Limk1 Promotes Mt1-mmp Expression And Localization To The Plasma Membrane

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LIM KINASE 1 PROMOTES MT1-MMP EXPRESSION AND LOCALIZATION TO THE PLASMA MEMBRANE

by

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ABSTRACT

LIM Kinase 1 (LIMK1), a serine/threonine kinase, modulates actin polymerization and microtubule assembly. The function of LIMK1 is regulated by kinases that are activated by Rho and Rac GTPases. LIMK1 is overexpressed in various cancerous cell types and tissues and its overexpression promotes increased invasion and metastasis of breast and prostate cancer cells. Membrane-Type Matrix Metalloproteinase 1 (MT1-MMP) is a member of the zinc-binding collagenase family, which is involved in extracellular matrix breakdown and activation of secreted MMP-2. The balance between activation and inhibition of MT1-MMP and MMP-2 helps maintaining normal extracellular matrix turnover. However, it has been shown that elevated MT1-MMP expression can cause excessive ECM digestion and promote tumor invasion and metastasis. Since RhoA and Rac1 have been implicated in metastasis and invasion along with LIMK1 activation, we investigated a possible link between LIMK1 and MT1-MMP. Our results show that the level of MT1-MMP expression is correlated with that of LIMK1 and LIMK1 acts as a transcriptional regulator of MT1-MMP. Additionally, we show that LIMK1 physically associates with MT1-MMP and promotes its translocation to the plasma membrane.

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1. INTRODUCTION

1.1 LIM Kinase 1

LIM Kinase 1 (LIMK1) is a dual specificity serine/threonine and tyrosine kinase involved in modulating actin and microtubule dynamics. It is a member of the small family of LIM domain containing kinases, which also includes LIM Kinase 2. LIMK1 contains two LIM domains in tandem at the N-terminus, a PDZ domain, and a kinase domain at the C-terminus (Figure 1) [Okano et al., 1995]. LIM domains are zinc -coordinating loops, which are involved in protein- protein interactions. The residues involved in zinc interactions are highly conserved while the residues in the center of the loop are more variable and provide the diversity for specific interactions with different proteins. In addition to the LIM domains, the PDZ domain is also believed to be involved in mediating protein-protein interactions. LIMK1 also contains a nuclear localization sequence in its kinase domain along with two nuclear exit signals in the PDZ domain [Yang and Mizuno, 1999].

Inactive LIMK1 exists in a closed conformation state, which is a result of its LIM domains associating with its C-terminal kinase domain. Activating phosphorylation at Thr^{508}, located within the kinase domain, induces a conformational change that allows its kinase domain to be able to interact with its substrates (Figure 2) [Edwards et al., 1999]. The activity of LIMK1 is also modulated through association with Hsp90 leading to transphosphorylation

and an increased half-life (Figure 2) [Li et al., 2006]. LIMK1 is phosphorylated by kinases that are activated by the Rho subfamily of small GTPases [Lou et al., 2001].

Two members of the Rho family, Rac1 and Cdc42 mediate activation of LIMK1. Rac1 and Cdc42 in their GTP bound form bind to an inactive PAK1/4 homodimer. This binding causes the inhibitory domain of one PAK protein to disassociate from the kinase domain of the other PAK protein [Stofega et al., 2004]. Activated PAK1/4 can then phosphorylate LIMK1, which promotes formation of lamellipodia and filopodia. In addition to Rac1 and Cdc42, GTP-bound RhoA also activates LIMK1 through recruitment and activation of Rho kinase ROCK. Activated ROCK phosphorylates LIMK1 at T^{508} and activates it [Lin et al., 2003]. The RhoA signaling pathway induced activation of LIMK1 leads to the formation of actin stress fibers.Activation of LIMK1 is involved in stabilizing the actin cytoskeleton through the inactivating phosphorylation of Cofilin at Ser^{3} [Arber et al., 1998]. This phosphorylation prevents Cofilin from severing actin filaments to globular (G) actin and results in the accumulation of filamentous (F) actin (Figure 2), [Moriyama et al., 1996]. Slingshot Phosphatase functions in opposition to this action by removing the phosphate group on Cofilin, restoring its activity and causing the increase in G-actin. In addition to stabilizing actin filaments, LIMK1 can cause a reduction of microtubule stability through its association with Tubulin-Polymerization Promoting Protein alpha (TPPP-α). The inhibitory phosphorylation of TPPP-α on serine residues by LIMK1 promotes the disassembly of tubulin polymers [Acevedo et al., 2007].

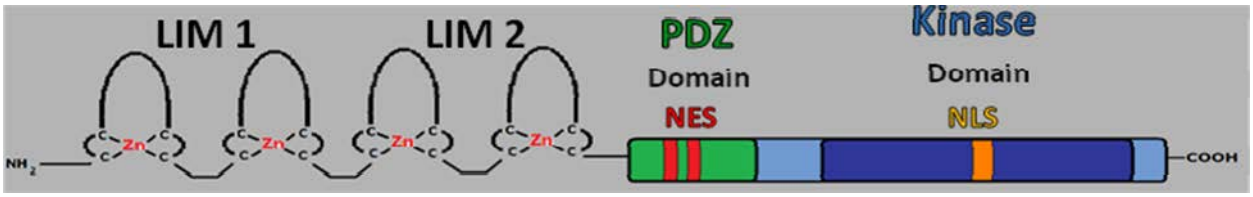
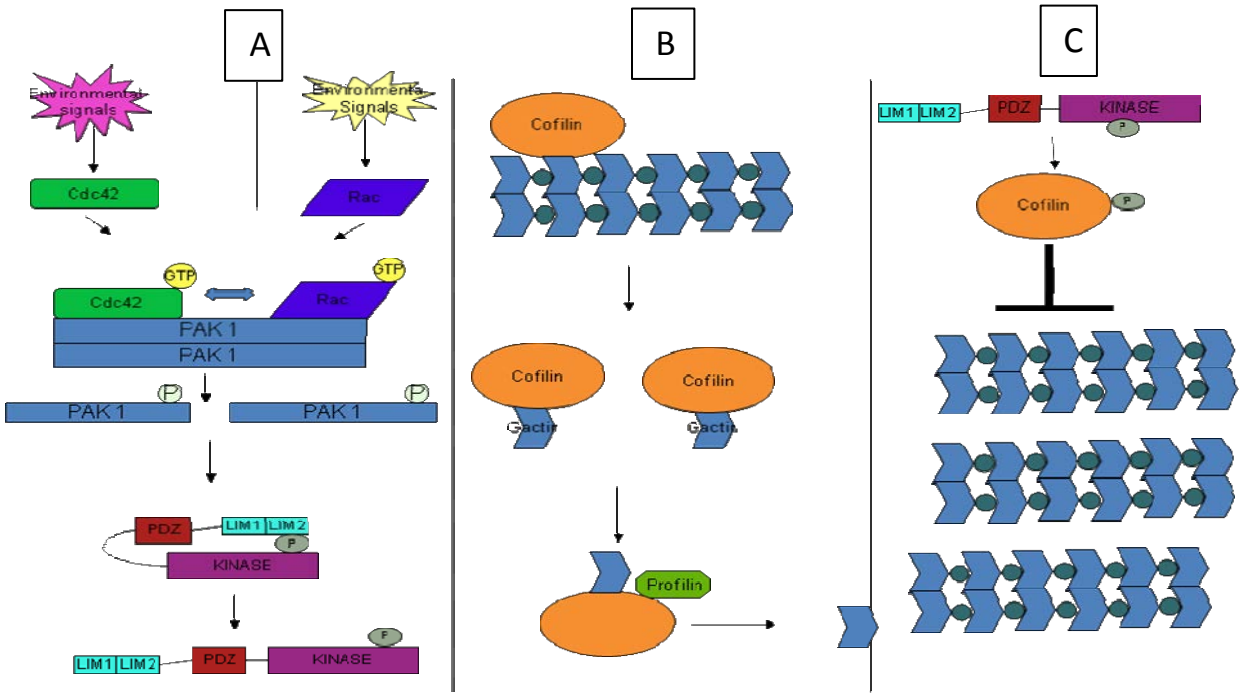


Figure 1: Structure of LIM Kinase 1

LIM Kinase 1 contains two N-terminal LIM domains, a PDZ domain, and a C-terminal Kinase domain. A nuclear localization signal is located in the Kinase domain and two nuclear exit signals are located in the PDZ domain.



(Tapia T., 2007)

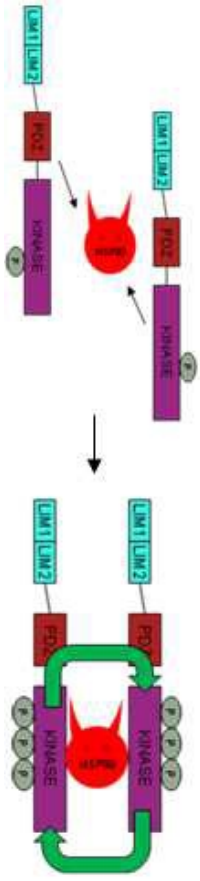


Figure 2: Activation and Function of LIM Kinase 1

A) GTP bound Cdc42 and Rac1 bind to PAK1 homodimers resulting in dissociation and transphosphorylation of PAK1. Active PAK1 phosphorylates LIMK1 at T508, causing a conformational change that activates LIMK1 and allows binding of Hsp90 leading to trans-phosphorylation and increased stability. B) Phosphorylation of cofilin at Ser3 by LIMK1 inactivates cofilin. As a result cofilin is unable to bind to and induce severing of filamentous actin. C) Dephosphorylation of cofilin by slingshot restores its activity. Resulting in the disassociation of actin subunits.

1.2 Role of LIM Kinase 1 in Cancer Progression

The progression of cancer to a metastatic state is associated with loss of contact inhibited growth, increased cell mobility, and acquiring the ability to evade the primary tumor site and invade secondary organs. Increased activity of Rho GTPases is noted in a variety of cancers, which results in deregulation of pathways involved in modulation of actin cytoskeleton. Loss of regulation can lead to cell polarization and induce structures associated with cell mobility and invasion. These processes rely on stabilized actin filaments to provide the necessary forces need to drive changes in cell morphology. The actin depolymerizing family includes the protein cofilin, which is a known substrate of LIMK1. Inactivation of cofilin by LIMK1 results in local stabilization of actin filaments. LIMK1 and cofilin localize to focal adhesions and to the membrane ruffles of lamellipodia at the plasma membrane. Overexpression of LIMK1 is associated with an invasive cancer and was shown to promote formation of invadopodia. Although actin cytoskeleton reorganization is a key step in invasion, degradation of extracellular matrix is an important initial event that allows tumor cells to escape the primary site, which leads to distant metastasis. One group of proteolytic proteins integral in these events is the family members of Matrix Metalloproteinase (MMP). Additionally, increased activity of Rho signaling cascades are associated with enhanced expression, activation, and localization of MMPs.

