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ORIGINAL ARTICLE

C3KO mouse expression analysis: downregulation of the muscular dystrophy Ky protein and alterations in muscle aging

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Abstract Mutations in CAPN3 gene cause limb–girdle muscular dystrophy type 2A (LGMD2A) characterized by muscle wasting and progressive degeneration of scapular and pelvic musculature. Since CAPN3 knockout mice (C3KO) display features of muscle pathology similar to those features observed in the earliest-stage or preclinical LGMD2A patients, gene expression profiling analysis in C3KO mice was performed to gain insight into mechanisms of disease. Two different comparisons were carried out in order to determine, first, the differential gene expression between wild-type (WT) and C3KO soleus and, second, to identify the transcripts differentially expressed in aging muscles of WT and C3KO mice. The up/downregulation of two genes, important for normal muscle function, was identified in C3KO mice: the Ky gene, encoding a protease implicated in muscle development, and Park2 gene encoding an E3 ubiquitin ligase (parkin). The Ky gene was downregulated in C3KO muscles suggesting that Ky protease may play a complementary role in regulating muscle cytoskeleton homeostasis in response to changes in muscle activity. Park2 was upregulated in the aged WT muscles but not in C3KO muscles. Taking into account the known functions of parkin E3 ligase, it is possible that it plays a role in ubiquitination and degradation of atrophy-specific and damaged proteins that are necessary to avoid cellular toxicity and a cellular stress response in aging muscles.

Keywords C3KO · LGMD2A · Calpain-3 · Gene expression · Muscular dystrophy · Ky · Park2

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Introduction

Limb–girdle muscular dystrophy type 2A (LGMD2A) is caused by mutations in calpain 3, a muscle-specific calcium-dependent cysteine protease. Patients with LGMD2A show muscle wasting and cell death that lead to progressive degeneration of scapular and pelvic musculature, while the facial muscles are preserved [1, 2]. The mechanism by which mutations in a proteolytic enzyme might lead to muscle cell death has been so far elusive [3–5].

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Patients with mild or preclinical LGMD2A show almost normal muscle histology except for small areas of focal necrosis [6, 7]. The histological appearance of muscles of CAPN3 knockout mice (C3KO), which completely lack both CAPN3 mRNA and protein in skeletal muscles, showed similar evidence of muscle pathology. Cross sections of the gastrocnemius, soleus, tibialis anterior, and diaphragm muscles showed rare and small foci of necrosis and regeneration surrounded by primarily healthy-looking tissue. The cross-sectional area of both slow and fast fibers was significantly reduced. The soleus and diaphragm were the most affected muscles among those examined in C3KO mice [8]. In LGMD2A patients, weakness and atrophy predominate in proximal groups of muscles, extending in the upper limbs, to the triceps brachialis and, to a lesser degree, to the radialis and cubital muscles. In the lower limbs, the weakness extends to the quadriceps and, to a minor degree, to the tibialis anterior and triceps surae [1, 2].

Although calpain 3 was identified in 1989 [9], its specific function remains unknown. Calpain 3 has been ascribed several different roles in skeletal muscle; for example, it plays a role in myofibrillar protein turnover, due to its placement on titin in the sarcomere [8, 10–15].

Gene expression profiling by microarray technology is a useful and unbiased approach to mine data and hopefully open new perspectives on molecular pathways involved in the pathogenesis of muscular disorders [14, 16–21]. The principal aim of the present study was to determine the differential gene expression between wild-type (WT) and C3KO soleus in order to identify pathways in which calpain 3 is implicated. Furthermore, because calpainopathy is a progressive disease, this study also sought to identify transcripts that were differentially expressed in aging muscles (2–3 vs 11–12 months old) in WT and C3KO mice.

Materials and methods

Microarray technology

Muscle samples were taken from 11 wild-type and 11 C3KO mice [8]. Two types of muscles were dissected from each mouse: soleus, representing one of the most affected muscles, and quadriceps, representing a less affected muscle [8]. Moreover, soleus showed the greatest molecular similarities with human muscles [22]. Four out of 11 mice from each genotype were 2–3 months old (young group) while the other seven were 11–12 months old (adult group). All the mice were males to minimize the inter-gender variability. Muscle tissues were snap frozen and stored at −80 °C. All experimental protocols were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the UCLA Institutional Animal Care and Use Committee.

The quality of RNA extracted from muscles was analyzed using the spectrophotometer and the Bioanalyzer system (Agilent), and only samples with acceptable quality and integrity (RIN above 7) were selected for further experiments.

The microarray experiments were performed according to the manufacturers' protocol. Briefly, cDNA was generated from RNA samples, and biotinylated cRNA was transcribed in vitro. Fragmented cRNA was hybridized with *GeneChip MouseGenome 430 2.0* microarrays (Affymetrix, Santa Clara, CA). These microarrays analyze the expression of over 39,000 transcripts from over 34,000 well-characterized mouse genes using 45,000 probes.

