PNAS
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Supplementary Information for

PtdIns(3,4,5)P_3-dependent Rac exchanger 1 (P-Rex1) promotes mammary tumor initiation and metastasis.

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www.pnas.org/cgi/doi/10.1073/pnas.2006445117

Supplementary Materials and Methods

Materials

Antibodies were from: pERK1/2 (4377), pAKT (Thr^{308}, 2965), pS6 ($Ser^{235/236}$, 4858), cleaved caspase 3 (Asp^{175}, 9664), p4EB-P1 ($Thr^{37/46}$, 2855), cyclin D1(2978), ErbB2 (2165), P-Rex1 (rabbit monoclonal, 13168) and pERM (3141) Cell Signaling Technology (Boston, MA), HA (MMS-101) Covance (Princeton, NJ), Ki67 (RM-9106-SO), GAPDH (AM4300) ThermoFisher Scientific (Waltham, MA), E-cadherin (610181) BD Biosciences (San Jose, CA), CK8 (2031-1) Epitomics, CK14 (ab7800) Abcam (Cambridge, MA), GM130 Sigma (G7295), PyMT (DP10L) Calbiochem (San Diego, CA) and Myc (A190-104A) Bethyl Laboratories (Montgomery, TX), HRP-conjugated secondary antibodies (Merck Millipore, Burlington, MA), fluorescently labelled secondary antibodies and Alexa-Fluor 488 phalloidin Molecular Probes (ThermoFisher Scientific). P-Rex1 antibody (rabbit polyclonal) was generated in house (1). DAPI was from Sigma-Aldrich (Merck Millipore, Burlington, MA).

Quantitative Real-Time Polymerase Chain Reaction

RNA was extracted using an Isolate II RNA kit (Bioline, London, UK) according to the manufacturer's instructions. cDNA synthesis and qRT-PCR was performed using QuantiTect Reverse Transcription and QuantiTect SYBR® Green PCR Kits (Qiagen, Venlo, Netherlands), according to the manufacturer's instructions.

Droplet digital Polymerase Chain Reaction

Droplet digital PCR was performed using a Bio-Rad QX100 Droplet Digital PCR system. 20 µl reactions containing 1x ddPCR EvaGreen Supermix, 1x primers (QuantiTect Primer Assay, Qiagen) and 8.3 ng/µl cDNA were loaded into the middle wells of a droplet generator cartridge and 70 µl Droplet Generation Oil for EvaGreen (Bio-Rad) into the lower wells then individual droplets generated. 40 µl of droplets were transferred to a 96-well PCR plate, sealed and subjected to thermal cycling. After PCR, detection of the completed reactions in individual droplets was detected using a droplet reader. The data was analyzed using QuantaSoft software (Bio-Rad) with the thresholds for detection set manually based on the results from the no template control well.

Immunoblotting

Total cell lysates obtained by direct cell lysis in SDS-PAGE sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue) were separated by 7.5% SDS-PAGE followed by immunoblotting. Signals were visualized by enhanced chemiluminescence. Commercial antibodies were utilized according to the manufacturer's instructions. Rabbit polyclonal P-Rex1 antibodies were utilized at 1:1000 dilution.

Immunohistochemistry

5 µm sections from formalin fixed, paraffin embedded (FFPE) tissues/organoids were dewaxed in three changes of xylene then rehydrated in three changes of ethanol. Antigen retrieval was performed in a pressure cooker for 10 min in Novocastra Epitope Retrieval solution pH 9 (Leica Microsystems, Wetzler, Germany) or 10 mM citrate buffer pH 6. Sections were incubated in 1% BSA/50 mM Tris pH 8/150 mM NaCl blocking buffer for 1 h then incubated with primary antibodies diluted in blocking buffer overnight at 4°C. Sections were washed thrice with 50 mM Tris pH 8/150 mM NaCl then endogenous peroxidase activity was quenched with 0.3% hydrogen

peroxide for 10 min prior to incubation with EnVision^{+} HRP-conjugated antibodies and immunoreactivity detection by DAB staining (Dako Agilent, Santa Clara, CA). Sections were counterstained with hematoxylin and imaged with an Olympus Provis or dotSlide light microscope.
For immunofluorescence staining, sections were washed thrice with 50 mM Tris pH 8/150 mM NaCl after incubation with primary antibody then incubated in fluorescent-conjugated secondary antibodies for 1 hour in the dark at room temperature. Sections were washed thrice, counterstained with DAPI (Sigma-Aldrich) and imaged using a Nikon Upright or Leica Microsystems SP5 confocal microscope.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assay
5 μm sections from FFPE tissues were dewaxed in three changes of xylene then rehydrated in three changes of ethanol. Apoptotic cells were identified using the *In situ* Cell Death Detection Kit, Fluorescein (Roche, Sigma-Aldrich) according to the manufacturer's instructions. Cell nuclei were co-stained with DAPI (Sigma-Aldrich). Cells were imaged on a Nikon C1 confocal microscope.

Mammary fat pad Carmine alum staining
Murine inguinal mammary fat pads were spread onto Superfrost slides and fixed overnight with Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid). Mammary fat pad whole mounts were stained with Carmine Alum (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions then cleared in xylene and coverslips mounted with Permount (Thermo Scientific). Slides were imaged with an Olympus dotSlide microscope.

Morphometric analysis
Ductal invasion was determined by measuring the mean length of the three longest ducts of each mammary gland from the branch point closest to the nipple to the ends. Data were expressed as a percentage of the length to the end of the mammary fat pad. Branching was assessed by scoring the total number of branch points in a box of defined area next to the lymph node, distal to the nipple. Mice were assessed for estrus cycle stage by vaginal cytology (2) and mice in diestrus were excluded from this analysis.

Mammary tumor latency and growth
MMTV-*neu* and MMTV-*neu;PREX1* transgenic mice were monitored once per week for tumor incidence by physical palpation. Palpable tumors were calliper measured 3 times per week. Tumor volume was determined by: (width^2 x length)/2. Mice were determined to be no longer tumor-free once the primary tumor reached 100-200 mm^3. Tumor-free survival was assessed by Kaplan-Meier analysis of the whole cohort. The Log-rank test was performed to determine median time to tumor and statistical significance. Mice were sacrificed when tumors reached 1000-1500 mm^3 in size or when moribund.

Lung metastases
10 μm sections from FFPE lungs were dewaxed and stained with haematoxylin and eosin. The number of metastases were scored in 5 lung sections at least 200 μm apart per mouse as described (3).

FLIM-FRET imaging of Rac1 activity

FLIM-FRET imaging was performed as described (4-6). Multiphoton time correlated single photon counting (TCSPC) FLIM was performed on a Nikon Eclipse TE2000-U inverted microscope with an Olympus long working distance 20x 0.95 NA water immersion lens. A Titanium:Sapphire femtosecond pulsed laser (Chameleon) was used as an excitation source at 840 nm wavelength. A dichroic filter (Chroma 455 nm) was used to separate the second harmonic generation signal (SHG) from the ECFP donor fluorescence and passed through band pass filters (Semrock 435/60 and 460/60 respectively). The signal was detected by non-descanned detectors (Hamamatsu) and for FLIM a 16-anode PMT (FLIMx16, LaVision Biotech) was used for TSCPC. Fluorescent lifetimes were analyzed using ImSpectorPro (Versions 262+, LaVision Biotech) by selecting ROIs around single cell membranes and recording the τ of a fitting routine to the florescence decay data. Lifetime maps were further generated with intensity thresholds set to the average background pixel values for each recording, smoothed 3 x 3 and a standard rainbow color look up table (LUT) applied with bounds from 1.0 – 3.0 ns. Rac1 activity is displayed by blue to green colored regions, inactivity by yellow to red and areas where no lifetime measurements above the background could be achieved are displayed in black.

MCF-10A cell and acini culture

MCF-10A cells (ATCC, Manassas, VA) were maintained in acini growth media (DMEM supplemented with 10% FCS (Invitrogen), 5 μg/ml insulin, 10 ng/ml EGF, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin). MCF-10A acini were cultured as described (7). Trypsinized MCF-10A cells were washed with DMEM/F12 + 20% (v/v) horse serum, 2 mM L-glutamine, 100 units/ml penicillin, 1% (v/v) streptomycin then suspended in acini growth media (DMEM/F12 + 2% (v/v) horse serum, 2 mM L-glutamine, 100 units/ml penicillin, 1% (v/v) streptomycin, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, 5 ng/ml EGF, 2% (v/v) Matrigel) ± 1 μg/ml doxycycline. MCF-10A cells were plated at 5000 cells/well in 400 μl acini growth media ± 1 μg/ml doxycycline and incubated in a 5% CO_2 humidified 37°C incubator for 7, 14 or 21 days. Media was replaced every 4-5 days.

MCF-10A cell transduction

HA-tagged PREX1 was subcloned into the pLVX-TRE3G-mCherry (Clontech, Mountain View, CA) vector and lentiviral particles generated in HEK293T cells using a Lenti-X HTX packaging mix (Clontech). MCF-10A cells (ATCC, Manassas, VA) were transduced with lentiviral transduction particles containing a pLVX-Tet3G plasmid encoding the Tet-On® 3G transactivator protein and a pLVX-TRE3G plasmid encoding either mCherry alone or HA-PREX1 at an MOI of 1 in growth media + 8 μg/ml hexadimethrine bromide overnight at 37°C. Transduced cells were selected in growth media supplemented with 1 μg/ml puromycin/600 μg/ml G418 then subsequently maintained in growth media containing 0.5 μg/ml puromycin/300 μg/ml G418.

