Supporting Information

Liu et al. 10.1073/pnas.1314239110

SI Materials and Methods

1. High-Throughput Screening and Reporter Gene Assay.

a. Mouse leydig cell TM3 cells (American Type Culture Collection) were cultured in a 1:1 mixture of Ham's F-12 medium and DMEM (Gibco/Invitrogen) supplemented with 2.5% (vol/vol) FBS (Gibco/Invitrogen), 5% (vol/vol) horse serum (Gibco/Invitrogen), 50 unit/mL penicillin, and 50 μg/mL streptomycin (Gibco/Invitrogen). TM3 cells in a 10-cm dish were cotransfected with 8 μg STF-reporter plasmid containing a luciferase gene driven by Wnt-responsive elements and 2 μg pcDNA3.1-Neo (Gibco/Invitrogen) with 30 μL FuGENE6 (Roche Diagnostics) following the manufacturer's protocol. Stable cell lines (TM3 Wnt-Luc) were selected with 400 μg/mL G418 (Gibco/Invitrogen). The TM3 Wnt-Luc cells and L-cell Wnt3A cells (American Type Culture Collection) were cocultured in a 384-well plate with DMEM supplemented with 2% (vol/vol) FBS and treated with different concentrations of compounds. After 24 h, the firefly luciferase activities were assayed with the Bright-Glo Luciferase Assay System (Promega). The IC_{50} is measured when the effect of the compound reduces the luminescence signal by 50%.

b. Hedgehog (HH) coculture assay was set up similarly with the coculture of HH ligand secretion cells, 293 EcR HH (American Type Culture Collection), and an HH-responsive reporter cell line, TM3Hh12 (1).

2. Cell Culture, Tissue Samples, TaqMan, and Colony Formation Assay.

a. HN30 cells (Wayne State University) and UMSCC cells (University of Michigan) are derived from human head and neck squamous cell carcinoma (HNSCC) patient tumor samples (2–4). Other cell lines, including A-253, CAL27, Detroit 562, FaDu, Hs840.T, SCC-4, SCC-9, SCC-25, and U2OS, are from American Type Culture Collection; SNU-1066 and SNU-1076 (5) are from a Korean cell line bank.

b. Normal human oropharynx tissues were purchased from commercial sources (two independent samples from Asterand and one sample from Biochain). Human neonatal primary keratinocytes were purchased from Invitrogen.

c. For TaqMan assay, 2×10^6 cells per well were plated into six-well cell culture plates and treated with or without compound in a multipoint dose–response. RNA samples were collected after 48 h.

d. For colony formation assays, 2×10^3 cells per well were plated into six-well cell culture plates with or without compound treatment. Cells were stained with crystal violet 1 wk later.

3. Drug Treatment.

a. Acetyl-CoA was purchased from Sigma.

b. γ-Secretase inhibitor (RO4929097) was purchased from Selleck Chemicals.

4. RNA and DNA Extraction.

a. Total RNA or DNA was isolated using the Qiagen RNeasy or DNeasy blood and tissue kit according to the manufacturer's instruction, respectively.

5. TaqMan, GeneCard, Cloning, Mutagenesis, Notch Reporter Gene Assay, and Western Blot Analysis.

a. Two-step TaqMan RT-PCR analysis was performed on a PTC-200 peltier thermal cycler (MJ Research) and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). cDNA was synthesized using the High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. TaqMan analyses were performed using TaqMan Universal Master Mix (Applied Biosystems) and *AXIN2* and *GAPDH* probes (Applied Biosystems) according to the manufacturer's instructions.

b. mRNA expression levels for the target genes were normalized to *GAPDH* mRNA levels, and data were analyzed using SDS 2.0 software (Applied Biosystems) to calculate relative RNA quantities. Curve fitting was performed using Prism.

c. For GeneCard analysis, a 96-well TaqMan array gene card was used according to the manufacturer's instructions. The gene card assays were run on the ABI 7900HT Real-Time PCR System (Applied Biosystems) and analyzed with the ΔΔCt method.

d. Full-length human *Porcupine* (*PORCN*) was cloned into pcDNA 3.1 (Invitrogen); the full-length *Notch1* (Origen) or the Notch1 intracellular domain was cloned into the pLVX-IRES-Neo vector (Clontech).

e. Site-directed mutagenesis was conducted using QuikChange (Qiagen) according to the manufacturer's instructions. All constructs were reconfirmed with Sanger sequencing.