In-depth quality controls were used to confirm the validity of the hybridization processes in accordance with four criteria: (1) a presence of the signal corresponding to the spike control BioB, (2) expression ratio between 3′ and 5′ ends of the housekeeping GAPDH should not exceed a value of 3, (3) the full percentage of presence detected by the Affymetrix Detection algorithm for each array must be in the range of 40–60, and (4) percentage of the outlier probe sets detected within each microarray should be less than 5 %. Only one microarray failed to follow these criteria, and therefore, it was excluded from posterior data analysis.

The hybridized arrays were scanned, and raw data were extracted using the Microarray Analysis Suite 5.0 (MAS5; Affymetrix). The raw data were normalized using robust multichip average (RMA) expression summary in Bioconductor [23]. RMA consists of three steps: a background adjustment, quantile normalization, and finally, summarization [24–26].

Analysis

First, in order to identify significantly different gene expression, a geometric fold change analysis was used [18, 27]. The threshold was set at a twofold change value. Second, class comparison difference analyses were performed using BRB-ArrayTools developed by Dr. Richard Simon and the BRB-ArrayTools Development Team. In order to identify probe sets with significant intensity differences between disease classes, a two-sample univariate *t* test was applied to all the performed comparisons. The threshold was set at $p<0.001$. To minimize false positives, only the probe sets commonly yielded by both methodologies were included into the final list of genes differentially expressed in each comparison. Two different comparisons were performed: (a) between WT and C3KO adult soleus and (b) between young and adult muscles (soleus and quadriceps) in WT and C3KO mice.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [28] and are accessible through GEO Series accession number GSE33931 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33931).

Real-time PCR

To validate array data, expression levels of some of the differentially expressed genes were measured using the TaqMan quantitative real-time (RT)-PCR assay. Quantitative RT-PCR was performed using the 7900 HT Fast Real-Time PCR System (Applied Biosystems). Because of the limiting RNA amount isolated from muscle biopsies used for microarray analysis, only 20 muscles were used for validation of 64 genes with quantitative RT-PCR experiments.

The TaqMan low-density arrays (TLDA) and the TaqMan probes were purchased from Applied Biosystems, and the protocol recommended by the manufacturer was used. Expression levels for all transcripts in the TLDAs were determined relative to the internal housekeeping control gene 18S, which did not show modified expression in the different comparisons. In order to identify probe sets with significant intensity differences, the Benjamini–Hochberg method was applied to the unaffected control data set vs the LGMD2A data set using the Stat Miner program (Integromics).

Muscle extract preparation and Western blot analysis

The following antibodies were used for Western blot analysis: anti-Ky, anti-Ddit4l (REDD2), and anti-Park2 from Abcam and anti-GAPDH from Santa Cruz Biotechnology.

For Ddit4l Western Blot analysis, muscles were homogenized in a Dounce homogenizer in reducing sample buffer (80 mM Tris, pH 6.8; 0.1 M dithiothreitol; 2 % sodium dodecyl sulfate (SDS); and 10 % glycerol with protease inhibitor cocktail (Sigma)).

For Ky and Park2 Western Blot analysis, frozen tissue samples were weighed and homogenized with 19 *w/v* of treatment buffer (0.125 mol/L Tris, 4 % SDS, 10 % glycerol, 0.1 mol/L ethylenediaminetetraacetic acid, and 5 % β-mercaptoethanol) in a TissueLyser mixer-mill disruptor (Qiagen).

Muscle histology and immunohistochemistry

Muscles to be used for immunohistochemistry were dissected from the mice and frozen in isopentane that was cooled in liquid nitrogen. Frozen sections were cut at 10 μm and kept frozen until use. After thawing, sections were treated with 0.3 % H_2O_2 for 5 min (if horseradish peroxidase was used for color reaction) and blocked in PBS with 0.2 % gelatin, 0.5 % Tween-20, and 3 % bovine serum albumin for 30 min. Anti-CD11b antibody (BD Biosciences) was used as a primary antibody. After primary and biotin-conjugated secondary antibodies, sections were incubated with avidin-conjugated horseradish peroxidase and stained using AEC substrate kits (Vector Laboratories).

Results

Figure 1 shows histological and immunohistochemical staining of the muscles that were used for analysis. CD11b staining shows focal areas of regeneration and a few inflammatory cells present in solei from young C3KO mice similar to the LGMD2A patients at the presymptomatic and early stages of the disease. No signs of pathology were usually found in quadriceps muscle from young C3KO mice. In the older C3KO animals, however, both solei and quadriceps showed some pathological features such as centrally nucleated fibers, lobulated fibers, fiber size variability, and splitting, which were more pronounced in solei.

Using WT and C3KO mice soleus and quadriceps, different comparisons were carried out. Transcripts were classified according to their potential involvement in different biological processes, as suggested by LocusLink (www.ncbi.nlm.nih.gov/LocusLink/).

