Supporting Online Material for

Seeding and Propagation of Untransformed Mouse Mammary Cells in the Lung

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Supporting Online Material

Supplementary Table S1. **Development of lung metastases in IV recipients of mouse mammary cells upon induction of *MYC* and *Kras^{D12}* oncogenes.** For experiment #1, gross primary tumors from *TOM:TOR:MTB* animals exposed to doxycycline for 1 month, glands with many microscopic tumors from *TOM:TOR:MTB* animals exposed to doxycycline for 1 week, or phenotypically normal mammary glands from 10-week-old virgin *TOM:TOR:MTB* animals unexposed to doxycycline, or from their mono-transgenic littermates, were dissociated as described in Materials and Methods. $5x10^4$ dissociated cells were injected in the lateral tail veins of immunodeficient *Rag1$^{-/-}$* mice. Half of the recipients were placed on doxycycline 1 day before injection, and the other half did not receive doxycycline. Mice were euthanized one month after injection, and the lungs were fixed and paraffin-embedded for histological analysis. Ten sections were collected throughout the thickness of the lungs and solid tumor foci were counted. For experiment #2, the 5th pairs of mammary glands from 4-week-old *TOM:TOR:MTB* animals unexposed to doxycycline or from mono-transgenic littermates were removed by biopsy and dissociated. All of the recovered cells from individual preparations were injected into the lateral tail veins of the same animal from which the biopsy was taken. All recipients were placed on doxycycline starting 1 day before injection. One month after injection, mice were euthanized, and lungs fixed and paraffin-embedded for histological analysis. Ten sections were collected throughout the thickness of the lungs and solid tumor foci counted.

Supplementary Fig. S1: **Mammary tumors induced by coordinate expression of *MYC* and mutant *Kras* display malignant characteristics.** (A) Primary tumor cells from

TOM;TOR;MTB mice receiving doxycycline can be transplanted to immunodeficient hosts. $1x10^5$ primary tumor cells from a *TOM;TOR;MTB* donor (4 wks on doxycycline) were prepared and orthotopically injected in a fat pad containing mammary glands 2 and 3 as described in Materials and Methods. The recipient animal in the experiment shown was kept on doxycycline for 6 weeks starting one day prior to grafting. The tumor graft, but not host mammary epithelium (arrow), contains MYC protein, as shown by staining with rabbit anti-c-Myc, and displays mitotic activity (rabbit anti-pH3). Staining was performed as described in Materials and Methods. Top row, bar = 1mm. Bottom row, bar = 0.2 mm. (B) Tumor-bearing *TOM:TOR:MTB* mice develop spontaneous metastases on doxycycline. The H/E-stained lung section shows a mammary tumor focus in the lung of a *TOM;TOM;MTB* animal exposed to doxycycline for 6 weeks. Bar = 0.2 mm. (C) Primary tumor cells from *TOM;TOR;MTB* mice receiving doxycycline establish experimental metastases in immunodeficient hosts. Mammary tumor foci in the lung of a *Rag1*$^{-/-}$ female after IV injecton of $3x10^5$ dissociated primary tumor cells from a *TOM;TOR;MTB* donor (4 wks on doxycycline). The recipient animal received doxycycline for 3 weeks, starting one day prior to injection. Left panel, bar = 1mm. Right panel, bar = 0.2 mm.

Supplementary Fig. S2: **Mixed-lineage phenotype of mammary tumors induced by coordinated expression of *MYC* and mutant *Kras* is retained in experimental metastases induced from previously untransformed cells.** (A) Typical appearance of the primary mammary tumors in *TOM:TOR:MTB* animals on doxycycline (top row). Sections were stained with antisera against SMA (mouse anti-SMA), K8 (rat anti-K8, TROMA), and K6 (rabbit anti-K6) as described in Materials and Methods. Similar

findings were observed in experimental metastases in *Rag1*$^{-/-}$ immunodeficient mice (bottom row). 1x10^{6} dissociated mammary cells from doxycycline-naïve *TOM:TOR:MTB* donors were injected in lateral tail veins of a *Rag1*$^{-/-}$ recipient. Recipient was placed on doxycycline 1 day prior to injection. Bar = 0.2 mm.

