Supporting Information

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SI Materials and Methods

Cell Culture. MCF-7, HCC1937, H1299, and Normal Murine Mammary gland (NMuMG) cells were cultured in DMEM (Lonza), 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, and 10% FBS. Medium of NMuMG was additionally supplemented with 10 μg/mL insulin.

Real-Time PCR. Total RNA was isolated (RNeasy Micro Kit; Qiagen) and reverse-transcribed using QuantiTect Reverse Transcription (Qiagen). RT-quantitative PCR (qPCR) reactions were performed using the 7500 Fast Real-Time PCR System apparatus (Applied Biosystems), with TaqMan Gene Expression Master Mix (Applied Biosystems). For RT-qPCR, the following primers were used: m*TAp63* (forward), CACCCAGACAAGCGAGTTC; m*TAp63* (reverse), TCCAGAAAATCCCAGATATGC; mΔ*Np63* (forward), CTGGCAAAACCCTGGAAG; mΔ*Np63* (reverse), CAACATGTTAGCAGTGAGACTGG; h*TAp63* (forward), 5′-TCAGAAGATGGTGCGACAAAC-3′; h*TAp63* (reverse), 5′-GTTCAGGAGCCCCAGGTTCG-3′; hΔ*Np63* (forward), 5′-GAAGAAAGGACAGCAGCATTG-3′; hΔ*Np63* (reverse), 5′-GGGACTGGTGGACGAGGAG-3′; *hShh* (forward), 5′-GGAGCGGACAGGCTGATG-3′; *hShh* (reverse), 5′-GATGGCCAAAGCGTTCAACT-3′; h*Gli2* (forward), 5′-CAGCTGCGCAAACACATGA-3′; and h*Gli2* (reverse), 5′-TTGAGTGACTTGAGCTTCTCCTTCT-3′. Predesigned real-time PCR assays were purchased (Applied Biosystems) for the following genes: m*Shh* (Mm00436528_m1), m*Ihh* (Mm00439613_m1), m*Ptch1* (Mm00436026_m1), m*Gli2* (Mm01293117_m1), m*Bmi-1* (Mm00776122_gh), m*JunB* (Mm00492781_s1), m*EGF-R* (Mm00433023_m1), m*Notch1* (mm00435245_m1), m*Notch3* (Mm00435270_m1), m*Hes1* (mm00468601_m1), m*Hey2* (Mm00469280_m1), m*Fzd7* (Mm00433409_s1), and h*Bmi1* (hs00180411_m1). All of the experiments were run in triplicate, and the results were normalized to *TBP* or 18S expression.

Western Blot Analysis. Cells were lysed in radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors. Total cell extracts were subjected to SDS/PAGE, followed by immunoblotting with mouse monoclonal anti-p63 Ab4 (clone 4A4, 1:400; NeoMarkers) and mouse monoclonal antivinculin (1:1,000; Sigma) antibodies. Densitometric analysis of the blots was performed using ImageLab software, version 5.0 (BioRad).

Immunohistochemical Staining. Tissues were surgically removed, fixed with 4% (wt/vol) paraformaldehyde, paraffin-embedded, and sectioned. Sections were deparaffinized by xylene and rehydrated in graded alcohol. Slides were preincubated with blocking solution [2% (wt/vol) BSA, 2% (vol/vol) normal goat serum, and 0.02% Tween 20 in TBS] and then stained with primary antibodies overnight at 4 °C. Slides were incubated with secondary antibody (HRP rabbit or mouse antibody; DAKO EnVision System) for 30 min at room temperature. After washing, sections were incubated in peroxidase substrate solution (DAB; DAKO), rinsed in water, and counterstained with hematoxylin. Immunohistochemistry was applied to localize anti-p63 (clone 4A4, 1:500; Neomarkers), anti–Ki-67 (clone B56, 1:1,000; BD Biosciences), anti-SHH (clone H160, 1:200; Santa Cruz Biotechnology), and anti-GLI2 (clone ab7195, 1:200; Abcam). For quantification of Ki-67 staining, randomly taken images (at least five fields per animal) were captured from tissue sections and processed with ImageJ software (National Institutes of Health).