Comparison of WT vs C3KO adult soleus

Differential gene expression between WT and C3KO adult soleus was analyzed in order to identify pathways in which calpain 3 might be involved. Besides the expected downregulation of calpain 3 gene, the analysis identified six differentially expressed genes, four of which were overexpressed and two showed a reduced expression in C3KO mice compared to the WT controls. Almost all genes that were deregulated in the C3KO soleus were involved in signal transduction or metabolism. Additionally, the Ky gene, implicated in muscle development, was downregulated in C3KO muscles compared to WT muscle (Table 1).

Young vs adult comparison in WT and C3KO muscles

LGMD2A patients have inherited mutations in calpain 3; however, the clinical onset of the disease usually occurs during the second decade of life. It has been shown that histological changes precede clinical symptoms of LGMD2A and may differ at different stages of disease progression. For example, inflammatory infiltrates are usually present at earlier stages and disappear with aging and disease progression [29]. It is likely that transcriptomic changes also depend on age and stage of disease [19]. Thus, comparisons between young and adult muscles were performed to identify the transcripts differentially expressed in aging muscles (2–3 vs 11–12 months old) in WT and C3KO mice.

In the comparison between young vs adult WT soleus, differential expression of 43 genes was detected. In the comparison of young vs adult C3KO soleus, 38 deregulated genes were identified; however, only five genes changed the same with aging as in WT: Cyfip2, Amph, Pln

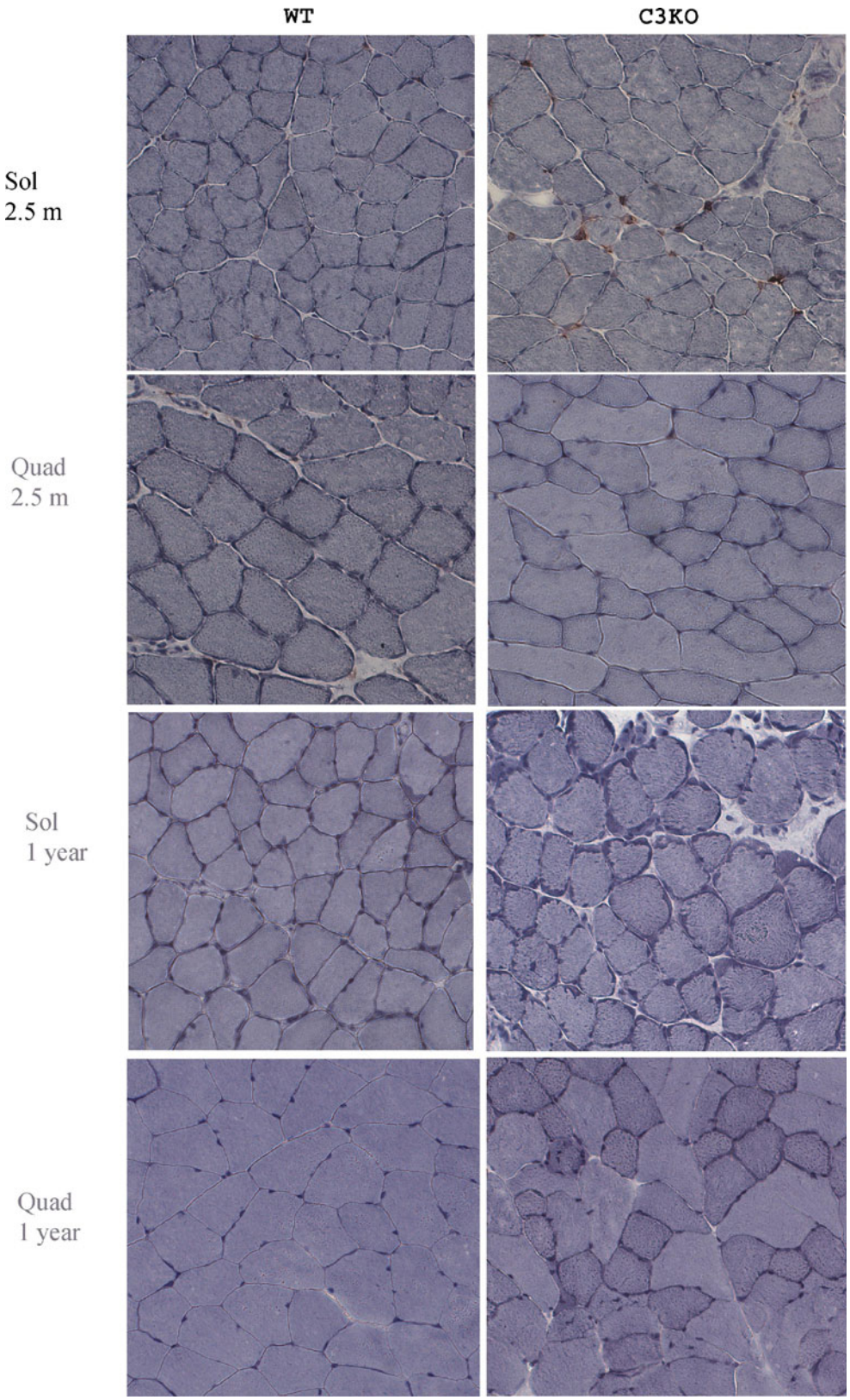

Fig. 1 Muscle histology and immunohistochemistry stained with CD11b antibody (a marker of immune cells, stained *red*) and counterstained with hematoxylin (*blue*) of soleus and quadriceps at 2.5 months and at 1 year in WT and C3KO mice

(upregulated), and Tfrc (downregulated). The Dct gene, implicated in metabolic processes, was deregulated in both genotypes in the young vs adult comparison; however, it was downregulated in the WT mice soleus with aging, while in the C3KO, it was upregulated with aging (Table 2, Supplementary material Table A).