Supplementary Fig. S3: **Doxycycline-dependent and mammary-specific expression of the *PyMT* transgene can be monitored by bioluminescence and results in diffuse malignant transformation of the mammary gland.** (A) Components of the tetracycline-inducible *PyMT* transgene. PCR-amplified *PyMT* DNA was cloned between a tetracycline operator sequence and the internal ribosomal entry site (*IRES*), followed by cDNA encoding firefly luciferase (*Luc*) in a *TMILA* construct obtained from Lewis Chodosh (U. Penn) (*1*). (B) Doxycycline-naïve animals do not express the *TOMT:IRES:Luc* transgene. When females from *TOMT:IRES:Luc;MTB* transgenic line are placed on doxycycline, expression of *PyMT* is accompanied by a bioluminescence signal. Representative images of a bi-transgenic *TOMT:IRES:Luc;MTB* mouse (on the left) demonstrating signal-emitting cells in areas of the mammary glands and of a bioluminescence-free mono-transgenic *TOMT:IRES:Luc* mouse (on the right). Graph: Expression of the transgene by mammary cells in doxycycline-fed *TOMT:IRES:Luc;MTB* mice after the indicated number of days was measured by bioluminescence and quantified in relative luminescence units (RLU). (n = 5, error bars represent SD). (C) When females from *TOMT:IRES:Luc;MTB* transgenic line are placed on doxycycline, expression of *PyMT* induces diffuse expansion of mammary tissue and development of palpable tumors within 3-4 weeks. Diffuse transformation observed in whole mount preparations (see Materials and Methods) of the inguinal mammary gland from *TOMT:IRES:Luc;MTB*

mice 1 and 3 weeks after beginning of a doxycycline exposure. (D) Histological progression of mammary gland transformation observed in *TOMT:IRES:Luc;MTB* mice before and 1 and 5 weeks after addition of doxycycline to the diet (Bar=0.2mm), followed by an image of spontaneous lung metastases observed in doxycycline-fed bi-transgenic animals (Bar=1mm). H/E.

Supplementary Fig. S4: **Morphological and functional mammary characteristics of ectopic epithelial cells in the lungs**. (A) Ectopic cell clusters in the lungs (outlined in blue) display mammary epithelial biomarkers. Typical appearance of ectopic cells derived from mammary glands of a *β–actin-GFP* transgenic mouse in the lung of *Rag1*$^{-/-}$ recipient. Ectopic cells stain for K8 (red, rat anti-K8, TROMA, Developmental Studies Hybridoma Bank, 1:100) and SMA (white, mouse anti-SMA, Dako, #M0851, 1:100) as well as GFP (green, rabbit-anti-GFP, 1:100). Each of the antibodies also stains cells in an intact mammary gland in *β–actin-GFP* mouse (right column). "B"- bronchus, K8 positive, "V"- vessel, SMA positive. (B-C) Ectopic epithelial structures in the lungs display functional mammary characteristics. (B) Cells from ectopic foci develop into characteristic mammary acinar structures that fluoresce green under excitation light. Collagen gels were seeded with dissociated lung preparations from animals IV-injected with mammary cells derived from *β–actin-GFP* mice 3 weeks prior to lung harvest. A heterogeneous mixture of recipient- and donor-derived cells from the lung was plated in collagen gels at high density ($2x10^4$ cells per 100 µl gel) and maintained in culture for 7-10 days under mammary gland culture conditons (see Materials and Methods) before photography under light (left) and fluorescent (right) microscopes.

Materials and methods

Generation of transgenic mice

PyMT DNA was amplified by PCR using primers containing *EcoRI* and *ClaI* sites and digested with the EcoRI and ClaI restriction enzymes. The *TMILA* vector (*1*), containing the tetracycline operator, internal ribosomal entry site, firefly luciferase coding region, and SV40 polyadenylation signal, was linearized at the *EcoRI* and *ClaI* sites located between the promoter and the luciferase sequence. The *PyMT* coding sequence was ligated into *pTetO-Luc* and released from the *TMILA* vector backbone by digestion with NotI and resuspended for pronuclear injection, which was performed by the MSKCC Transgenic Core.

