NEOPLASIA
www.neoplasia.com

Volume 16 Number 2 February 2014 pp. 158–167 158

Tumor-Suppressive Activity of Lunatic Fringe in Prostate through Differential Modulation of Notch Receptor Activation[1]

Shubing Zhang*, Wen-cheng Chung*, Guanming Wu†, Sean E. Egan‡ and Keli Xu*,§

*Cancer Institute, University of Mississippi Medical Center, Jackson, MS; †Department of Medical Informatics and Clinical Epidemiology and Oregon Clinical and Translational Research Institute, Oregon Health and Science University, Portland, OR; ‡Program in Developmental and Stem Cell Biology, The Hospital for Sick Children and Department of Molecular Genetics, University of Toronto, Toronto, Canada; §Department of Neurobiology and Anatomical Sciences, University of Mississippi Medical Center, Jackson, MS

Abstract

Elevated Notch ligand and receptor expression has been associated with aggressive forms of prostate cancer, suggesting a role for Notch signaling in regulation of prostate tumor initiation and progression. Here, we report a critical role for *Lunatic Fringe* (*Lfng*), which encodes an *O*-fucosylpeptide 3-β-*N*-acetylglucosaminyltransferase known to modify epidermal growth factor repeats of Notch receptor proteins, in regulation of prostate epithelial differentiation and proliferation, as well as in prostate tumor suppression. Deletion of *Lfng* in mice caused altered Notch activation in the prostate, associated with elevated accumulation of Notch1, Notch2, and Notch4 intracellular domains, decreased levels of the putative Notch3 intracellular fragment, as well as increased expression of *Hes1, Hes5*, and *Hey2. Loss of Lfng resulted in expansion of the basal layer, increased proliferation of both luminal and basal cells, and ultimately, prostatic intraepithelial neoplasia. The Lfng*-null prostate showed down-regulation of prostatic tumor suppressor gene *NKX3.1* and increased androgen receptor expression. Interestingly, expression of *LFNG* and *NKX3.1* were positively correlated in publically available human prostate cancer data sets. Knockdown of *LFNG* in DU-145 prostate cancer cells led to expansion of $CD44^+CD24^-$ and $CD49f^+CD24^-$ stem/progenitor-like cell population associated with enhanced prostatosphere-forming capacity. Taken together, these data revealed a tumor-suppressive role for Lfng in the prostate through differential regulation of Notch signaling.

Neoplasia (2014) 16, 158–167

Introduction

Prostate cancer is the most common malignancy in males. Despite recent progress on defining the cellular origin of prostate cancer and oncogenic events associated with tumor initiation, it remains unclear how the normal prostatic epithelial hierarchy is established and maintained and how it is subverted during oncogenic transformation of prostate tissue. In recent years, Notch has emerged as a critical regulator of epithelial differentiation and proliferation in the prostate [1–5]. Indeed, Notch1 is selectively expressed in basal epithelial cells, and elimination of Notch1-expressing cells inhibited the branching morphogenesis, growth, and differentiation of early postnatal prostate in culture and impaired prostate regeneration following hormone replacement in castrated mice [3,4]. Interestingly, expression of a Notch1 gain-of-function allele during mouse embryogenesis or postnatal prostate development induced proliferation and expansion of the progenitor cells in basal epithelium, whereas loss of Notch signaling through deletion of canonical Notch transcriptional

Address all correspondence to: Keli Xu, PhD, Cancer Institute, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216. E-mail: kxu@umc.edu

[1]This work was supported by start-up funds from the Cancer Institute of the University of Mississippi Medical Center and by the National Institutes of Health (R21 CA175136) to K.X. and by funds from the Canadian Cancer Society Research Institute to S.E.E. The authors declare no conflict of interest.
Received 7 November 2013; Revised 6 January 2014; Accepted 17 January 2014

DOI 10.1593/neo.131870

effector *RBPJκ* led to decreased cell proliferation and loss of epithelial progenitors [2]. Paradoxically, a recent study showed that Notch signaling may suppress proliferation and expansion of basal stem/progenitor cells during regeneration of the murine prostate tissue [5]. Thus, Notch signaling plays complex roles during development, homeostasis, and regeneration of the prostate gland.

Fringe genes code for *O*-fucosylpeptide 3-β-*N*-acetylglucosaminyltransferases that can add *N*-acetylglucosamine to *O*-linked fucose residues on epidermal growth factor repeats of Notch [6]. This modification modulates specificity and sensitivity of Notch receptors for different ligands. Therefore, Fringes are powerful regulators of ligand-mediated Notch signaling. There are three *Fringe* genes in mammals, namely, *Lunatic Fringe* (*Lfng*), *Manic Fringe*, and *Radical Fringe* [7,8]. Expression of *Lfng* in the prostate is relatively low compared with *Manic Fringe* and *Radical Fringe* but more restricted to basal epithelium [5]. Given the existence of multipotent basal progenitors during prostate postnatal development, regeneration, and homeostasis [9,10] and the fact that Notch signaling regulates basal cell proliferation and differentiation, Lfng may control Notch activation in these cells. Because the prostate basal cell is a cell of origin for prostate cancer in both mouse and human [10–14], modulation of Notch signaling by Lfng in these cells could play a critical role in prostate tumor initiation and/or progression. Interestingly, Lfng-dependent Notch signaling controls basal stem/progenitor cell self-renewal and differentiation in mammary gland, and *Lfng* deficiency induces basal-like breast cancer [15]. We therefore sought to test for similar activities of this gene in prostate gland.

In an attempt to define the function of Lfng in the prostate, we studied activation of different Notch receptors in the *Lfng*-null prostate as well as epithelial development in this context. Our data show that Lfng inhibits activation of Notch1 and Notch4 in basal cells of the mouse prostate gland while enhancing Notch3 activation. This is associated with increased expression of *Nkx3.1* tumor suppressor gene and prevention of basal stem/progenitor cell expansion and prostatic intraepithelial neoplasia (PIN) formation. In the human prostate cancer cell line DU-145, *LFNG* knockdown increased cancer stem/progenitor cell activity. Finally, a positive correlation was observed between expressions of *LFNG* and *NKX3.1*, as well as negative correlation between LFNG expression and Gleason scores in human prostate cancer data sets. Thus, *Lfng* gene deficiency or silencing may contribute to prostate cancer initiation through Notch-dependent expansion of normal basal stem/progenitor cells and promote tumor progression through Notch-dependent enrichment of cancer stem/progenitor cells.

Materials and Methods

Histology and Immunohistochemistry

The $Lfng^{-/-}$ mice have been previously described [16]. Formalin-fixed paraffin-embedded prostate tissues were processed for histology and immunohistochemistry by standard procedures. Staining was carried out on two sections per prostate from at least three mice for each group, and representative images were acquired with a Nikon Eclipse 80i microscope (Nikon, Melville, NY). Primary antibodies used for immunohistochemistry were Notch1 (1:100; Cell Signaling Technology, Danvers, MA, No. 3608), Notch4 (1:100; Millipore, Billerica, MA, 09-089), cytokeratin 14 (K14) (1:200; Panomics, Santa Clara, CA, E2624), cytokeratin 5 (K5) (1:1600; Covance, Princeton, NJ, PRB-160P), cytokeratin 8 (K8) (1:800; Covance, MMS-162P), smooth muscle actin α (Sma) (1:200; Abcam, Cambridge, MA, ab5694), p63 (1:100; Santa Cruz Biotechnology, Dallas, TX, sc-8431), aldehyde dehydrogenase (Aldh1) (1:100; Abcam, ab52492), and Ki-67 (1:100; Abcam, ab16667).

