Online Data Supplement

Title: Protease activity sensors enable real-time treatment response monitoring in lymphangioleiomyomatosis

Authors: Jesse D. Kirkpatrick, Ava P. Soleimany, Jaideep S. Dudani, Heng-Jia Liu, Hilaire C. Lam, Carmen Priolo, Elizabeth P. Henske, Sangeeta N. Bhatia

Supplementary Methods

Immunofluorescence staining

Kidneys were excised from 7-month-old *Tsc2*$^{+/-}$ and WT AJ mice and fresh frozen in optimal cutting temperature (OCT) media (Sakura; Torrance, CA, USA). Sections were stained with rabbit IgG isotype control (ThermoFisher, #31235; Waltham, MA, USA) (2 µg/ml in block buffer) and primary antibodies against mouse MMP9 (Abcam, ab137867; Cambridge, MA, USA) (1:250 dilution in block buffer), MMP2 (Abcam, ab37150, 1:200 dilution in block buffer), and CTSK (Abcam, ab19027, 1:250 dilution in block buffer) for 2.5 hours at RT. Lungs were excised from either healthy female nude mice or 19 days after intravenous inoculation with $5x10^5$ 105K cells. Lungs were filled with OCT media, embedded, cryosectioned, and stained for Ki-67 (Abcam, ab15580, 1 ug/mL in block buffer) and cathepsin K (Abcam, ab19027, 1 ug/mL in block buffer) for 1 hour at RT. Slides were imaged using a Pannoramic P250 whole slide scanner (3DHistech; Budapest, Hungary).

Cell lines

Tsc2-null 105K cells were previously derived from a *Tsc2*$^{+/-}$ C57BL/6J mouse renal tumour as described (17). 105K+*Tsc2* cells were generated via retroviral reintroduction of *Tsc2*, as previously described (18, 19). 105K and 105K+*Tsc2* cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin.

Western blot for CTSK

105K cells were lysed in RIPA buffer supplemented with protease inhibitors. Normal kidney and lung were harvested from healthy mice, snap frozen in liquid nitrogen, and subsequently homogenized in ice cold RIPA buffer with protease inhibitors. Recombinant active mouse cathepsin K (Biovision; Milpitas, CA, USA) was used as a positive control. Samples were loaded

into NuPAGE Bis-Tris Mini Gels (Invitrogen; Carlsbad, CA, USA) and western blot was performed. Membranes were subsequently stained for cathepsin K (ab19027, Abcam, 1:1000 in 5% milk) and β-actin (ab8227, Abcam, 1:1000 in 5% milk).

Rapamycin dose response in 105K cells

Rapamycin (LC Labs) was reconstituted in ethanol. 105K cells were plated at 5,000 cells/well in 24-well plates and treated with either vehicle (0.002% ethanol) or rapamycin at 2 nM, 20 nM, or 200 nM. Cells were trypsinized and counted at each time point.

***Ex vivo* protease substrate screening**

To establish flank tumours, $1x10^6$ 105K cells (P20) were injected subcutaneously in female nude mice (3-4 weeks old) and grown until tumour volumes were approximately 500 mm^3. Mice were euthanized and tumours and healthy lungs were extracted, flash frozen in liquid nitrogen, and homogenized in PBS using MACS M tubes (Miltenyi Biotec; Somerville, MA, USA) according to manufacturer's protocol. Sixteen previously reported protease-cleavable substrates (14) flanked by a fluorophore (5-carboxyfluorescein) and quencher (CPQ2) were synthesized by CPC Scientific. Homogenates were diluted to 200 mg of tissue/ml in PBS and incubated with fluorogenic substrates (0.33 μM final concentration) at 30 μl final volume at 37 ºC for 30 min. For the protease inhibitor ablation study, homogenates were further diluted 5-fold in either pH 7.5 buffer containing 50 mM Tris, 10 mM $CaCl_2$, 300 mM NaCl, 20 μM $ZnCl_2$, 0.02% Brij-35, and 1% bovine serum albumin (BSA) or pH 5.25 buffer containing 50 mM sodium acetate, 2.5 mM EDTA, 1 mM DTT, 0.01% Triton X-100, and 1% BSA and pre-incubated with either marimastat (1 mM), 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (400 μM), E64 (100 μM), or pepstatin (4 μM) for 30 minutes. Fluorogenic substrates were then added at 10 μM final concentration and cleavage was allowed to proceed for 60 min at 37 ºC. For BALF experiments,

the pulmonary LAM model was initiated, as described in the main methods. After 13 and 19 days, mice were euthanized by isoflurane overdose, and BALF was collected as previously described, with a single 1 ml injection of cold PBS (20). Centrifugation at 8,000xg for 5 minutes was performed at 4 ºC to remove debris. BALF was incubated with a panel of 16 fluorogenic substrates (0.33 μM final concentration) at 30 μl final volume at 37 ºC for 45 min. For all *ex vivo* protease activity experiments, proteolytic cleavage of substrates was quantified by increases in fluorescence over time by fluorimeter (Tecan Infinite M200 Pro; Männedorf, Switzerland).

Recombinant protease screening

Recombinant human MMP2 (Enzo), PRSS3 (R&D Systems), NAPSA (R&D Systems) and CTSK (Enzo) were incubated with each fluorogenic peptide substrate (5 μM final concentration) at enzyme concentrations ranging from 10 pM to 10 nM and fluorescence increase over time was measured for 90 min by fluorimeter at 37 ºC. MMP2 buffer consisted of 50 mM Tris, 10 mM $CaCl_2$, 300 mM NaCl, 20 μM $ZnCl_2$, 0.02% Brij-35, and 1% BSA (pH 7.5); PRSS3 buffer consisted of 50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij-35, and 1% BSA (pH 7.5); NAPSA buffer consisted of 100 mM NaOAc, 200 mM NaCl, and 1% BSA (pH 3.5); CTSK buffer consisted of 50 mM NaOAc, 2.5 mM EDTA, 1 mM DTT, 0.01% Triton X-100, and 1% BSA (pH 5.5).

