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Analysis Of The Structure And Function Of A Timp-1/cd63 Complex And Its Relationship To An Mt1-Mmp/cd63 Complex

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ANALYSIS OF THE STRUCTURE AND FUNCTION OF A TIMP-1/CD63 COMPLEX AND ITS RELATIONSHIP TO AN MT1-MMP/CD63 COMPLEX

by

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DEDICATION

This work is dedicated to my wonderful family. Beginning with my wife and three children, I want to acknowledge how important my family has been to me throughout this major undertaking in my life. Paramount in my life is my relationship to God and my spirituality, which I apply in all things that I do; my wife, Stephanie, has been an unfailing support in this aspect. It is a truly wonderful and humbling experience that I have had as a spouse and parent together with her. My three children, Corban, Collette, & Camille are choice spirits who have impressed me with their intelligence, surprised me with their creativity, individuality and curiosity, and caused me to laugh and step back from aspects of work that are seemingly too serious.

As well, I owe a large debt of gratitude to my loving parents Gayle and JoLynn Warner, for raising me up to become who I am. They have urged me to be and do my best from grade school art and science projects up through this graduate work. I would also like to dedicate this work to all of my siblings and their spouses (Michael & Robyn, Stacy, David & Ceanne, Douglas, and Rebecca & Jesse Frost) and to my nieces and nephews (Jessica, Benjamin, Bronte, Joshua, Oriana, Spencer, Eva, Ian, Ember, and Andrew) for all of their love and support by visiting us in Michigan, or at their homes, or with phone calls and pictures. It has been a long road to finish my PhD and my family has been with me every step of the way to cheer me on. I could not have done it without them.

I also dedicate this work to all of the women diagnosed with breast cancer past, present, and future. I hope that in the future my work will have contributed to curative treatment for breast cancer.

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PREFACE

This work was supported by the Ruth L. Kirschstein National Research Service Award T32-CA009531 (to Richard B. Warner); a Thomas C. Rumble University Graduate Fellowship (to Richard B. Warner); and a National Institute of Health RO1 grant (to HRC Kim). Richard B. Warner is a member of the Cancer Biology Graduate Program at Wayne State University. The purpose of this project is to map the TIMP-1 and CD63 interaction domains and to identify functional consequences of TIMP-1/CD63 complex formation with particular focus on their relationship with MT1-MMP and an MT1-MMP/CD63 complex.

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LIST OF ABBREVIATIONS

AA (or aa)	**Amino acid**
ADAM	**A disintegrin and metalloproteinase**
ALA	**Alanine**
β-ME	**Beta mercaptoethanol**
CTL	**Control**
CYS	**Cysteine**
DDO	**Double dropout media**
DMEM	**Dulbecco's modified eagle medium**
ECL	**Enhanced chemiluminescence**
ECM	**Extra-cellular matrix**
EGF	**Epidermal growth factor**
EPA	**Erythroid potentiating activity**
EMT	**Epithelial-mesenchymal transition**
GLucC	**C-terminal fragment of hGLuc**
GLucN	**N-terminal fragment of hGLuc**
GLY	**Glycine**
H2	**Helix 2**
H3	**Helix 3**
hGLuc	**Humanized *gaussia* luciferase**
HNF4	**Hepatocyte nuclear factor 4**
LEL	**Large extra-cellular loop**
MMP	**Matrix metalloproteinase**

MT1-MMP (or MT1)	**Membrane type-1 MMP**
PBS	**Phosphate buffered saline**
PBS-CM	**Phosphate buffered saline with calcium and magnesium**
PCA	**Protein complementation assay**
PNGaseF	**Peptide: N-Glycosidase type F**
PTC	**Papillary thyroid carcinoma**
QDO	**Quadruple dropout media**
Ref	**Reference**
RLU	**Relative luciferase unit**
rTIMP-1	**Human recombinant TIMP-1**
SEL	**Short extra-cellular loop**
SER	**Serine**
SF	**Serum-free**
TBST	**Tris buffered saline with tween 20**
TfR	**Transferrin receptor**
TIMP-1 (or T1)	**Tissue inhibitor of metalloproteinases-1**
TIMP-2	**Tissue inhibitor of metalloproteinases-2**
TM	**Transmembrane domain**
VAL	**Valine**
YPD	**Yeast extract peptone dextrose**

Chapter 1

Introduction and Hypothesis

1.1 Tissue inhibitor of metalloproteinases-1 (TIMP-1) as an inhibitor of proteolysis

The balance of enzyme activity in the extra-cellular matrix (ECM) is maintained by regulation of extra-cellular proteases' expression and activity. A large family of proteases and major contributors to the degradation and remodeling of the ECM is the matrix metalloproteinases (MMPs). These enzymes are secreted in inactive zymogen form and following activation are regulated by competitive inhibition at their active sites by endogenous inhibitors which include the tissue inhibitors of metalloproteinases, or TIMPs. There are four known TIMPs (TIMP-1 thru -4); which are known to inhibit MMPs via 1:1 stoichiometric reversible complexes [1,2]. The first of the TIMP family to be discovered was tissue inhibitors of metalloproteinases-1 (TIMP-1); which has been known for the role of specific protease inhibition for at least the past thirty years [3-6]. Another function of TIMP-1 that has been long-known is erythroid potentiating activity (EPA) for its growth effects toward blood cells [7]; more will be said about this function in section 1.3. TIMP-1 has been very widely studied as an MMP inhibitor and therefore as a molecule that prevents ECM degradation which may occur in diseases including cancer invasion in advanced stages of tumor progression [8-10]. However, quite surprisingly clinical studies [11-23] have shown TIMP-1 to be correlated with poor prognosis.

1.2 TIMP-1 associates with poor prognoses in cancer

Deregulation of MMPs and MMP-based proteolysis, not surprisingly, occurs in cancer progression for increased ECM degradation along with other reasons. However, the question as

to why increased TIMP-1 expression occurs and is associated with poor prognosis in many types of cancer including breast cancer [11-23] has not been well answered beyond the assumption that it is upregulated as cells and tissues to try to keep up with increased MMP expression or activity.

Previously, the functional role of TIMP-1 has generally been characterized in relation to its MMP inhibition. However, mounting evidence in recent years has illustrated the need for better understanding of the whole functionality of TIMP-1; which contributes to its overall value as an indicator of poor prognosis. In review of the utility of TIMP-1 as a biomarker, Wurtz et al. stated, "...a substantial amount of data is present pointing to a potential role of TIMP-1 as a prognostic marker, however, probably only when studied at the protein level" [24]. This was contradicted by one study [25] finding a favorable prognosis associated to TIMP-1 protein expression, but confirmed by numerous studies above (Refs 11-23) and again in a study comparing the prognostic values of TIMP-1 mRNA expression and protein, in which the protein level but not the RNA was associated with poor prognosis [26]. Thus increased TIMP-1, especially in serum and tissue protein levels, is widely accepted as a marker of poor prognosis and the evidences to the contrary are few –discussed further by Wurtz et al [24]. In addition, TIMP-1 has been found to mediate chemoresistance and to be a predictive marker for response to chemotherapy [24,27-33] as well as endocrine therapy [34].

As with any biomarker, there is a window in disease progression for which its value is the most predictive. Due to better clarification of MMP-dependent and -independent effects of TIMP-1, this valuable prognostic window and the potential for TIMP-1-based therapies are closer to being realized. In this study, we will examine the dual nature of the TIMP-1 protein as

an MMP inhibitor and its functions apart from MMP inhibition –for further information see also the following reviews [35-40].

1.3 MMP-dependent vs. MMP-independent actions of TIMP-1

TIMP-1 is widely known for its regulation of MMPs and therefore regulation of tissue function and providing balance and inhibition toward MMP proteolysis of the extracellular matrix (ECM) [41-43]. However, in pathological conditions like cancer progression, both MMP-dependent and MMP-independent activities of TIMP-1 are utilized and altered in tumor development and metastasis. Hence, therapeutic attempts with synthetic active-site MMP inhibitors based on TIMP-1 have met with unforeseen side effects and toxicities [44-49]. Prior *in vitro* evidence and animal studies suggested that TIMP-1 was basically tumor suppressive and reduced tumor cell invasion (reviewed by Jiang et al. [37]), however, this has turned out not to be a complete picture of TIMP-1 functions as we will explain.

At practically the same time of the discovery of TIMP-1 as a protease inhibitor, a molecule was discovered to cause erythroid potentiation of a T-lymphoblast cell line and an erythroleukemia cell line –resulting in the label Erythroid Potentiating Activity (EPA) [50-55]. Before long, these two molecules TIMP-1 and EPA were identified as one and the same [7]. Chesler et al. illustrated that EPA function of TIMP-1 and MMP inhibition are independent functions, since mutation of His7 & Gln9 removed its ability for MMP inhibition, but the growth effect on erythroid cells was comparable to that of wild type TIMP-1. As well, the authors noted that synthetic hydroxamate-based MMP inhibitors, including GI213, which is identical to a well-known MMP inhibitor, BB94, did not induce EPA [56]. That EPA and MMP-inhibition are separate functions has also been shown by reductive alkylation of TIMP-1; where disruption of

disulphide bridges removes MMP-inhibitory function but EPA function of TIMP-1 is still operable [57,58].

Others have addressed the relationship of TIMP-1 stimulation of proliferation and MMP inhibition, however, in contrast their results suggest in MDA-MB-435 cancer cells TIMP-1-stimulated cell proliferation, gene expression changes, and metabolic changes, were duplicated by a synthetic MMP-inhibitor GM6001 but less well by an inactive compound, GM6001* [59,60].

Interestingly, TIMP-1 and also TIMP-2 have demonstrated signaling ability for negative regulation on the cell cycle by upregulation or stabilization of p27 and induction of G1 arrest [61,62] –for both TIMP-1 and TIMP-2 these effects were independent of MMP-inhibition. At present, it is not clear whether growth inhibition or promotion by TIMP-1 has a more predominant role in cancer progression, or whether this depends on cell type, tumor stage, and other factors. For further reading, these excellent reviews also examine the cell proliferation/growth promotion activities and growth retardation by TIMP-1 [36,63].

Another intriguing TIMP-1 function relating to cancer, and also fraught with controversy, is its role in angiogenesis –reported to mediate both induction and inhibition of angiogenesis. Using a transgenic mouse model de Lorenzo *et al.* found high TIMP-1 levels in circulation increased the growth of early melanoma tumors accompanied by an augmentation of angiogenic response [64]. This reported induction of angiogenesis, was contrary to earlier reports where TIMP-1 inhibited angiogenesis via its MMP-inhibitory function and prevention of endothelial cell migration [65-68]. Inhibition of endothelial cell migration was confirmed by Akahane et al. in 2004; however, in this case, MMP-dependent and -independent actions of TIMP-1 were

implicated. Briefly, they reported increased VE-cadherin and also PECAM-1 expression in the endothelial cells –suggesting protection by TIMP-1 from proteolytic cleavage. Meanwhile, the PTEN signaling pathway, inhibitory to cell migration, was also down-regulated by TIMP-1 but this was not observed with synthetic MMP-inhibitors [69].

Anti-apoptotic signaling by TIMP-1

Our research into TIMP-1 functions during cancer progression was initiated when we and others discovered cell protective anti-apoptotic activity of TIMP-1 [27,58,70].

While studying Bcl-2-mediated anti-apoptotic signaling in MCF10A cells, our lab made novel findings that TIMP-1 mediates inhibition of apoptosis when cells were challenged with death stimuli [27]. We found that overexpression of Bcl-2 increased TIMP-1 expression to protect cells from insult of hydrogen peroxide, Adriamycin, and also cytotoxic irradiation. Similar to Bcl-2, TIMP-1 protected cells from detachment-induced cell death; which suggests that the anti-apoptotic activity of TIMP-1 does not rely on stabilization of cell-matrix interactions. Subsequently, we found that TIMP-1 was very diverse in its ability to protect cells from apoptosis, offering protection from both extrinsic death signals (anoikis, staurosporine, and growth factor withdrawal) and intrinsic TRAIL-induced death signaling [71,72]. This signal transduction by TIMP-1, occurred through activation of downstream kinases particularly of FAK, PI3K, and ERK kinases. Importantly, in our studies, either TIMP-1 or a non-MMP-inhibitory mutant, T2G [73], increased cell survival, but neither synthetic A-Disintegrin-And-Metalloproteinase (ADAM) nor MMP inhibitors repeated the effects [71,72]. As mentioned above, this protection from apoptosis may contribute to resistance to chemotherapy [27,28].

Much like the anti-apoptotic effects observed by us in MCF10A breast epithelial cells, anti-apoptotic activity has been observed by others in many different cell types [40,58,68,70,73-78]. In Burkitt's lymphoma, TIMP-1 mediated protection from apoptosis and regulation of B-cell differentiation and lymphomagenesis via signal regulation including induction of IL-10 secretion [58,79]. In these studies, the authors demonstrated independence from TIMP-1's MMP-inhibitory function with reduced and alkylated TIMP-1 (which has lost MMP-inhibition but retains growth factor activity). The authors also demonstrated saturable TIMP-1 binding to the cell surface and speculated about a TIMP-1 receptor which mediates these signaling mechanisms.

1.4 TIMP-1/CD63 interaction: a proposed ligand and receptor

TIMP-1 has been suggested as a possible mediator of signal transduction at the cell surface since the early studies of its EPA function. Multiple groups have reported evidence of receptor-mediated signaling by TIMP-1. Specifically, Avalos *et al.* were able to demonstrate binding of TIMP-1 to K562 cells (an erythroleukemia cell line which displays secretion of and potentiation by TIMP-1). This binding at 37°C peaked at 1hr and was rapidly lost afterward; however, binding persisted at lower temperatures -as would be typical of receptor binding [80]. Also, TIMP-1 bound to the surface of human keratinocytes and induced growth stimulation [81]. Furthermore, as mentioned above, TIMP-1 was found to have saturable cell surface binding in JD38 a Burkitt lymphoma cell line [58].

This line of evidence which suggested receptor binding, invited researchers to further study whether TIMP-1 has significant roles involving a receptor or other molecular interactions aside from metalloproteases. When TIMP-1 was added to a breast carcinoma cell line, BC-61, it

caused increased tyrosine phosphorylation accompanied by growth stimulation of the cells [82]. And again in support of receptor-mediated TIMP-1 biology, others observed the trafficking of cell-surface associated TIMP-1 to the nucleus [83] leading to nuclear accumulation of TIMP-1 [84,85]. Similarly, the potential of TIMP-1 for cell surface association/receptor-mediated function was observed in neurons that were protected from excitotoxic cell death associated with excess intra-cellular calcium levels after glutamic acid stimulation [86]. Interestingly, synthetic MMP-inhibitors did not offer the same protection, and secretion of TIMP-1 was required. Therefore, the authors proposed that a TIMP-1/X (X=unknown) complex may down-regulate neuronal glutamate receptors.

Thus, the identification by our lab of an interaction between TIMP-1 and CD63, a transmembrane protein within tetraspanin family, proffers a probable receptor and a reasonable biological mechanism for TIMP-1 signal transduction [87]. A member of the tetraspanin family, CD63 (238aa) crosses the membrane four times with cytosolic N- and C-terminal tails and also short and large extracellular loops commonly referred to as SEL and LEL, respectively. Though the main biological functions of CD63 are not well-known, it is known that CD63 is involved in protein trafficking to lysosomes and cycles between the endosomal/lysosomal vesicles and the plasma membrane [88-90]. Endocytosis of CD63 from the plasma membrane is mainly due to a tyrosine-based sorting signal in its C-terminal tail which allows binding by adapter protein 2 (AP-2) and clathrin-dependent endocytosis [91,92]. Early studies on CD63 showed its increased expression in early melanoma; hence, it was also known as ME491, a melanoma antigen [93,94]. CD63 is known to be involved in integrin regulation and signaling as it and other tetraspanins interact with multiple integrins [95-99].

In our yeast-2-hybrid screen, TIMP-1 and CD63 identified with multiple positive interactions. We further validated their interaction by immunoprecipitation and confocal microscopic analysis; which, as well, confirmed their co-localization to the cell surface of MCF10A cells. Using shRNA-mediated downregulation of CD63, we demonstrated reduced TIMP-1 binding to the cell surface, and consequently reversal of TIMP-1-mediated cell survival signaling and protection from apoptosis [87].

We hypothesize that TIMP-1 bears multiple pro-oncogenic features, including the activities mediated by CD63, which increase the likelihood of tumor metastasis and may account for its overwhelmingly poor prognostic value. In the aftermath of our discovery of the TIMP-1 and CD63 interaction, their relationship and functions have been probed by an increasing body of researchers testing the hypothesis of a TIMP-1/CD63 (ligand/receptor) interaction and functionality. In this regard, the preponderance of data to date, favors their interaction for signaling as we will report in the following sections.

1.5 Recent discoveries of TIMP-1 functions with CD63 and regulation of MT1-MMP

The function of TIMP-1 and CD63 together in apoptosis inhibition has been analyzed in a variety of cell types since our finding of reduced apoptosis in MCF10A breast epithelial cells. TIMP-1 and CD63 together have been implicated in anti-apoptotic function in: cells within ovarian tissue [100], MDCK kidney epithelial cells [101], hematopoietic stem and progenitor cells [78], and anoikis resistant melanocytes [77].

Recently, Bommarito et al. have implicated TIMP-1 and CD63 in sustaining aggressive papillary thyroid carcinoma (PTC) cells' survival and invasion. Specifically, the activating mutation $BRAF^{V600E}$ (constitutive) in PTC tumor tissue was associated with increase of both

TIMP-1 and CD63 expression. TIMP-1 is ostensibly induced by NF-κB which displays increased DNA binding in $BRAF^{V600E}$ tumors. Using a PTC cell line which bears the $BRAF^{V600E}$ mutation, BCPAP, the authors examined survival and invasion. BCPAP cells have increased CD63 (flow cytometry) and TIMP-1 (flow cytometry & qRT-PCR); notably, these cells displayed increased survival after drug insult, increased proliferation, and increased invasion –which was dependent on TIMP-1 expression [102].

In 2012, Stilley *et al.* demonstrated an association of TIMP-1 and CD63 with reproductive organ function/failure [100]. In this study and their previous work [103] the authors suggested an MMP-dependent stabilization of ECM and protection from apoptosis by TIMP-1. They also found that CD63 co-immunoprecipitated with recombinant TIMP-1 (rTIMP-1) from the ovaries of treated rats, with a slight increase in CD63 binding by non-MMP-inhibitory Ala-TIMP-1 –with an alanine appended to the N-terminus. Thus, they suggested that TIMP-1/CD63 complex formation is independent of inhibitory MMP binding.

Additionally, Egea *et al.* have connected TIMP-1 and CD63 with the Wnt signaling pathway and with maintenance of stemness in human mesenchymal stem cells (hMSC)s. They demonstrated that TIMP-1 in hMSCs suppresses proliferation, metabolic activity, and differentiation to an osteogenic lineage -in a CD63-dependent manner. They showed this by TIMP-1 targeted siRNA which caused an increase in each of these processes. As well, upon TIMP-1 knockdown they observed an increase in β-catenin expression, its translocation to the nucleus, and downstream transcriptional activity. Knockdown of CD63 in these cells abrogated β-catenin signaling activity. They found let-7f miRNA levels were significantly increased upon TIMP-1 knockdown and that let-7f was acting to downregulate β-catenin signaling activity likely

thru suppression of axin 2 –a negative regulator of β-catenin [104]. These authors have previously suggested in a review of Wnt signaling in stem cells and cancer that the Wnt/β-catenin pathway may be critical to EMT and cancer cell invasion [105]. However, as I will detail in the following paragraphs, when we did our studies in epithelial cells, surprisingly, we observed increased EMT-like characteristics in these cells associated with TIMP-1 signaling. This unexpected result may be explained by the difference in cell types: mesenchymal stem cells (the TIMP-1 signaling study by Egea et al.) and epithelial cells (our studies with TIMP-1 signaling). Additionally, the effects associated with TIMP-1/CD63 signaling may be dependent on temporal status of the tumor and microenvironmental cues such as the presence or absence of repressive/activating proteins. Other researchers have implicated the Wnt/β-catenin pathway as a critical part of cancer stem cell-like behavior in colorectal tumors [106]. Interestingly, they also noted an interdependence of cancer stem cells and tumor associated myofibroblasts (TAMs); which TAMs were found to secrete high amounts of trophic factors including HGF, TIMP-1, and TIMP-2 [106].

The role of TIMP-1 in EMT-like signaling in epithelial cells was reported by our group. We observed phenotypic and genotypic changes associated with EMT which followed after over-expression of TIMP-1 in both kidney and breast epithelial cells [[101] and Rosemarie D'Angelo unpublished observations]. As part of this EMT-like activity by TIMP-1 we observed a novel upregulation of membrane type-1 matrix metalloproteinase (MT1-MMP), a cancer invasion associated MMP [107-109]. MT1-MMP, also known as MMP-14, is one of six total membrane-tethered MMPs [110], or MT-MMPs, and is a collagenase [111]. MT1-MMP has been well studied for it role in activating other metalloproteases especially pro-MMP-2 via the formation of

a trimeric complex with MT1-MMP binding TIMP-2 (thru the active site and inhibitory site, respectively) and with pro-MMP-2 binding c-terminally the TIMP-2 molecule positioning the pro-MMP-2 for cleavage and activation by a free-MT1-MMP [111-114].

This upregulation of MT1-MMP by TIMP-1 implicates a major paradigm shift in regulation MMP activity by TIMP-1. In the aforementioned studies (section 1.2) TIMP-1 has only been known to regulate MMPs as an inhibitor. However, we observed in MDCK kidney epithelial cells that TIMP-1 overexpression stimulates MT1-MMP and MMP-2 expression at the transcript and protein levels [101]. We also saw increased activation of pro-MMP-2; which indicates upregulation of the trimeric complex (also referred to as the quaternary complex): dimerized MT1-MMP, TIMP-2, and pro-MMP-2 [111-113,115,116]. Interestingly, this inductive effect specifically increased MT1-MMP and MMP-2; however, MMP-9 expression was not increased, but rather was decreased at the mRNA and protein levels and for gelatin zymographic activity. The resulting effect of an upregulated MT1-MMP/MMP-2 axis diminished the ability of MDCK cells to form polarized structures in 3D culture and increased migration, invasion, and resistance to apoptosis. Moreover, TIMP-1 induced the characteristic genetic switch from expression of epithelial genes to mesenchymal-cell-associated genes. Thus, taken together, the effect of high TIMP-1 expression in MDCK cells drives an MT1-MMP/MMP-2-mediated epithelial-mesenchymal transition (EMT). Importantly, this function of TIMP-1 was independent of MMP-inhibition by TIMP-1, since a non-MMP-inhibitory mutant of TIMP-1 dubbed, T1D (described in section 3.1.2), duplicated the induction of these pathways in MDCK cells [101].

The induction of EMT-like mechanisms by TIMP-1 was also observed by us in MCF10A cells. Over-expression of TIMP-1 was accompanied by a loss of E-cadherin and of epithelial

catenins as well as an increase of N-cadherin, vimentin, and fibronectin expression. These EMT-like changes were coordinated at the genetic level by an increase in the EMT transcription factor, Twist, and its activity. All TIMP-1-induced EMT-like changes in MCF10A cells were proven to be dependent on CD63 as the effects were reduced or lost upon CD63 knockdown [Rosemarie D'Angelo –unpublished observations].

Hence, TIMP-1 induces an EMT-like response when highly expressed in kidney and breast epithelial cells, and the regulatory relationship of TIMP-1 with MT1-MMP is largely in contrast to the relationship of TIMP-1 with almost all other MMPs. Specifically, TIMP-1 exhibits poorer inhibition affinity for MT1-MMP [43,117-122] compared to most other MMPs, and as we have shown upregulates MT1-MMP expression/activity. Our finding, therefore, identifies TIMP-1 as an activator of MT1-MMP; which, as mentioned above, promotes MT1-MMP proteolysis of Pro-MMP-2.

