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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 deletion to biliary epithelia demonstrating the activating cancer program and demonstrate signaling occurs via a noncanonical pathway independent of the mediator of classical Notch, Reconstituent 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. Notch3 drives development and progression of cholangiocarcinoma; however, we hypothesize how these orchestrate cancer, and how they can be targeted for therapy.

cholangiocarcinoma | Notch | noncanonical | bile duct | cancer

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.

In the embryo, hepatoblast specification to a biliary fate and tubulogenesis are dependent on Reconstituent Signal Binding Protein for Immunoglobulin Kappa J Region (RBPJ)-driven effectors transcription, i.e., canonical Notch signaling (1). Furthermore, niche-derived ligand reactivate Notch during biliary injury in the adult to expand the hepatic progenitor cell (HPC) pool for repair (2). The four receptors play distinct roles, as evidenced by the spectrum of phenotypes seen after transgenic KO, as well as in vitro and in vivo studies of HPC differentiation (3). aberrant activation of Notch paralogs results in a spectrum of cancer phenotypes (4), implying differing potentials for therapeutic targeting. Their individual contribution to biliary carcinogenesis remains unclear.

A population of periportal hepatocytes has been identified enriched for biliary gene expression, with special replicative capacity and potential for parenchymal regeneration during hepatocyte injury (5). Introduction of transgenically activated, supraphysiological levels of Notch1 intracellular domain (N1-ICD) in hepatocytes can redirect cell identity to a ductular lineage, activating the cancer program (6, 7). There is further evidence that after damage, hepatocytes can contribute to the HPC pool, adopting biliary-specific functions, and that this reverses during recovery (8). This potential for hepatocyte plasticity may explain the appearance of perivascular CC in chronic hepatitis C virus (HCV) infection. We used lineage tracing to demonstrate CC can arise from CK19+ ductal cells; however, the contribution from biliary vs. hepatocyte-derived HPCs and the CC cell of origin is still hotly debated.

Oncogenic Notch1 is a driver of a proportion of T-cell acute lymphoblastic leukemias, whereas other tumors rarely exhibit mutated Notch; rather, WT signaling is disregulated. Sequencing of CC has failed to identify NOTCH1 mutations, and therefore we sought to evaluate the contribution of endogenous WT Notch. As the role of Notch in cancer depends on somatic context, we aimed to use a range of models to reflect the mutational heterogeneity of CC. Both pan-receptor and Notch1 inhibition are associated with off-target effects, so we hypothesized that characterizing the signal might identify specific drivers to enable targeting to bypass toxicity.

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 Reconstituent 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.
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. S24). 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. 2B, 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 (52.01-fold by qRT-PCR; \( P = 0.0022 \)), 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, \( P = 0.3095 \)), and unlike in human disease, no change in effector transcription: \( \text{Hes}1, 0.67$-fold (\( P = 0.3095 \)); \( \text{Hey}1, 0.70$-fold (\( P = 0.3095 \)); \( \text{Hey}2, 0.77$-fold (\( P = 0.3939 \); and \( \text{Hey}L, 2.10$-fold (\( P = 0.00649 \). 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).

Reports demonstrated an inhibitory effect using \( \gamma \)-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 \( N\)-(3,5-difluorophenacetyl)-L-alanyl-\( S\)-phenylglycine \( \tau \)-butyl 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 \( 19 \pm 0.53 \% (P = 0.0121; \text{Fig. S3C}) \), and the proportion of liver infiltrated by the tumor was reduced by \( 78 \pm 0.84 \% (P = 0.0148; \text{Fig. S3B}) \). 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.

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 (\( P = 0.105153 \)); \( 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 (\( P \leq 0.0001 \)), with \( NOTCH1, 1.1$-fold (\( P \leq 0.0011 \)); \( NOTCH2, 7.5$-fold (\( P \leq 0.0001 \)); \( NOTCH4, 2.0$-fold (\( P \leq 0.0001 \)); \( JAG1, 363.3$-fold (\( P \leq 0.0001 \)); \( JAG2, 938.6$-fold (\( P \leq 0.0001 \)); \( HES1, 483.7$-fold (\( P \leq 0.0001 \)); \( HES4, 304.2$-fold (\( P \leq 0.0001 \)); \( HEY1, 46.8$-fold (\( P \leq 0.0001 \)); \( HEY2, 384.4$-fold (\( P = 0.0005 \)); \( HEYL, 160.6$-fold (\( P \leq 0.0001 \)) (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. S1A 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 \( \pm \) 0.77% displayed \( \geq 10$% coverage; 31 \( \pm \) 0.84% displayed \( >20$% ; 1.6 \( \pm \) 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 \( \pm \) 3.17% tumors vs. 2.03 \( \pm \) 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 \( \alpha\)-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 \( \beta\)-actin) was 95 \( \pm \) 74.66 times greater in tumor vs. matched nontumor lysates (\( P = 0.0286 \); Fig. S1E). Almost all tumours exhibited stromal expression of JAGGED1 (Fig. S1F).

