Notch-Nrf2 Axis: Regulation of *Nrf2* Gene Expression and Cytoprotection by Notch Signaling

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The Notch signaling pathway enables regulation and control of development, differentiation, and homeostasis through cell-cell communication. Our investigation shows that Notch signaling directly activates the Nrf2 stress adaptive response pathway through recruitment of the Notch intracellular domain (NICD) transcriptosome to a conserved Rbpjk site in the promoter of *Nrf2*. Stimulation of Notch signaling through Notch ligand expression in cells and by overexpression of the NICD in *Rosa^{NICD/-}::AlbCre* mice *in vivo* induces expression of *Nrf2* and its target genes. Continuous and transient NICD expression in the liver produces a Notch-dependent cytoprotective response through direct transcriptional activation of Nrf2 signaling to rescue mice from acute acetaminophen toxicity. This response can be reversed upon genetic disruption of *Nrf2*. Morphological studies showed that the characteristic phenotype of high-density intrahepatic bile ducts and enlarged liver in *Rosa^{NICD/-}::AlbCre* mice could be at least partially reversed after *Nrf2* disruption. Furthermore, the liver and bile duct phenotypes could be recapitulated with constitutive activation of Nrf2 signaling in *Keap1^{EF/EF}::AlbCre* mice. It appears that Notch-to-Nrf2 signaling is another important determinant in liver development and function and promotes cell-cell cytoprotective signaling responses.

Nfe2l2 (*Nrf2*) is a ubiquitously expressed basic leucine zipper transcription factor that induces prosurvival responses within the cell through induction of ~200 genes that function to prevent macromolecular damage mediated by electrophiles and oxidants; enhances the recognition, repair, or removal of damaged macromolecules; and fosters tissue regeneration (1). The formation of heterodimeric complexes with Nrf2 (NCBI accession no. GSE15633) and small Maf (sMaf) regulates the expression of Nrf2 target genes through binding to antioxidant response element (ARE) sequences that act as enhancers on target gene promoters. Under basal conditions, metabolic turnover of the Nrf2 protein is rapid via effective proteasomal degradation mediated by a degasome consisting of a Keap1-Cul3 complex. When cells are subjected to electrophilic or oxidative stressors, efficient degasome formation is disrupted due to conformational changes within the Keap1-Cul3 complex, allowing newly synthesized Nrf2 to translocate into the nucleus, where target genes are expressed through the formation of a functional transcriptosome consisting of an ARE-Nrf2-sMaf-polymerase II (Pol II) complex on the chromatin (2).

Inasmuch as the liver plays a central role in the metabolism and excretion of reactive intermediates of endogenous and environmental chemicals, the Nrf2-ARE signaling system in the liver regulates an adaptive response against these stressors. Disruption of *Nrf2* enhances the susceptibility of mice to hepatic damage following exposure to toxins (3). Conversely, the conditional disruption of *Keap1* in mouse hepatocytes evokes resistance to toxin-induced liver damage (4, 5). Similarly, pretreatment of wild-type mice with inducers of Nrf2 signaling (e.g., dithiolethiones, triterpenoids,

and isothiocyanates) mitigates damage of the liver and other tissues from exposure to toxins (1).

Recently, Nrf2 has been shown to participate in cytoprotective actions through interaction with other target genes that influence the repopulation and regeneration of the liver. The observed phenotype of a delayed early regrowth phase in *Nrf2* null mice following partial hepatectomy led to hypotheses of links with both Notch (6) and insulin-like growth factor (IGF) (7) signaling. Notch signaling is an evolutionarily conserved intracellular signaling pathway involved in cell fate decisions, lineage commitment, and maintenance of stem/progenitor cells in both the early developmental phases and the adult animal (8). The Notch family consists of intermembrane receptors that can be bound by Notch ligands, which are present on the surface of adjacent cells. Formation of the Notch ligand receptor complex initiates proteolytic cleavage by a member of the ADAM family of metalloproteases, followed by cleavage by γ -secretase, to release the Notch intracellular domain (NICD) from the cytoplasmic membrane. The NICD then translocates to the nucleus, where it interacts with the common DNA

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binding partner for the Notch receptor family, recombination signal binding protein for the immunoglobulin kappa J region (Rbpjk). This interaction converts Rbpjk from a transcriptional repressor to an activator, resulting in target gene expression. In mammals, there are two families of canonical Notch ligands (Jagged1 and -2 and Delta-like-1, -3, and -4) and four Notch receptors (Notch1 to -4). Notch1 and Notch2 are central in the maintenance of the liver based on studies using mice with genetic disruption of these transcription factors (9, 10). Nrf2 is evolutionarily conserved among animals (11), as is Notch (8), suggesting that there is potential for cross talk between the two molecules and their effector pathways. Indeed, the gene regulatory region of the major *Notch1* transcript possesses a functional ARE through which Nrf2 can regulate *Notch1* gene expression directly (6). *In silico* analyses indicated that the Rbpjk core binding sequence is conserved in the *Nrf2* gene regulatory region among various species, prompting us to investigate whether NICD-forced expression, using both cell culture and mouse models, affects Nrf2 signaling. Our findings indicate the functional importance of Notch-Nrf2 interactions in both stress responses of the liver and liver development.

