Supplemental Materials and Methods

In situ hybridization

In situ hybridization analysis of HFE2 and neogenin mRNA in rat liver tissues was performed as previously described (1). Briefly, the digoxigenin-labeled antisense and sense riboprobes for rat HFE2 and neogenin were synthesized by *in vitro* transcription using either MEGAscript SP6 kit or MEGAscript T7 kit (Ambion, Austin, TX). The fragments of rat HFE2 and neogenin cDNA used for riboprobe synthesis were amplified from a rat liver cDNA preparation by PCR using the Expand High Fidelity PCR system (Roche Applied Science), followed by cloning into pGEM-T vector (Promega). The primers used for HFE2 cDNA amplification were 5'- CTATGAAGCCCGGTTTTCCA-3' (forward) and 5'-GGAAAAGGTGCAAGTTCTCCAA-3' (reverse). The primers used for rat neogenin cDNA amplification were 5'-CTCATGCCCAGACCATCAAA-3' (forward) and 5'-CTGGTGGCCTCCTGTACCTC-3' (reverse). The amplicons were confirmed by DNA sequencing.

Immunohistochemistry.

Immunohistochemistry was used to localize the expression of HJV and neogenin proteins in rat liver tissues. Formalin-fixed and paraffin-embedded rat liver sections (5 μm thick) were processed for the analysis of HJV, neogenin and glial fibrillary acidic protein (GFAP, a specific marker for HSC in liver) (2). Briefly, tissue sections were fixed with 1% paraformaldehyde in PBS for 15 min at room temperature, followed by permeabilization with 0.2% Triton X-100 for another 15 min. After 1 hr blocking in PBS with 3% bovine serum albumin (BSA) (blocking buffer), tissue sections were incubated with affinity-purified rabbit anti-HJV antibody (1 μg/ml), rabbit anti-neogenin (1 μg/ml, Santa Cruz Biotechnology), or mouse anti-GFAP-CY3 conjugate (1 μg/ml, Sigma) in blocking buffer at 4°C overnight. For anti-GFAP-CY3 conjugate, tissues were directly mounted with ProLong Antifade (Molecular Probes, OR) and imaged by a Nikon TE200 microscope (Meridian Instrument Company, Inc., Kent, WA) at the magnifications indicated in the text. For anti-HJV and neogenin antibodies, tissue sections were further incubated with Alexa 488-labeled goat anti-rabbit antibody (1:500 dilution; Molecular Probes, OR) for 1 h at room temperature, followed by soaking in 50 mM ammonium acetate buffer (pH 5.0) with 5 mM CuSO4 for 10 min to quench the autofluorescence (3). Rabbit IgG (1 μg/ml) and soluble HJV preabsorbed-rabbit anti-HJV antibody (1 μg/ml) were used as negative controls. The rabbit anti-HJV antibody cross-reacts with rat HJV (4).

References

1. Zhang, A. S., Xiong, S., Tsukamoto, H., and Enns, C. A. (2004) *Blood* **103**, 1509-1514
2. Knittel, T., Kobold, D., Piscaglia, F., Saile, B., Neubauer, K., Mehde, M., Timpl, R., and Ramadori, G. (1999) *Histochem. Cell Biol.* **112**, 387-401
3. Schnell, S. A., Staines, W. A., and Wessendorf, M. W. (1999) *J. Histochem. Cytochem.* **47**, 719-730
4. Zhang, A. S., Anderson, S. A., Meyers, K. R., Hernandez, C., Eisenstein, R. S., and Enns, C. A. (2007) *J. Biol. Chem.* **282**, 12547-12556