Table 1 Comparison between adult WT and C3KO soleus (six genes differentially regulated)

Affymetrix ID	Biological process/gene title	Gene symbol	Fold change arrays	Fold change RT-PCR
Metabolic process				
1442466_a_at	Histidine acid phosphatase domain containing 2A	Hisppd2a	0.36	NV
1429862_at	Phospholipase A2, group IVE	Pla2g4e	2.49	3.2
1451355_at	Alkaline ceramidase 2	Acer2	2.35	2.21
Signal transduction				
1434140_at	Mcf2 transforming sequence-like	Mcf2l	2.01	NV
1425098_at	Zinc finger protein 106	Zfp106	2.42	2.31
Muscle development				
1440435_at	Kyphoscoliosis peptidase	Ky	0.42	0.465
Proteolysis				
1426043_a_at 1433681_x_at	Calpain 3	Capn3	0.01 0.02	NV

NV not validated

We also performed a comparison between expression profiles of quadriceps muscles in addition to solei, because quadriceps muscles are much less affected by the absence of CAPN3 than solei. In the comparison between WT quadriceps (young vs adult), a differential expression of 78 genes was detected. In the comparison between C3KO quadriceps (young vs adult), 26 deregulated genes were identified (Table 2, Supplementary material Table A).

Considering only the biological functions that presented gene up/downregulation in all the performed comparisons (Table 2a), six genes (Vegfc, Fgl2, Rgs5, Nfil3, Nr4a2, Ctgf) showed the same regulation pattern in both WT muscles, soleus and quadriceps, during maturation (underlined in Table 2a). However, no such changes were observed in C3KO muscles. On the other hand, five genes (Dkk3, Npr3, Rasd2, Amy1, Papola) were upregulated in aged quadriceps from both C3KO and WT mice (Table 2a, highlighted).

Additionally, in the comparison between young vs adult C3KO soleus, none of the genes participating in biological functions such as ubiquitination, collagen fibril organization, and immune response were deregulated (Table 2b).

Validation of microarray data by quantitative RT-PCR

Quantitative RT-PCR was carried out to validate microarray data. The genes used for the technical validation were selected based on the comparisons performed in the present work as well as on other comparisons that finally were not included in this work. The selected gene list and the main correlation between the two assays (R=0.8538) are shown in Supplemental material Table B. The mean fold changes in expression levels obtained by this analysis were directionally similar to those determined by microarray analysis. Overall, the fold change observed by quantitative RT-PCR was lower when assessed by microarray than by quantitative real-time PCR (Table 1 and Supplementary material Table A).

Western blot

Western blot analyses of Ky, Ddit4l, and Park2 proteins were performed in two muscles, quadriceps and soleus, of the young and adult WT and C3KO mice. As Ky was the only gene related to muscle identified in the WT vs C3KO comparison, it was selected for further analysis. The Western blot of Ky protein was performed in order to analyze if the downregulation observed at the RNA level was also present at the protein level. Figure 2 shows that Ky protein is also downregulated significantly in the soleus of adult C3KO mice compared to their WT counterparts (p=0.0015).

Ddit4l was also selected because it is involved in the mTOR pathway which is implicated in muscle growth. Ddit4l protein was not detected in WT or C3KO quadriceps (Fig. 3). In soleus, the downregulation observed at the RNA level in the young vs adult comparison in C3KO soleus was not observed in the Western blot analysis. Nevertheless, Ddit4l protein showed statistically significant (p<0.05) expression level differences between WT and C3KO young mice showing a lower amount in the young C3KO mice (Fig. 3).

Finally, because ubiquitinization is impaired in C3KO mice [12], the E3 ubiquitin ligase parkin was also selected for Western blot analysis, which confirmed that the upregulation of parkin in the aging soleus of the WT mice also occurs at the protein level (Fig. 4). However, the quadriceps did not show protein-level differences even if the RNA level was also upregulated in this muscle (data not shown). In accordance with the RNA results which did not show any change in expression in the aging muscles in C3KO mice, no differences in parkin protein expression were detected in the young vs adult comparison of these mice (Fig. 4).

