

Chemical tools for selective activity profiling of endogenously expressed MMP-14 in multicellular models

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Abstract

Matrix metalloproteases (MMPs) are a large family of zinc-dependent endopeptidases, involved in a diverse set of physiological and pathological processes, most notably in cancer. Current methods for imaging and quantifying MMP activity lack sufficient selectivity and spatiotemporal resolution to allow studies of specific MMP function *in vivo*. Previously, we reported a strategy for selective targeting of MMPs by engineering a functionally silent cysteine mutation that enables highly specific covalent modification by a designed activity-based probe (ABP). Here we describe the translation of that technology into a mouse model of breast cancer and subsequent demonstration of the utility of the approach for studies of MMP-14 activation in the tumor microenvironment. Using this approach, we find that MMP-14 is active in late stage tumors and is predominantly associated with stromal cell populations that have been activated by specific signaling molecules (e.g. TGF β) produced by tumor cells. Our data demonstrates the applicability of this approach for studies of MMP function in whole organisms and identifies important regulatory mechanisms for MMP-14 activity in the tumor microenvironment.

Introduction

MMPs are a family of zinc-dependent endopeptidases, consisting of more than 25 members in humans.¹ This large family of proteases is responsible for the degradation of most extracellular proteins and has long been associated with many types and stages of cancer.² Proteolysis by MMPs is essential for a variety of physiological activities⁴ and signaling events^{5, 6} and is tightly regulated at several levels. In addition to regulation at the transcriptional level, all MMPs are produced as latent pro-enzymes that require enzymatic activation for catalytic function.¹ Their activity is regulated by compartmentalization and trafficking and is inhibited by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs).⁷

MMPs promote tumor progression and regulate the tumor microenvironment through degradation of the extracellular matrix (ECM) as well as through additional signaling functions.^{6, 8} However, attempts to target MMPs for therapeutic purposes have been hampered by a general poor understanding of the specific roles of individual MMP members and inadequate target validation.^{3, 9} Contradicting roles have emerged for MMPs both as tumor promoters and tumor suppressors.¹⁰ Depending on the circumstances, different members of the MMPs regulate tumorigenesis during the process of microenvironment remodeling. Despite mounting evidence of elevated MMP activity at the interface of a tumor and its stroma,^{2, 8} our understanding of the specific roles, physical location and dynamics of proteolytic activation of individual MMPs remains elusive. Current methods to visualize MMP activity, such as fluorogenic substrates,^{11, 12} radiolabeled inhibitors^{13, 14} or fluorescent matrix proteins¹⁵ lack the specificity and spatiotemporal precision required to map the activity of individual MMPs in the context of a native tissue during tumor development.

Our group previously reported the design and validation of a methodology to specifically image and selectively inhibit individual MMPs.¹⁶ This approach makes use of a combination of protein engineering and a corresponding activity-based probe (ABP) to selectively label active forms of MMPs in intact cells (Figure 1a). A proximity-induced interaction between an engineered cysteine and an electrophilic warhead on the ABP results

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3 in irreversible labeling and selective inhibition of the engineered 'probe-sensitive' MMP,
4 while any non-specific interaction of the probe with wild type MMPs remains reversible and
5 results in weak, reversible inhibition (Figure 1b). Since ABP binding relies on the
6 accessibility of the catalytic zinc ion and the active site, this strategy provides a means to
7 distinguish active enzyme from pro-enzyme forms or inhibited complexes in the context of
8 live cells or intact tissues with high spatial resolution.¹⁶

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15 The membrane-anchored MMP-14 (MT1-MMP) is thought to be one the main
16 proteases involved in the process by which tumor cells penetrate the basement membrane,
17 a process that marks the transition to invasive carcinoma.^{2, 17, 18} High expression levels of
18 MMP-14 by tumor cells, and cells within the tumor stroma are associated with rapid tumor
19 progression and increased invasiveness.¹⁹⁻²¹ The coordinated compartmentalization of
20 MMP-14 in invadopodia or podosomes, membrane protrusions specialized for ECM-
21 adhesion and degradation²², is evident for both the invading tumor cells and stromal cells in
22 the tumor microenvironment such as cancer-associated fibroblasts^{18, 23} (CAFs),
23 macrophages^{24, 25} and other myeloid cells.²⁶ However, the identity of soluble signaling
24 molecules produced by malignant cancer cells and the surrounding stroma that induces
25 expression and activation of MMP-14 remain largely undefined. Understanding the
26 regulation, activity and function of MMP-14 is essential for defining the cell invasion
27 programs of specific cancers. Here, we describe the construction of a new mouse model in
28 which the native MMP-14 gene is engineered to express a cysteine mutant MMP-14 that can
29 be targeted with a corresponding ABP through a covalent labeling reaction. This mutation
30 was also crossed with MMTV-PyMT mice to generate a breast cancer model in which all
31 cells express the probe-sensitive MMP-14. Using an activity-based probe, we were able to
32 visualize MMP-14 activity patterns and cellular localization within components of the tumor
33 microenvironment. We find elevated levels of expression and activation of MMP-14 primarily
34 in stromal cells, while MMP-14 is expressed at the protein level within the tumor cells but
35 remains largely inactive within the tumor bulk. The induction of MMP-14 activity within the
36 stroma is orchestrated by soluble signals produced by cancer cells. We identify TGF- β 1 as
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3 one of the potential cytokines that regulates this activity. Overall, our data demonstrate the
4 applicability of this methodology to visualize and quantify the activity of a specific protease in
5 the context of the complex tumor microenvironment. It also shows that, while MMP-14 is
6 expressed at the protein level by both tumor and stromal derived immune cells, it is primarily
7 activated in the immune cell populations as the result of response to tumor cell-derived
8 cytokines.
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16 **Results and Discussion**

