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(54) Title: ACTIVATING MUTATIONS IN NOTCH-1

(57) Abstract: The present invention relates to certain methods and compositions for diagnosing, preventing, treating, and/or ameliorating the effects of various conditions in a mammal, such as T-cell lymphoblastic leukemia and lymphoma. The present invention also relates, inter alia, to methods of determining whether reducing or blocking NOTCH 1 activation will be effective to treat, prevent, or ameliorate the effects of a cancer in a patient. Such methods may include determining if the patient harbors one or more mutations in exon 28 or intron 27 of the NOTCH1 gene.

ACTIVATING MUTATIONS IN NOTCH-1

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is related to, and incorporates by reference, U.S. provisional patent application serial number 60/937,981, filed June 29, 2007, and U.S. provisional patent application serial number 61/065,804, filed February 15, 2008.

FIELD OF THE INVENTION

[0002] The field of the present invention relates to methods and compositions for diagnosing, preventing, treating, and/or ameliorating the effects of various conditions in a mammal, such as cancer, for example, T-cell lymphoblastic leukemia and lymphoma. The present invention further relates to screening assays as well as patient stratification methods.

BACKGROUND OF THE INVENTION

[0003] NOTCH signaling plays a critical role in lineage specification decisions that enable multipotential precursor cells to become committed to specific cell lineages during development, and therefore has important roles in cell differentiation, proliferation and apoptosis. The fundamental components of the NOTCH pathway include the DSL ligands (Delta-like 1, 3 and 4; and Jagged 1 and 2), the NOTCH receptors (NOTCH1-4), and the CSL DNA binding protein, a transcription factor that mediates the conversion of NOTCH-activating signals at the cell surface to changes in gene expression in the nucleus.

[0004] The mature NOTCH1 receptor is a heterodimeric class I transmembrane glycoprotein generated by proteolytic processing of a precursor polypeptide in the trans-Golgi network. This first protease cleavage (S1) is catalyzed by a furin protease, which

cuts the NOTCH1 precursor protein approximately 70 amino acids external to the transmembrane domain to generate extracellular (N^{EC}) and transmembrane and intracellular (N^{TM}) NOTCH1 subunits. These two polypeptides remain noncovalently associated in the resting receptor through the interaction of the sequences lying immediately N-terminal and C-terminal of the S1 furin cleavage site, which constitute the so-called heterodimerization (HD) domain.

[0005] In addition, the extracellular subunit of NOTCH1 contains 36 epidermal growth factor (EGF)-like repeats involved in ligand-receptor interaction, followed by three LIN-12/NOTCH repeats (LNR), which stabilize the interaction between the extracellular and transmembrane subunits and help keep the receptor in the resting state in the absence of ligand. The N^{TM} NOTCH1 subunit consists of a short extracellular juxtamembrane peptide followed by a transmembrane sequence and a series of cytoplasmic domains, including a RAM domain, a membrane proximal to a nuclear localization signal, a series of ankyrin repeats, a distal nuclear localization signal, a transactivation domain and a carboxy-terminal PEST sequence, which together function as a ligand-activated transcription factor.

[0006] Current models of the mechanism of NOTCH1 activation provide that ligand interaction is followed by a conformation change in the LNR repeats-HD domain complex, which leads to the proteolytic cleavage of the N^{TM} NOTCH1 subunit, first by an ADAM metalloprotease, which cuts the extracellular juxtamembrane just 12 amino acids proximal to the membrane (S2 site); and subsequently by γ-secretase, an aspartyl protease multiprotein complex, which cuts the receptor at several different positions within the transmembrane domain. The final cleavage is catalyzed by the γ-secretase

complex at position Val 1744 (site S3), which releases the intracellular fraction of NOTCH1 (ICN1) from the membrane, allowing it to translocate to the nucleus where it activates the transcription of target genes in a complex with the DNA-binding factor CSL and members of the mastermind family of coactivators.

[0007] The NOTCH signaling pathway plays a critical role in the hematopoietic system by maintaining stem cell homeostasis and participating at multiple stages of T-cell development. During early hematopoiesis, NOTCH signaling is required for the commitment of multipotent hematopoietic progenitors to the T-cell lineage. In addition, NOTCH1 is required later in T-cell development for progression through the early DN1, DN2 and DN3 stages of thymocyte maturation; participates in the regulation of TCRB gene rearrangement; and regulates lineage decisions between αβ *vs.* γδ lineages and, at least in some systems, between CD4 vs. CD8 lineages.

[0008] Aberrant activation of NOTCH1 signaling induces transformation of T-cell progenitors and plays a prominent role in the pathogenesis of T-cell lymphoblastic leukemia (T-ALL). Thus, mice reconstituted with hematopoietic progenitors transduced with viruses driving the expression of constitutively active forms of NOTCH1 (and transgenic mice expressing dominant active forms of NOTCH1 in hematopoietic progenitor cells or in immature thymocytes) rapidly develop T-cell neoplasias. In human leukemias, NOTCH1 activation was first demonstrated in T-ALL cases harboring the t(7;9)(q34;q34.3), a rare chromosomal translocation, which juxtaposes a truncated NOTCH1 gene next to the TCRB locus, leading to the aberrant expression of an intracellular constitutively active form of NOTCH1.

[0009] More recent studies have demonstrated the major role of NOTCH1 in the pathogenesis of human leukemias by showing the presence of activating mutations in NOTCH1 in over 50% of human T-ALLs. Activating mutations in NOTCH1 in human T-ALL are concentrated in exons 26 and 27, which encode the homodimerization domain; and in the 3' end of exon 34, which encodes the PEST domain in the C-terminal region of the protein. HD mutations are typically single-amino-acid substitutions and small in-frame deletions and insertions, which probably operate by inducing ligand-independent activation of NOTCH1. In contrast, PEST mutations result in premature stop codons, the loss of the PEST domain, and increased levels of intranuclear active NOTCH1 due to impaired degradation of the activated receptor by the proteasome. Importantly, HD and PEST mutations are often found in *cis* in the same NOTCH1 transcript. These HD-PEST-double mutant alleles result in synergistic activation of NOTCH1 signaling and are 10-times more active than NOTCH1 alleles containing an HD or a PEST mutation alone.

[0010] In view of the foregoing, it would be beneficial to identify other mutations in NOTCH1 that contribute to ligand-independent activation of NOTCH1.

SUMMARY OF THE INVENTION

[0011] According to certain embodiments of the invention, assays for identifying candidate compounds that may be used to treat or ameliorate the effects of cancer are provided. Such assays generally comprise the steps of (a) contacting cells from a cell line comprising a juxtamembrane expansion (JME) mutation with a candidate compound and (b) determining whether NOTCH1 expression is modulated in the cell

line contacted with the candidate compound relative to control cells from the same cell line that have not been contacted with the candidate compound.

[0012] According to additional embodiments of the invention, methods of making a cell line that over-expresses NOTCH1 are provided. These methods comprise introducing a mutation into a region of DNA that encodes the extracellular juxtamembrane region of a NOTCH1 receptor.

[0013] According to additional embodiments of the invention, assays for identifying candidate compounds that may be used to treat or ameliorate the effects of cancer are provided. These assays generally employ the steps of contacting cells from a cell line that over-expresses a NOTCH1 receptor, which comprises a mutation in the extracellular juxtamembrane region thereof, with a candidate compound. The assays further comprise the step of determining whether NOTCH1 expression is modulated in the cell line contacted with the candidate compound, compared to control cells from the same cell line that have not been contacted with the candidate compound.

[0014] According to further embodiments of the invention, isolated polypeptides, and isolated nucleic acid sequences that encode such polypeptides, are provided. These polypeptides comprise a mutation in the extracellular juxtamembrane region of a NOTCH1 receptor.

[0015] According to yet further embodiments of the invention, methods of determining whether reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of a cancer in a patient are provided. These methods generally comprise (a) determining if the patient harbors one or more mutations in exon

28 or intron 27 of a NOTCH1 gene or (b) determining if NOTCH1 comprises the amino acid sequence of SEQ ID NO:2 proximal to the transmembrane domain of NOTCH1.

[0016] According to still further embodiments of the invention, methods of (a) determining whether reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of a cancer, (b) identifying whether a patient is sensitive to one or more γ-secretase inhibitors, and (c) identifying a patient population for inclusion in a clinical trial of a drug candidate for treating cancer are provided. Such methods comprise carrying out a screen for mutations in exon 26, exon 27, intron 27, exon 28, and exon 34 of a NOTCH1 gene in a sample of DNA from a patient. According to such embodiments, the presence of a mutation identified by the screen in the patient's DNA sample (i) is indicative that reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of the cancer, (ii) is indicative that a patient will be sensitive to γ-secretase inhibitors, and (iii) should be considered in determining whether to include each patient in a particular clinical trial (in view of the mode of action of the candidate compound to be tested in the clinical trial).

[0017] According to further embodiments of the invention, kits for determining whether reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of a cancer are provided. These kits comprise a set of PCR primers that may be used to generate an amplicon from patient DNA which comprises intron 27 and/or exon 28 of the NOTCH1 gene.

BRIEF DESCRIPTION OF THE FIGURES

[0018] The file of this patent application contains at least one drawing executed in color. Copies of any patent to issue from this application with color drawing(s) will be

provided by the United States Patent and Trademark Office upon request and payment of the necessary fee.

[0019] **Figure 1.** Identification of internal tandem duplications in the extracellular juxtamembrane domain of NOTCH1 in T-ALL. **A.** Western blot analysis of T-ALL cell lines lacking HD and PEST domain mutations in NOTCH1 demonstrating high levels of activated NOTCH1 in the Jurkat cell line. ICN1 levels were detected with the NOTCH1 Val1744 antibody. Tubulin is shown as loading control. **B.** Sequence analysis of the NOTCH1 transcripts showing the mutation identified in the Jurkat cell line. The wild type NOTCH1 transcript sequence is shown at the bottom and inserted sequences on top. S2 and S3 indicate the sites for ADAM metalloprotease and γ-secretase cleavage, respectively. Duplicated nucleotides are shown in italics and underlined in the wild type sequence. The common 4 amino acids (QLHF) present in all three JME mutants in this series are highlighted in yellow. **C.** PCR amplification of exon 28 of NOTCH1 and adjacent intronic sequences. Three samples corresponding to Jurkat cells and two independent primary T-ALL cases show bands of increased size compared to the control genomic DNA indicating the presence of insertions in the extracellular juxtamembrane domain of NOTCH-1.

