



(51) International Patent Classification:

A61K 38/00 (2006.01) C12N 15/00 (2006.01)
A61K 48/00 (2006.01) A61P 25/28 (2006.01)
C07K 14/47 (2006.01) A61P 31/18 (2006.01)
C07K 19/00 (2006.01)

(21) International Application Number:

PCT/IL2010/000295

(22) International Filing Date:

8 April 2010 (08.04.2010)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/202,824 8 April 2009 (08.04.2009) US

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

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

Published:

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

[Continued on next page]

(54) Title: ISOLATED PEPTIDES FOR REGULATING APOPTOSIS

FIG. 1A

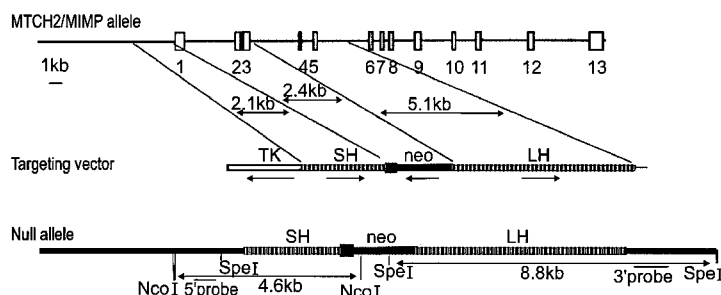
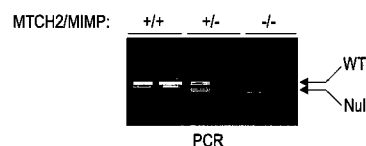


FIG. 1B



(57) Abstract: Provided are isolated peptides consisting of the amino acid sequence selected from the group consisting of SEQ ID NOs:111, 30, 31, 32, 115, 114, 112, 113, 108, 109, 110, 116 and 117 which can be used to increase the level of apoptosis in a cell and treat pathologies such as cancer. Also provided are isolated peptides consisting of the amino acid sequence set forth by SEQ ID NO:106, 107, 16, 19, 23, 17, 18, 20, 21, 22, 24, 25, 26, 136, 137 or 138, which can decrease the level of apoptosis in a cell and treat pathologies such as neurodegenerative diseases.



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ISOLATED PEPTIDES FOR REGULATING APOPTOSIS

5 FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to isolated peptides which can increase or decrease apoptosis in a cell, and to methods of using same for treating disorders associate with abnormally low or high levels of apoptosis.

10 Programmed cell death, or apoptosis, is essential for the development and maintenance of tissue homeostasis in multicellular organisms. Defects in apoptosis contribute to a variety of diseases, including cancer and neurodegenerative diseases. Thus, while oncogenesis and maintenance of the malignant phenotype of cancer cells involves blocking of death signaling, maintenance of the neurodegenerative phenotype of neuronal cells involves constitutive activation of death signaling.

15 The most common abnormalities of cancer and neurodegenerative diseases are related to the mitochondrial apoptotic pathway, which involve the mitochondrial outer membrane permeabilization (MOMP). MOMP results in release of proteins from the intermembrane space to the cytosol (e.g., cytochrome c), leading to caspase protease activation and cell death. BCL-2 family members are the major regulators of
20 mitochondrial apoptosis, affecting the decision of "MOMP or no MOMP", which is translated into death or survival of the cell. BAX and BAK are the pro-apoptotic effectors directly responsible for MOMP, and are antagonized by pro-survival proteins, including BCL-2, BCL-X_L, and MCL-1. A third class of BCL-2 proteins, the activator BH3-only proteins (BIM and BID), control the "MOMP or no MOMP" decision by
25 activating BAX and BAK.

The translocation t(14;18), which is found in follicular lymphoma and some diffuse large B cell lymphoma (DLBCL), drives overexpression of BCL-2, which inhibits MOMP. BCL-2 and other pro-survival proteins may sequester BAX or BAK, making them unavailable for activation, or alternatively they may sequester BH3-only
30 proteins that would otherwise bind and activate BAX and BAK. Several drugs that act as BH3-mimetics and antagonize the functions of pro-survival BCL-2 family proteins have been identified. These include the potent ABT-737/ABT-263 (Abbott Laboratories) and GX15-070 (Obatoclax, Gemin X) drugs, which exhibit pro-apoptotic activities in experimental tumors. However, not all cancers cell types express high

levels of pro-survival BCL-2 family proteins (Deng, J., et al., 2007). In addition, it remains largely unknown how apoptosis is constitutively activated in neurodegenerated cells (Vila M, and Przedborski S, 2003).

BID has emerged as a key regulator of neuronal apoptosis, and several recent studies report the development of small-molecule BID inhibitors that provide a promising therapeutic strategy in neurodegenerative diseases (Becattini, B., et al., 2006; Culmsee and Plesnila, 2006).

The mitochondrial carrier homolog 2 (MTCH2/MIMP) [also called met-induced mitochondrial protein (MIMP)] is an evolutionary conserved protein, which carries six α -helices that cross the outer mitochondrial membrane and interacts with the activated form of the BH3-only protein BID (tBID) in cells signaled to die by tumor necrosis factor-alpha (TNF α) or FAS (Grinberg M., et al., 2005; Gross A, 2005). In addition, MTCH2/MIMP was shown to act as a tumor suppressor gene in mice (Leibowitz-Amit R., et al., 2006).

PCT Patent Application IL2006/000021 discloses methods and pharmaceutical compositions for regulating apoptosis and treating pathologies associated with disregulated apoptosis using agents capable of modulating the expression of MTCH2/MIMP and/or the tBID binding activity thereof.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided an isolated peptide consisting of the amino acid sequence set forth by SEQ ID NO:106, 107, 16, 19, 23, 17, 18, 20, 21, 22, 24, 25, 26, 136, 137 or 138.

According to an aspect of some embodiments of the present invention there is provided an isolated peptide comprising the amino acid sequence selected from the group consisting of SEQ ID NOs:106, 107, 16, 19, 23, 17, 18, 20, 21, 22, 24, 25, 26, 136, 137 and 138, wherein the amino acid sequence is less than 60 amino acids in length and whereas the peptide decreases a level of apoptosis in a cell, with the proviso that the amino acid sequence is not the amino acid sequence set forth by SEQ ID NO:36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68 or 69.

According to an aspect of some embodiments of the present invention there is provided an isolated peptide comprising the amino acid sequence set forth by SEQ ID NO:111, 30, 31, 32, 115, 114, 112, 113, 108, 109, 110, 116 or 117, wherein the amino acid sequence is less than 60 in length and whereas the peptide increases a level of apoptosis in a cell.

According to an aspect of some embodiments of the present invention there is provided an isolated peptide consisting of the amino acid sequence selected from the group consisting of SEQ ID NOs:111, 30, 31, 32, 115, 114, 112, 113, 108, 109, 110, 116 and 117.

According to an aspect of some embodiments of the present invention there is provided an isolated molecule comprising the isolated peptide of the invention, attached to an amino acid sequence which enhances penetration of the peptide into a cell.

According to an aspect of some embodiments of the present invention there is provided an isolated molecule comprising the isolated peptide of the invention attached to an amino acid sequence which enhances penetration of the peptide into a cell.

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide comprising a nucleic acid sequence encoding an amino acid sequence consisting of the amino acid sequence of the invention, or of the isolated molecule of the invention.

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide comprising a nucleic acid sequence encoding an amino acid sequence consisting of the amino acid sequence of the invention or of the isolated molecule of the invention.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid construct comprising the isolated polynucleotide of the invention and a promoter for directing expression of the amino acid sequence in a host cell.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid construct comprising the isolated polynucleotide of the invention and a promoter for directing expression of the amino acid sequence in a host cell.

According to an aspect of some embodiments of the present invention there is provided a pharmaceutical composition comprising as an active ingredient the isolated peptide of the invention, the isolated molecule of the invention, the isolated polynucleotide of the invention or the nucleic acid construct of the invention.

5 According to an aspect of some embodiments of the present invention there is provided a method of downregulating apoptosis in a cell, comprising contacting the cell with the peptide of the invention, the isolated molecule of the invention, the isolated polynucleotide of the invention or the nucleic acid construct of the invention, thereby downregulating the apoptosis in the cell.

10 According to an aspect of some embodiments of the present invention there is provided a method of upregulating apoptosis in a cell, comprising contacting the cell with the peptide of the invention, the isolated molecule of the invention, the isolated polynucleotide of the invention or the nucleic acid construct of the invention, thereby upregulating the apoptosis in the cell.

15 According to an aspect of some embodiments of the present invention there is provided a method of treating a pathology associated with abnormally high levels of apoptosis in a subject, comprising administering to the subject a therapeutically effective amount of the peptide of the invention, the isolated molecule of the invention, the isolated polynucleotide of the invention or the nucleic acid construct of the invention, thereby treating the pathology associated with abnormally high levels of apoptosis in the subject.

20 According to an aspect of some embodiments of the present invention there is provided a method of treating a pathology associated with abnormally low levels of apoptosis in a subject, comprising administering to the subject a therapeutically effective amount of the peptide of the invention, the isolated molecule of the invention, the isolated polynucleotide of the invention or the nucleic acid construct of the invention, thereby treating the pathology associated with abnormally low levels of apoptosis in the subject.

 According to some embodiments of the invention, the peptide is cyclic.

30 According to some embodiments of the invention, the pathology associated with abnormally high levels of apoptosis is a degenerative disorder.

According to some embodiments of the invention, the degenerative disorder is selected from the group consisting of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) and retinitis pigmentosa.

According to some embodiments of the invention, the pathology associated with abnormally high levels of apoptosis is human immunodeficiency virus (HIV)-induced acquired immunodeficiency syndrome (AIDS).

According to some embodiments of the invention, the pathology associated with abnormally low levels of apoptosis is selected from the group consisting of cancer, an autoimmune disorder, a bacterial infection, and a viral infection.

According to some embodiments of the invention, the amino acid sequence which enhances penetration of the peptide into a cell is set forth by SEQ ID NO:131.

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Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

15

BRIEF DESCRIPTION OF THE DRAWINGS

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Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

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In the drawings:

FIGs. 1A-B are a diagram (Figure 1A) and a PCR analysis (Figure 1B) depicting the generation of MTCH2/MIMP knockout embryos. Figure 1A – A diagram depicting the MTCH2/MIMP genomic locus, the targeting vector and the homologous recombinant, with the restriction enzyme sites (NcoI and SpeI) and the position of

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external probes. Figure 1B - PCR analysis of wild-type, MTCH2/MIMP^{+/-} heterozygote and MTCH2/MIMP^{-/-} homozygote embryos. Two parallels are shown for each condition.

FIGs. 2A-E are histological analyses depicting morphological characteristics of the Mtch2 knock-out embryos. Figures 2A and B are Sagittal sections of representative E7.5 wild-type (Figure 2A) and MTCH2/MIMP^{-/-} (Figure 2B) embryos stained with hematoxylin and eosin. The MTCH2/MIMP^{-/-} embryos lack some of the typical structures of this stage: the chorion, amnion and ectoplacental cone are undetectable. Moreover, the extraembryonic region is unorganized and the mesoderm fails to migrate. Em (Embryonic region), ExEm (Extra-embryonic region), M (Mesoderm), Ec (Ectoderm), En (Endoderm), Ch (Chorion), Am (Amnion), EPC (Ectoplacental cone). Figure 2C – a histogram depicting the number of cells in MTCH2/MIMP^{-/-} E7.5 and wild-type E7.5 embryos. The cells of the ectodermal and mesodermal layers were counted in two wild-type and two knockout embryos. For each embryo three adjacent 4 μm sections were counted and the average value obtained. Note that MTCH2/MIMP^{-/-} E7.5 embryos consist three times less cells than wild-type E7.5 embryos. Figures 2D and E are images depicting Brachyury (T) mRNA expression patterns in E7.5 embryos using whole mount RNA *in situ* hybridization. Brachyury expression was detected by RNA in situ hybridization using an antisense dig-labeled ribo-probes. Figure 2D - E7.5 wild-type embryos; Figure 2E – E7.5 MTCH2/MIMP^{-/-} embryos. In the wild-type embryo Brachyury mRNA is highly expressed in the primitive streak at the posterior side of the embryonic (Em) region (Figure 2D, marked by arrow). These results demonstrate that E7.5 MTCH2/MIMP^{-/-} embryos are significantly smaller than the wild-type embryos and lack structures that are typical to the developmental stage.

FIG. 3A – MTCH2/MIMP Western blot analysis demonstrating the generation of MTCH2/MIMP^{-/-} embryonic stem (ES) stable lines. Wild-type (+/+) and MTCH2/MIMP^{-/-} (-/-) E3.5 blastocytes were isolated and cultured as described under the “General Materials and Experimental Methods” of the Examples section which follows. Cells from both lines were lysed, subjected to SDS-PAGE and Western blot analyzed using anti-MTCH2/MIMP antibodies.

FIG. 3B - MTCH2/MIMP Western blot analysis. The MTCH2/MIMP^{-/-} ES cells were transfected with either an empty pcDNA3.1 vector (clone V42) or a pcDNA3.1

vector carrying MTCH2/MIMP (clone R5). Cells from two single stable clones (V42 and R5) were lysed, subjected to SDS-PAGE and Western blot analyzed using anti-MTCH2/MIMP antibodies. Lane 1 – cells from wild-type (+/+) ES cells; lane 2 – cells from MTCH2/MIMP^{-/-} (-/-); lane 3 – cells from MTCH2/MIMP^{-/-} which were transformed with the empty vector (clone V42); lane 4 – cells from MTCH2/MIMP^{-/-} which were transformed with the MTCH2/MIMP vector (clone R5). The results demonstrate the presence of MTCH2/MIMP in cells rescued with a vector carrying the MTCH2/MIMP coding sequence.

FIG. 3C – tBID Western blot analysis. V42 and R5 cells were infected with an adenovirus vector expressing the tBID coding sequence (Ad-HA-tBID), and mitochondria prepared from these cells were treated with the BS³ cross-linker followed by Western blot analysis using anti-HA antibodies. The results demonstrate that tBID cross-linked mitochondrial complex is generated in rescued cells (R) which express wild-type MTCH2/MIMP but not in cells devoid of MTCH2/MIMP (V cells).

FIGS. 3D-E are graphs depicting the effect of MTCH2/MIMP on mitochondrial membrane potential. Experiments were carried out with 1.2 mg of cells in 750 µl of an intracellular medium (passed through a Chelex column to reduce ambient Ca²⁺) composed of 120 mM KCl, 10 mM NaCl, 1 mM KH₂PO₄, 20 mM Hepes/Tris, pH 7.2, supplemented with 1 µg/ml each of antipain, leupeptin, pepstatin and with 40 µg/ml digitonin at 35 °C under continuous stirring in a fluorometer. The following additions were made: 2 µM TMRE (10s), 5 mM succinate (50s), 2 mM ATP + CP/CPK (100s), 12 µg/ml oligomycin (150s), purified recombinant histidine-tagged murine tBID (300s), FCCP 5 µM (600s). Presented are actual raw data (from representative experiments) TMRE fluorescence (ex. 545, em. 580) recordings of one pair of V (V42; Figure 3D; MTCH2/MIMP^{-/-} ES cells rescued with an empty vector) and R (R5; Figure 3E; MTCH2/MIMP^{-/-} ES cells rescued with a vector containing wild-type MTCH2/MIMP) clones treated with 0.3, 0.5, and 2 nM recombinant tBID. Similar results were obtained with the two additional pairs of V and R clones. The results demonstrate that the R cells (which include wild-type MTCH2/MIMP) are more sensitive than V cells (which are devoid of wild-type MTCH2/MIMP) to tBID-induced mitochondrial depolarization.

FIG. 3F is a Western blot analysis demonstrating cytochrome c (Cyt c) release as a function of wild-type MTCH2/MIMP. At the end of the recordings described in

Figures 3D, E, the suspensions of the V42 and R5 clones were centrifuged and the pellets were separated from the supernatants. 30 μ l of the supernatants were subjected to SDS-PAGE, followed by Western blot analysis using anti-Cyt c mAbs (7H8.2C12; PharMingen; Catalogue number 556432). Similar results were obtained with two additional pairs of V and R clones. Note that the R cells are more sensitive than V cells to tBID-induced Cyt c release, demonstrating that MTCH2/MIMP is a positive regulator of tBID-induced MOMP.

FIG. 3G is a Western blot analysis depicting dimerization of BAX as a function of wild-type MTCH2/MIMP. At the end of the recordings described in Figures 3D, E, the suspensions of the V42 and R5 clones were centrifuged and the pellet fractions were treated with the Sulfo-BSOCOES cross-linker and lysed. Equal amounts of protein (30 μ g per lane) were subjected to SDS-PAGE, followed by Western blot analysis using anti-BAX Abs (651; gift from Stan Korsmeyer, DFCI, Boston, USA). * marks an intra-molecular cross-linked product of activated BAX. The results shown in this Figure are representative of four independent experiments. Similar results were obtained with the two additional pairs of V and R clones. Note that BAX is homodimerized in the R cells at the low concentration of tBID.

FIGS. 4A-G demonstrate that conditional knockout of *MTCH2/MIMP* in MEFs reduces the sensitivity to tBID-induced apoptosis. Figure 4A - Generation of the *MTCH2/MIMP* conditional targeting vector. Indicated are loxP sites (black triangles), Frt sites (gray triangles), the neomycin (Neo) positive selection cassette, and the thymidine kinase (TK) negative selection cassette. Figure 4B - Western blot analysis of conditional deletion of *MTCH2/MIMP* in MEFs. *MTCH2/MIMP*^{fl/fl} MEFs +/- Cre-recombinase were lysed, and the mitochondria-enriched fractions were analyzed by Western blot for MTCH2/MIMP using an anti-MTCH2/MIMP antibody (Grinberg, M., Schwarz, M., Zaltsman, Y., Eini, T., Niv, H., Pietrokovski, S., and Gross, A. Mitochondrial Carrier Homolog 2 Is a Target of tBID in Cells Signaled To Die by Tumor Necrosis Factor Alpha. Mol Cell Biol, 25: 4579-4590, 2005). BAX was used as an internal standard. Figure 4C - Western blot analysis. *MTCH2/MIMP*^{fl/fl} MEFs were treated as in Figure 4B, infected with Ad-tBID and the mitochondria-enriched fractions were treated (+) or not (-) with the BSOCOES cross-linker followed by Western blot analysis. CL, cross-linker. * mark cross-reactive bands. Porin was used as an internal

standard (bottom panel). The results show that tBID cross-linked complex is not generated in *MTCH2/MIMP*-deficient MEFs. Figure 4D - *MTCH2/MIMP*^{fl/fl} (fl/fl; left panel) and *MTCH2/MIMP*^{fl/+} (fl/+; right panel) MEFs +/- Cre-recombinase were infected with Ad-tBID and cell death was monitored by PI dye exclusion. Data are the mean $\pm$ s.d. of three independent experiments. The results show that *MTCH2/MIMP*-deficient MEFs are less sensitive to Ad-tBID-induced apoptosis. Figure 4E - Fl/fl MEFs +/- Cre-recombinase were infected with the indicated adenoviruses, and cell death was monitored as above. Data are the mean $\pm$ s.d. of three independent experiments, * $P < 0.00005$. The results show that the reduced susceptibility of *MTCH2/MIMP*-deficient MEFs to Ad-tBID is due to the absence of MTCH2/MIMP. Figure 4F - Fl/fl MEFs +/- Cre-recombinase were infected with the indicated adenoviruses, and cell death was monitored as above. Data are the mean $\pm$ s.d. of three independent experiments. The results show that *MTCH2/MIMP* deletion has no effect on apoptosis induced by other pro-apoptotic BCL-2 family members. Figure 4G - MTCH2/MIMP was knocked down in U2OS cells as described in the Methods, and cells were either left untreated (N/T) or infected with Ad-tBID and cell death was monitored as above. Data are the mean $\pm$ s.d. of three independent experiments, * $P < 0.005$. The results show that knocking down MTCH2/MIMP in U2OS cells reduces the sensitivity to Ad-tBID.

FIGS. 5A-E demonstrate that conditional knockout of *MTCH2/MIMP* in MEFs hinders the recruitment of tBID to mitochondria. Figure 5A - fl/fl and fl/+ MEFs +/- Cre-recombinase were treated with each of the indicated apoptotic stimuli for 14 hours: Fas (1 ng/ml) and cycloheximide (CHX; 1 μ g/ml), Etop (100 μ M), and Cis (33 μ M). Cell death was monitored as above. Data are the mean $\pm$ s.d. of three independent experiments, * $P < 0.0005$. The results show that *MTCH2/MIMP*-deficient MEFs are less sensitive to apoptosis induced by DNA-damaging reagents. Figure 5B - Fl/fl MEFs +/- Cre-recombinase were either infected with Ad-tBID, treated with Etop (100 μ M; 8 hours), or treated with Fas (5 ng/ml) and cycloheximide (CHX; 1 μ g/ml; 6 hours). Cells were then lysed, and the mitochondria-enriched fractions, the cytosolic fractions, and total cell lysates were Western blot analyzed with anti-HA (left top panel) or anti-BID (all other panels) Abs. * mark cross-reactive bands. Porin and actin were used as internal standards. The results show that deletion of *MTCH2/MIMP* hinders the

recruitment of tBID to mitochondria. Figure 5C - Fl/fl MEFs were treated as in Figure 5B, lysed, and the mitochondria-enriched fractions were treated with trypsin as described in the Methods, followed by Western blot analysis. The middle panel shows a short exposure of the blot shown in the top panel. Porin was used as an internal
 5 standard. The results show that deletion of *MTCH2/MIMP* reduces the levels of NH₂-terminus exposed/activated BAX. Figure 5D - Fl/fl MEFs +/- Cre-recombinase were treated with the indicated death stimuli. Cells were then fixed, immunostained for Cyt c, and the percentage of cells with Cyt c released was quantified. Data are the mean $\pm$ s.d. of three independent experiments. Approximately 300 cells of each treatment were
 10 analyzed. a: $P < 0.01$; b: $P < 0.05$; c: $P < 0.01$. The results show that deletion of *MTCH2/MIMP* reduces Cyt c release. Figure 5E - Fl/fl MEFs were either infected or not with Ad-BCL-2 and then treated with the indicated death stimuli for 14 hrs: Fas and cycloheximide [as in (a)], and Etop (10 μ M). Cell death was monitored as in (a) and data are the mean $\pm$ s.d. of three independent experiments, * $P < 0.005$. The results show
 15 that fl/fl MEFs are type I cells.

FIGs. 6A-H demonstrate that *MTCH2/MIMP* deletion in the liver reduces the sensitivity of mice to Fas-induced hepatocellular apoptosis and hinders the recruitment of tBID to mitochondria. Figure 6A - Western blot analysis of *MTCH2/MIMP* in liver lysates demonstrates its absence in livers prepared from *MTCH2/MIMP^{fl/ Δ} ; Alb-Cre*
 20 (*fl/ Δ*) mice. BCL-X_L was used as an internal standard (bottom panel). * marks a cross reactive band. Figure 6B - Conditional knockout of *MTCH2/MIMP* in the liver significantly reduces the sensitivity of mice to Fas-induced hepatocellular apoptosis. Figure 6B - Kaplan-Meier survival curves of *MTCH2/MIMP^{fl/+}; Alb-Cre* mice (*fl/+*; n = 15) and *MTCH2/MIMP^{fl/ Δ} ; Alb-Cre* mice (*fl/ Δ* ; n = 14) in response to a single i.p.
 25 injection of 0.55 μ g g⁻¹ anti-Fas antibody (Jo2; PharMingen). The Kaplan-Meier survival curves were compared using the long-rank test and were found statistically different from each other ($P < 0.05$). Figures 6C-D - Haematoxylin-eosin staining of paraffin-embedded liver sections from a *fl/+* mouse (Figure 6C) and a *fl/ Δ* mouse (Figure 6D) 4 hours after anti-Fas antibody injection. Note the condensed and
 30 fragmented nuclei and the haemorrhage in the *fl/+* liver. The bars represent 50 μ m. Figures 6E-F - *fl/+* and *fl/ Δ* mice (four from each) were either left untreated (-) or injected with anti-Fas Abs for the indicated times. Following treatment, S100 fractions

were prepared from all eight livers and analyzed by Western blot for caspase-8 cleavage/activation (Figure 6E, top panel; the p18 represents the activated protease), BID cleavage/activation (Figure 6E, middle panel), and caspase-3 activity using the fluorogenic peptide substrate DEVE-AMC [Figure 6F; the results are presented in arbitrary units (AU) as the mean $\pm$ s.d. of three independent experiments; a: $P < 0.01$; b: $P < 0.02$]. Actin was used in both blots as an internal standard. The results show a significant decrease in caspase-3 activation in fl/ Δ liver cytosolic fractions in response to anti-Fas Ab. Figure 6G - Liver mitochondria fractions prepared from the mice described in Figures 6E-F were lysed, and Western blot analyzed using anti-BID Abs. Figure 6H - Liver mitochondria fractions treated with trypsin, lysed, and Western blot analyzed using anti-BAX Abs. Porin was used in both blots as an internal standard. Note the significant decrease in tBID recruitment to mitochondria and BAX activation in fl/ Δ liver mitochondrial fractions in response to anti-Fas Ab.

FIGs. 7A-B are Western blot analyses demonstrating that *MTCH2/MIMP* deletion in the liver prevents the *in vitro* import of tBID. Kinetics of HA-tBID import into mitochondria (Figure 7A) and Cyt c release (Figure 7B). Cytosolic fractions of 293T cells expressing HA-tBID and depleted of Cyt c (using anti-Cyt c Abs) were incubated with purified, intact mitochondria isolated from mouse liver prepared from either fl/+ mice (top panels) or fl/ Δ mice (bottom panels). At the indicated time points, mitochondria were separated from the soluble fraction by centrifugation, and both fractions were lysed and analyzed by Western blot with anti-HA (Figure 7A) or anti-Cyt c (Figure 7B) Abs. Actin and porin were used as internal standards for the soluble/cytosolic and mitochondrial fractions, respectively.

FIGs. 8A-C are Western blot analyses of mouse liver mitochondria using antibodies directed against *MTCH2/MIMP* (Figure 8A), AIF (Figure 8B) and ANT (Figure 8C). Mouse liver mitochondria were either left untreated (-) or treated with a low (0.1 μ g/ml; +) or a high concentration (1 μ g/ml; ++) of proteinase K, lysed, size-fractionated by SDS-PAGE and analyzed by Western blot using anti-*MTCH2/MIMP* Abs (Figure 8A), anti-AIF (apoptosis inducing factor) Abs (Figure 8B), or anti-ANT (adenine nucleotide translocator) Abs (Figure 8C). The results demonstrate that *MTCH2/MIMP* is exposed on the surface of mitochondria.

FIGs. 8D-G are Western blot analyses of submitochondrial membrane vesicles using antibodies directed against cytochrome c oxidase subunit IV (Figure 8D), ANT (Figure 8E), Tom20 (Figure 8F) and MTCH2/MIMP (Figure 8G). Submitochondrial membrane vesicles were prepared from rat liver mitochondria, lysed, size-fractionated by SDS-PAGE and analyzed by Western blot using anti-cytochrome c oxidase subunit IV (Cyt Oxi; Figure 8D) Abs, anti-ANT Abs (Figure 8E), anti-Tom20 Abs (Figure 8F), and anti-MTCH2/MIMP Abs (Figure 8G). The low-density fractions enriched in outer membrane vesicles are marked "OMM" and the high-density fractions enriched in inner membrane vesicles are marked "IMM". The results show that MTCH2/MIMP is enriched in the OMM.

FIGs. 9A-B are immunoblot analyses depicting binding of recombinant tBID (Figure 9A) or recombinant BID (Figure 9B) to a MTCH2/MIMP – derived peptide array. Cellulose-bound peptide array consisting of overlapping peptides derived from MTCH2/MIMP was screened by immunoblot experiments with recombinant tBID/BID proteins. A dark spot represents binding of tBID/BID to a specific peptide as specified by the peptide reference number (rows E, F or G; columns 1-24). The MTCH2/MIMP-derived peptides which bind the recombinant tBID/BID (resulted in a positive signal, dark spot) are provided in Table 4, Example 6 of the Examples section which follows.

FIG. 9C is a schematic presentation of the MTCH2/MIMP secondary structure with the position of the MTCH2/MIMP-derived peptides which bind BID or tBID according to the immunoblot results presented in Figures 9A-B. The colors represent the degree of binding the BID/tBID proteins: Pink – peptides that do not bind BID or tBID; Dark green – Peptides which strongly bind tBID; Light green – peptides that bind tBID moderately; Light blue – peptides that bind weakly to tBID; Brown – peptides which bind full BID.

FIGs. 9D-F are schematic illustrations of the MTCH2/MIMP tertiary structure with the position of the tBID/full BID binding sites. The tBID/full BID binding sites that were discovered in the peptide array screening (Figures 9A-C, Table 4) are highlighted on the three-dimensional (3D) model structure that was recently generated [Schwarz, M., Andrade-Navarro, M. A., and Gross, A. (2007). Mitochondrial carriers and pores: key regulators of the mitochondrial apoptotic program? *Apoptosis* 12, 869-876]. Figure 9D - The tBID binding sites on the MTCH2/MIMP tertiary structure

model. The peptide that binds the tightest to tBID is colored using strong red and the other peptides are colored using light red; Figure 9E - The full length BID binding sites on the MTCH2/MIMP tertiary structure model. The peptide that binds the tightest to full length BID is colored with green; Figure 9F - The tBID and full length BID binding sites on the MTCH2/MIMP tertiary structure. The peptide that binds tBID are colored with red, the peptides that bind full BID are colored with green; For peptide sequences and residue numbers see Table 4 in the Examples section which follows. "aa" (amino acid) residues marks the position of the peptides on the MTCH2/MIMP polypeptide sequence (SEQ ID NO:1).

FIG. 10 is a schematic illustration depicting a proposed model according to some embodiments of the invention for the regulation of tBID-induced MOMP by MTCH2/MIMP. Cleavage by caspase-8 generates tBID (light purple), which rapidly migrates to the membrane. At the membrane, tBID induces a conformational change in BAX (pink; which includes N-terminus exposure) leading to its insertion into the membrane. MTCH2/MIMP (deep purple), localized at the OMM, interacts with tBID and assists it in BAX activation. The resulting activated BAX can oligomerize resulting in membrane permeabilization.

FIG. 11 depicts the amino acid sequence of mouse BID (SEQ ID NO:3) with the identified peptides following mass spectrometry results of cross-linking experiments using HA-tagged full length BID immunoprecipitated from transfected cells. Sequences of the seven peptides from the mouse BID protein that were identified in the MS analysis [highlighted with yellow, with a red box delineating each peptide; SEQ ID NOs:97, 100, 101, 102, 103, 104 and 105], and the five peptides that were not identified by mass-spec [non-highlighted, each peptide is delineated with a red box; SEQ ID NOs:96, 98, 99, 28, 29]. Marked in red is the cleavage site of BID to tBID, and in gray the potential sites of cross-linker binding in one of none-identified peptides.

FIGs. 12A-C are immunoblot analyses depicting binding of MTCH2/MIMP biotinylated peptide 240-290 (SEQ ID NO:106) to a tBID/BID – derived peptide array. Cellulose-bound peptide array consisting of overlapping peptides derived from tBID/BID was screened by immunoblot experiments with biotinylated peptide 240-290 (SEQ ID NO:106) in different ionic strengths: 150 mM (Figures 12A), 100 mM (Figures 12B), 50 mM (Figures 12C). Dark spots represent binding of MTCH2/MIMP

biotinylated peptide 240-290 (SEQ ID NO:106) to a specific peptide as specified by the peptide reference number (rows A-B colored red; columns 1-24). The tBID/BID derived peptides which bind the MTCH2/MIMP Biotinylated peptide 240-290 (SEQ ID NO:106) (resulted in a positive signal, dark spot) are provided in Table 8, Example 9 of the Examples section which follows.

FIG. 13A is a schematic presentation of the tBID/BID secondary structure with the position of the tBID/BID -derived peptides which bind MTCH2/MIMP peptide 240-290 (SEQ ID NO:106) according to the immunoblot results presented in Figures 12A-C. The colors represent the degree of binding the MTCH2/MIMP peptide 240-290: black – peptides that do not bind MTCH2/MIMP peptide 240-290 AA; Dark blue – Peptides which strongly bind MTCH2/MIMP peptide 240-290; Cyan – peptides that bind moderately-weakly to MTCH2/MIMP peptide 240-290.