Western Blot Analysis

Prostate tissues were homogenized and lysed in RIPA buffer (Boston BioProducts, Ashland, MA) supplemented with protease inhibitor (Roche, Indianapolis, IN). Supernatants were clarified by centrifugation, and total protein was quantified. Whole-cell lysates from DU-145 cells were prepared using the same buffer. Equivalent protein amounts from experimental and control lysates were loaded for Western blot analysis, performed according to standard methodology. Prostate tissues from three pairs of wild-type and mutant littermates were analyzed with similar results. Primary antibodies used for Western blot analyses were Notch1 (1:1000; Cell Signaling Technology, No. 3608), Cleaved Notch1 (1:1000; Cell Signaling Technology, No. 4147), Notch2 [1:2000; Developmental Studies Hybridoma Bank (DSHB), University of Iowa (Iowa City, IA), C651.6DbHN], Cleaved Notch2 (1:1000; Abcam, ab52302), Notch3 [1:1000; Proteintech Group (Chicago, IL), 55114-1-AP], Notch4 (1:1000; Millipore, 07-189), Cleaved Notch4 [1:1000; GeneTex (Irvine, CA), GTX86910], Hes1 (1:1000; Millipore, AB5702), pan-Akt (1:1000; Cell Signaling Technology, No. 4691), Phospho-Akt (Ser473) (1:1000; Cell Signaling Technology, No. 4060), PTEN (1:1000; Cell Signaling Technology, No. 9188), Smad2 (1:1000; Cell Signaling Technology, No. 5339), Phospho-Smad2 (Ser465/467) (1:1000; Cell Signaling Technology, No. 3101), c-Met (1:1000; Cell Signaling Technology, No. 3127), and androgen receptor (AR) (1:1000; Santa Cruz Biotechnology, sc-816). Blots were reprobed with anti–β-actin antibody (1:4000; Santa Cruz Biotechnology, sc-81178) as control for equal loading.

Quantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was prepared from anterior prostate glands or cultured cells using RNeasy Mini Kit (Qiagen, Valencia, CA,) and reverse transcribed using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed on a Bio-Rad CFX96 Real-Time System using the RT^2 SYBR Green qPCR Mastermixes (Qiagen). We used primer sequences for mouse Hes1, Hes5, Hey1, *Hey2*, and Klkb1 and human LFNG, HES1, HES5, HEY1, and HEY2 as previously reported [17–20]. Mouse Nkx3.1 primer sequences were 5′ TGTCTTTGCCAGCCCTGAA (forward) and 5′ TAGTATACACGGAGACCAAGGAGGTA (reverse). The relative abundance of mRNA for each gene to *GAPDH* was determined by the equation $2^{-\Delta CT}$ (ΔCT = threshold cycle $(CT)_{Tested\ Gene} - CT_{GAPDH}$). Data were derived from triple reactions for each sample.

Generation of Stable LFNG *Knockdown Cell Lines*

The LFNG-small hairpin RNA (shRNA) construct in retroviral red fluorescent protein (RFP) vector targeting 5′-AGCAGGTGACGCTGAGCTACGGTATGTTT-3′ sequences of human *LFNG* gene was purchased from OriGene Technologies (Rockville, MD). DU-145 cells were transfected with the LFNG-shRNA plasmid or the scrambled shRNA plasmid using FuGENE 6 Transfection Reagent (Promega,

Madison, WI). Stable cell lines expressing LFNG-shRNA or scrambled shRNA were established by selection with 1 μg/ml Puromycin (Sigma, St Louis, MO).

Cell Proliferation and Prostatosphere Formation Assays

DU-145 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. CellTiter96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega) was used for cell proliferation assay, according to the manufacturer's instructions. For prostatosphere formation assays, DU-145 cells from monolayer cultures were re-suspended in serum-free Dulbecco's modified Eagle's medium/F12 supplemented with MEGM SingleQuot Kit (Lonza, Allendale, NJ, CC-4136) and plated onto individual wells of ultralow-attachment six-well plate (2×10^3 cells in 2 ml per well) (Corning, Tewksbury, MA). The spheres formed after 2 weeks of culture were quantified using Celigo cytometer (Brooks, Chelmsford, MA). Secondary sphere formation was tested by replating trypsin-dissociated cells from primary spheres.

Flow Cytometry Analysis

Flow cytometry was performed by standard procedures using the following fluorochrome-conjugated antibodies: PE-Cyanine7 Anti-Human/Mouse CD44 (1:80; eBioscience, San Diego, CA, 25-0441), PE-Cy7 anti-human/mouse CD49f (1:20; BioLegend, San Diego, CA, 313621), and Alexa Fluor 488 anti-human CD24

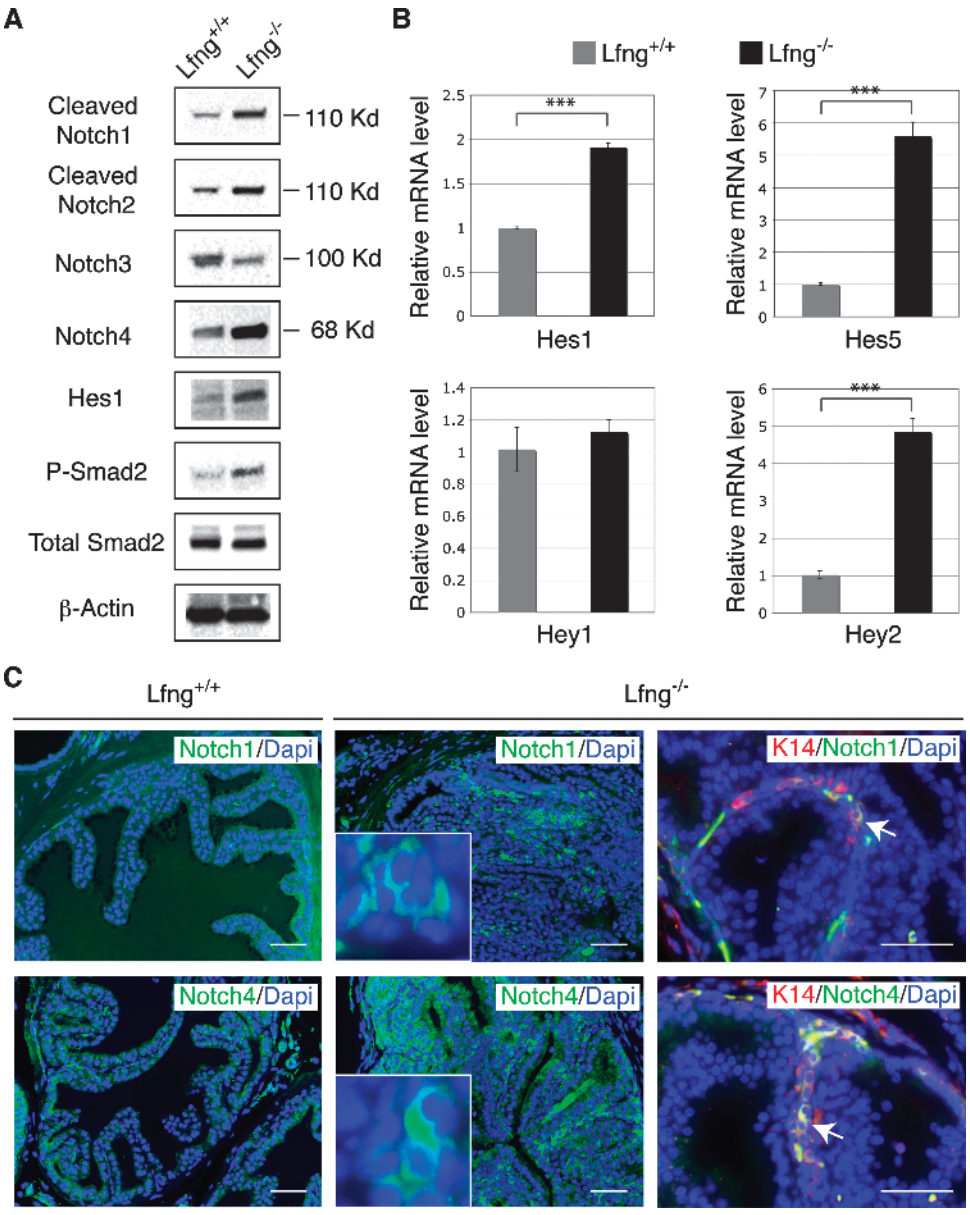

Figure 1. Deletion of *Lfng* caused dysregulation of Notch signaling in the prostate. (A) Western blot analysis for Notch receptors, Hes1, and Smad proteins in prostate tissues from 10-week-old wild-type and $Lfng^{-/-}$ mice. (B) Quantitative RT-PCR of Notch downstream targets Hes1, Hes5, Hey1, and Hey2 in prostates of 10-week-old mice. ***$P < .001$. (C) Immunofluorescence staining of Notch1, Notch4, Notch1/K14, and Notch4/K14 in prostate sections from 10-month-old mice. Insets are high magnification images showing Notch1 and Notch4 staining in cell membrane and cytoplasm. Arrows point to cells showing costaining of Notch1 and K14 and Notch4 and K14, respectively. Scale bars, 50 μm.

