Loss-of-function mutations in Notch receptors in cutaneous and lung squamous cell carcinoma

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Squamous cell carcinomas (SCCs) are one of the most frequent forms of human malignancy, but, other than *TP53* mutations, few causative somatic aberrations have been identified. We identified *NOTCH1* or *NOTCH2* mutations in ∼75% of cutaneous SCCs and in a lesser fraction of lung SCCs, defining a spectrum for the most prevalent tumor suppressor specific to these epithelial malignancies. Notch receptors normally transduce signals in response to ligands on neighboring cells, regulating metazoan lineage selection and developmental patterning. Our findings therefore illustrate a central role for disruption of microenvironmental communication in cancer progression. *NOTCH* aberrations include frameshift and nonsense mutations leading to receptor truncations as well as point substitutions in key functional domains that abrogate signaling in cell-based assays. Oncogenic gain-of-function mutations in *NOTCH1* commonly occur in human T-cell lymphoblastic leukemia/lymphoma and B-cell chronic lymphocytic leukemia. The bifunctional role of Notch in human cancer thus emphasizes the context dependency of signaling outcomes and suggests that targeted inhibition of the Notch pathway may induce squamous epithelial malignancies.

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Squamous cell carcinomas (SCCs) collectively are the most common ectodermal cancers, resulting in >300,000 deaths per year (1, 2). SCCs arise from renewable squamous epithelial cells that serve to create an environmental barrier in the skin, esophagus, lung, and cervix. In normal squamous epithelia, basal progenitors give rise to more superficial daughter cells that terminally differentiate into keratinized cells as they migrate toward the surface, coupling terminal differentiation with microanatomic position. An early feature of squamous neoplasia of all types is disrupted differentiation to variable degrees, typically associated with thickening of the epithelium and increased proliferation. Therefore, although SCCs from different sites demonstrate varying epidemiologic associations—UV radiation in skin cancers, alcohol and tobacco exposure in esophageal cancers, and human papillomavirus infection in cervical and head and neck cancers—they likely share disruption of pathways that coordinate microenvironment-dependent squamous differentiation (3).

Although emerging targeted therapies show promise in epithelial cancers, few recurrent genetic aberrations have been identified in lung SCCs or cutaneous SCCs (cSCCs) (4). Activating *HRAS* mutations are highly prevalent in murine SCC-like malignancies but rare in their human counterparts (5, 6). Fewer than 10% of head and neck SCCs and cSCCs carry *EGFR* and *PIK3CA* gain-of-function mutations (7–9), and the oncogenic kinase *DDR2* is activated in ∼5% of lung SCC primary tumors and cell lines (10). In fact, most SCCs lack an identifiable classical driver mutation, stalling deployment of targeted treatments. Although virtually all SCCs harbor *TP53* mutations, additional tumor suppressors have proved elusive. Recent studies have implicated loss of function in *NOTCH1*, *IRF6*, and *TP53* in head and neck SCCs (11, 12). Several models suggest that inactivation of Smad proteins up-regulates TGF signaling, but somatic mutations have not been detected in primary cancers (13).

We recently integrated whole-exome DNA sequence and allele-specific copy number data to examine somatically acquired genomic aberrations in eight primary cSCCs (14). Given the very high mutation burdens in these cancers, we sought to increase specificity for genes with a causative role in tumorigenesis. A search for mutations accompanied by loss of the wild-type allele—a pattern shared by known tumor suppressors in our series, including *TP53* and *CDKN2A*—identified multiple mutations in Notch receptors.

