Role for α-dystrobrevin in the pathogenesis of dystrophin-dependent muscular dystrophies

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A dystrophin-containing glycoprotein complex (DGC) links the basal lamina surrounding each muscle fibre to the fibre's cytoskeleton, providing both structural support and a scaffold for signalling molecules. Mutations in genes encoding several DGC components disrupt the complex and lead to muscular dystrophy. Here we show that mice deficient in α-dystrobrevin, a cytoplasmic protein of the DGC, exhibit skeletal and cardiac myopathies. Analysis of double and triple mutants indicates that α-dystrobrevin acts largely through the DGC. Structural components of the DGC are retained in the absence of α-dystrobrevin, but a DGC-associated signalling protein, nitric oxide synthase, is displaced from the membrane and nitric-oxide-mediated signalling is impaired. These results indicate that both signalling and structural functions of the DGC are required for muscle stability, and implicate α-dystrobrevin in the former.

A multimolecular complex called the dystrophin–glycoprotein complex (DGC) spans the membrane of striated (skeletal and cardiac) muscle fibres, linking the intracellular cytoskeleton to the overlying basal lamina[1,2]. The best-characterized component of the DGC is the cytoplasmic protein dystrophin, mutant forms of which underlie Duchenne and Becker muscular dystrophies and X-linked cardiomyopathy[3–7]. Dystrophin binds both to cytoskeletal actin and to the cytoplasmic tail of a transmembrane DGC protein, β-dystroglycan[8,9]. The extracellular domain of β-dystroglycan binds α-dystroglycan, which in turn binds to the α2 chain of laminin, a major component of the basal lamina[10,11]. Thus, the DGC maintains the structural integrity of the muscle fibre by linking the basal lamina to the cytoskeleton (Fig. 1). The importance of this linkage is underscored by the fact that mutations of laminin-α2 or any of four transmembrane DGC components (α-, β-, γ- or δ-sarcoglycan) also lead to muscular dystrophies in humans and rodents[2,7].

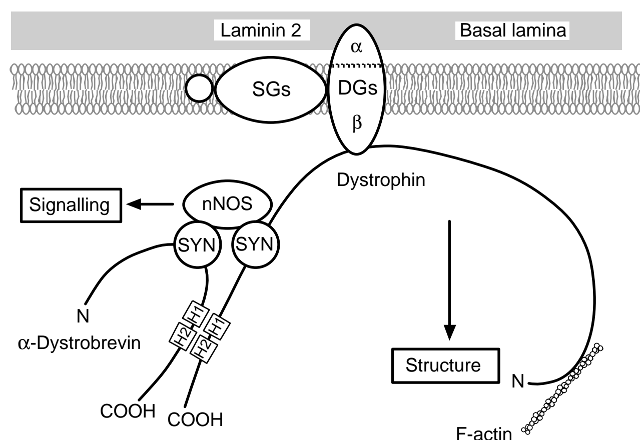

Figure 1 **Model of the DGC in skeletal muscle.** Extracellular-matrix components include laminin-2; membrane components include the sarcoglycans (SGs) and dystroglycans (DGs); and cytoplasmic components include dystrophin, α-dystrobrevin and syntrophin (SYN). We propose that dystrophin mediates structural functions, by binding to F-actin, and signalling functions, by binding to α-dystrobrevin and syntrophin, which in turn anchor signalling molecules such as neuronal nitric oxide synthase (nNOS) to the DGC. H1 and H2 are helical-repeat structures.

It has been suggested that the DGC has signalling as well as structural roles. Several lines of evidence support this idea. First, the core DGC components, dystrophin and dystroglycan, both bind signalling molecules: β-dystroglycan binds Grb2, a signal-transducing adaptor protein, and dystrophin interacts with calmodulin, a regulator of calcium-dependent kinases[12–14]. Second, the DGC contains two groups of cytoplasmic proteins that have been implicated in signalling, the syntrophins (α1, β1 and β2)[15,16] and the dystrobrevins (α and β)[17–23]. α1-Syntrophin contains a PDZ domain (a protein–protein-interaction motif) that interacts with at least two sarcolemmal proteins involved in signal transduction, neuronal nitric oxide synthase (nNOS)[24] and voltage-gated sodium channels[25,26]. α-Dystrobrevin binds both syntrophins and dystrophin, and is a substrate for tyrosine kinases[18,27–29]. Third, the regulation of intramuscular blood flow by contractile activity is disrupted in dystrophin-deficient (*mdx*) mouse muscles, implicating the DGC in the production or transmission of an intercellular signal from muscle fibres to the vascular endothelium (refs 30–32 and see below).

Despite these data, the ways in which the DGC mediates signalling functions and the extent to which signalling dysfunction contributes to dystrophy have remained unclear. Here we address these issues through generation and analysis of α-dystrobrevin-deficient ($adbn^{-/-}$) mutant mice. Our results provide evidence that both signalling and structural functions of the DGC contribute to muscle stability, and show that α-dystrobrevin has a role in signalling.

