Title
Activation of Notch1 synergizes with multiple pathways in promoting castration-resistant prostate cancer

Permalink
https://escholarship.org/uc/item/0n57d752

Journal
Proceedings of the National Academy of Sciences of the United States of America, 113(42)

ISSN
0027-8424

Authors
Stoyanova, T
Riedinger, M
Lin, S
et al.

Publication Date
2016-10-18

DOI
10.1073/pnas.1614529113

Peer reviewed
Activation of Notch1 synergizes with multiple pathways in promoting castration-resistant prostate cancer

Tanya Stoyanova, Mireille Riedinger, Shu Lin, Claire M. Faltermeyer, Bryan A. Smith, Kelvin X. Zhang, Catherine C. Going, Andrew S. Goldstein, John K. Lee, Justin M. Drake, Meghan A. Rice, En-Chi Hu, Kenneth J. Pienta, Robert E. Reiter, Sharon J. Pitteri, Jiaoti Huang, and Owen N. Witte

Contributed by Owen N. Witte, August 31, 2016 (sent for review May 16, 2016; reviewed by Warren S. Pear and Robert L. Vessella)

University of California, Los Angeles, CA 90095; Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, CA 90095; Department of Molecular and Medical Pharmacology, University of California, Los Angeles, CA 90095; Department of Urology, University of California, Los Angeles, CA 90095; Department of Biological Chemistry, University of California, Los Angeles, CA 90095; Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, University of California, Los Angeles, CA 90095; Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, CA 90095; Division of Hematology and Medical Oncology, University of California, Los Angeles, CA 90095; Rutgers Cancer Institute of New Jersey, New Brunswick, NJ, 08903; Department of Medicine, Rutgers-Robert Wood Johnson Medical School, New Brunswick, NJ, 08901; Department of Pathology, University of California, Irvine, CA 92697; Department of Pathology and Laboratory Medicine, University of California, Irvine, CA 90095; Department of Internal Medicine, Brigham and Women’s Hospital, Boston, MA 02115; Brady Urological Institute, Johns Hopkins School of Medicine, Baltimore, MD 21287; Department of Pathology, Duke University School of Medicine, Durham, NC 27710; and Howard Hughes Medical Institute, University of California, Los Angeles, CA 90095

Metastatic castration-resistant prostate cancer (CRPC) is the primary cause of prostate cancer-specific mortality. Defining new mechanisms that can predict recurrence and drive lethal CRPC is critical. Here, we demonstrate that localized high-risk prostate cancer and metastatic CRPC, but not benign prostate tissues or low/intermediate-risk prostate cancer, express high levels of nuclear Notch homolog 1, translocation-associated (Notch1) receptor intracellular domain. Chronic activation of Notch1 synergizes with multiple oncogenic pathways altered in early disease to promote the development of prostate adenocarcinoma. These tumors display features of epithelial-to-mesenchymal transition, a cellular state associated with increased tumor aggressiveness. Consistent with its activation in clinical CRPC, tumors driven by Notch1 intracellular domain in combination with multiple pathways altered in prostate cancer are metastatic and resistant to androgen deprivation. Our study provides functional evidence that the Notch1 signaling axis synergizes with alternative pathways in promoting metastatic CRPC and may represent a new therapeutic target for advanced prostate cancer.

Significance

A high nuclear Notch homolog 1, translocation-associated (Notch1) intracellular domain level distinguishes high-risk prostate cancer and castration-resistant prostate cancer from benign and low/intermediate-risk prostate cancer. Chronic activation of Notch1 cooperates with multiple oncogenic pathways altered in early prostate cancer, including AKT, Myc, and Ras/Raf/MEK, to promote progression to androgen ablation-resistant prostate adenocarcinoma.


Reviewers: W.S.P., University of Pennsylvania; and R.L.V., University of Washington. The authors declare no conflict of interest.

Freedly available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE87448).

1To whom correspondence may be addressed. Email: stoyan@stanford.edu or owenwitte@mednet.ucla.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1614529113/-/DCSupplemental.
Components of the Notch signaling pathway are altered in multiple cancers (21–32). Interestingly, Notch signaling previously has been reported to function as both a tumor suppressor and an oncogene (21–32). The dependency of Notch1 function in cancer may be tissue specific and context dependent (21–32). Loss-of-function mutations in Notch receptors support their tumor-suppressive role in multiple malignancies, including bladder cancer and squamous cell carcinoma (22–24). Constitutive activation of the Notch receptors through gene rearrangements or mutations leads to Notch receptors’ oncogenic function in 55–60% of patients with T-cell acute lymphocytic leukemia (26, 27). An oncogenic role of Notch1 also has been demonstrated in chronic lymphocytic leukemia and in solid tumors such as lung adenocarcinoma and others (28–31). Mutations within Notch1 receptor are rare (3% frequency) in metastatic prostate cancer (32, 33).

Here we report significantly elevated levels of nuclear NICD1 in hormone-naive high-risk prostate cancer and nearly all metastatic CRPC specimens but not in benign tissues or low- and intermediate-risk localized prostate cancer. Although overexpression of NICD1 alone was not sufficient to drive prostate tumorogenesis, NICD1 in combination with components of pathways commonly altered in early prostate cancer, such as myristoylated AKT, Myc, and Ras/Raf/MAPK, promoted the development of aggressive prostate adenocarcinoma and progression to CRPC. Consistent with their aggressiveness, these tumors displayed an epithelial-to-mesenchymal (EMT) transition phenotype, high self-renewal, and the potential for metastatic colonization. Tumors driven by NICD1 in combination with myristoylated AKT, Myc, and the Ras/Raf/MAPK pathway are also resistant to androgen deprivation. Our results indicate that Notch1 receptor signaling plays a central role in the development and progression of prostate cancer and may serve as a rational therapeutic target in high-risk prostate cancer and metastatic CRPC.

