

NF- κ B-dependent expression of the antiapoptotic factor c-FLIP is regulated by calpain 3, the protein involved in limb-girdle muscular dystrophy type 2A

Béatrice Benayoun,* Stephen Baghdiguian,[†] Alicia Lajmanovich,[‡] Marc Bartoli,* Nathalie Daniele,* Evelyne Gicquel,* Nathalie Bourg,* Fabrice Raynaud,[§] Marie-Anne Pasquier,[‡] Laurence Suel,* Hanns Lochmuller,^{||,1} Gérard Lefranc,^{||} and Isabelle Richard*²

*Généthon CNRS FRE 3018, Evry, France; [†]Université Montpellier 2, CNRS UMR 5554, Montpellier, France; [‡]The Research Group on Lymphoma, INSERM, EMI 353, Albert Bonniot Institute, La Tronche, France; [§]IGF, UMR 5203, Département de Neurosciences, Montpellier, France; ^{||}Friedrich-Baur-Institute and Department of Neurology, Ludwig-Maximilians-University, Munich, Germany; and ¹Laboratoire d'Immunologie Moléculaire, Institut de Génétique Humaine, CNRS UPR 1142, and Université Montpellier 2, Montpellier, France

ABSTRACT Limb-girdle muscular dystrophy type 2A (LGMD2A) is a recessive genetic disorder caused by mutations in the cysteine protease calpain 3 (CAPN3) that leads to selective muscle wasting. We previously showed that CAPN3 deficiency is associated with a profound perturbation of the NF- κ B/I κ B α survival pathway. In this study, the consequences of altered NF- κ B/I κ B α pathway were investigated using biological materials from LGMD2A patients. We first show that the antiapoptotic factor cellular-FLICE inhibitory protein (c-FLIP), which is dependent on the NF- κ B pathway in normal muscle cells, is down-regulated in LGMD2A biopsies. In muscle cells isolated from LGMD2A patients, NF- κ B is readily activated on cytokine induction as shown by an increase in its DNA binding activity. However, we observed discrepant transcriptional responses depending on the NF- κ B target genes. I κ B α is expressed following NF- κ B activation independent of the CAPN3 status, whereas expression of c-FLIP is obtained only when CAPN3 is present. These data lead us to postulate that CAPN3 intervenes in the regulation of the expression of NF- κ B-dependent survival genes to prevent apoptosis in skeletal muscle. Deregulations in the NF- κ B pathway could be part of the mechanism responsible for the muscle wasting resulting from CAPN3 deficiency.—Benayoun, B., Baghdiguian, S., Lajmanovich, A., Bartoli, M., Daniele, N., Gicquel, E., Bourg, N., Raynaud, F., Pasquier, M.-A., Suel, L., Lochmuller, H., Lefranc, G., Richard, I. NF- κ B-dependent expression of the antiapoptotic factor c-FLIP is regulated by calpain 3, the protein involved in limb-girdle muscular dystrophy type 2A. *FASEB J.* 22, 1521–1529 (2008)

Key Words: muscle • apoptosis • FLICE inhibitory protein • *lgmd2A*

LIMB-GIRDLE MUSCULAR DYSTROPHY type 2A (GMD2A) is an autosomal recessive disorder leading to atrophy

and weakness of the proximal limb muscles, especially those of posterior compartments (1). This phenotype results from defects in the human calpain 3 gene (CAPN3), which encodes a member of the calpain family of intracellular nonlysosomal cysteine proteases, predominantly expressed as a 94 kDa protein in adult skeletal muscle (2, 3). Analyses of LGMD2A patient biopsies revealed an increase in myonuclear apoptosis associated with subsarcolemmal localization of the NF- κ B p65 transcription factor and nuclear accumulation of its inhibitor I κ B α (4). Myonuclear apoptosis was confirmed by transmission electron microscopy and has been found to be associated with lobulated fibers (5).

NF- κ B is a transcription factor family involved in the inflammatory response and in cell survival (6, 7). In resting cells, NF- κ B is generally found in the cytoplasm, associated with its inhibitor, I κ B α . On stimulation, activation of the I κ B α kinase complex (IKK) leads to phosphorylation of the I κ B α molecules. Subsequent ubiquitination and degradation of this inhibitor by the proteasome liberates NF- κ B, which migrates into the nucleus and binds to target gene promoters. The transcriptional activity of NF- κ B p65 is controlled by post-translational modifications (8) and requires recruitment of cofactors or chromatin remodeling for promoter accessibility to ensure proper expression of NF- κ B-dependent genes (9).

In skeletal muscle, molecular mechanisms governing the activation of the NF- κ B pathway and its downstream targets have not been fully elucidated. Activation of this signaling pathway was demonstrated to occur in this tissue through tumor necrosis factor (TNF)- α stimula-

¹ Current address: Institute of Human Genetics, University of Newcastle upon Tyne, Newcastle, UK.

² Correspondence: Généthon CNRS FRE3018, 1, rue de l'internationale, 91000 Evry, France. Email: richard@genethon.fr
doi: 10.1096/fj.07-8701com

tion, oxidative and mechanical stresses, unloading, or exercise (10–12). The best documented role of NF- κ B is as a potent mediator of muscle atrophy. Indeed, muscle fibers of mice engineered for inactivation of the NF- κ B pathway are resistant to atrophic signals classically induced by muscle unloading and denervation (13–15). In addition, mice transgenic for a muscle-specific activated form of IKK β leading to constitutive activation of the NF- κ B target genes exhibit significant muscle wasting (16). However, only a few NF- κ B target genes have been identified to date in the skeletal muscle, including the proatrophic interleukin (IL)-6 and the ubiquitin ligase Muscle RING Finger 1 (16, 17).

Following our previous observations of the perturbation of the NF- κ B pathway in LGMD2A, we took advantage of CAPN3-deficient biopsy material to gain further insight into the regulation of this pathway in mature muscles. The present study reports that loss of CAPN3 function is associated with a down-regulation of the antiapoptotic factor, cellular-FLICE inhibitory protein (c-FLIP), in LGMD2A biopsies. We also show that c-FLIP expression is dependent on the NF- κ B pathway in muscle cells and that TNF- α and IL-1 β induce the activation of NF- κ B even in the absence of calpain 3. However, we observed different transcriptional responses depending on the target promoter: I κ B α but not c-FLIP expression is induced in CAPN3-deficient cells. These observations pinpoint CAPN3 as a potent regulator of the NF- κ B cell survival pathway in skeletal muscle.

MATERIALS AND METHODS

Biological samples and cell culture

Muscle biopsies were obtained from the patients, their parents and other relatives, and from controls. They provided informed consent in accordance with French bioethical laws or after obtaining approval from the relevant Lebanese institutional ethics committees. A30, A31, and A32 correspond to control individuals; A1 and A2 to patients homozygous for a 257C-to-T transition resulting in a Ser-86-to-Phe substitution (S86F); A10, A11, and A12 to patients homozygous for a deletion in exon 5 (717deltaT); A3 and A4 to patients who are compound heterozygotes for S86F and for a 956C-to-T transition resulting in a Pro319-to-Leu substitution (P319L); and A34 to a patient heterozygous for a 1477C-to-T transition resulting in a Arg493-to-Trp substitution (R493W) and a complex mutation in exon 22 (2362AG->TCATCT).

