Notch1 activation or loss promotes HPV-induced oral tumorigenesis

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Abstract

Viral oncogene expression is insufficient for neoplastic transformation of human cells, so HPV-associated cancers will also rely upon mutations in cellular oncogenes and tumor suppressors. However, it has been difficult so far to distinguish incidental mutations without phenotypic impact from causal mutations that drive the development of HPV-associated cancers. In this study, we addressed this issue by conducting a functional screen for genes that facilitate the formation of HPV E6/E7-induced squamous cell cancers in mice using a transposon-mediated insertional mutagenesis protocol. Overall, we identified 39 candidate driver genes including Notch1, which unexpectedly was scored by gain or loss of function mutations that were capable of promoting squamous cell carcinogenesis. Autochthonous HPV-positive oral tumors possessing an activated Notch1 allele exhibited high rates of cell proliferation and tumor growth. Conversely, Notch1 loss could accelerate the growth of invasive tumors in manner associated with increased expression of matrix metalloproteinases and other pro-invasive genes. HPV oncogenes clearly cooperated with loss of Notch1, insofar as its haploinsufficiency accelerated tumor growth only in HPV-positive tumors. In clinical specimens of various human cancers, there was a consistent pattern of NOTCH1 expression that correlated with invasive character, in support of our observations in mice. While Notch1 acts as a tumor suppressor in mouse skin, we found that oncogenes enabling any perturbation in Notch1 expression promoted tumor growth, albeit via distinct pathways. Our findings suggest caution in interpreting the meaning of putative driver gene mutations in cancer, and therefore therapeutic efforts to target them, given the significant contextual differences in which such mutations may arise, including in virus-associated tumors.
Introduction

While viral oncogenes are often necessary for carcinogenesis, they are not sufficient to cause cancer. Several approaches have sought to identify the cellular perturbations that facilitated the development of virally induced cancers. To this end, groups have extensively mapped cellular pathways reprogrammed by viral oncogenes (1) and sequenced cervical cancers as well as head and neck squamous cell cancers (HNSCCs) that are frequently associated with Human Papillomavirus (HPV) infections (2-5). Together, these studies have identified mutations in and/or activation of genes including NOTCH1, PIK3CA, PTEN and KRAS among others. However, these studies have primarily catalogued the mutations associated with HPV cancers and, therefore, cannot distinguish between incidental mutations with no phenotypic impact from causal mutations that mediate disease. For example, evidence supports conflicting functions of NOTCH1 as either a tumor suppressor or an oncogene based on the tissue context (6-8). Furthermore, previous observations did not account for how additional driving mutations, which are commonly found in cancers, may impact the tissue specific functions of these candidates. Finally, validation of these candidates may benefit from primary tumor models that better reflect the human condition (9) because previous reports using in vitro passaged cell models have shown contradictory observations for the same gene (10, 11). By identifying and validating the genetic events that impact HPV-positive tumor growth in vivo, we will better prognose outcomes and tailor therapies accordingly.
Here, we used transposon-based insertional mutagenesis as a functional screen to identify cellular genes that promoted the growth of autochthonous HPV-positive tumors. We identified 39 candidate genes, of which \textit{Notch1} was frequently mutated. Forward and reverse genetic approaches revealed that gain or loss of Notch1 promoted tumorigenesis in the skin. Thus, by identifying candidates that promoted HPV-positive tumorigenesis, we observed that Notch1 functioned unlike a traditional oncogene or tumor suppressor where gain or loss of activity accelerated tumor growth.
Methods

Animals

All mice were maintained under specific pathogen-free conditions and used according to protocols approved by The University of Chicago (Chicago, IL) Institutional Animal Care and Use Committee and Institutional Biosafety Committee. KH mice expressing the K14-CreER\textsuperscript{tam} transgene (12) and the Lox-Stop-Lox-HPV-Luc (LSL-E6E7) transgene have been described (13). Onc3 mice (STOCK Gt(ROSA)26Sor\textsuperscript{tm2(sb11)Njen} TgTn(sb-T2/Onc3)12740Njen/Nci and STOCK Gt(ROSA)26Sor\textsuperscript{tm2(sb11)Njen} TgTn(sb-T2/Onc3)12775Njen/Nci) and Onc2 mice (STOCK Gt(ROSA)26Sor\textsuperscript{tm2(sb11)Njen} TgTn(sb-T2/Onc2)6113Njen/Nci and STOCK Gt(ROSA)26Sor\textsuperscript{tm2(sb11)Njen} TgTn(sb-T2/Onc2)6070Njen/Nci) were obtained from NCI Mouse Repository (14-16). B6.129S4-Kras\textsuperscript{tm4Tyj}/J (LSL-Kras\textsuperscript{G12D}) mice (17), STOCK Gt(ROSA)26Sor\textsuperscript{tm1(Notch1)Dam}/ (LSL-NICD) (18), B6.129X1-Notch1\textsuperscript{tm2Rko}/GridJ (Notch1\textsuperscript{flox/flox}) (19) were purchased from The Jackson Laboratory. Details of mouse strains are described in the Supplementary Methods and Supplemental Table 1.

Carcinogenesis models

For skin carcinogenesis, one week after TAM treatment, the dorsal surface of mice was shaved and 25 \(\mu\)g of DMBA in 200 \(\mu\)l was topically administered. One week after DMBA treatment, 7.5 \(\mu\)g of TPA in 200 \(\mu\)l was administered twice per week for 8 weeks. Papilloma formation was monitored twice per week. Papillomas greater than 2 mm in diameter were scored. For oral tumor carcinogenesis, mice were treated with TAM and
one week later 4-NQO treatment was initiated at 20 μg/mL in drinking water. 4-NQO water was replaced weekly.

**Sleeping Beauty transposon mapping and identification of Common Insertion Sites**

Transposon mapping was adapted from a previously reported protocol using ligation-mediated PCR with modifications described in the Supplementary Methods (16).

