Materials and Methods

Animals. Male Fisher rats weighing 250–300 g were obtained from Harlan (Madison, WI). They were housed in cages at 22 ± 2°C under a 12-hour light-dark cycle and fed a commercial diet and water *ad libitum*. All animal study protocols conformed to the guidelines by the Institutional Animal Care and Use Committee (IACUC) of the Animal Facility, University of Pittsburgh.

Animal Experiments. The rats were subjected to two-third resection of the liver under methoxyflurane anesthesia and sterile conditions, as described previously.[1] For the immediate time points of 0–15 minutes after surgery, the incision was covered with sterile gauze, saturated with sterile saline, before harvesting liver remnant. For longer time points, incisions were sutured closed until the liver was harvested. In sham-operated rats, the abdomen was cut open under the same anesthesia, and after removal of the xyphoid, the liver was briefly exposed outside the peritoneal cavity. For harvesting the liver, rats were anesthetized again and portions of the liver were immersed in 20% neutral buffered formalin solution for immunohistochemical examination. Portions of the liver were homogenized in TE-buffer (20 mM Tris, 1 mM EDTA, pH 7.4) or IP-buffer (1% Triton-X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40) containing protease- and phosphatase-inhibitors and processed for plasma membrane-, nuclear protein- or whole-cell lysate preparation. The remaining liver was frozen in liquid nitrogen and stored at –80°C for RNA isolation.

Primary Hepatocyte Cultures. Rat hepatocytes were isolated from normal liver using Seglen's two-step collagenase perfusion technique[2] as previously described from our laboratory.[3] Hepatocytes were cultured on collagen coated (Vitrogen® 100 Collagen; Cohesion, CA) culture dishes and cultured in hepatocyte growth medium (HGM)[4] containing dexamethasone and ITS (insulin, transferrin, selenium) with or without hepatocyte growth factor (HGF) (40 ng/ml) and epidermal growth factor (EGF) (20 ng/ml).

Protein Isolation. Native protein extracts were isolated and subjected to Western blot analysis as described.[5] Lysates were centrifuged at 10,000*g* in a table top centrifuge for 20 minutes. Jagged-1 and Notch were detected in the supernatant of the 20 minutes × 10,000*g* centrifugation.

Plasma Membrane Preparation. For the detection of membrane bound Jagged-1 and Notch-1, isolation of plasma membranes was performed after modified sucrose gradient centrifugation.[6] Tissue homogenized in Tris-ethylenediaminetetraacetic acid (EDTA)-sucrose buffer was centrifuged at 4°C at 17,400*g* for 30 minutes. The pellet was resuspended in Tris-EDTA-sucrose (TES) buffer and subjected to sucrose gradient centrifugation (38.3% sucrose) and centrifuged at 4°C at 23,700*g* in a swinging bucket Ti 60 SW rotor in a Beckmann centrifuge. The plasma membrane containing interphase was collected and centrifuged again. The pellet was resuspended in Tris EDTA buffer. All buffer and solutions were supplemented with protease- and phosphatase-inhibitor cocktails I and II from Sigma (St. Louis, MO).

Nuclear Extract Preparation. Liver tissue was homogenized and washed in 40 mM Tris/HCl pH 7.6, 14 mM NaCl and 1 mM Na_2EDTA with protease and phosphatase inhibitors (Sigma, St. Louis, MO). The pellet was resuspended in hypotonic buffer [10 mM Hepes pH 7.9, 10 mM NaH_2PO_4, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5 mM spermidine, 1 mM NaF, 1% nonfat dry milk and protease- and phosphatase-inhibitors], incubated at 4°C for 10 minutes, and homogenized in a Dounce homogenizer. Homogenates were examined under the light microscope to verify breakage of the cells. The released nuclei were pelleted by centrifugation (5 minutes, 800*g*). Nuclear proteins were extracted in 50–100 &mgr;l hypertonic buffer (30 mM HEPES pH 7.9, 25% glycerol, 450 mM NaCl, 12 mM $MgCl_2$, 6 mM DTT, 0.3 mM Na_2EDTA and protease- and phosphatase-inhibitors) for 45 minutes at 4°C. After centrifugation at 30,000*g* for 30 minutes, the supernatant was recovered and dialyzed for 2 hours against the same solution, but containing 150 mM NaCl.

Western Blot Analysis and Co-immunoprecipitation. For protein determination, the Bicinchoninic Acid Protein Assay (Sigma, St. Louis, MO) was used. Whole cell lysate (100 &mgr;), 50 μg of plasma membrane proteins or 20 μg of nuclear protein extracts were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) according to Laemmli.[7] Notch was detected by a hamster anti-Notch monoclonal and rabbit anti-Notch polyclonal antibody (Upstate Biotechnology, Lake Placid, NY), rabbit anti-Notch or goat anti-Notch polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Jagged-1 was detected with rabbit anti-Jagged polyclonal antibody (Santa Cruz Biotechnology; Upstate Biotechnology). Horseradish peroxidase (HRP)-conjugated purified secondary antibodies (Chemicon, CA; Abcam, UK) and Super Signal (Pierce, IL) chemiluminescence reagents were used to visualize blots.

Immunohistochemical Staining. Formalin fixed paraffin embedded liver tissue was used for immunohistochemical staining of Notch (1:100, H-131; Santa Cruz, CA) recognizing an epitope within the extracellular domain and Jagged-1 (1:200, C-20; Santa Cruz, CA). Primary antibody to Notch was incubated for 18 h; primary antibody to Jagged was incubated also for 18 hours. Sections were blocked with 10% normal goat or donkey serum for 20 minutes and then incubated with primary antibodies. This was followed by washing in Tris-buffered saline and incubation with biotinylated antibodies. Visualization was performed with diaminobenzoic acid (DAB) (Vector Stain Laboratories, Burlingame, CA) peroxidase kit and Elite Vectastain ABC Kit (Vector Stain Laboratories).

Immunofluorescence. Frozen liver tissues were used for immunofluorescence detection of Notch and Jagged-1 in colocalization studies. Cryostat sections (5 μm) were fixed in methanol for 20 minutes (–20&°C) and unspecific binding was blocked using normal goat or donkey serum and washed with bovine serum albumin (BSA) solution (0.5% [wt/vol] BSA, 0.15%[wt/vol] glycine in phosphate buffered saline [PBS]). After 3 washes, primary antibodies were diluted 1:400 and incubated for 2 hours at room temperature.

Sections were washed 3 times with BSA solution, then incubated for 1 hour for each labeled secondary antibody with intermediate washing steps (CY3- 1:2,000; Alexa-488 1:3,000) (Jackson Immunoresearch Laboratories, West Grove, PA; Molecular Probes, Eugene, OR). Nuclei were stained with Hoechst dye (bis-benzimide, Sigma, St. Louis, MO) for 30 s. Visualization was performed using Olympus Provis fluorescence microscope (Melville, NY) and digitized with a Sony 3CCD video camera (Tokyo, Japan). Immunofluorescence localization of NICD was performed with polyclonal antibody C-20 anti-Notch (Santa Cruz, CA). Negative control was performed using blocking peptide sc-6014P (Santa Cruz, CA), which prevented nuclear and membrane staining for Notch (NICD) in frozen liver sections.

References:

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