Notch receptors participate in a highly conserved signal transduction pathway that regulates many aspects of development through context-dependent effects on cell fate determination (15, 16), growth (17), and survival (18). Cognate ligands expressed on the surface of signal-sending cells bind the EGF-like repeats of the Notch ectodomain on signal-receiving cells, initiating a series of proteolytic cleavage events that allow the Notch intracellular domain (NICD) to translocate to the nucleus and form a tran-

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scription activation complex with the DNA binding factor RBPJ (also known as CSL) and coactivators of the Mastermind-like (MAML) family (19). Although activating mutations in Notch1 occur in the majority of T-cell lymphoblastic leukemia/lymphoma (20) and are also often present in poor-prognosis B-cell chronic lymphocytic leukemia (21–23), loss of Notch activity can also produce basal cell carcinoma-like cancers (24) or squamous cancers (25) in mice, suggesting that the consequence of specific somatic Notch aberrations in cancer is strongly influenced by epigenetic context. Current models propose that Notch transcriptional down-regulation, possibly due to loss of p53 function, is a causative event in human epithelial malignancies (26), but disabling somatic aberrations have not been reported in cSCCs or lung SCCs. Here, we describe and functionally characterize frequent Notch loss-of-function mutations in these malignancies.

Results

Identification of Notch Mutations. Three additional cSCCs were sequenced on the whole-exome level, in addition to the eight originally reported (14). For these 11 samples, ~1,300 mutations per genome were identified; >85% were made up of G>A transitions consistent with UV damage. Nine samples showed at least one *NOTCH1* or *NOTCH2* mutation, including two samples, cSCCs P8 and P10, derived from solid organ transplantation patients exposed to long-term immune suppression (Fig. 1 and Table 1; ref. 14). Six of these mutations were duplicated truncations with loss of the wild-type allele. One additional primary tumor and 14 cSCC cell lines were Sanger sequenced for 30/34 exons of *NOTCH1* and 30/34 exons of *NOTCH2*, revealing 15 additional missense mutations and yielding an overall nonsynonymous mutation prevalence of *NOTCH1* or *NOTCH2*, per sample, of 19/26 (~75%). Thirty-three of 43 total missense or nonsense mutations in Notch genes resulted from a G>A transition. By contrast, exome or transcriptome sequencing of five primary basal cell cancers (BCCs) and directed Sanger sequencing of three BCCs did not identify missense mutations in *NOTCH1* or *NOTCH2* (Table S1). No association with clinical subtype or *TP53* status was noted (Table S2).

Recent exome-sequencing data from 40 lung SCCs acquired by The Cancer Genome Atlas (TCGA) project were also examined. Three missense mutations and one splice site mutation were identified in *NOTCH1*, as well as one missense and one nonsense mutation in *NOTCH2*—a combined mutation frequency of 5/40 samples or 12.5% (Table S1). Sanger sequencing for *NOTCH1* and *NOTCH2* in lung SCC cell lines SW900 and HCC95, esophageal SCC lines TT and TE10, and lung adenocarcinoma lines H549 and A549 revealed a single heterozygous nonsense *NOTCH1* mutation in TE10.

Analysis of Unique Notch1 Loss-of-Function Mutants. Amino acid (missense) changes were analyzed by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) for potential structural effects. Eighteen of 27 mutations were identified as "probably damaging" with the remainder being labeled "probably benign" (Table S3). Besides overt loss-of-function frameshift and nonsense mutations, *NOTCH1* point mutations involved the extracellular EGF-like repeats, the juxtamembrane heterodimerization domain, and the intracellular RAM domain. To exclude the possibility that the observed changes are nonpathogenic passenger events, we tested the functional consequence of mutations involving each of these domains. An EGF-like repeat mutation (D469G) and a unique heterodimerization domain mutation (R1594Q) interfered with ligand-mediated activation of Notch1 (Fig. 2*A*). D469, a residue within EGF repeat 12 (Fig. 2*B*), contributed to binding of a Ca^{2+} ion (27), which fixed the orientation of EGF repeat 11 with respect to repeat 12 (28). Prior genetic studies have shown that EGF repeats 11–12 are necessary and sufficient for ligand binding by *Drosophila* Notch (29), and biochemical studies have implicated D469G in binding of recombinant Notch1 EGF repeats 11–14 to the human Delta-like-1 ligand (30).

