ΔNp63 promotes stem cell activity in mammary gland development and basal-like breast cancer by enhancing Fzd7 expression and Wnt signalling

Rumela Chakrabarti¹, Yong Wei¹, Julie Hwang¹, Xiang Hang¹, Mario Andres Blanco¹,², Abrar Choudhury¹, Benjamin Tiede¹, Rose-Anne Romano³, Christina DeCoste¹, Laura Mercatali⁴, Toni Ibrahim⁴, Dino Amadori⁴, Nagarajan Kannan³, Connie J. Eaves⁵, Satrajit Sinha⁵ and Yibin Kang¹,⁶,⁷

Emerging evidence suggests that cancer is populated and maintained by tumour-initiating cells (TICs) with stem-like properties similar to those of adult tissue stem cells. Despite recent advances, the molecular regulatory mechanisms that may be shared between normal and malignant stem cells remain poorly understood. Here we show that the ΔNp63 isoform of the Trp63 transcription factor promotes normal mammary stem cell (MaSC) activity by increasing the expression of the Wnt receptor Fzd7, thereby enhancing Wnt signalling. Importantly, Fzd7-dependent enhancement of Wnt signalling by ΔNp63 also governs tumour-initiating activity of the basal subtype of breast cancer. These findings establish ΔNp63 as a key regulator of stem cells in both normal and malignant mammary tissues and provide direct evidence that breast cancer TICs and normal MaSCs share common regulatory mechanisms.

The mechanisms and pathways regulating self-renewal and differentiation of MaSCs are of great interest for their potential application in the prevention and treatment of breast cancer¹. TICs, also known as cancer stem cells, play key roles in treatment resistance, recurrence, and metastasis of breast cancer and other malignant diseases²–⁴, and may share similar features and regulatory mechanisms with normal stem cells. However, despite significant progress in recent years, the molecular basis underlying the putative link between TICs and normal tissue stem cells remains poorly characterized.

Recent studies have highlighted several transcription factors, such as Bmi-1, Oct1, p53, Snail, Gata3 and Elf5, as key regulators with multiple roles in mammary cell fate determination, stem cell activity, and tumour initiation and progression⁵–¹⁰. Transformation-related protein 63 (Trp63, or p63) is a member of the p53/p63/p73 family of transcription factors that is highly expressed in stratified epithelia such as cervix, skin, prostate and breast¹¹. p63 proteins include isoforms of two main groups: those possessing a full-length transactivation domain (referred to as the TA isoforms), and those lacking this domain (the ΔN isoforms)¹¹,¹². The role of p63 in development and cancer has remained perplexing largely because of these multiple isoforms of p63. Both increased and reduced expression of p63 have been observed in human cancers¹³,¹⁴. Disparate results regarding the role of p63 in cancer have also been reported using different p63 mouse models¹⁵,¹⁶. It has been suggested that the TA isoforms are most similar to p53 in their tumour-suppressive functions¹⁷,¹⁸ and the ΔNp63 isoforms generally exhibit oncogenic functions, such as in skin and bladder¹⁹,²⁰. In the normal mammary gland, ΔNp63 is shown to be expressed at much higher levels than TAp63 (refs 21–24). Recent studies have also revealed a role for ΔNp63 in maintaining basal lineage cell fate in vitro in mammary epithelia cells²⁵. Despite these findings, rigorous functional studies of p63 isoforms in regulating MaSCs and TICs remain scarce and further investigation is warranted.

The role of p63 in regulating epithelial homeostasis has been linked to its influence on several signalling pathways such as Notch, Wnt and Shh (refs 26–29), which have all been shown to be important regulators of normal and cancerous stem cells³⁰,³¹. The extent to which these pathways are involved in p63-dependent regulation of...
**Figure 1** ΔNp63 is enriched in MaSCs and promotes MaSC activity. (a) Heatmap of differential expression of 26 transcriptional regulators, including *p63* (arrow), in different mouse MEC subpopulations (upper panel), qRT-PCR analysis of ΔNp63 and TAp63 mRNAs in MECs (lower panel; *n* = 3 samples; data represent mean ± s.d.), qRT-PCR values were normalized to Gapdh. (b) Immunoblot of ΔNp63 expression in different mouse MEC subpopulations. (c) Immunohistochemical analysis of ΔNp63 and TAp63 expression in the terminal end buds (TEBs) and ducts of mammary gland serial sections. (d) qRT-PCR analysis of ΔNp63 and TAp63 mRNAs in normal human mammary epithelium (left panel, *n* = 3 samples; data represent mean ± s.d.) and CD49f<sup>hi</sup>EpCAM<sup>−</sup>MaSC-enriched population (right panel, *n* = 3 samples; data represent mean ± s.d.). qRT-PCR values were normalized to GAPDH. (e) Strategy of lentiviral transduction of different MEC subpopulations. (f) The MaSC-enriched Lin<sup>−</sup>CD24<sup>−</sup>CD29<sup>−</sup> population (P4) was transduced with the indicated lentivirus constructs and transplanted into cleared fat pads (1,000 cells). (g) 1,000 luminal cells (Lin<sup>−</sup>CD24<sup>+</sup>CD29<sup>+</sup>) (P5) were transduced with the indicated lentivirus constructs and injected into cleared fat pad. Representative mammary outgrowths are shown in f and g. In the pie graph below the representative images, each circle represents one mammary gland, with the blackened area representing the degree of gland filling with outgrowth. (h) 0–30%, (i) 30–80%, (j) 80–100%, (k) 0–30% and (l) no reconstitution. (h,i) Pie graph (h) and representative fluorescence images (i) of the mammary fat pads after a competitive reconstitution assay. (j) Representative images of 3D Matrigel colonies of P4 population from GFP− and dsRED−MECs mixed equally. (k) Number of colonies formed in three generations in a 3D Matrigel assay (*n* = 3 samples; data represent mean ± s.d.). (l) Table showing reconstitution rate of the mammary gland over successive generations after transplantation of 10,000 unsorted MECs transduced with control or ΔNp63-expressing lentiviruses. *P* < 0.05, **P** < 0.01 by Student’s *t*-test. Scale bars, 40 μm in c, 1 mm in f and g, 2 mm in i, and 3 mm in j. Uncropped images of blots are shown in Supplementary Fig. 9.
Figure 2 ΔNp63 deficiency reduces MaSC activity. (a,b) Box plot (a) and western blot (b) of ΔNp63 expression in P4 cells from WT and ΔNp63<sup>Gfp/+</sup> mice. The boxes in a represent the 75th, 50th and 25th percentile of the values. The whiskers represent maximum and minimum data points within the 1.5 x IQ (inner quarter) range. P value computed by Mann–Whitney U test (n = 5 samples per genotype). (c) Representative mammary outgrowths from WT and ΔNp63<sup>Gfp/+</sup> mice. (d) Representative FACS profiles of MECs from WT and ΔNp63<sup>Gfp/+</sup> mice. (e) Representative mammary outgrowths from f. Each circle below the images represents one mammary gland, with the blackened area representing the degree of gland filling with outgrowth. • 80–100%, ○ 50–80%, ▼ 0–30% and □ no reconstitution. (f) Table showing transplantation of limiting numbers of P4 cells into cleared mammary fat pads from the indicated mice (n = number of mammary fat pad injections as indicated in the table). P value was obtained by Pearson’s χ<sup>2</sup> test using ELDA software. (g) Fold change (FC) of ΔNp63 mRNA level in primary MECs transduced with control and two ΔNp63 shRNA lentiviral constructs (∆N KD1 and ∆N KD2) (left panel). Fold change of TAp63 mRNA level in control or ΔNp63-KD MECs (right panel). (h) Western blot of ΔNp63 in control and ΔNp63-KD MECs. (i) Fold change of TAp63 mRNA level in primary MECs transduced with control and two TAp63 shRNA lentiviral constructs. TA isoform-specific KD of TAp63 was confirmed by qRT-PCR because TAp63 protein is undetectable in MECs. (j) Quantification of mammospheres formed by control, ΔNp63-KD and TAp63-KD P4 cells (20,000 cells) from WT mouse mammary glands. In g, i and j, n = 3 samples; data represent mean ± s.d. *P < 0.05 by Student’s t-test. (k) GSEA demonstrating enrichment of MaSC gene signatures in WT versus ΔNp63<sup>Gfp/+</sup> MECs using MaSC signatures derived from the current study (left panel) or a previously reported MaSC gene signature<sup>63</sup> (right panel). Scale bars, 2 mm and 4 mm in c and e respectively. Uncropped images of blots are shown in Supplementary Fig. 9.