Immunofluorescence. Immunofluorescence analysis of ErbB2 tumor sections was performed as previously described (1). Briefly, tissues were fixed in 4% (wt/vol) paraformaldehyde and embedded in paraffin. Sections were deparaffinized and rehydrated stepwise in alcohol/distilled water. Microwave-assisted antigen retrieval was performed in 0.01 M sodium citrate (pH 6.0) for three cycles of 5 min (300 W), followed by cooling at 50 °C. Nonspecific antigens were blocked by incubation in 5% (vol/vol) goat serum in PBS for 1 h in a humidified atmosphere at room temperature. Subsequently, sections were incubated overnight with primary antibodies. Sections were then washed three times with PBS and incubated for 1 h with anti-mouse Cy3 antibody or anti-rat Alexa-488 antibody (Molecular Probes; Invitrogen). After two washes in PBS, the tissue sections were counterstained with DAPI to highlight nuclei.

Transplantation Assays. For transplantation experiments, p63 expression was silenced in two independent mammosphere preparations. shSCR or shp63 mammospheres were dissociated, pelleted, and resuspended in PBS. One hundred thousand mammary cells were injected into the inguinal mammary fat pad of 3-wk-old syngeneic female mice. For each independent preparation, shSCR and shp63 mammary cancer progenitors were used for injecting a total of $n = 2$ and $n = 5$ mice, respectively. The occurrence of palpable tumors was monitored twice a week. Due to ethical regulatory issues at our institute, all mice were euthanized, 10 wk after transplantation, when the tumor mass in the first control (shSCR) animal reached ∼2 cm in diameter. Tumors were then measured and processed for analyses. One mouse in each experimental group (shSCR and shp63) was excluded from the analysis because these mice died from causes other than cancer.

ChIP Assays. ChIP experiments were performed using the MAGnify Chromatin Immunoprecipitation System (Invitrogen). Briefly, cells were cross-linked for 10 min at room temperature with 1% (wt/vol) formaldehyde (Merck). The cross-linking reaction was stopped by addition of 125 mM glycine for 5 min, followed by a washing step with PBS. The pellet was lysed and sonicated using a sonicator (Bioruptor UCD-200; Diagenode), shearing the chromatin into 500- to 1,000-bp fragments. The chromatin extract was incubated with Dynabeads Protein A/G coupled, respectively, to 10 μg of rabbit anti-p63 antibody (Clone H-129; Santa Cruz Biotechnology) or rabbit anti-HA tag antibody (ab9110; Abcam) and rabbit IgG (Invitrogen) as negative controls at 4 °C with rotation for 2 h. The immunocomplexes were washed and treated with proteinase K (20 mg/mL) at 55 °C for 15 min to reverse the cross-linking. DNA was purified with the DNA purification magnetic beads, dissolved in elution buffer, and used for PCR analysis. The following oligos were used: *hShh* promoter: forward 5′-CCTTCCCATGTGGCCTCTT-3′, reverse 5′-ACAGGAGAGGCTGCGTTTAGG-3′; *mShh* promoter: forward 5′-TTGCCCAGCCTCCAAAACCTTC-3′, reverse 5′-AGACCTACCAACTTCATCACCAGTG-3′; peak 1 *hGli2* promoter: forward 5′-CCTGGGCATGCAGAGGAA-3′, reverse 5′-CTTCATGATGAGGTAGGAGGTAGCT-3′; peak 2 *hGli2* promoter: forward 5′-CACTTCCTTCCCTTTCTCTCACA-3′, reverse, 3′-GGAGGAGAAAGGAGGGCATCT-3′; peak 5 *hGli2* promoter: forward 5′-CGTTTTCACTTCTCTTGGGTATGTG-3′,

reverse 5′-ATGGAGCAGCGTTTTTGGAA-3′; and *hPtch1* promoter: forward 5′-GCCGGGTGGCATTTGTC-3′, reverse 5′-CTGGGCCTGTGCTCATTGAT-3′. PCR products were analyzed by electrophoresis on agarose gels.

Luciferase Reporter Assay. PGL3-basic luciferase reporter vectors were constructed by amplification of the genomic regions containing the p63-binding sites (+22, +191, and +30 kb from the TSS for *Shh*, *Gli2*, and *Ptch1* genes, respectively). The following primers were used for the *Shh*, *Gli2*, and *Ptch1* genomic regions, respectively: forward, 5′-CTAGCTAGCTTGCCCAGCCTCCAAAACCTTC-3′; reverse, 5′-CCGCTCGAGAGACCTACCAACTTCATCACCAGTGC-3′; forward, 5′-CTAGCTAGCCTGTATGGATTTGCCTGTTCTGG-3′; reverse, 5′-CCGCTCGAGTCATTCATTGTGGGTGGAAAGG3-3′; forward, 5′-CTGGGCCTGTGCTCATTGAT-3′; and reverse, 5′-CCGCTCGAGCAGCTCGCCTATGCCTGTC-3′. The amplified regions were cloned within NheI and XhoI sites in PGL3 promoter. H1299 cells were transfected with 300 ng of luciferase reporters and 2 ng of internal control plasmid TK-*Renilla* reporter (Promega), in the absence or presence of 900 ng of cDNAs expressing p63 isoforms using Effectene (Qiagen). Twenty hours after transfection, cells were lysed and luciferase activity was measured by using the Dual Luciferase Reporter Assay System protocol (Promega) according to the manufacturer's instructions. The pRL-TK vector was included to normalize transfection efficiency, and reporter basal luciferase activity was normalized as 1.

