



Functional disparities within the TIMP family in cancer: hints from molecular divergence

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Abstract

The members of the tissue inhibitor of metalloproteinase (TIMP) family (TIMP-1, 2, 3, 4) are prominently appreciated as natural inhibitors of cancer-promoting metalloproteinases. However, clinical and recent functional studies indicate that some of them correlate with bad prognosis and contribute to the progression of cancer and metastasis, pointing towards mechanisms beyond inhibition of cancer-promoting proteases. Indeed, it is increasingly recognized that TIMPs are multi-functional proteins mediating a variety of cellular effects including direct cell signaling. Our aim was to provide comprehensive information towards a better appreciation and understanding of the biological heterogeneity and complexity of the TIMPs in cancer. Comparison of all four members revealed distinct cancer-associated expression patterns and distinct prognostic impact including a clear correlation of TIMP-1 with bad prognosis for almost all cancer types. For the first time, we present the interactomes of all TIMPs regarding overlapping and non-overlapping interaction partners. Interestingly, the overlap was maximal for metalloproteinases (e.g., matrix metalloproteinase 1, 2, 3, 9) and decreased for non-protease molecules, especially cell surface receptors (e.g., CD63, overlapping only for TIMP-1 and 4; IGF-1R unique for TIMP-2; VEGFR2 unique for TIMP-3). Finally, we attempted to identify and summarize experimental evidence for common and unique structural traits of the four TIMPs on the basis of amino acid sequence and protein folding, which account for functional disparities. Altogether, the four TIMPs have to be appreciated as molecules with commonalities, but, more importantly, functional disparities, which need to be investigated further in the future, since those determine their distinct roles in cancer and metastasis.

Keywords TIMP · Cancer · Metastasis · Interactome · Proteases

1 Introduction

The four members of the family of tissue inhibitor of metalloproteinases (TIMPs), TIMP-1, 2, 3, and 4, were originally described as secreted endogenous inhibitors of metalloproteinases (MPs) [1], which are able to degrade most of the proteins of the extracellular matrix [2]. Proteolytic activity of MPs can transiently occur during tissue turnover and is tightly regulated by the canonical anti-proteolytic function

of TIMPs [3]. In many diseases, upregulation and activation of MPs are observed as an important disease-driving mechanism [4], while it was thought that the concomitant increased expression of TIMPs is a reflection of attempted but failed balancing of this ECM-degrading activity [5]. In cancer, proteolytic activity supports invasion and metastasis [6–9], while protease inhibition was considered to prevent these aggressive features of malignancies [10–14]. Therefore, TIMPs are generally considered as disease-protective molecules, especially in metastasis, the fatal aspect of cancer progression. However, clinical observations [15, 16] and recent studies [17–20] point at the fact that TIMPs, rather than being protective, can promote cancer progression. At least in the case of TIMP-1, this could be attributed to the emerging non-canonical signaling functions [21–23]. This indicates a far more complex role of TIMPs in cancer than originally thought. However, this notion is still not fully appreciated [24, 25]. This underestimation of the multi-functionality of TIMPs limited our view on the complex biology of individual TIMPs in cancer and possibly other diseases.

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In this review, we attempt to relate the knowledge on functional disparities and molecular divergence of the four members of the TIMP family to distinct cancer-associated tissue expression patterns as well as blood levels, and unexpected prognostic correlations in cancer. We will not only focus on the canonical anti-proteolytic activity of the TIMPs with regard to possible indirect cancer-modifying effects [25]. Instead, building on historical and recently gained knowledge on the multi-functionality of TIMP-1, we here compared commonalities and differences among all four TIMPs in respect to their differential expression in healthy tissue and cancer, their differential interactomes, as well as their structural traits. In this context, TIMP-1 must serve as the prototype for assumed structure-function relationships responsible for multi-functionality, as the other TIMPs have been not extensively studied in this respect so far. Therefore, this review provides a basis for new research avenues, which will provide us with a far better understanding of the complex biology of the four TIMPs and their multi-functionality in disease.

2 Early evidence for multi-functionality of TIMPs

The first-discovered molecule of the TIMP family was purified from amniotic fluid, biochemically characterized as a collagenase inhibitor, and termed TIMP (later known as TIMP-1) in 1981 [26]. Molecular cloning and gene sequencing revealed that TIMP-1 was identical to EPA (erythroid-potentiating activity) [27]. It was independently isolated from supernatants of T lymphoblasts, exhibited pro-proliferative activity, and was unknown to have metalloproteinase inhibitory activity [28]. This early history of the discovery of TIMP-1 already points at multi-functionality (anti-proteolytic and pro-proliferative activities) of this molecule. However, its pro-proliferative activity somehow vanished from scientific focus and was superimposed by the notion that it could play a protective role in cancer [29], probably due to its ability to inhibit cancer-associated MPs including matrix metalloproteinases (MMPs) such as MMP-2 or 9 [30, 31]. TIMP-2, which also possesses erythroid-potentiating activity [32], was cloned in 1990 [33], TIMP-3 was cloned in 1992 [34], and TIMP-4 was identified as the last TIMP family member in 1996 [35]. In contrast to the initial dogma of TIMPs as cancer-protective molecules, TIMP-1 expression was found to correlate positively with cancer progression [36–38], giving a first hint that at least TIMP-1 may not be protective at all. In order to address the clinical relevance of the four TIMPs in different tissues and cancer settings, we updated and comprehensively presented available patient data on each of the four members of the TIMP family and linked their respective levels in tissues and blood with clinical outcome.