mammary epithelium remains unclear. Notably, Wnt signalling has been shown to be instrumental for mammary gland development and MaSC activity<sup>32–34</sup>. aberrant Wnt signalling has also been reported in tumours from patients with different types of cancer such as colorectal cancer, hepatocellular carcinoma, hepatoblastoma and breast cancer<sup>35–37</sup>. In breast cancer, Wnt signalling has been found to be particularly hyperactive in the basal-like subtype and predicts poor prognosis<sup>38,39</sup>. However, what regulates Wnt signalling in normal MaSCs and the highly aggressive basal subtype of breast cancer remains poorly understood. In general, oncogenic mutations in Wnt pathway components, such as β-catenin, APC and Axin, are relatively rare in breast cancer<sup>40,41</sup>. In this study, we dissect the role of different p63 isoforms in MaSCs and breast cancer TICs. Using multiple isoform-specific molecular and genetic tools and models, we demonstrate the important function of ΔNp63 in regulating MaSC activity and promoting breast cancer initiation in basal-like breast cancer through direct transcriptional activation of Fzd7 and subsequent enhancement of Wnt signalling.
RESULTS

**ΔNp63 but not TAp63 regulates MaSC activity**

To identify candidate regulators of MaSCs, we performed transcriptomic analyses of different mammary epithelial cell (MEC) subpopulations isolated by fluorescence-activated cell sorting (FACS) from the mouse mammary epithelium, including Lin−CD24+CD29high (P4, representing MaSC-enriched basal populations) and Lin−CD24+CD29low (P5, representing luminal cells). Global transcriptome analyses revealed 26 transcriptional regulators that have a >3-fold higher expression in P4 versus P5 populations (Fig. 1a, upper panel, and Supplementary Table 1). Among these, p63 expression is most strongly elevated by more than 45-fold in the P4 population. Isoform-specific messenger RNA and protein analyses confirmed that ΔNp63, but not TAp63, is the primary p63 isoform expressed in primary MECs and MaSC-enriched MECs in both mice and humans (Fig. 1a–d and Supplementary Fig. 1a).

Figure 3 ΔNp63 but not TAp63 increases Wnt signalling through direct activation of Fzd7 expression. (a) GSEA of Wnt gene signatures in unsorted (upper panels) or P4 (lower panels) WT versus ΔNp63gfp/+ MECs. (b) Relative expression of Wnt luciferase reporter in P4 cells after lentiviral transduction (upper panels) or P4 (lower panels) WT versus ΔNp63gfp/+ (P4) subpopulations isolated by fluorescence-activated cell transcriptomic analyses of different mammary epithelial cell populations) and Lin−CD24+CD29high (P5, representing luminal to the housekeeping GAPDH. Data represent mean ± s.d. (i) ChIP-seq data from human keratinocytes showing the location of the ΔNp63 binding region (highlighted by a red rectangle around −40,000) in a putative Fzd7 enhancer. The orange rectangle highlights various histone modification marks, DNA hypersensitivity sites (DNAse) and mammalian conserved areas (mamm cons). (j) ChIP analysis of p63 (using H129 and 4A4 antibody) binding to the Fzd7 enhancer in human MECs (n = 3 samples; data represent mean ± s.d.). (k,l) Relative expression of Fzd7 enhancer-driven luciferase reporter in HMLE cells (I) and immortalized murine MECs (m) transiently transfected with the indicated expression plasmids (n = 3 samples; data represent mean ± s.d.). *P < 0.05 by Student’s t-test, # not statistically significant. Uncropped images of blots are shown in Supplementary Fig. 9.
To explore the functional importance of ΔNp63 in MaSCs, ΔNp63- and TAp63-expressing lentiviral vectors were transduced into MECs (Fig. 1e and Supplementary Fig. 1b,c), and robust ectopic overexpression of both genes was confirmed (Supplementary Fig. 1d,e). Interestingly, overexpression of ΔNp63, but not TAp63, in P4 cells led to a significant increase in MaSC activity, as reflected by increased reconstitution and ductal outgrowth after transplantation into cleared fat pads (Fig. 1f and Supplementary Fig. 1f,g). Importantly, ΔNp63 overexpression also conferred stem cell activity to P5 cells, indicating that ΔNp63 is able to induce luminal cells to enter a stem-like state (Fig. 1g and Supplementary Fig. 1h,i). We next measured ΔNp63-induced enhancement of MaSC activity using a more stringent competitive reconstitution assay (Supplementary Fig. 1j). Transplantation of ΔNp63−dsRED+ and control−GFP+ P4 cells in an equal ratio resulted in outgrowths that were predominantly dsRED+, suggesting that ΔNp63-overexpressing MaSCs had a competitive growth advantage over their normal counterparts (Fig. 1h,i). In contrast, overexpression of TAp63 did not augment MaSC ability (Fig. 1h,i), Collectively, these studies demonstrate that ΔNp63, but not TAp63, promotes MaSC activity.

We next examined the function of ΔNp63 in different subpopulations of luminal cells. CD61 has been described to be a luminal progenitor marker among luminal epithelial cells (P5). Overexpression of ΔNp63 increased the repopulating frequency in both P5-CD61+ and P5-CD61− populations (Supplementary Fig. 2a,b). Furthermore, immunostaining of three-dimensional (3D) Matrigel colonies and mammary outgrowths from ΔNp63-overexpressing CD61+ and CD61− luminal subsets with cytokeratin 14 and 8 (K14 and K8) markers revealed cells from both basal and luminal lineages, suggesting the capability to differentiate into both lineages (Supplementary Fig. 2c,d). Interestingly, ΔNp63 overexpression failed to significantly increase reconstitution in Lin−CD24low (P6) stromal-enriched cells (Supplementary Fig. 2e,f), suggesting that its function is restricted to epithelial cells.

ΔNp63-overexpressing outgrowths showed increased tertiary branching and an increased percentage of Ki67−positive cells (Fig. 1f).

Table 1. Competitive reconstitution assay

| Number of cells (P4) | Control | ΔNp63 | ΔNp63 + Fzd7 KD |
|----------------------|---------|-------|----------------|
| 1,000                | 12/12   | 6/12  | 8/12           |
| 500                  | 4/8     | 8/8   | 6/8            |
| 250                  | 0/8     | 5/6   |                |
| Repopulating frequency (95% interval) | 1/384 (1/209–1/709) | 1/1,787 (1/806–1/3,964) | 1/642 (1/349–1/1,189) |
| P value              | 0.002   | 0.03  |                |

Figure 4 ΔNp63-mediated MaSC function is dependent on Fzd7. (a) Quantification of colonies formed by P4 cells transduced with indicated lentiviruses (n=3 samples; data represent mean ± s.d.). (b) P4 cells were transduced with control or Fzd7 lentiviruses and limiting numbers of cells were transplanted into cleared fat pads. The table shows take rate quantifications. (c) Quantification of colonies formed by P4 cells from WT and ΔNp63gfp/+ mice with or without Fzd7 overexpression (n = 5 samples; data represent mean ± s.d.). (d) Representative images of colonies from c. (e) Table showing reconstitution efficiency at limiting dilution of P4 cells into WT and ΔNp63gfp/+ mice with Fzd7 overexpression. (f) Representative images and pie graph summary of outgrowths from e. (g) Table showing reconstitution efficiency at limiting dilution of P4 cells from control and ΔNp63-overexpressing cells with or without Fzd7 KD. (h) Representative images and pie graph summary of outgrowths from g. © 2014 Macmillan Publishers Limited. All rights reserved.
Relative Wnt signalling in human breast cancer. (a) ΔNp63 expression in clinical samples of basal and luminal type human tumours. The box represents the 75th, 50th and 25th percentile of the values. The whiskers represent maximum and minimum data points within the 1.5 x IQR (inter quartile) range (n = 286 tumours). (b) Scatter plot showing correlation between ΔNp63 and FZD7 expression in breast tumours from the TCGA-BRCA-RNAseqV2 data set (n = 1,000 tumours). (c) Analysis of ΔNp63 and FZD7 expression in MDA-MB-231 and SUM-1315 cells. (d) Western blot of ΔNp63, TAp63 and FZD7 expression in MDA-MB-231 and SUM-1315 cells. (f,g) Western blots of ΔNp63 and FZD7 expression in MDA-MB-231 and SUM-1315 cells after transduction with indicated shRNAs. (h) Fold change of the TAp63 mRNA level in SUM-1315 and MDA-MB-231 cells respectively, after transduction with control and TAp63 shRNA. (i) Relative expression of Wnt luciferase reporter in MDA-MB-231 cells after lentiviral transduction of a Wnt reporter (7TFNC), together with indicated shRNAs. (j) Tumour incidence of MDA-MB-231 cells expressing the indicated shRNAs. (k) Tumour incidence of SUM-1315 cells expressing the indicated shRNAs. (l) Tumour incidence of MDA-MB-231 cells showing a 1.5 x IQR (inter quartile) range (n = 3 samples, data represent mean ± s.d.). (m) Representative images of tumourspheres from control and TAp63-KD cells (10,000 cells) from PDX-2 (HCI002; ref. 48). (n) Quantification of tumourspheres formed by control, p63-KD and Fzd7-KD tumours (10,000 cells) from PDX-2 (HCI002) (n-o) and PDX-3 tumours (HCI009) (p). In l,n-p, n = 4 tumours; data represent mean ± s.d.

As shown in Fig. 9, the increased proliferation may in part account for the enhanced branching associated with ΔNp63 overexpression. An increased presence of ΔNp63- and K14-positive basal cells (Supplementary Fig. 3c–e) is consistent with FACS analysis of outgrowths showing a ~2-fold increase of P4 population in the ΔNp63-overexpressing mammary epithelium (Supplementary Fig. 3f,g).