Normal human skeletal muscle cells (SkMC) were purchased from Cambrex Corp., (East Rutherford, NJ, USA). Human myoblast control cells (A33) and LGMD2A cells (A35, A36) were obtained from the Muscle Tissue Culture Collection (MTCC) at the Friedrich-Baur-Institute (Department of Neurology, Ludwig-Maximilians-University, Munich, Germany). The patients from whom the LGMD2A cells originated are both compound heterozygotes for mutations in the CAPN3. A35 carries: 1) a deletion of a nucleotide in exon 4 (550delA), resulting in frameshift and creation of a premature stop codon, and 2) a 1063C-to-T transition, resulting in an Arg355-to-Trp (R355W) substitution. A36 carries the same deletion (550delA) and a

2288A-to-G transition, resulting in a Tyr763-to-Cys (Y763C) substitution.

All cell lines were maintained in SkMC growth medium (Promocell, Heidelberg, Germany) until confluence was reached. Medium then was replaced by SkMC differentiation medium (Promocell) and changed every 2–3 days. After 14 days (SkMC cells) or 7 days (A33, A35, and A36 cells) in differentiation medium, when extensive syncytium development could be observed, cells were treated for 150 min with TNF- α or IL-1 β , at a final concentration of 10 ng/ml, before mRNA or protein extraction.

Luciferase reporter assay of c-FLIP promoter activity

Myogenic C2 cells were seeded in 6-well plates and transfected with 1 μ g of a pGL3-FLIP promoter luciferase reporter plasmid with an escalating dose of pSORT6-hNF- κ B (0–5 μ g), using 40 μ l FuGENE 6, according to the manufacturer's protocol (Roche, Neuilly sur Seine, France). In all experiments, a variable quantity of pcDNA3.1D/V5-His/LacZ plasmid was added to keep the amount of transfected DNA at 6 μ g. The pGL3-FLIP plasmid carries the sequence corresponding to positions –1179 to –281 relative to the proposed transcriptional start site of FLIP (kindly provided as a gift by Dr. Wafik S. El-Deiry, University of Pennsylvania, Philadelphia, PA, USA) (18). Twenty-four hours after transfection, cells were collected and luciferase activity was measured using the Luciferase assay system (Promega Corp., Madison, WI, USA) on a Victor multilabel reader (Wallac, Neuilly sur Seine, France). Results shown are triplicates from 4 separate experiments.

Measurement of caspase-3 activity

The caspase-3 protease activity of myogenic cells was evaluated using the PhiPhiLux G2D2 kit, according to the manufacturer's instructions (Onco Immunin, Inc., Gaithersburg, MD, USA). Briefly, 1×10^5 cells at the myoblast stage were incubated with 50 μ l of a 10 μ M G2D2 substrate solution supplemented with 10% fetal calf serum for 1 h at 37°C. Cells were washed once in ice-cold PBS and resuspended in 500 μ l of PBS, and caspase-3 activity was analyzed by fluorescence-activated cell-sorting flow cytometry.

Western blotting

Muscle biopsies were solubilized in lysis buffer (1% Triton X-100, 20 mM Tris-HCl pH 8, 137 mM NaCl, 10 mM EDTA, 10% glycerol) in the presence of protease inhibitors (1 mM PMSF, 100 μ M iodoacetamide, 10 μ M leupeptin) for 30 min at 4°C. Cell debris were removed by centrifugation at 10,000 *g* for 30 min at 4°C. For each sample, a volume of 30 μ l was loaded onto an 8% SDS-polyacrylamide gel, and normalization of protein amounts was carried out following immunoblotting. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Millipore, St. Quentin-Yvelines, France) by electroblotting.

Total cell lysates were prepared using the TransAM extraction buffer (Active Motif, Rixensart, Belgium). The nuclear and cytosolic proteins were extracted with the NucBuster protein extraction kit (Novagen, Madison, WI, USA), according to the manufacturer's instructions. Protein concentration was determined by the Amido Schwarz methodology (19). Proteins (25 μ g) were resolved on a precast 4–12% NuPAGE[®] Novex Bis-Tris Gel with MOPS SDS running buffer (Invitrogen, Carlsbad, CA, USA) and were transferred to nitrocellulose membranes by electroblotting.

After blocking nonspecific binding sites overnight with nonfat milk, the membranes were incubated at room temperature with primary antibodies: anti-I-FLICE monoclonal antibody (Dave-2; Alexis Biochemicals, Lüufelingen, Switzerland) or anti-skeletal fast myosin heavy-chain IIA monoclonal antibody (MHCIIA; A4.74; Alexis Biochemicals). Antibody labeling was revealed using HRP-conjugated goat anti-rat or rabbit anti-mouse secondary antibodies (Dako, Rixensart, Belgium) and was visualized using chemiluminescence (Amersham Life Science or Super Signal West Pico chemiluminescent kit, Pierce). For semiquantitative analysis of the immunoblots, subsaturated autoradiograms were scanned, and the signals were analyzed by densitometry using ImageJ (<http://rsb.info.nih.gov/ij/>). Statistically significant comparisons were treated by a Student's *t* test.

Indirect immunofluorescence confocal microscopy

Immunofluorescence was performed as described previously (20). For c-FLIP analysis, the polyclonal anti-FLIP antibody AL129 (21) was used at a concentration of 25 µg per ml in

TBS 0.2% gelatin. Mouse monoclonal antibody directed against dystrophin was used as recommended (22). Affinity-purified secondary antibodies conjugated to either fluorescein or rhodamine were obtained from Jackson ImmunoResearch Laboratory (West Grove, PA, USA). Detection of apoptosis was performed using the TUNEL method (Boehringer, Mannheim, Germany), and the number of apoptotic myonuclei used for correlation was previously published (4). The slides were viewed using a Leica TCS 4D laser confocal microscope (Leica, Mannheim, Germany).

RNA extraction and real-time quantitative RT-PCR

Total RNA from cells and muscle tissue was isolated using Trizol Reagent (Life Technologies, Inc., Mannheim, Germany) according to the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA using the SuperScript first-strand synthesis system for the RT-PCR kit (Invitrogen) and random oligonucleotides. Expression of the CAPN3 and c-FLIP gene was monitored by a real-time quantitative RT-PCR method using TaqMan probes (PerkinElmer, Wellesley, MA, USA). The