Additional regulation of MT1-MMP by CD63

CD63 has an established regulatory relationship with MT1-MMP as well. CD63 is known to associate with a wide variety of proteins [90,123] at the plasma membrane and at endosomal/lysosomal vesicles, and, as mentioned above, CD63 aids in trafficking and delivery of proteins to lysosomes (reviewed in [90,91]). Takino et al. have reported that CD63 performs such regulation on MT1-MMP. They reported that MT1-MMP interacts directly with CD63 and that CD63 likely delivers MT1-MMP to lysosomes for degradation [88]. Direct MT1-MMP interaction with CD63, as well as interactions and complexes formed with other tetraspanins have been reported by other groups as well [124-126].

The main objective of this dissertation work has focused on efforts to bring to light the nature of the protein-protein interactions, relationships, and functional regulation between TIMP-1, CD63, and MT1-MMP. The main hypothesis being that TIMP-1 interacts with CD63 thru an alternative site to its MMP-inhibitory site, and that TIMP-1/CD63 interaction at the cell surface is in competition to the formation of an MT1-MMP/CD63 complex. The specific aims are as follows: 1a) To map the domain of TIMP-1 responsible for binding to CD63. 1b) To map the domain of CD63 responsible for binding to TIMP-1. 2a) To determine the domains involved in CD63 and MT1-MMP binding. 2b) To clarify the cooperative or competitive nature of TIMP-1 and MT1-MMP binding with CD63. 3) To establish the expression pattern of TIMP-1, CD63, and MT1-MMP in normal and cancerous breast cells.

First, we have attempted to map the interaction sites involved in the TIMP-1 and CD63 interaction. Domain mapping for TIMP-1 and CD63 is important because although it has been known that TIMP-1 and CD63 interact for transduction of a variety of cellular signals, there has been no clarification of how they interact or of the structural features of such a complex. Also, we have attempted to map the interaction sites involved in the MT1-MMP and CD63 interaction. Additionally, we have attempted to define the favorability, or more appropriately the unfavorability, of simultaneous TIMP-1 and MT1-MMP binding to CD63. We further evaluated the possible proteolytic cleavage of TIMP-1 or CD63 by MT1-MMP, but we find no evidence to support such proteolytic activity by MT1-MMP.

We have evaluated the TIMP-1 and CD63 interaction in LIVE HEK293FT cells and find their interaction to be robust in live cells; including the suggestion of principally cell surface localization. As well, we report potential for therapeutic design/approach towards the C-terminal

region of TIMP-1 which could disrupt interaction with CD63, yet maintain MMP-inhibitory interactions. Further, we have evaluated TIMP-1, CD63, and MT1-MMP localization to the cell surface in vitro and in vivo within xenografted tumors. These three molecules are readily observed at the cell surface in MCF10DCIS.com xenografts –with particular cell surface staining in progressing and more invasive regions. Also, from in vitro analysis it is apparent that increased TIMP-1 expression results in an increase of CD63 presence at the cell surface. These are the main conclusions that will be illustrated in this dissertation work.

Chapter 2

Materials & Methods

2.1 Vectors, antibodies, and other reagents

For yeast-2-hybrid analyses we used the Yeast Matchmaker 3 system (Clontech; Mountain View, CA); including the pGADT7 vector containing the GAL4 activation domain and the pGBKT7 vector containing the GAL4 DNA-binding domain. For PCA, we used p-EYFP-N1 vector and pECFP-C1 vector (Clontech) in which the genes encoding fluorescent proteins were replaced with humanized GLucN and GLucC fragments (see section 2.5) –these vectors were a kind gift from Dr. James Granneman. MT1-MMP gene or modified MT1-MMP inserts in either the pcDNA3.1/myc-His (-) A vector (Invitrogen; Carlsbad, CA) or in the pGADT7 and pGBKT7 vectors from the above mentioned Clontech system, were a kind gift from Dr. Rafael Fridman. The G.Luc substrate, Native Coelenterazine, was purchased from Nanolight Technology (Pinetop, AZ). The white 96-well plates were from Thermo Scientific (Rockford, IL). Dulbecco's Phosphate Buffered Saline was purchased from Hyclone (Logan, UT). Anti-TIMP-1 Ab-2 (102 D1) monoclonal antibody (mAb) was purchased from Thermo Scientific (Fremont, CA). anti-TIMP-1 (EP1549RY) rabbit mAB, anti-CD63 mouse mAb, anti-MT1 Cat mouse mAB (LEM-2/15.8) and Hinge rabbit polyclonal antibodies and focal adhesion staining kit containing DAPI, phalloidin, and vinculin were purchased from Millipore (Billerica, MA). Anti-β-actin mAb, anti-mouse IgG peroxidase conjugate, and anti-rabbit IgG peroxidase conjugate antibodies were purchased from Sigma (St. Louis, MO). Anti-transferrin receptor mAB was purchased from BD Transduction Laboratories (San Jose, CA). Anti-GAPDH mAB and normal mouse IgG

were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal Anti-Gaussia Luciferase antibody was purchased from Nanolight Technology.

2.2 Primers and mutation of genes

All mutations were made to proteins of interest by mutational PCR either by use of truncating primers or using the QuikChange Mutagenesis II Kit (Agilent Technologies; Santa Clara, CA) as per kit instructions. All primers used, including mutagenesis primers, follow in Table 1: the Table of Primers.

TABLE 1. TABLE OF PRIMERS		
CD63 MUTATION PRIMERS		
7AA	F	5'-GGG ACT GAT TGC CGT GGG TGT CGG GGC AGC GGC TGC CGC GGC TGC GGC CAT AAT CCA GGG GGC TAC CCC-3'
	R	5'-GGG GTA GCC CCC TGG ATT ATG GCC GCA GCC GCG GCA GCC GCT GCC CCG ACA CCC ACG GCA ATC AGT CCC-3'
IIQ	F	5'-CAG CTT GTC CTG AGT CAG ACC GCA GCC GCG GGG GCT ACC CCT GGC TCT CTG TTG-3'
	R	5'-CAA CAG AGA GCC AGG GGT AGC CCC CGC GGC TGC GGT CTG ACT CAG GAC AAG CTG-3'
TPGS	F	5'-ATA ATC CAG GGG GCT GCC GCT GCC GCT CTG TTG CCA GTG G-3'
	R	5'-CCA CTG GCA ACA GAG CGG CAG CGG CAG CCC CCT GGA TTA T-3'
C145S	F	5'-GAT GCA GGC AGA TTT TAA GAG CTG TGG GGC TGC TAA CT-3'
	R	5'-AGT TAG CAG CCC CAC AGC TCT TAA AAT CTG CCT GCA TC-3'
C146S	F	5'-GAT GCA GGC AGA TTT TAA GTG CAG TGG GGC TGC TAA CT-3'
	R	5'-AGT TAG CAG CCC CAC TGC ACT TAA AAT CTG CCT GCA TC-3'
C145,146S	F	5'-GAT GCA GGC AGA TTT TAA GAG CAG TGG GGC TGC TAA CT-3'

	R	5'-AGT TAG CAG CCC CAC TGC TCT TAA AAT CTG CCT GCA TC-3'
C169S	F	5'-CCG AGT CCC CGA CTC CAG CTG CAT TAA TGT TAC TGT G-3'
	R	5'-CAC AGT AAC ATT AAT GCA GCT GGA GTC GGG GAC TCG G-3'
C170S	F	5'-CCG AGT CCC CGA CTC CTG CAG CAT TAA TGT TAC TGT G-3'
	R	5'-CAC AGT AAC ATT AAT GCT GCA GGA GTC GGG GAC TCG G-3'
C169,170S	F	5'-CCG AGT CCC CGA CTC CAG CAG CAT TAA TGT TAC TGT G-3'
	R	5'-CAC AGT AAC ATT AAT GCT GCT GGA GTC GGG GAC TCG G-3'
C177S	F	5'- GCA TTA ATG TTA CTG TGG GCA GTG GGA TTA ATT TCA ACG AG-3'
	R	5'-CTC GTT GAA ATT AAT CCC ACT GCC CAC AGT AAC ATT AAT GC-3'
C191S	F	5'-GAT CCA TAA GGA GGG CAG TGT GGA GAA GAT TGG-3'
	R	5'-CCA ATC TTC TCC ACA CTG CCC TCC TTA TGG ATC-3'
CD63 YEAST-2-HYBRID TRUNCATION PRIMERS		
SEL+	F	5'-CAG AAT TCA TGA AAT GTG TGA AG-3'
SEL+	R	5'-GAG GAT CCG TTC TCC TTG CAG GC-3'
LEL	F	5'-CAG AAT TCG GCT ATG TGT TTA GA-3'
LEL	R	5'-GAG GAT CCT ACC ACC AGC ACA TT-3'
LEL+	F	5'-CAG AAT TCT ATT GTC TTA TGA TC-3'
LEL+	R	5'-GAG GAT CCC TAC ATC ACC TCG TAG CC-3'
PCA SUB-CLONING AND TRUNCATION PRIMERS		
CD63-H3	F	5'-GAA AGC TTG CCA TGG CGG TGG AA-3'
CD63-AGE-I	R	5'-GAA CCG GTT CTG AGC CTC CTC CGC CTG AAC CTC CTC CTC CTA CCA CCA GCA CAT T-3'
CD63-ΔLEL	R	5'-GAA CCG GTT CTG AGC CTC CTC CGC CTG AAC CTC CTC CTC CAA ACA CAT AGC CAG C-3'
AGE-I-KOZ-GLUC-SIG	F	5'-CGC CCA CCG GTC ACC ATG GGA GTC AAA GTT CTG TTT GCC-3'

TIMP-1-H3	F	5'-GAA AGC TTG CAC CTG TGT CCC A-3'
T1ΔC	R	5'-GAT CTA GAC AGG CAG GCA AGG TG-3'
T1ΔC2	R	5'-GAT CTA GAG GTG CAC AGC CCT GG-3'
MT1-H3	F	5'-GTA AGC TTA CGC CAT CCA G-3'
MT1-XBA-I	R	5'-GAT CTA GAT CAG ACC TTG-3'
MT6-GPI-XBA-I	R	5'-GAT CTA GAT CAG CGG GAG GCT AC-3'
FOR TIMP-1 DELETION IN EXPRESSION VECTOR		
T1ΔC-W/STOP	R	5'-GAT CTA GAC TAC AGG CAG GCA AGG TG-3'
T1ΔC2-W/STOP	R	5'-GAT CTA GAC TAG GTG CAC AGC CCT GG-3'

2.3 Cell culture

All cell lines were cultured at 37°C in a humidified incubator with 5% CO_2, and all media was supplemented with 100units/mL penicillin and 100ug/mL streptomycin. Human Embryonic Kidney cells, HEK293FT, were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. MCF10A breast epithelial cells were maintained in DMEM/F12 (which is a 1:1 mixture of DMEM and F12 nutrient mix) supplemented with 5% horse serum, 0.5ug/mL hydrocortisone, 20ng/mL epidermal growth factor (EGF), 10ug/mL insulin, and 0.1ug/mL cholera enterotoxin. TIMP-1 over-expressing (T29), TIMP-1 stable knockdown (AST1), T29 with stable CD63 knockdown (T29shCD63) or control (T29shctl) cell lines were established as previously described [87]. Culture reagents were purchased from Invitrogen.

2.4 Yeast-2-Hybrid mating analysis

To assess the difference between the TIMP-1 N- or C- terminal domains for binding to CD63, we generated truncation mutants. For the N-terminal domain we truncated TIMP-1 to retain the first three disulphide bonds which included aa 1-126 (T1N). As a complementary truncation we included the remaining three disulphide bonds which are of the C-terminal domain, aa 127-184 (T1C). To make comparison with a larger C-terminal portion of TIMP-1, we made a truncation which included aa 66-184 but was still deficient for MMP inhibition -dubbed T1D.

TIMP-1 deletion mutants were compared with full-length TIMP-1 for CD63 interaction by yeast-2-hybrid analysis. Complementary yeast strains were transformed with TIMP-1 constructs and CD63 in vectors containing the GAL4 transcription factor binding or activation domain, respectively. Transformed yeast strains were spot-plated for mating on rich YPD agar. Colonies were allowed to grow at 30°C, colonies were then replica plated to double-dropout (DDO) selective agar and finally to quadruple dropout (QDO). QDO selective agar, in addition to absent leucine and tryptophan to indicate presence of experiment vectors, lacks histidine and adenine, which are lethal and cause a change in color from white to red, respectively, if not produced upon interaction of target proteins. As a third reporter, interacting yeast actively make β-galactosidase to process X-GAL substrate producing a blue color.

As well, we tested truncations of CD63 for TIMP-1 interaction with yeast-2-hybrid. We made the following truncations: SEL+, comprising the first portion of CD63 including the short extracellular loop (aa 7-79); LEL, comprising only the large extracellular loop (aa 104-206); LEL+, comprising the LEL with flanking transmembrane domains (aa 80-238). However, upon transformation of these constructs into yeast for mating, we observed toxicity of the mutants

toward the yeast to varying degrees; this made domain analysis by truncation of CD63 not feasible for yeast-2-hybrid.

2.5 Protein Complementation Assay design and sub-cloning

For protein complementation assay (PCA) analyses, chimeric DNA constructs were made from cDNAs for the interacting proteins of interest fused at their 5' or 3' to one of a pair of complementary fragments of humanized *Gaussia* Luciferase. Vectors were a kind gift by Dr. James Granneman containing G.Luc fragments optimized for recombination and reconstitution of luciferase activity during protein complementation assay as described by I. Remy and S.W. Michnick [127]. Briefly, the G.Luc enzyme naturally contains a 16 amino acid secretion leader sequence; which sequence was removed by Remy and Michnick when GLucN (aa 1-93) and GLucC (aa 94-169) fragments yielded optimal recombination over all other bifurcated combinations. In cases where we desired protein secretion, the secretion peptide was retained; this may affect recombination ability –though we have not observed any negative effect. For our purpose, GLucN including the secretion peptide was fused to the N-terminus of TIMP-1 at Cys^{1}; while GLucC was fused to the C-terminal region of CD63 truncated at Val^{206} (removing the C-terminal tail including the 'GYEVM' internalization motif (recognized by adapter proteins) hence there should be increased retention of CD63 at the plasma membrane [91,128,129]. Also, for MT1-MMP PCA analysis active form MT1-MMP without pre- and pro- domains beginning at Tyr^{112} was attached N-terminally to GLucN including the secretion peptide. For MT1-MMP domain analysis we included the E240A catalytic site mutant (EA-MT1), which lacks catalytic activity [130], an MT1-MMP (112-540) MT6-MMP GPI anchor (541-562) chimera (MT1-GPI),

and a mutant with deletion of the hemopexin repeats (112-318, 536-582) (ΔH-MT1) (a kind gift from Dr. Rafael Fridman).

For a positive interaction control, we used nuclear protein hepatocyte nuclear factor 4α (HNF4); which is known to readily homodimerize. Again the GLucN (this time without secretion peptide) or GlucC fragments were appended by subcloning to either 5' or 3' end of HNF4 similar to TIMP-1 and CD63; the HNF4 vectors were a kind gift of Dr. Todd Leff. For all cases, experimental protein fusions and positive controls, the GLuc fragments were fused indirectly to the gene of interest via a flexible linker (also optimized by Remy and Michnick [127]) consisting of a 10 amino acid sequence (Gly.Gly.Gly.Gly.Ser –in duplicate).

2.6 Protein Complementation Assay in cell lysate

PCA fusion plasmids were co-transfected in a 1:1 ratio (400ng DNA total/well) into HEK293FT cells in 24-well plates, using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Transfected cells were incubated for 5hr then given fresh media and incubated for an additional 17-19hrs to allow for expression of the fusion proteins. At this point, there were approximately 3-4 x10^5 cells per well. Medium was exchanged for 220ul/well of DMEM phenol-red free (Invitrogen) containing Complete Protease Inhibitor Cocktail (Roche; Indianapolis, IN) at manufacturers recommended dilution. Cells were then lysed by two freeze and thaw cycles at -80 deg and room temperature (preserving G. Luc enzymatic capacity). For each sample, 100ul was transferred to a white 96-well plate (Thermo Scientific) for luminescence measurements. Native coelenterazine (Nanolight Technology) was reconstituted as a stock solution at 1mg/mL in 100mM HCl in Methanol; this was diluted in DMEM phenol-red free at a final concentration of 20uM was injected (100ul per injection) to the samples. Signal

intensities (integrated over 10 seconds, with an injection delay of 2 seconds) were read on either a MicroLumat 96 LB+ plate reader (Berthold Technologies; Oak Ridge, TN) or a Glomax 96 microplate luminometer (Promega; Madison, WI).

2.7 Protein Complementation Assay in live cells

PCA fusion plasmids were co-transfected in a 1:1 ratio (200ng DNA total/well) into HEK293FT cells in 96-well clear-bottom white plates (Thermo Scientific), using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Transfected cells were incubated for 5hr then given fresh media and incubated for an additional 17-19hrs to allow for expression of the fusion proteins. At this point, there were approximately 6-7x10^4 cells per well. Medium was exchanged for 100ul/well of DMEM phenol-red free (Invitrogen). Prior to luminometric analysis, a white backing tape (Perkin Elmer; Boston, MA) was applied to the bottom of the plate. The native coelenterazine stock solution was diluted in DMEM phenol red-free at a final concentration of 20uM was injected (100ul per injection) to the samples. Signal intensities (integrated over 10 seconds, with an injection delay of 2 seconds) were read on a MicroLumat 96 LB+ plate-reader (Berthold Technologies) or a Glomax 96 microplate luminometer (Promega).

2.8 Protein Complementation Assay with TIMP-1 antibodies

PCA fusions plasmids were co-transfected as with other live cell PCAs. After the 17-19hr overnight growth, transfected cells were washed once with PBS + calcium/magnesium, and then given 5ug/mL antibody in DMEM phenol-red free (100uL/well); antibodies including: C-terminal TIMP-1 antibody (CT1AB), a non-C-terminus directed TIMP-1 ab (T1AB2), or a non-immune IgG. Cells were incubated with antibody-containing media at 37°C for an 8hr incubation

period. Media was replaced with fresh DMEM phenol-red free, white backing was applied to the plate and luminescence detected as with other live cell PCA experiments.

2.9 Protein Complementation Assay with TIMP-1 peptide

We conducted PCA with addition of a synthetic TIMP-1 peptide from the sequence of the last 9aa of TIMP-1 (WQSLRSQIA). This peptide was resuspended in DMSO to make a stock solution (28mM). PCA fusion plasmids were co-transfected in a 1:1 ratio (400ng DNA total/well) into HEK293FT cells in 24-well plates, using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. After the 17-19hr. overnight growth, transfected cells were switched to serum free (SF) DMEM and returned to 37°C for a 40min. incubation. Then peptide in SF-DMEM was added at the following concentrations: 50, 100, 200, or 400uM with a DMSO vehicle control. Cells were incubated with peptide dilutions for 1hr. at 37°C and plates were gently rocked over 10-15min. intervals. Media was then replaced with 220uL/well fresh DMEM phenol-red free containing Complete Protease Inhibitor Cocktail (Roche) and F/T lysis and luminescence detection were carried out as in section 2.6.

2.10 Deglycosylation with PNGaseF treatment

PCA was carried out as for cell lysate analysis (section 2.6) with the following changes. After lysis of the cells by freeze/thaw cycles at room temp/-80°C, 100uL was transferred for each sample to a white 96-well plate and prepared for PNGaseF reactions. To ensure de-glycosylation of proteins it's best to have optimal exposure of glycosylation sites. Normally for PNGaseF treatment exposure of the base of glycosylations would be ensured by addition of SDS and β-ME (to 0.5% and 1% final percentages, respectively) to lysates and boiling prior to PNGaseF treatment –adding PNGaseF and NP-40 after boiling to protect enzymatic activity. However, we

could not boil and denature the lysates as that would also denature the luciferase reporter and NP-40 interferes with assay detection. When we tested the effect of SDS and β-ME by boiling samples from CD63-transfected lysates with or without denaturing buffer (SDS and β-ME), we did not observe any difference for de-glycosylation dependent on addition of SDS and β-ME. In that experiment, 1ul PNGaseF (1mg/mL) was added to a 60uL volume including 20ug of protein from lysates and 0.05M phosphate buffer and reactions were incubated at 37°C for 2hr. For treatment of PCA experiment samples we added 100uL of phosphate buffer (0.1M) to 100uL aliquots of lysates to achieve a final concentration of 0.05M and an excess of PNGaseF (3uL at 1mg/mL), and the reaction was incubated at 37°C for 4hr. with hourly vortexing. The PNGaseF was prepared in the following buffer: 20mM Tris-HCl(pH7.5), 50mM NaCl, 5mM EDTA and lypohilized to powder, then resuspended in equal volume (to original) with dH2O and Glycerol to final amount of 50% Glycerol; and for each reaction comparison was made for incubation with PNGaseF or buffer alone. After incubation, luminescence was measured on the plate reader as described (see section 2.6).

2.11 Production of recombinant TIMP-1

Human recombinant TIMP-1 (rTIMP-1) was expressed in HeLa cells using a vaccinia virus expression system and purified to homogeneity as described previously [27]. Upon collection and pooling of TIMP-1 purified fractions, a final dialysis was conducted against HA buffer containing 25mM HEPES (pH7.5), 20mM NaCl, 0.01% Brij-35, & 10% Glycerol. The protein concentration of rTIMP-1 was determined using spectrophotometric measurement and TIMP-1's molar extinction coefficient of 26,500 $M^{-1}cm^{-1}$. As well, purified rTIMP-1 homogeneity and concentration were assessed using EZ-Blue Gel Staining Reagent (Sigma)

staining of SDS-polyacrylamide gel separated TIMP-1 in comparison with standards of BSA protein.

2.12 TIMP-1 activity assay in MCF10A cells

To assay the activity of rTIMP-1 we employed AST1 MCF10A cells, bearing TIMP-1 anti-sense shRNA, and used ERK phosphorylation as a relevant readout activated by TIMP-1 signaling [71]. AST1 cells were seeded into 6-well plates at ~1.8*10^5 cells per well and allowed to grow in MCF10 growth media 45-68hrs. until cells were ~90% confluent. Medium was removed and cells were washed twice with PBS-CM then replaced with serum-free (SF) DMEM/F12 for 24hr. Following the incubation with SF media, the rTIMP-1 (100-500ng/mL) or EGF (50ng/mL) in SF media and cells were incubated for 30min. or 10min. for rTIMP-1 or EGF treatment, respectively. For experiments in which TIMP-1 blocking antibodies were used, the blocking antibody or antibody buffer alone were mixed with the rTIMP-1 in SF media 5 to 10 minutes before addition to the cells for activation assay. After the desired incubation period the media was aspirated and cells were washed with cold (4°C) PBS twice. Then ~200uL lysis buffer was added to the cells [lysis buffer: 1X RIPA, Complete Protease Inhibitor Cocktail (Roche – according to manufacturer's instructions), 10uM PMSF, 10uM Na_3VO_4, 10uM NaF]. Cells were scraped and transferred to cold eppendorf tubes and incubated on ice ~30min. with intermittent vortexing. Lysates were then clarified by centrifugation at 4°C for 20min. at ~12,000rpm.

