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Evidence for a cooperative role of gelatinase A and membrane type-1 matrix metalloproteinase during Xenopus laevis development.

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SUMMARY

Matrix metalloproteinases (MMPs) are a large family of extracellular or membrane-bound proteases. Their ability to cleave extracellular matrix (ECM) proteins has implicated a role in ECM remodeling to affect cell fate and behavior during development and in pathogenesis. We have shown previously that membrane-type 1 (MT-1)-MMP is coexpressed temporally and spatially with the MMP gelatinase A (GelA) in all cell types of the intestine and tail where GelA is expressed during Xenopus laevis metamorphosis, suggesting a cooperative role of these MMPs in development. Here we show that Xenopus GelA and MT1-MMP interact with each other in vivo and that overexpression of MT1-MMP and GelA together in Xenopus embryos leads to the activation of pro-GelA. We further show that both MMPs are expressed during Xenopus embryogenesis, although MT1-MMP gene is expressed earlier than the GelA gene. To investigate whether the embryonic MMPs play a role in development, we have studied whether precocious expression of these MMPs alters development. Our results show that overexpression of both MMPs causes developmental abnormalities and embryonic death by a mechanism that requires the catalytic activity of the MMPs. More importantly, we show that coexpression of wild type MT1-MMP and GelA leads to a cooperative effect on embryonic development and that this cooperative effect is abolished when the catalytic activity of either MMP is eliminated through a point mutation in the catalytic domain. Thus, our studies support a cooperative role of these MMPs in embryonic development, likely through the activation of pro-GelA by MT1-MMP.

Keywords

Matrix metalloproteinase (MMP)-2 (gelatinase A; GelA); MMP-14 (membrane type 1-MMP; MT1-MMP); *Xenopus laevis*, development

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INTRODUCTION

Matrix metalloproteinases (MMPs) have long been implicated to play critical roles in vertebrate development and pathogenesis due to their ability to cleaving extracellular matrix (ECM) proteins (Coussens et al., 2002;MacDaougall and Matrisian, 1995;Mott and Werb, 2004;Sang, 1998;Seiki, 1999;Shapiro, 1998;Stetler-Stevenson et al., 1993;Tryggvason et al., 1987;Uria and Werb, 1998;Vu and Werb, 2000). MMPs are a large family of Zn^{2+}-dependent extracellular or membrane bound proteases that include collagenases, gelatinases (Gel), stromelysins, and membrane type MMPs (MT-MMPs) (Barrett et al., 2004;Birkedal-Hansen et al., 1993;McCawley and Matrisian, 2001;Parks and Mecham, 1998;Sternlicht and Werb, 2001). Most MMPs are synthesized as full length or pre-enzymes and secreted as inactive latent or pro-enzymes with the cleavage of the propeptide (signal peptide), and the proenzymes are activated upon the removal of the propeptide through various mechanisms (Kleiner and Stetler-Stevenson, 1993;Murphy et al., 1999;Nagase, 1998;Nagase et al., 1992;van Wart and Birkedal-Hansen, 1990). MT-MMPs and stromelysin-3, however, are activated intracellularly through a furin-dependent process (Pei, 1999;Pei and Weiss, 1995;Seiki, 1999). The mature or activated MMPs are capable of cleaving both ECM components and non-ECM extracellular and membrane-bound proteins, such as growth factor precursors and cell surface receptors (Amano et al., 2005;Barrett et al., 2004;Birkedal-Hansen et al., 1993;McCawley and Matrisian, 2001;Mott and Werb, 2004;Overall, 2002;Parks and Mecham, 1998;Sternlicht and Werb, 2001;Uria and Werb, 1998). Thus, it is believed that MMPs can influence cell fate and behavior through multiple mechanisms, including alteration of cell-ECM interaction and regulation of the levels and function of growth factors, etc.

We are studying the role of MMPs during the development of the South African frog Xenopus laevis. We and others have previously identified a number of MMP genes that are upregulated during metamorphosis in Xenopus laevis and Rana catesbeiana (Brown et al., 1996;Hasebe et al., 2006;Jung et al., 2002;Oofusa et al., 1994;Patterton and Shi, 1994;Shi et al., 2001;Stolow et al., 1996), a thyroid hormone-dependent process that transforms essentially every organ/tissue as the animal changes from a tadpole into a frog (Shi, 1999). The expression of these MMP genes, including GelA, MT1-MMP, stromelysin-3, and collagenases, are induced either directly or indirectly by thyroid hormone and is associated with cell death during metamorphosis in various tissues/organs, such as the tail and intestine (Berry et al., 1998a;Berry et al., 1998b;Brown et al., 1996;Damjanovski et al., 1999;Fu et al., 2005;Hasebe et al., 2006;Ishizuya-Oka et al., 2000;Ishizuya-Oka et al., 1996;Jung et al., 2002;Oofusa et al., 1994;Patterton and Shi, 1994;Stolow et al., 1996). Among them, the Xenopus laevis MT1-MMP and GelA are coexpressed spatially and temporally in different tissues of the tail and intestine where GelA is expressed, although MT1-MMP but not GelA is also expressed in the longitudinal muscle of the metamorphosing intestine (Hasebe et al., 2006). Given the a number of in vitro and in vivo studies in mammals have implicated a role of MT1-MMP in the activation of pro-GelA into mature GelA (Nagase, 1998;Seiki, 1999;Zhou et al., 2000), the coexpression of GelA and MT1-MMP suggest that they function together in ECM remodeling and regulation of cell fate and behavior during frog development.

Toward studying the role of GelA and MT1-MMP during Xenopus development in vivo, we have made use of Xenopus embryogenesis as an in vivo model. We first show that both MMPs are expressed early during embryogenesis. To investigate the function of these MMPs in vivo, we have then introduced wild type and mutant MT1-MMP and GelA either individually or together into developing embryo. Our data suggest that MT1-MMP and GelA function together to affect Xenopus laevis development.

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RESULTS

Both GelA and MT1-MMP genes are expressed early during Xenopus embryogenesis

We have recently shown that the mRNA levels of both GelA and MT1-MMP are upregulated in the intestine and tail during metamorphosis and when premetamorphic tadpoles are treated with thyroid hormone to induced metamorphic changes (Hasebe et al., 2006). To determine how the MMP genes are regulated throughout Xenopus development, RT-PCR was carried out on total RNA isolated from whole embryos from stage 1 (fertilized egg) to stage 65, just before the completion of metamorphosis at stage 66. The result showed that MT1-MMP mRNA was detectable in whole embryos by stage 12, during gastrulation, and reached high levels by stage 34, just prior to hatching at stage 35/36 (Fig. 1). Its mRNA levels remained high throughout the rest of the development (Fig. 1). GelA mRNA was detectable a little later in development, starting around stage 30, tailbud stage (Fig. 1). Like MT1-MMP, GelA expression was high throughout tadpole and metamorphic stages (Fig. 1). The mRNA levels of both MT1-MMP and GelA in whole animals during metamorphosis contrast sharply with those in the intestine and tail. During metamorphosis, both genes are expressed at low levels prior to stage 60 but at high levels during intestinal remodeling and tail resorption from stages 62–64 (Hasebe et al., 2006), suggesting that these MMP genes are regulated in a tissue and developmental stage dependent manner during metamorphosis. Furthermore, the expression of the MMP genes, especially MT1-MMP, during early embryogenesis, suggests that they play a role in embryonic development.

Functional characterization of Xenopus GelA and MT1-MMP and their mutants

To investigate the possible role of these MMPs in Xenopus development, we asked the question whether precocious expression of these MMPs may affect Xenopus embryogenesis. For this purpose, we constructed a series of expression constructs for wild type and mutant GelA and MT1-MMP. First, we placed the FLAG tag at the carboxyl terminus of GelA (Fig. 2A). For MT1-MMP, we placed a FLAG or Myc tag between the propeptide domain and the catalytic domain since the carboxyl terminus is known to be important for membrane targeting while blocking the amino terminus would affect the function of the prepeptide (Urena et al., 1999) (Fig. 2A). In addition to the wild type MMPs, we also generated catalytically inactive mutants of both MMPs by mutating the conserved E residue in the catalytic domain to an “A”, at the position 401 for GelA and 231 for MT1-MMP, respectively (Crabbe et al., 1994). Finally, we also made two auto-activating mutants of GelA by replacing the N at position 100 within the propeptide domain to an R or G (Sanchez-Lopez et al., 1988).

To ensure that the wild type and mutant MMPs indeed have the desired catalytic properties, we overexpressed them in E. coli and analyzed their MMP activity by using gelatin zymography. As a positive control, we analyzed tail extract from metamorphosing tadpoles on the same gel. Similar to what observed before (Shi and Ishizuya-Oka, 1997), the tail extract produced several distinct gelatin-degrading bands, whose identifies are not known, although the middle and lowest bands of molecular weights similar to pro- (latent) and activated GelA, respectively (Fig. 2B). The wild type GelA expressed in E. coli gave predominantly a single band of expected size (full length plus the tag from the cloning vector) and a minor, smaller band, likely representing autoactivated GelA with part or all of the propeptide cleaved, possibly due to either the presence of an E. coli enzyme/protein capable of facilitating the processing of full length GelA or inappropriate folding of full length GelA in E. coli which allowed auto-activation (Fig. 2B). The auto-activating mutants GelA N100R and N100G had two gelatin-degrading bands, corresponding to the full length GelA and activated form (upon partial to complete removal of the pre- and propeptide) (Fig. 2B). In contrast, the inactive mutant GelA E401A failed to give any gelatin degrading band, even though all proteins were expressed to a similar level based on western blot of an identical gel. Thus, both the wild type GelA and its

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mutants had the intended catalytic properties. In addition, while there were two bands for wild type GelA and its auto-activating mutants on the zymogram, only a single peptide, the full length protein, was detectable by western blot. This was most likely because that the activated GelA was completely active while the full length GelA was probably only partially activated under our renaturation/activation conditions used in zymography. Thus much less activated GelA, not detectable by western blot compared to full length GelA, could have significant activity on a zymogram.