(1:20; BioLegend, 311108). Fluorescence was recorded using Gallios Flow Cytometer (Beckman Coulter, Indianapolis, IN) and analyzed with Kaluza (Beckman Coulter) flow cytometry analysis software version 1.2.

Analysis of LFNG *Expression in Human Prostate Cancer*

The Memorial Sloan Kettering Cancer Center (MSKCC) data set was downloaded from http://cbio.mskcc.org/cancergenomics/prostate/data/. The MSKCC_PCa_mRNA_data.txt file was used without further processing. Values in column PathGGS, combined Gleason score in the radical prostatectomy specimen, were used as Gleason scores for correlation analysis. Only normal and primary cancer samples were used in *LFNG* and *NKX3.1* gene expression correlation analyses. The Cancer Genome Atlas (TCGA) prostate cancer RNA Sequencing (RNA-seq) and clinical data were downloaded from the Broad Firehose (The Broad Institute, Cambridge, MA) website (https://confluence.broadinstitute.org/display/GDAC/Dashboard-Stddata) (November release, 2013). All plots and analyses were done using R (http://www.r-project.org/).

Statistics

All data are presented as means ± SEs. Statistical analysis was performed using the two-tailed Student's *t* test. *P* value of .05 or less was considered statistically significant.

Results

Lfng *Regulates Notch Signaling in the Prostate*

Multiple Notch ligands, receptors, and Fringes are expressed in murine prostate epithelia [5]. To test for Lfng control of Notch receptor activation in this context, we analyzed Notch intracellular domain ($Notch^{ICD}$) accumulation in prostate glands of adult *Lfng* mutant ($Lfng^{-/-}$) mice [16]. Western blot analysis using antibody for cleaved Notch1 (Val^{1744}) or cleaved Notch2 (Asp^{1733}) revealed a dramatic increase in the level of $Notch1^{ICD}$ (~110 kDa) as well as $Notch2^{ICD}$ (~110 kDa). The mutant glands also showed elevated levels of Notch4 C-terminal peptide that are consistent in size with $Notch4^{ICD}$ (~68 kDa). In contrast, decreased levels of a putative Notch3 intracellular protein fragment (~100 kDa) were observed in the mutant glands (Figure 1*A*). Thus, Lfng differentially regulates activation through different Notch receptors in the prostate gland. Next, we assessed mRNA levels of selected Notch downstream targets by quantitative RT-PCR. In agreement with increased accumulation of Notch1, Notch2, and Notch4 intracellular domains, the mutant gland showed dramatically increased levels of Hes5 and Hey2 mRNA and Hes1 mRNA to a lesser extent (Figure 1*B*). We also performed immunofluorescence staining for Notch1 and Notch4 receptors, in combination with a basal cell marker K14. Consistent with the Western blot analysis results, $Lfng^{-/-}$ glands showed accumulation of cells with very high levels of Notch1 and Notch4 proteins. Interestingly, most, if not all, $Notch1^{+}$ and $Notch4^{+}$ cells expressed K14 (Figure 1*C*), indicating that Notch1- and Notch4-expressing cells are located in the basal epithelium. Notch activation can facilitate a transforming growth factor β (TGFβ)–mediated positive feedback loop in the murine prostate by upregulating TGFβ ligands and receptors [5]. Indeed, we detected elevated levels of phospho-Smad2, but not total Smad2 proteins, in *Lfng* mutant prostates (Figure 1*A*). Thus, loss of *Lfng* in the prostate leads to hyperactivated signaling through Notch1, Notch2, and Notch4, as well as enhanced activation of the TGFβ pathway.

Deletion of Lfng *Caused Expansion of the Basal Cell Compartment*

The basal prostate epithelial compartment contains multipotent stem/progenitor cells [9,10]. Intriguingly, deletion of *Lfng* caused hyperactivation of Notch1 and Notch4 receptors in this layer (see above). We therefore investigated whether Lfng-regulated Notch signaling plays a role in self-renewal and/or differentiation of basal stem/progenitor cells of the prostate gland. Immunostaining for K14 and p63 demonstrated a remarkable increase in the number of basal lineage cells in adult $Lfng^{-/-}$ mutant prostates (Figure 2, *A* and *B*). We next stained for another basal cell marker, K5, and a luminal cell marker, K8. The mutant gland showed a small increase in the number of $K5^{+}$ basal cells and more intense K8 staining in luminal cells (Figure 2, *A* and *B*). By comparing K5 and K14 staining, it is clear that extra basal cells in *Lfng* mutants are mostly $K14^{+}$ rather than $K5^{+}$. Interestingly, a lineage-tracing study by Ousset and colleagues revealed a higher frequency of labeled luminal cells after marking $K14^{+}$ cells as opposed to $K5^{+}$ cells, suggesting that $K14^{+}$ basal cells are less restricted in their multilineage potential than $K5^{+}$ basal cells [9,21]. Thus, deletion of *Lfng* resulted in expansion of basal cells, in particular, of the $K14^{+}$ bipotential progenitor cells. Stem/progenitor cells in the prostate gland show high levels of ALDH activity [22]. Indeed, $Lfng^{-/-}$ prostates contained a dramatically increased number of $Aldh1^{+}$ cells, most of which were basally located (Figure 2, *A* and *B*). c-Met is specifically expressed in basal and intermediate cells of the prostate, and activation of c-Met induced a stemlike phenotype in human prostate cancer [23,24]. Interestingly, the *Lfng* mutant gland showed dramatically increased c-Met protein level, suggesting an accumulation of basal and/or intermediate cells. Taken together, examination of expressions of multiple basal stem/progenitor cell markers revealed an expansion of the basal cell compartment in *Lfng*-null prostate.

Notch signaling has also been shown to regulate differentiation of smooth muscle cells in the prostate [2]. We therefore examined smooth muscle differentiation by staining for Sma. Both wild-type and $Lfng^{-/-}$ mutant prostates showed a single layer of smooth muscle cells surrounding most epithelial ducts (Figure 2*D*). However, three of four mutant glands contained abnormal ducts with a large number of glandular structures showing basally located Sma^{+} cells (Figure 2*D*, *inset*), suggesting that Notch hyperactivation caused enhanced smooth muscle cell specification, differentiation, or recruitment.

Increased Epithelial Proliferation and PIN in the Lfng*-Null Mutant Gland*

PIN, a form of carcinoma *in situ*, is thought to represent a precursor of prostate adenocarcinoma. We followed male $Lfng^{-/-}$ mutants for an extended period of time and found that the majority developed PIN, starting at 6 months of age (Figure 3*A*). *Lfng*-null prostates show largely intact overall architecture, where prostatic epithelial cells are fully contained within the ducts. However, the mutant glands contain multiple layers of atypical cells, which appear to have increased nuclear size and nuclear pleomorphism. In some cases, foci of atypical cells almost filled the ductal lumen. Unlike prostatic adenocarcinoma, in which abnormal cells spread beyond the boundary of ducts and form clusters without basal cell layer, *Lfng*-null glands show expansion of basal cell layer. Using the criteria established by Park et al. [25], we

