Supplementary Data and Synthesis Procedures

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

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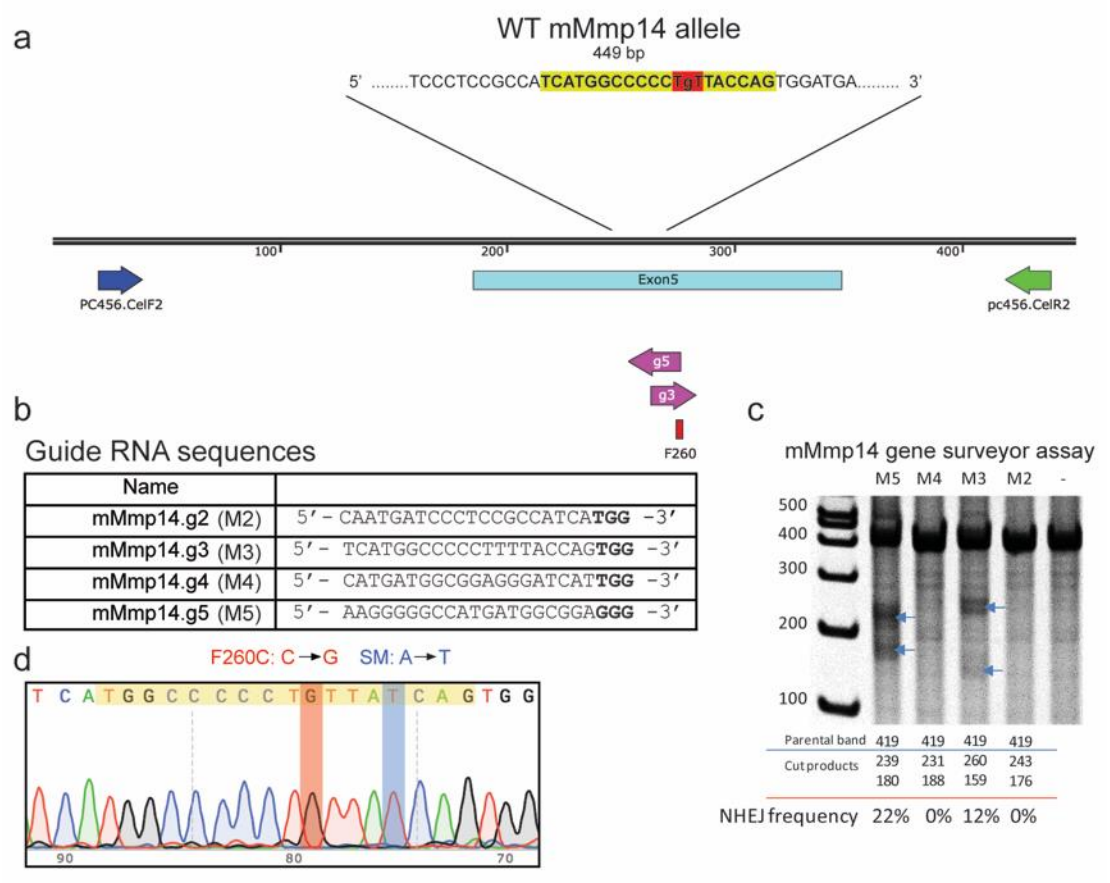


Figure S1: Producing MMP-14 F260C KI mouse line using CRISPR Cas9 technology. a) Schematic illustration of F260C editing in mMmp14 gene. The genomic loci targeted by gRNA M3 is highlighted in yellow and the mutation site is highlighted in red. The blue and green arrows indicate the PCR primers, pc456.CelF2 and pc456.CelR2, used for screening of the targeting site. b) The sequences of the candidate gRNAs tested for targeting the NT6688 region and the protospacer adjuscent motif (PAM). c) In vitro mMmp14 gene surveyor assay of gRNAs targeting the NT6688 region. d) Representative PCR-sequencing genotyping of mMmp14-F260C F1 homozygous mice. Data generated by Applied StemCell Inc. CA, USA.

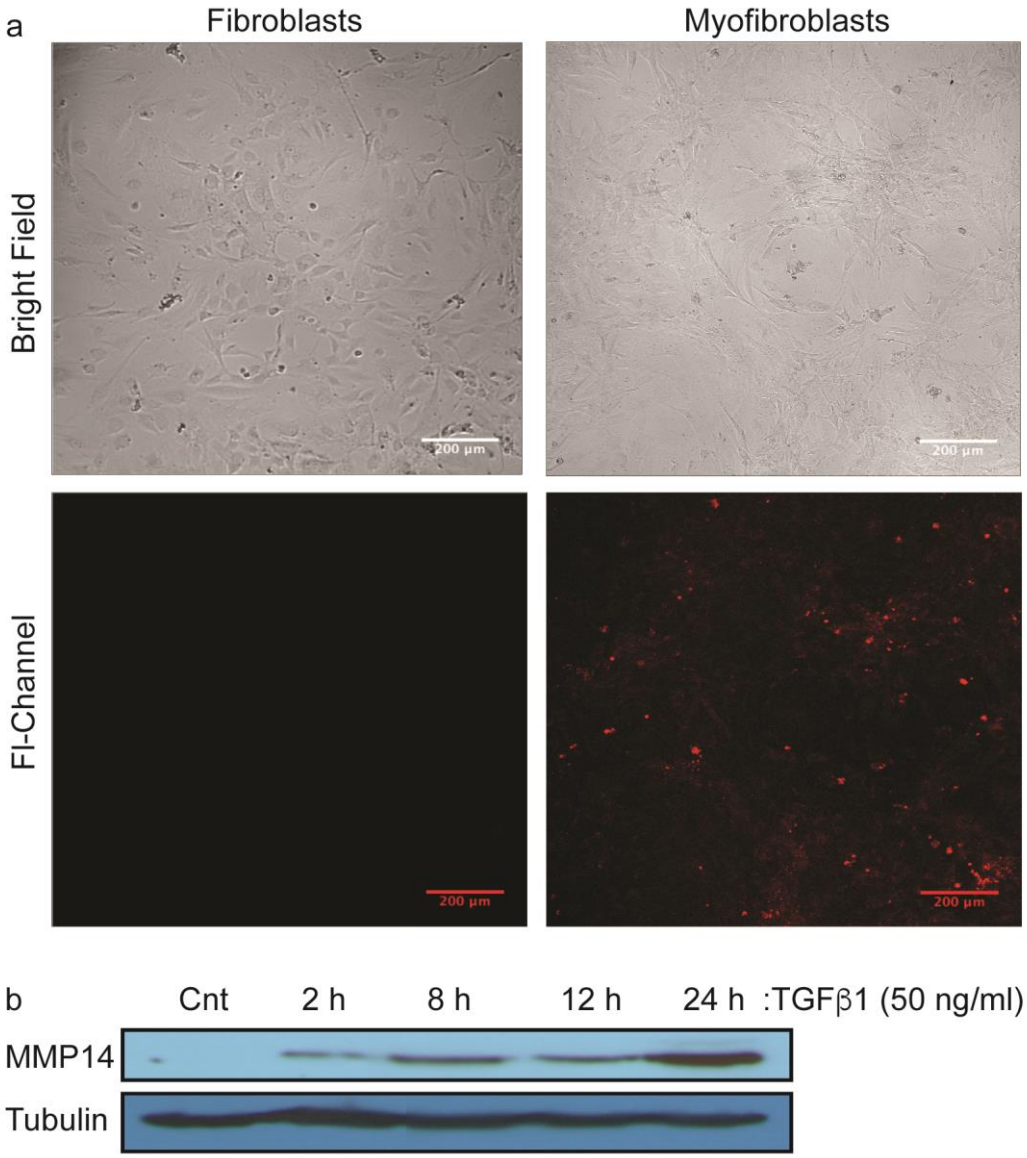


Figure S2: TGFβ1 induces expression and activation of MMP-14 in myofibroblasts. a) Primary fibroblasts and myofibroblasts (induced by 24 hours incubation with 50 ng/ml TGFβ1) grown in culture, and labeled with NAP8 (10 μM). Fluorescence imaging shows activation of MMP-14 in myofibroblasts. b) Westen blot analysis for MMP-14 of fibroblast cells incubated with TGFβ1 (50 ng/ml) over 24 hours.

