(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 April 2013 (11.04.2013)

(10) International Publication Number
WO 2013/052155 A1

(51) International Patent Classification:
C07K 16/28 (2006.01) *A61P 1/16* (2006.01)

(21) International Application Number:
PCT/US2012/032621

(22) International Filing Date:
6 April 2012 (06.04.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/543,483 5 October 2011 (05.10.2011) US

(71) Applicant *(for all designated States except AL, AT, BE, BG, CH, CN, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IN, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR, US)*: **GENENTECH, INC.** [US/US]; 1 DNA Way, South San Francisco, California 94080 (US).

(71) Applicant *(for AL, AT, BE, BG, CH, CN, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IN, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR only)*: **F. HOFFMANN-LA ROCHE AG** [CH/CH]; Grenzacherstrasse 124, CH-4070 Basel (CH).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **FRENCH, Dorothy** [US/US]; c/o Genentech, Inc., 1 DNA Way, South San Francisco, California 94080 (US). **SIEBEL, Christian W.** [US/US]; c/o Genentech, Inc., 1 DNA Way, South San Francisco, California 94080 (US). **HUNTZICKER, Erik** [US/US]; c/o Genentech, Inc., 1 DNA Way, South San Francisco, California 94080 (US).

(74) Agents: **VOM WEGE, Julia** et al.; c/o Genentech, Inc., 1 DNA Way, MS49, South San Francisco, California 94080 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: METHODS OF TREATING LIVER CONDITIONS USING NOTCH2 ANTAGONISTS

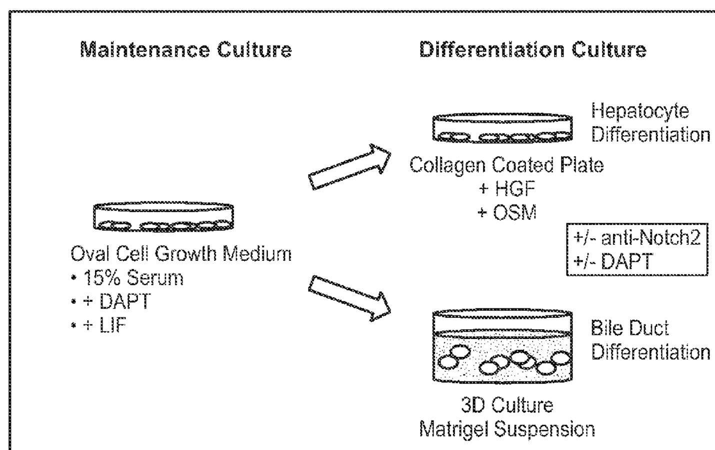


FIG. 7A

(57) Abstract: Methods and compositions for the treatment of liver conditions are provided, such methods and compositions comprising Notch2 antagonists, e.g., anti-Notch2 antibodies. Liver conditions include, but are not limited to, chronic liver disease.

WO 2013/052155 A1

Published:

— *with international search report (Art. 21(3))*

METHODS OF TREATING LIVER CONDITIONS USING NOTCH2 ANTAGONISTS

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of U.S. Provisional Application No. 61/543,483, filed October 5, 2011, the disclosure of which is incorporated herein by reference as if set forth in its entirety.

FIELD OF THE INVENTION

The present invention relates to methods of treating liver conditions using Notch2 10 antagonists. Compositions for the treatment of such conditions are also provided.

BACKGROUND

The Notch receptor family is a class of evolutionarily conserved transmembrane receptors that transmit signals affecting development in organisms as diverse as sea urchins and humans. Notch receptors and their ligands Delta and Serrate (known as Jagged in 15 mammals) are transmembrane proteins with large extracellular domains that contain epidermal growth factor (EGF)-like repeats. The number of Notch paralogues differs between species. For example, there are four Notch receptors in mammals (Notch1-Notch4), two in *Caenorhabditis elegans* (LIN-12 and GLP-1) and one in *Drosophila melanogaster* (Notch). Notch receptors are proteolytically processed during transport to the cell surface by 20 a furin-like protease at a site S1, which is N-terminal to the transmembrane domain, producing an extracellular Notch (ECN) subunit and a Notch transmembrane subunit (NTM). These two subunits remain non-covalently associated and constitute the mature heterodimeric cell-surface receptor.

Notch2 ECN subunits contain 36 N-terminal EGF-like repeats followed by three 25 tandemly repeated Lin 12/Notch Repeat (LNR) modules that precede the S1 site. Each LNR module contains three disulfide bonds and a group of conserved acidic and polar residues predicted to coordinate a calcium ion. Within the EGF repeat region lie binding sites for the activating ligands.

The Notch2 NTM comprises an extracellular region (which harbors the S2 cleavage 30 site), a transmembrane segment (which harbors the S3 cleavage site), and a large intracellular

portion that includes a RAM23 domain, six ankyrin repeats, a t
carboxy-teminal PEST sequence. Stable association of the ECN and NTM subunits is dependent on a heterodimerization domain (HD) comprising the carboxy-terminal end of the ECN (termed HD-N) and the extracellular amino-terminal end of NTM (termed HD-C). Before ligand-induced activation, Notch is maintained in a resting conformation by a negative regulatory region (NRR), which comprises the three LNRs and the HD domain. The crystal structure of the Notch2 NRR is reported in Gordon *et al.*, (2007) *Nature Structural & Molecular Biology* 14:295-300, 2007.

Binding of a Notch ligand to the ECN subunit initiates two successive proteolytic cleavages that occur through regulated intramembrane proteolysis. The first cleavage by a metalloprotease (ADAM17) at site S2 renders the Notch transmembrane subunit susceptible to a second cleavage at site S3 close to the inner leaflet of the plasma membrane. Site S3 cleavage, which is catalyzed by a multiprotein complex containing presenilin and nicastrin and promoting γ-secretase activity, liberates the intracellular portion of the Notch transmembrane subunit, allowing it to translocate to the nucleus and activate transcription of target genes. (For review of the proteolytic cleavage of Notch, see, e.g., Sisodia *et al.*, *Nat. Rev. Neurosci.* 3:281-290, 2002.)

Five Notch ligands of the Jagged and Delta-like classes have been identified in humans (Jagged1 (also termed Serrate1), Jagged2 (also termed Serrate2), Delta-like1 (also termed DLL1), Delta-like3 (also termed DLL3), and Delta-like4 (also termed DLL4)). Each of the ligands is a single-pass transmembrane protein with a conserved N-terminal Delta, Serrate, LAG-2 (DSL) motif essential for binding Notch. A series of EGF-like modules C-terminal to the DSL motif precede the membrane-spanning segment. Unlike the Notch receptors, the ligands have short cytoplasmic tails of 70-215 amino acids at the C-terminus. In addition, other types of ligands have been reported (e.g., DNER, NB3, and F3/Contactin). (For review of Notch ligands and ligand-mediated Notch activation, see, e.g., D'Souza *et al.*, *Oncogene* 27:5148-5167, 2008.)

The Notch pathway functions during diverse developmental and physiological processes including those affecting neurogenesis in flies and vertebrates. In general, Notch signaling is involved in lateral inhibition, lineage decisions, and the establishment of boundaries between groups of cells. (See, e.g., Bray, *Mol. Cell Biol.* 7:678-679, 2006.) A variety of human diseases, including cancers and neurodegenerative disorders have been

shown to result from mutations in genes encoding Notch recept
Nam *et al.*, *Curr. Opin. Chem. Biol.* 6:501-509, 2002.)

Certain anti-Notch2 antagonist antibodies having therapeutic efficacy have been described. (See U.S. Patent Application Publication No. US 2009/0081238 A1, expressly
5 incorporated by reference in its entirety herein.) For example, such antibodies bind to the negative regulatory region (NRR) of Notch2, block Notch2 signaling, and inhibit the growth of melanoma cell lines, diffuse large B-cell lymphoma (DLBCL) cell lines, and marginal zone B cells. Certain anti-Notch2 antibodies described therein bind to the LNR-A domain (the first of the three LIN12/Notch Repeats) and the HD-C domain of Notch2 NRR.

10 Adult liver has the capacity to regenerate after injury. It has been speculated that biliary-hepatocyte progenitor cells (oval cells) in or near intrahepatic bile ducts can differentiate into adult hepatocytes (Brues and Marble, J. Exp. Med., 65(1):15 (1937); Zajicek *et al.*, Liver, 5(6):293 (1985)), which subsequently mature as they move toward the central vein and eventually undergo apoptosis and elimination (Benedetti *et al.*, J. Hepatol., 7(3):319
15 (1988)). Recent lineage-tracing studies have supported a role of progenitor cells in liver homeostasis and repair, but the signals that govern precursor differentiation into hepatocytes are poorly understood. While Notch signaling is known to be critical for the proper formation of the intrahepatic biliary system during development (Lozier *et al.*, PLoS One 3(3):e1851 (2008); McCright *et al.*, Development 129(4):1075 (2002)), it was not known what role, if
20 any, Notch signaling plays in adult hepatocyte formation and in adult hepatobiliary disease.

Chronic liver disease is marked by gradual destruction of liver tissue, especially of hepatocytes and the functional lobular unit, leading to fibrosis (replacement of liver tissue with scar tissue) and cirrhosis (fibrosis with ineffective nodular regeneration and associated loss of liver function). Moreover, chronic liver disease often includes pathological biliary
25 hyperplasia and may increase the risk of liver cancer.

There is a need in the art for further therapeutic methods of treating liver conditions. The invention described herein meets the above-described needs and provides other benefits.

SUMMARY

The present invention relates to the treatment of liver conditions using Notch2
30 antagonists. The present invention is based, in part, on the observation that anti-Notch2 NRR antibodies (a) improve liver histologic appearance and hepatocyte function in an acute liver

damage model *in vivo* and (b) reduce biliary damage and impro
chronic liver damage model *in vivo*.

In one aspect, a method of treating a liver condition characterized by liver damage is provided, the method comprising administering to a patient having such condition an effective amount of a Notch2-specific antagonist. In certain embodiments, the liver condition is chronic liver disease. In certain embodiments, the liver condition is liver fibrosis.

In any of the above embodiments, the Notch2-specific antagonist may be an anti-Notch2 antagonist antibody. In certain embodiments, the anti-Notch2 antagonist antibody is an anti-Notch2 NRR antibody. In one such embodiment, the anti-Notch2 NRR antibody binds to the LNR-A and HD-C domains of Notch2 NRR. In another such embodiment, the anti-Notch2 NRR antibody is Antibody D, Antibody D-1, Antibody D-2, or Antibody D-3. In another such embodiment, the anti-Notch2 NRR antibody comprises the heavy and light chain variable region CDRs of Antibody D, Antibody D-1, Antibody D-2, or Antibody D-3. In certain embodiments, the anti-Notch2 antagonist antibody is an anti-Notch2 antibody that binds to one or more EGF-like repeats of Notch2.

The above and further aspects and embodiments of the invention are provided herein.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-G show transcriptional profiling of hepatic progenitor cells and Identification of active Notch signaling in hepatic progenitors *in vivo*.

Figures 2A-O show that Notch signaling inhibition promotes hepatocyte differentiation of hepatic progenitors *in vitro*.

Figures 3A-J show that inhibition of Notch2 signaling *in vivo* promotes hepatocyte differentiation and improved liver function in chronic and acute liver damage models.

Figures 4A-E illustrate a liver progenitor cell isolation strategy.

Figures 5A-H show an analysis of oval cell-specific gene expression signature.

Figures 6A-E show a validation of oval cell gene signature and expression pattern of putative hepatic stem cell markers.

Figures 7A-B show a strategy for *in vitro* differentiation.

Figures 8A-F show the efficacy of anti-Notch2 antibody treatment *in vivo* and its effect on liver growth and proliferation following partial hepatectomy.

Figures 9A-H show the effect of anti-Notch2 antibody t
hepatobiliary function markers following partial hepatectomy.

Figures 10A-F show the effect of anti-Notch2 antibody treatment on hepatobiliary and Notch signaling gene expression following partial hepatectomy.

Figures 11A-I show serum hepatobiliary function markers following 4 weeks of antibody administration in normal and DDC-fed mice.

Figure 12 shows the H1, H2, and H3 heavy chain hypervariable region (HVR) sequences of anti-Notch2 NRR monoclonal antibodies designated Antibody D, Antibody D-1, Antibody D-2, and Antibody D-3. Amino acid positions are numbered according to the Kabat numbering system as described below.

Figure 13 shows the L1, L2, and L3 light chain HVR sequences of anti-Notch2 NRR monoclonal antibodies designated Antibody D, Antibody D-1, Antibody D-2, and Antibody D-3. Amino acid positions are numbered according to the Kabat numbering system as described below.

Figure 14 shows an alignment of the heavy chain variable region sequences of Antibody D, Antibody D-1, Antibody D-2, and Antibody D-3. HVRs are enclosed in boxes.

Figure 15 shows an alignment of the light chain variable region sequences of Antibody D, Antibody D-1, Antibody D-2, and Antibody D-3. HVRs are enclosed in boxes.

Figures 16A-B show exemplary acceptor human variable heavy (VH) consensus framework sequences for use in practicing the instant invention. Sequence identifiers are as follows:

- human VH subgroup I consensus framework “A” minus Kabat CDRs (SEQ ID NOs:32, 33, 34, 35).
- human VH subgroup I consensus frameworks “B,” “C,” and “D” minus extended hypervariable regions (SEQ ID NOs:36, 37, 34, 35; SEQ ID NOs:36, 37, 38, 35; and SEQ ID NOs:36, 37, 39, 35).
- human VH subgroup II consensus framework “A” minus Kabat CDRs (SEQ ID NOs:40, 41, 42, 35).
- human VH subgroup II consensus frameworks “B,” “C,” and “D” minus extended hypervariable regions (SEQ ID NOs:43, 44, 42, 35; SEQ ID NOs:43, 44, 45, 35; and SEQ ID NOs:43, 44, 46, and 35).
- human VH subgroup III consensus framework “A” minus Kabat CDRs (SEQ ID NOs:47, 48, 49, 35).

- human VH subgroup III consensus frameworks "B," hypervariable regions (SEQ ID NOs:50, 51, 49, 35; SEQ ID NOs:50, 51, 52, 35; and SEQ ID NOs:50, 51, 53, 35).
- human VH acceptor framework "A" minus Kabat CDRs (SEQ ID NOs:54, 48, 55, 35).
- human VH acceptor frameworks "B" and "C" minus extended hypervariable regions (SEQ ID NOs:50, 51, 55, 35; and SEQ ID NOs:50, 51, 56, 35).
- human VH acceptor 2 framework "A" minus Kabat CDRs (SEQ ID NOs:54, 48, 57, 35).
- human VH acceptor 2 framework "B," "C," and "D" minus extended hypervariable regions (SEQ ID NOs:50, 51, 57, 35; SEQ ID NOs:50, 51, 58, 35; and SEQ ID NOs:50, 51, 59, 35).

Figure 17 shows exemplary acceptor human variable light (VL) consensus framework sequences for use in practicing the instant invention. Sequence identifiers are as follows:

- human VL kappa subgroup I consensus framework (κv1): SEQ ID NOs:60, 61, 62, 63
- human VL kappa subgroup II consensus framework (κv2): SEQ ID NOs:64, 65, 66, 63
- human VL kappa subgroup III consensus framework (κv3): SEQ ID NOs:67, 68, 69, 63
- human VL kappa subgroup IV consensus framework (κv4): SEQ ID NOs:70, 71, 72, 63

Figure 18 shows framework sequences of huMAb4D5-8 light and heavy chains. Numbers in superscript/bold indicate amino acid positions according to Kabat.

Figure 19 shows framework sequences of huMAb4D5-8 light and heavy chains with the indicated modifications. Numbers in superscript/bold indicate amino acid positions according to Kabat.

DETAILED DESCRIPTION OF EMBODIMENTS

I. DEFINITIONS

For purposes of interpreting this specification, the following definitions will apply and whenever appropriate, terms used in the singular will also include the plural and vice versa. In

the event that any definition set forth below conflicts with any (
by reference, the definition set forth below shall control.

The term "Notch," as used herein, refers, unless specifically or contextually indicated otherwise, to any native or variant (whether native or synthetic) Notch polypeptide (Notch1-4). The term "native sequence" specifically encompasses naturally occurring truncated forms (e.g., an extracellular domain sequence or a transmembrane subunit sequence), naturally occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants. The term "wild-type Notch" generally refers to a polypeptide comprising an amino acid sequence of a naturally occurring, non-mutated Notch protein. The term "wild-type Notch sequence" generally refers to an amino acid sequence found in a naturally occurring, non-mutated Notch.

The term "Notch2," as used herein, refers, unless specifically or contextually indicated otherwise, to any native or variant (whether native or synthetic) Notch2 polypeptide. The term "native sequence" specifically encompasses naturally occurring truncated forms (e.g., an extracellular domain sequence or a transmembrane subunit sequence), naturally occurring variant forms (e.g., alternatively spliced forms) and naturally occurring allelic variants. The term "wild-type Notch2" generally refers to a polypeptide comprising an amino acid sequence of a naturally occurring, non-mutated Notch2 protein. The term "wild type Notch2 sequence" generally refers to an amino acid sequence found in a naturally occurring, non-mutated Notch2.

The term "Notch2 ligand," as used herein, refers, unless specifically or contextually indicated otherwise, to any native or variant (whether native or synthetic) Notch2 ligand (for example, Jagged1, Jagged2, Delta-like1, Delta-like3, and/or Delta-like4) polypeptide. The term "native sequence" specifically encompasses naturally occurring truncated forms (e.g., an extracellular domain sequence or a transmembrane subunit sequence), naturally occurring variant forms (e.g., alternatively spliced forms) and naturally occurring allelic variants. The term "wild-type Notch2 ligand" generally refers to a polypeptide comprising an amino acid sequence of a naturally occurring, non-mutated Notch2 ligand. The term "wild type Notch2 ligand sequence" generally refers to an amino acid sequence found in a naturally occurring, non-mutated Notch2 ligand.

The term "Notch2 NRR," as used herein, refers, unless specifically or contextually indicated otherwise, to any native or variant (whether native or synthetic) polypeptide region of Notch2 consisting of the 3 LNR modules and the amino acid sequences extending from the

carboxy-terminus of the LNR modules to the transmembrane d(including the HD domain (HD-N and HD-C). An exemplary Notch2 NRR consists of the region from about amino acid 1422-1677 of human Notch2 (SEQ ID NO:73). An exemplary human Notch2 NRR is also shown in SEQ ID NO:74. The term "native sequence Notch2 NRR" specifically encompasses naturally occurring truncated forms, naturally occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of a Notch2 NRR. The term "wild-type Notch2 NRR" generally refers to a naturally occurring, non-mutated Notch2 NRR. In some embodiments, a Notch2 NRR is contained in a Notch2, such as, for example, a Notch2 processed at the S1, S2 and/or S3 site(s), or an unprocessed Notch2. In some embodiments, a Notch2 NRR contains two or more non-covalently linked fragments of a Notch2 NRR amino acid sequence, e.g., a fragment containing amino acids 1422 to 1608 of SEQ ID NO:73 non-covalently linked to a fragment containing amino acids 1609 to 1677 of SEQ ID NO:73.

The term "increased Notch2 signaling," as used herein, refers to an increase in Notch2 signaling that is significantly above the level of Notch2 signaling observed in a control under substantially identical conditions. In certain embodiments, the increase in Notch2 signaling is at least two fold, three fold, four fold, five fold, or ten fold above the level observed in the control.

The term "decreased Notch1 signaling," as used herein, refers to a decrease in Notch2 signaling that is significantly below the level of Notch2 signaling observed in a control under substantially identical conditions. In certain embodiments, the decrease in Notch2 signaling is at least two fold, three fold, four fold, five fold, or ten fold below the level observed in the control.

In certain embodiments, Notch2 signaling (i.e., increased or decreased Notch2 signaling) is assessed using a suitable reporter assay, *e.g*, as described in U.S. Patent Application Publication No. US 2010/0080808 A1.

The term "anti-Notch2 antibody" or "an antibody that binds to Notch2" refers to an antibody that is capable of binding Notch2 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting Notch2. Preferably, the extent of binding of an anti-Notch2 antibody to an unrelated, non-Notch protein is less than about 10% of the binding of the antibody to Notch2 as measured, e.g., by a radioimmunoassay (RIA). In certain embodiments, an antibody that binds to Notch2 has a dissociation constant (Kd) of ≤ 1μM, ≤ 0.5 μM, ≤ 100 nM, ≤ 50 nM, ≤ 10 nM, ≤ 5 nM, ≤ 1 nM, ≤ 0.5 nM, or ≤ 0.1 nM. In

certain embodiments, an anti-Notch2 antibody binds to an epitc
among Notch2 from different species, e.g., rodents (mice, rats) and primates.

The term “anti-Notch2 NRR antibody” or “an antibody that binds to Notch2 NRR” refers to an antibody that is capable of binding Notch2 NRR with sufficient affinity such that
5 the antibody is useful as a diagnostic and/or therapeutic agent in targeting Notch2. Preferably, the extent of binding of an anti-Notch2 NRR antibody to an unrelated, non-Notch protein is less than about 10% of the binding of the antibody to Notch2 NRR as measured, e.g., by a radioimmunoassay (RIA). In certain embodiments, an antibody that binds to Notch2 NRR has a dissociation constant (Kd) of $\leq 1\mu M$, $\leq 0.5\ \mu M$, ≤ 100 nM, ≤ 50 nM, ≤ 10
10 nM, ≤ 5 nM, ≤ 1 nM, ≤ 0.5 nM, or ≤ 0.1 nM. In certain embodiments, an anti-Notch2 NRR antibody binds to an epitope of Notch that is conserved among Notch from different species, e.g., rodents (mice, rats) and primates.

The term “Notch2-specific antagonist” refers to an agent that effects decreased Notch2 signaling, as defined above, and does not significantly affect signaling by another
15 Notch receptor (Notch1, 3, or 4 in mammals).

An “anti-Notch2 antagonist antibody” is an anti-Notch2 antibody (including an anti-Notch2 NRR antibody) that effects decreased Notch2 signaling, as defined above.

The term “antagonist” refers to an agent that significantly inhibits (either partially or completely) the biological activity of a target molecule.

20 The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (*e.g.* bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

An “isolated” antibody is one which has been identified and separated and/or
25 recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In some embodiments, an antibody is purified (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method,
30 and in some embodiments, to greater than 99% by weight; (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of, for example, a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or silver stain. Isolated antibody

includes the antibody *in situ* within recombinant cells since at l
antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as "VH." The variable domain of the light chain may be referred to as "VL." These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions (HVRs) both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a beta-sheet configuration, connected by three HVRs, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The HVRs in each chain are held together in close proximity by the FR regions and, with the HVRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, Fifth Edition, National Institute of Health, Bethesda, MD (1991)). The constant domains are not

involved directly in the binding of an antibody to an antigen, bι
functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the
5 amino acid sequences of their constant domains.

Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2 . The
10 heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas *et al. Cellular and Mol. Immunology*, 4th ed. (W.B. Saunders, Co., 2000). An antibody may be part of a larger fusion molecule, formed by
15 covalent or non-covalent association of the antibody with one or more other proteins or peptides.

The terms "full length antibody," "intact antibody" and "whole antibody" are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains
20 that contain an Fc region.

A "naked antibody" for the purposes herein is an antibody that is not conjugated to a cytotoxic moiety or radiolabel.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen binding region thereof. Examples of antibody fragments include Fab, Fab',
25 $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$
30 fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv)

species, one heavy- and one light-chain variable domain can be
peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three HVRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six HVRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three HVRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

"Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv, see, *e.g.*, Pluckthün, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., (Springer-Verlag, New York, 1994), pp. 269-315.

The term "diabodies" refers to antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 404,097; WO 1993/01161; Hudson *et al.*, *Nat. Med.* 9:129-134 (2003); and Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson *et al.*, *Nat. Med.* 9:129-134 (2003).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies

comprising the population are identical except for possible mut
occurring mutations, that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. In certain embodiments, such a monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, or recombinant DNA clones. It should be understood that a selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity *in vivo*, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins.

The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler and Milstein, *Nature*, 256:495-97 (1975); Hongo *et al.*, *Hybridoma*, 14 (3): 253-260 (1995), Harlow *et al.*, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567), phage-display technologies (see, e.g., Clackson *et al.*, *Nature*, 352: 624-628 (1991); Marks *et al.*, *J. Mol. Biol.* 222: 581-597 (1992); Sidhu *et al.*, *J. Mol. Biol.* 338(2): 299-310 (2004); Lee *et al.*, *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee *et al.*, *J. Immunol. Methods* 284(1-2): 119-132(2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences

(see, e.g., WO 1998/24893; WO 1996/34096; WO 1996/33735;
et al., *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits *et al.*, *Nature* 362: 255-258 (1993); Bruggemann *et al.*, *Year in Immunol.* 7:33 (1993); U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks *et al.*, *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild *et al.*, *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (see, *e.g.*, U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)). Chimeric antibodies include PRIMATIZED® antibodies wherein the antigen-binding region of the antibody is derived from an antibody produced by, e.g., immunizing macaque monkeys with the antigen of interest.

"Humanized" forms of non-human (*e.g.,* murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. In one embodiment, a humanized antibody is a human immunoglobulin (recipient antibody) in which residues from a HVR of the recipient are replaced by residues from a HVR of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and/or capacity. In some instances, FR residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications may be made to further refine antibody performance. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin, and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see, *e.g.*, Jones *et al., Nature* 321:522-525 (1986); Riechmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*

2:593-596 (1992). See also, *e.g.*, Vaswani and Hamilton, *Ann.*
1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5:428-433 (1994); and U.S. Pat. Nos. 6,982,321 and 7,087,409.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art, including phage-display libraries. Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks *et al.*, *J. Mol. Biol.*, 222:581 (1991). Also available for the preparation of human monoclonal antibodies are methods described in Cole *et al.*, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boerner *et al.*, *J. Immunol.*, 147(1):86-95 (1991). See also van Dijk and van de Winkel, *Curr. Opin. Pharmacol.,* 5: 368-74 (2001). Human antibodies can be prepared by administering the antigen to a transgenic animal that has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled, *e.g.*, immunized xenomice (see, *e.g.*, U.S. Pat. Nos. 6,075,181 and 6,150,584 regarding XENOMOUSE™ technology). See also, for example, Li *et al., Proc. Natl. Acad. Sci. USA*, 103:3557-3562 (2006) regarding human antibodies generated via a human B-cell hybridoma technology.

The term "hypervariable region," "HVR," or "HV," when used herein refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). In native antibodies, H3 and L3 display the most diversity of the six HVRs, and H3 in particular is believed to play a unique role in conferring fine specificity to antibodies. See, *e.g.*, Xu *et al.*, *Immunity* 13:37-45 (2000); Johnson and Wu, in *Methods in Molecular Biology* 248:1-25 (Lo, ed., Human Press, Totowa, NJ, 2003). Indeed, naturally occurring camelid antibodies consisting of a heavy chain only are functional and stable in the absence of light chain. See, *e.g.*, Hamers-Casterman *et al., Nature* 363:446-448 (1993); Sheriff *et al.*, *Nature Struct. Biol.* 3:733-736 (1996).

A number of HVR delineations are in use and are encompassed herein. The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (Kabat *et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). Chothia refers

instead to the location of the structural loops (Chothia and Lesk (1987)). The AbM HVRs represent a compromise between the Kabat HVRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The "contact" HVRs are based on an analysis of the available complex crystal structures. The residues from each of these HVRs are noted below.

Loop	Kabat	AbM	Chothia	Contact
L1	L24-L34	L24-L34	L26-L32	L30-L36
L2	L50-L56	L50-L56	L50-L52	L46-L55
L3	L89-L97	L89-L97	L91-L96	L89-L96
H1	H31-H35B	H26-H35B	H26-H32	H30-H35B
	(Kabat Numbering)			
H1	H31-H35	H26-H35	H26-H32	H30-H35
	(Chothia Numbering)			
H2	H50-H65	H50-H58	H53-H55	H47-H58
H3	H95-H102	H95-H102	H96-H101	H93-H101

HVRs may comprise "extended HVRs" as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2) and 89-97 or 89-96 (L3) in the VL and 26-35 (H1), 50-65 or 49-65 (H2) and 93-102, 94-102, or 95-102 (H3) in the VH. The variable domain residues are numbered according to Kabat *et al*., supra, for each of these definitions.

"Framework" or "FR" residues are those variable domain residues other than the HVR residues as herein defined.

The term "variable domain residue numbering as in Kabat" or "amino acid position numbering as in Kabat," and variations thereof, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat *et al*., *supra*. Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or HVR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g. residues 82a, 82b, and 82c, etc. according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The Kabat numbering system is generally used when ref
variable domain (approximately residues 1-107 of the light chain and residues 1-113 of the heavy chain) (*e.g.*, Kabat *et al.*, *supra*). The "EU numbering system" or "EU index" is generally used when referring to a residue in an immunoglobulin heavy chain constant region (e.g., the EU index reported in Kabat *et al., supra*). The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody. Unless stated otherwise herein, references to residue numbers in the variable domain of antibodies means residue numbering by the Kabat numbering system. Unless stated otherwise herein, references to residue numbers in the constant domain of antibodies means residue numbering by the EU numbering system (e.g., see United States Patent Application Publication US 2008/0181888 A1, Figures for EU numbering).

An "affinity matured" antibody is one with one or more alterations in one or more HVRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). In one embodiment, an affinity matured antibody has nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies may be produced using certain procedures known in the art. For example, Marks *et al. Bio/Technology* 10:779-783 (1992) describe affinity maturation by VH and VL domain shuffling. Random mutagenesis of HVR and/or framework residues is described by, for example, in Barbas *et al. Proc Nat. Acad. Sci. USA* 91:3809-3813 (1994); Schier *et al. Gene* 169:147-155 (1995); Yelton *et al. J. Immunol.* 155:1994-2004 (1995); Jackson *et al., J. Immunol.* 154(7):3310-9 (1995); and Hawkins *et al, J. Mol. Biol.* 226:889-896 (1992).

Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (*e.g.* B cell receptor); and B cell activation.

"Binding affinity" generally refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (*e.g.*, an antibody) and its binding partner (*e.g.*, an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (*e.g.*, antibody and antigen). The affinity of a molecule X for its partner Y can generally

be represented by the dissociation constant (Kd). Affinity can t
methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of
5 measuring binding affinity are known in the art, any of which can be used for purposes of the present invention. Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

In one embodiment, the "Kd" or "Kd value" according to this invention is measured by a radiolabeled antigen binding assay (RIA) performed with the Fab version of an antibody
10 of interest and its antigen as described by the following assay. Solution binding affinity of Fabs for antigen is measured by equilibrating Fab with a minimal concentration of (^{125}I)-labeled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate (see, *e.g.*, Chen, *et al.*, *J. Mol. Biol.* 293:865-881(1999)). To establish conditions for the assay, MICROTITER® multi-well plates (Thermo
15 Scientific) are coated overnight with 5 μg/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-adsorbent plate (Nunc #269620), 100 pM or 26 pM [^{125}I]-antigen are mixed with serial dilutions of a Fab of interest (*e.g.*, consistent with assessment of the anti-VEGF antibody,
20 Fab-12, in Presta *et al.*, *Cancer Res.* 57:4593-4599 (1997)). The Fab of interest is then incubated overnight; however, the incubation may continue for a longer period (e.g., about 65 hours) to ensure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature (e.g., for one hour). The solution is then removed and the plate washed eight times with 0.1% TWEEN-20™ in PBS. When the plates
25 have dried, 150 μl/well of scintillant (MICROSCINT-20 ™; Packard) is added, and the plates are counted on a TOPCOUNT ™ gamma counter (Packard) for ten minutes. Concentrations of each Fab that give less than or equal to 20% of maximal binding are chosen for use in competitive binding assays.

According to another embodiment, the Kd or Kd value is measured by using surface
30 plasmon resonance assays using a BIACORE®-2000 or a BIACORE ®-3000 (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIACORE, Inc.) are activated with *N*-ethyl-*N'*- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-

hydroxysuccinimide (NHS) according to the supplier's instructi
10 mM sodium acetate, pH 4.8, to 5 μg/ml (~0.2 μM) before injection at a flow rate of 5 μl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics
5 measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% TWEEN-20™ surfactant (PBST) at 25°C at a flow rate of approximately 25 μl/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIACORE® Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant
10 (Kd) is calculated as the ratio k_{off}/k_{on}. See, *e.g.*, Chen *et al.*, *J. Mol. Biol.* 293:865-881 (1999). If the on-rate exceeds $10^6\ M^{-1}\ s^{-1}$ by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH
15 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophometer (Aviv Instruments) or a 8000-series SLM-AMINCO™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

An "on-rate," "rate of association," "association rate," or "k_{on}" according to this invention can also be determined as described above using a BIACORE®-2000 or a
20 BIACORE®-3000 system (BIAcore, Inc., Piscataway, NJ).

The term "substantially similar" or "substantially the same," as used herein, denotes a sufficiently high degree of similarity between two numeric values (for example, one associated with an antibody of the invention and the other associated with a reference/comparator antibody), such that one of skill in the art would consider the difference
25 between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (*e.g.*, Kd values). The difference between said two values is, for example, less than about 50%, less than about 40%, less than about 30%, less than about 20%, and/or less than about 10% as a function of the reference/comparator value.

30 The phrase "substantially reduced," or "substantially different," as used herein, denotes a sufficiently high degree of difference between two numeric values (generally one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of

statistical significance within the context of the biological chara

values (e.g., Kd values). The difference between said two values is, for example, greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, and/or greater than about 50% as a function of the value for the reference/comparator molecule.

5 An "acceptor human framework" or a "human acceptor framework" for the purposes herein is a framework comprising the amino acid sequence of a VL or VH framework derived from a human immunoglobulin framework or a human consensus framework. An acceptor human framework "derived from" a human immunoglobulin framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain 10 pre-existing amino acid sequence changes. In some embodiments, the number of pre-existing amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less. Where pre-existing amino acid changes are present in a VH, preferably those changes occur at only three, two, or one of positions 71H, 73H and 78H; for instance, the amino acid residues at those positions may be 71A, 73T and/or 78A. In one embodiment, 15 the VL acceptor human framework is identical in sequence to the VL human immunoglobulin framework sequence or human consensus framework sequence.

A "human consensus framework" is a framework which represents the most commonly occurring amino acid residues in a selection of human immunoglobulin VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH 20 sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat et al., *supra*. In one embodiment, for the VL, the subgroup is subgroup kappa I as in Kabat et al., *supra*. In one embodiment, for the VH, the subgroup is subgroup III as in Kabat et al., *supra*.

A "VH subgroup III consensus framework" comprises the consensus sequence 25 obtained from the amino acid sequences in variable heavy subgroup III of Kabat et al., *supra*. In one embodiment, a human acceptor framework is derived from the VH subgroup III consensus framework and comprises an amino acid sequence comprising at least a portion or all of each of the following sequences: (SEQ ID NO:50)-H1-(SEQ ID NO:51)-H2-(SEQ ID NO:57 or 59)-H3-(SEQ ID NO: 35). In some embodiments, the last residue (S11) of SEQ ID 30 NO:35 is substituted with an alanine.

A "VL subgroup I consensus framework" comprises the consensus sequence obtained from the amino acid sequences in variable light kappa subgroup I of Kabat et al., *supra*. In one embodiment, the VH subgroup I consensus framework amino acid sequence comprises at

least a portion or all of each of the following sequences: (SEQ I
NO:61)-L2-(SEQ ID NO:62)-L3-(SEQ ID NO:63).

A “disorder” is any condition or disease that would benefit from treatment with a composition or method of the invention. This includes chronic and acute disorders including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include conditions such as cancer.

The terms “cell proliferative disorder” and “proliferative disorder” refer to disorders that are associated with some degree of abnormal cell proliferation. In one embodiment, the cell proliferative disorder is cancer.

“Tumor,” as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. The terms “cancer,” “cancerous,” “cell proliferative disorder,” “proliferative disorder,” and “tumor” are not mutually exclusive as referred to herein.

