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A mouse model offers novel insights into the myopathy and tendinopathy often associated with pseudoachondroplasia and multiple epiphyseal dysplasia

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Pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED) are relatively common skeletal dysplasias belonging to the same bone dysplasia family. PSACH is characterized by generalized epi-metaphyseal dysplasia, short-limbed dwarfism, joint laxity and early onset osteoarthritis. MED is a milder disease with radiographic features often restricted to the epiphyses of the long bones. PSACH and some forms of MED result from mutations in cartilage oligomeric matrix protein (COMP), a pentameric glycoprotein found in cartilage, tendon, ligament and muscle. PSACH-MED patients often have a mild myopathy characterized by mildly increased plasma creatine kinase levels, a variation in myofibre size and/or small atrophic fibres. In some instances, patients are referred to neuromuscular clinics prior to the diagnosis of an underlying skeletal dysplasia; however, the myopathy associated with PSACH-MED has not previously been studied. In this study, we present a detailed study of skeletal muscle, tendon and ligament from a mouse model of mild PSACH harbouring a COMP mutation. Mutant mice exhibited a progressive muscle weakness associated with an increased number of muscle fibres with central nuclei at the perimysium and at the myotendinous junction. Furthermore, the distribution of collagen fibril diameters in the mutant tendons and ligaments was altered towards thicker collagen fibrils, and the tendons became more lax in cyclic strain tests. We hypothesize that the myopathy in PSACH-MED originates from an underlying tendon and ligament pathology that is a direct result of structural abnormalities to the collagen fibril architecture. This is the first comprehensive characterization of the musculoskeletal phenotype of PSACH-MED and is directly relevant to the clinical management of these patients.

INTRODUCTION

Pseudoachondroplasia (PSACH: MIM 177170) is an autosomal dominant skeletal dysplasia characterized by short-limbed dwarfism, epi-metaphyseal dysplasia, joint laxity and early onset osteoarthritis (OA) (1,2). Multiple epiphyseal dysplasia (MED: MIM 132400) is predominantly an autosomal dominant skeletal dysplasia belonging to the same bone dysplasia family (3), but it is generally milder, and the radiographic features are primarily restricted to the epiphyses. MED patients can also suffer from joint laxity and early onset OA.

PSACH and some forms of MED (EDM1) result from mutations in the gene encoding cartilage oligomeric matrix

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protein (COMP: MIM 600310) (4,5), which is a 550 kDa pentameric extracellular matrix (ECM) glycoprotein (6) found in cartilage, tendon (7), ligament (8) and skeletal muscle (9). COMP is thought to act as a bridging molecule within the ECM (10). Indeed, COMP interacts with numerous ECM proteins including types I, II, IX, XI and XII collagen, decorin, fibronectin and matrilin-3 (11–15), and a role for COMP as a catalyst in collagen fibrillogenesis has been proposed (16).

Each COMP monomer consists of a coiled-coil oligomerization domain, four EGF-like domains, eight TSP type 3 (T3) repeats and a large C-terminal globular domain (CTD). PSACH-MED mutations in COMP cluster in two distinct regions: the type 3 repeats (~85% of the mutations identified to date) and the CTD (~15% of the mutations identified to date) (17). Extensive research over the last 10 years has demonstrated that mutant COMP protein harbouring structural changes in the type 3 repeats is retained within patient chondrocytes, eventually resulting in rER stress and increased cell death both *in vitro* and *in* vivo (reviewed in 18). In contrast, some of the COMP-CTD mutations allow the secretion of mutant protein but low levels of ER/cell-stress are still observed, both *in vitro* and *in vivo* (19,20).

PSACH-MED patients have a well-characterized ligamentous laxity (2), and the ligaments from patients with COMP-T3 mutations can exhibit a disorganized collagen fibril network, with both variable fibril diameters and lateral fusion of neighbouring fibrils (unpublished data) (15). Conflicting data exist concerning the retention of mutant COMP in tendon and ligament cells. For example, no apparent intracellular retention of mutant COMP was noted in ligament cells cultured *in vitro* (21), although the retention of mutant protein was observed *in vivo* in ligament cells from a PSACH patient with a COMP-T3 mutation (p.Gly465Ser; unpublished data). In common with ligament cells, tenocytes from patients with *COMP* mutations retain mutant COMP in the rER *in vivo* and in cells cultured in monolayer (22,23), whereas in another study, no protein retention was evident (24).

An overlooked clinical complication in some patients with PSACH-MED, specifically those with mutations in the CTD of COMP, is mild myopathy. This can be characterized clinically by a difficulty in standing up and a tendency to tire easily, and biochemically by mildly increased plasma creatine kinase (PCK) levels, differences in fibre diameters and/or atrophic fibres observed in muscle samples obtained through biopsy (17,25,26). Furthermore, MED patients with mutations in the α3(IX) (27) or α2(IX) (M.D.B., submitted for publication) chains of type IX collagen have also been reported as having mild myopathy. However, the muscular, tendon and ligament complications of the PSACH-MED phenotype have not yet been studied in detail, primarily because of the difficulty in obtaining suitable pathological samples.

Here we report a detailed study of the phenotypic and morphological effect of a C-terminal COMP mutation on skeletal muscle, Achilles tendon and spinal ligament in a mouse model of mild PSACH-MED (20). We show that the mutant mice exhibit a progressive mild myopathy, localized specifically to the perimysium and myotendinous junction (MTJ) and characterized by a dramatic increase in the number of fibres with central nuclei (i.e. indicative of remodelling). We also present evidence that this CTD mutation in COMP affects the morphology and biomechanical characteristics of murine Achilles tendon and spinal ligament, which is consistent with a proposed role for COMP in collagen fibrillogenesis. Our results led us to the hypothesis that the PSACH-MED associated myopathy is a secondary consequence of an underlying tendinopathy and that joint laxity seen in patients is a direct consequence of structural abnormalities to the tissue's ECM.

This is the first comprehensive characterization of the musculoskeletal phenotype of the PSACH-MED dysplasia family, and it may directly influence the clinical management of PSACH-MED patients in the future.

RESULTS

Comp T585M mutant mice exhibit a mild and progressive muscle weakness

To assess muscle weakness in the *Comp* T585M mutant mouse model, we performed forelimb grip-strength tests on male mice at 3 and 9 weeks of age (Fig. 1A). At 3 weeks of age, mutant mice and wild-type controls had a comparable maximum forelimb strength (defined as the highest force recorded during the experiment), but the force at which mutant mice released their grip was significantly lower than in wild-type mice, suggesting that the mutant mice tired easier (Fig. 1B; 12.6% reduction, $P < 0.05$ by one-way ANOVA, $n = 3$). By 9 weeks of age, the maximum strength and the force at which the mutant mice released their grip were both significantly lower than the values recorded for the wild-type mice, indicating that the muscle weakness in mutant mice progressed with age (Fig. 1B; a decrease of 26.7 and 23.3%, respectively; $P < 0.001$ by one-way ANOVA, $n = 3$).

