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(54) Title: ISOLATED BID POLYPEPTIDES, POLYNUCLEOTIDES ENCODING SAME AND ANTIBODIES DIRECTED THEREAGAINST AND METHODS OF USING SAME FOR INDUCING CELL CYCLE ARREST OR APOPTOSIS

(57) Abstract: The present invention relates to a PIKK-phosphorylated BID polypeptide which can be used to induce cell cycle arrest. Specifically, the present invention provides isolated polypeptides comprising a BID amino acid sequence which can be used to induce cell cycle arrest and treat diseases associated with genomic instability. Alternatively, the present invention provides isolated polypeptides comprising a BID amino acid sequence capable of inhibiting the PIKK-mediated phosphorylation of endogenous BID which can be used to induce apoptosis and treat cancer.

# ISOLATED BID POLYPEPTIDES, POLYNUCLEOTIDES ENCODING SAME AND ANTIBODIES DIRECTED THEREAGAINST AND METHODS OF USING SAME FOR INDUCING CELL CYCLE ARREST OR APOPTOSIS

## FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to double strand break (DSB) – induced ATM phosphorylation of BID which induces cell cycle arrest. More particularly, the present invention relates to BID polypeptides capable of inducing cell cycle arrest which can be used to treat diseases associated with genomic instability as well as to BID polypeptides capable of preventing the ATM-mediated BID phosphorylation which can be used to induce apoptosis and treat cancer.

In eukaryotic cells the genetic material is constantly subjected to DNA damage. Following induction of DNA damage, the cell may activate a survival system that enables repair and continuation of its normal life cycle, or it may activate its apoptotic machinery in the face of extensive or irreparable damage. Cell cycle arrest can occur following various stimuli such as exposure to DNA damaging agents such as radiation [e.g., IR, which induces double strand breaks (DSB), ultra violet (UV), which induces single strand breaks (SSB) and thymine dimers], various chemicals (e.g., chemotherapeutic agents such as cisplatin) which induce SSB or DSB in the DNA and/or following exposure to agents inducing replication stress such as inhibitors of topoisomerase II (Etoposide), mitomycin C and hydroxyurea. While a DSB is not the major DNA lesion, it is certainly among the most harmful.

One of the major responses associated with the cell survival network is the temporary arrest of cell cycle progression, which reflects the activation of cell cycle checkpoints. The best-documented, damage-induced cell cycle checkpoints operate in the G1/S boundary, and at the S and G2 phases. The very early events that take place at the site of a DNA DSB and precede activation of the response network involve several proteins that are rapidly recruited to the damaged site, act as DSB sensors and convey a damage signal to transducers, which in turn deliver it to numerous downstream effectors.

A prototype transducer of the DSB response is ATM, a nuclear serine-threonine protein kinase, which is absent or inactivated in patients with the genomic instability syndrome Ataxia-Telangiectasis (A-T). Cells from A-T patients exhibit

genomic instability, radiosensitivity and defective activation of the entire DSB response, most notably, the cell cycle checkpoints. ATM is a member of a group of conserved large proteins, several of which are protein kinases involved in mediating DNA damage responses. These proteins, including the ATM and RAD3-related protein (ATR), share several motifs, among them a domain containing a PI3-kinase signature, which gives this group the title, "PI3-kinase-related protein kinases" (PIKKs). Activated ATM phosphorylates a wide spectrum of substrates, many of them at the sites of damage. The functional consequences of some of ATM phosphorylation events have been associated with the activation of the cell cycle checkpoints. However, not all of the phenotypic abnormalities in A-T patients can be explained by a lack of these phosphorylation events, implying that additional ATM targets exist, which have not yet been identified.

Many cancers of the lymphoid origin bear oncogenic chromosomal rearrangements that have arisen as a consequence of defective DSB DNA damage repair. In particular, in childhood or early adulthood, 10-15 % of A-T patients present a lymphoid malignancy of a B- or T-cell origin such as non-Hodgkin's lymphoma, Hodgkin's lymphoma and several forms of leukemia. Thus, defects in cellular responses to DSBs leading to genetic instability may be a frequent initiating event of lymphoid tumors. However, to date, the molecular mechanisms leading to DSB-induced tumor formation is only partially understood.

Apoptosis is regulated by proteins which function to promote or inhibit programmed cell death. Apoptosis can be in response to diverse signals such as stimulation by growth factors (e.g., TNFα and Fas), limb and neural development, neurodegenerative diseases, radiotherapy and chemotherapy as well as environmental conditions which induce DNA damage. 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. Several proteins which modulate the apoptotic process have now been identified, including members of the BCL-2 family which are major regulators of the apoptotic process. The BCL-2 family is comprised of both pro-apoptotic proteins, such as BAX, as well as anti-apoptotic proteins, such as BCL-2. Most BCL-2 family members share three

conserved domains; BH1, BH2, and BH3, of which BH3 functions as a death domain in the pro-apoptotic members of the family. One subset of such pro-apoptotic molecules comprises the BH3-only proteins (*i.e.*, proteins which contain only the BH3 domain out of the three conserved BH domains) including the major regulator of apoptosis BID (BH3 interacting death agonist). Deletion and mutagenesis analysis studies have argued that the amphipathic α-helical BH3 domain serves as a critical death domain in these proteins.

BID is believed to be relatively inactive in the cytosol until proteolytically cleaved by caspase-8. Cleavage of cytosolic BID at Asp59 yields a p15 C-terminal truncated fragment (tBID) that translocates to the mitochondria, where it activates BAX and BAK, resulting in the release of cytochrome c. Phosphorylation seems to regulate BID activity, since it was recently demonstrated that the phosphorylation of BID by casein kinase 1 and/or 2 inhibits its cleavage by caspase-8. However, the apoptotic pathways in which BID plays a role are not yet fully characterized. Studies with $BID^{-/-}$ mice have demonstrated that BID is required for Fas-induced apoptosis. However, no significant difference was observed between mouse embryonic fibroblasts (MEFs) of $BID^{-/-}$ or $BID^{+/+}$ mice in response to a wide range of intrinsic damage signals. On the other hand, $BID^{-/-}$ MEFs were found to be less susceptible than $BID^{+/+}$ MEFs to the DNA damage reagent adriamycin, as well as to the nucleotide analogue 5-fluorouracil (Sax et al., 2002), suggesting that BID may contribute to the DNA damage response. However, to date the mechanisms by which BID is involved in the response to DNA damage have not been identified.

There is thus a widely recognized need for, and it would be highly advantageous to understand the mechanisms involved in the response to DNA damage and to identify agents and methods suitable for treating diseases associated with genomic instability and cancer.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided an isolated polypeptide comprising a BID amino acid sequence having a mutation in a PIKK phosphorylation site.

According to another aspect of the present invention there is provided an isolated polypeptide comprising a BID amino acid sequence having a PIKK phosphorylation site and being capable of inhibiting PIKK-mediated phosphorylation of endogenous BID.

According to yet another aspect of the present invention there is provided an isolated polypeptide comprising a BID amino acid sequence which comprises a phosphorylated PIKK phosphorylation site.

According to still another aspect of the present invention there is provided an isolated polypeptide comprising a BID amino acid sequence wherein the isolated polypeptide being incapable of inducing apoptosis.

According to an additional aspect of the present invention there is provided an isolated polypeptide comprising a BID amino acid sequence attached to a nuclear targeting moiety.

According to yet an additional aspect of the present invention there is provided an isolated polynucleotide encoding the polypeptide.

According to still an additional aspect of the present invention there is provided a transformed cell expressing the polypeptide.

According to a further aspect of the present invention there is provided a method of inducing cell cycle arrest comprising introducing into-, or expressing in the cell a polypeptide comprising a BID amino acid sequence incapable of inducing apoptosis, thereby inducing cell cycle arrest.

According to yet a further aspect of the present invention there is provided a method of treating a disease associated with a genomic instability comprising introducing into-, or expressing in cells of an individual in need thereof a polypeptide comprising a BID amino acid sequence incapable of inducing apoptosis, thereby inducing cell cycle arrest and treating the disease associated with the genomic instability.

According to still a further aspect of the present invention there is provided a method of inducing apoptosis, comprising introducing into-, or expressing in a cell a

polypeptide comprising a BID amino acid sequence incapable of inducing cell cycle arrest, thereby inducing apoptosis.

According to still a further aspect of the present invention there is provided a method of treating cancer, comprising introducing into-, or expressing in cells of an individual in need thereof a polypeptide comprising a BID amino acid sequence incapable of inducing cell cycle arrest, thereby inducing apoptosis and treating the cancer.

According to still a further aspect of the present invention there is provided an antibody comprising an antigen recognition domain capable of specifically binding a BID polypeptide phosphorylated on a serine residue corresponding to amino acid 78 of human BID as set forth in SEQ ID NO:15 but does not bind the BID polypeptide when not phosphorylated on this respective position.

According to still a further aspect of the present invention there is provided an antibody comprising an antigen recognition domain capable of specifically binding a BID polypeptide phosphorylated on a serine residue corresponding to amino acid 78 of mouse BID as set forth in SEQ ID NO:16 but does not bind the BID polypeptide when not phosphorylated on this respective position.

According to still a further aspect of the present invention there is provided an antibody comprising an antigen recognition domain capable of specifically binding a BID polypeptide phosphorylated on a serine residue corresponding to amino acid 61 of mouse BID as set forth in SEQ ID NO:16 but does not bind the BID polypeptide when not phosphorylated on this respective position.

According to still a further aspect of the present invention there is provided a kit for detecting a presence and/or level of a PIKK-mediated phosphorylated BID comprising the antibody.

According to still a further aspect of the present invention there is provided a method of detecting cellular exposure to DNA damaging agents, comprising detecting in a biological sample a presence and/or level of a PIKK-mediated phosphorylated BID, thereby detecting the cellular exposure to the DNA damaging agents.

According to still a further aspect of the present invention there is provided a kit for detecting cellular exposure to DNA damaging agents, comprising at least one reagent capable of detecting a presence and/or level of a PIKK-mediated phosphorylated BID.

According to still a further aspect of the present invention there is provided a method of diagnosing a disease associated with an abnormal PIKK-mediated phosphorylation of BID, comprising detecting in cells of an individual in need thereof a presence and/or level of a PIKK-mediated phosphorylated BID, thereby diagnosing the disease associated with the abnormal PIKK-mediated phosphorylation of BID.

According to still a further aspect of the present invention there is provided a kit for diagnosing a disease associated with an abnormal PIKK-mediated phosphorylation of BID, comprising at least one reagent capable of detecting a presence and/or level of a PIKK-mediated phosphorylated BID.

According to still a further aspect of the present invention there is provided a method of inducing apoptosis comprising contacting a cell with the antibody, thereby inducing apoptosis in the cell.

According to still a further aspect of the present invention there is provided a pharmaceutical composition comprising as an active ingredient the antibody and a pharmaceutically acceptable carrier.

According to still a further aspect of the present invention there is provided a method of identifying an agent capable of inducing cell cycle arrest, comprising: (a) contacting a plurality of cells with a plurality of molecules; (b) identifying at least one molecule from the plurality of molecules capable of increasing a level of a phosphorylated BID polypeptide, the at least one molecule being the agent capable of inducing cell cycle arrest.

According to still a further aspect of the present invention there is provided a multicellular organism comprising a genome which comprises a genetically modified BID gene which comprises a mutation in a PIKK phosphorylation site.

According to further features in preferred embodiments of the invention described below, the mutation abolishes phosphorylation at the phosphorylation site.

According to still further features in the described preferred embodiments the mutation mimics phosphorylation at the phosphorylation site.

According to still further features in the described preferred embodiments the BID amino acid sequence does not exceed 20 amino acids in length.

According to still further features in the described preferred embodiments the polypeptide is capable of inducing apoptosis.

According to still further features in the described preferred embodiments the nuclear targeting moiety is an NLS.

According to still further features in the described preferred embodiments the BID amino acid sequence is at least 20 amino acids long.

According to still further features in the described preferred embodiments the polypeptide is capable of inducing cell cycle arrest.

According to still further features in the described preferred embodiments the PIKK phosphorylation site comprises a residue corresponding to a serine residue at position 78 of a BID polypeptide set forth by SEQ ID NO:15.

According to still further features in the described preferred embodiments the PIKK is an ATM.

According to still further features in the described preferred embodiments the PIKK is an ATR.

According to still further features in the described preferred embodiments the cell cycle arrest is induced following DSB in a DNA.

According to still further features in the described preferred embodiments the cell cycle arrest occurs at an S phase.

According to still further features in the described preferred embodiments the polypeptide is the isolated polypeptide.

According to still further features in the described preferred embodiments the isolated polypeptide is as set forth in SEQ ID NOs:20-22.

According to still further features in the described preferred embodiments the isolated polypeptide is as set forth in SEQ ID NOs:32-34.

According to still further features in the described preferred embodiments the disease associated with genomic instability is associated with an abnormal S phase checkpoint.

According to still further features in the described preferred embodiments the disease associated with genomic instability is selected from the group consisting of Ataxia-Telangiectasia, Fanconi anemia, Bloom's syndrome, hereditary breast and ovarian cancer syndromes involving BRCA1 and Nijmegen breakage syndrome (NBS1).