A cancer that “responds” to a therapeutic agent is one that shows a significant decrease in cancer or tumor progression, including but not limited to, (1) inhibition, to some extent, of tumor growth, including slowing down and complete growth arrest; (2) reduction in the number of cancer or tumor cells; (3) reduction in tumor size; (4) inhibition (i.e., reduction, slowing down or complete stopping) of cancer cell infiltration into adjacent peripheral organs and/or tissues; and/or (5) inhibition (i.e. reduction, slowing down or complete stopping) of metastasis.

As used herein, “treatment” (and variations such as “treat” or “treating”) refers to clinical intervention in an attempt to alter the natural course of the individual or cell being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, Notch2 antagonists of the invention are used to delay development of a disease or disorder or to slow the progression of a disease or disorder.

An “individual,” “subject,” or “patient” is a vertebrate. In certain embodiments, the vertebrate is a mammal. Mammals include, but are not limited to, farm animals (such as cows), sport animals, pets (such as cats, dogs, and horses), primates, mice and rats. In certain embodiments, a mammal is a human.

The term "pharmaceutical formulation" refers to a prepa
as to permit the biological activity of the active ingredient to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered. Such formulations may be sterile.

5 An "effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

The term "progenitor," "hepatic progenitor," "liver progenitor" or "oval cell" refers to small epithelial cells that can differentiate into both hepatocytes and intra-hepatic bile duct cells.

10 **II. EMBODIMENTS OF THE INVENTION**

The present invention relates to the treatment of liver conditions using Notch2 antagonists. The present invention is based, in part, on the observation that anti-Notch2 NRR antibodies (a) improve liver appearance and hepatocyte function in an acute liver damage model *in vivo* and (b) reduce biliary damage and improve hepatocyte function in a chronic 15 liver damage model *in vivo*. Without being bound by any particular theory or operation, the Notch2 antagonist might improve liver conditions by promoting hepatocyte differentiation and/or by decreasing aberrant bile duct proliferation.

In various aspects of the invention, a method of treating a liver condition characterized by liver damage is provided, the method comprising administering to a patient 20 having such condition an effective amount of a Notch2-specific antagonist. In certain embodiments, the liver condition is chronic liver disease, including but not limited to fibrosis, cirrhosis, viral hepatitis (e.g., hepatitis A, B, C, D, E, or G), autoimmune liver diseases (e.g., autoimmune hepatitis, primary biliary cirrhosis, or primary sclerosing cholangitis), genetic liver diseases (e.g., alpha-1 antitrypsin deficiency, Crigler-Najjar syndrome, familial 25 amyloidosis, Gilbert's syndrome, Dubin-Johnson syndrome, hereditary hemchromatosis, primary oxalosis, or Wilson's disease), alcoholic hepatitis or nonalcoholic fatty liver disease. In certain embodiments, the liver condition is an acute liver condition, such as acute liver failure, acute liver injury, or acute liver toxicity, *e.g.,* acetaminophen toxicity. In certain embodiments, the liver condition is liver cancer, *e.g.*, hepatocellular carcinoma (HCC), 30 intrahepatic cholangiocarcinoma (bile duct cancer), or hepatoblastoma.

In some embodiments, treatment results in improved liver histological appearance, including but not limited to, *e.g.*, larger cell size, lower nuclear-to-cytoplasmic ratio, two nuclei, as compared to cell size, nuclear-to-cytoplasmic ration and nuclei number in cultured

adult oval cells. In some embodiments, treatment results in a m morphology, *e.g.*, as compared to morphology of cultured adult oval cells.

In some embodiments, treatment results in decreased expression of Keratin-19 biomarker in liver cells, *e.g.*, decreased expression relative to expression of Keratin-19 biomarker in cultured adult oval cells. Methods for detecting keratin-19 biomarker (e.g., Keratin-19 gene expression, *e.g.*, mRNA expression) are well known in the art and are also exemplified herein.

In some embodiments, treatment results in increased expression of albumin and AFP e.g., increased expression relative to expression of albumin and/or AFP biomarkers in cultured adult oval cells. Methods for detecting albumin and/or AFP biomarkers (e.g., gene expression, e.g., mRNA expression) are well known in the art and are also exemplified herein.

In some embodiments, treatment results in a reduced number of Hes1 positive intrahepatic bile duct cells.

In some embodiment, treatment results in reduced liver progenitor cells (*e.g.*, adult liver oval cell) proliferation within the bile ducts. Reduced proliferation may be determined, *e.g.*, by determining average cross-sectional area of K19-positive tissue as compared to the total liver cross sectional area.

In some embodiments, treatment results in improved hepatocyte function. Hepatocyte function may be measured by methods known in the art, including but not limited to: no significant elevation of biomarkers associated with biliary dysfunction, such as those biomarkers described in Figure 11. In some embodiments, a biomarker associated with biliary dysfunction is total and/or direct serum bilirubin level. In some embodiments, a biomarker associated with biliary dysfunction is the differentiation quotient, as further described and exemplified herein.

In some embodiments, improved hepatocyte function is determined, *e.g.*, by assessment of heptobiliary function biomarkers, including but not limited to the serum heptobiliary function biomarker described in Figures 2 and 5. In some embodiments, serum heptobiliary function biomarker is serum albumin level.

In some embodiments, improved hepatocyte function is increased rate of recovery of liver function.

The invention also provides methods for promoting hepatocyte differentiation and/or by decreasing aberrant bile duct proliferation, the method comprising administering to a

patient in need of such treatment an effective amount of a Notcl
some embodiments, the patient has a liver condition characterized by liver damage. In certain embodiments, the liver condition is chronic liver disease, including but not limited to fibrosis, cirrhosis, viral hepatitis (*e.g.*, hepatitis A, B, C, D, E, or G), autoimmune liver diseases (*e.g.*,
5 autoimmune hepatitis, primary biliary cirrhosis, or primary sclerosing cholangitis), genetic liver diseases (*e.g.*, alpha-1 antitrypsin deficiency, Crigler-Najjar syndrome, familial amyloidosis, Gilbert's syndrome, Dubin-Johnson syndrome, hereditary hemchromatosis, primary oxalosis, or Wilson's disease), alcoholic hepatitis or nonalcoholic fatty liver disease. In certain embodiments, the liver condition is an acute liver condition, such as acute liver
10 failure, acute liver injury, or acute liver toxicity, *e.g.,* acetaminophen toxicity. In certain embodiments, the liver condition is liver cancer, *e.g.*, hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (bile duct cancer), or hepatoblastoma. In some embodiment, treatment results in reduced liver progenitor cell (*e.g.*, adult liver oval cell) proliferation. Reduced proliferation may be determined, *e.g.*, by determining average cross-sectional area of
15 K19 positive tissue as compared to the total liver cross sectional area.

The invention also provides methods for improving liver histological appearance, the method comprising administering to a patient in need of such treatment an effective amount of a Notch2-specific antagonist. In some embodiments, treatment results in improved liver histological appearance, including but not limited to: larger cell size, lower nuclear-to-
20 cytoplasmic ratio, two nucleic, *e.g.*, as compared to cell size, nuclear-to-cytoplasmic ratio and nuclei number in cultured adult oval cells. In some embodiments, treatment results in a more differentiated morphology, *e.g.*, as compared to morphology of cultured adult oval cells.

In some embodiments, treatment results in decreased expression of Keratin-19 biomarker in liver cells, *e.g.*, decreased expression relative to expression of Keratin-19
25 biomarker in cultured adult oval cells. Methods for detecting keratin-19 biomarker (e.g., Keratin-19 gene expression, *e.g.*, mRNA expression) are well known in the art and are also exemplified herein.

In some embodiments, treatment results in increased expression of albumin and AFP *e.g.*, increased expression relative to expression of albumin and/or AFP biomarkers in
30 cultured adult oval cells. Methods for detecting albumin and/or AFP biomarkers (*e.g.*, gene expression, *e.g.*, mRNA expression) are well known in the art and are also exemplified herein.

In some embodiments, treatment results in reduced num
intrahepatic bile duct cells.

The invention also provides methods for reducing serum bile acids, serum bilirubin, serum alkaline phosphatase, serum ALT, and/or serum AST following hepatic injury, the method comprising administering to a patient in need thereof an effective amount of a Notch2-specific antagonist.

The invention also provides methods for reducing the number of CK19-positive cells in cell population that comprises an oval cell, the method comprising the step of contacting the oval cell with a Notch2-specific antagonist.

The invention also provides methods for reducing the expression or secretion of bile acids, bilirubin, alkaline phosphatase, ALT, and/or AST, the method comprising contacting an oval cell with an effective amount of a Notch2-specific antagonist.

The invention provides methods for identifying a patient eligible for receiving treatment of a liver condition characterized by liver damage by administering to a patient having such condition an effective amount of a Notch2-specific antagonist, the method comprising determining expression of one or more of the genes listed in Table 2 in a sample obtained from the patient. In some embodiments, the genes belong to the Notch pathway, *e.g.*, JAG1. In some embodiments, a sample or biopsy from the patient is analyzed for mRNA expression of one of the genes listed in Table 1 using methods well known in the art, such as, *e.g.*, quantitative PCR analysis, and compared to expression of the same gene or genes in a biopsy obtained from a control individual or compared to a reference value. In some embodiments, elevated expression of one or more genes listed in Table 1 in the biopsy obtained from the patient, relative to the control, identifies the patient as suitable for receiving treatment with a Notch2-specific antagonist, as described herein. In some embodiments, additional parameters, such as, *e.g.*, examination by a physician, histologic evaluation of a biopsy, determination of serum levels indicative of liver damage, etc. are employed to identify the patient for receiving the treatment. Also, elevated hepatic expression by a patient of one or more of the genes identified in Table 2 is specifically contemplated as one possible embodiment of any of the methods provided herein.

In some embodiments patients are selected for treatment with a Notch2-specific antagonist as described herein by measuring other known markers of oval cells or aggressive HCC (*see, e.g.,* Woo et al 2011 Mol Carcinog. 2011 Apr;50(4):235-43). In some

embodiments, patients are selected for treatment by analyzing h
example by detection of the activated form of Notch2 as described herein.

Examples of Notch2-specific antagonists include, but are not limited to, soluble Notch receptors, soluble Notch ligand variants, *e.g.,* dominant negative ligand variants, aptamers or oligopeptides that bind Notch2 or Notch2 ligands, organic or inorganic molecules that interfere specifically with Notch2 signaling, anti-Notch2 antagonist antibodies and anti-Notch2 ligand antagonist antibodies. Examples of Notch2-specfic antagonists include those described in U.S. Patent Application Publication No. US 2010/0111958.

In certain embodiments, the Notch2-specific antagonist is an anti-Notch2 antagonist antibody. In one such embodiment, the anti-Notch2 antagonist antibody is an antibody that binds to the extracellular domain of Notch2 and effects decreased Notch2 signaling. In one such embodiment, the anti-Notch2 antagonist antibody is an anti-Notch2 NRR antibody. Anti-Notch2 NRR antibodies include, but are not limited to, any of the anti-Notch2 NRR antibodies disclosed in U.S. Patent Application Publication No. US 2010/0080808 A1, which is expressly incorporated by reference herein in its entirety. Such antibodies include, but are not limited to anti-Notch2 NRR antibodies that bind to the LNR-A and HD-C domains of Notch2 NRR. Exemplary anti-Notch2 NRR antibodies are monoclonal antibodies designated Antibody D, Antibody D-1, Antibody D-2, or Antibody D-3 that were derived from a phage library, as disclosed in US 2010/0080808. Antibody D that binds to Notch2 NRR was isolated. That antibody was affinity matured to generate Antibody D-1, Antibody D-2, and Antibody D-3. The sequences of the heavy chain and light chain hypervariable regions (HVRs) of Antibody D, Antibody D-1, Antibody D-2, and Antibody D-3 are shown in Figures 12 and 13. The sequences of the heavy and light chain variable domains of Antibody D, Antibody D-1, Antibody D-2, and Antibody D-3 are shown in Figures 14 and 15. Further embodiments of anti-Notch2 NRR antibodies are provided as follows.

In one aspect, an antagonist antibody that specifically binds to Notch2 NRR is provided, wherein the antibody comprises at least one, two, three, four, five, or six HVRs selected from:

(a) an HVR-H1 comprising an amino acid sequence that conforms to the consensus sequence of SEQ ID NO:3;

(b) an HVR-H2 comprising the amino acid sequence of SEQ ID NO:4;

(c) an HVR-H3 comprising the amino acid sequence of SEQ ID NO:5;

(d) an HVR-L1 comprising an amino acid sequence that sequence of SEQ ID NO:10;

(e) an HVR-L2 comprising an amino acid sequence that conforms to the consensus sequence of SEQ ID NO:14; and

5 (f) an HVR-L3 comprising an amino acid sequence that conforms to the consensus sequence of SEQ ID NO:19.

In a further aspect, the antibody comprises an HVR-H3 comprising the amino acid sequence of SEQ ID NO:5 and at least one, two, three, four, or five HVRs selected from (a), (b), (d), (e), and (f) above. In a further aspect, the antibody comprises (a), (b), (c), (d), (e), and (f) 10 above. With respect to (a), (d), (e), and (f), any one or more of the following embodiments are contemplated: HVR-H1 comprises an amino acid sequence selected from SEQ ID NOs:1-2; HVR-L1 comprises an amino acid sequence selected from SEQ ID NOs:6-9; HVR-L2 comprises an amino acid sequence selected from SEQ ID NOs:11-13; and HVR-L3 comprises an amino acid sequence selected from SEQ ID NOs:15-18.

15 In another aspect, an antibody that specifically binds to Notch2 NRR is provided, wherein the antibody comprises an HVR-H1 comprising an amino acid sequence that conforms to the consensus sequence of SEQ ID NO:3, an HVR-H2 comprising the amino acid sequence of SEQ ID NO:4, and an HVR-H3 comprising the amino acid sequence of SEQ ID NO:5. In one embodiment, HVR-H1 comprises an amino acid sequence selected from 20 SEQ ID NOs:1-2.

In another aspect, an antibody that specifically binds to Notch2 NRR is provided, wherein the antibody comprises an HVR-L1 comprising an amino acid sequence that conforms to the consensus sequence of SEQ ID NO:10, an HVR-L2 comprising an amino acid sequence that conforms to the consensus sequence of SEQ ID NO:14, and an HVR-L3 25 comprising an amino acid sequence that conforms to the consensus sequence of SEQ ID NO:19. The following embodiments are contemplated in any combination: HVR-L1 comprises an amino acid sequence selected from SEQ ID NOs:6-9; HVR-L2 comprises an amino acid sequence selected from SEQ ID NOs:11-13; and HVR-L3 comprises an amino acid sequence selected from SEQ ID NOs:15-18. In one embodiment, an antibody that binds 30 to Notch2 NRR comprises an HVR-L1 comprising the amino acid sequence of SEQ ID NO:6; an HVR-L2 comprising the amino acid sequence of SEQ ID NO:11; and an HVR-L3 comprising the amino acid sequence of SEQ ID NO:15. In another embodiment, an antibody that binds to Notch2 NRR comprises an HVR-L1 comprising the amino acid sequence of

SEQ ID NO:7; an HVR-L2 comprising the amino acid sequenc
HVR-L3 comprising the amino acid sequence of SEQ ID NO:16. In another embodiment, an antibody that binds to Notch2 NRR comprises an HVR-L1 comprising the amino acid sequence of SEQ ID NO:8; an HVR-L2 comprising the amino acid sequence of SEQ ID NO:12; and an HVR-L3 comprising the amino acid sequence of SEQ ID NO:17. In another embodiment, an antibody that binds to Notch2 NRR comprises an HVR-L1 comprising the amino acid sequence of SEQ ID NO:9; an HVR-L2 comprising the amino acid sequence of SEQ ID NO:13; and an HVR-L3 comprising the amino acid sequence of SEQ ID NO:18.

In one embodiment, an antibody that specifically binds to Notch2 NRR is provided, wherein the antibody comprises:

(a) an HVR-H1 comprising the amino acid sequence of SEQ ID NO:1;

(b) an HVR-H2 comprising the amino acid sequence of SEQ ID NO:4;

(c) an HVR-H3 comprising the amino acid sequence of SEQ ID NO:5;

(d) an HVR-L1 comprising the amino acid sequence of SEQ ID NO:6;

(e) an HVR-L2 comprising the amino acid sequence of SEQ ID NO:11; and

(f) an HVR-L3 comprising the amino acid sequence of SEQ ID NO:15.

In another embodiment, an antibody that specifically binds to Notch2 NRR is provided, wherein the antibody comprises:

(a) an HVR-H1 comprising the amino acid sequence of SEQ ID NO:2;

(b) an HVR-H2 comprising the amino acid sequence of SEQ ID NO:4;

(c) an HVR-H3 comprising the amino acid sequence of SEQ ID NO:5;

(d) an HVR-L1 comprising the amino acid sequence of SEQ ID NO:7;

(e) an HVR-L2 comprising the amino acid sequence of SEQ ID NO:11; and

(f) an HVR-L3 comprising the amino acid sequence of SEQ ID NO:16.

In another embodiment, an antibody that specifically binds to Notch2 NRR is provided, wherein the antibody comprises:

(a) an HVR-H1 comprising the amino acid sequence of SEQ ID NO:2;

(b) an HVR-H2 comprising the amino acid sequence of SEQ ID NO:4;

(c) an HVR-H3 comprising the amino acid sequence of SEQ ID NO:5;

(d) an HVR-L1 comprising the amino acid sequence of SEQ ID NO:8;

(e) an HVR-L2 comprising the amino acid sequence of SEQ ID NO:12; and

(f) an HVR-L3 comprising the amino acid sequence of SEQ ID NO:17.

In another embodiment, an antibody that specifically bi
provided, wherein the antibody comprises:

(a) an HVR-H1 comprising the amino acid sequence of SEQ ID NO:2;
(b) an HVR-H2 comprising the amino acid sequence of SEQ ID NO:4;
(c) an HVR-H3 comprising the amino acid sequence of SEQ ID NO:5;
(d) an HVR-L1 comprising the amino acid sequence of SEQ ID NO:9;
(e) an HVR-L2 comprising the amino acid sequence of SEQ ID NO:13; and
(f) an HVR-L3 comprising the amino acid sequence of SEQ ID NO:18.

In certain embodiments, any of the above antibodies further comprises at least one framework selected from a VH subgroup III consensus framework and a VL subgroup I consensus framework.

In certain embodiments, an anti-Notch2 NRR antibody is affinity matured. For example, any one or more of the following substitutions in the indicated HVR positions (Kabat numbered) may be made in any combination:

- in HVR-H1 (SEQ ID NO:1): S28T; T30S;
- in HVR-L1 (SEQ ID NO:6): S28N; I29N or V; S30R or K; S31R; Y32F
- in HVR-L2 (SEQ ID NO:11): G50R; S53I or T; A55E
- in HVR-L3 (SEQ ID NO:15): S93I or R; L96W or H

The specific antibodies disclosed herein, i.e., Antibody D as well as affinity matured forms of Antibody D (D-1, D-2, and D-3), may undergo further affinity maturation. Accordingly, affinity matured forms of any of the antibodies described herein are provided.

In certain embodiments, an anti-Notch2 NRR antibody having any of the above HVR sequences can further comprise any suitable framework variable domain sequence, provided binding activity to Notch2 NRR is substantially retained. In certain embodiments, an anti-Notch2 NRR antibody comprises a human variable heavy (VH) consensus framework sequence, as in any of the VH consensus framework sequences shown in Figures 16A and 16B. In one embodiment, the VH consensus framework sequence comprises a human subgroup III heavy chain framework consensus sequence, e.g., as shown in Figures 16A and 16B. In another embodiment, the VH consensus framework sequence comprises an "Acceptor 2" framework sequence, e.g., as shown in Figures 16A and 16B. In a particular embodiment, the VH framework consensus sequence comprises FR1-FR4 of Acceptor 2B or Acceptor 2D, wherein the FR4 comprises SEQ ID NO:35 (Figures 16A and 16B), with the last residue of SEQ ID NO:35 (S11) optionally being substituted with alanine. In a further

particular embodiment, the VH framework consensus sequence SEQ ID NOs:50; 51; 57 or 59; and 35, wherein S11 of SEQ ID NO:35 is optionally substituted with alanine.

In certain embodiments, an anti-Notch2 NRR antibody having any of the above HVR sequences can further comprise a human variable light (VL) consensus framework sequence as shown in Figure 17. In one embodiment, the VL consensus framework sequence comprises a human VL kappa subgroup I consensus framework (κv1) sequence, e.g., as shown in Figure 17. In another embodiment, the VL framework consensus sequence comprises FR1-FR4 of huMAb4D5-8 as shown in Figures 18 or 19. In a particular embodiment, the VL framework consensus sequence comprises the sequences of SEQ ID NOs:60, 61, 62, and 63.

In another aspect, an anti-Notch2 NRR antibody comprises a heavy chain variable domain (VH) sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NOs:20-21. In certain embodiments, a VH sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-Notch2 NRR antibody comprising that sequence retains the ability to bind to Notch2 NRR. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in an amino acid sequence selected from SEQ ID NOs:20-21. In certain embodiments, substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs). In a particular embodiment, the VH comprises one, two or three HVRs selected from: (a) an HVR-H1 comprising an amino acid sequence that conforms to the consensus sequence of SEQ ID NO:3, (b) an HVR-H2 comprising the amino acid sequence of SEQ ID NO:4, and (c) an HVR-H3 comprising the amino acid sequence of SEQ ID NO:5. In one such embodiment, HVR-H1 comprises an amino acid sequence selected from SEQ ID NOs:1-2.

In another aspect, an antibody that specifically binds to Notch2 NRR is provided, wherein the antibody comprises a light chain variable domain (VL) having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NOs:22-25. In certain embodiments, a VL sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-Notch2 NRR antibody comprising that sequence retains the ability to

bind to Notch2 NRR. In certain embodiments, a total of 1 to 1(
substituted, inserted and/or deleted in an amino acid sequence selected from SEQ ID NOs:22-25. In certain embodiments, the substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs). In a particular embodiment, the VL comprises one, two or three HVRs selected from (a) an HVR-L1 comprising an amino acid sequence that conforms to the consensus sequence of SEQ ID NO:10; (b) an HVR-L2 comprising an amino acid sequence that conforms to the consensus sequence of SEQ ID NO:14; and (c) an HVR-L3 comprising an amino acid sequence that conforms to the consensus sequence of SEQ ID NO:19. In one such embodiment, the VL comprises one, two or three HVRs selected from (a) an HVR-L1 comprising an amino acid sequence selected from SEQ ID NOs:6-9; (b) an HVR-L2 comprising an amino acid sequence selected from SEQ ID NOs:11-13; and (c) an HVR-L3 comprising an amino acid sequence selected from SEQ ID NOs:15-18. In one such embodiment, the VL comprises one, two or three HVRs selected from (a) an HVR-L1 comprising the amino acid sequence of SEQ ID NO:6; (b) an HVR-L2 comprising the amino acid sequence of SEQ ID NO:11; and (c) an HVR-L3 comprising the amino acid sequence of SEQ ID NO:15. In another such embodiment, the VL comprises one, two or three HVRs selected from (a) an HVR-L1 comprising the amino acid sequence of SEQ ID NO:7; (b) an HVR-L2 comprising the amino acid sequence of SEQ ID NO:11; and (c) an HVR-L3 comprising the amino acid sequence of SEQ ID NO:16. In another such embodiment, the VL comprises one, two or three HVRs selected from (a) an HVR-L1 comprising the amino acid sequence of SEQ ID NO:8; (b) an HVR-L2 comprising the amino acid sequence of SEQ ID NO:12; and (c) an HVR-L3 comprising the amino acid sequence of SEQ ID NO:17. In another such embodiment, the VL comprises one, two or three HVRs selected from (a) an HVR-L1 comprising the amino acid sequence of SEQ ID NO:9; (b) an HVR-L2 comprising the amino acid sequence of SEQ ID NO:13; and (c) an HVR-L3 comprising the amino acid sequence of SEQ ID NO:18.

In certain embodiments of the variant VH and VL sequences provided above, substitutions, insertions, or deletions may occur within the HVRs. In such embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antibody to bind antigen. For example, conservative alterations that do not substantially reduce binding affinity may be made in HVRs. In certain instances, alterations in HVRs may actually improve antibody affinity. Such alterations may be made in HVR "hotspots" (i.e., residues encoded by codons

that undergo mutation at high frequency during the somatic mat
increase antibody affinity. (See, e.g., Chowdhury, *Methods Mol. Biol.* 207:179-196, 2008.) In certain embodiments of the variant VH and VL sequences provided above, each HVR either is conserved (unaltered), or contains no more than a single amino acid substitution,
5 insertion or deletion.

In another aspect, an antibody that specifically binds Notch2 NRR is provided, wherein the antibody comprises a VH as in any of the embodiments provided above, and a VL as in any of the embodiments provided above. In one embodiment, the antibody comprises a VH having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or
10 100% sequence identity to the amino acid sequence of SEQ ID NO:20, and a VL having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO:22. In one such embodiment, the VH comprises one, two or three HVRs selected from: (a) an HVR-H1 comprising the amino acid sequence of SEQ ID NO:1, (b) an HVR-H2 comprising the amino acid sequence of SEQ ID NO:4, and (c)
15 an HVR-H3 comprising the amino acid sequence of SEQ ID NO:5, and the VL comprises one, two or three HVRs selected from (a) an HVR-L1 comprising the amino acid sequence of SEQ ID NO:6; (b) an HVR-L2 comprising the amino acid sequence of SEQ ID NO:11; and (c) an HVR-L3 comprising the amino acid sequence of SEQ ID NO:15. In a particular embodiment, the antibody comprises a VH comprising the amino acid sequence of SEQ ID
20 NO:20, and a VL comprising the amino acid sequence of SEQ ID NO:22.

In another embodiment, an anti-Notch2 NRR antibody that specifically binds Notch2 NRR comprises a VH having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO:21, and a VL having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence
25 identity to an amino acid sequence selected from SEQ ID NOs:23-25. In one such embodiment, the VH comprises one, two or three HVRs selected from: (a) an HVR-H1 comprising the amino acid sequence of SEQ ID NO:2, (b) an HVR-H2 comprising the amino acid sequence of SEQ ID NO:4, and (c) an HVR-H3 comprising the amino acid sequence of SEQ ID NO:5, and the VL comprises one, two or three HVRs selected from (a) an HVR-L1
30 comprising an amino acid sequence selected from SEQ ID NOs:7-9; (b) an HVR-L2 comprising an amino acid sequence selected from SEQ ID NOs:11-13; and (c) an HVR-L3 comprising an amino acid sequence selected from SEQ ID NOs:16-18. In particular

embodiments, the antibody comprises a VH comprising the am
NO:21 and a VL comprising an amino acid sequence selected from SEQ ID NOs:23-25.

In certain embodiments, an affinity-matured form of any of the above antibodies is provided. In further embodiments, a recombinant protein that specifically binds Notch2 NRR is provided, wherein the recombinant protein comprises an antigen binding site(s) of any of the above antibodies. In one such embodiment, a recombinant protein comprises any one or more of the HVRs provided above.

In certain embodiments, a polynucleotide encoding any of the above antibodies is provided. In one embodiment, a vector comprising the polynucleotide is provided. In one embodiment, a host cell comprising the vector is provided. In one embodiment, the host cell is eukaryotic. In one embodiment, the host cell is a CHO cell. In one embodiment, a method of making an anti-Notch2 NRR antibody is provided, wherein the method comprises culturing the host cell under conditions suitable for expression of the polynucleotide encoding the antibody, and isolating the antibody.

In another embodiment, an isolated antibody is provided that binds to the same epitope as an antibody provided herein. In one embodiment, an isolated anti-Notch2 NRR antibody is provided that binds to the same epitope as an antibody selected from Antibody D, Antibody D-1, Antibody D-2, and Antibody D-3. In another embodiment, the invention provides an anti-Notch2 NRR antibody that competes for binding with an antibody selected from Antibody D, Antibody D-1, Antibody D-2, and Antibody D-3. In another embodiment, an isolated antibody is provided that binds to at least one domain selected from the LNR-A domain and the HD-C domain of Notch2. In one such embodiment, the antibody binds to both the LNR-A domain and the HD-C domain. In another such embodiment, the antibody further binds to the LNR-B and/or HD-N domains.

Any of the Notch2-specific antagonists provided herein may be used in therapeutic methods. In one aspect, a Notch2-specific antagonist for use as a medicament is provided. In further aspects, a Notch2-specific antagonist for use in treating a liver condition characterized by liver damage is provided. In certain embodiments, a Notch2-specific antagonist for use in a method of treatment is provided. In certain embodiments, the invention provides a Notch2-specific antagonist for use in a method of treating an individual having a liver condition characterized by liver damage comprising administering to the individual an effective amount of the Notch2-specific antagonist. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic

agent, e.g., as described below. An "individual" according to a
is preferably a human.

In a further aspect, the invention provides for the use of a Notch2-specific antagonist in the manufacture or preparation of a medicament. In one embodiment, the medicament is for treatment of a liver condition characterized by liver damage. In a further embodiment, the medicament is for use in a method of treating a liver condition characterized by liver damage comprising administering to an individual having a liver condition characterized by liver damage an effective amount of the medicament. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, e.g., as described below. An "individual" according to any of the above embodiments may be a human.

In a further aspect, the invention provides pharmaceutical formulations comprising any of the Notch2-specific antagonists provided herein, e.g., for use in any of the above therapeutic methods. In one embodiment, a pharmaceutical formulation comprises any of the Notch2-specific antagonists provided herein and a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical formulation comprises any of the Notch2-specific antagonists provided herein and at least one additional therapeutic agent, e.g., as described below.

Antibodies of the invention can be used either alone or in combination with other agents in a therapy. For instance, an antibody of the invention may be co-administered with at least one additional therapeutic agent.

Such combination therapies noted above encompass combined administration (where two or more therapeutic agents are included in the same or separate formulations), and separate administration, in which case, administration of the antagonist of the invention can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant. Antagonists of the invention can also be used in combination with radiation therapy.

The antagonist can be administered to a human patient by any known method, such as intravenous administration, *e.g.*, as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The Notch2-specific antagonist might be administered as a protein or as a nucleic acid encoding a protein (see, for example, WO96/07321). Other therapeutic regimens may be combined with the administration of the

Notch2-specific antagonist. The combined administration inclu
separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. In some embodiments, such combined
5 therapy results in a synergistic therapeutic effect.

The dosage and mode of administration will be chosen by the physician according to known criteria. The appropriate dosage of antibody, oligopeptide or organic molecule will depend on the type of disease to be treated, the severity and course of the disease, whether the antibody, oligopeptide or organic molecule is administered for preventive or therapeutic
10 purposes, previous therapy, the patient's clinical history and response to the Notch2-specific antagonist, and the discretion of the attending physician. The Notch2-specific antagonist can be administered to the patient at one time or over a series of treatments.

Success of treatment of liver disease can be monitored by assessing parameters of liver function and recovery. Such parameters include, but are not limited to, improved liver
15 function tests, (*e.g.,* assessing serum albumin, bilirubin, bile acids, total protein, clotting times), liver enzymes (*e.g.,* alanine transaminase, aspartate transaminase, alkaline phosphatase, gamma glutamyl transpeptidase), histologic appearance (*e.g.,* needle biopsy showing improved hepatic architecture), and imaging modalities (*e.g.,* ultrasound, magnetic resonance imaging for fibrosis and liver size).

20 In a further aspect, an anti-Notch2 antibody used in any of the above embodiments may incorporate any of the features, singly or in combination, as described in Sections 1-7 below.

1. Antibody Affinity

In certain embodiments, an antibody provided herein has a dissociation constant (Kd)
25 of $\leq 1\mu M$, ≤ 100 nM, ≤ 10 nM, ≤ 1 nM, ≤ 0.1 nM, ≤ 0.01 nM, or ≤ 0.001 nM (*e.g.*, 10^{-8} M or less, *e.g.* from 10^{-8} M to 10^{-13} M, *e.g.*, from 10^{-9} M to 10^{-13} M). For example, the exemplary phage Antibody D-3 binds to Notch2 with a Kd of 5 nM.

In one embodiment, Kd is measured by a radiolabeled antigen binding assay (RIA) performed with the Fab version of an antibody of interest and its antigen as described by the
30 following assay. Solution binding affinity of Fabs for antigen is measured by equilibrating Fab with a minimal concentration of (^{125}I)-labeled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate

(*see, e.g.*, Chen et al., *J. Mol. Biol.* 293:865-881(1999)). To est
assay, MICROTITER® multi-well plates (Thermo Scientific) are coated overnight with 5 μg/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-adsorbent plate (Nunc #269620), 100 pM or 26 pM [^{125}I]-antigen are mixed with serial dilutions of a Fab of interest (e.g., consistent with assessment of the anti-VEGF antibody, Fab-12, in Presta et al., *Cancer Res.* 57:4593-4599 (1997)). The Fab of interest is then incubated overnight; however, the incubation may continue for a longer period (e.g., about 65 hours) to ensure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature (e.g., for one hour). The solution is then removed and the plate washed eight times with 0.1% polysorbate 20 (TWEEN-20®) in PBS. When the plates have dried, 150 μl/well of scintillant (MICROSCINT-20 ™; Packard) is added, and the plates are counted on a TOPCOUNT ™ gamma counter (Packard) for ten minutes. Concentrations of each Fab that give less than or equal to 20% of maximal binding are chosen for use in competitive binding assays.

According to another embodiment, Kd is measured using surface plasmon resonance assays using a BIACORE®-2000 or a BIACORE ®-3000 (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIACORE, Inc.) are activated with *N*-ethyl-*N'*- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10 mM sodium acetate, pH 4.8, to 5 μg/ml (~0.2 μM) before injection at a flow rate of 5 μl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% polysorbate 20 (TWEEN-20™) surfactant (PBST) at 25°C at a flow rate of approximately 25 μl/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIACORE ® Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (Kd) is calculated as the ratio k_{off}/k_{on}. See, e.g., Chen et al., *J. Mol. Biol.* 293:865-881 (1999). If the on-rate exceeds 10^6 M^{-1} s^{-1} by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent

quenching technique that measures the increase or decrease in f
(excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophometer (Aviv
5 Instruments) or a 8000-series SLM-AMINCO ™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

2. Antibody Fragments

In certain embodiments, an antibody provided herein is an antibody fragment. Antibody fragments include, but are not limited to, Fab, Fab', Fab'-SH, $F(ab')_2$, Fv, and scFv
10 fragments, and other fragments described below. For a review of certain antibody fragments, see Hudson et al. *Nat. Med.* 9:129-134 (2003). For a review of scFv fragments, see, e.g., Pluckthün, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., (Springer-Verlag, New York), pp. 269-315 (1994); see also WO 93/16185; and U.S. Patent Nos. 5,571,894 and 5,587,458. For discussion of Fab and $F(ab')_2$ fragments
15 comprising salvage receptor binding epitope residues and having increased in vivo half-life, see U.S. Patent No. 5,869,046.

Diabodies are antibody fragments with two antigen-binding sites that may be bivalent or bispecific. See, for example, EP 404,097; WO 1993/01161; Hudson et al., *Nat. Med.* 9:129-134 (2003); and Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993).
20 Triabodies and tetrabodies are also described in Hudson et al., *Nat. Med.* 9:129-134 (2003).

Single-domain antibodies are antibody fragments comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody (Domantis, Inc., Waltham, MA; *see,* e.g., U.S. Patent No. 6,248,516 B1).

25 Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells (e.g. *E. coli* or phage).

3. Chimeric and Humanized Antibodies

In certain embodiments, an antibody provided herein is a chimeric antibody. Certain
30 chimeric antibodies are described, e.g., in U.S. Patent No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)). In one example, a chimeric antibody comprises a non-human variable region (e.g., a variable region derived from a mouse, rat,

hamster, rabbit, or non-human primate, such as a monkey) and
further example, a chimeric antibody is a "class switched" antibody in which the class or subclass has been changed from that of the parent antibody. Chimeric antibodies include antigen-binding fragments thereof.

