The transcription coactivator ASC-1 is a regulator of skeletal myogenesis, and its deficiency causes a novel form of congenital muscle disease

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Abstract

Despite recent progress in the genetic characterization of congenital muscle diseases, the genes responsible for a significant proportion of cases remain unknown. We analysed two branches of a large consanguineous family in which four patients presented with a severe new phenotype, clinically marked by neonatal-onset muscle weakness predominantly involving axial muscles, life-threatening respiratory failure, skin abnormalities and joint hyperlaxity without contractures. Muscle biopsies showed the unreported association of multi-minicores, caps and dystrophic lesions. Genome-wide linkage analysis followed by gene and exome sequencing in patients identified a homozygous nonsense mutation in TRIP4 encoding Activating Signal Cointegrator-1 (ASC-1), a poorly characterized transcription coactivator never associated with muscle or with human inherited disease. This mutation resulted in TRIP4 mRNA decay to around 10% of control levels and absence of detectable protein in

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patient cells. ASC-1 levels were higher in axial than in limb muscles in mouse, and increased during differentiation in C2C12 myogenic cells. Depletion of ASC-1 in cultured muscle cells from a patient and in Trip4 knocked-down C2C12 led to a significant reduction in myotube diameter ex vivo and in vitro, without changes in fusion index or markers of initial myogenic differentiation. This work reports the first TRIP4 mutation and defines a novel form of congenital muscle disease, expanding their histological, clinical and molecular spectrum. We establish the importance of ASC-1 in human skeletal muscle, identify transcriptional co-regulation as novel pathophysiological pathway, define ASC-1 as a regulator of late myogenic differentiation and suggest defects in myotube growth as a novel myopathic mechanism.

**Introduction**

Congenital muscular dystrophies (CMDs) and congenital myopathies (CMs) are the two most important groups of congenital-onset muscle disease (1). They share common clinical manifestations such as early-onset presentation (typically within the first year of life), delayed motor development, stable or slowly progressive muscle weakness leading to impaired or absent ambulation, orthopaedic deformities and, in some cases, respiratory failure or heart disease associated with early mortality (2). No specific treatments are available thus far for these often devastating inherited disorders. Classically, the CMD and the different forms of CM are defined by characteristic histological findings in the patients’ muscle biopsies. CMDs are characterized by dystrophic features [small round fibres, marked increase in endomyosial connective tissue and regenerating or (uncommonly) necrotic muscle fibres] without histological evidence of another disease (1). In contrast, the CMs are defined by a non-dystrophic muscle biopsy with the presence of distinctive structural changes in the architecture of muscle fibres (3). Along these lines, the presence in muscle fibres of multiple small foci of sarcomere disorganization and mitochondria depletion, termed ‘minicores’, defines multi-minicore disease (MmD), and crescent-shaped peripheral (subsarcolemmal) abnormal arrays of myofibrils, called ‘caps’, lead to the diagnosis of cap myopathy (6) (for a detailed review of the different CMs, see (7)).

From the genetic point of view, most forms of CMD are autosomal recessive and associated with structural or glycosylation defects of a number of extracellular or sarcolemmal membrane proteins (such as collagen VI, laminin alpha-2 or alpha-dystroglycan), which lead to alteration of the transmembrane linkage between the extracellular matrix and the cytoskeleton (2,8). A rare, dominant form of CMD is due to mutations in lamin A/C, an intermediate filament of the nuclear envelope encoded by LMNA (9). The modes of inheritance and pathophysiological mechanisms involved in CM are more heterogeneous but, in accordance with their histological presentation, generally involve intracellular proteins. The vast majority of genetically characterized CM forms are due to mutations in genes that encode (i) contractile, scaffolding or regulatory components of the sarcomere involved in myofibrillar force generation (such as alpha-actin, nebulin, myosin, tropomyosin, troponin or titin) (10,11); or (ii) proteins primarily or secondarily implicated in excitation–contraction coupling, including triadic junction proteins (the ryanodine receptor RyR1 (12), Stac3 (13,14)) or phosphoinositide phosphatases involved in membrane remodelling (15,16). To our knowledge, primary defects in intranuclear proteins or transcription regulation have never been associated with either congenital or late-onset muscle disease.

Despite recent progress in the identification of the genetic bases of CMD and CM, a significant proportion of affected families is not associated with defects of the known genes and remains genetically uncharacterized. This is the case of MmD, a phenotypically and genetically heterogeneous autosomal recessive CM (5,17,18). We have previously reported that the classical and most common MmD phenotype presents with axial muscle weakness, spinal rigidity, scoliosis and early respiratory failure and is most often due to mutations in the SEPN1 gene encoding selenoprotein N, a putative antioxidant enzyme of the endoplasmic reticulum (19). Mutations in the RyR1 or the titin genes (RyR1 and TTN) are also common causes of MmD (18,20–22), while changes in MYH7, DOK7, MEGF10 or CCDC78 have been associated with minicores in only a few families (23–27). Overall, up to 40% cases of core myopathy (28) and personal observation, AF remain genetically uncharacterized. Similarly, mutations in the genes encoding collagen VI (COL6A1, COL6A2 or COL6A3) have been excluded in a proportion of patients presenting with clinical features characteristic of collagen VI-related CMD, which include proximal weakness, joint hyper laxity, finger flexor contractures and skin abnormalities (keratosis pilaris, hyperelasticity and hypertrophic scars) (29–32). As for the rare CM cap disease (33), only in five patients have mutations in the thin filament protein-encoding genes TPM2, TPM3 and ACTA1 been identified (6,34–37). Consequently, genetic diagnosis and counselling are not always possible for the affected families, and the pathomechanisms involved in these conditions are far from fully understood.