RNAi-Mediated Gene Silencing. The siRNA duplexes were synthesized by Sigma-Aldrich. For p63 knockdown, siRNA sequences were as follows: 5′-GCGACAAACAAGAUUGAGA-3′ for TAp63 no. 1, 5′-CGACAAACAAGAUUGAGAUU-3′ for TAp63 no. 2, and 5′-GCAGCAUUGAUCAAUCUUA-3′ for ΔNp63. Transfection was performed using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's protocol and optimized for a six-well plate. In brief, 30 nmol of siRNA was mixed with 5 μL of Lipofectamine RNAiMAX in 0.4 mL of OptiMEM medium (Invitrogen). The mixture was added to the cells. Twenty-four hours after transfection, the cell culture medium was replaced with fresh medium.

Lentiviral-Mediated Transduction of shRNAs. For murine progenitors, disaggregated spheres were plated at a density of 50,000 cells per milliliter in standard conditions. Lentiviral particles (multiplicity of infection = 1) bearing shSCR and shp63 (Santa Cruz Biotechnology) were added to the culture medium, along with 8 μg/mL polybrene. After two infection cycles, mammospheres were collected, dissociated to single cells, and incubated with 1 μg/mL puromycin for 3 d. Human breast cancer cell lines were transduced with SMARTchoice lentiviral shRNA particles (clone SH-003330-02-10; Thermo Scientific) targeting p63 mRNA. Cells at density of 1×10^5 cells per well were seeded in a six-well plate, and 24 h, they were infected with virus suspension containing-medium supplemented with 8 μg/mL polybrene. Forty-eight hours after infection, cells were selected in medium containing 1 μg/mL puromycin.

Sphere-Forming Efficiency Assay. For analysis of sphere formation, following transfection with Lipofectamine RNAiMAX or lentiviral infection, single-cell suspensions of breast cancer cells were plated in 24-well culture plates covered with poly-2-hydroxyethylmethacrylate (Sigma) to prevent cell attachment, at a density of 1,000–2,000 cells per milliliter, in serum-free DMEM supplemented with 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 2% (vol/vol) B27 (Invitrogen), 20 ng/mL EGF and bFGF (BD Biosciences), and 4 μg/mL heparin (Sigma). The medium was made semisolid by the addition of 1% (vol/vol) methylcellulose to prevent cell aggregation. The number of spheres for each well was evaluated 7 d after seeding, and sphere formation rate was counted. Sphere-forming efficiency was calculated as the number of spheres formed divided by the original number of single cells seeded and expressed as a percentage.

Bioinformatics Analysis. METABRIC gene expression data (mRNA) have been used (2). The Pearson correlation coefficient was computed as a measure of correlation between mRNA profiles of two genes [p63 and a partner (i.e., *Gli2*, *Fzd7*)]. We used these data as the observed distribution for correlation coefficients between *p63* and a gene in the METABRIC dataset and estimated the *P* value of correlation between *p63* and a partner gene using this distribution. In this case, the *P* value is an observed probability of randomly selecting a gene that would correlate with *p63* on the same or a better level.

1. Candi E, et al. (2007) DeltaNp63 regulates thymic development through enhanced expression of FgfR2 and Jag2. *Proc Natl Acad Sci USA* 104(29):11999–12004.