5 In certain embodiments, a chimeric antibody is a humanized antibody. Typically, a non-human antibody is humanized to reduce immunogenicity to humans, while retaining the specificity and affinity of the parental non-human antibody. Generally, a humanized antibody comprises one or more variable domains in which HVRs, e.g., CDRs, (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are derived from
10 human antibody sequences. A humanized antibody optionally will also comprise at least a portion of a human constant region. In some embodiments, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the HVR residues are derived), e.g., to restore or improve antibody specificity or affinity.

15 Humanized antibodies and methods of making them are reviewed, e.g., in Almagro and Fransson, *Front. Biosci.* 13:1619-1633 (2008), and are further described, e.g., in Riechmann et al., *Nature* 332:323-329 (1988); Queen et al., *Proc. Nat'l Acad. Sci. USA* 86:10029-10033 (1989); US Patent Nos. 5, 821,337, 7,527,791, 6,982,321, and 7,087,409; Kashmiri *et al.*, *Methods* 36:25-34 (2005) (describing SDR (a-CDR) grafting); Padlan, *Mol.*
20 *Immunol.* 28:489-498 (1991) (describing "resurfacing"); Dall'Acqua et al., *Methods* 36:43-60 (2005) (describing "FR shuffling"); and Osbourn et al., *Methods* 36:61-68 (2005) and Klimka et al., *Br. J. Cancer*, 83:252-260 (2000) (describing the "guided selection" approach to FR shuffling).

Human framework regions that may be used for humanization include but are not
25 limited to: framework regions selected using the "best-fit" method (see, e.g., Sims et al. *J. Immunol.* 151:2296 (1993)); framework regions derived from the consensus sequence of human antibodies of a particular subgroup of light or heavy chain variable regions (see, e.g., Carter et al. *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); and Presta et al. *J. Immunol.*, 151:2623 (1993)); human mature (somatically mutated) framework regions or human
30 germline framework regions (see, e.g., Almagro and Fransson, *Front. Biosci.* 13:1619-1633 (2008)); and framework regions derived from screening FR libraries (see, e.g., Baca et al., *J. Biol. Chem.* 272:10678-10684 (1997) and Rosok et al., *J. Biol. Chem.* 271:22611-22618 (1996)).

4. *Human Antibodies*

In certain embodiments, an antibody provided herein is a human antibody. Human antibodies can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk and van de Winkel, *Curr. Opin. Pharmacol.* 5: 368-74 (2001)
5 and Lonberg, *Curr. Opin. Immunol.* 20:450-459 (2008).

Human antibodies may be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge. Such animals typically contain all or a portion of the human immunoglobulin loci, which replace the endogenous
10 immunoglobulin loci, or which are present extrachromosomally or integrated randomly into the animal's chromosomes. In such transgenic mice, the endogenous immunoglobulin loci have generally been inactivated. For review of methods for obtaining human antibodies from transgenic animals, see Lonberg, *Nat. Biotech.* 23:1117-1125 (2005). See also, e.g., U.S. Patent Nos. 6,075,181 and 6,150,584 describing XENOMOUSE™ technology; U.S. Patent
15 No. 5,770,429 describing HUMAB® technology; U.S. Patent No. 7,041,870 describing K-M MOUSE® technology, and U.S. Patent Application Publication No. US 2007/0061900, describing VELOCIMOUSE® technology). Human variable regions from intact antibodies generated by such animals may be further modified, e.g., by combining with a different human constant region.

20 Human antibodies can also be made by hybridoma-based methods. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described. (See, e.g., Kozbor *J. Immunol.*, 133: 3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner et al., *J. Immunol.*, 147: 86 (1991).) Human
25 antibodies generated via human B-cell hybridoma technology are also described in Li et al., *Proc. Natl. Acad. Sci. USA*, 103:3557-3562 (2006). Additional methods include those described, for example, in U.S. Patent No. 7,189,826 (describing production of monoclonal human IgM antibodies from hybridoma cell lines) and Ni, *Xiandai Mianyixue*, 26(4):265-268 (2006) (describing human-human hybridomas). Human hybridoma technology (Trioma
30 technology) is also described in Vollmers and Brandlein, *Histology and Histopathology*, 20(3):927-937 (2005) and Vollmers and Brandlein, *Methods and Findings in Experimental and Clinical Pharmacology*, 27(3):185-91 (2005).

Human antibodies may also be generated by isolating Fv clone variable domain sequences selected from human-derived phage display libraries. Such variable domain sequences may then be combined with a desired human constant domain.

5. Library-Derived Antibodies

5 Antibodies of the invention may be isolated by screening combinatorial libraries for antibodies with the desired activity or activities. For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies possessing the desired binding characteristics. Such methods are reviewed, e.g., in Hoogenboom et al. in *Methods in Molecular Biology* 178:1-37 (O'Brien et al., ed., Human
10 Press, Totowa, NJ, 2001) and further described, e.g., in the McCafferty et al., *Nature* 348:552-554; Clackson et al., *Nature* 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1992); Marks and Bradbury, in *Methods in Molecular Biology* 248:161-175 (Lo, ed., Human Press, Totowa, NJ, 2003); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34):
15 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284(1-2): 119-132(2004).

In certain phage display methods, repertoires of VH and VL genes are separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be screened for antigen-binding phage as described in Winter et al., *Ann. Rev. Immunol.*, 12: 433-455 (1994). Phage typically display antibody fragments, either as single-
20 chain Fv (scFv) fragments or as Fab fragments. Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned (e.g., from human) to provide a single source of antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths et al., *EMBO J*, 12: 725-734 (1993). Finally, naive
25 libraries can also be made synthetically by cloning unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement *in vitro*, as described by Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-388 (1992). Patent publications describing human antibody phage libraries include, for example: US Patent No. 5,750,373, and US Patent Publication
30 Nos. 2005/0079574, 2005/0119455, 2005/0266000, 2007/0117126, 2007/0160598, 2007/0237764, 2007/0292936, and 2009/0002360.

Antibodies or antibody fragments isolated from human ;
considered human antibodies or human antibody fragments herein.

6. Multispecific Antibodies

In certain embodiments, an antibody provided herein is a multispecific antibody, e.g. a
5 bispecific antibody. Multispecific antibodies are monoclonal antibodies that have binding specificities for at least two different sites. In certain embodiments, one of the binding specificities is for Notch2 and the other is for any other antigen. In certain embodiments, bispecific antibodies may bind to two different epitopes of Notch2. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express Notch2. Bispecific antibodies
10 can be prepared as full length antibodies or antibody fragments.

Techniques for making multispecific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein and Cuello, *Nature* 305: 537 (1983)), WO 93/08829, and Traunecker et al., *EMBO J.* 10: 3655 (1991)), and "knob-in-hole" engineering (see, e.g., U.S.
15 Patent No. 5,731,168). Multi-specific antibodies may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules (WO 2009/089004A1); cross-linking two or more antibodies or fragments (see, e.g., US Patent No. 4,676,980, and Brennan et al., *Science*, 229: 81 (1985)); using leucine zippers to produce bi-specific antibodies (see, e.g., Kostelny et al., *J. Immunol.*, 148(5):1547-1553
20 (1992)); using "diabody" technology for making bispecific antibody fragments (see, e.g., Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993)); and using single-chain Fv (sFv) dimers (see,e.g. Gruber et al., *J. Immunol.*, 152:5368 (1994)); and preparing trispecific antibodies as described, e.g., in Tutt et al. *J. Immunol.* 147: 60 (1991).

Engineered antibodies with three or more functional antigen binding sites, including
25 "Octopus antibodies," are also included herein (see, e.g. US 2006/0025576A1).

The antibody or fragment herein also includes a "Dual Acting FAb" or "DAF" comprising an antigen binding site that binds to Notch2 as well as another, different antigen (see, US 2008/0069820, for example).

7. Antibody Variants

30 In certain embodiments, amino acid sequence variants of the antibodies provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of an

antibody may be prepared by introducing appropriate modificat
sequence encoding the antibody, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and
5 substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., antigen-binding.

a) Substitution, Insertion, and Deletion Variants

In certain embodiments, antibody variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the HVRs
10 and FRs. Conservative substitutions are shown in Table 1 under the heading of "conservative substitutions." More substantial changes are provided in Table 1 under the heading of "exemplary substitutions," and as further described below in reference to amino acid side chain classes. Amino acid substitutions may be introduced into an antibody of interest and the products screened for a desired activity, e.g., retained/improved antigen binding,
15 decreased immunogenicity, or improved ADCC or CDC.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.* a humanized or human antibody). Generally, the
20 resulting variant(s) selected for further study will have modifications (e.g., improvements) in certain biological properties (e.g., increased affinity, reduced immunogenicity) relative to the parent antibody and/or will have substantially retained certain biological properties of the parent antibody. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated, e.g., using phage display-based affinity maturation techniques
25 such as those described herein. Briefly, one or more HVR residues are mutated and the variant antibodies displayed on phage and screened for a particular biological activity (e.g. binding affinity).

TABLE 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

Amino acids may be grouped according to common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

5 (3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

Alterations (e.g., substitutions) may be made in HVRs, e.g., to improve antibody
10 affinity. Such alterations may be made in HVR "hotspots," i.e., residues encoded by codons that undergo mutation at high frequency during the somatic maturation process (see, e.g., Chowdhury, *Methods Mol. Biol.* 207:179-196 (2008)), and/or SDRs (a-CDRs), with the

resulting variant VH or VL being tested for binding affinity. Af
constructing and reselecting from secondary libraries has been described, e.g., in Hoogenboom et al. in *Methods in Molecular Biology* 178:1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, (2001).) In some embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (e.g., error-prone PCR, chain shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any antibody variants with the desired affinity. Another method to introduce diversity involves HVR-directed approaches, in which several HVR residues (e.g., 4-6 residues at a time) are randomized. HVR residues involved in antigen binding may be specifically identified, e.g., using alanine scanning mutagenesis or modeling. CDR-H3 and CDR-L3 in particular are often targeted.

In certain embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antibody to bind antigen. For example, conservative alterations (e.g., conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. Such alterations may be outside of HVR "hotspots" or SDRs. In certain embodiments of the variant VH and VL sequences provided above, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

A useful method for identification of residues or regions of an antibody that may be targeted for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989) *Science*, 244:1081-1085. In this method, a residue or group of target residues (e.g., charged residues such as arg, asp, his, lys, and glu) are identified and replaced by a neutral or negatively charged amino acid (e.g., alanine or polyalanine) to determine whether the interaction of the antibody with antigen is affected. Further substitutions may be introduced at the amino acid locations demonstrating functional sensitivity to the initial substitutions. Alternatively, or additionally, a crystal structure of an antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues may be targeted or eliminated as candidates for substitution. Variants may be screened to determine whether they contain the desired properties.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of

terminal insertions include an antibody with an N-terminal metl
insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

5

b) Glycosylation variants

In certain embodiments, an antibody provided herein is altered to increase or decrease the extent to which the antibody is glycosylated. Addition or deletion of glycosylation sites to an antibody may be conveniently accomplished by altering the amino acid sequence such that one or more glycosylation sites is created or removed.

10 Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region. See, e.g., Wright et al. *TIBTECH* 15:26-32 (1997). The oligosaccharide may include various carbohydrates, e.g., mannose, N-acetyl glucosamine
15 (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the “stem” of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antibody of the invention may be made in order to create antibody variants with certain improved properties.

In one embodiment, antibody variants are provided having a carbohydrate structure
20 that lacks fucose attached (directly or indirectly) to an Fc region. For example, the amount of fucose in such antibody may be from 1% to 80%, from 1% to 65%, from 5% to 65% or from 20% to 40%. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (e. g. complex, hybrid and high mannose structures) as measured by MALDI-TOF
25 mass spectrometry, as described in WO 2008/077546, for example. Asn297 refers to the asparagine residue located at about position 297 in the Fc region (Eu numbering of Fc region residues); however, Asn297 may also be located about ± 3 amino acids upstream or downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in antibodies. Such fucosylation variants may have improved ADCC function. See,
30 e.g., US Patent Publication Nos. US 2003/0157108 (Presta, L.); US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Examples of publications related to “defucosylated” or “fucose-deficient” antibody variants include: US 2003/0157108; WO 2000/61739; WO 2001/29246;

US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2
2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO2005/053742; WO2002/031140; Okazaki et al. *J. Mol. Biol.* 336:1239-1249 (2004); Yamane-Ohnuki et al. *Biotech. Bioeng.* 87: 614 (2004).
5 Examples of cell lines capable of producing defucosylated antibodies include Lec13 CHO cells deficient in protein fucosylation (Ripka et al. *Arch. Biochem. Biophys.* 249:533-545 (1986); US Pat Appl No US 2003/0157108 A1, Presta, L; and WO 2004/056312 A1, Adams *et al.*, especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, *FUT8,* knockout CHO cells (see, e.g., Yamane-Ohnuki et al. *Biotech. Bioeng.* 87: 614
10 (2004); Kanda, Y. et al., *Biotechnol. Bioeng.*, 94(4):680-688 (2006); and WO2003/085107).

Antibodies variants are further provided with bisected oligosaccharides, e.g., in which a biantennary oligosaccharide attached to the Fc region of the antibody is bisected by GlcNAc. Such antibody variants may have reduced fucosylation and/or improved ADCC function. Examples of such antibody variants are described, e.g., in WO 2003/011878 (Jean-
15 Mairet et al.); US Patent No. 6,602,684 (Umana et al.); and US 2005/0123546 (Umana *et al.*). Antibody variants with at least one galactose residue in the oligosaccharide attached to the Fc region are also provided. Such antibody variants may have improved CDC function. Such antibody variants are described, e.g., in WO 1997/30087 (Patel et al.); WO 1998/58964 (Raju, S.); and WO 1999/22764 (Raju, S.).

20 **c) Fc region variants**

In certain embodiments, one or more amino acid modifications may be introduced into the Fc region of an antibody provided herein, thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence (*e.g.*, a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (*e.g.* a substitution) at one or more
25 amino acid positions.

In certain embodiments, the invention contemplates an antibody variant that possesses some but not all effector functions, which make it a desirable candidate for applications in which the half life of the antibody *in vivo* is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. *In vitro* and/or *in vivo* cytotoxicity
30 assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability.

The primary cells for mediating ADCC, NK cells, express FcγI

express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-492 (1991). Non-limiting examples of *in vitro* assays to assess ADCC activity of a molecule of interest is
5 described in U.S. Patent No. 5,500,362 (see, e.g. Hellstrom, I. et al. *Proc. Nat'l Acad. Sci. USA* 83:7059-7063 (1986)) and Hellstrom, I et al., *Proc. Nat'l Acad. Sci. USA* 82:1499-1502 (1985); 5,821,337 (see Bruggemann, M. et al., *J. Exp. Med.* 166:1351-1361 (1987)). Alternatively, non-radioactive assays methods may be employed (see, for example, ACTI™ non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View,
10 CA; and CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, *e.g.*, in a animal model such as that disclosed in Clynes et al. *Proc. Nat'l Acad. Sci. USA* 95:652-656 (1998). C1q binding assays may also be carried out to
15 confirm that the antibody is unable to bind C1q and hence lacks CDC activity. See, e.g., C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996); Cragg, M.S. et al., *Blood* 101:1045-1052 (2003); and Cragg, M.S. and M.J. Glennie, *Blood* 103:2738-2743 (2004)). FcRn binding and *in vivo*
20 clearance/half life determinations can also be performed using methods known in the art (see, e.g., Petkova, S.B. et al., *Int'l. Immunol.* 18(12):1759-1769 (2006)).

Antibodies with reduced effector function include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Patent No. 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions
25 265, 269, 270, 297 and 327, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (US Patent No. 7,332,581).

Certain antibody variants with improved or diminished binding to FcRs are described. (See, e.g., U.S. Patent No. 6,737,056; WO 2004/056312, and Shields et al., *J. Biol. Chem.* 9(2): 6591-6604 (2001).)

30 In certain embodiments, an antibody variant comprises an Fc region with one or more amino acid substitutions which improve ADCC, e.g., substitutions at positions 298, 333, and/or 334 of the Fc region (EU numbering of residues).

In some embodiments, alterations are made in the Fc re;
either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in US Patent No. 6,194,551, WO 99/51642, and Idusogie et al. *J. Immunol.* 164: 4178-4184 (2000).

5 Antibodies with increased half lives and improved binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)), are described in US2005/0014934A1 (Hinton et al.). Those antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. Such Fc variants
10 include those with substitutions at one or more of Fc region residues: 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434, e.g., substitution of Fc region residue 434 (US Patent No. 7,371,826).

See also Duncan & Winter, *Nature* 322:738-40 (1988); U.S. Patent No. 5,648,260; U.S. Patent No. 5,624,821; and WO 94/29351 concerning other examples of Fc region
15 variants.

d) Cysteine engineered antibody variants

In certain embodiments, it may be desirable to create cysteine engineered antibodies, e.g., "thioMAbs," in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the
20 antibody. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described further herein. In certain embodiments, any one or more of the following residues may be substituted with cysteine: V205 (Kabat numbering) of the light chain; A118
25 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc region. Cysteine engineered antibodies may be generated as described, e.g., in U.S. Patent No. 7,521,541.

e) Antibody Derivatives

In certain embodiments, an antibody provided herein may be further modified to
30 contain additional nonproteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization of the antibody include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not

limited to, polyethylene glycol (PEG), copolymers of ethylene g
carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer are attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, etc.

In another embodiment, conjugates of an antibody and nonproteinaceous moiety that may be selectively heated by exposure to radiation are provided. In one embodiment, the nonproteinaceous moiety is a carbon nanotube (Kam et al., *Proc. Natl. Acad. Sci. USA* 102: 11600-11605 (2005)). The radiation may be of any wavelength, and includes, but is not limited to, wavelengths that do not harm ordinary cells, but which heat the nonproteinaceous moiety to a temperature at which cells proximal to the antibody-nonproteinaceous moiety are killed.

B. Recombinant Methods and Compositions

Antibodies may be produced using recombinant methods and compositions, e.g., as described in U.S. Patent No. 4,816,567. In one embodiment, isolated nucleic acid encoding an anti-Notch2 antibody described herein is provided. Such nucleic acid may encode an amino acid sequence comprising the VL and/or an amino acid sequence comprising the VH of the antibody (e.g., the light and/or heavy chains of the antibody). In a further embodiment, one or more vectors (e.g., expression vectors) comprising such nucleic acid are provided. In a further embodiment, a host cell comprising such nucleic acid is provided. In one such embodiment, a host cell comprises (e.g., has been transformed with): (1) a vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and an amino acid sequence comprising the VH of the antibody, or (2) a first vector comprising a

nucleic acid that encodes an amino acid sequence comprising tl
second vector comprising a nucleic acid that encodes an amino acid sequence comprising the VH of the antibody. In one embodiment, the host cell is eukaryotic, e.g. a Chinese Hamster Ovary (CHO) cell or lymphoid cell (e.g., Y0, NS0, Sp20 cell). In one embodiment, a method of making an anti-Notch2 antibody is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the antibody, as provided above, under conditions suitable for expression of the antibody, and optionally recovering the antibody from the host cell (or host cell culture medium).

For recombinant production of an anti-Notch2 antibody, nucleic acid encoding an antibody, e.g., as described above, is isolated and inserted into one or more vectors for further cloning and/or expression in a host cell. Such nucleic acid may be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody).

Suitable host cells for cloning or expression of antibody-encoding vectors include prokaryotic or eukaryotic cells described herein. For example, antibodies may be produced in bacteria, in particular when glycosylation and Fc effector function are not needed. For expression of antibody fragments and polypeptides in bacteria, see, e.g., U.S. Patent Nos. 5,648,237, 5,789,199, and 5,840,523. (See also Charlton, *Methods in Molecular Biology, Vol. 248* (B.K.C. Lo, ed., Humana Press, Totowa, NJ, 2003), pp. 245-254, describing expression of antibody fragments in *E. coli.*) After expression, the antibody may be isolated from the bacterial cell paste in a soluble fraction and can be further purified.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been "humanized," resulting in the production of an antibody with a partially or fully human glycosylation pattern. See Gerngross, *Nat. Biotech.* 22:1409-1414 (2004), and Li et al., *Nat. Biotech.* 24:210-215 (2006).

Suitable host cells for the expression of glycosylated antibody are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures can also be utilized as hosts. *See,* e.g 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIES™ technology for producing antibodies in transgenic plants).

Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293 cells as described, e.g., in Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK); mouse sertoli cells (TM4 cells as described, e.g., in Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK; buffalo rat liver cells (BRL 3A); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); TRI cells, as described, e.g., in Mather et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982); MRC 5 cells; and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including DHFR$^-$ CHO cells (Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); and myeloma cell lines such as Y0, NS0 and Sp2/0. For a review of certain mammalian host cell lines suitable for antibody production, see, e.g., Yazaki and Wu, *Methods in Molecular Biology, Vol. 248* (B.K.C. Lo, ed., Humana Press, Totowa, NJ), pp. 255-268 (2003).

C. Assays

Anti-Notch2 antibodies provided herein may be identified, screened for, or characterized for their physical/chemical properties and/or biological activities by various assays known in the art.

1. Binding assays and other assays

In one aspect, an antibody of the invention is tested for its antigen binding activity, e.g., by known methods such as ELISA, Western blot, etc.

In another aspect, competition assays may be used to identify an antibody that competes with Antibody D, Antibody D-1, Antibody D-2, or Antibody D-3 for binding to Notch2. In certain embodiments, such a competing antibody binds to the same epitope (e.g., a linear or a conformational epitope) that is bound by Antibody D, Antibody D-1, Antibody D-2, or Antibody D-3. Detailed exemplary methods for mapping an epitope to which an

antibody binds are provided in Morris (1996) "Epitope Mappin
Molecular Biology vol. 66 (Humana Press, Totowa, NJ).

In an exemplary competition assay, immobilized Notch2 is incubated in a solution comprising a first labeled antibody that binds to Notch2 (e.g., Antibody D, Antibody D-1, Antibody D-2, or Antibody D-3) and a second unlabeled antibody that is being tested for its ability to compete with the first antibody for binding to Notch2. The second antibody may be present in a hybridoma supernatant. As a control, immobilized Notch2 is incubated in a solution comprising the first labeled antibody but not the second unlabeled antibody. After incubation under conditions permissive for binding of the first antibody to Notch2, excess unbound antibody is removed, and the amount of label associated with immobilized Notch2 is measured. If the amount of label associated with immobilized Notch2 is substantially reduced in the test sample relative to the control sample, then that indicates that the second antibody is competing with the first antibody for binding to Notch2. See Harlow and Lane (1988) *Antibodies: A Laboratory Manual* ch.14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

2. Activity assays

In one aspect, assays are provided for identifying anti-Notch2 antibodies thereof having biological activity. Biological activity may include, e.g., inhibition or reduction of Notch2 activity, e.g., Notch2 signaling. Antibodies having such biological activity in vivo and/or in vitro are also provided.

In certain embodiments, an anti-Notch2 NRR antibody of the invention is tested for its ability to inhibit generation of marginal zone B cells. An exemplary assay is provided in the Examples. In certain other embodiments, an antibody of the invention is tested for its ability to inhibit expression of a reporter gene that is responsive to Notch2 signaling.

D. Immunoconjugates

The invention also provides immunoconjugates comprising an anti-Notch2 antibody herein conjugated to one or more cytotoxic agents, such as chemotherapeutic agents or drugs, growth inhibitory agents, toxins (e.g., protein toxins, enzymatically active toxins of bacterial, fungal, plant, or animal origin, or fragments thereof), or radioactive isotopes.

In one embodiment, an immunoconjugate is an antibody-drug conjugate (ADC) in which an antibody is conjugated to one or more drugs, including but not limited to a maytansinoid (see U.S. Patent Nos. 5,208,020, 5,416,064 and European Patent EP 0 425 235

B1); an auristatin such as monomethylauristatin drug moieties I
MMAF) (see U.S. Patent Nos. 5,635,483 and 5,780,588, and 7,498,298); a dolastatin; a calicheamicin or derivative thereof (see U.S. Patent Nos. 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, and 5,877,296; Hinman et al., *Cancer Res.* 53:3336-3342 (1993); and Lode et al., *Cancer Res.* 58:2925-2928 (1998)); an anthracycline such as daunomycin or doxorubicin (see Kratz et al., *Current Med. Chem.* 13:477-523 (2006); Jeffrey et al., *Bioorganic & Med. Chem. Letters* 16:358-362 (2006); Torgov et al., *Bioconj. Chem.* 16:717-721 (2005); Nagy et al., *Proc. Natl. Acad. Sci. USA* 97:829-834 (2000); Dubowchik et al., *Bioorg. & Med. Chem. Letters* 12:1529-1532 (2002); King et al., *J. Med. Chem.* 45:4336-4343 (2002); and U.S. Patent No. 6,630,579); methotrexate; vindesine; a taxane such as docetaxel, paclitaxel, larotaxel, tesetaxel, and ortataxel; a trichothecene; and CC1065.

In another embodiment, an immunoconjugate comprises an antibody as described herein conjugated to an enzymatically active toxin or fragment thereof, including but not limited to diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes.

In another embodiment, an immunoconjugate comprises an antibody as described herein conjugated to a radioactive atom to form a radioconjugate. A variety of radioactive isotopes are available for the production of radioconjugates. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu. When the radioconjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example tc99m or I123, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, mri), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

Conjugates of an antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-

azido compounds (such as bis (p-azidobenzoyl) hexanediamine
(such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., *Science* 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of a cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari et al., *Cancer Res.* 52:127-131 (1992); U.S. Patent No. 5,208,020) may be used.

The immunuoconjugates or ADCs herein expressly contemplate, but are not limited to such conjugates prepared with cross-linker reagents including, but not limited to, BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (e.g., from Pierce Biotechnology, Inc., Rockford, IL., U.S.A).

III. EXAMPLES

Example 1: Isolation and transcriptional profiling of liver progenitor cells.

To identify the signals that regulate hepatocyte differentiation from liver progenitor cells, transcriptional profiling was performed on adult liver progenitors (oval cells) from mice fed a choline deficient, ethionine supplemented (CDE) diet (FIG. 1A; FIG. 5A). This CDE model is known in the art as a model of chronic liver disease. CDE is also a steatohepatitis model (NASH). Chronic CDE can also lead to hepatocellular carcinoma (HCC), thereby also serving as a model for HCC. To induce an oval cell response, 8-12 week-old female C57BL/6N mice (Charles River) were fed a choline deficient diet (20% Lard; Teklad TD.04523) supplemented with 0.15% (w/v) Ethionine in the drinking water (Akhurst *et al.*, Hepatology 34(3):519 (2001)). Liver non-parenchymal cells were isolated according to the protocol of del Castillo (del Castillo *et al.*, Am. J. Pathol., 172(5):1238 (2008)) with the addition of 0.04% Hyaluronidase (Sigma) to the *in vitro* dissociation step.

Epithelial Cell Adhesion Molecule (EpCAM)-expressing progenitor cells and normal bile duct cells from livers of CDE-fed and control mice were isolated by fluorescence activated cell sorting (FACS; FIG. 4A-B). C57BL/6 mouse livers were perfused with a Collagenase/Pronase solution and the liver was dissociated and further incubated in the

presence of DNase and Hyaluronidase (FIG. 4B). The resulting
over a 30%/70% Percoll density gradient by centrifugation at 2500 RPM for 30 minutes. Cells from the 30%/70% Percoll interface, consisting mostly of non-parenchymal cells, were stained with fluorescently labeled antibodies to EpCAM (BioLegend) and CD45 (BD Pharmingen). Flow cytometry was used to isolate EpCAM^{+}/CD45^{-} cells from mice fed CDE or standard rodent diet. The majority of EpCAM^{+}/CD45^{-} cells from CDE-fed mice were oval cells, while the majority of EpCAM^{+}/CD45^{-} cells from standard diet-fed mice were bile duct cells. QRT-PCR analysis on RNA from FACS sorted cells confirmed that EpCAM^{+}/CD45^{-}-sorted cells were greatly enriched for EpCAM as well as CK19 (FIG. 4B), indicating a successful positive selection of EpCAM^{+} progenitor and bile duct cells.

In addition to isolating progenitor cells by FACS sorting, progenitor and normal bile duct cells were isolated by laser capture microdissection (LCM) from hematoxylin and eosin (H&E) stained liver sections. Livers from C57BL/6N 8-12 week-old female mice fed normal chow or CDE diet were removed and immediately flash frozen in liquid Nitrogen. Flash frozen liver pieces were placed in prechilled plastic molds, embedded in TISSUE-TEK OCT Compound (Sakura, The Netherlands) and immediately placed on a dry ice/2-methylbutane bath until frozen. The embedded frozen liver pieces were cut into 7-8 μm sections at -14°C, adhered to metal frame membrane slides (MMI, Eching, Germany), fixed, stained with hematoxylin and eosin, and dried. Laser microdissection was used to isolate 1-2 mm^2 of oval cell or normal bile duct tissue per sample.

RNA from flow-sorted and laser microdissected tissue was isolated using the RNEASY Micro Kit (Qiagen). For microarray analysis RNA from flow-sorted and laser microdissected tissue, as well as whole liver controls, spiked with Agilent RNA Spike-In RNA (Agilent), was submitted to two rounds of amplification with Message AmpII (Ambion) and hybridized to Whole Mouse Genome Oligo 44k microarrays. Log expression ratios were exported and analyzed using Partek Genomic Suite (Partek). Quantitative real-time PCR (QRTPCR) was performed using the TAQMAN One-Step RT-PCR Kit for one step reactions using the 7900 HT RT-PCR system (Applied Biosystems) with TAQMAN probes (Applied Biosystems) or High Capacity cDNA RT kit with TAQMAN Fast Advanced Master Mix using the Viia7 RT-PCR system (Applied Biosystems) with custom designed low density arrays.

Enrichment of bile duct and progenitor cell-associated transcripts, such as EpCAM and Keratin19, confirmed effective isolation of bile duct and progenitor cells (FIG. 1E).

Using supervised analysis (FIG. 5B), genes expressed more hig
than in
closely related normal bile duct cells were identified (Table 2). This supervised analysis correlated highly with progenitor cell associated transcripts identified by unbiased principle components analysis (FIG. 5C-H). The expression pattern of these genes was validated on independent samples by QRT-PCR (FIG. 6) and immunofluorescence, which confirmed that members of the Notch signaling pathway, including Jag1 and Notch2, as well as Hes1 and Hey1, among other target genes, were upregulated in liver progenitor cells compared to bile duct cells (FIG. 1C-G). A selection of microarray probes for Notch pathway-associated transcripts distinguished flow-sorted oval cells from flow-sorted biliary cells and whole liver by supervised clustering (FIG. 1B). Supervised hierarchical clustering of 29 Notch pathway-associated genes, including receptors, ligands, transcription factors, and select target genes, differentiated whole liver, bile ducts, and oval cells into separate clusters (Rand index=1, 3 clusters; Rand index=0.7607, 3 clusters, for randomly generated list of 29 genes) and revealed differences between normal bile duct cells and progenitors. The data showed significant upregulation of Jag1(p=0.0006; FIG. 1C), Notch2 (p=0.0006; FIG. 1D), Hes1(p=0.0035; FIG. 1E), and Hey1(p=0.0087; FIG. 1F) in liver progenitor cells. Immunofluorescence staining confirmed that Jag1 was more highly expressed in $EpCAM^{+}$ oval cells radiating out from the portal vein in a CDE liver than in adjacent normal bile duct cells. Immunhistochemistry for Hes1 confirmed that Hes1 positive cells were largely confined to the oval cell and bile duct compartments, with some staining in other non-parenchymal cells of the liver (FIG. 1G).

UNQ_Short_Name	ProbeID	t-statistic	pvalue	
ADAMTS9	A_52_P49321	5.272	0	2.132
ANXA9	A_51_P451482	6.839	0	2.578
APP	A_52_P381311	5.045	0	2.531
BMP8B	A_51_P411926	6.273	0	3.562
CHRNB1	A_51_P475342	5.32	0	1.912
CTGF	A_51_P157042	7.146	0	3.336
DTNA	A_52_P108607	5.585	0	3.558
Embigin	A_51_P382849	6.196	0	3.246
Epdr2	A_52_P577388	5.703	0	2.431
EPHA7	A_52_P504787	5.855	0	2.255
FADS3	A_52_P451796	5.809	0	2.163
Foxc1	A_51_P107686	5.098	0	2.77
GSPT1	A_52_P354785	5.672	0	2.726
Hig2l	A_52_P321150	5.522	0	1.696
ID2	A_52_P240542	5.054	0	1.504
Ifrd1	A_51_P367060	5.888	0	1.609
JAG1	A_52_P634090	5.368	0	2.377
LTB	A_51_P302358	6.15	0	2.428
MAL	A_52_P562661	5.619	0	3.02
Mex3a	A_52_P706060	5.228	0	3.582
MFI2	A_51_P324351	7.735	0	3.397
MYC	A_51_P102096	5.498	0	1.522
NFAM1	A_52_P686701	5.472	0	2.09
NFKB1	A_52_P32733	5.348	0	2.398
Nrarp	A_51_P504354	5.261	0	2.585
peg3	A_51_P206037	6.329	0	2.84
RASL11A	A_51_P340699	5.425	0	2.303
SLIT2	A_51_P496569	5.964	0	3.261
SPATA7	A_52_P134680	5.187	0	1.926
SPRR1A	A_51_P139678	7.825	0	2.8
TNFAIP8	A_51_P435968	6.322	0	2.403
TNFRSF12A	A_51_P131408	6.641	0	2.397
tp53	A_52_P957260	5.399	0	2.388
TRIM47	A_51_P437176	5.633	0	1.592
Trio	A_51_P319662	6.257	0	2.81
TTYH1	A_52_P475052	5.668	0	2.442
USP47	A_52_P610967	5.021	0	2.018
VCAM1	A_51_P210956	5.696	0	1.847

Table 2: Oval Cell Associated Genes

Example 2: Identification of expression patterns of oval cell-associated genes.

5 The microarray data from RNA isolated from oval and bile duct cells isolated FACS (Flow) or microdisection (LCM) and control and CDE livers were examined for the expression of putative markers of oval cells and markers of other select cell types. Albumin

transcript was detected at relatively high levels in all groups (FI
marker of immature hepatocytes that is upregulated during chronic liver damage, was greatly enriched in LCM oval cells, but virtually absent from the other cell types examined (FIG. 6A-1). To determine why AFP was present only in LCM oval cells, immunofluorescence was performed on 7μm frozen CDE liver sections that had been briefly air-dried and fixed in 4% paraformaldehyde in PBS. After blocking with normal horse serum in PBS, sections were incubated with fluorescently labeled antibodies to EpCAM (BioLegend), Sca1 (BD Pharmingen), CD90 (BioLegend), or with unlabeled primary antibodies to AFP (R&D Systems) and CK19 (Santa Cruz Biotech) followed by incubation with fluorescently labeled secondary antibodies (Invitrogen). AFP was expressed in only a subset of hepatocytes, often in close proximity to $EpCAM^{+}$ oval cells. However, AFP expression could not be observed in oval cells themselves.

LCM isolates expressed high levels of the myofibroblast marker SMA and the mesenchymal cell marker CD90/Thy1 (FIG. 6A-3), possibly as a result of inclusion of periportal myofibroblasts, which are positive for both CD90/Thy1 and SMA. Thus, it appears that the LCM samples contain a heterogeneous mixture of cell types that at least include myofibroblasts, and in the case of LCM samples from CDE livers, AFP positive hepatocytes adjacent to cords of microdissected oval cells. Though Sca1 also marks mesenchymal cells, it also appears to be expressed in bile duct cells and oval cells themselves, as the transcript is found at high levels in both the FACS-sorted and LCM samples (FIG. 6A-2). The oval cell markers CD13, Sox9, FoxL, and FoxJ1 were also expressed in both FACS-sorted and LCM samples. Except for Sox9, which was more highly expressed in FACS oval cells, each of these markers was expressed at comparable levels in CDE oval cells and in normal bile ducts. Expression patterns of oval cell-associated genes and genes marking other hepatic cell types were confirmed in independent samples by QRT-PCR. For these experiments, CDE oval cells were enriched by Magnetically Activated Cell Sorting (MACS), first by depleting $CD45^{+}$ cells from the lower band of a 30%/70% Percoll gradient followed by positive selection for $EpCAM^{+}$ cells (FIG. 6B). Purity of the resulting cell suspensions was >95%. **c**, Relative to CD45-/EpCAM- cells, CD45-/EpCAM+ cells expressed high levels of EpCAM, CK19, Trop2, and CD133 and low levels of AFP, LGR5, CD90, and Vimentin (FIG. 6C). Albumin was expressed at comparable levels in $CD45^{-}/EpCAM^{-}$ and $CD45^{-}/EpCAM^{+}$ fractions.

