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HUMAN MYOTONIC DYSTROPHIES: PROTEOME PROFILING AND DIFFERENTIATION STUDIES

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INDEX

1. ABSTRACT	5
2. INTRODUCTION	
2.1 Mutations	6
2.2 Symptoms	7
2.3 Molecular mechanism	10
2.4 Transgenic mice	12
2.5 Atrophy correlates with augmented protein degradation or decreased protein synthesis	14
2.6 Mitochondrial involvement in Atrophic conditions	21
3. MATERIAL AND METHODS	
3.1 Patients and primary cultures	25
3.2 Subcellular fractionation	25
3.3 Two dimensional gel electrophoresis (2D-GE)	26
3.4 Protein identification by Matrix Assisted laser desorption/ionization Time of Flight	26
3.5 Immunoblot analysis	27
3.6 FISH	27
3.7 Reagent and antibodies	28
3.8 JC-1	28
4. RESULTS	
4.1 Proteomic analysis	29

4.2 Result clusterization	32
4.3 Western blotting confirmations	
33	
4.4 Functional aspects	35
4.5 Evaluation of cultured fibroblasts as a cellular model for the mitochondrial and degradative issues of the pathology	
37	
5. DISCUSSION	42
6. BIBLIOGRAPHY	46
1 ^b . ABSTRACT	50
2 ^b . INTRODUCTION	
2.1 Differentiation overview	
51	
2.2 DM1 differentiative issues and role for CUGBP1	53
2.3 GSK3beta implications in the Cyclin D3-CUGBP1-eIF2 pathway	54
3 ^b . MATERIALS AND METHODS	
3.1 Cell culture	56
3.2 Subcellular fractionation	
56	
3.3 HPLC	56
3.4 Immunoprecipitation	56
3.5 Western blotting analysis	
57	
3.6 RT-PCR	57
4 ^b . RESULTS	

4.1 Expression of the CTG amplification in HeLa cells affects the expression level of the differentiation complex Cyclin D3/pRb/Cdk4 core components	58
4.2 The interaction between pRb and Cyclin D3 is undetectable in immortalized HeLa cells as well as in double stable clones expressing CUG-914	62
4.3 In vivo evidencing of the Cyclin D3/pRb complex formation	64
4.4 Overexpression of GSK3 beta in the CTG-250 mouse model	65
5 ^b . DISCUSSION	69
6 ^b . BIBLIOGRAPHY	74
APPENDIX, Neurobiology of Disease Manuscript	76

1. ABSTRACT

Proteome profile in Myotonic Dystrophy type 2 myotubes reveals dysfunction in protein processing and mitochondrial pathways.

Myotonic Dystrophy type 2 (DM2) is caused by a DNA microsatellite expansion within the ZNF9 gene leading to an abnormal splicing pattern largely responsible for the pathological condition. To better define the functional changes occurring in human DM2 myotubes, we performed a quantitative proteome comparison between myotubes of DM2 and control patients using two-dimensional gel electrophoresis followed by mass spectrometry. Our results indicate that the proteins, altered in DM2 cultures, belong to two major functional categories: i) mitochondrial components, with a reduction of Elongation factor Tu (EFTu), Heat Shock Protein 60 (HSP60), Glucose Regulated Protein 75 (GRP75) and Dienoyl-CoA-Isomerase, an enzyme involved in fatty acids degradation; ii) the ubiquitin proteasome system, with increase of the 26S proteasome regulatory subunit 13 and a reduction of Proteasome subunit Apha 6 and of Rad23B homolog. Altered ubiquitin-proteasomal activity is supported by a global reduction of cytosolic ubiquitinated proteins, nonetheless the accumulation of ubiquitin-protein conjugates after proteasomal inhibitor MG-132 treatment is maintained in DM2 and control cells, suggesting a higher degradation rate for the proteasome in myoblasts from patients affected by the disease. Although future work is required to clarify how these changes affect the protein degradation machinery and mitochondrial function and to evaluate if these changes also occur in the biopsies of DM2 patients, these results identify the mitochondrial proteins and the ubiquitin-proteasomal system as candidates potentially relevant to DM2 pathogenesis. Further analysis performed in Human skin fibroblasts primary cultures, obtained from patients biopsies, revealed an Hyperpolarization of the mitochondrial membrane potential involving DM2 cells, indicating a putative functional issue for mitochondria. As the evaluation of the Cytochrome *c* release following hydrogen peroxide treatment showed a differential response to this stress inducing compound, we pointed out a functional involvement of the DM2 mitochondrial-mediated cellular response to oxidative stress.

Keywords:

Myotonic Dystrophy; Proteomics; Myotubes, Mitochondria, Ubiquitin-Proteasome System, Ubiquitination, Protein Folding, Protein Disulfide Isomerase, Chaperone Proteins.

2. INTRODUCTION

2.1 Mutations

Myotonic Dystrophy (DM) is the most common form of adult muscular dystrophy, (Day and Ranum 2005) it is inherited as an autosomic dominant disease. Up to now we recognize two different forms of this complex multisystemic neuromuscular disorder: Myotonic Dystrophy type 1 (DM1) and type 2 (DM2). Both forms can be referred to as "Repeats Amplification Diseases" since the genetic mutation underlying these pathologies consist on a microsatellite amplification and in particular a CTG amplification for the DM1 and a CCTG amplification for the DM2. Normally microsatellite regions map at the level of non-coding regions of the DNA: for the DM pathology the CTG and CCTG amplification have been found in the 3'UTR of the Myotonic Dystrophy Protein Kinase (DMPK) gene, (Brook et al. 1992) on chromosome 19, and at the level of the first intron of the Zinc Finger 9 (Znf9) gene, on chromosome 3, (Liquori et al. 2001) respectively. The genetic mutations causing these two pathologies were discovered recently. In 1992 the CTG amplification has been identified as the cause of DM1, whose symptoms and implications were known since a long time. In particular Hans Steinert described the clinical features of DM1, also called Steinert disease, in 1909. In 2001 the genetic mutation underlying DM2 was recognized in the CCTG repeats amplification. Since DM2 was only recently discovered, this form has been less frequently diagnosed than DM1 and its different manifestations are still object of research. In general, DM2 tends to be less severe than DM1, with fewer systems affected and symptoms appearing later in life. For what concerns the DM1, we already defined the DMPK gene as the CTG amplification host. The mutation lies in the 3'UTR, a non-coding region of DMPK mRNA, defining its stability. The long mutated DMPK mRNA is retained into the nucleus. Thus it cannot be translated and the gene product is undetectable in the cell. Anyway, the function of the DMPK gene product is currently unknown and up to now no correlations were established between the absence of this kinase in patient's cells, and the pathogenesis of the DM1. The case of DM2 is different: the scientific community is actually divided into two currents of thought. In 2006 Margolis et al., proposed a substantial independence between the mutation and the expression of Znf9. They measured the levels of this protein and found them unaltered. Their suggestion was that since the mutation lies in the first intron of the Znf9 gene, it does not affect the gene expression, reasoning on the hypothesis of an unaltered splicing process. In 2009, Huichalaf et al., proposed a different view after demonstrating the presence of lower levels of Znf9 protein in the cytoplasm of DM2 cells. Recently a role in CAP dependent and independent translation

promotion was attributed to this RNA binding protein, a small polypeptide of 19 KDa, characterized by seven Zinc Fingers domains^(Finisterer et al.2002). It binds to a particular motif that can be found at the level of the 5'UTR of specific mRNAs, and it is called Terminal Oligopyrimidine (TOP). The group of Dr. Timchenko evidenced a reduced capability of Znf9 to bind the 5'UTR of important mRNAs encoding the Human Ribosomal Protein 17 (RPS17), the PolyA-binding protein 1 (PABP1) and two elongation factors (eEF1A and eEF2), and in turn promoting their translation. These findings shed some light on the reduced level of these proteins of the translation machinery, further suggesting that the reduced protein synthesis in myoblasts from DM2 patients, could return to the physiological translational rate by exogenous Znf9.

2.2 Symptoms

Myotonic Dystrophy type 1 is the most diffuse neuromuscular disease in adult, with the incidence of 1/8000 in the whole world^(Cho & Tapscott. 2006). 5 to 38 CTG repeats constitute the physiologic length of the microsatellite region in the 3'UTR of DMPK gene while amplified repeats ranging from 50 to 1500 are encountered in the pathologic DM1 condition^(Ranum & Cooper 2006). Depending on the importance of the CTG triplet amplification patients develop a precocious or delayed Muscular Dystrophy, and a severe or milder pathology, in other words a positive correlation exists between the amplification length and both the age of onset, and the gravity of symptoms. The case of DM2 is different: if on the one hand the incidence seems to be slightly lower, it is plausible an underrated determination of the number of patients. Often, the pathologic symptoms of the milder cases turn out to be disregarded, particularly in poor countries^(Liquori et al. 2001). The CCTG tetraplet is considered as normal till 75 repeats, overcoming this threshold means pathogenic condition. Patients with 11000 CCTG tetraplet repeats have been diagnosed. The symptoms of these two Human Pathologies are very similar to each other although it is important to define the major severity of the DM1 with respect to the DM2. In both forms patients develop Cataracts and display Heart Conduction System impairment (Conduction Defects, Heart Trouble, Cardiomyopathy, Arrhythmias), Insulin Resistance, Atrophy and Muscle Weakness^(Meola and Moxley, 2004). Myotonia characterizes both forms of DM as well, and consists in the persistence of the muscle contraction even after voluntary stimuli are over. In other words, muscles are not only weaker, but are hardly released once contracted, and a patient must toil whenever he needs to drop an object he got hold of.

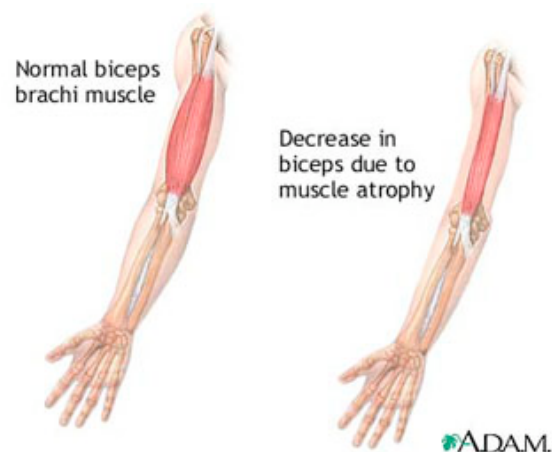


Figure. 1 (<http://nicka1.wordpress.com/2008/09/09/muscle-atrophy/>) This figure highlights the overall effect of the number and diameter reduction of muscle fibers during the atrophic degeneration.

The Myotonic Phenomenon is triggered by an expression alteration of a Chloride Channel (CLCN1), and a consequent reduced presence of it at the level of the sarcomembrane. Moreover this channel displays a slightly reduced Chloride permeability^(Lueck et al. 2007). The simple evidence that DM1 and DM2 are caused by different nucleotide amplification, lying on different loci, suggests a common molecular aetiology, nonetheless differences in the symptomatology has been observed. In the DM1 the muscular system is affected mainly at the level of distal districts (forearm, hand, leg, foot) and the facial muscular system, even if an involvement of the whole system is characterized by generalized weakness. In the DM2 the muscular involvement is mainly proximal (shoulder, arm, pelvis, thigh), and anyway, the pathology is less severe compared to DM1, in particular for the Heart affections^(Day et al. 2003). Only in DM1 patients cerebral alterations, leading to mental retardation in particular in the congenital form of the pathology, were observed.

At the ultrastructural level, similarities and differences can be appreciated: both pathologies display centralized nuclei at the level of the muscle fibers, small myofibers, and a reduced diameter of the muscular cells sections, which witnesses for muscular atrophy^(Rotondo et al. 2005). But also significant differences can be pointed out, one of the most important being an involvement of the type 1 muscular fibers on a atrophic point of view for DM1, while the atrophic phenomenon mainly affecting DM2 patients has its origins at the level of type 2 muscular fibers. Slow fibers or type 1 muscular fibers also referred to as red fibers, are capable of slow but protracted contractions compared to fast fibers, and rely upon the aerobic metabolism, that is the use of the mitochondrial produced ATP.

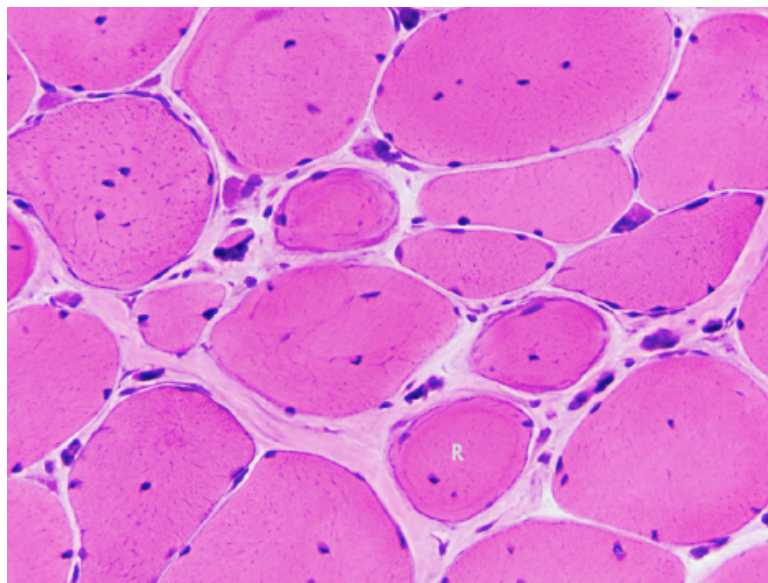


Figure. 2 (www.neuropathologyweb.org/chapter13/chapter13cDystrophy.html) One of the fundamental characteristics of the DM patient's muscle fibers are the centralized nuclei which normally can be found only at the periphery of muscle cells. The letter R, in white, marks a reduced-diameter fiber.

Fast fibers can contract faster, but for a limited interval of time, and they are also referred to as white, type two fibers. They mainly use the anaerobic metabolism relying upon the glycolysis-produced ATP. It is worth noting that the CTG and the CCTG repeats amplifications (respectively in DM1 and DM2) are present on both muscular fiber kinds^(Bassez et al. 2008). To conclude the symptoms dissertation we wish to spend some words about the "Anticipation phenomenon": children of DM1 patients tend to develop the pathology earlier and in more severe form compared to their parents, this is due to the genetic instability of the CTG repeats amplification, and the number of repeats progressively grows in the succeeding generations. After some generations DM1 patients can develop the most severe form of the DM1, the so called Congenital Myotonic Dystrophy, associated to an extremely long CTG amplification. Differently from DM1, the CCTG repeats amplification underlying the DM2, tends to grow during the life span of the patient. By analyzing the blood samples, it was possible to establish a correlation between the age of the patient and the number of CCTG repeats. This phenomenon was named Tetraplet Expansion Instability^(Day et al. 2003). A further proof of the tetraplet instability came from the positive correlation between the amplification length and the age of onset of the DM2 pathology.

2.3 Molecular Mechanism

The pathogenetic hypothesis is based on a mechanism called “Toxic Gain of Function Model”. This model is based on the accumulation of the pre-mRNAs carrying the CTG (DMPK RNA) and CCTG amplifications (first intron spliced of Znf9), raising the formation of ribonuclear amplifications called “Foci” (Mankodi et al. 2003).

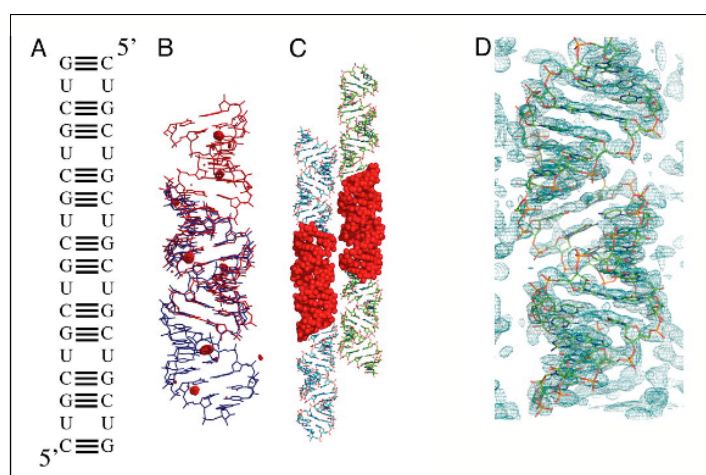


Figure. 3 (PNAS November 15, 2005 vol. 102 no. 46 16626-16631). (A,B) Secondary CTG repeats structure and double strand formation. (C) Two double helices are sticking each other showing the initial Foci formation event (D) Three-dimensional prediction of the Ribonuclear Inclusions.

At the level of the Foci, the mutated RNA acquires a secondary structure, in particular hairpin structures, and aggregates reaching considerable dimensions (Napierala et al. 1997). Even if the binding between the repeats at the level of the stem, shows a U-U mismatch, the hairpin structure is very stable and close to 100% complementarity, anyway the presence of mismatch could trigger a periodic alteration of the surface electrostatic potential able to strengthen the interactions between this structure and the RNA binding proteins sequestered at the level of the Foci. Two classes of RNA binding proteins, referred to as Splicing Regulators, seem to play a pivotal role in the pathogenesis of the disease, by virtue of the sequestration of one of it inside the Foci: the Muscleblind 1-like family (MBNLs) and the CUG-binding protein, or ETR3-like family (CELF) of splicing regulators, in particular CUGBP1. The Muscleblind family of proteins plays an important role in the alternative splicing process. Activated after birth, it leads to the excision of foetal exons and to the expression of the appropriate isoforms instead of the foetal ones (Ho et al. 2004).

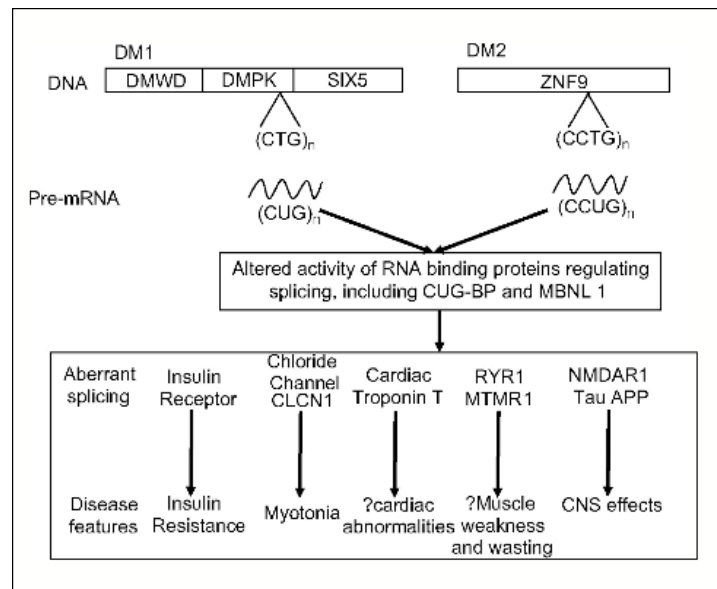


Figure. 4 (J Neurol Neurosurg Psychiatry 2010;81:358-367 doi:10.1136/jnnp.2008.158261). Schematic representation of the mechanism of the DM pathology, showing the impairment of the splicing regulator equilibrium due to Foci sequestration of MBNL-1 protein, and the pathologic splicing pattern involving important genes.

This family of proteins was first discovered in *Drosophila*, where it is responsible for the terminal development of the photoreceptors at the level of the insect eyes, and leads to the correct development of the Skeletal muscle apparatus^(Artero et al. 1998). Up to now three isoforms of Muscleblind have been discovered in Mammals: Muscleblind-like 1 (MBNL1 or MBNL), Muscleblind-like 2 (MBNL2 or MBL2) and Muscleblind-like 3 (MBNL3 or MBXL). While the first two isoforms are mainly expressed in the Skeletal muscles, the third one is expressed in the Placenta^(Miller et al. 2000). The CELF family of splicing regulators, and in particular CUGBP1 acts in an antagonistic fashion with respect to MBNL-1, promoting the inclusions of foetal exons in particular mRNAs during the pre-RNA maturation. These two antagonistic proteins do not compete directly with each other, since they recognize different consensus sequences at the level of the transcripts^(Dansothong et al. 2005). CUGBP1, differently from MBNL1 does not colocalize within the foci, but is overexpressed in myoblast cell cultures and muscular biopsies from DM1 patients. This is a molecular signature of the DM^(Timchenko et al. 2001). The Muscleblind 1 and 2 sequestration at the level of the ribonuclear inclusions, and the CUGBP1 overexpression seem to be the main reasons for the abnormalities of the splicing processes of a number of genes which can be associated to the clinical manifestations of the Myotonic Dystrophy. Up to now 22 genes incorrectly spliced have been recognized at the level of DM patients skeletal and cardiac muscles. These genes play a role in the multisystemic

sympomatology of the disease: among the other Myotonia, triggered by an alteration of the splicing process of the Chloride Channel 1 (CLCN1) RNA^(Mankodi et al.2002); Insulin Resistance, caused by a disequilibrium between the IR-A and IR-B splicing isoforms of the Insulin Receptor (IR) in which exon 11 is skipped ^(Savkur et al. 2004); the Heart Arrhythmias, probably correlated with the embrional splicing-isoform prevalence of the Cardiac Troponin T mRNA, transcribed from the TNNT2^(Philips et al. 1998).

2.4 Transgenic mice

In order to define the pathogenetic mechanisms underlying the Myotonic Dystrophy and to correlate the molecular basis of the disease with the multisystemic symptomatology a number of genetically modified mice has been created. With the idea to test the hypotesis that the CTG repeats amplification could be responsible itself for the DM onset, a mice in which the CTG amplification lies in a position with no correlation to the DMPK gene was produced. Mice of this kind have the characteristic 250 CTG mutation at the level of the 3'UTR of the Skeletal Actin ^(Mankodi et al. 2000). The results demonstrated that the pathology originates following the expression of the transgenic Skeletal Actin mRNA, supporting the accuracy of the "Toxic RNA Gain of Function" model, predicating the sufficiency of the amplified CTG repeats either localized in the DMPK 3'UTR or everywhere else, to generate a DM phenotype in agreement with the expression of that gene at the level of the skeletal muscles. This mouse shows atrophy, centralized nuclei, small myofibers, and a reduced walking and running ability at the Rota Road test, strictly ricapitulating the Human DM1 phenotype. To establish a role for the MBNL1 protein and to demonstrate the relationships between the clinical manifestations of the DM and the sequestration of this protein at the level of the toxic ribonuclear inclusions, a Muscleblind 1 KO mouse was generated^(Kanadia et al. 2003). The skeletal muscle spliceopathy affecting MBNL1 KO mice is very similar, if not identical, to the one triggered by the CTG repeats amplification in Human, and in CTG-250 transgenic mouse, in other words, the genetic silencing toward Muscleblind-1 evokes clinical features characteristic of the Myotonic Dystrophy: Skeletal Muscles alterations, Cataracts, alternative splicing affection of the Chloride Channel-1, and the consequent Myotonia. Consequences of the MBNL-1 protein inactivation are further supported by the evidence, obtained in the mouse model of the DM1, in which the CTG amplification lies in the 3'UTR of the DMPK gene. The mutation complementation obtained thanks to adenoviral ectopic expression of the MBNL-1 protein, at the level of the skeletal muscles, settles myotonia, and restores the splicing pattern abnormalities. Adult isoform of CLCN-1 replaces the foetal one, and the expression of this

Chloride Channel at the level of the sarcomeric membranes turns out to be augmented compared to the adenoviral untreated mice. Anyway, ultrastructural analysis of the adenoviral infected mice revealed abnormalities lingering on at the level of the muscle fibers: centralized nuclei are still present, as well as the CUGBP1 overexpression and the atrophic features. This evidence demonstrates the complexity of the pathogenesis of Myotonic Dystrophy, since it is not possible to define a single mechanism (like the inactivation of MBNL-1 protein) as the only critical event leading to the manifestation of the disease, but as an important event contributing to the pathogenetic process. Transgenic mice that recapitulates every Human symptom of the DM1 have been obtained in the lab of Thomas Cooper (University of Texas in Dallas) and are characterized by the presence of a long CTG amplification (960 repeats) at the level of the exon 15 of the DMPK gene. It is an inducible mouse model. Importantly, this DM1 model recapitulates severe muscle wasting, which has not been reported in models in which depletion of MBNL1 is the main feature.

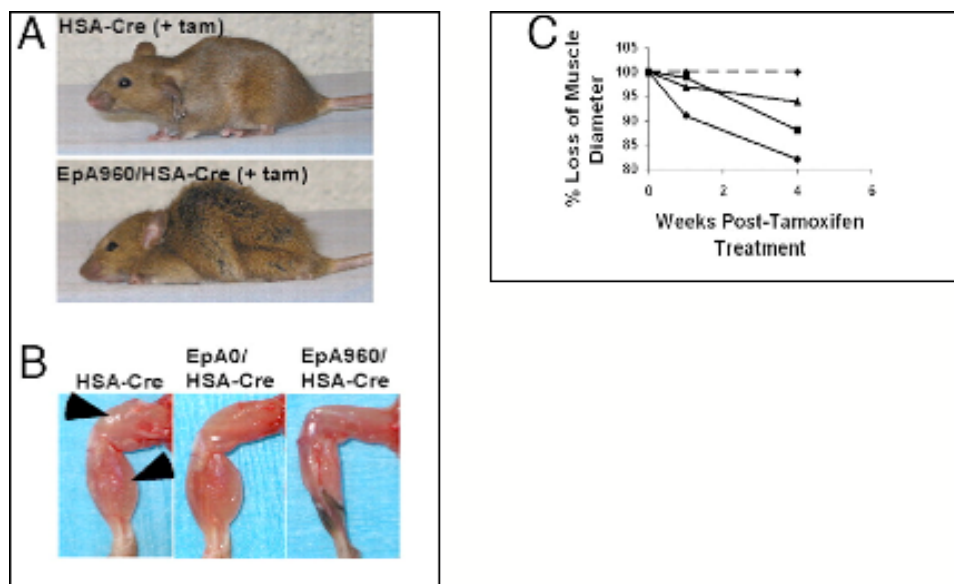


Figure. 5 (PNAS February 19, 2008 vol. 105 no. 7 2646-2651). EpA960/HSA-Cre-ERT2 mice exhibit severe and progressive skeletal muscle wasting and dysfunction after tamoxifen administration. (A) HSA-Cre-ERT2 (Upper) and EpA960/HSA-Cre-ERT2 (Lower) littermates 4 weeks after tamoxifen administration. (B) From left to right, the hind limbs of age- and sex-matched HSA-Cre-ERT2, EpA0/HSA-Cre-ERT2, and EpA960/HSA-Cre-ERT2 mice 4 weeks after tamoxifen administration. Arrows indicate quadriceps and gastrocnemius muscle bodies. (C) Wasting of the paraspinal muscle determined by MRI from one HSA-Cre-ERT2 (dashed line) and three EpA960/HSA-Cre-ERT2 (solid lines) mice during a 4-week time course after tamoxifen administration. Measurements were normalized to the kidney diameter.

In these transgenic mice expression of DMPK exon 15 containing either 960 CTG repeats (EpA960) or no repeats (EpA0) was induced by Cre-mediated recombination in cardiomyocytes: the authors crossed the EpA lines with HSA-Cre-ERT2 mice, expressing the tamoxifen-dependent Cre-ERT2 recombinase selectively in skeletal muscle tissue. To induce recombination and expression of mRNA containing the expanded CTG repeats [EpA960(R)], EpA960/HSA-Cre-ERT2 3 to 4 months old bitransgenic mice were injected daily for 5 days with 1 mg of tamoxifen. EpA0/HSA-Cre-ERT2 bitransgenic mice were also treated with tamoxifen to induce expression of the identical mRNA containing human DMPK exon 15, but lacking CTG repeats [EpA0(R)]. These EpA960/HSA-Cre-ERT2 mice showed RNA Foci colocalizing with MBNL-1, misregulation of the developmentally regulated alternative splicing events, Myotonia, and Atrophy, and CUGBP1 overexpression. The presence of Atrophy and the concomitant CUGBP1 overexpression suggest a possible correlation between this pathologic protein behaviour and the phenomenon of the progressive reduction of the muscle fibers diameter. Severe skeletal muscle wasting is the most debilitating symptom experienced by individuals with myotonic dystrophy type 1^(Orengo et al. 2007), increased CUGBP1 protein levels are associated with DMPK-CUG RNA expression, suggesting a role for CUGBP1-specific splicing or cytoplasmic functions in muscle wasting.

2.5 Atrophy correlates with augmented protein degradation or decreased protein synthesis.

Skeletal muscles are characterized by a consistent plasticity, and represent the major amino acids storage of the organism. Muscular proteins can be mobilized as aminoacids in different conditions: nutrient deficiency, immobility, and a number of pathologic conditions^(Lecker et al. 2006). Protein mobilization represents a transient phenomenon, but it can be fundamental in the adaptative response finalized to the protein synthesis maintenance in those organs whose activity is essential, like Heart, Brain, Liver. On the contrary Muscular Atrophy is a dangerous epiphenomenon of many pathological states characterized by an intense and substained catabolic activity. Muscular mass reduction could depend either on a reduction of protein synthesis or on an augmented proteolysis. Recent clinical studies revealed proteolysis as the major cause leading to Muscular Atrophy underlying pathologic conditions and catabolic states like Muscular Dystrophies and Myopathies.^(Lecher et al.2006) As every mammalian tissue does, skeletal muscle has a number of proteolytic systems including the Ubiquitin-Proteasome System (UPS), the Lysosomal degradation, the Ca⁺⁺ dependent degradation, and the Metalloproteinase degradation. While Lysosomal degradation and Ca⁺⁺ dependent degradation do not play an important role in Muscular Atrophy, since they are not systematically activated during Atrophy, and

are not responsible for the major contractile protein degradation, the Caspases and Metalloproteinases role in the Atrophy phenomenon has not yet been elucidated.

