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(54) **Title**: NOTCH 3 MUTANTS AND USES THEREOF

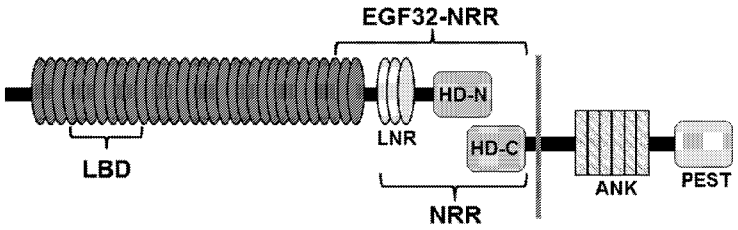

Figure 1

(57) **Abstract**: The present invention relates to the field of pharmacogenomics. In particular, the present invention relates to Notch 3 mutants and uses thereof.

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NOTCH 3 MUTANTS AND USES THEREOF

Related Applications

This application claims priority to US Provisional Application No. 61/781,396 filed on March 14, 2013, the contents of which are incorporated herein by reference in their entirety.

Field of the Invention

The present invention relates to the field of pharmacogenomics. In particular, the present invention relates to Notch 3 mutants and uses thereof.

Background of the Invention

Notch signaling is an evolutionarily conserved pathway that regulates a diverse set of biological functions including stem cell maintenance, cell differentiation and proliferation in both embryonic development and adult tissues (Kopan *et al.,* (2009) Cell 137: 216-233, Guruharsha *et al.,* (2012) Nat Rev Genet. 13: 654-66, and Andersson *et al.,* (2001) Development 138: 3593-3612). In mammals, four Notch receptors have been described (Notch1-4), which have a conserved domain architecture. The extracellular domain (ECD) consists of a series of EGF-like repeats followed by a negative regulatory region (NRR) which contains 3 LNR repeats and a heterodimerization domain. Canonical Notch signaling is activated when a Notch receptor on one cell interacts with a ligand on a neighboring cell. In mammals there are five trans-membrane ligands, three Delta-like ligands (DLL1, DLL4, and DLL3) and two Jagged ligands (Jagged1, Jagged2). Ligand binding results in cleavage of Notch by ADAM proteases at the S2 site within the NRR domain. This initial cleavage generates the substrate for subsequent cleavage of the Notch receptor at the S3 site by the γ-secretase complex. Following γ-secretase cleavage, the intracellular domain of Notch (ICD) translocates to the nucleus where it interacts with a CSL transcription factor (CBF-1/RBP-Jk in mammals) and the co-activator mastermind (MAML1) to activate target gene transcription. The HES/HEY family of transcription factors are well-characterized Notch target genes, however a large number of transcriptional targets are cell-type specific.

To date, the evidence for Notch receptors in cancer has focused primarily on alterations in Notch1 signaling, but very little on other Notch receptors. Accordingly, a need exists to study

and identify methods and compositions that alter other Notch receptor signaling, such as Notch 3 signaling.

Summary of the Invention

The disclosure pertains to a number of Notch 3 mutants that activate Notch 3 signal transduction ("activating mutants"), and uses thereof.

Accordingly, in one aspect, the disclosure pertains to a mutant Notch 3 receptor comprising at least one activating mutaton set forth in Table 1, or combinations thereof , where the presence of the activating mutation is determined using an assay comprising a Notch 3 intracellular domain 3 (ICD3) antibody or fragment thereof that detects SEQ ID NO: 3.

In another aspect the disclosure pertains to a mutant Notch 3 receptor comprising at least one activating mutation located in the NRR of Notch 3, where the activating mutation activates Notch 3 signal transduction, and wherein the presence of the activating mutation is determined using an assay comprising a Notch 3 intracellular domain 3 (ICD3) antibody or fragment thereof that detects SEQ ID NO: 3. In one embodiment, the mutation in the NRR domain is selected from the group consisting of S1580L, D1587N, Y1624H, L1518M, A1537T, N1597K, L1547V, R1526C (HD) and G1487D (LNR-C)]. In one embodiment, the mutant Notch 3 receptor further comprises at least one mutation located in the PEST domain of Notch 3. In one embodiment, the mutation in the PEST domain is selected from the group consisting of P2034fs, P2067fs, p2177fs, Q2075*, W2172*, G2112D, L2212M, F2121L, G2038S, G2059R, R2022H, Y2127H, Y2211C, V2202I, S2096L, P2089L, P2209L, R1981C, R2145Q, and P2178S.

In another aspect the disclosure pertains to a mutant Notch 3 receptor comprising an amino acid sequence that is at least 90% identical to SEQ ID NO:1, wherein the amino acid sequence of the mutant Notch 3 receptor differs from SEQ ID NO:1 by virtue of containing a Leu at position 1580 rather than Ser in an NRR domain of Notch 3, and wherein the mutation in the Notch 3 polypeptide activates Notch 3 signal transduction.

In another aspect the disclosure pertains to a mutant Notch 3 receptor comprising an amino acid sequence that is at least 90% identical to SEQ ID NO:1, wherein the amino acid sequence of the mutant Notch 3 receptor differs from SEQ ID NO:1 by virtue of containing D at

position 1487 rather than G in an NRR domain of Notch 3, and wherein the mutation in the Notch 3 polypeptide activates Notch 3 signal transduction.

In another aspect the disclosure pertains to a method of determining the increased likelihood of having or developing a cancer in a subject, comprising:

assaying a biological sample obtained from a subject for the presence of a Notch 3 activating mutation using an assay comprising a Notch 3 intracellular domain 3 (ICD3) antibody or fragment thereof that detects SEQ ID NO: 3; and

comparing the biological sample from subject with a non-cancerous or normal control cell, wherein the presence of the Notch 3 mutation indicates the likelihood of developing cancer. In one embodiment, the biological sample is selected from the group consisting of blood, serum, urine, hair follicle, ascites, and tumor biopsy In one embodiment, the subject is a human and the cancer is selected from the group consisting of breast cancer, colorectal cancer, lung cancer, multiple myeloma, ovarian cancer, liver cancer, gastric cancer, pancreatic cancer, prostate cancer, acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, t-cell acute lymphoblastic leukemia, mantle cell lymphoma, chronic lymphocytic leukemia, Ewings sarcoma, lymphoma, osteosarcoma, squamous cell carcinoma, peripheral nerve sheath tumors , schwannoma, head and neck cancer, bladder cancer, esophageal cancer, glioblastoma, clear cell sarcoma of soft tissue, malignant mesothelioma, neurofibromatosis, renal cancer, and melanoma. In one embodiment, the cancer is T-cell acute lymphoblastic leukemia (TALL).

In another aspect the disclosure pertains to a method for detecting the presence of an activated form ofNotch 3 receptor in a biological sample, the method comprising:

contacting the biological sample with a Notch 3 intracellular domain 3 (ICD3) antibody or fragment thereof that detects SEQ ID NO: 3;

incubating the sample and the ICD3antibody or fragment thereof under conditions to induce binding of the ICD3antibody or fragment thereof to a Notch 3 receptor if present in the sample to form a complex; and

detecting the ICD3 antibody, thereby detecting the presence of activated form of the Notch 3 receptor in a sample.

Brief Description of Figures

Figure 1: Domain structure of Notch 3;

Figure 2: Domain structure of Notch 3 NRR with amino acid positions of each region;

Figure 3A-B: Notch 3 NRR (Top) and PEST (Bottom) mutations;

Figure 4A-C: Notch 3 reporter assay and graphs showing the characterization of Notch 3 NRR mutations;

Figure 5A-B: Graphs showing TALL-1 mRNA and inhibition of proliferation in the presence of Notch 3 antibodies;

Figure 6A-B: Photographs of Western blots showing the presence of a neo-epitope ICD3 antibody in TALL-1 cells only;

Figure 7A-B: Photographs of Western blots showing decreased Notch 3 signaling with Notch 3 antibody treatment in TALL-1 cells and MDA-MB468 cells;

Figure 8A-C: Photographs of Western blots showing decreased Notch 3 signaling with Notch 3 antibody treatment in Ishikawaheraklio02_ER cells, TE-11 cells, and A549 cells;

Figure 9: Photographs of Western blots showing decreased Notch 3 signaling with Notch 3 antibody treatment in a Notch 3 amplified cell-line, HCC1143;

Figure 10A-B: Photographs of Western blots and IHC photographs of in vivo PD studies in TALL-1;

Figure 11A-B: Photographs of Western blots of in vivo PD studies in MDA-MB468;

Figure 12: Photographs of Western blots in an in vivo PD HLUX1823 model;

Figure 13A-B: Photographs of mice showing TALL-1 in vivo efficacy; and

Figure 14A-D: Structure of Notch3 NRR. (A) and (B), structures of Notch3 NRR in complex with Fab of Ab-A or Ab-B. The structures of Notch3 NRR are almost identical (RMSD 0.42Å) in these two complexes; (C), domain boundaries of Notch3 NRR; (D), surface and ribbon representation of Notch3 NRR structure, labeled are 1) N- and C-terminus of the proteins; 2) the three LNR repeats and the coordinated Ca2+ ions; 3) L1419, the autoinhibitory plug; 4) S1 and S2 sites; 5) secondary structures within HD domain; and 6) the

two regions in Notch3 with significantly different conformation (RMSD > 2Å) than Notch1 and Notch2 (LNR-B/C linker plus first half of LNR-C, and β4-α3 loop in HD domain).

Detailed Description

Definitions

In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

The phrase "Notch 3 activating mutation" or "activating mutation" as used herein refers to a mutation in a Notch 3 receptor that switches on Notch 3 signal transduction.

The terms "marker" or "biomarker" are used herein refers to a nucleic acid or polypeptide, of a mutation in a Notch 3 receptor. The presence or absence of the biomarker is used to determine the presence of a Notch 3 mutation. For example, Notch 3 is a biomarker when the presence of a mutation in a cancer cell when as compared to non-cancerous or normal control cell. Examples of mutations that represent a biomarker include, but are not limited to a mutation selected from the group consisting of S1580L, A1476T, G1487D, or combinations thereof.

The phrase "signal transduction" or "signaling activity" as used herein refers to a biochemical causal relationship generally initiated by a protein-protein interaction such as binding of a growth factor to a receptor, resulting in transmission of a signal from one portion of a cell to another portion of a cell. For Notch 3, ligand binding results in cleavage of Notch 3 by ADAM proteases at the S2 site within the NRR domain. This initial cleavage generates the substrate for subsequent cleavage of the Notch receptor at the S3 site by the γ-secretase complex. Following γ-secretase cleavage, the intracellular domain of Notch (ICD) translocates to the nucleus where it interacts with a CSL transcription factor (CBF-1/RBP-Jk in mammals) and the co-activator mastermind (MAML1) to activate target gene transcription.

The term "Notch 3" or "Notch 3 receptor" as used herein refers to mammalian human Notch 3 protein. The domain structure of Notch 3 is depicted in Figure 1, which shows the ligand binding domain (LBD), negative regulatory region (NRR) comprising the Lin Notch Repeats (LNR) and the N-, C-terminal heterodimerization domain (HD-N and HD-C, respectively), as

well as the ankarin domain (ANK) and PEST domains. Figure 2 shows the overall structure of Notch 3 NRR and the corresponding amino acid residues: LNR-A has amino acid residues E1383-G1422; LNR-A-B linker has amino acid residues Asp1423-Leu-1431; LNR-B has amino acid residues Gln1432-Ala1460; LNR-B-C linker has amino acid residues; LNR-B-C linker has amino acid residues Gly1461-Asn1468; LNR-C has amino acid residues Pro1469-Ser1502; LNR-HD linker has amino acid residues Glu1503-Arg1510; HD-N has amino acid residues Gly1511-Arg1571; and HD-C has amino acid residues1572-Ser1640.

Human Notch 3, as represented below as SEQ ID NO: 1.

MGPGARGRRRRRRPMSPPPPPPPVRALPLLLLLAGPGAAAPPCLDGSPCANGGRCTQ
LPSREAACLCPPGWVGERCQLEDPCHSGPCAGRGVCQSSVVAGTARFSCRCPRGFRG
PDCSLPDPCLSSPCAHGARCSVGPDGRFLCSCPPGYQGRSCRSDVDECRVGEPCRHGG
TCLNTPGSFRCQCPAGYTGPLCENPAVPCAPSPCRNGGTCRQSGDLTYDCACLPGFEG
QNCEVNVDDCPGHRCLNGGTCVDGVNTYNCQCPPEWTGQFCTEDVDECQLQPNAC
HNGGTCFNTLGGHSCVCVNGWTGESCSQNIDDCATAVCFHGATCHDRVASFYCACP
MGKTGLLCHLDDACVSNPCHEDAICDTNPVNGRAICTCPPGFTGGACDQDVDECSIG
ANPCEHLGRCVNTQGSFLCQCGRGYTGPRCETDVNECLSGPCRNQATCLDRIGQFTCI
CMAGFTGTYCEVDIDECQSSPCVNGGVCKDRVNGFSCTCPSGFSGSTCQLDVDECAS
TPCRNGAKCVDQPDGYECRCAEGFEGTLCDRNVDDCSPDPCHHGRCVDGIASFSCAC
APGYTGTRCESQVDECRSQPCRHGGKCLDLVDKYLCRCPSGTTGVNCEVNIDDCASN
PCTFGVCRDGINRYDCVCQPGFTGPLCNVEINECASSPCGEGGSCVDGENGFRCLCPP
GSLPPLCLPPSHPCAHEPCSHGICYDAPGGFRCVCEPGWSGPRCSQSLARDACESQPC
RAGGTCSSDGMGFHCTCPPGVQGRQCELLSPCTPNPCEHGGRCESAPGQLPVCSCPQ
GWQGPRCQQDVDECAGPAPCGPHGICTNLAGSFSCTCHGGYTGPSCDQDINDCDPNP
CLNGGSCQDGVGSFSCSCLPGFAGPRCARDVDECLSNPCGPGTCTDHVASFTCTCPPG
YGGFHCEQDLPDCSPSSCFNGGTCVDGVNSFSCLCRPGYTGAHCQHEADPCLSRPCL
HGGVCSAAHPGFRCTCLESFTGPQCQTLVDWCSRQPCQNGGRCVQTGAYCLCPPGW
SGRLCDIRSLPCREAAAQIGVRLEQLCQAGGQCVDEDSSHYCVCPEGRTGSHCEQEV
DPCLAQPCQHGGTCRGYMGGYMCECLPGYNGDNCEDDVDECASQPCQHGGSCIDL
VARYLCSCPPGTLGVLCEINEDDCGPGPPLDSGPRCLHNGTCVDLVGGFRCTCPPGYT
GLRCEADINECRSGACHAAHTRDCLQDPGGGFRCLCHAGFSGPRCQTVLSPCESQPC
QHGGQCRPSPGPGGGLTFTCHCAQPFWGPRCERVARSCRELQCPVGVPCQQTPRGPR
CACPPGLSGPSCRSFPGSPPGASNASCAAAPCLHGGSCRPAPLAPFFRCACAQGWTGP
RCEAPAAAPEVSEEPRCPRAACQAKRGDQRCDRECNSPGCGWDGGDCSLSVGDPWR

QCEALQCWRLFNNSRCDPACSSPACLYDNFDCHAGGRERTCNPVYEKYCADHFADG
RCDQGCNTEECGWDGLDCASEVPALLARGVLVLTVLLPPEELLRSSADFLQRLSAILR
TSLRFRLDAHGQAMVFPYHRPSPGSEPRARRELAPEVIGSVVMLEIDNRLCLQSPEND
HCFPDAQSAADYLGALSAVERLDFPYPLRDVRGEPLEPPEPSVPLLPLLVAGAVLLLV
ILVLGVMVARRKREHSTLWFPEGFSLHKDVASGHKGRREPVGQDALGMKNMAKGE
SLMGEVATDWMDTECPEAKRLKVEEPGMGAEEAVDCRQWTQHHLVAADIRVAPA
MALTPPQGDADADGMDVNVRGPDGFTPLMLASFCGGALEPMPTEEDEADDTSASIIS
DLICQGAQLGARTDRTGETALHLAARYARADAAKRLLDAGADTNAQDHSGRTPLHT
AVTADAQGVFQILIRNRSTDLDARMADGSTALILAARLAVEGMVEELIASHADVNAV
DELGKSALHWAAAVNNVEATLALLKNGANKDMQDSKEETPLFLAAREGSYEAAKL
LLDHFANREITDHLDRLPRDVAQERLHQDIVRLLDQPSGPRSPPGPHGLGPLLCPPGAF
LPGLKAAQSGSKKSRRPPGKAGLGPQGPRGRGKKLTLACPGPLADSSVTLSPVDSLDS
PRPFGGPPASPGGFPLEGPYAAATATAVSLAQLGGPGRAGLGRQPPGGCVLSLGLLNP
VAVPLDWARLPPPAPPGPSFLLPLAPGPQLLNPGTPVSPQERPPPYLAVPGHGEEYPA
AGAHSSPPKARFLRVPSEHPYLTPSPESPEHWASPSPPSLSDWSESTPSPATATGAMAT
TTGALPAQPLPLSVPSSLAQAQTQLGPQPEVTPKRQVLA (SEQ ID NO: 1)

Cynomolgus monkey Notch 3 is represented below as SEQ ID NO: 2.

MGPGARGRRRRRRPMSPPPPPVRALPLLLLLAGPGAAVPPCLDGSPCANGGRCTQLP
SREAACLCPPGWVGERCQLEDPCHSGPCAGRGVCQSSVVAGTARFSCRCPRGFRGPD
CSLPDPCLSSPCAHSARCSVGPDGRFLCSCPPGYQGRSCRSDVDECRVGEPCRHGGTC
LNTPGSFRCQCPAGYTGPLCENPAVPCAPSPCRNGGTCRQSGDLTYDCACLPGFEGQ
NCEVNVDDCPGHRCLNGGTCVDGVNTYNCQCPPEWTGQFCTEDVDECQLQPNACH
NGGTCFNTLGGHSCVCVNGWTGESCSQNIDDCATAVCFHGATCHDRVASFYCACPM
GKTGLLCHLDDACVSNPCHEDAICDTNPVNGRAICTCPPGFTGGACDQDVDECSIGA
NPCEHLGRCVNTQGSFLCQCGRGYTGPRCETDVNECLSGPCRNQATCLDRIGQFTCIC
MAGFTGTYCEVDIDECQSSPCVNGGICKDRVNGFSCTCPSGFSGSTCQLDVDECASTP
CRNGAKCVDQPDGYECRCAEGFEGMLCERNVDDCSPDPCHHGRCVDGIASFSCACA
PGYTGTRCESQVDECRSQPCRHGGKCLDLVDKYLCRCPSGTTGVNCEVNIDDCASNP
CSFGVCRDGINRYDCVCQPGFTGPLCNVEINECASSPCGEGGSCVDGENGFRCLCPPG
SLPPLCLPPSHPCAHEPCSHGICYDAPGGFRCVCEPGWSGPRCSQSLARDACESQPCR
AGGTCSSDGMGFHCTCPPGVQGRQCELLSPCTPNPCEHGGRCESAPGQLPVCSCPQG
WQGPRCQQDVDECAGPAPCGPHGICTNLAGSFSCTCHGGYTGPSCDQDINDCDPNPC
LNGGSCQDGVGSFSCSCLLGFAGPRCARDVDECLSNPCGPGTCTDHVASFTCTCPPG

YGGFHCEQDLPDCSPSSCFNGGTCVDGVNSFSCLCRPGYTGAHCQHEADPCLSRPCL
HGGVCSAAHPGFRCTCPQSFTGPQCQTLVDWCSRQPCQNGGRCVQTGAYCLCPPGW
SGRLCDIRSLPCREAAAQIGVRLEQLCQAGGQCVDEDSSHYCVCPEGRTGSHCEQEV
DPCLAQPCQHGGTCRGYMGGYMCECLPGYNGENCEDDVDECASQPCQHGGSCIDL
VARYLCSCPPGTLGVLCEINEDDCGPGPPLDSGPRCLHNGTCVDLVGGFRCTCPPGYT
GLRCEADINECRSGACHAAHTRDCLQDPGGGFRCLCHAGFSGPRCQTVLSPCESQPC
QHGGQCRPSPGPGGGLTFTCHCAQPFWGPRCERVARSCRELQCPVGVPCQQTPRGPR
CACPPGLSGPSCRSFSGSPPGASNASCAAAPCLHGGSCRPAPLAPFFRCACAQGWTGP
RCEAPAAAPEVSEEPRCPRAACQAKRGDQRCDRECNSPGCGWDGGDCSLSVGDPWR
QCEALQCWRLFNNSRCDPACSSPACLYDNFDCHAGGRERTCNPVYEKYCADHFADG
RCDQGCNTEECGWDGLDCASEVPALLARGVLVLTVLLPPEELLRSSADFLQRLSAILR
TSLRFRLDAHGQAMVFPYHRPSPGSEPRARRELAPEVIGSVVMLEIDNRLCLQSPEND
HCFPDAQSAADYLGALSAVERLDFPYPLRDVRGEPLEPPEPSVPLLPLLVAGAVLLLV
ILVLGVMVARRKREHSTLWFPEGFSLHKDVAAGHKGRREPVGQDALGMKNMAKGE
SLMGEVATDWMDTECPEAKRLKVEELGMGAEEAVDCRQWTQHHLVAADIRVAPA
MALTPPQGDADADGMDVNVRGPDGFTPLMLASFCGGALEPMPTEEDEADDTSASIIS
DLICQGAQLGARTDRTGETALHLAARYARADAAKRLLDAGADTNAQDHSGRTPLHT
AVTADAQGVFQILIRNRSTDLDARMADGSTALILAARLAVEGMVEELIASHADVNAV
DELGKSALHWAAAVNNVEATLALLKNGANKDMQDSKEETPLFLAAREGSYEAAKL
LLDHFANREITDHLDRLPRDVAQERLHQDIVRLLDQPSGPRSPPGTHGLGPLLCPPGA
FLPGLKVTQSGSKKSRRPPGKAGLGPQGPRGRGKKLTLACPGPLADSSVTLSPVDSLD
SPRPFGGPPASPGGFPLEGPYAAATATAVSLAQLGGPGRAGLGRQPPGGCVLSLGLLN
PVAVPLDWARLPPPAPPGPSFLLPLAPGPQLLNPGTPVSPQERPPPYLAVPGHGEEYPA
AGAHSSPPKARFLRVPSEHPYLTPSPESPEHWASPSPPSLSDWSESTPSPATATGAMAT
ATGALPAQPLPLSVPSSLAQAQTQLGPQPEVTPKRQVLA (SEQ ID NO: 2)

The terms “polypeptide” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer. Unless otherwise indicated, a particular polypeptide sequence also implicitly encompasses conservatively modified variants thereof.

The phrase “conservatively modified variant” applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified

variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence.

For polypeptide sequences, "conservatively modified variants" include individual substitutions, deletions or additions to a polypeptide sequence which result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles disclosed herein. The following eight groups contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (see, *e.g.*, Creighton, Proteins (1984)). In some embodiments, the term "conservative sequence modifications" are used to refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the amino acid sequence.

The phrases "percent identical" or "percent identity," in the context of two or more nucleic acids or polypeptide sequences, refers to two or more sequences or subsequences that are the same. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, 60% identity, optionally 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identity over a specified region, or, when not specified, over the entire sequence), when compared and aligned for maximum

correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides (or 10 amino acids) in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200 or more amino acids) in length.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman, (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch, (1970) J. Mol. Biol. 48:443, by the search for similarity method of Pearson and Lipman, (1988) Proc. Nat'l. Acad. Sci. USA 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, *e.g.*, Brent *et al.*, (2003) Current Protocols in Molecular Biology).

Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, (1977) Nuc. Acids Res. 25:3389-3402; and Altschul *et al.*, (1990) J. Mol. Biol. 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when

aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, (1989) Proc. Natl. Acad. Sci. USA 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin and Altschul, (1993) Proc. Natl. Acad. Sci. USA 90:5873-5787). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

The percent identity between two amino acid sequences can also be determined using the algorithm of E. Meyers and W. Miller, (1988) Comput. Appl. Biosci. 4:11-17) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (1970) J. Mol. Biol. 48:444-453) algorithm which has been incorporated into the GAP program in the GCG software package (available at www.gcg.com), using either a Blossom 62 matrix or a

PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

Other than percentage of sequence identity noted above, another indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequence.

The phrase "nucleic acid" is used herein interchangeably with the term "polynucleotide" and refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, as detailed below, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, (1991) Nucleic Acid Res. 19:5081; Ohtsuka *et al.*, (1985) J. Biol. Chem. 260:2605-2608; and Rossolini *et al.*, (1994) Mol. Cell. Probes 8:91-98).

The term "subject" includes human and non-human animals. Non-human animals include all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, sheep, dog, cow, chickens, amphibians, and reptiles. Except when noted, the terms "patient" or "subject" are used herein interchangeably.

The phrase "differentially expressed" as used herein refers to the differential production of the mRNA transcribed and/or translated from the gene or the protein product encoded by the gene. A differentially expressed gene may be overexpressed or underexpressed as compared to the expression level of a normal or control cell. However, as used herein, overexpression is an increase in gene expression and generally is at least 1.25 fold or, alternatively, at least 1.5 fold or, alternatively, at least 2 fold, or alternatively, at least 3 fold or alternatively, at least 4 fold expression over that detected in a normal or control counterpart cell or tissue. As used herein, underexpression, is a reduction of gene expression and generally is at least 1.25 fold, or alternatively, at least 1.5 fold, or alternatively, at least 2 fold or alternatively, at least 3 fold or alternatively, at least 4 fold expression under that detected in a normal or control counterpart cell or tissue. The term "differentially expressed" also refers to where expression in a cancer cell or cancerous tissue is detected but expression in a control cell or normal tissue (e.g. non cancerous cell or tissue) is undetectable.

A high expression level of the gene can occur because of over expression of the gene or an increase in gene copy number. The gene can also be translated into increased protein levels because of deregulation or absence of a negative regulator. Lastly, high expression of the gene can occur due to increased stabilization or reduced degradation of the protein, resulting in accumulation of the protein.

A "gene expression profile" or "gene signature" refers to a pattern of expression of at least one biomarker that recurs in multiple samples and reflects a property shared by those samples, such as mutation, response to a particular treatment, or activation of a particular biological process or pathway in the cells. A gene expression profile differentiates between samples that share that common property and those that do not with better accuracy than would likely be achieved by assigning the samples to the two groups at random. A gene expression profile may be used to predict whether samples of unknown status share that common property or not. Some variation between the biomarker(s) and the typical profile is to be expected, but the overall similarity of biomarker(s) to the typical profile is such that it is statistically unlikely that the similarity would be observed by chance in samples not sharing the common property that the biomarker(s) reflects.

Various aspects of the disclosure are described in further detail in the following sections and subsections.

Notch 3 Receptor

Notch signaling is an evolutionarily conserved pathway that regulates a diverse set of biological functions including stem cell maintenance, cell differentiation and proliferation in both embryonic development and adult tissues (Kopan *et al.,* (2009) Cell 137: 216-233, Guruharsha *et al.,* (2012) Nat Rev Genet. 13: 654-66, and Andersson *et al.,* (2001) Development 138: 3593-3612). In mammals, four Notch receptors have been described (Notch1-4), which have a conserved domain architecture. The extracellular domain (ECD) consists of a series of EGF-like repeats followed by a negative regulatory region (NRR) which contains 3 LNR repeats and a heterodimerization domain as shown in Figure 1.

In solid tumors, the role of Notch signaling in tumor initiation and progression is not well understood (Ranganathan *et al.,* (2011) Nat Rev Cancer 11:338-51). Early evidence for Notch receptors in transformation of epithelial cells came from mouse mammary tumor virus (MMTV) insertional mutagenesis studies. For example, activation of Notch4 (initially known as *Int3*) by MMTV, resulted in mammary tumorigenesis (Gallahan *et al.,* (1987) J Virol 61:218-220, Gallahan *et al.,* (1997) Oncogene 14: 1883-1890). In 2011, rearrangements of Notch1 or Notch2 in estrogen receptor (ER) negative breast cancer were identified (Robinson *et al.,* (2011) Nat Med 17:1646-51). These rearrangements of the Notch receptor result in production of a membrane tethered form of the receptor lacking an intact NRR domain or an ICD-like protein.

Notch3 NRR has a similar overall folding as that of Notch1 (Gordan *et al.*, (2009) Blood 113:4381-4390; Gordon *et al.*, (2009) 4:e6613; Wu *et al.*, (2010) Nature 464:1052-1057) and Notch2 (Gordon *et al.*, (2007) Nat Struct Mol Biol 14:295-300). It is composed of three Lin12/Notch repeats (LNR), namely LNR-A, LNR-B and LNR-C; and a heterodimerization (HD) domain divided into N-terminal part (HD-N) and C-terminal part (HD-C) by furin cleavage at S1 site (between R1571 and E1572) (see Figure 2).

NRR domains regulate the activation of Notch receptors, which involves three proteolysis steps. Furin-like convertase cleaves at S1 site within NRR during maturation of Notch precursor, to prime the activation. ADAM proteases cleave at S2 site, also within NRR, to create the substrate for intramembrane proteolysis at S3 site by gamma secretase. Following S3 cleavage, the intracellular part of Notch then enters nucleus to activate transcription. S2 cleavage is the key step of this activation series and is negatively regulated by NRR domains. The mechanism of this so called autoinhibition can be explained by NRR structures below.

Although not bound to provide a theory, one possible model for the mechanism of action is that Notch 3 NRR typically exists in an autoinhibited conformation in which the three LNRs, each coordinating a Ca^{2+} ion, wrap around HD to protect S2 site from access by ADAM proteases. The stability of the interactions between LNRs and HD, as well as those within these regions, is critical to maintain the autoinhibited conformation of NRR. Mutations in the Notch 3 NRR alter the autoinhibited conformation, thereby exposing the HD domain, such that the S2, and subsequently the S3 site is available for cleavage by proteases, thereby activating downstream Notch 3 signal transduction. Therefore, mutations destabilizing NRR, like those found in relevant cancers (disclosed herein), could enhance activation of Notch 3. On the other hand, reagents like antibodies or fragments thereof that can stabilize LNR-HD interaction can potentially inhibit Notch 3 signaling. Antibodies or fragments thereof such as Ab-B, and Ab-C bind the autoinhibited conformation of Notch 3 and stabilizes (directly maintains, holds, locks,) the autoinhibited conformation thereby preventing exposure of the S2 site to protease cleavage, and subsequent downstream Notch 3 signaling.

