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(54) Titre : MUTATIONS DE NOTCH CONDUISANT A UNE SIGNALISATION DE RECEPTEUR ACCRUE
(54) Title: NOTCH MUTATIONS LEADING TO INCREASED RECEPTOR SIGNALING

(57) Abrégé/Abstract:
The present invention is based upon the identification of regions within the NOTCH-1 receptor that, when mutated, lead to increased receptor signaling. The mutations are associated with uncontrolled cellular growth and this growth may be arrested using agents that interfere with NOTCH-1 activity, such as inhibitors of gamma-secretase. Assays for the NOTCH-1 mutations may be used diagnostically or as part of a treatment regimen for cancer patients.

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NOTCH Mutations Leading to Increased Receptor Signaling

Cross Reference to Related Applications

The present application claims priority to, and the benefit of, United States provisional applications 60/598,546, filed on August 4, 2004 and 60/672,053, filed on April 18, 2005. The contents of these prior applications are hereby incorporated by reference in their entirety.

Field of the Invention

The present invention is directed to human NOTCH receptors that have undergone mutations resulting in either increased responsiveness to biological stimuli or constitutive activation independent of the need for other stimuli. The mutations occur in specific, well-defined, regions of NOTCH receptors and are somatically acquired by cancer cells as part of the process of malignant transformation. The growth of these cancer cells can be slowed or stopped using agents that interfere with receptor signaling.

Background of the Invention

NOTCH receptors 1-4 are transmembrane receptor proteins that signal through a pathway that relies on regulated proteolysis. Following ligand-binding, the receptor is sequentially: i) cleaved extracellularly by metalloproteases of the ADAM family (Brou, *et al.*, *Mol. Cell 5*:207-216 (2000); Mumm, *et al. Mol Cell 5*:197-206 (2000)); ii) mono-ubiquitinated on a lysine residue lying just internal to the transmembrane domain (Gupta-Rossi, *et al.*, *J. Cell Biol. 166*:73-83 (2004)); iii) endocytosed (Gupta-Rossi, *et al.*, *J. Cell Biol. 166*:73-83 (2004)), and iv) proteolytically cleaved by a gamma-secretase enzyme (De Strooper, *et al.*, *Nature 398*:518-522 (1999)). This final step in the activation process permits the intracellular portion of NOTCH receptors to translocate to the cell nucleus where it interacts with transcription factors to alter gene activity. NOTCH receptor signaling appears to play an important role in the differentiation and proliferation of cells and in controlling apoptosis, three processes that are important with respect to neoplastic transformation (see U.S. 6,703,221).

The *NOTCH-1* gene was discovered through its involvement in a (7;9) chromosomal translocation found in fewer than 1% of T-cell acute lymphoblastic leukemias (T-ALLs) (Ellisen, *et al.*, *Cell 66*:649-661 (1991)). NOTCH-1 is highly expressed in thymocytes (Ellisen, *et al.*, *Cell 66*:649-661(1991)), where it induces common lymphoid progenitors to adopt a T cell fate (Radtke, *et al.*, *Immunity* 10:547-558). Subsequently, it promotes the assembly of pre-T cell receptor complexes which play a critical role in driving a proliferative burst that accompanies maturation of CD4-/CD8- thymocytes to the CD4+/CD8+ developmental stage (Wolfer, *et al.*, *Immunity 16*:869-879 (2002)).

In its resting state, mature NOTCH-1 is a heterodimeric receptor comprised of a ligand-binding extracellular subunit (NEC) and a non-covalently associated transmembrane subunit (NTM) (Rand, *et al.*, *Mol. Cell. Biol. 20*:1825-1835; Logeat, *et al.*, *Proc. Natl. Acad. Sci. USA 95*:8108-8112 (1998)). NEC consists of a ligand-binding domain comprised of epidermal growth factor-like repeats, three iterated Lin12/NOTCH repeats, and a conserved 103 amino acid sequence (hereafter termed HD, for heterodimerization domain) that is sufficient for association with the extracellular portion of NTM.

Physiologic activation of NOTCH receptors occurs when a ligand of the Delta-Serrate-Lag2 (DSL) family binds to the NEC subunit and initiates a cascade of successive proteolytic cleavages in the NTM subunit. The final cleavage, which is catalyzed by γ-secretase, a multiprotein complex containing presenilin-1 or -2, nicastrin, APH-1, and PEN-2 (Francis, *et al.*, *Dev. Cell 3*:85-97 (2002); Kimberly, *et al.*, *Proc. Natl. Acad. Sci. USA 100*:6382-6387 (2003)) releases the intracellular part of NTM (called intracellular NOTCH, or ICN) from the membrane, permitting it to translocate to the cell nucleus. There, it associates with the DNA-binding factor CSL and co-activators of the Mastermind family to form a short-lived transcriptional activation complex (Wallberg, *et al.*, *Mol. Cell Biol. 22*:7812-7819 (2002); Fryer, *et al.*, *Genes Dev. 16*:1397-1411 (2002); Nam, *et al.*, *J. Biol. Chem. 278*:21232-21239 (2003)) Degradation and turnover of the complex is apparently regulated by F-box factors of the SEL-10 family (Oberg, *et al.*, *J. Biol. Chem. 276*:35847-35853 (2001)).

The (7;9) translocation creates a *NOTCH*-T cell receptor β fusion gene that encodes N-terminally-deleted, constitutively active NOTCH-1 polypeptides similar to the ICN (Ellisen, *et al.*, *Cell 66*:649-661 (1991); Aster, *et al.*, *Cold Spring Harb. Symp. Quant. Biol. 59*:125-136 (1994); Das, *et al.*, *J. Biol. Chem.*, epublished May 3, 2004)) and these truncated and constitutively active forms of NOTCH-1 induce T-ALL in mouse models (Aster, *et al.*, *Mol. Cell Biol. 20*:7505-7515 (2000)). NOTCH-1 is also the site of frequent retroviral insertions that cooperate with the E2A-PBX1 and cMYC transgenes in multistep pathways leading to the development of murine T-ALL (Hoemann, *et al.*, *Mol. Cell. Biol. 20*:3831-3842 (2000); Feldman, *et al.*, *Blood 96*:1906-1913(2000)). Further, NOTCH inhibitors cause a G_O/G_1 cell cycle arrest in cell lines derived from human and murine NOTCH-1-associated T-ALLs, indicating that NOTCH signaling is required for the sustained growth of these leukemias (Weng, *et al.*, *Mol. Cell Biol. 23*:655-664 (2003)).

The (7;9) translocation has only been associated with a very small percentage of patients having T-ALL. The identification of additional mutations associated with NOTCH-1 would have important implications for the pathogenesis of this type of cancer. Although mutations in NOTCH-2, NOTCH-3, and NOTCH-4 have not been identified in human cancer, it is known that abnormal increases in function of these NOTCH receptors in other mammals can cause T-ALL (NOTCH-2 and -3, Bellavia, *et al.*, *Embo J. 19*:3337-3348 (2000); Rohn, *J. Virol. 70*:8071-8080 (1996); Weng, *et al.*, *Mol. Cell. Biol. 23*:655-664)) and breast cancer (NOTCH-4, Callahan and Rafat, *J. Mammary Gland Biol Neoplasia 6*:23-36 (2001)). Identification of novel mutations in human tumors should be useful diagnostically in helping to identify the presence of cancer and in identifying cancer cells that respond to inhibitors of NOTCH signaling, thereby making it possible to direct rational cancer treatment with NOTCH signaling pathway inhibitors.

Summary of the Invention

The present invention is based upon the discovery that mutations in two specific regions of the NOTCH-1 receptor lead to increased receptor signaling and are associated with uncontrolled cellular growth. The first is a region within the heterodimerization domain that is characterized by the presence of many amino acids that are highly conserved between species. The amino acids (residues 1571-1622 in SEQ ID NO:1, see Figures 1and 3 for sequence identification numbers) are encoded by exon 26 and are found within the NEC

subunit (*i.e.* the extracellular portion) of the NOTCH-1 protein. In intact NOTCH-1, they are non-covalently bound to amino acids encoded by exon 27 (amino acids 1674-1730) which lie on the NTM subunit (*i.e.*, the transmembrane portion of the receptor). It is believed that mutations, especially non-conservative mutations, in these "hotspots" of the HD domain, *i.e.*, 1571-1622 of the NEC subunit and 1674-1730 of the NTM subunit, destabilize the NOTCH-1 heterodimer. This leads to dissociation of NEC and successive metalloprotease cleavage, ubiquination, endocytosis, and cleavage by gamma-secretase, permitting nuclear translocation of the intracellular portion of NOTCH-1 and activation of target genes.

A second region involved by mutations that lead to uncontrolled cellular growth is the C-terminal portion of NOTCH-1 (amino acid 2311 to 2556), which includes the PEST region (amino acid 2423 to amino acid 2556). These mutations appear to reduce the rate at which the receptor is degraded intracellularly after activation, *i.e.*, after it has been released due to gamma-secretase cleavage. As a result, the transcription of NOTCH-1-responsive genes continues for an inappropriately long period of time, again, leading to increased signaling and, ultimately, uncontrolled growth.

It has been discovered that mutations in the HD domain and in the PEST domain are often found in leukemic cell lines and in biological samples obtained from patients with T cell acute lymphoblastic leukemia (T-ALL). Uncontrolled growth in these cells may be arrested using agents that interfere with NOTCH signaling, such as gamma-secretase inhibitors. Overall, the experiments performed have led to the conclusion that there is a group of cancer cells that depend upon abnormal NOTCH activity to maintain uncontrolled growth and that mutations in the HD domain and in the PEST domain of NOTCH-1 may be used diagnostically to identify cancer cells likely to respond to gamma-secretase inhibitors or other factors that diminish NOTCH activity.

In its first aspect, the invention is directed to a substantially purified mutant NOTCH-1 receptor which has the amino acid sequence of wild type NOTCH-1, shown herein as SEQ ID NO:1, except for mutations within the hotspots of the HD domain. These mutations lead to increased receptor signaling relative to unmutated NOTCH-1, due to increased susceptibility to cleavage by gamma-secretase. The specific hotspot regions of

SEQ ID NO:1 in which the mutations occur are amino acids 1571-1622 and/or amino acids 1674-1730.

As used herein, the term "substantially purified" means that the receptor protein (or the polynucleotide encoding the protein) has been separated from the biological components associated with NOTCH-1 as found in nature. Typically, a substantially pure protein (or polynucleotide) will comprise at least 85% of a sample, with greater percentages being preferred. Many means are available for assessing the purity of protein or nucleic acid samples, including analysis by electrophoresis, chromatography and analytical centrifugation. The term "unmutated NOTCH-1" refers to NOTCH-1 receptors having the sequence of SEQ ID NO:1 and to polynucleotides that encode receptors having this sequence. The term also includes allelic variations in these sequences that are normally found in cells and which are not associated with abnormal NOTCH-1 signaling or with abnormal, uncontrolled cellular growth.

In order to determine whether a mutation leads to increased gamma-secretase signaling, the assays described herein in the Examples section may be used. The most preferred method involves the use of cells transfected with a reporter gene, *e.g.*, a polynucleotide encoding luciferase, that is under the control of a regulatory element turned on in response to NOTCH-1 signaling. Activation of the reporter gene upon exposure of cells to gamma-secretase can be readily measured and reflects NOTCH activity (see, *e.g.*, Hsieh, *et al.*, *Mol. Cell. Biol. 16*:952-959 (1996)).

Comparison of HD amino acid sequences from a variety of species has resulted in the identification of certain specific amino acid positions that are highly conserved in hotspots. Mutations in these positions, particularly non-conservative mutations, are particularly likely to destabilize the interaction between the NEC and NTM subunits of NOTCH-1. The term "non-conservative mutation" refers to a mutation in which wild type amino acids are replaced with amino acids having different chemical characteristics. Examples of a non-conservative substitution include the substitution of a proline for other amino acids within a well-structured protein domain (*i.e.*, the HD domain), the substitution of a basic amino acid for a non-basic amino acid, the substitution of an acidic amino acid for a non-acidic amino acid, the substitution of a polar amino acid for a non-polar amino

acid, etc. The distinction between conservative and non-conservative mutations is well recognized by those of skill in the art. Conserved amino acids in the HD domain where mutations are associated with increased NOTCH-1 signaling are found at positions: 1571; 1573; 1575; 1576; 1578; 1579; 1583; 1586; 1588; 1590; 1593; 1594; 1595; 1598; 1600-1607; 1610-1618; 1674; 1675; 1677; 1679-1684; 1686; 1695; 1696; 1703; 1706; 1715; 1720; 1725; and 1728. All of these positions are identified with reference to SEQ ID NO:1.

The invention includes specific mutations that are associated with the uncontrolled growth of cancer cells. With respect to the HD domain, this occurs in cases where amino acids 1571-1618 are replaced with a sequence selected from the group consisting of SEQ ID NO:12 – SEQ ID NO:22, SEQ ID NO:102-118 or SEQ ID NO:145. Other mutations associated with the growth of cancer cells include the replacement of amino acids 1571-1622 with a sequence selected from the group consisting of SEQ ID NO:23 – SEQ ID NO:29 or SEQ ID NO:119-127. Similarly, the sequence from amino acid 1674 to amino acid 1730 may be replaced with a sequence selected from the group consisting of SEQ ID NO:70 - SEQ ID NO:75 or SEQ ID NO:128-137.

The inventors have also found that mutations in SEQ ID NO:1 occurring in the C-terminal portion of NOTCH-1, *i.e.*, from amino acid 2311 to amino acid 2556, lead to increased NOTCH-1 signaling in response to exposure of cells to gamma-secretase. All of these mutations cause the appearance of a premature stop codon or a shift in the translational reading frame, and thus lead to the loss of some or all of the sequences corresponding to the PEST region (amino acid 2423 to 2556). It is believed that these mutations lead to a reduced rate at which receptor is degraded. Specific mutations include the replacement of the portion of SEQ ID NO:1 corresponding to amino acids 2311-2556 with a sequence selected from the group consisting of SEQ ID NO:30 – SEQ ID NO:59 or SEQ ID NO:139-144.

Mutations in the HD domain and the PEST domain may occur independently of one another or may be present in a single NOTCH-1 receptor. For example, a substantially purified mutant NOTCH-1 receptor, may comprise or consist of the amino acid sequence of SEQ ID NO:1, but wherein:

a) the receptor has at least one of the following mutations:

i) the portion of said amino acid sequence of SEQ ID NO:1 from amino acid 1571 to amino acid 1618 is replaced with a sequence selected from the group consisting of SEQ ID NO:12 – SEQ ID NO:22, SEQ ID NO:102-118, or SEQ ID NO:145;

ii) the portion of the amino acid sequence of SEQ ID NO:1 from amino acid 1571 to amino acid 1622 is replaced with a sequence selected from the group consisting of SEQ ID NO:23 – SEQ ID NO:29, SEQ ID NO:119-127;

iii) the portion of the amino acid sequence of SEQ ID NO:1 from amino acid 1674 to amino acid 1730 is replaced with a sequence selected from the group consisting of SEQ ID NO:70 – SEQ ID NO:75 or SEQ ID NO:128-137; and

b) and, in addition, the receptor is further mutated such that the portion of the amino acid sequence of SEQ ID NO:1 from amino acid 2311 to amino acid 2556 is replaced with a sequence selected from the group consisting of SEQ ID NO:30 – SEQ ID NO:59 or SEQ ID NO:138-144.

Alternatively, a substantially purified mutant NOTCH-1 receptor, may comprise or consist of the amino acid sequence of SEQ ID NO:1, but wherein:

a) the amino acid sequence of SEQ ID NO:1 is mutated in one or more amino acid positions selected from the group consisting of: 1571; 1573; 1575; 1576; 1578; 1579; 1583; 1586; 1588; 1590; 1593; 1594; 1595; 1598; 1600-1607; 1610-1618; 1674; 1675; 1677; 1679-1684; 1686; 1695; 1696; 1703; 1706; 1715; 1720; 1725; and 1728; and

b) the amino acid sequence of SEQ ID NO:1 is further mutated such that the portion of said amino acid sequence from amino acid 2311 to amino acid 2556 is replaced with a sequence selected from the group consisting of SEQ ID NO:30 – SEQ ID NO:59.

When mutations are present in both the HD and PEST regions, this leads to receptors in which signaling in response to gamma-secretase is especially enhanced.

In another aspect, the invention includes polynucleotides that encode any of the mutant NOTCH-1 receptors described above. Nucleic acid sequences coding for the mutant receptors may be incorporated into a vector in which they are operably linked to a promoter. The term "operably linked" refers to genetic elements that are joined in a manner that enables them to carry out their normal functions. For example, a gene is operably linked to a promoter when its transcription is under the control of the promoter and the protein normally encoded by the gene is ultimately produced. The invention includes the vectors coding for the mutant receptors as well as host cells transformed with the vectors.

In another aspect, the invention is directed to methods of diagnostically testing a biological sample for the presence of cancer cells by assaying the sample for any of the mutant NOTCH-1 receptors described above. The term biological sample, as used herein includes all of the different types of samples commonly used in the art of medicine in making diagnoses. Thus, the term encompasses, biopsy samples of tissue or fluid, blood, plasma, serum, lymph, urine, etc. Depending on the type of cancer being considered, certain types of samples will be the ones predominantly used. For example, for leukemia, samples of blood, plasma, or serum would tend to be favored. The choice of appropriate samples for testing is routine in clinical medicine

Also included are the same methods applied to the NOTCH-2, NOTCH-3 and NOTCH-4 receptors, which contain regions with high levels of identity to the regions with mutations described for NOTCH-1. The NOTCH-1, -2, -3 and -4 receptors are very highly related and likely perform very similar or identical functions in a variety of cell types, differing mainly in that different members of this receptor family are expressed and used preferentially by different tissues and cell types. Cancers arising in different tissues may thus acquire very similar types of mutations that affect any of the NOTCH-1,-2, -3 or -4 receptors with identical consequences in terms of promoting tumor cell growth, depending on which of the receptor subtypes are expressed by the tissues from which these cancers arise. Thus, in this application, unless otherwise indicated, the term "NOTCH" receptor

will be used to refer to diagnostic applications applied collectively to each of the NOTCH-1, -2, -3, and –4 receptors.

The presence of mutant receptor may be detected either by assays directed at the protein or by assays directed at the gene encoding the protein. With respect to assays directed at genes, the preferred method of testing is to amplify either the full-length polynucleotide encoding a mutant NOTCH receptor, or a portion of this polynucleotide, using the polymerase chain reaction (PCR). Oligonucleotide primers that can be used for amplification are described in the Examples section, but one of skill in the art can readily identify other appropriate primers based upon sequence information included herewith and known in the art.

Alternatively, gene mutations may be detected by performing hybridizations using probes under conditions that permit hybrids to form with mutant, but not wild type, nucleic acid. Both the assays and the oligonucleotides used in the assays are encompassed by the invention. The oligonucleotides should be at least 15 bases in length and hybridization assays should be carried out under stringent conditions. As used herein, the term "stringent conditions" refers to hybridizations carried out at approximately 60-70°C and at a low salt concentration (equivalent to, for example, 0.02 - 0.15 M NaCl).

Assays designed to detect the mutant protein per se may be carried out using antibodies that bind to mutant NOTCH receptors with at least a hundredfold greater affinity than to unmutated NOTCH receptors. These antibodies may be either monoclonal or polyclonal in nature and can be made by injecting an antibody-producing animal with peptides derived from regions of NOTCH receptor containing mutations. Antibodies made in this manner and which preferentially bind to mutant receptor are also a part of the invention.

The invention is also directed to methods for slowing or stopping the growth of cells with the mutant receptors described above by exposing the cells to an effective amount of an agent, such as a gamma-secretase inhibitor, that interferes with NOTCH receptor signaling. Examples of appropriate inhibitors include III-31-C, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]*S*-phenylglycine *t*-butyl ester) (DAPT), compound E,

D-helical peptide 294, isocoumarins, BOC-Lys(Cbz)Ile-Leu-epoxide, and $(Z\text{-}LL)_2$-ketone (see Kornilova, *et al.*, *J. Biol. Chem. 278*:16479-16473 (2003)). The term "effective amount" indicates a sufficient concentration of inhibitor or other agent to significantly reduce NOTCH-1 activity, for example, a sufficient amount to reduce activity by at least 20%. The presence of an effective amount of an agent that inhibits Notch receptor activity should also be reflected clinically. For example, the rate growth of cancer cells should be slowed to a significant extent (*e.g.*, by at least 20%) relative to the rate of growth occurring prior to treatment.

Although gamma-secretase inhibitors have been found to be effective as therapeutics, other agents that inhibit NOTCH activity may also be used. The mutations described herein should cause several effects, including: i) increased metalloprotease cleavage; ii) increased mono-ubiquitination (an enzymatic step carried out by an E3 ligase); iii) increased endocytosis, iv) increased gamma-secretase cleavage (as already discussed); v) increased nuclear translocation; and vi) increased formation of a nuclear NOTCH transcriptional activation complex. Drugs that act at any of these steps should stop the growth of cancer cells. One agent, an inhibitor of the nuclear complex, a dominant negative form of a protein called mastermind-like-1, has been previously described and inhibits all four NOTCH receptor subtypes (Weng, *et al.*, *Mol Cell Biol 23*:655-664 (2003)).

Also encompassed by the invention are methods of assaying a test compound for its ability to inhibit abnormal cellular growth induced by the presence of a mutated NOTCH receptor. This involves incubating the test compound with cells that express any of the mutant NOTCH-1 receptors described above. Such cells may either be isolated from a biological sample or created by transfecting a cell with an expression vector encoding the mutant receptor. The incubation is carried out in the presence of gamma-secretase and the amount of NOTCH receptor activity that occurs is determined. Any method for measuring receptor activity may be used, but the luciferase reporter gene assay described in Hsieh, *et al.* (*Mol. Cell. Biol. 16*:952-959 (1996)) is preferred. The results obtained are then compared with those from assays carried out under essentially the same conditions but in the absence of the test compound. A reduction in activity caused by the test compound suggests that it is a NOTCH receptor inhibitor and may be useful in limiting the growth of cells expressing

the receptor. This can be confirmed directly by comparing the growth of such cells in the presence and the absence of increasing concentrations of the test compound.

In addition to being of value in assays designed to evaluate the ability of a test compound to inhibit NOTCH receptor activity, polynucleotides encoding mutant NOTCH receptors may serve as a source for probes that can be used in the hybridization assays discussed above and as controls in diagnostic assays. The mutant receptor protein can be used to generate antibodies specific for mutated forms of NOTCH and can serve as a control in immunoassays designed to either quantitate the amount of NOTCH receptor present in a sample or to distinguish between normal and mutated forms of receptor. Both the mutant proteins and the polynucleotides encoding the mutant proteins should also be of interest to researchers studying biological processes such as differentiation and apoptosis.