We next investigated the effect of ΔNp63 and TAp63 overexpression on colony-forming abilities. A significant increase in clonogenic potential was observed in MaSCs overexpressing ΔNp63 compared with TAp63 in primary 3D Matrigel cultures (Supplementary Fig. 4a,b). Monochromatic colonies formed by mixed P4 cells from actin–GFP and actin–DsRed mammary glands confirmed that each colony originates from single cells instead of aggregates (Fig. 1j). We next performed serial passage and transplantation assays both in vitro and in vivo, which confirmed that ΔNp63 can induce MaSC self-renewal activity (Fig. 1k,l and Supplementary Fig. 4c).

To further test whether the observed ΔNp63 phenotype is dependent on its transcriptional activity, we tested a DNA-binding mutant (MT) form of ΔNp63 in P4 cells. Although similar levels of MT and wild-type (WT) ΔNp63 were overexpressed in these cells (Supplementary Fig. 4d,e), only WT ΔNp63 induced the increased MaSC activity as shown by the Matrigel colony formation and reconstitution assays (Supplementary Fig. 4f–h).
Loss of ΔNp63 leads to MaSC deficiency

To complement our gain-of-function analysis of ΔNp63 as described above, we next used a ΔNp63–GFP knock-in loss-of-function mouse model (Supplementary Fig. 5a). As homozygous loss of ΔNp63 leads to embryonic lethality, we performed all of our experiments using heterozygous ΔNp63<sup>+/−</sup> animals with reduced expression of ΔNp63 (Fig. 2a,b). We observed a significant reduction of ductal elongation in ΔNp63<sup>+/−</sup> mammary glands compared with those of WT counterparts at both 6 weeks and 9–10 weeks (Fig. 2c and Supplementary Fig. 5b–d). FACS and limiting dilution assays also demonstrated a reduced number and activity of MaSCs in ΔNp63<sup>+/−</sup> mice (Fig. 2d–f and Supplementary Fig. 5b,d,e).

To further evaluate the loss-of-function effect of individual p63 isoforms, we next performed isomeric-specific knockdown (KD) of...
Figure 7 Loss of ΔNp63 and Fzd7 attenuates tumour initiation in MMTV-Myc tumours. (a) Western blot analysis of Fzd7 expression in primary MMTV-Myc tumour cells transduced with the indicated shRNAs. (b,c) Reduced level of ΔNp63 mRNA (b) and protein (c) expression in primary MMTV-Myc tumour cells with or without p63 KD. *P < 0.05 by Mann–Whitney U test and n = 5 tumours per group in b. The box represents the 75th, 50th and 25th percentiles of the values. The whiskers lines represent the maximum and minimal data points within the 1.5×IQR (inter quarter range). (d,e) Freshly isolated tumour MECs with or without p63 KD were used for tumoursphere assays in low adherent plates. Bar graphs show quantification of tumoursphere numbers in control and p63-KD MECs (10,000 cells; n = 4 tumours; data represent mean ± s.d.). (f,g) Kaplan–Meier curves showing mammary gland tumour-free survival using MMTV-Myc tumour MECs transduced with control and p63 shRNAs (p63 KD1 and p63 KD2). 10,000 cells (n = 6 mice per group) and 5,000 cells (n = 10 mice per group) were injected in f and g, respectively. Log rank test was used for statistical analysis and P values computed for both plots are <0.001. (h,i) Tumour incidence (h) and volume (i) were calculated at the end point. P value was obtained by Pearson’s chi² test using ELDA software in h (n = number of mammary fat pad injections as indicated in the table). *P < 0.05 by Mann–Whitney U test in i (n = 6 tumours per group; data represent mean ± s.d.). P value was computed by Student’s t-test. *P < 0.05. Scale bar, 40 μm in c. Uncropped images of blots are shown in Supplementary Fig. 9.

ΔNp63 and TAp63 in mouse MECs (Fig. 2g–i). As expected, only ΔNp63 KD led to significantly reduced mammosphere numbers, suggesting decreased stem cell activity (Fig. 2j). Together, these results indicate that ΔNp63 but not TAp63 is a crucial regulator of normal MaSCs.

To obtain a global view of the molecular events underlying the loss of the ΔNp63 phenotype, we next performed a microarray analysis of control and ΔNp63ΔNp63KD MECs (Fig. 2k). Gene-set enrichment analysis (GSEA) demonstrated that MaSC-enriched genes were significantly downregulated in the transcriptome of ΔNp63ΔNp63KD MECs (Fig. 2k), a finding consistent with the decreased MaSC activity of ΔNp63ΔNp63KD MECs.

Wnt receptor Fzd7 is a ΔNp63 target that mediates its effect on MaSCs

Multiple lines of evidence suggest that Notch, Wnt and Shh signalling is important for stem cell activity in different tissues32,33. Therefore, we next investigated whether any of these pathways were altered in ΔNp63ΔNp63KD MECs. GSEA demonstrated that only Wnt signalling gene sets were significantly downregulated in both MECs and P4 cells from ΔNp63ΔNp63KD mice compared with WT (Fig. 3a). To directly test whether ΔNp63-regulated Wnt signalling is important for MaSC function, we performed colony-formation assays with and without the Wnt signalling inhibitor Dickkoph 1 (Dkk1). Dkk1 inhibited the ΔNp63-mediated increase in colony formation of both primary cells and cells in subsequent serial passages (Supplementary Fig. 5f–h), suggesting that ΔNp63-induced MaSC activity is indeed mediated by Wnt signalling. Moreover, a Wnt reporter assay confirmed the positive regulation of canonical Wnt signalling in MaSCs by ΔNp63 but not TAp63 (Fig. 3b).

We next sought to identify Wnt signalling genes regulated by ΔNp63. Transcriptome profiling indicated that nine Wnt pathway genes, including several Wnt ligands and receptors, have reduced expression in ΔNp63ΔNp63KD P4 cells compared with WT (Fig. 3c). Quantitative real-time PCR (qRT-PCR) analysis confirmed four of these nine genes to be significantly downregulated in ΔNp63ΔNp63KD MaSCs (Fig. 3d). Among these, only one receptor and one ligand gene, Fzd7 and Wnt5a, have reduced expression after acute lentivirus-
mediated KD of p63 in primary MECs (Fig. 3e). Notably, both Wnt5a and Fzd7 belonged to the 'enrichment cores' of two WNT gene signatures used in the GSEA study (Supplementary Fig. 6a). Therefore, we reasoned that ΔNp63 may regulate MaSCs through direct activation of either Fzd7 or Wnt5a, or both. To further investigate this possibility, we next performed isoform-specific KD of p63. Reduced

**Figure 8** Loss of ΔNp63 attenuates Wnt1-driven hyperplasia and tumorigenesis. (a) Representative mammary gland whole mounts from 8-week-old WT; ΔNp63+/+, MMTV–Wnt1; ΔNp63+/− and MMTV–Wnt1; ΔNp63gfp/+ mice. (b) Representative FACS profiles of mammary epithelial cells from the indicated mammary glands from different genotypes. (c) Quantification of mammospheres formed by P4 cells (10,000 cells) from 8-week-old mice of the indicated genotypes (n = 3 samples, data represent mean ± s.d.). *P < 0.05 by Student’s t-test. (d) Table showing reconstitution efficiency at limiting dilution of P4 cells from mice of the indicated genotypes (n = number of mammary fat pad injections as indicated in the table). P value was obtained by Pearson’s χ² test using ELDA software. (e) Representative images of outgrowths from d. (f) Kaplan–Meier curve analysis of tumour-free mammary glands at the indicated ages (n = 12 mice for MMTV–Wnt1; ΔNp63+/− and n = 10 mice for MMTV–Wnt1; ΔNp63gfp/+). Log rank test was used for statistical analysis. (g) Schematic model for function of the ΔNp63–Fzd7 axis in mammary cell fate regulation and basal breast cancer initiation. High expression of ΔNp63 in mammary stem cells and TICs maintains their self-renewal abilities by activating Fzd7 expression and Wnt signalling. High expression of ΔNp63 can also confer luminal differentiated/progenitor cells with MaSC-like properties. TICs in basal-like breast cancer may arise from the oncogenic transformation of MaSCs with intrinsically elevated expression of ΔNp63 or from progenitor cells that have acquired high ΔNp63 expression during their transformation. Scale bars, 3 mm and 2 mm respectively in a and e.
levels of Fzd7 and not Wnt5a were observed with ΔNp63 KDs but not Tap63 KDs (Fig. 3f,g). Conversely, enforced overexpression of ΔNp63 but not Tap63 led to a significant increase in the expression of both WNT5A and FZD7 in the HMLE human mammary epithelial line (Fig. 3h, right panel, and Supplementary Fig. 6b). Interestingly, only FZD7 showed strong activation by ΔNp63 within 48 h post-infection, suggesting it to be a direct target of ΔNp63 (Fig. 3h, left panel). Indeed, examination of chromatin immunoprecipitation sequencing (ChiP-seq) data from human keratinocytes revealed p63 binding in a conserved active enhancer region ~40 kilobases (kb) upstream from the FZD7 gene (Fig. 3i, region highlighted in red). To confirm binding of p63 to this enhancer, we performed ChiP analysis of primary human MECs and primary mouse MMTV-Wnt1 tumour cells using anti-p63 antibodies, and observed strong enrichment of the FZD7 enhancer in p63-bound chromatin (Fig. 3j,k). We next performed luciferase assays using a reporter driven by the FZD7 enhancer, together with ΔNp63, Tap63, or a DNA-binding mutant of ΔNp63. As expected, only ΔNp63 overexpression significantly activated FZD7 enhancer reporter activity in HMLE cells and immortalized murine MECs (ref. 45; Fig. 3l,m).