According to still further features in the described preferred embodiments the cancer is of a lymphoid origin.

According to still further features in the described preferred embodiments the disease associated with the abnormal PIKK-mediated phosphorylation of BID is selected from the group consisting of Ataxia-Telangiectasia, Fanconi anemia, Bloom's syndrome, hereditary breast and ovarian cancer syndromes involving BRCA1 and Nijmegen breakage syndrome (NBS1).

According to still further features in the described preferred embodiments the detecting in the cells the presence and/or level of the PIKK-mediated phosphorylated BID comprises using the antibody.

According to still further features in the described preferred embodiments the at least one reagent comprises the antibody.

According to still further features in the described preferred embodiments the phosphorylated BID polypeptide is present in a cell nucleus.

According to still further features in the described preferred embodiments the phosphorylated BID polypeptide being phosphorylated on a PIKK phosphorylation site.

According to still further features in the described preferred embodiments the mutation abolishes phosphorylation at the PIKK phosphorylation site.

According to still further features in the described preferred embodiments the mutation comprises a substitution of a serine residue with an alanine residue at a position corresponding to amino acid residue 78 of a BID polypeptide set forth by SEQ ID NO:16.

According to still further features in the described preferred embodiments the mutation comprises a substitution of a serine residue with an alanine residue at a position corresponding to amino acid residue 61 of a BID polypeptide set forth by SEQ ID NO:16.

The present invention successfully addresses the shortcomings of the presently known configurations by providing polypeptides, polynucleotides and expression vectors encoding same and antibodies directed thereagainst which can be used to induce cell cycle arrest or apoptosis.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention,

suitable methods and 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 not intended to be limiting.

## BRIEF DESCRIPTION OF THE DRAWINGS

The invention is 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 the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1a-d are graphs depicting the dose-response/death curves of MEFs following treatment with DNA damaging agents. $BID^{+/+}$ and $BID^{-/-}$ MEFs were subjected to treatment with various doses of etoposide (Etop; 20, 50 and 100 μM Etop for 24 hours; Figure 1a), cisplatin (Cis; 10, 20 and 50 μM Cis for 14 hours; Figure 1b), ultraviolet radiation (UV; 5, 10 and 20 $J/m^2$ for 14 hours; Figure 1c), and ionizing radiation (IR; 20, 50 and 100 Gy for 24 hours; Figure 1d) and the effect on cell death was monitored by FACScan using propidium-iodide (PI) dye exclusion. The results presented as the means ± SEM of pooled results from three independent experiments. Note that $BID^{-/-}$ MEFs are less susceptible than $BID^{+/+}$ MEFs to apoptosis induced by DNA-damaging reagents.

FIGs. 2a-c are graphs depicting the dose-response/death curves of $BID^{+/+}$ and $BID^{-/-}$ splenocytes (Figure 2a) or SV40-transformed $BID^{-/-}$ and $BID^{+/+}$ MEFs (Figures 2b-c) in response to treatment with Etop (Figure 2a), UV (Figure 2b) or IR (Figure 2c). $BID^{-/-}$ or $BID^{+/+}$ splenocytes were treated for 40 hours with 0, 5, 10 or 50 μM Etop; SV40-transformed $BID^{-/-}$ and $BID^{+/+}$ MEFs were treated for 18 hours with 0, 2.5, 5 or 10 $J/m^2$ UV radiation or for 24 hours with 10, 20 and 50 Gy IR radiation.

Following treatment cell death was monitored by FACScan using PI dye exclusion. The data shown represent the means ± SEM of pooled results from three independent experiments. Note that $BID^{-/-}$ splenocytes are less susceptible than $BID^{+/+}$ splenocytes to cell death induced by etoposide (Figure 2a). Also note that SV40-transformed $BID^{-/-}$ MEFs are less susceptible than $BID^{+/+}$ MEFs to cell death induced by DNA-damaging reagents.

FIGs. 3a-c are bar graphs depicting the effect of BID on colony survival following IR treatment (Figure 3a) or cell death following either Etop (Figure 3b) or IR (Figure 3c) treatments. Figure 3a - 1000 cells from $BID^{+/+}$ and $BID^{-/-}$ MEFs were seeded per well and irradiated with 1, 3, 5, or 8 Gy of IR. Cells were then incubated for 10 days and the percent of colony survival was calculated as the ratio between the No. of colonies formed after IR treatment and the No. of colonies formed in untreated cells. Asterisk (*) represents significant differences ($p < 0.05$) based on student's t-test. Note that $BID^{-/-}$ MEFs have increased clonogenic survival compared to $BID^{+/+}$ cells following DNA damage. Figures 3b-c - $BID^{+/+}$ or $BID^{-/-}$ MEFs were either left untreated (N/T) or treated with either Etop (100 μM for 24 hours; Figure 3b) or ionizing radiation (IR; 100 Gy for 24 hours; Figure 3c). Alternatively, $BID^{-/-}$ MEFs were infected with recombinant adenoviruses carrying a tetracycline-inducible BID vector (Sarig et al., 2003 JBC 278:10707-10715). Two hours after the addition of doxycyclin, the cultures were washed three times and treated with either Etop (Figure 3b) or IR (Figure 3c). Cell death was monitored by FACScan using the PI dye exclusion. The data represent the means ± SEM of pooled results from three independent experiments. Note that the reduced susceptibility of $BID^{-/-}$ MEFs to DNA-damaging reagents is due to the absence of BID.

FIGs. 4a-e are Western blot analyses demonstrating the effect of DNA damaging agents on BID expression pattern. Figures 4a-b - $BID^{+/+}$ MEFs were either left untreated (N/T; lane 1), or treated for 1 hour with one of the indicated cell death stimuli: Etop (100 μM; lane 2), IR (50 Gy; lane 3), Cis (50 μM; lane 4), UV (20 $J/m^2$; lane 5), Thaps (2 mM; lane 6), TNFα (40 ng/ml together with 2 μg/ml actinomycin D; lane 7), and STS (4 μM; lane 8). Cells were collected, lysed, and equal amounts of protein (20 μg per lane) were subjected to SDS-PAGE, followed by Western blot analysis using anti-BID Abs (Figure 4a). The blot was stripped and reprobed with

anti-β-actin Abs to control for loading (Figure 4b). Asterisk marks a cross-reactive band; The question mark (?) marks the BID double electrophoretic mobility shift. Note that Etop and IR induce a double electrophoretic mobility shift in BID; Figure 4c - $BID^{+/+}$ MEFs were treated for 30 minutes with 100 μM Etop, lysed and either left untreated (-) (lane 1), or further treated for 30 minutes at 37 °C with alkaline phosphatase (+) (lane 2), followed by Western blot analysis using anti-BID Abs. BID-P marks the BID double electrophoretic mobility shift. Note that alkaline phosphatase treatment abolishes the Etop-induced double electrophoretic mobility shift in BID. Figures 4d-e - HeLa cells were transiently transfected with pcDNAIII-wt-BID and 18 hours post-transfection cells were metabolically labeled with $^{32}P$-orthophosphate, treated for 30 minutes with 100 μM Etop (+) (Figure 4d lane 2) or remained untreated (-) (Figure 4d lane 1), following which radiolabeled BID was immunoprecipitated with anti-BID Abs and evaluated by either autoradiography (Figure 4d) or by Western blot, using anti-BID Abs (Figure 4e). Note that exposure to Etop resulted in a marked increase in $^{32}P$-labeling of BID, which appears as a doublet (BID-P);

FIGs. 5a-b are Western blot analyses demonstrating that BID phosphorylation is mediated by the ATM kinase. Atm/Arf double knockout MEFs (Atm-/-) (lanes 1-3) and Atm+/+Arf-/- MEFs (Atm+/+) (lanes 4-6) were either left untreated (N/T) (lanes 1 and 4), or treated for 30 minutes with 100 μM Etop (lanes 2 and 5) or 50 Gy IR (lanes 3 and 6), collected, lysed and equal amounts of protein (20 μg per lane) were subjected to SDS-PAGE, followed by Western blot analysis using anti-BID Abs (Figure 5a). The blot was stripped and reprobed with anti-β-actin Abs to control for loading (Figure 5b). Note that the slower migrating forms of BID (BID-P) do not appear in ATM-deficient MEFs.

FIGs. 6a-b are Western blot analyses depicting the phosphorylation of exogenous BID (Figure 6a) or β-actin (Figure 6b) in Etop – treated HeLa cells in which the expression of LacZ or ATM were downregulated. Control HeLa cells (lanes 1-2) or stably transfected HeLa cells with LacZ siRNA (LacZ downregulated) (lane 3) or ATM siRNA (ATM downregulated) (lane 4) were transiently transfected with pcDNAIII-wt-BID and 18 hours post-transfection the cells were treated for 30 minutes with 100 μM Etop (lanes 2, 3 and 4) or remained untreated (lane 1). $BID^{-/-}$

MEFs, which were similarly treated with Etop, served as a positive control (lane 5). Cells were collected, lysed and subjected to Western blot analysis using anti-BID Abs (Figure 6a). The blots were stripped and reprobed with anti-β-actin Abs to control for loading (Figure 6b). Note the presence of the Etop-induced phosphorylation of exogenous BID in LacZ downregulated cells (Figure 6a lane 3, marked by BID-P), but not in ATM downregulated HeLa cells (Figure 6a lane 4).

FIGs. 7a-g are a sequence diagram (Figure 7a) and Western blot analyses (Figures 7b-d) demonstrating the phosphorylation of BID on Serine 61 and Serine 78 of mouse BID. Figure 7a – Sequence alignment of mouse (BID_mouse GenBank Accession No. NM_007544), human (BID_human; GenBank Accession No. CR_456389) and rat (BID_rat; GenBank Accession No. AF_136282) BID proteins depicting the PIKK consensus sites (SQ/TQ). Note that mouse BID carries two PIKK consensus sites (S61Q and S78Q) whereas human and rat BID carry only one (S78Q). Figures 7b-c – HeLa cells were transiently transfected with pcDNAIII-wt-BID (lanes 3-4), pcDNAIII-BID-S61A (lanes 7-8), pcDNAIII-BID-S78A (lanes 5-6), or left untransfected (-) (lanes 1-2). Eighteen hours post-transfection, cells were either left untreated (-) (lanes 1, 3, 5 and 7), or treated for 30 minutes with 100 μM Etop (+) (lanes 2, 4, 6 and 8), collected, lysed and subjected to Western blot analysis using anti-BID Abs (Figure 7b). The blot was stripped and reprobed with anti-β-actin Abs to control for loading (Figure 7c). Note that mutation of serine 61 to alanine abolishes the Etop-induced double electrophoretic mobility shift in BID (Figure 7b lane 8). Figures 7d-g - $BID^{+/+}$ (lanes 1-3) or $BID^{-/-}$ (lanes 4-5) MEFs were either left untreated (-) (lanes 1 and 4), or treated for 30 minutes with 100 μM Etop (+) (lanes 2, 3 and 5), lysed, and subjected to Western blot analysis using the phospho-specific Abs to either S61 (Figure 7d) or S78 Figure 7f). Alternatively, following Etop treatment, the $BID^{+/+}$ MEFs were further treated for 30 minutes at 37 °C with potato acid phosphatase (PAP; +) (lane 3), lysed and subjected to Western blot analysis as above. The blots were stripped and reprobed with anti-β-actin Abs to control for loading (Figures 7e and g). BID-P marks the phosphorylated form of BID. Asterisk marks a cross-reactive band. Note that the phospho-specific antibodies to serine 61 and serine 78 recognize endogenous BID in MEFs treated with Etop (Figures 7d and f, lane 2)

which is eliminated following treatment with potato acid phosphatase (Figures 7d and f, lane 3).

FIGs. 8a-d are Western blot analyses depicting the specificity of the anti-pS61 and anti-pS78 antibodies to phosphorylated BID. HeLa cells were transiently transfected with pcDNAIII-wt-BID (lanes 1-2), pcDNAIII-BID-S61A (lanes 3-4 in Figures 8a-b) or pcDNAIII-BID-S78A (lanes 3-4 in Figures 8c-d). Eighteen hours post-transfection, cells were either left untreated (-) (lanes 1 and 3), or treated for 30 minutes with 100 μM Etop (lanes 2 and 4), collected, lysed and subjected to Western blot analysis using either the anti-pS61 (Figure 8a) or anti-pS78 (Figure 8c) Abs. The blots were stripped and reprobed with anti-β-actin Abs to control for loading (Figures 8b and d). The asterisk in Figure 8a marks a cross-reactive band, whereas the asterisk in Figure 8c marks the phosphorylated form of endogenous human BID. Note that the anti-S61 Abs recognize phosphorylated BID (BID-P) only in HeLa cells transfected with WT BID (Figure 8a, lane 2) but not in HeLa cells transfecetd with the S61A mutant BID (Figure 8a, lane 4). Similarly, the anti-S78 Abs recognize phosphorylated species of BID (BID-P) in HeLa cells transfecetd with WT BID (Figure 8c, lane 2) but not in HeLa cells transfected with S78A mutant BID (Figure 8c, lane 4). Note that the anti-pS78 Abs also recognize the lower of the three bands in HeLa cells that were not treated with Etop, indicating a basal level of phosphorylation in healthy cells.