FIG. 13B is a schematic illustration of the tBID/BID tertiary structure with the position of the MTCH2/MIMP peptide 240-290 (SEQ ID NO:106) binding sites. The tBID/full BID binding sites that were discovered in the peptide array screening (Figures 12A-C,) are highlighted on the three-dimensional (3D) model structure PDB ID:2Bid. The peptide that binds the tightest to MTCH2/MIMP peptide 240-290 is colored using strong blue and the other peptides are colored using cyan. For peptide sequences and residue numbers see Table 8, Example 9 of the Examples section which follows. “aa” (amino acid) residues marks the position of the peptides on the tBID/BID polypeptide sequence

FIGs. 14A-C are immunoblot analyses depicting binding of MTCH2/MIMP Biotinylated peptide 140-161 (SEQ ID NO:107) to a tBID/BID – derived peptide array. Cellulose-bound peptide array consisting of overlapping peptides derived from tBID/BID was screened by immunoblot experiments with biotinylated peptide 140-161 in different ionic strengths: 150 mM (Figures 14A), 100 mM (Figures 14B), 50 mM (Figures 14C). Dark spots represent binding of MTCH2/MIMP biotinylated peptide 140-161 to a specific peptide as specified by the peptide reference number (rows A-B colored red; columns 1-24). The tBID/BID derived peptides which bind the MTCH2/MIMP biotinylated peptide 140-161 (resulted in a positive signal, dark spot) are provided in Table 9, Example 9 of the Examples section which follows.

FIG. 15A is a schematic presentation of the tBID/BID secondary structure with the position of the tBID/BID -derived peptides which bind MTCH2/MIMP peptide 140-161 (SEQ ID NO:107) according to the immunoblot results presented in Figures 14A-C. All peptides that bound both the MTCH2 240-290 AA and the MTCH2 140-161 AA are highlighted as in Figure 13A, amino acid numbers indicated only the peptides that bound the MTCH2 140-161. Most peptides that bound the MTCH2 140-161, also bound the MTCH2 240-290, except for one additional peptide (tBID 62-76) which only bind to MTCH2 140-161 and is colored in pink.

FIG. 15B are schematic illustrations of the tBID/BID tertiary structure with the position of the MTCH2/MIMP peptide 140-161 (SEQ ID NO:107) binding sites. The tBID/full BID binding sites that were discovered in the peptide array screening (Figures 14A-C,) are highlighted on the three-dimensional (3D) model structure PDB ID:2Bid. The peptide that binds the tightest to MTCH2/MIMP peptide 140-161 is colored using strong blue and the other peptides are colored using cyan and one additional peptide that showed binding only to MTCH2 140-161 colored in pink. For peptide sequences and residue numbers see Table 9 in the Examples section which follows. "aa" (amino acid) residues marks the position of the peptides on the tBID/BID polypeptide sequence

FIG. 16 is a schematic illustration of the tBID/BID tertiary structure with the position of the MTCH2/MIMP peptides 140-161 (SEQ ID NO:107) and 240-290 (SEQ ID NO:106) binding sites. The tBID/full BID binding sites that were discovered in the peptide array screening (Figures 12-15,) are highlighted on the three-dimensional (3D) model structure PDB ID:2Bid. The binding site of the peptides that bind the cleavage part of the protein (tBID) are colored in magenta, and the binding site represented by three peptides that bind the N-terminus of full length Bid are colored in blue. For peptide sequences and residue numbers see Tables 8 and 9 in the Examples section which follows. "aa" (amino acid) residues marks the position of the peptides on the tBID/BID polypeptide sequence

FIGs. 17A-B demonstrate the conditional gene targeting of murine *MTCH2/MIMP*. Figure 17A – A schematic illustration depicting the generation of the *MTCH2/MIMP* conditional targeting vector. Shown are the wild-type allele and the homologous recombinant product. Also indicated are loxP sites (which are excised by Cre recombinase; black triangles), Frt sites (which are excised by Flp recombinase; gray

triangles), the neomycin (Neo) positive selection cassette, the thymidine kinase (TK) negative selection cassette, the restriction enzyme sites XbaI and NcoI and the position of the probes used to screen for *MTCH2/MIMP*^{+/-} ES clones by Southern blot. Figure 17B - Southern blot analysis demonstrating homologous recombination within the *MTCH2/MIMP* locus of one of the ES clones that was subsequently aggregated with tetraploid embryos. Left panel: XbaI-digested genomic DNA extracted from the ES clone was hybridized with 5'-probe, and the band obtained represents the homologous recombination of the long homology (LH) arm. Right panel: NcoI-digested genomic DNA of the ES clone was hybridized with 3'-probe, and the band obtained represents the homologous recombination of the short homology (SH) arm.

FIGS. 18A-I demonstrate the conditional knockout of *MTCH2/MIMP* in MEFs. Figure 18A - A histogram depicting percentage of cell death in fl/fl MEFs following several apoptotic stimuli. fl/fl MEFs were either transduced or not with Cre-recombinase, and treated with each of the indicated apoptotic stimuli: TNF α (0.02 ng/ml) and actinomycin D (ActD; 2 μ g/ml; 14 hours), Staurosporine (STS; 0.1 μ M; 13 hours), and ultra-violet (UV; 10J/m²; 8 hours). Cell death was monitored as in Figure 5A and the results are presented as mean $\pm$ SD. Note that *MTCH2/MIMP*-deficient MEFs are equally sensitive to several apoptotic stimuli. Figure 18B - Western blot analysis with anti-cleaved caspase antibodies. fl/fl MEFs were treated as above, lysed, and Western blot analyzed using anti-cleaved caspase-3 Abs. Actin was used as an internal standard (bottom panel). Note that deletion of *MTCH2/MIMP* reduces Ad-tBID- and Etop-induced caspase-3 cleavage/activation. Figure 18C - Western blot analysis with anti-Bax antibodies. fl/fl MEFs were either transduced or not with Cre-recombinase, and then treated with Ad-tBID, Etop or Fas as described in Figure 5C. Cells were then lysed, and the mitochondria-enriched fractions were treated with the BSOCOES cross-linker, followed by Western blot analysis. Note that deletion of *MTCH2/MIMP* hinders BAX dimerization. Figures 18D-I - immuno-fluorescence analyses using cytochrome c antibodies. fl/fl MEFs were either transduced (Figures 18E, 18G and 18I) or not (Figures 18D, 18F and 18H) with Cre-recombinase, and then plated on coverslips in a 12 well plate (80,000 cells/well). Cells were then exposed to the indicated death stimuli [Ad-tBID (10 MOI), Figures 18D-E; Etop (100 μ M), Figures 18F-G; Fas (1 ng/ml) plus cycloheximide (CHX; 1 μ g/ml), Figures 18H and I, for 14, 8

and 7 hours, respectively]. Cells were then fixed, immunostained for Cyt c. The white arrows represent cells that have released Cyt c. Note that deletion of *MTCH2/MIMP* hinders Cyt c release. Altogether, the results show that the conditional knockout of *MTCH2/MIMP* in MEFs hinders BAX dimerization and Cyt c release.

FIGs. 19A-C are PCR analyses depicting genotyping of mouse tails of progeny that were generated from crosses between *MTCH2/MIMP*^{+Δ}; Alb-Cre and *MTCH2/MIMP*^{fl/fl} mice. The liver specific knockout mouse (No. #66) carries a floxed allele (fl), a deleted allele (Δ) and the Alb-Cre gene that allows the specific deletion of the floxed allele only in hepatocytes. The control heterozygote littermate (No. #69) carries a wild-type allele (+), a floxed allele (fl), and the Alb-Cre gene. Mice numbers (#) refer to: #65: *MTCH2/MIMP*^{+Δ}; Alb-Cre; #66: *MTCH2/MIMP*^{fl/Δ}; Alb-Cre; #67: *MTCH2/MIMP*^{fl/Δ}; #68: *MTCH2/MIMP*^{+Δ}; #69: *MTCH2/MIMP*^{fl/+}; Alb-Cre.

FIG. 20 is a histogram depicting the level of serum liver enzymes aspartate aminotransferase (AST) and alanineaminotransferase (ALT) in fl/+ and fl/Δ mice.

Serum was collected after over night fast. Data are presented as mean ± SEM.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to isolated peptides which can increase or decrease apoptosis in a cell and to methods of using same for treating cancer or neurodegenerative diseases.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

The present inventors have uncovered novel *MTCH2/MIMP*-derived peptides which bind BID and/or tBID and which can decrease the level of apoptosis in cells and treat pathologies associated with excessive apoptosis such as neurodegenerative diseases. In addition, the present inventors have uncovered BID- and tBID-derived peptides which can increase the level of apoptosis in cells and treat pathologies associated with abnormally low levels of apoptosis such as cancer.

Thus, as shown in the Examples section which follows, the present inventors found that *MTCH2/MIMP* is required for embryonic development and that a complete

absence of MTCH2/MIMP is embryonic lethal (Example 1, Table 3, Figures 1A-B and 2A-E). In addition, using embryonic stem (ES) cells homozygote to the MTCH2/MIMP-knock-out allele (devoid of MTCH2/MIMP expression or activity) it was found that MTCH2/MIMP is a positive regulator of tBID-induced BAK/BAX activation and mitochondrial outer membrane permeabilization (MOMP) (Example 2, Figures 3A-G). Moreover, using a conditional knock-out approach, the present inventors have found that conditional knockout of MTCH2/MIMP in MEFs reduces the sensitivity to tBID-induced apoptosis (Example 3, Figures 4A-G, 17A-B), and hinders the recruitment of tBID to mitochondria (Example 4, Figures 5A-E; 18A-I). In addition, as shown in Example 5, MTCH2/MIMP deletion in the liver reduces the sensitivity of mice to fas-induced hepatocellular apoptosis and hinders the recruitment of tBID to mitochondria (Figures 6A-H, 19-20). Moreover, as shown in Example 6, MTCH2/MIMP deletion in the liver prevents the *in vitro* import of tBID (Figures 7A-B). In addition, it was found that MTCH2/MIMP is exposed on the surface of mitochondria (Example 5, Figures 8A-G). Furthermore, the present inventors identified MTCH2/MIMP-derived peptides which bind tBID or BID (SEQ ID NOs:106, 107, 16-26 for human MTCH2/MIMP-derived peptides; Example 8, Tables 4 and 5, Figures 9A-D) and which can reduce apoptosis in cells, and novel BID- and tBID-derived peptides which bind MTCH2/MIMP (SEQ ID NOs:30, 31, 32, 115, 111, 114, 112, 113, 108, 109 or 110 for human BID- and tBID-derived peptides; Examples 9, 10 and 11, Tables 6, 7, 8, and 9, Figures 11, 12, 13, 14, 15 and 16) and which can increase apoptosis in cells.

Thus, according to an aspect of some embodiments of the invention, there is provided an isolated peptide comprising the amino acid sequence set forth by SEQ ID NO:111, 30, 31, 32, 115, 114, 112, 113, 108, 109, 110, 116 or 117, wherein the amino acid sequence is less than 60 in length and whereas the peptide increases a level of apoptosis in a cell.

According to some embodiments of the invention, the isolated peptide is less than 60 amino acids in length, e.g., 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41 or 40 amino acids, e.g., less than 40 amino acids in length, e.g., 39, 38, 37, 36, 35, 34, 33, 32, 31 or 30 amino acids, e.g., less than 30 amino acids in length, e.g., 29, 28, 27, 26, 25, 24, 24, 22, 21 or 20 amino acids, e.g., less than 20 amino acids in length, e.g., 19, 18, 17, 16, 15, 14, 13, 12, 11 or 10 amino acids, or less.

According to some embodiments of the invention the isolated peptide consists of the amino acid sequence selected from the group consisting of SEQ ID NOs:111, 30, 31, 32, 115, 114, 112, 113, 108, 109, 110, 116 or 117.

According to some embodiments of the invention the isolated peptide which
 5 comprises the amino acid sequence selected from the group consisting of SEQ ID NOs:111, 30, 31, 32, 115, 114, 112, 113, 108, 109, 110, 116 or 117 is capable of increasing the level of apoptosis in a cell.

According to an aspect of some embodiments of the invention, there is provided an isolated peptide comprising the amino acid sequence selected from the group
 10 consisting of SEQ ID NOs:106, 107, 16, 19, 23, 17, 18, 20, 21, 22, 24, 25, 26, 136, 137 and 138, wherein the amino acid sequence is less than 60 in length and whereas the peptide decreases a level of apoptosis in a cell, with the proviso that the amino acid sequence is not the amino acid sequence set forth by TYALDSGVSTMNEMKSYSQA (SEQ ID NO:36), YALDSGVSTMNEMKSYSQAV (SEQ ID NO:37),
 15 ALDSGVSTMNEMKSYSQAVT (SEQ ID NO:38), LDSGVSTMNEMKSYSQAVTG (SEQ ID NO:39), DSGVSTMNEMKSYSQAVTGF (SEQ ID NO:40), SGVSTMNEMKSYSQAVTGFF (SEQ ID NO:41), YPFVLVSNLMAVNNCGLAGG (SEQ ID NO:42), PFVLVSNLMAVNNCGLAGGC (SEQ ID NO:43), FVLVSNLMAVNNCGLAGGCP (SEQ ID NO:44), VLVSNLMAVNNCGLAGGCPP
 20 (SEQ ID NO:45), LVSNLMAVNNCGLAGGCPPY (SEQ ID NO:46), VSNLMAVNNCGLAGGCPPYS (SEQ ID NO:47), AVNNCGLAGGCPPYSPIYTS (SEQ ID NO:48), VNNCGLAGGCPPYSPIYTWS (SEQ ID NO:49), NNCGLAGGCPPYSPIYTWSI (SEQ ID NO:50); SWIDCWCMLQKEGNMSRGNS (SEQ ID NO:51), WIDCWCMLQKEGNMSRGNSL (SEQ ID NO:52),
 25 IDCWCMLQKEGNMSRGNSLF (SEQ ID NO:53), DCWCMLQKEGNMSRGNSLFF (SEQ ID NO:54), CWCMLQKEGNMSRGNSLFFR (SEQ ID NO:55), WCMLQKEGNMSRGNSLFFRK (SEQ ID NO:56), CMLQKEGNMSRGNSLFFRKV (SEQ ID NO:57), PPYSPIYTWSIDCWCMLQKE (SEQ ID NO:58), PYSPIYTWSIDCWCMLQKEG (SEQ ID NO:59), YSPIYTWSIDCWCMLQKEGN
 30 (SEQ ID NO:60), SPIYTWSIDCWCMLQKEGNM (SEQ ID NO:61), PIYTWSIDCWCMLQKEGNMS (SEQ ID NO:62), IYTWSIDCWCMLQKEGNMSR (SEQ ID NO:63), YTWSIDCWCMLQKEGNMSRG (SEQ ID NO:64),

TSWIDCWCMLQKEGNMSRGN (SEQ ID NO:65), GNSLFFRKVPFGKTYCCDLK (SEQ ID NO:66), NSLFFRKVPFGKTYCCDLKM (SEQ ID NO:67), SLFFRKVPFGKTYCCDLKML (SEQ ID NO:68) or LFFRKVPFGKTYCCDLKMLI (SEQ ID NO:69).

5 According to some embodiments of the invention, the isolated peptide consists of the amino acids sequence set forth by SEQ ID NO:106, 107, 16, 19, 23, 17, 18, 20, 21, 22, 24, 25, 26, 136, 137 or 138.

According to some embodiments of the invention, the isolated peptide which comprises the amino acid sequence selected from the group consisting of SEQ ID
10 NOs:106, 107, 16, 19, 23, 17, 18, 20, 21, 22, 24, 25, 26, 136, 137 and 138 is capable of decreasing the level of apoptosis in a cell.

The term "isolated" refers to at least partially separated from the natural environment e.g., the human body.

According to some embodiments the term "isolated" refers to a soluble
15 molecule.

The term "peptide" as used herein encompasses native peptides (either degradation products, synthetically synthesized peptides or recombinant peptides) and peptidomimetics (typically, synthetically synthesized peptides), as well as peptoids and semipeptoids which are peptide analogs, which may have, for example, modifications
20 rendering the peptides more stable while in a body or more capable of penetrating into cells. Such modifications include, but are not limited to N terminus modification, C terminus modification, peptide bond modification, including, but not limited to, CH₂-NH, CH₂-S, CH₂-S=O, O=C-NH, CH₂-O, CH₂-CH₂, S=C-NH, CH=CH or CF=CH, backbone modifications, and residue modification. Methods for preparing
25 peptidomimetic compounds are well known in the art and are specified, for example, in Quantitative Drug Design, C.A. Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992), which is incorporated by reference as if fully set forth herein. Further details in this respect are provided hereinunder.

As used herein the term "mimetics" refers to molecular structures, which serve
30 as substitutes for the peptide of the invention in performing the biological activity (Morgan et al. (1989) Ann. Reports Med. Chem. 24:243-252 for a review of peptide mimetics) such as peptoids and oligopeptoids, which are peptides or oligomers of N-

substituted amino acids [Simon et al. (1972) Proc. Natl. Acad. Sci. USA 89:9367-9371]. Peptide mimetics may or may not contain amino acids and/or peptide bonds, but retain the structural and functional features of the peptide. Further included as peptide mimetics are peptide libraries, which are collections of peptides designed to be of a given amino acid length and representing all conceivable sequences of amino acids corresponding thereto. Methods of producing peptide mimetics are described hereinbelow.

Peptide bonds (-CO-NH-) within the peptide may be substituted, for example, by N-methylated bonds (-N(CH₃)-CO-), ester bonds (-C(R)H-C-O-O-C(R)-N-), ketomethylen bonds (-CO-CH₂-), α -aza bonds (-NH-N(R)-CO-), wherein R is any alkyl, e.g., methyl, carba bonds (-CH₂-NH-), hydroxyethylene bonds (-CH(OH)-CH₂-), thioamide bonds (-CS-NH-), olefinic double bonds (-CH=CH-), retro amide bonds (-NH-CO-), peptide derivatives (-N(R)-CH₂-CO-), wherein R is the "normal" side chain, naturally presented on the carbon atom.

These modifications can occur at any of the bonds along the peptide chain and even at several (2-3) at the same time.

Natural aromatic amino acids, Trp, Tyr and Phe, may be substituted for synthetic non-natural acid such as TIC, naphthylelanine (Nol), ring-methylated derivatives of Phe, halogenated derivatives of Phe or o-methyl-Tyr.

In addition to the above, the peptides of the present invention may also include one or more modified amino acids or one or more non-amino acid monomers (e.g. fatty acids, complex carbohydrates etc).

The term "amino acid" or "amino acids" is understood to include the 20 naturally occurring amino acids; those amino acids often modified post-translationally *in vivo*, including, for example, hydroxyproline, phosphoserine and phosphothreonine; and other unusual amino acids including, but not limited to, 2-amino adipic acid, hydroxylysine, isodesmosine, nor-valine, nor-leucine and ornithine. Furthermore, the term "amino acid" includes both D- and L-amino acids.

Tables 1 and 2 below list naturally occurring amino acids (Table 1) and non-conventional or modified amino acids (e.g., synthetic, Table 2) which can be used with the invention.

Table 1

<i>Amino Acid</i>	<i>Three-Letter Abbreviation</i>	<i>One-letter Symbol</i>
alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
glycine	Gly	G
Histidine	His	H
isoleucine	Ile	I
leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
tryptophan	Trp	W
tyrosine	Tyr	Y
Valine	Val	V
Any amino acid as above	Xaa	X

Table 1.

5

Table 2

<i>Non-conventional amino acid</i>	<i>Code</i>	<i>Non-conventional amino acid</i>	<i>Code</i>
α -aminobutyric acid	Abu	L-N-methylalanine	Nmala
α -amino- α -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
aminocyclopropane-	Cpro	L-N-methylasparagine	Nmasn
carboxylate		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-	Norb	L-N-methylglutamine	Nmgin
carboxylate		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisoleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Das	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg

<i>Non-conventional amino acid</i>	<i>Code</i>	<i>Non-conventional amino acid</i>	<i>Code</i>
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α -methyl-aminoisobutyrate	Maib
D-valine	Dval	α -methyl- γ -aminobutyrate	Mgab
D- α -methylalanine	Dmala	α -methylcyclohexylalanine	Mchexa
D- α -methylarginine	Dmarg	α -methylcyclopentylalanine	Mcpen
D- α -methylasparagine	Dmasn	α -methyl- α -naphthylalanine	Manap
D- α -methylaspartate	Dmasp	α -methylpenicillamine	Mpen
D- α -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D- α -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D- α -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D- α -methylisoleucine	Dmile	N-amino- α -methylbutyrate	Nmaabu
D- α -methyllleucine	Dmleu	α -naphthylalanine	Anap
D- α -methylllysine	Dmlys	N-benzylglycine	Nphe
D- α -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D- α -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D- α -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D- α -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D- α -methylserine	Dmser	N-cyclobutylglycine	Ncbut
D- α -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D- α -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D- α -methyltyrosine	Dmtty	N-cyclodecylglycine	Ncdec
D- α -methylvaline	Dmval	N-cyclododecylglycine	Ncdod
D- α -methylalnine	Dnmala	N-cyclooctylglycine	Ncoct
D- α -methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D- α -methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D- α -methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D- α -methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylleucine	Dnmleu	N-(3-indolyl)ethyl glycine	Nhtrp
D-N-methylllysine	Dnmlys	N-methyl- γ -aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nva
D-N-methyltyrosine	Dnmtyr	N-methyl- α -naphthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ -aminobutyric acid	Gabu	N-(p-hydroxyphenyl)glycine	Nhtyr
L-t-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L- α -methylalanine	Mala
L- α -methylarginine	Marg	L- α -methylasparagine	Masn

<i>Non-conventional amino acid</i>	<i>Code</i>	<i>Non-conventional amino acid</i>	<i>Code</i>
L- α -methylaspartate	Masp	L- α -methyl- <i>t</i> -butylglycine	Mtbug
L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
L- α -methylglutamine	Mgln	L- α -methylglutamate	Mglu
L- α -methylhistidine	Mhis	L- α -methylhomo phenylalanine	Mhphe
L- α -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolethyl)glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl- γ -aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyl- α -naphthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ -aminobutyric acid	Gabu	N-(<i>p</i> -hydroxyphenyl)glycine	Nhtyr
L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L- α -methylalanine	Mala
L- α -methylarginine	Marg	L- α -methylasparagine	Masn
L- α -methylaspartate	Masp	L- α -methyl- <i>t</i> -butylglycine	Mtbug
L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
L- α -methylglutamine	Mgln	L- α -methylglutamate	Mglu
L- α -methylhistidine	Mhis	L- α -methylhomophenylalanine	Mhphe
L- α -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L- α -methylleucine	Mleu	L- α -methyllysine	Mlys
L- α -methylmethionine	Mmet	L- α -methylnorleucine	Mnle
L- α -methylnorvaline	Mnva	L- α -methylornithine	Morn
L- α -methylphenylalanine	Mphe	L- α -methylproline	Mpro
L- α -methylserine	mser	L- α -methylthreonine	Mthr
L- α -methylvaline	Mtrp	L- α -methyltyrosine	Mtyr
L- α -methylleucine	Mval Nnbhm	L-N-methylhomophenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbamylmethyl-glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl(1)glycine	Nnbhe
1-carboxy-1-(2,2-diphenylethylamino)cyclopropane	Nmbc		

Table 2.

The peptides of the invention can be utilized in a linear or a cyclic form.

According to some embodiments of the invention, the peptide is a cyclic

Peptides with a cyclic backbone have been described in the art for increasing their ability to penetrate a cell-of-interest (see e.g., Hariton-Gazal E, et al., 2005).

Since the present peptides are preferably utilized in therapeutics or diagnostics which require the peptides to be in soluble form, the peptides of the invention may include one or more non-natural or natural polar amino acids, including but not limited to serine and threonine which are capable of increasing peptide solubility due to their hydroxyl-containing side chain.

The peptides of the invention may be synthesized by any techniques that are known to those skilled in the art of peptide synthesis. For solid phase peptide synthesis, a summary of the many techniques may be found in J. M. Stewart and J. D. Young, Solid Phase Peptide Synthesis, W. H. Freeman Co. (San Francisco), 1963 and J. Meienhofer, Hormonal Proteins and Peptides, vol. 2, p. 46, Academic Press (New York), 1973. For classical solution synthesis see G. Schroder and K. Lupke, The Peptides, vol. 1, Academic Press (New York), 1965.

These methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation and classical solution synthesis. These methods are preferably used when the peptide is relatively short (i.e., 10 kDa) and/or when it cannot be produced by recombinant techniques (i.e., not encoded by a nucleic acid sequence) and therefore involve different chemistry.

Solid phase peptide synthesis procedures are well known in the art and further described by John Morrow Stewart and Janis Dillaha Young, Solid Phase Peptide Syntheses (2nd Ed., Pierce Chemical Company, 1984).

Synthetic peptides can be purified by preparative high performance liquid chromatography [Creighton T. (1983) Proteins, structures and molecular principles. WH Freeman and Co. N.Y.] and the composition of which can be confirmed via amino acid sequencing.

In general, these methods comprise the sequential addition of one or more amino acids or suitably protected amino acids to a growing peptide chain. Normally, either the amino or carboxyl group of the first amino acid is protected by a suitable protecting group. The protected or derivatized amino acid can then either be attached to an inert solid support or utilized in solution by adding the next amino acid in the sequence having the complimentary (amino or carboxyl) group suitably protected, under

conditions suitable for forming the amide linkage. The protecting group is then removed from this newly added amino acid residue and the next amino acid (suitably protected) is then added, and so forth. After all the desired amino acids have been linked in the proper sequence, any remaining protecting groups (and any solid support) are removed
5 sequentially or concurrently, to afford the final peptide compound. By simple modification of this general procedure, it is possible to add more than one amino acid at a time to a growing chain, for example, by coupling (under conditions which do not racemize chiral centers) a protected tripeptide with a properly protected dipeptide to form, after deprotection, a pentapeptide and so forth. Further description of peptide
10 synthesis is disclosed in U.S. Pat. No. 6,472,505.

A preferred method of preparing the peptide compounds of the invention involves solid phase peptide synthesis. Large scale peptide synthesis is described by Andersson *Biopolymers* 2000;55(3):227-50.

Combinatorial chemical, antibody or peptide libraries may be used to screen a
15 plurality of peptides or mimetics thereof.

It will be appreciated that when utilized along with automated equipment, the above-described method can be used to screen multiple agents both rapidly and easily.

Large peptides (e.g., above 25 amino acids) can be generated using recombinant DNA techniques. For example, to generate the isolated peptide of the invention, an
20 isolated polynucleotide sequence encoding the amino acid sequence of the isolated peptide of the invention (e.g., SEQ ID NOs:106, 107, 16, 19, 23, 17, 18, 20, 21, 22, 24, 25, 26, 136, 137 or 138) is preferably ligated into a nucleic acid construct suitable for expression in a host cell. Such a nucleic acid construct includes a promoter sequence for directing transcription of the polynucleotide sequence in the cell in a constitutive or
25 inducible manner

The nucleic acid construct of the invention may also include an enhancer, a transcription and translation initiation sequence, transcription and translation terminator and a polyadenylation signal, a 5' LTR, a tRNA binding site, a packaging signal, an origin of second-strand DNA synthesis, and a 3' LTR or a portion thereof; a signal
30 sequence for secretion of the peptide from a host cell; additional polynucleotide sequences that allow, for example, the translation of several proteins from a single

mRNA such as an internal ribosome entry site (IRES) and sequences for genomic integration of the promoter-chimeric polypeptide;

Examples for mammalian expression vectors include, but are not limited to, pcDNA3, pcDNA3.1(+/-), pGL3, pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pSinRep5, DH26S, DHBB, pNMT1, pNMT41, pNMT81, which are available from Invitrogen, pCI which is available from Promega, pMbac, pPbac, pBK-RSV and pBK-CMV which are available from Strategene, pTRES which is available from Clontech, and their derivatives.

Expression vectors containing regulatory elements from eukaryotic viruses such as retroviruses can be also used. SV40 vectors include pSVT7 and pMT2. Vectors derived from bovine papilloma virus include pBV-1MTHA, and vectors derived from Epstein Bar virus include pHEBO, and p2O5. Other exemplary vectors include pMSG, pAV009/A⁺, pMTO10/A⁺, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV-40 early promoter, SV-40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. Thus, the type of vector used by the present invention will depend on the cell type transformed. The ability to select suitable vectors according to the cell type transformed is well within the capabilities of the ordinary skilled artisan and as such no general description of selection consideration is provided herein. For example, bone marrow cells can be targeted using the human T cell leukemia virus type I (HTLV-I) and kidney cells may be targeted using the heterologous promoter present in the baculovirus *Autographa californica* nucleopolyhedrovirus (AcMNPV) as described in Liang CY et al., 2004 (Arch Virol. 149: 51-60).

Recombinant viral vectors are useful for *in vivo* expression since they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single

infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

Various methods can be used to introduce the expression vector of the present invention into stem cells. Such methods are generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al., Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et al. [Biotechniques 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

Introduction of nucleic acids by viral infection offers several advantages over other methods such as lipofection and electroporation, since higher transfection efficiency can be obtained due to the infectious nature of viruses.

Currently preferred *in vivo* nucleic acid transfer techniques include transfection with viral or non-viral constructs, such as adenovirus, lentivirus, Herpes simplex I virus, or adeno-associated virus (AAV) and lipid-based systems. Useful lipids for lipid-mediated transfer of the gene are, for example, DOTMA, DOPE, and DC-Chol [Tonkinson et al., Cancer Investigation, 14(1): 54-65 (1996)]. The most preferred constructs for use in gene therapy are viruses, most preferably adenoviruses, AAV, lentiviruses, or retroviruses. A viral construct such as a retroviral construct includes at least one transcriptional promoter/enhancer or locus-defining element(s), or other elements that control gene expression by other means such as alternate splicing, nuclear RNA export, or post-translational modification of messenger. Such vector constructs also include a packaging signal, long terminal repeats (LTRs) or portions thereof, and

positive and negative strand primer binding sites appropriate to the virus used, unless it is already present in the viral construct. In addition, such a construct typically includes a signal sequence for secretion of the peptide from a host cell in which it is placed. Preferably the signal sequence for this purpose is a mammalian signal sequence or the signal sequence of the polypeptide variants of the present invention. Optionally, the construct may also include a signal that directs polyadenylation, as well as one or more restriction sites and a translation termination sequence. By way of example, such constructs will typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second-strand DNA synthesis, and a 3' LTR or a portion thereof. Other vectors can be used that are non-viral, such as cationic lipids, polylysine, and dendrimers.

Other than containing the necessary elements for the transcription and translation of the inserted coding sequence, the expression construct of the present invention can also include sequences engineered to enhance stability, production, purification, yield or toxicity of the expressed peptide. For example, the expression of a fusion protein or a cleavable fusion protein comprising the protein of the invention (the gene product of the polynucleotide-of-interest) and a heterologous protein can be engineered. Such a fusion protein can be designed so that the fusion protein can be readily isolated by affinity chromatography; e.g., by immobilization on a column specific for the heterologous protein. Where a cleavage site is engineered between the protein of the invention and the heterologous protein, the protein of the invention can be released from the chromatographic column by treatment with an appropriate enzyme or agent that disrupts the cleavage site [e.g., see Booth et al. (1988) *Immunol. Lett.* 19:65-70; and Gardella et al., (1990) *J. Biol. Chem.* 265:15854-15859].

As mentioned hereinabove, a variety of prokaryotic or eukaryotic cells can be used as host-expression systems to express the polypeptides of the present invention. These include, but are not limited to, microorganisms, such as bacteria transformed with a recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vector containing the coding sequence; yeast transformed with recombinant yeast expression vectors containing the coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors, such as Ti plasmid,

containing the coding sequence. Mammalian expression systems can also be used to express the polypeptides of the present invention.