[0020] **Figure 2.** NOTCH1 JME mutants show high levels of NOTCH1 signaling. **A.** Transactivation activity of different NOTCH1 alleles in a CSL luciferase reporter assay. Three classic NOTCH1 alleles: HD L1600P, class 1 HD mutant; Δ-PEST, a C-terminal truncation of NOTCH1 lacking a PEST domain; L1600P+Δ-PEST, a double mutant allele of NOTCH1 harboring both a HD mutation and a PEST domain truncation; and Jurkat JME 17, NOTCH1 exon 28 mutant allele identified in Jurkat cells. Reporter

activity is shown as fold change compared with wild type NOTCH1 (WT). **B.** Transactivation activity of artificially generated NOTCH1 JME alleles. NOTCH1 constructs expressing juxtamembrane insertions of 5-14 amino acids corresponding to the VSV-G epitope as well as the Jurkat JME 17 allele were tested in a CSL luciferase reporter assay. Reporter activity is shown as fold change compared with wild type NOTCH1 (WT).

[0021] **Figure 3. A**. Western blot analysis of activated NOTCH1 (ICN1) levels in Jurkat cells treated with CompE (100 nM) for 24 hours shows inhibition of NOTCH1 processing and clearance of activated intracellular NOTCH1. **B.** Quantitative RT-PCR analysis of HES1 expression in Jurkat cells treated with CompE (100 nM) or vehicle only (DMSO). **C-D.** CSL luciferase reporter assay (**C**) and Western blot analysis (**D**) of ICN1 activity and expression in HeLa cells expressing NOTCH1 JME mutants treated with vehicle (DMSO) or a γ-secretase inhibitor (CompE 100 nM). **E-F.** Luciferase reporter assay with a CSL reporter construct shows effective inhibition of NOTCH1 signaling from the NOTCH1 Jurkat JME 17 allele (**E**) and the NOTCH1 VSVG JME 14 allele (**F**) upon mutation of the S2 cleavage site.

[0022] **Figure 4.** JME mutants do not affect the stability of the LNR-HD complex. **A.** Schematic representation of the soluble LNR-HD complex encoded by the NOTCH1 minigene. In addition to the LNR repeats (green), the N-terminal (HD-N) and C-terminal (HD-C) subunits of the HD domain (blue) and the extracellular juxtamembrane region (JM), this construct contains an N-terminal FLAG tag and a C-terminal HA tag. **B.** Immunoprecipitation immunoblot analysis of NOTCH1 minigene protein products recovered from conditioned media of 293T cells transfected with empty plasmid

(Control), a NOTCH1 wild type minigene (WT), a NOTCH1 minigene containing the Jurkat JME 17 amino acid insertion (Jurkat JME 17) and a NOTCH1 minigene containing the artificial VSVG-P JME 14 amino acid insertion. **C.** Immunoprecipitation immunoblot analysis of NOTCH1 minigene protein products from a NOTCH1 wild type minigene (WT) and NOTCH1 minigene containing the Jurkat JME 17 amino acid insertion (Jurkat JME 17) incubated with increased concentrations of urea to test the stability of the LNR-HD domain complex.

[0023] **Figure 5.** Schematic representation of NOTCH1 mutations leading to increased γ-secretase processing and activation in T-ALL. Functional domains of NOTCH1 are annotated in the structure of wild type NOTCH-1. EGF-like: EGF-like repeats. HD: heterodimerization domain. LNR: LNR repeats. RAM: RAM domain. Ankyrin: ankyrin repeats. TAD: transactivation domain. PEST: PEST domain. S2: metalloprotease cleavage site. S3: γ-secretase cleavage site. Sequences altered by the different NOTCH1 mutations are highlighted in red. HD class 1 mutants disrupt the HD domain structure. HD class 2 mutants displace the S2 cleavage site away from the HD-LNR complex. NOTCH1 JME mutants displace the HD-LNR repeat complex away from the cell surface.

[0024] **Figure 6** A table summarizing certain NOTCH1 mutations and the effects thereof on the predicted amino acid sequences of the resulting polypeptides.

DETAILED DESCRIPTION OF THE INVENTION

[0025] According to certain embodiments of the invention, assays for identifying candidate compounds that may be used to treat or ameliorate the effects of cancer are provided. Such assays generally comprise the steps of (a) contacting cells from a cell

line comprising a juxtamembrane expansion (JME) mutation with a candidate compound and (b) determining whether NOTCH1 expression is modulated in the cell line contacted with the candidate compound relative to control cells from the same cell line that have not been contacted with the candidate compound. According to such embodiments, the modulation of NOTCH1 expression may be represented by a decrease in NOTCH1 expression in the cells of the cell line containing the JME mutation. The invention provides that such a decrease in NOTCH1 expression is indicative that the candidate compound may be effective to treat cancer.

[0026] Candidate compounds identified by such assays may be used to treat or ameliorate the effects of various types of cancer, including without limitation T-cell leukemia, myeloleukemia, and solid tumors. Non-limiting examples of solid tumors include those implicated in neuroblastoma, breast cancer, and ovarian cancer. Preferably, the cancer is T-ALL leukemia.

[0027] In such embodiments, the invention provides that the JME mutation may be, for example, an internal tandem duplication that comprises a polynucleotide that encodes at least 11 amino acids, such at least 12, and preferably at least 14 or more, and is located in a region that encodes the extracellular juxtamembrane region of a NOTCH1 receptor. The mutation, *e.g.*, an internal tandem duplication, may be inserted in a region extending from about the distal part of intron 27 to about the proximal part of exon 28 of a NOTCH1 gene and, more specifically, within about 50 nucleotides (upstream or downstream) of nucleotide 1740 of a NOTCH1 gene (SEQ ID NO:1). In some cases, the mutation, *e.g.*, internal tandem duplication, will be inserted between about nucleotide 1738 to about 1741 of SEQ ID NO:1, and encodes from about 11 to

about 36 amino acids. More specifically, in certain embodiments, the mutation, *e.g.*, internal tandem duplication, comprises a polynucleotide that encodes a peptide sequence comprising the amino acids: QLHF (SEQ ID NO:2). In the present invention, when a range is recited, both endpoints recited in the range are included within the range.

[0028] The assays of the present invention may employ the use of a mammalian cell line, such as for example, a human cell line. In certain embodiments, however, the cell line is a human T-cell line, such as for example, a Jurkat, IARC 301, PER-117, AG-F, or MT-2 cell line.

[0029] According to another embodiment of the invention, methods of making cell lines that over-express NOTCH1 are provided. Such methods comprise introducing a mutation into a region of DNA that encodes the extracellular juxtamembrane region of a NOTCH1 receptor. Preferably, the mutation comprises an insertion that encodes at least 11 amino acids. By way of example only, the mutation may comprise an internal tandem duplication. The mutation, *e.g.*, internal tandem duplication, may be inserted from about the distal part of intron 27 to about the proximal part of exon 28 of a NOTCH1 gene. In certain embodiments, the mutation, *e.g.*, internal tandem duplication, will be inserted within about 50 nucleotides (upstream or downstream), such as within about 25 nucleotides, such as 15 or 10 nucleotides, of nucleotide 1740 of a NOTCH1 gene or, more specifically, between about nucleotide 1738 to about nucleotide 1741 of the NOTCH1 gene. In such embodiments, the NOTCH1 gene will preferably comprise the polynucleotide sequence of SEQ ID NO:1.

[0030] Although such a mutation will preferably comprise an insertion that encodes at least 11 amino acids, the invention provides that an internal tandem duplication encoding from about 11 to about 36 amino acids is preferred. More particularly, in certain preferred embodiments, the mutation, *e.g.*, internal tandem duplication, comprises a polynucleotide that encodes a tetrapeptide sequence comprising the amino acids: QLHF (SEQ ID NO:2).

[0031] The cell lines made by the processes of the present invention may be obtained from any appropriate source. Preferably, the source is a mammalian cell line, such as for example, a human cell line. More preferably, the source is a human T-cell line, such as for example, a Jurkat cell line. The invention further encompasses pure or substantially pure cell lines that are produced by the foregoing methods.

[0032] In another embodiment, the invention is an assay for identifying candidate compounds that may be used to treat or ameliorate the effects of cancer. This assay comprises contacting cells from a cell line made by any of the processes disclosed herein with a candidate compound and determining whether NOTCH1 expression is modulated in the cell line contacted with the candidate compound compared to control cells from the same cell line that have not been contacted with the candidate compound.

[0033] According to further embodiments of the invention, isolated polynucleotide sequences are provided that comprise a mutation in the extracellular juxtamembrane region of a NOTCH1 receptor. Preferably, the NOTCH1 receptor is obtained from a mammal. More preferably, the NOTCH1 receptor is obtained from a human.

[0034] As used herein, the term "isolated polynucleotides," and similar terms, means nucleotide sequences that are free of the genes and other sequences that flank the polynucleotide sequences in the naturally-occurring genome of the organism from which the isolated polynucleotide sequences of the invention are derived. The term therefore includes, for example, a recombinant nucleotide sequence which is incorporated into a vector; into an autonomously replicating plasmid or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (*e.g.*, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant nucleotide sequence which is part of a hybrid gene encoding additional polypeptide sequences.

[0035] The isolated polynucleotide sequences of the invention comprise an insertion of nucleotides that encode at least 11 amino acids. In certain embodiments, the insertion is an internal tandem duplication. The mutation, *e.g.*, internal tandem duplication, is inserted in a region extending from about the distal part of intron 27 to about the proximal part of exon 28 of a NOTCH1 gene, such as within about 50 nucleotides (upstream or downstream), as described in more detail above, of nucleotide 1740 of a NOTCH1 gene. More particularly, in certain embodiments, the isolated polynucleotide sequences of the invention comprise a mutation, *e.g.*, an internal tandem duplication, between about nucleotide 1738 to about 1741 of a NOTCH1 gene (SEQ ID NO:1). The mutation, *e.g.*, internal tandem duplication, of such isolated polynucleotide sequences will preferably encode from about 11 to about 36 amino acids, which may comprise a tetrapeptide sequence comprising the amino acids: QLHF (SEQ ID NO:2).

The isolated polynucleotide sequences of such embodiments will preferably include a mutation that promotes ligand-independent activation of NOTCH1.

[0036] According to yet further embodiments of the invention, methods are provided for determining whether reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of a cancer in a patient. These methods comprise determining if the patient harbors one or more mutations in exon 28 or intron 27 of the NOTCH1 gene. In such embodiments, NOTCH1 activation is reduced or blocked by providing the patient with one or more γ-secretase inhibitors, such as [(2S)-2-{[(3,5-Difluorophenyl)acetyl]amino}-N-[(3S)1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-yl] propanamide], N-[N-(3,5-difluorophenacetyl)-L-alanyl]-Sphenylglycine-t-butylester, [1,1'-Biphenyl]-4-acetic acid, 2-fluoro-alpha-methyl, NGX-555, LY-411575, Cellzome, LY-450139, E-2012, 2-Thiophenesulfonamide, 5-chloro-N-[(1S)-3,3,3-trifluoro-1-(hydroxymethyl)-2-(trifluoromethyl)propyl], NIC5-15, BMS, CHF-5074, or analogs, salts, and combinations thereof.

