the molecular mechanisms underlying the contrasting expression of Dp71 between neuronal and

muscular cells, Dp71 promoter activity was characterized in detail in both neuronal and muscular cell lines. It was found that a 224-bp proximal promoter region containing four Sp1 binding sites and one activating protein 2 (AP2) element is necessary and sufficient for baseline expression of Dp71 in both muscular and neuronal cells. Furthermore, it was demonstrated that the proximal promoter region controls the repression of Dp71 expression that occurs during myogenesis, as well as promoter activity induction in differentiated neuronal cells [35, 36]. In myoblasts, Sp1, together with specificity protein 3 (Sp3), transactivate the Dp71 promoter; however, Sp1 and −3 protein levels decrease during muscle cell differentiation, ultimately causing downregulation of the promoter in mature muscle cells [35] (Fig. 2a). Modulation of Dp71 expression during myogenesis is relevant in the physiology of muscle tissue.

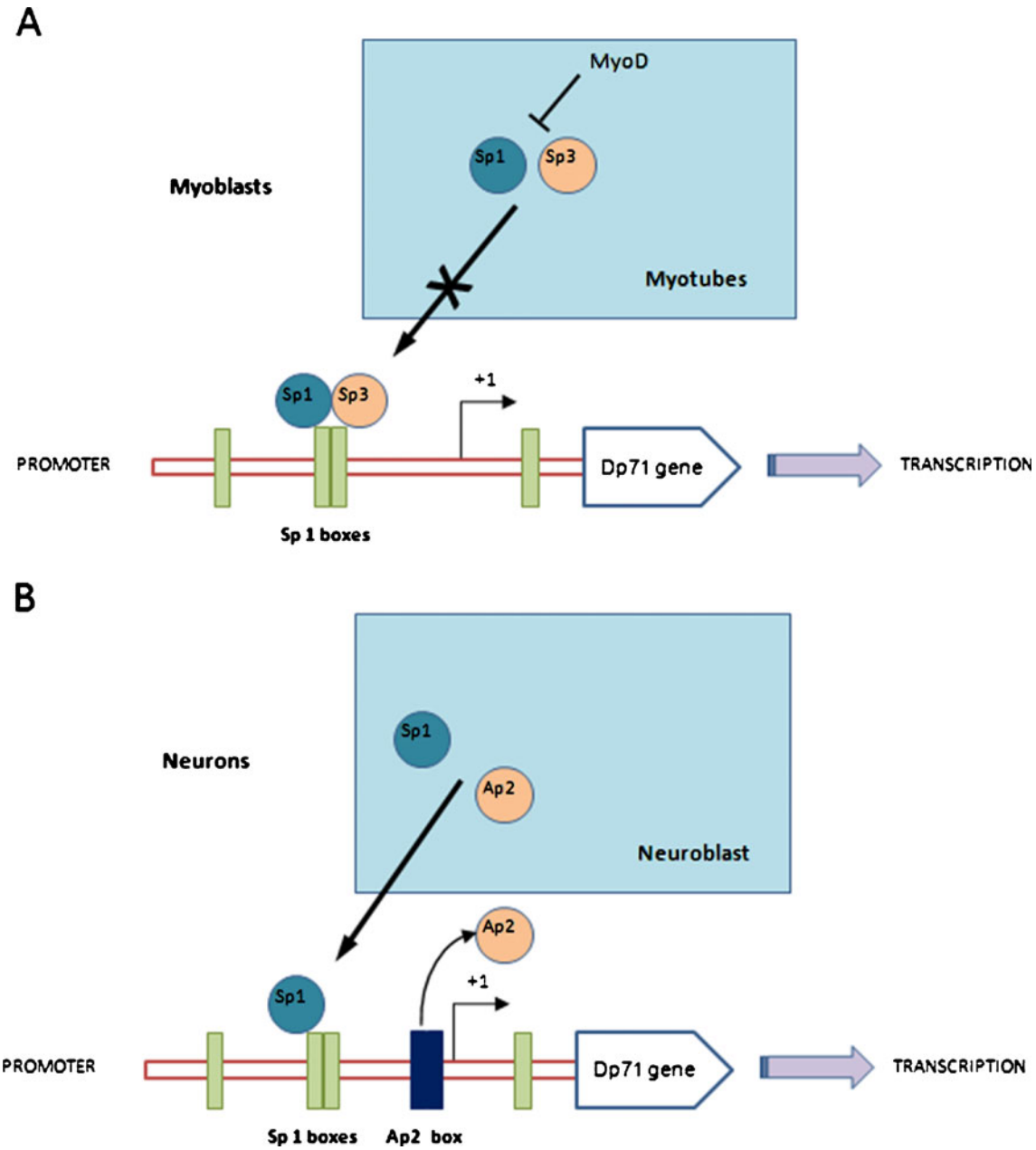

Fig. 2 Regulation of Dp71 expression. a Expression of Dp71 is downregulated during myogenesis. In myoblasts, Sp1 and Sp3 transcription factors activate the Dp71 promoter by binding to Sp1 boxes. In myotubes, MyoD represses the expression of Sp1 and Sp3 genes, and the lack of these factors results in promoter inactivation. **b** Expression of Dp71 is induced during neuronal differentiation. Baseline expression of Dp71 in neuroblasts is maintained by the combined action of Sp1 and AP2 transcription factors, working on the Dp71 promoter as activator and repressor, respectively. Induction of Dp71 promoter activity during neuronal differentiation is the result of the positive regulation exerted by Sp1 as well as of the loss of AP2α binding to the AP2 motif, releasing the promoter from repression

In early myogenesis, Dp71 is expressed for participation in cytoskeletal remodeling [37], while in mature muscle fibers its expression must be extinguished to allow dystrophin to be the predominant DMD gene product and to avoid competition among these for available DAPC binding sites. In fact, ectopic expression of Dp71 in skeletal muscle of transgenic mice with normal dystrophin expression gives rise to a muscular dystrophy phenotype [38]. On the other hand, baseline expression of Dp71 in undifferentiated neurons is controlled by the combined action of Sp1 and activating protein 2α (AP2α) as positive and negative regulators, respectively. In differentiated neurons, induction of Dp71 promoter activity results from the positive regulation exerted by Sp1 and the loss of binding of AP2α to the promoter, which ultimately releases it from repression [36] (Fig. 2b).