Supplemental Table 2 lists primers used in SB transposon mapping and used to make pooled PCR libraries that were sequenced using an Illumina HiSeq2500 (single-end 100 bp) runs at University of Chicago Functional Genomics Facility using standard methods. Sequencing reads were preprocessed to remove 5’ transposon tag, 3’ adaptor sequences and 3’ low quality bases (base quality<20) using Cutadapt 1.1 and eliminated final reads shorter than 36bp. We mapped preprocessed reads to the mm9 mouse genome assembly using Novoalign 3.00.05, allowing one best match per read. We removed reads with mapping quality lower than 20, reads with target sequences lacking the 5’ TA transposon insertion site and reads that mapped to the donor chromosome. We used the final reads to identify significant CIS using the TapDance pipeline (20). Genomic regions significantly enriched with CIS were identified across samples by a sliding window approach, which applies Poisson distribution statistics normalized by the genome size, total number of events (insertions) and the observed integrations within a defined window. The identified significant CISs were annotated with nearby genes located within a maximum of 20,000bp in distance.
Tumor growth measurements

Tumors were measured every 3 days as previously described (13). We measured the intercommissural distance (a), two additional orthogonal measurements (b and c) and the lip thickness on each side (d and e) to calculate the oral tumor volume (mm$^3$) using the equation: \[
\frac{1}{6}\pi^*a*b*c - \pi ((\frac{1}{2}(a-d-e))^2)*c.
\]

Immunohistochemistry and Immunofluorescence

Tumors were isolated and placed into 10% formalin solution for 24 hours and then dehydrated with 70% ethanol. Tissues were processed and stained with hematoxylin and eosin or the indicated antibodies at the University of Chicago Immunohistochemistry core facility. Histology was reviewed by a board-certified pathologist (M.W.L.). For immunofluorescence, tissues were embedded in OCT and immediately frozen in dry ice/2-methylbutane. Immunofluorescence images were captured on a Zeiss Axiovert 200M. We used a modified ImmunoRatio (21) Plugin installed in ImageJ to quantify Hes1, Hey1 or BrdU-positive nuclei. The percentage of positively stained nuclei was assessed using a color deconvolution algorithm and adaptive threshold for nuclei area segmentation.

Microarrays

Tissues were snap frozen in TRIzol (Invitrogen) and stored at -80 C until use. RNA was prepared and treated with DNase (Thermo Scientific). 4-6 distinct tumors were used per group covering 2-3 independent experiments. RNA quality was checked using an Agilent Bioanalyzer (Santa Clara, CA) and biotin-labeled using an Illumina TotalPrep-96
RNA Amplification Kit (Illumina). RNA expression detection was performed using the Illumina Mouse Ref-8 v2 BeadChip (Illumina, San Diego, CA). Microarrays were scanned using an Illumina HiScan. Raw signal intensities of each probe were obtained using data analysis software (Genome Studio; Illumina) and imported into Partek after quantile normalization and background subtraction. Differentially expressed genes were identified using Analysis of Variance (ANOVA) model. We filtered differentially expressed genes with ≥ ± 2.0-fold change and an FDR <0.05. Gene specific expression analysis were performed using the GSEA package (22). Pathway analysis on differentially expressed genes and CIS were assessed using Ingenuity IPA. Microarray data have been deposited in Gene Expression Omnibus (Accession numbers GSE59950 and GSE59951).

**Quantitative RT-PCR**

Total RNA was isolated using TRIzol (Invitrogen), and treated with DNase I. cDNA was generated using ProtoScript® First Strand cDNA Synthesis Kit (NEB) and subjected to qPCR using SYBR Green Supermix (Biorad). RT-PCR primer pairs for Mmp3, Mmp9, Mmp10, Mmp11, Mmp13, Twist2 and Col1a1 were obtained from Bio-Rad. qPCR reactions were run on an iQ 5 Multicolor Real-time QPCR Detection System Machine (Bio-rad). CT values for individual samples were subtracted from the corresponding CT values for Gapdh (ΔCT). Normalized fold expression was obtained using the –log2 ΔΔCT formula.

**Notch1 expression in clinical samples**
For cervical and breast cancer database analysis, Notch1, Hes1 and Hey1 expression data were downloaded from Oncomine (Life Technologies) using the Zhai cervix database (n = 37) and the Curtis breast database (n = 211). Notch1 expression in verrucous and invasive HNSCCs was conducted on prospectively acquired specimens in accordance with the University of Chicago IRB14-0729. IHC scoring was performed on a scale of 0 to 3+ for intensity. Notch expression was grouped into negative expression (0-1+) and positive expression (2+ to 3+). All histological diagnosis and IHC expression was assessed by a single pathologist (M.L.). Only patients with documented pathologies were included in analyses.

**Statistical and Database analysis**

Aside from statistical analysis of microarray and insertional mutagenesis experiments described elsewhere, statistical analysis was performed using JMP version 10 (SAS Institute). All tests to determine statistical significance were two-sided and statistical significance was defined as \( P < .05 \). Survival curves were plotted using the Kaplan-Meier method and comparisons between categorical risk factors were conducted using the log-rank test. The chi-square test was used to compare differences between discrete variables, and the \( t \) test was used to compare continuous variables. Differences between medians were assessed using the Wilcoxon test.
Results

Sleeping Beauty Transposon-Based Insertional Mutagenesis Accelerated HPV-positive Carcinogenesis

Since additional cellular mutations likely cooperate with HPV oncogenes to cause cancer, we used Sleeping Beauty (SB) transposon-based insertional mutagenesis which has identified candidate cancer genes in other models (14, 16, 23, 24). To study HPV carcinogenesis, we used transgenic mice to conditionally express the HPV16 oncogenes E6 and E7 as well as SB transposition in the skin. We bred KH mice, containing the K14-CreER<sup>tam</sup> and LoxP-STOP-LoxP (LSL)-E6E7 transgenes(13), to Onc mice, containing a Cre-regulated LSL-SB11 transgene (16) and a T2/Onc transposon transgene (KH x Onc2 and KH x Onc3 mice; Figure 1A) (14, 15). Tam treatment of KH x Onc mice activated the CreER<sup>tam</sup> transgene expressed in basal keratinocytes to excise the inhibitory LSL sequences in order to induce expression of HPV oncogenes and SB-transposition. As controls, we used K x Onc (K x Onc2 and K x Onc3) mice that only induced SB-transposition in the skin as well as KH mice.