We next determined the direct functional importance of Fzd7 in MaSCs. Fzd7 overexpression in P4 cells led to increased MaSC activities, phenocopying the effect of ΔNp63 (Fig. 4a,b). Moreover, overexpression of Fzd7 in ΔNp63(+/−) P4 cells was able to at least partially rescue the reduced MaSC phenotype close to that of the WT levels in both colony-formation (Fig. 4c,d) and repopulation assays (Fig. 4e,f). Similarly, Fzd7 overexpression rescued MaSC activity in ΔNp63-KD MECs (Supplementary Fig. 6c,d). Finally, Fzd7 KD attenuated the increase in MaSC activity driven by ΔNp63 overexpression (Fig. 4g,h). Collectively, these data suggest that ΔNp63-induced MaSC activity is primarily mediated by increasing the expression of the Wnt signalling receptor Fzd7.

Positive correlation of ΔNp63 and Fzd7 expression in human tumours

To evaluate the relationship between ΔNp63 and overall WNT signalling activity in the clinical setting, we interrogated two human breast cancer data sets. Consistent with the ΔNp63(+/−) mouse data (Fig. 3a), GSEA of the EMCA286 data set revealed a significant enrichment of WNT signalling gene sets in human breast tumour samples predicted to have high ΔNp63 activity on the basis of their ΔNp63-specific gene signature score (Supplementary Fig. 6e). High ΔNp63 signature expression is preferentially associated with the basal subtype over the luminal subtype (Fig. 5a) and the ΔNp63 signature score showed a positive correlation with Fzd7 expression (Supplementary Fig. 6f). Further confirming this observation, analysis of a TCGA data set containing isoform-specific RNA-Seq data revealed ΔNp63 as the primary p63 isoform expressed in tumours (Supplementary Fig. 6g) and a strong positive correlation between ΔNp63 and FZD7 mRNA levels (Fig. 5b). Consistently, immunostaining of ΔNp63 and FZD7 in 12 primary human breast tumours revealed a similar positive correlation (r = 0.47, P = 0.06) based on staining intensity score (Pearson correlation; Fig. 5c), and both proteins were found to be upregulated in the triple-negative (TN) breast tumours compared with the luminal subtype (P = 0.04 and P = 0.03 for ΔNp63 and FZD7 respectively; Fig. 5c). To further test the functional link between p63 and canonical Wnt signalling, we next performed Wnt reporter assays in the MDA-MB-231 breast cancer cell line. p63 KD led to a significant reduction of Fzd7 protein level (Supplementary Fig. 7a), as well as inhibition of WNT3A-induced canonical Wnt signalling (Fig. 5d), to an extent comparable to Fzd7 KD. As expected, p63 KD also led to reduced tumour engraftment of TN MDA-MB-231 and SUM-151 cells (Supplementary Fig. 7b,c). Furthermore, KD of ΔNp63 but not Tap63 (Fig. 5e–h) led to significant inhibition of Wnt signalling (Fig. 5i), similar to pan-p63 KD using short hairpin RNAs (shRNAs) targeting sequences encoding the DNA-binding domain (DBD). Importantly, ΔNp63 but not Tap63 KD reduced tumour incidence to a degree similar to pan-p63 KD or Fzd7 KD (Fig. 5j,k), and resulted in reduced Fzd7 expression (Fig. 5f,g).

To further strengthen the molecular connection between ΔNp63 and Fzd7 in human breast cancer, we next investigated the functional role of ΔNp63 and Fzd7 in three patient-derived xenografts (PDXs) of human TN breast tumours. Immunohistochemistry (IHC) on PDX tumours indicates strong expression of ΔNp63 and Fzd7 in all three PDX tumours compared with weak Tap63 staining in PDX-1 and PDX-3 tumours (Supplementary Fig. 7d). Lentiviral KD of ΔNp63 and Fzd7 in PDX tumours (PDX-2 and PDX-3) significantly reduced tumoursphere formation compared with control and Tap63 KD (Fig. 5l–p). Taken together, our data suggest that the ΔNp63–Fzd7–Wnt signalling axis is conserved in human breast cancer and regulates tumour formation.

ΔNp63 induces tumour-initiating ability in basal-like mouse mammary tumours

To more rigorously test the functional role of ΔNp63-regulated Fzd7 expression in controlling TIC activity of basal-like breast cancer cells, we next performed IHC on tumours derived from three different mouse models representing different subtypes of breast cancer based on gene expression profiling—MMTV-Wnt1 (basal), MMTV–Myc (mixed basal and luminal) and MMTV–PyMT (luminal). As expected, higher expression of ΔNp63 and Fzd7 was observed in the basal subtype (MMTV–Wnt1) compared with luminal subtype (MMTV–PyMT) tumours (Supplementary Fig. 7f,g), further supporting observations from human breast cancers. Modest staining for ΔNp63 and Fzd7 was also observed in MMTV–Myc tumours. In contrast, no positive Tap63 staining was observed in any tumour subtype (Supplementary Fig. 7e). Functionally, p63 KD in MMTV–Wnt1 tumour cells significantly reduced tumoursphere formation in vitro (Fig. 6a,b) and increased tumour latency and decreased tumour volume in vivo (Fig. 6c–e).

A small subset of CD45−CD24+Thy-1+ cells in the MMTV–Wnt1 tumours is thought to be endowed with TIC properties. We found that both p63-KD and Fzd7-KD tumour cells showed a significant reduction of the Thy-1+ population (Fig. 6f,g). Fzd7 expression was significantly decreased (Fig. 6h) after p63 KD, further suggesting that Fzd7 as a direct target of ΔNp63. Consistent with the isoform-specific role of ΔNp63 in regulating TIC activity, KD of ΔNp63 but not Tap63 reduced Fzd7 expression and tumoursphere formation (Fig. 6i–k).

We next directly investigated whether Fzd7 has any functional significance in Wnt1-driven tumour cells both in vitro and in vivo. Interestingly, Fzd7 KD in Wnt1 tumour cells decreased tumoursphere formation (Fig. 6l), phenocopying ΔNp63 KD. Furthermore, the
reduced tumoursphere-forming activity of ΔNp63-KD Wnt1 tumour cells was rescued by ectopic expression of Fzd7 (Fig. 6l). Corroborating the in vitro data, Fzd7-KD cells showed increased tumour latency and significantly reduced tumour burden, phenocopying ΔNp63 KD (Fig. 6m–o). However, one potential caveat of using the MMTV–Wnt1 tumour model to investigate the p63–Fzd7 regulatory axis is that Wnt1-induced tumours may depend on Fzd7 regardless of p63. To circumvent this problem, we performed p63 and Fzd7 KD in both MMTV–Myc and Blg–Cre–Brca1/I1/p53+/− tumours that are not induced by Wnt. ΔNp63 KD in MMTV–Myc tumours yielded similar results to ΔNp63 KD in MMTV–Wnt1 tumours, such as decreased tumoursphere formation, increased tumour latency, and significantly reduced tumour burden (Fig. 7a–i). Furthermore, Fzd7 KD also led to reduced tumoursphere-forming ability of these two additional basal-like mouse mammary tumour models (Fig. 7j,k), suggesting a common role for Fzd7 in regulating basal-like tumours.

To more definitively examine the role of ΔNp63 in basal-like breast cancer, we generated MMTV–Wnt1; ΔNp63β/β/+ mice. Expression of the Wnt1 oncogene under the MMTV promoter led to extensive ductal hyperplasia and expansion of the MaSC-enriched basal (P4) population32, a phenotype that is reduced in ΔNp63β/β/+ mice (Fig. 8a,b). Consistently, MMTV–Wnt1; ΔNp63β/β/+ MECs exhibited reduced MaSC activity (Fig. 8c–e). Reduced expression of ΔNp63 in ΔNp63β/β/+ mice also led to a significant delay in tumour initiation as well as a reduction of Fzd7 and basal marker K14 expression (Fig. 8f and Supplementary Fig. 8a–c). These results indicate that ΔNp63 activity contributes to the regulation of the TIC population in Wnt1-induced pre-neoplasia and basal tumours.