Results

Nuclear NICD1 Is Highly Elevated in High-Risk Prostate Cancer and CRPC. To determine if alterations in the Notch signaling axis are associated with prostate tumorigenesis, we first assessed the levels of Notch1 in benign prostate tissue and different stages of human prostate cancer including low- to intermediate-risk (Gleason score 6 and 7) prostate cancer and CRPC. Immunohistochemical analysis of human prostate tissue microarrays (TMAs) showed a significant (more than two-fold) increase in the average intensity of nuclear NICD1 staining in advanced human prostate cancer.

Fig. 1. The Notch1 receptor intracellular domain is highly elevated in advanced human prostate cancer. (A) Human prostate TMAs were stained with an antibody against NICD1 (Novus Biologicals). Representative images are shown. (Scale bars: 100 microns in Upper Row and 50 microns in Lower Row). (B) NICD1 expression was scored from 0–3 in benign tissue (n = 221), localized low- to intermediate-risk prostate cancer (Gleason score 6 or 7) (n = 207), localized high-risk prostate cancer (Gleason score 8–10) (n = 23), and CRPC (n = 19) specimens. (Left) The intensity of nuclear staining was plotted. ***P < 0.0001, **P < 0.005, *P < 0.05; ns, nonsignificant; one-way ANOVA. (Right) Percentage of patients exhibiting nuclear NICD1.

(C) Western blot analyses were performed with anti-NICD1 antibody (Epitomics/Abcam) and anti-Erk2 used as loading control using the following specimens. (i) Human specimens were separated into regions of benign tissue and low- to intermediate-risk prostate cancer by a urologic pathologist. (ii) Metastatic CRPC tissues were obtained from the rapid autopsy program at the University of Michigan. (iii) The Myc/myrAKT CRPC model initiated in primary human cells. Metastatic CRPC tissues were obtained from total of eight different patients (including the samples presented in Fig. S1). Twenty-three distinct metastatic sites are shown. Each distinct patient is indicated by a different color.
Fig. 2. NICD1 synergizes with multiple oncogenic pathways to drive prostate cancer. (A, Left) Schematic representation of the in vivo mouse tissue regeneration assay. Lentiviral transduction was used to overexpress GFP alone (GFP), human NICD1 and GFP (NICD1), mutant kRasG12D and RFP (kRasG12D), myristoylated/activated AKT and RFP (myrAKT), or Myc and RFP (Myc), alone or in combination with NICD1 (NICD1/kRasG12D, NICD1/myrAKT, NICD1/Myc). (B, Right) Transduced epithelial cells were combined with UGSM and implanted under the kidney capsule of SCID mice. Twelve weeks later the grafts were evaluated histologically. One of the five experiments performed is shown. (C) Representives of the recovered grafts from each condition were stained with H&E and were evaluated histologically. Representatives of the recovered grafts are shown. (Scale bars: 500 microns in upper panels; 100 microns in lower panels.)
High levels of nuclear NICD1 are observed predominantly in high-risk prostate cancer and CRPC, suggesting a role for the pathway in prostate cancer progression. We sought to determine whether NICD1 promotes prostate tumorigenesis through collaboration with early genetic alterations. Deletion of the PTEN tumor suppressor is observed in up to 70% of prostate cancers and results in the activation of AKT (37, 38). Other common alterations in advanced human prostate cancer include elevated expression of the Myc oncogene and activation of the Ras/Raf/MAPK signaling pathway (38, 39). To mimic the loss of PTEN, we used myrAKT. Activation of Ras/Raf/MAPK was achieved through overexpression of mutant kRasG12D. Dissociated mouse prostate cells can regrow prostate-like structures in vivo when combined with urogenital sinus mesenchyme (UGSM) followed by implantation under the kidney capsule of SCID mice (Fig. 2A) (40). This prostate regeneration model allows testing the functional role of single genes or combinations of genes in vivo (Fig. 2A) (40). Overexpression of a single oncogene such as myrAKT, kRasG12D, or Myc initiates prostate intraepithelial neoplasia (PIN) in our model but is not sufficient to drive prostate adenocarcinoma. Therefore these genes are suitable for addressing the role of NICD1 in promoting tumor progression (Fig. 2).

Overexpression of NICD1 alone or in combination with kRasG12D (NICD1/kRasG12D), myrAKT (NICD1/myrAKT), or Myc (NICD1/Myc) was investigated for its ability to drive prostate tumorigenesis using the in vivo mouse prostate regeneration model (Fig. 2A). NICD1 alone was not sufficient to drive prostate cancer initiation, because we observed normal prostate tubular structures similar to the GREX expressing control cells (Fig. 2). In contrast, NICD1 strongly synergized with kRasG12D, myrAKT, and Myc, giving rise to prostate adenocarcinoma (Fig. 2B and C). GFP signals in the recovered tissues were used as a control for infection efficiency (Fig. 2B). Importantly, the level of NICD1 overexpression in our in vivo regeneration assay was comparable to the levels observed in human CRPC specimens (Fig. S4). Oncogene expression or pathway activity was confirmed through immunohistochemical analysis (Fig. 3 and Fig. S5).

NICD1/kRasG12D, NICD1/myrAKT, and NICD1/Myc tumors were highly proliferative as measured by proliferating cell nuclear antigen (PCNA), exhibit loss of basal cell marker p63, and express androgen receptors (ARs) (Fig. 3 and Fig. S5). These results demonstrate the functional role of NICD1 in synergizing with alternative pathways to promote the development of advanced prostate adenocarcinoma.

