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# Mitochondrial dysfunction and apoptosis in myopathic mice with collagen VI deficiency

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**Collagen VI is an extracellular matrix protein that forms a microfilamentous network in skeletal muscles and other organs[1–3]. Inherited mutations in genes encoding collagen VI in humans cause two muscle diseases, Bethlem myopathy and Ullrich congenital muscular dystrophy[4,5]. We previously generated collagen VI–deficient (*Col6a1*$^{-/-}$) mice and showed that they have a muscle phenotype that strongly resembles Bethlem myopathy[6]. The pathophysiological defects and mechanisms leading to the myopathic disorder were not known. Here we show that *Col6a1*$^{-/-}$ muscles have a loss of contractile strength associated with ultrastructural alterations of sarcoplasmic reticulum (SR) and mitochondria and spontaneous apoptosis. We found a latent mitochondrial dysfunction in myofibers of *Col6a1*$^{-/-}$ mice on incubation with the selective $F_1F_O$-ATPase inhibitor oligomycin, which caused mitochondrial depolarization, $Ca^{2+}$ deregulation and increased apoptosis. These defects were reversible, as they could be normalized by plating *Col6a1*$^{-/-}$ myofibers on collagen VI or by addition of cyclosporin A (CsA), the inhibitor of mitochondrial permeability transition pore (PTP). Treatment of *Col6a1*$^{-/-}$ mice with CsA rescued the muscle ultrastructural defects and markedly decreased the number of apoptotic nuclei *in vivo*. These findings indicate that collagen VI myopathies have an unexpected mitochondrial pathogenesis that could be exploited for therapeutic intervention.**

Mechanical measurements showed that isolated *Col6a1*$^{-/-}$ muscles had less tension development (**Fig. 1**). Loss of contractile strength was most prominent in diaphragm, which is the most affected muscle[6], but was also detected in flexor digitorum brevis (FDB) and other hindlimb muscles (data not shown). In diaphragm strips, isometric tetanic tension was significantly lower (**Fig. 1a**). Twitch tension was even more reduced and relaxation time was prolonged (**Fig. 1b–d**), suggesting an abnormal SR function with less $Ca^{2+}$ released on stimulation or less effective $Ca^{2+}$ uptake during relaxation[7]. The decrease in tension development was not due to variations in fiber excitability (**Supplementary Table 1** online) or to changes in fiber type composition (data not shown). The decreased contractile strength of *Col6a1*$^{-/-}$ muscles was confirmed by mechanical measurements on single skinned fibers during maximal activation (**Fig. 1e**). We detected structural alterations and irregular shape in about one-third of the skinned *Col6a1*$^{-/-}$ fibers (**Fig. 1f**). These partially damaged fibers were never found in wild-type samples and developed significantly less tension than apparently healthy *Col6a1*$^{-/-}$ fibers (**Fig. 1g**). Taken together with the presence of Evans blue–positive fibers[6], this finding suggests that collagen VI–deficient muscles contain differently affected fibers that might represent various steps of the dystrophic process.

Electron microscopic analysis identified ultrastructural defects in various *Col6a1*$^{-/-}$ muscles. About 30% of fibers had structural alterations of mitochondria and SR (**Fig. 2b**). Mitochondria had abnormal cristae with tubular shape and altered matrix density associated with the presence of dense bodies (**Fig. 2d,e**). We observed marked dilations in SR, especially at the level of triadic system (**Fig. 2g**), but sarcomeres, sarcolemma and basal lamina appeared normal. We detected myonuclei with the typical hallmarks of apoptosis in *Col6a1*$^{-/-}$ fibers with organelle alterations (**Fig. 2i**). None of these defects were present in wild-type muscles (**Fig. 2a,c,f,h**).

We investigated mitochondrial function in cultured FDB myofibers. Mitochondrial transmembrane potential ($\varnothing\psi_m$) was monitored by tetramethylrhodamine methyl ester (TMRM), a fluorescent probe that accumulates in polarized mitochondria and is released when $\varnothing\psi_m$ decreases[8]. When measured for 1 h in standard conditions, the absolute TMRM fluorescence showed no significant differences between *Col6a1*$^{-/-}$ and wild-type fibers, indicating that $\varnothing\psi_m$ levels were the same. But a mitochondrial dysfunction of *Col6a1*$^{-/-}$ fibers could have been masked by the ATP synthase operating in a reverse mode, maintaining $\varnothing\psi_m$ during the experiment[9]. To test this hypothesis, we incubated the fibers with oligomycin, an inhibitor of mitochondrial $F_1F_O$-ATP synthase that is widely used to investigate mitochondrial function in cultured cells[9–11]. As expected, addition of oligomycin to wild-type fibers did not cause immediate $\varnothing\psi_m$ changes, and mitochondrial depolarization proceeded at slow rates even after extensive incubation (**Fig. 3a**). We observed fast mitochondrial depolarization in wild-type fibers only after addition of the protonophore carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP). In contrast, addition of oligomycin to the *Col6a1*$^{-/-}$ fibers quickly caused marked mitochondrial depolarization (**Fig. 3a,b**). Depolarization was fast, proceeding at a rate