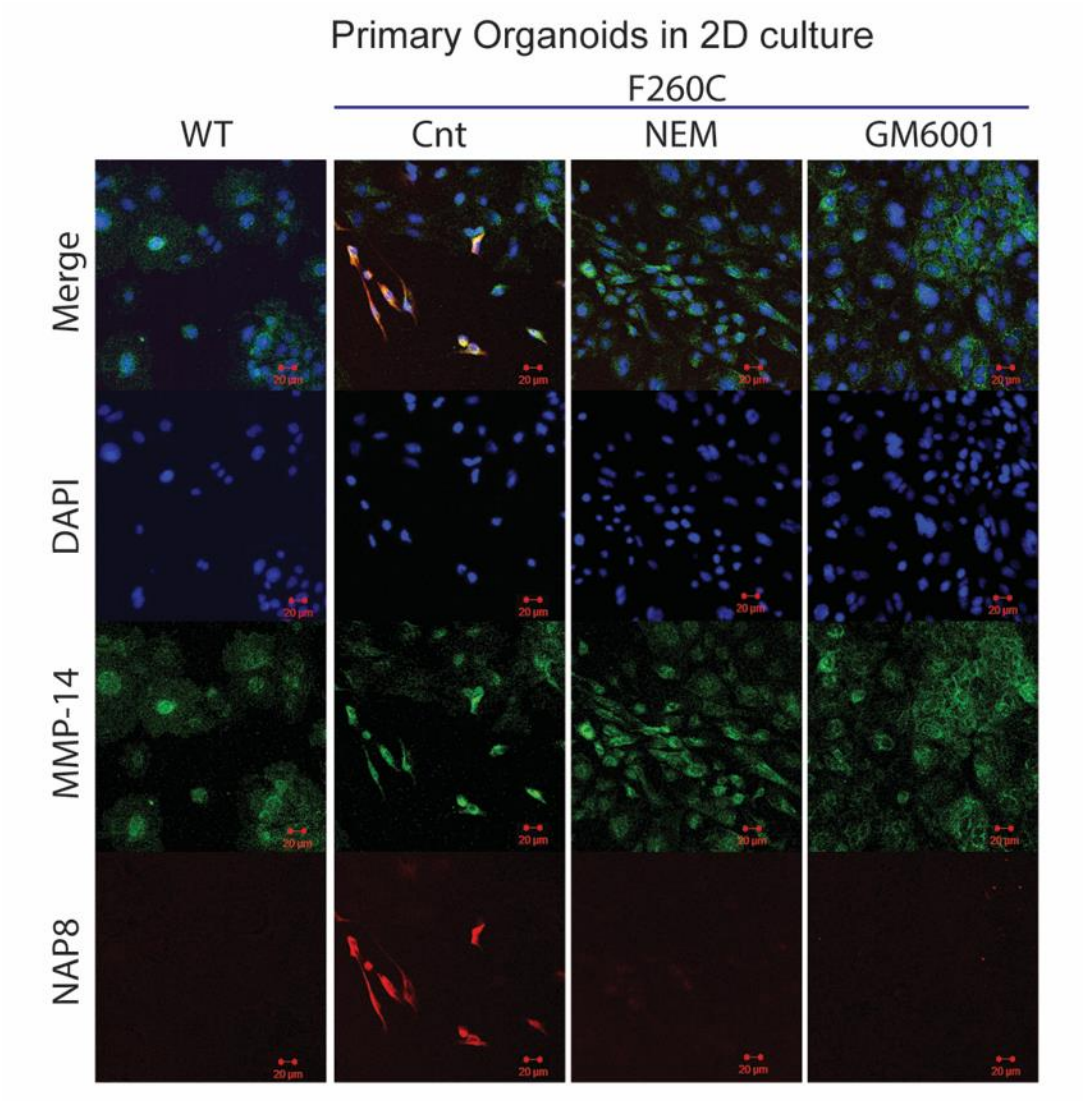


Figure S3: MMP14 activity is seen in stromal cell populations, but not in epithelial cells. Primary organoids grown in 2D-cultures were pretreated with GM6001 (10 µM), NEM (100 µM) or DMSO and then treated with NAP8 (10 µM). Cells were fixed and stained with anti-MMP-14 and DAPI and visualized by confocal microscopy. NAP8 (red), MMP14 (green), DAPI (blue), cells that have active MMP14 are marked with a white arrow (scale bar 20 µm).

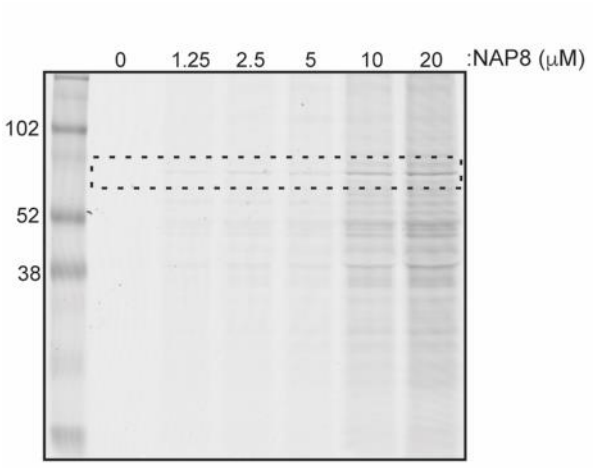


Figure S4: Uncropped in-gel fluorescence scanning image used in figure 3c. Serum starved primary mMMP14-F260C fibroblasts were incubated with TGFβ1 for 24 hours then NAP8 over a range of concentrations as indicated. Cell lysates were analyzed by SDS-PAGE followed by in-gel fluorescence scanning for Cy5. The dashed box indicated the cropped area used in figure 3c.

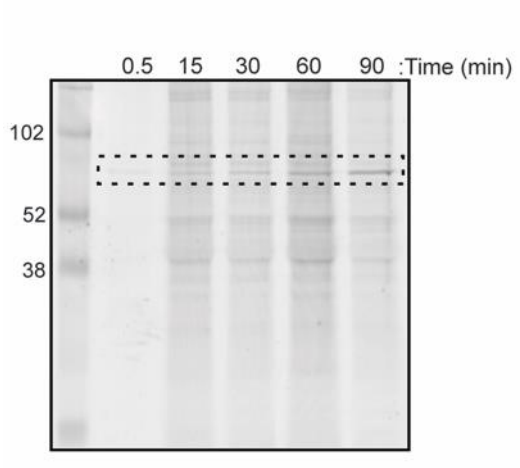


Figure S5: Uncropped in-gel fluorescence scanning image used in figure 3d. Serum starved primary mMMP14-F260C fibroblasts were incubated with TGFβ1 for 24 hours then NAP8 for the indicated incubation times. Cell lysates were analyzed by SDS-PAGE followed by in-gel fluorescence scanning for Cy5. The dashed box indicated the cropped area used in figure 3d.