Results

Muscular dystrophy in *adbn*$^{-/-}$ mice. Several alternatively spliced forms of α-dystrobrevin have been described[20,29,33] (Fig. 2a). We

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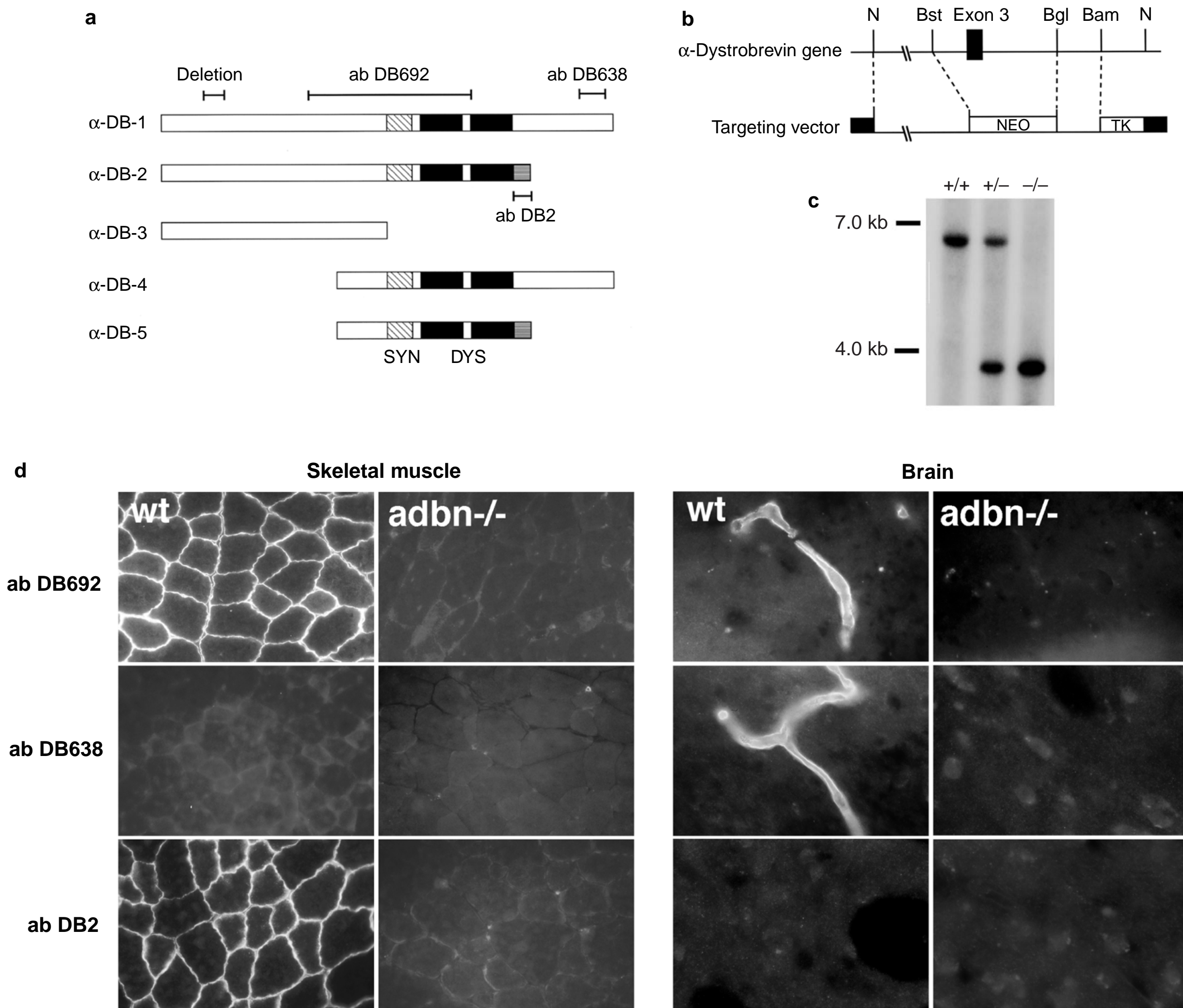