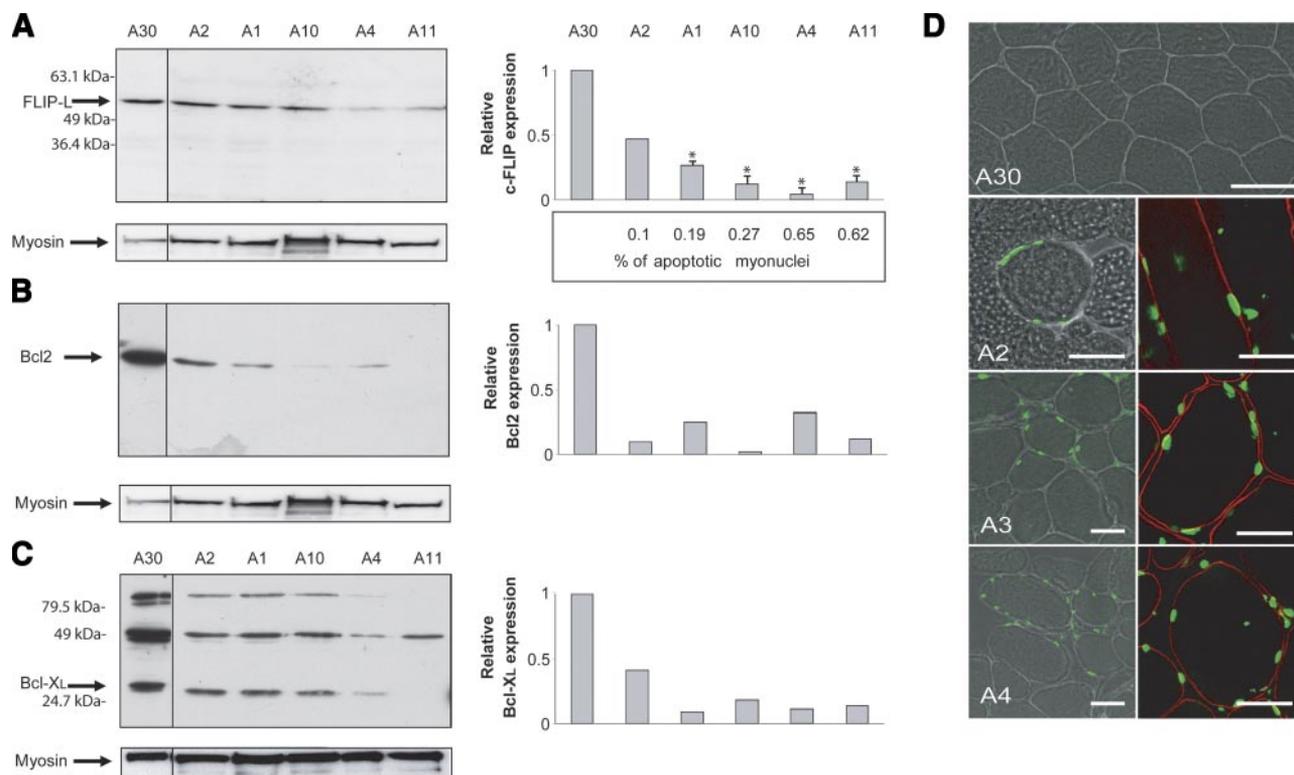


Figure 1. CAPN3 deficiency is associated with apoptosis and down-regulation of c-FLIP, *BclII*, and *Bcl-X_L* at protein level. *A*) c-FLIP expression detected by Western blot in LGMD2A muscles (lanes A2, A1, A10, A4, and A11). Lane A30 corresponds to a healthy muscle. Molecular weights are indicated on the left. The autoradiograms obtained for myosin (MHCIIA) were used as reference for protein loading. The graph represents the relative c-FLIP expression compared to myosin. The lane corresponding to A30 was used as the reference and accorded an arbitrary value of 1. The ratio for the other lanes compared to the control was determined and were reported as relative c-FLIP expression. *Significant difference compared to control; *P* < 0.05. For each patient, the corresponding percentage of apoptotic myonuclei is indicated. *B*) *BclII* expression detected by Western blot in LGMD2A muscles (lanes A2, A1, A10, A4, and A11). Lane A30 corresponds to a healthy muscle. Molecular weights are indicated on the left. The reference for protein loading was the same as the blot in *A*. *C*) *Bcl-X_L* expression detected by Western blot in LGMD2A muscles. Lane A30 corresponds to a healthy muscle. Molecular weights are indicated on the left. The autoradiogram obtained for myosin at bottom was used as reference for protein loading. *D*) Muscle sections of normal individual (A30) and 3 LGMD2A patients (A2, A3, and A4) were labeled for apoptosis by TUNEL staining (green myonuclei). Top and left pictures: TUNEL staining was superimposed over the phase-contrast field. Right pictures: TUNEL staining was associated with detection of dystrophin by indirect immunofluorescence to mark the sarcolemma and discriminate myonuclei from outer nuclei. No TUNEL positive nucleus was detected in the normal muscle whereas, in the 3 LGMD2A patients, positive nuclei can be observed in cluster in some fibers. Scale bars = 50 µM.

ubiquitous acidic ribosomal phosphoprotein (PO) was used to normalize the data across samples. PO expression was monitored by SYBRGreen incorporation. The primer pairs and Taqman probe used for CAPN3 amplification were: h269F: 5'-GCCAGAAGTTCCCCATCCA₃', h333R: 5'-TTCTG-GTTGGCTCCATCAATGATA₃', and h307P: 5'-CCGAAAA-TTTGCGAGAATCCCCG₃'. The primer pairs and Taqman probe used for c-FLIP amplification were: h1363F: 5'-AGCTTTGT-GTGTGCTCCTGGTGA₃', h1435R: 5'-GCCCTGAGTGAGTCT-GATCCA₃', and h1387P: 5'-CGAGGAGGCTCCAGAGTGTG-TATGG₃'. The primer pairs and Taqman probe used for IκBα amplification were 1171hIκB.F: 5'-GCACACTGCCTAGCCCCAA₃', 1249hIκB.R: 5'-CCTACAAAAGTTACAAAAGCAACA₃', and 1191hIκB.P: 5'-CGTCTTATTTTGTGGTAGGATCAGCCCTCA₃'. The primers pairs used for PO amplification were: h95F: 5'-GGCGACCTGGAAGTCCAAC₃' and h243R: 5'-CCATCAG-CACCACAGCCTTC₃'. Each experiment was performed in duplicate and repeated at least twice.

NF-κB activity assay

Transcription factor NF-κB activation was detected and quantified using an ELISA-based method, the TransAM NF-κB p65 kit, according to the manufacturer's instructions. Briefly, the activated NF-κB, present in nuclear fraction extracted with the NucBuster protein extraction kit (Novagen), specifically binds to oligonucleotides corresponding to NF-κB consensus binding sites and immobilized in a 96-well plate. Bound NF-κB is detected with an anti-p65 antibody. Addition of a secondary HRP-conjugated antibody provides sensitive colorimetric readout quantified by spectrophotometry at 450 nm.

RESULTS

Deficiency in CAPN3 is associated with a down-regulation of the antiapoptotic factor c-FLIP and myonuclear apoptosis in LGMD2A muscles

We previously demonstrated that muscle biopsies from LGMD2A patients present apoptotic features associated with perturbation of the IκBα/NF-κB pathway and nuclear exclusion of NF-κB (4). To analyze the consequences of this exclusion, we examined the expression level of antiapoptotic factors on deltoid biopsies of LGMD2A patients. We observed that the expression of c-FLIP, BclIII, and Bcl-X_L is reduced in LGMD2A muscle biopsies. Those proteins were previously shown to be under the control of NF-κB in nonmuscle cells (23–25), suggesting that the LGMD2A condition is associated with down-regulation of NF-κB-dependent genes that regulates apoptosis (Fig. 1). Moreover, for c-FLIP and Bcl-X_L, a direct inverted correlation between their expression level and the number of apoptotic nuclei is observed (Kendall rank correlation test, $P=0.0166$). The lower their expression, the higher the number of apoptotic nuclei (Fig. 1A, D). In this report, we focused on the analysis of the participation of c-FLIP in the pathogenesis of LGMD2A, considering that, among the different factors that are known to mediate the NF-κB antiapoptotic effect, c-FLIP was shown to participate in the resistance to apoptosis of the skeletal muscle cells (26).