**DISCUSSION**

p63 is a member of the p53 superfamily that is structurally similar but functionally distinct from p53 (ref. 11). The function of p63 has also been extended from regulating stem cell activity, differentiation, growth and survival to other aspects of cancer biology, such as cell adhesion52 and metastasis13. Despite these advances, the functional role of p63 in regulating MaSCs remains unclear. Overexpression of ΔNp63 in unsorted MECs in vitro was found to result in an increase in basal cells but reduced regenerative potential by antagonizing Notch25. In the present study, we used isoform-specific lentiviral overexpression in primary MaSCs to show that ΔNp63 overexpression promotes MaSC activity and genetic ablation of even one allele of ΔNp63 reduces MaSC function in vivo. We also demonstrated that the ΔNp63 isoform regulates these phenotypes through upregulating Wnt signalling whereas the TAp63 isoform does not have such effects, highlighting the importance of isoform-specific studies of the p63 gene in development and cancer. Furthermore, ΔNp63 overexpression can convert luminal cells to a stem-like state, supporting the emerging concept of reversible plasticity in epithelial cell fate3,4,8,25,33.

The role of ΔNp63 in breast cancer remained largely unclear despite some recent exploration of p63 functionality in breast cancer initiation54. We establish a functional role for ΔNp63 in regulating TICs in basal breast cancer, an aggressive form of breast cancer with few available treatment options55,56. The ΔNp63-specific gene signature is highly enriched in this subtype and ΔNp63-specific KD severely diminishes TIC activity of basal breast cancer cells. Taken together, our studies establish ΔNp63 as a functional driver of normal and malignant stem cells in the mammary epithelium (Fig. 8g).

FZD7 is one of the most abundant Frizzled family proteins expressed in basal/TN breast cancer, and has been shown to promote proliferation of these tumours48,57,58. However, the role of Fzd7 in mammary gland development and the mechanism of its elevated expression in basal/TN breast cancer remain poorly defined. We identified Fzd7 as a direct transcriptional target of ΔNp63 and demonstrated that Fzd7 functions downstream of ΔNp63 in regulating MaSC activity. Despite the strong correlation of Fzd7 and ΔNp63 expression in clinical samples, we do not rule out the possibility that Fzd7 expression could be regulated by additional mechanisms. Our findings thus establish a paradigm for Wnt pathway activation in basal-like breast cancer that does not depend on oncogenic mutation of Wnt pathway components, as commonly seen in colorectal cancer, hepatocellular carcinoma and other cancers36,40,41,59. The ΔNp63–FZD7–Wnt axis may represent a driving force for the initiation and maintenance of basal-like breast tumour, thus serving as a potential target for therapeutic intervention.

The cell of origin of TICs in different subtypes of breast cancer remains ill-defined despite some recent advances. Gene expression profiling and targeted disruption of BRCA1 in mouse models suggest that luminal progenitors are a likely target cell population of basal-like breast cancer harbouring BRCA1 mutations55,60,61. In the present study, heterozygous loss of ΔNp63 reduces expansion of the MaSC-enriched P4 population in the pre-neoplastic mammary gland hyperplasia, as well as a reduction of the Thy-1+ TIC population in MMTV–Wnt1 tumours. These findings suggest that MaSCs may be the cell of origin in another subset of basal-like breast cancers that is dependent on the ΔNp63–Fzd7–Wnt axis. Given the heterogeneity of basal-like breast cancers, it is not surprising that under the influence of different oncogenic events, distinct target cell populations in the normal gland may give rise to TICs in different subsets of basal-like breast cancer. It is also possible that during oncogenic transformation of luminal progenitors, MaSC-like features as well as elevated ΔNp63 expression might be acquired in TICs or their pre-neoplastic precursors that eventually give rise to basal-like cancers (Fig. 8g). Future studies are required to rigorously explore these various possibilities.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Supplementary Information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

R.C. and Y.K. designed experiments. R.C., Y.W., J.H., X.H., M.A.B., A.C., B.T., N.K. and S.S. performed the experiments. T.I., L.M., D.A., R.A.R., C.D., N.K. and C.J.E.
provided crucial samples and technical advice. R.C. and Y.K. wrote the manuscript. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.
METHODS

Animal studies. Animal procedures were conducted in compliance with the Institutional Animal Care and Use Committee (IACUC) of Princeton University. The ΔNp63-GFP knock-in mouse model has been described previously40. For all experiments, WT (ΔNp63−/−, shRNA−/− littermates, all genotypes were matched) and TAp63-KD (ΔNp63−/−, shRNA+/−, p63+−/−) mice were used. For Western blot analysis, cells from WT and ΔNp63−/− mice were cultured in DMEM/F12 media containing 10% fetal bovine serum, 20 ng/ml EGF, 20 ng/ml cholera toxin and 500 ng/ml insulin, 20 ng/ml insulin, 20 ng/ml cholera toxin and 500 ng/ml insulin. For orthotopic primary tumour formation, FVB mice were used, because MMTV-Wnt1 or MMTV-cMyc animals for this experiment were on the FVB background. MMTV-Wnt1 or MMTV-cMyc tumour cells were used, because MMTV-Wnt1 or MMTV-cMyc animals for this experiment were on the FVB background.

The targeting shRNA sequences are: 5′-GGAGCCTGAAATTTCTGTA-3′ for ΔNp63 KD1, 5′-GCCACCTGAATTCTGTA-3′ for ΔNp63 KD2, 5′-GGTTACTTGAAGACTCTTA-3′ for Tap63 KD1 and 5′-CCCCTCCTGTGTGATCAA-3′ for Tap63 KD2.

For general and isoform-specific p63-KD studies in human cells, the targeting shRNA sequences are: 5′-CAGAGTTGTTCTCTC-3′ for ΔNp63, 5′-CAGAGTTGTTCTCTC-3′ for ΔNp63, 5′-GCAGCTAATGAAATGCT-3′ for ΔNp63, 5′-GCCACCTGAAATTTCTGTA-3′ for ΔNp63, 5′-GCCACCTGAAATTTCTGTA-3′ for ΔNp63, 5′-GGGAACAGCCATGCCCAGTATG-3′ for p63 BD, and 5′-GCCGCTGTGCTGCTCTCCTGA-3′ for Fzd7.

Viral production and infection. For lentivirus-mediated overexpression or knockdown studies, expression plasmids and shRNA constructs are described above. All plasmids were packaged into viruses using HEK293T-17 cells as packaging cell lines and helper plasmids VSVG and dR8 following standard protocols. Primarily cells were spin-infected with virus-containing media supplemented with 2 μg/ml Polybrene for 2 h at 1,000 g at 4°C and then either plated in Matrigel or transplanted. Self-renewal activity of transduced MaSCs after transplantation was tested by their ability to regenerate functional mammary glandular tissues in virgin and pregnant mice as shown in Supplementary Fig. 1c. For HMLE cells, virally infected cells were selected with puromycin. The retroviral vectors were transduced into the H292 packaging cell line. Viruses were collected 48–72 h after transfection. Cells were infected with viral media in the presence of 5 μg/ml Polybrene. The infected cells were selected with puromycin.

Luciferase enhancer reporter assay. HMLE or iMMEC cells were seeded in 24-well plates 24 h before transfection. The following day, 600 ng of the WT-ΔNp63, WT-ΔNp63 and WT-TA-p63 expression plasmids was co-transfected along with 400 ng of enhancer reporter plasmid (Fzd7 enhancer or TK control) and 100 ng of internal control plasmid that constitutively expresses Renilla luciferase using Lipofectamine 2000 (Invitrogen). Cells were collected 24 h post-transfection and assayed for luciferase activity using the Glomax 96 microplate luminometer (Promega).

Wnt reporter assay. P4 cells were co-transduced with the 7TFC Wnt reporter41 and either control, ΔNp63 or TaP63 virus and then plated on gelatin. After 48 h, cells were treated with Wnt3a (R & D Systems) for 24 h. Cells were then selected for infected cells by sorting for mCherry and a luciferase assay was performed as previously described42. MDA-MB-231 cells were co-transduced with the 7TFC Wnt reporter41 and either control, ΔNp63-KD, TaP63-KD or Fzd7-KD virus and then plated. The luciferase assay was performed using a method similar to that for MaSCs.

Protein extraction and western blot analysis. Proteins were extracted from primary epithelial cell cultures and cell lines in RIPA buffer as previously described43. Western blot analysis was performed using a standard protocol. Antibodies and dilutions used are listed in Supplementary Table 2.

Histological analysis, immunohistochemistry and immunofluorescence. For histological analysis, mammary gland specimens were processed as previously described44. Antibodies and dilutions used are listed in Supplementary Table 2. DAPI was used to stain nuclei. Confocal images were taken using a Nikon A1 confocal microscope.