1.3 Matrix Metalloproteinases

Matrix metalloproteinases (MMP) are zinc-binding endopeptidases involved in maintaining the dynamic balance of the extracellular environment through regulated degradation of the ECM and basement membrane [Kessenbrock et al., 2010]. These enzymes play diverse roles in tissue remodeling, organ development, inflammation, and cancer. Twenty three MMP family members that are expressed in humans share common characteristics including structural elements, Zn dependence, and tight regulation. These shared structural features include the N-terminal signal peptide, propeptide, catalytic domain, and a C-terminal hemopexin-like domain connected by a flexible hinge region [Visse, Nagase, 2003]. The MMP family is sub-classified into two groups: soluble or membrane associated members. Membrane associated MMPs, in which MT1-MMP is classified, have a transmembrane domain ending in a short cytoplasmic tail or instead linked to a GPI anchor (Figure 3). MMP regulation begins at the transcriptional level and requires the activation of transcription factors, including Sp1 and AP-1, to initiate transcription of the pro-enzymes [Sroka et al., 2007][Benbow et al., 1996]. Like many other proteolytic enzymes, MMPs are expressed as a zymogen providing additional control over activity. Latent MMP molecules have the cysteine residue, present in the prodomain, associated with the zinc ion present at the catalytic site. This conformation prevents the catalytic domain from interacting and cleaving its substrates. The cysteine switch mechanism is the next regulatory

step that requires the cleavage or modification of the prodomain to activate the MMP [Van Wart et al., 1990]. Cleavage events for secreted and membrane associated MMPs occur at different locations. Both types have their signal sequence cleaved upon entry or integration into the ER, and are transported through the Golgi to specific areas of the membrane. Soluble MMPs can then be secreted into the extracellular environment where processing to the active form occurs. Alternatively, membrane-associated MMPs are activated by a convertase during transport and then are tethered to or integrated into the plasma membrane. Further regulation of MMP activity is modulated by expression of Tissue Inhibitors of Metalloproteinases (TIMPs). TIMPs bind MMPs and block their active site inhibiting their catalytic activity, although TIMP2 has an additional role in the activation of MMP-2.

MMPs participate in normal physiological processes including roles in tissue remodeling and angiogenesis. Under normal physiological conditions, MMP activity is tightly controlled at multiple levels but loss of this regulation can lead to excessive degradation of the ECM and events associate with cancer progression. MMPs have long been linked with cancer and invasion. Early research sought inhibitors to reduce the activity of these peptidases; unfortunately the inhibitors were not effective in significantly changing the disease outcome in patients. This may be partially explained by more recent research showing additional role of MMPs in cancerous tissue beyond degrading basement membranes. MMPs participate in the complex cross talk between the cancerous tissue and

stromal cell and alter intracellular signaling. These findings suggest MMPs can modify tumor vasculature, inhibit apoptosis, and promote cell proliferation. Additionally, MMPs can induce epithelial-to-mesenchymal transition (EMT), a hallmark of advanced stage cancers.

One important member of the MMP family is MT1-MMP, also known as MMP-14. MT1- MMP is anchored to the membrane by its transmembrane domain and positioned with the catalytic domain in the pericellular space. MT1-MMP has roles in normal development along with cancer progression. It is found to be overexpressed in a variety of tumor tissues (Visse et al., 2003). Functions of MT1-MMP include degradation of multiple ECM components, activation of MMP-2, promotion of cell migration, and regulation of growth in 3-D tissue environment. With many diverse functions, cells have evolved a complex means of regulation for MT1-MMP. Starting at the transcriptional level, activation of the transcription factor Sp1 drives expression of proMT1-MMP. Next, the propeptide is cleaved in the Golgi by furin convertase producing the active enzyme [Yana et al., 2000] that is targeted to the plasma membrane. This cleavage along with glycosylation, are speculated to modulate localization and substrate specificity of MT1- MMP [Wu et al., 2007][Wu et al., 2004]. The activity of membrane-integrated MT1-MMP undergoes further regulation by the levels of expressed TIMP2 [Strongin et al., 1995]. Additional regulatory processes include inactivation by autocatalytic shedding, and endocytosis [Osenkowski et al., 2004].

One of the major functions of MT1-MMP is the proteolytic activation of secreted proMMP-2. Elevated expressions of MT1-MMP, MMP-2, and MMP-9 are positively

correlated with tumor progression, invasiveness, and poor patient survival [Deryugina et al., 2006]. Activation of proMMP-2 by MT1-MMP requires sub saturation expression levels of TIMP2. TIMP2 binds and inhibits MT1-MMP but is still free to bind the hemopexin-like domain of proMMP-2. The resulting MT1-MMP/TIMP2/proMMP-2 complex can now associate with a neighboring TIMP2-free MT1-MMP molecule through the hemopexin domains of both MT1- MMP molecules, initiating cleavage of proMMP-2 (Figure 4), [Itoh et al., 2001]. Furthermore, MT1-MMP/TIMP2/MMP-2 complexes can cleave and activate secreted proMMP-9, in a TIMP-2 dependent activation cascade starting with MT1-MMP [Toth et al., 2003].

1.4 Role of MT1-MMP in Cancer Progression

MT1-MMP has many functions in the development of metastatic cancers, and its expression is associated with increased invasiveness of cancerous cells. Invasion begins at the invadopodia, similar to lamellipodia, which are specialized membrane protrusions containing actin scaffolding that penetrate the substrate below. This penetration requires the degradation of the extracellular matrix by secreted and membrane associated proteases. An essential mediator of this process, MT1-MMP is localized to these regions, and also promotes MMP-2 localization through binding to its hemopexin domain [Kim et al. 1998]. MT1-MMP can then degrade multiple ECM substrates including collagens, laminins, and

fibronectin. This physically allows the cells to migrate but also reveals cryptic binding sites on the cell surface that play additional roles. The cleavage of laminin-5 by MT1-MMP promotes increased cell motility and this effect is amplified by MMP-2, which is activated by MT1-MMP [Koshikawa et al., 2000]. In addition to cancer cell motility, cleavage of collagen-IV reveals a cryptic binding site, which promotes migration of vascular endothelial cells. The invasion and migration of vascular endothelial cells is a critical event required for angiogenesis. Additionally, MT1-MMP can degrade the fibrin matrix surrounding new vessels [Hiraoka et al., 1998]. This potentially allows for increased sprouting of new vessels and increased circulation to the tumor. During the development of new vasculature, newly formed vessels are leaky and have loose cellular junctions especially in the lymphatic vessels. This increases the ability of cancerous cells to become metastatic by escaping the tumor and entering circulation. Circulating tumor cells then need to escape the blood vessels and enter surrounding tissues. This process is called extravasation and expression of MT1-MMP is involved in these events {Tsunenzuka et al., 1996]. Extensive research has been conducted on regulation of expression and functions of MT1-MMP, some of which has been described, but still many aspects remain to be elucidated. This can partially be explained by the diverse roles of MT1-MMP and the impact other MMPs have on cell-cell cross talk. Although broad-spectrum inhibitors of MMP initially looked promising because of the structural similarities among MMPs, non-oncogenic MMPs are also inhibited by these inhibitors. As a result, significant side effects, high toxicity and limited effectiveness of

these inhibitors made them failed clinically. New therapeutics needs to be developed with reduced toxicity, which may be achieved through the inhibition of multiple oncogenic proteins through combination therapy or inhibiting specific MMP, such as MT1- MMP.

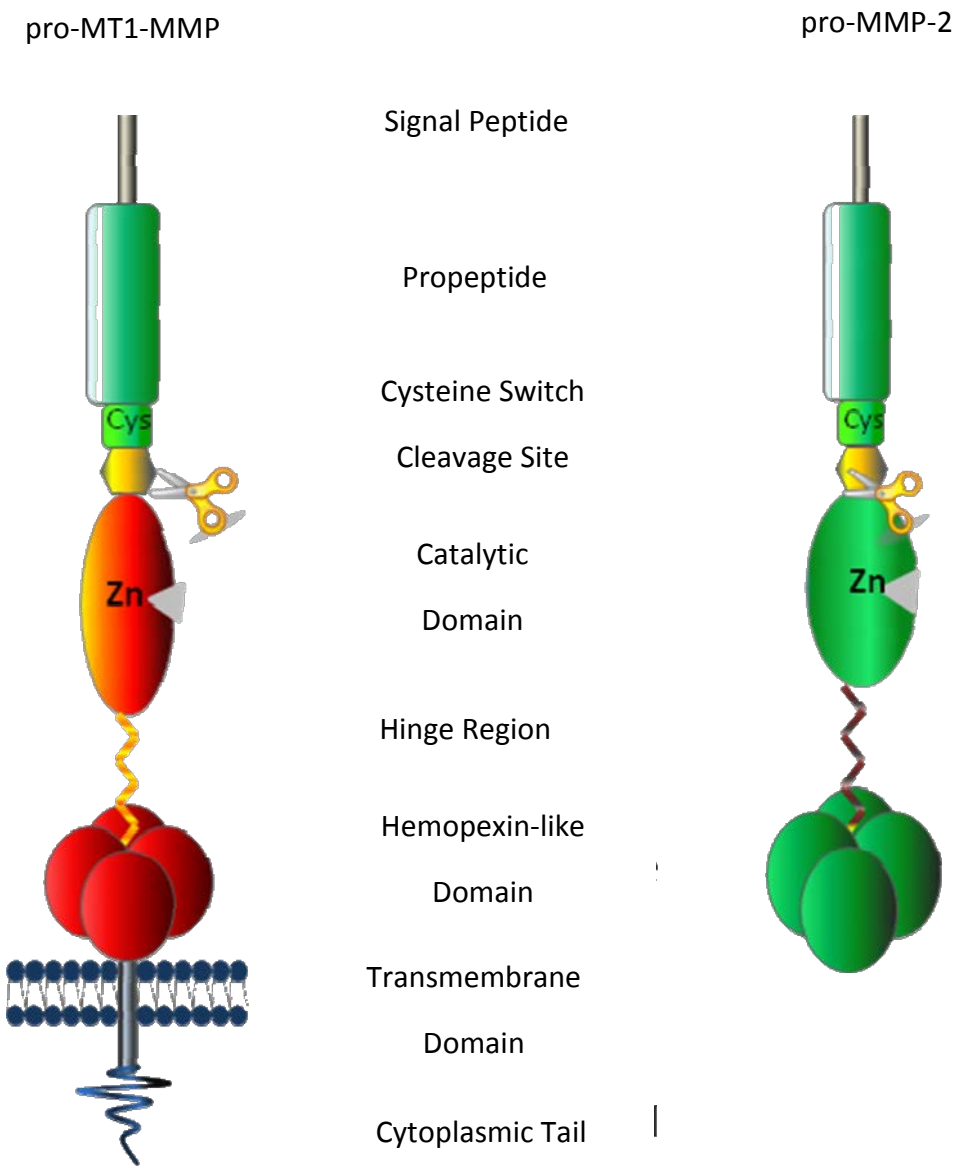


Figure 3: Structural Organization of proMT1-MMP and proMMP-2

Common structural elements shared by MMPs include the signal peptide, propeptide (containing Cys residue), and Zn binding catalytic domains.