3 The TIMP family in cancer patients












We arranged expression data of TIMPs according to their expression in various tissues under physiological conditions as well as in cancer (Fig. 1a). Data allowing a relative comparison of the expression of the four TIMPs in healthy tissue was accessed on the mRNA level in the human protein atlas (HPA) RNA-seq database (<https://www.proteinatlas.org> and [39]). Our approach revealed that all TIMPs are expressed at rather low levels (< 600 transcripts per million (TPM)) with the exception of gender-specific tissues (ovary, uterus, endometrium, cervix, vagina, fallopian tube, and placenta; prostate, testis, and epididymis) (Fig. 1a). Housekeeping genes (GAPDH, ribosomal protein L13a, and beta-actin) typically show a TPM > 600 [39]. Comparison of tissue-specific expressions revealed that TIMP-1, 2, and 3 show a similar pattern (ranging between < 50 TPM in pancreatic tissue and > 1000 TPM in female tissues), while TIMP-4 expression levels are always much lower (< 50 TPM) (Fig. 1a).

Since all TIMPs are able to inhibit several pro-tumorigenic and pro-metastatic MPs [1], one would have expected that their expression should consistently correlate with inhibition or attenuation of cancer progression. However, the clinical findings are in strong contrast to this assumption (Fig. 1). Rather, TIMP-1, with exception of cancers of male tissues (prostate, testis, epididymis) [40], is consistently elevated in cancerous tissues (Fig. 1a) as well as in the blood of cancer patients (Fig. 1b) and clearly correlates with bad prognosis. Specifically, increased systemic TIMP-1 levels are associated with the presence of the two major morbidities in cancer disease, namely metastasis [19, 41] and cachexia [42]. The mechanistic cause for this pro-metastatic effect of TIMP-1 is at least partly elucidated [19, 41]. Also, TIMP-1 emerges as a quite suitable new liquid prognostic biomarker in cancer [42–44].

In contrast to TIMP-1, the disease-associated expression profile as well as the prognostic trend of TIMP-2 is rather heterogeneous and seems to depend on the tissue of origin of the respective tumor (Fig. 1a). For example, bad prognosis of cancer patients correlates with decreased expression of TIMP-2 in lung cancer tissue [45], while it correlates with increased expression in breast cancer [46] (Fig. 1a). Although changes in TIMP-2 blood levels were described for many cancer types, a clear prognostic trend could only be established for lung cancers, where increased blood levels were associated with poor prognosis [47–49] (Fig. 1b). Since most studies revealed no correlation between blood levels and prognosis (Fig. 1b), TIMP-2 did not emerge as a useful liquid biomarker for cancer. Regarding metastasis formation, there is one study suggesting a protective function of TIMP-2. Specifically, elevated levels of TIMP-2 in cancerous tissue and serum of hepatocellular carcinoma patients were associated with decreased metastasis formation [50].

a

Tissue expression and prognosis

Tumor origin	TIMP-1			TIMP-2			TIMP-3			TIMP-4		
	Relative baseline expression	Cancer-associated expression change	Level associated with poor prognosis	Relative baseline expression	Cancer-associated expression change	Level associated with poor prognosis	Relative baseline expression	Cancer-associated expression change	Level associated with poor prognosis	Relative baseline expression	Cancer-associated expression change	Level associated with poor prognosis
Brain 	+++	↑	high	+++	↓	low	+++	↓	low	+	↑	high
Lung 	+++	↑	high	+++	↓	low	+++	↑↓	low	n.e.	n.d.	n.d.
Bone marrow & immune system 	+++	↑	n.d.	++/+++	n.d.	n.d.	++/+++	n.d.	n.d.	n.e.	↑	n.d.
GIT 	+++	↑	high	+++	↑	high	++	↑↓	low	+	n.d.	low
Pancreas 	+	↑	n.d.	+	↓	low	+	↓	low	n.e.	↑	n.d.
Liver 	+++	↑	high	+	↑	high	+	↓	low	n.e.	n.d.	n.d.
Kidney 	++	↑	high	++	↑↓	high	+++	↓	no corr.	+	↑	n.d.
Skin 	++	↑	high	+++	n.d.	n.d.	++	↓	no corr.	n.e.	n.d.	n.d.
Female Tissues 	+++++	↑	n.d.	+++++/+++++	↑	low	++++	↓	n.d.	+	↑	high
Breast 	+++	↑	high	+++	↑	high	+++	↓	low	+	↑	high
Male Tissues 	++++	↓	low	++++	↓	high	+++	↓	n.d.	+	↑	n.d.

Relative baseline expression (RNA expression based on HTA dataset)	
TPM < 50	+
50 - 100 TPM	++
100 - 300 TPM	+++
300 - 600 TPM	++++
600 - 1000 TPM	+++++
TPM > 1000	+++++
not expressed	n.e.

