Supplementary Data 3

A

| Physiological parameters of dams and offspring | Control | Maternal LT-GE |
|-----------------------------------------------|---------|---------------|
| Hair                                          | No hair loss | No hair loss  |
| Skin                                          | Healthy | Healthy       |
| Behavior                                      | Normal  | Normal        |
| Pregnant period                               | 3 weeks | 3 weeks       |
| Weaning period                                | 3-4 weeks | 3-4 weeks    |
| Pregnant complications/birth defects/dystocia/stillbirth | Not observed | Not observed |
| Pup numbers                                   | 6-8     | 6-8           |
| Pup weight at 4 weeks                         | ~8 g    | ~8 g          |

B

Supplementary Data 3 Impact of maternal GE treatment on general health indexes and mammary gland development. A. Physiological parameters in the dams and offspring of control and maternal LT-GE treatment groups. B. Whole mount analysis for mammary gland development in control and maternal LT-GE treated offspring. Unaffected mammary glands from SV40 offspring mice at 15-20 weeks were extracted for whole mount analysis. Localized epithelial branching density was measured and quantified by Sholl analysis. Square shape indicates defined mammary epithelial area and a skeletonized image of the area that were
evaluated. C. Maternal LT-GE did not affect mammary gland development in the offspring.

Epithelial branching density was determined by the ratio of the number of intersections (N)/gland area (mm²). Photographs (10 × magnification) were selected from at least 5 biological repeats/group to represent each experimental group with. Data were calculated from three randomly selected fields in each slide. Columns, mean; Bars, SD.

Supplementary Methods

Quantitative real-time RT-PCR

Total RNAs extracted from mammary tumors of the mouse offspring in each treatment group were reversely transcribed to cDNA using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA Biorad). Specific primers for selected target genes were synthesized by Integrated DNA Technologies (Coralville, Iowa). These selected target genes included transformation related protein 63 (Trp63), Myc, Cyclin D1, Cyclin A1, Twist1, Keratin 18, inhibitor of DNA binding 1 (Id1), retinoic acid receptor beta (Rarb), progesterone receptor (Pgr), snail family zinc finger 2 (Snai2), secreted frizzled-related protein (Sfrp1) and stratifin (Sfn), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. Quantitative real-time PCR were performed in triplicate by using SsoAdvanced Universal SYBR Green Supermix (BioRad) and analyzed in a BioRad CFX Connect Real-time System. Thermal cycling was optimized based on individual gene amplification conditions. GAPDH gene expression was used as an endogenous control, and the control was used as a calibrator. The relative changes of gene expression were calculated using the following formula: fold change in gene expression, \(2^{-\Delta \Delta Ct} = 2^{-\{\Delta Ct (treated\ tumor\ samples) - \Delta Ct (control\ tumor\ samples)\}}\), where \(\Delta Ct = Ct (test\ gene) - Ct (GAPDH)\) and \(Ct\) represents threshold cycle number.
Western blot analysis

Mouse breast tumors were homogenized to extract tissue proteins with T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s protocols. Breast tumor proteins from 3 randomly selective animals in either control or maternal LT-GE groups were used for western-blot analysis. Denatured proteins were separated through electrophoresis in BioRad SDS-polyacrylamide ready gels and transferred onto nitrocellulose membranes. Membranes were then probed with antibodies including p63 (Thermo Fisher Scientific), Cyclin D1, pPb, p27, Bcl-2, Bak and Cleaved caspase 3 (Cell Signaling Technology, Danvers, MA). The same membranes were stripped and reprobed with β-actin antibody as an internal control. Immunoreactive bands were visualized using the enhanced chemiluminescence detection system (Santa Cruz Biotechnology, Dallas, TX) and documented by an Amersham Imager 680 RGB. The protein expression levels were quantified by optical densitometry using ImageJ Software version 1.8.0_172 (https://imagej.nih.gov/ij/).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with the SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology) according to the manufacturer’s protocol. Approximately 25 mg of mammary tumor was used for each immunoprecipitation (IP). Tissues were cross-linked with formaldehyde, and chromatin was harvested and fragmented using sonication and enzymatic digestion. The ChIP-validated antibodies specific to cAMP Responsive Element Binding Protein 1 (CREB1), methyl CpG binding protein 2 (MeCP2), trimethyl-histone 3-lysine 4 (H3K4me3) and trimethyl-histone 3-lysine 27 (H3K27me3) were used to co-precipitate the DNA sequences that were associated with these proteins. After IP, the protein-DNA cross-links were reversed and the ChIP-purified DNAs were amplified by real-time PCR using primers specific for the
Trp63 promoter 1 (P1) ranging from -653 to -503, promoter 2 (P2) from -27 to +94, and intron 3 (P3) from +1210580 to +121175, yielding 150, 121, and 117 bp for P1, P2 and P3 fragments, respectively: P1 sense, 5’- GGAATTCCCTGTCTTTGCTATTT -3’ and anti-sense, 5’- TCTGCTGTGGCTACAATGTT -3’; P2 sense 5’- TGTTGGTATCAAAGAGAGTTGA -3’, anti-sense, 5’- TTTGACAGACGCCAGGT -3’; P3 sense 5’- CAGGCATCAGCTGTAGATTGA -3’, anti-sense, 5’-TCTTTTCTCCCAGTCCCTTCT -3’. ChIP results were quantified by real-time PCR as described above. 10 µl samples of the diluted chromatin were used for an input control and the data from control group were calibrated to 1. ChIP conditions were optimized by a positive control (histone H3) and a negative control (normal rabbit IgG) for kit-provided primer sets for PCR detection of the mouse ribosomal protein L30 (RPL30) prior to ChIP assay.