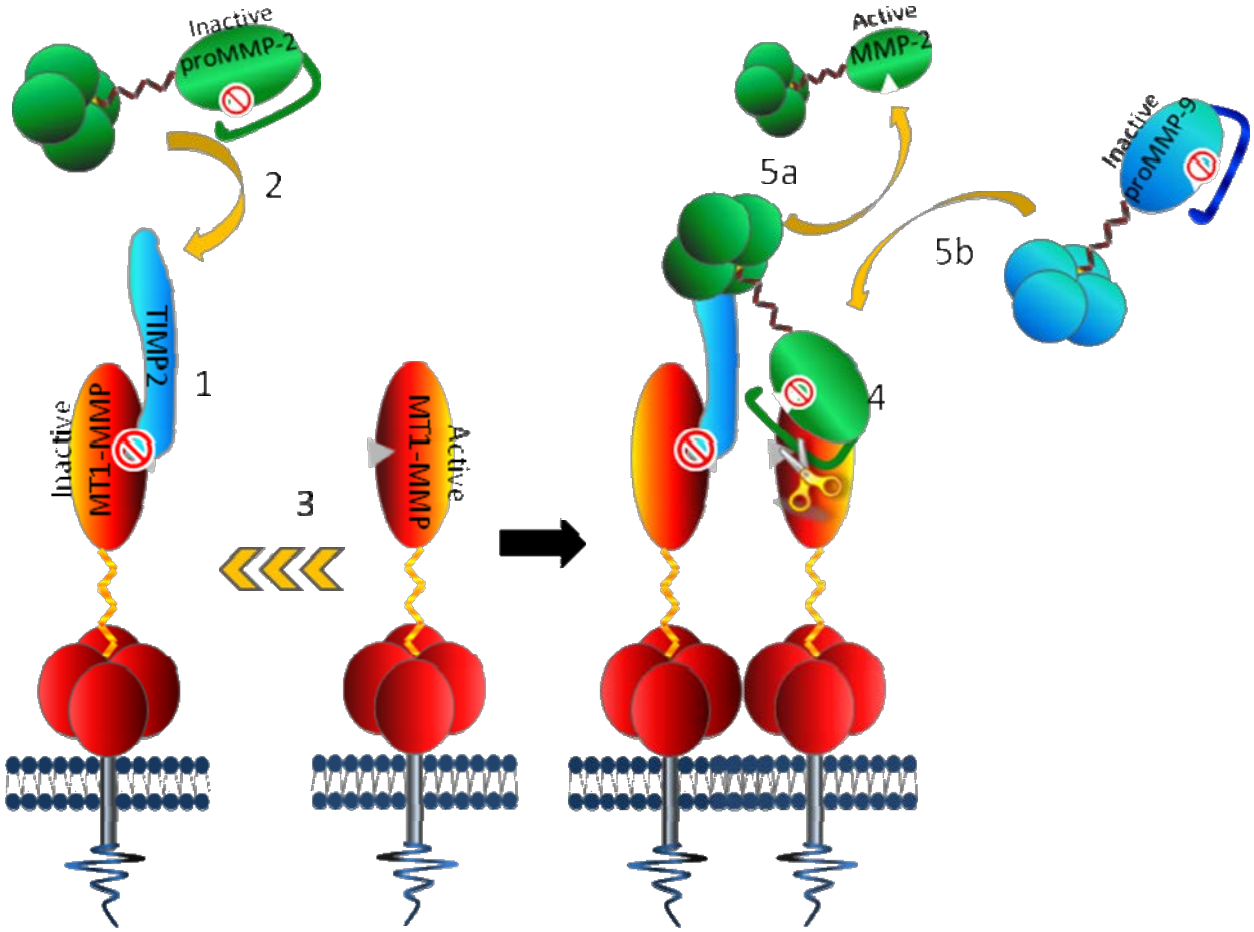


Figure 4: TIMP2 Mediated Activation of proMMP-2 by MT1-MMP

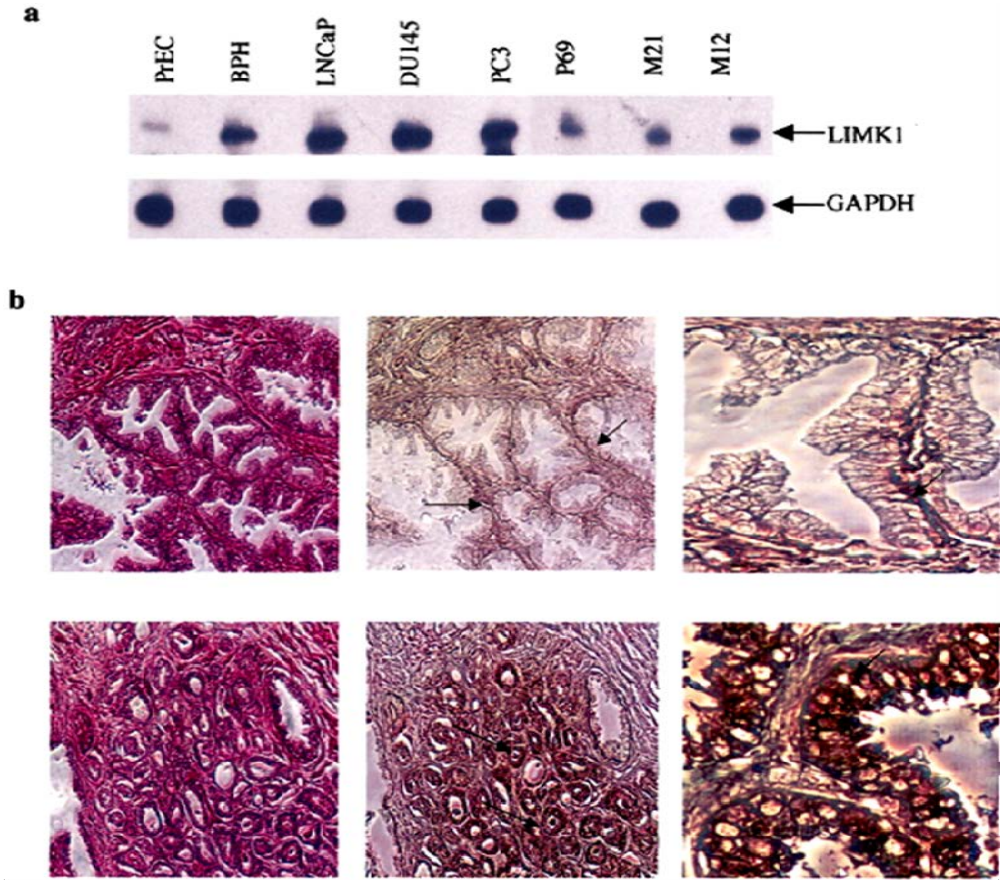
1) TIMP2 binds and inactivates MT1-MMP, 2) Soluble proMMP-2 binds TIMP2 through its hemopexin-like domain, 3) A neighboring active MT1-MMP molecule can now associate with the MMP/TIMP2 complex, 4) The active MT1-MMP interacts with the complexed MT1-MMP and initiates cleavage/activation of proMMP-2, 5a) Active MMP-2 disassociates from complex or 5b) Active MMP-2 remains complexed and activates proMMP-9.

1.5 Previous Studies

Earlier studies from our laboratory and others showed overexpression of LIMK1 in various types of cancerous tissues including cancerous prostate tissue. Differential expression of LIMK1 also was noted in a variety of prostate cancer (PCa) cell lines and normal prostate epithelial cells (PrEC). Our studies showed elevated LIMK1 expression in all cancer cell lines analyzed compared to PrEC (Figure 5). The highest expression of LIMK1 was noted in the metastatic prostate cancer line PC-3 and a low to moderate level in the Benign Prostatic Hyperplasia cell line BPH-1. A higher level phosphorylated cofilin, a bona fide LIMK1 substrate was also noted in PC-3 cells compared to BPH-1 cells . To study the function of LIMK1 BPH-1 cell line was used for ectopic expression of LIMK1. Several constructs were prepared to express full- length as well as mutant LIMK1. These include: Full length LIMK1; Kinase-dead LIMK1; or Constitutively-Active LIMK1. Kinase-dead LIMK1 was generated by mutating Asp^{460} into an alanine, and Constitutively-Active LIMK1 was generated by mutating Thr^{508} into two glutamic acid residues. Our studies further showed that the stable expression of full length LIMK1 was enough to induce an invasive phenotype in BPH-1 cells and the partial inhibition of LIMK1 in PC-3 cells using anti-sense RNA reduced the number of invasive cells [Davila et al. 2003]. Because matrix metalloproteinases (MMPs) are the key mediators involved in invasion, a possible correlation between LIMK1 expression and MMPs was studied next. BPH-1 cells expressing constitutively active LIMK1 ($BPHL^{CA}$) or the control

vector (BPH^{V}) were used to study the effect of LIMK1 expression on invasion. These assays were conducted with or without treatment with a broad-spectrum MMP inhibitor, ilomastat (GM6001). The results showed that $BPHL^{CA}$ cells had a 4-5-fold increase in the number of invasive cells, and that this invasion was largely blocked by treatment with the MMP inhibitor. This data lead us to speculate that a possible relationship exists between LIMK1 expression and MMPs.

To further elucidate this link we monitored the effect of LIMK1 expression on the secretion of proMMP-2 and proMMP-9. Elevated expression of MMP-2 and MMP-2 has been correlated with increased malignancy. Secretion of these MMPs was studied next using Zymography which showed increased gelatinolytic activity of both MMP-2 and MMP-9 in $BPHL^{CA}$ cells compared to vector expressing cells. We also noted increased concentrations of proMMP-2 and active MMP-2 in $BPHL^{CA}$ cells, while MMP-9 only showed an increase in its latent form. To verify that the increased expression of LIMK1 was responsible for these results, we monitored MMP-2 and MMP-9 mRNA levels in $BPHL^{CA}$ compared to vector expressing cells by RT-PCR. We observed a 10 fold increase in MMP-2 mRNA in $BPHL^{CA}$ compared to vector expressing cells, although the levels of MMP-9 mRNA remained relatively unaffected by LIMK1 expression. This data suggested a possible correlation between LIMK1 expression and MT1- MMP, because MT1-MMP is both an activator of MMP-2 and a target of the inhibitor,ilomastat.



Davila M et al. JBiol. Chem. 2003;278:36868-36875

Figure 5: LIMK1 is differentially expressed in human prostate cell lines and prostate tissues

a) Immunoblot analysis of LIMK1 in total lysates of human prostate cell lines. b) Expression of LIMK1 in prostate tissues. Upper panel, Normal/benign areas. Lower panel, cancerous glands. Upper and lower left, Hematoxylin/eosin-stained slides. Upper and lower right, areas from normal and cancerous tissues are shown in higher magnification (×200). Arrows indicate light staining in basal cells in normal/benign areas (upper panel) and intense staining in cells in cancerous areas (lower panel)

1.6 Hypothesis/Objectives

Based on published studies and preliminary experiments conducted in our laboratory, we hypothesize that LIMK1 is involved in the regulation MT1-MMP. To test our hypothesis we planned to study

1. The expression of MT1-MMP in cells differentially expressing LIMK1
2. A possible physical association of LIMK1 with MT1-MMP
3. Subcellular localization of LIMK1 and MT1-MMP
4. Effect of LIMK1 expression on MT1-MMP transport and localization
5. Effect of LIMK1 expression on MT1-MMP transcription

2. MATERIAL AND METHODS:

2.1 Cell Culture and Cell Lines

The parental BPH-1 cells (a gift for P Narayana, University of Florida) (Hayward et al., 1995) and its transfected sub-lines, were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) with 10% fetal bovine serum and 1% antibiotic/antimycotic (Invitrogen). BPH-1 cells stably overexpressing constitutively active (phosphomimic) LIMK1 with a C-terminal Flag tag ($BPHL^{CA}$) and the empty vector control (BPH^{V}) were produced previously in the lab. The LIMK1 phosphomimic (T^{508EE}) was produced by site directed mutagenesis. Stable cells were selected using hygromycin and stable populations were mixed to prevent clonal bias and continually maintained in media containing hygromycin. PC3 cells (ATCC) were maintained in F-12 HAM (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 2mM glutamine, and 1% antibiotic/antimycotic. All cells were in a humidified atmosphere containing 5% CO2, at 37°C.