In some embodiments, the antibody or fragment thereof binds to the conformational epitope such that it restricts the mobility of the LNR regions (LNR-A, LNR-B, LNR-C as well as corresponding linkers between LNR domains) relative to HD, stabilizing Notch 3 NRR in an autoinhibited conformation. The failure to form the active (uninhibited, open) conformation results in failure to activate signal transduction. In some embodiments, the antibody or fragment thereof binds to the conformational epitope such that it prevents the HD within the NRR from becoming exposed, thereby rendering it unavailable for cleavage at the S2, and/or S3 sites by proteases. The failure to cleave the S2 site results in failure to activate signal transduction.

Notch 3 Mutants

In one aspect, the disclosure pertains to mutations in the Notch 3 receptor. Activating mutations in Notch1 were identified in >50% of T-ALL patients in two general regions of the receptor (Weng et al., (2004) Science 306:269-71). One class of mutations was found to be clustered in the hydrophobic core of the HD domain of the NRR. Rare mutations have also been identified in the LNR domain (Gordon *et al.*, (2009) Blood 113:4381-4390). The NRR mutations likely act by partially, or completely unfolding the HD domain, altering the pocket that protects the S2 site and disrupting interactions with the LNR. This hypothesis is supported by biochemical data that HD domains with leukemia-associated mutations are less stable (Malecki *et al.*, (2006) Mol. Cell Biol. 26:4642-4651).

Mutations were also identified in the PEST (proline-glutamate-serine-threonine-rich) domain at the C-terminus of the protein. The levels of the ICD are tightly regulated and phosphorylation of the PEST domain and subsequent ubiquitination, target the ICD for degradation by E3 ligases such as Fbw7. Mutations are either nonsense mutations or frame-shift mutations that result in deletion of the PEST domain and result in an ICD with increased stability and longer protein half-life.

To date, the evidence for Notch receptors in cancer has focused primarily on alterations in Notch1 signaling. However, Notch 3 has been shown in several studies, including the TCGA analysis of serous ovarian cancer to be amplified in 11-25% of patient samples (Nakayama *et al.,* (2007) Int J Cancer 120:2613-17, Etemadmoghadam *et al.*, (2009) Clin Can Res 15: 1417-27, Bell *et al.,* (2011) Nature 474:609-615). Although mutations in Notch 3 have been reported in Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) syndrome, these mutations are generaly missense in nature and the link to alterations in Notch 3 function and disease pathology is not clear (see Ayata, (2010), Stroke 41:S129-S134). Comprehensive analysis of gene mutations in various cancer types has been performed by TCGA and other organizations. The standard technique used is exon-capture. As part of these studies, Notch 3 mutations have been reported in around 1% of head and neck squamous carcinomas, ovarian cancers and lung adenocarcinoma. However, the lack of sufficient exon coverage for Notch 3 exon 25, and 33 make it difficult for the skilled artisan to look for mutations in the Notch 3 gene. Further, the high GC content in the Notch 3 gene has discouraged the skilled artisan from looking at mutations. In addition, the mutations identified in squamous cell lung cancer have been suggested to be loss of function mutations (see Egloff & Grandis (2012) Clin Can Res 18:5188-519). In contrast, and contrary to previous studies, the disclosure herein shows a number of mutations that activate Notch 3 signal transduction ("activating mutations") and lead to increased cancer.

To identify Notch 3 mutations, 947 human cancer cell lines were characterized and mutation information was obtained for >1600 genes by massively parallel sequencing using a solution phase hybrid capture technology, as described in Example 5. In addition, primary tumor samples were sequenced with RNAseq (Wang Z. *et al.* (2009) Nature Reviews 10:57-63). Mutations were identified in both the NRR and PEST domain in multiple cell lines and tumor samples as shown in Table 1.

Activating mutations which interfere with the function of Notch 3 are involved in the pathogenesis of cancer. As the presence of an altered Notch 3 having a loss of function, gain

of function, or altered function, directly increases the risk of cancer, detection of such mutations lends itself to diagnostic and prognostic methods. The identification of such activating mutations may then be treated by antibodies or fragments thereof that bind to the mutant Notch 3.

Table 1: Notch 3 activating mutations

Mutation	Cellular data	Structure-based interpretation
Group 1		
S1580L	Activating	Lose intra-domain hydrogen bonds and thus destabilize HD domain
R1510H		
D1587N		
R1589Q		
Y1624H		
Group 2		
G1487D	Activating	Affect structural integrity, cause clash
A1476T		
A1609T		
L1518M		
A1537T		
Group 3		
N1597K	Activating	On the surface of NRR, no obvious interpretation, but might interfere with protein-protein interaction
L1547V		
R1526C		

Two mutations from the NRR domain from different cell-lines were selected for characterization: (i) TALL-1 cells, which are a T-cell acute lymphoblastic cell line with a S1580L mutation; and (ii) breast tumor (X-1004) with a G1487D mutation. The Examples show that introduction of either a S1580L mutation or a G1487D mutation into a Notch 3 receptor resulted in an approximately 10 fold increase in the basal signaling from the receptor relative to a wild-type control. In this system the wild-type and mutant receptors were expressed at approximately equivalent levels as determined by FACS assay. This data shows that these mutations active Notch3 signaling in cell lines and tumors expressing these and other similar mutations. This activation of Notch 3 signaling is inhibited by Notch 3 antibodies or fragments therof

In order to detect a Notch 3 mutant, a biological sample is prepared and analyzed for a difference between the sequence of the test sample thought to contain the mutant Notch 3 with the sequence of the wild-type Notch 3. Mutant Notch 3 can be identified by any of the techniques described herein. The mutant Notch 3 can be sequenced to identify the specific mutation (activating mutations that increase Notch 3 signal transduction). The mutations,

especially those which lead to an altered function of the protein, are then used for the diagnostic and prognostic methods of the present invention.

For further analysis, cancer mutations of the Notch 3 mutants were mapped onto Notch 3 NRR X-ray crystal structure. Structural analysis shows that some of these mutations can disrupt intra- and inter-domain interactions, destabilize the autoinhibitory conformation of Notch 3 NRR and cause Notch 3 activation and signal transduction.

A comparison of these mutations with Ab-B and Ab-C epitopes (described below) shows that most of them are not within the epitopes, indicating that the Ab-B and Ab-C antibody fragments can bind both wild type and mutant Notch3 NRRs in its autoinhibited conformation and inhibit Notch 3 signal transduction.

In some embodiments, mutants can be introduced into wild-type Notch 3 (SEQ ID NO: 1) to investigate the effect on Notch 3 binding agents such as small molecule drugs or biologics, e.g., antibodies or fragments thereof. Mutagenesis using known techniques such as alanine-scanning can help define functionally relevant epitopes. Mutagenesis utilizing an arginine/glutamic acid scanning protocol can also be employed (see, e.g., Nanevicz *et al.*, (1995), J. Biol. Chem. 270(37):21619-21625 and Zupnick *et al.,* (2006), J. Biol. Chem. 281(29):20464-20473). In general, arginine and glutamic acids are substituted (typically individually) for an amino acid in the wild-type polypeptide because these amino acids are charged and bulky and thus have the potential to disrupt binding between an antigen binding protein and an antigen in the region of the antigen where the mutation is introduced. Arginines that exist in the wild-type antigen are replaced with glutamic acid. A variety of such individual mutants can be obtained and the collected binding results analyzed to determine what residues affect binding. A series of mutant Notch 3 can be created, with each mutant Notch 3 having a single mutation. Binding of each mutant Notch 3 with various Notch 3 Notch 3 binding agents such as small molecule drugs or biologics, e.g., antibodies or fragments thereof, and can be measured and compared to the ability of the selected Notch binding agents to bind wild-type Notch 3 (SEQ ID NO: 1).

An alteration (for example a reduction or increase) in binding between a Notch 3 binding agents such as antibodies or fragments thereof f and a mutant or variant Notch 3 means that there is a change in binding affinity (e.g., as measured by known methods such as Biacore testing or the bead based assay described below in the examples), EC_{50}, and/or a change (for example a reduction) in the total binding capacity of the antigen binding protein (for example,

as evidenced by a decrease in B_{max} in a plot of antigen binding protein concentration versus antigen concentration). A significant alteration in binding indicates that the mutated residue is involved in binding to the antibody or fragment thereof.

In some embodiments, a significant reduction in binding means that the binding affinity, EC_{50}, and/or capacity between an antibody or fragments thereof and a mutant Notch 3 antigen is reduced by greater than 10%, greater than 20%, greater than 40%, greater than 50%, greater than 55%, greater than 60%, greater than 65%, greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90% or greater than 95% relative to binding between the an antibody or fragment thereof and a wild type Notch 3 (e.g., SEQ ID NO: 1).

In some embodiments, binding of an antibody or fragments thereof is significantly reduced or increased for a mutant Notch 3 having one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) mutations as compared to a wild-type Notch3 protein (e.g., SEQ ID NO: 1).

Although the variant forms are referenced with respect to the wild-type sequence shown in SEQ ID NO: 1, it will be appreciated that in an allelic or splice variants of Notch 3 the amino acids could differ. Antibodies or fragments thereof showing significantly altered binding (e.g., lower or higher binding) for such allelic forms of Notch 3 are also contemplated. The skilled artisan will appreciate that any one of the mutatnts described in Table 1 can be be combined with any other 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 of the other mutants in Table 1 to produce an expression pattern or expression signature that can be used to identify, diagnose, or monitor a subject.

In some embodiments, the expression signature comprises one or more group 1 mutations, for example a combination of S1580L, R1510H, D1587N, R1580Q, and Y1624H. In some embodiments, the expression signature comprises one or more group 2 mutations, for example a combination of G1487D, A1476T, A1609T, L1518M, and A1537T. In some embodiments, the expression signature comprises one or more group 3 mutations, for example a combination of N1597K, L1547V, and R1526C.

In some embodiments, the expression signature comprises one or more group 1 mutations, for example a combination of S1580L, R1510H, D1587N, R1580Q, and Y1624H; and one or more group 2 mutations, for example a combination of G1487D, A1476T, A1609T, L1518M, and A1537T. In some embodiments, the expression signature comprises one or more group 1 mutations, for example a combination of S1580L, R1510H, D1587N, R1580Q, and Y1624H; and

one or more group 3 mutations, for example a combination of N1597K, L1547V, and R1526C. In some embodiments, the expression signature comprises one or more group 2 mutations, for example a combination of G1487D, A1476T, A1609T, L1518M, and A1537T; and one or more group 3 mutations, for example a combination of N1597K, L1547V, and R1526C.

Notch 3 Structure and Conformational Epitopes

The three dimensional structure of the NRR domain (residues 1379-1640) of Notch 3 complexed with an antibody or fragment thereof is presented. The Notch 3 NRR/Ab-B Fab complex and the Notch 3 NRR/Ab-C Fab have been determined at 3.2 angstrom (Å) and 2.1Å resolution, respectively, and shown in Figure 14 A and B.

The disclosure herein shows that there are number of distinct conformational epitopes in the NRR to which different classes of Notch 3 antibodies or fragments thereof bind. In one embodiment, a first class of antibodies (e.g., Ab-B) binds to a first conformational epitope in the NRR domain; a second class of antibodies (e.g., Ab-C) binds to a second conformational epitope in the NRR domain; and a third class of antibodies binds to a third conformational epitope in the NRR domain.

To analyze the different conformational epitopes within the NRR, X-ray chrystallography and hydrogen-deuterium exchange experiments were conducted as described in detail in the experiements section. The crystals of Notch 3 can be prepared by expressing a nucleotide sequence encoding Notch 3 or a variant thereof in a suitable host cell, and then crystallising the purified protein(s) in the presence of the relevant Notch 3 targeted Fab.

Notch 3 polypeptides may also be produced as fusion proteins, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), histidine (HIS), hexahistidine (6HIS), GAL4 (DNA binding and/or transcriptional activation domains) and beta-galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences.

After expression, the proteins may be purified and/or concentrated, for example by immobilised metal affinity chromatography, ion-exchange chromatography, and/or gel filtration.

The protein(s) may be crystallised using techniques described herein. Commonly, in a crystallisation process, a drop containing the protein solution is mixed with the crystallisation buffer and allowed to equilibrate in a sealed container. Equilibration may be achieved by known techniques such as the "hanging drop" or the "sitting drop" method. In these methods, the drop is hung above or sitting beside a much larger reservoir of crystallization buffer and equilibration is reached through vapor diffusion. Alternatively, equilibration may occur by other methods, for example under oil, through a semi-permeable membrane, or by free-interface diffusion (See e.g., Chayen *et al.*, (2008) Nature Methods 5, 147 – 153)

Once the crystals have been obtained, the structure may be solved by known X-ray diffraction techniques. Many techniques use chemically modified crystals, such as those modified by heavy atom derivatization to approximate phases. In practice, a crystal is soaked in a solution containing heavy metal atom salts, or organometallic compounds, e.g., lead chloride, gold thiomalate, thimerosal or uranyl acetate, which can diffuse through the crystal and bind to the surface of the protein. The location(s) of the bound heavy metal atom(s) can then be determined by X-ray diffraction analysis of the soaked crystal. The patterns obtained on diffraction of a monochromatic beam of X-rays by the atoms (scattering centres) of the crystal can be solved by mathematical equations to give mathematical coordinates. The diffraction data are used to calculate an electron density map of the repeating unit of the crystal. Another method of obtaining phase information is using a technique known as molecular replacement. In this method, rotational and translational alogrithms are applied to a search model derived from a related structure, resulting in an approximate orientation for the protein of interest (See Rossmann, (1990) Acta Crystals A 46, 73-82). The electron density maps are used to establish the positions of the individual atoms within the unit cell of the crystal (Blundel et al., (1976) Protein Crystallography, Academic Press).

The conformational epitopes of Ab-B and Ab-C do not overlap as determined by superimposing the crystal structures of Notch 3 NRR/Ab-B complex and Notch3 NRR/Ab-C complex on Notch3 NRR

Notch 3 Inhibitors

In one aspect, the disclosure pertains to Notch 3 inhibitors that inhibit Notch 3 activation.

Antibodies

The Notch 3 inhibitor is an antibody or fragment thereof. Examples of antibodies include but are not limited to an antibody that binds a Notch protein or a Notch ligand protein and inhibits Notch ligand induced stimulation of Notch signaling. Such antibodies include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by a Fab expression library, bifunctional/bispecific antibodies, humanized antibodies, CDR grafted antibodies, human antibodies and antibodies which include portions of CDR sequences specific for a Notch protein or a Notch ligand protein.

Means for preparing and characterizing antibodies are well known in the art (see, e.g., Harlow and Lane, (1988). Antibodies: A Laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y). Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a non-human animal including rabbits, mice, rats, hamsters, goat, sheep, pigs or horses. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Antibodies, both polyclonal and monoclonal, specific for isoforms of antigen may be prepared using conventional immunization techniques, as will be generally known to those of skill in the art. As used herein, the term "specific for" is intended to mean that the variable regions of the antibodies recognize and bind a Notch protein or a Notch ligand protein and are capable of distinguishing a Notch protein or a Notch ligand protein from other antigens. A composition containing antigenic epitopes of a Notch protein or a Notch ligand protein can be used to immunize one or more experimental animals, such as a rabbit or mouse, which will then proceed to produce specific antibodies against the Notch protein or a Notch ligand protein. Polyclonal antisera may be obtained, after allowing time for antibody generation, simply by bleeding the animal and preparing serum samples from the whole blood.

Monoclonal antibodies to a Notch protein or a Notch ligand protein may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Koehler and Milstein (1975) Nature, 256(5517), 495-497; the human B-cell

hybridoma technique (Kosbor *et al.*, (1983) Immunol. Today, 4, 72-79; Cote *et al.*, (1983) Proc. Natl. Acad. Sci. USA., 80(7), 2026-2030; and the EBV-hybridoma technique (Cole *et al.*, (1985 Monoclonal Antibodies and Cancer Therapy. (eds. R. A. Reisfeld and S. Sell), Alan R Liss Inc, New York N.Y., pp 77-96).

Methods of making antibody fusion proteins are well known in the art. See, e.g., U.S. Pat. No. 6,306,393, the disclosure of which is incorporated herein by reference in its entirety. In certain embodiments of the invention, fusion proteins are produced which may include a flexible linker, which connects the chimeric scFv antibody to the heterologous protein moiety. Appropriate linker sequences are those that do not affect the ability of the resulting fusion protein to be recognized and bind the epitope specifically bound by the V domain of the protein (see, e.g., WO 98/25965, the disclosure of which is incorporated herein by reference in its entirety).

In addition to the production of monoclonal antibodies, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison *et al.*, (1984), Nature, 368(6474), 812-813; Neuberger *et al.*, (1984) Nature 312(5995), 604-608; Takeda *et al.*, (1985) Nature, 314(6010), 452-454. Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can be adapted to produce a Notch protein or a Notch ligand protein-specific single chain antibodies.

Antibodies may also be produced by inducing in vivo production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi *et al.* (1989) Proc Natl Acad Sci 86: 3833-3837; and Winter and Milstein (1991) Nature 349: 293-299, 1991.

Fully human antibodies relate to antibody molecules in which essentially the entire sequences of both the light chain and the heavy chain, including the CDRs, arise from human genes. Such antibodies are termed "human antibodies" or "fully human antibodies" herein. Antibodies against human Notch3 proteins were generated by selection of clones having high affinity binding affinities, using as the source of antibody variant proteins a commercially available phage display library - HuCAL PLATINUM® library (Prassler *et al.*, (2011) J Mol

Biol 413:261-278). Using the HuCAL PLATINUM® library, anti-Notch 3 antibodies, Ab-A, Ab-C, Ab-D, and others were identified. The three dimensional structure of the NRR domain (residues 1379-1640) of Notch 3 complexed with an antibody or fragment thereof is presented. The Notch 3 NRR/Ab-B Fab complex and the Notch 3 NRR/Ab-C Fab have been determined at 3.2 angstrom (Å) and 2.1Å resolution, respectively, and shown in Figure 14 A and B.

Othere methods for generating human monoclonal antibodies include, but are not limited to, trioma technique; the human B cell hybridoma technique (see Kozbor *et al.*, (1983) Immunol. Today, 4, 72-79); and the EBV hybridoma technique to produce human monoclonal antibodies (see Cole *et al.*, (1985)) In, Monoclonal Antibodies and Cancer Therapy. (eds. R. A. Reisfeld and S. Sell), Alan R Liss Inc, New York N.Y., pp 77-96. Human monoclonal antibodies may be utilized in the practice of the present invention and may be produced by using human hybridomas (see Cote *et al.*, (1983) Proc. Natl. Acad. Sci. USA., 80, 2026-2030) or by transforming human B cells with Epstein Barr Virus in vitro (see Cole *et al.,* 1985, *supra*).

In addition, human antibodies can also be produced using additional techniques, including phage display libraries (Hoogenboom and Winter (1992) J. Mol. Biol., 227(2), 381-388; Marks *et al.*, (1991) J. Mol. Biol., 222(3), 581-597). Similarly, human antibodies can be made by introducing human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in Marks *et al.* (1992) BioTechnology, 10, 779-783; Lonberg *et al.* (1994) Nature, 368(6474), 856-859; Morrison (1994) Nature, 368(6474), 812-813; Fishwild *et al.*, (1996); Neuberger (1996) Nature Biotechnology, 14, 845-851; and Lonberg and Huszar (1995) Rev. Immunol., 13(1), 65-93.

Human antibodies may additionally be produced using transgenic nonhuman animals which are modified so as to produce fully human antibodies rather than the animal's endogenous antibodies in response to challenge by an antigen. (See PCT publication WO94/02602). The endogenous genes encoding the heavy and light immunoglobulin chains in the nonhuman host have been incapacitated, and active loci encoding human heavy and light chain

immunoglobulins are inserted into the host's genome. The human genes are incorporated, for example, using yeast artificial chromosomes containing the requisite human DNA segments. An animal which provides all the desired modifications is then obtained as progeny by crossbreeding intermediate transgenic animals containing fewer than the full complement of the modifications. The preferred embodiment of such a nonhuman animal is a mouse, and is termed the Xenomouse™ as disclosed in PCT publications WO 96/33735 and WO 96/34096. This animal produces B cells which secrete fully human immunoglobulins. The antibodies can be obtained directly from the animal after immunization with an immunogen of interest, as, for example, a preparation of a polyclonal antibody, or alternatively from immortalized B cells derived from the animal, such as hybridomas producing monoclonal antibodies. Additionally, the genes encoding the immunoglobulins with human variable regions can be recovered and expressed to obtain the antibodies directly, or can be further modified to obtain analogs of antibodies such as, for example, single chain Fv molecules.

Antisense

In another embodiment, the Notch 3 inhibitor is an siRNAs, shRNAs or targeted synthetic oligonucleotides that hybridize with Notch3 mRNA (i.e. by RNA interference, RNAi), thus inhibiting the synthesis of Notch3 receptors (see e.g., US20100189775). Short hairpin RNA (shRNA) is an RNA molecule in the form of a stable hairpin which silences gene expression via RNA interference in vivo. The shRNA hairpin structure is cleaved by cellular processing machinery to produce the mature siRNA, whose anti-sense strand is specifically taken up by the RNA-induced silencing complex (RISC). The latter complex binds to and cleaves mRNAs which match the siRNA sequence contained in the RISC, thus guiding the targeted RNA to degradation. Hence, the said inhibition will result in a certain time, in the depletion of Notch3 receptors from the target cells as the pre-existing receptors will eventually turn over but will not be replenished aced by newly synthesized Notch 3 receptors.

A skilled person could design RNAs suitable for Notch 3 inhibition using protocols and services for designing siRNAs or shRNAs are available online from genelink, ambion, or psilencer. siRNAs that specifically target Notch3 would decrease Notch3 wild type and mutant Notch 3 receptors. In some embodiments, siRNA could be designed to only inhibit mutant Notch 3.

The shRNAs can be inserted in any vector suitable for gene therapy. shRNA expression vectors have been engineered using both viral (including retroviral, adenoviral and lentiviral

vectors), and plasmid systems. These vectors utilize promoters from a small class of pol. III promoters to drive the expression of shRNA. All vectors have to include a promoter for human Pol III. The Human U6 promoter is the best studied type III pol promoter frequently used in RNAi.

shRNAs are exported from the nucleus by Exportin 5, which recognizes short RNA loop. Once in the cytoplasm, both pre-miRNAs and shRNAs are processed into siRNA duplexes by cleavage with a second RNase III enzyme termed Dicer. Importantly, Dicer binds the base of the shRNA and cleaves 21 or 22 nt up the stem, leaving a second 2 nt 3' overhang and forming an siRNA duplex structure. RNA duplex is taken up by the RNAi-Induced Silencing Complex (RISC). RISC unwinds the double-strand RNA and the activated complex with the associated antisense.

The genetic material in retroviruses is in the form of RNA molecules, while the genetic material of their hosts is in the form of DNA. When a retrovirus infects a host cell, it will introduce its RNA together with some enzymes into the cell. This RNA molecule from the retrovirus must produce a DNA copy from its RNA molecule before it can be considered as part of the genetic material of the host cell. The process of producing a DNA copy from an RNA molecule is termed reverse transcription. It is carried out by one of the enzymes carried in the virus, called reverse transcriptase. After this DNA copy is produced and is free in the nucleus of the host cell, it must be incorporated into the genome of the host cell by using another enzyme carried in the virus called integrase. One of the problems of gene therapy using retroviruses is that the integrase enzyme can insert the genetic material of the virus in any arbitrary position in the host's genome. If genetic material happens to be inserted in the middle of one of the original genes of the host cell, this gene will be disrupted (insertional mutagenesis). If the gene happens to be one regulating cell division, uncontrolled cell division (i.e., cancer) can occur. The state of the art in this field has disclosed the use of retroviral vectors utilizing zinc finger nucleases or including certain sequences such as the beta-globin locus control region to direct the site of integration to specific chromosomal sites The skilled person would know, however, where to find indications in the state of the art for the construction of a vector suitable for the pharmaceutical composition of the invention. Vectors, kit construction vectors and services for the construction of vectors for the expression and the targeting of said RNAs are known in the art, such as, by way of example, the INGENEX GeneSuppressorRetro Construction Kit, or are available online, or are described in the art in: Arts, *et al.* ((2003) Genome Res. 13: 2325-2332), that demonstrates adenovirus-based

shRNA expression in a variety of cell types, including primary cells; Matta, *et al.* ((2003) Cancer Biol. Ther. 2: 206-210) where the authors use Invitrogen's pLenti6 backbone to express an shRNA cassette; Tiscornia, *et al.*((2003) Proc. Natl. Acad. Sci. USA 100: 1844-1848) demonstrates the utility of lentiviral vectors for delivery of shRNA to cells and mice.

The vector could comprise a tumour specific promoter driving shRNA or siRNA expression in cells only in the tumour. The oligonucleotides can be covered with lipids in an organized structure like a micelle or a liposome. When the organized structure is complexed with the nucleic acid it is called a lipoplex. There are three types of lipids, anionic (negatively charged), neutral, or cationic (positively charged). Initially, anionic and neutral lipids were used for the construction of lipoplexes for synthetic vectors. Cationic lipids, due to their positive charge, naturally complex with the negatively charged nucleic acids and they are also less time consuming to produce than anionic of neutral lipids. Moreover due to their positive charge they also interact with the cell membrane facilitating their endocytosis and subsequent release of the nucleic acid into the cytoplasm. The cationic lipids also protect against degradation of the nucleic acid by the cell.

Low Molecular Weight Compounds

Known inhibitors of Notch signaling include low molecular weight compounds that inhibit the gamma secretase enzyme (gamma secretase inhibitors) or the ADAM metalloprotease enzymes (metalloprotease inhibitors).

Inhibitors of Notch 3 that inhibit by cleavage by γ- secretase include but re not limited to,γ-secretase inhibitor I (GSI I) Z-Leu-Leu-Norleucine; γ-secretase inhibitor II (GSI II); γ-secretase inhibitor III (GSI III), N-Benzyloxycarbonyl-Leu- leucinal, N-(2-Naphthoyl)-Val-phenylalaninal; γ-secretase inhibitor III (GSI IV); γ-secretase inhibitor III (GSI V), N-Benzyloxycarbonyl-Leu- phenylalaninal; γ-secretase inhibitor III (GSI VI), 1-(S)-endo-N-(1,3,3)- Trimethylbicyclo[2.2.1]hept-2-yl)-4-fluorophenyl Sulfonamide; γ-secretase inhibitor III (GSI VII), Menthyloxycarbonyl-LL-CHO; γ-secretase inhibitor III (GSI IX), (DAPT), N--[N-(3,5- Difluorophenacetyl-L- alanyl)]-S-phenylglycine t- Butyl Ester; γ-secretase inhibitor X (GSI X), {1S-Benzyl-4R-[1-(1S- carbamoyl-2- phenethylcarbamoyl)-1S-3-methylbutylcarb-amoyl]-2R- hydroxy-5- phenylpentyl}carbamic Acid tert-butyl Ester; γ-secretase inhibitor XI (GSI XI), 7-Amino-4-chloro-3- methoxyisocoumarin; γ-secretase inhibitor XII (GSI XII), Z-Ile-Leu-CHO; γ-secretase inhibitor XIII (GSI XIII), Z-Tyr-Ile-Leu-CHO; γ-secretase inhibitor XIV (GSI XIV), Z-Cys(t-Bu)-Ile-Leu-CHO; γ-secretase inhibitor

XVI (GSI XVI), N--[N-3,5- Difluorophenacetyl]-L- alanyl-S-phenylglycine Methyl Ester; γ-secretase inhibitor XVII (GSI XVII); γ-secretase inhibitor XIX (GSI XIX), benzo[e][1,4]diazepin-3-yl)- butyramide; γ-secretase inhibitor XX (GSI XX), (S,S)-2-[2-(3,5-Difluorophenyl)acetylamino]- N-(5-methyl-6-oxo-6,7- dihydro--5H- dibenzo[b,d]azepin-7-yl)propionamide; γ-secretase inhibitor XXI (GSI XXI), (S,S)-2-[2-(3,5- Difluorophenyl)-acetylamino]-N-(1-methyl-2- oxo-5-phenyl-2-,3-dihydro-1H-benzo[e][1,4]diazepin-3- yl)-propionamide; Gamma40 secretase inhibitor I, N-trans-3,5- Dimethoxycinnamoyl-Ile-leucinal; Gamma40 secretase inhibitor II, N-tert-Butyloxycarbonyl- Gly-Val-Valinal Isovaleryl-V V-Sta-A-Sta- OCH_3; MK-0752 (Merck); LY450139 (Eli Lilly); RO4929097; PF-03084,014; BMS-708163; MPC-7869 (γ-secretase modifier), and semagacestat..

Inhibition of Notch 3 by inhibition by interference with Notch nuclear co- activator include, but are not limited to MAML1, MAML-CSL-Notch, Antennapedia/dominant- MAML. Inhibition of notch 3 inibition by interference with Dll4 ligand- receptor interaction include, but are not limited to OMP-21M18 (DLL4 antibody).

The γ-secretase inhibitors, γ-secretase inhibitor MK-0752 (Merck) has been administered to human subjects in single doses of 110 to 1000 mg (Rosen et al., 2006). MK-0752 is in Phase I clinical trials for patients with breast cancer tumors (ClinicalTrials.gov Identifier NCT00106145). The γ-secretase inhibitor LY450139 (Eli Lilly) has been administered to human subjects at doses ranging from 5 mg/day to 50 mg/day for 14 days (Seimers *et al.*, (2005) Clin Neuropharmacol., 28(3), 126-132). A longer term study with LY450139 has been conducted at a dose of 60 mg/day for 2 weeks, followed by 100 mg/day for 6 weeks, followed by either 100 mg/day or 140 mg/day for another 6 weeks (Beals, (2007) Reporting on press briefing by Dr. Siemers at Alzheimer's Association International Conference on Prevention of Dementia: Abstract HT-005. Presented Jun. 11, 2007-Medscape Medical News.

Diagnostic Uses

In one aspect, the disclosure encompasses diagnostic assays for determining Notch 3 protein and/or nucleic acid expression as well as Notch 3 protein function, in the context of a biological sample (*e.g.*, blood, serum, cells, tissue) or from individual afflicted with cancer, or is at risk of developing cancer.