2.13 TIMP-1 antibody blocking with C-terminal peptide

To verify specific recognition of the C-terminal TIMP-1 residues by the C-terminal antibody we did immunoblot analysis in which TIMP-1 antibodies were blocked with our synthetic C-terminal peptide. C-terminal or non-C-terminal TIMP-1 antibodies were pre-blocked

with peptide for 24hr. at 4°C in a 1mL volume of 3% TBST-Milk (for TBST and TBST-Milk recipes see Immunoblot analysis section 2.15). To these tubes 5x (by weight) TIMP-1 C-terminal peptide was added. Antibodies were set on a rotating nutator at 4°C overnight. A nitrocellulose membrane was prepared after transfer of protein from an SDS-PAGE gel where each lane contained equal loading of lysate from HEK293FT cells over-expressing huTIMP-1. This prepared membrane was stained with Ponceau S and promptly cut into nine strips (a separate strip for each lane of TIMP-1 lysate). These strips were stored at 4°C overnight, then they were blocked with 5% TBST-Milk for 45min. at room temperature, then rinsed in TBST –as with normal immunoblots, and added to separate 50ml conical tubes with the appropriate antibody either Milli C-term T1AB (Millipore), 1C6F (a TIMP-1 antibody which was a kind gift from Dr. Seung-Taek Lee), or T1AB2 (each at the same dilution from stock ie. 1:500 dilution) in 4mL volume of 3% TBST-Milk, or tubes to which 3mL of 3% TBST-Milk were mixed with the 1mL of antibody that was pre-blocked with peptide. The membrane strips were then incubated on a rotator at 4°C overnight. The strips were then washed individually 3 times in TBST and probed with respective Mouse (1C6F and T1AB2) or Rabbit (C-T1AB) 2°antibody in 5% TBST-Milk separately for 45min. at room temperature, then washed individually again 3 times in TBST. Western Lightning ECL Plus (Perkin Elmer) was applied and the strips were placed in order and according to best alignment in the cassette and developed. The strips were re-probed with GAPDH antibody as a loading control and developed according to the same protocol. Lanes of the blots were also aligned upon conversion to digital images; this made for optimal qualitative comparison of antibody efficiency and peptide blocking –not perhaps precise enough for specific protein size determinations but that was not necessary in this analysis.

2.14 Total cell surface biotinylation

Cells were cultured as described in section 2.3. For evaluation of TIMP-1, CD63 and MT1-MMP expression we examined whole cell lysate and surface levels in MCF10A with an empty vector (Neo), MCF10A with stable TIMP-1 over-expression (T29), or the T29 cells with unaltered CD63 expression (T29shCTL) or with shRNA targeting CD63 (T29shCD63). Cells were grown in 150mm dishes until approximately 85% confluence. Cells were washed gently with Dulbecco's PBS containing 1mM $MgCl_2$ and 0.1mM $CaCl_2$ (Hyclone; PBS-CM) twice. The second wash of PBS-CM was not aspirated, and cells were placed at 4°C and allowed to cool for 5min.. After cooling, the cells were given either 11mL of chilled 0.5 mg/mL EZ-Link Sulfo-NHS-Biotin (Pierce; Rockford, IL) in PBS-CM or PBS-CM alone and set on a rotating platform at slow speed for 30min. at 4°C and protected from light. Biotin solution was then removed and cells were washed once with cold PBS-CM. After this wash, 50mM NH_4Cl in PBS-CM was added to the dishes to quench the biotinylation –this was rotated for 10min. at 4°C in the dark. Cells were then washed twice with PBS-CM. After the aspiration of the second wash, leaving >1mL behind, the cells were quickly scraped with a rubber policeman and transferred into eppendorf tubes on ice. Collected cells were spun in a centrifuge for 3min. @ 2000rpm and at 4°C. Supernatant was aspirated and each cell pellet was resuspended with between 400-500uL of Brij-97 lysis buffer [1% Brij-97, 150mM NaCl, 5mM $MgCl_2$, 25mM HEPES, 2mM PMSF + Complete Protease Inhibitor Cocktail (Roche –according to manufacturer's instructions)]. To allow lysis to occur, suspensions were incubated for 30min. on ice with intermittent vortexing.

Lysates were analyzed using a BCA assay kit (Pierce) to determine protein concentrations, and from this presumed equal amounts of protein were loaded at 10ug per sample on SDS-PAGE

and immunoblot of TIMP-1, CD63, and GAPDH was done to verify protein concentration accuracy. Based on the immunoblot, minor adjustments were made to protein calculations and 1mg of protein for each lysate was used for pull-down with Neutravidin (Pierce) beads. Lysate volumes were equalized to 400uL using Brij-97 lysis buffer and to this was added 300uL (amount determined from manufacturer's recommendation) of Neutravidin bead slurry. Suspensions were incubated on a rotating nutator for 2hr at 4°C. Samples were then spun for 3min. @ 3000rpm at 4°C and washed with 700-800uL of cell harvest buffer [0.5% SDS, 60mM Tris-HCl –pH7.5, 2mM EDTA –pH8.0, 2.5% Triton-X 100]. Spin and wash steps were repeated until four washes of the beads with cell harvest buffer. Wash buffer was aspirated completely after the final spin and to the beads was added 220uL of Novex 2X Tris-Glycine SDS Sample Buffer (Invitrogen) and samples were boiled for 5min. at 100°C. Samples received a final spin for 3min. @ 3000rpm at room temperature and supernatants were collected for immunoblot analysis.

2.15 Immunoblot analysis

Cell lysates were obtained either by lysing the cells in monolayer or upon transfer to eppendorf tubes after collection by rubber policeman scraping. Cells were lysed with either Triton X-100 lysis buffer [1% Triton X-100, 50mM Tris pH 7.4, 150mM NaCl, Complete Protease Inhibitor Cocktail (Roche –according to manufacturer's instructions), 10uM PMSF, 10uM Na_3VO_4, 10uM NaF] or RIPA lysis buffer [1X RIPA, Complete Protease Inhibitor Cocktail (Roche –according to manufacturer's instructions), 10uM PMSF, 10uM Na_3VO_4, 10uM NaF] unless otherwise specified. The lysates were clarified by 20min. centrifugation at 4°C. Protein concentration was measured using the BCA protein assay reagent (Pierce). Equal

amounts of protein samples in Novex 2X Tris-Glycine SDS Sample Buffer with or without 5-10% β-mercaptoethanol were boiled for 5min. and subjected to SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane ((Optitran) GE Healthcare; Piscataway, NJ). Prepared membranes were stained with Ponceau S to verify quality of transfer. Membranes were then blocked with 5% nonfat dry milk in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.02% NaN_3 and 0.2% Tween 20 (TBST) for 45min.-1hr. at room temperature. Membranes were then incubated overnight at 4°C with 5% TBST-milk and the desired primary antibodies. After three washes with TBST, the blot was incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Detection of proteins was then completed using Western Lightning ECL Plus (Perkin Elmer), according to the manufacturer's instruction.

2.16 Immunohistochemical analysis

We performed immunohistochemical analysis (IHC) using a Ventana Discovery (Roche) staining machine in collaboration with Dr. Chong Jai Kim. Mouse-grown tumor tissue samples were from MCF10DCIS.com tumors xenografted in SCID mice and were a kind gift of Dr. Fred Miller. Tumors were stained for TIMP-1, CD63, and MT1-MMP with the antibodies listed in section 2.1. Slides were de-parafinized according to standard Discovery protocols and applications of EZ Prep, Deparafinizing, and Coverslip solutions for the Discovery machine at 75°C for a total of 16 min. (8min. prior to and 8min. following addition of solutions). Antigen retrieval was performed using citrate buffer at 100°C for approximately 30 min.. Following antigen retrieval, slides were incubated with primary antibody in antibody diluent for 1hr. at 37°C. An antibody blocking solution was then applied to the slides for two minutes, and the

secondary antibody was applied and incubated for 30 min.. Streptavidin horseradish peroxidase (SA-HRP) was applied for 16 min., slides were rinsed four times with Coverslip solution, and DAB D solution was applied with H_2O_2 for detection antibody with minimal background. Slides were counterstained with hematoxylin diluted in Coverslip solution for two minutes, rinsed, and bluing reagent was applied for an additional two minutes. Slides were swished in soapy water and rinsed with fresh running water for ~1min. then de-hydrated in 95% EtOH (30sec), 100% EtOH (30sec) twice, and Xylene (30sec) twice and cover-slips were applied by an automated applicator. Slides were analyzed on an Axioplan 2 microsope (Zeiss; Oberkochen, Germany).

2.17 Statistical analysis

For PCA analyses, T-tests were performed to determine differences significance between averages of measurements of reconstituted enzyme activity reported in relative luciferase units (RLUs). P-values of <0.05 were considered to indicate significant differences.

Chapter 3

Results

3.1 Structural features of the TIMP-1 and CD63 complex

3.1.1 Foundation for our analysis of structure/complexes

Protein structural analyses require structures to be viewed as changeable and context-dependent. Many factors can affect the tertiary structure of a protein, especially formation of and disruption of complexes with other proteins. With that in mind, we have attempted to map the binding sites of TIMP-1 and CD63, and also for interaction of CD63 with MT1-MMP. Our objective is to build on the available information regarding the structures and relationships of these proteins to each other and to clarify the binding sites involved in and integral to their signaling functions in cancer progression.

Many useful observations can be made using 3D structures from X-ray analysis of protein crystals. For TIMP-1, a full-length TIMP-1 crystal structure at high resolution was achieved in complex with the catalytic domain of MMP-3 [131]. Indeed, I will highlight how we have used this structural information as a basis for our conceptualization of CD63 binding to TIMP-1 which we have empirically tested. For CD63, no crystal structures have been successfully completed to this date. However, a portion of a tetraspanin family member, CD81 (LEL domain) has been so described [132,133]. This will be further discussed in the discussion chapter. In addition, all tetraspanins bear certain features including conserved cysteines in the LEL domain and conserved polar transmembrane amino acids [134-137].

3.1.2 TIMP-1/CD63 interaction mapping by yeast-2-hybrid

We began with yeast-2-hybrid analysis using truncated TIMP-1 and CD63 proteins to address the importance of TIMP-1's domains (N- or C- terminal) for CD63 interaction and vice versa. We generated TIMP-1 truncations including the N-terminal domain; which consists of the first three cysteine bridges and contains the prominent MMP-inhibitory site (aa 1-126, which we called T1N). For the C-terminus we made the complementary truncation to T1N retaining aa 127-184 (called T1C) and also a broader retention of the C-terminus of the protein aa 66-184 (called T1D) which still lacks MMP-inhibitory ability (FIG. 1A). We observed interaction of both C-terminal mutants with CD63 comparable to full-length TIMP-1; however, the T1N mutant fails to interact (FIG. 1B and [87]). The more inclusive T1D may have additional interaction sites to that of T1C and CD63 as we have shown that T1D, but not T1C, is capable of transmitting anti-apoptotic and EMT signaling mediated by TIMP-1 ([101] and observations by XuWen Liu).

We investigated domain involvement of CD63 by yeast-2-hybrid examining both the large extra-cellular loop (LEL) and short extra-cellular loop (SEL) of CD63 for involvement in the interaction with TIMP-1. We examined the extra-cellular loops of CD63 as they are the most reasonable sites for predicted TIMP-1 interaction due to TIMP-1 being a secreted protein. For the analysis, each domain was deleted separately and tested for TIMP-1 interaction over a series of trials. Truncations were as follows: SEL+, comprising the first portion of CD63 including the short extracellular loop and first two transmembrane (TM) domains (aa 7-79); LEL, comprising only the large extracellular loop (aa 104-206); and LEL+, comprising the LEL with the flanking 3rd and 4th TM domains (aa 80-238) (FIG. 1C). However, we were unable to get definitive results because upon transformation of these constructs the yeast exhibited drastically slowed growth

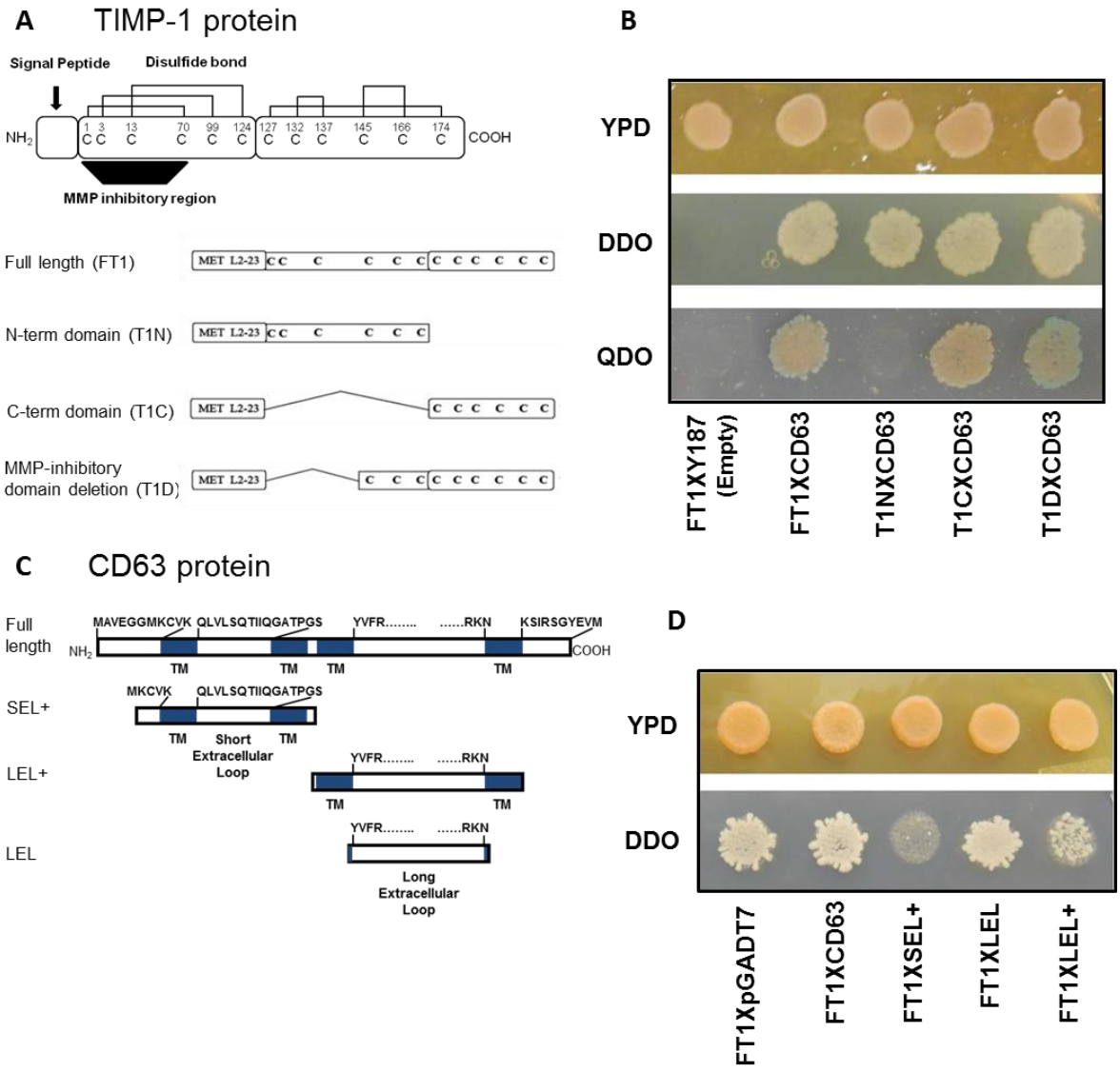


Figure 1. Domain analyses for TIMP-1 and CD63 by yeast-2-hybrid interaction analysis. **A)** Diagram of full-length TIMP-1 and domain deleting truncations. **B)** Yeast colonies were grown on rich YPD agar plates after spot-mating of complementary strains bearing TIMP-1 and CD63 reporter (GAL4) fusion constructs. Mated yeast strains were then replica plated to double drop-out (DDO) and reporter quadruple drop-out (QDO) selective nutrient deficient media. On selective QDO media yeast with non-interacting proteins turn red (due to lack of Adenine) or fail to grow (due to lack of histidine) (see section 2.4). **C)** Diagram of full-length CD63 and domain deleting truncations. **D)** Yeast colonies were again grown on YPD agar upon spot-mating of TIMP-1 and CD63 bearing strains. Yeast colonies were transferred in a similar manner to DDO media; however, growth was slowed for some matings with CD63 truncations and halted altogether for others indicating that truncated CD63 variants exhibited toxicity.

(insufficient for transfer to the reporter plate) due to toxicity of the mutants toward the yeast to varying degrees (FIG. 1D). Toxicity was especially noted with TM domain retaining mutants (SEL+ and LEL+); though LEL was not as toxic we observed only a few positive colonies with this mutant and TIMP-1. Therefore, very minimal information for the CD63 binding site was obtained by yeast analysis. Thus, we undertook further definition of TIMP-1 and CD63 binding domains through an alternate technique (see below).

3.1.3 TIMP-1/CD63 interaction mapping by PCA

To obtain more detailed information regarding the TIMP-1/CD63 interaction we conducted protein-protein interaction studies in human cells using an established protein complementation assay (PCA) system [127]; which offers more natural protein trafficking and post-translational modification. Mammalian cell based PCA utilizes reporter protein fragments that can be re-combined upon the interaction of proteins of interest. As opposed to a transcription factor reporter in yeast-2-hybrid, PCA utilizes reconstitution of a bi-furcated luciferase reporter to offer quantitative luminescence pertaining to the proteins of interest. To develop our PCA system, we generated the fusions of TIMP-1 and CD63 to complementary N-terminal (GLucN) and C-terminal (GLucC) fragments of humanized *Gaussia* Luciferase (hGLuc) [138], respectively. Specifically, GLucN including the natural Gaussia signal peptide for secretion was fused to the N-terminal Cys^{1} of TIMP-1 (ie. G-T1); while GLucC was fused to the C-terminal region of CD63 truncated at Val^{206} -removing most of the 4^{th} TM domain (ie. CD63-G) (see FIG. 2A). For the illustration of fusion protein size in panel C of figure 2 we have used the labels G-T1 and CD63-G to distinguish from non-fused proteins. Hereafter, they will simply be referred to as TIMP-1 and CD63; which names will represent the

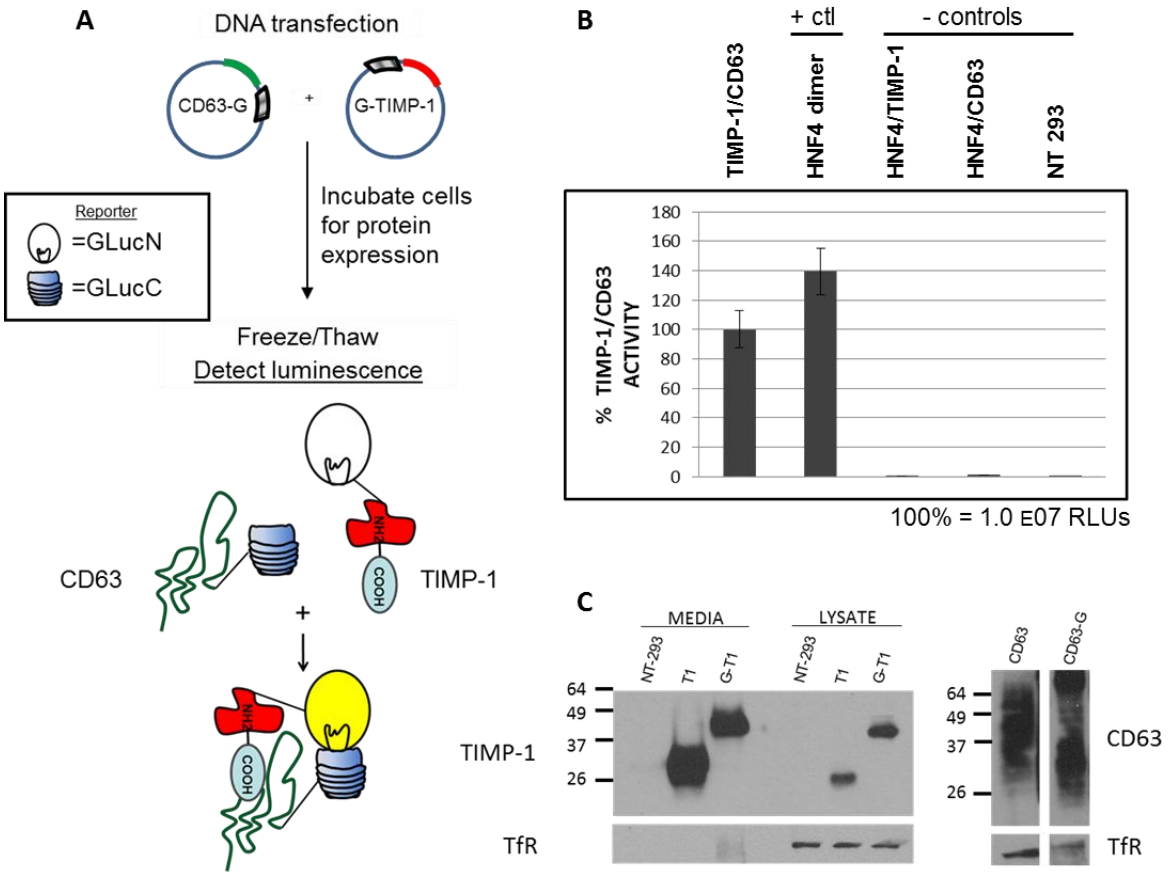


Figure 2. Design of protein complementation assay (PCA) system for TIMP-1/CD63 interaction analysis. **A)** Schematic showing transfection, expression, freeze/thaw lysis, and luminscence signal upon interaction of TIMP-1 and CD63 PCA constructs. Fusion genes were generated for TIMP-1 and CD63 to the N-term (GLucN) and C-term (GLucC) fragments of humanized *Gaussia* Luciferase (hGLuc), respectively. **B)** Vectors from panel (A) were co-transfected in HEK293FT cells and protein interaction and hGLuc reconstitution were measured in lysates by luminescence upon addition of coelenterazine. Shown is the TIMP-1/CD63 interaction signal quantified in relative luciferase units. HNF4, a transcription factor known to homodimerize, was used as a positive control. Values shown are the average of atleast triplicate measurements. Error bars represent standard deviation. **C)** Western blot showing protein sizes of normal TIMP-1 and CD63 proteins compared to hGLuc fragment fusion proteins labeled as G-T1 and CD63-G. Transferrin receptor (TfR) was used as loading control.

fusion proteins within the PCA system. We transfected these constructs into HEK293FT cells and observed stable expression of the fusion-protein products by western blot (FIG. 2C). TIMP-1 and CD63 interaction (and hGLuc reconstitution) was determined 22-24 hours after transfection and upon lysis by two cycles of freeze and thaw at -80°C and room temperature followed by manual membrane disruption by pipetting -avoiding use of detergents that would remove hGLuc activity. In a 96-well white plate, the coelenterazine substrate was injected into lysate-containing wells and luminescence signal integrated over 10 seconds after a 2 second delay. TIMP-1/CD63 interaction was substantial (well above an accepted minimum threshold for representing protein-protein interaction i.e. 1x10^5 RLUs) and easily measured in our PCA system –comparable to positive control HNF4 transcription factor fusions (G-HNF4 & HNF4-G) which readily homodimerize (FIG. 2B). Importantly, TIMP-1 and CD63 displayed specific binding which is not driven by hGLuc fragments, since co-transfection of either TIMP-1 or CD63 constructs with their complementary HNF4 construct did not yield any interaction in lysates after injection with coelenterazine (FIG. 2B).

