

Published in final edited form as:

Biochim Biophys Acta. 2010 January ; 1803(1): 55–71. doi:10.1016/j.bbamcr.2010.01.003.

The tissue inhibitors of metalloproteinases (TIMPs): An ancient family with structural and functional diversity

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Abstract

Tissue inhibitors of metalloproteinases (TIMPs) are widely distributed in the animal kingdom and the human genome contains four paralogous genes encoding TIMPs 1 to 4. TIMPs were originally characterized as inhibitors of matrix metalloproteinases (MMPs), but their range of activities has now been found to be broader as it includes the inhibition of several of the disintegrin-metalloproteinases, ADAMs and ADAMTSs. TIMPs are therefore key regulators of the metalloproteinases that degrade the extracellular matrix and shed cell surface molecules. Structural studies of TIMP–MMP complexes have elucidated the inhibition mechanism of TIMPs and the multiple sites through which they interact with target enzymes, allowing the generation of TIMP variants that selectively inhibit different groups of metalloproteinases. Engineering such variants is complicated by the fact that TIMPs can undergo changes in molecular dynamics induced by their interactions with proteases. TIMPs also have biological activities that are independent of metalloproteinases; these include effects on cell growth and differentiation, cell migration, anti-angiogenesis, anti- and pro-apoptosis, and synaptic plasticity. Receptors responsible for some of these activities have been identified and their signaling pathways have been investigated. A series of studies using mice with specific TIMP gene deletions has illuminated the importance of these molecules in biology and pathology.

Keywords

Matrix metalloproteinase; Extracellular matrix; Evolution; Multifunctional protein; Cell growth; Sorsby fundus dystrophy

1. Introduction

The appropriate assembly of the extracellular matrix (ECM) from its many components is essential in multicellular organisms for organizing cells and tissues and maintaining a suitable environment for cellular functions. Changes in ECM composition are crucial for embryonic development, morphogenesis, tissue remodeling and repair. The major proteinases involved in ECM catabolism are the matrix metalloproteinases (MMPs), which include collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and others [1] and the ADAMTS (a disintegrin and metalloproteinase with thrombospondin domains) metalloproteinases [2]. The membrane-anchored ADAM metalloproteinases, often called “sheddas”, also affect

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cellular behavior by proteolytically releasing the extracellular domains of cell surface molecules such as membrane-bound growth factors, cytokines and cell adhesion molecules [3]. The activities of these metalloproteinases are precisely regulated under physiological conditions at the levels of transcription, zymogen activation and inhibition by endogenous inhibitors. Disruption of the balance between the production of active enzymes and their inhibition may result in diseases associated with uncontrolled ECM turnover, inflammation, cell growth and migration, such as arthritis, cardiovascular disease, cancer, pulmonary disease, nephritis, neurological disorders and tissue ulceration. Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of these metalloproteinases and are consequently important regulators of ECM turnover, tissue remodeling and cellular behavior.

The inhibitory activity of TIMP-1 was discovered in the early 1970s in the form of a collagenase inhibitor in the media of cultured human skin fibroblasts [4], human serum [5], and in extracts of bovine cartilage and aorta [6]. The inhibitor was purified and found to be a 25–31 kDa protein in 1979 [7,8]. It was later designated “tissue inhibitor of metalloproteinases” or “TIMP” as it inhibited not only collagenases, but also gelatinases and proteoglycanase (now called matrix metalloproteinase 3/MMP-3) [9]. The human genome has 4 paralogous genes encoding TIMPs (TIMPs-1 to -4). All four TIMPs inhibit MMPs, but with affinities that vary for different inhibitor-protease pairs. Among the four TIMPs, TIMP-3 has the broadest inhibition spectrum as it inhibits several members of the ADAM and ADAMTS families [10]. It also differs from the others in being tightly bound to the extracellular matrix [11]. In addition to their metalloproteinase inhibitory activity, TIMPs have various biological activities such as promoting cell proliferation, anti-angiogenic, pro- and anti-apoptotic and synaptic plasticity activities, many of which are independent of metalloprotease inhibition.

In 2000, we reviewed the structure, function and evolution of TIMPs [12]. Since then considerable progress has been made in the field; genome projects have shown that the TIMP family is widely distributed in the animal kingdom and is structurally diverse. Structural studies have led investigators to generate TIMP mutants with selective inhibitory activities and we have begun to understand the molecular mechanisms through which TIMPs elicit metalloproteinase-independent biological activities. This review focuses on these topics by interlacing earlier “classical” work and recent advances.

2. Genes and protein structures of TIMPs

The four TIMPs encoded by the human genome are the most extensively characterized with respect to structure, activity and biological function. Although a comprehensive survey has not been made, it appears that orthologues of all four human TIMPs are present in other mammals. Homologues of the TIMPs are distributed widely among both invertebrate and vertebrate animals, but there is limited information available on the properties of non-mammalian TIMPs. Structural similarities between TIMPs and other proteins have also been noted, e.g. netrins, agrins and some bacterial proteins [13]. Before discussing the larger protein family, we review the structures and properties of the well-characterized mammalian TIMPs.

2.1. Mammalian TIMPs

Some of their general properties are summarized in Table 1. The genes for TIMPs-1, -3 and -4, but not TIMP-2, are each nested within an intron in the genes for synapsins; TIMP-1 is associated with synapsin 1, TIMP-3 with synapsin 3 and TIMP-4 with synapsin 2. Synapsins are peripheral neuronal proteins that coat the cytoplasmic surfaces of synaptic vesicles. This inter-relationship appears to be ancient because the gene for the single TIMP in *Drosophila melanogaster* is nested within an intron of the only *Drosophila* synapsin (*Syn*) gene [14]. In the genome of *Fugu rubripes*, the tiger blowfish, a pair of nested *Syn-Timp* genes are present together with a *Syn* gene that does not contain a *Timp*, but there are two *Timp* genes that are

separate from *Syn* genes [15]. The nested gene arrangement suggests the possibility of coordinated transcription or alternative splicing between TIMP and synapsin genes, but there appears to be no evidence that this occurs. The gene for TIMP-2 is not nested within a *Syn* gene, but hosts the gene DDC8 (differential display clone 8), a gene that is expressed in the testis at an enhanced level during spermatogenesis. Recent studies indicate that this gene is also expressed in other tissues in a manner similar to TIMP-2, e.g., in response to an injury to the cerebral cortex. A regulatory relationship between the two genes is suggested by an alternative spliced mRNA variant involving the two genes [16].

All mammalian TIMPs have two distinct domains, an N-terminal domain of about 125 amino acid residues and a C-terminal domain with about 65 residues; the conformation of each domain is stabilized by three disulfide bonds [17]. The four human TIMPs are about 40% identical in sequence to each other; the most similar pair is TIMP-2 and TIMP-4, which are 50% identical in sequence, whereas TIMP-1 is only 37 to 41% identical to the other TIMPs. Although the N- and C-terminal regions of TIMPs have been described as “subdomains” [18], the more definitive term “domain” is appropriate because of their separate evolutionary origin and ability of the N-terminal region to fold and function independently. Recombinant forms of the N-terminal domains of TIMPs, designated N-TIMPs, expressed in heterologous systems, have stable native structures and are fully active as inhibitors of matrix metalloproteinases (MMPs) and some disintegrin-metalloproteinases (ADAMs and ADAMTSs) [19–22]. N-TIMPs have been widely used in characterizing the biochemical and biophysical properties of TIMPs and for investigating structure–function relationships.

2.2. Functional specialization for metalloproteinase inhibition

The four human TIMPs are, in general terms, broad-spectrum inhibitors of the 23 MMPs found in humans, but there are some differences in specificity among them. TIMP-1 is more restricted in its inhibitory range than the other three TIMPs, having a relatively low affinity for the membrane-type MMPs, MMP-14, MMP-16, and MMP-24 as well as for MMP-19 [10]. Also, there are some relatively subtle differences between the affinities of different TIMPs for other MMPs. For example, TIMPs-2 and -3 are weaker inhibitors than TIMP-1 for MMP-3 and MMP-7, contrasting with their affinities for other MMPs [23]. TIMP-3 is unique among the mammalian TIMPs in inhibiting a broader array of metalloproteinases including several members of the ADAM and ADAMTS families [21,22,24–27]. Other TIMPs have limited inhibitory activities for ADAMs: TIMP-1 and TIMP-2 inhibit ADAM10 [24] and ADAM12 [27], respectively. TIMP-3 and N-TIMP-4, but not full-length TIMP-4, inhibit ADAM17 [28]. TIMP-4 was also reported to inhibit ADAM28 [29]. ADAM metalloproteinases differ from the MMPs in domain structures and are highly divergent in catalytic domain sequences: ADAMs are membrane-bound enzymes containing disintegrin, cysteine-rich, EGF-like and transmembrane domains C-terminal to their catalytic domains [3]; and ADAMTS (disintegrin-metalloproteinases with thrombospondin motifs) are secreted proteins with a disintegrin domain and variable numbers of thrombospondin type 1 motifs and other domains in their C-terminal regions [2]. In humans there are 21 members of the ADAM family, of which only 13 are functional proteases, and there are 19 ADAMTSs.

The first non-MMP metalloproteinase reported to be inhibited by TIMP-3 was ADAM17, also known as tumor necrosis factor- α converting enzyme or TACE [21]. ADAM17 catalyzes the release of soluble tumor necrosis factor α (TNF- α) from its membrane-bound precursor as well as the shedding of transforming growth factor (TGF)- α , various receptors, L-selectin and Notch and the non-pathogenic processing of amyloid precursor protein as an α -secretase [30]. It has now been established that TIMP-3 is an effective inhibitor of ADAM10, 12, 17, 28 and 33, and ADAMTS-1, -2, -4 and -5 [10]. ADAMTS-4 and -5 are also called aggrecanases 1 and 2, respectively. The inhibition of ADAM17 and aggrecanases by TIMP-3 appears to be important

in regulating inflammatory processes, affecting the progression of disease processes such as cancer, rheumatoid arthritis and osteoarthritis [31,32]. The interaction between TIMP-3 and ADAMTS-4 is enhanced about 4-fold by aggrecan [33]. N-TIMP-3 is a weak inhibitor of ADAMTS-2, procollagen N-proteinase, with a K_i of about 600 nM, but the inhibition is also enhanced by heparin to a K_i of 160 nM [26]. As a potentiator of TIMP-3 inhibitory action, the anti-arthritic agent, calcium pentosan polysulfate, is remarkable; it enhances the binding affinity between TIMP-3 and ADAMTS-4 or -5 more than 100-fold and also blocks the endocytosis of TIMP-3 by chondrocytes [34]. These results suggest that the anti-arthritic activity of pentosan polysulfate is dependent on the endogenous TIMP-3 of cartilage.

