Matrilin-1 is Essential for Zebrafish Development by Facilitating Collagen II Secretion*

Received for publication, October 24, 2013, and in revised form, November 22, 2013 Published, JBC Papers in Press, November 29, 2013, DOI 10.1074/jbc.M113.529933

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Background: Matrilin-1 is an abundant cartilage extracellular matrix protein.

Results: Morpholino knockdown of matrilin-1 in zebrafish results in growth defects, disturbed craniofacial cartilage formation, and decreased collagen II deposition.

Conclusion: Matrilin-1 is indispensable for zebrafish development, presumably by facilitating secretion of collagen II.

Significance: These results challenge the concept that matrilins only function as extracellular adaptor proteins.

Matrilin-1 is the prototypical member of the matrilin protein family and is highly expressed in cartilage. However, gene targeting of matrilin-1 in mouse did not lead to pronounced phenotypes. Here we used the zebrafish as an alternative model to study matrilin function in vitro. Matrilin-1 displays a multiphasic expression during zebrafish development. In an early phase, with peak expression at about 15 h post-fertilization, matrilin-1 is present throughout the zebrafish embryo with exception of the notochord. Later, when the skeleton develops, matrilin-1 is expressed mainly in cartilage. Morpholino knockdown of matrilin-1 resulted in both overall growth defects and in disturbances in the formation of the craniofacial cartilage, most prominently loss of collagen II deposition. In fish with mild phenotypes, certain cartilage extracellular matrix components were present, but the tissue did not show features characteristic for cartilage. The cells showed endoplasmic reticulum aberrations but no activation of XBP-1, a marker for endoplasmic reticulum stress. In severe phenotypes nearly all chondrocytes died. During the early expression phase the matrilin-1 knockdown had no effects on cell morphology, but increased cell death was observed. In addition, the broad deposition of collagen II was largely abolished. Interestingly, the early phenotype could be rescued by the co-injection of mRNA coding for the von Willebrand factor C domain of collagen IIα1α, indicating that the functional loss of this domain occurs as a consequence of matrilin-1 deficiency. The results show that matrilin-1 is indispensable for zebrafish cartilage formation and plays a role in the early collagen II-dependent developmental events.

The matrilins are a family of oligomeric adaptor proteins that are expressed in cartilage and many other types of extracellular matrix. They participate in the formation of fibrillar or filamentous structures and mediate interactions between collagen fibrils and other matrix constituents (1, 2). This adaptor function may be modulated by physiological proteolysis that causes the loss of single subunits and thereby a decrease in binding avidity (3). Although the matrilins have been extensively studied, their exact in vivo functions are not known.

Initial attempts to study matrilin function by gene inactivation in mouse were inconclusive as mice deficient for matrilin-1 (4), matrilin-2 (5), or matrilin-3 (6) did not show any obvious phenotype, whereas an independently generated matrilin-1 null mouse line showed ultrastructural abnormalities of cartilage collagen fibrils (7) and a second matrilin-3-deficient line appeared to have a premature maturation of growth plate chondrocytes, increased bone mineral density, and a predisposition for osteoarthritis (8). Later it was shown that peripheral nerve regeneration is disturbed in matrilin-2 deficient mice, pointing to a role for matrilin-2 in axonal growth (9). Even mice double deficient for matrilin-1 and matrilin-3, which are both highly expressed in cartilage, did not show any overt phenotype. However, in depth analysis of the cartilage matrix of such mice revealed moderately increased collagen fibril diameters and an increased collagen volume density, which could, although less marked, be detected also in the corresponding single mutant mice (10). Taken together the results demonstrate that matrilin-1 and matrilin-3 modulate cartilage collagen fibrillogenesis and that functional compensation between the two proteins may occur. As matrilins are well conserved in evolution (11), we decided to study matrilin function in a less complex animal model, the zebrafish (Danio rerio). Matrilins are present in all vertebrates studied, including the zebrafish, where the temporal and spatial expression has been partially explored (12). Furthermore, many interaction partners of matrilins, e.g. collagen II, collagen VI, collagen IX, or decorin, have been characterized in zebrafish (13–18). In contrast to mammals, no orthologue of matrilin-2 is present (11, 12). How-
**Zebrafish Matrilin-1**

**TABLE 1**

| Name              | Oligonucleotide primers and morpholinos                                                                 |
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Alexa 488 anti-guinea pig, Alexa 546 anti-guinea pig (Invitrogen), anti-digoxigenin AP (Roche Applied Science), biotin-IgG anti-rabbit, donkey anti-guinea pig HRP (Jackson ImmunoResearch), IRDye 700 DX anti-rabbit, IRDye 800 CW anti-guinea pig (Rockland), rabbit anti-guinea pig HRP (Sigma), and swine anti-rabbit HRP (Dako Cytomation).