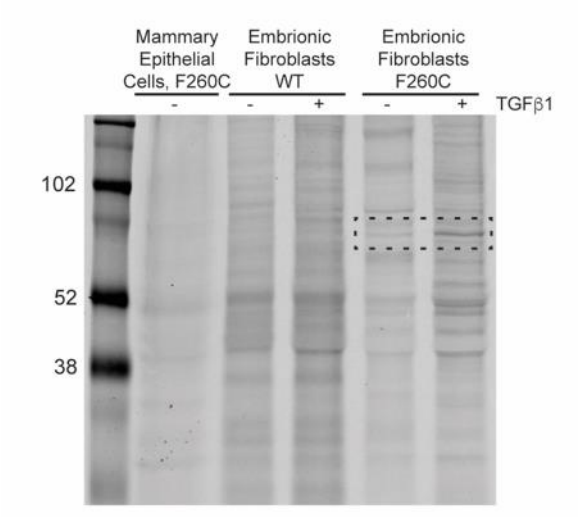


Figure S6: Uncropped in-gel fluorescence scanning image used in figure 3f. Serum starved primary mMMP14-F260C fibroblasts were incubated in the presence or absence of TGFβ1 (50 ng/ml) for 24 hours, and then treated with NAP8 (10 µM). Cell lysates were analyzed by SDS-PAGE followed by in-gel fluorescence scanning for Cy5. The dashed box indicated the cropped area used in figure 3f.

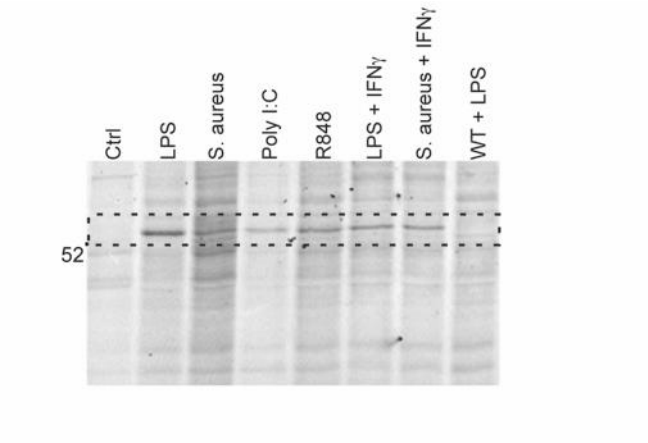


Figure S7: Uncropped in-gel fluorescence scanning image used in figure 4d. Primary mMMP14-F260C and WT BMDMs were primed with pathogen-associated molecular patterns (PAMPs); LPS (100 ng/ml), S. aureus (heat inactivated, 1×10^7 cells/ml), Poly I:C (1 µg/ml), R-848 (1 µg/ml), IFNγ (50 ng/ml). Cell cultures were treated with TND124 (5 µM) and lysed cells were analyzed by SDS-PAGE followed by in-gel fluorescence scanning for Cy5. The dashed box indicated the cropped area used in figure 4d.

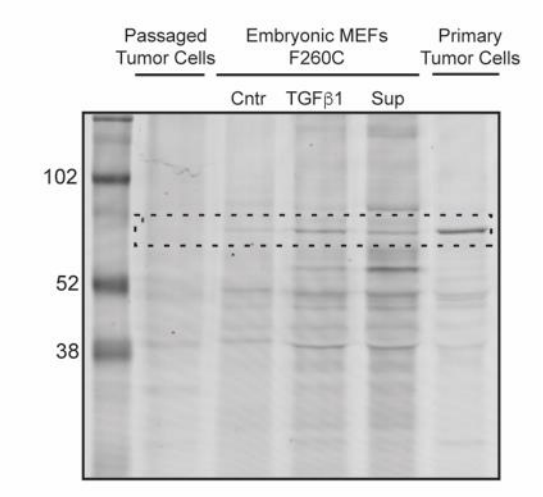


Figure S8: Uncropped in-gel fluorescence scanning image used in figure 6b. Primary tumor cultures, passaged tumor cultures (neoplastic epithelial cells) and primary fibroblasts from MMP14(F260C)$^{+/+}$MMTV^{+} mice were incubated with TGFβ1 (50 ng/ml) or supernatant of neoplastic epithelial cells (24 hours, serum free medium) for 24 hours, then treated with NAP8 (10 µM). Whole cell lysates were analyzed by SDS-PAGE and scanned for in-gel fluorescence of Cy5. The dashed box indicated the cropped area used in figure 6b.

Supplementary Methods

Protein Expression and Purification. Recombinant MMP-14 F260C catalytic domain was expressed in *Escherichia coli* and purified as described previously[1].

Enzymatic Activity. Recombinant MMP-14 F260C catalytic domain was diluted in reaction buffer [50 mM Tris-HCl pH-7.4, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3] to a final concentration of 100 nM and added to a black 384 well plate. NAP8 or TND124 were added at varying concentration between 100-0.54 μM and the plate was incubated for 1 hour at 37 °C, DMSO was used as control. To measure enzyme activity, 10 μl of the MMP FRET substrate Mca-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Agr-NH_2 (Anaspec) from a 100 μM stock solution was added and reactions were incubated for additional 30 minutes at 37 °C. Substrate turnover was then measured by recording the fluorescence emission (λ_{ex} 325 nm, λ_{em} 393 nm) and relative activity, compared to the fluorescence of the DMSO control was plotted vs. logarithm of probe concentration. IC_{50} values were determined by non-linear fit to a dose-response equation using Prism V.7.0d (GraphPad Software Inc.).

Mouse Line Construction. The mouse mMmp14 point mutation knock-in (mMmp14-F260C) was generated using Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) technology. Phenylalanine (F) of the 260th residue of mMMP14 protein was mutated to cysteine (C) by changing Thymidine to Guanine (T to G) at the 6688th nucleotide of mMmp14 cDNA. Four guide RNA (gRNA) sequences were selected for targeting nucleotide 6688 in the mouse mMmp14 gene using the CRISPR design tool created by Zhang et al.[2] (http://tools.genome-engineering.org). The genomic loci targeted by gRNAs and the Protospacer Adjacent Motif (PAM) are depicted in supplementary figure 1 (Fig. S1a,b). Individual gRNA sequences were cloned into a gRNA expression vector and transfected into mouse N2A cells to test the efficiency of Cas9 targeting. DNA was extracted and PCR performed with the primer pair pc456.CelF2 5' – GAGGGTAGTGACTGCCTTGG – 3' and pc456.CelR2 5' – ACTTCACACATGATGGAGGG – 3' yielded a 419 bp fragment. PCR products were tested in a SURVEYOR assay to evaluate the activity of gRNAs, using the SURVEYOR mutation detection kit (Transgenomic Inc., cat#: 706020), according to the manufacturer's instructions (Fig. S1c). Both gRNAs 3 and 5 qualified with NHEJ frequency of 12% and 22% respectively, and mMmp14.g3 sequence was referenced to design and generate a single strand oligo donor nucleotide (ssODN) for microinjection (5' – TTCTTGGTGGCTGTGCATGAGTTGGGGCATGCCCTAGGCCTGGAACATTCCAATGATC

CCTCCGCCATCATGGCCCCCTgTTACCAGTGGATGGACACAGAGAACTTCGTGTTGCCT GATGACGATCGCCGTGGCATCCAGCAACTTTATGGCGAGTTACC - 3'. C57BL/6 embryos were injected through cytoplasmic route with a CRISPR targeting cocktail (gRNA, ssODN and Cas-9 mRNA), and well-developed embryos were implanted into surrogate CD1 mice (conducted by Applied StemCell Inc, Milpitas, CA). Heterozygous male and female mMmp14-F260C founders were confirmed by PCR screening. F1 breeding was carried to obtain homozygous mMmp14-F260C KI mice (Fig. S1d). To generate a breast cancer MMTV-PyMT model mouse line, mMmp14-F260C homozygous females were crossbred with an MMTV-PyMT breast cancer model male (022974, The Jackson Laboratory). This murine line expresses the polyomavirus middle T antigen (PyMT) under the control of the mouse mammary tumor virus promoter (MMTV); female mice develop mammary tumors with lung metastases. Mouse genotypes from tail biopsies were determined using real time PCR with specific probes designed for each gene (Transnetyx, Cordova, TN).