As discussed above the invention includes isolated nucleic acid molecules encoding mutant Notch-1 receptors. Such nucleic acid molecules may comprise a nucleotide sequence selected from the group consisting of:

a) a sequence of nucleotides that encodes a mutant Notch-1 receptor comprising any of the mutant amino acid sequences described herein and wherein, except for the codons encoding the mutant amino acid(s), the nucleotide sequence is that of SEQ ID NO:76;

b) a sequence of nucleotides that encodes a mutant Notch-1 receptor having any of the amino acid sequences described herein;

c) a sequence of nucleotides degenerate with the mutant Notch-1 encoding sequence of (a) or (b).

In another aspect, the invention includes a method of determining the oncogenic potential of a biological sample by assaying the sample for the presence any of one or more mutant Notch-1 receptors as described herein. The presence of mutant receptors indicates increased oncogenic potential relative to a sample in which such mutant Notch-1 receptors are not present.

In addition, the invention includes a method for following the progress of a therapeutic regime designed to alleviate a condition characterized by abnormal expression of a mutant Notch-1 receptor comprising:

a) assaying a sample from a subject to determine the level of mutant Notch-1 receptor (or nucleic acid encoding a mutant Notch-1 receptor) at a first time point;

b) assaying the level of the same receptor (or nucleic acid) at a second time point; and

c) comparing the level at the second time point to the level determined at the first time point. Decreasing levels of mutant protein or nucleic acid is an indication that a therapy is working. Increasing levels are an indication that a therapy is not working.

The invention also encompasses a method for determining regression, progression or onset of a pathological disorder characterized by dysfunctional Notch-1 signal transduction. The method involves incubating a sample obtained from a patient having or suspected of having the disorder with a complementary nucleic acid hybridization probe having a sequence of nucleotides that is substantially homologous to that encoding one or more mutant Notch-1 receptors as described herein. The probe should generally be designed to interact with nucleic acid encoding the mutant form of the receptor and not the normal form as shown in SEQ ID NO:1. Binding between the probe and any complimentary mRNA that may be present in the sample is used to indicate the regression, progression or onset of the pathological disorder in said patient. Alternatively, assays may be performed using antibody (preferably monoclonal antibody) specific for a mutant form of the receptor. As the level of mutant receptor or nucleic acid encoding mutant receptor increases the likelihood that the disorder is present or progressing also increases. Reduced or absent levels of mutant receptor or nucleic acid is an indication of disorder regression or absence.

Brief Description of the Drawings

Figure 1-1 to 1-7: Aligned Full length amino acid sequences of human NOTCH receptors. The sequences shown are: NOTCH-1 (SEQ ID NO:1); NOTCH-2 (SEQ ID NO:2); NOTCH-3 (SEQ ID NO:3); and NOTCH-4 (SEQ ID NO:4).

Figure 2A-2C. Identification of T-ALL cell lines that require NOTCH signals for growth. (2A) Effect of the γ-secretase inhibitor compound E (GSI, 1 μM) or vehicle alone (mock) on the cell cycle distribution of sensitive cell lines. Cells were treated for 4-8 days, fixed in cold 70% ethanol, stained with propidium iodide, and analyzed by flow cytometry. Live cells were gated by forward/side scatter criteria. (2B) Rescue of GSI-induced cell cycle arrest by MSCV-GFP-ICN1 retrovirus. (2C) Induction of cell cycle arrest by dominant negative MSCV-GFP-Mastermind-like-1 retrovirus. In (2B) and (2C), control cultures were transduced with control empty MSCV-GFP retrovirus or the indicated test virus. Two days post-transduction, cultures were split in two and treated with either compound E (GSI) or DMSO vehicle (mock) for 7-10 days. Cultures were then harvested and stained with the DNA dye DRAQ5, which permits flow cytometric measurement of DNA content in unsorted GFP- and GFP+ subpopulations. A minimum of 15,000 events was collected to create each DNA content histogram.

Figure 3A-3G2. HD and PEST domain mutations in γ-secretase inhibitor-sensitive T-ALL cell lines and in primary human T-ALL samples. (3A) sequence conservation of the exon 26 HD domain mutational "hotspot" region among vertebrate NOTCH-1 receptors. Positions of recurrent mutations are in bold and italics. (3B) missense mutations and deletions identified in cell lines (underlined) and primary T-ALLs. One mutation SEQ ID NO:145, was associated with acute myelogenous leukemia. Mutation sites are in bold and italics. (3C) in-frame insertions in the HD domain identified in primary T-ALLs. (3D) PEST domain mutations. The amino acid sequences of mutated NOTCH-1 alleles in cell lines (underlined) and primary T-ALLs are shown. Stop codons are indicated by asterisks. (3E) Schematic representation of PEST domain mutations. The circles (white, primary T-ALLs; black, cell lines) correspond to the positions at which the NOTCH-1 polypeptide sequence terminates. Key: NEC, NOTCH-1 extracellular domain; LNR, Lin/NOTCH repeats; HD, heterodimerization domain; NTM, NOTCH transmem-brane subunit; TM, transmembrane domain; ANK, ankyrin repeat domain; TAD, transcriptional activation domain. In (A-D), amino acid residues correspond to those of human pro-NOTCH-1. (3F) Western blot analysis of lysates from NOTCH-dependent T-ALL cell lines. Whole cell extracts were analyzed with a NOTCH-1 antibody raised against the intracellular transcriptional activation domain (Aster, *et al.*, *Mol. Cell. Biol. 20*:7505-15). Positions of

pro-NOTCH-1 and the normal NTM subunit are denoted. The lower panel is a longer exposure of the same blot that accentuates the presence of additional NOTCH-1 polypeptides of smaller size than the normal NTM polypeptide. Each lane was loaded with 25 μg of total protein. (3G) Sequence conservation and mutations in exon 27, encoding the HD domain of the NOTCH-1 TMT subunit. This subunit of NOTCH is noncovalently bound to exon 26, the HD domain of the NEC subunit. Mutation sites are in bold and italics. For TALL 04.36 the i in the sequence indicates the site of insertion of the 14 amino acids shown immediately below.

Figure 4A-4B. HD and PEST domain mutations activate NOTCH-1 signaling synergistically. (4A) Reporter gene assays. U2OS cells were transiently co-transfected in 24-well format with the indicated pcDNA3 plasmids, a NOTCH-responsive luciferase reporter gene, and an internal *Renilla* luciferase internal control plasmid, as described previously (Aster, *et al.*, *Mol. Cell. Biol. 20*:7505-15). Twenty-five ng of pcDNA3 plasmid was used per well, except for experiments with pcDNA3-ICN1, in which 5 ng of plasmid per well were used. Normalized luciferase activities in whole cell lysates were determined in triplicate and expressed relative to the activity in lysates prepared from cells transfected with the empty vector control. (4B) Model for synergistic NOTCH-1 activation by dual mutations involving both the HD and PEST domains.

Figure 5-1 to 5-11: Full length human NOTCH-1 gene sequence (SEQ ID NO:76) and encoded amino acid sequence (SEQ ID NO:1).

Figure 6-1 to 6-3: Compilation of HD and PEST domain mutations.

Detailed Description of the Invention

The present invention is concerned with the identification of mutations in the human NOTCH-1 receptor that lead to an increase in receptor signaling. The mutations are associated with cancer cells that respond positively to treatment with agents that interfere with NOTCH-1 activation, such as gamma-secretase inhibitors and are located within two specific regions of the NOTCH-1 protein, the HD domain and the PEST domain. It is expected that analogous mutations in corresponding regions of NOTCH-2, NOTCH-3 and NOTCH-4 receptor subtypes should also lead to increased signaling and abnormal growth.

Assays designed to detect the presence of NOTCH-1 mutations, *e.g.*, assays based upon PCR amplification, may be used diagnostically to identify tissue samples with cells in which abnormal NOTCH-1 receptor signaling is occurring and which are, as a result, more likely to exhibit uncontrolled growth. Cancers characterized by this particular abnormality should be amenable to treatment with agents that reduce NOTCH-1 signaling, such as gamma-secretase inhibitors. Any of the inhibitors described herein or which have been described in the art (for example, in connection with potential use in the treatment of Alzheimer's disease) may be used in treatment regimens. The diagnostic assays and therapeutic methods may be applied to other types of cancer besides T-ALL. Such cancers include but are not limited to: carcinomas of the breast, prostate, lung, pancreas, ovary, colon, bladder, kidney and GI tract, B cell leukemias, B and T cell lymphomas, Hodgkin's lymphoma, acute myelogenous leukemias, melanomas, neuroblastomas, medulloblastomas, glioblastoma multiforme and other glial cancers of the brain, and mesotheliomas.

I. Production of Polynucleotides Encoding Mutant NOTCH-1 Receptors

The full length amino acid sequence of human NOTCH-1 is shown in Figure 1 and has been given the sequence identification number SEQ ID NO:1. This is the normal, unmutated form of receptor. Mutations in the HD domain and PEST domain that have been associated with samples from cancer patients or cancer cell lines are shown in Figure 3. It should be noted that certain positions in the hotspots of the HD domain are highly conserved among different species (see Figure 3A) and are especially sensitive to mutations that lead to increased gamma-secretase-generated activity.

Many methods are available for producing polynucleotides that encode NOTCH-1 receptors having the mutated sequences shown herein (see, *e.g.*, Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press (1989)). For example, a cDNA library prepared by reverse transcribing mRNA isolated from cells that express the mutant NOTCH gene may be screened using probes synthesized based upon the sequences shown in Figure 1. Alternatively, the desired sequences may be obtained by PCR amplifying reverse transcribed RNA. Primers for PCR may be constructed using the sequences shown herein and specific oligonucleotides that have been found to be suitable for amplification are described in the Examples section. Confirmation that the correct

sequence has been amplified and identification of the presence of specific mutations may be obtained by sequencing amplification products.

Alternatively, nucleic acids may be produced using standard chemical synthetic methods well known in the art. Once obtained, the polynucleotides can be used in recombinant methods for making receptor protein, expressed in cells for the purpose of screening for potential therapeutic agents, or used as controls in diagnostic assays.

II. Production of Mutant Receptor Protein

NOTCH-1 receptor protein can be synthesized using chemical methods or produced using the polynucleotides described above. In the latter case, the DNA sequence encoding the mutant receptor may be placed in a vector containing transcriptional and translational signals recognizable by an appropriate host. The cloned receptor sequences, in double-stranded form, are inserted into the expression vector in an operable linkage. Appropriate vectors and techniques for cloning and expressing receptor are well known in the art of molecular biology.

Expression vectors may be introduced into host cells, preferably mammalian host cells, by methods such as calcium phosphate precipitation, microinjection, electroporation or viral transfer. Cells expressing the recombinant mutant receptor can be selected using standard, well established methods. One simple method for confirming the presence of the mutant receptor nucleic acid in cells is to perform PCR amplification using primers known to flank the receptor-encoding nucleotide sequence. The presence of receptor may also be confirmed using functional assays of NOTCH-1 such as the luciferase assay described in the Examples section and in the art (see, Hsieh, *et al.*, *Mol. Cell. Biol. 16*:952-959 (1996)). Once cells producing recombinant mutant NOTCH-1 receptor have been identified, they may be used in assays designed to identify agents capable of reducing signaling, for example, by incubation with gamma-secretase, or in assays that measure the ability of NOTCH-1 inhibitors to arrest uncontrolled cellular growth. The NOTCH-1 receptor itself may be used in diagnostic assays or to generate antibodies specific for mutant receptor that can be used in such assays.

III. Antibodies to Mutant NOTCH Receptor

The present invention encompasses antibodies that bind specifically to mutant forms of NOTCH receptors and processes for producing such antibodies. Antibodies that "bind specifically to mutant NOTCH receptors" are defined as those that have at least a hundredfold greater affinity for the mutant form of the receptor than for the unmutated form. The process for producing such antibodies may involve either injecting the full length mutant receptor protein into an appropriate animal or, preferably, injecting short peptides that include regions where mutations occur. The peptides should be at least five amino acids in length and may be injected either individually or in combinations.

Methods for making and selecting antibodies are well known to those of skill in the art as evidenced by standard reference works such as Harlow, *et al.*, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. (1988); Klein, Immunology: The Science of Self-Nonself Discrimination (1982); ; Kennett, *et al.*, Monoclonal Antibodies and Hybridomas: A New Dimension in Biological Analyses (1980); and Campbell, "Monoclonal Antibody Technology," in: Laboratory Techniques in Biochemistry and Molecular Biology (1984).

The term "antibody," as used herein, is meant to include intact molecules as well as fragments that retain their ability to bind antigen such as Fab and $F(ab')_2$ fragments. The term "antibody" is also defined as referring to both monoclonal antibodies and polyclonal antibodies. Polyclonal antibodies are derived from the sera of animals immunized with an appropriate antigen. Monoclonal antibodies can be prepared using hybridoma technology as taught by references such as: Hammerling, *et al.*, in Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981). In general, this technology involves immunizing an immunocompetent animal, typically a mouse, with either intact protein or a fragment derived therefrom. Splenocytes are then extracted from the immunized animal and are fused with suitable myeloma cells, such as SP_2O cells. Thereafter, the resulting hybridoma cells are selectively maintained in HAT medium and then cloned by limiting dilution (Wands, *et al.*, *Gastroenterology 80*:225-232 (1981)). The cells obtained through such selection may then be assayed to identify clones that secrete antibodies binding preferentially to mutant forms of NOTCH-1 or mutant forms of other NOTCH receptors.

The antibodies, or fragments of antibodies, of the present invention may be used to detect the presence of mutant NOTCH receptor proteins using a variety of immunoassays. For example, the antibodies may be used in radioimmunoassays or immunometric assays, also known as "two site" or "sandwich" assays (see Chard, "An Introduction to Radioimmune Assay and Related Techniques" in: Laboratory Techniques in Biochemistry and Molecular Biology, North Holland Publishing Co., N.Y. (1978)). In a typical immunometric assay, a quantity of unlabeled antibody is bound to a solid support that is insoluble in the fluid being tested, *e.g.*, blood, lymph, cellular extracts, etc. After the initial binding of antigen to immobilized antibody, a quantity of detectably labeled second antibody (which may or may not be the same as the first) is added to permit detection and/or quantitation of bound antigen (see, *e.g.*, Kirkham, *et al.*, Radioimmune Assay Methods, pp. 199-206 (1970)). Many variations of these types of assays are known in the art and may be employed for the detection of mutant NOTCH receptors.

Antibodies to mutant NOTCH receptors may also be used in the purification of either the intact receptor or fragments of these receptors (see generally, Dean, *et al.*, Affinity Chromatograph, A Practical Approach, IRLP Press (1986)). Typically, antibody is immobilized on a chromatographic matrix such as Sepharose 4B. The matrix is then packed into a column and a preparation containing mutant receptor is passed through the column under conditions that promote binding, *e.g.*, under conditions of low salt. The column is then washed and bound receptor is eluted using a buffer that promotes dissociation from antibody (*e.g.*, a buffer having an altered pH or salt concentration). The eluted receptor protein may be transferred into a buffer of choice, *e.g.*, by dialysis and either stored or used directly. Purified receptor may be used in the immunoassays described above or for the generation of antibodies for use in assays.

IV. Assay Methods

Diagnostic assays for determining whether a biological sample contains cancer cells in which the mutations described herein contribute to uncontrolled growth may be performed either using the immunoassays described above to analyze protein or, preferably, by assaying a sample for the presence of nucleic acid sequences encoding a mutant form of receptor. The preferred method for nucleic acid analysis is by PCR amplifying polynucleic acid that has been extracted from cells using methods well known in the art. Amplification

may be performed using the oligonucleotide primers described in the Examples section below or using other oligonucleotides based upon the sequence of NOTCH-1 shown as SEQ ID NO:1. The amplified NOTCH receptor product may then be sequenced to determine whether it carries a mutation leading to increased signaling.

Alternatively, hybridizations may be performed under stringent conditions using probes that bind only to mutant sequences. The probes should be a minimum of 15 nucleotides in length and must cover the portion of the polynucleotide sequence that encodes the mutated region of NOTCH-1. Stringent conditions would typically involve hybridization at a temperature of 60-70°C with a low salt concentration (*e.g.*, 0.02 M to 0.15 M NaCl). Procedures for identifying and labeling probes as well as for carrying out hybridizations and analyzing results are well known in the art of molecular biology.

Host cells transformed with vectors encoding recombinant mutant NOTCH receptors may also be used in assays designed to identify test compounds that reduce inappropriate signaling by the receptor. The assays will typically involve incubating cells in the presence of gamma-secretase and test compound. NOTCH receptor signaling can then be quantitated using assays such as those described herein or using other assays that are known in the art (see also, Hsieh, *et al.*, *Mol. Cell. Biol. 16*:952-959 (1996)). As a simple alternative, cells known to be expressing mutated forms of NOTCH-1 that lead to uncontrolled growth may be cultured in the presence and absence of gamma-secretase and test compound to determine the extent to which the compound is capable of arresting growth.

V. Treatment Methods

Gamma-secretase inhibitors have been developed by pharmaceutical companies interested in their potential use as a treatment for Alzheimer's disease. These same inhibitors can be used to treat cancer cells in cases in which uncontrolled growth is associated with the NOTCH receptor mutations described herein.

The therapeutic regimen involves first testing a biological sample containing cancer cells removed from a patient to determine whether a mutated form of a NOTCH receptor is present. Patients whose samples evidence the presence of mutations would then be treated using a gamma-secretase inhibitor or another agent that interferes with NOTCH receptor

activity. The dosage administered will depend upon the particular condition being treated, the route of administration and clinical considerations that are well known in the art. Dosages can be gradually increased until a beneficial effect, *e.g.*, a slowing of tumor growth, is detected. Agents may be provided in either single or multiple dosage regimens and may be given either alone or in conjunction with other therapeutic agents.

Treatment of mutant NOTCH receptor-associated cancers is compatible with any route of administration and dosage form. Depending upon the particular condition being treated, certain dosage forms will tend to be more convenient or effective than others. For example, topical administration may be preferred in treating skin cancers, whereas parenteral administration might be preferred for leukemias. Apart from parenteral and topical preparations, agents may be administered orally, perorally, internally, intranasally, rectally, vaginally, lingually and transdermally. Specific dosage forms include tablets, pills, capsules, powders, aerosols, suppositories, skin patches, parenterals and oral liquids including suspensions, solutions and emulsions. Sustained release dosage forms may also be used. All dosage forms may be prepared using methods that are standard in the art (see, *e.g.*, Remington's Pharmaceutical Sciences, 16th ed., Easton, PA (1980)).

Examples

The present example provides results suggesting that approximately 65% of human T cell acute lymphoblastic leukemias (T-ALLs), including tumors from all major molecular oncogenic subtypes and those occurring in children and adults, have *NOTCH-1* mutations. Most of these mutations occur in regions encoding an extracellular heterodimerization domain and as truncations removing a C-terminal PEST destruction box. The mutations cause increased NOTCH-1 signaling, and T-ALL cell lines bearing such mutations are growth arrested by NOTCH-1 pathway inhibitors.

Introduction

T cell acute lymphoblastic leukemia (T-ALL) is an aggressive neoplasm that preferentially affects children and adolescents. It is commonly associated with acquired chromosomal translocations and other genetic or epigenetic abnormalities, which lead to aberrant expression of a select group of transcription factors, including the helix-loop-helix

proteins TAL1, TAL2, LYL1, and BHLHB1, the orphan homeobox proteins HOX11 and HOX11L2, and the fusion proteins MLL-ENL and CALM-AF10. Mis-expression of these proteins, alone or in combination, perturbs normal pathways of T cell development, growth, and survival, and defines subgroups of T-ALL with distinct gene expression profiles. Nevertheless, enforced expression of any one of these proteins alone in murine models is not sufficient to cause T-ALL, emphasizing the multistep molecular pathogenesis of this disease.

Materials and Methods

Cell Lines

All T-ALL cell lines were cultured in RPMI (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μ/ml streptomycin at 37°C under 5% CO_2. U2OS cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μ/ml streptomycin at 37°C under 5% CO_2.

Gamma-Secretase Inhibitor Treatment of Cell Lines

Gamma-secretase activity was inhibited by addition of compound E to growing cells to a final concentration of 1 μ/M. Mock-treated cultures were exposed to vehicle only, DMSO at a final concentration of 0.01%. The following human T-ALL cell lines were tested for sensitivity to compound E: T-ALL-1, ALL-SIL, KOPT-K1, HPB-ALL, DND-41, CCRF-HSB2, RPMI8402, THP-6, BE-13, JM-Jurkat, Jurkat-E6, REX, CEM, LOUCY, DU-528, KE-37, SKW-3, MKB-1, MOLT-3, MOLT-4, MOLT-13, MOLT-15, MOLT-16, SUPT-7, SUPT-11, SUPT-13, P12-Ichikawa, KARPAS-45, DSMZ-PEER and PF-382. The murine cell line T-6E, which was derived from a T-ALL induced with a membrane-tethered form of truncated NOTCH-1 that requires gamma-secretase cleavage for activation, was used as a positive control.

Retroviruses

Construction of retroviral expression plasmids (MSCV-GFP-ICN1 and MSCV-dominant negative Mastermind-like-1-GFP), production of high-titer pseudotyped,

amphotropic, replication-defective retroviruses and spinoculation procedure were performed as described previously (Weng, *et al.*, *Mol. Cell Biol. 23*:655-664).

Cell Cycle Analysis

Cells were stained with either propidium iodide after overnight fixation in cold 70% ethanol, or DRAQ5 (Biostatus, Leicester, UK) in complete medium (as suggested by the manufacturer) in instances where GFP fluorescence and DNA content measurement were measured simultaneously. DNA content was ascertained by flow cytometry and cell cycle factors determined using multicycle software (Phoenix Flow Systems, San Diego).

NOTCH-1 Expression Plasmids

NOTCH-1 expression plasmids in pcDNA3 have been described previously. To create a generic PEST deletion, the insertion mutation in the PEST domain of the T-ALL cell line ALL-SIL was amplified by PCR, identified by sequencing, and then sub-cloned into pcDNA3-NOTCH-1. Site-directed mutagenesis of the HD domain was conducted using the QuikChange kit (Stratagene). Mutagenic PCR primers were designed using the Stratagene web tool.

Receptor Gene Assays

NOTCH-1 expression plasmids (pcDNA3) were transiently transfected with U2OS cells with (i) an artificial luciferase reporter gene under the control of a promoter containing CSL binding sites and (ii) an internal Renilla luciferase control gene, as described previously (Aster, *et al.*, *Mol. Cell Biol. 20*:7505-7515 (2000)), using Lipofectamine Plus (Invitrogen). Total DNA was kept constant by adding empty vector as needed. All transfections were carried out in triplicate, and each experiment was repeated at least three times. Cell lysates were harvested 44-48 hours post-transfection and luciferase assays were carried out using the Dual Luciferase Assay System (Promega) on a Turner Systems luminometer, as per the manufacturer's suggestions.