f. Notch reporter gene assay was conducted as follows. Notch1 (1-1762) (WT or mutant) and GAL4 DNA binding domain/VP16 activation domain fusion constructs were transfected into U2OS cells along with Gal4–firefly luciferase reporter construct (Agilent Technologies) and human Renilla luciferase reporter genes (Promega); 24 h later, cells were replated and cocultured with rat DLL1-expressing L cells (Gerry Weinmaster, University of California, Los Angeles) or its parental L cells into 96-well plates. After an additional 24 h, cells were harvested and subjected to Dual-Glo Luciferase Assay (Promega). Gal4 firefly luciferase reporter genes readout was normalized to the control Renilla luciferase readout according to the manufacturer's instructions.

g. For immunoblots, cell/tumor lysates (25 μg per lane) were mixed with XT sample buffer and reducing agent (Bio-Rad), separated by 4–12% Gradient SDS Criterion Precast Gel (Bio-Rad), and transferred to a PVDF membrane (Millipore). Proteins were detected with primary antibodies and HRP-conjugated secondary antibodies by using an enhanced chemiluminescence kit (Amersham Biosciences). Primary antibodies used were mouse anti-HA monoclonal antibody (Abcam), mouse anti–β-actin monoclonal antibody (Sigma), and rabbit antiphospho-LRP6 and anti-LRP6 antibodies (Cell Signaling).

6. Radioligand Binding Assay.

a. Membrane preparation: 10^8 cells were transfected with pcDNA 3.1 constructs (Invitrogen) bearing human *PORCN* using Fugene 6 (Roche). After 48 h, cells were harvested by scraping in PBS and centrifuging at $1,000 \times g$ for 10 min. Cell pellets were frozen in a dry ice bath and then gently resuspended in 10 mL 50 mM Tris (pH 7.5) and 250 mM sucrose buffer containing an EDTA-free protease inhibitor mixture (Sigma). Cells were lysed using a polytron (Brinkman). Lysed cells were centrifuged at $1,600 \times g$ for 20 min at 4 °C, and supernatant was transferred and centrifuged at 20,000 rpm in an SS34 rotor for 20 min at 4 °C. Supernatants were discarded, and the pellets were resuspended in 10% (wt/vol) sucrose, 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, and 1 mM EDTA solution using three 10-s pulses with a polytron.

b. Radioligand labeling of GNF-1331: the GNF-1331 precursor, GNF-5326 (Fig. S1*H*), was radiolabeled through a hydrogenation

reaction done by AmBioslabs to make an ^{3}H-radiolabeled GNF-1331. GNF-1331 binds to PORCN and serves as a hot radioligand in the in vitro biochemical PORCN binding assay for competition with cold testing compounds.

c. Radioligand binding assay: using the aforementioned membrane preps, filtration binding assays were performed. To reduce nonspecific binding, 96-well filtration plates (PerkinElmer) were precoated as suggested by the manufacturer with 0.1% BSA and then washed four times with 0.1% BSA. Membrane preps (50 μg total protein) were incubated in polypropylene 96-well plates with 6.6 nM ^{3}H-GNF-1331 in the presence or absence of a testing compound in binding buffer (50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 0.1% BSA) plus EDTA-free protease inhibitor mixture (Sigma) in a final volume of 150 μL for 3 h at room temperature. Binding reaction mixtures were then transferred to the precoated 96-well filtration plates (PerkinElmer), filtered, and washed using a 96-pin FilterMate Harvester (PerkinElmer). Radioactive signals were obtained using a Microplate Scintillation Counter TopCount (PerkinElmer). Curve fitting was performed using Prism.

7. Animal Model Study.

a. All animal studies were conducted at the Genomics Institute of the Novartis Research Foundation and Novartis Oncology at Cambridge. The experimental protocols were in compliance with animal welfare regulations and approved by the Institutional Animal Care and Use Committee at Genomics Institute of the Novartis Research Foundation and Novartis Oncology.

b. Nude mice (or nude rats) bearing the mouse mammary tumor virus–Wnt1, HN30, or SNU1076 tumors were randomized according to tumor volume. LGK974 was formulated in 10% (vol/vol) citrate buffer (pH 2.8)/90% (vol/vol) citrate buffer (pH 3.0) or 0.5% MC/0.5% Tween 80 and administered by oral gavage at a dosing volume of 10 μL/g animal body weight. Body weight was monitored daily, and tumor sizes were assessed three times per week after the tumors were palpable. Tumor sizes were determined by using caliper measurements. Tumor volumes were calculated with a formula (length × width × height)/2.