P1770S is a mutation in the Notch1 RAM domain, a region that binds the downstream transcription factor RBPJ and

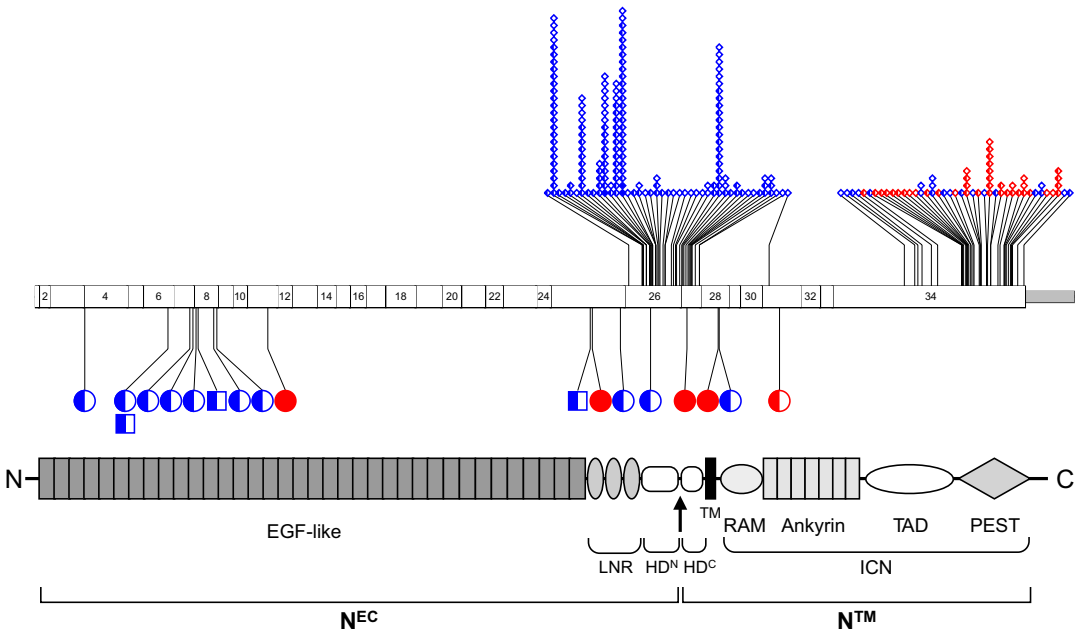

Fig. 1. Structural distribution of missense (blue) and nonsense (red) mutations in *NOTCH1*, organized by exon and protein domain. Below the domain graph, circles represent mutations in cSCCs, and squares represent mutations in lung SCCs. *NOTCH1* driver mutations from hematologic malignancies (Catalogue of Somatic Mutations in Cancer database) are displayed above the exon graph. In these activating changes, missense mutations clustered in the NRR domain, promoting ligand-independent processing, whereas truncations in the C-terminal PEST domain enhanced protein half-life. In contrast, mutations depicted below the graph, from SCCs, appeared in EGF repeats, the juxtamembrane heterodimerization domain, and the intracellular RAM domain.

Table 1. Identified amino acid substitution mutations in Notch receptors and pathway genes in primary and immortalized cSCCs

Sample	*NOTCH1*	*NOTCH2*	*NOTCH3*	*NOTCH4*	*JAG2*	*CREB1*	*EP300*
cSCCs, primaries							
cSCC P1	***Q610****	R1838*, R452C, W330*, P224L					P1452L
cSCC P2	C478F			R1333C			
cSCC P3			E1566K				
cSCC P4	***W1768****	T2278I, Q1634*, G313C		S1602F			L2303F
cSCC P5				***W309****			
cSCC P6	P1770S, R1594Q		P226S		Q1017*		
cSCC P7	Splice site	S1836F, E297K					
cSCC P8	Q1923*	Q1616*, G488D					
cSCC P9	R353C		C570*				
cSCC P10	C423F						
cSCC P11	***E1446****	N46S, E38K					Q974*
cSCC P12							
cSCCs, cell lines							
SCC4							
SCC12B							
SCC12F	S137L						
SCC25							
SCCRDEB2							
SCCRDEB3	***R353C***						
SCCRDEB4	D1517N						
SCCT1		C861Y					
SCCT2	E415D, C409F, D469G	C433Y, G1751D, P2343S					
SCCT3		D1451N					
SCCT8		C616F					
SCCIC1	N1809H	P1913S					
SCCIC8	***Q1687****						
SCCIC12							