**Fig. 1.** Notch3 is differentially activated in human CC. (A) Volcano plot of rt-PCR Notch array in human CC and patient-matched liver (\( n = 5, n = 5 \)). Gray line represents \( P \) value of 0.05. Red labels, up-regulation at least fourfold; green, down-regulation at least fourfold. (B) Notch expression in human CC (\( n = 48 \)) and healthy liver (\( n = 42 \)) (RT-PCR). Medians compared with Mann-Whitney \( U \) test. (C) Tissue microarray human CC (\( n = 77 \)) and noncancerous liver (\( n = 47 \)). Representative Notch1 and Notch3 immunostaining (positive and isotype controls; Fig. S1B and C). Filled arrowheads, Notch3$^+$ ductules and vascular smooth muscle in healthy liver. Pixel analysis of CC and controls compared with Mann-Whitney \( U \) test. (D) Dual fluorescence of Notch3 (green) in human CC with \( \alpha\)-SMA, CD31$^+$, or CD68$^+$ (red) (Scale bar, 100 \( \mu\)m.) (E) N3-ICD (green) in human CC (white filled arrowheads). (Scale bar, 50 \( \mu\)m.) Data are means \( \pm \) SEM. *\( P \leq 0.05 \), **\( P \leq 0.01 \), ***\( P \leq 0.001 \).
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).

**Genetic Deletion of Notch3 Reduces CC Formation and Progression.**

γ-Secretase is a large protease complex, and, although blockade results in total loss of Notch signal (single point mutation causes embryonic lethality) (10), Notch is only one of its substrates. Notch3 is an atypical receptor with structural differences to Notch1 and 2 and can be targeted without disrupting normal development (11). We therefore aimed to evaluate its potential as a nonredundant CC inhibitor (8). In pneumocytes, eYFP-positive 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).

Notch3 mRNA and to a lesser degree Notch2, but not Notch1 or Notch4 (undetectable), was overexpressed in CC in CK19CreYFPp53<sup>fl/fl</sup> mice compared with CK19CreYFPp53<sup>wt/wt</sup> and CK19CreYFPp53<sup>wt/wt</sup> mice, as well as CK19CreYFPp53<sup>fl/fl</sup> mice without CC (Fig. 4B). When normalized to CK19CreYFPp53<sup>wt/wt</sup> mice with 26 wk of TAA, Notch3 is up-regulated 85.92-fold (P = 0.00286) in CK19CreYFPp53<sup>wt/wt</sup> mice with CC, compared with Notch1 at 24.28-fold (P = 0.0286). In CK19CreYFPp53<sup>fl/fl</sup> 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<sup>T</sup>eYFPp53<sup>fl/fl</sup> mice on the TAA protocol to mice carrying constitutive deletion of the Notch3 gene (CK19CreER<sup>3p53<sup>N3</sup></sup>). A difference in livers in N3<sup>wt/wt</sup> mice compared with N3<sup>fl/fl</sup> and N3<sup>wt/wt</sup> animals was seen at 26 wk (Fig. 5A). Although macroscopic canicular 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<sup>wt/wt</sup> mice, as well as a reduction in the mean tumor number [28.78 ± 15.37 N3<sup>wt/wt</sup> mice (n = 9) vs. 0.0875 ± 0.38 N3<sup>fl/fl</sup> mice (n = 8)], indicating single copy loss of Notch3 is sufficient to inhibit CC formation (Fig. 5B and Fig. S5A). N3<sup>fl/fl</sup> mice exhibited a similar phenotype; there was no statistical difference in tumor burden to N3<sup>wt/wt</sup> animals (N3<sup>wt/wt</sup> mice, 0.035 ± 0.01 mean% tumor area vs. 0.086 ± 0.05 N3<sup>fl/fl</sup>). 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 CK19CreYFPp53<sup>fl/fl</sup> or CK19CreYFPp53<sup>wt/wt</sup> mice (Fig. S4A). 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 recombination in this model (14). In tumors, eYFP<sup>+</sup> 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>tg</sup>eYFPp53<sup>lf/f</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 sequences (Fig. S5E). Across multiple colonies, transfection inhibited NOTCH3 expression (Fig. S5 E and F). Almost total ablation of effectors was observed, suggesting functional signaling inhibition (Fig. S5G).

