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Notch3 drives development and progression of cholangiocarcinoma

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The prognosis of cholangiocarcinoma (CC) is dismal. Notch has been identified as a potential driver; forced exogenous overexpression of Notch1 in hepatocytes results in the formation of biliary tumors. In human disease, however, it is unknown which components of the endogenously signaling pathway are required for tumorigenesis, how these orchestrate cancer, and how they can be targeted for therapy. Here we characterize Notch in human-resected CC, a toxin-driven model in rats, and a transgenic mouse model in which p53 deletion is targeted to biliary epithelia and CC induced using the hepatocarcinogen thioacetamide. We find that across species, the atypical receptor NOTCH3 is differentially overexpressed; it is progressively up-regulated with disease development and promotes tumor cell survival via activation of PI3k-Akt. We use genetic KO studies to show that tumor growth significantly attenuates after Notch3 deletion and demonstrate signaling occurs via a noncanonical pathway independent of the mediator of classical Notch, Reombinant Signal Binding Protein for Immunoglobulin Kappa J Region (RBPJ). These data present an opportunity in this aggressive cancer to selectively target Notch, bypassing toxicities known to be RBPJ dependent.

Cholangiocarcinoma (CC) is an aggressive primary liver malignancy with an increasing global incidence. Surgery remains the only potential cure, but few patients present with operable disease. New adjuvant treatments are urgently required; however, few targets have been put forward and none have been shown to have efficacy.

Notch is a master regulator of cell fate in the mammalian liver. In the embryo, hepatoblast specification to a biliary fate and tubulogenesis are dependent on Reombinant Signal Binding Protein for Immunoglobulin Kappa J Region (RBPJ)-driven effector transcription, i.e., canonical Notch signaling (1). Furthermore niche-derived ligand reactivate Notch during biliary injury in the adult potential to induce CC when transgenically overexpressed, and this study aimed to characterize how endogenous Notch might drive tumorigenesis. We identify the atypical receptor Notch3 as differentially overactivated in CCs in humans, rats, and mice, with genetic deletion significantly reducing CC growth. Notch3 sustains tumor cell survival through PI3k/Akt activation via a noncanonical mechanism independent of Reombinant Signal Binding Protein for Immunoglobulin Kappa J Region (RBPJ), presenting an opportunity to target the pathway without disrupting classical Notch and bypassing toxicities associated with γ-secretase inhibitors.


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Significance

Clinical outcomes in cholangiocarcinoma (CC) are poor; few patients are candidates for curative resection, and palliative chemotherapy produces only modest effects on survival. With an increasing incidence, new targets are urgently needed. Notch has been identified as having potential to induce CC when transgenically overexpressed, and this study aimed to characterize how endogenous Notch might drive tumorigenesis. We identify the atypical receptor Notch3 as differentially overactivated in CCs in humans, rats, and mice, with genetic deletion significantly reducing CC growth. Notch3 sustains tumor cell survival through PI3k/Akt activation via a noncanonical mechanism independent of Reombinant Signal Binding Protein for Immunoglobulin Kappa J Region (RBPJ), presenting an opportunity to target the pathway without disrupting classical Notch and bypassing toxicities associated with γ-secretase inhibitors.
Tumor HES4 -2 NOT J B (12.6- = 0.0148; P = ≤ Jag1, Hes1, CD31 0.076371). and 3.17% tumors vs. 2.03% 0.016558); and 0.43% nontumors. In all CC PCR array to compare HES1 0.687197; Fig. 1 = JOCTH1 (Fig. 1C and Fig. S1 A and B) in contrast to NOTCH3, which was consistently seen on vascular smooth muscle (Fig. S1A) and on many, although not all, bile ducts (Fig. 1C and Fig. S1A). Large regions of almost all tumors stained positively for NOTCH3 (19 ± 0.77% displayed >10% coverage; 31 ± 0.84% displayed >20%; 1.6 ± 5.40% displayed >40%). Pixel analysis showed mean coverage of each core was 56.2% greater in tumors compared with noncancerous controls (Fig. 1C). NOTCH1 positivity was also greater in tumors, but not to the same extent (mean coverage, 4.49 ± 3.17% tumors vs. 2.03 ± 0.43% nontumors). In all CC samples, positivity colocalized with CK19, and a subset of tumors also exhibited stromal positivity, colocalizing with the myofibroblast marker α-SMA (Fig. 1D and Fig. S1D). NOTCH3 did not colocalize with endothelial or inflammatory cell markers (CD31 and CD68) (Fig. 1D). In malignant ductules, NOTCH3 was frequently nuclear; reactivity of the intracellular domain (N3-ICD) suggested functionality (Fig. 1E). To corroborate this, we performed N3-ICD immunoblotting; the mean signal of N3-ICD (normalized to β-actin) was 95 ± 74.66 times greater in tumor vs. matched nontumor lysates (P = 0.0026; Fig. S1E). Almost all tumors exhibited stromal expression of JAGGED1 (Fig. S1F).