Unlike GelA, wild type MT1-MMP failed to show any gelatin degrading activity under our zymography conditions, even though the protein was overexpressed in E. coli to a level similar to that of GelA (within a few fold) based on western blot analysis by using the anti-FLAG antibody (Fig. 2B). To investigate whether this lack of gelatin-degrading activity was due to the inability of E. coli produced MT1-MMP to properly fold, we overexpressed MT1-MMP and GelA as well as their mutants in developing Xenopus embryos by microinjecting their mRNA into fertilized egg. One day later, protein extracts were prepared from the embryos and analyzed by zymography as before. Again, we found that wild type and autoactive mutant GelA were able to degrade gelatin as expected (Fig. 3). Quantitative analyses based on four independent zymograms showed that the auto-activating mutants GelA(N100G) and GelA (N100R) had about 24 and 9% of gelatin degrading activity in activated form compared to the 6% for the wild type GelA (Fig. 3), supporting the mutations, especially GelA(N100G), enhanced activation in vivo, as intended. As in Fig. 2 and for similar reasons, only a single band was detected by western blot even though considerable activity was found for the activated GelA on the zymogram, especially for GelA(N100G). Again,, we found that neither the wild type MT1-MMP nor its inactive mutant E231A produced any gelatin degrading band on the zymogram, even though both proteins were overexpressed in the embryos at similar levels as GelA and their mutants based on western blot with the anti-FLAG antibody (Fig. 3) (Note that the band detected on the western blot was the predominant form in embryos, represented latent or pro-GelA and activated/mature MT1-MMP, respectively, since MT1-MMP is activated intracellularly while GelA is secreted in the latent form. Thus, the molecular weights of the proteins detected here would be smaller compared to those produced in E. coli as shown in Fig. 2 by the amount of the pre-peptide and prepro-peptides for GelA and MT1-MMP, respectively, in addition to the extra N-terminal tag present in the proteins produced in E. coli).

While it is expected that GelA would have much higher activity toward gelatin than MT1-MMP in the zymography assay, the lack of any catalytic activity by wild type MT1-MMP under the conditions prompted us to investigate the activity of MT1-MMP by using an in vivo assay. This is based on the findings that mammalian MT1-MMP is capable of activating pro-GelA (Nagase, 1998;Seiki, 1999;Zhou et al., 2000). Thus, we coinjected mRNAs for wild type GelA and MT1-MMP or its inactive mutant into fertilized eggs. One day later, embryo extracts were prepared and subjected to gelatin zymography or coimmunoprecipitation analysis. As shown in Fig. 4A, overexpressing wild type GelA alone led to predominantly a single gelatin degrading band, the latent or pro-GelA, on the zymogram while overexpressing wild type or mutant MT1-MMP failed to do so. Coexpressing wild type GelA with wild type MT1-MMP but not catalytically inactive mutant MT1-MMP (E231A) led to enhanced the formation of two additional bands, a fast migrating band with a molecular weight similar to activated/mature GelA and an intermediate (Fig. 4A, top). Quantitative analyses based on four independent zymograms showed that the regions corresponding to the latent, intermediate, and activated forms of GelA had 72%, 20.5%, and 7.5% of the activity on the zymogram when wild type GelA was expressed alone (Fig. 4A, bottom). The numbers changed to 45.2%, 35.4%, and 19.4%, respectively, when wild type MT1-MMP was coexpressed, while the distribution was not altered when the mutant MT1-MMP was coexpressed (Fig. 4A, bottom). Thus, wild type MT1-MMP enhanced the activation of latent GelA, leading to the formation of more intermediate (from 20.5% to 35.4%) and activated (from 7.5% to 19.4%) GelA.

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To determine whether MT1-MMP interacts with GelA in vivo, we microinjected into fertilized eggs mRNAs for wild type FLAG-tagged GelA and wild type Myc-tagged MT1-MMP either individually or together. Embryo extracts were prepared for anti-FLAG immunoprecipitation followed by western blot analysis of the immunoprecipitates. As shown in Fig. 4B, immunoprecipitation of GelA with the anti-FLAG antibody from the extracts of embryos injected with mRNAs for both wild type FLAG-tagged GelA and wild type Myc-tagged MT1-MMP but not individually led to the coprecipitation of MT1-MMP. These results indicate that the wild type MT1-MMP is catalytically active while its E231A mutant lacks MMP activity in vivo, as intended. They further demonstrate that Xenopus MT1-MMP is capable of binding to and activating Xenopus GelA in developing embryos.

Overexpression of MT1-MMP and GelA causes developmental defects

Having established that the wild type and mutant MMPs have the desired catalytic property, we then investigated the consequence of their overexpression on embryo development. The mRNAs of MT1-MMP and GelA as well as their mutants were injected into developing embryos. Gross morphology of the embryos was examined and quantified daily. Within the first 3 days of embryogenesis, about 90% of the uninjected embryos or embryos injected with mRNA encoding GFP (green fluorescent protein) developed normally (Fig. 5). In contrast, embryos injected with mRNA encoding wild type MT1-MMP developed abnormally in a dose-dependent manner with 50% or 70% embryos developed abnormally with 1 or 3 ng mRNA injection, respectively (Fig. 5A, B). Various morphological phenotypes were present in embryos injected with both doses of mRNA and the higher dose led to higher percentage of abnormal embryos. Mutating the catalytic domain of MT1-MMP with the E231A substitution reduced the effect of overexpression on embryogenesis (Fig. 5B). Similarly, overexpression of wild type GelA also led to embryonic defect (Fig. 5C), although at similar levels of mRNA injection, more embryos injected with GelA mRNA were normal compared to those injected with wild type MT1-MMP mRNA (Compared Fig. 5D to B). Furthermore, the autoactive mutants GelA (N100G) and GelA (N100R) had more dramatic effects on embryogenesis than the wild type GelA while the inactivating mutant GelA (E401A) had little effects on embryogenesis (Fig. 5C and D). While the underlying molecular bases of the observed developmental effects were unclear, these results indicate that inappropriate expression of MMPs interferes with embryogenesis and this effect requires the catalytic activity of the MMPs.

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MT1-MMP and GelA cooperate to affect embryonic development

As shown above, MT1-MMP and GelA were coexpressed during late embryogenesis (after stage 30, Fig. 1). In addition, MT1-MMP is capable of activating proGelA in vivo (Fig. 4). Thus, we investigated whether MT1-MMP and GelA function together to affect embryonic development. We injected mRNA for wild type or mutant MT1-MMP together with the mRNA for GelA into developing embryos. We kept the total mRNAs injected at 0.5 ng or 1 ng. Phenotypic analysis of the embryos revealed that again at the 1 ng/embryo, MT1-MMP led to a high percentage of embryos develop abnormally while 1 ng of wild type GelA or catalytically inactivate GelA and MT1-MMP had little effect on embryonic development (Fig. 6, right panel), in agreement with the data in Fig. 5. In addition, 0.5 ng of the mRNA for any individual MMPs failed to affect embryogenesis at any significant rate (Fig. 6, left panel). In contrast, 0.25 ng each of the mRNAs for wild type GelA and MT1-MMP produced a similar effect as 1 ng of wild type MT1-MMP mRNA (Fig. 6, compared the left and right panels). Similarly, 0.5 ng each of the mRNAs for wild type GelA and MT1-MMP produced a much greater effect than 1 ng of wild type MT1-MMP mRNA alone while 1 ng of GelA had little effect by itself (Fig. 6, right panel). These results indicate the MT1-MMP and GelA cooperate with each other to affect embryonic development, likely through the activation of proGelA by MT1-MMP.

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DISCUSSION

The extracellular matrix plays a critical role in the development and homeostasis of multicellular organisms such as vertebrates. It serves as the structural scaffold for various organs/tissues and functions as a regulator of cell fate and behavior through direct contacts with cells, or indirect effects by regulating cell–cell interactions and the availability of various factors such as cytokines and their precursors stored in the ECM (Brown and Yamada, 1995;Frishch and Ruoslahti, 1997;Hay, 1991;Hay, 1993;Schmidt et al., 1993;Shi et al., 1998;Vukicevic et al., 1992;Werb et al., 1996). MMPs can cleave ECM proteins and can regulate development and pathogenesis through ECM remodeling. In addition, MMPs can also affect cells by cleaving non-ECM extracellular and membrane-bound proteins, such as growth factor precursors and cell surface receptors (Barrett et al., 2004;McCawley and Matrisian, 2001;Mott and Werb, 2004;Overall, 2002;Parks and Mecham, 1998;Sternlicht and Werb, 2001;Uria and Werb, 1998). As MMPs often have overlapping substrate specificities, the regulation of their expression and activation into mature MMPs are critical factors that dictate the developmental and pathological roles of MMPs.