Recovery of the recombinant polypeptide is effected following an appropriate time in culture. The phrase "recovering the recombinant polypeptide" refers to
5 collecting the whole fermentation medium containing the polypeptide and need not imply additional steps of separation or purification. Notwithstanding the above, polypeptides of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction
10 chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

The peptide of the invention may be further conjugated to an amino acid sequence, which facilitates penetration of the peptide into a cell or further into a sub-cellular organelle such as the nucleus, nucleoli, mitochondria and the like. For example,
15 the peptide can be conjugated to a known sequence such as the P18 (LSTAADMQGVVTDGMASG; SEQ ID NO:70) or P28 (LSTAADMQGVVTDGMASGLDKDYLPDD; SEQ ID NO:71) azurin-derived peptides (Taylor BN, et al., Cancer Res. 69:537-546, 2009); the peptide can be conjugated to the cationic cell-penetrating peptides Tat [CRKKRRQRRR (SEQ ID
20 NO:72)], oligoarginine [r9; CRRRRRRRRR (SEQ ID NO:73)] or oligolysine [k9; CKKKKKKKKK (SEQ ID NO:74)] described in Patel LN, et al., Mol. Pharm. 2009, Feb 19, Epub ahead of print); the peptide can be conjugated to the nuclear localization signal (NLS) [KKKRKV (SEQ ID NO:75)] or to the basic TAT peptide [GRKKRRQRRR (SEQ ID NO:76)] described in Peitz, M., et al., 2002, "Ability of the
25 hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes". Proc Natl Acad Sci U S A 99, 4489-4494; the peptide can be conjugated to Penetratin™ 1 Peptide [QBiogene Molecular Biology; e.g., Activated Penetratin 1 Peptide (Cat. No. PENA0500; Biotinylated Activated Penetratin 1 Peptide (Cat. No. PENB0500)].

30 Conjugation of a cell-penetrating amino acid sequence to the isolated peptide of the invention can be performed using methods known in the art. For example, the cell-penetrating amino acid sequence can be conjugated via, for example, a disulfide bridge

to a d-isofom cysteine (c) present at the N-terminal of the isolated peptide of the invention.

Additionally or alternatively, the cell-penetrating amino acid sequence can be recombinantly synthesized along with the peptide of the invention from a nucleic acid construct encoding both sequences.

The peptide of the invention may be also conjugated to a non-proteinaceous moiety, which increases the stability (e.g., against protease activities) and/or solubility (e.g., within a biological fluid such as blood, digestive fluid) of the peptide while preserving the biological activity and prolonging the half-life of the peptide.

The non-proteinaceous moiety can be a polymer or a co-polymer (synthetic or natural) such as polyethylene glycol (PEG), Polyvinyl pyrrolidone (PVP), divinyl ether and maleic anhydride copolymer (DIVEMA; see for example, Kaneda Y, et al., 1997, Biochem. Biophys. Res. Commun. 239: 160-5) and poly(styrene comaleic anhydride) (SMA; see for example, Mu Y, et al., 1999, Biochem Biophys Res Commun. 255: 75-9).

Bioconjugation is advantageous particularly in cases of therapeutically useful proteins which exhibit short half-life and rapid clearance from the blood. The increased half-lives of bioconjugated proteins in the plasma results from increased size of protein conjugates (which limits their glomerular filtration) and decreased proteolysis due to polymer steric hindrance. Generally, the more polymer chains attached per peptide, the greater the extension of half-life. However, measures are taken not to reduce the specific activity of the isolated peptide of the invention.

Bioconjugation with PEG (*i.e.*, PEGylation) can be performed using PEG derivatives such as N-hydroxysuccinimide (NHS) esters of PEG carboxylic acids, monomethoxyPEG₂-NHS, succinimidyl ester of carboxymethylated PEG (SCM-PEG), benzotriazole carbonate derivatives of PEG, glycidyl ethers of PEG, PEG p-nitrophenyl carbonates (PEG-NPC, such as methoxy PEG-NPC), PEG aldehydes, PEG-orthopyridyl-disulfide, carbonyldimidazol-activated PEGs, PEG-thiol, PEG-maleimide. Such PEG derivatives are commercially available at various molecular weights [See, e.g., Catalog, Polyethylene Glycol and Derivatives, 2000 (Shearwater Polymers, Inc., Huntsville, Ala.)]. If desired, many of the above derivatives are available in a monofunctional monomethoxyPEG (mPEG) form. In general, the PEG added to the

amino acid sequence of the peptide of the invention should range from a molecular weight (MW) of several hundred Daltons to about 100 kDa (e.g., between 3-30 kDa). Larger MW PEG may be used, but may result in some loss of yield of PEGylated peptides. The purity of larger PEG molecules should be also watched, as it may be difficult to obtain larger MW PEG of purity as high as that obtainable for lower MW PEG. It is preferable to use PEG of at least 85 % purity, and more preferably of at least 90 % purity, 95 % purity, or higher. PEGylation of molecules is further discussed in, e.g., Hermanson, *Bioconjugate Techniques*, Academic Press San Diego, Calif. (1996), at Chapter 15 and in Zalipsky et al., "Succinimidyl Carbonates of Polyethylene Glycol," in Dunn and Ottenbrite, eds., *Polymeric Drugs and Drug Delivery Systems*, American Chemical Society, Washington, D.C. (1991).

Conveniently, PEG can be attached to a chosen position in the amino acid sequence by site-specific mutagenesis as long as the activity of the conjugate is retained (e.g., decrease of apoptosis). For example, a Cysteine residue can be a target for PEGylation. Computational analysis may be effected to select a preferred position for mutagenesis without compromising the activity.

Various conjugation chemistry of activated PEG such as PEG-maleimide, PEG-vinylsulfone (VS), PEG-acrylate (AC), PEG-orthopyridyl disulfide can be employed. Methods of preparing activated PEG molecules are known in the arts. For example, PEG-VS can be prepared under argon by reacting a dichloromethane (DCM) solution of the PEG-OH with NaH and then with di-vinylsulfone (molar ratios: OH 1: NaH 5: divinyl sulfone 50, at 0.2 gram PEG/mL DCM). PEG-AC is made under argon by reacting a DCM solution of the PEG-OH with acryloyl chloride and triethylamine (molar ratios: OH 1: acryloyl chloride 1.5: triethylamine 2, at 0.2 gram PEG/mL DCM). Such chemical groups can be attached to linearized, 2-arm, 4-arm, or 8-arm PEG molecules.

While conjugation to cysteine residues is one convenient method by which the peptide of the invention can be PEGylated, other residues can also be used if desired. For example, acetic anhydride can be used to react with NH₂ and SH groups, but not COOH, S-S, or -SCH₃ groups, while hydrogen peroxide can be used to react with --SH and --SCH₃ groups, but not NH₂. Reactions can be conducted under conditions

appropriate for conjugation to a desired residue in the peptide employing chemistries exploiting well-established reactivities.

For bioconjugation of the peptide of the invention with PVP, the terminal COOH-bearing PVP is synthesized from N-vinyl-2-pyrrolidone by radical polymerization in dimethyl formamide with the aid of 4,4'-azobis-(4-cyanovaleric acid) as a radical initiator, and 3-mercaptopropionic acid as a chain transfer agent. Resultant PVPs with an average molecular weight of Mr 6,000 can be separated and purified by high-performance liquid chromatography and the terminal COOH group of synthetic PVP is activated by the N-hydroxysuccinimide/dicyclohexyl carbodiimide method. The isolated peptide of the invention is reacted with a 60-fold molar excess of activated PVP and the reaction is stopped with amino caploic acid (5-fold molar excess against activated PVP), essentially as described in Haruhiko Kamada, et al., 2000, Cancer Research 60: 6416-6420, which is fully incorporated herein by reference.

Resultant conjugated peptide molecules (e.g., PEGylated or PVP-conjugated CCR2) are separated, purified and qualified using e.g., high-performance liquid chromatography (HPLC). In addition, purified conjugated molecules of this aspect of the invention may be further qualified using e.g., *in vitro* assays in which the level of apoptosis in a cell is tested (as is further described hereinbelow) in the presence or absence of the peptide-conjugates of the invention.

As mentioned above and further described in Examples 9-11 (Tables 6, 7, 8 and 9) of the Examples section which follows, the present inventors have uncovered BID-derived peptides which are capable of binding MTCH2/MIMP.

According to some embodiments of the invention, the BID derived peptide comprises the amino acid sequence set forth by SEQ ID NO:111, 30, 31, 32, 115, 114, 112, 113, 108, 109, 110, 116 or 117, wherein the amino acid sequence is less than 60 in length and whereas the peptide increases a level of apoptosis in a cell.

According to some embodiments of the invention the isolated peptide is set forth by SEQ ID NOs:111, 30, 31, 32, 115, 114, 112, 113, 108, 109, 110, 116 or 117.

As used herein the phrase "increase of apoptosis" refers to an increase in the rate of apoptosis in a cell(s) and/or an increase in the number of cells undergoing apoptosis in a tissue or subject.

As used herein the term "apoptosis" refers to a programmed cell death machinery whereby the cell executes a "cell suicide" program. Apoptosis plays a crucial role in ensuring the normal development and maintenance of cells, organs, and tissues and involves in a number of physiological events such as embryogenesis, regulation of the immune system, and homeostasis. Thus, apoptosis can be in response to diverse signals such as stimulation by growth factors (e.g., TNF α and Fas), limb and neural development, neurodegenerative diseases, radiotherapy and chemotherapy as well as environmental conditions. Apoptotic processes are usually characterized by uncoupling of mitochondrial oxidation, decreased levels of nicotinamide adenine dinucleotide phosphate [NAD(P)H], release of cytochrome c, activation of caspases, DNA fragmentation and externalization of phosphatidylserine (a membrane phospholipid normally restricted to the inner leaflet of the lipid bilayer) to the outer leaflet of the plasma membrane (described in length in the preceding background section).

Thus, according to an aspect of some embodiments of the invention, there is provided a method of upregulating apoptosis in a cell, comprising contacting the cell with the isolated peptide, isolated molecule and/or the isolated polynucleotide or nucleic acid construct encoding same of the invention, thereby upregulating the apoptosis in the cell.

The cell can be any cell such as an embryonic or adult cell, a stem cell, a progenitor cell, a fetal or adult blood cell, a bone marrow cell, a neuronal cell, a cardiac cell, a bone cell, a muscle cell, and the like.

It should be noted that contacting the cells with the peptide can be performed under *in vitro* or *in vivo* conditions. For example, under *in vitro* conditions, the cells are contacted with the peptide e.g., by adding the peptide to cells derived from a subject (e.g., a primary cell culture, a cell line) or to a biological sample comprising same (e.g., a fluid, liquid which comprises the cells) such that the peptide is in direct contact with the cells. According to some embodiments of the invention, the cells of the subject are incubated with the peptide. The conditions used for incubating the cells are selected for a time period/concentration of cells/concentration of peptide/ratio between cells and peptide and the like which enable the peptide to induce cellular changes, such as reduction in the rate of apoptosis.

The effect of the isolated peptides or molecules comprising same of the invention on apoptosis can be determined, for example, using functional assays, such the Ethidium homodimer-1 staining (Invitrogen-Molecular Probes), the Tunnel assay (Roche, Basel, Switzerland), the Live/dead viability/cytotoxicity two-color fluorescence
5 assay (Molecular Probes, Inc., L-3224, Eugene, OR, USA), FACS analysis [using molecules capable of specifically binding cells undergoing apoptosis, such as propidium iodide and Annexin V], and those of skills in the art are capable of assessing such levels in order to determine the standards of normal levels.

It should be noted that the agents of the invention which increase the level of
10 apoptosis in a cell can be used to treat a pathology associated with abnormally low levels of apoptosis in a subject, by administering to the subject a therapeutically effective amount of the isolated peptide (e.g., SEQ ID NO:111, 30, 31, 32, 115, 114, 112, 113, 108, 109, 110, 116 or 117), the isolated molecule comprising same and/or the isolated polynucleotide or the nucleic acid construct encoding same.

15 The term "treating" as used herein refers to inhibiting, preventing or arresting the development of a pathology (disease, disorder or condition) and/or causing the reduction, remission, or regression of a pathology. Those of skill in the art will understand that various methodologies and assays can be used to assess the development of a pathology, and similarly, various methodologies and assays may be
20 used to assess the reduction, remission or regression of a pathology.

As used herein, the term "subject" (or "individual" which is interchangeably used herein) refers to an animal subject e.g., a mammal, e.g., a human being at any age who suffers from or is at risk of developing the pathology.

As used herein the term "pathology" refers to any deviation from the normal
25 structure and/or function of a particular cell, cell type, group of cells, tissue or organ leading to a disease, a disorder, a syndrome or an abnormal condition.

As used herein, the phrase "abnormally low levels of apoptosis" relates to any pathology which is caused by, characterized by or associated with a rate and/or level of apoptosis which is below (*i.e.*, abnormally low) the level present in normal or
30 unaffected cells of the same type or developmental stage.

Normal or unaffected cells can be obtained from a subject who is devoid of the pathology, e.g., from a subject who does not suffer from the pathology or its symptoms, and/or is not predisposed to have the pathology.

Pathologies which are caused by, characterized by or associated with abnormally low levels of apoptosis and which can be treated using the agents of the present invention include, but are not limited to, cancer, autoimmune disorders associated with low level of apoptosis of auto-reactive lymphocytes, bacterial infection associated with downregulation of apoptosis in host cell [e.g., bacterial infections caused by *Chlamydia* sp., *Neisseria* sp., *Salmonella enterica*, *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, *Rickettsia rickettsii*, *Wolbachia* Neutrophils, *Bartonella* sp., *Helicobacter pylori*, *Porphyromonas gingivalis*, *Listeria monocytogenes*, *Shigella flexneri*, *Legionella pneumophila*, *Mycoplasma fermentans*, *Brucella suis*, *Escherichia coli* K1, and *Coxiella burnetii* (for further details see Faherty CS and Maurelli AT, Trends in Microbiology 16: 173-180)] and viral infections associated with downregulation of apoptosis [e.g., those caused by grouper iridovirus (GIV; family of Iridoviridae) which blocks apoptosis in host cells such as grouper kidney (GK) cells (Lin PW, Huang YJ, et al., 2008, Apoptosis, 13:165-76).

As mentioned, the peptide according to some embodiments of the invention decreases a level of apoptosis in a cell.

Thus, according to an aspect of some embodiments of the invention, there is provided a method of downregulating apoptosis in a cell, the method is effected by contacting the cell with the isolated peptide, the isolated molecule comprising same and/or the isolated polynucleotide or nucleic acid construct encoding same of the invention, thereby downregulating the apoptosis in the cell.

According to some embodiments of the invention, the peptide which is capable of decreasing the level of apoptosis in cells is selected from the group consisting of SEQ ID NOS:106, 107, 16, 19, 23, 17, 18, 20, 21, 22, 24, 25 and 26.

It should be noted that the human derived peptide MTCH2/MIMP 240-290 (SEQ ID NO: 136), the mouse derived peptide MTCH2 240-292 (SEQ ID NO:137) and/or the human derived peptide MTCH2/MIMP 2402-92 (SEQ ID NO:138) can be also used to reduce the level of apoptosis in a cell.

As used herein the phrase “decrease of apoptosis” refers to a decrease in the rate of apoptosis in a cell(s) and/or a decrease in the number of cells undergoing apoptosis in a tissue or subject.

The teachings of the invention can be used to treat a pathology caused by, associated with or characterized by abnormally high levels of apoptosis in a subject.

As used herein, the phrase “abnormally high levels of apoptosis” relates to any pathology which is caused by, characterized by or associated with a rate and/or level of apoptosis which is above (*i.e.*, abnormally high) the level present in normal or unaffected cells of the same type or developmental stage.

Pathologies which are caused by, associated with or characterized with abnormally high levels of apoptosis and which can be treated using the isolated peptide, the isolated molecule and/or the isolated polynucleotide or nucleic acid construct encoding same of the invention include, but are not limited to, degenerative disorders such as neurological disorders [e.g., a neurodegenerative disorder such as Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis (ALS) and retinitis pigmentosa], atherosclerosis (Mercer J., et al., 2007, Mutation Research 621: 75-86), or pathologies associated with viral infections such as central nervous system (CNS) diseases [e.g., human immunodeficiency virus (HIV)-induced associated dementia (Li, W., Galey D et al., 2005, Neurotox res. 8:119-134), herpes simplex virus -induced encephalitis (Perkins D., Gyure K., et al., 2003, J. Neurovirol. 9:101-111), cytomegalovirus-induced encephalitis (DeBiasi R.L., et al., 2002, J. Infect. Dis. 186: 1547-1557)], heart diseases [e.g., active and chronic myocarditis (Alter P., et al., 2001, Cardiovasc. Patholog. 10:229-234)], liver diseases [e.g., hepatitis B or C virus associated liver injury (Bantel H., et al., 2003, Cell Death Differ. 10 (suppl. 1): 48-58; Patel T., et al., 1998, Semin. Liver Dis. 18: 105-114)], foot and mouth disease virus which induces apoptosis of immature dendritic cells (Jin H, Xiao C, et al., 2007, J Cell Biochem. 102:980-91) and stroke [e.g., stroke-induced apoptosis in cerebral ischemia (Tagami M, et al., 1999, Lab Invest. 79:609-15)].

The isolated peptide, isolated molecule comprising same and/or isolated polynucleotide or nucleic acid construct encoding same of the invention can be administered to the subject per se, or in a pharmaceutical composition where it is mixed with suitable carriers or excipients.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

5 Herein the term "active ingredient" refers to the isolated peptide, isolated molecule comprising same and/or the isolated polynucleotide or the nucleic acid construct encoding same of the invention accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intracardiac, e.g., into the right or left ventricular cavity, into the common coronary artery, intravenous, intraperitoneal, intranasal, or intraocular injections.

Conventional approaches for drug delivery to the central nervous system (CNS) include: neurosurgical strategies (e.g., intracerebral injection or intracerebroventricular infusion); molecular manipulation of the agent [e.g., production of a chimeric fusion protein that comprises a transport peptide that has an affinity for an endothelial cell surface molecule in combination with an agent that is itself incapable of crossing the blood brain barrier (BBB)] in an attempt to exploit one of the endogenous transport

pathways of the BBB; pharmacological strategies designed to increase the lipid solubility of an agent (e.g., conjugation of water-soluble agents to lipid or cholesterol carriers); and the transitory disruption of the integrity of the BBB by hyperosmotic disruption (resulting from the infusion of a mannitol solution into the carotid artery or the use of a biologically active agent such as an angiotensin peptide). However, each of these strategies has limitations, such as the inherent risks associated with an invasive surgical procedure, a size limitation imposed by a limitation inherent in the endogenous transport systems, potentially undesirable biological side effects associated with the systemic administration of a chimeric molecule comprised of a carrier motif that could be active outside of the CNS, and the possible risk of brain damage within regions of the brain where the BBB is disrupted, which renders it a suboptimal delivery method.

Alternately, one may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a tissue region of a patient.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions,

and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g.,

gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose
5 containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous
10 solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of
15 the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with
20 a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The pharmaceutical composition of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of the present invention
25 include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients (e.g., the isolated peptide, the isolated molecule comprising same and/or the isolated polynucleotide or the nucleic acid construct encoding same) effective to prevent, alleviate or ameliorate symptoms of a
30 disorder (e.g., a neurodegenerative disease or cancer) or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from *in vitro* and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide levels of the active ingredient are sufficient to induce or suppress the biological effect (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from *in vitro* data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

For example, in order to treat degenerated neuronal cells associated with abnormally high level of apoptosis (as in the case, for example, of Alzheimer's disease) the isolated peptide and/or the isolated molecule comprising same can be administered

to the individual [e.g., systemically (e.g., intravenous, intramuscularly) or locally to the target tissue or organ, e.g., brain, using for example an implanted pump].

Additionally or alternatively, for treating a neurodegenerative disease the isolated polynucleotide or the nucleic acid construct encoding the peptide of the invention can be targeted to the brain using liposomal and viral vectors as described in de Lima MC., et al., 2005, *Curr Drug Targets CNS Neurol Disord.* 4(4):453-65, which is fully incorporated herein by reference, and/or using a neuron-specific promoter such as the neurofilament promoter [Byrne et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477].

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

The isolated peptides or molecules comprising same of the invention can be qualified for their ability to upregulate or downregulate apoptosis as needed. This can be done, for example, using functional assays, such as by monitoring the effect of the peptide/molecule of the invention on apoptosis in cells. The level of apoptosis in cells and tissues can be determined using various methods such as the Ethidium homodimer-1 staining (Invitrogen-Molecular Probes), the Tunnel assay (Roche, Basel, Switzerland), the Live/dead viability/cytotoxicity two-color fluorescence assay (Molecular Probes, Inc., L-3224, Eugene, OR, USA), FACS analysis [using molecules capable of specifically binding cells undergoing apoptosis, such as propidium iodide and Annexin

V], and those of skills in the art are capable of assessing such levels in order to determine the standards of normal levels.

The teachings of the invention can be used to screen for peptides which can upregulate or downregulate apoptosis. For example, peptides can be qualified for their ability to increase or decrease the binding of MTCH2 with BID or the binding of MTCH2 with tBID.

The peptides described hereinabove can be used to generate antibodies which bind the MTCH2/MIMP or the BID polypeptides in a biological sample of a subject. Such antibodies can be used for both diagnostic and therapeutic methods.

The biological sample can be any sample which contains proteins of the subject. For example, the biological sample can include cells or cell content such as a body fluid [e.g., whole blood, white blood cells, peripheral blood mononuclear cells (PBMCs), serum, plasma, cerebrospinal fluid, urine, lymph fluids, and various external secretions of the respiratory, intestinal and genitourinary tracts, tears, saliva or milk] or a tissue biopsy. It should be noted that a "biological sample of the subject" may also optionally comprise a sample that has not been physically removed from the subject (e.g., *in vivo* detection).

The term "antibody" as used in this invention includes intact molecules as well as functional fragments thereof, such as Fab, F(ab')₂, and Fv that are capable of binding to macrophages. These functional antibody fragments are defined as follows: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) (Fab')₂, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')₂ is a dimer of two Fab' fragments held together by two disulfide bonds; (4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (5) Single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy

chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

For example, to generate an antibody, the isolated peptide of the invention can be conjugated to an immunogenic moiety such as keyhole limpet haemocyanin (KLH) (Imject Maleimide-activated mCKLH from Pierce, according to the manufacturer's protocol) and be further subcutaneously injected in several places on the back of an experimental animal (e.g., rabbit). Conventionally, following a predetermined period, such as two weeks, a booster prepared with incomplete Freund's adjuvant (Sigma) is injected. Following 2-3 weeks, blood is collected from the animal, red blood cells are clotted and removed and the serum is centrifuged (e.g., for 30 minutes at 1500 g). The resulting supernatant contains the antibodies.

Methods of producing polyclonal and monoclonal antibodies as well as fragments thereof are well known in the art (See for example, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

Antibody fragments according to the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R. [*Biochem. J.* 73: 119-126 (1959)]. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be

used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Fv fragments comprise an association of VH and VL chains. This association may be noncovalent, as described in Inbar et al. [Proc. Nat'l Acad. Sci. USA 69:2659-62 (1972)]. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by [Whitlow and Filpula, *Methods* 2: 97-105 (1991); Bird et al., *Science* 242:423-426 (1988); Pack et al., *Bio/Technology* 11:1271-77 (1993); and U.S. Pat. No. 4,946,778, which is hereby incorporated by reference in its entirety.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry [*Methods*, 2: 106-10 (1991)].

Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab').sub.2 or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in

the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature* 332:323-327 (1988); Verhoeven et al., *Science*, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985) and Boerner et al., *J. Immunol.*, 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introduction of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely

resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., *Bio/Technology* 10,: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368 812-13 (1994); Fishwild et al., *Nature Biotechnology* 14, 845-51 (1996); Neuberger, *Nature Biotechnology* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.* 13, 65-93 (1995).

As used herein the term "about" refers to $\pm 10\%$.

10 The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of" means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

20 Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

30 Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges

from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John

Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

25

GENERAL MATERIALS AND EXPERIMENTAL METHODS

Generation of MTCH2/MIMP knockout mice - Designing the targeting vector:

The targeting vector was designed to delete 2.4 Kb, encompassing exons 1-3 of MTCH2/MIMP [GenBank Accession No. AF176009 (SEQ ID NO:34; gi:5815346); encoding GenBank Accession No. AAD52647; gi:5815347]. The targeting vector was constructed using PCR on ES genomic DNA (Galli-Taliadoros, L. A., J. D. Sedgwick, S. A. Wood, and H. Korner. 1995. Gene knock-out technology: a methodological

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overview for the interested novice. J Immunol Methods 181:1-15). First the short homology (SH), which contains 2.1 Kb upstream to exon-1 was ligated into the XhoI site in the pRapidflirt vector. The forward primer contained both the cloning XhoI site and an AflII site that was later used to linearize the targeting vector. The primers used for the SH-PCR reaction were:

5'-TAGCTCGAGCTCTTAAGAGAAACCCTGCCCAACAACCACATGAAGG-3'

(SEQ ID NO:77; forward primer) and

5'-GACGGTCGACCAGACGGAGTCACCAAGCGACACAGC-3' (SEQ ID NO:78;

reverse primer) which contains a SalI site. Next, a polylinker made of a double stranded

oligonucleotide containing an NheI and an AsiI sites was inserted into the NotI site of this vector. The polylinker sequence was: 5'-

GGCCGCATATGCTAGCTTACCACCTGCTTGCATTAAATGGCGATCGCAGC-

3' (SEQ ID NO:79). Finally, the long homology (LH) which contained 5.1 Kb downstream to exon-3 was ligated into the NheI and AsiI of this vector using PCR with

the following primers:

5'-GGAAAGCGGCGATCGCTCGCATATATCTTGGCTACCTTTTATGCGG-3'

(SEQ ID NO:80; forward primer) and

5'-GAGTCAGGAAGCTAGCTCCAATAGAATTAATGTTATATTTTTTCC-3' (SEQ

ID NO:81; reverse primer). The linearized targeting vector was introduced into R1 ES

cells (derived from 129/ola mice) by electroporation and ~1,000 neomycin resistant clones were picked. The individual clones described above were screened for homologous recombination by Southern blot analysis.

Southern blot analysis for the MTCH2/MIMP knockout mice - For Southern blot analysis, a 5' probe and a 3' probe were constructed. The 5' probe, which is located ~500 bp upstream to the SH, was prepared by a PCR reaction with the following primers: 5'-TATTTGGATCCTCCTAACCAGTTTAGATGGTTGC-3' (SEQ ID NO:82; forward primer) and

5'-TTGTTGAATTCATGGTCTCCCTATGTAGCTATGGCTGGTC-3' (SEQ ID NO:83; reverse primer). The 3' probe, which is located ~250 bp downstream to the LH,

was prepared by a PCR reaction with the following primers:

5'-CTTTAGGGATCCGCTCTCATGTTCTTTGTTTACC-3' (SEQ ID NO:84; forward

primer) and 5'-CTCGAATTCCAGGTATCTGACAACTCTTCTGGCCTC-3' (SEQ ID

NO:85; reverse primer). The PCR products were digested with EcoRI/BamHI and cloned into the EcoRI/BamHI sites of pcDNA3. Genomic DNA was digested with either NcoI or SpeI, separated on a 0.9 % agarose gel and transferred to Hybond-N⁺ membrane (Amersham) in 0.1 N NaOH. The probes were synthesized using PCR and labeled with α -³²P-dCTP (3,000 ci/mmol, Amersham) using the random primer DNA labeling kit (Biological Industries Beit-Haemek). The hybridization was performed in rapid-hybridization buffer (Amersham) according to manufacturer's protocol and specific binding was analyzed by autoradiography.

Generation of MTCH2/MIMP knockout mice - Two homologous recombinant R1 clones were identified, aggregated with tetraploid embryos and implanted into separate white-coated ICR foster mother mice. The first generation of black-coated mice were born, bred again to white ICR mice, to obtain the second generation of MTCH2/MIMP^{+/-} animals. Intercross of MTCH2/MIMP^{+/-} animals resulted in offspring homozygous for the Mch2/Mimp knock out (MTCH2/MIMP^{-/-}).

Timed pregnancies, isolation of embryos, and PCR analysis - Timed pregnancies were conducted with MTCH2/MIMP^{+/-} mice. Females with copulation plugs were considered to be at embryonic development day 0.5 (E0.5) of gestation. Pregnant females were sacrificed at different time points of gestation, and embryos were dissected from maternal tissue. Uteri from E6.5, E7.5, E8.5 and E9.5 pregnancies were isolated in ice-cold phosphate-buffered saline. Decidua were separated, embryos dissected out under a binocular, and pictures were taken. For genotyping of the E6.5 and E7.5 embryos, DNA was prepared using the Epicentre MasterPure purification kit and than analysed by PCR. For the E8.5 and E9.5 embryos, the yolk sac was separated and lysed using the REDExtract-N-Amp Tissue PCR Kit (Sigma), and the sets of primers that were used for genotyping of pups were employed. For histological preparation, the whole uterus was fixed in 4% paraformaldehyde for 48 hrs at RT. Sections were cut from paraffin blocks and stained with hematoxylin and eosin (H&E).

Generation of ES cells of MTCH2/MIMP knockout mice - Pregnant females from MTCH2/MIMP^{+/-} intercrosses were sacrificed at E3.5, and blastocysts were collected by flushing the uteri (Hogan, B., R. Beddington, F. Constantini, and E. Lacy. 1994. Manipulating the mouse embryo. Cold Spring Harbor Laboratory Press, Plainview, N.Y). Blastocysts were cultured individually in 96-well plates in DMEM

(Gibco) supplemented with 20 % fetal bovine serum (FBS) (Gibco), 1 mM NaPyruvate, 2 mM L-Glutamine, 100 u/ml Pen/Strep, 1 X non-essential Amino Acids, 0.1 mM β -mercaptoethanol and 5 mg/ml Uridine. The outgrowths were monitored daily and when clumps with ES morphology dominated the culture they were gradually expanded and their genotype was assessed by PCR. For the generation of ES stable clones, MTCH2/MIMP^{-/-} ES cells were transfected with either an empty pcDNA3.1 vector or a pcDNA3.1 vector carrying MTCH2/MIMP using Lipofectamine 2000 (Invitrogen). The cells were then cultured under selective conditions (medium containing 2 mg/ml hygromycin), and surviving clones were expanded and used as stable clones for the experiments described.

Generation of MTCH2/MIMP conditional knockout mice - Designing the MTCH2/MIMP floxed targeting vector: To generate this vector, a construct designed to excise the first three exons of MTCH2/MIMP was assembled in the pRapidflirt vector (a kind gift from Dr. Steffen Yung). This vector consists of all the elements needed for conditional gene targeting: two loxP sites, a PGKneo cassette (which provides neomycin resistant) flanked by two Frt sites (that enable the excision of the PGKneo cassette upon Flp recombinase expression), an ampicillin resistant cassette, and a thymidine kinase (TK) cassette (which serves as a negative selection against random, non-homologous integration of the construct to the genome). The targeting vector was constructed by PCR using 129/SVJ genomic DNA. First, each of the PCR products was ligated into the pGEM T-Easy vector (Promega), and colonies that carried the expected insert size were taken for sequencing. The "best" colony (the one with the sequence that most resembled the sequence that appears in the data base) was chosen and suspected mutations were corrected by site directed mutagenesis. The second step was to clone the isolated PCR products into the pRapidflirt vector. The long homology (LH) arm, which consists of 7 Kb upstream to exon-1 was ligated into XhoI and FseI sites in the pRapidflirt vector down stream to the TK cassette. The forward primer contained both the cloning XhoI site and an AflII site that was later used to linearize the targeting vector, and the reverse primer contained the FseI site. The primers used for the LH-PCR reaction were: 5'-CCGCTCGAGCTTAAGTGACCATATGACCTTTCCAT-3' (SEQ ID NO:86; forward) and 5'-CGACGTGGCCGGCCAAAGTTTGATGGTTGTTTTTC-3' (SEQ ID NO:87; reverse). A 2.9 Kb DNA fragment which consists of the 5'-UTR, the

first three exons of MTCH2/MIMP and a small portion of the third intron (named Ex; Figure 4A) was ligated into SalI and SbfI sites of the pRapidflirt vector between the two loxP sites (the first loxP site is located downstream to the 5'-UTR of MTCH2/MIMP and the other one is located upstream to the Frt site of the PGKneo cassette). The

5 primers used for the Ex-PCR reaction were: 5'-ACGCGTCGACTCTAGAACGTCGTCAAAGCCTGAAAG-3' (SEQ ID NO:88; forward) which contained the SalI cloning site and XbaI site [that is further used in the Southern blot screen for identifying MTCH2/MIMP^{lox/neo} positive embryonic stem (ES) cells], and 5'-CAGAGAACCTGCAGGAGAGATGCCATGCCAGAGTTA-3' (SEQ ID

10 NO:89; reverse) which contains the SbfI site. The short homologues (SH) arm which contains 2 Kb of the third intron was ligated into the NotI and ClaI restriction sites of the pRapidflirt vector. The primers used for the SH-PCR reaction were: 5'-AAGGAAAAAAGCGGCCGCTTCTCTTGAAAGACATTTTC-3' (SEQ ID NO:90; forward) and 5'-CCCATCGATTTCTTTGCCTTTTTCTCTTTC-3' (SEQ ID NO:91;

15 reverse). Subsequently, the complete targeting vector was subjected to sequence analysis, and the ~18.8 Kb linearized vector was introduced into R1 ES cells by electroporation.