**Notch3 is Differentially Up-Regulated During CC Development.** To determine the contribution of Notch to CC development, we used a well-characterized toxin-induced model in rat using the hepatocarcinogen thioacetamide (TAA) to induce injury followed by cancer (9). After 16 wk, multifocal foci of the invasive CC are seen with mucin production and desmoplasia. The model has a penetrance of 100% at 20 wk, when tumors are numerous, large, and coalescent (Fig. S2A). We used a Notch PCR array to compare expression in uninjured animals to those with inflammation (8- to 10-wk TAA) (Fig. 2A, Left), fibrosis (12–14 wk), early malignancy (20 wk), and invasive adenocarcinoma (26 wk) (Fig. 2A, Right, and Table S2). An induction in transcription was observed in line with tumor development as confirmed with qPCR (Fig. 2B); Notch3 was a highly up-regulated receptor at 26 wk (S2D-fold by qRTPCR; P = 0.0022), contrasted by modest up-regulation of Notch1 (5.32-fold, P = 0.0411), Notch2 (4.75-fold, P = 0.0022), and Notch4 (9.67-fold, P = 0.0022). Jag1 was up-regulated 24.00-fold (P = 0.0022). We saw nonsignificant up-regulation of Jag2 (2.35-fold, P = 0.3093), and unlike in human disease, no change in effector transcription: Hes1, 0.67-fold (P = 0.3095); Hey1, 0.70-fold (P = 0.3095); Hey2, 0.77-fold (P = 0.3939); and HeyL, 2.10-fold (P = 0.0649). Immunostaining the time course mirrored these data; up-regulation occurred in line with tumor expansion, with Jagged1 and Notch3 in stroma and malignant ducts (Fig. 2C).