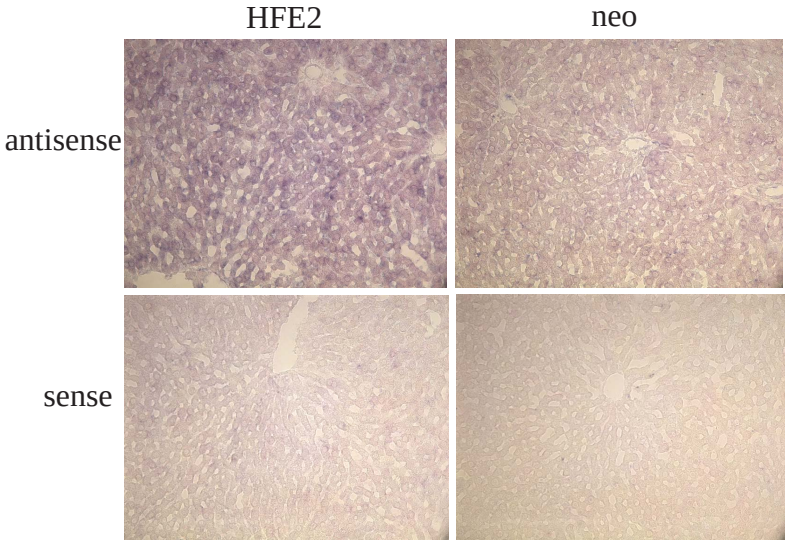
Supplemental Figure 1. *In situ* hybridization and immunohistochemistry analysis of HFE2 and neogenin expression in liver tissue sections. A. *In situ* hybridization analysis of HFE2 and neogenin mRNA localization in liver tissue sections. Digoxigenin-labeled antisense riboprobes for rat HFE2 and neogenin were used to probe HFE2 and neogenin mRNA in rat liver tissue sections, respectively. The corresponding sense riboprobes were used as negative controls. Images were taken under light microscope at 200x magnifications. B. Immunohistochemistry analysis of HJV and neogenin protein localization in rat liver tissue sections. Rabbit antibodies against HJV and neogenin were used to probe the HJV and neogenin proteins in rat liver sections, respectively. Mouse antibody against GFAP, a HSC marker protein, was used to stain the HSC. Anti-HJV antibody was used alone, and images were taken under fluorescent microscopy at 400x magnification. Antibodies against neogenin and GFAP were used for double labeling. Rabbit IgG was used as a negative control. C. Immunohistochemistry analysis of neogenin protein localization by confocal microscopy. The same rat liver tissue sections as described in B were visualized under the confocal microscopy at 1,000x magnification. Rabbit antibody against neogenin was used to probe the neogenin protein (neo), whereas the mouse antibody against GFAP, a HSC marker protein, was used to stain the HSC.

Supplemental Figure 2. Characterization of G99V, D172E and G320V mutant HJV. A. G99V HJV, but not D172E and G320V HJV, binds neogenin. myc-HJV (HJV with a N-terminal *myc* tag), HJV, G320V HJV, G99V HJV, or D172E HJV was co-transfected with neogenin into HEK293 cells. HJV in the cell lysates was immunoprecipitated (IP) with rabbit anti-HJV 18745 antibody and the Pansorbin beads. Immunoprecipiated proteins were separated by SDS-PAGE, followed by immunoblotting (IB) with both anti-HJV and anti-neogenin (neo) antibodies. HEK293 cells transfected with pcDNA3 empty vector (C) were used as a negative control. B. Released G99V HJV has a lower molecular weight. HepG2 cells stably expressing HJV, G320V HJV, D172E HJV, or G99V HJV were subcultured into 12-well plates with complete medium. After 48-hr, medium was changed to MEM/5% FCS (1 ml per well). About 18 hr later, conditioned medium (CM) was collected and cell lysates were prepared. About 120 μl of CM and the total cell lysate were subjected to SDS-PAGE, followed by immunodetection of HJV in CM and neogenin (neo), HJV and β-actin in the lysate (L). HepG2 cells transfected with pcDNA3 empty vector (C) were used as a negative control. C. Flow cytometry analysis of cell surface HJV. HepG2 cells stably expressing HJV, G320V HJV, D172E HJV, or G99V HJV were first detached from flasks with the cell dissociation buffer (Invitrogen). Cells were then incubated with affinity-purified rabbit anti-HJV antibody (4 μg/ml) in Hanks Buffer supplemented with 3% fetal bovine serum for 30 min at 4°C, followed by incubation with phycoerythrin-conjugated goat anti-rabbit IgG (1:500 dilution) (Caltag, Burlingame, CA) in the same buffer for 30 min at 4°C. Flow cytometry analysis was performed on a Becton Dickinson FACSCalibur flow cytometer. Rabbit IgG and control-HepG2 cells were used as negative controls. The levels of cell surface HJV are expressed as arbitrary units. All the experiments were repeated at least three times with consistent results. The qRT-PCR results in C were assessed by one-way ANOVA, and the statistical significant difference relative to HJV-HepG2 cells were determined by Tukey's post test. ** $P<0.01$.