Shaded regions were not assessed (cell lines were Sanger sequenced for 30/34 exons each of *NOTCH1* and *NOTCH2* only). *denotes stop codons; bold/italicized changes are homozygous. *RBJP*, *MAML1-3*, and *JAG1* mutations were not identified in any samples (data not shown).

thereby contributes to the assembly of Notch transcription complexes (31–33). The P1770S substitution greatly diminished signaling when scored in ΔEGFΔLNR, a truncated form of Notch1 that signals in a ligand-independent fashion (Fig. 3*A*). This mutation also prevented formation of stable ICN1/RBPJ complexes on DNA (Fig. 3*B*). Thus, P1770S appears to interfere with Notch signaling at the level of transcription complex assembly.

Discussion

We describe here a spectrum of inactivating somatic mutations of Notch receptors in lung and skin cancers, indicating that Notch loss of function plays a prominent role in multiple variants of SCC. These aberrations apparently occur significantly more often in cSCCs than in SCCs arising in the lung and occur in patients with and without a history of immunosuppression. cSCCs accumulate >100,000 nucleotide substitutions as a result of sun damage (14), perhaps more frequently inactivating tumor suppressors than their visceral counterparts. Indeed, 85% of Notch mutations in cSCCs resulted from the G>A transitions induced by UV radiation after homozygous *TP53* loss, consistent with evidence for a role in tumor progression rather than initiation, possibly through evasion of inhibitory stromal signaling (25).

Attenuated expression of Notch transcripts is well established in skin cancers (26). The high frequency of truncation mutations in our study suggests that some of these instances may represent nonsense-mediated decay. The distribution of missense changes in *NOTCH1* and *NOTCH2* also reflects abrogated function. Whereas gain-of-function mutations found in leukemias cluster in the negative regulatory region (34) and the C-terminal PEST domain (20–23), the disabling mutations identified in our study span Notch ectodomains and the N-terminal portion of the intracellular domains (Fig. 1).

All of the nonsense mutations lie within or N-terminal of the Notch ankyrin repeats, which are required for all known Notch functions (19). Truncations such as Q610* in Notch1 and W330* in Notch2 prevent expression of the EGF repeats 11–13 required for ligand interaction (35); thus, these mutations probably ablate signaling. Other mutations likely produce either secreted (e.g., E1446* in Notch1 and Q1634* in Notch2) or membrane-tethered (e.g., Q1924* in Notch1 and R1838* in Notch2) truncated polypeptides that retain ligand-binding EGF repeats and therefore have the potential for dominant negative activity (36). Similar truncated polypeptides have dominant negative activity in vivo when expressed from transgenes (29).

Our functional studies suggest that the D469G substitution causes loss-of-function by disrupting the structure of EGF repeats required for productive Notch1–ligand engagement. It is less clear how R1594Q, which lies in the activation switch of the receptor, might affect ligand-mediated Notch1 activation. This arginine residue lies in an α-helix within the heterodimerization domain that packs against the preceding LNR-C module, forming two charged hydrogen bonds to residues D1533 and E1555 (Fig. 2*C*). The most likely explanation for the observed loss of function is that the mutation interferes with proper folding of the receptor in a manner that prevents efficient delivery to the cell surface. Consistent with the folding defect idea, mutations of nearby residues in this α-helix identified in T-cell acute lymphoblastic leukemia (e.g., F1592S, L1593P, L1596H, R1598P) produce gain of function by destabilizing the heterodimerization domain, leading to ligand-independent cleavage of Notch1 at site S2 and subsequent receptor activation (37).

