Tip30 controls differentiation of murine mammary luminal progenitor to estrogen receptor-positive luminal cell through regulating FoxA1 expression

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Estrogen receptor-alpha positive (ER⁺) breast cancers comprise the majority of human breast cancers, but molecular mechanisms underlying this subtype of breast cancers remain poorly understood. Here, we show that ER⁺ mammary luminal tumors arising in Tip30⁻/⁻ MMTV-Neu mice exhibited increased enrichment of luminal progenitor gene signature. Deletion of the Tip30 gene increased proportion of mammary stem and progenitor cell populations, and raised susceptibility to ER⁺ mammary luminal tumors in female Balb/c mice. Moreover, Tip30⁻/⁻ luminal progenitors displayed increases in propensity to differentiate to mature ER⁻ luminal cells and FoxA1 expression. Knockdown of FoxA1 expression in Tip30⁻/⁻ progenitors by shRNA specific for FoxA1 reduced their differentiation toward ER⁺ mature luminal cells. Taken together, our results suggest that Tip30 is a key regulator for maintaining ER⁺ and ER⁻ luminal pools in the mammary luminal lineage, and loss of it promotes expansion of ER⁺ luminal progenitors and mature cells and ER⁺ mammary tumorigenesis.

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TIP30, also known as HTATIP2 or CC3, is implicated in the pathogenesis of several types of human cancers including breast cancer. There has been a body of evidence showing that it acts as a tumor suppressor involved in the regulation of multiple cellular processes including cell proliferation and apoptosis through influencing ER-mediated transcription, EGFR signaling and nuclear importins in cells. Particularly, loss of TIP30 in mice was found to cause ductal hyperplasia in mammary glands early in life and extensive mammary hyperplasia with age. In addition, deletion of the TIP30 gene in MMTV-Neu mouse was shown to result in increased ER luminal cells in the preneoplastic mammary glands and development of ER+/PR- mammary tumors. Despite these observations, the role of TIP30 in regulation of differentiation of mammary stem cells (MaSCs)/progenitors remains unknown. Here, we describe that loss of TIP30 not only promoted expansion of mammary stem and luminal progenitor cells in mammary gland, but also influenced luminal progenitor cells fate via upregulation of FoxA1, leading to an increased subpopulation of mature ER luminal cells, which may contribute to ER+ mammary tumor development.

Results

**Tip30**/MMTV-Neu tumors show enrichment of luminal progenitor gene signature and cluster with a subtype of human HER2+ breast cancer. We previously reported that Tip30 loss promotes development of ER luminal carcinomas in MMTV-Neu mouse model. To further explore the role of Tip30 in ER+ luminal carcinomas development, we arrayed mammary tumors arising in MMTV-Neu and Tip30/+/MMTV-Neu mice. Unsupervised hierarchical clustering revealed a significant difference in Tip30+/MMTV-Neu tumors as compared with control MMTV-Neu tumors (Figure 1a). Consistent with the previous report, EGFR and Ras signaling were further enhanced in Tip30+/MMTV-Neu tumors (Figure 1b). The gene expression profiles of the tumors were then clustered with human breast cancer profiles that had been annotated using intrinsic clustering. In agreement with previous studies, MMTV-Neu tumors do not cluster well with human HER2+ tumors. We noted that Tip30+/MMTV-Neu tumors clustered most closely with human luminal A and B types of breast cancers and secondly with human HER2+ tumors (Supplementary Figure 1A), indicating that Tip30+/MMTV-Neu tumors are mimicking human luminal types of breast cancers. We also used publicly available gene expression data to analyze for a role of these identified differentially expressing genes in overall patient survival. We observed that low levels of major histocompatibility complex (MHC) in HER+ breast cancer samples were associated with poor survival relative to HER+ breast cancer samples with high levels of MHC (Supplementary Figure 1B). Intriguingly, when the gene signatures of mouse luminal progenitor, mature luminal and stem/basal cells in Tip30+/MMTV-Neu tumors versus MMTV-Neu tumors were compared, Tip30 loss resulted in increased enrichment of luminal progenitor gene signature (Figure 1c, \(P = 0.0147\); FDR \(q = 0.0217\)), whereas without significantly affecting enrichment of mature luminal and stem/basal cell signatures (data not shown). This result indicates that Tip30 is a potential regulator controlling proliferation and differentiation of luminal progenitors in the mammary gland.