Figure 2 **Mutation of the α-dystrobrevin gene. a**, The five known products (α-DB-1 to α-DB-5) generated by alternative splicing of the α-dystrobrevin gene, showing regions recognized by anti-α-dystrobrevin antibodies (ab DB638, ab DB692 and ab DB2) and the portion of the protein encoded by the exon that we deleted to mutate the α-dystrobrevin gene. Also indicated are binding sites for syntrophin (SYN; hatched area) and dystrophin (DYS; filled area), and sequences unique to α-DB-2 and α-DB-5 (grey area). **b**, Targeting vector used to mutate the α-dystrobrevin gene. Bam, *Bam*HI; Bgl, *Bgl*I; *Bst*, *Bst*BI; N, *Not*I; NEO, neomycin-resistance gene; TK, thymidine kinase. **c**, Southern analysis of genomic DNA following germline transmission of the mutant α-dystrobrevin gene. The 6.8-kb fragment of the wild-type allele (+/+) was altered to a 3.8-kb fragment in the mutant (–/–). Both bands are present in heterozygotes (+/–). **d**, Immunostaining of skeletal muscle and brain from wild-type (wt) and $adbn^{-/-}$ mice using three different anti-α-dystrobrevin antibodies. In controls, α-DB1, α-DB3 and/or α-DB4 are present in cerebral vessels, and α-DB2, α-DB3 and/or α-DB5 is present in sarcolemma. None of the three antibodies stains either mutant tissue.

deleted a segment common to α-DB-1 and α-DB-2, the only forms reported to be present in muscle[29]. The mutation was introduced into embryonic-stem cells by homologous recombination and two independent recombinants were transmitted through the germ line (Fig. 2b,c). Heterozygous ($adbn^{+/-}$) and homozygous ($adbn^{-/-}$) mutant animals were viable, fertile and outwardly normal. None of the known alternatively spliced forms of α-dystrobrevin was detectable in non-synaptic portions of $adbn^{-/-}$ skeletal muscles or brains of homozygous mutants (Fig. 2d), and no compensation by β-dystrobrevin was detected (data not shown).

Muscles of $adbn^{-/-}$ mice appeared histologically normal during the first two postnatal weeks, but became dystrophic by 1 month of age. Pathological findings included small groups of degenerating myofibres and infiltrating monocytes (Fig. 3a,b). Regenerating fibres, characterized by expression of the embryonic myosin heavy chain[34] and central nuclei, were also present (Fig. 3b–d). Degenerating muscle cells were usually grouped in clusters of 3–10 fibres. Histologically, the myopathy of $adbn^{-/-}$ mice resembled that of *mdx* mice (Fig. 4a and ref. 35), although $adbn^{-/-}$ mice had fewer centrally nucleated fibres (Table 1). In $adbn^{-/-}$ mice, as in *mdx* mice, the diaphragm was the most severely affected skeletal muscle. Overall, however, both $adbn^{-/-}$ and *mdx* mice exhibited milder pathology than humans with Duchenne dystrophy and never developed the early myofibrosis characteristic of the human disease[7,35].

The number of necrotic clusters in $adbn^{-/-}$ muscles reached a plateau at ~6 months of age and remained constant for at least a year. Beyond 3 months of age, the percentage of centrally nucleated fibres also remained constant at ~50% of all fibres within the muscle. This observation indicated that some muscle-fibre types might be resistant to damage in $adbn^{-/-}$ mice, but staining with fibre-type-specific antibodies failed to support this idea (data not shown). Moreover, similar patterns of degeneration and regeneration were observed in muscles with varying fibre-type composition, such as tibialis anterior, quadriceps femoris, soleus and sternomastoid.

α-Dystrobrevin is expressed along with the other components of the DGC in heart, and cardiomyopathy is a common feature of

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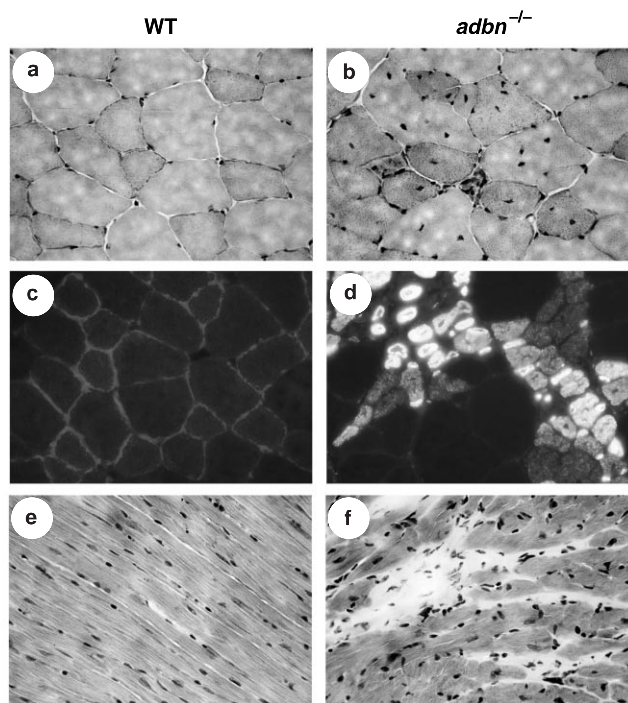

Figure 3 **Histology of *adbn*$^{-/-}$ muscle. a**, **b**, Haematoxylin and eosin stained sections of skeletal muscle from wild-type (WT) and *adbn*$^{-/-}$ mice. Small areas of necrosis and centrally nucleated fibres are seen in *adbn*$^{-/-}$ muscle. **c**, **d**, Sections of skeletal muscle stained with antibody to embryonic and fetal myosin heavy chains. The positively stained fibres seen in *adbn*$^{-/-}$ muscle indicate actively regenerating muscle fibres. **e**, **f**, Haematoxylin and eosin stained sections of cardiac muscle, showing a dystrophic area in *adbn*$^{-/-}$ tissue.