Animal husbandry and genotyping

All animals were kept in specific pathogen-free housing with abundant food and water under guidelines approved by the MSKCC Institutional Animal Care and Use Committee and Research Animal Resource Center. *Rag1*$^{-/-}$ (B6.129S7-Rag1tm1Mom/J, http://jaxmice.jax.org/strain/002216.html) and *β–actin-GFP* (C57BL/6-Tg(CAG-EGFP)1Osb/J http://jaxmice.jax.org/strain/003291.html) were purchased from Jackson Labs (Bar Harbor, ME). Mice bearing the *MTB*, *TOR*, and *TOM* transgenes have been previously described (*1-3*). Doxycycline was administered by feeding mice with doxycycline-impregnated food pellets (625 ppm; Harlan-Teklad). Mice were placed on doxycycline as indicated for particular experiments. Tail DNA was isolated using the DNeasy® 96 Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. Detection of the *TOM*, *TOR* and *MTB* transgenes was performed as described previously (*1-3*) and *TOMT:IRES:Luc* transgenic mice were genotyped primers: TOMT.F: 5'-ctgctactgcacccagacaa-3'; and TOMT.R: 5'-tccgccgttttggattatac-3' (468-bp product).

Preparation of mammary cells for injection

The tissue from 2 mammary glands without mechanical dissociation (or a 5 mm tumor piece minced with a piece of abdominal adipose tissue to simulate the fat pad of the untransformed mammary gland preparation; or a minced left lobe of the lung) was placed in 5 ml digestion medium (DMEM/F12 with L-glutamine, 15 mM Hepes (Biowhittaker, #12-719Q), supplemented with 1M Hepes (Biowhittaker, #17-737E) to 25 mM final concentration, 150 U/mL Collagenase type 3 (Worthington, #CLS3), and 20 μg/mL Liberase Blendzyme 2 (Roche, #11988425001) and digested for 15-16 h at 37 °C in loosely capped 50 mL polypropylene conical tubes. The resultant organoid suspension was washed with 45 mL phosphate-buffered saline (PBS) containing Ca^{++} and Mg^{++}, pelleted at 1000 rpm for 5 min at room temperature (RT), and resuspended in 5 ml 0.25% trypsin-EDTA. After incubation for 40 min at 37ºC in loosely capped 50 mL polypropylene conical tubes, cells were washed with 45 ml DMEM/F12 with L-glutamine, 15 mM Hepes, supplemented with 1M Hepes to 25 mM final concentration and with 10% Tet System Approved FBS (Clontech, #631101). When necessary, suspensions were treated with 5-15 mg/ml DNaseI (Sigma, #D4527). Preparations with visible pieces of remaining tissue were filtered using a 100 μm cell strainer. Dissociated cells were pelleted at 1000 rpm for 5 min at RT and resuspended in PBS containing Ca^{++} and Mg^{++}, counted, and were immediately used for IV injection, tumor grafting, or mammary gland repopulation assays, or plated onto collagen coated 10 cm plates (BioCoat, #356450) in preparation for *in vitro* morphogenesis assays.

In vitro morphogenesis assay of mammary cells

One day after plating, cultured cells were freed of unattached cells by washing with PBS without Ca^{++} and Mg^{++}, and the remaining cells were treated with 1 ml 0.25% trypsin-EDTA. After cell detachment, trypsin was inactivated with 9 ml DMEM/F12 with L-glutamine, 25 mM HEPES, supplemented with 10% Tet System Approved FBS. Cells were pelleted at 1000 rpm for 5 min at RT and resuspended in PBS containing Ca^{++} and Mg^{++} and counted. Cells were mixed rapidly on ice with Cultrex 3-D Culture Matrix Basement Membrane Extract (Trevigen, #3445-048-01) containing 1.5 mg/ml Cultrex 3-D Collagen I rat tail (Trevigen, #3447-020-01). 100-μl droplets containing between 8,000-10,000 cells from mammary gland preparations, or 20,000 cells from the lung preparations were dispensed into 17-mm wells. After solidification on a level surface at RT for 15–30 min, the gels were placed at 37°C in a CO_2 incubator with 1 ml of serum-free medium supplemented with the bullet kit (Mammary Epithelial Cell Medium BulletKit, #CC-3150, contains one 500 ml bottle of Mammary Epithelial Cell Basal Medium and the following growth supplements: Bovine Pituitary Extract, 2 ml; hEGF, 0.5 ml; Hydrocortisone, 0.5, 0.5 ml; GA-1000, 0.5 ml; Insulin, 0.5 ml, Cambrex). Medium was replaced every 2 days.