We and other have previously shown that the Xenopus laevis GelA and MT1-MMP genes are upregulated indirectly by thyroid hormone in the intestine and tail during metamorphosis (Hasebe et al., 2006;Jung et al., 2002;Patterton et al., 1995). Extensive cell death occurs in the intestine and tail as the intestine undergoes drastic remodeling while the tail completely resorbs (Dodd and Dodd, 1976;Shi, 1999;Yoshizato, 1989). Interestingly, unlike another MMP, stromelysin-3, both GelA and MT1-MMP genes are upregulated later, with highest levels occurring around stage 62 or later in the intestine when cell death is near completion. These results suggest that while stromelysin-3 appears to play a role in regulating cell death (Fu et al., 2005;Ishizuya-Oka et al., 2000), GelA and MT1-MMP likely participate in post-apoptosis ECM remodeling to facilitate adult intestinal morphogenesis during metamorphosis. Furthermore, in situ hybridization analyses have revealed that GelA and MT1-MMP are coexpressed spatially and temporally in the intestine and tail with the exception that MT1-MMP but not GelA is also expressed in the longitudinal muscle of the metamorphosing intestine, raising the possibility that like in mammals, MT1-MMP also plays a role in the activation of pro-GelA into mature GelA by facilitating the removal of the propeptide in pro-GelA. Indeed, by expressing wild type or mutant GelA and MT1-MMP individual or together in developing Xenopus embryos, we have shown here that Xenopus laevis MT1-MMP facilitate the activation of pro-GelA to mature GelA. We have further shown that this activation requires the catalytic function of MT1-MMP.

Most of the studies on MMPs have been on the correlation of their expression with development and pathogenesis. It has been much more difficult to study their functions in vivo. A number of MMPs have been knocked out in mouse. Interestingly, mice lacking individual MMP genes often have little or mild phenotype (Masson et al., 1998;Shapiro, 1998;Vu et al., 1998;Wilson et al., 1997), likely due to redundancy and overlapping nature of their substrate specificity. An exception is that mouse lacking MT1-MMP have severe developmental defect (Holmbeck et al., 1999;Zhou et al., 2000). While many factors may contribute to this severity of the MT1-MMP knockout, one may be due to the fact that MT1-MMP knockout also reduce the function of GelA (Zhou et al., 2000) due to the involvement of MT1-MMP in GelA activation. The expression profiles of MT1-MMP and GelA during frog development and our demonstration here that Xenopus MT1-MMP can activate GelA in developing Xenopus embryo suggest that the cooperative effect of MT1-MMP and GelA in development is conserved through evolution.

In support of a cooperative role of MT1-MMP and GelA during Xenopus development, we have shown here that both MMPs are expressed during Xenopus embryogenesis, although MT1-MMP mRNA is detectable earlier than GelA. Furthermore, precocious expression of

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either one results in abnormal embryos. While some abnormality was seen in embryos injected with the inactive mutant of GelA or MT1-MMP, it was not statistically significant. Such abnormality was likely due to the dominant-negative effects of these MMPs as observed in zebrafish (Zhang et al., 2003) or possibly unknown functions independent of their enzymatic activity. Regardless, the effects were much more dramatic with catalytically active GelA or MT1-MMP. It is likely that some wild type GelA was activated by endogenous MT1-MMP or through other processes to affect embryogenesis. In addition, autoactive mutants of GelA had bigger effects than wild type GelA, supporting that catalytic activity is important for the developmental effects. More importantly, coexpression of wild type GelA and MT1-MMP leads to synergistic effects on embryogenesis and this synergy requires that both MMPs contain a functional catalytic domain.

It is worthwhile to note that MT1-MMP is also likely to act in GelA independent manner during frog development as (1) MT1-MMP expression becomes detectable earlier than GelA during embryogenesis (Fig. 1), (2) MT1-MMP but not GelA is expressed in the longitudinal muscle layer of metamorphosing intestine (Hasebe et al., 2006), and (3) MT1-MMP itself can also cleave ECM and non-ECM proteins as well as intracellular proteins (Golubkov et al., 2005a;Golubkov et al., 2005b;McCawley and Matrisian, 2001;Overall, 2002;Takahashi et al., 2002). Thus, it may not be surprising that embryos injected with MT1-MMP mRNA alone exhibited abnormality by 2 day old (stage 30, Fig. 5), when endogenous GelA expression was relatively low (Fig. 1). A GelA-independent mechanism is also consistent with the fact that overexpression of MT1-MMP appeared to have a more severe effect on embryogenesis compared to overexpression of autoactive mutants of GelA at similar levels of mRNA injection (Fig. 5).

Although it is difficult to determine the exact biological processes/pathways affected by GelA and/or MT1-MMP overexpression that led to the embryogenesis defects, it is quite likely that many tissues/cell types were affected since proper cell-cell and cell-ECM interactions are critical for organogenesis and tissue morphogenesis during embryonic development. Our results support the view that temporal and spatial regulation of different MMP expression and function is likely a critical factor in regulating cell-cell and cell-ECM interactions. Our results further suggest that during late embryonic development and during metamorphosis, MT1-MMP and GelA function cooperatively to affect cell fate and tissue remodeling through the activation of pro-GelA by MT1-MMP.

EXPERIMENTAL PROCEDURES

Animals

Larval and adult *Xenopus laevis* were purchased from *Xenopus* I Inc (Dexter, MI) and Nasco (Fort Atkinson, WI). Tadpoles were reared in dechlorinated tap water and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). Animal rearing and treatment were done according to the guidelines set by the NICHD animal use and care committee.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from whole body of tadpoles or juveniles at different stages was extracted by using TRIZOL reagent (Invitrogen, Carlsbad, CA) followed by DNase treatment with DNA-free (Ambion, Austin, TX) to remove any DNA contamination. The integrity of RNA was checked based on 18S and 28S ribosomal RNAs by electrophoresis. □RT-PCR was performed by using Super-Script One-Step RT-PCR with Platinum Taq (Invitrogen) according to the manufacture's instructions with 200 ng of total RNA as the template. For MT1-MMP detection, primers□ 5'-TACCAGATGATGACCGCAGAGG-3' and 5'-TAATGGAGCTGGGAAGACCACG-3' were used. □For GelA detection, primers 5'-

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GCTGTTTACCTGGAATGAGC-3' and 5'-TCCCCAGTTTCAGGAAGAC-3' were used. □As a loading control, histone H4 was amplified in a separate reaction with primers 5'-CGGGATAACATTCAGGGTATCACT-3' and 5'-ATCCATGGCGGTAACTGTCTTCCT-3'(Friedle et al., 1998). RT-PCR was performed by using the following program: RT reaction at 42°C for 30 min followed by denaturation at 94°C for 2 min and amplification with 28 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, then final elongation at 72°C for 4 min. RT-PCR products were run on 2% agarose gels, visualized with ethidium bromide staining under UV lights, and photographed with Kodak imaging system (Gel Logic 100 Imaging System, Kodak, New Haven, CT).

Plasmid DNA constructs

MT1-MMP—FLAG or Myc epitope tag was inserted between propeptide and catalytic domain of MT1-MMP (Itoh et al., 1999;Itoh et al., 2001) via a two-step PCR process by using *PfuUltra* high-fidelity DNA polymerase (Stratagene, La Jolla, CA) and pCR-Blunt II-MT1-MMP (Hasebe et al., 2006) as the template. Briefly, the upstream fragment of MT1-MMP-FLAG/Myc was amplified with MT1-forward primer (5'-GATATACATATGCCCGGGGCCGCCACCATGGAGCCTCTGAGAGCAGCC-3') and MT1-FLAG-reverse primer (5'-CTTGTCGTCGTCATCCTTGTAGTCTCTCTTCCGTCTCACATTTGCC-3'). For Myc tag insertion, MT1-Myc-reverse primer (5'-CAGATCCTCTTCTGAGATGAGTTTTTGTTCTCTCTTCCGTCTCACATTTGCC-3') was used instead of MT1-FLAG-reverse primer. The downstream fragment of MT1-MMP-FLAG was amplified with MT1-FLAG-forward primer (5'-GACTACAAGGATGACGACGACAAGTACGCCATCCAGGGTTTGAAGTGG-3') and MT1-reverse primer (5'-ATATTAGAATTCACTAGTTCACACTTTATCCAGGAGGGACCGCTGCC-3'). For Myc insertion, MT1-Myc-forward primer (5'-ACTACAAGGATGACGACGACAAGTACGCCATCCAGGGTTTGAAGTGG-3') was used instead of MT1-FLAG-forward primer. Both upstream and downstream fragments were mixed and amplified without any primer for 5 cycles followed by another 20 cycles after addition of MT1-forward and MT1-reverse primers to obtain the full-length MT1-MMP-FLAG and MT1-MMP-Myc cDNAs. The addition of an adenine to each end of the PCR product was performed by using *Taq* DNA polymerase (Promega, Madison, WI). The resulting cDNA was cloned into pCRT7/NT-TOPO vector (Invitrogen) (producing the clones pCRT7-MT1-MMP-FLAG and pCRT7-MT1-MMP-Myc, which contained the 6x His tag and the Express tag at N-terminus) and sequenced. MT1-MMP-FLAG and MT1-MMP-Myc was excised, without the N-terminal tags, from pCRT7-MT1-MMP-FLAG and pCRT7-MT1-MMP-Myc, respectively, with SmaI and SpeI to insert into EcoRV-SpeI sites of the T7Ts expression vector (a gift from G. J. C. Veenstra, University of Nijmegen, Nijmegen, The Netherlands), which is based on the pGEM-4Z vector (Promega) and contains the 5'□ and 3'□ untranslated regions of the *X. laevis* □-globin gene flanking the multiple cloning sites. To generate the inactive mutant of MT1-MMP (MT1-MMP(E231A)-FLAG and MT1-MMP(E231A)-Myc), a two-step PCR was performed by using T7Ts-MT1-MMP-FLAG and T7Ts-MT1-MMP-Myc plasmids as templates. The upstream fragment was amplified with MT1-forward primer and MT1(E231A)-reverse primer (5'-CATGGCCAAGTGCATGTACAGCCAC-3'). The downstream fragment was amplified with MT1-reverse primer and MT1(E231A)-forward primer (5'-GTGGCTGTACATGCACTTGGCCATG-3'). The full-length MT1-MMP(E231A)-FLAG and MT1-MMP(E231A)-Myc were amplified, inserted into T7Ts and sequenced as above (resulting in T7Ts-MT1-MMP(E231A)-FLAG and T7Ts-MT1-MMP(E231A)-Myc).

