

## Cyclosporine A treatment for Ullrich congenital muscular dystrophy: a cellular study of mitochondrial dysfunction and its rescue

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Mutations in *COL6A1*, *COL6A2* and *COL6A3*, the genes which encode the extra-cellular matrix component collagen VI, lead to Bethlem myopathy and Ullrich congenital muscular dystrophy (UCMD). Although the *Col6a1*<sup>-/-</sup> null mouse has an extremely mild neuromuscular phenotype, a mitochondrial defect has been demonstrated, linked to dysregulation of the mitochondrial permeability transition pore (PTP) opening. This finding has been replicated in UCMD muscle cells in culture, providing justification for a clinical trial using cyclosporine A, an inhibitor of PTP opening. We investigated whether PTP dysregulation could be detected in UCMD fibroblasts (the predominant source of muscle collagen VI), in myoblast cells from patients with other diseases and its response to rescue agents other than collagen VI. Although we confirm the presence of PTP dysregulation in muscle-derived cultures from two UCMD patients, fibroblasts from the same patients and the majority of fibroblasts from other well-characterized UCMD patients behave normally. PTP dysregulation is found in limb girdle muscular dystrophy (LGMD) type 2B myoblasts but not in myoblasts from patients with Bethlem myopathy, merosin-deficient congenital muscular dystrophy, LGMD2A, Duchenne muscular dystrophy and Leigh syndrome. In addition to rescue by cyclosporine A and collagen VI, this cellular phenotype was also rescued by other extra-cellular matrix constituents (laminin and collagen I). As the muscle derived cultures demonstrating PTP dysregulation shared poor growth in culture and lack of desmin labelling, we believe that PTP dysregulation may be a particular characteristic of the state of these cells in culture and is not specific to the collagen VI defect, and can in any case be rescued by a range of extra-cellular matrix components. Further work is needed on the relationship of PTP dysregulation with UCMD pathology.

**Keywords:** Muscular dystrophy; MPTP; extracellular matrix; Membrane Potential; mitochondria

**Abbreviations:** BM = Bethlem myopathy; CsA = cyclosporine A; ECM = extra-cellular matrix; LGMD = limb girdle muscular dystrophy; MDC1A = merosin-deficient congenital muscular dystrophy type 1A; PTP = permeability transition pore; TMRM = tetramethyl rhodamine methyl ester; UCMD = Ullrich congenital muscular dystrophy

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## Introduction

Mutations in collagen VI cause the rare muscle disorders, Bethlem myopathy (BM; MIM 158810) and Ullrich congenital muscular dystrophy (UCMD; MIM 254090). BM and UCMD were initially thought to be distinct entities but are now considered to be opposite ends of a phenotypic spectrum caused by mutations in the same genes. BM is a relatively mild disorder characterized by proximal muscle weakness and contractures, usually affecting the distal joints, with an onset within the first two decades. In contrast, UCMD is a more severe disease, with congenital weakness and hypotonia, proximal joint contractures and hyperlaxity of distal joints (Kirschner and Bonnemann, 2004). Unlike BM, where respiratory involvement is relatively rare, patients affected by UCMD require ventilatory support by the second decade of life. Currently, therapeutic strategies for the collagen VI related disorders are limited to the management of symptoms by physiotherapy, nocturnal ventilation and surgery.

The collagen VI protein is composed of three different peptide chains  $\alpha 1(VI)$ ,  $\alpha 2(VI)$  and  $\alpha 3(VI)$  which undergo a complex assembly process (Furthmayr *et al.*, 1983; Colombatti *et al.*, 1995) prior to export from the cell into the extra-cellular matrix (ECM), where collagen VI forms a beaded microfibril matrix closely associated with the basal lamina (Sanes, 2003). It is hypothesized that collagen VI plays a role in anchoring the muscle basement membrane to the ECM, which is supported by the finding in some UCMD patients of a specific loss of collagen VI from the basement membrane (Ishikawa *et al.*, 2004). In addition, *in vitro* studies in fibroblast cells show that collagen VI may also play a role in ECM organization (Sabatelli *et al.*, 2001), which could be facilitated via binding to collagen IV (Kuo *et al.*, 1997), biglycan and decorin (Wiberg *et al.*, 2001), and other ECM components.

A somewhat unexpected twist in our understanding of the collagen VI-related muscular dystrophies came with the investigation of collagen VI-deficient mice (*Col6a1*<sup>-/-</sup>). Unlike patients with complete collagen VI deficiency presenting as severe UCMD, these mice had a very mild neuromuscular phenotype (Bonaldo *et al.*, 1998). Mitochondrial depolarization was demonstrated using the potentiometric dye tetramethyl rhodamine methyl ester (TMRM), which measures mitochondrial transmembrane potential, inferring opening of the mitochondrial permeability transition pore (PTP) (Irwin *et al.*, 2003). Opening of the PTP allows equilibration of the ionic charge between the mitochondrial matrix and the intermembrane space, causing depolarization of the electrochemical gradient normally used to generate ATP and signalling to the cell to progress down an apoptotic pathway (Crompton, 1999). Using the same assay, a latent mitochondrial depolarization was also observed in the cells derived from skeletal muscle of UCMD patients, along with altered mitochondrial ultrastructural morphology and an increase in apoptosis (Angelin *et al.*, 2007). Importantly for potential therapeutic applications, mitochondrial depolarization showed a positive response to cyclosporine A (CsA), a PTP inhibitor, *in vitro* (Angelin *et al.*, 2007), *in vivo* in the mouse model (Irwin *et al.*, 2003) and most recently in an open pilot trial of orally administered CsA in five patients with collagen VI myopathies (Merlini *et al.*, 2008). As CsA is a widely used drug, which is currently being

trials as an adjunctive treatment with steroids in Duchenne muscular dystrophy, a clear potential for development of a pharmacological treatment option for collagen VI-related disorders was suggested.