c. The plasma concentrations and exposures of LGK974 in the tumor-bearing nude mice ($n = 2$ per dosing group) were determined on day 14. Blood samples (50 μL) were collected by serial retroorbital sampling at 1, 3, 7, 16, and 24 h postdose. The blood samples were centrifuged, and plasma was separated and frozen until analysis by liquid chromatography/MS/MS.

d. For tolerability studies, LGK974 was administrated to nontumor-bearing Wistar rats one time per day by oral gavage at 3 or 20 mg/kg per day. Necropsies were performed at the end of the study. Tissues were fixed in 10% (vol/vol) neutral-buffered formalin, sectioned, and subjected to H&E staining.

8. Exome Capture Library Preparation and Sequencing.

a. Exome Capture Library Construction was done using the Roche NimbleGen V2 (44.1 Mbp) Exome Enrichment Kit (Otogenetics). Paired-end sequencing (2 × 100 bp) of the captured exons was carried out on an Illumina Genome Analyzer IIx Platform, with an average coverage of 50× (Otogenetics).

9. Sequence Data Processing.

a. Sequencing data were processed by a standard pipeline recommended by Broad Institute Genome Atlas Tool Kit (GATK).

1. The raw sequencing fastq files with reads and quality scores for each sample were aligned to the National Center for Biotechnology Information human reference genome GRCh37 using Burrows-Wheeler Aligner version 0.5.9 (http://bio-bwa.sourceforge.net). For each sample, a single-sorted binary alignment map (BAM) file with their alignments to the reference genome was generated.
2. The BAM files from the alignment were further refined by using sample level realignment with known indels and recalibration method (http://www.broadinstitute.org/gatk/guide).
3. The GATK unified genotyper was used for each recalibrated and cleaned BAM file to generate SNP, multiple nucleotide polymorphism (MNP), and indel. After variant calls for each sample, they were merged into multisample SNP and indel calling. Separated variant call format (VCF) files were produced for SNP and indel calling.
4. All variants calls were then processed by SnpEff (v2.1b http://snpeff.sourceforge.net/) to predict their functional impacts on corresponding protein products and GATK's variant annotator for additional annotations. Various publically available databases, including dbSNP v.135, COSMIC v.58, and ESP5400, were used to map the genomic changes to known variants.

10. Analysis of Variants.

a. To reduce the sequencing artifact, various filtrations were used, including variant quality < 30, mapping quality < 30, variant confidence < 2, and normalized Phred-scaled likelihoods > 80. There are a total of 18,349 genes harboring genomic changes compared with the reference exome. The total number of genomic changes in all 40 HNSCC cell lines is about 169,000. However, most of them (89%) are known variants annotated in the public databases mentioned above. Among the remaining variants, about one-half (6% of total) of variants result in protein sequence modifications, such as mutations, truncations, insertions, and deletions. Note that all of the sequencing was for cell lines where no matched normal was available. Therefore, some of the remaining variants seen here potentially could be SNPs. Mutations acquired during cell line passage are also possible and cannot be distinguished from those mutations acquired during cancer development and progression. The UMSCC lines each have unique genotypes and were used at the lowest possible passage number (usually less than 100 passages from initial culture).

b. The functional impacts of genomic changes were assessed by SnpEff. The high-impact mutations include frameshift, splice site mutations, start codon loss, stop codon gain, and stop codon loss. The moderate impact mutations include in-frame insertion or deletion and nonsynonymous mutations. The potential germ-line mutation was determined by whether the mutation is annotated in dbSNP with SNP allele origin < 2 or an ESP5400 entry and not in COSMIC.

c. To study the correlation between loss-of-function (LoF) mutations and LGK974 pharmacological function, we aggregated all likely LoF variants into one category, because it is known that LoF mutations of a gene may have mutations spreading across a wide range of the protein sequence. Mutations included high-impact variants, such as stop codon gain, frameshift, start codon loss, and splice site mutations. We also required combined sequence depth for WT and mutant allele to be at least five to remove extremely low-coverage variants. Using the variant calls from 40 HNSCC cell lines that were sequenced, we evaluated the enrichment effect of LoF variants on LGK974 pharmacodynamics response for each gene. The enrichment factor is defined as (i.e., odds ratio)