Table 2 Summary of functions deregulated in aging muscles

A)

Biological function	Soleus		Quadriceps	
	WT	C3KO	WT	C3KO
Signal Transduction	Vegfc	Cds1, Prkd1, Prkg2, Tiam2	Dkk3, Eda2r, Fgfbp1, Map2k6, Npr3, Ptger3, Rasd2, Rhpn2, Unc13c, Vegfc, Xpr1	Dkk3, Rcan1, Npr3, Rasd2
	Fgl2, Hbegf, Ms4a6b, Rgs5	Ddit4l, Frzb	Fgl2, Rgs5, Socs3	Aplnr, Dkk2
Metabolic process	Blvrb	Pmm1, **Dct**, Uck2, Leprel1, Acer2	Amy1, Gstm2, Sh3rf2, Lep, Pfkfb3, Ces3	Amy1
	Dct, Entpd4, Plod2, Rrm2	-	Aldh18a1, Herc4, Odc1, Pon1, Ppara, Smox	-
Transcription	Rora, Zfp30	Ankrd1, Atf3, Per2, Zfp697	Trim16, Papola, Synpo2	Papola, Nr4a3, Per2
	Nfil3, Nr4a2, Zkscan3	-	Fos2l, Irf2bp2, Klf6, Klf7, Nfil3, Nr4a2, Nrip1, Qk, Rbm5	Rcor3
Cell adhesion	-	Pkp2, Npnt	Pkp2	-
	Cd44, Ctgf, Lgals4, Postn, Spp1	-	Ctgf, Vcan	-
B)	**Biological functions that were not up/downregulated in C3KO soleus in young vs adult comparison**			
Protein ubiquitinization	Park2	No	Park2, Mib1	Cbl
Collagen fibril organization	Col1a1, Col5a2, Col11a1, Col22a1, Lox	No	Col1a1, Col1a2, Col1a3	Col1a1, Col1a2, Col1a3
Immune response	H2-Q7	No	H2-Q7, Cd1d1, Igh-6, Igk	Igh-6
	Ccl6, Ccl9, Fcgr2b		Ccl6, Ccl9, Ctla2a	Gbp6

Common deregulated biological functions in muscle comparisons (young vs adult). Shaded, upregulated genes; not shaded, downregulated genes. Underlined genes presented the same regulation pattern in both WT muscles, soleus and quadriceps, during maturation, not observed in C3KO mice muscles. Highlighted genes presented the same regulation pattern in the quadriceps of both genotypes (complete information in Supplementary material Table A)

Discussion

It is well documented that transcriptomes from different muscles could vary significantly and muscular dystrophies can affect different groups of skeletal muscle [22, 30]. When analyzing animal models, this raises a question of which mouse muscle more accurately represents particular (myopathic) characteristics of a given human muscle [22]. In limb–girdle muscular dystrophy type 2A, proximal muscles are the most severely affected; however, in the C3KO mouse, the soleus is one of the most affected muscles [8]. Interestingly, as previously mentioned, soleus, which is the only mouse hind limb muscle with a significant proportion of slow-twitch myofibers, showed the greatest molecular similarities with human muscles [22] that are usually composed of a mix of slow and fast fibers.

The comparison of the transcriptomes of the soleus vs the quadriceps muscles in C3KO and WT mice has been published elsewhere. In this comparison, we identified several slow phenotype-associated genes that were expressed at much lower levels in C3KO solei vs quadriceps compared with WT solei vs quadriceps [31].

Identification of differentially expressed transcripts in adult C3KO soleus compared to adult WT soleus

The p94KI mice (p94 knockin mice, p94:C129S) have a gradually progressive muscular dystrophy phenotype

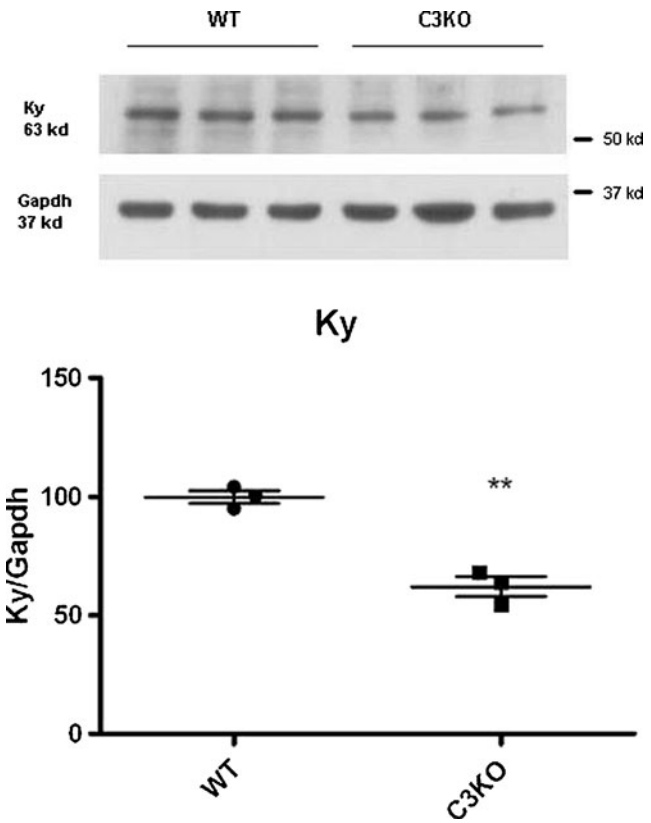

Fig. 2 Ky Western blot analysis. Ky protein level is lower in C3KO soleus of the adult mice than in the soleus of the adult WT mice (p= 0.0015). Arbitrary units