We report here the phenotypical and genetic study of a large consanguineous family presenting with a so-far unreported clinical and histological phenotype, overlapping CMD, MmD and cap disease. We provide evidence that this phenotype is associated with the first-reported mutation of TRIP4, leading to depletion of the transcription coactivator ASC-1. Ex vivo and in vitro studies demonstrate that the absence of ASC-1 leads to significant defects in myotube growth. Thus, our work expands the phenotypical and genetic spectrum of congenital muscle diseases, reports defects in transcriptional regulation as a novel pathomechanism and identifies ASC-1 as a novel key player in myogenic differentiation and skeletal myotube growth.

**Results**

Overlapping features of congenital muscular dystrophy and two forms of congenital myopathy: a novel clinical and histological phenotype

Initially, we examined three siblings with a severe form of CM (Table 1, Fig. 1) and a histological diagnosis of MmD. A second cousin of these patients was subsequently referred to us for uncharacterized congenital muscle disease. Both parental couples were consanguineous, originated from Eastern France and belonged to a group of itinerant fairground people with high rates of inbreeding (Fig. 2A), without known Romani ancestry.

The clinical phenotype in all the patients was identical, with mild intrafamilial variability only in severity (Table 1). They presented from birth with neonatal hypotonia particularly marked in axial (neck and trunk) muscles, severe head lag, poor
antigravity limb movements and, in three patients, respiratory failure and feeding difficulties which needed intermittent nasogastric feeding from the first 2 years of life. There were no congenital contractures. Motor development was severely delayed, but some degree of motor maturation was observed during the first decade. Only the mildest case (Patient II.2) achieved independent ambulation (at 4 years) and was able to walk short distances with waddling gait until the age of 11 years (Fig. 1A).

Life-threatening respiratory failure required assisted ventilation from the first year of life in three patients (two of them tracheotomized) and from the age of 11 years in the mildest case. Severely affected Patient II.5, who was not tracheotomised, died at 16 months of respiratory failure during a respiratory infection. The surviving patients are currently aged 25, 24 and 10 years. They are wheelchair-bound and require full assistance for all daily life activities. All cases show severe muscle weakness

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NG, nasogastric; WCB, wheelchair bound.
Figure 1. Clinical and histological presentation. Upper panel: clinical findings in the mildest (Patient II.2 at ages 9 (A) and 19 (B–D) years), the intermediate (II.1, E–J) and the most severe (III.1, K–Q) surviving patients. Only Patient II.2 was able to walk a few steps independently between the ages of 4 and 11 years (A). Major axial muscle weakness led to severe scoliosis (A) that required arthrodesis in patients beyond their first decade of life (B, E) and was associated with dorsal hyperlordosis (B, E, L) and cervical spine rigidity (H: maximum neck flexion). Note the identical spontaneous position of II.1 and III.1 (unknown to each other): they form a triangle with their lower body to support their highly hypotonic trunk (F, K). Limb muscles retained partial antigravity strength (N). Joint hyperlaxity was present in all patients (F, H–K, M, P). Limb joint contractures were absent in non-ambulant Patient III.1 and mild and localized in II.1 and II.2 (C). Finger flexor contractures upon wrist extension were very mild in Patient III.1 (Q) and absent in II.1 and II.2. Note follicular hyperkeratosis (G) and increased leg subcutaneous adipose tissue in females (C, D, M, O). Lower limb MRI in II.2 (D) showed major adipose replacement of muscles, predominantly in quadriceps, posterior thigh muscles and soleus (D). Lower panel: quadriceps muscle biopsies from Patients II.2 (R–T) and III.1 (U, W). Transversal frozen sections stained with hematoxylin–eosin (R, S), SDH (T) and modified Gomori trichrome (U); ultrastructural longitudinal sections (V, W). Note fibro-adipose replacement in Patient II.2 and rare fibres with nuclear centralisations (R). All samples showed multiple areas lacking oxidative activity in most fibres (T, arrow) which corresponded on EM to short zones of mitochondrial depletion and sarcomere disorganization (minicores) (V and W, arrows). In Patient III.1, abundant small angular fibres contained areas which stained red on Gomori trichrome (U, arrow) were dark blue on NADPH-TR and corresponded on EM to subsarcolemmal accumulations of disorganized sarcomere components lacking thick filaments (caps, W, arrowheads). Some scattered similar fibres were retrospectively observed in Patient’s II.2 biopsy (S, arrow) but could not be found on EM. Note coexistence in the same fibre of a minicore and a cap localized immediate under the sarcolemmal membrane (stars, W). Scale bars = 50 μm (R–U), 2 μm (V–W).
predominantly involving axial and proximal muscles (globally quoted around 2 on the Medical Research Council scale). Neck flexors are particularly weak (0–1), leading to head lag, poor head control and cervical rigid spine (Fig. 1H). Weakness of trunk muscles requires compensation by a peculiar ‘tripod’ posture in order to maintain stability of the sitting position (Fig. 1F).
and K). Generalized joint hyperlaxity (Fig. 1H–J, M and P) and a skin phenotype (mild hyperelasticity, dry skin with scratch lesions, follicular hyperkeratosis, Fig. 1G) which cosegregates strictly with the muscle phenotype are highly reminiscent of those observed in CMD due to collagen VI defects, except for the absence of hypertrophic scars. In the two older siblings (II.1 and II.2), slow muscular and respiratory deterioration was observed during the second decade. Severe scoliosis required spinal fusion in their early teens, but joint contractures (almost exclusively in lower limbs, sparing finger flexors) were absent before their mid-teens and remain remarkably mild for non-ambulant patients (Fig. 1C and I). The female patients show also a peculiar aspect of lower limbs, with abundant adipose tissue giving a pseudo-infiltrative aspect (Fig. 1C, M and O).

