Excessive activity of cathepsin K is associated with cartilage defects in a zebrafish model of mucolipidosis II

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SUMMARY
The severe pediatric disorder mucolipidosis II (ML-II; also known as I-cell disease) is caused by defects in mannose 6-phosphate (Man-6-P) biosynthesis. Patients with ML-II exhibit multiple developmental defects, including skeletal, craniofacial and joint abnormalities. To date, the molecular mechanisms that underlie these clinical manifestations are poorly understood. Taking advantage of a zebrafish model of ML-II, we previously showed that the cartilage morphogenesis defects in this model are associated with altered chondrocyte differentiation and excessive deposition of type II collagen, indicating that aspects of development that rely on proper extracellular matrix homeostasis are sensitive to decreases in Man-6-P biosynthesis. To further investigate the molecular bases for the cartilage phenotypes, we analyzed the transcript abundance of several genes in chondrocyte-enriched cell populations isolated from wild-type and ML-II zebrafish embryos. Increased levels of cathepsin and matrix metalloproteinase (MMP) transcripts were noted in ML-II cell populations. This increase in transcript abundance corresponded with elevated and sustained activity of several cathepsins (K, L and S) and MMP-13 during early development. Unlike MMP-13, for which higher levels of protein were detected, the sustained activity of cathepsin K at later stages seemed to result from its abnormal processing and activation. Inhibition of cathepsin K activity by pharmacological or genetic means not only reduced the activity of this enzyme but led to a broad reduction in additional protease activity, significant correction of the cartilage morphogenesis phenotype and reduced type II collagen staining in ML-II embryos. Our findings suggest a central role for excessive cathepsin K activity in the developmental aspects of ML-II cartilage pathogenesis and highlight the utility of the zebrafish system to address the biochemical underpinnings of metabolic disease.

INTRODUCTION
The autosomal recessive lysosomal disease mucolipidosis II (ML-II; also known as I-cell disease) is caused by defects in the biosynthesis of mannose 6-phosphate (Man-6-P) residues (Kollmann et al., 2010). These residues serve as the key recognition marker for the sorting of lysosomal hydrolases to lysosomes by Man-6-P receptors (Ghosh et al., 2003). ML-II arises from mutations in the single gene encoding the α and β subunits of the GlcNAc-1-phosphotransferase enzyme (GNPTAB) (Reitman et al., 1981; Kornfeld, 1986; Raas-Rothschild et al., 2000; Raas-Rothschild et al., 2004; Tiede et al., 2005). The clinical manifestations of this disorder are diverse, encompassing skeletal and craniofacial defects, impaired speech and cognitive function, and recurrent lung infections (Cathey et al., 2010). Indeed, many of the abnormalities associated with ML-II are noted at birth, highlighting the rapidly progressive nature of the disease and its impact on prenatal development (Sprigz et al., 1978; Herman and McAlister, 1996).

Although a clearer delineation of the genetic bases for this disorder has emerged in recent years, the molecular and cellular mechanisms that drive pathology in individuals with ML-II and the specific Man-6-P-modified proteins implicated in affected tissues remain incompletely understood. In an effort to address the developmental pathogenesis of this disorder, we previously generated and characterized a novel morpholino-based model for ML-II using the vertebrate organism zebrafish (Danio rerio) (Flanagan-Steet et al., 2009). GNPTAB-depleted embryos exhibited decreased mannose phosphorylation of lysosomal hydrolases, craniofacial and cardiac defects, impaired motility, and altered development of pectoral fins and otic vesicles. Focusing on the cellular and molecular bases for the craniofacial cartilage defects in this model, we demonstrated striking changes in the timing and expression of two chondrogenic factors [the extracellular matrix (ECM) protein type II collagen and the transcription factor Sox9] in craniofacial elements; these changes were associated with abnormal morphogenetic movements of the chondrocytes in cartilage elements. These findings suggested that loss of Man-6-P biosynthesis impaired the normal chondrocyte differentiation program in the ML-II zebrafish. Because the development of craniofacial cartilage relies heavily on the timed deposition and remodeling of ECM proteins, we hypothesized that disruption in the biosynthesis and/or proper maintenance of the ECM contributes to the disease process in ML-II.

In light of the fact that ECM deposition and turnover occurs at stages within developing cartilage that were most strongly affected in ML-II embryos, proteins and enzymes responsible for the biosynthesis, remodeling and clearance of proteins such as collagen are likely to play a key role in the altered craniofacial development
noted in this model. The consequences of impaired expression and activity of several classes of proteolytic enzymes, including ADAMTS proteases, matrix metalloproteinases (MMPs) and cathepsins, on the development and homeostasis of bone and cartilage is evidenced by animal models and humans with defects in these proteases (Yasuda et al., 2005; Holmbeck and Szabova, 2006; Lincoln et al., 2006). Moreover, recent work in animal models of mucopolysaccharidoses (MPS) has suggested a role for both cathepsins and MMPs in the pathogenesis of lysosomal disorders (Simonaro et al., 2005; Ma et al., 2008; Simonaro et al., 2008). Subsequent studies have also identified increases in both the transcript abundance and activity of these proteases, implicating the disruption of specific signaling pathways (Metcalf et al., 2009; Metcalf et al., 2010). Because most lysosomal cathepsins are modified by Man-6-P residues and might be hypersecreted when mannose phosphorylation is lost, these proteases are ideal candidates for the initiation and progression of the phenotypes associated with ML-II.

Taking advantage of methodologies that are highly amenable in the zebrafish system, we conducted a targeted investigation of molecules involved in ECM deposition, remodeling and turnover in wild-type (WT) and ML-II embryos. Our results showed that the transcript abundance of several ECM proteins, cathepsins and MMP enzymes were altered in chondrocyte-enriched cell populations isolated from ML-II embryos, as compared with WT. Focusing our subsequent analyses on the proteases, we further demonstrated that the activities of cathepsins K, L and S as well as MMP-13 were elevated and sustained during developmental stages and in tissues most affected in ML-II embryos. Suppression of the activity of one of these enzymes, cathepsin K, to near WT levels was not only sufficient to reduce the activity of several other proteases in the ML-II zebrafish but also partially corrected the craniofacial phenotypes in these embryos. Together, these data suggest a central role for excessive cathepsin K activity in the cartilage pathogenesis of ML-II and further highlight the utility of the zebrafish system to address both the developmental and biochemical underpinnings of metabolic disease.