We wanted to understand more about the context for CsA treatment in collagen VI-related disorders. Firstly, it was important to explore the TMRM assay in UCMD fibroblast cells, which are not only the major tissue resource for diagnostic testing of UCMD and BM (Hicks *et al.*, 2008) but also have recently been implicated as the main source of collagen VI in skeletal muscle (Zou *et al.*, 2008). Secondly, no TMRM assay data from disease control cell lines have so far been reported, even for muscular dystrophies such as MDC1A, where there is also a suggested apoptotic component to the pathology (Girgenrath *et al.*, 2004). Thirdly, whilst collagen VI had also been shown to be able to rescue the collapse of the transmembrane potential, it was not known how much this effect was specific rather than a general feature of culturing cells on a matrix component. These issues have direct bearing on the relationship between PTP opening as indirectly measured by the TMRM assay, the mitochondrial apoptotic pathway and the potential for modification of the pathogenic mechanism in collagen VI-related disorders.

The results presented in this manuscript indicate that PTP opening is neither common in UCMD fibroblasts, nor specific to UCMD myoblasts and may be rescued by a variety of matrix components, not only collagen VI. We conclude that PTP opening as measured by the TMRM assay is a non-specific pro-apoptotic phenotype *in vitro* and whilst it is clearly abnormal in UCMD myoblasts, this may not relate directly to the primary pathogenetic mechanism underlying collagen VI disease.

## Material and Methods

### Patients

The basic clinical features of the 15 patients that were analysed in this study are summarized in Table 1. All participants provided appropriate consent.

### Myoblast and fibroblast cultures

Skin biopsies were taken from the ventral forearm, collagenase digested and immersed in Ham's F-10 (Gibco) supplemented with 20% heat inactivated foetal bovine serum (PAA Laboratories), fungizone, penicillin-streptomycin and L-glutamine (Gibco). Human disease and control myoblasts were obtained from the Muscle Tissue Culture Collection, Friedrich-Baur-Institute, Munich, Germany and grown in skeletal muscle growth medium (PromoCell, Heidelberg, Germany) supplemented with 15% foetal bovine serum. The pre-plating technique was used to ensure the maximum potential purity of myoblast cells to fibroblast cells. Cells were incubated in trypsin-EDTA (Gibco) for 2–3 min before microscopic verification of detachment and neutralization with 2 ml growth media per 15 cm<sup>2</sup>. Cells were incubated for 10–15 min, which allows for fibroblast attachment to the culture flask whilst the myoblast-containing media was centrifuged and re-suspended in fresh growth media.

**Table 1 Summary of clinical and genetic information of patient cohort**

Patient	Disease	Major clinical features	Mutation
1	UCMD	Motor delay, distal joint laxity, elbow contractures. Loss of independent ambulation at 4 years.	Homozygous COL6A1e3 c.350_351delTG p.Val117GlyfsX34
2	UCMD	Developmental dysplasia of the hip. Scoliosis in infancy. Congenital torticollis, squint, hypotonia. Never achieved independent ambulation.	Heterozygous COL6A2e3 c.688_689dupAC p.Ile231ProfsX8 Heterozygous COL6A2e27 skipping c.2455_2461 + 37 del46ins33 p.Asp808_Thr820del
3	UCMD	At birth flexion deformities at wrists, talipes and congenital hip dislocation. Delayed motor development. Consanguineous parents. Loss of independent ambulation at 5 years.	Homozygous COL6A3e27 skipping c.6816 G>A p.Gly2252_Lys2272del
4	UCMD	Congenital hip dislocation, hyperlax fingers; contractures in ankles, knees and toes; spinal rigidity; walked 16 months to 10 years; sub-gravity power proximal lower limbs, partial antigravity power proximal upper limb and axial.	Homozygous COL6A1e27 skipping due to splice donor site mutation
5	UCMD	Frequent falls from 2 months. Walked 12 months to 11 years. Proximal contractures, talipes equinovagum, finger laxity, severe scoliosis.	Homozygous COL6A2e26 c.2329 T>C p.Cys777Arg
6	UCMD	Talipes at birth. Walked 2.5–3.5 years. Moderate to severe proximal contractures. Distal laxity.	Heterozygous COL6A3e16 skipping c.6210 + 1 G>A splice donor
7	BM	Delayed motor development. Contractures. Still ambulant at 19 years.	Heterozygous COL6A1e10 c.868 G>A p.Gly290Arg
8	UCMD	Congenital hypotonia, hip dysplasia, neck and feet contractures. Walked from 2 to 15 years of age. Scoliosis. Non-invasive nocturnal ventilation (13–15-year-old).	Heterozygous COL6A1e10 c.869 G>A p.Gly290Glu
9	UCMD	Congenital kypho-scoliosis. Never walked. Severe respiratory insufficiency (tracheostomy at 13 years of age).	Homozygous COL6A2e17 c.1402 C>T p.Arg468X
10	Duchenne	Proximal weakness. Biopsy showed a degenerative myopathy, absent dystrophin and upregulated utrophin.	Whole dystrophin gene deletion
11	LGMD2A	Onset at age 19 years (limb girdle weakness). Limb girdle weakness and atrophy upper and lower extremities. Muscle biopsy showed degenerative myopathy, negative calpain on immunoblot	Compound heterozygous CAPN3 Exon 4: c.550delA Exon 6: c.865C>T p.Arg289Trp
12	LGMD2B	Onset at age 17 years (inability to stand on tiptoes). Proximal weakness upper and lower limbs, wheelchair bound. Muscle biopsy showed degenerative myopathy; dysferlin negative on immunohistochemistry.	Compound het DYSF Exon 19 c.1663C>T p.Arg555Trp Exon 34 c.3708delA
13	MDC1A	Congenital hypotonia. Severe proximal muscle weakness and atrophy, inability to walk. Muscle biopsy showed degenerative myopathy, merosin negative on immunohistochemistry.	Not done
14	Leigh syndrome	Onset at age 1 year (vomiting, delayed motor development). Hypotonia, ataxia, elevated lactate. Sporadic inheritance. Muscle biopsy showed partial COX deficiency.	Homozygous SURF1 exon9 c.845delCT
15	BM	Onset at age 10 years (Achilles tendon contractures). Contractures of finger extensors, elbows, proximal weakness upper and lower extremities. Muscle biopsy shows degenerative myopathy.	Het COL6A2e13 c.1125_1133del9