MCF-10A acini immunofluorescence staining

Staining was carried out as described (7). Acini were fixed with 4% (w/v) paraformaldehyde for 30 minutes, and permeabilized in 130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 0.5% (w/v) Triton X-100 pH 8.0 in distilled H_2O for 20 minutes at 4°C. Samples were rinsed three times with 100 mM glycine in phosphate buffered saline (PBS) for 10 mins each, and blocked for 90 mins with primary block (10% goat serum in IF buffer: 130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 7.7 mM NaN_3, 0.1% (w/v) bovine serum albumin, 0.2% (v/v) Triton X-100, 0.05%

(v/v) Tween-20) then 40 mins with secondary block (10% goat serum, 20 μg/ml goat anti-mouse F(ab')2 fragment in IF buffer). Acini were incubated with primary antibodies diluted in secondary block overnight at 4°C, washed three times with IF buffer 20 mins each, then incubated with fluorescent secondary antibodies and Alexa-Fluor 488 phalloidin (1:200) diluted in primary block for 50 mins. Acini were washed three times with IF buffer for 20 mins each. Nuclei were stained by incubating acini in 2 μg/ml DAPI in PBS for 15 minutes. Acini were rinsed in PBS and coverslips mounted with Fluoromount (ThermoFisher Scientific). Acini were imaged using a Nikon upright fluorescent confocal microscope.

Mammary organoid culture

Mammary organoids were isolated and cultured as described (8). Fourth mammary fat pads of ~12 week old virgin mice were minced with a scalpel blade then incubated in 10 ml digestion solution (DMEM/F12 + 300 U/ml collagenase III, 0.25% (v/v) trypsin, 5% (v/v) FCS, 50 μg/ml gentamycin) at 37°C for 30 mins in an orbital shaker to isolate epithelial fragments. Fragments were enriched by centrifugation at 1250 xg for 10 mins and the top layer (including the fat layer) was transferred to a fresh tube containing 5 ml DMEM/F12 + 5% (v/v) FCS. 5 ml DMEM/F12 + 5% (v/v) FCS was also added to the bottom layer. Fragments were resuspended by vigorous pipetting then centrifuged at 1250 xg for 10 mins. Supernatants were aspirated and fragments from both layers were resuspended in a final volume of 5 ml DMEM/F12 + 5% (v/v) FCS. Fragments were transferred to a 10 cm dish and incubated in a 5% CO_2 humidified 37°C incubator for 30 mins to allow fibroblast attachment. After gently shaking the dish, the fragment-containing media was transferred to a 15 ml tube and centrifuged at 1250 xg for 10 mins. The pellet was resuspended in 2 ml PBS + 2% (v/v) FCS then 8 ml RBC lysis buffer (PBS + 0.8% (w/v) NH_4Cl, 0.1 mM EDTA) was added, fragments were incubated for 2 mins and centrifuged at 1250 xg for 2 mins. The supernatant was aspirated down to ~200 μl and the fragments resuspended. Isolated organoids were mixed with Matrigel at ~10,000 cells/50 μl of matrix and seeded into 24-well plates. The Matrigel was polymerized at 37°C for 30 mins then overlayed with organoid media (DMEM/F12 + 2 mM L-glutamine, 100 units/ml penicillin, 1% (v/v) streptomycin, 20 ng/ml EGF, 20 ng/ml FGF2, 1x ITS, 10 μM Y27632 (only added for the first 4 days in culture), 2.5% (v/v) R-spondin1 conditioned medium) and cultured in a 5% CO_2 humidified 37°C incubator. Mammary organoids were maintained in culture by passaging every 2-3 weeks. Organoids were treated with trypsin then centrifuged at 200 xg for 5 mins. Mammary cells were resuspended in Matrigel (10,000 cells/50 μl) and seeded in 24-well plates as above without the addition of Y27632.

Mammary organoid branching assay

Mammary fragments were isolated as for mammary organoid culture described above, then resuspended in a mixture of Matrigel (30%) and collagen I (70%)(Corning, Corning, NY). Prior to use, collagen I was neutralized to pH 7 using 1 M NaOH and left on ice for 1 hour to allow fibre formation. After plating, the matrix was polymerized at 37°C for 30 mins then overlayed with organoid media (DMEM/F12 + 2 mM L-glutamine, 100 units/ml penicillin, 1% (v/v) streptomycin, 20 ng/ml EGF, 20 ng/ml FGF2, 1x ITS, 10 μM Y27632 (only added for the first 4 days in culture), 2.5% R-spondin1 conditioned medium) and cultured in a 5% CO_2 humidified 37°C incubator. Organoids were cultured for 9 days to allow branch formation and elongation. For wild-type organoids, branches were defined as extensions >100 μm in length measured from the centre of the organoid. For MMTV-*PREX1*^{L1} organoids, branches were defined as extensions >100 μm in length to account for the increased organoid size.

Tumoroid culture
Tumor cells isolated from MMTV-*neu* or MMTV-*neu;PREX1* mouse mammary tumors as above were suspended in Matrigel as single cells at 10,000 cells/50 µl of Matrigel. Tumoroids were maintained in culture in the same manner as mammary organoid culture described above. For experiments involving tumoroid growth in the presence of inhibitors, NSC23766 or U0126 was added to the growth media at the specified concentration. Experiments on MMTV-*neu;PREX1*^{L1} *versus* MMTV-*neu* and MMTV-*neu;PREX1*^{L2} *versus* MMTV-*neu* tumoroids were conducted independently.

Mammary organoid and tumoroid fixation and staining
Organoids and tumoroids grown in Matrigel were fixed in 0.2% (w/v) paraformaldehyde in PBS at 4°C for 48 hours with gentle agitation then collected via centrifugation at 200 xg for 5 mins. Organoid pellets were resuspended in 50-75 µl of Histogel (ThermoFisher Scientific) and embedded in blocks of agarose. The Histogel was left on ice for 30 mins to set then fixed in 10% formalin overnight, and paraffin embedded. Blocks containing organoids were sectioned and stained according to the immunohistochemistry and immunofluorescence protocols described above.

Tumoroid invasion assay
Tumor cells isolated from MMTV-*neu* and MMTV-*neu;PREX1* mammary tumors were resuspended as single cells in collagen I. Prior to use, collagen I was neutralized to pH 7 with 1 M NaOH and incubated on ice for 1 hour to allow fibre formation. The matrix was polymerized at 37°C for 30 mins then overlayed with organoid media (DMEM/F12 + 2 mM L-glutamine, 100 units/ml penicillin, 1% (v/v) streptomycin, 20 ng/ml EGF, 20 ng/ml FGF2, 1x ITS, 10 µM Y27632 (only added for the first 4 days in culture), 2.5% (v/v) R-spondin1 conditioned medium) ± NSC23766 or U0126 and cultured in a 5% CO_2 humidified 37°C incubator. Tumoroids were cultured for 21 days to allow development of invasive phenotypes. Invasive tumoroids were defined as having at least several disseminated cells surrounding the tumoroid body. Experiments on MMTV-*neu;PREX1*^{L1} *versus* MMTV-*neu* and MMTV-*neu;PREX1*^{L2} *versus* MMTV-*neu* tumoroids were conducted independently.

Primary mammary tumor cell culture
Mice were humanely killed and mammary tumors removed aseptically. Tumors were finely minced with a scalpel blade and suspended in 5 ml DMEM supplemented with 10% (v/v) FCS, 2 mg/ml BSA, 0.9 U/ml collagenase D (Roche). Tumor cell suspensions were incubated at 37°C for 3 h with gentle agitation then centrifuged at 230 xg for 5 min. Pelleted cells were resuspended in DMEM supplemented with 10% (v/v) FCS, 5 µg/ml insulin (Sigma-Aldrich), 10 ng/ml EGF (Life Technologies), 500 ng/ml hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), 50 U/ml Nystatin (Sigma-Aldrich). Cell suspensions were plated into 3 x 10 cm dishes and incubated at 33°C. Cells were maintained in DMEM supplemented with 10% FCS, 5 µg/ml insulin, 10 ng/ml EGF, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin and incubated at 37°C.

Transwell cell migration/invasion assays
5 x 10^4 primary tumor cells were seeded into the top chamber of a Transwell in serum-free DMEM in duplicate. Cells were incubated at 37°C for 24 h to allow migration towards DMEM, 10% (v/v) FCS. A cotton swab was used to remove non-migrated cells from the upper chamber surface. Cells

that had migrated to the underside of the upper chamber were fixed and stained with a DiffQuick Staining Kit (Lab Aids P/L, Australia) then imaged using a ×20 objective on an Olympus CKX41 light microscope. The number of migrated cells was scored from 6 fields/Transwell. For invasion assays, the top chamber of the Transwell was coated with 50 µl of 1 mg/ml growth factor reduced Matrigel in DMEM prior to cell seeding.