The present disclosure provides methods for identifying a disease or disorder associated with the Notch 3 signaling pathway by administering to a subject in need thereof an effective

amount of the antibodies of the disclosure. In a specific embodiment, the present disclosure provides a method of treating or preventing cancers (e.g., breast cancer, colorectal cancer, lung cancer, multiple myeloma, ovarian cancer, liver cancer, gastric cancer, pancreatic cancer, prostate cancer, acute myeloid leukemia, chronic myeloid leukemia, t-cell acute lymphoblastic leukemia, mantle cell lymphoma, chronic lymphocytic leukemia, Ewings sarcoma, osteosarcoma, squamous cell carcinoma, peripheral nerve sheath tumors, schwannoma, head and neck cancer, bladder cancer, esophageal cancer, glioblastoma, clear cell sarcoma of soft tissue, malignant mesothelioma, neurofibromatosis, renal cancer and melanoma) by administering to a subject in need thereof an effective amount of the antibodies of the disclosure. In some embodiments, the present disclosure provides methods of treating or preventing cancers associated with a Notch 3 signaling pathway by administering to a subject in need thereof an effective amount of the antibodies of the disclosure.

In a specific embodiment, the present disclosure provides methods for identifying cancers associated with a Notch 3 signaling pathway that include, but are not limited to breast cancer, lung cancer, and T-cell acute lymphoblastic leukemia (TALL).

The detection of Notch 3 mutations can be done by any number of ways, for example: DNA sequencing, PCR based methods, including RT-PCR, microarray analysis, Southern blotting, Northern blotting and dip stick analysis.

The polymerase chain reaction (PCR) can be used to amplify and identify Notch 3 mutations from either genomic DNA or RNA extracted from tumor tissue. PCR is well known in the art and is described in detail in Saiki et al., Science 1988, 239:487 and in U.S. Patent No. 4,683,195 and U.S. Patent No. 4,683,203.

Detection of gene expression can be by any appropriate method, including for example, detecting the quantity of mRNA transcribed from the gene or the quantity of cDNA produced from the reverse transcription of the mRNA transcribed from the gene or the quantity of the polypeptide or protein encoded by the gene. These methods can be performed on a sample by sample basis or modified for high throughput analysis. For example, using Affymetrix™ \microarray chips.

In one aspect, gene expression is detected and quantitated by hybridization to a probe that specifically hybridizes to the appropriate probe for that biomarker. The probes also can be attached to a solid support for use in high throughput screening assays using methods known in the art. WO 97/10365 and U.S. Pat. Nos. 5,405,783, 5,412,087 and 5,445,934, for example,

disclose the construction of high density oligonucleotide chips which can contain one or more of the sequences disclosed herein. Using the methods disclosed in U.S. Pat. Nos. 5,405,783, 5,412,087 and 5,445,934, the probes of this invention are synthesized on a derivatized glass surface. Photoprotected nucleoside phosporamidites are coupled to the glass surface, selectively deprotected by photolysis through a photolithographic mask, and reacted with a second protected nucleoside phosphoramidite. The coupling/deprotection process is repeated until the desired probe is complete.

Alternatively any one of gene copy number, transcription, or translation can be determined using known techniques. For example, an amplification method such as PCR may be useful. General procedures for PCR are taught in MacPherson et al., PCR: A Practical Approach, (IRL Press at Oxford University Press (1991)). However, PCR conditions used for each application reaction are empirically determined. A number of parameters influence the success of a reaction. Among them are annealing temperature and time, extension time, Mg 2+ and /or ATP concentration, pH, and the relative concentration of primers, templates, and deoxyribonucleotides. After amplification, the resulting DNA fragments can be detected by agarose gel electrophoresis followed by visualization with ethidium bromide staining and ultraviolet illumination.

In one embodiment, the hybridized nucleic acids are detected by detecting one or more labels attached to the sample nucleic acids. The labels can be incorporated by any of a number of means well known to those of skill in the art. However, in one aspect, the label is simultaneously incorporated during the amplification step in the preparation of the sample nucleic acid. Thus, for example, polymerase chain reaction (PCR) with labeled primers or labeled nucleotides will provide a labeled amplification product. In a separate embodiment, transcription amplification, as described above, using a labeled nucleotide (e.g. fluorescein-labeled UTP and/or CTP) incorporates a label in to the transcribed nucleic acids.

Alternatively, a label may be added directly to the original nucleic acid sample (e.g., mRNA, polyA, mRNA, cDNA, etc.) or to the amplification product after the amplification is completed. Means of attaching labels to nucleic acids are well known to those of skill in the art and include, for example nick translation or end-labeling (e.g. with a labeled RNA) by kinasing of the nucleic acid and subsequent attachment (ligation) of a nucleic acid linker joining the sample nucleic acid to a label (e.g., a fluorophore).

Detectable labels suitable for use in the present disclosure include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include biotin for staining with labeled streptavidin conjugate, magnetic beads (e.g., Dynabeads™), fluorescent dyes (e.g., fluorescein, texas red, rhodamine, green fluorescent protein, and the like), radiolabels (e.g., ^{3H}, ^{125I}, ^{35S}, ^{14C}, or ^{32P}) enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and calorimetric labels such as colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads. Patents teaching the use of such labels include U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

Detection of labels is well known to those of skill in the art. Thus, for example, radiolabels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted light. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and calorimetric labels are detected by simply visualizing the coloured label.

The detectable label may be added to the target (sample) nucleic acid(s) prior to, or after the hybridization, such as described in WO 97/10365. These detectable labels are directly attached to or incorporated into the target (sample) nucleic acid prior to hybridization. In contrast, "indirect labels" are joined to the hybrid duplex after hybridization. Generally, the indirect label is attached to a binding moiety that has been attached to the target nucleic acid prior to the hybridization. For example, the target nucleic acid may be biotinylated before the hybridization. After hybridization, an avidin-conjugated fluorophore will bind the biotin bearing hybrid duplexes providing a label that is easily detected. For a detailed review of methods of labeling nucleic acids and detecting labeled hybridized nucleic acids see Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 24: Hybridization with Nucleic Acid Probes, P. Tijssen, ed. Elsevier, N.Y. (1993).

Notch 3 mutations when translated into proteins can be detected by specific antibodies. Expression level of Notch 3 mutations can also be determined by examining protein expression or the protein product of Notch 3 mutants. Determining the protein level involves measuring the amount of any immunospecific binding that occurs between an antibody that selectively recognizes and binds to the polypeptide of the biomarker in a sample obtained from a patient and comparing this to the amount of immunospecific binding of at least one

biomarker in a control sample. The amount of protein expression of the Notch 3 can be increased or reduced when compared with control expression.

Diagnostic assays, such as competitive assays rely on the ability of a labelled analogue (the "tracer") to compete with the test sample analyte for a limited number of binding sites on a common binding partner. The binding partner generally is insolubilized before or after the competition and then the tracer and analyte bound to the binding partner are separated from the unbound tracer and analyte. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the binding partner was precipitated after the competitive reaction). The amount of test sample analyte is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-response curves with known amounts of analyte are prepared and compared with the test results in order to quantitatively determine the amount of analyte present in the test sample. These assays are called ELISA systems when enzymes are used as the detectable markers. In an assay of this form, competitive binding between antibodies and Notch 3 antibodies results in the bound Notch 3 protein, preferably the Notch 3 epitopes of the disclosure, being a measure of antibodies in the serum sample, most particularly, neutralizing antibodies in the serum sample.

A significant advantage of the assay is that measurement is made of neutralizing antibodies directly (*i.e.*, those which interfere with binding of Notch 3 protein, specifically, epitopes). Such an assay, particularly in the form of an ELISA test has considerable applications in the clinical environment and in routine blood screening.

Assaying for Biomarkers

Another aspect of the disclosure provides methods for determining Notch 3 nucleic acid expression or Notch 3 protein activity in an individual to thereby select appropriate therapeutic or prophylactic agents for that individual (referred to herein as "pharmacogenomics"). Pharmacogenomics allows for the selection of agents (*e.g.*, small molecule drugs or biologics such as antibodies or fragments thereof) for therapeutic or prophylactic treatment of an individual based on the genotype of the individual (*e.g.*, the genotype of the individual examined to determine the ability of the individual to respond to a particular agent.)

Yet another aspect of the disclosure pertains to monitoring the influence of agents (*e.g.*, small molecule drugs or biologics such as antibodies or fragments thereof) on the expression or activity of Notch 3 protein in clinical trials.

Once a patient has been assayed for Notch 3 mutation and predicted to be sensitive to a Notch 3 inhibitor (e,g., a small molecule inhibitor or a biologic such as a Notch 3 antibody or fragment thereof) administration of any Notch 3 inhibitor to a patient can be effected by dose, continuously or intermittently throughout the course of treatment. Suitable dosage formulations and methods of administering the agents may be empirically adjusted baased on the presence and expression level of Notch 3 mutants.

Notch 3 mutations can be assayed for after Notch 3 inhibitor administration in order to determine if the patient remains sensitive to the Notch 3 treatment. In addition, Notch 3 mutations can be assayed for multiple timepoints after a single Notch 3 inhibitor administration. For example, an initial bolus of a Notch 3 inhibitor is administered, a Notch 3 mutation can be assayed for at 1 hour, 2 hours, 3 hours, 4 hours, 8 hours, 16 hours, 24 hours, 48 hours, 3 days, 1 week or 1 month or several months after the first treatment.

The patient could undergo multiple Notch 3 inhibitor administrations and then be assayed for Notch 3 mutations at different timepoints. For example, a course of treatment may require administration of an initial dose of Notch 3 inhibitor, a second dose after a specified time period later, and still a third dose hours after the second dose. Notch 3 mutations can be assayed for at 1 hour, 2 hours, 3 hours, 4 hours, 8 hours, 16 hours, 24 hours, 48 hours, 3 days, 1 week or 1 month or several months after administration of each dose of the Notch 3 inhibitor.

Kits for assessing the activity of any Notch 3 inhibitor (e.g., antibody or fragment thereof) can be made. For example, a kit comprising nucleic acid primers for PCR or for microarray hybridization for a Notch 3 mutation can be used for assessing the presence of Notch 3 mutants. Alternatively, a kit supplied with antibodies or fragments thereof for the Notch 3 mutations listed in Table 1.

It is possible to use the Notch 3 mutations to screen for Notch 3 inhibitor. This method comprises providing for a cell containing a Notch 3 mutation from Table 1, contacting the cell with a candidate Notch 3 inhibitor (e.g., a small molecule or a biologic such as an antibody or fragment thereof, and comparing the IC_{50} of the treated cell with a known Notch 3 inhibitor.

ICD3 Assay and Uses Thereof

In one apect, the disclosure pertains to an assay for detecting Notch 3 signal transduction. Notch signaling is activated by a series of proteolytic cleavages. The gamma secretase complex mediates the final cleavage of the Notch receptor ultimately releasing the Notch intracellular domain (ICD) that translocates to the nucleus to activate Notch target gene transcription. A neoepitope antibody (detection antibody) was generated to detect the gamma secretase cleaved form of the Notch3 ICD (ICD3) only when cleaved between amino acids Gly1661 and Val1662 of human Notch 3.

The assay comprises using a detection antibody that detects a neoepitope VMVARRK (SEQ ID NO: 3) in the gamma secretase cleaved domain of Notch 3 (ICD3). The ICD3 can be produced by cleavage at positions Gly1661-Val1662 of either wild type Notch 3 or mutant Notch 3.

Detection of the ICD3 by the assay disclosed herein indicates Notch 3 signal activation and transduction. An antibody or fragment thereof that prevents Notch 3 signal activation and transduction prevents the production of ICD3, and thereby detection of the neoepitope contained therein by the detection antibody. In one embodiment, the antibody or fragment thereof holds the Notch 3 in an autoinhibited conformation, thereby precluding exposure of the S2, and S3 cleavage sites to proteases, thereby preventing the formation of ICD3 comprising the neoepitope recognized by the detection antibody.

In one aspect, the disclosure encompasses diagnostic assays for determining Notch 3 protein and/or nucleic acid expression as well as Notch 3 protein function, in the context of a biological sample (*e.g.*, blood, serum, cells, tissue) or from individual afflicted with cancer, or is at risk of developing cancer.

The ICD3 assay can be used to detect the presence of activated Notch3 signaling. Activation of Notch3 signaling may be achieved by Notch 3 mutations or high Notch3 expression/gene amplification. A biological sample may be prepared and analyzed for the presence or absence of ICD3 protein. If the Notch 3 ICD is present, the NRR domain may contain a mutation that results in the autoinhibited conformation of the NRR being altered thereby exposing the HD domain to protease cleavage and the production of the ICD3, which can be detected by the detecting antibody of the disclosure. Results of these tests and interpretive information can be returned to the health care provider for communication to the tested individual. Such diagnoses may be performed by diagnostic laboratories, or, alternatively, diagnostic kits can

manufactured and sold to health care providers or to private individuals for self-diagnosis.

Another aspect of the disclosure provides methods for determining Notch 3 nucleic acid expression or Notch 3 protein activity in an individual to thereby select appropriate therapeutic or prophylactic agents for that individual (referred to herein as "pharmacogenomics"). Pharmacogenomics allows for the selection of agents (*e.g.*, small molecule drugs or biologics such as antibodies or fragments thereof) for therapeutic or prophylactic treatment of an individual based on the genotype of the individual (*e.g.*, the genotype of the individual examined to determine the ability of the individual to respond to a particular agent.)

Yet another aspect of the disclosure pertains to monitoring the influence of agents (*e.g.*, small molecule drugs or biologics such as antibodies or fragments thereof) on the expression or activity of Notch 3 protein in clinical trials.

Examples

Example 1: Cloning of cynomolgus monkey Notch3

As the sequence of cynomolgus monkey Notch3 was not available in public data bases, it was cloned as follows:

CynomolgusTotal RNA

All total RNAs were purchased from Zyagen (http://zyagen.com/index.php), San Diego, CA92121). Total RNAs were extracted from various tissues (brain, kidney, liver, lung, skeletal muscle, pancreas, spleen, skin, stomach, testis, thymus, thyroid, bone marrow) of cynomolgus monkeys. Origin and individual monkey's references were not specified by Zyagen. Total RNA was routinely extracted from tissues/cells using the guanidine isothiocyanate–phenol: chloroform extraction method which allows the rapid isolation of total RNA including microRNA. RNA was treated with RNase-free DNase to remove residual DNA, precisely quantified, and stored at -80°C. The integrity of each RNA sample, as indicated by intact ribosomal RNA, was verified by denatured agarose gel electrophoresis. The purity of RNA was assessed by spectrophotometer (A260/A280: 1.9-2.1). RNA was ideal for Northern blotting, ribonuclease protection assay, SI nuclease assay, RT-PCR/Q-PCR analysis, rapid amplification of cDNA ends (RACE) and purification of mRNA for library construction. Total RNA wasprovided in RNase-free water, 1mM sodium citrate, or 0.1 mM EDTA at a concentration of 1 mg/ml and shipped on dry ice. After receipt all Total RNAs samples are stored at -80°C.

Reverse transcription of RNA to cDNA and PCR amplification

All Total RNAs were reverse transcribed using the Thermo Script RT-PCR System (Invitrogen, Cat.11146-016) and oligodT. 2 µg of Total RNA was generally used for each cDNA pool and was eluted in 20 µl. 1 µl primer (50 µM Oligo (dT20), 2µg (tissue). Total RNA and 2 µl 10 mM dNTPs mix were combined and the volume adjusted to 12 µl with DEPC-treated water. After incubation at 65°C for 5 min, a master mix of 4 µl 5x cDNA Synthesis buffer, 1 µl of 0.1 M DTT, 1 µl RNaseOUT™ (40 U/µl), 1 µl DEPC-treated water and 1 µl ThermoScript™ RT (15 units/µl) was prepared and the 8 µl total volume was added to each previous reaction tube on ice. The reverse transcription phase of the total RNA sample was completed in 90 minutes at 55°C. This reaction was then stopped by incubating the whole reaction at 85 °C for five minutes. At last, 1 µl of RNase H was added and the samples were

incubated at 37°C for 20 minutes. The cDNA reactions were stored at -20 °C as base material for all polymerase chain reactions.

PCR amplifications were performed using 2 µl of cDNA. Primers were designed in the UTR regions and in the coding sequences. PCR products were directly gel extracted and analyzed by direct sequencing.

PCR primers for cynomolgus Notch3 gene fishing

The target sequences of non-human primates for example gorilla, ourangutan, rhesus were aligned to human sequence for primer design and specificity testing. Mouse and rat sequences of the target sequences may also be required. The target sequences for the alignment can be extracted from databases like NCBI, eEnsembl or UniProt.

Primers	Sequences
RS4242 UTR Fw	5'-CGGAGCCCAGGGAAGGAGGGAGGAGGGGAGGGTCGCGGCCGGCCGCC-3' (SEQ ID NO: 3)
RS4243 UTR Rev	5'-CAGGACGGGGGTCTCTTTAGGCCCCCAAGATCTAAGAACTGACGAGCGTCTCA-3'(SEQ ID NO: 4)
RS4244 CDS1825bp FW	5'-CCATGGCGGCAAATGCCTAGACCTGGTGG-3'(SEQ ID NO: 6)
RS4245 CDS 1999bp Rev	5'-CAAAGGGGCCCTGTGAAGCCAGGTTGGCAGACACAGTCG-3'(SEQ ID NO: 7)
RS4246 CDS 4384bp Fw	5'-CTTCAACAACAGCCGCTGCGACCCCGCCTGCAGCTCG-3'(SEQ ID NO: 8)
RS4247 CDS 4560bp Rev	5'-CAGCCGCACTCCTCCGTGTTGCAGCCCTGGTCG-3'(SEQ ID NO: 9)
RS4277 CDS 1137bp Rev	5'-GTCACAGATAGCATCCTCGTGGCAGGGGTTGCTGACACAGG-3'(SEQ ID NO: 10)
RS4278 CDS 821bp Fw	5'-GGGACATGCGTGGATGGCGTCAACACCTATAACTGCCAGTGCCC-3'(SEQ ID NO: 11)
RS4279 CDS 3136bp Rev	5'-GGCCCCAGTCTGGACGCAGCGACCCCCGTTTTGACAAGGC-3'(SEQ ID NO: 12)
RS4280 CDS 2905bp Fw	5'-GAACTCGTTCAGCTGCCTGTGCCGTCCCGGCTACACAGGAGCCCACTGC-3'(SEQ ID NO: 13)
RS4281 CDS 5692bp	5'-

Primers	Sequences
Rev	GCCTGAGTGGTCCTGGGCATTGGTGTCTGCCC CAGCATCC-3'(SEQ ID NO: 14)
RS4282 cds5501bp Fw	5'-GAAGAGGATGAGGCAGATGACACATCAGCTA GCATCATCTCC-3'(SEQ ID NO: 15)
RS4302 CDS 3091bp Fw	5'-TCACTGTGCCCAGCCGTTCT-3'(SEQ ID NO: 16)
RS4303 CDS 4147bp Fw	5'-CTTCTTCCGCTGCGCTTGCGCGCAG-3'(SEQ ID NO: 17)
RS4304 CDS 5046bp Rev	5'-ATGACCAGCAGCAAGACAGCGC-3'(SEQ ID NO: 18)
RS4305 CDS 5100bp Rev	5'-CAGAGGGTGCTGTGCTCGCGCTTG-3'(SEQ ID NO: 19)
RS4306 CDS 3901bp Fw	5'-ACAGTGCTGCTGCCGCCAGAGGAGCTAC-3'(SEQ ID NO: 20)
RS4361 CDS Seq. Fw	5'-CAGTCCCAGGACATGGCGAGGAGTAC-3'(SEQ ID NO: 21)
RS4362 UTR Fw	5'-AGCCCAGGGAAGGAGGGAGGAGGGGAGGGTC G-3'(SEQ ID NO: 22)
RS4363 CDS 861bp Rev	5'-ACTGGCAGTTATAGGTGTTGACGCCATCCACG C-3'(SEQ ID NO: 23)
RS4364 CDS 1950bp Rev	5'-GCACAGTCGTCAATGTTCACTTCGCAG-3'(SEQ ID NO: 24)
RS4365 CDS 2822bp Fw	5'-TACGGAGGCTTCCACTGCGAACAG-3'(SEQ ID NO: 25)
RS4366 CDS 4067bp Rev	5'-CGACCCCGAGAAACTGCGGCAGGAG-3'(SEQ ID NO: 26)
RS4367 UTR Rev	5'-CCCCAAGATCTAAGAACTGACGAGC-3'(SEQ ID NO: 27)

PCR and gel purification

PCR of the cDNA was achieved by the Corbett® Rotor-Gene 6000 (now QIAGEN® Rotor-Gene Q) RT-PCR using KAPA™ SYBR® FAST Master Mix (2X). The KAPA™ SYBR® FAST qPCR Master Mix (2X) Universal, a ready-to-use cocktail containing antibody-mediated hot start, SYBR® Green I fluorescent dye, MgCl2, dNTPs and stabilizers for the amplification and detection of DNA in qPCR (KAPABIOSYSTEMS).. For PCR, a reaction mix with a volume of 20 µl, consisting of 10 µl SYBR® green, 0.4 µl forward-primer (10 µM), 0.4 µl reverse-primer (10 µM), 2 µl template and 7.2 µl H_2O RNase-free was prepared to each 0.1 ml PCR tube and the tubes closed by caps. The PCR cycling was preceded by a hold

temperature of 95 °C for five minutes and the cycling steps were repeated 45 times. The denaturation consisted of heating the reaction to a temperature of 95°C for ten seconds. After that step the temperature was reduced to 60°C for 30 seconds, allowing annealing of the primers to the single-stranded DNA template. The elongation was obtained by increasing temperature to 72°C for 30 seconds and the cycling steps were repeated. All PCR products were then loaded on a 1 x TBE agarose gel, 1%, PCR fragment size and gel extracted and stained with Ethidium Bromide ($3x10^{-3}$ mg/ml).

Then gel extractions of target DNA fragments were then performed. In this case, a procedure based on the QIAquick® Gel Extraction Kit protocol in combination with a NucleoSpin® 8 / 96 Extract II by MACHEREY-NAGEL® was used to purify the DNA fragment. For the extraction of the PCR DNA fragment, 400 µl QG solubilization buffer of QIAGEN® were added to each piece of gel band in a 96-well plate. To melt down the gel bands, the Deep well plate was placed into hot water bath (50 to 60°C) for about 15 minutes. Before pipetting the solution onto the NucleoSpin® 8 / 96 Extract II filter plate, the solution was vortexed carefully. An additional 100 µl of Isopropanol was used if the DNA bands were lower than 400 bp. The solution was filtrated two times. After this step, the column was washed by 650 µl wash buffer NT3 two times and then dried by placing it under vacuum for 20 minutes before elution of DNA fragment with RNase-free water. For that the collection-reservoir below the NucleoSpin® 8 / 96 Extract II filter plate was replaced by an elution plate "U-bottom" and 100 µl of RNase-free water was added directly onto the middle of membrane without touching it. The extraction of DNA was achieved by the usage of vacuum filtrationand the eluate could finally be used for sequencing.

Sequencing and Data analysis

For sequencing purified DNA fragment, 8 µl of purified PCR sample was mixed with 4 µl H_2O RNase-free and 1 µl forward or 1 µl reversed primer (10 µM). The sequencing of the PCR fragments was completed with the Sanger method in combination with an Applied Biosystems® ABI 3730xl DNA Analyzer. The DNA sequence reads were imported to the program, trimmed and then assembled to a reference, in this case the sequence of the human gene. The sequence of the corresponding gene was directly copied from Ensembl or Swiss-Prot genome database browser into Vector NTI®. The use of the reference sequences allowed identification of full-length sequences.

Cynomolgus monkey Notch3 sequence. Three natural SNPs were identified at positions: 213S/N; 719E/D; and 2053V/A

MGPGARGRRRRRRPMSPPPPPVRALPLLLLLAGPGAAVPPCLDGSPCANGGRCTQLP
SREAACLCPPGWVGERCQLEDPCHSGPCAGRGVCQSSVVAGTARFSCRCPRGFRGPD
CSLPDPCLSSPCAHSARCSVGPDGRFLCSCPPGYQGRSCRSDVDECRVGEPCRHGGTC
LNTPGSFRCQCPAGYTGPLCENPAVPCAPSPCRNGGTCRQSSGDLTYDCACLPGFEGQ
NCEVNVDDCPGHRCLNGGTCVDGVNTYNCQCPPEWTGQFCTEDVDECQLQPNACH
NGGTCFNTLGGHSCVCVNGWTGESCSQNIDDCATAVCFHGATCHDRVASFYCACPM
GKTGLLCHLDDACVSNPCHEDAICDTNPVNGRAICTCPPGFTGGACDQDVDECSIGA
NPCEHLGRCVNTQGSFLCQCGRGYTGPRCETDVNECLSGPCRNQATCLDRIGQFTCIC
MAGFTGTYCEVDIDECQSSPCVNGGICKDRVNGFSCTCPSGFSGSTCQLDVDECASTP
CRNGAKCVDQPDGYECRCAEGFEGMLCERNVDDCSPDPCHHGRCVDGIASFSCACA
PGYTGTRCESQVDECRSQPCRHGGKCLDLVDKYLCRCPSGTTGVNCEVNIDDCASNP
CSFGVCRDGINRYDCVCQPGFTGPLCNVEINECASSPCGEGGSCVDGENGFRCLCPPG
SLPPLCLPPSHPCAHEPCSHGICYDAPGGFRCVCEPGWSGPRCSQSLARDACESQPCR
AGGTCSSDGMGFHCTCPPGVQGRQCELLSPCTPNPCEHGGRCESAPGQLPVCSCPQG
WQGPRCQQDVDECAGPAPCGPHGICTNLAGSFSCTCHGGYTGPSCDQDINDCDPNPC
LNGGSCQDGVGSFSCSCLLGFAGPRCARDVDECLSNPCGPGTCTDHVASFTCTCPPG
YGGFHCEQDLPDCSPSSCFNGGTCVDGVNSFSCLCRPGYTGAHCQHEADPCLSRPCL
HGGVCSAAHPGFRCTCPQSFTGPQCQTLVDWCSRQPCQNGGRCVQTGAYCLCPPGW
SGRLCDIRSLPCREAAAQIGVRLEQLCQAGGQCVDEDSSHYCVCPEGRTGSHCEQEV
DPCLAQPCQHGGTCRGYMGGYMCECLPGYNGENCEDDVDECASQPCQHGGSCIDL
VARYLCSCPPGTLGVLCEINEDDCGPGPPLDSGPRCLHNGTCVDLVGGFRCTCPPGYT
GLRCEADINECRSGACHAAHTRDCLQDPGGGFRCLCHAGFSGPRCQTVLSPCESQPC
QHGGQCRPSPGPGGGLTFTCHCAQPFWGPRCERVARSCRELQCPVGVPCQQTPRGPR
CACPPGLSGPSCRSFSGSPPGASNASCAAAPCLHGGSCRPAPLAPFFRCACAQGWTGP
RCEAPAAAPEVSEEPRCPRAACQAKRGDQRCDRECNSPGCGWDGGDCSLSVGDPWR
QCEALQCWRLFNNSRCDPACSSPACLYDNFDCHAGGRERTCNPVYEKYCADHFADG
RCDQGCNTEECGWDGLDCASEVPALLARGVLVLTVLLPPEELLRSSADFLQRLSAILR
TSLRFRLDAHGQAMVFPYHRPSPGSEPRARRELAPEVIGSVVMLEIDNRLCLQSPEND
HCFPDAQSAADYLGALSAVERLDFPYPLRDVRGEPLEPPEPSVPLLPLLVAGAVLLLV
ILVLGVMVARRKREHSTLWFPEGFSLHKDVAAGHKGRREPVGQDALGMKNMAKGE
SLMGEVATDWMDTECPEAKRLKVEELGMGAEEAVDCRQWTQHHLVAADIRVAPA
MALTPPQGDADADGMDVNVRGPDGFTPLMLASFCGGALEPMPTEEDEADDTSASIIS

DLICQGAQLGARTDRTGETALHLAARYARADAAKRLLDAGADTNAQDHSGRTPLHT
AVTADAQGVFQILIRNRSTDLDARMADGSTALILAARLAVEGMVEELIASHADVNAV
DELGKSALHWAAAVNNVEATLALLKNGANKDMQDSKEETPLFLAAREGSYEAAKL
LLDHFANREITDHLDRLPRDVAQERLHQDIVRLLDQPSGPRSPPGTHGLGPLLCPPGA
FLPGLKVVTQSGSKKSRRPPGKAGLGPQGPRGRGKKLTLACPGPLADSSVTLSPVDSL
DSPRPFGGPPASPGGFPLEGPYAAATATAVSLAQLGGPGRAGLGRQPPGGCVLSLGLL
NPVAVPLDWARLPPPAPPGPSFLLPLAPGPQLLNPGTPVSPQERPPPYLAVPGHGEEYP
AAGAHSSPPKARFLRVPSEHPYLTPSPESPEHWASPSPPSLSDWSESTPSPATATGAMA
TATGALPAQPLPLSVPSSLAQAQTQLGPQPEVTPKRQVLA (SEQ ID NO:28).

Example 2: Screening for Notch3 antibodies and evaluation of protein/cell binding

For selection of antibodies recognizing human Notch 3, several recombinant proteins representing key regions of the Notch 3 receptor were used (see extracellular domain structure schematic in Figure 1) in pannings with a phage display library. The NRR, EGF32-NRR and ligand binding (LBD) regions of Notch 3 were used in pannings. In addition, cell lines expressing either exogenous or endogenous Notch3 were used in either whole cell panning or differential whole cell panning as described below. Antibodies against human Notch3 proteins were generated by selection of clones having high affinity binding affinities, using as the source of antibody variant proteins a commercially available phage display library (HuCAL PLATINUM® library – (Prassler *et al.,* (2011) J Mol Biol 413:261-278).

A number of anti-Notch Antibodies were identified and designated A-F.

Example 3: Characterization of Notch3 antibodies in a ligand-driven reporter gene assay

Canonical Notch signaling is activated when a Notch receptor on one cell interacts with a ligand on a neighboring cell. In mammals there are five trans-membrane ligands, three Delta-like ligands (DLL1, DLL4, and DLL3) and two Jagged ligands (Jag1, Jag2). To determine the capacity of anti-Notch3 antibodies to inhibit Notch3 ligand-induced signaling, a reporter gene assay (RGA) using the double stable reporter cell line HLR-huNotch3-Gal4-NLS-VP16 / Gal4-UA-Luciferase was developed. Using this assay the inhibition of Notch3 signaling activated by either Jag1 or DLL1 was examined. Similar assays were developed for human Notch1 and Notch2 receptors. Testing of Notch3 antibodies in this series of Notch receptor-specific RGA assay allowed specificity assessment of the antibodies for inhibition of Notch3.

To determine the capacity of anti-Notch3 antibodies to inhibit Notch3 ligand-induced signaling, a reporter gene assay (RGA) using the double stable reporter cell line HLR-huNotch3-Gal4-NLS-VP16 / Gal4-UA-Luciferase was developed.