Isolation and Culture of Primary Mouse Embryonic Fibroblasts (MEFs). Primary MEF cells were isolated from embryos of mMmp14-F260C mice at 12.5 days after coitus. Embryos were removed and separated from maternal tissues and yolk sack and were finely minced, digested with 0.25% trypsin for 15 minutes at 37 °C, and centrifuged for 5 min at 500 × g. The pellet was resuspended in culture medium before plating. Cells were cultured at 37 °C with 5% CO_2 in DMEM (Invitrogen) supplemented with 10% (v/v) FetalPlex™ animal serum (Gemini Bio Products), 100 units/ml penicillin/streptomycin (Invitrogen) and 2 mM L-Glutamine. MEF cells were frozen in liquid nitrogen at passage 1 in aliquots of 3×10^6 cells/vial. For analysis of MMP-14 activity, MEFs were seeded on tissue culture treated 24-well plates at 150,000 cells per well, in DMEM supplemented with 10% animal serum, 100 units/ml penicillin/streptomycin and 2 mM L-Glutamine and incubated at 37 °C with 5% CO_2. After overnight incubation, cells were serum-starved for 12 hours, and stimulated with growth factors in serum-free DMEM for 24 hours; mEGF, mFGF, mKGF 10 ng/ml (Gemini Bioproducts) and TGFβ1 10-50 ng/ml (BioLegend).

Isolation and Culture of Primary Bone Marrow Derived Macrophages (BMDM). BMDMs were isolated by culturing mouse bone marrow in DMEM with 2 mM L-glutamine, 10% animal serum and 10 ng/ml recombinant mouse m-CSF (eBioscience) for 6 days at 37 °C with 5% CO_2. After 6 days cells were washed several times with PBS, harvested using CellStripper (Corning CellGro) and frozen in liquid nitrogen at passage 1 in aliquots of 1×10^6 cells/vial. For analysis of MMP-14 activity, BMDMs were seeded on tissue culture treated 24-well plates at 150,000 cells per well, in DMEM supplemented with 10% animal

serum, 100 units/ml penicillin/streptomycin and 2 mM L-Glutamine and incubated at 37 °C with 5% CO_2. After overnight incubation, were primed with pro-inflammatory cytokines; IFNγ 40 ng/ml (Biolegend), IL6 50 ng/ml (R&D Systems), IL4 10 ng/ml (R&D Systems), TGFβ1 10 ng/ml (Biolegend), or pathogen-associated molecular patterns (PAMPs) LPS 100 ng/ml (Sigma Aldrich), heat inactivated *S. aureus*, 1×10^7 cells/ml (ATCC3556), Poly I:C 1 µg/ml (Torcis), P-848/Resiquimod 1 µg/ml (Enzo).

Isolation and 3-D Culture of Mammary Organoids. Primary mammary organoids were isolated from the inguinal fat pads of WT and mMmp14-F260C 12-week old virgin females. Dissected fat pads were washed in cold PBS and minced with a scalpel to 0.5-1mm pieces. Minced tissue was digested in DMEM/F12 containing 5% FBS, 5 µg/ml insulin, 0.2% collagenase I and 0.1% trypsin for 45 minutes at 37 °C, then centrifuged at 400 × g for 10 minutes. The supernatant was pipetted to release cells from the fat layer and centrifuged again, combined pellets were resuspended in DMEM/F12 containing 20 units/ml DNAse I and gently mixed for 5 minutes. The suspension was then passed through a 100 µm filter to remove large tissue debris and the cells/organoids mixture was incubated 1 hour at 37 °C with 5% CO_2 in tissue a culture treated dish to allow fibroblasts and myeloid cells to adhere. After 1 hour, the organoid-containing supernatant was collected and remaining single-cell populations were removed by three rounds of pulse centrifugation (fast acceleration to 400 × g followed by rapid stop and collection of the pelleted organoids). Organoids were then suspended in DMEM/F12 growth medium containing 5% horse serum (Invitrogen), 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin and 100 units/ml penicillin/streptomycin and frozen in liquid nitrogen at a concentration of 20 organoids/µl. For 3-D cultures, organoids were mixed in Matrigel and plated on Matrigel coated µSlide chambered coverslips (Ibidi) at a concentration of 2 organoids/µl. Organoids were cultured for 9 days at 37 °C with 5% CO_2, replacing the growth media every 2-3 days until matured into polarized hollow spheroids. Growth media was replaced at day 9 with assay media that lacked EGF and at day 10 spheroids were incubated with probes for MMP-14 imaging. For analysis of MMP-14 activity in 2-D culture, organoids were seeded on a tissue culture treated 25 cm petri dish in growth media and incubated for 24 hours at 37 °C with 5% CO_2. Cell monolayers were harvested using trypsin and plated on 24-well plates at 150,000 cells per well and incubated overnight in assay medium. Cells were stimulated with growth factors for 24 hours: mEGF, mFGF, mKGF 10 ng/ml (Gemini Bioproducts) and TGFβ1 10 ng/ml (BioLegend).

Isolation and Culture of Tumor cells from Mammary Glands. Palpable tumors were dissected from the mammary glands of 20-23 weeks-old mMmp14-F260C$^{+/+}$MMTV-PyMT^{+} and mMmp14-F260C$^{-/-}$MMTV-PyMT^{+} virgin females. Tumor tissue was washed in cold PBS and minced with a scalpel to ~1mm pieces. Minced tissue was digested in DMEM containing 0.1% collagenase IV, 0.1% dispase and 20 units/ml DNAse I for 30 minutes. After incubation the solution was passed through a 100 μm filter to remove large tissue debris and cells were pelleted by centrifugation at 400 × g for 5 minutes. Cell pellet was resuspended in DMEM supplemented with 10% animal serum, 100 units/ml penicillin/streptomycin and 2 mM L-Glutamine and plated on a 15 cm petri dish. After overnight incubation the cell monolayer was washed to remove debris and non-adherent cells, harvested using trypsin and frozen in liquid nitrogen at passage 1 in aliquots of 3×10^6 cells/vial. To isolate neoplastic epithelial cells, tumor cell cultures were grown to confluence in supplemented DMEM at 37 °C with 5% CO_2 and continuously passaged 10-15 times until a uniform proliferating population of epithelial cell was observed. For analysis of MMP-14 activity, primary tumor cultures and passaged tumor cultures were seeded on tissue culture treated 24-well plates at 150,000 cells per well, in DMEM supplemented with 10% animal serum, 100 units/ml penicillin/streptomycin and 2 mM L-Glutamine and incubated at 37 °C with 5% CO_2. For analysis of stimulation of MEFs, passaged tumor cells were co-cultured with MEFs in 24-well plates, each cell population was seeded at 75,000 cells per well and incubated in supplemented DMEM for 24 hours. MEFs stimulated with tumor cells culture supernatant were firs serum starved for 12 hours then the supernatant of 24 hours-old tumor cell culture in serum free DMEM was added and incubated additional 24 hours.

Probe Labeling of Cells in Culture. Cells grown in 2-D or 3-D cultures were first washed several times with serum free DMEM and pre-incubated with DMSO, GM6001 (10-100 μM) or NEM (100 μM) for 1 hour at 37 °C with 5% CO_2. NAP8 or TND124 (10 μM) were then added from DMSO stocks and cells were incubated for additional 1-1.5 hours. After incubation, cells were washed twice with fresh DMEM containing 1% BSA and once with DMEM, with each wash step cells were incubated for 20 minutes. Labeled cells were fixed for immunofluorescence assay or lysed in sample buffer for SDS-PAGE analysis, then visualized on Typhoon flatbed fluorescent laser scanner (GE Healthcare, UK).

Western Blots. Cell lysates separated by SDS-PAGE were transferred to 0.2 μm nitrocellulose resin (BioRad) and western blots were performed with diluted antibodies; anti-MMP14 (1:2000, Abcam, ab51074), anti-E-cadherin (1:1000, Cell Signaling, 24E10), anti-α-

smooth muscle actin (1:500, Abcam, ab5694), anti-α-tubulin (1:4000, Sigma-Aldrich, B-512) and HRP-conjugated secondary antibodies (GE Healthcare).