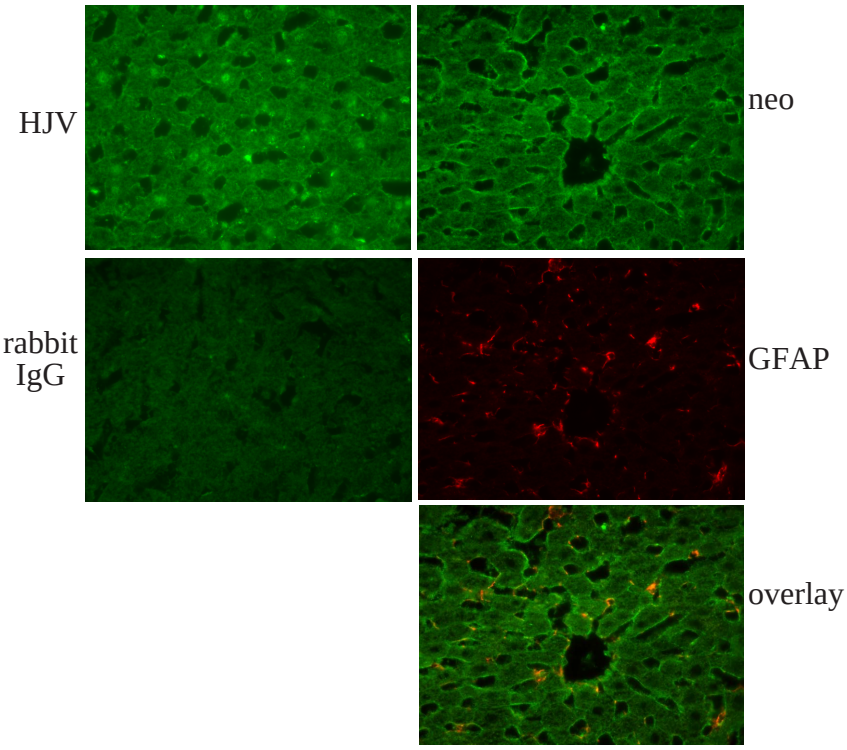
Supplemental Figure 3. Neo FNIII 5-6 disrupts the HJV/neogenin interactions. HEK293 cells stably expressing both HJV and neogenin were incubated in MEM (without met/cys)/2% FCS/^{35}S-(met/cys) (100 μCi/ml, Perkin Elmer) for 4 hours to metabolically label the cellular proteins. After washing the cells with PBS, cell lysates were prepared and incubated in the presence of 20 μM holo-Tf (Tf), 40 nM Neo FNIII 5-6 (N-FNIII), or 1 μM soluble neogenin ectodomain (N-ecto) for 1 hr at 4°C. Afterwards, HJV was immunoprecipitated with the Pansorbin precoated with either pre-immune serum (ctrl), rabbit anti-neogenin (anti-neo) or rabbit anti-HJV 18745 antibody (anti-HJV). Immunoprecipitated proteins were separated by SDS-PAGE. Image was obtained by exposure to X-ray film. During the process of gel drying, the gel was warped which caused the slanted neogenin bands. This experiment was repeated three times with consistent results.

sFigure 1.