MATERIALS AND METHODS

Animals. *Keap1^{FF}* and *Nrf2^{-/-}* mice were described previously (12). Gt(Rosa)26Sortm1(Notch1)Dam/J (*Rosa^{NICD/-}*) (13) and B6.Cg-Tg(AlbCre)21Mgn/J (*AlbCre*) (14) mice were obtained from the Jackson Laboratory. *Nrf2^{-/-}::Rosa^{NICD/NICD}* mice were generated by crossing *Nrf2^{-/-}::Rosa^{NICD/NICD}* mice with *Nrf2^{-/-}::AlbCre* mice, which were established by the consecutive mating of *Nrf2^{+/-}::AlbCre* and *Nrf2^{+/-}::Rosa^{NICD/-}* mice, correspondingly. *Nrf2^{FF}* mice were developed as described previously (15). They were consecutively mated with *Rosa^{NICD/NICD}* or *AlbCre* mice to establish *Nrf2^{FF}::Rosa^{NICD/NICD}::AlbCre* and *Nrf2^{FF}::Rosa^{NICD/NICD}* mice. All mice were established in a C57BL/6J albino background [B6(Cg)-*Tyr^{c-2/J}*] and genotyped by PCR as previously reported (12, 15) or as described in Table S1 in the supplemental material. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Delivery of a specific NICD expression vector or mock vector to mouse liver. pEF-Flag-mNICD was constructed as an NICD expression vector for use *in vivo* in which the Flag-mouse NICD (mNICD) cDNA fragment was separated from pSG5-NICD and pTracer/EF-BSD-A (Invitrogen, USA). Eight-week-old male B6(Cg)-*Tyr^{c-2/J}* mice were coinjected with 25 μ g of pEF-Flag-mNICD or pEF-Mock vector DNA with 5 μ g of pGFP-V-RS (Origene, USA), which carries a turbo green fluorescent protein (tGFP)-expressing unit to confirm delivery using TransIT-EE hydrodynamic delivery solution (Mirus, USA) through the tail vein (16). The liver genomic DNA was isolated from each mouse 5 days after hydrodynamic tail vein injection (HTI), and the NICD-expressing unit was detected by PCR (see Table S4 in the supplemental material).

APAP treatment and biochemical analysis. Male B6(Cg)-*Tyr^{c-2/J}* mice (7 weeks old), injected with the NICD expression vector or its control mock vector, were treated intraperitoneally (i.p.) with 250 mg/kg of body weight acetaminophen (APAP) (Sigma, USA) or saline as the vehicle 5 days after DNA delivery by HTI. Mice were fasted for 20 h prior to APAP treatment. Plasma alanine aminotransferase (ALT) activities were used as a marker for hepatic injury induced by APAP treatment and were measured with an automated biochemical analyzer (Dri-Chem 4000i; Fujifilm, USA). The same dosing and assay conditions were used for treatment and analysis of the different genetic lines of mice.

Resin casting. The common bile duct (CBD) was isolated and cannulated above the pancreas by using stretched polyethylene-10 tubing (Intramedic Clay Adams brand; Becton Dickinson, USA) while mice were under anesthesia. The cannula was fixed in the CBD by ligation of 7-0 silk. Phosphate-buffered saline (PBS) was manually flushed through the CBD to ensure proper cannulation. Liquid Mercor II resin (Ladd Industries,

USA) (2 ml) was mixed with 0.2 g of catalyst. Following cystic duct ligation by 10-0 nylon, the resin mixture (200 to ~400 μ l) was injected by manual perfusion into the CBD of 9-week-old male mice. At the point of feeling resistance, the CBD was ligated, and the cannula was then removed. Whole liver was resected from the abdominal cavity and floated in warm PBS (50°C overnight). Individual lobes were separated for minute observation among genotypes. Whole liver or individual lobes were placed into a 15% KOH solution at room temperature to macerate the tissue. Casts were rinsed several times with double-distilled, deionized water and allowed to dry before imaging with a stereoscope coupled with a camera (Olympus SZX12 and Olympus DP-70).

Cell culture. Mouse embryonic fibroblast (MEF) cells were previously established from the embryos of wild-type littermates (17) and maintained in Iscove's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS). HeLa cells and HEK293 cells were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Coculture. DsRed2WtB6js was used for the stable expression of red fluorescent protein (RFP) in MEFs by transfection with pCMVDSRed2 puro3, which was constructed through insertion of DsRed2 cDNA into pIRES Puro3 (TaKaRa Clontech, USA). This cell line was maintained with culture medium containing 5 μ g/ml puromycin. Approximately 4×10^5 DsRed2WtB6js cells and 7.5×10^5 HEK293 cells were plated onto 10-cm-diameter and 6-cm-diameter dishes, respectively. The next day, HEK293 cells were transfected with Lipofectamine (Invitrogen, USA) containing 10 μ g of pLZRS-hDl11 or pLZRS. Enhanced green fluorescent protein (EGFP) and puromycin-N-acetyltransferase gene-expressing units were included in the same constructs. Six hours later, cells were washed twice with culture medium and then refed with fresh medium without any antibiotics for 48 h. More than 95% of cells at that time expressed EGFP and were harvested for coculture. Transfected HEK293 cells (1.0×10^7 cells) were used for coculture with DsRed2WtB6js cells ($\sim 1.0 \times 10^6$ cells) in a 10-cm-diameter dish.