**Generation of Polyclonal Antibodies against a Fragment of Zebrafish Matrilin-1**—A twin-strep tagged fragment containing the EGF and the second VWA domain of zebrafish matrilin-1 was recombinantly expressed (primers listed in Table 1) in EBNA-HEK-293 cells and purified using a Strep-Tactin® column (IBA). Recombinant protein was used for immunization of rabbits and guinea pigs (Pineda Antikörper Service, Berlin). The immune serum obtained was depleted of cross-reactivity against zebrafish matrilin-1 VWA 1, matrilin-3a VWA, matrilin-3b VWA, and matrilin-4 VWA 1 domains (12) by cycling the serum over columns coupled with the recombinant protein for 2 days. The depleted serum underwent final affinity purification using a column coupled with the antigen used for immunization and elution of the bound antibody with 0.1 M glycine, pH 2.5.

**Photography and Whole Mount Staining**—Zebrafish larvae were anesthetized with a 1:100 dilution of a 4 mg/ml Tricaine® (Sigma) stock solution prior to photography of living zebrafish. After sacrifice but before fixation, all zebrafish embryos older than 24 hpf were bleached using 1.5% H2O2, 0.5% KOH for 20 min. After sacrifice but before fixation, all zebrafish embryos older than 24 hpf were bleached using 1.5% H2O2, 0.5% KOH for 20 min at room temperature. Where appropriate, embryos were manually dechorionated. Then embryos and larvae were fixed using 4% paraformaldehyde for 2 to 4 h at room temperature.

For whole-mount Alcian blue staining, the embryos were washed in 50% ethanol and stained with Alcian blue solution overnight (22). The stained embryos were then bleached as described above and cleared for 2 days in 0.25% KOH, 20% glycerol at room temperature. The samples were photographed using a Nikon SMZ 1500/Nikon DS-Fi1 setup.

For whole-mount immunohistochemical staining, fixed embryos were incubated in methanol overnight at −20 °C. The embryos were sequentially rehydrated (5 min 100, 100, 96, 90, 70, and 50% (v/v) EtOH in PBS) and subsequently blocked with 5% (w/v) normal goat serum in PBS for 2 h. The affinity purified antibodies were applied in 5% (w/v) normal goat serum in PBS for 1 h or overnight at room temperature. The primary antibodies were visualized by consecutive application of Alexa 488/546-conjugated secondary antibody (Sigma, diluted 1:500) and detected using polyclonal antibodies diluted in TBS. Bands were visualized using a Zeiss EM109 (80 kV, 500-m bar thickness), magnification was calibrated with a cross-grating replica (2160 lines/mm, d = 0.463 μm).

**Western Blot Analysis**—Western blot analysis was performed using a Laemmli-based discontinuous buffer system (23). Protein samples were obtained by sequential extraction as described (10). 1 volume of 6× SDS sample buffer (2% (w/v) SDS, 10% (v/v) glycerol, 0.04% (w/v) bromphenol blue, 80 mM Tris-HCl, pH 6.8) was added to the protein extract and the sample subjected to 4–12% (w/v) nonreducing SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and detected using polyclonal antibodies diluted in TBS. Bands were detected by chemiluminescence immunooassay using a
peroxidase-conjugated swine anti-rabbit secondary antibody (Dako).

**Whole Mount in Situ Hybridization**—Fragments of matrilin-1 cDNA (bp 475–1370, GenBank accession No. NM_001099740) were subcloned into pBluescript II SK, which contained T7 and T3 promoters and were used as probes for *in situ* hybridization. The plasmid DNA was linearized with SpeI (T7) or KpnI (T3), transcribed *in vitro* by T7 and T3 RNA polymerases (Roche Applied Science) and labeled with digoxigenin (Roche Applied Science). *In situ* hybridization was carried out according to Ref. 24. Wild type embryos were collected between 24 hpf and 5 days post-fertilization. After removal of the chorion, embryos were fixed in PBS with 4% paraformaldehyde. The hybridization was performed with 30–50 ng of anti-sense digoxigenin-labeled RNA probe in hybridization buffer (50% deionized formamide, 5 × SSC, 0.1% Tween 20, 50 μg/ml of heparin, 500 μg/ml of RNase-free tRNA adjusted to pH 6.0 with citric acid) at 70 °C overnight. Hybridization was detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:10,000) followed by incubation with BM-Purple (NBT/BZIP, Roche Applied Science) for 3–8 h. The embryos were finally mounted in 100% glycerol and observed on a Nikon SMZ1500/Nikon DS-Fi1 setup.