Generation of a cell line expressing human Notch3-Gal4-NLS-VP16/Gal4-UA-luciferase

Human Notch1, Notch2 and Notch3 as well as cyno Notch3 extracellular and trans-membrane portions followed by Gal4 DNA binding domain, VP16 and a nuclear localization sequence (NLS) were cloned into the retroviral vector pLNCX2 (Clontech, cat# 631503). Generation of these chimeric Notch receptors and corresponding reporter gene assays allowed for examination of the effects of Notch3 antibodies of Notch receptor specific signaling.

Expression vectors for Notch1-, Notch2-, and Notch3-Gal4-VP16

The coding sequence for Gal4-VP16 was gene synthesized and cloned into the SalI-ClaI sites of the vector pLNXC2 (Clontech) to make pLNXC2-Gal4-VP16. The extracellular (ECD) and transmembrane domains of cyno Notch3 (amino acids 1-1669), human Notch1 (amino acids 1-1762) and human Notch 2 (1-1704) were gene synthesized and cloned into the HindIII-SalI sites of pLNXC2-Gal4-VP16 to produce fusions of the respective Notch proteins to Gal4-VP16.

Constructs for Notch-Gal4-VP16 expression vectors

Human Notch3-Gal4-VP16

MGPGARGRRRRRRPMSPPPPPPPVRALPLLLLLAGPGAAAPPCLDGSPCANGGRCTQ
LPSREAACLCPPGWVGERCQLEDPCHSGPCAGRGVCQSSVVAGTARFSCRCPRGFRG
PDCSLPDPCLSSPCAHGARCSVGPDGRFLCSCPPGYQGRSCRSDVDECRVGEPCRHGG
TCLNTPGSFRCQCPAGYTGPLCENPAVPCAPSPCRNGGTCRQSGDLTYDCACLPGFEG
QNCEVNVDDCPGHRCLNGGTCVDGVNTYNCQCPPEWTGQFCTEDVDECQLQPNAC
HNGGTCFNTLGGHSCVCVNGWTGESCSQNIDDCATAVCFHGATCHDRVASFYCACP
MGKTGLLCHLDDACVSNPCHEDAICDTNPVNGRAICTCPPGFTGGACDQDVDECSIG
ANPCEHLGRCVNTQGSFLCQCGRGYTGPRCETDVNECLSGPCRNQATCLDRIGQFTCI
CMAGFTGTYCEVDIDECQSSPCVNGGVCKDRVNGFSCTCPSGFSGSTCQLDVDECAS
TPCRNGAKCVDQPDGYECRCAEGFEGTLCDRNVDDCSPDPCHHGRCVDGIASFSCAC
APGYTGTRCESQVDECRSQPCRHGGKCLDLVDKYLCRCPSGTTGVNCEVNIDDCASN
PCTFGVCRDGINRYDCVCQPGFTGPLCNVEINECASSPCGEGGSCVDGENGFRCLCPP
GSLPPLCLPPSHPCAHEPCSHGICYDAPGGFRCVCEPGWSGPRCSQSLARDACESQPC

RAGGTCSSDGMGFHCTCPPGVQGRQCELLSPCTPNPCEHGGRCESAPGQLPVCSCPQ
GWQGPRCQQDVDECAGPAPCGPHGICTNLAGSFSCTCHGGYTGPSCDQDINDCDPNP
CLNGGSCQDGVGSFSCSCLPGFAGPRCARDVDECLSNPCGPGTCTDHVASFTCTCPPG
YGGFHCEQDLPDCSPSSCFNGGTCVDGVNSFSCLCRPGYTGAHCQHEADPCLSRPCL
HGGVCSAAHPGFRCTCLESFTGPQCQTLVDWCSRQPCQNGGRCVQTGAYCLCPPGW
SGRLCDIRSLPCREAAAQIGVRLEQLCQAGGQCVDEDSSHYCVCPEGRTGSHCEQEV
DPCLAQPCQHGGTCRGYMGGYMCECLPGYNGDNCEDDVDECASQPCQHGGSCIDL
VARYLCSCPPGTLGVLCEINEDDCGPGPPLDSGPRCLHNGTCVDLVGGFRCTCPPGYT
GLRCEADINECRSGACHAAHTRDCLQDPGGGFRCLCHAGFSGPRCQTVLSPCESQPC
QHGGQCRPSPGPGGGLTFTCHCAQPFWGPRCERVARSCRELQCPVGVPCQQTPRGPR
CACPPGLSGPSCRSFPGSPPGASNASCAAAPCLHGGSCRPAPLAPFFRCACAQGWTGP
RCEAPAAAPEVSEEPRCPRAACQAKRGDQRCDRECNSPGCGWDGGDCSLSVGDPWR
QCEALQCWRLFNNSRCDPACSSPACLYDNFDCHAGGRERTCNPVYEKYCADHFADG
RCDQGCNTEECGWDGLDCASEVPALLARGVLVLTVLLPPEELLRSSADFLQRLSAILR
TSLRFRLDAHGQAMVFPYHRPSPGSEPRARRELAPEVIGSVVMLEIDNRLCLQSPEND
HCFPDAQSAADYLGALSAVERLDFPYPLRDVRGEPLEPPEPSVPLLPLLVAGAVLLLV
ILVLGVMVARRKRVDKLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPK
TKRSPLTRAHLTEVESRLERLEQLFLLIFPREDLDMILKMDSLQDIKALLTGLFVQDNV
NKDAVTDRLASVETDMPLTLRQHRISATSSSEESSNKGQRQLTVSKLKLLSSIEQACP
KKKRKVDEFPGISTAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDSPG
PG (SEQ ID NO: 29)

Cyno Notch3-Gal4-VP16

MGPGARGRRRRRRPMSPPPPPVRALPLLLLLAGPGAAVPPCLDGSPCANGGRCTQLP
SREAACLCPPGWVGERCQLEDPCHSGPCAGRGVCQSSVVAGTARFSCRCPRGFRGPD
CSLPDPCLSSPCAHSARCSVGPDGRFLCSCPPGYQGRSCRSDVDECRVGEPCRHGGTC
LNTPGSFRCQCPAGYTGPLCENPAVPCAPSPCRNGGTCRQSGDLTYDCACLPGFEGQ
NCEVNVDDCPGHRCLNGGTCVDGVNTYNCQCPPEWTGQFCTEDVDECQLQPNACH
NGGTCFNTLGGHSCVCVNGWTGESCSQNIDDCATAVCFHGATCHDRVASFYCACPM
GKTGLLCHLDDACVSNPCHEDAICDTNPVNGRAICTCPPGFTGGACDQDVDECSIGA
NPCEHLGRCVNTQGSFLCQCGRGYTGPRCETDVNECLSGPCRNQATCLDRIGQFTCIC
MAGFTGTYCEVDIDECQSSPCVNGGICKDRVNGFSCTCPSGFSGSTCQLDVDECASTP
CRNGAKCVDQPDGYECRCAEGFEGMLCERNVDDCSPDPCHHGRCVDGIASFSCACA
PGYTGTRCESQVDECRSQPCRHGGKCLDLVDKYLCRCPSGTTGVNCEVNIDDCASNP

CSFGVCRDGINRYDCVCQPGFTGPLCNVEINECASSPCGEGGSCVDGENGFRCLCPPG
SLPPLCLPPSHPCAHEPCSHGICYDAPGGFRCVCEPGWSGPRCSQSLARDACESQPCR
AGGTCSSDGMGFHCTCPPGVQGRQCELLSPCTPNPCEHGGRCESAPGQLPVCSCPQG
WQGPRCQQDVDECAGPAPCGPHGICTNLAGSFSCTCHGGYTGPSCDQDINDCDPNPC
LNGGSCQDGVGSFSCSCLLGFAGPRCARDVDECLSNPCGPGTCTDHVASFTCTCPPG
YGGFHCEQDLPDCSPSSCFNGGTCVDGVNSFSCLCRPGYTGAHCQHEADPCLSRPCL
HGGVCSAAHPGFRCTCPQSFTGPQCQTLVDWCSRQPCQNGGRCVQTGAYCLCPPGW
SGRLCDIRSLPCREAAAQIGVRLEQLCQAGGQCVDEDSSHYCVCPEGRTGSHCEQEV
DPCLAQPCQHGGTCRGYMGGYMCECLPGYNGENCEDDVDECASQPCQHGGSCIDL
VARYLCSCPPGTLGVLCEINEDDCGPGPPLDSGPRCLHNGTCVDLVGGFRCTCPPGYT
GLRCEADINECRSGACHAAHTRDCLQDPGGGFRCLCHAGFSGPRCQTVLSPCESQPC
QHGGQCRPSPGPGGGLTFTCHCAQPFWGPRCERVARSCRELQCPVGVPCQQTPRGPR
CACPPGLSGPSCRSFSGSPPGASNASCAAAPCLHGGSCRPAPLAPFFRCACAQGWTGP
RCEAPAAAPEVSEEPRCPRAACQAKRGDQRCDRECNSPGCGWDGGDCSLSVGDPWR
QCEALQCWRLFNNSRCDPACSSPACLYDNFDCHAGGRERTCNPVYEKYCADHFADG
RCDQGCNTEECGWDGLDCASEVPALLARGVLVLTVLLPPEELLRSSADFLQRLSAILR
TSLRFRLDAHGQAMVFPYHRPSPGSEPRARRELAPEVIGSVVMLEIDNRLCLQSPEND
HCFPDAQSAADYLGALSAVERLDFPYPLRDVRGEPLEPPEPSVPLLPLLVAGAVLLLV
ILVLGVMVARRKRVDKLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPK
TKRSPLTRAHLTEVESRLERLEQLFLLIFPREDLDMILKMDSLQDIKALLTGLFVQDNV
NKDAVTDRLASVETDMPLTLRQHRISATSSSEESSNKGQRQLTVSQLKLLSSIEQACP
KKKRKVDEFPGISTAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDSPG
PG (SEQ ID NO: 30)

Human Notch1-Gal4-VP16

MPPLLAPLLCLALLPALAARGPRCSQPGETCLNGGKCEAANGTEACVCGGAFVGPRC
QDPNPCLSTPCKNAGTCHVVDRRGVADYACSCALGFSGPLCLTPLDNACLTNPCRNG
GTCDLLTLTEYKCRCPPGWSGKSCQQADPCASNPCANGGQCLPFEASYICHCPPSFHG
PTCRQDVNECGQKPGLCRHGGTCHNEVGSYRCVCRATHTGPNCERPYVPCSPSPCQN
GGTCRPTGDVTHECACLPGFTGQNCEENIDDCPGNNCKNGGACVDGVNTYNCRCPP
EWTGQYCTEDVDECQLMPNACQNGGTCHNTHGGYNCVCVNGWTGEDCSENIDDC
ASAACFHGATCHDRVASFYCECPHGRTGLLCHLNDACISNPCNEGSNCDTNPVNGKA
ICTCPSGYTGPACSQDVDECSLGANPCEHAGKCINTLGSFECQCLQGYTGPRCEIDVN
ECVSNPCQNDATCLDQIGEFQCICMPGYEGVHCEVNTDECASSPCLHNGRCLDKINEF

QCECPTGFTGHLCQYDVDECASTPCKNGAKCLDGPNTYTCVCTEGYTGTHCEVDIDE
CDPDPCHYGSCKDGVATFTCLCRPGYTGHHCETNINECSSQPCRHGGTCQDRDNAYL
CFCLKGTTGPNCEINLDDCASSPCDSGTCLDKIDGYECACEPGYTGSMCNINIDECAG
NPCHNGGTCEDGINGFTCRCPEGYHDPTCLSEVNECNSNPCVHGACRDSLNGYKCDC
DPGWSGTNCDINNNECESNPCVNGGTCKDMTSGYVCTCREGFSGPNCQTNINECASN
PCLNQGTCIDDVAGYKCNCLLPYTGATCEVVLAPCAPSPCRNGGECRQSEDYESFSC
VCPTGWQGQTCEVDINECVLSPCRHGASCQNTHGGYRCHCQAGYSGRNCETDIDDC
RPNPCHNGGSCTDGINTAFCDCLPGFRGTFCEEDINECASDPCRNGANCTDCVDSYTC
TCPAGFSGIHCENNTPDCTESSCFNGGTCVDGINSFTCLCPPGFTGSYCQHDVNECDS
QPCLHGGTCQDGCGSYRCTCPQGYTGPNCQNLVHWCDSSPCKNGGKCWQTHTQYR
CECPSGWTGLYCDVPSVSCEVAAQRQGVDVARLCQHGGLCVDAGNTHHCRCQAGY
TGSYCEDLVDECSPSPCQNGATCTDYLGGYSCKCVAGYHGVNCSEEIDECLSHPCQN
GGTCLDLPNTYKCSCPRGTQGVHCEINVDDCNPPVDPVSRSPKCFNNGTCVDQVGGY
SCTCPPGFVGERCEGDVNECLSNPCDARGTQNCVQRVNDFHCECRAGHTGRRCESVI
NGCKGKPCKNGGTCAVASNTARGFICKCPAGFEGATCENDARTCGSLRCLNGGTCIS
GPRSPTCLCLGPFTGPECQFPASSPCLGGNPCYNQGTCEPTSESPFYRCLCPAKFNGLL
CHILDYSFGGGAGRDIPPPLIEEACELPECQEDAGNKVCSLQCNNHACGWDGGDCSL
NFNDPWKNCTQSLQCWKYFSDGHCDSQCNSAGCLFDGFDCQRAEGQCNPLYDQYC
KDHFSDGHCDQGCNSAECEWDGLDCAEHVPERLAAGTLVVVVLMPPEQLRNSSFHF
LRELSRVLHTNVVFKRDAHGQQMIFPYYGREEELRKHPIKRAAEGWAAPDALLGQV
KASLLPGGSEGGRRRRELDPMDVRGSIVYLEIDNRQCVQASSQCFQSATDVAAFLGA
LASLGSLNIPYKIEAVQSETVEPPPPAQLHFMYVAAAAFVLLFFVGCGVLLSRKRRRV
DKLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPKTKRSPLTRAHLTEVE
SRLERLEQLFLLIFPREDLDMILKMDSLQDIKALLTGLFVQDNVNKDAVTDRLASVET
DMPLTLRQHRISATSSSEESSNKGQRQLTVSQLKLLSSIEQACPKKKRKVDEFPGISTA
PPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDSPGPG (SEQ ID NO: 31)

Human Notch2-Gal4-VP16

MPALRPALLWALLALWLCCAAPAHALQCRDGYEPCVNEGMCVTYHNGTGYCKCPE
GFLGEYCQHRDPCEKNRCQNGGTCVAQAMLGKATCRCASGFTGEDCQYSTSHPCFV
SRPCLNGGTCHMLSRDTYECTCQVGFTGKECQWTDACLSHPCANGSTCTTVANQFS
CKCLTGFTGQKCETDVNECDIPGHCQHGGTCLNLPGSYQCQCPQGFTGQYCDSLYVP
CAPSPCVNGGTCRQTGDFTFECNCLPGFEGSTCERNIDDCPNHRCQNGGVCVDGVNT
YNCRCPPQWTGQFCTEDVDECLLQPNACQNGGTCANRNGGYGCVCVNGWSGDDCS

ENIDDCAFASCTPGSTCIDRVASFSCMCPEGKAGLLCHLDDACISNPCHKGALCDTNP
LNGQYICTCPQGYKGADCTEDVDECAMANSNPCEHAGKCVNTDGAFHCECLKGYA
GPRCEMDINECHSDPCQNDATCLDKIGGFTCLCMPGFKGVHCELEINECQSNPCVNN
GQCVDKVNRFQCLCPPGFTGPVCQIDIDDCSSTPCLNGAKCIDHPNGYECQCATGFTG
VLCEENIDNCDPDPCHHGQCQDGIDSYTCICNPGYMGAICSDQIDECYSSPCLNDGRCI
DLVNGYQCNCQPGTSGVNCEINFDDCASNPCIHGICMDGINRYSCVCSPGFTGQRCNI
DIDECASNPCRKGATCINGVNGFRCICPEGPHHPSCYSQVNECLSNPCIHGNCTGGLSG
YKCLCDAGWVGINCEVDKNECLSNPCQNGGTCDNLVNGYRCTCKKGFKGYNCQVN
IDECASNPCLNQGTCFDDISGYTCHCVLPYTGKNCQTVLAPCSPNPCENAAVCKESPN
FESYTCLCAPGWQGQRCTIDIDECISKPCMNHGLCHNTQGSYMCECPPGFSGMDCEE
DIDDCLANPCQNGGSCMDGVNTFSCLCLPGFTGDKCQTDMNECLSEPCKNGGTCSD
YVNSYTCKCQAGFDGVHCENNINECTESSCFNGGTCVDGINSFSCLCPVGFTGSFCLH
EINECSSHPCLNEGTCVDGLGTYRCSCPLGYTGKNCQTLVNLCSRSPCKNKGTCVQK
KAESQCLCPSGWAGAYCDVPNVSCDIAASRRGVLVEHLCQHSGVCINAGNTHYCQC
PLGYTGSYCEEQLDECASNPCQHGATCSDFIGGYRCECVPGYQGVNCEYEVDECQN
QPCQNGGTCIDLVNHFKCSCPPGTRGLLCEENIDDCARGPHCLNGGQCMDRIGGYSC
RCLPGFAGERCEGDINECLSNPCSSEGSLDCIQLTNDYLCVCRSAFTGRHCETFVDVCP
QMPCLNGGTCAVASNMPDGFICRCPPGFSGARCQSSCGQVKCRKGEQCVHTASGPR
CFCPSPRDCESGCASSPCQHGGSCHPQRQPPYYSCQCAPPFSGSRCELYTAPPSTPPAT
CLSQYCADKARDGVCDEACNSHACQWDGGDCSLTMENPWANCSSPLPCWDYINNQ
CDELCNTVECLFDNFECQGNSKTCKYDKYCADHFKDNHCDQGCNSEECGWDGLDC
AADQPENLAEGTLVIVVLMPPEQLLQDARSFLRALGTLLHTNLRIKRDSQGELMVYP
YYGEKSAAMKKQRMTRRSLPGEQEQEVAGSKVFLEIDNRQCVQDSDHCFKNTDAA
AALLASHAIQGTLSYPLVSVVSESLTPERTQLLYLLAVAVVIILFIILLGVIMAKRKRVD
KLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPKTKRSPLTRAHLTEVES
RLERLEQLFLLIFPREDLDMILKMDSLQDIKALLTGLFVQDNVNKDAVTDRLASVETD
MPLTLRQHRISATSSSEESSNKGQRQLTVSQLKLLSSIEQACPKKKRKVDEFPGISTAPP
TDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDSPGPG (SEQ ID NO: 32)

Generation of a retrovirus to expression Notch3-Gal4-VP16

Retrovirus was produced transfecting 293-GP2 Packaging Cell Line (Clontech, cat# 631458) with the appropriate retroviral vector (pLNCX2_hNotch1_Gal4-VP16, pLNCX2_hNotch2_Gal4-VP16 pLNCX2_hNotch3_Gal4-VP16 pLNCX2_cNotch3_Gal4-VP16). Promega's Fugene6 was used as the lipid-based transfection reagent. Transfection was

carried out according to manufacturer's instructions. Virus was collected at 48 h after transfection and immediately used to transduce HLR cells (HLR-PathDetect, Stratagene). Transduced cells were under selection for at least two weeks, before they were tested in a co-culture assay. Clonal populations for each cell line were selected.

Notch3-Gal4-NLS-VP16-UA-luciferase ligand-induced reporter gene assay

HLR-Notch3-Gal4-NLS-VP16 / Gal4-UA-TATA-Luciferase (HLR-N3) cells are activated byco-culture with L cells stably expressing either cell surface expressed rrJagged1 (SN3T9) or rrDelta1 (DLL1-19) (Hicks C *et al.* (2000) Nature Cell Bio 2:515-520; Lindsell C *et al.* (1995) Cell 80:909-917). Co-culture with ligand expressing cells results in activation of Notch3 signaling and proteolytic cleavage of the Notch3 chimeric receptors to release the Gal4-NLS-VP16. This Gal4-NLS-VP16 translocates to the nuclease where it binds to the Gal4-luciferase reporter resulting in production of luciferase. At 90% confluency HLR-N3 cells were detached using Trypsin-EDTA and diluted in assay medium (DMEM, High glucose, L-Glu, Invitrogen, Cat# 21063-029; supplemented with 10% FBS, 1% P/S) to a concentration of $2x10^5$ cells/ml. 50 µl HLR-N3 cells per well (= $1x10^4$ cells) were seeded into white flat-bottomed 96-well plates (Costar, Cat #:3917) and incubated at 37°C and 5% CO_2 overnight.

The next day, the anti-Notch antibodies were diluted at the desired concentrations in PBS. Per well 10 µl of antibody dilution were added to the seeded cells and incubated for 2 h at 37°C and 5% CO_2. Next Jagged1 and Delta1 ligand expressing mouse L-cells were detached using Trypsin-EDTA and diluted in assay media to a concentration of $8x10^5$ cells/ml. Per well 50 µl mouse L-cells (= $4x10^4$ cells/ well) were added to the cultured HLR-N3 cells (50 µl HLR cells + 10 µl antibody + 50 µl mouse cells = 110 µl final volume) and incubated over night at 37°C and 5% CO_2. As a control 50 µl mouse parental L-cells were added instead for the ligand independent setting.

After overnight incubation, 50 µl of freshly prepared Bright-Glo reagent was adapted to room temperature (Promega, Cat #E2610) and added to each well. After 5 min incubation time, the luminescence was read in a luminometer (GeniosPro, Tecan). IC_{50} values were calculated using Prism after full titration of the respective antibodies. Percentage inhibition relative to an IgG control is indicated. If increased signaling was detected upon antibody addition then a negative number is used.

Summary and discussion

In addition to the huNotch3 RGA, cynoNotch3 RGAs as well as huNotch1 RGA (only DLL1 ligand setting) and huNotch2 RGA (Jagged1 and DLL1) were performed as described above. None of the Notch3 antibodies described, showed any activity in the huNotch1 or huNotch2 RGAs up to a maximal concentration of 10μg/ml. Notch3 antibodies were identified that inhibit both Jagged1 and Delta1 induced Notch3 signaling. The percentage of inhibition and IC_{50} varied depending on the antibody and the ligand used for activation. Antibodies that were identified from pannings directed against the LBD domain (Ab-F, Ab-D, Ab-E) were most effective in inhibiting signaling from this ligand-driven RGA assay.

Example 4: Effects of Notch3 antibodies on Notch target gene mRNA levels

In order to identify Notch target genes in a series of breast cancer cell lines the effect of gamma secretase inhibitor (GSI) treatment on the mRNA expression of genes was evaluated. Affymetrix human U133A Arrays were used to profile treatment of HCC70, MDA-MB468 or HCC1143 cells with either DMSO or 10μM DAPT (Calbiochem 565770) for 72h. There were three replicates per time point. The R / Bioconductor framework was used and the Limma package was employed to determine differentially expressed genes between the DMSO treatment and the DAPT treatment. An adjusted P-value of .05 was used as the threshold to determine the set of differentially expressed genes. Ultimately, two target genes were selected per cell line, and are summarized in below. Hes1, MMP7 and VSNL1 mRNA levels are decreased upon inhibition of Notch signaling while DKK1 mRNA levels are increased upon inhibition of Notch signaling.

Gene	Cell Line
PP1A	All
Hes1	HCC1143, MDA-MB-468
MMP7	HCC1143
DKK1	MDA-MB-468
Hey2	HCC70
VSNL1	HCC70

To quantitate mRNA levels of the above genes, cell lines HCC70, MDA-MB-468 or HCC1143 were plated in 100 μL in 96-well plates (Costar, cat#3610) at a cell density of

$1x10^5$ cells/mL. Plates were incubated overnight at 37°C before treatment with antibodies at appropriate concentrations. Treated plates were returned to the incubator for an extra 72h before being lysed for RNA extraction using Qiagen's RNeasy kit (cat# 74181). cDNA was synthesized using Taqman Reverse Transcription Reagents (Applied Biosystems, cat# N808-0234). mRNA expression was determined by real-time PCR (Taqman Fast Advanced Master Mix, Applied Biosystems, cat# 4444557). Real-time PCR was run in a ViiA 7 Real-Time PCR System or 7900HT Fast Real-Time PCR System (Applied Biosystems). To quantitate the levels of each target gene, 2–[delta][delta]Ct method was employed. Calculation of delta delta Ct involves comparing the Ct values of the samples of interest with a control such as a non-treated sample or DMSO treated sample (Schmittgen and Livak 2008 Nature Protocols 3: 1101-1108)

Summary and discussion

Notch3 antibodies (Abs A-F) were identified that could inhibit endogenous Notch3 signaling in a series of breast cancer cell lines. Treatment of breast cancer cell lines with Notch3 antibodies resulted in decreased expression of HES1 or MMP7 mRNA and increased expression of DKK1 mRNA.

Example 5: Identification and Characterization of Mutations in Notch3 NRR and PEST Domains

To date, the evidence for Notch receptors in cancer has focused primarily on alterations in Notch1 signaling. Although Notch3 is amplified in ovarian cancer there is no direct evidence that its amplification leads to dependence on Notch3 signaling. In addition, there is no evidence for activating mutations in Notch3. Notch 3 was sequenced in a panel of cells lines to identify mutations in the gene for further characterization.

The Cancer Cell Line encyclopedia (CCLE) was used to characterize 947 human cancer cell lines (Barretina J. *et al.* (2012) Nature 483:603-7). Mutation information was obtained for >1600 genes by massively parallel sequencing using a solution phase hybrid capture technology. Multiplexed libraries for exome capture sequencing were constructed as described using the SureSelect Target Enrichment system (Aligent Technologies). Notch3 was one of the genes sequenced and the data was analyzed to identify any mutations in the NRR (exon 25, 26, amino acid 1378-1640) and PEST (exon 33 amino acid 1972-2322) domains of the protein. Upon close examination of the sequence data from the 947 cancer cell lines, it was determined that there was insufficient sequence coverage in exons 25 and 33 to identify

mutations. The table shows the average coverage of exons in Notch3. The numbers listed are the average number of reads per base pair in Table 2.

Table 2: Notch 3 Exon reads.

Exon of Notch3	Average coverage
e01	0.03
e02	-
e03	-
e04	10.49
e05	595.39
e06	277.47
e07	79.71
e08	99.63
e09	210.51
e10	0.58
e11	42.39
e12	558.77
e13	0.77
e14	0.66
e15	1.71
e16	168.88
e17	1.65
e18	1.13
e19	111.12
e20	53.03
e21	414.89
e22	12.79
e23	6.72
e24	-
e25	0.44
e26	171.70
e27	52.77
e28	3.44
e29	36.78
e30	280.90
e31	404.35
e32	223.13
e33	1.27

In order to determine whether any of these cell lines or primary tumors contain mutations in these regions, three approaches were used including Sanger Sequencing (Genewiz), RainDance (Tewhey *et al.* (2009) Nature Biotechnology 27:1025-1031) and RNAseq (Wang *et al.* (2009) Nature Reviews Genetics 10:57-63.Mutations were identified in both the NRR and PEST domain in multiple cell lines and tumor samples as shown in Figure 3. In Figure 3a

the upper panel shows cells lines with NRR mutations while the lower panel has PEST mutations. The NRR mutations identified in primary tumors are indicated in Figure 3b.

Isolation of primary tumors and generation of a bank of primary tumor xenografts

Data obtained from primary human tumor xenografts was generated in the following manner: tumor specimens were collected in RPMI supplemented with 1% penicillin/streptomycin from patients during surgical resection with ischemic time less than one hour. Fragments of 15-30 mm^3 free of necrotic tissue were grafted subcutaneously into interscapular fat pad of 6- to 8-week-old female nude mice under isoflurane anesthesia. Mice were maintained in specific pathogen-free animal housing and handled in accordance with approved protocols and regulations. Xenografts appeared at the graft site 2 to 8 months after grafting. They were subsequently transplanted from mouse to mouse once tumors reached 700-800 mm^3 until a reasonably consistent growth rate is achieved. Frozen stocks in RPMI supplemented with 50% FBS and 10% DMSO were generated during serial passage in mice and were tested to ensure successful establishment of a xenograft model. Fragments of 30-50 mg from patients and xenografts at each passage were snap frozen for gene expression profiling, copy number as well as mutation analyses. Fragments of 150 mg of each successfully engrafted xenograft model were also collected and subject to histological analysis. An established tumor xenograft model was further used for in vivo studies after passage four. For gene expression profiling, total RNA was isolated using affinity resin (QIAGEN RNeasy Mini Kit; QIAGEN AG). RNA integrity and purity were assessed with the RNA 6000 Nano LabChip system on a Bioanalyzer 2100 (Agilent Technologies).

Example 6: Characterization of Notch3 NRR mutations in a reporter gene assay

Generation of Notch3 expression vectors with Notch3 NRR mutations

Two mutations were selected for characterization. TALL-1 cells are a t-cell acute lymphoblastic cell line with a S1580L mutation. TALL-1 cells were purchased from DSMZ (#ACC 521). A breast tumor (X-1004) was also identified with a G1487D mutation. The RNA used for RNAseq analysis to detect mutations in the X-1004 sample was from a passage 5 mouse. These mutations were introduced into the vector pLNCX2_Notch3-GAL4-NLS-VP16.