PCR Amplification of NOTCH-1 cDNAs

RNA isolated from T-ALL cell lines (1) μ/g was reverse transcribed into cDNA using oligo-dT priming and Superscript reverse transcriptase (Invitrogen) in 20 μl reactions per the manufacturer's suggested conditions. PCR amplifications were performed using

1 µl of cDNA as template, 20 pMol of 5′-TTTGAATTCGGGCTGGACTGTGCGGAGCA TGTACCCGA (SEQ ID NO:78) and 5′-TTTGGATCCTCCGGAATGCGGGCGATCTG GGACTGCA (SEQ ID NO:79) NOTCH-1-specific primers, 0.25 µl of Ex-Taq Takara, Japan), 1X reaction buffer, 1XdNTPs and 7.5% DMSO in a 50 µl reaction volume. Cycle parameters were: 94°C for three minutes; 30 cycles of 94°C for one minute; 60°C for thirty seconds; 72°C for two minutes; and 72°C for eight minutes. PCR products were purified on Qiagen columns, digested with Bam HI and Eco RI, ligated into pSP72 and used to transform DH5 alpha cells. Miniprep DNA from individual colonies was sequenced using T7 and SP6 primers.

Patient Material

Samples of cryopreserved lymphoblasts from 96 children and young adults with T-ALL treated at St. Jude Children's Research Hospital and Dana Farber Cancer Institute were obtained with informed consent at the time of diagnosis. Genomic DNA from each sample was extracted with a commercial kit (GENTRA) following the manufacturer's instructions. RNA was prepared from cryopreserved lymphoblasts with RNAaqueous reagents (Ambion) according to the manufacturer's instructions.

Quantitative RT-PCR

Quantitative RT-PCR analysis of HOX11, HOX11L2, TAL1, LYL1, TAL2 and BHLHB1 was performed as previously described (Ferrando, *et al.*, *Cancer Cell 1*:75-87; Ferrando, *et al.*, *Lancet 363*:535-536) using an ABI PRISM 7700 Sequence Detection System instrument (Perkin Elmer Applied Biosystems). RT-PCR-based detection of MLL-ENL and CALM-AF10 fusion transcripts was also performed as previously described (Ferrando, *et al.*, *Lancet 363*:535-536; Asnafi, *et al.*, *Blood 102*:1000-1006).

Genomic DNA Preparation

Genomic DNA was prepared from primary T-ALLs by extraction with a commercial kit (GENTRA) following the manufacturer's instructions. DNA was prepared from cell lines by SDS lysis/proteinase K digestion, followed by phenol:chloroform extraction and ethanol precipitation. DNAs were resuspended in TE buffer, spectrophotometrically quantified and stored at -20°C.

Mutation Detection

Mutation detection in exons 26, 27, and 34 of *NOTCH-1* was performed via PCR-based denaturing HPLC using a WAVE DNA fragment analysis system (Transgenomic) equipped with a DNASep HT cartridge. Amplicons for mutation analysis were prepared with the following primer combinations. The N-terminal region of the HD domain of NOTCH-1, encoded in exon 26 was divided into two amplicons. Amplicon HD-N1 was amplified by PCR using primers HD-N1FW1: 5′-AGCCCCCTGTACGACCAGTA (SEQ ID NO:80), HD-N1RV1: 5′-CTTGCGCAGCTCCTCCTC (SEQ ID NO:81), and by nested PCR using primers HD-N1FW2: 5′-GACCAGTACTGCAAGGACCA (SEQ ID NO:82), HD-N1RV2: 5'-TCCTCGCGGCCGTAGTAG (SEQ ID NO:83). Amplicon HD2 was amplified by PCR using primers HD-N2FW1: 5′-GTGCTGCACACCAACGTG (SEQ ID NO:84), HD-N2RV1: 5′-GAGGGCCCAGGAGAGTTG (SEQ ID NO:85), and by nested PCR using primers HD-N2FW2: 5′-GCACACGGCCAGCAGATGAT (SEQ ID NO:86), and HD-N2RV2: 5′-CGCCGGGTCTCACTCAC (SEQ ID NO:87).

The C terminal region of the HD domain of NOTCH 1, encoded in exon 27, was amplified by PCR using primers HD-CFW1: GTGGCGTCATGGGCCTCA (SEQ ID NO:88) and HD-CRV1: TAGCAACTGGCACAAACAGC (SEQ ID NO:89), and by nested PCR using primers HD-CFW2: CATGGGCCTCAGTGTCCT (SEQ ID NO:90) and HD-CRV2: GCACAAACAGCCAGCGTGTC (SEQ ID NO:91).

Sequences of exon 34 encoding the PEST domain and the continuous N-region containing the TAD of NOTCH-1 were divided into three amplicons. Amplicon PEST1 and PEST2 were amplified by PCR using primers PESTFW1: 5′-GCAGCATGGCATGGTAGG (SEQ ID NO:92), and PESTRV1: 5′-AACATGTGTTTTAAAAAGGCTCCTC (SEQ ID NO:93), and by nested PCR using primers PEST1FW: 5′-AAACATCCAGCAGCAGC AAA (SEQ ID NO:94), PEST1RV: 5′-CACAGGCGAGGAGTAGCTGTG (SEQ ID NO:95), and PEST2FW: 5′-GTGACCGCAGCCCAGTTC (SEQ ID NO:96), and PEST2RV: 5′-AAAGGAAGCCGGGGTCTC (SEQ ID NO:97). Amplicon TAD1 was amplified by PCR using primers TAD1FW1: 5′-AGACTGGCCCACCTCGTCTCT (SEQ ID NO:98), TAD1RV1: 5′-GCTCTCCACTCAGGAAGCTC (SEQ ID NO:99), and by nested PCR using primers TAD1FW2: 5′-CGTCTCTCCCACCTGCCTGT (SEQ ID NO:100), and TAD1RV2: 5′-CTGAGCTCACGCCAAGGT (SEQ ID NO:101). Before

DHPLC analysis, nested PCR products were denatured at 95°C for five minutes and slowly ramped to 40°C using a Peltier Thermal Cycler (MJ Research, PTC-225) in order to allow the formation of homo- and hetero-duplexes in samples heterozygous for a mutant allele.

Fifteen to 30 microliters of annealed PCR fragments per sample were injected on to the DNASep HT cartridge for analysis. Products were eluted at a constant flow rate of 1.5 ml/min with a linear acetonitrile gradient determined by Navigator software (Transgenomic) based on the size and GC-content of the amplicon. The gradient was produced by combining 0.1 m trimethylammonium acetate (TEAA) buffer (pH 7) (Transgenomic) and Buffer B (0.1 m TEAA with 25% acetonitrile). The elution profiles of DNA fragments, monitored by the system's UV detector, were used to produce chromatographs. The analysis sequence for each sample was as follows: a 1.9-minute time lapse for the detector, a 0.1-minute loading stage with a 3% decrease in Buffer B, a 2.0-minute linear gradient with an increasing slope of 2% Buffer B per minute, a 0.1-minute cleaning stage using 75% acetonitrile, and a 0.1-minute equilibration before the next injection. Homo- and hetero-duplex peaks were detected between the initial injection peak, produced by residual nucleotides and primers in the reaction, and the wash stage.

Melting profiles for the nested PCR products, ranging from 276 to 452 based pairs, were constructed using the Navigator software. Appropriate partial denaturing temperatures for mutation detection, which produced percent helicities between 30% and 98% at each base contained in regions of interest, were predicted for all amplicons: PEST1, 66.5°C; PEST2, 65.0°C; HD1, 67.0°C and 64.5°C; HD2, 67.0°C and 64.0°C; and TAD1, 64.5°C and 63.9°C. Less stable heteroduplexes denature earlier than homoduplexes and, thus, appear first in elution profiles. Therefore, direct sequencing was performed on samples with chromatographs displaying species that eluted before normal homoduplexes.

Results

To evaluate the possible role of NOTCH-1 signaling in human T-ALL beyond the rare cases associated with the t(7;9), we first tested T-ALL cell lines lacking the t(7;9) for NOTCH dependency by treating these cells with a γ-secretase inhibitor (Weng, *et al.*, *Mol. Cell. Biol. 23*:655-64). We observed that five of thirty human T-ALL cell lines tested showed a G_0/G_1 cell cycle arrest, which equaled or exceeded that of the T6E cell line, a

reference NOTCH-1-dependent murine T-ALL (Fig. 2A). The growth suppression caused by gamma-secretase inhibitor was abrogated by retroviral expression of ICN1 (Fig. 2B), which is the fragment released after γ-secretase cleavage, and reproduced by retroviral expression of dominant negative Mastermind-like-1 (Fig. 2C). Taken together, these results indicated that the growth of these five cell lines depends on NOTCH-mediated signals.

Because NOTCH receptors can be activated by physical dissociation of the NOTCH extracellular domain (Rand *et al.*, *Mol. Cell. Biol. 20*:1825-35; Kramer, *Sci. STKE* 2000, PE1), we reasoned that a newly identified HD domain of NOTCH-1 could be the site of gain-of-function mutations. One class of retroviral insertion observed in murine T-ALL causes deletion of the negative regulatory C-terminal PEST sequences (Hoemann, *et al.*, *Mol. Cell. Biol. 20*:3831-42; Feldman, *et al.*, *Blood 96*:1906-13), making this a second candidate region for oncogenic NOTCH-1 mutations. Remarkably, DNA sequencing of these genomic regions revealed both HD domain and PEST domain mutations in 4 of the 5 NOTCH-dependent cell lines. Missense mutations affecting the HD domain (encoded by exon 26 of *NOTCH-1*) involved residues that are invariant in vertebrate NOTCH-1 receptors, and caused non-conservative changes in amino acid residues (Fig. 3A-C). One cell line, DND-41, had two different HD domain mutations within the same *NOTCH-1* allele.

The PEST mutations (found in exon 34) were short insertions or deletions causing shifts in reading frame that are predicted to result in partial or complete deletion of the negative regulatory, C-terminal PEST domain (Fig. 3D and 3E). PCR amplification, cloning, and sequencing of cDNAs revealed that the HD domain and PEST domain mutations lay in cis in the same *NOTCH-1* allele in each of the 4 cell lines tested. Normal *NOTCH-1* cDNA clones were also identified in each cell line, indicating that both alleles are expressed. This is consistent with the results of Western blot analysis (Fig. 3F), which revealed that cell lines with exon 26 and 34 mutations contained a polypeptide of the expected size of NTM and additional aberrant polypeptides of slightly smaller size than NTM. In contrast, the cell line TALL-1, which lacks mutations in exons 26 and 34, contained only NOTCH-1 polypeptides of the expected sizes.

We extended our mutational analysis of the HD and PEST domains of NOTCH-1 to cryopreserved primary T-ALL samples obtained from the bone marrow of ninety-six children and adolescents at the time of diagnosis. At least one mutation was identified in 49 tumors (51%); 20 tumors (20.8%) had HD domain mutations only, 15 tumors (15.6%) had PEST domain mutations only, and 14 tumors (14.6%) had mutations in both the HD and PEST domains. Mutations were seen in tumors associated with mis-expression of HOX11 (2 of 3 cases), HOX11L2 (10 of 13, or 77%), TAL1 (11 of 31, or 35%), LYL1 (7 of 14, or 50%), MLL-ENL (1 of 3), or CALM-AF10 (1 of 2), which together define the major molecular subtypes of T-ALL. The HD domain mutations in primary tumors were clustered in a "hotspot" spanning residues 1574-1622, and included each of the three L to P missense mutations originally identified in the NOTCH-dependent T-ALL cell lines, as well as deletions of 1-2 residues and short "in-frame" insertions (Fig. 3A-B). PEST domain mutations included insertions or deletions that induced a shift in reading frame, as well as point mutations that created premature stop codons (Fig. 3D-E, and Fig. 6).

In contrast to T-ALLs, no mutations were observed in 89 primary B-ALL samples. Mutations were also absent from four remission bone marrow samples obtained from patients whose T-ALLs harbored NOTCH-1 mutations, indicating that these mutations are not present in the germline, but rather acquired within the malignant clones. Polymorphisms have not been detected within the HD domain region of NOTCH-1 (NCBI SNP database), leading us to further suspect that the mutations in conserved residues we detected in T-ALLs were likely to have functional consequences.

To address this issue directly, we studied the effects of common HD domain mutations, with and without a PEST domain deletion, on activation of a NOTCH-sensitive luciferase reporter gene in transient expression assays (Fig. 4A). Single L to P mutations within the HD domain at residues 1575, 1594, or 1601 caused a 3-9-fold stimulation of luciferase activity, while an isolated PEST deletion (corresponding to the mutation found in the ALL-SIL cell line that produces a truncation at amino acid 2471) resulted in an approximate 1.5-2-fold stimulation. More strikingly, each HD mutation and the same PEST domain truncation in cis resulted in 20-40-fold stimulations of transcriptional activation. In contrast, the same mutations in trans produced lower levels of stimulation that were close to the average of each mutation acting alone.

Additional reporter gene assays carried out as described in Figure 4 have proven that all HD mutations tested to date convey increased function on NOTCH-1. The mutations tested in these functional assays are:

V1577E	Sequence ID NO:102
L1586Q	Sequence ID NO:106
F1593S	Sequence ID NO:117
L1597H	Sequence ID NO:109
R1599P	Sequence ID NO:15
I1617N	Sequence ID NO:115
I1617T	Sequence ID NO:116
V1677D	Sequence ID NO:135
L1679P	Sequence ID NO:71
I1681N	Sequence ID NO:73
A1702P	Sequence ID NO:129
I1719T	Sequence ID NO:128
ins(RLGSLNIPYKIEAV)	Sequence ID NO:137

These data suggest that increased function will be a general feature of mutations found in the HD domain of NOTCH1.

The synergistic interaction of HD and PEST domain mutations in cis is consistent with a model in which (i) HD domain mutations enhance γ-secretase cleavage and increase the rate of production of ICN1, and (ii) truncations removing the PEST domain of ICN1 increase the half-life of this activated form of NOTCH (Fig. 4B). The intermediate levels of activation produced by these mutations in trans presumably reflect competition between relatively weak and strong gain-of-function NOTCH-1 polypeptides for factors required for processing and signaling. The stimulatory effects of mutated transmembrane NOTCH-1 polypeptides were completely abrogated by a γ-secretase inhibitor, indicating a requirement for proteolysis at the juxtamembrane site of mutated NTM subunits for signal transduction (Fig. 4A). In contrast, the stimulation produced by ICN1, which is constitutively nuclear, was unaffected by γ-secretase inhibition (Fig. 4A).

More recently we have observed two mutations in tumors other than T-ALL. An insertional mutation in exon 26 of NOTCH-1 was found in a case of acute myelogenous leukemia that causes amino acid residues 1593-1594 to be changed from FL to LSL. This mutation stimulates the ability of NOTCH-1 to activate a CSL-sensitive luciferase gene in assays analogous to those shown in Figure 4A, indicating that it increases NOTCH-1 function. A novel NOTCH-1 mutation in exon 27 was found in a case of Langerhans cell histiocytosis, in which a highly conserved residue, amino acid 1693, was changed from a C to an R. Together, these data support the prediction that mutations identical or functionally similar to those discovered in T-ALL will be found in other forms of human neoplasia.

Discussion

The prevalence of gain-of-function NOTCH-1 mutations, which are found in every major molecular oncogenic subtype of T-ALL, strongly supports a pre-eminent role for upregulated NOTCH signaling in the pathogenesis of this tumor. The existence of at least one cell line, TALL-1, that apparently requires NOTCH signals for growth, yet lacks NOTCH-1 HD domain and PEST region mutations, suggests that other mechanisms exist that account for pre-T cell transformation involving the NOTCH pathway. It is conceivable that activating mutations affecting other regions of NOTCH-1 may be identified in human T-ALL cases.

Prior work has shown that activation of NOTCH receptors is achieved primarily through release of the NEC subunit (Rand, *et al.*, *Mol. Cell. Biol. 20*:1825-35; Kramer, *Sci. STKE* 2000, PE1), which normally represses proteolysis. Recombinant "mini-receptors" consisting of just the NEC HD domain and the extracellular portion of NTM form stable heterodimers after furin cleavage, indicating that critical inter-subunit contacts lie in these regions. Hence, HD domain mutations may act by destabilizing NEC/NTM heterodimers. It follows that similar effects might be imparted by mutations occurring in the analogous heterodimerization region of the NTM subunit.

Several factors may explain the high frequency of NOTCH-1 mutations in T-ALL. The absolute requirement for NOTCH-1 signals during several stages of normal early T cell development provides a functional basis for the frequent involvement of this pathway. Further, unlike the t(7;9), which is created by aberrant recombination during attempted V-

D-Jβ rearrangement in committed T cell progenitors, the more common point mutations and insertions described here could occur in multipotent hematopoietic progenitors, which also normally express NOTCH-1 (Calvi, *et al.*, *Nature* 425,841-6). If so, such mutations would be predicted to induce daughter cells to adopt a T cell fate (Allman, *et al.*, *J. Exp. Med. 194*:99-106) and thereby increase the pool of cells that are at risk for additional leukemogenic events, which could include additional mutations affecting the same NOTCH-1 allele, as well as the chromosomal, genetic and epigenetic events that lead to the mis-expression of other critical transcription factors. Analogous NOTCH mutations might be involved in the molecular pathogenesis of certain more common types of human cancer, as NOTCH receptors are expressed in many normal tissues and a variety of malignancies (Allenspach, *et al.*, *Cancer Biol. Ther. 1*:466-76).

Our findings identify the NOTCH pathway as a rational target for molecular therapy in T-ALL. Although approximately 75% of patients are currently cured of this disease with very intensive and cytotoxic chemotherapy regimens (Pui, *et al.*, *N. Engl. J. Med. 350*:1535-48), new therapies are needed for patients with refractory disease, and less toxic, more efficacious combinations of drugs would be beneficial to all T-ALL patients. The development of potent, specific inhibitors of γ-secretase (Wolfe, *Nat. Rev. Drug Discov. 1*:859-66), due to the involvement of this protease in the pathogenesis of Alzheimer's disease, should expedite clinical trials of cancer therapies aimed at blocking NOTCH-1 activity.

DEMANDE OU BREVET VOLUMINEUX

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What is Claimed is:

1. A substantially purified mutant NOTCH-1 receptor, comprising the amino acid sequence of SEQ ID NO:1 except that said amino acid sequence has one or more mutations in amino acids 1571-1622 and/or 1674-1730 and/or amino acid 2311 to amino acid 2556 such that, relative to unmutated NOTCH-1, said mutant NOTCH-1 receptor exhibits increased signaling in response to incubation with gamma-secretase.

2. The substantially purified mutant receptor of claim 1, wherein, except for said one or more mutations, said mutant receptor consists of the amino sequence of SEQ ID NO:1.

3. The substantially purified mutant NOTCH-1 receptor of claim 1, wherein said one or more mutations include one or more of the following:

a) the portion of the amino acid sequence of SEQ ID NO:1 from amino acid 1571 to amino acid 1618 is replaced with a sequence selected from the group consisting of SEQ ID NO:12 – SEQ ID NO:22, SEQ ID NO:102-118, and SEQ ID NO:145;

b) the portion of the amino acid sequence of SEQ ID NO:1 from amino acid 1571 to amino acid 1622 is replaced with a sequence selected from the group consisting of SEQ ID NO:23 – SEQ ID NO:29, or SEQ ID NO:119-127;

c) the portion of the amino acid sequence of SEQ ID NO:1 from amino acid 1674 to amino acid 1730 is replaced with a sequence selected from the group consisting of SEQ ID NO:70 – SEQ ID NO:75, or SEQ ID NO:128-137;

d) the portion of the amino acid sequence of SEQ ID NO:1 from amino acid 2311 to amino acid 2556 is replaced with a sequence selected from the group consisting of SEQ ID NO:30 – SEQ ID NO:59 or SEQ ID NO:138-144.

4. The substantially purified mutant receptor of claim 2, wherein, except for said one or more mutations, said mutant receptor consists of the amino sequence of SEQ ID NO:1.

5. The substantially purified mutant NOTCH-1 receptor of claim 1, wherein said sequence is mutated at one or more amino acid positions selected from the group consisting of: 1571; 1573; 1575; 1576; 1578; 1579; 1583; 1586; 1588; 1590; 1593; 1594; 1595; 1598; 1600-1607; 1610-1618; 1674; 1675; 1677; 1679-1684; 1686; 1695; 1696; 1703; 1706; 1715; 1720; 1725; and 1728.

6. The substantially purified receptor of claim 5, wherein, except for said one or more mutated amino acids, said receptor consists of the amino acid sequence of SEQ ID NO:1.

7. A substantially purified polynucleotide comprising a nucleotide sequence encoding the mutant NOTCH-1 receptor of claim 1.

8. The substantially purified polynucleotide of claim 7, wherein said polynucleotide consists of a nucleotide sequence encoding the mutant receptor of claim 1.

9. A substantially purified polynucleotide, at least 15 nucleotides in length which hybridizes to the polynucleotide of claim 8 under stringent conditions but which does not hybridize to a polynucleotide encoding unmutated NOTCH-1 under the same conditions.

10. A vector comprising a promoter operably linked to a polynucleotide encoding the mutant NOTCH-1 receptor of claim 1.

11. A host cell transformed with the vector of claim 10.

12. An antibody that binds to the mutant NOTCH-1 receptor of claim 1 with at least a hundredfold greater affinity than to the unmutated NOTCH-1 receptor of SEQ ID NO:1.

13. A method of diagnostically testing a biological sample for the presence of cancer cells, comprising assaying said biological sample for the mutant NOTCH-1 receptor of claim 1.

14. A method of diagnostically testing a biological sample for the presence of cancer cells, comprising assaying said biological sample for cells expressing the polynucleotide of claim 7.

15. A method of diagnostically testing a biological sample for the presence of cancer cells, comprising assaying said biological sample for cells expressing the polynucleotide of claim 8.

16. The method of claim 15, wherein said method is performed by PCR amplifying either the full length polynucleotide encoding said mutant NOTCH-1 receptor or a portion of the polynucleotide encoding said mutant NOTCH-1 receptor.

17. A method of slowing or stopping the growth of cells identified as having the mutant NOTCH-1 receptor of claim 1, comprising contacting said cells with an effective amount of a gamma-secretase inhibitor.

18. A method of treating a patient for cancer comprising:

a) assaying a biological sample from said patient for the presence of cancer cells having the mutant Notch-1 receptor of claim 1;

b) treating said patient with an effective amount of an agent that inhibits Notch-1 receptor signaling if said biological sample is found to have said mutant Notch-1 receptor.