MMPs and ADAMs are usually inhibited by the N-terminal domains of TIMPs, but Rapti et al. [35] found that while TIMP-1 and TIMP-3 both inhibit ADAM10, their isolated N-terminal domains do not. This indicates that the mechanism of inhibition of this enzyme by TIMPs differs from those of MMPs and other ADAMs and ADAMTSs. Full-length TIMP-3 and N-TIMP-3 are therefore useful agents for discriminating between the activities of ADAM10 and ADAM17 which often share similar substrates.

2.3. Three-dimensional structures of TIMPs and their complexes

The structures of full-length TIMP-1 and TIMP-2, N-TIMP-1 and N-TIMP-3 have been determined by X-ray crystallography [18,36–41] and the solution structures of N-TIMP-1 and N-TIMP-2 have been elucidated by NMR [42–44]. Most of the structures are of the bound forms of TIMPs in their inhibitory complexes with their target metalloproteinases, exceptions being the crystallographic structure of free TIMP-2 [37], solution structures of free forms of N-TIMP-1 [45] and N-TIMP-2 [42], and the structure of TIMP-2 in its complex with pro-MMP-2 which is stabilized by interactions between the C-terminal domain of the inhibitor and the hemopexin domain of the proenzyme [38] (Table 2).

The structures of full-length TIMP and N-TIMP have a “wedge-shaped” appearance [18,42]. The N-domain has an oligonucleotide and oligosaccharide binding (OB) fold [46], a five-stranded (β strands sA through sF) closed twisted β -barrel with a Greek key topology. This domain also contains three α -helices, one located close to the N-terminus (hI) and two (hII and hIII) near the C-terminus where they form part of the interface with the smaller C-domain. The C-domain has a pair of parallel β strands (sG and sH) connected by a loop, followed by a helix (hIV) and a pair of antiparallel β -strands (sI and sJ) connected by a β -hairpin (Fig. 1A).

The structure of the immediate N-terminal region is highly conserved in all TIMPs, reflecting its crucial function in inhibiting MMPs. The cysteines in the Cys¹-X-Cys³ sequence are attached by disulfide bonds to two other conserved cysteines, Cys⁷⁰ and Cys¹⁰⁰, respectively (numbering based on the sequence of TIMP-1) (Fig. 1B). The third Cys, residue 13, is disulfide bonded with Cys¹²³, a residue that delineates the C-terminus of the inhibitory domain. All structurally characterized inhibitory TIMP-metalloproteinase complexes, including the complex of N-TIMP-3 with ADAM17, are closely similar with respect to the core of the protein–protein interaction interface. The majority (60–75%) of the interactions between a TIMP and a metalloproteinase are made by the continuous ridge formed by the N-terminal five residues, Cys¹-Thr-Cys-Val-Pro⁵ in TIMP-1 (designated here IR I (interaction region I)) and the loop connecting sC and sD (CD loop), residues Met⁶⁶-Glu-Ser-Val-Cys⁷⁰ in TIMP-1 (IR II), that are covalently fastened by the disulfide bond between Cys¹ and Cys⁷⁰ (see Fig. 1). This ridge inserts into the metalloproteinase active site in such a way that the conserved N-terminal Cys¹ of the TIMP lies above the catalytic Zn²⁺ of the MMP. A key to the mechanism of inhibition is the bidentate coordination of the metal ion by the N-terminal α -amino group and carbonyl group of Cys¹, which displaces from the enzyme the water molecule needed for peptide bond hydrolysis. Residue 2, which is Thr or Ser in all TIMPs from vertebrates, interacts with the so-called S1' specificity pocket of the MMP, a subsite that has a dominant role in

determining enzyme specificity. Residues 3–5 interact with primed subsites (that interact with residues of the peptide substrate located to the C-terminal side of the scissile bond) in the protease active site, while Ser⁶⁸ and Val⁶⁹ interact with non-primed subsites S2 and S3 (that interact with residues of the peptide substrate located to the N-terminal side of the scissile bond) of the MMP (Fig. 1B).

Other regions that contribute to the interaction site are the AB loop (IR III) that lies to the right of the CD loop, and the EF loop (IR IV) (Fig. 1A). There is a large variation in the size of the AB loop among different TIMPs and this influences their interactions with MMPs. In the complex of N-TIMP-3 with the catalytic domain of ADAM17, there are also significant interactions involving the C-terminal end of sD (IR V) that are not observed in other structurally characterized TIMP complexes [41]. It is not clear whether these occur in complexes of TIMP-3 with MMPs or are unique to the interaction with ADAM17 and possibly other ADAMs.

In the complexes formed between full-length TIMPs and the catalytic domains of MMPs [18,36,40], there are some contacts between the C-domain of the TIMP and the protease, but these are on the periphery of the interaction site and do not appear to make important contributions to complex formation because the truncated N-TIMPs are high affinity MMP inhibitors. However, studies of the binding kinetics of TIMP-2 with full-length MMP-2 suggest a much higher affinity (6 fM) [47] compared with MMP-2 that lacks the hemopexin domain (275 nM) [48]. This affinity of N-TIMP-2 for full-length MMP-2 was less, although remaining high (40 pM), suggesting that the C-terminal domain of TIMP-2 makes some stabilizing interactions with the C-terminal hemopexin domain of MMP-2. Complexes of TIMP-1 or TIMP-3 with ADAM10 are also expected to have substantial contacts through the C-domain as N-TIMPs fail to inhibit ADAM10 [35]. In contrast, the C-terminal non-catalytic domains in ADAM17 have unfavorable effects on the interaction with N-TIMP-3 [49]. The interaction of TIMP-3 with ADAM17 also differs from those with MMPs in showing cooperative binding, which suggests the presence of multiple TIMP-3 binding sites, and also in the mode of interaction since an N-terminal Ala extension in N-TIMP-3 has a relatively small effect on the inhibition [50].

While close contacts between IR I and IR II and the metalloproteinase are found in all known TIMP complexes, the other regions have variable roles depending on the TIMP and on the target enzyme. Further detailed structural studies are required since such information will be useful for engineering TIMPs with restricted inhibitory spectra.

2.4. Interactions with pro-MMPs

The complexes of TIMPs with pro-MMPs are stabilized by interactions of the C-terminal domains of TIMP with the hemopexin domains of the zymogen. The interactions of TIMPs with pro-MMPs are relatively specific: TIMP-2, TIMP-3 or TIMP-4 with pro-MMP-2 and TIMP-1 or TIMP-3 with MMP-9 [10]. Since the interactions in these complexes do not involve the N-terminal domain of the TIMP, the complexes are capable of interacting with a second MMP molecule. However, with the exception of the pro-MMP-2-TIMP-2 complex, their functional significance is unclear.

The interaction of TIMP-2 with pro-MMP-2 is part of the activation mechanism of pro-MMP-2. In this process, membrane-type-1 MMP (MT1-MMP or MMP-14) plays a key role. The catalytic domain of MMP-14 interacts with the pro-MMP-2-TIMP-2 complex through the N-terminal domain of TIMP-2 and forms a cell membrane-associated ternary complex. No component of this complex is proteolytically active but the activation of the pro-MMP-2 component is triggered by cleavage of its pro-domain by a second MMP-14 molecule. This is facilitated by interactions between the two MMP-14 molecules through their hemopexin domains [51]. The activation is completed by autolytic cleavage by MMP-2 [52]. The

crystallographic structure of the complex of TIMP-2 with pro-MMP-2 shows they have two interaction interfaces. One is the loop between β -strands G and H of TIMP-2 and the 4th blade of the 4-bladed β -propeller structure of the hemopexin domain of pro-MMP-2, and the second involves the C-terminal tail region of TIMP-2 that follows the last cysteine in the sequence; this is inserted between the 3rd and 4th propeller blades [38]. TIMP-4 can also interact with pro-MMP-2 and even form a ternary MMP-14-TIMP-4-pro-MMP-2 complex, but this does not lead to the activation of pro-MMP-2 [53,54]. In fact, TIMP-4 is an effective inhibitor of pro-MMP-2 activation through its inhibitory action on MMP-14 [54]. A similar terminal complex that cannot be activated is formed with TIMP-3. TIMP-3 is therefore inhibitory for pro-MMP-2 activation. TIMP-3^{-/-} cells display enhanced activation of pro-MMP-2 by MMP-14 [55]. Domain swapping mutagenesis studies with TIMP-2 and TIMP-4 show that the C-domain of TIMP-2 is the key to pro-MMP-2 activation since a N-TIMP-4/C-TIMP-2 hybrid can support activation, and further deletion of the C-terminal tail of TIMP-2 (residues 186–194) leads to a loss of its ability to promote pro-MMP-2 activation, indicating the functional importance of this tail region [56].

3. Non-mammalian TIMPs

3.1. Vertebrates

Although four human TIMPs appear to be present in all mammalian species, their orthologues are not present in all vertebrates (Fig. 2). For example, there appears to be no orthologue of TIMP-1 in chickens, and the zebrafish (*Danio rerio*) has four TIMPs, all of which are most similar in sequence to human TIMP-2 [57]. On the other hand, a search of EST databases suggests that cartilaginous fishes have an orthologue of mammalian TIMP-3 but no other TIMPs, whereas bony fishes have orthologues of both TIMP-2 and TIMP-3. However, in more distantly related species the relationships of TIMPs with specific mammalian TIMPs are less clear.

3.2. Invertebrates

TIMPs from invertebrates are more variable in sequence than those from vertebrates. Apart from the homologues from nematodes, all are two-domain proteins that, with a few exceptions, have arrangements of cysteines that are similar to those of their mammalian relatives (see Fig. 3). However, a large proportion of invertebrate TIMPs have eight cysteines rather than six in their C-terminal domains. Some species, for example, *D. melanogaster*, have a single TIMP, whereas others have multiple TIMPs (e.g. hydra). Regions of the alignment show striking variations in length. For example, some species variants have large insertions between the third and fourth cysteines of the N-terminal domain (Fig. 3), that appear to reflect variations in length of the AB loop and in the loop between β strands B and C. A single 2-domain TIMP molecule in insects has been suggested to reflect the origin of the four mammalian TIMPs from a single common ancestor during vertebrate evolution. The four TIMPs found in the purple sea urchin and the three TIMPs in *Hydra magnipapillata* do not correspond to the mammalian subtypes. Nematodes have genes for multiple TIMP homologues that are generally single-domain proteins corresponding to the N-terminal inhibitory domains of mammalian TIMPs but have no region corresponding to the C-domains of mammalian TIMPs [12]. Although this suggests that the single-domain protein is a model for the ancestral protein, *H. magnipapillata*, which is thought to have diverged from the line leading to the vertebrates at a more ancient time than flies and nematodes [58], has a two-domain TIMP. Therefore, the single-domain nematode TIMP may have developed by loss of a domain from a two-domain ancestor or it could represent a distinct gene line. Based on this consideration and the variable numbers of subtypes in different species, TIMP evolution does not appear to have been a linear process in the invertebrates.