Multiplexed protein assay

105K and 105K+*Tsc2* cells were plated in 6-well plates (250,000 cells/well) and allowed to adhere for 1 day. Media was replaced with serum-free media and supernatant was collected after 1 day. Metalloprotease concentrations were quantified using a multiplexed protein assay (Eve Technologies; Calgary, Alberta, Canada).

ELISA

For quantification of pulmonary protease concentrations, BALF was collected as described above and concentrations of MMP9 (MMPT90, R&D Systems; Minneapolis, MN, USA) and MMP2 (MMP200, R&D Systems) were measured by ELISA, according to the manufacturer's protocol.

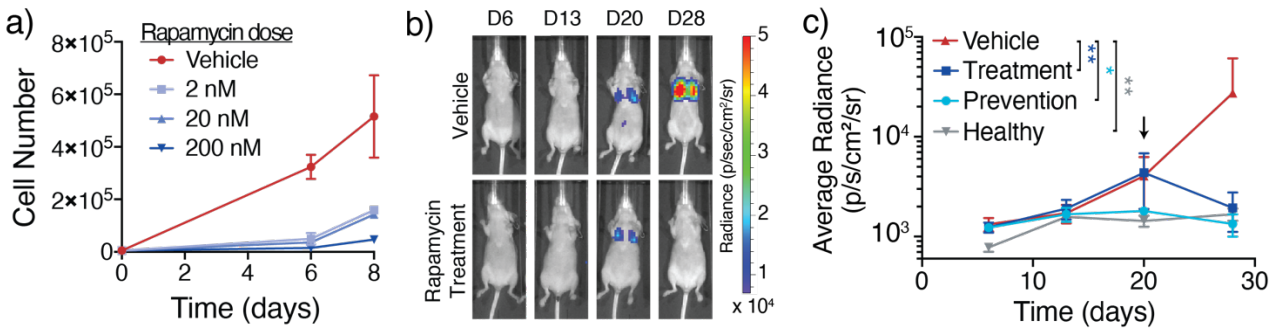

Figure S1: *Tsc2*-deficient 105K cells are responsive to rapamycin *in vitro* and *in vivo*. a) Cell growth over time is shown for 105K cells treated with various doses of rapamycin or vehicle (ethanol) (n = 2 wells each time point). b) Representative IVIS images of 105K-Luc-inoculated mice treated with either vehicle or rapamycin (1 mg/kg) 20 days after intravenous injection of 105K-Luc cells. c) Quantification of lung luminescence in 105K-Luc-injected mice treated with vehicle from day 20 ("Vehicle"), rapamycin from day 20 ("Treatment"), or rapamycin from day 0 ("Prevention"), and healthy controls ("Healthy") (n = 4 to 5 per group). Arrow denotes the time at which rapamycin was initiated in the "Treatment" group. *$P < 0.05$, **$P < 0.01$ from "Vehicle" at day 28 by Mann-Whitney test. Error bars are SD.

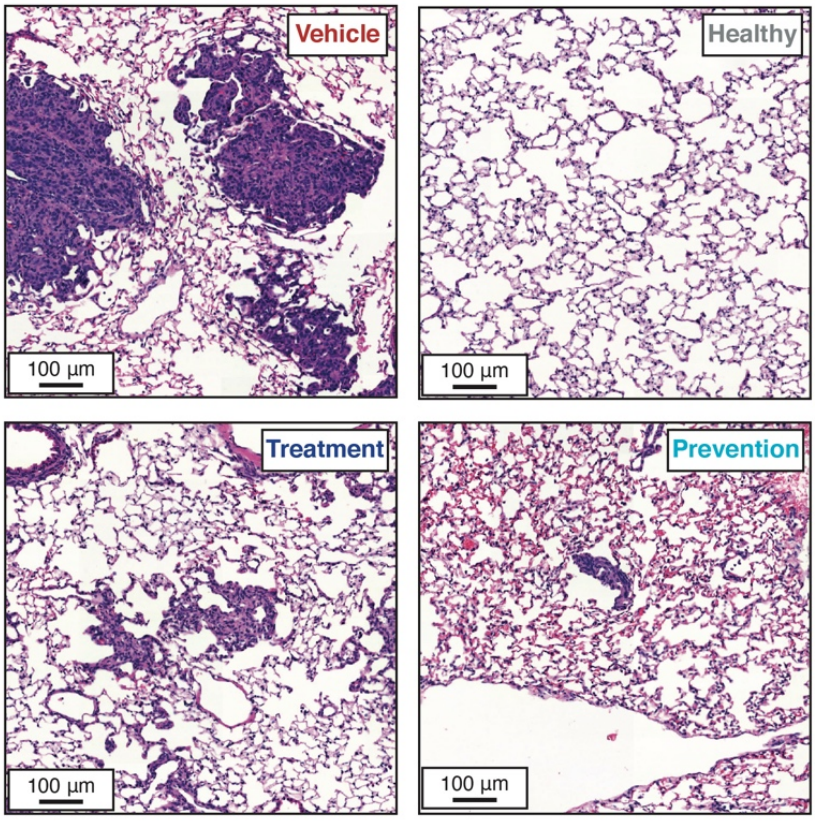

Figure S2: 105K cell lesions respond to rapamycin treatment. Representative H&E images of lungs from 105K-Luc-injected mice ("Vehicle"), healthy control mice ("Healthy"), and 105K-Luc-injected mice treated with rapamycin at either day 20 (3 mg/kg, "Treatment") or day 0 (1 mg/kg, "Prevention").