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18 **Optimization of the synthesis of probes for cysteine mutant MMP-14.** The ABP TND124
19 (Figure 2a) was previously designed and synthesized by our group to enable specific,
20 activity-dependent labeling of a mutant MMP-14 containing a functional silent cysteine
21 mutation near the active site. It is capable of irreversibly labeling engineered MMP mutants,
22 MMP-12 T184C and MMP-14 F260C. These point mutations do not interfere with enzymatic
23 activity, substrate specificity and protein stability as we have previously reported¹⁶. Its design
24 was based on the scaffold of the broad-spectrum MMP inhibitor Ilomastat (GM6001) that
25 employs an isobutylsuccinylhydroxamic acid motif as the affinity recognition element.
26
27 Difficulties with the chemical stability of the isobutylsuccinylhydroxamic acid and several low
28 yielding steps prompted us to modify the synthesis route. Specifically, our focus turned to
29 issues arising from 1) low yields for the formation of the N-terminal amide 2) low chemical
30 stability of the isobutylsuccinylhydroxamic acid intermediate and 3) considerable loss of
31 material due to the nucleophilic potential of the hydroxamic acid and its cross-reactivity with
32 the electrophilic warhead that is introduced in a click reaction. Replacing the terminal amide
33 of TND124 with a propyl amide in NAP8 (Figure 2), using a solution phase amidation
34 reaction instead of solid support, increased the yields for this step, and potentially improves
35 its metabolic stability. The precursor t-butyl (R)-2-isobutylsuccinate **7** used in the synthesis
36 of TND124 is intrinsically unstable with a high propensity to form a cyclic anhydride, leading
37 to the formation of multiple byproducts both in basic and in acidic reaction conditions. To get
38 around instability of **7** and avoid the cross-reactivity of the unprotected hydroxamate towards
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3 the electrophilic warhead of the probe, we sought to pursue a strategy that replaces **7** with a
4 protected hydroxamate. Thus, we synthesized precursor **5**, adopting a published strategy,²⁷
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7 ²⁸ but quickly discovered that its instability was even greater than that of **7** (Fig. 2b).
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9 Preparing the activated ester derivative **6** slightly improved stability but still resulted in
10 considerable byproduct formation and low reaction yields in the consequent coupling step.
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12 We determined that a hydroxylamine Wang resin could be used to both produce the final
13 hydroxamate product and also serve as a protecting group while introducing the electrophilic
14 warhead (Figure 2c). To improve reaction yields for the coupling of **7**, we used HBTU and
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DIPEA because it balances mild activation with fast reaction time, minimizing byproducts and increasing reaction yields. To facilitate the click reaction on the solid support (CuAAC-SP), we used anhydrous CuSO₄ dissolved in DMF as the source of Cu(II) ions along with ascorbic acid as the reducing agent. An overnight reaction with incubation at 37 °C with agitation increased the yields from 18% to 75% over four steps from **11** to **13**. Collectively, these modifications of the synthesis route enable the synthesis of NAP8 at multi-milligram scale.

Generation of a mouse line endogenously expressing probe-sensitive MMP-14. To confirm that the binding and inhibition properties of NAP8 do not differ from TND124 we incubated recombinant MMP-14 F260C active domain with each probe and compared the IC₅₀ inhibition values (Figure 3a). As expected, both TND124 and NAP8 had nearly identical IC₅₀ values towards the recombinant enzyme, roughly 100-fold higher than that of GM6001.²⁹ MMP-14 is tightly regulated by various mechanisms including compartmentalization and trafficking, enzymatic activation, inhibition by small molecules or TIMPs, degradation and clearance.^{7, 30} Therefore, it is essential to achieve expression of the cysteine mutant MMP-14 from the endogenous gene locus to be able to make relevant conclusions about its activation and regulation. Using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology, we generated a knock in mouse in which the native mMmp-14 gene was replaced with the gene containing the F260C mutation. This was achieved by changing

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3 Thymidine to Guanine at the 6688th nucleotide of mMmp-14 cDNA (Figure S1a-c), followed
4 by pronuclear microinjection of guide RNA (gRNA), single stranded oligo donor nucleotide
5 (ssODN) and Cas-9 mRNA into a C57BL/6 embryo (conducted by Applied StemCell Inc,
6 Milpitas, CA). After implantation and isolation of offspring, we identified heterozygous
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10 founders by PCR screening. These were used to generate homozygous mMmp14-F260C F1
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12 mice using standard breeding schemes (Figure S1d). Importantly, we observed no
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14 detectable phenotypes associated with the presence of the homozygous mutation with
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16 growth and breeding indistinguishable from that of the wild type (WT) mice. This confirms
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18 that the cysteine mutation on MMP-14 is functionally silent as shown in our prior studies.¹⁶
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22 **Engineered ABPs specifically label probe-sensitive MMP-14 in activated fibroblasts.**

