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Supplemental Information

Cell-State Transitions Regulated by SLUG Are Critical for Tissue Regeneration and Tumor Initiation

Sarah Phillips, Aleix Prat, Maja Sedic, Theresa Proia, Ania Wronski, Sohini Mazumdar, Adam Skibinski, Stephanie H. Shirley, Charles M. Perou, Grace Gill, Piyush B. Gupta, and Charlotte Kuperwasser

Inventory of Supplemental Information

1. **Figure S1. Inhibition of SLUG promotes epithelial cell differentiation.** Related to Figure 1:
This Figure supports Figure 1 in the main text. It provides brightfield images of the shControl and shSlug HMEC cells. It also provides additional analysis of microarray data from shControl and shSlug HMEC and MCF10A cells.

2. **Figure S2. Inhibition of SLUG results in increased proportions of luminal cells *in vitro.*** Related to Figure 2:
This Figure supports Figure 2A in the main text. It provides additional flow cytometry analysis (and representative dot plots) of luminal and basal populations in the shControl and shSlug HMEC and MCF10A cells.

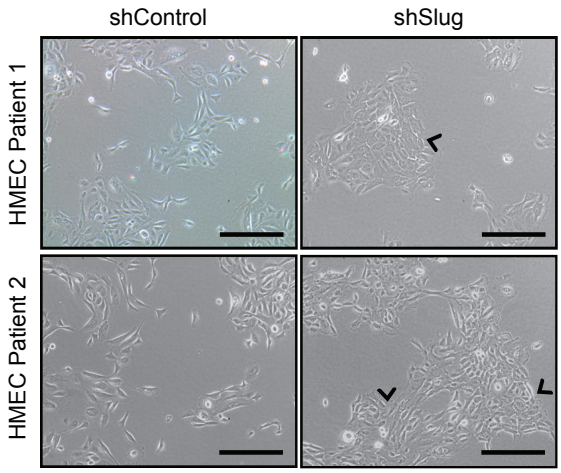
3. **Figure S3. SLUG and LSD1 interact in mammary epithelial cells.** Related to Figure 5:
This Figure supports Figure 5 in the main text. It provides additional data detailing the interaction between SLUG and LSD1. Included in this figure is data showing knockdown levels of SLUG and LSD1 (by protein and mRNA) in the HMEC cells, as well as microarray and flow cytometry analysis of shSlug and shLSD1 HMEC cells. Figure 5 includes this data for the MCF10A cells.

4. **Supplemental Figure Legends**

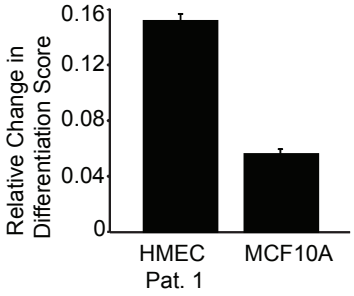
5. **Supplemental Experimental Procedures**: This section provides a comprehensive list of the methods, reagents, etc. used to generate the data in the main text as well as the supplemental materials.