Muscle weakness in Comp T585M mice results from a mild myopathy localized to the perimysium and the MTJ

To determine whether the muscle weakness was due to a generalized myopathy resulting from abnormal muscle morphology and structure, we undertook a detailed microscopic analysis of skeletal muscle from mutant mice and wild-type controls. To examine skeletal muscle morphology by histology, whole legs were dissected from 3 and 6 week-old mice and sectioned in a transverse plane until the gastrocnemius and soleus muscles were clearly visible. Tissue sections were stained with Gomori's trichrome stain, which stained the collagenous tissue blue, skeletal muscle red and the nuclei black. The number of fibres with central nuclei (i.e. indicative of fibre stress and remodelling (28,29)) was determined throughout the muscle tissue and expressed as a percentage of the total number of fibres (Fig. 2). In the mutant skeletal muscle, there was a ~33% increase in the number of fibres with central nuclei compared with the wild-type mice at 3 weeks of age [Fig. 2 (total tissue 3 weeks); $P < 0.05$ by independent samples t-test, $n = 3$]. This initial observation was indicative of a mild myopathy and we wished to establish the precise localization of the abnormal fibres seen in the mutant muscle. Following stratification, a larger proportion of fibres with central nuclei were present specifically at the

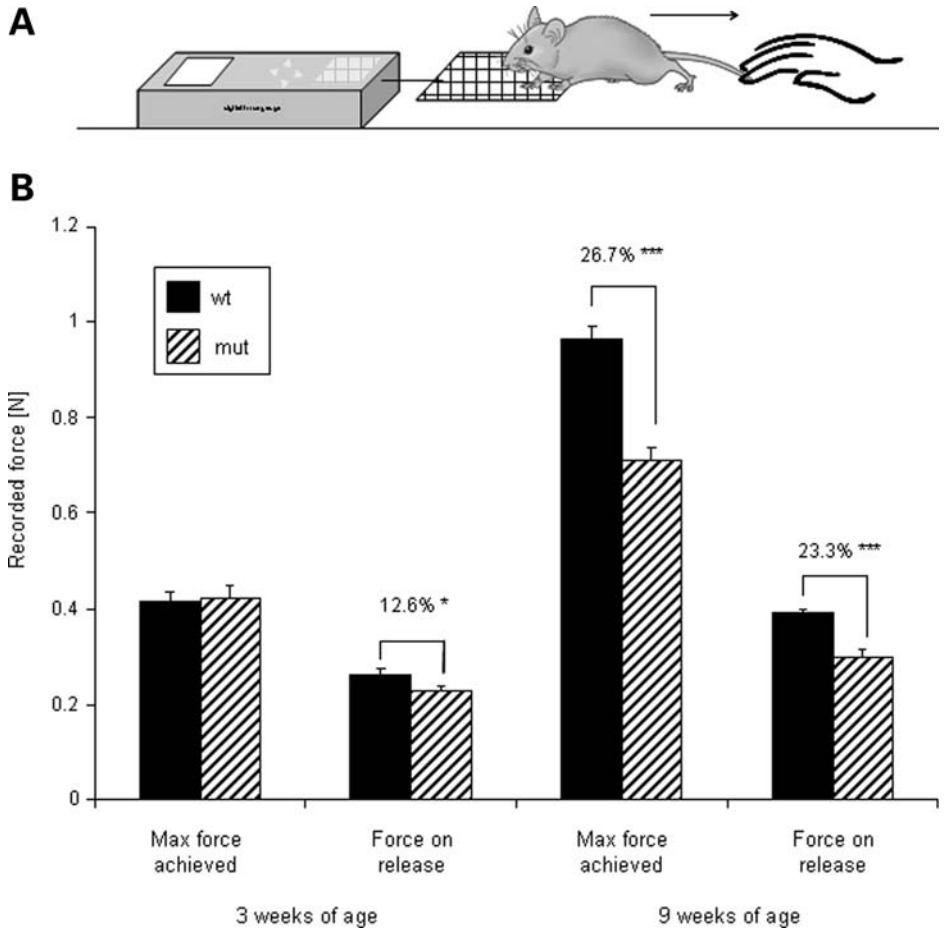

Figure 1. (**A**) Schematic of a grip strength test. (**B**) Grip measurements were performed on 3 and 9 week-old animals. At 3 weeks of age, there was no difference between the maximum strength of the wild-type and mutant animals, but there was a 12.6% difference in the final recorded strength, indicating that the mutant mice tired faster ($P < 0.05$, one-way ANOVA, $n > 5$). At 9 weeks of age, both the maximum recorded strength and the final recorded strength were lower for the mutant mice (26.7 and 23.3%, respectively, $P < 0.005$, one-way ANOVA, $n > 5$). wt, wild-type; mut, homozygous for the mutation; standard error of the mean, $^{*}P < 0.05$, $^{***}P < 0.005$.

MTJ and around the perimysium in the mutant tissue compared with the wild-type controls [Fig. 2 (MTJ 3 weeks); ~43%; $P < 0.005$ by independent samples *t*-test, $n = 3$]. No significant differences in the numbers of fibres with central nuclei were observed elsewhere in the muscle tissue of mutant mice when compared with wild-type controls [Fig. 2 (rest 3 weeks)]. Furthermore, at 6 weeks of age, the number of muscle fibres with central nuclei was >2.5-fold higher in mutant mice compared with wild-type controls [Fig. 2 (total tissue 6 weeks); $P < 0.01$ by independent samples *t*-test, $n = 3$]. The muscle fibres with central nuclei were still specifically localized to the perimysium and MTJ in the mutant muscle and there was no difference in the number of remodelling muscle fibres in the rest of the tissue [Fig. 2 (MTJ 6 weeks); $P < 0.01$ by independent samples *t*-test].

To identify an underlying reason for the increased stress and remodelling by myocytes in the mutant muscle, we determined the relative levels of the molecular chaperone BiP and the anti-apoptotic protein Bcl-2 by performing densitometry measurements on Western blots of total muscle proteins. BiP is a general marker of ER stress that is applicable to skeletal muscle cells (30,31), whereas Bcl-2 has a protective role in skeletal muscle and has been shown to be decreased in several myopathies (32,33). At 3 weeks of age, there was no difference in the relative levels of BiP or Bcl-2 between wild-type and mutant samples (Supplementary Material, Fig. S1A; independent samples *t*-test, $n = 5$). These data therefore suggested that the expression of the *Comp* mutation *per se* did not have a generalized effect on the myocytes of skeletal muscle.

Comp is expressed in murine tendon, ligament and skeletal muscle

Comp is known to be expressed in skeletal muscle in mice (9); however, conflicting data exist concerning its expression in tendon with reports of either its presence (9) or absence (34) in adult murine Achilles tendon. Therefore, before studying further the pathomolecular mechanisms of myopathy in the mutant mice, we undertook a detailed analysis of *Comp* expression in the relevant murine tissues (i.e. those of the musculoskeletal system).

The expression levels of *Col1a1* (a positive marker for tendon, ligament and skeletal muscle), *Col2a1* (a negative