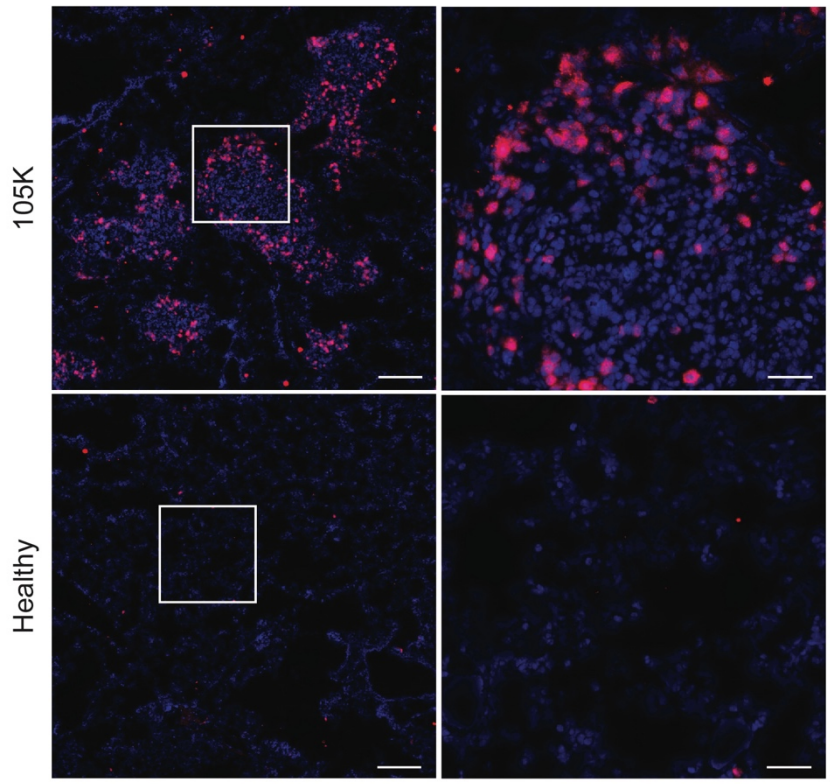

Figure S3: 105K lesions are proliferative. Immunofluorescence images of Ki-67 staining (red) in mouse lungs 19 days after intravenous injection with 105K cells (top) and in healthy control lungs (bottom). Images are shown at low (scale = 200 um, left) and high (scale = 50 um, right) magnification. Nuclei are shown in blue.