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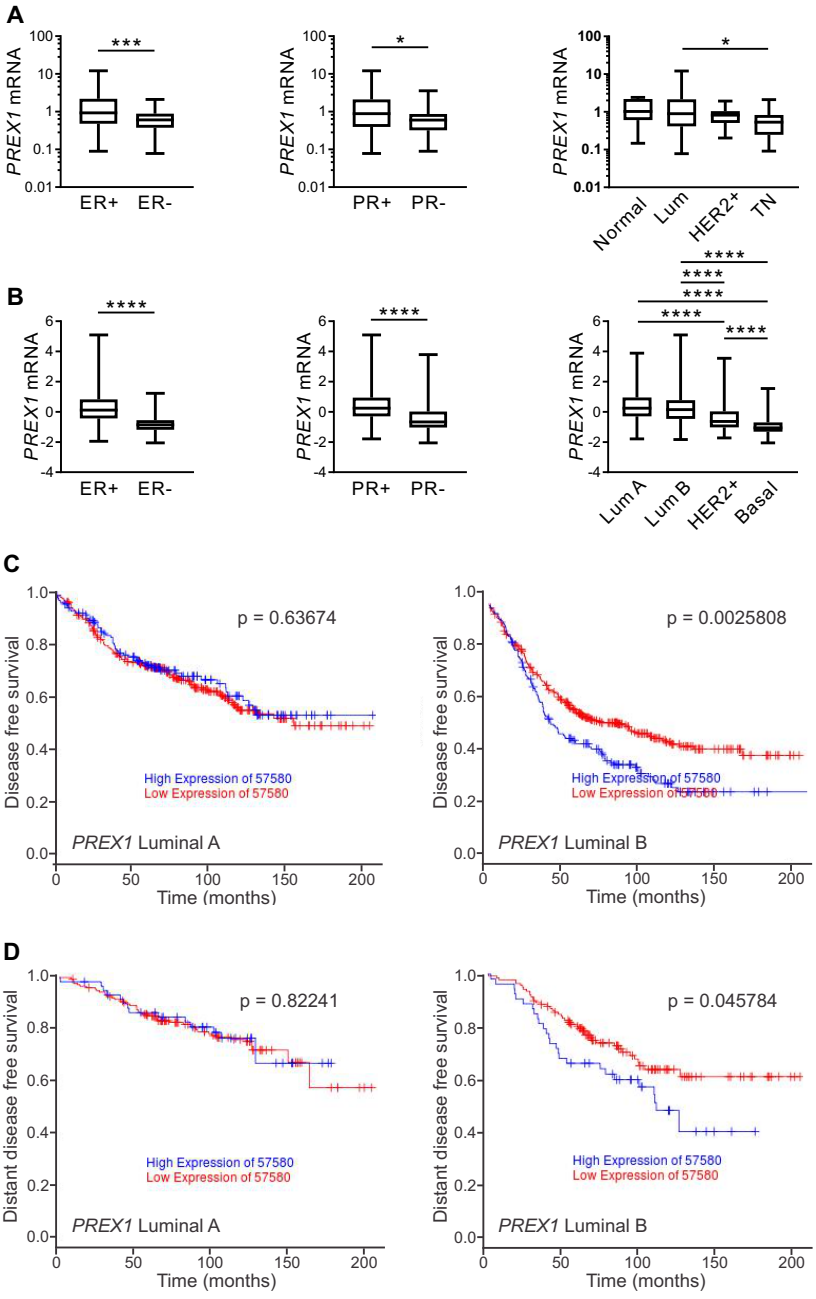
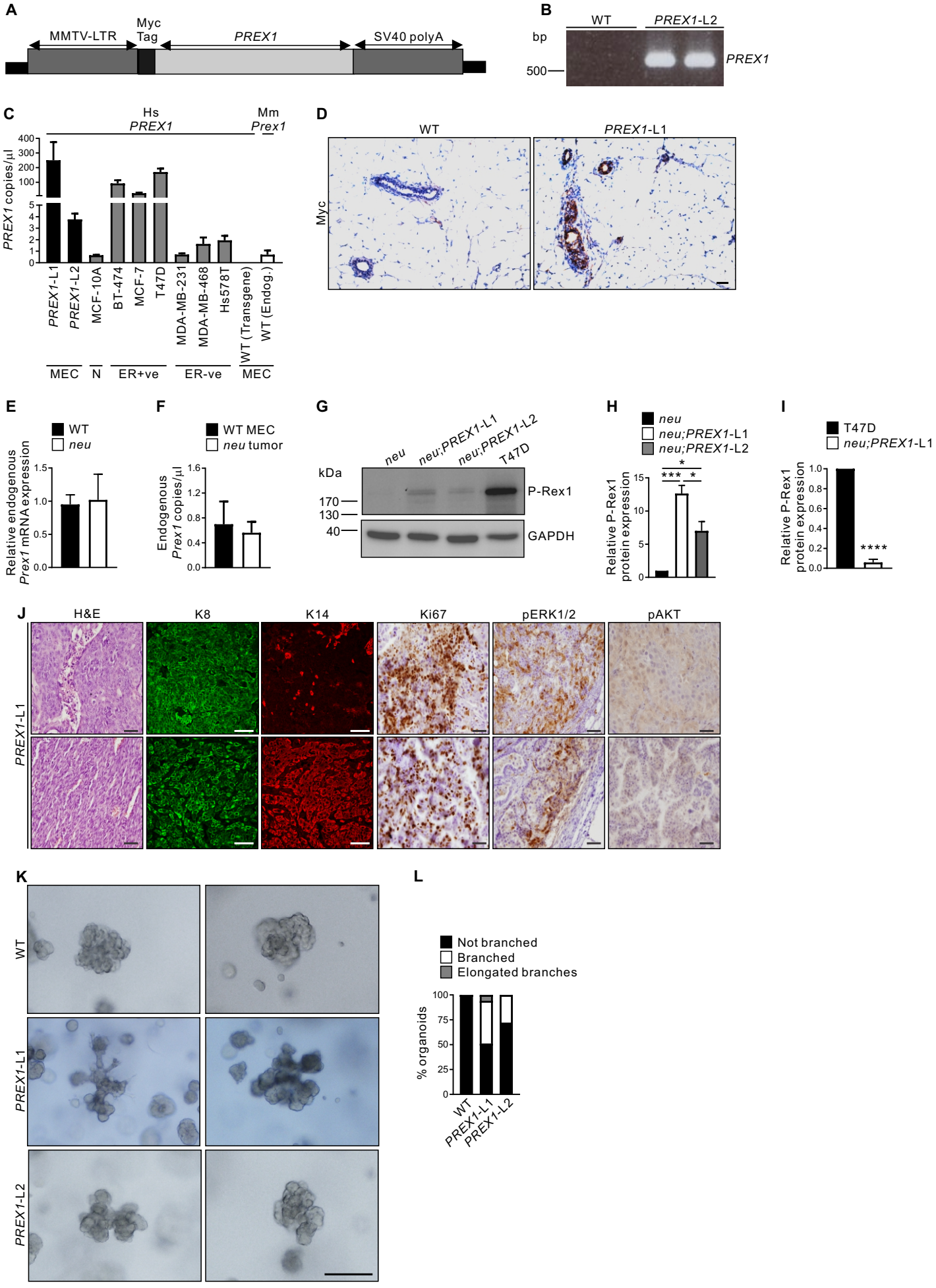

A
PREX1 mRNA
***
ER+ ER-
*
PR+ PR-
*
Normal Lum HER2+ TN
B
****
ER+ ER-
PR+ PR-
Lum A Lum B HER2+ Basal
C
Disease free survival
p = 0.63674
High Expression of 57580
Low Expression of 57580
PREX1 Luminal A
Time (months)
p = 0.0025808
PREX1 Luminal B
D
Distant disease free survival
p = 0.82241
p = 0.045784

Supplementary Figure S1. Higher *PREX1* mRNA expression correlates with reduced survival in patients with luminal B breast cancer.

(A) Normalized *PREX1* mRNA expression was determined by qRT-PCR using TissueScan Breast Cancer Arrays I-IV with *PREX1* and β-actin primers. The data are displayed as box and whiskers on a log scale. *PREX1* mRNA expression was correlated with ER (140 cases), PR (123 cases), HER2 (138 cases) or breast cancer subtype (134 breast cancer cases, 16 normal (non-cancerous tissue adjacent to the breast tumor) cases). p values were determined using an unpaired t test (ER, PR, HER2) or one-way ANOVA with Tukey's post-hoc test (subtype).

(B) *PREX1* mRNA expression in the METABRIC dataset. The data are displayed as box and whiskers. *PREX1* expression was correlated with ER (1904 cases), PR (1904 cases), HER2 (1904 cases) or breast cancer subtype (1559 cases). p values were determined using an unpaired t test (ER, PR, HER2) or one-way ANOVA with Tukey's post-hoc test (subtype).

(C-D) Survival analysis in patients with luminal breast cancer using the MTCI Breast Cancer Survival Analysis Tool. Samples in the BreastMark database were dichotomized for gene expression around a high cut-off using the pam50 classifier to subtype the samples. Kaplan-Meier plots of (C) disease free survival (Luminal A: n = 429, number of events = 154, hazard ratio = 0.9224 [0.6593-1.291], p = 0.63674, Luminal B: n = 545, number of events = 309, hazard ratio = 1.42 [1.129-1.787], p = 0.0025808) and (D) distant disease free survival (Luminal A: n = 241, number of events = 57, hazard ratio = 0.9332 [0.5105-1.706], p = 0.82241, Luminal B: n = 191, number of events = 69, hazard ratio = 1.636 [1.004-2.664], p = 0.04578) are shown. Kaplan-Meier plots shown in blue represent survival data for patients in whom *PREX1* (57580) mRNA expression is in the top 25% expression level whereas those in red represent *PREX1* (57580) expression in the bottom 75% expression level.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MMTV-LTR
Myc Tag
PREX1
SV40 polyA
B
WT
PREX1-L2
bp
500
PREX1
C
Hs PREX1
Mm Prex1
PREX1 copies/µl
400
300
200
100
5
4
3
2
1
0
PREX1-L1
PREX1-L2
MCF-10A
BT-474
MCF-7
T47D
MDA-MB-231
MDA-MB-468
Hs578T
WT (Transgene)
WT (Endog.)
MEC
N
ER+ve
ER-ve
MEC
D
WT
PREX1-L1
Myc
E
WT
neu
Relative endogenous Prex1 mRNA expression
1.5
1.0
0.5
0.0
F
WT MEC
neu tumor
Endogenous Prex1 copies/µl
1.6
1.2
0.8
0.4
0.0
G
neu
neu;PREX1-L1
neu;PREX1-L2
T47D
kDa
170
130
40
P-Rex1
GAPDH
H
neu
neu;PREX1-L1
neu;PREX1-L2
*
***
*
Relative P-Rex1 protein expression
15
10
5
0
I
T47D
neu;PREX1-L1
Relative P-Rex1 protein expression
1.0
0.8
0.6
0.4
0.2
0.0
****
J
H&E
K8
K14
Ki67
pERK1/2
pAKT
PREX1-L1
K
WT
PREX1-L1
PREX1-L2
L
Not branched
Branched
Elongated branches
% organoids
100
75
50
25
0
WT
PREX1-L1
PREX1-L2

Supplementary Figure S2. Generation of *PREX1* transgenic mice.