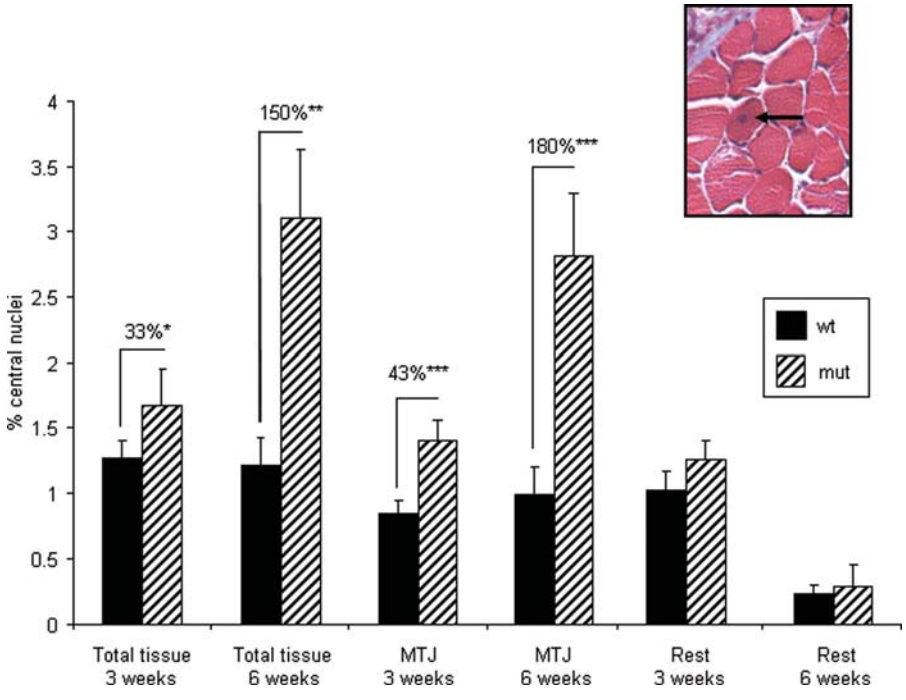

Figure 2. Muscle fibres with central nuclei (inset) were counted in the skeletal muscle of 3 and 6 week old wild-type and mutant mice and expressed as a percentage of total muscle fibres in the tissue. There was a 33% increase in the number of fibres with central nuclei in the mutant tissue (total tissue; $P < 0.05$, independent samples *t*-test, $n = 3$). When the localization of the central nuclei was taken into the account, a 43% increase in central nuclei was found at the perimysial and MTJ ($P < 0.005$, independent samples *t*-test, $n = 3$) but not elsewhere in the tissue (rest). By 6 weeks of age, the proportion of muscle fibres with central nuclei was increased to 150% in the total tissue and to 180% in the MTJ. wt, wild-type; mut, homozygous for the mutation; standard error of the mean, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

marker for the same tissues), *Myod1* (a positive marker for skeletal muscle and a negative marker for tendon) and *Comp* were determined at 3 weeks of age by quantitative real-time PCR (Supplementary Material, Table S1). The expression levels of these marker genes confirmed the accuracy of the dissection protocol since the relative levels of *Myod1* expression in the Achilles tendon and spinal ligament were negligible as was the expression of *Col2a1* in the skeletal muscle. The most abundant gene transcript was *Col1a1*, which was unsurprising since type I collagen is the most abundant protein of tendon and ligament and an abundant component of the skeletal muscle ECM.

Not surprisingly, *Comp* expression was lower in tissue samples from Achilles tendon, spinal ligament and skeletal muscle relative to the positive marker (*Col1a1*). Nevertheless, *Comp* was still expressed in all of these tissues at levels >10-fold higher than the negative controls (Supplementary Material, Table S1). The relative levels of *Comp* expression were also determined in Achilles tendon, spinal ligament, skeletal muscle and epiphyseal cartilage at 3 weeks of age (Supplementary Material, Table S2). The levels of *Comp* expression were comparable for tendon, ligament and skeletal muscle, whereas *Comp* expression was significantly higher in cartilage when compared with tendon, ligament and muscle samples ($P < 0.05$ by independent samples *t*-test, $n = 3$). There was no difference in *Comp* expression between wild-type and mutant samples in all of the tissues studied (data not shown).

The presence of COMP was also confirmed at the protein level by Western blotting of total protein isolated from the Achilles tendon and skeletal muscle of mice at 3 weeks of age (Fig. 3C). There were no differences in the relative levels of COMP between genotypes (i.e. WT versus M), but we did note that there were slight differences in the levels of COMP protein between tendon and muscle samples, which was in contrast to the quantitative RT–PCR analysis that showed comparative levels of *Comp* mRNA in these two tissues.

The localization of key structural molecules is not affected in the ECM of mutant skeletal muscle

We performed immunohistochemical (IHC) staining for COMP in mouse skeletal muscle and Achilles tendon to confirm that it was expressed in both tissues (Fig. 3). In both wild-type and mutant tendon, COMP was present in the ECM between individual collagen fibrils (Fig. 3A; data not shown), confirming previous studies (35,36). Furthermore, in both wild-type and mutant skeletal muscle, COMP was present on the surface of the myofibres and there were no apparent differences in the intensity and localization of the staining between genotypes (Fig. 3B; data not shown).

To investigate further the skeletal muscle stress and remodelling, additional markers such as desmin, vimentin and collagen types IV and VI were analysed at 3 weeks of age by IHC of saggital and transverse sections of the soleus and gastrocnemius muscles. There were no apparent differences in the localization of desmin, vimentin, type I, IV or VI collagen between wild-type and mutant tissues at 3 weeks of age, confirming that it was not a generalized myopathy (data not shown).

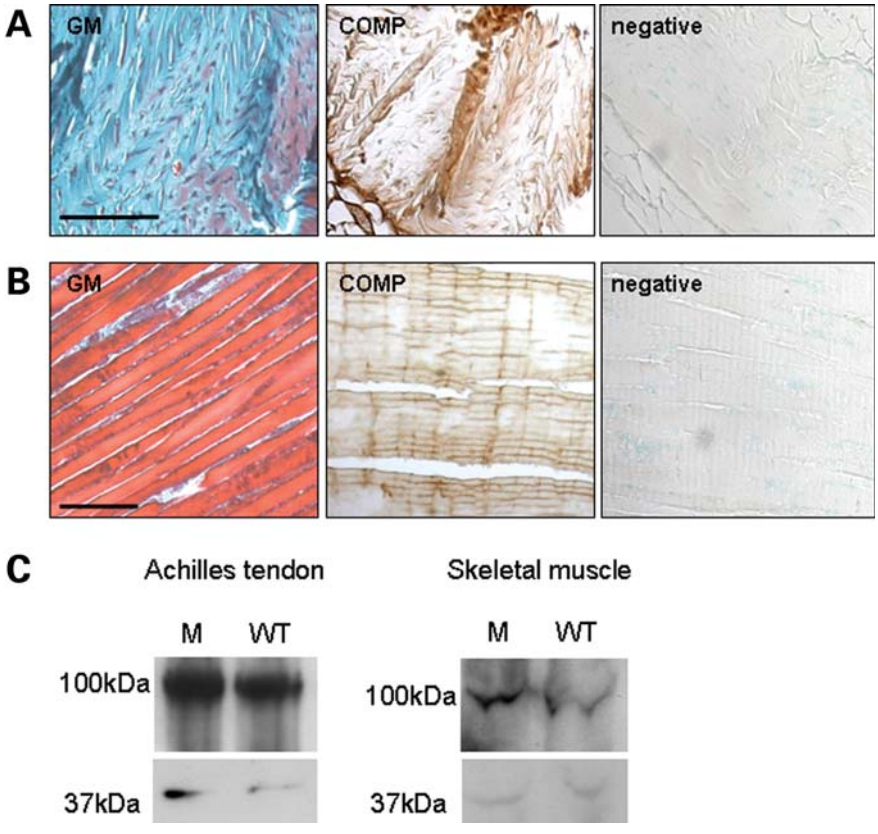

Figure 3. (**A**) IHC localization of COMP in wild-type tendon at 3 weeks of age. Gomori's trichrome staining (left panel) of tendon showing localization of the collagenous component (blue) and COMP immunostaining (brown) (central panel), with negative control (no primary antibody, right panel). (**B**) IHC localization of COMP in wild-type skeletal muscle at 3 weeks of age. Gomori's trichrome staining (left panel) showing the endomysial collagen (blue) and COMP immunostaining (central panel), with negative controls (no primary antibody, right panel). Scale bar: 100 μm. (**C**) Western blot of tissue homogenates at 3 weeks of age showing equal levels of COMP in wild-type and mutant tissues. Actin was used a loading control. WT, wild-type; M, mutant protein samples.