[0037] In these embodiments, the cancer may be, for example, T-cell leukemia, myeloleukemia, or solid tumors. In the present invention, the solid tumors may include, *e.g.*, neuroblastoma, breast cancer, and ovarian cancer. Preferably, the cancer is T-cell leukemia.

[0038] According to such embodiments, the one or more mutations in exon 28 or intron 27 of the NOTCH1 gene may be detected using PCR and DNA sequencing techniques, as well as other well-known detection techniques, *e.g.* hybridization reactions. For example, the one or more mutations in exon 28 or intron 27 of the NOTCH1 gene may be detected by (a) extracting DNA from a patient, (b) amplifying a

portion of the DNA that comprises the NOTCH1 gene to produce an amplicon, and (c) sequencing the amplicon and determining whether the amplicon comprises one or more mutations in exon 28 or intron 27 of the NOTCH1 gene. Alternatively, pre-mRNA may be extracted from the patient, which may be used to generate and amplify cDNA therefrom by performing an RT-PCR reaction. The cDNA may then be sequenced in order to determine whether the cDNA comprises one or more mutations in exon 28 or intron 27 of the NOTCH1 gene.

[0039] Still further, the one or more mutations in exon 28 or intron 27 of the NOTCH1 gene may be detected by (a) extracting DNA from the patient and (b) determining whether portions of the DNA in which exon 28 or intron 27 of the NOTCH1 gene resides hybridizes to one or more polynucleotides that are complementary to mutated forms of exon 28 or intron 27 under standard conditions, preferably high stringency conditions. Non-limiting examples of such hybridization reactions include southern blots and microarray analyses. As used herein, "standard conditions" refers to the conditions that are generally used by a person skilled in the art to detect specific hybridization signals, or preferably so-called stringent hybridization conditions used by a person skilled in the art. Thus, as used herein, the term "stringent hybridization conditions" means that hybridization will occur if there is 85% and preferably at least 90% or 95%, such as for example, 96%, 97%, 98% or 99% identity between the sequences. Stringent hybridization conditions may include, *e.g.*, overnight incubation at 42°C using, *e.g.*, a digoxygenin (DIG)-labeled DNA probe (constructed by using a DIG labeling system; Roche Diagnostics GmbH, 68298 Mannheim, Germany) in a solution comprising 50% formamide, 5xSSC (150 mM NaCl, 15 mM trisodium citrate), 0.2%

sodium dodecyl sulfate, 0.1% N-lauroylsarcosine, and 2% blocking reagent (Roche Diagnostics GmbH), followed by washing the filters in 0.1xSSC at about 60°C.

[0040] According to additional embodiments of the invention, methods of determining whether reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of a cancer in a patient are provided. These methods comprise determining if NOTCH1 comprises a tetrapeptide sequence of SEQ ID NO:2 proximal to the transmembrane domain of NOTCH-1. In such embodiments, determining whether NOTCH1 comprises the tetrapeptide sequence may comprise the steps of (a) extracting a protein sample from the patient, (b) contacting the protein sample with an antibody (or FAB fragment thereof) that selectively recognizes and binds to the tetrapeptide sequence or a portion thereof, and (c) determining whether the antibody (or FAB fragment thereof) recognized and bound to the tetrapeptide sequence within the protein sample. Such immunochemical methods of detecting the presence of the tetrapeptide sequence may be carried out using, *e.g.*, an immunoblot, ELISA, RIA, flow cytometry, or a combination thereof.

[0041] "Antibody," as used herein, includes an antibody of classes IgG, IgM, IgA, IgD, or IgE, or fragments or derivatives thereof, including Fab, F(ab')2, Fd, and single chain antibodies, diabodies, bispecific antibodies, and bifunctional antibodies. The antibody may be a monoclonal antibody, polyclonal antibody, affinity purified antibody, or mixtures thereof which exhibits sufficient binding specificity to a desired epitope or a sequence derived therefrom. The antibody may also be a chimeric antibody. The antibody may be derivatized by the attachment of one or more chemical, peptide, or polypeptide moieties known in the art. The antibody may be conjugated with a chemical

moiety. The antibody may be a human or humanized antibody. These and other antibodies are disclosed in U.S. Published Patent Application No. 20070065447, which is hereby incorporated by reference.

[0042] Suitable antibody mimetics generally can be used as surrogates for the antibodies and antibody fragments described herein. Such antibody mimetics may be associated with advantageous properties (*e.g.*, they may be water soluble, resistant to proteolysis, and/or be nonimmunogenic). For example, peptides comprising a synthetic beta-loop structure that mimics the second complementarity-determining region (CDR) of monoclonal antibodies have been proposed and generated. *See*, *e.g.*, Saragovi *et al.*, Science. Aug. 16, 1991;253(5021):792-5. Peptide antibody mimetics also have been generated by use of peptide mapping to determine "active" antigen recognition residues, molecular modeling, and a molecular dynamics trajectory analysis, so as to design a peptide mimic containing antigen contact residues from multiple CDRs. *See*, *e.g.*, Cassett *et al.*, Biochem Biophys Res Commun. Jul. 18, 2003;307(1):198-205. Additional discussion of related principles, methods, etc., which may be applicable in the context of this invention are provided in, *e.g.*, Fassina, Immunomethods. October 1994;5(2):121-9.

[0043] According to still further embodiments of the invention, methods of (a) determining whether reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of a cancer, (b) identifying whether a patient is sensitive to one or more γ-secretase inhibitors, and (c) identifying a patient population for inclusion in a clinical trial of a drug candidate for treating cancer are provided. These methods comprise carrying out a screen for mutations in exon 26, exon 27, intron

27, exon 28, and exon 34 of a NOTCH1 gene in a sample of DNA from a patient. The invention provides that screens for such mutations may be performed using the PCR / sequencing methods described above or, *e.g.*, hybridization-based assays (such as southern blots and/or microarray analyses). According to such embodiments, the presence of a mutation in the patient's DNA sample (i) is indicative that reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of the cancer, (ii) is indicative that a patient will be sensitive to γ-secretase inhibitors, and (iii) should be considered in determining whether to include each patient in the clinical trial (in view of the mode of action of the drug candidate to be tested).

[0044] According to further embodiments of the invention, kits for determining whether reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of a cancer are provided. The kits comprise a set of PCR primers, such as the primers used in the examples below, which may be used to generate an amplicon from patient DNA which comprises intron 27 and/or exon 28 of the NOTCH1 gene. The amplicon that is generated using such PCR primers may be subsequently sequenced in order to determine whether the amplicon contains a mutation in intron 27 and/or exon 28 of the NOTCH1 gene.

[0045] The following examples are provided to further illustrate the methods and compositions of the present invention. These examples are illustrative only and are not intended to limit the scope of the invention in any way.

EXAMPLES

[0046] **Methods**

[0047] *NOTCH1 expression plasmids.*

[0048] The pcDNA3 NOTCH1 expression plasmid is a full-length NOTCH1 expression construct containing codons 1-2555 of NOTCH1 followed by a Flag tag sequence. pcDNA3 NOTCH1 L1601P encodes a HD (substitution of L to P at position 1601) mutant allele of NOTCH1 tagged with a Flag tag epitope in the C-terminus. pcDNA3 NOTCH1 L1601P-ΔPEST encodes a double HD (substitution of L to P at position 1600) plus ΔPEST (truncation at position 2473) mutant allele of NOTCH1 tagged with a Flag tag epitope in the C-terminus. pcDNA3 NOTCH-1, pcDNA3 NOTCH1 L1601P and pcDNA3 NOTCH1 L1601P-ΔPEST constructs were provided by New York University. The pcDNA3 NOTCH1 Jurkat JME17 mutant was generated by cloning a partial NOTCH1 transcript (exons 19 to 29) amplified by PCR from Jurkat cells, which contain an internal tandem duplication of 51 bases within exon 28 of the NOTCH1 gene, in the unique BamH1 and NotI restriction sites of pcDNA3 NOTCH-1. The pcDNA3 NOTCH1 P12 mutant was generated by cloning a partial NOTCH1 transcript (exons 19 to 29) amplified by PCR from P12-ICHIKAWA cells, which contain an internal tandem duplication of 51 bases within exon 28 of the NOTCH1 gene, in the unique BamH1 and NotI restriction sites of pcDNA3 NOTCH-1.

[0049] A series of artificial NOTCH1 alleles (pcDNA3 NOTCH1 JME VSV-G 5, 8, 11-14) encoding insertions of 5 amino acids (NRLGK)(SEQ ID NO: 34), 8 amino acids (IEMNRLGK)(SEQ ID NO: 35), 11 amino acids (YTDIEMNRLGK)(SEQ ID NO: 36), 12 amino acids (QYTDIEMNRLGK)(SEQ ID NO: 37), 13 amino acids (EQYTDIEMNRLGK)(SEQ ID NO: 38) and 14 amino acids (KEQYTDIEMNRLGK)(SEQ ID NO: 39) in the extracellular juxtamembrane region of NOTCH1 at position 1740 were generated by cloning synthetic oligonucleotides containing the corresponding codons in

the unique NotI restriction site of pCDNA3 NOTCH-1. Mutant forms of NOTCH1 at the S2 cleavage site (AV 1720-1721 VH and AV 1720-1721 ED) were generated using the Quick Change II site directed mutagenesis kit (Stratagene, La Jolla, CA). NOTCH1 amino acid sequence NP_060087.3 (SEQ ID NO: 33) was used as the reference for the annotation of all JME constructs and mutations.

[0050] *Clinical samples.*

[0051] Samples of cryopreserved lymphoblasts from 210 children and young adults with T-ALL were collected with informed consent at the time of diagnosis. Genomic DNA from each sample was extracted using a commercial kit (GENTRA, Minneapolis, MN).

[0052] *Western blot.*

[0053] Antibodies against activated NOTCH1 (NOTCH1 Val 1774) (Cell Signaling), *Renilla* luciferase (Chemicon) and tubulin (SC-8035, Santa Cruz Biotechnology) were used in immunoblot assays following manufacturer instructions.