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24 Using primary fibroblasts generated from the mMmp14-F260C mice, we performed labeling
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26 studies with the NAP8 probe to assess its overall selectivity and to determine the levels of
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28 MMP-14 activity in those cells. We first labeled WT and mMmp14-F260C fibroblasts after
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30 incubation with multiple paracrine factors such as epithelial growth factor (EGF), fibroblast
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32 growth factor (FGF), keratinocyte growth factor (KGF) and transforming growth factor β 1
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34 (TGF β 1) over 24 hours (Figure 3b). We then treated intact cells with NAP8 and resolved
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36 lysates by SDS-PAGE. Surprisingly, in the absence of growth factors, no specific labeling
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38 was observed. However, labeling of fibroblasts that were stimulated with TGF β 1 showed a
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40 selective fluorescent band that corresponds to the active MMP-14. The labeled protein was
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42 only observed in the cells expressing the mutant MMP-14 F260C and was blocked by pre-
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44 incubation with excess of the non-specific hydroxamate inhibitor Ilomastat (GM6001) or the
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46 general cysteine reactive molecule N-ethylmaleimide (NEM) suggesting that it results from
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48 labeling of the probe sensitive MMP-14 by the NAP8 probe. Consistent with this finding,
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50 western blot of MMP-14 protein levels showed increased expression in fibroblasts only when
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52 treated with TGF β 1 and not with other cytokines such as EGF, FGF and KGF. Notably, the
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54 labeled band for active MMP-14 in figure 3b appears to migrate slightly higher than the
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3 expected ~57kDa for the mature active protein. The intensity of labeling was dependent on
4 both probe concentration (Figure 3c) and labeling time (Figure 3d) as expected for a
5 covalent modifier. In addition, incubation with TGF β 1 increased the expression of MMP-14 in
6 a dose dependent manner (Figure 3e). This increase in MMP-14 activity corresponds with
7 the TGF β -induced differentiation program of fibroblasts into myofibroblasts³¹ that is
8 accompanied by the production of α smooth muscle actin (SMA; Figure 3f and S2).³²
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17 **MMP-14 is active in primary macrophage cells.** We next assessed the labeling of MMP-
18 14 in bone marrow derived macrophages (BMDMs) isolated from either WT or mMmp14-
19 F260C mice. As observed for primary fibroblasts, we found no probe labeling of MMP-14 in
20 unstimulated BMDMs (Figure 4a). When we stimulated the cells with a range of cytokines
21 including TGF β 1, IFN- γ , IL6 and IL4 we again found no labeling by the NAP8 or TND124
22 probes. However, we did observe labeling of active MMP-14 in BMDMs treated with LPS,
23 and this labeling was blocked by pretreatment with GM6001 (Figure 4b). Western blot
24 analysis revealed that the mature 57kDa form of MMP-14 accumulated only upon stimulation
25 by LPS. Unstimulated cells and cells treated with the panel of cytokines showed
26 accumulation of only a 44kDa fragment. Previous studies have identified the 44kDa
27 fragment as the product of autocatalytic processing that takes place in the absence of TIMP-
28 2. Sequential cleavages at Ala255 and Gly284 of the 57kDa species generates an inactive
29 18kDa soluble species and a membrane bound 44kDa species, missing the entire catalytic
30 domain.³⁰ LPS activates macrophages to their pro-inflammatory, M1 functional state³³ and
31 results in MMP-14 activation. Stimulation by immune-regulatory cytokines such as IFN γ and
32 IL-6 (M1 activation) or IL-4 and TGF β 1 (M2 activation) do not result in increased activity of
33 MMP-14 (Figure 4b). To test if this is an LPS-specific response we tested the effect of
34 different agonists of toll-like receptors (TLRs). Stimulation of macrophages with multiple
35 different pathogen-associated molecular patterns (PAMPs) triggered a uniform response
36 resulting in activation of MMP-14 (Figure 4c). Sensing of pathogen-associated molecular
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3 patterns PAMPs is a general response that leads to the pro-inflammatory M1 activation, a
4 state that has been implicated with generally higher proteolytic activity.^{31, 34} Priming of
5 macrophages with PAMPs, either LPS or R-848, results in the increase in levels of the
6 mature form of MMP-14 over 24 hours (Figure 4d), suggesting that activation of pro-
7 inflammatory response through TLRs leads to an increase in levels of active MMP-14.
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14 **MMP-14 activity is tightly regulated on the surface of mammary epithelial cells.** After
15 determining that MMP-14 activity is found predominantly on activated fibroblasts and
16 macrophage cells, we wanted to examine how the activity of this protease may be regulated
17 on mammary epithelial cells during normal cell growth and morphogenesis. The
18 development of the mammary gland is an ideal model to study the physiologic roles of
19 MMPs. This complex organ undergoes continuous changes in structure and function from
20 initial development through cycles of lactation and involution.³⁵ MMPs play a crucial role in
21 ECM remodeling that takes place during ductal growth and morphogenesis.^{6, 36} Mammary
22 epithelial cells or epithelial organoids can be grown in three dimensional *ex-vivo* cultures
23 (3D-cultures). These 3D-cultures recapitulate numerous features of glandular epithelium *in*
24 *vivo*, including differentiation of myoepithelial and luminal cells and the formation of polarized
25 spheroids with a hollow lumen.^{37, 38} We therefore generated mammary organoids by isolation
26 of cells from the inguinal fat pads of WT or mMmp14-F260C virgin females. We treated
27 organoid cultures with NAP8 and lysed cells for analysis by fluorescence scanning.
28 Surprisingly, while the mature 57kDa MMP-14 species is present as confirmed by western
29 blot, we could not detect labeling of active MMP-14 in these cells with our probe (Figure 5a).
30 This suggests that while mature MMP-14 is present, its activity may be kept in check by
31 endogenous inhibitors. Incubation with different growth factors, among these TGFβ1, for 24
32 hours did not increase the expression of MMP-14 nor change its activation state. To
33 understand if chemical cues or stimuli are needed for MMP-14 activation, we seeded
34 mammary organoids on collagen matrix (Matrigel) and allowed development and maturation
35 of the spheroids over the course of 10 days. At day 10, we incubated spheroids with NAP8
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3 to label active MMP-14 and then performed fluorescence microscopy (Figure 5b). Active
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5 MMP-14 was not detected on the surface of the mature spheroids, and no significant
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7 differences in fluorescence were observed between the WT spheroids and the mMmp14-
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9 F260C spheroids. To further assess the presence and specific localization of MMP-14 on
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11 mature spheroids, we fixed probe labeled spheroids on slides for immunofluorescence
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13 analysis. Anti-MMP-14 staining showed its localization in small micro-domains on the apical
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15 membrane, where it co-localizes with E-cadherin forming tight junctions between
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17 neighboring cells (Figure 5c). The lack of probe fluorescence indicates that MMP-14 is
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19 inactive on the surface of the spheroids, suggesting that growth and expansion of the
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21 spheroid into the matrix is independent of MMP-14 or alternatively, MMP-14 activity may be
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23 contributed from cell populations present in the stroma, that are not present in our 3D
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25 cultures. It is important to note that Matrigel contains an undefined mixture of proteins and
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27 can include hormones, growth factors or enzymes that may influence the activation or
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29 inhibition of MMP14 on the surface of the spheroids. When organoids were plated and
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31 cultured in 2D-cultures, prior to separation from populations of stromal cells, probe labeling
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33 revealed that active MMP-14 is present on sub-populations of cells other than the epithelial
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35 cells (Figure 5d and S3). These labeled cells have spindle morphology resembling that of
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37 mesenchymal cells such as fibroblasts or myeloid cells that reside in the stroma. Probe
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39 labeling of these cell populations was specific to the probe sensitive MMP-14 in the
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41 mMmp14-F260C organoids. Preincubation with excess of Ilumastat (GM6001) or NEM
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43 blocked labeling by NAP8.
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46 **Construction of a cancer model to study the pathophysiologic role of MMP-14.** To
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48 better understand the role of MMP-14 in regulation of the tumor microenvironment, we
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50 crossed the mice expressing the probe sensitive MMP-14 with mice that express the
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52 Polyoma Virus middle T antigen under the direction of the mouse mammary tumor virus
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54 promoter (MMTV-PyMT). MMTV-PyMT⁺ females develop mammary tumors that become
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56 palpable typically at around 140 days of age and metastasize to the lung. This allowed us to
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3 harvest tumors from the mammary glands of mMmp14-F260C^{WT}MMTV-PyMT⁺ and
4 mMmp14-F260C^{+/+}MMTV-PyMT⁺ females. After the initial digestion and dissociation of
5 tumor tissue, we filtered cells and plated them for culture. Primary cell cultures treated with
6 NAP8 show specific labeling of MMP-14 only in the cells derived from tumors from the mice
7 expressing MMP14-F260C but not from mice expressing WT MMP-14, confirming the
8 presence of active MMP-14 in the tumor microenvironment (Figure 6a). However, after
9 multiple passages of the cells in culture, the MMP-14 activity was completely lost. While
10 primary, non-neoplastic cells (i.e. stromal cells) reach replicative senescence and perish
11 after 4-5 passages, transformed cancer cells proliferate and dominate the culture. After 10
12 passages the entire cell population consists of neoplastic epithelial cancer cells. This
13 population of cells, although clearly expressing MMP-14, shows no active MMP-14 as
14 assessed by probe labeling (Figure 6a). We therefore concluded that the observed labeled
15 active MMP-14 from the primary tumor cultures originates from non-neoplastic cells that
16 normally exist in the tumor microenvironment. These cell populations include fibroblasts,
17 bone marrow derived cells, leukocytes and endothelial cells.^{26, 39} Activated fibroblasts, also
18 known as cancer associated fibroblasts (CAFs) are myofibroblast populations present at the
19 tumor-stroma interface.³¹