Creatine phosphokinase level was normal or nearly normal (<2×N). Electromyography (Patient II.1) showed a myopathic pattern with normal conduction velocities. Electroencephalography (II.1 and II.5) and a neonatal brain CT scan (II.1) were normal. A muscle CT scan of Patient II.2 at 19 years showed major, diffuse fatty degeneration of most muscle groups, with relative preservation of adductors only (Fig. 1D). Echocardiography and electrocardiography were normal in all patients except for an incomplete right branch block in Patient II.1 at 20 years. Thyroid hormone levels were normal. Patient II.1 had normal testosterone and dihydrotestosterone and mildly elevated delta-4 androstenedione levels (2469 pg/ml, < 2000). Nolhydrotestosterone and mildly elevated delta-4 androstenedione levels (2469 pg/ml, N < 2000).

Muscle biopsies performed at ages 5 months (II.1), 6 years (II.2) and 4 years (III.1) showed an unreported combination of lesions characteristic of different congenital muscle diseases, in variable proportions. All samples showed fibre size variability, rounded fibres with mild increase of endomysial connective tissue and, in II.1 and II.2, a more marked adipose replacement (Fig. 1B), which typically define a CMD. However, there were also histological signs characteristic of at least two forms of CMD. Abundant minicore lesions were present in all cases and associated with a mild increase of centrally located nuclei (Fig. 1T, V and W, arrows). In Patient III.1, light and electron microscopy studies disclosed the presence of abundant angular fibres containing basophilic areas which stained red with Comori trichrome (Fig. 1U, arrow) and corresponded on electron microscopy to typical cap lesions (Fig. 1W, arrowheads). Scarce similar fibres were also retrospectively observed on hematoxylin–eosin staining in the biopsy from Patient II.2 (Fig. 1S, arrow). Immunostaining of muscle sections for dystrophin, merosin and collagen VI was compatible with secondary abnormalities whose signification was unclear (data not shown).

The parents and the two healthy siblings of Patients II.1, II.2 and II.5 were carefully examined and showed no muscle, respiratory or skin phenotype.

A nonsense mutation in TRIP4, encoding the poorly known transcription coactivator ASC-1, leads to severe protein depletion in patients

To identify the genetic defect underlying this new phenotype, we excluded by direct sequencing mutations of the known MmD or cap genes SEPN1, RYR1 and TPM2. Whole-genome screen using 6056 SNPs (Human Linkage Panel V, Illumina) excluded linkage to TTN, COL6A1, COL6A2 and COL6A3 and disclosed a unique haplotype that was homozygous by descent in all patients from both family branches, heterozygous in the healthy parents and absent in unaffected siblings. This new 2.6 Mb locus localized in chromosome 15q22 showed a logarithm of odds (LOD) score of 3.8 (Fig. 2B), and included 32 positional candidate genes. After Sanger sequencing of 10 candidate genes, we identified a nonsense variant in TRIP4 (Thyroid Hormone Receptor Interactor 4 gene, MIM#604501). TRIP4 is a 67.5 kbp gene that contains 13 exons and encodes the ubiquitous 65 kDa protein ASC-1 (Activating Signal Cointegrator-1), a poorly known transcription coactivator. The variant, c.G950A (NM_016213.4) or p.W297* (NP_057297.2), is localized in exon 7 and introduces a premature termination codon (PTC) (Fig. 2D). This change, which was homozygous in the four patients and heterozygous in both parental couples, was not found in 240 control chromosomes and is not reported in the dbSNP, 1000 Genomes Project database or in the Exome Aggregation Consortium database (ExAC, Cambridge, MA) (URL: http://exac.broadinstitute.org, accessed 23 December 2015). Furthermore, ExAC includes only 24 very rare (frequency < 0.01%) TRIP4 loss of function (LoF, frameshift or splice) variants, none of them present at the homozygous state in the canonical transcript, supporting the potential pathogenicity of the homozygous p.W297* change. To further confirm this, we performed in Patient II.2 whole-exome sequencing followed by analysis of the variants within the 15q22-linked region. This excluded non-synonymous changes in KBTBD13, a positional candidate associated with nemaline myopathy, and confirmed the presence of the homozygous TRIP4 c.G950A mutation. No other LoF variants were identified in the locus, which contained only one non-synonymous change with frequency < 1.5%. This variant, which was homozygous in the patient consistently with its location in the locus haplotype cosegregating with the disease in this family, was located in the LCTL (lactase-like) gene (NM_001278562, c.T792G:p.N264K or NM_207338, c.T1311G:p.N437K) and unreported in ExAC or in dbSNP, 1000 Genomes. LCTL encodes a member of family 1 glycosidases with alternative transcripts and unknown function, which is predominantly expressed in the brain and eye in humans; we have found no reports of skeletal muscle LCTL expression. The LCTL variant does not involve any of the known post-translational modification sites in the protein. Furthermore, predictions on its pathogenicity are inconsistent (predicted as deleterious by Polyphen2, as tolerated by FATHMM). Although we cannot formally exclude a potential contribution of this LCTL variant to the patients’ phenotype, the exome results as well as the expression and prediction findings supported pathogenicity of the nonsense p.W297* TRIP4 mutation in this novel form of muscle disease.

Searching to identify other families with TRIP4 mutations, we sequenced this gene in 83 phenotypically similar index cases with MmD, cap disease, collagen VI-like CMD or other genetically uncharacterized CMS. We found only a non-synonymous heterozygous variant (p.L201S), in a family with dominant disease, which did not cosegregate with the phenotype, suggesting that TRIP4 mutations are not a common cause of congenital muscle disease.

The p.W297* mutation changes a highly conserved amino acid localized in a coiled coil domain and predicts a truncated ASC-1, devoid of its 284 most C-terminal amino acids which represent 49% of the protein (Fig. 2D). However, no truncated or full-length protein was detectable by western blot using primary cultured fibroblasts from patients (Fig. 2E). Since the mutation fulfilled the conditions for nonsense-mediated decay (NMD), we performed RT-PCR and qRT-PCR on primary fibroblasts and cultured muscle cells. This excluded compensatory splicing mechanisms (Fig. 2F) and identified a severe depletion of TRIP4.
mRNA (8.8–12% residual transcript) in the homozygous patients and a reduction by 47% in the heterozygous mother I.2 (Fig. 2G), which explains the absence of detectable protein and categorizes these patients as a human equivalent of a TRIP4 knockout.