The down-regulation of c-FLIP at the protein level was confirmed by immunostaining (Fig. 2A). In control

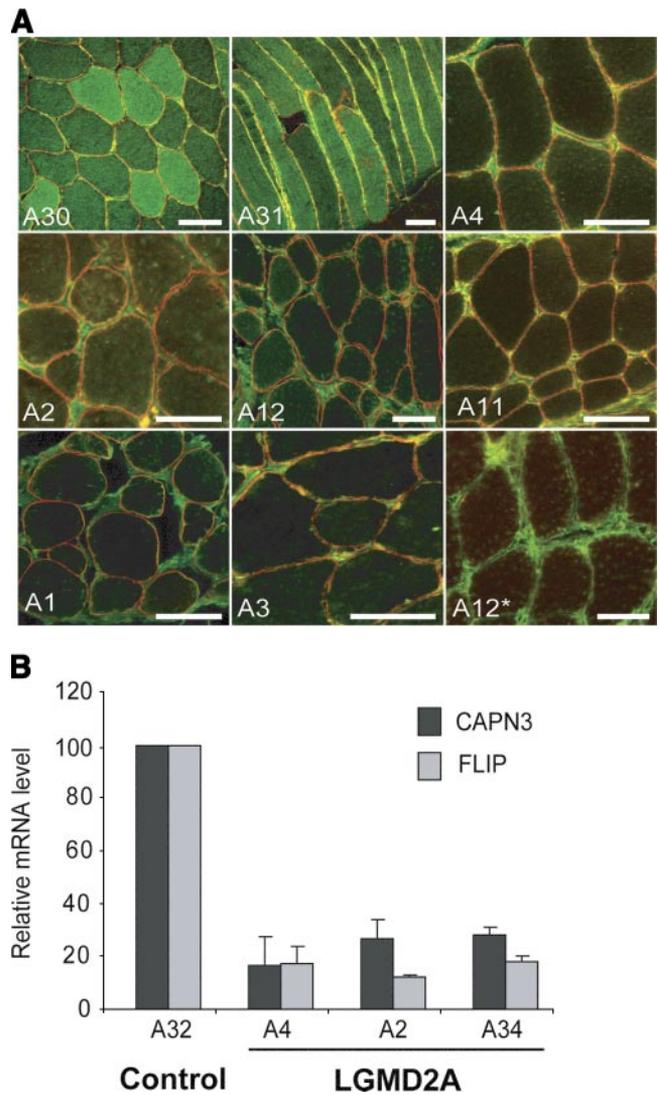


Figure 2. c-FLIP immunostaining and mRNA expression in LGMD2A muscles. A) c-FLIP (green) and dystrophin (red) double-labeling of sections from control individuals (A30 and A31) and LGMD2A patients (A4, A2, A12, A11, A1, and A3). *Nonspecific labeling was determined by incubation without c-FLIP and dystrophin primary antibodies. Variation in intensity could suggest an expression that is dependent on the muscle fiber type. Scale bars = 80 μM. B) CAPN3 and c-FLIP mRNA expression was detected by real-time quantitative RT-PCR in control (A32) and LGMD2A (A4, A2, A34) muscle biopsies. The relative mRNA level compared to control is shown. Error bars = sd.

deltoid muscle sections (A30, A31), c-FLIP labeling appears to have a cytoplasmic localization with some variation in intensity between myofibers. In contrast, in LGMD2A muscles, the staining intensity is decreased, indicating a weak expression of c-FLIP. However, as dots can still be seen, the subcellular localization of c-FLIP does not seem affected.

Finally, we measured the RNA level of c-FLIP by real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR; Fig. 2B). c-FLIP exists as both long (c-FLIP_L) and short (c-FLIP_S) isoforms (25). The Taqman probe and oligonucleotides used detect tran-

scripts of both isoforms, hence RT-PCR results represent a global profile of c-FLIP expression. The data obtained show that c-FLIP mRNA was 2- to 4-fold less abundant in LGMD2A than in control muscles (Fig. 2B). It should be noted that we performed the same experiment in affected muscle samples from CAPN3-deficient mouse model and observed a 25% down-regulation. Altogether, these data show that CAPN3 deficiency is correlated with a down-regulation of the antiapoptotic factor c-FLIP originating from a reduction of mRNA transcripts.

The NF- κ B pathway is activated by TNF- α and IL-1 β and controls c-FLIP expression in normal skeletal muscle cells

Considering the simultaneousness of NF- κ B and c-FLIP anomalies in LGMD2A, we investigated whether c-FLIP expression is under the control of NF- κ B in skeletal muscle. Several known activators of the NF- κ B pathway, such as members of the cytokine family (TNF- α , IL-1 β , IL-6, and IL-18), oxidative stress (H₂O₂), or growth factors (IGF1), were tested for induction of NF- κ B activity in differentiated primary skeletal muscle cell culture. They were added to the culture medium, and NF- κ B DNA binding activity was measured in protein extracts using an ELISA-based method. Among the cytokines and factors tested, only TNF- α and IL-1 β , the main cytokines regulating muscle protein degradation in wasting syndromes, were able to drive NF- κ B activation in myotubes under our conditions. The results presented in Fig. 3A illustrate the DNA binding activation of p65 NF- κ B transcription factor on treatment with these cytokines (Fig. 3A).

Next, we measured c-FLIP expression after activation of the NF- κ B pathway with TNF- α and IL-1 β in human

differentiated primary muscle culture cells. We showed that NF- κ B activation was correlated with an up-regulation of c-FLIP expression at the messenger and protein level as revealed by real time quantitative RT-PCR and Western blot experiments (Fig. 3B). To confirm that c-FLIP expression is under the control of NF- κ B, we treated cells with a salicylate derivate, sulfasalazine. This drug was previously shown to inhibit the phosphorylation and degradation of I κ B α , preventing the nuclear translocation of NF- κ B (27). Incubation with sulfasalazine results in a reduction of the NF- κ B activity of ~84%, as shown by TransAM analysis and a decrease of c-FLIP mRNA and protein levels (Fig. 3C).

Finally, a reporter plasmid carrying the luciferase gene under the control of the c-FLIP promoter was cotransfected with a plasmid coding for NF- κ B into myogenic C2 cells. The relative luciferase activity increased in a dose-dependent manner (Fig. 3D).

All these data suggest that expression of the antiapoptotic factor c-FLIP is NF- κ B-dependent in myogenic cells.