Constructs:

Notch3_S1580L_Gal4-VP16

MGPGARGRRRRRRPMSPPPPPPPVRALPLLLLLAGPGAAAPPCLDGSPCANGGRCTQ
LPSREAACLCPPGWVGERCQLEDPCHSGPCAGRGVCQSSVVAGTARFSCRCPRGFRG
PDCSLPDPCLSSPCAHGARCSVGPDGRFLCSCPPGYQGRSCRSDVDECRVGEPCRHGG
TCLNTPGSFRCQCPAGYTGPLCENPAVPCAPSPCRNGGTCRQSGDLTYDCACLPGFEG
QNCEVNVDDCPGHRCLNGGTCVDGVNTYNCQCPPEWTGQFCTEDVDECQLQPNAC
HNGGTCFNTLGGHSCVCVNGWTGESCSQNIDDCATAVCFHGATCHDRVASFYCACP
MGKTGLLCHLDDACVSNPCHEDAICDTNPVNGRAICTCPPGFTGGACDQDVDECSIG
ANPCEHLGRCVNTQGSFLCQCGRGYTGPRCETDVNECLSGPCRNQATCLDRIGQFTCI
CMAGFTGTYCEVDIDECQSSPCVNGGVCKDRVNGFSCTCPSGFSGSTCQLDVDECAS
TPCRNGAKCVDQPDGYECRCAEGFEGTLCDRNVDDCSPDPCHHGRCVDGIASFSCAC
APGYTGTRCESQVDECRSQPCRHGGKCLDLVDKYLCRCPSGTTGVNCEVNIDDCASN
PCTFGVCRDGINRYDCVCQPGFTGPLCNVEINECASSPCGEGGSCVDGENGFRCLCPP
GSLPPLCLPPSHPCAHEPCSHGICYDAPGGFRCVCEPGWSGPRCSQSLARDACESQPC
RAGGTCSSDGMGFHCTCPPGVQGRQCELLSPCTPNPCEHGGRCESAPGQLPVCSCPQ
GWQGPRCQQDVDECAGPAPCGPHGICTNLAGSFSCTCHGGYTGPSCDQDINDCDPNP
CLNGGSCQDGVGSFSCSCLPGFAGPRCARDVDECLSNPCGPGTCTDHVASFTCTCPPG
YGGFHCEQDLPDCSPSSCFNGGTCVDGVNSFSCLCRPGYTGAHCQHEADPCLSRPCL
HGGVCSAAHPGFRCTCLESFTGPQCQTLVDWCSRQPCQNGGRCVQTGAYCLCPPGW
SGRLCDIRSLPCREAAAQIGVRLEQLCQAGGQCVDEDSSHYCVCPEGRTGSHCEQEV
DPCLAQPCQHGGTCRGYMGGYMCECLPGYNGDNCEDDVDECASQPCQHGGSCIDL
VARYLCSCPPGTLGVLCEINEDDCGPGPPLDSGPRCLHNGTCVDLVGGFRCTCPPGYT
GLRCEADINECRSGACHAAHTRDCLQDPGGGFRCLCHAGFSGPRCQTVLSPCESQPC
QHGGQCRPSPGPGGGLTFTCHCAQPFWGPRCERVARSCRELQCPVGVPCQQTPRGPR
CACPPGLSGPSCRSFPGSPPGASNASCAAAPCLHGGSCRPAPLAPFFRCACAQGWTGP
RCEAPAAAPEVSEEPRCPRAACQAKRGDQRCDRECNSPGCGWDGGDCSLSVGDPWR
QCEALQCWRLFNNSRCDPACSSPACLYDNFDCHAGGRERTCNPVYEKYCADHFADG
RCDQGCNTEECGWDGLDCASEVPALLARGVLVLTVLLPPEELLRSSADFLQRLSAILR
TSLRFRLDAHGQAMVFPYHRPSPGSEPRARRELAPEVIGLVVMLEIDNRLCLQSPEND
HCFPDAQSAADYLGALSAVERLDFPYPLRDVRGEPLEPPEPSVPLLPLLVAGAVLLLV
ILVLGVMVARRKRVDKLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPK
TKRSPLTRAHLTEVESRLERLEQLFLLIFPREDLDMILKMDSLQDIKALLTGLFVQDNV

NKDAVTDRLASVETDMPLTLRQHRISATSSSEESSNKGQRQLTVSKLKLLSSIEQACP
KKKRKVDEFPGISTAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDSPG
PG (SEQ ID NO: 33)

Notch3_G1487D_Gal4-VP16

MGPGARGRRRRRRPMSPPPPPPPVRALPLLLLLAGPGAAAPPCLDGSPCANGGRCTQ
LPSREAACLCPPGWVGERCQLEDPCHSGPCAGRGVCQSSVVAGTARFSCRCPRGFRG
PDCSLPDPCLSSPCAHGARCSVGPDGRFLCSCPPGYQGRSCRSDVDECRVGEPCRHGG
TCLNTPGSFRCQCPAGYTGPLCENPAVPCAPSPCRNGGTCRQSGDLTYDCACLPGFEG
QNCEVNVDDCPGHRCLNGGTCVDGVNTYNCQCPPEWTGQFCTEDVDECQLQPNAC
HNGGTCFNTLGGHSCVCVNGWTGESCSQNIDDCATAVCFHGATCHDRVASFYCACP
MGKTGLLCHLDDACVSNPCHEDAICDTNPVNGRAICTCPPGFTGGACDQDVDECSIG
ANPCEHLGRCVNTQGSFLCQCGRGYTGPRCETDVNECLSGPCRNQATCLDRIGQFTCI
CMAGFTGTYCEVDIDECQSSPCVNGGVCKDRVNGFSCTCPSGFSGSTCQLDVDECAS
TPCRNGAKCVDQPDGYECRCAEGFEGTLCDRNVDDCSPDPCHHGRCVDGIASFSCAC
APGYTGTRCESQVDECRSQPCRHGGKCLDLVDKYLCRCPSGTTGVNCEVNIDDCASN
PCTFGVCRDGINRYDCVCQPGFTGPLCNVEINECASSPCGEGGSCVDGENGFRCLCPP
GSLPPLCLPPSHPCAHEPCSHGICYDAPGGFRCVCEPGWSGPRCSQSLARDACESQPC
RAGGTCSSDGMGFHCTCPPGVQGRQCELLSPCTPNPCEHGGRCESAPGQLPVCSCPQ
GWQGPRCQQDVDECAGPAPCGPHGICTNLAGSFSCTCHGGYTGPSCDQDINDCDPNP
CLNGGSCQDGVGSFSCSCLPGFAGPRCARDVDECLSNPCGPGTCTDHVASFTCTCPPG
YGGFHCEQDLPDCSPSSCFNGGTCVDGVNSFSCLCRPGYTGAHCQHEADPCLSRPCL
HGGVCSAAHPGFRCTCLESFTGPQCQTLVDWCSRQPCQNGGRCVQTGAYCLCPPGW
SGRLCDIRSLPCREAAAQIGVRLEQLCQAGGQCVDEDSSHYCVCPEGRTGSHCEQEV
DPCLAQPCQHGGTCRGYMGGYMCECLPGYNGDNCEDDVDECASQPCQHGGSCIDL
VARYLCSCPPGTLGVLCEINEDDCGPGPPLDSGPRCLHNGTCVDLVGGFRCTCPPGYT
GLRCEADINECRSGACHAAHTRDCLQDPGGGFRCLCHAGFSGPRCQTVLSPCESQPC
QHGGQCRPSPGPGGGLTFTCHCAQPFWGPRCERVARSCRELQCPVGVPCQQTPRGPR
CACPPGLSGPSCRSFPGSPPGASNASCAAAPCLHGGSCRPAPLAPFFRCACAQGWTGP
RCEAPAAAPEVSEEPRCPRAACQAKRGDQRCDRECNSPGCGWDGGDCSLSVGDPWR
QCEALQCWRLFNNSRCDPACSSPACLYDNFDCHAGGRERTCNPVYEKYCADHFADG
RCDQDCNTEECGWDGLDCASEVPALLARGVLVLTVLLPPEELLRSSADFLQRLSAILR
TSLRFRLDAHGQAMVFPYHRPSPGSEPRARRELAPEVIGSVVMLEIDNRLCLQSPEND
HCFPDAQSAADYLGALSAVERLDFPYPLRDVRGEPLEPPEPSVPLLPLLVAGAVLLLV

ILVLGVMVARRKRVDKLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPK
TKRSPLTRAHLTEVESRLERLEQLFLLIFPREDLDMILKMDSLQDIKALLTGLFVQDNV
NKDAVTDRLASVETDMPLTLRQHRISATSSSEESSNKGQRQLTVSKLKLLSSIEQACP
KKKRKVDEFPGISTAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDSPG
PG (SEQ ID NO: 34)

Retrovirus was produced by transfecting 293-GP2 Packaging Cell Line (Clontech, cat# 631458) with the appropriate retroviral vector. Promega's Fugene6 was used as the lipid-based transfection reagent. Transfection was carried out according to manufacturer's instructions. Virus was collected at 48 hs after transfection and immediately used to transduce HLR cells (HLR-PathDetect, Stratage). HLR cells (Stratagene) were transduced with either Notch3wt-Gal4-VP16, Notch3_p.S1850L-Gal4-VP16 or Notch3_p.G1487D-Gal4-VP16 retroviral particles. Cells were selected with G418 for 2 weeks before testing.

Notch3 reporter gene assay to assess basal activity of Notch3 wild-type and Notch3 NRR mutant receptors

Notch3 reporter gene assay: HLR-Notch3wt-Gal4-VP16, HLR-Notch3_p.S1580L-Gal4-VP16 and HLR-Notch3_p.G1487D-Gal4-VP16 cells were maintained in DMEM no phenol red, 10% FBS (Hyclone, cat# SH30071), 1% penicillin-streptomycin (Gibco cat# 15140-122), L-Glutamine (Gibco, cat#25030-081), 100 μg/mL hygromycin (Gibco, cat# 10687-010) and 400 μg/mL G418 (Gibco, cat# 10131-027). The HLR parental line was maintained in DMEM no phenol red, 10% FBS (Hyclone, cat# SH30071), 1% penicillin-streptomycin (Gibco cat# 15140-122), L-Glutamine (Gibco, cat#25030-081) and 100μg/mL hygromycin (Gibco, cat# 10687-010). Sub-confluent cells grown in complete medium were washed with PBS (Gibco, cat# 20012-027), trypsinized with TryplE (Gibco, cat# 12605010), and diluted into $4x10^4$ cells/mL; 100 μL of cell suspension was plated in 96-well clear bottom white plates (Costar, cat# 3610) at a density of 4000 cells/well. All plates were then incubated overnight at 37°C prior to treatment with DAPT (10 μM, CalBiochem). Plates were returned to the incubator for 24 hs before luciferase activity was determined using Bright-Glo (Promega). The Envision plate reader (PerkinElmer) was used to determine amount of luminescence.

FACS assay to assess cell surface levels of wild-type and mutant Notch3 receptors

To demonstrate expression of mutant Notch3 receptors in a cell line, flow cytometry was used. Cell lines expressing mutant Notch3 and wild-type Notch3 (grown under standard conditions) were mixed with an anti- Notch3 binding and detection antibody that contains an

APC fluorescein label (R&D cat# FAB1559A) in PBS containing 0.1% BSA and 0.01% sodium azide, and incubated for 1 h at 4°C. After washing, the cells were analyzed by BD FACSCanto instrument using light and side scatter properties to gate on single cells.

The level of Notch3 receptors on the cell surface was determined by binding of commercially available anti-Notch3 APC (R&D # FAB1559A) labeled antibody to cells expressing mutant and endogenous Notch3 and assessed by FACS. Cells were trypsinized (Invitrogen TrypLE cat# 12605-010) and diluted to $2x10^6$ cells/mL in FACS Buffer (PBS/3% FBS/0.01%NaN3). $2.5x10^5$ cells/well were added to each well of a 96 well plate (Corning cat#3610) and centrifuged at 1500 rpm for 5 min at 4°C before removing the supernatant. Anti-Notch3 APC antibody or Sheep IgG Isotype Control labeled with APC (R&D cat#IC016A) was added to the cell pellets at a final concentration of 0.1 μg in 100 μL of FACS buffer and incubated for 1 hour at 4°C. The cells were washed and pelleted 2 times with 100 μL FACS Buffer. Finally cells were resuspended in 200 μL FACS buffer and fluorescence values were measured with a BD FACSCanto (BD Biosciences). The amount of cell surface bound anti-Notch3 APC antibody was assessed by measuring the mean channel fluorescence.

Summary and Discussion

Introduction of either a S1580L mutation or a G1487D mutation into a Notch3 receptor resulted in an approximately 10 fold increase in the basal signaling from the receptor relative to a wild-type control (Figure 4B). In this system the wild-type and mutant receptors were expressed at approximately equivalent levels as determined by FACS assay (Figure 4C). This data suggests that these mutations activate Notch3 signaling in cell lines and tumors expressing these and other similar mutations (see further discussion in Examples 7, 9, 10, 11, 15).

Example 7: Effect of Notch3 antibodies on Notch3 signaling and in vitro proliferation in TALL-1 cells

The TALL-1 cell line has a mutation in the NRR domain of Notch3 at S1580L. Introduction of this mutation into a Notch3 expression construct resulted in activation of Notch3 signaling. To further characterize the effects of inhibition of Notch3 signaling in this cells line, the mRNA levels of Notch target genes were examined and the in vitro proliferation of the cells was monitored in the presence of Notch 3 antibodies.

TALL-1 in vitro proliferation assay

$1x10^4$ TALL-1 cells/well were seeded into 96-well tissue culture plates (Corning, Catalog #3610) in 100ul medium (RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin/ streptomycin). The same day, antibody dilutions were prepared in 1X PBS from which 5µl of 20X antibody dilution was added per well. Cells were incubated with antibody at 37°C/5 % CO_2. After incubation for 0 and 9 days at 37 °C/5 % CO_2, 100µl of CellTiter-Glo reagent (Promega) was added and the plates were incubated for 10min on plate shaker. The amount of luminescence was determined using a Perkin Elmer Envision plate reader. CellTiter-Glo luminescene values of cells treated with an IgG control were used to normalize the data and calculate percentage inhibition of proliferation due to treatment with Notch3 antibodies.

TALL-1 mRNA quantitation assay

Deltex1 is a well characterized target gene of Notch signaling in TALL lines (Weng *et al.*, 2006, Genes Dev. 20:2096-2109). $1x10^4$ TALL-1 cells/well were seeded into 96-well tissue culture plates (Corning, cat#3610) in 100µl medium (RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin streptomycin). The same day, antibody and compound dilutions were prepared in 1X PBS from which 5µl of 20X antibody or compound dilution was added per well. DAPT (Calbiochem, cat#565770) and DMSO (ATCC, Catalog #4-X-5) were the compounds used for this assay. Cells were incubated with antibody or compound at 37°C/5 % CO_2 for 72h. RNA was isolated using the Qiagen RNeasy 96 kit. cDNA was made using the TaqMan Reverse Transcription reagents (Life Technologies) and the MJ Research PTC-225 Thermal cycler. TaqMan gene expression assays were run using TaqMan Universal PCR Master Mix (Life Technologies) along with gene expression probes for Deltex1 (DTX1) (Hs00269995_m1, Life Technologies) and the housekeeping gene PP1A (Hs99999904_m1, Life Technologies). TaqMan gene expression assays were run on the Applied Biosystems ABI Prism 7900HT Fast Real-Time PCR system. To quantitate the levels of Deltex 1, 2–[delta][delta]Ct method was employed. Calculation of delta delta Ct involves comparing the Ct values of the samples of interest with a control such as a non-treated sample or DMSO treated sample (Schmittgen and Livak (2008) Nature Protocols 3: 1101-1108).

Summary and discussion

As shown in Figures 5A-B, Notch3 antibodies that were identified from pannings against NRR domain or EGF32-NRR domain (Ab-B, Ab-C, Ab-A) potently inhibited Deltex1 mRNA

expression in TALL-1 cells. In contrast antibodies directed to the LBD domain (Ab-D) did not significantly inhibit Deltex1 mRNA. In addition, Ab-B, Ab-C, Ab-A significantly inhibited TALL-1 proliferation in a dose-dependent manner. When Notch3 antibodies were tested in a panel of other TALL cell lines (DND41, P12-Ichikawa, SUPT1, SUPT11 and RPMI-8402), no effects on proliferation were detected.

Example 8: Generation of a neo-epitope antibody that detects the gamma secretase cleaved form of the Notch3 intracellular domain.

Notch signaling is activated by a series of proteolytic cleavages. The gamma secretase complex mediates the final cleavage of the Notch receptor ultimately releasing the Notch intracellular domain (ICD) that translocates to the nucleus to activate Notch target gene transcription. A neo-epitope antibody was generated to detect the gamma secretase cleaved form of the Notch3 ICD (ICD3) only when cleaved between amino acids Gly1661 and Val1662 (human Notch3).

Generation of a ICD3 rabbit polyclonal antibody

The peptides used for immunization and negative selection (depletion peptide) are indicated.

Immunization peptide: H_2N-VMVARRK(dPEG4)C-amide (SEQ ID NO: 35)

Depletion peptide: Ac-VILVLGVMVARRK(dPEG4)C-amide (SEQ ID NO: 36). A rabbit polyclonal antibody was generated at Covance using standard procedures. Briefly, a 77 day protocol was employed with a primary boost with 500µg of immunizing peptide and Freund's adjuvant. Additional boosts with 500µg of immunizing peptide were performed on day 21, 42 and 63. To deplete non-specific antibodies that recognize the VMVARRK (SEQ ID NO: 3) sequence of Notch3, but not the neo-epitope following gamma secretase cleavage, a depletion peptide was used for negative selection. The purified sample was depleted using the depletion peptide by "negative" affinity chromatography. Peptides were coupled to a column using terminal cysteine to properly orient the peptide. Cross reacting antibodies were removed from the sample and confirmed by ELISA. Serum from rabbit was tested by Western blot in TALL-1 cells to determine if a specific band was detected.

Conversion of the rabbit polyclonal ICD3 antibody to a rabbit monoclonal antibody

To convert the rabbit polyclonal antibody to a rabbit monoclonal antibody shown below, a final IV boost of immunizing peptide was performed on the selected rabbit. 4 days later a

splenectomy was performed and rabbit hybridomas were generated by standard procedures at Epitomics. Briefly all the lymphocytes from 1 rabbit spleen were isolated. Fusion and standard ELISA screen of 40 x 96 well plates was performed. All ELISA positive hybridomas were expanded to 24 well plates and an ELISA was again performed with both the immunizing peptide and the depletion peptide. Supernatant from the 139 positive hybridomas were analyzed by Western blotting in TALL-1 cells. Based on Western screening of the ELISA positive hybridomas, 3 hybridomas (73, 128, 95) were chosen for subcloning. To subclone hybridomas, a limited dilution of the selected parental hybridomas (0.5 cells/well) was perfomed and these sub-clones were plated in 4 x 96 well plates. Subclones were again screened by ELISA using both the immunizing peptide and the depletion peptide. Clones were expanded to 24 well plates and supernatants from ELISA positive sub-clones were screened by Western blotting in TALL-1 cells. Exemplary Western data from 3 sub-clones are shown (Figure 6A). The sequence of the rabbit polyclonal antibody was determinded using standard techniques and is shown in Table 3.

Table 3: Sequence of ICD3 antibody

ICD3 Ab		
SEQ ID NO: 37 (Kabat)	HCDR1	KNAYMC
SEQ ID NO: 38 (Kabat)	HCDR2	CIETGDGTTYYASWAKG
SEQ ID NO: 39 (Kabat)	HCDR3	ELYDDYGDYFNL
SEQ ID NO: 40 (Chothia)	HCDR1	GFSFTKNA
SEQ ID NO: 41 (Chothia)	HCDR2	ETGDGT
SEQ ID NO: 42 (Chothia)	HCDR3	ELYDDYGDYFNL
SEQ ID NO: 43	VH	QSLEESGGDLVKPGASLTLTCTASGFSFTKNAYMCWDRQAPGKRPEWIACIETGDGTTYYAS WAKGRFTVSKTSSTTVTLQMTSLTAADTATYFCARELYDDYGDYFNLWGPGTLVTVSS
SEQ ID NO: 44	DNA VH	CAGTCGTTGGAGGAGTCTGGGGGAGACCTGGTCAAGCCTGGGGCATCCCTGACACTCAC CTGCACAGCCTCTGGATTCTCCTTCACTAAGAACGCCTACATGTGCTGGGACCGCCAGGC TCCAGGGAAGAGGCCTGAGTGGATCGCATGCATTGAGACTGGTGACGGCACCACATATT ATGCGAGCTGGGCGAAAGGCCGATTCACCGTCTCCAAAACCTCGTCGACCACGGTGACT CTGCAAATGACCAGTCTGACAGCCGCGGACACGGCCACCTATTTCTGTGCGAGGGAATT ATACGATGACTATGGTGATTACTTCAATTTGTGGGGCCCAGGCACCCTGGTCACCGTCTC CTCA
SEQ ID NO: 45	Heavy Chain	QSLEESGGDLVKPGASLTLTCTASGFSFTKNAYMCWDRQAPGKRPEWIACIETGDGTTYYAS WAKGRFTVSKTSSTTVTLQMTSLTAADTATYFCARELYDDYGDYFNLWGPGTLVTVSSGQP KAPSVFPLAPCCGDTPSSTVTLGCLVKGYLPEPVTVTWNSGTLTNGVRTFPSVRQSSGLYSLSS VVSVTSSSQPVTCNVAHPATNTKVDKTVAPSTCSKPTCPPPELLGGPSVFIFPPKPKDTLMISR TPEVTCVVVDVSQDDPEVQFTWYINNEQVRTARPPLREQQFNSTIRVVSTLPIAHQDWLRG KEFKCKVHNKALPAPIEKTISKARGQPLEPKVYTMGPPREELSSRSVSLTCMINGFYPSDISVE WEKNGKAEDNYKTTPAVLDSDGSYFLYSKLSVPTSEWQRGDVFTCSVMHEALHNHYTQKSI SRSPGK

SEQ ID NO: 46	DNA Heavy Chain	CAGTCGTTGGAGGAGTCTGGGGGAGACCTGGTCAAGCCTGGGGCATCCCTGACACTCAC CTGCACAGCCTCTGGATTCTCCTTCACTAAGAACGCCTACATGTGCTGGGACCGCCAGGC TCCAGGGAAGAGGCCTGAGTGGATCGCATGCATTGAGACTGGTGACGGCACCACATATT ATGCGAGCTGGGCGAAAGGCCGATTCACCGTCTCCAAAACCTCGTCGACCACGGTGACT CTGCAAATGACCAGTCTGACAGCCGCGGACACGGCCACCTATTTCTGTGCGAGGGAATT ATACGATGACTATGGTGATTACTTCAATTTGTGGGGCCCAGGCACCCTGGTCACCGTCTC CTCAGGGCAACCTAAGGCTCCATCAGTCTTCCCACTGGCCCCCTGCTGCGGGGACACACC CAGCTCCACGGTGACCCTGGGCTGCCTGGTCAAAGGGTACCTCCCGGAGCCAGTGACCG TGACCTGGAACTCGGGCACCCTCACCAATGGGGTACGCACCTTCCCGTCCGTCCGGCAGT CCTCAGGCCTCTACTCGCTGAGCAGCGTGGTGAGCGTGACCTCAAGCAGCCAGCCCGTC ACCTGCAACGTGGCCCACCCAGCCACCAACACCAAAGTGGACAAGACCGTTGCGCCCTC GACATGCAGCAAGCCCACGTGCCCACCCCCTGAACTCCTGGGGGGGACCGTCTGTCTTCAT CTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCACGCACCCCCGAGGTCACATGCGT GGTGGTGGACGTGAGCCAGGATGACCCCGAGGTGCAGTTCACATGGTACATAAACAAC GAGCAGGTGCGCACCGCCCGGCCGCCGCTACGGGAGCAGCAGTTCAACAGCACGATCC GCGTGGTCAGCACCCTCCCCATCGCGCACCAGGACTGGCTGAGGGGCAAGGAGTTCAA GTGCAAAGTCCACAACAAGGCACTCCCGGCCCCCATCGAGAAAACCATCTCCAAAGCCA GAGGGCAGCCCCTGGAGCCGAAGGTCTACACCATGGGCCCTCCCCGGGAGGAGCTGAG CAGCAGGTCGGTCAGCCTGACCTGCATGATCAACGGCTTCTACCCTTCCGACATCTCGGT GGAGTGGGAGAAGAACGGGAAGGCAGAGGACAACTACAAGACCACGCCGGCCGTGCT GGACAGCGACGGCTCCTACTTCCTCTACAGCAAGCTCTCAGTGCCCACGAGTGAGTGGC AGCGGGGCGACGTCTTCACCTGCTCCGTGATGCACGAGGCCTTGCACAACCACTACACG CAGAAGTCCATCTCCCGCTCTCCGGGTAAA
SEQ ID NO: 47 (Kabat)	LCDR1	QTSENFYSNDILS
SEQ ID NO: 48 (Kabat)	LCDR2	EASTLAS
SEQ ID NO: 49 (Kabat)	LCDR3	QGSVLDSGWYDIS
SEQ ID NO: 50 (Chothia)	LCDR1	SENFYSNDI
SEQ ID NO: 51 (Chothia)	LCDR2	EAS
SEQ ID NO: 52 (Chothia)	LCDR3	SVLDSGWYDI
SEQ ID NO: 53	VL	ALVMTQTPSSVSAAVGGTVTINCQTSENFYSNDILSWYQQKPGQPPKLLIYEASTLASGVPSR FKGSGSGTQFTLTISDVQCDDAATYYCQGSVLDSGWYDISFGGGTEVVVK
SEQ ID NO: 54	DNA VL	GCCCTTGTGATGACCCAGACTCCATCGTCCGTGTCTGCAGCTGTGGGAGGCACAGTCACC ATCAATTGCCAGACCAGTGAGAATTTTTATAGTAACGACATCTTATCCTGGTATCAGCAG AAGCCAGGGCAGCCTCCCAAGCTCCTGATCTATGAAGCATCCACTCTGGCATCTGGGGTC CCCTCGCGATTCAAAGGCAGTGGATCTGGGACACAGTTCACTCTCACCATCAGCGACGTG CAGTGTGACGATGCTGCCACTTACTATTGTCAAGGCAGTGTTCTTGATAGTGGTTGGTAC GATATTTCTTTCGGCGGAGGGACCGAGGTGGTGGTCAAA
SEQ ID NO: 55	Light Chain	ALVMTQTPSSVSAAVGGTVTINCQTSENFYSNDILSWYQQKPGQPPKLLIYEASTLASGVPSR FKGSGSGTQFTLTISDVQCDDAATYYCQGSVLDSGWYDISFGGGTEVVVKGDPVAPTVLIFP PAADQVATGTVTIVCVANKYFPDVTVTWEVDGTTQTTGIENSKTPQNSADCTYNLSSTLTLT STQYNSHKEYTCKVTQGTTSVVQSFNRGDC
SEQ ID NO: 56	DNA Light Chain	GCCCTTGTGATGACCCAGACTCCATCGTCCGTGTCTGCAGCTGTGGGAGGCACAGTCACC ATCAATTGCCAGACCAGTGAGAATTTTTATAGTAACGACATCTTATCCTGGTATCAGCAG AAGCCAGGGCAGCCTCCCAAGCTCCTGATCTATGAAGCATCCACTCTGGCATCTGGGGTC CCCTCGCGATTCAAAGGCAGTGGATCTGGGACACAGTTCACTCTCACCATCAGCGACGTG CAGTGTGACGATGCTGCCACTTACTATTGTCAAGGCAGTGTTCTTGATAGTGGTTGGTAC GATATTTCTTTCGGCGGAGGGACCGAGGTGGTGGTCAAAGGTGATCCAGTTGCACCTAC TGTCCTCATCTTCCCACCAGCTGCTGATCAGGTGGCAACTGGAACAGTCACCATCGTGTG TGTGGCGAATAAATACTTTCCCGATGTCACTGTCACCTGGGAGGTGGATGGCACCACCCA AACAACTGGCATCGAGAACAGTAAAACACCGCAGAATTCTGCAGATTGTACCTACAACCT CAGCAGCACTCTGACACTGACCAGCACACAGTACAACAGCCACAAAGAGTACACCTGCA AGGTGACCCAGGGCACGACCTCAGTCGTCCAGAGCTTCAATAGGGGTGACTGT

In vitro screening of Notch3 signaling inhibition using an ICD3 antibody

An antibody targeting the Notch3 ICD was used to assess pathway activity. Cell line TALL-1 was purchased from DSMZ and routinely maintained in growth media supplemented with 10% FBS and 1% Penicillin-Streptomycin. Experimental set up: 5 million TALL-1 cells were plated in 10 mL of medium in a 25 cm^2 tissue culture flask (Corning, cat#430639). Cells were treated with either 0.5% DMSO or 10μM DAPT (Calbiochem, cat#565770) for 72h. TALL-1 cells were spun down and then washed in PBS. Cells were lysed in 60 μL of 1X Cell Lysis Buffer (CST, cat#9803) with the addition of N-ehtylmaleimide (Thermo Scientific, cat# 23030) and protease and phosphatase inhibitors (Pierce, Cat# 78444). Protein quantitation was performed using the BCA method and read in a Spectramax M5 microplate reader. 30 μL of protein samples were loaded per well in a 4-12% Bis-Tris gel (Invitrogen, cat# NP0006-1).

SDS-PAGE: Samples were run under standard conditions in 1X NuPage MOPS SDS running buffer (Invitrogen, cat# NP0001) for approximately 90 min at 180 V. Before transfer to a nitrocellulose membrane (iBlot, Invitrogen), gels were soaked in 2x Transfer Buffer (Invitrogen, cat#NP0006-1) with 20% methanol. Membranes were blocked in 4% milk-TBST for one hour; supernatants from hybidoma supernatants were diluted 1:4 in 2% milk-TBST and incubated ON at 4°C with gentle shaking. Secondary antibodies were added in 2% milk-TBST for 45 minutes, after a series of membrane washes with TBST. Membrane was developed using ECL Plus Western Detection System (GE healthcare, cat# RPN2232).

Screening of a panel of T-cell acute lymphoblastic leukemia cells lines with an ICD3 antibody.

Cell lines TALL-1 (# ACC521), RPMI8402 (#ACC290), DND41 (#ACC525), SUPT11 (#ACC605), and P12-Ichikawa (#ACC34) were purchased from DSMZ and routinely maintained in growth media supplemented with 10% FBS and 1% Penicillin-Streptomycin. Cell lines HPB-ALL and Jurkat cells were obtained commercially from Andreas Strasser (Walter and Eliza Hall Institute of Medical Research, Australia. 5 million TALL-1 cells were plated in 10 mL of medium in a 25 cm^2 tissue culture flask (Corning, cat#430639). Cells were spun down and then washed in PBS. Cells were lysed and Western performed as described above. Purified antibody from hybridoma sub-clone 73-8 was used for further

studies at a 1:5000 dilution. ICD1 protein levels were assessed using an antibody from Cell Signaling (#2421) at a dilution of 1:1000.

Summary and Discussion

As shown in Figures 6A-B, high levels of ICD3 protein was only detected in TALL-1 cells but not in a panel of other T-cell acute lymphoblastic leukemia cell lines. High ICD1 levels can be detected in several TALL lines including HPBALL, RPMI-8402, DND41, P12 Ichikawaand Jurkat, which are known to have activating mutations in Notch1 (Weng *et al.* (2004) Science 306:269-71). The ICD3 antibody does not cross-react with ICD1 as evidenced by lack of signal in these other TALL lines with Notch1 mutations.

Example 9: In vitro assessment of Notch3 signaling inhibition upon antibody treatment

Evaluation of Notch3 mutation status in the panel of CCLE lines resulted in identification of TALL-1 with an NRR mutation and MDA-MB468 with a PEST domain mutation. MDA-MB468 cells have a frameshift mutation at amino acid 2034 which results in introduction of a premature stop codon. Therefore the ICD3 has an altered molecular weight which can be detected as a faster migrating band on a Western blot.