2.2 Immunoblot, Immunoprecipitation, and Antibodies

For immunoblots, cells were lysed using RIPA lysis buffer (50mM Tris pH 8.0, 20mM NaCl, 2.5mM EDTA, 1mM PMSF, 1% NP-40, 10ug/mL leupeptin, 10ug/mL aprotinin) and freeze thaw cycles. 50ug of whole cell extract was separated by SDS-PAGE and subjected

to western blotting with the appropriate primary and horseradish peroxidase conjugated secondary antibodies. A chemiluminescence kit (Thermo Scientific) was used to detect target proteins. For immunoprecipitation, cells were lysed as describe above in RIPA lysis buffer containing a proteinase inhibitor mixture set III (Calbiochem EMD). LIMK1 or MT1-MMP was immunoprecipitated from 500ug whole cell lysate using 2ug of the appropriate primary antibody for 4 hours at 4°C. Antigen-antibody complexes were immunoprecipitated with 40ul protein A/G PLUS Sepharose beads (Santa cruz) for 16-18 hours at 4°C. Precipitated proteins were detected by immunoblot analysis and described above.

Table 1: Dilutions of antibodies and incubation times

Antibody	Company	Dilution	Incubation Time/Temperature
Flag	Sigma	1:2000	1hr/RT
LIMK1	BD biosciences	1:100	16hr/4°C
GAPDH	Sigma	1:1000	1hr/RT
MT1-MMP	Neomarker	7ug/ml	16hr/4°C

2.3 Gene Silencing Using Short Hairpin RNA

LIMK1 expression in PC3 cells was reduced by transfection of HuSH shRNA construct against LIMK1 (#3: AAGGACAAGA GGCTCAACTTCATCACTGA) in the pGFP-V-RS vector (Origene Technologies). Four different shRNA constructs targeting LIMK1 were screened and the construct with the highest reduction in LIMK1 was used in subsequent experiments. A non- targeting shRNA construct (scr) was created to control for off target effects. PC3 cells were transiently transfected with either construct using Lipofectamine or FuGENE HD for 55-72 hours.

2.4 Dual and Triple Label Immunofluorescence Analysis

Cells were seeded on poly-L-lysine coated coverslips in 24 well dishes and allowed to attach for 24 hours. For knockdown experiments, PC3 cells were transfected with LIMK1 shRNA or scrambled shRNA 24 hours after seeding and were maintained for an additional 24 hours. For immunostaining, cells were washed with phosphate buffer (0.1M) and fixed in 4% paraformaldehyde at room temperature for 10 minutes. Cells were then washed with either 0.2% Triton X-100 (BPH) or 0.1% Triton X-100 for 3 min or 0.1% Tween-20 for 4 min (PC3) in phosphate buffer. Cells were blocked in phosphate buffer containing 10% goat serum, 0.2% Triton X-100 for 1.5 hours at room temperature. Cells were stained with primary antibodies diluted in blocking solution for 1 hour at room temperature. Cells were then washed with blocking solution. Next, cells were stained with fluorophore-conjugated

secondary antibodies diluted in blocking solution for 30 minutes at room temperature. Coverslips were then washed in phosphate buffer containing Triton X-100 or Tween-20 as described above then post-fixed with 4% paraformaldehyde for 5 minutes at room temperature. Coverslips were mounted with gel mount (BioMeda) and visualized on a Zeiss 710 confocal microscope. Colocalization values were quantified by selecting specific regions of singly labeled cells to set the thresholds. The specific regions of interest (vesicles, entire cell, or entire membrane) were used for pixel quantification. Colocalization was quantified using Zeiss Zen 2009 software or Olympus FV1- ASW software, which calculates overlap and colocalization coefficient as derived from Mander's article based on Pearson's correlation coefficient.

$$\text{Overlap Coefficient}: \left[\Sigma(Ch1_i)(Ch2_i)\right] / \left[\sqrt{\left(\text{sum}(Ch1_i)^2(Ch2_i)^2\right)}\right]$$

The values for the overlap coefficient range from 0 to 1. An Overlap Coefficient with a value of 1 represents perfectly colocalized pixels.

$$\text{Pearson's Correlation}: \left[\Sigma\left(Ch1_i - Ch1_{avg}\right)\left(Ch2_i - Ch2_{avg}\right)\right] / \left[\sqrt{\left(\text{sum}\left(Ch1_i - Ch1_{avg}\right)^2\left(Ch2_i - Ch2_{avg}\right)^2\right)}\right]$$

Because each pixel is subtracted by the average pixel intensity, the value for Correlation R

can range from -1 to 1. A value of 1 would mean that the patterns are perfectly similar (colocalized), while a value of -1 would mean that the patterns are perfectly opposite.

Table 2: Dilutions of antibodies and incubation times

Primary Antibody	Company	Dilution	Incubation Time/Temperature
Flag	Sigma	35mg/mL	1hr/RT
LIMK1	BD biosciences	1:50	1hr/RT
MT1-MMP	Neomarker	1:200	1hr/RT
TGN46	Novus Biologicals	1:115	1hr/RT

2° Ab	Company	Dilution	Incubation Time/Temperature
Cy3	Jackson Labs	1:300	30min/RT
Cy5	Jackson Labs	1:300	30min/RT
Alexa 647	Invitrogen	1:300	30min/RT

2.5 Dual Luciferase Reporter Assay

Reporter assays were conducted with firefly luciferase driven by a 7.2 KB promoter fragment of MT1-MMP in a modified pGL3 parent vector (kindly provided by Jorma Keski-Oja, University of Helsinki). Cells were co-transfected with this construct and a transcription control construct containing Renilla luciferase driven by thymidine kinase promoter using Lipofectamine LTX according to our published protocol (Tapia et al., 2011). Stably transfected $BPHL^{CA}$ and BPH^{V} cells and PC3 cells transiently transfected with LIMK1 shRNA or scrambled shRNA were used. PC3 cells were harvested 62 hours post-transfection. Luciferase expression was determined using a Dual luciferase assay kit (Promega) according to manufacturer's protocol.

2.6 Statistical Analysis

Quantitative results are presented as mean ± SD of the number of independent experiments performed. A p value of < 0.05 was considered significant.

3. RESULTS:

3.1 Expression of MT1-MMP Correlates with the Expression of LIMK1

MT1-MMP expression has been shown to be elevated in tumors of increasing grade and malignancy, as is the expression of LIMK1. In order to elucidate a possible connection between the expression of LIM Kinase 1 and MT1-MMP, we first monitored the expression of MT1-MMP in cells differentially expressing LIMK1. Cell lysates from BPH-1, $BPHL^{CA}$, and PC-3 were immunoblotted with anti-LIMK1 and anti-MMP antibodies, and expression patterns were compared. We chose to use an antibody targeting the hinge region of MT1-MMP, because it recognized all forms of the protein. Our immunoblot results showed that with increasing concentrations of LIM Kinase 1, cells also expressed increasing levels of MT1-MMP (Figure 6). Increasing levels of all forms of MT1-MMP such as, latent, active, and autocatalytic forms were detected. To further confirm these findings, we employed shRNA mediated down regulation of LIMK1 expression in PC-3 cells.. LIM Kinase inhibition resulted in the reduced expression of all forms of MT1-MMP as noted by western blots.

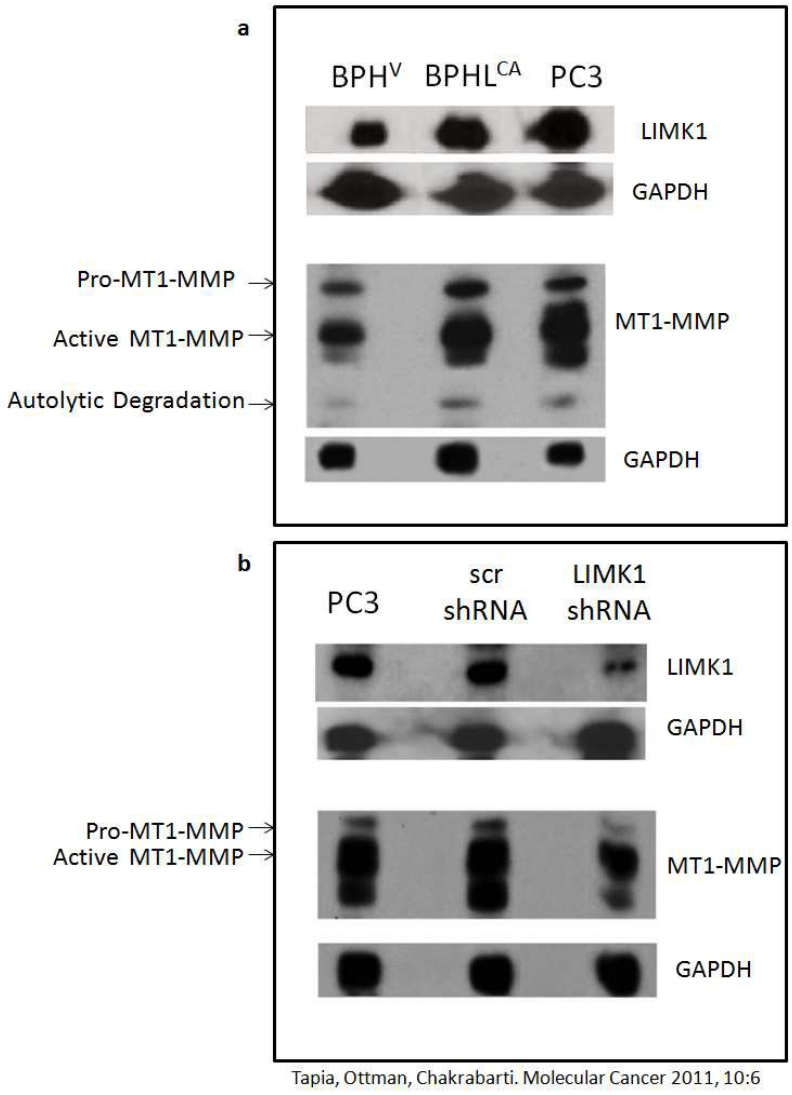


Figure 6: Expression of MT1-MMP correlates with LIMK1 Expression

Immunoblot analysis of LIMK1 and MT1-MMP, in total lysates of BPH^{V}, $BPHL^{CA}$, and PC-3 cell lines. a) Cells overexpressing LIMK1 display elevated MT1-MMP expression, b) PC-3 cells expressing LIMK1-shRNA display reduced MT1-MMP expression.

3.2 LIM Kinase 1 Colocalizes & Physically Associates with MT1-MMP

We next investigated whether LIMK1 physically interacts with MT1-MMP using immunoprecipitation and immunofluorescence analyses. LIMK 1 was immunoprecipitated using PC-3 cell lysates and immunoblotted with MT1-MMP. Detected polypeptide bands correlated with both active and latent forms of MT1-MMP. A polypeptide band corresponding to LIMK1 was also detected in the immunoblots of the reverse immunoprecipitation, which confirmed a physical association of LIMK1 with MT1-MMP in a complex (Figure 7). Additionally we noticed that LIMK1 was associating with both latent and active forms of MT1-MMP.