## TMRM assay of mitochondrial transmembrane potential

We dynamically monitored PTP dysregulation in cell culture using TMRM, a fluorescent lipophilic cation which accumulates in polarized mitochondria in proportion to their transmembrane potential ( $\Delta\Psi_m$ ). Dermal fibroblasts were grown to confluence in 35 mm dishes for 2 days in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal bovine serum, washed twice with 1 ml HEPES-buffered Tyrode's solution at pH 7.2–7.4 (Sigma) and loaded with 20 nM tetramethylrhodamine methyl ester perchlorate (TMRM; Molecular probes) for 20 min at 37°C. Following two further washes the fibroblasts were then re-incubated in 1.5 ml Tyrode's solution and placed on the stage of

the confocal microscope. Myoblasts were seeded in 35 mm dishes, either uncoated or coated with 5 µg/ml laminin (Sigma), 0.01% collagen I (Sigma) or 0.01% collagen VI (BD Biosciences) and grown for two days in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal bovine serum, washed twice in serum free Dulbecco's Modified Eagle's Medium and loaded with 20 nM TMRM as above. To control for the activity of the multi-drug resistance pump and normalize mitochondrial loading cells were incubated in Cyclosporin H in addition to TMRM and then incubated in serum free Dulbecco's Modified Eagle's Medium for 30 mins. Two micromolar CsA (BioChemica) was added at  $t = -30$  min; 5 µM oligomycin (Sigma) was added at  $t = 5$  min which unmask the PTP defect by preventing the  $F_1F_0$ ATP synthase operating in reverse mode maintaining  $\Delta\Psi_m$  in the presence of an open PTP; 5 µM carbonylcyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP; Sigma)

was added at  $t = 50$  min (fibroblasts) or  $t = 80$  min (myoblasts) which acts to increase the conductance of the membrane to protons, effectively collapsing the mitochondrial transmembrane potential (Nicholls and Ward, 2000), indicating baseline values for TMRM fluorescence. Imaging was performed with a real-time confocal system on an inverted Zeiss AXIOVERT 200M microscope fitted with a LSM510 META confocal head with a  $40\times/0.6$  korr LS Achromplan Zeiss objective and excitation wavelength/detection filter of 543/565–615 nm bandpass. Sequential confocal images were acquired at  $512 \times 512$  resolution at 60-s intervals for 60 min or at 120-s intervals for 90 min. The time course of the TMRM signal was calculated using the LSM510 real-time confocal system data acquisition software. The fluorescence signal was corrected for the background, identified as areas without cells, at each time point. Data were expressed as mean  $\pm$  SEM.

## Immunofluorescent labelling of fibroblasts for collagen VI

Fibroblast cells were immunofluorescently labelled for collagen VI as previously described (Hicks *et al.*, 2008). Slides were imaged using a Zeiss Axioplan 2ie MOT microscope, using a  $20\times/0.5$  or  $40\times/0.6$  korr Plan Neofluar objective. Images were captured using a Zeiss AxioCam HRm using Axiovision 4.5 software.

## Immunofluorescent staining of myoblasts for desmin

Cells were fixed in 4% paraformaldehyde for 10 min at room temperature before washing in  $1\times$  phosphate-buffered saline. The cells were then subjected to blocking reagent for 1 h (10% phosphate-buffered saline, 10% foetal bovine serum in  $dH_2O$ ) in the presence of 0.125% Triton X-100 (Sigma 21123). Monoclonal mouse anti-human desmin antibody (DAKO, M0760, clone D33) diluted 1:100 in blocking reagent was applied to the cells at room temperature for 1 h. Cells were then washed three times for 20 min. Alexa Fluor<sup>®</sup>-488 Goat anti-mouse antibody was applied for 1 h at room temperature, diluted 1:100 in blocking reagent. After washing as previously described, ice-cold methanol was applied to the cells for 7 min. Nuclei were stained with VectorShield with DAPI (4',6-diamidino-2-phenylindole) mounting media (Vector Laboratories H-1200). Slides were imaged using a Zeiss Axioplan 2ie MOT microscope, using a  $20\times/0.5$  Plan Neofluar objective. Images were captured using a Zeiss AxioCam HRm using Axiovision 4.5 software.

## Results

### Mitochondrial dysfunction is not common in UCMD fibroblasts

We studied mitochondrial depolarization in skin biopsy-derived fibroblast cells, a cell type readily available for diagnostic use (Hicks *et al.*, 2008) as well as the cell type suggested to be the primary source of collagen VI in muscle (Zou *et al.*, 2008). No depolarization was observed in the normal control cell line (Fig. 1A) and a BM culture was also normal (Fig. 1G). We observed mitochondrial depolarization rescued by CsA in only one out of six UCMD fibroblast cultures (Fig. 1H), whereas the other five UCMD fibroblast lines did not show this effect

(Fig. 1B–F). The cell line in which this phenomenon was noted shows a complete absence of collagen VI from the extra-cellular matrix and also intra-cellularly as judged by immunofluorescence analysis (Fig. 1P), unlike the other cell lines tested here (Fig. 1I–O) or over 100 proven collagen VI disorder fibroblast lines (data not shown). The mutation in this case (homozygous COL6A1 exon3 c.350\_351delTG) results in a shift in the reading frame and a premature termination codon (p.Val117GlyfsX34).