To examine the binding site of TIMP-1, we made deletions at the C-terminus of TIMP-1, since the C-terminal region of TIMP-1 was implicated by the yeast-2-hybrid analysis. The 3D structure illustrates a flexible C-terminal tail that is opposite the major MMP-binding pocket or "molecular edge" of the protein ([131] and FIG. 17A). TIMP-1 was truncated after L^{168} (deletion of 17aa, called T1ΔC) or else T^{175} (deletion of 9aa, T1ΔC2) (FIG. 3A). By comparison to full-length, these two truncations resulted in drastic loss of binding to CD63; returning only ~25% (for T1ΔC2) and only ~17% (for T1ΔC) of the signal for CD63 with full-length TIMP-1 (FIG. 3B, shown values- 21% and 13%). Thus, we concluded that for a strong TIMP-1/CD63

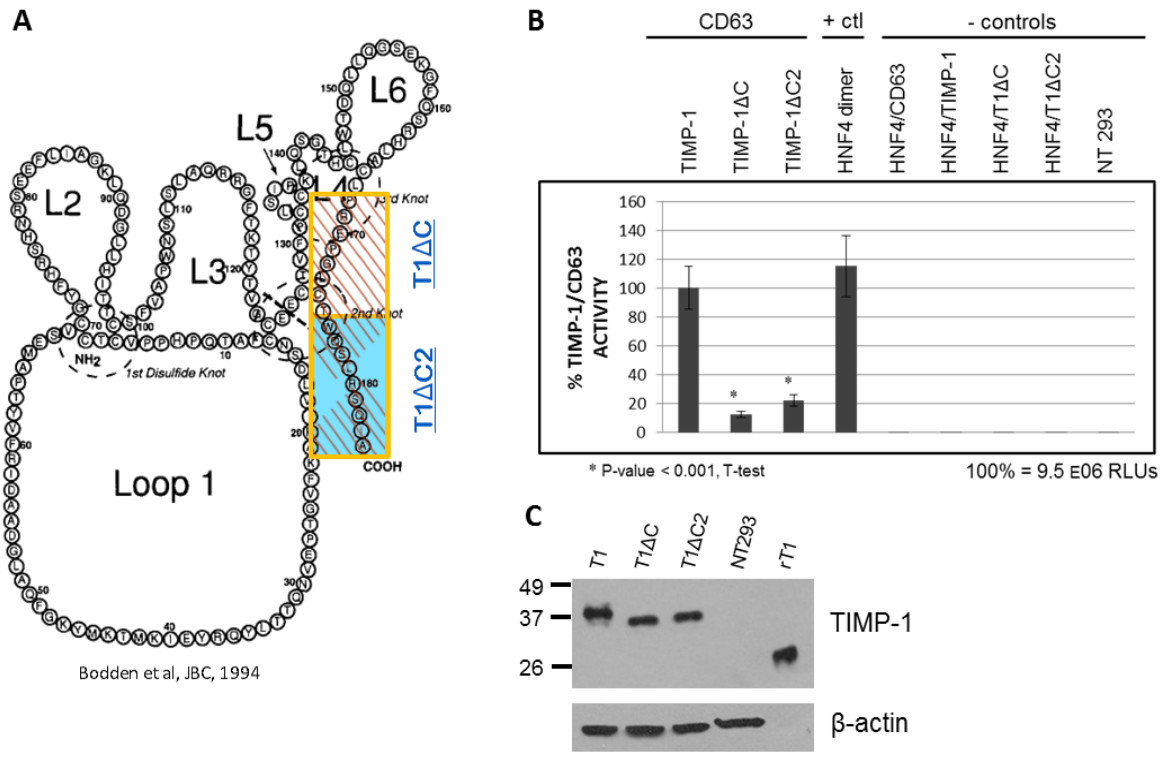


Figure 3. TIMP-1's C-terminus shown by PCA to have important role in CD63 binding. A) Amino acid diagram of TIMP-1 showing T1ΔC (17aa deletion) and T1ΔC2 (9aa deletion) deletions. **B)** PCA analysis showing TIMP-1 ΔC and ΔC2 interaction with CD63 as compared to TIMP-1/CD63 interaction, shown in the chart by relative luciferase activity. Values shown are the average of atleast triplicate measurements. Error bars represent standard deviation. Significance was assessed by T-test with P<0.05 being considered as significant. **C)** Western blot confirms the expression of TIMP-1 mutant constructs. For illustration of non-fused TIMP-1 protein size recombinant TIMP-1 (rT1) was used. For protein loading control β-actin was used.

interaction to occur the C-terminal residues (including the flexible tail) of TIMP-1 are critical; it was especially striking that for T1ΔC2, where only 9aa were removed which leaves all of TIMP-1's cysteine bridges intact, there was 75% less CD63 interaction. Again, stable protein expression was confirmed by western blot for these variants (FIG. 3C).

For CD63 domain analysis, we evaluated the SEL amino acids by serial mutation over the span of the SEL loop; changing the residues in turn to alanines over small intervals of the loop. This was done as follows: the predicted SEL runs from -Q^{36} LVLSQTIIQGATPGS^{51}; we mutated Q^{36}-T^{42} (called 7AA), from I^{43}-Q^{45} (called IIQ), and from T^{48}-S^{51} (called TPGS) (FIG. 4A). Two amino acids G^{46} and A^{47} were not changed as they are already generic amino acids without significant functional groups as side chains. Two of these SEL mutants caused significant, yet quite modest decreases in binding of CD63 to TIMP-1 (7AA, and TPGS mutants, FIG. 4D); while for IIQ the change was not a loss of binding but an unexpected significant increase. Stable protein expression was confirmed by western blot for these variants (FIG. 4B).

Mutational analysis of the LEL domain of CD63 is very challenging owing to the much larger size (99aa) and lack of reports of specific LEL domain interactions with proteins that could serve as comparison for TIMP-1. However, the LEL domain has potentially defining features (3 disulphide bonds and 3 N-glycosylation sites) and we have addressed these features in further experiments which are described in the following paragraphs.

Our initial approach was to make a deletion removing the entire LEL after F^{107} (FIG. 4A). This ΔLEL deletion accordingly reduced the size observed by western blot which confirmed stable expression of this mutant (FIG. 4B). Also it was noted that without the three glycosylation sites within the LEL domain the remaining N-terminal half of the protein appeared as two sharp

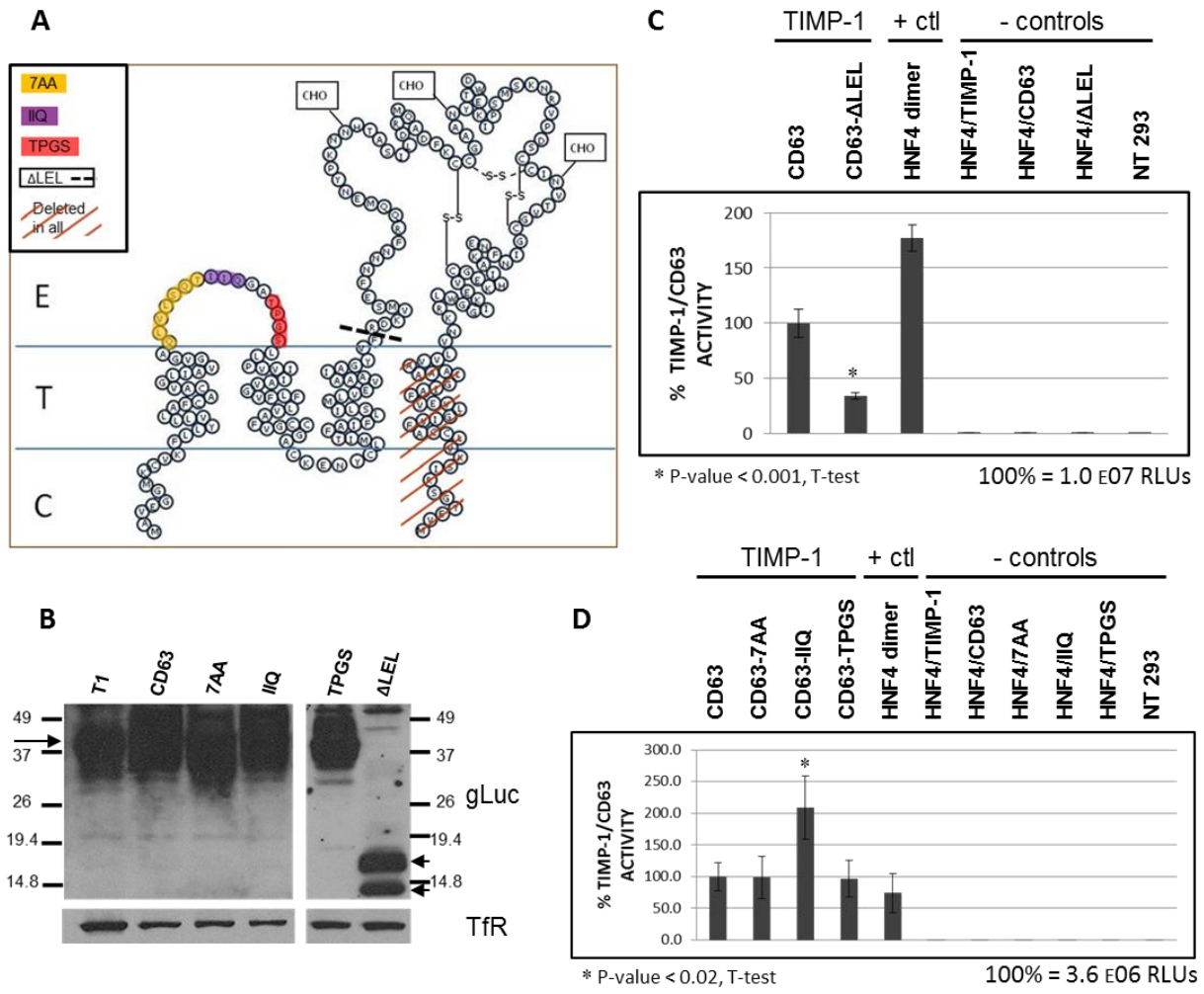


Figure 4. Interaction domain mapping of CD63: SEL and LEL evaluation by PCA. A) Diagram of CD63 showing modifications for analysis of the SEL and LEL domains. AA residues highlighted in color in the SEL were serially changed to Alanine residues to remove specificity. Separately the entire LEL domain was removed for PCA domain analysis. **B)** Western blot confirms the expression of CD63 mutants. TIMP-1 and CD63 fusions with hGluc fragments are indicated by a long arrow; CD63-ΔLEL fusion protein bands are indicated by short arrows. **C)** PCA analysis showing CD63-ΔLEL/TIMP-1 interaction as compared to CD63/TIMP-1 interaction, shown in the chart by relative luciferase activity. **D)** PCA analysis comparing CD63-SEL mutants for TIMP-1 interaction as compared to TIMP-1/CD63 interaction, shown in the chart by relative luciferase activity. All values shown are the average of atleast triplicate measurements. Error bars represent standard deviation. Significance was assessed by T-test with $P<0.05$ being considered as significant.

bands as opposed to a broad smear. This mutant had dramatically lower interaction with TIMP-1; just 36% luciferase activity compared to a standard interaction between CD63 and TIMP-1 (FIG. 4C). Taken together, our results show that the LEL domain is more centrally involved in TIMP-1 binding than is the SEL domain. The SEL does not appear to have direct sequence specific interaction with TIMP-1. One likely explanation is that the SEL may be involved through its proximity to the LEL in the tertiary protein structure, however further analysis of CD63 structure needs to be done to support this hypothesis.

As follow-up approaches to the LEL deletion analysis, we performed mutation toward the six cysteine residues in order to interfere with disulphide bridges and disrupt the general structure of the loop. Alternatively, we used PNGase F treatment to enzymatically deglycosylate N-glycosylation sites within the LEL domain. First, we mutated cysteines to serine residues singly (C145S, & C146S) or in groups of two (C145,146S; C169,170S; C145,191S; C146,170S), three (C145,146,169S; C146,169,170S), four (C145,146,169,170S), or five (C145,146,169,170,177S) (FIG. 5). However, after repeated analyses by PCA and western blot, the pattern emerged whereby the majority of changes to luciferase activity were due to protein instability –with the possible exception of the mutants shown in Figure 7 (see FIGS. 6-8). This confirms that the cysteine residues are important to the integrity of the protein structure as predicted by its homology to other tetraspanins with conserved LEL cysteines [135,137,139-144]; however, very little information toward defining the TIMP-1 binding site was gained.

We also attempted to determine the importance of N-glycosylation sites for TIMP-1/CD63 interaction by PCA analysis. Lysates of HEK293FT cells were treated with PNGase F de-glycosylase after co-transfection of TIMP-1 and CD63 prior to luminescence detection. Full

denaturation (addition of SDS, β-mercaptoethanol (β-ME), and boiling) of the proteins, which increases accessibility of the enzyme to the protein-sugar junction, where the enzyme cleaves, was not possible for this analysis since denaturing would also eliminate luciferase activity. Treatment of the un-boiled lysates in our system with PNGase F resulted in negligible changes to luminescence activity (data not shown). For more in-depth analysis of glycosylation effect on interaction a follow-up study should include mutational removal of N-glycosylation sites or an alternate method of analysis of binding; which does not involve an enzymatic reporter.

3.1.4 TIMP-1 binding to CD63 is present at the cell surface

Another important aspect of TIMP-1/CD63 interaction is confirmation that the interaction we observe is not merely a phenomenon of disruption of cellular compartments. Fortunately, an advantage offered by the PCA system is the ability for live cell analysis –demonstrated by Remy and Michnick [127]. The performance of the live assay is varied slightly from that in lysates (see sections 2.6 and 2.7); forgoing freeze/thaws and growing cells in a 96-well white plate with a clear well-bottom with application of a white backing tape prior to luminescence detection.

TIMP-1/CD63 interaction was indeed detectable in the live cell analysis and the level of detection remained at an amount of substantial interaction (FIG. 9A). By contrast, the HNF4 nuclear interaction was not detectable under assay conditions with live cells (FIG. 9A). This strongly supports observations for predominantly cell surface localization of TIMP-1/CD63 [78,87,102,145], however, we cannot exclude the possibility for intracellular interaction to occur as well. Additionally, we were able to confirm the importance of the TIMP-1 and CD63 domains that we previously determined in lysates (FIG. 9B). Hence, TIMP-1 and CD63 interaction at the cell surface is dependent on the C-terminal tail of TIMP-1 and the LEL domain of CD63.

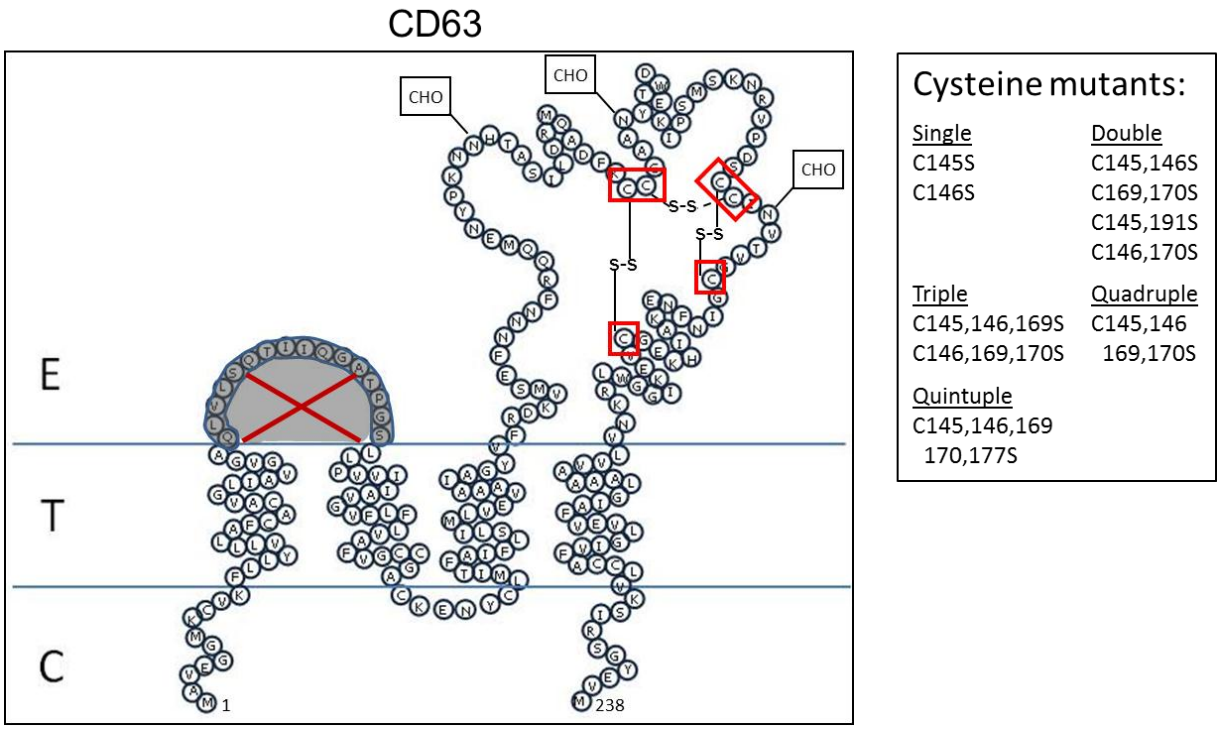


Figure 5. Schematic diagram of CD63 for cysteine mutation analyses.
The diagram highlights cysteines within the LEL of CD63 which were mutated to disrupt the structural conformation of the LEL. Mutations made were either single mutations or multiple from double to quintuple mutation. The cysteines are outlined by red boxes on the diagram and the numbers of the sequential locations are listed in the legend. The SEL domain is shaded in gray and marked with a red X to denote less importance for TIMP-1 binding.

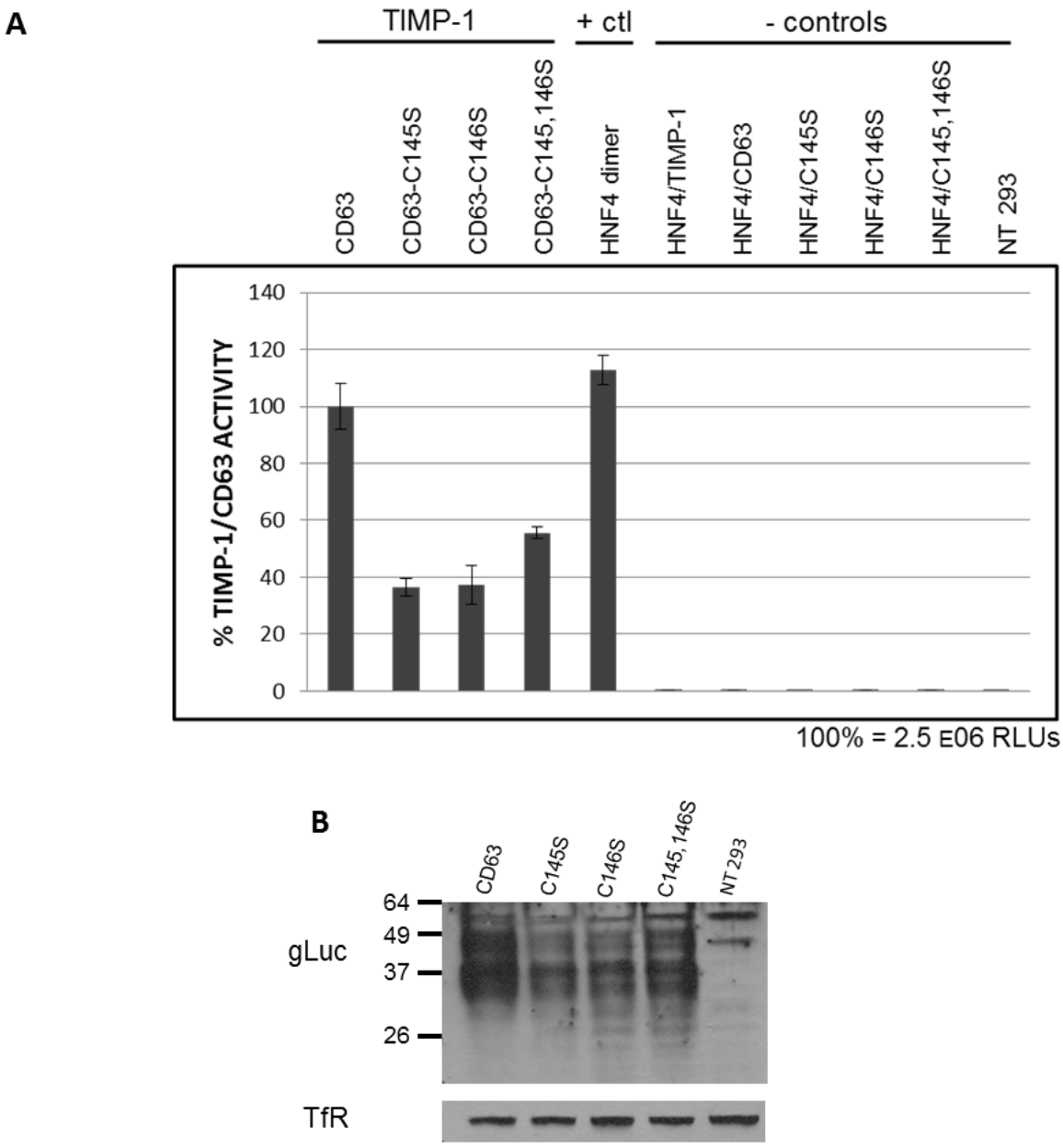


Figure 6. Comparison of cysteine mutants C145S, C146S, and C145,146S with CD63 by PCA. A) PCA analysis for cysteine mutants (C145S; C146S; C145,146S) as compared to TIMP-1/CD63 interaction, shown in the chart by relative luciferase activity. All values shown are the average of atleast triplicate measurements. Error bars represent standard deviation. **B)** Western blot shows the expression pattern/protein stability of cysteine mutants compared with CD63 (probed with polyclonal hGLuc antibody (gLuc)). Transferrin receptor (TfR) was used as loading control.

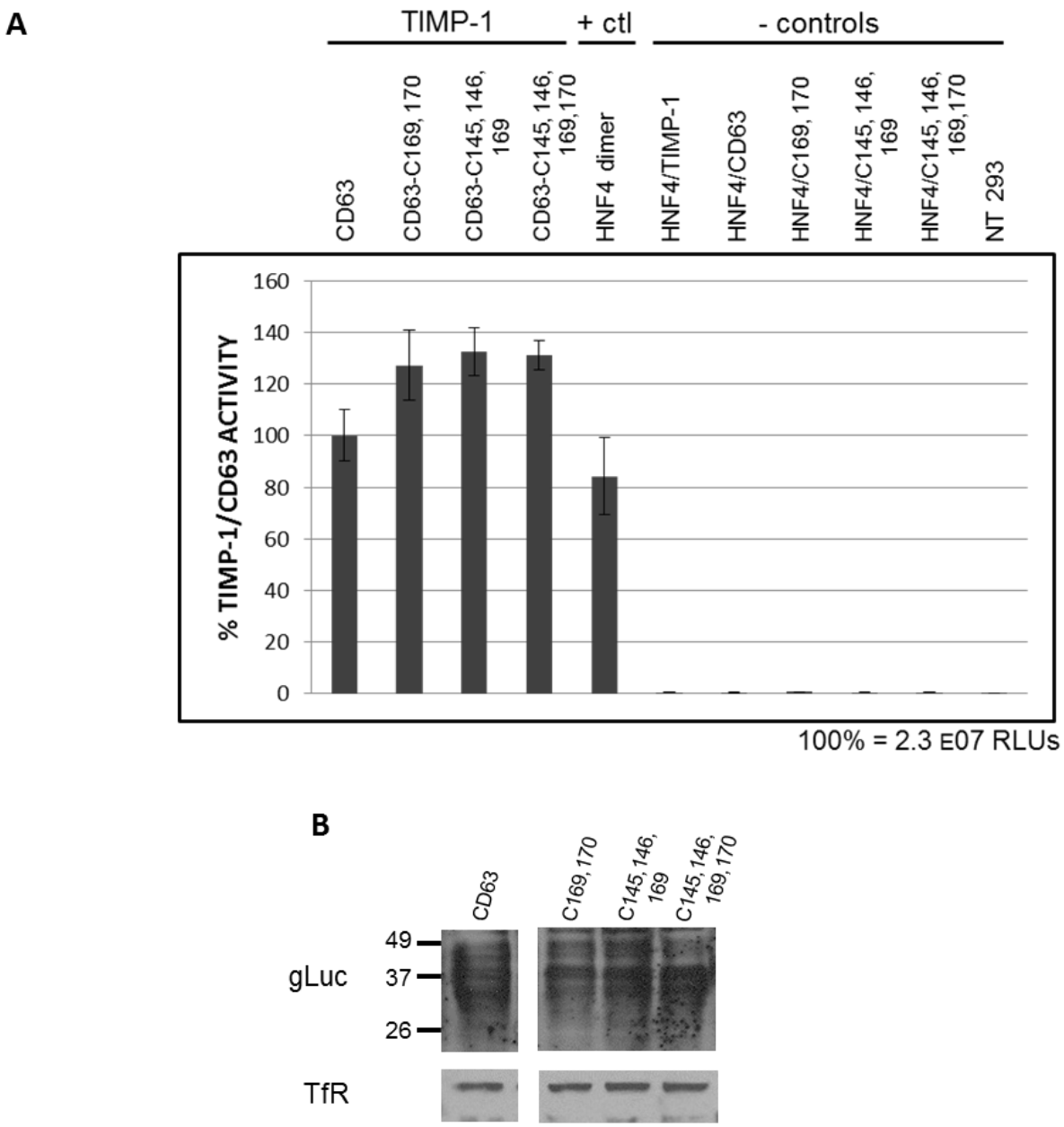


Figure 7. Comparison of cysteine mutants C169,170S; C145,146,169S, and C145,146,169,170S with CD63 by PCA. A) PCA analysis for cysteine mutants (C169,170S; C145,146,169S; C145,146,169,170S) as compared to TIMP-1/CD63 interaction, shown in the chart by relative luciferase activity. All values shown are the average of atleast triplicate measurements. Error bars represent standard deviation. **B)** Western blot shows the expression pattern/protein stability of cysteine mutants compared with CD63 (probed with polyclonal hGLuc antibody (gLuc)). Transferrin receptor (TfR) was used as loading control.

