UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

Sección Departamental de Bioquímica y Biología Molecular I

TESIS DOCTORAL

Búsquedas y caracterización de nuevas asociaciones moleculares de las colas citosólicas de las MT-MMPs

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Directora

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Madrid, 2012

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Memoria presentada para optar al grado de doctor europeo por:

María Victoria Hernández de Riquer

Bajo la dirección de la doctora:

Alicia García Arroyo

Madrid, 2012


A mi abuela
20·5·2001
RIQUER.

Agradecimientos

Una de las cosas buenas de llegar a la lectura de tu tesis doctoral es la oportunidad de poder agradecer públicamente a todas las personas su ayuda para esta consecución.
Considero la vida como algo lineal y, aunque esté dividida en etapas, los productos que se recogen en una de ellas no sólo provienen de lo que se ha sembrado en esa etapa si no que también se recogen frutos de etapas anteriores, tanto buenos como malos. Por esto mi agradecimiento abarca más que esta etapa de doctorado.

Así quisiera comenzar por mis abuelos. He tenido la suerte de conocer directamente a dos de ellos: a Ricardo y a Magdalena. Os agradezco enormemente vuestra vitalidad, las ganas de vivir y de disfrutar de la vida así como vuestra curiosidad por la ciencia y los inventos y sobre todo por vuestro amor.
A mis otros dos abuelos, Mariano y Rufina, no he tenido la suerte de conocerlos personalmente pero sí a través de sus hermanos, hijos, objetos… Os agradezco vuestro esfuerzo en una época dura y la educación y cariño que trasmitisteis a vuestros hijos que sigue patente y les acompaña en todo momento. Espero saber transmitir todas las cosas buenas que he aprendido de mis abuelos que no son pocas.
Mi agradecimiento es especialmente grande a mis padres. Todo lo que pueda conseguir en la vida es gracias a vosotros. Vosotros sois mi red, aquella red que está bajo los trapecistas, invisible en los buenos momentos y esencial en los malos. Con vuestro amor discreto he creído a veces que era yo quien conseguía las cosas, olvidándome que sois el fundamento de mi vida y que gracias a vosotros soy lo que soy.
Pablo, ¡Que ya he acabado la tesis! Te quiero mucho. Aunque estés en la distancia te tenemos presente y te sentimos a nuestro lado. Gracias también a Adriana, mi mejor cuñada, por tu forma de ser, tu trabajo y esfuerzo, tu gran conversación y por lo bien que le sienta a mi hermano estar contigo.
Tengo la suerte de que mi red se prolonga más allá. Quiero dar un agradecimiento especial a mis tías. A parte de agradeceros el poder disfrutar de piezas únicas en mi armario, os quiero agradecer vuestras enseñanzas, apoyo y gran cariño.
También quiero agradecer su apoyo a mis tíos y primos, particularmente a mi tía Esther. Tu actitud optimista y alegre es un ejemplo para mí.

De mi etapa "profesional" quiero dar las gracias en primer lugar a mi primer profe, a Juan. Allí en la escuela infantil Sueños aprendí a hacer palitos, a contar, a buscar diferencias… y todas esas típicas cosas que se siguen practicando durante el doctorado. Pocas etapas son tan importantes como esta. Tu dedicación y enseñanzas conforman la parte de mí que tengo automatizada, interiorizada y que parece que son cosas que naces sabiendo, pero no es así, alguien te las ha enseñado.

En el colegio tuve algunos profesores de los de verdad. A vosotros os quiero agradecer el vivir vuestra profesión, contagiar vuestro entusiasmo y prepararme un poco la cabeza para afrontar "la vida".
En el colegio también conocí a mis amigas: Daniela, Dedes, Teresa e Irene. Después han venido otros, pero los primeros son los primeros. Me conocéis a fondo y aún así después de tantos años no sólo me aguantáis si no que me queréis. Vuestro cariño lo llevo conmigo allá donde esté. Daniela, tu amistad es esencial en mi vida.

En la facultad conocí a mis famosas amigas de la facultad. Pues que os voy a contar. Os agradezco tantas cosas… Aquellas prácticas que se convertían en aventuras jamás vistas en las que cada una aportaba su toque, excursiones en las que las ovejas (transformadas en avutardas) levantaban el vuelo, trabajos… Elvira, Marta, Conchi, Tere, Mer, Ali, Diana, Sandra, Olga muchas gracias por todos esos buenos momentos y ¡por los que nos quedan!

La doctora García de Paco necesita un agradecimiento especial. Mi querida Elvira, me has ayudado tantas veces… Te agradezco mucho tu amistad y el ayudarme a conocerme mejor cada vez que escucho tus historias. Además eso de parecemos tanto y quererte mucho me ayuda a ser más beligerante conmigo misma.

No todo el mundo tiene la suerte de tener una amiga en el laboratorio desde que empieza la tesis. Marta, te agradezco muchísimo tu apoyo y ayuda en los momentos difíciles de esta tesis.

Desde aquí Conchi te mando un beso y te agradezco tu simpatía y alegría sin par ;) y otro para Tere no se nos ponga celosa.

Muchas gracias Eduardo por toda tu paciencia, apoyo, amor y complicidad. Gracias por haber tenido tu consuelo demasiadas veces durante esta tesis. Gracias por todo lo que hemos compartido juntos. Gracias por hacerme mejor persona.

También quiero dar las gracias a la familia de Eduardo. Ana, tus coloridos tapetitos de ganchillo tienen un sitio preferente en mi cuarto. Además de decorativos, me hablan de tu alegría y de tu ilusión por la vida. A Flora, Ángel, Alberto y María, gracias por acogerme y por hacerme sentir tan a gusto entre vosotros. Gracias también por muchas de las virtudes de Eduardo de las que disfruto y que se las habéis inculcado vosotros.

Quisiera agradecer también todo lo que he recibido de los amigos de Edu que, aunque no se lo he consultado a ellos, para mí son también mis amigos. Os agradezco todos los momentos que he pasado con vosotros, vuestra alegría e ilusión son contagiosas. Juan (el primero ¿eh?), Cristina, Pascu, Paula, Chema, Chivite, Miguel…

De mi etapa de doctorado quería dar las gracias a Alicia G. Arroyo por su dirección estrecha de esta tesis y por haberme animado a realizar una estancia en el extranjero.

También quiero dar las gracias a Juan Miguel Redondo por su interés en mi proyecto y sus sugerencias en mis seminarios.

A Jesús Pérez Gil, mi tutor de tesis. Lo primero que te quiero agradecer es que me escuchases. Sentirme escuchada fue importantísimo para mí. Además te agradezco tu apoyo. Muchísimas gracias.

Lo que me llevo de estos años son todas las vivencias con mis compañeros. Habéis sido lo mejor de esta etapa. Muchísimas gracias por todo lo que me habéis enseñado y por todo lo que hemos compartido. A mis compañeros de pupitre, Aga y Rubén -dianas predilectas de todas mis preguntas- a Vanessa -que me ha ayudado en este proyecto y sobretodo me ha escuchado y animado- y al resto del equipo Ángela, Pilar, Cristina, Mara y Laura. También quisiera agradecer a Alba, Nando y a Laura Genís -iniciadora del proyecto "de las ERMs"- todos los momentos compartidos. A los "Miguelángeles" antiguos y actuales, os agradezco mucho todo vuestra ayuda, consejos, material, cañas… Gracias también a Enara y Patricia, compañeras en el inicio de esta tesis.

También quería dar las gracias a Antonio Rodríguez. Recuerdo cómo te pusiste conmigo a analizar detenidamente qué podría estar fallando en el clonaje de los plásmidos. Muchas gracias por tu ayuda generosa.

Quisiera agradecer la ayuda de las unidades del CNIC que han participado en este proyecto. Especialmente a Sergio Callejas, de la Unidad de Genómica, por haberme enseñado el mundillo de la PCR cuantitativa con gran paciencia y disponibilidad. También quisiera dar las gracias a Enrique Clavo, de la Unidad de Proteómica, por su participación en este proyecto. Gracias a Elvira y a Antonio, de la Unidad de Microscopía, y a José Manuel Ligos y Mariano, de la Unidad de Citometría. También quería darte las gracias Raquel por escucharme y apoyarme, muchas gracias.

Gracias también a Esther, María y Eduardo por vuestra ayuda durante estos años.

Gracias Charo por tu alegría.

I would like to thank Pascale Zimmermann and Ilva Ivarsson (Belgium) and Matthew P. Hoffman and Ivan T. Rebustini (USA) for their collaboration in this project.

My short stay in Johanna Ivaska´s laboratory (Finland) has been one of the best things of this period. Johanna, I deeply thank you for giving me the opportunity to have such a great experience and for your collaboration in this project. I also thank you for all your help and for letting me to take part in your group from the scientific and social point of view. I am also very grateful to all members of your lab and especially to Saara Tuomi for "taking care" of me, for her collaboration in this project and for being so positive and cheerful. Kiitos!!
In Finland I also had the luck to make new friends. Thank you Henri for your support and help in the editing of this thesis. Gracias.

También quería dar las gracias a Maite García. Gracias por tus consejos y apoyo. Además, quiero agradecerte tu empuje para completar mi formación e introducirme en mundo de la Nutrición y Alimentación, lo que me ha permitido ver "más allá" personal y profesionalmente.

Finalmente me gustaría dar las gracias a Javier Gallego con quien empecé a trabajar en la investigación. Te agradezco muchísimo tu ayuda en aquella etapa y el seguir encontrando también tu puerta abierta durante el desarrollo de mi tesis. Me siento muy afortunada.

¡¡Muchísimas gracias a todos!!

Era un científico que estaba haciendo un experimento con una pulga.
Le dijo a la pulga: ¡Salta pulga! y la pulga saltó un metro.
Le quitó una pata y le dijo lo mismo, entonces saltó medio metro,
y así le fue quitando todas las patas hasta que no tenía patas
y lo anotó en su cuaderno.
Cuando ya no tenía patas le dijo que saltara y no saltó entonces anotó:
Pulga sin patas se queda sorda.

Anónimo

SUMMARY

Matrix metalloproteinases (MMPs) are involved in a variety of biological processes by their ability of remodelling the extracellular microenvironment, modulating the activity of transmembrane receptors and regulating signaling cascades. Membrane type-MMPs (MT1-, MT2-, MT3- and MT5-MMP) mainly act in pericellular proteolysis but they could also connect to intracellular events through their interaction with cytosolic proteins. We hypothesize that binding of MT-MMP cysolic tails to distinct partners can contribute to their specific functions. In this work, we have identified novel binding proteins of the MT-MMP cytosolic tails, characterized the residues involved in these interactions and explored their possible biological functions. The tyrosine present in the middle region of MT1-MMP cytosolic tail has been shown to be necessary for p130Cas binding and this binding participates in the promotion of myeloid progenitor migration and fusion. MT1-, MT2- and MT3-MMP have been shown to bind the ERM protein moesin; juxtamembrane and middle polybasic regions of MT1-MMP cytosolic tail act as key binding domains for this interaction that may be regulating MT1-MMP localization at the plasma membrane. Finally, ZO-1 has been identified as a differentially associated protein of MT-MMPs; MT2-, MT3- and MT5-MMP are able to bind ZO-1 in contrast to MT1-MMP. Essential residues in the PDZ binding motif responsible for this different association pattern have also been identified. We have also observed that MT2-MMP colocalizes with ZO-1 in MT2-MMP stably transfected MDCK cells and that MT2-MMP expression affects F-actin polarization in these epithelial cells. In summary, this work identifies three actin-linker proteins as new molecular partners differentially associated with MT-MMPs; this differential association may contribute to MT-MMP specific functions in diverse pathophysiological scenarios.

RESUMEN

Las metaloproteinasas de matriz (MMPs) están implicadas en varios procesos biológicos por su capacidad de remodelar el microambiente extracelular, modular la actividad de los receptores transmembrana y regular las cascadas de señalización celular. Las MMPs de membrana (MT1-, MT2-, MT3- y MT5-MMP) actúan principalmente en la proteolisis pericelular pero pueden además participar en los eventos intracelulares a través de su interacción con proteínas citosólicas. Nuestra hipótesis es que la interacción de las colas citosólicas de las MT-MMP a distintas proteínas puede contribuir a sus funciones específicas. En este trabajo hemos identificado nuevas proteínas que interaccionan con las colas citosólicas de las MT-MMPs, caracterizándose los residuos implicados en estas interacciones y explorándose sus posibles funciones biológicas. Se ha demostrado que la tirosina presente en la región central de la cola citosólica de MT1-MMP es necesaria para la unión de p130Cas y esta unión participa en la migración y fusión de los progenitores mieloides. Se ha detectado la unión de MT1-, MT2- y MT3-MMP a la proteína ERM moesina; las regiones polibásicas yuxtamembranal y central de la cola citosólica de MT1-MMP actúan como dominios clave y pueden regular la localización de MT1-MMP en la membrana plasmática. Finalmente, ZO-1 ha sido identificada como una proteína diferencialmente asociada a las MT-MMPs; MT2-, MT3- y MT5-MMP son capaces de interaccionar con ZO-1 al contrario que MT1-MMP. Los residuos en el motivo de unión a dominios PDZ responsables de este diferente patrón de asociación han sido también identificados. Además, hemos observado que MT2-MMP colocaliza con ZO-1 en células MDCK transfectadas establemente con MT2-MMP y que la expresión de MT2-MMP afecta la polarización de F-actina en estas células epiteliales. En resumen, en este trabajo se han identificado tres proteínas de unión a actina como nuevas proteínas asociadas diferencialmente a las MT-MMPs; esta asociación diferencial puede contribuir a las funciones específicas de las MT-MMP en distintos escenarios fisiopatológicos.

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Abbreviations

Aa:	Amino acid
Ab:	Antibody
Abl kinase:	Abelson leukemia virus kinase
ABP:	AMPA receptor Binding Protein
ADAM:	A Disintegrin and Metalloproteinase Domain
ADAMTS:	ADAM with a Thrombospondin Motif
AMPA:	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid
AP:	Adaptor Protein
APS:	Amonium Persulfate
BAEC:	Bovine Aortic Endothelial Cells
bFGF:	Basic Fibroblast Growth Factor
BHK cells:	Baby Hamster Kidney cells
BM:	Bone Marrow
BSA:	Bovine Serum Albumin
CCL2:	Chemokine (C-C motif) Ligand 2
CHO cells:	Chinese Hamster Ovary cells
Coll:	Collaborators
DMEM:	Dulbecco´s Modified Eagle Medium
DNA:	Deoxyribonucleic Acid
DTT:	Dithiothreitol
EBP50:	ERM-Binding Phosphoprotein 50
EC:	Endothelial Cell
ECL:	Enhanced Chemiluminescence
ECM:	Extracellular Matrix
EDTA:	Ethylenediamine Tetra-acetic Acid
EGF:	Epidermal Growth Factor
EGR-1:	Early Growth Response protein 1
ELISA:	Enzyme-Linked Immunosorbent Assay
EMMPRIN:	Extracellular Matrix Metalloproteinase Inducer
ER:	Endoplasmic Reticulum
ERK:	Extracellular signal-Regulated Kinase
ERM:	Ezrin, Radixin, Moesin
FAK:	Focal Adhesion Kinase
FBS:	Fetal Bovine Serum
FERM:	Four-point one, Ezrin, Radixin, Moesin
FGF:	Fibroblast Growth Factor
FITC:	Fluorescein Isothiocyanate
FL:	Full Length
gC1qR:	Globular C1q-binding protein
GFP:	Green Fluorescent Protein
GM-CSF:	Granulocyte-Macrophage Colony-Stimulating Factor
GPI:	Glycosylphosphatidylinositol
GRIP:	Glutamate Receptor Interaction Protein
GST:	Glutathione Sepharose-Transferase
HA:	Hemagglutinin
H&E:	Hematoxylin and Eosin
HMEC:	Human Microvascular Endothelial Cells
HRP:	Horse Radish Peroxidase
HSE:	Heat Shock Element
HUVEC:	Human Umbilical Vascular Endothelial Cell

ICAM: Intercellular Adhesion Molecule
Ig: Immunoglobulin
IL: Interleukin

JAM: Junctional Adhesion Molecule

kD: Kilodalton
Ki app: Apparent inhibition constant

LC-MS: Liquid Chromatography-Mass Spectrometry

MAGUK: Membrane Associated Guanylate Kinase
M-CSF: Macrophage Colony-Stimulating Factor
MDCK cells: Madin-Darby Canine Kidney cells
MMPs: Matrix Metalloproteinases
MS: Mass Spectrometry
MTCBP-1: Membrane-type 1 matrix metalloproteinase Cytoplasmic tail Binding Protein-1
MT-MMPs: Membrane Type Matrix Metalloproteinases
(-MMP ending has been suppressed in some figures for clarity purposes)

NF-kB: Nuclear Factor kappa-light-chain-enhancer of activated B cells
NHE: Sodium-Hydrogen Exchanger
NHE-RF: Sodium-Hydrogen Exchanger Regulatory Cofactor
nNOS: Neuronal Nitric Oxide Synthase

OC: Osteoclast

PBS: Phosphate Buffer Saline
p130Cas: p130 Crk-associated substrate
PCR: Polymerase Chain Reaction
PDGF: Platelet-Derived Growth Factor
PDZ: PDZ derives from the first three proteins in which these domains were identified:
- **PSD-95:** Post-Synaptic Density 95 kD protein
- **DLG:** *Drosophila melanogaster* Discs Large protein
- **ZO-1:** Zonula Occludens 1

PFA: Paraformaldehyde
PIP_2: Phosphatidylinositol bisphosphate
PKC: Protein Kinase C
PMSF: Phenylmethylsulfonylfluoride
PSGL-1: P-selectin glycoprotein ligand 1

RANKL: Receptor Activator of NF-jB Ligand
RNA: Ribonucleic Acid
Rpm: Revolutions per minute
RT: Room Temperature
RU: Response Units

SBD: Src-binding domain
SD: Substrate domain
SDF1: Stromal-cell Derived Factor-1
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM: Standard Error of the Mean
SF/HGF: Scatter Factor/Hepatocyte Growth Factor
S1P: Sphingosine-1-Phosphate
SPR: Surface Plasmon Resonance

SRR:	Serine-Rich Region
TBS:	Tris-Buffered Saline
TEMED:	Tetramethyl Ethylenediamine
TGFβ:	Tumor Growth Factor β
TIMPs:	Tissue Inhibitors of Metalloproteinases
TNFα:	Tumor Necrosis Factor α
TRAP:	Tartrate-Resistant Acid Phosphatase
VCAM:	Vascular Cell Adhesion Molecule
VEGF:	Vascular Endothelial Growth Factor
WB:	Western Blot
WT:	Wild Type

Introduction

1. The matrix metalloproteinases (MMPs)

The matrix metalloproteinases or matrixins (MMPs) are zinc dependent endopeptidases that belong to the metzincin superfamily (Nagase and Woessner, 1999). This superfamily is also formed of the following families: disintegrin and metalloproteinases (ADAMs) (White, 2003), the ADAMs with a thrombospondin motif (ADAMTS) (Tang, 2001), the bacterial serralysins (Nakahama *et al*., 1986) and other proteinases such as the astacins (including the meprins) (Bode *et al*., 1992).

The MMP family is composed of 23 members in humans. The first member of the matrix metalloproteinase family was identified in 1962 by Gross and Lapiere, who found that tadpole tails during metamorphosis contained an enzyme that could degrade fibrillar collagen (Fig. 1) (Gross and Lapiere, 1962). Subsequently, an interstitial collagenase, collagenase-1 or MMP-1, was found in diseased skin and synovium. MMPs have been since identified as the major enzymes responsible for turnover of extracellular matrix (ECM) (Birkedal-Hansen *et al*., 1993).

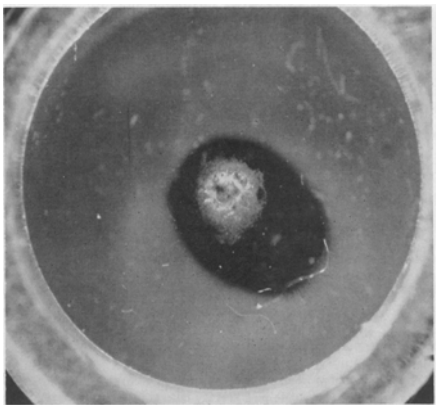


Figure 1: Photograph of culture of tadpole fin skin illustrating area of lysis around the explant. Remaining collagen gel substrate is seen as surrounding opalescent region within ring (magnification 9 X) (Taken from Gross and Lapiere, 1962).

1.1. MMP structure and classification

All MMPs share three main domains: a signal peptide, a propeptide and a catalytic domain. Additionally, some groups of MMPs can also have at their C-terminus the following domains: a hinge region, an hemopexin-like domain, a glycosylphosphatidylinositol (GPI) modification, a transmembrane domain or a cytosolic tail (Fig. 2).

Based on the presence or absence of membrane anchoring domains, the MMP family members can be divided in two groups: **secreted MMPs**, which do not present membrane anchoring, and **Membrane-Type MMPs (MT-MMPs)**, which are anchored to the membrane.

These MT-MMPs can further be subdivided in two groups:

1. **GPI-anchored MT-MMPs**: which bind to the membrane through a GPI modification. These proteins include MT4-MMP (MMP-17) and MT6-MMP (MMP-25).

2. The **transmembrane MT-MMPs:** which are type I proteinases with a short cytosolic tail. This subfamily is formed of: MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16) and MT5-MMP (MMP-24).

   Another MT-MMP protein called MMP-23 has been described. This protein exists as a type II transmembrane protein (N-terminus inside the cell) or as a secreted form if it is cleaved (Velasco *et al*., 1999).

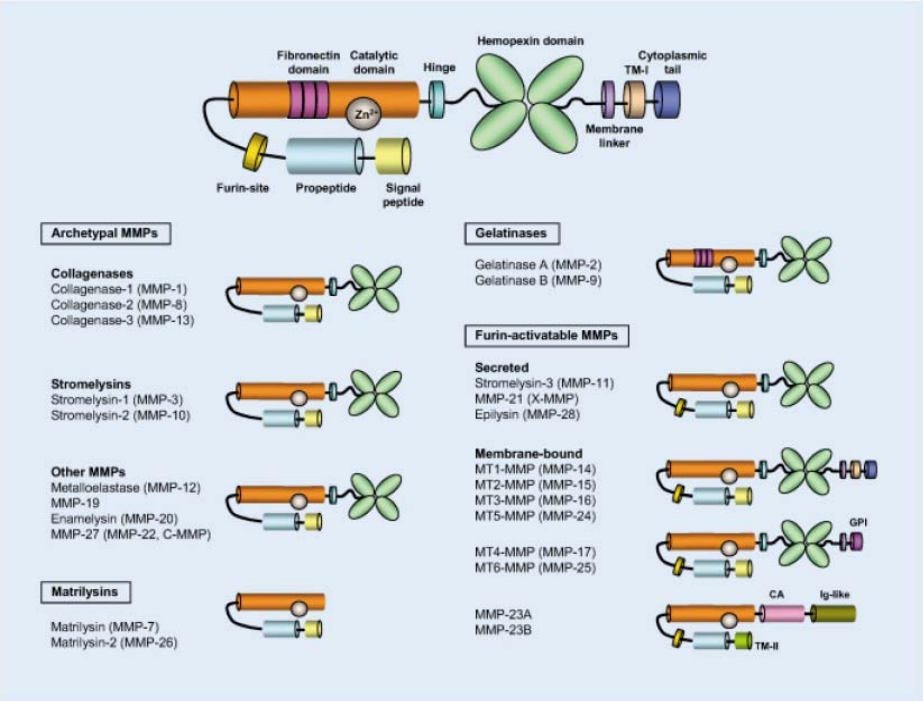


Figure 2: Structural MMP classification based on the organization of their domains (Taken from Folgueras *et al*., 2004).

As mentioned, mammalian MMPs share a conserved domain structure that consists of an autoinhibitory propeptide and a catalytic domain. The **propeptide** is formed of about 80 amino acids and contains a conserved cysteine residue that coordinates the active-site zinc to inhibit catalysis. When the propeptide is destabilized or removed, the active site becomes available to cleave substrates.

The **catalytic domain** comprises about 167-170 residues. Its active site is a 2 nm groove that runs across the catalytic domain. The zinc atom is located in this active site and it is bound to three histidine residues.

All MT-MMPs and some soluble ones have a **furin recognition motif** inserted between the propeptide and the catalytic domain. This motif contains the basic sequence Arginine108-Arginine109-Lysine110-Arginine111 that is recognized and cleaved by convertases like furin (Pei and Weiss, 1996). These convertases are calcium-dependent transmembrane serine proteinases of the subtilisin family and are prominently displayed in the trans Golgi network (Steiner *et al*., 1998). It is there, in the Golgi network, where the propeptide is released from the catalytic domain

by its cleavage between the Arginine111-Tyrosine112 (Rozanov *et al*., 2002a; Yana and Weiss, 2000).

Most MMP family members also contain an **hemopexin-like domain** attached at the C-terminus of their catalytic domain by a flexible **hinge region**. The hemopexin-like domain is formed of about 200 residues and encodes a four-bladed β-propeller structure that resembles hemopexin (an heme binding serum protein). This domain has been described to have a role in many functions: matrix binding, substrate specificity recognition, protein-protein interactions, activation of the enzyme, proteinase localization, internalization and degradation (Page-McCaw *et al*., 2007). For example, the hemopexin-like domain plays a functional role in MMP binding to tissue inhibitors of metalloproteinases (TIMPs), a family of specific MMP protein inhibitors (Borden and Heller, 1997). In the case of MT-MMPs it has been observed that hemopexin domain of MT1-MMP affects its collagenolytic activity by its binding to tetraspanin CD151 (Yanez-Mo *et al*., 2008) and moreover, it is essential for MT1-MMP induced cell migration (Cao *et al.*, 2004).

After the hemopexin repeats, which usually mark the end of the protein in most MMPs, all MT-MMPs contain a C-terminal hydrophobic extension rich in hydrophobic residues involved in the attachment of these proteinases to the cell surface (Cao *et al*., 1995). As mentioned, MT4- and MT6-MMP have a **GPI modification**, a short hydrophobic domain that binds to the membrane in a non-permanent manner through a GPI bridge (Itoh *et al*., 1999; Kojima *et al*., 2000).

By contrast, MT1-, MT2-, MT3- and MT5-MMP contain a long stretch of hydrophobic residues that are predicted to act as a **transmembrane anchor** in all of them. This sequence is followed by a **cytosolic tail** whose functional importance in different contexts has been addressed by several independent studies (Jiang *et al*., 2001; Lohi *et al*., 2000; Uekita *et al*., 2001). The aim of this work is to analyze the interactions of the cytoplasmic domain of the MT-MMPs with different proteins. The details of this domain will be explained later in the introduction.

1.2. MMP regulation

MMPs are regulated at different levels: gene expression, posttranslational modifications, proenzyme activation, inhibition, complex formation and compartmentalization.

Induction of MMP **gene expression** is controlled by different growth factors and cytokines, and may be suppressed by transforming growth factor β and glucocorticoids (Nagase and Woessner, 1999). Moreover, an important modulatory role of epigenetic processes in the expression of MMPs has been described (Chernov *et al*., 2009). Besides soluble factors, MMP expression may also be regulated by cell–cell contact or interaction of cells with ECM components such as extracellular matrix metalloproteinase inducer (EMMPRIN) (Biswas *et al*., 1995).

MMPs can also have **postranslational modifications** than can regulate their activity. MT1-MMP can be palmitoylated and phosphorylated at different sites and that these modifications can

influence MT1-MMP internalization or cell migration (Anilkumar *et al*., 2005; Moss *et al*., 2009; Nyalendo *et al*., 2007). This point will be explained in more detail later in the introduction.

The expressed MMPs are secreted as inactive proenzymes with the propeptide effectively limiting entrance into and catalysis of a substrate in the catalytic pocket by blocking the catalytic zinc ion via the cysteine switch mechanism. **ProMMP activation** can occur through several mechanisms, all of which lead to disruption of the cysteine switch. For MT-MMPs, removal of the prodomain by convertases like furin has been described (Stawowy *et al*., 2005). Alternatively, the prodomain can be proteolytically removed by plasmin and other serine proteases, or even other MMPs (reviewed in Ra and Parks, 2007).

MMPs can also be regulated by specific inhibitors. An important family of MMP inhibitors is the **TIMPs**. Currently four TIMPs have been identified: TIMP-1, -2, -3 and -4. TIMP-1, -2 and -4 are secreted in soluble form, whereas TIMP-3 is associated with the ECM (Westermarck and Kahari, 1999). TIMPs act to inhibit MMP activity by forming complexes with them. MT-MMPs exhibit differential inhibition by members of TIMP family; transmembrane MT-MMPs are poorly inhibited by TIMP-1, but they are relatively well inhibited by TIMP-2, TIMP-3 and TIMP-4. On the other hand, the GPI anchored MT-MMPs are inhibited by TIMP-1 and TIMP-2 (Seiki, 1999). In addition, MMPs can be also inactivated by other proteins such as the **plasma membrane α2 macroglobulin** (Baker *et al*., 2002).