To assess the impact of insertional mutagenesis on HPV oncogenesis, we treated mice with DMBA/TPA to accelerate skin tumorigenesis (Figure 1A). Papillomas occurred earlier in KH x Onc mice compared to control K x Onc mice and KH mice (P < .0001; Figure 1B). Furthermore, more papillomas developed in KH x Onc mice (P < .0001) compared to K x Onc mice or KH mice. For KH x Onc tumors, 70.7% (n=29) were invasive cancers, 4.9% (n=2) were verrucous carcinomas and 24.4% (n=10) were
non-invasive lesions (Figure 1D&E). The majority of tumors displayed SB-mediated transposition and HPV oncogene recombination (Supplemental Figure 1A). From 150 KH x Onc tumors treated with DMBA/TPA, 145 tumors (96.7%) contained a recombined LSL-E6E7 oncogene indicating HPV oncogene expression and 119 tumors (79.3%) contained SB-excision at the donor chromosome (Supplemental Figure 1A&B). As DMBA induces \( Hras^{Q61K} \) mutations, we found that 98.0% of tumors (\( n=147 \)) tumors contained this mutation.

In a second carcinogenesis model, we employed 4-NQO to induce oral tumors (Supplemental Figure 2A). Compared to control KH x Onc mice that did not receive Tam, both KH x Onc mice (\( P = .003 \)) and K x Onc mice (\( P = .005 \)) more frequently developed oral tumors (Supplemental Figure 2B). 77% of oral tumors were invasive squamous cell carcinomas while the remainders were premalignant lesions or normal tissue (Supplemental Figure 2C&D). Thus, in two distinct HPV models, we have utilized insertional mutagenesis in order to accelerate tumor development.

Identification of Common Insertion Sites (CIS) in HPV-Positive and HPV-Negative Tumors

To identify transposon insertions, we applied a high-throughput analysis to map T2/Onc transposon integration sites in mutagenized tumors (16). In DMBA/TPA treated mice, we isolated T2/Onc integration sites from 69 KH x Onc tumors and 3 K x Onc tumors. In 4-NQO treated mice, we isolated T2/Onc integration sites from 27 KH x Onc tumors and 15 K x Onc tumors. High-throughput sequencing of pooled libraries resulted
In 12,706,111 reads that were successfully mapped to the mouse genome and were used for identification of common insertion sites (CIS). In total, 39 significant regions were identified in both carcinogenesis models (Figure 2 and Supplemental Table 3). 50 coding genes were within these regions of which 39 genes possessed transposon insertions. Of these 39 genes only 2, \textit{Notch1} and \textit{Gphn}, were orthologous with known cancer associated genes as determined by the Cancer Gene Consensus. Thus, we have identified several known and novel candidate genes involved in HPV carcinogenesis of which some human orthologs possess were cancer associated genes.

**Notch1 Contained Transposon Insertions with Opposing Orientations that Correlated with Heterogeneous Gene Expression**

In both DMBA/TPA and 4-NQO models, \textit{Notch1} was located in one of the most significant CIS regions identified. In 25 distinct DMBA/TPA treated KH x Onc tumors, there were 26 transposon insertions (Figure 3A, Supplemental Table 3&4; $P = 9.1 \times 10^{-32}$). In 6 distinct 4-NQO treated KH x Onc tumors there were 17 transposon insertions ($P = 2.0 \times 10^{-5}$). Similarly, 15 of the 39 candidate genes identified using transposon mutagenesis were associated with the Notch pathway indicating the importance of this pathway during HPV tumorigenesis (Figure 3B). Supplemental Table 5 summarizes the transposon insertion frequency and orientation for genes in Figure 3B. Using PCR to amplify the transposon-gene junction, we confirmed that these \textit{Notch1} insertions were tumor specific in 15 KH x Onc3 tumors tested (Figure 3C).
Previous studies (14, 23) have used these transposon orientations to infer gene function because the transposon payload has a directional promoter and polyadenylation site that respectively induces or inhibits gene expression depending on the orientation of the transposon insertion. Here, we observed that in DMBA/TPA treated tumors, transposons were oriented to induce Notch1 expression in 14 distinct tumors as well as to inhibit Notch1 expression in 12 tumors (Figure 3A; Supplemental Table 4). In tumors with transposons oriented to induce gene expression, we detected Notch1 expression by quantitative RT-PCR (qRT-PCR) as well as by immunohistochemistry (Figure 3D; Supplemental Table 4; Supplemental Figure 3). By contrast, in tumors with transposons oriented to inhibit gene expression, we detected decreased Notch1 mRNA expression and less intense Notch1 staining in tissue sections. Thus, even though the Notch1 pathway was a significant target for insertional mutagenesis, transposon insertions were predicted to induce and to inhibit Notch1 activity.