Immunohistochemical analysis of breast tumour specimens. Normal mammary tissues and breast tumour specimens used in the study were de-identified samples and were considered exempt by Institutional Review Boards of Princeton University and the Rutgers New Jersey Medical School, as well as the Area Vasta Romagna Ethics Committee (CEIAB IRB IRCCS), Modena, Italy. Immunohistochemistry of ΔNp63 and Fzd7 was performed by the Tissue Analytic Core Facility in the Rutgers Cancer Institute of New Jersey. Anti-ΔNp63, anti-TaP63 and anti-Fzd7 (Sigma-Aldrich, Cat. no. AV14251) were first optimized...
on regular human breast tissue slides using a Ventana Medical Systems Discovery XT automated immunostainer. Slides were processed as previously described66. Anti-Np63, anti-TAp63 and anti-Fzd7 antibody were applied at dilutions of 1:80, 1:80 and 1:100 respectively and incubated at room temperature for two hours. Twelve primary human breast tumour samples were used for immunohistochemical staining in our ΔNp63/Fzd7 expression correlation study. Each sample was scored as negative (0), low (1), medium (2) or high (3) according to ΔNp63 and Fzd7 staining abundance. A tumour with a mean score larger than 1 was considered to be positive for the expression of the gene. Pearson’s coefficient test was performed to assess statistical significance.

qRT-PCR analyses. Total RNA was isolated from primary cells using the Qiagen RNA extraction kit in accordance with the manufacturer’s instructions. Real-time RT-PCR was performed on an ABI 7900HT 96 series PCR machine (Applied Biosystems) using SYBR Green Supermix (Bio-Rad Laboratories). The gene-specific primer sets were used at a final concentration of 0.2μM and their sequences are listed in Supplementary Table 3. All qRT-PCR assays were performed in duplicate in at least three independent experiments using three different tissue samples.

Microarray analysis. The P4, P5 and P6 subpopulations of MECs were isolated from the mammary glands (4 mammary glands from each group) of virgin mice. MECs were isolated using FACS as described in ref. 32. The unsorted whole MECs or sorted P4 cells from WT and ΔNp63+/− mice (C57/B6 strain) at 7 weeks of age were prepared as described. RNA was collected from these samples using the RNAeasy kit (Qiagen) according to the manufacturer’s instructions. The gene expression profiles of various populations of MECs from the WT and ΔNp63−/+ mice were determined using the Agilent mouse GE 4×44K two-colour microarrays system (Agilent, G4122F), following the manufacturer’s instructions. Briefly, the RNA samples and universal mouse reference RNA (Agilent 741010) were labelled with CTP-cyt and CTP-cyt3, respectively, using the Agilent Quick Amp Labelling Kit. Labelled test and reference RNA samples were mixed in equal proportions, and hybridized to the mouse GE 4×44K array. The arrays were scanned with an Agilent G2565BA scanner and raw data were extracted using Agilent Feature Extraction software (v9.5). Data were analysed using the GeneSpring GX software (Agilent). The expression value of individual probes refers to the Log2(Cy5/Cy3) ratio.

Gene set enrichment analysis (GSEA). Gene set enrichment analysis (GSEA) v2.0 was used to perform the GSEA on various functional and/or characteristic gene signatures as described in a previous study33. Normalized microarray expression data were rank-ordered by differential expression between cell populations and/or genetic background as indicated, using the provided ratio of classes (that is, fold change) metric. Two independent MaSC-specific gene signatures were used to characterize MaSC-characteristics. Both are defined by significantly upregulated genes (< 0.05 and FDR > 3) in MaSC-enriched subpopulations from MECs of WT mice. The MaSC signature from current study is derived from the microarray data collected from our laboratory as described in this study (GSE47493). The gene qualify by showing a >3-fold upregulation in P4 compared with both P5 and P6 of WT mice. The other MaSC signature is derived from published data set GSE22446. Other gene signatures used in GSEA were obtained from the MSigDB database v3.0 (September 2010 release).

Clinical data set analysis. To develop a conserved gene expression readout for ΔNp63 activity, we first interrogated microarray data sets comparing both MECs as well as the MaSC-enriched (P4) subpopulation of WT and ΔNp63−/+ mice. Using log-transformed and median-centred microarray data, we defined the ΔNp63 signature as the gene expression values of all genes with >1.5-fold change between MECs and P4 cells and compared with their corresponding WT control cells. This corresponded to 125 genes upregulated or downregulated in both MECs and P4 cells compared with the WT. To reduce each gene to one expression value, we took the average of its expression in MECs and in P4 cells. We then applied this signature to log-transformed and median-centred microarray data from the EMC286 clinical breast cancer data set32. To predict ΔNp63 activity in a given patient, we used overall Pearson correlation of patient gene expression values and ΔNp63 signature gene expression values for all genes in the experimentally derived ΔNp63 signature. The Pearson correlation coefficient values were used as a given patient’s ΔNp63 signature score. We then defined ΔNp63-high and ΔNp63-low patients as those with above and below median ΔNp63 signature scores, respectively.

To analyse the expression of different isoforms of p63 in breast cancer patients, we used the TCGA breast invasive carcinoma (BRCA) exon expression RNAseq data set64. We downloaded the expression data set (data set ID: TCGA_BRCA_exp_HiSeqV2_exon) and patient sample characteristics from the UCLA cancer browser (https://genome-cancer.ucsc.edu/). The expression levels of isoform-specific exons were used to represent the expression of specific isoforms. The Pearson correlation coefficient was calculated to measure the correlation between genes. For grouping, 1 base unit RPKM (Reads Per Kilobase of exon model per Million mapped reads) was set as the cutoff, so fewer than 1 RPKM is defined as absent (or undetectable) and more than 1 RPKM is defined as present (or detectable).

In silico analysis. The sequence conservation between the mouse and human Fzd7 enhancer was examined using the VISTA computational tools for comparative genomics (http://genome.lbl.gov/vista/index.shtml).

Chromatin immunoprecipitation. Human MECs (Lonza) or MTMV-Wnt1 primary cells were grown to 80% confluency and cells were then crosslinked with 1% formaldehyde and processed. The crosslinking, immunoprecipitation, washing, elution, reverse crosslinking, and proteinase K treatment were performed according to the manufacturer’s directions described in the Magna ChIP G Chromatin Immunoprecipitation Kit from Millipore. Antibodies used were anti-4A4, H-129 (Santa Cruz) or normal rabbit IgG. Purified immunoprecipitated DNA was used for real-time qPCR. Primers for ChIP PCR are: 5’-TATCGAGATTCGCGCCCAC-3’ (forward) and 5’-TCCCTGGGAGAACAATCGCC-3’ (reverse) for human Fzd7; 5’-AATGGGGAACACACCCTCCT-3’ (forward) and 5’-CTCGCGGGGATTTAAGGTTGGG-3’ (reverse) for mouse Fzd7; 5’-CGAGATCCCTCACATCCA-3’ (forward) and 5’-CCAGCTCTTCTCAGTG-3’ (reverse) for human Gapdh and 5’-AACATCAATAGGGTGAGAG-3’ (forward) and 5’-GCTCTTCTCCATGGTGTTG-3’ (reverse) for mouse Gapdh.

Statistical analysis. Results were generally reported as mean ± s.d. (standard deviation) as indicated in the figure legends. For comparisons of central tendencies, normally distributed data sets were analysed using unpaired (with the exception of analyses of cellular populations from paired contralateral injections) two-sided Student’s t-tests assuming equal variance. Non-normally distributed data sets were analysed using non-parametric Mann–Whitney U tests. Pearson’s χ2 tests were used for tumour incidence using ELDA software. To adjust for host effects, paired two-sided Student’s t-tests assuming equal variance were used for experiments in which cellular populations were compared following matched contralateral injections of control and experimental cell types into the same mouse (Supplementary Fig. 3g). For tumour-free survival analyses, data were shown as Kaplan–Meier plots and significance was assessed by log-rank tests. Statistical analyses specific to limiting dilution Assays and GSEA are described above. All of the experiments with representative images (including western blot, FACS plot, histology and immunofluorescence) were repeated at least three times and representative images are shown.

Accession numbers for data sets. Microarray data generated in this study have been deposited at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the accession code GSE47493. Other published data sets used in this study are GSE22446 (MaSC signature)65 and GSE2034 (EMC286 data set)64. The processed TCGA RNAseq data were obtained from the UCLA Cancer Browser (https://genome-cancer.ucsc.edu/). Data set ID: TCGA_BRCA_exp_HiSeqV2_exon, version 2013-12-18.