Regulation of Dp71 expression ensures its presence in defined cell types and/or developmental stages, permitting the proper functioning of cellular processes in which Dp71 plays a role. Furthermore, it appears that the DMD gene needs to be tightly regulated to avoid redundancy in function and harmful competition among different dystrophins.

Formation and Distribution of the Dp71-Containing Dystrophin-Associated Protein Complex

Although similarity between Dp71 and dystrophin protein sequences predicts that the former protein might also be a cytoskeletal plasma membrane-associated molecule, the groups of Yaffe and Nudel, as well as that of Rendon [39, 40], provided the first evidence on Dp71 localization, employing a monoclonal antibody directed against the C-terminal domain of both dystrophin and Dp71. Immunoblotting analysis of subcellular fractions from rat liver and hepatoma HepG2 cells revealed that the majority of Dp71 is present in the purified plasma membrane fraction together with the plasma membrane protein marker *N*-cadherin. Likewise, immunofluorescence analysis of HeLa and HepG2 cells (two cell lines that do not express dystrophin) as well as of differentiated *mdx* myogenic cells expressing exogenous Dp71 revealed Dp71 immunostaining associated with the cell membrane. In agreement, Dp71 was found to be located in the cell membrane of cultured rat brain astrocytes [29].

Blake and collaborators [41] showed for the first time that Dp71 is able to form DAPC-like complexes in a non-muscle tissue, the brain. They found β-dystrobrevin, Dp71d isoform, and α- and β-2 syntrophin to be highly enriched in the rat forebrain post-synaptic density fraction, and revealed by immunoprecipitation assays that Dp71d is associated with α-dystrobrevin-1 in rat brain. A little later, Rendon and collaborators characterized a Dp71-containing DAPC in rat retinal Müller glial cells [42]. Dp71 was found interacting with β-dystroglycan, δ-sarcoglycan, and α1-syntrophin, while β-dystroglycan is associated with α-dystrobrevin-1 and PSD-93. In a more recent work, the latter group showed that the Dp71–DAP-dependent complex could be, at least partially, associated with a specific, specialized membrane microdomain fraction of Müller glial cells endfeet, the so-called detergent-resistant membranes or lipid rafts [43]. After that, a growing piece of evidence has undoubtedly shown the existence of Dp71-containing, DAPC-like complexes in diverse tissues and cell types. In mouse liver, Dp71 and syntrophins co-purified with immunoisolated β-dystrobrevin and, reciprocally, anti-dystrophin immunoisolates contained β-dystrobrevin and syntrophins [44]. Likewise, immunoprecipitation experiments revealed the assembly of a DAPC-like complex in mouse kidney, which is composed of Dp71d/Dp71f, β-dystrobrevin, and syntrophins [45]. In addition, biochemical characterization of β-dystrobrevin-null mice helped these authors to demonstrate that β-dystrobrevin is required for the assembly of Dp71 and syntrophins to the baseline membrane of kidney cells. Microscope analysis with an antibody directed against the C terminus of dystrophin revealed that Dp71 co-localizes with α-dystrobrevin-1, α1- and β2-syntrophin, and α- and β-dystroglycan in glial endfeet structures and vascular endothelial cells, suggesting the assembly of a DAPC-like complex in these brain regions [46]. Furthermore, immunoprecipitation assays on a membrane preparation from *Xenopus* cardiac muscle with anti-β-dystroglycan antibodies showed the interaction of Dp71f and β-dystroglycan [47].

Characterization of Dp71-containing DAPC has also been approached in blood and germinal cells. Two alternative DAPC-like complexes were identified by confocal microscope and by immunoprecipitation analyses in both platelets [48] and neutrophils [49], which were composed of Dp71d or $Dp71_{\Delta 110}$, and β-dystroglycan, α-syntrophin, and α-dystrobrevin-1 and −2. These complexes were found associated with the actin-based cytoskeleton in neutrophils, likely by the interaction of actin with the actin-binding motifs present in Dp71. On the other hand, Dp71 was found together with β-dystroglycan, α-syntrophin, and neuronal nitric oxide synthase (nNOS) in the membrane of mouse spermatozoa, in which Dp71 appears to be critical for proper localization of α-syntrophin, nNOS, the voltage-dependent sodium ion (Na^+) and potassium ion (K^+) channels and, ultimately, for flagellar morphology [50]. Furthermore, Dp71f and several members of the DAPC including dystroglycans, δ-, γ-sarcoglycans, α-dystrobrevin-1, and α-1syntrophin are expressed in pituicytes, resident glia of the neurohypophyses, implying the formation of a DAPC-like complex in these particular cells [51]. Likewise, expression of Dp71d and mRNAs for β-dystroglycan, α- and β-dystrobrevins, and α-, βI-, βII-, and γII-syntrophins was found in both visceral and subcutaneous rat adipose depots, suggesting a role for this complex in adipose biology

[52]. Finally, Dp71-containing, DAPC-like complexes have also been identified in different cell lines, including PC12 [53] and C2C12 cells [54]. In C2C12 myoblasts, Dp71 associates with β-dystroglycan, α-sarcoglycan, α-dystrobrevin, and α-syntrophin [54], while in PC12 cells, Dp71 interacts with β-dystroglycan, β1-syntrophin, β-dystrobrevin, and α-, β-, and γ-sarcoglycan [53]. In the latter study, the authors found Dp71–DAPC composition changes during NGF-induced neuronal differentiation, Dp71 being associated with β-dystroglycan, α1-syntrophin, β-dystrobrevin, δ-sarcoglycan, and nNOS in differentiated PC12 cells.