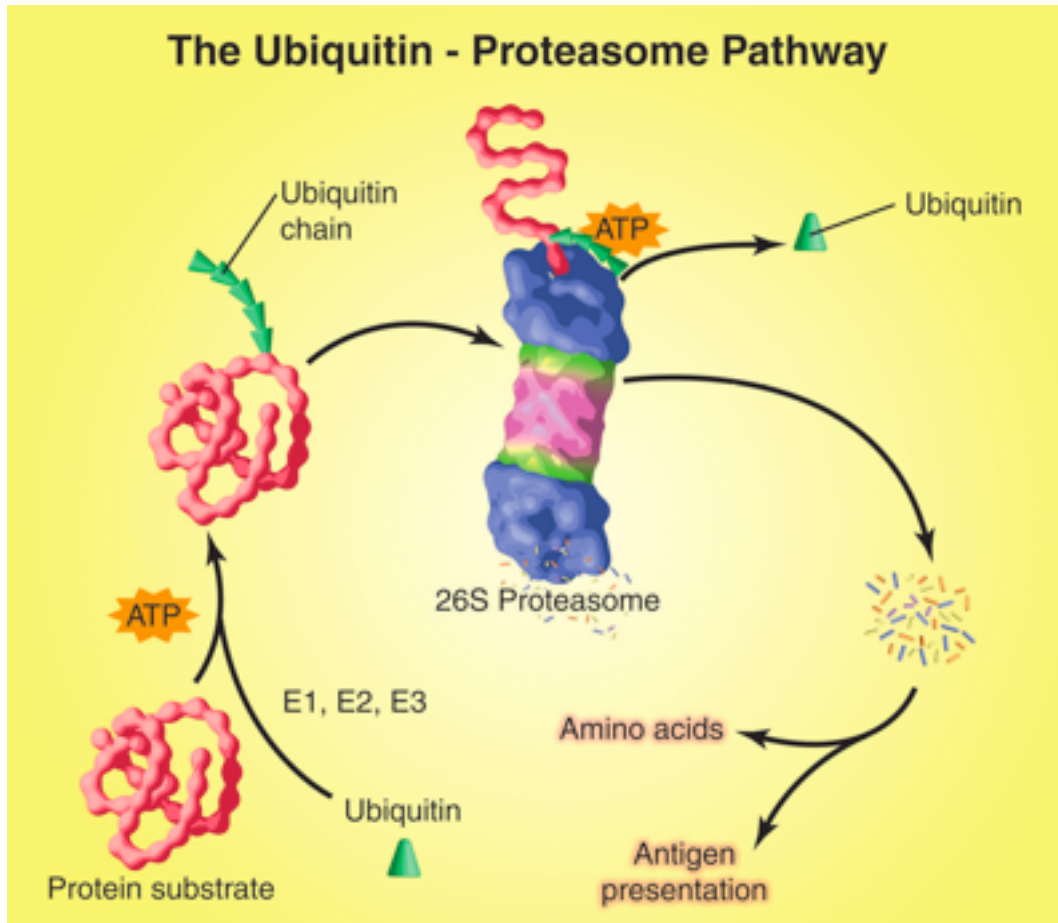


Figure. 6 (Lecker et al. 17 (7): 1807. (2006)) The ubiquitin (Ub)-proteasome System (UPS) of protein degradation. Ub is conjugated to proteins that are destined for degradation by an ATP-dependent process that involves three enzymes. A chain of five Ub molecules attached to the protein substrate is sufficient for the complex to be recognized by the 26S proteasome. In addition to ATP-dependent reactions, Ub is removed and the protein is linearized and injected into the central core of the proteasome, where it is digested to peptides. The peptides are degraded to amino acids by peptidases in the cytoplasm or used in antigen presentation. Illustration by Josh Gramling—Gramling Medical Illustration.

The most important player in the Atrophic conditions is the Ubiquitin-Proteasome pathway. The Proteasome is found in the nucleus and the cytosol of all cells and constitutes approximately 1 to 2% of cell mass (Voges et al. 2000). The 26S particle is composed of approximately 60 subunits and therefore is approximately 50 to 100 times larger than the typical proteases that function in the

extracellular environment (e.g., in digestion, blood clotting) and differs in critical ways. The most fundamental difference is that proteasome is a proteolytic machine in which protein degradation is linked to ATP hydrolysis. The 26S complex is composed of a central barrel-shaped 20S proteasome with a 19S regulatory particle at either or both of its ends (Figure 6). The 20S proteasome is a hollow cylinder that contains the machinery for protein digestion. It is composed of four stacked, hollow rings, each containing seven distinct but related subunits. The two outer {alpha} rings are identical, as are the two inner beta rings. Three of the subunits in the beta rings contain the proteolytic active sites that are positioned on the interior face of the cylinder. The outer {alpha} subunits of the 20S particle surround a narrow, central, and gated pore through which substrates enter and products exit ^(Grol et al. 2000). Substrate entry is a complicated process that is catalyzed by the 19S particle. This complex architecture evolved to isolate proteolysis within a nano-sized compartment and prevents the nonspecific destruction of cell proteins. One can view protein ubiquitination and the functioning of the 19S particle as a mechanisms that ensure proteolysis as an exquisitely selective process; only certain molecules are degraded within the 20S proteasome. In atrophic muscles expression of some specific subunits which are part of the 20S and 19S Proteasomal complexes are subjected to variation, strongly suggesting these protein species might be limiting entities in the Proteasomal assembly ^(Lecker et al. 2006). One possibility is that different transcription factors, or coregulators could govern particular Proteasomal subunit subgroups expression at the level of the muscles ^(Attaix et al. 2003). The Ubiquitin-Proteasome degradative pathway involves two consecutive steps: the target protein is first poly-Ubiquitinated, thanks to the E1,2&3 Ubiquitin ligases, (E3 Ligases determine protein specificity) and then recognized by specific receptor-proteins (like *hRD23B*, a cytosolic soluble protein containing an N-terminal ubiquitin-like domain, which has been reported to interact with 26S proteasome) involved in the Ubiquitin mediated proteolytic pathway targeting the degradation candidate to the 26S Proteasome regulative subunits for physical entrance in the 20S catalytic portion and degradation. In a number of experiments performed in transgenic mice it turned out that poly-ubiquitinated proteins preferentially accumulate at the level of the contractile proteic system of the muscular fibers rather than in the soluble protein fraction. Moreover, only ATP depletion or 26S Proteasomal inhibition, (for example by MG-132 inhibitor) ^(Lecker et al 2006) can efficiently block the release from atrophic muscles of 3-Methyl-Histidine. This post-translationally modified amino acid represents a marker of the Skeletal Actin and Myosin degradation, reflecting the catabolic rate of the myofibril ^(Attaix et al. 2005). Studies in rodent models have also established that accelerated muscle protein catabolism that is induced by uremia involves similar cellular mechanisms causing muscle wasting in a variety of other catabolic conditions, such as cancer cachexia, starvation, insulin deficiency, or sepsis ^{(Mitch et al.}

¹⁹⁹⁶). Atrophying muscles from such animals show accelerated proteolysis *via* the UPS, higher levels of mRNAs for certain components of this proteolytic system, and a similar pattern of changes (both increases or decreases) in the expression of approximately 100 atrophy-related genes, also termed atrogenes ^(Lecker et al. 2004).

Increased ATP-dependent proteolysis by the ubiquitin-proteasome System
Greater sensitivity to proteasome inhibitors Associated with activation of caspase-3 which provides substrates for the ubiquitin-proteasome pathway
Increased conjugation of ubiquitin to muscle proteins
Activation of a common transcriptional program: induction/suppression of a set of atrogenes
Upregulation of the muscle-specific Ub-protein ligases, atrogin-1/MAFbx and MuRF1
Suppression of the IGF-1/PI-3 kinase/Akt signaling pathway and activation of FoxO transcription factors
MuRF-1, muscle ring finger-1; PI3-K, phosphatidylinositol 3-kinase; Ub, ubiquitin; UPS, ubiquitin-proteasome System.

Table 1. Biochemical adaptations found in atrophying skeletal muscles

In humans in similar conditions, there is also evidence for activation of the UPS in muscle (*e.g.*, an increase of mRNAs that encode Ubiquitination machinery and proteasome subunits) ^(Pickering et al. 2002). In these catabolic states, the increase in mRNA levels for these Atrophy-related genes in muscle occurs through increased gene transcription ^(Lecker et al. 2004). As part of this common transcriptional program, the expression of various growth-related genes decreases in the Atrophic tissues. Therefore, multiple transcriptional factors seem to change coordinately to induce the loss of muscle mass. The strongest evidence for activation of the UPS in muscles that undergo Atrophy as a result of uremia (or other catabolic diseases) is that when studied *in vitro*, muscles from these mouse models, exhibit increased proteolysis that can be blocked by inhibitors of the proteasome ^(Tawa et al. 1997). For these reasons, muscle wasting represents a specific, carefully orchestrated response that is triggered by various stimuli in various pathologic conditions (*e.g.*, acidosis in renal failure, low insulin levels in fasting and diabetes, inactivity, glucocorticoids or cytokines in sepsis). In fasting and presumably in other disease states, an identifiable function of accelerated proteolysis is to mobilize amino acids from dispensable muscle proteins to provide the organism with precursors for hepatic gluconeogenesis or for new protein synthesis ^(Mitch et al 1996). However, these losses can have deleterious effects with time, especially in renal failure, in which disposal of nitrogenous waste is compromised. Because the UPS serves for many essential functions in cell regulation and homeostasis, its activation in these states must be highly selective and precisely regulated to avoid the unwanted removal of muscle proteins that are essential for cell function in muscle and other

organs. Whereas atrophy seems to affect all muscle cell components, contractile proteins are lost differentially. The specificity of the UPS in protein breakdown is determined by the cell's content of Ubiquitin ligases (E3s), which, as discussed, target specific sets of proteins to degradation. The cellular content of E3s varies among tissues and physiologic states. Two Ubiquitin ligases, atrogin-1 (also known as MAFbx) and MuRF-1, are specific constituents of muscle: their expression increases dramatically (eight- to 20-fold) in catabolic states, and they play a critical role in mediating the loss of muscle proteins.

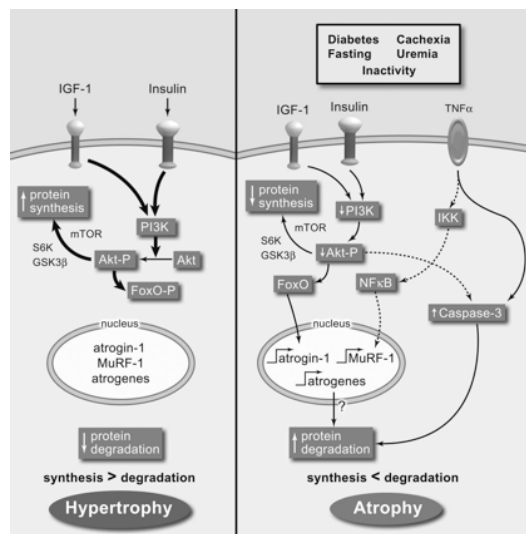


Figure. 7 Lecker et al. 17 (7): 1807. (2006)) The balance between protein synthesis and degradation determines whether muscles undergoes hypertrophy or atrophy. With hypertrophy, protein synthesis exceeds degradation because the phosphatidylinositol 3 kinase (PI3-K)/Akt pathway stimulates protein synthesis and phosphorylates the FoxO transcription factors that stimulate protein degradative processes. In diseases that suppress insulin/IGF-1 signaling, the PI3-K/Akt pathway is depressed, leading to decreased protein synthesis and FoxO phosphorylation. This allows FoxO to stimulate the expression of the E3 enzymes atrogin-1 and muscle ring finger- 1 (MuRF-1) and protein degradation

In mice that lack genes for atrogin-1 or MuRF-1, muscles grow normally, but if they are denervated, the rate of muscle atrophy is reduced^(Bodine et al. 2001). Muscles of these knock-out mice also show reduced atrophy upon fasting. In wild-type animals that are subjected to muscle denervation or disuse, expression of atrogin-1 and MuRF-1 rises quickly just when muscle atrophy occurs, and in cultured muscle cells, the content of atrogin-1 mRNA tightly correlates with the rate of protein breakdown^(Sandri et al 2004). Therefore, the muscle's content of atrogin-1 mRNA is an excellent biomarker for the rate of proteolysis and rapid wasting.

The *COP9* signalosome (*CSN*) complex is highly conserved from yeast to human. Although the plant *CSN* was first identified as a negative regulator of photomorphogenesis, the mammalian *CSN* is linked to different biological responses such as checkpoint control, signal transduction,

development and the cell cycle^(Kato et al. 2009). Frequent over-expression of the CSN subunit in a variety of human cancers suggests its involvement in cell transformation and tumorigenesis. The best-known biochemical function associated with the CSN is the control of protein stability via the ubiquitin-proteasome system through regulation of cullin-RING-E3 ubiquitin ligase activity by deneddylation, by controlling the activity of COP1 E3 ligase, or by counteracting ubiquitin-mediated degradation through a CSN-associated deubiquitinating enzyme. In addition to affecting the stability of transcription factors, CSN may regulate gene transcription by directly associating with chromatin. Recent studies were directed to understand which transduction pathway influences, at the skeletal muscles level, the Ubiquitin-Proteasome System: it seems that Insulin, and Insulin like Growth Factors^(Glass et al. 2005) negatively regulate this type of degradation, while Cytokines and Myostatin activate the system. As noted, once again activation of the UPS in various types of muscle wasting and the coordinated changes in the expression of a set of genes in muscle, suggest that these catabolic states activate a common cellular signaling pathway^(Lecker et al. 2004). Recent studies have established that in fasting and, presumably, in other insulin-deficient conditions, the fall in protein synthesis and the rise in proteolysis are coupled events that occur through decreased signaling of the phosphatidylinositol 3-kinase/Akt (PI3-K/Akt) pathway, which in normal individuals is activated by Insulin or IGF-1^(Lee et al. 2004). IGF-1 is released by the liver and mediates the anabolic effects of the growth hormone, but it is also an autocrine factor released by muscle after exercise. In fasting and disease, when insulin signaling is low, the activity of PI3-K is reduced and there is less production of phosphatidylinositol-3,4,5 phosphate, the active product of PI3-K; the decrease in this regulatory molecule decreases the phosphorylation and activity of the serine/threonine protein kinase Akt (Figure 7). Activated Akt is a major activator of growth-related processes, and, when overproduced, Akt causes muscle hypertrophy *via* phosphorylation of the downstream kinases glycogen synthase kinase 3 beta (GSK3beta) and mammalian target of rapamycin (mTOR)/S6kinase, which enhances global protein synthesis. In many types of atrophy, the reduction of PI3-K/Akt pathway leads to reduced mRNA translation and protein expression. However, rapid atrophy also requires enhanced proteolysis and induction of E3 atrogen-1 and MuRF-1, leading somehow to enhanced protein degradation in muscle cells^(Stitt et al. 2004). These results show that activation of protein degradation and induction of these E3 enzymes in atrophying muscles also result from a decrease in the activated Akt (*i.e.*, phosphorylated Akt). One of the targets of activated Akt is the forkhead family of transcription factors (FoxO1, 3, and 4). When they are not phosphorylated, they migrate into the nucleus and catalyze the transcription of atrogen-1^(Sandri et al. 2004). Conversely, insulin and IGF-1, by activating PI3-K and Akt, phosphorylate FoxO and suppress atrogen-1 expression.

As we described above, Insuline resistance is one of the main sympomatical feature of the DM pathology. The IR-A splicing isoform of Insulin Receptor, which is referred to as the foetal isoform, is characterized by the exclusion of the exon 11, and this negatively affect the receptor affinity for Insulin and Insulin-like Growth Factors. It could be therefore important, to investigate whether this is one of the triggering causes of Atrophy in DM patient's skeletal muscles. Besides controlling the expression of E3 Ubiquitin ligases, activation of the insulin/IGF-1--PI3-K/Akt pathway suppresses activation of caspase-3. This would decrease the breakdown of the complex structure of muscle proteins. In insulin-deficient rats that exhibit accelerated muscle protein degradation, it has been evaluated that the proapoptotic factor *Bax* is activated, leading to the release of cytochrome *c* from mitochondria, which in turn activates caspase-3 and increases production of the 14-kD actin fragment^(Lee et al. 2004). Similar changes were observed in cultured muscle cells by genetic or pharmacologic inhibition of PI3-K activity. Together, these data provide evidence that activation of muscle protein loss occurs through a common signaling pathway that alters transcription of key enzymes modulating protein synthesis and degradation in a complementary way to cause muscle wasting. A recent work by Huichalaf et al., (2009) revealed that DM2 muscle cells are characterized by reduced rate of global translation. These data provide additional evicence that CCUG repeats might cause pathological alteration of the steady state between protein synthesis and degradation leading to the Atrophic phenomenon. The authors present evidence for Znf9 protein to bind to the 5' UTR of a number of TOP mRNAs, including Ribosomal Protein RPS17, PABP1 and two elongation factors eEF1A and eEF2. Under normal conditions Znf9 supports translation of TOP-containing mRNAs since direct inhibition of Znf9 by siRNA in normal cells leads to the reduction of these proteic species. The reduction of these proteins correlates with the reduction of the protein synthesis rate. Dr. Timchenko's group demonstrated that the reduction of the cytosolic levels of Znf9 protein in myoblasts can be triggered by the CCTG expansion, since ectopic expression of the pure mutant CCUG repeats reduces the level of Znf9 and of proteins such as RPS17, PABP1, and two elongation factors, eEF1A and eEF2. Although the main result of this work was the evaluation of a reduction of the total rate of protein synthesis in DM2 myoblasts, which occurs after Znf9 depletion, the authors cannot exclude multiple causes for this feature, as they previously showed CUGBP1 as a translational regulator in DM1 myoblasts^(Salisbury et al. 2008). Depending on the CUGBP1 phosphorylation state, it may have positive or negative effects on the translation machinery. In DM2 cells CUGBP1 might reduce translation, or increase degradation of mRNAs encoding translational apparatus factors. CCUG repeats interact with CUGBP1-eIF2 complex and with the proteasome, hence, the reduction of the translational rates in DM2 cells, could also depend on the sequestration of the initiation factor eIF2, or as a result of proteasome dysfunction.

2.6 Mitochondrial involvement in atrophic conditions.

Mitochondrial protein translation is a complex process performed within mitochondria by an apparatus composed of mitochondrial DNA (mtDNA)-encoded RNAs and nuclear DNA-encoded proteins. Although the latter by far outnumber the former, the vast majority of mitochondrial translation defects in humans have been associated with mutations in RNA-encoding mtDNA genes, whereas mutations in protein-encoding nuclear genes have been identified in a handful of cases. Genetic investigation involving patients with defective mitochondrial translation gave the possibility to discover novel mutations in the mitochondrial elongation factor G1 (EFG1) in one affected baby and, for the first time, in the mitochondrial *Elongation Factor Tu* (EFTu) in another one. Both patients were affected by severe lactic acidosis at the muscular level and rapidly progressive, fatal encephalopathy. Sarasman et al. 2008 showed that EFTu as well as the translocation factor EFG2, but not EFG1 or EFTs, can partially suppress MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis, Stroke-like episodes) a Human pathology triggered by a heteroplasmic A3243G mutation in the mitochondrial tRNA^{Leu} (UUR). The rescue of mitochondrial tRNA mutant phenotypes by the overexpression of EFTu can be attributed to its function in the translation process, namely providing charged tRNAs to the ribosomal A-site during elongation of peptide chains, and hence, promoting protein synthesis. Because of the direct interaction with the charged tRNAs, overexpression of EFTu might additionally contribute to the stabilization of the mutant tRNA. In a patient with a mutation in the translocation factor EFG1, the levels of EFTu increase as part of an adaptive response in patient heart, the tissue least affected by the presence of the mutant protein^(Antonicka et al. 2006). However, overexpression of EFTu in control cells has a dominant negative effect^(Smeltinik et al. 2006) on mitochondrial translation, steady-state levels of COX subunits COX activity and assembly of OXPHOS complexes, suggesting that the relative ratios of the mitochondrial translation factors are important for efficient protein synthesis, and this may explain, in part, why suppression of the mutant phenotype is relatively modest.

The organ most frequently affected in mitochondrial disorders is the skeletal muscle (mitochondrial myopathy)^(Finisterer 2009). Mitochondrial Myopathies may be part of syndromic as well as non-syndromic mitochondrial disorders. Involvement of the skeletal muscle may remain subclinical, may manifest as isolated elevation of the creatine-kinase, or as weakness and wasting of one or several muscle groups. The course of Mitochondrial Myopathies is usually slowly progressive and only rarely rapidly progressive leading to restriction of mobility and requirement of a wheel chair or even muscular respiratory insufficiency.

Frequently reported symptoms of mitochondrial myopathies are permanent tiredness, easy fatigability, muscle aching at rest or already after moderate exercise, muscle cramps, muscle stiffness, fasciculations and muscle weakness. Sarcopenia, the progressive loss of muscle mass with age, is characterized by a deterioration of muscle quantity and quality leading to a gradual slowing of movement and a decline in strength and power. Sarcopenia is a highly significant public health problem. Since these age-related changes in skeletal muscle are largely attributed to various molecular mediators affecting fiber size, mitochondrial homeostasis, and apoptosis, the mechanisms responsible for these deleterious changes present numerous therapeutic target for new drugs. Some researchers demonstrated that a disruption of Akt-mTOR and RhoA-SRF signaling but not Atrogin-1 or MuRF1 contributes to sarcopenia. In addition, sarcopenia seems to include a marked loss of fibers attributable to apoptosis. Some of these changes can be attributed specifically to biological processes such as oxidative stress^(Hagen et al. 2004). This theory states that a proportion of the age-related changes to skeletal muscle are due to the accumulation of intracellular damage caused by free radical generation across the lifespan^(Nagley et al. 1992; Sohal and Weindruch 1996). Based on the findings from numerous studies, Hagen et al. (2004) concluded that “oxidative damage over time leads to mitochondrial DNA mutation deletions that result in dysfunctional mitochondria, and that skeletal muscle is one tissue that is particularly susceptible to this phenomenon”^(Hagen et al. 2004; Hepple et al. 2006). Thus, independently of age-related changes in physical activity patterns, other deleterious effects of advancing age on skeletal muscle structure, function and metabolism, can be attributed to oxidative damage and other biological processes. Ann, in 2007, described *Mortalin* (mtHSP70/GRP75) a heat uninducible member of hsp70 family of proteins. Mortalin is expressed in all cell types and tissues examined so far^(Wadhwa et al 1995; Kaul et al 1997) and is expected to perform some essential functions. Expression levels of Mortalin correlated with muscle activity, mitochondrial activity and biogenesis^(Ornatsky et al 1995). Some of the established features of mortalin include its various subcellular sites, multiple binding partners, and differential subcellular distribution in normal and immortal cells. Overexpression of Mortalin leads to extended life span in nematode and normal human fibroblasts. On the other hand, it serves as a major target for oxidation and was shown to be involved in old age pathologies including Parkinson's and Alzheimer's disease. Proteomic studies consistently identified oxidatively-damaged mortalin as potential biomarker. It is possible that mitochondrial dysfunction coincides with the collapse of the mitochondrial chaperone network that is responsible not only to import, sort and maintain integrity of protein components within the mitochondria, but also to act as a buffer for the molecular heterogeneity of damaged and aging

mitochondrial proteins within a ROS-rich microenvironment. Since mortalin interacts with many proteins, its modification in response to stress and damage caused by intracellular oxidation is likely to generate pleiotropic effects. For example, (a) inefficient import of mitochondrial proteins by Mortalin-Tim complexes may result in an inefficient mitochondrial biogenesis, energy generation, and functional decline and (b) inefficient chaperoning of proteins can result into a garbage catastrophe.

However, whether the cause of the progressive muscle wasting, in particular atrophy of fast type muscle fibers, rather than general muscle atrophy, could depend on impaired steady state between protein synthesis and degradation, or on mitochondrial dysfunction, or on impaired differentiation of satellite muscular stem cells into muscle fibers, has yet to be completely explained. Therefore the effects of CCTG repeat expansion are likely to extend beyond spliceopathy to include a more general disturbance leading to important changes for the physiological role of this muscle tissue. Many studies on DM physiopathology are focusing on the alterations occurring at the transcriptional and post-transcriptional level, however, the functionality of the biological system depends on the availability and activity of the proteins. Accessibility of wide-screening techniques for the analysis of global protein expression profile is therefore a suitable approach for the identification of altered protein pattern in the pathologic state as compared to the normal one. To investigate this issue, we have used cultured satellite cells obtained from biopsies of DM2 and unrelated patients. Under the experimental conditions we have previously set up, these cells can differentiate into myotubes with morphologic features and expression of specific differentiation markers similarly to control cultures. Moreover, DM2 myoblasts show ribonuclear inclusions, MBNL1 nuclear sequestration and splicing abnormalities characteristic of DM2 muscle.

Our large scale proteome analysis reveals that proteins belonging to two major functional classes are altered in DM2 myotubes: components of the mitochondrial compartment with particular emphasis to mitochondrial proteins involved in import mechanisms such as Hsp60 and GRP75 [also referred to as Stress-70 protein, mitochondrial precursor (mtHSP70) or Mortalin] and the translational machinery of mitochondria such as EFTu, and proteins belonging to the ubiquitin-mediated proteasomal system (UPS) suggesting that DM2 pathology affects biological processes relevant for muscular function. Moreover, in order to obtain an alternative Human cellular model for the study of the DM pathology we produced a skin fibroblasts bank with cells derived from DM1, DM2 and control patients, and validated DM2 fibroblasts as a potential candidate for DM2 mitochondrial impairment, evaluating a differential mitochondrial membrane potential for DM1 and

DM2 fibroblasts compared to control cultures, and a faster and more abundant cytochrome *c* release from the mitochondrial membranes of DM2 fibroblasts after Hydrogen peroxide treatment.

3. MATERIAL AND METHODS

3.1 Patients and primary muscle cell cultures

Human muscle biopsies from biceps brachii muscle were taken under sterile conditions from DM2 patients (n = 4) and from unaffected individuals (n = 4). The biopsies were used for this study after informed consent from patients. The diagnosis of DM2 was based upon the clinical diagnostic criteria set by the International Consortium for Myotonic Dystrophies (Moxley et al., 2002). The histological diagnosis was performed on serial sections processed for routine histological or histochemical stainings. Fluorescence in situ hybridization was performed on frozen sections using a (CAGG)₅ probe as previously reported (Cardani et al., 2004). The biopsies from donors were dissected and cells were propagated at 37°C as described (Cardani et al., 2009). Both DM2 and control myoblasts were plated at a density of 60,000 per 35 mm dishes and grown in proliferative medium at 37°C. Myoblasts were allowed to grow until 80% confluent. To initiate differentiation, the proliferative medium was replaced with differentiation medium consisting of DMEM supplemented with 7% FBS.

3.2 Subcellular fractionation

The cell cultures of each patient were lysed in proper conditions to obtain two fractions: one fraction enriched in cytosolic/membrane proteins, and one in nuclear proteins. The cells, scraped and pelleted, were treated with an hypotonic buffer (10mM Hepes pH 7.5, 0,5% NP-40, 10mM KCl, 1 mM EDTA, 1 mM DTT, additioned with proteases and phosphatases inhibitors). After 15 minutes of mild agitation, cells were centrifuged at 400xg at 4°C. The supernatant was then centrifuged at 15000xg to clarify the extract and the supernatant representing the cytosolic/membrane enriched fraction, was collected. The pellet obtained from the 400xG centrifugation was treated with an hypertonic buffer, (20mM TRIS-HCl pH 7.5, 0,6 M NaCl, 0,2 mM EDTA, 1,4 mM MgCl₂ 25% glicerol, and proteases and phosphatases inhibitors) with mild agitation for 15 min at 4°C and then centrifuged at 80.000xg at 4°C. The supernatant was collected and represent the nuclear-enriched fraction.

In order to obtain the cytosolic fraction of soluble proteins from fibroblast cultures the cells were washed 3 times with PBS then homogenized in 1 ml of ice-cold buffer A [20 mM HEPES, pH 7.5, 320 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol tetraacetic acid (EGTA), 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, supplemented with complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim,

Germany)]. 8 up and down syringe cycles were performed at 4° on the cell extract in order to destroy the cells properly, the collected homogenate was then centrifuged at 100,000°—g at 4°C for 1 h. Soluble cytosolic proteins were found at the level of the supernatant.

3.3 Two-dimensional Gel Electrophoresis (2D-GE)

Protein were processed essentially as described ^(Colombo et al., 2009), Each sample containing 100 µg proteins for the citosol and 50 mg for the nuclei was precipitated using a chloroform/methanol protocol and resuspended in a solution containing 7 M urea, 2 M thiourea, and 4% CHAPS. The first dimension was carried out with an 18-cm pH 3–10 non-linear gradient IPG strip (GE Healthcare). Strips were focused at 20°C for a total of 70,000 Vh at a maximum of 8,000 V using the Ettan IPGphor II system. For the second dimension, IPG strips were equilibrated in a solution containing 6 M urea, 2% SDS, 30% glycerol, and 50 mM Tris/HCl pH 8.8. DTT (10 mg/ml) and iodoacetamide (25 mg/ml) were respectively added to the first and to the second steps. IPG strips were placed on top of a polyacrylamide gel (12% T, 3,3% C) and run using the Ettan DALTsix system (GE Healthcare). Gels were then fixed and stained with 1:200 dilution of Epicocconone fluorescent stain (Deep Purple, GE Healthcare). Gel images were acquired with a Typhoon scanner, using green laser (532 nm) for the excitation of the fluorophore and 610BP filter to capture emitted light. The digitalized gel images were analyzed with Image Master 2D Platinum software (GE Healthcare). Normalized spot volumes of individual proteins expressed as mean ± standard deviation were used to perform Student's t test setting the significance level at p<0.05. Only spots present in all four samples of DM2 and control cultures (both for the cytosolic and nuclear fraction) that show statistically significant differential expression were considered.

3.4 Protein Identification by Matrix-assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) MS Analysis

Proteins were identified after excising bands from the stained gels and processed as described ^(Gorla et al., 2006). MALDI-TOF mass spectrometry was carried out using a Voyager-DE STR (Applied Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm). Mass spectra of tryptic peptides were acquired operating in the positive ion, delayed extraction and reflectron modes using -cyano-4-hydroxycinnamic acid (5 mg/mL in 50% acetonitrile with 0.1% trifluoroacetic acid) as the UV-absorbing matrix. Analytes were prepared by spotting 2 µL of supernatant on the target (384-sample plate with hydrophobic surface, Applied Biosystems), drying with a gentle air flow, and addition of 0.35 µL of matrix solution. Experimental conditions were as follows: accelerating voltage 20 000 V; grid voltage, 68%; guide-wire voltage, 0.000%; delay time, 250 ns; laser power,

2400 a.u. After acquisition, the spectra (resolution 15 000) were processed by Data Explorer software (version 4.0, Applied Biosystems) applying default parameters for advance baseline correction (peak width, 32; flexibility, 0.5; degree, 0.1), deisotoping algorithm, internal mass calibration (using autolysis trypsin peptides 842.5100 and 2211.1046 m/z) and peak detection.