Gene-Expression Profiling of Tumors Driven by NICD1 in Combination with Alternative Pathways Reveals the EMT Signature. To gain insight into how NICD1 promotes advanced prostate cancer, we performed genome-wide transcriptome profiling of NICD1-driven tumors. Tumors initiated by the combination of NICD1/kRasG12D, NICD1/myrAKT, or NICD1/Myc and normal mouse prostate were subjected to high-throughput RNA-sequencing and differential gene-expression analysis (Fig. 4A and Fig. S6A). We identified 4,238 genes significantly up-regulated or down-regulated in NICD1/kRasG12D tumors, 4,433 genes in NICD1/myrAKT tumors, and 3,502 genes in NICD1/Myc tumors compared with normal mouse prostate. Of the differentially expressed genes, 1,944 were common for all three tumors (Fig. 4A and Fig. S6A). Ingenuity pathway analysis identified “cell movement” and “migration” as one of the top regulatory networks across all NICD1-driven tumors (Fig. 4A). As expected, we observed a significant increase in direct transcriptional targets of Notch1 such as Hey2, Hey1, Heyl, Notch3, and Nrp1 in NICD1/kRasG12D, NICD1/myrAKT, and NICD1/Myc tumors compared with normal mouse prostate (Fig. S6B).
Gene set enrichment analysis (GSEA) (41) of differentially expressed genes across all NICD1-driven tumors revealed enrichment of previously published EMT signatures (Fig. 4B and Fig. S7). Our results demonstrate that tumors driven by NICD1 in combination with kRasG12D, myrAKT, and Myc exhibit EMT features, a phenotype that may characterize invasive, poorly differentiated carcinoma (42–45).

**Tumors Driven by NICD1 in Combination with Pathways Altered in Prostate Cancer Exhibit High Self-Renewal Activity.** EMT is a morphological change in which epithelial cells acquire mesenchymal features and is commonly associated with self-renewal activity, an invasive tumor phenotype, and metastasis (46). EMT has been previously demonstrated to stimulate cancer stem cell self-renewal (47). We performed limiting dilution experiments to assess functionally the acquisition of cancer self-renewal activity and stem cell properties and to evaluate the minimum number of cells required to regenerate new tumors upon transplantation. Regenerated NICD1/kRasG12D and NICD1/Myc tumors were dissociated into single cells and subjected to FACS based on expression of RFP and GFP (Fig. 5A and Fig. S8A). The GFP color marker identified cells expressing kRasG12D or Myc, and GFP allowed the detection of NICD1-expressing cells. GFP/RFP double-positive cells were isolated, and 10, 100, 1,000, or 10,000 GFP/RFP double-positive cells were combined with Matrigel and transplanted s.c. into SCID mouse recipients (Fig. 5A and Fig. S8A). By 4 wk as few as 10 cancer cells were sufficient to regenerate the original tumor, demonstrating the high frequency of cancer cells with self-renewing activity within the tumors (Fig. 5B and Fig. S8B and C). Histological analysis identified a phenotype consistent with the original NICD1/kRasG12D- and NICD1/Myc-driven tumors (Fig. 5B and Fig. S8B).

**Tumors Driven by NICD1 in Combination with Pathways Altered in Prostate Cancer Exhibit High Metastatic Colonization Potential.** Self-renewal activity of tumor cells is a necessary property during metastasis that allows cancer cells to colonize at new sites. The metastatic potential of tumors driven by NICD1 in combination with alternative pathways was assessed using an in vivo lung-colonization assay. Regenerated NICD1/kRasG12D and NICD1/Myc tumors were dissociated to single cells and infected with a luciferase-expressing lentivirus. As negative control, we used a previously characterized Myc-CAP cancer cell model derived from Myc transgenic mouse prostates (48) expressing luciferase. Immunocompromised mice were subjected to tail-vein injection with 8 x 10^5 cells. Twenty-eight days after injection we observed large lung tumors in animals injected with NICD1/kRasG12D and NICD1/Myc tumor cells but not in the control animals injected with Myc-CAP cells (Fig. 5C and D and Fig. S9). Histological and immunohistochemical analyses showed that these tumors closely resembled the primary tumors and exhibited high levels of nuclear AR (Fig. S9A). These results demonstrate that NICD1/kRasG12D and NICD1/Myc tumor cells can survive and regrow tumors at a distant site, revealing the metastatic potential of NICD1 combination tumors.

**Tumors Driven by NICD1 in Combination with Pathways Altered in Prostate Cancer Exhibit a Castration-Resistant Phenotype.** The frequent activation of NICD1 in clinical CRPC samples coupled with the high frequency of cancer stem cells and metastatic phenotype of NICD1-driven tumors led us to investigate the role of NICD1 in the development of castration resistance. To test if castration can affect tumor growth, we used cells derived from hormone-naive NICD1/kRasG12D, NICD1/myrAKT, and NICD1/Myc tumors (Fig. 6A and Fig. S10A). Myc-CAP mouse prostate cancer cells, previously demonstrated to be castration sensitive, were used as a control (Fig. 6C) (48). Tumor cells (6 x 10^5) were implanted s.c. into immunocompromised recipients. Upon detection of palpable tumors (average tumor volume, 50 mm^3), recipients were subjected to surgical castration. Myc-CAP cells grew only in intact mice and failed to grow in castrated mice, whereas NICD1/kRasG12D, NICD1/myrAKT, and NICD1/Myc tumors continued to grow rapidly after androgen deprivation (Fig. 6A and C and Fig. S10A).

To establish the capacity of NICD1/kRasG12D, NICD1/myrAKT, and NICD1/Myc tumors to resist androgen deprivation, cancer cells were implanted into castrated SCID mouse recipients. No significant difference in size and histology was seen in the NICD1/kRasG12D, NICD1/myrAKT, and NICD1/Myc secondary tumors recovered from intact or castrated recipients (Fig. 6B and Fig. S10B and C). These results establish that NICD1 in combination with the activation of other pathways promotes the development of prostate cancer and the progression to CRPC.