Immunofluorescence staining and Confocal Microscopy. Probe labeled cells were fixed with 4% paraformaldehyde in PBS, washed once with PBS containing 0.1M glycine and twice with PBS. Cells were permeabilized with PBS containing 0.5% Triton X-100, blocked with 10% goat serum in IF buffer [PBS, 0.1% BSA, 0.2% Triton X-100, 0.04% Tween-20, 0.02% NaN_3] and incubated with primary antibodies; anti-MMP14 (1:200, Abcam, ab51074) and anti-E-cadherin (1:200, Cell Signaling, 24E10) diluted in IF buffer. Samples were washed with PBS (× 3) and incubated with Alexa 594 or Alexa 488-conjugated secondary antibodies (1:1000, Invitrogen). Cells were washed with PBS, mounted in Vectashield with DAPI (Vector Labs) and imaged oz a Zeiss LSM700 confocal microscope. Snapshots were taken with 10x or 20x air objectives, typically 6 random fields per sample. Magnified regions of spheroids were taken with 63x oil objective.

Biological and Technical Replicates. Biological replicates indicate the replicate of the same experiment conducted upon separately prepared biological samples, typically ≥ 3. Cells, organoids or tumor tissue samples were isolated from multiple mice in independent batches. Cell cultures used for probe labeling of IFA were repeated from different sample batches, seeded at different days. Each experiment was conducted in three technical replicates, in which cells were seeded and treated simultaneously.

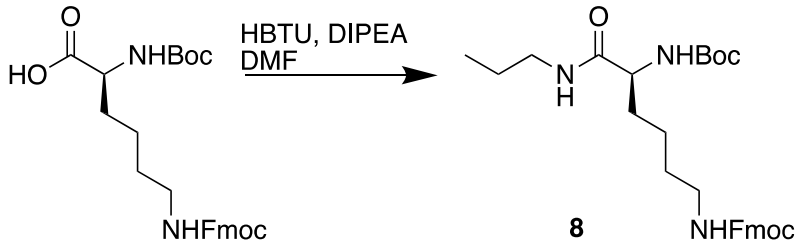
Detailed synthesis procedures and characterization of analogues shown in Figure 1

Materials

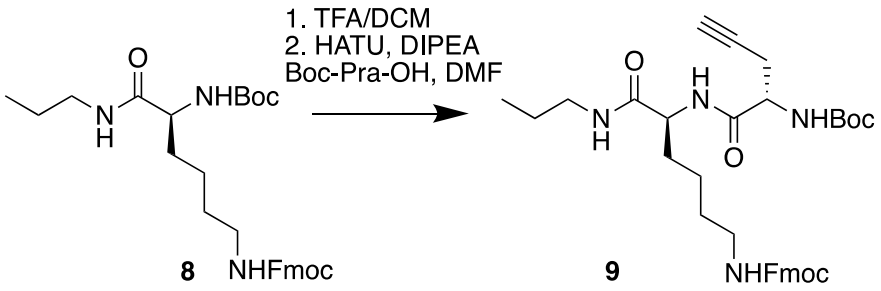
All reagents were purchased from commercial sources and were used without further purification. All solvents were purchased from Fisher Scientific (HPLC grade). Reaction products were purified by normal phase silica-gel flash column chromatography (60 Å, 230-400 mesh) or by high performance liquid chromatography (Agilent 1260 Infinity System, Agilent Technologies) using a ZORBAX 300SB-C18 reverse phase column. Structure characterization was based on NMR spectroscopy, recorded on a 500 MHz Bruker AVANCE system or a Varian 400 MHz (400/100) or a Varian 500 MHz (500/125), as well as mass spectrometry, measured using Surveyor Plus liquid chromatography system coupled to a Finnigan LTQ mass detector (Thermo-Fisher Scientific) or an Agilent 1100 series LCMS using an API 150EX single-quadrupole mass spectrometer. Spectroscopic data was analyzed using MestReNova 11.0.1 (Mestrelab Research). Chemical shifts are given in ppm

(δ) relative to tetramethylsilane as an internal standard, coupling constantans are given in Hz. Recombinant protein purification was done on a ÄKTAexplorer system, Amersham Pharmacia Biotech.

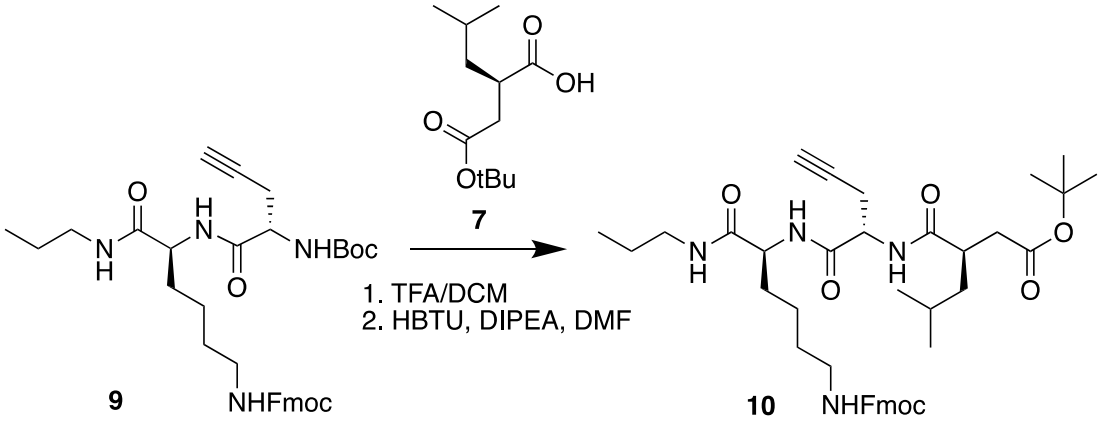
Synthesis Procedures



(9*H*-fluoren-9-yl)methyl *tert*-butyl (6-oxo-6-(propylamino)hexane-1,5-diyl)(*S*)-dicarbamate (8): Boc-Lys(Fmoc)-OH (2.34 gr, 5 mmol) was dissolved in dry DMF under argon and stirred at RT. HBTU (2.84 gr, 7.5 mmol) and DIPEA (1.74 ml, 10 mmol) were added and the reaction was stirred for 4 minutes, then propylamine (0.82 ml, 10 mmol) was added to the stirring solution. The reaction was stirred additional 1.5 hours and then DMF was evaporated under vacuum. Ethyl acetate (200 ml) was added and the solution was washed with water (50 ml) and 0.1M HCl (50 mL) and brine (50 ml) and dried with magnesium sulfate. The solvent was evaporated under vacuum and the crude was purified with column chromatography (5-30% ethyl acetate in DCM) to yield 2.52 gr (99%) of pure compound **8**. ^{1}H NMR (500 MHz, Chloroform-d) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 7.5 Hz, 2H), 7.40 (t, *J* = 7.5 Hz, 2H), 7.31 (td, *J* = 7.4, 1.1 Hz, 2H), 6.12 (s, 1H), 5.07 (s, 1H), 4.87 (s, 1H), 4.41 (d, *J* = 7.4 Hz, 2H), 4.21 (t, *J* = 7.0 Hz, 1H), 4.02 – 3.98 (m, 1H), 3.83 (h, *J* = 6.5 Hz, 2H), 3.21 (t, *J* = 6.9 Hz, 4H), 1.85 (dd, *J* = 14.3, 6.9 Hz, 1H), 1.52 (h, *J* = 7.3, 6.9 Hz, 4H), 1.43 (s, 9H), 1.40 – 1.35 (m, 1H), 0.91 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.14, 163.21, 153.26, 143.96, 141.30, 127.65, 127.02, 125.04, 119.95, 86.91, 77.28, 77.23, 77.03, 76.77, 60.39, 47.28, 41.16, 40.39, 38.60, 31.83, 29.49, 28.30, 21.04, 14.19, 11.29. $[M+H]^+$ calculated 510.29 found 510.4