Fig. 1 Comparison of TIMP levels in cancer-afflicted tissue and blood from patients and their prognostic trends. **a** First vertical rows: basal expressions of TIMP-1 (yellow background), 2 (blue background), 3 (green background), and 4 (red background) in healthy human tissues were compared based on RNA expression data as derived from the HTA dataset (<https://www.proteinatlas.org> and [39]). Second vertical rows: cancer-associated TIMP expression changes (up arrow: increase, down arrow: decrease of expression) compared with tissues from healthy individuals or patients with less-advanced cancer. Third vertical rows: poor prognosis (e.g., shortened survival, disease progression, disease

relapse) correlating with either increased (high) or decreased (low) TIMP expression in cancer tissues (n.d.: no data available, no corr.: no correlation existing). Pertaining references are listed in Supplementary information S1. **b** First vertical rows: cancer-associated changes of TIMP blood levels (up arrow: increase, down arrow: decrease) compared with samples from healthy individuals or patients with less-advanced cancer. Second vertical rows: poor prognosis correlating with either increased (high) or decreased (low) TIMP levels in the blood (n.d., no data available; no corr., no correlation existing). Pertaining references are listed in Supplementary information S2

TIMP-3 is considered to act as a tumor-suppressive protein, which is often epigenetically silenced in tumor cells [51]. This protective role of TIMP-3 is also reflected by the finding that its expression is consistently downregulated in cancerous tissues and this downregulation is associated with poor prognosis (Fig. 1a). There is only limited data on circulating levels of

TIMP-3 in cancer patients indicating that in certain cancer types (brain, skin, female tissues; Fig. 1b) loss of TIMP-3 expression in the tissue is accompanied with decreased TIMP-3 levels in the blood (Fig. 1b). One recent study in ovarian carcinoma showed a clear correlation between low TIMP-3 blood levels and bad prognosis [52]. Regarding

b

Blood levels and prognosis












Tumor origin	TIMP-1		TIMP-2		TIMP-3		TIMP-4	
	Cancer-associated change in blood levels	Level associated with poor prognosis	Cancer-associated change in blood levels	Level associated with poor prognosis	Cancer-associated change in blood levels	Level associated with poor prognosis	Cancer-associated change in blood levels	Level associated with poor prognosis
Brain 	↑	high	n.d.	n.d.	↓	n.d.	n.d.	n.d.
Lung 	↑	high	↑↓	high	n.d.	n.d.	n.d.	n.d.
Bone marrow & immune system 	↑	high	↑↓	n.d.	n.d.	n.d.	n.d.	n.d.
GIT 	↑	high	↓	no corr.	n.d.	n.d.	n.d.	n.d.
Pancreas 	↑	high	↓	no corr.	n.d.	n.d.	n.d.	n.d.
Liver & gallbladder 	↑	high	↑	high or low	n.d.	n.d.	n.d.	n.d.
Kidney 	↑	high	↓	n.d.	n.d.	n.d.	n.d.	n.d.
Skin 	↑	high	↑↓	n.d.	↓	n.d.	n.d.	n.d.
Female Tissues 	↑	high	↑↓	no corr.	↓	low	n.d.	n.d.
Breast 	↑	high	↑	n.d.	n.d.	n.d.	n.d.	n.d.
Male Tissues 	↑	high	↑↓	no corr.	n.d.	n.d.	n.d.	n.d.

Fig. 1 (continued)

metastasis formation, there is one study suggesting a protective function of TIMP-3, where decreased expression in cancerous tissue of hepatocellular carcinoma patients was associated with increased metastasis formation [53].

TIMP-4 is the least-studied member of the TIMP family in the context of cancer. In the investigated cancerous tissues, TIMP-4 expression levels are increased (Fig. 1a) and positively correlate with poor prognosis. So far, no data on blood

levels of TIMP-4 in cancer patients are available. Also, there are no data available indicating an impact of TIMP-4 on metastasis in cancer patients.

Taken together, the clinical observations revealed three rather clear associations between members of the TIMP family and cancer progression. Only TIMP-3 seems to behave as expected from the rationale that TIMPs should be protective due to their anti-proteolytic activity. Its expression positively

correlates with good prognosis in almost all investigated tumor types (Fig. 1). In contrast, TIMP-1 clearly positively correlates with bad prognosis of virtually all tumor types (Fig. 1), which led us to consider it to be the “black sheep” of the TIMP family. Interestingly, we here describe the few available indications that TIMP-4 may also be involved in tumor progression, since its expression is associated with bad prognosis as well (Fig. 1). On the other hand, TIMP-2 seems to be prone to act heterogeneously (Fig. 1). These disparities among the TIMPs shed an interesting light on this family, which contradicts both the initial concept that the whole family functions in a homogenous cancer-protective manner and that TIMP-1 is a black sheep. Instead, it is necessary to more carefully investigate the specific functional and structural traits of each member of the family, which obviously cannot be lumped together.

Below, we will discuss functional disparities as possible reasons for the clinical differences. Since functional disparities between the TIMPs are likely a result of differences in their interaction partners, we will describe available data on commonalities and disparities of the respective interactomes in the following chapter. The molecular divergence, which may

account for these interactomes, will be discussed in the third chapter.

4 Linking the interactome of the TIMP family members to their molecular functions

The above-mentioned associations and correlations identifying TIMPs to different degrees as clear markers for good or bad prognosis in cancer do not necessarily imply that the individual TIMPs are mechanistically and causally involved in the regulation of cancer progression. Indeed, studies in mice and in tissue culture revealed that TIMPs critically impact on cancer progression [17, 54–58]. To identify potential differences in molecular functions of the TIMP family members, we compared their respective interactomes, focusing only on so far experimentally verified interactions (Fig. 2).