Next we monitored localization of these proteins within PC-3 and $BPHL^{CA}$ cells using dual-label immunofluorescence analysis (Figure 8). Our results indicated that both LIMK1 and MT1-MMP were colocalized in the Golgi area as well as at the periphery of the cell. To confirm that these proteins were indeed colocalized, quantitative analysis of overlapping pixels and intensities was conducted using both Zeiss Zen and Leica LasAF Software (Figure 9). The colocalization values were generated on the basis that photons from both intensities/wavelengths, each wavelength correlating to either LIMK1 or MT1-MMP, were striking the same photo detector cell. The generated values strongly suggest a physical interaction between LIMK1 and MT1-MMP at the Golgi area and at the plasma

membrane in both PC-3 and BPHL^{CA} cells. This strengthened the co-immunoprecipitation results showing that LIMK1 was interacting, directly or indirectly, with both latent and active MT1-MMP.

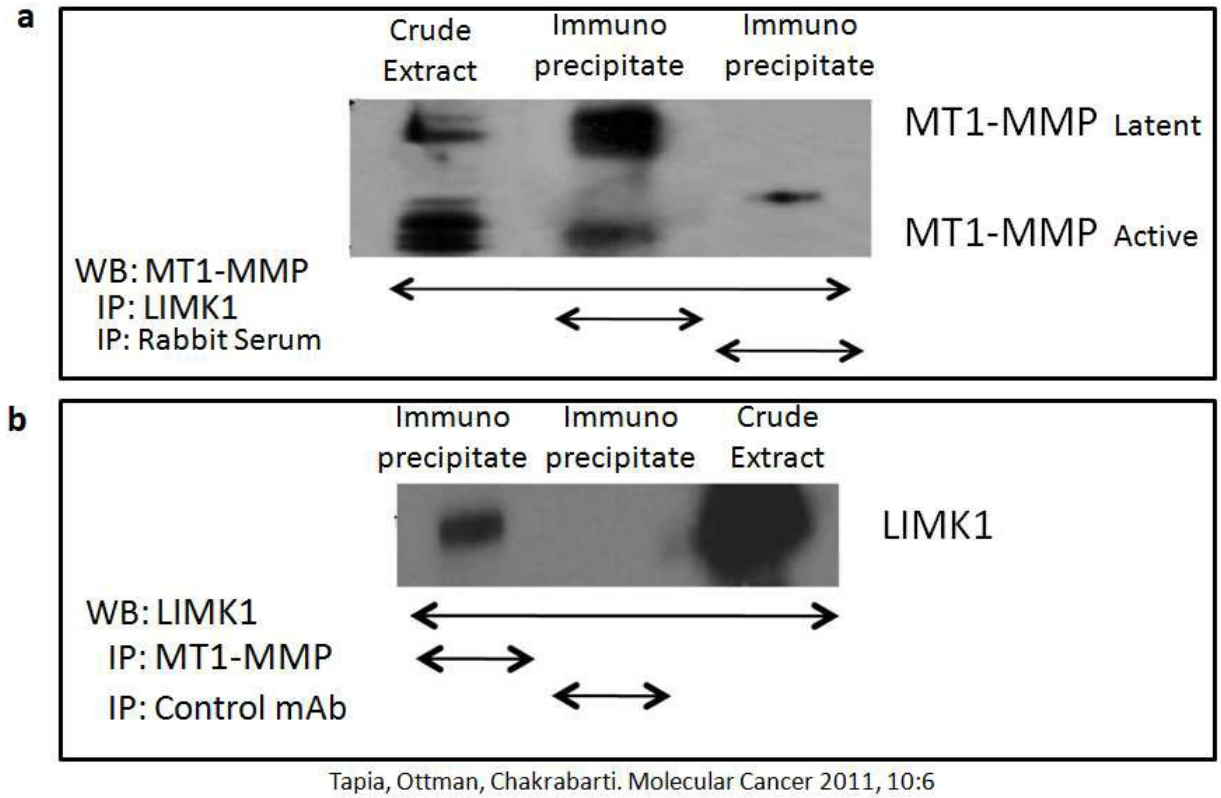


Figure 7: LIMK1 and MT1-MMP Physically Associate

a) Coimmunoprecipitation (Co-IP) and b) Reverse Co-IP of MT1-MMP and LIMK1 using anti-LIMK1 (mouse monoclonal) and anti-MT1-MMP (rabbit polyclonal) antibodies and PC3 cell extracts (500 µg) showing pull down of MT1-MMP and LIMK1 in immunoprecipitates. Nsp mAb: Nonspecific mouse monoclonal antibodies. Rabbit serum and nonspecific mouse mAb were used as the negative controls.

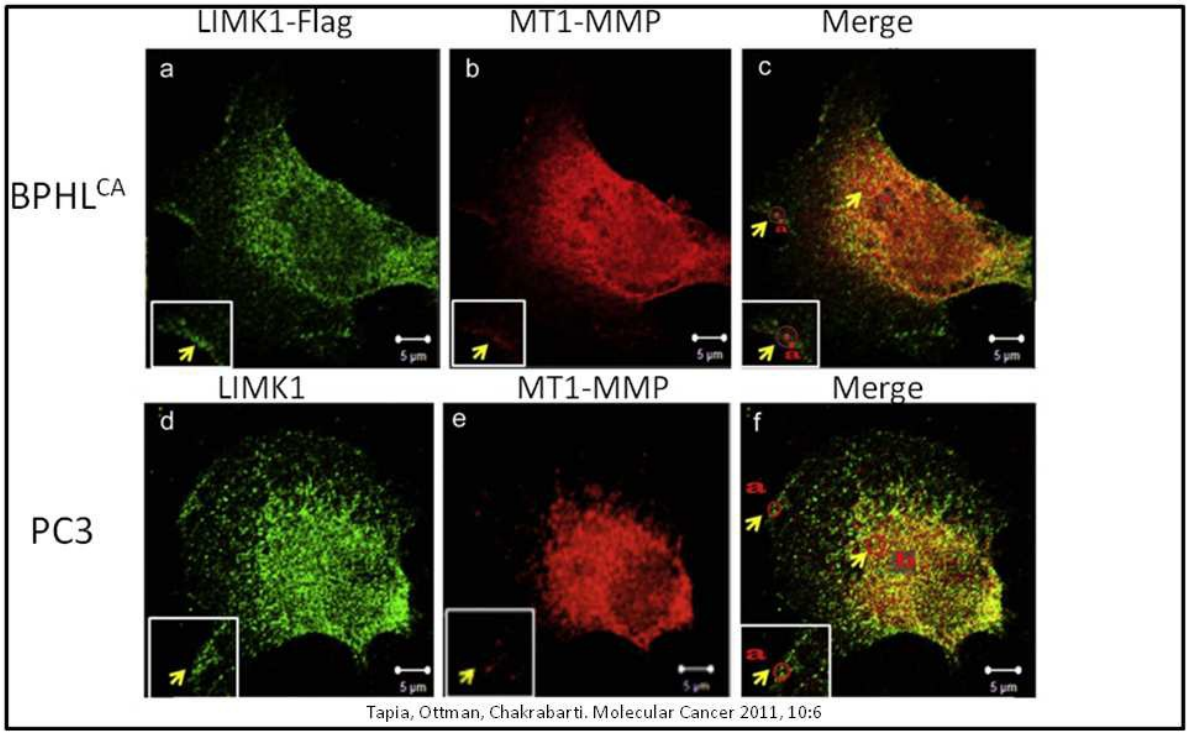


Figure 8: LIMK1 Colocalizes with MT1-MMP

Immunofluorescence analysis of LIMKT508EE and MT1-MMP in BPHLCA (a-c) and PC3 (d-f) cells. a-c: Co-localization of MT1-MMP (red) and LIMK1 (green)(merged image) in these cells was mainly to the perinuclear regions at the ER/Golgi area. Colocalization of MT1-MMP with LIMK1 was also at the plasma membranes (yellow arrows).These cells showed intense staining and accumulation of LIMK1 in the ruffling membranes (Insert: yellow arrows). Scale bar 5 µm. d-f:Colocalization of LIMK1 and MT1-MMP in PC3 cells. Immunofluorescence analysis of MT1- MMP and LIMK1 showed strong staining of both proteins in the Golgi areas and in transport vesicles (yellow arrows). Scale bar 5 µm.

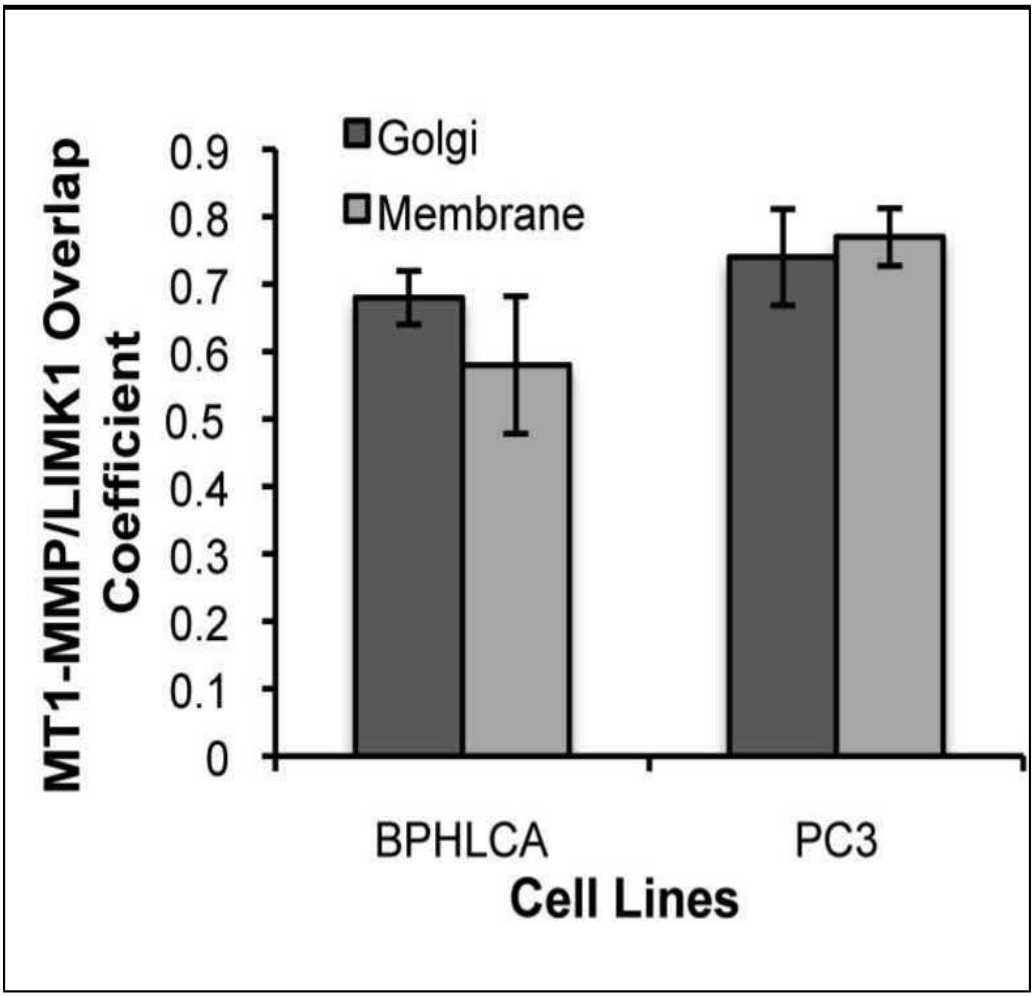


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Figure 9: Quantitative Analysis of LIMK1 and MT1-MMP Colocalization

Overlap coefficient of actual pixels in designated areas (red circles) at the Golgi region and at the membrane for both BPHLCA and PC3 cells.