FIGs. 9a-e are Western blot analyses depicting the *in vitro* phosphorylation of BID by ATM and ATR. 293T cells were transiently transfected with empty Flag vector (Figures 9a-c; lane 1), Flag-tagged ATM (Figures 9a-c - lane 2; Figures 9d-e – lane 1), Flag-tagged ATR (Figures 9a-c - lane 4; Figures 9d-e – lane 2), Flag-tagged kinase-inactive (KI) ATM (Figures 9a-c; lane 3), Flag-tagged KI ATR (Figures 9a-c; lane 5) and the cells overexpressing the Flagged vectors were immunoprecipitated with anti-Flag M2 Abs (Sigma Cat. No. F3165), and incubated in the presence of purified recombinant mouse wt-BID (Figures 9a-c) or BID-S61A/S78A (Figures 9d-e) and [$\gamma$-$^{32}$P] ATP. Proteins were separated by SDS-PAGE, transferred to PVDF membrane, and phosphorylated proteins were visualized by autoradiography ($^{32}$P) (Figure 9b). Flag-tagged proteins were subjected to immunoblotting with anti-Flag M2 Abs (Figure 9a) and BID levels were monitored by Coomassie staining (Figure

9c). The asterisk marks a cross-reactive band. Note that both human ATM (Figure 9b, lane 2) and ATR (Figure 9b, lane 4) phosphorylate purified recombinant mouse BID *in vitro*, but not the recombinant unphosphorylatable mouse BID (Figure 9d, lanes 1 and 2).

FIGs. 10a-d are Western blot analyses depicting Etoposide - induced phosphorylation of BID on both S61 and S78 in mouse splenocytes. Splenocytes purified from either $BID^{+/+}$ (lanes 1 and 2) or $BID^{-/-}$ (lane 3) mice were either left untreated (-) (lane 1), or treated for 40 minutes with 50 μM Etop (lanes 2 and 3) and lysed. Lysates from equal amounts of cells were subjected to SDS-PAGE followed by Western blot analysis using anti-BID Abs (Figure 10a), anti-pS61 (Figure 10b), or anti-pS78 (Figure 10c) Abs. The blots were stripped and reprobed with anti-β-actin Abs to control for loading (Figure 10d). Note the specific S61 (Figure 10b, lane 2) and S78 (Figure 10c, lane 2) phosphorylated BID species following Etop treatment.

FIGs. 11a-c are Western blot analyses depicting the phosphorylation of human BID on S78 in response to Etop treatment. HEK293 cells were either left untreated (-) (lane 1), or treated for 1 hour with 100 μM Etop (+) (lane 2), lysed, and equal amounts of protein (20 μg per lane) were subjected to SDS-PAGE, followed by Western blot analysis using either anti-pS78 Abs (Figure 11a) or anti-human BID Abs (Figure 11b). The blot was stripped and reprobed with anti-β-actin Abs to control for loading (Figure 11c). The asterisks mark cross-reactive bands.

FIGs. 12a-c are Western blot analyses depicting the specificity of the anti-pS78 Abs to Human wt-BID, but not human BID-S78A mutant. HeLa cells were transiently transfected with pcDNAIII-wt-BID (lanes 3 and 6), pcDNAIII-BID-S78A (lanes 2 and 5) or remained untransfected (lanes 1 and 4). Eighteen hours post-transfection, cells were either left untreated (-) (lanes 1-3), or treated for 1 hour with 100 μM Etop (lanes 4-6), collected, lysed and subjected to Western blot analysis using either the anti-pS78 Abs (Figure 12a) or the anti-human BID Abs (Figure 12b). The top blot was stripped and reprobed with anti-β-actin Abs to control for loading (Figure 12c).

FIGs. 13a-d are Western blot analyses depicting the time course of Etop – induced phosphorylation of endogenous mouse BID on S61 and S78. $BID^{+/+}$ MEFs were either left untreated (N/T) (lane 1), or treated with 100 μM Etop, collected at the

indicated time points: 15 minutes (lane 2), 30 minutes (lane 3), 1 hour (lane 4), 2 hours (lane 5), 3 hours (lane 6), 5 hours (lane 7), 7 hours (lane 8) and 9 hours (lane 9), lysed, and equal amounts of protein (20 μg per lane) were subjected to SDS-PAGE followed by Western blot analysis using either anti-BID (Figure 13a), anti-pS61 (Figure 13b), or anti-pS78 (Figure 13c) Abs. The blot was stripped and reprobed with anti-β-actin Abs to control for loading (Figure 13d). The asterisk marks a cross-reactive band. Note that BID phosphorylation on S61 or S78 is transient, appearing at 15 minutes or 1 hour, respectively, after initiation of Etop treatment and decreasing following 9 hours.

FIG. 14 is a bar graph depicting the time course of Etop-induced apoptosis of MEFs. $BID^{+/+}$ MEFs were treated with 100 μM Etop or remained untreated (N/T), collected at the indicated time points (2, 4, 8, 12 and 22 hours), and cell death was monitored by FACScan using the PI dye exclusion. Note that Etop-induced cell death begins at 12 hours post Etop treatment.

FIGs. 15a-d are Western blot analyses depicting that endogenous mouse BID is phosphorylated on S61 and on S78 in an ATM-dependent manner. Atm/Arf double knockout MEFs (Atm-/-) (lanes 1-2) and Atm+/+Arf-/- MEFs (Atm+/+) (lanes 3-4) were either left untreated (-) (lanes 1 and 3), or treated for 30 minutes with 100 μM Etop (lanes 2 and 4), collected, lysed and subjected to Western blot analysis using either anti-pS61 (Figure 15a) or anti-pS78 (Figure 15c) Abs. The blots were stripped and reprobed with anti-β-actin Abs to control for loading (Figures 15b and d). The asterisk marks a cross-reactive band.

FIGs. 16a-b are Western blot analyses depicting the phosphorylation of mouse BID on S61 only in response to reagents that induce double-strand breaks in DNA. $BID^{+/+}$ MEFs were either left untreated (N/T) (lane 1) or treated for 1 hour with the death stimuli: Etop (100 μM; lane 2), IR (50 Gy; lane 3), Cis (50 μM; lane 4), UV (20 $J/m^2$; lane 5), Thaps (2 mM; lane 6), TNFα (40 ng/ml together with 2 μg/ml actinomycin D; lane 7), and STS (4 μM; lane 8). Cells were collected, lysed, and equal amounts of protein (20 μg per lane) were subjected to SDS-PAGE followed by Western blot analysis using anti-pS61 Abs (Figure 16a). The blot was stripped and reprobed with anti-β-actin Abs to control for loading (Figure 16b). The asterisk marks a cross-reactive band.

FIGs. 17a-b are Western blot analyses depicting the phosphorylation of endogenous human BID on S78. HEK293 cells stably transfected with the LacZ siRNA (LacZ downregulated) (lanes 1-5) or ATM siRNA (ATM downregulated) (lanes 6-10) were either left untreated (N/T) (lanes 1 and 6), or treated for 1 hour with Etop (100 μM), IR (50 Gy), UV (20 $J/m^2$) or STS (4 μM). Cells were collected, lysed, and equal amounts of protein (20 μg per lane) were subjected to SDS-PAGE followed by Western blot analysis using anti-pS78 Abs (Figure 17a). The blots were stripped and reprobed with anti-β-actin Abs to control for loading (Figure 17b). Note the presence of endogenous S78 phosphorylated BID in LacZ downregulated cells in response to DNA damaging agents that induce double-strand breaks in DNA (Etop and IR, Figure 17a lanes 2 and 3) but not in ATM downregulated cells (Figure 17a lanes 7 and 8) nor in LacZ downregulated cells treated with US or STS (Figure 17 lanes 4 and 5). These results demonstrate that phosphorylation of S78 in endogenous human BID is ATM-dependent, and occurs only in response to reagents that induce double-strand breaks in DNA.

FIGs. 18a-h are immunofluorescence analyses depicting the intracellular expression pattern of BID. $BID^{+/+}$ MEFs (Figures 18a, c, e and g) or $BID^{-/-}$ MEFs (Figures 18b, d, f, and h) grown on glass cover slips, were either left untreated (Figures 18a-d), or treated for 3 hours with 100 μM Etop (Figures 18e-h), fixed and immunostained with anti-BID Abs (green, shown in Figures 18a, b, e and f) followed by DAPI nuclear staining (blue, shown in Figures 18c, d, g and h). Note the positive staining of BID in the cytoplasm and the nucleus of healthy MEFs.

FIGs. 19a-d are Western blot analyses depicting the subcellular localization of BID. $BID^{+/+}$ MEFs were either left untreated (-) (lanes 1-2), or treated with formaldehyde (+) (lanes 3-4), and subfractionated. Aliquots of the cytosolic (C) (lanes 1 and 3) and nuclear (N) (lanes 2 and 4) fractions were subjected to SDS-PAGE followed by Western blot analysis using anti-BID (Figure 19a), anti-MEK (Figure 19b), anti-BAX (Figure 19c), and anti-lamin B (Figure 19d) Abs. The asterisk marks a cross-reactive band that might represent a modified form of BID. Note that following cross linking with paraformaldehyde a small fraction of cellular BID can be detected in the nuclear fraction (Figure 19 lane 4).

FIGs. 20a-c are Western blot analyses depicting the phosphorylation of BID following IR treatment. $BID^{+/+}$ MEFs were either left untreated (N/T) (lane 1), or treated with 50 Gy IR (lane 2), collected 5 minutes later, and lysed. Equal amounts of protein (20 μg per lane) were subjected to SDS-PAGE followed by Western blot analysis using either anti-BID Abs (Figure 20a) or anti-pS61 Abs (Figure 20b). The blot was stripped and reprobed with anti-β-actin Abs to control for loading (Figure 20c). Note the presence of S61 phosphorylated BID in MEFs treated with IR (Figure 20b, lane 2), demonstrating the rapid phosphorylation of BID in response to IR – induced DNA damaging.

FIGs. 21a-c are Western blot analyses depicting the phosphorylation of BID following IR treatment. $BID^{+/+}$ (lanes 1-5) or $BID^{-/-}$ (lane 6) MEFs were either left untreated (N/T) (lane 1), or treated for 30 minutes with the indicated doses of IR: 0.2 Gy (lane 2), 0.5 Gy (lane 3), 1 Gy (lane 4) or 5 Gy (lanes 5 and 6), collected, lysed, and analyzed as described in Figures 20a-c. Note that phosphorylation of BID occurs in response to extremely low, non-apoptotic levels of IR.

FIGs. 22a-i are raw data of flow cytometry analyses (Figures 22a-f) and quantification of the percentage of cells in the G1 (Figure 22g), S (Figure 22h) or G2/M (Figure 22i) cell cycle phases of MEFs following Etop treatment. $BID^{+/+}$ or $BID^{-/-}$ MEFs were either left untreated (N/T) or treated for 2 hours with 20 μM Etop, rinsed, and then released into drug-free medium. At the indicated time points the DNA content was analyzed by flow cytometry. Figures 22a-f - The actual raw data from a representative experiment together with multi-line plots generated by the ModFit LT computer software program. The dark histograms represent the percent of cells in the G1 and G2/M phases and the hatched histograms represent the percent of cells in S phase. Figures 22a-c - $BID^{+/+}$ MEFs (N/T – Figure 22a; 8 hours – Figure 22b; and 24 hours – Figure 22c); Figures 22d-f – $BID^{-/-}$ MEFs (N/T – Figure 22d; 8 hours – Figure 22e; and 24 hours – Figure 22f). Figures 22g-i – Bar graphs depicting the quantification of the percentages of cells in G1 (Figure 22g), S (Figure 22h) and G2/M (Figure 22i) cell cycle phases as determined from the flow cytometry analyses. The data represent the means ± SEM of pooled results from three independent experiments. Filled columns - $BID^{+/+}$ MEFs; Empty columns – $BID^{-/-}$ MEFs. Note

that $BID^{-/-}$ MEFs do not accumulate in the S and G2 phases of the cell cycle following etoposide treatment.

FIGs. 23a-c are bar graphs depicting the percentage of DNA synthesis in $BID^{+/+}$ or $BID^{-/-}$ MEFs following Etop treatment. $BID^{+/+}$ or $BID^{-/-}$ MEFs were either left untreated (N/T) or treated for 2 hours with 20 μM Etop, rinsed, and then released into drug-free medium. At the indicated time points following release, the cells were pulse labeled for 30 minutes with BrdU to determine DNA synthesis. Figure 23a – The percentages of BrdU positive cells at eight hours after release as determined in $BID^{+/+}$ (filled bars) or $BID^{-/-}$ (empty bars). The data represent the means ± SEM of pooled results from three independent experiments. Note that $BID^{-/-}$ MEFs fail to decrease DNA synthesis following Etop treatment. Figures 23b-c – The percentages of BrdU positive cells was determined in $BID^{+/+}$ (Figure 23b) or $BID^{-/-}$ (Figure 23c) MEFs at early S (empty bars) or the late S/G2 (filled bars) phases of cell cycles at the indicated time points following drug release (*i.e.*, 0. 4. 6 and 24 hours). The data represent the means ± SEM of pooled results from three independent experiments. Note that $BID^{-/-}$ MEFs are not delayed in their progression from S to G2/M following Etop treatment.