19. The method of claim 18, wherein said cancer is T-cell acute lymphoblastic leukemia.

20. The method of claim 18, wherein said agent is a gamma-secretase inhibitor.

21. A method of assaying a test compound for its ability to inhibit abnormal cellular growth induced by the presence of a mutated NOTCH-1 receptor, comprising:

a) incubating said test compound with cells that express the mutant NOTCH-1 receptor of claim 1, said incubation being carried out in the presence of gamma-secretase;

b) comparing the amount of receptor activity that occurs as the result of the incubation of step a) with the amount of activity that occurs in a similar incubation carried out in the absence of said test compound; and

c) concluding that said test compound should inhibit the growth of cells expressing mutant NOTCH-1 receptor if the amount of activity observed in the presence of said test compound is less than the amount of activity observed in the absence of said test compound.

```
NOTCH1    ----------MPPLLAPLLCLALLPALAARGPRCSQPGETCLNGGKCEAAN 41
NOTCH2    ----------MPALRPALLWALLALWLCCAAPAHALQCRDGYEPCVNEGMCVTYH 45
NOTCH3    MGLGARGRRRRRRLMALPPPPPPMRALPLLLLLAGLGAAAPPCLDG-SPCANGGRCTHQQ 59
NOTCH4    ----------MQPPSLLLLLLLLLLCVSVVRPRGLLCGSFPEPCANGGTCLSLS 44
                          .  :  *  *        .  .  *     ..* * * * *

NOTCH1    --GTEACVCGGAFVGPRCQDPNPCL-STPCKNAGTCHVVDRR----------GVADYACS 88
NOTCH2    N-GTGYCKCPEGFLGEYCQHRDPCE-KNRCQNGGTCVAQAML----------GKAT--CR 91
NOTCH3    PSLEAACLCLPGWVGERCQLEDPCH-SGPCAGRGVCQSSVVA----------GTAR---- 104
NOTCH4    -LGQGTCQCAPGFLGETCQFPDPCQNAQLCQNGGSCQALLPAPLGLPSSPSPLTPSFLCT 103
                * *   .::*  **  :**     * . * *                    .

NOTCH1    CALGFSGPLCLTPLDNACLTN-PCRNGGTCDLLTLTEYKCRCPPGWSGKSCQQADPCASN 147
NOTCH2    CASGFTGEDCQYSTSHPCFVSRPCLNGGTCHMLSRDTYECTCQVGFTGKECQWTDACLSH 151
NOTCH3    ----------------------------------FSCRCLRGFQGPDCSQPDPCVSR 127
NOTCH4    CLPGFTGERCQAKLEDPCPPS-FCSKRGRCHIQASGRPQCSCMPGWTGEQCQLRDFCSAN 162
                                              .* *  *: * .*.  * * :.

NOTCH1    PCANGGQCLP-FEASYICHCPPSFHGPTCRQDVNECGQKPGLCRHGGTCHNEVGSYRCVC 206
NOTCH2    PCANGSTCTT-VANQFSCKCLTGFTGQKCETDVNECD-IPGHCQHGGTCLNLPGSYQCQC 209
NOTCH3    PCVHGAPCSVGPDGRFACACPPGYQGQSCQSDIDECR-SGTTCRHGGTCLNTPGSFRCQC 186
NOTCH4    PCVNGGVCLA-TYPQIQCHCPPGFEGHACERDVNECFQDPGPCPKGTSCHNTLGSFQCLC 221
          **.:*. *         * * ..: *  *. *:**      * :* :* *  **:*.*

NOTCH1    RATHTGPNCERPYVPCSPSPCQNGGTCRPTGD---VTHECACLPGFTGQNCEENIDDCPG 263
NOTCH2    PQGFTGQYCDSLYVPCAPSPCVNGGTCRQTGD---FTFECNCLPGFEGSTCERNIDDCPN 266
NOTCH3    PLGYTGLLCENPVVPCAPSPCRNGGTCRQSSD---VTYDCACLPGFEGQNCEVNVDDCPG 243
NOTCH4    PVGQEGPRCELRAGPCPPRGCSNGGTCQLMPEKDSTFHLCLCPPGFIGPGCEVNPDNCVS 281
               *  *:    **.*  * *****:   :      . * * *** *  ** * *:* .

NOTCH1    NNCKNGGACVDGVNTYNCRCPPEWTGQYCTEDVDECQLM-PNACQNGGTCHNTHGGYNCV 322
NOTCH2    HRCQNGGVCVDGVNTYNCRCPPQWTGQFCTEDVDECLLQ-PNACQNGGTCANRNGGYGCV 325
NOTCH3    HRCLNGGTCVDGVNTYNCQCPPEWTGQFCTEDVDECQLQ-PNACHNGGTCFNLLGGHSCV 302
NOTCH4    HQCQNGGTCQDGLDTYTCLCPETWTGWDCSEDVDECEAQGPPHCRNGGTCQNSAGSFHCV 341
          :.* ***.* **:.**.* **  ***  *:******    *  *:***** *  *.. **

NOTCH1    CVNGWTGEDCSENIDDCASAACFHGATCHDRVASFYCECPHGRTGLLCHLNDACISNPCN 382
NOTCH2    CVNGWSGDDCSENIDDCAFASCTPGSTCIDRVASFSCMCPEGKAGLLCHLDDACISNPCH 385
NOTCH3    CVNGWTGESCSQNIDDCATAVCFHGATCHDRVASFYCACPMGKTGLLCHLDDACVSNPCH 362
NOTCH4    CVSGWGGTSCEENLDDCIAATCAPGSTCIDRVGSFSCLCPPGRTGLLCHLEDMCLSQPCH 401
          **.** *  .*.:*:***  * *  *:** ***.** * ** *:.******:* *:*:**:
```

FIG.1-1

```
NOTCH1  EGSNCDTNPVNGKAICTCPSGYTGPACSQDVDECSLG---ANPCEHAGKCINTLGSFECQ 439
NOTCH2  KGALCDTNPLNGQYICTCPQGYKGADCTEDVDECAMAN--SNPCEHAGKCVNTDGAFHCE 443
NOTCH3  EDAICDTNPVSGRAICTCPPGFTGGACDQDVDECSIG---ANPCEHLGRCVNTQGSFLCQ 419
NOTCH4  GDAQCSTNPLTGSTLCLCQPGYSGPTCHQDLDECLMAQQGPSPCEHGGSCLNTPGSFNCL 461
          .: *.***:.*  :* *  *:.*  * :*:*** :.    ..**** * *:** *:* *

NOTCH1  CLQGYTGPRCEIDVNECVSNPCQNDATCLDQIGEFQCICMPGYEGVHCEVNTDECASSPC 499
NOTCH2  CLKGYAGPRCEMDINECHSDPCQNDATCLDKIGGFTCLCMPGFKGVHCELEINECQSNPC 503
NOTCH3  CGRGYTGPRCETDVNECLSGPCRNQATCLDRIGQFTCICMAGFTGTYCEVDIDECQSSPC 479
NOTCH4  CPPGYTGSRCEADHNECLSQPCHPGSTCLDLLATFHCLCPPGLEGQLCEVETNECASAPC 521
        *  **:*.*** * *** * **:  :**** :. * *:* .*  *  **:: :** * **

NOTCH1  LHNGRCLDKINEFQCECPTGFTGHLCQYDVDECASTPCKNGAKCLDGPNTYTCVCTEGYT 559
NOTCH2  VNNGQCVDKVNRFQCLCPPGFTGPVCQIDIDDCSSTPCLNGAKCIDHPNGYECQCATGFT 563
NOTCH3  VNGGVCKDRVNGFSCTCPSGFSGSMCQLDVDECASTPCRNGAKCVDQPDGYECRCAEGFE 539
NOTCH4  LNHADCHDLLNGFQCICLPGFSGTRCEEDIDECRSSPCANGGQCQDQP------------ 569
        :: . * * :* *.* *  .**:*  *: *:*:* *:** **.:* * *

NOTCH1  GTHCEVDIDECDPDPCHYGSCKDGVATFTCLCRPGYTGHHCETNINECSSQPCRHGGTCQ 619
NOTCH2  GVLCEENIDNCDPDPCHHGQCQDGIDSYTCICNPGYMGAICSDQIDECYSSPCLNDGRCI 623
NOTCH3  GTLCERNVDDCSPDPCHHGRCVDGIASFSCACAPGYTGIRCESQVDECRSQPCRYGGKCL 599
NOTCH4  ----------------------------GAFHCKCLPGFEGPRCQTEVDECLSDPCPVGASCL 604
                                     :: * * **: *  *. :::** *.**  .. *

NOTCH1  DRDNAYLCFCLKGTTGPNCEINLDDCASSPCDSGTCLDKIDGYECACEPGYTGSMCNINI 679
NOTCH2  DLVNGYQCNCQPGTSGVNCEINFDDCASNPCIHGICMDGINRYSCVCSPGFTGQRCNIDI 683
NOTCH3  DLVDKYLCRCPPGTTGVNCEVNIDDCASNPCTFGVCRDGINRYDCVCQPGFTGPLCNVEI 659
NOTCH4  DLPGAFFCLCPS-------------------------------GFTGQLC--EV 625
        *   . : * *                                  *:**  *  ::

NOTCH1  DECAGNPCHNGGTCEDGINGFTCRCPEGYHDPTCLSEVNECNSNPCVHGACRDSLNGYKC 739
NOTCH2  DECASNPCRKGATCINGVNGFRCICPEGPHHPSCYSQVNECLSNPCIHGNCTGGLSGYKC 743
NOTCH3  NECASSPCGEGGSCVDGENGFHCLCPPGSLPPLCLPANHPCAHKPCSHGVCHDAPGGFRC 719
NOTCH4  PLCAPNLCQPKQICKDQKDKANCLCPDG--SPGCAPPEDNCT---CHHGHCQRSS----C 676
          **  .  *     * :  :   * ** *   * *  .  .*    * ** *  .     *

NOTCH1  DCDPGWSGTNC--DINNNECESNPCVNGGTCKDMTSGYVCTCREGFSGPNCQTNINECAS 797
NOTCH2  LCDAGWVGINC--EVDKNECLSNPCQNGGTCDNLVNGYRCTCKKGFKGYNCQVNIDECAS 801
NOTCH3  VCEPGWSGPRCSQSLAPDACESQPCQAGGTCTSDGIGFRCTCAPGFQGHQCEV------- 772
NOTCH4  VCDVGWTGPEC--EAELGGCISAPCAHGGTCYPQPSGYNCTCPT--------------- 718
         *: ** *  .*   .    . * * **  ****     *: ***
```

FIG.1-2

```
NOTCH1  NPCLNQGTCIDDVAGYKCNCLLPYTGATCEVVLAPCAPSPCRNGGECRQSEDYESFSCVC 857
NOTCH2  NPCLNQGTCFDDISGYTCHCVLPYTGKNCQTVLAPCSPNPCENAAVCKESPNFESYTCLC 861
NOTCH3  ----------------------------------LSPCTPSLCEHGGHCESDPD-RLTVCSC 799
NOTCH4  ------------------------------------------------------------

NOTCH1  PTGWQAGQTCEVDINECV-LSPCRHGASCQNTHGGYRCHCQAGYSGRNCETDIDDCRPNP 916
NOTCH2  APGWQG-QRCTIDIDECI-SKPCMNHGLCHNTQGSYMCECPPGFSGMDCEEDIDDCLANP 919
NOTCH3  PPGWQG-PRCQQDVDECAGASPCGPHGTCTNLPGNFRCICHRGYTGPFCDQDIDDCDPNP 858
NOTCH4  ----------------------------------------GYTGPTCSEEMTACHSGP 736
                                                  *:.*  *. ::  * ..*

NOTCH1  CHNGGSCTDGINTAFCDCLPGFRGTFCEEDINECASDPCRNGANCTDCVDSYTCTCPAGF 976
NOTCH2  CQNGGSCMDGVNTFSCLCLPGFTGDKCQTDMNECLSEPCKNGGTCSDYVNSYTCKCQAGF 979
NOTCH3  CLHGGSCQDGVGSFSCSCLDGFAGPRCARDVDECLSSPCGPG-TCTDHVASFTCACPPGY 917
NOTCH4  CLNGGSCNPSPGGYYCTCPPSHTGPQCQTSTDYCVSAP---------------------- 774
        * :****  . .   * *  .. *  *  . : * * *

NOTCH1  SGIHCENNTPDCTESSCFNGGTCVDGINSFTCLCPPGFTGSYCQHDVN-ECDSQPCLHGG 1035
NOTCH2  DGVHCENNINECTESSCFNGGTCVDGINSFSCLCPVGFTGSFCLHEIN-ECSSHPCLNEG 1038
NOTCH3  GGFHCEIDLPDCSPSSCFNGGTCVDGVSSFSCLCRPGYTGTHCQYEAD-PCFSRPCLHGG 976
NOTCH4  ---------------CFNGGTCVNRPGTFSCLCAMGFOGPRCEGKLRPSCADSPCRNRA 818
                        ********:  .:*:***  *: *. *  .    * . ** : .

NOTCH1  TCQDGCGSYRCTCPQGYTGPNCQNLVHWCDSSPCKNGGKCWQTHTQYRCECPSGWTGLYC 1095
NOTCH2  TCVDGLGTYRCSCPLGYTGKNCQTLVNLCSRSPCKNKGTCVQKKAESQCLCPSGWAGAYC 1098
NOTCH3  ICNPTHPGFECTCREGFTGSQCQNPVDWCSQAPCQNGGRCVQTG--AYCICPPGWSGRLC 1034
NOTCH4  TCQDSPQGPRCLCPTGYTGGSCQTLMDLCAQKPCPRNSHCLQTGPSFHCLCLQGWTGPLC 878
         *       .* *  *:** .**. :. *   **  . . * *.     * *  **:*  *

NOTCH1  DVPSVSCEVAAQRQGVDVARLCQHGGLCVDAGNTHHCRCQAGYTGSYCEDLVDECSPSPC 1155
NOTCH2  DVPNVSCDIAASRRGVLVEHLCQHSGVCINAGNTHYCQCPLGYTGSYCEEQLDECASNPC 1158
NOTCH3  DIQSLPCTEAAAQMGVRLEQLCQEGGKCIDKGRSHYCVCPEGRTGSHCEHEVDPCTAQPC 1094
NOTCH4  NLPLSSCQKAALSQGIDVSSLCHNGGLCVDSGPSYFCHCPPGFQGSLCQDHVNPCESRPC 938
        ::   .*  **   *: :  **:..* *:: * ::.* *  *  ** *:. :: * . **

NOTCH1  QNGATCTDYLGGYSCKCVAGYHGVNCSEEIDECLSHPCQNGGTCLDLPNTYKCSCPRGTQ 1215
NOTCH2  QHGATCSDFIGGYRCECVPGYQGVNCEYEVDECQNQPCQNGGTCIDLVNHFKCSCPPGTR 1218
NOTCH3  QHGGTCRGYMGGYVCECPAGYAGDSCEDNIDECASQPCQNGGSCIDLVARYLCSCPPGTL 1154
NOTCH4  QNGATCMAQPSGYLCQCAPGYDGQNCSKELDACQSQPCHN-------------------- 978
        *:*.**     .** *:* .** * .*. ::* * .:**:*
```

FIG.1-3

```
NOTCH1  GVHCEINVDDCNPPVDPVSRSPKCFNNGTCVDQVGGYSCTCPPGFVGERCEGDVNECLSN 1275
NOTCH2  GLLCEENIDDC-------ARGPHCLNGGQCMDRIGGYSCRCLPGFAGERCEGDINECLSN 1271
NOTCH3  GVLCEINEDDCDLG-PSLDSGVQCLHNGTCVDLVGGFRCNCPPGYTGLHCEADINECRPG 1213
NOTCH4  ----------------------------HGTCTPKPGGFHCACPPGFVGLRCEGDVDECLDQ 1012
                                       * *    **: * * **:.* :**.*::**

NOTCH1  PCDARGTQNCVQRVN-DFHCECRAGHTGRRCESVINGCKGKPCKNGGTCAVASNTARG-- 1332
NOTCH2  PCSSEGSLDCIQLTN-DYLCVCRSAFTGRHCETFVDVCPQMPCLNGGTCAVASNMPDG-- 1328
NOTCH3  ACHAAHTRDCLQDPGGHFRCVCHPGFTGPRCQIALSPCESQPCQHGGQCRHSLGRGGGLT 1273
NOTCH4  PCHPTGTAACHSLAN-AFYCQCLPGHTGQWCEVEIDPCHSQPCFHGGTCEATAGSPLG-- 1069
         .* .  :  * .  .  : * * ..** *:  :. *   ** :** *  :  .   *

NOTCH1  FICKCPAGFEGATCENDARTCGSLRCLNGGTCISGPRS---PTCLCLGPFTGPECQFPAS 1389
NOTCH2  FICRCPPGFSGARCQS---SCGQVKCRKGEQCVHTASG---PRCFCPSPRD---CESGCA 1379
NOTCH3  FTCHCVPPFWGLRCERVARSCRELQCPVGIPCQQTARG---PRCACPPGLSGPSCRVSRA 1330
NOTCH4  FICHCPKGFEGPTCSHRAPSCGFHHCHHGGLCLPSPKPGFPPRCACLSGYGGPDCLTPPA 1129
        * *:*   * *  *.    :*   :* * *  .     * * *       *   :

NOTCH1  SPC------LGGNPCYNQGTCEPTS--ESPFYRCLCPAKFNGLLCHILDYSFGGGAGRDI 1441
NOTCH2  S----------SPCQHGGSCHPQR--QPPYYSCQCAPPFSGSRCELYT----------A 1416
NOTCH3  SPSGATNASCASAPCLHGGSCLPVQ--SVPFFRCVCAPGWGGPRCETPS---------AA 1379
NOTCH4  PKG-----CGPPSPCLYNGSCSETTGLGGPGFRCSCPHSSPGPRCQKPG---------- 1173
          .          **   *:*       * : * *.    *  *.

NOTCH1  PPPLIEEACELPECQEDAGNKVCSLQCNNHACGWDGGDCSLNFNDPWKNCTQSLQCWKYF 1501
NOTCH2  PPSTPPATCLSQYCADKARDGVCDEACNSHACQWDGGDCSLTMENPWANCSSPLPCWDYI 1476
NOTCH3  PEVPEEPRCPRAACQAKRGDQNCDRECNTPGCGWDGGDCSLNVDDPWRQC-EALQCWRLF 1438
NOTCH4  ----------AKGCEGRSGDGACDAGCSGPGGNWDGGDCSLGVPDPWKGCPSHSRCWLLF 1223
                  *     :  *.  *.  .  ******** . :**  *  .   **  :

NOTCH1  SDGHCDSQCNSAGCLFDGFDCQ--RAEGQCNPLYDQYCKDHFSDGHCDQGCNSAECEWDG 1559
NOTCH2  NN-QCDELCNTVECLFDNFECQ--GNSKTCK--YDKYCADHFKDNHCDQGCNSEECGWDG 1531
NOTCH3  NNSRCDPACSSPACLYDNFDCYSGGRDRTCNPVYEKYCADHFADGRCDQGCNTEECGWDG 1498
NOTCH4  RDGQCHPQCDSEECLFDGYDCE---TPPACTPAYDQYCHDHFHNGHCEKGCNTAECGWDG 1280
         : :*.  *.:  **:*.::*        *.  *:**  *** :..*:***:  ** ***

NOTCH1  LDCAEHVPERLAAGTLVVVVLMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIFP 1619
NOTCH2  LDCAADQPENLAEGTLVIVVLMPPEQLLQDARSFLRALGTLLHTNLRIKRDSQGELMVYP 1591
NOTCH3  LDCASEVPALLARGVLVLTVLLPPEELLRSSADFLQRLSAILRTSLRFRLDARGQAMVFP 1558
NOTCH4  GDCRPEDGDPEWGPSLALLVVLSPPALDQQLFALARVLSLTLRVGLWVRKDRDGRDMVYP 1340
         **   .          *.: *:.* *  ..   : : *.  *:..:  .: *  *. *:*
```

FIG.1-4

```
NOTCH1  YYGREEELRKHPIKRAAEGWAAPDALLGQVKASLLPGGSEGGRRRRELDPMDVRGSIVYL 1679
NOTCH2  YYGEKSAAMKK----------------QRMTRRSLPGEQE----------QEVAGSKVFL 1625
NOTCH3  YHRPSPGSESR----------------VRRELGP--------------EVIGSVVML 1585
NOTCH4  YPGARAEEKLG----------GTRDPTYQERAAPQTQPLG----KETDSLSAGFVVVM 1384
        *                                *                  * * :

NOTCH1  EIDNRQCVQ--ASSQCFQSATDVAAFLGALASLGSLN--IPYKIEAVQSETVEPPPPAQL 1735
NOTCH2  EIDNRQCVQ--DSDHCFKNTDAAAALLASHAIQGTLS--YP--LVSVVSESLTP---ERT 1676
NOTCH3  EIDNRLCLQSAENDHCFPDAQSAADYLGALSAVERLD--FPYPLRDVRGEPLEAPE-QSV 1642
NOTCH4  GVDLSRCGPDHPASRCPWDPGLLLRFLAAMAAVGALEPLLPGPLLAVHPHAGTAPPANQL 1444
         :*   *      .:*  ..     *.: :   *.  *  :  *  ..  .

NOTCH1  HFMYVAAAAFVLLFFVG---CGVLLSRKRRRQHGQLWFPEGFKVS---EASKKKRREPLG 1789
NOTCH2  QLLYLLAVAVVIILFII---LLGVIMAKRKRKHGSLWLPEGFTLRR--DASNHKRREPVG 1731
NOTCH3  PLLPLLVAGAVFLLIIF---ILGVMVARRKREHSTLWFPEGFALHKDIAAGHKGRREPVG 1699
NOTCH4  PWPVLCSPVAGVILLALGALLVLQLIRRRRREHGALWLPPGFTRRPRTQSAPHRRRPPLG 1504
           :     .:::        :  :*:*:*. **:* **       :. :**  *:*

NOTCH1  EDSVGLKPLKN-ASDGALMDDNQNE--WGDEDLETKKFRFEEPVVLPDLDDQTDHRQWTQ 1846
NOTCH2  QDAVGLKNLSVQVSEANLIGTGTSEHWVDDEGPQPKKVKAEDEALLSEEDDPIDRRPWTQ 1791
NOTCH3  QDALGMKNMAKGESLMGEVVTDLND----SECPEAKRLKVEEPGMGAE--EPEDCRQWTQ 1753
NOTCH4  EDSIGLKALKPKAEVDEDGVVMCSGPE----------EGEEVGQAEETGPPSTCQLWS- 1552
        :*:*:* :    .          .        . *:    :       : *:

NOTCH1  QHLDAADLR-MSAMAPTPPQGEVDADCMDVNVRGPDGFTPLMIASCSGGGLE-TGNSEE- 1903
NOTCH2  QHLEAADIRRTPSLALTPPQAEQEVDVLDVNVRGPDGCTPLMLASLRGGSSD-LSDEDED 1850
NOTCH3  HHLVAADIRVAPATALTPPQGDADADGVDVNVRGPDGFTPLMLASFCGGALEPMPAEEDE 1813
NOTCH4  --LSGGCGALPQAAMLTPPQ-ESEMEAPDLDTRGPDGVTPLMSAVCCG---EVQSGTFQG 1606
          * ..      :   **** : : :  *:****** **** *   *   :      :

NOTCH1  -EEDAPAVISDFIYQGASLHNQTDRTGETALHLAARYSRSDAAKRLLEASADANIQDNMG 1962
NOTCH2  AEDSSANIITDLVYQGASLQAQTDRTGEMALHLAARYSRADAAKRLLDAGADANAQDNMG 1910
NOTCH3  ADDTSASIISDLICQGAQLGARTDRTGETALHLAARYARADAAKRLLDAGADTNAQDHSG 1873
NOTCH4  AWLGCPEPWEPLLDGGACPQAHTVGTGETPLHLAARFSRPTAARRLLEAGANPNQPDRAG 1666
           ..     ::  **    :*  *** .******:.:*. **:***:*.*:.*  *. *

NOTCH1  RTPLHAAVSADAQGVFQILIRNRATDLDARMHDGTTPLILAARLAVEGMLEDLINSHADV 2022
NOTCH2  RCPLHAAVAADAQGVFQILIRNRVTDLDARMNDGTTPLILAARLAVEGMVAELINCQADV 1970
NOTCH3  RTPLHTAVTADAQGVFQILIRNRSTDLDARMADGSTALILAARLAVEGMVEELIASHADV 1933
NOTCH4  RTPLHAAVAADAREVCQLLLRSRQTAVDARTEDGTTPLMLAARLAVEDLVEELIAAQADV 1726
        * ***:**:***: * *:*:*.* * :***  **:*.*:******** .:: :** .:***
```