The TIMP homologues from nematodes have the characteristic pattern of cysteines and other conserved regions of sequence that are found in the N-domain of TIMPs and some netrin-fold proteins. They also have a secretion signal sequence. However, the amino acid residue present at position 2 in the mature forms of some of these proteins are atypical for a TIMP, for example, Lys, Arg or Gln. Substitution of these amino acids for Thr 2 in human TIMP-1 is known to produce a major loss of affinity for MMPs [59]. This suggests the possibility that these proteins, although homologous with the TIMP N-domains, may not function as MMP inhibitors. Recently, a TIMP homologue from the hookworm, *Acylostoma caninum*, was cloned and expressed as a His-tagged form cytoplasmically in *E. coli* [60]. It was soluble and, after purification, was found to have inhibitory activity against some human MMPs. The inhibition studies were conducted at a single relatively high inhibitor concentration of about 500 nM [60]. The implications of these results are unclear, because disulfide bonds do not readily form in proteins expressed in the cytoplasm of *E. coli* (other TIMPs have been found to be expressed in *E. coli* as denatured inclusion bodies and require oxidative *in vitro* folding to become active), and also because such a high concentration was required for MMP inhibition. One possible interpretation is that the TIMP preparation is unfolded and inhibits by acting as an alternative substrate.

3.3. Characterization of invertebrate TIMPs

There have been some studies of expression patterns of TIMPs in non-mammalian species including invertebrates [61–63], but only limited information is available regarding the functional properties of invertebrate TIMPs. Wei et al. cloned the N-terminal inhibitory domain of the single TIMP from a *D. melanogaster* embryonic cell library (dN-TIMP) and expressed it in *E. coli* as inclusion bodies. The protein was folded and purified and found to be an effective inhibitor of the two MMPs from *Drosophila in vitro* [64]. These studies were only semi-quantitative because of the relatively low activities of *Drosophila* MMPs on synthetic substrates, but more rigorous studies with mammalian MMPs showed that dN-TIMP inhibits MMPs-1, -2, and -14. It resembles human N-TIMP-3 in being a potent inhibitor of ADAM17, but it is a relatively weak inhibitor of ADAM10; this is not surprising because of the weak inhibitory activity of human N-TIMP-1 and N-TIMP-3 for ADAM10 [35]. The relatively low activity against mammalian MMP-1, a collagenase, was proposed to reflect an adaptation to differences in the composition of the extracellular matrix between insects and vertebrates. The *Drosophila* genome contains no genes for fibrillar collagens. Thus, *Drosophila* TIMP (dTIMP) would not be under selective pressure to acquire or maintain features required to bind effectively to the active sites of collagenases that cleave fibrillar collagens. Based on modeling, dN-TIMP appears similar in surface charge properties to N-TIMP-3. It is also closer to mammalian TIMP-3 in sequence, functional properties and predicted structure than to the other three TIMPs [64]. Disruption of *Timp* in *Drosophila* generates a subviable phenotype. In adult flies, this mutation produces inflated wings, bloated guts and tissue autolysis, impaired phototactic responses, and premature death, indicating that active dTIMP is needed for balanced ECM turnover, appropriate cell–cell or cell–matrix interactions and a functional nervous system in *Drosophila* [65].

In contrast to the situation in insects, fibrillar collagens have a wide distribution in many other invertebrate species, being present in cnidarians such as hydra and sea anemone as well as the choano-flagellate, *Monosiga brevicollis* and the demosponge, *Amphimedon queenslandica*. The most recent common ancestor of cnidarians is thought to be more ancient than that of insects [57], so that it appears that fibrillar collagen genes may have been lost in the line leading to insects. Most or all metazoans appear to have genes that encode TIMPs that might be expected to function as metalloproteinase inhibitors. While there is little information about their functional properties, it is reasonable to speculate that they also regulate turnover of ECM components including fibrillar collagens.

3.4. Bacteria

Structural studies show similarities between TIMPs and other OB-fold proteins from vertebrates that have distinct functions. The latter includes netrins which have a N-TIMP-like netrin (NTR) domain, the laminin-binding domain of agrin, the C-terminal C345C domain of complement proteins C3, C4 and C5, and a domain of type I procollagen C-proteinase enhancer [13,66]. These domains have similar 3D structures to the N-domains of TIMPs but their sequences do not align readily with those of the TIMPs. In contrast, several bacterial proteins show significant similarities in sequence to TIMPs, particularly to TIMP-2; their amino acid sequences fish out vertebrate TIMP sequences in BLAST searches and *vice versa*. The sequences of these proteins all start with a bacterial secretion signal sequence; an alignment of the predicted sequences of the mature forms of the proteins with N-TIMP-2 is shown in Fig. 4. Some of these proteins are designated as having a cbiN-domain, but their functions appear to be unknown at present.

4. TIMP engineering

The uncontrolled activities of MMPs, ADAMs and ADAMTSs have been linked to multifarious diseases in humans including cancer, rheumatoid arthritis, osteoarthritis, encephalomyelitis, heart, lung and kidney diseases. Thus, they are attractive targets for the development of specific inhibitors with potential clinical applications. At the same time, these metalloproteinases share generally similar active site structures and have overlapping specificities and numerous key roles in important biological processes. This may have contributed to the lack of success in clinical trials of synthetic MMP inhibitors [67]. An alternative approach to the therapy of metalloproteinase-related diseases could be to use engineered TIMPs with restricted inhibitory specificities. Natural TIMPs are broad-spectrum inhibitors, particularly for MMPs, but they have relatively large interaction interfaces and differ in the types of interactions that they make with different metalloproteinases as seen in the crystallographic structures of TIMP-1 and TIMP-2 with different MMPs [37,39–41]. This suggests that their affinities for different MMPs can be manipulated by mutagenesis. Most mutagenesis studies have used N-TIMPs as they are readily expressed in bacterial systems and folded from the inclusion bodies.

Earlier mutational studies of N-TIMP-1 focused on Thr2 as a residue that influences the affinity for different MMPs [59]. This was based on the fact that residue 2 of TIMP-1 interacts with the S1' subsite of MMPs, the specificity pocket that has a key role in substrate specificity [68,69]. Analysis of the effects of fifteen different substitutions for Thr2 on the inhibition of MMPs-1, -2 and -3 indicated that side chain size, charge and hydrophobicity are properties that make key contributions to MMP selectivity. However, the effects of these substitutions on MMP selectivity showed little correlation with the effects of the same amino acid in the P1' position of a peptide substrate, indicating that structural constraints of TIMP around the reactive site is different from flexible peptide substrates. The importance of residue 2 in MMP inhibition was also indicated by the fact that the removal of the side chain (a mutation to glycine) resulted in a loss of 33–55% of the free energy of complex formation while other substitutions differentially affected binding to the three MMPs. This suggested that residue 2 of this TIMP can be considered to be a "hot spot" in the TIMP–MMP interaction interface [59]. More recent studies, however, have shown that the Thr 2 to Gly substitution has a relatively small effect on the affinity of N-TIMP-1 for MMP-9, suggesting that interactions outside of the S1' pocket are more important in the interaction with MMP-9 than with other MMPs [23]. Other mutational studies have shown that substitutions for other residues in the TIMP reactive site also affect specificity, although less dramatically those involving residue 2. These studies have been extensively reviewed by Nagase and Murphy [10] and will not be discussed in great detail here. In brief, it can be said that residues 4, 68 and 98 (TIMP-1 numbering) have relatively large effects on inhibitory specificity.

The AB loop varies considerably in length between different TIMPs. The shortest is in TIMP-1, and this is extended by 1 residue in TIMP-3, by 6 residues in TIMP-4 and by 7 residues in TIMP-2. The long loop of TIMP-2 makes extensive contacts with MT1-MMP (MMP-14) in their inhibitory complex [36]; the shortest loop in TIMP-1 might be thought to provide an explanation of why TIMP-1 is a weak inhibitor of this and some other MT-MMPs. However, grafting the longer AB loop from TIMP-2 into the N-TIMP-1 structure does not enhance its binding to MMP-14 [23]. Lee et al. [70] have reported that residue 98 (TIMP-1) which is Thr in TIMP-1 and Leu in TIMP-2 is a key to the high affinity binding to MMP-14 and additional substitutions of Ala for Val4 and Leu for Pro6 further enhanced binding. The N-TIMP-1(T98L) mutant inhibits ADAM17 and MMP-19 as well as MT-MMPs [71]. Grafting the AB loop from TIMP-3 into the N-TIMP-1(V4S/V69L) mutant greatly improved the inhibitory action on ADAM17 [71]. In this case the mutant TIMP has better inhibitory affinity for ADAM17 than N-TIMP-3. Other studies support the view that these residues, and others that have not yet been identified, contribute to the weak binding of TIMP-1 to membrane-type MMPs [23].

These studies represent the successful application of rational structure-based engineering of TIMPs for developing variants with selective inhibitory action on MMPs and other metalloproteinase family members. However, protein-protein interactions are complex phenomena involving dynamic molecules that undergo binding-induced structural changes that can have varying effects on the thermodynamics of binding. Structural and calorimetric studies of the N-TIMP-1-MMP-3 interaction have illustrated this. Structural changes that occur during this interaction include the displacement of the N-terminal region of the MMP by disrupting a salt bridge [18], while NMR studies indicate that structural adjustments occur in the TIMP component throughout the enzyme reactive ridge formed by the two regions linked by the Cys¹-Cys⁷⁰ disulfide [45]. Calorimetric studies have shown that the N-TIMP-1-MMP-3 interaction is associated with a positive (unfavorable) change in enthalpy and that, thermodynamically, the interaction is driven by a strongly positive (favorable) entropy change. Generally a positive entropy change in a protein-protein interaction arises from increased solvent entropy due to the release of ordered water molecules from non-polar regions of the interaction interfaces; this would be reflected in a large negative change in heat capacity (ΔC_p) for the interaction. The small ΔC_p for the N-TIMP-1-MMP-3 interaction together with NMR studies suggests that much of the entropy contribution to binding arises from increased dynamics in the core of the TIMP β -barrel [72]. The relatively high crystallographic B-factors observed in the N-TIMP-3 component of the N-TIMP-3-ADAM17 complex, as compared with the ADAM17 component, suggests that this interaction might also be associated with an increase in conformational entropy in the TIMP component [41]. Although B-factors are a measure of dynamics, they can be affected by molecular contacts in a crystal as well as the intrinsic mobility of components of the structure. The fact that the entropy-driven binding, in at least one system, is independent of the hydrophobic effect is somewhat problematic for structure-based engineering.