Nrf2 reporter constructs. The regulatory region of the *Homo sapiens* *NRF2* gene including the initiation codon sequence was isolated from human genomic DNA (Promega, USA) by nested PCR. The first PCR product of primer-51 and primer-31 (~2 kb) was used as the template DNA for a second PCR with both primers -690hNRF2 and 3'NcoI-hNRF2. This region (~0.8 kb) was directly cloned between the KpnI and NcoI sites in pGL3 basic (Promega, USA). Consequently, the ATG of *NRF2* was replaced with the ATG of luciferase cDNA. Primer -690 mut-hNRF2 was used for the point-mutated constructs in the Rbpjk element. The *Mus musculus* Nrf2 reporter gene, which includes the region from positions -830 to +135 of mouse *Nrf2* (p-830 mNrf2 Luc), was cloned as described previously (18). This construct was utilized as the template for producing the fragment at positions -719 to -442 containing a point mutation or the intact Rbpjk element by PCR with primer -719 mNrf2 or -719 mut Nrf2 combined with primer 3-442PstI mNrf2. Each PCR product and the region from positions -442 to +315 produced by PstI and EcoRI digestion of p-830 mNrf2 Luc were inserted between KpnI and EcoRI sites in pGL3 basic. All constructs were confirmed by sequence analysis. Plasmids used in transfection experiments were purified with Endo Free Qiagen plasmid kits (Qiagen, USA). All primers are listed in Table S5 in the supplemental material.

Reporter gene assay. HeLa, HEK293, or MEF cells were plated at a density of 2×10^5 cells/60-mm dish for 24 h prior to DNA transfection. Reporter genes (2 μ g) were transfected together with 10 μ g pSG5-Mock or pSG5-NICD and 20 ng the pRLTK- Δ ARE normalizing vector by calcium phosphate coprecipitation (19). Cells were harvested 36 h after transfection. Luciferase activity was measured according to the manufacturer's instructions (Promega, USA) and normalized to *Renilla* luciferase activity derived from pRLTK- Δ ARE.

Analysis of DNA-nuclear factor binding. Nuclear extracts were prepared from HEK293 cells (20). Protein concentrations of each nuclear

FIG 1 Rbpjk binding sequences are conserved across mammalian species. (A) Alignment of proximal Nrf2 promoters among various animals. Asterisks indicate nucleotides conserved among all animals (NCBI accession numbers [NM_006164.4](#), [XM_001145876.2](#), [XM_001096024.2](#), [XM_002749316.2](#), [NM_001011678.2](#), [NM_031789.1](#), and [NM_010902.3](#)). (B) Human and mouse Nrf2 reporter gene constructs and relative luciferase activities (RLA). The human Nrf2 reporter gene and NICD expression vector or control mock vector were cotransfected into HEK293 or HeLa cells. The mouse Nrf2 reporter gene and NICD expression vector or mock control vector were cotransfected into MEF cells. (C) Human Nrf2 reporter gene activity influenced by EBNA2 expression instead of the NICD in HeLa cells. Luciferase activity was normalized by *Renilla* luciferase activity derived from pRLTK-ΔARE, which was transfected simultaneously. The relative luciferase activity derived from cells cotransfected with the mock vector is set as 1. Values are means ± SE of 3 or 4 experiments, each in duplicate. *, $P < 0.05$ by one-way ANOVA.

extract were determined by the Bio-Rad protein assay (Bio-Rad, USA) using bovine serum albumin (BSA) to generate a standard curve. 3'-Biotinylated probes were synthesized by Invitrogen Custom Oligo Service (Invitrogen, USA). Probes were annealed with the complementary oligonucleotide in a solution containing 10 mM Tris-HCl, 1 mM EDTA, and 0.5 M NaCl (pH 8.0). The annealed probes were then subjected to electrophoresis in 6% polyacrylamide gels in a solution containing Tris-borate-EDTA (TBE) buffer (45 mM Tris-borate and 1 mM EDTA), recovered, and purified as double-strand probes. For standard electrophoretic mobility shift assays (EMSAs), a 25-μl reaction mixture containing approximately 20 fmol of probe nucleotide was incubated with 7.5 μg protein of the nuclear extract in a buffer consisting of 10 mM HEPES (pH 7.9), 25 mM KCl, 1 mM EDTA, 1 mM ZnCl₂, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 4 μg poly(dI-dC), 0.05% Nonidet P-40, and 10% glycerol at 4°C for 30 min. For the competition experiments, each competitor oligonucleotide (see Table S2 in the supplemental material) was also added to the standard EMSA reaction mixture at a 50-fold molar excess to the probe, containing approximately 1 pmol, with the final volume adjusted to 25 μl. The reaction products were loaded onto a 6.0% acrylamide gel and run at 20 mA in 0.5× TBE. For the supershift assay, anti-Rbpjk antibody was used and loaded onto a 4.0% PAGE gel. All EMSAs were repeated three times, utilizing separate nuclear extracts from HEK293 cells. Detection of EMSA products was performed by using the LightShift chemiluminescent EMSA kit (Pierce Thermo-Scientific, USA) according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) assay. Formaldehyde cross-linking and chromatin fragmentation were performed as described previously (21). A diluted chromatin solution with an antibody to Rbpjk (H-50) (sc-28713X; Santa Cruz Biotechnology, USA) or nonspecific immunoglobulin G (normal rabbit IgG) (sc-2027; Santa Cruz Biotechnology, USA) or a solution without antibody was incubated for 18 h at 4°C with rotation. After washing and elution, precipitated DNA was dissolved in 60 μl of Milli-Q water. DNA solution (2 μl) was used as a template for PCR with the following primers: -807 hNRF2-CP (5'-CGCTTTGGTGG

GAAGAGGTTCTC-3') and -580 hNRF2-CP (5'-CTGGGGCCAGTGG GCCCTGCC-3'). For the *HES1* Rbpjk core sequence in the promoter area, primers 5'-GGTCCGCGTGTCTCCTCTC-3' and 5'-ATCAGT AGCGCTGTTCAGGAC-3' were used.