Loss of Tip30 increases susceptibility to spontaneous ER+/PR+ luminal subtype of mammary tumors. To further study the role of Tip30 in mammary development and tumorigenesis, we monitored a cohort of Tip30+/+ and Tip30+/+ Balb/c female mice for spontaneous tumor development for 18 months. Kaplan–Meier plot analysis revealed that 28.6% of Tip30+/+ mice spontaneously developed mammary tumors with an average latency of 16 months, whereas Tip30+/+ mice did not (Figure 2a,
Histological analysis showed that all tumors arising from Tip30\(^{-/-}\) female mice are moderately differentiated infiltrating ductal carcinomas consisting of atypical epithelial cells arranged as ducts, glands, nests and micropapillary patterns (Figure 2b). Immunohistochemical staining of the mammary tumors arising in Tip30\(^{-/-}\) mice demonstrated that almost all of them were K8-positive, but \(\alpha\)SMA-negative, ER-positive and PR-positive (Figures 2c and d). These results suggest that Tip30 deletion increases susceptibility to the development of ER\(^{+}\)/PR\(^{+}\) luminal subtype of mammary tumors in Balb/c mice.

**Tip30 deletion promotes expansion of stem cell and progenitor cell subpopulations.** To investigate whether Tip30 loss influences the stem/progenitor cell compartment and epithelial differentiation in the mammary gland, we first used an *in vitro* system, in which primary MaSCs are propagated on non-adherent substrata to form mammospheres. The number of mammospheres in the medium reflects the number of stem cells that were plated in the system, whereas the size of mammospheres is a reflection of progenitor cell proliferation. 31 Mammary cells from 5-month-old Tip30\(^{-/-}\) and Tip30\(^{+/+}\) mouse mammary glands were isolated and subsequently plated in the mammosphere medium. Compared with Tip30\(^{+/+}\) mammary cells, Tip30\(^{-/-}\) mammary cells displayed an increased number of mammospheres (Figure 3a, \(P<0.05\)). The average size of mammospheres formed by Tip30\(^{-/-}\) mammary epithelial cells was larger than those formed by Tip30\(^{+/+}\) mammary cells (\(P<0.05\)). Moreover, colony-forming cell assay by seeding mammary cells at low-cell density in matrigel substrata revealed that mammary cells isolated from Tip30\(^{-/-}\) mammary glands generated more colonies with larger size in diameter as compared with mammary cells from Tip30\(^{+/+}\) mammary glands (Figure 3b, \(P<0.05\)). These results indicated that Tip30 deletion

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**Figure 2**  Tip30\(^{-/-}\) balb/c mice developed mammary tumors. (a) Kaplan–Meier curves showing palpable tumors of Tip30\(^{-/-}\) \((n=28)\) and Tip30\(^{+/+}\) \((n=19)\) mice until 18 months. Histogram showing incidence of mammary tumor in Tip30\(^{-/-}\) and Tip30\(^{+/+}\) mice. (b) Representative images of H&E stained sections of mammary tumors in Tip30\(^{-/-}\) mice. Scale bar: 20 \(\mu\)m. (c) Representative images of immunostaining section for CK8 and \(\alpha\)SMA in mammary tumor of Tip30\(^{-/-}\) mice. Scale bar: 10 \(\mu\)m. (d) Representative immunofluorescent staining of ER\(\alpha\) and PRs in mammary tumors of Tip30\(^{-/-}\) mice. Scale bar: 20 \(\mu\)m.
promotes expansion of stem cell and progenitor cell subpopulations.