GelA—FLAG epitope tag was fused to C-terminus of GelA by PCR. Briefly, using cDNA from stage 62 intestine (Hasebe et al., 2006) as the template, GelA-FLAG was amplified with

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GelA-forward primer (5'-AACACCGGTGCCGCCACCATGCGGACAATTAAA-3') and GelA-FLAG-reverse primer (5'-CCCGGGTCACTTGTCGTCGTCATCCTTGTAGTCGCAGCGTAGCCAGTCATTTTGAC-3'). The resulting cDNA was inserted into pCRT7/NT-TOPO vector and sequenced as above (pCRT7-GelA-FLAG, which contained the 6x His tag and the Express tag at N-terminus of the resulting fusion protein). The inactive mutant (E401A) and the autoactive mutants (N100R and N100G) were generated via a two-step PCR process as above. The upstream fragment of E401A, N100R or N100G was amplified with GelA-forward primer and either GelA(E401)-reverse primer (5'-GCATGACCAAATGCATGAGCAGCTA-3'), GelA(N100R)-reverse primer (5'-TACATCGGGTCTCCCACATCTTGG-3') or GelA(N100G)-reverse primer (5'-TACATCGGGGCCCCCACATCTTGG-3'), respectively. The downstream fragment was amplified with GelA-FLAG-reverse primer and either GelA(E401)-forward primer (5'-TAGCTGCTCATGCATTTGGTCATGC-3'), GelA(N100R)-forward primer (5'-CCAAGATGTGGGAGACCCGATGTA-3') or GelA(N100G)-forward primer (5'-CCAAGATGTGGGGGCCCCGATGTA-3'), respectively. The full-length GelA(E401A)-FLAG, GelA(N100R)-FLAG and GelA(N100G)-FLAG were amplified with GelA-forward and GelA-FLAG-reverse primers, inserted into pCRT7/NT-TOPO vector and sequenced as above (pCRT7-GelA(E401A)-FLAG, pCRT7-GelA(N100R)-FLAG and pCRT7-GelA(N100G)-FLAG). The cDNAs encoding GelA and its mutants were excised from pCRT7, without the N-terminal tags, by SmaI and AgeI and inserted into T7Ts vector (T7Ts-GelA-FLAG, T7Ts-GelA(E401A)-FLAG, T7Ts-GelA(N100R)-FLAG and T7Ts-GelA(N100G)-FLAG).

Green fluorescent protein (GFP)—pCGCG (Fu et al., 2002) was digested with EcoRI followed by blunt-ending and cDNA encoding GFP was excised by AgeI and inserted into T7Ts vector as above.

Overexpression of MMPs in *E. coli*

Plasmids (pCRT7 vector) driving MT1-MMP-FLAG, GelA and its mutants were transformed into *E. coli* BL21 (DE3) strain (Invitrogen) and protein expression was induced according to the manufacture's instructions. Briefly, the cells were cultured in LB until OD reached 0.6. Protein expression was induced by adding 1 mM isopropyl-1-thio-beta-D-galactoside followed by incubation for 4 hours at 30°C. The pellet of bacteria was obtained by centrifugation at 15,000 x *g* for 3 min and proteins were extracted in 1X SDS-lading buffer (50 mM Tris, pH 7.5, 2% SDS, 0.1% BPB and 10% glycerol) (2 ml culture/100 μl buffer) followed by sonication and centrifugation. The supernatant was analyzed by western blotting (5 μl after adding 1% of 2-mercaptoethanol) or zymography (20 μl without 2-mercaptoethanol).

Embryo injection

The mRNA encoding MT1-MMP-FLAG, MT1-MMP-Myc, GelA-FLAG and their mutants were synthesized from the plasmid DNA constructs linearized with XmaI by using mMESSAGE mMACHINE (Ambion) according to the manufacture's instructions. Fertilized *Xenopus* eggs were dejellied in 0.1x Marc's modified ringers (0.1x MMR: 10 mM NaCl, 0.2 mM KCl, 0.1 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 0.5 mM HEPES, pH 7.6) containing 2% L-cysteine, washed 5 times with 0.1x MMR and kept in 0.1x MMR-6% Ficoll 400. At stage 2 (Nieuwkoop and Faber, 1956), embryos were injected with the indicated mRNAs into one blastomere. Dead or abnormal embryos found at 2 to 4 hours after injection were removed. The embryos were kept at 18°C overnight and transferred to 0.1x MMR at room temperature thereafter.

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Quantification of the percentage of normal embryo

At least 30 embryos were injected with each mRNA. The percentage of normal embryos (100% at day 0) was quantified every day until 3 days after injection and the mean values were plotted. Data were analyzed by ANOVA followed by Scheffe's posthoc test.

Protein preparation from embryos

Embryos injected with indicated mRNA were subject to protein extraction 1 day after injection. Briefly, 20 embryos were lysed by pipetting in 200 μl of IP buffer (20 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM β -glycerophosphate, 50 mM NaCl, 0.1% NP-40, protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN)). After centrifugation at 15000 x *g* for 15 min at 4°C, the supernatant was either diluted 1:1 with 2x SDS-loading buffer and then subjected to Western blotting (8 μl) and zymography (2 μl) analyses, or subjected to immunoprecipitation (IP) assay.

IP assay

80 μl of the supernatant obtained after centrifugation above was mixed with 320 μl of IP buffer and 10μl slurry of anti-FLAG-M2 agarose beads (EZview Red ANTI-FLAG M2 Affinity Gel, Sigma-Aldrich, St. Louis, MO). After the incubation overnight at 4°C, the beads were washed 3 times with IP buffer. Immunoprecipitates were eluted with 20 μl of 1x SDS-loading buffer-1% 2-mercaptoethanol and 8 μl was loaded for Western blotting.

Western blot analysis

The protein samples were electrophoresed on a 4-20% or 8–16% Novex Tris-Glycine gel (Invitrogen) followed by transferring onto PVDF membrane (Bio-Rad, Hercules, CA). The membrane was immediately washed with Tris-buffered saline containing 0.5% Tween-20 (TBST), blocked with TBST-5% non-fat dry milk (Bio-Rad) for 30 min, and incubated for 2 hours at room temperature with the indicated primary antibody diluted in TBST-5% milk. The antibodies used were anti-FLAG M2 mAb (Sigma-Aldrich, 1/5000 dilution), and anti-Myc pAb (Novus Biologicals, Littleton, CO, 1/1000 dilution). After washing 3 times with TBST, the membrane was incubated for 1 hour at room temperature with the secondary antibody against either mouse or rabbit IgG, which is conjugated with peroxidase (GE Healthcare Life Sciences, Piscataway, NJ), depending on the primary antibody used. After washing 3 times with TBST, peroxidase activity was detected by using Amersham ECL plus Western blotting detection reagents (GE Healthcare Life Sciences) with an imaging film (BioMax XAR Film, Kodak).

Gelatin zymography

Protein samples were electrophoresed on a 10% Novex gelatin zymogram gel (Invitrogen), followed by in-gel renaturation and substrate cleavage for two days by using zymogram renaturing and developing buffers (Invitrogen) according to the manufacture's instructions. The gelatinolytic activity was visualized as a clear band in the blue background after staining with Coomassie Blue. As a positive control, 1 μg of total protein extract, which was dissolved in 1x SDS-loading buffer, from stage 62 tadpole tail was loaded on to the gel. The intensity of the positive signal on the zymogram was quantified by the densitometry tool of Image J software (http://rsb.info.nih.gov/ij/) and the percentages of latent, intermediate, and activated forms of GelA were calculated based on zymograms from four independent experiments and presented as mean ± standard error.