The presence of Dp71 in numerous tissues confers versatility upon its function as a result of the particular DAPC composition present in each cell type and the interaction of Dp71 with different partners in local microenvironments.

Nuclear Localization of Dp71 and DAPC Components

For a long time, Dp71 was considered as a cytoplasmic, plasma membrane-associated protein, and this idea was largely accepted as dogma; therefore, the circumstantial visualization of Dp71 in the nucleus had been overlooked or taken simply as an experimental artifact. Nevertheless, the group of Cisneros and other colleagues have provided compelling evidence of the nuclear localization of Dp71. Alemán and collaborators [55] found that Dp71d and Dp71f are present in nuclear extracts obtained from hippocampal neurons, using antibodies directed specifically to each splice isoform. Confocal microscope immunofluorescence analysis confirmed these findings: Dp71d immunolabeling decorated prominent nuclear granules in both hippocampal neurons and forebrain astrocytes, while nuclear immunostaining of Dp71f was observed exclusively in neurons. Interestingly, it was demonstrated that alternative splicing determined the subcellular localization of Dp71 isoforms [56]. Confocal microscope analysis of HeLa, C2C12, and N1E-115 cell lines expressing different Dp71 splicing variants fused to GFP revealed that Dp71 isoform lacking amino acids encoded by exons 71 and 78 (the Dp71f isoform) localized exclusively in cytoplasm, whereas Dp71 variants containing amino acids encoded by exon 71 and/or exon 78 (the Dp71d isoforms) exhibited predominant nuclear localization. Consistent with this, immunofluorescence and immunoblotting assays on PC12 cells demonstrated that Dp71d exhibits predominant nuclear localization, while Dp71f is localized exclusively in the cytoplasm [31].

Aside from Dp71, the nuclear presence of some DAPC components, including dystrobrevins [41, 57] and syntrophins [58], was reported, which encouraged us to determine whether DAPC components coexist in the same cell-type nucleus and, more importantly, if they might form a DAPC within this organelle. It was found by cell fractionation and immunoprecipitation of nuclear extracts that Dp71 concurs in the nucleus with sarcoglycans, β-dystroglycan, syntrophins, and dystrobrevins, and that all these are assembled into a nuclear DAPC in HeLa [59], C2C12 [54], and PC12 [60] cell lines. Selective nuclear import of proteins involves the recognition of a conventional nuclear localizing signal (NLS), generally short stretches of basic amino acids either alone (monopartite) or separated by a linker region of 10–12 amino acids (bipartite), located in the cargo protein, by a cytoplasmic receptor that consists of importins α and β. Proteins carrying a classical or bipartite NLS are bound by importin α, which serves as adaptor for importin β−NLS binding. Importin β in turns targets the complex to the NPC, where it is translocated into the nucleus [61].

Although Dp71 lacks a canonical nuclear localization signal (NLS), its phosphorylation by $Ca2^{+}$ calmodulin-dependent protein kinase II appears to facilitate its nuclear transport [62]. Recently, a functional NLS in the cytoplasmic tail of β-dystroglycan was identified [63], which makes it plausible to propose that Dp71 gains access to the nucleus through its association with β-dystroglycan. Finally, it is noteworthy that a Dp71f-like protein was observed in the mitochondria of *mdx* and *mdx*^{3cv} mice [64]. Nevertheless, neither the identity of this Dp71f-related molecule nor its potential mitochondrial role has been approached.

The presence of Dp71 and DAPC components in the nucleus and the potential involvement of this particular localization with DMD development open a new and exciting area of study. Because the cell possesses a continuous network linking the plasma membrane to the nuclear envelope that potentially translates biomechanical signaling into nuclear reorganization, it is possible that both cytoplasmic and nuclear Dp71-containing DAPC participate in this cytoplasmic–nucleus pathway.

Experimental Models for Dp71

Advances in the definition of Dp71 function has been accomplished by the use of different study models, including patients with DMD and animal and cell models. However, due to ethical principles, the study of Dp71 in patients with DMD has been focused exclusively on the genetic screening of DMD gene deletions in an attempt to correlate cognitive or visual impairment with Dp71 dysfunction (see later).

Animal Models

Identification of an X chromosome-linked muscular dystrophy in the mouse (*mdx* mouse) opened a fructiferous avenue in the genetic and molecular study of DMD [65]. The *mdx* mouse has a point mutation in exon 23 of the mouse homologous DMD gene that eliminates the expres-

sion of full-length dystrophin but maintains unaltered that of short dystrophins, including Dp71. Later, a new *mdx* strain (*mdx*^{3cv}) was isolated from female progeny of an *N*-ethylnitrosourea-mutagenized *mdx* male. These mice bear a mutation in the exon 65 splice acceptor site that must impair the expression of all DMD gene products. This mutant mouse maintains *mdx*-associated skeletal muscle pathology but also exhibits reduced neonatal survival [66].

The ability of the knockout of specific genes in mice via embryonic stem, cell-based gene targeting technology has significantly enriched our understanding of gene function in normal and disease phenotypes. Applying this technology, mouse models that affect the expression of all known DMD gene products were generated: the *Dmd*$^{mdx\text{-}\beta geo}$ mouse was obtained by random insertion of the β-galactosidase–neomycin-resistant gene fusion (*βgeo*) at 3′ of exon 63 of the DMD gene [67], while the *DMD-null* mouse was generated by deleting the entire genomic region of the DMD gene on mouse chromosome X in mouse embryonic stem cells via the recombination system based on Cre site-specific DNA recombinase and loxP DNA sequences (Cre-loxP) [68]. Specifically, a *Dp71-null* mouse strain was generated by replacing via homologous recombination the first and unique exon of Dp71 with a sequence encoding a *βgeo* chimeric protein. Then, expression of Dp71 was abolished without interfering with the remaining DMD gene products [33], which renders this mouse model ideal for associating undoubtedly mutant phenotypes with Dp71 deficiency. On the other hand, several *mdx* transgenic mice ectopically expressing Dp71 in skeletal muscle were generated to test specifically whether Dp71 can functionally replace dystrophin in skeletal muscle and alleviate muscular dystrophy [69, 70]. Finally, some interesting attempts have been made to establish the zebrafish as the model for studying Dp71 expression during early development [71, 72]. The zebrafish has many advantages for developmental analysis and genetic studies, including short generation time, large and transparent embryos, and well-established large-scale chemical mutagenesis screens to produce mutants with motility defects [73]. Characterization of murine models in general and *Dp71-null* mice in particular has allowed identification of new mutant phenotypes associated with Dp71 deficit (see later).