(A) Schematic representation of the MMTV-*PREX1* transgene construct.

(B) Identification of transgenic mice by PCR of genomic DNA. Representative PCRs from wild-type (WT) and MMTV-*PREX1*^{L2} transgenic (Tg) F1 littermates are shown.

(C) Absolute *PREX1* mRNA expression was quantified by droplet digital PCR in mRNA extracted from MMTV-*PREX1*^{L1} and MMTV-*PREX1*^{L2} mammary epithelial cell (MEC) organoids (human (Hs) *PREX1* transgene, n = 3 mice/genotype), wild-type MEC organoids (human *PREX1* transgene, endogenous murine (Mm) *Prex1*, n = 4 mice/genotype) and human breast cancer cell lines (N, normal, ER+ve, ER-ve) (n = 3 – 4 replicates/cell line). Myc-tagged human *PREX1* transgene and endogenous murine *Prex1* mRNA were detected with species-specific primers. The data represent the mean ± SEM.

(D) Confirmation of Myc-PREX1 transgene expression in FFPE mammary glands from 7 week-old wild-type and MMTV-*PREX1*^{L1} mice by immunohistochemistry with a Myc antibody.

(E) Endogenous murine *Prex1* mRNA expression was examined in mammary epithelial cell organoids from wild-type and MMTV-*neu* mice by qRT-PCR relative to *Gapdh*. The data represent the mean ± SEM (n = 3 mice/genotype).

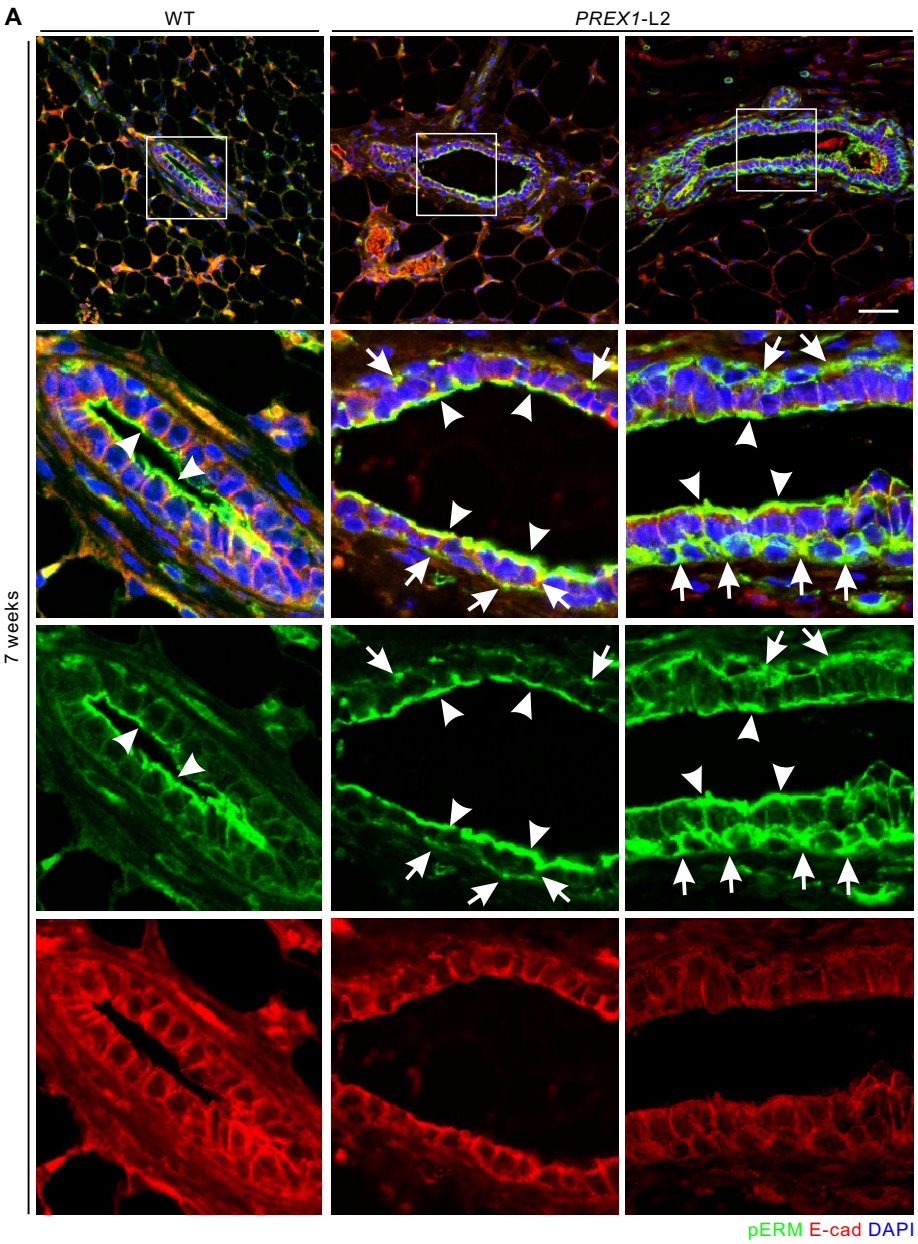
(F) Absolute endogenous *Prex1* expression was quantified by droplet digital PCR in mRNA extracted from wild-type mammary epithelial and MMTV-*neu* tumor cells. Data represent mean *Prex1* expression ± SEM (n = 4 mice/genotype).

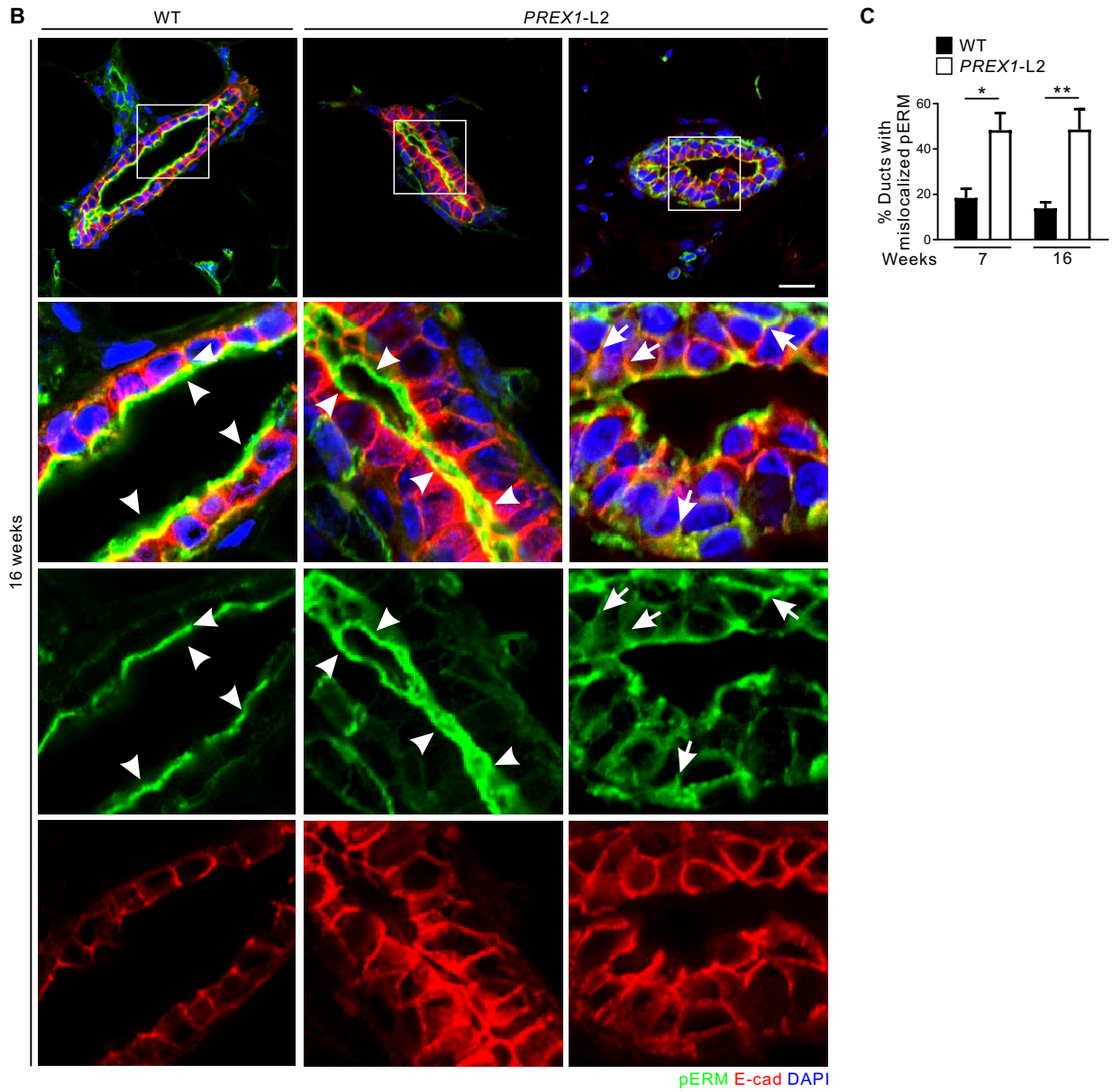
(G-I) Whole-cell lysates of mammary tumoroids derived from MMTV-*neu*, MMTV-*neu*;*PREX1*^{L1} and MMTV-*neu*;*PREX1*^{L2} transgenic mice and human T47D breast cancer cells were immunoblotted with antibodies specific for P-Rex1 or GAPDH as a loading control (G). Graphs show densitometric analysis of P-Rex1 expression normalized to GAPDH. Results are expressed relative to *neu* control (One-way ANOVA and Tukey's multiple comparisons test) (H) or T47D (Student's t test) (I) cells which were arbitrarily assigned a value of one. Data represents mean ± SEM from 3 independent experiments.