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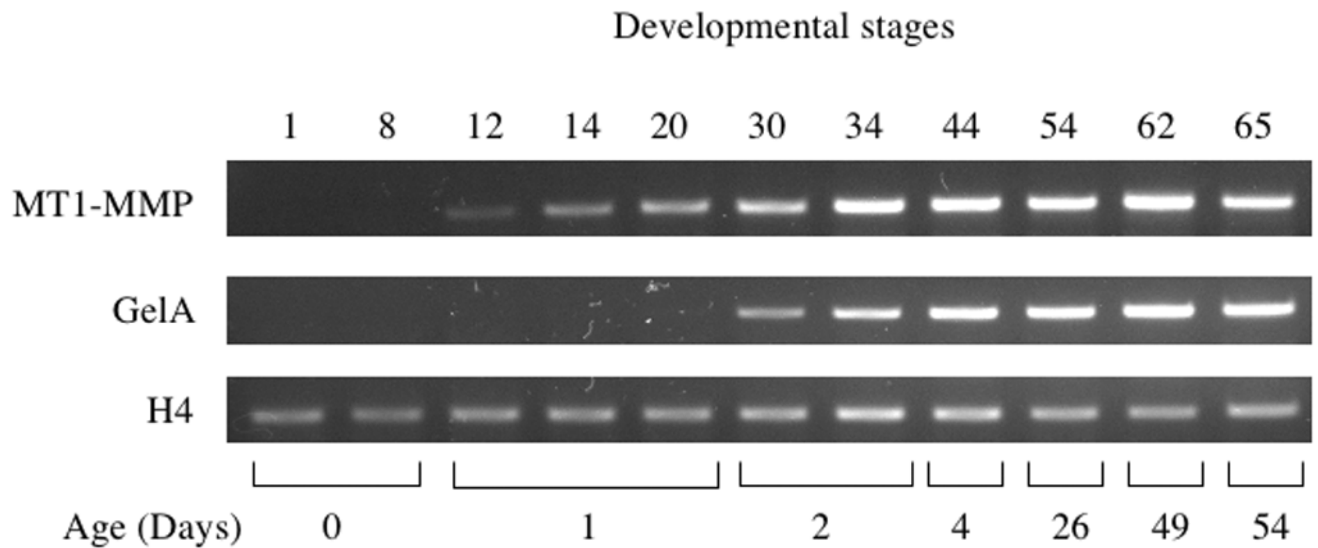

Figure 1.
MT1-MMP and GelA expression is activated early during Xenopus laevis development. Total RNA (200 ng/reaction) from whole animals at indicated stages was analyzed by RT-PCR using specific primers for MT1-MMP, GelA, and histone H4 (as a loading control). Note that the expression of MT1-MMP was detected in gastrulae (by stage 12) while GelA becomes detectable by stage 30, prior to hatching around stages 35/36. The age in days when the embryos or tadpoles develop into the indicated stages are indicated at the bottom.

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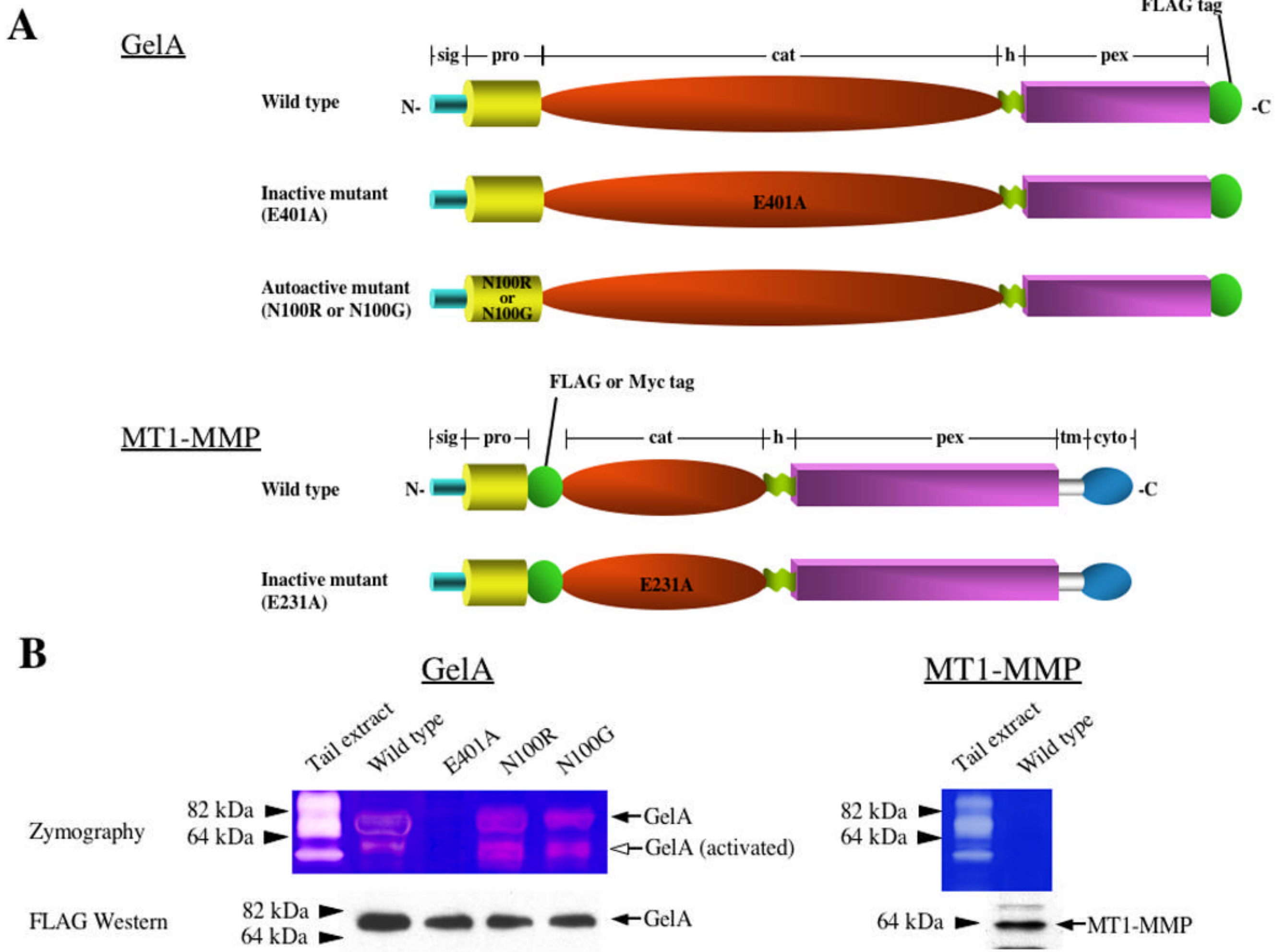

Figure 2.

In vitro analysis of gelatinolytic activity of wild type and mutant GelA and MT1-MMP produced in *E. coli*. **(A)** Diagrams of various MMPs used for the study. MMPs consist of the signal or pre-peptide (sig), propeptide (pro), catalytic domain (cat), hinge region (h), and hemopexin-like domain (pex) from N- to C-terminus. MT1-MMP has 2 additional domains: the transmembrane domain (tm) and cytoplasmic tail (cyto). For detection, a FLAG or Myc epitope tag was fused to the C-terminus of GelA or inserted between propeptide and catalytic domain of MT1-MMP as indicated. Amino acid substitutions from Glu_{401} to Ala of GelA (E401A) and from Glu_{231} to Ala of MT1-MMP (E231A) were performed to generate their inactive mutant, respectively. To generate autoactive mutants of GelA, Asn_{100} was substituted to Arg (N100R) or Gly (N100G).

(B) Gelatin zymography. FLAG-tagged GelA, MT1-MMP, and their mutants were overexpressed in *E. coli*. Gelatinolytic activities of the *E. coli* extract were examined by gelatin zymography with the tail extract from stage 62 tadpoles (the metamorphic climax when tail resorption occurs) used as a positive control (top panels). Duplicated gels were also analyzed by western blotting with anti-FLAG antibody to show the expression of the desired protein (bottom panels). Note that MT1-MMP did not show any gelatinolytic activity and as expected, both GelA wild type and autoactive mutants (N100G and N100R) but not inactive mutant (E401A) had activity. Both latent form (arrow) and the activated form (open arrow) of GelA (except for the inactive mutant E401A) were detected on the zymogram gel, although only the

latent form was detected on western blot, suggesting that the activated form was, expectedly, much more active than the latent or full length form under the zymography conditions. The locations of the molecular weight standards are indicated with arrowheads.