MMP **complex formation** constitutes another mechanism of MMP regulation. For example, proMMP-9 monomer is more rapidly activated by MMP-3 than proMMP-9 homodimer (Olson *et al*., 2000). Another example of MMP activity regulation by its homodimerization is MT1-MMP (Galvez *et al*., 2005). Moreover, complex formation of MMPs with other proteins can also regulate their activity. For instance, when proMMP-9 forms a dimer with collagenase, binding to TIMP-1 is prevented (Strongin *et al*., 1993).

Compartmentalization also regulates MMP activity by locating and concentrating them close to potential substrates. For example, intracellular association of MMP-1 with mitochondria and nucleus has implications for the control of cell growth, and may contribute to the association of this enzyme with tumor cell survival and spreading (Boire *et al*., 2005; Yang *et al*., 2009). Another example is the storage of MMPs in intracellular vesicles. Polymorphonuclear leukocytes and mast cells can store MMPs, as well as other proteinases, in exocytic vesicles and release them into the extracellular environment upon activation of the cells. Moreover, it has also been seen that endothelial cells, chondrocytes and various cancer cells can store MMPs in intracellular vesicles (Hadler-Olsen *et al*., 2011).

1.3. MMP functions

Historically, MMPs were thought to function mainly as enzymes that degrade structural components of the ECM. However, MMP proteolysis can have different functions: it can create space for cells to migrate, can produce specific substrate-cleavage fragments with independent biological activity, can regulate tissue architecture through effects on the ECM and intercellular

junctions, and can activate, deactivate or modify the activity of signaling molecules, both directly and indirectly (Fig. 3) (Sternlicht and Werb, 2001).

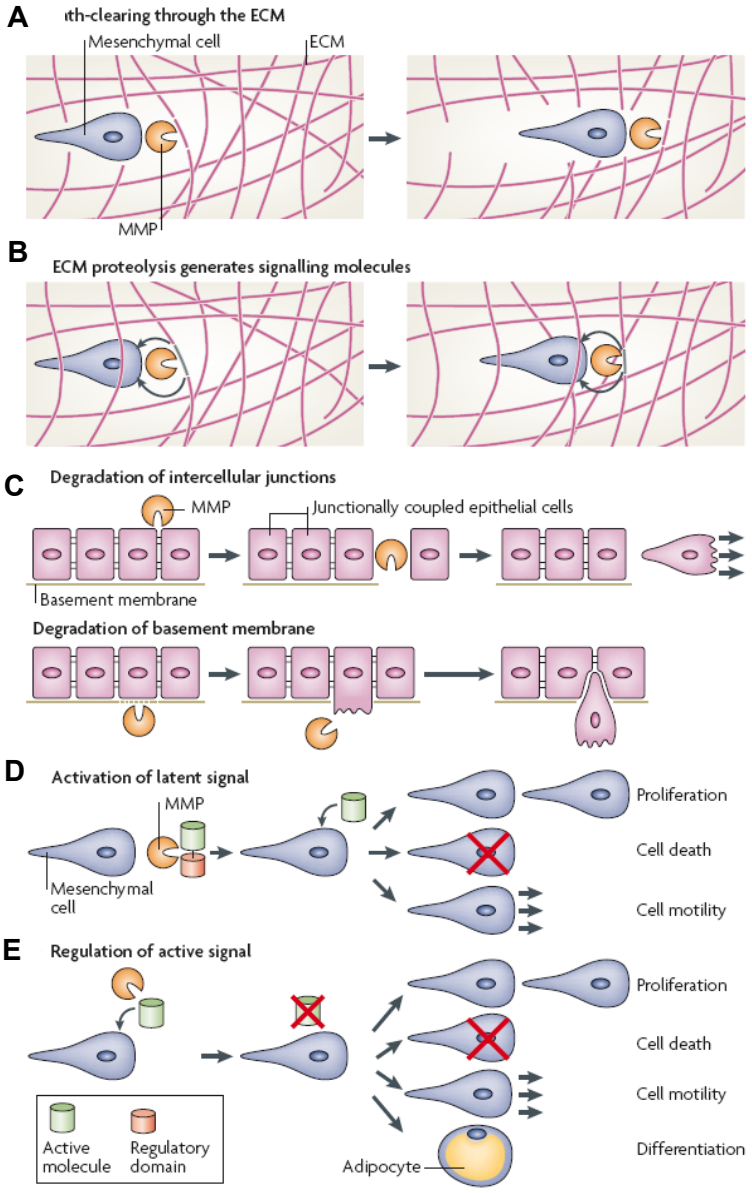


Figure 3: Possible modes of MMP action (Taken from Page-McCaw *et al.*, 2007). A) MMPs can cleave components of the ECM, resulting in increased space for cell or tissue movement. B) Alternatively, MMP proteolysis can generate specific cleavage products that then signal in an autocrine or paracrine manner (for example, cleavage of collagen IV α3 chain by MMP-9 yields tumstatin, an anti-angiogenic peptide that functions by binding to the αvβ3 integrin (Hamano *et al.,* 2003). C) MMPs can also directly regulate epithelial tissue architecture through cleavage of intercellular junctions or the basement membrane. D) MMPs can activate or modify the action of latent signaling molecules, resulting in diverse cellular consequences. For example, cleavage of vascular endothelial growth factor (VEGF) by MMPs changes angiogenic outcome by modifying the binding and diffusion properties of VEGF (Lee *et al*., 2005). E) MMPs can deactivate or modify the action of active signaling molecules, resulting in changes in proliferation, cell death, differentiation or cell motility. For example, MMP-2 cleavage of stromal-cell derived factor-1 (SDF1) results in its inactivation (McQuibban *et al.*, 2001).

This diverse MMP activity leads to different MMP functions in development, which can be dissected by the analysis of MMP knockout mice. Studies of single-MMP-mutant mice have shown that MMPs are not essential for embryonic development but have a key role in postdevelopmental remodelling. In this sense MMP have been seen to be involved in the development of the mammary gland, skeleton and vascular system, three prominent sites of postnatal tissue ECM remodelling (Page-McCaw *et al*., 2007).

MMP-2 and MMP-3 have been seen to be involved in **postnatal mammary development**. The epithelial ductal network of the mammary gland expands during puberty. This process requires degradation of the basement membrane and ECM, restructuring of endogenous vascular network and large-scale epithelial morphogenesis. It has been described that these two metalloproteinases are involved in branching morphogenesis in the mammary gland, participating in terminal end bud elongation and secondary branching (Wiseman *et al*., 2003).

Several MMP knockout mice present **bone remodelling** defects. Deletion of MMP-9 (Vu *et al*., 1998) or MMP-13 (Stickens *et al*., 2004) results in expansion of the zone of hypertrophic chondrocytes in the growth plate because of a failure of apoptosis, indicating that these MMPs are required for the transition from cartilage to bone at the growth plates of long bones. MT1-MMP also plays a role in skeletal development (Holmbeck *et al*., 1999; Zhou *et al*., 2000). Interestingly, human skeletal diseases have been seen to be associated with MMP-2, MMP-13 or MMP-20 loss-of-function MMP mutations (Kennedy *et al*., 2005; Kim *et al*., 2005; Martignetti *et al*., 2001).

MMP-9-mutant mice show defects in **angiogenesis** at the growth plates of long bones (Vu *et al*., 1998). Similarly, MT1-MMP mutant mice lack appropriate vascular invasion at secondary ossification centres. The reduced irrigation of these areas due to the defects in vascular development is also related with the mentioned bone remodelling defects of these mice (Zhou *et al*., 2000).

Moreover, the role of MMPs is not limited to developmental processes. Mouse-mutant experiments show that MMPs are also required to maintain homeostasis in response to environmental challenges, such as **infection**. In this regard it has been described that MMP-7 is involved in innate immunity. Studies with MMP-7 knockout mice showed that they were more easily infected with intestinal bacteria due to their inability to proteolytically activate an endogenous antibiotic peptide, pro-cryptdin (Wilson *et al*., 1999). **Inflammation** is another response that requires MMPs. For example, recruitment of inflammatory cells to alveoli following lung injury is defective in MMP-7 deficient mice due to impaired shedding release of chemokines. Finally, MMPs are related with **cancer**. Tumor cells are believed to use their pericellular degrading activity to spread to distant sites (John and Tuszynski, 2001). Moreover, MMPs also promote tumor angiogenesis (Deryugina *et al*., 2002). In humans high levels of MMPs in tumors often correlate with poor prognosis. However, the relationship between MMPs and disease is not simple and increased MMP activity can enhance tumor progression or can inhibit it (Coussens *et al*., 2002). For example, studies with mutant mice deficient in MMP-8 demonstrated the *in vivo* anti-tumor properties of this proteinase; the absence of MMP-8 strongly increased the incidence of skin tumors in these animals (Balbin *et al*., 2003).

2. The transmembrane MT-MMPs

Observations derived from analysis of the structure, exon–intron organization, and chromosomal localization of MT-MMP genes, suggest that MT-MMPs diverged early in the evolutionary history of MMPs. A view of the evolutionary history of MT-MMPs can be obtained from the analysis of the presence of these proteinases in diverse organisms. MT-MMPs have been identified in many mammalian species, as well as in birds and in fish (Kimura *et al*., 2001; Yang *et al*., 1996). From comparative studies it has been observed that there is a strikingly increased complexity of the MT-MMP system in vertebrates, both in the number of proteins belonging to the family and in the domain structure and anchoring systems used by these enzymes for membrane binding. Presumably, this increased complexity of MT-MMPs in vertebrates is necessary to deal with a series of vertebrate innovations including those derived from the occurrence of more complex cell–cell and cell–matrix interactions in vertebrates, or from the prominent development of complex immunological, neural, and vascular systems in these organisms (Zucker *et al*., 2003).

As explained previously, MT-MMP members can be anchored to the membrane though a GPI (MT4- and MT6-MMP) or by a transmembrane domain (MT1-, MT2-, MT3- and MT5-MMP). This second group is the focus of this work. In this section, general characteristics of each transmembrane metalloproteinase and the most important features of their cytosolic tails will be described.

2.1. MT1-MMP

2.1.1. General features

MT1-MMP was discovered in 1994 and is by far the best characterized MT-MMP. It is ubiquitously expressed. It is formed of 583 amino acids and it has a molecular weight of 65kDa (Sato *et al*., 1994).

MT1-MMP can be **regulated** by transcription factors, growth factors, cytokines, nitric oxide, cell-cell contact, ectodomain processing, ECM, compartmentalization and internalization (MT1-MMP internalization is explained in the MT1-MMP cytosolic tail section).

Haas and coll. have characterized the murine MT1-MMP gene promoter establishing that there are consensus binding sites for transcription factors including early growth response protein 1 (EGR-1), adaptor protein 4 (AP-4) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) but not stress response elements such as heat shock element (HSE) (Haas *et al*., 1999).

Other molecules, such as growth factors and cytokines, are also involved in the regulation of MT1-MMP (Foda *et al*., 1996; Lohi *et al*., 1996; Yang *et al*., 1996). These include interleukin 1β (IL1β), tumor necrosis factor α (TNFα), basic fibroblast growth factor (bFGF), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Rajavashisth *et al*., 1999; Tomita *et al*., 2000), scatter factor/hepatocyte growth factor (SF/HGF) (Wang and Keiser, 2000), chemokine C-C motif

ligand 2 (CCL2) and interleukin 8 (IL-8) (Galvez *et al*., 2005). Moreover, our group has also identified MT1-MMP as a key molecular effector of nitric oxide, a critical modulator of angiogenesis (Genis *et al*., 2007).

Cell-cell contact has also been reported to modulate MT1-MMP expression. MT1-MMP mRNA appears to decrease in cells following achievement of cell confluency (Tanaka *et al*., 1997). In this regard, it has been seen that transfection of E-cadherin cDNA into squamous carcinoma cells led to a decrease in MT1-MMP mRNA as well as decreased amounts of activated MMP-2 (Ara *et al*., 2000).

Proteolytic ectodomain processing of MT1-MMP on the cell surface also represents an important mechanism for regulating enzyme activity. Active MT1-MMP undergoes autocatalytic processing on the cell surface leading to the formation of an inactive 43/45 kD fragment remaining on the cell surface and the release of soluble catalytic domain fragments; both autocatalytic (18 kD) and non autocatalytic (56, 50, 31–35 kD) fragments have been identified (Toth *et al*., 2002).

MT1-MMP can be also regulated by the ECM. For example, it has been characterized that plating cells on fibronectin leads to MT1-MMP activation in fibrosarcoma cells without apparent change in MT1-MMP or TIMP-2 levels (Stanton *et al*., 1998).

MT1-MMP compartmentalization is also a relevant mechanism for its regulation. Compartmentalization can be dependent on MT1-MMP association with other proteins such as CD44, which leads MT1-MMP to the leading edge (Mori *et al*., 2002). Other proteins that regulate MT1-MMP are integrins (reviewed in Gonzalo *et al*., 2010b). MT1-MMP can be associated with αvβ3 integrin and caveolin-1 in EC membrane protrusions where MT1-MMP is active (Galvez *et al*., 2002; Galvez *et al*., 2004). Moreover, MT1-MMP can also associate with α3β1 integrin in tetraspanin(Tspan)-organized membrane microdomains (Yanez-Mo *et al*., 2009). Notably, the ternary complex constituted by MT1-MMP, α3β1integrin and Tspan CD151 at the junctions of human EC resulted in a reduced MT1-MMP proteolytic activity at these sites (Yanez-Mo *et al*., 2008).

The substrate specificity of MT1-MMP **activity** is well described in literature. This enzyme is capable of proteolytic degradation of type I, II and III collagen, and many other ECM components, such as fibronectin, vitronectin, tenascin, nidogen, aggrecan, fibrin, fibrinogen and laminin-5 (reviewed in Barbolina and Stack, 2008).

Apart from ECM components, MT1-MMP is capable of cleaving many membrane-anchored proteins such as E- and N-cadherin, integrins, CD44, receptor activator of NF-κB ligand (RANKL) and several cell surface proteoglycans and their receptors (Barbolina and Stack, 2008). MT1-MMP also cleaves α3 and α5, but not α2, integrin chains. Ratnikov and coll. proposed that MT1-MMP-mediated integrin processing in the same cell may be a general mechanism by which cells selectively regulate the functionality of integrins in promoting cell adhesion, migration, and focal adhesion kinase phosphorylation (Ratnikov *et al*., 2002).

Moreover, MT1-MMP can cleave other MMPs. MT1-MMP was originally identified as the extracellular proteinase responsible for activation of proMMP-2, and this process remains the best-described proteolytic function of the enzyme. In this process, one of the units of an MT1-MMP dimer forms a trimeric complex with proMMP-2 and TIMP-2 at the cell surface, leading to proteolytic removal of the propeptide of the proMMP-2 by the 'free' MT1-MMP unit (Cao *et al*., 1996). Besides proMMP-2, also proMMP-13 (Knauper *et al*., 2002) and proMMP-8 (Holopainen *et al*., 2003) have been identified as possible targets for activation through this mechanism.

Although the main proteolytic function of MT1-MMP lies in the cleavage of extracellular substrates, some studies indicate a role in intracellular proteolysis after incorporation and accumulation of active MT1-MMP in the centrosomal compartment, where it could contribute to development of mitotic spindle changes by degradation of pericentrin (Golubkov *et al*., 2005).

To define the *in vivo* **function** of MT1-MMP several groups have generated MT1-MMP deficient mice (Holmbeck *et al*., 1999; Sakamoto and Seiki, 2009; Zhou *et al*., 2000). These mice are the only lethal MMP-mutant mice due to their severe defects in skeletal development and angiogenesis that cause a marked deceleration of postnatal growth and death within 3–16 weeks.

The main defect of MT1-MMP-deficient mice is in skeletal development. MT1-MMP mutants are grossly defective in remodelling of connective tissue. Loss of an ECM-degrading enzyme would be expected to result in increased bone deposition; paradoxically, MT1-MMP mutants instead show secondary effects of increased bone resorption and defective secondary ossification centres (Holmbeck *et al*., 1999; Zhou *et al*., 2000). These skeletal defects are also related with the defective vascular invasion of the cartilage observed in these mice. The defects in angiogenesis and vascular component of the MT1-MMP deficient mice may be due to the impairment or delay of different processes taking place in the absence of MT1-MMP. Our group has demonstrated that MT1-MMP is involved in migration of endothelial cells into the ECM (Galvez *et al*., 2001). Moreover, Robinet and coll. have described that the formation and stabilization of new formed capillary tubes may also be dependent on MT1-MMP activity (Robinet *et al*., 2005). MT1-MMP is also able to release vascular endothelial growth factor A (VEGF-A), again promoting neovascularisation (Sounni *et al*., 2004). Besides, it has been observed that postnatal angiogenesis in response to FGF-2 in MT1-MMP null mice is also markedly deficient in a corneal model (Zhou *et al*., 2000). Defects in angiogenesis also affect postnatal development of the alveolar septum, leading to lung secondary expansion failure (Oblander *et al*., 2005). Finally, MT1-MMP deficient mice present defects in myeloid cell fusion (Gonzalo *et al*., 2010a), in submandibular gland (Oblander *et al*., 2005), adipose tissue (Chun *et al*., 2006) and kidney development (Riggins *et al*., 2010).

MT1-MMP also plays a role in **pathology**. There is clear evidence that MT1-MMP is involved at different stages of tumor progression from initial tumor development, growth and angiogenesis to invasion, metastasis and growth at secondary sites (Gilles *et al*., 1997; Seiki *et al*., 2003; Sounni *et al*., 2003; Zucker *et al*., 2003). Moreover, association between MT1-MMP expression and poor prognosis in cancer patients has been established for different cancer types such as

breast cancer (Vizoso *et al*., 2007), neuroblastoma (Sakakibara *et al*., 1999) or small cell lung cancer (Michael *et al*., 1999).

2.1.2. MT1-MMP cytosolic tail

MT1-MMP cytosolic tail consists of 20 amino acids (RRHGTPRRLLYCQRSLLDKV) and it has been described to have a role in MT1-MMP localization, invasive activity, trafficking, signaling and dimerization. For some of these functions posttranslational modifications of cytosolic residues or cytosolic tail interacting partners have been identified (Table 1).

Table 1: MT1-MMP cytosolic tail binding proteins.

Cytosolic tail interacting proteins	Function	Reference
Cortactin	Cell migration	(Artym *et al*., 2006)
p130Cas	Cell migration and fusion	(Gingras *et al*., 2008; Gonzalo *et al.*, 2010a)
MTCBP-1	Cell migration	(Uekita *et al.*, 2004)
MT1-MMP	MT1-MMP activity	(Kazes *et al*., 2000; Rozanov *et al.*, 2001)
AP-2 complex	MT1-MMP endocytosis	(Uekita *et al.*, 2001)
P-caveolin 1	MT1-MMP endocytosis and Src regulation	(Labrecque *et al.*, 2004)
Abl kinases	MT1-MMP endocytosis	(Smith-Pearson *et al*., 2010)
Tubulin	MT1-MMP endocytosis	(Radichev *et al*., 2009)
p59	Intracellular MT1-MMP trafficking	(Kuo *et al.*, 2000)
gC1qR	Intracellular MT1-MMP trafficking	(Rozanov *et al.,* 2002b)
Radixin	Unknown	(Terawaki *et al.*, 2008)

The first studies that analyzed the role of MT1-MMP cytosolic tail in MT1-MMP **localization** were performed by Nakahara and collaborators. They demonstrated that MT1-MMP required its cytoplasmic domain to localize to the invadopodia of tumor cells, protruding membrane structures associated with invasive cancer cells (Nakahara *et al.*, 1997).

Later, Lehti and coll. reported that MT1-MMP cytosolic tail was not only important for MT1-MMP localization but also for its **invasive activity**. This group showed that, when the last 10 amino acids of MT1-MMP were deleted, the invasive activity of human melanoma cells was reduced (Lehti *et al.,* 2000). Moreover, it has been described that MT1-MMP cytosolic tail is not only important in tumor cell invasion but also plays a role in physiological contexts such as MT1-MMP non-proteolytic activity to boost macrophage **migration** (Sakamoto and Seiki, 2009). Other studies have shown that when MT1-MMP is localized in these migrating cell domains, it interacts with signal-transducing intermediates such as cortactin (Artym *et al.,* 2006) or the focal adhesion protein p130Crk-associated substrate (CAS) (Gingras *et al.*, 2008; Gonzalo *et al.,* 2010a). Moreover, Uekita and coll. saw that the cytosolic tail of MT1-MMP can bind to MTCBP-1, a member of cupin superfamily, and both colocalized at the adherent membrane edge. This group also saw that an enforced expression of MTCBP-1 could inhibit the activity of MT1-MMP in cell migration and invasion promotion (Uekita *et al.,* 2004). Finally, it has been seen that tyrosine 573 and threonine 567 of MT1-MMP cytosolic tail can be phosphorylated and that these modifications influence cell migration and invasion (Moss *et al.,* 2009; Nyalendo *et al.*, 2007).

In contrast to the results pointing to a role of MT1-MMP cytosolic tail in migration and invasion, Rozanov and coll. saw that, although the cytosolic tail was involved in invasion of MCF7 breast carcinoma cells, when using the extremely migratory U-251 glioma cells, MT1-MMP cytosolic tail did not affect cell locomotion (Rozanov *et al.*, 2001). Moreover, Hotary and coll. were unable to detect MT1-MMP cytosolic tail function in COS or MDCK cell invasion of basement membranes or collagen gels (Hotary *et al.,* 2000; Hotary *et al.*, 2006). Thus, it seems probable that the discrepancy obtained in this issue can be attributed to the different cellular assays used to address this question.

Homophilic complex formation of MT1-MMP on the cell surface has been demonstrated. This mechanism fits the requirement for two adjacent MT1-MMP molecules cooperating to cleave one proMMP-2 molecule. Moreover, it was observed that MT1-MMP dimerization positively regulates its activity (Galvez *et al.*, 2005). A cysteine residue (C574) present in the cytosolic tail of MT1-MMP is apparently involved in an intermolecular disulfide bond linking monomers of this proteinase and generating stable covalent dimers of MT1-MMP on the cell surface (Kazes *et al.,* 2000; Rozanov *et al.,* 2001). In contrast, Itoh and coll. proposed that the hemopexin like domain of MT1-MMP was the responsible for homophilic complex formation (Itoh *et al.,* 2001). However, Overall and coll. were unable to detect homodimer formation of MT1-MMP hemopexin domains (Overall *et al.,* 2000). Later, Lehti and coll. concluded that both the hemopexin-like and cytoplasmic domains of MT1-MMP were involved in the formation of enzyme oligomers that function in intermolecular proteolytic events at the cell surface (Lehti *et al.,* 2002).

The redistribution of MT1-MMP to the sites of ECM degradation to promote migration involves a dynamic interplay of exocytic (Bravo-Cordero *et al.,* 2007) and endocytic (Jiang *et al.,* 2001; Uekita *et al.*, 2001) trafficking events. MT1-MMP cytosolic tail has a role in its **exocytic trafficking** to discrete regions of the cell surface. In this sense, it has been shown that MT1-MMP C-terminus mutants accumulate in an early exocytic compartment and do not reach the Golgi compartment. Moreover, for the correct trafficking of MT1-MMP to the cell membrane the last valine of its cytosolic tail is critical (Urena *et al.,* 1999).

The cytoplasmic domain of MT1-MMP also plays an important role in the regulation of its **internalization** (Jiang *et al.,* 2001; Lehti *et al.,* 2000; Nakahara *et al.,* 1997; Uekita *et al.,* 2001). Endocytosis of MT1-MMP occurs primarily through clathrin-dependent mechanisms (Jiang et al., 2001; Remacle *et al.,* 2003). MT1-MMP is palmitoylated at Cys574 and this modification is critical for its promotion of clathrin-mediated internalization (Anilkumar *et al.,* 2005). Moreover, the efficient endocytosis of MT1-MMP is related, at least in part, to its interaction with the μ2 subunit of adaptor protein 2 (AP-2), a key component of clathrin-coated vesicles (Uekita *et al.,* 2001).

In addition to MT1-MMP endocytosis through clathrin-dependent mechanisms (Jiang *et al.*, 2001; Remacle *et al.,* 2003), a significant proportion of the enzyme is associated with caveolae (Annabi *et al.,* 2001; Galvez *et al.*, 2004; Labrecque *et al.*, 2004; Remacle *et al.,* 2003). In this regard, it has been seen that overexpression of the cytosolic tail-deleted form of MT1-MMP resulted in an increased association of this mutant with caveolae that correlates with impaired internalization and reduced cell migration *in vitro* and tumor growth *in vivo* (Rozanov *et al.*, 2004).

Since endocytosis via caveolae is dependent on phosphorylation of caveolin-1 at Tyr14 and MT1-MMP specifically interacts with tyrosine phosphorylated caveolin-1 (Labrecque *et al.,* 2004), it is probable that this interaction could thus participate in regulating caveolae-mediated internalization of the enzyme. Interestingly, the association of MT1-MMP with phosphorylated caveolin-1 has been seen to induce the recruitment of Src, the kinase responsible of caveolin phosphorylation, and a concomitant inhibition of the kinase activity of the enzyme, suggesting that this complex may be involved in the negative regulation of Src activity (Labrecque *et al.,* 2004).

Another MT1-MMP cytosolic partner involved in its internalization process was described by Smith-Pearson and coll. They showed that active Abl kinases form complexes with MT1-MMP and that loss of Abl kinase signaling induced internalization of MT1-MMP from the cell surface and promoted its accumulation in the perinuclear compartment (Smith-Pearson *et al.,* 2010). Regarding intracellular trafficking, two more proteins have been seen to interact with MT1-MMP cytosolic tail and being involved in its intracellular trafficking: the Golgi protein p59 (Kuo *et al.,* 2000) and gC1qR, a chaperone-like compartment-specific regulator (Rozanov *et al.,* 2002b).

Additionally, the cytosolic tail of MT1-MMP is involved in MT1-MMP **recycling**. Wang and coll. showed that the deletion of the last three residues (DKV) of the cytosolic tail of MT1-MMP significantly impaired its recycling after an apparently normal internalization process (Wang *et al.,* 2004b).

Finally, MT1-MMP cytosolic tail can also regulate intracellular **signaling** pathways. MT1-MMP can contribute to cell proliferation by its activation, through the tyrosine-protein kinase Src, of focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) (Takino *et al.,* 2010). Moreover, Deryugina and coll. observed that overexpression of MT1-MMP in tumor cells up-regulated VEGF production, increasing the endothelial cell response and subsequent tumor vascularisation and growth (Deryugina *et al.,* 2002).

To conclude, it is important to highlight the systematic whole-cell analysis of MT1-MMP-associating proteins carried out by Dr. Seiki´s laboratory in which they identify numerous candidate proteins to interact directly or indirectly with MT1-MMP. Interaction of MT1-MMP cytosolic tail with some cytosolic proteins and membrane proteins identified in these studies is expected. However, no further analyses in this sense were performed (Niiya *et al.,* 2009; Tomari *et al.,* 2009).

2.2. MT2-MMP

2.2.1. General features

MT2-MMP was first described in 1995 as the second member of the MT-MMP subfamily. It is formed of 670 amino acids and it has a molecular weight of 75 kD. Although it is a ubiquitously expressed enzyme (Takino *et al.,* 1995), some studies have pointed to a higher expression in epithelial cells. Plaisier and coll., by analyzing the expression of MT2-MMP in endometrial cells *in vitro*, observed that MT2-MMP was much more expressed in human endometrial epithelial cells

than in endothelial ones (Plaisier *et al.,* 2006). In submandibular gland this cell type specific expression has also been observed; MT2-MMP is predominantly expressed in the epithelium and little expression in mesenchyme is detected (Rebustini *et al.,* 2009).

About MT2-MMP **regulation**, it has been described that TNFα increases its expression in cultured human retinal epithelial cells (Eichler *et al.,* 2002) and trophoblasts (Hiden *et al.,* 2007).

Regarding MT2-MMP **activity** this metalloproteinase can degrade gelatin, laminin and fibrin (Hotary *et al.,* 2002). Moreover, MT2-MMP is also capable of activating MMP-2 proenzyme in a mechanism not dependent on the presence of TIMP-2 (Morrison *et al.,* 2001; Morrison and Overall, 2006).

Several MT2-MMP **functions** have been described. Lafleur and coll. determined that MT2-MMP affects the formation of capillary-like tubes *in vitro*, indicating a possible role of MT2-MMP in angiogenesis (Lafleur *et al.,* 2002). Additionally, MT2-MMP plays a role in submandibular gland development; downregulation of MT2-MMP in explant cultures produces an impaired submandibular gland branching morphogenesis due to disruption of collagen IV metabolism and reduction of cell proliferation (Rebustini *et al.,* 2009). Moreover, antiapoptotic properties of MT2-MMP have been described (Abraham *et al.,* 2005). Finally, it has been observed that MT2-MMP plays a role in follicle rupture during ovulation (Ogiwara *et al.*, 2005).

In vitro studies have shown a role of MT2-MMP in invasion. It has been observed that COS cells expressing MT2-MMP acquire the ability to perforate and transmigrate through peritoneal membrane, epithelial basement membranes (Hotary *et al.,* 2006) and collagen I gels (Hotary *et al.*, 2000). However, when MT2-MMP MDCK transfectants were used different results were obtained. When these cells where plated on top of peritoneum high invasion was detected. But, on the contrary, MT2-MMP MDCK transfectants did not show nearly any invasive activity in a collagen I gel system. Instead, MT2-MMP-overexpressing cells formed a disorganized multilayered structure on top the collagen matrix (Hotary *et al.,* 2000). These differences, in authors opinion, may indicate that COS cells regulate MT2-MMP in a more balanced fashion than MDCK and/or that MT2-MMP MDCK transfectants penetrate the peritoneum by degrading extracellular matrix components other than type I collagen (Hotary *et al.,* 2000).