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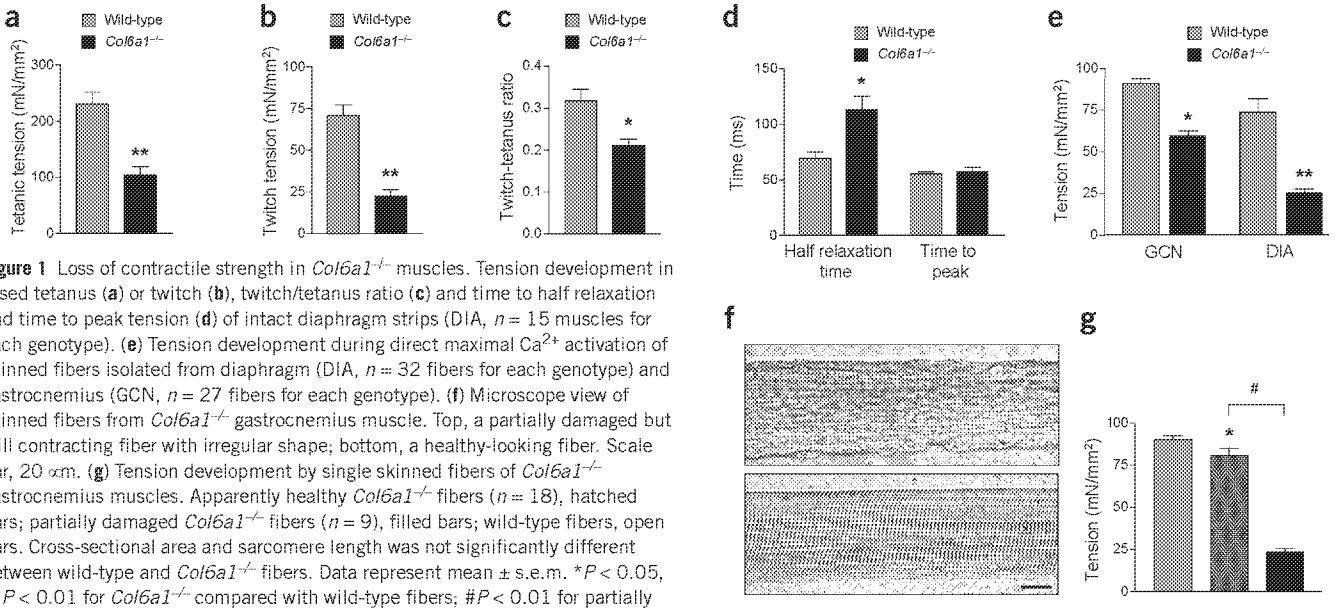


**Figure 1** Loss of contractile strength in *Col6a1*$^{-/-}$ muscles. Tension development in fused tetanus (**a**) or twitch (**b**), twitch/tetanus ratio (**c**) and time to half relaxation and time to peak tension (**d**) of intact diaphragm strips (DIA, $n = 15$ muscles for each genotype). (**e**) Tension development during direct maximal $Ca^{2+}$ activation of skinned fibers isolated from diaphragm (DIA, $n = 32$ fibers for each genotype) and gastrocnemius (GCN, $n = 27$ fibers for each genotype). (**f**) Microscope view of skinned fibers from *Col6a1*$^{-/-}$ gastrocnemius muscle. Top, a partially damaged but still contracting fiber with irregular shape; bottom, a healthy-looking fiber. Scale bar, 20 μm. (**g**) Tension development by single skinned fibers of *Col6a1*$^{-/-}$ gastrocnemius muscles. Apparently healthy *Col6a1*$^{-/-}$ fibers ($n = 18$), hatched bars; partially damaged *Col6a1*$^{-/-}$ fibers ($n = 9$), filled bars; wild-type fibers, open bars. Cross-sectional area and sarcomere length was not significantly different between wild-type and *Col6a1*$^{-/-}$ fibers. Data represent mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$ for *Col6a1*$^{-/-}$ compared with wild-type fibers; $\#P < 0.01$ for partially damaged *Col6a1*$^{-/-}$ fibers compared with apparently healthy *Col6a1*$^{-/-}$ fibers.

comparable to that observed in wild-type fibers treated with FCCP, indicating that *Col6a1*$^{-/-}$ mitochondria were very permeable to protons.