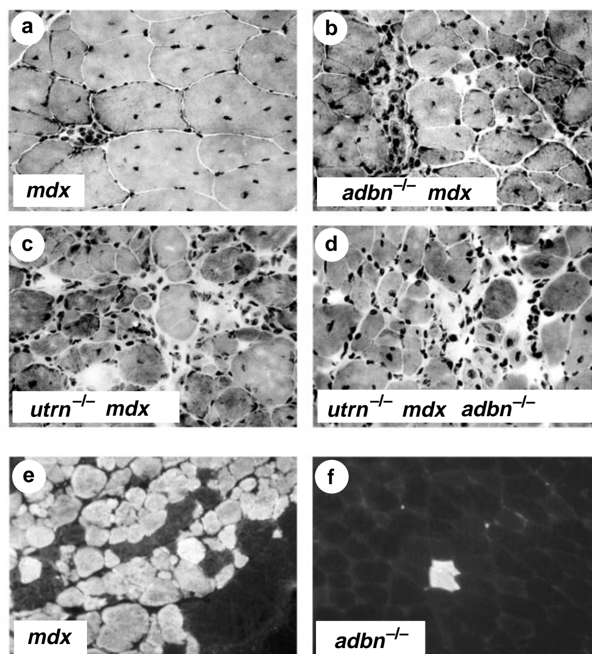

Figure 4 **Skeletal muscle from DGC mutants. a–d**, Haematoxylin and eosin stained sections of skeletal muscle from single, double and triple mutants. All genotypes have areas of necrosis, mononuclear cell infiltration and centrally nucleated fibres, but the process is more widespread in *utrn*$^{-/-}$ *mdx* and *utrn*$^{-/-}$ *mdx adbn*$^{-/-}$ tissue than in *mdx* or *adbn*$^{-/-}$ *mdx* tissue. **e**, **f**, Skeletal muscle from *mdx* and *adbn*$^{-/-}$ mice injected with Evans blue dye, which stains fibres with damaged membranes. Compromised cells are more numerous in *mdx* than in *adbn*$^{-/-}$ muscle.

Table 1 Dystrophy in seven mutant genotypes

Genotype	*n*	Lifespan	Skeletal dystrophy*	Central nuclei†	Cardio-myopathy
Wild type	20	>1 yr	None	0	None
utrn$^{-/-}$	5	>1 yr	None	0	None
adbn$^{-/-}$	45	>1 yr	Mild	~50%	Mild
adbn$^{-/-}$ *utrn*$^{-/-}$	4	>1 yr	Mild	~50%	Mild
mdx	55	>1 yr	Mild/moderate	>90%	Mild
adbn$^{-/-}$ *mdx*	12	8–10 months	Moderate	>90%	Moderate
utrn$^{-/-}$ *mdx*	24	4–20 wks	Severe	>90%	Moderate/ severe
utrn$^{-/-}$ *mdx adbn*$^{-/-}$	11	3–11 wks	Severe	>90%	Moderate/ severe

* Severity of dystrophy was scored by the average amount of dystrophic changes, including necrotic fibres, mononuclear infiltrate, fibre-size variability and fibrosis seen in haematoxylin and eosin stained muscle sections from each genotype.
† The percentage of centrally nucleated skeletal muscle fibres, a feature of regenerated cells, reflects the proportion of affected fibres seen within a haematoxylin and eosin stained cross-section of tibialis anterior muscle.

Duchenne dystrophy[6,7]. Myopathic changes were seen in 61% (19/31) of *adbn*$^{-/-}$ hearts studied from animals older than 1 month of age. Pathological findings included degenerating myocytes, mononuclear-cell infiltration and fibrosis, usually associated with plaques (Fig. 3e,f). These plaques were scattered through both ventricles and were surrounded by myocytes that appeared normal. Despite the pathological changes, *adbn*$^{-/-}$ hearts did not appear significantly dilated or hypertrophic.