FIGs. 24a-c are Western blot analyses of $BID^{-/-}$ MEFs stably expressing the -S61A/S78A mutant depicting absence of phosphorylation in response to Etop treatment. $BID^{-/-}$ MEFs stably expressing either wt-BID (lanes 1-2) or the BID-S61A/S78A mutant (lanes 3-4) were treated for 1 hour with 20 μM Etop (+) (lanes 2 and 4) or remained untreated (-) (lanes 1 and 3), lysed, and equal amounts of protein (20 μg per lane) were subjected to SDS-PAGE, followed by Western blot analysis using either anti-BID Abs (Figure 24a) or the S61 (Figure 24b) or S78 (Figure 24c) phospho-specific Abs. The blots were stripped and reprobed with anti-β-actin Abs to control for loading (Figures 24d-f). The asterisks mark cross-reactive bands. Note that BID-S61A/S78A expressed in $BID^{-/-}$ MEFs is not recognized by the phospho-specific Abs.

FIGs. 25a-i are the raw data of flow cytometry analyses (Figures 25a-f) and quantification of the percentages of cells in the G1 (Figure 25g), S (Figure 25h) or G2/M (Figure 25i) phases of cell cycle. $BID^{-/-}$ MEFs stably expressing either wt-BID or BID-S61A/S78A (two clones from each) were treated for 2 hours with 20 μM

Etop, rinsed and then released into drug-free medium. At the indicated time points the DNA content was analyzed by flow cytometry. Figures 25a-f - Actual raw data from a representative experiment together with multi-line plots generated by the ModFit LT computer software program. The dark histograms represent the percent of cells in the G1 and G2/M phases and the hatched histograms represent the percent of cells in S phase. Figures 25g-i – Bar graphs depicting the exact percentage of cells in each phase of the cell cycle (in each of the four clones). The data represent the means ± SEM of pooled results from three independent experiments. Note that $BID^{-/-}$ MEFs stably expressing BID-S61A/S78A do not accumulate in the S phase following Etop treatment.

FIGs. 26a-c are a bar graph (Figure 26a) and Western blot analyses (Figures 26b-c) demonstrating that $BID^{-/-}$ MEFs expressing the BID-S61A/S78A are more susceptible to Etop-induced apoptosis than those expressing WT BID. Figure 26a - $BID^{-/-}$ MEFs stably expressing either wt-BID or BID-S61A/S78A (the four clones shown in Figure 25g-i) were either left untreated (N/T) or treated with Etop (50 μM; 18 hours), UV (20 $J/m^2$; 24 hours) or TNFα (40 ng/ml together 2 μg/ml with actinomycin D; 4.5 hours). Cell death was monitored by FACScan using PI dye exclusion. The data represent the means ± SEM of pooled results from three independent experiments. Note that the percentage of cell death following Etop treatment is more pronounced in $BID^{-/-}$ MEFs expressing BID-S61A/S78A than in $BID^{-/-}$ expressing wild-type BID. Figures 26b-c - The two wt-BID and two mutant BID clones were treated with either 50 μM Etop (for 18 hours) or TNFα/ActD (for 4.5 hours), lysed and equal amounts of protein (20 μg per lane) were subjected to SDS-PAGE, followed by Western blot analysis using anti-BID Abs (Figure 26b). For the TNFα treatment, only clones #1 are shown. The blots were stripped and reprobed with anti-β-actin Abs to control for loading (Figure 26c). Note that the enhanced death obtained with the BID-S61A/S78A clones is not due to higher expression of mutant BID or to more cleavage to tBID.

FIGs. 27a-b are Western blot analyses depicting the effect of whole-animal irradiation on BID phosphorylation. Seven-week old $BID^{+/+}$ (lanes 1-14) or $BID^{-/-}$ (lane 15) female mice were either left untreated (-) (lanes 1-3) or subjected to whole-body γ-radiation (+; 3 Gy) (lanes 4-14). One hour after being exposed to the radiation

the mice were sacrificed by cervical dislocation and the indicated organs were removed and immediately frozen in liquid nitrogen. The organs were then thawed, homogenized, and equal amounts of protein (50 μg from each organ) were subjected to SDS-PAGE, followed by Western blot analysis using either anti-pS78 (Figure 27a) or anti-BID (Figure 27b) Abs. Lanes 1 and 7 – thymus; lanes 2, 8 and 15 – spleen; lanes 3 and 9 – bone marrow; lane 4 – brain; lane 5 – heart; lane 6 – lung; lane 10 – liver; lane 11 – pancreas; lane 12 – intestine; lane 13 – kidney; lane 14 – ovary; BID-P marks the phosphorylated form of BID. The asterisk marks a cross reactive band. Note that IR treatment induced prominent phosphorylation of BID in lymphoid organs (thymus, spleen, bone marrow) as well as in the kidney.

FIGs. 28a-b are Western blot analyses depicting the effect of dose-dependent whole-animal irradiation on BID phosphorylation in the thymus. Seven-week old $BID^{+/+}$ (lanes 1-10) or $BID^{-/-}$ (lanes 11-12) female mice were either left untreated (-) (lanes 1-2 and 11) or subjected to whole-body γ-radiation [3 Gy (lanes 3-6) or 6 Gy (lanes 7-10, 12)]. The mice were sacrificed by cervical dislocation at 1 hour (lanes 3-4, 7-8) or 4 hours (lanes 5-6, 9-10, 12) after being exposed to the radiation and the thymus was removed, homogenized and analyzed by Western blot as described in Figures 27a-b. BID-P marks the phosphorylated form of BID. The asterisk marks a cross reactive band. Note that phosphorylation of BID *in vivo* is transient, and it increases in an ionizing radiation dose-dependent manner.

FIGs. 29a-c are a diagram (Figure 29a) and Southern blot analyses (Figures 29b-c) depicting the generation of BIDS61A/S78A knock-in mice by gene targeting. Figure 29a - Diagram of the Bid genomic locus, the targeting vector and the homologous recombinant locus. Also indicated are the restriction enzyme cutting sites (X, *Xba*I; E, *Eco*RI; H, *Hind*III; C, *Cla*I; N, *Not*I; Xh, *Xho*I) and the position of external probes. Figures 29b-c are Southern blots of one ES clone that was subsequently aggregated with tetraploid embryos using the 5' external probe (probe-L; Figure 29b) and the 3' external probe (probe-S; Figure 29c) demonstrating homologues recombination within the Bid locus. The 5' external probe (probe-L; Figure 29b) recognizes a 10.1-kb *Eco*RI/*Xba*I fragment for wild-type loci (lane 1) and a 6.8-kb *Eco*RI/*Xba*I recombinant fragment for a targeted loci (lane 2). The 3'

external probe (probe-S; Figure 29c) recognizes a 9-kb *Hind*III fragment for wild-type loci (lane 1) and a 6-kb *Hind*III recombinant fragment for a targeted loci (lane 2).

FIGs. 30a-b are PCR analyses depicting the excision of the LoxP-Neo-LoxP cassette. Animals depicted are progeny of a BIDS61A/S78A(neo)/+ male crossed to a PGK-Cre transgenic female. EtBr-stained agarose gels of PCR flanking the Neo locus are presented. PCR primers used were: For 5'-GCCAGGTAAGCATCCTTCAAT (SEQ ID NO:11) and Rev 5'-TCAGCAATGGTTCTGTCAACA (SEQ ID NO:12) for the 1.6 and 0.6 kb bands presented in Figure 30a; and For 5'-GGGCAAAGTAGTGGCTTCAG (wild type; SEQ ID NO:13) and 5'-ACGAAGTTATAAGCTTGCGGC (KI; SEQ ID NO:4) and Rev 5'-GGAGTTCGCCACAAGAGAAG (SEQ ID NO:14) for the 848 and 684 bp bands presented in Figure 30b. Note that the presence of the homozygote KI mouse (BID61A/78A) is detected as a single band corresponding to the 0.6 kb KI excision product (Figure 30a, lane 3) and a single band corresponding to 724 bp (Figure 30b, lane 3).

FIGs. 31a-b are Western blot analyses depicting initial characterization of BIDS61A/S78A knock-in mice. Heterozygous mutant BIDS61A/S78A/+ animals were mated to obtain offspring homozygous for the mutant BID gene (BIDS61A/S78A). Three-week old $BID^{+/+}$, BIDS61A/S78A/+, and BIDS61A/S78A female mice (+/+, +/KI, KI/KI, respectively) were either left untreated (-) (lanes 1-2) or subjected to whole-body γ-radiation (+; 3 Gy; lanes 3-8). The mice were sacrificed by cervical dislocation 1 hour after being exposed to the radiation and the indicated organs were removed, immediately homogenized, and equal amounts of protein (50 μg from each organ) were subjected to SDS-PAGE, followed by Western blot analysis using either anti-pS78 (Figure 31a) or anti-BID (Figure 31b) Abs. Lanes 1 and 3-5 – thymus; lanes 2 and 6-8 – spleen. BID-P marks the phosphorylated form of BID. * marks a crossreactive band. Note that in the homozygote KI mice BID is expressed but is not phosphorylated following IR treatment.

FIGs. 32a-g demonstrate that BID carries a nuclear export signal that is involved in regulating its cellular location. Figure 32a is a sequence alignment of mouse (BID_mouse; GenBank Accession No. NM_007544), human (BID_human; GenBank Accession No. CR_456389) and rat (BID_rat; GenBank Accession No.

AF_136282) BID proteins depicting that the classical nuclear export signal (NES) is shared between all three BID homologues. The NES consensus motif (shown in the bottom line) contains four closely spaced leucine residues, which can be substituted by other large hydrophobic residues (Isoleucine/Valine). Figures 32b-e are immunofluorescence analyses (Figures 32b-c) or DAPI nuclear staining (Figures 32d-e) of HeLa cells transfected with wt-BID (Figures 32b and d) or NLS-BID-3LA (Figures 32c and e). HeLa cells were transfected with wt-BID and NLS-BID-3LA and the transfected cells fixed and stained with anti-BID Abs (green) and with DAPI (blue). Note that transfection of HeLa cells with the NLS-BID-3LA resulted in BID staining which is more confined to the nucleus than wt-BID. Figures 32f-g are Western blot analyses depicting BID expression in the cytosolic (C) or nuclear (N) fractions of HeLa cells which were transfected with either wt-BID (Figure 32f) or NLS-BID-3LA (Figure 32g). Transfected cells were treated with formaldehyde subfractionated and aliquots of the cytosolic and nuclear fractions were subjected to SDS-PAGE followed by Western blot analysis using anti-BID Abs. Asterisk marks a cross reactive band.

FIGs. 33a-c are bar graphs depicting cell cycle analyses of $BID^{-/-}$ MEFs which were transfected with either wt-BID or NLS-BID-3LA. $BID^{-/-}$ MEFs stably expressing either wt-BID or NLS-BID-3LA (two clones from each) were either left untreated (N/T), or treated with 10 μM Etop and following 8 hours the DNA content was analyzed by flow cytometry. The exact percentage of cells in each phase of the cell cycle is shown. The data represent the means ± SEM of pooled results from three independent experiments. Note that $BID^{-/-}$ MEFs expressing a "nuclear-trapped" BID mutant (NLS-BID-3LA) do not arrest in S phase following Etop treatment.

FIG. 34 is a bar graph depicting the effect of the "nuclear-trapped" BID mutant (NLS-BID-3LA) on apoptosis. $BID^{-/-}$ MEFs stably expressing either wt-BID or NLS-BID-3LA (the four clones used in Figures 33a-c) were either left untreated (N/T), or treated with Etop (100 μM; 24 hours). Cell death was monitored by FACScan using PI dye exclusion. The data represent the means ± SEM of pooled results from three independent experiments. Note that the "nuclear-trapped" BID mutants (NLS-BID-3LA) are less susceptible to Etop-induced apoptosis.

FIG. 35 is a schematic model depicting the "double agent" role of BID in the DNA damage response. Following DNA damage, ATM and ATR are activated and, through several protein targets, can cause either cell cycle arrest (and subsequent DNA repair), or apoptosis. Based on the findings of the present invention BID is an ATM target, which is important for both cell cycle arrest at the S phase and apoptosis (new steps are marked in red). BID clearly induces apoptosis at the mitochondria following activation of the TNF/Fas death-receptor pathway, however it remains an open question whether BID plays a similar role at the mitochondria in the DNA damage pathway (dotted arrow).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to a PIKK-phosphorylated BID polypeptide which can be used to induce cell cycle arrest. Specifically, the present invention relates to isolated polypeptides comprising a BID amino acid sequence which can be used to induce cell cycle arrest and treat diseases associated with genomic instability. Alternatively, the present invention discloses isolated polypeptides comprising a BID amino acid sequence capable of inhibiting the PIKK-mediated phosphorylation of endogenous BID which can be used to induce apoptosis and treat cancer.