Under various pathological conditions, mitochondria undergo a large increase in inner membrane permeability due to opening of the PTP. This process depends on mitochondrial $Ca^{2+}$ accumulation and leads to mitochondrial depolarization and other modifications that may trigger apoptosis[12]. Treatment with the PTP inhibitor CsA (**Fig. 3c**) or with the membrane-permeant $Ca^{2+}$ chelator BAPTA-AM (**Fig. 3d**) greatly improved the $\Delta\psi_m$ response to oligomycin in *Col6a1*$^{-/-}$ fibers, but cyclosporin H (CsH), an analog that does not inhibit PTP[13], was ineffective (**Fig. 3c**). The $\Delta\psi_m$ changes in *Col6a1*$^{-/-}$ fibers also normalized when we plated the fibers on collagen VI, indicating that the abnormal mitochondrial response to oligomycin is the result of a reversible alteration (**Fig. 3d**). These experiments indicate that the mitochondrial dysfunction unmasked by oligomycin in *Col6a1*$^{-/-}$ fibers involves PTP opening.

Increased PTP opening may alter the dynamics of intracellular $Ca^{2+}$ fluxes[14,15]. Therefore, we monitored the resting $[Ca^{2+}]_c$ of FDB fibers with Fura-2 (ref. 16; **Fig. 4**). Basal $[Ca^{2+}]_c$ levels in wild-type and *Col6a1*$^{-/-}$ fibers were not significantly different when measured in standard conditions (data not shown) or in the presence of 20 mM extracellular $Ca^{2+}$ (**Fig. 4a**). Addition of oligomycin caused a marked

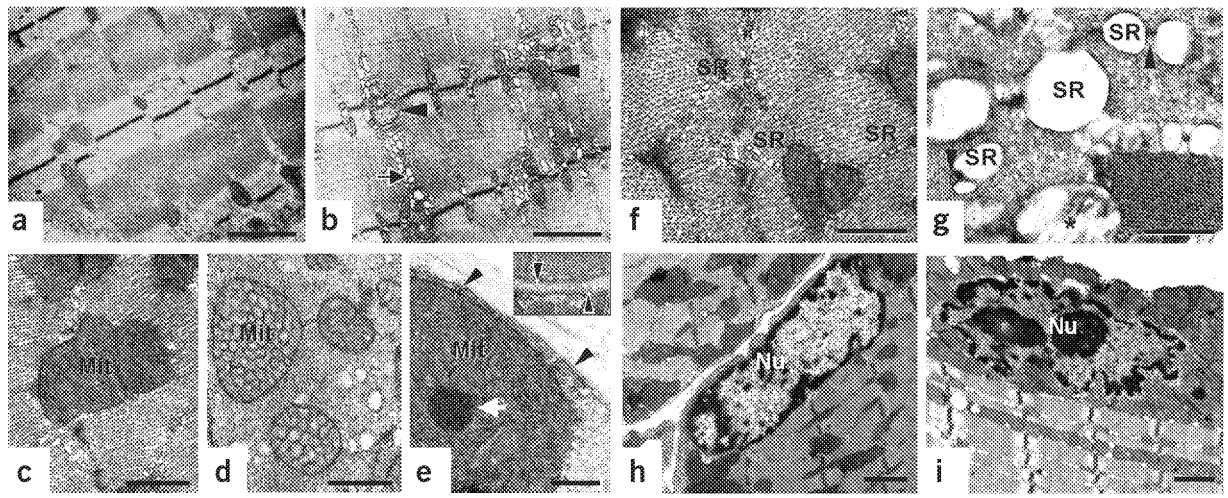


**Figure 2** Ultrastructural defects in collagen VI–deficient muscles. Electron micrographs of diaphragm and FDB muscles from wild-type (**a**,**c**,**f**,**h**) and *Col6a1*$^{-/-}$ (**b**,**d**,**e**,**g**,**i**) mice. (**a**,**b**) Low-power view of FDB longitudinal sections showing alterations in SR (arrows) and mitochondria (arrowheads) in *Col6a1*$^{-/-}$ fibers. Myofibrillar network is comparable in *Col6a1*$^{-/-}$ and wild-type mice. (**c**–**e**) High-power view of diaphragm transverse sections showing abnormal mitochondria (Mit) with altered cristae and dense bodies (white arrow) in *Col6a1*$^{-/-}$ fibers. Sarcolemma and basal lamina appear normal (arrowheads and inset in **e**). (**f**,**g**) High-power view of the triadic system showing dilation of the terminal cisternae of SR and normal T-tubules (arrowheads) in *Col6a1*$^{-/-}$ fibers. Swelling is visible in some *Col6a1*$^{-/-}$ mitochondria (asterisk). (**h**,**i**) Peripheral chromatin condensation and irregular shape characteristic of apoptosis are detected in *Col6a1*$^{-/-}$ myonuclei (Nu). Scale bars: **a**,**b**,**h**,**i**, 1 μm; **c**–**g**, 0.5 μm.