**RESULTS**

**Matrilin-1 Expression in Early Zebrafish Development**—First, we extended the previous analysis of zebrafish matrilin-1 expression (12). To determine the temporal expression during development, mRNA was collected at time points between 3 and 120 hpf and quantitative real-time PCR was performed. Thereby, a distinct early expression of matrilin-1 with its maximum at about 15 hpf was detected (Fig. 1A), however, 1000-fold weaker than later when the skeleton is formed. The second phase of expression starts at about 64 hpf, when the development of the craniofacial cartilage begins, and reaches a first maximum at about 88 hpf, and a second at about 112 hpf, the time the ossification of the spinal column begins (Fig. 1B). Proteins were sequentially extracted from mechanically disrupted whole zebrafish with TBS, high salt solution with 10 mM EDTA, and 4 M guanidinium HCl at 24, 48, and 72 hpf. Western blot analysis after nonreducing SDS-PAGE indicated that matrilin-1 is most strongly present at 72 hpf (Fig. 1C), but matrilin-1 protein could also be detected at 24 hpf. However, whereas at 24 hpf matrilin-1 could only be extracted using 4 M guanidinium HCl, the main fraction was at 72 hpf extracted already using high salt buffer. Moreover, non-reduced matrilin-1 extracted at 24 hpf yields a double band at about 55 kDa, the size of matrilin-1 subunits, whereas matrilin-1 extracted at 72 hpf migrates at about 250 kDa, indicating that in zebrafish matrilin-1 also forms oligomers.

In 4 and 5 dpf and 4-month-old zebrafish, matrilin-1 is mainly expressed in the skeleton (12). We now used whole mount *in situ* hybridization and immunohistochemical staining on whole fish and on sections to determine the sites of early expression (Fig. 2). At 18 hpf, matrilin-1 mRNA was detected throughout the embryo, with a higher expression in the head and the emerging tail (Fig. 2A, left panel). At 24 hpf, expression was mainly in the head and posterior part, dorsally and ventrally of the notochord, with a more intense signal in the distal tail (Fig. 2A, right panel).

Whole mount immunohistochemistry on 24-hpf-old embryos (Fig. 2B) gave a diffuse signal throughout the body, which was in contrast to the mRNA distribution, weaker in the head. Immunostaining on sections of 24-hpf zebrafish revealed matrilin-1 throughout the trunk of the embryo, with a greater deposition...
in the skin (Fig. 2C). At 72 hpf, matrilin-1 only remains present in the craniofacial cartilage (Fig. 2D and E).

**Gross Morphology of Matrilin-1 Knockdown Zebrafish** — The morpholino knockdown of matrilin-1 in zebrafish led to a broad spectrum of phenotypes, ranging from mild to severe (Fig. 3). At 24 hpf, just after the early expression phase, all morphants were smaller and the head was malformed. The yolk sac extension was shorter, the trunks of mildly affected fish (Fig. 3A, 24 hpf, upper “as”) failed to straighten entirely, and the tail was misshapen and curly. At 48 hpf the morphants retained all gross phenotypic hallmarks present at 24 hpf, but also showed pigment defects in the skin as well as their sharp, regular appearance. The areas distal and proximal to the most severe defects were less affected. C, photographs of living 18-hpf-old zebrafish embryos after neural crest cell migration has ceased. GFP expressing neural crest cells were studied in untreated (u) and matrilin-1 knockdown (as) transgenic Sox10:egfp embryos at the 13 somites stage. D, whole mount Alcian blue glycosaminoglycan staining. Only parts of the neurocranium (ethmoid plate, parasymphysis, and Meckel cartilage) were weakly stained.

At 72 hpf, the trunk above the yolk sac was less affected than the rest of the hind body axis, pointing to either a delay in trunk formation or a partial amelioration of the phenotype (Fig. 3A, 72 hpf). Closer examination showed a loss of the floor plate and notochord integrity. The somite borders lost their chevron shape as well as their sharp, regular appearance (Fig. 3B).