The distribution of collagen fibril diameters is altered in mutant Achilles tendon

Although we demonstrated that COMP is expressed in skeletal muscle tissue, a quantifiable pathology was only apparent at the perimysial and MTJ of mutant muscle. Since the overall skeletal muscle morphology was not affected in the mutant mice, and because the perimysium and MTJ are important for the transmission of force between the muscle and tendon (37–39), we considered the possibility that the 'mild myopathy' in the mutant mice may, in fact, arise from structurally abnormal tendons. We therefore used transmission electron microscopy (TEM) to measure the collagen fibril diameters in Achilles tendons from wild-type and *Comp* T585M mutant mice at 3 and 9 weeks of age (Fig. 4A and B). There was a significant difference in the distribution of collagen fibril diameters between wild-type and mutant Achilles tendons with a relative increase in the numbers of thicker collagen fibrils in the mutant samples at both 3 and 9 weeks of age (Fig. 4A and B; $P < 0.05$ and $P < 0.005$, respectively, by Mann–Whitney U test, $n > 3$). The total number of collagen fibres per mm^2 was also reduced in mutant tendons compared with wild-type tendons at 3 weeks of age (13%, $P < 0.05$; data not shown). A similar change in the distribution of collagen fibrils was also seen for wild-type and mutant spinal ligament at 9 weeks of age (Fig. 4C), further supporting a role for COMP in collagen fibrillogenesis.

Mutant Achilles tendons contained more fused/bifurcating collagen fibrils than wild-type tendon and were significantly thinner overall

Fused collagen fibrils were observed previously in a ligament sample from a PSACH patient who had undergone bilateral hip replacement (Fig. 5B; unpublished data; (15)). Therefore, the number of fused (or branching) collagen fibrils was determined in the wild-type and mutant tendon samples at 3 weeks of age and expressed as a percentage of total fibrils per cross-sectional area. There was a significant increase in the number of fused fibrils in the mutant tendon when compared with the wild-type tendon [Fig. 5A; ∼398% (>3-fold), $P < 0.05$ by independent samples t-test, $n > 5$]. A similar observation was also made in mouse spinal ligament at 9 weeks of age (data not shown). Furthermore, at 3 weeks of age, mutant Achilles tendons were significantly thinner (by 65%) than wild-type tendons ($P < 0.01$ by independent samples t-test, $n > 5$; data not shown). The total area occupied by collagen fibrils was also calculated from the TEM images of wild-type and mutant Achilles tendons and was comparable for

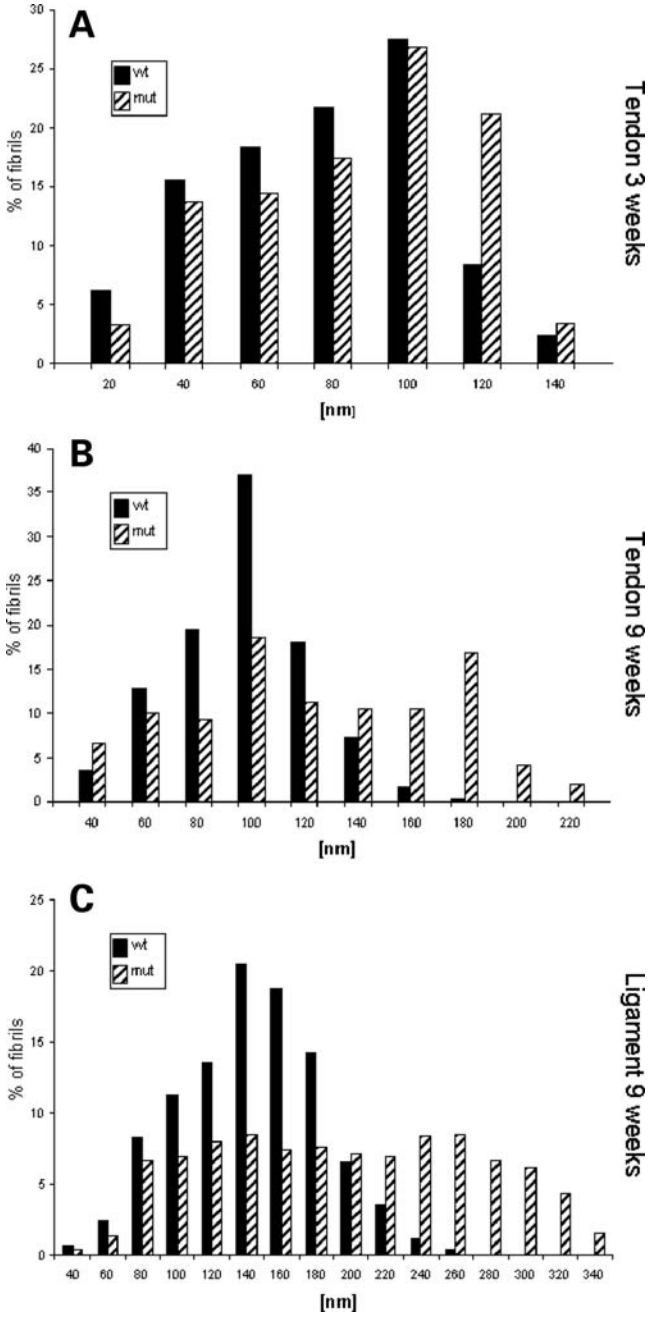

Figure 4. (**A**) Quantification of the collagen fibril diameters in the wild-type and mutant Achilles tendon at 3 weeks of age showing an altered distribution in the mutant tissue ($P < 0.05$, Mann–Whitney U test, $n = 5$). (**B**) Quantification of collagen fibril diameters in wild-type and mutant Achilles tendon at 9 weeks of age, showing a greater difference in the distribution of fibril diameters in the mutant tissue ($P < 0.005$, Mann–Whitney U test, $n = 5$). (**C**) Quantification of the collagen fibril diameters in the wild-type and mutant spinal ligament at 9 weeks of age, showing a further difference in the diameter distribution in the mutant tissue ($P < 0.005$, Mann–Whitney U test, $n = 5$). wt, wild-type; mut, homozygous for the mutation.

wild-type and mutant tendons, demonstrating that the proportion of interfibrillar matrix between the collagen fibrils was similar (data not shown). We considered the possibility that the ECM in the mutant tendons might contain under sulphated proteoglycans (PGs) (similar to that observed in the cartilage growth plate of mutant mice). However, DMMB (1,9,dimethyl-dimethylene blue) analysis revealed that the amount of sulphated PGs in the ECM was comparable with no significant differences between the wild-type and mutant tendons (Fig. 5C; independent samples t-test, $n > 10$).

Achilles tendons from mutant mice were more lax in biomechanical testing

The fibrillar organization and cross-sectional diameter of mutant Achilles tendons were dramatically altered compared with wild-type tendons. We therefore used tensile strength measurements (Table 1 and Fig. 6A) and cyclic strain testing (Fig. 6B) to analyse the biomechanical properties of wild-type and mutant Achilles tendons from 3 week-old animals. In the tensile strength test, the tendons were stretched at a set strain rate of 0.08/s [with strain measured as $(l-l_o)/l_o$, where l is the length of the sample, and l_o the original sample length] until breaking point, and a stress/strain curve was plotted [stress being the F/csa, where F is the force (N), and csa the cross-sectional area (mm^2)] (Table 1 and Fig. 6A). The data obtained were in agreement with the values published for various mouse strains (40). Interestingly, mutant tendons were capable of more 'stretch to failure' (27%, $P < 0.05$ by independent samples t-test, $n > 5$) and could sustain more stress (64%, $P < 0.01$ by independent samples t-test, $n > 5$); however, the force applied to failure (failure load) was comparable for wild-type and mutant tendons (Table 1). In addition, the elastic modulus and stiffness of the mutant tendons were comparable with wild-type tendons. Since mutant Achilles tendons were significantly thinner than the wild-type tendons, they were also capable of storing less potential kinetic energy during the tensile stretch experiment (Table 1; 34% for 1 mm stretch, $P < 0.05$ by independent samples t-test, $n > 5$).