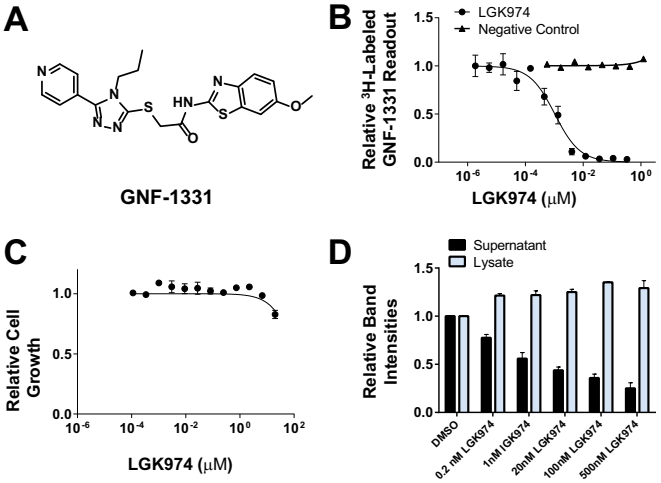
$$E(LoF) = \frac{P(responding|mutant)[1 - P(responding)]}{P(responding)[1 - P(responding|mutant)]},$$

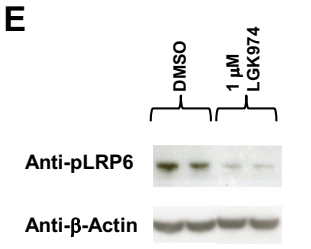
where $P(responding|mutant)$ represents the probability of responding to LGK974 treatment given the gene harboring an LoF mutation and $P(responding)$ represents the probability of responding to LGK974 treatment by chance. The candidate genes were selected based on E > 2 and the number of LoF

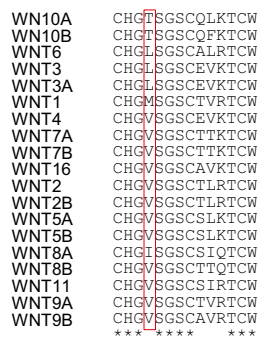
occurrences larger than three. Among the top candidates, Notch1 LoF has one of the highest enrichment factors (three-fold enrichment over random selection) (Table S3).

d. Notch1 mutations/variants were validated by sequencing of cDNA or genomic DNA of the cancer cell lines (GeneWiz). Sequences were analyzed using Sequencher (GeneCodes).

1. Pan SF, et al. (2010) Discovery of NVP-LDE225, a potent and selective smoothened antagonist. *ACS Med Chem Lett* 1(3):130–134.
2. Carey TE (1994) Head and neck tumor cell lines. *Atlas of Human Tumor Cell Lines*, eds Hay R, Gazdar A, Park J-G (Harcourt Brace Jovanovich, San Diego), pp 79–120.
3. Lansford C, et al. (1999) Head and neck cancers. *Human Cell Culture*, eds Masters J, Palsson B (Kluwer, Dordrecht, The Netherlands), Vol 2, pp 185–255.
4. Brenner JC, et al. (2010) Genotyping of 73 UM-SCC head and neck squamous cell carcinoma cell lines. *Head Neck* 32(4):417–426.
5. Rhee CS, et al. (2002) Wnt and frizzled receptors as potential targets for immunotherapy in head and neck squamous cell carcinomas. *Oncogene* 21(43):6598–6605.







G

Assay	LGK974 IC_{50} (nM)
Wnt1 (RGA)	1.1
Wnt2 (RGA)	0.2
Wnt3 (RGA)	0.6
Wnt3a (RGA)	0.9
Wnt6 (RGA)	0.2
Wnt7a (RGA)	2.4
Wnt9a (RGA)	0.05

Fig. S1. PORCN as a target for Wnt secretion inhibitors. (*A*) The structure of GNF-1331. (*B*) LGK974 competes off [3H]-GNF-1331 in a dose-dependent manner, where a negative control, Acetyl-CoA, has no effect. PORCN is a membrane-bound O-Acyl transferase that is responsible for palmitoylation (long-chain fatty acid modification) of Wnt proteins. Short-chain fatty acid CoA (Acetyl-CoA) here serves as a negative control. (*C*) LGK974 showed minimal cytotoxicity up to 20 μM in 293T cells. (*D*) Quantification of Western blotting result shown in Fig. 1*F*. Relative intensities for the corresponding H-Wnt3a bands in the supernatant and lysates are shown. (*E*) LGK974 strongly inhibited Wnt3A-dependent LRP6 phosphorylation in L-Wnt3A cells. (*F*) The conserved palmitoylation sites among 19 Wnts. The palmitoylation Serine residue is highlighted in red (sequences are aligned using ClustalW2). (*G*) The IC_{50} of LGK974 against different Wnts in STF reporter gene assays. (*H*) The GNF-1331 precursor, GNF-5326, was used for radiolabeling through a hydrogenation reaction.