FIG.1-5

```
NOTCH1  NAVDDLGKSALHWAAAVNNVDAAVVLLKNGANKDMQNNREETPLFLAAREGSYETAKVLL 2082
NOTCH2  NAVDDHGKSALHWAAAVNNVEATLLLLKNGANRDMQDNKEETPLFLAAREGSYEAAKILL 2030
NOTCH3  NAVDELGKSALHWAAAVNNVEATLALLKNGANKDMQDSKEETPLFLAAREGSYEAAKLLL 1993
NOTCH4  GARDKWGKTALHWAAAVNNARAARSLLQAGADKDAQDNREQTPLFLAAREGAVEVAQLLL 1786
        .* *. **:********** *:  **: **:.* *:.:*:********** *.*:.**

NOTCH1  DHFANRDITDHMDRLPRDIAQERMHHDIVRLLDEYNLVRSPQLHGAPLGGTPTLSPPLCS 2142
NOTCH2  DHFANRDITDHMDRLPRDVARDRMHHDIVRLLDEYNVTPSP--PGTVL--TSALSPVICG 2086
NOTCH3  DHLANREITDHLDRLPRDVAQERLHQDIVRLLDQPSGPRSPS-------GPHGLGPLLCP 2046
NOTCH4  GLGAARELRDQAGLAPADVAHQRNHWDLLTLLEG------------------------- 1820
          . * *:: *:  .  * *:*:.* * *:: **:

NOTCH1  PNGYLGSLKPGVQG-KKVRKPSSK-----GLACGSKEAKDLK-ARRKKSQDGKGCLLDSS 2195
NOTCH2  PNRSFLSLKHTPMG-KKSRRPSAKSTMPTSLPNLAKEAKDAKGSRRKKSLSEKVQLSESS 2145
NOTCH3  PGAFLPGLKAVQSGTKKSRRPPGK---------TGLGPQGTRGRGKKLTLACPGPLADSS 2097
NOTCH4  -----------AGPPEARHKATP------------------ GREAGPFPRARTVSVS 1848
                  *  : *:  .                          ::          . *

NOTCH1  GMLSPVDSLESPHGYLSDVASPPLLPSP-FQQSPSVPLNHLPGMPDTHLGIGHLNVAAKP 2254
NOTCH2  VTLSPVDSLESPHTYVSDTTSSPMITSPGILQASPNPMLATAAPPAPVHAQHALSFSNLH 2205
NOTCH3  VTLSPVDSLDSPRPFSGPPASP----------GGFPLEGPYATTATAVSLAQLGASRAG 2146
NOTCH4  VPPHGGGALPRCRTLSAGAGPR----------GGGACLQARTWSVDLAARGGGAYSHCR 1897
              .:*   :   .   .                .       .   .      :

NOTCH1  EMAALGGGGRLAFETGPPRLSHLPVASGTSTVLGSSSGGALNFTVGGSTSLNGQCEWLSR 2314
NOTCH2  EMQPLAHGASTVLPSVSQLLSHHHIVS-----PGSGSAGSLSR----LHPVPVPADWMNR 2256
NOTCH3  PLGRQPPGGCVLSFG-------------------------------LLNPVAVPLDWAR- 2174
NOTCH4  SLSGVGAGG-------------------------------------------------- 1906
          :     *.

NOTCH1  LQSGMVPNQYNPLRGSVAPGPLSTQAPSLQHGMVGPLHSSLAASALSQMMSYQGLPSTRL 2374
NOTCH2  MEVNET--QYNEMFGMVLAPAEGTHP-----GIA---------------------PQSRP 2288
NOTCH3  ------------------------------------------------------LPPPA 2179
NOTCH4  ------------------------------------------------------GPT 1909

NOTCH1  ATQPHLVQTQQVQPQNLQMQQQNLQPANIQQQQSLQPPPPPPQPHLGVSSAASGHLGRSF 2434
NOTCH2  PEGKHITTPREPLPPIVTFQ--------LIPKGSIAQPAGAPQPQSTCPPAVAGPLPTMY 2340
NOTCH3  PPGPSFLLPLAPGPQ-------------LLNPGAPVSPQERPPPYLAAPGHG-------- 2218
NOTCH4  PRGRRFSAGMR----------------------GPRPNPAIMRGRYG-------- 1934
          .    :                          * *
```

FIG.1-6

```
NOTCH1  LSGEPSQADVQPLGPSSLAVHTILPQESPALPTSLPSSLVPPVTAAQFLTPPSQHSYSSP 2494
NOTCH2  QIP-----EMARLPSVAFPTAMMPQQDGQVAQTILPAYHPFPASVGKYPTPPSQHSYASS 2395
NOTCH3  -------EEYPAAGTRSSPTKARFLRVPSEHPYLTPSPESPEHWASPSPPSLSDWSDSTP 2271
NOTCH4  ----------------------------------------VAAGRGGRVSTDDWPCD 1951
                                                  ..      *   . .

NOTCH1  --VDNTPSHQLQVP-EHPFLTPSPESPDQWSSSSPHSNVSDWSEGVSSPPTSMQSQIARI 2551
NOTCH2  NAAERTPSHSGHLQGEHPYLTPSPESPDQWSSSSPHS-ASDWSDVTTSPTPGGAGGGQRG 2454
NOTCH3  SPATATNATASGALPAQPHPISVPSLPQSQTQLGPQPEVTPKRQVMAZ------------ 2319
NOTCH4  WVALGACGSASNIPIPPPCLTPSPERGSPQLDCGPPALQEMPINQGGEGKK--------- 2002
           . : .          *   . *. .  . .* .      :

NOTCH1  PEAFK------------ 2556
NOTCH2  PGTHMSEPPHNNMQVYA 2471
NOTCH3  ------------------
NOTCH4  ------------------
```

FIG. 1-7

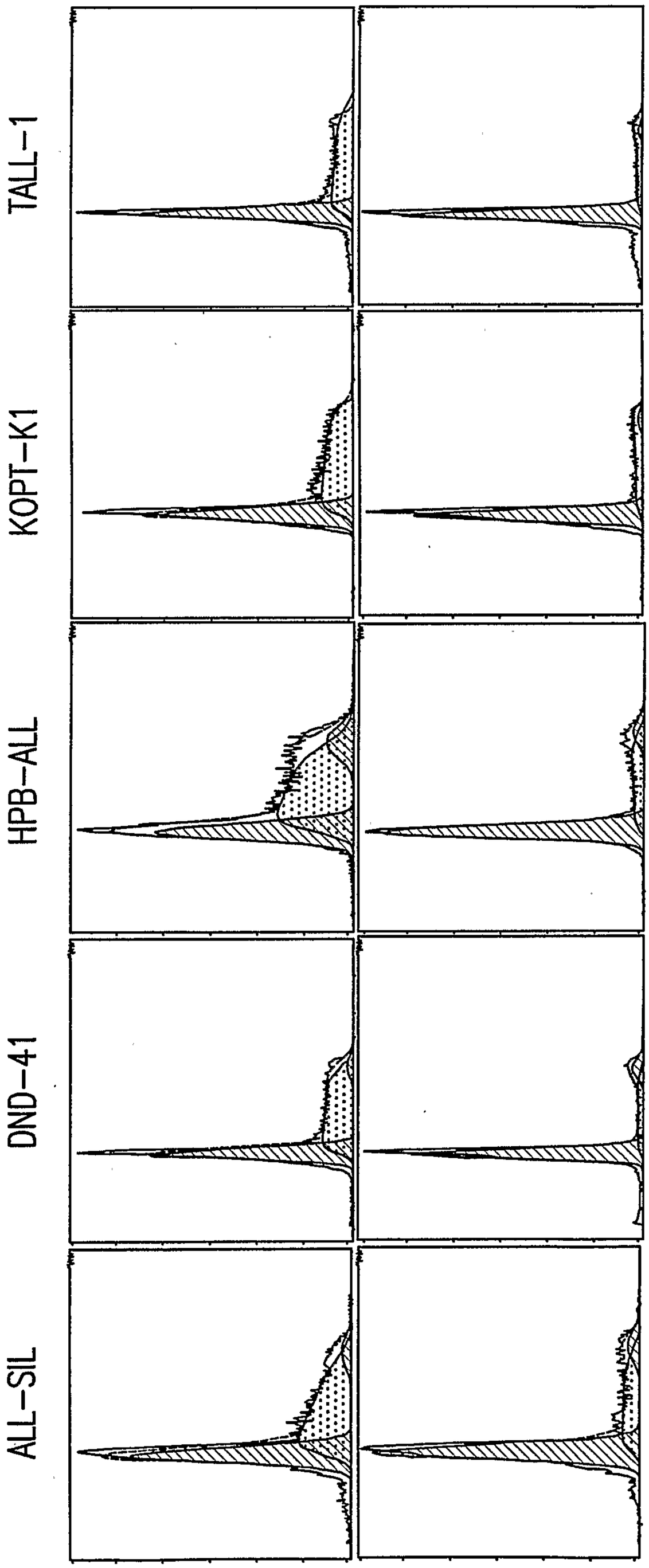


FIG.2A

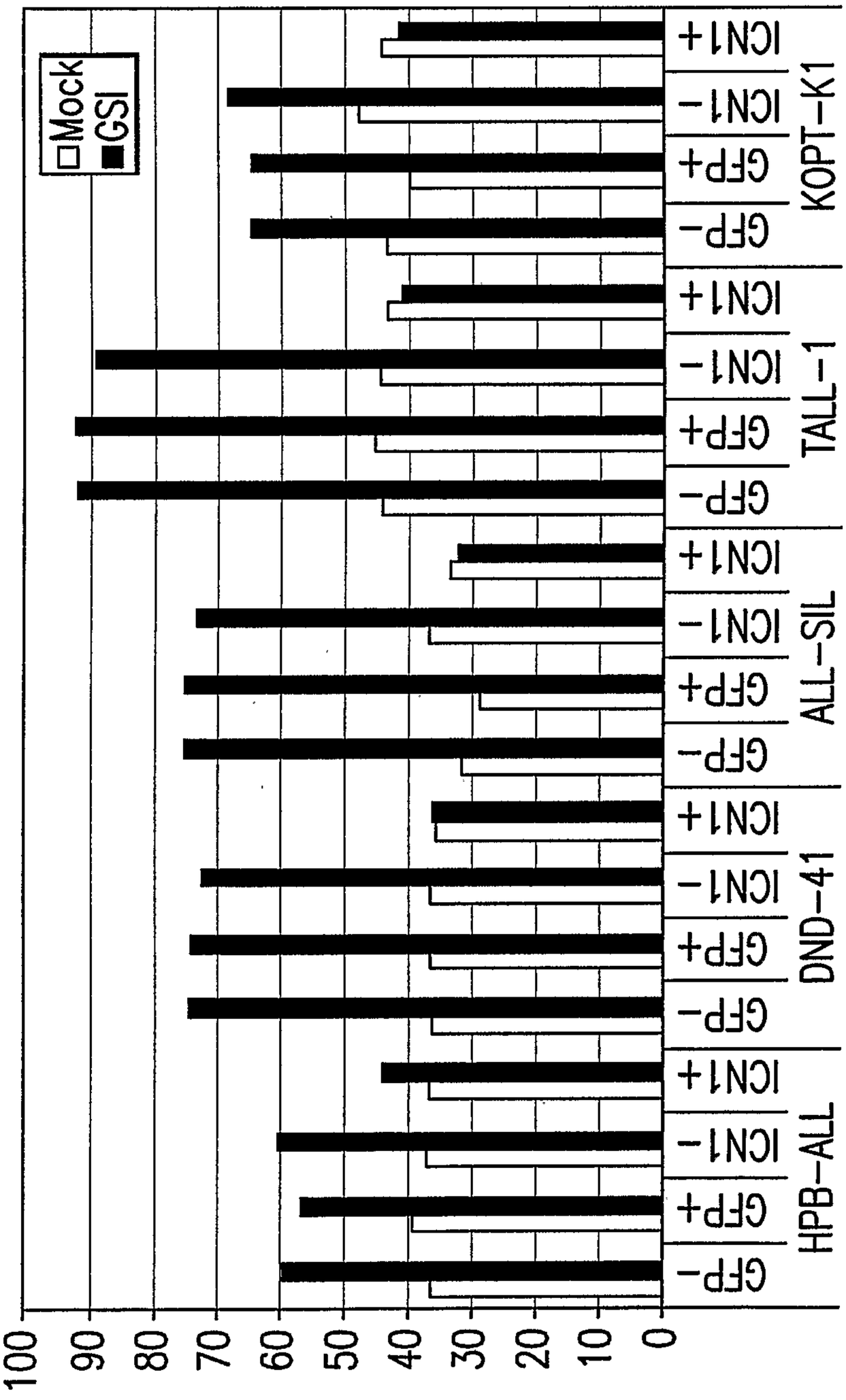

□Mock
■GSI
KOPT-K1
TALL-1
ALL-SIL
DND-41
HPB-ALL
+ICN1
-ICN1
+GFP
-GFP
100
90
80
70
60
50
40
30
20
10
0
%G1/G0

FIG.2B

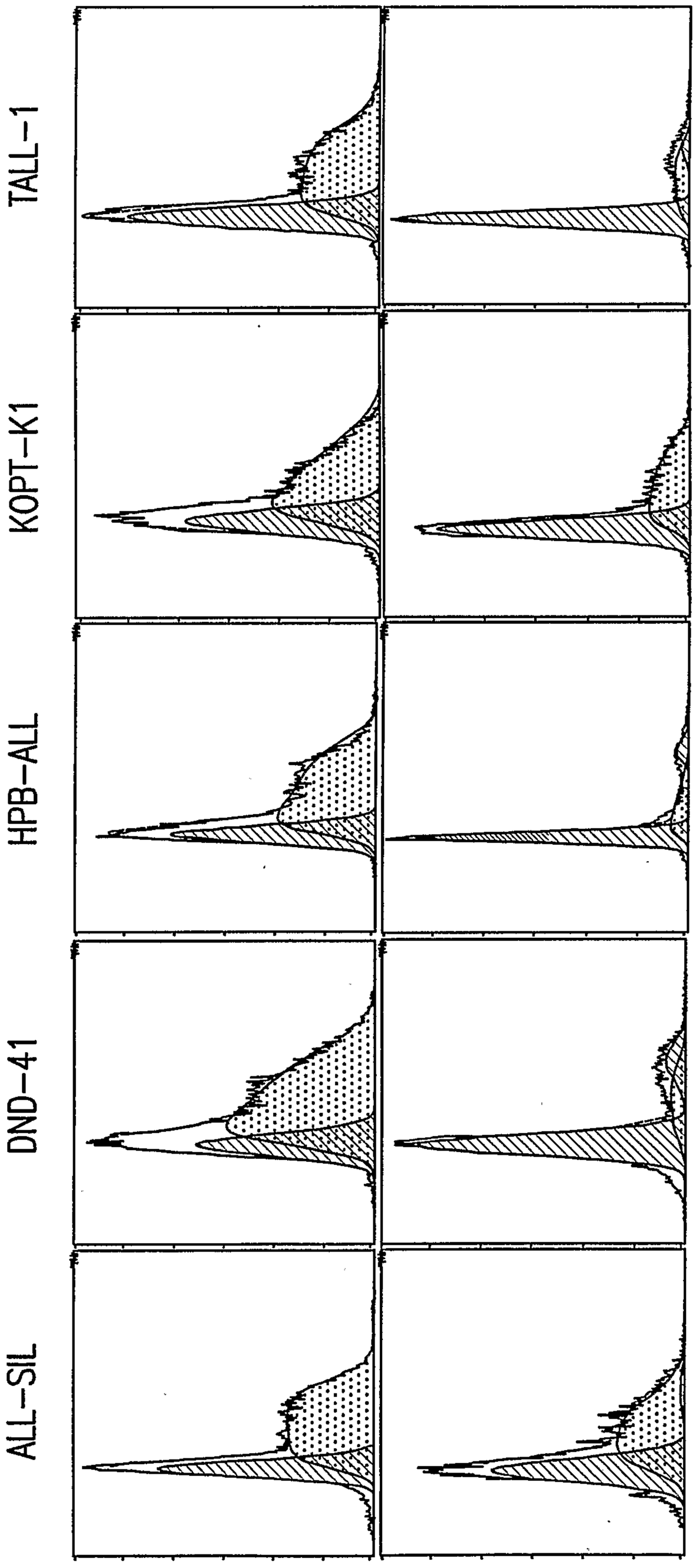


FIG.2C

		1571	1618	
human	NOTCH1	AAGT*L*VVV*V*LMPPEQ*L*RNSSFH*FL*RELS*RV L*HTNV*V*FK*R*DAHGQQMIF		SEQ ID NO:5
rat	notch1	AAGT*L*VLV*V*LPPDQ*L*RNNSFH*FL*RDVS*HV L*HTNV*V*FK*R*DAHGQQMIF		SEQ ID NO:6
mouse	notch1	AAGT*L*VLV*V*LPPDQ*L*RNNSFH*FL*RELS*HV L*HTNV**V**FK*R*DAHGQQMIF		SEQ ID NO:7
chicken	notch1	ADGT*L*V V*V*LITPEN*L*KNNSFN*FL*RELS*RV L*HTNV*V*FKKNAHGQQMIF		SEQ ID NO:8
frog	notch1	AEGT*L*VLV*V*LMPPER*L*KNNSVN*FL*RELS*RV L*HTNV*V*FKKDAHGQQMIF		SEQ ID NO:9
fish	notch1	AVGL*L*VVV*V*HIHPDQ*L*RNNSFG*FL*RELS*RV L*HTNV*V*FR*R*DAHGQQMIF		SEQ ID NO:10
consensus		A-G-LV-VV---P--L-N-S--FLR--S-VLHTNVVF--DAHGQQMIF		SEQ ID NO:11

FIG.3A

	1571	1618	
human NOTCH1	AAGTLVVVVLMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF		SEQ ID NO:5
KOPTK1	AAGTLVVVVLMPPEQLRNSSFHFLRELSRV*P*HTNVVFKRDAHGQQMIF		SEQ ID NO:12
TALL 07	AAGTLVVVVLMPPEQLRNSSFHFLRELSRV*P*HTNVVFKRDAHGQQMIF		SEQ ID NO:12
TALL 40	AAGTLVVVVLMPPEQLRNSSFHFLRELSRV*P*HTNVVFKRDAHGQQMIF		SEQ ID NO:12
TALL 41	AAGTLVVVVLMPPEQLRNSSFHFLRELSRV*P*HTNVVFKRDAHGQQMIF		SEQ ID NO:12
TALL 47	AAGTLVVVVLMPPEQLRNSSFHFLRELSRV*P*HTNVVFKRDAHGQQMIF		SEQ ID NO:12
TALL 68	AAGTLVVVVLMPPEQLRNSSFHFLRELSRV*P*HTNVVFKRDAHGQQMIF		SEQ ID NO:12
TALL 83	AAGTLVVVVLMPPEQLRNSSFHFLRELSRV*P*HTNVVFKRDAHGQQMIF		SEQ ID NO:12
ALL-SIL	AAGTLVVVVLMPPEQLRNSSFHF*P*RELSRVLHTNVVFKRDAHGQQMIF		SEQ ID NO:13
DND-41	AAGTLVVVVLMPPEQLRNSSFHF*P*RELSRVLHTNVVFKRVAHGQQMIF		SEQ ID NO:14
TALL 12	AAGTLVVVVLMPPEQLRNSSFHF*P*RELSRVLHTNVVFKRDAHGQQMIF		SEQ ID NO:13
TALL 18	AAGTLVVVVLMPPEQLRNSSFHF*P*RELSRVLHTNVVFKRDAHGQQMIF		SEQ ID NO:13
TALL 44	AAGTLVVVVLMPPEQLRNSSFHF*P*RELSRVLHTNVVFKRDAHGQQMIF		SEQ ID NO:13
TALL 45	AAGTLVVVVLMPPEQLRNSSFHF*P*RELSRVLHTNVVFKRDAHGQQMIF		SEQ ID NO:13
TALL 55	AAGTLVVVVLMPPEQLRNSSFHFLRELSPVLHTNVVFKRDAHGQQMIF		SEQ ID NO:15
TALL 64	AAGTLVVVVLMPPEQLRNSSFHFLRELSPVLHTNVVFKRDAHGQQMIF		SEQ ID NO:15
TALL 87	AAGTLVVVVLMPPEQLRNSSFHFLRELSPVLHTNVVFKRDAHGQQMIF		SEQ ID NO:15
TALL 89	AAGTLVVVVLMPPEQLRNSSFHFLRELSPVLHTNVVFKRDAHGQQMIF		SEQ ID NO;15
TALL 91	AAGTLVVVVLMPPEQLRNSSFHFLRELSPVLHTNVVFKRDAHGQQMIF		SEQ ID NO:15
HPB-ALL	AAGT*P*VVVVLMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF		SEQ ID NO:16
TALL 03	AAGT*P*VVVVLMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF		SEQ ID NO:16
TALL 79	AAGT*P*VVVVLMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF		SEQ ID NO:16
TALL 96	AAGT*P*VVVVLMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF		SEQ ID NO:16
TALL 05	AAGTLVVV-LMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF		SEQ ID NO:17