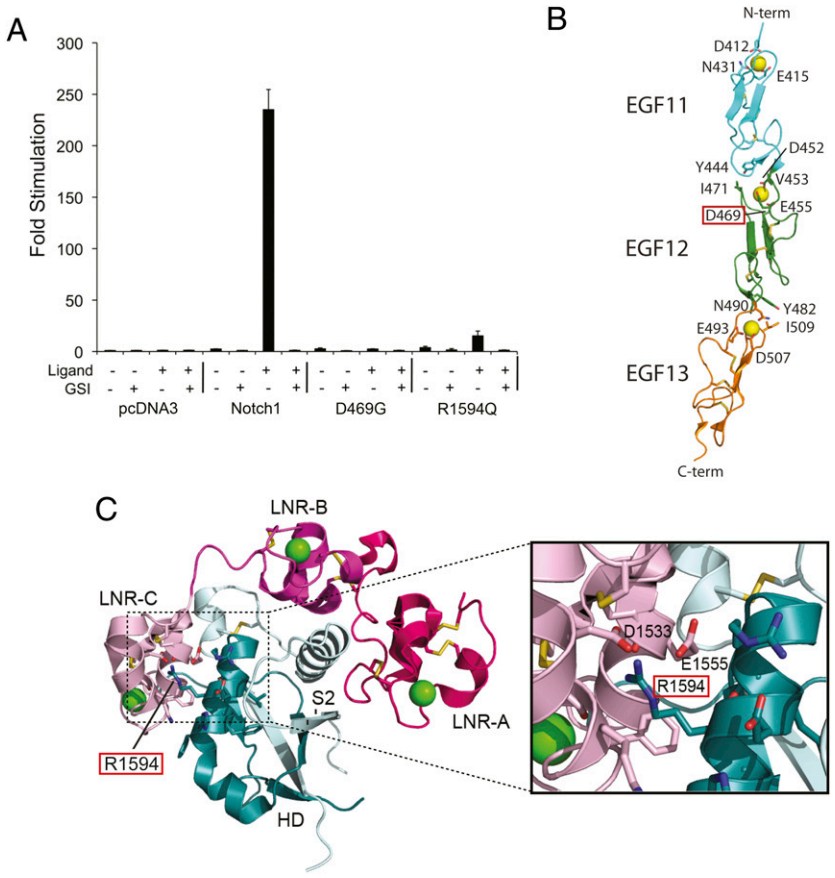

Fig. 2. Effects and structural context of various Notch1 ectodomain mutations found in cSCC. (*A*) Reporter gene assay. U2OS cells lines were transfected with a Gal4-responsive luciferase reporter plasmid and with chimeric receptors that contained the indicated wild-type or mutated Notch1 extracellular domain and an intracellular Notch1/Gal4 chimera that activates transcription under the control of Gal4 binding sites. Receptors were activated by coculture with 3T3 feeder cells lacking (–) or expressing (+) the ligand Jagged2 in the absence (–) or presence (+) of a gamma secretase inhibitor (GSI). Reporter gene activity was measured after 24 h. (*B*) Structural context of the D469G mutation. Ribbon representation of the structure of EGF repeats 11–13 from human Notch1 (Protein Data Bank ID code 2VJ3). EGF11, blue; EGF12, green; EGF13, orange. Calcium ions are yellow; coordinating residues and interdomain contact residues are labeled and rendered as sticks; D469 is boxed. (*C*) Structural context of the R1594Q mutation. The Notch1 NRR is rendered in ribbon representation, with the LNRs in different shades of pink and the HD domain in either turquoise (before the S1 site), or light blue (after the S1 site). The R1594 side chain and residues within 4 Å are rendered as sticks. *Right* shows a magnified region of R1594 and identifies the side chains of D15343 and E1555, both of which are close enough to form charged hydrogen bonds to the R1594 side chain.

Alternative possibilities, such as stabilization of the autoinhibited conformation of the receptor, seem less likely.

The majority of the identified mutations are heterozygous. Notch proteins demonstrate haploinsufficiency in tissue patterning (38), suggesting that loss of a single copy functionally impedes signaling and therefore could plausibly impact tumorigenesis. Specific aberrations, such as the P1770S mutant involving the RAM domain, have the potential to form nonproductive complexes with ligands and thereby act in a dominant-negative fashion. Our observation of inactivation of multiple Notch receptors in the same tumor aligns with data from compound knockout mice, in which conditional deletion of multiple Notch genes (e.g., *NOTCH1* and *NOTCH2*) or of *RBPJ*, the single downstream mediator of canonical Notch signaling, yields more profound differentiation and barrier function defects than deletion of any single gene (25, 39). Multiple mutations may act in concert to progressively disable a common downstream set of signaling targets. Alternatively, different Notch receptors might each suppress tumor progression through distinct mechanisms that have yet to be delineated, as the functional interchangeability of individual Notch receptors has not been established.