5. TIMPs are multifunctional proteins

TIMPs have various biological activities including the modulation of cell proliferation, cell migration and invasion, anti-angiogenesis, anti- and pro-apoptosis and synaptic plasticity. These activities may partially arise from metalloproteinase inhibition, as the regulation of ECM catabolism can influence cellular behavior, but many of them have been shown to be independent of MMP inhibition, because synthetic MMP inhibitors or TIMP mutants that do not inhibit MMPs can exhibit a similar activity. In the latter case, TIMPs interact directly with specific cell surface receptors to induce cellular responses (see Table 1 and a recent review by Stetler-Stevenson [73]).

5.1. Cell growth promotion

When the cDNA for TIMP-1 was first cloned [74], it was found to be identical to a factor that has erythroid potentiating activity [75]. Later, TIMP-1 was shown to have cell growth promoting activity on various cell types including keratinocytes and fibroblasts [76,77]. TIMP-2 also has erythroid potentiating activity [78] and cell growth promoting activity on many cell types [79] including metanephric mesenchyme cells during nephron morphogenesis [80]. These effects are independent of MMP inhibition, because TIMPs that lack MMP inhibitory activity either by mutations or reduction and alkylation retained cell growth promoting activity [81] and they were not produced by synthetic MMP inhibitors. In an investigation of the cellular events associated with TIMP-induced cell growth, Wang et al. [82] found that both TIMP-1 and TIMP-2 increased the level of Ras-GTP, but utilize different signaling pathways: TIMP-1 activates the tyrosine kinase/mitogen activated protein kinase (MAPK) pathway, whereas TIMP-2 signaling is mediated by protein kinase A activation which is directly involved in Ras/phosphoinositide 3-kinase (PI3-K) complex formation. This suggests that TIMP-1 and TIMP-2 have distinct receptors. Recent studies have shown that TIMP-1 binds to CD63 [83] and TIMP-2 to $\alpha_3\beta_1$ integrin [84], but these interactions have been found to inhibit apoptosis and arrest cell growth, respectively. Thus, the mitogenic activities of the two TIMPs are not explained by the involvement of these receptors, although there seem to be some similarities in the activation of signaling molecules and anti-apoptotic effects may be, in part, related to an effect on cell growth. Recently, D'Alessio et al. [85] have suggested that the effect of TIMP-2 on cell growth is mediated through its binding to MMP-14 on the cell surface and subsequent extracellular regulated kinase (ERK)1/2 activation. Since a TIMP-2 mutant with an extra alanine added to the N-terminus (Ala+TIMP-2), which does not bind to the active site of MMP-14, also activated ERK1/2, it was suggested that the interaction between TIMP-2 and MMP-14 does not involve the active site of the enzyme [85]. This is, however, inconsistent with the observation that a hydroxamate MMP inhibitor that blocks TIMP-2 binding to the active site of MMP-14 inhibited MMP-14-dependent ERK1/2 activation and abrogated cell growth. Further studies are needed to clarify the mechanism by which the TIMP-2–MMP-14 system promotes cell proliferation.

5.2. Cell growth arrest and anti-apoptotic activity of TIMP-1 and TIMP-2 lead to cell differentiation

In addition to their mitogenic activities, TIMP-1 and TIMP-2 suppress the growth of some cells. *In vitro* studies with human breast epithelial (MCF10A) cells showed that TIMP-1 reduces their growth rate by inducing cell cycle arrest at G₁ through the down-regulation of cyclin D₁ via the up-regulation of cyclin-dependent kinase inhibitor p27^{KIP1} [86]. TIMP-1 also has anti-apoptotic effects on other cell types [87–90]. While this effect of TIMP-1 on hepatic stellate cells was reported to be due to the inhibition of MMPs [90], the effects on other cells are not mediated by MMP inhibition [87–89]. Using the MCF10A cell system, Jung et al. [83] found that the anti-apoptotic activity is mediated through TIMP-1 binding to CD63, a member of the tetraspanin family. CD63 interacts with the β_1 subunit of integrins and the TIMP-1–CD63–integrin β_1 complex thus constitutively turns on survival signals through the activation of focal adhesion kinase (FAK), PI3-K and ERK pathways [89,91], similar to transformed cell survival signaling. In a 3D matrigel matrix, MCF10A cells form polarized acinar-like structures. The maintenance of these structures requires apoptosis in centrally located cells [83]. The over-expression of TIMP-1 in these cells disrupts the structures by inhibiting caspase-mediated apoptosis and fills the luminal space in the glandular epithelial structure by reducing cell polarization. This is one of the features of ductal cancer development and over-expression of TIMP-1 and its anti-apoptotic effect may be associated with oncogenic cellular transformation.

TIMP-1 levels in serum and TIMP-1 expression in cancerous tissues are associated with poor clinical outcomes in many types of cancer [92–95]. One possible explanation for this is that an elevated level of mesenchymal TIMP-1 increases hepatocyte growth factor (HGF; scatter factor) signaling by blocking a sheddase (possibly ADAM10) of the HGF receptor, cMet, making the liver more susceptible to metastasis [96]. The over-expression of TIMP-1 also elevates HGF and HGF-activating proteases in the liver [96]. Another potential cause is a combination of cell cycle arrest and anti-apoptotic activities of TIMP-1, which leads to cellular transformation. Guedez et al. [87] found that TIMP-1-positive Burkitt's lymphoma cell lines were resistant to Fas-dependent and Fas-independent (cold-shock, serum deprivation and γ -irradiation) apoptosis, but TIMP-1 negative cells were not. TIMP-1 promotes germinal center B cell survival and differentiation to plasma cells by up-regulating CD40, CD23 and IL-10, and down-regulating CD77 [97]. It also promotes plasmacytic/plasmablastic differentiation in Burkitt lymphoma cells [98], as well as hematopoietic cell differentiation [99].

TIMP-2 suppresses growth factor-stimulated cell proliferation at low to sub-nanomolar concentrations, similar to the concentrations that enhance cell growth [79,100]. This suppressive effect was shown to be mediated by the activation of adenylate cyclase, increased intracellular cAMP levels and the activation of SH2-protein tyrosine phosphatase-1 (SHP-1) which dampens the activity of growth factor receptors [100] and inhibits p42/44 MAPK activation [101]. Stetler-Stevenson et al. [84] identified $\alpha_3\beta_1$ integrin as the receptor of TIMP-2, and showed that vascular endothelial cell growth factor (VEGF)-A-stimulated or fibroblast growth factor (FGF)-2-stimulated endothelial cell proliferation is inhibited through the interaction of TIMP-2 with this integrin; this results in the transfer of the β_1 integrin-associated SHP-1 to the VEGF or FGF receptor and its de-phosphorylation. In addition, TIMP-2 induces G₁ growth arrest in endothelial cells through the *de novo* synthesis of p27^{KIP1}, which results in the inhibition of cyclin-dependent kinases 4 and 2 and subsequent hypophosphorylation of retinoblastoma protein [102]. These observations may explain how TIMP-2 arrests cell growth and inhibits angiogenesis.

TIMP-2 is expressed in post-mitotic neurons [103] and promotes cell differentiation and neurite outgrowth [104], as the result of cell cycle arrest through decreased expression of cyclins B and D and increased production of cyclin-dependent kinase inhibitor p21^{Cip}. TIMP-2 expression is found only in α_3 integrin-positive cells [105], suggesting that TIMP-2- $\alpha_3\beta_1$ integrin interactions participate in neurogenesis by withdrawing progenitor cells from the cell cycle and permitting their terminal neuronal differentiation.

While cell cycle arrest and the inhibition of apoptosis appear to be closely related to cell differentiation, as in case of TIMP-1, it is not clear whether the anti-apoptotic activity of TIMP-2 is essential for its differentiation-promoting activity because of the limited availability of information about the apoptotic activity of TIMP-2. Nonetheless, the over-expression of TIMP-2 in B16F10 murine melanoma cells rendered these cell more resistant to apoptosis [106] and in a mouse model of atherosclerosis, the over-production of TIMP-2 reduced plaque instability and inhibited the apoptosis of macrophages and macrophage-derived foam cells [107]. Lim et al. [108], on the other hand, reported that TIMP-2 causes the apoptosis of activated T cells and that this is due to inhibition of the cleavage of Fas ligand. Clearly, further investigation of the effects of TIMP-2 on apoptosis in other cell systems would be of considerable interest.

5.3. TIMP-3 and apoptosis

TIMP-3 induces apoptosis in a number of cancer cell lines [109–112] and rat vascular smooth muscle cells [113]. Smith et al. [111] proposed that the pro-apoptotic effects of TIMP-3 result from its inhibition of TACE (ADAM17) with subsequent stabilization of TNF receptors. TIMP-3 inhibits the shedding of a number of cell surface molecules. Ahonen et al. [114]

confirmed that TIMP-3 stabilizes three distinct death receptors (TNF receptor 1, FAS, and TNF-related apoptosis inducing ligand receptor 1 (TRAIL-R1)) in melanoma cells in culture and *in vivo*. TIMP-3 binds to the ECM after secretion [11]. However, recent studies by Troeberg et al. [34] indicate that TIMP-3 secreted from cultured chondrosarcoma cells and chondrocytes does not accumulate in the extracellular space or medium as it is endocytosed by a LDL receptor related protein (LRP). The sequestration of TIMP-3 by binding to the ECM or through endocytosis may help to protect cells from apoptosis while still allowing it to control pericellular proteolysis through its potent inhibition of MMPs, ADAMs and ADAMTSs.

5.4. Angiogenesis and TIMPs

Cartilage, an avascular tissue, contains an anti-angiogenic activity that is closely associated with anti-proteolytic activity, and the latter activity was identified as a collagenase inhibitor [115]. Moses et al. subsequently isolated the anti-angiogenic activity from bovine cartilage and found that its N-terminal sequence is similar to that of human TIMP-1 [116]. It is now well established that all four TIMPs have anti-angiogenic activity (see [117]). Because a number of MMPs, particularly gelatinases and MT1-MMP, are involved in endothelial cell migration and capillary formation, the inhibition of MMPs by TIMPs can prevent angiogenesis [117]. However, TIMP-2 and TIMP-3 also exhibit anti-angiogenic activity that is independent of their MMP inhibitory activities.