The principles and operation of the isolated polypeptides and methods of inducing cell cycle arrest or apoptosis according to the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not 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. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Genomic instability associated diseases such as Ataxia-Telangiectasia (A-T), Fanconi anemia, Bloom's syndrome, hereditary breast and ovarian cancer syndromes involving BRCA1 and Nijmegen breakage syndrome (NBS1) are associated with the inability of cells to activate the survival system which enables DNA repair and continuation of normal cell cycle following exposure to DNA damage. As a result, cells with damaged DNA often become tumorigenic. For example, cells from A-T

patients, in which the ATM gene is mutated or inactive, exhibit genomic instability, radiosensitivity and defective activation of the entire DSB response. Consequently, 10-15 % of A-T patients, present early in childhood a lymphoid malignancy of a B- or T-cell origin such as non-Hodgkin's lymphoma, Hodgkin's lymphoma and several forms of leukemia. ATM is a member of a group of conserved large proteins, several of which are protein kinases (e.g., ATM and ATR) involved in mediating DNA damage responses and which share a domain containing a PI3-kinase signature (PIKK). Activated ATM phosphorylates a wide spectrum of substrates, many of them at the sites of damage. The functional consequences of some of ATM phosphorylation events have been associated with the activation of the cell cycle checkpoints. However, not all of the phenotypic abnormalities in A-T patients can be explained by a lack of these phosphorylation events, implying that additional, yet un-identified, ATM targets exist.

When the DNA damage is extensive or irreparable, the apoptotic machinery is activated. Apoptosis is regulated by proteins which function to promote or inhibit programmed cell death such as members of the BCL-2 family (e.g., the pro-apoptotic BAX), as well as anti-apoptotic BCL-2 protein. A subset of the pro-apoptotic molecules comprises the BH3-only proteins including the major regulator of apoptosis BID. Cleavage of cytosolic BID yields a p15 C-terminal truncated fragment (tBID) that translocates to the mitochondria, where it activates BAX and BAK, resulting in the release of cytochrome c. The apoptotic pathways in which BID plays a role are not yet fully characterized. Studies with $BID^{-/-}$ mice have demonstrated that BID is required for Fas-induced apoptosis. On the other hand, $BID^{-/-}$ MEFs were found to be less susceptible than $BID^{+/+}$ MEFs to the DNA damage reagent adriamycin and to the nucleotide analogue 5-fluorouracil (Sax et al., 2002), suggesting that BID may contribute to the DNA damage response. Prior studies performed by the present inventors suggested that full-length BID is a player in the DNA damage pathway, since a caspase-8 non-cleavable BID mutant sensitized $BID^{-/-}$ mouse embryonic fibroblasts (MEFs) to DNA damage-induced apoptosis (Sarig et al., 2003). However, to date the mechanisms by which BID is involved in the response to DNA damage have not been identified.

While reducing the present invention to practice, the present inventors have uncovered that BID is a target for phosphorylation by PIKK proteins such as ATM or

ATR mediating cell-cycle arrest. Interestingly, the inhibition of such phosphorylation induces apoptosis. These findings suggest a yet unknown dual activity of BID in cell-cycle arrest and apoptosis.

As is shown in the Examples section which follows, the present inventors have uncovered that the involvement of BID in DNA damage-induced apoptosis is mediated by ATM phosphorylation (Figures 1-6; Example 1) which occurs, in a transient manner, hours prior to the onset of apoptosis and in response to reagents that induce double-strand breaks (Figures 7-17; Example 2); BID is partially localized to the nucleus and its phosphorylation occurs rapidly in response to extremely low non-apoptotic levels of ionizing radiation (IR) (Figures 18-21; Example 3); BID phosphorylation is required for the accumulation of cells in the S phase following Etoposide treatment and that cells expressing an unphosphorylatable BID variant (S61A/S78A) are more susceptible to Etoposide-induced apoptosis than cells expressing phosphorylatable BID (Figures 22-26; Example 4); BID carries a nuclear export signal (NES) that regulates the "shuttling" of BID from the nucleus to the cytosol and that export of BID from the nucleus is required for both the accumulation of cells in the S phase and for apoptosis inducement following Etoposide treatment (Figures 32-34; Example 7); and exposure of mice to IR results in phosphorylation of BID in lymphoid organs in a transient and IR dose-dependent manner (Figures 27-28; Example 5). Altogether, these results demonstrate that BID is an ATM effector having a pro-survival activity that is dependent on BID phosphorylation on PIKK sites (S61/S78 of mouse BID or S78 of human BID) and on its export from the nucleus. Thus, these results suggest the use of agents capable of modulating BID PIKK-mediated phosphorylation in order to induce cell cycle arrest or apoptosis.

Without being bound to any theory, these findings uncovered a dual role for BID in the cell. On one hand, it has a pro-apoptotic role, which is activated by the TNF/Fas death-receptor pathway, and on the other hand, as an ATM target, it is capable of inducing cell cycle arrest following exposure to DNA damaging agents. Thus, and without being bound to any theory, it is conceivable that modulation of one BID activity will affect the level of the other BID activity in the cell (see schematic model in Figure 35).

Thus, according to one aspect of the present invention, there is provided a method of inducing cell cycle arrest. The method is effected by introducing into-, or

expressing in the cell a polypeptide comprising a BID amino acid sequence incapable of inducing apoptosis, thereby inducing cell cycle arrest.

As used herein the phrase "cell cycle arrest" refers to the delay and/or halt of a cell in any stage of the cell cycle resulting in an abnormal accumulation of the cell in that cell cycle stage. The cell cycle includes 4 main stages: the M phase - cell mitosis; the G1 phase - interphase; the S phase – occurs towards the end of interphase and includes DNA synthesis; and the G2 phase – final stage of interphase in which the cell continues to grow and duplicates in preparation for mitosis. Preferably, the cell cycle arrest according to this aspect of the present invention occurs at the S phase, entrance to S phase, the G2 phase and/or the G2/M phase.

As used herein the phrase "inducing cell cycle arrest" refers to initiating or upregulating (e.g., increasing) the rate and/or level of cell cycle arrest at a specific phase (e.g., the S phase).

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.

As used herein the phrase "a polypeptide comprising a BID amino acid sequence" refers to any natural or chemically synthesized polypeptide, peptide or mimetic thereof which comprises at least a portion of a mammalian (e.g., human, mouse) BID protein [e.g., as set forth in GenBank Accession No. P55957 (SEQ ID NO:15; human BID) or GenBank Accession No. P70444 (SEQ ID NO:16; mouse BID)].

According to one embodiment of this aspect of the present invention the polypeptide is incapable of inducing apoptosis.

Such a polypeptide is selected of an amino acid length and composition (*i.e.*, at least encompassing PIKK phosphorylation site) sufficient to induce cell cycle arrest.

Methods of detecting apoptotic activity or cell cycle arrest are known in the art and are further described hereinunder.

As is mentioned hereinabove, the present inventors have uncovered that BID exhibits a dual activity in the cell. On one hand it exhibits a pro-apoptotic activity (Figures 1-3), and on the other hand, it is capable of inducing cell cycle arrest

following induction of DNA damage (Figures 22-23). In addition, the induction of cell cycle arrest is regulated by phosphorylation of BID on a PIKK site (e.g., the serine residue at position 78 as set forth by SEQ ID NO:15; Figures 24-25) and by the export of BID from the nucleus (Figures 33a-c).

Preferably the polypeptide comprises a phosphorylated PIKK phosphorylation site.

As used herein the phrase "PIKK phosphorylation site" refers to the conserved motif of serine or threonine residues followed by glutamine residues, a motif commonly named "SQ/TQ" (Kim et al., 1999) and which is found on proteins that are subject to phosphorylation by "PI3-kinase-related protein kinases" (PIKK) proteins. Such PIKK proteins can be, for example, ATM (GenBank Accession No. NP_000042), ATR (GenBank Accession No. NP_001175) or DNA-PK (GenBank Accession No. NP_008835).

Methods of phosphorylating polypeptides at specific amino acid residues are well known in the art (see for example, Oguchi K. et al., Blood, 2003, 101: 3622-3627).

A non-limiting example of a phosphorylated polypeptide which comprises the BID amino acid sequence and which can be used according to this aspect of the present invention is the isolated polypeptide set forth by SEQ ID NO:17.

According to another preferred embodiment of this aspect of the present invention the polypeptide comprises a mutation in the PIKK phosphorylation site, which mimics phosphorylation at that phosphorylation site. Such a mutation can be, for example, a substitution of a serine residue with an aspartic acid residue essentially as described in Germann UA., et al., 1996, The Journal of Biological Chemistry, 271: 1708-1716. A mutation in the PIKK phosphorylation site can be introduced to the polypeptide using recombinant techniques or solid phase synthesis methods which are well known in the art and further described hereinbelow.

A non-limiting example of a polypeptide which comprises a mutation which mimics phosphorylation at the PIKK site and which can be used according to this aspect of the present invention is the isolated polypeptide set forth by SEQ ID NO:18.

As mentioned, the present inventors have uncovered that BID is phosphorylated by ATM and that such phosphorylation occurs in the nucleus. In addition, a "nuclear trapped" polypeptide (e.g., the NLS-BID-3LA polypeptide

described in Example 7 of the Examples section which follows) was less efficient in sensitizing $BID^{-/-}$ cells to apoptosis following exposure to Etoposide than a polypeptide of the wild-type BID.

Since natural BID polypeptides (e.g., human BID as set forth by SEQ ID NO:15) lack a nuclear localization signal (NLS) it is conceivable that endogenous BID is subject to nuclear import via an NLS-containing protein. Thus, in order to facilitate the import of the isolated polypeptide of the present invention into the cell nucleus, the polypeptide of this aspect of the present invention preferably comprises a BID amino acid sequence attached to a nuclear targeting moiety.

As used herein the phrase "nuclear targeting moiety" refers to a moiety which is capable of directing the polypeptide to the nucleus. Once inside the nucleus, the polypeptide can be further subjected to PIKK-mediated phosphorylation (e.g., by endogenous ATM or ATR). A non-limiting example of such a moiety is a nuclear localization signal (NLS) such as that of the SV40 large-T antigen (Kalderon, D., et al., Nature, 311: 33-38, 1984) as set forth by SEQ ID NO:19 (Lys-Lys-Lys-Arg-Lys-Val-Glu). Alternatively, the nuclear targeting moiety can be a protein interaction domain which may mediate signal to an NLS containing protein. The nuclear targeting moiety can be covalently attached to the isolated polypeptide of the present invention using e.g., solid phase synthesis or can be recombinantly expressed along with the isolated polypeptide as is further described hereinbelow.

The abovedescribed isolated polypeptides which are incapable of inducing apoptosis can be of any length which enables induction of cell cycle arrest and those of skills in the art are capable of determining the length needed for that activity based on, for example, functional assays (e.g., *in vitro* assays) capable of detecting apoptosis and/or cell cycle arrest essentially as described in the "General Materials and Experimental Methods" and Example 4 of the Examples section which follows.

Preferably, the polypeptide which is used by the method of this aspect of the present invention comprises at least 20 amino acids, more preferably, at least 40 amino acids, more preferably, at least 60 amino acids, more preferably, at least 80 amino acids, more preferably, at least 100 amino acids, more preferably, at least 120 amino acids, more preferably, at least 140 amino acids, more preferably, at least 160 amino acids, more preferably, at least 180 amino acids, even more preferably, about 195 amino acids of the BID polypeptide set forth by SEQ ID NO:15.

Non-limiting examples of such polypeptides include for example, polypeptides which comprise a phosphorylated PIKK phosphorylation site and includes 50-195 amino acids of the BID polypeptide set forth by SEQ ID NO:15 and thus are incapable of inducing apoptosis. For example, such a polypeptide can be any of the polypeptides set forth by SEQ ID NOs:20-22.

Additionally or alternatively, the polypeptides which comprise a mutation in the PIKK phosphorylation site which mimic phosphorylation at that site and include 50-195 amino acids of the BID polypeptide set forth by SEQ ID NO:15 and thus are incapable of inducing apoptosis can be also used according to this aspect of the present invention. For example, such a polypeptide can be any of the polypeptides set forth by SEQ ID NOs:23-25.

Still additionally or alternatively, unmodified polypeptides including 50-202 amino acids of the BID polypeptide set forth by SEQ ID NO:15 and an NLS, and which are incapable of inducing apoptosis can be also used according to this aspect of the present invention. For example, such a polypeptide can be any of the polypeptides set forth by SEQ ID NOs:26-28.

The term "polypeptide" or "peptide" as used herein encompasses native polypeptides (either degradation products, synthetically synthesized polypeptides or recombinant polypeptides), peptidomimetics (typically, synthetically synthesized peptides), as well as peptoids and semipeptoids which are peptide analogs, which may have, for example, modifications rendering the polypeptides 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 of preparing 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.

Peptide bonds (-CO-NH-) within the polypeptide 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 Phenylglycine, TIC, naphthylelanine (Nol), ring-methylated derivatives of Phe, halogenated derivatives of Phe or o-methyl-Tyr.

In addition to the above, the polypeptides 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).

As used herein in the specification and in the claims section below 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-aminoadipic 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 (Table 2) which can be used with the present invention.