Monoisotopic peptide masses were analyzed using ProFound software (http://129.85.19.192/profound_bin/webprofound.exe 4.10.5) or Aldente software (<http://www.expasy.ch/tools/aldente/>). Input was searched according to the following databases: (1) ProFound: NCBI (nonredundant protein sequence database); taxonomy category: mammalian; digest chemistry: trypsin; max missed cut: 1; modification: cysteine alkylation with iodoacetamide (complete), oxidation at methionine (partial); charge state MH⁺; monoisotopic tolerance: 20 ppm. Protein peaks recognized by the software were removed automatically based on a Z score greater than 1.65 (95% percentile); and (2) Aldente: UniProtKB/SwissProt; predefined taxon: Mammalia; Spectrometer internal error max: 25.

3.5 Immunoblot Analysis

For monodimensional immunoblot analysis, samples were then resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose Protran Membranes (Schleicher Shuell). The membrane were probed with different primary and the proper peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Chemiluminescent signals were detected using the ECL detection system (GE Healthcare). Band intensities were quantified by densitometry with Scion Image Program (Scion Corporation) using GAPDH signals as internal control.

3.6 FISH

For fluorescence *in situ* hybridization (FISH) fibroblasts were fixed with 2% formaldehyde in PBS for 30' at 4°C. A Texas Red labelled (GAGG)₅ probe (IDT, Coralville, IA, USA) was used as previously reported by Cardani et al (2004, 2009). Fixed fibroblasts were permeabilized with 2% acetone in PBS (pre-chilled at -20°C) for 5'. After washing in PBS, cells were incubated in 30% formamide, and 2% SSC for 10' at RT, and then hybridized with the probe (1ng/ml) for 2 hours at 37°C in 30% formamide, 2% SSC, 0,02% BSA, 67 ng/ul yeast tRNA, 2 mM vanadyl ribonucleoside complex. Cells were washed in 30% formamide, 2X SSC at 45°C for 30' then washed 5 times in OBS for 3' at RT, and pre-incubated with normal goat serum (DAKO, Denmark) at the dilution 1:20 in PBS containing 2% BSA.

3.7 Reagents and antibodies

The reagents for 2D-GE were from GE Healthcare. Mouse monoclonal anti-PDI were purchased from Santa Cruz Biotechnology, anti ubiquitinated proteins (clone FK2) were from Affiniti, anti PARP were from BD Pharmingen . Anti actin (rabbit polyclonal) anti Tubulin (mouse monoclonal) anti GAPDH (rabbit polyclonal) were purchased from Sigma. Polyclonal antibodies directed against EFTu/Ts were a generous gift from L. Spremulli (Chapel Hill, NC, USA) and antibodies against CLIC1 were a kind gift from M Berryman (Ohio University College of Osteopathic Medicine Athens, Ohio USA)

3.8 JC1

Fibroblasts were grown in 96 well plates at the average concentration of 10000 cells/plates in DMEM 10% FBS, washed 3 times with PBS before adding the JC1 working solution. 100 ul/well of this solution were added. The cells were incubated with the dye at 37°C for 1 hour, then the compound was removed and the cells washed 3 times with PBS. With the fluorimeter the fluorescence intensity was evaluated. Excitation wave length: 490 nm, fluorescence emitted at 590 nm (orange), and 525 nm (green) were evaluated as a ratio Orange/Green.

4. RESULTS

4.1 Proteomic analysis

In this study we have used myotubes derived from satellite cells of DM2 patients and of unrelated control biopsies, as an experimental *in vitro* model to search for global alteration of human DM 2 muscle cells protein expression profile. For our large scale proteome analysis we used cells that have been differentiated *in vitro* along the myogenic lineage for 10 days.

To investigate alterations of single proteins in DM2 myotubes as compared to the control ones, we used two-dimensional gel electrophoresis (2D-GE) and mass spectrometry analysis (MS). Moreover, we fractionated cellular extracts to cytosol/membrane enriched, and also nuclear fractions to allow the enrichment of low abundance proteins.

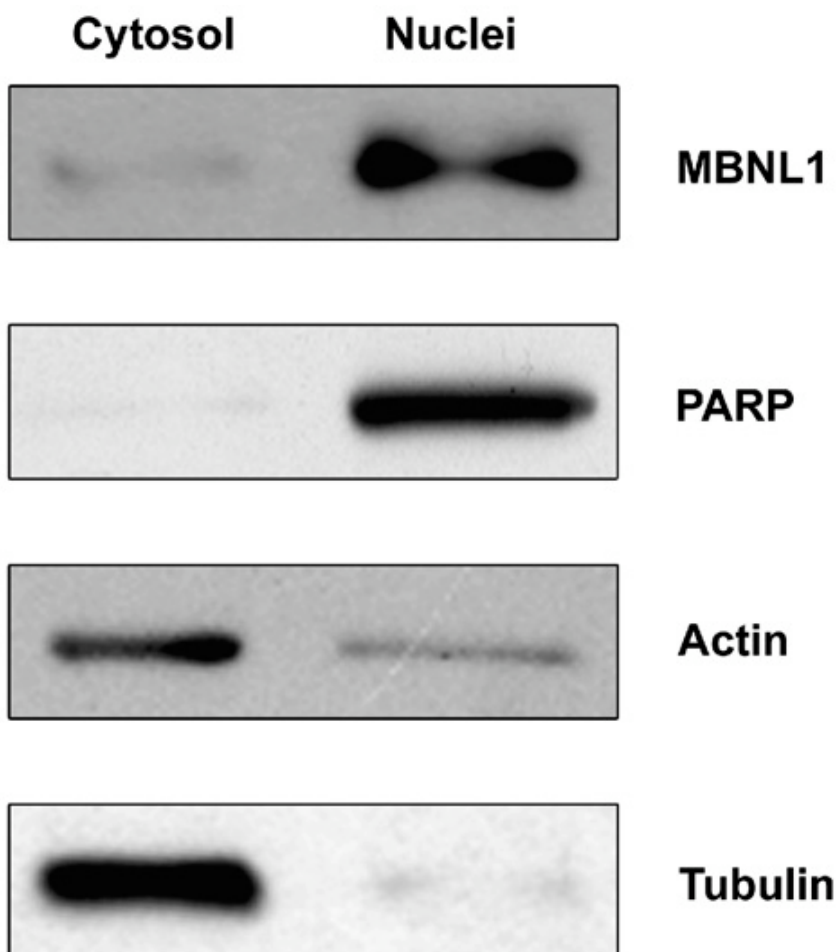


Figure. 8 Subcellular fractionation allows to enrich for specific compartment-localized proteins. Myotubes were fractionated according to the protocol described under Material and methods and analysed by immunoblotting using specific antibodies.

Both fractions were initially checked for the expression of specific markers. As shown in Fig. 8, Poly-(ADP-ribose) polymerase (PARP) and muscleblind, two nuclear proteins, are highly enriched in the nuclear fraction, while tubulin is mainly present in the cytosol confirming data from the literature and indicating that our procedure allowed the enrichment of specific compartment-localized proteins. As expected, Actin was present in both nuclei and cytosol. Cultures obtained from 4 DM2 and 4 control biopsies were fractionated and the different fractions (i.e. cytosolic and nuclear) of the 8 samples were independently separated by 2D-GE, stained with Deep Purple and the 2D-gel maps were acquired as digitalized gel images. We decided to use this fluorescent compound to visualize the proteic spot as it is characterized by a wide dynamic range, allowing a quantitatively reliable comparative expression analysis. Only spots present in all the gels (for each fraction) were analyzed and the normalized spot percentage of volume of individual proteins was statistically evaluated (see methods) QRepresentative two dimensional images are shown in fig 9 and fig 10, respectively referred to the cytosolic and to the nuclear fractionated proteins.

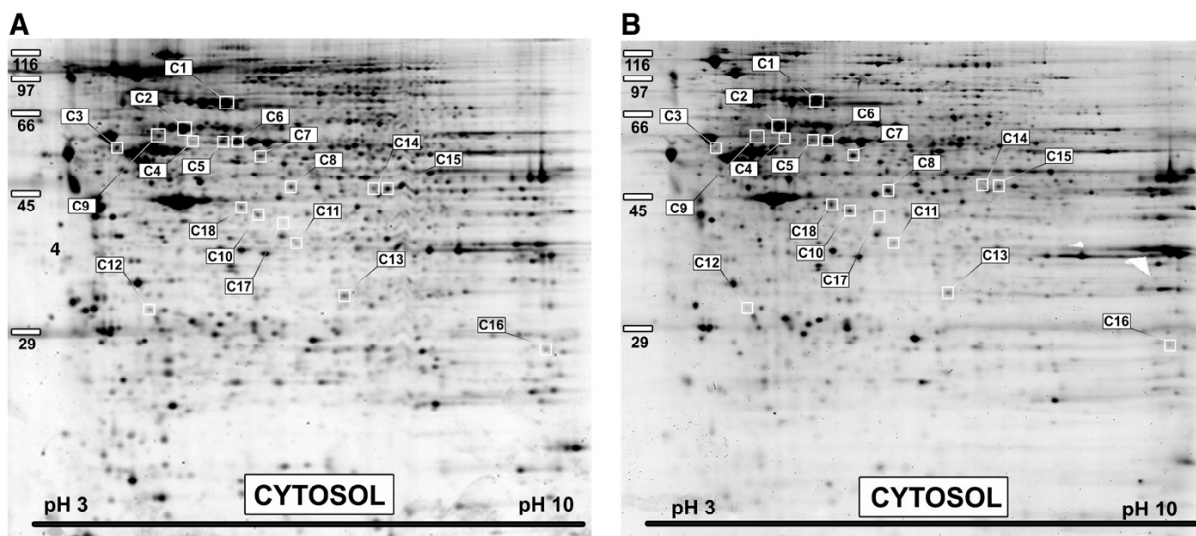


Figure. 9 Two-dimensional map of cytosolic enriched proteins. A representative two-dimensional gel electrophoresis image obtained using cytosolic-enriched fraction from myotubes. Cytosolic proteins obtained from 10 days differentiated myotubes were focused on pH gradient (3–10 NL) separated on 12% polyacrylamide gel and stained with deep purple. Image analysis performed as described under Material and methods, evidences 17 significantly altered spots ($p < 0.05$) in DM2 cultures as compared to control one. The differentially expressed spots were numbered and identified by mass spectrometry (with the exclusion of spots C9, C10 and C11) and correspond to the data presented in Table 2.

About 2000 proteic spots were detected in the cytosol while 1200 spots were identified in the nuclei. Those spots that were statistically significantly altered ($p < 0,05$) (17 spots for the cytosolic fraction and 7 from the nuclear one) were cut, analyzed by MALDI-TOF, identified by data base

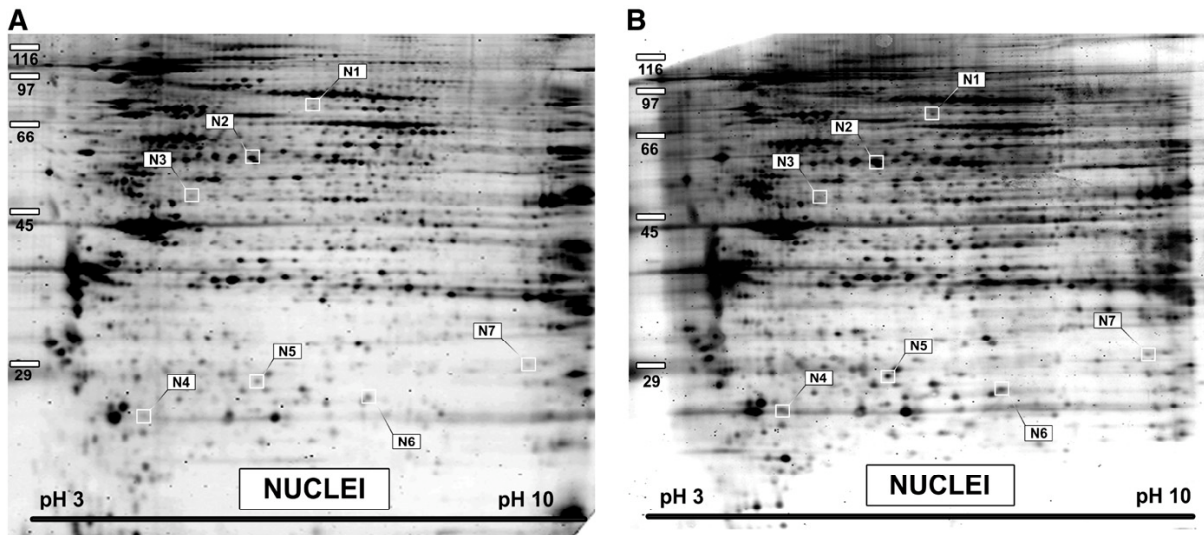


Figure. 10 Two-dimensional map of nuclear enriched proteins from myotubes . A representative two-dimensional gel electrophoresis image obtained using nuclear-enriched fraction from myotubes. Nuclear enriched proteins were analysed as above and image analysis evidences significantly altered spots ($p < 0.05$). The differentially expressed spots, were numbered and identified by mass spectrometry (with the exclusion of spots N1, N3, N4, N7) and correspond to the data presented in Table 2.

search and classified into functional categories according to Gene Ontology. The data are reported in Table 2 and Figure 11.

ID	SPOT	SCORE	AC	DE	MW	PI	COV	CTRL	DM2	P-VALUE B	PERCENT VARIATION	
GRP75_HUMAN	C1	47.13	P38646	Stress-70 protein, mitochondrial.	69	5.5	44	0.6	0.022	0.01	-	96%
CH60_HUMAN	C2	34.33	P10809	60 kDa heat shock protein, mitochondrial...	58	5.2	32	1.2	0.79	0.05	-	34%
RD23B_HUMAN _s	C3	33.39	P54727	UV excision repair protein RAD23 homolog...	43	4.8	25	0.073	0.021	0.01	-	71%
K1C16_HUMAN	C4	30.44	P08779	Keratin, type I cytoskeletal 16.	51	5	36	0.02	0.05	0.05	+	150%
PDIA3_HUMAN	C5	98.72	P30101	Protein disulfide isomerase A3.	54	5.6	40	0.11	0.072	0.01	-	34%
PDIA3_HUMAN	C6	60.92	P30101	Protein disulfide isomerase A3.	54	5.6	33	0.25	0.16	0.01	-	36%
AL9A1_HUMAN	C7	80.04	P49189	4-trimethylaminobutyraldehyde dehydrogen...	54	5.7	35	0.05	0.086	0.001	+	72%
VAT1_HUMAN	C8	31.59	Q99536	Synaptic vesicle membrane protein VAT-1 ...	42	5.9	28	0.068	0.024	0.01	-	64%
CLIC1_HUMAN	C12	97.05	O00299	Chloride intracellular channel protein 1...	27	5.1	60	0.036	0.016	0.05	-	55%
ECH1_HUMAN	C13	52.4	Q13011	Delta(3,5)-Delta(2,4)-diele noyl-CoA isomer...	32	6	44	0.58	0.22	0.01	-	62%
EFTU_HUMAN	C14	79.09	P49411	Elongation factor Tu, mitochondrial.	45	6.3	34	0.045	0.024	0.01	-	46%
EFTU_HUMAN	C15	245.39	P49411	Elongation factor Tu, mitochondrial.	45	6.3	60	0.045	0.023	0.05	-	48%
K1C16_HUMAN	C16	110.32	P08779	Keratin, type I cytoskeletal 16.	51	5	68	0.02	0.041	0.05	+	105%
PSD13_HUMAN	C17	177.54	Q9UNM6	26S proteasome non-ATPase regulatory sub...	43	5.5	42	0.01	0.039	0.05	+	290%
CSN4_HUMAN	C18	36.39	Q9BT78	COP9 signalosome complex subunit 4.	46	5.5	29	0.038	0.067	0.05	+	76%
TCPA_HUMAN	N2	66.11	P17987	T-complex protein 1 subunit alpha.	60	5.8	27	0.12	0.18	0.05	+	50%
ERP29_HUMAN	N5	47.35	P30040	Endoplasmic reticulum protein ERp29.	26	6.1	40	0.04	0.08	0.05	+	100%
PSA6_HUMAN	N6	52.36	P60900	Proteasome subunit alpha type-6.	27	6.3	49	0.12	0.06	0.05	-	50%

Table. 2 Spots differentially expressed in DM2 myotubes. Relative spot on 2D MAP (Fig. 2 and Fig. 3); AC indicates SwissProt Accession Number; Cov, sequence coverage (%); DE, description; ID, identified protein; Mw, molecular weight; pI, isoelectric point, p value confidence value obtained from t test with 6 degrees of freedom, volume: volume of the spot in the two conditions considered; percent variation: increase or decrease in DM2 cultures relative to control one.

4.2 Results clusterization

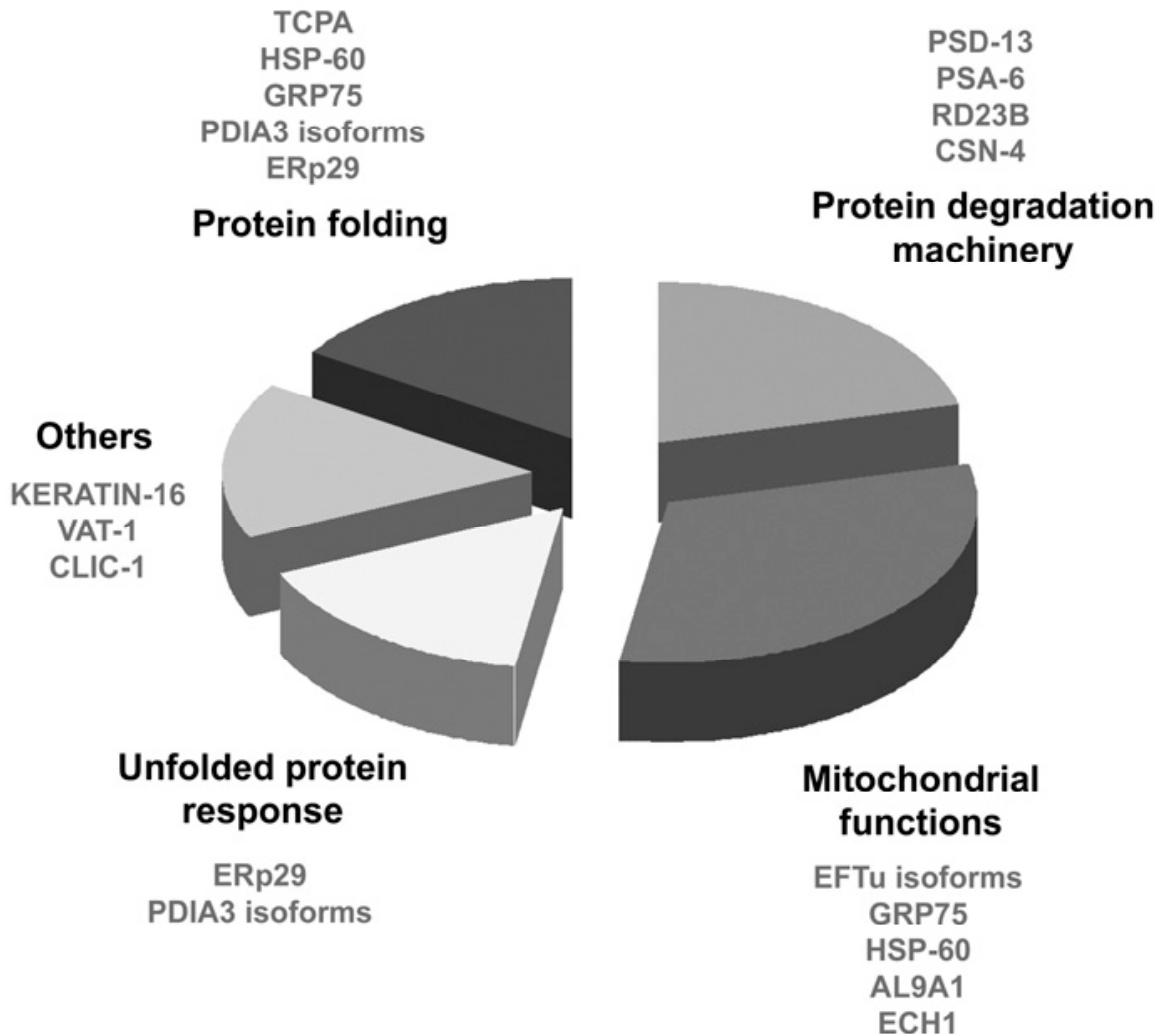


Figure. 11 Distribution of the identified proteins. Graphic representation of the proteins listed in Table 2 grouped by their GO function.

As one can observe in Figure. 11 we could cluster the variations we found, in a narrow number of functional categories: the most important were the mitochondrial related proteins, and the proteins related to the UPS. In the first category we isolated EFTu (mitochondrial elongation factor), Mortalin (GRP75, or mtHSP70), mtHSP60, and two enzymes related to the fatty acids beta-oxidation, and to the biosynthesis of Carnitine, the fatty acid transporter along the mitochondrial membranes. Among the UPS proteins we found 2 unrelated subunits of the Proteasome: PSD13 (26S Proteasome non ATP regulatory subunit 13) a component of the 20S core Proteasomal complex with proteolysis activity and PSA6, (Proteasome subunit alpha, type 6) a subunit of the 19S proteasomal regulatory particle. Moreover we identified as changing its level hRD23B (a receptor protein that recognizes ubiquitinated proteins and targets them to the regulatory 19S

proteasomal particle), and a subunit of the COP9 signalosome (that regulates the E3 Ubiquitin ligases and hence the proteasome mediated protein degradation). Also proteins taking part to the Unfolded Protein Response (UPR), and in particular ERp29 (Endoplasmic Reticulum protein 29) and PDI were isolated as proteins changing their expression levels in DM2 myoblasts. These results suggest an implication of the UPR in the pathogenesis of the DM2.

4.3 Western blotting confirmations

We choose a representative proteic species for every functional compartment found out to be involved in the expressive variations, confirming CLIC-1 which is a shuttling anion channel which is normally soluble but inserts into membranes during oxidative stress events and was ascribed on the category “others” and PDI, part of the Reticulum Stress Response together with ERp29.

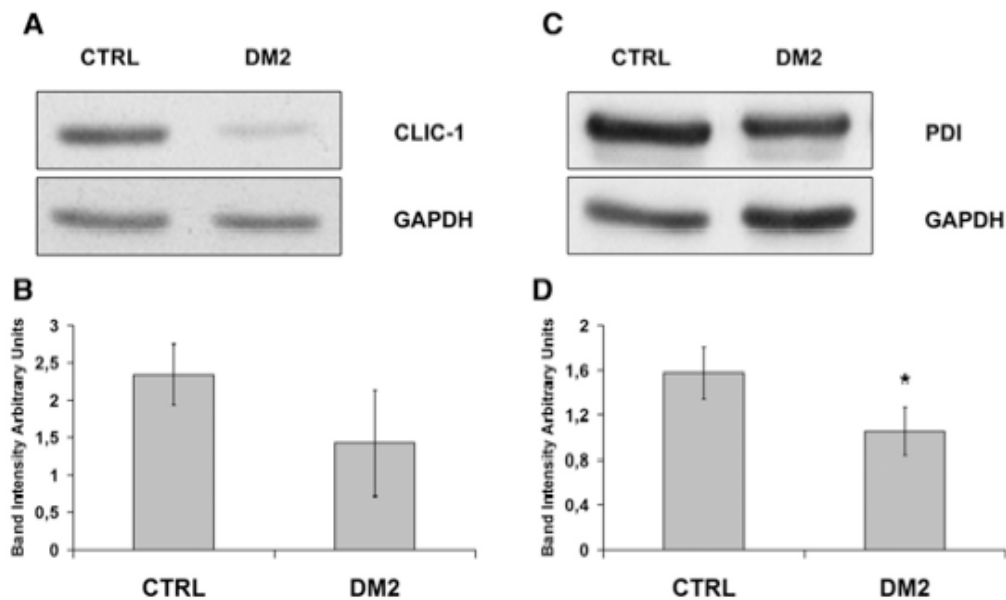


Figure. 12 Confirmation of the proteomic data by immunoblot analysis. Panel A and C show immunoreactive bands of CLIC1 and PDI, respectively. Panel B and D show the band intensity of PDI and CLIC1 normalized with the intensity of GAPDH. The quantification was done on 4 samples of DM2 and 4 samples of the control cultures. Comparative analysis revealed significant reduction of PDI ($p < 0.05$) and CLIC1 ($p < 0.01$) in DM2 cultures consistent with the proteomic data shown. Bars represent the s.d.

EFTu set down for the mitochondrial implications of the DM2 patient’s myoblasts. For elongation factor Tu (EFTu) we identified by bidimensional gel analysis two spots that were recognized with EFTu/Ts antibodies (not shown) and confirmed a global reduction of both isoforms of EFTu (figure. 13 A and B).

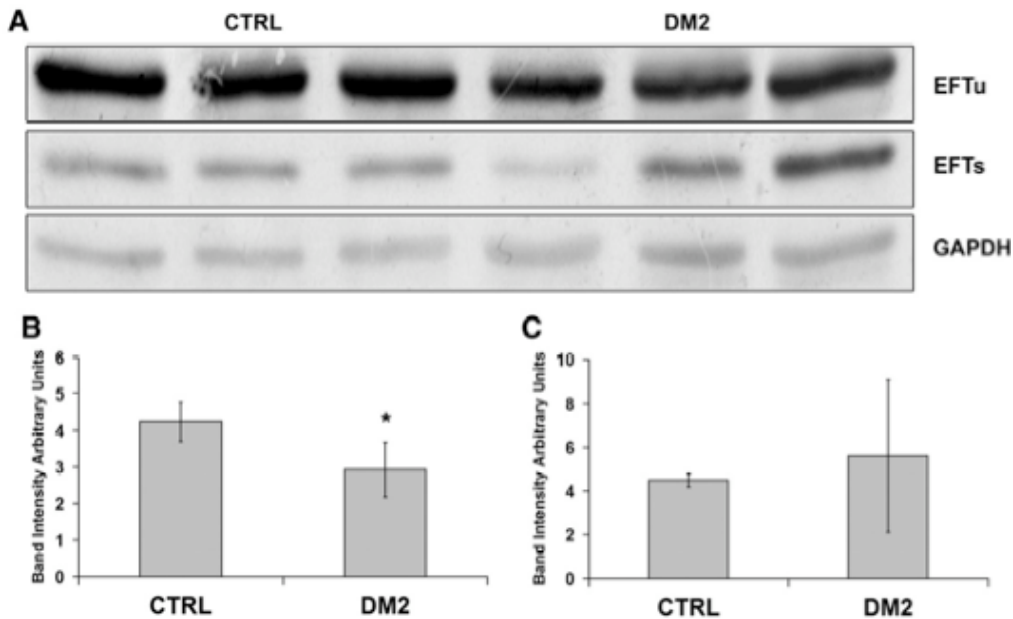


Figure. 13 Level of EFTu and EFTs proteins in DM2 cultures. Panel A: immunoblot analysis with antibodies against Tu/Ts to detect the level of EFTu and EFTs in cytosolic extracts of DM2 and control cultures. Panels B and C show the average intensity level of EFTu and EFTs after normalization for GAPDH intensity, respectively. The quantification was done on 3 samples of DM2 and 3 samples of control cultures in two independent experiments. Comparative analysis revealed significant reduction of EFTu (pb0.05), while the level of EFTs was highly variable in DM2 cultures. The error bar represents s.d.

EFTu mitochondrial elongation factor is involved in the organelle specific translation of proteins. The consistent reduction of two differently phosphorylated isoforms of this protein suggests a possible functional involvement of Mitochondria in the pathogenesis of the DM2. Every mitochondrial protein evaluated as differentially expressed in DM2 patient's myoblasts compared to controls, tuned out to be downregulated (see the Table 2 and Fig. 11). A possibility is that a similar downregulation can involve the mitochondrial EFTu as we found out, and the cytoplasmic elongation factors, eEF1A and eEF2, as the group of Dr. Timchenko showed. These factors may be implicated in a lowered global translation rate in DM2 myoblasts. Further analyses are required to understand whether mitochondrial elongation factor EFTu mRNA includes a TOP containing element in its 5'UTR, and could be regulated by Znf9 binding.

Since the same antibodies used to detect EFTu, recognize also the EFTs proteins, we measured the level of EFTs in the different cultures. Interestingly, we found (fig13 A and B) that while the level of EFTs in the different control cultures is constant, it is extremely variable in DM2 cultures therefore affecting the Tu/Ts ratio. (4 ± 0.028 for control and 4 ± 0.862 for DM2 cultures). The EFTs factor acts as a nucleotide exchange factor for EFTu, and the stoichiometry between the two factors is important in order to determine the translation process efficiency.

4.4 Functional aspects

A consistent group of proteins that were altered in our proteomic analysis, belong to the ubiquitin-proteasome system. For this category we decided to use a different method in order to confirm the accuracy of the proteomic analysis: the indirect evaluation of the functional involvement of the UPS through the evaluation of a differential amount of ubiquitinated proteins. To investigate if these alterations affect the ubiquitination state of proteins we used antibodies that recognize ubiquitin-protein conjugates (both mono and poly-ubiquitinated).

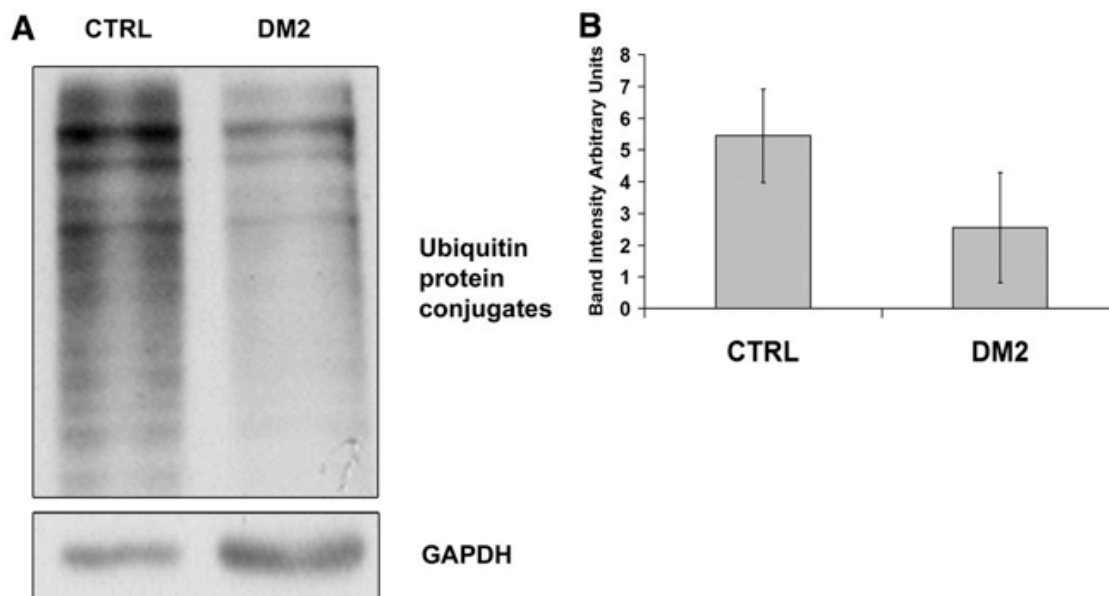


Figure. 14 *The level of ubiquitinated cytosolic proteins is reduced in DM2 cultures.* Cytosolic extracts of control and DM2 cultures were analyzed with antibodies against ubiquitin-protein conjugates. The quantification was done on 3 samples of DM2 and 3 samples of the control culture. The relative intensity of the blot was normalized for GAPDH and the data are shown in panel B. ($p < 0.01$). The bar represents the s.d.