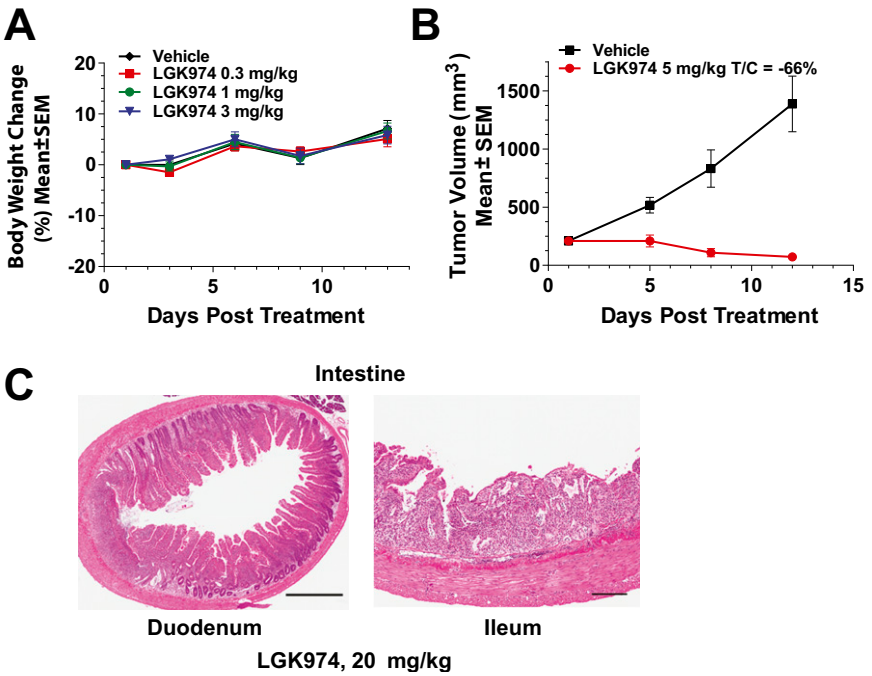


Fig. S2. LGK974 tolerability and efficacy in vivo. (*A*) Mouse body weight change after continuous dosing of LGK974 for 13 d. (*B*) LGK974 showed strong efficacy in a murine mouse mammary tumor virus–Wnt3 tumor model. Single-agent LGK974 dosed at 5 mg/kg two times per day induced 66% tumor regression. C, control; T, treated. (*C*) H&E staining of the intestine from the rats dosed with LGK974 for 14 d at 20 mg/kg per day. (Magnification: duodenum, 2×; ileum, 10×. Scale bars: duodenum, 1 mm; ileum, 200 μM.)