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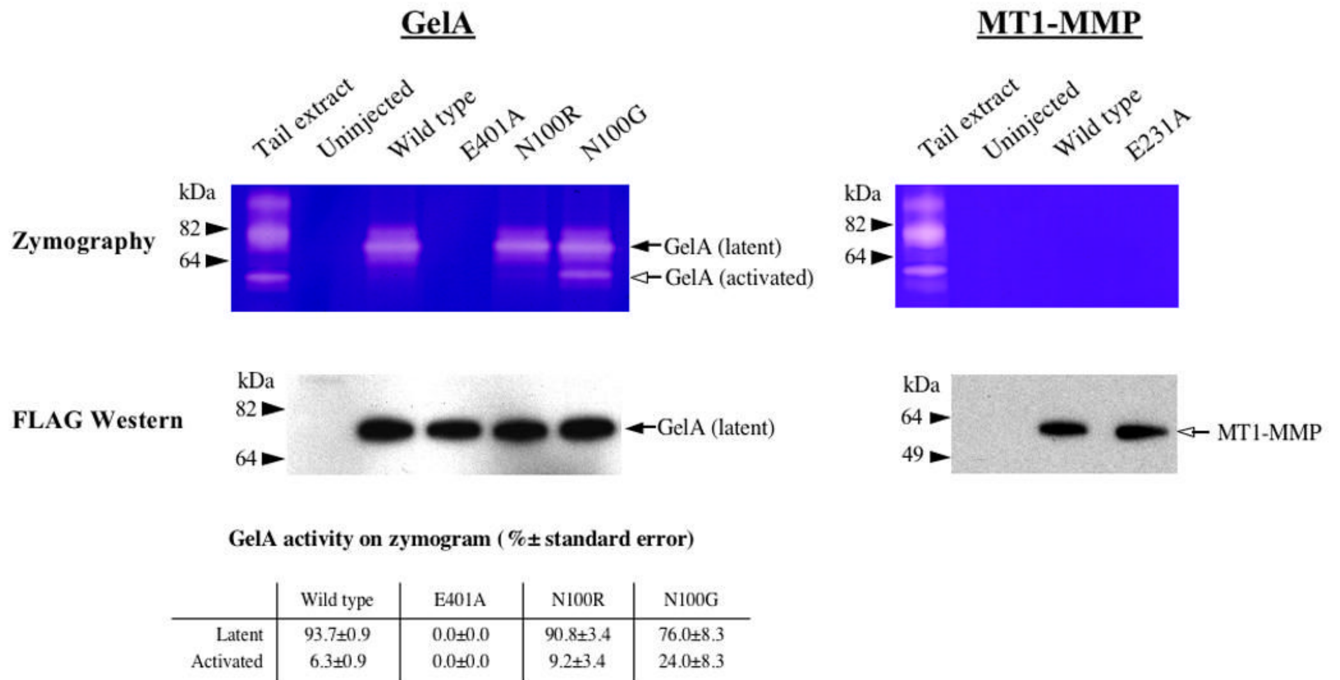

	Wild type	E401A	N100R	N100G
Latent	93.7±0.9	0.0±0.0	90.8±3.4	76.0±8.3
Activated	6.3±0.9	0.0±0.0	9.2±3.4	24.0±8.3

Figure 3.

In vitro analysis of gelatinolytic activity of wild type and mutant GelA and MT1-MMP produced in *Xenopus* embryos. The mRNAs encoding FLAG-tagged GelA, MT1-MMP or their mutants were injected into one blastomere of stage 2 embryos (3 ng/embryo). Proteins were extracted 1 day after injection and subjected to gelatin zymography (top panels) or Western blotting (bottom panels) as in Fig. 2. Note that again wild type MT1-MMP (as well as the inactive mutant E231A) did not exhibit any gelatinolytic activity and that GelA wild type and autoactive mutants (N100G and N100R) but not inactive mutant (E401A) had activity. Both latent form (arrow) and the activated form (open arrow) of GelA were detected on the zymogram gel (the activated form was much weaker for wild type and autoactive mutant N100R, and no activity was detected for the inactive mutant E401A), although only the latent form was detected on western blot. The locations of the molecular weight standards are indicated with arrowheads. The percentage of each form of GelA on the zymogram was calculated by densitometry from four independent experiments and shown at the bottom.

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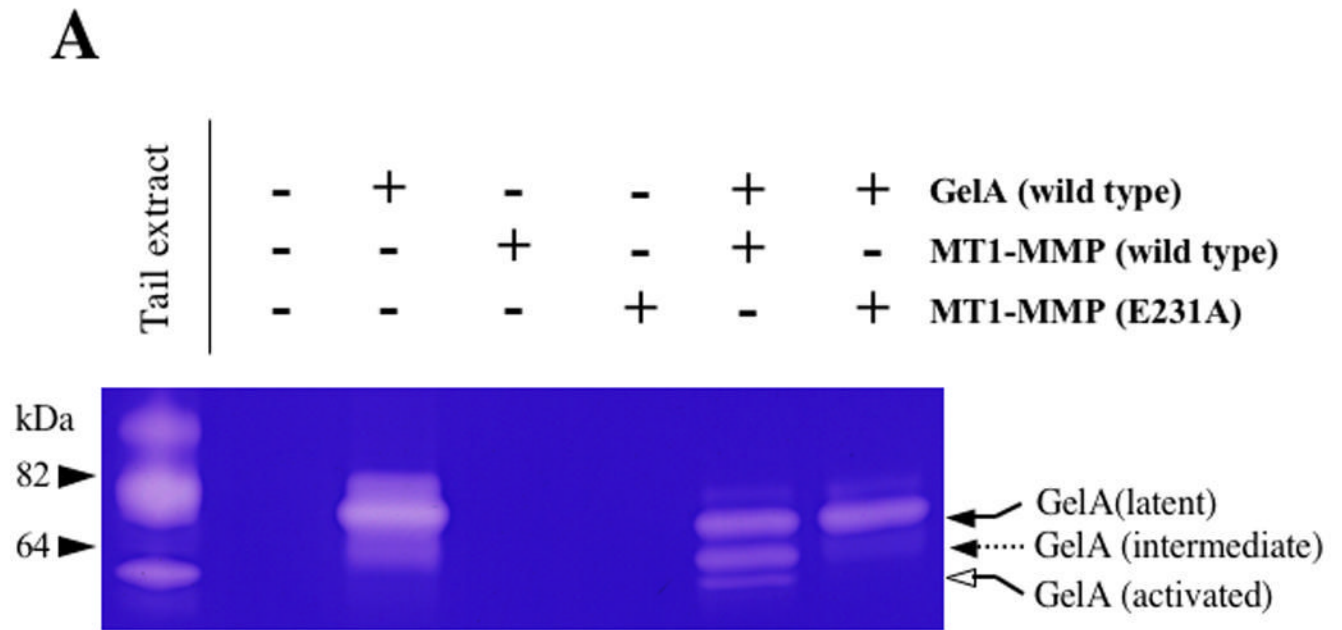

GelA activity on zymogram (%± standard error)

	GelA alone	GelA+MT1-MMP (wild type)	GelA+MT1-MMP (E231A)
Latent	72.0±3.2	45.2±5.3	76.8±3.6
Intermediate	20.5±5.7	35.4±1.2	17.7±2.8
Activated	7.5±2.8	19.4±5.1	5.5±3.8

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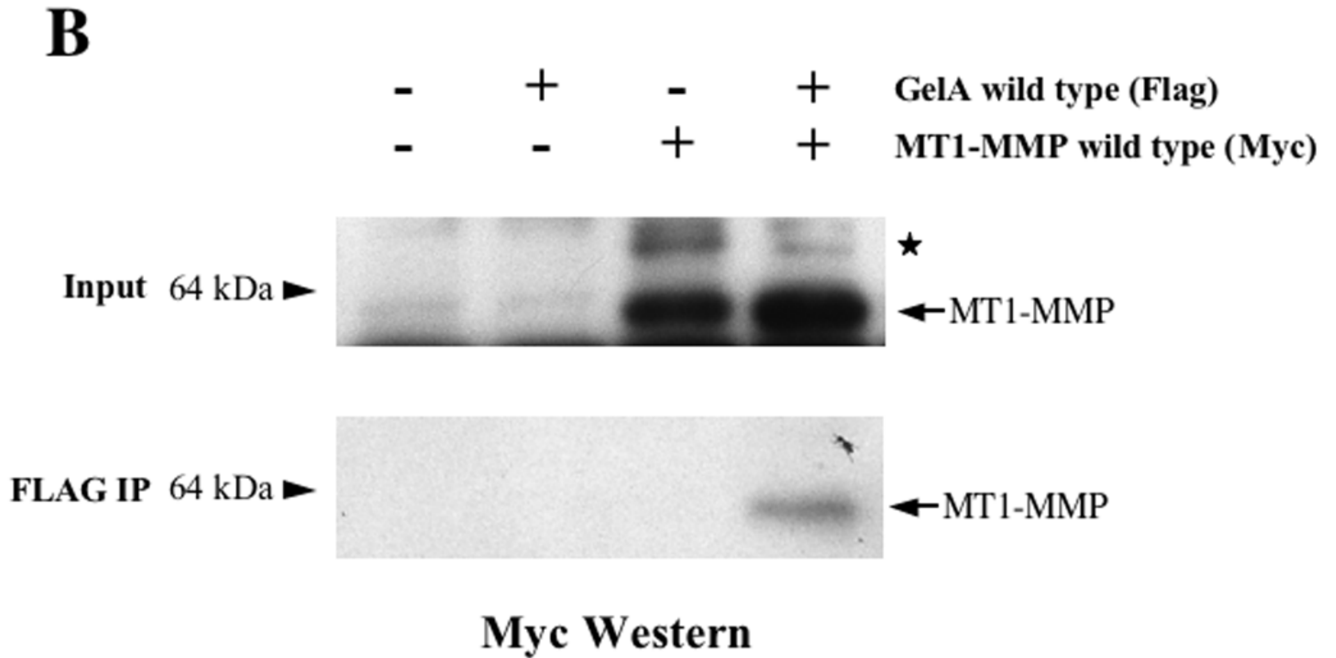

Figure 4.

MT1-MMP interacts with GelA and facilitates its activation *in vivo*. **(A)** Coexpression of MT1-MMP enhances the formation of activated (mature) GelA in vivo. The mRNA encoding FLAG-tagged GelA wild type was injected either alone or together with the mRNA encoding MT1-MMP wild type or inactive mutant (E231A) into one blastomere of stage 2 embryos. Proteins were extracted 1 day after injection and subjected to gelatin zymography with the tail extract from stage 62 tadpoles (the metamorphic climax when tail resorption occurs) used as a positive control. The latent, activation intermediate, and activated forms are indicated with an arrow, dashed arrow, and open arrow, respectively. The percentage of latent, intermediate, and activated form of GelA was calculated by densitometry from four independent experiments and shown at the bottom.