α-Dystrobrevin functions through the DGC. The skeletal and cardiac myopathies seen in *adbn*$^{-/-}$ mice might reflect either impairment of the DGC or a DGC-independent function of α-dystrobrevin. If α-dystrobrevin's function requires interaction with dystrophin, one might expect that mice lacking both dystrophin and α-dystrobrevin would have the same degree of dystrophy as *mdx* mice, which are deficient in dystrophin alone. This genetic analysis is complicated, however, by the fact that *mdx* mice have relatively mild muscle disease as a result of compensation by the dystrophin homologue, utrophin, which can also bind to α-dystrobrevin[29]. In contrast, mice deficient in both dystrophin and utrophin (*utrn*$^{-/-}$ *mdx* mice) have a severe phenotype comparable to that of Duchenne patients, with widespread loss of myocytes, myofibrosis and premature death[36,37]. To obviate the compensatory effects of utrophin, we therefore generated mice lacking α-dystrobrevin, dystrophin and utrophin and compared these triple mutants (*utrn*$^{-/-}$ *mdx adbn*$^{-/-}$) to double mutants (*utrn*$^{-/-}$ *mdx*) with intact α-dystrobrevin genes.

The triple mutants were born at the expected frequency and appeared outwardly normal at birth. Of 11 triple mutants studied, eight lived beyond weaning (three weeks) but developed the same abnormalities seen in *utrn*$^{-/-}$ *mdx* mice, including poor growth, kyphosis and severe limb contractures[36,37]. Four of these eight mice were killed between 7 and 11 weeks of age. Skeletal muscles in these triple mutants exhibited necrosis, fibrosis and central nuclei, but were no more dystrophic than muscles of *utrn*$^{-/-}$ *mdx* mice (Fig. 4c,d and Table 1). In addition, triple mutant mice developed a cardiomyopathy similar to that of *utrn*$^{-/-}$ *mdx* mice[36]. The shortened lifespan of triple mutants compared with double mutants (Table 1) may reflect a DGC-independent role for α-dystrobrevin in other tissues. However, the similar muscle pathology of *utrn*$^{-/-}$ *mdx* and triple mutants indicates that the functions of α-dystrobrevin in muscle may depend largely on its association with the DGC. Con-

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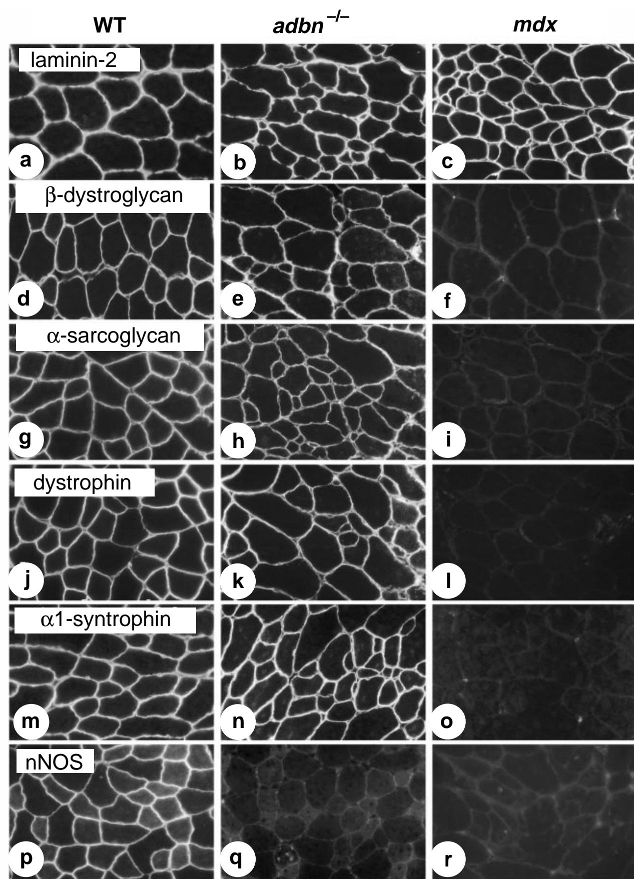

Figure 5 **Immunostaining of skeletal muscle from wild-type (WT), *adbn*$^{-/-}$ and *mdx* mice with antibodies to DGC-associated proteins. a–c**, Levels of laminin-α2 were similar in all three genotypes. **d–o**, Levels of the DGC proteins β-dystroglycan, α-sarcoglycan, dystrophin and α1-syntrophin were markedly reduced in *mdx* muscle, but normal in *adbn*$^{-/-}$ muscle. **p–r**, In contrast, levels of nNOS were greatly reduced in both *mdx* and *adbn*$^{-/-}$ muscle.

versely, dystrophy in *mdx* mice may result partly from loss of α-dystrobrevin.