As discussed above, the anti-angiogenic activity of TIMP-2 is mediated through its interaction with $\alpha_3\beta_1$ integrin. Feldman et al. [118] found that the over-expression of TIMP-2 in murine colon cancer cells inhibited tumor growth and angiogenesis *in vivo* by up-regulating MAP kinase phosphatase 1 which dephosphorylates p38 MAPK, a molecule involved in endothelial cell proliferation and migration. In addition, TIMP-2 enhances the expression of RECK (the reversion-inducing-cysteine-rich protein with Kazal motif), a membrane-anchored protein with inhibitory activity for MMP-2, -9 and -14 and ADAM10 [119,120]. Enhanced RECK expression is associated with the inhibition of endothelial cell migration [121]. The interaction of TIMP-2 with $\alpha_3\beta_1$ integrin reduces Src tyrosine kinase levels which alters the pattern of paxillin phosphorylation. This leads to a signaling switch from Rac 1 to Rap 1, resulting in enhanced RECK expression and loss of a migratory phenotype [122]. The anti-angiogenic activity of TIMP-2 has been localized to a region of sequence in the C-terminal domain [123], which may be the site of interaction with $\alpha_3\beta_1$ integrin.

TIMP-3 is also a potent inhibitor of angiogenesis. It binds directly to VEGF receptor 2 and blocks the action of VEGF on endothelial cells [124]. TIMP-3 also binds to angiotensin II type 2 receptor, and the over-expression of both TIMP-3 and angiotensin II type 2 receptor additively inhibits angiogenesis [125].

TIMP-2 and TIMP-3 also play an important role in the stabilization of capillary networks during angiogenesis. This process is important as newly formed capillary networks are predisposed to undergo regression, and their stabilization requires pericyte recruitment. Saunders et al. [126] have shown that endothelial cell-pericyte interactions stimulate the expression of TIMP-3 in pericytes. The TIMP-3 from this source together with TIMP-2 expressed in endothelial cells participates in vascular stabilization by inhibiting a number of MMPs and ADAMs [126] and probably by interacting with VEGFR-2 [124]. These actions of TIMPs inhibit endothelial cell tube formation and tube regression, which leads to the cessation of endothelial cell activation and the development of their quiescence [126]. The recruitment of pericytes and their interaction with endothelial cells also stimulate the assembly of vascular basement membrane matrix assembly which is a crucial step in vessel maturation [127].

5.5. Synaptic plasticity

TIMP-1 was found as a candidate plasticity-related gene whose expression is greatly increased in the hippocampus on stimulation with a glutamate analogue, kainate [128]. The hippocampus participates in synaptic plasticity such as long-term potentiation, seizures, kindling, learning and memory. *TIMP-1*-null mice, subjected to a learning and memory test in an olfactory maze, exhibited a significant impairment in forming and remembering order-reward associations [129,130]. This effect is not due to a direct action of *TIMP-1* on memory but is probably mediated by an effect on neuronal development arising from abnormal ECM turnover during development of the central nervous system.

Another interesting observation is that *TIMP-2*-null mice do not exhibit prepulse inhibition of the startle reflex and fear-potentiated startle [131]. The ability to learn potential dangers and to recall such stimuli requires long-term changes in synaptic transmission and structure. This type of synaptic plasticity is thought to be accompanied by changes in neuronal ECM structure, and *TIMP-2* probably affects neuronal ECM turnover as it is highly expressed in the amygdala, a neural structure implicated in synaptic plasticity associated with fear conditioning. Neurite outgrowth is greatly reduced in newborn *TIMP-2*^{-/-} mice, resulting in defects in neuronal development [105].

TIMP-2-null mice also exhibit symptoms of motor dysfunction, e.g., decreased time on a motorized rotating rod, reduced hind limb extension, and splayed and lengthy gait compared with wild-type mice [132]. These effects are more prominent during postnatal development and juvenile *TIMP-2*^{-/-} mice have increased nerve branching and acetylcholine receptor expression [132]. Adult end-plates are enlarged and more complex in *TIMP-2*^{-/-} mice than in the wild-type, whereas cerebella Purkinje cells have decreased neurite outgrowth, suggesting that the *TIMP-2*^{-/-} motor phenotype is due to both peripheral and central nerve defects resulting from abnormal ECM turnover [132].

6. Physiological and pathological functions proposed from studies with *TIMP* deficiency mice

Studies with mice deficient in specific *TIMP* genes provide insights into the physiological and pathological functions of individual *TIMPs* *in vivo* and indicate how ablation of these genes affects ECM remodeling in the whole animal. Table 3 summarizes the phenotypes observed in single *TIMP* deficient mice, and we discuss them here briefly.

6.1. *TIMP-1*^{-/-} mice

Nothnick and colleagues found that *TIMP-1*-null mice exhibit a number of alterations in processes associated with reproduction and steroidogenesis (see Table 3), suggesting that *TIMP-1* has a multifaceted role in regulating the murine reproductive cycle involving the uterus, ovary and testis. *TIMP-1* is also an important regulator of heart tissue remodeling. *TIMP-1*^{-/-} mice display changes in left ventricular structure and cardiac function [133]; they also display exacerbated left ventricular remodeling after myocardial infarction [134,135].

Partial hepatectomy in mice increases *TIMP-3* mRNA within 6 h and *TIMP-1* and *TIMP-4* mRNAs by 48 h. A number of MMPs are also upregulated in regenerating livers in these time periods. Transgenic mice that underexpress *TIMP-1* display accelerated hepatocyte cell cycle progression, and in *TIMP-1*^{-/-} mice HGF activity release from the ECM is increased in regenerating liver, leading to an increase in the phosphorylation of cMet receptor and activation of p38 MAPK [136]. *TIMP-1* over-expressing mice delayed cell cycle progression, suggesting that *TIMP-1* negatively regulates HGF activity during liver regeneration by regulating the activities of MMPs that activate and release pro-HGF from the ECM [136].

TIMP-1-null mice have increased resistance to corneal and pulmonary infection by *Pseudomonas aeruginosa* [137]. This was reversed by a MMP inhibitor, indicating that it results from a loss of MMP inhibitory activity. MMP-9, MMP-7 and MMP-3 are considered to be important for resistance to infections, but the mechanism of their influence is not known. Possibilities include the alteration of innate immunity by MMP-7-catalyzed conversion of pro-defensin to active defensin [138] or the cleavage of syndecan-1 and mobilization of chemokines [139].

Another interesting phenotype of TIMP-1^{-/-} mice is a decrease in adipose tissue weight when on a high fat diet [140]. This appears to contrast with the increased food intake presented by TIMP-1^{-/-} female mice despite their hyperleptinemia [141]. Since leptin promotes the expression of TIMP-1 mRNA in the hypothalamus, TIMP-1 contributes to the regulation of feeding and energy balance. It is also notable that TIMP-1^{-/-} mice have impaired learning and memory as discussed above.

6.2. TIMP-2^{-/-} mice

TIMP-2-null mice display a number of phenotypes associated with defects in the central nervous system and myogenesis (see Table 3). As discussed earlier, TIMP-2^{-/-} mice exhibit a deficiency in prepulse inhibitor of the startle reflex (a measure of pre-attentional sensorimotor gating) and in the acquisition of fear-potentiated startle, a model of synaptic plasticity in the amygdala [131]. They also have motor defects, decreased cerebellar neurite outgrowth [132], and delayed neuronal differentiation [104]. These effects are probably due to an abnormal turnover of neuronal ECM and indicate that TIMP-2 is an important promoter of neuronal differentiation.

Altered myotube formation by TIMP-2^{-/-} myoblasts [142] and weakened muscle and reduced fast-twitch muscle mass in TIMP-2^{-/-} mice have been reported [143]. In both cases the level of $\beta 1$ integrin is altered; also, its level is reduced in the fast-twitch muscle of TIMP-2^{-/-} mice [143], suggesting that ECM-cytoskeletal interactions required for muscle contraction are destabilized.

6.3. TIMP-3^{-/-} mice

Khokha et al. generated TIMP-3^{-/-} mice and initially reported two prominent phenotypes: air space enlargement in lungs [144] and enhanced apoptosis during mammary gland involution [145]. Both result from enhanced matrix degradation resulting from a lack of metalloproteinase inhibition, suggesting that TIMP-3 is an important regulator of matrix-degrading metalloproteinases.

Unbalanced matrix degradation is also seen in other tissues causing dilated cardiomyopathy [146], abnormal vascularization with dilated capillaries [147], impaired bronchiole branching morphogenesis [148], and spontaneous development of arthritis in aged mice [149]. When challenged with disease models linked to inflammation and ECM remodeling, the inflammatory process and tissue destruction were accelerated due to a large increase in metalloproteinase activities. TIMP-3^{-/-} mice challenged with mechanical stress to the heart, induced by constriction (aortic banding) of the aorta, exhibit accelerated development of dilated cardiomyopathy due to increased pro-tumor necrosis factor- α (TNF α) processing by ADAM17 (TACE) and enhanced MMP-mediated ECM degradation [150]. These mice also manifest excessive myocardial fibrosis accompanied by a parallel increase of TGF $\beta 1$ and TNF α signaling, amplified by their cross-talk, suggesting that TIMP-3 is a common innate regulator of TGF $\beta 1$ and TNF α in tissue responses to injury [151]. Liver regeneration after partial hepatectomy in TIMP-3^{-/-} mice was impaired because of abnormal inflammation that increases the release of TNF α , suggesting that ADAM17 is one of the major targets of TIMP-3

in this system [152]. The loss of TIMP-3 impacts innate immunity by dysregulating the cleavage of TNF α and its receptors [153]; in response to lipopolysaccharide (LPS), TIMP-3^{-/-} macrophages released more TNF α and TIMP-3^{-/-} mice became more susceptible to endotoxin shock as the result of greatly increased serum levels of TNF α .

Insulin receptor-haploinsufficient (*Insr*^{+/-}) mice that are diabetic have a defect in TIMP-3 expression and increased levels of ADAM17 activity, resulting in elevated levels of soluble TNF α . Doubly heterozygous *Insr*^{+/-}/*Timp3*^{+/-} mice exhibited more pronounced symptoms of diabetes and vascular inflammation resulting from increased TNF α [154]. Blocking ADAM17 activity with a synthetic metalloproteinase inhibitor or anti-TNF α antibody treatment led to marked reduction of hyperglycemia and vascular inflammation in *Insr*^{+/-} diabetic mice [154]. TIMP-3^{-/-} mice fed a high fat diet developed glucose-intolerance and insulin-resistance [155]. These studies suggest that TIMP-3 is an important protectant against type 2 diabetes by regulating TNF α shedding.