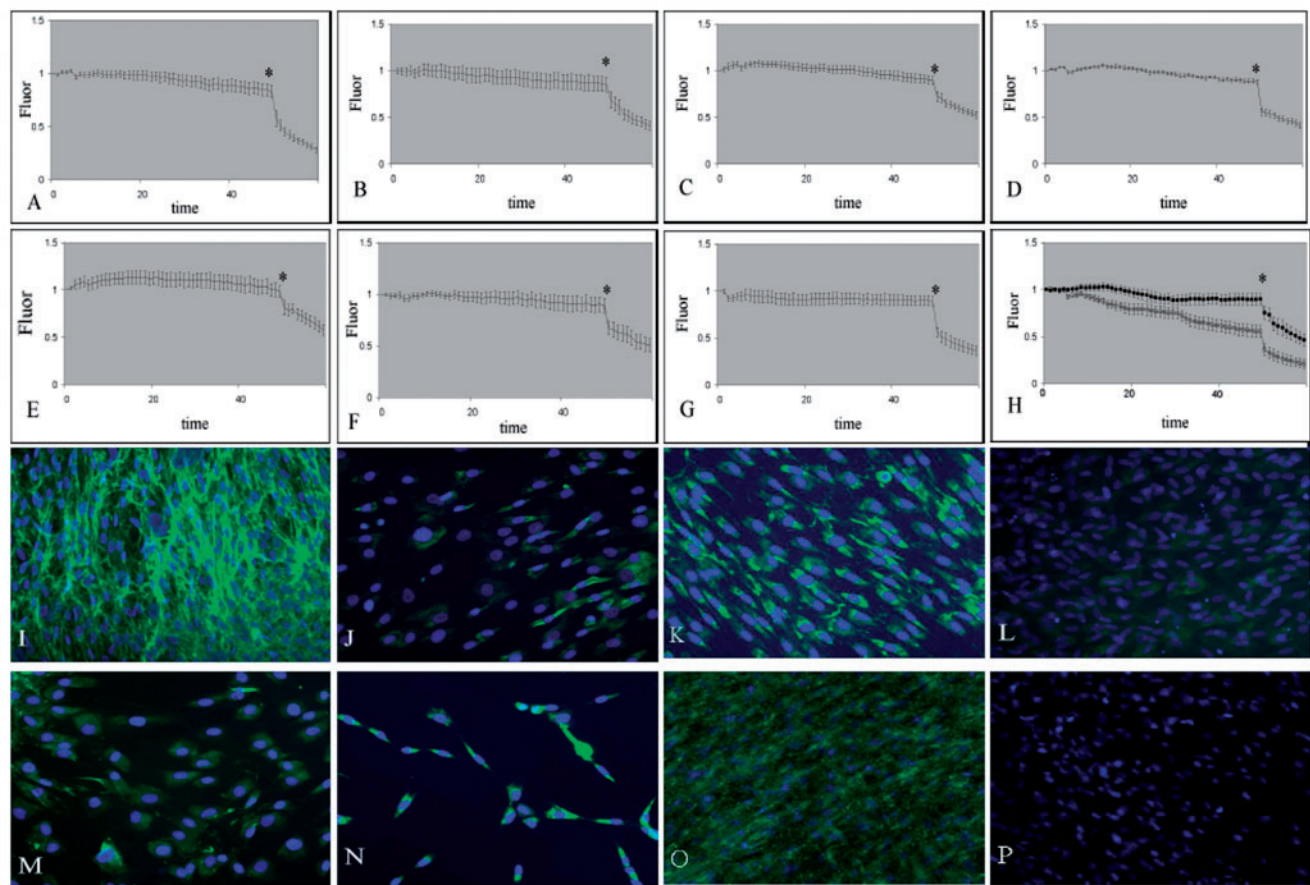
### Mitochondrial dysfunction is found in UCMD and LGMD2B muscle-derived cultures, but not in cultures from patients with other muscular dystrophies

To assess whether mitochondrial depolarization is specific to collagen VI-related muscle disorders we studied myoblasts from two UCMD patients and five patients with other conditions (Fig. 2). The patients were selected to represent other forms of muscular dystrophy in which there is a disruption of the ECM-cell link and/or increased apoptosis (BM, MDC1A and LGMD2A); a Leigh syndrome patient with a mutation in SURF1 (predicted to lack COX activity and have impaired respiratory chain function) and pathologically unrelated muscular dystrophies (Duchenne muscular dystrophy and LGMD2B). In concordance with previously published work (Angelin *et al.*, 2007; Merlini *et al.*, 2008), mitochondrial depolarization was found in muscle-derived cultures from UCMD Patients 8 and 9 (Fig. 2M and N). All of the other cell lines (BM, MDC1A, LGMD2A, Duchenne muscular dystrophy and Leigh syndrome) behaved normally with the exception of the LGMD2B cell line (Patient 12; Fig. 2O), where there was a loss in mitochondrial transmembrane potential similar to or even more severe than the response elicited in UCMD myoblasts. Of the muscle-derived cultures tested by TMRM assay, both UCMD muscle cultures and the LGMD2B culture were found to be largely negative for the specific muscle cell marker, desmin (Fig. 2Q, R and S).

### Mitochondrial depolarization in UCMD muscle-derived cell cultures is not shared by fibroblast cultures from the same patients and can be rescued by ECM constituents other than collagen VI

Paired myoblast and fibroblast cell cultures from the same UCMD patients were tested by the TMRM assay. For both UCMD Patients 8 and 9, depolarization was observed in UCMD myoblast mitochondria but not in the mitochondria of their fibroblast cells (Fig. 3B and C; solid squares represent fibroblast data, open squares represent muscle-derived culture data). Depolarization of all of the abnormal cell lines (Patient 1 fibroblasts, UCMD Patient 8 muscle-derived cultures, UCMD Patient 8 muscle-derived cultures and LGMD2B Patient 12 muscle-derived cultures) could be