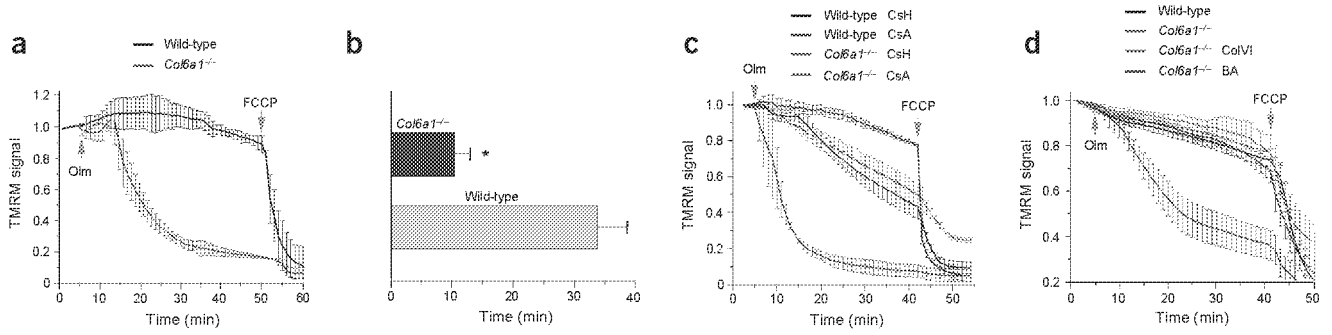


**Figure 3** Altered mitochondrial response to oligomycin in collagen VI–deficient myofibers. FDB muscle fibers were loaded with TMRM (20 nM) for 10 min at 37 °C. TMRM accumulates in mitochondria that maintain $\Delta\psi_m$. Oligomycin (Olm, 5 μM) and the protonophore FCCP (5 μM) were added at the indicated time points. (**a**) Measurement of $\Delta\psi_m$ in *Col6a1*$^{-/-}$ and wild-type fibers incubated with oligomycin. (**b**) Statistical analysis of the time required for a 20% decrease of the TMRM signal after addition of oligomycin in *Col6a1*$^{-/-}$ (five experiments) and wild-type (four experiments) fibers. (**c**) Effect of the PTP inhibitor CsA (2 μM). CsH (4 μM), an analog that does not inhibit PTP, was used as a control. CsA or CsH was added at $t = 0$. (**d**) Effect of the intracellular $Ca^{2+}$ chelator BAPTA-AM and of collagen VI. Where indicated, BAPTA-AM (BA, 5 μM) was added 15 min before the experiment or fibers were cultured on a substrate of collagen VI (ColVI) instead of laminin. Neither treatment changed the response of wild-type fibers (data not shown). Data represent mean ± s.e.m., $n = 2$–6 fibers for each time point and all results were confirmed in two or more independent experiments. *$P < 0.01$ for *Col6a1*$^{-/-}$ compared with wild-type fibers.

increase of $[Ca^{2+}]_c$ in *Col6a1*$^{-/-}$ but not wild-type fibers (**Fig. 4b,c**). As for mitochondrial depolarization, the $[Ca^{2+}]_c$ response of *Col6a1*$^{-/-}$ fibers was normalized by plating on collagen VI (**Fig. 4b**). To investigate the mechanisms of oligomycin-dependent $[Ca^{2+}]_c$ increase, we incubated *Col6a1*$^{-/-}$ myofibers with EGTA and various $Ca^{2+}$ channel antagonists (**Fig. 4d**). The $[Ca^{2+}]_c$ increase was still detected in nominally $Ca^{2+}$-free buffer or in the presence of sarcolemmal voltage-dependent $Ca^{2+}$ channel antagonists, such as verapamil, but it was completely prevented by dantrolene, a specific inhibitor of the SR $Ca^{2+}$ release channels[17] (**Fig. 4d**). CsA, but not CsH, delayed and reduced the oligomycin-dependent $[Ca^{2+}]_c$ increase (data not shown). These data indicate that SR and PTP are involved in the abnormal $[Ca^{2+}]_c$ response of *Col6a1*$^{-/-}$ fibers to oligomycin, in keeping with the mutual regulatory role of mitochondria and SR for $Ca^{2+}$ handling in excitation-contraction coupling[18].

We evaluated the occurrence of DNA strand breaks and detected TUNEL-positive (apoptotic) nuclei in both *Col6a1*$^{-/-}$ muscles and cultured *Col6a1*$^{-/-}$ FDB fibers, but not in the corresponding wild-type samples (**Fig. 5a,b**). *Col6a1*$^{-/-}$ fibers had approximately seven times more apoptotic nuclei than wild-type samples (**Fig. 5c**). Addition of oligomycin increased the fraction of apoptotic nuclei and caused a significant decrease of the total number of myonuclei in *Col6a1*$^{-/-}$ fibers; both events were prevented by CsA (**Fig. 5c,d** and **Supplementary Table 2** online). As adjacent fiber segments are controlled by individual nuclei[19], progressive depletion of *Col6a1*$^{-/-}$ myonuclei may contribute to muscle fiber loss and dystrophic processes.