**RESULTS**

**Cells isolated from Tg(fli1a:EGFP) zebrafish embryos express high levels of transcripts encoding ECM proteins and ECM remodeling enzymes**

Transcript abundance profiling in isolated populations of zebrafish cells has emerged as an effective way to identify changes in gene expression that are associated with the development of specific cell types and tissues. This methodology has proven useful in both normal embryos as well as disease models (Sumanas et al., 2005; Covassin et al., 2006). In an effort to further explore the molecular basis of the cartilage phenotypes in the ML-II model, GFP-positive (GFP+) and -negative (GFP−) cells were isolated by fluorescence activated cell sorting (FACS) from dissociated WT and ML-II Tg(fli1a:EGFP) embryos, and quantitative real-time PCR (qRT-PCR) analysis was performed on a targeted set of transcripts. Tg(fli1a:EGFP) embryos express EGFP in endothelial cells, certain hematopoetic cells, and pharyngeal arch neural crest-derived cells (Covassin et al., 2006). Owing to the expansion of craniofacial structures in embryos 2 and 3 days post fertilization (dpf), GFP+ cells isolated from dissociated embryos are highly enriched for chondrocytes and their precursors. The genes targeted for qRT-PCR analyses included...
several collagens and other ECM proteins, enzymes involved in collagen biosynthesis, and multiple classes of proteases capable of modifying and/or degrading the ECM (supplementary material Table S1). The choice of targets was primarily guided by our earlier assessment of the craniofacial phenotypes in the ML-II embryos, which indicated that stages of cartilage development that rely heavily on the deposition and/or remodeling of ECM proteins (such as collagens) were particularly sensitive to reduced Man-6-P biosynthesis (Flanagan-Steet et al., 2009). GFP+ and GFP– cells were effectively separated from dissociated embryos, with GFP+ cells representing ~8% and ~20% of the total cells isolated from 2- and 3-dpf embryos, respectively (Fig. 1A). Diagnostic FACS analysis of the sorted GFP+ and GFP– cell populations revealed that they were 99.2% and 99.9% pure, respectively. Consistent with the enrichment of chondrocytes within the GFP+ cell population and the requirement for active synthesis and turnover of the ECM during chondrocyte development, the relative level of several transcripts, including collagens I, II and X and the matrix metalloproteinases, in WT embryos were found to be higher in the GFP+ cells compared with GFP– cells (Fig. 1B).

**Transcript abundance of several proteases and ECM proteins was increased in chondrocyte-enriched cell populations isolated from ML-II zebrafish**

Comparison of WT and ML-II GFP+ cells at 2 and 3 dpf revealed differences in the transcript abundance of several target genes (Fig. 2). Overall transcript levels of several genes were shown to increase in WT embryos between 2 and 3 dpf, suggesting that dynamic changes in gene expression occur during this developmental period (supplementary material Fig. S1). Significant increases were detected in the transcripts of several cathepsins and MMPs (but not ADAMTS proteases) in ML-II embryos at these stages, with cathepsin L having the most striking elevation measured. Notable changes in the ECM targets analyzed included a profound decrease in aggrecan expression in ML-II GFP+ cells, probably reflecting the abnormal differentiation of chondrocytes in the ML-II embryos (Knudson and Knudson, 2001). Consistent with our previous analyses, which demonstrated the sustained expression of type II collagen in ML-II craniofacial cartilages, higher levels of col2a1 transcripts were also observed in ML-II embryos at 3 and 5 dpf (Fig. 2; supplementary material Fig. S1). Importantly, no significant changes were seen in the apparent expression of genes related to endoplasmic reticulum (ER) stress (bip and hsp70; data not shown) and inflammation (tlr4; Fig. 2), and the transcript abundance of several growth factors (tgfβ; data not shown) was comparable in WT and ML-II sorted cell populations. Most of the transcripts analyzed were elevated in ML-II embryos (GFP+ and GFP–) at 5 dpf, compared with WT. This increase might relate to the compromised health and impaired yolk utilization of the ML-II embryos by this later stage. It is unlikely, however, that global effects on health would account for the increased transcript abundance in ML-II embryos at 2 and 3 dpf, because the embryos are viable and, with the exception of specific phenotypes, do not display the same signs of deteriorating health noted in 5-dpf embryos.

Although differences in the transcript abundance of several genes were evident in multiple individual samples, a collective analysis of four independent biological replicates indicated that only a subset of these transcript changes were statistically significant. The disparity in transcript abundance between data sets probably reflects biological variability, because technical replicate analysis from the same biological sample was highly reproducible. Thus, the high degree of variability between the biological replicates might mask additional important differences for some of the transcripts analyzed, but those transcripts indicated with P-values in Fig. 2 were consistently and significantly altered in abundance in the biological replicates.
Cathepsin activity was greatly increased and sustained during early development of ML-II zebrafish embryos

To determine whether the increased abundance of cathepsin transcripts was associated with a corresponding increase in enzymatic activity, in vitro enzyme assays were performed on WT and ML-II zebrafish lysates. For all enzymes tested, the normalized activity values shown represent only the activity that could be specifically blocked with the respective inhibitors. As shown in Fig. 3A, statistically significant differences in the activity of several cathepsins were detected between WT and ML-II embryos across a developmental timeline spanning 1-4 dpf. With the exception of the aspartyl protease cathepsin D, the activity of all cathepsins tested was relatively low in WT embryos at 1 dpf. By 2 dpf, an increase in the activity of cathepsin K, but not S and L, was detected in WT embryos. The disappearance of cathepsin K activity by 3 dpf suggested that this protease is subject to tight regulation in normally developing embryos. In contrast to WT, the activities of cathepsins K, L and S all increased substantially at 2 dpf in ML-II embryos. Although these activities also decreased at 3 and 4 dpf, the levels in ML-II embryos remained significantly higher than that detected in WT embryos. The activity of cathepsin D was also moderately elevated in ML-II embryos at all four stages but did not exhibit the same sustained activity profile as the other cathepsins tested. To gauge the specificity of these effects, we also tested cathepsin activity in ML-II embryos that had been rescued by overexpression of WT GNPTAB mRNA (Fig. 3B). Rescued embryos exhibited cathepsin activity levels that were similar to WT embryos, indicating that the increased activity of these enzymes was specifically due to loss of GNPTAB activity. Moreover, overexpression of GNPTAB mRNA in WT embryos did not result in any significant change in cathepsin activity.

As an additional gauge of the specificity of increased cathepsin activity, several glycosidase enzyme activities were measured in 3-dpf WT and ML-II whole embryos. These analyses were important to assess whether the increased cathepsin activity was indicative of a general stimulation in lysosomal enzymes. We did not, however, detect any substantial changes in the activity of these glycosidases in the ML-II embryos (supplementary material Fig. S2). These data suggest that the increases in cathepsin protease activity probably occur independent of a global increase in the expression and/or activity of other lysosomal proteins.