The absence of ASC-1 does not prevent interaction of ASCC2 and ASCC3 in patient cells

ASC-1 is known as the key component of a 650 kDa tetrameric protein complex including three partners [the ASC-1 associated proteins ASCC1 (p50), ASCC2 (p100) and ASCC3 (p200)] and termed the ASC-1 complex (Fig. 3A). ASC-1 binds to both ASCC2 and ASCC3, linking these two partners which have no known direct interactions. However, it is not known if the absence of endogenous ASC-1 has an impact on the formation and stability of the ASC-1 complex. To investigate this, we used primary cultured fibroblasts from TRIP4-mutant patients as a first model of ASC-1 depletion and performed in situ proximity ligation assay (PLA), a sensitive method to detect close proximity compatible with interaction of two proteins. Fluorescent dots revealing ASCC2–ASCC3 complexes were observed in the ASC-1-depleted patient cells as well as in control fibroblasts, both in nuclei and cytoplasm (Fig. 3C). This suggests that ASC-1 is dispensable for the interaction of ASCC2 and ASCC3 in human cells (Fig. 3C). Moreover, an increased number of protein complexes were observed in ASC-1-depleted fibroblasts compared with controls, supporting the notion that alterations of this pathway are involved in the pathogenesis of disease (Fig. 3B).

ASC-1 is a novel actor in late myogenesis and regulates myotube growth

It has been reported that TRIP4 is ubiquitously expressed, but thus far it had never been associated with skeletal muscle development or function. To clarify this potential new role of ASC-1, we first analysed by western blot the expression profile of ASC-1 in adult murine tissues, including different skeletal muscles. ASC-1 protein was expressed at low levels (2–20% relative to GAPDH—Fig. 4A—or tubulin, data not shown) in all the muscles, but it was more abundant in axial [paravertebral muscles and particularly diaphragm, 10-fold compared with extensor digitorum longus (EDL)] than in limb muscles (Fig. 4A). This distribution is consistent with the clinical phenotype in patients. However, the overall low levels suggest that ASC-1 does not have a major role in adult skeletal muscle maintenance.

Next we investigated a putative role of ASC-1 in myogenesis in vitro and ex vivo. We quantified the expression of Trip4 during myogenic differentiation using a C2C12 murine myoblastic cell line. Although ASC-1 was expressed in proliferating cells, protein levels increased during differentiation and reached significant variation at 3 and 4 days upon serum withdrawal (Fig. 4B). Consistently, primary cultured muscle cells from Patient III.1 showed no obvious defects of proliferation or fusion and formed multinucleated myotubes.

Supporting Information

Figure 3. ASCC2 and ASCC3 interaction in the absence of ASC-1. (A) Schematic representation of the ASC-1 tetrameric complex. (B) PLA in fibroblasts from Patient II.1 compared with a healthy control showed significant differences in the number of interacting events between ASCC2 and ASCC3 both in cytosol (P-value = 2.2e−16) and nucleus (P-value = 0.009). (C) In situ PLA probed human fibroblasts for visualization of ASCC2/ASCC3 interactions (red dots). Nuclei are labelled in blue (DAPI). The negative control was performed by incubating with the anti-ASCC3 antibody alone. Scale bar = 20 μm.
Figure 4. ASC-1 expression profile and impact on myofibre growth. (A) ASC-1 quantification by western blot in adult murine striated muscles, compared with GAPDH. (B) ASC-1 levels (compared with histone H3) during differentiation of murine myoblastic C2C12 cells, in confluent proliferative cells (basal) and 1–4 days after differentiation induction by serum withdrawal (D1–D4). (C) Brightfield pictures of primary human myotubes from a healthy control (top panel) and from Patient III.1 (bottom panel) after 7 days of differentiation. Myotube aspect was comparable between control and patient in some fields (left column), but the largest myotubes observed in each sample (middle and right columns) showed different sizes. (D–J) siRNA knock-down of Trip4 in C2C12 cells showing defective myofibre growth. Brightfield pictures (D) and quantitative studies (E–G, J) were performed immediately after plating to confirm comparable cell density (basal, D), in confluent proliferative cells prior to serum withdrawal (D0, D–G, J) and/or 1–4 days after switch into differentiation medium (D1–D4, D–G, J). Trip4 KD cells showed no differences in the percentage of Pax7 or Myogenin-positive nuclei (E, F) or fusion index (G) compared with the scramble control, but formed myotubes which at D3 had a significantly smaller mean diameter (H), comparable length (I) and dramatically reduced MHC levels (by western blot, J). (K) Induction of expression of a SRE-luciferase 3DA.Luc reporter (SRF-controlled luciferase) in proliferating C2C12 transfected with a Trip4-expressing construct (pCITO-Trip4) relatively to the corresponding empty vector pCITO (control). QUAD, quadriceps; TA, tibialis anterior; EDL, extensor digitorum longus; SOL, soleus; GAS, gastrocnemius; Dia, diaphragm; PV, paravertebral muscles; Scbl, scramble control; siRNA, Trip4 siRNA knock-down. Scale bars = 10 μm (c) and 100 μm (d). $P < 0.06, ^*P < 0.05, ^{**}P < 0.01.
containing up to 15–20 nuclei after 7 days of differentiation, like an age-paired control sample (Fig. 4C, left column). However, the latter contained several thick branching myotubes (around 10 μm of maximum diameter), which were not present in the patient culture (Fig. 4C, middle and right columns). Taken together, our findings suggest that ASC-1 might have a role in late myogenesis and/or myoblast growth.