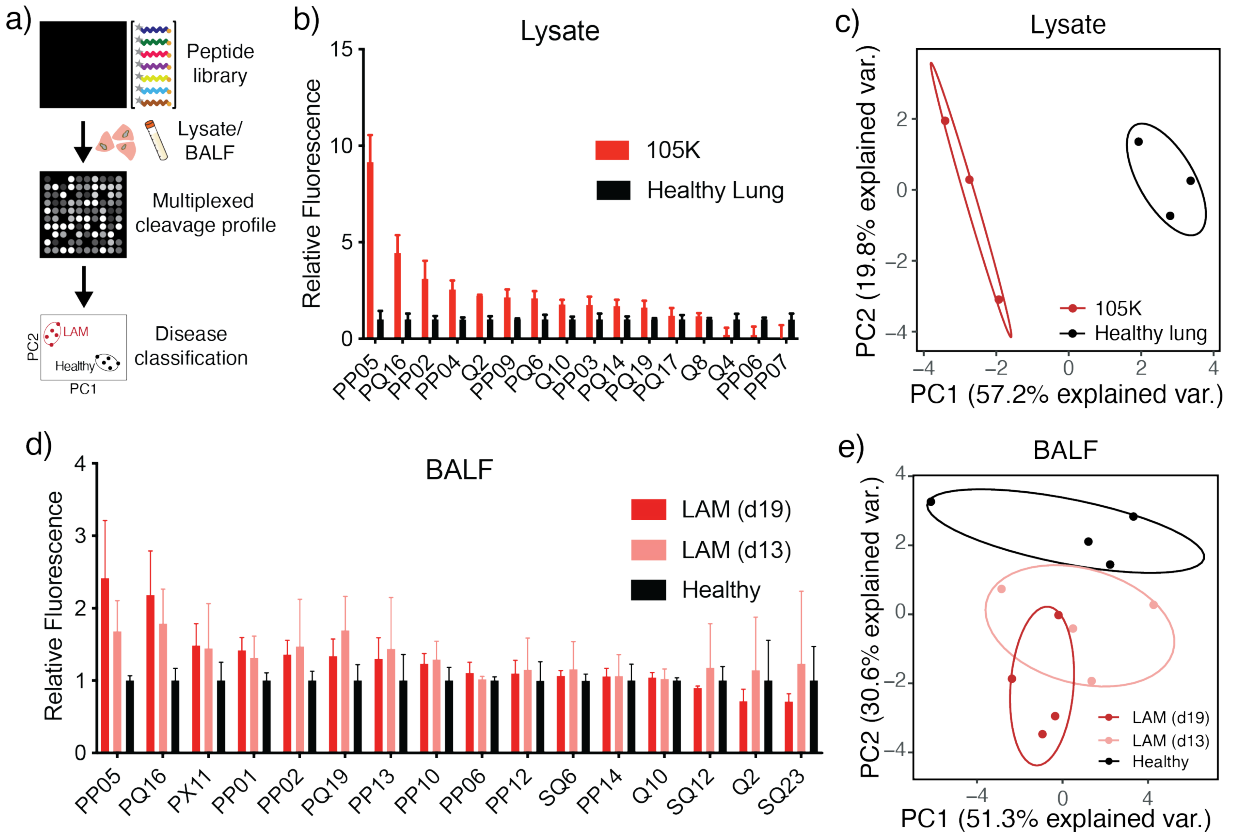

Figure S4: Protease activity is dysregulated in LAM mouse model. a) Tissue lysates and bronchoalveolar lavage fluid (BALF) were incubated with quenched fluorescent protease substrates, enabling quantification of protease activity dysregulation and assessment of global differences by PCA. b) Cleavage of peptides incubated with homogenates of 105K cell flank tumors for 30 minutes, relative to cleavage by homogenates of healthy lung. c) PCA of lysate cleavage data at 30 minutes. d) Cleavage of peptides incubated with BALF from LAM mice at day 13 (d13) or day 19 (d19) for 45 minutes, relative to cleavage by BALF from healthy mice. e) PCA of BALF cleavage data at 45 minutes.

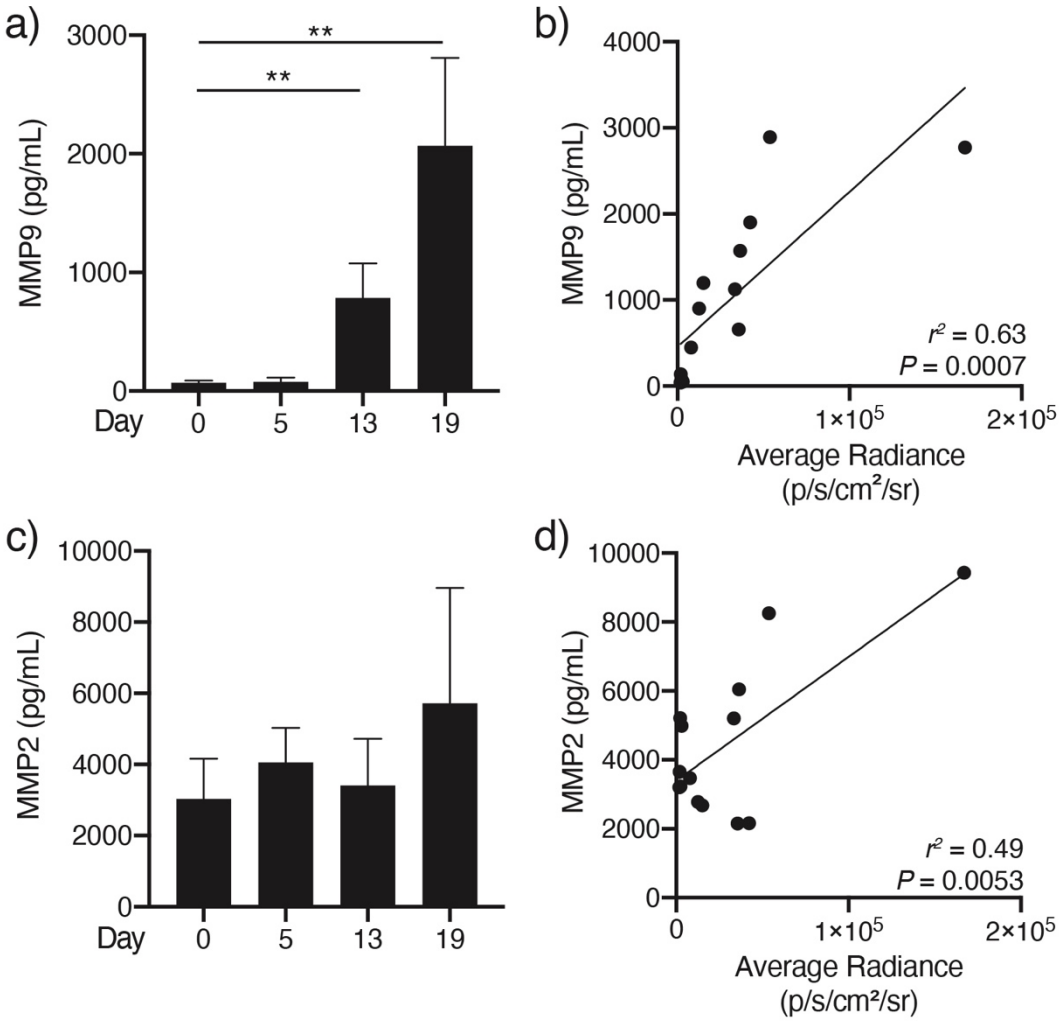

Figure S5: MMPs are dysregulated in mouse model of pulmonary LAM. a) Concentration of MMP9 (pg/ml) in BALF from mice intravenously inoculated with 105K cells. $^{**}P < 0.01$ by unpaired two-tailed *t* test. b) Linear regression of MMP9 concentration (y-axis) with disease burden as assessed by IVIS (x-axis). c) Concentration of MMP2 (pg/ml) in BALF from mice intravenously inoculated with 105K cells. d) Linear regression of MMP2 concentration (y-axis) with disease burden as assessed by IVIS (x-axis). n = 4-5 mice per time point.