***Table 1***

| *Amino Acid* | *Three-Letter Abbreviation* | *One-letter Symbol* |
|---|---|---|
| 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 | Iie | 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 2*

| *Non-conventional amino acid* | *Code* | *Non-conventional amino acid* | *Code* |
|---|---|---|---|
| α-aminobutyric acid | Abu | L-N-methylalanine | Nmala |
| α-amino-α-methylbutyrate | Mgabu | 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-methylisolleucine | Nmile |
| D-alanine | Dal | L-N-methylleucine | Nmleu |
| D-arginine | Darg | L-N-methyllysine | Nmlys |
| D-aspartic acid | Dasp | 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 |
| 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 | Mgabu |
| D-α-methylalanine | Dmala | α-methylcyclohexylalanine | Mchexa |
| D-α-methylarginine | Dmarg | α-methylcyclopentylalanine | Mcpen |
| D-α-methylasparagine | Dmasn | α-methyl-α-napthylalanine | 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-methylleucine | Dmleu | α-napthylalanine | Anap |
| D-α-methyllysine | 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 | Dmty | N-cyclodecylglycine | Ncdec |
| D-α-methylvaline | Dmval | N-cyclododeclglycine | Ncdod |
| D-α-methylalnine | Dnmala | N-cyclooctylglycine | Ncoct |
| D-α-methylarginine | Dnmarg | N-cyclopropylglycine | Ncpro |
| D-α-methylasparagine | Dnmasn | N-cycloundecylglycine | Ncund |
| D-α-methylasparatate | Dnmasp | N-(2,2-diphenylethyl)glycine | Nbhm |
| D-α-methylcysteine | Dnmcys | N-(3,3-diphenylpropyl)glycine | Nbhe |
| D-N-methylleucine | Dnmleu | N-(3-indolylyethyl) glycine | Nhtrp |
| D-N-methyllysine | Dnmlys | N-methyl-γ-aminobutyrate | Nmgabu |
| N-methylcyclohexylalanine | Nmchex | 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-methyla-napthylalanine | 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 |
| L-α-methylaspartate | Masp | L-α-methyl-*t*-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-(imidazolylethyl)glycine | Nhis |
| D-N-methylleucine | Dnmleu | N-(3-indolylyethyl)glycine | Nhtrp |
| D-N-methyllysine | Dnmlys | N-methyl-γ-aminobutyrate | Nmgabu |
| N-methylcyclohexylalanine | Nmchex | 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-methyla-napthylalanine | 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 |

| | | | |
|---|---|---|---|
| L-α-methylaspartate | Masp | L-α-methyl-*t*-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-diphenyl ethylamino)cyclopropane | Nmbc | | |

***Table 2 Cont.***

For use in clinical applications which require the polypeptides to be in soluble form, the polypeptides of the present invention preferably include one or more non-natural or natural polar amino acids, including but not limited to serine and threonine which are capable of increasing polypeptide solubility due to their hydroxyl-containing side chain.

The polypeptides of the present invention are preferably utilized in a linear form, although it will be appreciated that in cases where cyclicization does not severely interfere with peptide characteristics, cyclic forms of the peptide can also be utilized.

As mentioned, the isolated polypeptide of the present invention (e.g., the phosphorylated polypeptide set forth by SEQ ID NO:17) can be biochemically synthesized using standard solid phase techniques. 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 polypeptide is relatively short such as a small a peptide of a few amino acids (e.g., 3-20 amino acids) 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 polypeptide 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 polypeptides 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.

As used herein the term "mimetics" when made in reference to polypeptides refers to molecular structures which serve as substitutes for the polypeptides of the present invention in inducing cell cycle arrest [Morgan et al. (1989) Ann. Reports Med. Chem. 24:243-252 for a review of peptide mimetics].

Polypeptide or peptide mimetics include synthetic structures (known and yet unknown), which may or may not contain amino acids and/or peptide bonds, but retain the structural and functional features of a polypeptide ligand. Types of amino acids which can be utilized to generate mimetics are described hereinabove.

The term, "peptide mimetics" also includes 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]. 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.

Generation of peptide mimetics can be effected using various approaches, including, for example, display techniques as well as computational biology techniques.

In cases where large amounts and/or large polypeptides (e.g., above 25 amino acids) are desired, the polypeptides of the present invention can be generated using recombinant techniques such as described by Bitter et al., (1987) Methods in Enzymol. 153:516-544, Studier et al. (1990) Methods in Enzymol. 185:60-89, Brisson et al. (1984) Nature 310:511-514, Takamatsu et al. (1987) EMBO J. 6:307-311, Coruzzi et al. (1984) EMBO J. 3:1671-1680; Brogli et al., (1984) Science 224:838-843; Gurley et al. (1986) Mol. Cell. Biol. 6:559-565 and Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463.

For example, to generate the isolated polypeptide of the present invention (e.g., the polypeptide set forth by SEQ ID NO:17 or 18), a polynucleotide sequence encoding the polypeptide 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 inducible manner.

Constitutive promoters suitable for use with the present invention are promoter sequences which are active under most environmental conditions and most types of cells such as the cytomegalovirus (CMV) and Rous sarcoma virus (RSV). Inducible promoters suitable for use with the present invention include for example the tetracycline-inducible promoter [Zabala M, et al., Cancer Res. 2004, 64(8): 2799-804].

The nucleic acid construct (also referred to herein as an "expression vector") of the present invention includes additional sequences which render this vector suitable for replication and integration in prokaryotes, eukaryotes, or preferably both (e.g., shuttle vectors). In addition, a typical expression vector may also contain a transcription and translation initiation sequence, enhancers (e.g., SV40 early gene enhancer; see also Enhancers and Eukaryotic Expression, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. 1983), transcription and translation terminator, and a polyadenylation signal which may increase the efficiency of mRNA translation (e.g., the GU or U rich sequences located downstream from the polyadenylation site and a highly conserved sequence of six nucleotides, AAUAAA, located 11-30 nucleotides upstream). It will be appreciated that in order to secret the recombinant polypeptide from the host cell (*i.e.*, a cell in which the polynucleotide of the present invention is expressed) the expression vector of the present invention typically includes a signal sequence for secretion.

The expression vector of the present invention can further include 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.

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 polypeptide.

As mentioned hereinabove, a variety of cells can be used as host-expression systems to express the recombinant polypeptide of the present invention (e.g., the

polypeptide set forth by SEQ ID NO:17 or 18). 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 polypeptide coding sequence, mammalian expression systems, yeast transformed with recombinant yeast expression vectors containing the coding sequence (see for example, U.S. Pat. Application No: 5,932,447); 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 [for suitable plant expression vectors see for example, Brisson et al. (1984) Nature 310:511-514; Takamatsu et al. (1987) EMBO J. 6:307-311; Coruzzi et al. (1984) EMBO J. 3:1671-1680; Brogli et al., (1984) Science 224:838-843; Gurley et al. (1986) Mol. Cell. Biol. 6:559-565; Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463]. Bacterial systems are preferably used to produce recombinant polypeptides since they enable a high production volume at low cost.

In bacterial systems, a number of expression vectors can be advantageously selected depending upon the use intended for the polypeptide expressed. For example, when large quantities of polypeptide are desired, vectors that direct the expression of high levels of the protein product, possibly as a fusion with a hydrophobic signal sequence, which directs the expressed product into the periplasm of the bacteria or the culture medium where the protein product is readily purified may be desired. Certain fusion protein engineered with a specific cleavage site to aid in recovery of the polypeptide may also be desirable. Such vectors adaptable to such manipulation include, but are not limited to, the pET series of E. coli expression vectors [Studier et al., Methods in Enzymol. 185:60-89 (1990)].

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 Stratagene, 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.

Various methods can be used to introduce the expression vector of the present invention into host 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 at. [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 and specificity can be obtained due to the infectious nature of viruses.

Transformed cells are cultured under effective conditions, which allow for the expression of high amounts of the recombinant polypeptide. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. An effective medium refers to any medium in which a cell is cultured to produce the recombinant polypeptide of the present invention. Such a medium typically includes an aqueous solution having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes and petri plates. Culturing can be carried out at a temperature, pH and oxygen

content appropriate for a recombinant cell. Such culturing conditions are within the expertise of one of ordinary skill in the art.

Depending on the vector and host system used for production, resultant polypeptides of the present invention may either remain within the cell, secreted into the fermentation medium, secreted into a space between two cellular membranes, such as the periplasmic space in E. coli; or retained on the outer surface of a cell or viral membrane.

Following a predetermined time in culture, recovery of the recombinant polypeptide is effected. The phrase "recovery of the recombinant polypeptide" used herein refers to collecting the whole fermentation medium containing the polypeptide and need not imply additional steps of separation or purification.

Thus, 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 chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

To facilitate recovery, the expressed coding sequence can be engineered to encode the polypeptide of the present invention and a fused cleavable moiety. Such a fusion protein can be designed so that the polypeptide can be readily isolated by affinity chromatography; e.g., by immobilization on a column specific for the cleavable moiety. Where a cleavage site is engineered between the polypeptide and the cleavable moiety, the polypeptide can be released from the chromatographic column by treatment with an appropriate enzyme or agent that specifically cleaves the fusion protein at this site [e.g., see Booth et al., Immunol. Lett. 19:65-70 (1988); and Gardella et al., J. Biol. Chem. 265:15854-15859 (1990)].

The polypeptide of the present invention is preferably retrieved in "substantially pure" form. As used herein, the phrase "substantially pure" refers to a purity that allows for the effective use of the recombinant polypeptide (*i.e.*, the polypeptide of the present invention) in inducing cell cycle arrest.

It will be appreciated that for *ex vivo* or *in vivo* gene therapy applications which are further described hereinunder the polynucleotide encoding the polypeptide of the present invention is administered to the cell-of-interest (e.g., a cell of a subject

who suffers from or is predisposed to a genomic instability associated disease) to thereby induce cell cycle arrest. As used herein, the phrase "ex vivo gene therapy" refers to the process of expressing the polypeptide of the present invention in cell cultures derived from a subject (e.g., autologous or allogeneic cells) followed by administration of such cells (which express the polypeptide of the present invention) back into the subject in need of therapy. The phrase "*in vivo* gene therapy" refers to the process of expressing the polypeptide of the present invention in cells of the subject in need of therapy.

It will be appreciated that the type of viral vector and the specific promoter used for *ex vivo* or *in vivo* gene therapy 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.

Recombinant viral vectors are useful for *in vivo* expression of recombinant proteins 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.

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. 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.

It will be appreciated that other agents capable of inducing cell cycle arrest can be identified using the teachings of the present invention.

This can be effected by: (a) contacting a plurality of cells with a plurality of molecules; (b) identifying at least one molecule from the plurality of molecules capable of increasing a level of a phosphorylated BID polypeptide, the at least one molecule being the agent capable of inducing cell cycle arrest.

The "at least one molecule" or the agent described hereinabove which are capable of inducing cell cycle arrest can be for example a peptide, an oligonucleotide, a carbohydrate or any chemical which is capable of inducing BID phosphorylation, preferably in a PIKK site.

Presence or level of a phosphorylated BID polypeptide can be detected using immunological detection methods known in the art such as Western blot analysis, immunohistochemistry and the like. It will be appreciated that since BID-induced cell cycle arrest is mediated by BID phosphorylation on a PIKK site, the method according to this aspect of the present invention preferably detects the level of a PIKK-phosphorylated BID polypeptide. Such PIKK-phosphorylated BID polypeptide can be detected using, for example, the anti-phosphospecific BID antibodies (αpS61, αpS78) described in the Examples section which follows.

In addition, since BID phosphorylation, which is associated with induction of cell cycle arrest occurs in the nucleus, the method according to this aspect of the present invention preferably detects the level of phosphorylated BID in the cell nucleus. Detection of phosphorylated BID in the cell nucleus can be effected using antibodies which bind phosphorylated BID (e.g., αpS61, αpS78) which are employed in Western blot analyses of sub-cellular fractions (see for example, Figures 19a-d) or in immunofluorescence analyses (see for example, Figures 18a-h).

The polypeptides as well as other agents generated using the above-teachings can be qualified by functional assays, such as by monitoring the effect of the polypeptides on cell cycle arrest or apoptosis in cells. The induction of cell cycle arrest can be determined following treatment of cells with various agents known to induce DNA damage (e.g., etoposide, mitomycin C, hydroxyurea, UV, or IR) and detecting the fraction of cells in each of the cell cycle phases using, for example, FACS analysis, BrDU labeling, essentially as described in the Examples section which follows (see for example, Figures 22a-i, 23a-c, 25a-i and 33a-c). The effect of the polypeptides on apoptosis can be determined following exposure of cells to DNA damaging agents 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.

It will be appreciated that the polypeptides described hereinabove which are incapable of inducing apoptosis and thus are capable of inducing cell cycle arrest can be used to treat diseases associated with a genomic instability.

Thus, according to an additional aspect of the present invention there is provided a method of treating a disease associated with a genomic instability. The method is effected by introducing into-, or expressing in cells of an individual in need thereof the BID polypeptide which is incapable of inducing apoptosis, thereby inducing cell cycle arrest and treating the disease associated with the genomic instability.

The term "treating" refers to inhibiting, preventing, curing, reversing, attenuating, alleviating, minimizing, suppressing or halting the deleterious effects of a pathology or a disease and/or causing the reduction, remission, or regression of a pathology or a disease. Those of skill in the art will understand that various methodologies and assays can be used to assess the development of a pathology or a disease, and similarly, various methodologies and assays may be used to assess the reduction, remission or regression of the pathology or the disease.