We also analysed double mutant mice deficient in both α-dystrobrevin and dystrophin (*adbn*$^{-/-}$ *mdx*) or α-dystrobrevin and utrophin (*adbn*$^{-/-}$ *utrn*$^{-/-}$) (Fig. 4b and Table 1). The *adbn*$^{-/-}$ *mdx* mice showed a more severe dystrophy than *mdx* mice, but not as severe as that of *utrn*$^{-/-}$ *mdx* or triple mutants. On the other hand, *adbn*$^{-/-}$ *utrn*$^{-/-}$ mice had a similar dystrophy to *adbn*$^{-/-}$ mice. These phenotypes support the idea[36,37] that utrophin is unnecessary for muscle stability but can compensate for dystrophin deficiency.

Mechanism of dystrophy in *adbn*$^{-/-}$ mice. We next asked how the loss of α-dystrobrevin from the DGC leads to dystrophy. The absence of dystrophin in mice and humans results in a severe reduction in the sarcolemmal levels of DGC components[2,4], leading to a breakdown of the sarcolemma and eventual cell death. We used two approaches to test whether a similar mechanism accounts for the dystrophy of *adbn*$^{-/-}$ mice. First, to assess the structural integrity of the sarcolemma, we injected mice with the dye Evans blue. This dye is membrane impermeant and is therefore taken up only by cells with structurally impaired membranes[38,39]. A dramatic difference between genotypes was apparent at 1–3 months of age, which is when degeneration and regeneration peak in *mdx* mice. At this age, up to 20% of all muscle fibres in the tibialis anterior were stained positively in *mdx* mice (>500 fibres per cross-section; data not shown; see refs 36, 39), whereas <1% of fibres were stained positively in *adbn*$^{-/-}$ mice (mean±s.e.m.=7.8±4.8 positive fibres per

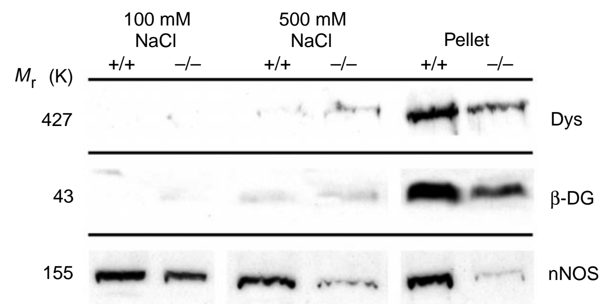

Figure 6 **Immunoblots of skeletal-muscle proteins from wild type (+/+) and *adbn*$^{-/-}$ (–/–) mice.** Soluble proteins (100 mM NaCl), a high-salt extract (500 mM NaCl) and the salt-insoluble fraction (pellet) were probed with antibodies to dystrophin (Dys), β-dystroglycan (β-DG) and nNOS. Dystrophin and β-dystroglycan were primarily found in the pellet in both genotypes. nNOS was distributed equally to all three fractions in wild-type muscle, but was primarily soluble in *adbn*$^{-/-}$ muscle.

cross-section, *n*=6 muscles). Consistent with previous reports[38,39], both degenerating fibres and fibres that lacked signs of necrosis stained positively with Evans blue. This difference was also observed at later stages, after active degeneration had waned in *mdx* mice. In 5–7-month-old animals, for example, the number of dye-positive fibres per muscle was fivefold higher in *mdx* than in *adbn*$^{-/-}$ mice (75±29 positive fibres per cross-section in mdx muscle, *n*=12, versus 14±4.8 in *adbn*$^{-/-}$ muscle, *n*=6; Fig. 4e,f). These results indicate that *adbn*$^{-/-}$ muscle fibres may be less structurally compromised than *mdx* fibres, and that this difference reflects the nature of the dystrophy as well as its severity.

Second, to determine whether the DGC is disassembled in *adbn*$^{-/-}$ mice, we stained muscle sections with antibodies to the DGC components dystrophin, α- and β-sarcoglycan and β-dystroglycan. The distribution of these DGC proteins appeared normal in *adbn*$^{-/-}$ muscle (Fig. 5a–l). Immunoblotting of muscle confirmed that dystrophin and β-dystroglycan remained primarily associated with the membrane in *adbn*$^{-/-}$ muscle (Fig. 6). Thus, *adbn*$^{-/-}$ muscle fibres, unlike *mdx* muscle fibres, appear to maintain the DGC and thereby preserve membrane integrity.

These results raised the possibility that muscle destruction in *adbn*$^{-/-}$ mice reflected non-structural roles of the DGC. We therefore investigated the association between α-dystrobrevin and a well-studied DGC-associated signalling protein, nNOS. Both dystrophin and α-dystrobrevin bind α1-syntrophin, which in turn binds nNOS[15,16,24,28,40,41]. Accordingly, levels of membrane-associated α-dystrobrevin, α1-syntrophin and nNOS are dramatically reduced in *mdx* muscle[29,40,41]. In *adbn*$^{-/-}$ skeletal muscle, α1-syntrophnin levels were only slightly reduced but levels of membrane-associated nNOS were markedly reduced (Fig. 5m–r). Cytosolic levels of nNOS were, however, normal in mutant muscle (Fig. 6), indicating a specific effect on the membrane-associated pool of nNOS. This reduction was not secondary to the loss of dystrophin or α1-syntrophin as both of these proteins retained their sarcolemmal distribution (Fig. 5k,n). The mechanism of loss of nNOS is not known, but clearly α-dystrobrevin has a role in localizing nNOS to the sarcolemma.