Cyclic strain testing was used to determine the performance of 3 week-old wild-type and mutant Achilles tendon in more relevant physiological conditions (Fig. 6B). The cyclic test was performed in the toe region (reflecting the tendon crimp) of the stress/strain curve for the wild-type and mutant tendons (41). The tissues were cycled nine times to a strain of 0.5 and the force applied was measured. The cycles for wild-type and mutant Achilles tendons were normalized to the first cycle force (100%), then plotted and compared. In the cyclic testing, the initial forces recorded for the wild-type and mutant Achilles tendon were comparable, but mutant Achilles tendons became more lax with an increasing number of cycles until, at cycle 8, significantly less stress was needed to stretch them to the same length as the wild-type tendons (Fig. 6B; 16.3%, $P > 0 < 0.05$ by independent samples t-test, $n = 5$). These data suggest that *in vivo* mutant tendons are more lax and as such are likely to convey less elastic energy to the muscle during walking.

ER stress and apoptosis are not increased in mutant tenocytes

On the basis of our previous findings in the mutant cartilage growth plate, where BiP was significantly upregulated and Bcl-2 was downregulated (20) owing to the expression of

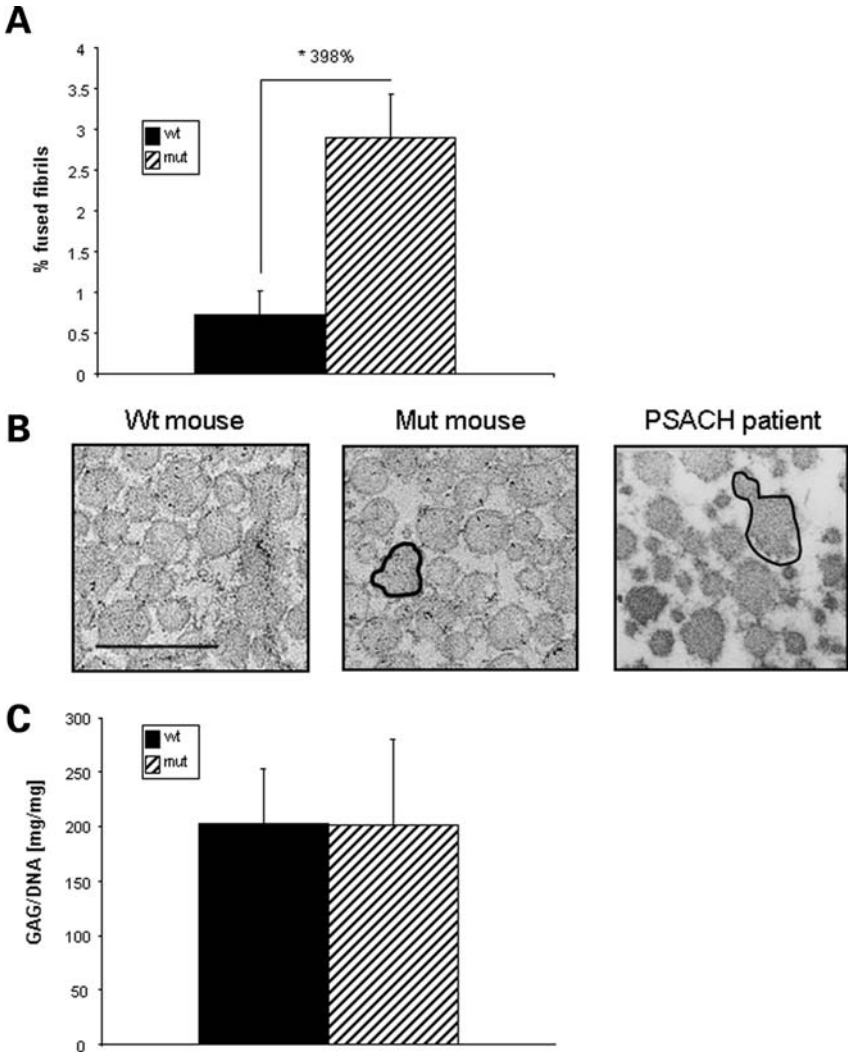

Figure 5. (**A**) Quantification of the fused/branching collagen fibrils expressed as a percentage of total collagen fibrils in the Achilles tendon at 3 weeks of age. There was a dramatic increase in the number of fused fibrils in the mutant tissues (398%, $P < 0.05$, independent samples *t*-test, $n = 5$). (**B**) TEM image of collagen fibrils in wild-type and mutant Achilles tendon, showing fused/bifurcating fibrils in mutant tissue (black line), and TEM image of a PSACH patient ligament (with COMP p.G465S mutation) with a similar pathology. Scale bar: 0.425 μm. (**C**) DMMB assay for sulphated PGs normalized to the DNA content of cells. There was no difference in the PG content of wild-type and mutant Achilles tendons at 3 weeks of age (independent samples *t*-test, $n > 10$). wt, wild-type; mut, homozygous for the mutation; $^{*}P < 0.05$.

mutant *Comp* T585M, we considered the possibility that the expression of mutant *Comp* T585M was eliciting an rER/cell stress response in tenocytes that might eventually be causing increased cell death. Densitometry measurements of Western blots was performed for Bcl-2 and BiP in protein samples from 3 week-old mutant and wild-type tendons. However, we found no differences in the relative levels of Bcl-2 and BiP in mutant and wild-type tenocytes (Supplementary Material, Fig. S1B; independent samples *t*-test, $n = 5$). These data suggest that either the expression levels of mutant COMP may not be high enough in tenocytes to elicit a classical rER stress response, or that tenocytes may respond differently to the expression of mutant COMP, which has been proposed previously (24). Alternatively, adult tenocytes may also not be as metabolically active as chondrocytes at 3 weeks of age (42,43).

DISCUSSION

PSACH is a skeletal dysplasia that in the most severe form is debilitating for the patient's well-being and lifestyle owing to the dramatic skeletal phenotype, which includes short-limbed dwarfism and early onset degenerative joint disease (44,45). In contrast, the related, but milder MED is sometimes misdiagnosed or not reported until later in life (17). In this study, we showed that mutations in *Comp* that cause PSACH-MED result in altered collagen fibril diameters in force-loaded tendons and ligaments and is associated with a mild muscle myopathy in the absence of a detectable muscle pathology.