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67. Fuerer, C. & Nusse, R. LentiVir vectors to probe and manipulate the Wnt signaling pathway. Plos ONE 5, e9370 (2010).
Supplementary Figure 1 ∆Np63 is enriched in MaSCs and promotes mammary stem cell activity. (a) Oocyte in mouse ovary sections stained with TAp63 antibody as positive control for the antibody. (b) Phase contrast and fluorescence images of GFP-expressing MECs 72h post-lentiviral transduction. (c) Whole mounts of GFP+ ductal outgrowths derived from P4 cells transduced with the GFP control vector. (d, e) Overexpression of ∆Np63 and TAp63 in primary mammary epithelial cells (MECs) transduced with ∆Np63 and TAp63 compared to control empty vector, as demonstrated by qRT-PCR (n = 3 samples; data represents mean ± s.d.) (d) and western blot (e). qRT-PCR values were normalized to the housekeeping gene Gapdh. *p < 0.05 by Student’s t test. (f) P4 population were transduced with control or ∆Np63 expressing lentiviruses and were transplanted into cleared fat pads and take rate was compared after using limiting numbers of cells in injections (n= number of mammary fat pad injections as indicated in the table). P value was obtained by Pearson’s Chi-squared test using ELDA software. (g) Percentage of fat pad filled from experiments in f. (h) P5 population were transduced with control or ∆Np63 expressing lentiviruses and were transplanted into cleared fat pads and take rate was compared after using limiting numbers of cells in injections (n= number of mammary fat pad injections as indicated in the table). P value was obtained by Pearson’s Chi-squared test using ELDA software. (i) Competitive reconstitution assay strategy for MaSCs (2000 cells per injection) after lentiviral transduction of ∆Np63, TAp63 or control expression plasmids. Size bars, 75 µm in a, and 40 µm in b and c respectively. Uncropped images of blots are shown in Supplementary Fig. 9.
Supplementary Figure 2 ∆Np63 increases mammary stem cell-like functions in P5-CD61+ and P5-CD61- luminal cells but not in the P6 population. (a) Table showing reconstitution efficiency of limiting dilution of CD61+ or CD61- cells from Lin-CD24+CD29lo (P5) cells with or without ∆Np63 overexpression (n= number of mammary fat pad injections as indicated in the table). (b) Representative alum carmine stained mammary outgrowths from (a). (c) Representative images of 3-D matrigel colonies formed by control and ∆Np63 overexpressing P5 cells (upper panels) as well as K14 and K8 staining of colonies (lower panels). (d) K14 and K8 staining in mammary outgrowths from control and ∆Np63 overexpressing CD61+ or CD61- cells from Lin-CD24+CD29lo (P5) population. (e) Table showing reconstitution efficiency of limiting dilution of P6 cells with or without ∆Np63 or TAp63 (n= number of mammary fat pad injections as indicated in the table). (f) Representative alum carmine stained mammary outgrowths from (e). Size bar, 1 mm in b, f and 40 μm in c and d. p value was obtained by Pearson's Chi-squared test using ELDA software in a and e.
Supplementary Figure 3 ∆Np63 increases proliferation and blocks differentiation in the mammary epithelium. (a) H&E stained outgrowth sections from control and ∆Np63 overexpressing cells. Quantification of ductal branching (tertiary branch points in defined area) is shown at the bottom panel (n = 10 areas scored for branch points from 3 mammary outgrowths per group). p-value computed by Mann-Whitney U test. Box represents 75th, 50th and 25th percentile of the values. The top and bottom lines represent the maximum and minimal data points within 1.5x IQ (inter quarter) range, respectively. (b) Ki67 staining in outgrowth sections from control and ∆Np63 overexpressing cells. Quantification of Ki67+ cell percentage over total epithelial cells in field of view is shown in the bottom panel (n = 25 areas scored from 3 mammary outgrowths per group, data represents mean ± s.d.). (c) ∆Np63 staining in outgrowth sections from control (n = 10 ducts and TEB sections scored from 3 mammary outgrowths; data represents mean ± s.d.) and ∆Np63 overexpressing (n = 12 ducts and TEB sections scored from 3 mammary outgrowths; data represents mean ± s.d.) cells. Quantification of the percentage ∆Np63+ basal cells among all basal cells was shown in the bottom panel. (d) K14 and K8 staining in mammary outgrowths from control (n = 10 ducts and TEB sections scored from 3 mammary outgrowths; data represents mean ± s.d.) and ∆Np63 overexpressing (n = 12 ducts and TEB sections scored from 3 mammary outgrowths; data represents mean ± s.d.) cells. Quantification of the number of K14+ basal cells in the field of view is shown in the bottom panel. P value computed by Student’s t test. (e) Western blot analyses of ∆Np63 and K14 in mammary epithelial cells from control and ∆Np63 overexpressing outgrowths. (f, g) Representative FACS plot of mammary epithelial cells from control and ∆Np63 overexpressing outgrowths after staining with CD24 and CD29. Results were quantified in g. P value computed by paired t test. Size bar, 100 μm in a, 40 μm in b, c and d. Uncropped images of blots are shown in Supplementary Fig. 9.
Supplementary Figure 4 ΔNp63 regulation of MaSC activity is dependent upon its DNA-binding activity. (a, b) Representative images (a) and quantification (b) of 3-D matrigel colonies (1st generation) formed by control, ΔNp63 and TAp63 overexpressing P4 cells (n = 5 samples; data represents mean ± s.d.). Colony images from lower panel are representative single colonies in higher magnifications from upper panel images were in a. (c) Representative image of outgrowths from 3rd generation shown in Fig. 1k. (d, e) qRT-PCR analysis (n = 6 technical replicates pooled from 2 independent experiments; data represents mean ± s.d.) (d) and western blot analyses (e) of the expression of ΔNp63 in control, WT ΔNp63 (replicate populations) and DNA binding mutant form of ΔNp63 overexpressing MECs 72 hrs post-transduction. qRT-PCR values were normalized to the housekeeping gene Gapdh. (f, g) Bar graphs (f) and representative images (g) of colonies formed in 3-D matrigel colony formation assay from control, WT and DNA binding mutant of ΔNp63 overexpressing Lin CD24+ CD29hi (P4) cells (n = 4 samples; data represents mean ± s.d.) in f. (h) P4 cells were transduced with control, WT, or mutant ΔNp63 expressing lentiviruses were transplanted into cleared fat pads and take rate was compared (n= number of mammary fat pad injections as indicated in the table). *p < 0.05 by Student’s t test. P value was obtained by Pearson’s Chi-squared test using ELDA software in h. Size bar, 1 mm (upper panel) and 40 µm (lower panel) in a, 2 mm in c and 1 mm in g, respectively. Uncropped images of blots are shown in Supplementary Fig. 9.
Supplementary Figure 5. ∆Np63<sup>grp+/</sup> mice exhibit defects in ductal morphogenesis and MaSCs defect at 9-10 weeks. (a) Schematic diagram of generation of ∆Np63<sup>grp+/</sup> mice. Targeting vector showing the insertion of a GFP coding cassette in-frame with the start codon of ∆Np63 located in exon 3’, followed by transcription termination signal (PolyA), leading to loss of expression of the endogenous ∆Np63. (b) Quantification of ductal elongation (arbitrary units) (left) (n = 5 mammary glands per group) and percentage (right) of MaSC-enriched (P4) cells in WT and ∆Np63<sup>grp+/</sup> mammary glands (6-7 weeks). For the right panel, WT (n = 8 mammary glands) and ∆Np63<sup>grp+/</sup> mice (n = 12 mammary glands). p-value computed by Mann-Whitney U test. Box represents 75th, 50th and 25th percentile of the values. The top and bottom lines represent the maximum and minimal data points within 1.5x IQ (inter quarter) range, respectively. (c) Representative alum carmine stained whole mount mammary outgrowths from WT and ∆Np63<sup>grp+/</sup> mice at 9-10 weeks. (d) Quantification of ductal elongation (arbitrary units) (left) and percentage (right) of MaSC-enriched (P4) cells in WT and ∆Np63<sup>grp+/</sup> mammary glands (9-10 weeks) (n = 5 mammary glands per group), p-value computed by Mann-Whitney U test. Box represents 75th, 50th and 25th percentile of the values. The top and bottom lines represent the maximum and minimal data points within 1.5x IQ (inter quarter) range, respectively. (e) Representative FACS profiles of mammary epithelial cells from WT and ∆Np63<sup>grp+/</sup> mice after dissociation and staining with CD24 and CD29. (f) Bar graph showing the number of colonies formed in Matrigel from control or ∆Np63 overexpressing P4 cells (20,000 cells) treated with Dikkopf-1 (Dkk1) for 7 days (n = 3 samples; data represents mean ± s.d.). (g) Bar graph showing colonies from 1st generation that were dissociated and passaged with equal cell numbers for 3 generations with Dkk1 treatment (n = 3 samples; data represents mean ± s.d.). *p < 0.05 by Student’s t test in f and g. (h) Representative images of 3-D matrigel colonies from 3<sup>rd</sup> generation P4 cells with or without ∆Np63 overexpression. Size bar, 1 mm (left panel), 2 mm (right panel) in c, respectively and 1 mm in h.
Supplementary Figure 6 Wnt signaling is necessary for ∆Np63-induced mammary stem cell activity. (a) Venn diagram showing significant overlap of core genes from two Wnt signaling GSEA signatures. Common core genes between 2 sets are presented in the table. (b) qRT-PCR analyses of the expression of ∆Np63 and TAp63 in control, ∆Np63, or TAp63 overexpressing HMLE cells (n = 3 samples; data represents mean ± s.d.). *p < 0.05 by Student’s t test. (c, d) Representative alum carmine stained mammary outgrowth images (c) and quantification table (d) showing reconstitution efficiency of P4 cells of indicated groups (n= number of mammary fat pad injections as indicated in the table). P value was obtained by Pearson’s Chi-squared test using ELDA software. (e) GSEA data showing positive enrichment of published WNT gene signatures in ∆Np63 signature\(^{6}\) patients compared to ∆Np63 signature\(^{6}\) patients. Patients were stratified using an experimentally-derived ∆Np63-specific gene expression signature as described in methods section. (f) Scatter plot showing the correlation between ∆Np63 signature scores and FZD7 expression in clinical patients from EMC286 dataset (n = 286 patients). Pearson Coefficient test was performed for statistical significance. (g) Percentage of tumors with positive detection of ∆Np63 and TAp63 expression from the TCGA-BRCA-RNAseqV2 clinical dataset (n = 1000 patients). Size bar, 1 mm in c.
Supplementary Figure 7 ΔNp63 and Fzd7 expression and function in human and mouse mammary tumors. (a) Western blot analysis of ΔNp63 and FZD7 expression in MDA-MB-231 cells transduced with shRNAs targeting p63 or FZD7, or control shRNA. (b, c) Tumor incidence of MDA-MB-231 (b) and SUM-1315 cells (c) with or without p63 KD. Tumor incidence was determined 70 days post injection (n= number of mammary fat pad injections as indicated in the table). P value was obtained by Pearson’s Chi-squared test using ELDA software. (d) Confocal images of immunofluorescence analysis of ΔNp63, TAp63 and Fzd7 protein expression in human patient derived xenografts (PDX). (e) Immunostaining of protein expression showing predominant expression of ΔNp63 and Fzd7 in basal (MMTV-Wnt1) compared to luminal (MMTV-PyMT) type mammary gland tumors. Mixed (MMTV-cMyc) type has modest expression of ΔNp63 and Fzd7. (f) qRT-PCR analysis of the RNA expression of ΔNp63 and TAp63 in MMTV-Wnt-1 tumor cells (n = 3 tumors; data represents mean ± s.d.). qRT-PCR values were normalized to the housekeeping gene Gapdh. *p < 0.05 by Student’s t test. Size bar, 40 μm in d and 100 μm in e respectively. Uncropped images of blots are shown in Supplementary Fig. 9.
Supplementary Figure 8 Reduced Fzd7 expression in MMTV-Wnt1; ΔNp63gfp/+ tumors compared to MMTV-Wnt1; ΔNp63 +/+ tumors. (a) qRT-PCR analysis of ΔNp63, Fzd7 and Wnt5a in the in MMTV-Wnt1; ΔNp63+/+ and MMTV-Wnt1; ΔNp63gfp/+ tumors (n = 5 tumors per genotype). *p < 0.05 by Mann Whitney U test. (b) Western blot analyses of indicated protein expression in MMTV-Wnt1; ΔNp63+/+ and MMTV-Wnt1; ΔNp63gfp/+ tumors. (c) Confocal immunofluorescence images of ΔNp63, K14 and K8 and Fzd7 protein expression in MMTV-Wnt1; ΔNp63+/+ and MMTV-Wnt1; ΔNp63gfp/+ tumors. Size bar, 40 μm in c. Uncropped images of blots are shown in Supplementary Fig. 9.
Supplementary Figure 9 Western blot scanned films. Boxes highlight lanes used in figures.
Supplementary Figure 9 continued Western blot scanned films. Boxes highlight lanes used in figures.
## Supplementary Table 1. Expression of transcriptional regulators in different MEC subpopulations