Southern blot analysis for the MTCH2/MIMP conditional knockout mice -

Homologues recombinant candidates were screened by Southern blot analysis using two

20 probes that were designed to detect wild-type and conditional alleles. The 5'-probe which is located 20 bp upstream to the LH arm was prepared by a PCR reaction with the following primers: 5'-TGAGCATGGAAGCAATGAAG-3' (SEQ ID NO:92; forward) and 5'-TGTTCTGGTTTGCTCTGTGG-3' (SEQ ID NO:93; reverse). The 3' probe, which is located 280 bp downstream of the SH arm, was prepared by a PCR reaction

25 with the following primers: 5'-AACCCGTCTTGCTTCTACCAG-3' (SEQ ID NO:94; forward) and 5'-GGTGGGCACTACCATACTG-3' (SEQ ID NO:95; reverse). The PCR products were cloned into the pGEM T-Easy vector. Genomic DNA was digested with either XbaI or NcoI restriction enzymes, separated on a 0.8 % agarose gel and transferred to Hybond-N⁺ membrane (Amersham) in 0.1 N NaOH. The probes were

30 labeled with α -³²P-dCTP (3000 ci/mmol, Amersham) using the random primer DNA labeling kit (Biological Industries Beit-Haemek). The hybridization was performed in rapid-hybridization buffer (Amersham) according to the manufacturer's instructions and

the radioactive signal of the specific binding of the labeled probe was analyzed by exposure to a high sensitive film (Kodak). ~750 neomycin resistant clones were picked, 400 individual ES clones were screened for homologous recombination by Southern blot analysis and ten clones showed the correct homologous recombination event.

5 **Generation of MTCH2/MIMP conditional knockout mice** - Two homologous recombinant R1 clones were identified, aggregated with tetraploid embryos and implanted into separate white-coated ICR foster mother mice. The first generation of black-coated mice were born, bred again to white ICR mice, to obtain the second generation of MTCH2/MIMP^{flx/+} (MTCH2/MIMP^{fl/+}) animals. To obtain
10 MTCH2/MIMP^{fl/+} mice with a pure 129 inbred background (both the ES cells and the tetraploid embryos that were used to create the chimeras have the 129 background), confirmed chimeras with germ-line transmission were mated to wild-type mice from the 129/SVJ line. Finally, The PGKneo cassette was excised by crossing the MTCH2/MIMP^{fl/+} mouse to a general Flp deleter mouse that expresses the Flp
15 recombinase in all tissues (e.g., the Rosa-Flp mouse).

Preparation and transduction of MEFs with Cre-recombinase - MTCH2/MIMP^{fl/fl} and MTCH2/MIMP^{fl/+} primary MEFs were prepared from 11- to 13-day-old embryos, and maintained in ISCOVE's medium containing 10 % fetal bovine serum. SV40 transformation of primary MEFs was performed by transfecting cells with
20 the SV40 whole genome using Lipofectamine 2000 (Invitrogen). Stable clones were collected 14-to-18 days post transfection. All the studies with MEFs described in the paper were performed with SV40-immortalized MEFs. Recombinant His-TAT-NLS-Cre (HTNC) fusion protein was expressed and purified as described previously (Peitz, M., K. Pfannkuche, K. Rajewsky, and F. Edenhofer. 2002. Ability of the hydrophobic
25 FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes. Proc Natl Acad Sci U S A 99:4489-94). HTNC was diluted in DMEM/PBS to a final concentration of 3–5 µM, sterile-filtered, and applied to MEFs grown in cell culture dishes. Cells were incubated for 20 hours, washed with PBS, and supplemented with
30 growth medium.

Cell viability assays - Cell viability was determined by propidium iodide (PI) dye exclusion. PI (25 µg/ml) was added to the cells immediately prior to analysis by

FACScan (Beckton Dickinson). All reagents used to induce cell death were from Sigma.

Isolation of mouse liver mitochondria, and proteinase K treatment - Mouse liver mitochondria were isolated as previously described (Grinberg, M., M. Schwarz, Y. Zaltsman, T. Eini, H. Niv, S. Pietrokovski, and A. Gross. 2005. Mitochondrial Carrier Homolog 2 Is a Target of tBID in Cells Signaled To Die by Tumor Necrosis Factor Alpha. Mol Cell Biol 25:4579-90). For proteinase K treatment, the mitochondrial pellet was resuspended in SEM (250 mM sucrose, 10 mM MOPS/KOH, 2.5 mM EDTA) together with 0.1 or 1 µg/ml proteinase K, and incubated at 4 °C for 20 minute. The reaction was stopped with 1 mM PMSF and the mitochondria were centrifuged at 10,000 X g for 10 minutes, resuspended in HIM buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES, pH 7.5) containing 1 mM PMSF, and again recentrifuged at 10,000 X g for 10 minutes.

Isolation and sub-fractionation of rat liver mitochondria - Mitochondria were isolated from rat liver and sub-fractionated as previously described (Bathori, G., G. Csordas, C. Garcia-Perez, E. Davies, and G. Hajnoczky. 2006. Ca²⁺-dependent control of the permeability properties of the mitochondrial outer membrane and voltage-dependent anion-selective channel (VDAC). J Biol Chem 281:17347-58). For the isolation of OMM, mitochondria were further purified using self-performing Percoll gradient centrifugation (30 % Percoll in 250 mM sucrose, 1 mM EGTA, and 2.5 mM Tris/HCl, pH 7.4). After a washing step, the pellet was resuspended and incubated in a hypotonic potassium phosphate buffer (swelling medium, 10 mM KH₂PO₄, pH 7.4) for 30 minutes on ice. Subsequently, a shrinking medium (32 % sucrose, 30 % glycerol, 10 mM MgCl₂) was added to the suspension (33 % volume). After 30 minutes of shrinking, the mitochondrial membranes were disrupted by ultrasonication (4 x 1 minute of irradiation, 1-minute break between each run). The resulted material was spun down at 12,000 X g for 10 minutes. The supernatant contains a mixture of mitochondrial membrane vesicles and was used as a reference for the further purified membranes. To separate the OMM and IMM fractions, a discontinuous sucrose gradient was used (from bottom to top, 70, 45.6, 34.2, and 26 % sucrose steps, 200,000 X g for 240 minutes). The OMM was concentrated in the interface between the 26 and 34.2 % steps, and the IMM

was collected between 45.6 and 70 %. The protein concentration was determined in each fraction.

Isolation and purification of the OMM from mouse liver mitochondria -

Livers from four female mice were used to prepare each OMM sample. Livers were
5 excised and mitochondria were prepared as was previously described [Grinberg, M. et al. Mitochondrial Carrier Homolog 2 Is a Target of tBID in Cells Signaled To Die by Tumor Necrosis Factor Alpha. Mol Cell Biol 25, 4579-90 (2005)] with several modifications. The final mitochondria-enriched pellet was gently resuspended in 50 ml of MB buffer (210 mM Mannitol, 70 mM Sucrose, 10 mM Hepes, 1 mM EDTA, pH
10 7.5) and purified on a discontinuous Nycodenz (Sigma) gradient according to [Da Cruz, S. et al. Proteomic analysis of the mouse liver mitochondrial inner membrane. J Biol Chem 278, 41566-71 (2003)]. Next, OMM were isolated by the swell-shrink-sonication procedure according to [Bathori, G., Csordas, G., Garcia-Pérez, C., Davies, E. & Hajnoczky, G. Ca²⁺-dependent control of the permeability properties of the
15 mitochondrial outer membrane and voltage-dependent anion-selective channel (VDAC). J Biol Chem 281, 17347-58 (2006)] with several modifications. The purified mitochondria pellet was resuspended (5 mg protein/ml) and incubated in hypotonic potassium phosphate buffer (swelling medium; 10 mM KH₂PO₄ pH 7.4 for 30 min on ice while stirring). Subsequently, a shrinking medium (32% sucrose, 30% glycerol, 10
20 mM MgCl₂) was added to the suspension (33% volume). After 30 min of shrinking the mitochondrial membranes were disrupted by ultrasonication 4 x 20 seconds with a 1 min break between runs. The resultant material was spun down at 12,000 x g for 10 min. The supernatant containing a mixture of mitochondrial membrane vesicles was carefully layered on a discontinuous sucrose gradient (from bottom to top 70, 45.6,
25 34.2, and 26% sucrose, 2 ml each in SW41 rotor test tubes), and centrifuged for 12-to-16 hrs (200,000 x g, 4°C). The OMM was concentrated in the interface between the 26 and 34.2% steps and further washed in 70 ml MB buffer and centrifuged (141,000 x g, 4 hrs). The resulting pellet was resuspended in MB buffer and immediately frozen in liquid N₂ for Western blot analysis.

30 ***Caspase-3 activity assay in hepatocytes -*** Caspase-3 activity assays were performed as previously described with some modifications [Sarig, R. et al. BID-D59A is a potent inducer of apoptosis in primary embryonic fibroblasts. J Biol Chem 278,

10707-15 (2003)]. Livers were minced, washed in ice-cold PBS, and homogenized in lysis buffer (20 mM HEPES pH 7.3, 5 mM EGTA, 5 mM EDTA, 10 μ M digitonin, 2 mM DTT) using a 2-ml Wheaton Dounce glass homogenizer and a glass "B"-type pestle. The lysates were clarified by centrifugation and the supernatants were used for
5 the assays. Enzymatic reactions were carried out in lysis buffer containing 20 μ g of protein and 50 μ M acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (DEVD-AMC; Alexis) to assess caspase-3 activity. Each sample was divided into three parts: One of them included in addition to the extract and substrate, 50 μ M zVAD-fmk (BioMol) to inhibit caspase activity and two replicates that included extract and substrate, without
10 inhibitor. The reaction mixtures were incubated at 37 °C and fluorescent AMC formation was measured at excitation 380 nm and emission 460 nm using a Wallac Victor2 1420 Multilabel Counter (PerkinElmer). Specific activity was calculated for each sample as the mean of the duplicate sample minus the value obtained for the sample containing zVAD-fmk.

15 ***Knockdown of MTCH2/MIMP in U2OS cells*** - Human MTCH2/MIMP was knockdown using siRNA On-TargetPlus smart pools (Dharmacon). Cells were transfected with siRNA [44 nM; a mix of 4 Mch2 siRNAs: GCACAUUGCCAGUAUCGAU (SEQ ID NO:132); UGACAAGGGUGAGGAGUUA (SEQ ID NO:133); GAGCCGAGGAAAUAGCUUA (SEQ ID NO:134); and
20 GAAGAGUUAUUCUCAAGCU (SEQ ID NO:135); ON-TARGETplus SMARTpool, Catalog number: L-007371-00-0010, for human MTCH2, NM_014342] using the Dharmafect 1 reagent according to the manufacturer's instructions. 72 hours post transfection the cells were either collected for Western blot analysis or infected with Ad-tBID for 14 hrs and cell death was monitored by propidium iodide (PI) dye
25 exclusion.

Preparation of recombinant adenoviruses and infection of MEFs - tBID and GFP recombinant adenoviruses were prepared as was previously described [Sarig, R. et al. BID-D59A is a potent inducer of apoptosis in primary embryonic fibroblasts. J Biol Chem 278, 10707-15 (2003), which is hereby incorporated by reference in its entirety].
30 MTCH2/MIMP, BAX, Noxa, and BCL-2 recombinant adenoviruses were prepared as was previously described for preparing tBID recombinant adenoviruses [Sarig, R. et al. 2003]. Bim recombinant adenoviruses were prepared as was previously described for

preparing Nbk recombinant adenoviruses [Gillissen, B. et al. Induction of cell death by the BH3-only Bcl-2 homolog Nbk/Bik is mediated by an entirely Bax-dependent mitochondrial pathway. *Embo J* 22, 3580-90 (2003), which is hereby incorporated by reference in its entirety]. Viruses were grown using 293T-TR cells. Virus preparations were made from freeze/thaw lysis of the cells, and virus titers were done on 293T-TR cells. In experiments, cells were generally seeded at 70-80% confluence. Cells were infected with an MOI (multiplicity of infection) of 10. Efficiency of infection was determined using the GFP recombinant adenovirus and was in the range of 70-to-90%.

Studies with permeabilized ES cells - Experiments were carried out with 1.2 mg of cells in 750 μ l of an intracellular medium (passed through a Chelex column to reduce ambient Ca^{2+}) composed of 120 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4 , 20 mM Hepes/Tris, pH 7.2, supplemented with 1 μ g/ml each of antipain, leupeptin, pepstatin and with 40 μ g/ml digitonin at 35 $^\circ\text{C}$ under continuous stirring in a fluorometer. The following additions were made: 2 μ M TMRE (10s), 2 mM succinate (50s), 2 mM ATP + 5 mM Phosphocreatine + 5 U/ml Creatine Phosphokinase (100s), 5 μ M oligomycin (150s), purified recombinant histidine-tagged murine tBID (300s), FCCP 5 μ M (600s). TMRE fluorescence recordings were carried out at ex. 545 nm, em. 580 nm. At the end of the recordings, the suspensions of the cells were centrifuged and the pellets were separated from the supernatants. Supernatants were used to analyze Cyt c release, and pellets were used to analyze BAK and BAX dimerization.

Cross-linking - BS^3 [bis (sulfosuccinimidyl) suberate], Sulfo-BSOCOES [sulfo-Bis[2-(sulfosuccinimidooxy-carbonyloxy)ethyl]sulfone], and BMH [bismaleimido-hexane] cross linking agents (obtained from Pierce), from a 10-fold stock solution were added to obtain a final concentration of 10 mM. The cross-linker was added to the pellet/mitochondrial fraction suspended in isotonic HIM buffer. After incubation at room temperature for 30 minutes, the cross-linker was quenched by the addition of 1 M Tris-HCl (pH 7.5) to a final concentration of 20 mM. After-quenching, samples were lysed and Western blot-analyzed with the indicated antibody.

Recombinant tBID, Western blot analysis and antibodies - Purified recombinant histidine-tagged murine tBID was prepared as previously described (Wei, M. C., T. Lindsten, V. K. Mootha, S. Weiler, A. Gross, M. Ashiya, C. B. Thompson, and S. J. Korsmeyer. 2000. tBID, a membrane-targeted death ligand, oligomerizes BAK

to release cytochrome c. Genes Dev 14:2060-71). For Western blot analysis, proteins were size-fractionated by SDS-PAGE, then transferred to PVDF membranes (Immobilon-P, Bio-Rad), and Western blots were developed by use of the enhanced chemiluminescence reagent (NEN). Antibodies used for Western blot included anti-MTCH2/MIMP Ab (Grinberg, M., et al., 2005. Mol Cell Biol 25:4579-90), anti-BID Ab (Grinberg, M., et al., 2005. Mol Cell Biol 25:4579-90), anti-mBAX Ab (651; gift from Stan Korsmeyer, DFCI, Boston, USA), anti-BAK Ab (Upstate Biotechnology, Inc.), anti-HA mAb (3F10; Roche), anti-Cyt c mAb (7H8.2C12, PharMingen), anti-Tom20 Ab (a present from Gordon Shore), anti-ANT Ab (Santa Cruz), Anti-AIF Ab (Santa Cruz), anti-cytochrome c oxidase subunit IV (Cyt Oxi) Ab (a present from Jim Hare), anti-cleaved caspase-3 (Cell Signaling), anti-BCL-X_L Ab (Santa Cruz), anti-caspase-8 Ab (Alexis), anti-porin Ab (Calbiochem), and anti-actin Ab (Santa Cruz).

Generation of MTCH2/MIMP liver-specific knockout mice - The existing Cre systems provide variable efficiencies which are rather weak in most cases. In order to ensure high efficiency of this system the present inventors generated mice in which one of the *MTCH2/MIMP* alleles was fully deleted and the other one knocked out only in the organ of target. These mice were generated by first mating the *MTCH2/MIMP^{fl/fl}* mice with mice bearing P_{gk}-Cre, a general deleter transgene [Lallemand, Y., Luria, V., Haffner-Krausz, R. & Lonai, P. Maternally expressed PGK-Cre transgene as a tool for early and uniform activation of the Cre site-specific recombinase. Transgenic Res 7, 105-12 (1998)] to create *MTCH2/MIMP^{+/-Δ}* mice. These mice were then mated with mice bearing Alb-Cre, a transgene for Cre-recombinase under control of the liver albumin promoter [Postic, C. et al. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. J Biol Chem 274, 305-15 (1999)]. Offspring that expressed both the *MTCH2/MIMP* deleted allele and the Alb-Cre transgene (*MTCH2/MIMP^{+/-Δ};Alb-Cre*) were mated with *MTCH2/MIMP^{fl/fl}* homozygous mice, to generate the *MTCH2/MIMP^{fl/Δ};Alb-Cre* and *MTCH2/MIMP^{fl/+};Alb-Cre* mice (liver specific knockout and its control littermate, respectively).

Genotyping the mice: Genotyping of the various *MTCH2/MIMP* alleles was performed by PCR analyses of tail DNA. For the PCR analyses, we used the oligonucleotides 5'- TGTTCACAGGCTTGACTCCA-3' (sense; SEQ ID NO:118) and

5'-CAAACGTGTATAGGTGAATGGCTCT-3' (antisense; SEQ ID NO:119) for the wild-type allele, 5'-CGGAATAGGAACTTCGTCGAG-3' (sense; SEQ ID NO:120) and 5'-CAAACGTGTATAGGTGAATGGCTCT-3' (antisense; SEQ ID NO:121) for the floxed allele, 5'-TCCCAAGTGCTGGATTAAGG-3' (sense; SEQ ID NO:122) and 5'-CAAACGTGTATAGGTGAATGGCTCT-3' (antisense; SEQ ID NO:123) for the deleted allele, and 5'-TGCCTGCATTACCGGTCGATGC-3' (sense; SEQ ID NO:124) and 5'-CCATGAGTGAACGAACCTGGTCG-3' (antisense; SEQ ID NO:125) for the *Alb-Cre* transgene.

Western blot analysis of cytosolic fractions of *Mtch2*^{-/-}*Mtch2* or *Mtch2*^{-/-}Vector ES cells – To establish the kinetics of tBID-induced MOMP/Cyt c release (Madesh M, et al., 2002), permeabilized *Mtch2*^{-/-}Vector or *Mtch2*^{-/-}*Mtch2* cells were used. In these experiments, a rapid filtration method was applied to obtain cytosolic fractions for Western blotting (Madesh M, et al., 2002). *Mtch2*^{-/-}Vector and *Mtch2*^{-/-}*Mtch2* lines were permeabilized using digitonin followed by the addition of recombinant tBID (a concentrations of 1 nM and 40 nM). Cytochrome c release was induced by recombinant tBID. At the end of the experiment, the suspension was centrifuged and the cell pellet was separated from the supernatant.

Purification of yeast mitochondria and in vitro import assays - Wild-type yeast mitochondria were isolated from cultures grown at 30 °C to an OD₆₀₀ of 2 in rich lactate medium (1 % yeast extract, 2 % tryptone, 0.05 % dextrose, and 2 % lactic acid, 3.4 mM CaCl₂ 2H₂O, 8.5 mM NaCl, 2.95 mM MgCl₂ 6H₂O, 7.35 mM KH₂PO₄, and 18.7 mM NH₄Cl). Mitochondria were isolated as previously described (Claypool, S. M., et al., 2006. Mitochondrial mislocalization and altered assembly of a cluster of Barth syndrome mutant tafazzins. J Cell Biol 174:379-90). Temperature-sensitive yeast mitochondria were isolated from cultures grown in YPEG at 25 °C. MTCH2/MIMP was cloned into a pcDNA3.1 vector (Grinberg, M., et al., 2005, Mol Cell Biol 25:4579-90), linearized using XmaI, and transcribed using the T7 polymerase. The mRNA was then translated in rabbit reticulocyte lysate in the presence of ³⁵S-methionine. The reticulocyte lysate containing the radiolabeled precursor was incubated at 30 °C with isolated mitochondria in import buffer (1 mg/ml bovine serum albumin, 0.6 M sorbitol, 150 mM KCl, 10 mM MgCl₂, 2.5 mM EDTA, 2 mM ATP, 2 mM NADH, and 20 mM Hepes-KOH, pH 7.4). Where indicated, the potential across the mitochondrial inner

membrane was dissipated using 1 μ M valinomycin and 25 μ M FCCP. Unimported radiolabeled precursor was removed by treatment with 10 μ g/ml trypsin for 30 minutes on ice. Trypsin was inhibited with 100 μ g/ml soybean trypsin inhibitor. For carbonate extraction, radiolabeled precursor was incubated at 30 °C with isolated mitochondria in import buffer, and then a single import reaction was divided into four tubes. Mitochondria were collected by spinning at 8,000 X g for 5 minutes at 4 °C and resuspended in 0.1 M sodium carbonate at various pHs or a total fraction in Thorner buffer (10 % glycerol, 8 M urea, 5 % SDS, 40 mM Tris, pH 6.8, 4 mg/ml bromophenol blue, and 5 % β -mercaptoethanol). After 30 minutes on ice, the mitochondria were pelleted at 20,000 X g for 15 minutes at 4 °C. The pellet containing the membrane fraction was resuspended in Thorner buffer, and the supernatants were precipitated with 20 % trichloroacetic acid for 30 minutes on ice. The supernatants were then spun at 20,000 X g for 15 minutes at 4 °C. These pellets were then resuspended in Thorner buffer.

Bacterial expression of MTCH2/MIMP and transport assays - The coding sequences for human and murine MTCH2/MIMP (accession numbers AY380792.1 and NP_062732.1, respectively) were amplified from human and murine liver cDNA by PCR. The oligonucleotide primers corresponded to the extremities of the coding sequences with additional *NdeI* and *EcoRI* restriction sites. The amplified products were cloned into the pMW7 expression vector (Fiermonte, G., et al., 1998, J Biol Chem 273:24754-9) and sequenced. The human and the murine MTCH2/MIMP were overexpressed as inclusion bodies in the cytosol of *E. coli* C0214(DE3) (Fiermonte, G., et al., 1998, J Biol Chem 273:24754-9). Control cultures with the empty vector were processed in parallel. Inclusion bodies were purified on a sucrose density gradient [Fiermonte, G., et al., 1993, Biochem J 294 (Pt 1):293-9] and analyzed by SDS-PAGE. The proteins were solubilized in 1.8 % sarkosyl (w/v). Small residues were removed by centrifugation (258,000 X g, 1 hour). Solubilized proteins were reconstituted into liposomes followed by four different procedures, specific for the functional reconstitution of the bovine oxoglutarate (OGC) [Fiermonte, G., et al., 1993, Biochem J 294 (Pt 1):293-9], the human aspartate/glutamate (isoform 2, AGC2) (Palmieri, L., et al., 2001, Embo J 20:5060-9), the ATP-Mg/Pi (isoform 1, APC1) (Fiermonte, G., et al., 2004, J Biol Chem 279:30722-30), and the yeast NAD⁺ (isoform 1, Ndt1p) (Todisco, S.,

et al., 2006, J Biol Chem 281:1524-31) recombinant mitochondrial carriers, used as positive controls. Each substrate was tested in homo-exchange reactions (same substrate at both sides of the proteoliposomal membranes). Transport at 25 °C was started by adding the labeled substrate to proteoliposomes and terminated after 1 minute or 30 minutes by the addition of a mixture containing pyridoxal 5'-phosphate and bathophenanthroline, 30 and 10 mM final concentration, respectively [this is the "inhibitor-stop" method (Palmieri, F., et al., 1995, Methods Enzymol 260:349-69)]. In controls, the inhibitor was added with the labeled substrate. Extraliposomal labeled substrate was removed from quenched samples on Sephadex G-75, and eluted radioactivity was measured (Palmieri, F., et al., 1995, Methods Enzymol 260:349-69). Transport activities were calculated from the experimental values minus the controls.

Immunofluorescence and imaging - For imaging, cells on coverslips were fixed with 3 % formaldehyde in PBS in permeabilized with 0.2 % Triton X-100/PBS. Cells were immunostained with anti-cytochrome c 6H2.B4 monoclonal antibodies (PharMingen) followed by Cy3-conjugated goat anti-IgG (Jackson ImmunoResearch). Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 10 µg/ml). Images were collected on an Olympus IX70 microscope, equipped with Deltavision imaging system, using a 40 x PLAN-APO 1.42NA objective. Images were processed by constrained iterative deconvolution on softWoRx™ software (Applied Precision).

Purification of the HA-tBID cross-linked complex – 100 x 10 cm plates of 293T cells were transiently transfected with pcDNA3-HA-mBID (GenBank Accession No. MMU75506; SEQ ID NO:33 (mRNA of mouse BID); gi:1669513). Eighteen hours post-transfection, cells were harvested and subcellularly fractionated by differential centrifugation, as described above. The mitochondria-enriched heavy membrane fractions were treated with sulfo-BSOCOES (Sulfo-Bis[2-(sulfosuccinimidooxycarbonyloxy)ethyl]sulfone; (Pierce)) at a final concentration of 10 mM. After incubation at room temperature for 30 minutes, the cross-linker was quenched by the addition of 1 M Tris-HCl (pH 7.5) to a final concentration of 20 mM.

Following quenching, the membrane fraction was separated from the soluble fraction by centrifugation, and lysed in Laemli sample buffer without reducing agents. The resulting lysate was diluted in binding buffer [20 mM Tris (pH 7.5), 0.1 M NaCl,

0.1 M EDTA] to reach a final concentration of 0.2 % SDS. The diluted lysate was incubated for 16 hours with 5 mg anti-HA mAbs coupled to agarose beads (Roche), followed by extensive washing of the beads with binding buffer containing 0.05 % Tween-20. The material that remained bound to the beads was eluted by incubation
5 with 1 ml (1 mg/ml) HA peptide at 37 °C for 15 minutes. Elution was repeated twice more, and the three elutents were pooled and concentrated, using a Centricon tube with a 3K cutoff (Amicon). The concentrated material was loaded onto a single lane, separated by SDS-PAGE, and then stained with Coomassie blue.

In gel proteolysis and mass spectrometry analysis - The stained protein bands
10 or spots in the gel were cut with a clean razor blade. The proteins in the gel were then reduced with 10 mM DTT, and modified with 100 mM iodoacetamide in 10 mM ammonium bicarbonate. The gel pieces were treated with 50 % acetonitrile in 10 mM ammonium bicarbonate to remove the stain from the proteins; the gel pieces were then dried. The dried gel pieces were rehydrated with 10 % acetonitrile in 10 mM
15 ammonium bicarbonate containing about 0.1 µg trypsin per sample. The gel pieces were then incubated overnight at 37 °C and the resulting peptides were recovered with 60 % acetonitrile with 0.1 % trifluoroacetate. The tryptic peptides were resolved by reverse-phase chromatography on 0.1 X 300-mm fused silica capillaries (100 micrometer ID, J&W) filled with porous R2 (Perspective). The peptides were then eluted using a 80-
20 min linear gradient of 5 to 95 % acetonitrile with 0.1 % acetic acid in water, at a flow rate of 1 µl/minute. The liquid from the column was electrosprayed into an ion-trap mass spectrometer (LCQ, Finnegan, San Jose).

Mass spectrometry was performed in the positive ion mode, utilizing a repetitively full MS scan, followed by collision-induced dissociation (CID) of the most
25 dominant ion selected from the first MS scan. The mass spectrometry data was compared to simulated proteolysis and CID of the proteins in the NR-NCBI database, using Sequest software (J. Eng, University of Washington, and J. Yates, Finnegan, San Jose). The amino terminal of the protein was sequenced on Peptide Sequencer 494A (Perkin Elmer) according to the manufacturer's instructions.

30 *Statistical analysis* - Data are presented as the mean $\pm$ s.d. Student's unpaired two-tailed *t*-test was performed using Microsoft Excel statistical analysis functions. Differences were considered statistically significant at $P < 0.05$. The Kaplan-Meier

survival curves were compared using the long-rank test (PASW Statistics 17.0 software).

EXAMPLE 1

5 **LOSS OF MTCH2/MIMP RESULTS IN EMBRYONIC LETHALITY AND MORPHOLOGICAL ABERRATIONS**

Experimental Results

Loss of MTCH2/MIMP results in embryonic lethality - To determine the role and importance of MTCH2/MIMP *in vivo*, the present inventors disrupted the
10 MTCH2/MIMP gene in mice. The MTCH2/MIMP gene spans ~23 Kb on chromosome 2 and consists of 13 exons. The wild-type MTCH2/MIMP allele, the targeting construct, and the targeted allele are illustrated in Figure 1A. In the targeted allele, the first three exons were replaced with the neomycin resistant cassette, thereby creating an MTCH2/MIMP null allele. Two ES cell clones containing the targeted MTCH2/MIMP
15 allele were isolated based on Southern-blot analysis (data not shown), and independent lines of genetically modified mice were generated from these two clones. Analysis of the offspring of heterozygote intercrosses revealed that heterozygotes for the targeted allele (MTCH2/MIMP^{+/-}) are viable and fertile and exhibit no obvious phenotypic abnormalities. However, no homozygote null (MTCH2/MIMP^{-/-}) animals were
20 observed, indicating that the homozygous disruption of MTCH2/MIMP leads to prenatal death. To determine the precise time point at which MTCH2/MIMP^{-/-} embryos die, timed pregnancies were set up, and the embryos were genotyped by polymerase chain reaction (PCR; Figure 1B). The analysis started with embryos of embryonic day E10.5. As shown in Table 3 below, no embryos of the -/- genotype were found at
25 E10.5. Next, embryos of embryonic day E9.5 and E8.5 were analyzed and very low percentages of the -/- genotype were found (~5 %; Table 3 below). On the other hand, expected Mendelian inheritance ratios between the offsprings were detected at E6.5 and E7.5 (~25 % of all embryos were MTCH2/MIMP^{+/-}). These results indicate that MTCH2/MIMP is critical for embryonic development.

Table 3

Distribution of genotypes during embryonic development of MTCH2/MIMP knock out mice

<i>Stage</i>	<i>Total</i>	<i>+/+</i>	<i>+/-</i>	<i>-/-</i>	<i>N/D</i>
E10.5	20	4	13	0 (0 %)	3
E9.5	19	4	10	1 (5 %)	4
E8.5	76	16	50	4 (5 %)	6
E7.5	42	10	20	11 (26 %)	1
E6.5	24	7	10	6 (25 %)	1

Table 3: Heterozygote mice carrying the MTCH2/MIMP knock out allele (MTCH2/MIMP^{+/-}) were intercrossed (timed pregnancies) and the frequency of each genotype in all embryos was determined during embryonic development from embryonic day 6.5 (E6.5) through embryonic day 10.5 (E10.5). +/+ homozygote wild-type embryos carrying two wild-type alleles (i.e., two copies of the normal, functional MTCH2/MIMP); +/- heterozygote embryos carrying one wild-type allele and one MTCH2/MIMP-knock out allele; -/- homozygote MTCH2/MIMP-knock out embryos carrying two alleles with MTCH2/MIMP-knock-out, i.e., devoid of a functional MTCH2/MIMP. N/D – genotype not determined.

E7.5 Mch2/Mimp^{-/-} embryos are significantly smaller than the wild-type embryos and lack structures that are typical to this stage - To begin to assess the exact defect(s) in these embryos the present inventors have focused on E7.5 embryos. Pregnant females were sacrificed at E7.5, and the whole uterus was subjected to histological sectioning. Figures 2A-B show sections of a representative wild-type (Figure 2A) and MTCH2/MIMP^{-/-} (Figure 2B) E7.5 embryo stained with hematoxylin and eosin. Several major morphological differences were noted between the wild-type and the MTCH2/MIMP-knock-out embryos. These included: 1) The wild-type embryo has an oval and elongated morphology, whereas the MTCH2/MIMP^{-/-} embryo is significantly smaller in size and rounder in shape. Counting the number of cells in the ectodermal and mesodermal layers in two wild-type and two knockout embryos indicated that the knockout embryos have three times less cells than the wild-type embryos (Figure 2C); 2) The wild-type embryo has well defined extraembryonic (ExEm) and embryonic (Em) regions as expected (Kaufman, M. 1999. The anatomical basis of mouse development. Academic Press, San Diego, CA), whereas in the knockout embryo there is no formal organization of the ExEm region (only one large cavity can be detected; Figures 2A-B). Moreover, it seems that the chorion (Ch) as well as amnion (Am) structures that divide the embryonic internal space are not formed; 3) The wild-type embryo has completed gastrulation and formed three definitive embryonic germ layers (ectoderm, endoderm and mesoderm) as expected (Tam, P.P.,

and Behringer R.R. 1997. Mouse gastrulation: the formation of a mammalian body plan. *Mech Dev* 68:3-25), whereas in the knockout embryo the mesoderm is hardly detectable (Figure 2B). To confirm these observations, whole mount RNA in situ hybridization was performed using an antisense probe to Brachyury. Brachyury is one of the earliest markers of mesoderm formation expressed at the onset of gastrulation at E6.5 (Tam, P.P., and Behringer R.R. 1997, *Supra*). As expected, intense and specific Brachyury expression was detected in the nascent primitive streak of wild type embryos, whereas a diffused and mislocalized expression of Brachyury was detected in the MTCH2/MIMP^{-/-} embryo (Figures 2D-E). Thus, there is a clear defect in mesoderm formation in the Mtch2/Mimp^{-/-} embryos in addition to multiple other defects that are likely to account for the embryonic lethality.