Despite of these functions, reported MT2-MMP knockout mice do not show defects in development or in postnatal growth. Moreover, these mice have mendelian ratios and neither female nor male mice display any significant difference in body-weight compared with wild-type littermates. Consistenty, mice likewise display no overt aberration in size and general appearance. Moreover, the lifespan, behaviour and grooming habits are normal and MT2-MMP-deficient mice reproduce normally. However, the combined loss of MT1- and MT2-MMP produces a major impact in mice development and leads to arrest of gestation at embryonic day 10.5. This arrest appeared to be due to the inability of trophoblasts to form the syncitya required for the development of a functional placental labyrinth (Szabova *et al.,* 2010).

Involvement of MT2-MMP in **pathology** is still unclear. It has been seen that MT2-MMP is present in different types of tumors, such as glioblastoma (Zhang *et al.,* 2005), non-small cell lung carcinoma (Atkinson *et al.,* 2007), ovarian (Davidson *et al.*, 2001) and breast carcinoma (Ueno *et al.,* 1997), and seems to correlate with tumor invasiveness (Zhang *et al.,* 2005). Moreover, a MT2-MMP role in tumor angiogenesis has also been reported (Chen *et al.,* 2010).

2.2.2. MT2-MMP cytosolic tail

MT2-MMP cytosolic tail consists of 20 amino acids (QRKGAPRVLLYCKRSLQEWV). No roles or interacting proteins of MT2-MMP cytoplasmic domain have been described to date.

2.3. MT3-MMP

2.3.1. General features

MT3-MMP was first described in 1997 (Matsumoto *et al.,* 1997) but is still poorly described in literature. MT3-MMP is formed of 608 amino acids and presents a molecular weight of 69 kD. The crystal structure of MT3-MMP has been elucidated and shows extensive similarity to MT1-MMP (Lang *et al.,* 2004). Its expression has been analyzed in mice where it has been found in brain, kidney, heart, lung and testis. It is also expressed in mesenchymal tissues of the skeleton and in peri-skeletal soft connective tissue (Nuttall *et al.,* 2004).

Although MT3-MMP is a transmembrane metalloproteinase, homology screening for human MT-MMPs has resulted in the identification of a minor cDNA encoding a soluble type of MT3-MMP lacking the transmembrane domain that is considered to be an alternatively spliced variant of MT3-MMP (Shofuda *et al.,* 1997). Soluble MT3-MMP has the same proteolytic effect on extracellular matrix substrates and proMMP-2 as native MT3-MMP (Matsumoto *et al.,* 1997).

MT3-MMP can be **regulated** at different levels. Expression of MT3-MMP mRNA is strongly increased by platelet-derived growth factor (PDGF) and fibronectin (Shofuda *et al.,* 1998). Moreover, HGF has been shown to stimulate MT3-MMP activity (Kang *et al.,* 2000). MT3-MMP is also regulated by its recycling which occurs together with MT1-MMP. Both proteins are transported to trans-Golgi by early endosomes and driven together again to the cell surface (Wang *et al.,* 2004b). Finally, was observed that MT3-MMP is rapidly degraded after maturation in COS-7 transfected cells (Shofuda *et al.,* 1997).

Regarding MT3-MMP **activity**, this metalloproteinase is able to degrade fibronectin, type I and type III collagen and activate proMMP-2, by a TIMP-2-dependent mechanism, and proMMP-13 (Zhao *et al.*, 2004).

Relating MT3-MMP **function** a role in invasion has been described. It has been observed that COS cells expressing MT3-MMP acquire the ability to transmigrate peritoneal or epithelial basement membranes. Moreover, siRNA-mediated silencing of MT3-MMP in MDA-MB-231 cells decreases their ability to perforate and invade basement membranes (Hotary *et al.,* 2006). On the

contrary, it has been recently found that MT3-MMP can reduce melanoma cell collagen invasion (Tatti *et al.,* 2011). Furthermore, Kang and coll. have shown that MT3-MMP has potential to directly enhance the growth of MDCK cells (Kang *et al.,* 2000). In addition, it has been shown that capillary-like tube formation by human endometrial microvascular endothelial cells is mediated by MT3-MMP, suggesting that MT3-MMP may be a potential regulator of endometrial angiogenesis (Plaisier *et al.*, 2004). MT3-MMP can also regulate neuronal responsiveness to myelin though its cleavage of Nogo-66 receptor 1 (Ferraro *et al.,* 2011).

As mentioned, MT3-MMP is expressed in mesenchymal tissues of the skeleton and in peri-skeletal soft connective tissue. Moreover, MT3-MMP works as a major collagenolytic enzyme, enabling cartilage and bone cells to cleave high-density fibrillar collagen and modulate their resident matrix to make it permissive for proliferation and migration (Shi *et al.,* 2008). Consistent with this, MT3-MMP deficient mice display growth inhibition tied to a decreased viability of mesenchymal cells in skeletal tissues and deficiencies in bone formation in the cranium and long bones. These results indicate that MT3-MMP is required for extracellular matrix remodelling (Shi *et al.,* 2008).

As the deficit in proteolytic capacity of MT3-MMP knockout mice may be offset, at least partially through the activity of close related enzymes like MT1-MMP, Shi and coll. generated MT1-MMP/MT3-MMP double-deficient mice (Shi *et al.,* 2008). In this case, the mice were born with severe developmental defects in collagen-rich tissues, including a severe dysfunction in palatal shelf formation leading to cleft palate that caused the demise of mice shortly after birth.

Finally, regarding MT3-MMP implication in **pathologies** it has been described that it plays a role in the invasiveness of malignant tumor tissues such as renal carcinoma (Kitagawa *et al.,* 1999) or melanoma (Jaeger *et al.,* 2007).

2.3.2. MT3-MMP cytosolic tail

MT3-MMP cytosolic tail consists of 20 amino acids (KRKGTPRHILYCKRSMQEWV). No roles or interacting proteins of MT3-MMP cytoplasmic domain have been described to date, although a role in recycling due to its similarities and co-recycling with MT1-MMP could be expected (Wang *et al.,* 2004b).

2.4. MT5-MMP

2.4.1. General features

MT5-MMP was first described in 1999 and it is expressed predominantly in brain, and at low levels in kidney, pancreas and lung (Llano *et al.,* 1999). MT5-MMP structural domains follow the general structure of the other MT-MMPs. However, it contains two small segments with divergent sequences: the hinge and the stem region with two dibasic motifs that can be potentially recognized by convertases. Through this mechanism MT5-MMP tends to be shed from the cell surface as a soluble enzyme (Pei, 1999). This characteristic appears to be unique to MT5-MMP since others MT-MMPs lack such a cryptic furin motif in the analogous positions.

Regarding MT5-MMP **regulation,** very little is known. Wang and coll. have determined that MT5-MMP can be regulated by its recycling to the cell surface through its interaction with Mint-3 (Wang *et al.,* 2004a).

About MT5-MMP **activity**, it has been shown to degrade several ECM components, such as fibronectin, gelatin or inhibitory chondroitin sulphate proteoglycans (Hayashita-Kinoh *et al.,* 2001). Moreover, MT5-MMP appears capable of mediating the cleavage of cell-adhesion molecules such as N-cadherin (Monea *et al.*, 2006). However, the best established function for MT5-MMP is its ability to activate proMMP-2 (Pei, 1999).

To analyze MT5-MMP **functions** two strains of MT5-MMP knockout mice have been generated. The first one was generated by Komori and coll. (Komori *et al.,* 2004). This group observed that MT5-MMP-deficient mice were born without obvious morphological abnormalities and no apparent histological defects in the nervous system. Then, they examined the effect of MT5-MMP deficiency on the development of a severe, long-lasting neuropathic pain caused by lesions in the nervous system. One characteristic feature of neuropathic pain is mechanical allodynia, which is defined as pain evoked by non-noxious mechanical stimuli. They observed that MT5-MMP deficient mice did not develop neuropathic pain with mechanical allodynia after sciatic nerve injury, though responses to acute noxious stimuli were normal (Komori *et al.,* 2004; Woolf and Mannion, 1999).

Later, Folgueras and coll. generated another strain of MT5-MMP deficient mice (Folgueras *et al.,* 2009). In their studies, they described that MT5-MMP plays an important role in cell-cell interactions between nociceptive neurites (neurites from a sensory neuron) and mast cells. Their data demonstrated that the absence of MT5-MMP leads to a phenotype of hyperinnervation and enhanced sensitivity to noxious thermal stimuli in MT5-MMP knockout mice. Their results also indicated that mast cell degranulation requires physical cell-cell interaction with sensory neurites and that MT5-MMP regulates this interaction by a mechanism involving N-cadherin (Folgueras *et al.*, 2009).

Moreover, the expression of MT5-MMP has been related with **pathology** as it has been detected in various cancer cells and tissues, most importantly in aggressive brain tumors (Llano *et al.,* 1999).

2.4.2. MT5-MMP cytosolic tail

MT5-MMP cytosolic tail consists of 20 amino acids (KNKTGPQPVTYYKRPVQEWV) and has been seen to play a role in several MT5-MMP functions. For some of these functions, MT5-MMP cytosolic tail interacting proteins have been described (Table 2).

As previously mentioned, Wang and coll. determined that MT5-MMP cytosolic tail is involved in its **recycling** to the cell surface by its interaction with Mint-3 in the trans-Golgi network. Moreover, they concluded that Mint-3 binds to the PDZ (Post-Synaptic Density 95 kD protein/ *Drosophila melanogaster* Discs Large protein/ Zonula Occludens 1) binding motif, EWV, of MT5-MMP cytosolic tail (Wang *et al.,* 2004a).

Table 2: MT5-MMP cytosolic tail binding proteins.

Cytosolic tail interacting proteins	Function	Reference
Mint-3	MT5-MMP recycling	(Wang *et al.,* 2004a)
AMPA receptor Binding Protein (ABP)	Possible role in axon path finding or synapse remodelling	(Monea *et al.*, 2006)
Glutamate Receptor Interacting Protein (GRIP)	Unknown	(Monea *et al.,* 2006)

MT5-MMP has also been reported to bind to glutamate receptor interaction protein (GRIP) and AMPA receptor binding protein (ABP), two related PDZ domain proteins that target AMPA receptors to synapses (Monea *et al.,* 2006). Characterization of MT5-MMP and ABP interaction showed that ABP PDZ5 was the binding site for MT5-MMP (Feng *et al.,* 2003). Moreover, Monea and coll. described that deletion of the PDZ binding motif of MT5-MMP impaired its membrane trafficking to filopodia tips of growth cones in cultured embryonic hippocampal neurons and to synapses in mature neurons (Monea *et al.,* 2006). Taking all these data together, they concluded that ABP could be leading MT5-MMP proteolytic activity to growth cones and synaptic sites in neurons, where it might be regulating axon path finding or synapse remodelling (Monea *et al.,* 2006).

2.5. Comparative analysis of MT1-, MT2-, MT3- and MT5-MMP

2.5.1. Specificity *versus* redundancy

In this section reported similarities *versus* differences of transmembrane MT-MMPs will be discussed, highlighting their possible redundancy and specificity.

To dissect transmembrane MT-MMP functions, mouse mutants of each MT-MMP have been generated. All of these knockout mice can survive to birth and only show, with the exception of MT1-MMP, subtle phenotypes. Possible explanations for the dispensability of the transmembrane MT-MMPs during embryonic development are enzymatic redundancy and enzymatic compensation. These MT-MMPs have many overlapping substrates *in vitro*, such as gelatin or fibronectin, which indicates a possible redundancy *in vivo* (Page-McCaw *et al.,* 2007). Indeed, **redundancy** has been determined with the generation of MT-MMP double mutants. MT2-MMP deficient mice do not show any apparent defects. However, when both MT1- and MT2-MMP are not expressed, mice die during the embryonic development (Szabova *et al.,* 2010). Similar results have been obtained in the case of MT3-MMP. MT3-MMP knockout mice show growth inhibition due to deficiencies in bone formation; but in MT1-MMP/MT3-MMP double-deficient mice these bone formation deficiencies are aggravated, leading to demise of mice shortly after birth (Shi *et al.,* 2008).

Enzymatic compensation between transmembrane MT-MMPs has also been found. For example, fibroblasts that lack MT1-MMP and show a defect in fibrin gel invasion are able to overcome this defect by MT2- or MT3-MMP compensation (Hotary *et al.,* 2002). Another example has been shown by Rebustini and coll., who observed a coordinated transcription regulation of

MT-MMPs in submandibular gland. They saw that reducing MT1- or MT3-MMP expression upregulated MT2-MMP, while reducing MT2-MMP expression upregulated MT3-MMP expression. In concordance, they also observed that in the submandibular gland of MT1-MMP knockout mice MT2-MMP was highly upregulated (Rebustini *et al.,* 2009).

Enzymatic regulation between MT-MMPs has been recently reported. Tatti and coll. have observed that MT3-MMP can affect MT1-MMP localization and its promotion of invasion in melanoma cells. Coimmunoprecipitation of these proteins and induced processing of MT1-MMP by MT3-MMP have been also described (Tatti *et al.*, 2011).

Nevertheless, transmembrane MT-MMPs are not merely redundant and **specific functions** have been described. The major example is MT1-MMP knockout mice that can not survive by the compensatory activity of other MT-MMPs (Holmbeck *et al.,* 1999; Sakamoto and Seiki, 2009; Zhou *et al.,* 2000). Moreover, Dr. Weiss´ laboratory has also shown specific roles of these proteinases. Although MT1-, MT2- and MT3-MMP can degrade collagen *in vitro*, it has been observed that MDCK cells overexpressing each of these metalloproteinases showed different invasion phenotypes in a three-dimensional collagen gel system. MDCK cells overexpressing MT1- or MT3-MMP presented an invasive response after SF/HGF stimulation in contrast to MT2-MMP overexpressing cells that formed a disorganized multilayered structure atop the collagen matrix (Hotary *et al.,* 2000).

Although MT-MMPs have overlapping functions and target common substrates, their **differential expression** within specific tissues may specify their functions. For example, during submandibular gland development expression of MT2-MMP is higher in the epithelium and MT1- and MT3-MMP are more abundant in the mesenchyme. Moreover, Rebustini and coll. showed that MT2-MMP-siRNA had the greatest effect on epithelial morphogenesis of the submandibular gland (number of end buds x duct length) compared with MT1- and MT3-MMP-siRNA. Similar results were obtained when epithelial proliferation was checked; MT2-MMP-siRNA significantly decreased epithelial cell proliferation whereas MT1- and MT3-MMP-siRNA had minimal effects on epithelial proliferation (Rebustini *et al.,* 2009).

MT-MMP activity **regulation** by TIMPs can be another mechanism to explain different MT-MMP functions. All transmembrane MT-MMPs can activate proMMP-2; however, their shedding mechanism is not the same. MT2-MMP, contrary to MT1-MMP, activates proMMP-2 in a pathway that is not dependent on the presence of TIMP-2, but rather on interaction with the hemopexin-like domain of MMP-2. Based on this different shedding mechanism, it can be speculated that MT2-MMP may be the main metalloproteinase for proMMP-2 shedding in tissues or pathologies characterized by low TIMP-2 expression (Morrison *et al.,* 2001; Morrison and Overall, 2006). Regarding regulation, it has been also observed that MT3-MMP is regulated by its internalization and that this internalization occurs together with MT1-MMP. Both proteins are transported to trans-Golgi by early endosomes and they are also again driven together to the cell surface (Wang *et al.,* 2004b).

MT5-MMP seems to have several differences compared with the rest of transmembrane MT-MMPs. It is the only one that presents two dibasic domains that can be recognized by convertases leading to shedding of MT5-MMP from the cell surface as a soluble enzyme. Moreover, its function seems to be mainly restricted to nervous system where it is predominately expressed (Pei, 1999). In spite of this apparent specificity, it has been observed that MT3-MMP expression pattern is similar to MT5-MMP in mouse carcinoma P19 cells differentiated to neurons by retinoic acid treatment. On the contrary, expression of MT1- and MT2-MMP was not induced by this treatment (Hayashita-Kinoh *et al.,* 2001). For MT5-MMP possible redundancy with other MT-MMPs can be speculated, as it has been described for the rest of transmembrane MT-MMPs. Nevertheless, no double knockout mice of MT5-MMP and another MT-MMP to study *in vivo* redundancy have been generated to date.

2.5.2. Cytosolic tail comparison

MT-MMP cytosolic tails can be also involved in the redundancy and specificity of the transmembrane MT-MMPs. Studies about MT-MMP cytosolic tails have mainly been focused on MT1- and MT5-MMP. Because of this, very little about common or specific features of MT-MMP cytosolic tails can be concluded. However, indirect analysis to predict possible redundant or specific functions can help to clarify this issue.

Comparison of the cytosolic tail residues of the MT-MMPs can give us clues about the existence of common or different motifs than can affect MT-MMP regulation or function. Moreover, based on MT-MMP cytosolic binding motifs, interacting proteins can be predicted. As shown in Figure 4A, cytosolic domains of MT-MMPs share part of their sequence. The four of them have two or three polybasic regions, two hydrophobic regions and a PDZ binding sequence at their C-termini.

A

Metalloproteinase	Cytoplasmic domain
MT1-MMP	RRHGTPRRLLYCQRSLLDKV
MT2-MMP	QRKGAPRVLLYCKRSLQEWV
MT3-MMP	KRKGTPRHILYCKRSMQEWV
MT5-MMP	KNKTGPQPVTYYKRPVQEWV

B

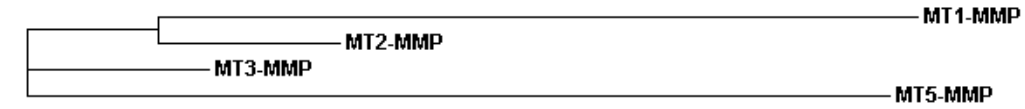


Figure 4: Human MT-MMP cytosolic tail comparison. A) Amino acid sequences of the cytoplasmic domain of the MT-MMPs. Negative residues are coloured in blue, positive residues in red, non polar residues in green and uncharged polar residues are in black. PDZ binding motif is underlined. B) Phylogram (ClustalW EBI) obtained by analyzing and comparing these cytosolic sequences.

To analyze the relationship between the MT-MMP cytosolic tails we first compared their amino acidic sequence with a phylogram (Fig. 4B). By this analysis, it can be observed that MT1-MMP and MT2-MMP are closely related, MT2-MMP and MT3-MMP also share a high percentage of their sequence and MT5-MMP is the one that differs more from the rest. As mentioned, MT5-

MMP has been described to have other differences with the rest of the transmembrane proteins such a unique dibasic motif that can let MT5-MMP to behave as a soluble protein. This phylogram obtained by cytosolic tail comparison shows the same relations between the four proteins that when their catalytic sequence is compared (Nie and Pei, 2003).

Moreover, as explained previously, MT1-MMP cytosolic tail has a role in MT1-MMP internalization. MT1-MMP LLY573 motif of the cytosolic tail acts as a binding site for AP-2 complex that mediates incorporation of target proteins into clathrin-coated pits. This sequence motif is present in MT2- and MT3-MMP which are also effectively internalized from the cell surface, but is absent in MT5-MMP that is less efficiently internalized (Uekita *et al.*, 2001). Based on this, binding of MT2- and MT3-MMP, but not MT5-MMP, to AP-2 can be suggested.

In regard to MT-MMP recycling, similar functions of MT1- and MT3-MMP cytosolic tails have been observed. Wang and coll. showed that the deletion of the three last residues of the cytosolic tail of MT1-MMP (DKV) or MT3-MMP (EWV) significantly impaired their recycling after an apparently normal internalization process (Wang *et al.,* 2004b).

The relevance of the amino acid sequence of the MT-MMP cytosolic tails can be indirect evaluated by their degree of conservation along evolution. For this purpose, we compared MT-MMP cytosolic residues of the species whose DNA has been sequenced. As it can be seen in the Table 3, the MT-MMP cytosolic tails are highly conserved along evolution, being nearly identical in separated taxons. This can indicate a key role of these amino acids for the correct function of the metalloproteinases. Moreover, this analysis can also point to conserved interactions of the MT-MMPs with cytosolic partners along evolution.

Table 3: Comparison of the amino acids of MT-MMPs cytosolic tails. Different amino acids to the human sequence are highlighted in red. Animals belonging to Class Mammalia are coloured in green.

Taxon	Animal	Scientific name	Sequence of the cytosolic tail amino acids			
			MT1-MMP	MT2-MMP	MT3-MMP	MT5-MMP
Primates	human	*Homo sapiens*	RRHGTPRRLLYCQRSLLDKV	QRKGAPRVLLYCKRSLQEWV	KRKGTPRHILYCKRSMQEWV	KNKTGPQPVTYYKRPVQEWV
Rodents	mouse	*Mus musculus*	RRHGTP**K**RLLYCQRSLLDKV	QRKGAPR**M**LLYCKRSLQEWV	KRKGTPRHILYCKRSMQEWV	KNK**A**GPQPVTYYKRPVQEWV
	rat	*Rattus norvegicus*	RRHGTP**K**RLLYCQRSLLDKV	QRKGAPR**M**LLYCKRSLQEWV	KRKGTPRHILYCKRSMQEWV	KNKTGPQPVTYYKRPVQEWV
Even-toed ungulates	cow	*Bos Taurus*	RRHGTP**K**RLLYCQRSLLDKV	QRKGAPR**M**LLYCKRSLQEWV	KRKGTPRHILYCKRSMQEWV	KNK**A**GPQPVTYYKRPVQEWV
Lagomorphs	rabbit	*Oryctolagus cuniculus*	RRHGTP**K**RLLYCQRSLLDKV			KNK**A**GPQPVTYYKRPVQEWV
Carnivores	dog	*Canis familiaris*	RRHGTP**K**RLLYCQRSLLDKV		KRKGTPRHILYCKRSMQEWV	
Chiropterous	bat	*Rhinolophus ferrumequinum*				KNK**A**GPQPVTYYKRPVQEWV
Fish	zebrafish	*Danio rerio*			KRK**D**T**Q**RHILYCKRSMQEWV	KNK**NVQ**Q**H**VTYYK**H**PVQEWV
	medaka	*Oryzias latipes*		Q**S**KGAPRLL**VH**CKRSLQ**D**WV	K**K**KGTPRHILYCKRSMQEWV	
Birds	chicken	*Gallus gallus*			KR**E**GTPRHILYCKRSMQEWV	KNK**EVQ**Q**N**V**V**YYKRPVQEWV
Amphibians	western frog	*Xenopus tropicalis*	R**QC**GTP**K**RI**L**Y**W**QRSLLDKV	**H**RKGP**K**AL**R**YCKRSLQEWV	KRKGTPRHILYCKRSMQEWV	

3. Linker proteins to actin machinery

One key mechanism for MT-MMP function and activity regulation is the control of their localization at specific cellular microdomains and dynamic shuttling between these distinct compartments. In this regard, MT-MMP-integrin crosstalk has been seen to play an important role. For example, MT1-MMP is detected together with integrin αvβ3 in caveolae at membrane protrusions (filopodia and lamellipodia) of migrating cells where MT1-MMP was found to be active. Moreover, the association of these proteins is involved in several events as for example tumor cell locomotion (reviewed in Gonzalo *et al.,* 2010b). Integrin receptors connect the extracellular matrix to the actin cytoskeleton. However, integrin binding to actin filaments is not direct but requires adaptor proteins that form, together with signaling proteins, macromolecular complexes. Then, they not only anchor the cell to the substratum but also can transmit signals in both directions. These complexes have been seen to be important for different cellular functions such as cell motility or polarization (Geiger *et al.,* 2001). For example, in healing skin wounds integrin-mediated cues promote the reorganization of the cytoskeleton of keratinocytes at the wound edge resulting in directed migration and wound closure (Brakebusch and Fassler, 2003). As MT-MMPs have been seen to participate in integrin associated protein complexes, a role of membrane protein linkers to actin cytoskeleton could be suggested for MT-MMP regulation. In this section three of these linker proteins will be explained in detail.

3.1. p130Cas

MT1-MMP cytosolic tail was reported to bind to the focal adhesion protein p130 Crk-associated substrate (p130Cas) at the leading edge of migrating endothelial cells and this association was induced by sphingosine-1-phosphate (S1P) (Gingras *et al.,* 2008).

p130Cas is **localized** at cellular sites of dynamic interaction between the actin cytoskeleton and the plasma membrane. In fibroblasts, tyrosine-phosphorylated p130Cas is prominently localized at mature focal adhesions, while the unphosphorylated protein appears to be largely cytoplasmic. In migrating cells, tyrosine-phosphorylated p130Cas is enriched at the cell periphery in nascent cell-matrix adhesion sites (focal complexes) and/or plasma membrane ruffles (Abassi *et al.,* 2003). In the case of osteoclast-like cells, p130Cas is localized in the peripheral sealing zone (Lakkakorpi *et al.*, 1999).

p130Cas is a non-enzymatic 'docking protein' consisting of multiple protein-protein interaction **domains** and serve as an important anchoring point for protein-protein interactions (Nojima *et al.,* 1996). It presents an N-terminal Src homology 3 (SH3) domain that binds FAK and Pyk2, an interior substrate domain (SD) characterized by 15 Tyr-X-X-Pro (YxxP) motifs, a C-terminal Src binding domain and a highly conserved C-terminal region that binds to the Nsp family of proteins (Nsp1, And-34, and Chat) (Fig. 5A) (Defilippi *et al.,* 2006).

The 15 YxxP motifs of the substrate domain represent the major sites of p130Cas tyrosine phosphorylation. Tyrosine phosphorylation creates docking sites for recruitment of SH2-containing signaling effectors. Notably, recruitment of **Crk adaptor proteins** to this domain sites has been strongly implicated in promoting **Rac activation and cell motility** (Fig. 5B) (Pratt *et al.,*

2005). Moreover, repetitive YxxP motifs have been suggested to mainly function in signal amplification to maximize the Crk output pathways, but also to diversify signaling, as functional cooperation in the Crk assemblages would also increase the repertoire of output signals, for example during integrin-mediated spreading and migration on ECM molecules (Fig. 5C) (Cary *et al.,* 1998).

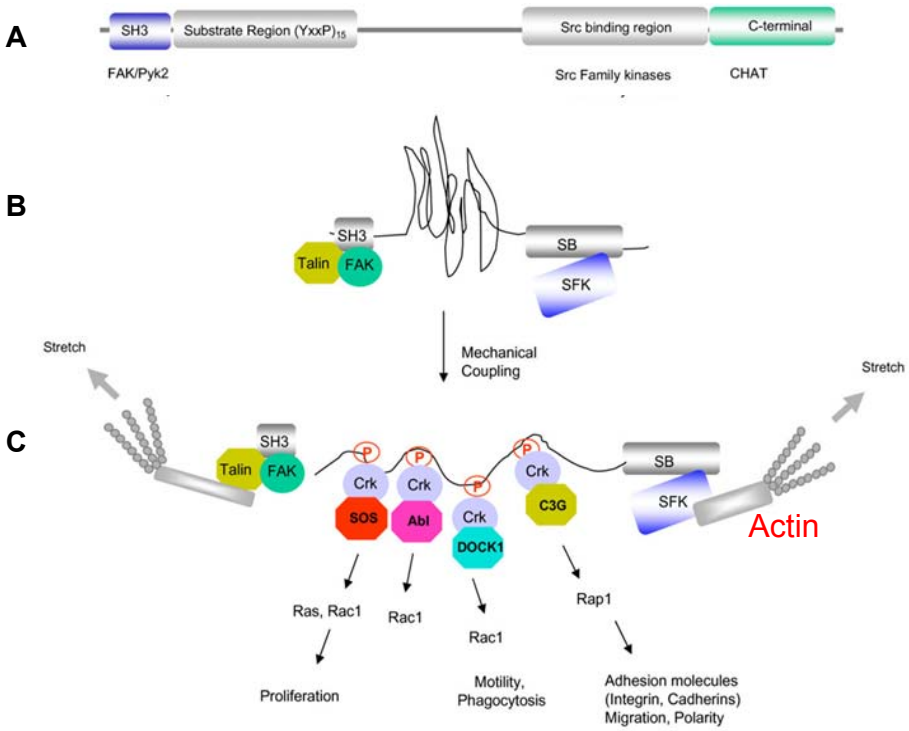


Figure 5: Structural characteristics and interacting proteins of p130Cas. A) p130Cas is a nonezymatic scaffolding protein that contains, an N-terminal SH3 domain that binds FAK and Pyk2, 15 repeats of a YxxP motif, a serine-rich motif that binds Src kinases, and a conserved C-terminal region that binds members of the Chat family of proteins. B) Signal transduction by the p130Cas scaffold protein. The central substrate region of p130Cas (shown in panel B as a compressed configuration) is activated by mechanical force and 'extension' of the central region. C) This would activate Src, induce tyrosine phosphorylation of the repetitive YxxP motifs, and recruit Crk through its SH2 domain. Further, by recruiting different proteins via the CrkSH3N, this signaling strategy would spatially integrate divergent signals, for example, after the recruitment of various GTPase pathways such as DOCK1, SOS, and C3G (Taken from Birge *et al.*, 2009).