3.3 LIMK1 Facilitates MT1-MMP Transport Through the Golgi to the Plasma Membrane

From the previous results we were able to infer that LIMK1 was indeed interacting with MT1-MMP, and these interactions were occurring at regions of the cell consistent with the location of the Golgi network and plasma membrane. To further confirm that these proteins were indeed colocalized at the Golgi vesicles we employed triple labeled immunofluorescent analysis using antibodies against TGN46, a Golgi/Trans-Golgi Network marker. PC-3 cells were labeled with LIMK1, MT1-MMP, and TGN46 antibodies and the colocalization of LIMK1 with MT1-MMP along with the colocalization of TGN46 with LIMK1/MT1-MMP was analyzed. The staining patterns of these proteins indicated transport of all three proteins from the Golgi through the Trans-Golgi vesicles to the membrane (Figure 10). Colocalization of these proteins was analyzed using the Leica LasAF software, which generated Pearson's Correlation values for our selected areas of interest. Pearson's Correlation values range from -1 to +1, meaning that 0% of either molecule is associating with the other to 100% association respectively. The Pearson's Correlation values analyzing the association of LIMK1 or MT1-MMP with TGN46 at the Golgi/Membrane were as follows: LIMK1-TGN46 +0.76/+0.71; MT1-MMP-TGN46 +0.63/+0.68. Additionally analysis of LIMK1 association with MT1-MMP at the Golgi/Membrane generated average Pearson's Correlation values of +0.75/+0.80. This data strongly suggests that LIMK1 is associating with

MT1-MMP at the Golgi and membrane.

To investigate the effect of inhibition of LIMK1 expression on the transport of MT1-MMP, PC-3 cells were transfected with either a plasmid expressing LIMK1-shRNA or non-targeting shRNA. After 48 hrs cells were fixed and stained for immunofluorescent microscopy. The reduction in LIMK1 and MT1-MMP expressions was confirmed by measuring staining intensities by immunofluorescent-microscopy and by western blotting of the lysates of PC-3 cells transfected at the same time. Slides were prepared and images were taken using the exact experimental conditions (antibody concentrations, laser power, gain, filter settings, etc) between LIMK1 knock-down and control slides. PC-3 cells expressing LIMK1-shRNA displayed an overall decrease in LIM Kinase 1 and MT1-MMP staining intensity compared to cells expressing the control shRNA (Figure 11). We also noticed that cells expressing the LIMK1-shRNA have MT1-MMP staining intensity restricted to the perinuclear region compared to control shRNA expressing cells.

To verify that reduction in LIMK1 expression correlates with an inhibition of MT1-MMP transport to the membrane, we next monitored MT1-MMP intensity at the membrane and at Golgi vesicles stained with TGN46. Our data showed an overall reduction in cellular MT1-MMP staining intensity following knockdown of LIMK1 (Figure 12). Specific quantification of MT1- MMP intensities at Golgi vesicles and at the membrane was then conducted (Figure 13). PC-3 cells expressing LIMK1-shRNA displayed an average reduction of 1.8-fold in the intensity of MT1-MMP staining at Golgi vesicle compared to control

shRNA-expressing cells. Moreover, the average reduction in MT1-MMP staining was further decreased at the membrane (3.1 fold). Additionally there was no significant change in the staining intensity or pattern of TGN46 following LIMK1 knockdown and no significant change in the colocalization of MT1-MMP with TGN46 at the Golgi or membrane. This suggests that LIMK1 expression is promoting MT1-MMP transport to the membrane.

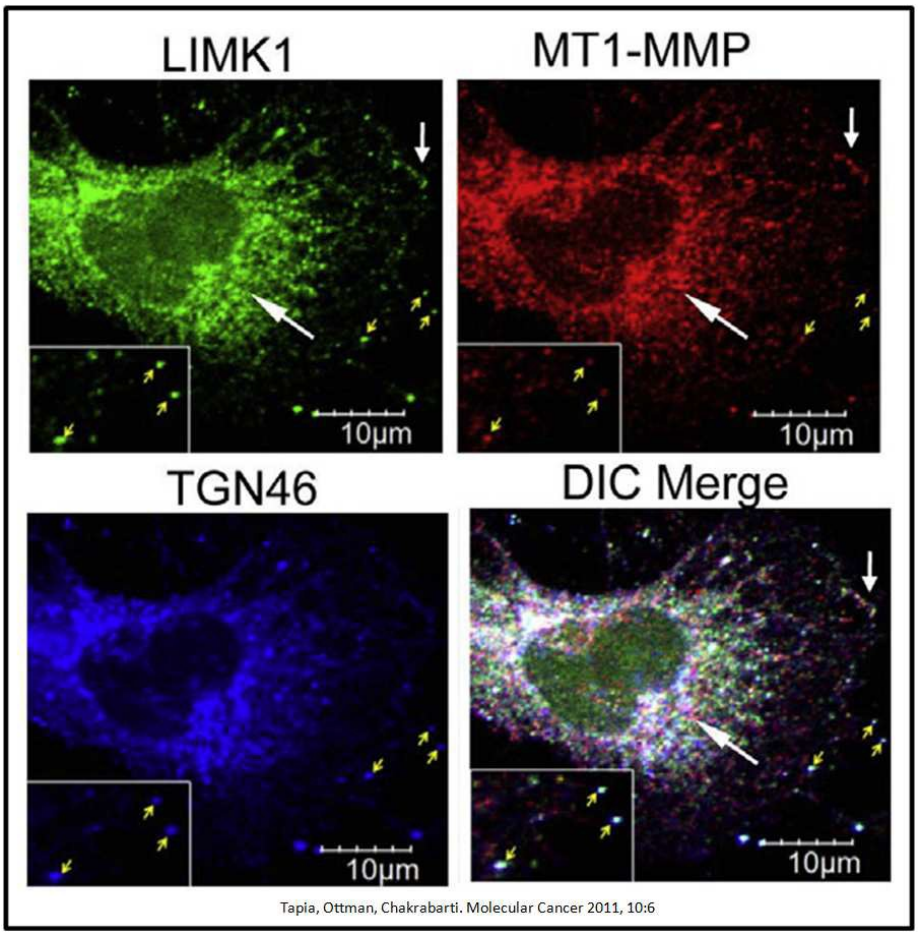

Figure 10: Colocalization of LIMK1 and MT1-MMP at Golgi Vesicles

Immunolocalization of MT1-MMP and LIMK1 showed strong staining of both proteins in the Golgi areas (White staining and long white arrows) along with Golgi marker: TGN46. Yellow arrows: Colocalization of MT1-MMP and LIMK1 in Golgi vesicles at various distances towards plasma membrane. White arrows: Colocalization of MT1-MMP and LIMK1 to the plasma membrane.

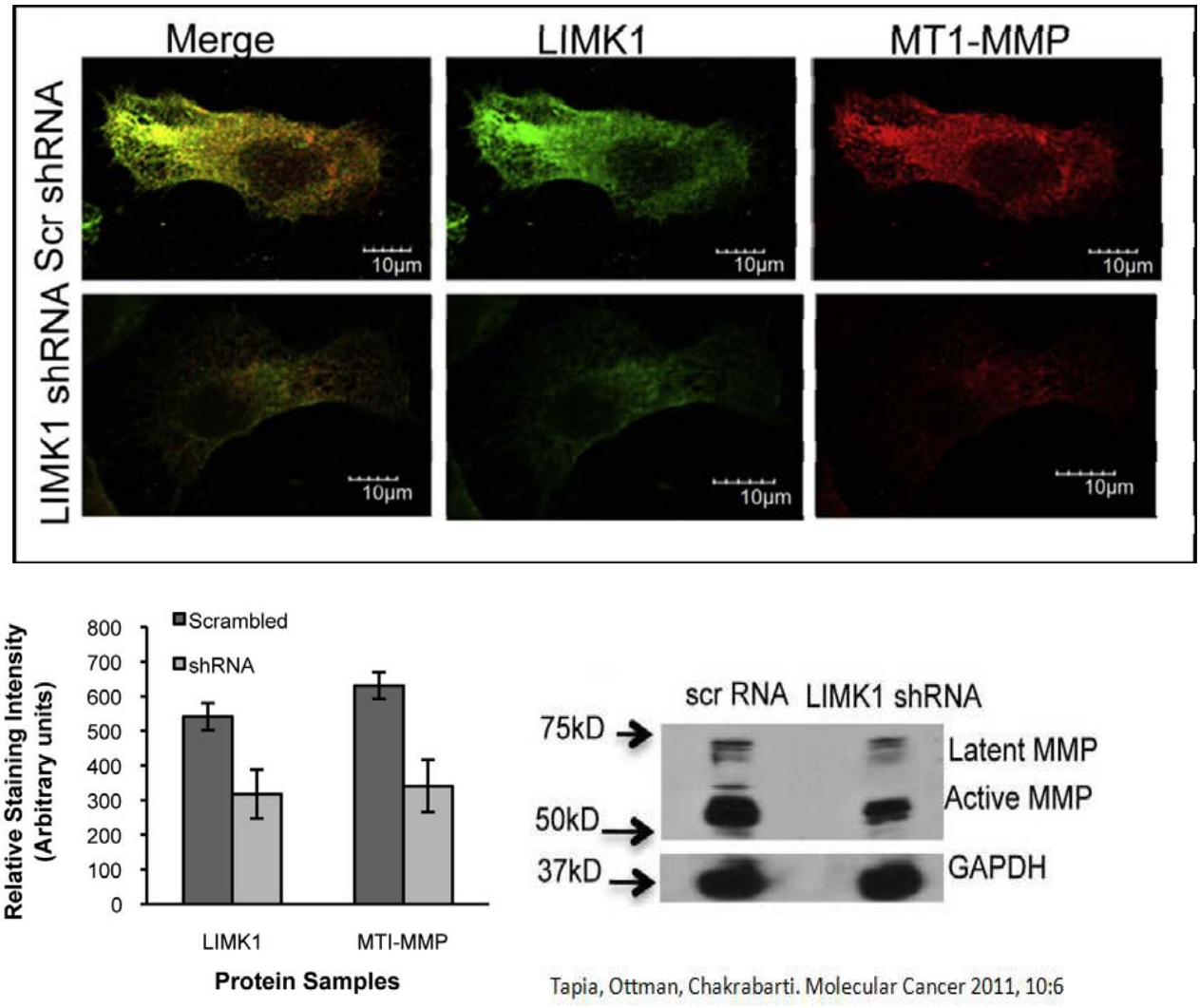

Figure 11: Reduced Expression of MT1-MMP Following LIMK1 Knockdown in PC-3 Cells

Immunofluorescence and quantitative analysis of the reduction of LIMK1 and MT1-MMP expression in LIMK1 shRNA but not scrambled RNA expression vector transfected cells. Immunoblot of transfected PC-3 cell lysate confirms loss in expression of MT1-MMP following LIMK1 knockdown compared to cells expressing scr shRNA.