Mammary fat pad repopulation assay

Mammary fat pads of 3-week-old immunodeficient *Rag1*$^{-/-}$ females were surgically cleared of the endogenous mammary epithelium as described (http://mammary.nih.gov/tools/mousework/index.html). Briefly, mice were anesthetized, skin on the abdominal area was shaved and disinfected, and mice were placed on their backs. A skin incision was made across lower abdomen between 4th and 5th nipples. Skin was lifted and separated from the fat pads on the left and the right side by blunt circular

scissor motions. Fat pads 4 and 5 were pulled slightly from the incision and separated at the bridge between the 4^{th} and 5^{th} fat pads, taking care not to damage the major blood vessel in the bridge. The tips of the 4^{th} fat pads on each side were pulled out of the skin incision and separated from the skin and the abdominal wall. The proximal part of the 4^{th} fat pads were dissected behind the inguinal lymph node, and the entire triangular portion from the bridge to the lymph node were removed and mounted on the glass slide to be stained to document successful clearance of the fat pad. Dissociated cells prepared from lungs as described above were injected into the remaining distal portion of the inguinal fat pads. 4-5 weeks after transplantation, the glands were harvested or host animals were mated. Harvested glands were mounted on a glass slide and inspected under the fluorescent microscope to document presence or absence of green fluorescence and then divided for paraffin embedding and for carmine staining as whole mounts.

Mammary tumor transplantation

Mammary tumors from *TOM:TOR:MTB* animals on doxycycline were collected and dissociated as described above. $1x10^5$ cells were injected in the surgically exposed inguinal or thoracic mammary fat pads of immunodeficient *Rag1*$^{-/-}$ animals placed on doxycycline one day prior to cell injection. The recipient animals were kept on doxycycline and monitored for tumor graft growth.

Traditional and modified experimental metastasis assays

For traditional experimental metastasis assays, mammary tumors from *TOM:TOR:MTB* animals on doxycycline were collected and dissociated as described above. The dissociated cells were injected into the lateral tail veins of immunodeficient *Rag1*$^{-/-}$ mice

placed on doxycycline one day prior to cell injection. The recipient animals were kept on doxycycline and lungs were harvested as indicated for individual experiments.

For modified experimental metastasis assay tumor-free mammary glands as described for individual experiments were dissected from female mice by biopsy or at necropsy, and dissociated as described above. For the majority of experiments, the dissociated cells (in numbers indicated for the specific experiments) were injected into the lateral tail veins of immunodeficient *Rag1*$^{-/-}$ mice. The recipient animals were either placed on doxycycline one day prior to cell injection, or 1.5, 8, or 17 weeks after the injection, as indicated for the particular experiments.

In vivo bioluminescent imaging

Mice were anesthetized with 3% isoflurane and injected retroorbitally with 50 μl of 30 mg/ml D-luciferin (Caliper Life Sciences, #XR-1001) in sterile water. Bioluminescence images were acquired with the IVIS Imaging System (Xenogen) 2–5 minutes after injection. For mammary gland imaging, mice were not shaved and acquisition time was set to 1 sec. For experimental metastasis imaging, chest hair was shaved and acquisition time was set to 120 sec. Analysis was performed using LivingImage software (Xenogen) by measurement of photon flux (measured in photons/s/cm^2/steradian) with a region of interest (ROI) drawn around the bioluminescence signal to be measured, or on a corresponding region from control mice.