Due to the high intrinsic variability of primary human cell cultures, investigation of other models is mandatory to reduce variability and to confirm the specificity and significance of the ex vivo cell phenotype. In addition, chronic ASC-1 depletion in patients might have triggered compensatory mechanisms that modulate this phenotype. Thus, we generated by RNA silencing (siRNA) a transient Trip4 knock-down in C2C12, to explore the impact of an acute depletion of ASC-1 in myogenic differentiation in vitro and particularly in its more advanced stages (between 2 and 4 days in differentiation medium, D2–D4). Minimum Trip4 transcript and ASC-1 levels were obtained at D2 and D3 and were consistently between 26 and 35% compared with scramble (Supplementary Material, Table S1 and data not shown). Interestingly, the cell phenotype in Trip4 KD cells at Days 3 and 4 was comparable with that observed in patients’ myotubes (Fig. 4D).

The percentage of nuclei expressing Fox7 or Myogenin (Fig. 4E and F) or the fusion index (Fig. 4G) was not significantly different between the Trip4 KD C2C12 cells and those transfected with the scramble siRNA. Consistently, Trip4 KD was not associated with significant changes at the mRNA or protein levels of myogenic factors such as MyoD, myogenin, myf5 or MRF4/myf6/herculin or of the early myogenic differentiation marker desmin (Supplementary Material, Table S1 and Supplementary Material, Table S1). This suggests that ASC-1 reduction is not associated with major abnormalities of the progenitor cell population, initiation of the myogenic differentiation programme or myoblast fusion. In contrast, upon Trip4 KD, the diameter of the myotubes was significantly reduced at D3 (Fig. 4H).

Consistently, these myotubes had dramatically reduced levels of the contractile protein and marker of late myogenic differentiation myosin heavy chain [reduced by more than half, as shown by fluorescence intensity quantification (data not shown) and western blot, Fig. 4J]. These results confirm that ASC-1 is a new key player in late myogenic differentiation and/or myotube growth.

One of the factors regulating growth and differentiation of myogenic cells is serum response factor (SRF), a crucial transcription factor for muscle-specific gene expression (38,39). The ASC-1 complex is known to enhance SRF transactivation in HeLa cells (40). Thus, to explore whether a direct regulation of SRF by ASC-1 happens also in myogenic cells and might be part of the disease mechanism, we analysed the transactivation of SRF by ASC-1 in C2C12 using a SRF reporter construct containing a SRF-controlled luciferase gene (Fig. 4K). Co-transfection of a Trip4-expressing construct induced a modest but significant and robust induction of SRF expression (1.3-fold, \( P < 0.05 \)) compared with the empty vector. This suggests that SRF expression in muscle cells is to some extent regulated by ASC-1 and might participate in the pathogenesis of the disease.

**Discussion**

Despite recent progress in the molecular characterization of congenital muscle diseases, up to 40% of CMs are at present genetically unresolved. We report and characterize here a novel and particularly severe form of autosomal recessive congenital muscle disease with a distinctive, unique phenotype and genetic basis. Histologically, patients present with the so-far unreported association of multiminicores, caps, and dystrophic lesions. The minicores typical of MmD have been reported to coexist in some patients with central nuclei (18,21,22), protein inclusions (41), nemaline rods (42) or mild dystrophic lesions (1,19,22), which classically define other forms of congenital muscle disease. In one case, cap lesions were associated with nemaline rods (43). The novel pattern reported here stresses the histologic-overlap not only between two different forms of CM (MmD, cap disease) but also between two different nosological groups (CM, CMD), contributing to blur the classic boundaries in the field (11). Strikingly, the patients show also an unreported and recognizable clinical phenotype which is marked by: (i) severe neonatal hypotonia, often associated with feeding difficulties and potentially lethal respiratory failure from infancy, all of which have been reported in cap disease; (ii) predominantly axial muscle weakness and contractures (rigid spine), dorsal lordosis, progressive scoliosis and relative respect of facial muscles, as observed in SEPN1-related MmD; and (iii) joint hyperlaxity, skin abnormalities and absence/loss of ambulation reminiscent of severe collagen VI-related CMD, although without the limb contractures typical of the latter. The leg appearance in female patients and the learning difficulties identified in some cases are atypical findings and might reflect adipose tissue or CNS involvement, which needs to be clarified (especially considering poor school attendance due to the family lifestyle). But, globally, the clinical phenotype accurately reflects the histological overlap, suggesting that some of the mechanistic pathways involved in cap disease, SEPN1-related myopathy or collagenopathies might also be implicated secondarily in this novel condition.

Along these lines, immunocytochemical analyses of collagen VI secretion showed no abnormalities on muscle biopsies or on a first study of cultured fibroblasts from two siblings, indicating that collagen VI deficiency is not characteristic of ASC-1 deficiency. However, partial abnormalities were observed on a second study of fibroblasts from one of these same patients, suggesting that partial, secondary collagen VI changes in a given sample might be non-specific, dependant on the cell state and do not exclude the possibility of Trip4 defects.