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22 To test our hypothesis, we compared the expression and activity profile of MMP-14 in
23 primary tumor cultures to that of TGFβ1-stimulated myofibroblasts (Figure 6b). An increase
24 in MMP-14 expression as well as probe labeling was observed in primary tumor cultures,
25 similar to TGFβ1-stimulated fibroblasts. The expression of SMA in the primary tumor culture
26 confirmed the presence of myofibroblasts. Although the total expression of MMP-14 was
27 lower than that of fibroblasts, primary tumor cultures consists primarily of cancer cells that
28 express lower levels of MMP-14, thus the observed increase results from a minority
29 population of cells. Nevertheless, MMP-14 activity is highest in the primary tumor cultures as
30 evidenced by probe labeling, presumably by stimulation through TGFβ1 signaling or in
31 conjunction with additional factors. A similar enhancement in expression of MMP-14 was
32 achieved by incubation of fibroblasts with tumor cell culture supernatant. Stimulation through
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3 soluble factors also resulted in increased activity of MMP-14, although not to the extent seen
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5 in the primary tumor cultures.

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7 While TGF β 1 produced by tumor cells may act on fibroblasts to increase MMP-14
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9 expression and activation, additional signals such as physical contact of tumor cells with the
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11 surrounding CAFs may be needed for substantial activation of MMP-14. To recapitulate the
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13 interactions between tumor cells and fibroblasts in the tumor microenvironment, we co-
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15 cultured tumor cells with embryonic fibroblasts on cover slides, followed by incubation with
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17 NAP8 to label active MMP-14 before cells were fixed and stained with an MMP-14 antibody
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19 and DAPI (Figure 6c). Similar to the biochemical results, epithelial cancer cells, although
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21 expressing MMP-14, do not show labeling by the probe. Embryonic fibroblasts that are not
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23 stimulated express high levels of MMP-14 but virtually all of this is in an inactive state. On
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25 the other hand, fibroblasts that are co-cultured with cancer cells have substantial levels of
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27 active MMP-14, as assessed by NAP8 labeling. This increase is likely due to signaling
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29 events that involve soluble factors given that fibroblasts that are cultured with the
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31 supernatant of cancer cells exhibit a similar induction of MMP-14 activity that can be blocked
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33 by excess GM6001.

34 35 36 **Conclusions**

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38 MMP-14 is associated with multiple human cancers and is considered the major cell-
39
40 associated protease necessary to confer invasive properties to normal or neoplastic cells.¹⁸
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42 Its role in breaching the basement membrane during the early stages of invasion is well
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44 established, both in the physiologic and pathologic states.^{18, 40} However, its role in infiltration
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46 and migration through the interstitial tissue at the later stages of tumorigenesis is still subject
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48 to debate, as are the molecular cues that stimulate its activity. MMP-14 has been implicated
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50 in tissue-invasive activity but mounting evidence suggests that the majority of MMP-14 is
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52 expressed at the tumor-stroma interface rather than within the bulk of the tumor.^{8, 19, 21, 41, 42}
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54 Significant proteolytic activity is attributed to the stromal cell populations that are stimulated
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56 through the cross talk between the tumor and its microenvironment.^{26, 39, 43-45} While many of
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3 these studies make use of expression of mutants or deletion of MMP-14, there remains an
4 insufficient understanding of its activation pattern in the context of complex multi-cellular
5 tissues.^{3, 46, 47} Using an engineered probe-sensitive MMP-14 and a corresponding ABP, we
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7 were able to profile the activity levels of this protease *in vivo* in several cell types as well as
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9 in the components of the microenvironment of late stage breast cancer tumors.
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12 Our findings support the notion that MMP-14 activity is tightly regulated in the
13 absence of stimulatory signals. This regulation is well documented in the literature with
14 TIMP-2 as the major endogenous inhibitor of MMP-14.^{6, 7, 36} The interaction between MMP-
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16 14 and TIMP-2 has been suggested to increase stability of MMP-14 and prevent
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18 autoproteolytic degradation.^{30, 48} While the autoproteolytic degradation product was the
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20 predominant form of MMP-14 observed in non-differentiated (M0) or alternatively activated
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22 (M2) macrophages, classically activated (M1) macrophages had increased levels of mature
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24 MMP-14 and active MMP-14 was detected by our probe only in classically activated (M1)
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26 macrophages. This finding is consistent with previous findings that M1 macrophages exhibit
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28 enhanced matrix degradation capabilities, and increased localization of MMP-14 in
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30 filopodia.²⁴ The compartmentalization of MMP-14 in filopodia or invadopodia has also been
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32 associated with increased stability of MMP-14 and elevated collagenase activity.²²
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34 Interestingly, the activation of MMP-14 in M1 macrophages requires stimulation through
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36 TLRs with pro-inflammatory cytokines such as IFN γ and IL6 not eliciting MMP-14 activation.
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40 MMP-14 activity was also low in non-stimulated fibroblasts. We have identified
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42 TGF β 1 as a paracrine factor that not only increases expression⁴⁹ but also induces the
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44 activation of MMP-14. This activation correlates with the initiation of the myofibroblast
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46 differentiation program, which is accompanied by elevated MMP-14 protein levels.
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48 Interestingly, we found that labeled MMP-14 migrated as a slightly higher molecular weight
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50 species in fibroblasts compared to macrophages. This difference in migration could be due
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52 to modifications of the enzyme that alter its regulatory activities in different cell types.
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54 Consistent with this hypothesis, various post translational modifications and protein-protein
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56 interactions have been attributed to the short cytoplasmic tail of MMP-14 which could result
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3 in the observed differences in migration in the gel⁵⁰⁻⁵⁴. While additional studies will be
4 required to confirm such modifications and their biological significance, these data highlight
5 the value of the active site probe for identifying the specific active form a target protease.
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9 In primary tumor cultures, we observed high activity of MMP-14, however this activity
10 originated from non-neoplastic fibroblasts and other stromal cell populations and not from
11 transformed cancer cells. We found that MMP-14 activation is stimulated by soluble factors
12 secreted by the tumor cells. One such effector is TGF β 1, which acts to differentiate
13 fibroblasts in the stroma and stimulate expression and activation of MMP-14. TGF β 1
14 signaling has been suggested to promote tumor growth, progression and migration at later
15 stages of multiple carcinomas.⁵⁵⁻⁵⁸ Santibanez et al. describe TGF β signaling in the tumor
16 microenvironment as a regulatory loop, where TGF β is overproduced by late stage cancer
17 cells causing increased expression of MMPs, which in turn mediate the activation of more
18 latent TGF β ⁵⁹. Thus, through balancing levels of MMPs and TIMPs, both TGF β and MMPs
19 contribute to tumor progression by the release of cytokines sequestered by the ECM.
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21 Similarly, TGF β increases the expression of Furin, a protease known to activate Pro-MMP-
22 14 as well as Pro-TGF β but not the expression of TIMPs, thus potentially enhancing the
23 proteolytic response^{32, 60}. Fibroblasts that were co-cultured with tumor cells show similar
24 patterns of MMP-14 activity, suggesting that CAFs similarly stimulated by tumor cells not
25 only express more MMP-14 but also increase levels of active MMP-14. TGF β 1 and perhaps
26 additional soluble factors are responsible for the activation of MMP-14 in fibroblasts that are
27 cultured with supernatants of tumor cells. As tumor cells invade through the basement
28 membrane and make contact with the three-dimensional collagenous matrix of the interstitial
29 tissue, paracrine signaling leads to the recruitment of stromal cells, mostly fibroblasts and
30 macrophages. These cells within the invasion front support tumor progression, help evade
31 the immune system and sustain the flow of nutrients.^{2, 17, 61, 62} Our data supports this model
32 in which the invading cancer cells mobilize and activate MMP-14 in these recruited stromal
33 populations to negotiate the ECM barrier.^{39, 43}
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3 Herein we demonstrate the utility of a methodology that involves the coupling of protein
4 engineering with ABP design to specifically and selectively label active MMP-14 in the
5 context of live cells. Generation of mice endogenously expressing the probe sensitive
6 cysteine mutant MMP-14 F260C followed by crossing of the mice into a model of breast
7 cancer provides a means to selectively image MMP-14 in complex cellular environments.
8 Specifically, it enables the dissection of specific roles of MMP-14 at various stages of
9 tumorigenesis as well as the contribution of specific cell populations within the tumor
10 microenvironment. We have shown that signaling cascades initiated by TGF β 1 or through
11 TLRs lead to the activation of MMP-14 on fibroblasts and macrophages and furthermore that
12 these are the cells that predominantly express active MMP-14. Further studies may
13 elucidate additional signaling pathways that lead to the activation of MMP-14 on other cell
14 types. A clear understanding of the activity profile of MMP-14 is paramount to determine
15 specific cell populations to be targeted for therapeutic applications. In addition, this general
16 methodology is applicable to additional MMP members as well as other large enzyme
17 families that lack selective inhibitors and dynamic reporters of enzyme activity levels.
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34 **Materials and Methods**