Magnetic Resonance Imaging

Mice were anesthetized with 2% isoflurane oxygen gas. Respiratory-gated lung MR images were acquired on a Bruker 4.7T Biospec scanner (Bruker Biospin Inc.) using a

custom-made 36-mm quadrature birdcage coil in the Small Animal Imaging MR Core Facility at MSKCC. Initial Fast Spin Echo scout images in 3 orthogonal directions were acquired for positioning the animal. Subsequently axial and coronal multi-slice lung T1 Spoiled Recalled Gradient Echo images were acquired with a slice thickness of 0.7 mm and a slice gap of 0.2 mm. The TR (time of repetition) was approximately 247 msec and the TE (echo time) was 2.5 msec. An acquisition matrix of 256 x 192 was used, with a field of view of 3 x 2.5 cm or 3 x 2.2 cm for coronal and axial images respectively, yielding a corresponding spatial resolution of 117 x130 μm and 117 by 115 μm. Each image used 8 excitations per phase-encoding step with a total scanning time of 6.4 minutes.

Whole-lung fluorescence imaging

To observe lung seeding by cells derived from *β-actin-GFP* mice, freshly dissected, saline-perfused whole lungs were placed on a glass slide and inspected for green fluorescence under the fluorescent microscope.

Histology, immunofluorescence, and immunohistochemistry

Mammary tissues were obtained by either excision biopsy or postmortem examination according to institutional guidelines. Mammary glands and tumors were excised; some were flash-frozen in liquid nitrogen for molecular analyses, and some fixed in 10% buffered formalin for 16-48 h at room temperature, placed in 70% ethanol, and sent for paraffin embedding and sectioning (Histoserv, Inc.). Whole mount preparations were made as described previously (http://mammary.nih.gov/tools/histological/wholemounts/index.html).

For immunofluorescence, the sections were deparaffinized in Histo-Clear (Richard-Allan Scientific, #6901) and passed through graded alcohols followed by antigen retrieval with citric acid (Vector Laboratories #H-3300) in a boiling-water bath for 15 min. After two washes in PBS and permeabilization in 0.2% Triton in PBS for 45 min, sections were incubated in PBS containing 2% bovine serum albumin (BSA), 5% normal donkey serum, and 0.1% Triton for 1 h at room temperature. For mouse antibodies, Mouse-On-Mouse (M.O.M.™) kit (Vector Laboratories, #BML-2202) was applied. Sections were incubated overnight at 4°C in PBS plus 1% BSA with 1:100 dilution of the following antibodies: rabbit anti-GFP (Invitrogen, #A11122), rat anti-K8 (TROMA, Developmental Studies Hybridoma Bank), and mouse anti-SMA (Dako, #M0851). Sections were washed in PBS and the secondary antibodies conjugated to different Alexa-Fluor dyes (488, 555, 647, Invitrogen, Molecular Probes®) at a dilution of 1:500 in PBS were applied for 1 hr. After the second wash in PBS, 4',6-diamidino-2-phenylindole (DAPI, 5 µg/ml in PBS) was used for nuclear counter stain, sections were mounted with 70% glycerol, and inspected and photographed under fluorescent microscope.

For 5-bromo-2-deoxyuridine (BrdU) labeling assay, mice were injected intraperitoneally with 100 mg/kg BrdU one hour prior to sacrifice. Immunohistochemistry was performed with Vectastain® Avidin: Biotinylated enzyme Complex (ABC) and M.O.M.™ kits (Vector Laboratories, #BMK-2202) according to the manufacturer's instructions. The following antibodies were used: anti–phospho (Ser10) histone H3 (Cell Signaling Technology, #9701, 1:200 dilution,); rat anti-BrdU (Serotec, #MCA2060, 1:400 dilution); rabbit anti-c-Myc (Cell Signaling Technology, #9402, 1:100 dilution), rabbit anti-GFP (Invitrogen, #A11122, 1:300 dilution); rat anti-K8 (TROMA, Developmental Studies Hybridoma Bank, 1:400 dilution); mouse anti-SMA (Dako, #M0851, 1:100 dilution).

To observe intratumoral vasculature, anesthesized mice were injected retroorbitally with biotin-conjugated lectin (Vector Laboratories, #B-1175) and sacrificed in 3 min. Lungs were perfused with PBS, fixed in formalin and paraffin embedded. Sections were stained with Vectastain® ABC kit (Vector Laboratories).