The obvious difference between the matrilin-1 knockout phenotypes in mouse (4, 7) and the matrilin-1 knockdown phe-

notypes in zebrafish necessitated extensive control experiments to exclude off-target effects of the antisense morpholinos. Two different translation blocking, one splice site blocking morpholino and 5-mismatch control morpholinos were used. The efficacy of the knockdown was assessed by Western blot...
Zebrafish Matrilin-1

**FIGURE 4.** A, immunofluorescence staining for matrilin-1 and matrilin-3a in matrilin-1 morphants. A paraffin-embedded section of the head of a matrilin-1 morphant at 72 hpf was stained with affinity-purified antibodies directed against zebrafish matrilin-1 and -3a followed by incubation with Alexa 546 (matrilin-1, green) and Alexa 488 (matrilin-3a, red) coupled secondary antibodies. Whereas matrilin-3a was still detected in cartilage remnants (arrows) a signal for matrilin-1 is lacking. p, parachordal cartilage; n, notochord. B, Western blot analysis of extracts from heads of untreated (u) and morphant (as) embryos at 72 hpf. The arrow indicates the matrilin-1 signal. Ponceau staining is shown to demonstrate equal protein load. C, morpholino knockdown statistics. Fertilized zebrafish eggs were injected with different amounts of matrilin-1 splice site morpholinos and matrilin-1 5-mismatch morpholinos as indicated. Rescue experiments were performed by co-injection of 3 pg of matrilin-1 mRNA into fertilized zebrafish eggs injected with 10 ng of antisense splice site morpholino (as). In the control, only the carrier solution (water and phenol red) was injected. u, untreated; as, antisense morpholino; mis, mismatch morpholino; res, matrilin-1 mRNA rescue. The numbers indicate the amount of morpholino injected. n ≥ 60.

Analysis of tissue extracts of untreated larvae and morphants as well as by immunohistochemical stainings on sections. In both cases, almost no matrilin-1 could be detected in morphants (Fig. 4, A and B). Moreover, mRNA rescue experiments and p53 morpholino co-injections, used to rescue unspecific toxic effects of morpholinos (25), were performed. All matrilin-1 morpholinos gave similar phenotypical changes. All figures show morphants that were injected with the splice site morpholino. Co-injection of zebrafish matrilin-1 mRNA could rescue the phenotype (Figs. 3A and 4C). Interestingly, p53 morpholino co-injections caused an even more pronounced phenotype, including severe growth retardation, but the pigmentation pattern was normal (Fig. 3A). Increased amounts of antisense morpholinos led to a dose-dependent increase in both the proportion of phenotypic larvae and phenotype severity. Control experiments using 5-mismatch morpholinos (Fig. 4C) also gave a less pronounced but dose-dependent increase in phenotype frequency, indicating that the introduction of 5 mismatch bases reduces, but does not eliminate, the binding of the morpholino to the target mRNA. Co-injection of 3 pg of full-length matrilin-1 mRNA with 10 ng of antisense splice site morpholino rescued the phenotype and reduced the phenotype frequency by about 60% (Fig. 4C).

**Analysis of the “Late Phenotype”—**Matrilin-1 is strongly expressed in the developing skeleton of the zebrafish (12). However, neural crest cell migration, which is important for craniofacial skeletal development (26), is not altered in matrilin-1 morphants (Fig. 3C). Furthermore, whole mount Alcian blue staining was performed at 72 hpf to investigate the development of the craniofacial cartilage (Fig. 3D). Matrilin-1 knockdowns showed an increased loss of glycosaminoglycan staining in the craniofacial cartilage according to the severity of the phenotype. In milder phenotypes, glycosaminoglycan staining in the ceratobranchial and basibranchial cartilage was lost. The parachordal, palatoquadrate, trabecular, and Meckel cartilage as well as the ethmoid plate showed a decreased or, in case of the palatoquadrate, only a partial staining of the distal part compared with the untreated larvae (Fig. 3D, upper right panel). Severe phenotypes lost nearly all glycosaminoglycan staining. Only a weak and disrupted staining in the neurocranium (ethmoid plate, parachordal, and trabecular cartilage) could be detected (Fig. 3D, lower right panel). A closer inspection of sections from morphants at the area where the trabecular cartilage should be located revealed that a connective tissue was present that did not show any cartilaginous features, such as the typical stacked orientation of the cells and Alcian blue positive extra-cellular matrix deposition (Fig. 3E), although chondroitinase ABC generated chondroitin sulfate and/or dermatan sulfate fragments could still be detected in immunofluorescence stainings with the C-4-S antibody, albeit with a broader tissue distribution pattern (Fig. 5A). Moreover, this tissue did not contain collagen II, which is the main collagen in cartilage (Fig. 5A, lower panel, encircled). Expression of collagen IX, which is present on the surface of collagen II fibrils (27, 28) was also lost (not shown). In contrast, deposition of matrilin-3a, another member of the matrilin family expressed in cartilage (6) was nearly unaffected by the knockdown of matrilin-1 (Fig. 5A). Interestingly, co-immunostaining with antibodies directed against collagen II and matrilin-1 did not only show complete co-localization of the two proteins in the cartilaginous extracellular matrix, but also inside the chondrocytes (Fig. 5B), indicating that the binding of matrilin-1 to collagen II (29) can occur already in the secretory pathway.