Increased MMP activity was observed in ML-II embryos and was a general feature of the disease

The qRT-PCR results indicated that the transcript abundance of MMP-13 and other MMPs was elevated in both GFP+ and GFP– cell populations from ML-II embryos. This increase was particularly evident at later developmental time points (5 dpf). To explore whether MMP activity was also increased in the ML-II embryos, total MMP activity was assayed using a FRET-based substrate with broad specificity for this class of proteases. As shown in Fig. 4A, general MMP activity in WT embryos was high at 1 and 2 dpf but had decreased by later stages. In ML-II embryos, this activity was increased and sustained, mirroring that of cathepsins K and L. As with the cathepsins, the increased MMP activity was largely abated in mRNA-rescued embryos (Fig. 4B). Because MMPs are synthesized as inactive proenzymes that require processing for
Role of cathepsins in ML-II pathogenesis

proteolytic activation, tests were performed to determine whether the MMP detected in the embryos existed as a mature enzyme or as an inactive proenzyme. Treatment of embryo lysates with APMA, a general activator of MMP activity, resulted in a 26% increase in MMP activity in both WT and ML-II embryos (Fig. 4C). The modest degree of APMA-stimulated activation suggested that most of the MMPs assayed in both WT and ML-II embryos were already in the mature form. Consistent with its dependence on metal ions, general MMP activity in embryo lysates could be effectively inhibited in the presence of EDTA. In light of the broad specificity of the MMP substrate used in the initial experiments, the source of the increased MMP activity was investigated further by utilizing a second substrate with specificity for MMP-12 and MMP-13. As shown in Fig. 4D, increases in MMP-12/13 activity were comparable to that detected with the general MMP substrate observed in ML-II lysates. This suggested that one or both of these proteases accounts for the MMP activity. Using an MMP-13-specific antibody, western blot analysis of detergent lysates was performed (Fig. 4E). Not only were total MMP-13 levels increased in ML-II embryos, but the enzyme was also primarily detected in its mature, activated form, confirming the APMA experiment and the MMP-12/13 substrate results described above.

Upregulation of MMP activity has been noted in several tissues from animal models of lysosomal storage disorders, including in models of the mucopolysaccharidoses (MPS I, VI and VII) (Simonaro et al., 2005; Ma et al., 2008; Simonaro et al., 2008). Because the increase in MMP activity in ML-II embryos could represent a zebrafish-specific phenomenon, this activity was investigated in fibroblast-like synoviocytes isolated from feline models of ML-II and MPS VI (Maroteaux-Lamy syndrome). As shown in Fig. 4F, the level of cell-associated MMP activity was significantly higher in ML-II synoviocytes relative to WT. As noted in zebrafish samples, this activity was fully inhibited by EDTA and was not substantially affected by APMA treatment (data not shown). General MMP activity was also elevated in MPS-VI cells but to a lesser extent compared with ML-II cells. Together, these data show that MMP activity, in particular that of MMP-13, is abnormally increased in ML-II embryos at developmental stages that correspond to the maturation and formation of craniofacial cartilage, and that MMP upregulation is a general feature of ML-II tissues.

**Cathepsin K is temporally and spatially expressed in developing cartilage during zebrafish embryogenesis**

In an effort to determine whether any of the assayed cathepsins were candidate contributors towards the cartilage defects in ML-II embryos, we assessed whether their individual activities were globally or regionally increased. WT and ML-II embryos were separated into head and tail sections (diagrammed in Fig. 5A) and each of these pools assayed for protease activity. The results of this analysis, shown in Fig. 5B,C, clearly demonstrated that the vast majority of elevated cathepsin activity was present in the heads of ML-II embryos, suggesting that these enzymes might be upregulated in cell types that are enriched within this region (i.e. precursors of cartilage and bone). Interestingly, MMP activity was increased in both the head and tail extracts of ML-II embryos, indicating that elevated MMP activity could contribute to disease onset or progression in other affected tissues.

In light of its known role in the maintenance of bone and cartilage homeostasis and its sustained activity during stages of cartilage development, further investigation of the role of cathepsin K in the ML-II cartilage morphogenesis defects was warranted. To specifically localize the expression of cathepsin K within the head, in situ hybridization and immunohistochemical experiments were performed (Fig. 5). Consistent with a role for the enzyme in the development and maturation of cartilage, *cathepsin K* transcript was detected primarily in the head at 2 and 3 dpf, with strong staining present in regions of craniofacial development (Fig. 5D-G). At 2 dpf, *cathepsin K* transcript was particularly prominent in the ventral portion of tissues posterior to the eye. By 3 dpf, *cathepsin K* transcript was visible throughout regions that generally correspond to Meckels cartilage and the ceratobranchials (CBs). Although the overall staining pattern was similar between WT and ML-II embryos at both 2 and 3 dpf, cathepsin K staining was notably absent from...
the pectoral fins (Fig. 5D,E, arrows) of ML-II embryos. This is probably due to the fact that, although the fin itself can form, it lacks fli1a:EGFP-positive chondrocytes in morphant embryos. Interestingly, in situ analyses also demonstrated a consistent increase in cathepsin K transcript within the developing heart of ML-II embryos at 3 dpf (Fig. 5F,G, arrows). Immunohistochemical analyses performed on sections of WT fli1a:EGFP embryos at 3 and 4 dpf confirmed that cathepsin K protein is expressed in the developing chondrocytes and, to a lesser extent, in the perichondrial fibroblasts that surround them. This was true for multiple structures, including the Meckels and trabecular cartilages (Fig. 5H-J). Within chondrocytes, cathepsin K expression was most evident in discrete cellular puncta, possibly corresponding to lysosomes. Its expression was also detected in the cellular sheath surrounding elements of the notochord (data not shown). Collectively these data suggest a role for cathepsin K during cartilage development and support the idea that increases in its activity might underlie the craniofacial defects noted in ML-II.

### Increased processing of cathepsin K underlies its sustained activity in ML-II embryos

The increased activity of the cathepsins could arise from several different mechanisms, including increased expression of the protein, decreased expression of endogenous protein inhibitors or enhanced conversion of inactive proenzymes into their mature active forms. To further explore the biochemical basis for the sustained increase in cathepsin K activity in ML-II embryos, cathepsin K protein was analyzed by western blot in embryos at 3 dpf (Fig. 6A). Although the 42-kDa procathepsin K band was present in both lysates at this stage, there were clear differences in the extent of proteolytic processing to lower molecular weight intermediates and the 32-kDa mature form of the enzyme. In particular, the mature form of cathepsin K was highly enriched in ML-II compared with WT samples. The increased abundance of the mature form corresponds with the detection of excessive cathepsin K activity in ML-II embryos. In order to further establish this relationship between proteolytic conversion of procathepsin K and enzymatic activity, additional experiments were performed in which both recombinant human procathepsin K (supplementary material Fig. S3) and 3-dpf WT and ML-II zebrafish lysates were acid treated prior to activity and western blot analysis. These results demonstrated that acid treatment completely shifts recombinant procathepsin K to the mature form, which corresponds with an increase in cathepsin K activity (supplementary material Fig. S3). Whereas acid treatment of WT embryos also resulted in a tenfold increase in cathepsin K activity, this level was still much lower than the robust activity noted in the ML-II embryos. Moreover, acid treatment only slightly increased cathepsin K activity in ML-II lysates. Although treatment of WT lysates with acid did convert the 42-kDa procathepsin K band to lower molecular weight
intermediates, it did not generate the mature 32-kDa form. Because the mature form was below the limit of detection, it is possible that the measured activity in acid-treated WT lysates resulted from weak catalytic activity of the intermediate forms. Highly similar results were obtained when heads from 3-dpf WT and ML-II embryos were subjected to the same analysis (Fig. 6B). From these data, we concluded that, although acid treatment leads to procathepsin K activation, it seems that other mechanisms might be involved in the processing of intermediate forms of the enzyme to the 32-kDa mature band.

