Two novel COLVI long chains in zebrafish that are essential for muscle development

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Abstract

Collagen VI (COLVI), a protein ubiquitously expressed in connective tissues, is crucial for structural integrity, cellular adhesion, migration and survival. Six different genes are recognized in mammalians, encoding six COLVI-chains that assemble as two ‘short’ (α1, α2) and one ‘long’ chain (theoretically any one of α3–6). In humans, defects in the most widely expressed heterotrimer (α123), due to mutations in the COL6A1-3 genes, cause a heterogeneous group of neuromuscular disorders, collectively termed COLVI-related muscle disorders. Little is known about the function(s) of the recently described α4-6 chains and no mutations have been detected yet. In this study, we characterized two novel COLVI long chains in zebrafish that are most homologous to the mammalian α4 chain; therefore, we named the corresponding genes col6a4a and col6a4b. These orthologues represent ancestors of the mammalian Col6a4-6 genes. By in situ hybridization and RT-qPCR, we unveiled a distinctive expression kinetics for col6a4b, compared with the other col6a genes. Using morpholino antisense oligonucleotides targeting col6a4a, col6a4b and col6a2, we modelled partial and complete COLVI deficiency, respectively. All morphant embryos presented altered muscle structure and impaired motility. While apoptosis was not drastically increased, autophagy induction was defective in all morphants. Furthermore, motoneuron axon growth was abnormal in these morphants. Importantly, some phenotypical differences emerged between col6a4a and col6a4b morphants, suggesting only partial functional redundancy. Overall, our results further confirm the importance of COLVI in zebrafish muscle development and may provide important clues for potential human phenotypes associated with deficiency of the recently described COLVI-chains.

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Introduction

Collagens are major components of extracellular matrices (ECM) (1), complex structures that ensure tissue architecture and play diverse biological functions. Collagen VI (COLVI), a unique member of this large family, is ubiquitously expressed and has been involved in tissue integrity, cell survival and signaling (2–10). To date, six different genes are recognized in mammalians, encoding six different α chains (11). COLVI assembly is a complex, multi-step process that begins with the formation of monomers composed of two short (invariably α1 and α2) and one long chain that may vary (either one of α3–6) (12–14). These monomers further form dimers and tetramers that are secreted into the ECM where they align into beaded microfibrils (15–19).

While the most widespread and best characterized COLVI heterotrimer contains the α1, α2 and α3 chains, the α4–6 long chains have been described more recently in human and mouse (14,20). In vertebrates, orthologues of the corresponding genes are organized in tandem at the same locus (5′ Col6a4, Col6a5, Col6a6 3′), with the exception of human, gorilla and chimpanzee where the COL6A4 gene is only partial due to a pericentric inversion that occurred before the branching of hominoids (11). The two halves of the human COL6A4 gene, located on both arms of chromosome 3, were initially considered as pseudogenes; nevertheless, the 5′ part of the split COL6A4 gene on chromosome 3p24.3 has been associated with osteoarthritis as the DVWA gene, and appears to be expressed intracellularly (21,22).

Similar to the α3 chain, the α4–6 mature proteins are composed of seven N-terminal von Willebrand factor A-like (vWFA) domains (N7-N1), followed by a 336-amino acid-long collagen triple helical domain (THD) classically containing [Gly-X-Y] triplets. They display two consecutive vWFA domains (C1 and C2) as well as unique sequences (C3) toward the C-terminus. In addition, the murine α4 chain carries 17 amino acid residues at its C-terminal end that constitute a partial Kunitz protease inhibitor domain. The C-terminus of α5 further extends with the presence of a third vWFA domain (C4) and a second unique stretch of 130 amino acids (CS) (reviewed in (11)).

The murine Col6a4-6 genes are positioned on chromosome 9. Their expressions differ in their spatial pattern, and are developmentally regulated (14,20). Importantly, α4 is not expressed in adult murine skeletal muscle, but both α5 and α6 are, only partially co-localizing with α3.

Mutations in either of the COL6A1, COL6A2 or COL6A3 gene lead to COLVI-related muscle disorders, a heterogeneous group of clinical presentations ranging from Ullrich Congenital Muscular Dystrophy [UCMD; MIM#158810; (23)] to Bethlem Myopathy [BM; MIM#101350; (24)], with intermediate phenotypes. The lar Dystrophy [UCMD; MIM#254090; (23)] to Bethlem Myopathy [BM; MIM#101350; (24)] is similar (33). Somites are separated by vertical myosepta, equivalent to tendons, to which myofilaments attach through myotendinous junctions (MTJs) (34). Slow muscle cells form a superficial monolayer in embryos and later remain at the periphery of the myotome, while fast muscle cells are located deep within the myotome, making up most of the trunk musculature (35).

Here, we report and characterize the orthologues, and we believe ancestors, of mammalian Col6a4-6 in the zebrafish genome. We examined their temporal and spatial expression at different developmental stages. We further showed that their knockdown leads to muscle defects, reminiscent of those observed in col6a2 deficiency, although some specific features emerged. We thus confirm the importance of the col6 genes in zebrafish muscle development and provide new insights on the potential roles of the recently described α4–6 chains.