Our approach revealed an intersection of interaction partners shared by all TIMPs consisting exclusively of activated (MMP-1, 2, 3, 9) and non-activated (proMMP-2) MMPs (Fig. 2). The intersections of interaction partners that are shared by

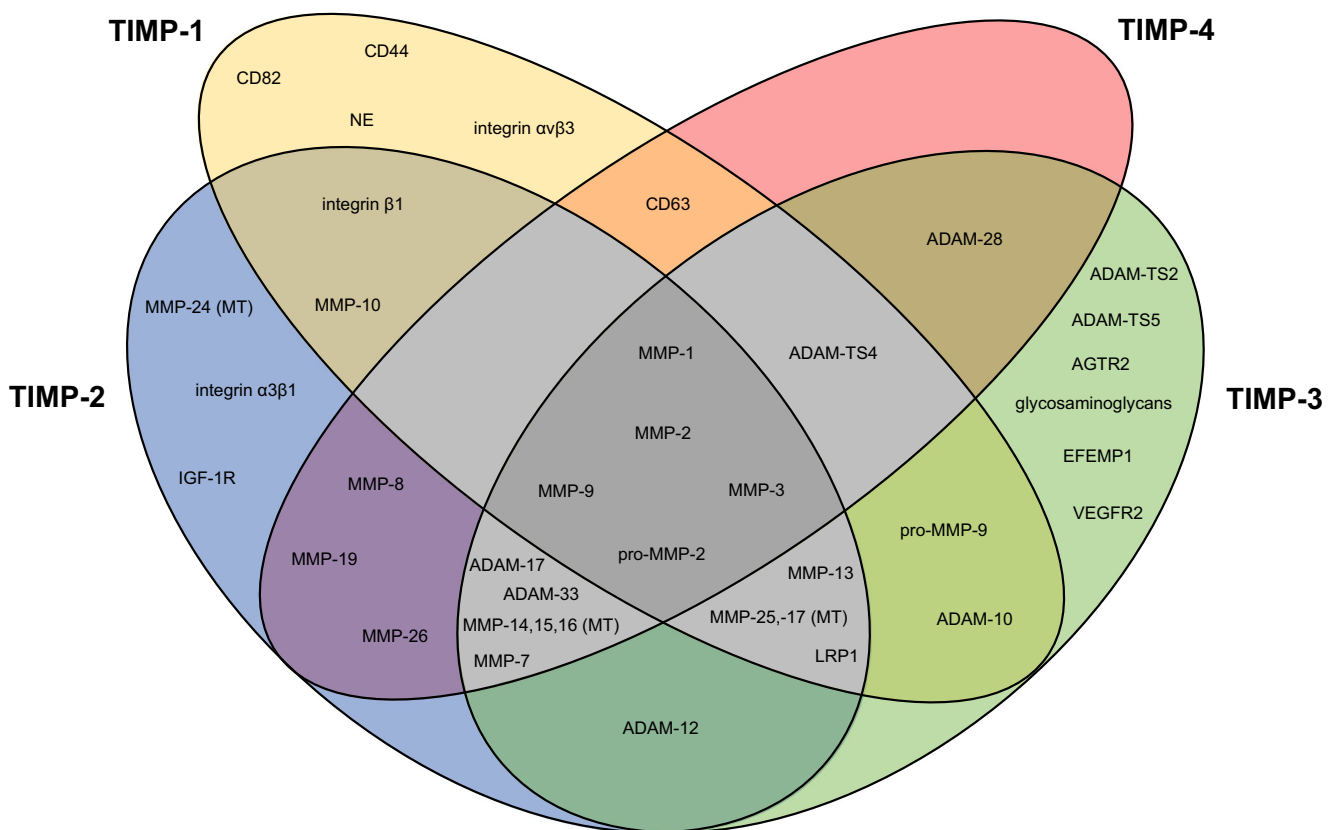


Fig. 2 Intersection diagram depicting the interactomes of the four TIMPs. Only experimentally verified interaction partners were included (yellow background: TIMP-1, blue background: TIMP-2, green background: TIMP-3, red background: TIMP-4; mixed colors indicate overlapping interactomes between two TIMP members; light grey: interaction partners of three TIMPs; dark grey: interactome of all four TIMPs). Pertaining references are listed in Supplementary information S3. ADAM a

disintegrin and metalloproteinase, ADAM-TS ADAM with thrombospondin motif, AGTR2 angiotensin II receptor type 2, EFEMP1 EGF-containing fibulin-like extracellular matrix protein 1, IGF-1R insulin-like growth factor 1 receptor, LRP1 low-density lipoprotein receptor-related protein 1, MMP matrix metalloproteinase, MT membrane-type, NE neutrophil elastase, VEGFR2 vascular endothelial growth factor receptor 2

three different TIMPs contain other MMPs including MT-MMPs, a disintegrin and metalloproteinases (ADAMs), as well as ADAM with thrombospondin motifs 4 (ADAM-TS4) (Fig. 2). Interestingly, low-density lipoprotein-related protein (LRP-1) is the only non-protease molecule known to interact with three TIMPs (TIMP-1, 2, and 3) (Fig. 2). Since binding of TIMPs to LRP-1 results in endocytosis of the respective TIMP molecules [59–61], this interaction is discussed as a dynamic mechanism to regulate the pericellular localization of TIMPs [62]. The intersections of interaction partners that are shared by two different TIMPs also contain mainly proteases, namely MMPs (including proMMP-9), as well as ADAMs and two non-proteases, namely the tetraspanin CD63 and the integrin β_1 (Fig. 2). In contrast, and importantly, the unique interaction partners for each TIMP are in the majority non-metalloproteases, namely cell surface and ECM-associated proteins including the tetraspanin CD82, CD44, neutrophil elastase (NE), and the integrin $\alpha_v\beta_3$ (unique for TIMP-1); integrin $\alpha_3\beta_1$ and insulin growth factor 1 receptor (IGF-1R) (unique for TIMP-2); glycosaminoglycans, vascular endothelial growth factor receptor 2 (VEGFR2), angiotensin II receptor type 2 (AGTR2), and EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1) (unique for TIMP-3) (Fig. 2). Only three MPs, namely MMP-24 (TIMP-2), ADAM-TS2, and ADAM-TS5 (TIMP-3) are so far described unique interaction partners of the respective TIMPs. Although several MPs can interact with more than one TIMP, each of the TIMPs exhibits a unique spectrum of MP interactions.