From the molecular point of view, our results represent the first description of Trip4 mutations, thereby identifying defects in transcriptional regulation as a novel mechanism and pathophysiologic pathway in congenital muscle disease. Severe weakness of axial muscles and restrictive respiratory failure with diaphragmatic involvement in our patients correlate with relatively high Trip4 expression in murine paravertebral muscles and diaphragm compared with limb muscles. High levels of ASC-1 murine protein were also found in the lung, skin and liver (data not shown), corresponding with the skin phenotype but not with the absence of signs of lung parenchyma abnormalities in patients. Trip4 is also expressed in the heart, suggesting that later-onset myocardial involvement cannot be excluded in these so-far young patients. The Trip4 mutation reported here leads to total ASC-1 depletion and a purely recessive phenotype. But the phenotypic spectrum associated with ASC-1 abnormalities remains to be determined, since other (i.e. missense) Trip4 mutations might potentially be associated with milder and/or dominant disorders. Thus, we propose that Trip4 should be screened for mutations in patients with a variety of genetically uncharacterized congenital muscle diseases, particularly (but not exclusively) in MmD, collagen VI-like and/or cap disease, with or without partial collagen VI deficiency. Additionally, our results suggest that genes encoding other proteins of the ASC-1 complex represent novel candidate genes for congenital muscle disease.
TRIP4 encodes ASC-1, an incompletely characterized transcription co-activator. Transcriptional co-regulators either bridge transcription factors and the basal transcriptional apparatus and/or remodel the chromatin structures (44). Some of them are bi-functional proteins that can also participate in RNA splicing (45). ASC-1 (also known as p65) was initially isolated as a protein partner of the transcription factor thyroid hormone receptor (THR) (46). Subsequently, it was found to contain a highly conserved E1A-type zinc finger domain that interacts with basal transcription factors, transcription integrators (CBF, SRC1) and other nuclear receptors (such as the retinoid acid receptor RXRA) in a ligand-independent manner in vitro (47). Upon interaction with ASCC1, ASCC2 and ASCC3 (or p50, p100 and p200), ASC-1 forms the ASC-1 complex, which bridges the transcription regulators mentioned above to the basal transcriptional apparatus (40). In addition, ASC-1 contains a conserved C-terminal domain, which defines a superfamily [the ASC-1 homology (ASCH) domains] with putative RNA-binding activity and has been predicted in silico to mediate some interactions between RNA and the ASC-1 complex (48). Experimentally, ASC-1 has been reported to localize to nuclei or cytoplasm of rat fibroblasts under different cellular conditions (47), to modulate expression of the anti-apoptotic plasminogen activator inhibitor-2 (PAI2) in gastric cancer-derived AGS cells (49) and to act as a coactivator of the transcriptional plasminogen activator inhibitor-2 (PAI2) in gastric cancer (40). In addition, it has been suggested that ASC-1 is involved in protection (40), although this had never been experimentally investigated. Furthermore, it has also been proposed as a locus for Alzheimer disease (51). Despite these limited data, the precise role of ASC-1 in human cell physiology and the underlying mechanisms in vivo remain unknown, and this protein has never been associated with skeletal muscle tissue.

Integrin of the ASC-1 complex seems to be critical for its transactivation effects (40,49). ASCC1 and ASCC3 interact directly, and both ASCC3 and ASCC2 bind to ASC-1. There is no known direct interaction between ASCC2 and any of the other members of the complex, suggesting that ASC-1 presence might be indispensable for linking the different components of the complex (40), although this had never been experimentally investigated. Using primary cultured cells from patients as a first model of ASC-1 depletion in humans, we found evidence of close proximity compatible with interaction of ASCC2 and ASCC3 despite the absence of ASC-1. These results suggest that ASC-1 is dispensable for complex formation in humans, and indicate that other so-far unknown factors are potentially involved in ASCC2 and ASCC3 interaction. Along these lines, we found a significant increase in PLA fluorescent signals indicative of ASCC2–ASCC3 interaction in the patient’s cells compared with age-paired controls, supporting the implication of the ASC-1 complex in the pathogenesis of disease. Additional investigations are needed to clarify whether this change indicates a defective turnover of the complex in the absence of ASC-1, or the activation of compensatory mechanisms involving these proteins.

The severe congenital muscle phenotype in our TRIP4-mutant patients revealed for the first time that ASC-1 is indispensable for normal skeletal muscle function. Trip4 transcription is initiated at E11.5 in muscle structures in mice (52), suggesting a role of ASC-1 in muscle development. Myogenesis is a sequential process during which myoblasts formed upon activation and differentiation of muscle progenitors [satellite cells (SC)] align and then fuse to form multinucleated myotubes, which in turn grow and mature to become new muscle fibres. In the past years, mutations of genes involved in the early steps of myogenesis, namely maintenance of the SC pool and myoblast fusion, have been associated with congenital muscle disease. This includes STAC3, encoding a triad junction protein that regulates negatively the differentiation and fusion of SC into myotubes (53), but also HACD1/PTPLA (54), encoding a 3-hydroxacyl-CoA dehydratase 1 that promotes myoblast fusion (55). Defects in SEPN1 or MEGF10, which cause recessive myopathies with minimal core lesions, have also been associated with abnormalities in SC regulation (26,56,57). Contrary to MEGF10 (57), Trip4 transcription does not increase significantly during SC activation (58,59), the initial stage of myogenesis. Consistently, we observed knock-down model disclosed no changes in the percentage of cells expressing the SC marker Pax7, the fusion rate or the expression of myogenic factors including myogenin, which drives the terminal differentiation and fusion of myoblasts into myotubes. Moreover, multinucleated myotubes formed adequately in a primary muscle culture from a TRIP4-mutant patient. These results suggest that ASC-1 does not play a major role in regulating SC or initial myogenic differentiation. As we establish here, ASC-1 levels increase with differentiation, and deficiency of this protein is associated with reduced myotube growth both in vitro and ex vivo. These results identify ASC-1 as a novel regulator of myogenesis, and describe defects in late myogenic differentiation and myotube growth as a novel mechanism in a human inherited disease.

Muscle growth is regulated by multiple factors, only partially understood, which include insulin-like growth factor (IGF-I) (60) or SRF. SRF is a transcription factor that binds to the serum response element (SRE) in the promoter region of many target genes, plays a central role during myogenesis and is crucial for the growth of cultured muscle cells (61) and skeletal muscle (38,39). SRF has been identified as a downstream target of the ASC-1 complex in HeLa cells. We found that ASC-1 consistently induces SRE expression in vitro in myogenic cells, suggesting that this pathway might be implicated in the myotube growth defect associated with ASC-1 depletion and be part of the disease mechanism. However, the magnitude of this modulation was relatively small, suggesting that it might involve other regulatory factors (i.e. other members of the ASC-1 complex) or so-far unknown pathways. Further investigations, including the effect of differentiation or TRIP4 mutations on SRF induction, are in progress to clarify this point.