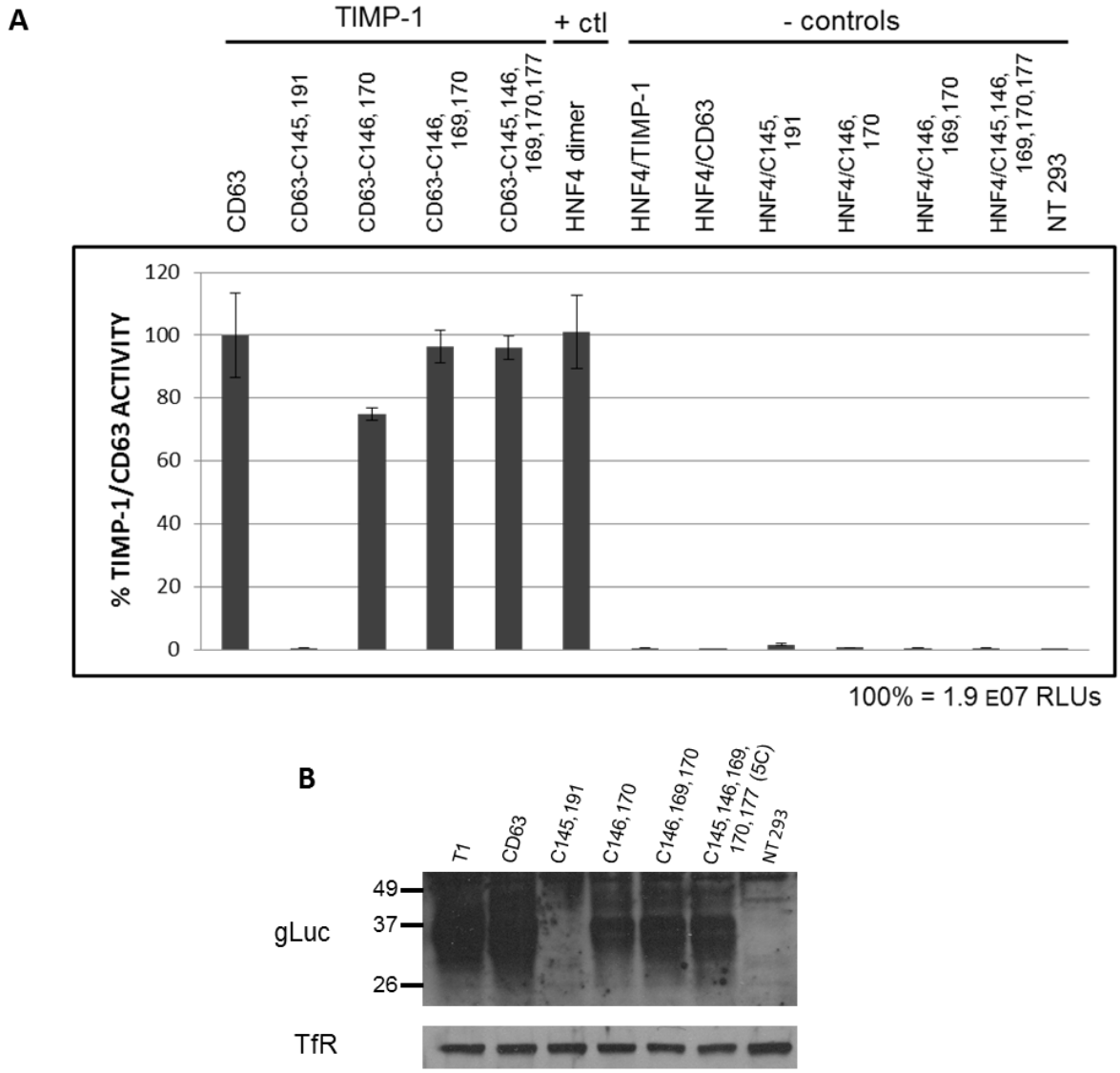


Figure 8. Comparison of cysteine mutants C145,191S; C146,170S; C146,169,170S, and C145,146,169,170,177S with CD63 by PCA. A) PCA analysis for cysteine mutants (C145,191S; C146,170S; C146,169,170S; C145,146,169,170,177S) as compared to TIMP-1/CD63 interaction, shown in the chart by relative luciferase activity. All values shown are the average of atleast triplicate measurements. Error bars represent standard deviation. **B)** Western blot shows the expression pattern/protein stability of cysteine mutants compared with CD63 (probed with polyclonal hGLuc antibody (gLuc)). Transferrin receptor (TfR) was used as loading control.

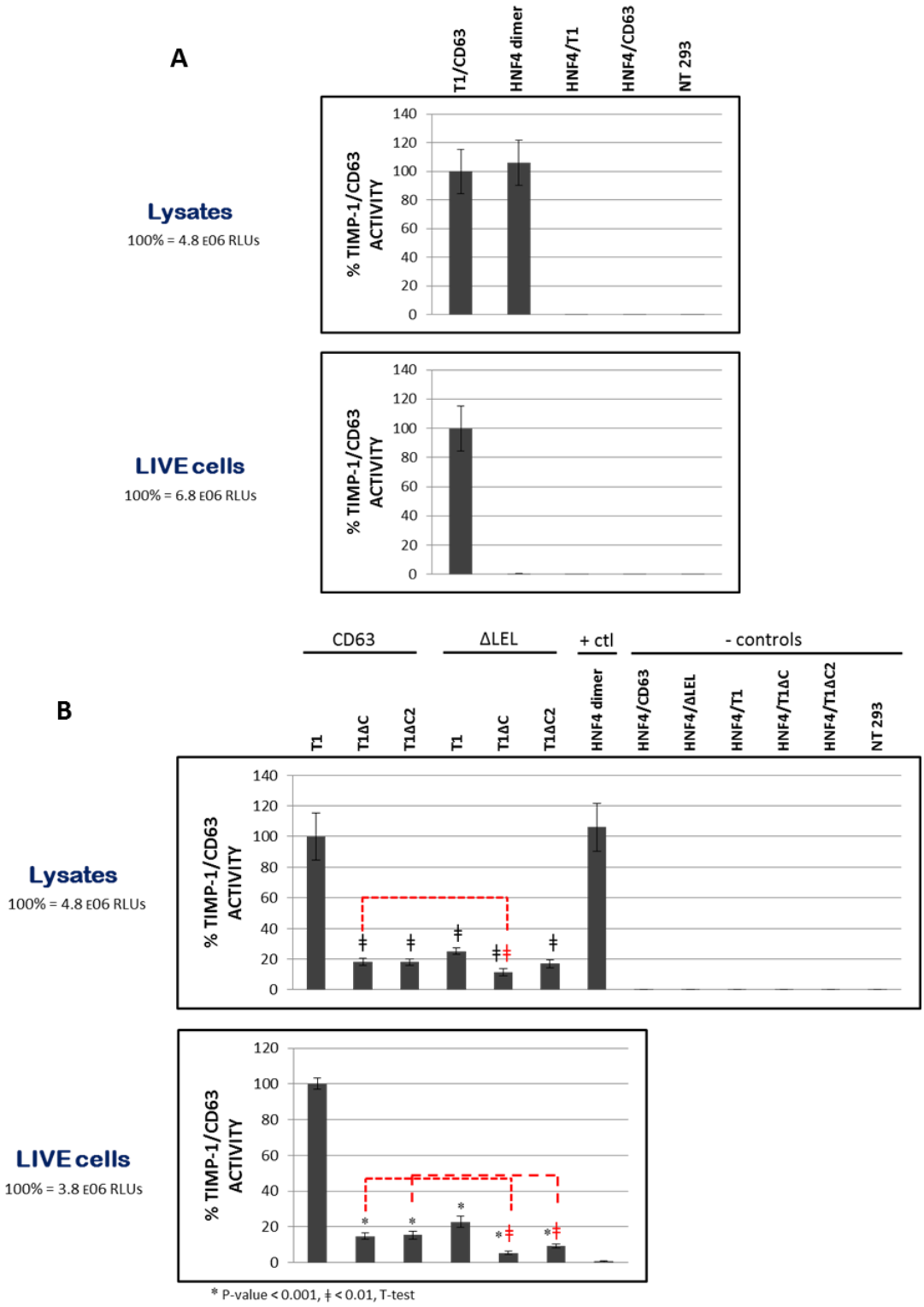


Figure 9. Comparison of TIMP-1/CD63 interactions by PCA in lysates and LIVE cell analysis. A) PCA comparison of TIMP-1/CD63 interaction with the HNF4 dimer interaction (nuclear) as measured in lysates and LIVE cells (described in sections 2.6 and 2.7), shown in the chart by relative luciferase activity. **B)** PCA comparison of TIMP-1/CD63 and interacting domain deletion mutants in lysates and LIVE cells, shown in the chart by relative luciferase activity. All values shown are the average of atleast triplicate measurements. Error bars represent standard deviation. Significance was assessed by T-test with $P<0.05$ being considered as significant.

3.1.5 TIMP-1 binding to CD63 can be disrupted by a C-terminal TIMP-1 antibody

In order to evaluate potential for disruption of the TIMP-1 and CD63 interaction and potential for therapeutic targeting, we performed peptide competition and neutralizing antibody experiments toward the C-terminus of TIMP-1. We first developed a synthetic peptide Acetyl-WQSLRSQIA-CooH based on the last 9aa of TIMP-1. The peptide was added to TIMP-1 and CD63 co-transfected cells in increasing amounts: 50, 100, 200, 400uM or DMSO (VEH) for 1hr. incubation. After media replacement, cells were lysed by normal freeze/thaw cycles for PCA analysis. However, we observed unexpected increases in detected TIMP-1/CD63 interaction (data not shown). We consider that it may be necessary to modify the peptide to a more rigid arrangement, or to extend the peptide in length to further resemble the C-terminus of native TIMP-1 in order to effectively compete with the native TIMP-1/CD63 interaction.

We then tested the effect of a neutralizing TIMP-1 antibody toward TIMP-1/CD63 interaction. First, to verify antibody recognition of TIMP-1's C-terminus we performed western analysis toward human TIMP-1 overexpressed in HEK293FT cells. We compared three different antibodies (1C6F, EP1549RY, & T1AB2) for recognition of human TIMP-1 with or without antibody-pre-blocking using our synthetic C-terminal TIMP-1 peptide. Our results verified EP1549RY as a C-terminal TIMP-1 antibody having an epitope that is blocked directly by the synthetic C-terminal peptide (FIG. 10A). Thus, to test the effect of the antibody on binding we performed live cell PCA analysis comparing EP1549RY with T1AB2 (non-C-terminal) for effect on the TIMP-1/CD63 interaction (FIG. 10B-C). We observed a significant reduction in the signal for TIMP-1/CD63 interaction upon EP1549RY treatment –decreasing the response by nearly 30%. Though the effect was significant, a greater effect was expected; one potential reason for a

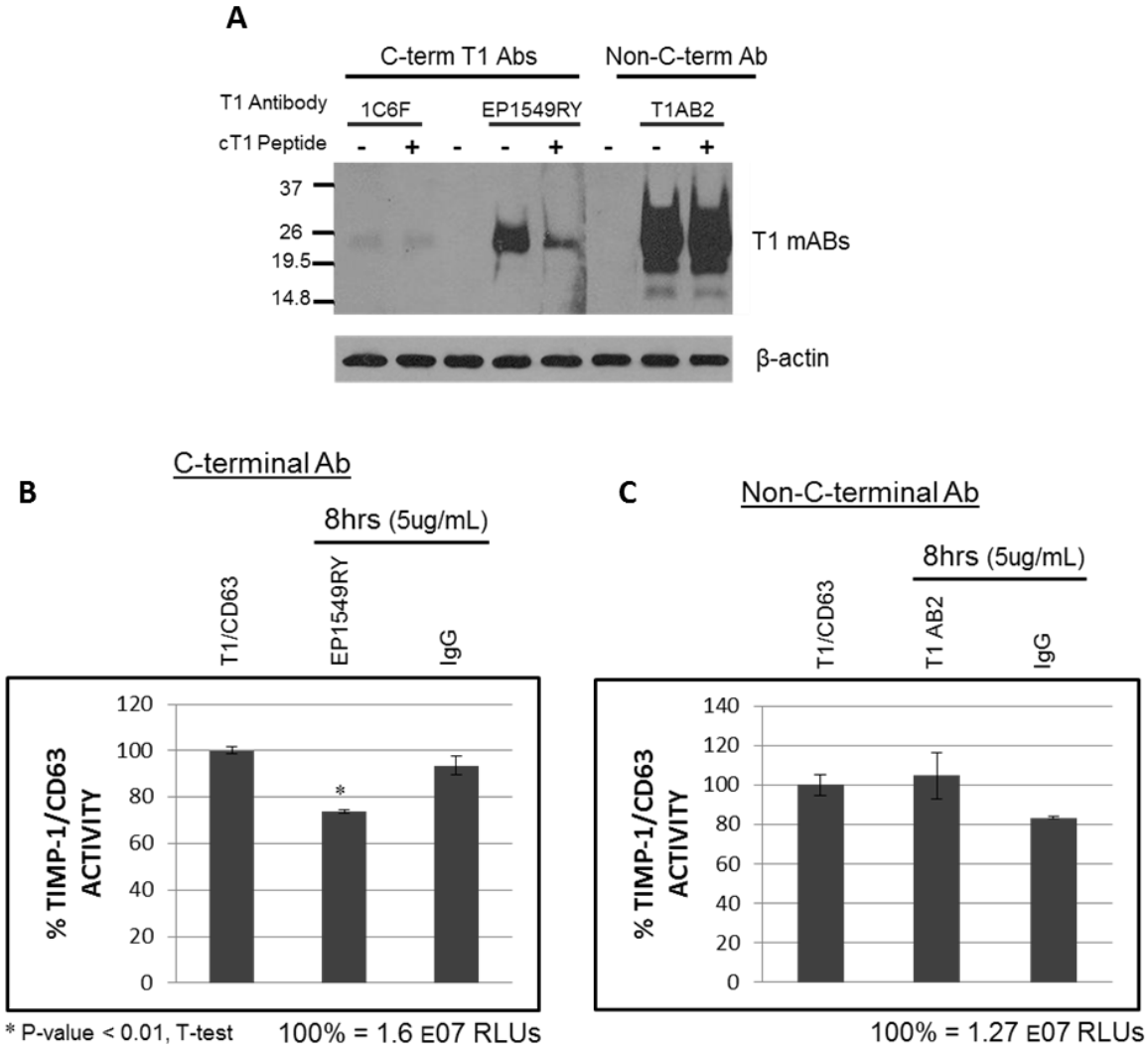


Figure 10. Disruptive effect of a C-terminal TIMP-1 antibody on TIMP-1/CD63 interaction. **A)** Western blot of TIMP-1 over-expressing HEK293FT lysates probed with different TIMP-1 antibodies (1C6F; EP1549RY; T1AB2) after pre-blocking with the C-terminal peptide (cT1 peptide). β-actin was used for protein loading control. **B+C)** PCA analyses comparing TIMP-1/CD63 interaction after challenge with a C-terminal or Non-C-terminal TIMP-1 antibody (normalized to treatment with each antibody buffer alone), as compared to typical TIMP-1/CD63 interaction, shown in the charts by relative luciferase activity. All values shown are the average of atleast duplicate measurements. Error bars represent standard deviation. Significance was assessed by T-test with $P<0.05$ being considered as significant.

dampened effect is the antibody buffer which contains 50% glycerol –limiting the achievable concentration and treatment parameters. In a clear contrast to the inhibitory effect of the EP1549RY antibody, we observed no reduction in interaction signal when cells were treated with T1AB2 (FIG. 10C).

3.2 CD63 and MT1-MMP interaction: relationship to TIMP-1/CD63

3.2.1 Binding of MT1-MMP's active form preferred over inactive

Using Yeast-2-Hybrid analysis we were able to demonstrate interaction of MT1-MMP with CD63. We compared the latent-form MT1-MMP (L-MT1-MMP) (containing Pre- and Pro-domains) with the active-form of MT1-MMP (A-MT1-MMP) for interaction with CD63 and found A-MT1-MMP able to interact while L-MT1-MMP showed weaker to no interaction (FIG. 11A). Analysis was also attempted for CD63 truncation mutants as described above (see section 3.1.2) with TIMP-1/CD63 yeast-2-hybrid analysis; which met with a similar fate and yielded no definitive results due to toxicity. Hence, the contribution of yeast-2-hybrid analysis toward our endeavor of clarifying the MT1-MMP/CD63 interaction was only a confirmation of the interaction previously observed by others [88,126] and a suggestion of differential CD63 binding to A-MT1-MMP more than L-MT1-MMP.

We then established a PCA system for further analysis of the MT1-MMP/CD63 interaction; MT1-MMP was cloned into the GLucN vector in similar fashion to TIMP-1 including the secretion peptide of hGLuc for PCA analysis (FIG. 11B). Since our design would append the luciferase fragment to the N-terminal portion of our MT1 protein, we used the activated form of MT1-MMP, without the Pre- and Pro- domains, beginning at Y^{112} to ensure that the fusion protein would not be separated by natural cleavage events. Also, for comparison

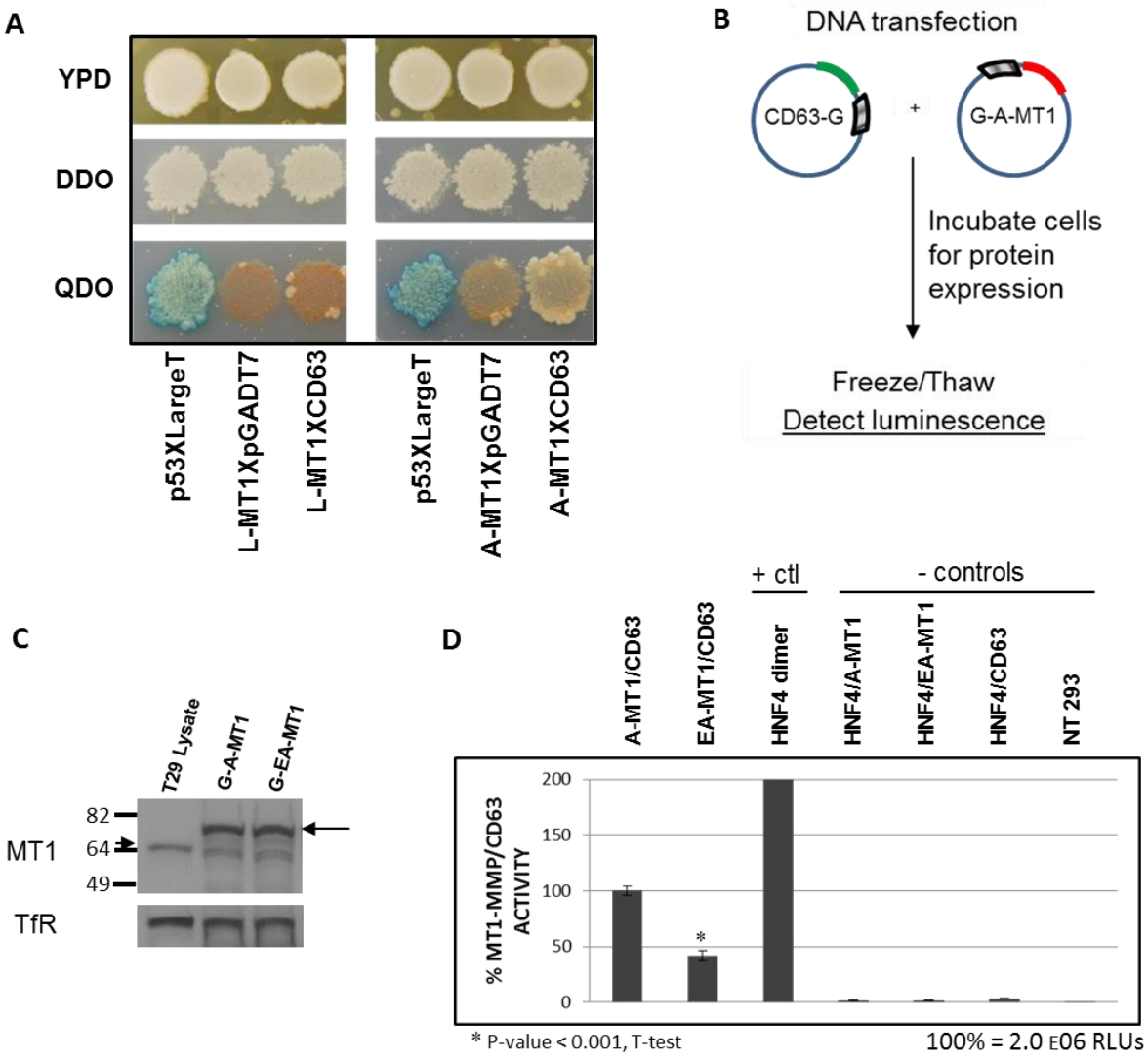


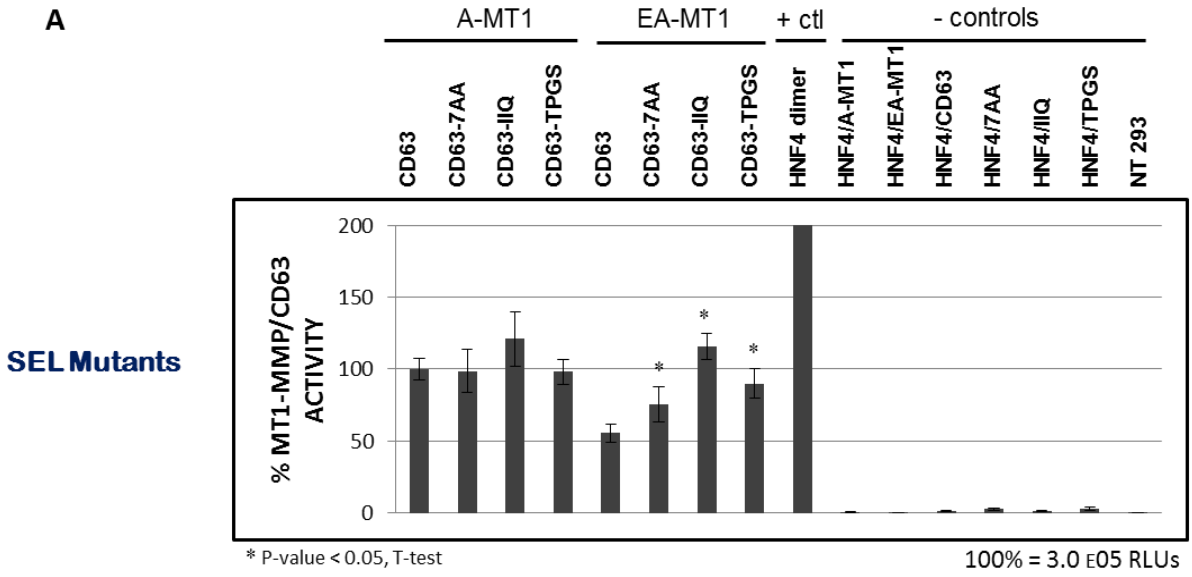
Figure 11. Establishment of PCA system for analysis of MT1-MMP/CD63 interaction. A) Yeast colonies were grown on rich YPD, selective DDO, and reporter QDO agar plates for spot-mating of complementary strains bearing MT1-MMP and CD63 reporter (GAL4) fusion constructs (see method in section 2.4). **B)** Schematic diagram for transfection of CD63 and MT1-MMP hGLuc fusion vectors and PCA assay. For our purpose, GLucN was fused to the N-terminus of MT1-MMP without the pre- and pro- domains (A-MT1) and the same construct with a catalytic mutation (EA-MT1; lacking cat. activity). **C)** Western blot confirms expression of MT1 fusions. The short arrow indicates non-fused MT1-MMP protein size; the protein size for A-MT1 and EA-MT1 with hGLuc fragment fusions is indicated by a long arrow. **D)** PCA analysis showing comparison of A-MT1/CD63 interaction and EA-MT1/CD63 interaction, shown in the chart by relative luciferase activity. All values shown are the average of atleast triplicate measurements. Error bars represent standard deviation. Significance was assessed by T-test with $P<0.05$ being considered as significant.