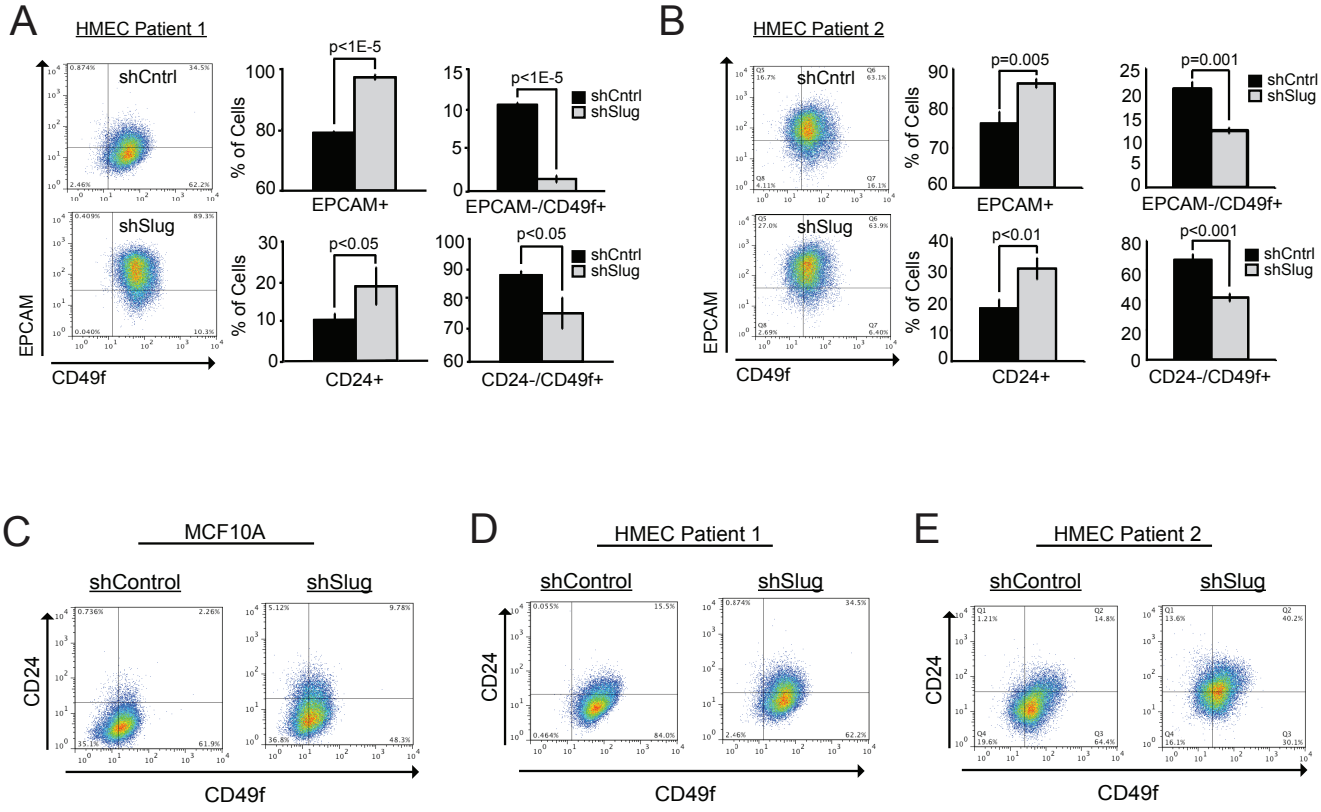
6. **Supplemental References**

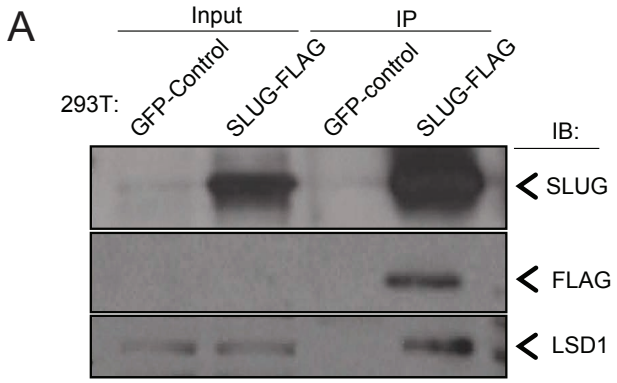
A

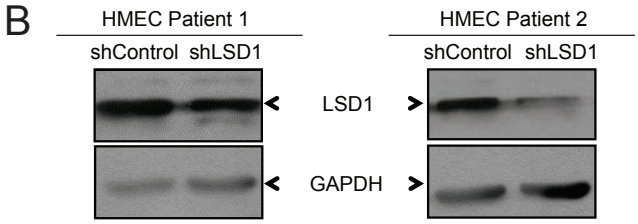

shControl
shSlug
HMEC Patient 1
HMEC Patient 2

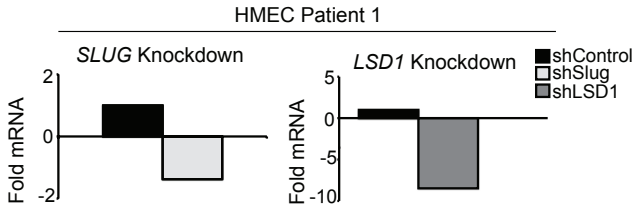
B

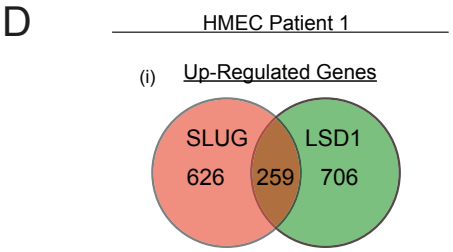

Relative Change in Differentiation Score
0.16
0.12
0.08
0.04
0
HMEC Pat. 1
MCF10A