TIMP-3^{-/-} tumor cells do not show changes in tumorigenesis (tumor growth and angiogenesis) when compared with TIMP-3^{+/+} tumor cells, but B16F10 melanoma cells that had been subcutaneously injected into TIMP-3^{-/-} mice grew faster and displayed increased angiogenesis relative to those injected into wild-type mice [156]. In response to FGF-2, TIMP-3^{-/-} endothelial cells invaded more effectively and formed functional blood vessels *in vitro* [156]. Enhanced dissemination of lymphoma and melanoma cells to multiple organs was reported in TIMP-3^{-/-} mice [157]. These observations indicate that TIMP-3 expressed within the tumor microenvironment has suppressive effects on tumorigenesis and metastatic colonization.

Together, these studies show that TIMP-3 is a key endogenous regulator of metalloproteinases involved in ECM turnover/tissue destruction, inflammation, innate immunity and cancer progression.

7. Sorsby fundus dystrophy

Mutations in the region of the human TIMP-3 gene that encodes the C-terminal domain are the cause of Sorsby fundus dystrophy (SFD), an autosomal dominant retinal disorder that results in macular degeneration and blindness in the 3rd and 4th decades of life [158]. The clinical symptoms are similar to those of age-related macular degeneration (AMD), except the earlier age of SFD onset. The mutations that cause SFD in different families variously result in the substitution of different residues in the C-terminal domain by a cysteine [159], a nonsense mutation [160], a splice mutation [161] or a His158Arg mutation [162]. Apart from the His158Arg mutation, which is associated with a later onset of disease (5th to 7th decades) than other SFD mutations, all SFD mutations result in an odd number of cysteines in the TIMP-3 C-terminal domain. The mutated proteins appear to dimerize or multimerize through intermolecular disulfide bridges [159] or form aberrant TIMP-3–ECM complexes [163], which may explain the increased deposition of SFD TIMP-3 mutants in Bruch membrane. The MMP inhibitory activities of SFD mutants are variable depending on mutants and enzymes tested but do not consistently show losses of inhibitory activity [163–166], and a loss of function does not correlate well with the autosomal dominant nature of SFD. Cells expressing SFD TIMP-3 mutants are more adherent to the ECM than those expressing wild-type TIMP-3 [163] and SFD TIMP-3 mutants induce apoptosis of retinal pigment epithelial (RPE) cells more readily than the wild-type protein [167]. Another proposal is that TIMP-3 SFD mutants may have greater stability than wild-type TIMP-3 and accumulate in the ECM, affecting its turnover and producing thickening of the Bruch membrane [168]. This would become a barrier to the diffusion of nutrients leading to atrophy of the RPE and photoreceptor death. A possible effect of the SFD mutation on the half-life of TIMP-3 in the matrix could be significant and may

correlate with the finding that patients with AMD have more TIMP-3 protein in their Bruch membranes than age-matched controls [169].

Studies with a mouse model of SFD generated by introducing the SFD Ser156Cys mutation in the murine *Timp3* gene support the proposal that site-specific excess rather than absence or deficiency of functional TIMP-3 may be the primary consequence of the *Timp3* gene mutation [170]. Qi et al. [166] recently reported that over-expression of the Ser156Cys mutant in VEGFR-2-transfected endothelial cells enhanced VEGF-induced cell proliferation, migration and angiogenesis as a consequence of a post-transcriptionally regulated increase in VEGFR-2 on the cell surface, although the Ser156Cys mutation in TIMP-3 did not alter the anti-angiogenic activity of TIMP-3 [165].

Malattia leventinese or Doyme honeycomb retinal dystrophy is an autosomal dominant disorder that also causes early onset macular degeneration and is linked in an interesting manner with TIMP-3. This disease is caused by a mutation in the gene for fibulin 3, an ECM protein also known as EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1) [171], that results in the substitution of Trp for Arg345 in one of the EGF-like domains. EFEMP1 has been identified as a binding partner of TIMP-3 [172] and this interaction was shown by yeast two-hybrid studies to be mediated by the C-terminal region of EFEMP1 (residues 256–493) and the C-domain of TIMP-3, both of which include the mutation sites in their respective diseases. Later studies have shown that the mutant form of EFEMP1 is not effectively secreted. When over-expressed in a RPE cell line, it was found to accumulate in the endoplasmic reticulum, activate the unfolded protein response and stimulate the production of VEGF [173].

8. Conclusions and future prospects

Because of the relationship of TIMPs with multiple human diseases and their roles in ECM homeostasis, research on their structures, functions, and biological and pathological roles continues to flourish, as reflected in the continued growth of the scientific literature in this area (702 papers featuring TIMPs in 2008). TIMPs were originally characterized as MMP inhibitors, but we now know that their inhibitory spectra are much broader and include a number of ADAMs and ADAMTSs. This diverse array of metalloproteinases may, in part, reflect similar active site topologies in these enzymes and also the extended surfaces in TIMPs that interact with their targets. These features of TIMPs provide an opportunity to employ mutational methods to alter their inhibitory specificities. The recent success in solving the structure of the complex of N-TIMP-3 with the catalytic domain of ADAM17 has indicated that mechanism of inhibition of ADAMs by TIMPs is similar to that for MMPs [41]. Nevertheless, we have also learned that the non-catalytic domains of ADAMs and ADAMTSs affect their interactions with TIMP-3. For example, the inhibition of the complete extracellular region of ADAM17 by TIMP-3 is 10-fold weaker than the inhibition of the truncated catalytic domain [49], whereas the presence of the C-terminal domains of ADAMTS-4 and -5 enhances their binding to TIMP-3 [174]. Furthermore, N-TIMP-3 displays cooperative binding to the longer form of ADAM17, suggesting the presence of at least two interactive binding sites for N-TIMP-3 [50]. Some mutations that largely inactivate TIMP-3 as a MMP inhibitor have little effect on ADAM17 inhibition [50]. Other TIMPs have varying patterns of inhibition for certain ADAMs as listed in Table 1. Therefore, elucidation of the characteristics, including the structural basis, of the molecular interactions of TIMPs with various forms of ADAMs and ADAMTSs will produce information that may be useful for engineering selective inhibitors of disintegrin-metalloproteinases. Only limited information is available about the functional properties of non-mammalian TIMPs particularly those from invertebrates. It will be interesting to see how the properties of these TIMPs relate to evolutionary changes in the ECM and the specificities of the metalloproteinases produced by different groups of animals.

Significant progress has been made towards understanding the metalloproteinase inhibition-independent mechanisms of action of TIMPs that control cell growth, apoptosis, differentiation and anti-angiogenesis, through identification of their receptors, particularly CD63 for TIMP-1, $\alpha 3\beta 1$ integrin for TIMP-2, and VEGF receptor 2 and angiotensin II receptor for TIMP-3. Recent studies demonstrating the endocytosis of TIMP-2 [175] and TIMP-3 [34] through the LRP-mediated pathway and the age-dependent extracellular accumulation of TIMP-3 in the kidney, lung and eye [176] have added further complexity to the biology and pathology of TIMPs. Genetic approaches have been fruitful in elucidating the physiological and pathological roles of TIMPs *in vivo*. Some studies clearly demonstrate that TIMPs function as regulators of ECM turnover by inhibiting metalloproteinase activities, but in other cases it is unclear whether specific TIMPs alter ECM catabolism or exert direct effects on cells. The phenotypes of TIMP-3-null mice indicate that this inhibitor largely functions as a metalloproteinase inhibitor *in vivo*. On the other hand, the molecular bases for some features of the phenotypes of TIMP-1-null and TIMP-2-null mice remain obscure. For example, memory and learning deficits in TIMP-1-null and TIMP-2-null mice are postulated to arise from abnormal ECM turnover during neuronal development, but the exact molecular events underlying these interesting phenomena are yet to be elucidated. The engineering of TIMP variants that interact with the previously discussed receptors but lack metalloproteinase inhibitory activity, as well as TIMP variants with selective inhibitory activity towards specific metalloproteinases will be useful tools for further functional studies. TIMP variants that inhibit restricted arrays of MMPs and disintegrin-metalloproteinases may also be useful for developing treatments for human diseases related to unregulated metalloproteinase activity. Further structural and functional studies of TIMPs, particularly on their interactions with other binding partners including non-MMP metzincins, would significantly advance the field toward these goals.

Acknowledgments

We thank Professor Gillian Murphy for critical reading of the manuscript. This work is supported by NIH/NIAMS grant AR40994, the Wellcome Trust grant 075473, and a grant from Arthritis Research Campaign in the UK.

Abbreviations

TIMP	tissue inhibitor of metalloproteinases
MMP	matrix metalloproteinase
ADAM	a disintegrin and metalloprotease
ADAMTS	ADAM with thrombospondin motifs
ECM	extracellular matrix
TACE	tumor necrosis factor α converting enzyme
MAPK	mitogen activated protein kinase
PI3-K	phosphoinositide 3-kinase
FAK	focal adhesion kinase
ERK	extracellular signal-regulated kinases
HGF	hepatocyte growth factor
IL-1	interleukin 1
TNF α	tumor necrosis factor α
TGF	transforming growth factor
VEGF	vascular endothelial cell growth factor

FGF	fibroblast growth factor
LDL	low-density lipoprotein
LRP	LDL receptor related protein
RECK	the reversion-inducing cysteine-rich protein with Kazal motif
LPS	lipopolysaccharide
SFD	Sorsby fundus dystrophy
AMD	age-related macular degeneration
EFEMP	EGF-containing fibulin-like extracellular matrix protein