The term "preventing" refers to keeping a pathology or a disease from occurring in a subject who may be at risk for having the pathology, but has not yet been diagnosed as having the pathology or the disease.

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. Non-limiting examples of individuals who are at risk of developing the pathology of the present invention include individuals who are genetically predisposed to develop the pathology (e.g., individuals who carry a mutation associated with the pathology, e.g., a mutation in the BRCA1 gene), and/or individuals who are at high risk to develop the pathology due to other factors such as environmental hazard or other pathologies (e.g., individuals who are exposed to DNA damaging agents as described hereinabove).

As used herein the term "pathology" refers to any deviation from the normal 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.

According to the method of this aspect of the present invention the disease is associated with a genomic instability.

As used herein, the phrase "disease associated with a genomic instability" refers to any pathology caused from or characterized by instable genome in at least a portion of the cells of the individual suffering from or predisposed to the pathology. In cells with instable genome, the DNA checkpoints can be abnormal, leading to the accumulation of irreparable mutations which may lead to tumorigenic processes.

Preferably, the disease associated with genomic instability is associated with an abnormal S phase checkpoint which may result in severely damaged genome leading to tumorigenic cells.

Preferably, the disease associated with genomic instability can be Ataxia-Telangiectasia, Fanconi anemia, Bloom's syndrome, hereditary breast and ovarian cancer syndromes involving BRCA1 and Nijmegen breakage syndrome (NBS1).

Polypeptides and other agents which are capable of inducing cell cycle arrest according to the teachings of the present invention can be administered to the subject per se, or in a pharmaceutical composition where it is mixed with suitable carriers or excipients as is further described hereinbelow.

As is mentioned hereinabove, the present inventors have uncovered that expression of a PIKK non-phosphorylatable BID polypeptide in cells lacking endogenous BID (e.g., $BID^{-/-}$ MEFs) resulted in increased levels of apoptosis following exposure to a DNA damaging agent such as Etoposide (Figure 26a; Example 4). These results demonstrate that agents capable of inhibiting PIKK-mediated BID phosphorylation can be used to induce apoptosis.

Thus, according to another aspect of the present invention there is provided a method of inducing apoptosis. The method is effected by introducing into-, or expressing in a cell a polypeptide comprising a BID amino acid sequence incapable of inducing cell cycle arrest, thereby inducing apoptosis.

Preferably, the polypeptide used by the method according to this aspect of the present invention comprises a mutation in the PIKK phosphorylation site which abolishes phosphorylation at the PIKK phosphorylation site, while still allowing apoptosis. Such a mutation can be, for example, a serine to alanine substitution such as the BID-S61A/S78A mutant described in Example 4 of the Example section which follows. Over expression of such a polypeptide in the cell can saturate the BID binding domain on the PIKK protein (e.g., ATM), thus, downregulating the level of endogenous PIKK-phosphorylated BID in the cell. A non-limiting example of such a polypeptide is the polypeptide set forth by SEQ ID NO:5.

It will be appreciated that inhibition of PIKK-mediated BID phosphorylation can be also effected using a polypeptide having a PIKK phosphorylation site which is capable of inhibiting PIKK-mediated phosphorylation of endogenous BID. Such a polypeptide can be a short peptide (e.g., of 5-25 amino acids) having a BID amino acid sequences and includes the PIKK site (e.g., the peptides set forth by SEQ ID NOs:29-31) and thus competes with endogenous BID on the BID binding domain in the PIKK protein (dominant negative).

Additionally or alternatively, the polypeptide used by the method according to this aspect of the present invention can be a short phosphorylated peptide (e.g., of 5-30 amino acids in length) comprising a BID amino acid sequence, which includes the PIKK phosphorylation site. Such a peptide can bind to proteins capable of recognizing phosphorylated BID and thus block the BID-mediated cell cycle arrest and induce apoptosis. Preferably, such peptides comprise a BID amino acid sequence

which does not exceed 20 amino acids in length. Non-limiting examples of such peptides are set forth by SEQ ID NOs:32-34.

Still additionally or alternatively, the polypeptide used by the method according to this aspect of the present invention can be a short peptide (e.g., of 5-30 amino acids in length) comprising a BID amino acid sequence, which includes a mutation which mimics phosphorylation at the PIKK phosphorylation site. Such a peptide can bind to proteins capable of recognizing phosphorylated BID and thus block the BID-mediated cell cycle arrest and induce apoptosis. Preferably, such peptides comprise a BID amino acid sequence which does not exceed 20 amino acids in length. Non-limiting examples of such peptides are set forth by SEQ ID NOs:35-37.

Preferably, the polypeptide used by the method of this aspect of the present invention is capable of inducing apoptosis. Qualified polypeptides can be evaluated using apoptosis functional assays as described hereinabove.

As described in the Examples section which follows, the present inventors have generated phospho-specific antibodies against BID polypeptides being phosphorylated on serine 61 (αpS61) or serine 78 (αpS78) by immunizing rabbits with synthetic peptides being phophorylated on a serine residue corresponding to the serine residue at position 61 of mouse BID or at position 78 of mouse BID. The resulting anti phospho-specific serine 61 (αpS61) or anti phospho-specific serine 78 (αpS78) antibodies were affinity-purified using the phosphopeptide immobilized on solid support. Such antibodies were qualified by Western blot analysis for being specific to their respective phosphorylated mouse BID proteins (αpS61 - Figure 7d; αpS78 – Figure 7f) as well as for phosphorylated human BID (αpS78 – Figure 11a).

Thus, according to yet an additional aspect of the present invention there is provided an antibody comprising an antigen recognition domain capable of specifically binding a BID polypeptide phosphorylated on a serine residue corresponding to amino acid 78 of human BID as set forth in SEQ ID NO:15 but does not bind the BID polypeptide when not phosphorylated on this respective position.

According to still an additional aspect of the present invention there is provided an antibody comprising an antigen recognition domain capable of specifically binding a BID polypeptide phosphorylated on a serine residue

corresponding to amino acid 78 of mouse BID as set forth in SEQ ID NO:16 but does not bind the BID polypeptide when not phosphorylated on this respective position.

According to still a further aspect of the present invention there is provided an antibody comprising an antigen recognition domain capable of specifically binding a BID polypeptide phosphorylated on a serine residue corresponding to amino acid 61 of mouse BID as set forth in SEQ ID NO:16 but does not bind the BID polypeptide when not phosphorylated on this respective position.

The term "antibody" as used in this invention includes intact molecules as well as functional fragments thereof, such as Fab, F(ab')2, Fv or single domain molecules such as VH and VL to an epitope of an antigen. 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; (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; and (6) Single domain antibodies are composed of a single VH or VL domains which exhibit sufficient affinity to the antigen.

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 and the Examples section which follows).

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 (19720]. 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); Verhoeyen 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).

It will be appreciated that targeting of particular compartment within the cell can be achieved using intracellular antibodies (also known as "intrabodies"). These are essentially SCA to which intracellular localization signals have been added (e.g., for nuclear expression a polynucleotide encoding the NLS peptide (SEQ ID NO:19) can be added). This technology has been successfully applied in the art (for review, see Richardson and Marasco, 1995, TIBTECH vol. 13). Intrabodies have been shown to virtually eliminate the expression of otherwise abundant cell surface receptors and to inhibit a protein function within a cell (See, for example, Richardson et al., 1995, Proc. Natl. Acad. Sci. USA 92: 3137-3141; Deshane et al., 1994, Gene Ther. 1: 332-337; Marasco et al., 1998 Human Gene Ther 9: 1627-42; Shaheen et al., 1996 J. Virol. 70: 3392-400; Werge, T. M. et al., 1990, FEBS Letters 274:193-198; Carlson, J.R. 1993 Proc. Natl. Acad. Sci. USA 90:7427-7428; Biocca, S. et al., 1994, Bio/Technology 12: 396-399; Chen, S-Y. et al., 1994, Human Gene Therapy 5:595-601; Duan, L et al., 1994, Proc. Natl. Acad. Sci. USA 91:5075-5079; Chen, S-Y. et

al., 1994, Proc. Natl. Acad. Sci. USA 91:5932-5936; Beerli, R.R. et al., 1994, J. Biol. Chem. 269:23931-23936; Mhashilkar, A.M. et al., 1995, EMBO J. 14:1542-1551; PCT Publication No. WO 94/02610 by Marasco et al.; and PCT Publication No. WO 95/03832 by Duan et al.).

To prepare an intracellular antibody expression vector, the cDNA encoding the antibody light and heavy chains specific for the target protein of interest are isolated, typically from a hybridoma that secretes a monoclonal antibody specific for the marker. Hybridomas secreting anti-marker monoclonal antibodies, or recombinant monoclonal antibodies, can be prepared using methods known in the art. Once a monoclonal antibody specific for the marker protein is identified (e.g., either a hybridoma-derived monoclonal antibody or a recombinant antibody from a combinatorial library), DNAs encoding the light and heavy chains of the monoclonal antibody are isolated by standard molecular biology techniques. For hybridoma derived antibodies, light and heavy chain cDNAs can be obtained, for example, by PCR amplification or cDNA library screening. For recombinant antibodies, such as from a phage display library, cDNA encoding the light and heavy chains can be recovered from the display package (e.g., phage) isolated during the library screening process and the nucleotide sequences of antibody light and heavy chain genes are determined. For example, many such sequences are disclosed in Kabat, E. A., et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242 and in the "Vbase" human germline sequence database. Once obtained, the antibody light and heavy chain sequences are cloned into a recombinant expression vector using standard methods.

It will be appreciated that the antibodies of this aspect of the present invention can be used as neutralizing antibodies capable of inhibiting the cell cycle arrest activity of the endogenous BID polypeptide within the cells.

It will be appreciated that the abovedescribed polypeptides, which are capable of inducing apoptosis, and the neutralizing antibodies which are capable of inhibiting the cell cycle arrest activity the endogenous BID polypeptide can be used to treat cancer.

Thus, according to yet an additional aspect of the present invention there is provided a method of treating cancer. The method is effected by introducing into-, or

expressing in cells of an individual in need thereof a polypeptide comprising a BID amino acid sequence incapable of inducing cell cycle arrest, thereby inducing apoptosis and treating the cancer.

As is shown in Figures 27a-b and is described in Example 5 of the Examples section which follows, PIKK-mediated phosphorylation of BID following exposure to IR was prominent in the thymus, spleen, bone marrow and kidney. Therefore, it is conceivable that prevention of PIKK-mediated BID phosphorylation in cancer cells of such tissues can be used to treat the cancer. Preferably, the cancer treated by the BID polypeptides according to this aspect of the present invention is of a lymphoid origin.

As mentioned hereinabove, the isolated polypeptides or polynucleotides encoding same and/or the neutralizing antibodies of the present invention can be administered to the individual as part of a pharmaceutical composition.

Herein the term "active ingredient" refers to the isolated BID polypeptide, the polynucleotide and/or expression vector encoding the BID polypeptide of the present invention and/or the neutralizing antibody of the present invention which is 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, neurosurgical strategies (e.g., intracerebral injection, intrastriatal infusion or intracerebroventricular infusion, intra spinal cord, epidural), transmucosal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary

injections as well as intrathecal, direct intraventricular, intracardiac, intravenous, inrtaperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the pharmaceutical composition in a local rather than a 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 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 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 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 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 include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose (*i.e.*, a therapeutically effective amount as described hereinabove).

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 tissue levels of the active ingredient that are sufficient to regulate cell cycle arrest or apoptosis (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.

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 for 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.

As is shown in the Examples section which follows, the level of PIKK-mediated BID phosphorylation was upregulated in response to DNA damaging agents such as IR and Etoposide. Thus, such a level can indicative of the response of cells to DNA damaging agents.

Thus, according to another aspect of the present invention, there is provided a method of detecting cellular exposure to DNA damaging agents. The method is effected by detecting in a biological sample a presence and/or level of a PIKK-mediated phosphorylated BID, thereby detecting the cellular exposure to the DNA damaging agents.

As used herein the phrase "cellular response" refers to the phosphorylation events occurring in a cell following exposure to DNA damaging agents which induce DNA breaks such as IR, UV, replication stress agents (e.g., Etoposide, mitomycin C and hydroxyurea) (for further details see the Examples section which follows and Zinkel SS., et al., 2005, Cell 122:579-591 which is incorporated herein by reference).

As used herein "biological sample" refers to a sample of cells isolated from a tissue or fluid of a subject, including but not limited to, for example, blood, tissue biopsy (e.g., from a tumor such as of a lymphoid origin), as well as cells which are derived from the subject and are cultured *ex vivo*.

The level of PIKK-mediated phosphorylated BID can be detected using various techniques using e.g., Western blot analysis, radio immuno assay, immunohistochemistry, FACS analysis with specific anti-BID antibodies (see for example, Figures 4a-e, 5a-b and 6a-b). Preferably, such detection utilizes phospho-specific antibodies such as the anti-pS61 and/or the anti-pS78 antibodies of the present invention (see for example, Figures 7d-g, 13a-d, 16a-b).