Example 3: Notch signaling in liver progenitors *in vitro*

To elucidate the role of Notch signaling in liver regeneration, an *in vitro* culture system was developed. Cell cultures were established by culturing primary oval cells on Sw-3T3 fibroblasts (ATCC), arrested with Mitomycin C (Sigma), in High Glucose Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 15% Fetal Calf Serum (Sigma), non-essential amino acids (Invitrogen), Glutamax (Invitrogen), and ITS (Invitrogen).

The anti-activated Notch2 antibody (clone 40-2-7) was generated against the peptide VIMAKRKRKHGSLW, corresponding to amino acids 1697-1710 of the human Notch2 protein sequence (SEQ ID NO:73), coupled to KLH (YenZym Custom Antibodies, LLC). Splenocytes from a rabbit producing an antibody with the appropriate specificity were used to generate hybridomas (Epitomics, Inc.). Clone 40-2-7 was identified by screening the resulting rabbit monoclonal antibodies by immunoblotting and immunohistochemistry. This antibody recognizes both human (FIG. 7B, left panel) and mouse (FIG. 7B, right panel) active Notch2 at endogenous levels.

Progenitors cultured in the culture system maintained growth (FIG. 2A) and formed colonies consisting of small, tightly packed cells with a high nuclear-cytoplasmic ratio and a distinct raised edge (FIG. 2A, left panel). The cells within these colonies were uniformly EpCAM positive (FIG. 2A, right panel). Progenitor cultures also maintained the characteristic progenitor expression signature *in vitro* (FIG. 2C). Activated Notch2 was detected by Western blot analysis using a rabbit monoclonal antibody raised against the S3 cleaved form of the human Notch2 protein (FIG. 7B). Activated Notch2 signal was increased upon ligand stimulation (Jag) or EDTA stimulation (EDTA), and the activated form was greatly enriched in the nuclear fraction (FIG. 7B; lanes "N").

Treatment of the primary cultures with the γ-secretase and Notch pathway inhibitor N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester (DAPT) unexpectedly enhanced colony formation approximately ten-fold, from approximately 1 colony formed per 100,000 plated CD45 negative, non-parenchymal cells to approximately 1 colony per 10,000 cells plated (FIG. 2B) suggesting that inhibition of Notch pathway activity promoted either liver progenitor cell maintenance or proliferation. Treatment with DAPT resulted in a small increase in progenitor cell proliferation (FIG. 2D).

To determine if Notch signaling inhibition also suppresses the differentiation of progenitor cells, thereby allowing for long-term maintenance, the biliary and hepatic differentiation potential of these cells *in vitro* was analyzed (FIG. 7). Cultured oval cells

were maintained on a mitomycin-C treated feeder layer of Swis
the presence of 15% Fetal Bovine Serum (FBS) and the γ-secretase inhibitor DAPT.

Differentiation along the hepatocyte lineage was induced by plating oval cells without feeder cells onto tissue-culture treated plastic coated with diluted Rat Tail Collagen (BD) in the presence of 10 ng/ml Oncostatin M (R&D) and 25 ng/mL HGF (Lonza). For some experiments, DAPT or vehicle (DMSO) and/or the anti-Notch2 NRR antibody, Antibody D-3 (also referred to herein as anti-N2, anti-Notch2, or anti-NRR2) or an isotype control antibody were added to the medium at the time of cell plating and replenished every three days. Bile duct differentiation was induced by suspending oval cells in a 1:1 mixture of MATRIGEL and oval cell growth medium supplemented with 7.5% FBS and plating onto plastic tissue culture dishes. Following solidification, the MATRIGEL cultures were overlain with growth medium supplemented with 15% FBS. In some experiments, DAPT or vehicle and/or anti-Notch2 antibody or isotype control were added to the MATRIGEL as well as the overlying medium, which was replenished every three days.

Progenitor cells that were cultured on a collagen substrate in the presence of Hepatocyte Growth Factor and Oncostatin M displayed a changed cellular morphology consistent with hepatocyte differentiation, including larger cell size, lower nuclear-cytoplasmic ratio, and two nuclei (FIG. 2E-F). Hepatocyte-associated transcripts Albumin and α-Fetoprotein (AFP; FIG. 2G) were also increased in these cells. Notch2 signaling was active in cultured oval cells, as transcriptionally active Notch2 intracellular domain (ICD) could be detected using an antibody specific to the γ-secretase cleaved form of this receptor (FIG. 2H). The appearance of the cleaved form was dependent on γ-secretase activity, as it was absent from DAPT treated cells (FIG. 2H). Specific binding of an anti-Notch2 inhibitory antibody also blocked formation of this active form of Notch2 (FIG. 2H). As expected, treatment with the Notch2 inhibitory antibody leads to a decrease in Notch target gene Hes1 (p=6E-05) (FIG. 2I). Surprisingly, progenitor cells that were cultured on a collagen substrate in the presence of Hepatocyte Growth Factor and Oncostatin M in the presence of an anti-Notch2 inhibitory antibody (Wu *et al.*, Nature 464(7291):1052 (2010)) (FIG. 2H-I) resulted in a more pronounced hepatocyte-like morphology (FIG. 2 K) and increased albumin expression level (FIG. 2L), compared to cells cultured with the isotype control (FIG. 2J, L).

In contrast, differentiation of cultured oval cells along the biliary lineage, assessed in three dimensional culture, was decreased by inhibition of Notch2. Cells cultured in the presence of an anti-Notch2 inhibitory antibody displayed a less differentiated morphology

(FIG. 2N) and a 50% decreased expression of the biliary marke
05) (FIG. 2O) compared to cells grown in the absence of the inhibitory antibody (FIG. 2M, O). Together, these results are consistent with the notion that Notch2 inhibition biases differentiation away from the biliary lineage and toward hepatocyte formation.

Example 4: Inhibition of Notch2 signaling *in vivo*.

To determine the effect of Notch2 inhibition in liver damage, a rodent model of liver damage was employed. Mice were partially hepatectomized by removal of left lateral and median lobes according to Yokoyama *et al.* (Yokoyama *et al.*, Cancer Research 13(1):80-85 (1953)), which results in compensatory proliferation of hepatocytes and recovery of liver mass within 7-10 days (Higgins and Anderson, Arch. Pathol., 12:186 (1931); Yokoyama *et al.*, Cancer Res. 13(1):80 (1953)). Mice were injected intraperitoneally with an anti-Notch2 NRR antagonist antibody (Wu *et al.*, Nature 464(7291):1052 (2010)) or an anti-Ragweed isotype control antibody at a dose of 5mg/kg twice per week, including twice prior to hepatectomy. Two hours prior to liver harvest, mice were injected intraperitoneally with Bromodeoxyuridine at 50mg/kg. Immunohistochemistry was performed on 5μm liver sections using antibodies for BrdU (DAKO), pan-Cytokeratin (WSS; DAKO), and Hes1 (MBL International). Antibody binding was detected using standard streptavidin-HRP/DAB for BrdU and pan-Cytokeratin and tyramide signal amplification (TSA)/DAB for Hes1.

Treatment with anti-Notch2 antibody resulted in effective Notch2 inhibition as determined by significant splenic Marginal Zone B-cell (MZB) depletion ($p<0.0001$, FIG. 8A-C) (Wu *et al.*). MZB represent approximately 5% of the splenic B lymphocytes in normal C57Bl/6 mice. Treatment with Notch2 antagonist (5mg/kg, 2x/week) resulted in a virtual disappearance of MZB (FIG. 8A-C). Notch2 inhibition was maintained through the course of partial hepatectomy experiments as indicated by persistent depression in MZB population (FIG. 8C). Inhibition of Notch2 did not significantly alter the rate of liver mass recovery immediately following partial hepatectomy (FIG. 8D), which in early stages is due largely to hepatocyte hypertrophy and division of pre-existing polyploid hepatocytes (St. Aubin and Bucher, The Anatomical Record 112(4):797 (1952); Higgins and Ingle, The Anatomical Record,. 73(1):95 (1939)). Notch2 inhibition caused a small decrease in overall BrdU incorporation at 40 hours (FIG. 8E; ($p=0.027$)) and a much larger and more significant decrease in BrdU incorporation in intrahepatic bile ducts (FIG. 8F), suggesting that anti-Notch2 treatment affected liver progenitor cells within the bile ducts. Consistent with this observation, expression of the Notch target gene Hes1 was detected primarily in intra-hepatic

bile duct cells (FIG. 3C-1) rather than in hepatocytes. Moreove
intrahepatic bile duct cells was significantly reduced in anti-Notch2 treated mice 40 hours after surgery, compared to control antibody treated mice, and in many portal areas Hes1 staining was not observed (FIG. 3C-2). Despite the reduction in Hes1-postive cells, bile duct morphology was unaffected (FIG. 3, compare panels A-1-D-1 on left (isotype) to panels A-2-D-2 on right (anti-Notch2)) and no significant elevation of markers of biliary dysfunction was observed, even after one month of ongoing Notch2 inhibition (FIG. 11F-H).

Example 5: Effects of Notch2 inhibition on liver regeneration *in vivo.*

To determine whether Notch2 inhibition affects the recovery of hepatocyte function following partial hepatectomy, serum hepatobiliary function markers were assessed following 2/3 partial hepatectomy in mice treated with anti-Notch2 or control antibody. Unexpectedly, recovery in liver function began earlier in anti-Notch2-treated animals compared to controls. Serum albumin levels started increasing by 40 hours post surgery (FIG. 9A) and reached significantly ($p<0.05$) higher levels in anti-Notch2 antibody-treated mice at both the 40 hour and 72 hour time points (FIG. 3E; FIG. 9A), compared to serum levels in control animals that started to increase by 72 hours after surgery (2.8 versus 2.4 g/dL, $p<0.02$). This improvement in recovery of pre-operative serum albumin levels was accompanied by reduced expression of Hes1 ($p<0.02$; FIG. 3F). These results suggest that Notch2 inhibition results in enhanced recovery of hepatocyte function. Markers of hepatocyte damage were not significantly different between treatment and control groups (FIG. 5B-F**.** However, a consistent and significant increase in the ratio of albumin to K19 transcripts, referred to herein as Differentiation Quotient, was observed (FIG. 10E). The average Differentiation Quotient of anti-Notch2 antibody-treated livers, normalized to pre-surgical levels, recovered more quickly, regaining 75% of pre-surgical values 3 days after surgery and 100% of pre-surgical values between 3 and 6 days after surgery (FIG. 10E). In contrast, Differentiation Quotient values in isotype control antibody-treated animals did not recover to pre-surgical levels until 14 days after surgery (FIG. 10E). These results suggest that inhibition of Notch2 biases the differentiation of bipotent liver progenitor cells away from the biliary (K19-positive) towards the hepatic (albumin-positive) lineage. Differences between anti-Notch2 and isotype control antibody-treated livers in apparent de novo hepatocyte formation could be detected as early as 24 hours after partial hepatectomy. Transcript levels of in the form of the immature hepatocyte marker alpha-fetoprotein (AFP) were significantly elevated in anti-Notch2 antibody-treated mice (FIG. 10B). Also, morphological and functional differences between

anti-Notch2 and control antibody-treated livers persisted for up
anti-Notch2 antibody-treated livers appeared more robust, with a noticeably more uniform parenchymal architecture (FIG. 3A-2) compared to control-treated livers (FIG 3A-1).

The effects of Notch2 inhibition on liver regeneration were also studied in a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) model of chronic liver disease. This DDC model is known in the art as a model of chronic liver disease. The mechanism of liver damage in response to DDC is abnormal heme metabolism with accumulation of protoporphyrin which is toxic to the hepatocytes. Thus, the DDC model can also serve as model for hereditary or acquired defects in the heme metabolic pathway.

C57BL/6N female mice 8-12 weeks of age (Charles River) were fed a choline deficient diet (20% Lard; Teklad TD.04523) supplemented with 0.15% (w/v) Ethionine (supplier) in the drinking water to induce oval cells (Akhurst *et al*., Hepatology 34(3):519 (2001)). The prolonged hepatotoxic influence of a DDC diet led to a proliferation of cytokeratin (CK)19-positive progenitor cells (FIG 3D-1), referred to as oval cell response, reminiscent of the ductular reaction common in human hepatobiliary disease (Farber, Cancer Research, 16(2):142 (1956)). After four weeks of DDC, the oval cell reaction had increased such that CK19-positive oval cells occupied an average of about 15% (10-20%) of the total hepatic cross sectional area (FIG. 3D-1; FIG. 3G), while serum markers of hepatobiliary injury were greatly elevated ($p<0.0001$, FIG. 3H; FIG. 11 A-H). However, treatment with the anti-Notch2 inhibitory antibody significantly impeded the oval cell reaction (FIG. 3D-2), reducing the average cross-sectional area of CK19-positive tissue from approximately 15% to only 5% of total liver cross sectional area ($p<0.0001$, FIG. 3G). Despite this striking reduction in oval cell proliferation, hepatic architecture was not adversely affected by anti-Notch2 antibody treatment and was grossly indistinguishable from control-treated tissue (FIG. 3D-2). The decrease in CK19-positive oval cells associated with Notch 2 inhibition was accompanied by improved hepatobiliary function, with significantly decreased total and direct serum bilirubin levels, a marker of cholestasis and other forms of hepatobiliary damage ($p=0.0003$, FIG. 3H). Also, the Differentiation Quotient was significantly elevated in livers from mice treated with the anti-Notch2 antibody ($p<0.0001$, FIG. 3J) suggesting improved hepatocyte function. Inhibition of Notch2 signaling by treatment with an anti-Notch2 antibody was confirmed by a greater than 70% reduction in Hes1 expression in the biliary and progenitor cells in anti-Notch2 antibody-treated livers ($p<0.0001$, FIG. 3I), reflecting the central role of Notch2 signaling in governing oval cell fate choice.

Taken together, these results show that treatment with a
facilitates the recovery of liver function after two different types of liver damage, one by partial hepatectomy and one by chemical damage (choline-limiting diet). Mechanistically, anti-Notch2 NRR antibody facilitates liver recovery by favoring hepatocyte differentiation
5 and by preventing aberrant (or pathologic) bile duct proliferation. Accordingly, treatment with anti-Notch2 NRR antibody could, *e.g.*, prevent progression of chronic liver disease, such as liver fibrosis.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and
10 examples should not be construed as limiting the scope of the invention. The disclosures of all patent and scientific literatures cited herein are expressly incorporated in their entirety by reference.

WHAT IS CLAIMED IS:

1. A method of treating a liver condition characterized by liver damage, the method comprising administering to a patient having such condition an effective amount of a Notch2-specific antagonist.

2. The method of claim 1, wherein the liver condition is chronic liver disease.

3. The method of claim 2, wherein the chronic liver disease is liver fibrosis.

4. The method of claim 2 wherein the chronic liver disease is liver cirrhosis.

5. The method of claim 1, wherein the liver condition is an acute liver condition.

6. The method of claim 5, wherein the acute liver condition is acetaminophen toxicity.

7. The method of claim 1, wherein the Notch2-specific antagonist is an anti-Notch2 antagonist antibody.

8. The method of claim 7, wherein the anti-Notch2 antagonist antibody is an anti-Notch2 NRR antibody.

9. The method of claim 8, wherein the anti-Notch2 NRR antibody binds to the LNR-A and HD-C domains of Notch2 NRR.

10. The method of claim 8, wherein the anti-Notch2 NRR antibody is Antibody D, Antibody D-1, Antibody D-2, or Antibody D-3.

11. The method of claim 8, wherein the anti-Notch2 NRR antibody comprises the heavy and light chain variable region CDRs of Antibody D, Antibody D-1, Antibody D-2, or Antibody D-3.

12. The method of claim 7, wherein the anti-Notch2 antagonist antibody is an anti-Notch2 antibody that binds to one or more EGF-like repeats of Notch2.

13. The method of claim 1, wherein the Notch2-specific antagonist is administered by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation route.

14. A method of inducing hepatic differentiation, the m
contacting an oval cell with an effective amount of a Notch2-specific antagonist, thereby inducing hepatic differentiation of the oval cell.

15. The method of claim 14, wherein the oval cell is contacted with the Notch2-
5 specific antagonist *in vitro*.

16. The method of claim 14, wherein the oval cell is contacted with the Notch2-specific antagonist *in vivo*.

17. A method of reducing aberrant bile duct proliferation in a patient in need thereof, the method comprising the step of administering to the patient an effective amount of a
10 Notch2-specific antagonist, thereby reducing aberrant bile duct proliferation.

Whole Liver
Oval Cells
Bile Duct Cells
Oval Cells

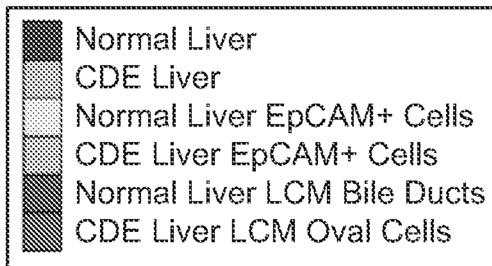


FIG. 1A

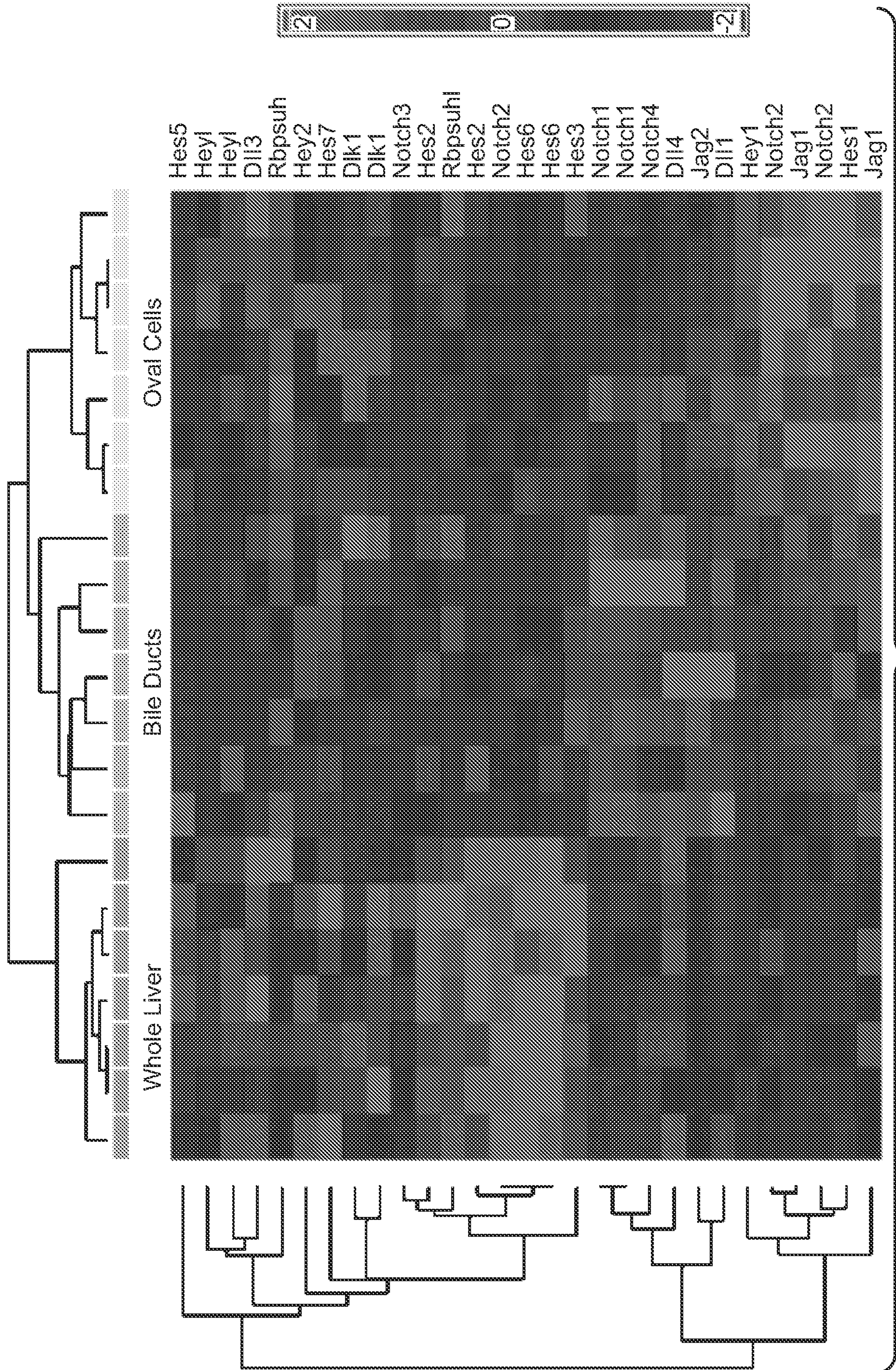


FIG. 1B

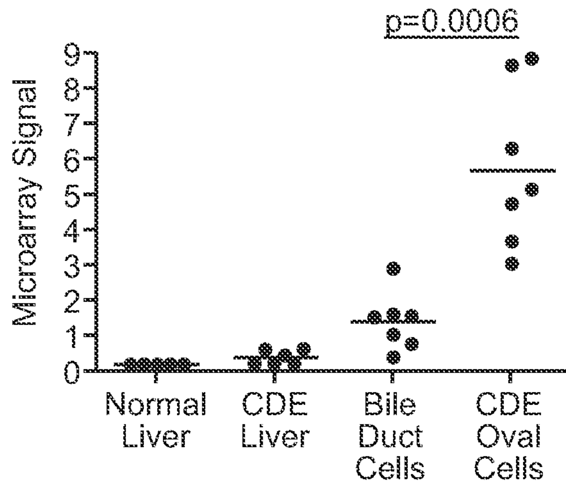


FIG. 1C

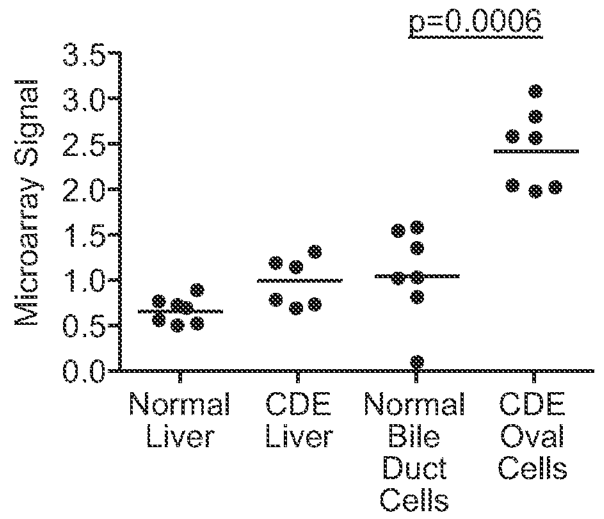


FIG. 1D

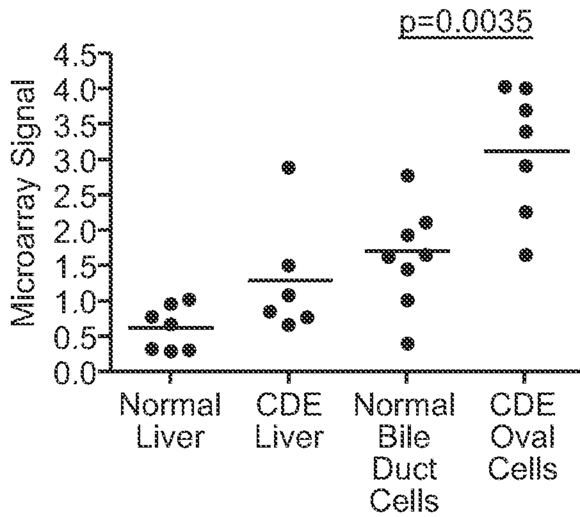


FIG. 1E

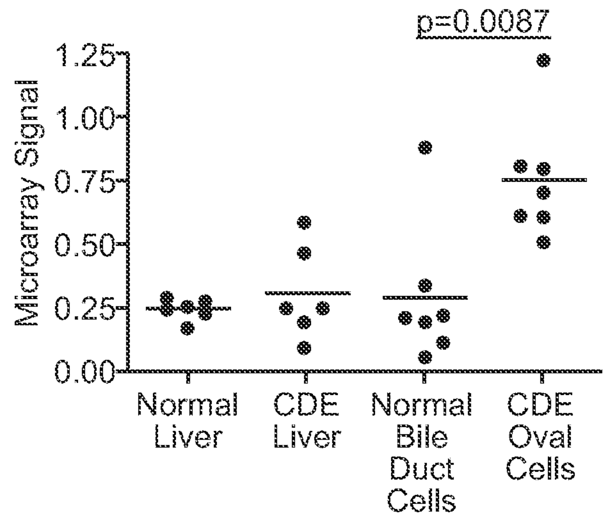


FIG. 1F

Hes1 IHC

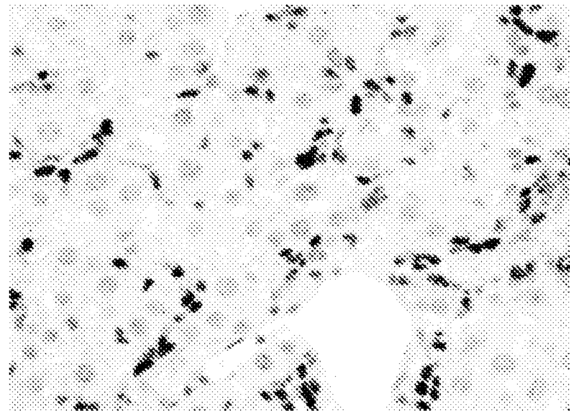
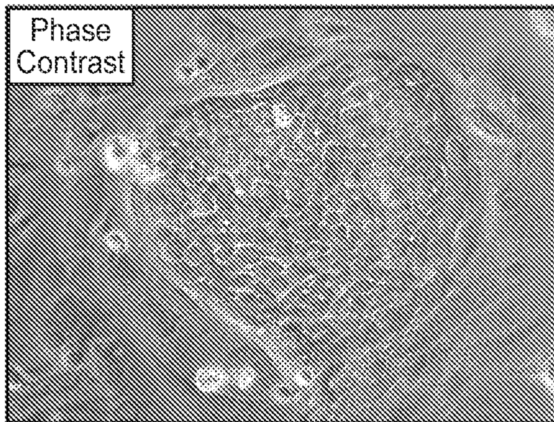