Results

Identification of two novel col6 genes in zebrafish

To identify zebrafish orthologues of the mammalian α4–6 chains [reviewed in (11)], the protein sequences of human α5–6 (NP_001265227.1 and NP_001096078.1, respectively) and mouse α4-6 (NP_081039.2, NP_001161395.1 and NP_001096071.1, respectively) were used in a BLAST search against the zebrafish genome at ENSEMBL. Two novel proteins were identified. Since their C-terminus domains were similar in size and organization, these parts of the proteins were aligned to other vertebrate Col6 proteins and a phylogenetic tree was inferred using the ClustalX software, revealing that the zebrafish proteins are phylogenetically closer to Col6a4 than to either Col6a5 or Col6a6, or even Col6a3 (Fig. 1A and Supplementary Material, Fig. S1 and S2). The corresponding genes, located on chromosomes 16 and 13, were thus named col6a4a and col6a4b, respectively (Fig. 1B). These genes likely represent ancestors of the mammalian Col6a4-6 genes. The zebrafish α4a (2568 amino acids) and α4b (2497 amino acids) chains present common features of all COLVI peptides, such as the conserved THD containing [Gly-X-Y] repeats (336 and 303 amino acids long for α4a and α4b, respectively), flanked by large globular domains with homologies to vWFA domains (eight and two in N- and C-terminal, respectively). In addition, they also possess two Kunitz domains in C-terminal, which are only present in mammalian α3 and α4 chains (Fig. 1B). In contrast, no fibronectin III domain was found in C-terminal, contrary to α3 (Supplementary Material, Fig. S1). Multiple sequence alignments between human, mouse and zebrafish α3–6 proteins are presented in Supplementary Material, Fig. S2.

COLVI expression during zebrafish development

We first examined the levels of all col6a transcripts by RT quantitative PCR (RT-qPCR) on whole embryos at different stages post-fertilization: 24–96 h, 10 days, as well as in juvenile animals (around 1 month). At all developmental stages considered, col6a1 and col6a2 transcript levels were most abundant (Supplementary Material, Fig. S3A). When normalized to the 48 hpf stage, it appeared that col6a transcript levels are rather stable, with the striking exception of col6a4b, which expression is dramatically increased at 72 and 96 hpf compared with 48 hpf, followed by a relative decrease at later stages (Fig. 2A). Among the long-chain-encoding transcripts, col6a4a levels were significantly higher between 24 and 72 hpf. During this period, col6a3 levels steadily increased, while col6a4b levels were barely detectable until 72 hpf and increased between 72 and 96 hpf, at which stage all transcripts could be detected at similar levels before decreasing (Supplementary Material, Fig. S3B).
In addition, the spatial patterns of expression of col6a2, col6a4a and col6a4b were assessed by whole-mount and section in situ hybridization using WT embryos at 48hpf and 5 dpf (Fig. 2B and data not shown). Overall, these analyses confirmed the RT-qPCR data, showing col6a4b was less expressed than col6a4a and col6a2 at 48 hpf. At this stage, col6a2 and col6a4a expression could be detected in pectoral fin buds, and all transcripts were localized throughout the myotome. Labeling of the myosepta appeared at 48 hpf for col6a4a (72 hpf for col6a2 and col6a4b, data not shown). At 5dpf, col6a4b was
strongly expressed and all transcripts were detected in the myotome and in pectoral fins muscles. Expression of the three genes was also detected in lens and sclera at 48 hpf, and in cornea at 5dpf.

Knock-down of col6a genes in zebrafish embryos

To further understand the function of the col6a4a and col6a4b gene products, we designed acceptor splice-site morpholinos (MOs).
targeting col6a4a exon11 (MO-a4a) and col6a4b exon 12 (MO-a4b) (Fig. 1B). The resulting splicing events induce frameshifts that create premature termination codons (PTC). We also used this strategy to knock-down col6a2 expression with a MO against exon 2 (MO-a2; Fig. 1C), to obtain reference morphants deficient in this crucial short chain present in all heterotrimers. A mismatch MO (scramble) with no biological target was injected as a control of specificity.

Knock-down efficacy was assessed by Northern blotting using total RNA extracted from 48 hpf whole embryos. A specific diminution of the targeted transcripts for col6a2 and col6a4a was detected: 5 and 8% of WT, respectively (Fig. 3). These results were also confirmed by RT-qPCR on cDNA from 48 hpf embryos (data not shown). Unfortunately, we failed to detect col6a4b transcripts by Northern blot at this stage, and by RT-qPCR in col6a4b mutants. We also investigated whether the knock-down of one of the genes affected the levels of the other transcripts. In col6a2 morphants, col6a4a levels were slightly increased (163% of WT), while a 53% reduction in the levels of col6a2 transcript was detected in col6a4a morphants (Fig. 3). By RT-qPCR, no significant variation in the levels of col6a1 and col6a3 were noted (data not shown).