Deficiency of CAPN3 prevents the NF- κ B-dependent induction of c-FLIP expression

We examined the consequence of CAPN3 deficiency on the NF- κ B cascade from receptor activation to transcription of target genes. For this purpose, we used muscles cells isolated from LGMD2A patients (Fig. 4A). Of note, these CAPN3-deficient cells show a number of vacuoles and a significantly higher caspase-3 activity than muscle cells isolated from a healthy donor (Fig. 4A, B), indicative of an increase of apoptosis. This observation correlates perfectly with the phenotypic alterations observed in LGMD2A muscles (4). These cells were treated with TNF- α and IL-1 β , and NF- κ B

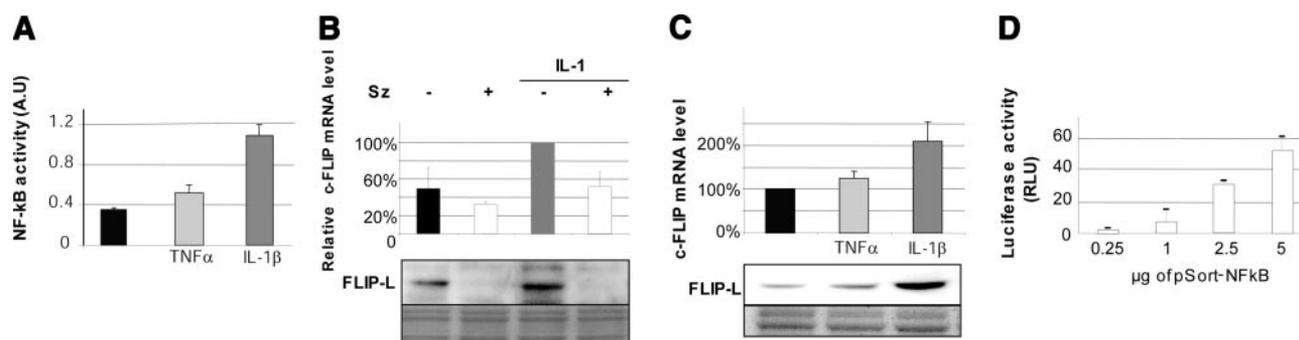


Figure 3. TNF- α and IL-1 β activate NF- κ B and induce an up-regulation of c-FLIP expression at the messenger and protein level in normal human skeletal muscle cells. *A*) Four micrograms of nuclear extracts from control, TNF- α - or IL-1 β -treated SkMC cells, was subjected to TransAM analysis that detected the DNA binding activity of NF- κ B p65. A.U. = arbitrary unit; error bars = sd. *B*) Top panel: c-FLIP mRNA expression was detected by real-time quantitative RT-PCR in control, TNF- α - or IL-1 β -treated SkMC cells. The value for the nontreated cells was considered 100%. Error bars = sd. The bottom panel represents the c-FLIP protein detected by Western blot with monoclonal antibody to c-FLIP. Images of Red Ponceau staining are shown as control for equivalent loading. *C*) The relative amount of c-FLIP was quantified in SkMC cells treated or not with sulfasalazine in the presence or absence of IL-1 β . The value for IL-1 β -treated cells was considered 100%. The corresponding amount of c-FLIP protein was shown (bottom panel). Error bars = sd. Images of Ponceau Red staining are shown as control for equivalent loading. *D*) Activity of the c-FLIP promoter in the presence of increasing amounts of pSort-NF- κ Bp65 plasmid was detected by luciferase activity on myogenic C2 cell extracts. RLU = relative luminosity unit; error bars = sd.

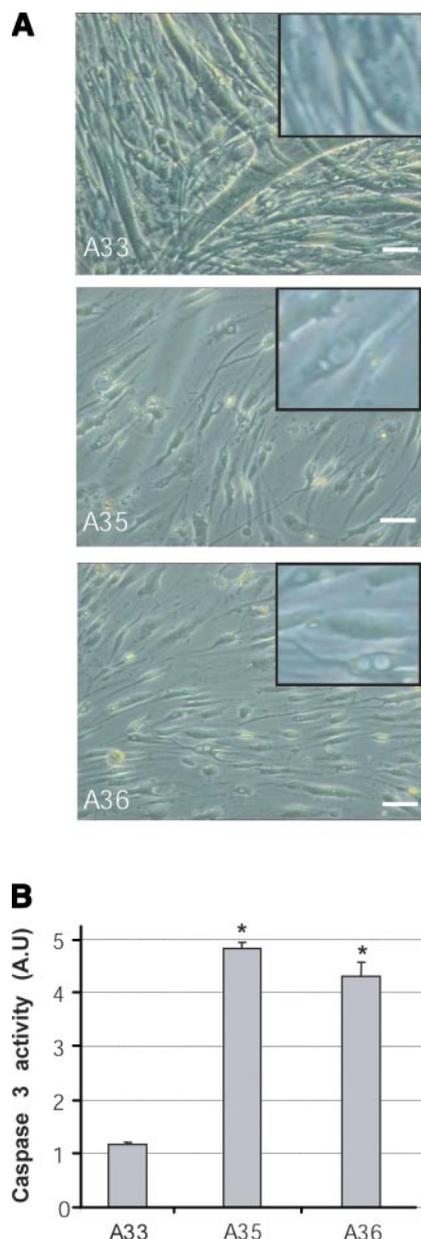


Figure 4. Apoptosis is induced in LGMD2A cells. *A*) Phase-contrast images of normal (A33) and LGMD2A cells observed after 5 days of differentiation. A lot of vacuoles are observed in the LGMD2A cells, bringing another argument toward the increase in apoptosis in those cells (insets). Scale bars = 10 μ M. *B*) Normal cells (A33) and LGMD2A cells (A35, A36) were analyzed by the PhiPhiLux methods for quantifying the frequency of cells with a high level of caspase-3 proteolytic activity, a signature of apoptosis induction. * $P < 0.05$; error bars = SD.

activation was measured. We observed that TNF- α or IL-1 β induce NF- κ B DNA binding activity in CAPN3-deficient cells, reaching levels even higher than in control cells (Fig. 5A). This activation is associated with a high increase in mRNA level of I κ B α , a NF- κ B responsive gene (Fig. 5B). However, stimulation with TNF- α or IL-1 β did not lead to an increase in c-FLIP messengers and protein in LGMD2A cells whereas it did in controls (Fig. 5C, D).

DISCUSSION

The results presented in this report confirm and further extend our previous observation of the association of CAPN3 deficiency in LGMD2A with a profound perturbation of the NF- κ B survival pathway (4, 28). Indeed, we report that NF- κ B responsive antiapoptotic proteins, c-FLIP, BclII, and Bcl-X_L are down-regulated in LGMD2A biopsies. The down-regulation of 3 NF- κ B-responsive proteins is another argument in agreement with the perturbation of the NF- κ B pathway in LGMD2A. Among these 3 factors, we were particularly interested by c-FLIP, considering that this factor was reported to participate in the resistance to apoptosis of skeletal muscle (26). In fact, c-FLIP was shown to be up-regulated in skeletal muscle under inflammatory conditions and that it contributed to resistance to cell death (26). Investigation of the BclII family members in the pathogenesis of LGMD2A would require further investigation.