(B) GelA and MT1-MMP interacts in vivo. The mRNA encoding FLAG-tagged GelA wild type and Myc-tagged MT1-MMP wild type were injected either alone or together into one blastomere of stage 2 embryos. Proteins were extracted 1 day after injection and subjected to Western blotting using anti-Myc antibody before (input, top) or after immunoprecipitation with anti-FLAG M2 antibody (FLAG IP, bottom). The arrow indicates the position of Myc-tagged MT1-MMP. The origin of the minor band labeled with a star above the MT1-MMP band is unclear. It might be a form of MT1-MMP modified post-translationally, although we did not detect such a band when the MT1-MMP was labeled with the FLAG tag (Fig. 3). Regardless, it does not affect the conclusion about GelA-MT1-MMP interaction. The arrowheads in A and B indicate the positions of molecular weight markers.

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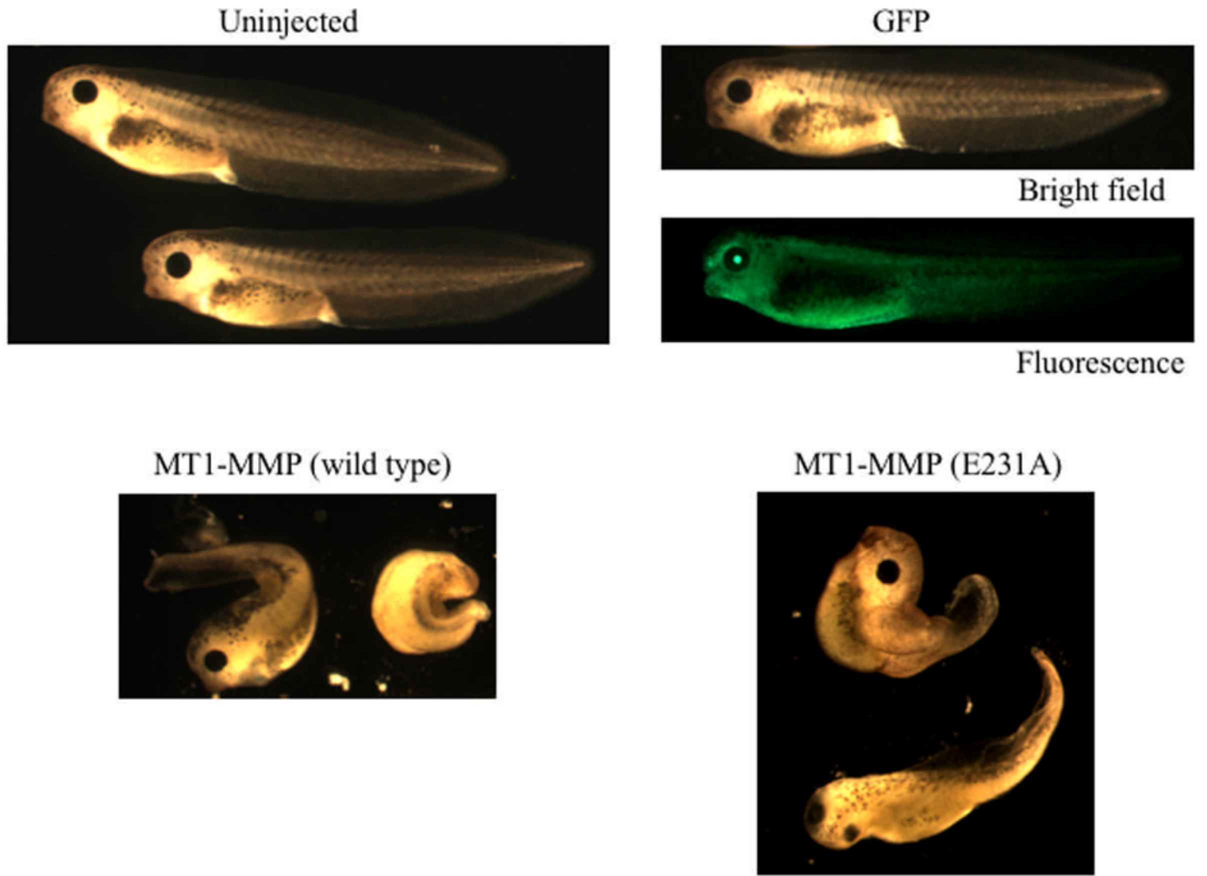

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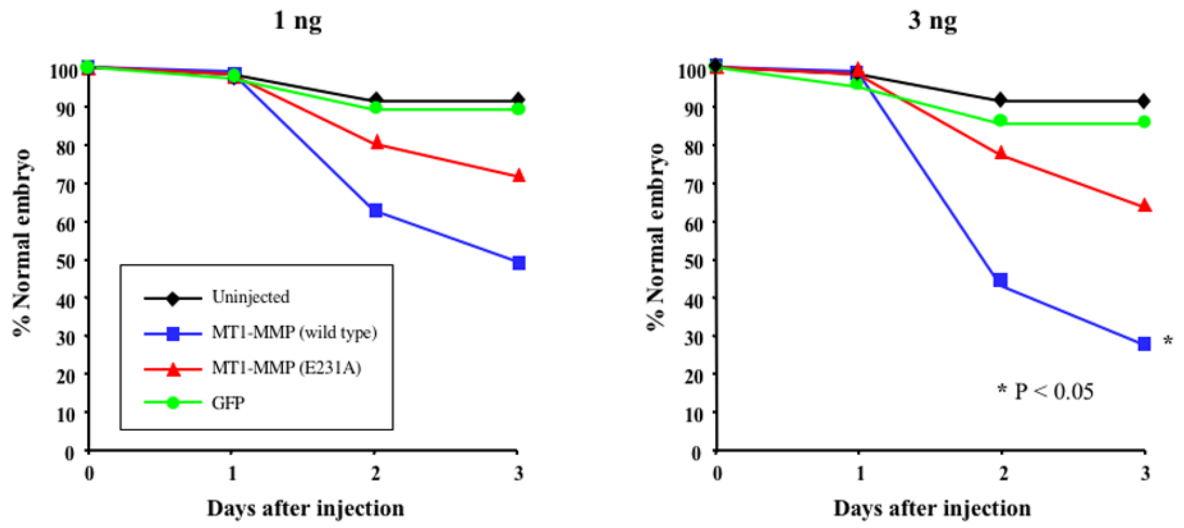
B



Standard error (1 ng)

	1	2	3 (Days)
Uninjected	2.74	15.12	15.12
MT1-MMP (wild type)	1.56	17.20	24.30
MT1-MMP (E231A)	3.20	16.89	23.41
GFP	4.81	11.11	11.11

Standard error (3 ng)

	1	2	3 (Days)
Uninjected	2.74	15.12	15.12
MT1-MMP (wild type)	1.28	7.20	21.97
MT1-MMP (E231A)	3.03	14.79	29.23
GFP	8.88	14.53	14.53

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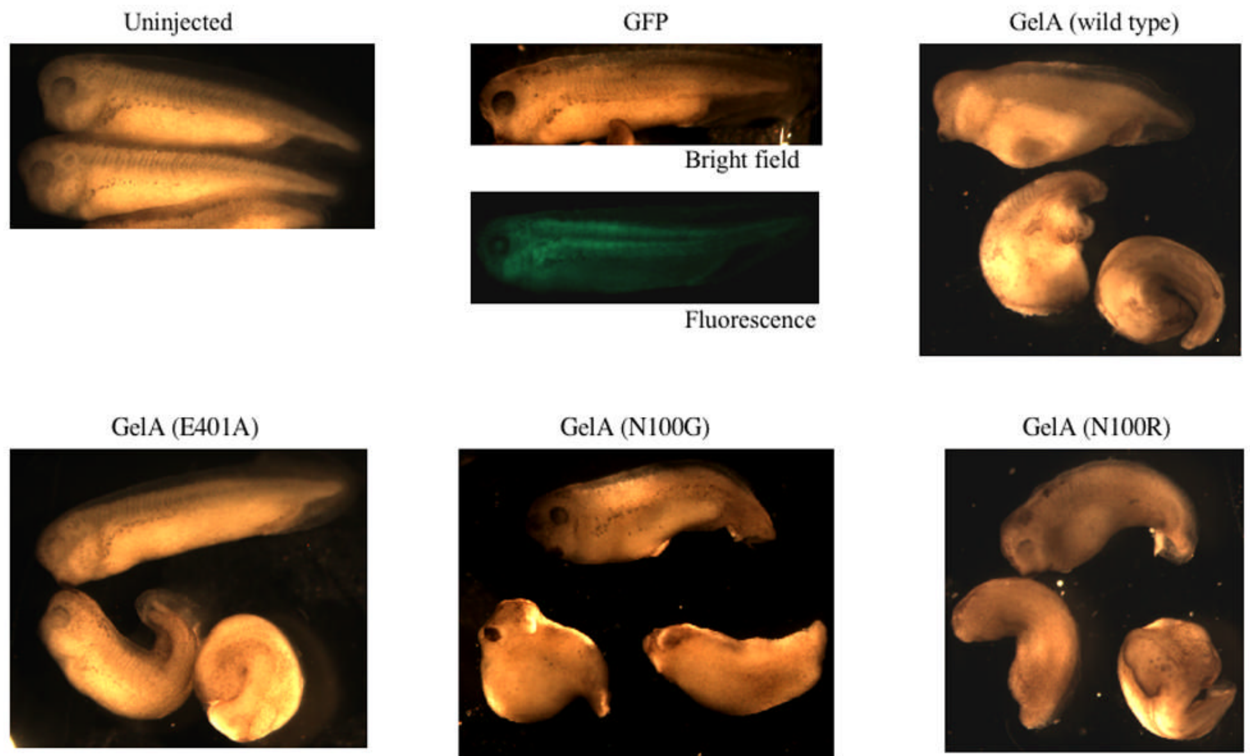