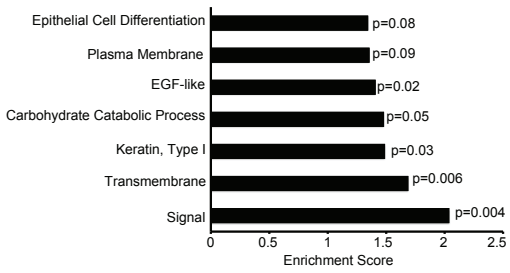

A
HMEC Patient 1
shCntrl
shSlug
EPCAM
CD49f
% of Cells
p<1E-5
EPCAM+
p<1E-5
EPCAM-/CD49f+
p<0.05
CD24+
p<0.05
CD24-/CD49f+
shCntrl
shSlug
B
HMEC Patient 2
p=0.005
p=0.001
p<0.01
p<0.001
C
MCF10A
shControl
shSlug
CD24
CD49f
D
HMEC Patient 1
E
HMEC Patient 2

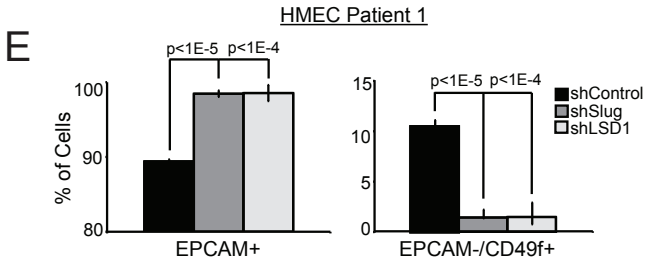

A
Input
IP
293T:
GFP-Control
SLUG-FLAG
GFP-control
SLUG-FLAG
IB:
SLUG
FLAG
LSD1

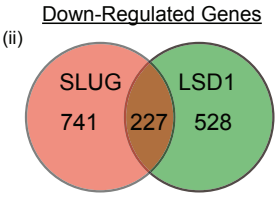

B
HMEC Patient 1
shControl shLSD1
HMEC Patient 2
shControl shLSD1
LSD1
GAPDH

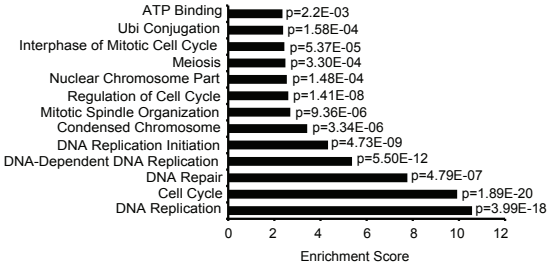

C
HMEC Patient 1
SLUG Knockdown
LSD1 Knockdown
shControl
shSlug
shLSD1
Fold mRNA

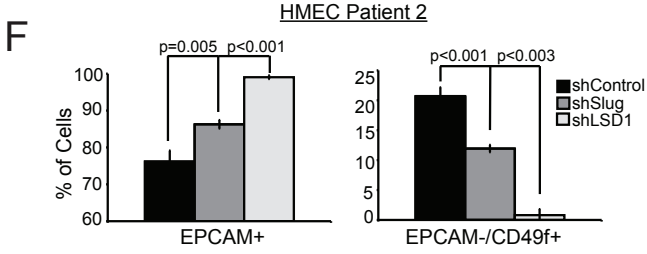

D
HMEC Patient 1
(i)
Up-Regulated Genes
SLUG
LSD1
626
259
706


Epithelial Cell Differentiation
p=0.08
Plasma Membrane
p=0.09
EGF-like
p=0.02
Carbohydrate Catabolic Process
p=0.05
Keratin, Type I
p=0.03
Transmembrane
p=0.006
Signal
p=0.004
Enrichment Score