Activity **regulation** of p130Cas occurs as a consequence of phosphorylation of multiple YxxP tyrosine residues in the SD. p130Cas SD tyrosine phosphorylation appears to be mediated primarily by Src-family kinases and occurs as a consequence of integrin-mediated cell adhesion. The SD is characterized by 15 YxxP motifs and the most N-terminal of these YxxP tyrosines, are subject to phosphorylation by Src. The 10 most C-terminal YxxP sites appear to be the most efficiently phosphorylated. Phosphospecific antibodies directed against SD YxxP phosphotyrosines indicate p130Cas is activated within focal adhesions and at the cell periphery of motile cells (Cabodi *et al.,* 2010).

p130Cas is involved in different **cellular functions.** p130Cas-null cells show defects in stress fiber formation, cell spreading, impaired actin bundling and cell migration, which are restored by the addition of full-length p130Cas (Honda *et al.,* 1998). By contrast, mutants with deletions of the YxxP motifs in the SD and of the C-terminal Src-binding domain can not recover the migratory

phenotype, probably because of their inability to bind Crk and to associate and regulate Src kinase (Huang *et al.,* 2002). p130Cas presents also a role in cell survival and apoptosis. p130Cas is an important transducer of survival signals. Pro-survival signals emanating from the ECM, soluble growth factors and hormones proceed through their respective receptors, then through FAK and Src to p130Cas, activating the small GTPases Ras and Rac. Moreover, p130Cas is also required for integrin-dependent EGF-receptor activation, which in turn leads to cell survival (Moro *et al.,* 2002). However, p130Cas has also a direct role in death signaling. Cell detachment triggers rapid dephosphorylation of p130Cas in anoikis-(apoptosis induced by cell detachment) sensitive normal epithelial but not in anoikis-resistant lung adenocarcinoma cells (Wei *et al.,* 2002). Finally, p130Cas is involved in cell transformation, invasion and **cancer**. For example, p130Cas has been seen to be hyperphosphorylated in Src- and Crk-transformed cells (Reynolds *et al.,* 1989).

p130Cas-deficient mouse embryos die *in uterus* and are phenotypically abnormal at 11.5-12.5 days *post coitum* with systemic congestion, growth retardation and heart and blood vessel abnormalities (Honda *et al.,* 1998).

3.2. Moesin

As mentioned, radixin has been described to interact *in vitro* with the MT1-MMP cytosolic tail (Terawaki *et al.,* 2008). Due to the known functions of ERM proteins and the established preferences in their binding features, it is probable that MT1-MMP could interact not only with radixin but also with other members of ERM family.

Moesin and the rest of ERM family members display the same **structure** (Fig. 6). They are formed of three domains; an N-terminal globular domain, also called FERM domain (Four-point one, Ezrin, Radixin, Moesin), which allows ERM proteins to interact with integral proteins of the plasma membrane or scaffolding proteins localized beneath the plasma membrane, an extended alpha-helical domain (coiled coil) and a C-terminal domain (ERM Actin and FERM-Binding Domain; A/FBD) which mediates the interaction with F-actin.

ERM proteins are conformationally **regulated**; the full-length (FL) dormant molecule has masked activities that can be revealed by activation of the full-length protein or expression of separated fragments. For example, soluble full-length dormant ezrin does not bind F-actin or the ERM-binding phosphoprotein 50 (EBP50), whereas the N-terminal domain alone binds EBP50, and the C-terminal domain alone binds F-actin (Gary and Bretscher, 1995). The molecular basis for this phenomenon was uncovered by the finding that in dormant ezrin the N-terminal domain binds very tightly to its C-terminal domain and thereby masks the binding sites for EBP50 and F-actin. Because these domains can mediate interactions of the different ERM members, they were given the functional names N- and C-ERMADs (N- and C-ERM-association-domains) (Gary and Bretscher, 1995).

Activation of ERM proteins was predicted to require the separation of the N- and C-ERMADs, thereby exposing the C-terminal F-actin binding site and potential membrane association sites in the N-ERMAD (Berryman and Rood, 1995). *In vitro* activation of dormant moesin by protein kinase

C-θ (PKC-θ), phosphorylates a specific C-terminal threonine (Pietromonaco *et al.,* 1998), which reduces the N-/C-ERMAD interaction (Matsui *et al.,* 1998) and simultaneously unmasks both the EBP50 and F-actin binding sites, validating the conformational activation model (Fig. 6) (Simons *et al.,* 1998).

ERM proteins have been shown to participate in two types of **interactions** with membrane proteins. Direct associations involve interactions with the cytoplasmic domains of adhesive type I membrane proteins, whereas indirect associations through the adaptor EBP50 involve the cytoplasmic domains of proteins that span the membrane many times (Fig. 6).

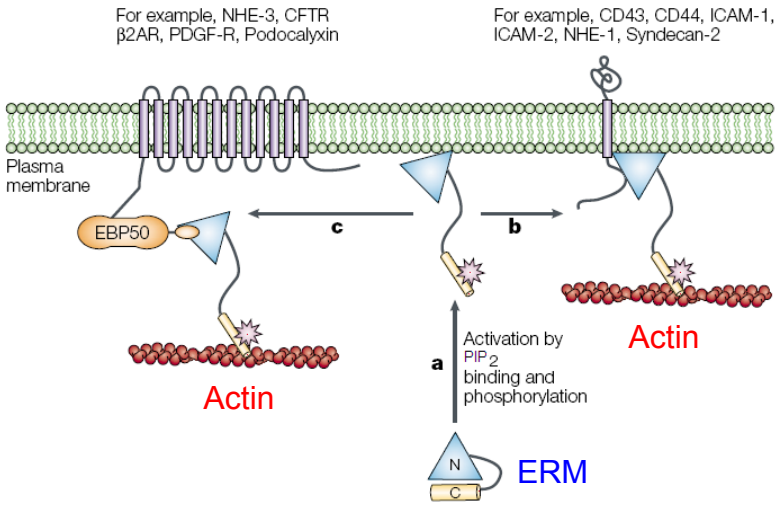


Figure 6: A model of the activation and function of ERM proteins. (a) ERM proteins exist in a dormant, monomeric form in which the FERM/N-ERMAD domain is associated with the C-ERMAD. (b) Local production of phosphatidylinositol bisphosphate (PIP_2) recruits ERM proteins to the plasma membrane, which places them in a location to be phosphorylated, and thereby activated, by Protein Kinase Cθ. Activated ERM proteins can then participate in microfilament-membrane linkage by direct association with transmembrane proteins, (c) or indirectly through scaffolding molecules such as EBP50. ICAM: intercellular adhesion molecule, NHE: sodium-hydrogen exchanger, NHE-RF: regulatory cofactor of NHE-3, PDGF-R: platelet-derived growth factor receptor (Adapted from Bretscher *et al.*, 2002).

By mapping the regions of interaction of ERM proteins, it has been observed that positively charged regions adjacent to the membrane of type I proteins play an important role for their affinity for ERMs. A positively charged juxtamembrane cluster binding to ERM proteins was found in CD43, CD44 and ICAM-2 (Legg and Isacke, 1998; Yonemura *et al.,* 1998).

ERM proteins are widely distributed membrane-associated proteins that regulate the structure and function of specific domains of the cell cortex. As linkers of transmembrane proteins to actin cytoskeleton, ERMs have been involved in different **cellular functions**. ERMs are implicated not only in cell-shape determination but also in migration, membrane-protein localization, membrane transport and signal transduction (Bretscher *et al.,* 2002). For example, ezrin interacts with the cytoplasmic tail of ICAM-2 (Fig. 6) and this has been shown of functional importance. Natural killer cells need to recruit ICAM-2 into a bud-like projection known as uropod before they can be activated by IL-2 binding. This recruitment of ICAM-2 is dependent on ezrin, as cells that lack ezrin have uniform ICAM-2 distribution (Helander *et al.,* 1996). Another example of ERM function

is their participation in Rho and PKC signaling, ERM activation has a crucial role in the cellular cytoskeletal response to Rho-pathway activation (Mackay *et al.,* 1997). Moreover, ezrin has been seen to participate in endocytic recycling system. In the acid-secreting parietal cells of the stomach the H^+-ATPase is sequestered in vesicles in resting cells, and upon stimulation vesicles fuse with plasma membrane. Ezrin becomes phosphorylated during this process, and apical microvilli are greatly enlarged to accommodate the added membrane (Agnew *et al.,* 1999; Hanzel *et al.,* 1989). In addition, moesin has been shown to play a crucial role in the generation of a rigid and uniform cell cortex during mitosis (Carreno *et al.,* 2008), in the establishment of embryo polarity (Jankovics *et al*., 2002) and in the maintenance of epithelial integrity (Speck *et al.*, 2003). Finally, a role of ERM in pathology has also been found. Estecha and coll. work showed that moesin plays a role in cell **tumor** invasion (Estecha *et al.,* 2009).

Regarding ERM-deficient mice, **moesin-deficient mice** are the only ones generated to date and no abnormalities were detected. Interestingly, no compensatory up-regulation of ezrin or radixin in these mice was seen (Doi *et al.,* 1999).

3.3. Zonula Occludens 1

As explained before, MT-MMPs have in the C-terminus of their cytosolic tails a PDZ binding motif and therefore, proteins with PDZ domains are likely to interact with them. In fact, this kind of interactions have been already described in the case of MT5-MMP binding to Mint-3 (Wang *et al.,* 2004a), ABP or GRIP (Monea *et al.,* 2006).

PDZ domains are protein-protein recognition modules that play a central role in organizing multiprotein complexes that function in signaling, as well as in the establishment and maintenance of cell polarity (Harris *et al.,* 2001). PDZ domains recognize specific C-terminal motifs, usually about five residues in length, of their partner proteins, most often in the cytoplasmic tails of transmembrane receptors (Kornau *et al.,* 1995).

Among the different proteins that contain PDZ domains we will focus in this work on Zonula Occludens 1 (ZO-1). ZO-1 belongs to the membrane associated guanylate kinase family (MAGUK) and it has the following **structure**: three PDZ domains, a Src homology (SH3) domain, a guanylate kinase (GK) domain and a praline-rich region that binds to the actin cytoskeleton (Fig. 7) (Guillemot *et al.,* 2004).

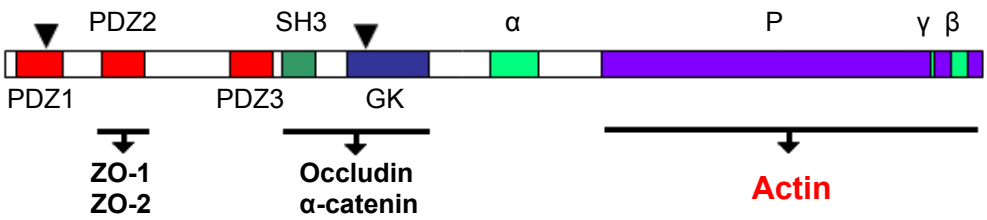


Figure 7: Diagram of ZO-1 domains. From the N-terminus to the C-terminus ZO-1 contains a core of PDZ domains, a basic Maguk core of Src homology (SH3), an enzymatically inactive guanylate kinase (GK) domain and a proline-rich domain (P). The alternative splice of regions of ZO-1 are indicated by Greek characters. Nuclear localization signals, arrowheads, and some binding proteins are also indicated (Adapted from Gonzalez-Mariscal *et al*., 2000).

ZO-1 is a tight junction (TJ)-associated protein, with a molecular weight between 210 and 225 kD, which is **located** at the submembranous region of the junction where acts as a linker between the proteins that constitute the TJ strands and the actin-based cytoskeleton. It is specifically enriched at the TJ of epithelial and endothelial cells. The most characterized **function** of ZO-1 is its role in TJ conformation (Fig. 29 of the discussion). ZO-1 has been shown to be indispensable for TJ formation in epithelial cells; targeted disruption of the ZO-1 gene results in deficient TJ formation (Umeda *et al.* 2006).

ZO-1 can be also localized in the membrane of subconfluent epithelial cells or at the edge of wounded monolayers (Gottardi *et al.,* 1996). Tuomi and coll. showed that ZO-1 preferentially interacts with α5β1 integrin at the lamella of migrating cells. Moreover, disruption of ZO-1 binding to α5 cytoplasmic tail prevented the localization of ZO-1 at the leading edge. Furthermore, silencing of ZO-1 resulted in reduced directional cell motility (Tuomi *et al.*, 2009).

Moreover, ZO-1 has also been detected at the nucleus and nuclear localization and exporting signals have been identified in its sequence (Fig. 7). ZO-1 has been seen to associate to different proteins and participate in the control of gene expression. ZO-1 can bind the transcription factor ZONAB and regulate ErbB-2 promoter in a cell density-dependent manner (Balda and Matter, 2000).

ZO-1-deficient mice are embryonic lethal. Embryos exhibit massive apoptosis in the notochord, neural tube area and allantois at E9.5. In the yolk sac, the ZO-1-deficiency induces defects in vascular development with impaired formation of vascular trees. In E8.5 WT mice embryos ZO-1 is expressed in almost all embryonic cells, presenting tight junction-localizing patterns. ZO-1 may affect cell remodelling and participate in tissue organization in both embryonic and extraembryonic regions, playing an essential role for embryonic development (Katsuno *et al.,* 2008).

Objectives

General objective:

To get further insights into the specific functions of MT1-MMP *versus* other MT-MMP subfamily members by the analysis of their cytosolic tail interactions.

Specific objectives:

1. Characterization of MT1-MMP cytosolic tail association with the adaptor protein p130Cas in myeloid progenitors.

2. Characterization of MT1-MMP, MT2-MMP and MT3-MMP cytosolic tail binding to ERM proteins.

3. Search of new molecular associations of MT-MMP cytosolic tails and analysis of their possible cellular function.

Materials and Methods

1. Antibodies

Primary and secondary antibodies used in this work are summarized in the following tables:

Primary antibody	Origin	Company
Annexin I	mouse	Iowa Gift from Mª Antonia Lizarbe (UCM)
Annexin II	mouse	BD Transduction Laboratories Gift from Mª Antonia Lizarbe (UCM)
β-Actin	mouse	Sigma-Aldrich
Caveolin-1	mouse	BD Transduction Laboratories
Ezrin	rabbit	Upstate
GST	rabbit	Molecular Probes
HA	mouse	Covance
Integrin β1	rabbit	Abcam
Moesin	rabbit	Upstate
MT1-MMP	mouse	LEM 2/63. Generated in our group (Galvez *et al.,* 2001)
MT2-MMP	mouse	R&D Systems
MT2-MMPcyt	rabbit	Gift from Kaisa Lehti (University of Helsinki)
p130CAS	rat	Pharmingen
Rho-GDI	rabbit	Santa Cruz Biotechnology
Tubulin	mouse	Abcam
ZO-1	rabbit	Zymed

Secondary antibody	Origin	Company
Anti mouse/rabbit/rat peroxidase conjugated	goat	Jackson Immuno-Research Laboratories
Anti mouse/rabbit/rat conjugated with Alexa 488 or 647	goat	Jackson Immuno-Research Laboratories

2. Cell cultures

MT1-MMP bone marrow progenitors (BM) were obtained from MT1-MMP-deficient mice (MT1-MMP$^{-/-}$). MT1-MMP$^{-/-}$ mice in the C57BL/6 background were generated as described (Zhou *et al.,* 2000). BM cells were cultured in α-MEM (Lonza) supplemented with 10% FBS, 50 IU/ml penicillin, 50 µg/ml streptomycin. During 3 days cells were exposed to 50 ng/ml nuclear factor-kB ligand (RANKL) (PreproTech) and 50 ng/ml macrophage colony-stimulating factor (M-CSF) (PreproTech). After, for 9 days more cells were exposed to 50 ng/ml RANKL and 25ng/ml of M-CSF. These conditions differentiate progenitors toward osteoclast (OC), giving rise to typical multinucleated cells expressing characteristic phenotypic markers, including tartrate-resistant acid phosphatase (TRAP) (Sigma) (Yagi *et al.,* 2005).

Human umbilical vein endothelial cells (HUVECs) were obtained and cultured as described previously (Galvez *et al.,* 2001). Cells were grown in plates covered with 0.5% or 1% gelatin (Sigma-Aldrich) in 199 Bio Whittaker Medium (Walkersville, MD) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin and 2.5 µg/ml of fungizone. After the first passage, 50 µg/ml of growth factor extracted from bovine cerebrum

and 10 UI/ml of heparin was also added to the medium. Cells at passage 3 or below were used in all assays.

Bovine aortic endothelial cells (BAEC) were obtained with the following protocol. Briefly, aortic adventicia layers were removed and small vessels arising from the aorta were closed with a string. After washing with PBS, 0.1% collagenase was added into the aorta cavity. After 10 minutes of incubation at RT detached cells were collected. BAEC were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin over 0.1% gelatin coated plates. Cells at passage 5 or below were used in all assays.

Human microvascular endothelial cells (HMECs) were cultured in MCDB131 medium (Gibco, Invitrogen Corporation) supplemented with 10% FBS, 2 mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin, 10 ng/ml EGF (Promega) and 100 nM hydrocortisone (Sigma).

HeLa and MDA-MB-231 cells were cultured in DMEM (Gibco, Invitrogen Corporation) 10% FBS, 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin.

NCI-H441 and NCI-H441 were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin.

MDCK type II were cultured in MEM (Gibco, Invitrogen Corporation) supplemented with 5% FBS, 2 mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin. In the case of the stable transfected MDCK 400 µg/ml neomycin (G418) (Sigma-Aldrich) was also added to the culture medium.

3. Cell interference

One day after seeding, cells were transfected with 100nM or 200nM moesin/control interference RNA (Ambion) using Oligofectamine reagent (Invitrogen). After 48, 72 or 96 h cells were lysed (lysis buffer: 10 mM Tris-HCL, 1% Triton X-100, 0.5% sodium deoxycolate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 10 mM $MgCl_2$, proteinase inhibitor cocktail (Roche), 0.2 mg/ml PMSF, 25 mM NaF and 1 mM Na_3VO_4) and membrane fractionation or western blot was performed.

4. MDCK stable transfection and clone selection

MDCK were transfected with pCR3.1 empty vector (mock) or pCR3.1 containing the cDNA fragments: MT1-MMP-HA, MT1-MMP-K581W-HA, MT2-MMP-HAFlag or MT2-MMP-W668K-HAFlag. For transfection FuGENE 6 (Roche) was used. Transfected MDCK cells were cultured in the presence of 400 µg/ml of G418 and single cell colony selection was performed by limited cell dilution in 96-well plates. Each cell clone was tested for the corresponding MT-MMP expression by immunoflourescence and western blot.

5. Cell infection

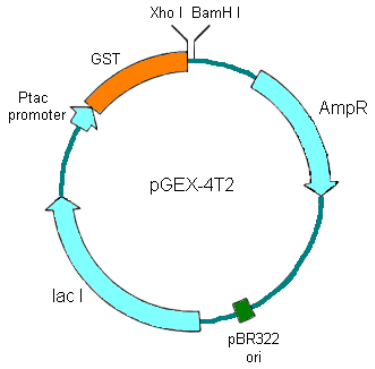
Retroviral particles were collected from conditioned medium after transfection of plasmids into the proper packaging cell lines (Viral Vector Unit, CNIC). As a control, BM cells were infected with retroviral particles obtained with the empty plasmids (pMSCV/pRETRO). BM cells were infected on day 0 and washed 48 h after infection.

6. Expression vectors and plasmid construction

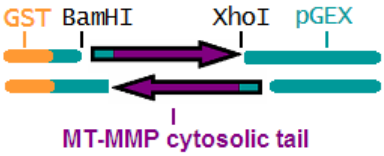
The MT1-MMP retroviral construct has been previously described (Bartolome *et al.*, 2009). The pR-MT1-MMP-Y573F mutant was generated from the pRETRO-MT1-MMP plasmid with the Quick Change site-directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. The primers used for this mutation were: fw: GACTGCTCTTCTGCCAGCG and rv: CGCTGGCAGAAGAGCAGTC.

GST and MT1-, MT2-, MT3- and MT5-MMP cytosolic tail GST fusion proteins were obtained by direct cloning (Fig. 8). PGEX-4T2 vector was digested with BamH I and Xho I and the DNA sequences corresponding to the cytosolic tails flanked with BamH I and Xho I sites were ordered (Fig. 8B and C). The oligonucleotides were heated for 5 min at 94ºC and cooled slowly for hybridization. Hybridized oligonucleotides were added to digested pGEX-4T2 and ligation was performed by T4 ligase.

A



B



C

Construct	Oligonucleotides
MT1-MMPcyt	Fw: GATCCAGACGCCATGGGACCCCCAGGCGACTGCTCTACTGCCAGCGTTCCCTGCTGGACAAGGTCTGAC Rv: TCGAGTCAGACCTTGTCCAGCAGGGAACGCTGGCAGTAGAGCAGTCGCCTGGGGGTCCCATGGCGTCTG
MT2-MMPcyt	Fw: GATCCCAGCGCAAGGGTGCGCCACGTGTCCTGCTTTACTGCAAGCGCTCGCTGCAGGAGTGGGTCTGA Rv: TCGATCAGACCCACTCCTGCAGCGAGCGCTTGCAGTAAAGCAGGACACGTGGCGCACCCTTGCGCTGG
MT3-MMPcyt	Fw: GATCCAAGAGGAAAGGAACACCCCGCCACATACTGTACTGTAAACGCTCTATGCAAGAGTGGGTGTGA Rv: TCGATCACACCCACTCTTGCATAGAGCGTTTACAGTACAGTATGTGGCGGGGTGTTCCTTTCCTCTTG
MT5-MMPcyt	Fw: GATCCAAGAACAAGACAGGCCCTCAGCCTGTCACCTACTATAAGCGGCCAGTCCAGGAATGGGTGTGA Rv: TCGATCACACCCATTCCTGGACTGGCCGCTTATAGTAGGTGACAGGCTGAGGGCCTGTCTTGTTCTTG

Figure 8: Cloning of the MT-MMP cytosolic tails in pGEX-4T2 vector. A) Diagram of pGEX-4T2 vector. B) Cloning strategy. C) Oligonucleotides used for the cloning of MT-MMP cytosolic tail cDNA sequences in pGEX-4T2 vector.

The cytosolic tail mutants of MT1- and MT2-MMP, K581W and W668K respectively, were performed with Quick Change site-directed Mutagenesis kit (Stratagene). The primers used for

these mutations were; fw: CAGCGTTCCCTGCTGGACTGGGTCTGATAGAAGCCG and rv: CGGCTTCTATCAGACCCAGTCCAGCAGGGAACGCTG for MT2-MMP-K581W and fw: GCGC TCGCTGCAGGAGAAGGTCTGAAATTCTGCAG and rv: CTGCAGAATTTCAGACCTTCTCCTG CAGCGAGCGC for MT2-MMP-W668K. All constructs were finally sequenced at the Genomic Service of the Spanish National Cancer Research Centre (CNIO).

To construct a putative catalytically inactive MT2-MMP, the putative amino acid of its active site E267 was changed for an alanine. This mutation was performed with Quick Change site-directed Mutagenesis kit (Stratagene). The primers used for this mutation were; fw: CTGGTGGC AGTGCATGCGCTGGGCCACGCGCTG and rv: CAGCGCGTGGCCCAGCGCATGCACTGCCA CCAG.

7. Glutathione Sepharose-Transferase (GST) fusion protein production

pGEX-4T2 constructs were used to transform *Escherichia coli* BL21 cells. For protein production, cells induced for 3 h with 500 mM isopropyl β-D-thiogalactoside were lysed using Cell Lytic Express (Sigma-Aldrich) supplemented with a proteinase inhibitor cocktail (Roche) and 0.2 mg/ml PMSF. Fusion proteins were recovered by glutathione-sepharose 4B beads (GE Healthcare). The amount of fusion proteins was estimated by Coomassie Blue staining. Bands were quantified using Image J software.

8. Pulldown assays

8.1. Pulldown with GST fusion proteins

Cells were lysed (lysis buffer: 50 mM Tris pH7.5, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxyicholate, 500 mM NaCl, 10 mM $MgCl_2$, proteinase inhibitor cocktail (Roche), 0.2 mg/ml PMSF, 25 mM NaF and 1 mM Na_3VO_4) and the obtained lysates were precipitated with GST fusion proteins or GST alone. After washing (10-50 mM Tris ClH pH7.5, 0.5% Triton X-100, 150 mM NaCl and 1 mM DTT), bound proteins were eluted by boiling in Laemmli buffer. Samples were run in SDS-PAGE and western blot or protein identification by mass spectrometry (MS) was performed.

8.2. Pulldown with biotinylated peptides

Cells were lysed with 1% NP40-TBS (with proteinase inhibitor cocktail (Roche), 0.2 mg/ml PMSF, 25 mM NaF and 1 mM Na_3VO_4) and N-terminal biotinylated peptides corresponding to the cytosolic tails of the different WT or MT-MMP mutants coupled with neutravidin beads were added. After overnight incubation, the precipitated was washed 6 times with 0.1% NP40-TBS and resuspended in Laemmli buffer for SDS-PAGE and western blot analysis.

9. Coimmunoprecipitation

For coimmunoprecipitation assays HeLa cells were transfected with FuGENE 6 (Roche) with MT1-MMP-HA, MT1-MMP-K581W-HA, MT2-MMP-HAFlag and MT2-MMP-W668K-HAFlag sequences all of them in PCR3.1. After 24 h, cells were lysed in 1% NP40-TBS with proteinase inhibitor cocktail (Roche), 0.2 mg/ml PMSF, 25 mM NaF and 1 mM Na_3VO_4. Lysates were

incubated for 1 h at 4ºC with 5 µg of anti-HA antibody. Immunoprecipitates were washed 6 times with 0.1% NP40-TBS and separated by 10% SDS-PAGE under reducing conditions. After transferring to nitrocellulose membrane (Bio-Rad) western blot was performed.

10. Western blot

For western blot, membranes were blocked with 5% BSA-PBS. Next, primary antibodies, diluted in 2% BSA-PBS, were incubated for 1h at RT. After, membranes were washed with 0.2% Tween 20-PBS and then they were incubated for 1 h at RT with a horseradish peroxidase-conjugated antibody. Finally, membranes were again washed and protein bands were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Densitometric analysis of band intensity was done using Image J software.

11. Mass Spectrometry

Pulldown SDS-PAGE was stained with Coomasie Blue and the proteins corresponding to the observed bands, and the equivalent regions or the other lanes, were identified by Liquid Chromatography/Mass Spectrometry (LC-MS) in the CNIC Proteomic Unit (Fig. 9). For protein identification fragmentation spectra were searched against MSDB database using the MASCOTTM program. All identifications were performed by Data Analysis software (Bruker).

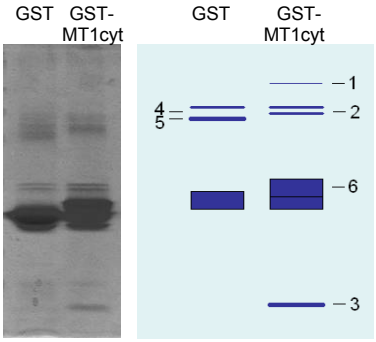


Figure 9: Example of a pulldown experiment. GST fusion proteins and control ones (only GST) were added to HMEC lysates. In the left the resulting pulldown resolved in a SDS-PAGE and stained with Coomasie Blue is depicted. In the right a diagram of the specific bands excised for LC-MS is shown. Equivalent regions of the observed bands in the other lines were also sequenced.

12. Subcellular fractionation

For subcellular fractionation two methods were used. In p130Cas experiments, particulate and soluble fractions were obtained from cultured OC progenitors as previously explained (Del Pozo *et al.*, 2002). Following this protocol two cell fractions corresponding to the plasma membrane proteins and the cytosolic ones were obtained. Briefly, cells were lysed with ice-cold hypotonic lysis buffer (10 mM Tris-HCl pH 7.4, 1.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, 1 mM Na_3VO_4, 0.2 mg/ml PMSF, 1 µg/ml aprotinin and 1 µg/ml leupeptin) for 5 min. Homogenates were centrifuged at 1700 rpm for 3 min and the supernatants were then spun at 14000 rpm for 30 min at 4°C. Sediment (particulate fraction) and supernatant (cytosolic fraction) were collected. Equal protein amounts were analyzed by western blot and densitometry analysis was performed with

Quantity One or ImageJ software. The amount of p130Cas in the particulate fraction (normalized to β1 integrin levels) was calculated.

For MT1-MMP analysis in moesin interfered cells subcellular fractionation using Triton X-114 was performed. With this method two cell fractions are obtained; one is formed of caveolae enriched membrane compartment and the other is formed of cytosolic proteins. Briefly, cells were lysed in 1.5% Triton X-114-TBS with proteinase inhibitor cocktail (Roche), 0.2 mg/ml PMSF, 25 mM NaF and 1 mM Na_3VO_4. After centrifugation at 14000 rpm for 15 min at 4ºC, supernatants were incubated at 37ºC for 2 min. Afterwards, samples were spun at 10000 rpm for 5 min at RT. The two phases formed, corresponding to the aqueous and the hydrophobic phase, were separated and analyzed by western blot. Densitometry analysis was performed with Quantity One or ImageJ software.

13. Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde in PBS for 10 min at RT, permeabilized for 15 min with 0.1% Triton-X-100 in blocking buffer (2% BSA, 10% FBS, 1 mM $MgCl_2$ and 1 mM EDTA in PBS) at 4ºC and then blocked for 1 h in the same buffer. Primary antibodies and Alexa-conjugated secondary antibodies were incubated for 1 h at RT. For nuclear staining Hoescht 33342 (Sigma) was used. Coverslips were mounted with Prolong mounting medium (Invitrogen). Samples were analyzed in a SPE Leica photomicroscopy and images were obtained with Leica LAS AF Software.

14. ELISA

Streptavidin binding plates (96 wells, Pierce) were incubated at 4ºC for 3 h in 0.1 M Na_2CO_3 buffer (pH 9.6) containing 40 μM of biotinylated peptides (GenScript) corresponding to the MT-MMP cytosolic tails: MT1-MMPcyt (563RRHGTPRRLLYCQRSLLD KV582), MT1-MMPcyt-K581W, MT1-MMPcyt-RRH563AAA, MT1-MMPcyt-RR569AA, MT1-MMPcyt-R576A, MT1-MMPcyt-S577A, MT2-MMPcyt (650QRKGAPRVLLYCKRSLQEWV669), MT2-MMPcyt-E667A, MT2-MMPcyt-W668K, MT2-MMPcyt-669ΔV or MT3-MMPcyt (588KNKTGPQPVTYYKRPVQEW V607). To avoid non-specific binding to biotinylated peptides samples were blocked with 2% BSA/TBS-Tween containing 10% FBS overnight at 4ºC. GST-tagged recombinant ZO-1PDZ1-3, ZO-1PDZ1, ZO-1PDZ2, ZO-1PDZ3, moesin, N-moesin, C-moesin or GST alone was added to the wells in equal concentrations and incubated at RT for 1 h. After extensive washing, GST protein binding to the cytosolic tail sequences was detected using anti-GST Ab and HRP-based detection (Fig. 10). For the analysis of the results optical density of an empty well was subtracted from all the absorbance data obtained.

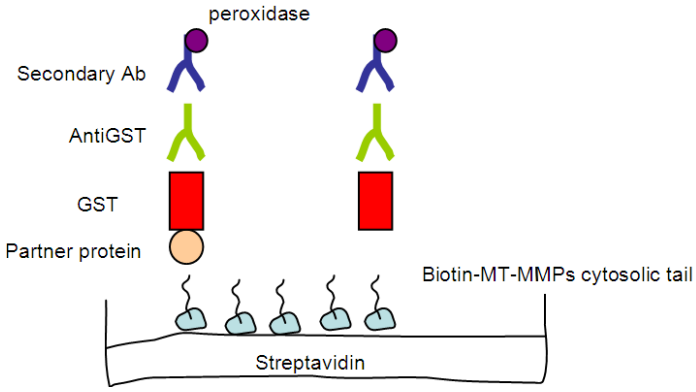


Figure 10: Diagram of ELISA components

15. Surface plasmon resonance

N-terminal biotinylated synthetic peptides corresponding to the cytoplasmic domain of MT1-, MT2-, MT3-MMP or MT2 mutant MT2-MMPcyt-669ΔV were immobilized on a streptavidin sensor chip. GST-ZO1-PDZ2 in running buffer (10 mM Hepes pH 7.4, 100 mM NaCl, 0.005% NP40) was perfused (30 µl/min) over these analytes. Binding was monitored with a BIAcore 2000 instrument (BIAcore AB. Uppsala, Sweden) and MT2-MMPcyt-669ΔV binding was subtracted from each curve. After 4 min of association, the sample solution was replaced by running buffer, allowing the complex to dissociate. The surface was regenerated through 1 min pulses of 1 M NaCl, 0.05 M NaOH (Fig. 11). The dissociation rate constants were analyzed using BIAevaluation 3.1 software from BIAcore AB (Uppsala, Sweden).

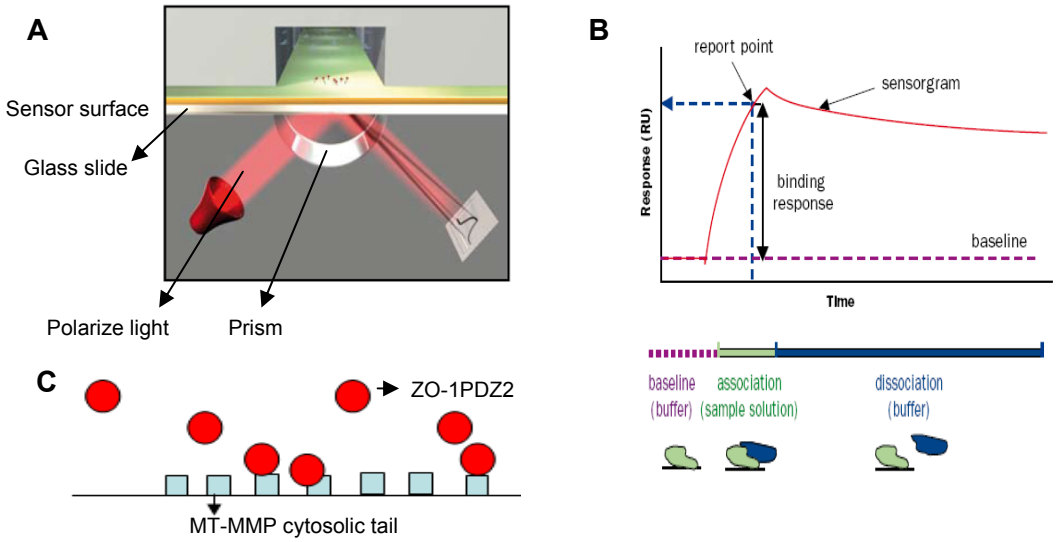


Figure 11: Surface plasmon resonance methodology (SPR). A) Fundamentals of how the interaction between two molecules is detected by SPR. As molecules are immobilized on a sensor surface, the refractive index at the interface between the surface and the solution flowing over the surface changes, altering the angle at which reduced-intensity polarized light is reflected from the supporting glass plane. The change in angle caused by association or dissociation of molecules from the sensor surface is proportional to the mass of bound material and is recorded in a sensorgram. B) Sensorgram. It provides real-time information about an interaction, with binding responses measured in response units (RU). C) Representation of MT-MMP cytosolic tail and ZO-1PDZ2 interaction in the SPR assays.

16. RT-PCR

Total RNA was extracted with Trizol (Invitrogen) and cDNA was prepared with Omniscript reagent (Qiagen). RT-PCR was conducted with primers for mouse MT1-MMP and GADPH, and human MT2-MMP and actin (Hikita *et al.,* 2006; Jung *et al.*, 2003). Products of PCR reactions were separated on 2% agarose gels.

17. Cellular functional models

17.1. MDCK cyst formation

MDCK cysts were grown in 3D Matrigel cultures (BD, San Jose, CA). Cells were trypsinized to a single cell suspension at 1.5×10^4 cells/ml in complete medium containing 2% Matrigel. Suspensions were plated into 8-well coverglass chambers (Nunc, Rochester, NY) pre-coated with 5 µl of 100% Matrigel. Cells were grown for 4 days before fixation with 4% PFA.

17.2. MDCK polarization

For MDCK cell polarization cells were cultured on transwell filters (Costar) for 3-5 days (Oztan *et al.,* 2008).

18. F-actin quantification

Polarized MDCK cells were fixed with 4% PFA and stained for F-actin (Phalloidin-Texas Red). Pictures for Z axis were taken and apical and basal actin was measured with Metamorph software.

19. Statistical analysis

Test and control samples were compared for statistical significance by using the Student´s t test. Differences were considered statistically significant at $p<0.05$.

Results

1. Characterization of MT1-MMP cytosolic tail binding to p130Cas in myeloid progenitors

1.1. The mutation of the unique tyrosine of MT1-MMP cytosolic tail decreases its binding to p130Cas

MT1-MMP/p130Cas binding has been reported in endothelial cells (Gingras *et al.,* 2008). Pulldown assays in myeloid progenitors confirmed that p130Cas could bind to the cytoplasmic domain of MT1-MMP (Fig. 12). It had been shown that S1P induced phosphorylation of MT1-MMP on its unique cytoplasmic tyrosine residue (Nyalendo *et al.,* 2007). Moreover, Gingras and coll. saw that p130Cas/MT1-MMP complex contained pMT1-MMP, indicating a possible interaction of the tyrosine phosphorylated form of MT1-MMP with p130Cas (Gingras *et al.*, 2008). To test this hypothesis we generated a fusion protein in which the unique tyrosine of the MT1-MMP cytosolic tail was mutated to a phenylalanine, which is unable to be phosphorylated. Pulldown assays were performed with this mutated fusion protein (GST-MT1-MMPcyt-Y573F) and WT one (GST-MT1-MMPcyt). As shown in Figure 12, this MT1-MMP mutant bound p130Cas less efficiently, showing that MT1-MMP Tyr 573 participates in MT1-MMP association with p130Cas.

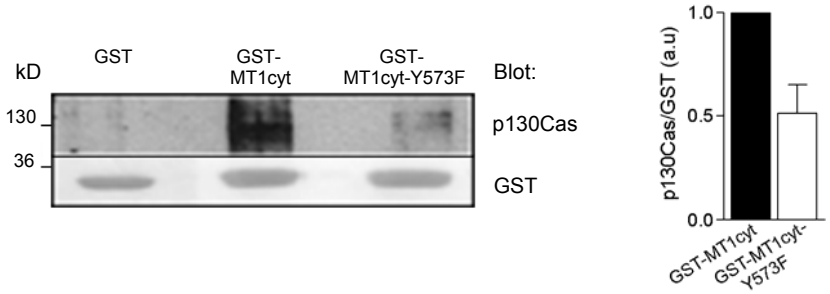


Figure 12: The mutation of MT1-MMP cytosolic tail tyrosine decreases its binding to p130Cas. GST fusion proteins of the MT1-MMP cytosolic tail (GST-MT1cyt) and a mutated version in which Y573 is replaced by F (GST-MT1cyt-Y573F) were used in pulldown assays; lysates from BM cells treated for 4 days with RANKL and M-CSF were used in these assays. A representative blot of p130Cas is shown. The histogram shows arithmetic means ± SE of densitometric quantification of the p130Cas/GST ratio normalized to GST-MT1cyt values (n = 3).

1.2. p130Cas binding to MT1-MMP is required for proper p130Cas localization at the cell membrane

We analyzed the possible contribution of MT1-MMP to p130Cas subcellular localization. For this purpose p130Cas localization in WT and MT1-MMP null myeloid progenitors was determined by cell fractionation and immunofluorescence assays. As it is depicted in Figure 13A, the amount of p130Cas in the plasma membrane was significantly decreased in MT1-MMP null progenitors.

To check that this decrease was directly due to the absence of MT1-MMP, retroviral reexpression of MT1-MMP was performed. After infection, human MT1-MMP expression was detected by RT-PCR and western blot (Fig. 13B). Significant increase in the amount of p130Cas in the membrane fraction of null myeloid progenitors was obtained when MT1-MMP was re-expressed (Fig. 13C). These data indicate that MT1-MMP is required for proper p130Cas localization at the cell membrane of these cells.

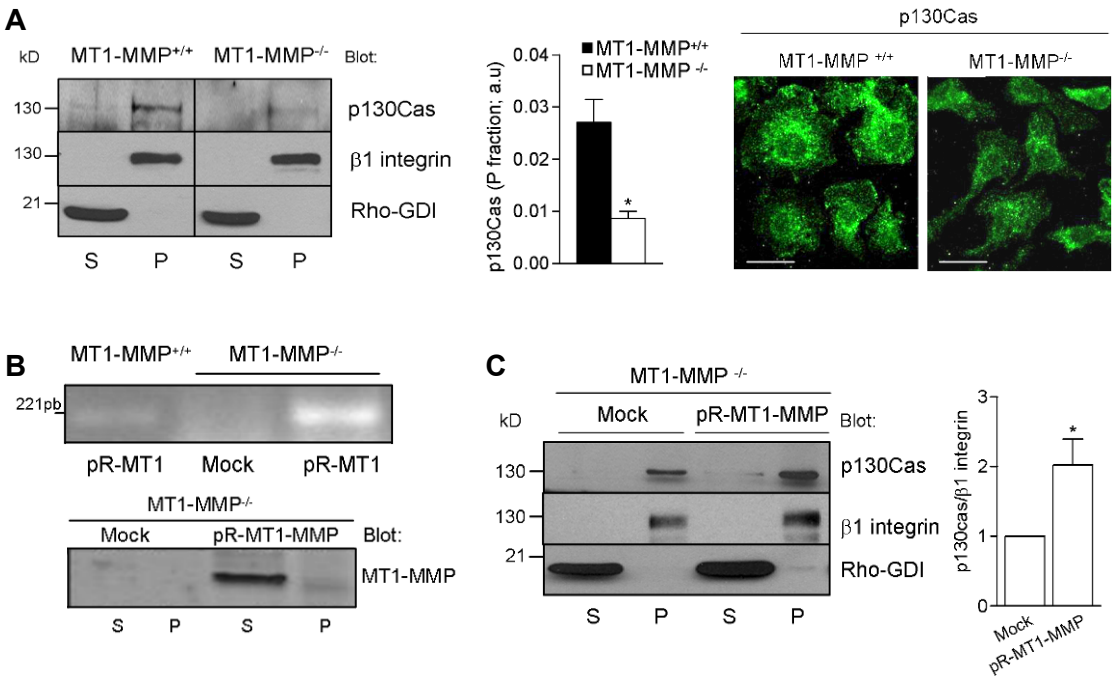


Figure 13: MT1-MMP is required for proper p130Cas localization at the cell membrane. A) p130Cas localization in WT and MT1-MMP null myeloid progenitors was analyzed by cell fractionation. Cells were fractionated and particulate/membranous (P) and soluble/cytosolic (S) fractions were analyzed by western blot; a representative blot is shown. The histogram shows arithmetic means ± SE of densitometric quantification of the p130Cas/β1 integrin ratio in the particulate fraction (n = 4 experiments from 20 mice). Images of cells stained for p130Cas (green) are shown. The scale bar indicates 20 μm. B) BM progenitors from WT or MT1-MMP null mice were infected with retrovirus encoding human MT1-MMP (pR-MT1-MMP) and cultured with M-CSF and RANKL for 4 days. Human MT1-MMP expression was detected by RT-PCR (upper panel) and western blot (botton panel). Mock, infection with the empty virus. C) Infected cells were fractionated, and particulate (P) and soluble (S) fractions were analyzed by western blot for p130Cas; a representative blot is shown. The histogram shows arithmetic means ± SE of densitometric quantification of the p130Cas/β1 integrin ratio in the particulate fraction (n = 5 independent experiments from 15 mice).

1.3. MT1-MMP cytosolic tyrosine is important for myeloid progenitor fusion

To test the relevance of the MT1-MMP cytosolic tyrosine Y573, involved in p130Cas assembling, in MT1-MMP function in myeloid cell fusion, rescue experiments with MT1-MMP or MT1-MMP tyrosine mutant were performed. Retroviral expression of MT1-MMP was able to rescue the OC fusion phenotype of MT1-MMP null cells. However, MT1-MMP-Y573F could not rescue this phenotype. Since we have shown that MT1-MMP cytosolic tail tyrosine is important for MT1-MMP interaction with p130Cas, this result indicates that MT1-MMP Y573 might be required for myeloid progenitor fusion by its binding to p130Cas (Fig. 14).

In conclusion, we have identified a novel signaling pathway for MT1-MMP regulation of myeloid progenitor fusion. This pathway involves the association of the MT1-MMP cytosolic tail with p130Cas. Our data not only confirm MT1-MMP-p130Cas interaction previously shown in endothelial cells (Gingras *et al.*, 2008), also shows that the phosphorylation of MT1-MMP Tyr573, in the cytosolic tail, plays a role in its association with p130Cas. Moreover, MT1-MMP participates in the recruitment of p130Cas to myeloid progenitor cell membrane, as it is shown by the decrease in p130Cas membrane targeting in MT1-MMP null progenitors and the rescue of this targeting by reexpression of MT1-MMP in null cells. The importance of the interaction of Tyr573

with p130Cas for myeloid cell fusion is demonstrated by the inability of the MT1-MMP Y573F mutant to rescue the multinucleated phenotype.

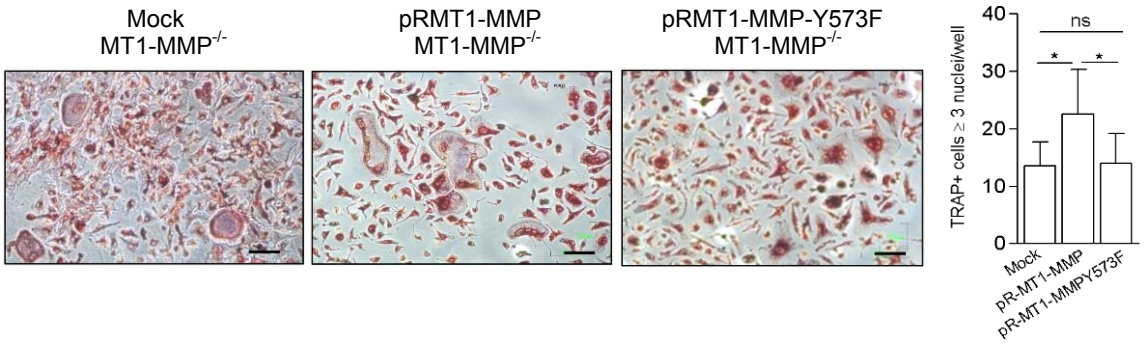


Figure 14: MT1-MMP cytosolic tyrosine is important for myeloid progenitor fusion. MT1-MMP null BM progenitors were infected with empty retrovirus (Mock) or retrovirus encoding non-mutated or Y573F mutated human MT1-MMP (pR-MT1-MMP or pR-MT1-MMP-Y573F) and cultured under osteoclastogenic conditions for 12 days. Images of TRAP+ cells are shown. The scale bars indicate 100 μm. The histogram shows arithmetic means ± SE of the number of TRAP+ cells containing ≥ 3 nuclei (n = 10).

2. Characterization of MT1-, MT2- and MT3-MMP binding to ERM proteins

2.1. MT1-, MT2- and MT3-MMP cytosolic tails bind to N-moesin *in vitro*

Transmembrane MT-MMPs have two or three positive clusters in their cytosolic tails (Fig. 15A) that make them good candidates for interacting with ERM proteins (Legg and Isacke, 1998). Moreover, crystal structure of MT1-MMP/radixin complex has been reported (Terawaki *et al.,* 2008). To investigate possible MT-MMP/ERM interactions ELISA assays were first performed (Fig. 10 of Materials and Methods). As depicted in Figure 15B, MT1-, MT2- and MT3-MMPs cytosolic tails can directly bind to moesin. To further characterize these interactions, we analyzed which domain of moesin was involved in the binding to MT1-, MT2- and MT3-MMP cytosolic tails. As expected, the cytosolic tails of these metalloproteinases bound to N-terminal moesin and no binding to C-terminal moesin was detected. Moreover, the cytosolic tails had much more affinity for N-terminal moesin that for FL-moesin (Fig. 15B). This increased affinity fits with the ERM activation model previously explained (Fig. 6 of the Introduction) (Tsukita *et al.,* 1994). According with this model, in inactive FL-ERM their N-terminal domain is bounded tightly to its C-terminal domain masking their binding sites to other proteins (Gary and Bretscher, 1995). Because of this, when C-terminal moesin was removed, interaction of N-terminal moesin with MT-MMPs was increased.

2.2. Characterization of MT1-MMP residues involved in moesin interaction

We focused on MT1-MMP for detailed analysis of MT-MMMP/moesin interaction. To analyze which residues of the MT1-MMP cytosolic tail were involved in its interaction with moesin, ELISA assays with MT1-MMP mutants were performed. As ERM proteins bind preferably to polybasic clusters of transmembrane type 1 cytosolic tails (Legg and Isacke, 1998; Yonemura *et al.*, 1998), we generated MT1-MMP mutants in which each of the polybasic and single positive residues were mutated to alanines (Fig. 15C). As it is can be seen in Figure 15C, the juxtamembrane polybasic region of MT1-MMP (RRH563) is essential for its binding to N-moesin. In the case of the absence of the middle polybasic region of MT1-MMP cytosolic tail (RR569), significantly

reduced binding was also obtained. Finally, when distal MT1-MMP arginine 576 was mutated, no differences in moesin binding were detected.

In addition to polybasic regions, serine residues have been also described to play a role in the binding of transmembrane type 1 proteins to ERMs (Serrador *et al.,* 2002a). To test the relevance of MT1-MMP cytosolic tail unique serine, a mutant in which this serine was substituted for an alanine was used. As it is depicted in Figure 15C, no changes in moesin affinity for this mutant compared with the WT sequence were observed.

Finally, when MT1-MMP or MT2-MMP mutants of the PDZ binding motif (Fig. 19D) were studied no differences in binding of these proteins to moesin were obtained (data not shown); similar results were obtained with a mutant in which MT1-MMP last positive residue (K581) was mutated. Thus, it can be concluded that this MT1-MMP last positive residue does not affect moesin binding.

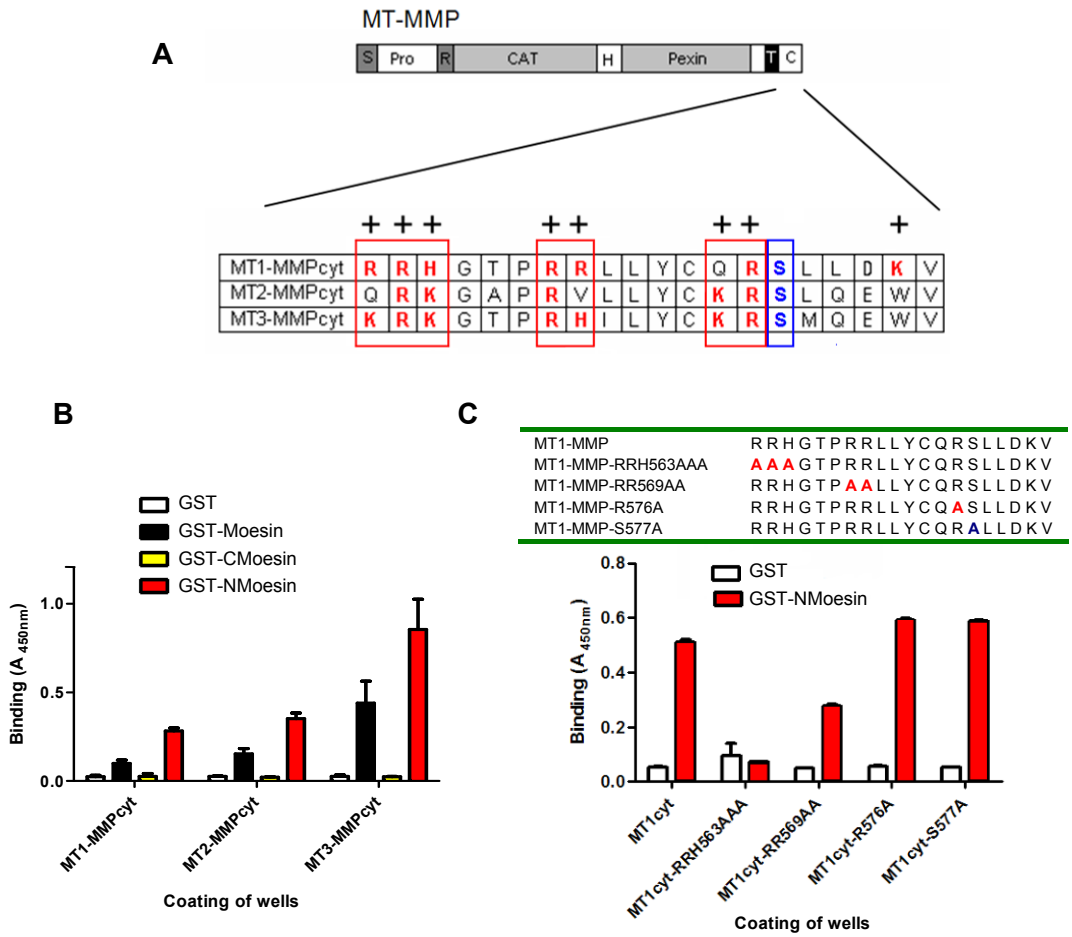


Figure 15: Moesin binds to MT1-, MT2- and MT3-MMP cytosolic tails. A) Diagram of the cytosolic residues of MT1-, MT2- and MT3-MMP. Positive amino acids are marked in red and serine residue is in blue. B) Binding of equal amounts of GST-fused moesin, N-terminus-moesin, C-terminus-moesin or GST alone was measured on microtiter plate wells coated with the indicated biotinylated peptides from MT-MMP cytosolic tails native or mutant sequences. Bound GST was detected using anti-GST antibody in an ELISA assay. Error bars represent standard error of the mean (SEM) from three independent experiments. C) Table of the native and mutant MT1-MMP cytosolic tail sequences used in the assays. Binding of equal amounts of GST fused N-terminus-moesin or GST alone was measured on microtiter plate wells coated with the indicated biotinylated peptides from MT1-MMP cytosolic tail native or mutant sequences. Bound GST was detected using anti-GST antibody in an ELISA assay. Error bars represent standard error of the mean (SEM) from three independent experiments.

For all mentioned before, it can be concluded that MT1-, MT2- and MT3-MMP bind *in vitro* to N-terminal domain of moesin. Moreover, the juxtamembrane polybasic cluster of MT1-MMP is a key region for its binding to moesin and MT1-MMP middle polybasic cluster also contributes to this binding.

2.3. Analysis of MT1-MMP moesin binding in the cellular context

To analyze MT1-MMP interaction in the cellular context coimmunoprecipitation assays were performed. HUVEC lysates were precipitated with an MT1-MMP or an IgG control antibody and a western blot against moesin was performed. As it is depicted in Figure 16A, moesin coimmunoprecipitates with MT1-MMP but not with the IgG control. These preliminary data need to be confirmed.

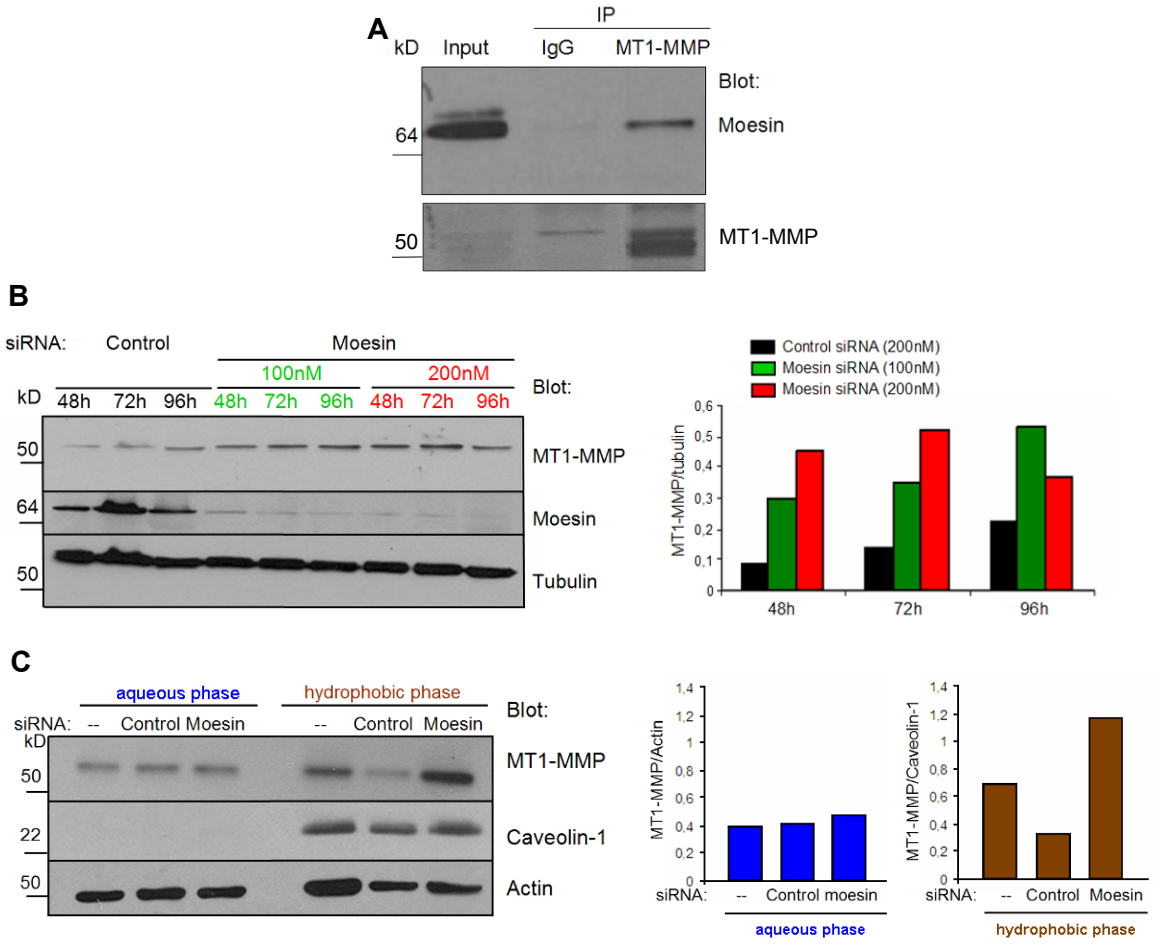


Figure 16: Analysis of MT1-MMP moesin binding in the cellular context. A) Moesin coimmunoprecipitates with MT1-MMP. HUVEC lysates were immunoprecipitated (IP) with MT1-MMP antibody or an IgG control. Immunoprecipitates and 5% of lysate (input) were resolved by SDS-PAGE and immunoblotted for moesin and MT1-MMP. B) MT1-MMP is increased in moesin-interfered cells. HUVEC were interfered with siRNA control or moesin siRNA. After 48, 72 or 96 cells were lysed and an SDS-PAGE was performed with equivalent protein amount of each condition. Afterwards, a western blot against MT1-MMP, moesin and tubulin was done. MT1-MMP corresponding bands were quantified (Image J) and normalized to tubulin ones. C) MT1-MMP is increased in the hydrophobic phase of moesin-interfered cells. HUVEC were interfered with an siRNA control or moesin siRNA. Cells were lysed and subcellular fractionation was performed. Aqueous and hydrophobic phase were analyzed by SDS-PAGE and western blot against MT1-MMP, caveolin-1 and actin. Corresponding bands were quantified (Image J) and MT1-MMP/actin and MT1-MMP/caveolin-1 ratios were calculated.