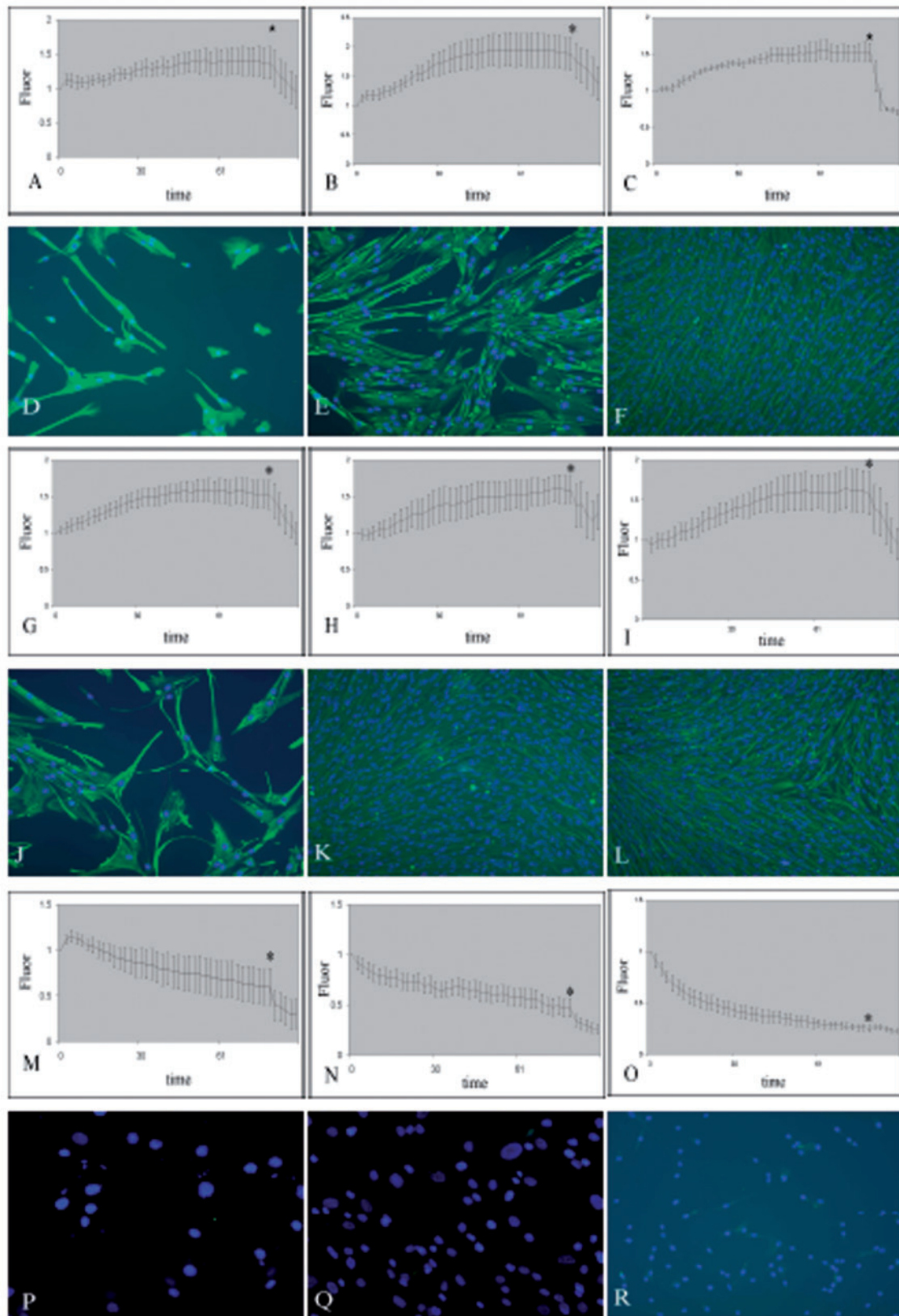
**Fig. 1** Effect of mitochondrial TMRM fluorescence (Fluor) in response to oligomycin over time in fibroblast cultures from six UCMD patients and one BM patient. Oligomycin elicited no depolarization of the mitochondrial membrane in normal human dermal fibroblast cells (A), or in the fibroblasts of UCMD Patients 4 (B), 2 (C), 3 (D), 5 (E) and 6 (F). No depolarization was also observed in fibroblasts from BM Patient 7 (G). Only UCMD Patient 1 showed a decrease in the TMRM signal in response to oligomycin (H, open symbols), which was rescued with CsA (closed symbols). As expected, mitochondrial depolarization resulted from addition of the protonophore FCCP, denoted by asterisk (\*). Data are represented as the mean,  $\pm$  standard error of the mean and are representative of 10 experiments or more per line. Artificial permeabilization revealed intra-cellular retention of collagen VI by immunofluorescence in fibroblast cultures from UCMD Patients 4 (J), Patient 2 (K), Patient 3 (L), Patient 5 (M), Patient 6 (N), compared to the normal control (I) which showed an abundant and well-organized collagen VI matrix. As expected, BM Patient 7 (O), showed a less severe abnormality of the collagen VI matrix, with normal exportation of collagen VI but a perturbed organization characterized by stippling. Collagen VI expression could not be detected either extra- or intracellularly in the fibroblasts from UCMD Patient 1 (P). Images were taken at  $\times 20$  magnification with the exception of M and N ( $\times 40$  magnification).

rescued by pre-incubation with CsA (Fig. 3E–H). To test the specificity of the collagen VI lesion with regards to inappropriate PTP opening and mitochondrial depolarization, we tested components of the ECM for their ability to rescue mitochondrial depolarization. Mitochondrial dysfunction in dermal fibroblasts from UCMD Patient 1 and muscle-derived cultures from UCMD Patients 8 and 9 and LGMD2B Patient 12 could be rescued by plating onto collagen I (Fig. 3M–P) and laminin coated dishes (Fig. 3Q–T), as well as collagen VI (Fig. 3I–L).