2. Curtis C, et al.; METABRIC Group (2012) The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 486(7403):346–352.

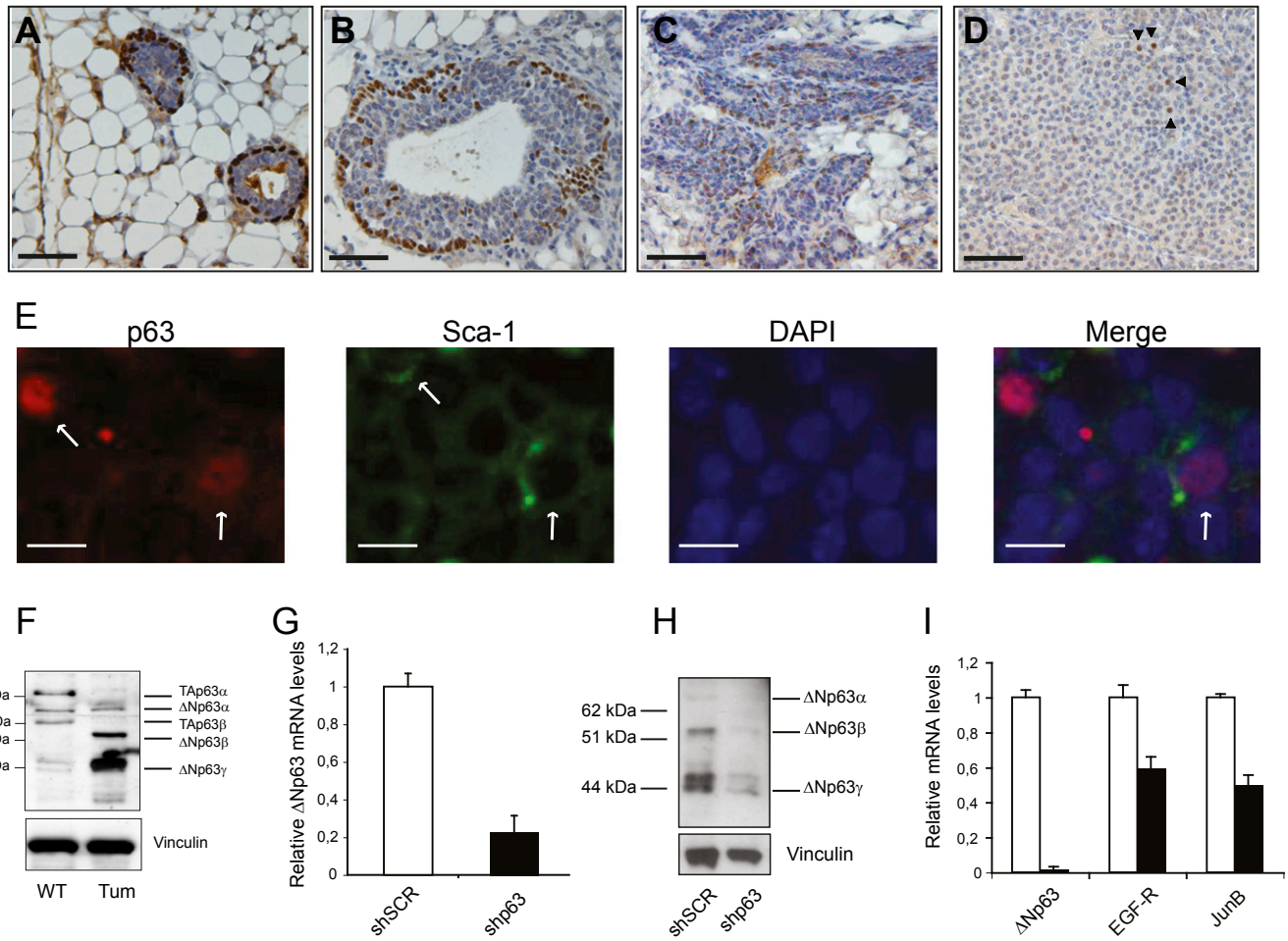


Fig. S1. (*A–D*) Immunohistochemical analysis of p63 expression in the mouse mammary gland and breast lesions occurring during ErbB2-driven carcinogenesis. (*A*) In the normal murine mammary gland, p63 is expressed in the basal/myoepithelial cells. (*B*) Hyperplastic benign lesions retain a continuous peripheral rim of myoepithelial cells expressing p63. (*C*) Discontinuous p63 immunoreactivity is present in the peripheral rim of myoepithelial cells in in situ ductal carcinomas. (*D*) Invasive ductal carcinomas show p63 immunoreactivity in a rare fraction of neoplastic cells. (Scale bars: *A–D*, 100 μm.) (*E*) Representative immunofluorescence staining of sections of ErbB2 invasive ductal carcinomas analyzed for p63 and stem cell antigen 1 (Sca-1) expression. Arrows indicate cells immunoreactive for either Sca-1 (red), p63 (green), or both antigens (red/green). (Scale bars: 2 μm.) (*F*) Immunoblot (IB) analysis of p63 protein levels in total cellular extracts of control (WT) and ErbB2 (Tum) mammospheres. Vinculin amounts were used as a loading control. (*G* and *H*) Knockdown of p63 in ErbB2 mammospheres upon lentiviral-mediated delivery of shRNAs against p63. (*G*) ΔNp63 mRNA levels were measured in shSCR and shp63 tumor spheres. RNA levels were normalized to TATA box-binding protein (TBP) mRNA amounts. Error bars represent mean ± SD of six independent experiments. (*H*) IB showing p63 down-regulation in ErbB2 spheres expressing shRNAs against p63. (*I*) Transcript levels of EGF receptor (EGF-R) and JunB in shSCR and shp63 ErbB2 spheres. mRNA levels were normalized to TBP mRNA amounts. Data are presented as mean ± SD.