FIG.3B-1

1571 1618

TALL 21	AAGTLVVV-LMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF	SEQ ID NO:17
TALL 23	AAGTLVVV-LMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF	SEQ ID NO:17
TALL 93	AAGTLVVV-LMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF	SEQ ID NO:17
TALL 30	AAGTLVVVVLMPPEQLRNSSFH*S*LRELSRVLHTNVVFKRDAHGQQMIF	SEQ ID NO:18
TALL 04	AAGTLVVVVLMPPEQ*P*RNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF	SEQ ID NO:19
TALL 76	AAGTLVVVVLMPPEQLRNSSFHFLRELSRVLHTNVVFK*S*DAHGQQMIF	SEQ ID NO:20
TALL 02	AAGTLVVVVLMPPEQLRNSSFHFLRELSRVLHTNV-FKRDAHGQQMIF	SEQ ID NO:21
TALL 42	AAGTLVVVVLMPPEQLRNSSFHFLRELSRVLHTNVVFK*T*--HGQQMIF	SEQ ID NO:22
TALL 04.1	AAGTLV*E*VVLMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF	SEQ ID NO:102
TALL 04.2	AAGTLVV*E*VLMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF	SEQ ID NO:103
TALL 04.3	AAGTLVVV*G*LMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF	SEQ ID NO:104
TALL 04.4	AAGTLVVVVLMPPEQ*P*RNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF	SEQ ID NO:105
TALL 04.5	AAGTLVVVVWLMPPEQ*Q*RNSSFHFLRELSRVLHTNVFKRDAHGQQMIF	SEQ ID NO:106
TALL 04.6	AAGTLVVVVLMPPVQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF	SEQ ID NO:107
TALL 04.7	AAGTLVVVVLMPPEQLRNSSFHFLRE*P*SRVLHTNVVFKRDAHGQQMIF	SEQ ID NO:108
TALL 04.8	AAGTLVVVVLMPPEQLRNSSFHFLRE*H*SRVLHTNVVFKRDAHGQQMIF	SEQ ID NO:109
TALL 04.9	AAGTLVVVVLMPPEQLRNSSFHFLREL*I*RVLHTNVVFKRDAHGQQMIF	SEQ ID NO:110
TALL 04.10	AAGTLVVVVLMPPEQLRNSSFHFLRELSRVLH*M*NVVFKRDAHGQQMIF	SEQ ID NO:111
TALL 04.11	AAGTLVVVVLMPPEQLRNSSFHFLRELSRVLHTN*E*VFKRDAHGQQMIF	SEQ ID NO:112
TALL 04.12	AAGTLVVVVLMPPEQLRNSSFHFLRELSRVLHTN*G*VFKRDAHGQQMIF	SEQ ID NO:113
TALL 04.13	AAGTLVVVVLMPPEQLRNSSFHFLRELSRVLHTNVVFK*S*DAHGQQMIF	SEQ ID NO:114
TALL 04.14	AAGTLVVVVLMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMNF	SEQ ID NO:115
TALL 04.15	AAGTLVVVVLMPPEQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQM*T*F	SEQ ID NO:116

FIG.3B-2

FIG. 3B-3

TALL 04.16	AAGTLVVVVLMPPEQLRNSSFRSLRELSRVLHTNVVFKRDAHGQQMIF SEQ ID NO:117
TALL 04.17	AAGTLVVVVLMP-EQLRNSSFHFLRELSRVLHTNVVFKRDAHGQQMIF SEQ ID NO:118
AML 04.1	AAGTLVVVVLMPPEQLRNSSFHFLSRELSRVLHTNVVFKRDAHGQQMIF SEQ ID NO:145

```
                1571                                                                1622
NOTCH1             AAGTLVVVVLMPPE.QLRNSSFHF.LRE...LSRVLHTNVVFK....RDAHGQQMIFPYY.G     SEQ ID NO:77
TALL 09            AAGTLVVVVLMPPE.QLRNSSFHF.LRE...LSRVLHTNVVFK....RDAHGQQMIFPPGGG     SEQ ID NO:23
TALL 19            AAGTLVVVVLMPPAQQLRNSSFHF.LRE...LSRVLHTNVVFK....RDAHGQQMIFPYY.G     SEQ ID NO:24
TALL 24            AAGTLVVVVLMPPE.QLRNSSFHF.LRE...LSRVLHTNVVLGR...RDAHGQQMIFPYY.G     SEQ ID NO:25
TALL 46            AAGTLVVVVLMPPE.QLRNSSFHLALRE...LSRVLHTNVVFK....RDAHGQQMIFPYY.G     SEQ ID NO:26
TALL 53            AAGTLVVVVLMPPE.QLRNSSFHF.LREKEDLSRVLHTNVVFK....RDAHGQQMIFPYY.G     SEQ ID NO:27
TALL 86            AAGTLVVVVLMPPE.QLRNSSFHF.LRE...LSRVLHTNVVFKSMPPRDAHGQQMIFPYY.G     SEQ ID NO:28
TALL 95            AAGTLVVVVLMPPE.QLRNSSFHTALRE...LSRVLHTNVVFK....RDAHGQQMIFPYY.G     SEQ ID NO:29

                1571                                                                1622
NOTCH1             AAGTLVVVVLMPPEQLRNSSFHF  LRELSRVLHTNV    VF     KRDAHGQQMIF PYYG    SEQ ID NO:77
TALL 04.18         AAGTLVVVVLMPPEQLRNSSFHFA LRELSRVLHTNV    VF     KRDAHGQQMIF PYYG    SEQ ID NO:119
TALL 04.19         AAGTLVVVVLMPPEQLRNSSFHLY LRELSRVLHTNV    VF     KRDAHGQQMIF PYYG    SEQ ID NO:120
TALL 04.20         AAGTLVVVVLMPPEQLRNSSFHSHLKRELSRVLHTNV    VF     KRDAHGQQMIF PYYG    SEQ ID NO:121
TALL 04.21         AAGTLVVVVLMPPEQLRNSSFHF  LRELSRVLHTNVLPPPAF     KRDAHGQQMIF PYYG    SEQ ID NO:122
TALL 04.22         AAGTLVVVVLMPPEQLRNSSFHF  LRELSRVLHTNVLGR  F     KRDAHGQQMIF PYYG    SEQ ID NO:123
TALL 04.23         AAGTLVVVVLMPPEQLRNSSFHF  LRELSRVLHTNV    VFERARSKRDAHGQQMIF PYYG    SEQ ID NO:124
TALL 04.24         AAGTLVVVVLMPPEQLRNSSFHF  LRELSRVLHTNV    VFH    KRDAHGQQMIF PYYG    SEQ ID NO:125
TALL 04.25         AAGTLVVVVLMPPEQLRNSSFHF  LRELSRVLHTNV    VFD    KRDAHGQQMIF PYYG    SEQ ID NO:126
TALL 04.26         AAGTLVVVVLMPPEQLRNSSFHF  LRELSRVLHTNV    VF     KRDAHGQQMILGPYYG    SEQ ID NO:127
```

FIG.3C

```
        2311
NOTCH1      WLSRLQSGMVPNQYNPLRGSVAPGPLSTQAPSLQHGMVGPLHSSLAASALSQMMSYQGLPSTRLATQPHLVQTQQVQPQNLQMQQQNLQP      SEQ ID NO:60
TALL 05     ..........................................................................................*      SEQ ID NO:30
TALL 13     ..........................................................................................*      SEQ ID NO:31
TALL 13     ..........................................................................................*      SEQ ID NO:32
TALL 44     ..............................*                                                                      SEQ ID NO:33

NOTCH1          WLSRLQSGMVPNQYNPLRGSVAPGPLSTQAPSLQHGMVGPLHSSLAASALSQMMSYQGLPSTRLATQPHLVQTQQVQPQNLQMQQQNLQP      SEQ ID NO:60
TALL 04.37      ..........................................................................................*      SEQ ID NO:138

      2401
NOTCH1      ANIQQQQSLQPPPPPPQPHLGVSSAASGHLGRSFLSGEPSQADVQPLGPSSLAVHTILPQESPALPTSLPSSLVPPVTAAQFLTPPSQHS      SEQ ID NO:60 (cont)
TALL 35     ..........................................................................................*        SEQ ID NO:34
ALL-SIL     ..................................................................................AHP*        SEQ ID NO:35
TALL 53     ...............................................................................GCHPRWSHP*        SEQ ID NO:36
TALL 96     ..............................................................................*                SEQ ID NO:37
TALL 40     ...........................................................................PCPRRCHPRWSHP*        SEQ ID NO:38
TALL 08     ..........................................................................*                     SEQ ID NO:39
TALL 37     ....................................................LSRDLCSHWAPAAWRCTLFCPRRAPPCPRRCHPRWSH*        SEQ ID NO:40
DND41       ....................................................CCSHWAPAAWRCTLFCPRRAPPCPRRCHPRWSHP*        SEQ ID NO:41
HPB-ALL     ...................................................EGRGRCSHWAPAAWRCTLFCPRRAPPCPRRCHPRWSHP*        SEQ ID NO:42
TALL 23     ..................................................GGGGMGSHWAPAAWRCTLFCPRRAPPCPRRCHPRWSHP*        SEQ ID NO:43
TALL 91     ..................................................ERARQTCSHWAPAAWRCTLFCPRRAPPCPRRCHPRWSHP*        SEQ ID NO:44
TALL 84     ..................................................RVRARQTCSHWAPAAWRCTLFCPRRAPPCPRRCHPRWSHP*        SEQ ID NO:45
TALL 07     ..................................................*                                                SEQ ID NO:46
```

FIG.3D-1

TALL 01 .....APS* SEQ ID NO:47
TALL 36 .....AA* SEQ ID NO:48
TALL 32 .....RHHHHHSRTLVA* SEQ ID NO:49
TALL 68 .....* SEQ ID NO:50
TALL 57 ...* SEQ ID NO:51

2401
ANIQQQQSLQPPPPPPQPHLGVSSAASGHLGRSFLSGEPSQADVQPLGPSSLAVHTILPQESPALPTSLPSSLVPPVTAAQFLTPPSQHS SEQ ID NO:60

(cont)
TALL 04.38 .....LILWSHP* SEQ ID NO:139
TALL 04.39 .....VL* SEQ ID NO:140
TALL 04.40 .....CGLSPRRPHHHHSRTLA* SEQ ID NO:141
TALL 04.41 .....* SEQ ID NO:142
TALL 04.42 ....* SEQ ID NO:143

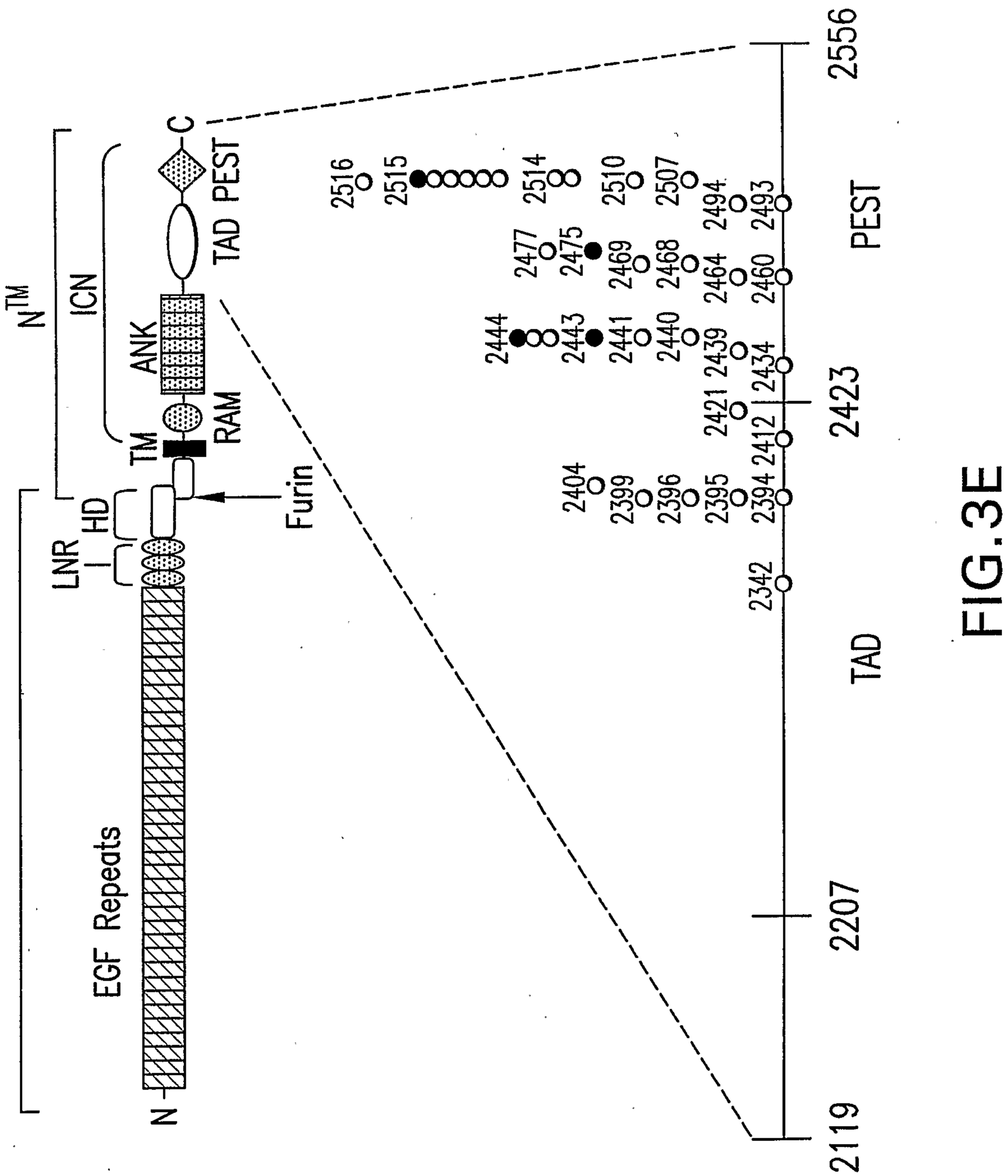
2491 2556
NOTCH1 YSSPVDNTPSHQLQVPEHPFLTPSPESPDQWSSSSPHSNVSDWSEGVSSPPTSMQSQIARIPEAFK* SEQ ID NO:60 (cont)
TALL 41 .....DSGPARPRIPTSPTGPRASPALPPACSPRSPAFRRPSSKRRAPRDPGFLSQAFGRLCALCGCQGRPEEPF* SEQ ID NO:52
TALL 30 .....SVP* SEQ ID NO:53
KOPTK1 .....RVP* SEQ ID NO:54
TALL 83 .....RVP* SEQ ID NO:54
TALL 94 .....RVP* SEQ ID NO:54
TALL 80 .....RVP* SEQ ID NO:54
TALL 81 .....RVP* SEQ ID NO:54
TALL 47 .....VP* SEQ ID NO:55

FIG.3D-2

```
TALL 52      .....................VP*                                                    SEQ ID NO:55
TALL 41      ..................Y*                                                      SEQ ID NO:56
TALL 93      ................*                                                        SEQ ID NO:57
TALL 63      ...GRQHPQPPATGA*                                                        SEQ ID NO:58
TALL 92      ..*                                                                     SEQ ID NO:59

      2491                                                                  2556
           YSSPVDNTPSHQLQVPEHPFLTPSPESPDQWSSSSPHSNVSDWSEGVSSPPTSMQSQIARIPEAFK   SEQ ID NO:60(cont)
TALL04.43  ....................................PAFQRLRLVRGRLQPSHQHAVPDRPHSGGLQVNGAPHETPASFPKPSGVCVRS   SEQ ID NO:144
           VDARADQRSLFKTHVFIQNKNEDFNFF*                                    SEQ ID NO:144 (cont)
```

FIG.3D-3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ICN
C
TAD PEST
ANK
TM
RAM
Furin
LNR
HD
N^EC
EGF Repeats
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2510
2507
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2441
2440
2439
2434
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2395
2412
2394
2423
PEST
2342
TAD
2207
2119

FIG.3E

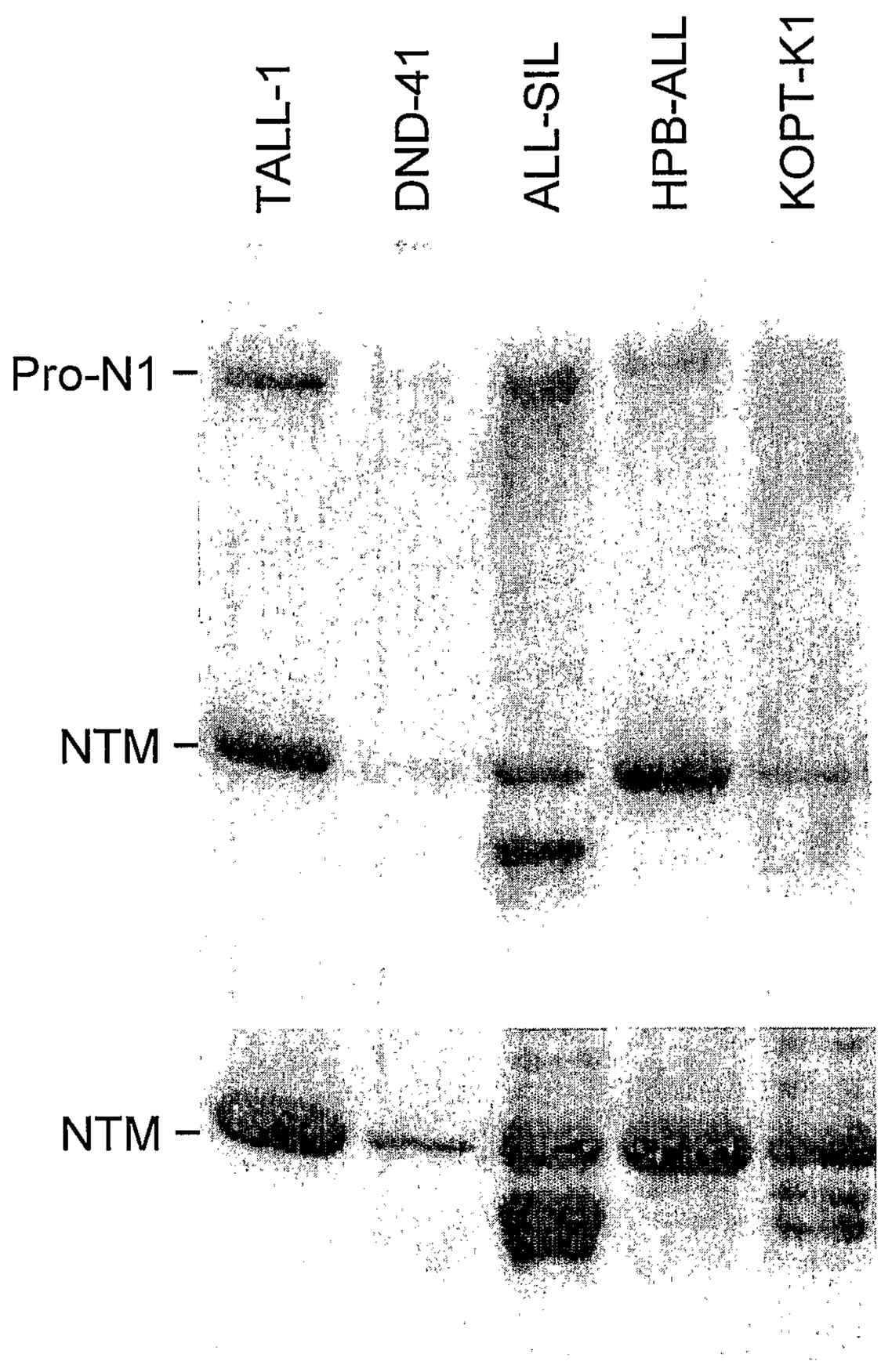

FIG.3F

Fish	GSIVYLEIDNRLCSQ--GSDDCFRNADSAAEYLGALSAREMLR-FPYPTKEVTSEKREPS	SEQ ID NO:61
hN3	GSVVMLEIDNRLCLQSAENDHCFPDAQSAADYLGALSAVERLD-FPYPLRDVRGEPLEAP	SEQ ID NO:62
Mouse	GSIVYLEIDNRQCVQ--SSSQCFQSATDVAAFLGALASLGSLN-IPYKIEAVKSEPVEPP	SEQ ID No:63
Rat	GSIVYLEIDNRQCVQ--SSSQCFQSATDVAAFLGALASLGSLN-IPYKIEAVKSETVEPP	SEQ ID NO:64
Human	GSIVYLEIDNRQCVQ--ASSQCFQSATDVAAFLGALASLGSLN-IPYKIEAVQSETVEPP	SEQ ID NO:65
Chicken	GSIVYLEIDNRQCIQ--SSSQCFQSATDVAAFLGALASLGNLN-IPYKIEAVKSETAEPA	SEQ ID NO:66
Frog	GSIVYLEIDNRQCYK--SSSQCFNSATDVAAFLGALASLGSLDTLSYKIEAVKSENMETP	SEQ ID NO:67
HN2	GSKVFLEIDNRQCVQ--DSDHCFKNTDAAAALLASHAIQGTLS---YPLVSVVSESLTPE	SEQ ID NO:68
Concsensus	GS-V-LEIDNR-C--------CF------A--L--------L----Y----V--E-----	SEQ ID NO:69

1674 1730

Human NOTCH1	GSIVYLEIDNRQCVQASSQCFQSATDVAAFLGALASLGSLNIPYKI-EAVQSETVEPP	SEQ ID NO:65
Mutation 1	GSIVYLEIDNRQCVQASSQCFQSATDVAAFLGALASLGSLNIPYKI*P*EAVQSETVEPP	SEQ ID NO:70
Mutation 2	GSIVY*P*EIDNRQCVQASSQCFQSATDVAAFLGALASLGSLNIPYKI-EAVQSETVEPP	SEQ ID NO:71
Mutation 3	GSI*D*YLEIDNRQCVQASSQCFQSATDVAAFLGALASLGSLNIPYKI-EAVQSETVEPP	SEQ ID NO:72
Mutation 4	GSIVYLE*N*DNRQCVQASSQCFQSATDVAAFLGALASLGSLNIPYKI-EAVQSETVEPP	SEQ ID NO:73
Mutation 5	GSIVYLE*T*DNRQCVQASSQCFQSATDVAAFLGALASLGSLNIPYKI-EAVQSETVEPP	SEQ ID NO:74
Mutation 6	GSIVYLEIDNRQCVQASSQCFQSA*N*DVAAFLGALASLGSLNIPYKI-EAVQSETVEPP	SEQ ID NO:75