Having defined the molecular forms of cathepsin K, the electrophoretic mobility of this protease was then analyzed in WT and ML-II embryo lysates at 2, 3 and 4 dpf (Fig. 6C). As shown, the extent of processing of cathepsin K was generally increased in ML-II embryos at these stages, with the 32-kDa active form uniquely present in ML-II lysates at both 3 and 4 dpf. Although activity was highest in all samples at 2 dpf, low levels of protein were detected, possibly owing to the instability of the mature form of the protein relative to the intermediate and pro-forms. Collectively, these observations are highly consistent with the increased activity of this protease during development (Fig. 3) and indicate that abnormal processing of cathepsin K underlies its sustained activity in ML-II embryos.

**Inhibition of cathepsin K effectively reduces the activity of other cathepsins and MMPs**

The above experiments establish that cathepsin activity was increased in ML-II embryos and that this increase correlated temporally and spatially with the craniofacial defects noted in these embryos. In order to begin defining the role of cathepsin K in the onset and progression of these phenotypes, its activity and expression were independently suppressed in ML-II embryos using either a cathepsin-K-specific enzyme inhibitor or one of two cathepsin-K-targeting morpholinos. The morpholinos used included a splice blocker (SB), which inhibited processing of cathepsin K mRNA, and a translation blocker (TB), which spanned the start codon. Both approaches were first titrated to determine an effective dose of the inhibitor or morpholino that would reduce cathepsin K activity to levels comparable with WT (see Methods and supplementary material Fig. S4 for details of this titration). It was important to avoid doses that would eliminate the activity or expression of cathepsin K because this could result in additional phenotypes not relevant to ML-II.

The effects of the inhibitor treatment were dose dependent and varied with the timing of inhibitor administration. For example, addition of 5 μM cathepsin K inhibitor to WT and ML-II embryos at 1 dpf resulted in developmental abnormalities. Although cathepsin K activity might be required at these early time points for normal development, control experiments demonstrated that the observed toxicity was primarily due to the presence of DMSO (data not shown). By contrast, this same concentration of inhibitor applied at 2 dpf effectively reduced the cathepsin K activity in ML-II embryos to WT levels without an observed increase in developmental defects. Surprisingly, addition of inhibitor was not only found to effectively reduce cathepsin K activity, but also the activities of the other cathepsins and general MMP activity (Fig. 7A). In vitro analyses of the cathepsin K inhibitor demonstrated that, at the low doses used, it specifically affects cathepsin K activity, with no significant effect on the other proteases (supplementary material Fig. S4). This suggests that the corresponding reductions noted in the activity of the additional proteases following in vivo administration of the inhibitor might stem from loss of cathepsin K activity itself. As with pharmacological manipulation, inhibition of cathepsin K by morpholino knockdown in the ML-II embryos also resulted in a reduction in cathepsin K activity albeit not to WT levels (see supplementary material Fig. S4). This was true for both of the cathepsin-K-specific MOs tested. Similarly, when the other protease activities were measured in the cathepsin-K/ML-II double morphants at 3 dpf, reductions comparable to those noted in the inhibitor-treated embryos were seen (Fig. 7B).

**Cathepsin K inhibition rescues multiple aspects of the craniofacial phenotypes in ML-II zebrafish**

We next tested whether the reduction in cathepsin K activity and the corresponding decrease in the activities of the other proteases would improve the craniofacial phenotypes in ML-II morphant embryos. For these experiments, ML-II embryos were either treated at 2 dpf with the cathepsin K inhibitor for a period of 2 days or sequentially injected at the one-cell stage with morpholinos to inhibit both GNPTAB and cathepsin K expression. To ensure specificity with morpholino-based inhibition of cathepsin K, similar
In vivo inhibition of cathepsin K reduces the activities of multiple cathepsins. (A) In vivo administration of a cathepsin K inhibitor at 2 dpf leads to reduced cathepsin and MMP activity levels measured at 4 dpf; for DMSO only treatment, n=3; for samples treated with cathepsin K inhibitor, n=6. (B) Inhibition of cathepsin K expression by MO knockdown also reduces the activity of multiple cathepsins and MMP; n=3 experiments per group with 45 embryos per experimental sample. *P<0.05, **P<0.01, ***P<0.001, Student’s t-test.

analyses were independently performed using both the SB and TB cathepsin K MOs (phenotypic data for SB MO is shown). All of the treated embryos were initially analyzed at 4 dpf by Alcian blue staining (Fig. 8A,B). Importantly, several aspects of the craniofacial phenotypes that typify ML-II embryos were significantly corrected following reduction of cathepsin K activity or expression. The degree of phenotypic correction was quantified by multiple parameters, which are shown schematically in Fig. 8C and the results detailed in Fig. 8D,E. Parameters scored included: (1) whether Meckels cartilage reached the palate, (2) the shape of the anterior jaw, as represented by the ratio of the long and short axes (see Fig. 8 legend), (3) the angle between the two ceratohyal (CH) cartilages, and (4) whether the pectoral fins contained Alcian-blue-positive cartilage. Suppression of cathepsin K activity following addition of either 2.5 μM or 5 μM inhibitor resulted in significant amelioration of all of these phenotypes in 13.9% and 22.2% of the animals treated, respectively. Interestingly, following pharmacological inhibition, the craniofacial structures of ML-II animals were either completely rescued (indistinguishable from WT) or were unaffected by the treatment, perhaps suggesting variable penetrance of the inhibitor. By contrast, MO inhibition of cathepsin K expression yielded a broader range of corrected phenotypes. In the case of the SB MO, we found that 15.9% of the embryos tested exhibited full rescue of craniofacial phenotypes, whereas 69.2% exhibited partial rescue, most often due to incomplete recovery of either the angle between the CH cartilages or the presence of pectoral fin cartilage. Together, these results suggested that cathepsin K plays a central role in the onset of the craniofacial phenotypes in ML-II embryos.

Inhibition of cathepsin K leads to recovery of ML-II cellular morphology and reduces type II collagen expression

Previous work on the zebrafish ML-II model revealed large disruptions in the distribution of cells within multiple structures, including the trabecular and Meckels cartilages. In particular, ML-II cells were drastically underintercalated compared with the WT cells (Flanagan-Steet et al., 2009). In addition, ML-II chondrocytes expressed substantially higher levels of type II collagen. This was most evident at later time points (4-6 dpf). To further assess whether cathepsin K inhibition also improved these aspects of the ML-II craniofacial phenotypes, ML-II morphants and cathepsin-K/ML-II double morphant chondrocytes were compared with WT and WT fli1a:EGFP transgenic background. A subset of the WT and ML-II fli1a:EGFP embryos was also treated with the cathepsin K pharmacological inhibitor. Embryos were collected at 4 dpf and stained immunohistochemically for the presence of type II collagen (Fig. 9). In most cases, striking decreases in the expression of type II collagen were noted in cathepsin-K-inhibited ML-II embryos. Less of a reduction in collagen staining was, however, noted in the inhibitor-treated embryos. This might reflect either the overall permeability of the inhibitor or the need for additional dosing to obtain a maximal effect. Although MO treatment was slightly more effective, inhibition of cathepsin K expression by either method (MO or inhibitor) also resulted in significant recovery of the morphology and distribution of chondrocytes within the ML-II cartilages. For example, although 85±5% of the WT trabecular chondrocytes were fully intercalated, only 6±4% of the cells of the morphant cartilages had completed this process. MO inhibition of cathepsin K in ML-II embryos significantly improved this phenotype, with 60±9% intercalation noted. The improved cellular distribution was also associated with changes in cell shape. Unlike morphant cells, which often lacked the elongated shape of mature chondrocytes, cathepsin-K/ML-II double morphant chondrocytes reverted to a flat, narrow cell (Fig. 9A,B). Similar improvements were noted when fli1a:EGFP WT and ML-II embryos were treated with 5 μM cathepsin K inhibitor. Here again, the degree of recovery was somewhat less than with MO inhibition, but the chondrocytes of drug-treated morphants showed increased intercalation.
compared with DMSO-treated morphant embryos (0% and 48±10%, respectively; Fig. 9C,D). These data suggest an intimate link between increased cathepsin K activity and the persistent expression of type II collagen.