The high prevalence of somatic aberration adds context to noncell autonomous models in which Notch loss is theorized to compromise skin barrier function and promote chronic injury in adjacent cells (25). We cannot exclude the possibility that primary cancers arose in fields of Notch-disabled cells, analogous to patches of p53-mutant cells in sun-damaged skin (40). However, such field mutations have not yet been described for Notch, and

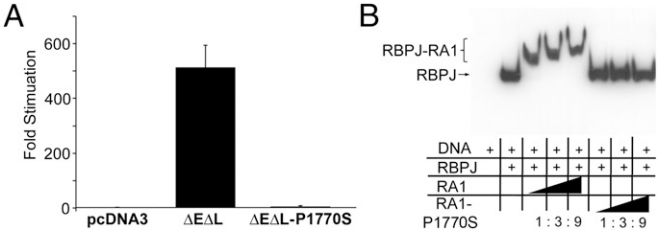

Fig. 3. Functional characterization of the P1770S mutation. (*A*) Reporter gene assay. U2OS cells lines were cotransfected with a Notch-responsive luciferase reporter plasmid and plasmids encoding a constitutively processed form of the wild-type or P1770S Notch1 receptor. Reporter gene activity was measured after 24 h. (*B*) Electrophoretic mobility shift assay. Purified RBPJ and purified polypeptides corresponding to the RAM and ankyrin-repeat (ANK) regions of either wild-type or P1770S Notch1 were incubated in the presence of radiolabeled Hes-1 cognate DNA, as described (55).

the substantial mutant allele frequency in cSCCs suggests at least some cell-autonomous function.

Notably, recurrent mutations were not identified in genes encoding Notch ligands, RBPJ, or Mastermind-like coactivators, other key components of the canonical Notch signaling pathway. This absence may simply stem from the limited number of tumors analyzed. However, some components of the canonical Notch signaling pathway, particularly RBPJ (41) and MAMLs (42), may execute Notch-independent functions required for effective oncogenesis. Finally, genetic evidence suggests that noncanonical Notch functions in skin may also contribute to carcinogenesis (25).

Notch transcription complexes transactivate via multiple mechanisms, including directly through monomeric and dimeric complexes (as for CD25 and Hes1). The most consequential direct targets of Notch transcription complexes in leukemic cells appear to be *Myc* (43–45) and *Hes1* (46), and Notch signaling enhances PI3-kinase/Akt and mTOR signaling (47, 48). The mechanism of tumor suppression in epithelial cells is less well defined. In mouse skin, Notch tumor suppression has been hypothesized to occur downstream of RBPJ-dependent expression of $p21^{WAF1/Cip1}$, possibly through down-regulation of Wnt proteins (49, 50). However, $p21^{WAF1/Cip1}$ up-regulation is not seen in human skin cancers. Other evidence implicates a distinct pathway involving Hes1-dependent derepression of ROCK1/2 and MRCK-α kinases, which normally activate the RhoA and CDC42 GTPases (26). Activation of this pathway (often via loss of suppression) appears to generate the dedifferentiation and increased motility characteristic of epithelial malignancies. Discriminating among these possibilities awaits sequencing of additional tumors and a more detailed knowledge of the mechanisms of tumor suppression in squamous epithelia, which should be aided by the study of Notch-inactivated cSCC cell lines identified in this report.

Gamma-secretase inhibitors now in development to treat hematologic malignancies associated with oncogenic increases in Notch signaling may induce some characteristics of somatic loss of function in squamous epithelia (34). Our results suggest that vigilant screening for epithelial malignancies is warranted upon use of these agents.