EXAMPLE 2

MTCH2/MIMP IS A POSITIVE REGULATOR OF TBID-INDUCED MOMP

To test the effect of loss of MTCH2/MIMP on apoptosis, wild-type and Mtch2/Mimp^{-/-} embryonic stem (ES) cells were prepared from day E3.5 blastocytes.

Experimental Results

MTCH2/MIMP deficiency reduces the sensitivity to tBID-induced MOMP – Mtch2/Mimp^{-/-} ES cells generated from E3.5 blastocytes were confirmed to lack the Mtch2 protein by Western blot analysis using anti-MTCH2/MIMP antibodies (Figure 3A). The Mtch2/Mimp^{-/-} ES cells were then transfected with either an empty vector or a vector carrying MTCH2/MIMP fused to a Myc-His (MH) tag, and several stable lines carrying either the empty vector (V clones) or MTCH2/MIMP-MH (Rescue or R clones) were generated (Figure 3B; note that the levels of MTCH2/MIMP-MH in the R clones were significantly lower than the levels of the endogenous MTCH2/MIMP in wild-type ES cells). Previous studies performed by the present inventors demonstrated that tBID forms a ~45 kDa cross-linkable complex in mitochondria prepared from apoptotic cells, and that MTCH2/MIMP is the protein that associates with tBID in this complex (Grinberg, M., et al., 2005, *Mol Cell Biol* 25:4579-90). To confirm that R cells can form the tBID complex whereas V cells cannot, both cell lines were infected with adeno-HA-tagged tBID (Ad-tBID; Sarig, R., et al., 2003, *J Biol Chem* 278:10707-15), and mitochondria prepared from these cells were subjected to cross-linking

treatment followed by Western blot analysis using anti-HA antibodies. As expected, the tBID cross-linkable complex appeared in the R cells but not in the V cells (Figure 3C; note that the tBID complex migrates at ~50 kDa due to the addition of the HA and Myc-His tags). These results definitively indicate that MTCH2/MIMP is the mitochondrial protein that forms the cross-linkable complex with tBID, and that the V and R lines are an appropriate tool to assess the importance of this interaction for the function tBID.

To begin to assess the importance of MTCH2/MIMP for tBID-induced MOMP, the sensitivity of the V and R cells to tBID-induced Cyt c release and mitochondria depolarization was examined. Permeabilized HepG2 cells have been previously used to establish at a resolution of seconds the kinetics of tBID-induced Cyt c release/depolarization of mitochondria, and the mitochondrial membrane potential ($\Delta\Psi_m$) was monitored fluorimetrically using TMRE and applied a rapid filtration method to obtain cytosolic/membrane fractions for Western blot analysis (Madesh, M., et al., 2002, J Biol Chem 277:5651-9). A similar protocol was followed now to monitor the mitochondrial membrane potential (except that centrifugation was used instead of filtration for rapid separation of cytosol from the membrane fraction). V and R cells were permeabilized followed by the addition of TMRE and recombinant tBID (three concentrations: 0.3 nM, 0.5 nM or 2 nM). The presence of an F_1F_0 ATPase inhibitor, oligomycin in the bathing medium allowed to observe depolarization when the oxidative metabolism became impaired. Prior to tBID addition the $\Delta\Psi_m$ was slightly more negative in the R cells than in V cells, and both R and V cells responded to 2 nM tBID by rapid depolarization (Figures 3D and E). However, the R cells were more sensitive than V cells to tBID-induced mitochondrial depolarization [Figure 3D and E; identical results were obtained from two additional pairs of single stable clones (data not shown)]. At the end of the recording, the suspension was centrifuged, and the membrane pellet was separated from the supernatant. Western blot analysis of the supernatants using anti-Cyt c antibodies confirmed that the presence of MTCH2/MIMP sensitized the R cells to tBID-induced Cyt c release (Figure 3F). The sensitization to tBID-induced mitochondrial depolarization is probably the result of the sensitization to Cyt c release since the loss of $\Delta\Psi_m$ lagged slightly behind Cyt c release [data not shown]. To assess whether the absence of MTCH2/MIMP affects the ability of tBID to induce BAK/BAX dimerization, cross-linking experiments using the cell

pellets/mitochondrial fractions were performed and it was found that already at low concentrations of tBID, BAX were homodimerized/activated in the R cells (Figure 3G and data not shown). Thus, these results demonstrate that MTCH2/MIMP is a positive regulator of tBID-induced BAX activation and MOMP.

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EXAMPLE 3

CONDITIONAL KNOCKOUT OF MTCH2/MIMP IN MEFS REDUCES THE SENSITIVITY TO TBID-INDUCED APOPTOSIS

Experimental Results

10 **Conditional gene targeting of murine MTCH2/MIMP** - The conventional gene knockout approach demonstrated that knocking out MTCH2/MIMP in mice results in embryonic lethality. In order to be able to dissect the role of MTCH2/MIMP in viable mice and cells, the Cre-loxP system was used to generate a conditional gene knockout mouse (Figure 4A). ES cell clones containing the targeted *MTCH2/MIMP* allele were
15 isolated, and independent lines of genetically modified mice were generated (Figures 17A-B). To define functional consequences of a deletion of MTCH2/MIMP, mouse embryonic fibroblasts (MEFs) were isolated from homozygous *Mtch2/Mimp*^{fl/fl} embryos and transduced with purified Cre-recombinase, leading to efficient deletion of MTCH2/MIMP *in vitro* (Figure 4B). Cross-linking experiments confirmed that tBID
20 forms the ~45kD tBID-MTCH2/MIMP cross-linkable complex in *MTCH2/MIMP*^{fl/fl} cells but not in the same cells transduced with purified Cre-recombinase (Fig. 4C).

To assess the sensitivity to tBID-induced cell death, homozygous *MTCH2/MIMP*^{fl/fl} and heterozygous *MTCH2/MIMP*^{fl/+} MEFs were transduced with Cre-recombinase, infected with HA-tagged tBID [Ad-tBID; Sarig, R. et al. BID-D59A is a
25 potent inducer of apoptosis in primary embryonic fibroblasts. J Biol Chem 278, 10707-15 (2003)] and cell death was monitored. Whereas Cre-transduction did not affect the apoptosis sensitivity in heterozygous *MTCH2/MIMP*^{fl/+} (fl/+) cells, *MTCH2/MIMP*^{fl/fl} (fl/fl) cells transduced with Cre-recombinase were significantly less sensitive to Ad-tBID (Figure 4D). Importantly, reintroduction of MTCH2/MIMP into fl/fl MEFs
30 transduced with Cre-recombinase fully restored susceptibility to tBID-induced cell death (Figure 4E). Thus, MTCH2/MIMP plays an important role in tBID-induced cell death.

To assess whether the effects described above were specific to tBID cells were infected with recombinant adenoviruses carrying BAX, Bim or Noxa vectors. Infection with all three viruses induced high levels of cell death in fl/fl MEFs (as compared to control Ad-GFP), however Cre-recombinase transduction had no effect on their ability to induce cell death (Figure 4F). Thus, MTCH2/MIMP does not play a role in the pro-apoptotic action of other BCL-2 family members. Finally to assess whether the effect of MTCH2/MIMP is conserved in different mammalian species, MTCH2/MIMP was knocked down in human U2OS cells, and this knockdown resulted in a ~40% reduction in Ad-tBID-induced cell death (Figure 3G). Thus, MTCH2/MIMP plays a role in the pro-apoptotic action of tBID also in human cells.

EXAMPLE 4

CONDITIONAL KNOCKOUT OF MTCH2/MIMP IN MEFS HINDERS THE RECRUITMENT OF tBID TO MITOCHONDRIA

To assess the sensitivity to apoptotic signals, fl/fl MEFs were treated as above and exposed to Fas, TNF α , staurosporine (STS), etoposide (Etop), cisplatin (Cis), and ultra-violet (UV). This analysis demonstrated that fl/fl cells after Cre-transduction were less sensitive to cell death induced by Etop and Cis but equally sensitive to the other death reagents (Figure 5A, left panel, and Figure 18A). The decreased sensitivity to Etop and Cis was due to deletion of *MTCH2/MIMP* since fl/+ MEFs transduced with Cre-recombinase did not show decreased sensitivity to these stimuli (Figure 5A, right panel). Caspase-3 cleavage analysis confirmed that deletion of *MTCH2/MIMP* reduces apoptosis induced by Ad-tBID or Etop (Figure 18B).

Next, the status of tBID was examined and it was found that deletion of *MTCH2/MIMP* significantly hindered the recruitment of tBID to mitochondria following treatment with Ad-tBID, Etop, and Fas (Figure 5B, top panels). Western blot analysis of the cytosolic fractions and of total cell lysates indicated that deletion of *MTCH2/MIMP* had no effect on the levels of either full-length BID or tBID (Figure 5B, middle and bottom panels, respectively). Thus, the presence of *MTCH2/MIMP* facilitates the recruitment of tBID to mitochondria.

Next, the affect of *MTCH2/MIMP* deletion on BAX activation and Cyt c release was assessed. BAX undergoes an activating conformational change prior to membrane

integration and oligomerization that includes exposure of its NH₂-terminus, which becomes accessible to protease cleavage [Goping, I. S. et al. Regulated targeting of BAX to mitochondria. J Cell Biol 143, 207-15 (1998)]. It was found that fl/fl MEFs transduced with Cre-recombinase showed significantly less cleavage of BAX by trypsin following treatment with all three stimuli (Figure 5C). The present inventors also found that deletion of *MTCH2/MIMP* significantly reduced the formation of BAX homodimers in cells treated with all three stimuli (Figure 18C). Based on these results the present inventors anticipated that *MTCH2/MIMP* deletion also reduces MOMP, and indeed it was found that fl/fl MEFs transduced with Cre-recombinase showed significantly less Cyt c release following treatment with all three stimuli (Figure 5D and Figures 18D-I). Thus, deletion of *MTCH2/MIMP* hinders tBID recruitment to mitochondria, resulting in less BAX activation and MOMP.

Finally, the fact that Cre-transduction had little effect on apoptosis induced by Fas but hindered Fas-induced tBID recruitment, BAX activation and Cyt c release suggested that fl/fl MEFs are type I cells (i.e., cells in which the mitochondrial pathway does not determine the time course of death receptor-induced apoptosis). Indeed, the present inventors found that fl/fl MEFs infected with recombinant adenoviruses carrying the BCL-2 vector were protected from Etop but not from Fas-induced cell death (Figure 5E).

EXAMPLE 5

MTCH2/MIMP DELETION IN THE LIVER REDUCES THE SENSITIVITY OF MICE TO FAS-INDUCED HEPATOCELLULAR APOPTOSIS AND HINDERS THE RECRUITMENT OF TBID TO MITOCHONDRIA

It was previously demonstrated that BID is a critical substrate *in vivo* for signaling by death-receptor agonists, which mediates a mitochondrial amplification loop essential for the apoptosis of hepatocytes [Yin, X. M. et al. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature 400, 886-91 (1999)]. To determine whether *MTCH2/MIMP* is a critical component of the tBID-death pathway *in vivo*, the present inventors generated *MTCH2/MIMP* liver-specific knockout mice using the Alb-Cre transgene (Figure 19). *MTCH2/MIMP^{fl/Δ};Alb-Cre* (liver-specific knockout) and *MTCH2/MIMP^{fl/+};Alb-Cre* (liver-specific heterozygote) were used for these studies

and Western blotting confirmed efficient and specific deletion of the *MTCH2/MIMP* allele in the liver of the knockout but not of the heterozygous animals (Figure 6A). In addition, analysis of serum liver enzymes indicated that *MTCH2/MIMP* deletion in the liver does not significantly alter the function of hepatocytes (Figure 20).

5 To determine the sensitivity of these mice to Fas, *MTCH2/MIMP^{fl/+}; Alb-Cre* (fl/+) and *MTCH2/MIMP^{fl/Δ}; Alb-Cre* (fl/Δ) mice were injected intraperitoneally with anti-Fas antibody (Jo2; 0.55 μg g⁻¹). Most fl/+ mice (12/15; 80%) died within ~5 hours of acute liver failure associated with massive hepatic apoptosis and haemorrhagic necrosis (Figures 6B-D). In contrast, only ~20% (3/14) of the fl/Δ mice died within 5
10 hours of anti-Fas antibody injection, and the rest either died with delayed kinetics (5/14; 35%), or survived (6/14; ~45%) with no apparent liver injury (Figures 6B-D).

To determine the molecular basis for the differences in sensitivity, three fl/+ and three fl/Δ mice were injected with anti-Fas antibodies, and the mice were sacrificed at three time points post injection (the fl/Δ mice were sacrificed at a 2 hour delay due to
15 their delayed death). Once sacrificed, the eight livers (including livers from two non-injected mice) were lysed, and the cytosolic and mitochondrial fractions were taken for analysis. Analysis of the cytosolic fractions of the fl/+ livers indicated that caspase-8, BID, and caspase-3 have been cleaved/activated (Figure 6E, top, middle, and bottom panels, respectively). Analysis of the cytosolic fractions of the fl/Δ livers indicated that
20 caspase-8 and BID have been cleaved/activated in all three mice to a similar extent as in the fl/+ mice (Figure 6E, top and middle panels). However there was significantly less caspase-3 activation in all three fl/Δ cytosols (Figure 6F, right panel). The caspase-8 and -3 results obtained here are similar to the results obtained with *BID* deficient mice [Yin, X. M. et al. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature 400, 886-91 (1999)], indicating that *MTCH2/MIMP* acts downstream of BID
25 cleavage and upstream of caspase-3 activation.

To determine whether *MTCH2/MIMP* acts at the level of tBID recruitment to mitochondria, the liver mitochondrial fractions were examined for tBID content. Strikingly, the present inventors found that the mitochondrial levels of tBID were
30 significantly lower in all three fl/Δ mitochondrial fractions as compared to the three fl/+ mitochondrial fractions (Figure 6G). The state of BAX activation was also examined and it was found that BAX cleavage by trypsin was also significantly reduced in all

three fl/ Δ mitochondrial fractions (Figure 6H). Of note, *MTCH2/MIMP* deficiency results in increased levels of mitochondrial BAX, which may indicate of an attempt to compensate for the lack of *MTCH2/MIMP*. However, despite this increase, mitochondrial BAX remained largely inactive.

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EXAMPLE 6

MTCH2/MIMP DELETION IN THE LIVER PREVENTS THE IN VITRO IMPORT OF tBID

The results described above suggest that *MTCH2/MIMP* plays a critical role in the recruitment of tBID to liver mitochondria *in vivo*. To verify these findings the present inventors performed *in vitro* import of tBID into liver mitochondria. Cytosolic fractions of 293T cells expressing HA-tBID were incubated with purified intact mouse liver mitochondria prepared from either fl/+ or fl/ Δ mice, followed by centrifugation to separate the mitochondria from the soluble fraction. Fifteen, thirty and sixty minutes after adding HA-tBID to mitochondria, a substantial amount of HA-tBID was incorporated into fl/+ mitochondria, whereas significantly less was incorporated into the fl/ Δ mitochondria (Figure 7A). As expected, the decreased recruitment of HA-tBID to fl/ Δ mitochondria resulted in less/delayed Cyt c release (Figure 7B). Thus, conditional knockout of *MTCH2/MIMP* in the liver prevents the recruitment of tBID to liver mitochondria both *in vivo* and *in vitro*.

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EXAMPLE 7

MTCH2/MIMP IS EXPOSED ON THE SURFACE OF MITOCHONDRIA AND IS LOCALIZED TO THE OMM FRACTION

MTCH2/MIMP is related to members of the mitochondrial carrier (MC) protein family and therefore is likely to be localized to the inner mitochondrial membrane (IMM). To assess the mitochondrial location of *MTCH2/MIMP*, purified mouse liver mitochondria were exposed to proteinase K and it was found that *MTCH2/MIMP* is sensitive to cleavage. These results suggest that either *MTCH2/MIMP* is cleaved due to a broken OMM (enabling proteinase K to reach the IMM) or that *MTCH2/MIMP* is localized to the OMM and exposed to the cytosol. To differentiate between these two possibilities, the studies were repeated and the sensitivity of two additional

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mitochondrial proteins to proteinase K was analyzed: apoptosis inducing factor [AIF, localized to the intermembrane space (IMS)], and adenine nucleotide translocator (ANT, localized to the IMM). Treatment of mitochondria with a low concentration of proteinase K resulted in the cleavage of the entire pool of MTCH2/MIMP, whereas only
5 a low percentage of the AIF and ANT protein molecules was cleaved (Figure 8A, compare lanes 1 and 2 in Figures 8A-C). Treatment of mitochondria with a 10-fold-higher concentration of proteinase K resulted in further cleavage of AIF and some cleavage of ANT, however the majority of the AIF and ANT protein molecules remained intact (Figure 8B and C, compare lanes 1-3). These results suggest that the
10 majority of the mitochondria used in these experiments retained an intact OMM, and therefore argue that MTCH2/MIMP most likely localizes to the OMM and is exposed to the cytosol.

To further assess whether MTCH2/MIMP localizes to the OMM, submitochondrial membrane fractionation studies were performed. Submitochondrial
15 membrane vesicles were prepared from rat liver mitochondria by sonication and separated by centrifugation through a discontinuous sucrose gradient, as was previously described (Bathori, G., et al., 2006, J Biol Chem 281:17347-58). The high-density fractions are enriched in inner membrane vesicles as demonstrated by the presence of the inner membrane proteins cytochrome c oxidase and ANT, whereas the low-density
20 fractions are enriched in outer membrane vesicles as demonstrated by the presence of the outer membrane protein Tom20 (Figures 8D-G). MTCH2/MIMP is most prominent in the low-density fractions, indicating that it is enriched at the outer membrane. Altogether, Figures 68-G show that MTCH2/MIMP is exposed on the surface of mitochondria and is localized to the OMM fraction.

25 *Analysis and Discussion*

The results presented here demonstrate that MTCH2/MIMP is a critical positive regulator of tBID-induced BAX activation and MOMP (see proposed model in Figure 10).

The present inventors have previously found that MTCH2/MIMP interacts with
30 tBID in mitochondria of apoptotic cells, and that this protein is found as part of a large complex in native mitochondria and that tBID and BAX are recruited to this complex and that BCL-X_L inhibits this recruitment, suggesting that MTCH2/MIMP plays a

positive role in regulating tBID/BAX-induced apoptosis (Grinberg, M., et al., 2005, Mol Cell Biol 25:4579-90). In the present study two cellular systems were used in order to explore the role of MTCH2/MIMP in the apoptotic process. In the first *Mtch2/Mimp*^{-/-} ES cell system the present inventors found that *Mtch2/Mimp*^{-/-} ES V cells are less sensitive than R cells expressing MTCH2/MIMP to tBID-induced Cyt c release and loss of $\Delta\Psi_m$ (Figures 3D-F). Importantly, the V and R cells showed similar mitochondrial targeting of tBID but the V cells showed less tBID-induced dimerization of BAX and BAK (at the low concentrations of tBID; Figure 3G), suggesting that MTCH2/MIMP is involved in regulating tBID-induced activation of BAX and BAK, thus MTCH2/MIMP is positively regulating tBID-induced MOMP.

In the second system, conditional knockout MEFs, the physiological role for MTCH2/MIMP that is specific for the pro-apoptotic activity of tBID was established (Figures 4A-G). The present inventors also demonstrated that *MTCH2/MIMP*-deficient MEFs are less sensitive to Etop- and Cis-induced apoptosis, as previously demonstrated for *BID*-deficient cells [Kamer, I. et al. Proapoptotic BID Is an ATM Effector in the DNA-Damage Response. Cell 122, 593-603 (2005); Shelton, S. N., Shawgo, M. E. & Robertson, J. D. Cleavage of Bid by Executioner Caspases Mediates Feed Forward Amplification of Mitochondrial Outer Membrane Permeabilization during Genotoxic Stress-induced Apoptosis in Jurkat Cells. J Biol Chem 284, 11247-55 (2009)]. In addition, the present inventors found that *MTCH2/MIMP*-deficient MEFs showed a significant reduction in tBID recruitment to mitochondria following Ad-tBID, Etop and Fas treatment (Figure 5A-E). *MTCH2/MIMP*-deficient MEFs also hindered BAX activation and Cyt c release following all three stimuli. Thus, MTCH2/MIMP acts as a tBID receptor-like protein in the OMM to facilitate tBID recruitment, resulting in accelerated/effective BAX activation and MOMP.

Using *MTCH2/MIMP* liver-specific knockout mice the present inventors demonstrated that the absence of MTCH2/MIMP decreases the sensitivity of mice to anti-Fas antibody-induced hepatocellular apoptosis (Figures 6A-H). The present inventors also performed a biochemical analysis of livers following Fas treatment and found that *MTCH2/MIMP*-deficiency significantly reduced the mitochondrial recruitment of tBID and the activation of BAX. Moreover, using purified liver mitochondria, the present inventors demonstrated that *MTCH2/MIMP* deletion in the

liver prevents the *in vitro* import of tBID (Figures 7A-B). These results establish a physiological role for MTCH2/MIMP in the Fas-death pathway *in vivo*.

It was previously reported that ~80% of the *BID*-deficient mice injected with anti-Fas antibody were resistant to the injection [Yin, X. M. et al. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature* 400, 886-91 (1999)], whereas the present inventors found that only ~45% of the *MTCH2/MIMP* liver-deficient mice were resistant and most of the rest died with delayed kinetics. Without being bound by any theory, these results suggest that either the absence of MTCH2/MIMP is less cytoprotective than the absence of BID or that Fas might kill mice not only by effects on hepatocytes but also by effects on non-hepatocytes. In favor of the first hypothesis, it was previously demonstrated that caspase-8 liver-conditional knockout mice (also generated using the Alb-Cre transgene) are resistant to anti-Fas antibody injection [Tan, K. O. et al. MAP-1 is a mitochondrial effector of Bax. *Proc Natl Acad Sci U S A* 102, 14623-8 (2005)]. Thus, the absence of MTCH2/MIMP is less cytoprotective than the absence of either BID or caspase-8.

The present inventors have demonstrated that MTCH2/MIMP acts at the very early stages of MOMP by facilitating the recruitment of tBID, the initiator of this process. The studies in mice demonstrate that MTCH2/MIMP is an indispensable player in the tBID-death pathway required for effective hepatocellular apoptosis.

EXAMPLE 8

PEPTIDES OF THE MTCH2/MIMP PROTEIN ARE CAPABLE OF BINDING TBID

To identify Mtch2-derived peptides that bind to tBID the present inventors have screened an Mtch2-peptide library with recombinant tBID, as follows.

Experimental Results

To identify the interaction sites of Mtch2/Mimp with tBID, a peptide array containing 37 overlapping peptides derived from the sequence of the full-length mouse Mtch2/Mimp (SEQ ID NO:1) was designed. Peptide length was between 10-30 residues. Peptides were designed based on the predicted secondary structure of the Mtch2/Mimp protein, and recombinant tBID or BID were screened for binding the peptide array using the procedure described in (Hayouka, Z., et al., 2007). tBID bound

peptides are derived from two sites that are distant from each other according to their linear position on the Mtch2 sequence but are in spatial proximity according to the Mtch2/Mimp model (Figures 9A-D and Table 4, hereinbelow). The first site consists of helix 6, between residues 140-161 (SEQ ID NO:5), and the second site consists of the whole C-terminal part of the protein, between residues 240-300 (SEQ ID NOs:8-12). This second site is represented by several peptides that bind Mtch2/Mimp with different strengths, with peptide F17 (SEQ ID NO:8; located at position 240-254 of the mouse Mtch2 protein set forth by SEQ ID NO:1) binding the tightest to tBID (Figure 9D, colored strong red).

10

Table 4

Peptide array screening results: peptides derived from mouse Mtch2/Mimp (SEQ ID NO:1) which bind the tBID protein

<i>Peptide number in array</i>	<i>Residues in mouse Mtch2 (SEQ ID NO:1)</i>	<i>Amino acid sequence</i>	<i>Binds to tBID/BID</i>	<i>SEQ ID NO:</i>	<i>Length</i>
F6	140-161	PFHVITLRSMVQFIGRESKYCG	BID and tBID	5	22
F7	153-167	IGRESKYCGLCDSIV	tBID	6	15
F13	210-224	SGVSTMNEMKSYSQA	tBID	7	15
F17	240-254	VSNLMAVNNCGLAGG	tBID	8	15
F19	247-264	NNCGLAGGSPPIYTS	tBID	9	18
F21	270-283	CMLQKAGNMSRGNS	tBID	10	14
F22	265-275	WIDCWCMLQKA	tBID	11	11
G1	284-300	LFFRKVPCGKTYCYDLR	tBID and BID	12	17
E23	90-104	KVLQYYQESEKPEEL	BID	13	15
E24	75-99	TPRLCSGVLGTVVHGKVLQYYQESE	BID	14	25
F2	105-119	GSVTVQKEYSSSFDR	BID	15	15

Table 4: Peptides from the mouse MTCH2/MIMP (SEQ ID NO:1; GenBank Accession No. GenBank accession number AAD52647; gi:5815347) which bind the mouse tBID or BID proteins according to the peptide-array immunoblot (Figures 9A-B).

Table 5

Peptides derived from human Mtch2/Mimp (SEQ ID NO:2) which bind the tBID protein

<i>Residues in human Mtch2 (SEQ ID NO:2)</i>	<i>Amino acid sequence</i>	<i>SEQ ID NO:</i>	<i>Length</i>
140-161	PFHVITLRSMVQFIGRESKYCG	16	22
153-167	IGRESKYCGLCDSII	17	15
210-224	SGVSTMNEMKSYSQA	18	15

<i>Residues in human Mtch2 (SEQ ID NO:2)</i>	<i>Amino acid sequence</i>	<i>SEQ ID NO:</i>	<i>Length</i>
240-254	VSNLMAVNNCGLAGG	19	15
247-264	NNCGLAGGCPPYSPIYTS	20	18
270-283	CMLQKEGNMSRGNS	21	14
265-275	WIDCWCMLQKE	22	11
284-300	LFFRKVPFGKTYCCDLK	23	17
90-104	KVLQHYQESDKGEEL	24	15
75-99	TPRLCSGVLGTVVHGKVLQHYQESD	25	25
105-119	GPGNVQKEVSSSFHD	26	15

Table 5: Peptides from the human MTCH2/MIMP (SEQ ID NO:2; GenBank Accession No. NP_055157; gi:7657347) which are predicted to bind the human tBID or BID proteins based on the homology to the mouse Mtch2/Mimp.

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EXAMPLE 9

PEPTIDES OF THE tBID PROTEIN ARE CAPABLE OF BINDING MTCH2

To identify tBID-derived peptides that bind to Mtch2 the present inventors have employed a cross-linking strategy followed by mass spectroscopy analysis, as follows.

Experimental Results

10

Identification of BID-derived peptides that bind Mtch2 – The present inventors have previously demonstrated that HA-tBID is capable of forming a 45 kDa complex in 293T cells that represents a complex with MTCH2/MIMP. The HA-BID complex (which included mouse BID) was purified and the peptides that are involved in the interaction with Mtch2 were identified. The mitochondria-enriched heavy membrane fraction prepared from 293T cells transfected with HA-BID was treated with cross-linker and then lysed. The mitochondrial lysate was then incubated with anti-HA antibodies coupled to agarose beads, and the HA-BID monomers/complexes were eluted with an HA peptide (see “General Materials and Experimental Methods”). In this gel, HA-BID and HA-tBID were clearly visible as a ~22 kDa and ~15 kDa bands (confirmed by mass spectrometry). Mass spectrometry analysis of the ~50 kDa band revealed that it included seven peptides from BID (Figure 11, highlighted in yellow). Five BID peptides were not identified and three of them are peptides from tBID (Figure 11, see peptides that were not highlighted; tBID is generated from cleavage of BID at Asp59 marked in red). A possible reason that these peptides were not identified by the

15

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MS analysis is that they were "trapped" by the cross-linker (potential sites of cross-linker binding in one of these peptides are marked in gray).

Tables 6 and 7 present peptides from tBID that interact with MTCH2/MIMP.

5

Table 6
Peptides from human BID (SEQ ID NO:4) which bind Mtch2

<i>Residues in human BID (SEQ ID NO:4)</i>	<i>Amino acid sequence</i>	<i>SEQ ID NO:</i>	<i>Length</i>
61-71	GNRSSHSRLGR	30	11
125-140	RDLATALEQLLQAYPR	31	16
188-195	SLARNGMD	32	8

Table 6: Peptides from human BID (SEQ ID NO:4; GenBank Accession No. NP_001187) encoded by human BID mRNA (GenBank Accession No. NM_001196.2; gi: 37574724; SEQ ID NO:35).

10

Table 7
Peptides from mouse BID (SEQ ID NO:3) which bind Mtch2

15

<i>Residues in human BID (SEQ ID NO:4)</i>	<i>Amino acid sequence</i>	<i>SEQ ID NO:</i>	<i>Length</i>
60-71	GSQASRSFNQGR	27	12
125-140	NCLAKALDEVKTAFFPR	28	16
188-195	NLVRNEMD	29	8

Table 7: Peptides from mouse BID (SEQ ID NO:3; GenBank Accession No. AAC71064) encoded by mouse BID mRNA (GenBank Accession No. MMU75506; gi:1669513; SEQ ID NO:33).

20

EXAMPLE 10

IDENTIFICATION OF HUMAN BID-DERIVED PEPTIDES WHICH BIND TO MTCH2/MIMP 240-290

25

The MTCH2/MIMP 240-290 peptide exhibits high affinity towards tBID – The present inventors synthesized peptides and determined their binding affinity toward tBID using fluorescence anisotropy. The results show 2 peptides that bind tBID in the μ M range: MTCH2/MIMP 240-290 (SEQ ID NO:106; VSNLMAVNNCGLAGGSPYSPITYSWIDCWCMLQKAGNMSRGNSLFFRKVP) and MTCH2/MIMP 140-161 (SEQ ID NO:107; Pro Phe His Val Ile Thr Leu Arg Ser Met Val Gln Phe Ile Gly Arg Glu Ser Lys Tyr Cys Gly). The MTCH2/MIMP 240-290

peptide, which can be considered as a domain of the C terminal part of Mtch2, exhibits a higher binding affinity to tBID protein relative to the Mtch2 140-161 peptide. The MTCH2/MIMP 240-290 and MTCH2/MIMP 140-161 peptides were labeled with fluorescein at their N terminal, which allows examination of their ability to penetrate
5 cells using a confocal microscope. Both peptides did not penetrate cells, and therefore were further manipulated using a known cell penetrating peptide (penetratinTM peptide; RQIKIWFQNRRMKWKK; SEQ ID NO:131).

EXAMPLE 11

10 ***BINDING OF BIOTINYLATED HUMAN MTCH2 PEPTIDES TO A BID- DERIVED PEPTIDE ARRAY***

Experimental Methods

Biotinylated MTCH2/MIMP peptides were used to identify BID derived peptides which specifically interact therewith. The bound peptides were identified using
15 avidin conjugated to HRP. The binding experiment was performed with different peptide concentrations (e.g., using 20 and 40 μ M peptide concentration) and in the presence of a buffer which contains: 50 mM Tris PH = 7.5, 50, 100 or 150 mM NaCl, milk 2.5 %, Tween 0.05 %. The different concentrations of NaCl provide a range of ionic strengths (IS). The array contains peptides derived from six different proteins, of
20 which the first row are BID-derived peptides. Each peptide array is a double array, with two replicas of the array on the same slide.