FIG. 1G



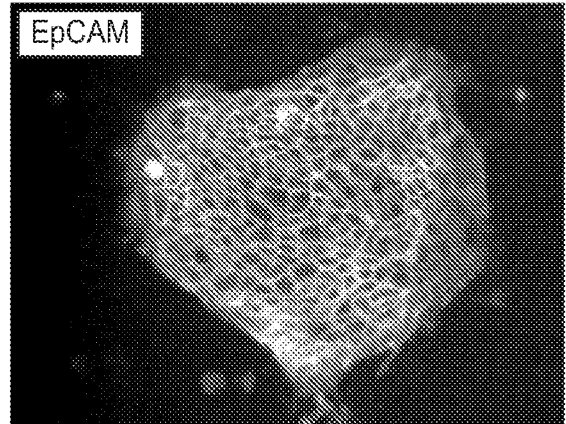


FIG. 2A

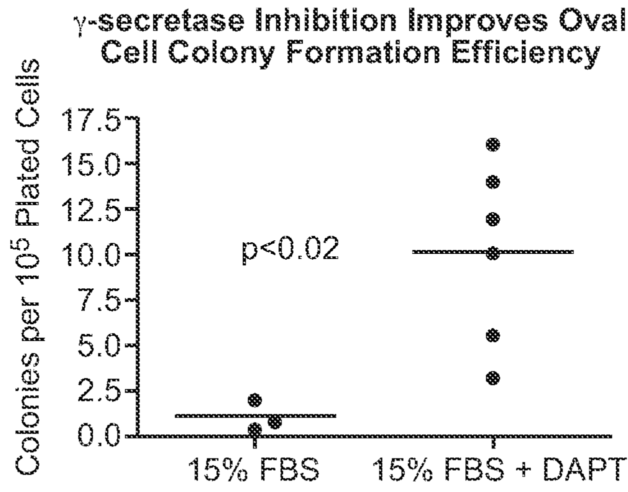


FIG. 2B

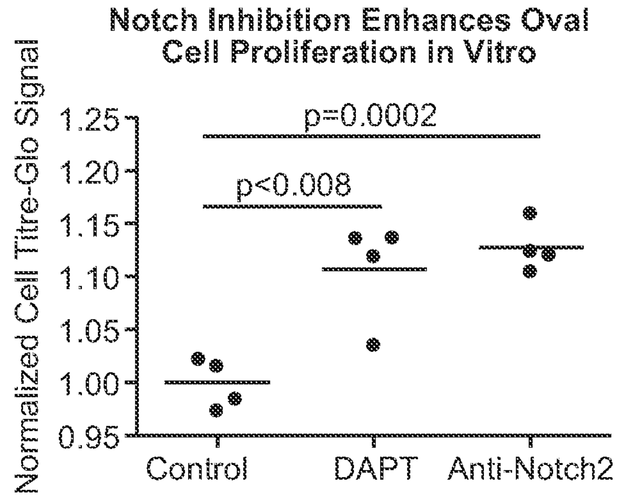


FIG. 2D

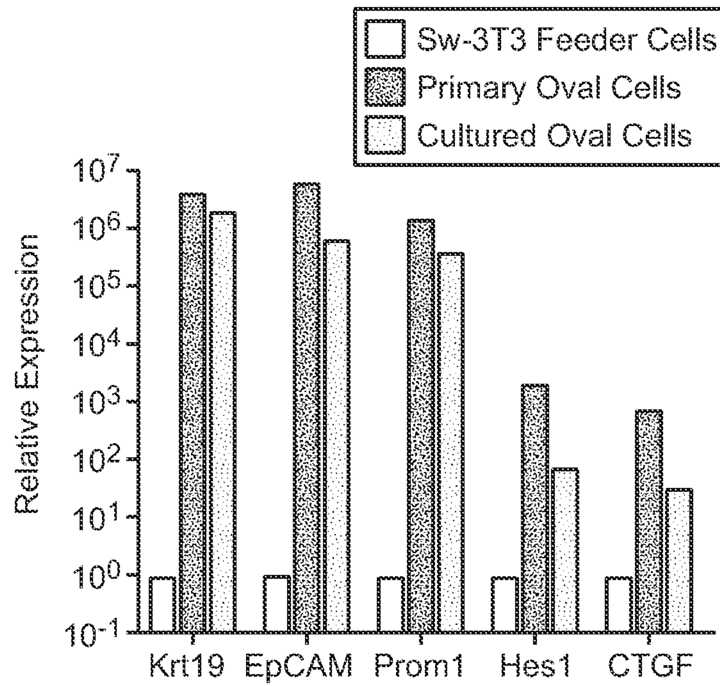


FIG. 2C

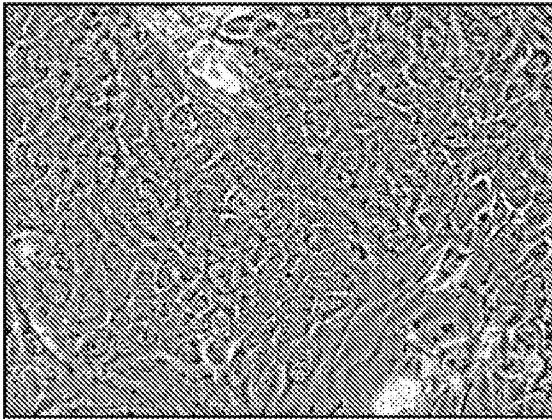


FIG. 2E

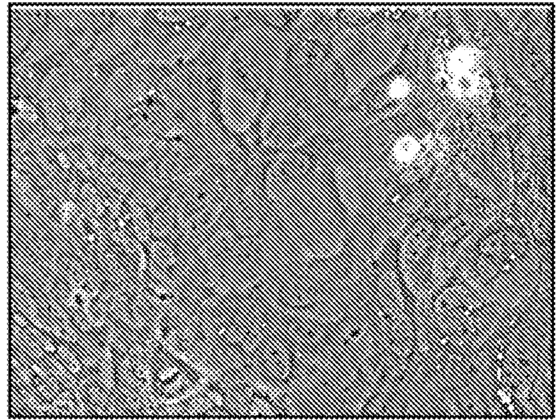


FIG. 2F

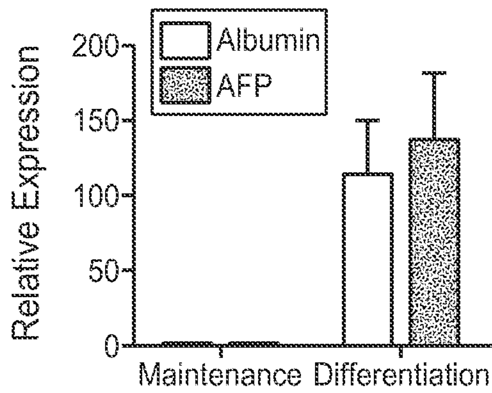


FIG. 2G

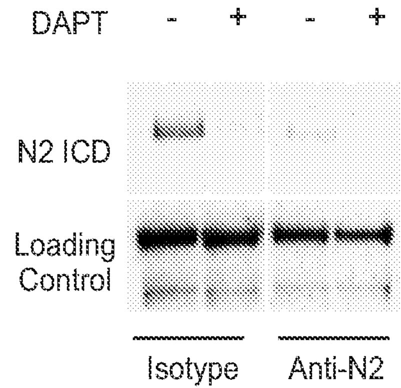


FIG. 2H

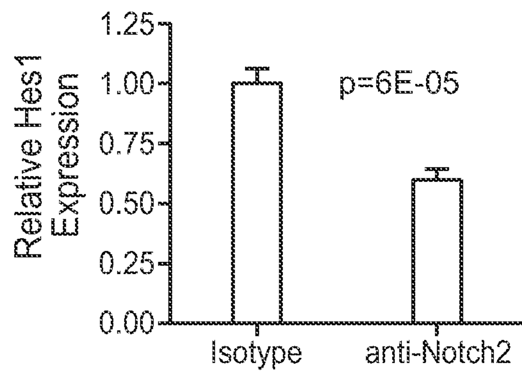


FIG. 2I

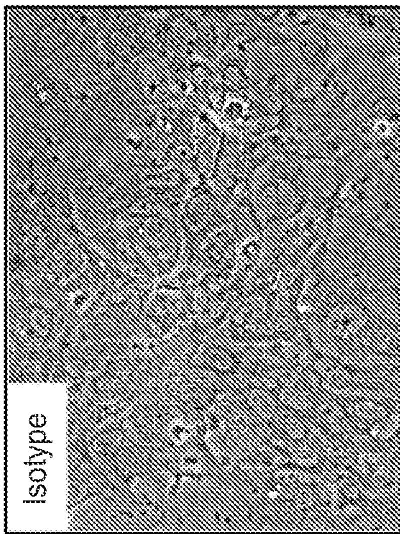


FIG. 2J

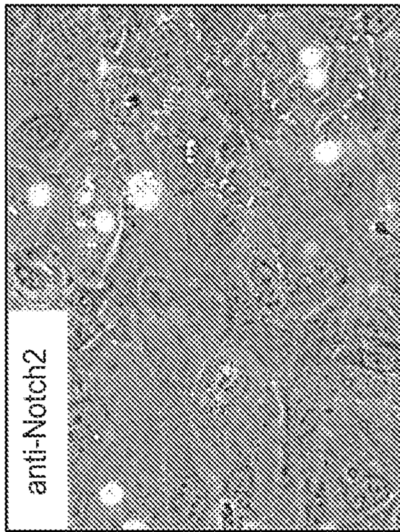


FIG. 2K

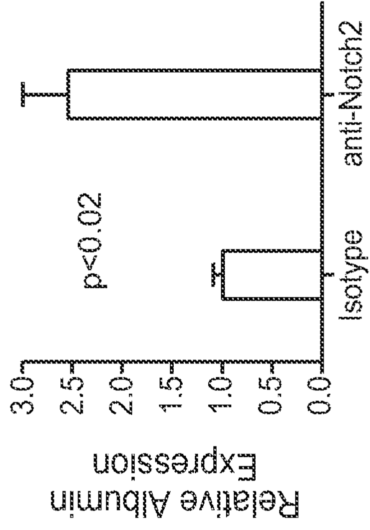


FIG. 2L

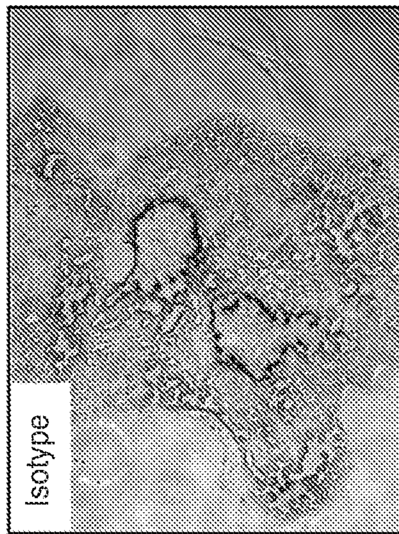


FIG. 2M

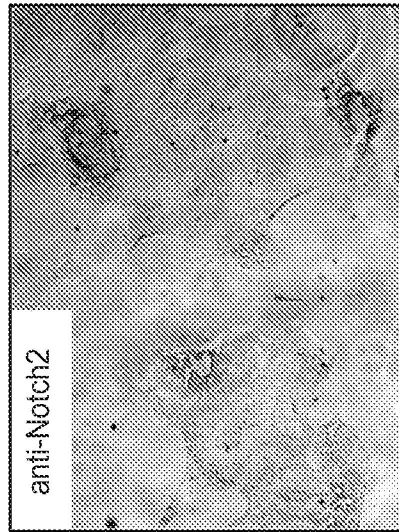


FIG. 2N

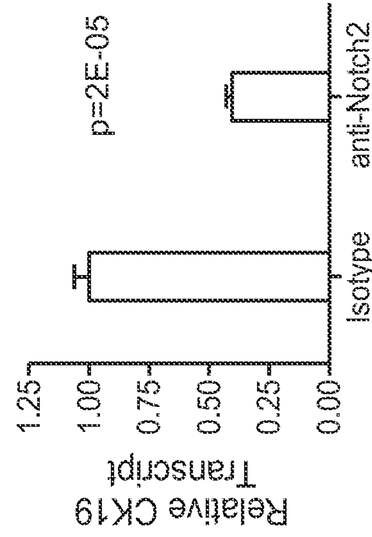


FIG. 2O

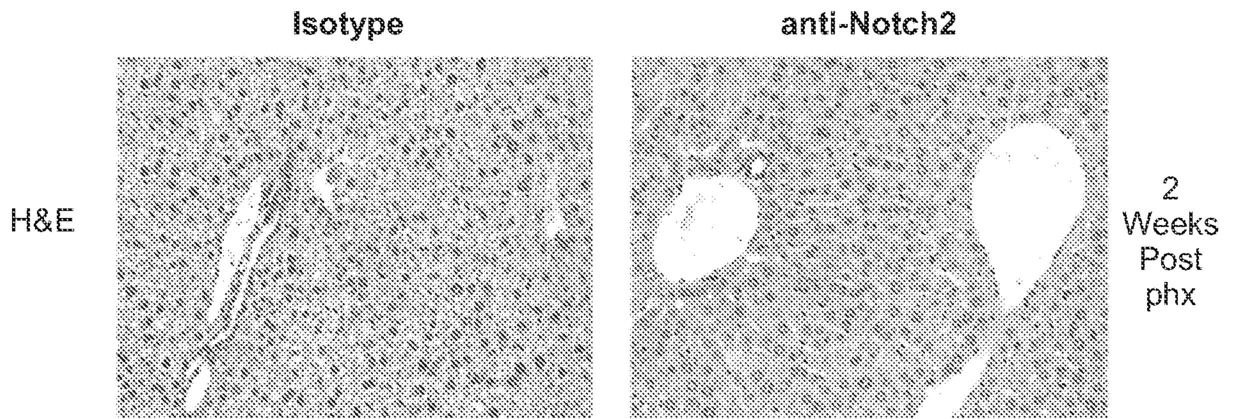


FIG. 3A-1 FIG. 3A-2

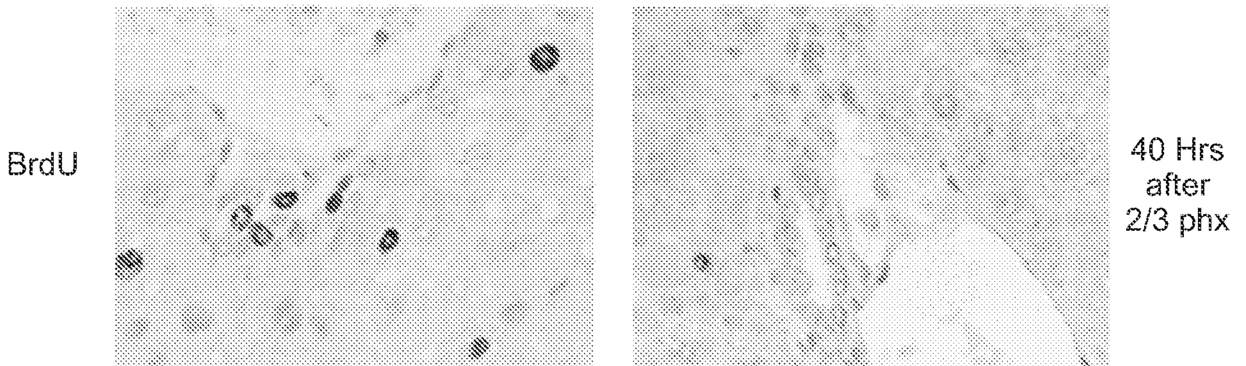


FIG. 3B-1 FIG. 3B-2

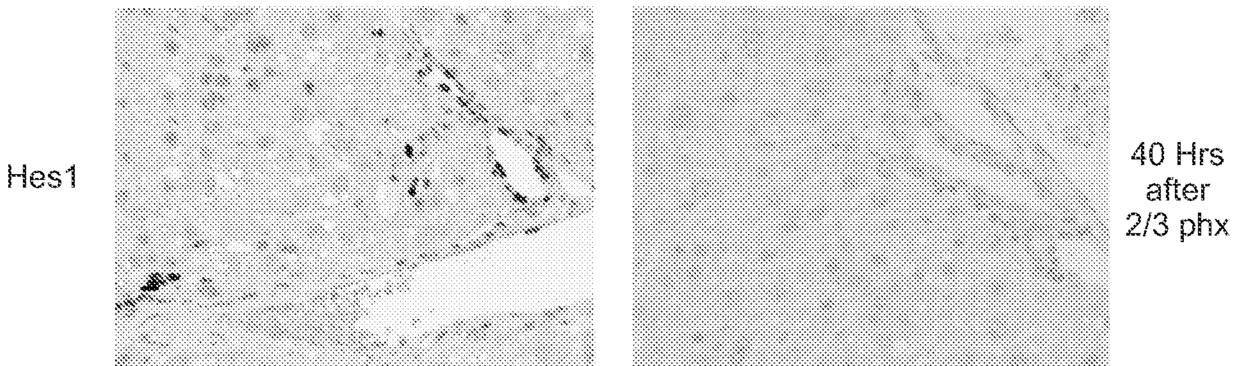


FIG. 3C-1 FIG. 3C-2

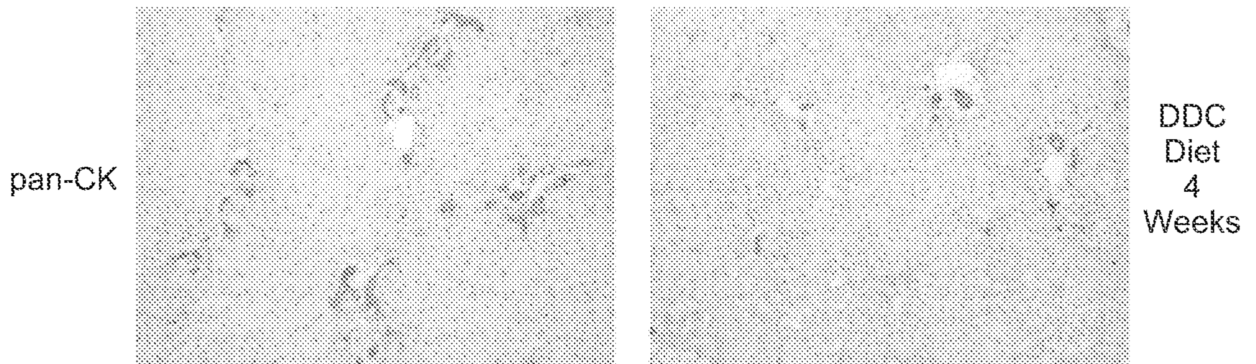


FIG. 3D-1 FIG. 3D-2

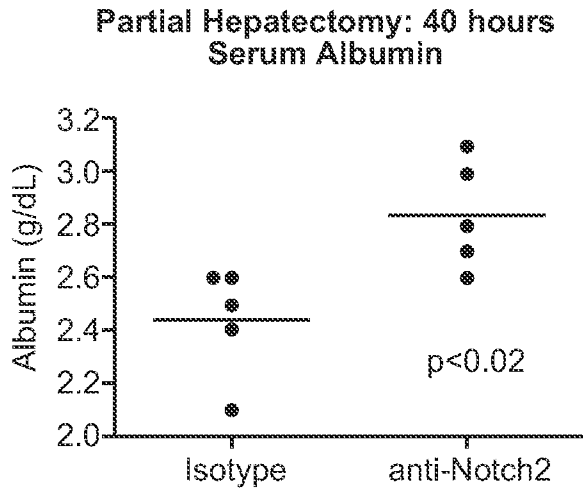


FIG. 3E

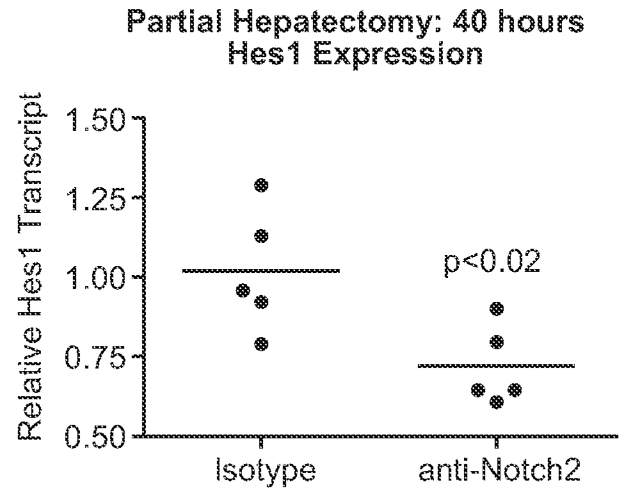


FIG. 3F

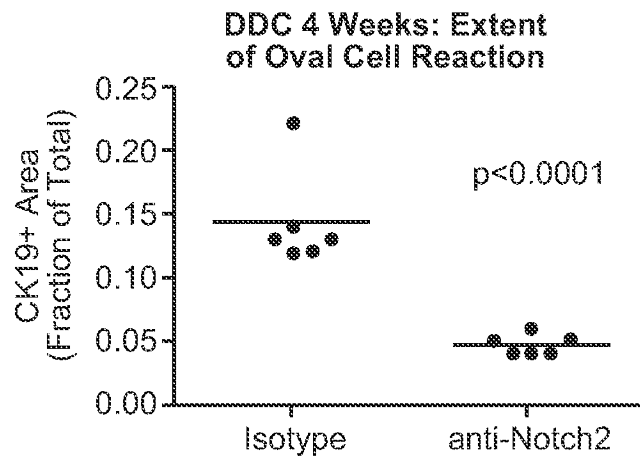


FIG. 3G

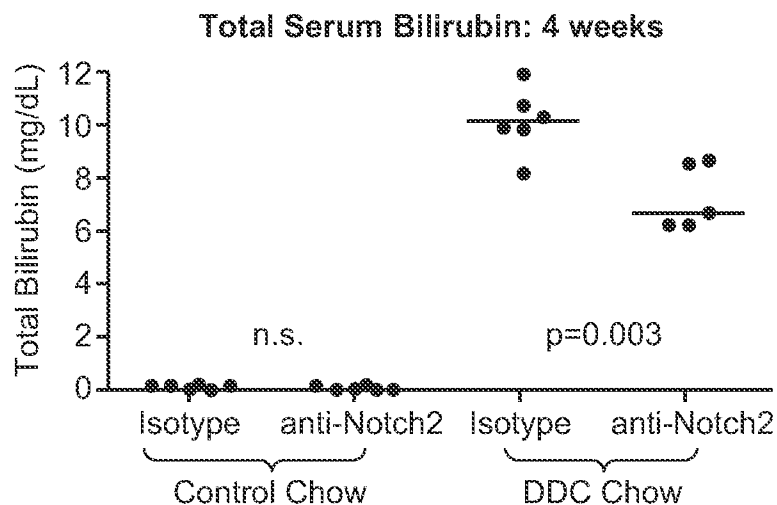


FIG. 3H

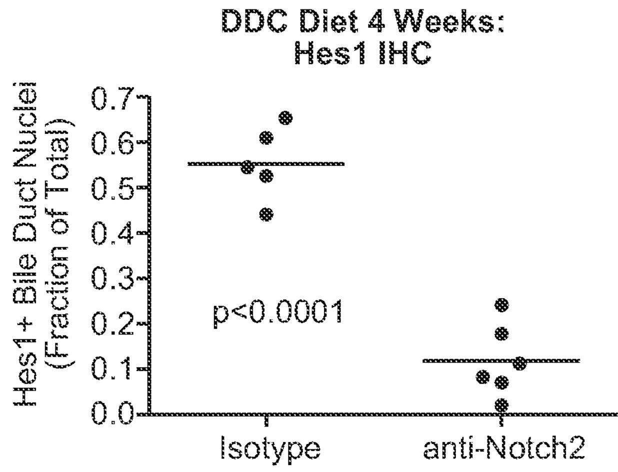


FIG. 3I

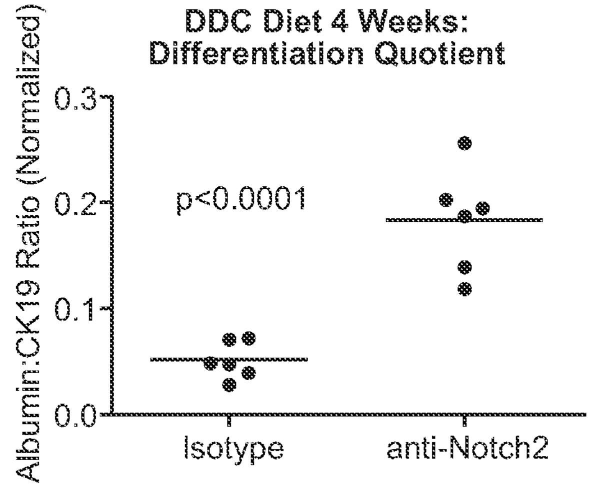


FIG. 3J

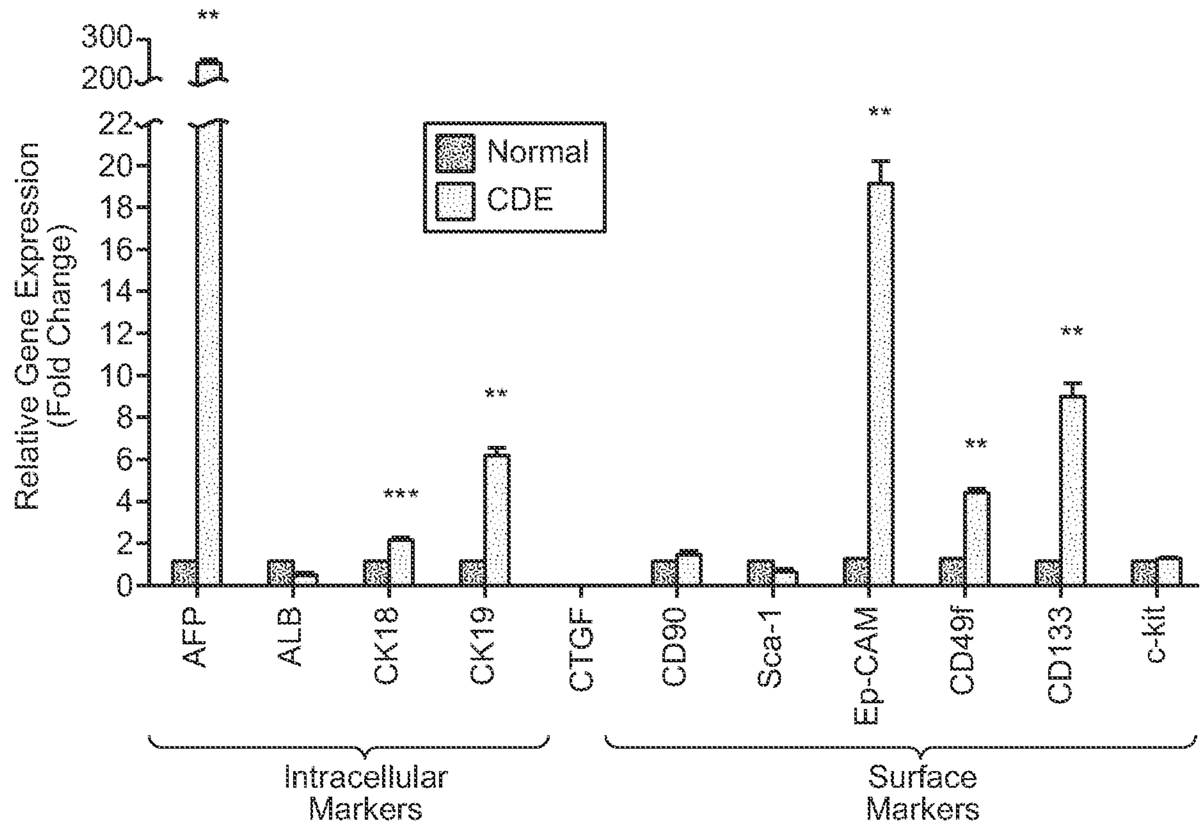


FIG. 4A

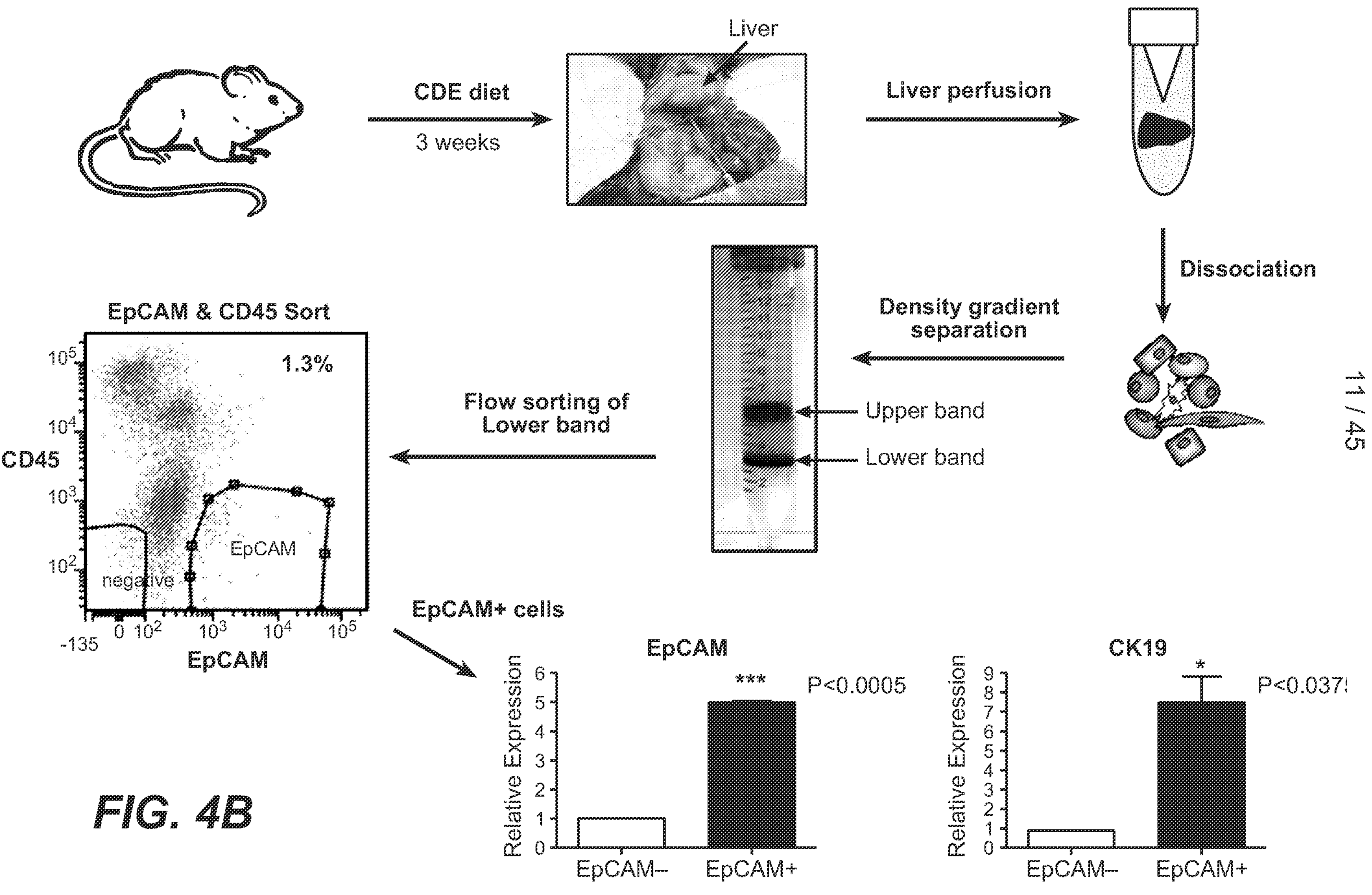
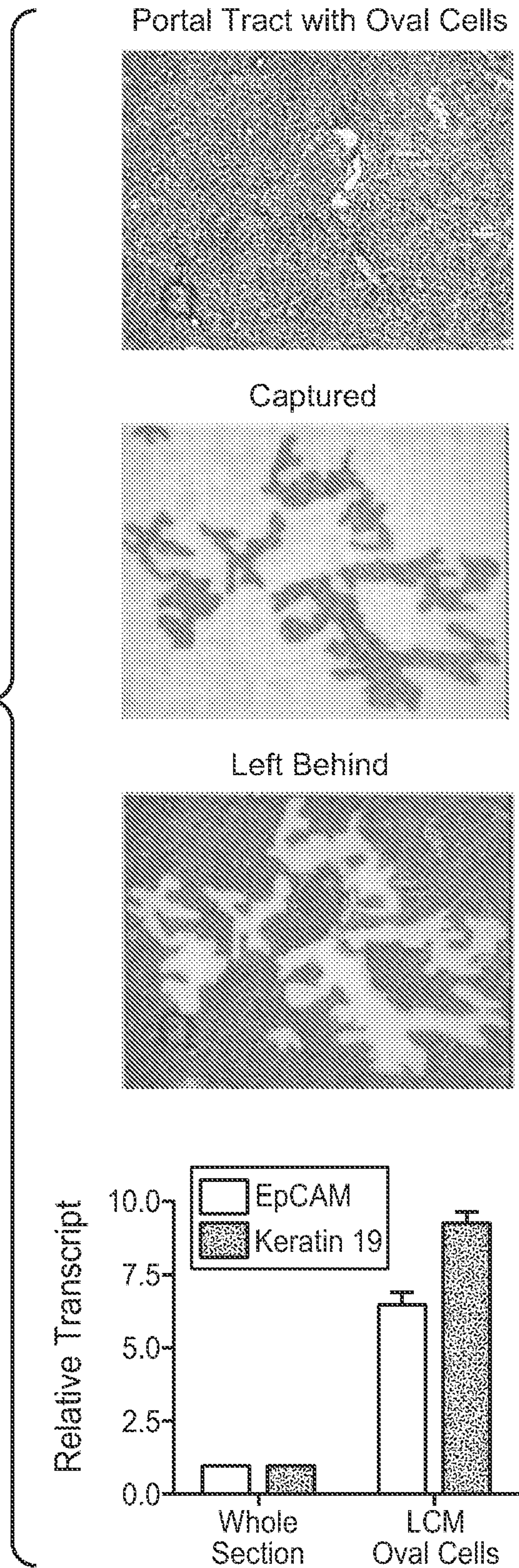


FIG. 4B

FIG. 4C



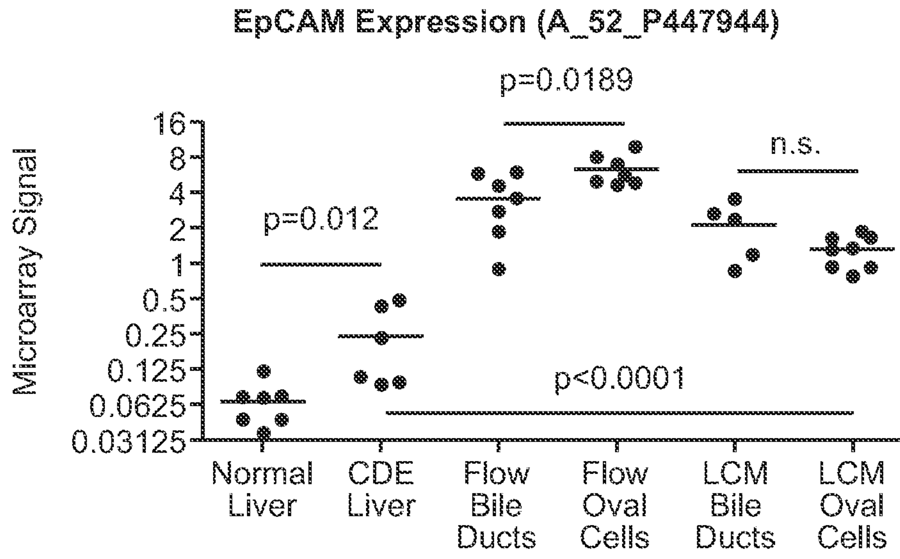


FIG. 4D

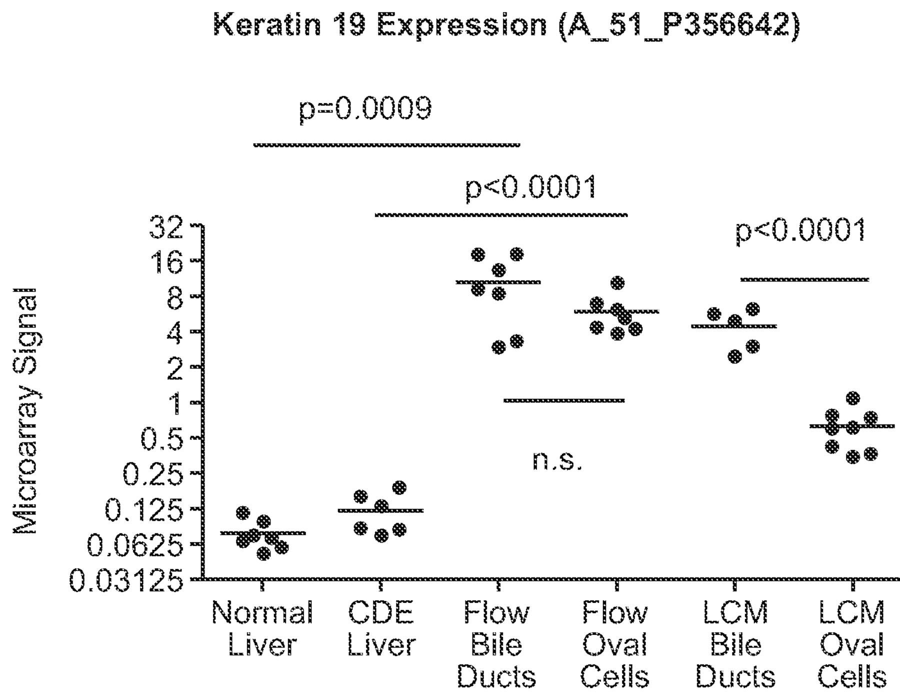
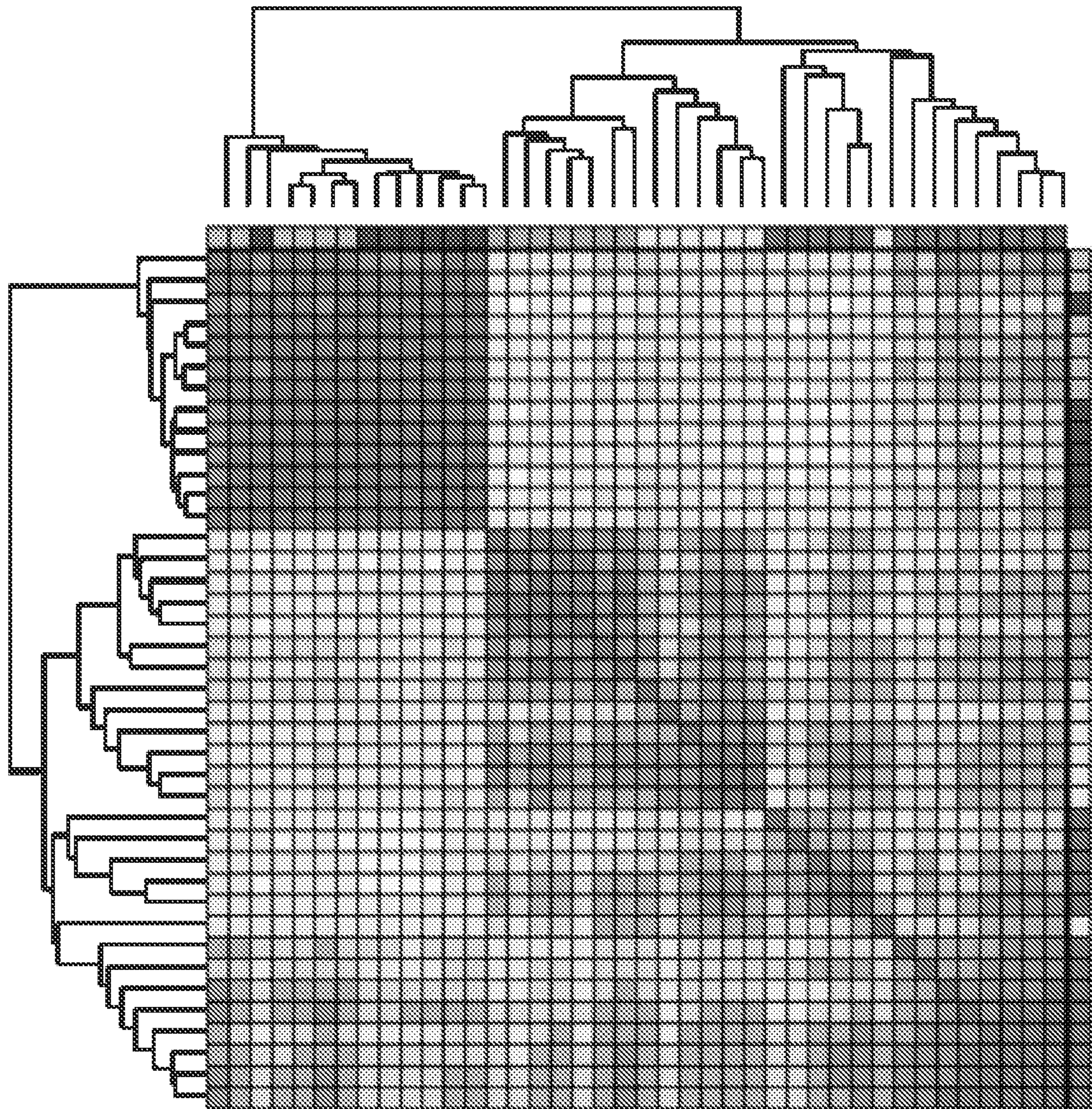


FIG. 4E



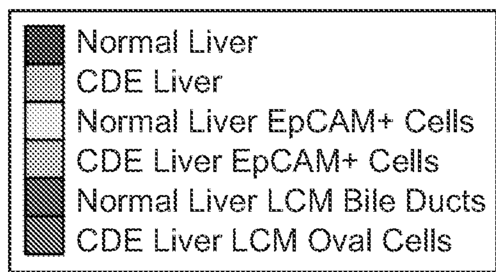
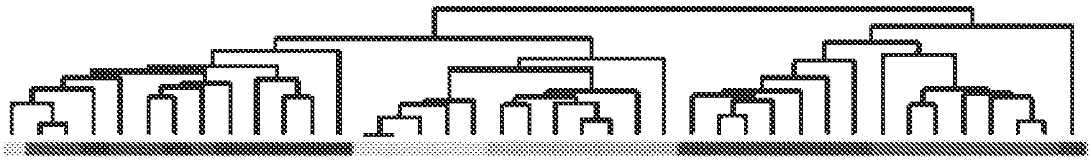