Isolation and purification of total RNA and RT-PCR. Mouse livers were perfused with ice-cold PBS prior to isolation of total RNA by using TRIzol (Invitrogen, USA). Total RNA was then purified by using the RNeasy minikit (Qiagen, USA). RNA integrity was confirmed by electrophoresis before the reverse transcriptase (RT) reaction. RNA quantification was performed by spectrophotometry at 260 nm. Several genes were analyzed by SYBR green real-time quantitative RT-PCR (qPCR), and the *Cre*, *Egfp*, and *Gapdh* genes were analyzed by semiquantitative RT-PCR. cDNA was synthesized by using the iScript system (Bio-Rad, USA). Real-time PCR was performed on a Bio-Rad MyIQ real-time PCR machine using Applied Biosystems SYBR green PCR master mix in triplicate 20-μl reaction volumes. The PCR efficiency was determined from a standard curve, and the Pfaffl method was used for calculations of fold changes (22). Melt curves and agarose gel electrophoresis were employed to ensure the specificity of the amplified product. Primers are shown in Table S3 in the supplemental material.

Protein preparation and immunoblotting analyses. Proper-size cut tissues were homogenized in RIPA-I buffer, which contained a protease inhibitor cocktail (Roche, USA). Cultured cells were harvested in radio immunoprecipitation assay buffer with protease inhibitor (RIPA-I) buffer directly. An equal volume of 2× SDS sample buffer was added, and the samples were denatured by boiling for 5 min. Samples were applied onto SDS-PAGE gels and transferred onto an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore, USA). The membranes were blocked with Tris-buffered saline with 0.05% Tween 20 and 5% skim milk and then treated with a primary antibody. The preparative membranes were reacted with appropriate secondary antibodies conjugated to horseradish peroxidase (Invitrogen, USA). The immunocomplexes were visualized with ECL (PerkinElmer, USA). All antibodies are listed in Table S6 in the supplemental material.

Statistical analysis. All values are expressed as means $\pm$ standard errors (SE). Statistical analysis was performed with unpaired Student's *t* test or one-way analysis of variance (ANOVA) (with the Tukey-Kramer *post hoc* test) for comparison of multiple groups. Differences between groups were considered statistically significant when the *P* value was <0.05 .

RESULTS

Rbpjk binding core sequences are present and functional in the *Nrf2* gene regulatory region. The Rbpjk binding core sequence identified by Tun et al. (23) is conserved in the promoter region of *Nrf2* in higher vertebrates, as shown in Fig. 1A. To test whether this conserved recognition sequence consists of a functional *cis* element for *Nrf2* transcriptional regulation by Rbpjk-NICD, both human and mouse *Nrf2* luciferase reporter constructs, with or without Rbpjk core point mutations, were prepared. Cotransfection of the *Nrf2* reporter genes with a NICD expression vector led to higher luciferase activity than cotransfection of the control vector into HEK293, HeLa, or MEF cells (Fig. 1B). Point mutation of the Rbpjk core sequence led to abrogation of the induction of luciferase activity by NICD overexpression (Fig. 1B). Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus, and adenovirus have been reported to utilize Notch signaling to establish infection in their target cells (24). One of them, Epstein-Barr virus nuclear antigen 2 (EBNA2), an NICD mimic (25–27), was tested in our cellular system. When EBNA2 was overexpressed instead of NICD, a similarly high hNrf2 reporter gene activity was detected in HeLa cells (Fig. 1C). These results demonstrate that the putative Rbpjk core element embedded within the *Nrf2* promoters can interact directly with the Notch signaling transcriptional machinery.

The NICD-Rbpjk complex binds to the *Nrf2* promoter region. In order to verify a direct interaction of Rbpjk with its core sequence on the *Nrf2* promoter, an EMSA using nuclear extracts from HEK293 cells was performed. As shown in Fig. 2A, the probe, human *NRF2* promoter-Rbpjk, could form complexes with the HEK293 nuclear extract. Addition of Rbpjk *cis* elements in the *HES1* promoter and the mouse or human *Nrf2* promoter-Rbpjk regions were able to compete for Rbpjk binding and eliminated the specific band. Moreover, when oligonucleotides containing mutations in the Rbpjk core sequences in human and mouse *Nrf2* promoters were used as competitors, the intensity of this band was not affected. A supershift assay with anti-Rbpjk antibody confirmed that the lower-migrating band included Rbpjk as a component of the complex (Fig. 2B). Thus, the Rbpjk core sequences in both mouse and human *Nrf2* promoter regions participate directly in the formation of the Rbpjk complex. To assess whether the actual *NRF2* genomic chromosome allows for an Rbpjk-NICD association, ChIP assays using anti-Rbpjk or anti-NICD antibodies were conducted in HEK293 cells. The human *NRF2* promoter with the Rbpjk core sequence was detected in both cases by using antibodies against Rbpjk or NICD (Fig. 2C). Detection of the *HES1*-Rbpjk core sequence served as a positive control. These results indicated that the putative Rbpjk core sequence in the *NRF2* promoter functions as a *cis*-regulatory element.

Notch ligand induces expression of *Nrf2* and *Nrf2* target genes. A transient coculture model was developed to determine whether expression of *Nrf2* and its target genes in naturally immortalized mouse embryonic fibroblasts (MEFs) was affected by stimulation of Notch signaling. HEK293 cells expressing a Notch ligand, DLL1, were cocultured with MEFs as the acceptor cells of

FIG 2 Rbpjk core sequences in the human *NRF2* promoter interact with nuclear proteins. (A) The complex (arrowhead) formed by nuclear factors from HEK293 cells and human *NRF2* Rbpjk core sequences could be competed away with human *HES1* and mouse *Nrf2* Rbpjk core sequences but not by mutant Rbpjk core sequences. A 50-fold molar excess of DNA was used as a competitor. (B) Supershifted migration complexes indicated by an arrow were detected only with anti-Rbpjk specific antibody. (C) The human *NRF2* promoter was detected in the precipitate by a ChIP assay using anti-Rbpjk or anti-Notch1 intracellular domain antibody. Detection of the human *HES1* promoter region was used as a positive control for the quality of antibodies and accuracy of the ChIP assay. DNA sizes detected by PCR are 233 bp and 206 bp for *NRF2* and *HES1*, respectively.