One of the clinical complications recently recognized as a part of the PSACH-MED phenotype is a mild myopathy, which in some cases may manifest earlier than the skeletal phenotype (17,25–27). In these instances, the patients are

Table 1. Biomechanical parameters of the tensile test on wild-type and mutant Achilles tendons at 3 weeks of age

	wt ($n = 5$)	mut ($n = 5$)	Change	*P*-value
Cross-sectional area (mm^2)	0.25 ± 0.02	0.14 ± 0.01	44% ↓	0.0012
Failure load (N)	10.11 ± 1.16	9.34 ± 1.08		0.64
Failure stress (MPa)	40.59 ± 3.85	66.62 ± 5.38	64% ↑	0.0077
Failure strain	1.95 ± 0.29	2.68 ± 0.37	37% ↑	0.05
Elastic modulus (MPa)	45.43 ± 4.47	53.68 ± 11.5		0.53
Stiffness (N/mm)	21.94 ± 3.19	26.7 ± 5.27		0.47
Potential kinetic energy for 1 mm strain (J)	5.76 ± 0.92	3.8 ± 0.96	34% ↓	0.05

Cross-sectional area and the potential kinetic energy were significantly decreased, and failure stress and failure strain were increased in the mutant tendons at 3 weeks (independent samples *t*-test, $n = 5$). wt, wild-type; mut, homozygous for the mutation.

reported as having difficulties with standing up or tire easily during exercise and are often referred to the clinic with an unclassified 'neuromuscular disorder' prior to the diagnosis of a underlying skeletal dysplasia. On the basis of these and other reported cases, it has been suggested that if a child presents with a 'difficult to explain' myopathy (i.e. waddling gait, increasing muscle weakness, but with none or only mild changes in a muscle biopsy), the child should be referred for a skeletal survey with a view to identifying an underlying skeletal dysplasia (46). Interestingly, in many PSACH-MED patients with a reported myopathy, a causative mutation was identified in the CTD of COMP. In these patients, the myopathy was not comprehensively documented, but included features such as mildly elevated PCK levels (25), basophilic and/or atrophic muscle fibres (17). Furthermore, a mild myopathy has also been reported in several MED families with type IX collagen mutations, a protein which is not expressed in skeletal muscle, but is present in tendons at the insertion of the tendon into the bone (i.e. the enthesis) (47). In these families, the proband was also referred to a neuromuscular clinic for the evaluation of proximal muscle weakness prior to the diagnosis of MED (27) (M.D.B., submitted for publication).

The musculoskeletal complications of the PSACH-MED phenotype have not been studied in detail, primarily due to the difficulty in obtaining suitable pathological samples and aged/site-matched controls. A detailed analysis of the myopathy associated with *COMP* mutations would therefore enable earlier diagnosis of mild PSACH-MED, improve our understanding of the disease mechanisms and ultimately improve patient care (48). In this study, we have clinically demonstrated muscle weakness in mutant mice and analysed the gastrocnemius and soleus muscles and Achilles tendon to gain insights into the key pathological features and disease mechanisms of this mild myopathy.

Comp T585M mutant mice, which we have previously characterized as a relevant model of mild PSACH (20), also suffer from a progressive myopathy as demonstrated by grip strength testing. Furthermore, when we analysed skeletal muscle from the mutant mice, we found a progressive increase in the number of myofibres with central nuclei, specifically around the perimysium and the MTJ. This observation is indicative of skeletal muscle stress and the subsequent remodelling of the gastrocnemius and soleus muscles in these specific areas (28,29). To test that the myopathy in mutant mice was not a generalized muscle pathology, we analysed the skeletal muscle tissue of wild-type and mutant mice in detail. We used immunohistochemistry to determine the localization of several important ECM proteins in murine skeletal muscle and did not detect any differences in the distribution of wild-type and mutant COMP, or in the distribution of types I, IV and VI collagen, desmin or vimentin. We have also confirmed that COMP was present in the ECM of skeletal muscle, specifically in the endo- and perimysium, and that there were no differences in its localization between wild-type and mutant tissues. Interestingly, these observations are in direct contrast to changes in the localization of mutant COMP seen in the cartilage growth plates of *Comp* T585M mutant mice (20) and suggests that the ECM of muscle and cartilage may assemble and respond differently to the presence of mutant COMP. We have previously shown that mild ER stress was detected in chondrocytes from mutant mice expressing *Comp* T585M. However, the analysis of an ER stress marker (BiP) and an apoptosis marker (Bcl-2) showed no differences between the wild-type and mutant cells. Overall, these data suggest that although secreted by myocytes and present in the ECM, mutant COMP did not have an effect on general skeletal muscle architecture and did not elicit an ER stress response in the mutant cells.

It has been shown that the transmission of forces between the skeletal muscle and tendon depends on the interaction of the muscle fibres with the surrounding ECM and also on the collagen fibrillar organization of the tendon (37). Perimysium has been shown to form a connective tissue with a 'lattice-like' structure and is important for conveying forces between the tendon and skeletal muscle (37–39,49). These observations led us to hypothesize that myopathy in the mutant mice may, in fact, be the result of an underlying tendinopathy. Therefore, to gain insight into the mechanisms underlying the restricted localization of the myopathic changes, we studied the ultrastructure of the Achilles tendon from wild-type and mutant mice at 3 and 9 weeks of age. Tendons are an important skeletal tissue since they act as buffers for muscle stretch during locomotion (50). We have shown that *Comp* is expressed in murine tendon and ligament by a variety of techniques, and that the localization of mutant COMP was not altered in mutant tissues. To determine the effect of mutant COMP on collagen organization, we measured the diameters of collagen fibrils in the Achilles tendon of wild-type and *Comp* T585M mutant mice. Interestingly, we found that the distribution of collagen fibril diameters in the mutant tendon was dramatically altered, with a higher proportion of larger diameter fibrils. There was a similar difference in the distribution of collagen fibrils in wild-type and mutant spinal ligament, further supporting a role for COMP in collagen fibrillogenesis. The total area occupied by the collagen fibrils was not altered between the wild-type and mutant tendons, but the number of fibrils per unit area of tendon was decreased in the mutant mice. Furthermore, the overall 'interfibrillar area' was not altered in the mutant tendons, suggesting that the amount of inter-territorial matrix in the wild-type and mutant tendons was similar. PGs comprise