Sequences of portions of WT and MDAMB468 PEST domain

Constructs:

WT Notch3 sequence (NP_000426) amino acid 2034-end

PSGPRSPPGPHGLGPLLCPPGAFLPGLKAAQSGSKKSRRPPGKAGLGPQGPRGRGKKL TLACPGPLADSSVTLSPVDSLDSPRPFGGPPASPGGFPLEGPYAAATATAVSLAQLGGP GRAGLGRQPPGGCVLSLGLLNPVAVPLDWARLPPPAPPGPSFLLPLAPGPQLLNPGTP VSPQERPPPYLAVPGHGEEYPAAGAHSSPPKARFLRVPSEHPYLTPSPESPEHWASPSP PSLSDWSESTPSPATATGAMATTTGALPAQPLPLSVPSSLAQAQTQLGPQPEVTPKRQ VLA (SEQ ID NO: 57)

MDA-MB468 sequence amino acid 2034-end

PSGPRSPPRSPRPGASALSSRGLPPWPQSGTVGVQEEQEAPREGGAGAAGAPGAGQE ADAGLPGPPG. (SEQ ID NO: 58)

Initially these 2 cell line models were used to characterize the effects of Notch3 inhibitory antibodies on Notch3 signaling. Western blots with the ICD3 antibody were used to monitor

signaling inhibition. Experimental set up: one million MDA-MB468 cells were plated in a 60 mm dish (Corning, cat# 430196) in 3 mL of medium or 5 million TALL-1 cells in 10 mL of medium in a 25cm^2 tissue culture flask (Corning, cat#430639). Plates were incubated overnight at 37°C prior to treatment with 10 μg/mL final concentrations of Notch3 inhibitor antibodies Ab-A, Ab-B, Ab-C and Ab-E as well as an IgG control. Antibodies were added directly to the plate and they were further incubated for 72h at 37°C, 5% CO_2. In addition some cells were treated with either 0.5% DMSO or 10μM DAPT (Calbiochem, cat#565770) for 72h. Cells were harvested by aspirating the media and rinsing in 1 mL PBS (Gibco, cat#20012-027), scraping the cells off the plate, and spinning down on a bench top centrifuge. Suspension cells were spun down and then washed in PBS. Western blots were performed with the purified ICD3 antibody as described previously.

In addition, three other cells lines were characterized for ICD3 levels and signaling inhibition upon Notch3 antibody treatment:- (i) Ishikawaheraklio02_ER has a NRR mutation at N1597R, (ii) A549 has a PEST frameshift mutation at 2034, while (iii) TE-11 has a PEST frameshift mutation at 2260.

Summary and discussion

As shown in Figures 7 and 8, in addition to the previously described ligand-driven RGA and Notch target gene mRNA quantitation, Notch3 signaling can also be monitored by measuring levels of ICD3. ICD3 levels are a membrane proximal readout of Notch3 signaling activity. Treatment of TALL-1 cells with Notch3 antibodies Ab-A, Ab-B, Ab-C resulted in decreased levels of ICD3 (Figure 7A). Level of ICD3 was equivalent in the IgG control sample and the DMSO samples. This data is consistent with inhibition of Deltex1 mRNA and TALL-1 proliferation upon treatment with these antibodies. In contrast no effect on ICD3 levels was detected with Ab-F treatment. As shown in Figure7B, in MDA-MB468 cells, the frame-shift mutation at amino acid 2034 results in a premature stop codon and smaller ICD3. This ICD3 can be detected as a faster migrating band on a Western blot. Upon treatment with Notch3 NRR antibodies Ab-A, Ab-B, Ab-C, decreased levels of ICD3 were detected. In contrast, treatment with Ab-E, a LBD antibody, did not alter ICD3 levels relative to a control IgG. As shown in Fig 8 A-C, varying effects on ICD3 levels were detected upon Notch3 antibody treatment in Ishikawaheraklio02_ER, TE-11, and A549 cells. However in all cell lines tested, Ab-B treatment consistently results in significantly decreased ICD3 levels.

Example 10: In vitro assessment of Notch3 signaling inhibition upon antibody treatment in a Notch3 amplified cell line

HCC1143 cells were described to have an amplification of Notch3 (Yamaguchi *et al.* (2008) Cancer Res. 68:1881). The levels of active Notch3 signaling were examined in this cell line using the ICD3 antibody. Western blots with the ICD3 antibody were used to monitor signaling inhibition.

Experimental set up: one million HCC1143 cells were plated in a 60 mm dish (Corning, cat# 430196) in 3 mL of medium in a 25cm^2 tissue culture flask (Corning, cat#430639). Plates were incubated overnight at 37°C prior to treatment with 10 μg/mL final concentrations of Notch3 inhibitor antibodies Ab-A, Ab-B, Ab-C and Ab-F as well as an IgG control. Antibodies were added directly to the plate and they were further incubated for 72h at 37°C, 5% CO_2. Cells were harvested and Western blots performed as described previously.

Summary and discussion

As shown in Figure 9, HCC1143 cells are amplified for Notch3 and exhibit high levels of ICD3. All Notch3 antibody treatments resulted in decreased ICD3 levels. At 10μg/ml, Ab-B treatment resulted in the largest reduction of ICD3 levels.

Example 11: In vivo PD Assessment

PD modulation was interrogated in three xenograft models harboring genetic abberations in Notch3: the NRR mutant TALL-1 human leukemia model, the PEST mutant MDA-MB-468 human breast model, and the Notch3-amplified HLUX1823 patient-derived lung model.

In vivo PD in the TALL-1 human leukemia xenograft model

Female SCID-beige mice harboring TALL-1 xenografts were treated with a single dose of Notch3 antibodies. Mice were inoculated with 10 x10^6 cells injected subcutaneously in a suspension of Hank's balanced salt solution. Once tumors reached between 300 and 500 mm^3 (n=3/group), mice were randomly assigned to receive a single intravenous 20 mg/kg dose of 3207 (IgG control), Ab-B or Ab-C. Following treatment, tumors were harvested at selected time points and ICD3 was evaluated by Western blot and IHC, as described below.

In vivo PD in the MDA-MB-468 human breast cancer xenograft model

Female SCID-beige mice harboring MDA-MB-468 xenografts were treated with a single dose of Notch3 antibodies. A 3 x 3 x 3 mm^3 tumor fragment was passaged from a MDA-MB-468 tumor bearing mouse (donor) and implanted subcutaneously into SCID-beige recipient mice on both the left and right flank. Once tumors reached between 300 and 500 mm^3 (n=3/group), mice were randomly assigned to an untreated control group or received a single intravenous 20 mg/kg dose of Ab-B. In additional studies, the effects of a single intravenous 20 mg/kg dose of Ab-B, Ab-C, Ab-A and Ab-E relative to PBS or 3207 non-targeting IgG controls was assessed. Following the various treatments, tumors were harvested and ICD3 was evaluated by Western blot, as described below.

In vivo PD in the HLUX1823 patient derived lung cancer xenograft model

The activity of anti-Notch3 antibodies was also evaluated in a Notch3-amplified patient-derived primary lung cancer tumor xenograft model, HLUX-1823. In these studies, nu/nu mice were implanted subcutaneously with 3 x 3 x 3 mm^3 tumor fragments containing 50% phenol-red free matrigel (BD Biosciences) in DMEM and reached approximately 250 mm^3 at 30 days post-implantation. Once tumors reached between 300 and 500 mm^3 (n=3/group), mice were randomly assigned to receive either PBS or a single 20 mg/kg intravenous dose of either the 3207 non-targeting control antibody, or Ab-C or Ab-F (the parental antibody from which Ab-D and Ab-E were derived). Following the various treatments, tumors were harvested and ICD3 was evaluated by Western blot, as described below.

Preparation of tumor cell lysates and ICD3 Western

Tumor samples were lysed in 200-400 µL of T-PER Tissue Protein Extraction Reagent (Pierce, cat# 78510) with Complete mini EDTA free protease inhibitor cocktail tablets (Roche, Cat# 04693159001), using a Tissue Lyser II (Qiagen) for 1 min at 30 Hz. One 5 mm stainless steel bead (Qiagen, cat# 69965) was placed per tube to help with tissue lysis. After bead removal, samples were then centrifuged on a bench-top centrifuge at top speed for 15 min at 4°C. Supernatants were collected and either stored at -80°C for studies at a later time or protein concentration as assessed using the BCA method (Pierce, cat# 232550) and a Western blot for ICD3 was run as described previously. Where applicable, Western was also performed with a full-length Notch3 antibody to detect total levels of Notch3 (Cell Signaling #2889, 1:1000 dilution).

Detection of ICD3 levels by IHC

Xenograft tumors were fixed in 10% formalin and embedded in paraffin. 5μm sections were placed on charged polylysine-coated slides. Immunohistochemistry protocol was optimized on an automated system Discovery ULTRA (Ventana Medical System).

Sections were baked at 60°C for 8 minutes, followed by deparaffination. Antigen retrieval was achieved in Cell Conditioning 1 (CC1, a TRIS based buffer with a slightly basic pH) at high temperature for 76 minutes. Blocking of non-specific binding of antibody was carried on using a specific Antibody Blocking (cat#760-4204). Primary antibody Notch3 ICD (20μg/ml) was incubated at 37°C for 60 minutes followed by incubation in secondary antibody for 32 minutes. Amplification step was performed using a specific Discovery Amplification HQ kit #760-052 (Ventana Medical Systems) as per manufacture specifications. Detection was achieved with diaminobenzydine (DAB) and counterstain with Hematoxylin. All these steps were run on Ventana Discovery ULTRA (Ventana Medical Systems).

Summary and Discussion

Figures 10-12 show in vivo PD studies in several xenograft models. As described earlier in this application, in vitro treatment of TALL-1 cells with Notch3 antibodies resulted in inhibition of signaling as assessed by both Deltex1 mRNA levels and ICD3 protein. TALL-1 cells were grown as a xenograft and mice were treated with Notch3 antibodies. Changes in Notch3 signaling in TALL-1 tumors was monitored by assessing ICD3 levels by Western blotting or IHC. Treatment with antibodies Ab-B or Ab-C resulted in decreased levels of ICD3 as shown in Figures 10A-B. ICD3 staining by IHC is indicated by the black/dark grey cells in the tumor section as shown in Figure 10B. ICD3 levels in tumors were assessed 72 h following the last Notch antibody administration, and there were still some cells within the tumor that showed strong ICD3 expression. In the MDA-MB468 model, as assessed by Western blotting, animals treated with Ab-B yielded a marked decrease in ICD3 24 h and 72 h post dose relative to untreated control mice (Figure 11A). It was found that, at the 72 h timepoint, Ab-B, Ab-C and Ab-A, all of which target the Notch3 NRR, induced decreases in ICD3 levels relative to the PBS and 3207 (IgG) controls. In contrast, following treatment with Ab-E, which targets a region of Notch3 outside of the NRR, ICD3 levels appeared similar to control levels (Figure 11B). In the HLUX1823 Notch3-gene amplified model, as assessed by Western blotting, animals treated with either Ab-C or Ab-F yielded a marked decrease in ICD3 at 72 h post dose relative to control mice (Figure 12). Taken together, these data demonstrate that the Notch3 NRR antibodies can inhibit Notch3 signaling in the presence of Notch3 gene-amplification or mutations in either the NRR or PEST domains, whereas Notch3

antibodies raised outside of this region can only inhibit Notch3 signaling in the presence of the gene-amplification and have more limited activity in the presence of mutations.

Example 12: In vivo efficacy in TALL-1 xenografts

Generation of a TALL-1 cell line with constitutive expression of luciferase

The TALL-1 cell line was transduced with pMMP-LucNeo retrovirus (see US 7399851) and selected in 1mg/mL of Geneticin (G418) for several weeks. TALL-1_Luc cells express high levels of luciferase compared to TALL-1 cells, where it was absent. Wild-type and luciferased cells were subjected to a proliferation experiment with Notch3 antibody inhibitors, showing identical results; suggesting that the infection did not interfere with TALL-1 sensitivityto Notch3 inhibition.

Assessment of *in vivo* activity of Notch3 antibodies in a TALL-1 cell-line xenograft model

Mice were inoculated with 10 x10^6 T-ALL1_Luc cells injected subcutaneously in a suspension of Hank's balanced salt solution and the presence of tumors was monitored using the Xenogen *in vivo* imaging system (Caliper Life Sciences). The presence of tumors was detectable by day 7. On day 11, tumor-bearing animals were randomly assigned to receive intravenous doses of either PBS or 20 mg/kg of 3207 negative control IgG antibody or the Notch3 antibodies Ab-A, Ab-B, Ab-C or Ab-E as single agents twice per week. Tumor size was monitored using the Xenogen in vivo imaging system.

Summary and Discussion

As shown in Figure 13, Ab-C and Ab-B showed the most anti-tumor activityof the antibodies evaluated in this study. Figure 13A shows a graphical representation of the luminescent signal obtained following the various treatments over the time-course of the study and Figure 13B shows the luminescent signal of the control groups at day 29 (the last time point that it was possible to image due to tumor size) and of the anti-Notch3 antibody treatment groups at day 43.

Example 13: Epitope binning of Notch 3 antibodies with Biacore

Epitope binning via Biacore was performed to classify Notch 3 antibodies (IgG or Fab fragments) into groups of identical, or significantly overlapping epitopes, i.e. antibodies which were able to inhibit each other's binding.

Experimental set-up epitope binning with Biacore

For epitope binning in Biacore, a sensor chip with a low density of immobilized or captured antigen was used (comparable to a kinetic experiment). The same sample prerequisites as for KD determination applied (i.e. monomer content). Experimental conditions, concerning preparation of chip (antigen immobilization/capture), as well as regeneration conditions were identical to KD determination in Biacore. To achieve saturation of an epitope, only one (high) concentration per antibody was used (e.g. 250nM for 90 s).

Antibody samples were injected pair wise in a full factorial assay design, e.g. for two antibody samples, A and B, the following pair wise injections were required: A-A, A-B, B-A, B-B.

The sensor chip had to be saturated with antibody by the first injection, so that the second antibody was only able to bind in case of a different epitope. Complete regeneration of bound antibodies had to be performed after each double injection.

For evaluation of the controls, i.e. double injections of the identical antibodies (A-A, B-B), their binding levels at the end of each injection were evaluated: The second injection was expected to give no additional binding. Double injections of different antibody sample pairs were compared for consistency, e.g. if the injection A-B resulted in additional binding of B (different epitopes) the injection of B-A was expected to result in additional binding of A, too. Possible causes for creating such inconsistencies were e.g. partially overlapping epitopes, or large differences in KD.

Summary and Discussion:

Anti Notch 3 antibodies identified from phage display screening have different conformational epitopes.

Example 14: Co-crystal structure studies with Ab-B and NRR as well as Ab-C and NRR

Two crystal structures of human Notch3 Negative Regulatory Region (NRR, SEQ ID NO: 282) bound to Fab fragment of Ab-B or Ab-C were determined. As detailed below, Notch3 NRR was expressed, purified and mixed with Ab-B or Ab-C Fab to form complex. Protein crystallography was employed to generate atomic resolution data for Notch3 NRR bound to Ab-B or Ab-C Fab, respectively, to define their epitopes (as Notch3 NRR residues within 5Å distance to the antibody residues).

Protein production

The sequences of Notch3 NRR, Ab-B Fab, and Ab-C Fab produced for crystallography are shown below. Construct of Notch3 NRR comprises residues 1378 to 1640 (underlined) of human Notch3 (UniProt identifier Q9UM47, SEQ ID NO: 1), along with N- and C-terminal residues from recombinant expression vector (shown in lower case letters, SEQ ID NO: 59). The N-terminal signal sequence from mouse IgG kappa light chain was used for secreted expression and was cleaved during expression, leaving intact N-terminus of Notch3 NRR. Proteins used for crystal structure determination

Construct:

Human Notch3 NRR (Q9UM47)

MGPGARGRRRRRRPMSPPPPPPPVRALPLLLLLAGPGAAAPPCLDGSPCANGGRCTQ
LPSREAACLCPPGWVGERCQLEDPCHSGPCAGRGVCQSSVVAGTARFSCRCPRGFRG
PDCSLPDPCLSSPCAHGARCSVGPDGRFLCSCPPGYQGRSCRSDVDECRVGEPCRHGG
TCLNTPGSFRCQCPAGYTGPLCENPAVPCAPSPCRNGGTCRQSGDLTYDCACLPGFEG
QNCEVNVDDCPGHRCLNGGTCVDGVNTYNCQCPPEWTGQFCTEDVDECQLQPNAC
HNGGTCFNTLGGHSCVCVNGWTGESCSQNIDDCATAVCFHGATCHDRVASFYCACP
MGKTGLLCHLDDACVSNPCHEDAICDTNPVNGRAICTCPPGFTGGACDQDVDECSIG
ANPCEHLGRCVNTQGSFLCQCGRGYTGPRCETDVNECLSGPCRNQATCLDRIGQFTCI
CMAGFTGTYCEVDIDECQSSPCVNGGVCKDRVNGFSCTCPSGFSGSTCQLDVDECAS
TPCRNGAKCVDQPDGYECRCAEGFEGTLCDRNVDDCSPDPCHHGRCVDGIASFSCAC
APGYTGTRCESQVDECRSQPCRHGGKCLDLVDKYLCRCPSGTTGVNCEVNIDDCASN
PCTFGVCRDGINRYDCVCQPGFTGPLCNVEINECASSPCGEGGSCVDGENGFRCLCPP
GSLPPLCLPPSHPCAHEPCSHGICYDAPGGFRCVCEPGWSGPRCSQSLARDACESQPC
RAGGTCSSDGMGFHCTCPPGVQGRQCELLSPCTPNPCEHGGRCESAPGQLPVCSCPQ
GWQGPRCQQDVDECAGPAPCGPHGICTNLAGSFSCTCHGGYTGPSCDQDINDCDPNP
CLNGGSCQDGVGSFSCSCLPGFAGPRCARDVDECLSNPCGPGTCTDHVASFTCTCPPG
YGGFHCEQDLPDCSPSSCFNGGTCVDGVNSFSCLCRPGYTGAHCQHEADPCLSRPCL
HGGVCSAAHPGFRCTCLESFTGPQCQTLVDWCSRQPCQNGGRCVQTGAYCLCPPGW
SGRLCDIRSLPCREAAAQIGVRLEQLCQAGGQCVDEDSSHYCVCPEGRTGSHCEQEV
DPCLAQPCQHGGTCRGYMGGYMCECLPGYNGDNCEDDVDECASQPCQHGGSCIDL
VARYLCSCPPGTLGVLCEINEDDCGPGPPLDSGPRCLHNGTCVDLVGGFRCTCPPGYT
GLRCEADINECRSGACHAAHTRDCLQDPGGGFRCLCHAGFSGPRCQTVLSPCESQPC

QHGGQCRPSPGPGGGLTFTCHCAQPFWGPRCERVARSCRELQCPVGVPCQQTPRGPR
CACPPGLSGPSCRSFPGSPPGASNASCAAAPCLHGGSCRPAPLAPFFRCACAQGWTGP
RCEAPAAAPEVSEEPRCPRAACQAKRGDQRCDRECNSPGCGWDGGDCSLSVGDPWR
QCEALQCWRLFNNSRCDPACSSPACLYDNFDCHAGGRERTCNPVYEKYCADHFADG
RCDQGCNTEECGWDGLDCASEVPALLARGVLVLTVLLPPEELLRSSADFLQRLSAILR
TSLRFRLDAHGQAMVFPYHRPSPGSEPRARRELAPEVIGSVVMLEIDNRLCLQSPEND
HCFPDAQSAADYLGALSAVERLDFPYPLRDVRGEPLEPPEPSVPLLPLLVAGAVLLLV
ILVLGVMVARRKREHSTLWFPEGFSLHKDVASGHKGRREPVGQDALGMKNMAKGE
SLMGEVATDWMDTECPEAKRLKVEEPGMGAEEAVDCRQWTQHHLVAADIRVAPA
MALTPPQGDADADGMDVNVRGPDGFTPLMLASFCGGALEPMPTEEDEADDTSASIIS
DLICQGAQLGARTDRTGETALHLAARYARADAAKRLLDAGADTNAQDHSGRTPLHT
AVTADAQGVFQILIRNRSTDLDARMADGSTALILAARLAVEGMVEELIASHADVNAV
DELGKSALHWAAAVNNVEATLALLKNGANKDMQDSKEETPLFLAAREGSYEAAKL
LLDHFANREITDHLDRLPRDVAQERLHQDIVRLLDQPSGPRSPPGPHGLGPLLCPPGAF
LPGLKAAQSGSKKSRRPPGKAGLGPQGPRGRGKKLTLACPGPLADSSVTLSPVDSLDS
PRPFGGPPASPGGFPLEGPYAAATATAVSLAQLGGPGRAGLGRQPPGGCVLSLGLLNP
VAVPLDWARLPPPAPPGPSFLLPLAPGPQLLNPGTPVSPQERPPPYLAVPGHGEEYPA
AGAHSSPPKARFLRVPSEHPYLTPSPESPEHWASPSPPSLSDWSESTPSPATATGAMAT
TTGALPAQPLPLSVPSSLAQAQTQLGPQPEVTPKRQVLA (SEQ ID NO: 1)

Notch3 NRR

metdtlllwvlllwvpgstgAPEVSEEPRCPRAACQAKRGDQRCDRECNSPGCGWDGGDCSLS
VGDPWRQCEALQCWRLFNNSRCDPACSSPACLYDNFDCHAGGRERTCNPVYEKYC
ADHFADGRCDQGCNTEECGWDGLDCASEVPALLARGVLVLTVLLPPEELLRSSADFL
QRLSAILRTSLRFRLDAHGQAMVFPYHRPSPGSEPRARRELAPEVIGSVVMLEIDNRL
CLQSPENDHCFPDAQSAADYLGALSAVERLDFPYPLRDVRGEPLEPPEPSgshhhhhh
(SEQ ID NO: 59)

Production of Notch 3 NRR

Notch3 NRR wasexpressed as a secreted protein in HEK293S GnTI- cells (ATCC). 1mg of Notch3 NRR construct DNA was diluted into 50ml of OptiMEM I medium (Life Technologies), and incubated with 2.5mg of PEI (Polysciences) in 50ml of the same medium for 30min. The mixture was then added into 1L of HEK293S GnTI- cells growing in suspension in FreeStyle™ 293 Expression medium (Life Technoligies) at 1 million cells/ml at 37 °C with 8% of CO_2 for transfection. After 72 hours, the medium which contains Notch3

NRR was harvested by centrifugation. 3ml of Ni-NTA Superflow resin (Qiagen) was added into the medium and continuously stirred at 4 °C overnight. The next day the resin was packed into a gravity column and washed with 50mM Hepes pH 7.4, 500mM NaCl, 20mM imidazole. The target protein was eluted with the same buffer plus 300mM imidazole and dialyzed in 20mM Hepes pH 7.4, 150mM NaCl, 10mM $CaCl_2$ at 4°C overnight. The protein was then concentrated to 1mg/ml and diluted by 3 fold in 50mM Tris pH 8.0, 10mM $CaCl_2$ (buffer A). The diluted protein was loaded onto HiTrap Q HP column (GE Healthcare) equilibrated in buffer A plus 4% of 50mM Tris pH 8.0, 1M $CaCl_2$ and 10mM $CaCl_2$ (buffer B). The Q column was eluted by a gradient of buffer A plus 2% - 100% of buffer B. The major peak containing Notch3 NRR was collected and treated with furin (NEB) at 30 units/mg of target protein at 4°C overnight. The furin treated protein was then concentrated and loaded onto Superdex 75 10/300 GL (GE Healthcare) equilibrated in 20mM Hepes pH 7.4, 150mM NaCl. Peak fractions wereanalyzed by SDS-PAGE and LCMS, and pooled to complex with Fabs.

Production of Ab-B and Ab-C Fabs

1L of HEK293F cells (Life Technologies) growing at 1 million cells/ml were transfected with 1mg of DNA construct containing full-length IgG of Ab-B (or Ab-C) for three days. The full-length IgGwas purified from the medium by ProSep–vA High Capacity Chromatography Media resin (Millipore) according to manufacturer's protocol. The purified IgGwas then digested by immobilized papain (Pierce) to generate Fab fragments. Specifically, IgG at 20 mg/ml in 20 mM sodium phosphate pH 7.0 and 10 mM EDTA was mixed with immobilized papain at a weight ratio of 80:1. The mixture was rotated in a 15 ml tube at 37 °C overnight. The next day the immobilized papain was removed by gravity flow column; the flow-through, which contains both Fab and Fc segments, was collected and loaded onto HiTrap MabSelect SURE column (GE Healthcare) to remove Fc segment. The flow-through from this step, which contains only Fab fragment, was concentrated and loaded onto HiLoad 16/60 Superdex 75 (GE Healthcare) equilibrated in 20mM Hepes pH 7.4, 150mM NaCl. Peak fractions were analyzed by SDS-PAGE and LCMS, then pooled to form complex with Notch3 NRR.

Crystallization and structure determination

The Notch3 NRR/Ab-B complex or the Notch3 NRR/Ab-C complex was prepared in the same way. Purified Notch3 NRR was mixed with the Fab at a 2:1 molar ratio (concentration measured via LCUV). The Notch3 NRR/Fab complex was incubated on ice for 30 min, and loaded onto a HiLoad 16/60 Superdex 75 (GE Healthcare) equilibrated in 20mM Hepes pH

7.5, 150mM NaCl. Peak fractions were analyzed by SDS-PAGE and LCMS. Fractions containing Notch3 NRR/Fab complex were concentrated to about 25 mg/ml for the Notch 3 NRR/Ab-B complex, or 18 mg/ml for the Notch 3 NRR/Ab-C complex. The Notch3 NRR/Fab complexwas immediately centrifuged and screened for crystallization.

Crystals were grown by sitting drop vapor diffusiontechnique Specifically for the NRR/Ab-B complex , 0.1 µl of the complex was mixed with 0.1 µl of reservoir solution which contains 0.1M NaAc pH 5.6, 17.5% PEG3000; and the drop was equilibrated against 45 µl of the reservoir solution at 20 °C.

For the NRR/Ab-C complex,, 0.1M Hepes pH 7.5, 10% PEG8000, 10% ethylene glycol was used; and the drop was equilibrated against 45 µl of the reservoir solution at 20 °C.

Before data collection, the Notch3 NRR/Fab crystals were transferred to reservoir solution containing additional 22.5% glycerol for Notch3 NRR /Ab-B complex; or 20% ethylene glycol for Notch3 NRR /Ab-C complex prior to being flash cooled in liquid nitrogen.

Diffraction data was collected at beamline 17-ID at the Advanced Photon Source (Argonne National Laboratory, USA). Data was processed and scaled using HKL2000 (HKL Research). The data of Notch3 NRR /Ab-B complex was processed to 3.2Å in space group C2 with cell dimensions a= 91.92 Å, b=104.35 Å, c=92.85 Å, alpha=90°, beta=113.17°, gamma=90°. The data of the Notch3 NRR/Ab-C complex was processed to 2.1 Å in space group $P2_12_12_1$ with cell dimensions a= 88.34 Å, b=123.86 Å, c=150.57 Å, alpha=90°, beta=90°, gamma=90°. The structures of Notch3 NRR/Fab complexes were solved by molecular replacement using Phaser (McCoy *et al.*, (2007) J. Appl. Cryst. 40:658-674) with Notch1 NRR structure (PDB ID: 3ETO) and in-house Fab structures with highest sequence identity with Ab-B or Ab-C Fab as search models. The final models were built in COOT (Emsley & Cowtan (2004) Acta Cryst. 60:2126-2132) and refined with Buster (Global Phasing, LTD). For the Notch3 NRR /Ab-B complex, the R_{work} and R_{free} values were 23.0% and 26.9%, respectively; and rmsd values of bond lengths and bond angles are 0.008Å and 1.17°, respectively. For the Notch3 NRR /Ab-C complex, the R_{work} and R_{free} values were 19.2% and 22.6%, respectively; and rmsd values of bond lengths and bond angles were 0.010Å and 1.13°, respectively.

Residues of Notch3 NRR that contain atoms within 5 Å of any atom in Ab-B or Ab-C Fab are identified by PyMOL (Schrödinger, LLC). The buried surface area between Notch3 NRR and Fabs are calculated by AREAIMOL from the CCP4 program suite (Winn *et al.*, (2011) Acta. Cryst. D67:235-242).

Structure of Notch 3 NRR

The structures of Notch3 NRR are very similar between Notch3 NRR /Ab-B complex and Notch3 NRR /Ab-C complex. The root-mean-square distance (RMSD) of superposing Notch3 NRR from the two complexes is 0.42 Å, indicating almost identical structures. Therefore, Notch3 NRR /Ab-C complex is used as a representative to analyze the structure further.

Notch3 NRR has a similar overall folding as that of Notch1 (Gordan *et al.*, (2009) Blood 113:4381-4390; Gordon *et al.*, (2009) 4:e6613; Wu *et al.*, (2010) Nature 464:1052-1057) and Notch2 (Gordon *et al.*, (2007) Nat Struct Mol Biol 14:295-300). It is composed of three Lin12/Notch repeats (LNR), namely LNR-A, LNR-B and LNR-C; and a heterodimerization (HD) domain divided into N-terminal part (HD-N) and C-terminal part (HD-C) by furin cleavage at S1 site (between R1571 and E1572).

NRR domains regulate the activation of Notch receptors, which involves three proteolysis steps. Furin-like convertase cleaves at S1 site within NRR during maturation of Notch precursor, to prime the activation. ADAM proteases cleave at S2 site, also within NRR, to create the substrate for intramembrane proteolysis at S3 site by gamma secretase. Following S3 cleavage, the intracellular part of Notch enters nucleus to activate transcription. S2 cleavage is the key step of this activation series and is negatively regulated by NRR domains. The mechanism of this so called autoinhibition can be explained by NRR structures.

Figure 14D shows the overall X-ray structure of Notch3 NRR. Labeled are 1) N- and C-terminus of the proteins; 2) the three LNR repeats and the coordinated Ca^{2+} ions; 3) L1419, the autoinhibitory plug; 4) S1 and S2 sites; 5) secondary structures within HD domain; and 6) the two regions in Notch3 with significantly different conformation than Notch1 and Notch2 (LNR-B/C linker plus first half of LNR-C, and β4-α3 loop in HD domain).