Experimental Results

Incubation of MTCH2/MIMP 240-290 with peptides derived from human-Bid
25 ***protein*** - Figures 12A-C show the selective binding of the MTCH2/MIMP 240-290 peptide at a concentration of 20 μ M on the human BID-derived peptide array under 3 different ionic strengths obtained in the presence of 150 mM NaCl (Figure 12A), 100 mM NaCl (Figure 12B) and 50 mM NaCl (Figure 12C). The binding results are summarized in Table 8, below.

Table 8

Bid-derived peptides that binds MTCH2/MIMP 240-290

<i>Position of peptide on the array</i>	<i>Amino acid residues on human BID</i>	<i>Sequence</i>	<i>SEQ ID NO:</i>	<i>Length (amino acids)</i>	<i>IS = 50 mM NaCl</i>	<i>IS = 100 mM NaCl</i>	<i>IS = 150 mM NaCl</i>	
Peptide Conc.					20 μ M	20 μ M	20 μ M	40 μ M
A2	6-20	NNGSSLRDEC ITNLL	108	15		Strong	Medium	
A4	20-34	LVFGFLQSCS DNSFR	109	15		Strong	Medium	
A5	27-34	SCSDNSFR	110	8	medium	Strong	Strong	
A11	59-73	TDGNRSSHRL LGRIE	111	15		Weak	Strong	Medium
A20	111-125	LQLRNTSRSE EDRNR	112	15			Medium	Weak
5B	180-191	QNLRTYVRSL AR	113	12		Weak	Weak	

Table 8. The position of peptide on the array refers to the BID-derived peptide array shown in Figures 12A-C.

Figures 13A and 13B demonstrate the binding of MTCH2/MIMP 240-290 peptide in relation to the secondary structure (Figure 13A) and three-dimensional structure (Figure 13B) of BID.

Incubation of MTCH2/MIMP 140-161 with peptides derived from human-Bid protein - Figures 14A-C show the selective binding of the MTCH2/MIMP 140-161 peptide at a concentration of 40 μ M on the human BID-derived peptide array under 3 different ionic strengths obtained in the presence of 150 mM NaCl (Figure 14A), 100 mM NaCl (Figure 14B) and 50 mM NaCl (Figure 14C). The binding results are summarized in Table 9, below.

Table 9

BID-derived peptides that binds Mtch2 140-161

<i>Position of peptide on the array</i>	<i>Amino acid residues on human BID</i>	<i>Sequence</i>	<i>SEQ ID NO:</i>	<i>Length (amino acids)</i>	<i>IS = 50 mM NaCl</i>	<i>IS = 100 mM NaCl</i>	<i>IS = 150 mM NaCl</i>
A5	27-34	SCSDNSFR	110	8	weak	Strong	Medium
A11	59-73	TDGNRSSHRL GRIE	111	15			Strong

<i>Position of peptide on the array</i>	<i>Amino acid residues on human BID</i>	<i>Sequence</i>	<i>SEQ ID NO:</i>	<i>Length (amino acids)</i>	<i>IS = 50 mM NaCl</i>	<i>IS = 100 mM NaCl</i>	<i>IS = 150 mM NaCl</i>
A12	62-76	NRSSHSRLGRIE ADS	114	15			Medium
5B	180-191	QNLRTYVRSLA R	113	12		Very weak	Weak

Table 9. MTCH2/MIMP peptide concentration was 40 μ M.

It should be noted that a Trp amino acid (W) was added to the peptides in order to measure the peptides concentration using UV spectroscopy for all assays.

1. tBID W+ 59-73 - WTDGNRSSHSRLGRIE (SEQ ID NO:126);
2. tBID W+ 62-76 – WNRSSHSRLGRIEADS (SEQ ID NO:127);
3. tBID W+ 61-73 – WGNRSSHSRLGRIE (SEQ ID NO:128);
4. tBID W+ 62-73 – WNRSSHSRLGRIE (SEQ ID NO:129);
5. tBID W+111-125 – WLQLRNTSRSEEDRNR (SEQ ID NO:130);

Figures 15A and 15B demonstrate the binding of MTCH2/MIMP 140-161 peptide in relation to the secondary structure (Figure 15A) and three-dimensional structure (Figure 15B) of BID.

In summary:

MTCH2/MIMP 240-290 domain binds to the following BID peptides:

1. Full length BID 27-34 AA (SEQ ID NO:110); (“AA” = amino acid”);
2. Full length Bid 20-34 AA (SEQ ID NO:109);
3. Full length Bid 6-20 AA (SEQ ID NO:108);
4. tBid 59-73 AA (SEQ ID NO:111);
5. tBid 111-125 AA (SEQ ID NO:112);
6. tBid 180-191 AA (SEQ ID NO:113);

Mtch2 140-161 peptide binds an additional peptide that was not found to bind for MTCH2/MIMP 240-290:

7. tBid 62-76 AA (SEQ ID NO:114);

MTCH2/MIMP 140-161 also binds some of the peptides that MTCH2/MIMP 240-290 bound:

1. Full length BID 27-34 AA (SEQ ID NO:110);
4. tBid 59-73 AA (SEQ ID NO:111);
6. tBid 180-191 AA (SEQ ID NO:113);

Full-length human BID is cleaved between D60/G61 (mouse BID is cleaved
5 between D59/G60) to generate tBID, and tBID (61- 195 aa) is the active part that
interacts with MTCH2/MIMP and induces cytochrome c release. Thus, the BID
peptides 59-73 (SEQ ID NO:111) / 62-76 (SEQ ID NO:114), 61-73
(GNRSSHSRLGRIE; SEQ ID NO:116) 62-73 (NRSSHSRLGRIE, SEQ ID NO:115),
111-125 (SEQ ID NO:112), and 180-191 (SEQ ID NO:113) are important for binding
10 to MTCH2/MIMP. Full-length BID seems also to interact with MTCH2/MIMP as is
evidenced by the binding of the N-terminal peptides 6-20 (SEQ ID NO:108), 20-34
(SEQ ID NO:109) and 27-34 (SEQ ID NO:110).

15 Although the invention has been described in conjunction with specific
embodiments thereof, it is evident that many alternatives, modifications and variations
will be apparent to those skilled in the art. Accordingly, it is intended to embrace all
such alternatives, modifications and variations that fall within the spirit and broad scope
of the appended claims.

20 All publications, patents and patent applications mentioned in this specification
are herein incorporated in their entirety by reference into the specification, to the same
extent as if each individual publication, patent or patent application was specifically and
individually indicated to be incorporated herein by reference. In addition, citation or
identification of any reference in this application shall not be construed as an admission
25 that such reference is available as prior art to the present invention. To the extent that
section headings are used, they should not be construed as necessarily limiting.

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WHAT IS CLAIMED IS:

1. An isolated peptide comprising the amino acid sequence set forth by SEQ ID NO: 111, 30, 31, 32, 115, 114, 112, 113, 108, 109, 110, 116 or 117, wherein said amino acid sequence is less than 50 in length and whereas the peptide increases a level of apoptosis in a cell.
2. An isolated peptide consisting of the amino acid sequence selected from the group consisting of SEQ ID NOs: 111, 30, 31, 32, 115, 114, 112, 113, 108, 109, 110, 116 and 117.
3. An isolated peptide consisting of the amino acid sequence set forth by SEQ ID NO:106, 107, 16, 19, 23, 17, 18, 20, 21, 22, 24, 25, 26, 136, 137 or 138.
4. An isolated peptide comprising the amino acid sequence selected from the group consisting of SEQ ID NOs:106, 107, 16, 19, 23, 17, 18, 20, 21, 22, 24, 25, 26, 136, 137 and 138, wherein said amino acid sequence is less than 60 in length and whereas the peptide decreases a level of apoptosis in a cell, with the proviso that said amino acid sequence is not the amino acid sequence set forth by SEQ ID NO:36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68 or 69.
5. An isolated molecule comprising the isolated peptide of claim 1 or 2, attached to an amino acid sequence which enhances penetration of the peptide into a cell.
6. An isolated molecule comprising the isolated peptide of claim 3 or 4 attached to an amino acid sequence which enhances penetration of the peptide into a cell.

7. An isolated polynucleotide comprising a nucleic acid sequence encoding an amino acid sequence consisting of the amino acid sequence of claim 1 or 2, or of the isolated molecule of claim 5.

8. An isolated polynucleotide comprising a nucleic acid sequence encoding an amino acid sequence consisting of the amino acid sequence of claim 3 or 4 or of the isolated molecule of claim 6.

9. A nucleic acid construct comprising the isolated polynucleotide of claim 7 and a promoter for directing expression of said amino acid sequence in a host cell.

10. A nucleic acid construct comprising the isolated polynucleotide of claim 8 and a promoter for directing expression of said amino acid sequence in a host cell.

11. A pharmaceutical composition comprising as an active ingredient the isolated peptide of claim 1, 2, 3 or 4, the isolated molecule of claim 5 or 6, the isolated polynucleotide of claim 7 or 8 or the nucleic acid construct of claim 9 or 10.

12. A method of upregulating apoptosis in a cell, comprising contacting the cell with the peptide of claim 1 or 2, the isolated molecule of claim 5, the isolated polynucleotide of claim 7 or the nucleic acid construct of claim 9, thereby upregulating the apoptosis in the cell.

13. A method of downregulating apoptosis in a cell, comprising contacting the cell with the peptide of claim 3 or 4, the isolated molecule of claim 6, the isolated polynucleotide of claim 8 or the nucleic acid construct of claim 10, thereby downregulating the apoptosis in the cell.

14. A method of treating a pathology associated with abnormally low levels of apoptosis in a subject, comprising administering to the subject a therapeutically effective amount of the peptide of claim 1 or 2, the isolated molecule of claim 5, the

isolated polynucleotide of claim 7 or the nucleic acid construct of claim 9, thereby treating the pathology associated with abnormally low levels of apoptosis in the subject.

15. A method of treating a pathology associated with abnormally high levels of apoptosis in a subject, comprising administering to the subject a therapeutically effective amount of the peptide of claim 3 or 4, the isolated molecule of claim 6, the isolated polynucleotide of claim 8 or the nucleic acid construct of claim 10, thereby treating the pathology associated with abnormally high levels of apoptosis in the subject.

16. The isolated peptide of claim 1, 2, 3 or 4, the isolated molecule of claim 5 or 6, the pharmaceutical composition of claim 11 or the method of claim 12, 13, 14 or 15, wherein the peptide is cyclic.

17. The method of claim 15, wherein the pathology associated with abnormally high levels of apoptosis is a degenerative disorder.

18. The method of claim 17, wherein said degenerative disorder is selected from the group consisting of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) and retinitis pigmentosa.

19. The method of claim 15, wherein the pathology associated with abnormally high levels of apoptosis is human immunodeficiency virus (HIV)-induced acquired immunodeficiency syndrome (AIDS).

20. The method of claim 14, wherein the pathology associated with abnormally low levels of apoptosis is selected from the group consisting of cancer, an autoimmune disorder, a bacterial infection, and a viral infection.

21. The isolated molecule of claim 5 or 6, wherein said amino acid sequence which enhances penetration of the peptide into a cell is set forth by SEQ ID NO:131.

FIG. 1A

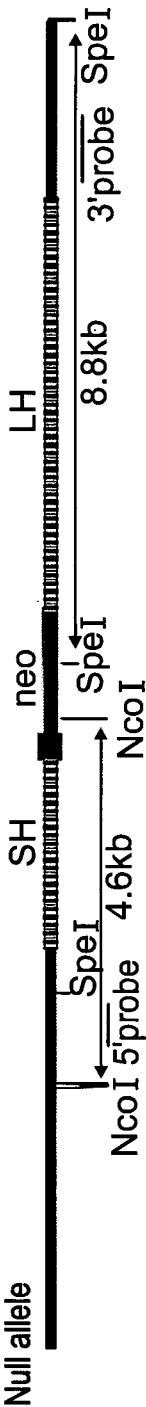
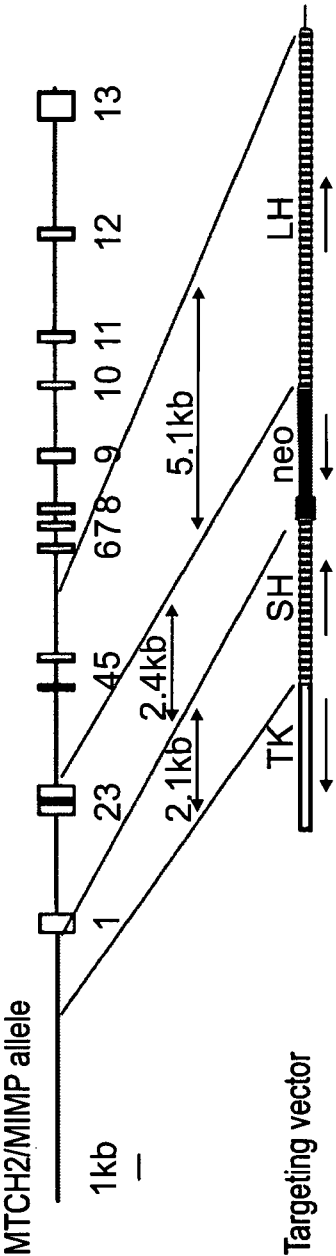


FIG. 1B

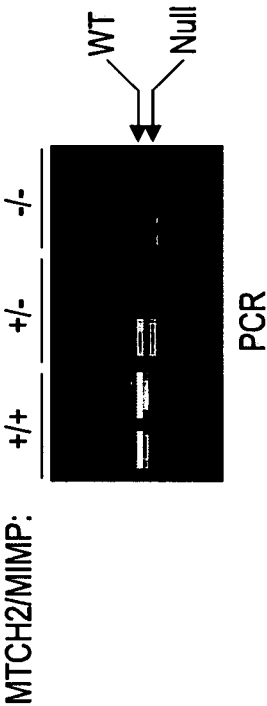


FIG. 2B

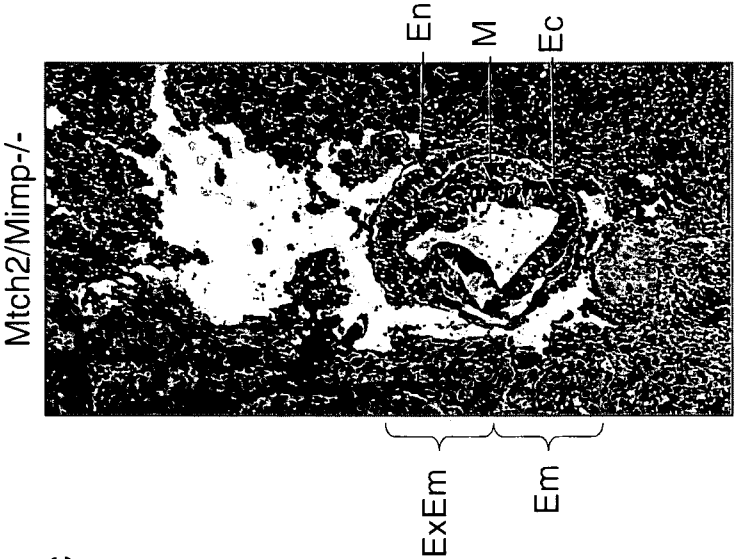


FIG. 2A

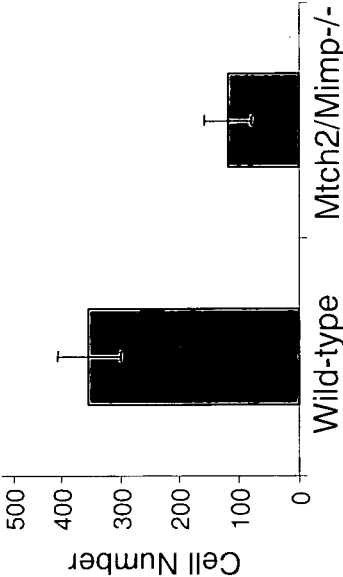
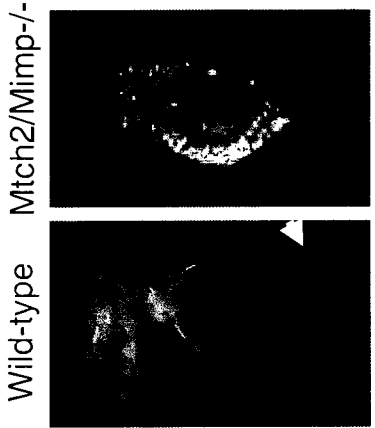
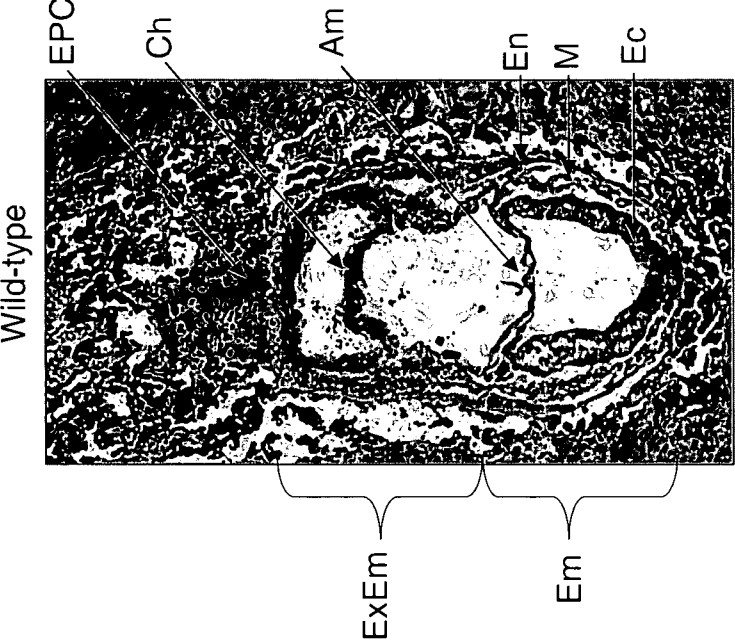


FIG. 2D

FIG. 2E

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FIG. 3A

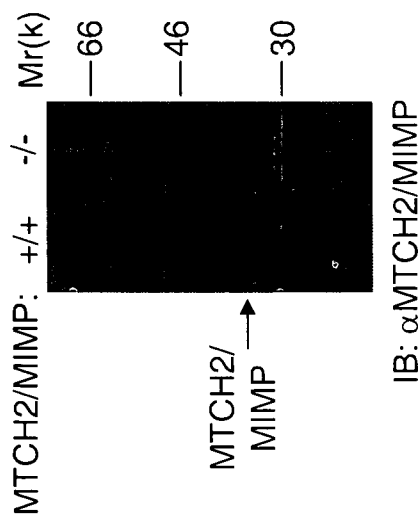


FIG. 3B

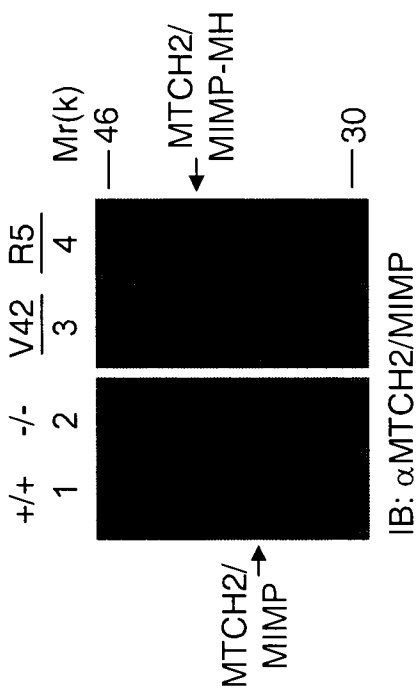


FIG. 3C

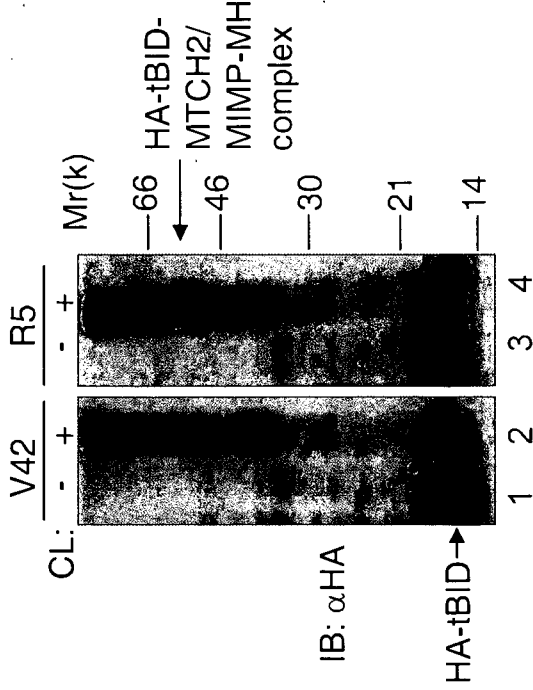


FIG. 3D

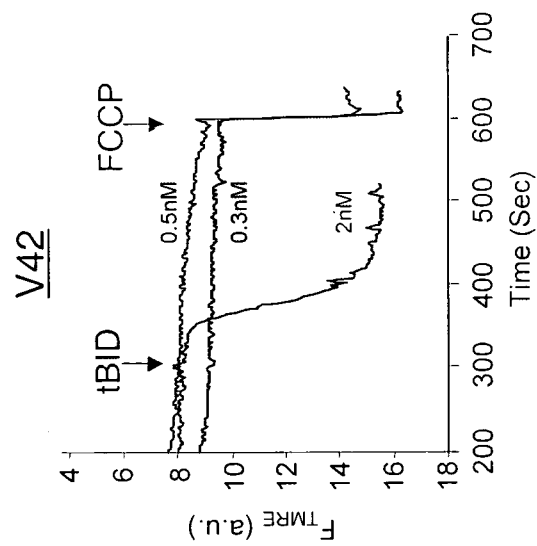
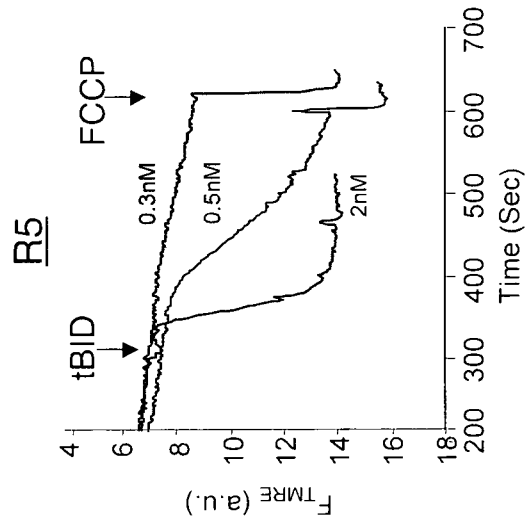


FIG. 3E



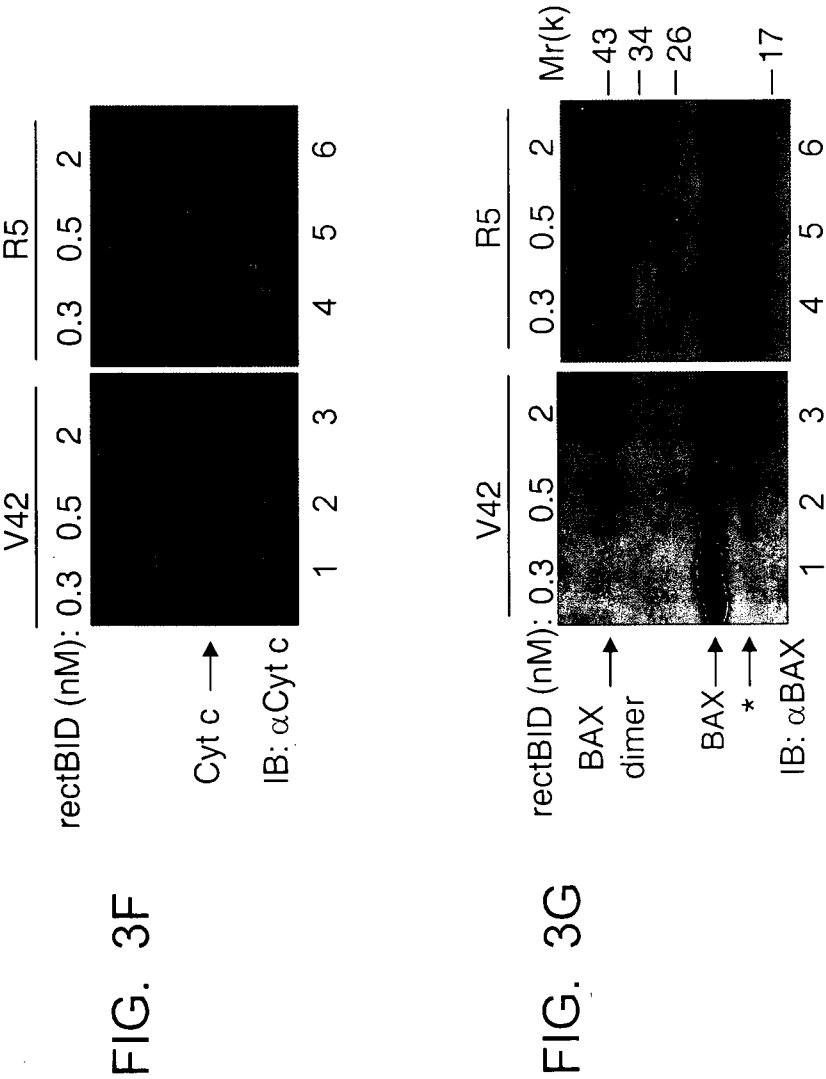


FIG. 4A

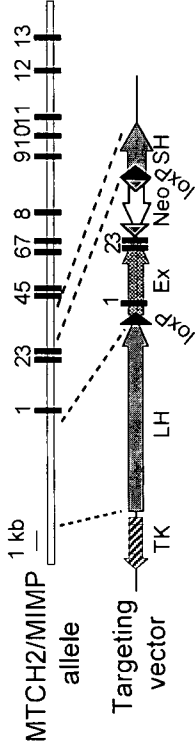


FIG. 4B

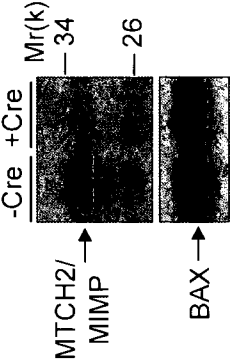


FIG. 4C

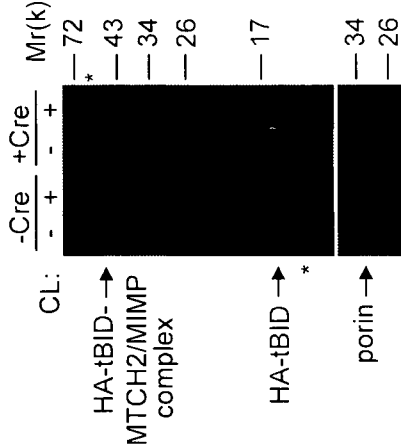


FIG. 4D

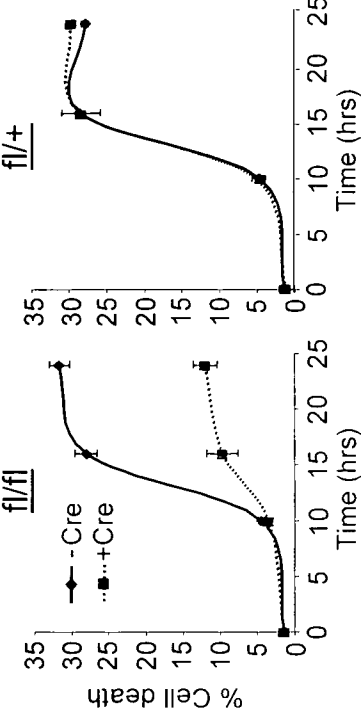


FIG. 4E

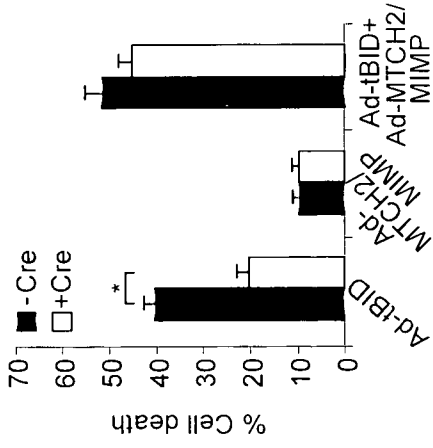


FIG. 4F

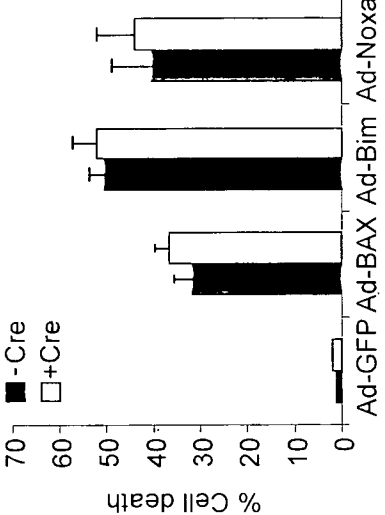
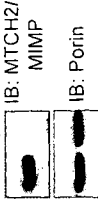
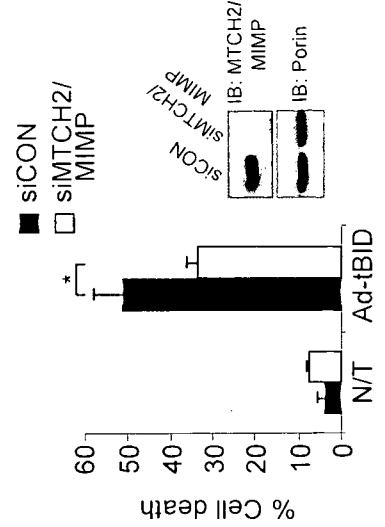