While the anti-proteolytic activity of individual TIMPs in cancer was reviewed previously [63, 64], we here focus on rather specific non-protease interaction partners, which attribute distinct pro- or anti-cancer activities to different TIMPs. For example, the formation of a ternary complex between TIMP-1, proMMP-9, and the cell surface receptor CD44 was shown to activate the JAK2/PI3K/Akt signaling pathway, resulting in increased survival of acute myeloid leukemia cells [65]. In addition, interaction of TIMP-1 with the integrin $\alpha_v\beta_3$ induced resistance against TNF α -mediated apoptosis in osteosarcoma cells [66]. TIMP-1 was also shown to bind to the tetraspanins CD82 [67] and CD63 [68] (Fig. 2). Interaction with CD82 seems to contribute to the internalization of TIMP-1 [67]; however, so far, only little is known about direct cellular effects resulting from this interaction. Since CD63 is a ubiquitously expressed protein [69], TIMP-1/CD63-induced signaling effects were intensively studied in tumor cells from different origins and several pro-tumorigenic effects of this interaction were described. In lung adenocarcinoma cells, TIMP-1 induced PI3K/AKT signaling *via* CD63 leading to HIF-1 α stabilization, miR-210 accumulation, and increased angiogenesis [20]. In addition, TIMP-1 was able to promote the formation of a hepatic pre-metastatic niche *via* CD63-dependent activation of hepatic stellate cells in the context of

pancreatic cancer [41]. Another study showed that TIMP-1 promotes survival and invasion of acute myeloid leukemia cells through CD63-mediated activation of PI3K/AKT signaling [57]. Altogether, these pro-tumorigenic as well as pro-metastatic effects of TIMP-1 mediated by interaction with non-proteases may explain the consistent clinical correlation of elevated TIMP-1 levels with metastasis and poor clinical outcome (Fig. 1). Interestingly, CD63 is also an interaction partner of the less-studied TIMP-4 (Fig. 2), which is increased in cancer tissues (Fig. 1a). So far, no cellular effects mediated by TIMP-4/CD63 interaction are known. Interestingly, TIMP-1 and TIMP-4 show comparable clinical trends in distinct cancer types (Fig. 1) including tumors of the brain tissue, where TIMP-1 [70], TIMP-4 [71], as well as expression of their common receptor CD63 [71] were shown to be associated with bad prognosis. In future studies, it has to be determined, whether the poor clinical outcome related to TIMP-4 overexpression is causally linked to its interaction with CD63.

Since low expression levels of TIMP-3 are associated with poor survival of patients (Fig. 1a), TIMP-3 is discussed as a tumor-suppressive molecule [51]. The clinical observations are in line with studies showing that TIMP-3 induces apoptosis [72] and decreases invasion [14, 72] as well as metastasis [73] of tumor cells of different origins. Analogous to the previous rationale, we should assume that the interaction partners of TIMP-3 account for its cancer-protective functions. In addition to possible anti-proteolytic activities [74–76], the unique non-protease interaction of TIMP-3 with VEGFR2 was shown to inhibit angiogenesis by antagonistically blocking the binding of VEGF to its receptor [77]. Similarly, the interaction of TIMP-3 with another unique non-protease interaction partner, namely AGTR2, was described to negatively affect angiogenesis [78]. Since angiogenesis is a central event occurring during cancer progression [79], these non-protease interactions of TIMP-3 likely contribute to its tumor-suppressive effects. So far, no direct pro- or anti-tumorigenic effects were described resulting from interaction between TIMP-3 and glycosaminoglycans or the glycoprotein EFEMP1, respectively. However, the unique interactions between TIMP-3 and these ECM-associated molecules determine the pericellular localization of TIMP-3 within the ECM [80, 81], which is a specific feature of this TIMP family member.

In contrast to the consistently increased expression of TIMP-1 and TIMP-4 or decreased expression of TIMP-3 in several cancer types, expression of TIMP-2 and its prognostic trends are rather heterogeneous between different cancer types (Fig. 1a). This ambiguity of TIMP-2 is reflected in *in vitro* studies as well as in pre-clinical studies in mice, where TIMP-2 can context-dependently stimulate [82] or suppress [83] tumor cell growth. On the molecular level, TIMP-2 interacts with integrin $\alpha_3\beta_1$, which is a unique feature within the TIMP family. This interaction induced signaling pathways

leading to the expression of differentiation markers [84] as well as to cell cycle arrest [85]. Furthermore, the unique antagonistic interaction of TIMP-2 with IGF-1R was shown to decrease cell proliferation and angiogenesis *via* inhibition of IGF-1R downstream signaling [86]. These findings show the tumor-suppressive effects of TIMP-2. In contrast, binding of TIMP-2 to MT1-MMP induces invasion-promoting signaling in tumor cells [54, 87]. Therefore, the impact of TIMP-2 on tumor progression is context dependent.