E
HMEC Patient 1
p<1E-5 p<1E-4
% of Cells
shControl
shSlug
shLSD1
EPCAM+
EPCAM-/CD49f+


Down-Regulated Genes
(ii)
SLUG
LSD1
741
227
528


ATP Binding
p=2.2E-03
Ubi Conjugation
p=1.58E-04
Interphase of Mitotic Cell Cycle
p=5.37E-05
Meiosis
p=3.30E-04
Nuclear Chromosome Part
p=1.48E-04
Regulation of Cell Cycle
p=1.41E-08
Mitotic Spindle Organization
p=9.36E-06
Condensed Chromosome
p=3.34E-06
DNA Replication Initiation
p=4.73E-09
DNA-Dependent DNA Replication
p=5.50E-12
DNA Repair
p=4.79E-07
Cell Cycle
p=1.89E-20
DNA Replication
p=3.99E-18
Enrichment Score


F
HMEC Patient 2
p=0.005 p<0.001
p<0.001 p<0.003
% of Cells
shControl
shSlug
shLSD1
EPCAM+
EPCAM-/CD49f+

Supplemental Figure Legends

Figure S1. Inhibition of SLUG promotes epithelial cell differentiation. Related to Figure 1

(A) Phase contrast images of shControl and shSlug HMECs (Patient 1 and Patient 2). Arrowheads highlight the tightly packed, cobblestone-like morphology of shSlug cells. Scale bars represent 100 μm.

(B) Genomic Differentiation Scores for HMEC (Patient 1) and MCF10A cells, calculated from microarray gene expression data (Prat et al., 2010).

Figure S2. Inhibition of SLUG results in increased proportions of luminal cells *in vitro*. Related to Figure 2

(A and B) Flow cytometry analysis of luminal (EPCAM and CD24) and basal (CD49f) cell surface markers in shControl (n=3) and shSlug (n=3) HMECs (A) Patient 1 and (B) Patient 2. Representative dot plots for EPCAM vs. CD49f expression are shown. Data represent the mean ±SD of three independent experiments and p values were calculated by Student's two-tailed t test.

(C-E) Representative dot plots for CD24 vs. CD49f expression in (C) MCF10A cells, (D) HMEC Patient 1 and (E) HMEC Patient 2 cells.

Figure S3. SLUG and LSD1 interact in mammary epithelial cells. Related to Figure 5

(A) Immunoblot analysis of proteins immunoprecipitated with anti-FLAG beads from 293T cells transfected with FLAG-SLUG or a control GFP vector and probed with anti-FLAG, anti-SLUG and anti-LSD1 antibodies.

(B) Western Blot analysis of LSD1 expression in shControl and shLSD1 HMECs (Patient 1 and Patient 2).

(C) Quantitative Real-time PCR analysis of *SLUG* and *LSD1* mRNA levels in shSlug and shLSD1 HMEC cells (Patient 1) relative to control cells.

(D) Above: Venn Diagrams showing the overlap of microarray-identified genes commonly (i) up-regulated or (ii) down-regulated in shSlug and shLSD1 HMEC (Patient 1) cells compared to control cells. Below: Gene ontology biological process categories commonly (i) up-regulated or (ii) down-regulated in shSlug and shLSD1 HMEC (Patient 1) cells compared to control cells. The DAVID Functional Annotation Tool was used to define categories with an enrichment score >1.5; the enrichment score and the p value of genes differentially expressed in each category are shown.

(E and F) Flow cytometry analysis of luminal (EPCAM) and basal (CD49f) cell surface marker expression in shControl, shSlug and shLSD1 HMEC cells, (E) Patient 1 and (F) Patient 2. Data represent the mean ±SD of three independent experiments and p values were calculated by Student's two-tailed t test.

Supplemental Experimental Procedure

Lentiviral Infection

The VSV-G-pseudotyped lentiviral vectors were generated by transient cotransfection of the vector construct with the VSV-G-expressing construct pCMV-VSVG and the packaging construct pCMV DR8.2Dvpr (Miyoshi et al., 1998) into 293T cells together with Mirius transfection reagent (Invitrogen). Lentiviral shRNA constructs targeting *SLUG* (Addgene plasmids 10904 and 10905), *LSD1* (Sigma-Aldrich, NM_015013.1-1812s1c1) and a scramble control (plKO.1 puro, Addgene) were prepared as previously described (Gupta et al., 2005).