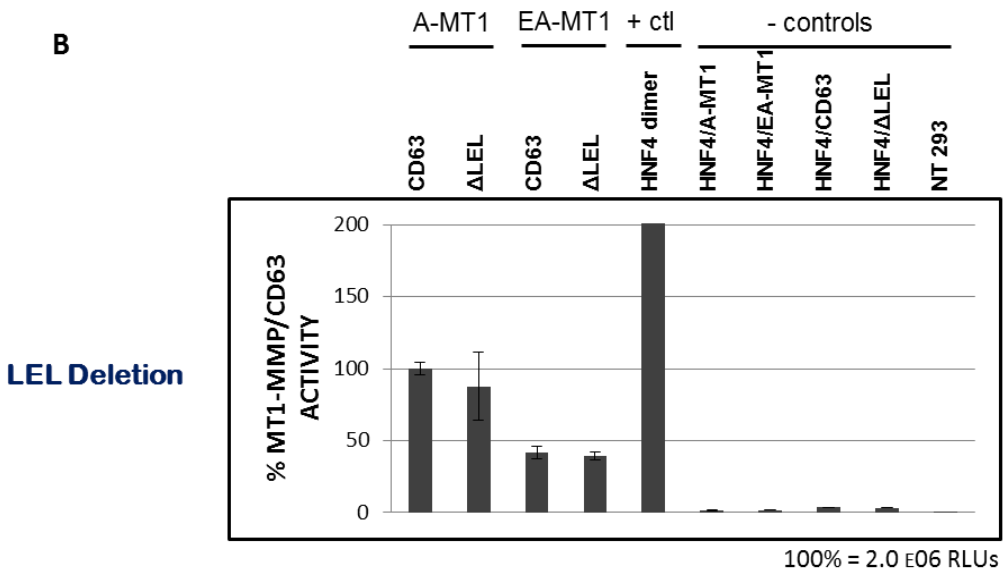
of active MT1-MMP (A-MT1) protease with an inactive form we included the E240A catalytic site mutant (EA-MT1) which lacks catalytic activity [130]. With expression of these MT1 constructs we observed consistent detections of reconstituted luciferase activity within the reliable range for specific interactions above background. Interestingly, the catalytic domain mutant EA-MT1 demonstrated approximately 50% less interaction with CD63 compared to A-MT1 in the PCA analysis (FIG. 11D). Also, we saw stable protein expression of our MT1 fusion proteins by western blot analysis (FIG. 11C).

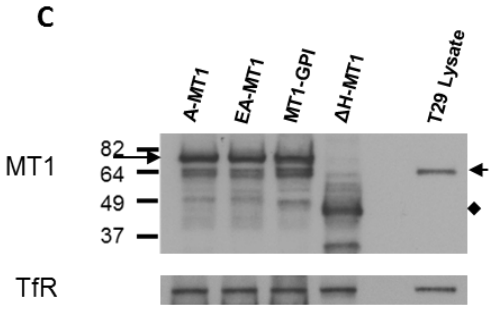
3.2.2 Analysis of CD63 and MT1-MMP interacting domains

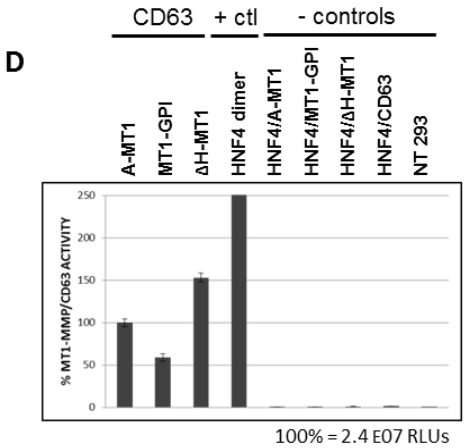
We performed CD63 domain analysis for MT1-MMP interaction similar to the above described analysis with TIMP-1. For this we compared either SEL domain mutants or the ΔLEL deletion mutant to the standard CD63 interaction with either A-MT1 or EA-MT1. Surprisingly, in contrast with the reductions in TIMP-1 binding caused by these CD63 alterations, we did not observe a reduction in binding of A-MT1 or the EA-MT1 catalytic mutant with these variants (FIG. 12A-B). Noteworthy from this PCA analysis, we saw almost no effect (positive or negative) on MT1 binding with the removal of 99aa via the LEL deletion. Interestingly, the mutation of the SEL amino acids had a positive effect on MT1 binding (significantly positive for EA-MT1 binding); which would suggest that the SEL domain may affect MT1 binding, though not in a manner which requires the specific SEL amino acid sequence.

Moreover, we continued our comparison of MT1-MMP domain involvement by the use of two additional MT1 variants. We tested the embedded and intracellular portions (TM domain and cytoplasmic tail) with a variant wherein those two domains are replaced by the GPI anchor from MT6-MMP (MT1-GPI); as well, we tested deletion of the hemopexin-like domain (ΔH-









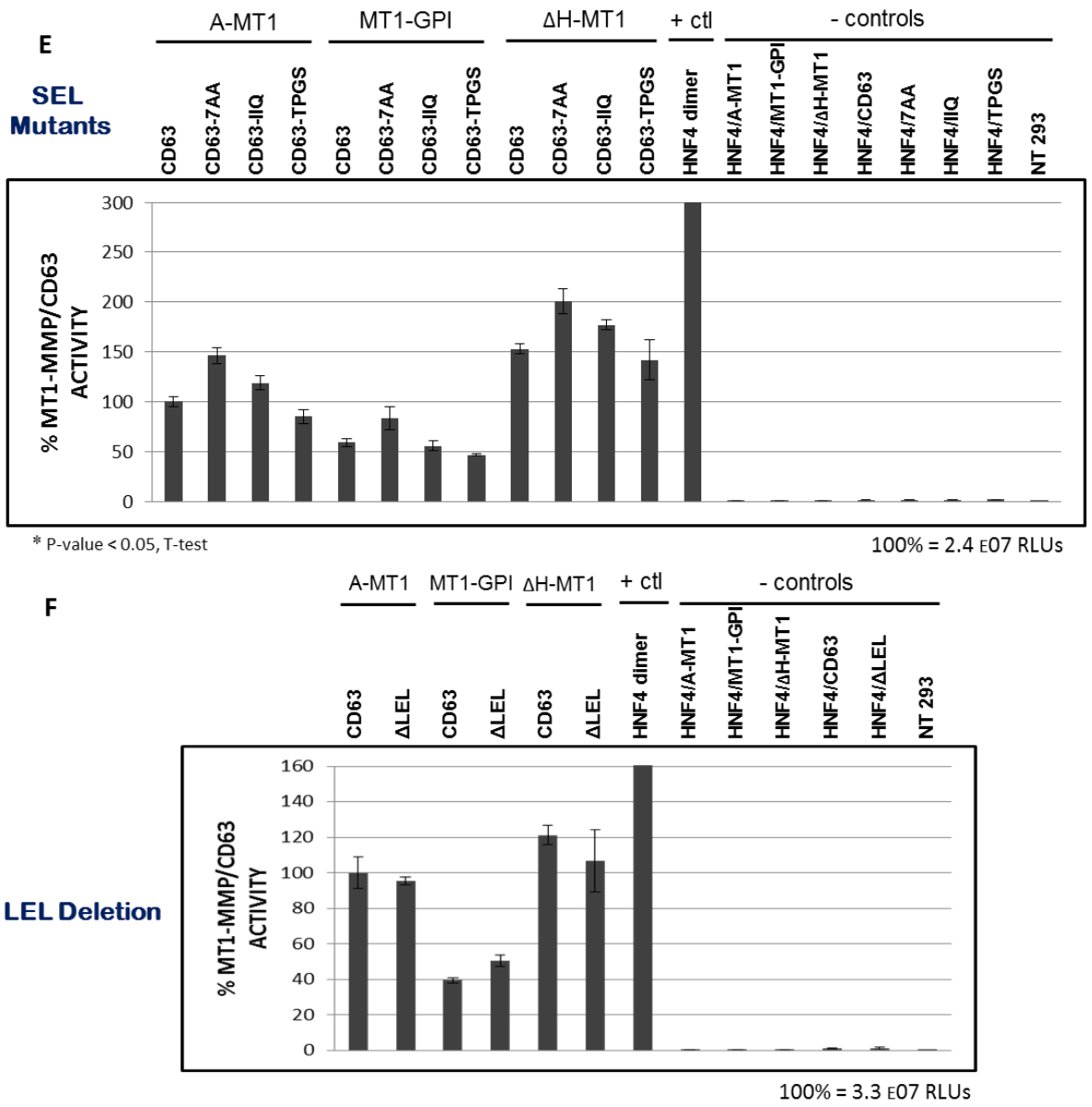


Figure 12. Domain mapping of MT1-MMP/CD63 interaction by PCA. A+B) PCA analyses showing A-MT1 and EA-MT1 interaction with SEL mutants or ΔLEL mutant as compared to interaction with un-mutated CD63, shown in the charts by relative luciferase activity. **C)** Western blot showing stable expression of A-MT1, EA-MT1, MT1-GPI, and ΔH-MT1 PCA fusion proteins. The short arrow indicates non-fused MT1-MMP protein size; the protein size for A-MT1, EA-MT1, and MT1-GPI with hGLuc fragment fusions is indicated by a long arrow; ΔH-MT1 fushion protein size is indicated with a diamond. **D)** PCA showing comparison of MT1-GPI and ΔH-MT1 interactions with CD63 to A-MT1/CD63 interaction, shown in the chart by relative luciferase activity. **E+F)** PCA analyses showing MT1-GPI and ΔH-MT1 interaction with SEL mutants or ΔLEL mutant as compared to interaction with un-mutated CD63, shown in the charts by relative luciferase activity. All values shown are the average of atleast triplicate measurements. Error bars represent standard deviation. Significance was assessed by T-test with P<0.05 being considered as significant.

MT1) to assess involvement of these MT1 domains in CD63 binding. Replacement of the TM and cytoplasmic tail gave a noteworthy reduction in CD63 interaction –a negative effect of similar magnitude to that of catalytic mutation (EA-MT1); while deletion of the hemopexin-like domain surprisingly increased the interaction signal (FIG. 12D). Furthermore, when comparing with the SEL mutants and LEL deletion of CD63 with MT1-GPI and (ΔH-MT1) we again saw no significant decreases (as with A-MT1 and EA-MT1) –with SEL mutations causing slight increases in binding (FIG. 12E-F). Stable protein expression of these constructs was observed by western blot analysis (FIG. 12C).

3.2.3 TIMP-1 and MT1-MMP compete for CD63 binding

Using PCA analysis, we evaluated whether the binding of TIMP-1 and MT1-MMP to CD63 are compatible with one another (may occur simultaneously) or if they are competitive one to another. We tested this by adding these three proteins together by co-transfection for transient expression in HEK293FT cells; two of them (X and CD63) with luciferase reporter fusions and the third with increasing amounts in its natural form without the reporter fusion. When TIMP-1 expression vector was added in increasing amounts to G-MT1 and CD63-G co-transfection we observed a dose dependent decline in signal from the A-MT1/CD63 interaction, however, TIMP-1 did not readily compete with the EA-MT1/CD63 interaction (FIG. 13A). Likewise, when WT-MT1-MMP was increased in conjunction with G-TIMP-1 and CD63-G expression we again observed clearly diminished signal in a dose dependent manner, but for EA-MT1-MMP competition was much weaker (FIG. 13B-C). We also did these competition analyses with MT1-GPI and ΔH-MT1. ΔH-MT1 binding was diminished competitively by TIMP-1 (similar to A-MT1); however, TIMP-1 did not compete with MT1-GPI (similar to EA-MT1) (FIG. 14).

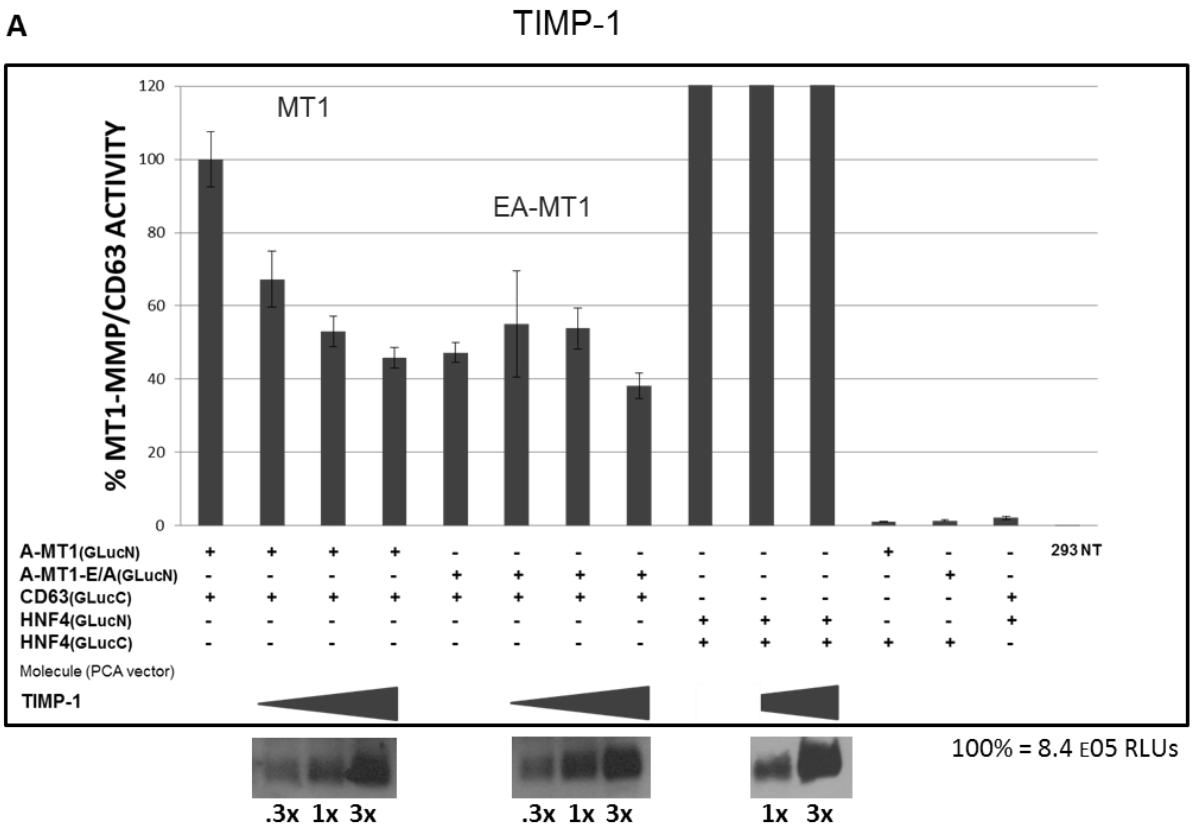
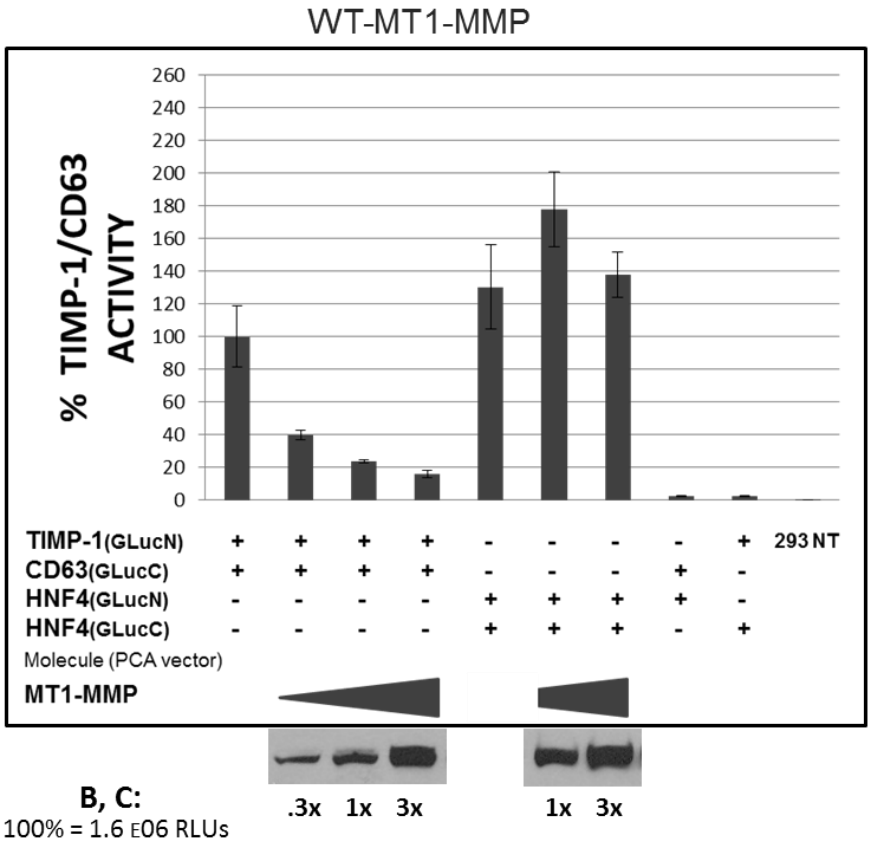


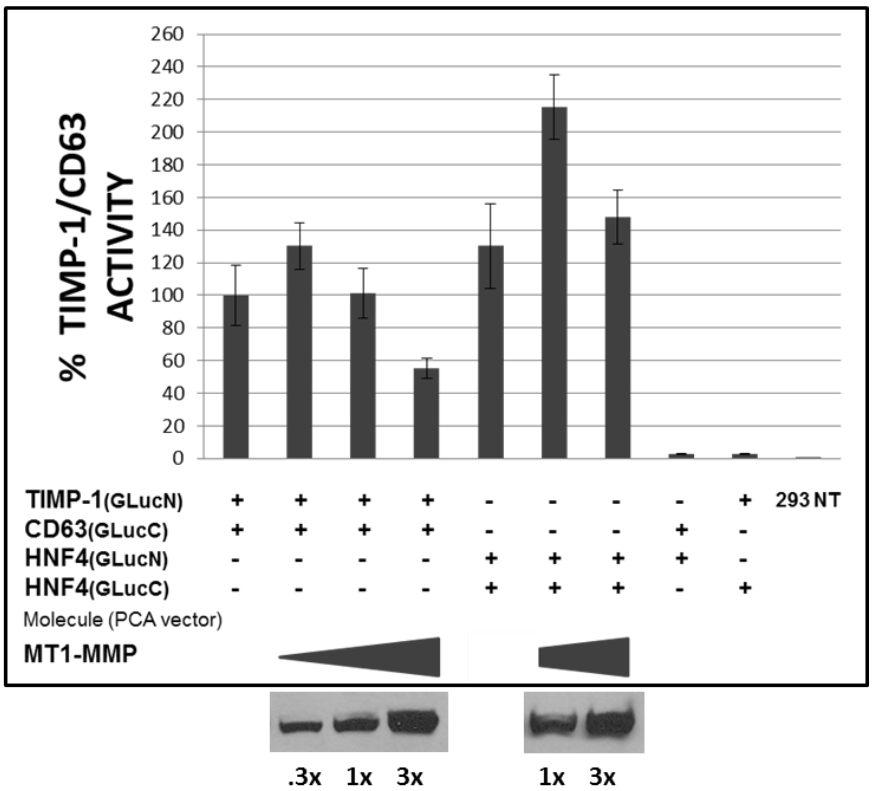
Figure 13. CD63 binding competition between TIMP-1 and A-MT1 or EA-MT1. A) PCA analysis of co-transfection of TIMP-1 in the pcDNA3.1 expression vector with A-MT1/CD63 and EA-MT1/CD63 interactions or the HNF4 control interaction, shown in the chart by relative luciferase activity. **B+C)** Co-transfection with WT-MT1-MMP or E/A-MT1-MMP in the pcDNA3.1 expression vector with TIMP-1/CD63 interaction or the HNF4 control interaction, shown in the charts by relative luciferase activity. All values shown are the average of atleast triplicate measurements. Error bars represent standard deviation. In all cases expression of the protein in the pcDNA3.1 expression vector at the proper dosage was verified at the protein level by western blot.