As one can observe in Fig. 14, it is obvious that an altered steady state of the ubiquitinated proteins occurs in DM2 myoblast compared to controls, further supporting implications of the UPS in the DM2 disease. We were also interested in further understanding the mechanisms underlying the results observed. A diminished amount of Ubiquitin-Protein conjugates, infact, suggests either an impairment of the ubiquitinations enzymatic machinery, or an augmented proteasomal functionality, able to more efficiently degrade the Ubiquitinated proteins. Administration of the proteasomal inhibitor MG-132 to the culture medium, could give some more indications about the processes involved. In treated DM2 myoblasts we found out increase of the conjugates after proteasomal inactivation, suggesting not only a higher efficiency, enhanced degradation rates for the proteasome

accomplishing its task, compared to control cultures, but also higher Ubiquitination rates, correlating with a sealed overexpression of CSN4 subunit of COP9 signalosome. This is a cellular multiproteic complex controlling the Ubiquitination process. The COP9 signalosome may play an important role in mediating E3 ubiquitin ligase-mediated responses since it is implicated in the regulation of cullin-RING type E3 ubiquitin ligases.

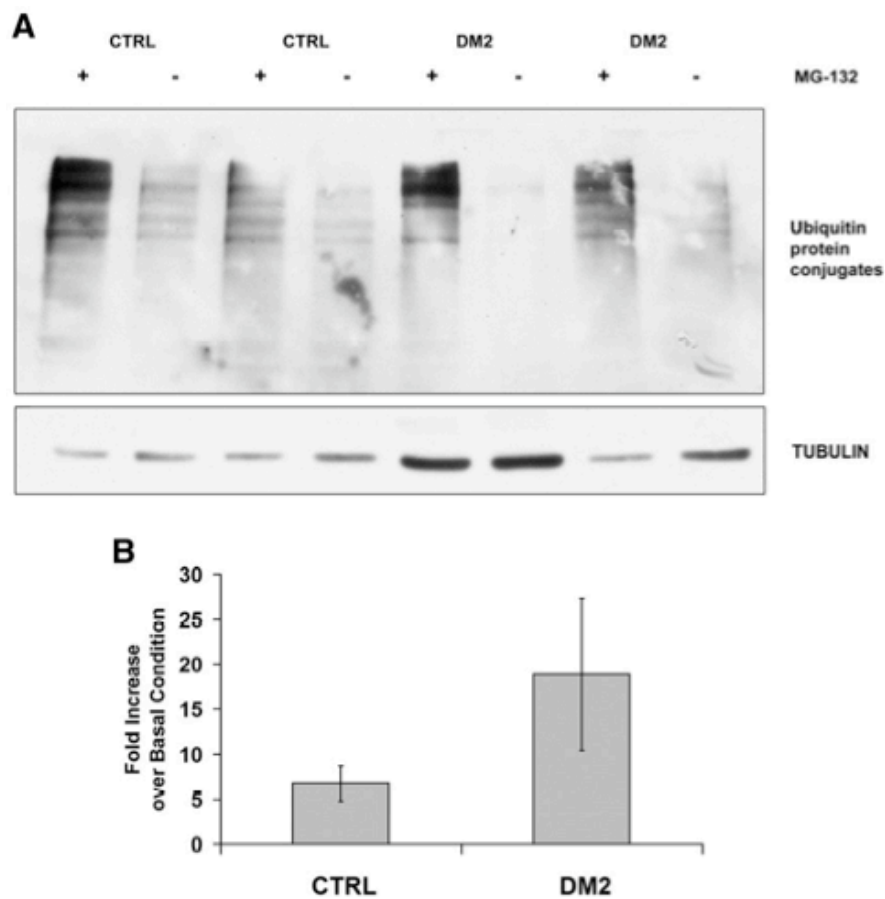


Figure. 15 Effect of proteasome inhibitor on the level of ubiquitinated cytosolic proteins. Control and DM2 myotubes were treated (or not) with MG132 (10 μ M) for 4 h. Cytosolic extracts were then prepared as described in Materials and methods and analyzed with antibodies against ubiquitin-protein conjugates and tubulin. A: a representative gel. B: Densitometric analysis: the average intensities of ubiquitinated proteins normalized for tubulin were determined for control and DM2 samples and the data were expressed as fold increase over the untreated condition. The quantification was done on 4 DM2 cultures and 4 control samples. The error bar represents s.d.

4.5 Evaluation of cultured fibroblasts as cellular model for the Mitochondrial and Degradative issues of the DM pathologies.

The data reported above with their interesting results and mechanistic objective to shed light on those cellular compartments, systems and functions affected in the DM2 myoblast, and hence hypothetically also candidate to play a role in the pathogenesis of the Human Myotonic Dystrophy, represent important preliminary results which anyway raise further questions.

In order to use DM2 myoblast from patients, biopsies from Biceps Brachii were obtained, the Satellite Stem Cells were expanded to Myoblasts, and then differentiated. The number of cells that can be obtained from patient's biopsies is limited and several analyses have to be performed on human material, as the diagnostic evaluation of the ribonuclear inclusions, by FISH analysis, the immunofluorescence attesting the MBLN-1 colocalization with the Foci, morphological assessments of atrophic condition, by muscle fiber diameter measurements and specific stainings. Human myoblasts undergo senescence after a reduced number of cultural passages. Obtaining a biopsy means a hard work which starts from convince the patients on the research importance, taking into account that a blood analysis is sufficient to diagnose the DM pathology. Last but not the least a biopsy means human pain. For all these reasons before planning a series of cellular and molecular experiments, it turns out to be wiser to search for an alternative cellular model to work on. The use patients cells is of primary importance. Fibroblasts represent most of the times a good choice to investigate the different human pathologies. The proliferative capability is higher in these cells than in myoblasts. Moreover multiple fibroblast extractions from the same skin fragment are allowed. Having the possibility to obtain a small piece of patient's skin, derived during the muscular biopsies, we started to evaluate cultured fibroblasts as a Human model for the study of Myotonic Dystrophy. The idea that prompted us to undertake this approach, is the presence in fibroblast nuclei, of the ribonuclear Foci indicating that the mutant RNA carrying the repeats amplification is expressed in this cell type. Moreover, fibroblasts can be converted to the myogenic lineage through MYO-D adenoviral infection, potentially reproducing in human myoblasts. These observations justified our efforts to create a fibroblast bank, including DM1, Congenital Myotonic Dystrophy (CDM), DM2 and control cells. The FISH technique was performed to verify the presence of the ribonuclear inclusions in the nuclei. Fig. 16 compares fibroblasts obtained from DM2 patients with control cells. The fluorescence probe used was

an antisense (CAGG)₅ complementary to a fragment of 5 CCUG repeats. It is possible to observe at the fluorescence microscope the of ribonuclear inclusions only in the skin fibroblasts from DM2 patients, and are absent in control cells.

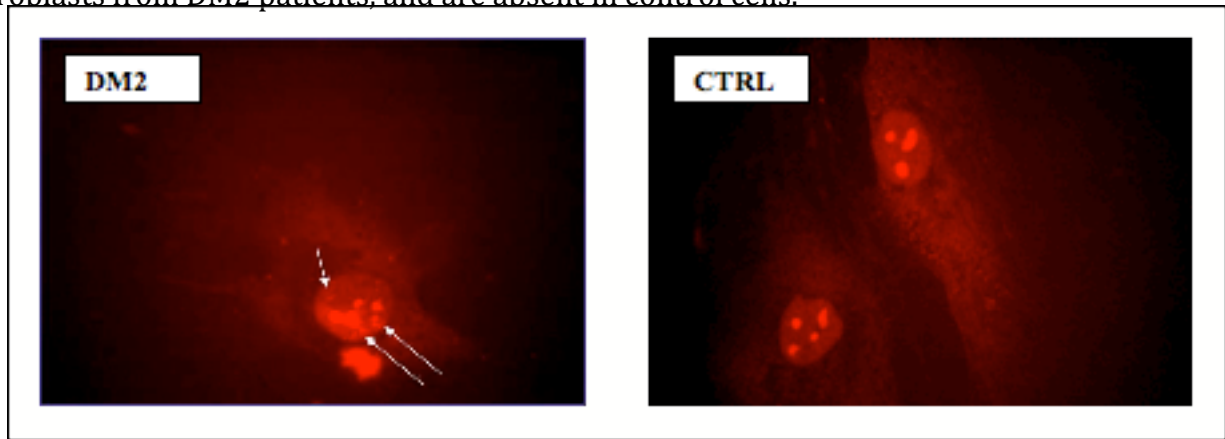


Figure. 16 FISH localization of ribonuclear inclusions. Foci can be observed in the DM2 fibroblast nucleus. Probe: (CAGG)₅ fluorophore, Texas Red.

We decided to test whether DM fibroblasts, had a functional impairment in the protein degradation processes. As we evaluated for DM2 myotubes.

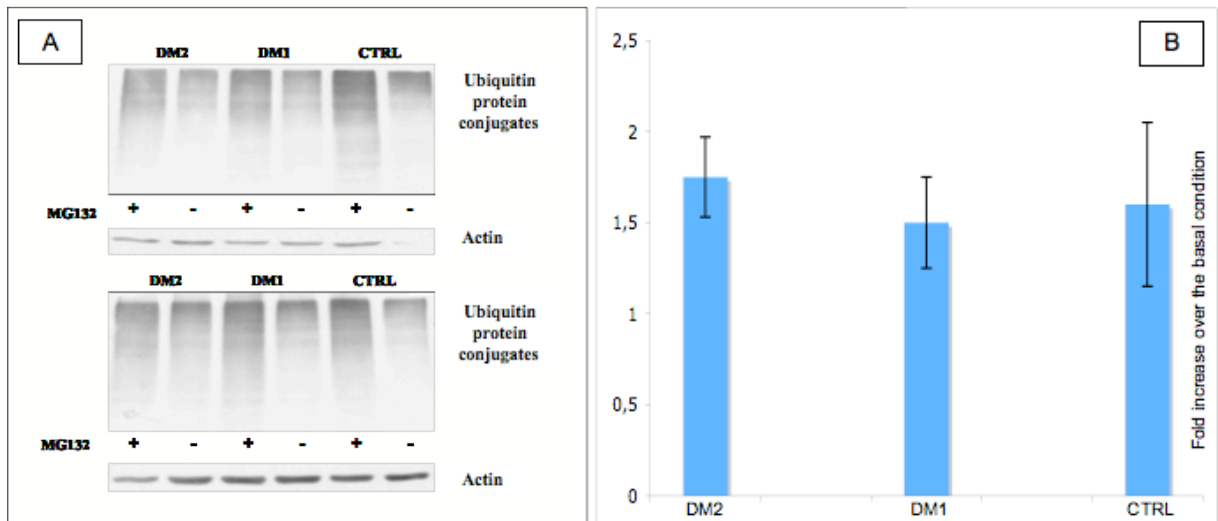


Figure. 17 Effect of proteasome inhibitor on the level of ubiquitinated protein. A total extract was analyzed. DM2 DM1 and control fibroblasts were treated (or not) with MG132 (10 μ M) for 4 h. A: a representative immunoblot. B: Densitometric analysis: the average intensities of ubiquitinated proteins normalized to tubulin were determined for DM2 DM1 and control samples and the data were expressed as fold increase over the untreated condition. The quantification was done on 4 DM2, 4 DM1 cultures and 4 control samples. The error bar represents s.d. Fibroblast obtained from DM2 and DM1 patients do not reveal an increased Ubiquitin-protein conjugates amount over the basal condition after MG-132 treatment compared to control fibroblasts.

A positive response would have been used to further understand the mechanisms underlying such an impairment in the new cellular model, like the behaviour of important muscular E3 Ubiquitin ligases like Atrogin-1 or MuRF-1, or the caspases-3 activation.

Fibroblasts obtained from DM1, DM2 and control patients were grown to 80% confluence and treated with MG-132 proteasomal inhibitor, (10 μ M for 4hs). Untreated control cells for all of the three experimental conditions were used in parallel. We extracted the total protein and performed SDS-PAGE. The immunoblot analysis was done using the antibody against the mono- and poly-Ubiquitinated protein conjugates, the results presented in figure 17 do not indicate an altered level of ubiquitinated conjugates over the basal condition after the MG-132 treatments. Differently from what observed in myoblasts it is possible that in the fibroblast model the presence of Ribonuclear inclusions (RNP) does not affect the degradation machinery.

Two major cellular systems were indicated by the proteomic analysis as possibly involved in the pathogenesis of the Myotonic dystrophy type 2. The Ubiquitin Proteasome System (UPS) and the Mitochondrial compartment. We noticed that while human myoblasts show a functional peculiarity, compared to controls, for what concerns the degradation processes, human DM fibroblasts do not follow a similar pattern either for Ubiquitin-conjugates downregulation or for higher increase of Ubiquitin-conjugates after blocking proteasomal activity. This suggests that proteasomal abnormalities could be triggered in a cellular specific fashion, depending on the myogenic genes, or on the genes specifically expressed in muscle cells. We are confident about the possibility in the future to evaluate proteasomal involvement at the level of the fibroblasts once these cells are adenovirally converted to the myogenic lineage, being this research our further planned step.

Mitochondria are involved in DM2 at the level of different gene products. As we already described above two isoforms with different isoelectric point of the Translation Elongation Factor Tu, a key enzyme acting in the metabolic mitochondrial process of the beta-oxidation of fatty acids, and two important HSP proteins, Mortalin and mtHSP60 turned out to be downregulated in DM2 myoblasts. A fast and interesting test to gain information on the mitochondrial functionality is the evaluation of the mitochondrial resistance to oxidative stress, in terms of release of cytochrome *c* from the mitochondrial inner membranes. Release of cytochrome *c* from mitochondria is therefore considered a key initial step in the apoptotic process, although the precise mechanisms regulating this event remain elusive. Mitochondria anyway have received considerable attention as principal source and target of ROS. Cytosolic superoxide may modulate cytosolic factors such as Bax, Bid, or caspase-8, all of which have been linked to the release of cytochrome *c* and the initiation of

apoptosis (Jurgensmeier et al., 1998; Narita et al., 1998). Bax mRNA has been shown to be up-regulated in insulin resistant mice, but more likely, the pathologic behaviour of DM2 mitochondria witnesses their inability to respond to a oxidative stress source. In order to preliminarily evaluate whether fibroblasts obtained from DM2 patients could present macroscopic mitochondrial differences with respect to control cells, we decided to test the mitochondrial membrane potential of our cells, using a

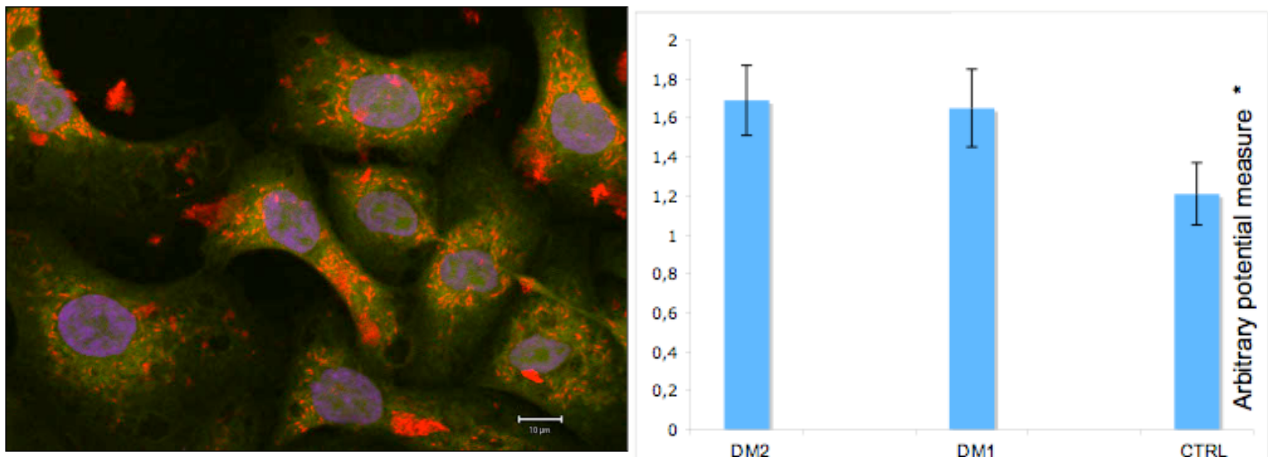


Figure. 18 Fibroblasts derived from DM2 and DM1 patients reveal a hyperpolarization of the membrane potential. The photograph shows a representative fluorescence microscope field, of fibroblasts treated with the JC1 compound. The histogram shows the ratio orange/green fluorescence arbitrary intensity* for DM2, DM1 and control patients derived fibroblasts.

colorimetric compound called JC1. This dye is capable to aggregate in a potential dependent manner. Being monomeric in the cytosol, it emits green fluorescence, and aggregates in the mitochondria in a potential proportional fashion, emitting on orange fluorescence. The JC-1 compound enables to normalize the data on the total amount of cytosol volume, because it is capable to enter the mitochondria, but it also stains the cytosolic compartment, where the signal emitted is green. This is important since the ratio of the orange/green fluorescence emitted, is a value independent on the number of cells, and the great number of cells evaluated at the fluorimeter renders a statistical randomization of the cytosolic emitted fluorescence even if the shapes of the cells, or the standard deviation of the cellular size are considerably wide. In our evaluation the tested fibroblasts from patient with respect to control show an average 35% hyperpolarization in the mitochondrial membrane potential, both from DM2 and DM1 patients as compared to controls.

We also treated DM2, DM1 and control fibroblasts with H₂O₂ to induce the release of cytochrome *c* from the mitochondria to the cytosol. Cells were fractionated to isolate cytosolic fractions that were analyzed by immunoblot analysis using antibodies against cytochrome *c*. As shown in Fig. 19,

H₂O₂ treatment induces the release of cytochrome *c* in the cytosol. This release was markedly higher for DM2 fibroblasts. However it is interesting to note that in DM2 mitochondria, the basal cytosolic cytochrome *c* is lower than that of DM1 cells. These results might suggest substantial differences between DM1 and DM2 mitochondria that requires further analysis.

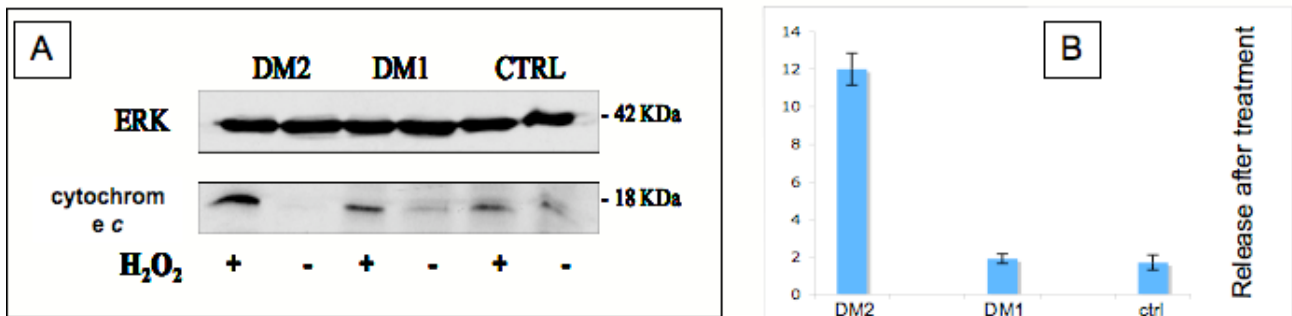


Figure. 19 – DM2 fibroblasts release more cytochrome *c* compared to DM1 and controls, after treatment with H₂O₂. Moreover the basal release of this small protein seems to be lower. Panel A: immunoblot image of cytosolic extracts of DM2, DM1 and control fibroblasts, treated and untreated with hydrogen peroxide, cytochrome *c* is evidenced thanks to the specific antibody. Panel B: densitometric obtained intensity comparison normalized to the ERK intensity. The histogram represents the ratio between the cytochrome *c* signal after the treatment with peroxide, and the basal release of this protein. The experiment was performed three times on the same fibroblasts extracts (Peroxide treatment: 4 hs, 1mM). Bars represent s.d.

5. DISCUSSION

In this study we have undertaken a proteomic approach to identify functional differences at the protein level between DM2 cell cultures and control ones. Proteome analysis of skeletal muscle cells has been used under physiological and pathological conditions, however such a study has never been undertaken to analyze the Myotonic Dystrophy pathology. We have used satellite cells from DM2 biopsies according to the criteria set by the International Consortium for Myotonic Dystrophies (Moxley, et al. , 2002). The cells were grown *in vitro* and induced to differentiate into myotubes. This cellular model retains many of the features of adult DM2 muscle cells with the advantage to allow a wide screen proteome analysis not possible with the small dimension of the biopsies. Our proteome analysis revealed that 25 spots were altered in DM2 cultures as compared to control ones. Of these 7 were isolated in the nuclear enriched fraction while 18 were mainly cytosolic proteins. Interestingly, these changes were statistically significant, even if the biopsies from which the cells were derived were neither matched for age, gender or length of the microsatellite expansion. It is also possible that using satellite cells (i.e muscle-specific stem cells) induced to differentiate *in vitro* we worked with a more homogeneous population, thus reducing the differences. Interestingly, for some of the spots that we did not analyze (since they were not statistically different in the wise pair comparison) we also found spots that were highly similar in their volume in the control condition but significantly divergent in DM2 cultures (see the results for EFTs). A greater sampling and a improved sorting of DM2 patients may be relevant to this issue. Our analysis was done by fractionating the cellular extracts in a cytosolic/membrane (Triton X-100 soluble) enriched fraction and a nuclear one to enrich for low abundance proteins. By this approach we have however discarded the cellular Triton insoluble fraction that contains mainly sarcomeric proteins (Myosin, etc) and of which we did not carry out any functional proteome comparison. The proteome analysis points to variation in the spot volume in some cases also greater than two-fold with a general tendency to a reduction rather than increase as compared to control cultures. The proteins identified by mass spectrometry were 17 out of 25. The overall picture that emerge from our analysis is that the proteins changing their level of expression can be clustered in a reduced number of functional categories. As shown in figure 11 we found proteins involved in mitochondrial biogenesis and function, proteins of the degradation machinery and proteins involved in the protein folding. Among the proteins involved in folding machinery of the ER we detected reduction of two isoforms of protein disulfide isomerase (PDI-A3), a chaperon protein that mediates protein folding by promoting formation of disulfide bonds. In parallel we found an increase of ERp29, another chaperone protein of the ER that does not have enzymatic activity and is usually induced following ER stress. Secretory proteins become folded and acquire stabilizing disulfide

bonds in the ER. Correct disulfide bond formation is a key step in ER quality control, where proteins with incorrect disulfide bonds are recognized by the quality control machinery and are retrotranslocated into the cytosol where they are degraded by the proteasome (Ellgaard et al., 1999). Reduced expression of PDI may impair the normal capability of the ER to correctly fold the ER proteins, while ERp29 level increase is consistent with the Dr. Timchenko's group results (Salisbury et al. 2009) concerning the increase of Bip, and its concomitant sequestration by CCUG the repeats amplified RNA, suggesting issues in the Unfolding Protein Response by the DM2 myoblasts ER. Our results also indicate changes in proteins involved in the ubiquitin-proteasomal system. We found increase of 26S proteasome non-ATPase regulatory subunit 13, as well as for a subunit of the 19S proteasomal regulatory particle that binds, unfolds and translocates ubiquitinated proteins into the proteasome core (Coux et al., 1996). Also a subunit of COP9 signalosome complex, part of a regulatory particle of the ubiquitin-proteasomal system whose function has not yet completely defined (WeinandDeng, 2003), is increased in DM2 cultures. It seems that COP9 signalosome could be implicated in the regulation of the ubiquitination process via the control of E3 ubiquitin ligases, as we discussed in the Introduction. In parallel we found reduction of one of the catalytic subunits of the 20S core (alpha-6) indicated as a sequestered protein as well by Salisbury et al. in 2009. A possible explanation is that we evaluated this protein as reduced because during the fractionations performed prior to our analysis large RNA protein complexes are pelleted and discarded as insoluble factors. Also RD23B, an ubiquitin receptor that binds ubiquitinated proteins and delivers these proteins to the regulatory proteasomal particle (Dantuma et al., 2009) turned out to be downregulated. We can speculate this downregulation as a cellular attempt to regulate an augmented proteasomal activity. The picture emerging from our results is at the moment far from being clear. However, alteration in the level of cytosolic ubiquitinated proteins in DM2 cultures are consistent with a modified ubiquitin mediated degradative machinery. Alteration in the ubiquitin-protein conjugates has also been recently reported (Salisbury et al., 2009) although in that case an increase rather than decrease has been observed. However, differences in the experimental protocols (isolation of cytosolic proteins in the presence of 0.5% detergent in our case as compared to total extract as reported by (Salisbury et al., 2009) could justify the discordances. Alteration in the ubiquitin-mediated proteasomal system may impair the degradation machinery affecting protein stability. Although our present results do not allow to define if this is the case, very recent work (Huichalaf et al., 2009) has demonstrated reduction of the rate of protein translation in patients with myotonic dystrophy 2. An altered degradation machinery in DM2 myotubes cultures it is not completely unforeseen. In fact, the ubiquitin-proteasome proteolytic system is involved in muscle wasting (Attaix et al., 2005, Murton et al., 2008) which is one of the features of dystrophic muscles. It is feasible that the altered splicing of

insulin receptor may affect insulin mediated signaling acting on the proteasomal degradation machinery. It is also possible that the alteration in the level of proteins involved in protein degradation may become an early biomarker for the wasting process. The mitochondria is probably the most relevant compartment for which we found a significant reduction in the protein profile of DM2 cultures. The proteins identified belong to different biological processes required for the biogenesis and function of the mitochondria. Among the proteins we detected, two spots (possibly postrationally modified proteins as reported by He et al., in 2001) were identified as EFTu. For both of them we found a global reduction further confirmed by western blotting. EFTu is a nuclear encoded protein that plays a major role in mitochondrial translational machinery. Mutation and/or reduction of this protein has been detected in infantile encephalopathy (Valente et al., 2007) with muscular hypotonia and degradation of motor skills. EFTu is a GTPase that participates in the formation of the ternary complex (EFTu:GTP:aminoacyl tRNA). The switch between an active (GTP-bound) and inactive (GDP-bound) forms requires the EFTs factor and the efficiency of mitochondrial translation is critically dependent on the relative ratios of these forms and other mitochondrial translation elongation factors. Interestingly our data points to a constant ratio between EFTu and EFTs in control cultures while the relative reduction of EFTu in DM2 cultures is not associated with the reduction of EFTs level. As reported in fig. 13, in the DM2 cytosolic extracts the level of EFTs fluctuates, thus causing the Tu/Ts ratio to be highly variable. Interestingly, alterations in the EFTu/Ts ratio has been reported in patients with fatal hepatopathy (Coenen et al., 2004). Future experiments will allow to understand if the changes in EFTu depicted in DM2 cultures affect the mitochondrial translation efficiency. Among the mitochondrial proteins found altered in DM2 cultures, we detected a reduction of the mitochondrial Heat Shock Protein 60 and Glucose-Regulated Protein 75 (also referred to as Stress-70 Protein). These proteins participate in the mitochondrial translocation reaction of matrix-target precursor proteins and in protein folding within the mitochondrial matrix, assisting in the maintenance of mitochondrial proteome integrity (Rassow et al., 1999). Together these two proteins perform mitochondrial import, chaperoning and quality control functions in mitochondria.. We also depicted a reduced level of delta dienyol-Co-A isomerase, a mitochondrial enzyme involved in beta oxidation of odd fatty acids. Therefore it is possible that folding, import processes, and metabolic processes necessary for the bioenergetic function of the mitochondria are altered. Interestingly, we found an increase of-trimethylaminobutyraldehyde dehydrogenase, an enzyme involved in the biosynthesis of carnitine a mitochondrial carrier for fatty acid and this might be a compensatory response to the reduced level of some mitochondrial proteins. In conclusion our functional proteome analysis suggests that other molecular functions and biological processes, in addition to spliceopathy, are affected in the

DM2 disease. The effects of CCTG repeat expansion extend therefore beyond spliceopathy to include a more general disturbance leading to changes, important for the physiological role of the muscular tissue. Further studies on the role of both mitochondria and the ubiquitin-proteasomal machinery may lead to the development of new therapeutic approaches that can be useful to block or partially prevent muscle wasting in human DM2 patients. To this extent we evaluated Human DM fibroblasts as a model system for further investigations, directed to a better understanding of the Mitochondrial and Degradation-related problems potentially underlying the DM2 pathology. We produced a fibroblast bank from an average of 10 DM1, DM2 and control patients. Several cryotubes filled with cells were stored for future studies. Our preliminary data showed no correspondance between the degradation abnormalities displayed by DM2 myoblasts, and the fibroblasts degradative behaviour. In particular, while myoblasts from patients affected showed diminished amounts of Ubiquitin-protein conjugates in the basal condition, and a higher increase of the levels of these conjugates after MG-132 proteasomal inhibitor treatment, the fibroblasts did not. We are confident on the MYO-D adenoviral conversion to the muscular lineage of the fibroblasts in order to obtain a good model to study this feature. The use of fibroblast cultures to study possible mitochondrial issues is definitely more promising, since not only this patient's cell type displays a different steady state of the membrane potential, but also shows a differential ability to respond to hydrogen peroxide mediated oxidative stress. A marked release of cytochrome *c* from the mitochondrial inner membrane is readily released in DM2 fibroblasts, at peroxide concentrations able to elicit a basal release from DM1 and control cells, suggesting a deep difference between the two pathologies, at the level of the mitochondrial involvement.