Functional evidence for α-dystrobrevin-dependent signalling. On the basis of the results presented so far, we sought direct evidence that α-dystrobrevin is required for signalling in muscle. Muscle-derived nitric oxide regulates blood flow during exercise: contractile activity stimulates nNOS activity in muscle fibres, producing nitric oxide which diffuses to the intramuscular vasculature where it locally opposes the reflex-driven increase in sympathetic tone that would otherwise decrease blood flow in active muscle[30,31,42]. It appears that the nitric oxide acts by stimulating production of cyclic GMP throughout the muscle, including the vascular smooth muscle, thereby inhibiting phosphorylation of smooth-muscle myosin

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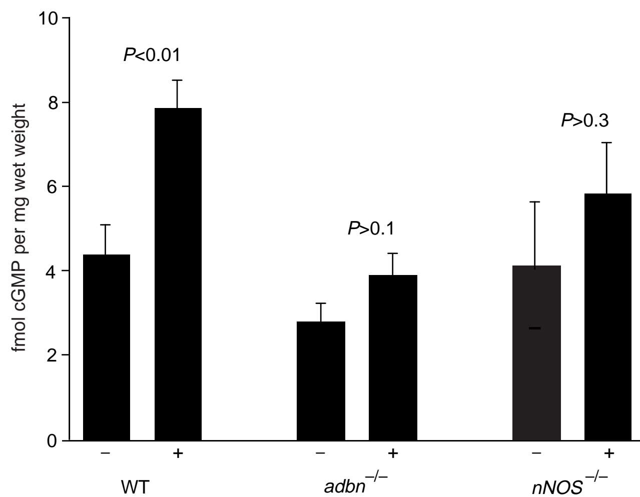

Figure 7 **cGMP levels in control and mutant muscle.** Amounts of cGMP in isolated extensor digitorum muscles from unstimulated (–) or electrically stimulated (+; 30 Hz for 15 s) control (n=4), *adbn*$^{-/-}$ (n=6) or *nNOS*$^{-/-}$ (n=6) mice. Bars show means±s.e.m. The significance of differences between stimulated and unstimulated muscles was assessed by Student's *t*-test.

and opposing vasoconstriction[32]. In *mdx* muscles, nNOS is no longer associated with the sarcolemma and direct electrical stimulation neither increases cGMP levels within the muscle nor blunts vasoconstriction[32,33]. We hypothesized that loss of α-dystrobrevin might lead to a similar defect.

To test this idea, we assayed the effect of electrical stimulation on cGMP levels in a hindlimb muscle. In initial experiments, we compared control and *nNOS*$^{-/-}$ muscles[43]. cGMP levels were roughly doubled by 30 seconds of 15-Hz stimulation in controls (P<0.01), but did not change significantly in *nNOS*$^{-/-}$ muscles (P>0.3) (Fig. 7). This result supports the assumption[32] that nNOS is required for the activity-dependent increase in cGMP. Finally, we performed similar assays on *adbn*$^{-/-}$ muscles. Stimulation did not significantly affect cGMP levels in these muscles (P>0.1), a finding similar to those obtained with *mdx* muscle[32]. Thus, the loss of α-dystrobrevin, and the subsequent displacement of nNOS from the DGC, mimicked total loss of nNOS in blocking the generation or transmission of an activity-dependent signal from muscle fibres.

Discussion

The structure and binding partners of the dystrobrevins have been studied in detail[17–23,27–29,33,44], but, to our knowledge, no information on their cellular functions has been reported so far. Our analysis of *adbn*$^{-/-}$ mutant mice provides direct evidence that α-dystrobrevin is required for the stability of skeletal and cardiac muscle fibres. *Adbn*$^{-/-}$ mice also exhibit defects in postsynaptic differentiation at the neuromuscular junction, consistent with enrichment of α-dystrobrevin at the synapse[17]; these defects will be described elsewhere (R.M.G., H. Zhou and J.R.S., unpublished observations). No defects in other tissues are apparent, but we have not yet sought subtle phenotypes, and it may be that non-muscle functions of α-dystrobrevin are masked by the presence of its widely distributed homologue β-dystrobrevin[21–23].