DISCUSSION

The mechanisms that underlie the molecular and cellular pathogenesis of lysosomal storage disorders are beginning to emerge, in part owing to the investigation of animal models for these diseases (Hubler et al., 1996; Gelfman et al., 2007; Haskins, 2007; Simonaro et al., 2008; Metcalf et al., 2009; Vogel et al., 2009; Moro et al., 2010; Boonen et al., 2011). The use of zebrafish as a model system to study these disorders is particularly attractive because the initial pathogenic mechanisms that arise during development can be studied, taking advantage of the genetic and experimental accessibility of this system. The previous generation and characterization of a zebrafish model for ML-II revealed multiple phenotypes within tissues, such as craniofacial cartilage, that are also affected in humans with ML-II (Flanagan-Steet et al., 2009). To further explore the molecular basis for these phenotypes, a targeted set of gene expression changes were analyzed in chondrocyte-enriched cell populations isolated from WT and ML-II embryos. These analyses revealed increases in the transcript abundance and activity of several proteases involved in ECM turnover and remodeling, including the cathepsins and MMPs. The subsequent analyses of these enzymes uncovered a key role for excessive cathepsin K activity in the cartilage lesions noted in ML-II embryos. Surprisingly, inhibition of cathepsin K activity not only resulted in phenotypic correction of the cartilage defects but also led to a general suppression of multiple protease activities in ML-II embryos.

The ability to isolate and biochemically analyze specific cell populations using transgenically labeled zebrafish embryos, such as the fli1a:EGFP line, is a promising means to address the mechanisms that account for tissue-specific pathology in disease models with this organism. In light of the increasing number of transgenic lines that have been generated in recent years, the investigation of most major organ systems and tissues within these models is possible. The expression of fli1a in zebrafish is detected in endothelial cells and angioblasts as well as in a subset of neural crest cells, including precursors of craniofacial chondrocytes (Covassin et al., 2006). Because not all labeled cells are chondrocytes, we believe that some of the changes in transcript abundance detected in GFP+ cells might be relevant to
pathogenesis outside of craniofacial cartilage. It is worth noting the significant transcript abundance increases in the GFP− cell populations, in particular with regards to cathepsin L and the MMPs in 2 dpf and 3 dpf embryos, respectively. Because these enzymes are known to play roles in ECM remodeling in many tissues, including the brain and heart (Felbor et al., 2002; Spira et al., 2007; Reiser et al., 2010), it will be of interest to determine whether their inappropriate expression and activity mediates additional aspects of ML-II pathogenesis. Parallel experiments on ML-II embryos generated in transgenic lines that label different cell populations are ongoing and should further address such tissue-specific mechanisms.

The basis for the increased transcript abundance of the cathepsins and MMPs in the cell populations isolated from ML-II fli1a:EGFP embryos is unclear. These data might reflect inappropriate stimulation of gene-specific transcription. Transcriptional stimulation could arise in response to accumulation of lysosomal storage and the need for increased lysosomal biogenesis, a response that has recently been shown to be coordinated by the transcription factor TFEB (Sardiello and Ballabio, 2009; Sardiello et al., 2009). The increase observed in the cathepsins would be consistent with a TFEB-dependent mechanism. However, no stimulation in other lysosomal components was noted, including the activity of several glycosidases (supplementary material Fig. S2). Furthermore, no obvious signs of either intralysosomal storage or lysosomal proliferation in the ML-II zebrafish embryos were detected (Flanagan-Steet et al., 2009). Owing to the fact that transcriptional upregulation of specific cathepsins has been observed in the context of cancer cells as well as tissues of animal models of MPS disorders (Ma et al., 2008; Reiser et al., 2010), it is plausible that the changes in cathepsin expression are independent of a global increase in lysosomal biogenesis. For enzymes such as MMP-13, increased transcript abundance was shown to correlate with both elevated activity and protein level. However, this was not the case with cathepsin K, for which post-translational modes of regulation (i.e. proteolytic activation) probably represent the primary mechanism leading to increased activity within the ML-II embryo.
The results demonstrated that substantial cathepsin K activity was required in WT embryos at 2 dpf and this enzyme was subject to tight regulation via its proteolytic activation. Because cathepsin K is a very potent collagenase with the ability to cleave triple-helical collagens at multiple sites (Kafienah et al., 1998; Lecaillé et al., 2003; Selent et al., 2007), it is likely that this activity is needed during discrete developmental time points to assist in the degradation and turnover of collagens, which are continually replaced and remodelled during embryonic development (Goldring et al., 2006). Our results indicate that multiple cell types, including craniofacial chondrocytes, express and/or secrete cathepsin K in the early stages of development in zebrafish embryos. In situ analysis of cathepsin K expression (Fig. 5) at 2 and 3 dpf revealed an abundance of transcripts throughout the craniofacial region, and the immunostaining experiments confirm its expression in chondrocytes. The complete range of cathepsin-K-expressing cells in the developing embryo is not currently known, but osteoclasts, the major cathepsin-K-expressing cells in postnatal mammals, are not a plausible source because these cells do not seem to arise at these early stages (Witten et al., 2001).

The data suggests that the increased activity of cathepsin K in the ML-II embryos at later stages (3 and 4 dpf) arises owing to sustained processing of cathepsin K, a mechanism that is supported by the unique presence of mature cathepsin K on western blots of whole embryo lysates (Fig. 6). The mechanism underlying this apparent sustained activation in the morphants is not known but, because this enzyme typically undergoes autocatalytic activation at low pH, this phenomenon might indicate abnormal acidification of cathepsin-K-containing vesicles (McQueney et al., 1997; Dodds et al., 2001; Rieman et al., 2001). Importantly, however, we found that acid treatment was capable of reducing procathepsin K to its intermediate forms but not to the mature 32-kDa form. We believe that the increased activity of cathepsin K in the ML-II background more likely reflects additional processing to its highly active mature form. This additional processing might result from decreased mannos phosphorylation of cathepsin K, its subsequent hypersecretion and contact with cell surface proteases within the extracellular space. Unlike cathepsin D, cathepsin K was recently shown to be hypersecreted from osteoclasts isolated from GNP TA B−/− mice, indicating enzyme-specific sorting of acid hydrolases in mice (van Meel et al., 2011). Exploring whether cathepsin-specific missorting is evident in zebrafish, how enzyme hypersecretion and activation are related, and what mechanisms control this process are all necessary areas for future investigation.