35 All materials and methods can be found in the Supporting Information.
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50 **Supporting Information.** Supplemental figures, detailed synthetic procedures and
51 compound characterizations can be found in the Supporting Information. This material is
52 available free of charge via the Internet at <http://pubs.acs.org>.
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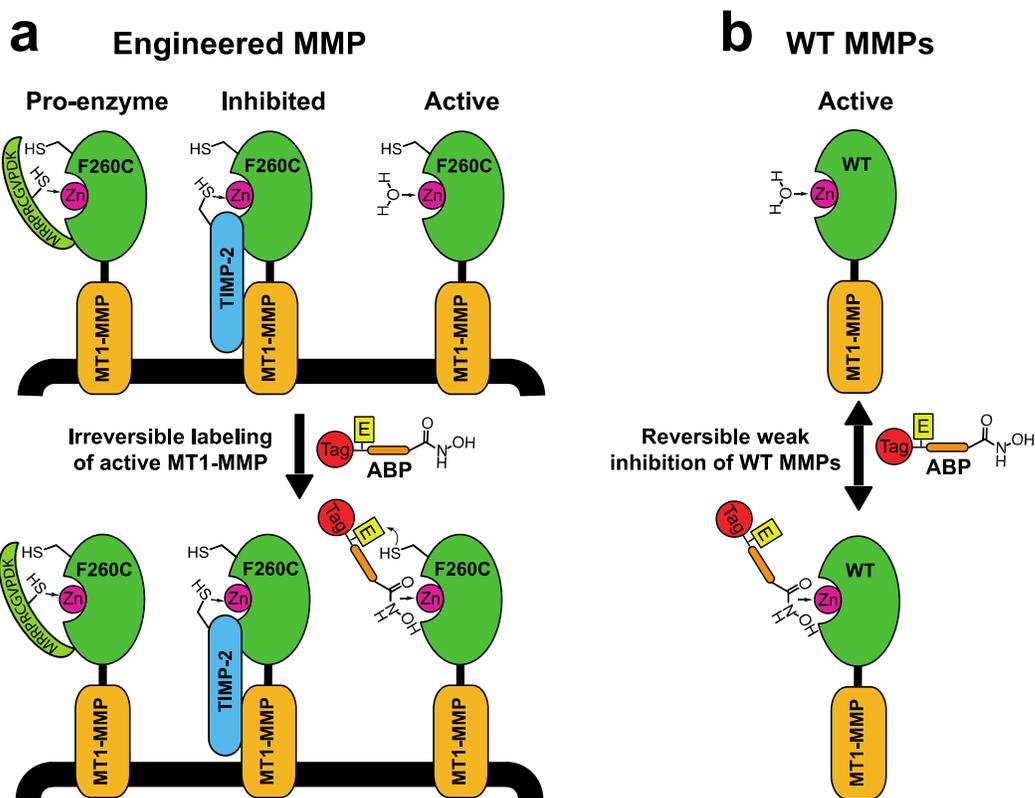


Figure 1: Specific labeling of active MMP-14 using protein engineering coupled with ABPs. a) Engineered MMP-14 contains a cysteine nucleophile that is irreversibly labeled by the ABP. Covalent binding occurs only with the active form of MMP-14 following affinity targeting of the catalytic zinc by the hydroxamate-containing ABP, but not with the proenzyme or the inhibited form in which the zinc is inaccessible. b) ABP labeling is selective to the probe sensitive MMP while any interactions with native MMPs result in reversible weak inhibition.