We next used flow cytometric analyses to identify cell populations and define their differentiation hierarchy in mammary glands. MaSCs are enriched in CD24⁺ CD49fhi population containing mammary repopulating units that are able to generate multilineage outgrowths in vivo, whereas progenitors are enriched in CD24hi CD49fhi population with mammary colony-forming cells that produce discrete colonies of mammary cells. The mammary glands of 5-month-old virgin wild-type and Tip30⁻/⁻ mice were dissected and processed to single-cell suspensions; cells were separated by FACS according to their CD24 and CD49f expression. As shown in Figures 4a and b, CD24⁺ CD49fhi stem cell enriched population was significantly increased by about twofold in mammary epithelium of Tip30⁻/⁻ mice compared with mammary epithelium of wild-type littermates (P < 0.05). Similarly, the CD24hi CD49fhi progenitor cell-enriched population of Tip30⁻/⁻ mammary epithelium was about 3.5-fold higher than that of wild-type mammary epithelium (Figures 4a and b, P < 0.01). These results suggest that Tip30 controls expansion of both MaSCs and progenitor cells.

Loss of Tip30 promotes luminal progenitor cells to ER⁺ luminal cells differentiation. Previous studies have defined Sca1⁺ CD24hiCD49fhi or Sca1⁻ CD24hiCD49fhi cells as ER⁺ or ER⁻ luminal progenitor cells, respectively. To determine whether ER⁺ or ER⁻ luminal progenitors were increased in Tip30⁻/⁻ mammary epithelium, we used Sca1 expression as a sorting marker to separate cells between ER⁺ and ER⁻ luminal progenitors in the CD24hi CD49fhi population. As shown in Figures 4c and d, Sca1⁻ luminal progenitor cells were significantly increased in Tip30⁻/⁻ mammary glands compared with that in Tip30⁺/+ mammary glands (P < 0.05). Consistent with the previous observations on ERx expression in Sca1⁻ luminal progenitor cells, we also observed that the majority of (~77%) of Sca1⁺ CD24hi CD49fhi luminal cells and only a minor portion (~26%) of Sca1⁻ CD24hi CD49fhi luminal cells were ER⁺ cells. To test whether these progenitor population committed to ER⁺ cell differentiation fate, CD24hi CD49fhi populations from Tip30⁻/⁻ and Tip30⁺/+ mammary glands were sorted out and subjected to in vitro colony-forming cell assay. Indeed, colonies formed by Tip30⁻/⁻ progenitor cells had a significantly higher percentage of ER⁺ cells compared with those formed by Tip30⁺/+ progenitors (Figures 5a and b, P < 0.05). However, percentage of PR⁻ cells in colonies
formed by Tip30\(^{-/-}\) progenitors was slightly, but not significantly increased when compared with Tip30\(^{+/+}\) progenitors. Consistent with these observations, we found that the number of ER\(^+\) cells was significantly increased in Tip30\(^{-/-}\) mammary glands as compared with Tip30\(^{+/+}\) mammary glands, whereas PR\(^+\) cells were slightly, but not significantly, increased (Figures 5c and d). As suggested by previous studies,\(^{24,34,35}\) statistically insignificant increase in PR\(^+\) cells observed in Tip30\(^{-/-}\) mammary glands might be due to PR proteins being rapidly turned over in cells.\(^{24}\) Taken together, these results indicated that loss of Tip30 promotes luminal progenitor cells to ER\(^+\) mature luminal cell differentiation.