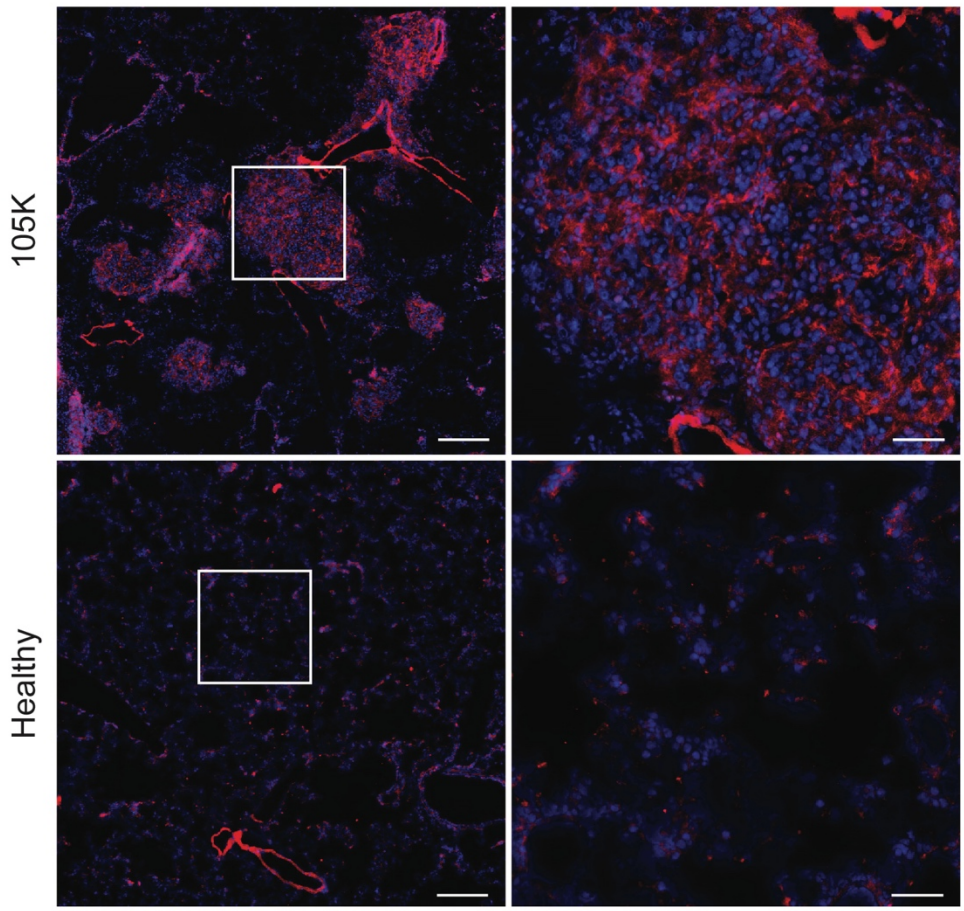

Figure S6: 105K lesions express cathepsin K. Immunofluorescence images of cathepsin K staining (red) in mouse lungs 19 days after intravenous injection with 105K cells (top) and in healthy control lungs (bottom). Images are shown at low (scale = 200 um, left) and high (scale = 50 um, right) magnification. Nuclei are shown in blue.