Cell Models

Development of cell models represents a useful strategy to identify gene function due to their simplicity of structure and advantageous features, such as ready availability, rapid proliferation, and relatively easier genetic manipulation on comparison with tissues and whole organisms. Established cell lines possess additional benefits, such as unlimited renewal source and the possibility of deriving clones with desirable genetic alterations. Different primary cultured cells and cell lines have been employed to study Dp71, including primary mouse hippocampal neurons and astrocytes, primary rat retinal MGC, mouse N1E115 neuronal cells, mouse C2C12 muscular cells, rat Hepa-1 hepatic cells, and mouse P19 embryonic carcinoma cells. Among these, PC12 and MGC cells are highlighted because they have been extensively validated as Dp71 models by long-term studies and constitute an important basis of the current knowledge of this protein.

(a) PC12 neuronal cells. Because Dp71 has been related with the cognitive impairment present in a fraction of patients with DMD, PC12 neuronal cells were chosen as *in vitro* model for studying the potential neuronal function of Dp71. PC12 cells derive from a rat adrenal pheochromocytoma and have the peculiarity of responding to NGF by differentiating into sympathetic-like neurons [74]. Regarding the study of Dp71, PC12 cells present the advantage of expressing Dp71d and Dp71f isoforms as the sole DMD gene products [31], which would avoid the interference of other dystrophin variants in defining Dp71 function. It was found that both Dp71 variants are upregulated during NGF-induced differentiation of PC12 cells but exhibit different subcellular distribution from each other: Dp71f is a cytoplasmic protein that localizes to the cell body and neurites, while Dp71d accumulates mainly in nuclei [31]. To gain insight into the functional role of Dp71 in PC12 neuronal cells, PC12-derivative clones with depleted levels of Dp71, obtained by stable transfection of a vector expressing an antisense RNA against Dp71, were obtained (Dp71-antisense cells) [75]. Employment of the PC12-based model has been decisive in revealing the role of Dp71 in several biological processes, including cell adhesion, neuronal differentiation, nuclear architecture, and cell division (see later).

(b) Retinal Müller glial cells. Retinal electrophysiology is abnormal in patients with DMD with mutations in the central and distal part of the DMD gene as well as in *mdx*^{3cv} mouse strains. Retinal Müller glial cells comprise the main glial cells of the neuronal retina; while astrocytes surround blood vessels in the nerve fiber layer, RMG are the only macroglial cells that span the entire thickness of the retina and enter into contact with virtually all retinal neurons. They also support photoreceptor and ganglion survival, playing a crucial role in the maintenance of retinal functional integrity [76]. Taking these advantages into consideration, retinal Müller glial cells were selected as *in vivo* and *in vitro* model to ascertain the role of Dp71 and DAPC components in the DMD-associated retinal phenotype. Specifically, the use of this cell model has been determinant in revealing the role of Dp71 in

clustering and anchoring ion and water channels to the plasma membrane, thus modulating water homeostasis (see later).

In conclusion, the utilization of the experimental model has allowed defining the role of Dp71. While cellular models have provided molecular details of Dp71 expression and function, the use of animal model has revealed its physiological relevance to DMD.

Function of Dp71

Early in the study of Dp71, it was revealed that this protein is able to restore the normal expression and localization of DAPC components at the sarcolemma of *mdx* mice but that it fails to alleviate muscular dystrophy [69, 70]. Thus, it was clear from the beginning that Dp71 might play a particular role different from that of dystrophin. In recent years, a growing piece of evidence has established that Dp71 is indeed crucial for understanding DMD physiopathology. Specifically, Dp71 has been involved in the two main non-muscular DMD phenotypes: mental retardation and retinal dysfunction. In addition, experimental data obtained from Dp71 murine and cellular models have clearly evidenced the participation of Dp71 in several cellular processes including ion and water homeostasis, cell signaling, cell adhesion, cell cycle division, and nuclear architecture.

Involvement of Dp71 in Cognitive Impairment

The presence of non-progressive mental retardation is now widely accepted and well recognized as a common feature in a substantial proportion of patients with DMD. Analyses carried out in patients with DMD reveal that their overall intelligence quotient (IQ) is significantly lower than that of the mean population, with a specific deficit in verbal rather than in performance IQ [1, 77, 78]. Nevertheless, a meta-analysis of intellectual functioning and DMD conducted by Cotton and colleagues revealed that both verbal and non-verbal intelligence are affected indeed in children with DMD [79].

Several lines of evidence suggest that weakness in IQ is likely related with mutations in the DMD gene [77]. Because 60–70% of patients with DMD and BMD have an intragenic deletion, there have been several genotype–phenotype studies, but no consistent correlation between deletion type, site, and extent and IQ has emerged [80–82]. A recent comprehensive study evidenced how the clinical heterogeneity of DMD patients, namely severity of muscle and brain dysfunction, impacts genotype–phenotype correlation studies, and showed that each brain dystrophin might contribute to the DMD-associated cognitive impairment [83]. However, a number of findings have suggested that rearrangements in the second part of the gene appear to be associated preferentially with cognitive impairment than with mutations in the proximal part [84–86]. Particularly, genetic studies on patients with DMD and BMD revealed a significant correlation between mutations affecting the promoter sequence or the coding region corresponding to Dp140 with cognitive impairment [87, 88], which highlights the potential implication of this DMD gene product in DMD-associated mental retardation. More recently, consistent correlation with mental retardation has emerged for mutations located in the Dp71 coding region. Moizard and collaborators [86] analyzed a cohort of 49 patients and found altered Dp71 transcripts in two patients with DMD with severe mental deficiency. The same laboratory extended the study to an additional 11 patients with DMD with mental retardation who carried no detectable deletion or duplication and detected five point mutations causing premature translation termination of Dp71 [89]. In agreement with this, a recent study based on the comparison of clinical, cognitive, and molecular data in a large cohort of 81 patients affected with DMD or BMD revealed that patients with BMD with mental retardation have mutations that significantly affected Dp71 expression or location in exons 75 and 76, while patients with DMD with mutations upstream of exon 62, predicted to lead to loss-of-function of all dystrophin products except Dp71, are associated predominantly with normal or borderline cognitive performances [90].