As moesin is a membrane linker of transmembrane proteins to actin cytoskeleton, we analyzed the effect of decreased moesin levels in MT1-MMP subcellular protein pools. Firstly, global MT1-MMP levels were checked. HUVEC were interfered for 48, 72 or 96h with 100nM or 200nM of moesin siRNA or 200nM of control siRNA. Interestingly, MT1-MMP total protein level was increased correlating with the decrease of moesin in a dose dependent manner (Fig. 16B). Subsequently, we analyzed what MT1-MMP pool was increased in moesin-interfered cells. For this purpose, HUVEC control or moesin interfered cell lysates were subcellular fractionated with Triton X114 and MT1-MMP levels of the aqueous and hydrophobic phase were analyzed. As shown in Figure 16C, the increased MT1-MMP levels observed in the interfered cells are due to an increase of the MT1-MMP protein amount in the hydrophobic phase (caveolin-1-rich regions). These preliminary data require to be confirmed.

3. Search of new molecular associations of MT-MMP cytosolic tails and analysis of their possible cellular function

3.1. Search of new molecular associations of MT-MMP cytosolic tails

To search for new molecular partners of MT-MMP cytosolic tails a proteomic approach was performed. Firstly, the cDNA corresponding to the cytosolic tails of MT1-, MT2-, MT3- and MT5-MMP was directly cloned in pGEX-4T2 (Fig. 8 of Materials and Methods) to generate GST-MT-MMPcyt fusion proteins that were used in pulldown assays with HMEC lysates. The obtained bands were excised from the gel and identified by MS in collaboration with the Proteomic Unit at CNIC. The proteins identified to interact with the MT-MMP cytosolic tails are listed in Figure 17A.

A

	Candidate partners
MT1-MMPcyt	Vimentin
MT2-MMPcyt	ZO-1 ZO-2 Vimentin
MT3-MMPcyt	ZO-1 ZO-2 Annexin A1 Annexin A2 β-tubulin
MT5-MMPcyt	ZO-1 ZO-2

B

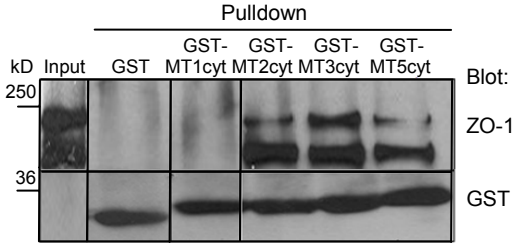


Figure 17: Identification of MT-MMP cytosolic tail candidate interacting proteins. A) Identified proteins associated with MT-MMP cytosolic tails. HMEC lysates were precipitated with GST or GST fused with MT1-, MT2-, MT3- and MT5-MMP cytosolic tails. Precipitates were run in a 10% SDS-PAGE and bands detected by Coomassie Blue staining were excised and identified by MS. B) Confirmation of ZO-1 association detected by MS. Lysates prepared from HUVEC were subjected to pulldown with the indicated GST or GST fusion proteins. The precipitates and 5% of lysate (input) were resolved by SDS-PAGE and immunoblotted for ZO-1. The immunoblot shown is representative of two experiments.

Next, to validate the candidate proteins obtained by MS, pulldown with the fusion proteins and western blot for some of the identified proteins was performed. No signal was obtained for annexin A1 and A2 binding to MT3-MMP by pulldown-WB (data not shown). Validation of ZO-1 association with MT2-, MT3- and MT5-MMP was however obtained (Fig. 17B). Taking this into

account, and the fact that ZO-1 contains 3 PDZ domains that are susceptible to bind to the PDZ binding motif of the MT-MMP cytosolic tails, we focus on ZO-1 for further analysis.

3.2. MT2- and MT3-MMP interact directly with the N-terminus domain of ZO-1

To characterize the interaction of MT2- and MT3-MMP with ZO-1, ELISA assays were performed. For this purpose, N-terminal domain of ZO-1, including its 3 PDZ domains, was used. This construct was added to streptavidin wells coated with the different biotinylated MT-MMP cytosolic tails (Fig. 10 of Materials and Methods). As it is depicted in Figure 18, we observed a direct binding of the cytosolic tails of MT2- and MT3-MMP to ZO-1. MT1-MMP could also bind to ZO-1 but much less efficiently than MT2- and MT3-MMP, according to MS and pulldown-WB results.

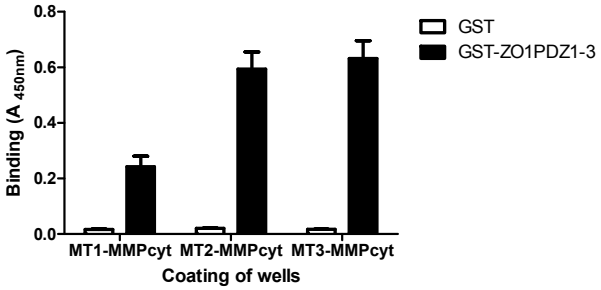


Figure 18: Binding of N-terminus of ZO-1 (PDZ1-3) to MT1-, MT2- and MT3-MMP cytosolic tails. Binding of equal amounts of GST-fused ZO1-PDZ1-3 or GST alone was measured on microtiter plate wells coated with the indicated peptides corresponding to MT-MMP cytosolic tails. Bound GST was detected using anti-GST antibody in an ELISA assay. Error bars represent standard error of the mean (SEM) from five independent experiments.

3.3. The three PDZ domains of ZO-1 bind MT2-MMP and MT3-MMP

To discriminate which ZO-1 PDZ domains were responsible for the interaction with MT2- and MT3-MMP, fusion proteins of each of the three PDZ domains of ZO-1 with GST were used. As it is shown in Figure 19A the 3 PDZ domains of ZO-1 were able to bind to MT2- and MT3-MMP cytosolic tails, although PDZ3 presented less affinity. In the case of MT1-MMP, as expected, nearly no binding to any PDZ domain was detected.

To deeper analyze MT2- and MT3-MMP interaction with ZO-1, the binding of the cytosolic tails of these MT-MMPs to PDZ2 of ZO-1 was also characterized by Surface Plasmon Resonance experiments (SPR) (Currie *et al.,* 1999) in collaboration with Pascale Zimmerman and Ylva Ivarsson from the Faculty of Medicine, K.U.Leuven (Belgium). Biotinylated peptides corresponding to MT1-, MT2- and MT3-MMP cytosolic tails were coupled to the surface of the sensor chip and GST-ZO-1PDZ2 in solution was injected. In Figure 19B (left) the analysis of the concentration dependence of GST-ZO1PDZ2 for its binding to MT1-, MT2 and MT3-MMP cytosolic tails is depicted. From these curves, it was selected a concentration of 30 μM of GST-ZO1PDZ2, close to saturation, for performing the following analysis. In Figure 19B (right) SPR measurements are shown. For this analysis the binding of ZO1PDZ2 to MT2-669ΔV (data not shown) was used as a negative control and the values obtained for this peptide were substracted from MT2- and MT3-MMP results. MT2- and MT3-MMP binding to ZO-1 confirmed the previous

data obtained by ELISA and in the case of MT1-MMP very weak interaction with ZO-1PDZ2 was detected (data not shown).

3.4. Analysis of the key residues of MT2-MMP involved in its binding to ZO-1

As MT2-MMP has the closest cytosolic sequence to MT1-MMP (Fig. 4 of the Introduction) but it shows a different pattern regard to ZO-1 binding, we decided to focus on MT2-MMP and analyzed which amino acids of MT2-MMP cytosolic tail were responsible for its interaction with ZO-1. As MT2-MMP was binding to the PDZ domains of ZO-1 and it has a PDZ binding motif, the possibility that the five residues forming the PDZ binding motif were the ones responsible for the binding to ZO-1 arose as the most probable one.

A PDZ domain consists of six β-strands (βA-βF) and two α-helices and peptide ligands have been seen to bind in an extended groove between strand βB and helix αB (Harrison, 1996). For this binding last residue of the PDZ binding motif, called P_0, plays a key role (Harris and Lim, 2001). Moreover, the side chain of the third residue of the PDZ binding motif starting from the C-terminus, called P_{-2}, directly points into the base of a second peptide-binding groove (Daniels *et al*., 1998) (Fig. 19C). Because of this, regarding what residue the ligand has at P_{-2} the affinity to different types of PDZ can be determined (Fig. 29 of the Discussion).

To analyze the relevance of the P_0 and P_{-2} residues in the interaction of MT2-MMP cytosolic tail and ZO-1, ELISA assays using MT2-MMP mutants were performed (Fig. 19D). As shown in Figure 19E, when last valine of MT2-MMP cytosolic tail was deleted no interaction with any of the PDZ domains of ZO-1 was detected. As P_0 residue is essential for the peptide binding to PDZ domains, it can be concluded that MT2-MMP cytosolic tail is binding to the PDZ domains of ZO-1 through its PDZ binding motif. However, no differences in binding were obtained when P_{-2} residue was mutated (MT2-MMP-E667A).

Zhang and coll. analyzed in detail ZO-1PDZ1 and PDZ3 domain binding properties and their results revealed that not only P_0 and P_{-2} residues were important for ligand recognition but also the penultimate residue of the ligand (P_{-1}) (Zhang *et al.,* 2006). Because of this we decided to explore the relevance of P_{-1} residue of MT2-MMP PDZ binding motif for the binding to ZO-1. By using MT2-MMPcyt-W668K and MT1-MMPcyt-K581W mutants we saw that, when lysine 581 of MT1-MMP was changed to a tryptophan, mimicking MT2-MMP cytosolic tail, MT1-MMP cytosolic tail was able to bind to the three PDZ domains of ZO-1. On the contrary, when the tryptophan 668 of MT2-MMP was changed for a lysine, mimicking MT1-MMP cytosolic tail, interaction with ZO-1 was abolished (Fig 19E). These results indicate that MT2-MMP tryptophan 668 is a key residue for its binding to ZO-1.

To analyze if the behaviour of these residues was the same in a more physiological context in which other proteins are present and ZO-1 has its complete sequence, precipitation of HUVEC lysates with MT1- and MT2-MMP biotinylated cytosolic tails and their mutants was performed. As shown in Figure 19F, similar results to ELISA experiments were obtained.

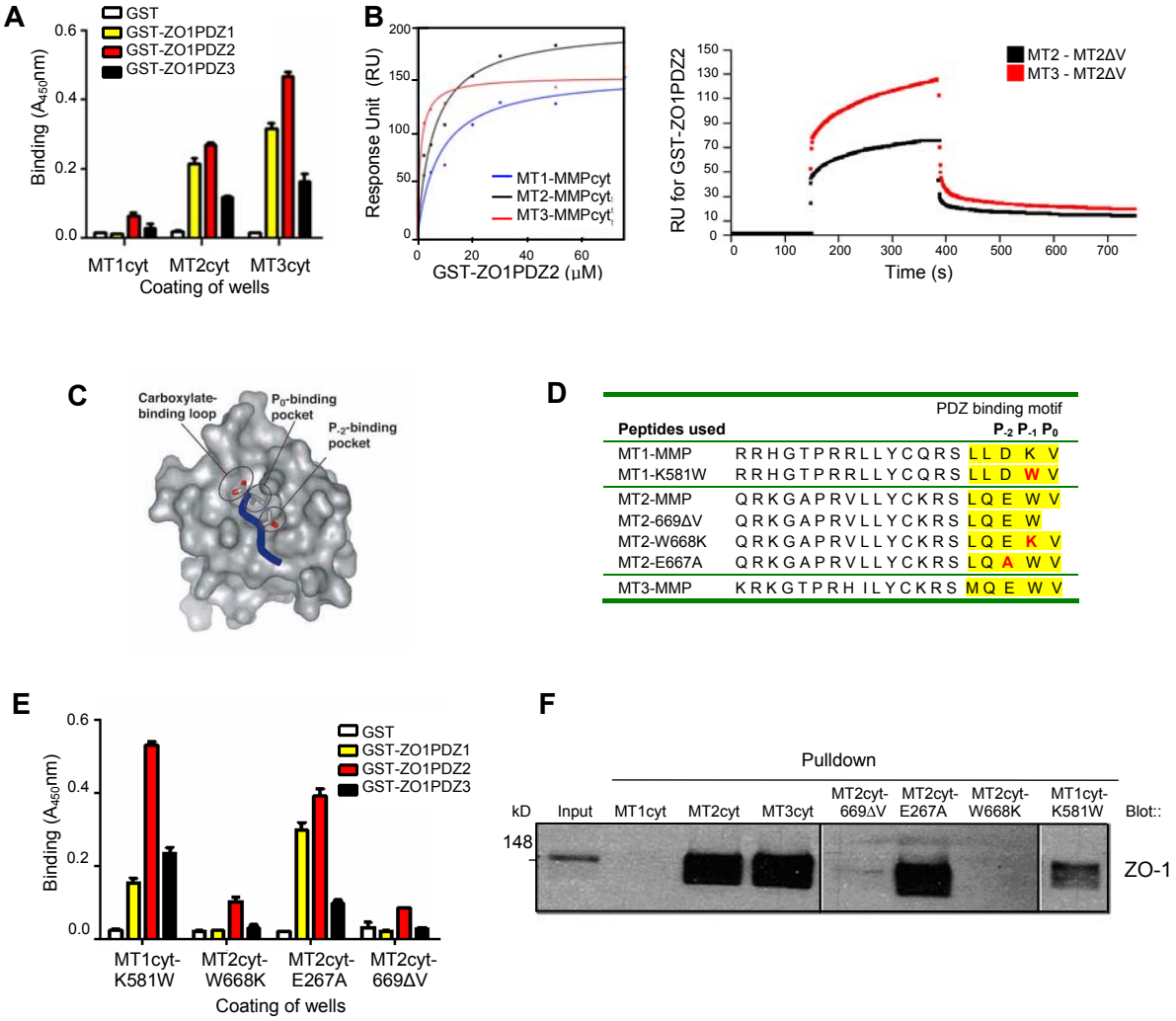


Figure 19: Molecular characterization of MT2-MMP and ZO-1 binding. A) Binding of equal amounts of GST fused ZO1-PDZ1, ZO1-PDZ2 and ZO1-PDZ3 or GST alone was measured on microtiter plate wells coated with the indicated peptides from MT-MMPs cytosolic tails. Bound GST was detected using anti-GST antibody in an ELISA assay. Error bars represent standard error of the mean (SEM) from three independent experiments. B) ZO-1 PDZ2 domain interacts with MT2- and MT3-MMP in SPR experiments. Left: purified GST-ZO1PDZ2 was perfused at different concentrations over a sensorchip coated with MT1-, MT2- and MT3-MMPcyt biotinylated peptides. Concentration dependence of GST-ZO1PDZ2 for its binding to the peptides was measured. Right: purified GST-ZO1PDZ2 was perfused at 30µM over a sensorchip coated with biotinylated peptides of MT2-MMP, MT2-MMP-669ΔV and MT3-MMP cytosolic tails. Values correspond to response units (RU) measured 4 minutes after perfusion of the protein. For negative control MT2-MMP-669ΔV was used and the values obtained for this peptide were subtracted (n=2). C) Diagram of a PDZ domain (PSD-95 PDZ domain 3) with a bound peptide (NH2-KQTSV-COOH, shown in blue). The side chains of the peptide P_0 residue (valine) and P_{-2} residue (threonine) are shown in stick form, as is the terminal carboxylate (Doyle *et al.*, 1996). D) Diagram of native and mutant MT-MMP cytosolic tails used in the assays. E) Binding of equal amounts of GST fused ZO1-PDZ1, ZO1-PDZ2 and ZO1-PDZ3 or GST alone was measured on microtiter plate wells coated with the indicated peptides from MT-MMP mutant cytosolic tails. Bound GST was detected using anti-GST antibody in an ELISA assay. Error bars represent standard error of the mean (SEM) from three independent experiments. F) Lysates prepared from HUVEC were subjected to precipitation with the indicated biotinylated peptides. Precipitates and 5% of lysate (input) were resolved by SDS-PAGE and immunoblotted for ZO-1. The immunoblot shown is representative of two experiments.

Based in all these results it can be concluded that MT2-MMP and MT3-MMP bind through their PDZ binding motif to the three PDZ domains of ZO-1, although with higher affinity for PDZ1 and PDZ2. In the PDZ binding motif the tryptophan 668 of MT2-MMP is essential for the interaction and it is the responsible of the different affinity of MT1-MMP and MT2-MMP for ZO-1.

3.5. Analysis of MT2-MMP/ZO-1 association in the cellular context

To confirm the interaction between MT2-MMP and ZO-1 in the cellular context, we expressed MT1-MMP-HA or MT2-MMP-HAFlag constructs in HeLa cells. These proteins were immunoprecipitated with HA antibody and the immunoprecipitates were resolved by SDS-PAGE. Immunoblotting analysis demonstrated that ZO-1 coimmunoprecipitates with MT2-MMP but not with MT1-MMP (Fig. 21A).

To analyze the importance of the tryptophan of MT2-MMP PDZ binding motif in its binding to ZO-1 in the cellular context, we expressed MT1-MMP-HAK581W and MT2-MMPHAFlag-W668K constructs in epithelial HeLa cells. These proteins were immunoprecipitated with HA antibody and the immunoprecipitates were resolved by SDS-PAGE. Immunoblotting analysis demonstrated that MT2-MMP 668 tryptophan is essential for MT2-MMP and ZO-1 coimmunoprecipitation. When this tryptophan is not present, MT2-MMP cytosolic tail interaction with ZO-1 is significantly decreased (Fig. 20B).

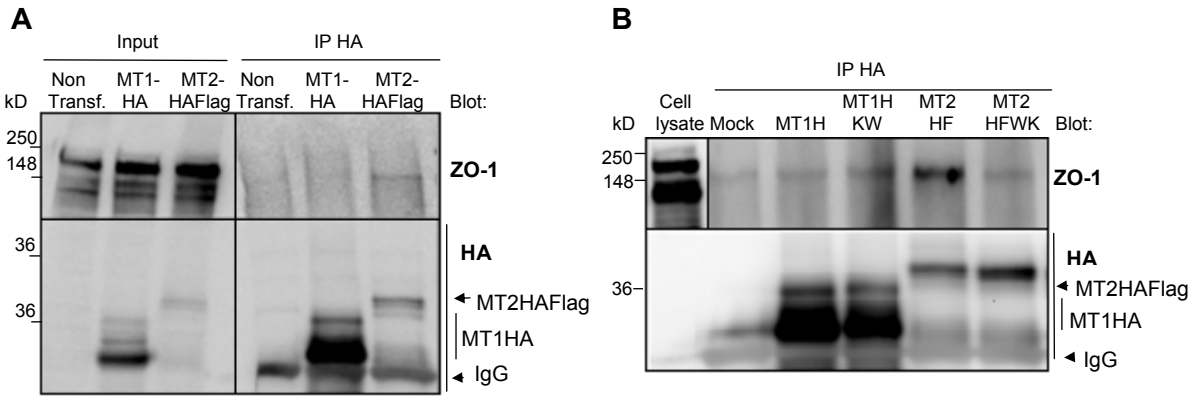


Figure 20: ZO-1 coimmunoprecipitates with MT2-MMP but not with MT1-MMP. Tryptophan 668 of MT2-MMP cytosolic tail is essential for MT2-MMP and ZO-1 coimmunoprecipitation. A) Lysates of HeLa non transfected or ectopically expressing MT1-MMP-HA or MT2-MMP-HAFlag were immunoprecipitated (IP) with HA antibody and blotted for ZO-1 and HA. The immunoblot shown is representative of two experiments. B) Lysates of HeLa cells transfected with the empty vector (Mock) or ectopically expressing MT1-MMP-HA (MT1H), MT1-MMP-HA-W581K (MT1H-WK), MT2-MMP-HAFlag (MT2HF) or MT2-MMP-HAFlag-W668K (MT2HF-WK) were IP with HA antibody and blotted for ZO-1 and HA. The immunoblot shown is representative of two experiments.

MT2-MMP/ZO-1 association was also analyzed by immunofluorescence in a model of transformed epithelial cells (breast carcinoma cell line MDA-MB-231) that express high levels of endogenous MT2-MMP, since MT2-MMP antibodies do not display high affinity. Firstly, MT2-MMP and F-actin double staining was performed. As it can be seen in Figure 21A, MT2-MMP is localized in cell protrusions. Double staining of MT2-MMP and ZO-1 shows that these two proteins colocalize in lamellipodia and in the perinuclear region of these cells (Fig. 21B).

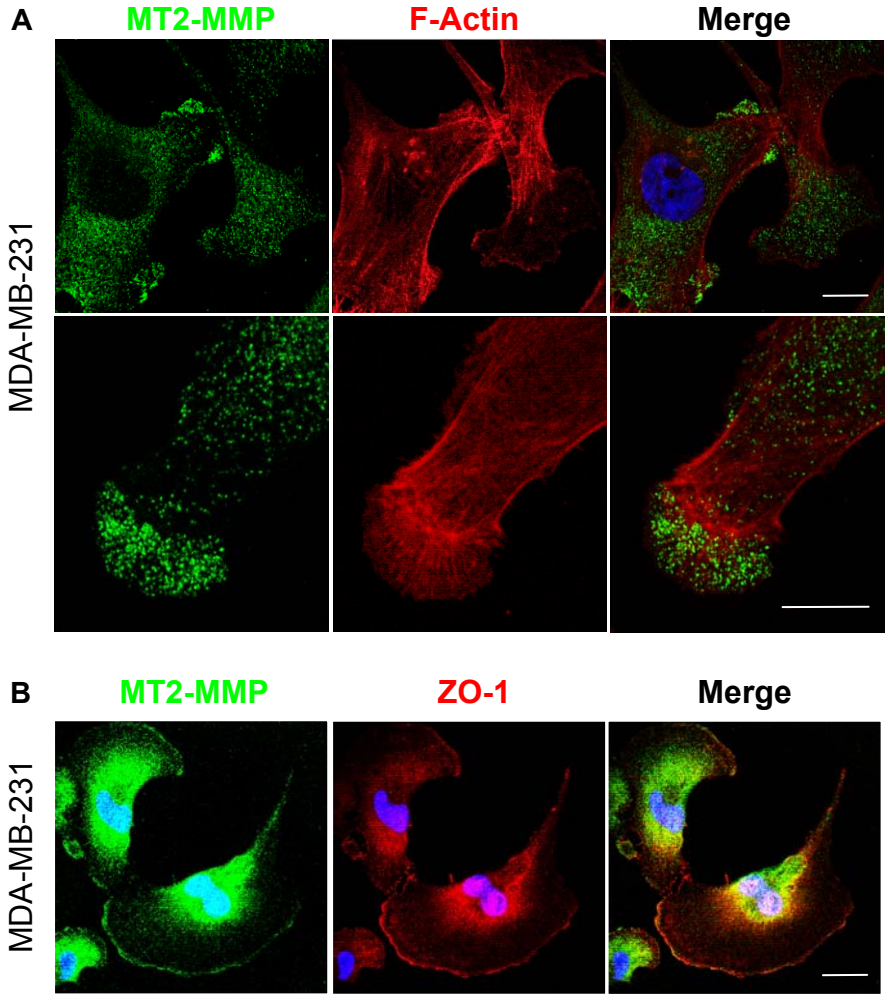


Figure 21: MT2-MMP and ZO-1 colocalize in breast cancer cells (MDA-MD-231). A) Image of cells stained for MT2-MMP (green) and F-actin (red). In the botton panels a cell protrusion detail is shown. Scale bar 10µm. B) Image of cells stained for MT2-MMP (green) and ZO-1 (red). Nuclei were stained with Hoescht 33342 (blue). MT2-MMP and ZO-1 colocalize at the cell lamella and in the perinuclear region. Scale bar 10µm.

3.6. Search for a proper cell model to analyze MT2-MMP/ZO-1 interaction

MT2-MMP is not as well characterized as other metalloproteinases such as MT1-MMP. We then first analyzed and compared its expression and cell localization in several primary and tumor cells to search for a good model to study MT2-MMP/ZO-1 interaction in a physiological scenario. As it is depicted in Figure 22, two endothelial primary cell types were analyzed; human umbilical endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC). In the case of HUVEC MT2-MMP expression was detected by PCR (Fig. 22A). However, neither in HUVEC nor in BAEC MT2-MMP protein could be detected by western blot (data not shown) or immunofluorescence (Fig. 22B). Lung cancer cells, H460 (human epithelial large lung cancer cell line) and H441 (human lung adenocarcinoma epithelial cell line), were also analyzed; despite the fact that in both cell types MT2-MMP expression was detected by PCR, MT2-MMP protein staining was faint or diffuse (Fig. 22B).

A

	Primary cells		Transformed cells		
	HUVEC	**BAEC**	**H460**	**H441**	**MDA-MB-231**
MT2-MMP expression (RT-PCR)	+	NT	+	+	+
MT2-MMP cellular localization	No detectable/ Faint	No detectable/ Faint	No detectable	Diffuse/spotty in the cytoplasm	Lamella, perinuclear

B

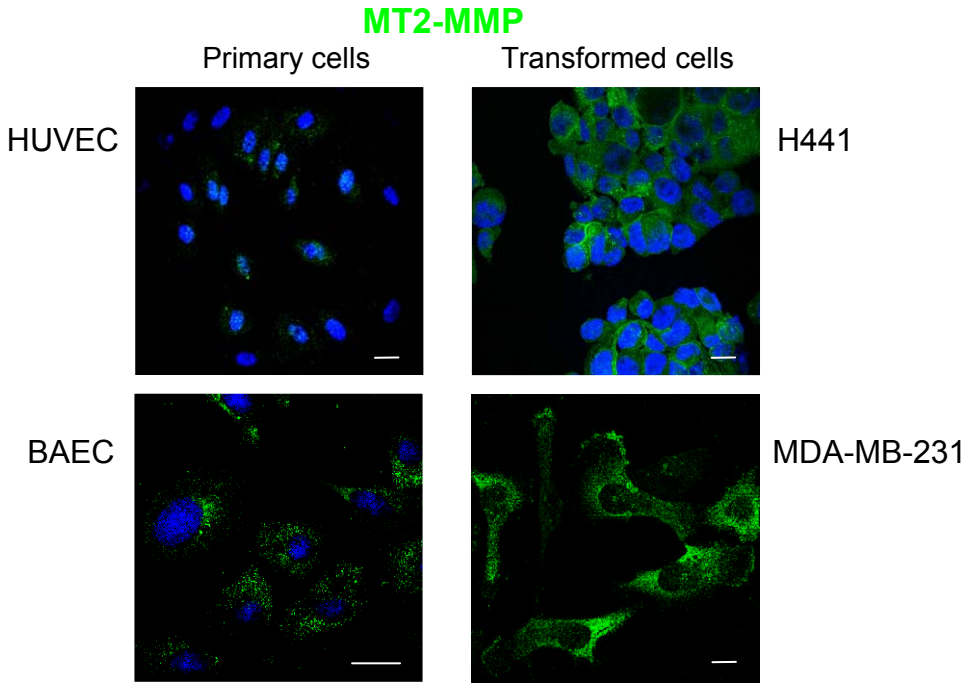


Figure 22: MT2-MMP expression and cellular localization in primary and transformed cells. A) Summary of the analysis of MT2-MMP expression (RT-PCR) and subcellular localization (immunoflourescence) in primary cells (HUVEC and BAEC), lung cancer cells (H460 and H441) and breast cancer cells (MDA-MB-231). B) Images of cells stained for MT2-MMP (green) are shown. Nuclei were stained with Hoescht 33342 (blue). Scale bar 10μm.

Taking into account the difficulty to detect and analyze subcellular localization of endogenous MT2-MMP in primary cells, and the fact that MT2-MMP is higher expressed in tumor epithelial cells and in epithelial tissues during development (Rebustini *et al.*, 2009), we moved to analyze MT2-MMP/ZO-1 interaction in the epithelial context. For this purpose, Madin-Darby Canine Kidney cells (MDCK), a commonly used non-transformed epithelial cell line, were selected. Stable clones expressing MT1-MMP-HA, MT1-MMP-KW-HA, MT2-MMP-HAFlag, MT2-MMP-WK-HAFlag or transfected with the empty vector (Mock) were selected and checked for MT-MMP expression by immunofluorescence (Fig. 23A) and western blot (Fig. 23B).