FIG. 5A

Top Oval Cell Genes



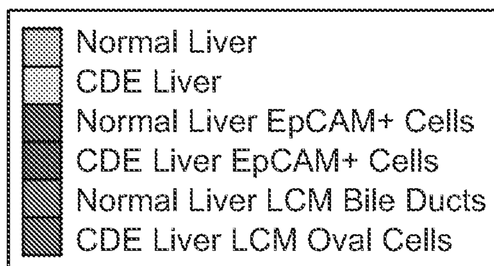


FIG. 5B

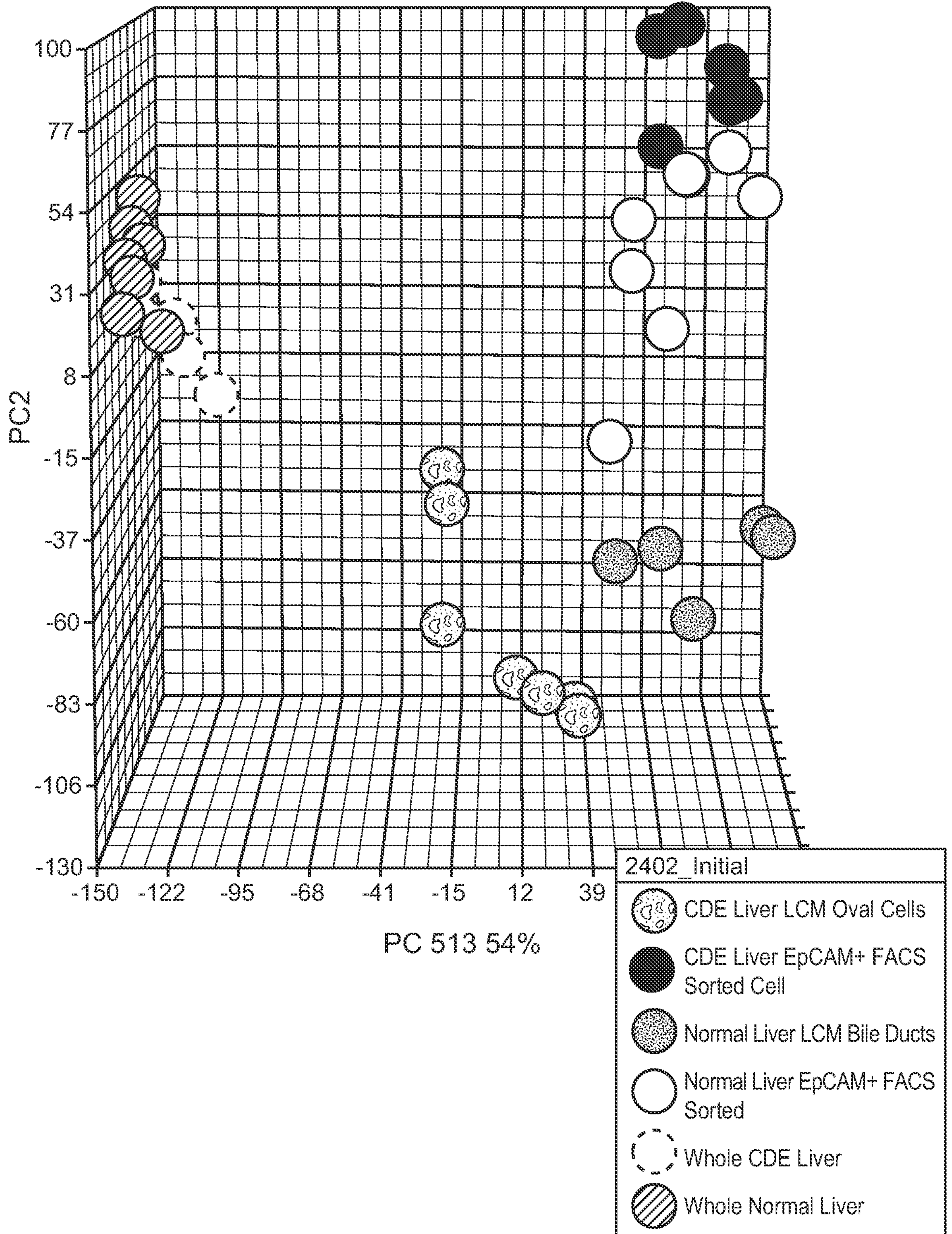


FIG. 5C

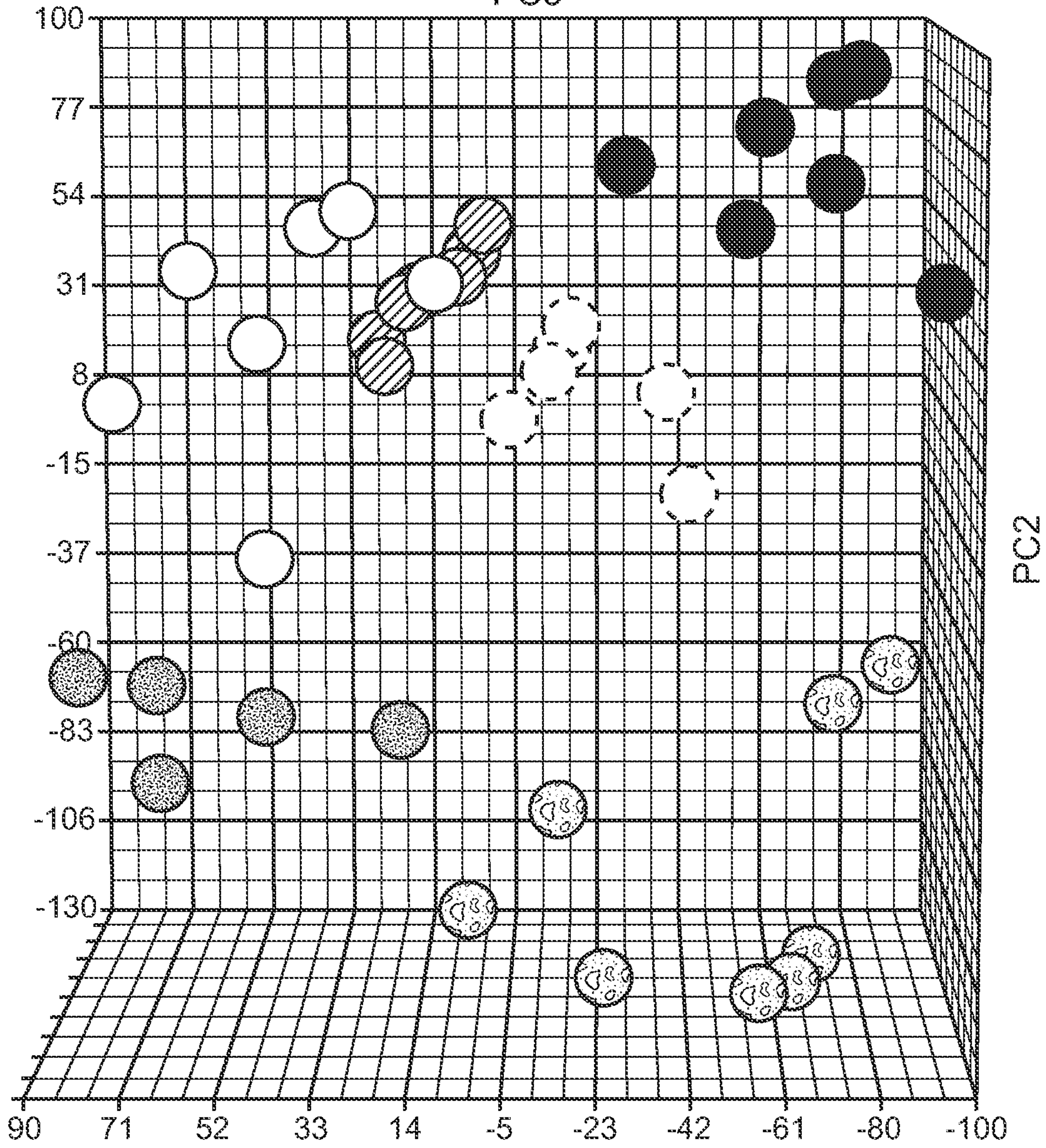


FIG. 5D

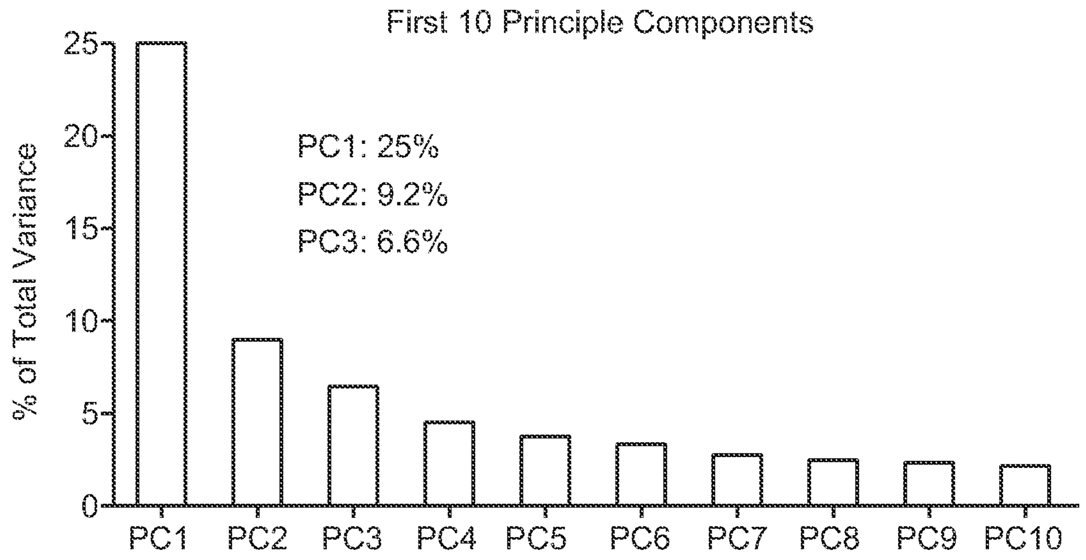


FIG. 5E

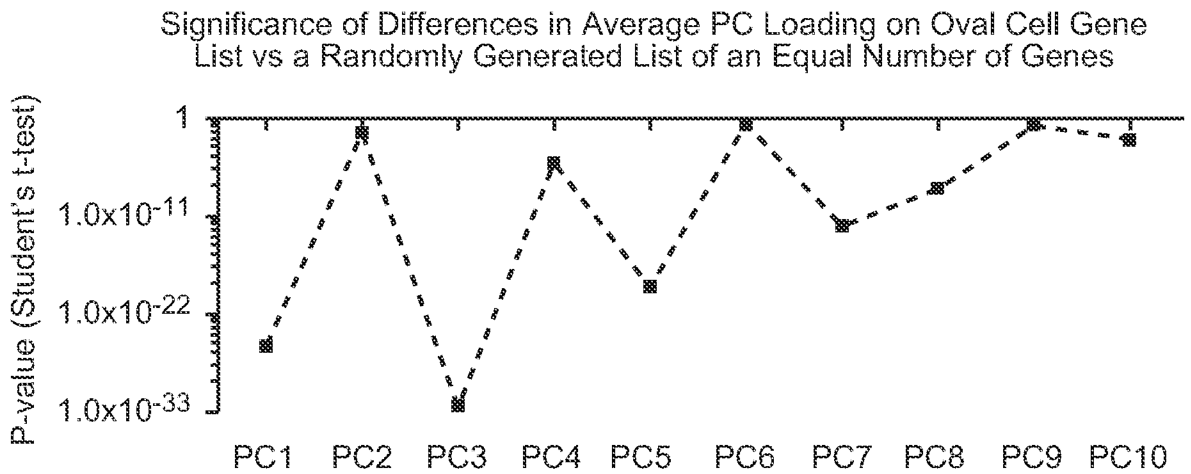


FIG. 5F

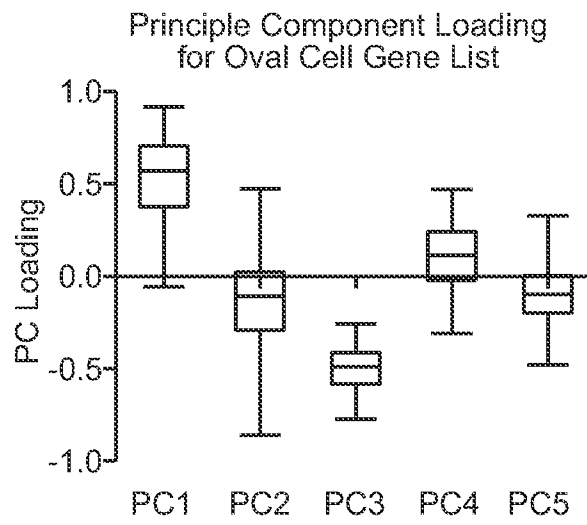


FIG. 5G

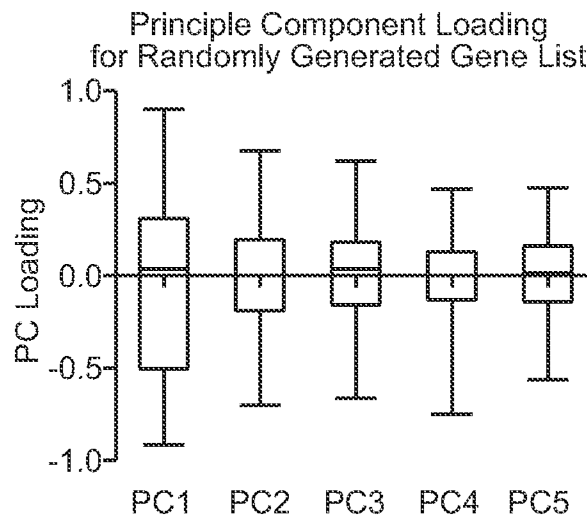


FIG. 5H

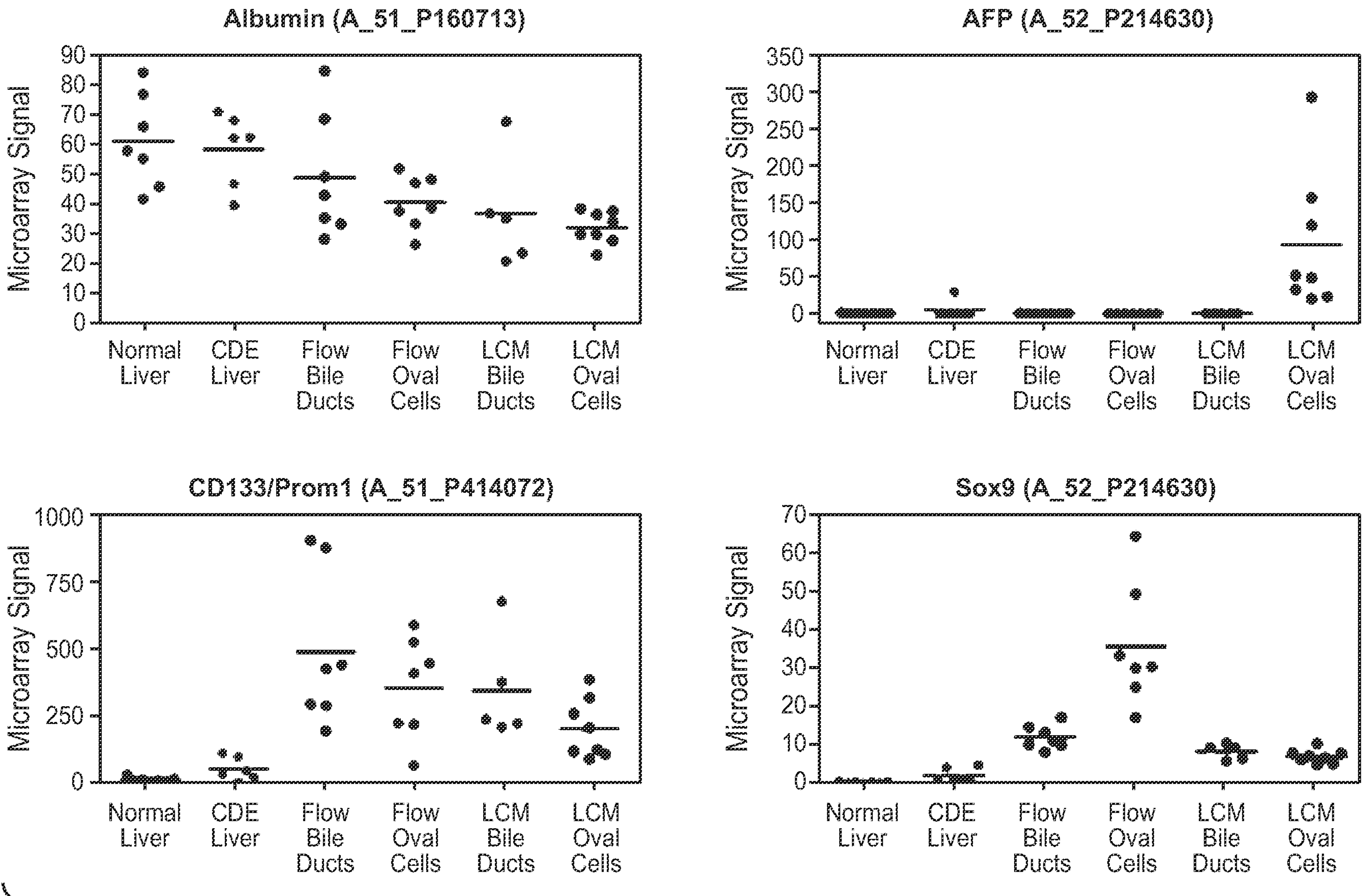


FIG. 6A-1

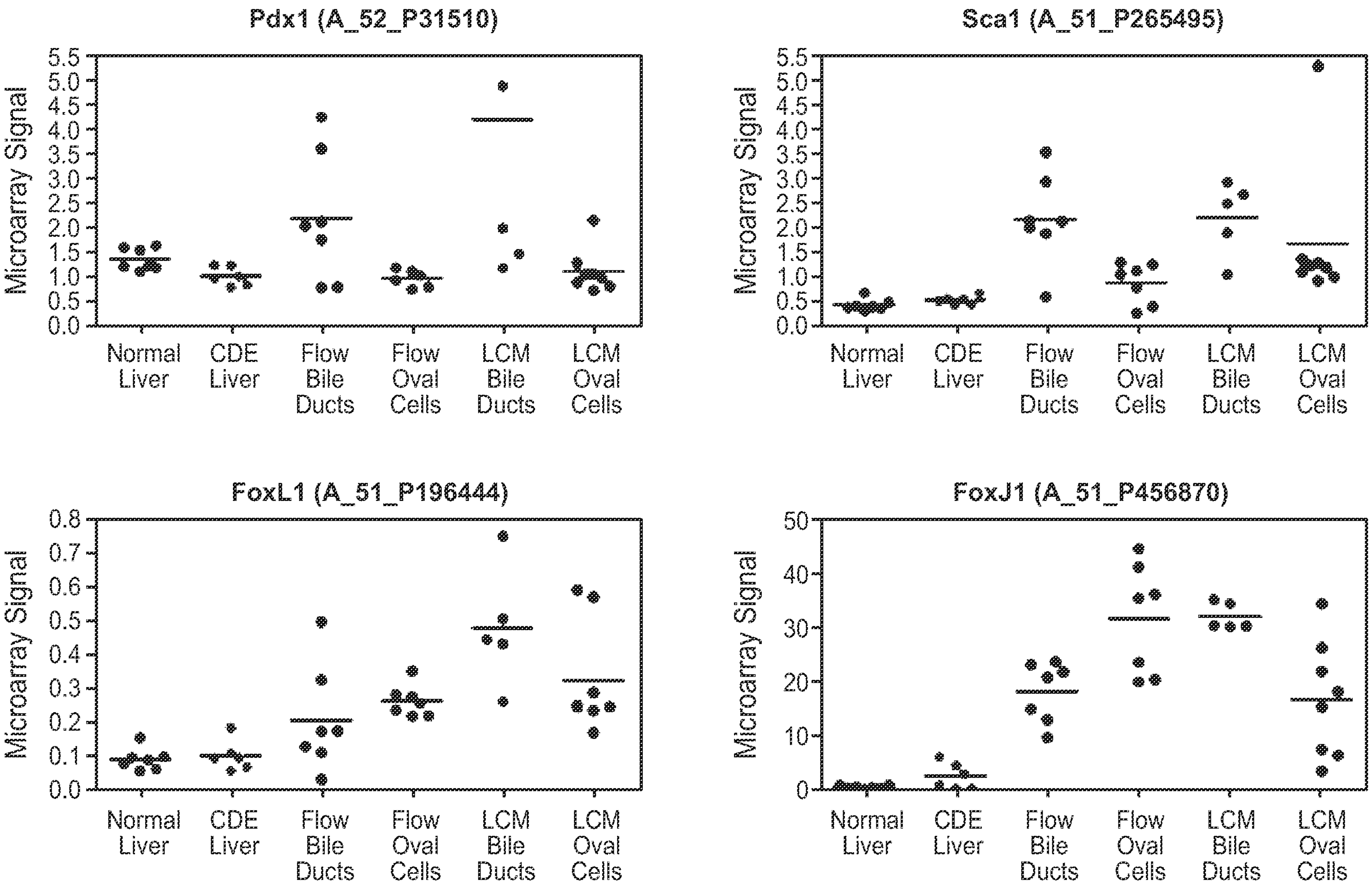


FIG. 6A-2

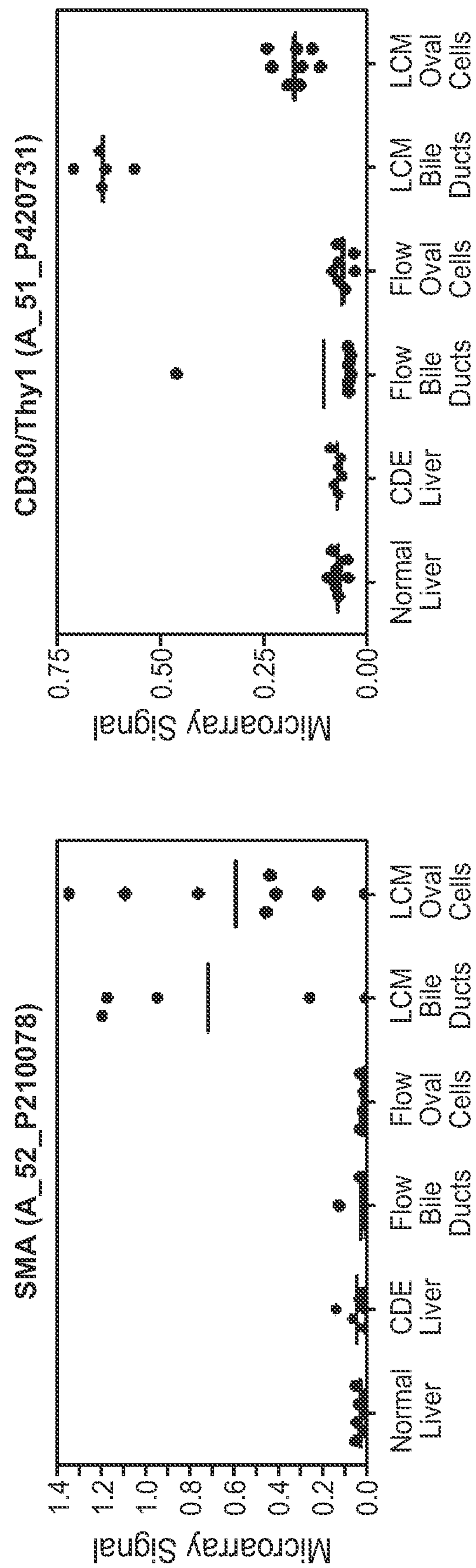


FIG. 6A-3

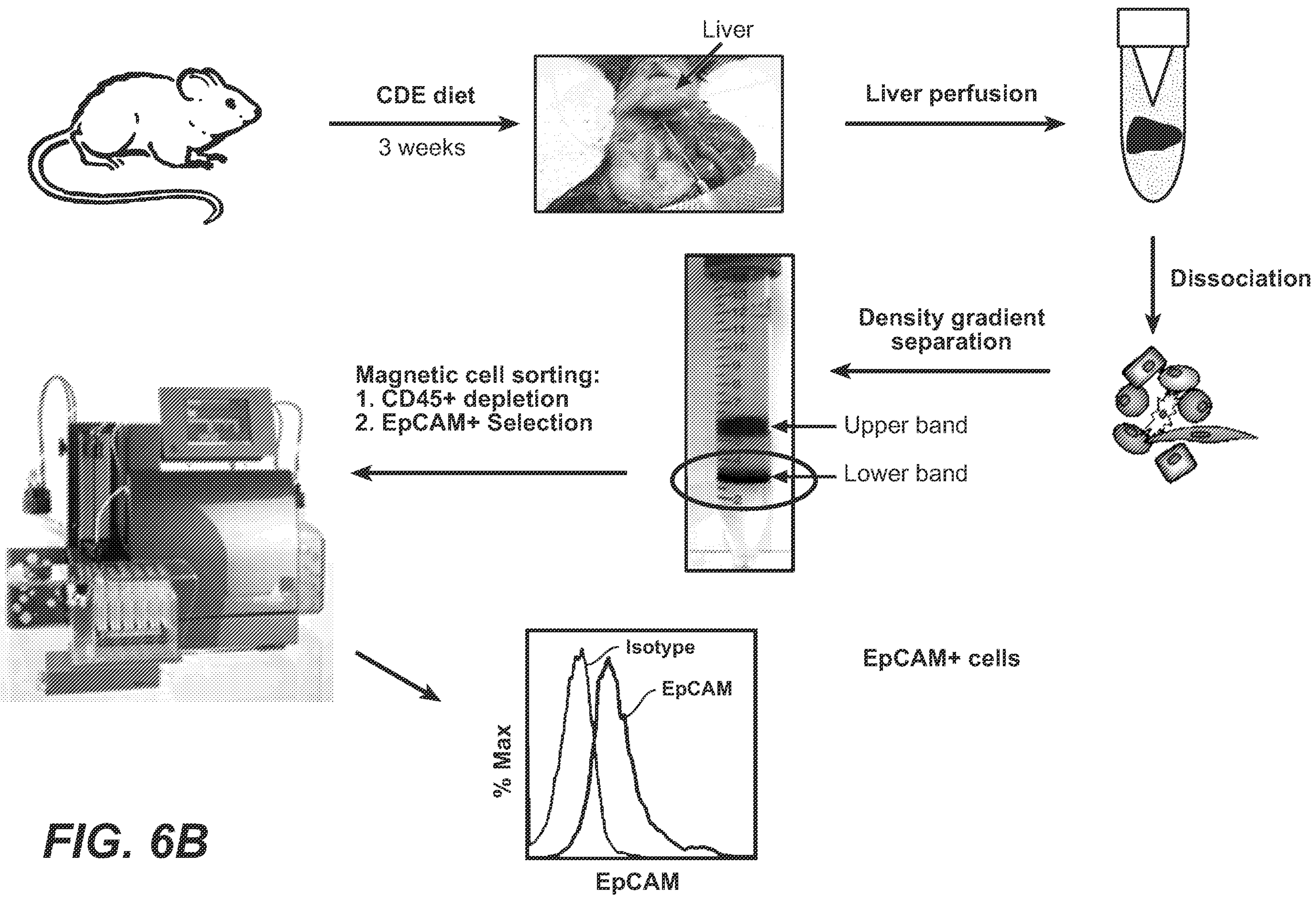


FIG. 6B

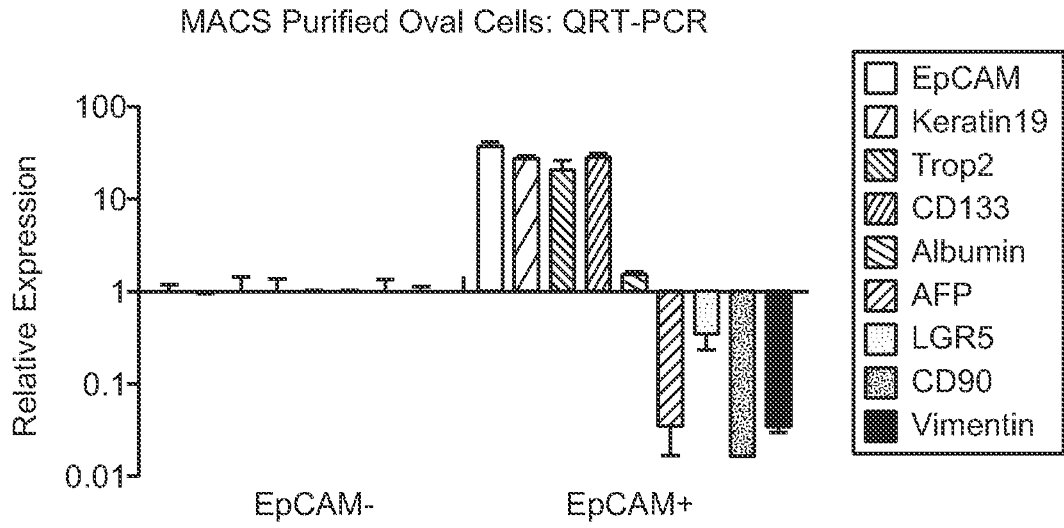


FIG. 6C

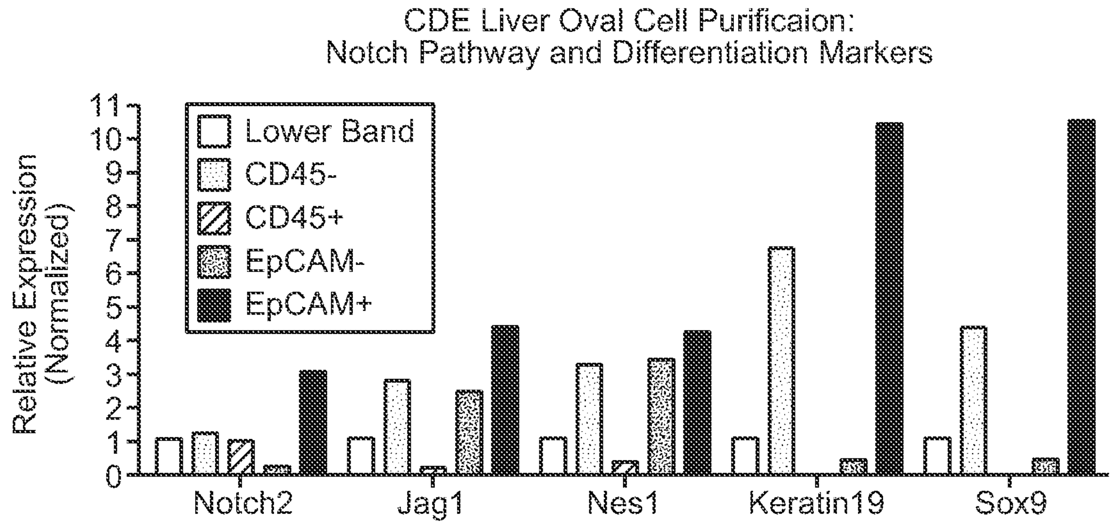


FIG. 6E

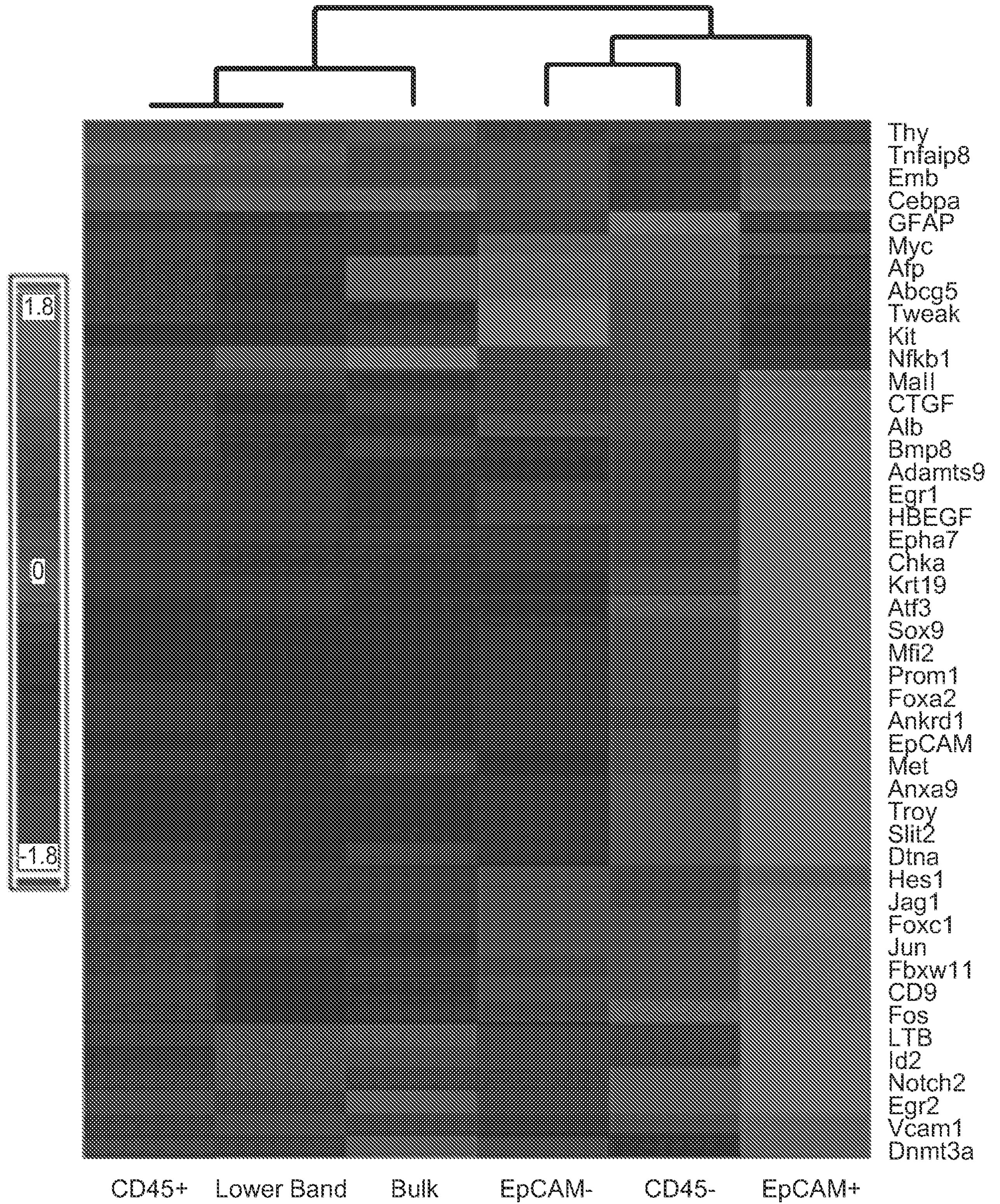


FIG. 6D

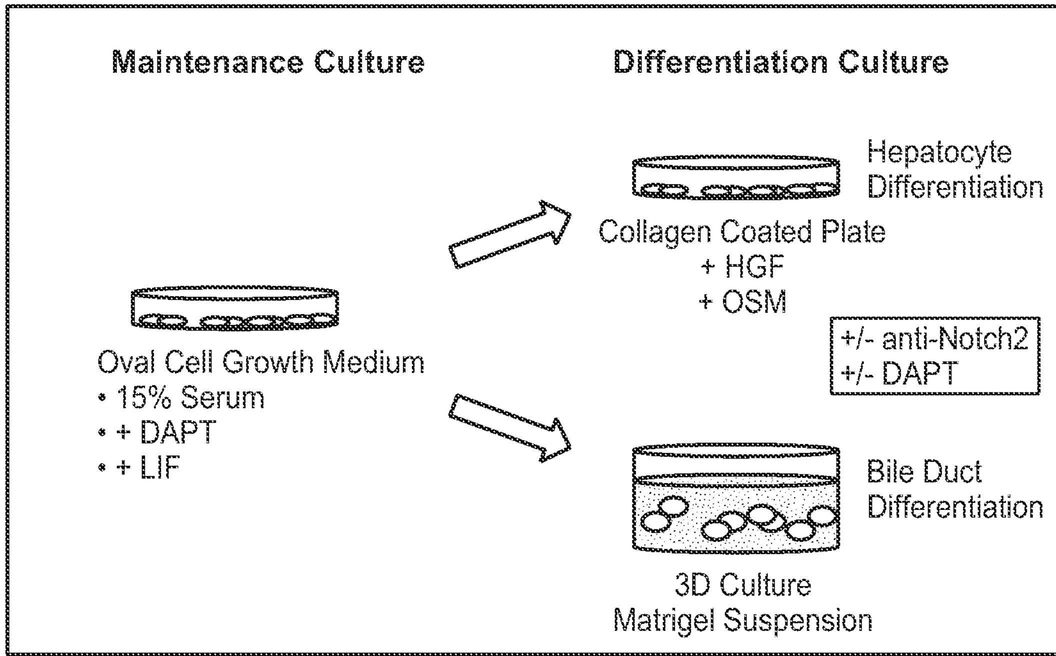


FIG. 7A

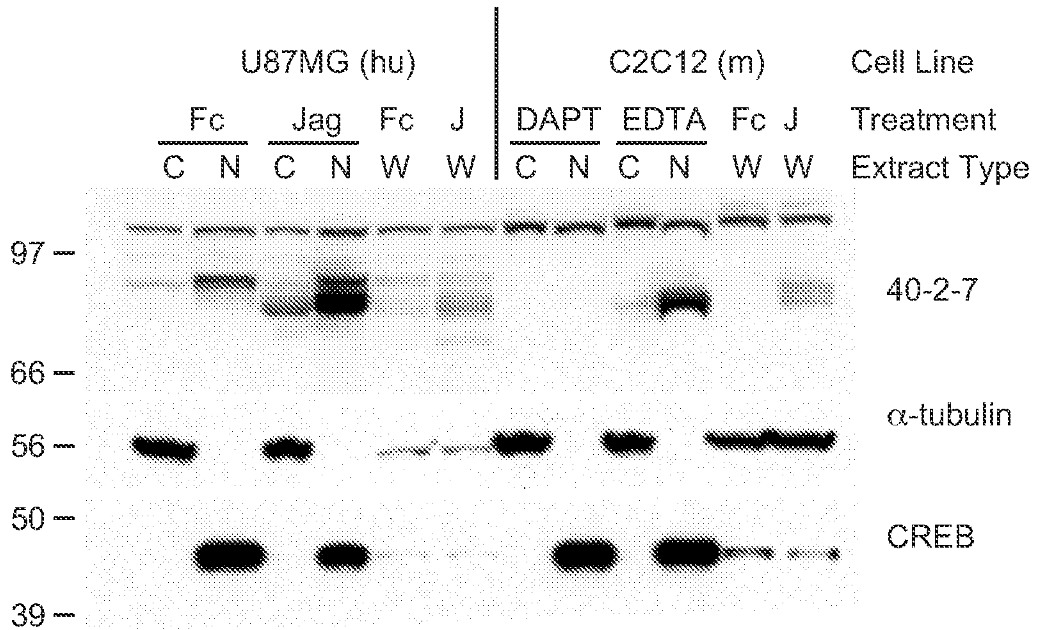


FIG. 7B