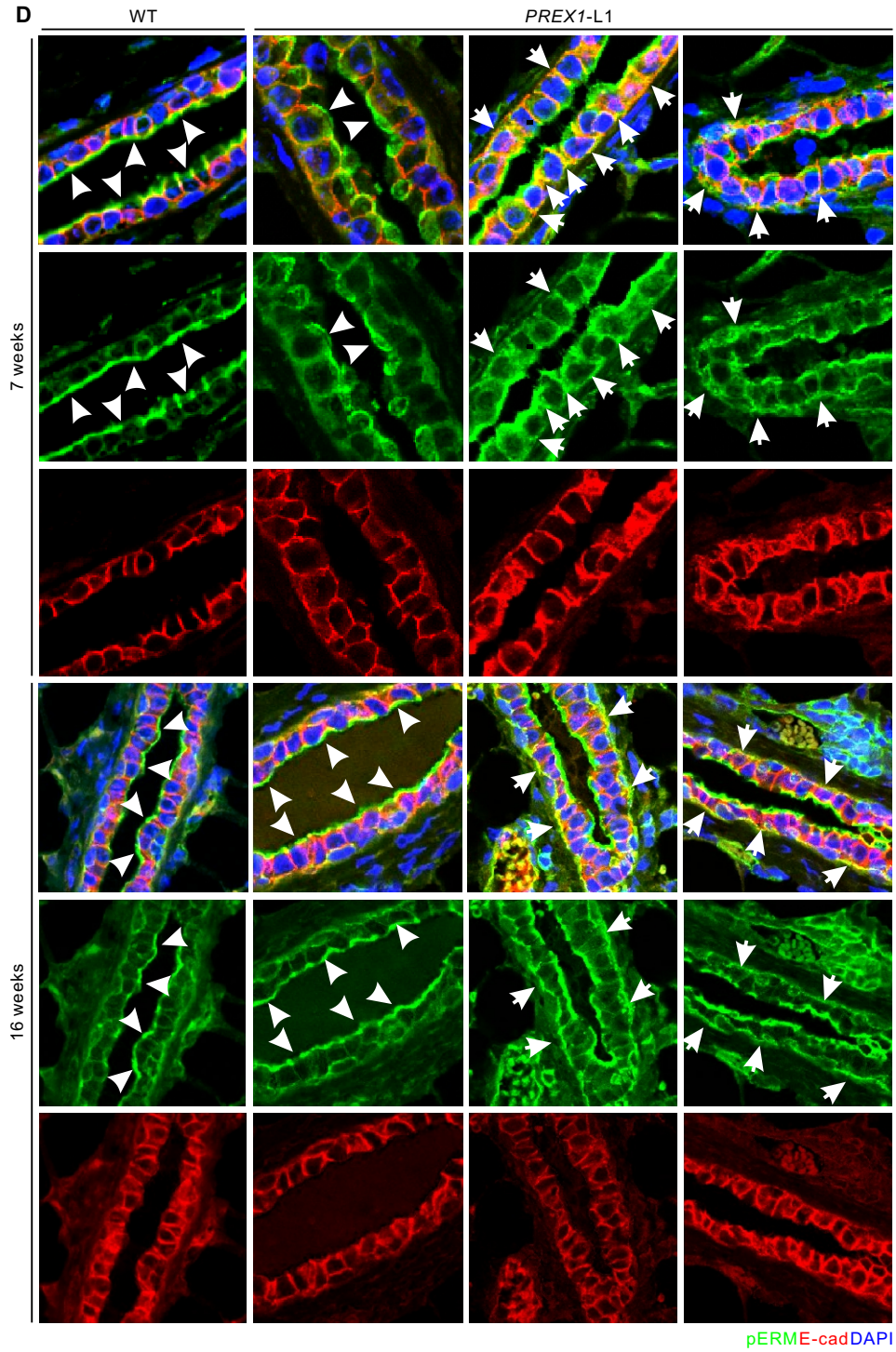
(J) FFPE sections of mammary tumors from 18-22 month-old MMTV-*PREX1*^{L1} mice were stained with H&E or immunostained with K8, K14, Ki67, pERK1/2, or pAKT antibodies. Representative images of tumors from two MMTV-*PREX1*^{L1} mice are shown.

(K-L) Mammary epithelial organoids derived from wild-type, MMTV-*PREX1*^{L1} and MMTV-*PREX1*^{L2} mice were cultured in growth factor reduced Matrigel in DMEM (K). Data represent the percentage of organoids from wild-type, MMTV-*PREX1*^{L1} and MMTV-*PREX1*^{L2} mice exhibiting no branching or elongated branches (n = 3 mice/genotype, >20 organoids/mouse) (L).

Scale bars, 50 μm (D and J) 100 μm (K). *p<0.05, ***p<0.001, ****p<0.0001


A
WT
PREX1-L2
7 weeks
pERM E-cad DAPI


B
WT
PREX1-L2
16 weeks
pERM E-cad DAPI
C
WT
PREX1-L2
% Ducts with mislocalized pERM
60
40
20
0
*
**
Weeks
7
16


D
WT
PREX1-L1
7 weeks
16 weeks
pERM E-cad DAPI

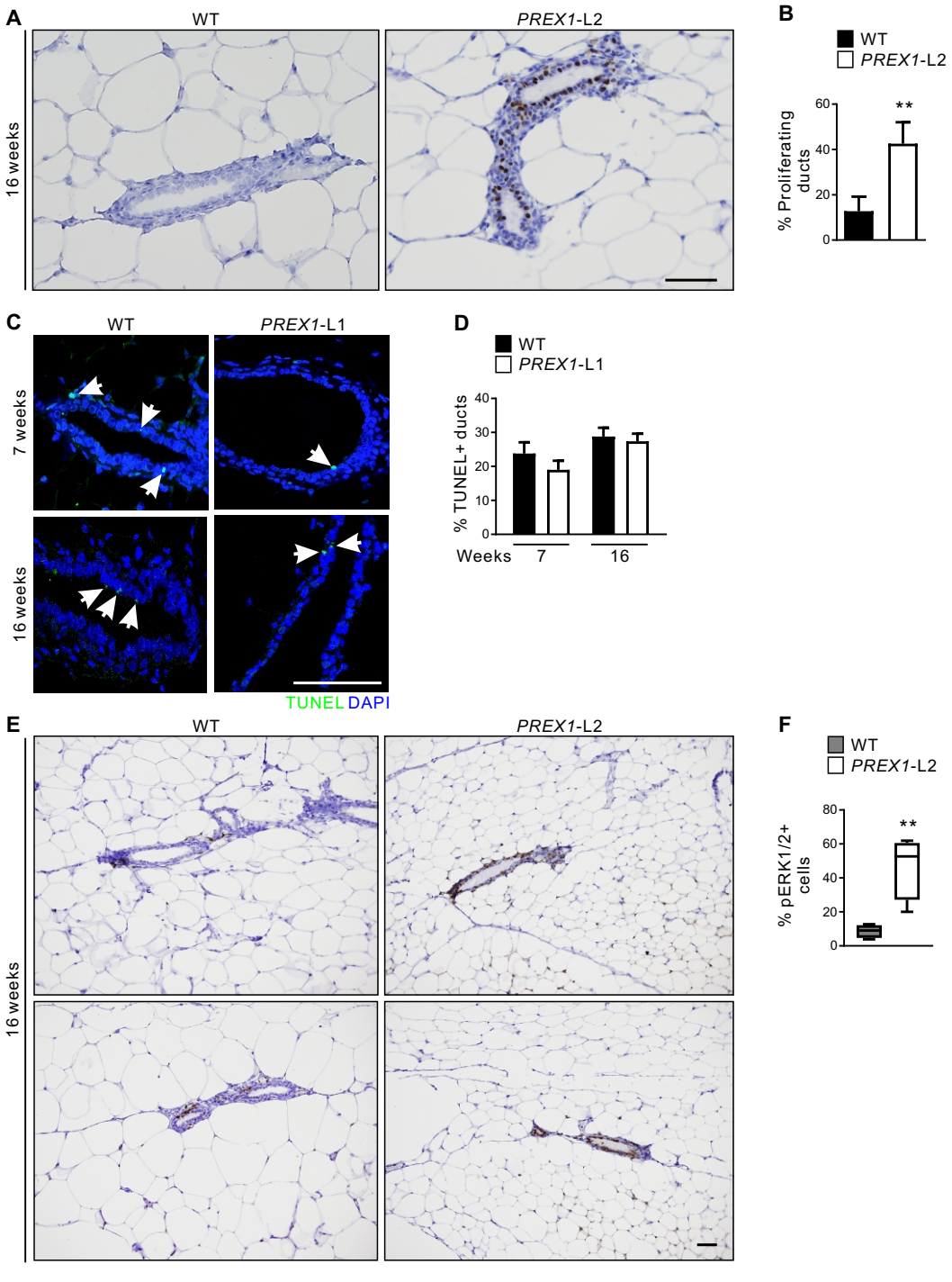
Supplementary Figure S3. P-Rex1 overexpression disrupts epithelial cell polarity in MMTV-*PREX1* mice.