**Activated Notch1 Accelerated HPV-Positive Primary Oral Tumor Growth**

Given that tumor development was associated with transposon insertions predicted to upregulate Notch1 expression, we assessed the impact of Notch1 activation on HPV tumor growth. To account for the role of Hras mutations during HPV tumorigenesis, we used a KHR oral tumor model (13) where a tamoxifen-regulated Cre recombinase drives HPV and KrasG12D oncogene expression in the epidermis and
reflects the KRAS mutations which have been identified in HPV-associated cancers (3, 5). We generated KHR mice containing a LSL-Rosa<sup>NICD</sup> transgene (18), where the expression of the active Notch1 intracellular domain (NICD) was also dependent on Cre recombinase (KHR<sup>NICD</sup> mice). After Tam treatment, KHR<sup>NICD</sup> oral tumors expressed the NICD transgene (Supplemental Figure 4). KHR<sup>NICD</sup> oral tumors grew faster compared to control KHR mice or KHNICD mice (Figure 4A). Similarly, anal tumors were larger in KHR<sup>NICD</sup> mice (18.2±0.7 mm<sup>2</sup>) compared to KHR mice (11.4±0.5 mm<sup>2</sup>; P < .0001) or KHNICD mice (5.6±0.9 mm<sup>2</sup>; P < .0001; Figure 4B). Furthermore, compared to KHR mice, KHR<sup>NICD</sup> tumors possessed increased nuclear localization for the Notch1 transcriptional targets Hey1 and Hes1 (Figure 4C; Supplemental Figure 5).

Since NICD overexpression regulated basal cell differentiation (25), we assessed tumor cell proliferation and differentiation markers in oral tumors. Using BrdU incorporation to assess proliferation, KHR<sup>NICD</sup> oral tumors contained more BrdU-positive cells compared to KHR oral tumors (29.8±5.6% vs. 19.8±5.2%; P < .0001; Figure 4D&E). KHR<sup>NICD</sup> anal tumors also contained more BrdU-positive cells compared to KHR anal tumors (27.5±5.5% vs. 19.2±5.2%; P < .0001). Consistent with increased BrdU incorporation in KHR<sup>NICD</sup> tumors, gene enrichment analysis revealed that KHR<sup>NICD</sup> tumors were enriched in genes associated with DNA replication and cell cycle mitosis (Figure 4F). While KHR tumors already displayed impaired differentiation as demonstrated by CK14 expression throughout the epithelial layers, expression of NICD did not further impact tumor differentiation as measured by CK14 intensity (Figure 4G & Supplemental Figure 6). Thus, expression of activated Notch1 accelerated the
growth of tumors displaying impaired differentiation profiles indicating that Notch1 possessed oncogenic activities in primary HPV tumors.

**Haploinsufficiency of Notch1 Accelerated HPV-Positive Primary Oral Tumor Growth**

Since transposon orientation in HPV-positive tumors was also predicted to inhibit Notch1 expression, we assessed the impact of Notch1 loss on KHR tumor growth. We generated HPV-positive KHR mice and HPV-negative KR mice with hemizygous (KHR-N+/− and KR-N+/−, respectively) or homozygous (KHR-N−/− and KR-N−/−, respectively) loss of Notch1. KHR-N−/− oral tumors grew similarly as KHR-N+/− tumors (P = .44; Figure 5A) but grew faster than control KHR tumors (P = .0002). While KHR tumors haploinsufficient for Notch1 grew similarly as KHR tumors with complete loss of Notch1, HPV-negative KR tumors required loss of both Notch1 alleles for accelerated tumor growth. Namely, oral tumors in KR-N−/− mice grew faster than oral tumors in KR-N+/− mice (P < .0001) or oral tumors in KR mice (P = .002; Figure 5B). While Notch1 loss promoted oral tumor growth, it also facilitated the appearance of non-mucosal squamous tumors in KHR-N+/− mice and KHR-N−/− mice (P = .0003; Figure 5C). We did not observe Notch1 expression in KHR-N−/− and KR-N−/− tumors consistent with their respective genotypes (Figure 5D). While KHR-N+/− and KR-N−/− tumors did express the downstream Notch target Hes1 in suprabasal cells, we did not observe Hes1 expression in the basal epithelial layer of KHR-N−/− or KR-N−/− tumors consistent with the primary location for Notch1 activity (Figure 5E). KHR-N−/− oral tumors incorporated similar levels
of BrdU as KHR-N^+/- oral tumors (51.9±5.7% vs. 52.5±4.2%; P = .62) but incorporated more BrdU compared to KHR oral tumors (29.2±4.2%; P < .0001; Figure 5F&G). Loss of Notch1 did not further impact differentiation defects already present in KHR tumors as tumors had similar CK14 staining intensity and distribution (Figure 5H & Supplemental Figure 7). Thus, haploinsufficient loss of Notch1 cooperated with HPV oncogenes to accelerate proliferation and tumor growth.

**Loss of Notch1 Correlated with Increased Expression of Genes Associated with Invasion in KHR Tumors and an Invasive Phenotype in Human Cancers**

To further understand how loss of Notch1 impacted KHR tumor growth, we performed gene expression profiling in primary oral tumors. Since KHR-N^-/- and KHR-N^+/- tumors grew similarly, we combined both genotypes in order to compare their transcriptional profiles to the wild type KHR tumors. Here, we detected 77 differentially expressed genes in the KHR-N^-/- and KHR-N^+/- group compared to the KHR group (Supplemental Table 6). Using these differentially expressed genes to perform unsupervised hierarchical clustering, we observed that KHR-N^-/-, KR-N^-/- and KHR-N^+/- tumors clustered similarly and apart from KHR and KR-N^+/- tumors (Figure 6A). The clustering KHR-N^-/-, KR-N^-/- and KHR-N^+/- tumors was consistent with the observation that Notch1 haploinsufficiency was sufficient to accelerate tumor growth only in HPV-positive mice. When only KHR-N^-/- tumors were compared to KHR tumors, we observed increased expression of the non-canonical Notch ligand Thbs2 (Supplemental Table 6) but not other Notch family members suggesting that KHR-N^-/- tumors may partially
compensate for Notch1 loss. Of the 50 genes differentially expressed between KHR\textsuperscript{NICD} and KHR-N\textsuperscript{-/-} tumors (Supplemental Table 7), 22 genes overlapped with genes differentially expressed between KHR-N\textsuperscript{-/-} and KHR tumors. Pathway analysis of KHR\textsuperscript{NICD} and KHR-N\textsuperscript{-/-} tumors identified enrichment of distinct pathways that promote tumor growth. Namely, KHR-N\textsuperscript{-/-} tumors were enriched for epithelial-mesenchymal transition and angiogenesis pathways (Supplemental Table 8&9). By contrast, KHR\textsuperscript{NICD} tumors were enriched in E2F and Myc pathways (Supplemental Table 9), consistent with the activation of these pathways by Notch1 (26, 27). In contrast to KHR tumors, KHR-N\textsuperscript{-/-} tumors were enriched for matrix metalloproteinase (MMP), cell adhesion, epithelial-mesenchymal and metastasis pathways (Figure 6B; Supplemental Table 8). qRT-PCR analysis confirmed overexpression of Mmp3, Mmp9, Mmp10, Mmp11, Mmp13, Col1A1 and Twist2 in KHR-N\textsuperscript{-/-} and KHR-N\textsuperscript{+/-} tumors compared to KHR tumors (Figure 6C). By contrast, Mmp3, Mmp9 and Mmp13 were upregulated in KR-N\textsuperscript{-/-} but not KR-N\textsuperscript{+/-} or KR tumors (Figure 6D). Furthermore, Notch1 activation resulted in downregulation of Mmp9, Mmp10 and Mmp13 in KHR\textsuperscript{NICD} tumors compared to KHR tumors (Figure 6E). Thus, HPV oral tumors with haploinsufficiency or complete loss of Notch1 had increased expression of genes associated with invasion.