**Loss of Notch1 and Pharmacological Inhibition of γ-Secretase Delay Prostate Cancer Cell and Tumor Growth.** The γ-secretase complex cleaves Notch cell-surface receptors within the transmembrane domains, leading to the release of the NICD from the membrane to the nucleus, where it is referred to as “activated Notch” (13–21). To evaluate the therapeutic potential of Notch1 inhibition, we first used the γ-secretase inhibitor (GSI) (S)-tert-butyl 2-(S)-2-(2-(3,5-difluorophenyl)acetamido)propanamido)-2-phenylacetate.
Fig. 5. Tumors driven by NICD1 in combination with pathways altered in prostate cancer exhibit the acquisition of self-renewal activity and metastatic potential. (A) Primary NICD/kRas<sup>G12D</sup> tumor was dissociated into single cells and subjected to FACS. (Scale bars: 1 cm.) GFP/RFP double-positive cells were sorted, and 10, 100, 1,000, or 10,000 cells were implanted into SCID recipient mice. (B, Left) Representative recovered tumors initiated from 10, 100, 1,000, or 10,000 cells. (Scale bar: 1 cm.) (Right) Histology of each tumor is shown. (Scale bar: 50 microns.) One of the three independent experiments is presented. (C) Tumors driven by NICD1 in combination with pathways altered in prostate cancer exhibit metastatic potential. Primary tumors driven by NICD1/kRas<sup>G12D</sup> and NICD1/Myc were dissociated into single cells, infected with lentivirus expressing luciferase and YFP, and cultured. One week after infection cells were sorted by FACS for YFP. As a negative control, we used Myc-CAP cells expressing luciferase with YFP. Cells (8 × 10<sup>5</sup>) were introduced to immunocompromised mice via tail vein injection. Bioluminescent images taken on the day of injection are shown. One of the three independent experiments is shown. (D, Upper) Bioluminescent images taken day 28 after injection are shown. (Lower) Recovered lungs are presented. (Scale bars: 1 cm.) The size of the recovered lungs, histology of the lungs, and immunohistochemistry for NICD1, pErk, myc, and AR are presented in Fig. S9.
Fig. 6. NICD1 in combination with the Ras/Raf/MAPK, myrAKT, or Myc pathways drives CRPC. (A, Upper) Schematic representation of the experimental design. Primary tumors driven by NICD1/kRasG12D, NICD1/myrAKT, or NICD1/Myc were dissociated into single cells. NICD1/kRasG12D, NICD1/myrAKT, or NICD1/Myc tumor cells ($6 \times 10^5$) were mixed with Matrigel and transplanted s.c. into six to eight mice per condition. Upon detection of palpable tumors (50 mm$^3$), six to eight mice carrying a total of six to eight tumors were castrated (marked as day 1). Tumor length, width, and height were measured every 3 d for a total of 10–20 d. (Lower) Tumor volumes were calculated by multiplying length $\times$ width $\times$ height/2 and were plotted. One of the two independent experiments for each tumor type (NICD1/kRasG12D, NICD1/myrAKT, or NICD1/Myc) is shown. (B, Upper) The experimental design. NICD1/myrAKT or NICD1/Myc tumor cells ($6 \times 10^5$) were mixed with Matrigel and transplanted s.c. into six castrated or six intact recipient mice. (Lower Left) Upon detection of palpable tumors (50 mm$^3$), marked as day 1, tumor volumes were measured every 3 d. (Lower Right) Recovered NICD1/Myc secondary tumors from intact or castrated recipients are shown. (Scale bars: 1 cm.) One of the two independent experiments for each tumor type (NICD1/myrAKT or NICD1/Myc) is presented. (C, Left) Myc-CAP cells ($6 \times 10^5$) were mixed with Matrigel and implanted s.c. into eight intact or four castrated immunodeficient recipient mice. Upon detection of palpable tumors, one group of intact mice ($n = 4$) was subjected to surgical castration. (Right) Images of the recovered tumors. (Scale bars: 1 cm.)
Fig. 7. Notch1 down-regulation and γ-secretase inhibition delay prostate cancer cell growth. (A) C4-2B, DU145, 22Rv1, and PC3 cells were lysed and subjected to Western blot analysis with antibodies against NICD1 and GAPDH. (B) C4-2B, DU145, and PC3 cells were subjected to a colony-formation assay. Plated cells were treated with DMSO (vehicle) or 25 μM GSI-IX (DAPT) every 48 h for 9 d. The mean colony-formation rate is plotted. Statistical analysis was performed by Student t test. ****P < 0.0001; ***P < 0.005; **P < 0.01; *P < 0.05; ns, nonsignificant. One of the two independent experiments is shown. (C) 22Rv1 and 22Rv1 ΔNotch1 cells were lysed and subjected to Western blot analysis with antibodies against NICD1 and Erk2. (D) 22Rv1 or 22Rv1/ΔNotch1 cells (1 × 10⁶) were plated. Cell number was counted 1, 3, and 6 d after plating. Student t test was used for statistical analysis. **P < 0.01. (E) 22Rv1 and 22Rv1 ΔNotch1 cells were subjected to a colony-formation assay. Cells were treated with DMSO (vehicle) or 25 μM GSI-IX (DAPT) every 48 h for 9 d. The mean colony-formation rate is shown. Student t test was used for statistical analysis. **P < 0.01; ns, nonsignificant.