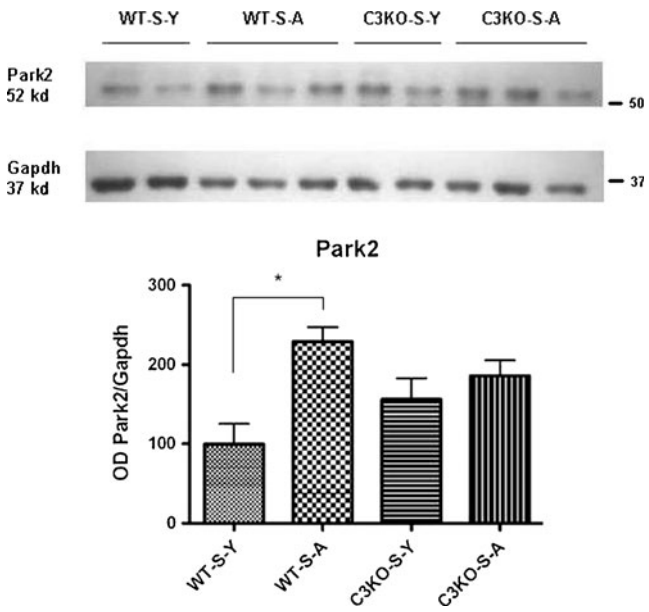

Fig. 4 Park2 Western blot analysis. Parkin protein showed statistically significant upregulation (p<0.05) in the aging soleus of the WT mice; however, in the aging soleus of the C3KO mice, insignificant changes were observed

that worsens with age without severe sarcolemmal disruption. DNA array analysis was performed for this mouse, and it was shown that mRNA levels were unchanged for the proteins affected by the proteolytic

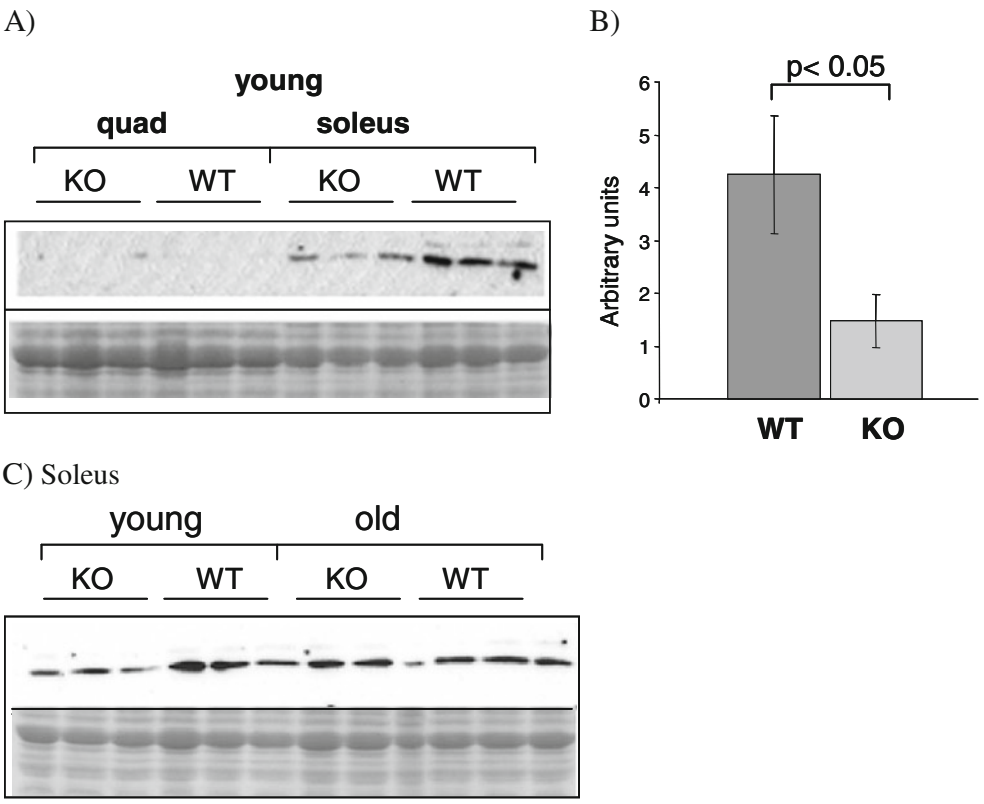

Fig. 3 Ddit4l Western blot analysis. Ddit4l was not expressed in quadriceps in all mice. Difference in the protein expression quantity between young WT and C3KO soleus is statistically significant (p< 0.05) (**a** and **b**). The y-axis on the graph shows arbitrary units of the ratio between the Western blot signal and the Ponceau red signal (protein loading) for each *lane*

activity defect, indicating that the changes were largely at the protein level [32].