Using a myogenic cell culture system, we further showed that expression of c-FLIP is under the control of NF- κ B. It should be noted that we observed a variability of c-FLIP expression between fibers, a heterogeneity that was previously reported (26). This piece of information must be viewed in light of the greater NF- κ B activation in slow- compared with fast-type muscle (29). The subcellular localization of c-FLIP in the muscle cells appears to be cytoplasmic, as it is in other cell types (30, 31). This localization corresponds to its supposed role as inhibitor of caspase 8 (32). Finally, we observed that, in the absence of CAPN3, NF- κ B activation by TNF- α or IL-1 β is maintained, and subsequent I κ B α transcription is normally achieved. However, up-regulation of c-FLIP mRNA seems to be abolished. The variability that we observed in this phenomenon may be related to either apoptosis itself or a variation in terminal differentiation, as it was reported that CAPN3-deficient cells present a delayed myofibrillogenesis (33). To conclude, it appears that NF- κ B would be able to participate in muscle cell survival through induction of antiapoptotic factors.

Importantly, the discrepancy between NF- κ B-dependent expression of I κ B α *vs.* c-FLIP suggests that only a subset of NF- κ B dependent genes is sensible to the deficiency in CAPN3. Three mechanisms can be proposed to describe how the NF- κ B pathway can be perturbed in the absence of CAPN3: 1) stabilization of c-FLIP mRNA, 2) regulation of NF- κ B nuclear shuttling, and 3) modification of NF- κ B interactions with cofactors resulting in increased transcription of c-FLIP.

1) The stability of a given mRNA transcript is often determined by the presence of specific cis-acting elements such as AU-rich regions in the 3'UTR, which are binding sites for trans-acting RNA-binding proteins to inhibit or enhance mRNA decay (34). Examination of c-FLIP mRNA shows, however, that it does not carry this type of sequence. In addition, in recent years, numerous studies on noncoding RNA have highlighted their importance in regulating the fate of transcribed RNA

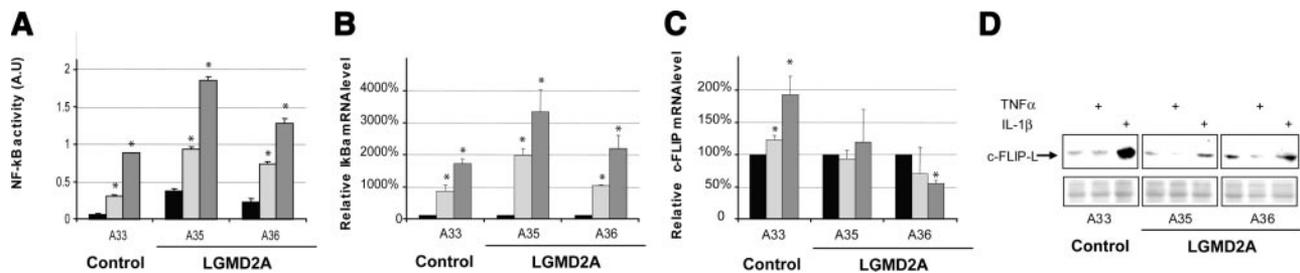


Figure 5. TNF- α and IL-1 β activate NF- κ B in LGMD2A cells, which promotes expression of I κ B α but not c-FLIP. *A*) Four micrograms of total extracts from control, TNF- α , or IL-1 β -treated normal (A33) or LGMD2A (A35, A36), cells were subjected to TransAM NF- κ B p65 analysis. Black boxes = no treatment; light gray = TNF- α treatment; dark gray = IL-1 β treatment; * P < 0.05; error bars = sd; A.U. = arbitrary unit. *B*) Relative I κ B α mRNA expression detected by real-time quantitative RT-PCR in control, TNF- α , or IL-1 β -treated normal (A33) or LGMD2A (A35, A36) cells (100% = nontreated cells). * P < 0.05; error bars = sd. *C*) Relative c-FLIP mRNA expression detected by real-time quantitative RT-PCR in control, TNF- α , or IL-1 β -treated normal (A33) or LGMD2A (A35, A36) cells (100% = nontreated cells). * P < 0.05; error bars = sd. *D*) c-FLIP expression by Western blot. Images of Ponceau Red staining are shown as control for equivalent loading.

molecules, adding another level of complexity in the control of gene expression (35, 36). Whether and how CAPN3 could intervene in these regulations remain to be investigated.

2) The first event in NF- κ B activation is its translocation from the cytoplasm to the nucleus on phosphorylation and degradation of its inhibitor I κ B α . Our results show that NF- κ B is able to shuttle correctly in the nucleus in the absence of CAPN3, revealed by the increased DNA-binding activity present in this compartment in LGMD2A cells. This result is an important new insight on the pathological mechanism that extends our previous observations. In LGMD2A muscle fibers, NF- κ B was found to be excluded from a subset of nuclei, thus raising the hypothesis that the CAPN3 deficiency would impair the nuclear shuttling of NF- κ B (4). In fact, in most cases, an overlap of NF- κ B staining with I κ B α -positive nucleus is observed in LGMD2A biopsies (4). Our results explained also why a high level of the I κ B α protein is present in the LGMD2A biopsies (4), an event that would necessitate the presence of NF- κ B into the nucleus. The coexisting presence of NF- κ B-negative and -positive nucleus in the biopsies might reflect the complexity of physiological context

compared to the artificial cytokine stimulation of cultured cells and the importance of timing in NF- κ B activation.

3) A large body of work has shown that transcription of NF- κ B target genes is dependent on several mechanisms, including post-translational modification of NF- κ B and interaction with other transcription factors, coactivators, or corepressors (37–40). We propose that limited proteolytic processing of NF- κ B regulators by CAPN3 could be a potent mechanism in determining the panel of genes transcribed in response to individual NF- κ B stimuli. Of particular interest, it has been shown that NF- κ B activation induces I κ B α , which in turn generates a negative feedback on the NF- κ B pathway and modulates its activity temporally (9). This process, which is dependent on the duration of the input signal promoting NF- κ B activation, leads to the induction of different sets of NF- κ B target genes into the nucleus. In this context, it is conceivable that CAPN3, through proteolysis of I κ B α , as previously proposed (4, 41), could generate subtle variations of NF- κ B activation and ultimately promote the expression of specific NF- κ B target genes. In addition, susceptibility to calpain proteolytic cleavage has been demonstrated for