Notch ligand-mediated signaling (Fig. 3A). Immunoblots of relative expression of the exogenous DLL1 from pLZRS-hDll1 or its negative-control mock vector in HEK293 cells are shown in Fig. 3B. Transcript levels of *Nrf2* and its target genes, *Nqo1* and *Gsta1*, increased following 3 h of coculture, as did transcript levels of *Hes1*, a gene directly downstream of Notch (Fig. 3C). Thus, canonical Notch signaling activated through ligand-receptor interactions likely produced NICD molecules in the ligand acceptor cells, leading to induction of *Nrf2* expression via transcriptosome formation with Rbpjk-NICD complexes on its promoter.

***Nrf2* expression is increased in *Rosa^{NICD}/NICD*;*AlbCre* mouse liver.** To elucidate the relevance of Notch1-Nrf2 cross talk *in vivo*, *Rosa^{NICD}/NICD*;*AlbCre* mice were generated from *Rosa^{NICD}/NICD* and *AlbCre* mice. *Rosa^{NICD}/NICD*;*AlbCre* mice have been shown to have constitutive postnatal expression of the NICD in cells of hepatocyte lineage, due to the excision of a repressor element between *loxP* sequences by *Albumin* promoter-driven Cre recombinase expression (14). To confirm the genomic structure for these transgenic mice, PCR was performed by using liver genomic DNA from

FIG 3 Stimulation of Notch signaling leads to Nrf2 signaling in target cells. (A) Scheme of the MEF coculture system with forced human DLL1 (hDLL1) expression in HEK293 cells as effector cells and MEFs as target cells. (B) Confirmation of hDLL1 expression in transfected HEK293 cells. (C) Time course for murine RNA expression following coculture with ligand-overexpressing cells. *Gapdh* expression was utilized for normalization. The ratio of the gene of interest to *Gapdh* at time zero was set to a value of 1. Values are means $\pm$ SE ($n = 3$). *, $P < 0.05$ by one-way ANOVA.

9-week-old males as a template. The Cre-inactive band was detectable in both *Rosa^{NICD}/-* and *Rosa^{NICD}/::AlbCre* mice; however, only *Rosa^{NICD}/::AlbCre* mice have the appropriate genomic structure to express the NICD from the *Rosa26* locus (Fig. 4A). In accordance with the genomic structural alterations, both *Egfp*, which is linked to the NICD cDNA-IRES (internal ribosomal entry site) unit, and Cre transcripts were detected in total RNA isolated from *Rosa^{NICD}/::AlbCre* mouse livers (Fig. 4B).

Nrf2 transcript levels were increased (about 3-fold) in livers of *Rosa^{NICD}/::AlbCre* mice compared to their *Rosa^{NICD}/-* or *AlbCre* counterparts. Accordingly, the mRNA levels of *Nqo1*, a canonical *Nrf2* target gene (28), were also increased (~ 2 -fold) in the livers of these mice. mRNA levels of representative Notch target genes (*Hes1*, *Herp1*, and *Nrarp*) (29) were increased in the livers of the *Rosa^{NICD}/::AlbCre* mice, as expected (Fig. 4C). mRNA levels of *Atg7*, which has neither functional ARE nor Rbpjk recognition sequences in its proximal promoter region (NCBI accession number NM_001253717.1), did not show any differences among the three genotypes (Fig. 4C). Therefore, it is apparent that constitutive NICD expression led to increased levels of *Nrf2* transcripts in murine liver, in accordance with the transcriptional activation observed in the cell line studies.

To verify whether the increased hepatic *Nrf2* mRNA levels in NICD transgenic mice were reflected in altered protein levels, immunoblot analyses were conducted. Cre recombinase was detected only in mice bearing *AlbCre* transgenes (Fig. 4E). *Nrarp*, a direct Notch signaling target gene, was strongly induced in *Rosa^{NICD}/::AlbCre* mice. Protein levels of *Nqo1*, an *Nrf2* target gene that does not have an Rbpjk recognition sequence in its proximal promoter, were also induced in these mice (Fig. 4E and F).

Hepatic nuclear extracts from *Rosa^{NICD}/::AlbCre* mice exhibited a 3-fold increase in the level of *Nrf2* protein compared to *AlbCre* or *Rosa^{NICD}/-* mice (Fig. 4D and F). In contrast, autophagy-related proteins (*Atg5*, *Atg7*, and *Beclin1*) (30), which increase *Nrf2* stability by mediating *Keap1* turnover, did not show significant differences among the three genotypes.