To test whether PTP has a crucial role in the pathogenesis of the myopathy *in vivo*, we subjected wild-type and *Col6a1*$^{-/-}$ mice to intraperitoneal injection of 5 mg CsA per kg body weight every 12 h. We chose this dose and frequency of CsA treatment on the basis of *in vivo* experiments in which PTP inhibition was estimated from the calcium retention capacity of liver mitochondria[20] (M. E. Soriano and P. Bernardi, unpublished data). After 4 d of CsA treatment, we observed substantially fewer apoptotic nuclei in both diaphragm sec-

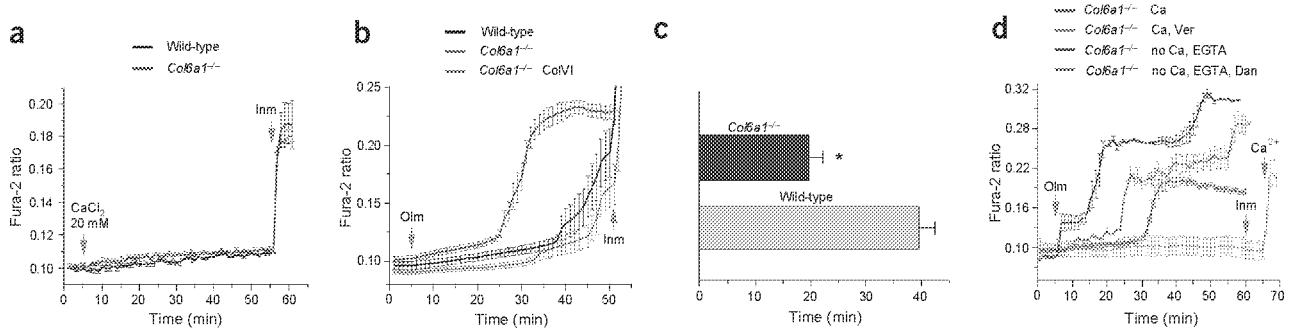


**Figure 4** Altered $[Ca^{2+}]_c$ response to oligomycin in collagen VI–deficient myofibers. FDB muscle fibers were loaded with Fura-2/AM (5 μM) for 20 min at 37 °C. The ratio of emission signals after excitation at 340 nm and 380 nm (Fura-2 ratio) increases with $Ca^{2+}$ levels. Oligomycin (Olm, 5 μM) and ionomycin (Inm, 5 μM) were added where indicated. (**a**) Measurement of $[Ca^{2+}]_c$ in *Col6a1*$^{-/-}$ and wild-type fibers using oligomycin-free Tyrode buffer supplemented with 20 mM $CaCl_2$. (**b**) Measurement of $[Ca^{2+}]_c$ in *Col6a1*$^{-/-}$ and wild-type fibers incubated with oligomycin and effect of collagen VI addition. Where indicated, fibers were cultured on a substrate of collagen VI (ColVI) instead of laminin. Collagen VI addition had no effect on wild-type fibers (data not shown). (**c**) Statistical analysis of the time required for a 50% increase of Fura-2 signal after addition of oligomycin in *Col6a1*$^{-/-}$ (nine experiments) and wild-type (six experiments) fibers. (**d**) Effect of EGTA, verapamil and dantrolene. Verapamil (Ver, 150 μM), dantrolene (Dan, 100 μM) or EGTA (5 mM) was added at $t = 0$ in standard Tyrode buffer (Ca) or in Tyrode buffer without $CaCl_2$ (no Ca). $CaCl_2$ ($Ca^{2+}$, 10 mM) was added at the end of the experiment as a control. Data represent mean ± s.e.m., $n = 2$–6 fibers for each time point and all results were confirmed in two or more independent experiments. *$P < 0.01$ for *Col6a1*$^{-/-}$ compared with wild-type fibers.