**Regulation of luminal progenitor cell fate by Tip30 is mediated by FoxA1.** Transcription factors Gata-3 and FoxA1 as well as several components in Notch, Hedgehog and Wnt mediated signaling pathways are critical in the regulation of differentiation of MaSCs to the luminal cell lineage in both humans and mice.\(^{36,37}\) To test whether expression of these factors in mammary glands is influenced by Tip30 loss, quantitative RT-PCR analysis was performed to measure mRNA levels of Notch1-4, iHh, sHh, Wnt1, Gata-3 and FoxA1 in wild-type and Tip30\(^{-/-}\) mammary glands. We found the mRNA level of FoxA1 was markedly elevated in Tip30\(^{-/-}\) mammary gland compared with Tip30\(^{+/+}\), whereas mRNA level of Notch1-4, iHh, sHh, Wnt1 or Gata-3 was not significantly changed (Figure 6a). Western blot analysis of FoxA1 protein levels in wild-type and Tip30\(^{-/-}\) mammary glands further confirmed increased FoxA1 expression in Tip30\(^{-/-}\) mammary glands (Figures 6b and c). Importantly, knockdown of FoxA1 expression significantly reduced proportion of ER\(^+\) cells in colonies formed by Tip30\(^{-/-}\) mammary stem/progenitor cells (Figures 6d–f, \(P < 0.05\)). In agreement with the role of Tip30 in regulation of EGFR/PI3K signaling, FoxA1 expression in Tip30\(^{-/-}\) mammary epithelial cells was significantly reduced after the cells were treated with LY294002, a specific PI3K kinase inhibitor or NVP-BEZ235, a PI3K/mTOR dual kinase inhibitor (Supplementary Data). Taken together, these results

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**Figure 5** Tip30 deletion predisposed mammary luminal progenitors to commitment of ER\(^+\) luminal cell differentiation. (a) Representative immunofluorescent staining of ER\(\alpha\) and PR in colonies formed by progenitor cells from Tip30\(^{-/-}\) and Tip30\(^{+/+}\) mammary gland. Mammary epithelial cells isolated from 5-month-old virgin mice were processed to single-cell suspensions and stained with CD24 and CD49f. Progenitor cell-enriched population (CD24\(^{high}\)CD49f\(^{+}\)) was sorted out and plated in Matrigel-coated 48-well plates. After cultured for 9 days, colonies were immuno stained for ER\(\alpha\) and PR-A. Scale bar: 50 \(\mu\)m. (b) The histogram shows the percentage of ER\(\alpha\)-positive and PR-A-positive cells in the colonies. Error bars represent the mean \(\pm\) S.E.M. of three mice per group. (c) Representative immunofluorescent staining of ER\(\alpha\) and PR-A in mammary glands from 5-month-old Tip30\(^{-/-}\) and Tip30\(^{+/+}\) mice. Scale bar: 20 \(\mu\)m. (d) The histogram shows the percentage of ER\(\alpha\)-positive and PR-A-positive cells in mammary glands. The bar graphs denote the mean \(\pm\) S.E.M. of three animals per group. Scale bar: 20 \(\mu\)m.
suggest that loss of Tip30 predisposes luminal progenitor cells to the commitment of ER$^+$ cells differentiation, in part, through an increase in FoxA1 expression.

**Discussion**

Elucidating the regulation of mammary cell differentiation hierarchy may lead to the identification of novel factors in the regulation of initiation and progression of various subtypes of breast cancer. Our present data demonstrated that loss of Tip30 in the mammary epithelium results in increased proportion of stem/progenitor cells in murine mammary epithelium and higher propensity of differentiation of luminal progenitors to ER$^+$ mature luminal cells both in vivo and in ex vivo. The effect of Tip30 loss on the propensity of cell differentiation to ER$^+$ mature luminal cells was likely
mediated by the transcription factor FoxA1. Thus, our study uncovers a critical role for Tip30 in the maintenance of the normal differentiation of luminal progenitors. In addition, we show that Tip30 deletion promotes spontaneous development of ER⁺ PR⁺ luminal types of mammary tumors in aged mice.