An intriguing finding in this work is the observation that reduction of cathepsin K activity results in decreased activity of other proteases such as cathepsin L, a phenomenon that we confirmed is not the result of non-specific drug inhibition (supplementary material Fig. S4). Because cathepsins are known to activate other cathepsins as well as MMPs (Okada and Nakanishi, 1989), it is possible that the reduction observed was due to a block in the proteolytic activation of proteases by cathepsin K. Owing to the extended timeframe of the rescue experiments, however, it is also plausible that inhibition of cathepsin K activity reversed a broader pathogenic cascade. Nonetheless, cathepsin K inhibition, by two separate methods, resulted in significant phenotypic correction of the craniofacial phenotypes as assessed by both Alcian blue staining and type II collagen expression. These results are encouraging from a clinical standpoint because they support cathepsin K as a potential therapeutic target for alleviation of the developmental defects associated with ML-II. We are actively investigating whether cathepsin K inhibition also leads to correction of other phenotypes and whether inhibition of the other elevated cathepsins and MMPs will impact ML-II pathogenesis.

Taken together, our results demonstrate that cathepsin K plays a crucial role in the development of the cartilage phenotypes in ML-II zebrafish, and provide the basis for investigating the role of cathepsins in non-craniofacial defects. They also highlight the importance of this class of enzymes during normal craniofacial development, as evidenced by the tight control of cathepsin K activation within the developing embryo. To our knowledge this work provides the first demonstration of a role for cathepsin K during the development of embryonic cartilages. Further studies are needed to better define the physiological function of cathepsin activity at these stages and how these activities are intertwined in the maturation program of chondrocytes and other cell types.

**METHODS**

**Fish strains**

Wild-type zebrafish were obtained from Fish 2U (Gibsonton, FL) and maintained using standard protocols. Embryos were staged according to the criteria established by Kimmel (Kimmel et al., 1995). In some cases, 0.003% 1-phenyl-2-thiourea was added to the growth medium to block pigmentation. All MO-generated phenotypes were tested in several genetic backgrounds, including a wild-type strain from a commercial source (Fish 2U). Analyses of craniofacial phenotypes were performed in both the F2U wild-type strain and Tg(fli1a:EGFP)y1 transgenic line (Lawson and Weinstein, 2002). Handling and euthanasia of fish for all experiments were carried out in compliance with the University of Georgia’s policies. This protocol has been approved by the University of Georgia Institutional Animal Care and Use Committee (permit number: A2009 8-144).

**Antisense MO injection and mRNA rescue**

Expression of N-acetylgalactosamine-1-phosphotransferase (αβ subunit; GNP TA B) was inhibited by injection of MO as previously described (Flanagan-Stee et al., 2009). Experiments involving mRNA rescue in the morphant background were performed following injection of full-length phosphotransferase mRNA as previously described (Flanagan-Stee et al., 2009). The expression of cathepsin K (ctsk) was inhibited using either 0.2 nl of a 500 μM (0.1 μM) solution of a SB MO (5’-TGTAACAATACCTACCATTGCACA-3’) directed against the exon-1–intron-1 junction or 0.2 nl of a 500 μM (0.1 μM) solution of a TB MO (5’-GAGGGAATCCGCAAAATCTACCAT-3’) directed at the ctsk ATG. The specificity of the ctsk MOs and the concentrations necessary to reduce cathepsin K activity (in ML-II embryos) to WT levels was determined by introducing a range of MO (0.01-0.5 μM) concentrations into both the WT and ML-II morphant backgrounds. For experiments involving inhibition of ctsk in the ML-II background, the MOs were injected sequentially at the one-cell stage. The degree of ctsk inhibition for various MO concentrations was then determined by RT-PCR analysis of the ctsk mRNA (in the case of the splice blocker) and/or activity assays (used in both cases as described below) (see supplementary material Fig. S4). In light of the fact that neither the SB nor the TB MOs resulted in control this process are all necessary areas for future investigation.

An intriguing finding in this work is the observation that reduction of cathepsin K activity results in decreased activity of other proteases such as cathepsin L, a phenomenon that we confirmed is not the result of non-specific drug inhibition (supplementary material Fig. S4). Because cathepsins are known to activate other cathepsins as well as MMPs (Okada and Nakanishi, 1989), it is possible that the reduction observed was due to a block in the proteolytic activation of proteases by cathepsin K. Owing to the extended timeframe of the rescue experiments, however, it is also plausible that inhibition of cathepsin K activity reversed a broader pathogenic cascade. Nonetheless, cathepsin K inhibition, by two separate methods, resulted in significant phenotypic correction of the craniofacial phenotypes as assessed by both Alcian blue staining and type II collagen expression. These results are encouraging from a clinical standpoint because they support cathepsin K as a potential therapeutic target for alleviation of the developmental defects associated with ML-II. We are actively investigating whether cathepsin K inhibition also leads to correction of other phenotypes and whether inhibition of the other elevated cathepsins and MMPs will impact ML-II pathogenesis.

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in embryonic phenotypes when injected alone into WT embryos, we did not assess off-target effects by mRNA recovery. It is important to note that, for both cases, the goal was reduction not elimination of ctsk expression.

Embryo dissociation and cell sorting

Wild-type and morphant flia:EGFP embryos were collected at the indicated stages in Ca2+-free Ringers solution. Embryonic yolks were removed by gentle passage through a flame-polished Pasteur pipette. Embryos were subsequently rinsed for 15 minutes in Ca2+-free Ringers solution. Dissociated cellular suspensions were generated by soaking the embryos in 0.25% trypsin, followed by repeated passage through 23- and 25-gauge syringes. Cellular dissociation was monitored microscopically. When cellular aggregates were no longer visible, the suspensions were filtered through sterile 40-μm Falcon filters to remove debris. Cells were collected following centrifugation at –80°C until RNA could be prepared (via RNeasy Plus kit, Qiagen).

qRT-PCR analysis of transcript abundance

Total RNA was isolated from sorted cell populations using the RNeasy Plus kit (Qiagen). Samples were quantitated with a NanoDrop spectrophotometer (Thermo) and stored at –80°C. First-strand cDNA synthesis was performed using the SuperScript VILO cDNA synthesis kit with 125 ng of total RNA. A tenfold dilution of the cDNA synthesis reaction was used as the template source for qRT-PCR reactions. RNA samples were checked for genomic DNA (gDNA) contamination using a control cDNA synthesis reaction without reverse transcriptase.

Sequences for D. rerio genes used in this study were obtained from ZFIN and NCBI databases. Primer pairs used for quantitative real time PCR (qRT-PCR) were designed within a single exon sequence of an individual gene as described previously for mouse genes (Nairn et al., 2008). Primer pairs were validated for specificity (amplification of a single product) and efficiency using D. rerio gDNA as a template. Sequences for primers used in this study are presented in supplementary material Table 1.