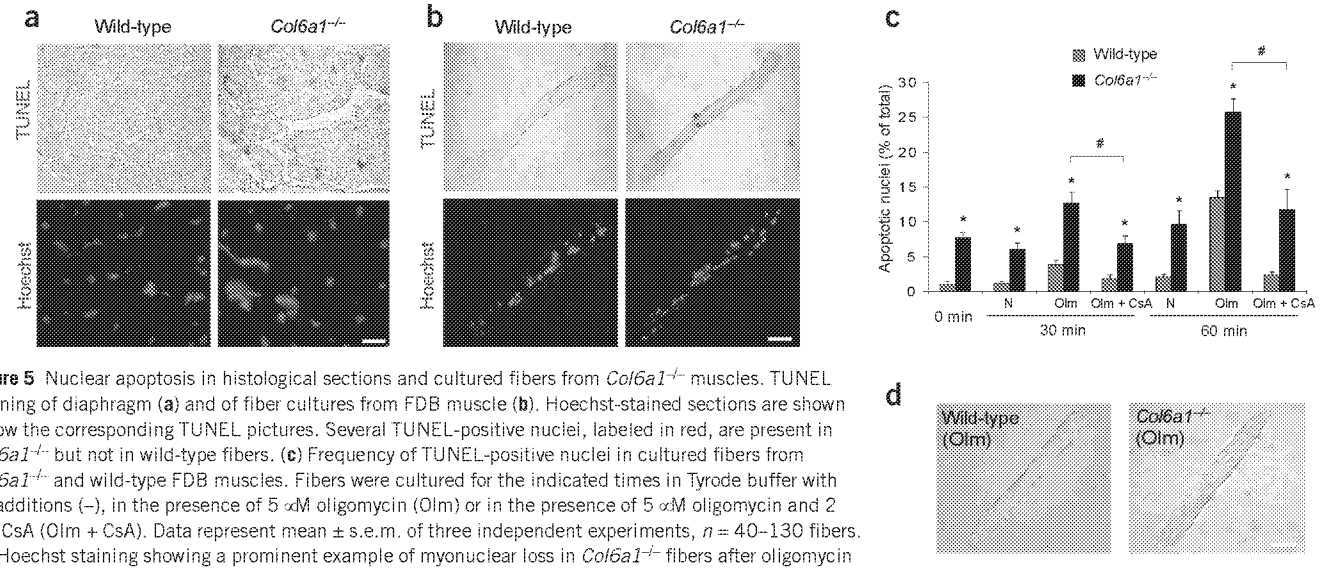


**Figure 5** Nuclear apoptosis in histological sections and cultured fibers from *Col6a1*$^{-/-}$ muscles. TUNEL staining of diaphragm (**a**) and of fiber cultures from FDB muscle (**b**). Hoechst-stained sections are shown below the corresponding TUNEL pictures. Several TUNEL-positive nuclei, labeled in red, are present in *Col6a1*$^{-/-}$ but not in wild-type fibers. (**c**) Frequency of TUNEL-positive nuclei in cultured fibers from *Col6a1*$^{-/-}$ and wild-type FDB muscles. Fibers were cultured for the indicated times in Tyrode buffer with no additions (–), in the presence of 5 αM oligomycin (Olm) or in the presence of 5 αM oligomycin and 2 αM CsA (Olm + CsA). Data represent mean ± s.e.m. of three independent experiments, $n = 40$–130 fibers. (**d**) Hoechst staining showing a prominent example of myonuclear loss in *Col6a1*$^{-/-}$ fibers after oligomycin addition. Scale bars: **a**, 50 αm; **b**,**d**, 10 αm. $^*P < 0.01$ for *Col6a1*$^{-/-}$ compared with wild-type fibers; $\#P < 0.01$ for *Col6a1*$^{-/-}$ (Olm + CsA) compared with *Col6a1*$^{-/-}$ (Olm) fibers.



tions (**Fig. 6a**,**b**) and isolated FDB fibers of *Col6a1*$^{-/-}$ mice (**Fig. 6c**). Occurrence of PTP inhibition *in vivo* was confirmed by measurements of the calcium retention capacity of isolated mitochondria, which was always higher in mice treated with CsA (data not shown). We also treated mice with FK506, which inhibits calcineurin but not PTP[13]. FK506 increased rather than decreased the incidence of apoptotic nuclei in both wild-type and *Col6a1*$^{-/-}$ mice (**Fig. 6a–c**). When compared with diaphragm fibers from *Col6a1*$^{-/-}$ mice treated with vehicle (**Fig. 6d–f**), those from *Col6a1*$^{-/-}$ mice treated with CsA recovered considerably from the ultrastructural defects, as both mitochondrial and SR abnormalities disappeared (**Fig. 6g–i**). Rescue was virtually complete; we detected a small residual SR dilation in 1 of 300 fibers examined for diaphragm and 9 of 300 fibers for FDB muscle.