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1^b. ABSTRACT

*GSK3 beta, a potential target in Myotonic Dystrophy type 1. ****

Differentiation of skeletal muscle depends at least in part on the formation of a multiprotein complex. Cyclin D3 is the only cyclin supporting growth arrest and differentiation. Hypophosphorylated pRb binds to Cyclin D3 protecting this protein against GSK3beta degradative phosphorylation and promoting the formation a complex, capable to sequester Cdk4 and PCNA. This triggers the irreversible exit from the cell cycle and promotes specific differentiative gene expression. The differentiation process is impaired in DM1 primary myoblasts. CUGBP1, is a multifunctional protein involved in splicing control in the nucleus, mRNA stability, and translation rate in the cytosol. While its level increases during normal myoblast differentiation, in DM1 cells it does not. Moreover, it fails to bind eIF2 and in turn is not able to promote translation initiation of important gene products involved in the differentiation process. Phosphorylation at different sites drives CUGBP1 to bind specific proteins and hence mRNAs targets. CyclinD3/cdk4 complex is responsible for the addition of a phosphate at CUGBP1 Ser-302 committing this protein to the differentiation task. In DM1 cells, CyclinD3 and Cdk4 do not increase their level and possibly do not phosphorylate CUGBP1.

To better understand the relationship between DM1 CTG microsatellite amplification and the differentiation issues of the disease, and to assess whether or not the differentiation problem could be defined as early pathogenic event, we used CTG 914 inducible HeLa stable clones. With these cells we found that the expression of the amplification itself is sufficient to downregulate the level of Cyclin D3 and Cdk4, in both cytosol and nuclei. Moreover we used a transgenic mouse model expressing the CTG amplification to test if the mutation could also affect the levels of GSK3beta. We found a strong overexpression of GSK3beta in skeletal muscle, and of both mRNA and protein. This overexpression could be pivotal for the impairment of Cyclin D3 turnover and for the abnormal development of myotubes. Taken together, these data suggest a correlation between the expression of the CTG amplification and the impairment of the differentiation machinery in both cellular and animal models of the DM1 pathology.

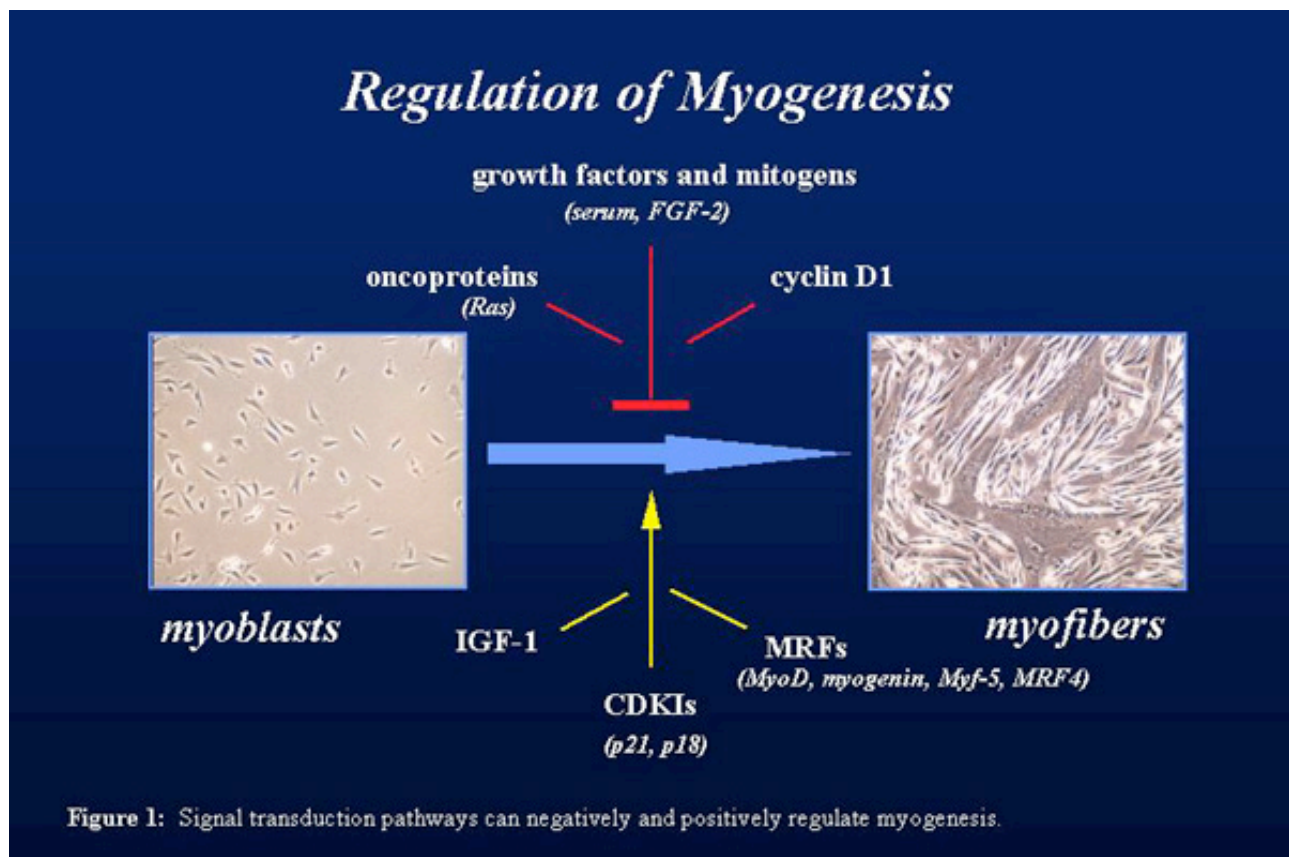
Keywords: Muscle differentiation, pRB, Cyclin D3, GSK3 beta, Myotonic Dystrophy type 1, CTG repeats.

****This work was performed in the Laboratory of Professor Lubov Timchenko, at the Baylor College of Medicine, Houston, (TX) USA.*

2^b. INTRODUCTION

2.1 Differentiation overview:

Skeletal muscle differentiation involves terminal withdrawal from the cell cycle, induction of muscle-specific gene expression and fusion of myoblast into polynucleated myotubes. For the majority of the cell types, including myoblasts, proliferation and differentiation are mutually exclusive events. Muscle specific transcription factors, belonging to the MyoD family, are responsible for the initiation and the maintenance of the myogenic program. Muscle regulatory factors (MRFs) are bHLH transcription factors. They promote differentiation by binding specific consensus sequence (E-box) at the regulatory regions of muscle-specific genes^{1,2,3}. For what concerns the induction of the cell cycle arrest, we know that MyoD induces transcription of Retinoblastoma (pRb), that can mediate the induction of transcription of the cell cycle inhibitor p21. Muscle differentiation is associated with induced expression of pRb⁴, and with enhanced nuclear affinity in its active hypophosphorylated state.



Rb is known to be inactivated by the Cyclin dependent kinases (Cdks) phosphorylation. These kinases act in conjunction with the Cyclins^{5,6}, which display a regulatory activity on the kinase and promote the progression of the cell cycle. The expression of the majority of the Cyclins is downregulated during terminal differentiation of muscle cells. This brings about the arrest of the cell cycle progression and the entry in a G₁/G₀ state. Only Cyclin D3 acts differently. Since its expression is induced during terminal differentiation.^{7,8} Cdks are negatively regulated by the Cdk. These inhibitors can bind either Cdks or Cyclin/Cdks complexes blocking their activity and blocking therefore the cell cycle. p21 is an important Cdk inhibitor which can also function as a direct DNA polymerase inhibitor by binding the Proliferating Cell Nuclear Antigen (PCNA)⁹.

Cenciarelli et al.¹⁰ reported a direct involvement of the hormone-activated MyoD, in the induction of Myogenin, p21, pRb, and Cyclin D3. They proposed a model in which MyoD activates target genes by two consequential molecular mechanisms, the first requiring p300 and the second requiring pRb. p300 is essential for the direct induction of MyoD itself, pRb, p21 and Cyclin D3 which are referred to as early differentiation markers. pRb is required for Myogenin-mediated induction of late differentiation genes as well for the irreversible cell cycle arrest needed for the terminal differentiation¹⁰.

With regard to Cyclin D3, Cenciarelli et al. reported that in differentiating C2C12 myoblasts not only that Cyclin D3 mRNA is induced, but also a stabilization of Cyclin D3 at the protein level occurs. Protein accumulation requires the function of pRb, since pRb^{-/-} cells induced to differentiate fail to accumulate Cyclin D3 protein, despite a normal induction of Cyclin D3 mRNA. The low level of Cyclin D3 in differentiating muscle cells lacking pRb is supported by the intrinsic instability of the protein, since the administration of proteasomal inhibitors increased the steady state levels of this Cyclin. In addition this data shed some light on the role of pRb in the protection of Cyclin D3 from the Ubiquitin-proteasome mediated degradation. But which is the pathway that controls the Cyclin D3 levels in myogenic cell lines? What about the mechanisms underlying the pRb protection toward Cyclin D3? To address these questions, De Santa et al¹³., worked to understand the rules governing the formation of the multiprotein complex in which the bulk of Cyclin D3 is pRb-associated. Their results suggest that Cyclin D3 acts as a bridging molecule, allowing pRb to sequester inactive Cdk4 complexes as well as PCNA into insoluble nuclear structures. They discovered the important role of GSK3beta, a multifunctional protein kinase, initially identified as a regulator in insulin-dependent glycogen synthesis. Over the last decade it turned out that GSK3beta is involved in a number of functions, including the regulation of cell fate (role in the Wnt and Hedgehog signalling pathways), signal transduction, protein synthesis, mitosis

and apoptosis¹¹. Ubiquitin-mediated proteasomal degradation of Cyclin D3 is triggered by the phosphorylation of Cyclin D3 Thr-286, since replacement of Thr-286 with Alanine (T286A) markedly stabilized this protein, preventing it from polyubiquitination. Interestingly, GSK3beta is responsible for this phosphorylation at least *in vitro*¹². Moreover the treatment of pRb negative myoblasts with LiCl a specific inhibitor of GSK3beta, resulted in Cyclin D3 stabilization, and could further prolong the half-life also of Cyclin D3 in pRb^{+/+} myocytes, indicating additive effects of pRb and GSK3beta inactivation on this stabilization¹². GSK3 beta is normally active in cells, and is physiologically regulated through inhibition of its activity in response to multiple signals¹³, the most of which are the insulin, and insulin-like growth factors. Insulin-like growth factors potentially stimulate muscle differentiation and skeletal muscle hypertrophy through the activation of the PI3K/Akt pathway¹⁴. Akt is a serine-threonine protein kinase that phosphorylates a number of substrates including GSK3beta which is in turn inactivated. It is worth mentioning that a classic clinical feature in both DM1 and DM2 patients is insulin resistance¹⁵. Alternative splicing of the insulin receptor (IR) pre-mRNA is aberrantly regulated in DM1 skeletal muscle and exon 11 is preferentially excluded, resulting in predominant expression of the insulin insensitive splice form, IR-A¹⁶.

2.2 DM1 differentiative issues and role for CUGBP1

It has been proposed that impaired differentiation of skeletal muscle is one of the critical events in DM1 muscle cells²⁴. The expression of mutant DMPK mRNA with long CUG repeats, alters the physiological behaviour of CUGBP1^{17,18}. This multifunctional RNA binding protein is involved in the regulation of multiple RNA processing stages: from the cap-dependent and cap-independent translation, to RNA stability and splicing¹⁹. Investigations in mouse model of the DM1 pathology showed an overexpression of CUGBP1 leading to impairment of muscle development and differentiation²⁰, Muscular Dystrophy, and Myotonia²¹. During differentiation the complex myogenin/E2/p300 binds to CUGBP1 promoter E-box, increasing its transcription²². Important target gene for CUGBP1 are p21, a Cdk inhibitor as described before, Myocyte enhance factor 2A (Mef2A), Insulin receptor and the Chloride ion channel.

CUGBP1 is phosphorylated by different kinases and specific phosphorylation of this protein changes its RNA binding affinity toward different mRNAs: Akt phosphorylates CUGBP1 at Ser-28 and this particular posttranslational isoform of the protein shows a higher affinity for Cyc D1 mRNA. On the contrary, phosphorylation by Cyclin D3-Cdk4 increases the affinity of CUGBP1 for p21 and C/EBPbeta²³. Studying protein-protein interactions, Salisbury et al.²³ found that during myoblasts proliferation, CUGBP1 interacts with active Akt while, during differentiation, it binds to

Cyclin D3/Cdk4. Moreover, the phosphorylation by CyclinD3/Cdk4 increases the affinity of CUGBP1 for eIF2, a factor mediating translation initiation. All these data shed some light on the role of Cyclin D3 as one of the critical regulator of CUGBP1 activities in myotubes. A possible correlation between the lack of early muscle differentiation markers expression and low or undetectable levels of Cyclin D3 in pRb^{-/-} myoblasts, is supported by the incorrect phosphorylation of CUGBP1 and hence the consequent inability to work properly during the differentiation process.

In particular, proliferating DM1 cells show a much stronger CUGBP1/Akt interaction than that found in control cultures. Moreover, while normal differentiation of control myoblasts is characterized by increased levels of Cyclin D3 and of Cdk4, and a corresponding increase of CUGBP1/eIF2 complex formation, DM1 cells do not display such an increase. The lack of elevation of Cyclin D3 in DM1 cells seems to be a critical event leading to impaired myoblast fusion, since normalization of these levels corrects early step of differentiation²³.

2.3 GSK3beta implications in the Cyclin D3-CUGBP1-eIF2 pathway

Studies performed in mice liver revealed a critical role for GSK3beta in the epigenetic regulation of the hepatocytes proliferation via reduction of Cyclin D3/Cdk4 complex. Cytoplasmic Cyclin D3/Cdk4 complex interacts with CUGBP1 triggering its phosphorylation at Ser-302, and this promotes its binding to eIF2²⁴. The accumulation of Cyclin D3 in liver of old mice, which leads to a dramatic elevation of the CUGBP1/eIF2 complex²⁵, is accompanied by reduced levels of GSK3beta. This correlates with activation of Cdk4 and CUGBP1 hyperphosphorylation. On the contrary, in the liver of young mice GSK3beta phosphorylates Cyclin D3 at Ser-283 which causes its degradation via 26S proteasome. Interestingly, the GSK3beta pathway takes place not only in the liver, but also in lung, brain and adipose tissue. But which is the biological meaning of the binding between eIF2 and CUGBP1? This complex increases the translation of two proteins, C/EBPbeta and HDAC, involved in the epigenetic control of translation. These proteins work by repressing the translation of specific target genes among which GSK3beta itself²⁶. In fact, the GSK3beta promoter contains three C/EBPbeta binding sites which are located in close proximity of the transcription initiation site. C/EBP/HDAC1 represses transcription by the deacetylation of the K9 residue of Histone H3 and subsequent trimethylation: the analysis of Histone H3 at the level of the GSK3beta promoter shows this pattern of repression.

In this work we tried to clarify the relationship between the expression of the CTG-914 amplification, and the deregulation of proteins such as Cyclin D3, pRb, Cdk4 and GSK3beta, participating in the differentiation process. We used stable clones of HeLa cells expressing the

amplification in an inducible manner, and showed how the CTG amplification by itself is sufficient to reduce the Cyclin D3 and Cdk4 levels in both cytosol and nuclei. We also found overexpression of GSK 3 beta both at the level of mRNA and protein, in muscles of a transgenic mouse model of DM1. These results can suggest a possible mechanism which could be responsible for the impairment of the differentiation process in DM1.

3^b. MATERIALS AND METHODS

3.1 Cell Culture

The double-stable HeLa cells expressing CUG-914 were grown in DMEM containing 10% tet-free FBS (Clontech Laboratories, Mountain View, CA, USA) supplemented with Geneticin and Hygromycin B to 80% density. Cytoplasmic and nuclear fractions were collected from cells in 100 mm plates, either without the addition of DOX or maintained for 48 hours in medium containing DOX (500ng/ul). Expression of GFP and CUG-914 RNA was verified by fluorescent analysis and by northern blot. C2C12 myoblasts were cultured in F10 medium (Gibco), containing 15% FBS, 5% defined complemented calf serum (Hyclone) and 1% penicillin/streptomycin, the myoblast growth medium was changed after two days. In order to promote differentiation, myoblasts were grown to 80% confluency, and at that point the growth medium was replaced with fusion medium, containing 2% Horse serum, 0,01M insulin in DMEM. The fusion medium was replaced every day. The cells were differentiated five days.

3.2 Subcellular fractionation

The HeLa double-stable clones were lysed in proper conditions to obtain two fractions: one fraction enriched in cytosolic/membrane proteins, and one in nuclear proteins. The cells, scraped and pelleted, were treated with a hypotonic buffer (10 mM Hepes pH 7.5, 0.5% NP-40, 10 mM KCl, 1 mM EDTA, 1 mM DTT, additioned with proteases and phosphatases inhibitors). After 15 min of mild agitation, cells were centrifuged at 400X g at 4 °C. The supernatant was then centrifuged at 15,000X g to clarify the extract and the supernatant, representing the cytosolic/membrane enriched fraction, was collected. The pellet obtained from the 400X g centrifugation was treated with a hypertonic buffer, (20 mM Tris-HCl pH 7.5, 0.6 M NaCl, 0.2 mM EDTA, 1.4 mM MgCl₂ 25% glycerol, and proteases and phosphatases inhibitors) with mild agitation for 15 min at 4 °C and then centrifuged at 80,000X g at 4 °C. The supernatant was collected, representing the nuclear enriched-fraction.

3.3 HPLC

Nuclear extracts of HeLa stable clones treated and untreated with DOX were subjected to size exclusion chromatography, on a SEC400 column, precalibrated with molecular weight markers. The chromatography fractions (300 ul) were analyzed by western blot.

3.4 Immunoprecipitation

Immunoprecipitation was performed using e-Bioscience TrueBlot. Mouse IgG TrueBlot ULTRA is ideal for use in protocols involving immunoblotting of immunoprecipitated proteins. TrueBlot

preferentially detects the non-reduced form of mouse IgG over the reduced, SDS-denatured form of IgG. When the immunoprecipitate is fully reduced immediately prior to SDS-gel electrophoresis, reactivity of Mouse IgG TrueBlot ULTRA with the 55 kDa heavy chains and the 23 kDa light chains of the immunoprecipitating antibody is minimized thereby eliminating interference by the heavy and light chains of the immunoprecipitating antibody in IP/immunoblotting applications. 500ug-1mg of total protein obtained from homogenization of i) HeLa stable clones, treated and untreated with DOX, ii) C2C12 myoblasts used as positive control, iii) Mice biopsies (muscle MIX) (buffer 50mM Tris-HCl pH 8.0, 150mM NaCl, 0,5-1% NP-40). 1-10 ug of Cyclin D3 antibody (Santacruz biotechnology, C-16 sc-182) was used to immunoprecipitate Cyclin D3, while western blot was performed using anti pRb (Santacruz Biotechnology, C-2 sc-74562 1:600)

3.5 Western blot analysis

Western blot analysis were performed with cytoplasmic and nuclear proteins, as described previously, 20 to 80 ug of proteins were separated by gel electrophoresis, blotted on a nitrocellulose membrane, and and incubated with different antibodies. Polyclonal anti-Cyclin D3 (sc182, C-16) 1:600; polyclonal anti-pRb, (sc50,C-15) 1:700; monoclonal anti-GSK3alpha/beta (sc7291, 0011-A) 1:1000; polyclonal anti-Cdk4 (C-22, 1:750) polyclonal anti-alphaTubulin (H-300 5546) 1:1000, were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Monoclonal anti-betaActin antibodies (AC-15) were from Sigma-Aldrich (St.Louis, MO, USA)

3.6 Semiquantitative RT-PCR

RNA was extracted from transgenic mice muscle biopsies (200-CTG in the 3'UTR of (HSA) Human Skeletal Actin) gene, using TRIzol reagent, (Invitrogen). Total RNA was run to determine the degradation state, and the 28S and 18S ribosomal subunits were observed. RNA retrotranscription was performed using *Taqman* reverse transcription reagents kit (Applied Biosystems, Baranchburg, NJ) with 10 ng of starting RNA (1ul). The RT-PCR was performed at 93°C, 57°C, 72°C for 20 cycles using 1,2 and 4 ul of c-DNA from the retrotranscribed total mRNA, and Red-MIX (Roche) PCR-buffer, on a total volume of 25 ul each tube. GSK3beta was analyzed with the specific primers whose sequence is: forward, 5'-TTCCTTTGGAATCTGCCATC-3', reverse, 5'-TCAGTGCCTCCAAAGATCAA-3'. The GSK3beta production was normalized by the analysis of GAPDH expression with the following primers: forward, 5'-AACTTTGGCATTGTGGAAGGGCTC-3' and reverse, 5'-TGGAAGAGTGGGAGTTGCTGTTGA-3'.

4^b. RESULTS

4.1 Expression of the CTG amplification in HeLa cells affects the expression level of the differentiation complex Cyclin D3/pRb/Cdk4 core components.

Cyclin D3 and Cdk4 are consistently expressed in both cytosolic and nuclear fractions of HeLa cells, C2C12, Cos7 cells (data not shown) while pRb is highly expressed only in the nuclear fractions of these cell types. In this study we used HeLa CTG-914 double stable clones which transcribe the CTG amplification in an inducible fashion, after somministration of Dox (Doxycyclin) according to TET-ON technology. It is worth noting that the CTG amplification lays in a different position at the level of the DNA, compared to the human mutation, and this give the possibility to study the effect of this simple mutation, without the interference of the positional implications of it. The CTG amplification is under the control of a CMV promoter.



Figure.2:

Schematic representation of the CUG-914, CUG-25 cassette in HeLa stable clones. rtTA: Tetracyclin controlled transactivator.

We decided to investigate whether or not the differentiation complex components could be influenced at the level of protein expression, by the induction of the CTG-914 transcription, in this HeLa double stable clones. Surprisingly we found a strong decrease of Cyclin D3 level following CTG-914 Dox-induction, indicating a tight correlation between the mutation and the physiological behavior of this protein both in the cytoplasmic environment and in the nucleus. It is interesting to appreciate that the major decrease in the levels of Cyclin D3 affects the nuclear fraction, while a minor decrease is evident in the cytosol. As we expected, from the Cyclin D3 total extract asset after DOX treatment, we detected an average decrease with respect to untreated cell culture extract. This result provide a further proof of the statistical robustness of the data presented, which anyhow display a $p < 0,001$. We can speculate a functional implication of the different fold increase affecting the Cyclin D3 levels in the different subcellular fractions taken into consideration, since diverse localizations of these proteins correspond to different functions and roles as described under Introduction.

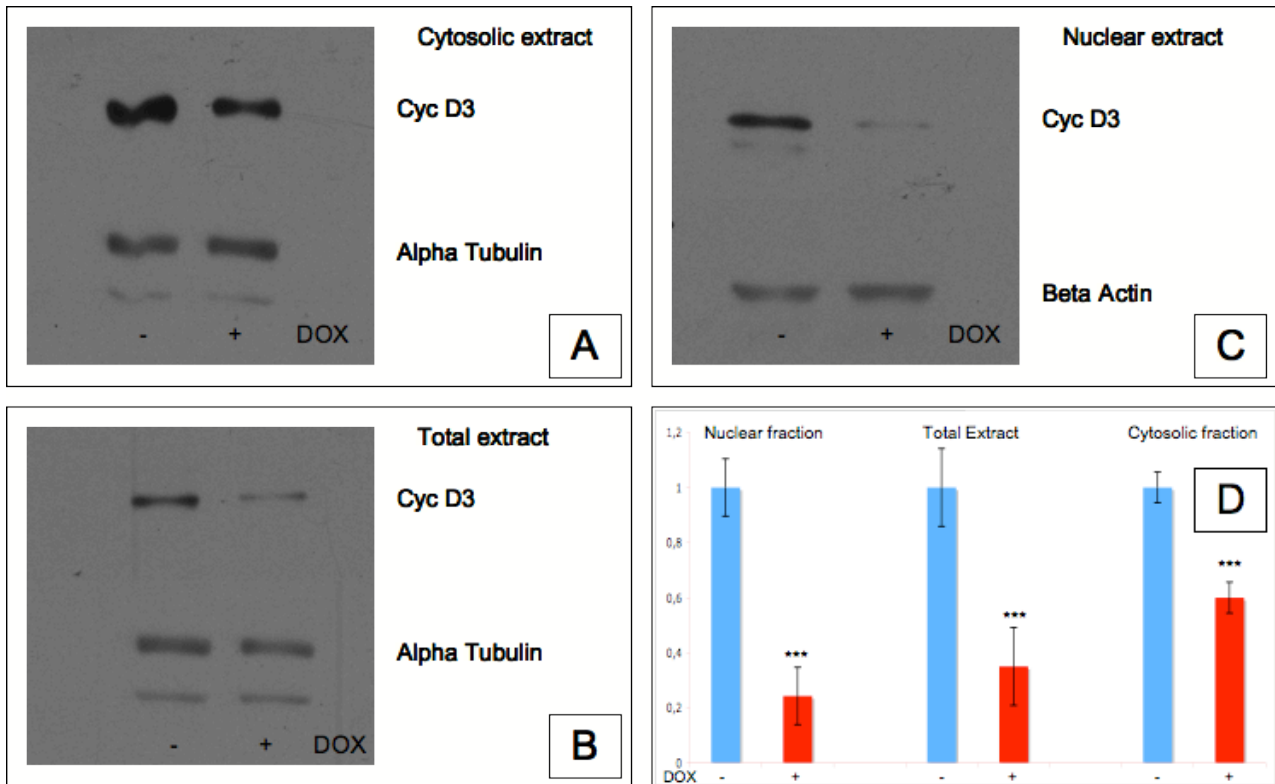


Figure. 3: Panels A, B, C show respectively immunoreactive bands of Cyclin D3 in Cytosolic, Total and Nuclear extracts of HeLa double stable clones expressing the CTG amplification and untreated with Doxycyclin. Panel D shows the average intensity level of Cyclin D3 after normalization with the internal control. 4 different cell extracts (treated and untreated with Dox) were evaluated for the Cyclin D3 levels ($p < 0,001$).

Since the Cyclin D3/pRb/Cdk4 complex which is present in C2C12 cells, promotes the withdrawal from the cell cycle and the induction of early muscle differentiation markers, and involves a protective role of pRb toward Cyclin D3, we decided to estimate the levels of Retinoblastoma

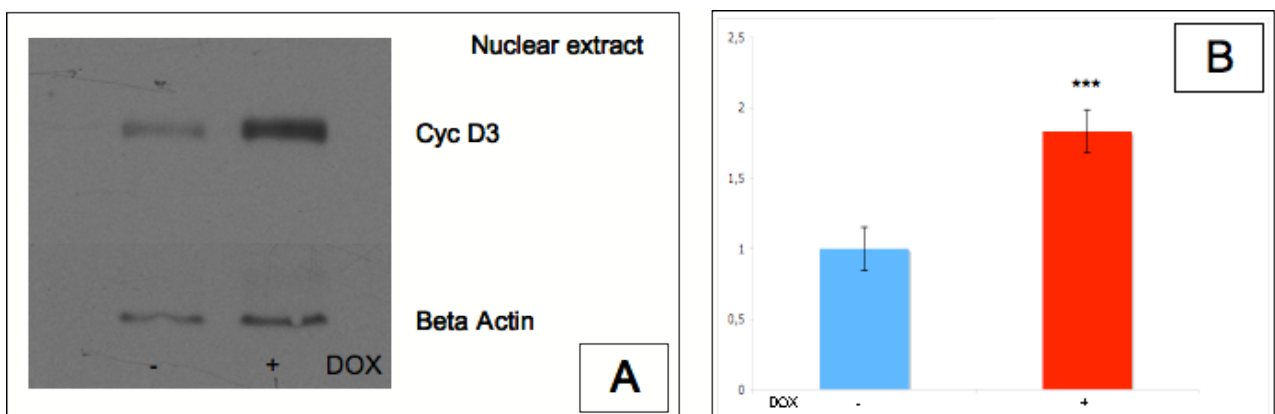


Figure. 4: Immunoblot analysis showing the pRb levels dependence on the CTG amplification expression (panel A) in HeLa double stable clones. Average intensity levels of pRb respectively with and without the Dox treatment, after Beta Actin normalization are shown in panel B, 3 cellular enriched nuclear fractions either treated or untreated for the expression of the CTG-914 amplification were used ($p < 0,001$)

protein and whether this level could be perturbed by the CTG expression on HeLa stable clones. We found an effect on the pRb levels, which showed a consistent overexpression following amplification expression.

We proceeded by analysing the levels of CDK4, the third protein known to play a role in conjunction with cyclin D3 in the phosphorylation of CUGBP1, at Ser-302. CDK4 commits it to accomplish its roles in the differentiation process of the muscle cells. It was very intriguing to find out a very similar trend with respect to Cyclin D3. As one can observe in figure 5, while the cytosolic levels of this protein underwent a slight decrease tendency following the translation of the CTG amplification, the nuclear enriched proteic fraction showed a dramatic decrease of the expression of Cdk4 when the mutant RNA was present in the cells.