Western Blot Analysis

Cultured cells were harvested by trypsinization, pelleted and incubated in RIPA buffer supplemented with protease inhibitor (Roche) to obtain whole cell lysates. Cellular debris was removed by centrifugation at 13,000 rpm for 10 min. 40 μg of the whole cell lysate was used per sample. Western blotting was performed according to the manufacturers protocol (BioRad). Briefly, 4-12% pre-cast gels and XT-MOPS running buffer were used for SDS-PAGE electrophoresis. 0.45 μm nitrocellulose membranes were used for protein transfer. Membranes were incubated overnight at 4°C with primary antibodies diluted in 1% bovine serum albumin in TBS-T or 5% milk in TBS-T. Secondary antibodies (HRP-conjugated) were applied for 1 hr at room temperature (Cell Signaling; #7076 and #7074). The antibodies used for these experiments included: anti-SLUG (Cell Signaling; #9585), anti-LSD1 (Abcam; ab62582), anti-LAMIN AC **(**Cell Signaling; #2032) and anti-GAPDH (Millipore; MAB374).

Immunoprecipitation (IP)

HMECs and MCF10A cells were lysed in IP buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Roche). For immunoprecipitation assays, protein lysates (1mg) were combined with 10 µg of antibody: anti-SLUG (Cell Signaling; #9589) or mouse normal IgG (Santa Cruz; SC2025) and 25 µl of Protein A-Plus agarose beads (Repligen). Following an overnight incubation at 4°C, agarose beads were extensively washed in IP buffer, resuspended in SDS sample buffer (125 Mm Tris pH 6.8, 2.5% SDS, 10% glycerol, 2.5% 2-mercaptoethanol, 0.01% bromo-phenol-blue) and loaded onto a protein gel. Antibodies used for immunoblotting included: anti-SLUG (Cell Signaling; #9585) and anti-LSD1 (Abcam; ab62582).

293T cells were transfected with pPGS-hSlug-FLAG or a control GFP construct using FuGene 6 ® Transfection Reagent (Roche). 48 hrs after transfection, cells were harvested and lysed as described above. 1 mg of protein was incubated overnight at 4°C with 40 µl of Anti-FLAG ® M2 Affinity Gel (Sigma). Following overnight incubation, beads were extensively washed in IP buffer, resuspended in SDS sample buffer and loaded onto a protein gel. Antibodies used for immunoblotting were as follows: anti-SLUG (Cell Signaling; #9585), anti-LSD1 (Abcam; ab62582) and Anti-FLAG ® M2 (Cell Signaling; 2044).

Quantitative RT-PCR

Total RNA from cultured cells was extracted with the RNeasy Mini Kit (QIAGEN). cDNA was prepared with an iScript kit (BioRad) and PCR was carried out with SYBR Green (BioRad). The following primers (human) were used in this study:

SLUG: Forward '5-GCATTTCTTCACTCCGAAGC-3' and Reverse '5-TGAATTCCATGCTCTTGCAG-3'

LSD1: Forward '5-CAAGTGTCAATTTGTTCGGG-3' and Reverse '5-TTCTTTGGGCTGAGGTACTG-3'

GAPDH: Forward '5-GAGTCAACGGATTTGGTCGT-3' and Reverse '5-GACAAGCTTCCCGTTCTCAG-3'

KRT18: Forward '5-TGATGACACCAATATCACACGAC-3' and Reverse '5-TACCTCCACGGTCAACCCA-3'

KRT19: Forward '5-ACCAAGTTTGAGACGGAACAG-3' and Reverse '5-CCCTCAGCGTACTGATTTCCT-3'

CD24: Forward '5-TGAAGAACATGTGAGAGGTTTGAC-3' and Reverse '5-AGAGTGAGACCACGAAGAGAC-3'

MUC1: Forward '5-CGCCGAAAGAACTACGGGCAGCTG-3' and Reverse '5-CAAGTTGGCAGAAGTGGCTGCCAC-3'

GATA3: Forward '5-GCGGGCTCTATCACAAAATGA-3' and Reverse '5-GCTCTCCTGGCTGCAGACAGC-3'