(A-C) FFPE sections of mammary glands from 7 (A) and 16 (B) week-old wild-type and MMTV-*PREX1*^{L2} mice were immunostained with the apical membrane marker pERM and E-cadherin antibodies and counterstained with DAPI. Higher power images of the boxed regions are shown in the lower panels with single channel images below. Representative images are shown demonstrating normal (white arrowheads) and mislocalized (white arrows) pERM staining in mammary ducts (A-B). Data represent mean percentage of ducts with mislocalized pERM ± SEM (n = 4 mice/genotype, >50 ducts/ mouse) (One-way ANOVA and Tukey's multiple comparisons test) (C).

(D) Higher power images of pERM and E-cadherin immunostaining of mammary glands from 7 and 16 week-old wild-type and MMTV-*PREX1*^{L1} mice from Figure 3C with single channel images shown below.

Scale bars, 50 μm.

*p<0.05, **p<0.01


A
WT
PREX1-L2
16 weeks
B
WT
PREX1-L2
% Proliferating ducts
60
40
20
0
**
C
WT
PREX1-L1
7 weeks
16 weeks
TUNEL DAPI
D
WT
PREX1-L1
% TUNEL+ ducts
40
30
20
10
0
Weeks 7 16
E
WT
PREX1-L2
16 weeks
F
WT
PREX1-L2
% pERK1/2+ cells
80
60
40
20
0
**

Supplementary Figure S4. P-Rex1 overexpression promotes epithelial cell proliferation in mammary glands from MMTV-*PREX1*^{L2} mice.

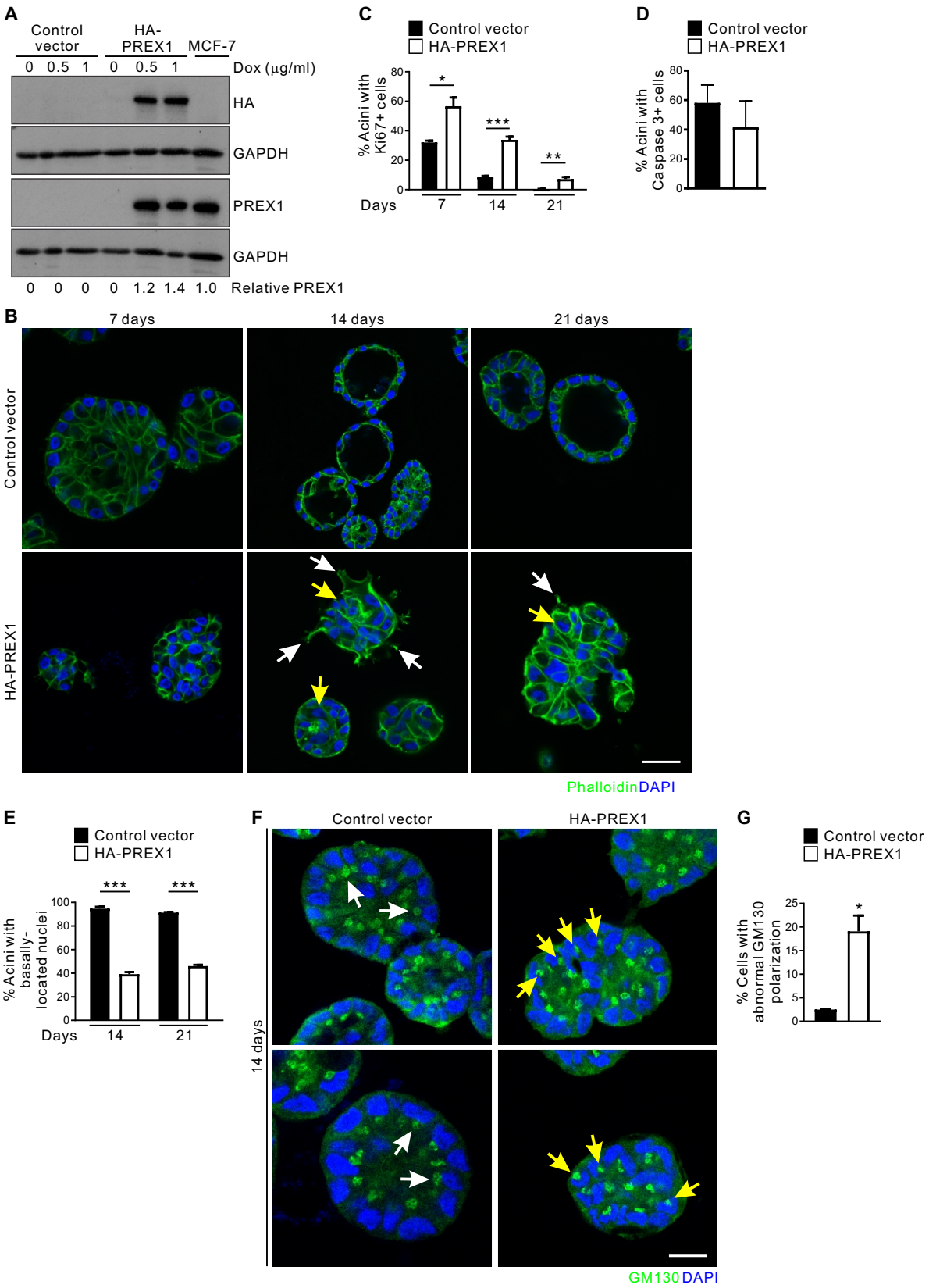
(A-B) FFPE sections of mammary glands from 16 week-old wild-type and MMTV-*PREX1*^{L2} mice were stained with a Ki67 antibody to identify proliferating cells (A). Data represent mean percentage of proliferating ducts, defined as ducts with > 15% Ki67-positive cells. (WT: n = 5, MMTV-*PREX1*^{L2}: n = 4 mice, >20 ducts) (Student's t test) (B).

(C-D) FFPE sections of mammary glands from 7 and 16 week-old WT and MMTV-*PREX1*^{L1} mice were stained with TUNEL (apoptotic cells, arrows) and DAPI (C). Data represent mean ± SEM (n = 5 mice/genotype, >15 ducts/mouse) (One-way ANOVA and Tukey's multiple comparisons test) (D).

(E-F) FFPE sections of mammary glands from 16 week-old wild-type and MMTV-*PREX1*^{L2} transgenic mice were immunostained with a pERK1/2 antibody (E). Data represent mean percentage of pERK1/2-positive epithelial cells ± SEM (n = 5 mice/genotype; >1500 mammary epithelial cells/mouse) (Student's t test) (F).

Scale bars, 50 μm.

**p<0.01


A
Control vector
HA-PREX1
MCF-7
0 0.5 1 0 0.5 1
Dox (μg/ml)
HA
GAPDH
PREX1
GAPDH
0 0 0 0 1.2 1.4 1.0 Relative PREX1
C
Control vector
HA-PREX1
% Acini with Ki67+ cells
Days
7
14
21
D
Control vector
HA-PREX1
% Acini with Caspase 3+ cells
B
7 days
14 days
21 days
Control vector
HA-PREX1
Phalloidin DAPI
E
Control vector
HA-PREX1
% Acini with basally-located nuclei
Days
14
21
F
Control vector
HA-PREX1
14 days
GM130 DAPI
G
Control vector
HA-PREX1
% Cells with abnormal GM130 polarization

Supplementary Figure S5. P-Rex1 overexpression disrupts MCF-10A acini morphology and polarity.

(A) MCF-10A cells stably transduced with lentivirus particles containing plasmids encoding the Tet-On 3G transactivator and either control vector or HA-PREX1 were treated with 0.5 or 1.0 μg/ml doxycycline (Dox) for 24 hours or left untreated then analysed by immunoblotting using HA, P-Rex1 or GAPDH antibodies. Normalized PREX1 protein expression relative to endogenous PREX1 levels in parental MCF-7 cells is shown below.

(B) MCF-10A cells stably expressing control vector or HA-PREX1 were grown on a Matrigel basement membrane for 7, 14 or 21 days in the presence of 1 μg/ml doxycycline then immunostained with Alexa-Fluor 488 phalloidin to detect polymerized F-actin and DAPI. White arrows indicate actin-rich protrusions, yellow arrows indicate apical localization of nuclei.

(C) MCF-10A cells stably expressing control vector or HA-PREX1 cultured as in (B) were immunostained with a Ki67 antibody and DAPI. Data represent the mean percentage of acini with Ki67-positive cells ± SEM (n = 3 independent experiments, 200 cells/cell line/experiment) (One-way ANOVA and Tukey's multiple comparisons test).