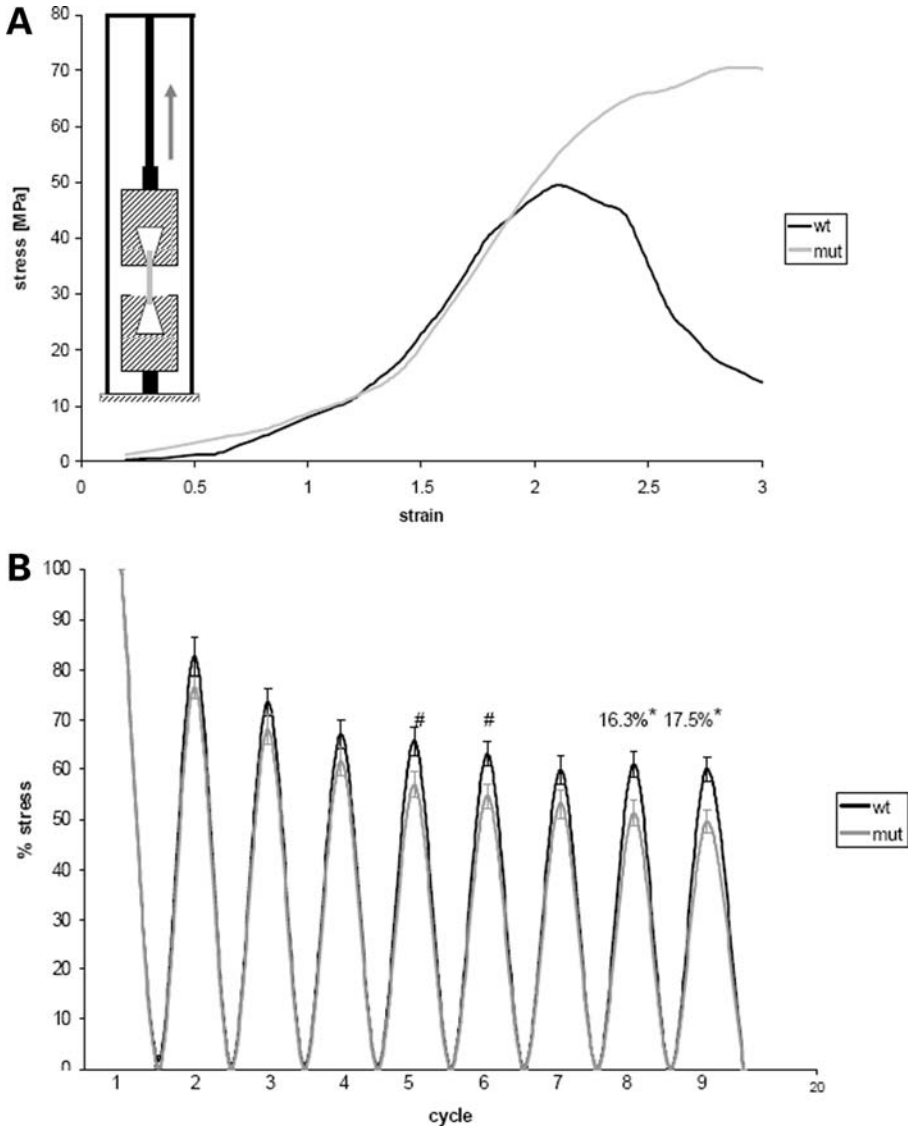

Figure 6. (**A**) Stress/strain curves of the stretch to failure experiment for wild-type and mutant Achilles tendon at 3 weeks of age. Note that the slopes of the curves in the linear phase are similar, indicating a comparable stiffness. Mutant tissue was more resilient since it stretched more to failure and could withstand higher tensile stress. (**B**) Cyclic strain experiment. Wild-type and mutant tendons were stretched and released nine times with a constant strain amplitude and the applied stress was measured. Mutant tendons became significantly more lax in cycles 8 and 9 (16.3 and 17.5%, respectively, $P < 0.05$, independent samples *t*-test, $n = 5$). wt, wild-type; mut, homozygous for the mutation; #approaching significance, $^{*}P < 0.05$.

~0.5% of tendon dry weight and play a role in tendon fibril spacing (37). We therefore analysed the PG content of wild-type and mutant Achilles tendons from 3 week-old mice and found that the PG content was similar for both genotypes.

The abnormal changes seen in collagen fibril diameters in the mutant tendon might be due directly to the mutation in the CTD of COMP. For example, this region of COMP contains a potential collagen-binding site (15), and a role for COMP as a catalyst in collagen fibrillogenesis has been proposed (16). It is therefore possible that this C-terminal COMP mutation (T585M), which is close to the potential collagen-binding site (15,17), has a detrimental effect on the 'catalyst' function of COMP that could alter collagen fibril diameter in the mutant tissue and ultimately its biomechanical properties. The detrimental effect of COMP mutations on collagen fibrillogenesis has been previously demonstrated *in vitro* (51). In addition, we also observed an increase in the number of fused/bifurcating collagen fibrils in mutant tendon and ligament compared with the wild-type tissues. Similar observations have previously been seen in a ligament sample from a PSACH. In wild-type animals, fibril bifurcation is abundant in mouse fetal tendon tissue, but decreases with age (52). Furthermore, increased fibril bifurcations have also been found in tendon scar tissue and at the scar to tissue junctions (53), and it has been suggested that an increase in fibril bifurcations may be indicative of wound healing and that they may be required to connect neighbouring fibrils to transform the force properly from the scar to the residual tissue (53). It has been proposed that the tendon biomechanical function depends on the precise collagen fibre alignment in the

tissue (54). It is therefore interesting to speculate that owing to the altered biomechanical properties of the mutant tendons, some microdamage could occur in the mutant tendon, and the increased number of fused fibrils may be indicative of repair mechanisms in the mutant tissue, which could in turn affect the biomechanical properties of the tissue.

Finally, the cross-sectional area of whole mutant tendons was also significantly less than that of the wild-type tendons. This may be due to tendon remodelling and/or disuse (55). It could also potentially be a compensatory mechanism for the thicker collagen fibrils in the mutant tendons, which would make the mutant tissue stiffer. The dimensions of tendons directly influence their ability to stretch, store and release kinetic energy (56). In physiological conditions, the cross-sectional area of the tendon, relative to that of the fascicles of the attached muscle, dictates the maximum tensile stress to which a tendon can be subjected (57). The smaller cross-sectional area of mutant tendons, and the abnormal structural similarities between mutant tendons and ligaments, may therefore explain the joint laxity seen in PSACH-MED patients (2). In addition, the variability in the diameters of individual collagen fibrils can have a dramatic impact on the tissue's biomechanical properties. The biomechanical properties of tendons are directly related to fibril length, diameter and modulus and inversely to interfibrillar spacing (58), and thick fibrils are predicted to withstand higher tensile forces owing to the higher number of intrafibrillar crosslinks (59).

To assess the biomechanical properties of the wild-type and mutant tendons, we performed a series of tensile strength experiments. In a stretch-to-failure experiment, mutant tendons were able to withstand higher stresses and were stretched more before failure. However, when the mutant tissues were tested in a cyclic strain experiment, they became more lax with an increasing number of cycles, which is analogous to the progressive joint laxity observed in PSACH-MED patients (2). Lax tendons would also be less suitable for conveying the appropriate forces from muscle to bone, which might help explain the neuromuscular symptoms seen in PSACH-MED patients. By analogy to the structural changes seen in mouse mutant tendon and ligament, we can hypothesize that joint laxity in PSACH-MED patients might also stem from an altered distribution of collagen fibrils.

Tendons are known to adapt to mechanical load requirements, and inactivity has been shown to decrease collagen turnover in tendon (37,60,61). It was also shown that training increased the cross-sectional area of tendons in pigs (62) and that an increase in cross-sectional area of tendons correlates with an increase in tendon stiffness (37). Collagen fibril diameters also shift towards thicker fibrils with age (63), resulting in less compliant tissues (50). Therefore, it is likely that the tendon and ligament pathology seen in this mouse model of mild PSACH, and in PSACH-MED patients, becomes exacerbated with age as short-limbed dwarfism and increasing joint pain greatly reduce the patients' mobility. The increased joint laxity may in turn have an effect on the joint stability and the progression of degenerative joint disease in PSACH patients (44,45).

In summary, we have shown that COMP is expressed in the Achilles tendon and skeletal muscle of adult mice. We have demonstrated that *Comp* T585M mutant mice suffer from a progressive myopathy that is specifically localized to the perimysium and the MTJ. The distribution of collagen fibril diameters is altered in the mutant tendon and ligament, whereas the PG content is comparable between the wild-type and mutant tissues. The biomechanical properties of mutant tendon are dramatically altered, making the tissue more resilient to failure stresses, but ultimately more lax following cyclic strain. We therefore hypothesize that the mild myopathy reported in PSACH-MED results from altered forces transmitted by the mutant tendon, and that the joint laxity may be directly due to the structural abnormalities in the ligament. Furthermore, our study suggests that the difficulties in walking and easy tiredness experienced by some PSACH-MED patients might be a combination of the skeletal phenotype and an underlying tendinopathy. Since tendons are able to adapt to mechanical load and environmental requirements (36,37,61,64), certain physiotherapeutic treatments may help alleviate some of clinical symptoms of PSACH-MED, such as muscle weakness and joint instability. Our detailed characterization of the mild myopathy and tendinopathy in the mouse model of mild PSACH may also help in the management and early diagnosis of some forms of PSACH-MED, specifically in those children that present with tiredness and muscle weakness prior to the diagnosis of an underlying skeletal dysplasia.