Table S1. **Development of lung metastases in IV recipients of mouse mammary cells upon induction of *MYC* and *Kras*^{D12} oncogenes.**

Source of donor cells	Donor receiving doxycycline	Recipient mouse	Recipient receiving doxycycline	Cell number injected	Number of tumor foci in 10 serial sections of lung
Experiment #1					
TOM;TOR;MTB tumors	1 month	*Rag1*$^{-/-}$	yes	5x10^4	46
					14
			no	5x10^4	0
					0
TOM;TOR;MTB tumors	1 week	*Rag1*$^{-/-}$	yes	5x10^4	49
					6
			no	5x10^4	0
					0
TOM;TOR;MTB mammary gland	no	*Rag1*$^{-/-}$	yes	5x10^4	12
					25
			no	5x10^4	0
					0
wt mammary gland	no	*Rag1*$^{-/-}$	yes	5x10^4	0
					0
			no	5x10^4	0
					0
Experiment #2					
TOM;TOR;MTB mammary gland	no	self	yes	105000	48
				47500	14
				36250	0
				21250	7
				20000	12
				10000	0
				10000	0
wt mammary gland	no	self	yes	101250	0
				107500	0
				76250	0
				31250	0
				30000	0
				27500	0
				23750	0
				13750	0
				10000	0
				10000	0
				6250	0
				5000	0

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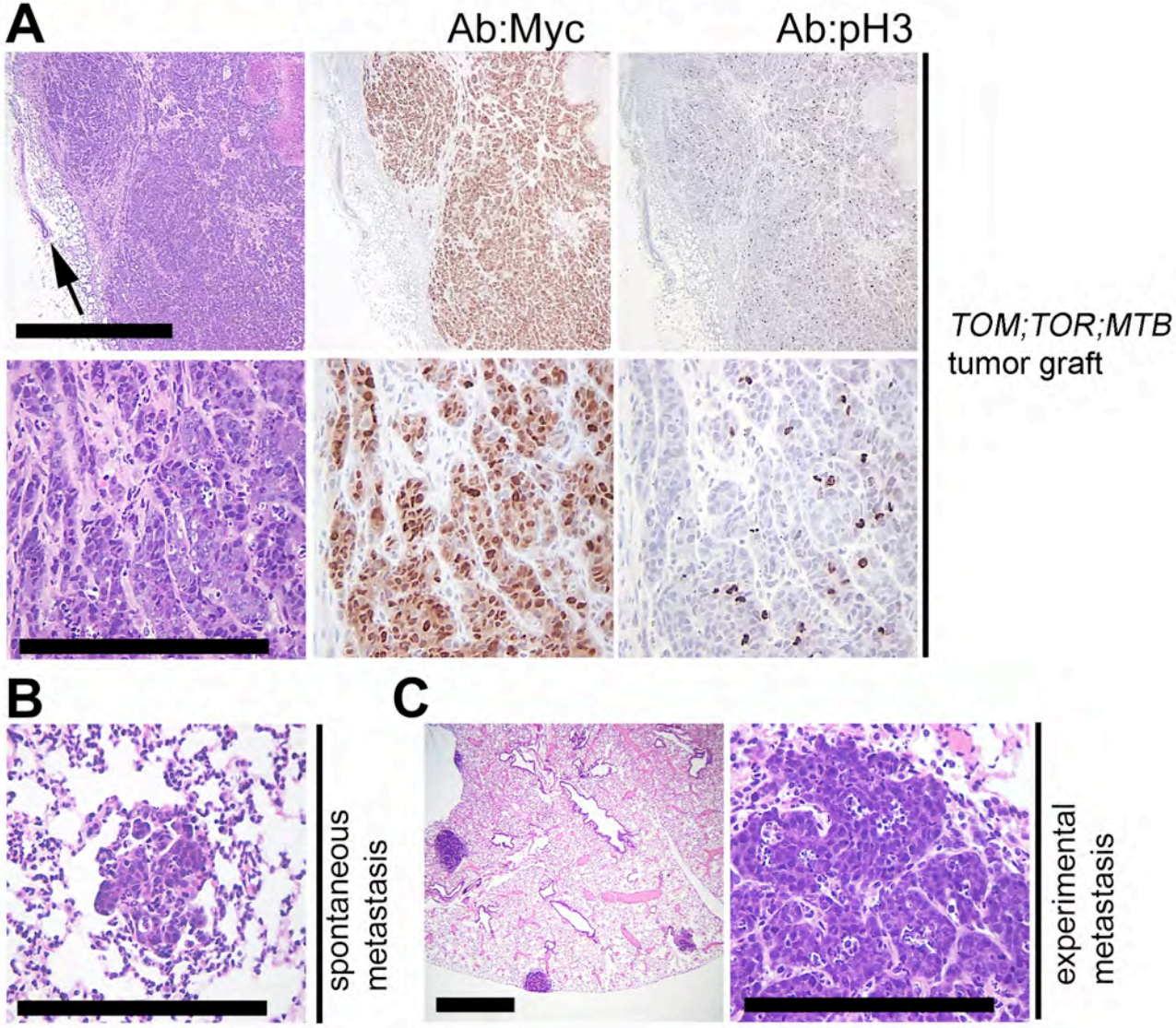
Podsypanina, Supplementary Fig.S1