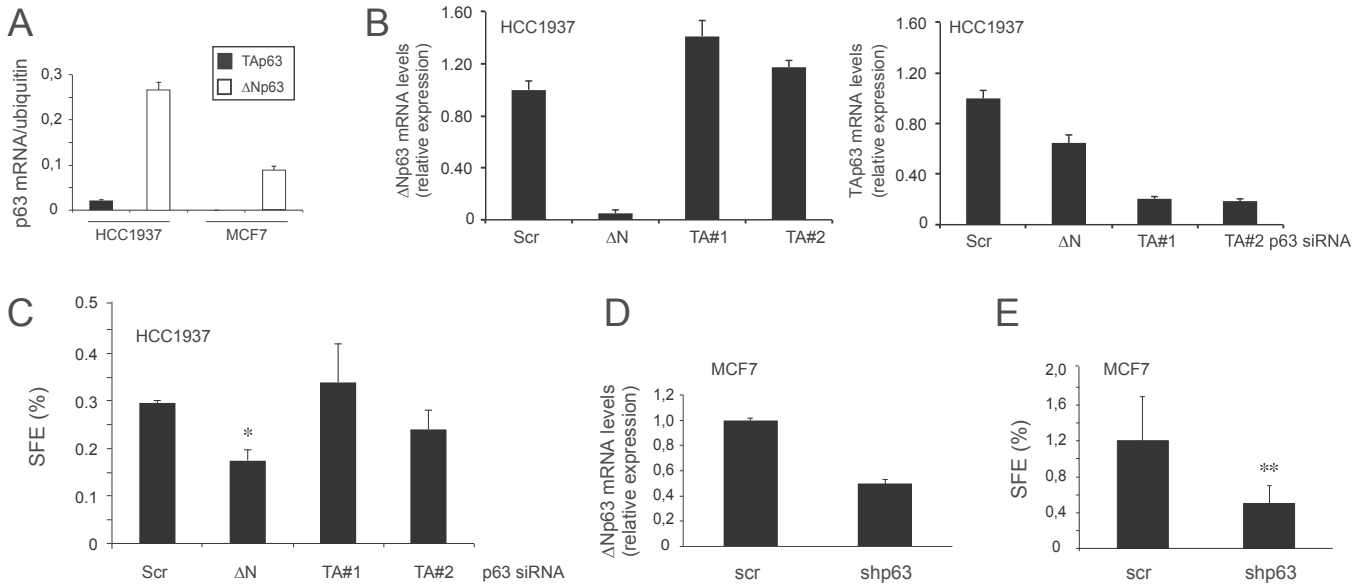


Fig. S2. Loss of ΔNp63 decreases the sphere-forming efficiency (SFE) of breast cancer cells. (*A*) RT-qPCR analysis of total cDNA from HCC1937 and MCF7 cells using primers specific to TAp63 (black bars) or ΔNp63 (white bars) isoforms. Expression of p63 isoforms was normalized to the ubiquitin amounts. Results are mean ± SD. (*B*) ΔNp63 (*Left*) and TAp63 (*Right*) isoform-specific RT-qPCR analysis of HCC1937 after treatment with nonsilencing control siRNA (Scr) or siRNA oligos specific to the ΔNp63 or to TAp63 isoforms (TAP#1 and TAP#2). Expression of p63 isoforms was normalized to the ubiquitin amounts. Results are mean ± SD. (*C*) Averages of the SFE of HCC1937 cells upon siRNA-mediated knockdown of TAp63 and ΔNp63 isoforms. HCC1937 cells were transfected with control siRNA, ΔNp63, or two TAp63 siRNAs for 48 h and then plated for the mammosphere-forming assay. The SFE was calculated as the percentage of the number of spheres per plated cell at every passage. Values represent the mean of three independent experiments ± SD (*$P = 0.016$). (*D*) ΔNp63 mRNA levels in MCF-7 cells transduced with control (Scr) and pan-p63 shRNA (shp63) lentiviral constructs. Expression of p63 was normalized to the ubiquitin mRNA levels. Results are mean ± SD. (*E*) SFE ± SD of MCF-7 cells infected with Scr and shp63 lentiviral shRNA constructs. The SFE was calculated as above. (**$P < 0.05$).