MATERIALS AND METHODS

Transgenic mice

The generation and phenotypic characterization of the mice used in this study is described in detail in Pirog-Garcia *et al*. (20). Wild-type mice and mice homozygous for the *Comp* T585M mutation were used in this study. We specifically chose to study the homozygous mutant mice in order to better accentuate the muscle and tendon pathology. All experiments were performed in compliance with the relevant Home Office and Institutional regulations governing animal breeding and handling.

Grip-strength analysis

Grip strength measurements were performed using a hand-held Chatillon digital force gauge (ChatillonDFE Series Digital Force Gauge, Ametek Inc.; Fig. 1A). Briefly, the mice were preconditioned 20 times on the cage lid, then held by the tail and gently lowered towards the apparatus. They were allowed to grip the grid with their forelimbs only and were pulled backwards in a horizontal plane. The highest force applied to the grid (maximum strength) and the force at the moment the grasp was released were recorded (N). The test was repeated two times per mouse, and 10 mice per genotype were tested at 3 and 9 weeks of age. One-way ANOVA was used for statistical analysis of the data.

RNA extraction and real-time PCR

Following snap-freezing of tissues in liquid nitrogen and their homogenization, RNA was extracted from the Achilles tendon, soleus/gastrocnemius skeletal muscle and posterior longitudinal spinal ligament tissue from 3 week-old mice,

using TriZOL reagent (Ambion Inc.). The RNA was DNaseI-treated (Ambion Inc.) and first-strand cDNA synthesized using Superscript III™ reverse transcriptase with random hexamers (Invitrogen Ltd). Real-time analysis of COMP, collagen I, collagen II and myoD expression was performed using SYBR® Green Kit (Eurogentech) on a Chromo4 sequence detector system (Bio-Rad). Each sample, including 'no template' controls, was run in duplicate and every sample had an 18S control. Each experiment was repeated at least three times with tissue from unrelated animals for statistical relevance, and the results were analysed by independent samples *t*-test.

Western blotting

Achilles tendon and gastrocnemius/soleus skeletal muscle tissue at 3 weeks of age was snap-frozen and homogenized, boiled in SDS-loading buffer containing DTT and loaded on an SDS–PAGE gel. The resolved gel was electroblotted onto a nitrocellulose membrane, which was blocked overnight with 2% skimmed milk powder in PBS-T. Primary antibodies [BiP (Cell Signalling Ltd), tenascin C, bcl-2 and actin (Abcam Plc), COMP (Genetex)] were diluted in PBS-T. An ECL detection kit (PerkinElmer Inc.) was used to develop the blots according to the manufacturer's protocol. The X-ray films were then scanned, and the densitometry of the bands was measured using Aida analysis software (Raytek Scientific Ltd). Independent samples *t*-test was used for statistical analysis.

Histology and immunohistochemistry

Full mouse limbs were skinned and fixed in ice-cold 10% neutral buffered formalin solution (Sigma-Aldrich Ltd; for histology) or in ice-cold 95% ethanol/5% acetic acid solution (for IHC), decalcified in 20% (w/v) EDTA, pH 7.4, paraffin-embedded and sectioned (6 μm sections). For haematoxylin and eosin (H&E) staining, the slides were dewaxed in xylene, rehydrated and H&E-stained using a ThermoShandon Ltd automated stainer, dehydrated in increasing concentrations of ethanol and in xylene and mounted using a xylene-based mounting solution. Gomori's trichrome staining (Polysciences Inc.) was performed according to the manufacturer's protocol and mounted using a xylene-based mounting solution (Pertex, Surgipath). Muscle fibres with central nuclei were counted and their number was expressed as a percentage of all the muscle fibres seen. Independent samples *t*-test was used for statistical analysis.

For IHC analysis, slides were dewaxed and rehydrated, endogenous peroxidase activity was quenched in H_2O_2/MetOH, followed by antigen unmasking in 0.2% bovine testes hyaluronidase (Sigma-Aldrich Ltd) in PBS. Samples were blocked in goat serum and BSA in PBS for 1 h and immediately incubated with primary antibody [COMP (Genetex Inc.), type I and type II collagen (Chemicon), type IV collagen, type VI collagen, desmin, vimentin and tenascin C (Abcam Ltd)] in PBS/BSA for 1 h. Slides were washed in PBS/BSA and incubated with FITC-conjugated secondary antibody (Abcam) and mounted in Vectashield™ containing DAPI (Vector Labs Ltd) or incubated with a biotinylated goat anti-rabbit IgG (Dako Cytomation Ltd) in PBS with goat serum, followed by incubation with ABC/HRP reagent (Dako Cytomation Ltd) and developed using DAB chromogen (Dako Cytomation Ltd), with methyl green as counter stain (Vector Labs Ltd). Vectamount™ (Vector Labs Ltd) xylene-free mounting medium was used for mounting the slides.

Ultrastructural analysis

Wild-type and mutant Achilles tendon and posterior longitudinal spinal ligament were dissected from mice at 3 and 9 weeks of age and immediately fixed in 2.5% glutaraldehyde in 1 M sodium cacodylate buffer for 2 h at 4°C. The tissues were washed three times in 0.1 M sodium cacodylate buffer and fixed in 2% OsO_4 in 0.1 M cacodylate buffer for 2 h. They were washed in distilled water and incubated for 2 h in 2% aqueous uranyl acetate at 4°C. The tendons were then washed in distilled water, dehydrated in increasing concentrations of acetone (50, 70, 90 and 100% for 30 min), incubated in propylene oxide to improve resin penetration and 1:1 solution of resin:propylene oxide and embedded in TAAB medium slow resin (TAAB Laboratories Equipment Ltd). Thin 70–80 nm sections were cut with a diamond knife on a Leica ultramicrotome and placed on electron microscope grids. Sections on the grids were stained with silver citrate solution and viewed in a FEI Tecnai 12 Twin transmission electron microscope operated at an accelerating voltage of 80 kV. The fibril diameters were measured, and the distribution was analysed for statistical significance using Mann–Whitney *U* test.

DMMB assay

PG content was analysed using the DMMB assay (65). Achilles tendons at 3 weeks of age were digested overnight in 50 μg/ml proteinase K in 100 mM K_2HPO_4 (pH 8.0) at 56°C. The samples were centrifuged at 12 000 g in Ultrafree filter (Amicon), and the flow through was used for DMMB assay (66). Serial dilutions of shark chondroitin sulphate (Sigma) in K_2HPO_4 were used as a calibration curve, and the absorbance was read at $\lambda = 525$. The retentate was used for DNA contents analysis using Hoechst dye, as described in Orioli *et al*. (66). The calculated GAG content was then normalized to the DNA content of the tissues, and the data were statistically analysed using independent samples *t*-test.

Biomechanical analysis

Tensile loading and cyclic stress tests were performed on an Instron 1122 tensile tester (Instron). Achilles tendons were harvested at 3 weeks of age and stored in PBS at −80°C. They were gently thawed and the cross-sectional area was assessed by measuring the thickness in three points along the tendon's length. The average mouse tendon length was ∼5 mm. The tendons were fitted into the tensile tester as shown in Fig 6A and were kept moist in PBS throughout the experiment. For the tensile loading test, the sample's starting length was 1 mm, and the samples were elongated at a constant strain rate of 0.08/s, until breaking point and force and displacement were measured. The toe region for the samples was established in the tensile loading test. In the cyclic

stress, the starting sample length was 2 mm; the samples were stretched and relaxed with a constant strain amplitude of 0.5 for $n = 9$ cycles at the strain rate of 0.04/s, and the force and displacement were recorded. The force was normalized against the cross-sectional area, and the decrease in stress at each cycle was compared between the wild-type and mutant samples. Independent samples *t*-test was used for statistical analysis.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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