Materials and Methods

Sample Acquisition. Tumor and matched normal tissue samples were obtained as part of an established skin cancer study protocol, with all subjects providing informed consent according to procedures approved by the University of California, San Francisco Committee on Human Research. Samples were either immediately frozen in liquid nitrogen or deposited in RNAlater preservative (Qiagen). Diagnosis of either cSCC or BCC was confirmed for all tumors through histological examination of a standard biopsy specimen by a board-certified dermatopathologist. Patient information and genomic profiling for lung SCCs analyzed here were obtained from sequencing completed by TCGA and deposited in the Database of Genotypes and Phenotypes. Further details are provided in *SI Materials and Methods*.

Isolation and culture details, as well as detailed characterization of cSCC lines SCCRDEB2-4, SCCT1-3, and -8 and SCCIC1, have been described (51). Two additional lines sequenced here, SCCIC8 and SCCIC12, were established from female immunocompetent patients aged 51 and 87, respectively. The tumor sites were, respectively, buttock (poorly differentiated spindle cell) and left calf (moderately to poorly differentiated SCC).

Sequencing. For exome sequencing of 11 cSCCs and matching normal tissue, ~40 megabases of coding region were selected from each genomic DNA sample by using oligonucleotide-based hybrid capture and sequenced by using the Illumina sequencing-by-synthesis platform. Three primary BCCs were similarly sequenced on the whole-exome level, and two additional samples were sequenced for transcribed sequences only. Standard methods for alignment, PCR duplicate removal, recalibration of base scores, and mutation calling were applied (detailed description is provided in *SI Materials and Methods*).

Three primary BCCs and 1 additional primary cSCC and matching tissue, as well as 14 cSCC cell lines, were capillary PCR-sequenced (Sanger) for *NOTCH1* and *NOTCH2* mutations. Thirty of 34 exons of *NOTCH1* and 30/34 exons of *NOTCH2* were reliably PCR amplified. Coverage of coding sequence was assessed by computing sequenced bases per exon with Phred-scaled quality scores, Q, ≥20. For the targeted exons of *NOTCH1*, a minimum of 64% exons met this threshold with a median of 93%; for *NOTCH2*, a minimum of 83% of exons met threshold with a median of 96%.

For cSCC cell lines, matching normal lines were not available; therefore, some variants discovered in these samples are likely germ-line in origin. To minimize inclusion of germ-line variants in the final results, all variants exhibiting overlap with markers from the dbSNP build 131 database (52) were filtered out, and three amino acid substitutions also present in other mammalian organisms were excluded.

Site-Directed Mutagenesis. QuikChange site-directed mutagenesis was performed according to the manufacturer's instructions (Stratagene), and mutagenized cDNA sequences were confirmed by resequencing.

Reporter Gene Assays. Notch1 reporter gene assays were as described (37, 53). Briefly, to assess ligand-mediated Notch1 activation, pcDNA3 plasmids encoding Notch1–Gal4 DNA binding domain fusion receptors (100 ng) were transfected into U2OS cells along with Gal4–firefly luciferase and human thymidine kinase *Renilla* luciferase reporter genes. After 24 h, transfected cells were split onto control NIH 3T3 cell feeders or feeders expressing the Notch ligand Jagged2 in the presence or absence of the gamma-secretase inhibitor compound E (1 μM). After an additional 24 h, cells were harvested, and dual luciferase assays were carried out. Results were normalized to the internal *Renilla* luciferase control and expressed relative to empty vector control, which was arbitrarily set to a value of 1. To assess effects of mutations on ligand-independent Notch1 activity, U2OS cells were cotransfected with the vector pcDNA3 encoding ΔEGFΔLNR, a form of Notch1 lacking the EGF and LNR repeats that is subject to constitutive ADAM metalloprotease and gamma-secretase cleavages, along with an artificial RBPJ–firefly luciferase and the internal control *Renilla* luciferase reporter genes, as described (54). Normalized luciferase activities were measured 48 h after transfection as described above.

Electrophoretic Mobility Shift Assays. Recombinant RBPJ and ICN1 polypeptides were expressed and purified to homogeneity from *Escherichia coli* as described (55). Electrophoretic mobility shift assays were performed in nondenaturing polyacrylamide gels as described (55).

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