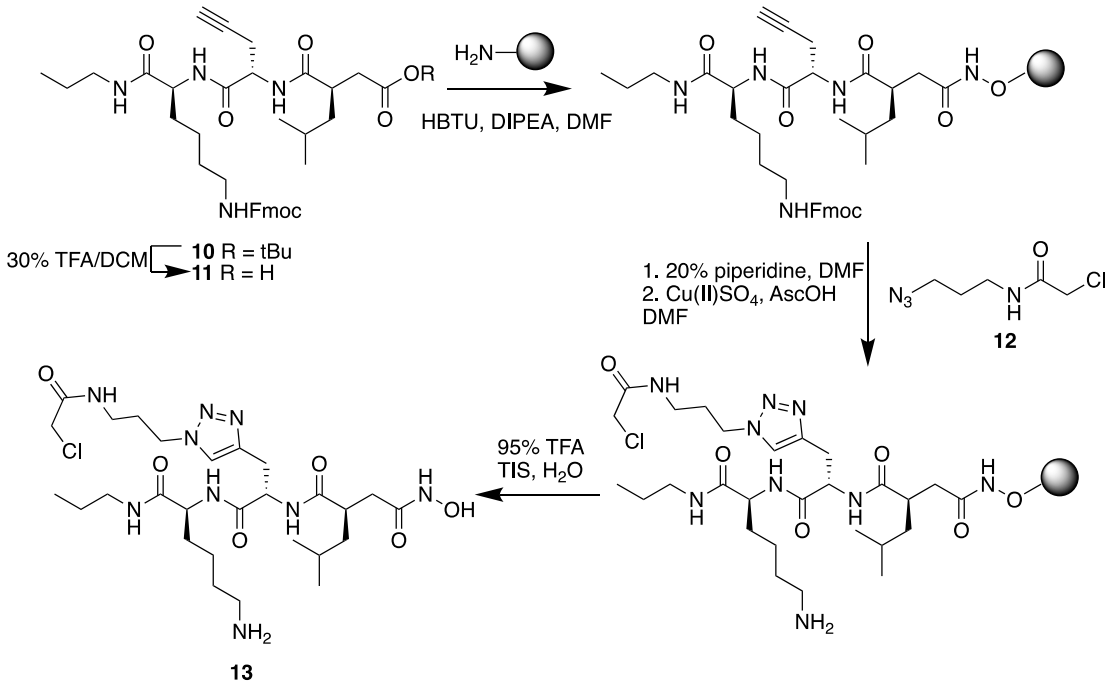


(9*H*-fluoren-9-yl)methyl ((*S*)-5-((*S*)-2-((*tert*-butoxycarbonyl)amino)pent-4-ynamido)-6-oxo-6-(propylamino)hexyl)carbamate (9): Compound **8** (509.6 mg, 1 mmol) was dissolved in a solution of DCM (3 ml) and TFA (3 ml) and stirred for 30 minutes at RT. The solvents were evaporated under vacuum, toluene was added on top of the resulting residue and again evaporated under vacuum. This was repeated 3 times to remove all the remaining TFA. The dry solid was then dissolved in dry DMF (50 ml) and kept under argon. Boc-Pra-OH (234.5 mg, 1.1 mmol) was premixed with HATU (418.3 mg, 1.1 mmol) and DIPEA (0.38 ml, 2.2 mmol) in DMF (5 ml), then added to the solution of deprotected **8**. The reaction was stirred for 4 hours at RT then solvents were evaporated under vacuum, redisolved in ethyl acetate (100 ml) and washed with water (25 ml), 0.1M HCl (25 ml) and brine (25 ml). The crude products solution was dried with magnesium sulfate, evaporated under vacuum and purified with column chromatography (2% methanol in DCM) to yield 550 mg (91%) of pure compound 9. ^{1}H NMR (500 MHz, Methanol-d4) δ 7.82 (d, *J* = 7.6 Hz, 2H), 7.67 (d, *J* = 7.5 Hz, 2H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.33 (t, *J* = 7.5 Hz, 2H), 5.52 (s, 1H), 4.37 (d, *J* = 6.9 Hz, 2H), 4.32 (dd, *J* = 8.7, 5.5 Hz, 1H), 4.22 (t, *J* = 6.8 Hz, 2H), 3.22 – 3.07 (m, 4H), 2.72 – 2.55 (m, 2H), 2.44 (s, 1H), 1.86 (d, *J* = 17.0 Hz, 1H), 1.70 (d, *J* = 10.6 Hz, 1H), 1.47 (s, 17H), 0.92 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (126 MHz, MeOD) δ 176.17, 173.14, 164.86, 158.23, 143.23, 141.08, 128.78, 128.14, 126.17, 120.93, 87.79, 73.27, 69.75, 67.63, 54.80, 51.60, 42.20, 38.90, 36.97, 31.67, 28.70, 11.72. $[M+H]^+$ calculated 605.33 found 605.5



(*R*)-2-(2-(*tert*-butoxy)-2-oxoethyl)-4-methylpentanoic acid (7): Compound **7** was synthesized according to published procedures[1]. ^{1}H NMR (500 MHz, Chloroform-d) δ 2.96 – 2.84 (m, 1H), 2.62 (dd, *J* = 16.4, 9.3 Hz, 1H), 2.41 (dd, *J* = 16.4, 5.1 Hz, 1H), 1.72 – 1.59 (m, 2H), 1.47 (s, 9H), 1.37 – 1.26 (m, 1H), 0.95 (dd, *J* = 16.3, 6.3 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 181.22, 171.22, 81.22, 40.89, 39.56, 37.64, 27.99, 25.75, 22.56, 22.25. $[M+H]^+$ calculated 231.15 found 231.3

***tert*-butyl (9*S*,12*S*,15*R*)-1-(9*H*-fluoren-9-yl)-15-isobutyl-3,11,14-trioxo-12-(prop-2-yn-1-yl)-9-(propylcarbamoyl)-2-oxa-4,10,13-triazaheptadecan-17-oate (10):** Compound **9** (604.3 mg, 1 mmol) was dissolved in a solution of DCM (3 ml) and TFA (3 ml) and stirred for 30 minutes at RT. The solvents were evaporated under vacuum, toluene was added on top of the resulting residue and again evaporated under vacuum. This was repeated 3 times to remove all the remaining TFA. The dry solid was then dissolved in dry DMF (50 ml) and kept under argon. Compound 7 (245.2 mg, 1.5 mmol) was premixed with HBTU (758.5 mg, 2 mmol) and DIPEA (0.35 ml, 2 mmol) in DMF (5 ml), then added to the solution of deprotected **9**. The reaction was stirred for 4 hours at RT then solvents were evaporated under vacuum, redisolved in ethyl acetate (100 ml) and washed with water (25 ml), 0.1M HCl (25 ml) and brine (25 ml). The crude products solution was dried with magnesium sulfate, evaporated under vacuum and purified with column chromatography (2% methanol in DCM) to yield 609.4 mg (85%). ^{1}H NMR (500 MHz, Methanol-d4) δ 7.82 (d, *J* = 7.6 Hz, 3H), 7.67 (d, *J* = 7.6 Hz, 2H), 7.41 (t, *J* = 7.5 Hz, 2H), 7.33 (t, *J* = 7.5 Hz, 2H), 7.12 (s, 1H), 5.37 (t, *J* = 5.0 Hz, 1H), 4.51 (s, 1H), 4.45 (q, *J* = 6.5 Hz, 1H), 4.37 (d, *J* = 7.0 Hz, 1H), 4.29 (dd, *J* = 9.1, 5.2 Hz, 1H), 4.22 (t, *J* = 7.0 Hz, 1H), 3.15 (dq, *J* = 18.9, 6.4, 5.8 Hz, 3H), 2.82 (ddd, *J* = 11.8, 8.3, 5.3 Hz, 1H), 2.78 – 2.64 (m, 2H), 2.57 (dd, *J* = 16.0, 8.6 Hz, 1H), 2.45 (t, *J* = 2.6 Hz, 1H), 2.35 (dd, *J* = 16.0, 6.0 Hz, 1H), 1.85 (t, *J* = 7.6 Hz, 1H), 1.75 – 1.64 (m, 1H), 1.57 (ddq, *J* = 35.1, 14.2, 6.8 Hz, 5H), 1.45 (s, 9H), 1.41 – 1.17 (m, 6H), 0.98 – 0.83 (m, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 185.46, 178.64, 175.62, 169.98, 156.71, 153.17, 143.93, 141.32, 134.37, 127.67, 127.05, 125.05, 119.97, 81.60, 81.44, 72.24, 69.47, 56.84, 52.60, 49.06, 47.25, 42.42, 41.35, 41.03, 37.96, 29.44, 28.98, 28.05, 25.74, 23.40, 22.82, 22.64, 22.50. $[M+H]^+$ calculated 717.41 found 717.5