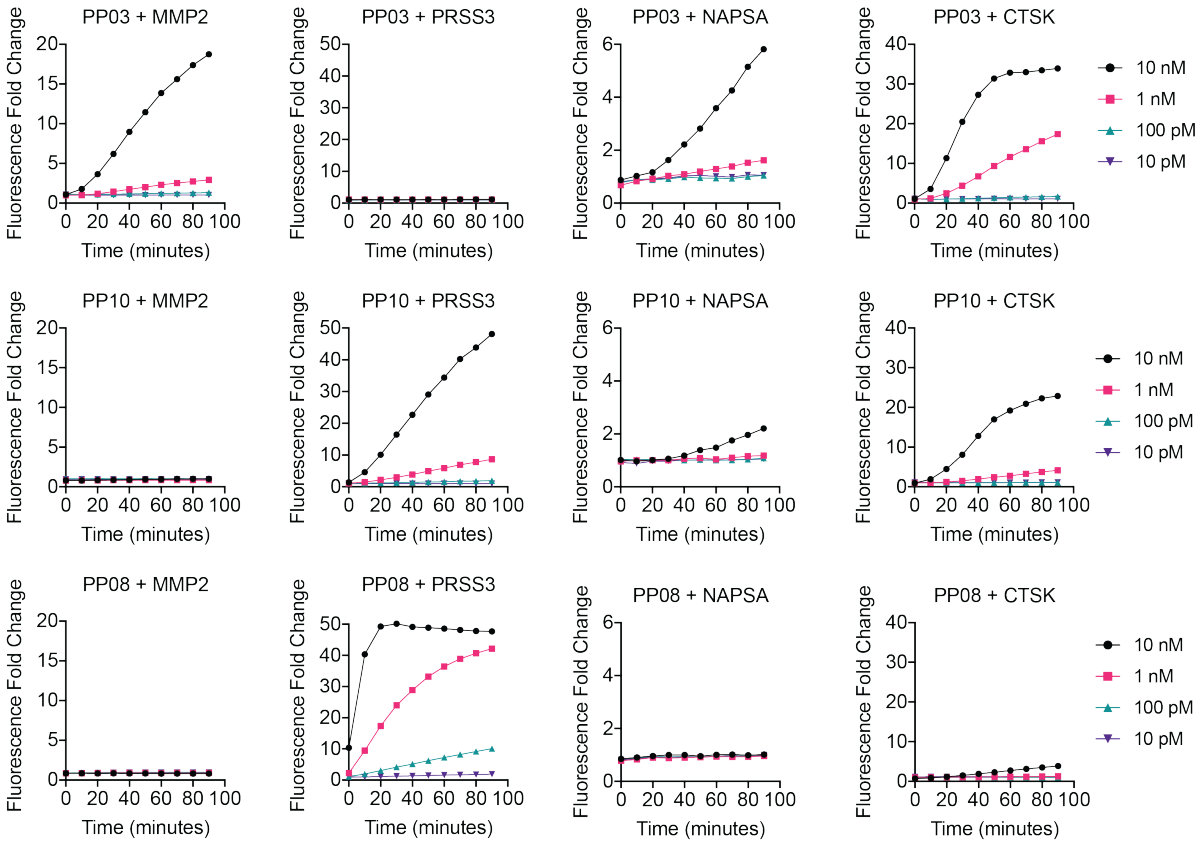

Figure S7: Protease nanosensors exhibit distinct specificities for proteases of different catalytic classes. Sample plots showing fluorescence increase over time after incubation of fluorogenic peptide substrates (PP03, top; PP10, middle; and PP08, bottom) with a metalloprotease (MMP2), serine protease (PRSS3), aspartic protease (NAPSA) and cysteine protease (CTSK) at enzyme concentrations ranging from 10 pM to 10 nM.

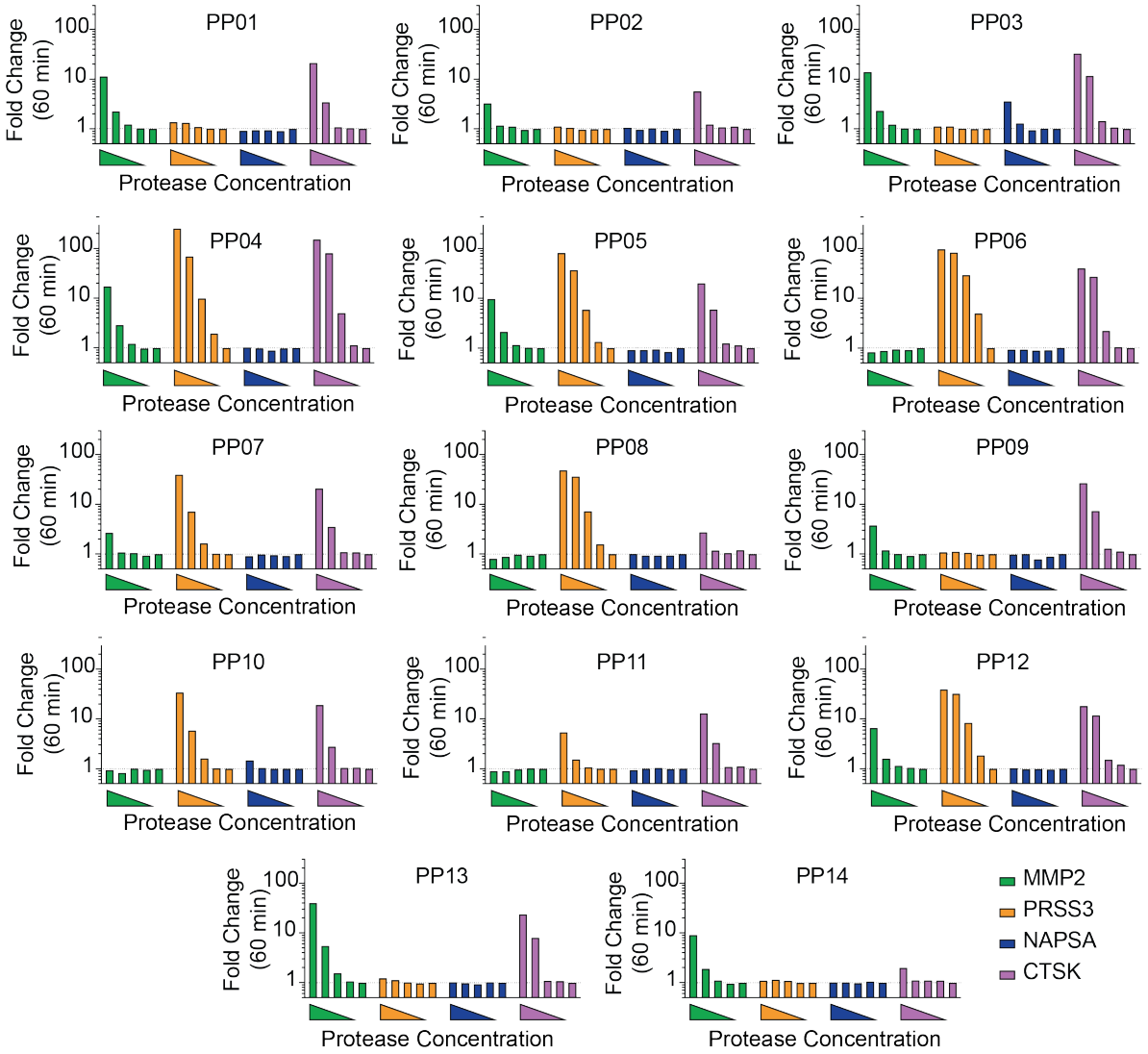

Figure S8: Protease nanosensors provide broad coverage of proteases across multiple catalytic classes. A metalloprotease (MMP2, green), serine protease (PRSS3, orange), aspartic protease (NAPSA, blue) and cysteine protease (CTSK, purple) were incubated at 10 nM, 1 nM, 100 pM, 10 pM, or 0 pM with quenched fluorescent versions of each nanosensor (PP01-14), such that fluorescence was activated in response to proteolytic cleavage. Fold change of fluorescence relative to peptide incubated without protease at 60 minutes is shown. Dotted line at fold change = 1.