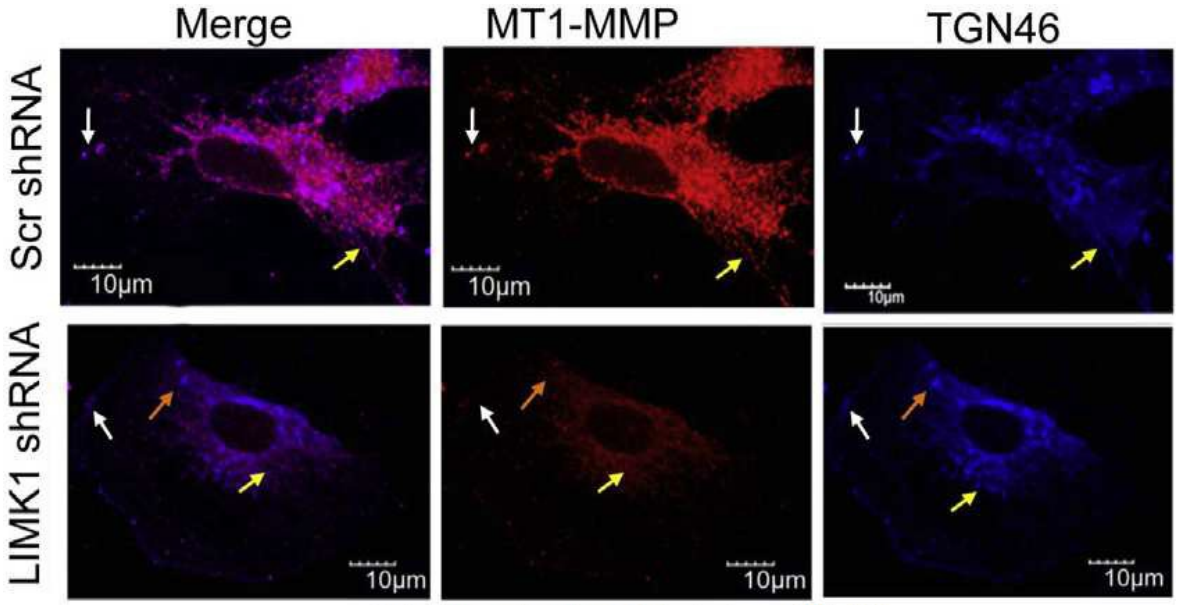

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Figure 12: LIMK1 Knockdown in PC-3 Cells Reduces MT1-MMP Expression and Localization to the Plasma Membrane

Immunofluorescence analysis of MT1-MMP, and TGN46 expression and localization in PC3 cells transfected with scrambled RNA (upper panel) or LIMK1 shRNA (lower panel) expressing vectors. Upper panel: Yellow arrows: Colocalization of MT1-MMP with TGN46 in Golgi vesicles moving towards the plasma membrane, White arrow: Targeting of MT1-MMP to the plasma membrane. Lower panel: Colocalization of MT1-MMP and TGN46 to the perinuclear region (yellow arrow) but not in the transport vesicles (orange arrow). White arrow: Transport of TGN46 positive Golgi vesicles to the plasma membrane. No targeting of MT1-MMP could be noted in these cells. A significant reduction in the MT1-MMP concentration was also evident in these cells. Scale: 10 µm.

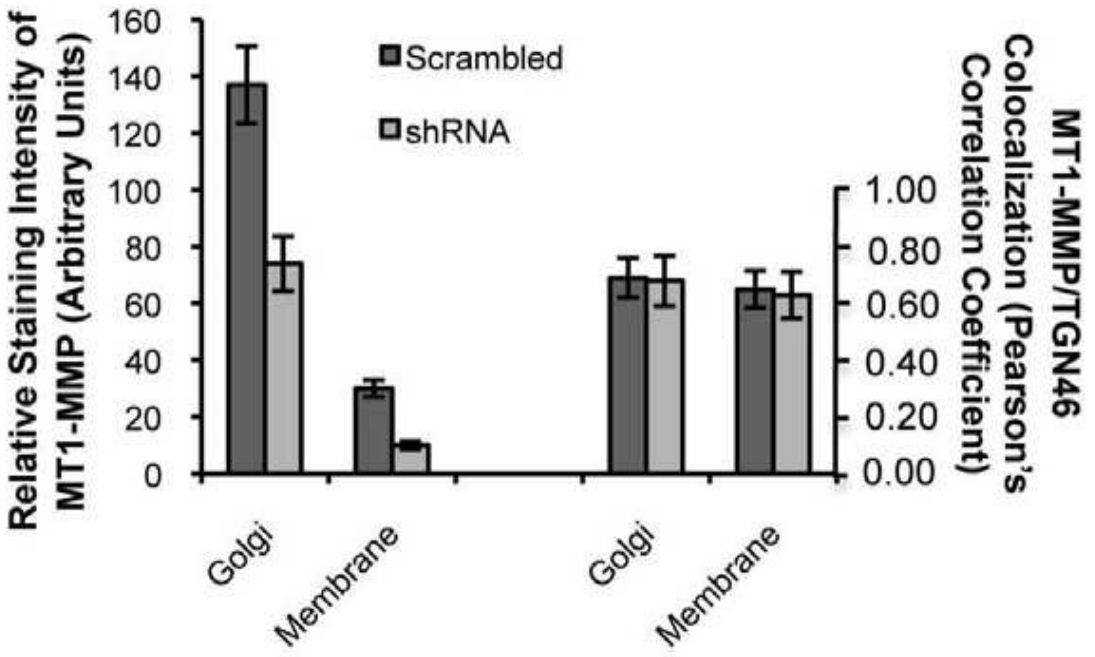

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Figure 13: Quantification of MT1-MMP Staining Intensities and MT1-MMP/TGN46 Colocalization at Areas of the Membrane and Golgi

Quantitative analysis of the staining intensity of MT1-MMP in the Golgi vesicles (red circles in DIC merge: yellow arrow) and in the membrane (entire membrane) in scrambled RNA and LIMK1 shRNA transfected PC3 cells. Pearson's correlation coefficient analysis confirms colocalization between MT1-MMP and TGN46 in Golgi vesicles at the same region used for the analysis of staining intensity.

3.4 LIMK1 Expression Promotes Surface Expression of MT1-MMP

Our immunofluorescent microscopy data showed a correlation between LIMK1 expression and MT1-MMP localization to the membrane. Surface labeling of MT1-MMP was next employed to verify if indeed surface localization of MT1-MMP is regulated by LIMK1. Surface MT1-MMP expression was first monitored in $BPHL^{CA}$ cells expressing constitutively active LIMK1, or in control BPH^{V} cells. Non-permeabilized cells were labeled with antibodies against MT1-MMP, to stain only active MT1-MMP on the plasma membrane. FITC-conjugated secondary antibodies were used monitor labeled cells by Flow Cytometry. $BPHL^{CA}$ cells displayed an increased number of MT1-MMP positive stained cells along with an increase in average fluorescence compared to BPH^{V} cells. This can be visualized by the increase in peak height and the shift towards the right, respectively, in stained $BPHL^{CA}$ cells compared to stained BPH^{V} cells (Figure 14).

We next examined the effect of LIMK1 knock down on MT1-MMP surface expression in PC-3 cells by biotin labeling. PC-3 cells expressing either LIMK1-shRNA or control shRNA, with intact membranes, were incubated with Sulfo-NHS-Biotin, a membrane impermeable biotin ester. In the presence of primary amine groups, the biotin molecule is transferred to the amine group, which results in the biotinylation of all surface proteins. The cells were then lysed, and biotinylated proteins were enriched through immunoprecipitation with strepavidin beads. The immunoprecipitates were resolved by SDS-PAGE and transferred to

a PVDF membrane to be blotted with the anti-MT1-MMP antibodies. Our results showed that PC-3 cells transfected with LIMK1-shRNA had reduced levels of biotin labeled MT1-MMP. These results show that LIMK1 expression positively correlates with the surface expression of MT1-MMP (Figure 15).

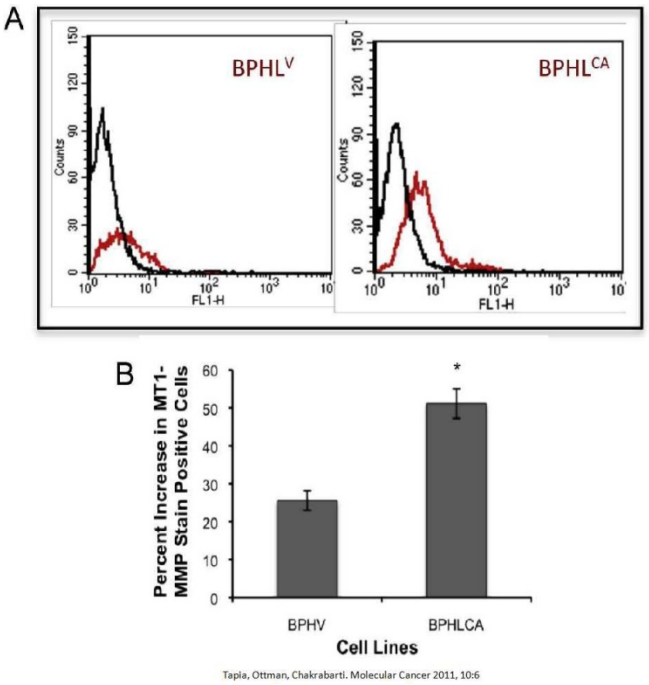

Figure 14: LIMK1 Expression Positively Regulates Surface Localization of MT1-MMP in BPH Cells

A) Flow cytometric analysis of MT1-MMP cell surface expression in transfected BPH cell lines. Two-parameter histogram of the surface staining of MT1-MMP with fluorescence intensities in the X-axis and number of cells in the Y-axis in BPH cells expressing LIMK1. Black histogram represents unstained cells. Red histogram represents population of fluorescent cells within each sample. B) Quantitative analysis of fluorescent cells. For densitometric analysis, the BPHLCA cells that were emitting fluorescence were gated out from the nonfluorescent cells and calculated as the percentage of the fluorescent control (BPHV) cells. Data represents the mean ± SD of three independent experiments. *P =0.0005.

