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No alteration in gene expression of components of the ubiquitin-proteasome proteolytic pathway in dystrophin-deficient muscles

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Abstract Increased expression of critical components of the ubiquitin-dependent proteolytic pathway occurs in any muscle wasting condition so far studied in rodents where proteolysis rises. We have recently reported similar adaptations in head trauma patients [Mansoor et al. (1996) Proc. Natl. Acad. Sci. USA 93, 2714–2718]. We demonstrate here that the increased muscle protein breakdown seen in mdx mice only correlated with enhanced expression of *m*-calpain, a Ca^{2+}-activated proteinase. By contrast, no change in mRNA levels for components of the ubiquitin-proteasome proteolytic process was seen in muscles from both mdx mice and Duchenne muscular dystrophy patients. Thus, gene expression of components of this pathway is not regulated in the chronic wasting that characterizes muscular dystrophy.

Key words: Skeletal muscle; Protein turnover; Ubiquitin; Proteasome; Calpain; Muscular dystrophy

1. Introduction

Duchenne muscular dystrophy (DMD) is the most common sex-linked lethal disease in man, affecting approximately 1 in 3500 boys and resulting in a fatal evolution before the end of the third decade of life [1]. This disease is characterized by the lack of detectable dystrophin, a very large (400 kDa) cytoskeletal protein [2,3]. In DMD patients and in mdx mice, the animal model that reproduces DMD, there is a severe muscle wasting. This wasting results from enhanced proteolysis in mdx mice [4,5].

Like all mammalian tissues, skeletal muscle contains multiple proteolytic systems. The best known system is the lysosomal pathway, which involves four major proteinases in muscle (cathepsins B, H, L and D). Increased specific activities of cathepsin B [6–8], H [8], L [7,8], and D [6] have been reported in dystrophic skeletal muscle. However, these increased activities of cathepsins were related to the infiltration of non-muscle cells in both dystrophic hamsters [7] and mdx mice [8], although some cathepsin L was co-localized in muscle cells as well as in invading macrophages [8]. Skeletal muscle also contains two major cytosolic proteolytic pathways, Ca^{2+}-dependent [9] and ATP-ubiquitin-dependent [10], respectively. Turner et al. [4] showed that the elevation of the concentration of intracellular Ca^{2+} in muscle fibers from young mdx mice resulted in an enhanced protein breakdown. Furthermore, MacLennan et al. [5] reported that the increased protein breakdown seen in mdx muscle was attributable to a non-lysosomal Ca^{2+}-activated proteolytic process. Recent data, however, demonstrated that both cathepsins and calpains (i.e. the Ca^{2+}-activated proteinases) do not contribute significantly to the overall muscle protein breakdown [11–15], and do not play an important role in the breakdown of actin and myosins [16–18]. By contrast, the ATP-ubiquitin-dependent proteolytic pathway which was previously believed to degrade short-lived and abnormal proteins [19,20] is responsible for the bulk of muscle protein breakdown [11–15,21–25] including the degradation of the long-lived contractile proteins [17,18].

Increased mRNA levels for critical components of the ubiquitin-proteasome-dependent proteolytic pathway have been systematically observed in various animal models of muscle wasting, such as fasting [11,25], denervation atrophy [25], cancer [13,21,22], acidosis [12], sepsis [14,18], simulated weightlessness [15], burn injury [26], and glucocorticoid administration [11,23]. We have also recently shown that similar adaptations prevailed in muscle biopsies from head trauma patients who exhibited negative nitrogen balance, increased whole body rate of protein breakdown, and enhanced urinary 3-methylhistidine excretion [24]. A concomitant stimulation of the ubiquitin-proteasome pathway with either the Ca^{2+}-dependent [13] or the lysosomal process [11,22] or both [14,15,17,24] seems to prevail in some of these catabolic states.

To our knowledge, the role of the ATP-ubiquitin-dependent proteolytic process has not been studied in muscular dystrophies, although an accumulation of ubiquitylated proteins was observed in muscle biopsies from patients with oculopharyngeal muscular dystrophy [27]. The present experiments were undertaken to determine (i) whether the ATP-ubiquitin-dependent proteolysis is activated in muscular dystrophy, and (ii) whether a coordinate activation of the lysosomal and Ca^{2+}-activated proteinases prevails in such conditions. To address this question we measured mRNA levels for cathepsins D and L, *m*-calpain, ubiquitin, 14-kDa ubiquitin conjugating enzyme E2 (14-kDa E2), and subunits of the 20S proteasome in muscles from mdx mice and in muscle biopsies obtained from control and dystrophin-deficient patients.