(DAPT) (GSI-IX). We tested multiple prostate cancer cell lines for expression of NICD1 (Fig. 7A). The androgen-independent prostate cancer cell lines C4-2B, DU145, 22Rv1, and PC3 express different levels of NICD1 (Fig. 7A). All four prostate cancer cell lines were subjected to a colony-formation assay in the presence of DAPT or vehicle (Fig. 7B). Treatment of C4-2B, DU145, 22Rv1, and PC3 cells with DAPT significantly inhibited cell growth as measured by colony formation in vitro (Fig. 7 B and E). Additionally, we generated a 22Rv1 Notch1-knockout cell line via CRISPR/Cas9 to demonstrate the effect of Notch1 loss on cell growth and colony formation (Fig. 7C). Deletion of Notch1 in 22Rv1 cells (22Rv1 ΔNotch1) significantly delayed cell growth (Fig. 7D). Comparable to the effect of DAPT on colony formation, the deletion of Notch1 in 22Rv1 cells led to a significant decrease in colony-formation potential. DAPT had no significant effect on cell growth in ΔNotch1 22Rv1 cells, demonstrating the specificity of the Notch1 effect on prostate cancer cell growth (Fig. 7E).

To evaluate the therapeutic potential of Notch1 inhibition in vivo, mice harboring androgen ablation-resistant 22Rv1 xenograft tumors were treated with either vehicle (n = 4) or DAPT (GSI-IX) (n = 4) at 50 mg/kg (Fig. S11A). Treatment was initiated upon the detection of palpable tumors, and tumor volume was assessed every 4 d after treatment induction. Treatment with DAPT delayed tumor growth of 22Rv1 xenografts. To confirm the on-target effect of DAPT in vivo, the 22Rv1 tumors were harvested and analyzed for NICD1 and cleaved Notch1 levels (Fig. S11B). These results demonstrate that loss or inhibition of Notch1 may represent a promising therapeutic strategy for CRPC.

Discussion

Defining the mechanisms that drive CRPC is critical for the development of new therapies for the lethal form of the disease. Here we demonstrate that NICD1 is localized to the nucleus in 70% of the high-risk prostate cancer and in 95% of CRPC samples analyzed. These findings suggest that nuclear NICD1 may distinguish between low- to intermediate-risk and high-risk prostate cancer and may predict prostate cancer that will recur as CRPC. Although activating mutations of Notch receptors are found in several cancers, there is no evidence of Notch1-activating mutations in advanced prostate cancer. Nuclear NICD1 in prostate cancer may arise as a consequence of Notch1 cleavage caused by elevated levels of Notch ligands, such as Jagged1, which has been reported to be overexpressed in metastasis (7). Mechanisms underlying the activation of Notch1 in advanced prostate cancer are yet to be elucidated.

Because the nuclear localization of Notch receptors reflects their activation state, we devised a strategy to mimic the high expression of Notch1 observed in clinical metastatic CRPC by engineering the overexpression of the NICD in primary mouse prostate epithelium. NICD1 strongly synergized with a broad range of pathways commonly altered in prostate cancer, such as myrAKT, Myc, and Ras/Raf/MAPK. These combinations resulted in advanced prostate adenocarcinoma (Fig. 8). Our observation that NICD1 alone is not sufficient to drive prostate cancer initiation but instead synergizes with other pathways that can initiate only low-grade PIN lesions to drive prostate adenocarcinoma suggests that the Notch 1 receptor has a critical role in the progression of prostate cancer. These results are consistent with previous findings in osteogenic sarcoma and lung adenocarcinoma in which NICD1 strongly synergizes with p53 loss or Myc overexpression to accelerate tumor progression (49, 50).

Transcriptome profiling of tumors driven by NICD1 in combination with myrAKT, Myc, and Ras/Raf/MAPK revealed features of EMT. During development, Notch1 is known to drive EMT (51), a process by which epithelial cells lose their polarity and acquire mesenchymal features. These changes are accompanied by down-regulation of epithelial and adhesion genes such as E-cadherin and cytokeratins and elevated expression of mesenchymal markers such as vimentin and N-cadherin. Acquisition of an EMT phenotype is characteristic of invasive carcinoma, metastasis, and increased tumor cell self-renewal (47). In prostate cancer, down-regulation of E-cadherin is associated with poor prognosis and survival (40), whereas increased expression of the mesenchymal marker N-cadherin correlates with a high Gleason

Fig. 8. NICD1 in combination with the myrAKT, Myc, or Ras/Raf/MAPK pathways promotes metastatic CRPC. The schematic representation summarizes our results.
score and drives CRPC (42, 44). Consistent with the acquisition of EMT features, we found NICD1/myrAKT, NICD1/Myc, and NICD1/RasG12D tumors to be highly metastatic and capable of self-renewal activity. Notch signaling may serve a more general role in cancer stem cell maintenance in multiple tumor types (47).

Promising strategies targeting individual Notch receptors, ligand-receptor interactions, and Notch transcriptional activity have been developed and have demonstrated anticancer activity in animal models (52–54). Humanized monoclonal antibodies that block the ligand–receptor interaction between Notch and its ligand DLL4 demonstrate potent anticancer activity in patient-derived xenograft models (52). Another class of antibodies that targets individual Notch receptors, locking them in an inactive conformation, has also demonstrated promising therapeutic potential (53). Another strategy to inhibit Notch receptors is the use of GSIs that block regulated intramembrane proteolysis and subsequent Notch receptor activation (55). Our finding that Notch1 cooperates with a range of common genetic alterations in prostate cancer suggests that Notch1 inhibition may represent an effective therapy for advanced prostate cancer. Indeed, here we demonstrate that GSI and loss of Notch1 decrease prostate cancer cell growth. Additionally, GSI treatment delays the tumor growth of prostate cancer xenografts. Consistent with our findings, previous study has shown that the GSI PF-03084014 results in a significant decrease in tumor growth in two xenograft models of prostate cancer (PC3 and DU145) (12). PF-03084014 demonstrated an even greater antitumor effect in prostate cancer growth when combined with the chemotherapeutic agent docetaxel, which is currently in clinical use (12).