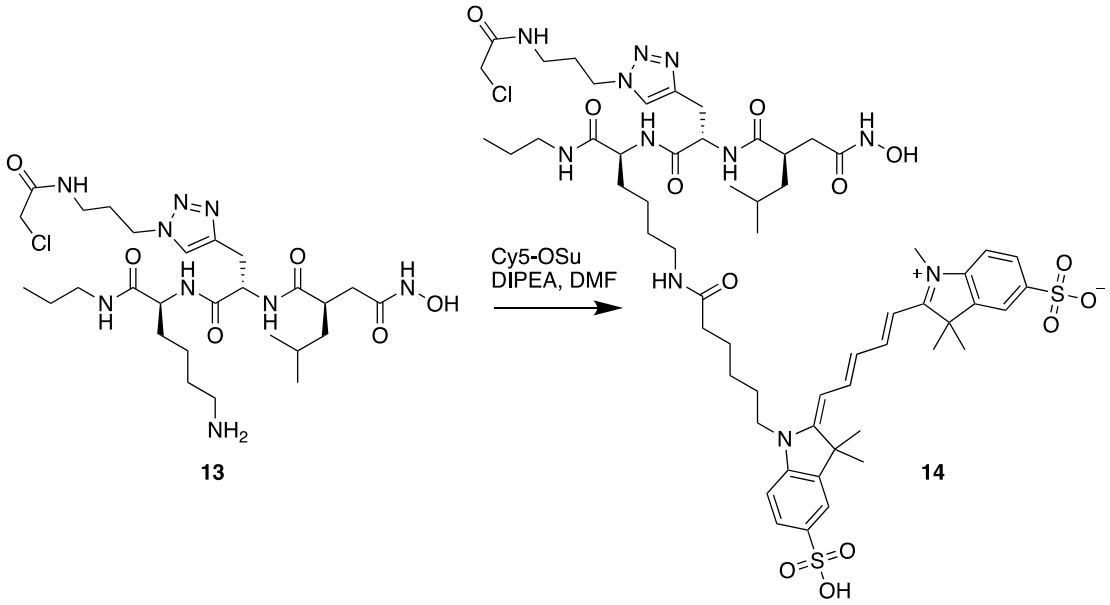


(9*S*,12*S*,15*R*)-1-(9*H*-fluoren-9-yl)-15-isobutyl-3,11,14-trioxo-12-(prop-2-yn-1-yl)-9-(propylcarbamoyl)-2-oxa-4,10,13-triazaheptadecan-17-oic acid (11): Compound **10** (71.7 mg, 0.1 mmol) was dissolved in DCM (1.4 ml) and TFA (0.6 ml) was added at RT. The removal of the Boc group was monitored by LC-MS (~2 hour) after which the solvents were evaporated under vacuum, toluene was added on top of the resulting residue and again evaporated under vacuum. This was repeated 3 times to remove all the remaining TFA. The dry solid was then used for the next reaction without further purification. $[M+H]^+$ calculated 661.35 found 661.6

***N*-(3-azidopropyl)-2-chloroacetamide (12):** Compound 12 was synthesized according to published procedures[1]. ^{1}H NMR (500 MHz, Chloroform-d) δ 6.74 (s, 1H), 3.91 (d, *J* = 1.0 Hz, 2H), 3.50 – 3.39 (m, 4H), 1.90 – 1.81 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.82, 49.57, 38.20, 29.46, 28.69. $[M+H]^+$ calculated 177.06 found 149.3 ($-N_2$)

(*R*)-*N*^{1}-((*S*)-1-(((*S*)-6-amino-1-oxo-1-(propylamino)hexan-2-yl)amino)-3-(1-(3-(2-chloroacetamido)propyl)-1*H*-1,2,3-triazol-4-yl)-1-oxopropan-2-yl)-*N*^{4}-hydroxy-2-isobutylsuccinamide (13): Compound **11** (66.1 mg, 0.1 mmol) was dissolved in dry DMF (3 ml), HBTU (76 mg, 0.2 mmol) and DIPEA (50 μl, 0.3 mmol) were added and the solution was immediately added on to a fritted syringe containing hydroxylamine Wang resin (750 mg, 0.15 mmol, [Novabiochem, 2.0 mmol/gr]). The syringe was capped and the reaction was mixed on a nutating mixer for 4 hours. The resin was then washed with DMF (6 × 5 ml), and a solution of 20% piperidine in dry DMF was added to remove Fmoc protection. After 30

minutes the resin was washed again with DMF (6 × 5 ml) and compound **12** (35.2 mg, 0.2 mmol) was added in a solution of DMF (3 ml). Ascorbic acid (8.8 mg, 0.05 mmol) was dissolved in DMF (1 ml) and added to the syringe. Copper(II) sulfate (8 mg, 0.05 mmol) was dissolved in water (400 μl) and added to the syringe. The reaction was mixed on a nutating mixer for 4 hours and then the resin was washed with DMF (6 × 5 ml), DCM (6 × 5 ml) and allowed to dry 3 hours. Compound **13** was cleaved from the resin with a solution of 95% TFA with 2.5% triisopropylsilane (TIS) and 2.5% water for 2 hours precipitated in cold ether and purified by HPLC using a gradient of 90% water 10% acetonitrile to 20% water 80% acetonitrile over 15 minutes. $[M+H]^+$ calculated 630.35 found 630.4