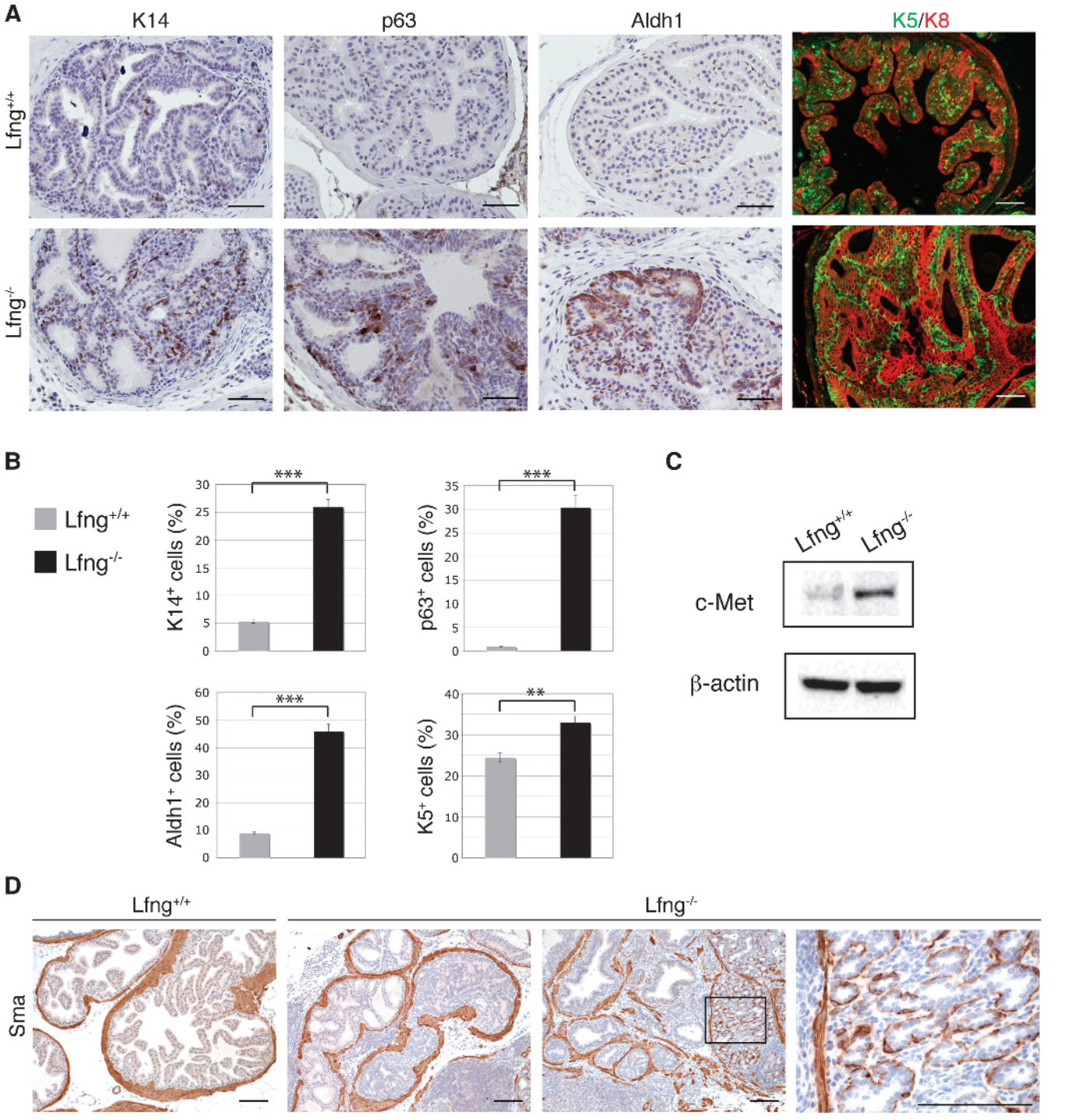

Figure 2. Deletion of *Lfng* caused expansion of basal compartment in the prostate. (A) Immunohistochemistry for K14, p63, Aldh1, and K5/K8 in prostates from 10-month-old wild-type and *Lfng*$^{-/-}$ mice. (B) Quantification of labeled cells. K14^{+}, p63^{+}, Aldh1^{+}, and K5^{+} cells are quantified and normalized as percentage of total cells. Data are presented as means ± SEs derived from staining of two sections per prostate from three mice of each group. $^{**}P < .01$ and $^{***}P < .001$. (C) Western blot analysis of c-Met in wild-type and *Lfng*$^{-/-}$ prostate tissues. (D) Anti-Sma staining in 10-month-old prostates. Occasional Sma staining in intraductal cells in the *Lfng*$^{-/-}$ prostate is shown by the rectangle and its high magnification image to the right. Scale bars, 50 μm in A and 100 μm in D.

determined that 13 of 15 *Lfng*-null mice (86.7%) developed low- to high-grade PIN (PIN1 to PIN3) at 6 to 12 months of age, whereas only 1 of 12 age-matched wild-type or *Lfng*$^{+/-}$ animals (8.3%) showed low-grade PIN (PIN1). The difference between these two groups is statistically significant (χ^2 test, $P < .001$). Immunostaining showed that up to 8% of epithelial cells were Ki-67^{+} in *Lfng* mutant glands, whereas less than 1% of cells were proliferating in prostates from wild-type littermates (Figure 3*B*). Because *Lfng* gene deletion caused expansion of the basal compartment, we used double labeling to identify dividing cells. Only 15% of Ki-67^{+} cells were K14^{+} (basal cell marker), whereas 80% were positive for K8 (luminal cell marker). This was true not only in *Lfng* mutants but also in wild-type glands

where few proliferating cells were found in either layer (Figure 3*C*). Thus, deletion of *Lfng* resulted in increased proliferation of basal as well as luminal cells.

The *Nkx3.1* homeobox gene is a key regulator of prostate epithelial cell differentiation and proliferation. Loss of Nkx3.1 function is an initiating event in prostate carcinogenesis in mouse models and an early event in humans [26–28]. By quantitative RT-PCR, we found that *Lfng*$^{-/-}$ glands expressed Nkx3.1 at 40% the level seen in wild-type glands (Figure 4*A*). In addition, *Lfng* mutant prostates showed up to seven-fold increased expression of *Klkb1*, a prostate-specific antigen (PSA)-related murine gene, in agreement with the report that NKX3.1 negatively regulates PSA promoter activity in humans [29]. We also detected increased levels of phospho-Akt in mutant glands (Figure 4*B*). Nkx3.1 has been shown to negatively regulate Akt activity through an AR/*PI3K*-dependent pathway [30]. Indeed, *Lfng* mutant prostates showed increased overall AR levels as indicated by Western blot analysis, consistent with a previous finding that Nkx3.1 negatively modulates AR transcription (Figure 4*C*) [30]. Immunohistochemistry demonstrated higher intensity of AR staining in the majority of luminal cells from *Lfng* mutant glands compared to luminal cells from wild-type prostates. However, mutant glands showed accumulation of a small number of AR^{-}K8^{+} luminal cells, in addition to expansion of the basal compartment as noted above (Figure 4*C*). Thus, dysregulated Notch signaling due to loss of *Lfng* resulted in elevated AR expression in the majority and silencing of AR in a small subset of luminal compartment cells. Given that Pten level in the *Lfng*$^{-/-}$ prostate was unchanged (Figure 4*B*), down-regulation of *Nkx3.1* may be the key event leading to Akt activation. Interestingly, *Nkx3.1* promoter contains a putative RBPJκ-binding element (data not shown), suggesting that an RBPJκ-dependent Notch signal may be responsible for altered regulation of Nkx3.1.

Positive Correlation between LFNG *and* NKX3.1 *Expressions in Human Prostate Cancer*

Lfng controls Notch activation in the mouse prostate, and deletion of *Lfng* resulted in basal cell expansion as well as PIN, suggesting a tumor-suppressive function for *Lfng* in this tissue. We therefore analyzed LFNG expression in two human prostate cancer gene