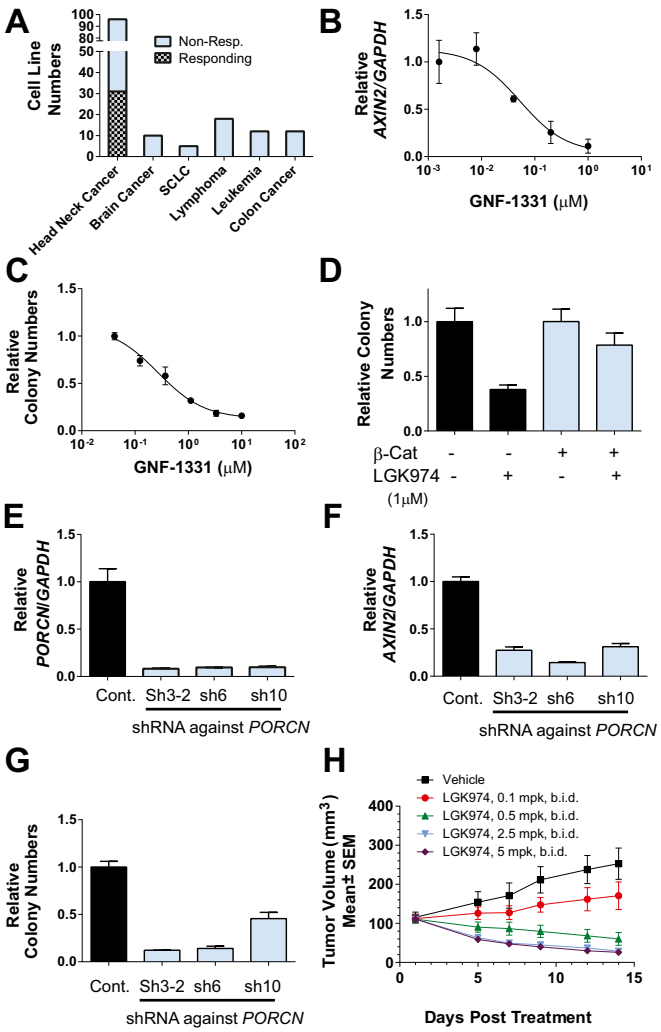


Fig. S3. PORCN and β-catenin dependency in a human HNSCC cell line (HN30). (*A*) Summary of the LGK974 responsiveness among different cancer types; 31 of 96 HNSCC cell lines showed pathway inhibition on treatment with LGK974, but none from the brain cancer, small cell lung cancer (SCLC), lymphoma/leukemia, or colon cancer cell lines responded. (*B*) GNF-1331, a PORCN inhibitor, inhibited Wnt signaling in HN30 cells. (*C*) GNF-1331 reduced HN30 cell colony formation in vitro. (*D*) The effect of LGK974 in HN30 cells can be partially rescued by expression of active β-catenin. (*E*) shRNA against PORCN reduced the PORCN mRNA levels. (*F*) *AXIN2* mRNA levels were reduced in PORCN knockdown cells. (*G*) PORCN knockdown reduced colony formation in HN30 cells. (*H*) LGK974 single agent-induced tumor regression in a human HNSCC tumor model HN30 in vivo. b.i.d., two times per day; Cont., control; mpk, mg per kg.

A

Genes	Mutation Occurrence
TP53	27/40 (68%)
CDKN2A	7/40 (18%)
Notch1	10/40 (25%)
PTEN	1/40 (3%)
HRAS	5/40 (13%)
PIK3CA	5/40 (13%)
Notch2	5/40 (13%)
Notch3	2/40 (5%)

B

Cell Line	Responsive to LGK974	Notch1 mutation
HN30	Yes	C478F
UMSCC 11A	Yes	R912W
UMSCC 8	No	R353C
UMSCC 69	No	H599HT

Fig. S4. Mutations in the human HNSCC cell lines. (*A*) Summary of the key mutations in the HNSCC cell lines identified by exome sequencing. (*B*) Summary of the missense mutations of Notch1 in the HNSCC cell lines.

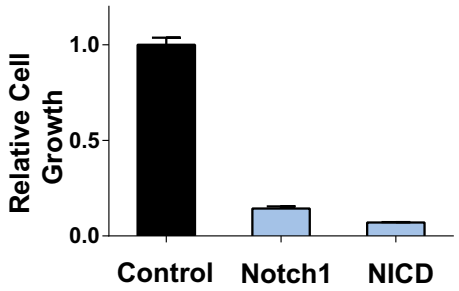


Fig. S5. Notch as a tumor suppressor gene in an HNSCC cell line (HN30). Expression of exogenous full-length Notch or Notch intracellular domain (NICD) inhibited HN30 cell growth in vitro.

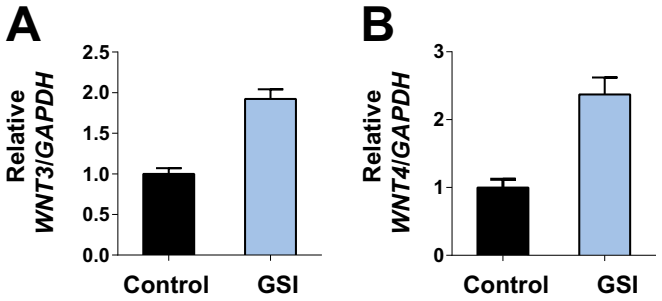


Fig. S6. Notch antagonizes *Wnt3/4* expression in human primary keratinocytes. (*A*) *Wnt3* and (*B*) *Wnt4* expression levels were increased on treatment of γ-secretase inhibitor (GSI), RO4929097, in human primary keratinocytes.