The present observations of FoxA1 effect on the luminal progenitor differentiation are in agreement with recent findings that FoxA1 is a key regulator in controlling expression of luminal and basal genes in ER⁺ and ER⁻ breast cancer cells.36,38,39 FoxA1 has been shown to be required not only for both ER expression and transcriptional activity, but also for ductal lineage expansion.38,39 Moreover, FoxA1 expression has been shown to positively correlate with ER⁺/PR⁺ luminal breast carcinomas.40 Using gene expression profiling and gene set enrichment analysis on breast cancer cell lines and breast cancer specimens, reducing FoxA1 expression has been demonstrated to cause a partial shift from luminal to basal gene signatures and basal cancer phenotypes.39 Our data are not inconsistent with these previous findings, but rather add further insight into FoxA1 function by suggesting a regulatory pathway mediated by FoxA1 and Tip30 in events controlling the expansion of ER⁺ luminal cells and ER⁺ mammary luminal tumor development. In support of this hypothesis, our results demonstrate that loss of Tip30 increases the number of Sca1⁺ CD24⁺/CD49f⁺ luminal progenitor that has been shown to be ER⁺-positive,32 suggesting that Tip30 regulates luminal progenitor cell fate. In addition, we previously reported that Tip30 acts in both luminal and basal gene signatures and basal cancer phenotypes.38 Our data further support our findings regarding a role for Tip30 in the maintenance of the basal gene signatures and basal cancer phenotypes.38

The present observations of FoxA1 effect on the luminal progenitor and mature luminal cells, which are rarely proliferative and generally growth-arrested by inhibitory factors.41,42 Evidently, the majority of human breast cancers at the time of diagnosis were ER⁺ ductal carcinomas containing proliferative ER⁻ cancer cells. Moreover, clustered ER⁺ proliferating cells were frequently detected in ductal hyperplasia and ductal carcinoma in situ, which are significantly associated with human ER⁺ breast cancer risk.43 It has been proposed that loss of such inhibitory factors may instigate carcinogenesis of ER⁻ breast cancers.44,45 Given the observations that loss of Tip30 resulted in mammary hyperplasia in female mice of various genetic backgrounds47 and increased expression of luminal progenitor gene signature in ER⁺ mammary tumors arising in MMTV-Neu mice, we speculate that Tip30 is one of the inhibitory factors in the suppression of differentiation and proliferation of ER⁺ luminal cells. Loss of Tip30 may result in expansion of ER⁺ luminal progenitor and mature luminal cells, which provides more cell targets for subsequent oncogenic events. Nonetheless, the present work has not provided any direct evidence supporting aberrantly increased ER⁺ progenitors as the cell of origin for ER⁺ luminal tumors. Thus, further studies will be needed to determine the expanded ER⁺ luminal progenitors in Tip30⁻/⁻ mammary glands are the cell of origin for ER⁺ luminal tumors.

In summary, we present the first evidence that Tip30 is a key factor regulating luminal progenitor differentiation by controlling FoxA1 expression. Loss of Tip30 increases susceptibility to ER⁺ mammary tumorigenesis. However, whether ER⁺ luminal progenitor is the target cell for ER⁺ mammary tumorigenesis remains undetermined. Future studies on determination of the cell of origin for ER⁺ luminal breast cancers may provide further insight to the understanding of the pathogenesis of ER⁺ breast cancers. Moreover, identification of the oncogenic pathways that are activated by impaired Tip30 function may prove to be useful in the development of new preventive and therapeutic strategies for ER⁺ breast cancers.

Materials and Methods

**Animal studies.** Tip30⁻/⁻ Balb/c mice were obtained by backcrossing Balb/c mice with Tip30⁻/⁻ C57BL/6 mice seven generations as described previously.46 All mice were housed in Animal Facility at Michigan State University. Animal protocols used for this study were approved by Michigan State University IACUC Committee.