It is apparent that the four TIMPs distinguish themselves significantly in their interactome. Regarding TIMP/MP interactions, it is important to note that each of the TIMPs is able to inhibit a distinct set of MPs. This indicates that each deregulation of the individual TIMPs likely has a distinct impact on the proteolytic balance within a given tissue. It can still be maintained that TIMPs are at the apex of the complex biological hierarchy [62]. This notion was established in the face of the TIMPs' ability to regulate those MPs, which, *via* the resulting degradome [88], impact decisively on cell functions [62]. This notion is now extended by the present analysis revealing that each TIMP exhibits a unique impact on the degradome. Consequently, depending on the presence and anti-proteolytic activity of each TIMP in different cancer contexts (Fig. 1), this gives rise to distinct TIMP (TIMP-1 *vs* TIMP-2 *vs* TIMP-3 *vs* TIMP-4)–modulated cancer degradomes determining quite different biological mechanisms. In addition to interactions with a unique repertoire of MPs, each of the TIMPs is able to bind to additional unique interaction partners, including cell surface receptors, thereby mediating distinct proteolysis-independent effects on tumor as well as host cells. Altogether, the unique interactomes of TIMP-1, 2, 3, and 4 likely determine their distinct functions in specific microenvironmental contexts.

The individual interactomes are a consequence of protein-protein interactions based on differences in peptide sequence and 3D-structure of the different TIMPs. Therefore, we will next provide examples for common and unique structural traits based on the amino acid sequence and folding, which, altogether, may account for distinct and common interactions of the four TIMPs.

5 Structural commonalities and disparities within the TIMP family

Information on the amino acid sequence, 3D structure, and functional motifs of TIMPs is extensively reviewed elsewhere [64, 89–92]. In brief, all TIMPs are quite similar in size and consist of two structurally distinguishable domains, the so-called N-terminal and C-terminal domains [93] (Fig. 3). Although the four TIMPs have a sequence identity of less than 40%, it is suggested that they all exhibit a rather similar 3D structure derived from the so far available crystallization data

on TIMP-1 [95], TIMP-2 [93], and the N-terminal domain of TIMP-3 [97]. Here, we make use of this information in order to identify common and unique structural traits, which, altogether, may account for the distinct interactomes of the four TIMPs. This is the first attempt to relate distinct structural traits (anti-proteolytic motif, exosites, posttranslational modifications, GH loop, and the C-terminal tail) to the spectrum of interaction partners of each TIMP.

5.1 The anti-proteolytic motif

The essential motif for the anti-proteolytic activity of all TIMPs is composed of the first three amino acids at the N-terminal end of the molecules. This motif (CTC-motif for TIMP-1 and 3; CSC-motif for TIMP-2 and 4) reaches into the active cleft of MPs, where the hydroxyl group of Thr² or Ser² prevents the coordination of the H₂O molecule, which is necessary for hydrolysis of the peptide bond of the substrate [95]. The second position (Thr² or Ser²) is likely one feature defining the repertoire of MP interactions for each TIMP, as it indeed was shown that exchange of Thr² by Ser in N-TIMP-1 leads to a shift in MP specificity and inhibitory potency [98]. Differences in the fourth position (Val⁴ for TIMP-1, Ser⁴ for TIMP-2 and 3, and Ala⁴ for TIMP-4), may also account for disparities of the spectrum of interactions with MPs [99], since this residue reaches into the specificity pocket S3' of the respective MP [95].

5.2 Exosites

The anti-proteolytic activity as well as target selectivity of TIMPs is, although to a lesser degree, also determined by exosites [100, 101], i.e., the regions adjacent to the anti-proteolytic motif.

One exosite important for TIMP-MP interaction is the C-connector loop (Met⁶⁶-Cys⁷⁰ in TIMP-1) (Fig. 3). The C-connector loop, first described for TIMP-1 [95], is a highly conserved structural trait of all four TIMPs and structurally linked to the anti-proteolytic motif *via* a Cys¹-Cys⁷⁰ disulfide bond. In the 3D structure, this connection positions the residues Ser⁶⁸ and Val⁶⁹ of TIMP-1 into specificity pockets S2 and S3 of the bound MP [95]. In TIMP-2, Ser⁶⁸ is exchanged to Ala [102], and in TIMP-3, Val⁶⁹ is exchanged to Leu [97] at the respective corresponding positions. These alterations are discussed to contribute to distinct interaction spectra of the different TIMPs with MPs [99, 103].

The AB-turn, connecting the first two beta strands and folding them to a beta sheet, is another common exosite contributing to the spectrum of MPs interacting with the four TIMPs. This structural trait, always starting at residue 31 in all TIMPs, is rather heterogeneous and varies in length (Fig. 3a). While it just forms a turn in TIMP-1 (encompassing Gln³¹-Leu³⁴) and TIMP-3 (Glu³¹-Gly³⁵), it forms a larger loop