We provide functional evidence that NICD1 synergizes with multiple pathways in driving poorly differentiated prostate adenocarcinoma and metastatic CRPC in vivo (Fig. 8). Our results suggest that activation of Notch1 may serve as a biomarker to predict the potential benefit of Notch1 inhibition and as a therapeutic target for patients suffering from metastatic CRPC.

Materials and Methods

TMAs. Seventy-five prostatectomy specimens from patients never treated with hormonal therapy were reviewed, and areas of normal prostate (indicated as benign tissue) and low- to intermediate-risk prostate cancer (Gleason patterns 6–7) were marked for sampling. Two cores per sample, each measuring 0.6 mm in diameter, were taken from selected regions in each paraffin block and transferred to a recipient paraffin block containing a total of 150 benign or cancer cores. Unstained sections of 4-μm thickness were used for immunohistochemical staining. Two additional TMAs were constructed containing (i) benign tissues and adjacent low- to intermediate-risk prostate cancer from 40 xenograft tumors and (ii) benign tissues and adjacent single-core samples (low- to intermediate-risk and high-risk) from 115 patients. Additional CRPC TMAs were constructed from blocks containing transurethral resection tissues from 20 patients who failed androgen-ablation therapy (i.e., patients with CRPC) and who developed urinary obstruction. Two cores from each patient block were transferred to a new TMA block (CRPC TMA). A section from the TMA blocks was obtained and used for immunohistochemical staining.

Mouse Strains. C57BL/6, CBA/Ig (wild type), and NSG (NOD-SCID-IL2Rγ-null) mice were purchased from the Jackson Laboratory. Animals were housed at the University of California, Los Angeles animal facilities according to the regulations of the Division of Laboratory Animal Medicine.

Prostate Tissue Regeneration Assays. Housing, breeding, and all surgical procedures were performed in agreement with the guidelines of the Division of Laboratory Animal Medicine of the University of California, Los Angeles. All experimental procedures were approved by the Division of Laboratory Animal Medicine of the University of California, Los Angeles. The details of the regeneration process have been explained previously (56).

Lung Colonization Assay. NICD1/Myc and NICD1/RasG12D secondary tumors were excised, minced, and incubated in DMEM containing 10% FBS, 1 mg/ml collagen, and 3 mg/ml dispase for 1 h at 37 °C. To achieve single-cell dissociation, tumor chunks were spun down, further treated with 0.05% Trypsin-EDTA for 5 min at 37 °C, passed through a 20-gauge syringe, and filtered through a 70-μm filter mesh. Cells were cultured for 2 wk followed by infection with lentivirus-expressing luciferase and YFP. Mice were injected with 8 × 106 NICD1/Myc, NICD1/RasG12D, and Myc-CAP cells expressing luciferase and YFP via tail vein injection. Thirty minutes after injection, animals were injected i.p. with 150 μL of 15 mg/ml luciferin followed by bioluminescence imaging with the IVIS Lumina II imaging system.

RNA Sequencing. RNA was extracted from mouse tumor tissue using the RNeasy Mini Kit from Qiagen. Libraries for RNA-sequencing were prepared with NuGen Ovation RNA-Seq System V2. The workflow consists of double-stranded cDNA generation using a mixture of random and poly (T) priming, fragmentation of double-stranded cDNA, and repair to generate blunt ends, A-tailing, adapter ligation, and PCR amplification. Different adapters were used for multiplexing samples in one lane. Sequencing was performed on an Illumina HiSeq 2500 for a pair-read 100 nt run. Data quality control was done on an Illumina Sequencing Analysis Viewer. Demultiplexing was performed with Illumina CASAVA 1.8.2. The reads were first mapped to the latest University of California, Santa Cruz transcript set using Bowtie2 version 2.1.0, and the gene-expression level was estimated using RSEM v1.2.15. TPM (transcript per million) was used as the normalized gene expression. Differentially expressed genes were identified using the DESeq package. Genes showing altered expression with a false-discovery rate \( P < 0.05 \) and more than twofold changes were considered differentially expressed.

Vector Production and Viral Packaging. The third-generation lentiviral vectors FUGW and FUCRW, derived from FUGW, were used for the construction of the human NICD1, kRasG12D, myrAKT, and Myc constructs.

Antibodies. The following antibodies were used: anti-Notch1 [Novus Biologicals J312; Abcam ab52627; Cell Signaling Technology C37C7; Cell Signaling Technology (Valip447) (D388); anti-PCNA (Cell Signaling), anti–AR-N-20 (Santa Cruz), anti-p63 (Santa Cruz), and anti-AKT (Cell Signaling).

Immunohistochemistry. Indicated tissues were fixed in 10% buffer and were embedded in formalin and paraffin. Four-micron sections were deparaffi- nized in xylene and rehydrated in 100, 95, and 70% ethanol. Antibegin retrieval was performed with citrate buffer (pH 6.0) at 95 °C for 20 min. Sections were blocked using mouse-on-mouse blocking reagents (BMK-2202; Vector Labs). The sections were incubated with the indicated antibodies overnight. Slides were washed with 1x PBS and incubated with anti-mouse HRP or anti-rabbit HRP antibodies (DAKO) for 1 h, developed with HRP substrate (DAKO), and counterstained with hematoxylin.