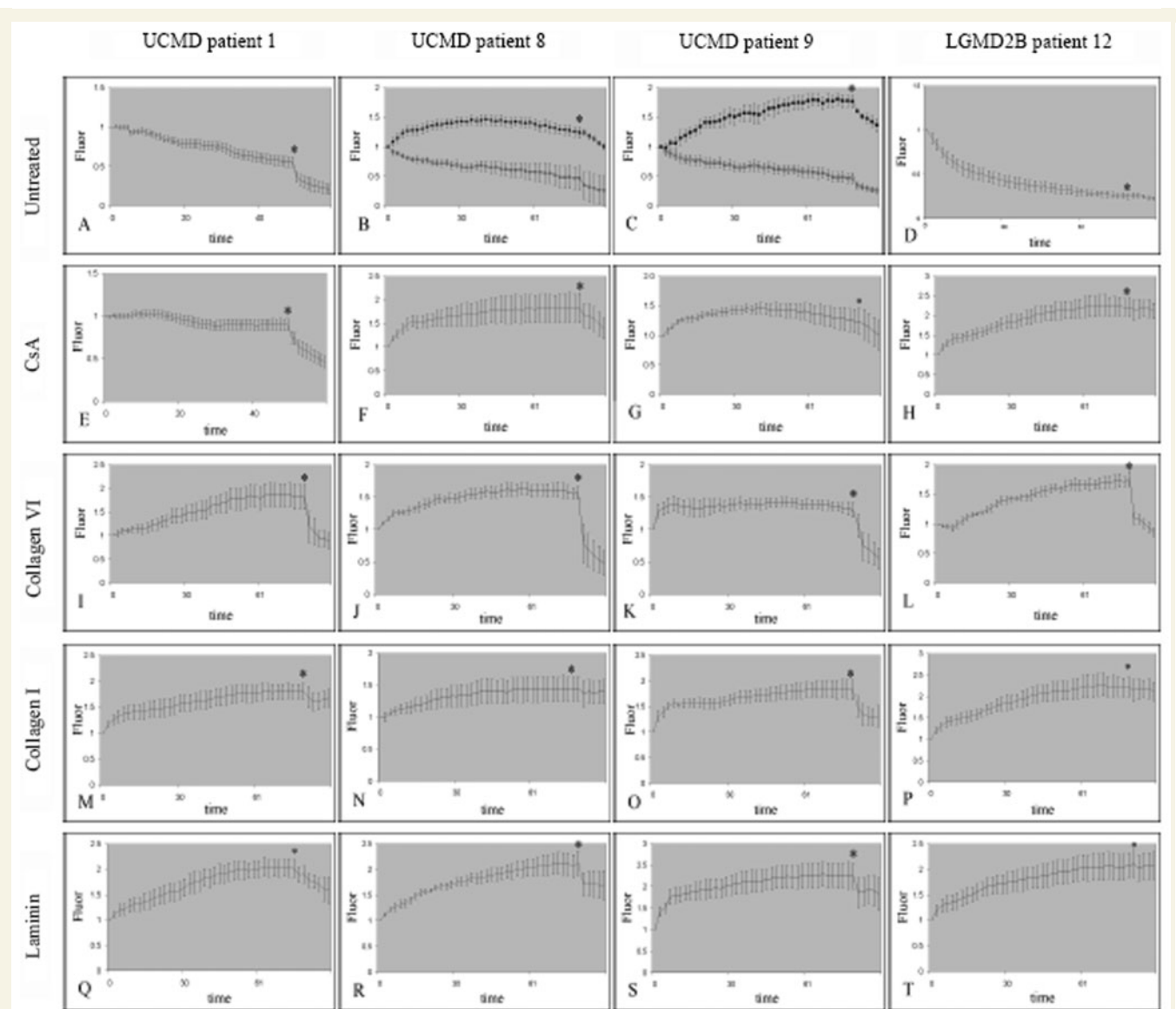
## Discussion

In this paper we have replicated previously published data that show aberrant mitochondrial depolarization in cultures derived

from UCMD muscle, but also show that this dysfunction is not present in fibroblast cultures from the same patients or in fibroblast cultures from other well-characterized UCMD and BM patients. The single fibroblast cell line showing oligomycin induced mitochondrial depolarization was from a patient whose fibroblast cells were completely negative, both intra- and extracellularly, for collagen VI by immunofluorescence, a unique finding in over 100 cell lines tested indicating that it is very rare indeed for patients to be completely 'null' for collagen VI. This patient had a COL6A1 mutation, as did patients with UCMD and BM but normal fibroblast TMRM results, suggesting that the phenotype does not relate to the gene mutated. We hypothesize that muscle cultures are more sensitive to a deficiency of collagen VI than fibroblasts, so that fibroblasts will only show abnormal PTP opening in the complete absence of collagen VI. The recent publication of the



**Fig. 2** Changes of mitochondrial TMRM fluorescence (Fluor) over time induced by oligomycin (A–C, G–I, M–O) and expression of desmin, detected by immunofluorescence, in muscle cultures (D–F, J–L, P–R). Normal healthy donor cells (A and D), BM Patient 15 (B and E), MDC1A Patient 13 (C and F), LGMD2A Patient 11 (G and J), Leigh syndrome Patient 14 (H and K), Duchenne MD Patient 10 (I and L) all show no depolarization of the mitochondrial membrane and are positive for desmin. However, UCMD Patient 8 (M and P), UCMD Patient 9 (N and Q) and LGMD2B Patient 12 (O and R) do depolarize and are negative for desmin. As expected, mitochondrial depolarization resulted from addition of the protonophore FCCP (denoted by asterisk).



**Fig. 3** TMRM fluorescence (Fluor) in fibroblasts from Patient 1 (A, E, I, M and Q) and cultures from UCMD Patient 8 (B, F, J, N and R), UCMD Patient 9 (C, G, K, O and S) and LGMD 2B Patient 12 (D, H, L, P and T) analysed over time. Mitochondrial depolarization was apparent in UCMD Patient 1 (A), UCMD Patient 8 muscle-derived cultures (B, open symbols), UCMD Patient 9 muscle cultures (C, open symbols) and LGMD 2B Patient 12 muscle cultures (D). However, fibroblast cells from UCMD Patient 8 (B, closed symbols) and UCMD Patient 9 (C, closed symbols) did not show any mitochondrial depolarization. Pre-incubation with CycA rescued the depolarizing phenotype in all patient cell lines (E–H) as did plating onto collagen VI (I–L), Collagen I (M–P) and laminin-coated (Q–T) plastic dishes. As expected, mitochondrial depolarization resulted from addition of the protonophore FCCP (denoted by asterisk).

role of muscle interstitial fibroblasts as the main source of collagen VI in skeletal muscle, rather than myoblasts (Zou *et al.*, 2008), suggests that fibroblasts contribute more collagen VI than myofibres. This presents a difficulty in relating the TMRM phenotype of muscle-derived cultures, which largely lack fibroblast cells, to the physiological state *in vivo*.