Studies at tissue and cellular levels support the potential participation of Dp71 in neuronal functions. Dp71 mRNA and protein have been shown to be expressed in different brain structures and cell types, including perivascular astrocytes [46, 91], MGC in retina [92], neurons in hippocampus and olfactory bulb [34], cultured hippocampal neurons and forebrain astrocytes [55], and post-synaptic densities *in vivo* [41]. Interestingly, an early study showed that activation of glutamate receptors by kainate alters mRNA expression of dystrophin and Dp71 in rat hippocampus, suggesting the involvement of the DMD gene in neuronal plasticity [93].

On the other hand, the employment of DMD mouse models has provided substantial evidence on the participation of dystrophins, and specifically Dp71, in nervous system development and maturation. The expression of α- and β-dystroglycan was found drastically reduced in total brain extracts of mdx^{3cv} mouse but not in liver, testis, and kidney [94]. Because Dp71 is the main dystrophin in the nervous system, these results suggest that Dp71 is crucial for the formation and/or stabilization of the DAPC in brain. Interestingly, it was reported that the *DMD-null* mouse model has defasciculated vomeronasal axons and that some of these altered axons aberrantly enter into the main olfactory bulb [95]. Because Dp71 is exclusively expressed in the mouse olfactory system and its deficit causes

decreased expression of β-dystroglycan and perlecan in olfactory ensheathing cells, these authors claimed that Dp71 participates in fasciculation of the vomeronasal nerve, most likely through interaction with the extracellular matrix. Recently, a comprehensive study on *Dp71-null* mice provides the first solid evidence for understanding the manner in which Dp71 loss may impair neuronal and cognitive functions [96]. It was also found in this work that cultured primary neurons from *Dp71-null* mice display abnormally large clusters of the post-synaptic scaffolding protein PSD-95 and a reduced number of mature synapses. Analysis at the ultrastructural level revealed that the adult brain exhibited a reduced number of excitatory synapses with greater cross-sectional, post-synaptic density length in *Dp71-null* mice, which is indicative of the reduced synapse density and altered morphology of the post-synaptic active zone. In agreement with this, electron microscopy analysis of the distribution of synaptic vesicles in CA1 hippocampal axospinous non-perforated excitatory synapses of mice lacking Dp71 demonstrated a decrease in the density of vesicles located in the vicinity of the active zone, and an increase in synaptic cleft vesicle size and width [97]. Furthermore, hippocampal slice preparations from mutant mice showed a drastic increase in α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid receptor (AMPAr)- and *N*-methyl-D-aspartate receptor (NMDAr)-mediated components of CA1 glutamatergic transmission, which was associated with reduced long-term potentiation (LTP), but not long-term depression (LTD) levels. In agreement with these findings, *Dp71-null* mice display selective behavioral disturbances characterized by reduced exploratory and novelty-seeking behavior, mild retention deficits in inhibitory avoidance, and impairments in spatial learning and memory [96].

Taken together, these findings establish that Dp71-containing DAPC works as a scaffold for proteins involved in membrane stabilization and transmembrane signaling in the brain. Thus, congenital loss of Dp71 appears to impair synaptic organization and maturation of central glutamatergic synapses significantly, which may explain some of the alterations of synaptic function and plasticity that contribute to intellectual disability in DMD. Moreover, as mentioned before, the group of Nico and collaborators reported, in a series of experiments performed with the *mdx* mouse strain [98–100], that disruption of Dp71 expression may alter the blood–brain barrier and water and K^+ homeostasis in perivascular astrocytes, which could indirectly affect neuronal function. It is interesting to note that although it has been extensively reported in the literature that in the *mdx* mouse strain the mutation exclusively affects 427-kDa dystrophin expression, this group found and reported a significant reduction in Dp71 at the glial endfeet. Thus, it is important to elucidate the molecular mechanism that could lie at the origin of this observation. Indeed, it appears from these results that although to our knowledge never before reported in the literature, the brain of the *mdx* mouse strain could be a model of Dp71 depletion.

Role of Dp71 in Retina

The abnormal electroretinogram (ERG) is the best characterized of all of the non-muscular manifestations of DMD. Analysis of the dark-adapted (scotopic) ERG revealed a reduction in the amplitude of the b-wave response in 80% of patients with DMD, as well as in dystrophic mouse mdx^{3cv} [101]. This characteristic phenotype has been attributed to altered expression of short products of the DMD gene because patients with deletions in the center region of the gene have an abnormal ERG, whereas the majority of patients with mutations at the 5′ end of the gene have normal ERG [101]. Nevertheless, studies with large cohorts of DMD patients are required to define the participation of each dystrophin in the DMD visual phenotype. In mouse retina, four DMD gene products are expressed: dystrophin; Dp260, Dp140, and Dp71 [16]. Dp260 is localized in the outer plexiform layer (OPL), expressed by photoreceptor cells [102, 103], while Dp71 is found surrounding retinal blood vessels and at the inner limiting membrane.