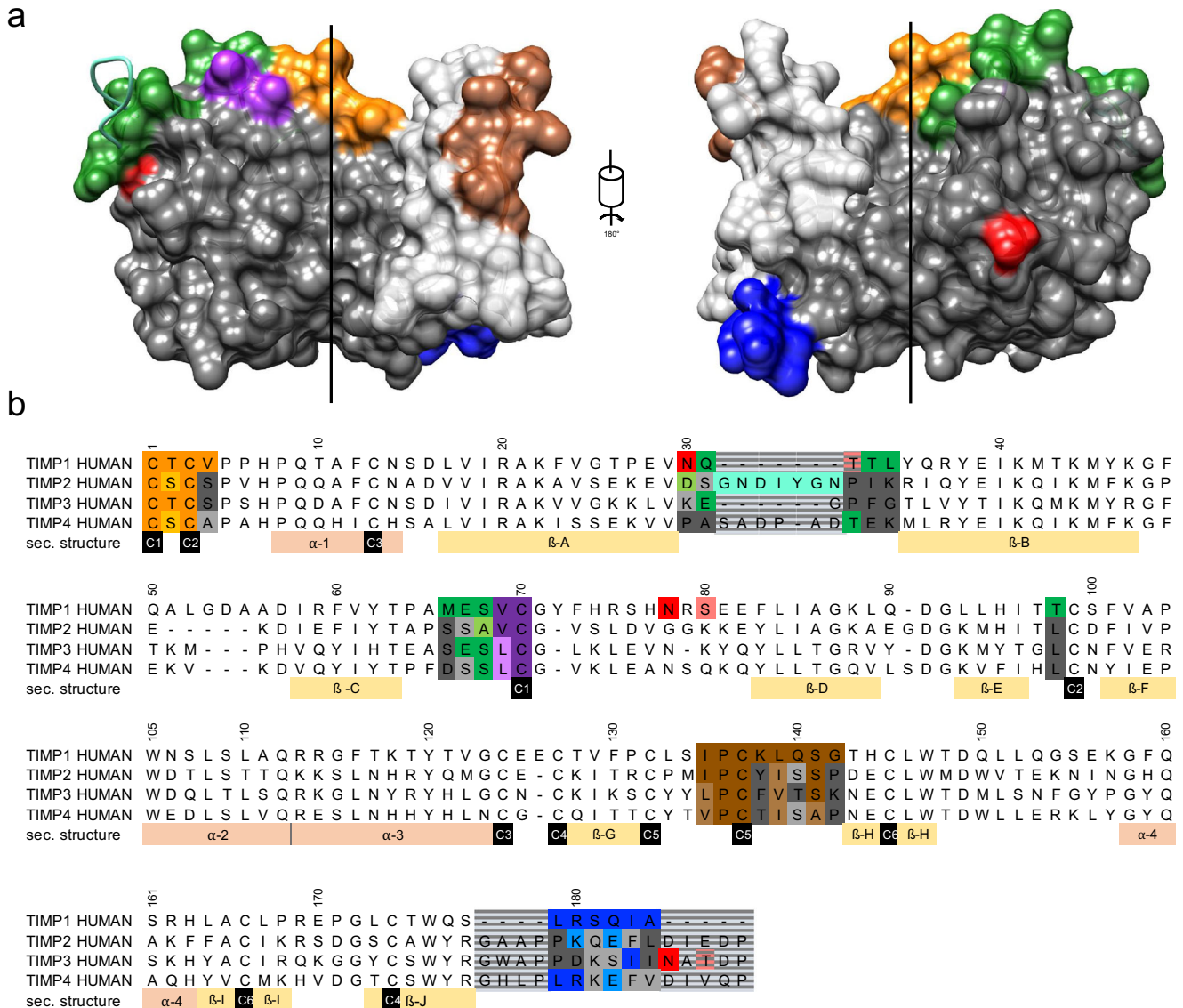


Fig. 3 Molecular commonalities and disparities (colored) within the TIMP family on the level of 3D structure and amino acid sequence (orange: anti-proteolytic motif; green: exosites; red: N-glycosylation sites (Asn); light red: Ser/Thr essential for the N-glycosylation sequon (N-X-S/T); purple: neutrophil elastase cleavage site; brown: GH loop; blue: C-terminal tail). **a** Structure of TIMP-1 as a surface model (created with UCSF Chimera [94]). Structure was modified by adding the last three amino acids based on X-ray information (1uea, [95]) (left panel) and turned by 180° (right panel). The aligned structure of the AB-loop (Glu³⁰-Lys⁴²) of TIMP-2 based on X-ray information (1br9 [93]) is shown in cyan blue. The N-terminal domain of TIMP-1 is shown in dark grey, and the C-terminal domain is depicted in light grey. **b** Amino acid

sequence alignment of the four human TIMPs. Colors correspond to the respective regions in the surface model. Variations of amino acids within these structural traits are color-coded according to their extent of divergence based on the BLOSUM62 score (BLOSUM62 score > 1: less color intensity, BLOSUM62 score = 0: light grey, BLOSUM62 score < 1: dark grey). Gaps are shown with grey stripes. Sequences were exported from uniprot (entries: P01033, P16035, P35625, Q99727) and aligned with MEGA7 (clustalW2 based on BLOSUM62) [96] on basis of the TIMP-1 sequence. Amino acids contributing to distinct secondary structural elements are marked in the line “secondary structure” (light yellow: beta-sheets; light red: alpha helices; black: cysteine bridges (C1, C2, C3, C4, C5, C6))

(AB-loop) in TIMP-2 (Ser³¹-Lys⁴¹) and TIMP-4 (Ala³¹-Lys⁴⁰) (Fig. 3b). Structures derived from TIMP-MP complexes revealed that the AB-turn/loop is directly located at the interface of the complex and thereby involved in the interaction of TIMPs with MPs [104]. Mutation approaches with respect to length as well as amino acid composition of the AB-

turn/loop resulted in a shift of the inhibitory capacity of TIMPs towards distinct MPs [100, 101, 105].