B



B, C:
100% = 1.6 E06 RLUs

C



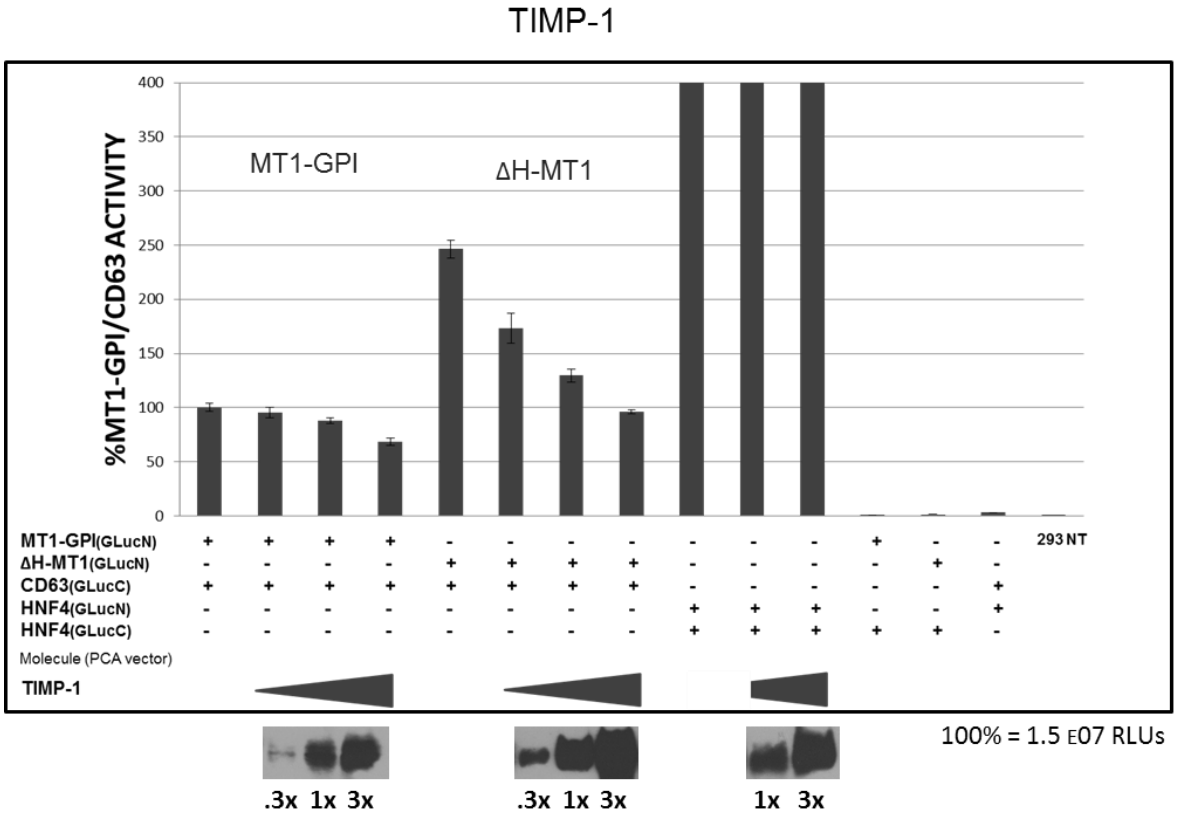


Figure 14. CD63 binding competition between TIMP-1 and MT1-GPI or ΔH-MT1. PCA analysis of co-transfection of TIMP-1 in the pcDNA3.1 expression vector with MT1-GPI/CD63 and ΔH-MT1/CD63 interactions or the HNF4 control interaction, shown in the chart by relative luciferase activity. All values shown are the average of atleast triplicate measurements. Error bars represent standard deviation. Expression of TIMP-1 in the pcDNA3.1 expression vector at the proper dosage was verified at the protein level by western blot.

At the time of this writing, the competition analyses for ΔH-MT1 and MT1-GPI to TIMP-1/CD63 interaction were still underway. Taken together, these co-expression experiments reveal that TIMP-1 and MT1-MMP don't enhance one another's CD63 binding, but compete for CD63 binding and can reduce existing interactions of each other with CD63 when presented in excess.

In order to show whether the negative regulation of TIMP-1/CD63 interaction by MT1-MMP is due to proteolytic processing of TIMP-1 or CD63 by MT1-MMP, we examined competition assay lysates for proteolytic processing by western blot analysis. We observed for both TIMP-1 dosage increase toward A-MT1/CD63 interaction and WT-MT1-MMP dosage increase toward TIMP-1/CD63 the fusion protein expressions remained unchanged. Importantly, neither CD63 nor TIMP-1 fusion proteins shifted in size or displayed cleavage fragments in the presence of active MT1-MMP protease (FIG. 15).

3.3 Relationship of TIMP-1, CD63, and MT1-MMP expression levels in xenografts & cells

3.3.1 Expression pattern in cancer tissue

Evaluation of xenografted breast tumors was done using serial sections from MCF10DCIS.com tumors to determine the pattern of expression for these three proteins in an *in vivo* cancer progression model. MCF10DCIS.com cells were grown in SCID mice and tumors were examined for protein expressions after 4 weeks (largely contained-tumors) and at 8 weeks (largely invasive tumors). CD63 was widely expressed in the cytoplasm but in several instances was drastically lost from the cytoplasm and, interestingly, still present at the cell surface. MT1-MMP was expressed at the cell surface throughout the whole tumor area with regions of very high expression near the edges of some of the ductal structures. TIMP-1 was also found easily across the tumors with cytoplasmic staining or with cell surface staining especially at edges of

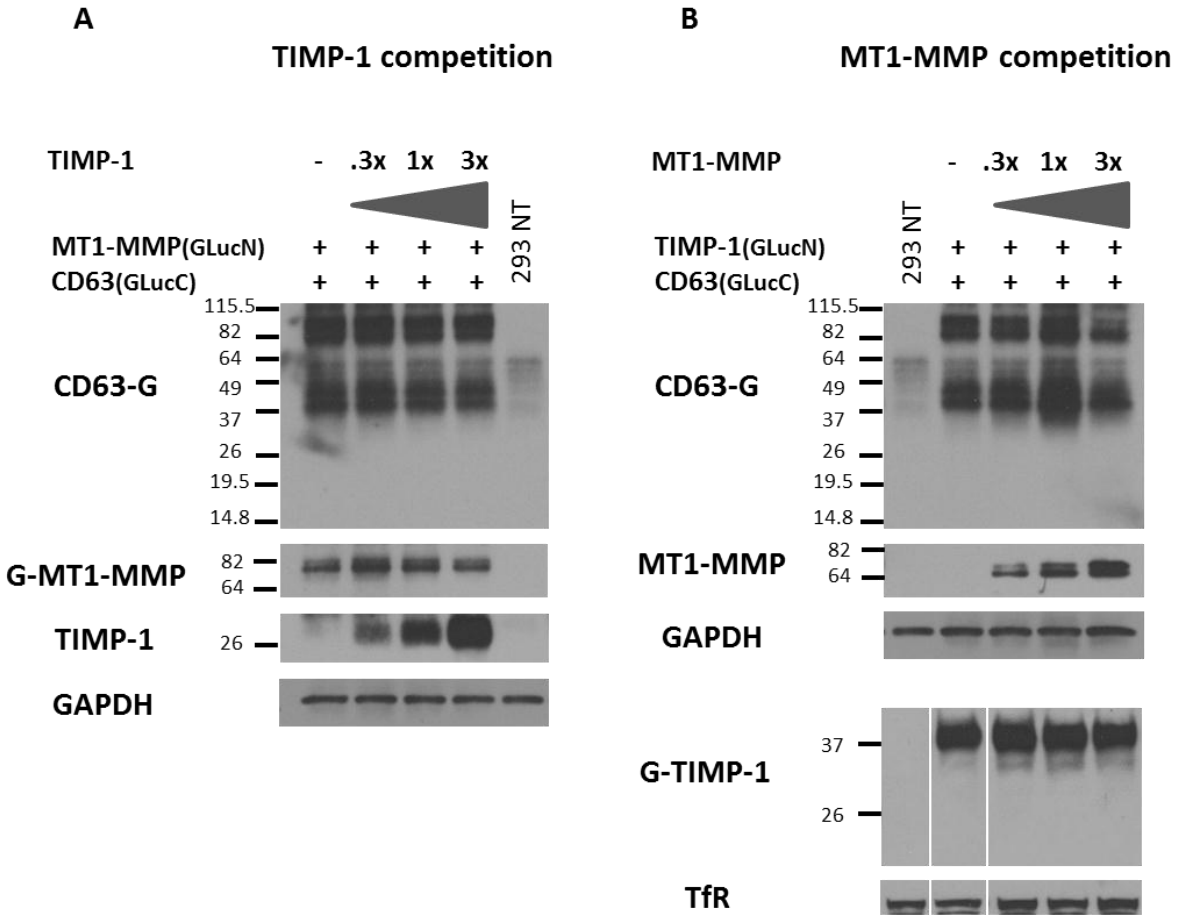


Figure 15. Analysis of TIMP-1 and CD63 for potential cleavage by MT1-MMP. A) Western blot analysis showing CD63-G protein size when co-transfected with G-A-MT1-MMP and in the presence of increasing amounts of TIMP-1 protein. **B)** Western blot analysis showing CD63-G and G-TIMP-1 protein sizes when co-transfected along with increasing amounts of WT-MT1-MMP. CD63-G was detected using a monoclonal CD63 antibody, MT1 and G-MT1 were detected using an MT1-MMP catalytic domain antibody, TIMP-1 and G-TIMP-1 were detected using a monoclonal TIMP-1 antibody. Transferrin receptor (TfR) and GAPDH were used as loading controls.

ductal structures (in agreement with MT1-MMP staining). Rather intriguingly, those ductal regions with the highest TIMP-1 and MT1-MMP cell surface staining were also those in which CD63 was absent from the cytoplasm but present on the cell surface (FIG. 16). Taken together, in ductal regions where intracellular CD63 was high there was less TIMP-1 and less MT1-MMP expression, and in ducts where CD63 is largely absent from the cytosol but has more of a cell surface localization there is increased TIMP-1 and MT1-MMP expression on the cell surface. Expression of these three proteins at the cell surface was maintained in invasive tumors with CD63 mainly displaying exclusive cell surface staining and with TIMP-1 and MT1-MMP displaying similar patterns of cell surface staining to CD63 in the invaded area (FIG. 16).

3.3.2 Expression at the cell surface in cultured cells

We then examined the levels of TIMP-1, CD63, and MT1-MMP at the cell surface in MCF10A cells in culture. Since, TIMP-1 is increased during cancer progression we examined stable TIMP-1 over-expressing MCF10A cells (T29) for detection of TIMP-1 at the cell surface; which would represent binding to CD63. Interestingly, when TIMP-1 was over-expressed in these cells, CD63 was increased at the cell surface compared to a Neo vector control (FIG. 17A) and in contrast to overall CD63 expression which was unchanged (FIG. 17B). To confirm that presence of TIMP-1 on the surface of the cells is mediated by CD63 we performed shRNA knockdown of CD63 in T29 cells and compared cell surface binding to T29 cells with scrambled shRNA (FIG. 17A). We also examined the levels of MT1-MMP at the cell surface in T29 cells with or without CD63 knockdown. However, there was not a drastic change in levels of MT1-MMP at the surface upon CD63 knockdown. This is not surprising as it has been reported that other tetraspanins interact directly with MT1-MMP and may have even greater effect on surface

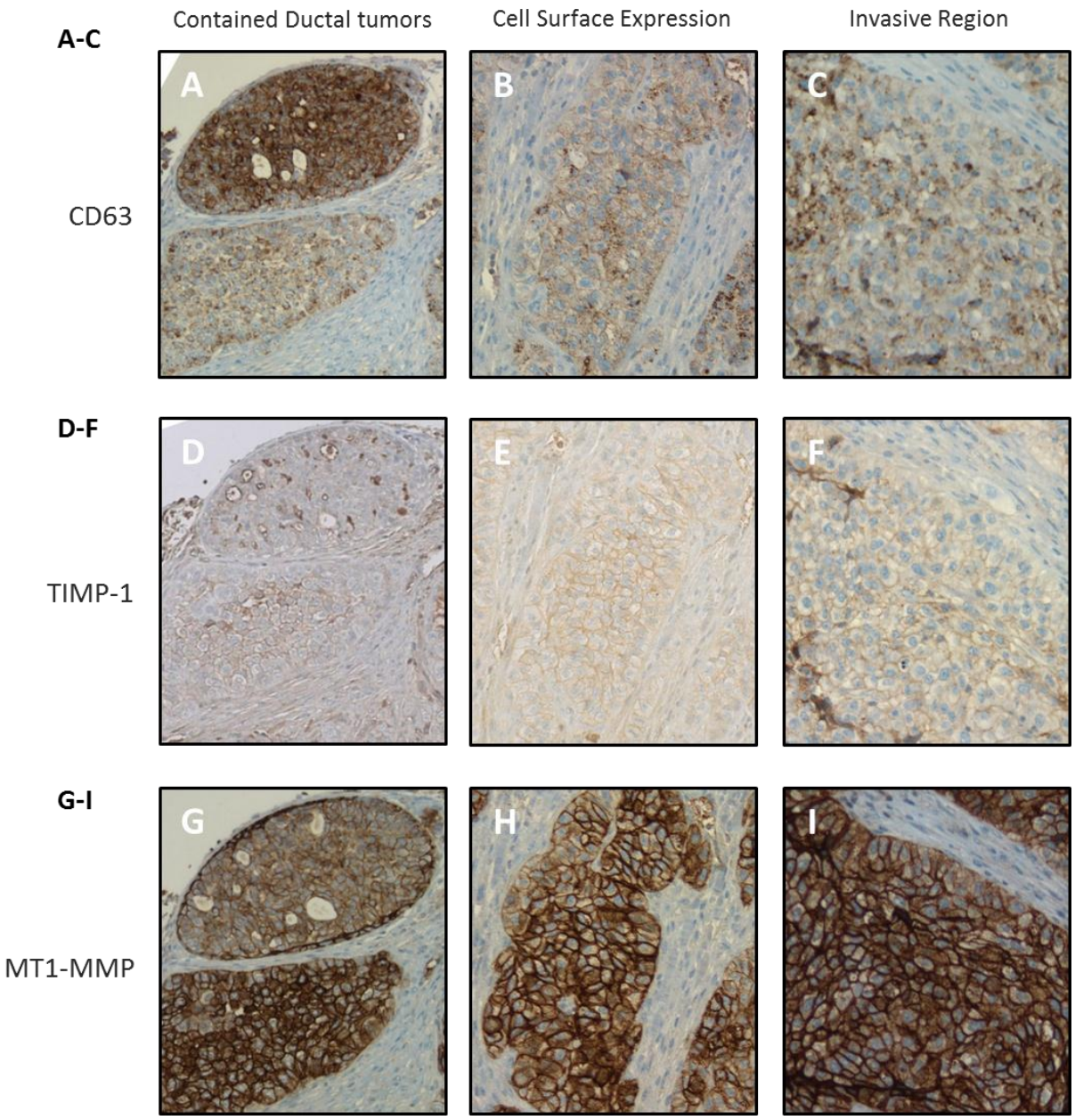


Figure 16. Immunohistochemistry analysis of CD63, TIMP-1, and MT1-MMP in xenografted MCF10DCIS.com tumors. A-C) Anti-CD63 antibody staining in contained ductal tumors (A) progressing ducts (B) and an invasive region (C) of MCF10DCIS.com tumor. **D-F)** Anti-TIMP-1 antibody staining in contained ductal tumors (D) progressing ducts (E) and an invasive region (F) of MCF10DCIS.com tumor. **G-I)** Anti-MT1-MMP antibody staining in contained ductal tumors (G) progressing ducts (H) and an invasive region (I) of MCF10DCIS.com tumor. All pictures were taken at 10X magnification.

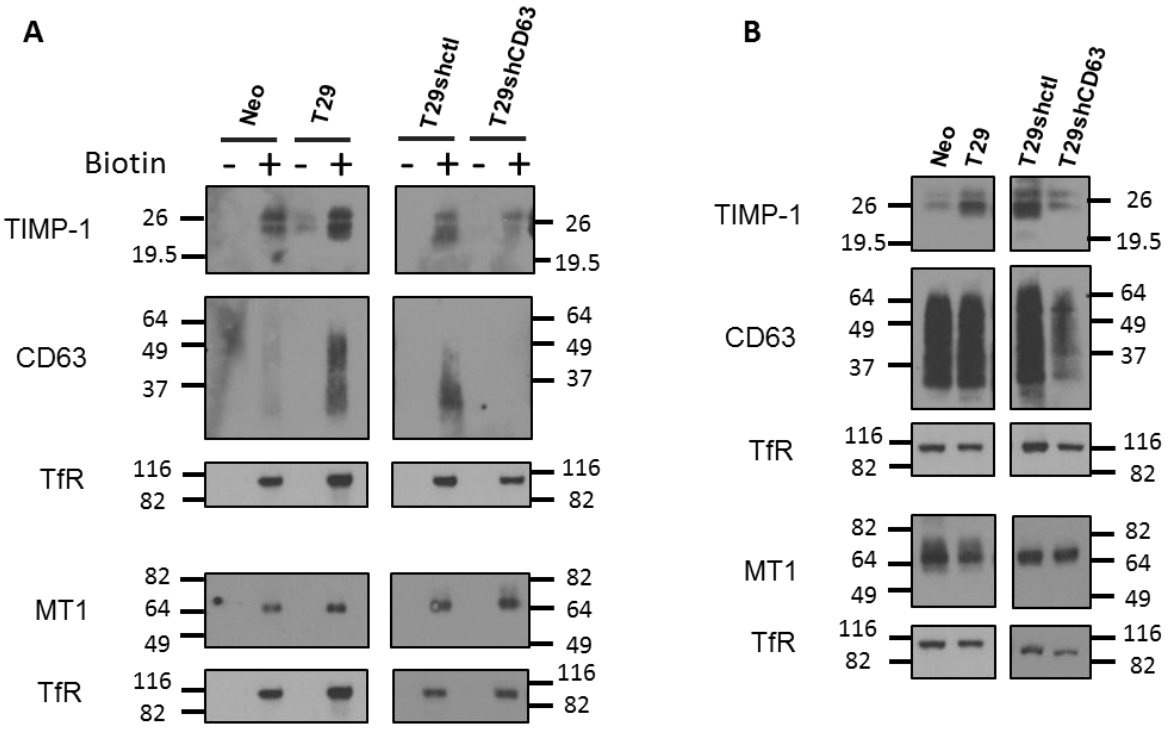


Figure 17. Evaluation of TIMP-1, CD63, and MT1-MMP expression at the cell surface upon TIMP-1 over-expression and CD63 knockdown. A) Determination of protein expression levels at the cell surface after total cell surface biotinylation of MCF10A cells bearing either TIMP-1 (T29), vector control (Neo) expression vectors; also with CD63 knockdown (T29shCD63) or scrambled shRNA control (T29shctl) **B)** Whole cell lysates pertaining to the cell surface determinations in panel A. Transferrin receptor (TfR) was used as protein loading control.

localization of MT1-MMP compared to CD63 [124-126]. The primary regulation of MT1-MMP by TIMP-1 and CD63 is likely through conformational changes or related to MT1-MMP ability to bind CD63, since we've seen increased activity in TIMP-1 over-expressing epithelial cells [101] and multiple MT1-MMP domains affect CD63 binding while the extra-cellular portions of CD63 ostensibly don't affect MT1-MMP binding as shown above (FIGS. 11-12).

Chapter 4

Discussion and Future Directions

4.1 Discussion of protein relationships and protein complexes

Herein, we've described the nature of a predicted complex between TIMP-1 and CD63 and implications of the complex toward cell function and the transmission of cell signaling. Additionally, we have described the interaction between MT1-MMP and CD63; which is likely not compatible with TIMP-1 binding. In review of our findings, we will go over the implications for each complex and their relationship together.

TIMP-1 and CD63 complex

Protein structural and protein complex analyses require that structure be viewed as changeable and context-dependent. Many factors can affect a protein's affinity for other protein partners, not the least of which are post-translational modifications, altered regulation of isoform expression, and creation of binding pockets or hindrance (i.e. sterics) upon interaction with other proteins.

TIMP-1 has been structurally characterized to have distinct N- and C-terminal domains. Gomis-Ruth et al, generated a crystal structure of TIMP-1 complexed with the catalytic domain of MMP-3. According to their description of the TIMP-1/MMP interaction, "…a wedge-shaped inhibitor slots with its edge into the active-site cleft of the MMP-3 catalytic domain." Also, the TIMP-1 molecule or, "…polypeptide chain folds into a contiguous elongated domain with the N- and C-terminal halves forming two opposing 'subdomains'." [131]. We've used this structure in our analysis of TIMP-1 and in identifying the CD63 binding site –see FIG. 18A, where TIMP-1 domains, MMP binding region, and CD63 potential binding regions have been highlighted.

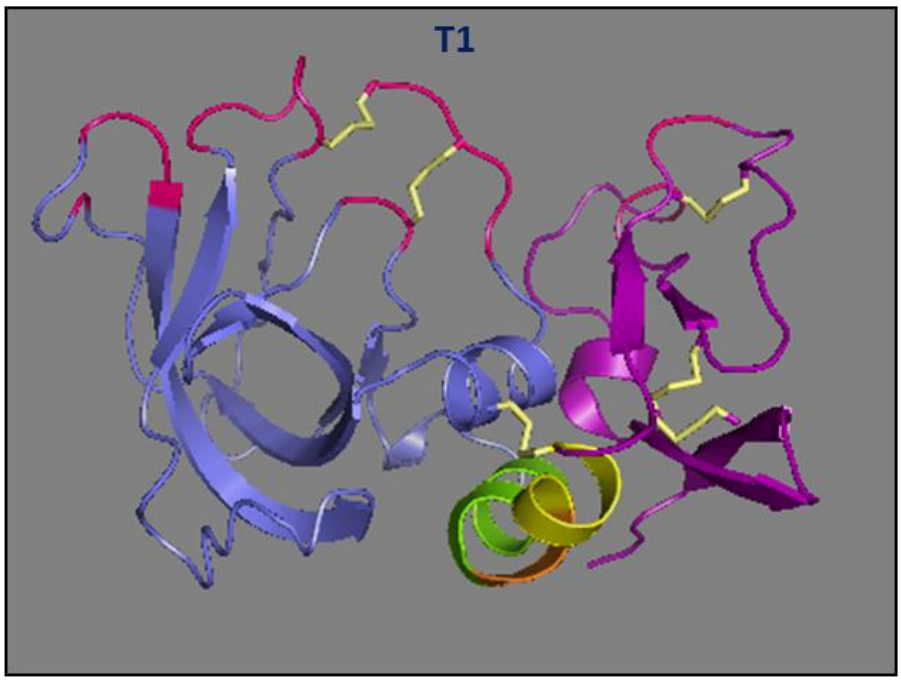


Figure 18. C-terminal mutants of TIMP-1 are predicted to have conserved C-terminal structure with full-length TIMP-1. A) The TIMP-1 structure is shown with highlights of important features; figure was prepared from PDB entry 1UEA -entered by Gomis-Ruth et al., Nature, 1997. Highlighted regions of the structure are as follows: site occupied by MMP-3 catalytic domain in crystal structure –Magenta; N-terminal domain (Loops 1-3, aa 1-126) –Blue; C-terminal domain of TIMP-1 (Loops 4-6, aa 127-184) –Purple; Helix 2 and Helix3 –Green and Yellow, respectively (connector –Orange); and disulphide bridges –Pale Yellow. Image generated with PyMOL structure viewer. **B)** Diagram of full-length TIMP-1 including secretion peptide, N-terminal domain, and C-terminal domain. Includes a 'C' for each cysteine in the protein sequence. Also, included are diagrams of T1C and T1D as generated by deletions in the N-terminal domain (represented by /\ ).

Thus, the TIMP-1 structure would suggest that a large portion of TIMP-1 is generally not involved in MMP-binding. Specifically, the C-terminal domain and opposing side to the MMP-binding edge are likely not engaged by MMPs and were thus our starting point when searching for the CD63 binding site. In our experiments, we have utilized truncation mutants of TIMP-1 to parse out the involvement of domains in TIMP-1 functions –specifically anti-apoptotic and EMT effects. We removed either part or else the entire N-terminal domain of TIMP-1; these truncations were called T1D and T1C, respectively (FIG. 18B). Through comparison of these two mutants to full-length TIMP-1, we have verified that non-MMP-inhibitory functions (anti-apoptotic and EMT signaling) of TIMP-1 can be carried out similarly by T1D, but not necessarily by T1C ([101] and observations by XuWen Liu). A potentially important difference between these two deletion mutants may be the retention of the 2^{nd} and 3^{rd} helices (H2 & H3: L^{110}-C^{124}) of TIMP-1 by T1D; which helices are prior to the C-terminal domain and on the opposing side of the MMP-inhibitory edge –highlighted green (H2), orange (connector), and yellow (H3) in FIG. 18A. As CD63 has been shown by our previous work to help mediate anti-apoptotic and EMT signaling by TIMP-1, we began this most recent endeavor to map the binding site considering it probable that C-terminal residues and H2 and H3 of TIMP-1 are involved in CD63 binding.