Quantitative RT-PCR reactions for individual genes were run in technical triplicate on four independent cell populations. Reactions consisted of 2.5 μl SYBR Green Supermix (Bio-Rad), 1.25 μl diluted cDNA synthesis reaction and 1.25 μl of gene specific-primer pair (125 mM final concentration). Amplification conditions and data analysis were performed as described previously (Nairn et al., 2010). Several housekeeping genes were evaluated as normalization controls and ribosomal protein L4 (rpl4) was determined to be the most uniformly expressed gene.

The relative transcript abundance of each gene (normalized to rpl4) was determined for each of the four biological samples at each growth stage. These values were then evaluated for statistically significant changes in abundance when comparing WT and morphant samples. A non-parametric Mann-Whitney test was used to determine statistically significant differences between samples with the InStat 3 software package (GraphPad Software).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as previously described (Flanagan-Steet et al., 2009). An L.M.A.G.E. clone containing the full-length cathepsin K mRNA (Accession # BC092901) was purchased from Thermo-Fisher. A probe plasmid was generated following PCR amplification of a 1.2 kb fragment that included the entire coding region. This fragment was cloned into the EcoRI site of the pCSII vector and its orientation determined by PCR. The mRNA probe was generated from (HindIII) linearized plasmid DNA using T3 RNA polymerase.

Immunohistochemistry

Whole-mount analysis of type II collagen expression was performed in the flia:EGFP transgenic background as previously described (Flanagan-Steet et al., 2009). For immunohistochemical analysis of normal ctsk expression, WT flia:EGFP embryos were harvested and fixed in 4% paraformaldehyde (PFA) at 4°C overnight. The PFA was rinsed out with several changes of phosphate buffered saline (PBS), and embryos were taken through an ascending series of sucrose solutions (7, 15, 30%). The sucrose-treated embryos were subsequently embedded and frozen in OCT freezing media (Tissue-Tek Corp.). 40-μm sections were cut on a Leica 1850 cryostat. The sections were incubated with blocking buffer (PBS+2% goat serum, 1% DMSO, 0.02% Triton X-100) for several hours at room temperature. This was followed by an overnight incubation at 4°C with rabbit anti-cathepsin-K primary antibody diluted (1:75; cat#ab19027, Abcam) in blocking buffer. Sections were rinsed with several changes of PBS+0.02% Triton X-100; anti-rabbit AlexaFluor-568-conjugated secondary antibody (diluted 1:400 in blocking buffer) was applied to the sections for 2 hours at room temperature. Sections were again rinsed and coverslips mounted with Prolong Gold mounting medium (Life Technologies) for microscopic analysis. In all cases, immunohistochemical stains were visualized using an Olympus FV-100 laser scanning confocal microscope using ideal image parameters as defined by a 40×W (N.A. 1.15) objective. Image acquisition and processing parameters were as previously described (Flanagan-Steet et al., 2009).

Protease activity assays

For the protease activity assays, WT and morphant embryos were dechorionated, deyolked (as described in the ‘Embryo dissociation and cell sorting’ section above), and homogenized on ice by sonication in 10 mM Tris, pH 6.5, 1% Triton X-100. Embryo lysates were centrifuged at 20,160 g for 10 minutes at 4°C and the protein concentration determined by Micro-BCA protein assay (Thermo Scientific). To gauge enzyme-specific substrate hydrolysis, equivalent samples were incubated with respective inhibitors or vehicle for 15 minutes at 4°C before starting the assay. Enzymatic activities of cathepsins D (cat# 72097) and S (cat# 72099) were obtained using enzyme-specific kits from AnaSpec (San Jose, CA) and assays were performed according to the manufacturer’s specifications. Thiolepine protease inhibitor E-64 (1 μM) was used to inhibit cathepsin S, and pepstatin A (1 μM) was used to inhibit cathepsin D. For cathepsins K and L, 10 μg of lysate was assayed in a 100 μl reaction buffer (100 mM sodium acetate, pH 5.5, 1 mM DTT and 1 mM EDTA) containing 10 μM of the respective substrates. The substrate for cathepsin K was (Z-Leu-Arg)2-Rhodamine 110 (cat# 219390) and the inhibitor Boc-Phe-Leu-NHNH-CO-NHNH-Leu-Z (1 μM; cat#
219373, Calbiochem, San Diego, CA). For the determination of cathepsin L activity, the substrate (Z-Phe-Arg)-enalysin (cat# 350014) was obtained from Abbiotec (San Diego, CA) and the cathepsin L inhibitor IV (1-naphthalenesulfonfyl-Ille-Trp-CHO; 1 μM; cat# 219433) was also obtained from Calbiochem. The enzymatic activity of the MMPs was determined from the rate of hydrolysis of a general MMP substrate, QXL520-γ-Abu-Pro-Cha-Abu-Smc-His-Ala-Dab (5-FAM)-Ala-Lys-NH2 (cat# 60581-01), from Anaspec. According to the manufacturer, the substrate is cleaved by MMP-1, -2, -3, -7, -9, -12 and -13. MMPs were activated using p-aminophenylmercuric acetate (APMA). The metal ion chelator EDTA was used as an inhibitor. Fluorescence units were measured at various time intervals -9, -12 and -13. MMPs were activated using acocktails (Sigma, St Louis, MO). Lysates were then homogenized on ice by probe sonication, centrifuged at 20,160 g for 10 minutes at 4°C and the protein concentration determined by Micro-BCA protein assay (Thermo Scientific). 100-125 μg of lysate was run on an SDS-PAGE gel and protein was transferred to a nitrocellulose membrane (Bio-Rad). Membranes were probed with either a rabbit polyclonal antibody (cat# ab19027, Abcam) or anti-MMP-13 antibody (cat# 55114, Anaspec). Secondary goat anti-rabbit antibodies tagged with HRP were used to detect protein by chemiluminescence (GE Healthcare, Piscataway, NJ).

In some cases, embryo lysates were treated with acid to reduce the pH to 4 for 1 hour at 4°C prior to subsequent analyses. The pH of the lysate was adjusted back to 5.5 prior to cathepsin K activity assays.

Small-molecule in vivo inhibition of cathepsin K activity

At 24 hpf, embryos were collected, dechorionated and 20-30 embryos placed per well into six-well tissue-culture plates. At 48 hpf, embryos were treated with either 2.5 μM or 5.0 μM cathepsin K inhibitor, or control treated with 0.3% DMSO (which represents the highest amount present with the inhibitor). After 2 days of continuous drug treatment, embryos were subsequently collected (96 hpf) and processed for either Alcian blue staining or cathepsin enzyme assays.

Alcian blue staining and quantification of craniofacial phenotypes

Embryos were stained with Alcian blue as described previously (Flanagan-Steet et al., 2009). Analysis of craniofacial structures was performed using the morphometric parameters outlined in the results section. Stained embryos were photographed on an Olympus SZ-16 dissecting scope outfitted with Q-capture software and a Retiga 2000R color camera.