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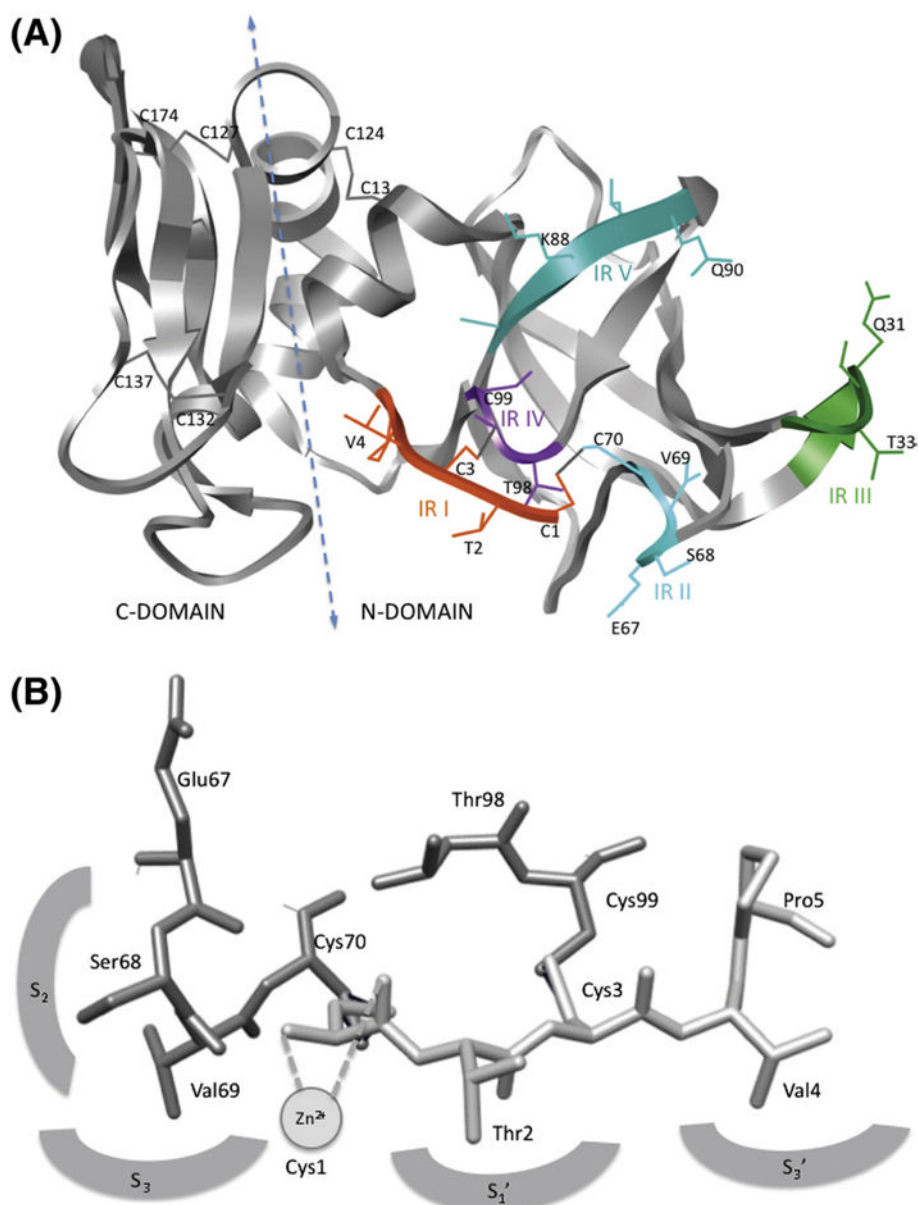


Fig. 1. Structure of TIMP-1 and its metalloproteinase interaction regions. (A) A ribbon structure of the three-dimensional structure of TIMP-1 showing the locations of the disulfide bonds, the two domains, domain interface and regions (IR I to V) that interact with metzincins and their component residues. This image was generated using Chimera [177]. (B) The structure of the core of the TIMP interaction site indicating interactions with individual subsites in MMP active sites.

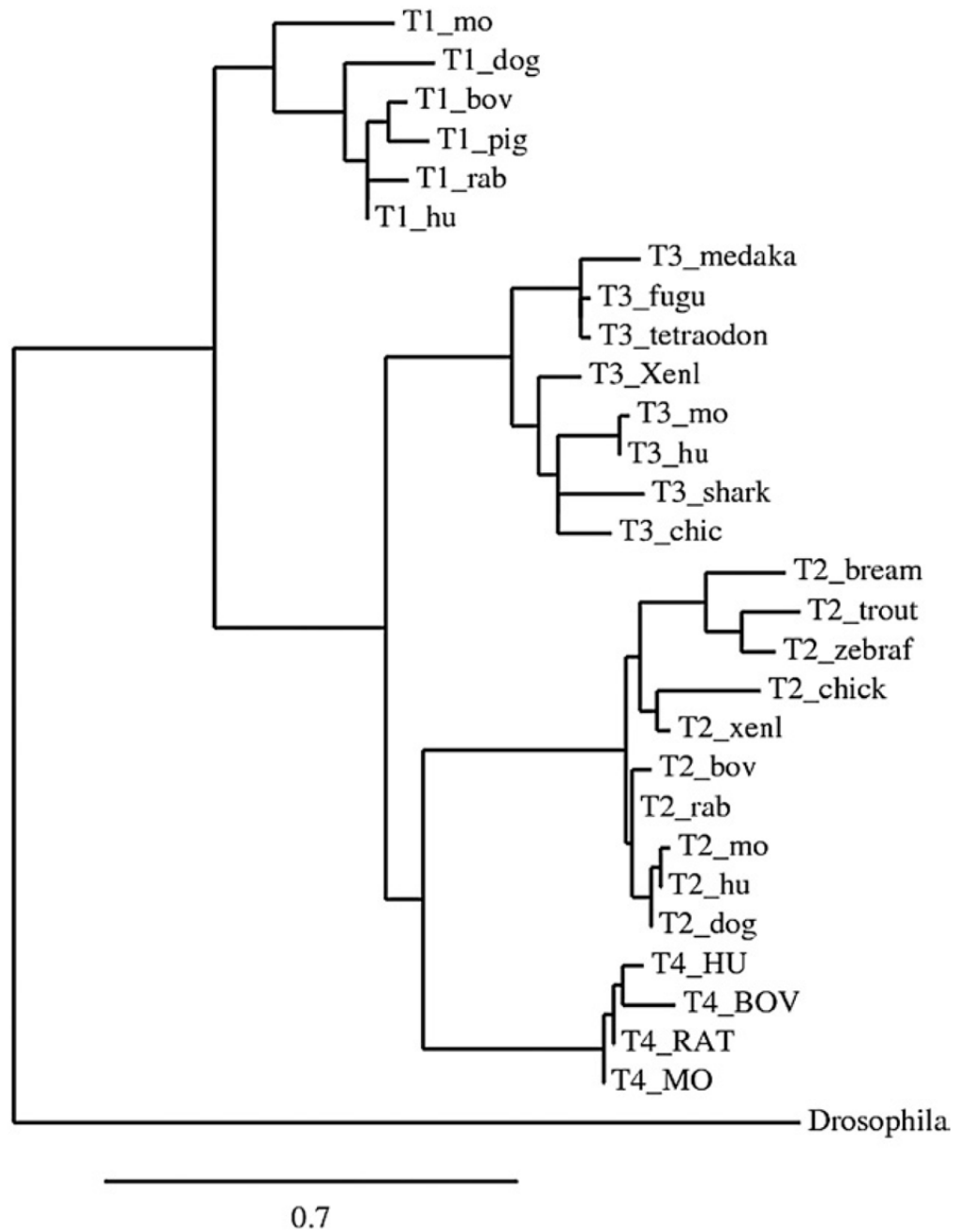


Fig. 2. A phylogenetic tree showing the inferred evolutionary relationships between vertebrate TIMPs-1, -2, -3, and -4, rooted by the single TIMP from *Drosophila melanogaster*. This was generated using Phylogeny.fr (www.phylogeny.fr) in “One Click” mode [178].

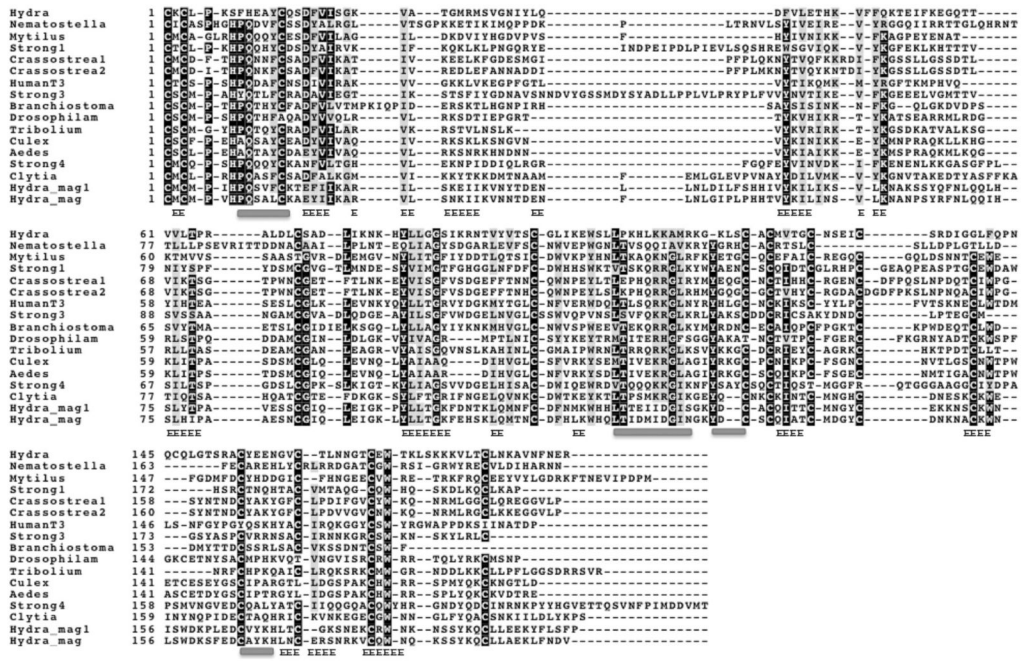


Fig. 3. An alignment of the complete amino acid sequences of TIMPs from invertebrates. The location of secondary structure elements projected from the known three-dimensional structures of mammalian TIMPs are marked underneath. "E" denotes residues in extended structures (β -strands) while bars identify α -helices. The species from which the TIMPs are derived are abbreviated as follows: Hydra, *Hydra magnipapillata*; Nematostella, *Nematostella vectensis* (sea anemone); Mytilus, *Mytilus californianus* (mussel); Strong, *Strongylocentrus purpuratus* (sea urchin); Crassostrea, *Crassostrea gigas* (oyster); HumanT3, human TIMP-3; Branchiostoma, *Branchiostoma floridae* (Florida lancelet); Drosophilam, *Drosophila melanogaster*; Tribolium, *Tribolium castaneum* (red flour beetle); Culex, *Culex quinquefasciatus* (Southern house mosquito); Aedes, *Aedes aegypti* (yellow fever mosquito); Clytia, *Clytia hemisphaerica* (jellyfish).