**Results**

**Notch3 is Differentially Activated in Human CC.** We used a targeted NOTCH PCR array in five surgically resected samples paired with matched noncancerous liver (Fig. 1A and Table S1; four perihilar and one mass-forming intrahepatic CC, all moderately differentiated adenocarcinoma). NOTCH3 was highly up-regulated: 18.2-fold (P ≤ 0.000025); NOTCH1, 1.9-fold (P = 0.015153); NOTCH2, 1.8-fold (P = 0.076917), and NOTCH4, 1.6-fold (P = 0.076371). Up-regulation of JAG1 (8.4-fold, P = 0.000426) and JAG2 (12.6-fold, P = 0.003088) indicated that signaling may be triggered by nearby ligand. This preliminary screen suggested pathway activity, with up-regulation of the Hes/Hey family of effectors: HEY1, 10.25-fold, (P = 0.016558); and HEYL, 6.0-fold (P = 0.000829), although in this cohort, there was no change in the archetypal effector of classical Notch, HES1 (0.9-fold, P = 0.687197; Fig. 1A). We expanded the analysis to a larger cohort of 48 CC cases and compared them with healthy livers using quantitative RT-PCR (qRT-PCR; n = 42), NOTCH3 was again up-regulated 38-fold (P ≤ 0.0001), with NOTCH1, 1.1-fold (P ≤ 0.0001); NOTCH2, 7.5-fold (P ≤ 0.0001); NOTCH4, 2.0 fold (P ≤ 0.0001); JAG1, 363.3-fold (P ≤ 0.0001); JAG2, 938.6-fold (P ≤ 0.0001); HES1, 483.7-fold (P ≤ 0.0001); HES4, 304.2-fold (P ≤ 0.0001); HEY1, 46.8-fold (P ≤ 0.0001); HEY2, 384.4-fold (P = 0.0005); HEYL, 160.6-fold (P ≤ 0.0001) (Fig. 1B). We stained the cohort and a tissue CC microarray for Notch receptors with a panel of cell-specific markers. In the healthy liver, we observed little expression of NOTCH1 (Fig. 1C and Fig. S1 A and B) and on many, although not all, bile ducts (Fig. 1C and Fig. S1A). Large regions of almost all tumors stained positively for NOTCH3 (19 ± 0.77% displayed >10% coverage; 31 ± 0.84% displayed >20%; 1.6 ± 5.40% displayed >40%). Pixel analysis showed mean coverage of each core was 56.2% greater in tumors compared with noncancerous controls (Fig. 1C). NOTCH1 positivity was also greater in tumors, but not to the same extent (mean coverage, 4.49 ± 3.17% tumors vs. 2.03 ± 0.43% nontumors). In all CC samples, positivity colocalized with CK19, and a subset of tumors also exhibited stromal positivity, colocalizing with the myofibroblast marker α-SMA (Fig. 1D and Fig. S1D). NOTCH3 did not colocalize with endothelial or inflammatory cell markers (CD31 and CD68) (Fig. 1D). In malignant ductules, NOTCH3 was frequently nuclear; reactivity of the intracellular domain (N3-ICD) suggested functionality (Fig. 1E). To corroborate this, we performed N3-ICD immunoblotting; the mean signal of N3-ICD (normalized to β-actin) was 95 ± 74.66 times greater in tumor vs. matched nontumor lysates (P = 0.0026; Fig. S1E). Almost all tumors exhibited stromal expression of JAGGED1 (Fig. S1F).

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Reports demonstrated an inhibitory effect using γ-secretase inhibitors (GSIs) in CC cell lines and xenograft models. We aimed to evaluate efficacy on in vivo CC growth in a model where desmoplastic CC arises from the liver without transgenic overactivation of Notch. We administered N-[N-3,5-difluorophenacetyl]-t-Alanyl]-S-phenylglycine t-buty] ester (DAPT) to rats on the TAA protocol, treating animals during the last 5 wk of injury, i.e., once tumors had established (Fig. S3A). TAA damage was equivalent in the two groups (Fig. S3B). Following DAPT, liver-to-body weight ratio was reduced by 19 ± 0.53% (P = 0.0121; Fig. S3C), and the proportion of liver infiltrated by the tumor was reduced by 78 ± 0.84% (P = 0.0148; Fig. 3B). There was no apparent difference in the microscopic appearance of DAPT-treated tumors; all cancerous foci exhibited features of well-differentiated adenocarcinoma with mucin production and desmoplasia, with no apparent difference in cell death or necrosis histologically. Moreover, tumor number was unchanged, consistent with the observation that by 21 wk, tumors are established...
Genetic Deletion of Notch3 Reduces CC Formation and Progression.

Cytokeratin, a major intermediate filament, was used as a ductular marker to identify ductular cells. Although Notch3 is a known mediator of ductular cell regeneration, we found that CK19CreYFPp53f/f animals exhibited a similar phenotype; the number of ductular cells formed in the livers of CK19CreYFPp53f/f mice was not significantly different from that in control mice (N3f/f, n = 3; n = 6 control). (C) Immunohistochemical staining of TAA time course. CK19 (DAB). Notch3, green; Jagged1, red; αSMA, green. (Scale bar, 100 μm.)

and DAPT after this point slows CC growth. To establish that inhibition of the γ-secretase complex resulted in a reduction in signaling via Notch3, we stained for the Notch3 protein and looked for nuclear positivity, i.e., Notch3 intracellular domain (Fig. 3D). Immunostaining for the proliferation marker Ki67 demonstrated a 38.15% reduction in cycling cells in tumor cells (P = 0.0005; 244.14 ± 10.03 vehicle vs. 150.99 ± 20.40 DAPT; Fig. 3D).