**Histopathology.** Mammary glands and other tissues were removed, fixed in 4% paraformaldehyde solution and then paraffin-embedded. Sections were stained with H&E and examined by two trained investigators and a pathologist who read slides blind to experimental detail. Fresh mammary tissues were frozen with liquid nitrogen after being rinsed with PBS and stored at −80 °C for western blot assay.

**Immunohistochemistry and immunofluorescence.** Immunohistochemistry and immunofluorescence for paraffin-embedded tissues were carried out as described previously.24 Primary antibodies against α-SMA (clone E184, 1:50; Millipore, Billerica, MA, USA), CK8 (ks8.7, 1:50; Santa Cruz Biotechnology, Dallas, TX, USA), ERα (MC-20, 1:50; Santa Cruz Biotechnology) or PR-A (hPRa7, 1:50; Labvision, Fremont, CA, USA) were used. Alexa-488- or Alexa-594-conjugated secondary antibody (1:200; Molecular Probes, Carlsbad, CA, USA) and 4′,6-diamidino-2-phenylindole for immunofluorescence were used. Immunofluorescence staining of in vitro cultures was as described previously.24 Briefly, colonies were extracted from matrigel by using PBS-EDTA and then spun to glass slides with a Cytospin4 centrifuge (Thermo Scientific, Kalamazoo, MI, USA). The colonies were fixed with 4% paraformaldehyde, followed by the immunofluorescence staining procedures. Slides were analyzed by confocal laser scanning microscopy (Carl Zeiss, Oberkochen, Germany).

**Mammary cell preparation.** Mammary glands from 5-month-old virgin female Balb/c mice were collected and the finely minced tissue was transferred to a digestion mix consisting of serum-free DMEM/F12 (Gibco, Grand Island, NY, USA) containing 0.3 Wünsch units/ml collagenase (Roche, Branford, CT, USA) and 1.5 mg/ml trypsin (Gibco) and then processed to single cells, as previously described.47

**Mammosphere assays.** Single-cell suspensions were grown (5000 cells/well) in 96-well Ultra Low Attachment plates (Corning, NY, USA) with serum-free DMEM/F12 medium (Gibco) containing 20 ng/ml EGF, 20 ng/ml bFGF, 5 mg/ml insulin, 2% B27 and 4 μg/ml heparin (Sigma, St. Louis, MO, USA) for 10 days in 37 °C, 5% CO₂ incubator. Mammospheres were imaged under microscopy and then calculated for the number and diameter from five randomly selected fields per sample at identical magnifications. Only mammospheres exceeding 60 μm in diameter were counted.

**Colony-forming assay.** 96-well plates were coated with 15 μl growth factor reduced Matrigel (BD Biosciences, San Jose, CA, USA) per well. Two thousand cells were seeded to a well with 1:1 DMEM/F12 medium (Invitrogen) containing...
10% growth factor reduced Matrigel, 10% FBS (Gibco), 5 μg/ml insulin (Sigma), 10 ng/ml cholera toxin (Sigma) and 10 ng/ml epidermal growth factor (Sigma) and cultured at 37 °C in 5% CO2 in an incubator for 10 days. Photomicrographs were taken and the colony number and size were calculated as described in Mammosphere assays.

Flow cytometry. CD45 /TER119 - , and CD31 - cells were removed from dissociated cells using the EasySep kit selection (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer’s instructions. Cells were resuspended in HBSS (Invitrogen) with 2% FBS and incubated with anti-CD44-PE (10 μg/ml, Mouse mammary cell enrichment kit, StemCell Technologies), anti-CD49f- FITC (10 μg/ml, Mouse mammary cell enrichment kit, StemCell Technologies) or anti-Scal-1-APC (0.06 μg/ml, ebioscience, San Diego, CA, USA) for 30 min on ice, followed by washing and resuspending in HBSS supplemented with 2% FBS. Analysis and sorting were performed by using a FACS Vantage Turbo Sort SE (BD Biosciences). The data were analyzed using BD FACS Software sorter software. For analysis of ERα expression in Scal-1 CD24-CD49f- and Scal-1 CD24+CD49f- luminal cell populations, sorted cells from Tip30 + /- virgin mice (n = 6) were collected and allowed to adhere to a collagen coated 96-well plate before fixation and then analyzed by immuno-cytometry with anti- ERα (MC-20, 1: 50; Santa Cruz Biotechnology) and inverted microscopy (Nikon Eclipse Ti, Melville, NY, USA).