The differences observed in both mice models may lie in the fact that in the KO mouse, there is a complete absence of the calpain 3 protein, whereas in the KI mouse, there is only an absence of proteolytic activity. The identification of only six differentially expressed genes may be due to the fact that we have been very restrictive with the selected fold change value (at least a twofold change value was required), and there could be genes, not included in our list, whose slight deregulation may cause important consequences for the muscle. It is also possible that most of the changes occur at the protein level.

On the other hand, metabolic differences are considered to be a significant feature of slow-twitch or type I and fast-twitch or type II muscle fibers; thus, if there is a fiber-type conversion in LGMD2A, it is expected that genes involved in energy metabolism will show a variation in their expression compared to the WT mice soleus. In this biological process, the general deregulation of these genes may confirm a conversion at the fiber-type level.

Several genes involved in muscle development are upregulated in LGMD2A patients compared to healthy controls [14]. However, we observed a downregulation of only one gene related to muscle (Ky) in C3KO mice compared to WT mice. Ky (*kyphoscoliosis*) encodes for a transglutaminase-like/protease protein [33], and a spontaneous mouse mutant in this gene has smaller muscles and slower contraction time, and is weaker than the control [34]. Several muscle-specific sarcomeric proteins, including TTN, MYBPC1, KYIP1, and FLNC, have been identified as interacting partners of KY. KY enzymatically targets FLNC in vitro, suggesting that KY may play a role in regulating FLNC in vivo. Interestingly, FLNC plays a pivotal role in the pathogenesis of several LGMD [35] and is a target for calpain-3 enzymatic activity (LGMD2A) [36, 37]. FLNC interacts with myotilin (LGMD1A) [38, 39], and γ- and δ-sarcoglycan (LGMD2C and 2F, respectively) [40–42]. FLNC is a crucial component of the Z-band [43]. Consistent with this role of FLNC, electron microscopy revealed clear myofibrillar alterations, including Z-line streaming and overlapping of thick filaments with loose A banding in dystrophic *ky* mutant soleus [35]. In C3KO mice, electron microscopy analysis also revealed abnormal A-bands suggesting a role for calpain 3 in sarcomere formation or maintenance of sarcomere alignment [8].

Both kyphoscoliosis peptidase and calpain 3 are proteases [9, 44, 35] underlying muscular dystrophies, and they both share FLNC and TTN as cytoskeletal partners. It is therefore possible that these proteins play a complementary role in regulating muscle cytoskeleton homeostasis in response to changes in muscle activity [35]. Further studies will be needed in order to establish direct interactions between calpain 3 and kyphoscoliosis peptidase.

Identification of transcripts differentially expressed in the young vs adult comparison in WT and C3KO mice

The comparison of young vs adult WT muscle transcriptomes allowed us to identify genes that are necessary for the normal muscle aging process. Interestingly, genes that should be up- or downregulated for a correct muscle maturation observed in the WT mice were not properly regulated in the adult C3KO mice (Table 2 and Supplementary material Table A).

In general terms, the young vs adult comparison showed that most of the genes in the adult WT soleus are downregulated during physiological muscle aging while most of the genes in the adult C3KO soleus are upregulated. Even though some biological functions are altered in both genotypes, the implicated genes are not the same (Table 2). For instance, in WT soleus, all differentially expressed genes involved in cell adhesion (Cd44, Ctgf, Lgals4, Postn, and Spp1) were downregulated in aged muscles. At the same time, in C3KO soleus, all the genes from this group were upregulated (Pkp2 and Npnt).

There are 11 genes whose regulation is tightly controlled in muscle maturation as shown in both WT muscle comparisons (underlined in Table 2). The up- or downregulation of these genes seems to be an essential requirement for normal muscle maturation given that it was observed in WT soleus as well as in WT quadriceps young vs adult comparison. Since these changes were not observed in any of the C3KO muscles, the lack of control of these genes may alter essential functions such as signal transduction (Vegfc, Fgl2, Rgs5), transcription (Nfil3, Nr4a2), and protein ubiquitination (Park2) that could be responsible for the impaired muscles in C3KO mice.

Ddit4l (Redd2) protein was not expressed in the quadriceps in any mice. Although the RNA expression data identified the downregulation of Ddit4l in the young vs adult comparison in the C3KO soleus, this alteration could not be confirmed at the protein level. Nevertheless, the Western blot analysis detected another interesting difference: in solei of the young mice, Ddit4l protein levels were significantly lower in C3KO compared to WT (Fig. 3a and b). Ddit4l is a protein of great interest because it diminishes mechanical stretch and inhibits basal mTOR signaling [45] whose activation is associated with muscle growth [46]. Thus, a downregulation in Ddit4l is expected to result in muscle growth. As this is only observed in young C3KO mice, it could be suggested that decreased Ddit4l represents a compensatory process that is trying to maintain muscle growth in the young C3KO muscles. As reported by Kramerova et al. [8], muscular mass is similar in C3KO and WT mice in the first 15 weeks of life, which could be related to this observation.