The authors of a recent publication of an open pilot trial of CsA in collagen VI myopathy patients were encouraged to find that mitochondrial depolarization of muscle biopsy-derived cells was somewhat normalized in repeat cultures taken after 1 month of oral CsA treatment. A limit to the interpretation of

these data however is that the cultures under test by TMRM assay contained twice as many fibroblasts than myoblast cells, which we have shown to be largely resistant to mitochondrial depolarization. None of the patients in the study had a total absence of collagen VI in fibroblasts, which is the only case in which we have found evidence of mitochondrial depolarization in this cell type. We feel that the heterogeneity of the TMRM assay results presented in this paper is a reflection of the heterogeneity of the cell population. We also note that the authors used laminin-coated plates for all TMRM assay experiments, which we have shown to rescue mitochondrial depolarization to



the same extent as CsA, as is the case for other ECM components, collagen I and collagen VI.

There are no previous studies comparing the findings in the *Col6a1*<sup>-/-</sup> null mouse model and UCMD patients to muscle-derived cell lines from patients with other forms of muscular dystrophy. We chose to study myoblast cultures from a range of different muscular dystrophies and were able to demonstrate that while the mitochondrial dysfunction phenotype was absent from four cases where mitochondrial and/or apoptotic abnormalities might have been expected (Leigh syndrome, LGMD2A, MDC1A and BM) (van Erven *et al.*, 1987; Jimenez-Mallebrera *et al.*, 2005; Lampe and Bushby, 2005; Zatz and Starling, 2005) and other unrelated muscular dystrophies, it was abnormal in a muscle culture from a patient with LGMD2B, a completely unrelated form of muscular dystrophy (Baghdiguian *et al.*, 1999). Taking together the TMRM assay data showing that PTP dysregulation is not common in fibroblast cultures and data showing that disease control cell lines with a suggested apoptotic component to pathology can perform well in the assay suggests that poor TMRM assay performance may be a particular characteristic of the state or nature of these cells in culture rather than related to the underlying pathophysiology of disease. Further supportive data for this hypothesis comes from the finding that both the UCMD and LGMD2B muscle-derived cells showing abnormal TMRM results were devoid of the muscle-specific intermediate filament protein, desmin, a well-established myogenic marker, hence our use of the term 'muscle-derived cells', rather than myoblasts. Our own studies have shown that myoblast cultures from LGMD2B patients fuse poorly and are generally difficult to differentiate (unpublished data). Myoblast cultures from UCMD patients are also difficult to establish, as the observation that international repositories completely lack UCMD myoblasts perhaps shows (<http://www.eurobiobank.org>). It is also noteworthy that previous papers reporting the use of UCMD myoblasts have not shown desmin labelling (Angelin *et al.*, 2007; Merlini *et al.*, 2008). We suggest that the latent pro-apoptotic phenotype of both LGMD2B and UCMD myoblasts as measured by the TMRM assay may reflect the general status of these cell cultures. This questions how far TMRM assay data can be extrapolated to the pathophysiological setting. While it was previously thought that cell death in culture was largely due to the passive process of necrosis it is now clearly established that many cell lines will respond to the stressful culture environment by undergoing cellular suicide or apoptosis (al-Rubeai and Singh, 1998) as may be the case in these cell lines.

Having defined the particular cell types where we were able to demonstrate an abnormality or PTP opening, we investigated the specificity of the response to CsA and collagen VI by plating cells on ECM components other than collagen VI. Normalization of the TMRM assay was obtained with plating on both collagen 1 and laminin, indicating that it was not replacement of collagen VI *per se* that rescued the cellular phenotype. The rescue of membrane depolarization by ECM components is best explained by the influence of the ECM on the execution of the apoptotic program (Abbott, 2003). One could hypothesize that re-establishing ECM links *in vitro* by plating on ECM components could act via the ERK pathway to phosphorylate and therefore activate

the anti-apoptotic factor, *Bcl-2* (Green and Reed, 1998), an inhibitor of PTP opening (Zamzami *et al.*, 1996). We still do not understand how mitochondria 'sense' a defect of the extra-cellular space. Genetic inhibition of a component of the PTP (pfif) which binds CsA attenuates pathology in mouse models of muscular dystrophy showing sarcolemmal fragility (Millay *et al.*, 2008). Our work shows that myoblast cultures from human diseases with sarcolemmal fragility fail to depolarise in the TMRM assay. In these models it is well recognized that reduced physical integrity of the sarcolemma leads to calcium influx (Mallouk *et al.*, 2000), whereas intracellular calcium levels are normal even in the presence of extremely high extracellular calcium levels in the *Col6a1*<sup>-/-</sup> null mouse (Irwin *et al.*, 2003) and therefore collagen VI deficiency is postulated to exert its deleterious effect on the mitochondria via signalling through adhesion molecules (Rizzuto, 2003).

So where does this leave the therapeutic option to treat UCMD/BM with cyclosporine A? We have shown that the cells which demonstrate abnormal PTP opening in culture share the characteristics of poor growth and differentiation rather than a shared pathophysiology. Rescue of the phenotype can be achieved by plating on a variety of ECM components. We conclude that the link between this cellular phenotype as measured by the TMRM assay and the development (and in the context of treatment, the progression) of disease needs to be better established.