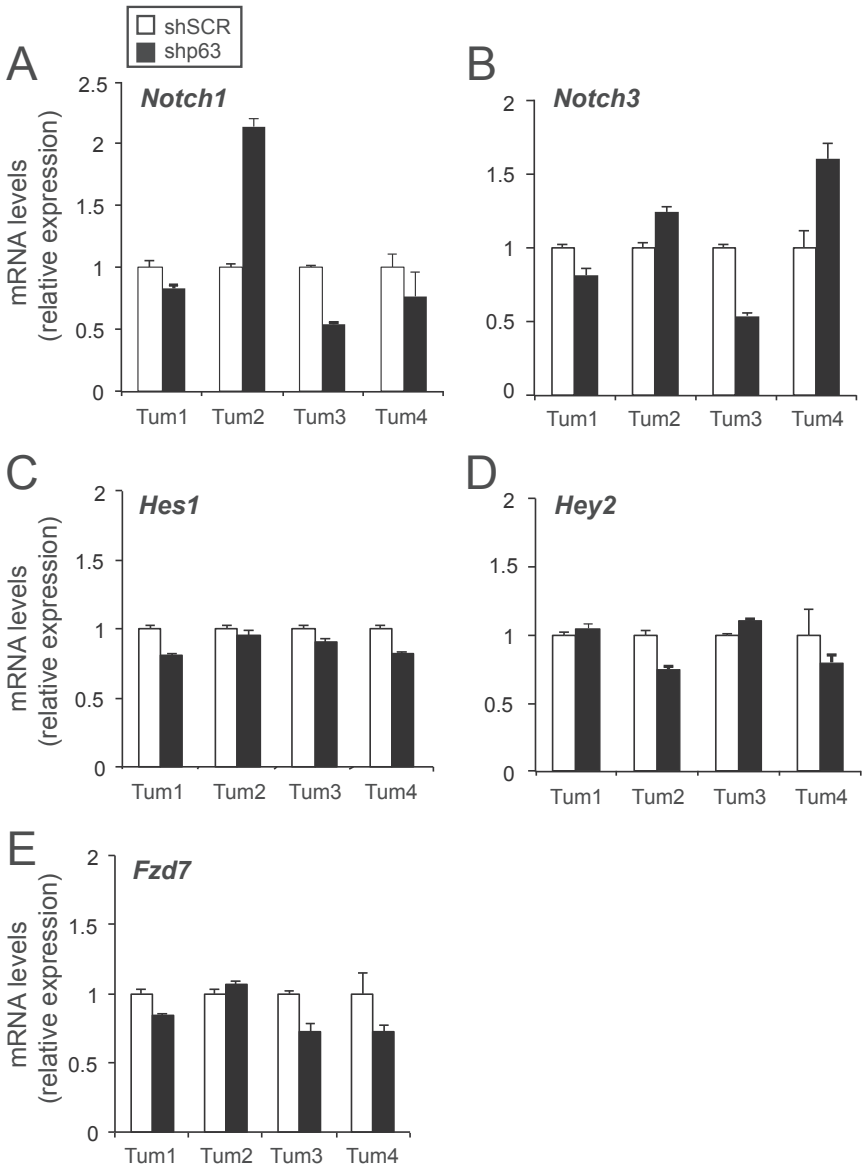


Fig. S3. Expression levels of components of the Notch and Wnt/β-catenin pathways in p63-depleted ErbB2 progenitors. (*A–E*) *Notch1*, *Notch3*, *Hes1*, *Hey*, and *Fzd7* transcript level expression was quantified by RT-qPCR using four independent preparations of RNA obtained from shSCR and shp63 ErbB2 mammospheres. Data are expressed as mean ± SD and are normalized to TBP expression. The resultant values for each group are normalized to the expression levels of the target gene in the shSCR controls.