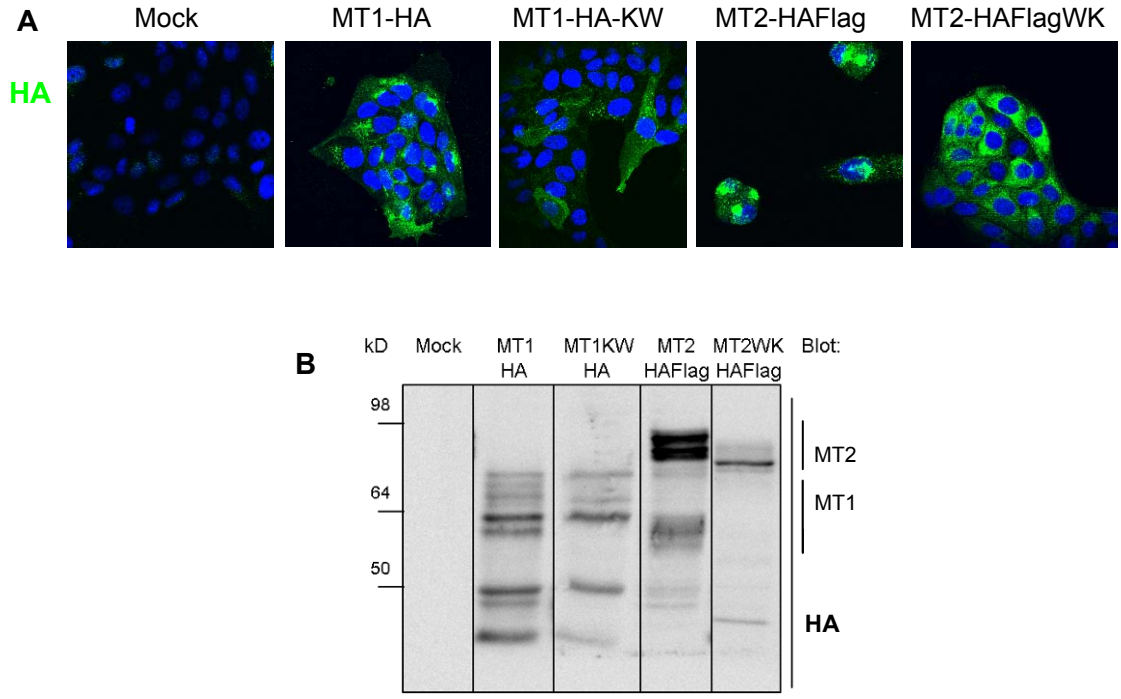


Figure 23: MDCK stable transfectant clone generation. MDCK cells were transfected with pCR3.1 empty vector (mock) or pCR3.1 containg each of the following tagged cDNA inserts: MT1-MMP-HA, MT1-MMP-KW-HA, MT2-MMP-HAFlag, MT2-MMP-WK-HAFlag. Transfected MDCK cells were cultured in the presence of 400 µg/ml of G418 and single cell colony selection was performed by limited cell dilution in 96-well plates. A) MT-MMP localization in stable transfectants. In each of the clones the corresponding MT-MMP levels was checked by immunoflourescence. B) MT-MMP protein expression in each clone was also checked by western blot analysis.

3.7. Cyst formation is not affected by MT2-MMP expression

Most internal epithelial organs consist of a monolayer of epithelial cells surrounding a central lumen. As MT2-MMP localization and function has been previously analyzed in 3D models and tissue explants, and MT2-MMP has been involved in submandibular gland development (Hotary *et al.,* 2000; Rebustini *et al.,* 2009), a possible role of MT2-MMP in the formation of three dimensional structures was first analyzed. For this purpose *in vitro* MDCK cyst formation was selected as a model. Formed cysts were counted and no differences were founded in the number of cyst formed by MDCK cells or MDCK cells expressing MT2-MMP.

Afterwards, cysts were stained for F-actin and Ezrin to check for correct apico-basal polarity of the cells forming the cyst, as these are two apical markers (Kivela *et al.,* 2000). As it is depicted in Figure 24A, MT2-MMP-MDCK cells forming the cysts display an apical F-actin and ezrin distribution as the MDCK control cells. Moreover, the number of lumens *per* cyst were counted as it can also indicate aberrant polarization of the cells (Martin-Belmonte *et al.,* 2008). We did not observe changes in MT2-MMP-MDCK cysts compared with control ones (677 cysts of Mock-MDCK and 680 cysts of MT2-MMP-MDCK were counted) (Fig. 24B).

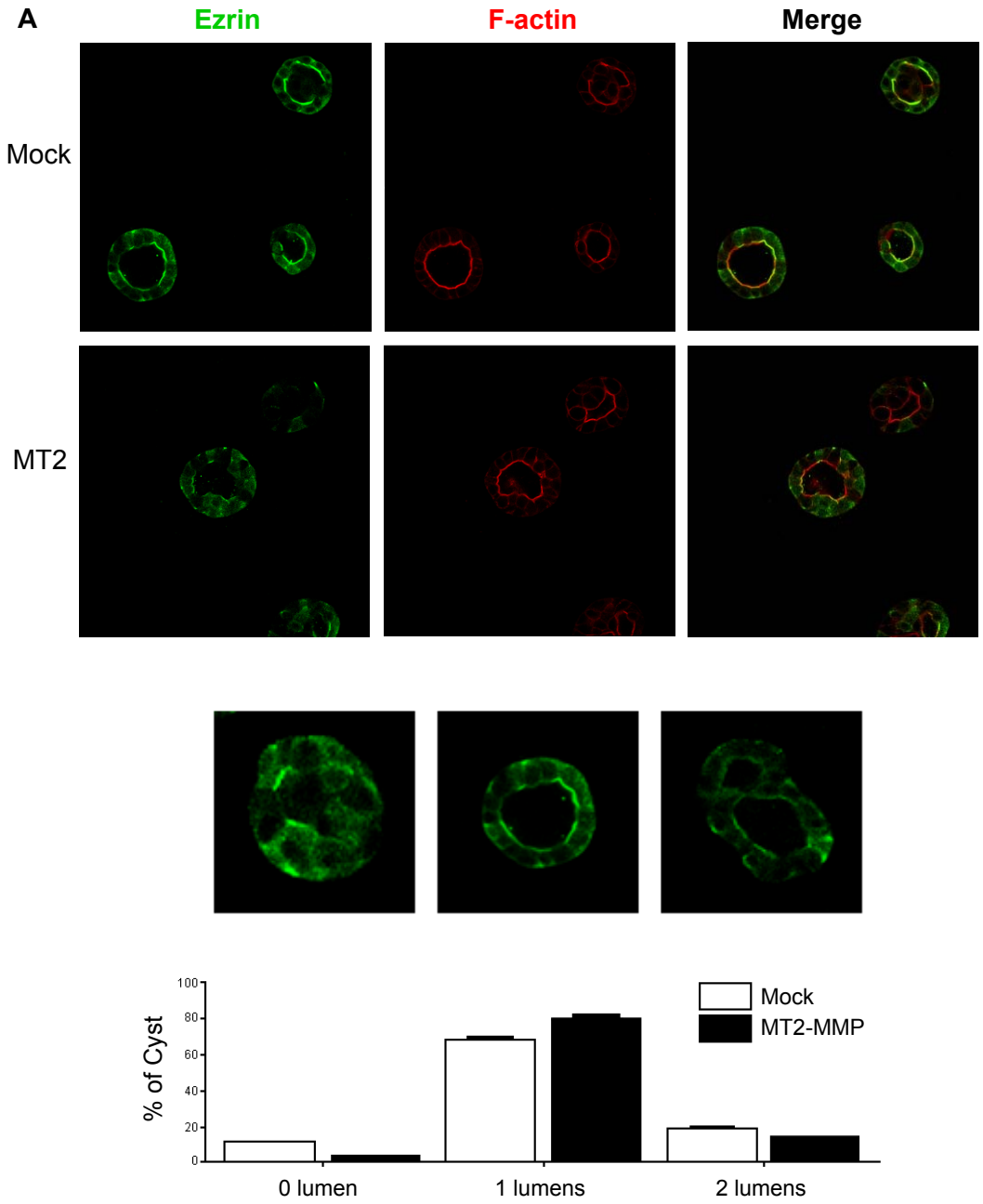


Figure 24: Cyst formation by MDCK control cells (Mock) and MT2-MMP-MDCK expressing cells. A) Mock and MT2-MMP-MDCK formed cysts were stained for ezrin and F-actin (Phalloidin-Texas Red). Pictures of cyst sections are shown. B) Percentage of cysts with 0, 1 or 2 lumens was calculated (677 cysts of Mock-MDCK and 680 cysts of MT2-MMP-MDCK from 2 independent experiments were counted).

3.8. MT2-MMP colocalizes with ZO-1 in polarized MDCK cells

Cyst formation involves many cellular functions besides cellular polarity such as migration, proliferation and apoptosis (Bryant and Mostov, 2008). Because of this we decided to move to a simpler model; MDCK polarized cells. When MDCK cells are cultured on transwell filters they get polarized mimicking a physiological scenario for an epithelial monolayer context. Firstly, MT2-MMP and ZO-1 localization was checked. As it can be seen in Figure 25A, MT2-MMP colocalizes with ZO-1 near the apical junctions (Maenders coefficient 0.51). MT2-MMP-WK mutant, unable to

bind to ZO-1, colocalizes with ZO-1 at the junctions at lower extent (Maenders coefficient 0.45) (Fig. 25B).

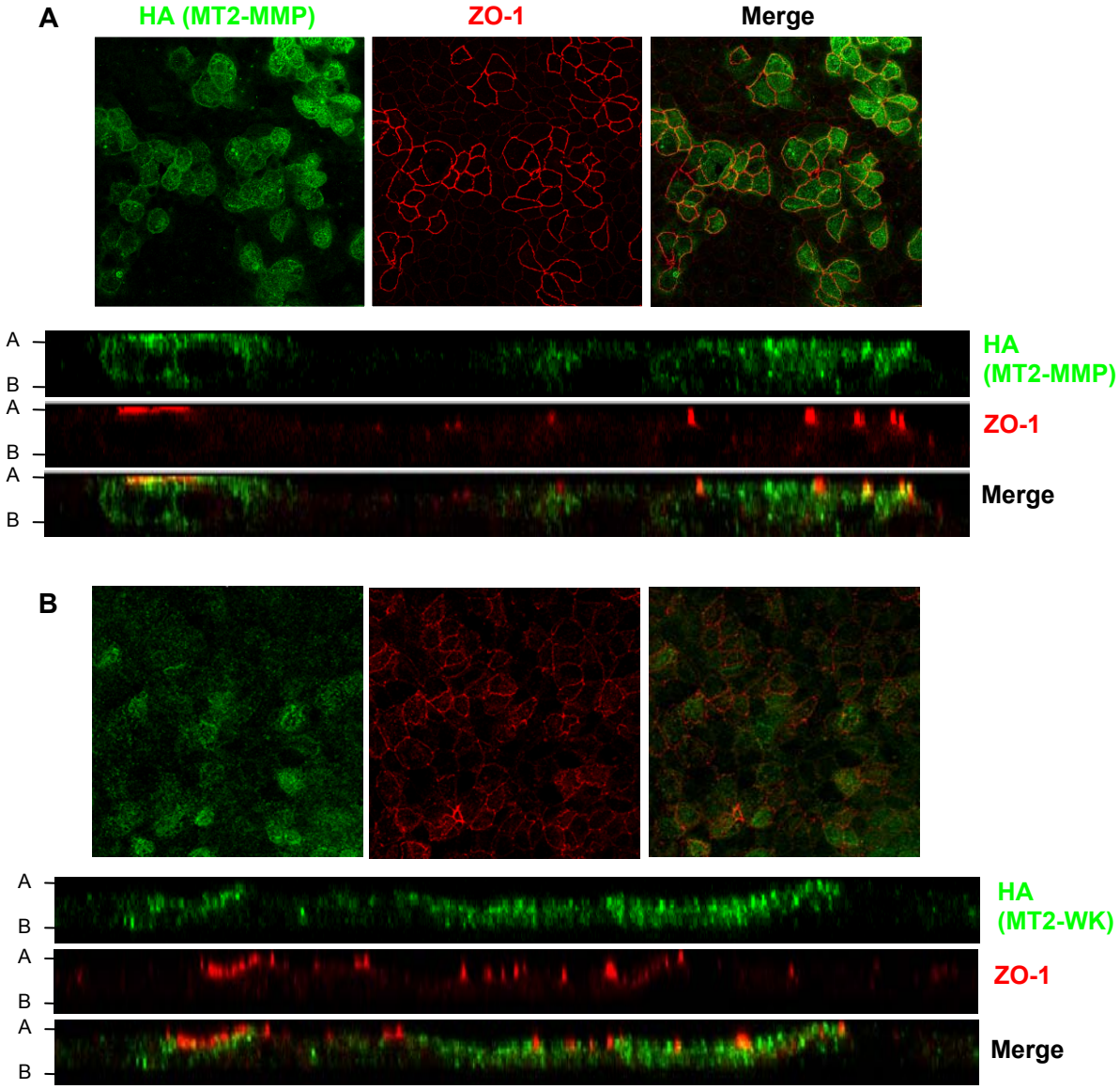


Figure 25: MT2-MMP, MT2-MMPWK and ZO-1 localization in MDCK polarized cells. MDCK cells were cultured on transwell filters (Costar). After 3-5 days cells were fixed and stained for HA (MT2-MMP) (green) and ZO-1 (red). A) MT2-MMP colocalizes with ZO-1 in the apical junctions of MDCK polarized cells (yellow). Top panels: XY axis. Bottom panels: XZ axis. B) In MT2-MMP-WK MDCK cells MT2-MMP colocalizes with ZO-1 at lower extent. Upper panels: XY axis. Bottom panels: XZ axis. A: Apical, B: basal.

3.9. MT2-MMP, but not MT2-MMP-WK, affects F-actin polarization of MDCK epithelial monolayers

In epithelial cells the cytoskeleton plays an important role in the generation and maintenance of the polarized phenotype. In this regard, apical F-actin has been shown to play a role in maintaining the apical localization of certain proteins in MDCK cells, even in the absence of cell-cell contacts (Ojakian and Schwimmer, 1988). As MT2-MMP was localized together with ZO-1 in cell-cell junctions, we next checked apical-basal F-actin in these MT2-MMP-MDCK cells. As shown in Figure 26, MDCK get polarized when cultured in transwell filters and a clear pattern of

apical (cortical actin), medium (cell-cell contacts) and basal (stress fibers) actin filaments can be distinguished.

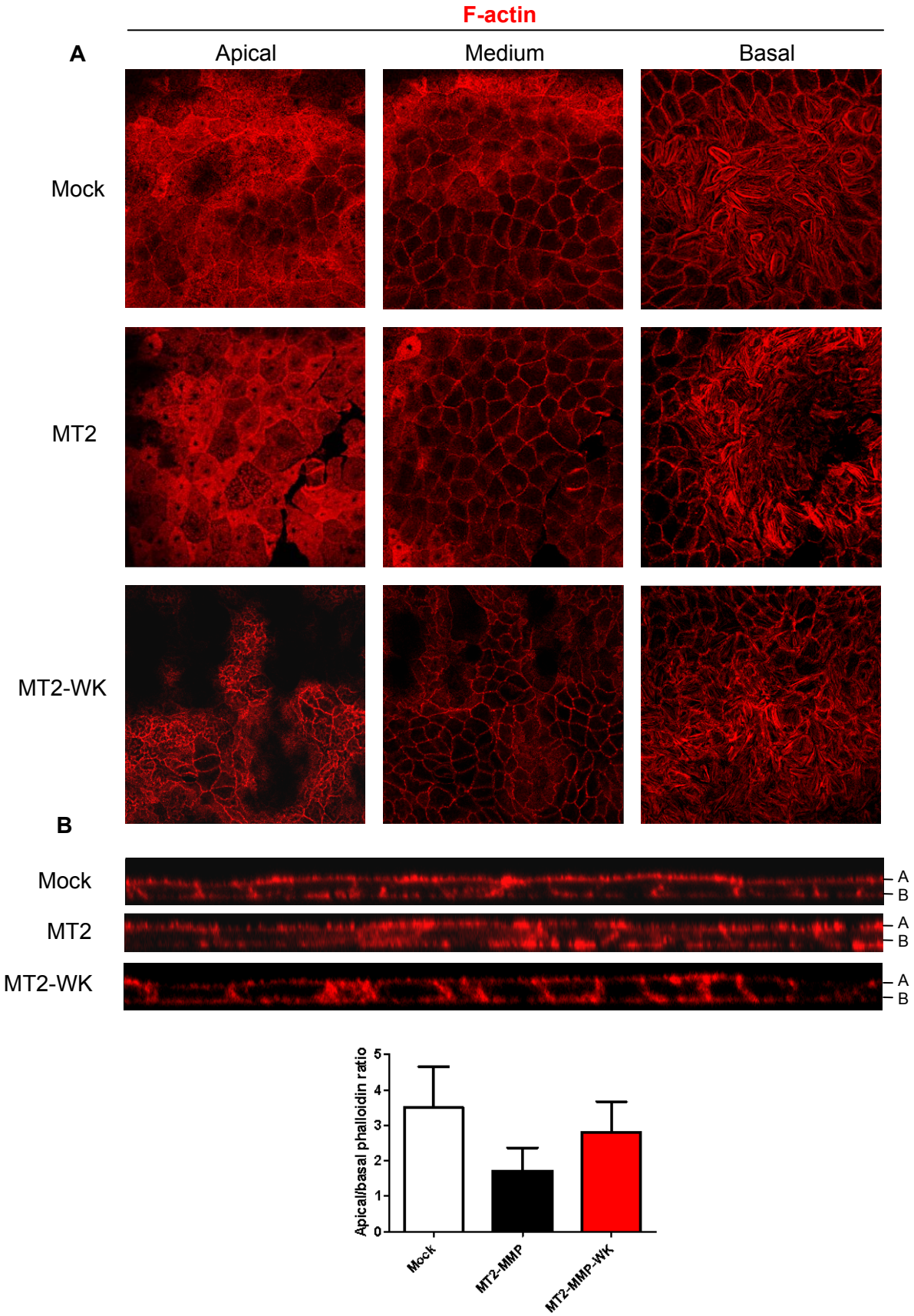


Figure 26: MT2-MMP, but not MT2-MMP-WK, affects F-actin apical-basal distribution. MDCK cells were cultured on transwell filters. After 3-5 days cells were fixed and stained for F-actin with Phalloidin-Texas Red. A) Pictures of XY axis corresponding to the apical, medium and basal regions of Mock, MT2-MMP and MT2-MMP-WK polarized monolayer. B) XZ axis of pictures in A. Histogram shows apical/basal F-actin ratio quantified with Image J (n=3). A: Apical, B: basal.

In the case of MT2-MMP, but not MT2-MMP-WK, basal actin filaments were less organized (Fig. 26A). To further analyze this observed difference in actin organization, F-actin apico/basal ratio was measured. In the case of the control MDCK cells (Mock) and MT2-MMP-WK cells, F-actin is mainly located in the apical compartment. On the contrary, in MT2-MMP MDCK cells the ratio between apical and basal F-actin is decreased, indicating that F-actin is less polarized. Moreover, F-actin pattern at cell junctions seemed less organized in MT2-MMP expressing cells compared with Mock or MT2-MMP-WK MDCK (Fig. 26B).

Discussion

1. Binding of the MT1-MMP cytosolic tail to p130Cas is involved in its regulation of myeloid progenitor fusion

The function of MT1-MMP cytosolic tail in migration and invasion is unclear and complex (Lehti *et al.,* 2000; Sabeh *et al.*, 2004). A potential problem is that many studies have used MT1-MMP mutants with the whole cytosolic tail deleted, thus removing putative positive and negative regulatory elements. This led to contradictory findings, with the cytosolic tail apparently dispensable for the catalytic activity of MT1-MMP in 3D contexts (Sabeh *et al.*, 2004), while partial deletion mutants (Δ567 and Δ573) pointed to these sequences as important for MT1-MMP localization at the leading edge for Matrigel invasion (Lehti *et al.*, 2000). Our findings clearly show the mechanism by which the MT1-MMP cytosolic tail contributes to myeloid cell fusion. In particular, our results show that the phosphorylation of Tyr573 is important for the association of MT1-MMP with p130Cas, an issue not addressed previously. Interestingly, MT1-MMP-Tyr573 can be phosphorylated by Src kinase (Nyalendo *et al.*, 2007), which is essential for normal OC development (Soriano *et al.*, 1991). We also show that MT1-MMP-p130Cas association is important for optimal membrane targeting and activity of Rac1 in myeloid progenitors. It seems that MT1-MMP, like integrins, is important for the proper regulation of GTPases in distinct contexts: MT1-MMP cooperates with Cdc42 in the formation of tunnels for invasion within 3D matrices (Fisher *et al.*, 2009), the MT1-MMP cytosolic tail can bind p27RF-Rho, a regulator of RhoA activity, and MT1-MMP deficiency decreases the amounts of Rac1 and Cdc42 and increases RhoA activity at the lamellipodia of myeloid progenitors (reviewed in Gonzalo and Arroyo, 2010).

Our results suggest a model (Fig. 27) in which MT1-MMP at the membrane of BM myeloid progenitors contributes to the efficient recruitment and activation of the p130Cas-Rac1 complex at lamellipodia. MT1-MMP is likely associated with by integrins at these sites. Among them, αvβ3 can be the integrin implicated in this process as it has been seen that MT1-MMP can be associated to αvβ3 in MT1-MMP protrusions (Galvez *et al.,* 2002; Galvez *et al.,* 2004). In such localization MT1-MMP would be phosphorylated in the tyrosine 573 of its cytosolic tail by Src kinases and this phosphorylation would contribute to the increase p130Cas and Rac1 targeting. p130Cas has 15 YxxP motifs in its substrate domain that can be phosphorylated (Fig. 5 of the Introduction). Binding of p130Cas to MT1-MMP might favour the phosphorylation of p130Cas at these sites. This phosphorylation would lead to Crk recruitment and then to Rac activation (Birge *et al.,* 2009). Impairment of this signaling pathway in the absence of MT1-MMP would decrease lamellipodia activity and cell migration, resulting in inefficient cell fusion.

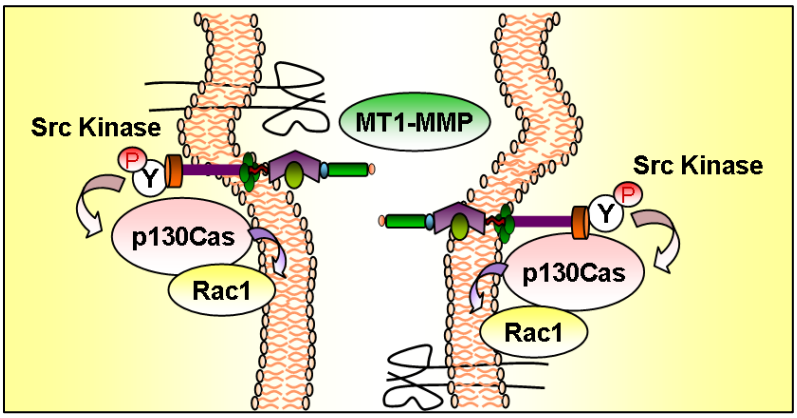


Figure 27: MT1-MMP is a novel component of the macrophage fusion machinery. The MT1-MMP cytosolic tail binds to the adaptor protein p130Cas thus contributing to optimal Rac1 membrane targeting and activity in myeloid progenitors. MT1-MMP tyrosine 573 is relevant to its association with p130Cas and it has been previously reported to be phosphorylated by Src kinase (Nyalendo *et al.*, 2007) (Taken from Gonzalo and Arroyo, 2010).

2. Moesin binds MT1-, MT2- and MT3-MMP cytosolic tails

2.1. Analysis of MT1-, MT2- and MT3-MMP moesin binding

The obtained MT1-MMP-moesin binding pattern is similar to the ones obtained by other authors. Ivetic and coll. analyzed in detail the binding of ERM proteins to L-selectin, a cell adhesion molecule that is expressed exclusively in leukocytes. L-selectin has three polybasic clusters and a fourth positive residue located near the C-terminus (Table 4). By mutating one positive residue of each polybasic cluster of L-selectin, this group saw that only when the arginine of the juxtamembrane polybasic region was mutated, a reduced binding to ERMs was obtained (Ivetic *et al.*, 2002). In other study, Serrador and coll. analyzed moesin binding to the intercellular adhesion molecule 3 (ICAM-3), a leukocyte-specific receptor involved in primary immune responses. They saw that the histidine and the second arginine of the juxtamembrane polybasic cluster of ICAM-3 (Table 4) were important for its binding to moesin because when these two residues were together mutated the interaction with moesin was significantly reduced (Serrador *et al*., 2002b).

In our study MT1-MMP seems to follow the same behaviour as L-selectin and ICAM-3. Taking also in to account the fact that MT2-MMP also binds to moesin and it lacks the first residue of the juxtamembrane polybasic cluster, it can be speculated that the second arginine and the histidine residue of the juxtamembrane polybasic cluster of MT1-MMP are the key residues involved in MT1-MMP binding to moesin.

The importance of serine motifs for ERM binding to transmembrane proteins has also been analyzed. P-selectin glycoprotein ligand 1 (PSGL-1) (Table 4), an adhesion receptor involved in the rolling of neutrophils on activated endothelium, has been seen to bind moesin. For this interaction just the juxtamembrane region of the cytosolic tail of PSGL-1 was sufficient (18 aa). By performing point mutations it has been reported an important role of the two first serines of PSGL-1 for its binding to moesin (and in the importance of the juxtamembrane positive cluster as

in the case of L-selectin and ICAM-3) (Serrador *et al.*, 2002a). The role of serine rich motifs of ICAM-3 for its binding to moesin has also been reported. Serrador and coll. determined that the serine residues of ICAM-3 are needed in its interaction with moesin and ezrin. They analyzed by single point mutations the fourth serines of ICAM-3; when each of the three first serines was changed to an alanine, reduced binding to moesin was obtained. However, when the fourth serine was mutated, a slightly increase of moesin binding was detected (Serrador *et al.,* 2002b). In our results, no decrease of N-moesin binding was obtained when MT1-MMP serine was mutated, thus it seems that this serine does not contribute to the regulation of the binding. We have to take into account that in the case of MT1-MMP there is only one serine and not a cluster as in PSGL-1. But we can not discard the possibility that this serine could have a role in the case of MT2- and MT3-MMP, because these proteins have partially the same sequence of PSGL-1 but in the opposite order (KRS) and they have a cluster (and not just one as MT1-MMP) of positive residues close to the serine for the binding of transmembrane proteins to ERMs.

Table 4: Cytosolic residues of MT1-, MT2-, MT3- and the proteins associated to ERMs commented in the discussion.

MT-MMP	Cytosolic residues
MT1-MMP	RRHGTPRRLLYCQRSLLDKV
MT2-MMP	QRKGAPRVLLYCKRSLQEWV
MT3-MMP	KRKGTPRHILYCKRSMQEWV

Transmembrane protein	Cytosolic residues
L-selectin	RRLKKGKKSQERMDDPY
ICAM-3	REHQRSGSYHVREESTYLPLTSMQPTEAMGEEPSRAE
PSGL-1	RLSRKGHMYPVRNYSPTEMVCISSLLPDGGEGPSATA NGGLSKAKSPGLTPEPREDREGDDLTLHSFLP

Positive residues are highlighted in red. Serine residues are highlighted in blue. Mutated residues are underlined.

In regard to MT3-MMP, more than 2-fold affinity for moesin in comparison with MT1- or MT2-MMP was detected. MT1- and MT3-MMP have both three juxtamembrane positive residues that as we have mentioned are essential for MT1-MMP/moesin interaction. There are not relevant differences in the sequence of MT3-MMP that can allow us to speculate why this increased affinity is obtained. We can hypothesize that probably this difference can be due to the accumulation of positive residue clusters. MT3-MMP has three positive clusters and, on the contrary, MT1- and MT2-MMP have only two.

2.2. MT1-MMP binding to moesin could link MT1-MMP to actin cytoskeleton and affect its internalization

When cells migrate they localize MT1-MMP at lamellipodia, the migration front of the cells, to degrade ECM barrier (Itoh *et al.*, 2001; Sato *et al.*, 1997). This localization was thought to be due to the interaction of MT1-MMP with CD44 through the hemopexin domain of the enzyme and stem region of CD44. CD44 in turn is associated with F-actin through its cytoplasmic domain by interacting with ERM proteins. Then, by this interaction, MT1-MMP is indirectly associated with F-

actin (Mori *et al.,* 2002). Without excluding this possibility, we propose that MT1-MMP could also be linked to the actin cytoskeleton though its direct binding to ERM proteins (Fig. 28). More experiments are needed to verify this point.