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## References

- Abbott A. Cell culture: biology's new dimension. *Nature* 2003; 424: 870–2.
- al-Rubeai M, Singh RP. Apoptosis in cell culture. *Curr Opin Biotechnol* 1998; 9: 152–6.
- Angelin A, Tiepolo T, Sabatelli P, Grumati P, Bergamin N, Golfieri C, et al. Mitochondrial dysfunction in the pathogenesis of Ullrich congenital muscular dystrophy and prospective therapy with cyclosporins. *Proc Natl Acad Sci USA* 2007; 104: 991–6.
- Baghdiguian S, Martin M, Richard I, Pons F, Astier C, Bourg N, et al. Calpain 3 deficiency is associated with myonuclear apoptosis and pro-found perturbation of the I $\kappa$ B $\alpha$ /NF- $\kappa$ B pathway in limb-girdle muscular dystrophy type 2A. *Nat Med* 1999; 5: 503–511.
- Bonaldo P, Braghetta P, Zanetti M, Piccolo S, Volpin D, Bressan GM. Collagen VI deficiency induces early onset myopathy in the mouse: an animal model for Bethlem myopathy. *Hum Mol Genet* 1998; 7: 2135–40.
- Colombatti A, Mucignat MT, Bonaldo P. Secretion and matrix assembly of recombinant type VI collagen. *J Biol Chem* 1995; 270: 13105–11.
- Crompton M. The mitochondrial permeability transition pore and its role in cell death. *Biochem J* 1999; 341: 233–49.
- Furthmayr H, Wiedemann H, Timpl R, Odermatt E, Engel J. Electron-microscopical approach to a structural model of intima collagen. *Biochem J* 1983; 211: 303–11.
- Girgenrath M, Dominov JA, Kostek CA, Miller JB. Inhibition of apoptosis improves outcome in a model of congenital muscular dystrophy. *J Clin Invest* 2004; 114: 1635–9.
- Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998; 281: 1309–12.
- Hicks D, Lampe AK, Barresi R, Charlton R, Fiorillo C, Bonnemann CG, et al. A refined diagnostic algorithm for Bethlem myopathy. *Neurology* 2008; 70: 1192–9.
- Irwin WA, Bergamin N, Sabatelli P, Reggiani C, Megighian A, Merlini L, et al. Mitochondrial dysfunction and apoptosis in myopathic mice with collagen VI deficiency. *Nat Genet* 2003; 35: 367–71.
- Ishikawa H, Sugie K, Murayama K, Awaya A, Suzuki Y, Noguchi S, et al. Ullrich disease due to deficiency of collagen VI in the sarcolemma. *Neurology* 2004; 62: 620–3.
- Jimenez-Mallebrera C, Brown SC, Sewry CA, Muntoni F. Congenital muscular dystrophy: molecular and cellular aspects. *Cell Mol Life Sci* 2005; 62: 809–23.
- Kirschner J, Bonnemann CG. The congenital and limb-girdle muscular dystrophies: sharpening the focus, blurring the boundaries. *Arch Neurol* 2004; 61: 189–99.
- Kuo HJ, Maslen CL, Keene DR, Glanville RW. Type VI collagen anchors endothelial basement membranes by interacting with type IV collagen. *J Biol Chem* 1997; 272: 26522–9.
- Lampe AK, Bushby KMD. Collagen VI related muscle disorders. *J Med Genet* 2005; 42: 673–85.
- Mallouk N, Jacquemond V, Allard B. Elevated subsarcolemmal Ca<sup>2+</sup> in mdx mouse skeletal muscle fibers detected with Ca<sup>2+</sup>-activated K<sup>+</sup> channels. *Proc Natl Acad Sci USA* 2000; 97: 4950–5.
- Merlini L, Angelin A, Tiepolo T, Braghetta P, Sabatelli P, Zamparelli A, et al. Cyclosporin A corrects mitochondrial dysfunction and muscle apoptosis in patients with collagen VI myopathies. *Proc Natl Acad Sci USA* 2008; 105: 5225–9.
- Millay DP, Sargent MA, Osinska H, Baines CP, Barton ER, Vuagniaux G, et al. Genetic and pharmacologic inhibition of mitochondrial-dependent necrosis attenuates muscular dystrophy. *Nat Med* 2008; 14: 442–7.
- Nicholls DG, Ward MW. Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts. *Trends Neurosci* 2000; 23: 166–74.
- Rizzuto R. The collagen-mitochondria connection. *Nat Genet* 2003; 35: 300–1.
- Sabatelli P, Bonaldo P, Lattanzi G, Braghetta P, Bergamin N, Capanni C, et al. Collagen VI deficiency affects the organization of fibronectin in the extracellular matrix of cultured fibroblasts. *Matrix Biol* 2001; 20: 475–86.
- Sanes JR. The basement membrane/basal lamina of skeletal muscle. [see comment]. *J Biol Chem* 2003; 278: 12601–4.
- van Erven PM, Cillessen JP, Eekhoff EM, Gabreels FJ, Doesburg WH, Lemmens WA, et al. Leigh syndrome, a mitochondrial encephalo(myo)pathy. A review of the literature. *Clin Neurol Neurosurg* 1987; 89: 217–30.
- Wiberg C, Hedbom E, Khairullina A, Lamande SR, Oldberg A, Timpl R, et al. Biglycan and decorin bind close to the N-terminal region of the collagen VI triple helix. *J Biol Chem* 2001; 276: 18947–52.
- Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M, et al. Mitochondrial control of nuclear apoptosis. [see comment]. *J Exp Med* 1996; 183: 1533–44.
- Zatz M, Starling A. Mechanisms of disease: calpains and disease. *N Engl J Med* 2005; 352: 2413–23.
- Zou YMD, Zhang R-ZMD, Sabatelli PB, Chu M-LP, Bonnemann CGMD. Muscle interstitial fibroblasts are the main source of collagen VI synthesis in skeletal muscle: implications for congenital muscular dystrophy types Ullrich and Bethlem. *J Neuropathol Exp Neurol* 2008; 67: 144–54.