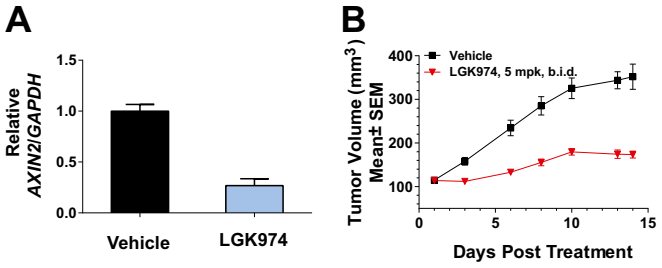


Fig. S7. LGK974 in vivo activities in a human HNSCC model (SNU1076). (*A*) LGK974 significantly reduced *AXIN2* expression in the SNU1076 xenograft model in vivo. (*B*) LGK974 single agent showed antitumor effects in the SNU1076 xenograft tumor model in vivo.

Cell Line	Responsive to LGK974	FAT1 mutation
UMSCC 63	Yes	P2559S
UMSCC 11A	Yes	D4109G
UMSCC 41	Yes	G4147C
UMSCC 35	Yes	P2559L
UMSCC-25	Yes	N524fs
UMSCC-43	Yes	M1150fs
UMSCC 8	No	Q3887X

Fig. S8. FAT1 mutations in the HNSCC cell lines. FAT1 mutations are enriched in LGK974-responsive HNSCC cell lines.

Cell Line	Responsive to LGK974	HRAS mutation
UMSCC 17B	Yes	Q61L
UMSCC 43	Yes	G12V
UMSCC 63	Yes	G13D
UMSCC 17A	No	Q61L
HN30	Yes	G12D

Fig. S9. HRAS mutations in the HNSCC cell lines. HRAS mutations are enriched in LGK974-responsive HNSCC cell lines.

Table S1. Thirty-one of ninety-six HNSCC cell lines showed pathway inhibition on treatment with LGK974

Numbers	HNSCC cell line names	Axin2 reduction response	Axin2 inhibition (1 treated/control)
1	A-253	No	0
2	CAL27	Yes	0.7
3	Detroit 562	No	0.2
4	FaDu	No	0
5	HN30	Yes	0.9
6	Hs 840.T	No	0
7	SCC-4	No	0.4
8	SCC-9	No	0
9	SCC-25	No	0.1
10	SNU-1066	No	0.3
11	SNU-1076	Yes	0.9
12	UMSCC 1	Yes	0.9
13	UMSCC 2	No	0.2
14	UMSCC 3	No	0.0
15	UMSCC 4	No	0.3
16	UMSCC 5	No	0.3
17	UMSCC 6	Yes	0.9
18	UMSCC 7	No	0.4
19	UMSCC 8	No	0.4
20	UMSCC 9	Yes	0.8
21	UMSCC 10A	Yes	0.5
22	UMSCC 10B	No	0.3
23	UMSCC 11A	Yes	0.6
24	UMSCC 11B	Yes	0.6
25	UMSCC 12	No	0.4
26	UMSCC 13	Yes	0.9
27	UMSCC 14A	No	0.0
28	UMSCC 14B	Yes	0.5
29	UMSCC 14C	Yes	0.7
30	UMSCC 16	No	0.3
31	UMSCC 17A	No	0.3
32	UMSCC 17B	Yes	0.6
33	UMSCC 19	Yes	0.5
34	UMSCC 21A	No	0.1
35	UMSCC 22A	No	0.3
36	UMSCC 22B	No	0.0
37	UMSCC 23	No	0.3
38	UMSCC 25	Yes	0.9
39	UMSCC 26	No	0.3
40	UMSCC 28	Yes	0.7
41	UMSCC 29	Yes	0.7
42	UMSCC 30	No	0.3
43	UMSCC 31	No	0
44	UMSCC 33	No	0.2
45	UMSCC 34	Yes	0.9
46	UMSCC 35	Yes	0.6
47	UMSCC 36	No	0.0
48	UMSCC 37	No	0.0
49	UMSCC 38	Yes	0.5
50	UMSCC 39	No	0.0
51	UMSCC 40	No	0.3
52	UMSCC 41	Yes	0.6

Table S1. Cont.