To investigate changes in matrilin-1 morphants at the ultrastructural level, transmission electron microscopy (TEM) was applied. At 72 hpf striking differences between wild type and morphant zebrafish ethmoid plate chondrocytes were observed. Morphant chondrocytes displayed an increased disruption of the endoplasmic reticulum (ER) and more dead chondrocytes with increasing phenotype severity (Fig. 6). The central chondrocytes of
untreated zebrafish larvae ethmoid plate showed the typical stacked arrangement (coin roll) and were of similar size and the ER displayed concentrically arranged, highly parallel “sheets” (Fig. 6, A and B). In contrast, chondrocytes from the weakest morphant phenotype, whereas still arranged in columns, lost their stacked orientation and displayed a variation in cell size. The parallel sheets of the ER were dilated, appeared undulated, and contained more material, leading to an increased thickness of the sheets (Fig. 6, C and D). More severe matrilin-1 morphant phenotypes had a reduced number of chondrocytes that were roundish and swollen. A significant portion of the intracellular space was occupied by distended ER cisternae. The ER had completely lost its parallel organization, resulting in a chaotic meshwork of membranes. (Fig. 6, E and F). In the most severe morphants, the structure of the ethmoid plate could not be distinguished. Single surviving cells were surrounded by patches of extracellular matrix and cellular debris, presumably originating from other dying chondrocytes. The intracellular space was completely filled with distended ER cisternae and ER sheets were no longer visible (Fig. 6, G and H). Occasionally,
exposed nuclei could be found, surrounded by cell debris and extracellular matrix (Fig. 6, I and J), pointing to a regulated cell death rather than necrosis (30–32). However, the cellular and subcellular morphology of non-cartilaginous cells was unaffected even in the most severe morphant phenotypes (Fig. 6, K and L).

As TEM analysis showed a significant change in the structure of the ER of morphant chondrocytes (Fig. 6), the importance of cellular stress responses in the establishment of the phenotype was investigated. Indeed, active p53 was detected in morphants but not in wild type fish at 68 hpf (Fig. 7 A). However, at 72 hpf the alternatively spliced transcription factor XBP-1, a marker for unfolded protein response and ER stress, was not present (Fig. 7 B), indicating that unfolded protein response and ER stress do not play a central role in the development of the late phenotype. Nevertheless, the distended ER cisternae point to a problem in protein turnover. We therefore applied the proteasome inhibitor bortezomib already at the time of morpholino injection into the embryo (Fig. 8, A and D) and also at 48 hpf to selected matrilin-1 morphants that did not exhibit gross morphological alterations at that time (Fig. 8 B). Indeed, the gated bortezomib treatment of morphants increased the frequency of craniofacial cartilage defects 10-fold, whereas the cartilage of untreated morphants was unaffected, indicating that the defects in the craniofacial cartilage were not induced by morphological changes resulting from matrilin-1 knockdown during the early expression phase (Fig. 8 C). These results clearly indicate impairment of protein degradation via the proteasome.

Analysis of the Early Phenotype—TEM was also used to investigate the early phenotype. At 24 hpf the morphants did not show any significant differences to untreated zebrafish (Fig. 9, A–C), neither in the notochord, the only primordial skeletal element that is present at this stage (Fig. 9 D), nor in the developing spinal cord (Fig. 9 F). However, in matrilin-1 knockdowns (Fig. 9 E, asterisk) the epidermis in some cases showed slight detachments from the underlying tissue, but no cells displayed...
the profound ER aberrations seen in chondrocytes in the late phenotype.