Real-time reverse transcription-PCR. Total RNA was isolated using the RNeasy mini kit (Qiagen, Germantown, MD, USA). Reverse transcription was performed using SuperScript III reverse transcriptase (Invitrogen) and Oligo(dT) primer (Invitrogen). The relative gene expressions were measured by real-time RT-PCR using the gene-specific primers and qSYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The RNA polymerase II (RPII) was the endogenous control for each cDNA sample. Primers used were as follows: Notch1, forward: 5'–AACCAACAGGAGTGTAGCCTG-3', reverse: 5’–ACCGCTGTCATCTGATATG-3'; Notch2, forward: 5’–TGAGCCTGTCCCTACTAGG-3’, reverse: 5’–CACGTCTTGCATTGCTCTGTCAAGT-3'; Notch3, forward: 5’–GAAGACCTGCTTGAAAGATTTGAC-3’, reverse: 5’–TGAGCTTCTCCCTCTTTATGCC-3'; Notch4, forward: 5’–CCTGAGTCTGCTGAAAGAACGCTTGC-3’, reverse: 5’–CTCTGATGGCGCCAACTCCTG-3'; shHh, forward: 5’–TGGGCGGTTACAAAGAACAT-3’, Gata-3, forward: 5’–AGGCCATCTTCTCTCCACG-3’, reverse: 5’–AGGGCCTGCTTCTCTAAAC-3'; FoxA1, forward: 5’–CCATTCTTCTCTCTTCCAC-3’, reverse: 5’–TGGGCTTTACAAACAAACA-3’; Wnt1, forward: 5’–ACAGCAACAAGCTGGTACG-3’, reverse: 5’–GAATTCCGTACACAGGTGTCG-3’.

Western blotting. Protein extraction from mouse mammary glands and primary cells, and western blotting were carried out as previously described.27 Primary antibodies for FoxA1 (H-120, 1: 500; Santa Cruz Biotechnology), pAkt (193H12, 1: 1000; Cell Signaling Technology) and Akt (11E7, 1: 1000; Cell Signaling Technology) were used. Gene expression analysis. Gene expression data was generated using Affymetrix mouse genome 430A 2.0 gene chips and will be available at the GEO at NCBI (USA). Unsupervised hierarchical clustering was conducted using Cluster 3.0 and results were viewed using Java Tree View (Free Software Foundation Inc., Boston, MA, USA software). Ras and EGFR pathway predictions were conducted as previously described28 Gene set enrichment analysis was conducted as described29 using a gene set derived from genes that are upregulated in luminal progenitor cells.30

Statistics. Results were represented as mean ± S.E.M. or ± S.D. as indicated. Statistical significance was assessed with a two-tailed, unpaired t-test. Tumor-free curves were compared using a log-rank (Mantel–Cox) test. Tumor incidences were evaluated with a χ2-test.

Conflict of Interest
The authors declare no conflict of interest.