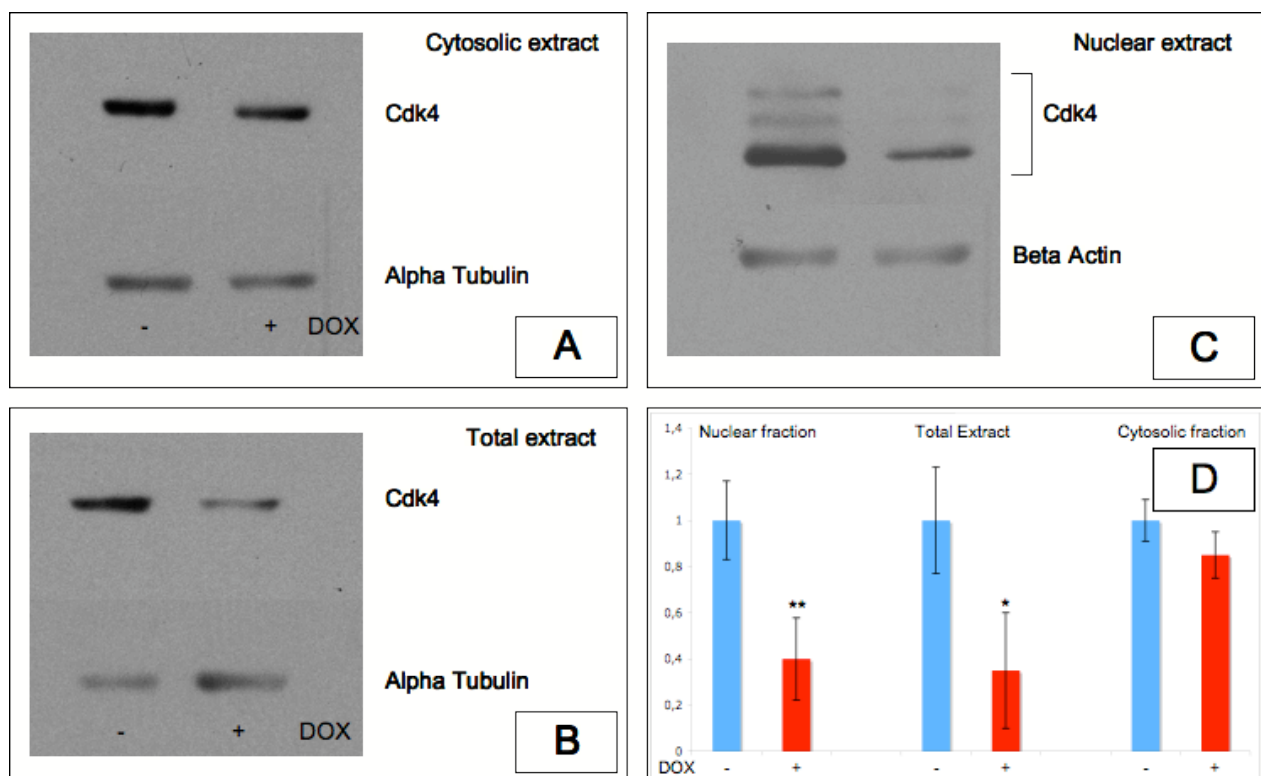


Figure. 5: Panels A, B, C show respectively immunoreactive bands of Cdk4 in Cytosolic, Total and Nuclear extracts of HeLa double stable clones expressing the CTG amplification and untreated with Doxycyclin. Panel D shows the average intensity level of Cdk4 after normalization with the internal control. 3 different cell extracts (treated and untreated with Dox) were evaluated for the Cdk4 levels (p<0,05; nuclear variation. p<0,1; total extract variation. Tendency to decrease for the cytosolic variation).

The last protein we were interested into was GSK3beta. Since we found downregulation of Cyclin D3 following the expression of the CTG amplification in HeLa stable clones, we thought that an increased activity or level of GSK3beta, could be responsible for the cyclin D3 reduction via

phosphorylation of its Thr-286, and consequent ubiquitination and 26S proteasome digestion. As it is easy to observe in Figure. 6, at least in cells, we did not find an increase in GSK3 beta with statistical significance, since in the total extract, and in the cytosolic extract, we observed a substantial invariance and only a slight tendency to GSK3beta upregulation in the cytosolic compartment, while in the nuclear environment this proteic species displays a tendency to downregulation.

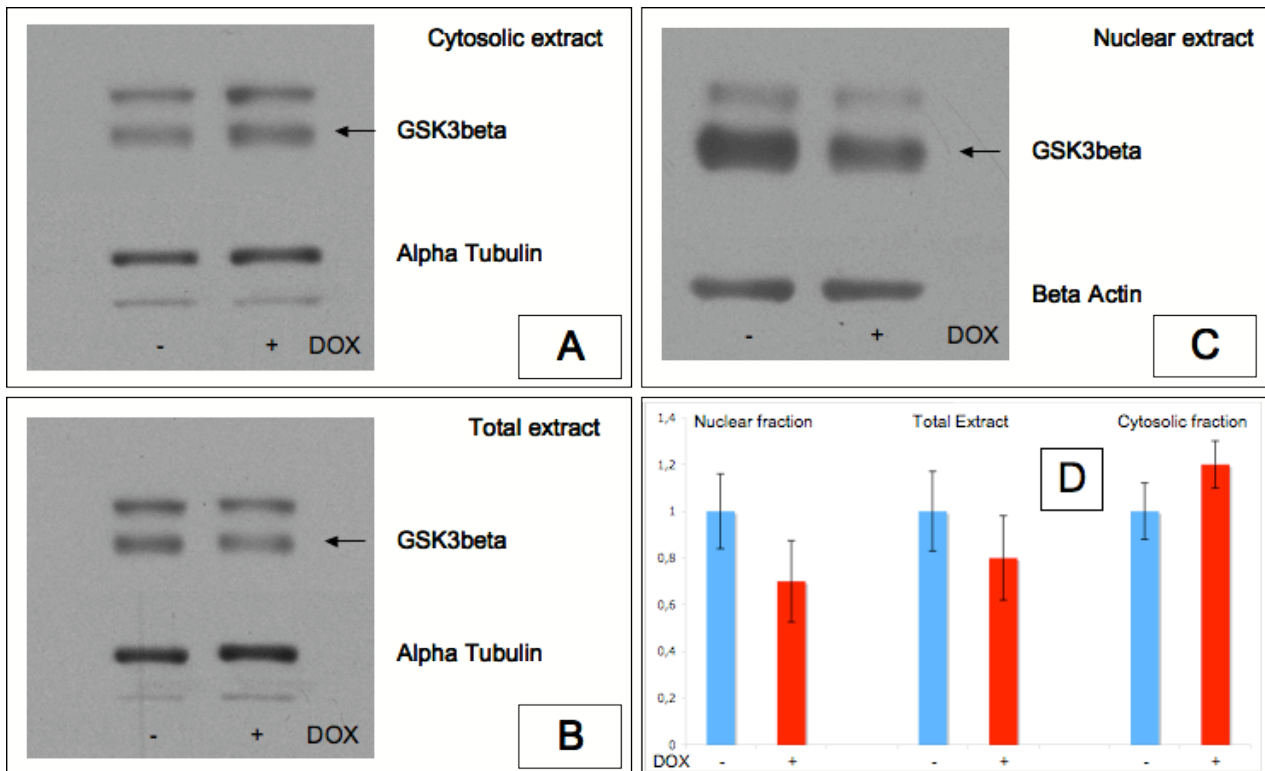


Figure. 6: Panels A, B, C show respectively immunoreactive bands of GSK3beta in Cytosolic, Total and Nuclear extracts of HeLa double stable clones expressing the CTG amplification and untreated with Doxycyclin. Panel D shows the average intensity level of GSK3beta after normalization with the internal control. 3 different cell extracts (treated and untreated with Dox) were evaluated for the Cdk4 levels (We couldn't obtain a strong statistical significance of this levels variation, we would better talk about a tendency of the trends we describe).

In conclusion the cellular model used in these studies, turned out to be important. It gave us the chance to identify a correlation between the expression of the mutant-toxic RNA and the levels of some proteins, like Cyclin D3, Cdk4, and pRb. This also proves that HeLa cells are able to recapitulate some of the most important biochemical and cellular aspects of the DM1 pathology.

4.2 The interaction between pRb and Cyclin D3 is undetectable in immortalized HeLa cells as well as in double stable clones expressing CTG-914

The CTG amplification itself causes protein expression changes of the components of the differentiation complex. We decided therefore to evaluate the capability of the Cyclin D3-Rb complex to form in order to understand whether the expression of the CTG-914 amplification could also interfere with this process. We decided to undertake two different solutions to address this issue: i) we performed HPLC (size exclusion chromatography) on the nuclear extract of HeLa cells, both expressing the CTG amplification and controls, ii) we co-immunoprecipitated Cyc D3 and Rb in a reciprocal manner. The multiprotein complex which forms during muscle cells differentiation, as described by De Santa et al. (2007), includes pRb, Cyclin D3, Cdk4, PCNA and represents a high molecular weight complex. The idea about the use of HPLC, was to identify those high molecular weight protein fractions characterized by the presence of both Cyclin D3 and pRb, as well as to evaluate whether the expression of CTG repeats could affect the association of the two proteins at the level of the HMW fractions. Unfortunately this approach turned out to be unsuccessful in assessing the differences in the complex formation. This was due to either experimental problems, like the high salt concentration needed in order to obtain the nuclear proteins affecting the protein-protein interactions, or cellular characteristics of the immortalized cell

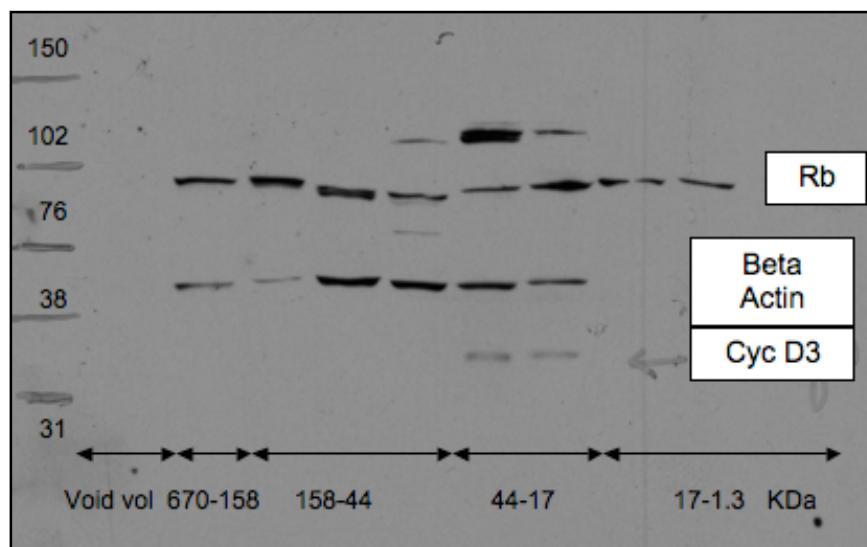


Figure. 7: Immunoblot analysis of the chromatographic fractions with antibodies against Rb, Cyclin D3 and beta Actin. Cyclin D3 is undetectable in the high molecular weight fractions of HeLa cells (nuclear extract) either expressing or not expressing the CTG repeats.

lines. Normally this type of cells cannot form complexes involved in the differentiation tasks, because one of the two interacting proteins, Rb, is an oncosuppressor which is probably inactivated,

or at least hyperphosphorylated (inactive isoform) in tumor cells. The second approach we sought to use to isolate the complex behaviour was the Co-immunoprecipitation (Co-IP) between the two proteins of interest, Cyclin D3 and Retinoblastoma protein.

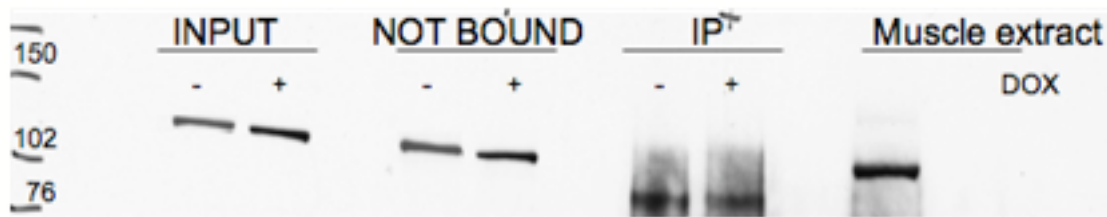


Figure. 8: Immunoblot analysis of the input, not bound and immunoprecipitate of HeLa cells treated and untreated with DOX in order to express the CTG amplification. Cyclin D3 antibody was used to precipitate the Cyclin while antibody against Rb was used to immunodecorate the nitrocellulose filter. No Rb bands are detectable in the immunoprecipitate lanes. In the last lane 25 ug of proteins extracted from mouse gastro muscle were used as a positive control.

As one can observe in figure 8, Cyclin D3 did not pull-down pRb. This result represents a second proof that the complex did not form in this particular cellular system. Defining a cellular model that could help in the comprehending how the differentiative mechanisms are affected by the CTG amplification, could be a very important task. It should be taken into account that understanding the relation between the mutation and the differentiative implications of it, could shed some light on a very important issue of the pathology, and on critical pathways important for a therapeutic approach. We decided so far to evaluate the behaviour of Cyclin D3/pRb complex in C2C12 myoblasts, which are able to differentiate, and in turn to form myotubes. As De Santa et al¹³. already proved. We evaluated a consistent increase of the expression of pRb, and Cyclin D3, during the differentiation process with respect to proliferating myoblasts (Fig. 9)

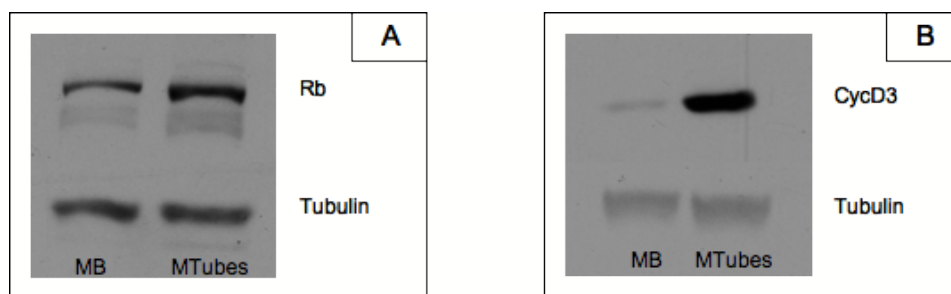


Figure. 9: Immunoblot analysis showing the increase in the levels of Cyclin D3 (panel A) and pRb (panel B) after 5 days of differentiation, total protein extracts of C2C12 cells were analyzed.

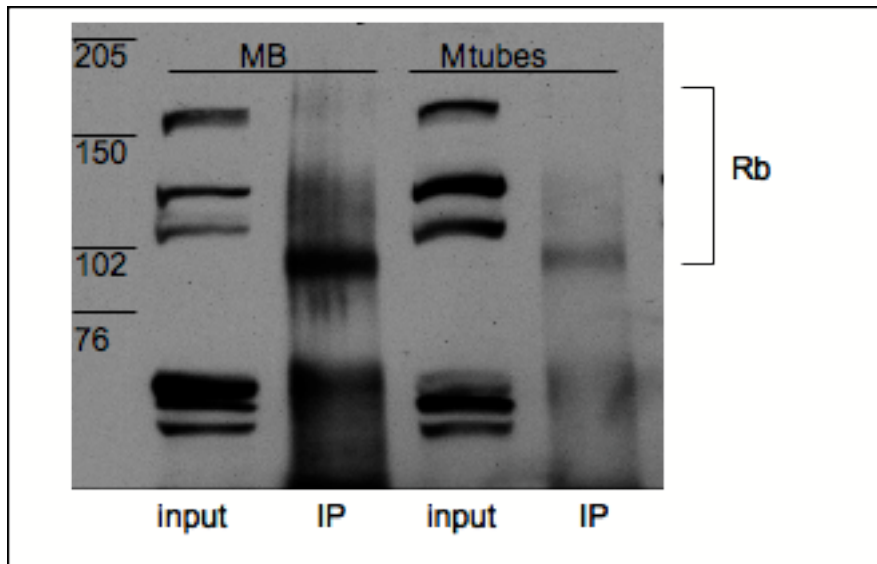


Figure. 10: Immunoblot analysis showing the impossibility to co-immunoprecipitate Cyclin D3 and pRb even in C2C12 cells. Cyclin D3 was immunoprecipitated and the precipitate was screened using the anti-pRb antibody.

Anyway once again it turned out to be impossible to evidence an interaction between these proteins in this cellular system. In the cell types which differentiate into myotubes we could observe the differentially phosphorylated pRb isoforms. The higher molecular weight ones represent hyperphosphorylated pRb, while the lower at ≈ 102 KDa, represents the hypophosphorylated pRb. (Fig. 10) None of them showed its co-immunoprecipitated counterpart. A further experiment we planned to perform was the co-transfection of Cyclin D3 and pRb in the HeLa stable clones expressing the CTG amplification, and the analysis of the complex in these conditions compared to controls, since we hypothesized that the interaction using larger amounts of proteins might become detectable.

4.3 In vivo evidentiatio of the Cyclin D3 pRb complex formation.

What supported the hypothesis about the lack or the difficulties in revealing the complex formation in HeLa cells and in C2C12 cells but not in animal models, was not only the positive control of figure 8, which shows that Rb protein is present in the Cyc D3 immunoprecipitate, but also the experiment performed on Gastro muscle from WT and transgenic CTG mice. For these reasons we decided to switch to a different model, a mouse model available in the lab, characterized by the constitutive expression of 250-CTG repeats mapping at the level of the skeletal Actin. Once again the mutation at the level of the DNA was not localized in the correct position in which we find it in the human DM1 pathology (3'UTR of the DMPK gene) and this aspect is important since the underlying idea is to demonstrate a role for the CTG amplification tout court, in the pathogenesis of the DM1, and not linked to a particular position and to its interference with the flanking genes mapping near the amplification. We used anti-pRb antibody in order to co-immunoprecipitate Cyc

D3, and this time we obtained the first insight about the possibility of a less efficient complex formation in the presence of the CTG amplification (figure 11).

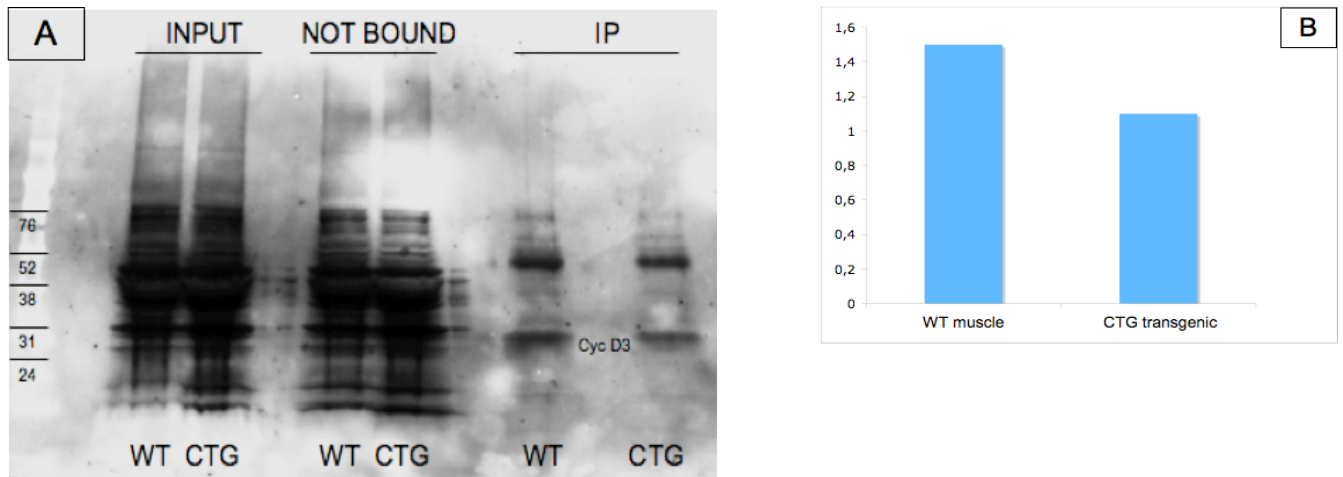


Figure. 11: Immunoblot analysis of the input, not bound and immunoprecipitate of Gastro muscle proteic extract obtained from WT and CTG transgenic mice. Immunoprecipitation: Anti-pRb antibody, Western Blot: Anti-CycD3 antibody (panel A). The differential intensity of Cyclin D3 bands show a tendency to reduction in the ability of pRb to pull-down Cyclin D3 in the gastro muscle of transgenic CTG expressing mouse (panel B).

4.4 Overexpression of GSK3 beta in the CTG-250 mouse model.

The cellular model we used so far gave us some indications connecting CTG amplification and the level of relevant proteins, as we showed, but it did not allow us to study neither the hypothetical implications of the CTG expression on the differentiative complex formation, nor the kinetic of the interactions between the molecular actors so far described. Moreover, we couldn't support our hypothesis about the GSK3beta toxic gain of function. On the contrary using the animal model, we demonstrated on the one hand that the differentiative complex forms, on the other we raise the possibility of an altered complex formation *in vivo*. We further decided to evaluate the levels of GSK3beta in a total protein extract obtained from a heterogeneous muscle biopsy that we called "Muscle Mix". This decision was taken to perform a randomization of the muscle used, since depending on the particular muscle of the animal, it is possible to evaluate different expression of specific gene products. The muscles included on this "mix" were primarily the diaphragm, the neck muscles, and the abdominal muscles. We planned to compare of the GSK3beta levels of the CTG transgenic mice, versus the control WT mice, and eventually get into the particular muscles once obtained preliminary results on the "Muscle Mix" biopsies. This experiments represented the second part of our research plan: the evaluation of GSK3beta levels in transgenic mouse was done

in order to propose a preliminar hypothesis about the differential Cyclin D3-pRb pull down property of CTG-250 mice. Our results indicate a strong overexpression of GSK3beta gene product in the CTG-250 transgenic mice compared to controls.

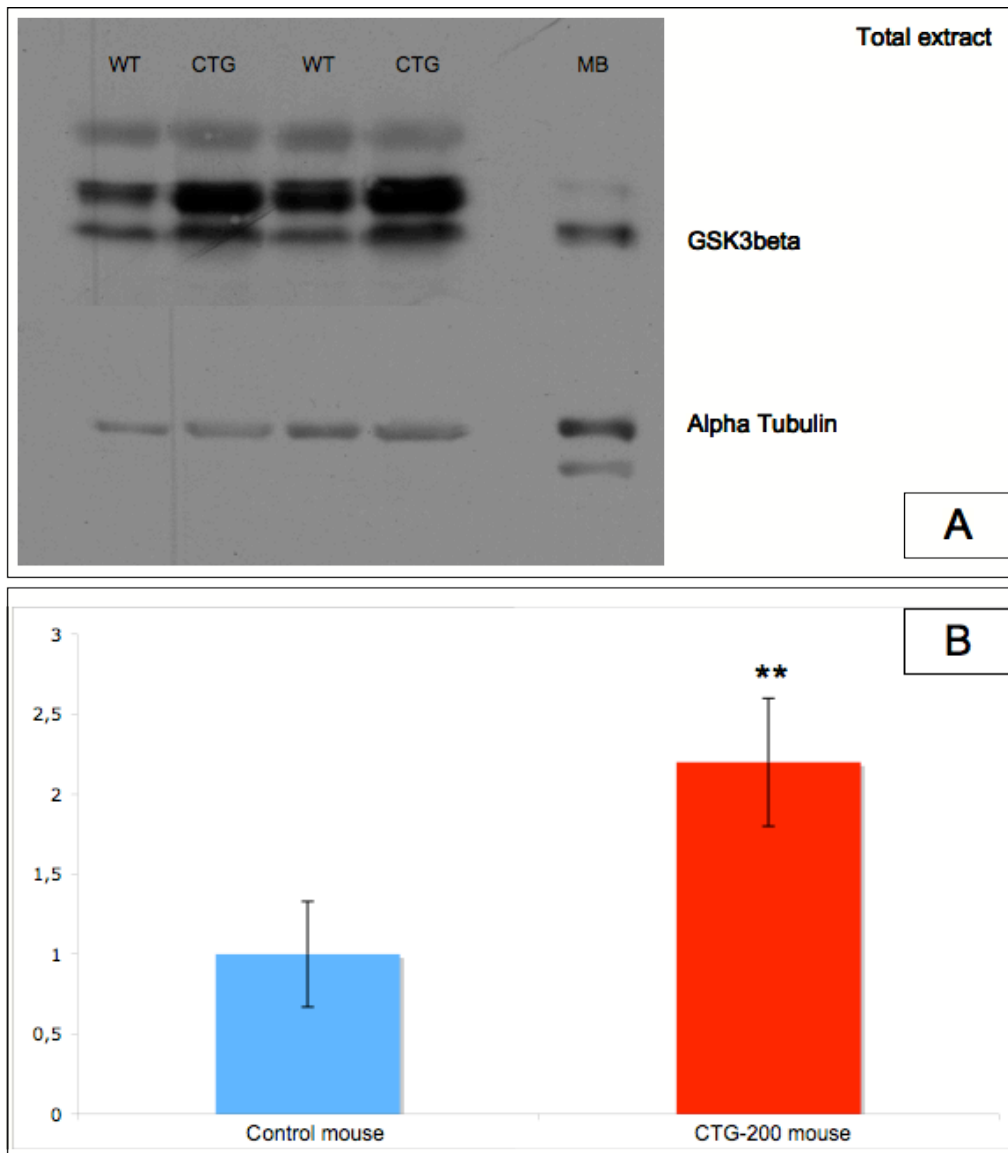


Figure. 12: Immunoblot analysis representing the immunoreactive bands of GSK3beta in a total proteic extract of control mice (lanes 1,3) and CTG-250 transgenic mice (lanes 2,4) (Panel A). In panel B, average densitometric intensity of GSK3beta after internal control normalization. We compared 4 total extracts from control mouse to 4 total extracts from CTG-250 transgenic mouse. ($p < 0,05$).

GSK3beta is known to be inactivated by AKT kinase following insulin or insulin like growth factor cell stimulation. A particular feature of DM is insulin-resistance, due to the uncorrect splicing of the insulin receptor (IR). Furthermore, the inactivating phosphorylation of Ser-9 by AKT creates a docking site for E3-Ubiquitin ligase, hence GSK3beta is degraded by the 26S proteasome. We decided to evaluate whether or not this increase could be related to an altered turnover of

GSK3beta, or eventually could play a role also the transcription process. We extracted RNA from “Muscle Mix” biopsies and after retrotranscription, we performed RT-PCR. In figure 13 we provide proof of the total RNA integrity after extraction with TRIzol reagent, as showed by the presence of 28S and 18S bands of the ribosomal RNA.

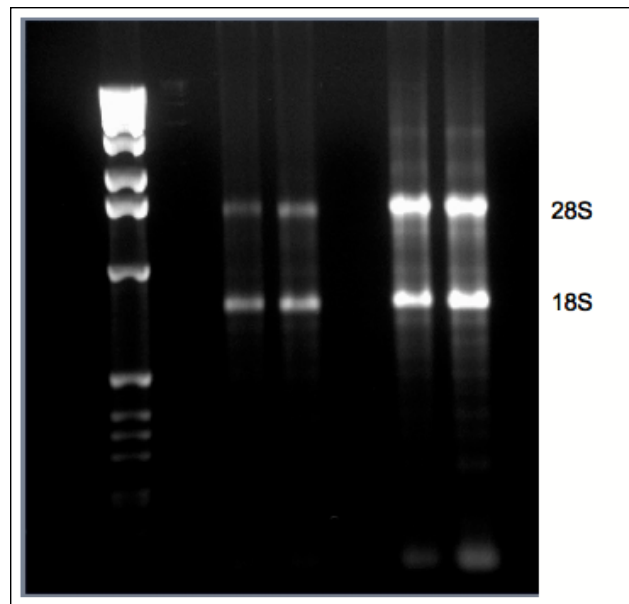


Figure. 13: RNA gel electrophoresis analysis to assess the quality of the total RNA obtained by TRIzol extraction.

Our results show that changes in mRNA levels are consistent with the increase in the protein levels of GSK3beta in transgenic CTG-250 mice. In figure 14 we provide PCR evidence of the difference in mRNA expression, anyway we are still not able to assess whether the transcription rate could be faster, or this kind of transgenic mouse could be characterized by different stability of the GSK3beta mRNA. A corresponding mRNA and protein level involvement in patient’s biopsies needs to be evaluated, and could represent an important feature both as a biomarker of the DM1 disease and a therapeutic target of the pathology.

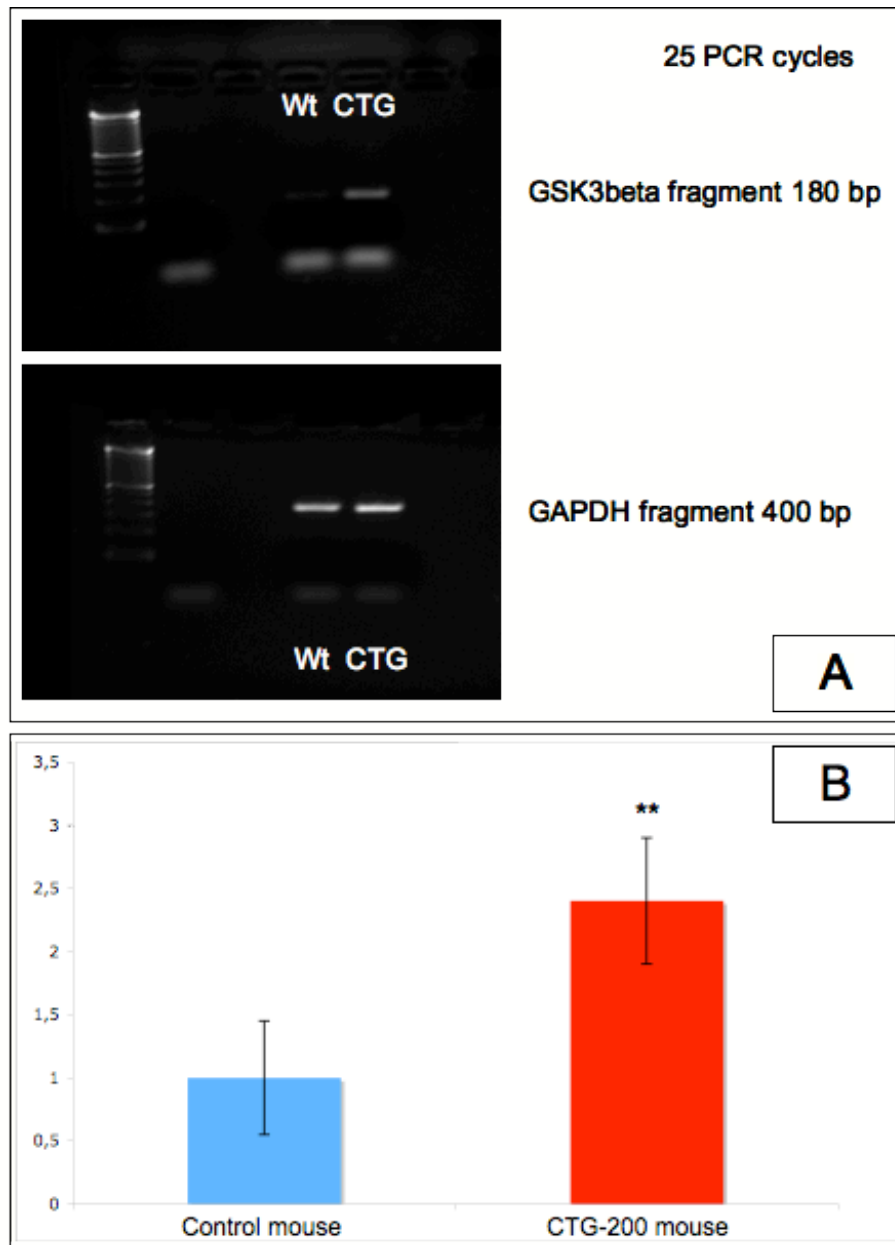


Figure. 14: PCR analysis of retrotranscribed c-DNA from control and CTG-200 transgenic mice (Panel A). PCR products of 180 bp and 400 bp are specific for GSK3beta and GAPDH respectively. GAPDH transcript was used to normalize the cDNA concentration. 2 ul of DNA preparation were used in order to evidence the GSK3beta amplicon, 1 ul was used to visualize GAPDH fragment. GSK3beta primers: forward, 5'-TTCCTTTGGAATCTGCCATC-3', reverse, 5'-TCAGTGCCTCCAAAGATCAA-3'. GAPDH primers: forward, 5'-AACTTTGGCATTGTGGAAGGGCTC-3' and reverse, 5'-TGGAAGAGTGGGAGTTGCTGTTGA-3'. We performed only 25 cycles of PCR to avoid GAPDH signal saturation. In panel B: average intensity levels of the GSK3beta PCR product after normalization on GAPDH, we performed 2 PCR reactions using the same retrotranscribed c-DNA from control and CTG-250 mouse once the PCR conditions were set.