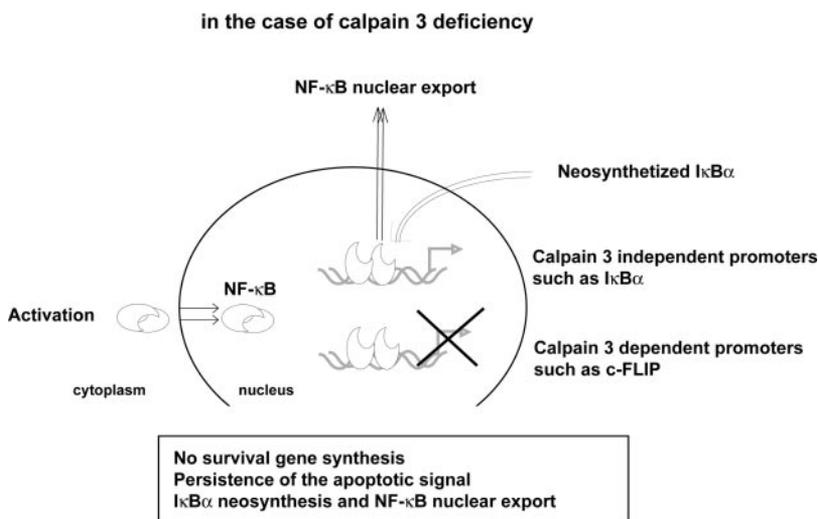


Figure 6. Hypothetical model for CAPN3 action on NF- κ B-dependent transcription of survival genes. The proposed sequence of events in the NF- κ B response in the absence of CAPN3 is as follows: activation of NF- κ B pathway leading to its nuclear translocation, induction of I κ B α transcription and other early response genes, absence of expression of antiapoptotic genes CAPN3-dependent such as c-FLIP, and persistence of the apoptotic signal and therefore of NF- κ B activation, leading to persistent synthesis of new I κ B α molecules (neosynthesis) that enter the nucleus to control the nuclear export of NF- κ B.

some NF- κ B cofactors, such as members of the signal transducers and activators of transcription (STAT) family STAT 3 and STAT5 (42), activating protein (AP-1), and CCAAT/enhancer binding protein family members (43, 44). Hypothetically, some of these cofactors could be cleaved by CAPN3 to modulate antiapoptotic signals mediated by NF- κ B in skeletal muscle.

In LGMD2A, a possible pathological mechanism following the deficiency in CAPN3 is an impairment of the antiapoptotic response of muscle, at least partly, because of insufficient levels of c-FLIP mRNA. The sequence of events could be as follows: NF- κ B nuclear translocation, induction of transcription of I κ B α and other early response genes, absence of expression of some antiapoptotic genes requiring the presence of CAPN3, and persistence of the apoptotic signal and therefore of NF- κ B activation, leading to a cycle of I κ B α over-expression and NF- κ B nuclear export *in vivo* (Fig. 6). Our results suggest a molecular mechanism, which at least partly explains the muscular wasting resulting from CAPN3 deficiency. A better understanding of NF- κ B pathway regulation by CAPN3 in skeletal muscle could therefore open the way to therapeutic strategies for LGMD2A. **[F]**

We acknowledge Dr. D. Stockholm for help in Taqman quantitative PCR, Dr. J. Tschopp for providing AL129 polyclonal antibody, and Dr. F. Willems for help in Western blot analysis. We thank Drs. F. Rousset and A. Sahuquet for imaging analysis as well as Dr. S. Cure for critical reading of the manuscript. MTCC is part of the German network on muscular dystrophies (MD-NET, service structure S1, 01GM0601) funded by the German Ministry of Education and Research (BMBF, Bonn, Germany). The Muscle Tissue Culture Collection is a partner of Eurobiobank (www.eurobiobank.org). This work was supported by grants from the Association Française contre les Myopathies to I.R. and S.B. The authors declare that they have no conflicts of interest.

REFERENCES

- Fardeau, M., Eymard, B., Mignard, C., Tome, F. M., Richard, I., and Beckmann, J. S. (1996) Chromosome 15-linked limb-girdle muscular dystrophy: clinical phenotypes in Reunion Island and French metropolitan communities. *Neuromuscul. Disord.* **6**, 447–453
- Richard, I., Broux, O., Allamand, V., Fougerousse, F., Chianilkulchai, N., Bourg, N., Brenguier, L., Devaud, C., Pasturaud, P., Roudaut, C., Hilaire, D., Passos-Bueno, M., Zatz, M., Tishfield, J., Fardeau, M., Jackson, C., Cohen, D., and Beckmann, J. (1995) Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. *Cell* **81**, 27–40
- Sorimachi, H., Imajoh-Ohmi, S., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y., and Suzuki, K. (1989) Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- and mu-types. Specific expression of the mRNA in skeletal muscle. *J. Biol. Chem.* **264**, 20106–20111
- Baghdiguian, S., Martin, M., Richard, I., Pons, F., Astier, C., Bourg, N., Hay, R. T., Chemaly, R., Halaby, G., Loiselet, J., Anderson, L. V., Lopez de Munain, A., Fardeau, M., Mangeat, P., Beckmann, J. S., and Lefranc, G. (1999) Calpain 3 deficiency is associated with myonuclear apoptosis and profound perturbation of the I κ B α /NF- κ B pathway in limb-girdle muscular dystrophy type 2A. *Nat. Med.* **5**, 503–511
- Chae, J., Minami, N., Jin, Y., Nakagawa, M., Murayama, K., Igarashi, F., and Nonaka, I. (2001) Calpain 3 gene mutations: genetic and clinico-pathologic findings in limb-girdle muscular dystrophy. *Neuromuscul. Disord.* **11**, 547–555
- Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996) Suppression of TNF- α -induced apoptosis by NF- κ B. *Science* **274**, 787–789
- Ghosh, S., May, M. J., and Kopp, E. B. (1998) NF- κ B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16**, 225–260
- Vermeulen, L., De Wilde, G., Notebaert, S., Vanden Berghe, W., and Haegeman, G. (2002) Regulation of the transcriptional activity of the nuclear factor- κ B p65 subunit. *Biochem. Pharmacol.* **64**, 963–970
- Hoffmann, A., Levchenko, A., Scott, M. L., and Baltimore, D. (2002) The I κ B-NF- κ B signaling module: temporal control and selective gene activation. *Science* **298**, 1241–1245
- Baldwin, A. S., Jr. (1996) The NF- κ B and I κ B proteins: new discoveries and insights. *Annu. Rev. Immunol.* **14**, 649–683
- Kumar, A., and Boriek, A. M. (2003) Mechanical stress activates the nuclear factor- κ B pathway in skeletal muscle fibers: a possible role in Duchenne muscular dystrophy. *FASEB J.* **17**, 386–396
- Ho, R. C., Hirshman, M. F., Li, Y., Cai, D., Farmer, J. R., Aschenbach, W. G., Witzack, C. A., Shoelson, S. E., and Goodyear, L. J. (2005) Regulation of I κ B kinase and NF- κ B in contracting adult rat skeletal muscle. *Am. J. Physiol.* **289**, C794–801
- Mourkioti, F., Kratsios, P., Luedde, T., Song, Y. H., Delafontaine, P., Adami, R., Parente, V., Bottinelli, R., Pasparakis, M., and Rosenthal, N. (2006) Targeted ablation of IKK2 improves skeletal muscle strength, maintains mass, and promotes regeneration. *J. Clin. Invest.* **116**, 2945–2954
- Hunter, R. B., and Kandarian, S. C. (2004) Disruption of either the Nfkb1 or the Bcl3 gene inhibits skeletal muscle atrophy. *J. Clin. Invest.* **114**, 1504–1511
- Judge, A. R., Koncarevic, A., Hunter, R. B., Liou, H. C., Jackman, R. W., and Kandarian, S. C. (2007) Role for I κ B α , but not c-Rel, in skeletal muscle atrophy. *Am. J. Physiol.* **292**, C372–382
- Cai, D., Frantz, J. D., Tawa, N. E., Jr., Melendez, P. A., Oh, B. C., Lidov, H. G., Hasselgren, P. O., Frontera, W. R., Lee, J., Glass, D. J., and Shoelson, S. E. (2004) IKK β /NF- κ B activation causes severe muscle wasting in mice. *Cell* **119**, 285–298
- Luo, G., Hershko, D. D., Robb, B. W., Wray, C. J., and Hasselgren, P. O. (2003) IL-1 β stimulates IL-6 production in cultured skeletal muscle cells through activation of MAP kinase signaling pathway and NF- κ B. *Am. J. Physiol.* **284**, R1249–1254
- Ricci, M. S., Jin, Z., Dews, M., Yu, D., Thomas-Tikhonenko, A., Dicker, D. T., and El-Deiry, W. S. (2004) Direct repression of FLIP expression by c-myc is a major determinant of TRAIL sensitivity. *Mol. Cell. Biol.* **24**, 8541–8555
- Schaffner, W., and Weissmann, C. (1973) A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* **56**, 502–514
- Martin, M., Roy, C., Montcourrier, P., Sahuquet, A., and Mangeat, P. (1997) Three determinants in ezrin are responsible for cell extension activity. *Mol. Biol. Cell* **8**, 1543–1557
- Bullani, R. R., Huard, B., Viard-Leveugle, I., Byers, H. R., Irmeler, M., Saurat, J. H., Tschopp, J., and French, L. E. (2001) Selective expression of FLIP in malignant melanocytic skin lesions. *J. Invest. Dermatol.* **117**, 360–364
- Pons, F., Nicholson, L. V., Robert, A., Voit, T., and Leger, J. J. (1993) Dystrophin and dystrophin-related protein (utrophin) distribution in normal and dystrophin-deficient skeletal muscles. *Neuromuscul. Disord.* **3**, 507–514
- Mitsiades, C. S., Mitsiades, N., Poulaki, V., Schlossman, R., Akiyama, M., Chauhan, D., Hideshima, T., Treon, S. P., Munshi, N. C., Richardson, P. G., and Anderson, K. C. (2002) Activation of NF- κ B and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. *Oncogene* **21**, 5673–5683
- Turco, M. C., Romano, M. F., Petrella, A., Bisogni, R., Tassone, P., and Venuta, S. (2004) NF- κ B/Rel-mediated regulation