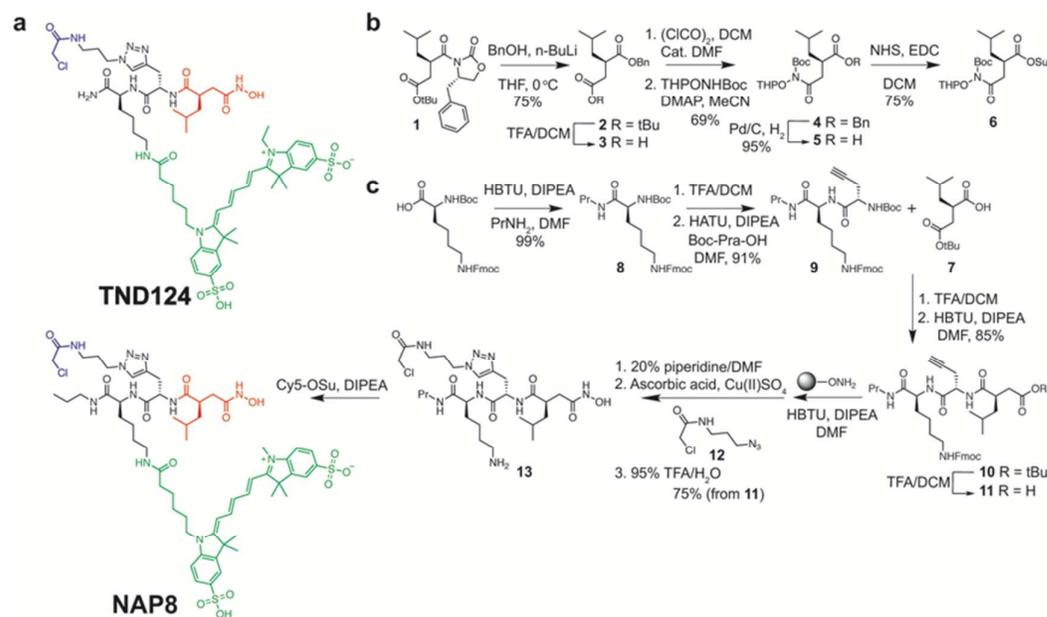


Figure 2: Chemical structure and optimized synthesis of an ABP that targets the cysteine-mutant MMP-14. a) ABPs TND124 and NAP8 are designed based on the core scaffold of GM6001 and comprise its high affinity isobutylsuccinylhydroxamic acid motif (red) with the addition of a chloromethyl amide electrophilic warhead (blue) and a Cy5 fluorescent tag (green). NAP8 has an additional propylamide to improve its metabolic stability. b) Optimized synthesis strategy to prepare double-protected hydroxylamine derivative **6**, synthesis procedures were adopted from Overkleeft *et al.*²⁸ c) Improved synthesis scheme for NAP8, employing an on-resin click reaction to add the electrophile **12** in the penultimate reaction step.

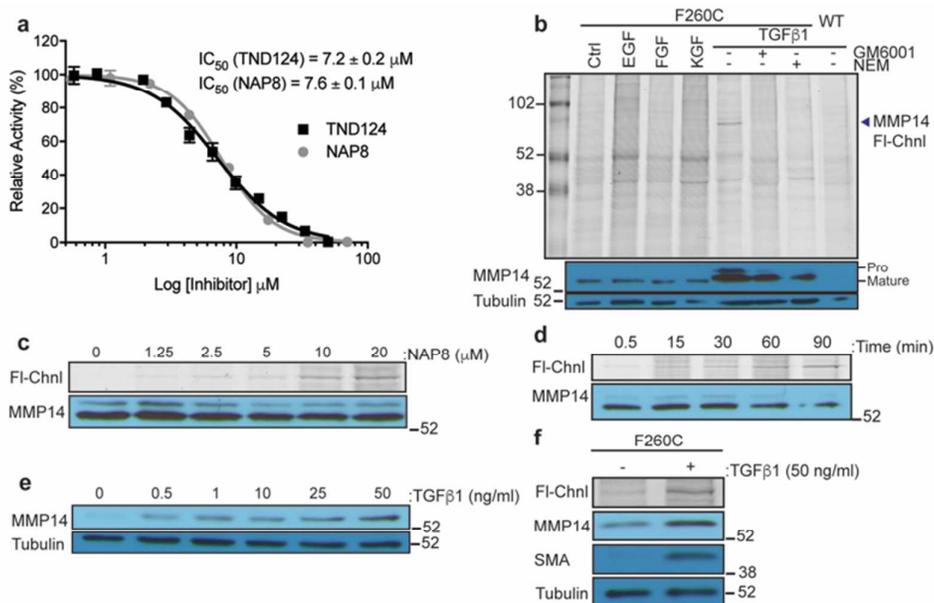


Figure 3: Active MMP-14 is selectively labeled by ABPs in stimulated fibroblasts. a) Recombinant MMP-14 F260C catalytic domain was incubated with TND124 or NAP8 over a range of concentrations. Enzyme activity was measured with the MMP FRET substrate MCA-PLGL-Dap(Dnp)-AR-NH₂ (Anaspec). Activity is relative to a DMSO control. Each data point represents an average of three replicates, error bars \pm SD. b) Primary mouse fibroblasts derived from the MMP14-F260C knock in mice and immortalized WT fibroblasts were serum starved for 12 hours followed by 24 hours incubation in the presence or absence of growth factors (all at 10 ng/ml), GM6001 (100 μM) and NEM (100 μM). Cell cultures were then incubated with NAP8 (10 μM) for 60 minutes and washed. Cell were lysed in sample buffer and resolved on SDS-PAGE followed by in-gel fluorescence scanning for Cy5 (FI-Chnl). Western blot analysis was performed with antibodies for MMP-14 and α -tubulin. Locations for pro-MMP14 (60kDa) and mature MMP14 (57kDa) are marked. c) Serum starved primary mMmp14-F260C fibroblasts were incubated with TGF β 1 for 24 hours then NAP8 over a range of concentrations as indicated. Cell lysates were analyzed by SDS-PAGE followed by in-gel fluorescence scanning for Cy5 (FI-Chnl). Western blot analysis was performed with MMP-14 antibody. d) Same as c) except cells were labeled with 10 μM NAP8 for the indicated incubation times. e) Serum starved WT fibroblasts were incubated with TGF β 1 over a range of concentrations for 24 hours. Western blot analysis was performed with antibodies for MMP-14 and α -tubulin. f) Serum starved primary mMmp14-F260C fibroblasts were incubated in the presence or absence of TGF β 1 (50 ng/ml) for 24 hours, then treated with NAP8 (10 μM). Cell lysates were analyzed by SDS-PAGE followed by in-gel fluorescence scanning for Cy5 (FI-Chnl). Western blot analysis was performed with antibodies for MMP-14, α -smooth muscle actin (SMA) and α -tubulin.