While tumors with either intact or deficient Notch1 displayed evidence of invasion (Supplemental Figure 8), our data suggested Notch1 loss promoted a more invasive phenotype due to the upregulation of genes important for invasion and migration. To further correlate Notch1 loss with tumor invasion, we assessed NOTCH1 expression in human head and neck cancers, cervical cancer and breast cancers with defined pre-
invasive/less invasive and invasive lesions. As HPV-positive head and neck cancers rarely present with pre-invasive disease, we assessed Notch1 expression in squamous cell carcinomas and exophytic verrucous carcinomas in retrospectively acquired head and neck specimens. Previous groups demonstrated that squamous cell carcinomas were associated with a more invasive phenotype and higher MMP expression while verrucous carcinomas were associated with less aggressive features and lower MMP expression (28). From 14 verrucous carcinomas and 72 invasive squamous cell carcinomas, 85.7% of verrucous carcinomas (12/14) expressed moderate to high levels of Notch1 compared to 22.2% of invasive squamous cell carcinomas (16/72; \( P < .0001 \); Figure 7A&B). To extend these results to other HPV-positive and HPV-negative cancers, we assessed Notch1 expression in cervical and breast cancers both of which present with clinically relevant pre-invasive and invasive lesions. In cervical cancer, Notch1 and its transcriptional targets Hes1 and Hey1 were downregulated in invasive cervical cancers compared to pre-invasive lesions (Figure 7C). Similarly, invasive breast cancer had lower Notch1 expression compared to normal breast tissue (Figure 7D). Thus, changes in Notch1 expression in cervix, breast and head and neck cancers supported our observations that loss of Notch1 resulted in a more invasive phenotype.
**Discussion**

Here, we identified several cellular genes that facilitated HPV carcinogenesis including Notch1 that functioned unlike a traditional tumor suppressor or oncogene. Our observation that Notch1 activation promoted primary tumor growth contrasts with previous observations where Notch1 inhibited squamous carcinogenesis (29). We draw our conclusions using both forward and reverse genetic approaches and, thereby, avoid the limitations of using a single model system. Since Notch1 gain or loss promoted the growth of tumors possessing the same driving mutations, the proto-oncogenic roles of Notch1 activation or loss occurs under similar genomic contexts. Furthermore, these genetically engineered mouse models avoided the impact of undefined genetic changes present during chemical carcinogenesis and/or in transplanted tumor models. In addition, we manipulated Notch1 activity in primary tumor models harboring defined oncogenic mutations which better reflects the genetic changes occurring in cancers. Finally, differences in Notch1 expression in human cancers and in HPV tumors undergoing insertional mutagenesis indicates that pathophysiologic activation or loss of Notch1 promoted distinct tumorigenic phenotypes. Therefore, tumors with multiple driving mutations, such as those associated with HPV, may negate the tumor suppressive activities of Notch1 and allow dichotomous Notch1 expression to stimulate distinct pro-growth pathways.

While we used multiple models where autochthonous tumors arose in mice with defined oncogenic events, our conclusions are limited by the intrinsic nature of any model system. First, the mixed genetic background could contribute to the manner by
which a Sleeping Beauty insertion impacts tumorigenesis, albeit by unknown mechanisms and undefined polymorphisms between C57BL/6 and FVB. For validation experiments, we used C57BL/6 and (C57BL/6xFVB)F1 mice with genetically defined backgrounds in order to avoid the impact of genetic differences on tumors possessing normal or altered Notch1 activity. In addition, our oral tumor models used a Kras\textsuperscript{G12D} transgene which is infrequently mutated in human HNSCCs or other cancers associated with HPV (2, 4, 5, 30, 31). While Seiwert et al. have reported cumulative mutations in HRAS and KRAS as high as 10\% in HPV-positive HNSCCs (5), others have observed the frequency of Ras mutations as low as 4\% or less in HNSCCs (2, 4, 30, 31). Similarly, Ras mutations are reported in only 10-20\% of cutaneous squamous cell carcinomas (32, 33). Finally, insertional mutagenesis in HPV transgenic mice relied on carcinogens to initiate tumorigenesis which differs from the clinical scenario as HPV tumors do not often display signatures of mutagen exposure. Therefore, our observations using models with defined HPV oncogene expression and Kras mutations may be applicable to only a fraction of HNSCCs that possess these mutations.