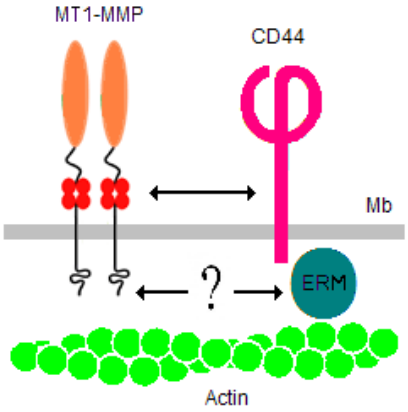


Figure 28: MT1-MMP is regulated by its association with CD44 followed by localization to migration front. Homodimers of MT1-MMP interact with CD44, which is bound to the actin cytoskeleton though the ERM proteins (Mori *et al.*, 2002). Binding of MT1-MMP to actin cytoskeleton through its binding to ERMs can be also expected.

This link to F-actin might regulate MT1-MMP localization. In this regard, our preliminary results point to a role of moesin in the regulation of MT1-MMP membrane pool. It can be thought that moesin could be affecting MT1-MMP recycling and in this way decrease of moesin levels will lead to MT1-MMP membrane accumulation. It has been seen that the cortical cytoskeleton regulates the membrane localization of several adhesion receptors, such as ICAMs, VCAM-1, CD43 and CD44, though ERM proteins (Barreiro *et al.,* 2002; Heiska *et al.,* 1998; Serrador *et al.,* 1997; Yonemura *et al.*, 1998). For example, transfection of human ezrin into natural killer resistant cells induces the redistribution of ICAM-2 (Helander *et al.*, 1996).

3. ZO-1 is a novel interacting partner of MT2-MMP cytosolic tail

3.1. MT2-, MT3- and MT5-MMP interact with ZO-1

Interaction with ZO-1 was detected for MT2-, MT3- and MT5-MMP, but not for MT1-MMP. Specifically, the cytosolic tail of these metalloproteinases could bind to each of the three PDZ domains of ZO-1. However, MT2- and MT3-MMP did not show the same affinity for the different PDZ domains of ZO-1. ZO-1 PDZ2 presented the highest binding to the metalloproteinases followed by PDZ1, and PDZ3 clearly showed the least binding to MT2- and MT3-MMP. Zhang and coll. observed that ZO1-PDZ3 had more affinity for leucine ending ligands and, in contrast, ZO1-PDZ1 preferentially bound to proteins ending in a valine residue (Zhang *et al.*, 2006). As MT2- and MT3-MMP cytosolic tail terminal residue is a valine, these different binding preferences of ZO-1 PDZ domains can be the factor by which these metalloproteinases have less affinity for ZO1-PDZ3.

MT5-MMP has been previously described to bind to the PDZ domains of two proteins; Mint-3 (Wang *et al.,* 2004a) and ABP (Monea *et al.,* 2006). Mint-3 contains two PDZ domains and

mutants lacking each PDZ domain remain associated with MT5-MMP, indicating that either of the PDZ domains is sufficient for binding MT5-MMP. Among the seven PDZ domains of ABP, MT5-MMP has been seen to bind to PDZ5, however PDZ4 acted as an accessory domain, which contributed to the generation of a compact, two PDZ domain structure; PDZ4-5. In this structure, PDZ5 provided the single functional binding site, and PDZ4 stabilized PDZ5 (Feng *et al.,* 2003). In our studies a higher interaction of MT2- and MT3-MMP cytosolic tails with ZO-1 N-terminus (PDZ1-3) compared with the single ZO-1 PDZ domains was detected. This suggests that, despite the fact that MT2- and MT3-MMP are able to bind to each of the three PDZ domains of ZO-1, the presence of the complete ZO-1 N-terminal domain positively modulates this binding. These observations point to a cooperative interaction of the different ZO-1 PDZ domains as in the case of ABP with MT5-MMP (Monea *et al.*, 2006). Moreover, when cell lysate precipitation or *in vitro* assays with ZO-1 N-terminus were performed, similar binding of ZO-1 to MT2- and MT3-MMP was obtained. In contrast, MT3-MMP showed more affinity than MT2-MMP when single PDZ domains of ZO-1 were used. Again, these results indicate the possible existence of a regulatory affinity mechanism included in ZO-1 N-terminus.

3.2. MT2-MMP binds to ZO-1 through its PDZ binding motif

Extensive peptide library screens pioneered by Songyang and coll. revealed the specificities of distinct PDZ domains (Schultz *et al.,* 1998; Songyang *et al*., 1997). Together, their results suggested that PDZ domains were extremely selective and, as it has been commented, P_0 and P_{-2} residues of the PDZ binding motifs were identified as the most critical residues for PDZ domain recognition. Therefore, PDZ domains can be divided into three main classes on the basis of their preferences for residues at P_0 and P_{-2}. Class I PDZ domains recognize the motif S/T-X-V/L (where X is any amino acid), class II domains recognize the motif Φ-X-Φ (where Φ is a hydrophobic amino acid) and class III domains recognize the motif D/E-X-Φ (Table 5) (Nourry *et al.*, 2003).

Table 5: PDZ domain classification (adapted from Nourry *et al*., 2003).

PDZ Domain	Consensus binding sequence			Ligand protein	Reference
	P_{-2}	P_{-1}	P_0		
Class I	**S/T**	**X**	**V/L**		
Syntrophin	S	L	V	Voltage-gated Na^+ channel	(Schultz *et al*., 1998)
PSD-95	T	D	V	Shaker-type K^+ channel	(Kim *et al.*, 1995)
Class II	**Φ**	**X**	**Φ**		
hCASK	Y	Y	V	Neurexin	(Songyang *et al.*, 1997)
Erythrocyte p55	Y	F	I	Glycophorin C	(Marfatia *et al.*, 2000)
Class III	**D/E**	**X**	**Φ**		
nNOS	D	S	V	Melatonin receptor	(Stricker *et al.,* 1997)
Mint-1	E	P	L	KIF17	(Setou *et al.*, 2000)

X denotes any amino acid (no specificity defined at this position for this class)
Φ denotes a hydrophobic amino acid, usually V, I or L

Based in this classification, C-terminal sequence of MT-MMPs, DKV in MT1-MMP and EWV in MT2-, MT3- and MT5-MMP, are typical Class III PDZ domain binding proteins. Consistently with Mint-3 and MT5-MMP binding (Wang *et al.*, 2004a), Mint proteins belong to Class III PDZ

domains, and both of their PDZ domains can recognize Class III PDZ motifs (Setou *et al.*, 2000). Subsequently, Bezprozvanny and Maximov proposed a novel classification of PDZ domains based on the nature of the amino acids in the two critical positions of the PDZ domain fold previously suggested to account for specificity of P_{-1} and P_{-2} positions of the PDZ binding motif (Daniels *et al.*, 1998). Their classification divided PDZ domains into 25 groups. Interestingly, ZO-1 and ZO-2, but not ZO-3, belonged to the same group as GRIP, which has been seen to bind MT5-MMP cytosolic tail (Bezprozvanny and Maximov, 2001).

Detailed *in vitro* analysis of ZO1-PDZ1 and PDZ3 domain binding properties using synthetic peptides was later performed by Zhang and coll. (Zhang *et al.*, 2006). Their results revealed the importance of all the C-terminal residues of the ligand, and not only P_0 and P_{-2} residues, for PDZ binding recognition. Interestingly, the results of this group highlighted the importance of P_{-1} of the PDZ binding motif. PDZ-1 and PDZ-3 of ZO-1 were found to accept tryptophan or tyrosine in P_{-1} position. Moreover, the binding affinity of a synthetic peptide when P_{-1} tryptophan was substituted for each of the rest amino acids was checked. For all the substitutions, except tyrosine, a great reduction in the binding was obtained (Zhang *et al.*, 2006). Among the combinations tryptophan substitution for a lysine, as we performed in MT2-MMP cytosolic tail, was included.

Finally, regarding P_{-2} residue, no preference was detected for ZO1-PDZ3. On the contrary, ZO1-PDZ1 exhibited class I binding specificity, as this domain preferentially bound to ligands with Thr/Ser at position P_{-2} (Zhang *et al.,* 2006). Our data shows that, despite the fact that neither MT2-, MT3- nor MT5-MMP have Thr/Ser at this position, they are able to interact with ZO1-PDZ1. Moreover, we showed that this position did not play a key role for ZO-1 binding because when it was substituted for an alanine the interaction with ZO-1 was maintained.

Regarding ZO-1 interactions with natural ligands, ZO1-PDZ domains have been shown to interact with several proteins, including members of the large families of claudins (Ikari *et al*., 2004), connexins (Kausalya *et al.*, 2001; Laing *et al.,* 2001) and also the ZO-1 homologues ZO-2 and ZO-3 (Wittchen *et al.,* 1999) (Fig. 30). ZO1-PDZ1 is responsible for interactions with members of the claudin family. Claudin C-termini, are atypical type I PDZ ligands as they do not contain the defining Thr/Ser at P_{-2}. In fact, P_{-2} is remarkably diverse among their sequences. This is another example that, although the consensus binding sequence of ZO-1 PDZ1 is described to correspond to class I, other PDZ binding motifs that do not belong to PDZ class I can be also partners of ZO-1. Moreover, regarding P_{-1}, nearly all of the 20 described claudin proteins present a tyrosine at this position. As we have commented tyrosine and tryptophan were described to be important for ZO1-PDZ1 binding affinity (Zhang *et al.,* 2006).

Taking together the identification of P_{-1} tyrosine and tryptophan as important players for ZO1-PDZ1 binding using synthetic peptides (Zhang *et al.,* 2006), the high degree of conservation of the tyrosine residue of claudins at this position (Ikari *et al.,* 2004) and the key role of P_{-1} tryptophan for MT2-MMP binding to ZO-1 that we have described in this work; it can be suggested that P_{-1} can be the key player for determining the ligand affinity for ZO-1 PDZ domains.

3.3. MT2-MMP/ZO-1 association affects F-actin polarization of epithelial monolayers

Our data show that MT2-MMP colocalizes with ZO-1 at cell-cell contacts of polarized epithelial MDCK cells. Moreover, MT2-MMP expressing cells, but not the ones expressing MT2-MMP-WK that is unable to bind to ZO-1, present a decreased F-actin apical polarization. These data point to a possible role of MT2-MMP in apical-basal polarity of these cells by its association with ZO-1. However, although the establishing of apico-basal polarity is important for MDCK cyst formation (Martin-Belmonte *et al.*, 2008), no differences in the number of cyst nor in the number of lumens of the cyst were observed in MT2-MMP-MDCK expressing cells compared with control ones. For cyst formation different events have to take place and in addition to polarization, cell proliferation and apoptosis play also an important role. It has been proposed that when the acquisition of polarity is efficiently achieved and coordinated with cell proliferation, lumen formation occurs without requirement of apoptosis. However, when apico-basal polarization is slow or inefficient or when cell polarity is uncoordinated with cell proliferation, apoptosis of cells becomes essential (Martin-Belmonte *et al.,* 2008). Thus, other mechanisms such as apoptosis can be compensating a possible defect in apico-basal polarity of these cells.

Cell polarity involves the asymmetric organization of most of the physical aspects of the cell, including the cell surface, intracellular organelles and the cytoskeleton. In an epithelial polarized tissue the apical surfaces of the cells provide the luminal interface and are specialized in regulating the exchange of materials. The lateral surfaces of epithelial cells contact adjacent cells and have specialized junctions and cell-cell adhesion structures. The basal surfaces of these cells contact the underlying basement membrane, ECM and, ultimately, underlying blood vessels. The basal and lateral surfaces are fairly similar in composition and organization, and are often referred together as the basolateral surface. The apical and basolateral surfaces, however, have very different compositions. In the maintenance of these differences, TJ play a key role (Bryant and Mostov, 2008).

TJ are found at the most-apical portion of the lateral surfaces, where they form barriers both between the apical and basolateral surfaces and between adjacent cells, limiting paracellular permeability. These junctions have an organizing role in epithelial polarization and establish an apico-lateral barrier to the diffusion of solutes through the intracellular space (gate function). They also restrict the movement of lipids and membrane proteins between the apical and the basolateral membrane (fence function). TJ are highly ordered membrane contact sites or ‘kissing points’, comprising a network of intra-membrane proteins (Aijaz *et al.*, 2006). They comprise at least four types of transmembrane proteins, including occludins, claudins, junctional adhesion molecules (JAMs) and crumb, and a number of cytoplasmic peripheral proteins. Whereas the transmembrane proteins mediate cell-cell adhesion, the cytosolic TJ plaque contains various types of proteins that link TJ transmembrane proteins to the underlying cytoskeleton. These adapters also recruit regulatory proteins, such as protein kinases, phosphatases, small GTPases and transcription factors, to the TJ. As a result, structural (actin and spectrin) and regulatory (actin-binding proteins, GTPases and kinases) proteins are juxtaposed with transmembrane proteins. This protein scaffolding facilitates the assembly of highly ordered structures, such as

junctional complexes or signaling patches that regulate epithelial cell polarity, proliferation and differentiation (Matter *et al.*, 2005) (Fig. 29).

ZO-1 is localized in these TJ plaque structures and it has an important role in creating and maintaining specialized membrane domains. Though its PDZ domains (Fig. 7 of the Introduction) ZO-1 binds to C-terminal ends of various proteins, especially transmembrane proteins. Thus, it can cross-link these transmembrane proteins at the cytoplasmic surface of plasma membranes to establish these specialized membrane domains (Fig. 29) (McNeil *et al.*, 2006).

One of these transmembrane proteins involved in TJs are claudins (Fig. 29A). Claudins appear to be involved in the homophilic and/or heterophilic interactions implicated in cell-cell adhesions. They have four hydrophobic transmembrane domains and two extracellular loops. The extracellular loops, whose sequences are distinct in different claudins, contribute to the formation of TJs. Several membrane proteins that participate in TJ-scaffolding bind to the C-terminal YV sequences of several claudins through their PDZ domains. As mentioned, among the proteins which directly interact with C-terminus of claudins ZO-1 has been described (Ikari *et al.,* 2004). Moreover, it has been observed that JAMs regulate several signaling mechanisms that control epithelial polarization. JAMs can also interact with ZO-1 and F-actin plaques are also found near the cytoplasmic domains of JAMs to promote firm adhesions (Fig. 29B). Finally, occludin is a transmembrane protein also localized in the TJs. Occludin is likely to be involved in establishing the seal at the sites of junctional strands and it also interacts directly with ZO-1 among other proteins (Fig. 29C) (Matter *et al.,* 2005).

Our hypothesis is that MT2-MMP can be taking part of the TJ complex by its anchoring to the actin cytoskeleton though its binding to ZO-1. In such position, it can be participating directly or indirectly in the cleaving of TJ transmembrane proteins thus disturbing apico-basal polarity. In this sense, it has been seen that claudin 5 promotes proMMP-2 processing by MT2-MMP. Miyamori and coll. analyzed the effects of claudin 5 expression on activation of proMMP-2 by MT-MMPs in HEK293T cells. Expression of claudin 5 or MT2-MMP alone did not have any effect on proMMP-2 processing. However, proMMP-2 activation was observed when claudin 5 cDNA was co-transfected with MT2-MMP. In the authors´ opinion claudins may recruit MT2-MMP and proMMP-2 favouring the cleavage of proMMP-2 (Miyamori *et al.*, 2001). To investigate the possibility that MT2-MMP is affecting apico-basal polarity by its catalytic activity, a putative catalytically inactive MT2-MMP mutant has been generated (see Materials and Methods). After confirming that the mutated amino acid impairs MT2-MMP activity, clones expressing catalytically inactive MT2-MMP will be generated and checked for F-actin apical-basal polarity. Another complementary approach would be to use an MMP inhibitor (as GM6001/Ilomastat) to analyze if the apical-basal reduced F-actin polarization is reverted when the inhibitor is present.

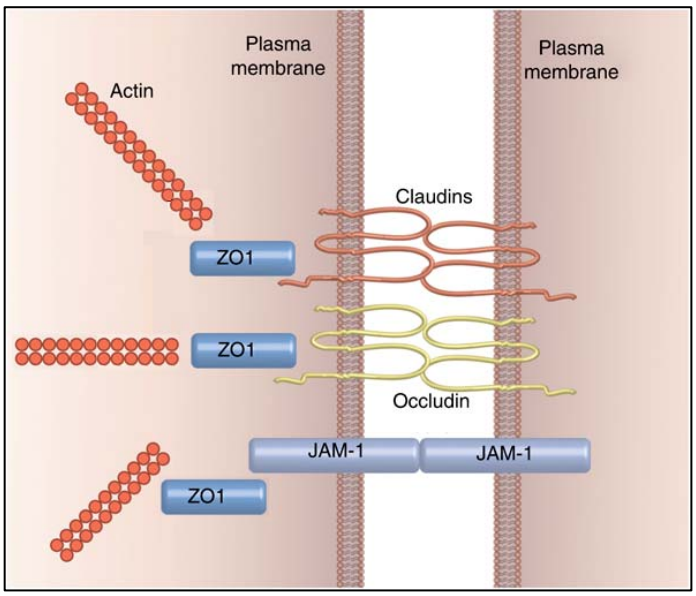


Figure 29: Schematic representation of the basic structural transmembrane components of tight junctions. ZO-1 is important for binding claudins, occluding and JAM-1 participating in the formation of tight junctional strands (Adapted from Niessen, 2007)

4. Global discussion

The original objective of this work was to get insight into redundant versus specific functions of MT-MMPs by their different association to cytosolic partners. Our work has identified three proteins that can bind some of them are common partners of all MT-MMPs and some others are specific for a subset of MT-MMPs. In Figure 30 the interacting partners of MT1- and MT2-MMP cytosolic tails described in this work and the cellular functions of these associations are summarized.

In the middle region of MT1-MMP the phosphorylation of its tyrosine is necessary for p130Cas binding and therefore Rac1 activation which leads to myeloid cell fusion. MT2-, MT3- and MT5-MMP have also a tyrosine residue in the same position as MT1-MMP, then, a similar function could be expected. However, MT1-MMP seems to have a specific role in OC fusion as MT2-, MT3- nor MT5-MMP are not able to compensate MT1-MMP myeloid fusion promotion in the MT1-MMP deficient mice. MT2-MMP expression in myeloid cells was checked and similar levels were found in WT and MT1-MMP deficient mice (data not shown). In conclusion, MT2-MMP although it shares this putative phosphorylable tyrosine with MT1-MMP, can not overcome the lack of MT1-MMP.

Juxtamembrane polybasic region of MT1-MMP acts as a key binding domain for moesin interaction and this association seems to be involved in the regulation of MT1-MMP presence at the plasma membrane. MT1-MMP/moesin binding could be involved in MT1-MMP endocytosis as different works have been previously shown the relevance of MT1-MMP cytosolic tail in MT1-MMP endocytosis and intracellular trafficking (Uekita *et al.,* 2001; Smith-Pearson *et al.,* 2010; Radichev *et al.,* 2009; Rozanov *et al.,* 2002b). Moreover, MT2- and MT3-MMP also bound *in vitro* to N-terminal moesin. As they have the same polybasic cluster structure than MT1-MMP it can be

thought that the findings obtained for MT1-MMP can also occur in the case of MT2- and MT3-MMP. Interestingly, MT3-MMP has been seen to co-recycle with MT1-MMP (Wang *et al.,* 2004b).

Finally, MT2-MMP C-terminus has been found to interact with ZO-1 PDZ domains and this binding affects F-actin polarity of epithelial (MDCK) monolayers. MT1-MMP, despite it also has a PDZ binding motif, is unable to bind to ZO-1. This difference of affinity of MT1-MMP and MT2-MMP to PDZ proteins can be relevant to determine not only the binding to ZO-1 but also their binding to other proteins with PDZ domains, which can therefore modulate their specific functions.

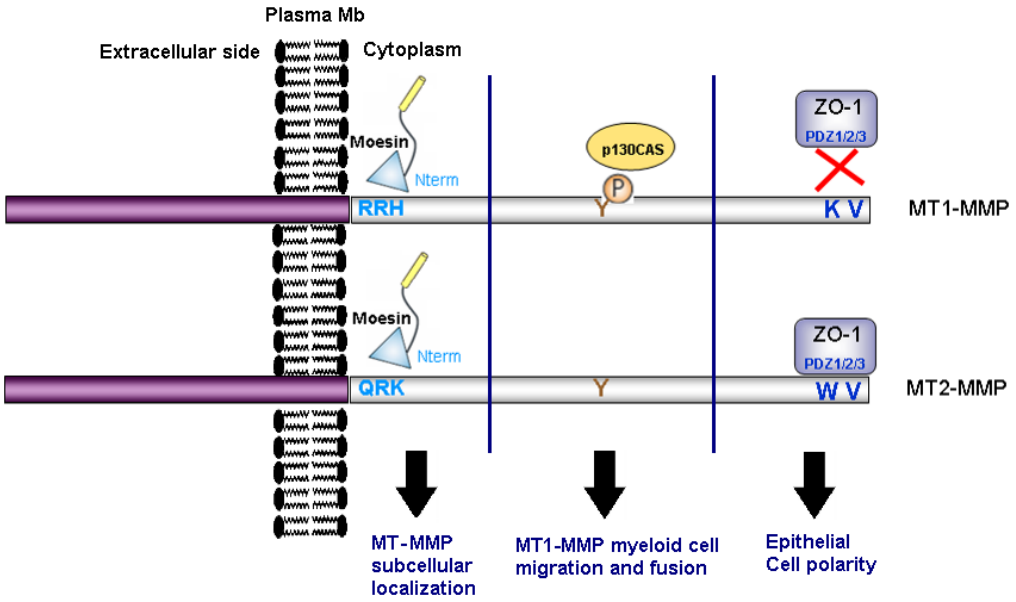


Figure 30: Diagram of the MT-MMP cytosolic tail interacting proteins described in this work. Identified MT-MMP key cytosolic residues for these interactions and their possible cellular function are also shown. p130Cas binds to the unique tyrosine residue of MT1-MMP cytosolic tail and this binding is important for myeloid cell migration and function. MT1-MMP binds to moesin N-terminal domain and juxtamembrane polybasic region of MT1-MMP cytosolic tail plays a key role for this interaction. Moreover, MT2-MMP also binds to moesin and it can be speculated as it shares the same moesin binding region binding can occur also at this site. MT1-MMP subcellular localization might be regulated by this association. ZO-1 does not bind to MT1-MMP cytosolic tail because of the presence of a lysine in its PDZ binding motif. On the contrary, MT2-MMP binds to the three ZO-1 PDZ domains and the last two residues (tryptophan and valine) of MT2-MMP cytosolic tail play a key role for this interaction. Moreover, this interaction affects the polarity of epithelial cells (MDCK).

Conclusions

1. MT1-MMP is required for proper p130Cas localization at the cell membrane.

2. The unique MT1-MMP cytosolic tyrosine is essential for MT1-MMP binding to p130Cas.

3. MT1-MMP cytosolic tyrosine participates in MT1-MMP promotion of myeloid progenitor fusion.

4. MT1-MMP, MT2-MMP and MT3-MMP cytosolic tails bind *in vitro* to moesin N-terminus.

5. Juxtamembrane polybasic cluster of MT1-MMP (RRH563) is essential for its binding to moesin. Middle MT1-MMP polybasic cluster (RR569) also participates in this interaction.

6. MT2-MMP, MT3-MMP and MT5-MMP interact with ZO-1. MT1-MMP affinity for ZO-1 is very low.

7. MT2-MMP can bind to each of the three PDZ domains of ZO-1 showing more affinity for PDZ2 and PDZ1 and less for PDZ3.

8. MT2-MMP binds to ZO-1 through its PDZ binding motif being the tryptophan residue located at this site the responsible of the different affinity of MT1-MMP and MT2-MMP for ZO-1.

9. MT2-MMP and ZO-1 colocalize in the lamella and in the perinuclear region of breast cancer cells.

10. MT2-MMP colocalizes with ZO-1 at cell-cell junctions of polarized stable transfectant MT2-MMP-MDCK cells affecting apico-basal polarity of F-actin.

1. MT1-MMP es necesaria para la correcta localización de p130Cas en la membrana celular.

2. La única tirosina citosólica de MT1-MMP es esencial para la unión de MT1-MMP a p130Cas.

3. La tirosina citosólica de MT1-MMP participa en la fusión de los progenitores mieloides potenciada por MT1-MMP.

4. Las colas citosólicas de MT1-MMP, MT2-MMP y MT3-MMP se unen *in vitro* al extremo N-terminal de moesina.

5. El grupo polibásico yuxtamembranal de MT1-MMP (RRH563) es esencial para su unión a moesina. El grupo polibásico central de MT1-MMP (RR569) participa también en esta interacción.

6. MT2-MMP, MT3-MMP y MT5-MMP interaccionan con ZO-1. La afinidad de MT1-MMP por ZO-1 es muy baja.

7. MT2-MMP se une a cada uno de los tres dominios PDZ de ZO-1 mostrando más afinidad por PDZ2 y PDZ1 y menos por PDZ3.

8. MT2-MMP se une a ZO-1 a través de su motivo de unión a dominios PDZ siendo el residuo de triptófano localizado en este lugar el responsable de la diferente afinidad de MT1-MMP y MT2-MMP por ZO-1.

9. MT2-MMP y ZO-1 colocalizan en la lamela y en la región perinuclear de células de cáncer de mama.

10. MT2-MMP colocaliza con ZO-1 en las uniones celulares de MDCK transfectantes estables de MT2-MMP afectando a la polaridad apico-basal de la F-actina.

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Appendix: Publication

Developmental Cell
Article

MT1-MMP Is Required for Myeloid Cell Fusion via Regulation of Rac1 Signaling

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DOI 10.1016/j.devcel.2009.11.012

SUMMARY

Cell fusion is essential for fertilization, myotube formation, and inflammation. Macrophages fuse under various circumstances, but the molecular signals involved in the distinct steps of their fusion are not fully characterized. Using null mice and derived cells, we show that the protease MT1-MMP is necessary for macrophage fusion during osteoclast and giant-cell formation in vitro and in vivo. Specifically, MT1-MMP is required for lamellipodia formation and for proper cell morphology and motility of bone marrow myeloid progenitors prior to membrane fusion. These functions of MT1-MMP do not depend on MT1-MMP catalytic activity or downstream pro-MMP-2 activation. Instead, MT1-MMP null cells show a decreased Rac1 activity and reduced membrane targeting of Rac1 and the adaptor protein p130Cas. Retroviral rescue experiments and protein binding assays delineate a signaling pathway in which MT1-MMP, via its cytosolic tail, contributes to macrophage migration and fusion by regulating Rac1 activity through an association with p130Cas.

INTRODUCTION

Cell fusion is fundamental in processes such as fertilization and vertebrate myogenesis (Chen et al., 2007), and may also be important in inflammation (Johansson et al., 2008; Nygren et al., 2008). Under certain circumstances, cells of the monocyte/macrophage lineage can fuse, giving rise to osteoclasts (OC) in bone or giant cells (GC) in inflamed soft tissues. These multinucleated derivatives acquire specialized functions in bone resorption and engulfment of pathogens and foreign bodies, respectively (Vignery, 2005). Several diseases of the adult skeleton are related to disturbances in OC function, either through increased activity (bone metastasis, osteoporosis, Paget's disease) or decreased activity (osteopetrosis).

Molecules can contribute to cell fusion by either directly participating in membrane fusion or by affecting earlier steps in the process (Oren-Suissa and Podbilewicz, 2007; Primakoff and Myles, 2007). The signaling pathways involved in cell-cell fusion have mostly been characterized in yeast and invertebrates; much less is known about the regulation of fusion by mammalian cells, and by macrophages in particular (Chen et al., 2007). For example, EFF-1 and AFF-1 act as direct membrane fusogens during *Caenorhabditis elegans* development (Mohler et al., 2002; Sapir et al., 2007), but the mechanisms by which proteins such as DC-STAMP, the d2 isoform v-ATPase, and CD200 contribute to macrophage fusion remain undefined (Cui et al., 2007; Lee et al., 2006; Yagi et al., 2005). One central issue is how competent cells come into contact. In the case of myotubes, the myogenic precursors are already in close proximity, whereas sperm-egg fusion is dependent on sperm motility; however, the mechanisms by which OC and GC precursors achieve proximity are poorly understood.

MMPs (matrix metalloproteinases) are endopeptidases capable of degrading a variety of extracellular matrix components and of modulating the activity of several secreted and cell-surface proteins (Page-McCaw et al., 2007). MT1-MMP (membrane type 1-MMP) is a membrane-anchored collagenase that plays important roles in pathophysiological settings, including the development of skeletal, lung, and adipose tissue, angiogenesis, and tumor invasion (Chun et al., 2006; Holmbeck et al., 1999; Oblander et al., 2005; Sabeh et al., 2004; Zhou et al., 2000). Here we report that MT1-MMP, independent of its catalytic activity, regulates Rac1 signaling in myeloid cells, thereby contributing to their migration and fusion during osteoclastogenesis and GC formation in vitro and in vivo.

RESULTS

MT1-MMP Null Myeloid Cells Are Defective for OC Multinucleation In Vitro and In Vivo

MT1-MMP participates in leukocyte migration (Matias-Roman et al., 2005; Yang et al., 2006), and we therefore analyzed hematopoietic development in MT1-MMP null mice. These mice die 2 weeks after birth, so 8-day-old mice were used for all analyses. Flow cytometry showed that the percentage of cells