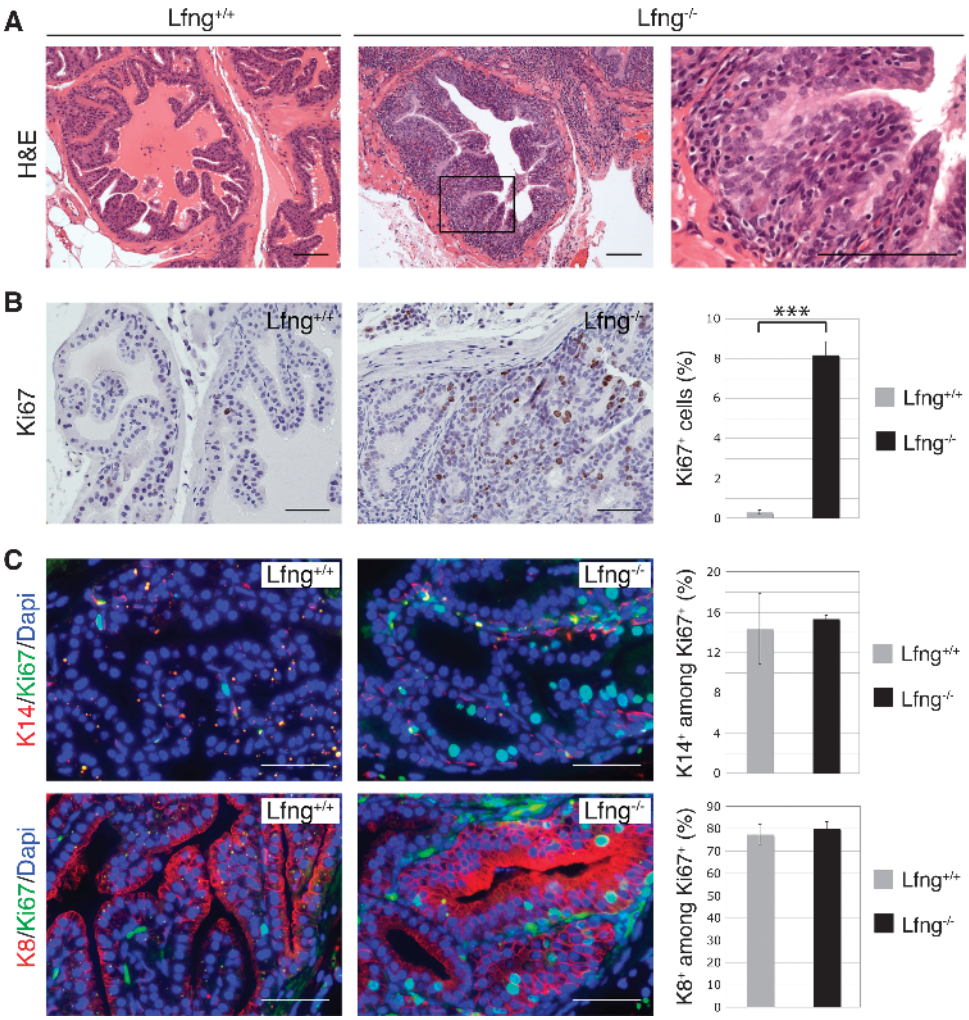

Figure 3. Increased epithelial proliferation and PIN in the *Lfng*$^{-/-}$ prostate. (A) Histology of anterior prostates from wild-type and *Lfng*$^{-/-}$ mice at 10 months of age. To the right is a high-magnification image of the area inside the rectangle. (B) Anti–Ki-67 staining in prostate sections of 10-month-old mice. (C) Immunofluorescence staining of K14/Ki-67 and K8/Ki-67 in prostates. Labeled cells are quantified and presented as percentage of Ki-67^{+} cells of total cells, K14^{+} among Ki-67^{+} cells, and K8^{+} among Ki-67^{+} cells, respectively. Data are derived from two sections per prostate from three animals of each group. Scale bars, 100 μm in A and 50 μm in B and C.

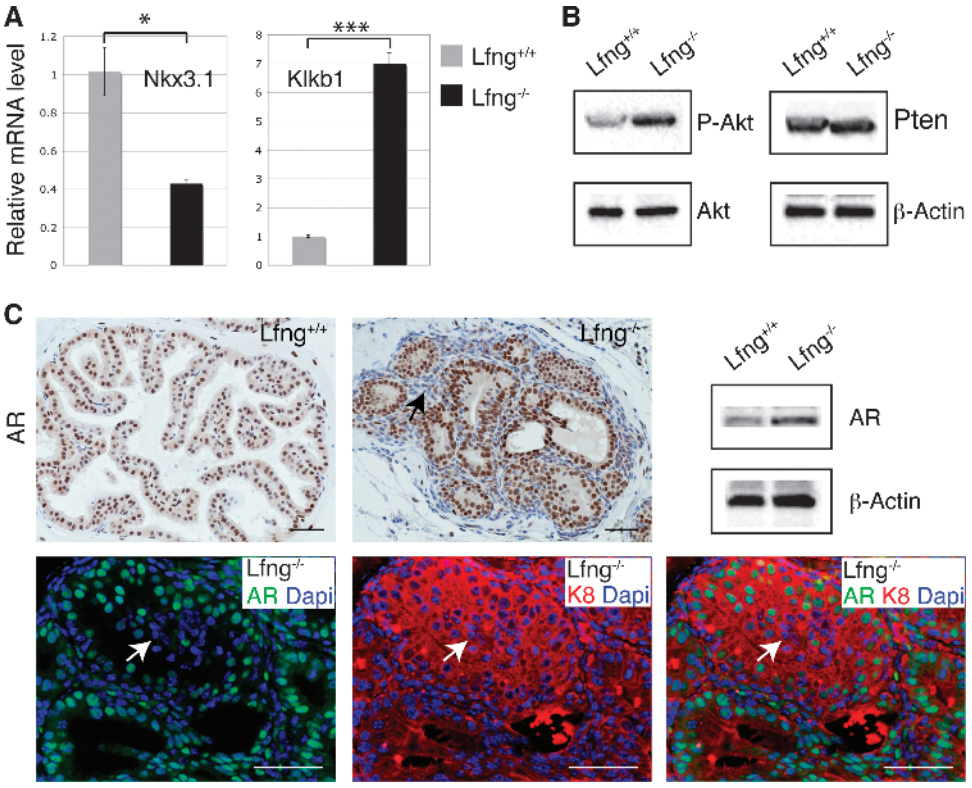

Figure 4. Down-regulation of Nkx3.1, increased AR expression, and elevated Akt phosphorylation in the *Lfng*$^{-/-}$ prostate. (A) Quantitative RT-PCR of Nkx3.1 and Klkb1 in prostates of wild-type and *Lfng*$^{-/-}$ mice at 10 weeks of age. *$P < .05$ and ***$P < .001$. (B) Western blot analysis for Akt, phospho-Akt, and Pten in prostate tissues from 10-week-old mice. (C) Anti-AR staining, Western blot analysis of AR, and double immunofluorescence staining of AR/K8. Black arrow points to accumulation of AR-negative basal cells. White arrows point to a small number of AR-negative, K8-positive luminal cells. Scale bars, 50 μm.

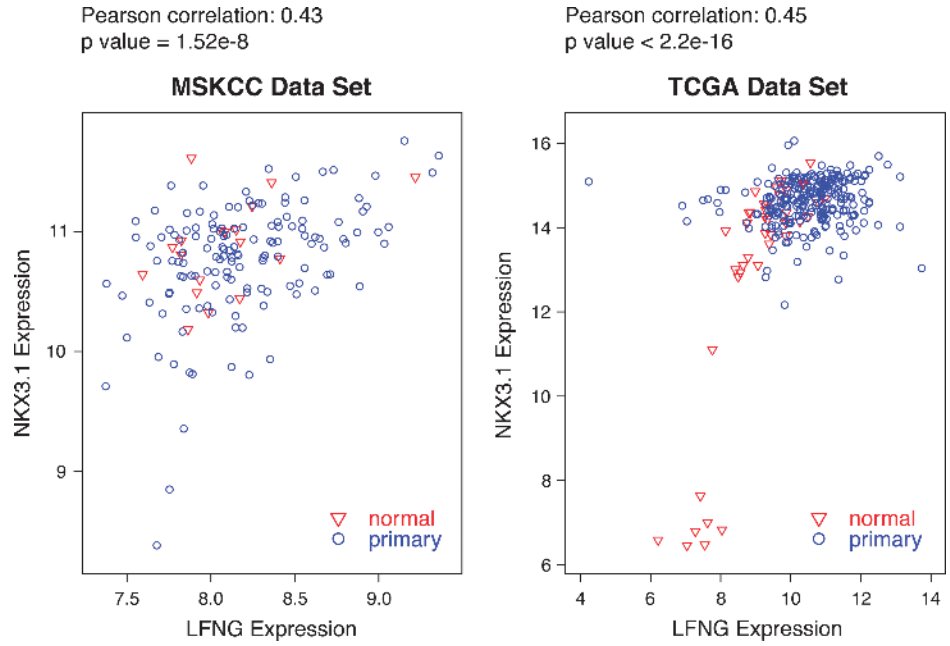

Figure 5. Scatterplots for *LFNG* and *NKX3.1* gene expressions in human primary prostate cancers and normal prostate tissues from two public data sets. Pearson correlation analysis showed positive correlation between LFNG expression and NKX3.1 expression: 0.43 for the MSKCC data set (left; $P = 1.52e\text{-}8$, 160 samples) and 0.45 for TCGA data set (right; $P < 2.2e\text{-}16$, 301 samples).