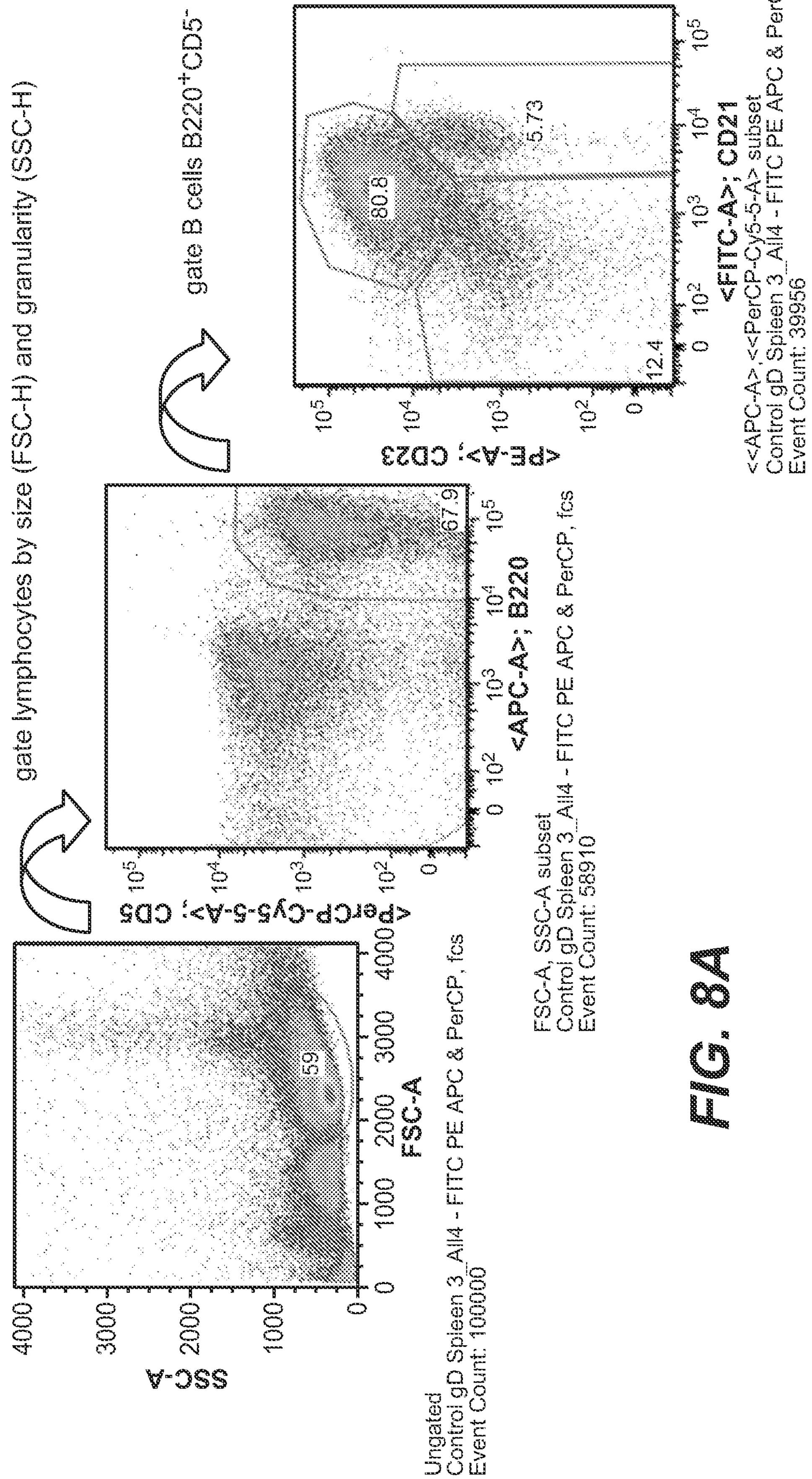


FIG. 8A

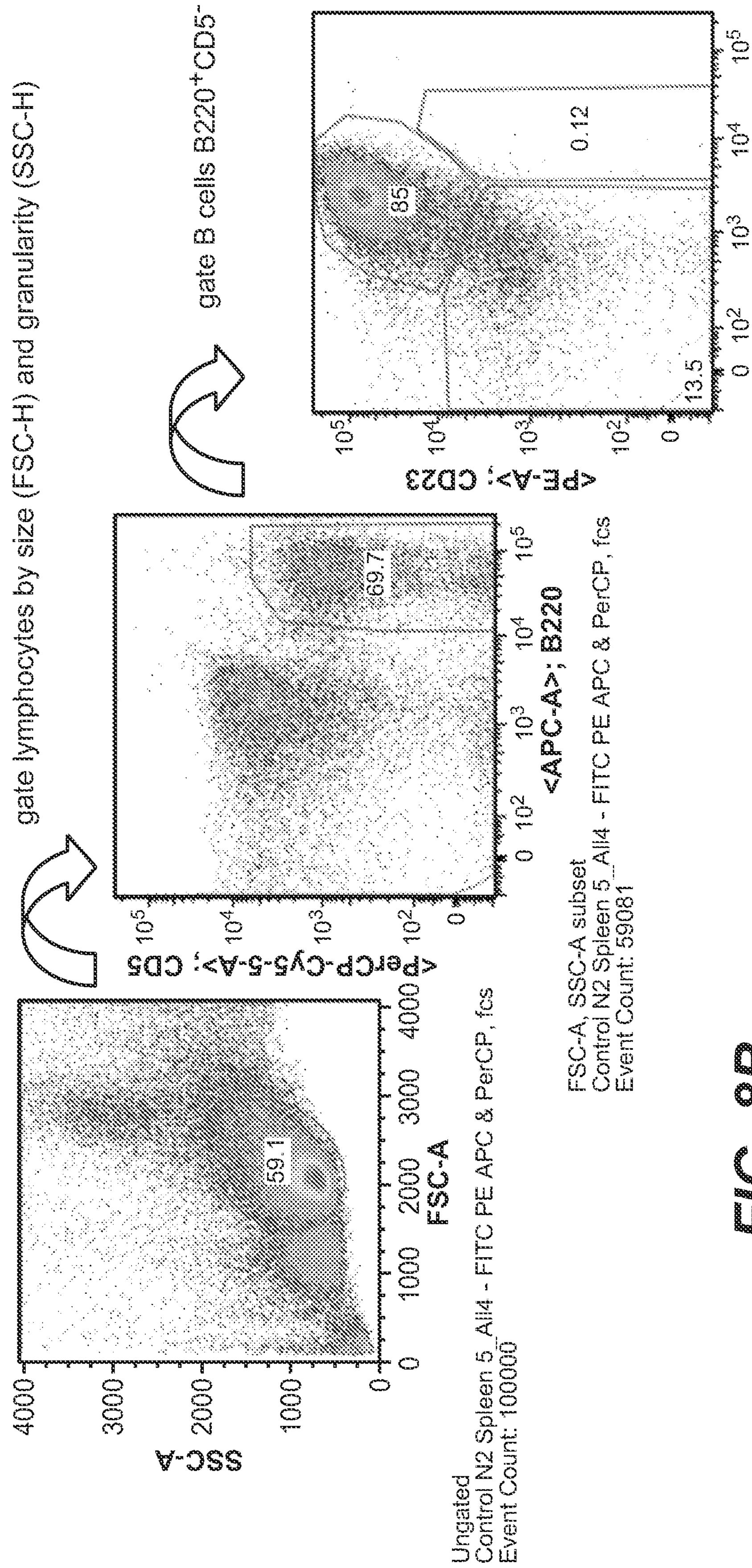


FIG. 8B

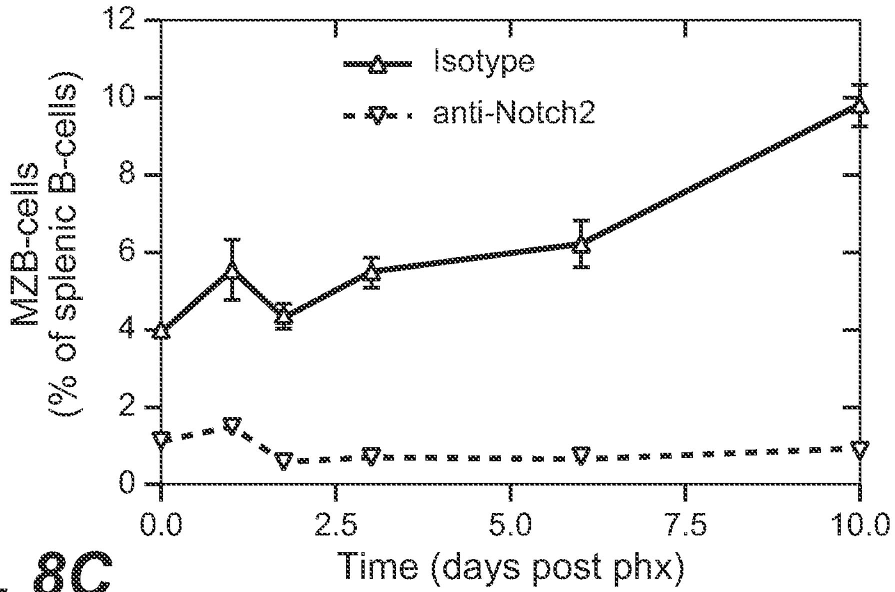


FIG. 8C

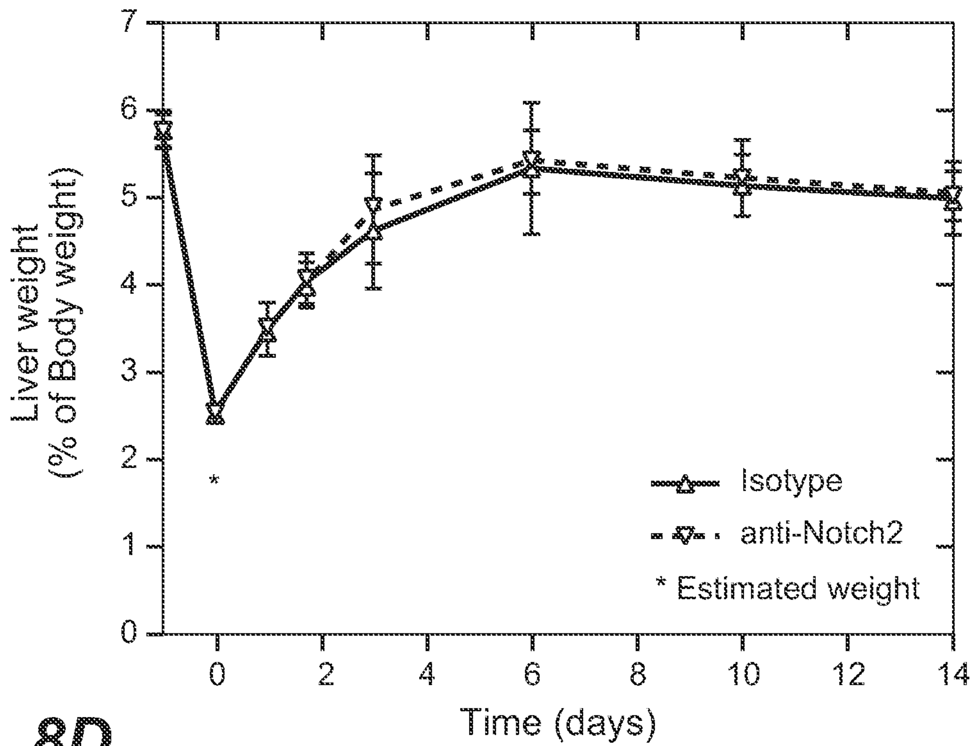


FIG. 8D

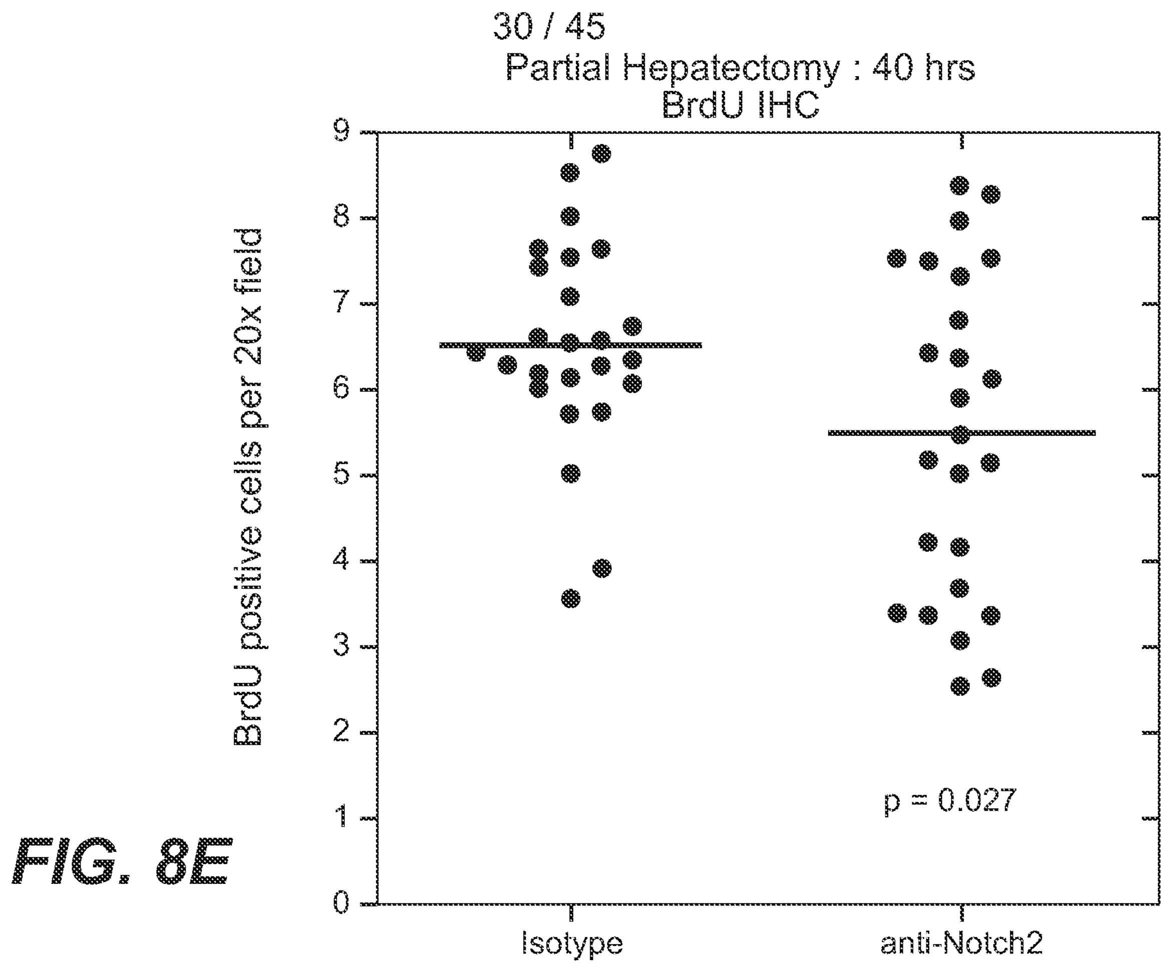


FIG. 8E

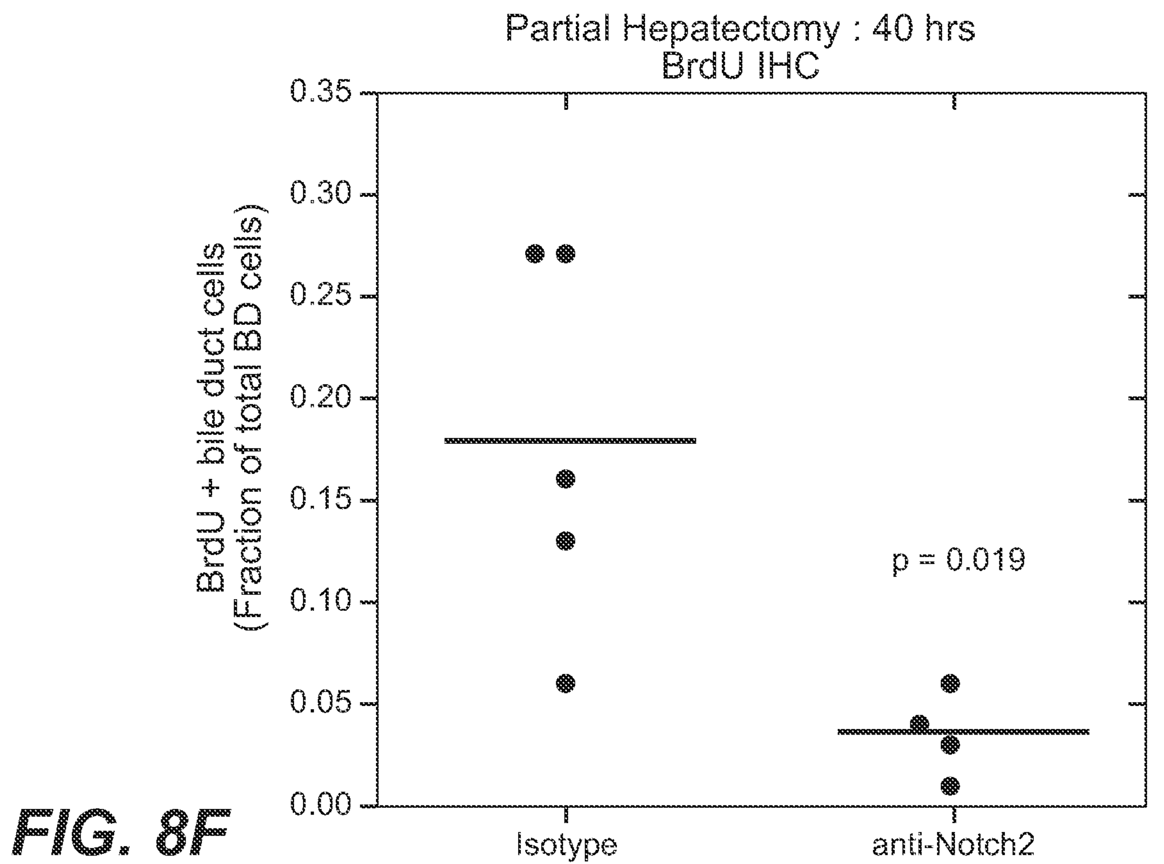


FIG. 8F

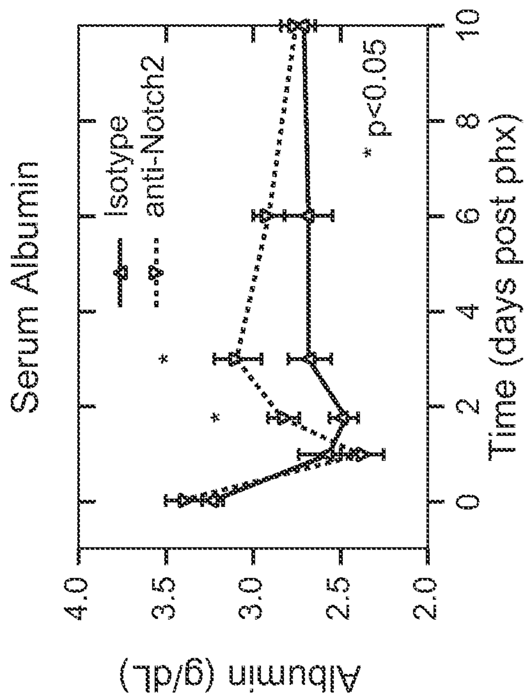


FIG. 9A

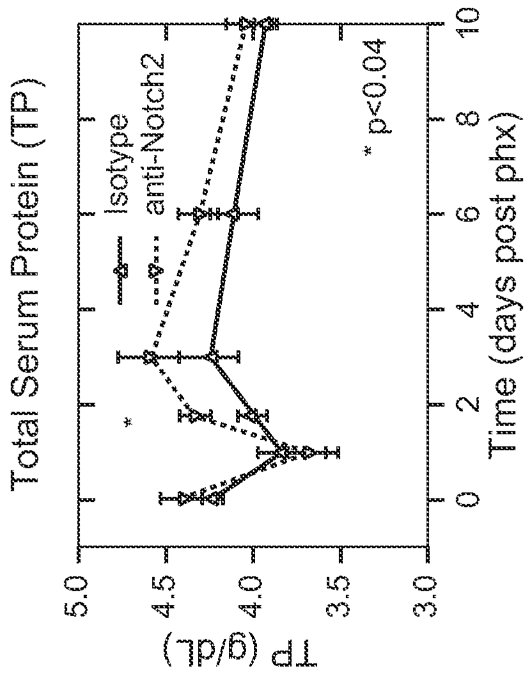


FIG. 9B

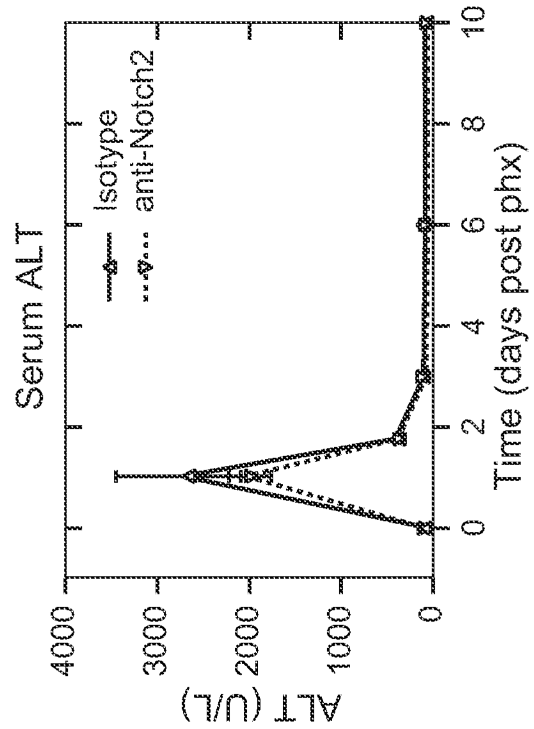


FIG. 9C

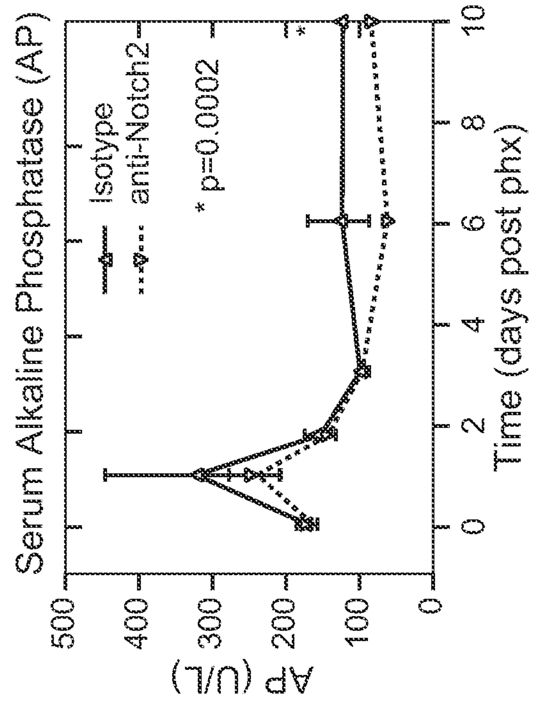


FIG. 9D

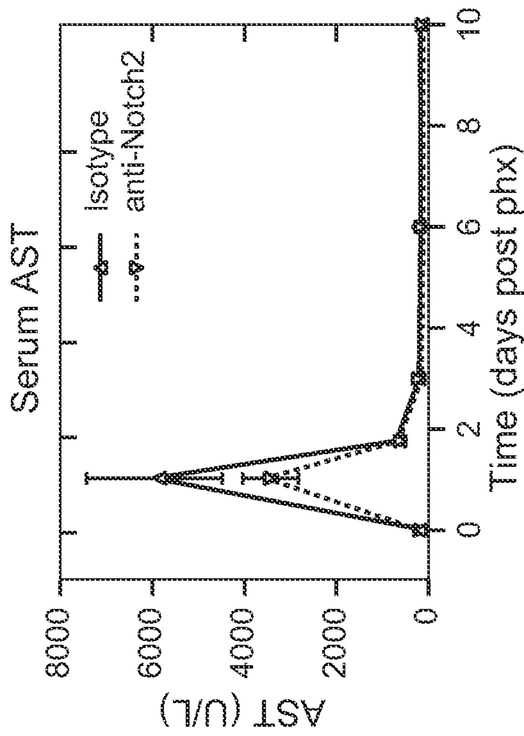


FIG. 9E

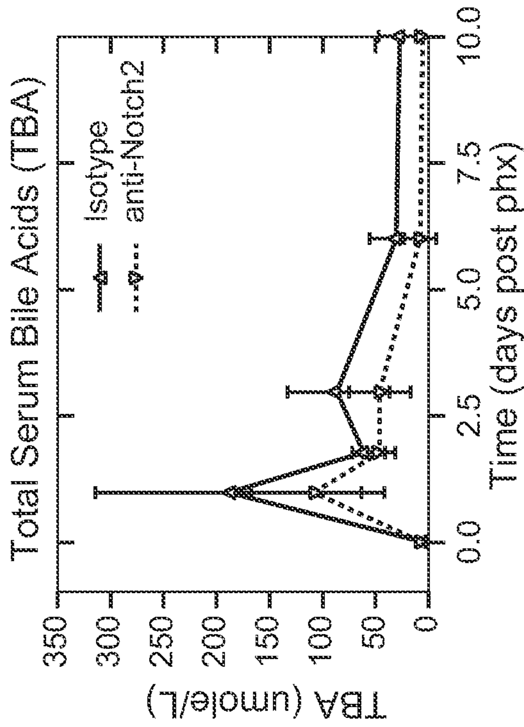


FIG. 9F

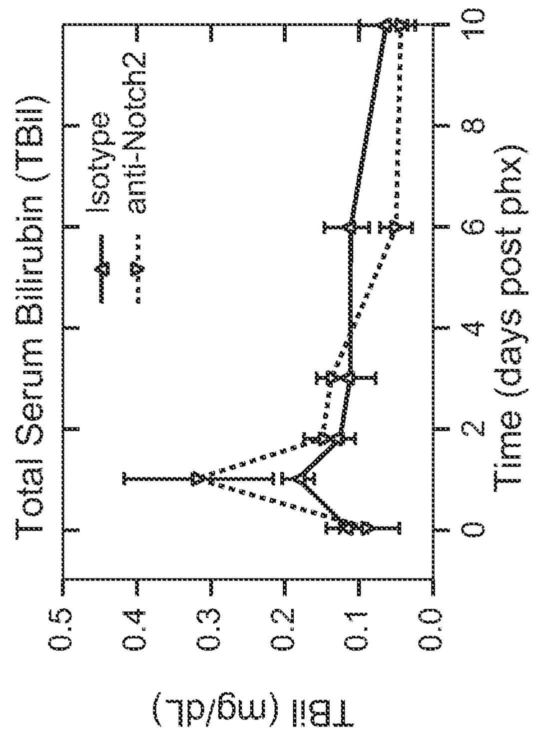


FIG. 9G

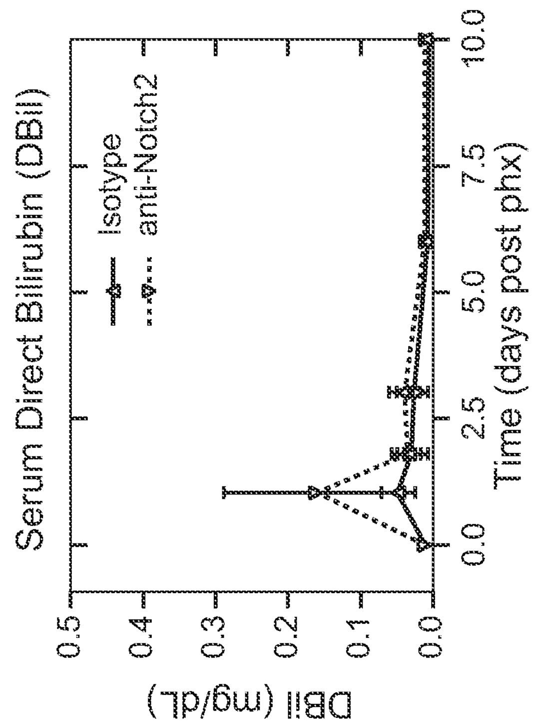


FIG. 9H

FIG. 10A

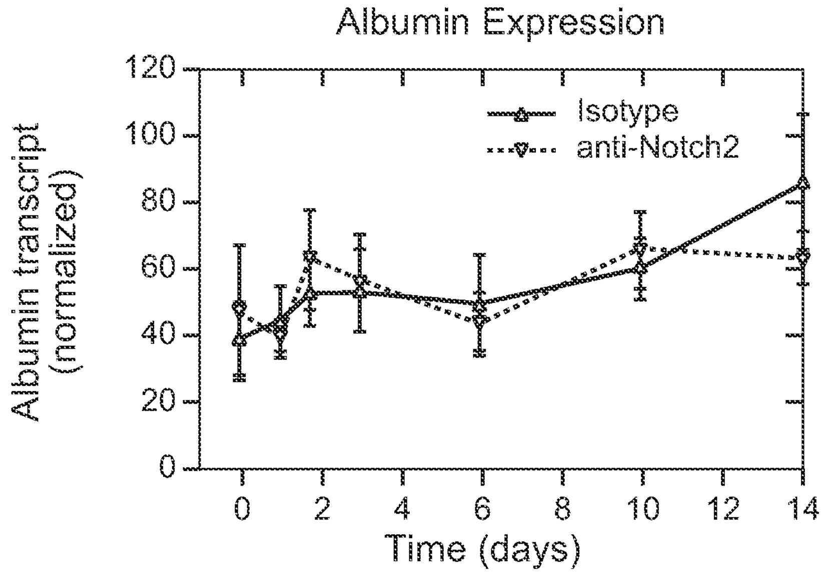


FIG. 10C

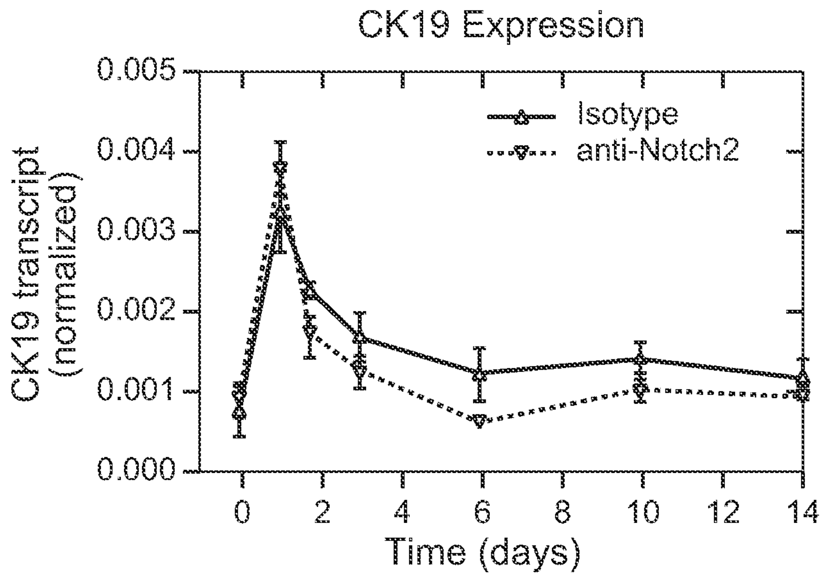
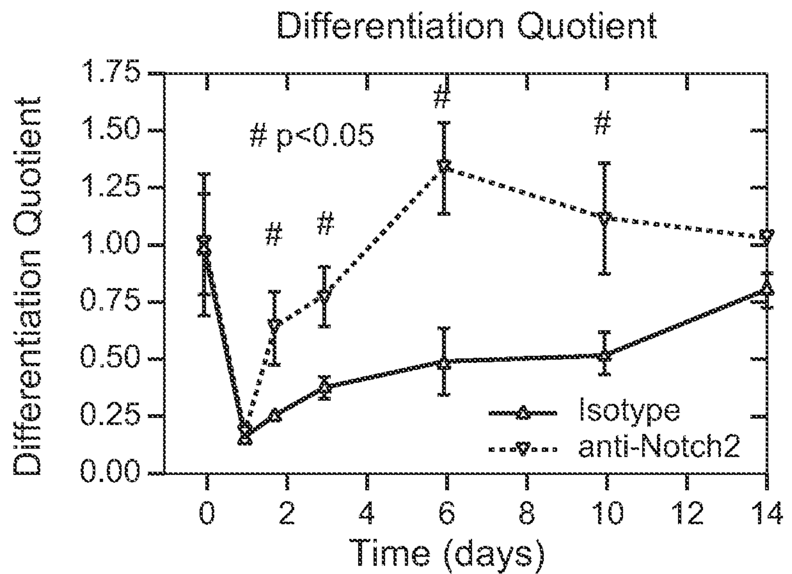