In summary, we report and characterize a novel form of congenital muscle disease for which we propose the term of ‘ASC-1-related myopathy’, which has the potential to include in the future conditions associated with defects in other proteins of the ASC-1 complex. Our work expands the histological, clinical and molecular spectrum of congenital muscle disease, identifies transcriptional co-regulation as a new pathophysiological pathway and reveals ASC-1 as a novel regulator of late myogenic differentiation and myotube growth. Loss of skeletal muscle mass or function is associated with significant morbidity in ageing and diseases such as cancer and diabetes. Therefore, this rare muscle disease may be useful as a model paradigm to identify new factors and mechanisms that control skeletal muscle mass and physiology, which has important implications for prevalent conditions affecting human health.

Materials and Methods

Patients and samples

We initially studied two branches of a French consanguineous family, including four affected patients. The three surviving patients, a couple of unaffected parents (I.1 and I.2) and two healthy siblings (II.3 and II.4) were specifically examined for this study by...
One of us (AF). Muscle samples, skin biopsies and peripheral blood samples (for DNA extraction) were obtained after informed consent in agreement with local ethic committees and with the Declaration of Helsinki. At least one diagnostic skeletal muscle biopsy was obtained from Patients II.1, II.2 and III.1, frozen or fixed and processed for standard histological, histochemical and electron microscopy studies. Primary fibroblast or myoblast cultures were obtained from skin biopsies (Patients II.1, II.2 and healthy mother I.2) or from surgically discarded tissues (skin and muscle from III.1 and age-paired unaffected controls).

Additionally, we screened TRIP4 through direct sequencing of genomic DNA samples from 83 other phenotypically similar index patients without mutations in the known genes (SEPN1, RYR1, ACTA1, TTN, COL6A1, COL6A2, COL6A3, TPM2, TPM3 or ACTA1), from our own series or collected through international collaborations, after informed consent. This included patients with the diagnosis of MmD (32), collagen VI-like CMD (19), cap Collaborations, after informed consent. This included patients with the diagnosis of MmD (32), collagen VI-like CMD (19), cap

Secondary ligation assay on primary fibroblasts

Fibroblasts from patients and age- and passage-paired controls were amplified in DMEM (1 g/L glucose, 1-glutamine, pyruvate) with 10% FBS and 2% penicillin-streptomycin, then seeded at 1.2 x 10^5 cells per well into micro slides (Angiogenesis ibiTreat BioValley) 48 h prior to PLA. Cells were fixed—permeabilized in methanol for 5 min at −20°C prior to 40 min-long incubation in blocking buffer: PBS 1x, 2% BSA, 0.1% Triton X100. Anti-ASCC2 (D16, Santa Cruz) and anti-ASCC3/HELCIC-1 (S20, Santa Cruz) diluted at 1:100 were incubated for 45 min at RT prior to the incubation with secondary antibodies—PLA coupled probes (In Situ PLA® Probes anti-Goat MINUS and anti-Rabbit PLUS). Samples incubated with the anti-ASCC3 antibody only were used as negative controls. Ligation and amplification were conducted accordingly to the manufacturer’s protocol (Duolink for PLA, Sigma). Finally, all samples were mounted in Vectashield and imaged on a Zeiss LSM 700 confocal microscope using the ZEN 2009 software. Over 90 nuclei were analysed per condition.

Primary human muscle cell culture

Myoblasts obtained from muscle explants from Patient III.1 (at P3) and an age-paired control (at P5) were maintained in proliferation in F-10/DMEM GlutaMAX (4.5 g/L glucose, pyruvate) with 20% FBS and 2% penicillin-streptomycin. At 85–90% confluence, the medium was changed for DMEM GlutaMAX (4.5 g/L glucose, pyruvate) with 2% horse serum (HS) and 2% penicillin-streptomycin. Myotube formation was analysed in both samples up to 7 days after medium switch (D7).

Murine myoblastic C2C12 and Trip4 knock-down

C2C12 cells were cultured in DMEM GlutaMAX (4.5 g/L glucose, pyruvate) with 10% FBS and 1% penicillin-streptomycin (Sigma-Aldrich). At confluence (85–90%), medium was changed for the differentiation medium DMEM GlutaMAX (4.5 g/L glucose, pyruvate) with 2% HS and 1% penicillin-streptomycin. Trip4 silencing was optimized to obtain maximum reduction of protein expression between differentiation Days D2 and D4. C2C12 cells were plated for 24 h at 5 x 10^5 cells per well (6-well plate) and then transfected with Lipofectamine RNAiMax (Invitrogen) and 20 x 10^-3 mM siRNA against Trip4 or scramble sequences (Trilencer-27, OriGene) in OptiMEM (GIBCO). After 48 h, cells were either collected for analysis (D0) or put in differentiation medium and analysed after 24, 48, 72 or 96 h (time points D1, D2, D3 and D4). Maximum reduction of ASC-1 expression was achieved at D2 and D3, with residual transcript and protein levels consistently between 26 and 35%. Each well was done in triplicate, and five independent transfection experiments were performed.