Our analysis of double and triple mutants indicates that α-dystrobrevin functions as part of the DGC, as expected from its tight association with dystrophin[28,29]. The best-studied functions of the DGC involve structural stabilization of the sarcolemma, and mutations of several DGC components appear to cause dystrophy by disassembling the complex and compromising the linkage of the muscle fibre's basal lamina to its cytoskeleton[2–7]. In contrast, the DGC is largely intact in *adbn*$^{-/-}$ mice, and our results indicate that dystrophy is largely a result of impaired DGC-dependent signalling. Thus, although α-dystrobrevin may have DGC-independent roles and loss of α-dystrobrevin may subtly destabilize the DGC, we favour the hypothesis that α-dystrobrevin acts primarily by mediating signalling rather than structural functions of the DGC (Fig. 1). Loss of α-dystrobrevin, and consequent disruption of signalling functions, may contribute, along with impairment of structural stability, to diverse DGC-related skeletal and cardiac myopathies. It is also possible that mutations of the α-dystrobrevin gene alone are directly responsible for some congenital human dystrophies.

Our analysis of α-dystrobrevin function focused on nNOS, because this is the best-studied signalling component of the DGC. Loss of nNOS alone is unlikely to account for the dystrophy in *adbn*$^{-/-}$ mice, as mice lacking *nNOS*$^{-/-}$ are not detectably dystrophic[43,45,46]. Likewise, α1-syntrophin-deficient mice lack sarcolemmal nNOS but are phenotypically normal[47]. On the other hand, studies of nitric-oxide-mediated modulation of intramuscular blood flow indicate that loss of nNOS from the DGC may contribute to the pathogenesis of dystrophy[29–31]. Moreover, the DGC binds other signalling proteins[12–14,25,26] whose membrane associations may be important, along with nNOS, for muscle viability. The function of these signalling molecules may be obscured in dystrophin or sarcoglycan mutants, as loss of the DGC also compromises the structural integrity of the sarcolemma. Thus, *adbn*$^{-/-}$ mice, in which the remainder of the DGC appears intact, may be useful for elucidating the signalling functions of the DGC in muscular dystrophy. □

Methods

Generation of mutant mice.

In the *adbn* targeting vector, a 2.5-kilobase (kb) *Bst*BI–*Bgl*I segment of the α-dystrobrevin gene containing exon 3 (ref. 48) was deleted and replaced with a neomycin-resistance gene (Fig. 2b). The vector was transferred to embryonic stem cells by electroporation, and homologous recombinants selected. Recombinants were injected into blastocysts of C57Bl/6 mice. Two independent clones gave rise to germline progeny. Phenotypes of both lines were identical and are described together. Mice were maintained on a 129SVJ × C57Bl/6 hybrid background. *Utrn*$^{-/-}$ mice have been described previously[49]. *Mdx* and *nNOS*$^{-/-}$ mice were obtained from Jackson Laboratories.

Histology.

Methods for immunostaining are as described in ref. 49. For nNOS staining, tissue sections were fixed for 5 min with 1% paraformaldehyde/PBS before incubating with primary antibody. For bright-field microscopy, frozen tissue sections were stained with haematoxylin and eosin. Tissue was obtained from mice of the eight genotypes listed in Table 1. Rabbit polyclonal antibodies to murine α-dystrobrevin (DB2 and DB638; ref. 29) and anti-β-dystrobrevin[21] were gifts from S. C. Froehner. DB692 was generated in our laboratory to a recombinant fragment of α-dystrobrevin. The antibodies to developmental myosin heavy chains and β-sarcoglycan were from Novacastra Laboratories (Newcastle on Tyne, UK). Anti-nNOS antibody for immunostaining was from Sigma; anti-nNOS antibody for immunoblotting was from Transduction Laboratories; and anti-α1-syntrophin antibody was a gift from S. C. Froehner. Sources of other antibodies are as described[36,49]. Evans blue (Sigma) (10 mg ml^{-1} in PBS) was injected intraperitoneally (0.1 ml per 10 mg body weight)[38,39]. Mice were killed 12–24 h later; the tibialis anterior was sectioned and dye-positive fibres were counted under a fluorescence microscope.

Immunoblotting.

For immunoblotting, tissue was prepared as described[40]. Skeletal muscle was homogenized in 100 mM NaCl buffer and centrifuged at 20,000*g*. The resulting pellet was extensively washed with buffer containing 500 mM NaCl and centrifuged at 15,000*g*, yielding a second supernatant and a pellet. Protein concentration of tissue extracts was determined by a BCA protein assay (Pierce). Equal amounts of protein were resolved by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies and visualized using enhanced chemiluminescence (NEN).

cGMP measurements.

Methods for stimulation are described in ref. 32. Extensor digitorum muscles were isolated and mounted on a Grass FT03.C force transducer and continuously bathed in an oxygenated, buffered solution. Muscles were electrically stimulated for 15 s and then quick-frozen. Later, frozen muscles were homogenized and centrifuged. The total cGMP content of the muscle soluble fraction was measured by a radioimmunoassay[32].

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