Phenotypic alterations of col6a morphants

From 24 hpf, col6a2, col6a4a and col6a4b MO-injected embryos displayed obvious macroscopic defects under light microscopy. As illustrated in Figure 4A, they present shorter and bent bodies, and vertical myosepta do not display the characteristic chevron shape seen in WT as well as in scramble MO-injected embryos; rather, myosepta of morphant embryos exhibited an abnormal ‘U-shape’ curvature. These features are quite characteristic of the knock-down of proteins involved in muscle development and/or myopathies. At 48 hpf, more than 80% of col6a MO-injected embryos presented a slight developmental delay characterized by a reduced pigmentation and aberrant head-to-body angle (36), in addition to curved bodies and smaller eyes, phenotypes which are not observed in scramble MO-injected embryos. To assess muscle integrity, embryos were examined under polarizing light. Decreased birefringence was observed in all morphants compared with WT, indicating compromised muscle fibres organization (Fig. 4B). At the functional level, all morphants also exhibited an absence of escape response to touch stimulus, or uncoordinated movements, compared with WT and embryos injected with the scramble MO (Supplementary Material, Movies S1–S5). It is worth mentioning that similar results were obtained when we injected donor splice-site MOs (data not shown), with the exception of col6a2 since the absence of a suitable sequence at the donor site prevented us from designing a MO. All col6a morphants died around 10dpf, most likely as a consequence of impaired motility which prevented them from feeding after yolk sac exhaustion (data not shown). As reported in other studies, MO injections yielded some phenotypic heterogeneity; nevertheless, col6a morphants mostly displayed distinctive muscle phenotypes, compared with scramble MO-injected embryos (Fig. 4C). All morphants used in the following experiments were sorted, so that ‘monster’ morphants were discarded.

Phenotypic rescue of col6a2 morphants by co-injection with col6a2 RNA

To confirm that the phenotype observed in col6a2 morphants was indeed specific to the knock-down of col6a2, zebrafish col6a2 RNA was co-injected with the col6a2-MO, leading to more than 85% of co-injected embryos with a phenotype similar to WT, and restored motility (Fig. 5A and B and Supplementary Material, Movie S6). Quantitative RT-PCR demonstrated that col6a2 transcript levels in co-injected embryos were restored to levels comparable with WT (Fig. 5C). On the other hand, co-injection of col6a2 RNA with the col6a4a-MO failed to rescue the phenotype of morphants (Supplementary Material, Fig. S4A and B), although col6a2 transcript levels were indeed increased (Supplementary Material, Fig. S4C). As anticipated, col6a4a transcripts were not modulated in co-injected embryos (data not shown). Unfortunately, similar experiments could not be performed for the col6a4 morphants due to the large size of the corresponding cDNAs that impeded their cloning.

Altered myofibrils and myotome boundaries components

In light of the macroscopic alterations induced by the down-regulation of col6a genes, we further examined the muscle structure...
by immunostaining on whole embryos. At 48 hpf, dystrophin, a sub-sarcolemmal protein, correctly accumulated to myotome boundaries in WT embryos as well as morphants, but this staining appeared slightly irregular and less defined in the latter, and revealed misshapen myotome boundaries, particularly in \textit{col6a2}-MO-injected embryos, and to a lesser extent in \textit{col6a4} morphants (Fig. 6). Collagen type XXII (COLXXII), a recently described MTJ marker (37), was also investigated, further demonstrating the abnormal shape of myosepta. COLXXII staining appeared to be most affected upon down-regulation of \textit{col6a2}, and to a lesser degree in \textit{col6a4} morphants (Fig. 6). Staining with an antibody against slow myosin revealed that in all morphants the labelled myofibres spanned entire somites, as in WT embryos, although they were less closely packed, possibly due to lateral detachment that appears as gaps between fibres. On the contrary, WT muscle fibres were tightly connected to each other along their length. These defects were still visible in embryos at 5dpf (Fig. 6).

**Muscle structure and ultrastructural analysis of 5dpf zebrafish muscle**

To further examine the effects of \textit{col6a2}, and both \textit{col6a4} knockdown on muscle structure, semi-thin sections were stained with toluidine blue (Fig. 7A). Light microscopy revealed the regular pattern of myofibrils and the fine, straight vertical myosepta in WT embryos. On the contrary, muscle fibres were clearly disorganized in \textit{col6a2} morphants, with abundant undifferentiated sarcoplasm, large nuclei and their enlarged nucleoli. The knockdown of \textit{col6a4a} did not alter significantly the sarcomeric organization, although some vacuolated areas are present at the myocyte periphery in \textit{col6a4b} morphant muscle. On the contrary, \textit{col6a2} morphant muscle was severely disorganized with ill-defined Z bands, enlarged T-tubules but no sarcoplasmic reticulum dilatation (arrows in Fig. 7B). In addition, large undifferentiated sarcoplasmic areas with altered mitochondria were also observed. These mitochondria showed an increased matrix electrodensity, an enlarged space between their outer and inner membranes and a pronounced cristae swelling.

As described above, vertical myosepta were affected by the down-regulation of all \textit{col6a} genes. In WT embryos, the regular and thin myosepta, which contained a dense matrix of collagen fibril bundles were outlined by the electron-dense sarclemma and subsarclemma and by the continuous, less electron-dense, lamina densa of the basement membrane (Fig. 7C). \textit{Col6a4a} morphant septa also showed a continuous basement...
membrane and a dense collagen network, but appeared wider and more ramified than in controls. In sharp contrast, col6a2 and col6a4b morphant myosepta were drastically altered; they were highly enlarged with discontinuous remnants of basal lamina bordering a vacuolated and nearly empty matrix with sparse collagen bundles. Moreover, col6a4b myosepta specifically exhibited quite irregular boundaries, with short additional ramifications (Insets in Fig. 7C).

The ultrastructure of the triangular zone of intersection between vertical septum and skin also showed the same type of alterations in col6a2 and col6a4b morphants (Supplementary Material, Fig. S5). While in WT and MO-col6a4a triangles, a characteristic orthogonally arranged, dense collagen stroma linked the deep epidermal surface and underlying myocytes, col6a2 and col6a4b-deficient triangles appeared disorganized and vacuolated, with sparse and loose collagen fibrils attached to the epidermis.