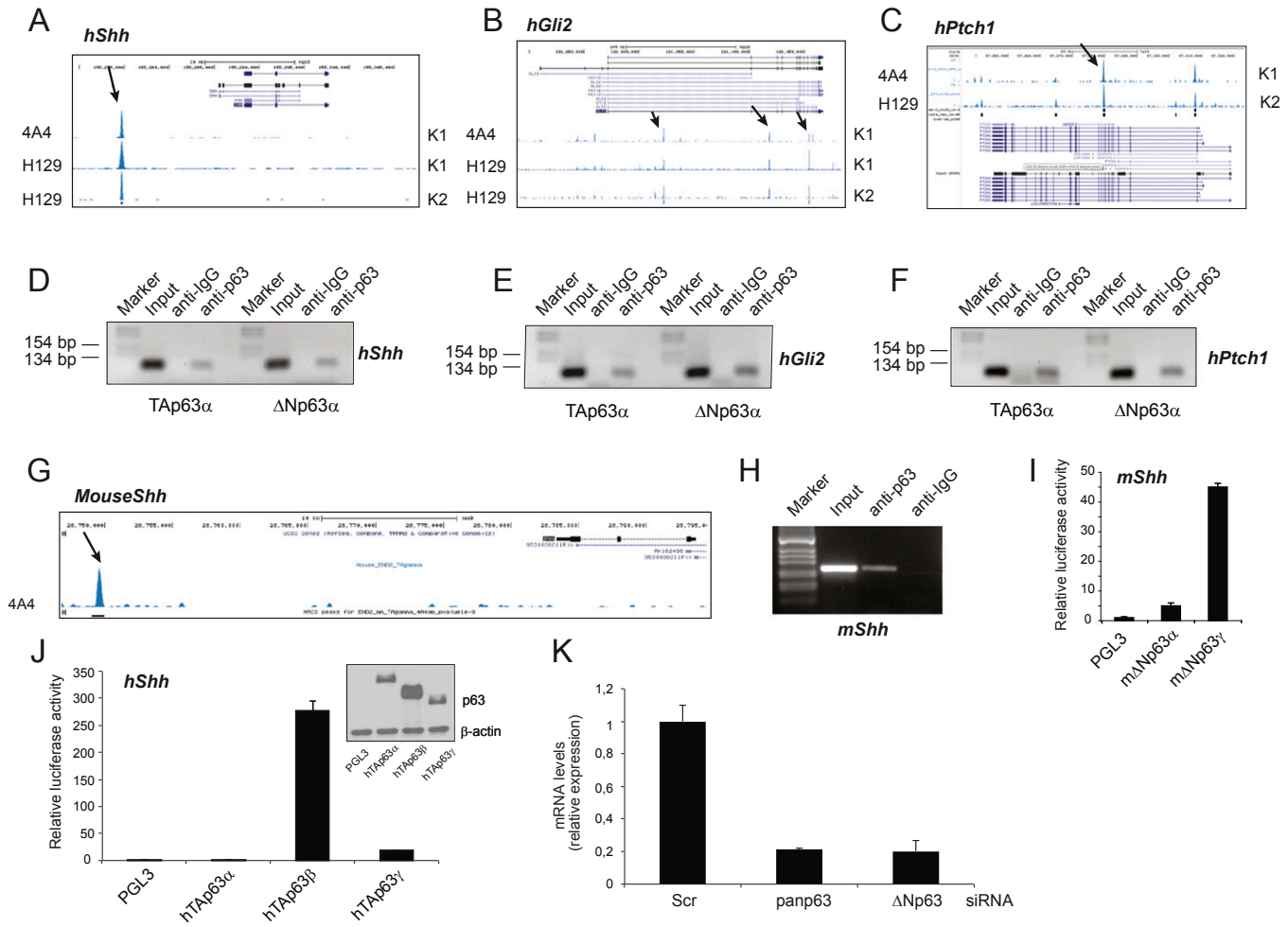
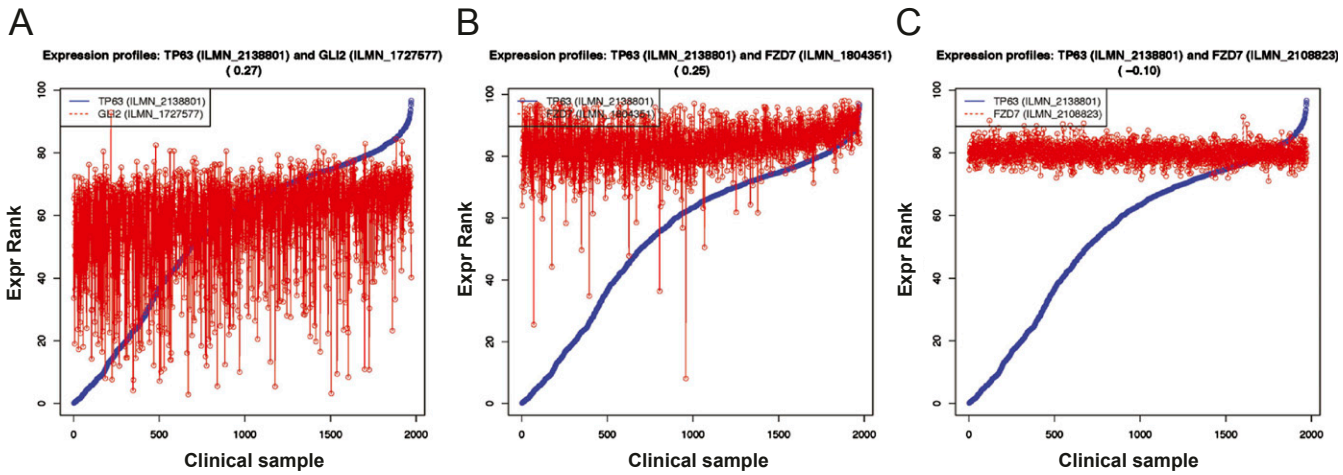