2. Materials and methods

2.1. Animals

Mice of the mdx strain were kindly provided by Dr. J.-L. Guénet (Institut Pasteur, Paris, France). They were compared to C57BL/6 control mice obtained from Iffa Credo (L'Arbresle, France). The mice were maintained in a temperature-controlled room (22 ± 1°C) on a 12-h light-dark cycle. They were fed ad libitum with a standard

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Abbreviations: 14-kDa E2, 14-kDa ubiquitin conjugating enzyme E2; DMD, Duchenne muscular dystrophy

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diet (R03; UAR, Epinay sur Orge, France) and had free access to water.

2.2. Rates of protein turnover

Animals were killed by cervical dislocation at 5 weeks of age. Extensor digitorum longus muscles were carefully dissected and incubated at approximately resting length by pinning their tendons on plastic supports. All tissues were incubated at 37°C in Krebs-Henseleit buffer (NaCl 120 mM, KCl 4.8 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM and $MgSO_4$ 1.2 mM, pH 7.4) equilibrated with 95% O_2 and 5% CO_2, and containing 5 mM glucose, 0.1 U/ml insulin, 0.17 mM leucine, 0.10 mM isoleucine, and 0.20 mM valine to improve protein balance [28]. After 1 h of preincubation, muscles were transferred to a fresh medium of identical composition and further incubated for 2 h.

The rate of protein synthesis was determined by incubating muscles in a medium containing 0.5 mM [U-^{14}C]phenylalanine (Amersham Corp., Amersham, UK; specific radioactivity in the medium 500 dpm/nmol), as previously described [13]. Tissues were homogenized in 10% trichloroacetic acid (TCA) and hydrolyzed in 1 N NaOH at 37°C. Tissue protein mass was determined using the bicinchoninic acid procedure [29]. Rates of phenylalanine incorporation were converted into tyrosine equivalents as described [28], and expressed in nmol tyrosine incorporated/mg protein/h.

Rates of protein breakdown were measured by following the rates of tyrosine release into the medium. Since muscle neither synthesizes nor degrades this amino acid, tyrosine release reflects the net breakdown of proteins. Thus, rates of total protein degradation were calculated by adding the rate of protein synthesis and the net rate of tyrosine release into the medium [28]. Tyrosine was assayed by the fluorometric method of Waalkes and Udenfriend [30].

2.3. Human muscle biopsies

Muscle biopsies were performed during orthopedic surgery, in the Department of Pediatric Surgery of the Hôtel Dieu Hospital in Clermont-Ferrand. The protocols were approved by an Ethical Committee (Consultative Committee for the Protection of Persons Undergoing Biomedical Research, Région d'Auvergne).

The characteristics of the patients and of the muscles sampled are given in Table 1. Five DMD patients presenting a lack of dystrophin and six control subjects were studied. The latter were not suffering from any neuromuscular pathology, and presented no abnormality in terms of muscle structure or fiber distribution. The age of the DMD patients studied ranged between 7 and 21 years. All DMD patients were wheelchair bound, except the youngest one.