We further investigated whether cell death during early development plays a role in the establishment of the phenotype. TUNEL detected an 7-fold increase in cell death in matrilin-1 knockdown embryos compared with the untreated and 5 mismatch-treated 18-hpf-old zebrafish (Fig. 10B). The highest frequency of DNA fragmentation events was found in and around the eye and in the developing spinal cord (Fig. 10A). Co-injection of the cell-permeant pan-caspase inhibitor Z-VAD-FMK resulted in a reduction of DNA fragmentation events to that of untreated levels (Fig. 10B). However, the reduction of apoptosis did not change the phenotype distribution in Z-VAD-FMK-treated morphants at 24 hpf (Fig. 10C).

Similar to the late phenotype, application of bortezomib already to the fertilized morphant eggs indicated a strong participation of the proteasome in the development of the early phenotype. Under the influence of bortezomib, the relative phenotype rate increased from 75 to 100% at 24 hpf (Fig. 10C). Active p53 was also detected in morphants at 15 hpf but not in wild type fish (Fig. 7A). Although the early phenotype displayed clear signs of a proteasome contribution, the underlying mechanism remained largely obscure.

As the analysis of the late phenotype pointed to a role of matrilin-1 in the secretion of collagen II, we asked which could be the consequences of the lack of collagen II at this early developmental point. It has been shown that the loss of an embryonic splice variant of the collagen IIα1 chain containing a VWC domain in mouse (33) leads to phenotypes similar to those observed in the zebrafish matrilin-1 knockdowns. Therefore we hypothesized that the early phenotype of the matrilin-1 morphants is caused by a disturbed secretion of VWC domain containing collagen II. Indeed, by RT-PCR we detected the col2a1a splice variant containing the VWC domain also in zebrafish and exclusively during early development. In addition, we could show that knockdown of matrilin-1 does not alter collagen II transcription at either of the investigated stages (Fig. 11A). We were also able to show that the knockdown of matrilin-1 resulted in a severe reduction of collagen II deposition at 24 hpf (Fig. 11B). To demonstrate that the loss of the collagen II VWC domain is important for the development of the early phenotype, we performed rescue experiments by co-injecting different amounts of mRNA coding only for the VWC domain of collagen IIα1a in matrilin-1 morphants. Indeed, after injection of even 2 pg of mRNA per fertilized egg the frequency of the early phenotype was markedly diminished (Fig. 12A) to the point where treated and untreated pools could barely be distinguished (Fig. 12B). Although co-injection of mRNA coding for the collagen IIα1a VWC domain was able to rescue the morphological phenotype, it did not restore the fibrillar collagen deposition in the embryo (Fig. 11B).

**DISCUSSION**

As in mammals, zebrafish matrilin-1 is strongly expressed in cartilaginous tissues (12). We therefore used morpholino knockdowns of matrilin-1 in zebrafish to study matrilin-1 function in skeletal development as an alternative to the matrilin-1 knockout mouse lines that did not show any overt phenotype. Matrilin-1 knockdown zebrafish, in addition to a late skeletal phenotype, showed a severe phenotype already during the first 24 hpf. This was surprising, as in our earlier work matrilin-1 expression was not detected at that time point (12) and cartilage is not formed so early in development. We therefore re-examined matrilin-1 expression by quantitative PCR and detected a weak but distinct early peak of expression at around 15 hpf. The analysis of the spatial distribution of matrilin-1 mRNA and protein during the first 24 hpf showed a diffuse mRNA expression in the whole embryo and increased protein deposition in the developing anterior and posterior body axis, as well as in the skin (Fig. 2, B and C).

The analysis of the morphants clearly indicates that matrilin-1 is indispensable for normal morphogenesis of zebrafish larvae and both mild and severe phenotypes could be observed within the first 24 hpf (Fig. 3). The enhancement of the early phenotype by p53 co-knockdown clearly indicates that the phenotype of the matrilin-1 morphant is not due to off-target effects that would attenuate upon loss of p53 (25).

The early phenotype is characterized by a general delay in development with a prominent malformation of the head. In the late phenotype, proper cartilage formation is abolished. Instead, at places where cartilage would develop in wild type fish, a mesenchymal tissue is present that lacks the typical morphology of cartilage. Indeed, the deposition of the major cartilage component collagen II is lost. The other major component is the proteoglycan aggrecan. Due to the lack of cross-reacting antibodies directed against epitopes in the aggrecan core protein, we could only determine the presence of glycosaminoglycan chains using Alcian blue staining or an antibody directed against a disaccharide neoepitope generated by chondroitinase ABC digestion.