As in the Notch3 NRR structure, three LNRs, each coordinating a Ca^{2+} ion, wrap around HD to protect S2 site from access by ADAM proteases. Notably the conserved L1419 from LNR-A/B linker directly plugs into S2 site and sterically occludes it from prrotease access. The stability of the interactions between LNRs and HD, as well as those within the domains, is critical to maintain the autoinhibited conformation of NRR. Therefore, mutations destabilizing NRR, like those found in relevant cancers, could enhance activation of Notch3. On the other hand, reagents like antibodies that can stabilize LNR-HD interaction can potentially inhibit Notch3 signaling.

Notch3 epitope for Ab-B and Ab-C

Ab-B epitope

The crystal structure of the Notch3 NRR/Ab-B Fab complex was used to identify the Notch3 epitope for Ab-B. The interaction surface on Notch3 NRR by Ab-B Fab was formed by several discontinuous (*i.e.* noncontiguous) sequences. These residues form the three-dimensional surface that is recognized by Ab-B Fab, as shown in Figure 14A. Interestingly, the β4-α3 loop in HD domain has a unique structure compared with Notch1 and Notch2, and a majority of this segment is within the Ab-B epitope. Furthermore, this loop is mostly unstructured (no electron density due to flexibility) in Notch3 NRR/Ab-C complex, but is stabilized and structured in this Ab-B complex by direct binding to the Fab.

Ab-B Fab binds across both LNR (mainly around LNR-B) and HD domains (mainly around β4-α3 loop) of Notch3 NRR. The buried surface area between Ab-B Fab and LNR is 554.9 $Å^2$, and 535.2 $Å^2$ between Ab-B Fab and HD domain. This positioning of the Fab indicates Ab-B can clamp LNR and HD domain together, stabilize the autoinhibitory conformation of Notch3 NRR, and inhibit Notch 3 activation.

Ab-C epitope

The crystal structure of the Notch3 NRR/Ab-C Fab complex was used to identify the Notch3 epitope for Ab-C. The interaction surface on Notch3 NRR by Ab-C Fab was formed by several discontinuous (*i.e.* noncontiguous) sequences: These residues form the three-dimensional surface that is recognized by Ab-B Fab, as shown in Figure 14. Interestingly, the LNR-B/C linker in the first half LNR-C has a unique structure compared with Notch1 and Notch2, and a majority of this segment is within Ab-C epitope.

Ab-C Fab binds across both LNR (mainly around LNR-B/C linker and LNR-C) and HD domains (mainly around α3-β5 loop) of Notch3 NRR. The buried surface area between Ab-C Fab and LNR is 729.6 $Å^2$, and 152.2 $Å^2$ between Ab-C Fab and HD domain. This positioning of the Fab indicates Ab-C can clamp LNR and HD domain together, stabilize the autoinhibitory conformation of Notch3 NRR, and inhibit Notch 3 activation.

Ab-B and Ab-C epitopes do not overlap

To determine whether the epitopes of Ab-B and Ab-C overlap, the crystal structures of Notch3 NRR/Ab-B complex and Notch3 NRR/Ab-C complex was superposed on Notch3

NRR. Ab-B and Ab-C bind to distinct separate conformational epitopes within the Notch 3 NRR that do not overlap.

Cancer mutation mapped on structure of Notch3 NRR

In order to gain additional mechanistic insight into the NRR of Notch 3, cancer mutations were mapped onto Notch3 NRR structure. Structural analysis suggested that some of these mutations disrupted intra- or inter-domain interactions, destabilize the autoinhibitory conformation of Notch3 NRR and cause Notch3 activation and signal transduction

Meanwhile, comparison of these mutations with Ab-B and Ab-C epitopes shows that most of them are not within the epitopes, indicating that Ab-B and Ab-C can bind to both wild type and mutant Notch3 NRRs in an autoinhibited conformation to inhibit Notch 3 signal transduction.

Table 4: Shows the structure-based interpretation of Notch3 mutations

Mutation	Cellular data	Structure-based interpretation
Group 1		
S1580L R1510H D1587N R1589Q Y1624H	Activating	Lose intra-domain hydrogen bonds and thus destabilize HD domain
Group 2		
G1487D A1476T A1608T L1518M A1537T	Activating	Affect structural integrity, cause clash
Group 3		
N1597K L1547V R1526C	Activating	On the surface of NRR, no obvious interpretation, but might interfere with protein-protein interaction

Group 1 (S1580L, R1510H, D1587N, Y1624H,R1589Q)

Mutations in this group lose hydrogen bonds within HD domain and thus cause destabilization.

A representative from this group is S1580L. It activates Notch3 signaling in cellular assays and is a driving force of T-ALL1. In the structure, the side-chain oxygen of S1580 (in HD-N) forms a hydrogen bond with the backbone nitrogen of P1521 (in HD-C). S1580L mutation can lose this hydrogen bond and destabilize HD domain. Considering S1580 is close to S2 site (~10 Å), this destabilization can make S2 site more accessible to ADAM proteases and thus enhance activation of Notch3.

Similarly, R1510H mutation in HD-N can lose hydrogen bond with D1603 in HD-C, D1587N and R1589Q mutations can lose the salt bridge originally existing between the two residues, and Y1624H mutation in HD-N can lose hydrogen bond with S1527 and D1530 in HD-C. All these mutations can destabilize the HD domain and potentially activate Notch3 signaling.

Group 2 (G1487D, A1476T, A1608T., , L1518M, A1537T)

Mutations in this group can affect structural integrity within domains or cause clash with surrounding residues, thus destabilize Notch3 NRR.

A representative from this group is G1487D. It activates Notch3 signaling in cellular assays. G1487 is adjacent to the C1475-C1488 disulfide bond of LNR-C, which is critical for the structural integrity and Ca^{2+} coordination within this domain. G1487D mutation can interfere with the correction positioning of this disulfide bond and destabilize LNR-C domain.

L1518 is in a hydrophobic pocket adjacent to S2 site, formed by side-chains of R1627, Y1558, and I1578. L1518M mutation can clash with this hydrophobic pocket and thus destabilize S2 site.

A1537 in HD-N is only 3.3 Å away from E1492 in LNR-C. A1537T mutation can clash with E1492 and destabilize LNR-HD interaction.

Group 3 (N1597K, L1547V, R1526C)

Mutations in this group are on the surface of Notch3 NRR. N1597K activates Notch3 signaling in cellular assays, indicating these surface mutations might function through mechanisms other than destabilization of NRR, *e.g.* interference with protein-protein interaction events.

Cancer mutations v.s. epitopes

Cancer mutations v.s. Ab-B epitope

The cancer mutations fall within or outside the majority of the Ab-B epitope, indicating Ab-B can still bind to both wild-type and mutant Notch3 NRRs.

The two cancer mutations within the epitope are R1510H and N1597K. For example, R1510H might weaken the binding of Ab-B to Notch3 NRR, because this mutation can lose several interactions with the light chain, including salt bridge with D50 and hydrogen bond with N31.

Cancer mutations v.s. Ab-C epitope

All cancer mutations except G1487D are outside of Ab-C epitope, indicating Ab-C can still bind to both wild-type and mutant Notch3 NRRs.

G1487D might weaken the binding of Ab-C to Notch3 NRR because this mutation can clash with and break the hydrogen bond between Y1471 (Notch3) and H55 (Ab-C heavy chain).

Equivalents

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the disclosure. The foregoing description and examples detail certain preferred embodiments of the disclosure and describe the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the disclosure may be practiced in many ways and the disclosure should be construed in accordance with the appended claims and any equivalents thereof.

Incorporation By reference

All references cited herein, including patents, patent applications, papers, text books, and the like, and the references cited therein, to the extent that they are not already, are hereby incorporated herein by reference in their entirety.

We Claim:

1. A mutant Notch 3 receptor comprising at least one activating mutaton set forth in Table 1, or combinations thereof, wherein the presence of the activating mutation is determined using an assay comprising a Notch 3 intracellular domain 3 (ICD3) antibody or fragment thereof that detects SEQ ID NO: 3.

2. A mutant Notch 3 receptor comprising at least one activating mutation located in the NRR of Notch 3, wherein the activating mutation activates Notch 3 signal transduction, and wherein the presence of the activating mutation is determined using an assay comprising a Notch 3 intracellular domain 3 (ICD3) antibody or fragment thereof that detects SEQ ID NO: 3.

3. The mutant Notch 3 receptor of claim 2, wherein the mutation in the NRR domain is selected from the group consisting of S1580L, D1587N, Y1624H, L1518M, A1537T, N1597K, L1547V, R1526C (HD) and G1487D, (LNR-C) .

4. The mutant Notch 3 receptor of claim 2, further comprising at least one mutation located in the PEST domain of Notch 3.

5. The mutant Notch 3 receptor of claim 4, wherein the mutation in the PEST domain is selected from the group consisting of P2034fs, P2067fs, p2177fs, Q2075*, W2172*, G2112D, L2212M, F2121L, G2038S, G2059R, R2022H, Y2127H, Y2211C, V2202I, S2096L, P2089L, P2209L, R1981C, R2145Q, and P2178S.

6. A mutant Notch 3 receptor comprising an amino acid sequence that is at least 90% identical to SEQ ID NO:1, wherein the amino acid sequence of the mutant Notch 3 receptor differs from SEQ ID NO:1 by virtue of containing a Leu at position 1580 rather than Ser in an NRR domain of Notch 3, and wherein the mutation in the Notch 3 polypeptide activates Notch 3 signal transduction.

7. A mutant Notch 3 receptor comprising an amino acid sequence that is at least 90% identical to SEQ ID NO:1, wherein the amino acid sequence of the mutant Notch 3 receptor differs from SEQ ID NO:1 by virtue of containing D at position 1487 rather

than G in an NRR domain of Notch 3, and wherein the mutation in the Notch 3 polypeptide activates Notch 3 signal transduction.

8. A method of determining the increased likelihood of having or developing a cancer in a subject, comprising:

assaying a biological sample obtained from a subject for the presence of a Notch 3 activating mutation using an assay comprising a Notch 3 intracellular domain 3 (ICD3) antibody or fragment thereof that detects SEQ ID NO: 3; and

comparing the biological sample from subject with a non-cancerous or normal control cell, wherein the presence of the Notch 3 mutation indicates the likelihood of developing cancer.

9. The method of claim 8, wherein the biological sample is selected from the group consisting of blood, serum, urine, hair follicle, ascites and tumor biopsy..

10. The method of claim 8, wherein the subject is a human and the cancer is selected from the group consisting of breast cancer, colorectal cancer, lung cancer, multiple myeloma, ovarian cancer, liver cancer, gastric cancer, pancreatic cancer, prostate cancer, acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, t-cell acute lymphoblastic leukemia, mantle cell lymphoma, chronic lymphocytic leukemia, Ewings sarcoma, lymphoma, osteosarcoma, squamous cell carcinoma, peripheral nerve sheath tumors , schwannoma, head and neck cancer, bladder cancer, esophageal cancer, glioblastoma, clear cell sarcoma of soft tissue, malignant mesothelioma, neurofibromatosis, renal cancer, and melanoma.

11. The method of claim 10, wherein the cancer is T-cell acute lymphoblastic leukemia (TALL).

12. A method for detecting the presence of an activated form ofNotch 3 receptor in a biological sample, the method comprising:

contacting the biological sample with a Notch 3 intracellular domain 3 (ICD3) antibody or fragment thereof that detects SEQ ID NO: 3;

incubating the sample and the ICD3antibody or fragment thereof under conditions to induce binding of the ICD3antibody or fragment thereof to a Notch 3 receptor if present in the sample to form a complex; and

detecting the ICD3 antibody, thereby detecting the presence of activated form of the Notch 3 receptor in a sample.

13. The method of claim 12, wherein the Notch 3 receptor comprises a mutation.

14. A kit for detecting the presence of a Notch 3 activating mutation comprising:i) means for detecting the Notch 3 activating mutation; and ii) instructions how to use the kit.

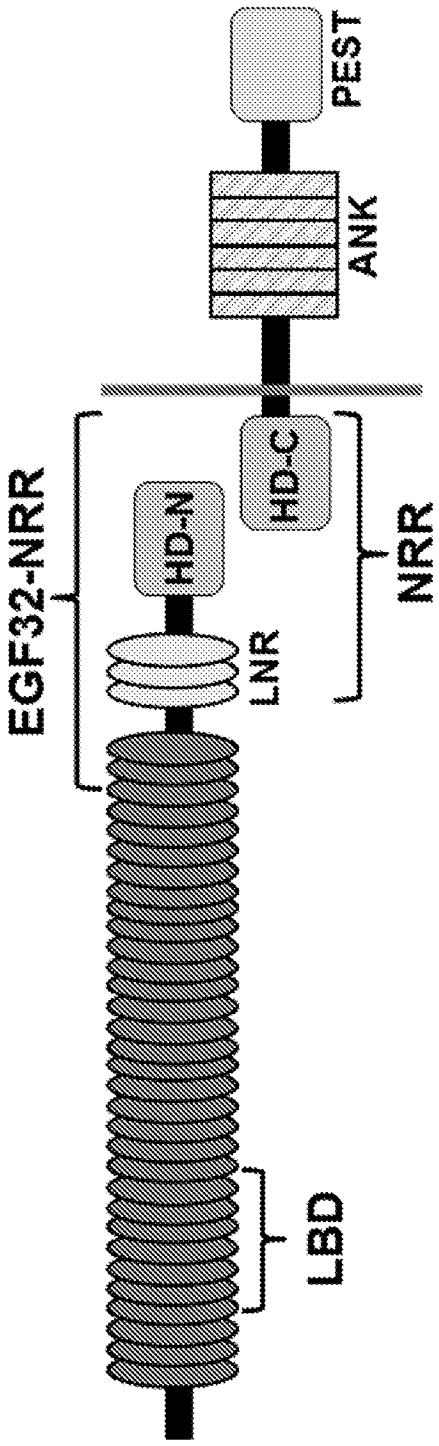
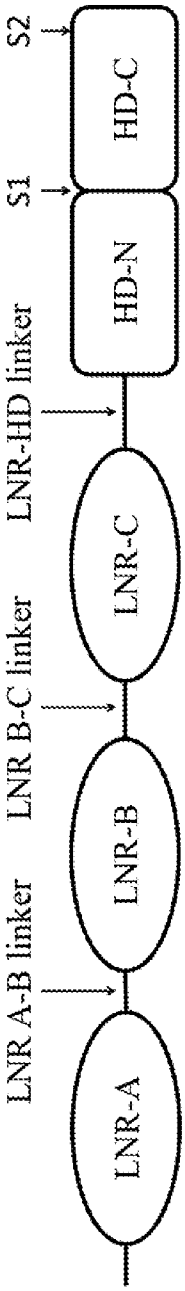


Figure 1



Domain	Residues
LNR-A	E1383-G1422
LNR-A/B linker	Asp1423-Leu1431
LNR-B	Gln1432-Ala1460
LNR-B/C linker	Gly1461-Asn1468
LNR-C	Pro1469-Ser1502
LNR-HD linker	Glu1503-Arg1510
HD-N	Gly1511-Arg1571
HD-C	Glu1572-Ser1640

Figure 2

CellLine_lineage	cDNA_Change	Codon_Change	Protein_Change
GMS10_CNS	c.4639C>G	c.(4639-4641)CTG>GTG	p.L1547V
HUH7_LIVER	c.4552C>A	c.(4552-4554)CTG>ATG	p.L1518M
ISHIKAWAHERAKLIO02ER_ENDOMETRIUM	c.4791T>A	c.(4789-4791)AAT>AAA	p.N1597K
KASUMI2_HAEMATOPOIETIC_AND_LYMPHOID	c.4552C>A	c.(4552-4554)CTG>ATG	p.L1518M
NCIH28_PLEURA	c.4552C>A	c.(4552-4554)CTG>ATG	p.L1518M
OVKATE_OVARY	c.4552C>A	c.(4552-4554)CTG>ATG	p.L1518M
SNU1040_LARGE_INTESTINE	c.4870T>C	c.(4870-4872)TAC>CAC	p.Y1624H
SNU175_LARGE_INTESTINE	c.4529G>A	c.(4528-4530)CGC>CAC	p.R1510H
SNU201_CENTRAL_NERVOUS_SYSTEM	c.4552C>A	c.(4552-4554)CTG>ATG	p.L1518M
TALL1_HAEMATOPOIETIC_AND_LYMPHOID	c.4739C>T	c.(4738-4740)TCG>TTG	p.S1580L
WM88_SKIN	c.4759G>A	c.(4759-4761)GAC>AAC	p.D1587N

Cell Line_Lineage	cDNA_Change	Codon_Change	Protein_Change	Type
A704_KIDNEY	c.6335G>A	c.(6334-6336)GGT>GAT	p.G2112D	Missense
CW2_LARGE_INTESTINE	c.6634C>A	c.(6634-6636)CTG>ATG	p.L2212M	Missense
FTC133_THYROID	c.6361T>C	c.(6361-6363)TTC>CTC	p.F2121L	Missense
FTC238_THYROID-Tumor	c.6112G>A	c.(6112-6114)GGC>AGC	p.G2038S	Missense
GCT_SOFT_TISSUE	c.6175G>A	c.(6175-6177)GGG>AGG	p.G2059R	Missense
GMS10_CNS	c.6239G>A	c.(6238-6240)CGG>CAG	p.R2080Q	Missense
HEC108_ENDOMETRIUM	c.6065G>A	c.(6064-6066)CGC>CAC	p.R2022H	Missense
IGROV1_OVARY	c.6379T>C	c.(6379-6381)TAT>CAT	p.Y2127H	Missense
KG1_HAEM_LYMP	c.6632A>G	c.(6631-6633)TAC>TGC	p.Y2211C	Missense
KYM1_SOFT_TISSUE	c.6223C>T	c.(6223-6225)CAG>TAG	p.Q2075*	Nonsense
LS411N_LARGE_INTESTINE	c.6604G>A	c.(6604-6606)GTC>ATC	p.V2202I	Missense
LS513_LARGE_INTESTINE	c.6287C>T	c.(6286-6288)TCG>TTG	p.S2096L	Missense
NCIH2347_LUNG	c.6515G>A	c.(6514-6516)TGG>TAG	p.W2172*	Nonsense
NCIH446_LUNG	c.6266C>T	c.(6265-6267)CCG>CTG	p.P2089L	Missense
NCIH716_LARGE_INTESTINE	c.6626C>T	c.(6625-6627)CCG>CTG	p.P2209L	Missense
NIHOVCAR3_OVARY	c.5941C>T	c.(5941-5943)CGC>TGC	p.R1981C	Missense
SF295_CNS	c.6434G>A	c.(6433-6435)CGG>CAG	p.R2145Q	Missense
SW1116_LARGE_INTESTINE	c.6532C>T	c.(6532-6534)CCT>TCT	p.P2178S	Missense
2313287_STOMACH	c.6201_6202insC	c.(6199-6204)CCCGGGfs	p.P2067fs	Frame shift
GP2D_LARGE_INTESTINE	c.6102delC	c.(6100-6102)CCCfs	p.P2034fs	Frame shift
HEC151_ENDOMETRIUM	c.6530_6531insC	c.(6529-6531)CCAfs	p.P2177fs	Frame shift
HEC59_ENDOMETRIUM	c.6530delC	c.(6529-6531)CCAfs	p.P2177fs	Frame shift
A549_LUNG-Tumor	c.6102_6103insC	c.(6100-6105)CCCGGTfs	p.P2034fs	Frame shift
MDAMB468_BREAST	c.6102_6103insC	c.(6100-6105)CCCGGTfs	p.P2034fs	Frame shift
SNUC2A_LARGE_INTESTINE	c.6201delC	c.(6199-6201)CCCfs	p.P2067fs	Frame shift

Figure 3a

Tumor_lineage	Protein_Change
X-1004_breast	G1487D
X-1407_breast	A1537T
X-1569_kidney	R1526C

Figure 3B

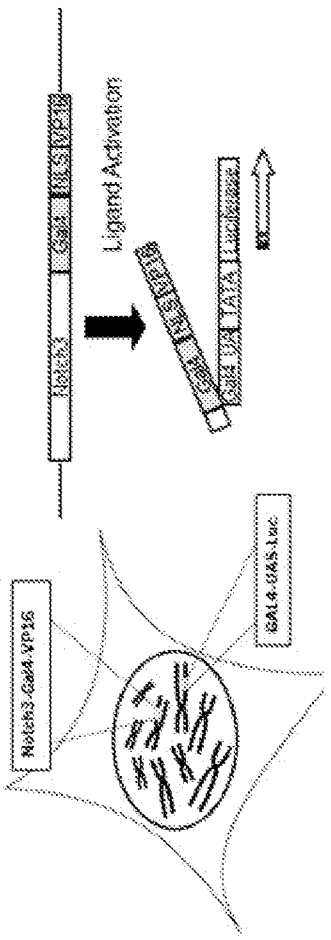


Figure 4a

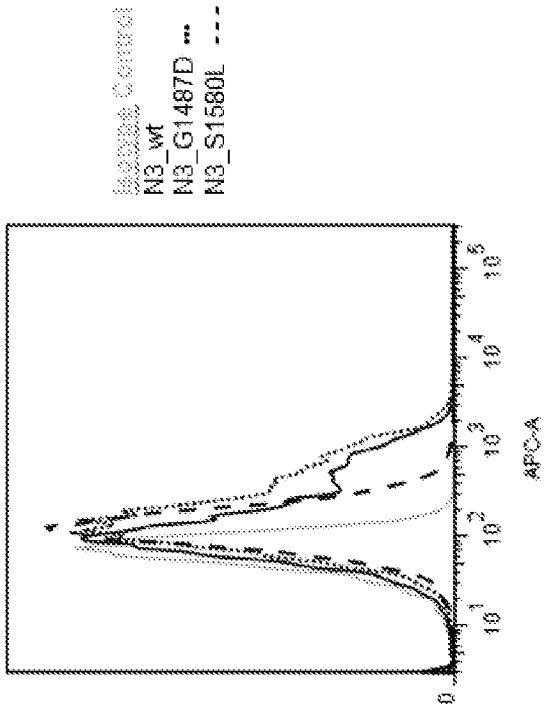


Figure 4c

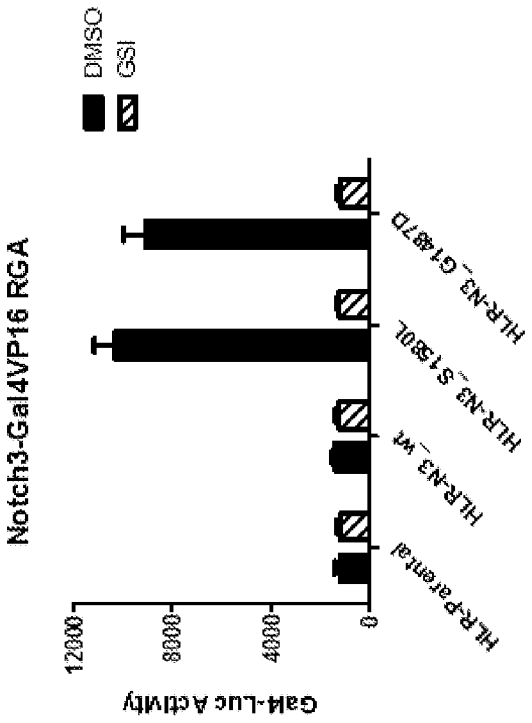


Figure 4b

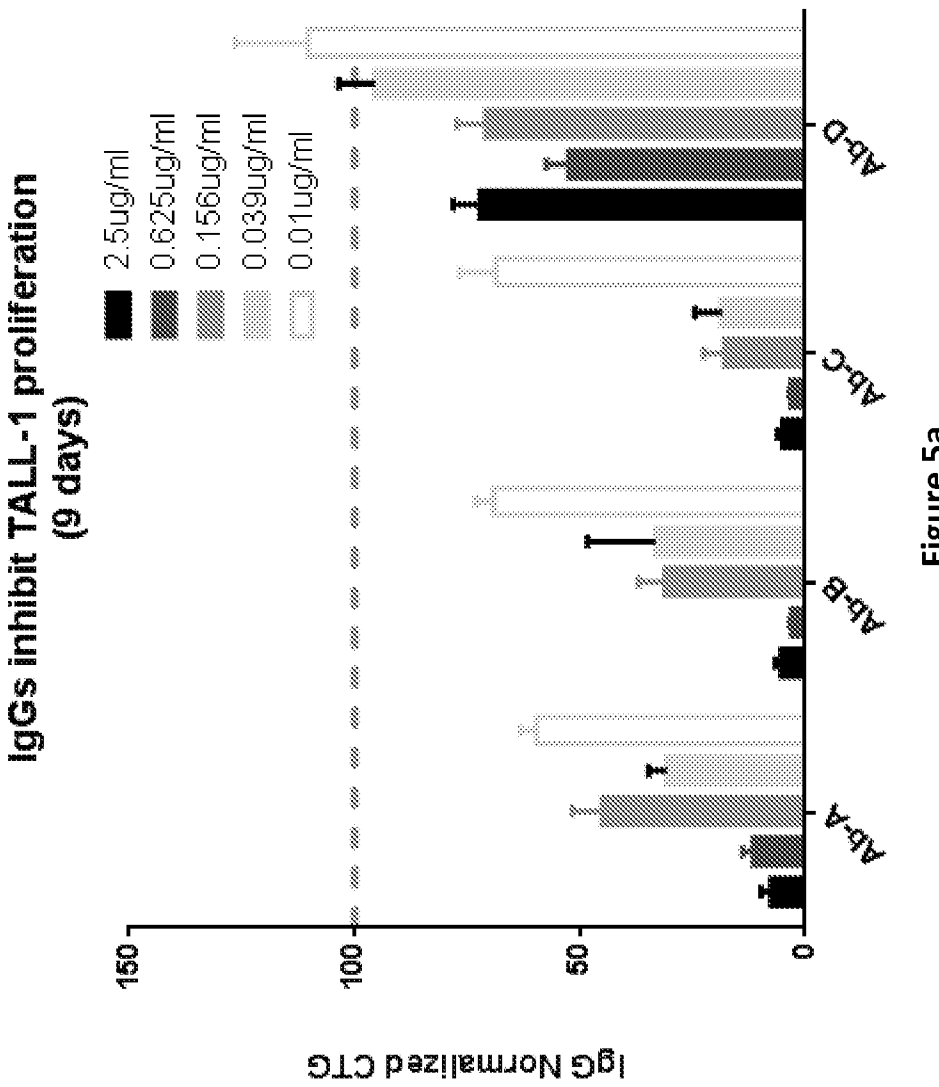

IgGs inhibit TALL-1 proliferation
(9 days)
2.5ug/ml
0.625ug/ml
0.156ug/ml
0.039ug/ml
0.01ug/ml
IgG Normalized CTG
150
100
50
0
Ab-A
Ab-B
Ab-C
Ab-D

Figure 5a

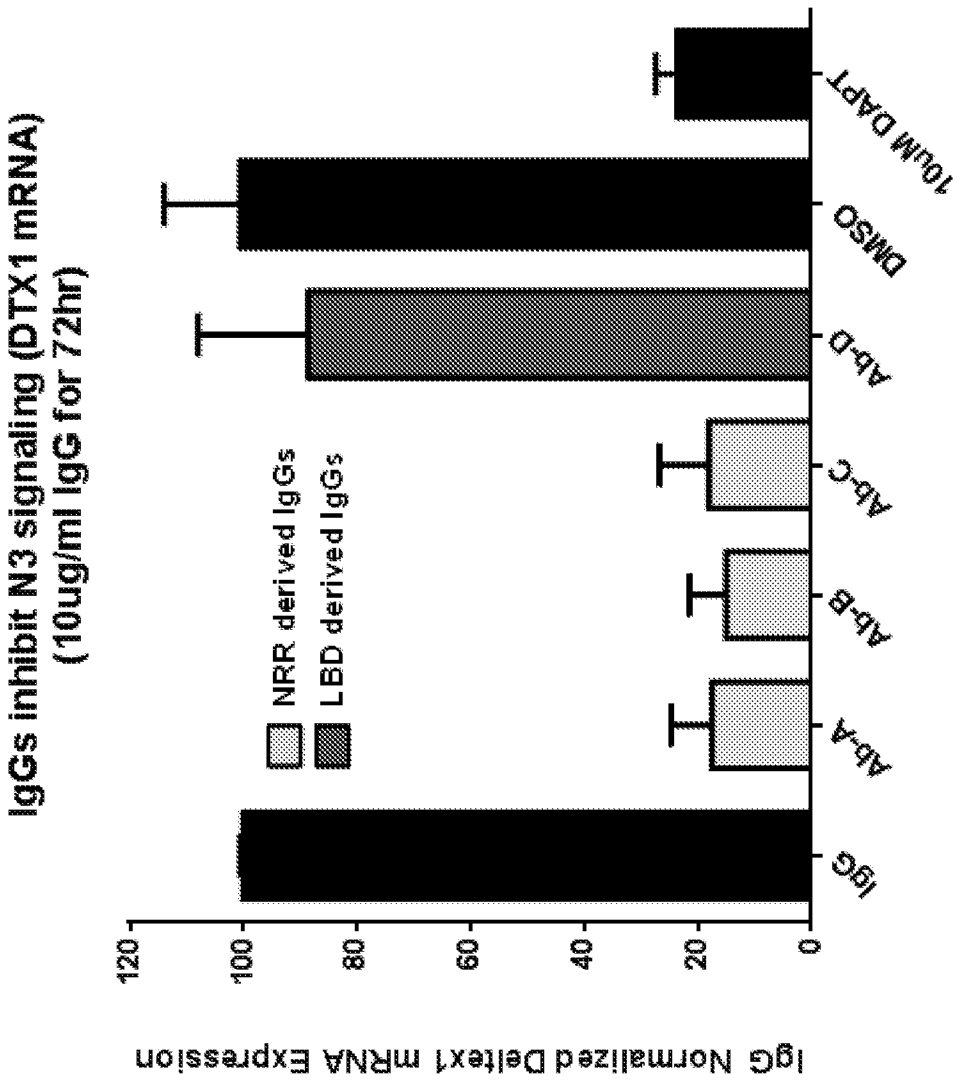

IgGs inhibit N3 signaling (DTX1 mRNA)
(10ug/ml IgG for 72hr)
NRR derived IgGs
LBD derived IgGs
IgG Normalized Deltex1 mRNA Expression
0
20
40
60
80
100
120
IgG
Ab-A
Ab-B
Ab-C
Ab-D
DMSO
10uM DAPT