It must be noted that the devastated aspect of col6a2 and col6a4b-deficient septa and triangles suggests a particularly loose organization of their collagenous matrix, which could have less resisted dehydration and embedding procedures.

Altered α2(VI) expression in col6a4 morphants

We developed a specific antibody against zebrafish α2 chain, which proved useful in western blotting experiments. The antibody detects a band at the appropriate size in protein extracts from adult WT zebrafish muscle and from 72 hpf whole embryos. The specificity of this antibody was assessed by immunogenic peptide competition, which abolished the detection of the bands (Fig. 8A). The levels of α2 were drastically decreased in embryos injected with MO-a2, as expected, but also in extracts from the col6a4 morphants (Fig. 8B).

Pathophysiological mechanisms

Since increased apoptosis had previously been reported in col6a1 morphants (38), we examined whether this was also the case in the three morphants, by whole-mount immunostaining of active caspase 3, a late marker of the apoptotic pathway. We observed a non-specific increase in the number of positive cells in the hindbrain, most likely due to the micro-injections themselves, as it was also detected with the scramble MO. On the other hand, some specific signal was visible in the trunk region of the different col6a morphants, in particular in col6a4b morphants (Fig. 9A).

Defective induction of autophagy has been implicated in the pathogenesis of COLVI-myopathies, in a murine model of α1 deficiency, as well as in biopsies and cultured cells from patients (39,40). Since a number of genes involved in this clearance mechanism are conserved in zebrafish (41), we investigated the levels of membrane-associated LC3 (LC3II) by western blotting and showed that they were decreased in all col6a morphants (Fig. 9B). This reduced LC3 conversion thus points to an alteration in autophagosome formation, an early step of autophagy. We also assessed whether col6a knock-down affected Akt signalling, a pivotal actor in numerous biological processes, including cell survival and autophagy (42–44). Reduced Akt activity (i.e. its phosphorylation) has previously been shown in muscle of
Col6a1-null mice (39). Phospho-Akt/total Akt ratios tend to be decreased in col6a2 and col6a4a morphants, and even more so in col6a4b morphants, although statistical significance was not reached (Fig. 9B).

Neuronal outgrowth defects

In addition to the muscle defects detailed above, whole-mount immunostaining with the SV2 antibody that labels synaptic vesicles revealed abnormal motor axons outgrowth in the three morphants: col6a2 and col6a4a deficient embryos frequently displayed no or very short ventral axons, while col6a4b morphants presented axons with hyperbranching (Fig. 10A). The severity of these defects was ranked according to the extent of growth with respect to the horizontal myoseptum, and to the degree of branching (following the scoring described in (45); Figure 10B). Overall, up to 40% of col6a2 and col6a4a deficient embryos displayed the most severe defects in motor axons outgrowth, classified as scores 0 and 1 (i.e. no growth or growth arrest before the horizontal myoseptum). On the contrary, over half of col6a4b morphants presented motor axons that grew beyond the horizontal myoseptum but were abnormally branched (score 2).

To further understand these defects, we investigated whether hdac6, a microtubule-associated histone deacetylase involved in migration processes (46,47), was differentially expressed upon col6a knock-down. Quantitative qPCR revealed that hdac6 transcripts levels were significantly reduced in col6a2 morphants only (Fig. 10C). This reduction was alleviated upon co-injection with col6a2 RNA, as were motor axons growth defects (Fig. 10C and data not shown).

Discussion

Although a significant proportion of patients with neuromuscular phenotypes reminiscent of COLVI-related disorders do not harbour mutations in the COL6A1–3 genes, no mutations have been reported in the human COL6A5 or COL6A6 genes to date.

In this study, we identified two zebrafish orthologues of the recently described α4, α5 and α6 chains, whose discovery has increased the complexity of COLVI (11,14,20). Phylogenetic and domain structure analyses led us to name the corresponding genes col6a4a and col6a4b, in light of their higher homology with murine α4 and the presence of Kunitz domains, which are present only in α3 and in murine α4 (14,48). Of note, in databases, a partial sequence of col6a4a had been computationally predicted as col6a6 from the zebrafish genome assembly, as were most of other fish col6a4 genes. This is most likely due to automated annotation of genome sequencing in reference to human genome in which the COL6A4 gene is rearranged. Another argument supporting the fact that zebrafish col6a4a is indeed part of the col6a4-a6 group is the presence on chromosome 16 of a small region of synteny (pik3r4-col6a4) with human chromosome 3 (PIK3R4-COL6A6-
COL6A5), mouse chromosome 9 (Pik3r4-Col6a6-Col6a5-Col6a4) and chicken chromosome 2 (pik3r4-col6a4). According to the phylogenetic tree, one evolutionary hypothesis would imply a col6a4 ancestor gene that underwent duplication in fish [as did many genes in the teleost genomes (49)], giving rise to col6a4a and col6a4b that evolved differently. This ancestor gene may have been triplicated in mammalian, on the same chromosome, and corresponds to the Col6a4-6 genes (11). In Xenopus tropicalis the presence of two genes, which C-terminal sequences allow clustering in two different groups may support this hypothesis and be a landmark of these later duplication events. Indeed one of these frog genes is closer to col6a4 in tetrapods and slow evolving fish (Latimeria chalumnae and Callorhinchus milii), while the second gene is clearly grouped with the mammalian Col6a5–6 cluster. The grouping by pairs of Col6a4 genes in Latimeria chalumnae and Callorhinchus milii is not explained but may be related to the fact that their genomes have been identified as very slow evolving ones (50,51).