2.4. Northern blot analysis

Tibialis anterior muscles from control and mdx mice and human muscle biopsies were rapidly excised, frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted as described by Chomczynski and Sacchi [31]. 20 μg of total RNA was electrophoresed in 1% agarose gel containing formaldehyde. RNA was electrophoretically transferred to a nylon membrane (GeneScreen, NEN Research Products, Boston, MA, USA) and covalently bound to the membrane following UV crosslinking. The membranes were hybridized with cDNA probes encoding chicken polyubiquitin [32], rat 14-kDa E2 [33], the rat [34] or human [35] C2 proteasome subunit, human cathepsin D [36], and human *m*-calpain [37]. The hybridizations were performed at 65°C with [^{32}P]cDNA fragments labelled by random priming. Following washes at the same temperature, the membranes were autoradiographed for 3–96 h at −80°C with intensifying screens on Hyperfilm-MP (Amersham International, Little Chalfont, UK). cDNA inserts encoding murine cathepsin L [38] and human *m*-calpain [37] were sub-cloned into *Pst*I sites of pGem-blue (Promega, Madison, WI, USA) and *Eco*RI-*Sma*I sites of pBluescript II KS$^+$ (Stratagene), respectively. The plasmids were linearized by *Hin*dIII (*m*-calpain) and *Pvu*II (cathepsin L), and [^{32}P]cRNAs were synthesized using T7 RNA polymerase. Hybridizations with *m*-calpain and cathepsin L [^{32}P]cRNAs were performed as previously described [15]. After stripping of the different probes, the filters were reprobed with a mouse 18S ribosomal probe (No. 63178, American Type Culture Collection, Rockville, MD, USA). Autoradiographic signals were quantified by digital image processing and analysis (NIH Image 1.54) and normalized using the corresponding 18S rRNA signals to correct for uneven unloading.

2.5. Statistical analysis

All data are expressed as means ± S.E.M. The unpaired Student's *t*-test was used for statistical analyses. Significance was defined at the 0.05 level.

3. Results and discussion

Recent lines of evidence strongly suggest that the ATP-ubiquitin-dependent proteolytic pathway plays a major role in skeletal muscle. First, in several instances of muscle atrophy, there is no evidence for an activation of either the lysosomal [13,21] or the Ca^{2+}-dependent [11,22] proteolytic process. Secondly, cathepsins and calpains only account for a minor part of overall protein breakdown in muscles from both control and cachectic animals where protein breakdown rises [11–15]. Thirdly, both lysosomal and Ca^{2+}-activated proteinases do not degrade myofibrillar proteins [16–18]. By contrast, in any catabolic state so far studied in rodents [11–15,18,21,22,26], it has been reported that the bulk of increased protein breakdown is due to the activation of a non-lysosomal and Ca^{2+}-independent process. This process is ATP-dependent [11–13,18,22,26], and increased expression of ubiquitin [11–15,18,21–26], 14-kDa E2 involved in the ubiquitylation of protein substrates [13–15,23,24,33], and subunits of the 20S proteasome [12–15,22–25], which is the proteolytic core of the 26S proteasome that degrades ubiquitin conjugates, systematically paralleled the changes in protein breakdown measured in incubated muscles. Furthermore, since the break-

Table 1
Characteristics of dystrophin-deficient and control patients

Age (years)	Sex	Muscle	Diagnosis	Dystrophin	Fiber necrosis and fibrosis
7	M	deltoid	DMD	−	+
10	M	triceps surae	DMD	−	++
12	M	hip adductor	DMD	−	+++
14	M	tensor fasciae latae	DMD	−	+++
21	M	deltoid	DMD	−	+++
5	M	deltoid	psychosis	+	−
8	F	triceps surae	tiptoes walker	+	−
10	M	piriformis	sciatic compression	+	−
10	M	hip adductor	cerebral palsy	+	−
11	M	hip adductor	femoral anteversion	+	−
15	M	paravertebral	kyphosis	+	−

M: male; F: female. The degree of fiber necrosis and fibrosis was evaluated by histoenzymatic analysis and classified as absent (−), moderate (+), intermediate (++) or severe (+++). All DMD patients were dystrophin (−). Control subjects were dystrophin (+) and fiber necrosis and fibrosis (−).