Fig. 2. Notch3 is differentially up-regulated during CC development. (A) PCR Notch pathway array in rats after 600 mg/kg TAA for 8–10 wk (inflamed) (n = 6) vs. control (Left) and 24–26 wk (adenocarcinoma) (n = 6) vs. control (n = 6) (Right). Red labels, at least fourfold up-regulation; green, at least fourfold down-regulation. (B) qRT-PCR of Notch expression in TAA rat liver normalized to uninjured controls at 8–10 (inflamed), 12–14 (fibrotic), 20 (early malignant), and 26 wk (late malignant) (n = 3; n = 6 control). (C) IHC of TAA time course. CK19 (DAB). Notch3, green; Jagged1, red; αSMA, green. (Scale bar, 100 μm.)

Fig. 3. Pan-inhibition of Notch reduces CC progression. (A) Tiled low power photomicrographs of rat liver after TAA with DAPT or vehicle during weeks 21–26. (Scale bar, 100 mm.) (B) (Left) Proportion of liver infiltrated by CC after DAPT (n = 8) vs. vehicle (n = 10) (P = 0.0148). (Right) Tumor number in rats treated with DAPT or vehicle (P = 0.2856). Data are means ± SEM. Medians compared with Mann–Whitney U test (**P < 0.05). (C) High- and low-power H&E sections of rat liver after vehicle (Upper) or TAA (Lower). Dashed lines are tumor boundary. (D) Ki67 immunostaining and quantification of rat liver sections after vehicle or TAA. (Scale bar, 100 μm.) Number of Ki67-positive tumor cells per x40 field (30 fields per rat) compared using Student t test. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

Notch3 mRNA and to a lesser degree Notch2, but not Notch1 or Notch4 (undetectable), was overexpressed in CC in CK19CreYFPp53f/f mice compared with CK19CreYFPp53wt and CK19CreYFPp53wt mice, as well as CK19CreYFPp53f/f mice without CC (Fig. 4B). When normalized to CK19CreYFPp53wt mice with 26 wk of TAA, Notch3 is up-regulated 85.92-fold (P = 0.0286) in CK19CreYFPp53wt mice with CC, compared with Notch1 at 24.28-fold (P = 0.0286). In CK19CreYFPp53f/f mice that did not develop CC, Notch3 was up-regulated 41.35-fold (P = 0.0286), compared with Notch1 at 14.94-fold (P = 0.0381). Nonsignificant increases in Jag1 and Jag2 were observed and the only effector to reach significance was Hey2: 45.47-fold (P = 0.0286; Fig. 4B).

We then compared tumor burden in CK19CreER−eYFPp53f/f mice on the TAA protocol to mice carrying constitutive deletion of the Notch3 gene (CK19CreER−p53N3). A difference in livers in N3+/− mice compared with N3−/− and N3+/− animals was seen at 26 wk (Fig. 5A). Although macroscopic cancerous nodules were not numerous on the liver surface of mice of any genotype, microscopic foci of invasive CC were clearly evident in all groups (Fig. 5 A and C). A 99.14 ± 0.48% reduction was seen in liver infiltrated by tumor in N3−/− mice, as well as a reduction in the mean tumor number [28.78 ± 15.37 N3−/− mice (n = 9) vs. 0.875 ± 0.38 N3+/− mice (n = 8)], indicating single copy loss of Notch3 is sufficient to inhibit CC formation (Fig. 5B and Fig. S5A). N3−/− mice exhibited a similar phenotype; there was no statistical difference in tumor burden to N3−/− animals (N3−/− mice, 0.035 ± 0.01% mean tumor area vs. 0.086 ± 0.05 N3−/−). Staining for pan-cytokeratin and pERK demonstrated an apparent reduction in proliferating malignant ductules in mice with Notch3 deletion (Fig. 5C). No significant compensatory up-regulation of Notch1, Notch2, or Notch4 was observed in CC19CreYFPp53wt or CC19CreYFPp53wt mice (Fig. S44). Tumors stained for ductular markers CK19 and Sox9, and these frequently but not exclusively colocalized with eYFP (Fig. 4A), in line with the weak efficiency of Cre recombinase in this model (14). In tumors, eYFP+ epithelia were almost always positive for NOTCH3, although not all NOTCH3+ cells carried the heritable eYFP label, indicating p53 loss is not required for Notch3 induction. In mice, we observed apparently less stromal Notch3 positivity (Fig. 4A, Bottom).
response to Notch3 deletion (Fig. S5B). To evaluate the role of Notch1 in CC development, CK19CreER<sup>Cre</sup>eYFPp53<sup>fl/fl</sup> mice were induced with tamoxifen and given TAA. These animals did not tolerate injury; they exhibited weight loss and signs of hepatic failure (jaundice and ascites), suggesting a failure of liver regeneration (Fig. S5C).