FIG. 4G



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FIG. 5A

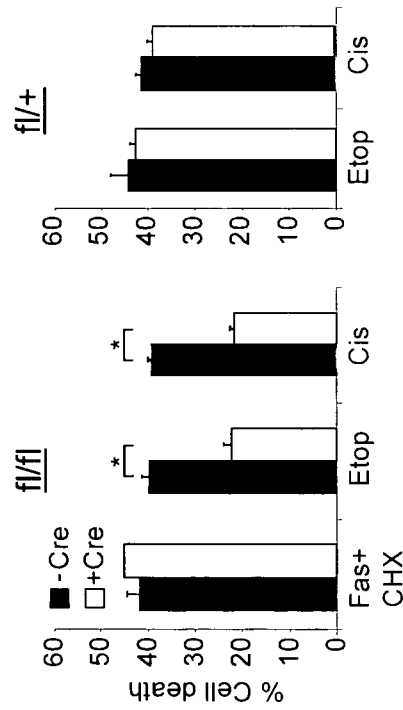


FIG. 5B

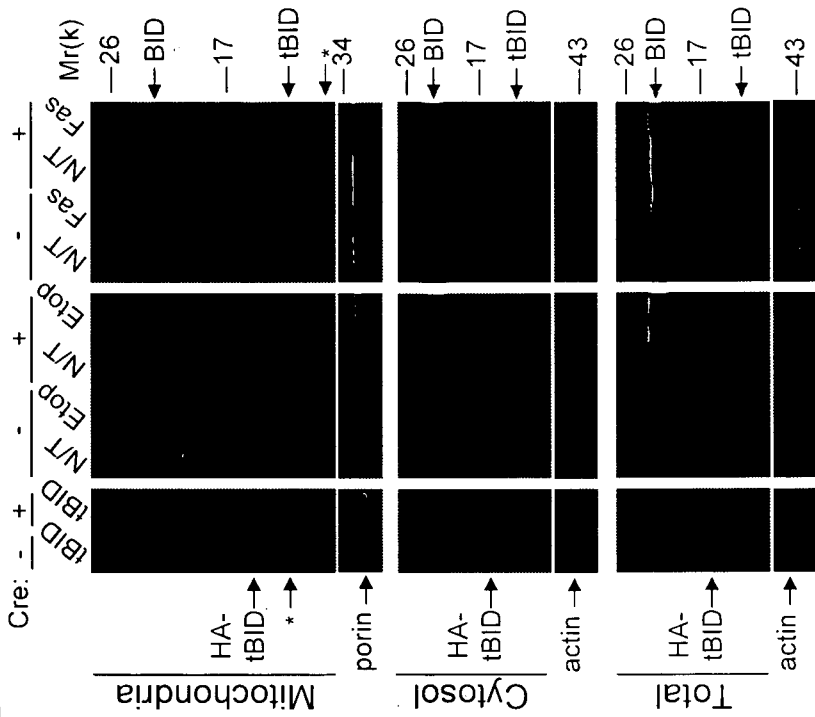


FIG. 5C

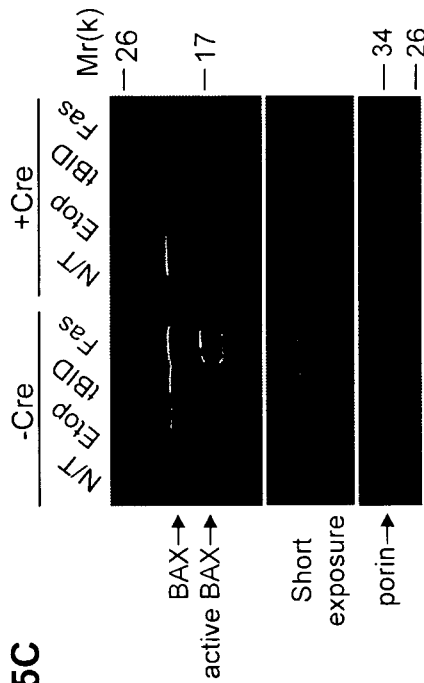


FIG. 5D

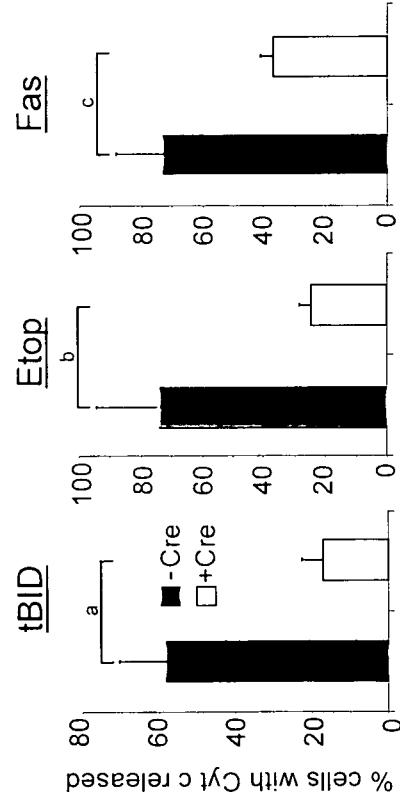
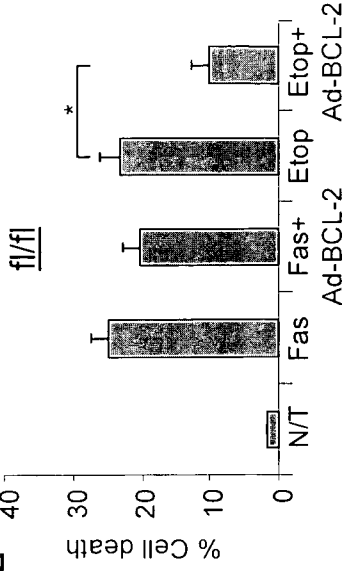


FIG. 5E



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FIG. 6A

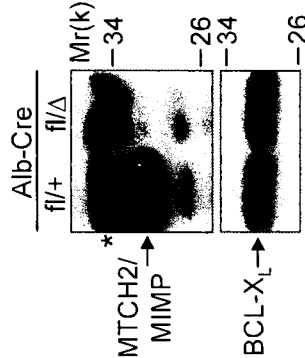


FIG. 6B

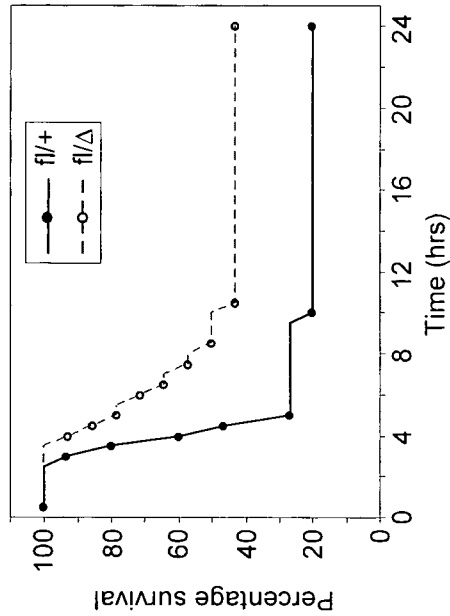


FIG. 6C

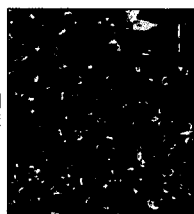
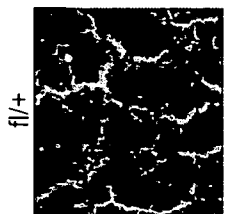


FIG. 6D

FIG. 6E

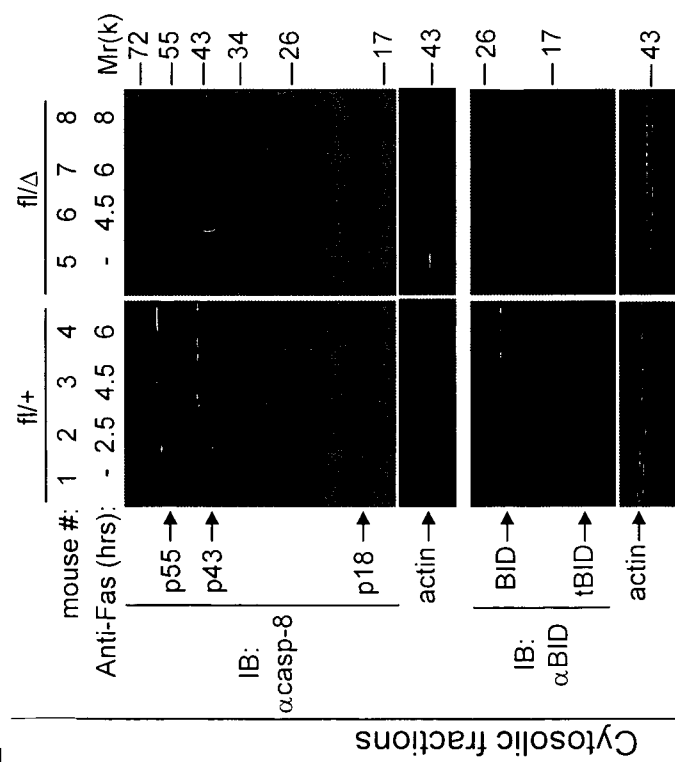


FIG. 6F

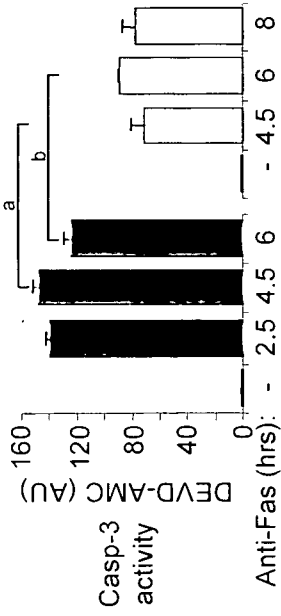


FIG. 6G

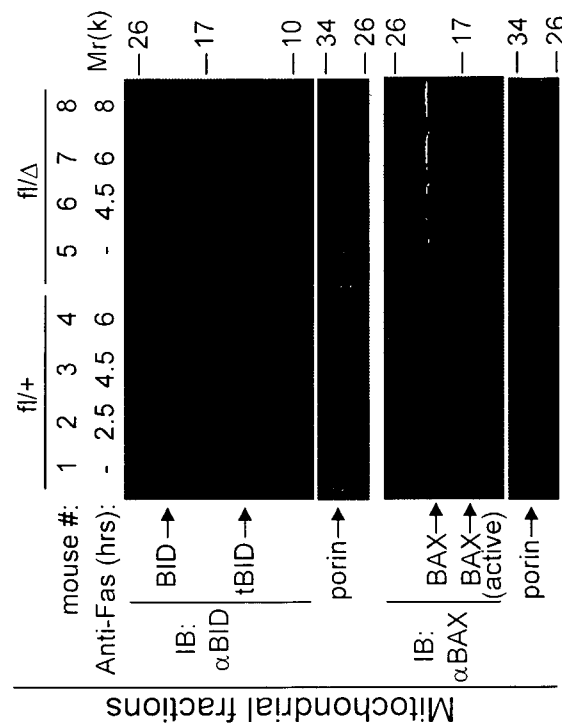


FIG. 6H

FIG. 7B

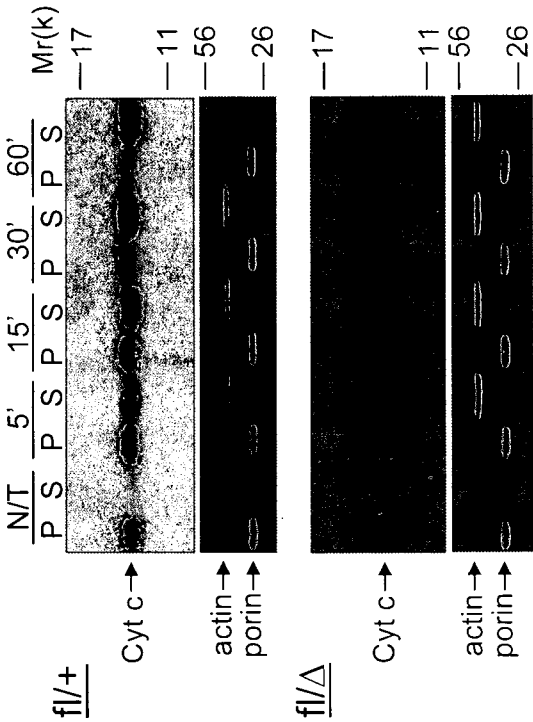


FIG. 7A

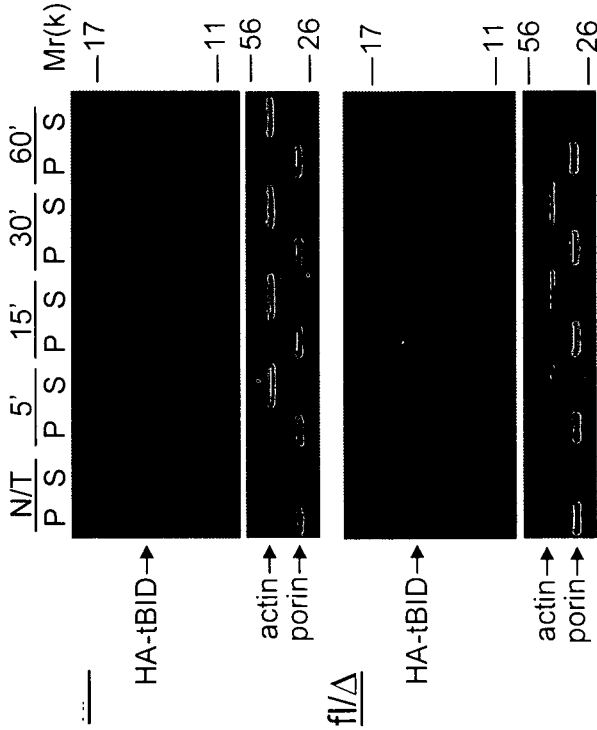


FIG. 8A

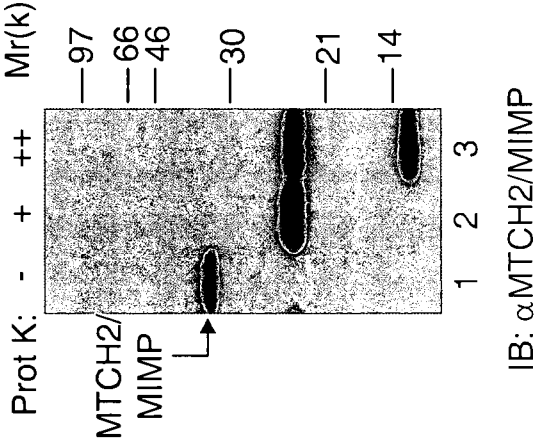


FIG. 8B

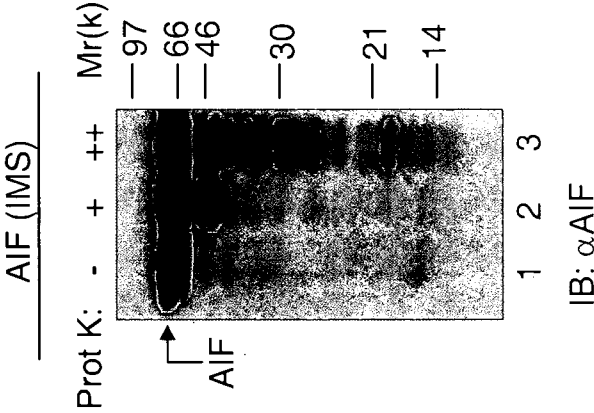
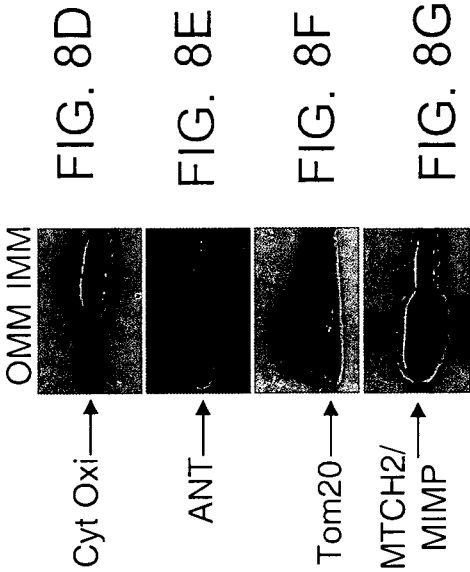
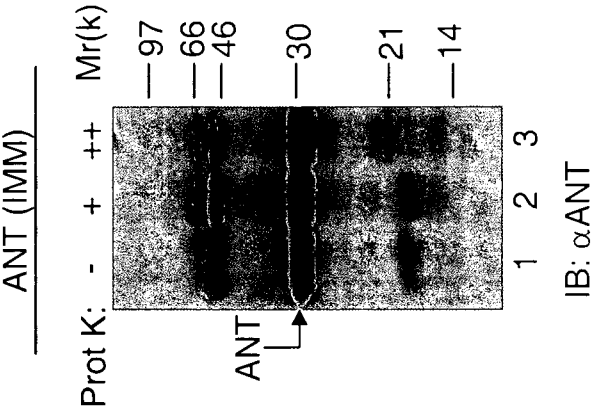


FIG. 8C



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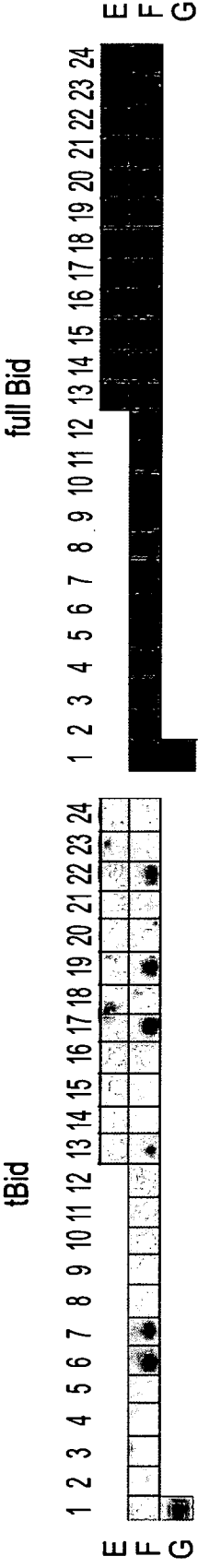
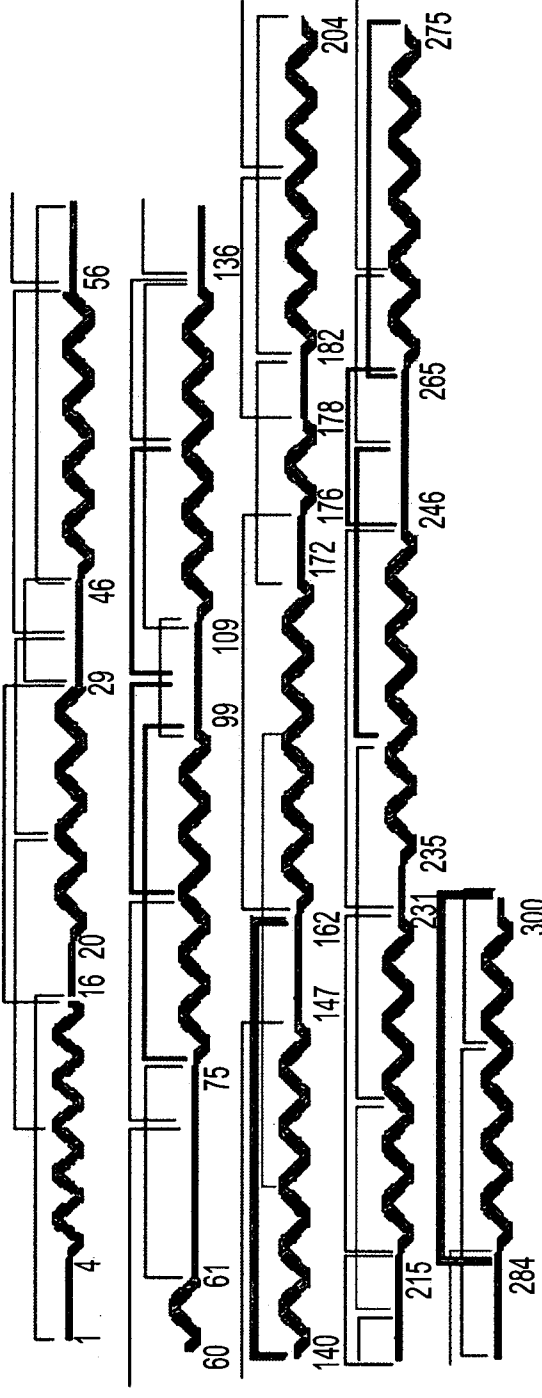


FIG. 9B

- Peptides that did not bind
- Strong binding from tBid
- Midum binding from tBid
- Weak binding from tBid
- Binding peptides from full Bid



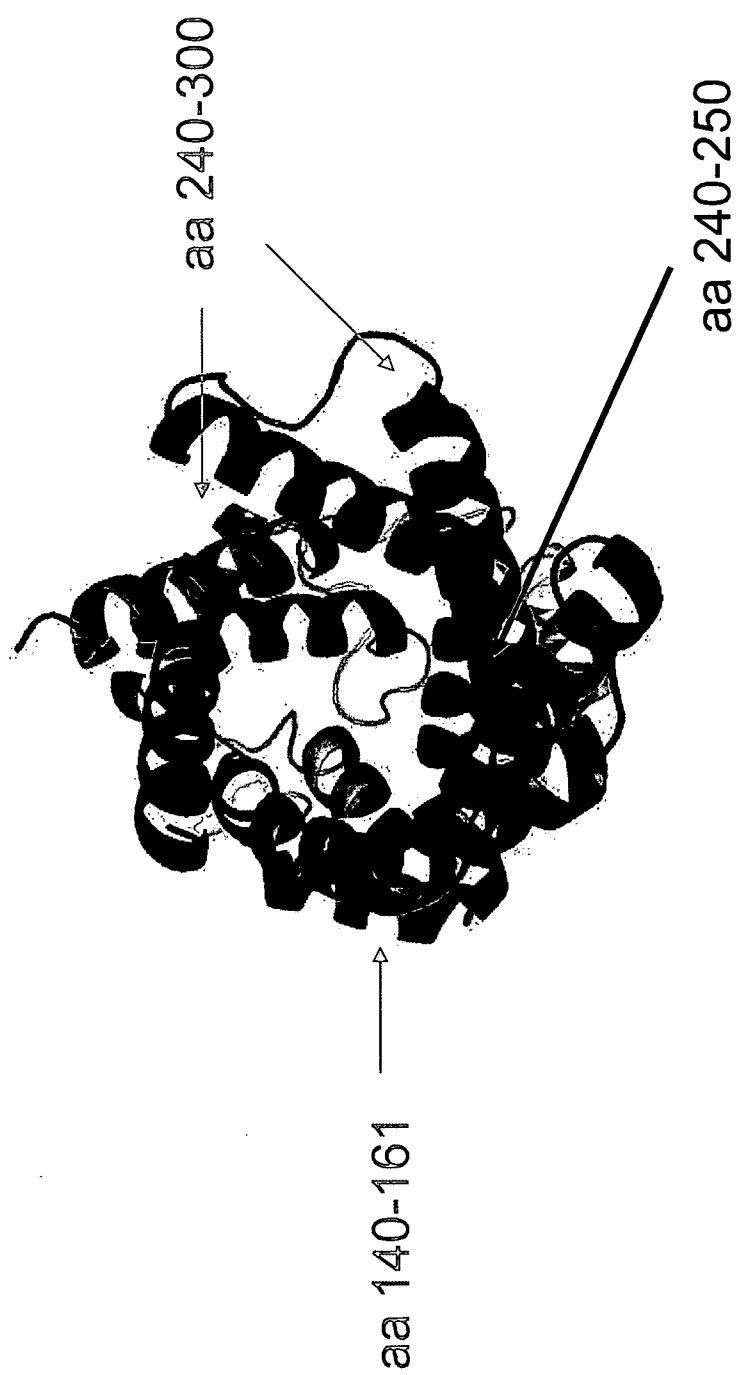


FIG. 9D

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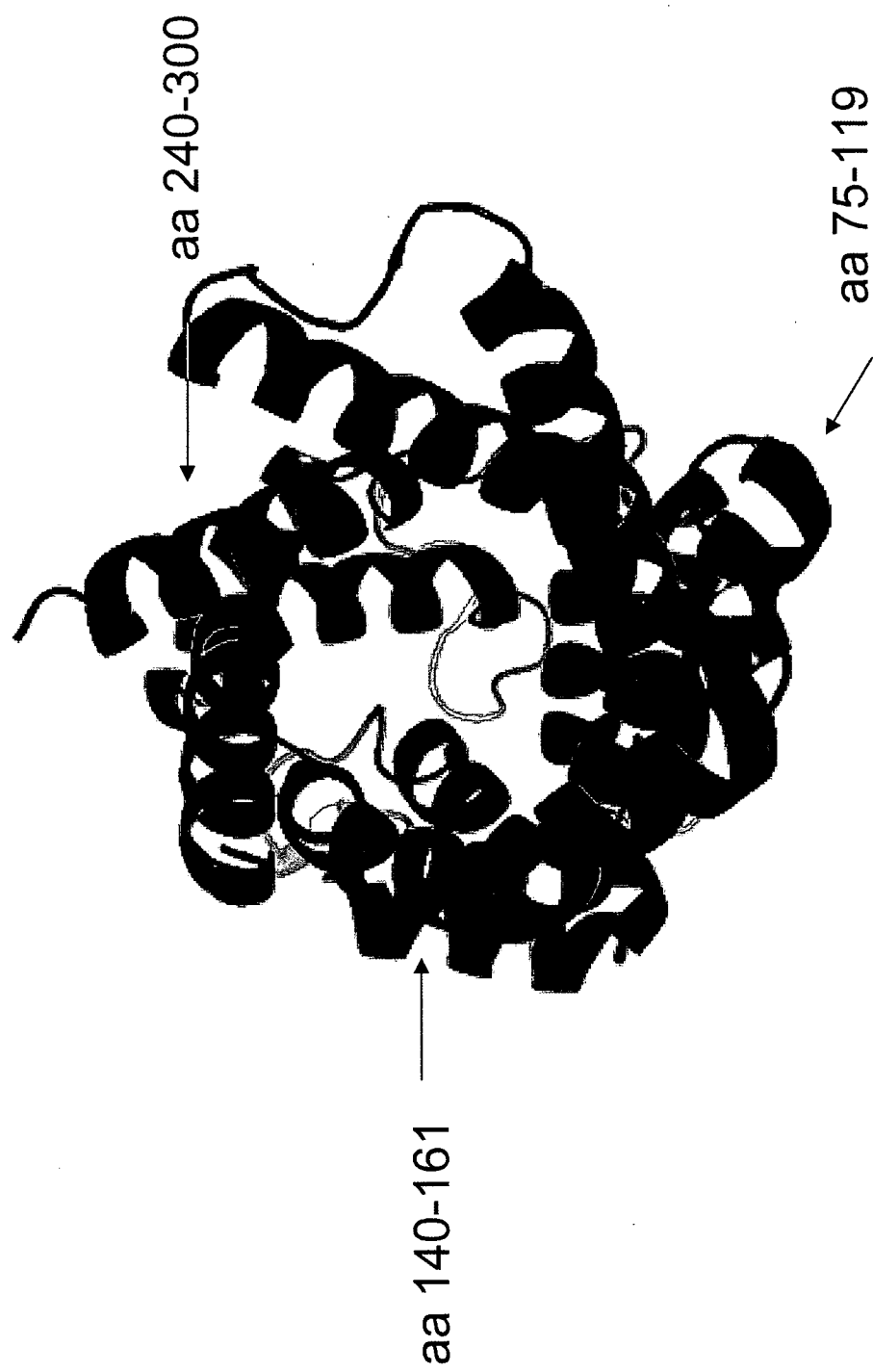


FIG. 9E

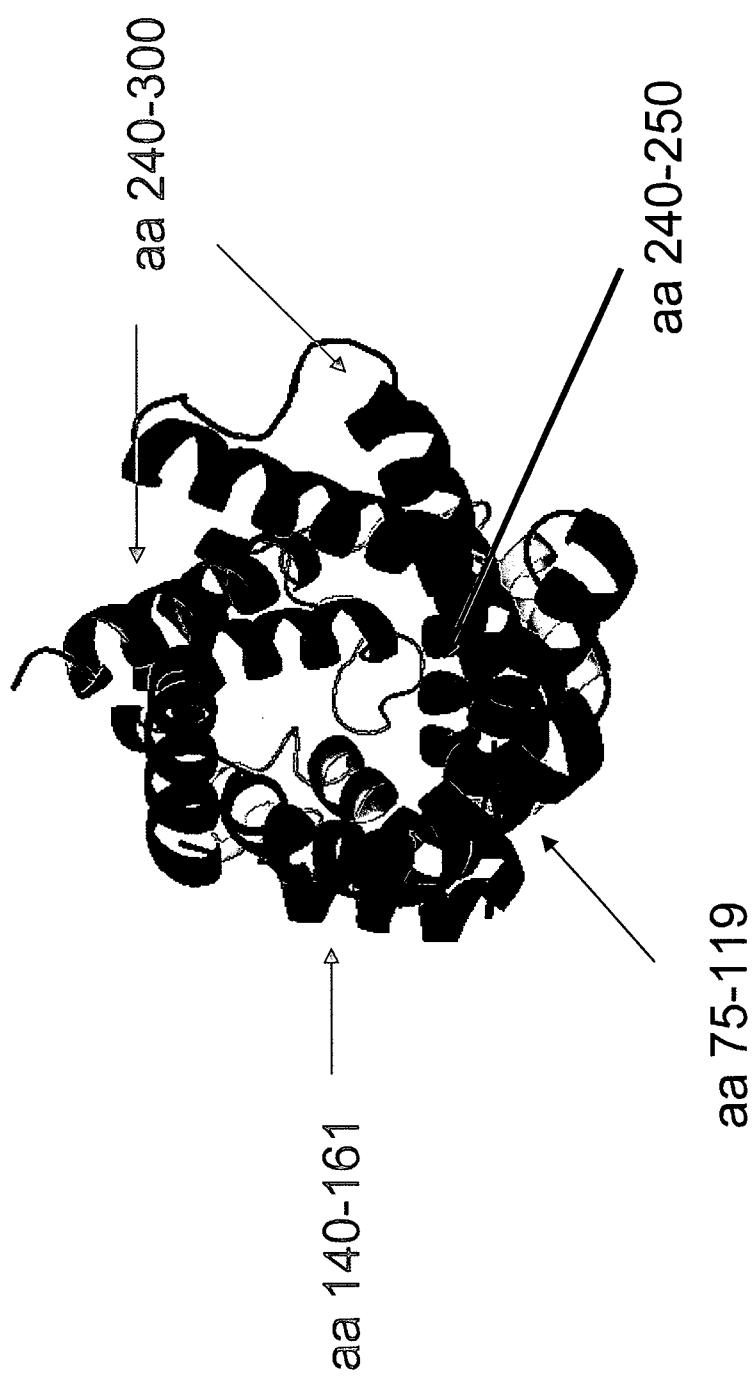


FIG. 9F

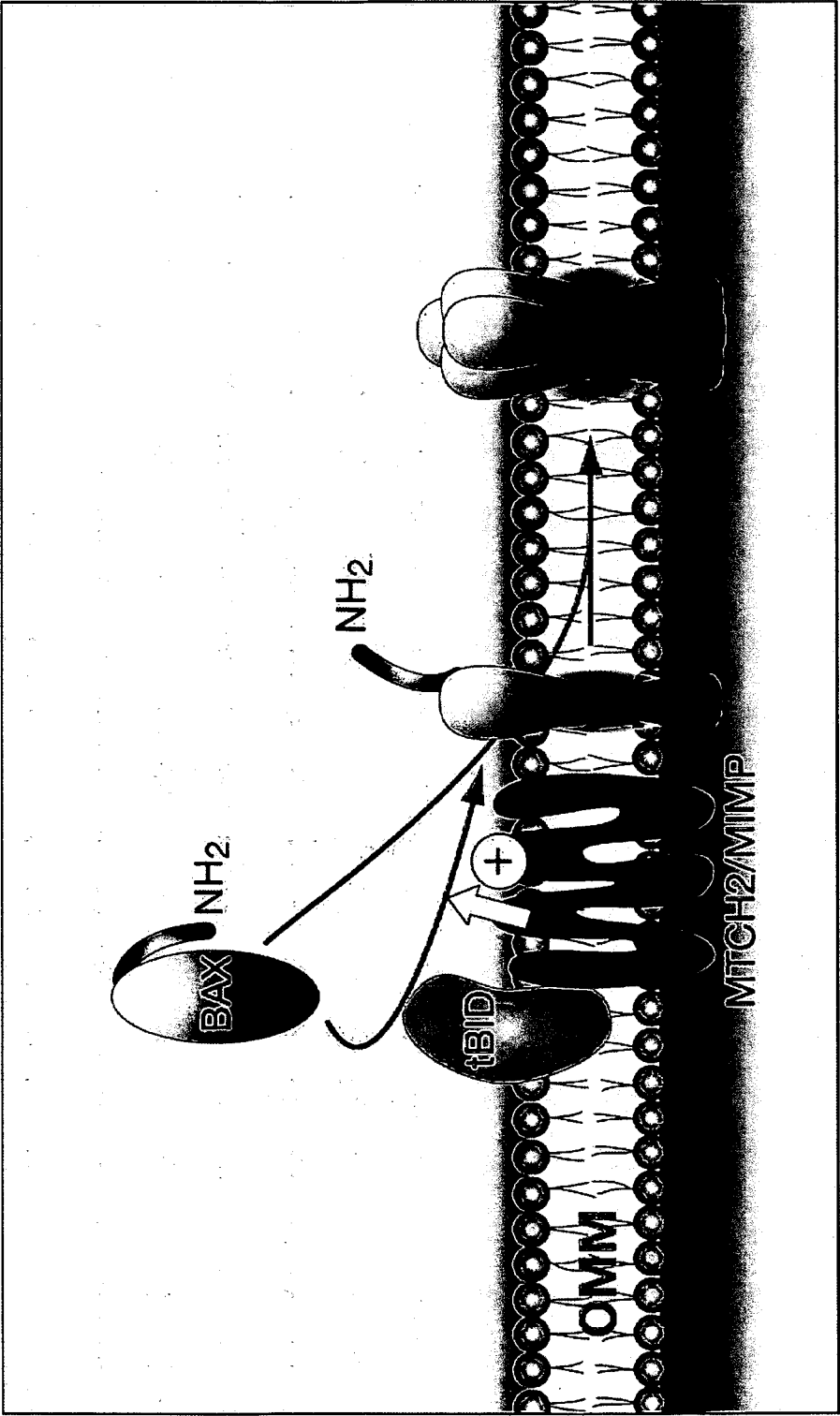


FIG. 10

mdsevsngsglgak	hitd11vfgflqssgctr	qelevlgrelpvqaywea
dledelqt	qgsqasrsfnggr	iepdseesqeeihniarhlaqiqdemdhn
iqptlvnqla	aqfmngslseedkrnc	lakaldevktafprdmendkamli
mtmllak	kvashapsllrdvfh	ttvnfinqnlfsyvvrnlvrnemd