Fig. S4. p63 DNA-binding profiles in the *Shh* (*A*), *Gli2* (*B*), and *Ptch1* (*C*) loci, obtained in human primary epidermal keratinocytes by ChIP-sequencing (ChIP-seq) using 4A4 and H129 anti-p63 antibodies in two normal human primary keratinocyte cell lines (K1 and K2) (1). The majority of p63 peaks are located within genes or in regions upstream of core promoters. (*D–F*) ChIP analysis of TAp63 and ΔNp63 occupancy at the regulatory regions of *Shh* (*Left*), *Gli2* (*Middle*), and *Ptch1 (Right)* genes. ChIP assays were performed in human MCF-7 breast carcinoma cells transfected with HA-TAp63α– or HA-ΔNp63α–expressing vectors using rabbit anti-HA antibody and control IgG. PCR validation was performed using primers spanning the p63-binding sites located within the genomic regions identified by ChIP-seq assays (1). (*G*) Identification of p63-binding regions in the *Shh* mouse locus. The position of the p63-binding site in a genomic region upstream of the mouse *Shh* core promoter (–30 kb from the TSS) was obtained by ChIP-seq using 4A4 anti-p63 antibody in murine endodermal END2 cells (2). (*H*) ChIP assays were carried out in the mouse mammary epithelial NMuMG cell line following overexpression of ΔNp63α using the H129 anti-p63 antibody. The results confirm specific binding of p63 to the putative p63-binding site identified by ChIP-seq analysis. (*I*) Luciferase reporter construct encompassing the p63-binding region within the mouse *Shh* (*mShh*) locus was efficiently induced by ectopic expression of murine ΔNp63α and ΔNp63γ isoforms in H1299. (*J*) H1299 cells were transfected with a pGL3-Luc reporter plasmid containing the p63-binding site within the human *Shh* (*hShh*) locus, along with the TAp63 isoforms. Luciferase assays were carried out 24 h posttransfection using a dual-luciferase reporter assay system. Relative luciferase units were normalized to transfection efficiency by cotransfecting the pRL-TK vector. Data are presented as mean ± SD and are representative of two independent experiments. (*Inset*) Protein extracts were prepared in parallel and subjected to IB for p63 and β-actin detection. (*K*) RT-qPCR analysis of ΔNp63 mRNA levels in HCC1937 cells after treatment with nonsilencing control (Scr), pan-p63, and ΔNp63 siRNA oligos. Expression of p63 isoforms was normalized to the ubiquitin amounts. Results are mean ± SD.

1. Kouwenhoven EN, et al. (2010) Genome-wide profiling of p63 DNA-binding sites identifies an element that regulates gene expression during limb development in the 7q21 SHFM1 locus. *PLoS Genet* 6(8):e1001065.
2. Wolchinsky Z, et al. (2014) Angiomodulin is required for cardiogenesis of embryonic stem cells and is maintained by a feedback loop network of p63 and Activin-A. *Stem Cell Res* 12(1): 49–59.



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TP63	Partner GENE	Pearson correlation	P-value
TP63 (ILMN_2138801)	GLI2(ILMN_1727577)	0.27	0.0085
TP63 (ILMN_2138801)	FZD7(ILMN_1804351)	0.25	0.013
TP63 (ILMN_2138801)	FZD7(ILMN_2108823)	-0.10	0.95

Fig. S5. *p63* expression positively correlates with *Gli2* expression in human patients with breast cancer. Association between p63 and Gli2 (*A*) and Fzd7 (*B* and *C*) mRNA levels in breast tumors from the METABRIC dataset (~2,000 samples). The expression rank (Expr Rank, *y* axis) reflects the relative expression level between *p63* and *Gli2* or *Fzd7*. The Pearson correlation coefficient was computed as a measure of correlation between mRNA profiles of two genes. (*D*) Pearson correlation coefficient and *P* values are indicated.