- of apoptosis in hematologic malignancies and normal hematopoietic progenitors. *Leukemia* **18**, 11–17
25. Irmeler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E., and Tschopp, J. (1997) Inhibition of death receptor signals by cellular FLIP. *Nature* **388**, 190–195
 26. Nagaraju, K., Casciola-Rosen, L., Rosen, A., Thompson, C., Loeffler, L., Parker, T., Danning, C., Rochon, P. J., Gillespie, J., and Plotz, P. (2000) The inhibition of apoptosis in myositis and in normal muscle cells. *J. Immunol.* **164**, 5459–5465
 27. Wahl, C., Liptay, S., Adler, G., and Schmid, R. M. (1998) Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. *J. Clin. Invest.* **101**, 1163–1174
 28. Baghdiguian, S., Richard, I., Martin, M., Coopman, P., Beckmann, J. S., Mangeat, P., and Lefranc, G. (2001) Pathophysiology of limb girdle muscular dystrophy type 2A: hypothesis and new insights into the IkappaBalpha/NF-kappaB survival pathway in skeletal muscle. *J. Mol. Med.* **79**, 254–261
 29. Phillips, T., and Leeuwenburgh, C. (2005) Muscle fiber specific apoptosis and TNF-alpha signaling in sarcopenia are attenuated by life-long calorie restriction. *FASEB J.* **19**, 668–670
 30. Zhou, X. D., Yu, J. P., Liu, J., Luo, H. S., Chen, H. X., and Yu, H. G. (2004) Overexpression of cellular FLICE-inhibitory protein (FLIP) in gastric adenocarcinoma. *Clin. Sci. (Lond.)* **106**, 397–405
 31. Valnet-Rabier, M. B., Challier, B., Thiebault, S., Angonin, R., Margueritte, G., Mouglin, C., Kantelip, B., Deconinck, E., Cahn, J. Y., and Fest, T. (2005) c-Flip protein expression in Burkitt's lymphomas is associated with a poor clinical outcome. *Br. J. Haematol.* **128**, 767–773
 32. Kataoka, T. (2005) The caspase-8 modulator c-FLIP. *Crit. Rev. Immunol.* **25**, 31–58
 33. Kramerova, I., Kudryashova, E., Tidball, J. G., and Spencer, M. J. (2004) Null mutation of calpain 3 (p94) in mice causes abnormal sarcomere formation in vivo and in vitro. *Hum. Mol. Genet.* **13**, 1373–1388
 34. Guhaniyogi, J., and Brewer, G. (2001) Regulation of mRNA stability in mammalian cells. *Gene* **265**, 11–23
 35. Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297
 36. Jovanovic, M., and Hengartner, M. O. (2006) miRNAs and apoptosis: RNAs to die for. *Oncogene* **25**, 6176–6187
 37. Hoffmann, A., Natoli, G., and Ghosh, G. (2006) Transcriptional regulation via the NF-kappaB signaling module. *Oncogene* **25**, 6706–6716
 38. Dutta, J., Fan, Y., Gupta, N., Fan, G., and Gelinas, C. (2006) Current insights into the regulation of programmed cell death by NF-kappaB. *Oncogene* **25**, 6800–6816
 39. Campbell, K. J., Witty, J. M., Rocha, S., and Perkins, N. D. (2006) Cisplatin mimics ARF tumor suppressor regulation of RelA (p65) nuclear factor-kappaB transactivation. *Cancer Res.* **66**, 929–935
 40. Ogawa, S., Lozach, J., Benner, C., Pascual, G., Tangirala, R. K., Westin, S., Hoffmann, A., Subramaniam, S., David, M., Rosenfeld, M. G., and Glass, C. K. (2005) Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. *Cell* **122**, 707–721
 41. Marcilhac, A., Raynaud, F., Clerc, I., and Benyamin, Y. (2006) Detection and localization of calpain 3-like protease in a neuronal cell line: possible regulation of apoptotic cell death through degradation of nuclear IkappaBalpha. *Int. J. Biochem. Cell Biol.* **38**, 2128–2140
 42. Oda, A., Wakao, H., and Fujita, H. (2002) Calpain is a signal transducer and activator of transcription (STAT) 3 and STAT5 protease. *Blood* **99**, 1850–1852
 43. Hirai, S., Kawasaki, H., Yaniv, M., and Suzuki, K. (1991) Degradation of transcription factors, c-Jun and c-Fos, by calpain. *FEBS Lett.* **287**, 57–61
 44. Welm, A. L., Timchenko, N. A., Ono, Y., Sorimachi, H., Radomska, H. S., Tenen, D. G., Lekstrom-Himes, J., and Darlington, G. J. (2002) C/EBPalpha is required for proteolytic cleavage of cyclin A by calpain 3 in myeloid precursor cells. *J. Biol. Chem.* **277**, 33848–33856

*Received for publication April 10, 2007.
Accepted for publication November 8, 2007.*