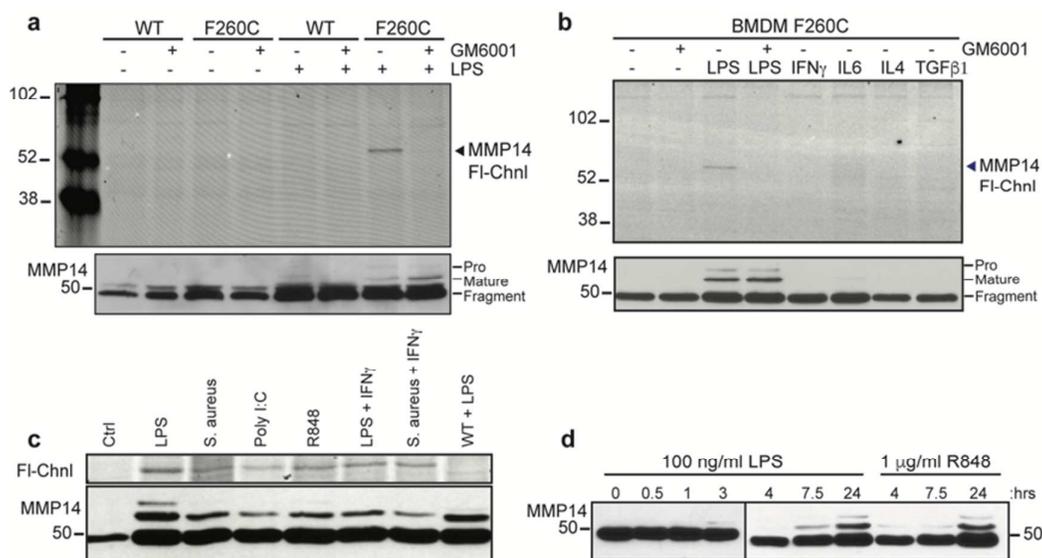


Figure 4: Active MMP-14 is detected only in M1 activated macrophages. Primary bone marrow-derived macrophages (BMDMs) from the WT or mMmp14-F260C knock in mice were primed in the presence of a) LPS (100 ng/ml) and b) other pro-inflammatory cytokines; IFN γ (40 ng/ml), IL6 (50 ng/ml), IL4 (10 ng/ml), TGF β 1 (10 ng/ml) for 16 hours, and treated with GM6001 (10 μ M) or DMSO vehicle control. Cell cultures were treated with TND124 (10 μ M) and lysed cells were analyzed by SDS-PAGE followed by in-gel fluorescence scanning for Cy5 (FI-Chnl). Western blot analysis was performed with antibodies for MMP-14 and α -tubulin. Locations for pro-MMP-14, mature MMP-14 and an inactive fragment are marked. c) Primary mMmp14-F260C and WT BMDMs were primed with different TLR agonists; LPS (100 ng/ml), *S. aureus* (heat inactivated, 1×10^7 cells/ml), Poly I:C (1 μ g/ml), R-848 (1 μ g/ml), IFN γ (50 ng/ml) for 16 hours. Cell cultures were treated with TND124 (5 μ M) and lysed cells were analyzed by SDS-PAGE followed by in-gel fluorescence scanning for Cy5 (FI-Chnl). Western blot analysis was performed with MMP-14 antibody. d) Western blot analysis for BMDMs primed with LPS (100 ng/ml) or R-848 (1 μ M) over 24 hours.

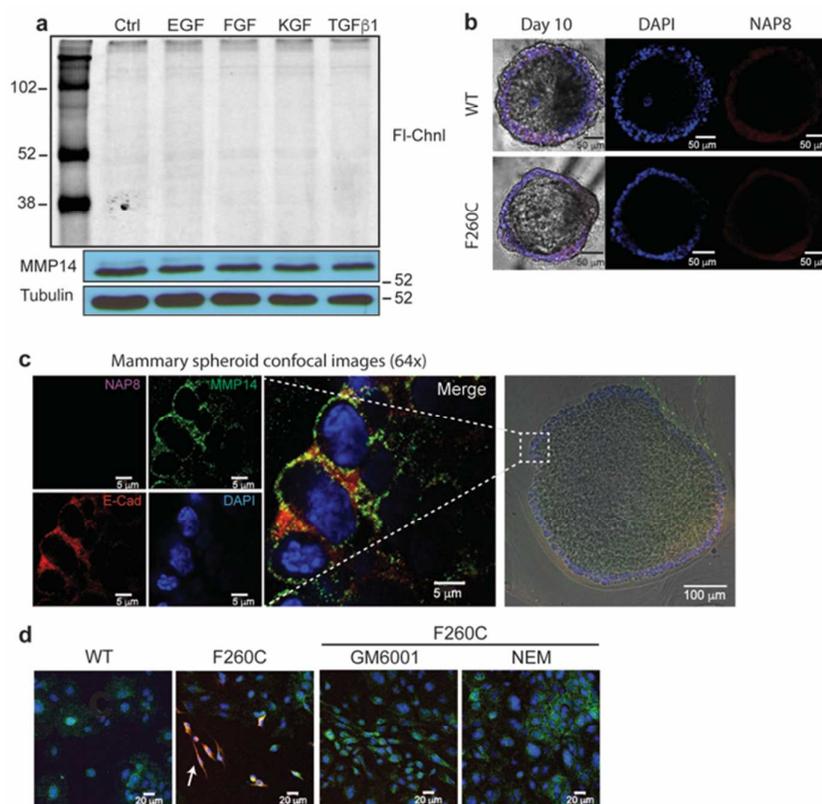


Figure 5: Active MMP-14 is not detected on the surface of primary epithelial cells. a) Primary mMp14-F260C epithelial cells were serum starved for 12 hours followed by 24 hours incubation in the presence of absence of growth factors (10 ng/ml). Cell cultures were treated with NAP8 (10 μM) and lysed cells were analyzed by SDS-PAGE followed by in-gel fluorescence scanning for Cy5 (FI-Chnl). Western blot analysis was performed with antibodies for MMP-14 and α-tubulin b) Confocal microscopy of mammary spheroids in 3D-cultures. Primary organoids isolated from WT and mMp14-F260C 14-week virgin females were cultured on Matrigel for 10 days. At day 10 spheroids were treated with NAP8 (10 μM) fixed, stained with the molecular marker DAPI and visualized by confocal microscopy (scale bar 50 μm). c) Magnified images (64X) of sections of mMp14-F260C spheroids treated with NAP8 (10 μM, purple) and stained with antibodies for MMP-14 (green), E-cadherin (red) and DAPI (blue). The magnified section is marked with a white box (scale bar 5 μm) on the white light image of the spheroid (bottom right). d) Crude primary organoid extract, grown in 2D-cultures were pretreated with GM6001 (10 μM), NEM (100 μM) or DMSO and then treated with NAP8(10 μM). Cells were fixed and stained with anti-MMP-14 and DAPI and visualized by confocal microscopy. NAP8 (red), MMP14 (green), DAPI (blue), cells that have active MMP14 are marked with a white arrow (scale bar 20 μm).