Constitutive NICD expression in the mouse liver: hepatomegaly and high-density intrahepatic biliary tract. Forced expression of NICD resulted in enlarged livers in *Rosa^{NICD}/::AlbCre* mice. At 9 weeks of age, total body weights of the *AlbCre*, *Rosa^{NICD}/-*, and *Rosa^{NICD}/::AlbCre* mouse lines were not significantly different and averaged 27.2 ± 0.5 g (mean $\pm$ SE) ($n = 9$), 26.7 ± 0.4 g ($n = 13$), and 26.3 ± 0.4 g ($n = 15$), respectively. However, average liver weights varied: 1.33 ± 0.04 g in *AlbCre*, 1.41 ± 0.05 g in *Rosa^{NICD}/-*, and 1.77 ± 0.04 g in *Rosa^{NICD}/::AlbCre* mice. The ratio of liver weight to total body weight was significantly higher in the *Rosa^{NICD}/::AlbCre* mice than in the two control lines (Fig. 5A). Similar elevated ratios of liver weight to body weight were observed for *Keap1^{F/F}* and *Keap1^{F/F}/AlbCre* mice, which express larger amounts of *Nrf2* in hepatocyte-lineage cells (31).

Resin corrosion casting was used to compare mice with altered NICD and *Nrf2* expression levels in order to observe structural alterations of the intrahepatic bile ducts (IHBDs). Increased IHBD density in *Rosa^{NICD}/::AlbCre* liver, previously reported by Sparks et al. (32), was confirmed (Fig. 5C and H). Higher-magnification observations revealed larger amounts of microbranching of the IHBD in the peripheral liver lobes of the *Rosa^{NICD}/::AlbCre* mice than in control mice (*AlbCre* and *Rosa^{NICD}/-* mice) (Fig. 5H to J). Two-dimensional histopathology (hematoxylin and eosin

FIG 4 RNA and protein expression in male *Rosa^{NICD/-}* mouse liver. (A) Cre recombinase regulated by the *Albumin* promoter created the NICD-expressing genomic structure only in *Rosa^{NICD/-}::AlbCre* mouse liver. Left lane, DNA ladder. (B) *Cre* and *Egfp* transcript levels determined by RT-PCR for each genotype. Three mice (9 weeks old) per genotype were used. (C) Expression of representative Notch signaling target genes. *Hes1*, *Herp1*, *Nrarp*, and *Nrf2* and its prototype target gene *Nqo1* were analyzed in liver of each genotype by qPCR. *Atg7* expression served as a control, while 18S expression was utilized for normalization. The ratio of the gene of interest to 18S in *AlbCre* mice was set to a value of 1. Values are means $\pm$ SE ($n = 3$). *, $P < 0.05$ by one-way ANOVA. (D) Immunoblotting analyses of Nrf2. Nuclear hepatic proteins (30 μ g) of each genotype were utilized. (E) Total hepatic proteins (50 μ g) were used for immunoblotting analyses. Coomassie brilliant blue (CBB) staining of the gel indicates the quantity of applied protein, and Gapdh immunoblotting indicates the quantity of the membrane transfer. Atg5, Atg7, and Beclin1 are autophagy-related proteins. Nrarp is a representative downstream target of Notch signaling. (F) Quantification of protein expression. Atg5, Atg7, Beclin1, and Nqo1 were normalized by Gapdh expression, and Nrf2 was normalized by Dnmt1 expression. The ratio of protein expression to Gapdh or Dnmt1 in *AlbCre* mice was set at a value of 1. Values are means $\pm$ SE ($n = 3$). *, $P < 0.05$ by one-way ANOVA.

[H&E] staining) corroborates the resin casting observations in that more cholangiocyte-lineage cells were detected in the peripheral regions of the liver lobes (Fig. 5M to O).

Phenotype reversal by deletion of the *Nrf2* gene from *Rosa^{NICD/-}::AlbCre* mice. In order to examine the role of *Nrf2* gene expression, if any, in the phenotype of hepatomegaly in *Rosa^{NICD/-}::AlbCre* mice, a genetic approach was utilized by generating *Nrf2^{-/-}::Rosa^{NICD/-}::AlbCre* mice. The liver size of *Nrf2^{-/-}::Rosa^{NICD/-}::AlbCre* was reduced compared to that of either of the *AlbCre* or *Rosa^{NICD/-}* controls (Fig. 5A). This reduction of total liver weight by disruption of *Nrf2* was also observed when *Keap1^{F/F}::AlbCre* and *Nrf2^{-/-}::Keap1^{F/F}::AlbCre* mice were compared (Fig. 5A). Resin casting of the IHBD within the *Nrf2^{-/-}::Rosa^{NICD/-}::AlbCre* mouse liver indicates that the microbranches of IHBD are reduced compared to those of *Rosa^{NICD/-}::AlbCre* mice (Fig. 5D). This reduction in the IHBD is reflected in the H&E staining analyses of the periphery of the left lobe; however, unlike the decrease in liver weight, the altered IHBD phenotype was not completely alleviated (Fig. 5H to J).

If hepatomegaly and structural alterations of the IHBD, after forced expression of the NICD in murine liver, are mediated through the Nrf2 pathway, a genetic amplification of Nrf2 signaling should provide a phenocopy. Indeed, as shown in Fig. 5A, livers of *Keap1^{F/F}::AlbCre* mice, in which there is constitutive ac-

tivation of Nrf2 signaling (4, 5), were larger than those of controls and the same size as those of the *Rosa^{NICD/-}::AlbCre* mice. Moreover, they exhibited the peripheral microbranches in the IHBD typical of NICD transgenic mice (Fig. 5K and P). Collectively, these results indicate that IHBD hyperplasia, previously associated with amplified Notch signaling (32), may be mediated through a Notch-Nrf2 axis.