Using transposon-based insertional mutagenesis, we identified 39 candidate genes associated with the development of HPV-positive cancers. While 15 of these candidates were previously identified in models of Ras induced papillomas (34), the physiological role of these candidates remained uncertain as SB-insertional mutagenesis did not significantly impact tumorigenesis in that model. By contrast, tumor development in our model required HPV expression and/or SB-insertional mutagenesis. In addition, other studies involving SB insertional mutagenesis identified Usp9x and
Arid1b as CIS in pancreatic adenocarcinomas (24) and medulloblastomas (35), respectively. Consequently, we have identified 22 additional candidates involved in HPV carcinogenesis. These additional CIS may reflect our increased sequencing depth where we analyzed 273,054 unique sequence reads compared to 50-85,000 unique sequences in previous reports (15, 16). As with transposon-based insertional mutagenesis, several groups have observed that the genomic integration of viral DNA may also drive carcinogenesis by inserting in proximity to known cancer-associated genes (3, 36-38). Similar to these reports, we did observe transposon integrations in regions containing Lipc and Usp9x that were also targets for integration by the HPV genome in human cancers. Thus, we have identified known and novel candidates involved in HPV carcinogenesis that provided novel leads for how these genes impacted tumor growth.

Our observation that Notch1 activation promoted primary tumor growth contrasts with previous observations where Notch1 inhibited squamous carcinogenesis. Classically, activating NOTCH1 mutations were observed in T cell acute lymphocytic leukemia targeting the Negative Regulatory Region (NRR, exons 26-28) as well as the inhibitory PEST domain (exon 34; Supplemental Figure 9). By contrast, in HNSCC and other squamous cancers, inhibitory mutations have been identified in the ligand binding domain and the Ankryn repeat domains. However, potentially activating mutations in the NRR and PEST domains as well as in another regulatory region, Abruptex domain have been identified and are displayed in Supplemental Figure 9 (2, 4, 39). While many of the transposon insertions that promoted Notch1 activity in our model did so most likely
by inducing gene expression, 5 transposons integrated into introns 27 and 33 that also likely mimic the mutations in the NRR or PEST domains, respectively.

While transgenic expression of NICD accelerated HPV tumor growth, expression of the activated NICD in cultured keratinocytes or in neonatal mouse skin resulted in growth arrest and the induction of differentiation (25, 40). While these studies relied on models of normal skin differentiation, we observed that Notch1 promoted the growth of tumors containing additional driving mutations. Since loss of $p21^{WAF1/CIP}$ or $p16^{INK4A}$ also abrogated the growth inhibitory properties of Notch1 in cultured keratinocytes (40, 41), inhibition of such pathways by HPV and/or Kras oncogenes in our model likely enabled Notch1 to promote tumor growth. Such additional oncogenic mutations in cancers may explain the opposing actions of Notch1 as a tumor suppressor or oncogene in cultured HPV-positive cells (10, 11). Namely, by impairing tumor differentiation, some oncogenes may enable other Notch1 functions to further enhance tumor growth. Thus, Notch1 may use multiple pathways to stimulate the growth of cancers harboring additional driving mutations.

While we observed that loss of Notch1 promoted tumorigenesis consistent with previous reports (29, 42-44), we also observed that the tumor genotype dictated the threshold level of Notch1 necessary to impact tumor growth. Namely, HPV-positive tumors grew faster with haploinsufficient or complete loss of Notch1. By contrast, HPV-negative tumors grew faster only with complete loss of Notch1 consistent with previous reports (29, 44). Analysis of 279 HNSCCs from The Cancer Genome Atlas contained
revealed that 46 of 197 HPV-negative cancers (18.9%) had at least loss of one Notch1 allele while HPV-positive cancers had 1 of 35 events (2.8%; $P = 0.005$) (30). While we caution conclusions due to the imbalanced and limited sample sizes, these results are consistent with the model where HPV oncogenes decrease the threshold of Notch1 loss necessary for carcinogenesis. HPV oncogenes may enable hemizygous loss of Notch1 to accelerate tumor growth via distinct mechanisms. While cutaneous HPV oncogenes such as HPV8 E6 may directly repress Notch1 activity (1, 45), similar findings have not been reported for high risk HPV16 oncogenes used in our model. By contrast, our observations suggest HPV oncogenes may facilitate the activation of pathways involved in cell migration and invasion that complement the impact of Notch1 haploinsufficiency. Therefore, Notch1 haploinsufficiency in cancers harboring multiple oncogenic drivers may induce an invasive phenotype that is distinct from the role Notch1 activation plays in tumors.

In both forward and reverse genetic experiments, we observed that Notch1 gain or loss promoted the growth of HPV-positive tumors. By contrast, other reports indicated that Notch1 can function either as an oncogene as observed in T cell acute lymphocytic leukemia (ALL) (46) and pancreatic cancer (47) or as a tumor suppressor as observed in head and neck cancer (2, 4) and chronic myelogenous monocytic leukemia (48) among others. Previous groups sought to rectify these opposing Notch1 functions based on the ability of Notch1 to control differentiation depending on the tissue context (6, 49). In our model, gain or loss of Notch1 promoted the growth of similar tumors that also harbored HPV and Kras oncogenes. Therefore, additional driving mutations that
commonly occur in cancers may impair Notch1’s ability to control cell fate and its resulting tumor suppressive function. Thus, given the paradoxical actions of Notch1 in similar tumors, therapeutic strategies targeting the Notch pathway should account for the impact of additional driving mutations in cancers.