We first characterized the temporal expression of col6a4a and col6a4b during zebrafish development. We showed a distinctive expression kinetic pattern for col6a4b, which appears delayed compared with that of col6a4a and the other long-chain encoding gene col6a3. The col6a1/a2 genes, encoding the two short chains, are expressed at higher levels throughout zebrafish development, in agreement with the requirement of α1 and α2 for assembly of heterotrimers with all the long chains. Using in situ hybridization, col6a4a/b transcripts could be detected in myotome, as well as in the eye, where mammalian COLVI is known to be expressed (4,52–54). Interestingly, while the murine α4 protein is not expressed in muscle, α5 and α6 are differentially expressed in skeletal muscle. In humans, α5 is specifically detected at the MTJ, while α6 is mainly interstitial with partial co-localization with α3 (29). In addition, α5 shows a unique, strong expression at the basal lamina of neuromuscular junctions (NMJ) and at the perineurium of intramuscular peripheral nerves (14,55). Indeed, one remaining question is whether zebrafish α4a and α4b may functionally be assimilated to mammalian α5 and α6.

We created models of α4a and α4b deficiency in zebrafish, using an anti-sense strategy against col6a4a and col6a4b. In parallel, we also targeted the col6a2 gene, to model loss of function of one of the chains involved in COLVI-related myopathy. The splice-site MO used here led to a truncated protein with a PTC occurring before the TH domain, and degradation of the corresponding mRNA. The resulting muscle phenotype was very similar to that obtained upon in-frame skipping of zebrafish col6a1 exon 9 (corresponding to human exon 10) as reported by Telfer et al. (38). Importantly, knock-down of col6a4a and col6a4b also caused muscle disorganization and dysfunction: all morphants displayed abnormal trunk curvature, misshapen vertical myosepta, defects in muscle organization and ultrastructure, leading to reduced motility. Although col6a4b transcript levels were very low in early developmental stages, the corresponding morphants displayed a clear muscle phenotype. One hypothesis

Figure 7. Skeletal muscle structure and ultrastructure of 5 dpf WT and col6a morphants. (A) Semi-thin sections showing the optical aspect of a hemi-myotome. Arrowheads indicate large nuclei with their enlarged nucleoli. Bar: 20 µm. (B) Sarcomeric ultrastructure. Triads are noted with arrows. Asterisks indicate large undifferentiated sarcoplasmic areas, with swollen mitochondria (M). Bar: 1 µm. (C) Vertical myosepta are pseudo-coloured in green. In all images, a dotted zone of the septum is enlarged in the inset below. N: nucleus. Bars: 2 µm for the main images and 200 nm for insets.
for this apparent contradiction could be that early on col6a4b expression is restricted to as of yet unidentified territories where it could play a crucial role for muscle development.

Collectively these results confirm the essential function(s) of all COLVI proteins in zebrafish muscle development. Since MOs remain active during approximately 5 days, we assessed whether a phenotype reversion could be obtained thereafter. This was not the case since morphant larvae died after yolk exhaustion, likely due to their inability to fetch food owing to the severe motility defects seen as early as 24 hpf.

Overall, col6a2 morphants presented the most severe muscle phenotype, a consequence of the ubiquitous expression of the α2 chain, and its presence in all monomers. Interestingly, col6a4a and col6a4b morphants displayed some specific phenotypes, with the latter showing a more pronounced muscle dysfunction as evidenced by birefringence and ME analyses. On the other hand, motoneuron axonal growth was more severely impaired in col6a4a and col6a2 morphants.

Over the last 10 years, numerous studies on Col6a1 knock-out mice have provided some clues on pathogenic mechanisms in skeletal muscle. In particular, increased apoptosis and defective autophagy, have been described in this animal model (39,40,56–58). The former was also reported in col6a1 MO-induced zebrafish models (38,59,60). Here, we assessed caspase-3 expression in col6a2, col6a4a and col6a4b morphants but did not detect a major increase in the number of apoptotic cells. Of note, no apoptosis could be detected in the recently described Col6a3 mutant

Figure 8. Altered α2(VI) chain expression in col6a morphants. (A) Total protein extracts from adult WT zebrafish muscle and whole WT 72 hpf embryos were separated by SDS-PAGE. PVDF membranes were hybridized with the specific anti-α2(VI) antibody (left panel). Competition with the antigenic peptide abolished the detection of the α2(VI) band (right panel). (B) Total proteins extracted from 72 hpf embryos were transferred to PVDF membranes, which were hybridized with the specific anti-α2(VI) antibody. A portion of the same membrane stained with Ponceau S Red prior to hybridization is shown as a loading control.