KRT14: Forward '5-CATGAGTGTGGAAGCCGACAT-3' and Reverse '5-GCCTCTCAGGGCATTCATCTC-3'

SMA: Forward '5-CAGGGCTGTTTTCCCATCCAT-3' and Reverse '5-GCCATGTTCTATCGGGTACTTC-3'

VIM: Forward '5-GAGTCCACTGAGTACCGGAGAC-3' and Reverse '5-TGTAGGTGGCAATCTCAATGTC-3'

CLDN4: Forward '5-GGGGCAAGTGTACCAACTG-3' and Reverse '5-GACACCGGCACTATCACCA-3'

SOX9: Forward '5-AGCGAACGCACATCAAGAC-3'and Reverse '5-CTGTAGGCGATCTGTTGGGG-3'

EPCAM: Forward '5-AATCGTCAATGCCAGTGTACTT-3' and Reverse '5-TCTCATCGCAGTCAGGATCATAA-3'

E-CADHERIN: Forward '5-GAACGCATTGCCACATACAC-3' and Reverse '5-GAATTCGGGCTTGTTGTCAT-3'

Gene Expression Microarray Analysis – Human Cell Lines

Total RNA for microarray expression studies was isolated from MCF10As and HMECs via the RNeasy Mini kit (QIAGEN). RNA was profiled as described previously using oligo microarrays (Agilent Technologies, Santa Clara, CA, USA; Hu et al., 2006). The probes or genes for all analyses were filtered by requiring the lowest normalized intensity values in both sample and control to be > 10. The normalized log2 ratios (Cy5 shSlug sample/Cy3 shControl sample) of probes mapping to the same gene (Entrez ID as

defined by the manufacturer) were averaged to generate independent expression estimates. Differentially expressed genes between shSlug sample and shControl sample were identified after performing a one class Significance Analysis of Microarrays (SAM; Tusher et al., 2001). All microarray data are available in the University of North Carolina (UNC) Microarray Database (https://genome.unc.edu/) and have been deposited in the Gene Expression Omnibus (GEO) under the accession number GSE54735.

Gene Expression nCounter Analysis

Total RNA for microarray expression studies was isolated from Basal, Luminal and Stem MCF10A cell sorted populations via the RNeasy Mini kit (QIAGEN). For each sample, ~100 ng of total RNA was used to measure gene expression of 110 selected genes using the digital color-coded nCounter platform (Nanostring Technologies, Seatlle). Raw data was log base 2 transformed and normalized using 5 house-keeping transcripts. All data is available in GSE54735.

Gene Expression Microarray Analysis – Mouse MECs

Mammary glands from *Snai2*$^{LacZ/LacZ}$ mice and WT controls were dissected, resuspended as single cell suspensions and sorted into basal/ME (Lin$^-$ /EPCAM^{lo}/CD49f^{hi}), luminobasal (Lin$^-$/EPCAM^{hi}/CD49f^{hi}) and unsorted fractions as described above. Pools of cells for each genotype and fraction were combined from 2-3 animals. RNA was extracted using the Qiagen RNeasy micro column with on-column DNA digestion as per manufacturer's instructions. All RNA samples were assayed on an Agilent 2100 Bioanalyzer to ensure high RNA quality (RIN > 8) prior to microarray analysis.

Microarray samples were submitted to the Tufts Center for Neuroscience Research Core. Sample preparation and analysis were conducted by the Yale Center for Genomic Analysis (Yale School of Medicine, CT) as follows: 5 ng of total RNA was amplified using the Ovation Pico WTA System v2 kit (Nugene, #3302) as per manufacturer's instructions. Amplified cDNA was purified using a QIAQuick PCR Purification kit (Qiagen) and the concentration of cDNA samples normalized to a total of 4 μg, as per Nanodrop readings (Thermo Scientific). cDNA was then labeled with the Encore BiotinIL Module Kit (Nugene, #4210) and purified with a MinElute Reaction Cleanup Kit (Qiagen) as per manufacturers' instructions. Labeled and purified cDNA was normalized to a total concentration of 750 ng and hybridized to a MouseRef v2 Expression BeadChip (Illumina) overnight at 48°C as per manufacturer's instructions. Beadchips were then washed using recommended buffers and stained with Amersham Cy3-Streptavidin (GE Healthcare #PA43001). BeadChips were scanned using the Illumina HiScan System and images analyzed using Beadstudio Software (Illumina). Quality control and data analysis were carried out according to the instructions provided by Illumina. Differential gene expression between WT and *Snai2*$^{LacZ/LacZ}$ samples for each fraction was computed by GenePattern Comparative Marker Selection module (Broad Institute, MIT). Gene signature comparison to the data from Lim et al. (Lim et al., 2009) was conducted as per Prat et al. (Prat et al., 2010).