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DmT          1  CSQPSH-DQTHFAQADVVQLRRLKRSDTIE-----PGRT-TKYVHKRKYRATSE
Acaryochloris 1  CSQMST-PEQMEQADVVTGRVDRKMKAIETPPFGRTWQWMTFVSD--HKGVS
HumanT3      1  CSQPSH-DQDAFCNSDVIIRAKVVGKLVKGC-----PFGTLVYTIHQMKMFRGPTK
HumanT2      1  CSQSPVH-DQAFACNADVIVIRAKVSEKEVDSGNDIYGN-PKIRIQYVIRHQMKMFRGPEK
Bacillus_cerQ1 1  CDINVS-AEBAFQKNDVVFEGQTEVGRKEGC-----GIEV-LFEVVKH--WKGTS
Bacillus_cer  1  CDIMKVS-DEBRLQKTVVFEQKYLEV-QEKGC-----KMEI-LFEVVKH--WKGTS
Bacillus_thur 1  CDIMKVS-DEBRLQKTVVFEQKYLEV-QEKGC-----KMEI-LFEVVKH--WKGTS
Bacillus_cereusG9241 1  CDVKAS-DKBRLLQNDVVFQKYLEV-QEKGC-----KMT-LFEVVKH--WKGTS
Geobacillus  1  CDGAPPEVASSLERSAATPAKQVTOIAGPPRQVMSSADPQVH-VLEVTEH--WKGELN
Plesiocystis 1  CSQMRAPVVAARGADVFSALVLSVAEADSG---TPDFPNKVPFVFAQT--LKGNL

DmT          51  ARMLRDGRLSQDDAMCGI-NLDLCK--VTVVAGRMP-----TLNICSVYREYTRMT
Acaryochloris 58  --EIV--TVESASNSAAGI-NQQLGE--RQVAFANQ--KTAIRASLCSGRARLRDGO
HumanT3      53  MPVQ---YIHBAESLQCG-RLEVK-YQDLTGRV--DQMYGLGNFVERWDLT
HumanT2      59  --DIE--FIVAPSSVCG--SLDVGKKEVFAKAEQ-DQKMTLGDPIVFDL
Bacillus_cerQ1 50  --SIL--IVYNGGD--QVF-HVEVCG--EVLVSSQSGSERQHNSCSGTRRLDE-A
Bacillus_cer  49  --SOV--IIVSFS--QTF-PFAEGG--EVLVSSYRG-EQQLERSMCSGTRRLDE-A
Bacillus_thur 49  --SOV--IIVSFS--QTF-PFAEGG--EVLVSSYRG-EQQLERSMCSGTRRLDE-A
Bacillus_cereusG9241 49  --SOV--IIVSFS--QTF-PFAEGG--EVLVSSYRG-EQQLERSMCSGTRRLDE-A
Geobacillus  58  --PVT--ELTAMSSASGVDQAVCS--EVLVFAAE--SRRLDGLDQTPPLTA-A
Plesiocystis 56  --PQV--RVIQHDGAAAGRDYDEACS--EVLVYARIND-DQQLRDNLCSRMPLDQ-A

DmT          102  ITRH-----GFSGGYAKATH-----
Acaryochloris 108  QEQSQAPSPCAGHGHRAPDATN-----
HumanT3      106  LSQK-----GNRYVHLGCN-----
HumanT2      112  TQKK-----SNHRYQMGCE-----
Bacillus_cerQ1 99  QADKV-----AQQIAKESVPTKKVDLKGEMVSG
Bacillus_cer  97  QADKV-----AQQIAKESVPTKKVDLKGERSV
Bacillus_thur 97  QADKV-----AQQIAKESVPTKKVDLKDMLN-
Bacillus_cereusG9241 97  QADKV-----AQQIAKESVPTKKVDL-
Geobacillus  108  QKELS-----EQAGYPPKAA-----
Plesiocystis 107  QADLE-----AQASKDSLSA-----
    
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Fig. 4.

A comparison of some bacterial homologues with the N-domains of human TIMP-2 and -3 and *Drosophila melanogaster* TIMP. The sequences are from the following sources: Acaryochloris, hypothetical protein AM1_44443 from *Acaryochloris marina* MBIC11017; Bacillus_cerQ1, cbiN-domain protein from *Bacillus cereus* Q1; Bacillus_cer, hypothetical protein from *Bacillus cereus* G9241; Bacillus_thur, hypothetical protein BT9727_1673 from *Bacillus thuringiensis* serovar *konkukian* str. 97-27; Bacillus_cereus9241, hypothetical protein from *Bacillus cereus* G9241; Geobacillus, hypothetical protein GYMC10DRAFT_3737 from *Geobacillus* sp. Y412MC10; Plesiocystis, hypothetical protein PPSIR1_33491 from *Plesiocystis pacifica* SIR-1. Other abbreviations are HumanT2, human N-TIMP-2; HumanT3, human N-TIMP-3; DmT, N-terminal domain of *D. melanogaster* TIMP.

Table 1

A summary of the general properties of the four human TIMPs.

Property	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Glycosylation	Yes	No	Partial	No
<i>pI</i>	8.47	6.48	9.14	7.21
No. of residues ^a	184	194	188	194
<i>M_r</i> ^b	20,709	21,755	21,690	22,329
MMP inhibition	Weak for MMP-14 -16, -19, and -24	All	All	Most
Other MMP inhibition	ADAM10	ADAM12	ADAM10, 12, 17, 28 and 33; ADAMTS-1, -4, and -5, ADAMTS-2 (weak)	ADAM17 ^d and 28, ADAM33 (weak)
Pro-MMP interactions	Pro-MMP-9	Pro-MMP-2	Pro-MMP-9 and pro-MMP-2	Pro-MMP-2
Other partners	CD63 and LRP-1 (MMP-9 complex)	$\alpha_3\beta_1$ integrin LRP-1	EFEMP1, VEGFR2 and Angiotensin II receptor	
Apoptotic effects	Negative	Positive Negative	Positive	
Angiogenesis	Negative	Negative	Negative	Negative
Chromosomal location: human	X11p11.23–11.4	17q23–25	22q12.1–q13.2	3p25
Mouse	X A1.3	11 E2	10C1–D1	6 E3
Synapsin gene ^c	I	None	III	II
Genetic disorder			Sorsby fundus dystrophy	

^a Mature protein.^b Excluding any glycans.^c Nested in intron V of the listed synapsin.^d Inhibited by N-terminal domain of TIMP-4.

Table 2

PDB codes of crystal structures of TIMP-metalloproteinase complexes and the involvement of interaction regions, IRs III–V. See the text and Fig. 2 for definition of IRs.

Complex	Pdb code	Buried surface (\AA^2)	IR III	IR IV	IR V
<i>Inhibitory</i>					
TIMP-1–MMP-3	1UEA	2000	+	+/-	-
N-TIMP-1–MMP-3	1UEA	1400 (calculated)	+	+/-	-
TIMP-2–MMP-14	1BUV	2500	+	+	-
TIMP-2–MMP-13	2E2D	2440	+	+	-
N-TIMP-1–MMP-1	2JOT	1500	-	+/-	-
N-TIMP-3–ADAMI7	3CKI	2120	+	+	+
<i>Non-inhibitory</i>					
TIMP-2–pro-MMP-2	1GXD	2489	-	-	-

Table 3

Mouse phenotypes resulting from deletion of individual TIMP genes.

Genotype	Phenotype	Ref.
TIMP-1 ^{-/-}	▪ Decreased serum testosterone levels	[179]
	▪ Reduced serum progesterone levels during copus lutereum development	[180]
	▪ Enhanced estrogen-induced uterine edema	[181]
	▪ Decrease in the length of estrus period and altered uterine morphology	[182]
	▪ Accelerated endometrial gland formation during early postnatal uterine development	[183]
	▪ Increased resistance to infection by <i>Pseudomonas aeruginosa</i> due to increased inflammatory and complement-dependent immune response	[137]
	▪ Resistant to kainite-induced excitotoxicity in hippocampus and showed the typical mossy fiber sprouting	[129]
	▪ Impaired learning and memory	[129,130]
	▪ Reduced luminal obliteration and increased re-epithelialization after tracked transplantation (reduced airway fibrosis)	[184]
	▪ Enhanced acute lung injury after bleomycin exposure (increased MMP-9 activity in alveoli)	[185]
	▪ Increased HGF activity in regenerating livers	[136]
	▪ Decreased adipose tissue weight in high fat diet induced obesity	[140]
	▪ Hyperphagia and obesity in female mice (standard chow-diet)	[141]
	▪ Altered left ventricular (LV) geometry and cardiac function	[133]
	▪ Exacerbated LV remodeling after myocardial infarction	[134,135]
TIMP-2 ^{-/-}	▪ Impairment of pro-MMP-2 activation by MMP-14	[186,187]
	▪ Motor dysfunction (increased nerve branching and acetylcholine receptor expression; decreased cerebellar neurite outgrowth)	[132]
	▪ Delayed neuronal differentiation (persistence of nestin-positive progenitors in the neocortical ventricular zone)	[104]
	▪ Defect in prepulse inhibition and fear-potentiated startle	[131]
	▪ Weakened muscle and reduced fast-twitch muscle (extensor digitorum longus) mass	[143]
TIMP-3 ^{-/-}	▪ Altered myotube formation	[142]
	▪ Spontaneous air space enlargement and impaired lung function in aged mice	[144]
	▪ Accelerated apoptosis during mammary gland involution	[145]
	▪ Impaired bronchiole branching morphogenesis	[148]
	▪ Chronic hepatic inflammation and failure of liver regeneration	[152]
	▪ Spontaneous dilated cardiomyopathy (left ventricular (LV) dilation, cardiomyocyte hypertrophy and contractile dysfunction with elevated MMP-9 and activation of the TNF α system)	[146]
	▪ LV dilation and dilated cardiomyopathy following aortic banding	[150]
	▪ Accelerated cardiac dilation and matrix disruption following a myocardial infarction	[188]
	▪ Resistance to a brief (30 min) middle cerebral artery occlusion and partial resistance against oxygen-glucose deprivation-induced neuronal cell death and increased Fas L shedding	[189]
	▪ Abnormal vascularization with dilated capillaries throughout the choroid and increased angiogenesis due to an unbalanced VEGF-mediated angiogenesis	[147]
	▪ Enhanced metastatic dissemination of cancer cells to multiple organs	[157]
	▪ Enhanced tumor growth and angiogenesis	[156]
	▪ Increased susceptibility to LPS-induced mortality	[153]
▪ Increased inflammatory response to intra-articular antigen-induced arthritis	[190]	
▪ Increased pulmonary compliance following LPS challenge, but not mechanical ventilation and hyperoxia	[191]	
▪ Spontaneous development of arthritis in aged mice	[149]	

Genotype	Phenotype	Ref.
TIMP-3 ^{+/-}	▪ Accelerated type 2 diabetes when combined with insulin receptor heterozygosity	[154]