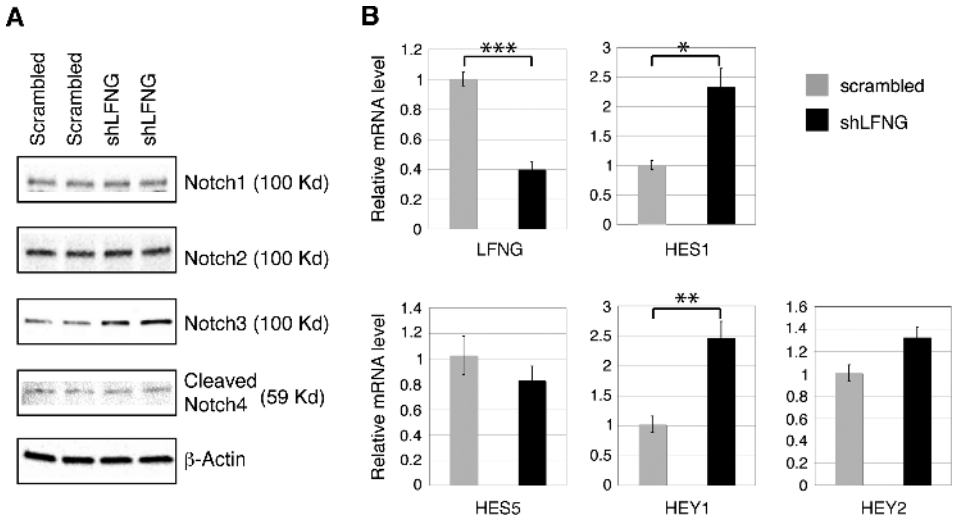

Figure 6. Knockdown of *LFNG* in DU-145 cells caused elevated Notch3 activation. (A) Western blot analysis for Notch receptors in DU-145 cells stably expressing LFNG-shRNA or scrambled control. Duplicate of cell lysates was loaded for each sample. (B) Quantitative RT-PCR of LFNG and Notch downstream targets HES1, HES5, HEY1, and HEY2 in these cells. Triple reactions were carried out for each sample. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

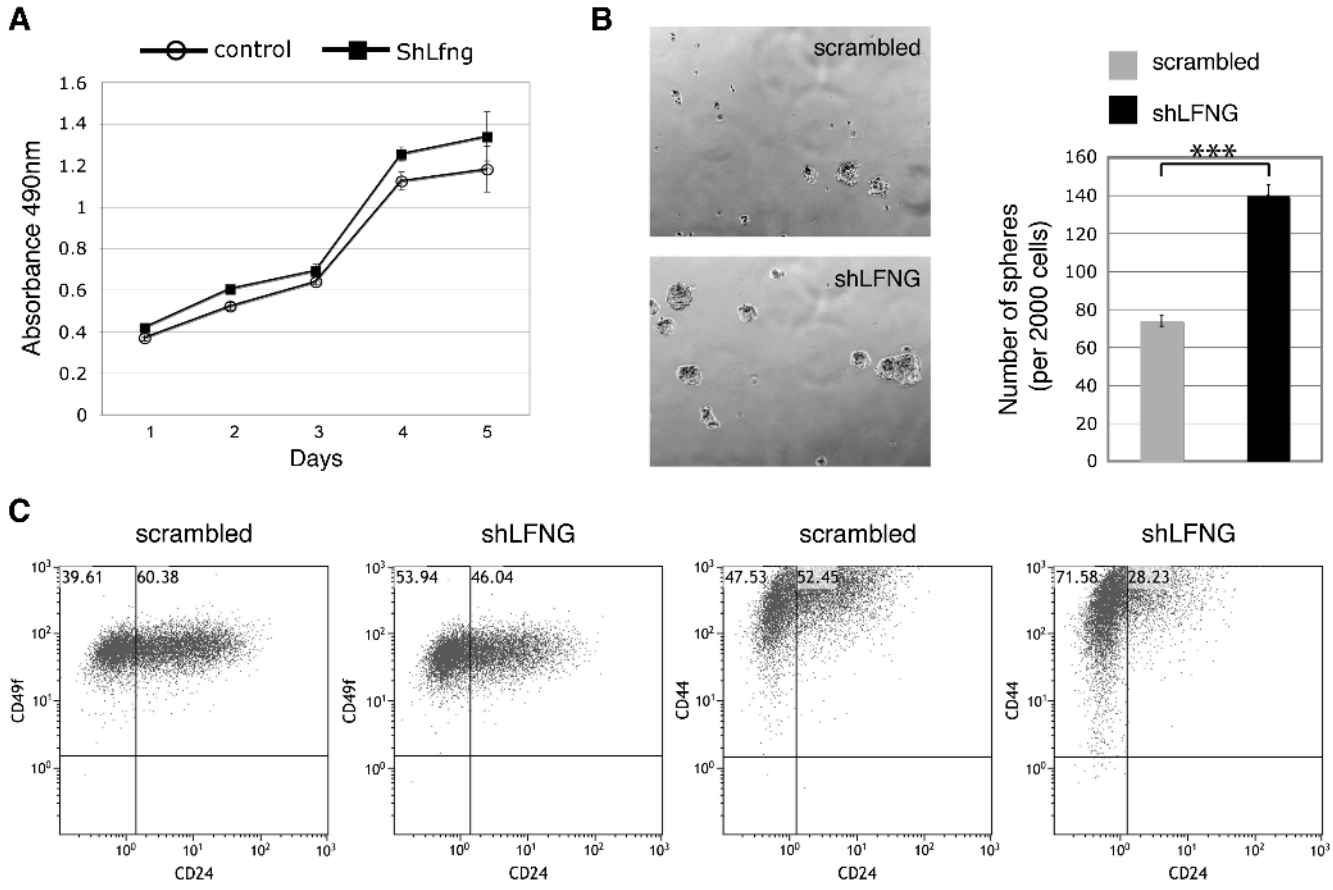

Figure 7. Knockdown of *LFNG* in DU-145 cells caused expansion of cancer stem/progenitor-like cells. (A) Proliferation assay for DU-145 cells stably expressing LFNG-shRNA or scrambled control in monolayer culture. (B) Prostatosphere formation assay for control and experimental DU-145 cells. Shown are representative photographs and quantification of secondary spheres formed after 2 weeks of culture. $^{***}P < .001$. (C) Representative flow cytometry analysis of CD24/CD49f and CD24/CD44 in control and experimental DU-145 cells.

expression data sets available from public sources, namely, MCKCC [31] and TCGA (http://cancergenome.nih.gov/cancersselected/prostatecancer) data sets. Gene expression correlation analysis showed a positive association between *LFNG* and *NKX3.1* gene expressions in both data sets (MSKCC data set: Pearson correlation r = 0.43, P value = 1.52e-8; TCGA data set: Pearson correlation r = 0.45, P value < 2.2e-16) (Figure 5). Interestingly, negative correlations between LFNG expressions and prostate cancer Gleason scores were observed in both data sets: −0.17 for the MSKCC data set (Kendall correlation, P value = 0.014) and −0.16 for the TCGA data set (P value = 0.0084). Although the correlation between *LFNG* gene expressions and Gleason scores is not strong, together with the results from our mouse experiments, these data suggest that LFNG may play a tumor-suppressive role in human prostate cancers as well.