Among *mdx* and knock-out mouse strains that aided in elucidating the specific participation of each of the four DMD gene short products in retinal electrophysiology, only the mdx^{3cv} strain bearing a mutation near the 3′ end of the DMD gene that affects all of the DMD gene products displays abnormal ERG with both increased implicit time and attenuation of the b-wave amplitude [104]. As the Rendon group demonstrated, *Dp71-null* mice display normal ERG with no significant changes in b-wave amplitude and kinetics; together, these results suggest that the abnormal ERG phenotype characterized by delayed b-wave implicit time and a reduction of b-wave amplitude is produced by the concomitant loss of dystrophin, Dp260, Dp140, and Dp71. Thus, DMD gene products at the OPL might be involved in presynaptic organization, anchoring receptors and/or channels implicated in b-wave generation.

In this context, because Müller glial cells the retina's major glial cell type are thought to play a crucial role in maintaining retinal homeostasis, Dr. Rendon's laboratory staff was encouraged to analyze the behavior of dystrophins and DAPC components in rat retinal Müller glial cells. It was found that Müller glial cells express Dp71 as the sole DMD gene product, as well as different DAPC components [105]. Furthermore, it was revealed by immunoprecipitation assays that Dp71 and/or utrophin form a DAPC-like complex in these glial cells (see previous material) [42]. In the neural retina, potassium buffering and water drainage via Müller glial cells are mediated by the cooperation of the inwardly rectifying potassium channels (Kir), especially

Kir4.1, with the selective water transport protein pore aquaporin-4 (AQP4) [106, 107]. Thus, the potential relationship of Kir4.1 and AQP4 with Dp71-containing DAPC in Müller glial cells was analyzed. In normal retina, Dp71 exhibits the same distribution pattern as those corresponding to Kir4.1 and AQP4; remarkably, genetic inactivation of Dp71 (in *Dp71-null* mice) alters Kir4.1 and AQP4 distribution in Müller glial cells, a mislocalization that increases the vulnerability of retinal nerve cells to transient ischemia [43, 92]. Similar studies in the *mdx*^{3cv} confirm the critical role of Dp71 for clustering Kir4.1 [108] and AQP4 [46] in glial endfeet structures. These data highlight the fact that even if the retina of *Dp71-null* mouse appears to be physiologically normal, it is indeed in a fragile steady state that renders it more vulnerable to challenges such as ischemic injury, compared with wild-type mouse retina. Interestingly, despite the close relationship between utrophin, the autosomal homologues of dystrophins [109] and dystrophins, Dp71 deficit is only partially compensated by the upregulation of utrophin that occurred in Müller glial cells of *Dp71-null* mouse.

Also, it was observed that experimental retinal detachment induces, concomitantly with a decrease in Dp71 expression, mislocalization of Kir4.1 and downregulation of AQP4, suggesting that Dp71 is one of the molecular targets responsible for physiological alterations of Müller glial cells in injured or diseased retinae. Consistent with these data, it was found that depletion of Dp71 in *Dp71-null* mice impairs the capability of volume regulation of Müller glial cells under osmotic stress and dramatically increases the permeability of the blood–retinal barrier (BRB) [110].

Collectively, these data support the view that Dp71 and DAP, by controlling the expression and subcellular distribution of K^+ and water channels, are essential for crucial glial cell functions such as retinal water homeostasis and maintenance of the BRB (Fig. 3a).

Participation of Dp71 in Cell Adhesion

Adhesion of cells to each other and to their substrate is crucial in generating cell shape and cell organization in tissues. The best studied molecules in adhesion are integrins, which are heterodimeric transmembrane molecules formed by several combinations of α and β subunits that mediate cellular binding to components of the extracellular matrix. Binding of the β1-integrin cytoplasmic domain to focal adhesion proteins such as talin, α-actinin, paxillin, and focal adhesion kinase (FAK) leads to recruitment of further proteins in order to assemble a complex hierarchical structure that directs cytoskeletal assembly and signal transduction cascades [111].

Previous studies have provided biochemical evidence of the interaction between DAPC components including dystrophin, dystroglycans, and sarcoglycans with integrand adhesion system proteins such as integrins, vinculin, talin, paxillin, and FAK [112–115]. Interestingly, knock-down regulation of α- and γ-sarcoglycan significantly decreased levels of associated focal adhesion proteins, which resulted in reduced adhesion of L6 muscular cells [113], while depletion of β-dystroglycan increased the number of fibrillar adhesions in myoblasts [115]. Hence, the existence of bidirectional communication between the DAPC and the integrin adhesion system to modulate cell adhesion is plausible.

Specifically, the first evidence linking Dp71 with cell adhesion was supplied by García-Tovar and collaborators [116]. By utilizing confocal microscope immunofluorescence analysis, the authors found that Dp71 is distributed to the leading borders of lamellipodia and the focal complex in U-373 MG cells, in which it co-localizes with β1-integrin, α-actinin, and vinculin. Later, the participation of Dp71-containing DAPC in actin-cytoskeleton remodeling of platelets was revealed [117], promoting shape change, adhesion, aggregation, and granule centralization. These authors also revealed that the platelet adhesion process requires the participation of microtubules and actin, and that the interplay between the two cytoskeletal systems is undertaken by β-DG and focal adhesion proteins [118]. Supporting the role of Dp71 in platelets, it was reported that adhesion to collagen in response to thrombin was significantly decreased in platelets isolated from *mdx*^{3cv} mice [119]. However, the deficit of Dp71 in *mdx*^{3cv} platelets appears to be compensated partially by utrophins [120], the autosomal homologues of dystrophins [109].