Figure 9. Assessment of pathophysiological mechanisms in col6a morphants. (A) Whole-mount immunostaining using an antibody against active caspase-3, in the head (left panel) and trunk (right panel) regions of 48 hpf embryos. E: eye. Bars: 50 μm. (B) Total proteins extracted from 72 hpf embryos were transferred to PVDF membranes, which were subsequently hybridized with antibodies against the membrane-associated form of LC3 (LC3II), the phosphorylated form of Akt (P-AktSer473) and total Akt. A portion of the same membrane stained with Ponceau S Red before the hybridization is shown as a loading control.
mice either, nor in human biopsies suggesting that apoptosis may not be a universal mechanism involved in the mediation of COLVI defects. Autophagy being a conserved process in zebrafish (41), we investigated whether COLVI deficiency had any effect on its early stages (i.e. autophagosome formation and/or elongation), by analogy to the murine Col6a1−/− findings. Similarly, we detected lower levels of the lipidated form of LC3 (LC3II) in all morphants; furthermore, beclin 1 levels, another factor involved in the autophagic process, also tended to be reduced (data not shown). One important mediator of muscle homeostasis is Akt/PKB, which is activated upon phosphorylation, and plays a regulatory role on autophagy (43). In Col6a1−/− mice, prolonged starvation (>24 h) enabled autophagy induction, but this type of experiment cannot be carried out in zebrafish larvae since nutrients are provided by the yolk sac. In fed Col6a1−/− mice, levels of activated Akt do not differ from those in WT mice, while upon starvation they are increased in tibialis anterior muscle, but not in diaphragm, suggesting a muscle type-specific response to nutrient depletion (39). In protein extracts from whole col6a2 and col6a4b morphants, we detected reduced levels of the activated form of Akt, while they were not altered in col6a4a morphants. This finding may reflect the rather mild muscle structure alterations in the latter model compared with the other two.

The importance of ECM molecules in axon guidance has been previously reported in zebrafish. For example, COLXV-deficient embryos present primary motoneurons guidance defects similar to those observed in the morphants presented here (61). In the col6a morphants presented here, the SV2 staining appeared weak and diffuse, which may indicate a defect of nicotinic acetylcholine receptor focalization, a process that is dependent on axonal growth (62). Furthermore, the importance of COLVI has already been demonstrated in neural crest development (63). Recently, nerve conduction defects and delayed response to acute pain stimuli have been described in Col6a1−/− mice. Indeed, COLVI is expressed by Schwann cells and its deficiency leads to hypermyelination of peripheral nervous system (64). How the primary neuron growth defects might relate (i.e. cause or consequence) to the muscle phenotypes remains to be determined in col6a morphants. The decrease in hdac6 expression does appear specific and may contribute to the neuronal outgrowth defects, as previously shown in Amyotrophic Lateral Sclerosis (65,66). Of interest, somewhat similar axonal growth defects were very recently reported in new col6a3 zebrafish models reproducing mutations identified in patients with dystonia (67). Collectively, our data point to the zebrafish α4a and α4b-encoding genes as being similar to an ancestor gene for vertebrate α4a-6. In mammals, this ancestor gene has likely been
triplicated into Col6a4−6. The expression pattern of zebrafish col6a4 and col6a2 genes overlap, and the kinetics of expression of zebrafish col6a4 suggest a role for these genes during muscle development and transient inactivation supports this hypothesis. Finally, the phenotypes observed in zebrafish col6a4 morphants strongly support the hypothesis that deficiency in COLVI-chains other than α1−3 may also lead to muscle dysfunction, and warrant screening of the human COL6A5 and/or COL6A6 genes in neuromuscular phenotypes of unknown aetiology.

Material and Methods

Zebrafish strains and maintenance

Different strains (AB, TU, ABC) were used for this study, with no difference in the experimental data obtained. They were kept at 28°C with a 14 h light cycle, using standard maintenance protocols. Animal care and experimentation were performed in compliance with French and European laws.

Sequence and phylogenetic analyses

COLVI phylogeny was performed using domains C-terminus to the collagen motif. The ClustalX software was used to perform the alignment and phylogeny (Neighbor-Joining method). The phylogenetic tree was drawn using FigTree v1.4.0. The same tree was obtained using the domains Nterminus to the collagen motif (data not shown).

In situ hybridization

Larvae were fixed overnight at 4°C in 4% PFA then progressively dehydrated in methanol and stored in 100% methanol at −20°C. Fragments of cDNA were produced by PCR targeting regions 5′ to the colagentic domain (Fig. 1B). Amplified fragments were cloned in pGEM-T (Promega) according to the supplier instructions. Clones suitable to produce anti-sense probes with T7 RNA polymerase (Roche) were selected and probes purified after synthesis using mini Quick Spin RNA columns (Roche).

Whole-mount in situ hybridizations (ish) were performed according to the protocol described in (68) with modifications as described in Zen (https://wiki.zen.org). Hybridized probes were revealed using anti-DIG antibody coupled to AP (Roche) and staining was obtained with NBT and BCIP (Roche). After staining, larvae were fixed overnight in 4% PFA then washed in PBSTw, transferred to glycerol at progressively increasing concentration and stored in 100% glycerol. Lateral and dorsal views were imaged in depression slides in 100% glycerol under dissecting microscope (SZX9 Olympus) using DFK 31AU03 camera and IC Capture software (both The Imaging Source).

For section ish, larvae fixed as above were progressively rehydrated and transferred in 30% sucrose then OCT medium (CellPath, CML). Larvae were transferred in mould (CML) and stored in liquid nitrogen-chilled isopentane. Ten µm sections were performed using a CMS5150S cryostat (Leica) and left to dry at room temperature. Ish were performed according to (69) at 65°C in humid chambers. After staining, sections were covered with Mo- wiol and a coverslip. Images were obtained using a microscope (BX41 Olympus), DMX31AU03 camera and IC Capture software (both The Imaging Source).

In vitro transcription

The full length col6a2 coding sequence was amplified from cDNA obtained from whole embryos by PCR using the Kapa HiFi ReadyMix (Kapabiosystems), then cloned into pGEM-t vector. RNA was synthetized from 1 µg of linearized col6a2 plasmid using the AmpliCap-Max T7 High Yield Message Maker Kit (Epicentre Biotechnologies) following the manufacturer’s instructions. Quantity and quality of the col6a2 RNA were assessed on agarose gel.