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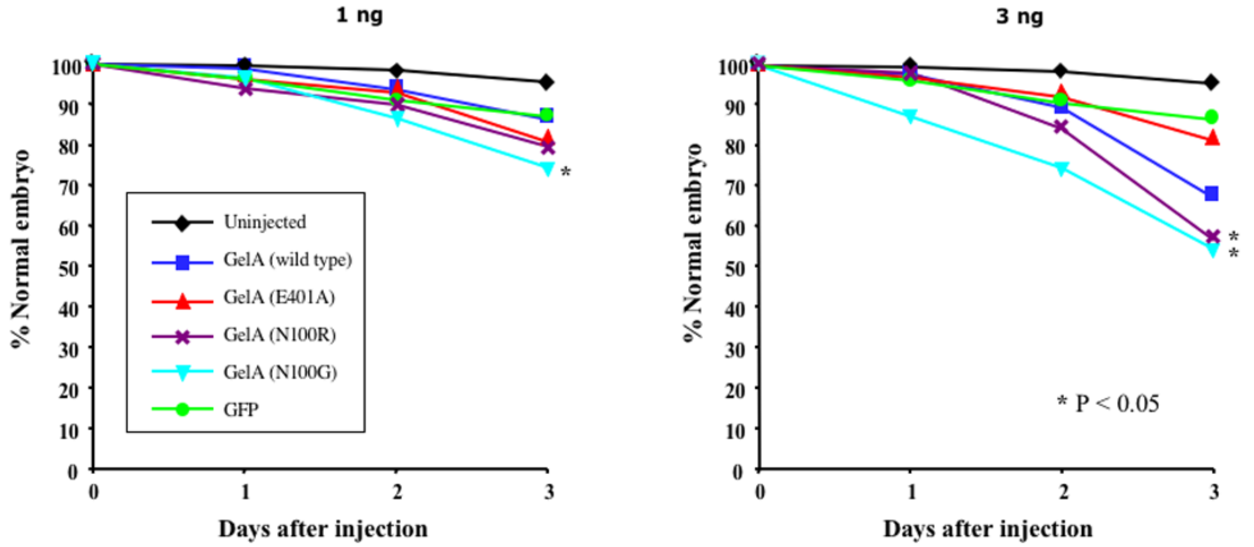
D



Standard error (1 ng)

	1	2	3	(Days)
Uninjected	0.79	1.77	1.44	
GelA (wild type)	2.63	5.41	1.57	
GelA (E401A)	4.86	7.28	8.57	
GelA (N100R)	6.07	7.76	4.34	
GelA (N100G)	4.14	10.89	8.23	
GFP	3.53	7.10	10.26	

Standard error (3 ng)

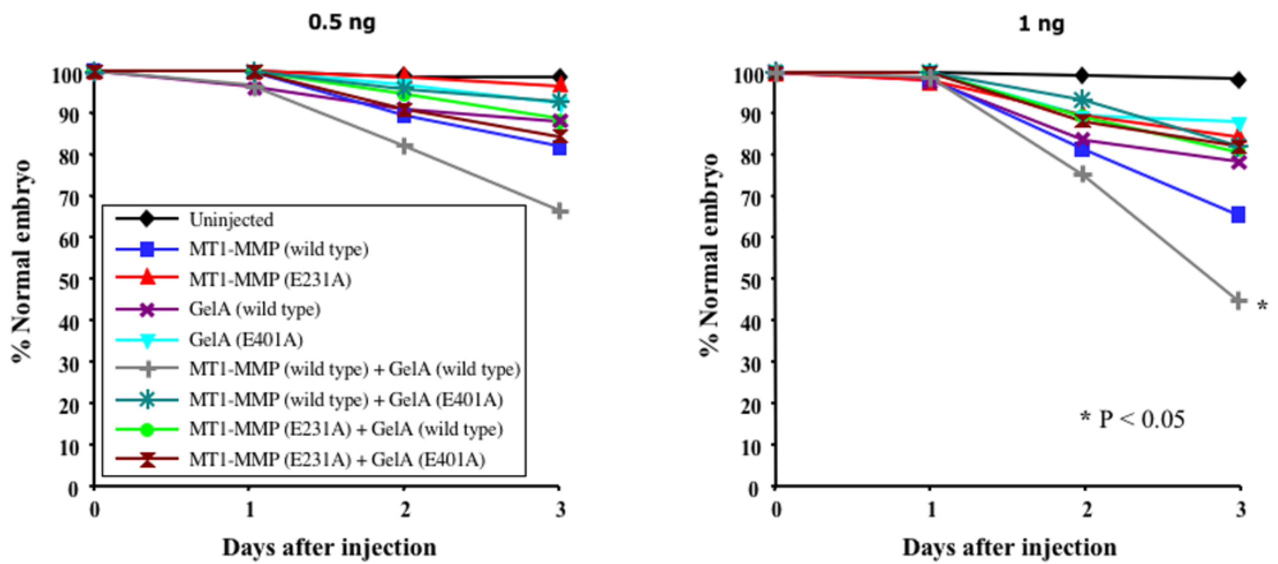
	1	2	3	(Days)
Uninjected	0.79	1.77	1.44	
GelA (wild type)	2.72	11.18	6.61	
GelA (E401A)	2.48	5.95	4.28	
GelA (N100R)	2.87	4.92	25.80	
GelA (N100G)	10.62	15.25	12.27	
GFP	2.60	5.47	10.47	

Figure 5.

Overexpression of GelA and MT-1 MMP leads to embryonic defects. 1 ng or 3 ng of mRNA encoding either GFP, GelA wild type, MT1-MMP wild type, or their mutants was injected into one blastomere of stage 2 embryos (day 0). Photographs were taken 3–4 days after injection (**A** and **C**) (with 3 ng mRNA). The percentage of embryos (relative to day 0) which were morphologically normal was determined each day and analyzed by ANOVA followed by Scheffe's posthoc test for embryos injected with 1 or 3 ng mRNA (**B** and **D**).

(A) and **(B)** Overexpression of both MT1-MMP wild type and inactive mutant E231A but not GFP caused deformation during early development. The quantification in (C) was based on 3 independent sets of experiments. Note that the wild type was more effective than the inactive mutant in affecting development.

(C) and **(D)** Overexpression of GelA wild type and autoactive mutants N100G and N100R caused severe deformation as compared to uninjected, GFP-injected, or E401A-injected animals. The quantification in (D) was based on 4 independent sets of experiments. Note that the autoactive mutants N100G and N100R were more effective than the wild type in affecting development.



Standard error (0.5 ng)

	1	2	3 (Days)
Uninjected	0	1.91	1.91
MT1-MMP (wild type)	0	2.86	7.36
MT1-MMP (E231A)	0	1.81	3.07
GelA (wild type)	4.97	5.79	7.53
GelA (E401A)	0	6.76	8.29
MT1 (wt) + GelA (wt)	3.93	13.94	23.03
MT1 (wt) + GelA (E401A)	0	3.86	7.06
MT1 (E231A) + GelA (wt)	0	9.65	10.27
MT1 (E231A) + GelA (E401A)	0	15.29	24.69

Standard error (1 ng)

	1	2	3 (Days)
Uninjected	0.00	1.91	1.91
MT1-MMP (wild type)	3.13	9.54	12.63
MT1-MMP (E231A)	3.57	11.56	8.85
GelA (wild type)	2.38	13.63	16.86
GelA (E401A)	0.00	15.29	16.52
MT1 (wt) + GelA (wt)	1.55	21.25	20.67
MT1 (wt) + GelA (E401A)	0.00	9.03	13.95
MT1 (E231A) + GelA (wt)	0.00	16.84	14.75
MT1 (E231A) + GelA (E401A)	0.00	16.15	25.25

Figure 6.

MT1-MMP and GelA cooperate to affect development. The mRNA encoding MT1-MMP wild type or E231A was injected either alone or together with the mRNA encoding GelA wild type or E401A into one blastomere of stage 2 embryos. Total amount of mRNA injected was 0.5 ng (left panel, e.g., 0.5 ng per mRNA if only one mRNA was injected or 0.25 ng per mRNA when two mRNAs were injected) or 1.0 ng (right panel). The percentage of normal embryo was analyzed as in Fig. 5 based on 4 independent sets of experiments. Note that coexpression of MT1-MMP wild type and GelA wild type had much more dramatic effect on development than either one alone, even though total RNA injected was the same. Mutation of the catalytic domain of MT1-MMP eliminated this cooperative effect.

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