FIG. 10E



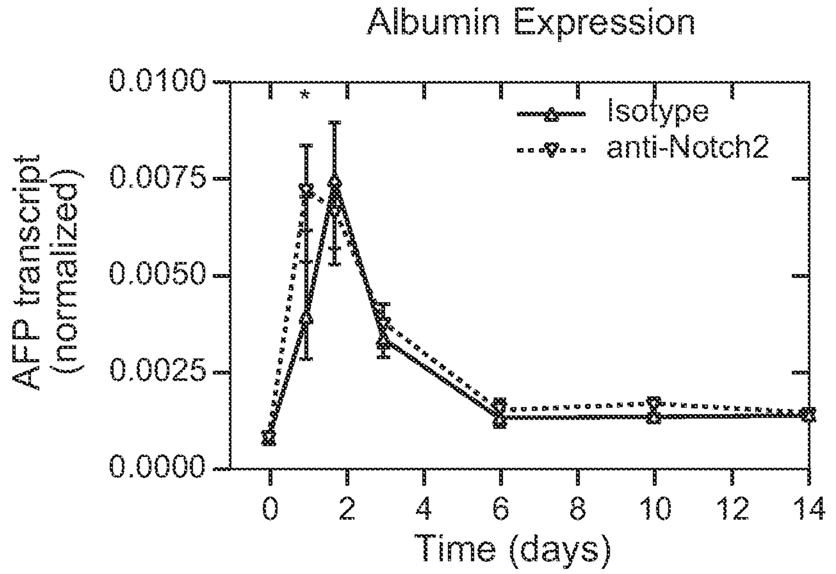


FIG. 10B

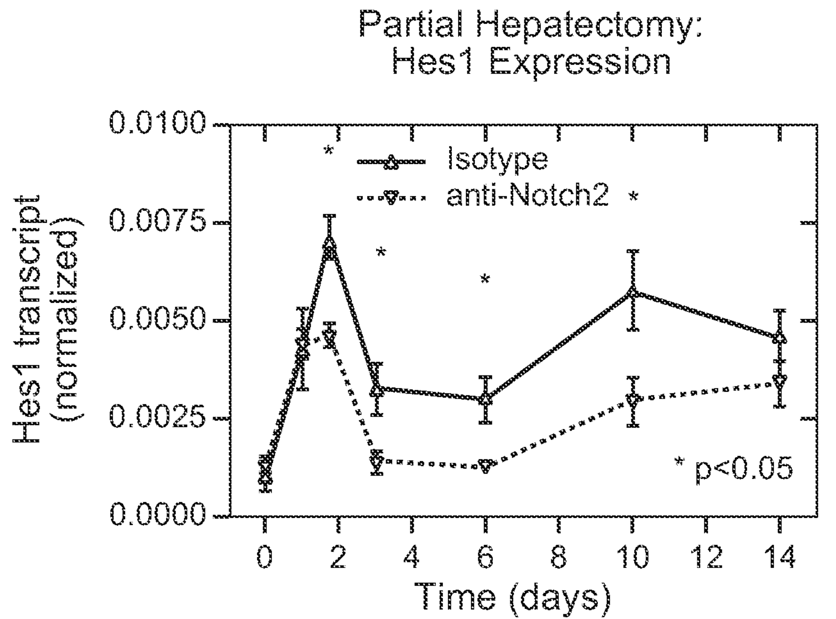


FIG. 10D

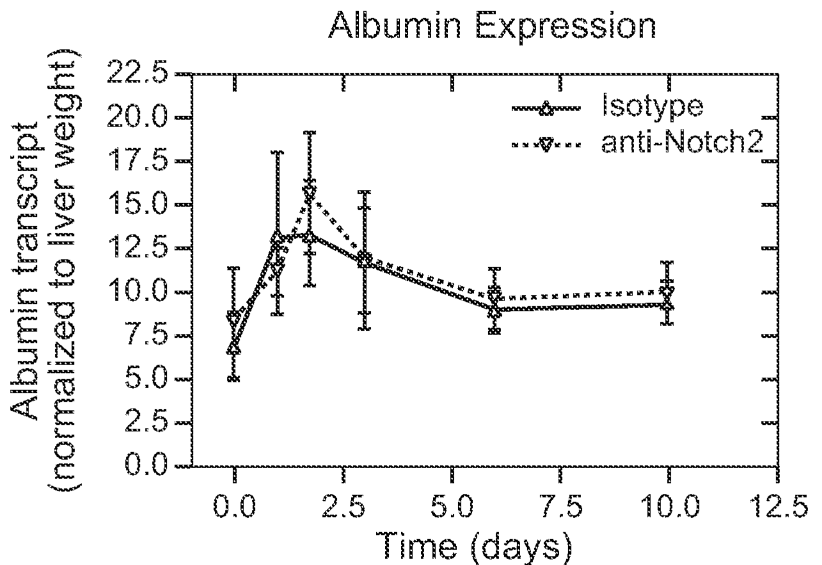


FIG. 10F

FIG. 11A

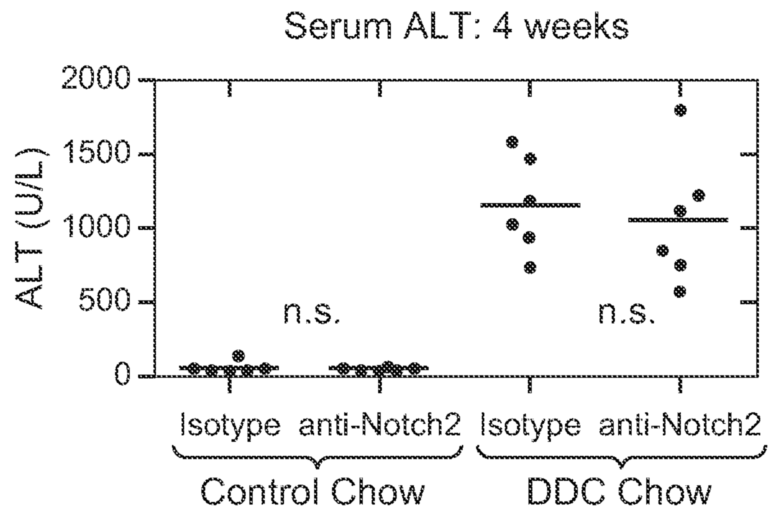


FIG. 11B

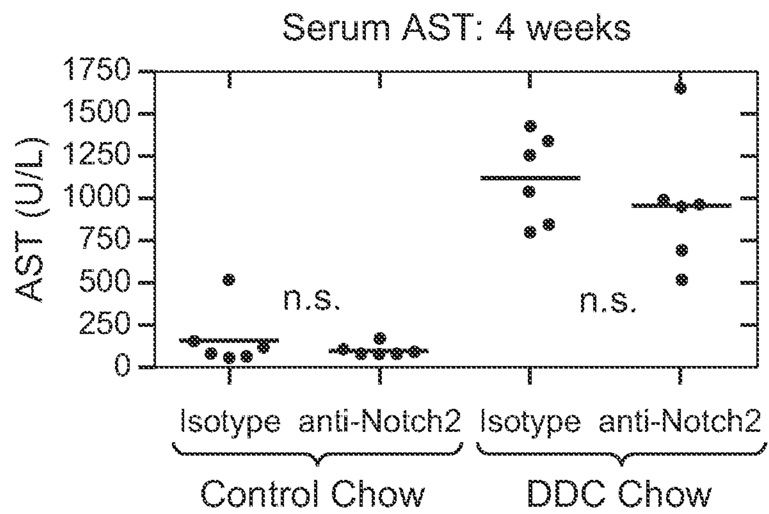


FIG. 11C

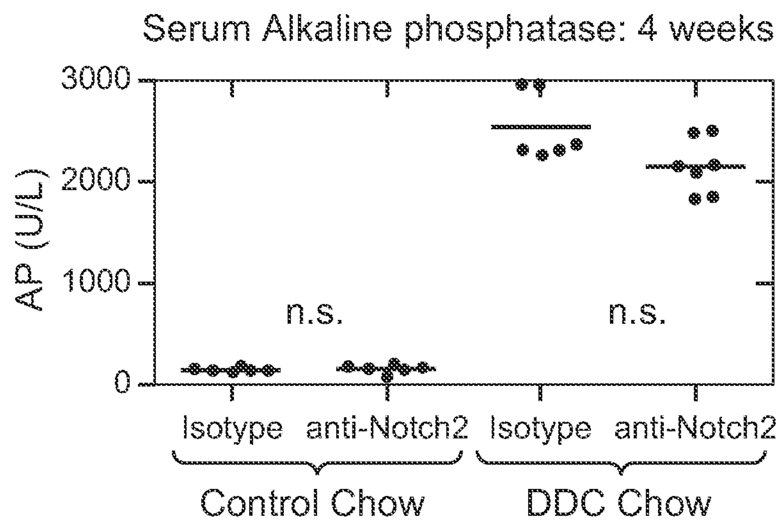


FIG. 11D

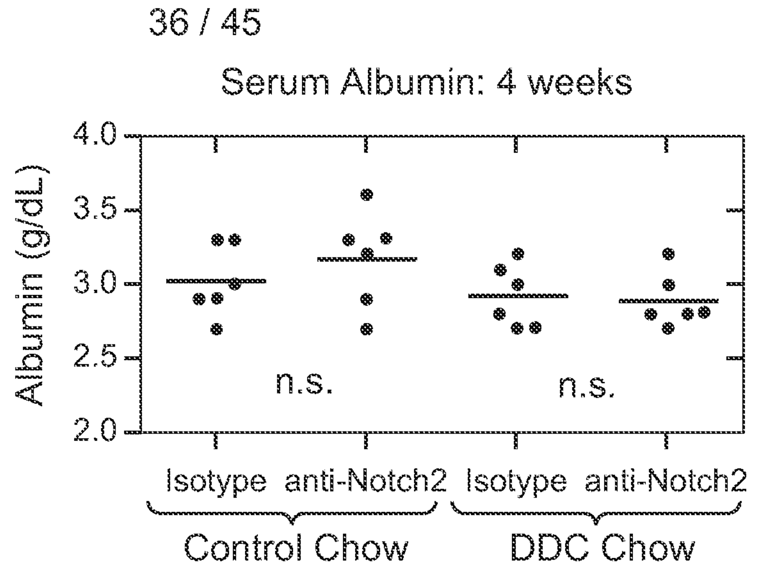


FIG. 11E

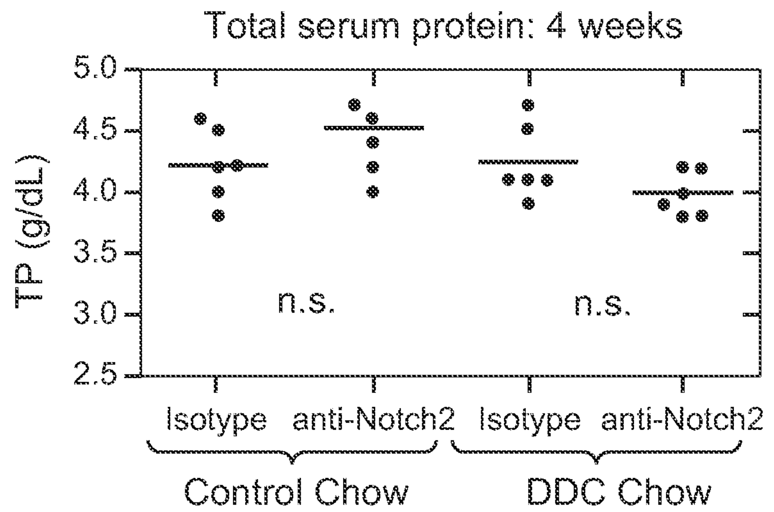


FIG. 11F

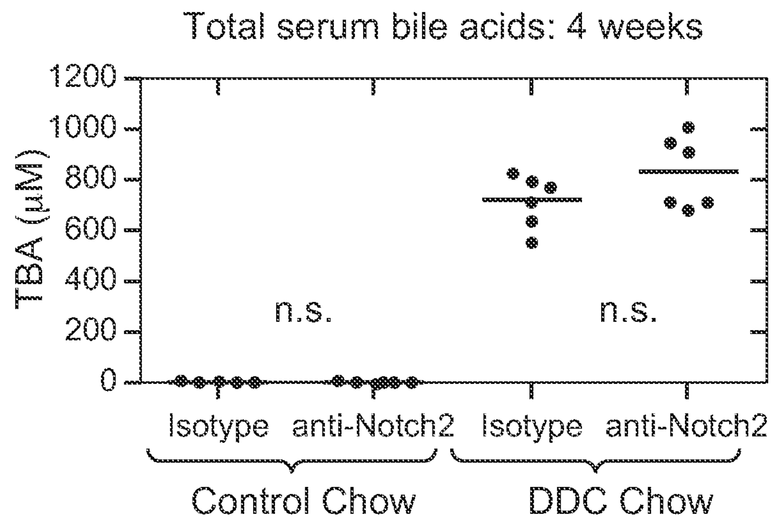


FIG. 11G

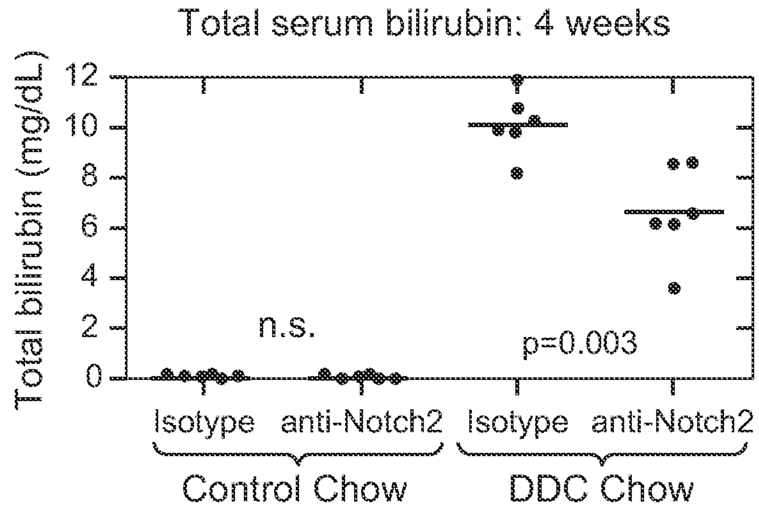


FIG. 11H

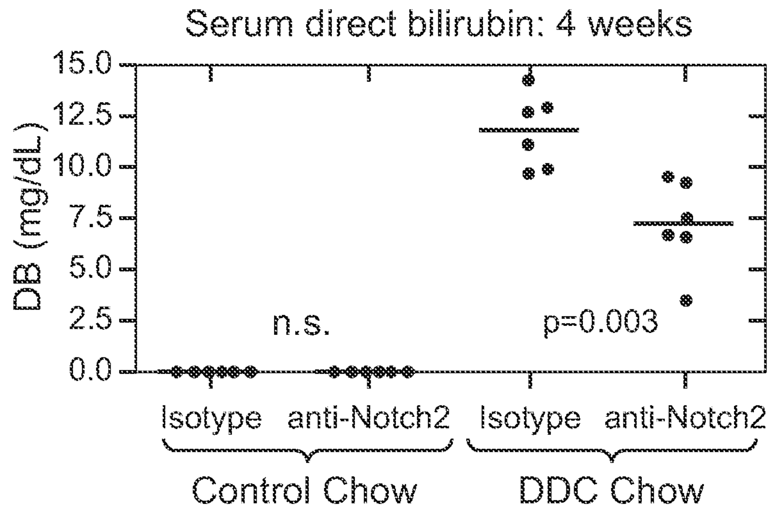
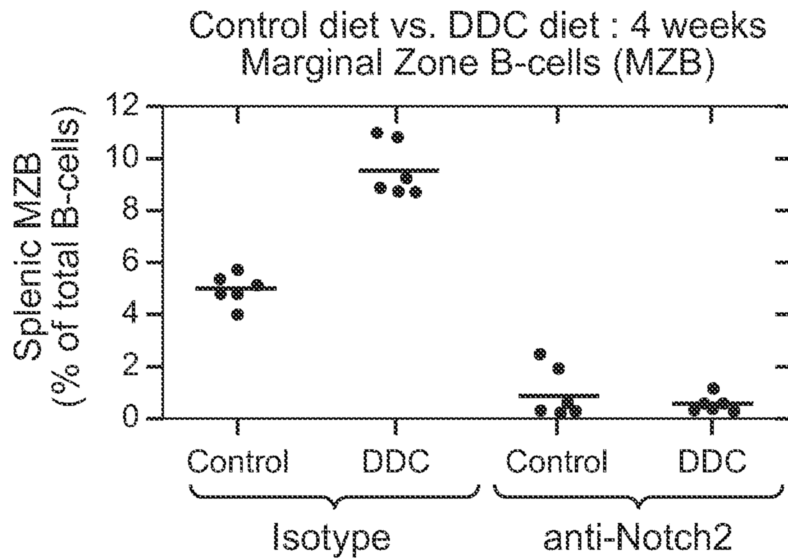


FIG. 11I



HVR-H1 Sequences - Antibody D and Affinity Matured Antibodies Derived from Antibody D

		Kabat Number									
Antibody #	SEQ ID NO:	26	27	28	29	30	31	32	33	34	35
D	1	G	Y	S	F	T	S	Y	G	M	S
D-1, D-2, D-3	2	G	Y	T	F	S	S	Y	G	M	S
Consensus	3	G	Y	S/T	F	S/T	S	Y	G	M	S

HVR-H2 Sequence - Antibody D and Affinity Matured Antibodies Derived from Antibody D

		Kabat Number																	
Antibody #	SEQ ID NO:	49	50	51	52	52a	53	54	55	56	57	58	59	60	61	62	63	64	65
D, D-1, D-2, D-3	4	S	Y	I	Y	P	Y	S	G	A	T	Y	Y	A	D	S	V	K	G

HVR-H3 Sequence - Antibody D and Affinity Matured Antibodies Derived from Antibody D

		Kabat Number												
Antibody #	SEQ ID NO:	95	96	97	98	99	100	100A	100B	100C	100D	100K	101	102
D, D-1, D-2, D-3	5	H	S	G	Y	Y	R	I	S	S	A	M	D	V

FIG. 12

HVR-L1 Sequences - Antibody D and Affinity Matured Antibodies Derived from Antibody D

		Kabat Number										
Antibody #	SEQ ID NO:	24	25	26	27	28	29	30	31	32	33	34
D	6	R	A	S	Q	S	I	S	S	Y	L	A
D-1	7	R	A	S	Q	S	N	R	R	F	L	A
D-2	8	R	A	S	Q	S	V	R	S	F	L	A
D-3	9	R	A	S	Q	N	I	K	R	F	L	A
Consensus	10	R	A	S	Q	S/N	I/N/V	S/R/K	S/R	Y/F	L	A

HVR-L2 Sequences - Antibody D and Affinity Matured Antibodies Derived from Antibody D

		Kabat Number						
Antibody #	SEQ ID NO:	50	51	52	53	54	55	56
D, D-1	11	G	A	S	S	R	A	S
D-2	12	R	A	S	I	R	A	S
D-3	13	G	A	S	T	R	E	S
Consensus	14	G/R	A	S	S/I/T	R	A/E	S

HVR-L3 Sequences - Antibody D and Affinity Matured Antibodies Derived from Antibody D

		Kabat Number								
Clone #	SEQ ID NO:	89	90	91	92	93	94	95	96	97
D	15	Q	Q	Y	Y	S	S	P	L	T
D-1	16	Q	Q	Y	Y	I	S	P	L	T
D-2	17	Q	Q	Y	Y	I	S	P	W	T
D-3	18	Q	Q	Y	Y	R	S	P	H	T
Consensus	19	Q	Q	Y	Y	S/I/R	S	P	L/W/H	T

FIG. 13

Kabat#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	35A	35B	36	37	38	39	40	41	42	43
Antibody D	E	V	Q	L	V	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L	S	C	A	A	S	G	Y	S	F	T	S	Y	G	M	S			W	V	R	Q	A	P	G	K
Antibody D-1	E	V	Q	L	V	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L	S	C	A	A	S	G	Y	T	F	S	S	Y	G	M	S			W	V	R	Q	A	P	G	K
Antibody D-2	E	V	Q	L	V	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L	S	C	A	A	S	G	Y	T	F	S	S	Y	G	M	S			W	V	R	Q	A	P	G	K
Antibody D-3	E	V	Q	L	V	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L	S	C	A	A	S	G	Y	T	F	S	S	Y	G	M	S			W	V	R	Q	A	P	G	K

Kabat#	44	45	46	47	48	49	50	51	52	A	B	C	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	A
Antibody D	G	L	E	W	V	S	Y	I	Y	P			Y	S	G	A	T	Y	Y	A	D	S	V	K	G	R	F	T	I	S	A	D	T	S	K	N	T	A	Y	L	Q	M	N
Antibody D-1	G	L	E	W	V	S	Y	I	Y	P			Y	S	G	A	T	Y	Y	A	D	S	V	K	G	R	F	T	I	S	A	D	T	S	K	N	T	A	Y	L	Q	M	N
Antibody D-2	G	L	E	W	V	S	Y	I	Y	P			Y	S	G	A	T	Y	Y	A	D	S	V	K	G	R	F	T	I	S	A	D	T	S	K	N	T	A	Y	L	Q	M	N
Antibody D-3	G	L	E	W	V	S	Y	I	Y	P			Y	S	G	A	T	Y	Y	A	D	S	V	K	G	R	F	T	I	S	A	D	T	S	K	N	T	A	Y	L	Q	M	N

Kabat#	B	C	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	A	B	C	D	E	F	G	H	I	J	K	101	102	103	104	105	106	107	108	109	110	111	112	113	
Antibody D	S	L	R	A	E	D	T	A	V	Y	Y	C	A	R	H	S	G	Y	Y	R	I	S	S	A							M	D	V	W	G	Q	G	T	L	V	T	V	S	A	SEQ ID NO: 20
Antibody D-1	S	L	R	A	E	D	T	A	V	Y	Y	C	A	R	H	S	G	Y	Y	R	I	S	S	A							M	D	V	W	G	Q	G	T	L	V	T	V	S	A	SEQ ID NO: 2[illegible]
Antibody D-2	S	L	R	A	E	D	T	A	V	Y	Y	C	A	R	H	S	G	Y	Y	R	I	S	S	A							M	D	V	W	G	Q	G	T	L	V	T	V	S	A	SEQ ID NO: [illegible]
Antibody D-3	S	L	R	A	E	D	T	A	V	Y	Y	C	A	R	H	S	G	Y	Y	R	I	S	S	A							M	D	V	W	G	Q	G	T	L	V	T	V	S	A	SEQ ID NO: [illegible]

FIG. 14

Kabat#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	A	B	C	D	E	F	28	29	30	31	32	33	34	35	36	37
Antibody D	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V	G	D	R	V	T	I	T	C	R	A	S	Q							S	I	S	S	Y	L	A	W	Y	Q
Antibody D-1	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V	G	D	R	V	T	I	T	C	R	A	S	Q							S	N	R	R	F	L	A	W	Y	Q
Antibody D-2	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V	G	D	R	V	T	I	T	C	R	A	S	Q							S	V	R	S	F	L	A	W	Y	Q
Antibody D-3	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V	G	D	R	V	T	I	T	C	R	A	S	Q							N	I	K	R	F	L	A	W	Y	Q

Kabat#	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
Antibody D	Q	K	P	G	K	A	P	K	L	L	I	Y	G	A	S	S	R	A	S	G	V	P	S	R	F	S	G	S	G	S	G	T	D	F	T	L	T	I	S	S	L	Q	P
Antibody D-1	Q	K	P	G	K	A	P	K	L	L	I	Y	G	A	S	S	R	A	S	G	V	P	S	R	F	S	G	S	G	S	G	T	D	F	T	L	T	I	S	S	L	Q	P
Antibody D-2	Q	K	P	G	K	A	P	K	L	L	I	Y	R	A	S	I	R	A	S	G	V	P	S	R	F	S	G	S	G	S	G	T	D	F	T	L	T	I	S	S	L	Q	P
Antibody D-3	Q	K	P	G	K	A	P	K	L	L	I	Y	G	A	S	T	R	E	S	G	V	P	S	R	F	S	G	S	G	S	G	T	D	F	T	L	T	I	S	S	L	Q	P

Kabat#	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	A	B	C	D	E	F	96	97	98	99	100	101	102	103	104	105	106	107	108	
Antibody D	E	D	F	A	T	Y	Y	C	Q	Q	Y	Y	S	S	P							L	T	F	G	Q	G	T	K	V	E	I	K	R	SEQ ID NO: 22
Antibody D-1	E	D	F	A	T	Y	Y	C	Q	Q	Y	Y	I	S	P							L	T	F	G	Q	G	T	K	V	E	I	K	R	SEQ ID NO: 23
Antibody D-2	E	D	F	A	T	Y	Y	C	Q	Q	Y	Y	I	S	P							W	T	F	G	Q	G	T	K	V	E	I	K	R	SEQ ID NO: 24
Antibody D-3	E	D	F	A	T	Y	Y	C	Q	Q	Y	Y	R	S	P							H	T	F	G	Q	G	T	K	V	E	I	K	R	SEQ ID NO: 25

FIG. 15

	FR1		FR2		
I					
A	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	-H1-	WVRQAPGQGLEWMG	-H2-	RVTIT
B	QVQLVQSGAEVKKPGASVKVSCKAS	-H1-	WVRQAPGQGLEWM	-H2-	RVTIT
C	QVQLVQSGAEVKKPGASVKVSCKAS	-H1-	WVRQAPGQGLEWM	-H2-	RVTIT
D	QVQLVQSGAEVKKPGASVKVSCKAS	-H1-	WVRQAPGQGLEWM	-H2-	RVTIT
II					
A	QVQLQESGPGLVKPSQTLSLTCTVSGGSVS	-H1-	WIRQPPGKGLEWIG	-H2-	RVTIS
B	QVQLQESGPGLVKPSQTLSLTCTVS	-H1-	WIRQPPGKGLEWI	-H2-	RVTIS
C	QVQLQESGPGLVKPSQTLSLTCTVS	-H1-	WIRQPPGKGLEWI	-H2-	RVTIS
D	QVQLQESGPGLVKPSQTLSLTCTVS	-H1-	WIRQPPGKGLEWI	-H2-	RVTIS
III					
A	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	-H1-	WVRQAPGKGLEWVS	-H2-	RFTIS
B	EVQLVESGGGLVQPGGSLRLSCAAS	-H1-	WVRQAPGKGLEWV	-H2-	RFTIS
C	EVQLVESGGGLVQPGGSLRLSCAAS	-H1-	WVRQAPGKGLEWV	-H2-	RFTIS
D	EVQLVESGGGLVQPGGSLRLSCAAS	-H1-	WVRQAPGKGLEWV	-H2-	RFTIS
Acceptor - 1					
A	EVQLVESGGGLVQPGGSLRLSCAASGFNIK	-H1-	WVRQAPGKGLEWVS	-H2-	RFTIS
B	EVQLVESGGGLVQPGGSLRLSCAAS	-H1-	WVRQAPGKGLEWV	-H2-	RFTIS
C	EVQLVESGGGLVQPGGSLRLSCAAS	-H1-	WVRQAPGKGLEWV	-H2-	RFTIS
Acceptor - 2					
A	EVQLVESGGGLVQPGGSLRLSCAASGFNIK	-H1-	WVRQAPGKGLEWVS	-H2-	RFTIS
B	EVQLVESGGGLVQPGGSLRLSCAAS	-H1-	WVRQAPGKGLEWV	-H2-	RFTIS
C	EVQLVESGGGLVQPGGSLRLSCAAS	-H1-	WVRQAPGKGLEWV	-H2-	RFTIS
D	EVQLVESGGGLVQPGGSLRLSCAAS	-H1-	WVRQAPGKGLEWV	-H2-	RFTIS

FIG. 16A

	FR3		FR4	SEQ ID NOs of FR1, FR2, FR3, FR4
I				
A	ADTSTSTAYMELSSLRSEDTAVYYCAR	-H3-	WGQGTLVTVSS	SEQ ID NO.: 32, 33, 34, 35
B	ADTSTSTAYMELSSLRSEDTAVYYCAR	-H3-	WGQGTLVTVSS	SEQ ID NO.: 36, 37, 34, 35
C	ADTSTSTAYMELSSLRSEDTAVYYCA	-H3-	WGQGTLVTVSS	SEQ ID NO.: 36, 37, 38, 35
D	ADTSTSTAYMELSSLRSEDTAVYYC	-H3-	WGQGTLVTVSS	SEQ ID NO.: 36, 37, 39, 35
II				
A	VDTSKNQFSLKLSSVTAADTAVYYCAR	-H3-	WGQGTLVTVSS	SEQ ID NO.: 40, 41, 42, 35
B	VDTSKNQFSLKLSSVTAADTAVYYCAR	-H3-	WGQGTLVTVSS	SEQ ID NO.: 43, 44, 42, 35
C	VDTSKNQFSLKLSSVTAADTAVYYCA	-H3-	WGQGTLVTVSS	SEQ ID NO.: 43, 44, 45, 35
D	VDTSKNQFSLKLSSVTAADTAVYYC	-H3-	WGQGTLVTVSS	SEQ ID NO.: 43, 44, 46, 35
III				
A	RDNSKNTLYLQMNSLRAEDTAVYYCAR	-H3-	WGQGTLVTVSS	SEQ ID NO.: 47, 48, 49, 35
B	RDNSKNTLYLQMNSLRAEDTAVYYCAR	-H3-	WGQGTLVTVSS	SEQ ID NO.: 50, 51, 49, 35
C	RDNSKNTLYLQMNSLRAEDTAVYYCA	-H3-	WGQGTLVTVSS	SEQ ID NO.: 50, 51, 52, 35
D	RDNSKNTLYLQMNSLRAEDTAVYYC	-H3-	WGQGTLVTVSS	SEQ ID NO.: 50, 51, 53, 35
Acceptor - 1				
A	ADTSKNTAYLQMNSLRAEDTAVYYCSR	-H3-	WGQGTLVTVSS	SEQ ID NO.: 54, 48, 55, 35
B	ADTSKNTAYLQMNSLRAEDTAVYYCSR	-H3-	WGQGTLVTVSS	SEQ ID NO.: 50, 51, 55, 35
C	ADTSKNTAYLQMNSLRAEDTAVYYCS	-H3-	WGQGTLVTVSS	SEQ ID NO.: 50, 51, 56, 35
Acceptor - 2				
A	ADTSKNTAYLQMNSLRAEDTAVYYCAR	-H3-	WGQGTLVTVSS	SEQ ID NO.: 54, 48, 57, 35
B	ADTSKNTAYLQMNSLRAEDTAVYYCAR	-H3-	WGQGTLVTVSS	SEQ ID NO.: 50, 51, 57, 35
C	ADTSKNTAYLQMNSLRAEDTAVYYCA	-H3-	WGQGTLVTVSS	SEQ ID NO.: 50, 51, 58, 35
D	ADTSKNTAYLQMNSLRAEDTAVYYC	-H3-	WGQGTLVTVSS	SEQ ID NO.: 50, 51, 59, 35

FIG. 16B

	FR1		FR2		FR3
kv1	DIQMTQSPSSLSASVGDRVTITC	-L1-	WYQQKPGKAPKLLIY	-L2-	GVPSRFSGSGSGTDFTLTISSLQP
kv2	DIVMTQSPLSLPVTPGEPASISC	-L1-	WYLQKPGQSPQLLIY	-L2-	GVPDRFSGSGSGTDFTLKISRVEA
kv3	EIVLTQSPGTLSLSPGERATLSC	-L1-	WYQQKPGQAPRLLIY	-L2-	GIPDRFSGSGSGTDFTLTISRLEP
kv4	DIVMTQSPDSLAVSLGERATINC	-L1-	WYQQKPGQPPKLLIY	-L2-	GVPDRFSGSGSGTDFTLTISSLQA

		FR4	SEQ ID NOs of FR1, FR2, FR3, FR4
EDFATYYC	-L3-	FGQGTKVEIK	SEQ ID NO.: 60, 61, 62, 63
EDVGVYYC	-L3-	FGQGTKVEIK	SEQ ID NO.: 64, 65, 66, 63
EDFAVYYC	-L3-	FGQGTKVEIK	SEQ ID NO.: 67, 68, 69, 63
EDVAVYYC	-L3-	FGQGTKVEIK	SEQ ID NO.: 70, 71, 72, 63

FIG. 17

Framework Sequences of huMAb4D5-8 Light Chain Variable Domain

LC-FR1 ^{1}Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys^{23} (SEQ ID NO: 60)

LC-FR2 ^{35}Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr^{49} (SEQ ID NO: 61)

LC-FR3 ^{57}Gly Val Pro Ser Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys^{88} (SEQ ID NO: 30)

LC-FR4 ^{98}Phe Gly Gln Gly Thr Lys Val Glu Ile Lys^{107} (SEQ ID NO: 63)

Framework Sequences of huMAb4D5-8 Heavy Chain Variable Domain

HC-FR1 ^{1}Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser^{25} (SEQ ID NO: 50)

HC-FR2 ^{36}Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val^{48} (SEQ ID NO: 51)

HC-FR3 ^{66}Arg Phe Thr Ile Ser Ala Asp Thr Ser Lys Asn Thr Ala Tyr Leu Gln Met Asn^{83} Ser^{83a} Leu^{83b} Arg^{83c} Ala Glu Asp Thr Ala Val Tyr Tyr Cys^{92} (SEQ ID NO: 59)

HC-FR4 ^{103}Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser^{113} (SEQ ID NO: 35)

FIG. 18

Framework Sequences of huMAb4D5-8 Light Chain Variable Domain Modified at Positions 66 and 99 (Underlined)

LC-FR1 ^{1}Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys^{23} (SEQ ID NO: 60)

LC-FR2 ^{35}Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr^{49} (SEQ ID NO: 61)

LC-FR3 ^{57}Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys^{88} (SEQ ID NO: 62)

LC-FR4 ^{98}Phe Arg Gln Gly Thr Lys Val Glu Ile Lys^{107} (SEQ ID NO: 31)

Framework Sequences of huMAb4D5-8 Heavy Chain Variable Domain Modified at Postions 71, 73 and 78 (Underlined)

HC-FR1 ^{1}Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser^{25} (SEQ ID NO: 50)

HC-FR2 ^{36}Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val^{48} (SEQ ID NO: 51)

HC-FR3 ^{66}Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu Gln Met Asn83 Ser83a Leu83b Arg83c Ala Glu Asp Thr Ala Val Tyr Tyr Cys^{92} (SEQ ID NO: 53)

HC-FR4 ^{103}Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser^{113} (SEQ ID NO: 35)

FIG. 19

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/032621

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28 A61P1/16
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, SCISEARCH, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2006/135949 A2 (UNIV WIEN MED [AT]; HUBMANN RAINER [AT]; SIEGHART WOLFGANG [AT]) 28 December 2006 (2006-12-28) page 19, paragraph 2	1-17
Y	WO 2010/005566 A2 (ONCOMED PHARM INC [US]; GURNEY AUSTIN L [US]; HOEY TIMOTHY CHARLES [US) 14 January 2010 (2010-01-14) page 26, paragraph 95 claim 8; example 3 page 72, paragraph 270	1-17
Y	WO 2010/039832 A1 (GENENTECH INC [US]; SIEBEL CHRISTIAN W [US]; WU YAN [US]) 8 April 2010 (2010-04-08) cited in the application pages 109-110	1-17

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 August 2012	10/08/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Domingues, Helena

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WU YAN ET AL: "Therapeutic antibody targeting of individual Notch receptors", NATURE: INTERNATIONAL WEEKLY JOURNAL OF SCIENCE, NATURE PUBLISHING GROUP, UNITED KINGDOM, vol. 464, no. 7291, 15 April 2010 (2010-04-15), pages 1052-1058, XP009137924, ISSN: 0028-0836, DOI: 10.1038/NATURE08878 cited in the application the whole document -----	1-17
Y	GEISLER FABIAN ET AL: "Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice", HEPATOLOGY, vol. 48, no. 2, August 2008 (2008-08), pages 607-616, XP002680875, ISSN: 0270-9139 the whole document -----	1-17
Y	TCHORZ JAN S ET AL: "Notch2 Signaling Promotes Biliary Epithelial Cell Fate Specification and Tubulogenesis During Bile Duct Development in Mice", HEPATOLOGY, vol. 50, no. 3, September 2009 (2009-09), pages 871-879, XP002680876, ISSN: 0270-9139 the whole document -----	1-17
Y	FIOROTTO R ET AL: "PROGENITOR CELL ACTIVATION AND LIVER REPAIR IS ALTERED IN NOTCH2-AND RBP-J kappa-DEFECTIVE MICE EXPOSED TO CHOLESTATIC INJURIES", JOURNAL OF HEPATOLOGY, vol. 52, no. Suppl. 1, 2010, page S45, XP002680877, & 45TH ANNUAL MEETING OF THE EUROPEAN-ASSOCIATION-FOR-THE-STUDY-OF-THE-LIVER; VIENNA, AUSTRIA; APRIL 14 -18, 2010 ISSN: 0168-8278 the whole document -----	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2012/032621

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006135949 A2	28-12-2006	AT 458487 T	15-03-2010
		AT 502055 A1	15-01-2007
		AU 2006261566 A1	28-12-2006
		CA 2612770 A1	28-12-2006
		EP 1901750 A2	26-03-2008
		US 2009029974 A1	29-01-2009
		WO 2006135949 A2	28-12-2006
WO 2010005566 A2	14-01-2010	AU 2009269095 A1	14-01-2010
		AU 2009269096 A1	14-01-2010
		CA 2729306 A1	14-01-2010
		CA 2729602 A1	14-01-2010
		CN 102112490 A	29-06-2011
		CN 102316897 A	11-01-2012
		CO 6390041 A2	29-02-2012
		CR 20110069 A	11-07-2011
		DO P2011000002 A	15-04-2011
		DO P2011000004 A	15-04-2011
		EA 201170157 A1	30-08-2011
		EA 201170158 A1	30-08-2011
		EP 2307051 A2	13-04-2011
		EP 2307459 A2	13-04-2011
		JP 2012501625 A	26-01-2012
		JP 2012501626 A	26-01-2012
		KR 20110028621 A	21-03-2011
		KR 20110031315 A	25-03-2011
		MA 32448 B1	01-07-2011
		MA 32449 B1	01-07-2011
		US 2010111958 A1	06-05-2010
		US 2011311552 A1	22-12-2011
		WO 2010005566 A2	14-01-2010
		WO 2010005567 A2	14-01-2010
WO 2010039832 A1	08-04-2010	AR 073717 A1	24-11-2010
		AU 2009298569 A1	08-04-2010
		CA 2736429 A1	08-04-2010
		CN 102170909 A	31-08-2011
		EP 2334331 A1	22-06-2011
		JP 2012504634 A	23-02-2012
		KR 20110061600 A	09-06-2011
		PE 00802012 A1	17-02-2012
		TW 201018484 A	16-05-2010
		US 2010080808 A1	01-04-2010
		WO 2010039832 A1	08-04-2010