Morpholinos and micro-injections:

Anti-sense MO oligonucleotides (MOs, Gene Tools LLC) targeted to interfere with col6a2 (MO-a2; 5′ ACTCTCTGAAAGGACAGAACA-CACT 3′), col6a4 (MO-a4a; 5′ TACACTGCGGATGATACAAAGGA 3′) and col6a4b (MO-a4b; 5′ GACCCCTGATTTACAAATCAAGACACT 3′) were designed over intron–exon junctions 1, 11 and 12, respectively. A mismatch (scramble) MO (scr; 5′ ACTCTCTGAAAGGACAGAACA-CACT 3′) with no sequence homology in the zebrafish genome was used as a control. MOs were dissolved in distilled water at a concentration of 500 µM along with 0.1% phenol red, which served as a positive marker for successful injections. Fertilized eggs were injected at the 1–2 cell stage with approximately 3–4 ng of MO. For the rescue experiment, col6a2 mRNA (100 ng/µl) was co-injected with the MO-a2. Injected embryos and non-injected controls were placed in a 28°C incubator to recover and euthanized with a lethal dose of benzocaine at various stages depending on the experiments.

Live image analysis

Embryos were photographed at 24 and 48 hpf using a Leica MZ8 Stereomicroscope. Embryos were embedded in methylcellulose to prevent movements.

RNA extraction and first-strand cDNA synthesis

RNA was extracted from whole embryos at different stages with the RNasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer’s instructions. RNA yield and quality were assessed with the Nanodrop system (Thermo Scientific). cDNAs were synthesized from 500 ng of RNA with oligo dT primers using the First-Strand Synthesis System for RT-PCR (Invitrogen), following the manufacturer’s instructions.

Northern blot

Northern blot analysis was carried out as previously described (70). Briefly, 8 µg of total RNA were separated on 1.3% agarose/ MOPS-gels containing 0.66 M formaldehyde and transferred overnight onto Hybond-N+ membrane (Amersham Pharmacia Biotech) by capillarity with 10× SSC. Blots were hybridized with random-primed 32P-labeled probes (from PCR amplicons; about 300 nucleotide-long) in a hybridization buffer (2% SDS, 10% dextran sulphate, 1× SSPE, 100 µg/ml salmon sperm DNA, 2% Denhardt’s) at 68°C overnight. Signals were analysed on a phospho-imager (Molecular Imager FX, Bio-Rad). Probe sequences are available upon request.

RT-quantitative PCR

RT-qPCR was performed on a Light Cycler 480 System (Roche) using the LightCycler480 SYBR Green I Master mix (Roche) with specific primers for col6a1-a4b. The programme included an initial denaturation step of 8 min at 95°C, followed by 50 amplification cycles of 10 sec denaturation at 95°C for, 15 sec hybridization at 58°C and 15 sec elongation at 72°C. Data were normalized to the expression levels of the elongation factor alpha 1 (elfa), a housekeeping gene.

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gene whose expression is acceptably stable throughout zebrafish development (71). Primers sequences are available upon request.

Birefringence

Muscle birefringence was analysed by placing living 48 hpf embryos in 3% methylcellulose under an Olympus BX41 microscope with a DMK 31AU03 camera using IC Capture software (both The Imaging Source).

Escape test

48 hpf embryos were subjected to a tactile stimulus: using a clamp, a gentle stimulus was applied at the extremity of the larval tail and its reaction was recorded with an Olympus S2X9 microscope with a DMK 31AU03 camera using the IC Capture software (both from The Imaging Source).

Zebrafish α2(VI) antibody synthesis

We obtained a polyclonal antibody against the zebrafish α2 chain from Interchim/Abgent. The C-terminal sequence RILTAQGDD- DERNAR was identified as the most immunogenic and specific peptide. It was injected (5-8 times) in rabbits and the serum obtained and purified with affinity or protein A purification. Competition experiments on western blotting were performed to verify the specificity of the antibody. Briefly, purified polyclonal rabbit anti-zebrafish COLVI antibody (diluted 1:250 in blocking solution) was incubated with 500 µg (250-fold more) of the purified immunogenic peptide overnight at 4°C. Subsequently, western blotting was performed and the signal was efficiently extinguished.

Whole-mount immunocytochemistry

Embryos were dechorionated at 48 hpf, euthanized with benzocaine and either directly stocked in RNA later or fixed in 4% paraformaldehyde, dehydrated and stored in 100% MetOH. Immunostaining was carried out on whole embryos with the following antibodies: anti slow myosin (F59; DSHB; 1:20), anti-dystrophin (MANDRA1; Sigma; 1:1000), anti-COLXXII (a gift from F. Ruggiero, Lyon, France; 1:250), anti-slow myosin (F59; DSHB; 1:20), anti-dystrophin (MANDRA1; Sigma; 1:1000), anti-COLXXII (a gift from F. Ruggiero, Lyon, France; 1:250), anti-apoptotic vesicle protein 2 (SV2, DSHB, 1:100). For apoptosis assessment, anti-cleaved caspase3 (559565, BD Pharmingen; 1:100) was used. Alexa Fluor secondary antibodies (goat anti-mouse A568, goat anti-rabbit A488; Jackson ImmunoResearch) were incubated at 1:1000 for 1 h at room temperature. Embryos were rinsed and mounted in 100% glycerol. Images were obtained under an Axiophot microscope (Zeiss) and images were captured using the Metaview software (Ropper Scientific) or on a confocal microscope (Olympus FV-1000).