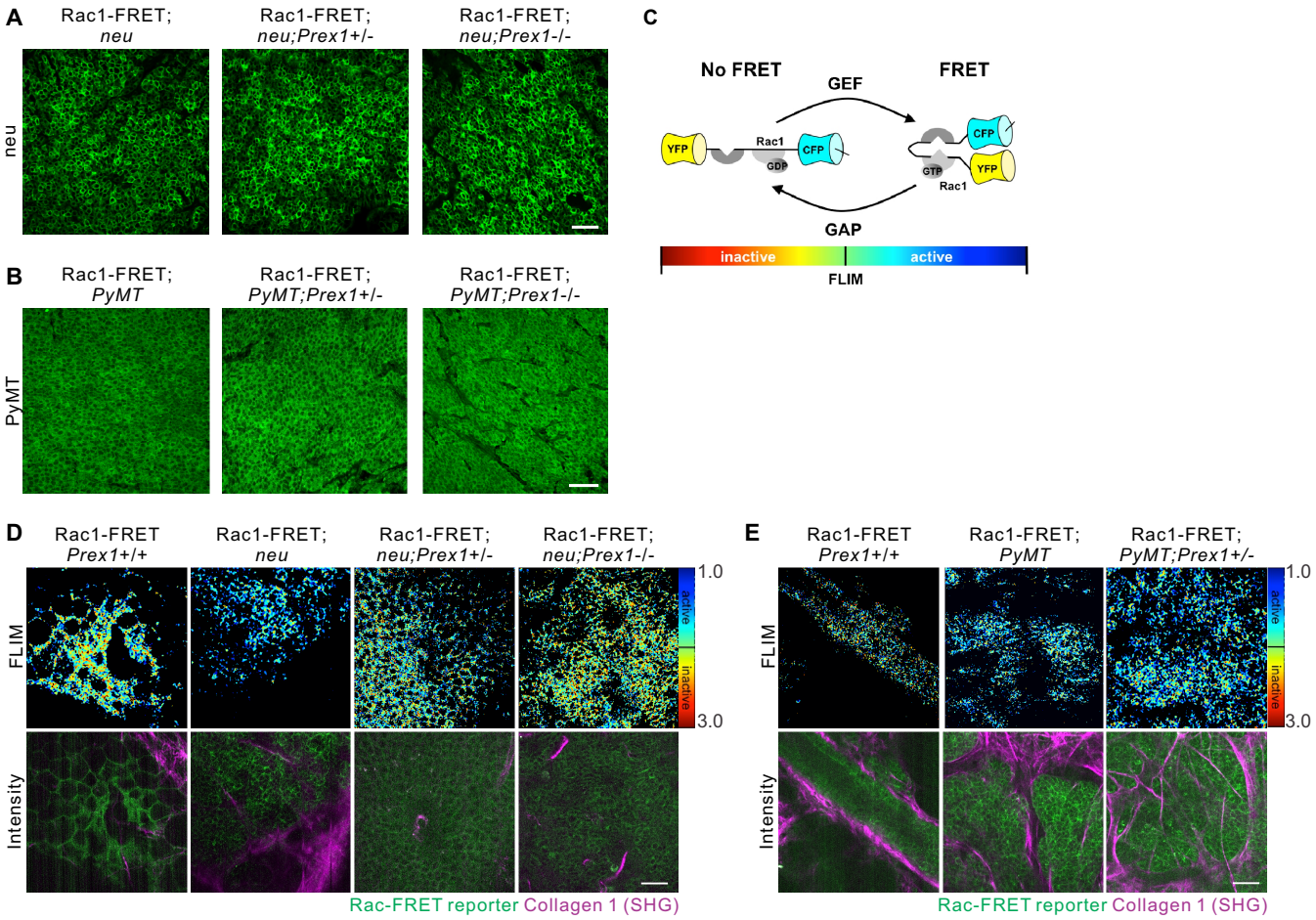
(D) MCF-10A cells stably expressing control vector or HA-PREX1 cultured as in (B) for 7 days were immunostained with a cleaved caspase 3 antibody and DAPI. Data represent the mean percentage of acini with cleaved caspase 3-positive cells ± SEM (n = 3 independent experiments, 100 cells/cell line/experiment) (Student's t test).

(E) MCF-10A cells stably expressing control vector or HA-PREX1 cultured as in (B) for 14 or 21 days were immunostained with Alexa-Fluor 488 phalloidin and DAPI. Cells were scored for the location of the nucleus towards the basal membrane. Data represent the mean percentage of cells with basally-located nuclei ± SEM (n = 3 independent experiments, 150 cells/cell line/experiment) (One-way ANOVA and Tukey's multiple comparisons test).

(F-G) MCF-10A cells stably expressing control vector or HA-PREX1 cultured as in (B) for 14 days were immunostained with a GM130-specific antibody and DAPI. White arrows indicate normally oriented Golgi apparatus, yellow arrows indicate aberrant Golgi localization (F). Data represent the mean percentage of cells with abnormal Golgi apparatus polarization ± SEM (n = 3 independent experiments, >100 cells/cell line/experiment) (Student's t test) (G).

Scale bars, 50 μm.

*p<0.05, **p<0.01, ***p<0.001



Supplementary Figure S6. *Prex1* ablation does not affect neu or PyMT transgene expression in mammary tumors.

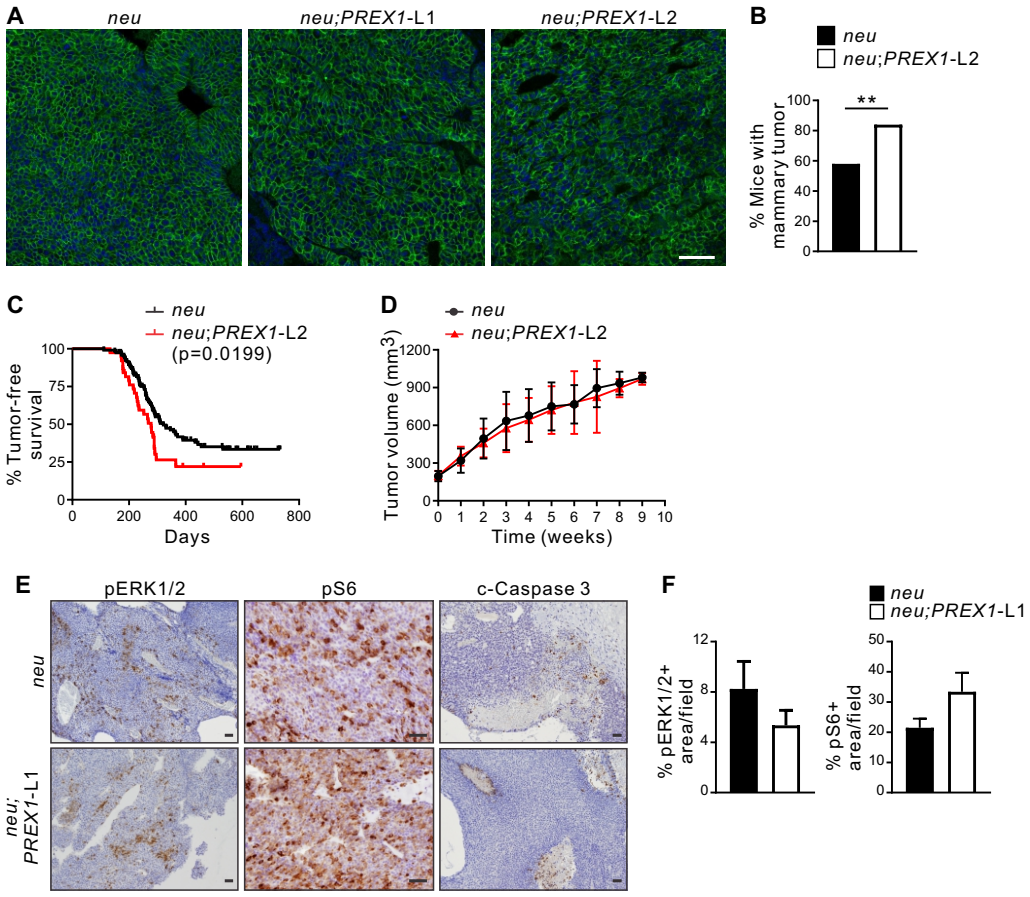
(A) Representative images of mammary tumor sections from Rac-FRET;MMTV-*neu*, Rac-FRET;MMTV-*neu*;*Prex1*$^{+/-}$ and Rac-FRET;MMTV-*neu*;*Prex1*$^{-/-}$ transgenic mice stained with an antibody specific for neu (n = 3 mice/genotype).

(B) Representative images of mammary tumor sections from Rac-FRET;MMTV-*PyMT*, Rac-FRET;MMTV-*PyMT*;*Prex1*$^{+/-}$ and Rac-FRET;MMTV-*PyMT*;*Prex1*$^{-/-}$ transgenic mice stained with an antibody specific for PyMT (n = 3 mice/genotype).

(C) Schematic diagram of the Rac1-FRET biosensor for Rac1 activation.

(D-E) Rac1-FRET;*neu;Prex1* (D) or Rac1-FRET;*PyMT;Prex1* (E) mice at clinical endpoint were imaged on a multiphoton system. Top panels show fluorescence lifetime maps where active Rac1 is indicated by blue-green colored regions, inactive Rac1 is shown by yellow to red areas and black indicates areas where no fluorescence lifetime measurements above background could be detected. Bottom panels show the corresponding fluorescence intensity images for the Rac1-FRET reporter (green) and second harmonic generation (SHG) signal from host ECM collagen (magenta).

Scale bars, 50 μm.



Supplementary Figure S7. P-Rex1 overexpression enhances tumor initiation but not growth in a *neu*-driven mouse model of breast cancer.

(A) Representative images of mammary tumor sections from MMTV-*neu*, MMTV-*neu*;*PREX1*^{L1} and MMTV-*neu*;*PREX1*^{L2} transgenic mice stained with an antibody specific for neu.

(B) Data represent tumor incidence defined as the percentage of MMTV-*neu* (n = 93) and MMTV-*neu;PREX1*^{L2} (n = 31) mice developing a palpable mammary tumor within the first year of life (Student's t test).