Interestingly, the neoepitope C-4-S antibody was still able to detect chondroitinase-generated disaccharide stubs in the tissue remnants occurring where a cartilage developed in control. Some chondroitin/dermatan sulfate is apparently still present in this tissue, even though immunofluorescence microscopy does not allow quantification. This finding is in apparent conflict with the loss of Alcian blue staining in these tissues (Fig. 3, D and E). Probably, either glycosaminoglycan...
elongation or sulfation is affected by the ER disruptions occurring upon matrilin-1 knockdown (Fig. 6) leading to decreased Alcian blue staining despite the presence of some chondroitin/dermatan sulfate chains. These results could implicate that the matrilin-1/collagen II secretion pathway may differ from that of aggrecan.

In general, the craniofacial cartilage is more strongly affected than the cartilage of the neurocranium, possibly a consequence of the order in which they develop (34, 35). Interestingly, collagen IX, which is present on the surface of collagen II fibrils (27, 28), is also absent. In contrast, matrilin-3a, another member of the matrilin family, could be detected and was used as a positive control to locate cartilage tissue remnants (Fig. 5A).

Although there are earlier studies on zebrafish collagen II (13–15), the cartilage proteoglycan aggrecan has to our knowledge not been studied at all in this species. In the database we found two aggrecan genes (acana and acanb). We were able to amplify transcripts of acana and acanb at 72 hpf. Interestingly, acana is still transcribed in matrilin-1 morphants, but acanb transcription is markedly reduced compared with untreated larvae (not shown). The reason for this is unclear. However, it was shown that the inhibition of chondroitin sulfate and hepato-
ran sulfate biosynthesis in zebrafish results in impaired cartilage formation, but the overall cellular phenotype was different from that observed by us (36). In contrast to our results, the extracellular matrix displayed an increased electron density in TEM and a more frequent cell death was not observed. The Wnt pathway may be involved in the phenotypic changes, as it has been reported that both chondroitin and heparan sulfate can influence Wnt signaling (37, 38). Deposition and integrity of the collagen network was not analyzed in these studies.

The loss of collagen II deposition in cartilage raised the question if the deposition is affected also at earlier time points, thereby contributing to the early phenotype. In zebrafish two collagen II genes, col2a1a and col2a1b, are present. The partially overlapping expression of the two collagen II isoforms starts at the 3-somite stage (15). The knockdown of collagen IIa1 in zebrafish (39) yielded phenotypes similar to the mild ones in matrilin-1 morphants.

In human and mouse, an alternatively spliced transcript of the collagen II gene, COL2A1A, carrying a VWC domain, is preferentially expressed in non-chondrogenic embryonic tissues, suggesting an isoform-specific function during embryogenesis (40, 41). We could show that this expression is conserved in zebrafish (Fig. 11A). Based on the similarity of the collagen II VWC domain to those of chordin or noggin, it was suggested that procollagen IIa may act as an antagonist of BMP signaling during embryogenesis. It was indeed shown that the N-terminal prodomain of COL2A1A, containing the VWC domain, can bind to TGFβ1 and BMP-2 (42). In the absence of this specific splice variant, the mutant mice displayed an inactivation of the sonic hedgehog signaling pathway and thus, similar to the early zebrafish matrilin-1 knockdown phenotype, a partially penetrant phenotype consisting of loss of head tissues, holoprosencephaly, and loss of mid-facial structures (33). Indeed, co-injection of mRNA coding for the zebrafish collagen IIa1a VWC domain rescued the early phenotype in matrilin-1 morphants in a dose-dependent manner. This showed that the lack of the VWC domain plays a crucial role in the early matrilin-1 morphant phenotype.

But which is the mechanism that leads to the loss of collagen II deposition in matrilin-1 knockdown zebrafish? A striking observation was that at 72 hpf dilated ER and distended ER cisternae could be detected by TEM. Such morphological changes have been described as “chondroptosis,” which is believed to be a variant of programmed cell death (apoptosis) in chondrocytes (44). The ER phenotype in the matrilin-1 mor-
phants is, however, different from that in the human chondrodysplasias PSACH and MED, where misfolded COMP or matrilin-3 accumulate in the ER causing an unfolded protein response and ER stress (45, 46). Interestingly, the secretion of some extracellular matrix proteins (fibromodulin, decorin, and collagens VI, XI, and XII) was affected in a PSACH patient carrying a mutation in COMP, but not that of large fibrillar collagens or aggrecan, indicating the existence of distinct secretory pathways or ER-sorting mechanisms for matrix molecules (47). Collagen II deposition is also affected in site-1 protease knock-out mice (48, 49).

