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

Abstract
Elevated Notch ligand and receptor expression has been associated with aggressive forms of prostate cancer, suggesting a 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.

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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
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; Abcam, Cambridge, MA, ab52302), Notch3 (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 RT2 SYBR Green qPCR Mastermixes (Qiagen). We used primer sequences for mouse Hes1, Hes5, Hey1, Hey2, and Klb1 and human LFNG, HES1, HES5, HEY1, and HEY2 as previously reported [17–20]. Mouse Nkx3.1 primer sequences were 5′-TGTCTTTTGCCACGCTCTAA (forward) and 5′-TAGTATACAGG-GAGACAGGAGGTA (reverse). The relative abundance of mRNA for each gene to GAPDH was determined by the equation 2−ΔCT (ACT = threshold cycle (CT)Tested Gene − CTGAPDH). Data were derived from triplicate 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′-AGCAGTGAGCGT-GAGCTACGCTATGTTTT-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, Princeton, NJ, PRB-160P), cytotekatin 8 (K8) (1:800; Covance, MMS-162P), smooth muscle actin (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).
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 AQqueous 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 resuspended 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 × 10^5 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 fluochrome-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

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**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 (NotchICD) accumulation in prostate glands of adult LFNG−/− mice [16]. Western blot analysis using antibody for cleaved Notch1 (Val1744) or cleaved Notch2 (Asp1733) revealed a dramatic increase in the level of Notch1ICD (∼110 kDa) as well as Notch2ICD (∼110 kDa). The mutant glands also showed elevated levels of Notch4 C-terminal peptide that are consistent in size with Notch4ICD (68 kDa). In contrast, decreased levels of a putative Notch3 intracellular protein fragment (∼100 kDa) were observed in the mutant glands (Figure 1A). 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 mRNAs and Hes1 mRNA to a lesser extent (Figure 1B). 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 1C), 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 prostate (Figure 1A). 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 Ouisset 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 multilayered 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 Aldh1a1+ 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 2D). However, three of four mutant glands contained abnormal ducts with a large number of glandular structures showing basally located Sma+ cells (Figure 2D, 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 3A). 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
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 (χ² 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 3B). 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 3C). 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 4A). In addition, Lfng mutant prostate 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 4B). 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 4C) [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 4C). 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 4D), 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

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**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.

Pearson correlation: 0.43
p value = 1.52e-8

Pearson correlation: 0.45
p value < 2.2e-16

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-8, 160 samples) and 0.45 for TCGA data set (right; P < 2.2e-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/cancersel ected/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 6B). Knockdown of LFNG caused only slight increase in cell proliferation in two dimensional (2D) culture (Figure 7A); however, it significantly increased prostatestroma-forming capacity (Figure 7B). Indeed, flow cytometry analysis showed an expansion of stem/progenitor cell–enriched CD44$^+$CD24$^-$ and CD49f$^+$CD24$^-$ populations in LFNG knockdown cells (Figure 7C) [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 6A) and increased expression of the Notch target genes HES1 and HEP1 (Figure 6B). 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 Notch1ICD in the postnatal prostate using PB-Cre4 caused expansion of the basal compartment [2]. Interestingly, expression of the same Notch1ICD 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 Notch1ICD 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$^{−/−}$ prostate showed a decreased level of putative Notch3 intracellular domains (Figure 1A), 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 1A) [39]. Lfng may also suppress tumor progression through inhibition of Notch-dependent cancer stem cell enrichment. In addition, down-regulation of a known prostate 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.

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