Protection of *Rosa^{NICD/NICD}::AlbCre* mice against APAP hepatotoxicity. There are many reports that Nrf2 plays a pivotal role in resistance against APAP hepatotoxicity through ARE-mediated regulation of enzymes influencing APAP metabolism and disposition (3–5, 33–35). Therefore, if Notch1 influences Nrf2 signaling *in vivo*, amplified Notch signaling should enhance the resistance of mice to APAP in an Nrf2-dependent manner. A series of mice with relevant genotypes were challenged with APAP to test this hypothesis (Fig. 6A). Plasma ALT levels were measured 6 h after intraperitoneal dosing with 250 mg/kg APAP. As positive controls, resistance against APAP hepatotoxicity was determined in mice under different conditions of *Keap1* knockdown (*Nrf2^{F/F}::Keap1^{F/F}*) and knockout (*Keap1^{F/F}::AlbCre*) (Fig. 6B). Floxing of the *Keap1* alleles was previously shown to lead to hypomorphic expression of *Keap1* (5), while floxing of the *Nrf2* alleles appears to have no effect on expression (see Fig. S2 in the supplemental material). Disruption of Nrf2 signaling in *Nrf2^{F/F}::Keap1^{F/F}::AlbCre* mice re-

FIG 5 Hepatic characterization of *Rosa^{NICD/-}::AlbCre* mice. (A) Comparison of ratios of liver weight to whole-body weight for various genotypes. Values are means $\pm$ SE. *, $P < 0.01$ by one-way ANOVA. (B to F) IHBD resin casting of whole liver. Scale bars, 5 mm. (G to K) High-power magnification of representative (6 or 7 per genotype) resin casts in the periphery of the left lobe for each genotype. Scale bars, 200 μ m. (L to P) H&E staining of peripheral left lobes. Scale bars, 50 μ m.

stores sensitivity to APAP toxicity, as seen by the huge elevation of ALT activity after APAP treatment (Fig. 6B). Since the NICD leads to increased Nrf2 signaling in hepatocytes, it is predicted that hepatocellular damage induced by APAP exposure will be attenuated in *Rosa^{NICD/NICD}::AlbCre* mice compared to control mice. Indeed, the mean plasma ALT activity (187 U/liter) in *Rosa^{NICD/NICD}::AlbCre* mice was markedly lower than the mean activity in control mice (11,391 U/liter in *Rosa^{NICD/NICD}* and 12,890 U/liter in *AlbCre* mice) (Fig. 6B). Thus, hyper-Notch signaling derived from the transgenic NICD locus protected against hepatic damage induced by APAP.

To confirm that the protection observed in *Rosa^{NICD/NICD}::AlbCre* mice was mediated through Nrf2 signaling, *Nrf2^{F/F}::Rosa^{NICD/NICD}::AlbCre* and *Nrf2^{F/F}::Rosa^{NICD/NICD}* mice were established (Fig. 6A) and challenged with APAP. Disruption of Nrf2 abrogated the protective action associated with NICD overexpression. As expected, *Nrf2^{F/F}::Keap1^{F/F}::AlbCre* mice were also sensitive to APAP (Fig. 6B). This genetic approach clearly provides *in vivo* evidence that Nrf2 is an important target of Notch signaling

and provides a mechanism by which Notch signaling can manipulate the xenobiotic detoxification machinery through Nrf2-ARE cascades.

Transient forced NICD expression in mouse liver protects against APAP hepatotoxicity. Genetic manipulation of constitutive expression of the NICD in hepatoprogenitors after birth gave rise to developmental consequences, with *Rosa^{NICD/-}::AlbCre* mice demonstrating enlarged livers (Fig. 5A) and hyperplasia of biliary microbranches (Fig. 5C, H, and M). To exclude confounding by these or possible additional effects of constitutive NICD expression, we conducted experiments using transient, forced expression of the NICD in hepatocytes of wild-type mouse liver by the HTI method. Five days after injection of the DNA expression vector through the tail vein, coinjected pGFP-V-RS resulted in expression of tGFP in almost all hepatocytes (Fig. 6C and D). Using microscopic analyses, we could not identify expression of tGFP in any other tissues, such as lung, intestine, heart, spleen, kidney, and brain. Thus, pEF-Flag-mNICD and its control vector DNA appear to be specifically expressed in hepatocytes when ad-

FIG 7 Summary of Notch signaling-dependent cytoprotection, cell fate, and cholangiogenesis through *Nrf2* gene expression. When Notch signaling is activated, the transcription machinery, including NICD, increases *de novo* Nrf2 synthesis. In hepatocytes, this Notch-induced Nrf2 production can lead to enhanced expression of cytoprotective genes. Modulation of Notch signaling in hepatoprogenitor cells may affect bile duct formation (cholangiogenesis) in an Nrf2-dependent manner. ROS, reactive oxygen species.

trengthening the evidence that NICD signaling influences *Nrf2* gene expression directly. Given the central role of Notch in the control of cell fate determination, survival, proliferation, and the maintenance of stem cells, activation of the cytoprotective response of Nrf2 could be critical to several of these actions (Fig. 7).