Our results identify an unexpected mitochondrial pathogenesis in collagen VI deficiency. The key issue is the mechanism leading to mitochondrial defects and apoptosis. $Ca^{2+}$ overload has been proposed as an early event in certain muscular dystrophies[21], and one possibility is that slight increases of sarcolemmal $Ca^{2+}$ influx in collagen VI–deficient fibers might result in intracellular $Ca^{2+}$ overload, eventually causing PTP opening and apoptosis. Our results suggest that this may not be the case, because $[Ca^{2+}]_c$ levels were indistinguishable in wild-type and *Col6a1*$^{-/-}$ fibers, even at 20 mM extracellular $Ca^{2+}$, and because addition of dantrolene or CsA, but not verapamil, ameliorated oligomycin-dependent $Ca^{2+}$ deregulation. Another possibility is suggested by the interaction of collagen VI with integrins and other cell surface receptors[2,3]. Collagen VI prevents

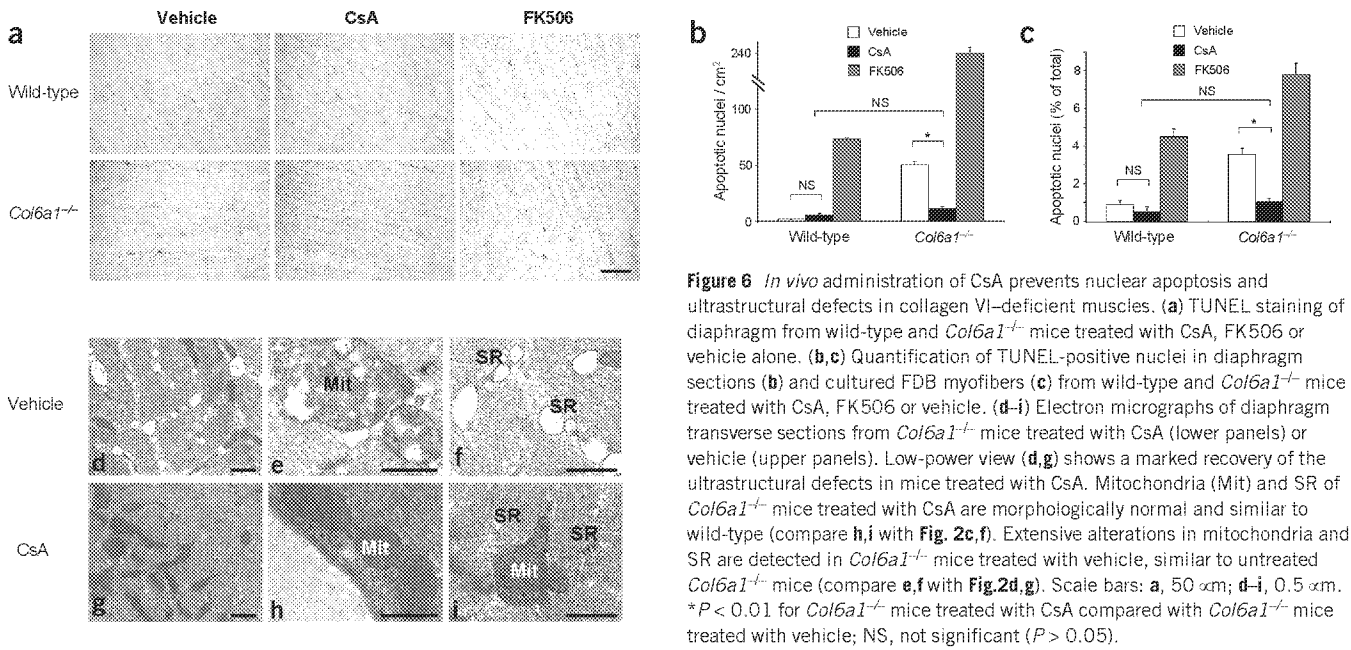


**Figure 6** *In vivo* administration of CsA prevents nuclear apoptosis and ultrastructural defects in collagen VI–deficient muscles. (**a**) TUNEL staining of diaphragm from wild-type and *Col6a1*$^{-/-}$ mice treated with CsA, FK506 or vehicle alone. (**b**,**c**) Quantification of TUNEL-positive nuclei in diaphragm sections (**b**) and cultured FDB myofibers (**c**) from wild-type and *Col6a1*$^{-/-}$ mice treated with CsA, FK506 or vehicle. (**d–i**) Electron micrographs of diaphragm transverse sections from *Col6a1*$^{-/-}$ mice treated with CsA (lower panels) or vehicle (upper panels). Low-power view (**d**,**g**) shows a marked recovery of the ultrastructural defects in mice treated with CsA. Mitochondria (Mit) and SR of *Col6a1*$^{-/-}$ mice treated with CsA are morphologically normal and similar to wild-type (compare **h**,**i** with **Fig. 2c**,**f**). Extensive alterations in mitochondria and SR are detected in *Col6a1*$^{-/-}$ mice treated with vehicle, similar to untreated *Col6a1*$^{-/-}$ mice (compare **e**,**f** with **Fig.2d**,**g**). Scale bars: **a**, 50 αm; **d–i**, 0.5 αm. $^*P < 0.01$ for *Col6a1*$^{-/-}$ mice treated with CsA compared with *Col6a1*$^{-/-}$ mice treated with vehicle; NS, not significant ($P > 0.05$).