Clone 1 exhibited efficient knockdown and was used for further experiments. In vitro, a modest attenuation in proliferation was observed (19.42 ± 2.87% reduction in 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) absorbance; P = 0.0765; Fig. S5H), and when xenografted, a 62 ± 28.74% reduction in size (P = 0.0237) and 76 ± 28.44% reduction in mass (P = 0.0237) was seen in Notch3 KD xenografts (Fig. 5D). We confirmed this was not due to reduced neoangiogenesis by quantification of CD31 (mean signal CD31 to DAPI, 0.0506 ± 0.0056 scrambled vs. 0.0285 ± 0.0079 N3shRNA xenografts; P = 0.329; Fig. S5J).

**Genetic Silencing of Notch3 but Not RBPJ Reduces Signaling Through the PI3K-AKT Cascade.** 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 (CC-LP-1) with siRNA against either NOTCH3 or the canonical effector RBPJ. Inhibition was confirmed with qRT-PCR and immunoblotting (Fig. S6 A and B). Eighty-four known drivers of hepatic carcinogenesis were screened with a PCR array (Tables S3 and S4). Almost all genes exhibiting changes in transcription (defined as at least fourfold) were either upstream mediators or downstream targets of the AKT cascade including MET, IRS1, 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 p-mTor (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, IRS1, FAS, and RAC1 (Fig. S6C).

To confirm this phenomenon was not an off-target effect of RNAi, we looked at Akt in CK19CreER<sup>tg</sup>eYFPp53<sup>lf/f</sup> mice on the TAA protocol with (n = 8) and without (n = 9) Notch3 deletion. A reduction in Fox, Fadd, and Rac1 gene expression was seen, although this did not reach significance (Fig. S7A). Immunoblots, however, revealed a 72% reduction in pAKT protein (N3<sup>−/−</sup>/N3<sup>+/+</sup> 0.41 ± 0.10 vs. N3<sup>−/−</sup>/N3<sup>+/+</sup> 0.12 ± 0.06; P = 0.0426), a 30% reduction in p-mTOR (N3<sup>−/−</sup>/N3<sup>+/+</sup> 1.55 ± 0.13 vs. N3<sup>−/−</sup>/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>/N3<sup>+/+</sup> 0.54 ± 0.16; P = 0.0127) and an 88% reduction in p70S6 (N3<sup>−/−</sup>/N3<sup>+/+</sup> 0.91 ± 0.34 vs. N3<sup>−/−</sup>/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 = 0.0288; Fig. S7B).

**Discussion**

Exogenous oncogene activation in mice can initiate carcinogenesis in many tissues and indeed often in tissues where these oncogenes...
Genetic silencing of Notch3, but not RBPJ, reduces PI3K-AKT transcription. (A) Human (CC-LP-1) cells transfected with NOTCH3 or RBPJ siRNA and analyzed with oncogene 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 pS6 with pixel analysis in Notch3 shRNA and scrambled CC-LP-1 xenografts. (Scale bar, 100 μm.)

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 β-fetoprotein 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 RBPP- or Hes1-deficient mice (21). Therefore, the possibility of a tumor-forming role via an RBPP-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 RBPP 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 RBPP; or activation by membrane-tethered receptors without GS cleavage (23). RBPP-independent signaling is characterized in T cells where N3-ICD interacts with IKKα to stimulate NF-κB 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 RBPP-independent route, given the restriction of tumor growth after GSI.

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
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 untreatable 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. C19CreER²R26ReYFP mice (14) were a kind gift from Guoquan Gu (Vanderbilt Medical Center, Nashville, TN). These mice were cross-bred with Trp53−/− mice (Ref. 85) (ref. 86.1292-2Trp53tm1Bnv), Notch3tm1Grid (N3−) mice (Ref. B6.129S1-Notch3−/−J) (11), or Notch3R (Notch1tmflk0 GRID) from Jackson Laboratories. Trp53−/− (Ref. 85) and Notch3tm1Grid (N3−) mice were on a C57BL/6J background; Notch3−/− mice were on a C57BL/6J background. Before experimental use, animals were cross-bred with the C19CreER²R26ReYFP line, which carried a CD1;C57BL/6 background. Progeny were bred with the CK19CreER mouse and with Notch3CreER mouse to ensure that Notch3 is not 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 untreatable cancer.

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 H&E sections from each block (five per liver) by a histopathologist blinded to the region. 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. Post hoc testing groups of nonparametric data were performed using Dunn’s multiple comparison test.

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