[0054] *NOTCH1 mutation analysis.*

[0055] NOTCH1 mutation analysis in the Jurkat cell line was performed by direct sequencing of RT-PCR-amplified NOTCH1 transcripts. Primary T-ALL samples were analyzed by direct sequencing of PCR products expanding NOTCH1 exons 26, 27, 28 and the distal part of exon 34 encompassing the sequences encoding the TAD and PEST domains. Primer sequences were as follows:

[0056] Exon 26 FW: GCTGAGGGAGGACCTGAACTTGG (SEQ ID NO: 3);

[0057] Exon 26 RV: CCTGAGCTGGAATGCTGCCTCTA (SEQ ID NO: 4);

[0058] Exon 27 FW: CATGGGCCTCAGTGTCCT (SEQ ID NO: 5);

[0059] Exon 27 RV:TAGCAACTGGCACAAACAGC (SEQ ID NO: 6);

[0060] Exon 28 FW:GCGTAGCCGCTGCCTGAT (SEQ ID NO: 7);

[0061] Exon 28 RV:CAGACTCCCGGTGAGGATGC (SEQ ID NO: 8);

[0062] Exon 34 FW1:GCTGGCCTTTGAGACTGG (SEQ ID NO: 9);

[0063] Exon 34 RV1:CTCCTGGGGCAGAATAGTGT (SEQ ID NO: 10);

[0064] Exon 34 FW2: ACAGATGCAGCAGCAGAACC (SEQ ID NO: 11); and

[0065] Exon 34 RV2:CCTGGGGCCAGATAAAACAGTACA (SEQ ID NO: 12).

[0066] *Reporter gene assays.*

[0067] NOTCH1 expression plasmids (pcDNA3) were transiently transfected using FuGEne (Roche, Basel, Switzerland) transfection reagent together with the pGaLUC artificial luciferase reporter construct provided by Kyoto University, which contains six tandem CSL binding sites; and pRL, a plasmid expressing the Renilla luciferase gene under the control of the CMV promoter (which was used as an internal control). Total DNA was kept constant by adding empty vector as needed. All transfections were carried out in triplicate. Cell lysates were harvested 48 hours post-transfection and luciferase assays were carried out using the Dual Luciferase Assay System (Promega, Madison, WI) on a LUMAT LB 9507 luminometer (Berthold Technologies, Oak Ridge, TN).

[0068] *Cell lines.*

[0069] Jurkat cells were obtained from the ATCC. LOUCY, MOLT16, SUPT11, SUPT13 and P12/ICHIKAWA cells were provided by Dana-Farber Cancer Institute. T-ALL cell lines were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum, 100U/ml penicillin G, and 100 µg/ml streptomycin at 37°C in a humidified

atmosphere under 5% CO_2. Hela cells and immortalized murine fibroblast lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100U/ml penicillin G, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere under 5% CO_2.

[0070] *Gamma-secretase inhibitor treatment of cell lines.*

[0071] Gamma-secretase activity was inhibited by addition of 100 nM Comp E (Alexis Biochemicals, Mountain View, CA). Mock-treated cultures were exposed to vehicle only (DMSO).

[0072] *Heterodimer stability assays.*

[0073] 293T cells were transfected in 60-mm dishes with 2 µg of empty pcDNA3 plasmid or with pcDNA3.1 plasmids encoding soluble N-terminal FLAG and C-terminal HA tags NOTCH1 mini-receptors corresponding to wild type NOTCH1 (amino acids 1446 to 1743) and Jurkat JME17 and VSVG JME 14 NOTCH1 mutant alleles. Three days after transfection, the conditioned media were collected, centrifuged at 500 g for 5 minutes and divided in two aliquots that were incubated with anti-FLAG and anti-HA beads at 4°C. Following 3 washes with wash buffer (50mM Tris, pH 7.5, 150 mM NaCl, 0.1% NP40), the precipitated proteins were eluted with FLAG or HA peptide and analyzed by Western blotting with antibodies against the epitope tags. For the experiment involving the presence of urea (described below), HA immunoprecipitates were prepared as described above and following three washes with wash buffer, were incubated with 1 ml of wash buffer with 0 to 4M urea for 30 minutes at 24°C. Following two additional washes, immunoprecipitated proteins were eluted and analyzed by Western blot as described above.

[0074] **Results**

[0075] *Internal tandem duplications in exon 28 of NOTCH1 in T-ALL.*

[0076] Activating mutations in the HD and PEST domains of NOTCH1 have been detected in over 70% of T-ALL cell lines and are associated with the expression of high levels of ICN1. To analyze the possible role of NOTCH1 signaling in T-ALL lines lacking known activating mutations in the NOTCH1 gene, the levels of ICN1 protein were measured by Western blot using the Val 1744 antibody, which specifically recognizes the γ-secretase cleaved activated form of NOTCH-1, in a panel of T-ALL lines lacking mutations in exons 26, 27 and 34 of the NOTCH1 gene. This analysis demonstrated high levels of ICN1 protein in the Jurkat T-ALL cell line but not in LOUCY, MOLT16, SUPT11 or SUPT13 cells, suggesting the presence of an unidentified activating mutation in the NOTCH1 gene in this cell line (**Figure 1A**). To screen for further activating mutations in NOTCH-1, direct sequencing analysis of RT-PCR products encompassing the complete coding sequence of the NOTCH1 gene in Jurkat cells was performed. This sequence analysis revealed an in-frame insertion of the CAGG tetranucleotide followed by an internal tandem duplication of 47 base pairs (bp) in exon 28 of NOTCH-1, resulting in the insertion of 17 amino acids (QAVEPPPPAQLHFMYVA) (SEQ ID NO: 13) at position 1740 in the extracellular juxtamembrane region of the NOTCH1 receptor (**Figure 1B**).

[0077] No further mutations in the entire NOTCH1 cDNA sequence from this cell line were detected. PCR amplification of exon 28 sequences from genomic DNA from 210 primary T-ALL samples identified seven patients showing an extra band of increased size in addition to the normal exon 28 PCR product (**Figure 1C**). Sequence

analysis of these aberrant PCR products demonstrated the presence of internal tandem duplications ranging from 33 to 108 bases in the region extending from the distal part of intron 27 to the proximal segment of exon 28, resulting in an in-frame insertion of 11 to 36 amino acids in the extracellular juxtamembrane segment of NOTHC1 (**Figure 6**).

[0078] A first patient showed an in-frame insertion of the TGAAGGGG octanucleotide (SEQ ID NO: 14) followed by an internal tandem duplication encompassing the distal 49 bases of intron 27 and the 45 proximal bases of exon 28. A second patient showed an in-frame internal tandem duplication encompassing the distal 54 bases of intron 27 and the proximal 54 bases of exon 28. The first of these mutations is predicted to encode an insertion of 35 amino acids (LKGSRCLMSGHLPLAPVPAGETVEPPPPAQLHFMY) (SEQ ID NO: 15) at position 1738 in the extracellular juxtamembrane segment of NOTCH-1, while the second one is predicted to encode an insertion of 36 amino acids (VRSRCLMSGHLPLAPVPAGETVEPPPPAQLHFMTWR) (SEQ ID NO: 16) at position 1741, also extending the extracellular juxtamembrane segment of NOTCH1 (**Figure 6**). The other five internal tandem duplications, and the corresponding predicted amino acid insertions, are listed in **Figure 6**. Direct sequence analysis of exon 28 and flanking intronic sequences of T-ALL samples showing normal size PCR products failed to detect any additional *NOTCH1* mutations in this region.

[0079] *Expansion of the extracellular juxtamembrane region of NOTCH1 induces strong receptor activation.*

[0080] To analyze the functional relevance of NOTCH1 JME mutants, luciferase reporter assays were performed to compare the activity of wild type NOTCH1, the

JME17 mutation isolated from Jurkat cells and a prototypical HD mutant allele of NOTCH1 (L1600P). Transfection of Hela cells with the HD (L1600P), ΔPEST, and HD (L1600P)ΔPEST NOTCH1 mutant alleles resulted in 12-, 2.5- and 105-fold activation of NOTCH1 signaling over basal levels, respectively. In contrast, expression of the NOTCH1 Jurkat JME17 mutant allele induced over 2000-fold activation of the NOTCH1 reporter compared to controls (**Figure 2A**).

[0081] Three outstanding features were in common in all eight JME mutant alleles identified in the series. First, the alleles all encoded NOTCH1 receptors with significantly extended extracellular juxtamembrane regions that would presumably leave intact, but displaced from the membrane, the ADAM protease S2 cleavage site and the complex formed by the two subunits of the HD domain and the LNR repeats. Second, the alleles all clustered in a specific location around amino acid 1740 of NOTCH-1. Finally, the alleles shared 4 common amino acids (QLHF) (SEQ ID NO: 2) in each peptide inserted in the extracellular juxtamembrane region of the receptor.

[0082] Based on these observations, it was hypothesized that NOTCH1 JME mutations could be related to two NOTCH1 HD mutant alleles previously identified in the distal region of exon 27 in the P12-ICHIKAWA cell line and in a primary T-ALL patient sample, which consisted of internal tandem duplications resulting in the displacement of the NOTCH1 S2 cleavage site away from the inhibitory LNR-HD complex. Thus, in such a case, NOTCH1 JME mutations might result in aberrant proteolytic processing of the extracellular juxtamembrane region of NOTCH1 by creating an alternative S2-like site, possibly located within their common QLHF (SEQ ID NO: 2) sequence. Alternatively, aberrant activation of NOTCH1 might result from

conformational changes in the HD-LNR complex upon its displacement away from the membrane.

[0083] To test these possibilities, a series of artificial NOTCH1 JME mutants were generated that included insertions of 5, 8, 11, 12, 13 and 14 amino acids derived from the VSV-G epitope in position 1740 of NOTCH1. The ability of these mutants to induce activation of a CSL-driven promoter was tested in a luciferase reporter assay. These amino acid sequences lacked any significant similarities with the NOTCH JEM insertions and are devoid of any valine or alanine residues to avoid the introduction of novel S2-like cleavage sites. These experiments demonstrated moderate (1.5-12 fold) gains in NOTCH1 signaling for JME mutants with insertions of 5, 8, or 11 amino acids. In contrast, the introduction of 12-14 amino acids resulted in marked increases in NOTCH1 signaling, ranging from 200-700 fold over baseline levels (**Figure 2B**). These results strongly suggest that the activation of NOTCH1 in JME mutants depends on the length of the spacing inserted between the HD-LNR repeat complex and the membrane, but is apparently independent of the specific amino acid sequence introduced in the juxtamembrane region of the receptor by these mutations.

[0084] *NOTCH1 JEM mutations in NOTCH1 require γ-secretase and S2 processing for activation.*

[0085] Activating mutations in the HD domain of NOTCH1 typically work by facilitating the S2 processing of the receptor and the subsequent S3 cleavage catalyzed by the γ-secretase complex. As a result, inhibition of S3 processing by small molecule inhibitors of the γ-secretase complex effectively abrogate the activity of these mutants. Treatment of Jurkat T-ALL cells with CompE resulted in almost complete clearance of

ICN1 at 24 hours, as determined by Western blot analysis with the NOTCH1 Val 1744 antibody; and in marked downregulation of NOTCH1 transcriptional activity - evidenced by a downregulation of HES1 (a direct target of NOTCH1) (**Figure 3A,B**).