| Gene Symbol | Full gene name                                      | P4   | P5   | P4/P5 |
|-------------|-----------------------------------------------------|------|------|-------|
| Atxn1       | Ataxin 1                                            | 2.274| -0.133| 5.3   |
| Bnc1        | Basonuclin 1                                        | -2.626| -5.799| 9     |
| Camta1      | Calmodulin binding transcription activator 1         | 5.714| -0.154| 58.4  |
| Ccdc85b     | Coiled-coil domain containing 85B                    | 1.139| -1.501| 6.2   |
| Csdc2       | Cold shock domain containing C2, RNA binding         | 2.426| -0.581| 8     |
| Ctnnd2      | Catenin (cadherin associated protein), delta 2        | 4.176| 1.951 | 4.7   |
| Dach1       | Dachshund 1                                         | 6.071| 2.702 | 10.3  |
| Egr3        | Early growth response 3                              | 2.538| -2.635| 36.1  |
| Egr4        | Early growth response 4                              | 4.929| 1.797 | 8.8   |
| Eya1        | Eyes absent 1 homolog                                | 0.792| -2.541| 10.1  |
| Foxj1       | Forkhead box J1                                      | 3.156| -0.943| 17.1  |
| Fryl        | Furry homolog-like                                   | 2.541| -0.213| 6.7   |
| Id4         | Inhibitor of DNA binding 4                          | 6.153| 0.256 | 59.6  |
| Irx4        | Iroquois related homeobox 4                         | 4.481| 1.221 | 9.6   |
| Irx1        | Neuregulin 1                                        | 1.728| -2.356| 17    |
| Pou3f1      | POU domain, class 3, transcription factor 1          | 4.084| 1.788 | 4.9   |
| Rbpb8       | Retinoblastoma binding protein 8                     | 1.1  | -1.403| 5.7   |
| Rbpms       | RNA binding protein gene with multiple splicing      | 3.631| -0.24 | 14.6  |
| Scx         | Scleraxis                                            | 2.153| -2.465| 24.6  |
| Sdpr        | Serum deprivation response                           | 1.803| -1.753| 11.8  |
| Tceal3      | Transcription elongation factor A (SII)-like 3        | 3.493| 0.886 | 6.1   |
| Tcf7        | Transcription factor 7, T-cell specific              | -1.779| -3.851| 4.2   |
| Tgif2       | TGFβ-induced factor homeobox 2                       | 2.26 | -0.226| 5.6   |
| Trp63       | Transformation related protein 63                    | 6.923| 1.355 | 47.4  |
| Trp73       | Transformation related protein 73                    | 4.309| -0.415| 26.4  |
| Zfp239      | Zinc finger protein 239                              | 1.482| -1.13 | 6.1   |
Supplementary Table 2. Antibodies used for immunohistochemistry (IHC), immunofluorescence (IF) and western blot (WB) analyses

| Antibodies | IHC/IF | WB     | Company and catalogue number                      |
|------------|--------|--------|---------------------------------------------------|
| ΔNp63      | 1:100  | 1:1000 | Romano et al, 2012                               |
| TAp63      | 1:100  | 1:1000 | Romano et al, 2012                               |
| Ki67       | 1:50   | -      | Novocastra, Cat # NC9025734, clone MM1           |
| K14        | 1:100  | -      | Abcam, Cat # ab53115                             |
| K8         | 1:10   | -      | Fitzgerald, Cat #10R-C177AX, clone Ks8.7         |
| Fzd7       | 1:50   | 1:1000 | Sigma Aldrich, Cat # AV41251                      |
| Wnt5a      | -      | 1:2000 | Abcam, Cat # ab86720, clone 3D10                 |
| β-actin    | -      | 1:10,000 | Abcam, Cat # ab6276, clone AC-15               |
### Supplementary Table 3. Quantitative RT-PCR primer sequences

| Gene     | Species | Forward            | Reverse                        |
|----------|---------|--------------------|--------------------------------|
| ΔNp63   | Mouse   | TGCCCAGACTCAATTTAGTA | GAGGAGCCGTTCCTGAATCTG         |
| TAp63   | Mouse   | CTGGTTGAAAGAAAGTTATTAC | GGCGTGTACTGAGCATATAG         |
| Fzd7    | Mouse   | GGAGGGCTGCCACATCCTCCT | TGGCCAGCTTCATGCGCAGC         |
| Fzd3    | Mouse   | ACCATGGCAACCTCCACAGGT | CGTGCATGCTGCCTGAGGT          |
| Fzd6    | Mouse   | AGTACCGCATCCCGTGCCCT | GCTTCCAACCAGAAAGACCAGCA      |
| Fzd8    | Mouse   | ATGGAGTGCGGTGTTACCTGTTG | CACCGTGATCTCTTGGCAG        |
| Lrp6    | Mouse   | TTGGTCTTTATGCAACAGACG | GTTCGTTTAATGGCTTCTCGC        |
| Wnt3    | Mouse   | CTCGCTGGGTACCAATAATTG | CTTCACACCTTCTGCTACGCT        |
| Wnt5a   | Mouse   | CAACTGGCAGGACTTCTCAA | CATCTCCGATGCGGAACCT         |
| Wnt5b   | Mouse   | CTGCTGACTGACGCAACTT | CCTGATACAACTGACGACTTTT       |
| Wnt11   | Mouse   | GCTGGCACTGTCCAGACTC | CCTCCGTCCTCCTCTCCCA         |
| Gapdh   | Mouse   | CCCCAATGTGTCGGCTCGT | GCCTGCTTCACCCCTTCT         |
| ΔNP63   | Human   | GGAAACCAATGCGCGACACT | GTGGAGATACGCCAGGGTGGC       |
| TAP63   | Human   | AAGATGGCTGCGCAAACAG | AGAGAGATCGAGGTTGAG          |
| WNT5A   | Human   | GGTGGAACGCACTGCTGGA | CGTGCCTGGCTCCAGCTCGTAT       |
| FZD7    | Human   | GCAGAGCAGCGAACTCGAGG | CAGCACACTGCTCCTCTCCTCTCTCCT |
| GAPDH   | Human   | GAAGGTGAGGGTCCGAGTC | GAAGATGGTGATGGGATTTTC       |