Colonoy-Formation Assay. Five hundred C4-2B, DU145, 22RV1, and PC3 cells per well were plated in triplicate in a six-well plate. Cells were treated with DMOS (vehicle) or 25 μM DAPT (GSI-IX; Selleck Chemicals LLC) every 48 h. Nine days after plating colonies were fixed with ice-cold methanol for 30 min at ~20 °C and were stained with 0.1% crystal violet in 1x PBS for 30 min at room temperature. Plates were washed with 1 h with water. Colonies were counted, and the colony formation rate (%) was calculated as the number of colonies per 500 cells × 100.

Generation of Notch1 22Rv1 Cell Lines. The 5′ UTR and first exons of Notch1 were used to design guide RNAs. Sequences were loaded on crispr.mit.edu. A sequence spanning the ATG was identified (guide #1 Notch1 CRISPR forward CACCCGGGGAGGCATGCCGCCGCTCC; guide #1 Notch1 CRISPR reverse AAACGGACGGGCCCCGATGCCTCC). Overhangs appropriate for cloning onto BbsI sites were added. Oligos were annealed and cloned into the PX58 vector. pSpCas9(BB)-2A-GFP (PX588) (Addgene plasmid no. 48138) was a gift from Feng Zhang, Broad Institute of MIT and Harvard, Cambridge, MA; McGovern Institute for Brain Research, Cambridge, MA; and Departments of Brain and Cognitive Sciences and Biological Engineering, MIT, Cambridge, MA (57). 22Rv1 cells (5 × 105 cells per well in 2% DMEM in a six-well plate) were transfected with 5 μg DNA using Lipofectamine 2000. GFP+ cells were sorted by FACS 24 h after transfection and were plated in a 96-well plate at four cells per well. Cells were grown until wells with single colonies were 50% confluent (4 wk). Loss of Notch1 was confirmed by Western blot.

In Vivo Studies with GSI. NSG mice (8–10 wk old) were injected s.c. in the flank with 1 × 105 22Rv1 cells in Matrigel. When tumors reached palpable size (50 mm3), animals were randomly assigned to treatment with vehicle (control) or with DAPT (50 mg/kg GSI-IX). Vehicle and DAPT were dissolved in 10% ethanol and 90% corn oil and were administered by oral gavage on the following schedule: 3 d on, 1 d off, 6 d on, and 1 d off. The schedule was...
repeated two times. The length (L), width (W), and height (H) of tumors were measured every 4 d. Tumor volume was calculated by \( V = \frac{1}{2} \times L \times W \times H \).

ACKNOWLEDGMENTS. We thank the University of California, Los Angeles (UCLA) Tissue Procurement Core Laboratories for tissue preparation. T.S. is supported by a Prostate Cancer Foundation Young Investigator Award and N\&I/Natural Cancer Institute (NCI) K99/R00 Pathway to Independence Award (principal investigator: Mitchell Wong). C.M.F. is supported by a UCLA Tumor Biology Training Grant. S.L. is supported in part by the American Urological Association/Urology Care Foundation Research Scholar Award Program. B.A.S. is supported by the Specialty Training and Advanced Research (STAR) Program at UCLA, a Prostate Cancer Foundation Young Investigator Award, and a UCLA Clinical and Translational Science Institute-KL2 Award KL2TR001882 (principal investigator: Mitchell Wong) from the NIH/National Center for Advancing Translational Sciences. J.M.D. is supported by Department of Defense Prostate Cancer Research Program Grant W81XWH-15-1-0236 and a Prostate Cancer Foundation Young Investigator Award. J.H. is supported by a Prostate Cancer Foundation Research Program Grant P50CA092131 (to the UCLA SPORE in prostate cancer). A.S.G and O.N.W. are supported by a Prostate Cancer Foundation Young Investigator Award. O.N.W. is an Investigator of the Howard Hughes Medical Institute. L.A. is partly supported by the Ely and Edythe Broad Center of Regenerative Medicine and Stem Cell Research. K.J.P. is supported by a PCF Challenge Award and by the NIH. R.E.R. is supported by NIH/NCI Grant P50CA092131 (to the UCLA SPORE in prostate cancer). A.S.G and O.N.W. are supported by a PCF Creativity Award (principal investigator: O.N.W.). A.S.G., J.H., and O.N.W. are supported by a PCF Challenge Award (principal investigator: O.N.W.) and the UCLA SPORE in prostate cancer (principal investigator: R.E.R.).

Fig. S1. Nuclear NICD1 is elevated in advanced human prostate cancer. The figure shows negative and positive controls for the human prostate TMA stained with an antibody against the intracellular domain of Notch1 presented in Fig. 1A. (Left) Benign human tissue; epithelial cells are negative for NICD1. (Center) Previously described mouse Myc-CAP xenografts (41). Myc-CAP xenografts are negative for nuclear NICD1. (Right) As a positive control we used tumors driven by the combination of NICD1 overexpression and active AKT overexpression (myrAKT). (Scale bars: 100 microns in the upper row; 50 microns in the lower row.)
**Fig. S2.** NICD1 is highly expressed in metastatic prostate cancer but not in benign prostate tissues and low- or intermediate-risk prostate cancer. Western blot analyses were performed using anti-NICD1 antibody (Epitomics; currently Abcam), and anti-Erk2 was used as a loading control. For the Western blot analyses we used (i) human prostate tissues separated into benign (B) and low- to intermediate-risk prostate cancer (T) regions; (ii) metastatic CRPC tissues obtained from the rapid autopsy program at the University of Michigan; and (iii) the Myc/myrAKT CRPC model initiated in primary human cells that express high levels of full-length Notch1 (Notch1 FL, ~300 kDa) and NICD1 (~100 kDa). Distinct patients with metastatic disease are shown in different colors.