To assess whether this role for Notch3 was reproducible in a human system, we stably inhibited the gene using shRNA in cultured human CC cells and xenografts. Immunofluorescence of receptors was performed on three lines, and one was selected (CC-LP-1) (Fig. S5D). Cells were transfected with four independent shRNA with puromycin resistance cassettes for stable selection or scrambled sequence dependent shRNA with puromycin resistance cassettes for stable selection or scrambled sequence, with four independent shRNA with puromycin resistance cassettes for stable selection or scrambled sequence. We then sought to identify potential targets preferentially activated by Notch3 that might drive cell survival or proliferation. To compare the immediate effects of knockdown on downstream signaling, we transfected human CC cells with one-way ANOVA and Dunn’s multiple comparison test for post hoc analyses. (C) Representative H&E, pan-Cx, and pERK-stained sections from Notch3 depletion. A 30% reduction in pmTor (1.465 ± 0.675 scrambled vs. 0.606 ± 0.211 N3 shRNA) and pS6 (1.194 ± 0.322 scrambled vs. 0.379 ± 0.996 N3 shRNA; Fig. 6B). At the gene level, qPCR results mirrored the reduced transcription of targets identified in the siRNA-treated cells using the PCR array: MET, I.RSI, FAS, and RAC1 (Fig. S6C).

To confirm this phenomenon was not an off-target effect of RNAi, we looked at Akt in CK19CreER<sup>Cre</sup>eYFPp53<sup>fl/fl</sup> mice on the TAA protocol with (n = 8) and without (n = 9) Notch3 deletion. A reduction in Fas, Fadd, and Rac1 gene expression was seen, although this did not reach significance (Fig. S7A). Immunoblotts, however, revealed a 72% reduction in pAKT protein (N3<sup>+/−</sup>/N3<sup>−/−</sup> 0.41 ± 0.10 vs. N3<sup>−/−</sup> 0.12 ± 0.06; P = 0.0426), a 30% reduction in pmTor (N3<sup>+/−</sup>/N3<sup>−/−</sup> 1.55 ± 0.13 vs. N3<sup>−/−</sup> 1.08 ± 0.13; P = 0.0426), a 54% reduction in pS6 (N3<sup>+/−</sup>/N3<sup>−/−</sup> 1.19 ± 0.13 vs. N3<sup>−/−</sup> 0.54 ± 0.16; P = 0.0127) and an 88% reduction in p70-S6 (N3<sup>+/−</sup>/N3<sup>−/−</sup> 0.91 ± 0.34 vs. N3<sup>−/−</sup> 0.11 ± 0.06; P = 0.0593; Fig. 7A). Finally, to independently verify Akt blockade reduces CC growth, we xenografted nude mice with WT CC cells, allowed tumors to establish, and systemically treated them with a small molecule inhibitor of PI3K, PI-103. At 28 d, we observed a 60.87% reduction in tumor size (mean volume, 228.07 ± 48.68 vs. 89.25 ± 32.54 mm<sup>3</sup> vehicle; P = 0.0238; Fig. S7B).

**Discussion**

Exogenous oncogene activation in mice can initiate carcinogenesis in many tissues and indeed often in tissues where these oncogenes (defined as at least fourfold) were either upstream mediators or downstream targets of the AKT cascade including MET, I.RSI, and XIAP and the death receptors FAS and FADD. Surprisingly no changes were observed in response to RBPJ inhibition (Fig. 6A and Tables S3 and S4).