In conclusion, we identified several cellular genes that facilitated HPV carcinogenesis including Notch1. The oncogenic role of Notch1 depended more on the tumor genotype than the tissue context that contrasts with the previous notions of tissue specific functions for Notch1. Furthermore, activation or loss of Notch1 likely stimulated tumor growth through distinct pathways. Therefore, our results suggest that traditional tumor suppressors or oncogenes may function differently in the context of additional driving mutations and promote tumor growth via distinct pathways. Thus, interpreting gene function in the presence of additional driving mutations will enable us to better predict tumor behavior and to tailor therapies accordingly.
Acknowledgments

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References


Figure Legends

Figure 1. Sleeping Beauty insertional mutagenesis and HPV oncogene expression accelerated tumorigenesis in DMBA treated mice. (A) Scheme for chemical carcinogenesis in HPV mice. Mice were generated carrying following transgenes: KH (K14-CreER<sup>tam</sup> x LSL-E6E7), K x Onc2 (K14-CreER<sup>tam</sup> x LSL-SB11x T2/Onc2), KH x Onc2 (K14-CreER<sup>tam</sup> x LSL-E6E7 x LSL-SB11x T2/Onc2), K x Onc3 (K14-CreER<sup>tam</sup> x LSL-SB11x T2/Onc3) and KH x Onc3 (K14-CreER<sup>tam</sup> x LSL-E6E7 x LSL-SB11 x T2/Onc3). Tam treatment induced HPV oncogene expression and/or SB11 mediated transposition in the skin. One week later, mice underwent DMBA-TPA treatment. (B) Papillomas developed faster in mice expressing HPV oncogenes and undergoing insertional mutagenesis. Kaplan-Meier analysis of KH mice (n=5), K x Onc2 mice (n=7), KH x Onc2 mice (n=25), K x Onc3 mice (n = 18) and KH x Onc3 mice (n=33). (C) Mice expressing HPV oncogenes and undergoing insertional mutagenesis developed more papillomas. (* denotes P < .0001). Data displayed in quantile box-and-whisker plots where upper and lower whiskers represent the 1<sup>st</sup> and 4<sup>th</sup> quartiles, respectively, and the upper and lower boxes represent the 2<sup>nd</sup> and 3<sup>rd</sup> quartiles, respectively. (D) Histology of papillomas developing in KH x Onc mice. Left panels scale bar = 500 μM; Right panels scale bar = 100 μM. Box in lower magnification field area depicted in the higher magnification field. (E) Summary of histologic types of papillomas arising in KH x Onc mice.
Figure 2. Identification of Common Insertion Sites (CIS) in HPV positive tumors.
(A) Circos plot of CIS from tumors arising in DMBA treated KH x Onc mice. (B) Circos plot of CIS from tumors arising in 4-NQO treated KH x Onc mice. Outer ring represents the chromosomal karyotype. Middle ring represents histogram plot of the individual transposon insertion clusters. Inner ring represents the number of transposon cluster orientations predicted to induce (green color) or to inhibit (orange color) gene expression. Distance from the black axis represents the number of individual transposon clusters. Size of the marker indicates number of tumors involved.

Figure 3. Notch1 was a CIS candidate displaying opposing transposon insertions that were associated with heterogeneous gene expression. (A) Gene view of transposon insertions in Notch1 locus in tumors arising in DMBA treated or 4-NQO treated KH x Onc mice. Red arrow indicates transposon orientation predicted to induce Notch1 expression. Blue arrow indicates transposon orientation predicted to inhibit Notch1 expression. (B) IPA analysis of CIS candidates associated with the Notch pathway. Red shading reflected relative number of insertions in the indicated gene. (C) Transposon insertions in Notch1 genomic loci were tumor specific. Transposon-genomic DNA junctions were amplified from the genomic DNA of the indicated KH x Onc tumor or the autologous skin. (D) Notch1 expression in DMBA tumors containing transposons predicted to induce or to inhibit gene expression. Scale bar = 300 μM.

Figure 4. Activated Notch accelerated HPV-positive oral tumorigenesis. (A) Oral tumors in mice with activated Notch (KHR^{NICD}; n=5) grew faster than control KHR
tumors (n=8). KHNICD mice (n=4) did not develop any tumors. Representative of 5 independent experiments giving similar results. Error bars represent standard deviation. (* denote $P < .02$ for comparison between KHR and KHR$^{NICD}$ tumors). (B) KHR mice containing activated Notch develop larger anal tumors. Data from two independent experiments using KH$^{NICD}$ (n=4), KHR (n=13) and KHR$^{NICD}$ (n=8) mice. Data represented with quantile box-and-whisker plots defined in Fig. 1C. Statistical significance was observed for KHNICD compared to KHR mice and for KHR compared to KHR$^{NICD}$ mice. (C) KHR$^{NICD}$ tumors have increased expression of Notch targets Hey1 and Hes1 as measured by immunohistochemistry. Scale bar = 100 μM. (D) Oral and anal tumors expressing NICD had more BrdU-positive cells. Scale bar = 100 μM. (E) Quantitation of BrdU positive cells in oral and anal tumors. For each group, 8 hpf from 5 separate tumors were analyzed. (* denotes $P < .0001$). Data represented with quantile box-and-whisker plots defined in Fig. 1C. (F) Oral tumors were enriched for DNA replication and cell cycle genes as determined by GSEA analysis. RNA from KHR (n=5) and KHR$^{NICD}$ (n=6) tumors was subjected to Illumina microarray analysis. 18,138 genes were subjected to GSEA analysis. (G) NICD expression did not impact differentiation of KHR oral tumors. KHR and KHR$^{NICD}$ oral tumors were stained for CK14 (green), CK10 (red) and DAPI (blue). Scale bar = 200 μM.