[0086] To further analyze the requirement of the γ-secretase complex for the activity of NOTCH1 JME alleles, luciferase reporter assays were performed in the presence and absence of CompE, a highly active γ-secretase inhibitor (GSI). Incubation of Hela cells transfected with the NOTCH1 Jurkat JME17, or with the artificial NOTCH1 JME VSV-G 14 mutant allele, with CompE, resulted in complete abrogation of NOTCH1 signaling (**Figure 3C**). Overall, these results demonstrate a strict requirement of the γ-secretase complex for the activity of NOTCH1 JME mutant alleles.

[0087] To test if NOTCH1 JME mutants are strictly dependent on a proteolytic processing at the prototypical S2 cleavage site, two different targeted mutations were generated at this position (AV 1720-1721 VH and AV 1720-1721 ED) in the context of the NOTCH1 Jurkat JME17 and VSV-G 14 alleles (**Figure 3D**). These mutations have been reported to partially (AV>VH) and completely (AV>ED) abrogate S2 cleavage of the wild type receptor and to effectively block NOTCH1 signaling. Functional analysis of NOTCH1 Jurkat JME17 and VSV-G 14 alleles with S2 cleavage site mutations showed abrogation of NOTCH1 activity in luciferase reporter assays (**Figure 3E,F**). These results demonstrate that activation of NOTCH1 by JME mutations requires cleavage at the canonical S2 site and does not support that these mutations generate alternative S2-like cleavage motifs in the juxtamembrane extracellular region of the receptor.

[0088] *NOTCH1 JME mutations do not affect the stability of the HD-LNR repeat complex.*

[0089] The strict requirement of a prototypical S2 cleavage site for the activation of NOTCH1 JME mutant alleles suggested that these insertions may work similarly to class 1 NOTCH1 HD mutations, by severely destabilizing the HD-LNR repeat complex, which normally protects the S2 site from the activity of ADAM proteases. To test this hypothesis, a construct was generated that encoded a secreted LNR-HD-juxtamembrane segment of NOTCH1 (amino acids 1446 to 1743) tagged with a FLAG epitope in the N-terminus and an HA epitope in the C-terminus (**Figure 4A**). Transfection of this construct in mammalian cells generates a soluble HD-LNR heterodimer that can be immunoprecipitated from the conditioned media with a FLAG antibody and detected by Western blot using the anti-HA tag antibody. In addition to a wild type LNR-HD construct, versions of the NOTCH1 minigene containing the NOTCH1 Jurkat JME17 and NOTCH1 VSV-G JME14 alleles were generated.

[0090] Immunoprecipitation of the wild type protein using the FLAG antibody recovered a 25 kd N-terminus fragment containing the FLAG epitope and a 14 kd C-terminus fragment with the HA tag (**Figure 4B**). This result demonstrates that the wild type LNR-HD protein was correctly processed by furin proteases and secreted into the media as a stable LNR-HD heterodimer. Notably, FLAG immunoprecipitation of Jurkat and VSV-G JME LNR-HD also recovered both the N-terminus and the C-terminus peptides consistent with the production and secretion of a stable soluble LNR-HD repeat complex in the context of activating JME insertions (**Figure 4B**). Treatment of NOTCH1 LNR-HD complexes encoded by these NOTCH1 minigenes with increased concentrations of urea showed only a slight increase in urea sensitivity for the dissociation of the LNR-HD repeat complex in Jurkat JME17 mutant compared to wild

type NOTCH1 (**Figure 4C**). Overall, these results demonstrate that highly active JME mutants do not significantly affect the stability of soluble LNR-HD complexes. This is in contrast with results previously reported for class 1 NOTCH1 HD mutations, which typically induce receptor activation by destabilizing the interaction between the two HD subunits making the S2 site accessible to metalloprotease cleavage.

[0091] Analysis

[0092] The extracellular HD and LNR repeat domains of NOTCH proteins play a critical role in the stabilization of the receptor in a resting state and are the target of activating mutations that result in aberrant NOTCH1 signaling in T-ALL. The resolution of the crystal structure of the NOTCH2 LNR-HD complex has provided mechanisms that mediate the inhibitory effects of these extracellular domains on the processing and activation of NOTCH receptors. These studies have shown that the N-terminal and C-terminal subunits of the HD domain are closely associated, an interaction that is further stabilized by the LNR repeats, which fold over the HD domain and hold the two HD subunits together. In addition, the third LNR domain buries the S2 cleavage site located in the C-terminal HD subunit, thereby protecting it from cleavage by metalloproteases.

[0093] According to this model, physiologic NOTCH1 signaling is initiated by the binding of a DSL ligand to the EGF repeats in the extracellular subunit of the receptor. This triggers a conformational change in the LNR-HD complex that exposes the S2 site to proteolytic cleavage by ADAM metalloproteases. Following S2 cleavage, the short extracellular juxtamembrane stub of the transmembrane-intracelluar subunit binds to nicastrin, a membrane protein that works as a receptor for the γ-secretase complex, which ultimately cleaves NOTCH1 at the S3 site located in the transmembrane domain

of the receptor. This final proteolytic cleavage activates the receptor by releasing the intracellular domains of NOTCH1 from the membrane, which then translocate to the nucleus and activate the expression of target genes.

[0094] Mutations in the heterodimerization domain of NOTCH1 induce ligand-independent activation of the receptor. Aberrant NOTCH1 activation by most HD mutations (class 1) occurs because of reduced heterodimer stability resulting in either overt heterodimer dissociation (class 1A mutations), or in some cases, more subtle weakening of the LNR-HD complex inducing a slow heterodimer dissociation and/or an increase in S2 site exposure (class 1B mutations) (**Figure 5**).

[0095] In rare cases, HD mutations do not affect the stability of the LNR-HD complex, but introduce extra amino acids between the distal part of the HD domain and the S2 cleavage site displacing it away from the protective effects of the LNR-HD complex (class 2 mutations) (**Figure 5**). Common features of both class 1 and class 2 HD mutations are: (i) their strict dependence on the integrity of the S2 cleavage site for activation, (ii) having ADAM10 as the main metalloprotease involved in S2 processing, and (iii) the requirement of γ-secretase activity for NOTCH1 activation. However, they differ in their relative activity with class 2 mutants being significantly more active than class 1 NOTCH1 alleles.

[0096] The foregoing examples reveal a new family of activating mutations within the extracellular juxtamembrane region of NOTCH1 in T-ALL (**Figure 5**). These Juxtamembrane Expansion Mutants or JME NOTCH1 mutant alleles are generated by an internal tandem duplications in the 3' end of intron 27 and/or in the proximal region of exon 28, and result in the insertion of relatively long peptides around position 1740 of

NOTCH1. Interestingly, each of the eight JME insertions identified in this series introduces a common tetrapeptide sequence (QLHF)(SEQ ID NO: 2) proximal to the transmembrane domain of the NOTCH1 receptor. However, activation of NOTCH1 by JME mutants is not dependent on the sequence of this common motif, but on the number of amino acid residues introduced proximal to the membrane domain of the NOTCH1 receptor.

[0097] Thus, artificially generated JME insertions of 5, 8 and 11 amino acids failed to induce significant activation of the NOTCH1 receptor, while the insertion of 14 amino acids induced marked increase in NOTCH1 signaling. Importantly, the amino acid sequences introduced in the extracelular juxtamembrane region of NOTCH1 by these constitutively-active artificial JME alleles were unrelated to the insertions found in leukemia-derived JME mutants. Based on these results, it was proposed that JME mutations could result in aberrant processing of NOTCH1 at the typical S2 metalloprotease cleavage site, or alternatively, in proteolytic cleavage of the receptor at an alternative S2-like cleavage site located in the inserted peptide sequences encoded by these NOTCH1 alleles, followed by γ-secretase cleavage.

[0098] The foregoing examples show that the activity of JME mutants seems to be dependent on the length of the inserted sequences and not on the specific amino acid sequence of the insertion. This suggests that these mutants probably work by inducing a conformational change in the LNR-HD complex that facilitates constitutive cleavage at the classic S2 site. Consistent with this hypothesis, activation of NOTCH1 signaling is strictly dependent on the integrity of the standard S2 cleavage site and is also effectively blocked by small molecule inhibitors of the γ-secretase complex.

[0099] NOTCH1 JME alleles and class 2 *HD* mutants have several features in common: (i) both are generated by relatively long insertions resulting from internal tandem duplications in the NOTCH1 gene, (ii) they result in high levels of NOTCH1 activity compared to class 1 HD alleles and (iii) their activity is dependent on the integrity of the S2 cleavage site and the function of the γ-secretase complex. In the case of class 2 HD NOTCH1 mutants, aberrant S2 cleavage is induced by the insertion of extra amino acids immediately proximal to the classic S2 cleavage site, which is displaced closer to the membrane and out of reach from the structure of the LNR-HD complex (**Figure 5**). In contrast, insertions in the JME mutants are distal to the S2 cleavage site and displace the LNR-HD complex and the S2 site away from the membrane, without altering the primary structure of any of these elements (**Figure 5**).

[00100] These results suggest that moving the LNR-HD complex away from the cell surface facilitates aberrant metalloprotease S2 cleavage of the NOTCH1 receptor. Importantly, analysis of the amino acid sequence of the extracellular juxtamembrane region of different NOTCH receptors showed little sequence homology, but a more strict conservation on the length of the segment that separates the LNR-HD complex from the membrane. Thus, the S2 cleavage site is located 11 amino acids distant from the membrane in NOTCH2 and NOTCH3, while in NOTCH1 and NOTCH4 the extracellular juxtamembrane region is 12 amino acids long. Overall, the foregoing examples demonstrate that a novel class of mutations involved in the aberrant activation of NOTCH1 signaling in T-ALL has been identified, and provide further insight in the mechanisms that control the activation of the NOTCH1 receptor.

[00101] Although illustrative embodiments of the present invention have been described herein, it should be understood that the invention is not limited to those described, and that various other changes or modifications may be made by one skilled in the art without departing from the scope or spirit of the invention.

What is claimed is:

1. An assay for identifying candidate compounds that may be used to treat or ameliorate the effects of cancer comprising:

a. contacting cells from a cell line comprising a juxtamembrane expansion (JME) mutation with a candidate compound; and

b. determining whether NOTCH1 expression is modulated in the cell line contacted with the candidate compound compared to control cells from the same cell line that have not been contacted with the candidate compound.

2. The method according to claim 1, wherein the modulation is a decrease in NOTCH1 expression in the cells of the cell line containing the JME mutation.

3. The method according to claim 2, wherein the decrease is indicative that the candidate compound may be effective to treat cancer.