Herein, we identified the critical role of the C-terminal residues of TIMP-1 in CD63 binding. The broad C-terminal region of TIMP-1 (aa 127-184) was confirmed to be central to CD63 interaction by yeast-2-hybrid analysis; which showed that upon deletion of these residues the T1N mutant (aa 1-126) failed to interact with CD63 and the yeast did not survive on reporter plates (FIG 1B). Following this analysis, we demonstrated by PCA a more specific requirement

of the C-terminal tail of TIMP-1, even down to the last 9aa being critical for CD63 interaction. Deletion of either the last 17aa or last 9aa had a very dramatic negative effect on TIMP-1's interaction with CD63 on average reducing it to 17% or 25% of full-length TIMP-1 measurements, respectively (FIG. 3B).

Additionally, we characterized the involvement of CD63 domains in TIMP-1 interaction. At the outset, our understanding of CD63 was rather limited and we had much less structural information to direct our hypothesis of the probable TIMP-1-interacting amino acids/domains. Without a crystal structure, the closest prediction of the 3D contour of CD63 domains comes from computer modeling based on a crystal isolated from an artificial dimer of CD81 LEL domains without the remainder of the protein [132]. In our view, the most plausible biological interaction with TIMP-1 would not largely involve TM residues but would mainly incorporate extracellular portions of the molecule (SEL or LEL) due to prominent secretion of TIMP-1. Deletion of these extracellular domains for yeast-2-hybrid analysis, however, yielded no substantial clue into SEL and LEL interaction with TIMP-1 as there were ranging levels of toxicity caused with expression of these mutants in the yeast. Thus, our primary option for acquiring knowledge of the CD63 binding region was mutational analysis in the mammalian PCA system. Alteration of either the SEL or LEL of CD63 had some effect on TIMP-1 binding. Mutation of a three amino acid sequence in the middle of the SEL (IIQ) resulted in increased TIMP-1 interaction shown to be significant by t-test. While the brunt of mutational impact on binding was negative: leaving 87 or 86% on average of full interaction for 7AA and TPGS mutants, respectively; and the much greater reduction upon LEL deletion to just 36% of a full interaction signal for the two proteins. Thus, we concluded that while the SEL domain may play

a lesser role or have an indirect impact on TIMP-1 interaction, the principal portion of CD63 driving the interaction with TIMP-1 is within the LEL domain.

It was also needful to show that the TIMP-1/CD63 interaction could be detected as well in living cells with intact membranes. We confirmed the maintenance of TIMP-1/CD63 interaction in live HEK293FT cells; with retention of a similar intensity of PCA signal to that observed in lysates. This undiminished signal would suggest a cell surface interaction as opposed to a decreasing signal for intracellular proteins -for HNF4 (intracellular (and nuclear)) the signal was almost completely lost in live cell analysis (FIG. 9A). Importantly, we were also able to confirm that TIMP-1/CD63 key binding residues for which dependence was shown in lysates maintained a critical role in the live HEK293FT cells (FIG. 9B). Taken together, the C-terminal end of TIMP-1 (and in the case of signaling, we predict H2 & H3 as well) interacts with the LEL of CD63 in a stable interaction within living mammalian cells as we observed over repeated experiments.

How might this information be used for therapy design? In our continuing study, we were able to confirm the necessity of the C-terminal tail residues for TIMP-1 non-MMP-mediated signaling functions. First, we confirmed that interaction with CD63 could be reversed by addition of an antibody recognizing the C-terminal tail of TIMP-1 including the most C-terminal residues (FIG 10B). Currently, we are examining the ability of T1ΔC and T1ΔC2, the TIMP-1 C-terminal deletions, for anti-apoptotic signaling in epithelial cells –to confirm the biological outcomes expected upon TIMP-1/CD63 complex disruption. These experiments were still underway at the time of the writing of this work. We expect the C-terminal deletions to have

diminished TIMP-1 signaling and reduced protection from apoptosis as we have observed the near complete loss of CD63 binding.

TIMP-1 and MMP complexes

TIMP-1, of course, interacts with and effectively inhibits a wide range of the MMP and ADAM families –not just MMP-3 as crystallized by Gomis-Ruth et al.. The most notable exceptions to this observation are: MT1-MMP, MT2-MMP, MT3-MMP, MT5-MMP, MMP19, and a number of ADAMs perhaps the most well-known being ADAM 17 [119,122,146-152]. TIMP-1 is perhaps most well-known for its inhibition of MMP-9 and closely related collagenases/gelatinases, and it is similar to TIMP-2 in many of these inhibitions [1,41,153-156].

A very important facet of TIMP-1 structure and complexes with MMPs is the ability of TIMP-1 to selectively bind to the latent pro-MMP-9; this is also seen with TIMP-2 and pro-MMP-2 [157]. Interestingly, the TIMP-1/pro-MMP-9 complex may obstruct pro-MMP-9 activation [41,158]; while the TIMP-2/pro-MMP-2 complex still allows for proteolytic activation and resulting proteolytic function [159]. These complexes have also been noted to retain MMP-inhibitory ability for MMPs aside from the complexed pro-form molecule [57,159,160]. Ward et al. suggested that active MMPs generally are likely to be able to outcompete the latent-MMP/TIMP interactions and in fact they demonstrated that active MMP-2 can effectively displace pro-MMP-9 from TIMP-1 [160]. As well, the zymogen/inhibitor complexes seem to increase the rate of proteolytic site inhibition perhaps because of appropriate positioning of the N-terminus of the TIMPs [1,161,162]. Intriguingly, Hayakawa et al., compared pro-MMP/TIMP complexes to complexed TIMP-1 or -2 with MMP-1 in its active form, which displayed no further MMP-inhibitory activity; they found that either form of MMP/TIMP complex had

decreased ability to promote cell growth [57]. Hence, TIMP-1 signaling for cell growth may occur through an N-terminal binding site which is blocked by MMP binding. This does not exclude CD63 as a potential mediator of cell growth signaling since the mediation of the anti-apoptotic/EMT signaling that we've reported involved a more N-terminal region (perhaps H2 and H3) present in the T1D mutant that is not present in T1C. Thus, CD63 may bind through the C-terminal residues of TIMP-1 as we've shown here (FIG 3), but not have a strong enough interaction to sustain a signaling complex without this further binding to a region in the N-terminal domain. It remains to be tested whether receptor binding (CD63) may still occur after MMP binding or may be sterically blocked by MMP binding. In contrast to our observations, Chesler and colleagues noted cell growth activity/EPA of a TIMP-1 mutant lacking the c-terminus (Δ128); hence they propose that the growth promoting activity of TIMP-1 is not reliant on any C-terminal domain involvement [56].

CD63 and MT1-MMP complex

The second major interaction that has been the focus of our study is that between MT1-MMP and CD63; which was first reported by others [88]. We were able to observe the interaction between A-MT1 and CD63 by yeast-2-hybrid, however, the full-length latent form L-MT1 did not interact (FIG. 11A). Thus, an accessible or active catalytic site of MT1-MMP may be important to CD63 interaction. Further, by PCA analysis we examined the active-form, A-MT1; without the activation cleavage site (removal of pre- and pro- domains) we could append the GLucN fragment to Y^{112} for use in our PCA system. We observed consistent measurements confirming a real interaction between A-MT1 and CD63; furthermore, we noted that the

mutation of the catalytic glutamate (E240A) in EA-MT1 caused a substantial loss (to just 51% on average of A-MT1) of interaction strength for the complex (FIG. 11D).

We then undertook to map the interacting portions of each protein. For CD63, we used the same mutants constructed for analysis against TIMP-1 binding. However, for the SEL mutants we recorded slight increases or no change in binding for both A-MT1 and EA-MT1, and more surprisingly, for LEL deletion there was no significant negative effect for binding (with A-MT1/CD63 for comparison (i.e. 100%) we observed A-MT1 vs. 7AA, IIQ, TPGS and ΔLEL had percentages of 122, 121, 97, and 113, respectively; for EA-MT1 vs. 7AA, IIQ, TPGS and ΔLEL percentages were 69, 81, 67, and 48, respectively, as compared to the 51% noted in the paragraph previous) (FIG. 12A-B).

Interestingly, we saw comparable outcomes for examination of the hemopexin-like domain and the TM domain of MT1-MMP to the A-MT1 and EA-MT1 forms, respectively. The effects of mutation of the SEL or deletion of the LEL were very similar to those mentioned in the previous paragraph (FIG. 12E-F). Taken together, our results demonstrate that the catalytic mutation in EA-MT1 renders MT1-MMP less capable of binding CD63 by nearly half, and also the replacement of the TM domain with a GPI anchor removes nearly 50% of the binding capacity. This suggests two sites for MT1-MMP interaction with CD63 one in the catalytic domain and one in the TM domain. Meanwhile, the deletion of the hemopexin-like domain was not an interaction diminishing factor but enhanced CD63 binding; this is likely because it made the catalytic and/or TM domain more accessible to CD63 and this may be related to altered structural proximity of the MT1 domains and/or removal of other MT1 interactions which interfere with CD63 binding. Alternatively, the catalytic site may be less directly involved in

CD63 interaction, since neither extra-cellular loop of CD63 displayed any critical effect on MT1-MMP binding (FIG. 12). The catalytic domain may indirectly affect CD63 binding through overall structure or conformational changes effected by interference with the catalytic pocket. Since TIMP-1 and in particular CD63, were not shown to be proteolytically degraded by MT1-MMP, the enzyme/substrate relationship does not seem to apply (FIG. 15). Thus, further study is warranted to address the underlying cause of preferential interaction between active MT1-MMP and CD63 as compared to latent MT1-MMP or catalytically deficient forms.

CD63 structure

The 3D structure of CD63 is not known, but as was mentioned, it is proposed to have certain conserved features with other tetraspanins within the LEL and TM domains [134-137], and the crystal of the CD81 LEL dimer is helpful in predicting the CD63 LEL characteristics [132,133]. Many tetraspanin researchers have generated structural models for the 3D structure based on the CD81 LEL crystal and predictions for TM clustering and hence structural conformation. In most of these models the four TM domains are predicted to cluster together tightly in a bundle and this likely positions the LEL in such close proximity to the SEL as to hang over the top of the SEL so as to make it largely un-exposed [140-142]. Thus, the LEL is the most likely functional site in the extra-cellular part of CD63. Our interaction analysis with TIMP-1 indicated it to be interacting with the LEL of CD63; however, due to the size (99aa), the LEL domain was not amenable to site mutational analysis without indication of most probable binding sequences/sites –which information is not available as of yet. We did attempt to define the binding site by involvement of structure created by the three disulfide bonds. However, while

the cysteine mutational analyses confirmed the overall importance of the cysteines for protein folding/stability, we were not able to make rational refinement of predicted binding of TIMP-1.

Proposed relationship of TIMP-1/CD63 and MT1-MMP/CD63 complexes

We have studied the interactions for a TIMP-1/CD63 complex and an MT1-MMP/CD63 complex in human cells. It is necessary to state that our PCA system does not offer enough proof for the actual affinities of these molecules in complexed form to determine which relationship is stronger between the two complexes. We were able to investigate the ability of each complex to exist under increasing amounts (competition) of the third molecule. We found that both TIMP-1 and MT1-MMP are exclusive of each other in CD63 binding and can competitively remove the luciferase signal generated by a CD63/X complex, with the other protein being represented by X. Thus, it is unlikely that a mutual complex between all three molecules can exist for any length of time. Interestingly, the ability of a catalytic mutant MT1-MMP, EA-MT1, to compete with TIMP-1 was greatly diminished compared to active MT1-MMP, A-MT1. As well, the vice versa was true that TIMP-1 did not much affect the signal of the EA-MT1/CD63 complex; which was already at half strength of the A-MT1 interaction. Interestingly, TIMP-1 was also able to effectively compete with the ΔH-MT1/CD63 interaction, but did not strongly compete with the MT1-GPI/CD63 interaction (FIGs. 13-14). At the time of this writing, the complementary competition for ΔH-MT1 and MT1-GPI toward the TIMP-1/CD63 interaction was not completed and so will not be commented on. As mentioned above, we observed that replacement of the MT1-MMP TM domain with a GPI anchor, MT1-GPI, yielded about a 50% reduction in interaction with CD63. Taken together, we propose two potential MT1 sites for CD63 interaction, the TM and catalytic domains. Our results suggest that MT1-MMP is much less

effective at CD63 binding if either one of these interaction sites is withdrawn. As well, TIMP-1 is likely able to compete with the MT1-MMP/CD63 complex due to their differing binding sites; which allows for TIMP-1 to still obtain access to CD63 with an MT1-MMP complex and vice versa. An observation from our results is that TIMP-1 can compete perhaps sterically with the extra-cellular portion of MT1-MMP (the catalytic domain), but when that domain is mutated (EA-MT1/CD63) TIMP-1 does not compete well with the TM domain interaction.

4.2 Future directions

Some future considerations that could add upon this dissertation work will be mentioned here. The main value of this work relates to the novel binding site mapping that we have completed for TIMP-1 and CD63 and similarly for MT1-MMP and CD63.

The fact that these molecules interact is not disputed by the majority of researchers in the field. We have clarified the binding sites that contribute to their interaction and developed a system for examining their relationships in a dynamic setting, such as in live cells –both of these points are critical to understanding the role of the TIMP-1/CD63 complex and have been lacking up to this point. Also, the information from our study provides mechanistic insight into the functions of TIMP-1, CD63, and MT1-MMP; which offers new possibilities for drug design to disrupt the survival signaling and aggressiveness of tumor cells that utilize these valuable pathways.

Investigation of other TIMP-1 partners: compatible or incompatible to CD63 binding?

Our understanding of a TIMP-1/CD63 complex is much clearer after binding analysis by PCA. However, we have mentioned as well, the important relationship of TIMP-1 to MMP-9. They exhibit a strong interaction as enzyme/inhibitor, the initiation of which is most likely to

occur as an interaction with zymogen form of MMP-9 even before secretion and dependent on both C-termini of TIMP-1 and pro-MMP-9. [1,41,156,157,163,164]. Thus, it will be critical to the understanding of the mechanism and activities of the TIMP-1/CD63 complex to also understand the definitive binding region of TIMP-1 occupied by pro-MMP-9. Also, it would be valuable to conduct a PCA analysis of TIMP-1 and pro-MMP-9 in the presence or absence of CD63, and vice versa, to determine if these three molecules are cooperative or exclusive of each other. This could as well be done for integrin β1; though the compatibility of integrin β1 with TIMP-1/CD63 is known [77,78,87]. These studies would address remaining actions of TIMP-1 complexes relating to non-MMP-inhibitory function, and they would reveal the role of MMP-9 and integrin β1 in regulating the TIMP-1/CD63 complex and thus the considerations that should be made for therapeutic design.

As well our studies have been simplistic with regard to cell type, done within the cellular environment of HEK293FT cells which may not express high levels of non-transfected molecules. Though we have proven that CD63 and TIMP-1 readily form a complex in HEK293FT cells, further PCA analysis in other cell types (such as epithelial or cancerous cells) would offer assessments of TIMP-1/CD63 complex in perhaps more therapeutically pertinent cellular backgrounds.

Some final thoughts on targeting TIMP-1 for therapy

Increased TIMP-1 expression is frequently concomitant with MMP increase and/or a result thereof. Based on the mounting evidence of the pleiotropic functions of TIMP-1 and its involvement in multiple signaling mechanisms it is reasonable to view TIMP-1 as an important molecule in tumor progression, which may have increased expression because of its pro-

oncogenic functions. Recently, TIMP-1 has also been found to participate in chemoresistance and has been rather uniquely indicated as a predictive marker of poor chemotherapeutic response [27-32]. In 2010, Gilbert and Hemann published a report of a chemoresistance niche based on coordinated mechanisms through IL-6 and TIMP-1. They found that these factors acted on Burkitt's lymphomas in mice, in a paracrine manner, to aid in the survival of cells after genotoxic chemotherapy (doxorubicin). B lymphoma cells survived chemotherapy only in small amounts except in thymus tissues where there was 6.5-fold higher survival of lymphoma cells relative to lymph nodes. Among cytokines that were upregulated in thymic lymphomas only two were confirmed to have pro-survival effects on the lymphoma cells in vitro as well, IL-6 and TIMP-1 [29]. The authors went into further detail on IL-6, but the implications of their findings for TIMP-1 are convincing toward a chemoresistance property in addition to TIMP-1's intrinsic pro-survival properties.

So, what are the potential therapeutic options for manipulation of TIMP-1 signaling? This is an important question considering the practical dead-end of synthetic MMP-targeting inhibitors in cancer [165,166]. Perhaps TIMP-1's MMP-inhibitory function can be left intact while modulating its other functions. This could be done using a blocking antibody directed to the C-terminal domain or Helix 2 and 3 of TIMP-1. Another means of modulation might be through synthetic peptides that resemble TIMP-1's C-terminus or other sites; as drug design toward the MMP-inhibitory site has proven difficult for including correct specificity. As we have shown, a synthetic peptide would likely need to involve more than the unimproved amino acid string resembling the most C-terminal residues of TIMP-1. Approaching TIMP-1 in these ways

might not only disrupt TIMP-1's pathologic signaling and receptor (CD63) binding, but may potentially increase TIMP-1's association with MMPs upon release from CD63.

Targeting TIMP-1 through its C-terminus has appeal due to the divergence of the C-terminal sequence of TIMP-1 as compared to the other 3 TIMPs. In an effort to examine the conservation of the C-terminal residues of TIMP-1 we have used BioEdit sequence alignment software to compare the TIMP-1 C-terminal residues across vertebrate species and also contrast human TIMP-1 with human TIMP-2,-3, & -4 (Table 2) –examining all of these sequences from the last sequential cysteine to the end of the protein sequence. The last 9aa of human TIMP-1, WQSLRSQIA, are highly conserved across vertebrate species. Meanwhile, the C-terminal sequence of the other TIMPs is generally conserved between TIMP-2 thru -4 but is quite dissimilar to the sequence of TIMP-1. For further information regarding the evolution of TIMP-1 (and the invertebrate TIMP sequence from Drosophila –which is not highly conserved with TIMP-1) see these papers [167-169]. Taken together, the C-terminus of TIMP-1 is a region that may provide unique targeting of TIMP-1 and not be amenable with the other TIMPs.

Another option for therapeutic intervention of TIMP-1 signaling is via manipulation of CD63 expression/functionality. CD63 is already established among melanoma researchers as a tumor-associated antigen with high potential for use as a biomarker/prognostic molecule [170,171]. Naturally, the concerted increase of CD63 to very high expression levels in the very early stages (pre-neoplastic) of melanoma is appealing as a potentially distinct cancer-progression associated event. Thus, as a tumor antigen, CD63 could be potentially exploited for enhanced immune recognition or response to tumor development. We and others observed very high CD63 expression in progressing breast carcinoma [145] and tumor xenografts (FIG. 16);

thus, targeting CD63 may disrupt TIMP-1 signaling in cancer while avoiding alteration of the proteolysis regulation by TIMP-1.

	TABLE 2. Comparative Analysis of TIMP-1's C-terminus	
	C-terminal Sequence	Sequence Conservation
TIMP-1 SPECIES COMPARISON	TIMP-1 Human CTWQSLRSQIA Cow CTWQSLRAQMA Dog CTWQSLRPRMA Horse CTWQSLRPRTA Mouse CTWRSLGAR Pig CTWQSLRPRVA Rabbit CAWQSLRPRKD Rat CTWQYLGVSMTRSLPLAKAEA Sheep CTWQSLRPRGA	
TIMP FAMILY	Human TIMP-1 CTWQSLRSQIA TIMP-2 CAWYRGAAPPKQEFLDIEDP TIMP-3 CSWYRGWAPPDKSIINATDP TIMP-4 CSWYRGHLPLRKEFVDIVQP	

4.3 Conclusions

In conclusion, the significance of this work is that it pin-points the C-terminus of TIMP-1, and CD63 interaction, as a novel potential therapeutic target. Hence, TIMP-1's anti-tumor effect of MMP inhibition may be preserved, and pro-tumor effects mediated by CD63 interaction may be disrupted. As well, we propose that TIMP-1 is a regulator of a variety of cellular processes including protease-inhibition/modulation, and importantly, cell communication/signal transduction as major parts of its roles in normal and pathologic biology. The pleiotropic activities of TIMP-1 (noted above) include positive regulation of MT1-MMP, and competition with MT1-MMP/CD63 interaction. This represents a paradigm shift for TIMP-1 (a broad MMP inhibitor) to be mediating MT1-MMP activation thru its C-terminal function. We propose that

much of TIMP-1 signaling is dependent on the tetraspanin, CD63, via the interaction we described herein and is also dependent on the amount of TIMP-1 expressed (increasing in pathology) in addition to cellular and microenvironmental cues. Taken together, the non-MMP-inhibitory roles of TIMP-1 mediated by CD63 interaction are very appealing for therapeutic intervention. Thus, the benefit of therapeutically targeting TIMP-1 in disease is becoming more appealing and continued commitment to this effort is logically warranted.

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ABSTRACT

ANALYSIS OF THE STRUCTURE AND FUNCTION OF A TIMP-1/CD63 COMPLEX AND ITS RELATIONSHIP TO AN MT1-MMP/CD63 COMPLEX

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The relationship of the extra-cellular matrix (ECM) and secreted proteins to intra-cellular functions has an important impact on disease progression. The tissue inhibitors of metalloproteinases (TIMPs), of which there are four, are known for their broad metalloprotease inhibitory abilities. TIMP-1 has been widely studied as an inhibitor of matrix metalloproteinases (MMPs) and therefore as a molecule that prevents ECM degradation and proteolytic cleavage of extra-cellular molecules, processes thought to be critical for tumor invasion and metastasis. However, TIMP-1 has shown in the clinic to have increased expression in cases of disease progression; especially, it is associated with poor prognosis in many types of cancer. Hence, there may be oncogenic functions of TIMP-1 that drives cancer progression. We have been studying non-MMP-inhibitory functions of TIMP-1 to better understand TIMP-1 as a pleiotropic molecule and its role in disease development. In this area, we and others have shown important signaling cues mediated by TIMP-1 including anti-apoptotic signaling and epithelial-to-mesenchymal transition (EMT) induction; which may play a role in cancer progression. Our current research has included direct protein-protein interaction analysis using protein

complementation assay (PCA) of the interaction of TIMP-1 and CD63. CD63 is a tetraspanin, whose major functional properties are not well understood. We have previously identified CD63 as a non-MMP protein and a transmembrane (TM) protein with which TIMP-1 has direct interaction, resulting in intra-cellular signal transduction for cell survival and EMT. We have characterized the interaction between TIMP-1 and CD63 including identification of their respective binding sites. Importantly, we have also demonstrated a robust interaction for TIMP-1 and CD63 at the cell surface in live cells.

In addition, we have utilized PCA methodology in studying the relationship of CD63 with an important disease-related MMP, namely membrane type-1-MMP (MT1-MMP). This MMP is known to be upregulated in cancer progression and has been shown to have interaction with CD63 in previous studies. As we did for TIMP-1, we have mapped the interacting sites of MT1-MMP and CD63. As well, to better understand the relationship of TIMP-1 and MT1-MMP to the binding of each other to CD63 we have studied these three proteins together. We observe that TIMP-1 and MT1-MMP are not compatible in CD63 binding but they compete one with another for interaction with CD63. Our study presents a paradigm shift in our understanding of TIMP-1 functions; we show that the C-terminal domain of TIMP-1, independent of its MMP-inhibitory domain, interacts with CD63 and induces intracellular signal transduction. Importantly, these interactions compete for MT1-MMP binding with CD63, known to mediate endocytosis and degradation of MT1-MMP. Thus, TIMP-1 interaction with CD63 results in MT1-MMP stabilization/accumulation on the cell surface, leading to activation of the MT1-MMP/MMP-2 proteolytic cascade. Our finding provides valuable information for the design of

therapeutic intervention of TIMP-1's oncogenic activity while preserving its tumor suppressive MMP-inhibitory functions.

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