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1. Boccirolfo WP, Korach KS. Mammary gland development and tumorigenesis in estrogen receptor knockout mice. J Mammary Gland Biol Neoplasia 1997; 2: 323–334.
2. Anderson E. The role of estrogen and progesterone receptors in human mammary development and tumorigenesis. Breast Cancer Res 2002; 4: 197–201.
3. Alred DC, Brown P, Medina D. The origins of estrogen receptor alpha-positive and estrogen receptor alpha-negative human breast cancer. Breast Cancer Res 2004; 6: 240–247.
4. Shackleton M, Vaillant F, Simpson KJ, Stirling J, Smyth GK, Asselin-Labat ML et al. Generation of a functional mammary gland from a single stem cell. Nature 2006; 439: 84–88.
5. Stirling J. Estrogen and progesterone in normal mammary gland development and in cancer. Horm Cancer 2011; 2: 85–90.
6. Stirling J, Racaud O, Elrew P, Evans CJ. Dephosphorylation of the mammary epithelial cell hierarchy. Cell Cycle 2006; 5: 1519–1522.
7. Villadsen R, Fridkisoktedt AJ, Ronnov-Jessen L, Gudjonsson T, Rank F, LeBarge MA et al. Evidence for a stem cell hierarchy in the adult human breast. J Cell Biol 2007; 177: 87–101.
8. Booth BW, Smith GH. Estrogen receptor-alpha and progesterone receptor are expressed in label-retaining mammary epithelial cells that divide asymmetrically and retain their template DNA strands. Breast Cancer Res 2006; 8: R49.
9. Shyamala G, Chou VC, Cardif RD, Vargas E. Effect of c-neu ErbB2 expression levels on estrogen receptor alpha-dependent proliferation in mammary epithelial cells: implications for breast cancer biology. Cancer Res 2006; 66: 10391–10398.
10. Shenata H, Teschendorf A, Sharp G, Novic N, Russell IA, Avril S et al. Phenotypic and functional characterisation of the luminal cellular hierarchy of the mammary gland. Breast Cancer Res 2012; 14: R34.
11. Liu BY, McDermott SP, Khwaja SS, Alexander CM. The transforming activity of c-neu correlates with their ability to induce the accumulation of mammary progenitor cells. Proc Natl Acad Sci USA 2004; 101: 4158–4163.
12. Li Y, Weim B, Podyapynska K, Huang M, Chamorro M, Zhang X et al. Evidence that transgenic encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. Proc Natl Acad Sci USA 2003; 100: 15853–15858.
13. Oakes SR, Naylor MJ, Asselin-Labat ML, Blazek KD, Gardner-Garden M, Hilton HH et al. The Ets transcription factor Elf5 specifies mammary alveolar cell fate. Genes Dev 2008; 22: 591–596.
14. Vaillant F, Asselin-Labat ML, Shackleton M, Forrest NC, Lindeman GJ, Visvader JE. The mammary progenitor marker CD61/β1 integrin identifies cancer stem cells in mouse models of mammary tumorigenesis. Cancer Res 2008; 68: 7711–7717.
15. Jesselnit R, Brown NE, Arnedt L, Kieba I, Hu MO, Kupferman C et al. Cyclin D1 kinase activity is required for the self-renewal of mammary stem and progenitor cells that are targets of MMTV-ErbB2 tumorigenesis. Cancer Cell 2010; 17: 65–76.
16. Henry MD, Triplett AA, Oh KB, Smith GH, Wagner KU. Parity-induced mammary epithelial cell proliferation precedes tumorigenesis. Cancer Res 2008; 68: 7711–7717.
17. Molyneux G, Geyer FC, Magnay FA, McCarthy A, Kendrick H, Natrajan R et al. BRCA1 basal-like breast cancers originate from luminal epithelial progenitors and not from basal stem cells. Cell Stem Cell 2010; 7: 403–417.
18. Xiao H, Tao Y, Greenblatt J, Roeder RG. A cofactor, TIP30, specifically enhances HIV-1 Tat-activated transcription. Proc Natl Acad Sci USA 1998; 95: 2146–2151.
19. Shriver E. A link between metastasis and resistance to apoptosis of variant small cell lung carcinoma. Oncogene 1997; 14: 2167–2173.
20. Ito M, Jiang C, Kumm K, Zhang X, Pechia J, Zhao J et al. TIP30 deficiency increases susceptibility to tumorigenesis. Cancer Res 2003; 63: 8765–8767.

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