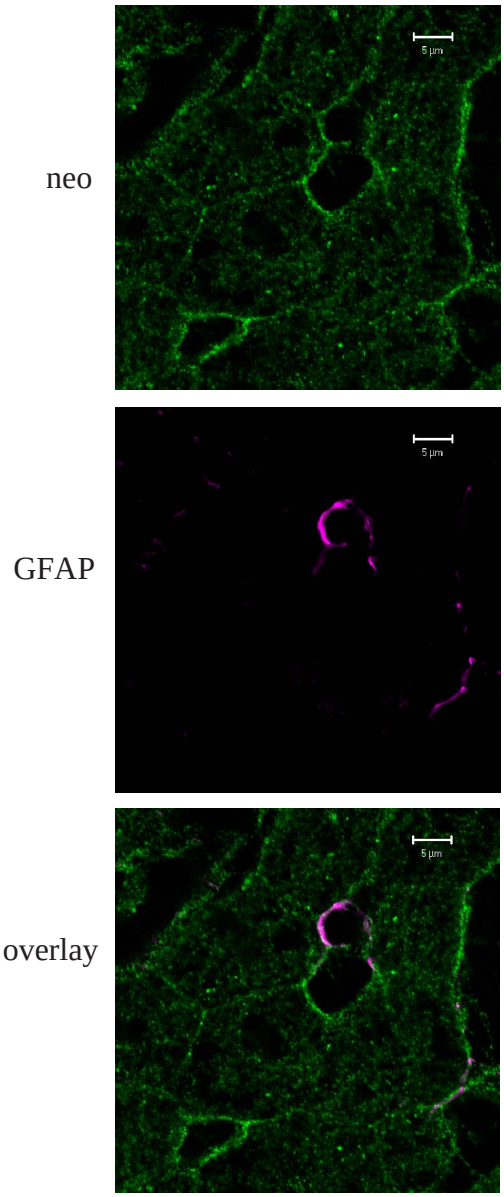
A. *In situ* hybridization



B. Immunohistochemistry

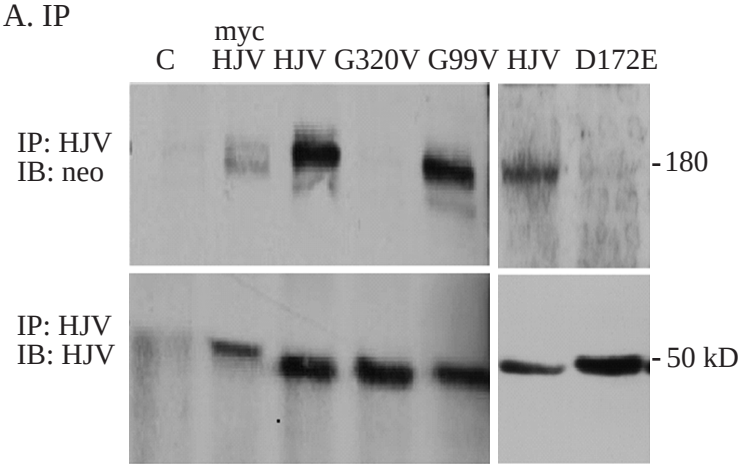


C. Confocal microscopy

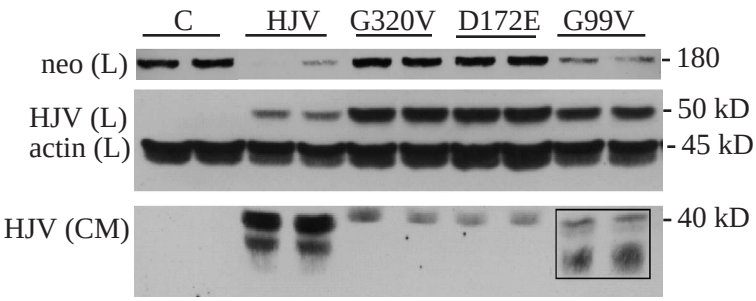


sFigure 2.

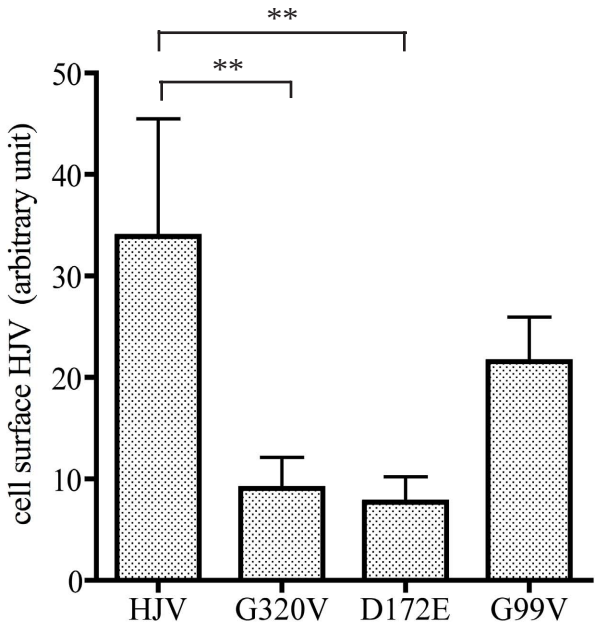
A. IP



B. western blot



C. Flow cytometry



sFigure 3