Figure 5b

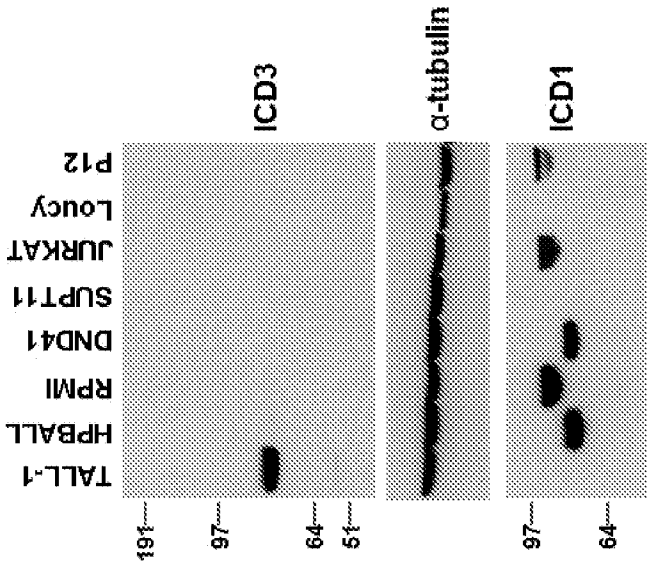


Figure 6b

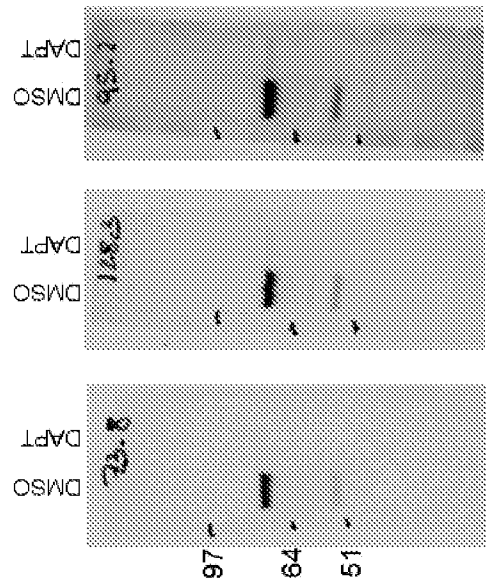


Figure 6a

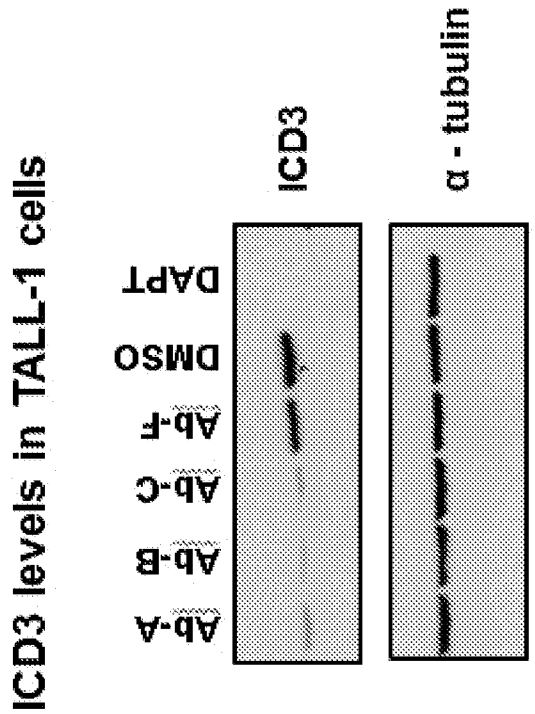

ICD3 levels in TALL-1 cells
Ab-A
Ab-B
Ab-C
Ab-F
DMSO
DAPT
ICD3
α - tubulin

Figure 7a

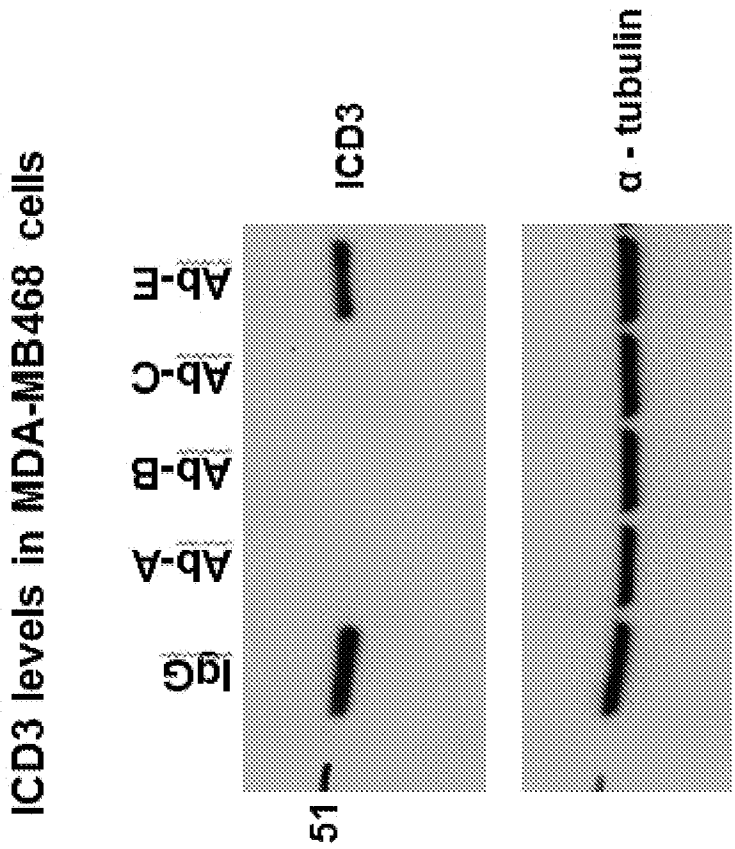

ICD3 levels in MDA-MB468 cells
IgG
Ab-A
Ab-B
Ab-C
Ab-M
ICD3
α - tubulin
51

Figure 7b

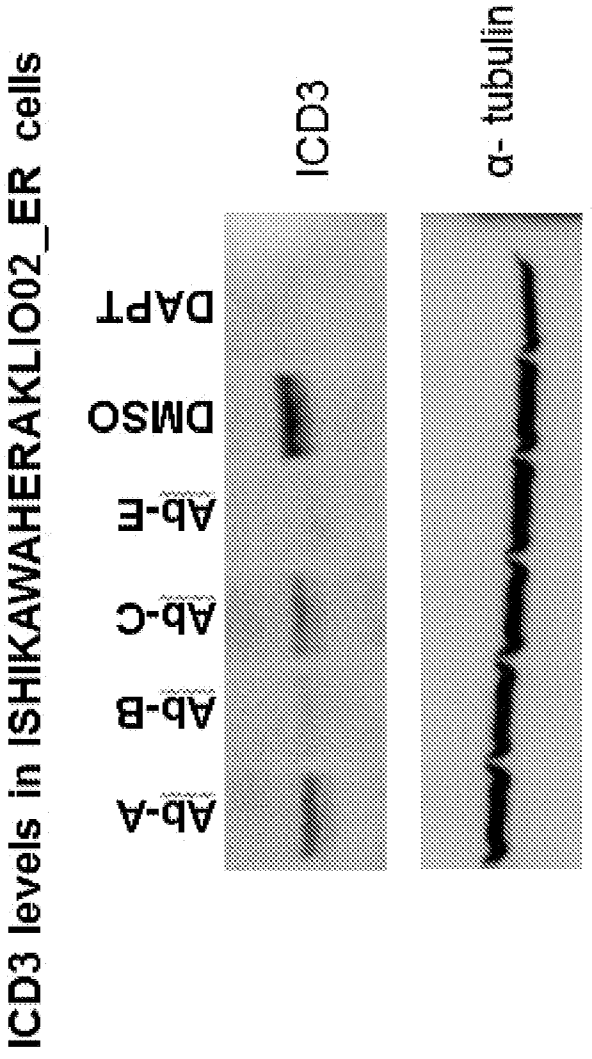

ICD3 levels in ISHIKAWAHERAKLIO02_ER cells
Ab-A
Ab-B
Ab-C
Ab-E
DMSO
DAPT
ICD3
α- tubulin

Figure 8a

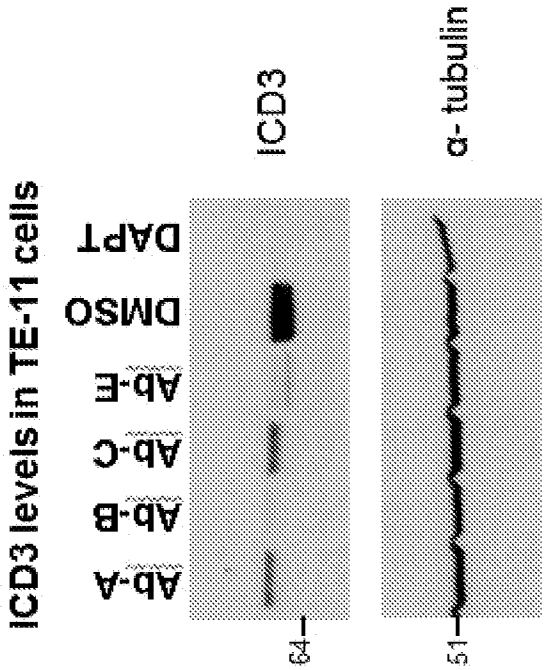

ICD3 levels in TE-11 cells
Ab-A
Ab-B
Ab-C
Ab-E
DMSO
DAPT
ICD3
α- tubulin
64
51

Figure 8b

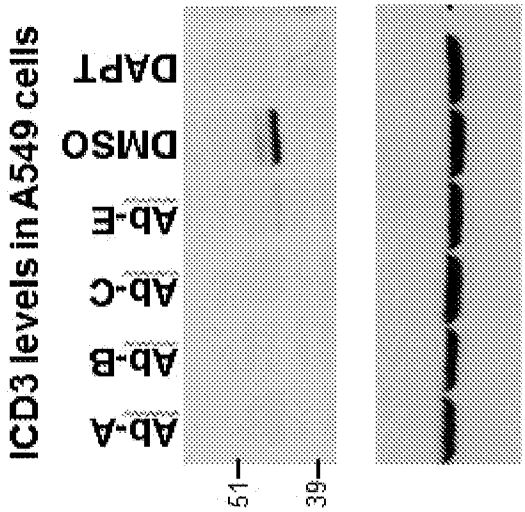


Figure 8c

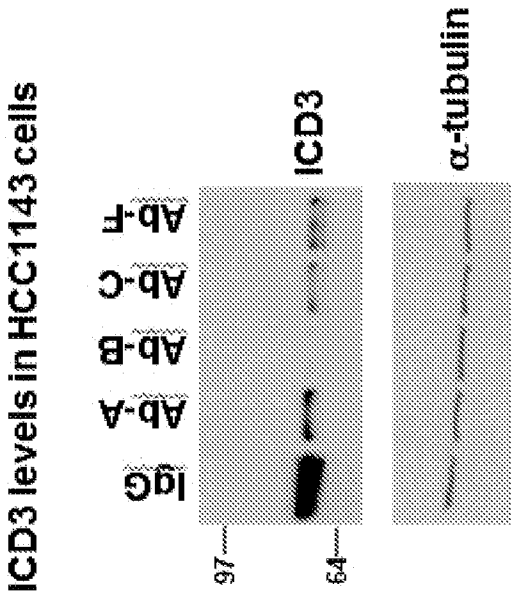

ICD3 levels in HCC1143 cells
IgG
Ab-A
Ab-B
Ab-C
Ab-F
97
64
ICD3
α-tubulin

Figure 9

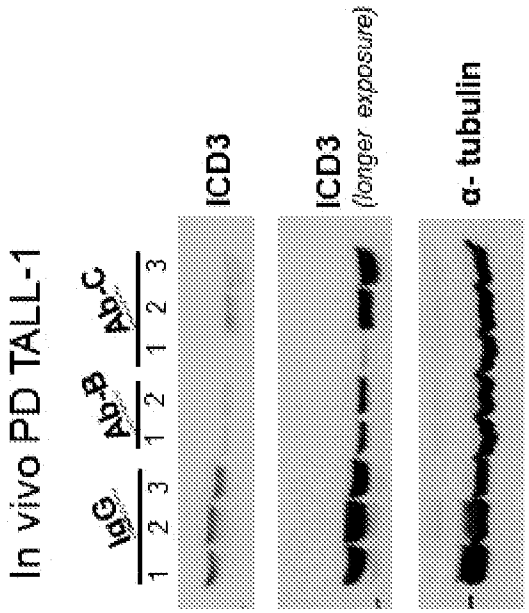

In vivo PD TALL-1
IgG
1 2 3
Ab-B
1 2
Ab-C
1 2 3
ICD3
ICD3
(longer exposure)
α- tubulin

Figure 10a

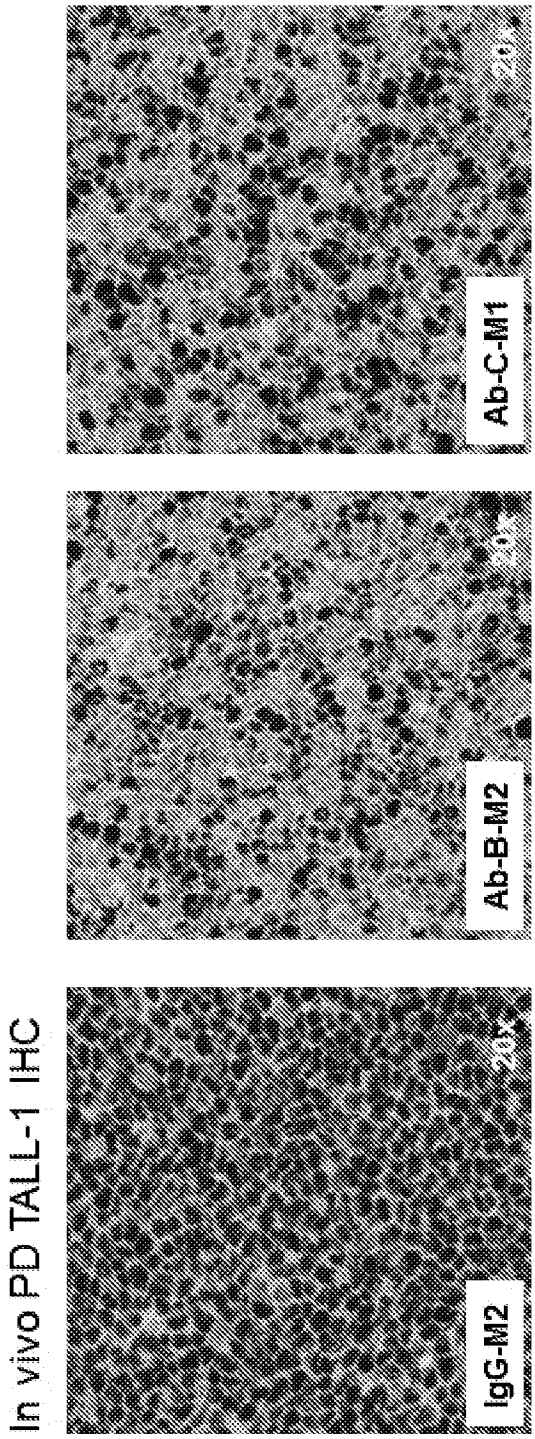


Figure 10b

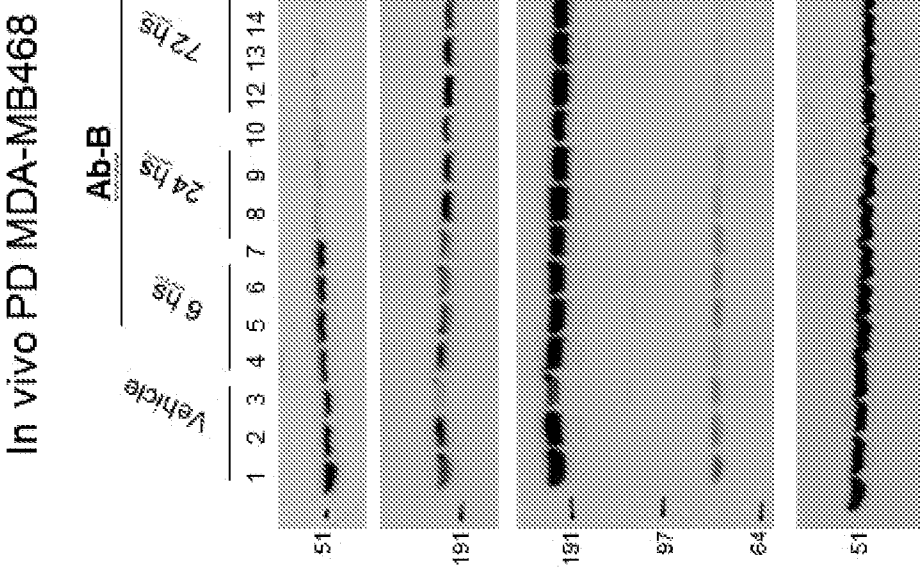

In vivo PD MDA-MB468
Ab-B
Vehicle
6 hs
24 hs
72 hs
1 2 3 4 5 6 7 8 9 10 12 13 14 15
ICD3
N3-FL
N3-FL
Longer exposure
α-tubulin
51
191
191
97
64
51

Figure 11a

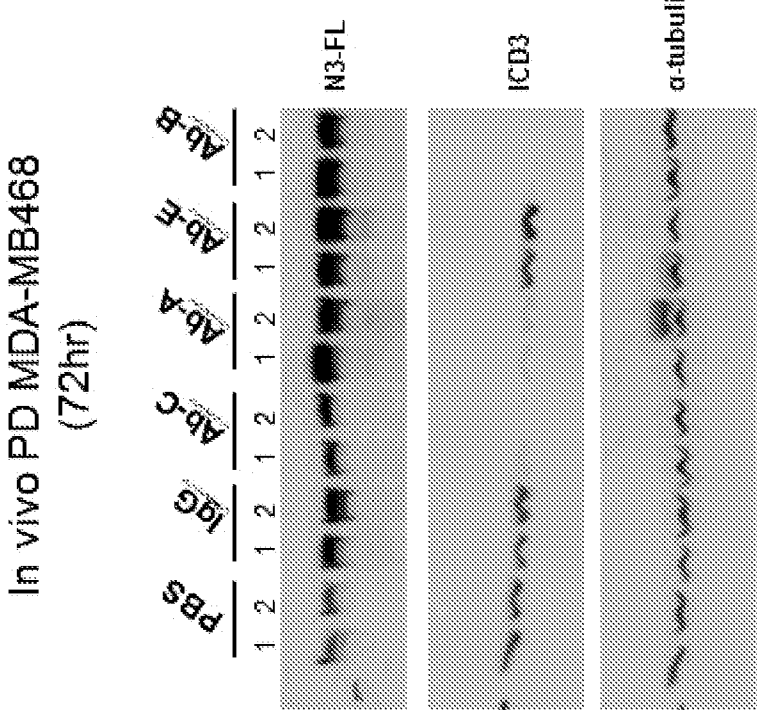

In vivo PD MDA-MB468
(72hr)
PBS
IgG
Ab-C
Ab-A
Ab-E
Ab-B
1 2 1 2 1 2 1 2 1 2 1 2
N3-FL
ICD3
α-tubulin

Figure 11b

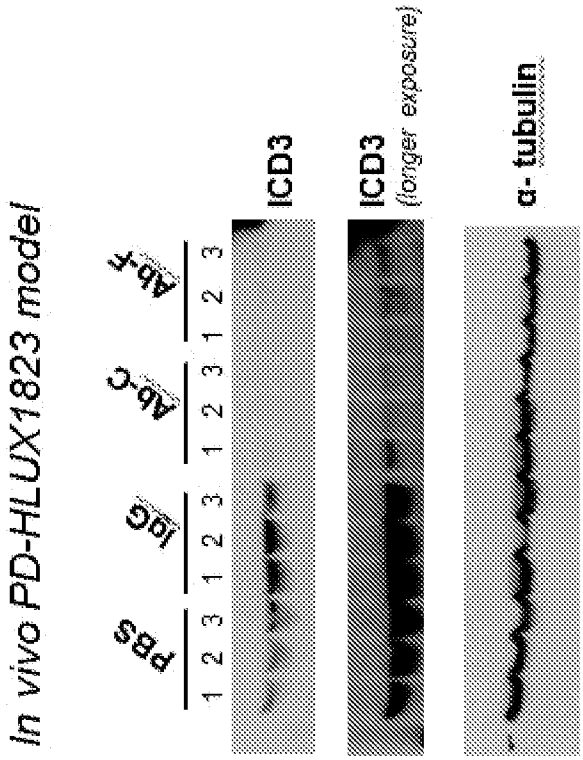

In vivo PD-HLUX1823 model
PBS
IgG
Ab-C
Ab-F
1 2 3 1 2 3 1 2 3 1 2 3
ICD3
ICD3
(longer exposure)
α- tubulin

Figure 12

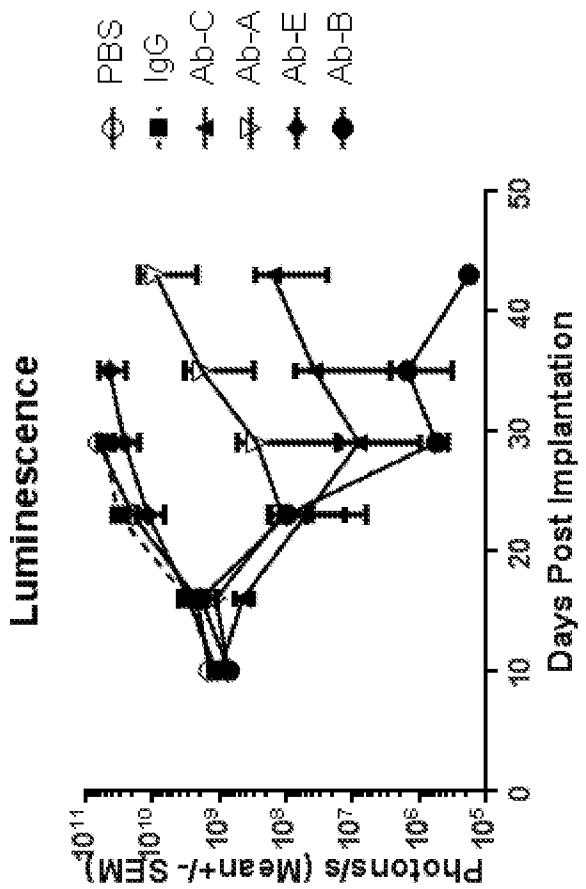

Luminescence
PBS
IgG
Ab-C
Ab-A
Ab-E
Ab-B
Photons/s (Mean+/- SEM)
10^11
10^10
10^9
10^8
10^7
10^6
10^5
0
10
20
30
40
50
Days Post Implantation

Figure 13a

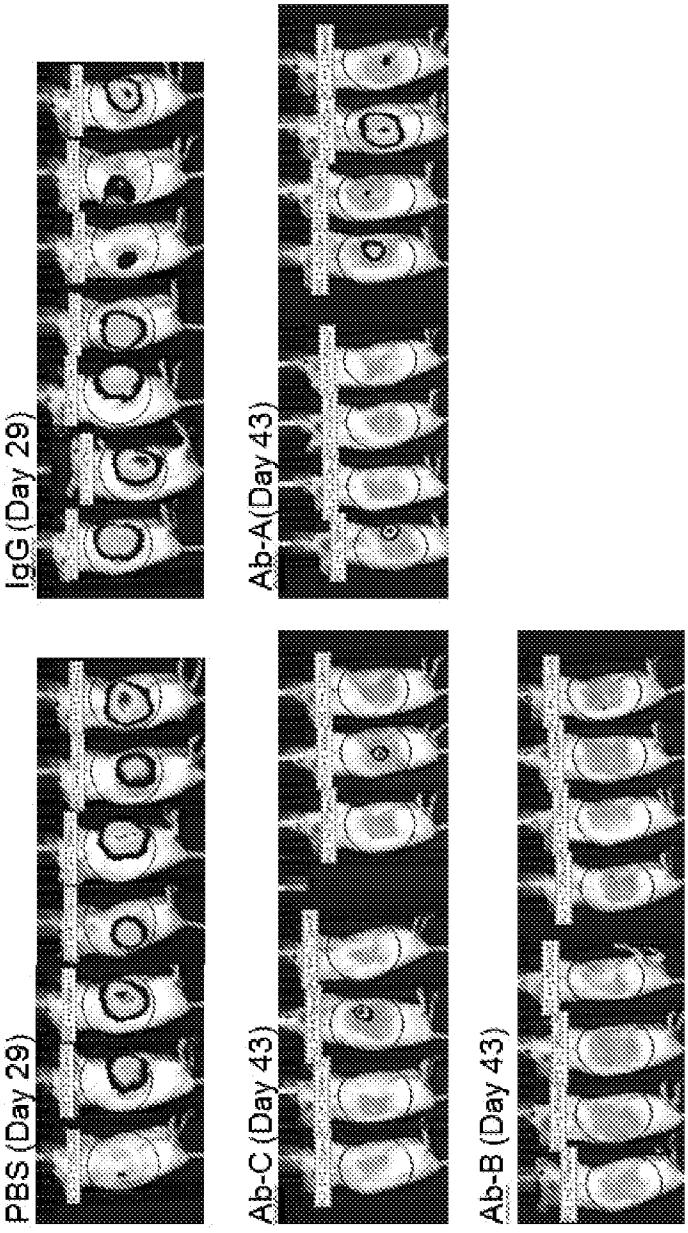


Figure 13b

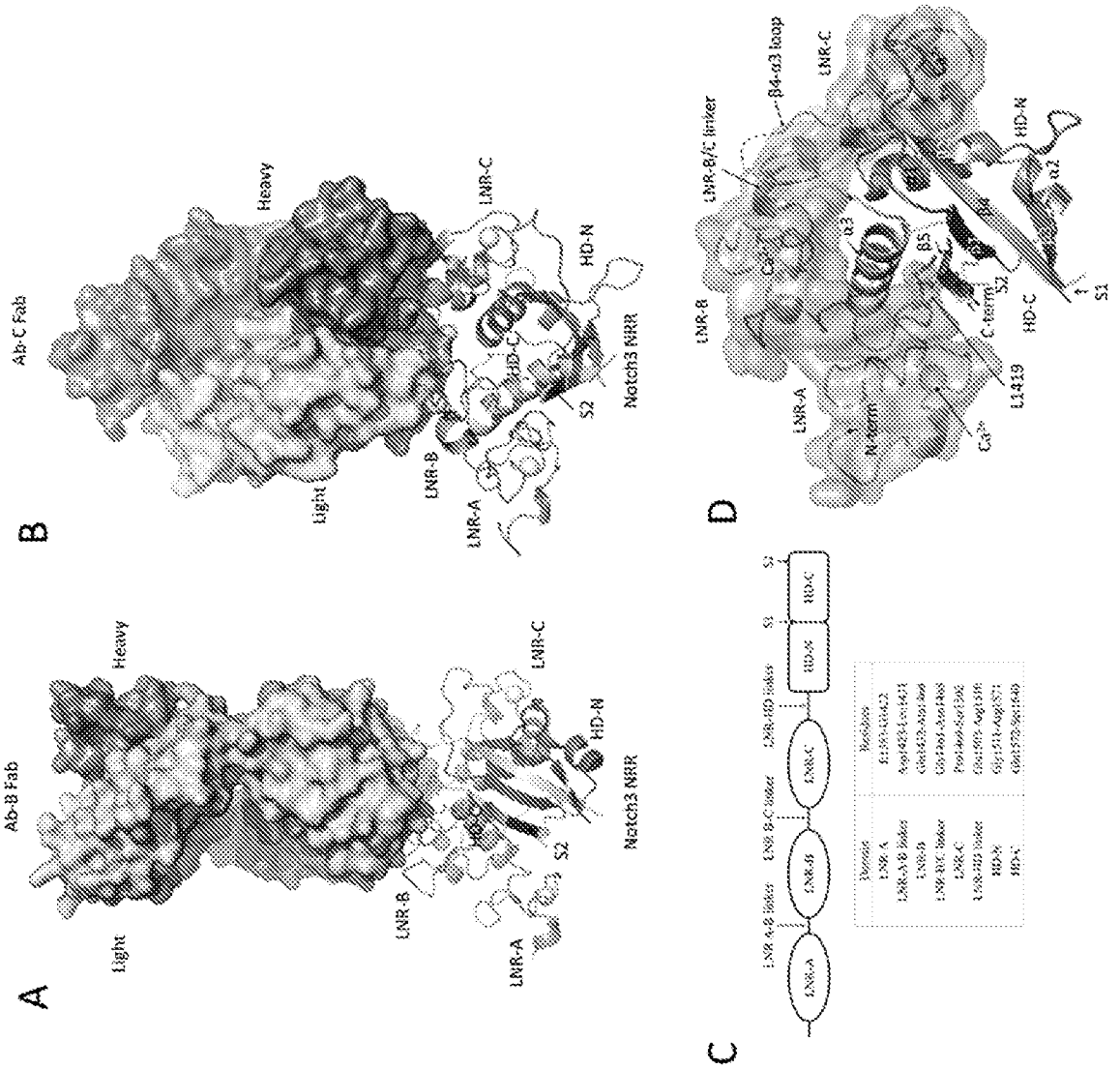

A
Ab-B Fab
Heavy
Light
LNR-B
LNR-A
S2
LNR-C
HD-N
Notch3 NRR
B
Ab-C Fab
Heavy
Light
LNR-B
LNR-A
LNR-C
HD-C
HD-N
S2
Notch3 NRR
C
LNR-A
LNR-B
LNR-C
HD-N
HD-C
S1
S2
D
LNR-B
LNR-B/C linker
β4-α3 loop
LNR-C
α3
β5
β4
α2
S2
S1
C-term
HD-C
HD-N
LNR-A
N-term
L1419

Figure 14

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/022658

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/705 C07K16/28
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, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/041336 A2 (GENENTECH INC [US]; HOFFMANN LA ROCHE [CH]; SIEBEL CHRISTIAN W [US]) 7 April 2011 (2011-04-07) page 14	12-14
X	CHARLES FOUILLADE ET AL: "Activating NOTCH3 mutation in a patient with small-vessel-disease of the Brain", HUMAN MUTATION, vol. 29, no. 3, 1 March 2008 (2008-03-01), pages 452-452, XP055130886, ISSN: 1059-7794, DOI: 10.1002/humu.9527 the whole document	2,12-14

☐ Further documents are listed in the continuation of Box C.

☒ 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

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"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
22 July 2014	30/07/2014

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	Scheffzyk, Irmgard

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2014/022658

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011041336 A2	07-04-2011	AU 2010300747 A1	26-04-2012
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		CN 102630229 A	08-08-2012
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		CR 20120213 A	09-07-2012
		EP 2483311 A2	08-08-2012
		JP 2013506675 A	28-02-2013
		KR 20120100952 A	12-09-2012
		MA 33973 B1	01-02-2013
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		WO 2011041336 A2	07-04-2011