We therefore returned to previous models to assess whether induction of AKT by Notch3 held true in these systems. In shRNA Notch3 KD CC xenografts, pixel analysis revealed reduced phosphorylated AKT(Thr308) (0.537 ± 0.078 rodamine: DAPI signal scrambled vs. 0.346 ± 0.115 N3shRNA tumors), as well as phosphorylated downstream targets pmTor (1.465 ± 0.675 scrambled vs. 0.606 ± 0.211 N3 shRNA) and pS6 (1.194 ± 0.322 scrambled vs. 0.379 ± 0.996 N3 shRNA; Fig. 6B). At the gene level, qPCR results mirrored the reduced transcription of targets identified in the siRNA-treated cells using the PCR array: MET, I.RSI, FAS, and RAC1 (Fig. S6C).
are not overexpressed or mutated in human cancer. Consistent with the role of Notch as a cell fate determinant, transgenic overactivation of Notch1 (N1-ICD) in albumin-expressing cells results in biliary tumor formation (6, 7). In an almost identical model, however, N1-ICD expression under albumin and α-feto-protein promoters produce HCC at 100% penetrance (16). Studies of KRAS and MYC show precise expression levels are critical to biological outcome. Because genomic analyses of CC conclude transforming Notch mutations are infrequent (17) and antibodies blocking Notch1 increase the number and extent of tumors (18), we aimed to elucidate the contribution of endogenous WT Notch to CC and identify components with potential for targeting.

We used CC models in three species not reliant on any one oncogenic alteration, and Notch3 is consistently overexpressed. As reported by others, Notch1 is barely detectable in the healthy adult liver (19). Conversely, Notch3 is consistently present around the vasculature, making the up-regulation observed in tumors all of the more striking. Notch3 up-regulation occurs with disease; the greatest increase occurs late during expansion and invasion. Overexpression is associated with functional activity as evidenced by consistent nuclear visualization of the intracellular domain. Inhibition in xenografted cells with shRNA or genetic KO in mice both result in attenuated tumor growth. This target, with many functions and interactions distinct from canonical signaling, offers an attractive prospect for therapy. Past work suggests antibody-mediated Notch3 inhibition has no effect on liver cancer; however, evidence of Notch3 activity in the model and antibody efficacy was lacking (18). In contrast, other work acknowledges that, in addition to Notch1, Notch3 is strongly expressed in human CC compared with the liver (7). Notch3 drives 40% of non-small-cell lung cancers (NSCLCs) and almost all T-cell acute lymphoblastic leukemia. Tumor-inhibiting effects of GSIs are lost after Notch3 silencing in NSCLCs, suggesting cell survival is mediated via Notch3 (20). Serial transplantation studies indicate Notch3 is a regulator of self-renewal in tumor-propagating cells, and with no essential function in development or homeostasis (Notch3-null mice have no liver phenotype), Notch3 inhibition appears a safe strategy (11). GSIs have been pursued as therapy in a range of cancers, but translation has been hampered by toxicity. Such effects arise not due to disrupting the GS complex; the same phenotype occurs in RBPI- or Hes1-deficient mice (21). Therefore, the possibility of a tumor-forming role via an RBPI-independent mechanism is appealing. Our data suggest activation of Akt by Notch3 might be one such route.

Using independent techniques of blockade, we identify the PI3K/AKT pathway as one route of Notch3-driven cell survival; these data in line with Fan et al. who showed enhanced biliary tumorigenesis with transgenic activation of Notch and AKT (6). Many studies show the PI3K/AKT/mTor axis is dysregulated in CC, with AKT phosphorylation correlating with poor survival, and dual treatment with AKT and mTOR inhibitors synergistically slowing tumor growth (22).

Although N-ICD translocation via RBPI to drive Hes/Hey transcription is the most studied pathway, alternative modes of signaling are described including GS activation independent of ligand; N-ICD activity independent of RBPI; or activation by membrane-tethered receptors without GS cleavage (23). RBPI-independent signaling is characterized in T cells where N3-ICD interacts with IKKα to stimulate NF-kB and drive leukemia (24). Indeed, noncanonical Notch signaling is not uncommonly described in cancer, triggering cascades including PI3K/AKT, Wnt, and HIF1-α (25). Our data in rats of profound receptor overexpression without concomitant effector up-regulation further suggest Notch-driven CC can arise via an RBPI-independent route, given the restriction of tumor growth after OSI.