4. The method of claim 1, wherein the cancer is selected from the group consisting of T-cell leukemia, myeloleukemia, and solid tumors.

5. The method of claim 4, wherein the solid tumors are selected from the group consisting of neuroblastoma, breast cancer, and ovarian cancer.

6. The method according to claim 4, wherein the cancer is T-cell leukemia.

7. The method according to claim 1, wherein the JME mutation is an internal tandem duplication that comprises a polynucleotide that encodes at least 11 amino acids and is located in a region that encodes the extracellular juxtamembrane region of a NOTCH1 receptor.

8. The method according to any one of claims 1 or 7, wherein the mutation is inserted in a region extending from about the distal part of intron 27 to about the proximal part of exon 28 of a NOTCH1 gene.

9. The method according to any one of claims 1 or 7, wherein the mutation is inserted within about 50 nucleotides of nucleotide 1740 of a NOTCH1 gene.

10. The method according to any one of claims 1 or 7, wherein the mutation is inserted between about nucleotide 1738 to about 1741 of SEQ ID NO:1.

11. The method according to any one of claims 1 or 7, wherein the mutation encodes from about 11 to about 36 amino acids.

12. The method according to any one of claims 1 or 7, wherein the mutation comprises a polynucleotide that encodes a peptide comprising the amino acids: QLHF (SEQ ID NO:2).

13. The method according to claim 1, wherein the cell line is a mammalian cell line.

14. The method according to claim 1, wherein the cell line is a human cell line.

15. The method according to claim 1, wherein the cell line is a human T-cell line.

16. The method according to claim 1, wherein the human T-cell line is a Jurkat cell line.

17. A method of making a cell line that over-expresses NOTCH1 comprising introducing a mutation into a region of DNA that encodes the extracellular juxtamembrane region of a NOTCH1 receptor.

18. The method according to claim 17, wherein the mutation comprises an insertion that encodes at least 11 amino acids.

19. The method of claim 18, wherein the insertion is an internal tandem duplication.

20. The method according to claim 17, wherein the mutation is inserted from about the distal part of intron 27 to about the proximal part of exon 28 of a NOTCH1 gene.

21. The method according to claim 17, wherein the mutation is inserted within about 50 nucleotides of nucleotide 1740 of a NOTCH1 gene.

22. The method according to claim 17, wherein the mutation is inserted between about nucleotide 1738 to about nucleotide 1741 of a NOTCH1 gene.

23. The method according to any one of claims 21 or 22, wherein the NOTCH1 gene comprises the polynucleotide sequence of SEQ ID NO:1.

24. The method according to claim 17, wherein the mutation encodes from about 11 to about 36 amino acids.

25. The method according to claim 17, wherein the mutation comprises a polynucleotide that encodes a peptide sequence comprising the amino acids: QLHF (SEQ ID NO:2).

26. The method according to claim 18, wherein the cell line is a mammalian cell line.

27. The method according to claim 18, wherein the cell line is a human cell line.

28. The method according to claim 18, wherein the cell line is a human T-cell line.

29. The method according to claim 28, wherein the human T-cell line is a Jurkat cell line.

30. A substantially pure cell line made by the method of claim 18.

31. An assay for identifying candidate compounds that may be used to treat or ameliorate the effects of cancer comprising:

a. contacting cells from a cell line made by the method of claim 18 with a candidate compound; and

b. determining whether NOTCH1 expression is modulated in the cell line contacted with the candidate compound compared to control cells from the same cell line that have not been contacted with the candidate compound.

32. An isolated polynucleotide sequence that comprises a mutation in the extracellular juxtamembrane region of a NOTCH1 receptor.

33. The isolated polynucleotide sequence of claim 32, wherein the mutation comprises an insertion of nucleotides that encode at least 11 amino acids.

34. The isolated polynucleotide sequence of claim 33, wherein the insertion is an internal tandem duplication.

35. The isolated polynucleotide sequence of claim 33, wherein the mutation is inserted in a region extending from about the distal part of intron 27 to about the proximal part of exon 28 of a NOTCH1 gene.

36. The isolated polynucleotide sequence of claim 33, wherein the mutation is inserted within about 50 nucleotides of nucleotide 1740 of a NOTCH1 gene.

37. The isolated polynucleotide sequence of claim 33, wherein the mutation is inserted between about nucleotide 1738 to about 1741 of a NOTCH1 gene.

38. The method according to any one of claims 36 or 37, wherein the NOTCH1 gene comprises the polynucleotide sequence of SEQ ID NO:1.

39. The isolated polynucleotide sequence of claim 33, wherein the mutation encodes about 11 to about 36 amino acids.

40. The isolated polynucleotide of claim 33, wherein the mutation comprises a polynucleotide that encodes a peptide sequence comprising the amino acids: QLHF (SEQ ID NO:2).

41. The isolated polynucleotide sequence of claim 33, wherein the mutation promotes ligand-independent activation of NOTCH-1.

42. The isolated polynucleotide sequence of claim 33, wherein the NOTCH1 receptor is from a mammal.

43. The isolated polynucleotide sequence of claim 42, wherein the mammal is human.

44. A method of determining whether reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of a cancer in a patient comprising determining if the patient harbors one or more mutations in exon 28 or intron 27 of a NOTCH1 gene.

45. The method of claim 44, wherein NOTCH1 activation is reduced or blocked by administering to the patient one or more γ-secretase inhibitors.

46. The method according to claim 45, wherein the γ-secretase inhibitors are selected from the group consisting of [(2S)-2-{[(3,5-Difluorophenyl)acetyl]amino}-N-[(3S)1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-yl] propanamide], N-[N-(3,5-difluorophenacetyl)-L-alanyl]-Sphenylglycine-t-butylester, [1,1'-Biphenyl]-4-acetic acid, 2-fluoro-alpha-methyl, NGX-555, LY-411575, Cellzome, LY-450139, E-2012, 2-Thiophenesulfonamide, 5-chloro-N-[(1S)-3,3,3-trifluoro-1-(hydroxymethyl)-2-

(trifluoromethyl)propyl], NIC5-15, BMS, CHF-5074, and analogs, salts, and combinations thereof.

47. The method according to claim 45, wherein the γ-secretase inhibitors are selected from the group consisting of [(2S)-2-{[(3,5-Difluorophenyl)acetyl]amino}-N-[(3S)1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-yl] propanamide], N-[N-(3,5-difluorophenacetyl)-L-alanyl]-Sphenylglycine-t-butylester, and analogs, salts, and combinations thereof.

48. The method of claim 44, wherein the cancer is selected from the group consisting of T-cell leukemia, myeloleukemia and solid tumors.

49. The method of claim 47, wherein the solid tumors are selected from the group consisting of neuroblastoma, breast cancer, and ovarian cancer.

50. The method according to claim 48, wherein the cancer is T-cell leukemia.

51. The method of claim 45, wherein one or more mutations in exon 28 or intron 27 of the NOTCH1 gene is detected by (a) extracting DNA from the patient, (b) amplifying a portion of said DNA that comprises the NOTCH1 gene to produce an amplicon, and (c) sequencing the amplicon and determining whether the amplicon comprises one or more mutations in exon 28 or intron 27 of the NOTCH1 gene.

52. The method of claim 45, wherein one or more mutations in exon 28 or intron 27 of the NOTCH1 gene is detected by (a) extracting NOTCH1 pre-mRNA from the patient, (b) generating and amplifying cDNA from the pre-mRNA by performing an RT-PCR reaction, and (c) sequencing the cDNA and determining whether the cDNA comprises one or more mutations in exon 28 or intron 27 of the NOTCH1 gene.

53. The method of claim 45, wherein one or more mutations in exon 28 or intron 27 of the NOTCH1 gene is detected by (a) extracting DNA from the patient and (b) determining whether portions of the DNA in which exon 28 or intron 27 of the NOTCH1 gene resides hybridizes to one or more polynucleotides that are complementary to mutated forms of exon 28 or intron 27.

54. The method of claim 52, wherein a southern blot or microarray analysis is carried out to determine whether the patient harbors one or more mutations in exon 28 or intron 27 of the NOTCH1 gene.

55. The method of claim 45, wherein one or more mutations in exon 28 or intron 27 of the NOTCH1 gene is detected by (a) extracting DNA from the patient and (b) determining whether portions of the DNA in which exon 28 or intron 27 resides hybridizes to one or more polynucleotides that are complementary to normal forms of exon 28 or intron 27.

56. The method of claim 54, wherein a southern blot or microarray analysis is carried out to determine whether the patient harbors the normal forms of exon 28 or intron 27 of the NOTCH1 gene.

57. A method of determining whether reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of a cancer in a patient comprising determining if a NOTCH1 sequence in the patient's genome comprises a tetrapeptide sequence of SEQ ID NO:2 proximal to the transmembrane domain of NOTCH-1.

58. The method of claim 56, wherein determining whether NOTCH1 comprises the tetrapeptide sequence comprises the steps of (a) extracting a protein sample from

the patient, (b) contacting the protein sample with an antibody that selectively recognizes and binds to the tetrapeptide sequence, and (c) determining whether the antibody recognized and bound to the tetrapeptide sequence within the protein sample.

59. The method of claim 57, wherein determining whether the antibody recognized and bound to the tetrapeptide sequence is carried out using an immunoblot, ELISA, RIA, or flow cytometry.

60. A method of determining whether reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of a cancer comprising carrying out a screen for mutations in exon 26, exon 27, intron 27, exon 28, and exon 34 of a NOTCH1 gene in a sample of DNA from a patient, wherein the presence of a mutation in the NOTCH1 gene of the patient's DNA sample is indicative that reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of the cancer.