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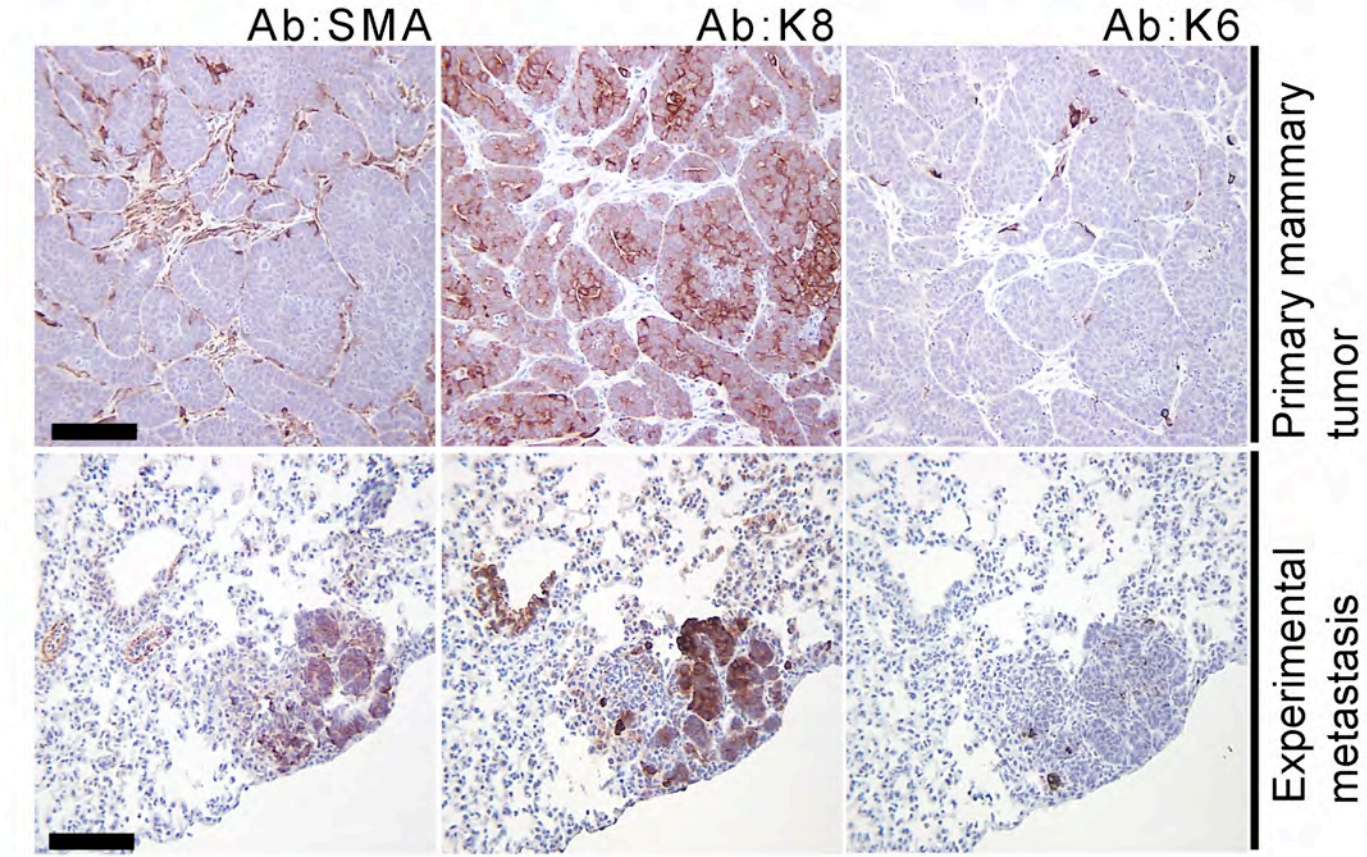