LFNG *Suppresses* In Vitro *Tumorsphere Formation in Human Prostate Cancer Cells*

To test the role of LFNG in human prostate cancer cells, we used shRNA to suppress *LFNG* expression in the DU-145 cell line (Figure 6*B*). Knockdown of *LFNG* caused only slight increase in cell proliferation in two dimensional (2D) culture (Figure 7*A*); however, it significantly increased prostatosphere-forming capacity (Figure 7*B*). Indeed, flow cytometry analysis showed an expansion of stem/progenitor cell–enriched $CD44^+CD24^-$ and $CD49f^+CD24^-$ populations in LFNG knockdown cells (Figure 7*C*) [24,32]. Thus, Lfng can inhibit self-renewal of stem/progenitor-like cells in the prostate under normal physiological condition as well as in the prostate cancer cell population. Interestingly, unlike germline deletion of *Lfng* in mice, *LFNG* knockdown in DU-145 cells resulted in increased accumulation of putative intracellular domain of Notch3 (Figure 6*A*) and increased expression of the Notch target genes *HES1* and *HEY1* (Figure 6*B*). Thus, although *Lfng* appears to suppress self-renewal of normal and cancer stem cells in the prostate, this may involve different Notch receptors and ligands during tumor initiation and progression.

Discussion

Prostate basal stem/progenitor cells are efficient targets for prostate cancer initiation [10–12,14]. Here, we show that deletion of *Lfng* caused a dramatic expansion of prostate basal stem/progenitor cells. Interestingly, the mutant glands showed increased expression and activation of Notch1 and Notch4 in basal cells (Figure 1, *A* and *C*). Given that both Jagged-family ligands, Jagged1 and Jagged2, are highly expressed in basal cells [5], Lfng likely inhibits Jagged-mediated activation of Notch1/Notch4 in these cells, thereby blocking their proliferation. Indeed, forced expression of a constitutively active $Notch1^{ICD}$ in the postnatal prostate using PB-Cre4 caused expansion of the basal compartment [2]. Interestingly, expression of the same $Notch1^{ICD}$ allele using APR2PB-Cre resulted in a decreased number of basal cells [5]. The apparent discrepancy between these studies may arise from use of different Cre-expression deleter strains. PB-Cre4 may cause $Notch1^{ICD}$ expression in prostate epithelium as well as in stroma, which may indirectly influence basal cell activity [33]. In our study, *Lfng* is deleted in the whole animal, and its effect on prostate basal cell activity may also involve cell-autonomous and non–cell-autonomous functions. In fact, a recent report showed that Lfng can repress Notch activity in neighboring cells by modulating Dll1 function [34]. In this context, Lfng could function in the stromal cells to repress Notch activation in adjacent basal cells of prostatic epithelium.

In addition to the expansion of basal compartment, the *Lfng* mutant prostate showed hyperproliferation of luminal cells and PIN formation. This could occur through differentiation of basal stem/progenitor cells into luminal progenitor cells during postnatal development [9]. The *Lfng* mutant prostate may contain increased number of luminal-committed basal cells and/or unipotent luminal progenitors, leading to increased proliferation in the luminal compartment. Loss of *Lfng* could have a direct impact on luminal cell differentiation and proliferation. Whereas Notch2 is highly expressed in basal as well as luminal compartment, Notch1 and Notch4 are preferentially basal, and Notch3 is the only Notch receptor that is almost exclusively luminal [5]. Intriguingly, $Lfng^{-/-}$ prostates showed a decreased level of putative Notch3 intracellular domains (Figure 1*A*), suggesting that Lfng-dependent Notch3 signaling might regulate luminal cell differentiation and/or proliferation.

We observed differential regulation of Notch receptor activation by Lfng in the mouse prostate gland and in a human prostate cancer cell line. For instance, $Lfng^{-/-}$ mutant gland showed elevated Notch1, Notch2, and Notch4 signaling but decreased Notch3 activation, whereas increased NOTCH3 activation occurred in response to *LFNG* gene knockdown in DU-145 cells. The reason for this is not clear. However, the result is not particularly surprising in that 1) Fringe proteins control activation of multiple Notch receptors in response to multiple ligands, 2) expression of specific Notch ligands and receptors is noted in different cells within the prostate epithelial hierarchy as well as in different stages of tumor progression, and 3) multiple cell types and layers interact *in vivo*, whereas this complexity is lost in cell lines grown *in vitro*.

Several studies have implicated dysregulated Notch activation in prostate cancer [35–38]. Negative correlation between *LFNG* expression and Gleason scores in human prostate cancer suggests that LFNG may function as a tumor suppressor through modulation of Notch signaling. Our functional analyses of *Lfng* in the mouse prostate as well as in human prostate cancer cell line support a tumor-suppressive role for this gene. Lfng may suppress tumor initiation by preventing basal cell expansion and PIN formation through inhibiting Jagged-mediated Notch1/Notch4 signaling in basal compartment and facilitating Notch3 signaling in luminal cells. Interestingly, the *Lfng*-null mice rarely develop invasive prostate tumor (only one case in more than 40 animals monitored), suggesting that prostate tumorigenesis in these mice is subject to restraint by other signaling pathways such as TGFβ (Figure 1*A*) [39]. Lfng may also suppress tumor progression through inhibition of Notch-dependent cancer stem cell enrichment. In addition, down-regulation of a known prostatic tumor suppressor gene *Nkx3.1* in the $Lfng^{-/-}$ prostate, as well as positive correlation between expressions of LFNG and NKX3.1 in human prostate cancer, reinforces Lfng as a tumor suppressor and suggests that Lfng-mediated regulation of Notch may well represent a node for therapeutic intervention of the disease. Although *LFNG* gene mutations do not typically occur in human prostate cancer, this study highlights the importance of LFNG in regulation of Notch signaling within the prostatic stem/progenitor hierarchy and as a potent suppressor of inappropriate stem cell self-renewal.

Acknowledgments

We thank the University of Mississippi Medical Center Flow Cytometry Core and Histology Core staff for their expertise.

References

[1] Wang XD, Leow CC, Zha J, Tang Z, Modrusan Z, Radtke F, Aguet M, de Sauvage FJ, and Gao WQ (2006). Notch signaling is required for normal prostatic epithelial cell proliferation and differentiation. *Dev Biol* **290**, 66–80.

[2] Wu X, Xu K, Zhang L, Deng Y, Lee P, Shapiro E, Monaco M, Makarenkova HP, Li J, Lepor H, et al. (2011). Differentiation of the ductal epithelium and smooth muscle in the prostate gland are regulated by the Notch/PTEN-dependent mechanism. *Dev Biol* **356**, 337–349.

[3] Wang XD, Shou J, Wong P, French DM, and Gao WQ (2004). Notch1-expressing cells are indispensable for prostatic branching morphogenesis during development and re-growth following castration and androgen replacement. *J Biol Chem* **279**, 24733–24744.

[4] Shou J, Ross S, Koeppen H, de Sauvage FJ, and Gao WQ (2001). Dynamics of notch expression during murine prostate development and tumorigenesis. *Cancer Res* **61**, 7291–7297.

[5] Valdez JM, Zhang L, Su Q, Dakhova O, Zhang Y, Shahi P, Spencer DM, Creighton CJ, Ittmann MM, and Xin L (2012). Notch and TGFβ form a reciprocal positive regulatory loop that suppresses murine prostate basal stem/progenitor cell activity. *Cell Stem Cell* **11**, 676–688.

[6] Haines N and Irvine KD (2003). Glycosylation regulates Notch signalling. *Nat Rev Mol Cell Biol* **4**, 786–797.

[7] Cohen B, Bashirullah A, Dagnino L, Campbell C, Fisher WW, Leow CC, Whiting E, Ryan D, Zinyk D, Boulianne G, et al. (1997). Fringe boundaries coincide with Notch-dependent patterning centres in mammals and alter Notch-dependent development in *Drosophila*. *Nat Genet* **16**, 283–288.

[8] Johnston SH, Rauskolb C, Wilson R, Prabhakaran B, Irvine KD, and Vogt TF (1997). A family of mammalian *Fringe* genes implicated in boundary determination and the *Notch* pathway. *Development* **124**, 2245–2254.

[9] Ousset M, Van Keymeulen A, Bouvencourt G, Sharma N, Achouri Y, Simons BD, and Blanpain C (2012). Multipotent and unipotent progenitors contribute to prostate postnatal development. *Nat Cell Biol* **14**, 1131–1138.

[10] Wang ZA, Mitrofanova A, Bergren SK, Abate-Shen C, Cardiff RD, Califano A, and Shen MM (2013). Lineage analysis of basal epithelial cells reveals their unexpected plasticity and supports a cell-of-origin model for prostate cancer heterogeneity. *Nat Cell Biol* **15**, 274–283.