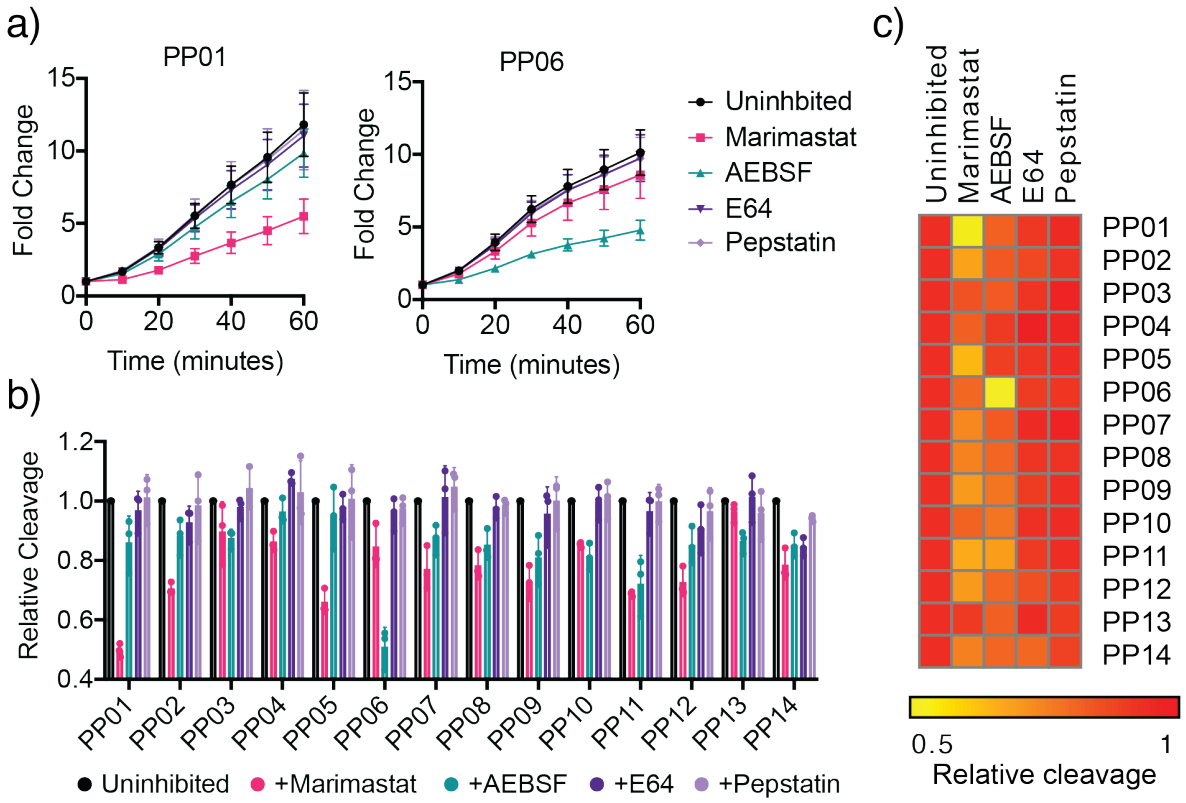

Figure S9: Metalloproteases and serine proteases are active in *Tsc2*-deficient 105K cell lesions at neutral pH. a) Fluorescence increase over time of a sample metalloprotease-sensitive (PP01, left) and serine protease-sensitive (PP06, right) peptide incubated with homogenates of 105K cell tumors at neutral pH with or without inhibitors against metalloproteases (marimastat), serine proteases (AEBSF), cysteine proteases (E64), or aspartic proteases (pepstatin). b-c) Substrate cleavage after 30 minutes in homogenates diluted in neutral pH buffer incubated with different protease inhibitor classes, relative to uninhibited homogenates.

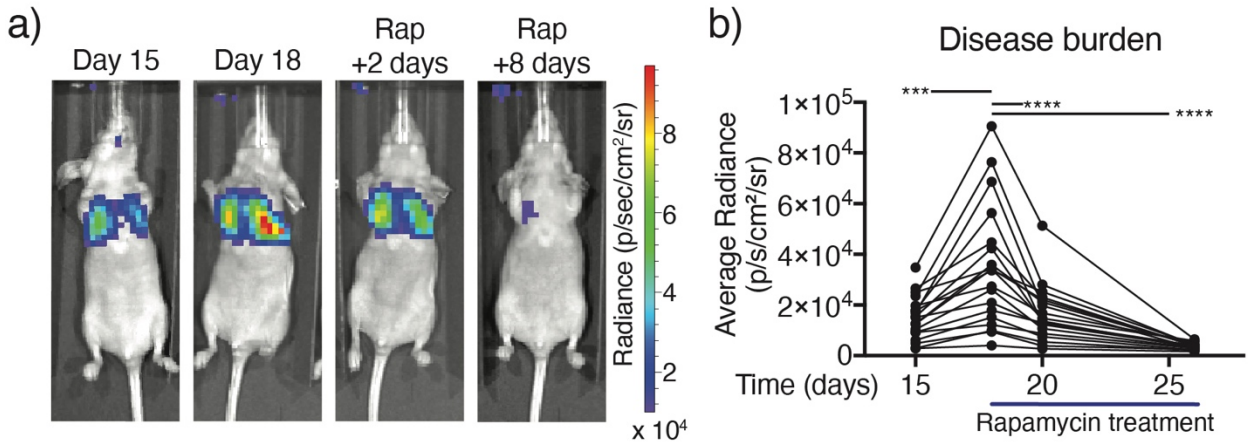

Figure S10: Rapamycin induces disease regression within two days after initiation. A) Representative IVIS images of a LAM mouse treated with rapamycin (3 mg/kg) after 18 days of disease development. B) Quantification of disease burden, as assess by IVIS imaging. ****$P <$ 0.0001 by paired two-tailed *t* test.