The stimuli for Notch3 up-regulation are as yet unknown. In our rat time course, early ligand up-regulation by fibroblasts tempts speculation that stroma-derived factors might be a trigger. However, as tumors evolve, Jagged1 appears on ductules, suggesting a switch to autonomous signaling or activation of an alternative pathway. In ovarian carcinoma where Notch3 gene amplification is common, Jagged1 is itself dependent on Notch3 activity; deletion and ectopic

**Fig. 6.** Genetic silencing of Notch3, but not RBPI, reduces PI3K-AKT transcription. (A) Human (CC-LP-1) cells transfected with NOTCH3 or RBPI siRNA and analyzed with oncoscene PCR array. Three independent siRNA sequences were used, and RNA was pooled from three replicate wells for each sequence. Gene expression measured 48 h after transfection and compared with scrambled controls (dotted line represents no change in transcription). Genes in color are at least fourfold down-regulated. (B) IHC of pAKT(Thr308), pmTor, and p56 with pixel analysis in Notch3 shRNA/scrambled CC-LP-1 xenografts. (Scale bar, 100 μm.)

**Fig. 7.** Genetic silencing of Notch3 reduces activity through the PI3K-AKT cascade. Immunoblots and corresponding densitometry for p-Akt, p-mTor, p-S6, and p-p70S6K with many functions and interactions distinct from canonical signaling, offers an attractive prospect for therapy. Past work suggests antibody-mediated Notch3 inhibition has no effect on liver cancer; however, evidence of Notch3 activity in the model and antibody efficacy was lacking (18). In contrast, other work acknowledges that, in addition to Notch1, Notch3 is strongly expressed in human CC compared with the liver (7). Notch3 drives 40% of non-small-cell lung cancers (NSCLCs) and almost all T-cell acute lymphoblastic leukemia. Tumor-inhibiting effects of GSIs are lost after Notch3 silencing in NSCLCs, suggesting cell survival is mediated via Notch3 (20). Serial transplantation studies indicate Notch3 is a regulator of self-renewal in tumor-propagating cells, and with no essential function in development or homeostasis (Notch3-null mice have no liver phenotype), Notch3 inhibition appears a safe strategy (11). GSIs have been pursued as therapy in a range of cancers, but translation has been hampered by toxicity. Such effects arise not due to disrupting the GS complex; the same phenotype occurs in RBPI- or Hes1-deficient mice (21). Therefore, the possibility of a tumor-forming role via an RBPI-independent mechanism is appealing. Our data suggest activation of Akt by Notch3 might be one such route.

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The stimuli for Notch3 up-regulation are as yet unknown. In our rat time course, early ligand up-regulation by fibroblasts tempts speculation that stroma-derived factors might be a trigger. However, as tumors evolve, Jagged1 appears on ductules, suggesting a switch to autonomous signaling or activation of an alternative pathway. In ovarian carcinoma where Notch3 gene amplification is common, Jagged1 is itself dependent on Notch3 activity; deletion and ectopic

**Fig. 6.** Genetic silencing of Notch3, but not RBPI, reduces PI3K-AKT transcription. (A) Human (CC-LP-1) cells transfected with NOTCH3 or RBPI siRNA and analyzed with oncoscene PCR array. Three independent siRNA sequences were used, and RNA was pooled from three replicate wells for each sequence. Gene expression measured 48 h after transfection and compared with scrambled controls (dotted line represents no change in transcription). Genes in color are at least fourfold down-regulated. (B) IHC of pAKT(Thr308), pmTor, and p56 with pixel analysis in Notch3 shRNA/scrambled CC-LP-1 xenografts. (Scale bar, 100 μm.)