Numbers	HNSCC cell line names	Axin2 reduction response	Axin2 inhibition (1 treated/control)
53	UMSCC 42	No	0.3
54	UMSCC 43	Yes	0.7
55	UMSCC 44	No	0.0
56	UMSCC 45	Yes	0.5
57	UMSCC 46	Yes	0.5
58	UMSCC 47	No	0.3
59	UMSCC 49	No	0.2
60	UMSCC 50	No	0.1
61	UMSCC 51	No	0.4
62	UMSCC 52	No	0.2
63	UMSCC 53	No	0.1
64	UMSCC 55	No	0.0
65	UMSCC 58	No	0.4
66	UMSCC 59	Yes	0.7
67	UMSCC 60	No	0.2
68	UMSCC 62	No	0.3
69	UMSCC 63	Yes	0.8
70	UMSCC 67	No	0.2
71	UMSCC 69	No	0.4
72	UMSCC 70	No	0.3
73	UMSCC 71	No	0.2
74	UMSCC 73B	No	0.4
75	UMSCC 74A	No	0.0
76	UMSCC 74B	No	0.0
77	UMSCC 76	Yes	0.7
78	UMSCC 77	No	0.2
79	UMSCC 78	No	0.0
80	UMSCC 80	No	0.0
81	UMSCC 81A	No	0.0
82	UMSCC 81B	No	0.3
83	UMSCC 83A	No	0.2
84	UMSCC 83B	No	0.4
85	UMSCC 85	No	0.0
86	UMSCC 90	Yes	0.5
87	UMSCC 92	Yes	0.9
88	UMSCC 93	Yes	0.6
89	UMSCC 94	No	0.3
90	UMSCC 97	Yes	0.8
91	UMSCC 98	No	0.1
92	UMSCC 103	No	0.0
93	UMSCC 104	No	0.0
94	UMSCC 105	No	0.3
95	UMSCV 1A	No	0.0
96	UMSCV 6	No	0.1

Table S2. Forty HNSCC cell lines subjected to exome sequencing

Numbers	Samples for exome sequencing
1	HN30
2	SNU-1076
3	UMSCC 1
4	UMSCC 2
5	UMSCC 4
6	UMSCC 6
7	UMSCC 8
8	UMSCC 9
9	UMSCC 10A
10	UMSCC 10B
11	UMSCC 11A
12	UMSCC 14A
13	UMSCC 14B
14	UMSCC 14C
15	UMSCC 17A
16	UMSCC 17B
17	UMSCC 13
18	UMSCC 23
19	UMSCC 25
20	UMSCC 28
21	UMSCC 29
22	UMSCC 30
23	UMSCC 34
24	UMSCC 35
25	UMSCC 38
26	UMSCC 40
27	UMSCC 41
28	UMSCC 43
29	UMSCC 46
30	UMSCC 47
31	UMSCC 53
32	UMSCC 55
33	UMSCC 59
34	UMSCC 63
35	UMSCC 69
36	UMSCC 76
37	UMSCC 78
38	UMSCC 81B
39	UMSCC 92
40	UMSCC 97

Table S3. The top candidate genes with aggregated LoF mutations that correlated the best with the LGK974 pharmacodynamics response data from the HNSCC cell lines

Gene	Genomic change	Amino acid change	Responsive mutants	Resistant mutants	Enrichment factor
FAM58A	g.chrX:152864477G > GC|g.chrX:152858079GC > G |g.chrX:152864513G > GC	A18fs, A181fs, A6fs	11	1	6.6
FLJ43860	g.chr8:142459777C > CA	L850fs	7	1	4.2
NOTCH1	g.chr9:139411813AC > A|g.chr9:139411793TG > T|g.chr9:139410488C > CT|g.chr9:139417470C > A|g.chr9:139412259CTGGCACGG > C|g.chr9:139417398C > A	E488fs, G192X, E216X, K538fs, A495fs, P460fs	5	1	3.0
OR7G3	g.chr19:9236916AG > A	A237fs	8	2	2.4
CCDC168	g.chr13:103386417C > A|g.chr13:103384147C > T	E5544X,W6300X	4	1	2.4
ZNF527	g.chr19:37879852C > CTGTG|g.chr19:37879854AT > A	P301fs, Y302fs	7	2	2.1
CDKN2A	g.chr9:21971123TGA > T|g.chr9:21968242C > T|g.chr9:21971186G > A|g.chr9:21971028C > T|g.chr9:21971120G > A	S78fs, W110X, R58X, R80X	4	0	NA

fs, frameshift; NA, not applicable; X, nonsense mutations.