apoptosis of fibroblasts when integrin-matrix interactions are perturbed or serum is withdrawn[22,23]. Notably, addition of collagen VI to fibroblasts causes downregulation of Bax[23], a proapoptotic protein that triggers PTP opening[12]. Lack of collagen VI might cause mitochondrial dysfunction and increased PTP opening through an abnormal engagement of integrins, in keeping with the recent finding that integrin-mediated signaling regulates mitochondrial function[24]. Increased PTP opening would alter $Ca^{2+}$ handling by SR[15], cause $Ca^{2+}$ deregulation with further increase of the PTP open time and thus set in motion a self-amplifying loop eventually leading to structural defects of both mitochondria and SR. Our finding that the structural alterations and apoptotic defects of *Col6a1*$^{-/-}$ muscles *in vivo* can be rescued by CsA through a mitochondrial mechanism may open the way to a pharmacological treatment for Bethlem myopathy and Ullrich congenital muscular dystrophy.

## METHODS

**Mice.** We backcrossed *Col6a1*$^{+/-}$ mice in a mixed 129/Sv × C57BL/6J background[6] into C57BL/6J strain for eight generations. We obtained data by comparing sex-matched, 8–32-wk-old *Col6a1*$^{-/-}$ mice with the corresponding wild-type littermates. Mouse procedures were approved by the ethics committee of the University of Padova.

**Drug treatment *in vivo*.** We injected wild-type and *Col6a1*$^{-/-}$ mice intraperitoneally every 12 h for 4 d with CsA (Novartis, 5 mg per kg body weight; $n = 8$), FK506 (Fujisawa, 2.5 mg per kg body weight; $n = 4$) or vehicle (olive oil; $n = 8$).

**Muscle mechanics and electrophysiology.** We dissected FDB, extensor digitorum longus and diaphragm strips from wild-type ($n = 20$) and *Col6a1*$^{-/-}$ ($n = 15$) mice and recorded twitches and tetani as described[25]. We isolated single myofibers from diaphragm and gastrocnemius muscles, chemically skinned them as described[25] and measured the tension during maximal isometric activation (pCa = 4.5, T = 20 °C, sarcomere length = 2.75 ∝m). We recorded resting and action membrane potentials intracellularly as described[26].

**Transmission electron microscopy.** We longitudinally stretched diaphragm and FDB muscles from wild-type ($n = 7$) and *Col6a1*$^{-/-}$ ($n = 7$) mice on wax, fixed them with 2.5% glutaraldehyde in phosphate buffer 0.1 M (pH 7.4) and embedded them in Epon E812 resin. We observed ultrathin sections, stained with uranyl acetate and lead citrate, in a Philips EM400 electron microscope at 100 kV.

**Isolation and culture of skeletal myofibers.** We isolated fibers from FDB muscles of wild-type ($n = 33$) and *Col6a1*$^{-/-}$ ($n = 28$) mice as described[8]. We plated intact myofibers onto glass coverslips coated with mouse EHS laminin (3 ∝g cm$^{-2}$) or collagen VI (15 ∝g cm$^{-2}$) and cultured them in Dulbecco's modified Eagle medium containing 10% fetal calf serum for 16–40 h before starting the experiment. Only apparently healthy fibers (*i.e.*, fibers without structural alterations and not stained by Evans blue) adhered to the coverslips.

**∅$\psi_m$ and $[Ca^{2+}]_c$ assay.** We placed FDB myofiber cultures in 1 ml glucose-free Tyrode buffer and loaded them with appropriate probes. We used TMRM (Molecular Probes, 20 nM) to monitor ∅$\psi_m$ as described[8] and Fura-2/AM (Sigma, 5 ∝M) to monitor $[Ca^{2+}]_c$ according to established procedures[16]. We used oligomycin, CsA, FCCP, ionomycin, dantrolene, verapamil (all Sigma), CsH (a gift from Novartis) or BAPTA-AM (Molecular Probes) at the indicated times and concentrations. Imaging was done with a Zeiss Axiovert epi-fluorescence microscope. We analyzed data with the MetaFluor Imaging software.

**TUNEL.** We prepared 7-∝m-thick sections of muscles from wild-type ($n = 6$) and *Col6a1*$^{-/-}$ ($n = 6$) mice after formalin fixation and paraffin embedding. We fixed FDB fiber cultures in 50% acetone/50% methanol. TUNEL was done using the ApopTag *in situ* apoptosis detection kit (Intergen). We stained samples with peroxidase-diaminobenzidine to detect TUNEL-positive nuclei and counterstained them with Hoechst 33258 (Sigma) to mark all nuclei. We determined the numbers of total and TUNEL-positive nuclei in randomly selected fibers using a Zeiss Axioplan microscope.

**Statistical analysis.** Data are expressed as mean ± s.e.m. We analyzed data with the unpaired Student's *t*-test. Values with $P < 0.05$ were considered significant.

*Note: Supplementary information is available on the Nature Genetics website.*

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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