**Fig. S3.** NICD1 is highly expressed in metastatic prostate cancer. Western blot analyses were performed on metastatic CRPC tissues obtained from the rapid autopsy program at the University of Michigan using anti-cleaved Notch1 V1744 antibody (Cell Signaling Technology). GAPDH was used as a loading control. Distinct patients with metastatic disease are shown in different colors. Different colors and metastatic sites match the patients' samples analyzed in Fig. 1C and Fig. S2.
Fig. S4. Expression levels of NICD1 in tumors driven by NICD1 in combination with kRas<sup>G12D</sup>, myrAKT, or Myc resemble levels of nuclear NICD1 in human CRPC specimens. Levels of nuclear NICD1 overexpression mimic the levels of nuclear NICD1 in human CRPC. Immunohistochemical analyses of 4-μm sections of paraffin-embedded human low- to intermediate-risk prostate cancer (Gleason score 6 or 7), localized high-risk prostate cancer (Gleason score 8–10), CRPC specimens, and NICD1/myrAKT tumors with antibodies against NICD1 (Novus Biologicals) are shown. (Scale bars: 100 microns in left panels; 50 microns in right panels.)
NICD1/myrAKT, NICD1/Myc, and NICD1/kRas\textsuperscript{G12D} tumors are highly proliferative. Immunohistochemical analysis of 4-μm sections of paraffin-embedded NICD1/myrAKT, NICD1/Myc, and NICD1/kRas\textsuperscript{G12D} tumors stained with H&E or with antibodies against NICD1, pErk, pAKT, Myc, or PCNA. (Scale bars: 100 microns.)
Fig. S6. Tumors driven by NICD1 in combination with pathways altered in prostate cancer express high levels of Notch1 target genes. (A) Heat map of common genes differentially expressed in NICD1/Myc, NICD1/myrAKT, and NICD1/kRasG12D tissue versus normal mouse prostate. Red represents genes overexpressed, and blue represents genes down-regulated across all NICD1/Myc, NICD1/myrAKT, and NICD1/kRasG12D tissues versus normal mouse prostate. (B) The fold change in reads per kilobase per million mapped reads (RPKM) of transcript of Notch1 target genes (Hey2, Hey1, Heyl, Notch3, and Nrarp) and GAPDH from our RNA-sequencing analysis is shown. The average of RPKM of each gene in the mouse prostate is considered as 1.
Fig. S7. Tumors driven by NICD1 in combination with pathways altered in prostate cancer exhibit EMT characteristics. Plots representing GSEA of genes differentially expressed in NICD1/myrAKT, NICD1/Myc, and NICD1/kRas$^G12D$ versus normal mouse prostate are shown. NES, normalized enrichment score.
Fig. S8. NICD1 in combination with alternative pathways exhibits high self-renewal activity and metastatic potential in vivo. (A) Dissociated single cells from a primary NICD1/Myc tumor were subjected to FACS. GFP/RFP double-positive cells were sorted. GFP/RFP cells were implanted into SCID recipient mice at limiting dilutions of 10, 100, 1,000, or 10,000. (Scale bars: 0.5 cm.) (B) Left) Representative tumors initiated from 10, 100, or 1,000 cells recovered 4 wk after implantation or from 10,000 cells recovered 3 wk after implantation. (Scale bars: 1 cm.) (Right) H&E staining of each tumor is shown. (Scale bars: 50 microns.) One of the two independent experiments is shown. (C) The Myc-CAP cell line (44) has lower self-renewal activity and was used as a control. Representative tumors initiated from 10, 100, or 1,000 Myc-CAP cells recovered 8 wk after implantation. (Scale bar: 1 cm.)
Fig. S9. Tumors driven by NICD1 in combination with other pathways altered in prostate cancer exhibit metastatic potential. (A) Immunohistochemistry analysis of paraffin-embedded lungs from the mice injected with NICD1/kRas<sup>G12D</sup> and NICD1/Myc cells shown in Fig. 5A stained with H&E or antibodies against NICD1, pErk, Myc, and AR. (Scale bars: 100 microns in left panels; 50 microns in center and right panels.) (B) Weight of the lungs recovered at day 28 after injection from the animals shown in Fig. 5C injected with Myc-CAP-Luciferase, NICD1/kRas<sup>G12D</sup>-Luciferase, or NICD1/Myc-Luciferase was measured in milligrams and plotted. **P < 0.005, one-way ANOVA.
Fig. S10. Tumors driven by NICD1 in combination with other pathways altered in prostate cancer are castration resistant. (A) Images of the transplanted tumors driven by NICD1/kRas\(^{G12D}\) plotted in Fig. 6A. (Scale bars: 1 cm.) (B) NICD1/kRas\(^{G12D}\) or NICD1/Myc tumor cells (6 × 10\(^5\)) were mixed with Matrigel and were transplanted s.c. into six castrated or six intact recipient mice. Upon detection of palpable tumors (50 mm\(^3\)), tumors volumes were measured and plotted. (C) Histology of NICD1/kRas\(^{G12D}\), NICD/myrAKT, and NICD1/Myc tumors recovered from intact or castrated recipients. (Scale bars: 100 microns.)

Fig. S11. γ-Secretase inhibition delays prostate cancer tumor growth. (A) 22Rv1 cells (1 × 10\(^6\)) were injected s.c. in NSG mice. Upon detection of palpable tumors, animals were treated with either vehicle (n = 4) or DAPT (GSI-IX) (n = 4) administered for 20 d according to the following schedule: 50 mg/kg DAPT for 3 d followed by 1 d off DAPT, then 50 mg/kg DAPT for 6 d and 1 d off DAPT. Day 1 is the day of treatment initiation. (B) Inhibition of Notch1 by DAPT in vivo was confirmed by Western blot for NICD1 and the cleaved Notch1 expression level.