Western blot

Total proteins from 72 hpf embryos were extracted using extraction buffer (0.2 M NaCl, 0.1 M Glycine, 0.1% Triton, 50 µm DTT, 10 mM EDTA, 20 mM Tris pH 7.5, 2% protease inhibitors and 2% phosphatase inhibitors). Fifty µg of these proteins were separated using 10% or 8–16% gradient gels for 4–5 h at 100 V at room temperature. They were then transferred on PVDF 0.45 µm membrane overnight, at 600 mA and 4°C. Membranes were blocked in 5% skim milk/PBS for 1 h and then hybridized overnight at 4°C with primary antibodies against LC3, Akt, P-AktSer (Cell Signalling) and α2(VI). Then, they were washed 3 x 15 min with 1% skim milk/PBS-Tween and hybridized with appropriate HRP-conjugated secondary antibodies (Jackson ImmunoResearch) for 1 h at room temperature. Membranes were washed again and revealed using the Immobilon Western chemiluminescent HRP substrate (Millipore) on the chemi-imager G-box (Syngene).

Electron microscopy

Embryos were anaesthetized with benzocaine, immediately immersed in 2.5% glutaraldehyde in PBS during 2 h, rinsed three times in PBS and finally stored in PBS at 4°C. After a 2% OsO₄ 40 min fixation, they were gradually dehydrated in acetone including a 1% uranyl staining step in 70% acetone, and finally flat embedded in Epon resin (EMS, Fort Washington, PA, USA). Then 500 nm parasagittal semi-thin sections were stained with 1% toluidine blue. After uranyl and lead citrate staining, parasagittal 70 nm ultrathin sections were examined with a Philips CM120 electron microscope (Philips, Eindhoven, The Netherlands) operated at 80 kV and photographed with a SIS Morada digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Statistical analysis

Data are means ± SD. ANOVA tests were performed to determine significant differences between groups. When normality or equal variance test failed, a Kruskal–Wallis one-way analysis was used. All tests were performed using SigmaStat (Systat, Erkath, Germany).

Accession numbers

Accession numbers of sequences used in this study were as follows: Mouse (Mus musculus) Col6a1 NP_340663.1, Col6a2 NP_666119.1, Col6a3 NP_001229937.1, Col6a4 NP_081039.2, Col6a5 NP_001161395.1, Col6a6 NP_001096077.1; Human (Homo sapiens) COL6A1 NP_001839.2, COL6A2 NP_001840.3, COL6A3 NP_004360.2, COL6A5 NP_101265227.1, COL6A6 NP_001096078.1; Zebrafish (Danio rerio) col6a1 XP_009304444.1, col6a2 XP_066164.2, col6a3 XP_009302963.1, col6a4 XP_692457.6, col28a1 NP_001153314.1; Rat (Rattus norvegicus) Col6a4 NP_001258111.1, Col6a5 XP_087561211.1, Col6a6 NP_008764827.1; Marmoset (Callithrix jaccus), Col6a4 XP_002759734.1, Col6a5 XP_00892098.1, Col6a6 XP_002759732.2; Frog ( Xenopus (Silurana) tropicalis) col6a1 XP_00292077.2, col6a2 NP_001120436.1, col6a3 XP_00292067.2, col6a4_1 XP_002937788.2, col6a2_2 XP_002942405.2; Chicken (Gallus gallus) Col6a1 NP_990438.1, Col6a2 NP_990679.1, Col6a3 NP_990865.1, Col64 XP_426088.4; Medaka (Oryzias latipes) col6a1 XP_004791441.2, col6a2 XP_00479072.1, col6a2b XP_00481925.1, col6a3 XP_00478095.1; African coelacanth (Latimeria chalumnae) col6a1 XP_005997002.1, col6a2 XP_006014655.1, col6a3 XP_005997469.1, col6a4 XP_006004266.1, col6a5 XP_006010064.1; Elephant shark (Callorhinchus milii) col6a1 XP_007898245.1, col6a2 XP_007896379.1, col6a3 XP_007904071.1, col6a4 XP_007878867.1, col6a5 XP_007878879.1; half-tongue sole (Cynoscion nebulosus) col6a1 XP_008332498.1, col6a2 XP_008326809.1, col6a3 XP_008327160.1, col6a4 XP_008321223.1; dog (Canis lupus familiaris) col6a1 XP_003494049.2, col6a2 XP_005639071.1, col6a3 NP_001096685.1, col6a4 XP_853242.2, col6a5 XP_853265.3, col6a6 XP_853279.3; northern pike (Esox lucius) col6a1 XP_010863420.1, col6a2 XP_010865210.1, col6a3 XP_010865851.1, col6a4 XP_010905337.1.

Author Contributions

L.R., X.C. and V.A. conceived and designed the experiments. L.R., C.R., J.L., A.K., L.J., C.G. and M.F. performed experiments. L.R., C.R., J.L., A.K., X.C. and V.A. analysed the data. L.R., J.L., X.C. and V.A. wrote the paper. L.L. was responsible for fish maintenance. E.K. and S.C. provided zebrafish embryos and access to the ICM facility.
All authors have read and approved submission of this work.

Supplementary Material
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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