Cell culture and treatment

Normal human mammary epithelial cells (HMECs) were obtained from Lonza (Basel, Switzerland) and maintained in serum-free Mammary Epithelial Growth Medium (MEGM) at 37 °C and 0.1% CO2. Precancer cells were established from normal HMECs that were stably transfected with SV40 and human telomerase reverse transcriptase, hTERT (27). Human triple negative breast cancer (TNBC) cell lines, MDA-MB-157 and MDA-MB-231, were purchased from ATCC (Manassas, VA). Precancer and TNBC cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin (Mediatech, Herndon, VA) in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. HMECs and precancer cells were treated with 40 µM of GE for 3 days as done previously (17). TNBC cells were transiently transfected by either Trp63 ORF expression plasmid (MR227530) or empty plasmid, pCMV6-Entry (Origene, Rockville, MD) by Lipofectamine™ 3000 reagent (Invitrogen) according to the
manufacturer’s protocol. Cell RNAs and proteins were collected 72 h post-transfection to detect relevant gene expression via real-time RT-PCR or western blot as described previously. HMECs and TNBC cells were obtained from commercially available companies and authentication was achieved by the vendors. Transgene expressions and relevant neoplastic characteristics in precancer cells were validated to ensure genetic and phenotypic stability prior to the tests.

**MTT assay**

To determine the effects of overexpressed p63 on cellular proliferation, aliquots of $5 \times 10^3$ post-transfected TNBC cells were seeded in triplicate in 96-well plates in regular growth medium that were replaced daily. After 72 h seeding, MTT solution (Sigma, St. Louise, MO) was added to the medium to achieve a final concentration of 1 mg/ml. The cells were incubated at 37 °C and dissolved in 100 µl DMSO after 2 h incubation. The absorbance of the cell lysates in DMSO solution was read at 570 nm by a microplate reader.

**Cell apoptosis analysis**

MDA-MB-231 and MDA-MB-157 cell that were transiently transfected with either control or Trp63 plasmid were collected after 72 h transfection and washed with cold PBS. Cells were then used for apoptosis analysis with the Annexin V Apoptosis Detection Kit FITC (Invitrogen). After washing with the annexin-binding buffer, cells were stained both with Annexin V FITC and propidium iodide (PI) according to the manufacturer’s instruction. Flow cytometry analyses were performed on a Cytek Aurora flow cytometer. The fluorescence intensity of the viable cells was analyzed using CellQuest software.

**Whole mount analysis for mammary gland development**

Mammary gland whole mount staining was followed by a standard protocol. Inguinal mammary glands from different developmental stages in control and treatment groups were removed and
placed on a microscope slide, and then fixed in carnoy’s fixative solution and strained in carmine aluminum staining solution. After overnight staining, mammary tissue was subjected to gradual dehydration process and cleared in xylene. In order to determine mammary gland development, branching density was quantified by Sholl analysis through an Image J plugin distributed with FIJI. Microscopic analysis of breast tissue sections was performed using an Olympus BX41 microscope fitted with a Q-color 5 Olympus camera. The microphotographs were taken at a final magnification of × 10.