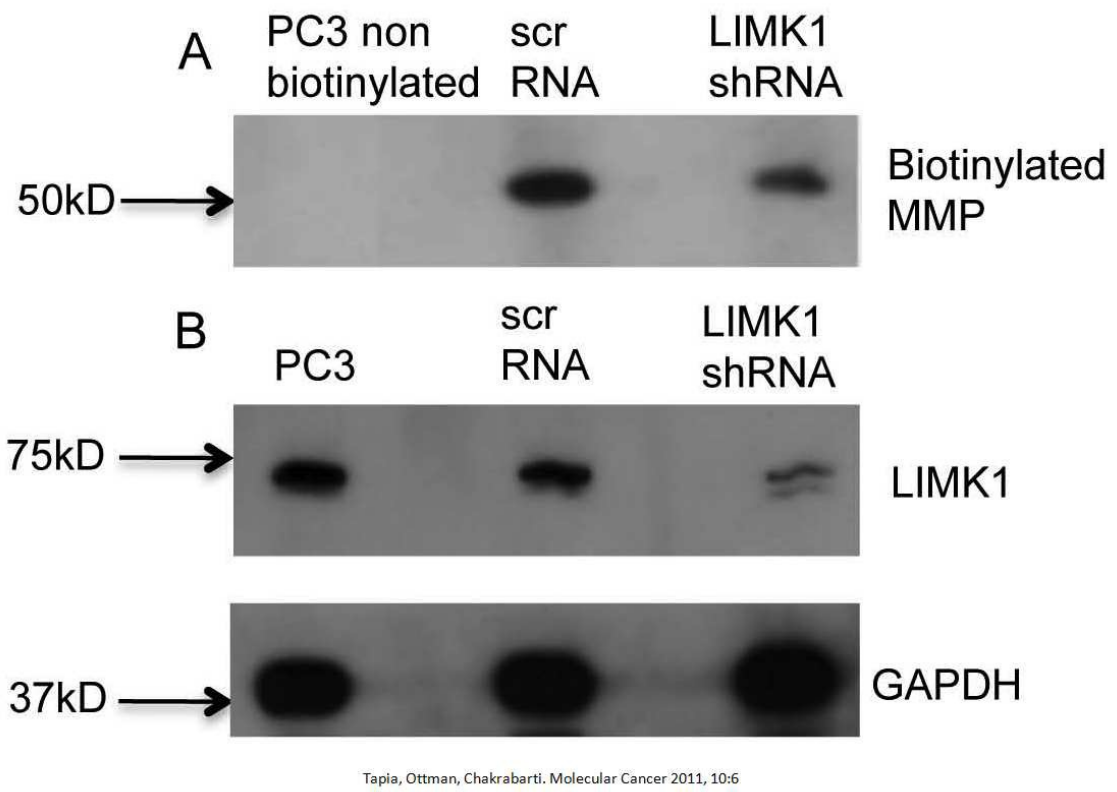

Figure 15: Inhibition of LIMK1 Expression Reduces Surface Localization of MT1- MMP in PC-3 Cells

A) Surface biotinylation of MT1-MMP following knock down of LIMK1 in PC3 cells. Immunoblot of biotinylated MT1-MMP in the streptavidin bead bound MT1-MMP. Lane 1 non-biotinylated cells, Lane 2: control shRNA expressing cells, Lane 3: LIMK1 shRNA expressing cells. B) Immunoblot analysis of LIMK1 in PC3 cells expressing control shRNA or LIMK1 shRNA used for surface biotinylation assays. Data show representative images of three independent experiments.

3.5 Expression of LIMK1 is Associated with Increased Transcriptional Activation of MT1-MMP

The expression of matrix-metalloproteinases including MT1-MMP is up-regulated in advanced prostate cancer tissues and invasive PCa cell lines. MT1-MMP expression has been correlated with the aggressiveness of prostate cancer cell types and shown to be overexpressed in the invasive PCa cell line PC-3. In PC-3 cells, MT1-MMP transcription is regulated through AKT/JNK signaling, leading to activation of the transcription factor Sp1. Additionally, treatment with bone morphogenic protein 2 was shown to activate PI3K/AKT signaling leading to PAK1/4 phosphorylation and activation of LIMK1. These reports suggest a possible link between LIMK1 activity and MT1-MMP transcription. To elucidate any connection with LIMK1 activity, we used prostate cells differentially expressing LIMK1 and monitored MT1-MMP promoter activity using a dual luciferase reporter assay. The Firefly luciferase reporter vector was constructed with the full 7.2kb MT1-MMP promoter positioned to drive the expression of Firefly luciferase. The MT1- MMP reporter vector was co-transfected with a plasmid constitutively expressing Renilla luciferase into $BPHL^{CA}$ and BPH^{V} cells along with PC-3 cells expressing LIMK1-shRNA, control shRNA, or only expressing the luciferase reporter vectors. Firefly luciferase activity was measured by the light emitted and these values were normalized to Renilla luciferase activity to compensate for inconsistencies in transfection and transcription efficiencies (Figure 16). $BPHL^{CA}$ displayed a

3-4 fold increase in relative Firefly luciferase activity compared to BPH^V cells. This suggests that LIMK1 activity positively correlates with MT1-MMP transcriptional activation. Furthermore, the knockdown of LIMK1 resulted in significant (95%) and consistent reduction in luciferase activity compared to PC-3 cells expressing only the luciferase reporter vectors. PC-3 cells expressing the control shRNA displayed some off target effects but LIMK1-shRNA cells still displayed reduced luciferase activity. Although, the luciferase activity of cells expressing the control shRNA was similar to the other LIMK1-shRNA vectors tested, which were not effective in reducing LIMK1 expression. This shows that the results generated using the LIMK1-shRNA in all of the experiments is not a consequence of off target effects. These observations indicate that MT1-MMP transcription is increased with increased expression of LIM Kinase 1.

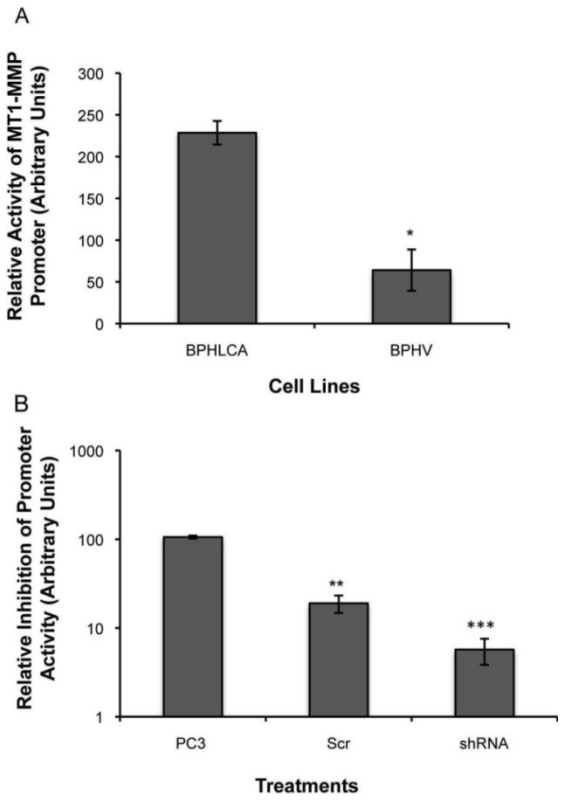

Figure 16: Effect of LIMK1 on transcription activation of MT1-MMP

A) Relative luciferase activity in BPHLCA and BPHV cells transfected with MT1-MMP promoter luciferase constructs. B) Relative luciferase activity in PC3 cells transfected with MT1-MMP promoter luciferase construct alone or in combination with scrambled shRNA or LIMK1 shRNA expressing plasmids. Results show Mean ± SD of at least three separate experiments. *P =0.0075 (BPHLCA vs. BPHV), **P = 0.004 (PC3 scr shRNA vs. PC3LIMK1 shRNA),***P = 0.0005 (PC3 vs. PC3LIMK1 shRNA).

4. DISCUSSION

The organization and modulation of actin filaments are key processes in cell motility and change in cell morphology. Formation of the membrane structures, lamellipodia, filopodia, and invadopodia, require the temporal and spatial regulation of proteins involved in actin cytoskeleton stability and organization. Abnormal regulation cytoskeletal dynamics would predispose cells to adopting characteristics associated with invasive and metastatic cancers. The Rho family of GTPases participates in signaling pathways affecting actin organization and was shown to have increased activity in advanced cancers. A downstream effector of this pathway is LIMK1, which is activated by Rho/ROCK signaling. Expression of LIMK1 has been linked with advanced metastatic cancers and shown to promote invasion in cancer cell lines. Additionally, activity of Rho family members, RhoA and Rac1, promotes expression and activation of MMPs involved in invasion and metastasis. Enhanced expression of MT1-MMP is correlated with tumor progression and malignancy.

Our studies presented here reveal a role for LIMK1 in the complex regulatory scheme of MT1-MMP. The functions of MT1-MMP in metastatic events are well described. Therefore, it is very plausible that the ability of LIMK1 to promote cell migration and invasion is facilitated by activation of MMPs. Our preliminary results confirmed the participation of MT1-MMP, MMP-2, and MMP-9 in the LIM Kinase 1 induced invasion of the benign prostatic hyperplasia cell line, ($BPHL^{CA}$). The broad inhibitor of MMP activity,

ilomastat/GM6001, significantly reduced the number of invasive cells induced by LIMK1 expression. This study is the first to report the novel functional association between LIMK1 and MT1-MMP.

To further investigate the link between LIMK1 and MMP, we first asked if LIMK1 expression was increasing the enzymatic activity of MMPs. Our results showing increased processing of MMP-2 in $BPHL^{CA}$ cells addresses this question. Furthermore, these cells had elevated secretion of both proMMP-2 and proMMP-9, a common phenomenon noted in prostate cancer patients. We next explored the possibility that LIMK1 expression was modulating the transcription of these MMPs. Our findings showed a significantly higher transcription of MMP-2 in LIMK1 expressing cells. These results lead us to investigate a possible connection between LIMK1 and a known activator of MMP-2, MT1-MMP.

From our immunoblot results we noted an enhanced expression of MT1-MMP in prostate cell lines over-expressing LIMK1. This effect was reversed when LIMK1 expression was reduced by LIMK1-shRNA in PC-3 cells. Upon further investigation into this relationship, we identified, through co-immunoprecipitation that LIMK1 was physically associating with active and latent forms of MT1-MMP. This suggests a possible association before and after processing of MT1-MMP in the Golgi. We next monitored the locations of these interactions with immunofluorescent microscopy. These results showed colocalization, verified by strong Pearson's correlation values, occurring in the Golgi, Trans-Golgi vesicles, and at the cell cortex. Furthermore, colocalization at Golgi vesicles at varying distances

from the perinuclear area to the cell membrane suggests a role of LIMK1 in regulating vesicular trafficking of MT1-MMP. To confirm this speculation, we inhibited LIMK1 expression in PC-3 cells and noted an overall reduction of MT1-MMP expression although this reduction was greater at the membrane than at the Golgi. Taken together, with previous reports on the role of LIMK1 in modulating Golgi vesicles and endocytic vesicles, strengthens the argument for the involvement of LIMK1 in regulating membrane expression of MT1-MMP (Rosso et al., 2004)(Nishimura et al., 2006). Confirmation that LIMK1 was indeed involved in the surface expression of MT1-MMP came from our surface labeling experiments. Using flow cytometry we monitored the effect of enhanced expression of LIMK1 in BPH-1 cells, which revealed an increase in surface labeled MT1-MMP. Conversely, the effect of reduced LIMK1 expression in PC-3 cells was monitored by immunoprecipitation of biotinylated surface proteins, which were then immunoblotted for MT1-MMP expression. These immunoblots show reduced MT1-MMP concentrations in the immunoprecipitates of cells expressing LIMK1 shRNA.

Finally our study showed that LIMK1 expression was involved in regulating the transcriptional activity of MT1-MMP. We employed the full length MT1-MMP promoter, containing a known Sp1 binding site, to drive transcription of Firefly Luciferase. We were than able to quantify the effect of LIMK1 expression on MT1-MMP transcription by monitoring the relative activity of Firefly luciferase. Although the effect of LIMK1 on MT1-MMP transcription was clearly observable, the underlying mechanism remains to be

elucidated. However the importance of this study lies in the possibility that, through regulation of MMPs, LIMK1 may be a more effective therapeutic target without the toxicity of inhibitors of MMP activity.

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