Human Notch1	GSIV YLEIDNRQCVQASSQCFQSATDVAAFLGALASLGSLNIPYKIEAVQSETVEPP	SEQ ID NO:65
TALL 04.27	GSIV YLEIDNRQCVQASSQCFQSATDVAAFLGALASLGSLNIPYK*T*EAVQSETVEPP	SEQ ID NO:128
TALL 04.28	GSIV YLEIDNRQCVQASSQCFQSATDVA*P*FLGALASLGSLNIPYKIEAVQSETVEPP	SEQ ID NO:129
TALL 04.29	GSIV YLEIDNRQCVQASSQCFQSATDVAAFLGALAS*P*GSLNIPYKIEAVQSETVEPP	SEQ ID NO:130
TALL 04.30	GSIV YLEIDNRQCVQASSQCFQ*L*ATDVAAFLGALASLGSLNIPYKIEAVQSETVEPP	SEQ ID NO:131
TALL 04.31	GSIV*STL*YLEIDNRQCVQASSQCFQSATDVAAFLGALASLGSLNIPYKIEAVQSETVEPP	SEQ ID NO:132

FIG.3G-1

TALL 04.32	GSIV	YLEIDNRQCVQASSQCFQSA*M*DVAAFLGALASLGSLNIPYKIEAVQSETVEPP	SEQ ID NO:133
TALL 04.33	GSIV	YLE*M*DNRQCVQASSQCFQSATDVAAFLGALASLGSLNIPYKIEAVQSETVEPP	SEQ ID NO:134
TALL 04.34	GSI*D*	YLEIDNRQCVQASSQCFQSATDVAAFLGALASLGSLNIPYKIEAVQSETVEPP	SEQ ID NO:135
TALL 04.35	GSIV	Y*P*EIDNRQCVQASSQCFQSATDVAAFLGALASLGSLNIPYKIEAVQSETVEPP	SEQ ID NO:136
Human		GSIVYLEIDNRQCVQASSQCFQSATDVAAFLGALASLGSLNIPYKIEAVQSETVEPP	SEQ ID NO:65
TALL 04.36		GSIVYLEIDNRQCVQASSQCFQSATDVAAFLGALASLGSLNIPYKIEiVQSETVEPP	SEQ ID NO:137

RLGSLNIPYKIEAV

FIG.3G-2

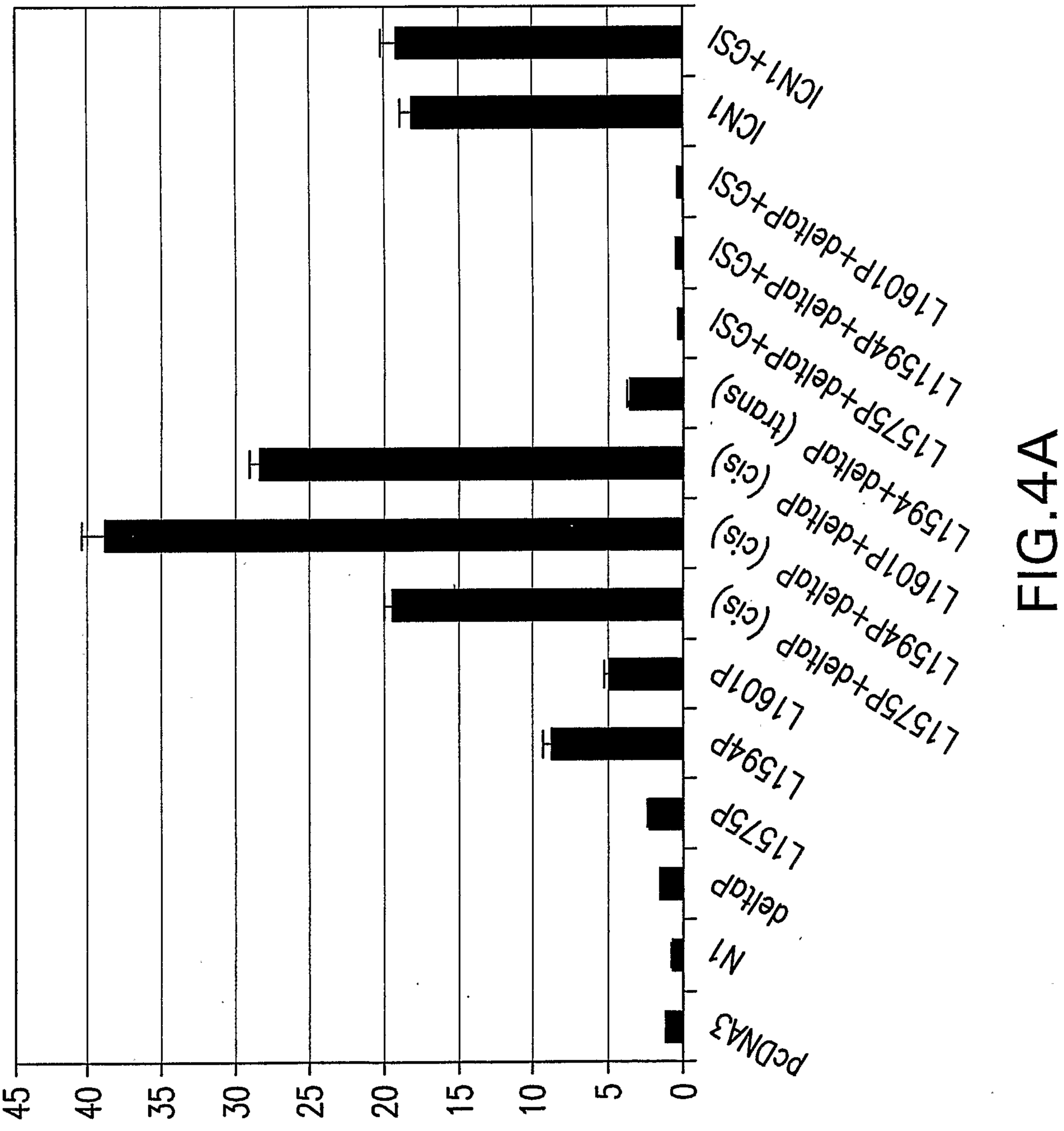
Fold Stimulation
0
5
10
15
20
25
30
35
40
45
pCDNA3
N1
deltaP
L157S
L160P
L159A
L157S+deltaP (cis)
L160P+deltaP (cis)
L159A+deltaP (cis)
L157S+deltaP (trans)
L159A+deltaP
L160P+deltaP
ICN1
ICN1+GSI

FIG.4A

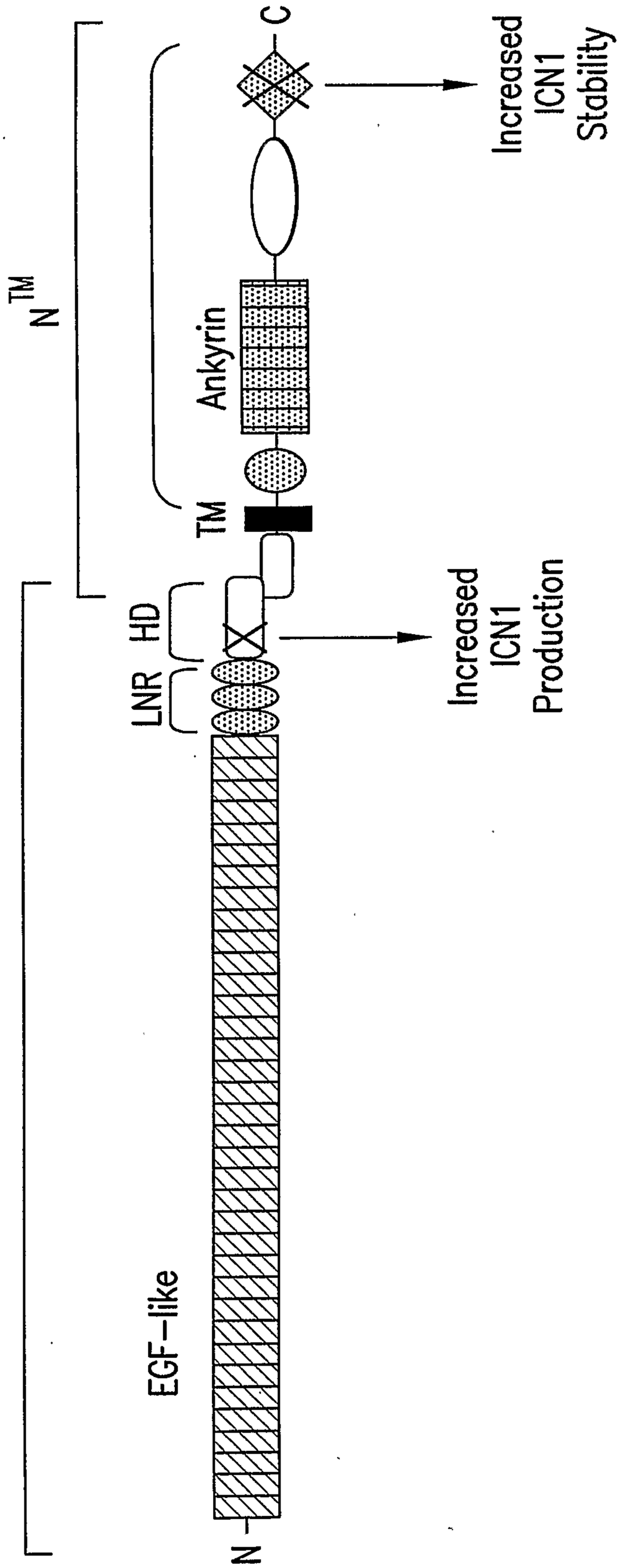
C
Increased ICN1 Stability
N TM
Ankyrin
TM
HD
LNR
Increased ICN1 Production
N EC
EGF-like
N

FIG.4B

```
1/1                                31/11                              61/21                              91/31
ATG CCG CCG CTC CTG GCG CCC CTG CTC TGC CTG GCG CTG CTG CCC GCG CTC GCC GCA CGA GGC CCG CGA TGC TCC CAG CCC GGT GAG ACC TGC CTG AAT GGC GGG AAG TGT GAA GCG GCC
M  P  P  L  L  A  P  L  L  C  L  A  L  L  P  A  L  A  A  R  G  P  R  C  S  Q  P  G  E  T  C  L  N  G  G  K  C  E  A  A

121/41                             151/51                             181/61                             211/71
AAT GGC ACG GAG GCC TGC GTC TGT GGC GGG GCC TTC GTG GGC CCG CGA TGC CAG GAC CCC AAC CCG TGC CTC AGC ACC CCC TGC AAG AAC GCC GGG ACA TGC CAC GTG GTG GAC CGC AGA
N  G  T  E  A  C  V  C  G  G  A  F  V  G  P  R  C  Q  D  P  N  P  C  L  S  T  P  C  K  N  A  G  T  C  H  V  V  D  R  R

241/81                             271/91                             301/101                            331/111
GGC GTG GCA GAC TAT GCC TGC AGC TGT GCC CTG GGC TTC TCT GGG CCC CTC TGC CTG ACA CCC CTG GAC AAT GCC TGC CTC ACC AAC CCC TGC CGC AAC GGG GGC ACC TGC GAC CTG CTC
G  V  A  D  Y  A  C  S  C  A  L  G  F  S  G  P  L  C  L  T  P  L  D  N  A  C  L  T  N  P  C  R  N  G  G  T  C  D  L  L

361/121                            391/131                            421/141                            451/151
ACG CTG AGG GAG TAC AAG TGC CGC TGC CCG CCC GGC TGG TCA GGG AAA TCG TGC CAG CAG GCT GAC CCG TGC GCC TCC AAC CCC TGC GCC AAC GGT GGC CAG TGC CTG CCC TTC GAG GCC
T  L  T  E  Y  K  C  R  C  P  P  G  W  S  G  K  S  C  Q  Q  A  D  P  C  A  S  N  P  C  A  N  G  G  Q  C  L  P  F  E  A

481/161                            511/171                            541/181                            571/191
TCC TAC ATC TGC CAC TGC CCA CCC AGC TTC CAT GGC CCC ACC TGC CGG CAG GAT GTC AAC GAG TGT GGC CAG AAG CCC GGG CTT TGC CGC CAC GGA GGC ACC TGC CAC AAC GAG GTC GGC
S  Y  I  C  H  C  P  P  S  F  H  G  P  T  C  R  Q  D  V  N  E  C  G  Q  K  P  G  L  C  R  H  G  G  T  C  H  N  E  V  G

601/201                            631/211                            661/221                            691/231
TCC TAC CGC TGC GTC TGC CGC GCC ACC CAC ACT GGC CCC AAC TGC GAG CGG CCC TAC GTG CCC TGC AGC CCC TCG CCC TGC CAG AAC GGG GGC ACC TGC CGC CCC AGG GGC GAC GTC ACC
S  Y  R  C  V  C  R  A  T  H  T  G  P  N  C  E  R  P  Y  V  P  C  S  P  S  P  C  Q  N  G  G  T  C  R  P  T  G  D  V  T
```

FIG.5-1

721/241 751/251 781/261 811/271
CAC GAG TGT GCC TGC CTG CCA GGC TTC ACC GGC CAG AAC TGT GAG GAA AAT ATC GAC GAT TGT CCA GGA AAC AAC TGC AAG AAC GGG GGT GCC TGT GTG GAC GGC GTG AAC ACC TAC AAC
H E C A C L P G F T G Q N C E E N I D D C P G N N C K. N G G A C V D G V N T Y N

841/281 871/291 901/301 931/311
TGC CGC TGC CCG CCA GAG TGG ACA GGT CAG TAC TGT ACC GAG GAT GTG GAC GAG TGC CAG CTG ATG CCA AAT GCC TGC CAG AAC GGC GGG ACC TGC CAC AAC ACC CAC GGT GGC TAC AAC
C R C P P E W T G Q Y C T E D V D E C Q L M P N A C Q N G G T C H N T H G G Y N

961/321 991/331 1021/341 1051/351
TGC GTG TGT GTC AAC GGC TGG ACT GGT GAG GAC TGC AGC GAG AAC ATT GAT GAC TGT GCC AGC GCC GCC TGC TTC CAC GGC GCC ACC TGC CAT GAC CGT GTG GCC TCC TTC TAC TGC GAG
C V C V N G W T G E D C S E N I D D C A S A A C F H G A T C H D R V A S F Y C E

1081/361 1111/371 1141/381 1171/391
TGT CCC CAT GGC CGC ACA GGT CTG CTG TGC CAC CTC AAC GAC GCA TGC ATC AGC AAC CCC TGT AAC GAG GGC TCC AAC TGC GAC ACC AAC CCT GTC AAT GGC AAG GCC ATC TGC ACC TGC
C P H G R T G L L C H L N D A C I S N P C N E G S N C D T N P V N G K A I C T C

1201/401 1231/411 1261/421 1291/431
CCC TCG GGG TAC ACG GGC CCG GCC TGC AGC CAG GAC GTG GAT GAG TGC TCG CTG GGT GCC AAC CCC TGC GAG CAT GCG GGC AAG TGC ATC AAC ACG CTG GGC TCC TTC GAG TGC CAG TGT
P S G Y T G P A C S Q D V D E C S L G A N P C E H A G K C I N T L G S F E C Q C

1321/441 1351/451 1381/461 1411/971
CTG CAG GGC TAC ACG GGC CCC CGA TGC GAG ATC GAC GTC AAC GAG TGC GTC TCG AAC CCG TGC CAG AAC GAC GCC ACC TGC CTG GAC CAG ATT GGG GAG TTC CAG TGC ATC TGC ATG CCC
L Q G Y T G P R C E I D V N E C V S N P C Q N D A T C L D Q I G E F Q C I C M P

FIG.5-2

1441/481 1471/491 1501/501 1531/511
GGC TAC GAG GGT GTG CAC TGC GAG GTC AAC ACA GAC GAG TGT GCC AGC AGC CCC TGC CTG CAC AAT GGC CGC TGC CTG GAC AAG ATC AAT GAG TTC CAG TGC GAG TGC CCC AGG GGC TTC
G Y E G V H C E V N T D E C A S S P C L H N G R C L D K I N E F Q C E C P T G F

1561/521 1591/531 1621/541 1651/551
ACT GGG CAT CTG TGC CAG TAC GAT GTG GAC GAG TGT GCC AGC ACC CCC TGC AAG AAT GGT GCC AAG TGC CTG GAC GGA CCC AAC ACT TAC ACC TGT GTG TGC ACG GAA GGG TAC ACG GGG
T G H L C Q Y D V D E C A S T P C K N G A K C L D G P N T Y T C V C T E G Y T G

1681/561 1711/571 1741/581 1771/591
ACG CAC TGC GAG GTG GAC ATC GAT GAG TGC GAC CCC GAC CCC TGC CAC TAC GGC TCC TGC AAG GAC GGC GTC GCC ACC TTC ACC TGC CTC TGC CGC CCA GGC TAC ACG GGC CAC CAC TGC
T H C E V D I D E C D P D P C H Y G S C K D G V A T F T C L C R P G Y T G H H C

1801/601 1831/611 1861/621 1891/631
GAG ACC AAC ATC AAC GAG TGC TCC AGC CAG CCC TGC CGC CAC GGG GGC ACC TGC CAG GAC CGC GAC AAC GCC TAC CTC TGC TTC TGC CTG AAG GGG ACC ACA GGA CCC AAC TGC GAG ATC
E T N I N E C S S Q P C R H G G T C Q D R D N A Y L C F C L K G T T G P N C E I

1921/641 1951/651 1981/661 2011/671
AAC CTG GAT GAC TGT GCC AGC AGC CCC TGC GAC TCG GGC ACC TGT CTG GAC AAG ATC GAT GGC TAC GAG TGT GCC TGT GAG CCG GGC TAC ACA GGG AGC ATG TGT AAC ATC AAC ATC GAT
N L D D C A S S P C D S G T C L D K I D G Y E C A C E P G Y T G S M C N I N I D

2041/681 2071/691 2101/701 2131/711
GAG TGT GCG GGC AAC CCC TGC CAC AAC GGG GGC ACC TGC GAG GAC GGC ATC AAT GGC TTC ACC TGC CGC TGC CCC GAG GGC TAC CAC GAC CCC ACC TGC CTG TCT GAG GTC AAT GAG TGC
E C A G N P C H N G G T C E D G I N G F T C R C P E G Y H D P T C L S E V N E C

FIG.5-3

2161/721 2191/731 2221/741 2251/751
AAC AGC AAC CCC TGC GTC CAC GGG GCC TGC CGG GAC AGC CTC AAC GGG TAC AAG TGC GAC TGT GAC CCT GGG TGG AGT GGG ACC AAC TGT GAC ATC AAC AAC AAT GAG TGT GAA TCC AAC
N S N P C V H G A C R D S L N G Y K C D C D P G W S G T N C D I N N N E C E S N

2281/761 2311/771 2341/781 2371/791
CCT TGT GTC AAC GGC GGC ACC TGC AAA GAC ATG ACC AGT GGC TAC GTG TGC ACC TGC CGG GAG GGC TTC AGC GGT CCC AAC TGC CAG ACC AAC ATC AAC GAG TGT GGG TCC AAC CCA TGT
P C V N G G T C K D M T S G Y V C T C R E G F S G P N C Q T N I N E C A S N P C

2401/801 2431/811 2461/821 2491/831
CTG AAC CAG GGC ACG TGT ATT GAC GAC GTT GCC GGG TAC AAG TGC AAC TGC CTG CTG CCC TAC ACA GGT GCC ACG TGT GAG GTG GTG CTG GCC CCG TGT GCC CCC AGC CCC TGC AGA AAC
L N Q G T C I D D V A G Y K C N C L L P Y T G A T C E V V L A P C A P S P C R N

2521/841 2551/851 2581/861 2611/871
GGC GGG GAG TGC AGG CAA TCC GAG GAC TAT GAG AGC TTC TCC TGT GTC TGC CCC ACG GGC TGG CAA GCA GGG CAG ACC TGT GAG GTC GAC ATC AAC GAG TGC GTT CTG AGC CCG TGC CG
G G E C R Q S E D Y E S F S C V C P T G W Q A G Q T C E V D I N E C V L S P C R

2641/881 2671/891 2701/901 2731/911
CAC GGC GCA TCC TGC CAG AAC ACC CAC GGC GGC TAC CGG TGC CAC TGC CAG.GCC GGC TAC AGT GGG CGC AAC TGC GAG ACC GAC ATC GAC GAC TGC CGG CCC AAC CCG TGT CAC AAC GGG
H G A S C Q N T H G G Y R C H C Q A G Y S G R N C E T D I D D C R P N P C H N G

2761/921 2791/931 2821/941 2851/951
GGC TCC TGC ACA GAC GGC ATC AAC ACG GCC TTC TGC GAC TGC CTG CCC GGC TTC CGG GGC ACT TTC TGT GAG GAG GAC ATC AAC GAG TGT GCC AGT GAC CCC TGC CGC AAC GGG GCC AAC
G S C T D G I N T A F C D C L P G F R G T F C E E D I N E C A S D P C R N G A N

FIG.5-4

2881/961 2911/971 2941/981 2971/991
TGC ACG GAC TGC GTG GAC AGC TAC ACG TGC ACC TGC CCC GCA GGC TTC AGC GGG ATC CAC TGT GAG AAC AAC ACG CCT GAC TGC ACA GAG AGC TCC TGC TTC AAC GGT GGC ACC TGC GTG
C T D C V D S Y T C T C P A G F S G I H C E N N T P D C T E S S C F N G G T C V

3001/1001 3031/1011 3061/1021 3091/1031
GAC GGC ATC AAC TCG TTC ACC TGC CTG TGT CCA CCC GGC TTC ACG GGC AGC TAC TGC CAG CAC GAT GTC AAT GAG TGC GAC TCA CAG CCC TGC CTG CAT GGC GGC ACC TGT CAG GAC GGC
D G I N S F T C L C P P G F T G S Y C Q H D V N E C D S Q P C L H G G T C Q D G

3121/1041 3151/1051 3181/1061 3211/1071
TGC GGC TCC TAC AGG TGC ACC TGC CCC CAG GGC TAC ACT GGC CCC AAC TGC CAG AAC CTT GTG CAC TGG TGT GAC TCC TCG CCC TGC AAG AAC GGC GGC AAA TGC TGG CAG ACC CAC ACC
C G S Y R C T C P Q G Y T G P N C Q N L V H W C D S S P C K N G G K C W Q T H T