3D Collagen Assay

For 3D collagen gel assays, rat-tail collagen was diluted to 1 mg/mL with 5 mM glacial acetic acid and brought to pH 7.0 with 0.01 M NaOH. Wells of 4-well chamber

slides (BD Falcon) were coated with 100 µl of collagen and allowed to set at room temperature for 20–30 min. Then, 10,000 MCF10A cells (shControl or shSlug) were plated in 1 mL of MEGM containing 2% Matrigel (BD Biosciences) per well, in triplicate. Structures were allowed to form on top of the collagen gels for 5-6 days with media changes every 3 days. Structures were quantified under a microscope at 100x magnification.

Immunostaining

For paraffin embedded tissues, samples were deparaffinized, rehydrated in graded alcohols and processed for antigen retrieval by incubation in citrate buffer (PH 6) at 95°C for 45 min. Slides were then washed in PBS before primary antibody incubation. For plated cells, samples were cultured on 8-well chamber-slides, fixed with methanol at −20°C for 10 min and washed 3x with 1x PBS before primary antibody incubation. All samples were incubated overnight at 4°C with primary antibodies: anti- (Abcam; ab8667) or anti-SMA (Vector Laboratories; VPS281) diluted in 1% bovine serum albumin in PBS. Alexa Fluor 546 and Alexa Fluor 488-conjugated secondary antibodies (Invitrogen; #A11010 and #AA11001) were applied for 1 hr at room temperature. Tissues/cells were counterstained with DAPI and mounted with the Slow-Fade mounting kit (Invitrogen). A Leica SP2 confocal microscope was used to capture these images.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections with sodium citrate or Tris EDTA antigen retrieval, followed by detection with biotinylated secondary antibodies (anti-mouse, Vector; BA-202 or anti-rabbit,

Vector Laboratories; BA 1000) and visualization with the Elite ABC Peroxidase Kit and DAB substrate (Vector Laboratories). Antibodies used on paraffin-embedded tumor and mammary gland sections were as follows: anti-ER-α, (Santa Cruz; SC542, clone M20).

Staining for Ki67 was performed by the Histology Special Procedures Laboratory at Tufts Medical Center.

Tissue samples from each tumor lesion were fixed for 24 hr in 4% neutral buffered formalin. After paraffin embedding, tumor specimens were cut into 5-μm sections and stained with H&E. A Nikon Eclipse 80t microscope and SPOT camera were used for analyzing and photographing the stained sections.

Preparation of MEC Single-Cell Suspensions

The 3rd, 4th and 5th mammary glands were harvested from 8 or 16-week old mice. Glands were mechanically disaggregated and then digested with collagenase (Sigma) and Hyaluronidase (Sigma) for 1 hr at 37°C. Red blood cells were lysed in Red Blood Cell Lysis Buffer (Sigma). The resultant organoids were further digested in 0.25% trypsin-EDTA (2 min) and Dispase/DNaseI (2 min), and then filtered through a 40 μm mesh filter.

Flow Cytometry and FACS

Nonconfluent cultures of MCF10A and immortalized HMEC cells were trypsinized into single-cell suspension, counted, washed with PBS and stained with antibodies specific for human cell CD24 (PE, BD Bioscience; #555427), EPCAM (APC, BD Bioscience; #347200) and CD49f (FITC, BD Bioscience; #555735). Conjugated

isotype-matching IgGs were used as negative controls (IgG2a-PE, BD Bioscience; 559319, IgG1-APC, BD Bioscience; 3404442 and IgG2a-FITC, BD Bioscience; 555573). Antibody-bound cells were washed and resuspended at 1x 10^6 cells/ml in 2% FBS in PBS and run on a FACS Calibur flow cytometer (BD Biosciences) or sorted on a BD Influx FACS sorter (BD Biosciences). Flow cytometry data was analyzed with the Flowjo software package (TreeStar).