Another important exosite is the amino acid immediately N-terminal of the second cysteine bridge (Fig. 3b). While TIMP-1, which is not able to interact with MMP-14 (Fig. 2), exhibits Thr⁹⁸ at this position (Fig. 3b), TIMP-2, 3, and 4

exhibit Leucine at the respective site (Leu⁹⁹, Leu⁹⁴, and Leu¹⁰⁰, respectively) (Fig. 3b) and are able to interact with MMP-14 (Fig. 2). Like a proof of concept for our approach, it was shown that experimental T98L substitution in TIMP-1 enabled it to efficiently inhibit MMP-14 [100].

All above-mentioned structural traits are located in the N-terminal domains of TIMPs. Since TIMP mutants comprising the N-terminal domain only (N-TIMP-1, 2, 3, and 4) show reduced inhibition of most MPs compared with their full-length counterparts [106], it is assumed that also exosites within the C-terminal TIMP domain exist, which are important to mediate their interactions with MPs. In fact, the finding that parts of the C-terminal domain of TIMP-1 are necessary for its full inhibitory capacity towards ADAM-10 [107] further substantiates the rationale that structural traits are decisive for the interactome, and thereby for the function of the TIMPs.

5.3 GH loop

The GH loop region is highly conserved among all TIMPs (Fig. 3). Crystallographic analyses revealed that the GH loop constitutes the interface during the interaction of TIMP-2 with the latent pro-form of MMP-2 (proMMP-2) [108]. Importantly, all TIMPs interact with proMMP-2 (Fig. 2), suggesting that the GH loop is responsible for this common feature of all TIMPs.

5.4 C-terminal tail

More recently, the C-terminal domain of the TIMPs gained a lot of attention, as it seems to contain an important structural trait mediating direct signaling effects, at least of TIMP-1 [41, 109, 110]. The C-terminal tail, i.e., the last few amino acids, is highly variable within the TIMP family (Fig. 3b) and seems to be involved in the interaction of TIMP-1 with the tetraspanin CD63 [111], via the C-terminal domain [68]. In fact, the interaction of the C-terminal domain of TIMP-1 is involved in many recently described pro-tumorigenic [109, 110] and pro-metastatic [41] signaling functions of TIMP-1. Although it was shown that TIMP-4 interacts with CD63 [71], no biological effects have so far been assigned to this interaction. Interestingly, the C-terminal ends of TIMP-1 and TIMP-4 are rather heterogeneous with respect to length as well as sequence (Fig. 3b). Future studies will have to substantiate and further explore these initial observations on TIMP-4/CD63 interactions.

5.5 Posttranslational modifications

The emerging appreciation of posttranslational modifications (PTMs) as crucial modifiers of protein function [112, 113] exhibits another highly relevant level of structural traits of proteins in general and TIMPs in specific. PTMs can change

the interface of proteins significantly and can therefore contribute to the selection of interaction partners. Therefore, PTMs vastly expand functionality as well as multi-functionality of proteins [114].

N-glycosylation represents the most complex PTM [115, 116]. TIMP-1 and TIMP-3 are glycoproteins carrying the essential N-glycosylation sequon Asn-X-Ser/Thr, while TIMP-2 and TIMP-4 do not exhibit such PTM and do not harbor any glycosylation sequon [62] (Fig. 3b). TIMP-1 has two glycosylation sites at positions Asn³⁰-Gln³¹-Thr³² and Asn⁷⁸-Arg⁷⁹-Ser⁸⁰ [28], and TIMP-3 harbors one potential glycosylation site at Asn¹⁸⁴-Ala¹⁸⁵-Thr¹⁸⁶ [34], which are altogether not characterized any further so far (Fig. 3b).

Proteolytic cleavage represents an irreversible PTM of proteins. In TIMP-1, the C-connector loop was identified as a proteolytic target of human neutrophil elastase [117] (Fig. 2). Although the overall structure of TIMP-1 seems to be maintained by Cys-bridges after NE-mediated cleavage between Val⁶⁹ and Cys⁷⁰ [118, 119], TIMP-1 forfeits its anti-proteolytic functions [120]. Interestingly, TIMP-2 possesses the same Val-Cys motif and the corresponding Val is conservatively replaced by a Lys in TIMP-3 and TIMP-4 (Fig. 3b). Given this similarity in protein sequence, it has to be investigated, whether TIMP-2, 3, and 4 can be cleaved as well, which may lead to loss of their MP inhibitory functions.

6 Conclusions

This review took a new approach towards the understanding of the clinical relevance of the four TIMPs in cancer and metastasis by evaluating functional disparities and relating them to their molecular divergence. The impact of the individuality of each TIMP runs like a golden thread through all levels that were examined. Even though they are all metallo-proteinase inhibitors with common features for anti-proteolytic activity, each TIMP exhibits rather specific features in respect to expression levels and prognostic trends in different cancer entities, their interactome including protease and non-protease molecules, as well as structural peculiarities. These disparities are often overlooked easily leading to an underestimation of the wide biological versatility of this protein family. In fact, in order to get a deep insight into the biology of TIMPs in cancer, but also in other diseases, it is necessary to study all aspects of their diversity in more detail. Studies on structure/function relationships for each TIMP are a starting point towards a more comprehensive understanding of these molecules and associated diseases. Ultimately, this knowledge may lead to the dissection of clear disease-protective or promoting activities of each TIMP and a more educated therapeutic interference targeting either their specific structures or their interaction with specific partners.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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