(SEQ ID NO:3)

FIG. 11

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FIG. 12A

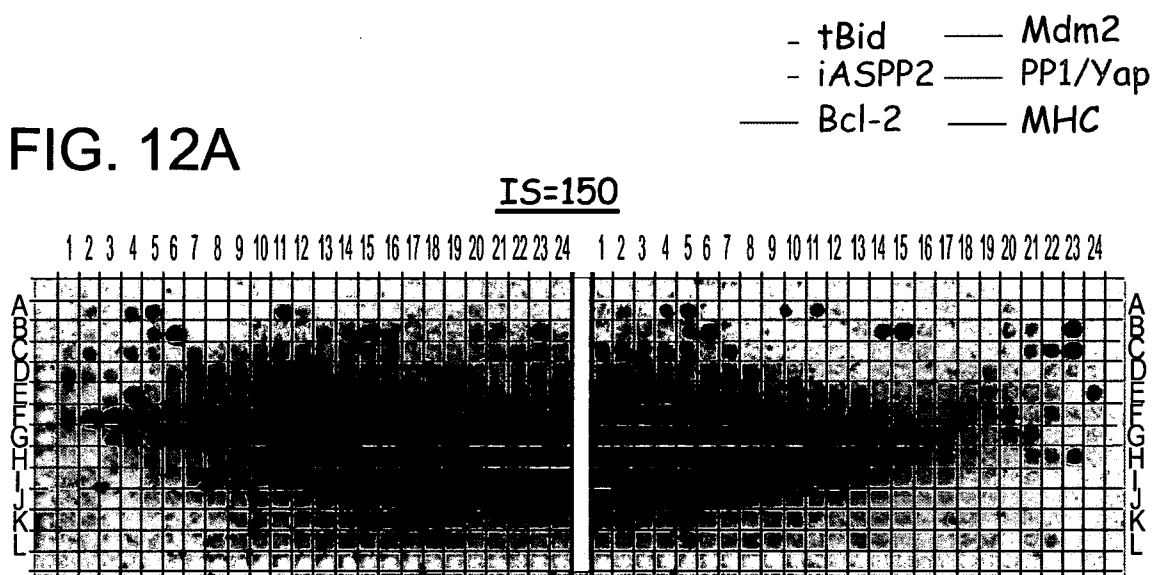


FIG. 12B

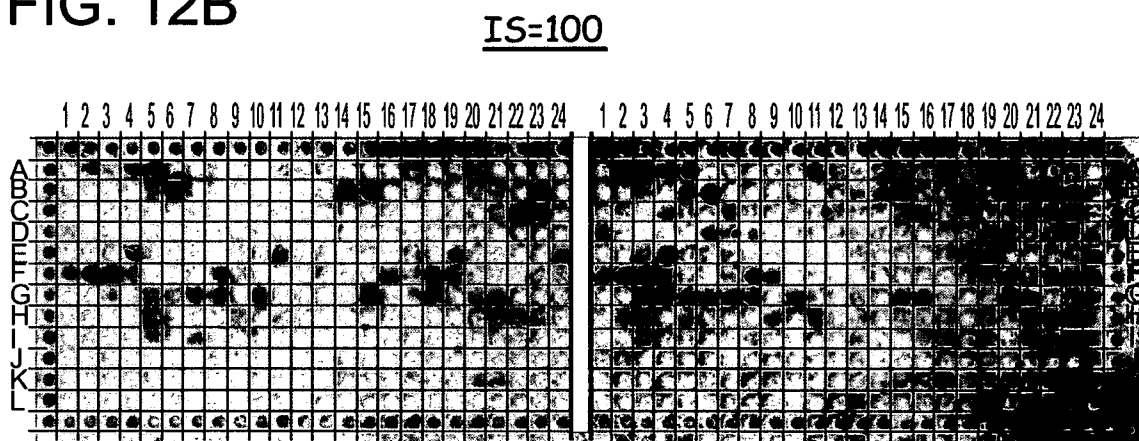
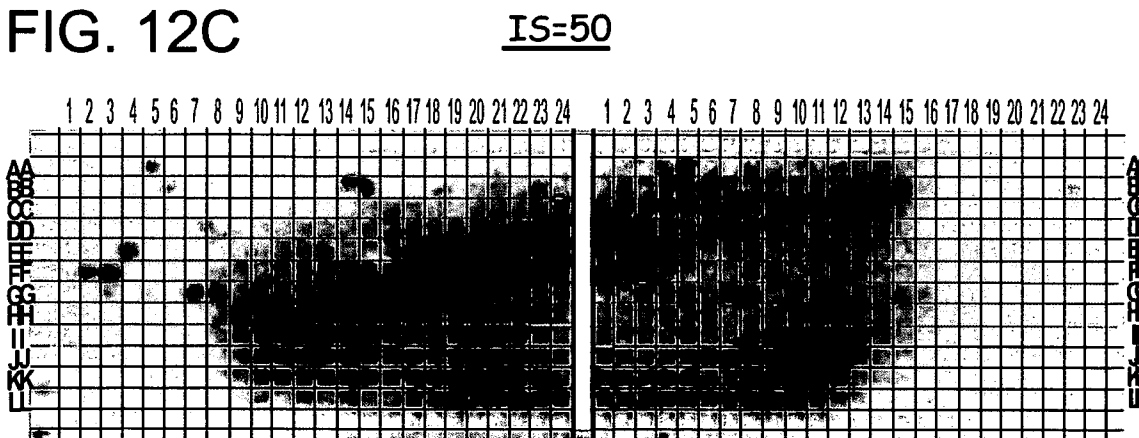


FIG. 12C



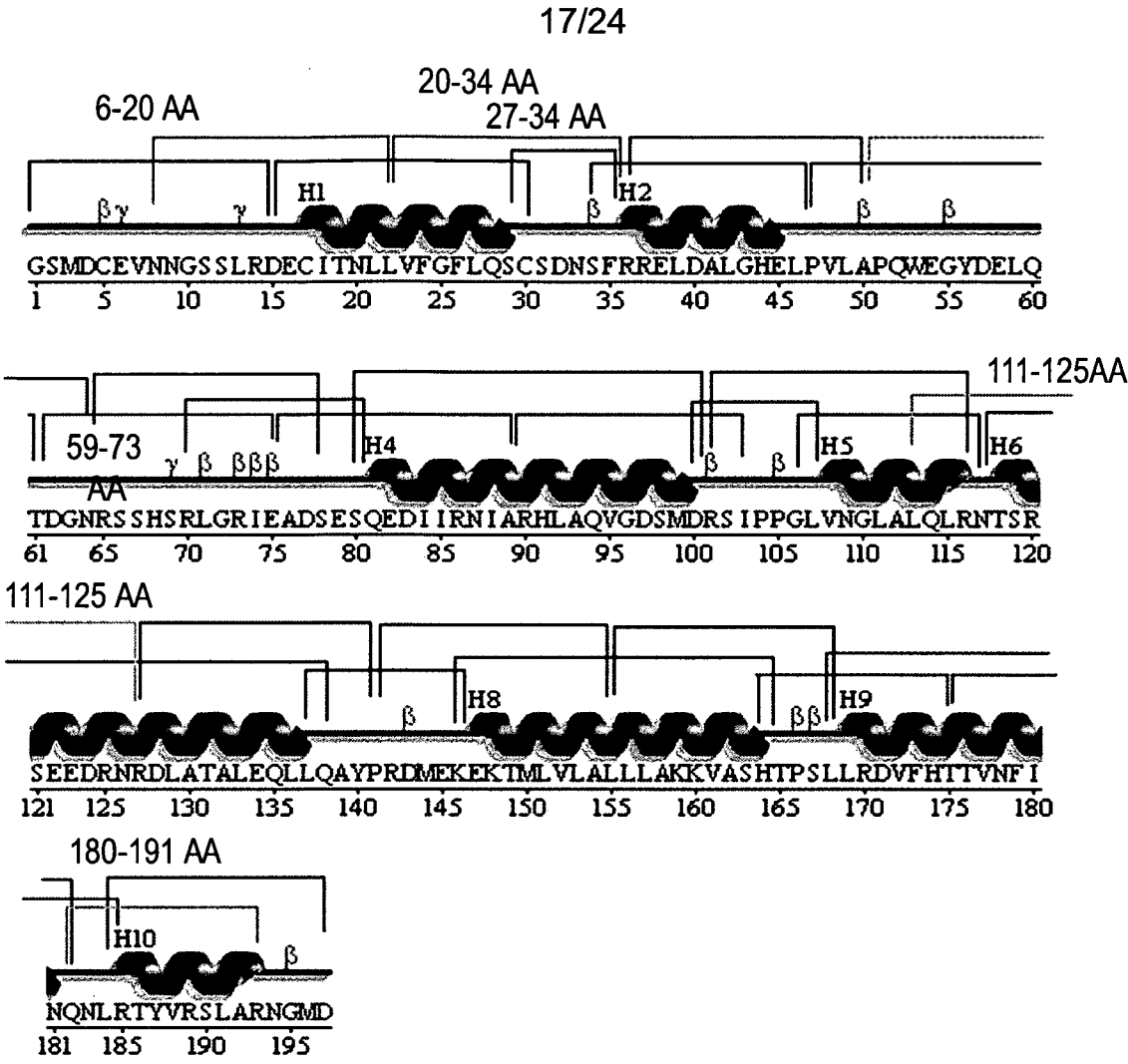


FIG. 13A

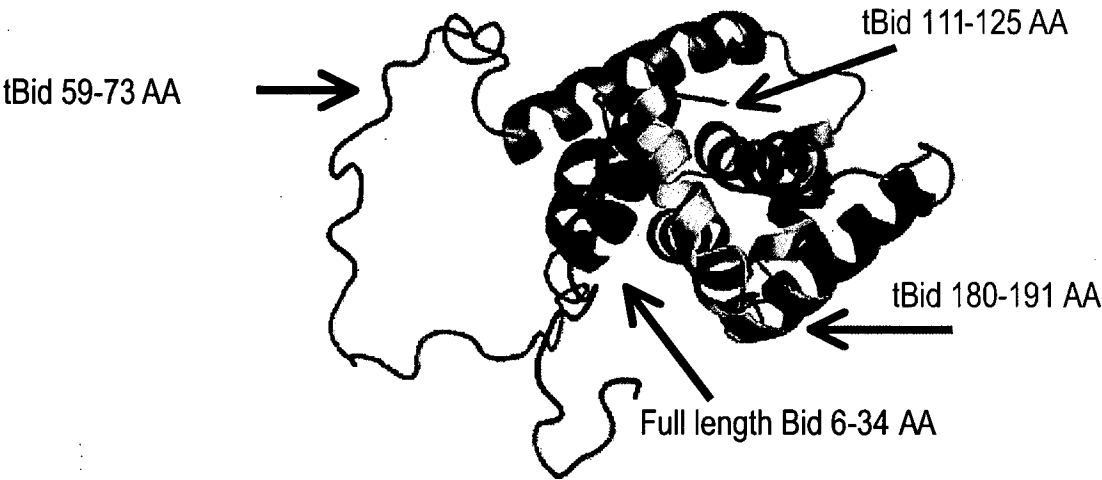


FIG. 13B

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FIG. 14A

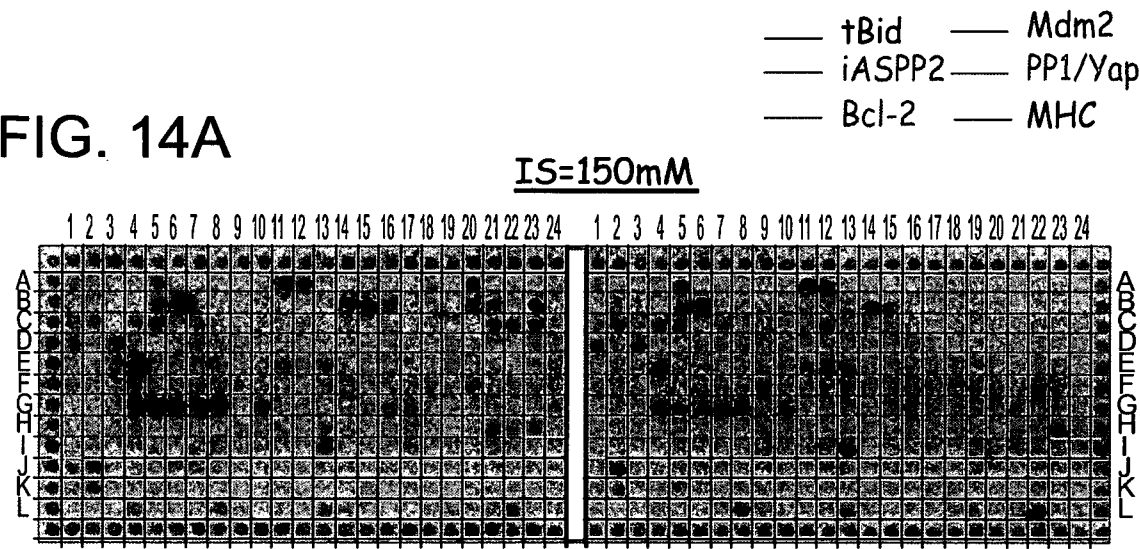


FIG. 14B

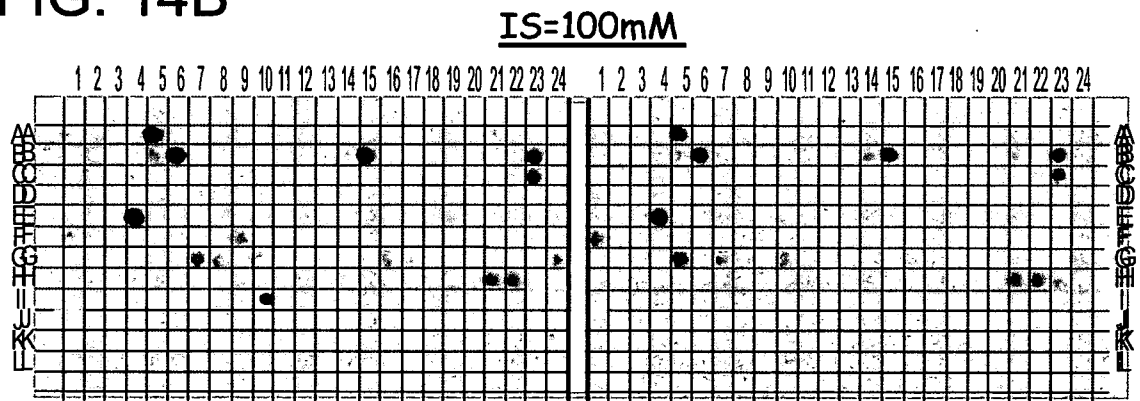


FIG. 14C

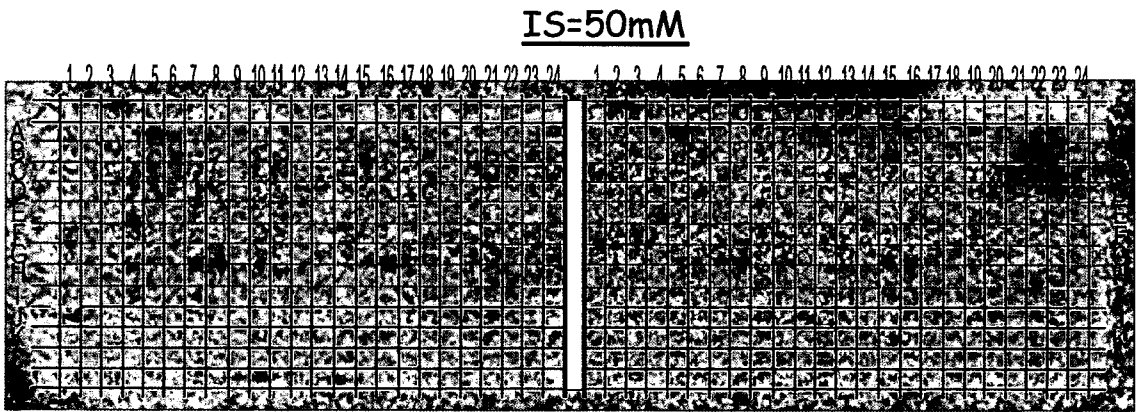


FIG.15A

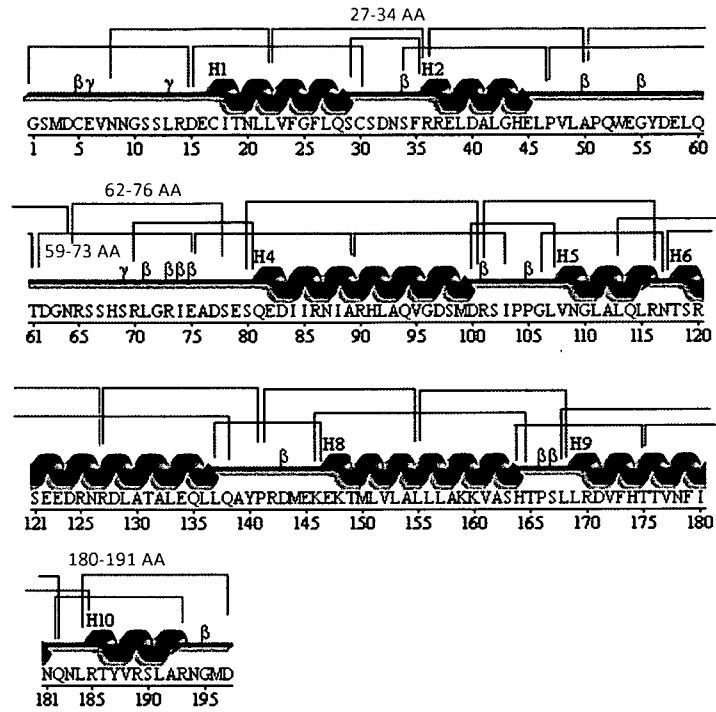


FIG.15B

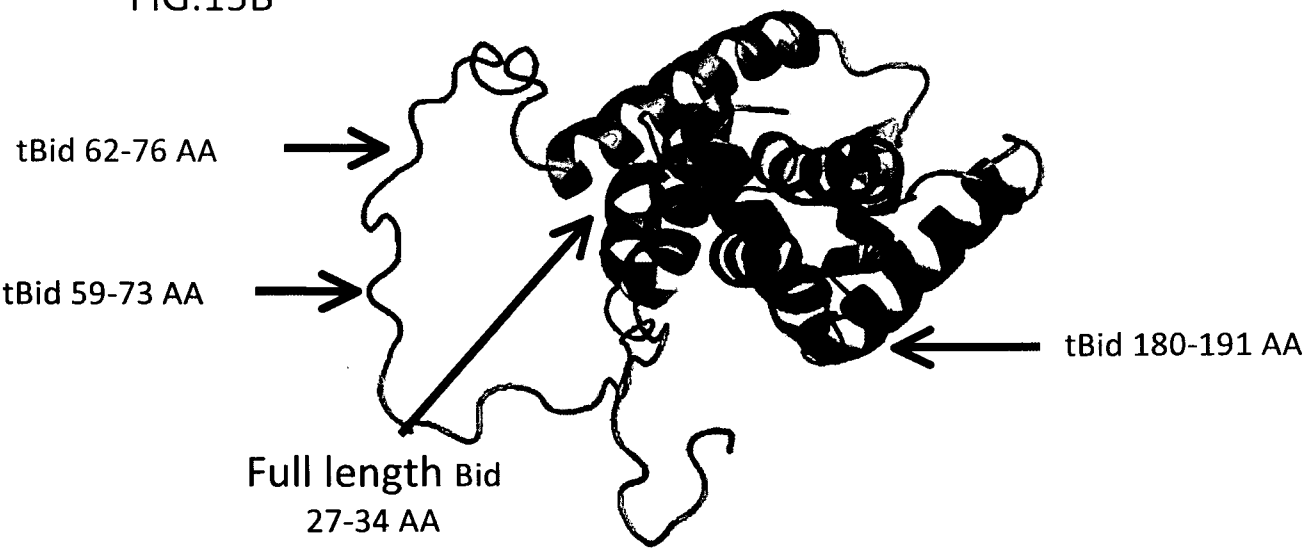


FIG.16

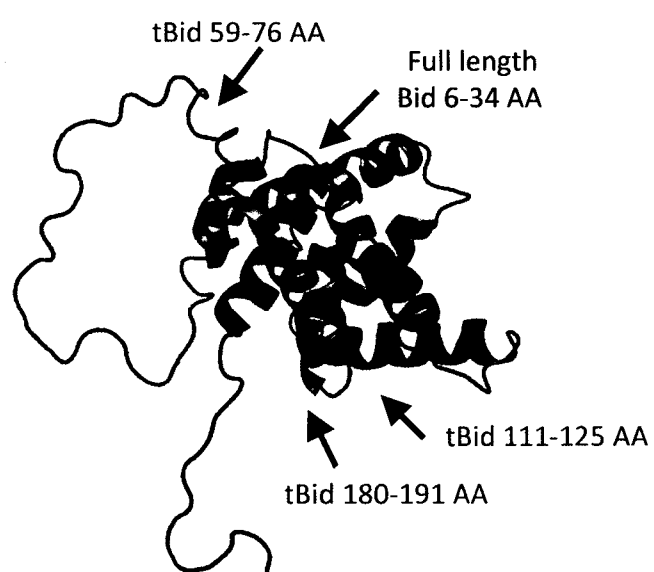


FIG. 17A

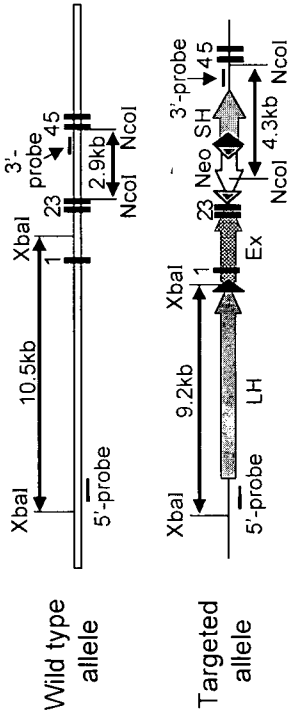


FIG. 17B

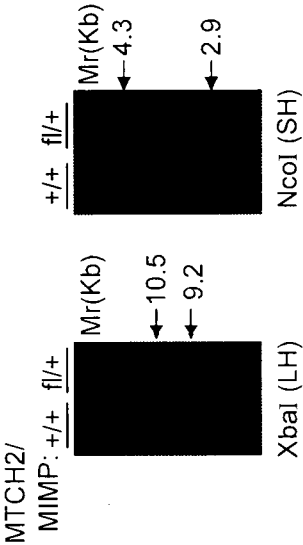


FIG. 18A

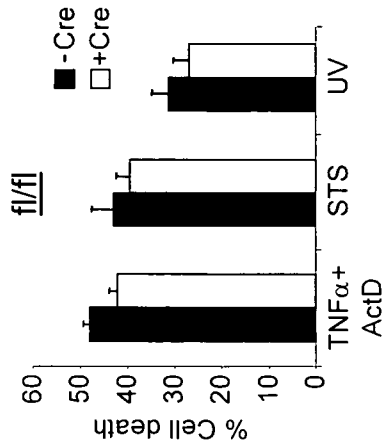


FIG. 18B

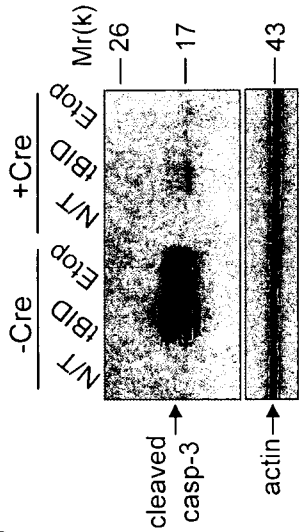


FIG. 18C

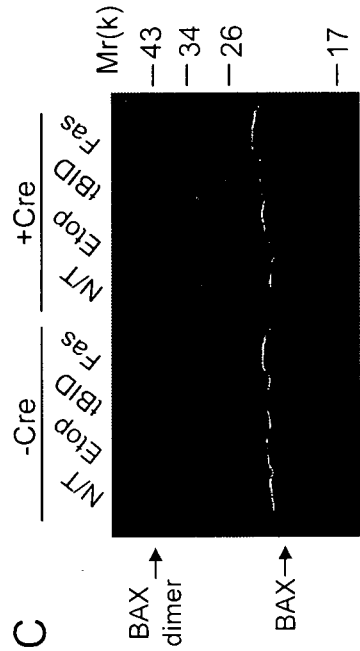


FIG. 18D

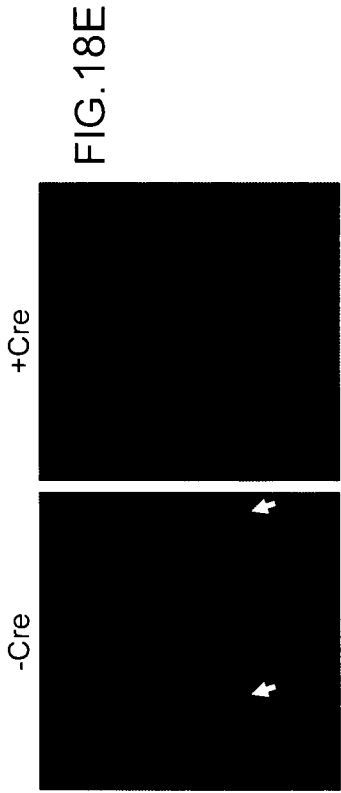


FIG. 18F

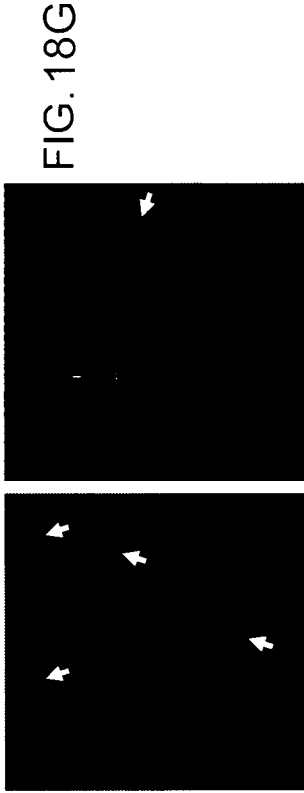


FIG. 18H

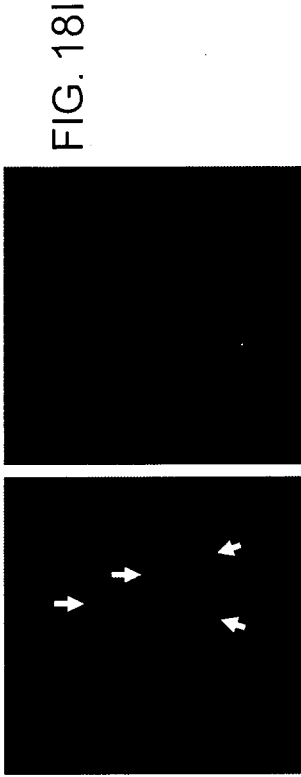


FIG. 18E

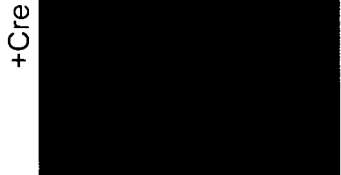


FIG. 18G

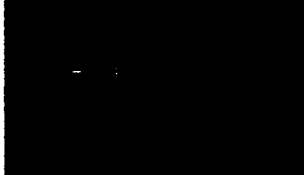
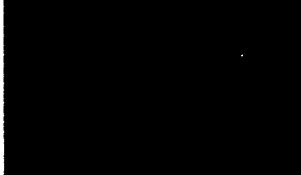
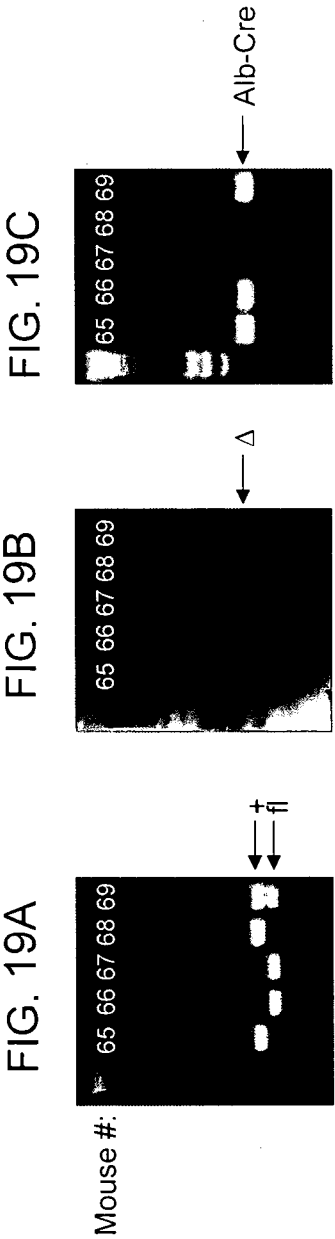


FIG. 18I





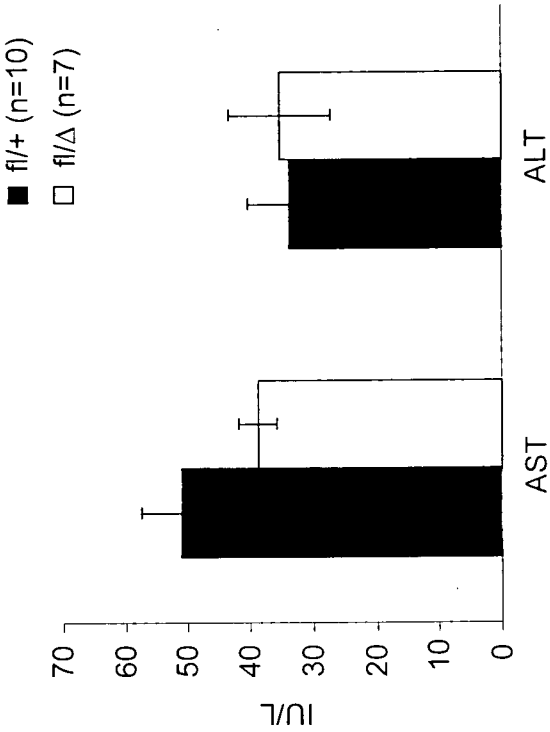


FIG. 20

INTERNATIONAL SEARCH REPORT

International application No

PCT/IL2010/000295

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/00 A61K48/00 C07K14/47 C07K19/00 C12N15/00
 A61P25/28 A61P31/18

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K C12N

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

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

EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/09980 A1 (UNIV WASHINGTON [US]) 12 March 1998 (1998-03-12) page 23	1,2,5,7, 9,11,12, 14,16, 20,21
X	WO 2006/103666 A2 (YEDA RES & DEV [IL]; GROSS ATAN [IL]; SARIG RACHEL [IL]; KAMER IRIS [I] 5 October 2006 (2006-10-05) sequence 30	1,2,5,7, 9,11,12, 14,16, 20,21



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

25 June 2010

Date of mailing of the international search report

09/09/2010

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040,
 Fax: (+31-70) 340-3016

Authorized officer

Rosin, Oliver

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2010/000295

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1, 2, 5, 7, 9, 11, 12, 14, 16, 20, 21(all partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Invention: 1; Claims: 1, 2, 5, 7, 9, 11, 12, 14, 16, 20, 21(all partially)

Peptides, molecules, polynucleotides, nucleic acids, pharmaceutical compositions and methods involving Seq ID N° 111.

Inventions: 2-13; Claims: 1, 2, 5, 7, 9, 11, 12, 14, 16, 20, 21(all partially)

Peptides, molecules, polynucleotides, nucleic acids, pharmaceutical compositions and methods involving Seq ID N° 30 (invention 2), 31 (invention 3) ... up to 117 (invention 13).

Inventions: 14-29; Claims: 3, 4, 6, 8, 10, 11, 13, 15-19, 21(all partially)

Peptides, molecules, polynucleotides, nucleic acids, pharmaceutical compositions and methods involving Seq ID N° 106 (invention 14), 107 (invention 15) ... up to 138 (invention 29).

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IL2010/000295

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9809980	A1	12-03-1998	
		AU 722622 B2	10-08-2000
		AU 4411197 A	26-03-1998
		CA 2264578 A1	12-03-1998
		EP 1007534 A1	14-06-2000
		JP 2001502894 T	06-03-2001
		US 5955593 A	21-09-1999
		US 5998583 A	07-12-1999
WO 2006103666	A2	05-10-2006	NONE