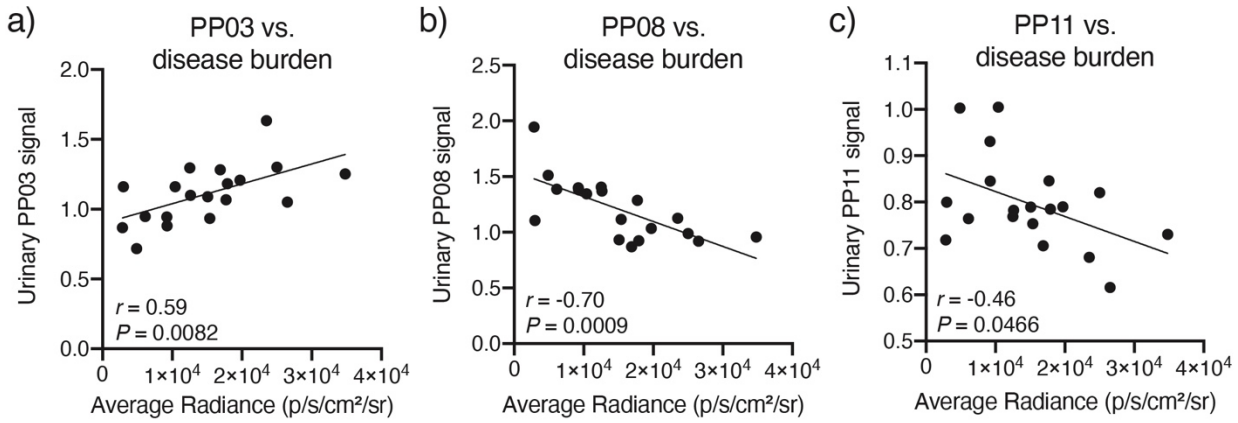

Figure S11: Multiple activity-based nanosensors correlate with disease burden. Correlation (Pearson r) of mean scaled urinary peak area ratio of PP03 (a), PP08 (b) and PP11 (c) with disease burden, as assessed by IVIS bioluminescence imaging, 14 to 15 days after 105K-Luc cell injection.

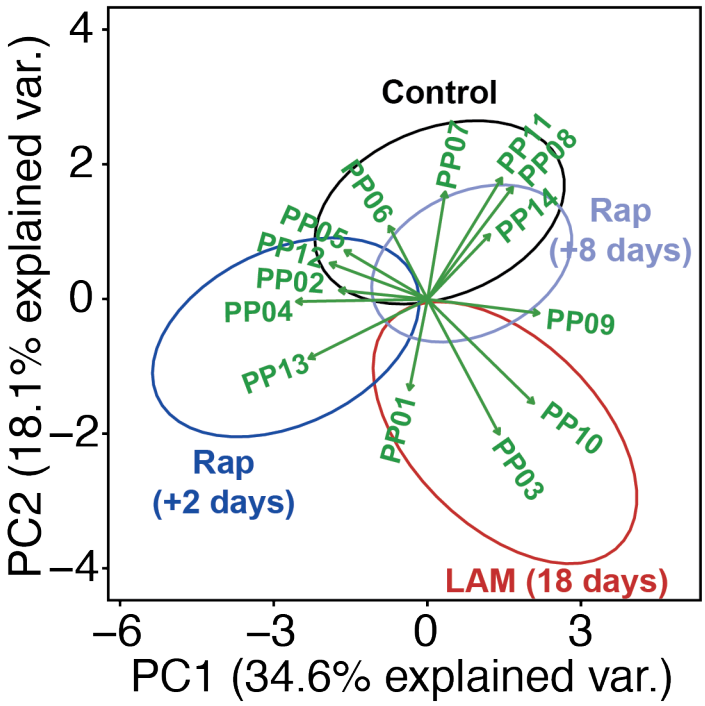

Figure S12: Disease progression and rapamycin treatment result in distinct nanosensor cleavage profiles. PCA loadings (green arrows) showing the contribution of each nanosensor to each principal component in figure 4c.

Nanosensor	Substrate sequence	Metallo-protease sensitivity (nM)	Serine protease sensitivity (nM)	Aspartic protease sensitivity (nM)	Cysteine protease sensitivity (nM)
PP01	GGPQGIWGQC	0.1	1	ND	1
PP02	GGPVGLIGC	10	ND	ND	1
PP03	GGPVPLSLVMC	0.1	ND	1	0.1
PP04	GGPLGLRSWC	0.1	0.01	ND	0.1
PP05	GGPLGVRGKC	1	0.01	ND	0.1
PP06	GGfPRSGGGC	ND	0.01	ND	0.1
PP07	GGLGPKGQTGC	10	0.1	ND	1
PP08	GGGSGRSANAKGC	ND	0.01	ND	10
PP09	GGKPISLISSGC	10	ND	ND	0.1
PP10	GGILSRIVGGGC	ND	0.1	10	1
PP11	GGSGSKIIGGGC	ND	1	ND	1
PP12	GGPLGMRGGC	1	0.01	ND	0.01
PP13	GGP-(Cha)-G-Cys(Me)-HAGC	0.01	10	ND	1
PP14	GGAPFEMSAGC	1	ND	ND	10

Table S1. Substrate sequences and protease class sensitivity. Cha, 3-Cyclohexylalanine; Cys(Me), methyl-cysteine; lowercase letters, D-amino acids; ND, no cleavage detected; Sensitivity is defined as minimum enzyme concentration required to yield a fluorescence fold change greater than 1.2 after 60 minutes.