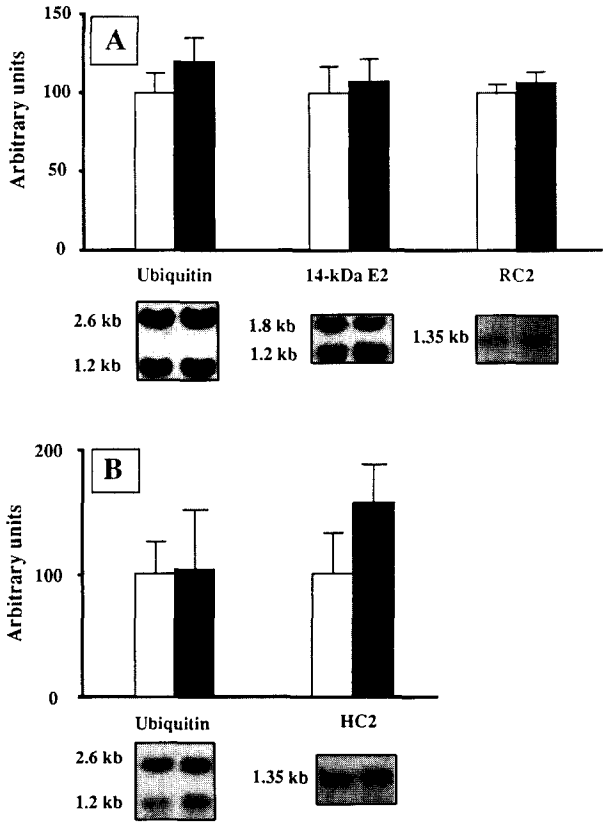

Fig. 1. Quantification of mRNA levels for components of the ubiquitin-proteasome proteolytic pathway in skeletal muscle from mdx mice (A) and DMD patients (B). Open bars, control muscles; black bars, dystrophic muscles. RNA was extracted, electrophoresed, transferred to a nylon membrane and hybridized with ^{32}P-labeled cDNAs encoding ubiquitin, 14-kDa E2, and rat (RC2) or human (HC2) C2 proteasome subunit, as described in Section 2. After stripping of the probes, blots were rehybridized with an 18S ribosomal oligonucleotide. Autoradiographic signals are expressed as a percentage of controls corrected for 18S rRNA abundance, to take into account slight variations in RNA loading. Data are means ± S.E.M. for $n=6$ mice and 5–6 patients. Representative Northern blots are also shown.

down of actin and myosin requires ATP [17,18], and because ubiquitylated proteins were found in the myofibrillar fraction [39], it has been suggested that the ubiquitin-proteasome proteolytic process is critical for the breakdown of the major contractile proteins.

In the present experiments we attempted to determine whether increased mRNA levels for components of the ATP-ubiquitin-dependent proteolytic pathway prevailed in dystrophin-deficient muscles. mdx animals were studied at 5 weeks of age, i.e. at a stage of development when total protein breakdown was elevated compared to control animals (Table 2), as described [4]. Since protein synthesis was also previously reported to be increased in mdx muscles [5], we verified that the net protein breakdown was also enhanced in such conditions (Table 2). However, in striking contrast with all various muscle wasting conditions so far studied in rodents where protein breakdown was elevated [11–15,21–23,25,26], we found no significant variation in mRNA levels for components of the ubiquitin-proteasome proteolytic pathway in muscle from mdx mice (Fig. 1A). Interestingly, the lack of change in mRNA levels for ubiquitin in mdx muscle did not result from a total blockade in the responsiveness of the ubiquitin genes due to the dystrophic state. Indeed, recent evidence has been provided that ubiquitin expression was increased to the same extent in normal and mdx muscle fibers after exercise [40]. mRNA levels for ubiquitin, 14-kDa E2 and proteasome subunits also increased in muscle biopsies from head trauma patients who exhibited rapid muscle wasting [24]. However, again, no similar changes prevailed in muscle biopsies from DMD patients (Fig. 1B). Ubiquitin-proteasome-dependent proteolysis is now recognized to be of major importance for the breakdown of contractile proteins [17,18]. Thus, the lack of detectable activation of this pathway in dystrophin-deficient muscles is presumably due to the very progressive changes in muscle mass in such conditions. For example, in many instances of induced cachexia in rodents, the loss of muscle proteins is ~5%/day [14,15,22]. In the present experiments, the difference in muscle mass between 5 weeks old dystrophic and control mice is at most 0.7%/day in the tibialis anterior muscle and 0.5%/day in the extensor digitorum longus muscle (Table 2). Furthermore, these differences do not entirely reflect muscle wasting, but impaired growth. Alternatively, some regulatory mechanisms may prevent sustained increased expression of proteolytic genes in muscular dystrophy, to avoid excessive and rapid muscle wasting. Finally, it was recently reported that increased mRNA levels for proteasome subunits in atrophying muscles either reflected increased transcription [41] or entered active translation [15]. However, we cannot rule out a possible activation of the ubiquitin-proteasome proteolytic pathway based on the lack of variation of mRNA levels for components of this pathway.