RT-PCR and qRT-PCR

RNA was extracted from cultured cells using the RNasy Mini Kit (Qiagen) according to the manufacturers’ instructions. Genomic DNA was eliminated by deoxyribonuclease treatment (RNase-Free DNase set, Qiagen), and samples were immediately stored at −80°C. cDNA was obtained using SuperScript III Kit (Invitrogen) according to the manufacturers’ protocol. For exclusion of alternative splicing, TRIP4 PCR was performed on patient primary cell cDNA using SuperMix Platinum (Invitrogen) and a couple of primers located within the 5’ UTR and 3’ UTR and amplifying the whole cDNA sequence (5’ TTGGCAGCTACGTGTT 3’ and 5’ CTCCCTATGTCTGATATCC 3’). Sequences of the primers used for qRT-PCR of Trip4, MyoD, Myog, Myf5, myf6 and Des in C2C12 are available upon request. Quantitative PCR was performed on a Light Cycler 480 II (Roche) using a compatible SYBR Green I Master 2x (Roche) in 20 μl reaction mix. Relative expression of a gene of interest (GOI) was calculated using the ‘delta–delta Ct’ method and normalization by geometric averaging of the transcript levels of three housekeeping genes (HKG: Hprt, 18S ribosomal RNA, Gapdh). The final results represent the mean of five independent qRT-PCR with biological triplicates for each experiment, comparing Trip4 siRNA-transfected versus siScramble-transfected cells.
Western blotting
All the available commercial antibodies against ASC-1 (ab 70627 Abcam, NBP1-89656, Novus Biologicals) directed against epitopes localized upstream the mutation site showed a non-specific pattern on immunocytochemistry but identified by western blot, among others, a band at the expected ASC-1 molecular size (65 kDa). Muscle extracts (30 μg) from 6-week-old mice [sacrificed according to the ‘Principles of laboratory animal care’ (NIH Publication No. 85–23, revised 1985) and to local ethical guidelines] were obtained from muscles immediately frozen in isopentane and grided prior to resuspension. Cell samples were collected in cell lysis buffer (Sigma-Aldrich) completed with 1% protease inhibitor cocktail (Sigma-Aldrich). C2C12 cell line extracts (15–20 μg) were then separated and transferred using either precast PAGE Criterion TGX 4–15% gels (BioRad) and polyvinylidene difluoride (PVDF) Trans-Blot Turbo membranes (BioRad), or 10% SDS-PAGE and nitrocellulose membranes (Amer sham protran, GE Healthcare). PVDF membranes were blocked in PBS 1×, 0.2% Tween-20, 0.25% SDS-20 and 2% skimmed milk, probed with primary antibodies [anti-ASC-1 (ab70627 Abcam), anti-GAPDH (ab140584 Sigma-Aldrich), anti-Histone 3 (H0164 Sigma), anti-Myosin Heavy Chain (MF20 DSHB) at 1:500 and anti-Tubulin (T5293 Sigma-Aldrich) at 1:1000] for 4 h and with secondary fluorescent-coupled antibodies [anti-Mouse and anti-Rabbit (A10038 and A11369 Life Technologies) at 1:7500] for 1 h at RT. Immunolabelled proteins were detected using the Odyssey CLx system. Nitrocellulose membranes were blocked in TBS (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 5% dry milk, probed with primary antibodies [anti-desmin (Sigma, D8281), anti-lamin B1 (Abcam, ab16048), anti-MyoD (Santa Cruz, sc-304) at 1:1000 and anti-myogenin (DSHB, FSD) at 1:500] and HRP-conjugated secondary antibodies [goat anti-rabbit HRP conjugate (Thermo Scientific, 31460) and goat anti-mouse HRP conjugate (Thermo Scientific, 31430) at 1:10000] for 1 h in TBS–1% milk at RT. Proteins were detected by enhanced chemiluminescence. Each well was run in triplicate, and at least three independent experiments were performed. Quantification of western blots was carried out using Image J densitometry tool (National Institute of Health, Bethesda, MS, USA).

Immunofluorescence analysis of myogenic markers in C2C12
Cells were washed in PBS 1× prior to fixation and blocking as described previously. They were then incubated with the primary antibodies anti-Myosin Heavy Chain (MF20 DSHB), Pax7 (sc-81648, Santa Cruz) and anti-Myogenin (FSD, DSHB) at 1:50 for 4 h and with the secondary antibodies anti-mouse IgG Alexa Fluor 488 and anti-mouse IgG2b Alexa Fluor 633 (A21146 and A3152, Life Technologies) at 1:1000 for 1 h at RT. Finally, all samples were mounted in Vectashield and imaged on a Zeiss LSM 700 confocal microscope using the ZEN 2009 software. Image acquisitions were analysed with MATLAB 7.10.0 Nuclear Segmentation program to quantify nuclei positively labelled for Myogenin or Pax7. Fluorescence intensity quantification of MF20 positive myofibres and myotube measurements (length and width) were conducted on Image J (National Institute of Health, Bethesda, MS, USA). Three independent experiments were performed.

Luciferase assays and Trip4 cloning
Proliferating C2C12 were plated for 24 h at 5 × 10^5 cells (96-well plate) prior to co-transfection with the SRF reporter vector SRE-Luciferase (3DA.Luc 2 ng) (41), a TK-Renilla-expressing vector (20 ng) and either an empty vector pCITO or a Trip4-expressing construct pCITO-Trip4 (178 ng) for 16 h, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. The Trip4-expressing vector was obtained from C2C12 Trip4 cDNA inserted into a pCITO expression vector—pCITO being a pcG-based plasmid expressing a tomato reporter. Cells were harvested 48 h post transfection and lysed for measure of luciferase and renilla activities using a dual-luciferase reporter assay system (Promega) in a Centro XS3 LB960 luminometer (Berthold). Results were calculated as ratios of luciferase counts over renilla counts and normalized to the empty pCITO values. Each well was done in triplicate, and at least three independent experiments were performed.

Computational and statistical analyses
All values are presented as ±SEM. Statistics were calculated using the PRISM software. All the experiments were analysed using the Kruskal–Wallis and Mann–Whitney tests for small samples. P-values <0.05 were considered statistically significant ($ P < 0.05, ** P < 0.01$).

Supplementary Material
Supplementary material is available at HMG online.

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