The second element of biological significance of this interaction is highlighted by the observation that *Nrf2* deletion rescues, at least partially, the phenotypes of hepatomegaly and high-density IHBDs characteristic of *Rosa^{NICD};⁺AlbCre* mice. Thus, the hepatostatic set point governing liver size appears to be regulated in part by Notch1 through Nrf2 signaling, a direction of cross talk opposite that affecting liver regeneration. The ratio of liver weight to total body weight of *Rosa^{NICD};⁺AlbCre* mice was increased compared to that of control mice. Interestingly, a murine model exhibiting increased stability of Nrf2 due to disrupted Keap1 expression and diminished Nrf2 degradation (*Keap1^{F/F};⁺AlbCre* mice) showed a similarly enlarged liver size as *Rosa^{NICD};⁺AlbCre* mice. Deletion of *Nrf2* in *Keap1^{F/F};⁺AlbCre* mice as well as in *Rosa^{NICD};⁺AlbCre* mice resulted in comparable reductions of the ratios of liver weight to body weight. As shown by Komatsu et al. (36), the autophagy system is utilized as an additional pathway to provide Nrf2 stabilization by controlling the turnover of Keap1 in a p62-dependent manner. Impaired autophagy accumulates p62 as well as Keap1, as seen in hepatocyte-specific *Atg7*-disrupted mice (31, 37). p62 masks the binding interface of Nrf2 within the functional Keap1 molecule, leading to Nrf2 accumulation. These *Atg7*-deficient mice show more severe hepatomegaly than our observations of enlarged livers in hepatocyte-specific NICD-overexpressing or *Keap1*-disrupted mice. However, analysis of expression of autophagy-related genes, including *Atg7*, by immunoblotting did not demonstrate differential regulation in our mice. Disruption of the autophagy system might not be a major pathway contributing to the enlarged-liver phenotype of the *Rosa^{NICD};⁺AlbCre* mice. It is noteworthy that the deletion of *Nrf2* did not lead to a reduction of the ratio of liver weight to body weight in *Rosa^{NICD};⁺AlbCre* mice to the levels in *Nrf2^{-/-};⁺Rosa^{NICD};⁺* mice. This attenuated effect may be due to the fact that the NICD

is expressed from the *Rosa* locus and does not carry a PEST domain (38), which is a mark for signaling proteasomal degradation. Thus, unlike the endogenous NICD, this model animal does not completely reflect the dynamics of Notch signaling (13).

Biliary tree architecture may also be influenced through the Nrf2-Notch axis. Sparks et al. reported previously that formation of the IHBD architecture in the liver is regulated by Notch signaling (32, 39). Impaired Notch signaling in bipotential hepatoblast progenitor cells dose-dependently decreased the density of peripheral IHBDs, whereas activation of Notch1 resulted in an increased density of peripheral IHBDs. Although Notch2 has a dominant role in IHBD formation, those authors demonstrated a compensated role for other Notch receptors in determining IHBD formation. Furthermore, they suggested that Notch plays a role in cooperation with other factors to influence lineage decisions of progenitor cells and to sustain peripheral IHBDs. We propose that this disturbed IHBD architecture along with the enlarged liver in *Rosa^{NICD};⁺AlbCre* mice are at least partially attributable to increased *Nrf2* gene expression levels promulgated by Notch signaling. Deletion of Nrf2 signaling in *Rosa^{NICD};⁺AlbCre* mice was found to reverse most, but not all, bile duct enhancement. The incomplete reversal of the phenotype may reflect a role for additional, Nrf2-independent signaling pathways. Indeed, enhanced peripheral branch density of IHBDs was also seen in *Keap1^{F/F};⁺AlbCre* mice. It should be noted that the transgenic intracellular domain of the Notch molecule promoted from the *Rosa* locus is Notch1 derived but not Notch2 derived, which is the major Notch receptor involved in bile duct development (10). However, domain-swapping experiments have shown that the C termini of Notch1 and Notch2 intracellular domains are functionally interchangeable *in vivo* (40). In humans, mutation in the Notch receptor ligand *JAG1* gene results in defective IHBD development in Alagille syndrome (41). This syndrome, a chronic hepatobiliary disease, is characterized by a paucity of IHBDs and is modeled with disruption of Notch signaling in mice. Improper maintenance of IHBD architecture also arises from irresolvable cholestasis (39). Nrf2, in turn, is an important modifier of susceptibility to

cholestasis. Sustained activation of Nrf2 signaling, such as that provoked in *Keap1^{F/F}* mice, protects against liver injury in a bile duct ligation model of cholestasis (42). Treatment of mice with lithocholic acid, a toxic bile acid, produces cholestatic liver injury that is exacerbated in *Nrf2*-disrupted mice (43). Nrf2 is also known to play a major role in the regulation of bile acid homeostasis in the liver and intestine (44). Thus, interactions between the Notch and Nrf2 pathways may influence bile duct structure and bile acid homeostasis.

In summary, we have demonstrated that a functional, conserved Rbpjk recognition sequence exists within the gene regulatory region of *Nrf2* using both *in vitro* and murine models. Notch is often considered to be a model hematopoietic proto-oncogene due to its role in T cell acute lymphoblastic leukemia (45); however, recent studies demonstrate that Notch signaling can also function as a potential tumor suppressor gene. Notch can be a tumor suppressor or an oncogene depending on the cell type (46). Interestingly, Nrf2 appears to function in a similar context. Nrf2 acts as a prosurvival factor through the expression of its cytoprotective target genes and has been an attractive target for chemopreventive interventions (47). Mutations in either Nrf2 or Keap1 as well as epigenetic silencing of Keap1 are seen in a number of cancer types (48). The roles of the Nrf2-Notch bidirectional interaction in driving or impeding a tumor phenotype are unclear but merit exploration.

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N.W. designed and conducted experiments and wrote the manuscript. J.J.S., D.V.C., and S.L.S. conducted experiments and assisted with manuscript preparation; P.A.B. and D.L.P. conducted experiments. S.K., K.N., Y.T., and N.S. implemented the general surgery techniques and resin casting experiments. M.F. cloned EBNA2 and constructed its expression vectors. M.Y. provided the Nrf2- and Keap1-related mice. S.B. provided the *Nrf2^{F/F}* mice. T.W.K. helped to initiate the project and assisted with research design and manuscript preparation. All authors reviewed the manuscript.

We declare that we have no conflicts of interest.

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