Freshly dissociated single cell suspensions of mouse mammary epithelial cells were stained with anti-CD24-APC (eBiosciences; #17-0242), anti-CD49f-FITC (StemCell #10111), anti-CD49f-PercpCy5.5 (Biolegend; #313618) and anti-EPCAM-APC (Biolegend; #118213) antibodies. Endothelial, lymphocytic and monocytic lineages were depleted with antibodies specific to mouse CD31 (PE, #12-0311-83), CD45 (PE, #12-0451-81) and Terr119 (PE, #12-5921) (eBiosciences). Conjugated isotype-matching IgGs were used as negative controls. They were as follows: APC-IgG2b (eBiosciences; 17-4031-81), APC-IgG2a (eBiosciences; 174321), PerCP-Cy5.5-IgG2a (Biolegend; 400531) and FITC-IgG1 (BD Biosciences; 553443).

Whole Mounts

Mammary glands were spread onto glass slides, fixed in 10% neutral buffered formalin, and stained overnight in 0.2% carmine red. Glands were subsequently dehydrated with graded ethanol solutions, cleared in xylene and mounted.

Mammary Fat-Pad Transplants:

Single cell suspensions (see above) of isolated MECs (previously harvested and frozen) were resuspended at 50,00 cells/25 µl in Matrigel:Mouse Mammary Medium

(1:3) solution, and injected into cleared inguinal fat pads of 3-week old nonobese diabetic/severe combined immunodeficient mice (Deome et al., 1959). *Snai2*$^{+/+}$ or *Snai2*$^{LacZ/LacZ}$ MECs were transplanted into contralateral inguinal fat pads. Twelve weeks after transplantation, glands were harvested and processed for whole mounting. Mouse Mammary Media: (DMEM/F12 + 2% CS, 10 µg/ml mouse-insulin, 5 ng/ml mEGF, 0.5 µg/ml hydrocortisone).

Chromatin Immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed by using the EZ-Magna ChIP A kit (Millipore, cat. no. 17–409) following the manufacturer's instructions. In brief, cells were grown to 60–70% confluence in 15cm dishes. Proteins were cross-linked to DNA by adding the formaldehyde directly to the culture medium to a final concentration of 1% for 10 min. The unreacted formaldehyde was quenched by incubating with 1× glycine buffer for 5 min. After rinsing twice with ice-cold PBS, the cells were resuspended in 1 ml of PBS with protease inhibitor by scraping the cells from the dishes. After brief centrifugation to pellet cells, cells were resuspended in 0.5 ml of cell lysis buffer and then nuclear lysis buffer. Sonication of cell lysate was performed four times for 25 sec each on a Branson Sonicator, followed by centrifugation at 4°C for 10 min. Supernatants were collected and diluted 1:10 with dilution buffer. A portion of diluted supernatant (1%) was kept and used as "input." Immunoprecipitation was carried out overnight at 4°C by adding the immunoprecipitating antibody and 20 µl of fully suspended protein A magnetic beads (Millipore). The antibodies used were: anti-SLUG (Cell Signaling; 9589), anti-LSD1 (Abcam; ab17721), anti-H3 (Millipore; #06-755), anti-H3K4me3 (Millipore; #NG18233938) and Mouse normal IgG (Santa Cruz; #SC2025).

After washing the protein A bead-antibody/chromatin complex, ChIP elution buffer with proteinase K was added and incubated at 62°C for 2 hrs. DNA was recovered and purified with DNA spin columns. Both the immunoprecipitated samples and the input samples were processed in the same way. After ChIP assays, we used custom-made ChIP arrays (Qiagen, EpiTect ® ChIP qPCR Array Human Custom) to assess localization of SLUG and LSD1 as well as enrichment of H3K4me3 along the proximal promoters of the *EPCAM*, *E-CADHERIN* and *MUC1* genes.

Markov Model

A description of the cell state transition Markov Model and computation of the stochastic matrix associated with a population of cells has been previously described (Gupta et al., 2011).

Supplemental References

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