The mechanism by which elevated protein breakdown results in muscle wasting in mdx mice is assumed to be Ca^{2+}-dependent, based on indirect evidence. First, elevated intracellular Ca^{2+} concentration resulted in increased rates of proteolysis in muscles from 5 weeks old mdx mice [4]. Secondly, the β2-agonist clenbuterol, which increases the activity of the calpain inhibitor calpastatin, can retard muscle wasting in mdx mice [42]. The enhanced mRNA levels for *m*-calpain observed here (Fig. 2A) provided strong direct evidence for a role of Ca^{2+}-dependent proteinases in muscle wasting in mdx mice.

Table 2
Muscle/body weight ratios and protein turnover in the extensor digitorum longus (EDL) of control and mdx mice

	Muscle mass/body weight ratio		Protein synthesis	Total protein breakdown	Net protein breakdown
	tibialis anterior	EDL			
Control mice	1.551 ± 0.049	0.469 ± 0.009	0.211 ± 0.007	0.842 ± 0.072	0.629 ± 0.073
mdx mice	1.165 ± 0.169*	0.378 ± 0.022**	0.427 ± 0.057**	1.558 ± 0.184**	1.130 ± 0.178*
Difference	−0.386	−0.091	+0.216	+0.716	+0.501

Values are means ± S.E.M. for six animals. Protein turnover data are expressed in nmol tyrosine/mg protein/h (for more details see Section 2).
*$P < 0.05$, **$P < 0.005$ vs. controls.

However, in human muscle biopsies, we observed no significant variation in mRNA levels for *m*-calpain (Fig. 2B) in accordance with recent observations by Sakamoto et al. [43]. The precise significance of changes in mRNA levels for Ca^{2+}-activated proteinases in different muscle wasting conditions is still unclear. Enhanced expression of *m*-calpain was associated with increased enzyme activity in the atrophying unweighted soleus muscle [15], suggesting a transcriptional regulation. By contrast, no change in μ- and *m*-calpain activity occurred in the muscles of fasted rabbits, although the mRNA levels for μ- and *m*-calpain were elevated [44]. Furthermore, a third calpain, p94, that is expressed abundantly only in skeletal muscle, has been characterized [45]. Mutations in p94 cause limb-girdle muscular dystrophy type 2A [46]. There is some evidence that p94 was expressed in proportion of dystrophin, whose absence causes DMD [43]. Interestingly, the expression of p94 was also down-regulated in the atrophying muscles from interleukin-6 transgenic mice [47]. Thus, muscle atrophy seems to be associated with decreased p94 mRNA levels.