A PC12 cell-based model was employed to define in detail the involvement of Dp71 in cell adhesion. It was demonstrated by confocal microscope immunofluorescence analysis and immunoprecipitation assays that Dp71f associates with different integrin complex components, including β1-integrin, FAK, α-actinin, talin, and actin [121, 122]. The binding of Dp71f to the adhesion complex appears to be functionally relevant because depletion of Dp71f results in a drastic decrease in the amount of β1-integrin adhesion complex components and, ultimately, in adhesion failure [122]. In that cell adhesion is intimately linked with neurite outgrowth, it is likely that the failure of Dp71-depleted PC12 to extend neurites in response to NGF [75] is mainly caused by their deficient adhesion activity. At present, it is difficult to establish how Dp71f interacts with the multiprotein adhesion complex. Because α-actinin binds directly with the C terminus region of dystrophin [123], a protein domain shared with Dp71, it is tempting to propose that α-actinin interacts with Dp71 in PC12 cells, thus establishing cross-communication between Dp71f-containing DAPC and the

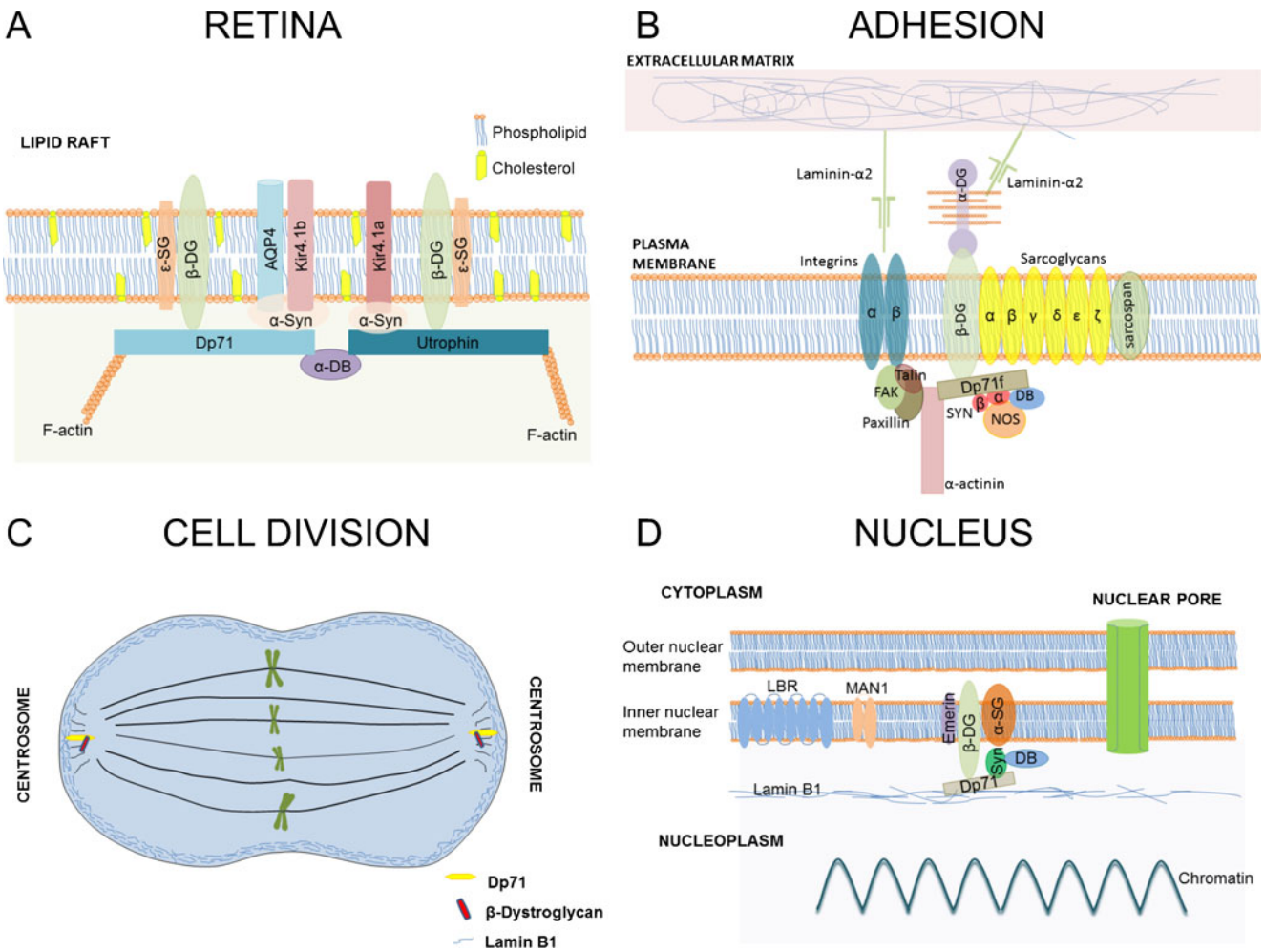

Fig. 3 Functional diversity of Dp71. **a** Retina. Hypothetical model for the binding of Dp71– and utrophin–DAPC complex to Kir4.1 and AQP4. It is known that Dp71 and utrophin can both be associated with β-dystroglycan (*β-DG*) and α-syntrophin (*α-Syn*). The Dp71–DAPC subcomplex is localized in the GM1-cholesterol-enriched plasma membrane domain in which α-Syn binds Kir4.1b and AQP4. In the utrophin–DAPC subcomplex, α-Syn is only associated with Kir4.1a. α-Dystrobrevin (*α-DB*) may function as a keystone of the molecular scaffold in order to permit the physical association of the two subcomplexes. The ε-sarcoglycan (*ε-SG*) and α-DB positions within the macromolecular complex requires confirmation. **b** Dp71 in cell adhesion. Dp71 modulates cell adhesion by facilitating cross-communication between the DAPC and the integrin-adhesion complex via its interaction with α-actinin. *SYN* syntrophin, *DB* dystrobrevin, *DG* dystroglycan. **c** Role of Dp71 in cell division. During mitosis, Dp71 binds to β-dystroglycan and lamin B1at the mitotic spindle poles, regulating their localization and stability. **d** Function of nuclear Dp71. The model proposes that β-dystroglycan (*β-DG*) and α-sarcoglycan (*α-SG*) are inserted into the inner nuclear membrane in proximity to emerin, with β-dystroglycan as a scaffold for the secondary association of Dp71 and the remaining DAPC components to the nuclear envelope. Nuclear envelope proteins MAN1 and Lamin B receptor (*LBR*) are also shown in the scheme. Interaction of Dp71 with β-dystroglycan and lamin B1 mediates communication between the nuclear envelope and nucleoplasm, thus modulating the localization and stability of emerin and lamin B1

β1-integrin adhesion system (Fig. 3b). Therefore, Dp71f appears to be a structural component of the β1-integrin adhesion complex of PC12 cells that modulates PC12 cell adhesion by conferring proper assembly and/or stability on the adhesion complex.