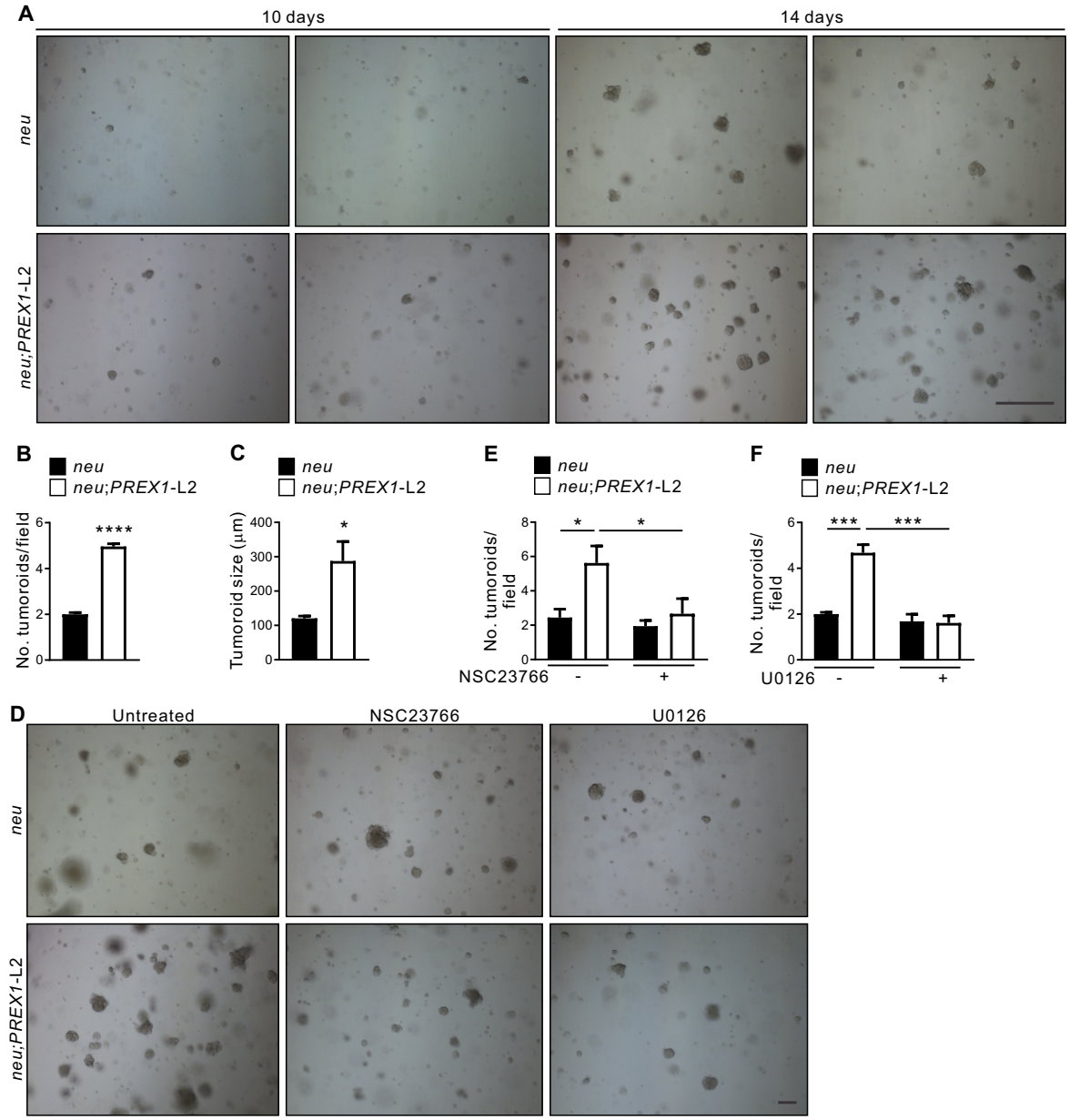
(C) Kaplan-Meier curve showing tumor-free mice in MMTV-*neu* (n = 117) and MMTV-*neu;PREX1*^{L2} (n = 38) mice (Log-rank Mantel-Cox test).

(D) Data represent the mean tumor volume (mm^3) ± SEM after diagnosis (time 0) from MMTV-*neu* (n = 38) and MMTV-*neu;PREX1*^{L2} mice (n = 16). The largest tumor in all mice exhibited similar sizes (100-200 mm^3) at diagnosis (time 0) (Log-rank Mantel-Cox test).

(E-F) FFPE sections of 1000 mm^3 mammary tumors from MMTV-*neu* and MMTV-*neu;PREX1*^{L1} mice were immunostained with pERK1/2, pS6 or cleaved caspase 3 antibodies (E). Data represent mean percentage of positive area/field (>10,000 cells/mouse) (F).

Scale bars, 50 µm (A), 100 µm (E).

**p<0.01



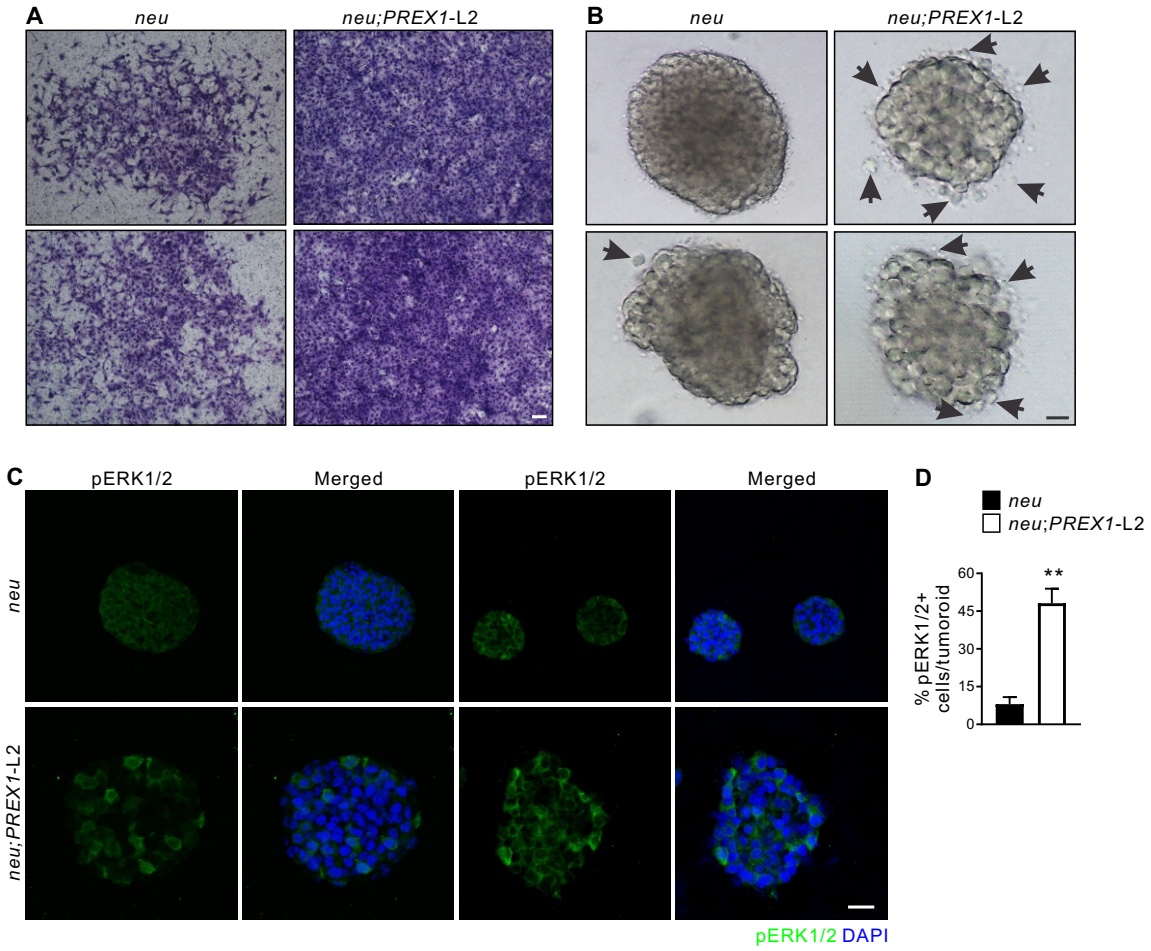
Supplementary Figure S8. P-Rex1 enhances mammary tumoroid colony formation in a Rac1- and ERK1/2-dependent manner.

(A-C) Mammary tumoroids derived from MMTV-*neu* and MMTV-*neu;PREX1*^{L2} transgenic mice were cultured in growth factor reduced Matrigel for 10 or 14 days and imaged by brightfield microscopy (A). Data represent the mean number of tumoroids/field ± SEM (n = 3 mice/genotype, >5 fields examined) (B) or mean tumoroid size ± SEM (n = 3 mice/genotype, > 12 tumoroids/mouse measured) (C) (Student's t test).

(D-F) Mammary tumoroids from MMTV-*neu* and MMTV-*neu*;*PREX1*^{L2} mice were cultured in growth factor reduced Matrigel ± 5 μM NSC23766 or 0.5 μM U0126 inhibitors for 14 days (D). Data represent the mean number of tumoroids/field ± SEM in untreated tumoroids *versus* tumoroids cultured in the presence of NSC23766 (E) or U1026 (F) (n = 3 mice/genotype, >5 fields were examined) (2-way ANOVA and Tukey's multiple comparisons test).

Scale bars, 100 μm.

*p<0.05, ***p<0.001, ****p<0.0001



Supplementary Figure S9: P-Rex1 enhances MMTV-*neu* tumoroid invasion.

(A) Invasion of epithelial tumor cell lines established from mammary tumors from MMTV-*neu* and MMTV-*neu*;*PREX1*^{L1} mice through Matrigel in Transwell assays. Representative images from 2 cell lines/genotype are shown.

(B) Mammary tumoroids derived from MMTV-*neu* and MMTV-*neu;PREX1*^{L2} transgenic mice were cultured in collagen for 21 days and imaged by brightfield microscopy (n = 3 mice/genotype).

(C-D) FFPE sections of mammary tumoroids derived from MMTV-*neu* and MMTV-*neu*;*PREX1*^{L2} mice cultured in collagen 1 were immunostained with a pERK1/2 antibody and counterstained with DAPI (C). Data represent the mean percentage of pERK1/2-positive cells/tumoroid ± SEM (n = 3 mice per genotype, 5 - 15 tumoroids/mouse) (D). **p<0.01 (Student's t test)

Scale bars, 100 µm.