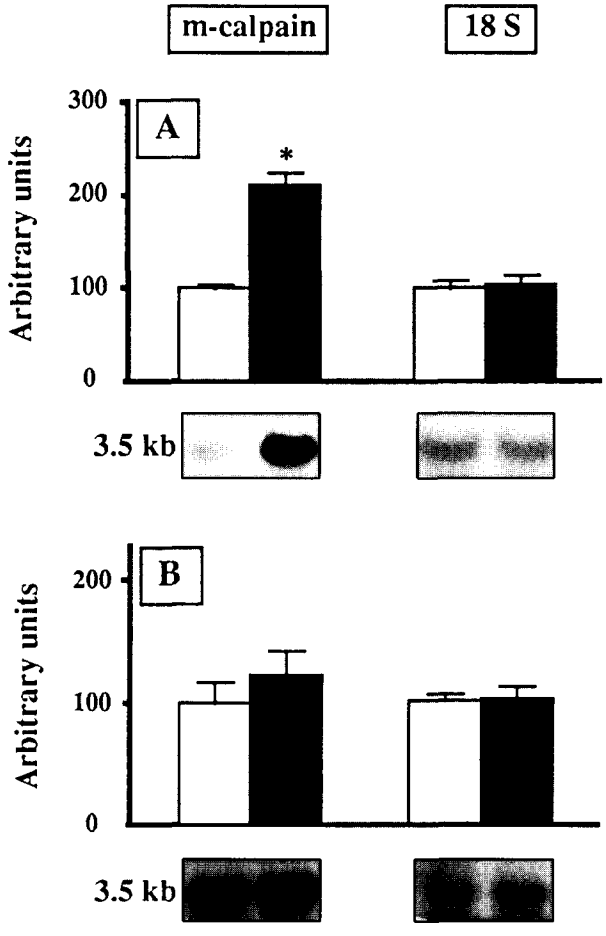

Fig. 2. Quantification of mRNA levels for *m*-calpain in skeletal muscle from mdx mice (A) and DMD patients (B). Open bars, control muscles; black bars, dystrophic muscles. RNA was extracted, electrophoresed, transferred to a nylon membrane and hybridized with a ^{32}P-labeled riboprobe (A) or cDNA (B) encoding *m*-calpain, as described in Section 2. After stripping of the probe, blots were rehybridized with an 18S ribosomal oligonucleotide. Autoradiographic signals are expressed as a percentage of controls corrected for 18S rRNA abundance, to take into account slight variations in RNA loading. Data are means ± S.E.M. for $n = 6$ mice and 5–6 patients. Representative Northern blots are also shown.

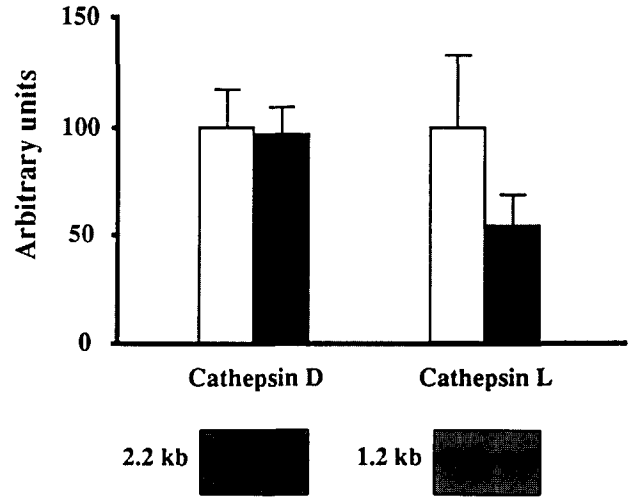

Fig. 3. Quantification of mRNA levels for cathepsins D and L in skeletal muscle from mdx mice. Open bars, control muscles; black bars, dystrophic muscles. RNA was extracted, electrophoresed, transferred to a nylon membrane and hybridized with a ^{32}P-labeled cDNA (cathepsin D) or a ^{32}P-labeled riboprobe (cathepsin L), as described in Section 2. After stripping of the probes, blots were rehybridized with an 18S ribosomal oligonucleotide. Autoradiographic signals are expressed as a percentage of controls corrected for 18S rRNA abundance, to take into account slight variations in RNA loading. Data are means ± S.E.M. for $n = 6$ mice. Representative Northern blots are also shown.

Finally, MacLennan et al. [5] demonstrated that enhanced muscle proteolysis in mdx muscle was attenuated by inhibitors of thiol proteases (e.g. leupeptin and E-64 which inhibit both the calpains and cathepsins B, H, and L), but not by the weak base methylamine, which only affects lysosomal protein breakdown. The lack of significant variations in mRNA levels for both cathepsins D and L in mdx muscle supported these observations (Fig. 3). Alternatively, these data may suggest that the possible involvement of cathepsins in elevated protein breakdown during muscular dystrophy [6–8] did not reflect a transcriptional regulation.

In conclusion, we have shown that in contrast with all other muscle wasting conditions so far studied, there is no detectable activation of the ubiquitin-proteasome proteolytic pathway in dystrophic muscle from mdx mice and DMD patients. We suggest that the lack of change in mRNA levels for critical components of this pathway resulted from the very progressive changes in muscle mass that characterize muscular dystrophies. Alternatively, some mechanisms may prevent sustained increased expression of proteolytic genes in muscular dystrophy, to avoid excessive and rapid muscle wasting. In contrast, the increased mRNA levels for *m*-calpain in mdx muscle strongly support a selective activation of the Ca^{2+}-dependent proteinases. These proteinases may selectively degrade soluble or membrane proteins. For example, they could be responsible for the dramatic loss of all the components of the dystrophin-glycoprotein complex which is characteristic of dystrophin deficiency [48].

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