Preparation of feline fibroblast-like synoviocytes

After removal of any excess tissue, synovial membranes were washed twice with PBS (supplemented with Pen/Strep) and minced into small pieces with a scalpel. Diced membranes were digested in RPMI-1640 media containing 0.5 mg/ml sterilized collagenase type IA (Sigma; C9891) at 37°C for 3 hours in a 15 ml conical tube. After incubation, suspended cells were pipetted through a sterile 100 μm nylon mesh into a new centrifuge tube. Cells were washed twice in complete RPMI-1640 media containing 15% heat-inactivated FBS and plated in a T-25 culture flask. After 6 hours to allow attachment, fresh media was placed on the cell monolayer to remove any residual tissue or debris. MMP activity assays in cell lysates was performed as described earlier.

Translational impact

Clinical issue

Mucolipidosis II (ML-II; also known as I-cell disease) is an autosomal recessive lysosomal storage disorder caused by defects in the enzyme that initiates the biosynthesis of mannose 6-phosphate residues, carbohydrate-based tags that target acid hydrolases to lysosomes. Patients with ML-II have diverse clinical manifestations, including skeletal and craniofacial defects, cardiac abnormalities, and impaired cognitive function. There are no available therapies for ML-II. Many fundamental questions regarding the pathogenic mechanisms of ML-II remain unanswered, particularly with respect to the abnormal development of craniofacial cartilage and the skeletal system. Defining these mechanisms might aid in the development of new therapeutic approaches.

Results

The authors previously developed a zebrafish model for ML-II and showed that the embryos exhibited many phenotypes consistent with the human disease, including craniofacial defects. The current study aims to investigate the molecular bases for these phenotypes, beginning with transcript abundance analysis of chondrocyte-enriched cell populations isolated from zebrafish ML-II embryos. The results demonstrate that the abundance of several transcripts – including those that encode cathepsins and matrix metalloproteinases (MMPs), which are enzymes that are important for the degradation and remodeling of the extracellular matrix – are increased in ML-II embryos compared with controls. Furthermore, the activity of cathepsins K and L, and MMP-13, is greatly increased in ML-II embryos, and is sustained during stages when cartilage development is occurring. Treatment with a cathepsin-K-specific inhibitor or suppression of cathepsin K activity by genetic means results in a broad reduction in protease activity in the developing embryo and substantial correction of the craniofacial cartilage phenotypes.

Implications and future directions

These results demonstrate that the activity of multiple proteases is increased in chondrocyte-enriched populations in ML-II zebrafish embryos, and that excessive activity of one of these proteases, cathepsin K, plays a central role in the cartilage morphogenesis defects and type II collagen accumulation observed in these animals. The findings also highlight the fact that activation of secondary biochemical pathways is a common feature of lysosomal diseases. Further investigation of this zebrafish model of ML-II will enhance our understanding of the normal role of proteases in early developmental processes, and how dysregulation of these enzymes can adversely impact chondrocyte maturation. Future studies will investigate the role of cathepsin proteases in other ML-II-associated phenotypes and assess the role of MMPs in the disease process.

Acknowledgements

We thank Julie Nelson (UGA Cell Sorting Facility) for her efforts in sorting dissociated zebrafish embryos and Sanjukta Sahu for technical assistance with immunohistochemistry.

Competing interests

The authors declare that they do not have any competing or financial interests.
AUTHOR CONTRIBUTIONS

FUNDING
This work was supported by the Office of Vice President for Research at the University of Georgia (to R.S.); National MPS Society (to R.S.); and a graduate fellowship from the Cousins Foundation (to A.C.P.).

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at doi:10.1242/dmm.008219/-/DC1

REFERENCES


Time course of relative transcript abundance of several cathepsin genes in wild-type (open bars) and ML-II (filled bars) sorted cells for both fli/GFP+ cells (panel A) and fli/GFP- cells (panel B). Data was normalized to a control gene (rpl4) and is plotted on a log$_{10}$ scale. Error bars represent the SEM from four independent biological samples. Asterisks denote a statistically significant difference (p<0.05) between the transcript abundance for that gene from WT and ML-II sorted cells.
A

WT fli/GFP+  ML-II fli/GFP+

2d

Relative Transcript Abundance (normalized to rpl4)

B

WT fli/GFP+  ML-II fli/GFP+

3d

Relative Transcript Abundance (normalized to rpl4)

C

WT fli/GFP+  ML-II fli/GFP+

5d

Relative Transcript Abundance (normalized to rpl4)
D

![Graph showing relative transcript abundance for WT fli/GFP- and ML-II fli/GFP- at 2d]

E

![Graph showing relative transcript abundance for WT fli/GFP- and ML-II fli/GFP- at 3d]

F

![Graph showing relative transcript abundance for WT fli/GFP- and ML-II fli/GFP- at 5d]
Supplemental Figure 1. Relative transcript abundance for a collection of genes in wild-type (open bars) and ML-II (filled bars) sorted cells for both fli/GFP+ cells (panels A-C) and fli/GFP- cells (panel D-F) at 2dpf (top panels, A and D), 3dpf (center panels, B and E) and 5dpf (bottom panels, C and F). Data was normalized to a control gene (rpl4) and is plotted on a log_{10} scale. Error bars represent the SEM from four independent biological samples. Asterisks denote a statistically significant difference (p<0.05) between the transcript abundance for that gene between WT and ML-II sorted cells.
Supplemental Figure 2. Total glycosidase activity is not altered in ML-II embryos. Whole embryo lysates from WT (white bars) and ML-II (black bars) zebrafish (3 dpf) were assayed for glycosidase activity using respective 4-methylumbelliferyl substrates.
Supplemental Figure 3. Acid activation of recombinant human cathepsin K. Recombinant cathepsin K was treated with acid to induce autocatalytic activation prior to Western blot analysis (panel A) and activity assay (panel B; n=3).
Supplemental Figure 4. Validation of cathepsin K inhibition methodology. A) Schematic representation of the cathepsin K gene. Dashes labeled 1 and 2 represent the translation blocking (TB) and splice blocking (SB) MOs, respectively. (*) represents location of the initiating ATG. Arrows represent the forward and reverse primers used to analyze mRNA expression of SB MO. B) RT-PCR analysis of ctsk expression at 1dpf in WT embryos following introduction of various concentrations of SB MO. RT-PCR analysis of rpl4 performed on each sample served as a normalizing control. C) Analysis of cathepsin K activity in lysates of 3dpf WT and ML-II embryos following introduction of a range of SB and TB MO concentrations. D) Specificity of the cathepsin K inhibitor was determined in vitro using whole embryo lysates. Analysis of cathepsin enzyme activities in the presence of 10µM inhibitor indicates that cathepsins D, L, S, and MMP are only minimally affected by this molecule (n=3). E) Dose-dependent inhibition of cathepsin K activity in inhibitor-treated embryo lysates. Based on this analysis, we chose to work with 2.5 and 5 µM for subsequent analyses.
### Table 1
Genes and primer sequences used for qRT-PCR

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*ADAMTS = A disintegrin and metalloproteinase with thrombospondin motifs 12-like