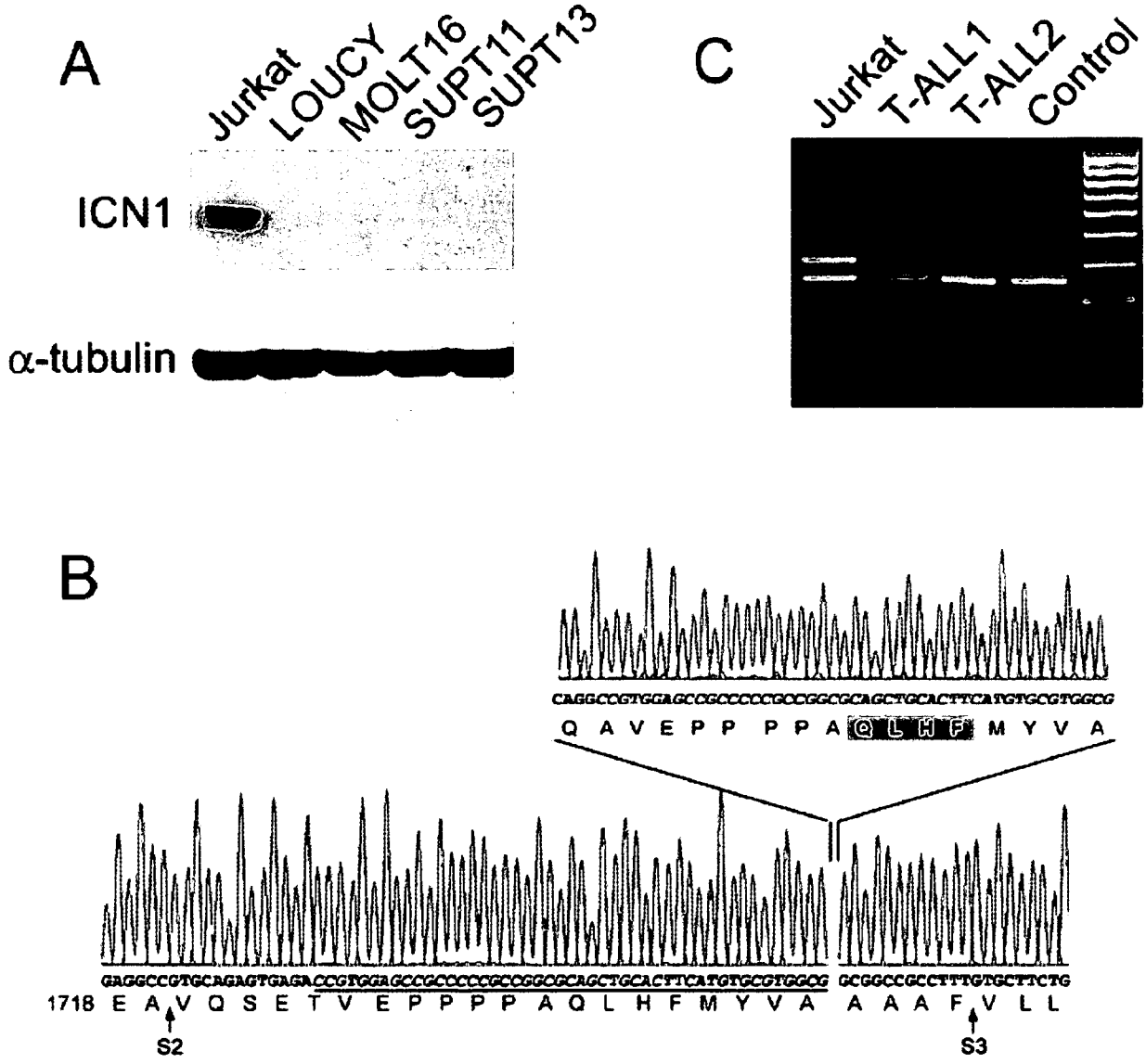
61. A method for identifying whether a patient is sensitive to one or more γ-secretase inhibitors comprising carrying out a screen for mutations in exon 26, exon 27, intron 27, exon 28, and exon 34 of a NOTCH1 gene in a sample of DNA from the patient, wherein the presence of a mutation identified by the screen is indicative of the patient being sensitive to a γ-secretase inhibitor.

62. A method for identifying a patient population for inclusion in a clinical trial of a drug candidate for treating cancer comprising (a) carrying out a screen for a mutation in exon 26, exon 27, intron 27, exon 28, and exon 34 of a NOTCH1 gene within a sample of DNA from each prospective patient, wherein the presence of a mutation identified by the screen of a patient's DNA sample is indicative of that patient being sensitive to γ-

secretase inhibitors, and (b) determining whether to include each patient in the clinical trial based on the patient's NOTCH1 mutation status determined by the screen.

63. A kit for determining whether reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of a cancer, which comprises a set of PCR primers that may be used to generate an amplicon from patient DNA which comprises intron 27 of the NOTCH1 gene.

64. A kit for determining whether reducing or blocking NOTCH1 activation will be effective to treat, prevent, or ameliorate the effects of a cancer, which comprises a set of PCR primers that may be used to generate an amplicon from patient DNA which comprises exon 28 of the NOTCH1 gene.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MOLT16
SUPT11
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ICN1
α-tubulin
C
Jurkat
T-ALL1
T-ALL2
Control
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Q A V E P P P P A Q L H F M Y V A
GAGGCCGTGCAGAGTGAGACCGTGGAGCCGCCCCCGCCGGCGCAGCTGCACTTCATGTGCGTGGCG GCGGCCGCCTTTGTGCTTCTG
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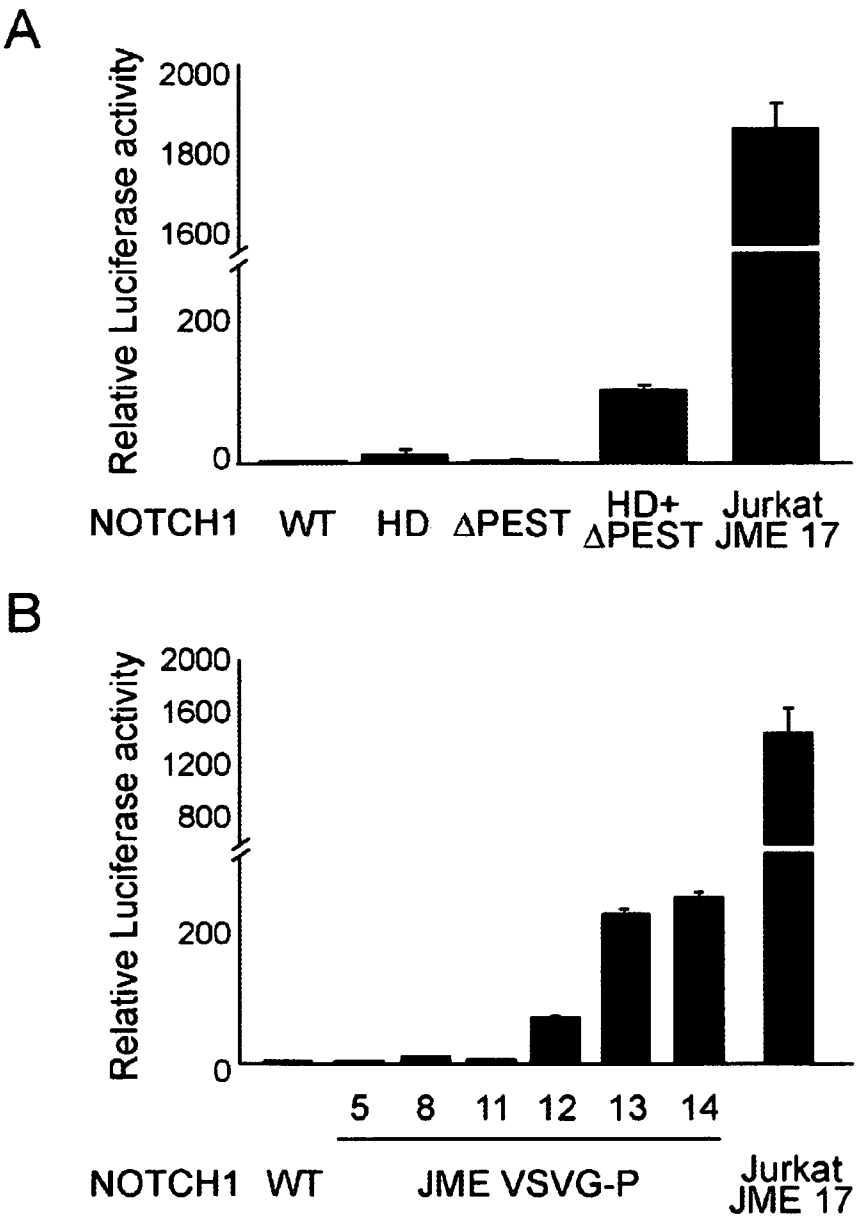
S2
S3

Figure 1 / 6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WT
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ΔPEST
HD+ ΔPEST
Jurkat JME 17
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Relative Luciferase activity
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1600
1200
800
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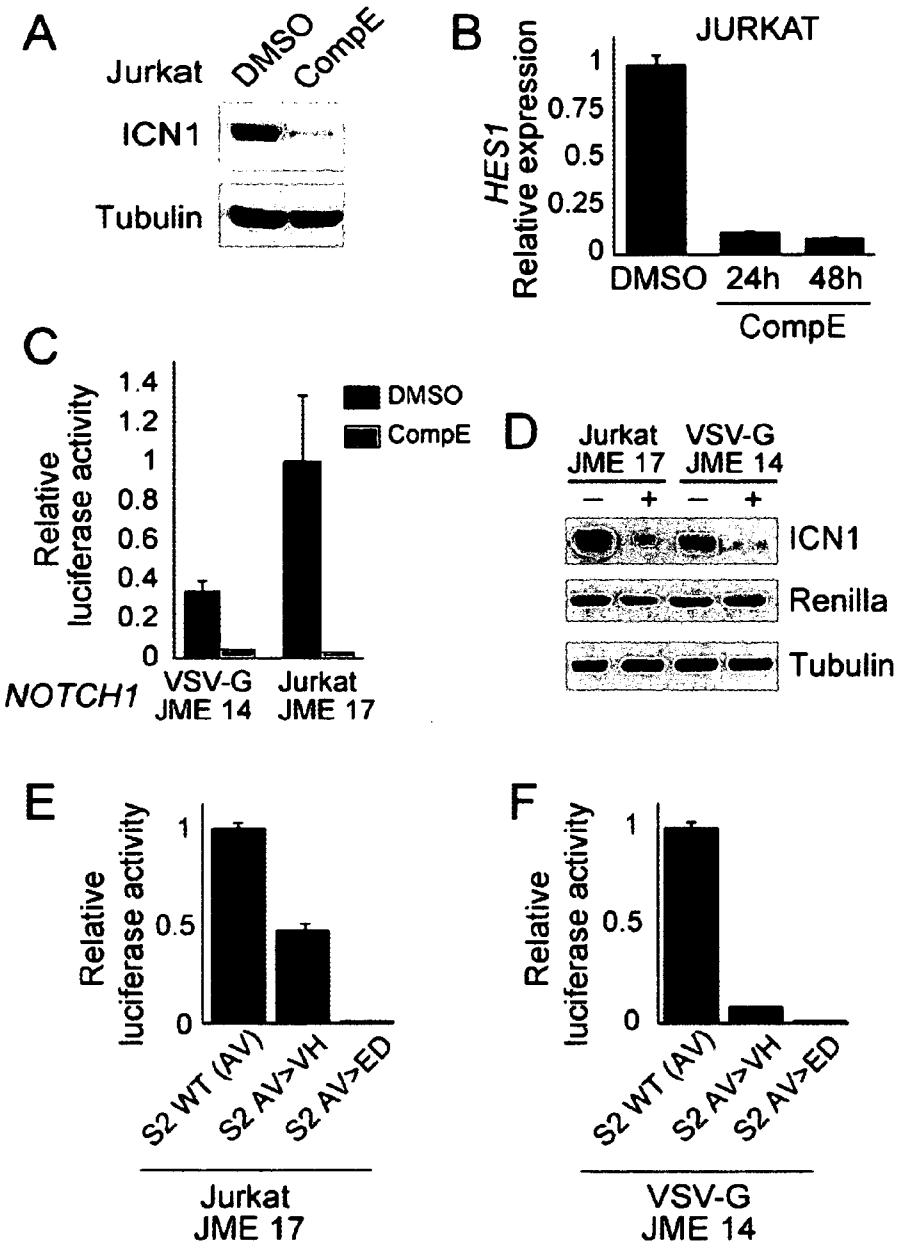
8
11
12
13
14
NOTCH1
WT
JME VSVG-P
Jurkat JME 17