5^b. DISCUSSION

Human Myotonic Dystrophy type 1 affects the capability of myoblasts obtained from patients biopsies to differentiate properly *in vitro*. In these cells the differentiation process, the phenomenon of myoblasts approaching, and the fusion of the plasma membranes leading to the formation of the multinucleated muscle fibers is poorly efficient, and the same can be said for the protein aspects of the differentiation process. Cells lack the burst of some proteic species, such as CUGBP1 which, as we already mentioned, plays a pivotal role in the process. Also they lack the Cyclin D3 and Cdk4 level increase, that is relevant to trigger and control the phosphorylated state of CUGBP1 at serine-302. The aim underlying the study of the differentiation issues in the Myotonic Dystrophy type 1 is easy to guess i.e. correcting the mechanisms which manage the proper differentiation of the DM1 muscle cells will be important on a therapeutic base. Symptoms like atrophy and muscle weakness. Defects in the differentiation process could cause an impairment of continuous renewal and substitution of damaged fibers, underlying the physiological behavior of skeletal muscles.

We already know from C2C12 immortalized mouse myoblasts studies that differentiation is mediated by the formation of a multiprotein complex made up of Cyclin D3/pRb/Cdk4/PCNA. We also know that its functions are manifold and span from Cyclin D3 protection against intrinsic instability, sequestration of cell cycle progression promoters, phosphorylation of proteins such as CUGBP1. We also know that CUGBP1 triggers translation of p21 mRNA, and in conjunction with eIF2, actively translates C/EBPbeta and HDAC1 mRNAs, pushing the cells to the silencing of transcription of factors involved in cell proliferation. We already know the role of GSK3beta kinase in the *in vitro* phosphorylation of Cyclin D3 which may trigger its nuclear export and degradation. Moreover Cyclin D3 levels could be restored in C2C12 myoblasts lacking pRb, (protective role on Cyclin D3) by using GSK3beta specific inhibitor LiCl, or MG-132 26S proteasomal inhibitor. At this point some questions still remain open: I) Is it possible to define a role for the differentiative complex Cyclin D3/pRb/Cdk4 in the impairment of the DM1 cells differentiation process? II) If so, is the impairment of the formation of the complex and/or of the levels of its members, an early event depending uniquely on the CTG repeats amplification expression, or this could be triggered by a complex interaction between different molecular aspects of the pathology? III) Is it possible to think of a toxic gain of function of GSK3beta, depending on a particular post-translational modification, and/or an overexpression of it depending on an augmented transcription, or mRNA stabilization, or translational rates, or impaired degradation? IV) Could we hypothesize a malfunction of the differentiation complex core formation (Cyclin D3/pRb)? V) Can we define a mechanism by which, if so, this differentiation complex cannot form properly, like a connection

between a peculiar phosphorylation state or post-translational modification of Cyclin D3 and/or pRb and the expression of the CTG repeats amplification which could affect the binding between them?

In this study we intended to trace a possible correlation between the expression of a particular DNA mutation, a CTG microsatellite amplification, causing the Myotonic Dystrophy type 1, and the pathologic behaviour of the multiprotein complex, involved in a number of differentiative functions. We needed to respond to a cause-effect issue, working in a simplified environment. We chose HeLa cells, expressing the mutation under CMV-TET-ON inducible promoter. In this cellular model we could demonstrate a direct correlation between the CTG-914 repeats RNA, and important variation in the expressive profile of the core components of this Cyclin D3/pRb/Cdk4 differentiation complex. In particular we detected an important downregulation of Cyclin D3, after the Doxycyclin administration to the culture medium. This level decrease characterizes both the nuclear and the cytosolic compartment, but is more prominent in the nucleus. It is of course also detectable at the level of the total proteic extract with a error threshold lower than 0,001. Cyclin D3 is the only Cyclin promoting, in appropriate conditions, the exit from the Cell Cycle and the commitment differentiation. Once the Myoblasts are prompted to form muscle fibers by serum deprivation, the physiological differentiation burst depends on the expression of early and late muscle differentiation markers, such as Myogenin, Myosin Heavy Chain, and p21¹², an important Cdk inhibitor, able to block the cell cycle progression. The same was shown for Cdk4, which is the cognate Cyclin Dependent Kinase of Cyclin D3. During differentiation Cyclin D3 and Cdk4 were shown to act together in the phosphorylation of important molecular targets²³, such as CUGBP1, a pivotal protein in the pathogenesis of the DM disease. A parallel decreased level of them is intriguing, since it turn out to be easy, to speculate about a common mechanism of augmented degradation eventually mediated by 26S proteasome, or at least a common involvement of their levels change. The decreasing trend of Cdk4 follows with a certain fedelity the Cyclin D3 one. Interestingly we found a decrease of Cdk4 level mainly in the nucleus, but a reduction also in the cytoplasm was observed. It will be interesting to further evaluate a time course of the combined level variations after Dox administration, in order to strictly couple these events. Our results indicated a strong correlation between the CTG triplets transcription, resulting in the formation of ribonuclear inclusions (data not shown), and the pronounced decrease of Cyclin D3 and Cdk4. This evidence shed some light on the role of the amplification itself, and not on the particular position of it at the level of the DNA, in order to affect the levels differentiation proteins. It would be very interesting as well, to investigate whether or not after CTG repeats expression in HeLa cells, CUGBP1 could decrease its phosphorylation levels at Ser-302, taking into account that this

arrangement pushes differentiation against proliferation, and hence this mechanism could be inhibited in this highly proliferating cellular model. In the determination of the levels of pRb, the “protector” of Cyclin D3 from degradation, it was kind of weird to note that pRb was modulated as well by the expression of CUG repeats RNA. We found pRb upregulated. It is difficult to speculate on the reason for that, since differentiation problems are eventually due to its decrease. One possibility is that pRb working as a oncosuppressor increases its levels to suppress the toxic CTG expression in the nucleus, even if a role as a oncosuppressor is unlikely in HeLa cell lines. An alternative explanation is a compensatory attempt to counteract the decrease of Cyclin D3. GSK3beta was the last protein we checked for the expression level. In HeLa CTG-914 we could not obtain a clear result (statistically significant) about the relation between GSK3beta levels and CTG repeats expression. The levels were substantially invariant, apart for a tendency to decrease for the nuclear fraction, and a tendency to increase in the cytosolic one. An analysis on how the CTG repeats could interfere with the GSK3beta levels could have been very important with the aim to provide a hypothetical mechanism about the decrease of Cyclin D3, since GSK3 mediates the degradation of it.

We know (data not shown) that HeLa CTG-914, as well as DM myoblasts from patients, express the fetal splice variant of the insulin receptor. This isoform, IR-A (without exon 11) is less sensitive to the insulin signal with respect to the physiologically expressed IR-B, and this in turn provide evidence of the insulin resistance characteristic of the DM. Since the insulin signaling activates Akt, via PI3K phosphorylation, and Akt is responsible for GSK3beta inactivation by adding a phosphate to Ser-9, and hence for its proteasomal degradation, we hypothesized a failure in this process, entailing augmented cellular levels of GSK3beta. However, our data obtained in HeLa cells do not give us the possibility to conclude whether or not such a mechanism could underlie the decrease of Cyclin D3/Cdk4.

In order to address our further questions, involving the possibility of impairment on the differentiation complex formation, first we needed to demonstrate the existence of the complex in our cellular model based on HeLa CTG-914 double stable clones and C2C12 cells as a positive control. At least in C2C12, we have been confident on this possibility since De Santa et al.¹² demonstrated *in vitro* a competition between pRb and GSK3beta binding to Cyclin D3. Unfortunately, while we were addressing this task, we encountered the first problem: in both C2C12 myoblasts, myotubes, and in HeLa stable clones we were working on, it turned out to be impossible to evidence a co-immunoprecipitation between endogenous proteins. To all intents and purposes we must underline De Santa et al. were able to evidence an *in vitro* co-

immunoprecipitation of Cyclin D3, pRb, and GSK3beta, in Sf9 insect cells, only after having used a huge amount of the Baculovirus expressed proteins. We tried to modulate the amount of proteins used, the antibody concentration, the concentration of detergents in the lysis buffer, but we were unable to obtain such a co-immunoprecipitation in these cell types. Another approach was to co-transfect the cells in order to make them express higher levels of the three proteins of interest, or alternatively, to use patients myoblasts and myotubes, since both C2C12 and HeLa are immortalized cells, characterized by certain peculiarities with respect to primary cultures. It is important to remark that we evidenced a co-immunoprecipitation of pRb-Cyclin D3 *in vivo*, using a protein extract from gastro muscle of adult WT and CTG transgenic mice, attesting for the first time an effective existence of this complex in mammals skeletal muscle. This transgenic mouse shows atrophy, small myofibers, centralized nuclei, and a reduced walking and running ability at the Rota-Road test on a time basis (data not shown). Although further confirmations are needed, preliminary results show a differential capability of pRb to pull down Cyclin D3, although it is far too early to assess whether it depends on a differential binding affinity or simply on the reduced level of Cyclin D3 that could be less available for the binding to pRb. We conclude that, if on the one hand, CTG-914 HeLa double stable clone, was important to establish a causal dependence between the expression of the CTG repeats mutation and the change in the expression levels of Cyclin D3, Cdk4, and pRb, on the other hand, its potential help in addressing part of the still open questions we placed was poor. Indeed we were not able to demonstrate either the differentiative complex formation, involving Cyclin D3 and pRb, or the mechanism potentially involving GSK3beta in the Cyclin D3 level decrease. On the contrary, we evaluated as a powerful tool the CTG-250 transgenic mouse model. In future we plan to define whether the differential Cyclin D3-pRb binding characterizing this animal model could be a matter of a change in the affinity between them, or could depend on a differential availability of one or both of them.

As we said, the power of *in vivo* system, involving CTG-250 transgenic mice was likely to be greater: for this reason we decided to evaluate the GSK3beta levels, with the attempt to propose an explanation for the differential capability of pRb to co-immunoprecipitate Cyclin D3. We found a marked and clear increase of the GSK3beta levels in muscle biopsies of CTG-250 expressing transgenic mice, compared to controls, and RT-PCR analysis shed also light on a transcript increase in this *in vivo* model. A lot of work still needs to be done: we need to extend of the number of the mice to be evaluated on this aspect, find the typology of muscles in which the overexpression is higher, and evaluate in the human myoblasts and biopsies of DM1 patients a confirmation of this results. It will be thereafter fundamental to define whether the treatment with LiCl, the specific GSK3beta inhibitor could recover the differentiation issues of the DM1 human myoblasts. It is clear

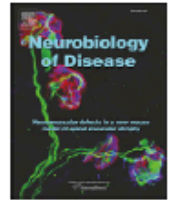
that this work could be important and propaedeutical to a possible therapeutic approach aimed to correct the differentiative defects involving Myotonic Dystrophy type 1.

I wish to add that this report was carried out in the frame of a bigger project on myopathies which is going on in the laboratory of Professor Lubov Timchenko. HeLa CTG-914/GFP double stable clones were obtained, at the Baylor College of Medicine, Houston, Texas, in order to study the early molecular and cellular events which follow the induction of the amplification of the micrisatellite triplet CTG. In particular they are studying the RNA foci kinetics of formation and the interference of this mutation with the cellular process of protein translation. They hypothesized that at least in part, the CTG amplification itself, (and the CCTG amplification in DM2) is responsible for the pathogenesis of the disease, independently on the position this mutation has in the DNA. It is important to declare that in the Human pathology Myotonic Dystrophy, the position of the amplification, (3'UTR of DMPK gene in DM1, and first intron of Znf9 in DM2) hardly matters, but on the one hand, part of the molecular and cellular problems characterizing the DM belongs to the interference with the correct expression of the genes we just enumerated, while on the other hand a number of symptoms and feature of Myotonic Dystrophy, could be related on the characteristics of the ribonuclear amplifications, its secondary structure, and the property to sequester a number of proteic species. This two overlapping mechanisms need to be separated in order to deeply understand their implications in the pathogenesis of the disease. Ribonuclear inclusions form following the translation of the CTG repeats, and CCTG repeats, in both Human, and cellular models of the disease. With the HeLa double stable clones it could be possible not only to study the kinetics of formation of the RNA foci, immediately after the expression induction of CTG repeats, but also to obtain information about their degradation as soon as the Doxycyclin is withdrawn from the culture medium. In the same way, different molecules could be tested in order to assess possible properties in promoting foci degradation, and hence, on a therapeutic point of view. With the same aim, a transgenic mouse model was obtained to better define the implications of the unique CTG amplification on the pathogenesis of a Dystrophic disease in mammals, and to assess whether muscle weakness, myotonia, and muscular atrophy, could raise independently on the position of the mutation.

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Proteome profile in Myotonic Dystrophy type 2 myotubes reveals dysfunction in protein processing and mitochondrial pathways

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Protein disulfide isomerase

ABSTRACT

Myotonic Dystrophy type 2 (DM2) is caused by a DNA microsatellite expansion within the Zinc Finger Protein 9 gene leading to an abnormal splicing pattern largely responsible for the pathological condition. To better define the functional changes occurring in human DM2 myotubes we performed a quantitative proteome comparison between myotubes of DM2 and control patients using two-dimensional gel electrophoresis followed by mass spectrometry. Our results indicate that the proteins, altered in DM2 cultures, belong to two major functional categories: i) mitochondrial components, with a reduction of EFTu, HSP60, GRP75 and Dienoyl-CoA-Isomerase, an enzyme involved in fatty acids degradation; ii) the ubiquitin proteasome system with increase of the 26S proteasome regulatory subunit 13 and a reduction of Proteasome subunit Alfa6 and of Rad23B homolog. Altered ubiquitin–proteasomal activity is supported by a global reduction of cytosolic ubiquitinated proteins. Although future work is required to clarify how these changes affect the degradation machinery and mitochondrial function and to evaluate if these changes also occur in the biopsies of DM2 patients, these results identify the mitochondrial proteins and the ubiquitin–proteasomal system as candidates potentially relevant to DM2 pathogenesis.

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Introduction

Myotonic Dystrophy type 2 (DM2) is an autosomal dominant genetic disease with multisystemic involvement. Characteristic features of this condition include myotonia, wasting, heart conduction defects and insulin insensitivity. The genetic basis of DM2 dystrophy results from an unstable (CCTG) repeat expansion within the first intron of the ZNF9 (Zinc Finger Protein 9) gene, on chromosome 3q21. The expanded CCTG-containing transcripts accumulate in distinct foci within nuclei of DM2 cells (Liquori et al., 2001). The disease is mainly

caused by an RNA gain-of-function in which mutant ZNF9 transcripts alter the function and localization of alternative splicing regulators, including CUG-BP1 and muscleblind protein1 (MBNL1) (Fardaei et al., 2002; Mankodi et al., 2001; Miller et al., 2000; Ranum and Day, 2004). In their splicing activities these proteins have antagonistic effects, while CUG-BP promotes the inclusion of exons normally expressed during fetal development, MBNL1 supports the adult splicing isoforms (Day and Ranum, 2005; Ho et al., 2005). This latter functions in the postnatal remodeling of skeletal muscle, controlling a key set of developmentally regulated splicing switches that include the insulin receptor (IR), muscle-specific chloride channel (CIC-1), and MBNL1 gene itself (for review see for example Day and Ranum, 2005). Among the symptoms of DM2, myotonia and insulin resistance are correlated with the disruption of the CIC-1 and IR alternative splicing, respectively (Charlet et al., 2002; Mankodi et al., 2002; Savkur et al., 2001, 2004). However, the cause of the progressive muscle wasting, in particular atrophy of fast type muscle fibers rather than general muscle atrophy, has yet to be completely explained. It is therefore likely that the effects of CCTG repeat expansion extend beyond spliceopathy to include a more general disturbance, leading to changes that can alter the physiology of this tissue. Many studies on

Abbreviations: DM2, Myotonic Dystrophy type 2; IR, insulin receptor; 2D-GE, 2D gel electrophoresis; GAPDH, glyceraldehyde-3-P-dehydrogenase; PDI, protein disulfide isomerase; CLIC1, chloride intracellular channel 1; EFTu, Elongation Factor Tu; PSA6, proteasome subunit alpha type-6; PSD13, 26S proteasome non-ATPase regulatory subunit 13; RD23B, UV excision repair protein RAD23 homolog B; GRP75, Stress 70 protein; MBNL1, muscleblind protein1; s.d., standard deviation; CIC-1, muscle-specific chloride channel; ER, endoplasmic reticulum; a.u., arbitrary units.

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DM pathophysiology are focused on the alterations occurring at the transcriptional and post-transcriptional level. However, the functionality of the biological system still depends on the availability and activity of proteins. Accessibility of wide-screening techniques for the analysis of global protein expression profiles is therefore a suitable approach for the identification of an altered protein pattern in the pathologic state, as compared to the normal one. To investigate this issue we have used cultured satellite cells obtained from biopsies of DM2 and unrelated patients. Under the experimental conditions we have previously set up, DM2 satellite cells can differentiate into myotubes with morphologic features and differentiation markers similar to control cultures. Moreover DM2 myoblasts show ribonuclear inclusions, MBNL1 nuclear sequestration and splicing abnormalities characteristic of DM2 muscle (Cardani et al., 2009).

Our large scale proteome analysis revealed that proteins belonging to two major functional classes were altered in DM2 myotubes: components of the mitochondrial compartment with particular relevance to proteins involved in import mechanisms, such as Hsp60 and GRP75 (also referred to as stress-70 protein, or mortalin), and in the translational machinery such as EFTu; and proteins belonging to the ubiquitin-mediated proteasomal system, suggesting that DM2 pathology affects biological processes relevant for muscular function.

Materials and methods

Patients and primary muscle cell cultures

Human biopsies from the biceps brachii muscle were taken under sterile conditions from DM2 patients ($n=4$) and from patients who underwent diagnostic biopsy that showed no pathological changes (used as control) ($n=4$). The biopsies were used for this study after informed consent from patients. The diagnosis of DM2 was based upon the clinical diagnostic criteria set by the International Consortium for Myotonic Dystrophies (Moxley et al., 2002) and genetically confirmed. The histological diagnosis was performed on serial sections processed for routine histological or histochemical stainings. Fluorescence *in situ* hybridization was performed on frozen sections using a (CAGG)₅ probe as previously reported (Cardani et al., 2004). The biopsies from donors were dissected and cells were propagated at 37 °C as described (Cardani et al., 2009). Both DM2 and control myoblasts were plated at a density of 60,000 cells per 35 mm dishes and grown in proliferative medium at 37 °C. Myoblasts were allowed to grow until 80% confluence. To initiate differentiation, the proliferative medium was replaced with differentiation medium consisting of DMEM supplemented with 7% FBS. By this protocol about 90% of the cells were desmin-positive as reported (Cardani et al., 2009).

Subcellular fractionation

The cell cultures of each patient were lysed in proper conditions to obtain two fractions: one fraction enriched in cytosolic/membrane proteins, and one in nuclear proteins. The cells, scraped and pelleted, were treated with an hypotonic buffer (10 mM Hepes pH 7.5, 0.5% NP-40, 10 mM KCl, 1 mM EDTA, 1 mM DTT, additioned with proteases and phosphatases inhibitors). After 15 min of mild agitation, cells were centrifuged at 400 ×g at 4 °C. The supernatant was then centrifuged at 15,000 ×g to clarify the extract and the supernatant, representing the cytosolic/membrane enriched fraction, was collected. The pellet obtained from the 400 ×g centrifugation was treated with an hypertonic buffer, (20 mM Tris-HCl pH 7.5, 0.6 M NaCl, 0.2 mM EDTA, 1.4 mM MgCl₂ 25% glycerol, and proteases and phosphatases inhibitors) with mild agitation for 15 min at 4 °C and then centrifuged at 80,000 ×g at 4 °C. The supernatant was collected and represented the nuclear-enriched fraction.

Two-dimensional gel electrophoresis (2D-GE)

Proteins were processed essentially as previously described (Colombo et al., 2009). Each sample containing 100 µg of proteins for the cytosolic fraction and 50 µg for the nuclear one was precipitated using a chloroform/methanol protocol and resuspended in a solution containing 7 M urea, 2 M thiourea, and 4% CHAPS. The first dimension was carried out with an 18-cm pH 3–10 non-linear gradient IPG strip (GE Healthcare). Strips were focused at 20 °C for a total of 70,000 Vh at a maximum of 8000 V using the Ettan IPGphor II system. For the second dimension, IPG strips were equilibrated in a solution containing 6 M urea, 2% SDS, 30% glycerol, and 50 mM Tris/HCl pH 8.8. DTT (10 mg/ml) and iodoacetamide (25 mg/ml) were respectively added to the first and to the second step. IPG strips were then placed on the top of a polyacrylamide gel (12% T, 3.3% C) and run using the Ettan DALTSix system (GE Healthcare). Gels were then fixed and stained with 1:200 dilution of Epicoconone fluorescent stain according to the manufacturer's specification (Deep Purple, GE Healthcare). Gel images were acquired with a Typhoon scanner, using a green laser (532 nm) for the excitation of the fluorophore and a 610BP filter to capture the emitted light. The digitalized gel images were analyzed with Image Master 2D Platinum software (GE Healthcare). Normalized spot volumes of individual proteins expressed as mean ± standard deviation were used to perform Student's *t*-test setting the significance level at $p<0.05$. Only spots present in all four samples of DM2 and control cultures (both for the cytosolic and nuclear fraction) that show statistically significant differential expression were considered.

Protein identification by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS analysis

Proteins were identified after excising bands from the stained gels and processed as described (Gorla et al., 2006). MALDI-TOF mass spectrometry was carried out using a Voyager-DE STR (Applied Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm). Mass spectra of tryptic peptides were acquired operating in the positive ion, delayed extraction and reflection modes using α -cyano-4-hydroxycinnamic acid (5 mg/mL in 50% acetonitrile with 0.1% trifluoroacetic acid) as the UV-absorbing matrix. Analytes were prepared by spotting 2 µL of supernatant on the target (384-sample plate with hydrophobic surface, Applied Biosystems), drying with a gentle air flow, and addition of 0.35 µL of matrix solution. Experimental conditions were as follows: accelerating voltage 20,000 V; grid voltage, 68%; guide-wire voltage, 0.000%; delay time, 250 ns; laser power, 2400 a.u. After acquisition, the spectra (resolution 15,000) were processed by Data Explorer software (version 4.0, Applied Biosystems) applying default parameters for advance baseline correction (peak width, 32; flexibility, 0.5; degree, 0.1), deisotoping algorithm, internal mass calibration (using autolysis trypsin peptides 842.5100 and 2211.1046 *m/z*) and peak detection.

Monoisotopic peptide masses were analyzed rising ProFound software (http://129.85.19.192/profound_bin/webprofound.exe 4.10.5) or Aldente software (<http://www.expasy.ch/tools/aldente/>). Input was searched according to the following databases: (1) ProFound: NCBI (nonredundant protein sequence database); taxonomy category: mammalian; digest chemistry: trypsin; max missed cut: 1; modification: cysteine alkylation with iodoacetamide (complete), oxidation at methionine (partial); charge state MH⁺; monoisotopic tolerance: 20 ppm. Protein peaks recognized by the software were removed automatically based on a Z score greater than 1.65 (95% percentile); and (2) Aldente: UniProtKB/SwissProt; predefined taxon: Mammalia; Spectrometer internal error max: 25.

Immunoblot analysis

For monodimensional immunoblot analysis, samples were then resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis

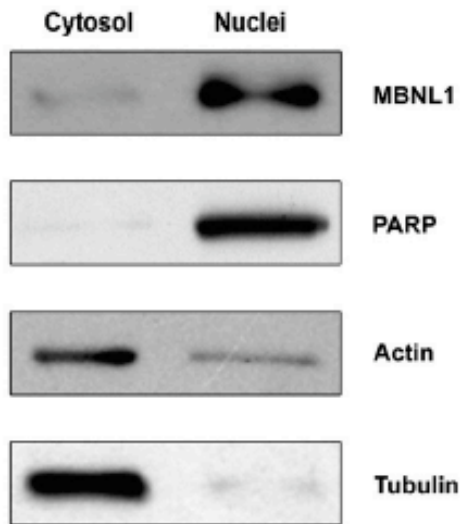


Fig. 1. Subcellular fractionation allows to enrich for specific compartment-localized proteins. Myotubes were fractionated according to the protocol described in Material and methods and analyzed by immunoblotting with specific antibodies. Equal amount of proteins of the two fractions were loaded on the gel.

(SDS-PAGE) and transferred to nitrocellulose Protran Membranes (Schleicher Shuell). The membranes were probed with different primary and the proper peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Chemiluminescent signals were detected using the ECL detection system (GE Healthcare). Band intensities were quantified by densitometry with Scion Image Program (Scion Corporation) using GAPDH or tubulin signals as internal control.

Reagents and antibodies

The reagents for 2D-GE were from GE Healthcare. Mouse monoclonal anti-PDI was purchased from Santa Cruz Biotechnology, anti ubiquitinated proteins (clone FK2) were from Affiniti, anti PARP were from BD Pharmingen. Anti-actin (rabbit polyclonal), anti-tubulin (mouse monoclonal) and anti-GAPDH (rabbit polyclonal)

were purchased from Sigma. Polyclonal antibodies directed against EFTu/Ts were a generous gift from L. Spemulli (Chapel Hill, NC, USA) and antibodies against CLIC1 were a kind gift from M. Berryman (Ohio University College of Osteopathic Medicine Athens, Ohio USA).

Results

In this study we have used myotubes derived from satellite cells of DM2 patients and of unrelated control biopsies, as an experimental “*in vitro*” model to search for global alterations of protein expression profiles in human DM2 muscle cells. For our large scale proteome analysis we have used cells that have been differentiated *in vitro* along the myogenic lineage for 10 days.

To investigate alterations of single proteins in DM2 myotubes as compared to control ones, we have applied two-dimensional gel electrophoresis and mass spectrometry analyses. Moreover, we fractionated cellular extracts in a cytosol/membrane enriched fraction and a nuclear one, to allow the enrichment of lower abundant proteins. Both fractions were initially checked for the expression of specific markers. As shown in Fig. 1, Poly-(ADP-ribose) polymerase (PARP) and MBLN1, two nuclear proteins are highly enriched in the nuclear fraction, while tubulin is mainly present in the cytosol, confirming literature data (Horovitz-Fried et al., 2008) and indicating that our procedural technique allowed the enrichment of specific compartment-localized proteins. As expected, actin was present in both nuclei and cytosol (Horovitz-Fried et al., 2008). Cultures obtained from 4 DM2 and 4 control biopsies were fractionated and the different fractions (i.e. cytosolic and nuclear) of the 8 samples were independently run on 2D-GE, stained with deep purple and acquired as digitalized gel images. For each fraction, only spots present in all the gels were analyzed and the normalized spot volumes of individual proteins were statistically evaluated (see Materials and methods). Representative two-dimensional images are shown in Figs. 2 and 3. About 2000 spots were detected in the cytosol while 1200 spots were identified in the nuclei. Those spots that were statistically significantly altered ($p < 0.05$) (18 spots for the cytosolic fraction and 7 from the nuclear one) were cut, analyzed by MALDI-TOF, identified by database search and classified into functional

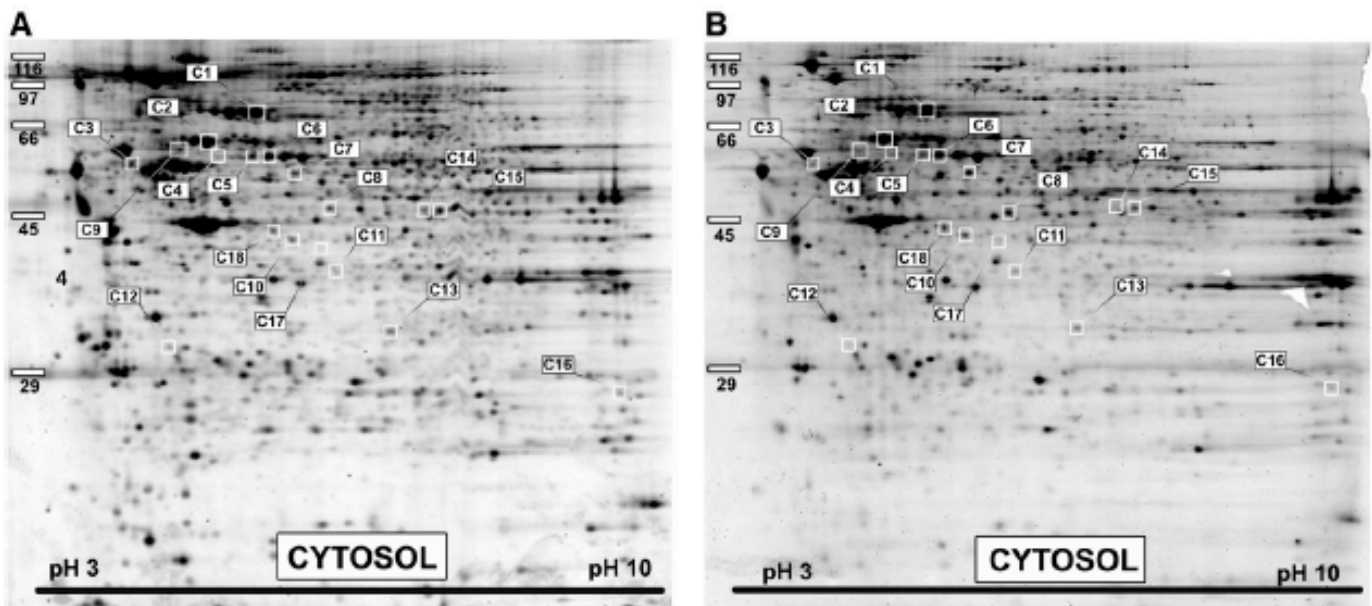


Fig. 2. Two-dimensional map of cytosolic-enriched proteins. Representative two-dimensional gel electrophoresis image of myotubes cytosolic-enriched fraction from control (A) and DM2 cultures (B). Cytosolic proteins obtained from 10 days differentiated myotubes were focused on pH gradient (3–10 NL) separated on 12% polyacrylamide gel and stained with deep purple. Image analysis performed as described in Materials and methods evidences 18 significantly altered spots ($p < 0.05$) in DM2 cultures as compared to control one. The differentially expressed spots were numbered and identified by mass spectrometry (with the exclusion of spots C9, C10 and C11). Table 1 represents protein identifications of significantly altered spots.