[11] Goldstein AS, Huang J, Guo C, Garraway IP, and Witte ON (2010). Identification of a cell of origin for human prostate cancer. *Science* **329**, 568–571.

[12] Lawson DA, Zong Y, Memarzadeh S, Xin L, Huang J, and Witte ON (2010). Basal epithelial stem cells are efficient targets for prostate cancer initiation. *Proc Natl Acad Sci USA* **107**, 2610–2615.

[13] Taylor RA, Toivanen R, Frydenberg M, Pedersen J, Harewood L, Australian Prostate Cancer B, Collins AT, Maitland NJ, and Risbridger GP (2012). Human epithelial basal cells are cells of origin of prostate cancer, independent of CD133 status. *Stem Cells* **30**, 1087–1096.

[14] Choi N, Zhang B, Zhang L, Ittmann M, and Xin L (2012). Adult murine prostate basal and luminal cells are self-sustained lineages that can both serve as targets for prostate cancer initiation. *Cancer Cell* **21**, 253–265.

[15] Xu K, Usary J, Kousis PC, Prat A, Wang DY, Adams JR, Wang W, Loch AJ, Deng T, Zhao W, et al. (2012). Lunatic fringe deficiency cooperates with the Met/Caveolin gene amplicon to induce basal-like breast cancer. *Cancer Cell* **21**, 626–641.

[16] Xu K, Nieuwenhuis E, Cohen BL, Wang W, Canty AJ, Danska JS, Coultas L, Rossant J, Wu MY, Piscione TD, et al. (2010). Lunatic Fringe-mediated Notch signaling is required for lung alveogenesis. *Am J Physiol Lung Cell Mol Physiol* **298**, L45–L56.

[17] Tsao PN, Wei SC, Wu MF, Huang MT, Lin HY, Lee MC, Lin KM, Wang IJ, Kaartinen V, Yang LT, et al. (2011). Notch signaling prevents mucous metaplasia in mouse conducting airways during postnatal development. *Development* **138**, 3533–3543.

[18] Lilla JN, Joshi RV, Craik CS, and Werb Z (2009). Active plasma kallikrein localizes to mast cells and regulates epithelial cell apoptosis, adipocyte differentiation, and stromal remodeling during mammary gland involution. *J Biol Chem* **284**, 13792–13803.

[19] Xiao Y, Ye Y, Zou X, Jones S, Yearsley K, Shetuni B, Tellez J, and Barsky SH (2011). The lymphovascular embolus of inflammatory breast cancer exhibits a Notch 3 addiction. *Oncogene* **30**, 287–300.

[20] Alcalay M, Meani N, Gelmetti V, Fantozzi A, Fagioli M, Orleth A, Riganelli D, Sebastiani C, Cappelli E, Casciari C, et al. (2003). Acute myeloid leukemia fusion proteins deregulate genes involved in stem cell maintenance and DNA repair. *J Clin Invest* **112**, 1751–1761.

[21] Goldstein AS and Witte ON (2012). A plethora of progenitors in the post-natal prostate. *EMBO Rep* **13**, 1036–1037.

[22] Burger PE, Gupta R, Xiong X, Ontiveros CS, Salm SN, Moscatelli D, and Wilson EL (2009). High aldehyde dehydrogenase activity: a novel functional marker of murine prostate stem/progenitor cells. *Stem Cells* **27**, 2220–2228.

[23] van Leenders G, van Balken B, Aalders T, Hulsbergen-van de Kaa C, Ruiter D, and Schalken J (2002). Intermediate cells in normal and malignant prostate epithelium express c-MET: implications for prostate cancer invasion. *Prostate* **51**, 98–107.

[24] van Leenders GJ, Sookhlall R, Teubel WJ, de Ridder CM, Reneman S, Sacchetti A, Vissers KJ, van Weerden W, and Jenster G (2011). Activation of c-MET induces a stem-like phenotype in human prostate cancer. *PLoS One* **6**, e26753.

[25] Park JH, Walls JE, Galvez JJ, Kim M, Abate-Shen C, Shen MM, and Cardiff RD (2002). Prostatic intraepithelial neoplasia in genetically engineered mice. *Am J Pathol* **161**, 727–735.

[26] Abdulkadir SA, Magee JA, Peters TJ, Kaleem Z, Naughton CK, Humphrey PA, and Milbrandt J (2002). Conditional loss of *Nkx3.1* in adult mice induces prostatic intraepithelial neoplasia. *Mol Cell Biol* **22**, 1495–1503.

[27] Kim MJ, Bhatia-Gaur R, Banach-Petrosky WA, Desai N, Wang Y, Hayward SW, Cunha GR, Cardiff RD, Shen MM, and Abate-Shen C (2002). *Nkx3.1* mutant mice recapitulate early stages of prostate carcinogenesis. *Cancer Res* **62**, 2999–3004.

[28] Abate-Shen C, Shen MM, and Gelmann E (2008). Integrating differentiation and cancer: the *Nkx3.1* homeobox gene in prostate organogenesis and carcinogenesis. *Differentiation* **76**, 717–727.

[29] Chen H, Nandi AK, Li X, and Bieberich CJ (2002). NKX-3.1 interacts with prostate-derived Ets factor and regulates the activity of the *PSA* promoter. *Cancer Res* **62**, 338–340.

[30] Lei Q, Jiao J, Xin L, Chang CJ, Wang S, Gao J, Gleave ME, Witte ON, Liu X, and Wu H (2006). NKX3.1 stabilizes p53, inhibits AKT activation, and blocks prostate cancer initiation caused by PTEN loss. *Cancer Cell* **9**, 367–378.

[31] Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, Arora VK, Kaushik P, Cerami E, Reva B, et al. (2010). Integrative genomic profiling of human prostate cancer. *Cancer Cell* **18**, 11–22.

[32] Hurt EM, Kawasaki BT, Klarmann GJ, Thomas SB, and Farrar WL (2008). $CD44^+$ $CD24^-$ prostate cells are early cancer progenitor/stem cells that provide a model for patients with poor prognosis. *Br J Cancer* **98**, 756–765.

[33] Goldstein AS and Witte ON (2013). Does the microenvironment influence the cell types of origin for prostate cancer? *Genes Dev* **27**, 1539–1544.

[34] Okubo Y, Sugawara T, Abe-Koduka N, Kanno J, Kimura A, and Saga Y (2012). Lfng regulates the synchronized oscillation of the mouse segmentation clock via *trans*-repression of Notch signalling. *Nat Commun* **3**, 1141.

[35] Bin Hafeez B, Adhami VM, Asim M, Siddiqui IA, Bhat KM, Zhong W, Saleem M, Din M, Setaluri V, and Mukhtar H (2009). Targeted knockdown of *Notch1* inhibits invasion of human prostate cancer cells concomitant with inhibition of matrix metalloproteinase-9 and urokinase plasminogen activator. *Clin Cancer Res* **15**, 452–459.

[36] Domingo-Domenech J, Vidal SJ, Rodriguez-Bravo V, Castillo-Martin M, Quinn SA, Rodriguez-Barrueco R, Bonal DM, Charytonowicz E, Gladoun N, de la Iglesia-Vicente J, et al. (2012). Suppression of acquired docetaxel resistance in prostate cancer through depletion of Notch- and Hedgehog-dependent tumor-initiating cells. *Cancer Cell* **22**, 373–388.

[37] Santagata S, Demichelis F, Riva A, Varambally S, Hofer MD, Kutok JL, Kim R, Tang J, Montie JE, Chinnaiyan AM, et al. (2004). JAGGED1 expression is associated with prostate cancer metastasis and recurrence. *Cancer Res* **64**, 6854–6857.

[38] Danza G, Di Serio C, Ambrosio MR, Sturli N, Lonetto G, Rosati F, Rocca BJ, Ventimiglia G, Del Vecchio MT, Prudovsky I, et al. (2013). Notch3 is activated by chronic hypoxia and contributes to the progression of human prostate cancer. *Int J Cancer* **133**, :2577–:2586.

[39] Bjerke GA, Yang CS, Frierson HF, Paschal BM, and Wotton D (2013). Activation of Akt signaling in prostate induces a TGFβ-mediated restraint on cancer progression and metastasis. *Oncogene*, E-pub ahead of print Sep 2.