**Fig. 7.** Genetic silencing of Notch3 reduces activity through the PI3K-AKT cascade. Immunoblots and corresponding densitometry for p-Akt, p-mTor, p-S6, and p-p70S6K with many functions and interactions distinct from canonical signaling, offers an attractive prospect for therapy. Past work suggests antibody-mediated Notch3 inhibition has no effect on liver cancer; however, evidence of Notch3 activity in the model and antibody efficacy was lacking (18). In contrast, other work acknowledges that, in addition to Notch1, Notch3 is strongly expressed in human CC compared with the liver (7). Notch3 drives 40% of non-small-cell lung cancers (NSCLCs) and almost all T-cell acute lymphoblastic leukemia. Tumor-inhibiting effects of GSIs are lost after Notch3 silencing in NSCLCs, suggesting cell survival is mediated via Notch3 (20). Serial transplantation studies indicate Notch3 is a regulator of self-renewal in tumor-propagating cells, and with no essential function in development or homeostasis (Notch3-null mice have no liver phenotype), Notch3 inhibition appears a safe strategy (11). GSIs have been pursued as therapy in a range of cancers, but translation has been hampered by toxicity. Such effects arise not due to disrupting the GS complex; the same phenotype occurs in RBPI- or Hes1-deficient mice (21). Therefore, the possibility of a tumor-forming role via an RBPI-independent mechanism is appealing. Our data suggest activation of Akt by Notch3 might be one such route.

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expression inhibit and promote Jagged1, respectively, implementing a self-sustaining signaling loop (26). This role for Jagged1 is an important question as ligands are attractive alternative therapeutic targets. In Drosophila, cis interactions (receptor stimulated by ligand from the same cell) inhibit receptor activity within the cell while stimulating activity in neighboring cells. Potential for Jagged1 to exert differential effects on Notch1 and Notch3 here is intriguing.

Stimulation of the ductular response by Notch1 in biliary regeneration requires classical signaling via Hes1. Further work is needed to understand whether this signal required in CC, how it is affected by Notch3, if at all (we see no change in Notch1 following Notch3 inhibition), and how Hes/Hey are involved. Our data suggest this role is complex; we observe Hes/Hey up-regulation in human disease and mice but to a much lesser extent in rats. The fact we observe reduced Hes/Hey expression with Notch3 silencing and yet the observed changes in Akt-related components do not occur with RBPJ inhibition suggests that at least two signaling routes are active downstream of the receptor, and further mechanistic work is needed to understand this better. Taken together, however, our data suggest Notch3 is an important driver in CC and drives cell survival independently of RBPJ, opening up new therapeutic targets for this largely untreated cancer.

Materials and Methods

Human Tissue. Human CC and liver were collected prospectively from patients undergoing resection at the Royal Infirmary Edinburgh with informed consent. The study was reviewed and approved by the Tayside Committee in Medical Research Ethics B. Retrospectively collected specimens were obtained from the National Health Service Lothian Scottish Academic Health Sciences Collaboration Bioresource and healthy liver from the Edinburgh Medical Research Council Sudden Death Tissue bank. Tissue CC microarrays were purchased from Pantomics.

Animal Models and Xenografts. CK19CreER<sup>R26ReYFP</sup> mice (14) were a kind gift from Guoquang Gu (Vanderbilt Medical Center, Nashville, TN). These mice were cross-bred with Trp53<sup>−/−</sup> mice (ref. B6.1292-2Trp53tm1Bnr), Notch3tm1Grid<sup>−/−</sup> mice (ref. B6.12951-Notch3<sup>tm1Grid</sup>), or Notch1tm1floflo (Notch1tm1floflo/Grind) from Jackson Laboratories. Trp53<sup>−/−</sup> (ref. B6.1292-2Trp53tm1Bnr) and Notch3tm1Grid<sup>−/−</sup> mice were on a C57BL/6J background; Notch1 mice were on a C57BL/6J background. Before experimental use, animals were cross-bred with the CK19CreER<sup>R26ReYFP</sup> line, which carried a CD1<sup>+</sup>C57BL/6 background. Progeny were subsequently on a mixed background and had a consistent mixed background.

Quantification of in Vivo Tumor Burden. Rat and mouse livers were cut into 3-mm slices before embedding and sectioning. Limits of malignancy were defined on HE sections from each block (five per liver) by a histopathologist blinded to the regime. Tumor area as a proportion of liver area was quantified with image J (NIH).

Statistical Analyses. Analyses were performed with Prism (GraphPad v5). Data are presented as mean ± SEM. Data distribution was assessed using the D’Agostino & Pearson normality test and comparisons between two groups using the Student t test or Mann–Whitney U test; multiple groups were compared with the one-way ANOVA or Kruskal–Wallis test. Posthoc testing groups of nonparametric data were performed using Dunn’s multiple comparison test.

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