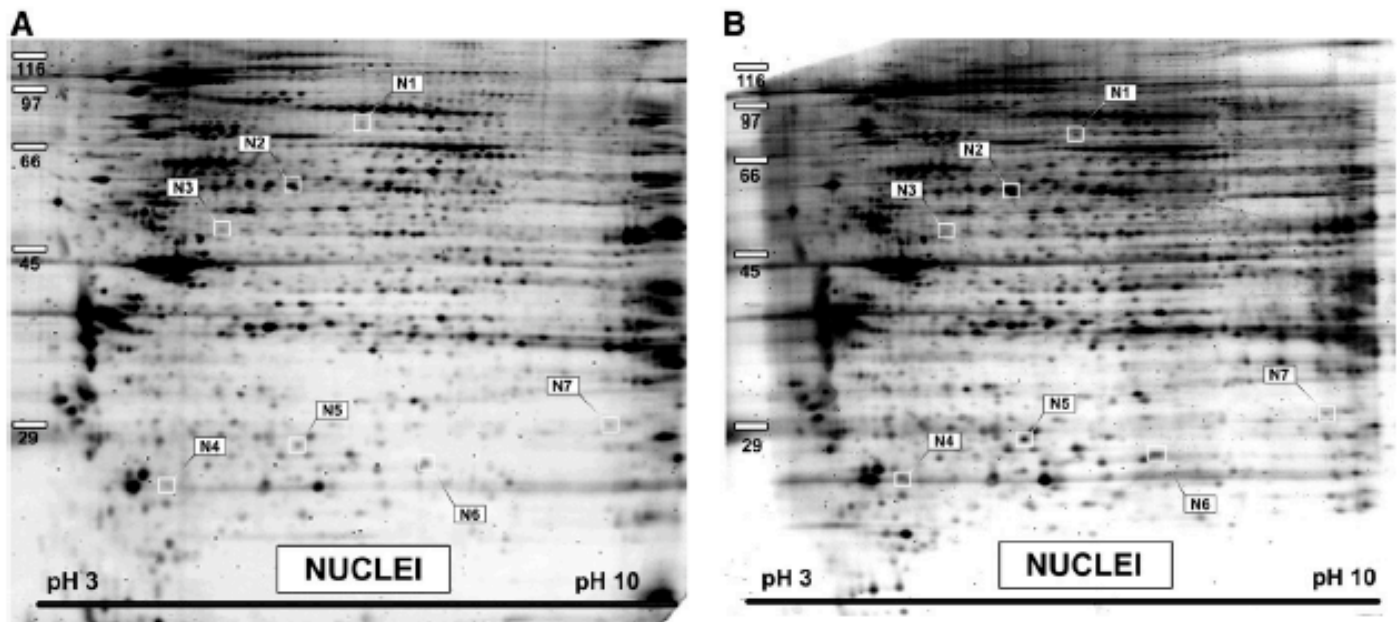


Fig. 3. Two-dimensional map of nuclear-enriched proteins from myotubes. Representative two-dimensional gel electrophoresis image of myotubes nuclear-enriched fraction from control (A) and DM2 cultures (B). Nuclear-enriched proteins were analyzed as above and image analysis evidences 7 significantly altered spots ($p < 0.05$). The differentially expressed spots were numbered and identified by mass spectrometry (with the exclusion of spots N1, N3, N4, N7) and correspond to the data presented in Table 1.

categories according to Gene Ontology. The results are reported in Table 1 and Fig. 4.

To validate some of the results obtained by the proteomic approach we carried out western blotting analysis with specific antibodies: we confirmed a reduction tendency of CLIC1, a redox-regulated chloride intracellular channel (Fig. 5B), and a statistical significant decrease of PDI ($p < 0.05$), a protein disulfide isomerase involved in endoplasmic reticulum (ER) protein folding (Fig. 5D).

For the Elongation Factor Tu (EFTu) we identified by bidimensional gel analysis, two spots that were recognized with EFTu/Ts antibodies (not shown) and confirmed a global reduction of EFTu by monodimensional gel analysis (Figs. 6A and B; $p < 0.05$). Since the same antibodies also recognize the EFTs proteins, we measured the level of EFTs in the different cultures. Interestingly, we found (Figs. 6A and C) that while the level of EFTs in the different control culture was similar, it was extremely variable in DM2 cultures (4 ± 0.028 a.u. for control and 4 ± 0.862 a.u. for DM2 cultures).

A consistent group of proteins which show altered levels in DM2 belong to the ubiquitin–proteasomal system. To investigate if these changes affect the ubiquitination state of proteins we analyzed the level of ubiquitinated proteins using antibodies that recognize ubiquitin–protein conjugates (both mono and poly-ubiquitinated). Fig. 7 depicts a global tendency to reduction of ubiquitinated proteins in the cytosolic fraction of DM2 cultures as compared to control ones.

To investigate if this alteration is associated with increased activity of the proteasome or with a reduced ubiquitination of the substrates we treated control and DM2 myotubes with MG132, a proteasomal inhibitor. Cytosolic extracts were then analyzed and the results are shown in Fig. 8. It is evident that inhibition of proteasomal activity increases the level of ubiquitinated proteins both in control and in DM2 population and this increase has a tendency to be higher (although not statistically significant) in DM2 cultures as compared to control one. All together the results described in Figs. 7 and 8 suggest that the process involved in substrate ubiquitination is not impaired

Table 1

Differentially expressed spots in DM2 myotubes. Relative spots on 2D Map (Figs. 2 and 3); AC: SwissProt Accession Number; Cov: sequence coverage (%); DE: description; ID: identified protein; Mw: molecular weight; pI: isoelectric point; p-value: confidence value obtained from *t*-test with 6 degrees of freedom; volume: volume of the spot in the two considered conditions; percent variation: increase or decrease in DM2 cultures relative to control ones. ([§] RD23B migrates at apparent molecular weight of 58 kDa).

ID	Spot	Score	AC	DE	Mw	pI	Cov	CTRL	DM2	p-value <	Percent variation
GRP75_HUMAN	C1	47.13	P38646	Stress-70 protein, mitochondrial.	69	5.5	44	0.6	0.022	0.01	– 96%
CH60_HUMAN	C2	34.33	P10809	60 kDa heat shock protein, mitochondriaL.	58	5.2	32	1.2	0.79	0.05	– 34%
RD23B_HUMAN [§]	C3	33.39	P54727	UV excision repair protein RAD23 homolog...	43	4.8	25	0.073	0.021	0.01	– 71%
K1C16_HUMAN	C4	30.44	P08779	Keratin, type I cytoskeletal 16.	51	5	36	0.02	0.05	0.05	+ 150%
PDI A3_HUMAN	C5	98.72	P30101	Protein disulfide isomerase A3.	54	5.6	40	0.11	0.072	0.01	– 34%
PDI A3_HUMAN	C6	60.92	P30101	Protein disulfide isomerase A3.	54	5.6	33	0.25	0.16	0.01	– 36%
AL9A1_HUMAN	C7	80.04	P49189	4-trimethylaminobutyraldehyde dehydrogen...	54	5.7	35	0.05	0.086	0.001	+ 72%
VAT1_HUMAN	C8	31.59	Q99536	Synaptic vesicle membrane protein VAT-1 ...	42	5.9	28	0.068	0.024	0.01	– 64%
CLIC1_HUMAN	C12	97.05	O00299	Chloride intracellular channel protein 1...	27	5.1	60	0.036	0.016	0.05	– 55%
ECH1_HUMAN	C13	52.4	Q13011	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomer...	32	6	44	0.58	0.22	0.01	– 62%
EFTU_HUMAN	C14	79.09	P49411	Elongation factor Tu, mitochondrial.	45	6.3	34	0.045	0.024	0.01	– 46%
EFTU_HUMAN	C15	245.39	P49411	Elongation factor Tu, mitochondrial.	45	6.3	60	0.045	0.023	0.05	– 48%
K1C16_HUMAN	C16	110.32	P08779	Keratin, type I cytoskeletal 16.	51	5	68	0.02	0.041	0.05	+ 105%
PSD13_HUMAN	C17	177.54	Q9UNM6	26S proteasome non-ATPase regulatory sub...	43	5.5	42	0.01	0.039	0.05	+ 290%
CSN4_HUMAN	C18	36.39	Q9BT78	COP9 signalosome complex subunit 4.	46	5.5	29	0.038	0.067	0.05	+ 76%
TCPA_HUMAN	N2	66.11	P17987	T-complex protein 1 subunit alpha.	60	5.8	27	0.12	0.18	0.05	+ 50%
ERP29_HUMAN	N5	47.35	P30040	Endoplasmic reticulum protein ERp29.	26	6.1	40	0.04	0.08	0.05	+ 100%
PSA6_HUMAN	N6	52.36	P60900	Proteasome subunit alpha type-6.	27	6.3	49	0.12	0.06	0.05	– 50%

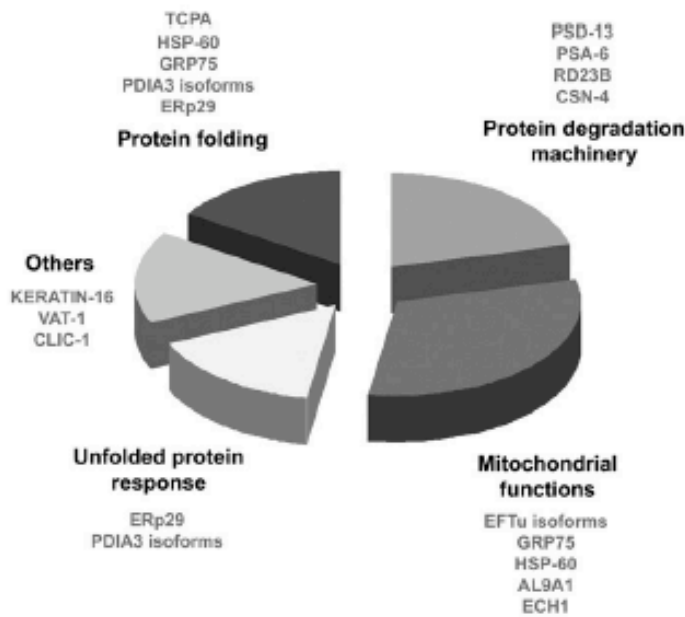


Fig. 4. Distribution of the identified proteins in functional categories. Graphic representation of all the proteins listed in Table 1 grouped by their Gene Ontology (GO) function.

in DM2 cultures while the reduction of ubiquitinated proteins might be due to an increased proteasomal activity.

Discussion

In this study we have undertaken a proteomic approach to identify functional differences at the protein level, between DM2 cell cultures and control ones. Proteome analysis of skeletal muscle cells under physiological and pathological conditions has already been used, however no such study has been undertaken to analyze the Myotonic Dystrophy pathology. We have used satellite cells from DM2 biopsies diagnosed according to the criteria set by the International Consor-

tium for Myotonic Dystrophies (Moxley et al., 2002). The cells were grown *in vitro* and induced to differentiate into myotubes. This cellular model retains many of the characteristic features of adult DM2 muscle cells with the advantage to allow a wide screen proteome analysis otherwise not possible due to the small dimensions of biopsies. Our proteome analysis revealed that 25 spots were altered in DM2 culture as compared to control ones. Out of these, 7 were isolated from the nuclear-enriched fraction while 18 were identified in the cytosol-enriched fraction. Interestingly, these spots were different with statistical significance, even if the biopsies used to derive cells were neither matched for age and gender nor for length of the microsatellite expansion. It is possible that using satellite cells (i.e. muscle-specific stem cells) induced to differentiate *in vitro* we have worked with a more homogeneous population, thus reducing the differences of adult tissues. Interestingly, for some of the spots that we did not analyze in this work (since they were not statistically significant in the pairwise comparison) we also found spots that were highly similar in their abundance in the control condition but significantly divergent in DM2 cultures (see the results for EFTs). A greater sampling and an improved sorting of DM2 patients may be relevant to understand this issue.

Our analysis was done fractionating cellular extracts in a cytosolic/membrane (NP-40 soluble) enriched fraction and a nuclear one to enrich for low abundant proteins. By this approach we have however discarded the cellular NP-40 insoluble fraction that contains mainly sarcomeric proteins (Myosin, etc) and of which we did not carry out any proteome comparison.

The proteome analysis points to variation in the spots % volume (in some cases also greater than two fold), with a general tendency to a reduction rather than an increase in DM2 as compared to control cultures. The proteins identified by mass spectrometry were 18 out of 25 total spots.

The general picture that emerges from our analysis is that the proteins changing their level of expression in DM2 patients can be clustered in a reduced number of functional categories. As shown in Fig. 4, we found proteins involved in mitochondrial biogenesis and function, proteins of the degradation machinery and proteins involved in the protein folding mechanisms.

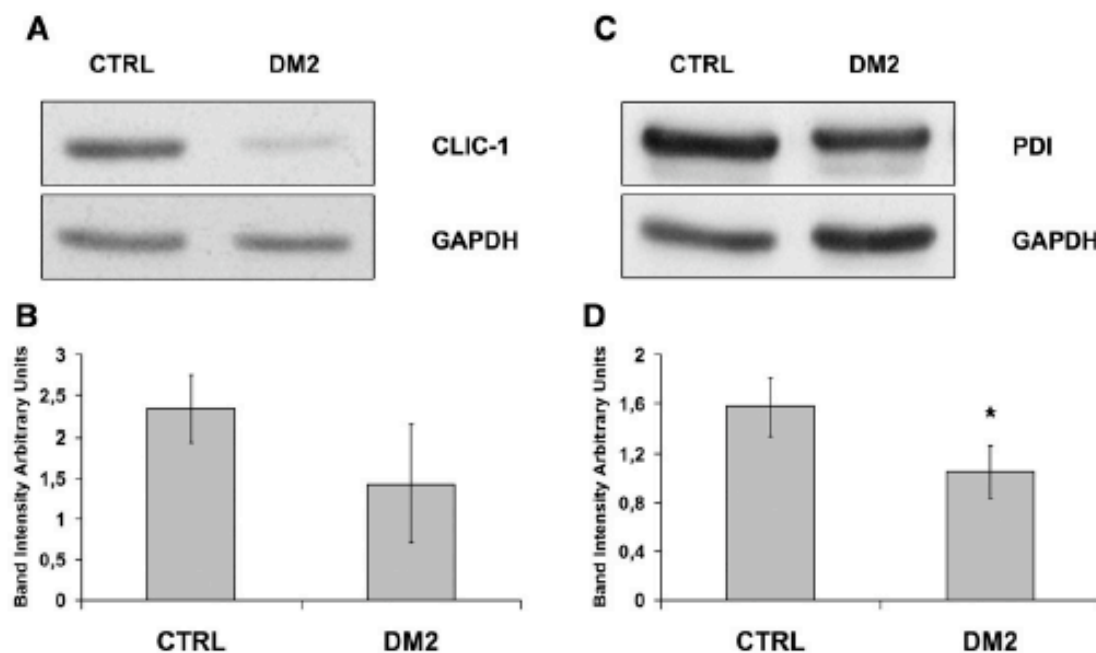


Fig. 5. Confirmation of the proteomic data by immunoblot analysis. Panels A and C show immunoreactive bands of CLIC1 and PDI respectively obtained from cytosolic-enriched fraction of DM2 and control cultures. Panels B and D show the average intensity level of CLIC1 and PDI after normalization with the GAPDH intensity. The quantification was done on 4 cytosolic fractions for DM2 and control cultures. Comparative analysis revealed a tendency to reduction of CLIC1 and a statistically significant decrease of PDI ($p < 0.05$) in DM2 cultures consistent with the proteomic data shown in Table 1. The error bar represents s.d.

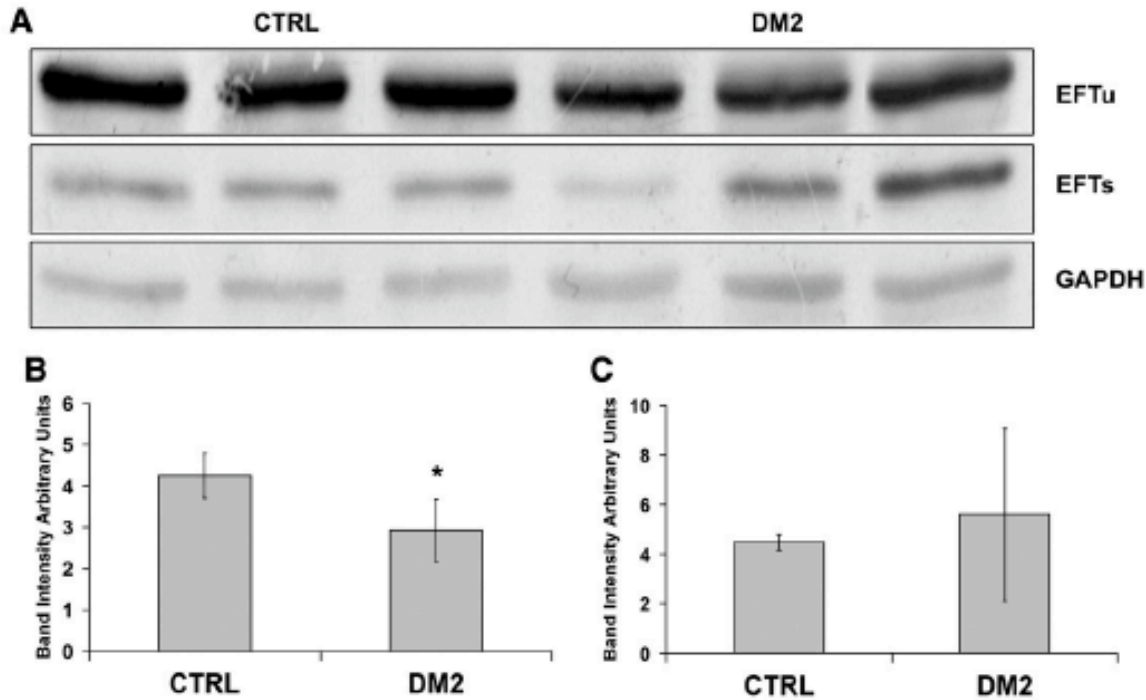


Fig. 6. Level of EFTu and EFTs proteins in DM2 cultures. Panel A: immunoblot analysis with antibodies against Tu/Ts to detect the level of EFTu and EFTs in cytosolic extracts of DM2 and control cultures. Panels B and C show the average intensity level of EFTu and EFTs after normalization with GAPDH intensity, respectively. The quantification was done on 3 samples of DM2 and 3 samples of control cultures in two independent experiments. Comparative analysis revealed significant reduction of EFTu ($p < 0.05$), while the level of EFTs was extremely variable in DM2 cultures. The error bar represents s.d.

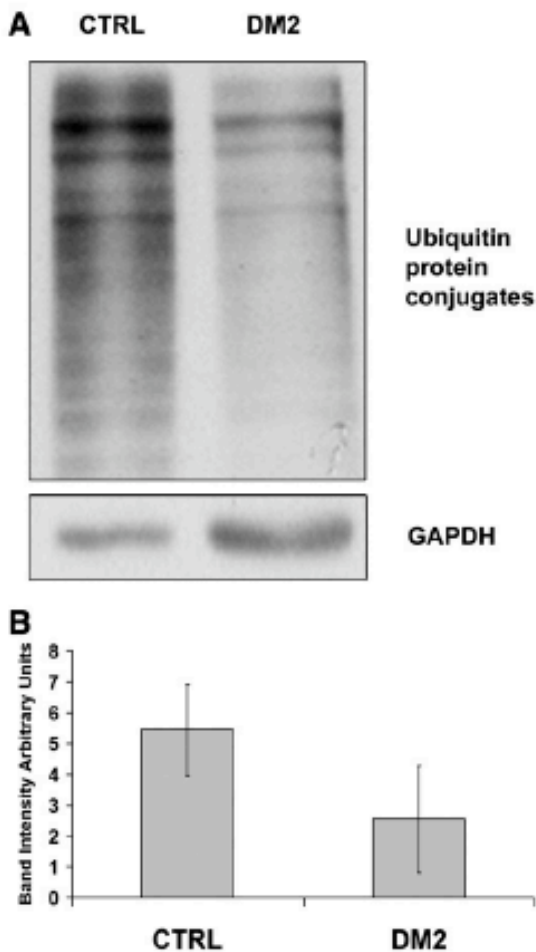


Fig. 7. The level of ubiquitinated cytosolic proteins is reduced in DM2 cultures. Cytosolic extracts of control and DM2 cultures were analyzed with antibodies against ubiquitin–protein conjugates. Panel B: average intensity of the whole blot after normalization with GAPDH intensity. The quantification was done comparing cultures from 3 DM2 versus 3 control samples in two independent experiments. The error bar represents s.d.

Among the proteins involved in the ER folding machinery, we detected a reduction of two protein disulfide isomerase (PDI-A3) isoforms, a chaperone protein that mediates protein folding promoting formation of disulfide bonds. In parallel, we found an increase of ERp29 another ER chaperone with no enzymatic activity, usually induced following ER stress. Secretory proteins become folded and acquire stabilizing disulfide bonds in the ER. Correct disulfide bond formation is a key step in the ER quality control: proteins with incorrect disulfide bonds are recognized by this machinery and are retrotranslocated into the cytosol, where they are degraded by the proteasome (Elgaard et al., 1999). Reduced expression of PDI may impair the normal capability of the ER to correctly fold proteins.

Our results also indicate changes in proteins involved in the ubiquitin–proteasomal system. We found increased 26S proteasome non-ATPase regulatory subunit 13, a subunit of the 19S proteasomal regulatory particle that binds, unfolds and translocates ubiquitinated proteins into the proteasome core (Coux et al., 1996). Also a subunit of COP9 signalosome complex, part of a regulatory particle of the ubiquitin–proteasomal system whose function has not yet completely defined (Wei and Deng, 2003), is increased in DM2 cultures. In parallel we found reduction of one of the catalytic subunits of the 20S core and of RD23B, an ubiquitin receptor that binds ubiquitinated proteins and delivers them to the regulatory proteasomal particle (Dantuma et al., 2009). The picture emerging from our results is at the moment far from being clear. However, alteration in the level of cytosolic ubiquitinated proteins in DM2 cultures is consistent with a modified ubiquitin-mediated degradative machinery and our data mainly point to an increased proteasomal activity rather than a reduced ubiquitination of the substrates in DM2 cultures. Alteration in the ubiquitin–protein conjugates has also been recently reported (Salisbury et al., 2009) although in that case an increase rather than a decrease, has been observed. However, differences between our experimental protocols (isolation of cytosolic proteins in the presence of 0.5% detergent in our case) and the total extract reported (Salisbury et al., 2009) could justify the discrepancies. Alteration in the ubiquitin-mediated proteasomal system may impair the degradation machinery affecting the protein stability. Although our present results

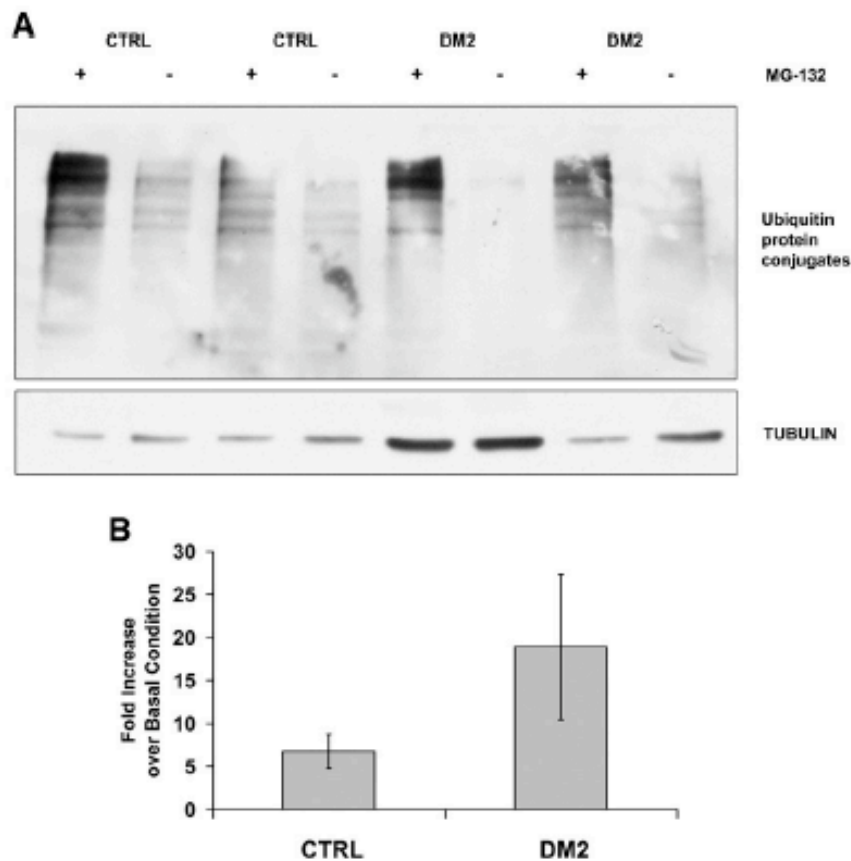


Fig. 8. Effect of proteasome inhibitor on the level of ubiquitinated cytosolic proteins. Control and DM2 myotubes were treated (or not) with MG132 (10 μ M) for 4 h. Cytosolic extracts were then prepared as described in Materials and methods and analyzed with antibodies against ubiquitin–protein conjugates and tubulin. A: a representative gel. B: Densitometric analysis: the average intensities of ubiquitinated proteins normalized for tubulin were determined for control and DM2 samples and the data were expressed as fold increase over the untreated condition. The quantification was done on 4 DM2 cultures and 4 control samples. The error bar represents s.d.

do not allow to define if this is the case, very recent work (Huichalaf et al., 2009) has demonstrated reduction of the rate of protein translation in patients with Myotonic Dystrophy 2.

An altered degradation machinery in DM2 myotubes cultures it is not completely unforeseen. In fact, the ubiquitin–proteasome proteolytic system is involved in muscle wasting (Attaix et al., 2005; Murton et al., 2008) which is one of the features of dystrophic muscles. It is feasible that the altered splicing of insulin receptor may affect the insulin-mediated signaling acting on the proteasomal degradation machinery. It is also possible that alterations in the level of proteins involved in protein degradation are early biomarkers for the wasting process.

Mitochondria are probably the most relevant compartment in which we found a significant reduction in the protein profile of DM2 cultures. The identified proteins belong to different biological processes required for the biogenesis and function of mitochondria. Among the proteins altered we detected two spots, possibly postrationally modified proteins as reported (He et al., 2001), identified as EFTu. For both of them we found a reduction in DM2 cultures also confirmed by western blotting. EFTu are nuclear encoded proteins that play a major role in mitochondrial translational machinery. Mutation and/or reduction of these proteins has been described in Infantile Encephalopathy (Valente et al., 2007) associated with muscular hypotonia and degradation of motor skills.

The Elongation Factor Tu is a GTPase that participates in the formation of the ternary complex (EFTu:GTP:aminoacyl-tRNA). The switch between an active (GTP-bound) and inactive (GDP-bound) form requires the EFTs factor and the efficiency of the mitochondrial translation is critically dependent on the relative ratios of these and other mitochondrial elongation factors (Smeitink et al., 2006).

Interestingly, we found that the level of EFTs in the different control cultures was similar, while it was extremely variable in DM2 cultures. This affects the Tu/Ts ratio, in fact we can infer that, while the ratio between EFTu and EFTs in control cultures is constant, the consistent reduction of EFTu in DM2 cultures is not correlated with a comparable change of EFTs levels in DM2 cultures causing the Tu/Ts ratio to be highly variable. Future experiments will allow to understand if the changes in EFTu and in Tu/Ts ratio observed in DM2 cultures affect the mitochondrial translation efficiency.

Among the mitochondrial proteins found altered in DM2 cultures we detected a reduction of the mitochondrial Heat Shock Protein 60 and Glucose-Regulated Protein 75 (also referred to as Stress-70 Protein). These proteins play a key role in the mitochondrial translocation reaction of matrix-targeted precursor proteins and in protein folding within the mitochondrial matrix, assisting in the maintenance of mitochondrial proteome integrity (Rassow et al., 1999). Together these two proteins are involved in import, chaperoning and quality control functions in mitochondria.

We also observed a reduced level of Delta Dienoyl-CoA Isomerase, a mitochondrial enzyme involved in the beta oxidation of odd-chain fatty acids. Therefore it is possible that folding, import and metabolic processes necessary for the bioenergetic function of the mitochondria are altered.

Interestingly, we found an increase of Trimethylaminobutyraldehyde Dehydrogenase, an enzyme involved in the biosynthesis of carnitine, a mitochondrial carrier for fatty acids. This modulation might be a compensatory response to the reduced level of some mitochondrial proteins.

In conclusion, this wide screen analysis has unraveled still undisclosed molecules that are altered in the DM2 cells. These data,

produced in an *in vitro* model for DM2 disorder, require further analysis on the biopsies of DM2 patients. In any case, our results suggest that other biological processes, in addition to spliceopathy, could be altered in the DM2 disease. This observation can open a novel field of research for the understanding of the DM2 pathophysiology that may also lead to the development of new pharmacological approaches useful to block or partially prevent muscle wasting in DM2 patients and ameliorate the life of these patients.

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