3241/1081 3271/1091 3301/1101 3331/1111
CAG TAC CGC TGC GAG TGC CCC AGC GGC TGG ACC GGC CTT TAC TGC GAC GTG CCC AGC GTG TCC TGT GAG GTG GCT GGG CAG CGA CAA GGT GTT GAC GTT GCC CGC CTG TGC CAG CAT GGA
Q Y R C E C P S G W T G L Y C D V P S V S C E V A A Q R Q G V D V A R L C Q H G

3361/1121 3391/1131 3421/1141 3451/1151
GGG CTC TGT GTG GAC GCG GGC AAC AGG CAC CAC TGC CGC TGC CAG GCG GGC TAC ACA GGC AGC TAC TGT GAG GAC CTG GTG GAC GAG TGC TCA CCC AGC CCC TGC CAG AAC GGG GCC ACC
G L C V D A G N T H H C R C Q A G Y T G S Y C E D L V D E C S P S P C Q N G A T

3481/1161 3511/1171 3541/1181 3571/1191
TGC AGG GAC TAC CTG GGC GGC TAC TCC TGC AAG TGC GTG GCC GGC TAC CAC GGG GTG AAC TGC TCT GAG GAG ATC GAC GAG TGC CTC TCC CAC CCC TGC CAG AAC GGG GGC ACC TGC CTC
C T D Y L G G Y S C K C V A G Y H G V N C S E E I D E C L S H P C Q N G G T C L

FIG.5-5

3601/1201 3631/1211 3661/1221 3691/1231
GAC CTC CCC AAC ACC TAC AAG TGC TCC TGC CCA CGG GGC ACT CAG GGT GTG CAC TGT GAG ATC AAC GTG GAC GAC TGC AAT CCC CCC GTT GAC CCC GTG TCC CGG AGC CCC AAG TGC TTT
D L P N T Y K C S C P R G T Q G V H C E I N V D D C N P P V D P V S R S P K C F

3721/1241 3751/1251 3781/1261 3811/1271
AAC AAC GGC ACC TGC GTG GAC CAG GTG GGC GGC TAC AGC TGC ACC TGC CGG CGG GGC TTC GTG GGT GAG CGC TGT GAG GGG GAT GTC AAC GAG TGC CTG TCC AAT CCC TGC GAC GCC CGT
N N G T C V D Q V G G Y S C T C P P G F V G E R C E G D V N E C L S N P C D A R

3841/1281 3871/1291 3901/1301 3931/1311
GGC ACC CAG AAC TGC GTG CAG CGC GTC AAT GAC TTC CAC TGC GAG TGC CGT GCT GGT CAC ACC GGG CGC CGC TGC GAG TCC GTC ATC AAT GGC TGC AAA GGC AAG CCC TGC AAG AAT GGG
G T Q N C V Q R V N D F H C E C R A G H T G R R C E S V I N G C K G K P C K N G

3961/1321 3991/1331 4021/1341 4051/1351
GGC ACC TGC GCC GTG GCC TCC AAC ACC GCC CGC GGG TTC ATC TGC AAG TGC CCT GCG GGC TTC GAG GGC GCC AGG TGT GAG AAT GAC GCT CGT ACC TGC GGC AGC CTG CGC TGC CTC AAC
G T C A V A S N T A R G F I C K C P A G F E G A T C E N D A R T C G S L R C L N

4081/1361 4111/1371 4141/1381 4171/1391
GGC GGC ACA TGC ATC TCC GGC CGG CGC AGC CCC ACC TGC CTG TGC CTG GGC CCC TTC AGG GGC CCC GAA TGC CAG TTC CCG GCC AGC AGC CCC TGC CTG GGC GGC AAC CCC TGC TAC AAC
G G T C I S G P R S P T C L C L G P F T G P E C Q F P A S S P C L G G N P C Y N

4201/1401 42311 1411 4261/1421 4291/1431
CAG GGG ACC TGT GAG CCC ACA TCC GAG AGC CCC TTC TAC CGT TGC CTG TGC CCC GCC AAA TTC AAC GGG CTC TTG TGC CAC ATC CTG GAC TAC AGC TTC GGG GGT GGG GCC GGG CGC GAC
Q G T C E P T S E S P F Y R C L C P A K F N G L L C H I L D Y S F G G G A G R D

FIG.5-6

4321/1441 4351/1451 4381/1461 4411/1471
ATC CCC CCG CCG CTG ATC GAG GAG GCG TGC GAG CTG CCC GAG TGC CAG GAG GAC GCG GGC AAC AAG GTC TGC AGC CTG CAG TGC AAC AAC CAC GCG TGC GGC TGG GAC GGC GGT GAC TGC
I P P P L I E E A C E L P E C Q E D A G N K V C S L Q C N N H A C G W D G G D C

4441/1481 4471/1491 4501/1501 4531/1511
TCC CTC AAC TTC AAT GAC CCC TGG AAG AAC TGC ACG CAG TCT CTG CAG TGC TGG AAG TAC TTC AGT GAC GGC CAC TGT GAC AGC CAG TGC AAC TCA GCC GGC TGC CTC TTC GAC GGC TTT
S L N F N D P W K N C T Q S L Q C W K Y F S D G H C D S Q C N S A G C L F D G F

4561/1521 4591/1531 4621/1541 4651/1551
GAC TGC CAG CGT GCG GAA GGC CAG TGC AAC CCC CTG TAC GAC CAG TAC TGC AAG GAC CAC TTC AGC GAC GGG CAC TGC GAC CAG GGC TGC AAC AGC GCG GAG TGC GAG TGG GAC GGG CTG
D C Q R A E G Q C N P L Y D Q Y C K D H F S D G H C D Q G C N S A E C E W D G L

4681/1561 4711/1571 4741/1581 4771/1591
GAC TGT GCG GAG CAT GTA CCC GAG AGG CTG GCG GCC GGC ACG CTG GTG GTG GTG GTG CTG ATG CCG CCG GAG CAG CTG CGC AAC AGC TCC TTC CAC TTC CTG CGG GAG CTC AGC CGC GTG
D C A E H V P E R L A A G T L V V V V L M P P E Q L R N S S F H F L R E L S R V

4801/1601 4831/1611 4861/1621 4891/1631
CTG CAC ACC AAC GTG GTC TTC AAG CGT GAC GCA CAC GGC CAG CAG ATG ATC TTC CCC TAC TAC GGC CGC GAG GAG GAG CTG CGC AAG CAC CCC ATC AAG CGT GCC GCC GAG GGC TGG GCC
L H T N V V F K R D A H G Q Q M I F P Y Y G R E E E L R K H P I K R A A E G W A

4921/1641 4951/1651 4981/1661 5011/1671
GCA CCT GAC GCC CTG CTG GGC CAG GTG AAG GCC TCG CTG CTC CCT GGT GGC AGC GAG GGT GGG CGG CGG CGG AGG GAG CTG GAC CCC ATG GAC GTC CGC GGC TCC ATC GTC TAC CTG GAG
A P D A L L G Q V K A S L L P G G S E G G R R R R E L D P M D V R G S I V Y L E

FIG.5-7

5041/1681 5071/1691 5101/1701 5131/1711
ATT GAC AAC CGG CAG TGT GTG CAG GCC TCC TCG CAG TGC TTC CAG AGT GCC ACC GAC GTG GCC GCA TTC CTG GGA GGG CTC GCC TCG CTG GGC AGC CTC AAC ATC CCC TAC AAG ATC GAG
I D N R Q C V Q A S S Q C F Q S A T D V A A F L G A L A S L G S L N I P Y K I E

5161/1721 5191/1731 5221/1741 5251/1751
GCC GTG CAG AGT GAG ACC GTG GAG CCG CCC CCG CCG GGG CAG CTG CAC TTC ATG TAC GTG GCG GCG GCC GCC TTT GTG CTT CTG TTC TTC GTG GGC TGC GGG GTG CTG CTG TCC CGC AAG
A V Q S E T V E P P P P A Q L H F M Y V A A A A F V L L F F V G C G V L L S R K

5281/1761 5311/1771 5341/1781 5371/1791
CGC CGG CGG CAG CAT GGC CAG CTC TGG TTC CCT GAG GGC TTC AAA GTG TCT GAG GCC AGC AAG AAG AAG CGG CGG GAG CCC CTC GGC GAG GAC TCC GTG GGC CTC AAG CCC CTG AAG AAC
R R R Q H G Q L W F P E G F K V S E A S K K K R R E P L G E D S V G L K P L K N

5401/1801 5431/1811 5461/1821 5491/1831
GCT TCA GAC GGT GCC CTC ATG GAC GAC AAC CAG AAT GAG TGG GGG GAC GAG GAC CTG GAG ACC AAG AAG TTC CGG TTC GAG GAG CCC GTG GTT CTG CCT GAC CTG GAC GAC CAG ACA GAC
A S D G A L M D D N Q N E W G D E D L E T K K F R F E E P V V L P D L D D Q T D

5521/1841 5551/1851 5581/1861 5611/1871
CAC CGG CAG TGG ACT CAG CAG CAC CTG GAT GCC GCT GAC CTG CGC ATG TCT GCC ATG GCC CCC ACA CCG CCC CAG GGT GAG GTT GAC GCC GAC TGC ATG GAC GTC AAT GTC CGC GGG CCT
H R Q W T Q Q H L D A A D L R M S A M A P T P P Q G E V D A D C M D V N V R G P

5641/1881 5671/1891 5701/1901 5731/1911
GAT GGC TTC ACC CCG CTC ATG ATC GCC TCC TGC AGC GGG GGC GGC CTG GAG ACG GGC AAC AGC GAG GAA GAG GAG GAC GGG CCG GCC GTC ATC TCC GAC TTC ATC TAC CAG GGC GCC AGC
D G F T P L M I A S C S G G G L E T G N S E E E E D A P A V I S D F I Y Q G A S

FIG.5-8

5761/1921 5791/1931 5821/1941 5851/1951
CTG CAC AAC CAG ACA GAC CGC ACG GGC GAG ACC GCC TTG CAC CTG GCC GCC CGC TAC TCA CGC TCT GAT GCC GCC AAG CGC CTG CTG GAG GCC AGC GCA GAT GCC AAC ATC CAG GAC AAC
L H N Q T D R T G E T A L H L A A R Y S R S D A A K R L L E A S A D A N I Q D N

5881/1961 5911/1971 5941/1981 5971/1991
ATG GGC CGC ACC CCG CTG CAT GCG GCT GTG TCT GCC GAC GCA CAA GGT GTC TTC CAG ATC CTG ATC CGG AAC CGA GCC ACA GAC CTG GAT GCC CGC ATG CAT GAT GGC ACG ACG CCA CTG
M G R T P L H A A V S A D A Q G V F Q I L I R N R A T D L D A R M H D G T T P L

6001/2001 6031/2011 6061/2021 6091/2031
ATC CTG GCT GCC CGC CTG GCC GTG GAG GGC ATG CTG GAG GAC CTC ATC AAC TCA CAC GCC GAC GTC AAC GCC GTA GAT GAC CTG GGC AAG TCC GCC CTG CAC TGG GCC GCC GCC GTG AAC
I L A A R L A V E G M L E D L I N S H A D V N A V D D L G K S A L H W A A A V N

6121/2041 6151/2051 6181/2061 6211/2071
AAT GTG GAT GCC GCA GTT GTG CTC CTG AAG AAC GGG GCT AAC AAA GAT ATG CAG AAC AAC AGG GAG GAG ACA CCC CTG TTT CTG GCC GCC CGG GAG GGC AGC TAC GAG ACC GCC AAG GTG
N V D A A V V L L K N G A N K D M Q N N R E E T P L F L A A R E G S Y E T A K V

6241/2081 6271/2091 6301/2101 6331/2111
CTG CTG GAC CAC TTT GCC AAC CGG GAC ATC ACG GAT CAT ATG GAC CGC CTG CCG CGC GAC ATC GCA CAG GAG CGC ATG CAT CAC GAC ATC GTG AGG CTG CTG GAC GAG TAC AAC CTG GTG
L L D H F A N R D I T D H M D R L P R D I A Q E R M H H D I V R L L D E Y N L V

6361/2121 6391/2131 6421/2141 6451/2151
CGC AGC CCG CAG CTG CAC GGA GCC CCG CTG GGG GGC ACG CCC ACC CTG TCG CCC CCG CTC TGC TCG CCC AAC GGC TAC CTG GGC AGC CTC AAG CCC GGC GTG CAG GGC AAG AAG GTC CGC
R S P Q L H G A P L G G T P T L S P P L C S P N G Y L G S L K P G V Q G K K V R

FIG.5-9

6481/2161 6511/2171 6541/2181 6571/2191

AAG CCC AGC AGC AAA GGC CTG GCC TGT GGA AGC AAG GAG GCC AAG GAC CTC AAG GCA CGG AGG AAG AAG TCC CAG GAC GGC AAG GGC TGC CTG CTG GAC AGC TCC GGC ATG CTC TCG CCC

K P S S K G L A C G S K E A K D L K A R R K K S Q D G K G C L L D S S G M L S P

6601/2201 6631/2211 6661/2221 6691/2231

GTG GAC TCC CTG GAG TCA CCC CAT GGC TAC CTG TCA GAG GTG GCC TCG CGG CCA CTG CTG CCC TCC CCG TTC CAG CAG TCT CCG TCC GTG CCC CTC AAC CAC CTG CCT GGG ATG CCC GAC

V D S L E S P H G Y L S D V A S P P L L P S P F Q Q S P S V P L N H L P G M P D

6721/2241 6751/2251 6781/2261 6811/2271

ACC CAC CTG GGC ATC GGG CAC CTG AAC GTG GCG GCC AAG CCC GAG ATG GCG GCG CTG GGT GGG GGC GGC CGG CTG GCC TTT GAG ACT GGC CCA CCT CGT CTC TCC CAC CTG CCT GTG GCC

T H L G I G H L N V A A K P E M A A L G G G G R L A F E T G P P R L S H L P V A

6841/2281 6871/2291 6901/2301 6931/2311

TCT GGC ACC AGC ACC GTC CTG GGC TCC AGC AGC GGA GGG GCC CTG AAT TTC ACT GTG GGC GGG TCC ACC AGT TTG AAT GGT CAA TGC GAG TGG CTG TCC CGG CTG CAG AGC GGC ATG GTG

S G T S T V L G S S S G G A L N F T V G G S T S L N G Q C E W L S R L Q S G M V

6961/2321 6991/2331 7021/2341 7051/2351

CCG AAC CAA TAC AAC CCT CTG CGG GGG AGT GTG GCA CCA GGC CCC CTG AGC ACA CAG GCC CCC TCC CTG CAG CAT GGC ATG GTA GGC CCG CTG CAC AGT AGC CTT GCT GCC AGC GCC CTG

P N Q Y N P L R G S V A P G P L S T Q A P S L Q H G M V G P L H S S L A A S A L

7081/2361 7111/2371 7141/2381 7171/2391

TCC CAG ATG ATG AGC TAC CAG GGC CTG CCC AGC ACC CGG CTG GCC ACC CAG CCT CAC CTG GTG CAG ACC CAG CAG GTG CAG CCA CAA AAC TTA CAG ATG CAG CAG CAG AAC CTG CAG CCA

S Q M M S Y Q G L P S T R L A T Q P H L V Q T Q Q V Q P Q N L Q M Q Q Q N L Q P

FIG.5-10

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7201/2401                    7231/2411                    7261/2421                    7291/2431
ACA AAC ATC CAG CAG CAG CAA AGC CTG CAG CCG CCA CCA CCA CCA CCA CAG CCG CAC CTT GGC GTG AGC TCA GCA GCC AGC GGC CAC CTG GGC CGG AGC TTC CTG AGT GGA GAG CCG AGC
A  N  I  Q  Q  Q  Q  S  L  Q  P  P  P  P  P  P  Q  P  H  L  G  V  S  S  A  A  S  G  H  L  G  R  S  F  L  S  G  E  P  S

7321/2441                    7351/2451                    7381/2461                    7411/2471
CAG GCA GAC GTG CAG CCA CTG GGC CCC AGC AGC CTG GCG GTG CAC ACT ATT CTG CCC CAG GAG AGC CCC GCC CTG CCC AGG TCG CTG CCA TCC TCG CTG GTC CCA CCC GTG ACC GCA GCC
Q  A  D  V  Q  P  L  G  P  S  S  L  A  V  H  T  I  L  P  Q  E  S  P  A  L  P  T  S  L  P  S  S  L  V  P  P  V  T  A  A

7441/2481                    7471/2491                    7501/2501                    7531/2511
CAG TTC CTG ACG CCC CCC TCG CAG CAC AGC TAC TCC TCG CCT GTG GAC AAC ACC CCC AGC CAC CAG CTA CAG GTG CCT GAG CAC CCC TTC CTC ACC CCG TCC CCT GAG TCC CCT GAC CAG
Q  F  L  T  P  P  S  Q  H  S  Y  S  S  P  V  D  N  T  P  S  H  Q  L  Q  V  P  E  H  P  F  L  T  P  S  P  E  S  P  D  Q

7561/2521                    7591/2531                    7621/2541                    7651/2551
TGG TCC AGC TCG TCC CCG CAT TCC AAC GTC TCC GAC TGG TCC GAG GGC GTC TCC AGC CCT CCC ACC AGC ATG CAG TCC CAG ATC GCC CGC ATT CCG GAG GCC TTC AAG TAA
W  S  S  S  S  P  H  S  N  V  S  D  W  S  E  G  V  S  S  P  P  T  S  M  Q  S  Q  I  A  R  I  P  E  A  F  K  *
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FIG.5-11

NOTCH1 exon 27 seq	NOTCH1 exon 27 mut
5030 T/A	1677 V/D
5032 INS<AGCACACTT>	1678 INS STL
5036 T/C	1679 L/P
5042 T/A	1681 I/N
5042 T/C	1681 I/T
5093 C/A	1698 T/N
5156 T/C	1719 I/T
5157 INS<TCC>	7120 INS P
5104 G/C	1702 A/P
5129 T/C	1710 L/P

5165 INS<CCCGGTTGGGCAGCCTCAACATCCCCTACAAGATCGAGGCCG> (SEQ ID NO:146) 1722 V/A, 1723 INS RLGSLNIPYKIEAV(SEQ ID NO:137)

Exon 26 seq	Exon 26 Mutation
4802 T/C	1601 L/P
4781 T/C	1594 L/P
4796 G/C	1599 R/P
4724 T/C	1575 L/P
4735 DEL(GTG)	1579 DEL V
4778 T/C	1593 F/S
4757 T/C	1586 L/P
4825 C/A	1609 R/S
4815 DEL(GGT)	1606 DEL V
4825 DEL (CGTGACGCA) INS<ACG>	1609 R/T, 1610 DEL D, 1611 DEL A
4857 DEL (TACTAC) INS<CCTGGGGGA>	1620 Y/P, 1621 Y/G, 1622 INS G

FIG.6-1

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4751 INS<CCC>
4821 DEL(CAAGC) INS<GG>
4779 INS<AGC>
4779 INS<TAAGGAGGA>
4777 DEL(TTCC) INS<ACTGCG>
4781 T/C, 4829 A/T
4821 INS<ATCAATGCCCCC> (SEQ ID NO:147)

Exon 34 Seq

7195 C/T
7183 C/T
7180 C/T
7025 INS<GAGCACACAGGCCCCCT> (SEQ IS NO:148)
7428 DEL(C)
7422 INS<GG>
7405 INS<GG>
7403 C/A
7389 DEL (C)
7378 C/T
7330 INS<TTAAGTAGAGACTT> (SEQ ID NO:149)
7330 DEL (G) INS<TGT>
7329 DEL (C) INS<GGGCCGTGGACG> (SEQ ID NO:150)
7331 DEL(T) INS<GTGGAGGGGGGATGG> (SEQ ID NO:151)
7317 INS<AGAAC>,7518 T/G
7316 INS<GGGTT>
7321 C/T

1584 E/A, 1585 INS Q
1607 F/L, 1608 K/G, 1609 INS R
1593 F/L, 1594 INS A
1594 INS K, 1595 INS E, 1596 INS D
1593 F/T, 1594 INS A
1594 L/P, 1610 D/V
1608 INS S, 1609 INS M, 1610 INS P, 1611 INS P

Exon 34 Mutation

2399 STOP
2395 STOP
2394 STOP
2342 STOP
2477 *STOP
2474 AHP*STOP
2469 GCHPRWSHP*STOP (SEQ ID NO:154)
2468 *STOP
2464 PCPRRCHPRWSHP*STOP (SEQ ID NO:155)
2460 *STOP
2444 LSRDLCSHWAPAAWRCTLFCPRRAPPCPRRCHPRWSH*STOP (SEQ ID NO:156)
2442 CCSHWAPAAWRCTLFCPRRAPPCPRRCHPRWSHP*STOP (SEQ ID NO:157)
2442 EGRGRCSHWAPAAWRCTLFCPRRAPPCPRRCHPRWSHP*STOP (SEQ ID NO:158)
2444 GGGGMGSHWAPAAWRCTLFCPRRAPPCPRRCHPRWSHP*STOP (SEQ ID NO:159)
2440 ERARQTCSHWAPAAWRCTLFCPRRAPPCPRRCHPRWSHP*STOP (SEQ ID NO:160)
2439 RVRARQTCSHWAPAAWRCTLFCPRRAPPCPRRCHPRWSHP*STOP (SEQ ID NO:161)
2441 *STOP

FIG.6-2

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37/38
PCT/US2005/027716

7299 INS <TGCCC>
7262 INS <CC>,
7233 INS<CC>
7216C/T
7210 C/T

7548 DEL(GTCCCCTGAC), 7561 T/C (SEQ ID NO:152)

7542 INS<GAGCGTC>
7544 DEL(CT)
7539 INS <C>
7522 C/T, 7529 DEL(TCCTCACCCC) (SEQ ID NO:153)
INS<GA>,
7519 G/T
7480 DEL (CCTGTG) INS<GGGC>
7478 C/A
7195 C/T

2434 APS*STOP
2421 AA*STOP
2412 RHHHHHSRTLVA*STOP (SEQ ID NO:162)
2406 STOP
2404 STOP

2516 DSGPARPRIPTSPTGPRASPALPPACSPRSPAFRRPSSKRRAPRSPGF
LSQAFGRLCALCGCQGRPEEPS*STOP (SEQ IS NO:163)
2515 SVP*STOP
2515 RVP*STOP
2514 VP*STOP
2508 H/Y, 2510*STOP

2507 *STOP
2494 GRQHPQPPATGA *STOP (SEQ ID NO:164)
2493 STOP
2399 STOP

FIG.6-3