Figure 5. Haploinsufficient loss of Notch1 increased tumor cell proliferation but did not affect tumor differentiation. (A) In HPV-positive mice, oral tumors grew faster with haploinsufficiency (KHR-N$^{+/-}$, n=5) or complete loss (KHR-N$^{-/-}$, n=5) of Notch1 compared to control mice (KHR, n=5). (* denotes $P < .001$ for comparison of KHR to
KHR-N\textsuperscript{−/+} and for comparison of KHR to KHR-N\textsuperscript{−/+}. (B) In HPV-negative mice, oral tumors grew faster with complete loss of Notch1 (KR-N\textsuperscript{−/+}, n=6) compared to tumors with intact Notch1 (KR-N\textsuperscript{+/+}, n=7 and KR, n=3). (* denotes $P < .01$ for comparison of KR to KR-N\textsuperscript{−/+} and for comparison of KR-N\textsuperscript{+/+} to KR-N\textsuperscript{−/+}). (A-B) Representative of 3 independent experiments. Error bars represent standard deviation. (C) Notch1 loss promoted outgrowth of non-mucosal epithelial tumors in KHR but not KR mice. Results combined 2 similar experiments using KHR (n=9), KHR-N\textsuperscript{+/+} (n=9), KHR-N\textsuperscript{−/+} (n=8), KR (n=8), KR-N\textsuperscript{+/+} (n=10) and KHR-N\textsuperscript{−/+} (n=8) mice. Data represented with quantile box-whisker plots defined in Fig. 1C. (D) Tumors with haploinsufficient loss of Notch1 still retained Notch1 expression. (E) Tumors with complete loss of Notch1 had decreased expression of the Notch1 target gene, Hes1, in the basal layer. KHR, KHR-N\textsuperscript{+/+}, KHR-N\textsuperscript{−/+}, KR, KR-N\textsuperscript{+/+} and KR-N\textsuperscript{−/+} oral tumors were stained for Notch1 (D) or Hes1 (E) expression. Scale bar = 100 μM for (D) and 50 μM for (E). (F) KHR-N\textsuperscript{+/+} and KHR-N\textsuperscript{−/+} tumors had more BrdU-positive cells compared to KHR tumors. Scale bar = 100 μM. (G) Quantitation of BrdU positive cells in KHR oral tumors. For each group, 8 hpf from 4 to 6 separate tumors were analyzed. (* denotes $P < .0001$). Data represented with quantile box-whisker plots defined in Fig. 1C. (H) Loss of Notch1 did not impact differentiation of KHR oral tumors. KHR, KHR-N\textsuperscript{+/+} and KHR-N\textsuperscript{−/+} oral tumors were stained for CK10 (red) and CK14 (green) expression. Scale bar = 200 μM.

**Figure 6. HPV oncogenes compensated for Notch1 haploinsufficiency to induce the expression of genes involved in invasion.** (A) Haploinsufficient KHR-N\textsuperscript{+/-} tumors (n=4, orange) possessed transcriptional profiles more similar to KHR-N\textsuperscript{−/+} tumors (n=5,
blue) and KR-N⁻/⁻ tumors (n=4, purple) than KHR tumors (n=4, black) and KR-N⁺/⁻
tumors (n=5, red) tumors. Heatmap of unsupervised hierarchical clustering of RNA
isolated from the indicated tumors. Columns represent individual genes. Rows
represent individual tumors. (B) GSEA analysis detected metallopeptidase expression
signatures enriched in KHR-N⁻/⁻ tumors but not in KHRNICD tumors. (C)
Metalloproteinases (Mmp3, Mmp9, Mmp10, Mmp11 and Mmp13) and other genes
involved in invasion (Twist2 and Col1a) were upregulated in KHR-N⁻/⁻ and KHR-N⁺/⁻
tumors as detected by qRT-PCR analysis (* denotes P < .05). (D) Mmp3, Mmp9 and
Mmp13 were upregulated in KR-N⁻/⁻ tumor but not KR-N⁺/⁻ and KR tumors. (E) Mmp9,
Mmp10 and MMp13 were downregulated in KHRNICD tumors compared to KHR tumors.
For (C-E), 3 separate tumors per group were assayed in duplicate. Error bars represent
standard deviation.

Figure 7. Notch1 was differentially expressed between invasive and non-invasive
lesions. (A) Notch1 was expressed in verrucous carcinoma but not in invasive
squamous cell carcinomas of the head and neck region as measured by
immunohistochemistry. Scale bar = 100 μM (B) Quantitation of Notch1 expression in
verrucous and invasive squamous cell carcinomas. (C) Notch1, Hes1 and Hey1
expression were downregulated in invasive cervical cancers compared to pre-invasive
disease. (D) Notch1 was downregulated in invasive breast cancers compared to normal
breast tissue. For C&D, data represented with quantile box-whisker plots defined in Fig.
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DMBA  

TPA  

Weeks

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B  

Tumor free mice (percent)  

C  

Number of tumors per mouse

D  

Hyperplasia  

Carcinoma in situ  

Squamous Cell Cancer

E  

Squamous cell carcinoma (n=29)  

Verrucous Carcinoma (n=2)  

Dysplasia/ Carcinoma in situ (n=8)  

Hyperplasia (n=2)

Zhong et al. Figure 1
A DMBA treated KH x Onc tumors

B 4-NQO treated KH x Onc tumors

Zhong et al. Figure 2
**Figure 4**

**A** Tumor Volume (mm³) over time for KHR, NICD, and KHR NICD.

**B** Anal Circumference (mm) for KHR, NICD, and KHR NICD.

**C** Immunohistochemistry images showing Hey1 and Hes1 expression.

**D** Reactome pathways for DNA replication and cell cycle mitosis.

**E** Box plot showing BrdU positive nuclei in Anus and Mouth.

**F** Enrichment scores for DNA replication and cell cycle mitosis.

**G** Immunofluorescence images for CK14, CK10, and DAPI.
Number of non-mucosal epithelial tumors

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Zhong et al. Figure 5
**Zhong et al. Figure 6**

**A** Enrichment Score for KHR-N-/ and KHR-

NER = 1.59, P = .001, FDR = .188

NER = 0.82, P = .700, FDR = 1.000

**B** Metalloendopeptidase

**C** Relative mRNA expression for Mmp3, Mmp11, Twist2, Col1a1, Mmp9, Mmp10, and Mmp13

**D** Relative mRNA expression for Mmp3, Mmp9, and Mmp13

**E** Relative mRNA expression for Mmp9, Mmp10, and Mmp13
NOTCH1 expression in:
Verrucous Carcinoma

B

NOTCH1 intensity in head and neck cancers

P < .0001

Cervical cancer

C

HES1

HEY1

D

Breast cancer

Zhong et al. Figure 7
Notch1 activation or loss promotes HPV-induced oral tumorigenesis

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