Recently, a mechanism for the secretion of large extracellular matrix molecules, like fibrillar collagens, in a subset of COP II-coated vesicles was suggested (50, 51). Interestingly, collagen II secretion in zebrafish was shown to be dependent on COP II proteins sec23a, sec24d, and sec13-sec31 (52–54). The corresponding zebrafish mutants bulldog, crusher, and feelgood, show severe craniofacial defects similar to those in matrilin-1 morphants (53–55). Moreover, electron micrographs of chondrocytes from these mutants show dilated ER and distended ER cisternae as in the matrilin-1 morphants. Collagen II secretion is also severely affected in human cranio-lenticulo-sutural-dysplasia, caused by mutations in SEC23A (56, 57). Nevertheless, we could exclude that alterations in migration of neural crest cells are responsible for the matrilin-1 knockdown phenotype (Fig. 3C), which is the case in the sec24d (crusher) mutant zebrafish. However, only the late matrilin-1 knockdown phenotype resembles that of the zebrafish COP II mutants.

Which are the consequences of the secretion defect? Upon cellular stress, p53 is activated preventing cell growth and cell division, indicating that processes such as DNA repair or autophagy are involved (58). Indeed, the knockdown of p53 together with matrilin-1 leads to more severe phenotypes, indicating that the p53 pathway is involved in counteracting the matrilin-1 phenotype. The increased phenotype penetration after application of the proteasome inhibitor bortezomib supports the participation of the proteasome in the repair of the phenotype at both developmental stages and was used to differentiate between the two in that hypomorphic morphants were “induced” at 48 hpf by application of bortezomib (Fig. 8B).

In addition, at early stages areas positive for matrilin-1 showed more DNA fragmentation events by TUNEL staining. Interestingly, blocking apoptosis by co-injecting the caspase inhibitor Z-VAD-FMK, whereas greatly reducing the apoptotic effects of the matrilin-1 knockdown, did not rescue the morphological phenotype (Fig. 10), indicating that apoptosis is a secondary effect of the knockdown phenotype, but not the primary cause.

But why does TEM analysis of 24-hpf-old morphants not show any significant changes in cellular appearance and ER morphology? At early stages collagen II is expressed by different cells than later. Furthermore, the early expression of collagen II expression is weak and therefore the absence of matrilin-1 may not markedly impair ER function. In contrast, at late stages, when bulk amounts of collagen II are synthesized and secreted, the cells are no longer able to compensate.

In addition to the difference between early and late expression matrilin-1 mRNA levels, a significant difference in electrophoretic mobility of the early and the late expressed matrilin-1 proteins was seen. As alternative splicing was not detected (not shown), proteolytic processing could be responsible. Extensive processing of matrilins was earlier demonstrated in mouse (2, 3), where it was shown that cleavage in the vicinity of the coiled-coil domain releases monomeric fragments that contain most of the subunit.

It is not clear why targeting matrilin-1 in mouse gives no overt phenotype, whereas the knockdown of matrilin-1 in zebrafish causes severe abnormalities. The zebrafish extracellular matrix and the secretion and assembly of its components may be different from that in mammals. Despite the lack of severe phenotypes in either of the two matrilin-1-deficient mouse lines, both strains displayed alterations in collagen II fibril formation (7, 10), pointing to some extent of conservation of matrilin-1 function between the two species.

Taken together, we propose that the difference of the phenotypes observed in the matrilin-1 knockdown zebrafish at early and late time points is linked to different functions of collagen II and different impacts on the cells that secrete the protein. Early, cells generate minute amounts of collagen II but its VWC domain has an important role as a morphogen. The secreting cells are not strongly affected by the secretion block. Later, chondrocytes are specialized on the bulk secretion of collagen II and when secretion is hindered they suffer secretory pathway collapse and ultimately die.

Acknowledgments—We thank Tom Carney and Matthias Hammerschmidt for the sox10:egfp strain and Attila Asszódi, Hamid Kashkar, and Matthias Hammerschmidt for valuable discussions.

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