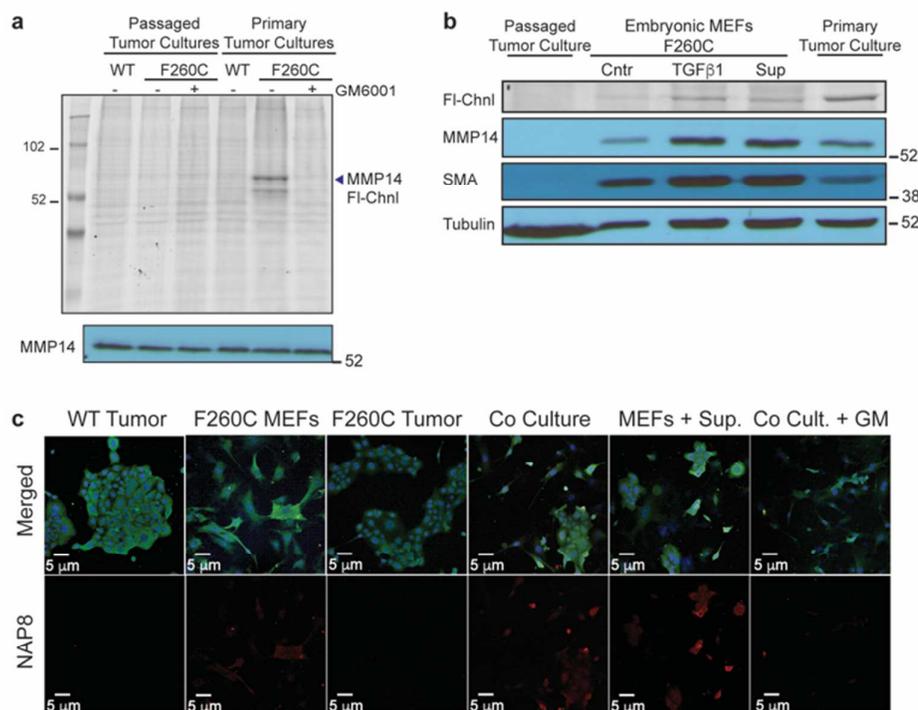


Figure 6: The majority of MMP-14 activity originates from stromal cells in the microenvironment of late-stage carcinoma that respond to stimulation by tumor cells. a) Tumors harvested from the mammary glands of $mMmp14(F260C)^{WT}MMTV-PyMT^+$ and $mMmp14(F260C)^{+/+}MMTV-PyMT^+$ mice were cultured in 2D-cultures and treated with NAP8 (10 μ M) at the first passage or after 10 passages. Whole cell lysates were analyzed by SDS-PAGE and scanned for in-gel fluorescence of Cy5 (FI-Chnl). Western blot analysis was performed with MMP-14 antibody. b) Primary tumor cultures, passaged tumor cultures (neoplastic epithelial cells) and primary fibroblasts from $mMmp14(F260C)^{+/+}MMTV-PyMT^+$ mice were incubated with TGF β 1 (50 ng/ml) or supernatant of neoplastic epithelial cells (24 hours, serum free medium) for 24 hours, then treated with NAP8 (10 μ M). Whole cell lysates were analyzed by SDS-PAGE and scanned for in-gel fluorescence of Cy5 (FI-Chnl). Western blot analysis was performed with antibodies for MMP-14, α -smooth muscle actin (SMA) and α -tubulin. c) 2D-cultures of $mMmp14(F260C)^{WT}MMTV-PyMT^+$ and $mMmp14(F260C)^{+/+}MMTV-PyMT^+$ passaged tumor cells, fibroblasts and co-cultures of passaged tumor cells and fibroblasts were pretreated with DMSO or GM6001 (10 μ M), or pre-incubated with supernatant of neoplastic epithelial cells (24 hours, serum free medium), then treated with NAP8 (10 μ M). Cells were fixed and stained with anti-MMP-14 and DAPI and visualized by confocal microscopy. NAP8 (red), MMP14 (green), DAPI (blue), each experiment was performed in three independent biological repeats (scale bar 20 μ m).

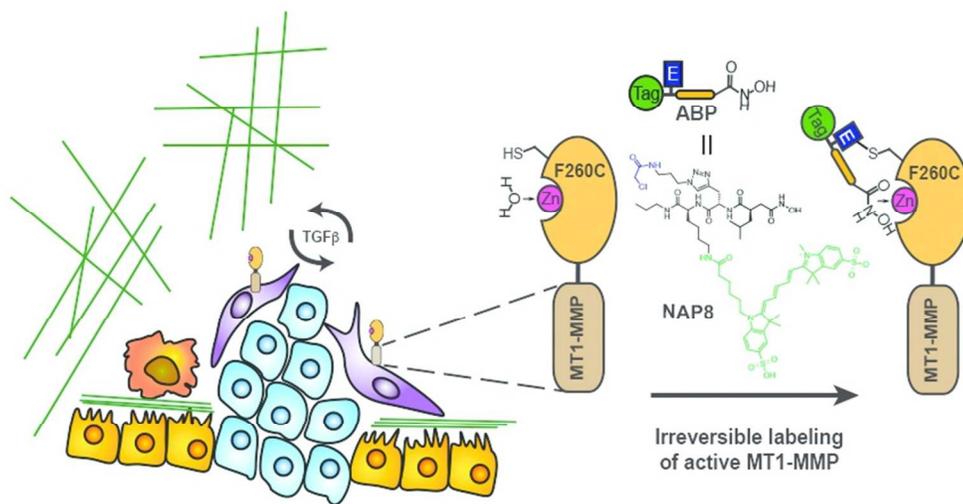


Table of Contents Graphic

74x39mm (300 x 300 DPI)