As β1-integrin-mediated cell adhesion modulates neuronal migration and synaptogenesis during development of the central nervous system [124], and because brain necropsies from DMD patients with DMD exhibited cerebral heterotopias [125, 126], the participation of Dp71f in modulating β1-integrin-mediated cell adhesion might constitute a clue to pursue in order to define the implication of Dp71 in DMD-associated cognitive impairment.

Potential Role of Dp71 in Cell Division

Cell cycle is an ordered, tightly regulated process that involves multiple checkpoints that assess extracellular growth signals, cell size, and DNA integrity. It is divided

into four phases. During two of these phases, the cell executes the basic events in cell division, such as generation of a single and faithful copy of its genetic material (synthetic or S phase) and portioning of all the cellular components between the two identical daughter cells (mitosis or M phase). The remaining two phases of the cell cycle represent gap periods (G1 and G2) during which the cells prepare themselves for the successful completion of the S and M phases, respectively.

Interestingly, researchers in the laboratory of Dr. Cisneros have recently provided experimental evidence linking Dp71 with the cell division cycle [60]. It was observed that Dp71-knockdown PC12 cells display a marked delay in cell growth with no alteration in cell death, suggesting a failure in the cell division cycle. Supporting this hypothesis, flow cytometry-based cell cycle analysis revealed an altered behavior of Dp71-depleted cells, which consists of a delay in G0/G1 transition and an increase in apoptosis during nocodazole-induced mitotic arrest. The abnormal response of Dp71-knockdown cells to nocodazole treatment evidenced that these cells might have altered mitosis. It was found by confocal microscopy analysis that Dp71 is targeted to the mitotic spindle-cleavage furrow and midbody of PC12 cells, where it is spatially associated with lamin B1 and β-dystroglycan [127]. These two proteins have been involved in aspects of the cell division cycle [128, 129]. It is noteworthy that these researchers observed drastically decreased staining for both lamin B1 and β-dystroglycan in the mitotic spindle–cleavage furrow and in the midbody of Dp71-depleted cells, as well as a marked reduction in their levels in total extracts [60, 127]. In light of this data, we postulate that Dp71 is a component of the mitotic spindle and cytokinesis multiprotein apparatuses that might modulate the cell-division cycle by binding to lamin B1 and β-dystroglycan and conferring proper localization and stability upon them (Fig. 3c). The role of Dp71 in PC12 cell division opens a new avenue in the study of Dp71 in neuronal function. It is tempting to speculate that alteration in cell division due to Dp71 deficit could be related with DMD-associated brain abnormalities such as neuronal loss, gliosis, and Purkinje cell loss [125, 126].

Dp71 in the Nucleus

As mentioned previously, the Dp71d isoform localizes in the nucleus of cultured neuronal cells and different cell lines, forming a nuclear DAPC-like complex. In this scenario, researchers in Dr. Cisneros's laboratory developed a long-term study aimed at determining the function of nuclear Dp71-containing DAPC. Previously, it was found that Dp71d and DAPC components are recovered in the nuclear matrix and nuclear envelope fractions of HeLa [59] and C2C12 [54] cells, respectively. Furthermore, interaction of Dp71d with the nuclear envelope protein lamin B1 was demonstrated in HeLa [59] and PC12 cells [60]. The nuclear envelope is composed of inner and outer nuclear membranes, the nuclear pore complex, and nuclear lamin. Lamin is a fibrous structure associated with the inner nuclear membrane through interaction with integral membrane proteins and is the main component of the nuclear matrix, which is a permanent network of core filaments underlying thick fibers. It was revealed that the fraction of Dp71d associated with the nuclear matrix increased during NGF-induced neuronal differentiation of PC12 cells [130] and, remarkably, that knockdown of Dp71 expression in PC12 cells caused alterations in nuclear envelope proteins, such as a severe decrease of lamin B1 levels and mislocalization of emerin [127].

It was proposed that nuclear DAPC might be functionally connected with the nuclear envelope via interaction between Dp71d and lamin B1 and, importantly, that Dp71d works as nuclear scaffolding protein that is critical for proper localization and stability of nuclear envelope proteins (Fig. 3d). Because the nuclear envelope and nuclear matrix are thought to comprise the platform for numerous nuclear processes including nuclei morphology, gene expression, and DNA repair, Dp71d might modulate these nuclear envelope-associated functions in neuronal cells. Hence, potential impairment in nuclear function as a result of Dp71 deficiency and the relationship of such an alteration with the DMD-associated neuronal phenotype merit analysis.

Final Remarks

For a long time, the study of DMD was being conducted exclusively on skeletal muscle, focusing on understanding the function of dystrophin as well as on the development of therapeutic strategies against the disease. The discovery of Dp71, the main DMD gene product in non-muscle tissues, did not change this tendency immediately, and this protein was regarded primarily as a potential therapeutic molecule that might compensate for dystrophin deficiency in muscle cells. However, the fact that Dp71 is nearly ubiquitously expressed implies that it might participate in basic functions that are common to all tissues or in specialized tasks in each cell type. It was not until Dp71-specific experimental models were developed that its function and direct implication in DMD-associated retinal dysfunction and cognitive impairment were unraveled. It is now well established that Dp71 works as a scaffolding protein, exerting physical and functional interaction with a number of signaling and structural

protein partners not only in numerous cell types but also in different cellular compartments including plasma membrane and nucleus, which confers functional diversity on this protein. Considering the physiological relevance of Dp71 to DMD and its versatility of function, it would not be surprising if new DMD non-muscular phenotypes linked with a Dp71 deficit were to emerge in the near future.

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Conflict of Interest The authors declare that they have no conflict of interest.

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