On the other hand, for some genes (implicated in ubiquitination, collagen fibril organization, and immune

response) whose expression was different in young vs adult WT solei, no difference was found in C3KO mice (Table 2b). One of those genes is Park2, and since it encodes a cytosolic E3 ubiquitin ligase, it would be very interesting to investigate whether it plays a role in the accumulation of ubiquitinated proteins during muscle aging. Our analysis of the parkin RNA and protein levels showed that in WT mice, there is an increase in the parkin protein amount in aged muscles. During aging, small increases in RNA expression of some components of the ubiquitin–proteasome pathway have been observed in rodent and human muscles [47, 48] suggesting a modest activation of this pathway. The difference in parkin protein levels was not observed between young and aged C3KO muscles, probably because there is already a higher level of parkin in the C3KO young mice. Therefore, it could be suggested again that a compensatory mechanism is taking place even at an early stage. Thus, in the adult C3KO mice, the obtained level would not reach a significant difference compared to the young C3KO mice because parkin level was already high (this is in agreement with the fact that no difference was observed at the RNA level between the young vs adult C3KO mice).

The absence of calpain 3 may cause accumulation of damaged proteins, and this malfunction could induce an upregulation of parkin protein as a compensatory effect initiating in the young mice. A similar mechanism has been observed in a model for Parkinson's disease induced by a mutated α-synuclein in *Drosophila*. As parkin can ubiquitinate α-synuclein, parkin can counteract the effects of α-synuclein on the premature loss of climbing activity [49].

Muscle-specific ubiquitin ligases (E3s), muscle RING-finger 1 (MuRF1), and atrogin-1/MAFbx, implicated in atrophy processes, are not deregulated in LGMD2A patients nor in C3KO mice, because they have not been identified as differentially expressed in microarray analysis performed by Saenz and colleagues [14] and in this work, respectively.

On the other hand, even though no significant changes in global protein synthesis or proteolysis were found in calpain 3-deficient mice [50], a reduction was observed in the expression of several components of proteolytic systems such as cathepsin L, the 14-kDa E2 conjugating enzyme, and the alpha-type C2 subunit of the 20S catalytic core of the proteasome. Conjugation mediated by this E2 is supposed to be a rate-limiting step in the N-end rule-mediated degradation of proteins [51, 52], suggesting that a perturbation of proteasomal degradation could occur in LGMD2A.

Even though parkin has mostly been related to Parkinson's disease, it has been reported that loss of parkin in *Drosophila* results in progressive degeneration of indirect flight muscles accompanied by apoptosis [53]. Moreover, Narendra and colleagues [54] have linked parkin to the regulation of mitochondria homeostasis. parkin translocates to depolarized mitochondria and promotes autophagy of damaged mitochondria. Suen and colleagues [55] reported that long-term overexpression of parkin can eliminate mitochondria with deleterious COXI mutations in heteroplasmic cybrid cells, thereby enriching cells for wild-type mtDNA and restoring cytochrome c oxidase activity. These data support the model that parkin is involved in selective removal of defective mitochondria within the cell and that it functions in a mitochondrial quality control pathway. Indeed, loss of parkin is associated with swollen mitochondria and muscle degeneration in *Drosophila melanogaster*, as well as mitochondrial dysfunction. Accordingly, swollen and disorganized mitochondria were observed by electron microscopy in C3KO muscles [8, 56], as well as in LGMD2A biopsies [57, 58]. Abundant and disorganized mitochondria can be visualized by light microscopy as lobulated fibers [57]. Lobulated fibers in muscles of young C3KO mice (2–3 months) were not observed, but they became apparent as the mice aged (16 months) [8].

In C3KO adult mice, our study showed that the parkin level is not as high as in the WT adult mice. Therefore, the putative compensatory mechanism suggested for the young C3KO mice could not be sufficient to maintain mitochondrial homeostasis in adulthood, justifying the appearance of mitochondrial morphological changes and loss of function of the muscle fiber. Therefore, it would be very interesting to investigate whether increased expression of Park2 in C3KO adult mice would ameliorate the disease phenotype.

In the dystrophin-deficient mdx mice, an animal model of Duchenne muscular dystrophy (DMD), damaged skeletal muscles are efficiently regenerated, and thus, the animals thrive [59] and appear to fare much better than their human counterparts. The phenotypic differences between LGMD2A patients and C3KO mice follow a similar pattern to the DMD model, suggesting that rescue mechanisms are different in humans and mice. In spite of the mild phenotype of the C3KO mice, these data suggest that Ky and Park2 modulate the muscle wasting in the mouse model.

As a summary, our data confirm the role of the ubiquitin proteasome system in the pathophysiology of the LGMD2A with some differences between the mouse model and the patients that could be due to differences in compensatory systems or alternative pathways. A better comprehension of these active alternative pathways in the mouse, apparently absent in LGMD2A patients, could offer useful information to design new therapeutic approaches.

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