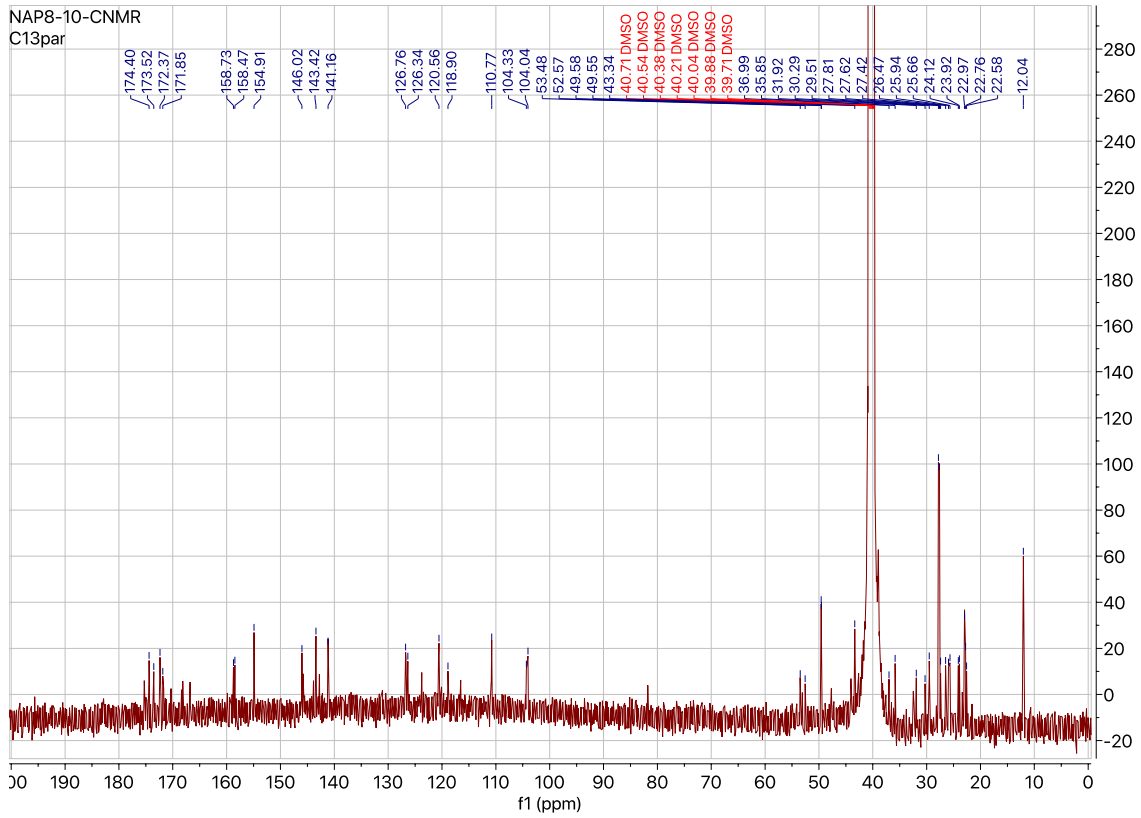


13 **14**

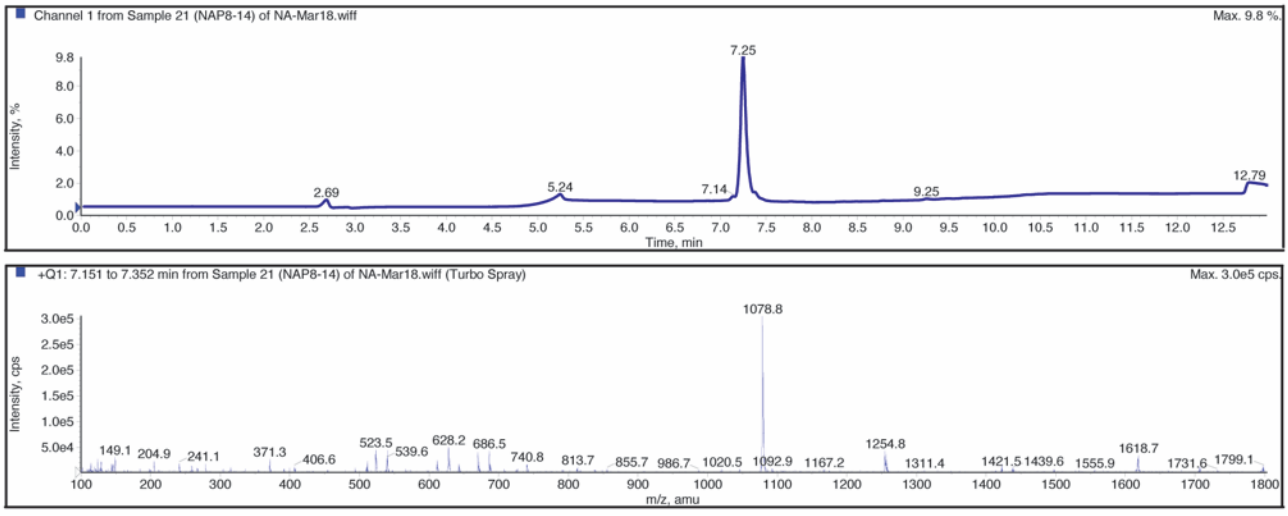
2-((1*E*,3*E*)-5-((*E*)-1-(6-(((*S*)-5-((*S*)-3-(1-(3-(2-chloroacetamido)propyl)-1*H*-1,2,3-triazol-4-yl)-2-((*R*)-2-(2-(hydroxyamino)-2-oxoethyl)-4-methylpentanamido)propanamido)-6-oxo-6-(propylamino)hexyl)amino)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3*H*-indol-1-ium-5-sulfonate (14) NAP8: Compound 13 (3.1 mg, 0.0049 mmol), was dissolved in dry DMF (1 ml) and the succinimide ester of sulfo-Cy5 (5.46 mg, 0.0074 mmol) was added together with DIPEA (0.83 μl, 0.0049 mmol). The reaction was stirred at RT for 2 hour then DMF was evaporated under vacuum and the crude product was purified by HPLC using a gradient of 95% water 10% acetonitrile to 30% water 70% acetonitrile over 15 minutes. ^{1}H NMR (500 MHz, DMSO-d6) δ 10.46 (s, 1H), 8.39 (d, *J* = 6.6 Hz, 1H), 8.35 (d, *J* = 12.6 Hz, 3H), 7.89 (d, *J* = 16.1 Hz, 1H), 7.85 – 7.75 (m, 4H), 7.73 (s, 1H), 7.65 (td, *J* = 5.5, 2.7 Hz, 3H), 7.32 (t, *J* = 7.5 Hz, 3H), 6.57 (t, *J* = 12.3 Hz, 2H), 6.30 (dd, *J* = 21.9, 13.8 Hz, 3H), 4.32 (t, *J* = 7.2 Hz, 2H), 4.13 (s, 1H), 4.08 (d, *J* = 12.4 Hz, 4H), 3.60 (s, 4H), 3.10 (s, 2H), 3.06 – 2.98 (m, 1H), 2.96 (dd, *J* = 12.8, 6.6 Hz, 3H), 2.73 (s, 1H),

2.59 (s, 1H), 1.99 (dt, *J* = 35.1, 7.1 Hz, 4H), 1.71 – 1.67 (m, 17H), 1.53 (t, *J* = 7.5 Hz, 1H), 1.39 (p, *J* = 7.3 Hz, 2H), 1.32 (d, *J* = 8.4 Hz, 3H), 1.19 (s, 1H), 0.80 (ddt, *J* = 13.6, 9.8, 6.8 Hz, 9H). ^{13}C NMR (126 MHz, DMSO) δ 174.40, 173.52, 172.37, 171.85, 158.73, 158.47, 154.91, 146.02, 143.42, 141.16, 126.76, 126.34, 120.56, 118.90, 110.77, 104.33, 104.04, 53.48, 52.57, 49.58, 49.55, 43.34, 36.99, 35.85, 31.92, 30.29, 29.51, 27.81, 27.62, 27.42, 26.47, 25.94, 25.66, 24.12, 23.92, 22.97, 22.76, 22.58, 12.04. $[M+H]^+$ calculated 1254.55 found 1254.8

^{1}H-NMR Spectrum of NAP8



^{13}C-NMR Spectrum of NAP8



LCMS trace and mass-spec analysis of NAP8 after HPLC purification

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1. Morell, M.; Nguyen Duc, T.; Willis, A. L.; Syed, S.; Lee, J.; Deu, E.; Deng, Y.; Xiao, J.; Turk, B. E.; Jessen, J. R.; Weiss, S. J.; Bogyo, M., Coupling protein engineering with probe design to inhibit and image matrix metalloproteinases with controlled specificity. *J Am Chem Soc* **2013,** *135* (24), 9139-48.

2. Ran, F. A.; Hsu, P. D.; Wright, J.; Agarwala, V.; Scott, D. A.; Zhang, F., Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* **2013,** *8* (11), 2281-2308.