Figure 2 / 6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JURKAT
HES1
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0
DMSO
24h
48h
CompE
C
Relative luciferase activity
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1.2
1
0.8
0.6
0.4
0.2
0
DMSO
CompE
NOTCH1
VSV-G JME 14
Jurkat JME 17
D
Jurkat JME 17
VSV-G JME 14
–
+
–
+
ICN1
Renilla
Tubulin
E
Relative luciferase activity
1
0.5
0
S2 WT (AV)
S2 AV>VH
S2 AV>ED
Jurkat JME 17
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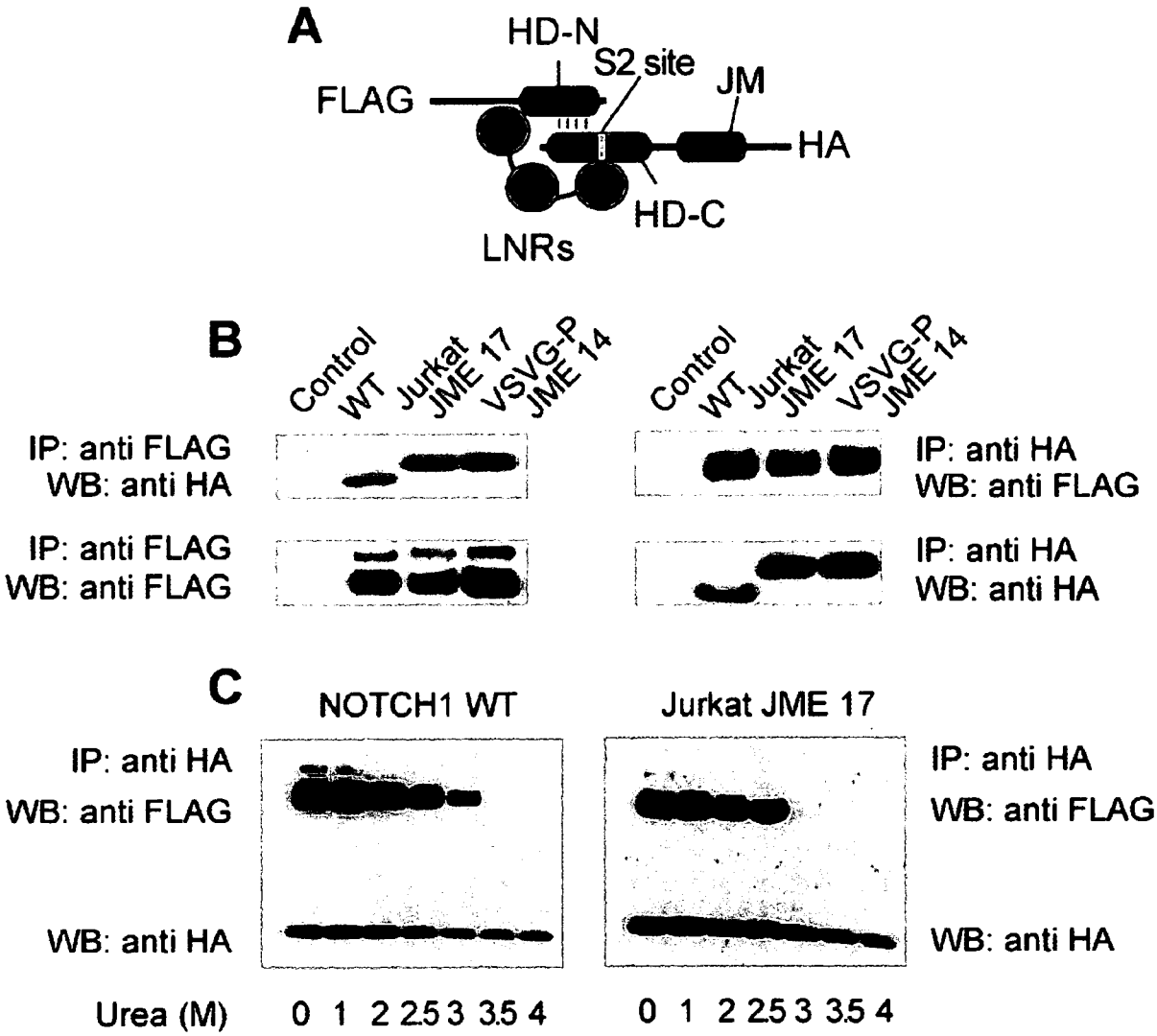
0
S2 WT (AV)
S2 AV>VH
S2 AV>ED
VSV-G JME 14

Figure 3 / 6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JM
FLAG
HA
HD-C
LNRs
B
Control
WT
Jurkat
JME 17
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JME 14
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WB: anti HA
IP: anti HA
WB: anti FLAG
IP: anti FLAG
WB: anti FLAG
IP: anti HA
WB: anti HA
C
NOTCH1 WT
Jurkat JME 17
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Urea (M)
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0 1 2 2.5 3 3.5 4

Figure 4 / 6

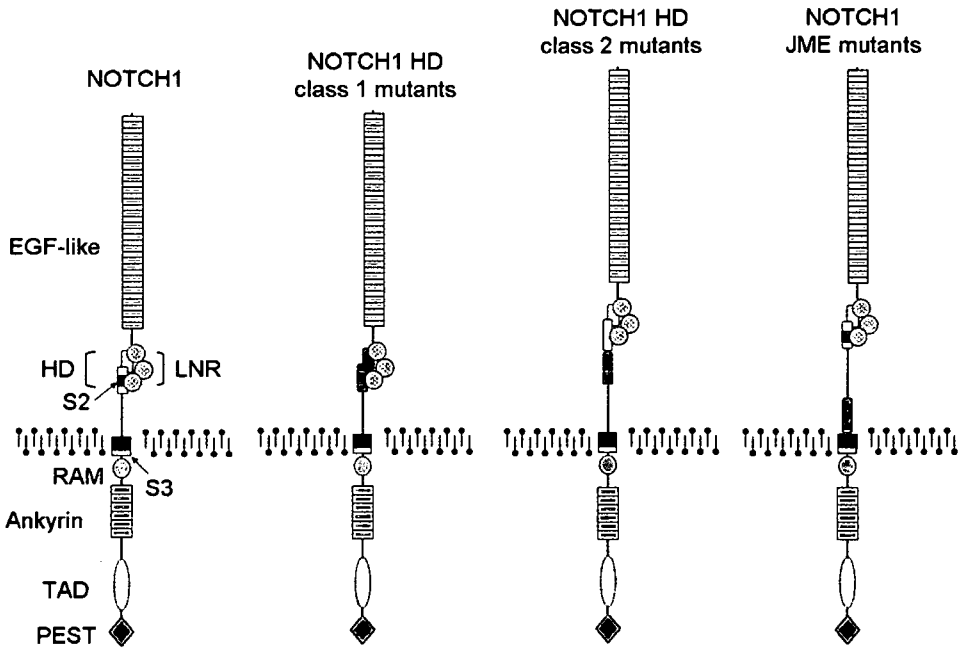
NOTCH1
NOTCH1 HD
class 1 mutants
NOTCH1 HD
class 2 mutants
NOTCH1
JME mutants
EGF-like
HD
LNR
S2
RAM
S3
Ankyrin
TAD
PEST

Figure 5 / 6

Sample	NOTCH1 Sequence (NM_017617.2)	Predicted amino acid change
Jurkat	5220 Ins CAGGCCGTGGAGCCGCCCCCGCC GGCGCAGCTGCACTTCATGTACGT GGCG (SEQ ID NO: 17)	1740 Ins QAVEPPPPAQLHFMYVA (SEQ ID NO: 20)
T-ALL 1	5212 Ins TGAAGGGGAGCCGCTGCCTGATGT CCGGGCACCTGCCCCTGGCCCCC GTGCCCGCAGGTGAGACCGTGGA GCCGCCCCCGCCGGCGCAGCTGC ACTTCATGT (SEQ ID NO: 18)	1738 Ins LKGSRCLMSGHLPLAPVPAG ETVEPPPPAQLHFMY (SEQ ID NO: 21)
T-ALL 2	5221 Ins TGCGTAGCCGCTGCCTGATGTCCG GGCACCTGCCCCTGGCCCCCGTG CCCGCAGGTGAGACCGTGGAGCC GCCCCCGCCGGCGCAGCTGCACT TCATGTACGTGGCGG (SEQ ID NO: 19)	1741 Ins VRSRCLMSGHLPLAPVPAGE TVEPPPPAQLHFMTWR (SEQ ID NO: 22)
T-ALL 3	5214 Ins CCGCCCCCGCCGGCGCAGCTGCA CTTCATGTAC (SEQ ID NO: 23)	1738 Ins PPPPAQLHFMY (SEQ ID NO: 24)
T-ALL 4	5209 Ins GGACCGTGGAGCCGCCCCCGCCG GCGCAGCTGCACTTCA (SEQ ID NO: 25)	1737 Ins RTVEPPPPAQLHF (SEQ ID NO: 26)
T-ALL 5	5221 Ins AGGCCCGGCAGCTGCACTTCATGT ACGTGGCGG (SEQ ID NO: 27)	1741 Ins EARQLHFMYVA (SEQ ID NO: 28)
T-ALL 6	5218 Ins GGGAGCCGCCCCCGCCGGCGCAG CTGCACTTCATGTACGTGG (SEQ ID NO: 29)	1740 Ins GEPPPPAQLHFMYV (SEQ ID NO: 30)
T-ALL 7	5218 Ins CCGTGGAGCCGCCCCCGCCGGCG CAGCTGCACTTCATGTACGTGG (SEQ ID NO: 31)	1740 Ins AVEPPPPAQLHFMYV (SEQ ID NO: 32)

Figure 6 / 6