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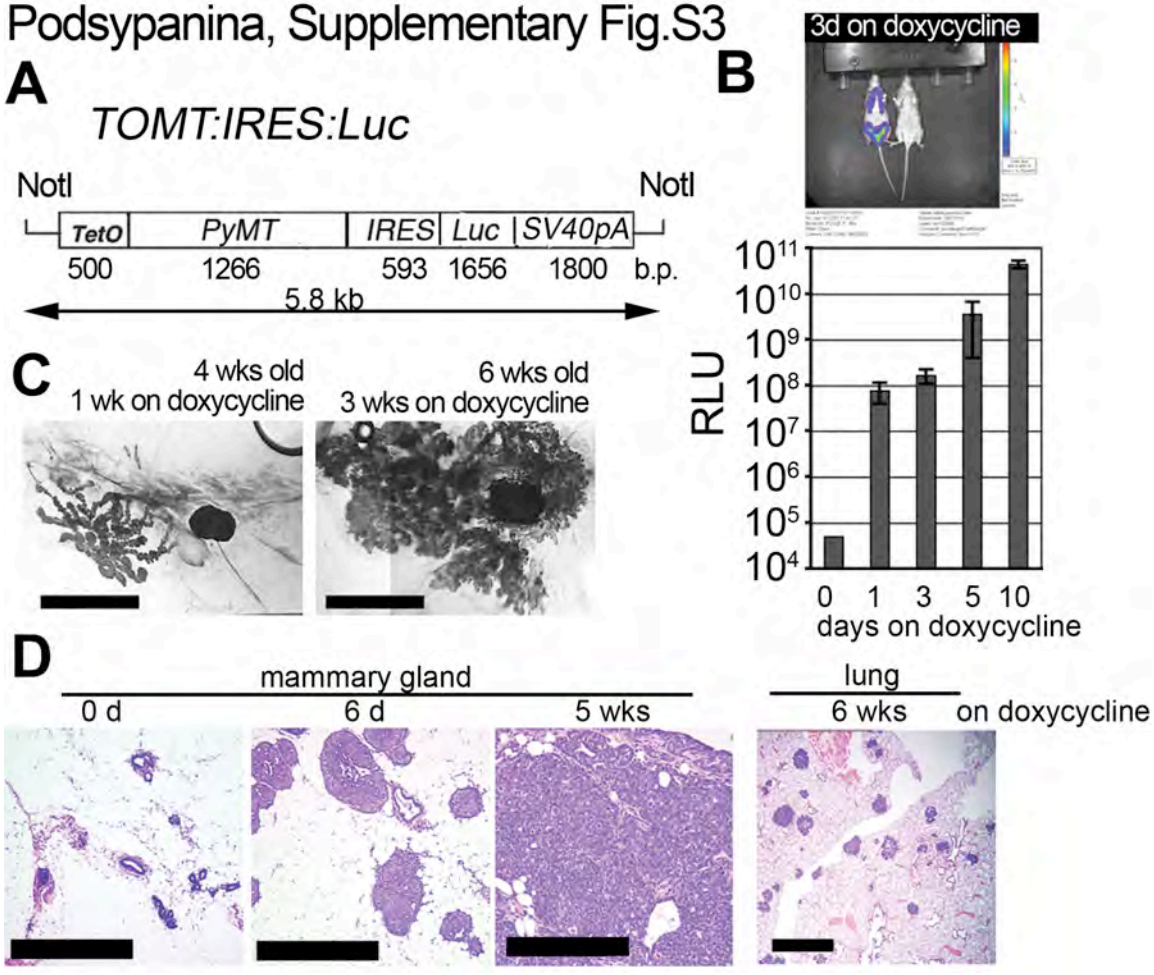

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