It will be appreciated that the presence and/or level of PIKK-mediated phosphorylation of BID can be used to diagnose disease(s) associated with abnormal PIKK-mediated phosphorylation of BID. Such diseases can be, for example, a disease associated with genomic instability as described hereinabove (e.g., A-T, breast and ovarian cancer syndromes associated with BRCA1).

Thus, according to still an additional aspect of the present invention there is provided a method of diagnosing a disease associated with an abnormal PIKK-mediated phosphorylation of BID. The method is effected by detecting in cells of an individual in need thereof a presence and/or level of a PIKK-mediated phosphorylated BID, thereby diagnosing the disease associated with the abnormal PIKK-mediated phosphorylation of BID.

As used herein the term "diagnosing" refers to classifying a disease or a symptom, determining a severity of the disease, monitoring disease progression, forecasting an outcome of a disease and/or prospects of recovery.

The agents capable of detecting the presence of PIKK-mediated phosphorylated BID (e.g., the phospho-specific antibodies of the present invention) which are described hereinabove for detecting cellular exposure to DNA damaging agents and/or diagnose disease(s) associated with abnormal PIKK-mediated phosphorylation of BID may be included in a diagnostic kit/article of manufacture preferably along with appropriate instructions for use and labels indicating FDA approval for use in detecting cellular exposure to DNA damaging agents and/or diagnosing disease(s) associated with abnormal PIKK-mediated phosphorylation of BID.

Such a kit can include, for example, at least one container including at least one of the above described diagnostic agents (e.g., the phospho-specific -αpS78 antibody) and an imaging reagent packed in another container (e.g., enzymes, secondary antibodies, buffers, chromogenic substrates, fluorogenic material) which is used to visualize the presence of bound antibodies. The kit may also include appropriate buffers and preservatives for improving the shelf-life of the kit.

As is further shown in Figures 29a-c, 30a-b and 31a-b and is described in Example 6 of the Examples section which follows, the present inventors have generated genetically modified mice in which the BID gene is incapable of PIKK-mediated phosphorylation (*i.e.*, knock-in mice).

Thus, according to another aspect of the present invention, there is provided a multicellular organism comprising a genome which comprises a genetically modified BID gene which comprises a mutation in a PIKK phosphorylation site.

As used herein the phrase "multicellular organism" refers to any organism having more than one cell, preferably, with differentiated cells that perform specialized functions (e.g., neuronal system, digestive system, cardiovascular system and the like). Preferably, the phrase "multicellular organism" refers to mammals and their fetuses, but human, such as rodents (e.g., mouse, rat, guinea pig), monkeys (e.g., gorilla, chimpanzee, gibbon, rhesus, apes in particular), pigs, sheep, cattle etc.

According to presently preferred embodiments of this aspect of the present invention, the multicellular organism is a mouse.

The phrase "genetically modified" refers to a sequence alteration which results in altered expression as compared with a wild type equivalent sequence. The sequence alteration (e.g., mutation) may be natural or man-made. Preferably, it is a mutation in a PIKK phosphorylation site (S/Q motif) which abolishes phosphorylation by PIKK proteins such as ATM and ATR. Preferably, the mutation comprises the substitution of a serine residue with an alanine residue at a position corresponding to amino acid residue 61 and/or 78 of mouse BID (SEQ ID NO:16). It will be appreciated, however, that other mutations can also result in a BID gene being incapable of PIKK-mediated phosphorylation [e.g., mutations in control sequences (e.g., promoter, enhancer) such as insertion, deletion and/or substitution of one or more nucleotides in one or more locations] and the phrase "genetically modified" is intended to include all such mutations.

As used here the term "gene" refers to a nucleic acid sequence from which a protein can be expressed or a part thereof suitable, for example, for directing homologous recombination. Thus, a gene can include, for example, a complementary DNA sequence, a genomic DNA sequence or a mixed sequence of genomic and cDNA. Additional sequences can be included, such as, polylinkers, positive and negative selection sequences, and genetically modified sequences such as sequence alterations and the like. The introns of the gene, can be for example modified, e.g., shortened. However, the term gene as used herein further relates to the control sequences flanking the nucleic acid sequence from which a protein can be expressed, in particular upstream (5') control sequences. Since a gene according to the present invention is expected to undergo homologous recombination, the term as used herein refers also to any portion of a gene that following the homologous recombination is capable of combining with endogenous sequences to reconstruct a nucleic acid sequence from which a protein can be expressed.

Preferably, the BID gene which is genetically modified in the multicellular organism of this aspect of the present invention is derived from mouse (nucleic acids 120942137-120917231 of GenBank Accession No. NC_000072.3).

Following homologous recombination (using e.g., the Cre/LoxP system as described in U.S. Pat. No. 4,959, 317 to Sauer, U.S. Pat. No. 6,924,146 to Wattler, et

al. which are fully incorporated herein by reference), the genetically modified BID gene is incapable of PIKK-mediated phosphorylation.

For example, as is shown in Figures 29b-c and 30a-b, the modified BID which comprises the S61A and S78A mutations in the targeting construct exhibits unique RFLF pattern in both Southern blot analysis (Figures 29b-c) and PCR analysis (Figures 30a-b).

It will be appreciated that such genetically modified BID organism (e.g., mouse) can be used as an *in vivo* system to test the efficacy of the agents of the present invention (e.g., the isolated BID polypeptides) in regulating cell cycle arrest or apoptosis in vivo. For example, a BID polypeptide being incapable of inducing apoptosis (e.g., the PIKK-phosphorylated BID polypeptide set forth by SEQ ID NO:17) can be administered to the BID pS61A/S78A mouse and the effect on cell cycle arrest can be tested on biological samples (e.g., of thymus, bone marrow, spleen and kidney tissues) derived from the mouse using e.g., FACS analysis. In addition, such a genetically modified BID organism can be used to screen for drugs capable of modulating BID activity in cell cycle arrest and/or apoptosis *in vivo*.

As used herein the term "about" refers to ± 10 %.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

## *EXAMPLES*

Reference is now made to the following examples, which together with the above descriptions, illustrate 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); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "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.

## *GENERAL MATERIALS AND EXPERIMENTAL METHODS*

***Mouse embryonic fibroblasts*** - *BID*$^{-/-}$ mice (originally kept on a mixed C57BL/6 x 129Sv background) had been bred to wild type C57BL/6 mice twelve times in order to obtain animals that are F12 on a C57BL/6 background. *BID*$^{-/-}$ MEFs were generated from the F12 mice. *BID*$^{+/+}$ and *BID*$^{-/-}$ primary MEFs were prepared from 11-13 day-old embryos and were maintained in ISCOVE's medium containing 10% fetal bovine serum (MEF medium). Atm/Arf double knockout (Atm-/-Arf-/-) and *Atm*$^{+/+}$*Arf*$^{-/-}$ MEFs were obtained from Chuck J. Sherr (St. Jude Children's Research Hospital).

***hTERT transformation of primary MEFs*** – The primary MEFs utilized by the present study were immortalized with the catalytic subunit of human telomerase (hTERT) (Wood et al., 2001) by viral transformation, as follows. PA317 packaging cells stably producing pBABE-puro hTERT viral particles (a generous gift from Tej Pandita, Washington University) were grown to 80 % confluence, following which the cells were rinsed and the medium was replaced with complete MEF medium. Following 16 hours of culturing the PA317 cells in complete MEF medium, the medium (i.e., infecting media) was collected, filtered through a 0.45 μm filter and stored at -80 °C until use. Primary BID-/- and BID+/+ MEFs were grown for 3 passages and were then infected at ~50 % confluence with 3 ml infecting media mixed with 3 ml MEF media and 4 μg/ml polybrene (Sigma). The infected MEFs were incubated for 16 hours, rinsed and incubated in fresh medium for an additional 8 hours. The cells were infected again as described above, rinsed and incubated in fresh medium for additional 48 hours. The cells were then split 1:3 and grown for 4 days in a selection medium containing 1 μg/ml puromycin. After selection, the cells were washed once and incubated with MEF medium (without puromycin). Stable clones were collected 14 to 18 days post-infection.

***Generation of hTERT BID*$^{-/-}$ *stable clones expressing wtBID or the BID-S61A/S78A mutant*** - ψNX cells (a 293T cell line carrying an ecotropic packaging plasmid) were seeded in a 100-mm plate at 60 % confluence and incubated for one day, following which the medium was replaced and the cells were incubated with a transfection cocktail containing 15 μg retroviral vector (pBABE-wtBID or pBABE-BID-S61A/S78A) prepared using a calcium phosphate kit (Promega). Cells were

incubated for 5 hours with the cocktail and rinsed; the medium was then replaced, and the cells were re-incubated in a fresh medium for another day. The conditioned medium containing the retroviruses was then collected, filtered through a 0.45 μm filter, divided to aliquots and frozen at -80 °C. For infection of MEFs, cells were grown in a 60-mm plate and incubated for 4 hours at 37 °C with a 3-ml retroviral supernatant, supplemented with 16 μg/ml polybrene, following which 7 ml of DMEM medium (Gibco-Invitrogen Corporation products, Grand Island, NY, USA) containing 10 % fetal bovine serum (FBS; Gibco-Invitrogen) was added. After 24 hours of incubation with the retroviral supernatant/DMEM medium, the medium was replaced with fresh DMEM (Gibco-Invitrogen) medium containing 10 % FCS and 1 μg/ml puromycin. Puromycin was replaced every day for three days. On the fourth day, cells were seeded (100 cells per 10-cm dish) and grown until single clones appeared.

***SV40 transformation of primary MEFs*** - SV40 transformation of primary MEFs was performed by transfecting cells with the SV40 whole genome using Lipofectamine 2000 (Invitrogen). Stable clones were collected 14-to-18 days post transfection.

***Preparation of splenocytes*** - The spleens from either *BID*$^{-/-}$ or *BID*$^{+/+}$ mice were pressed through stainless steel mesh to make a cell suspension in PBS. The cells were centrifuged at 1500 rpm for 5 minutes and then transferred to Ficoll-Paque Plus (Amersham) to separate between erythrocytes and splenocytes. After centrifugation at 1800 rpm for 25 minutes, the fraction of splenocytes was isolated and the cells were washed twice with PBS. The cell pellet was resuspended (1 x $10^6$ cells/ml) in RPMI 1640 medium supplemented with 50 U/ml penicillin, 50 mg/ml streptomycin, 1 mM Sodium pyruvate, 0.1 mM β-mercaptoethanol, 2 mM glutamine, 0.1 mM non-essential amino acids, and recombinant human IL-2 (40 U/ml).

***Preparation of BID recombinant adenoviruses and infection of MEFs*** - For the expression of BID in *BID*$^{-/-}$ MEFs adenovirus vectors expressing proteins under the control of tetracycline (tet)-regulatable promoters ("tet-on") were produced as previously described (Sarig et al., 2003). Briefly, in these constructs, which rely on the reverse tet transactivator (rtTA), the E1 region of the virus was replaced with either wild-type (wt) BID or GFP. Viruses were grown using 293T cells. Virus preparations were made from freeze/thaw lysis of the cells, and virus titers were done

on 293T cells. In experiments, cells were generally seeded at 70-80 % confluence. Cells were infected with an MOI (multiplicity of infection) of 100 with either the BID or GFP containing viruses and the rtTA containing virus. 1 μg/ml doxycycline (a synthetic analog of tetracycline; Sigma) was added to the medium 12-to-15 hours post infection to activate gene expression from the tet-inducible promoter. Efficiency of infection was determined using the recombinant adenovirus carrying the inducible expression vector of GFP and was in the range of 70-to-90 %.

***Clonogenic survival assays*** - Cells were seeded at a density of 1000 cells per well (6 well plate). The next day, cells were treated with the indicated DNA damage reagent, the medium was replaced with fresh medium, and cells were incubated for 10 days. Once colonies were formed, cells were fixed in 70 % methanol and stained with 0.5 % crystal Violet. The percent of colony survival was calculated as the ratio of the # of colonies after DNA damage to the # of colonies in untreated cells.

***DNA damage/death reagents and cell viability assays*** - TNFα, actinomycin D, and staurosporine were purchased from Sigma. Etoposide, cisplatin and thapsigargin were purchased from Alexis. 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).

***Human cell lines and transient transfection*** - 293, a human embryonic kidney cell line, and HeLa, a human cervical adenocarcinoma cell line were maintained in 10 % fetal bovine serum. Transient transfections were performed by the calcium phosphate method (Graham and van der Eb, 1973) or with lipofectamine 2000 (Gibco BRL).

***Generation of stable ATM knocked down cells*** - Stable ATM knocked down HeLa and 293 cells were generated by the siRNA approach using the pRETRO-SUPER viral vector (Brummelkamp et al., 2002). To generate the pRETRO-SUPER-ATM vectors, the pRETRO-SUPER vector was digested with *Bgl*II and *Hind*III and the annealed oligos (5'-gatccccctggttagcagaaacgtgcttcaagagagcacgtttctgctaaccagtttttggaaa–3' (atm7218; SEQ ID NO:1) and 5'-gatccccgataccagatccttggagattcaagagatctccaaggatctggtatctttttggaaa-3' (generously received from R. Agami, the Netherlands Cancer Institute; SEQ ID NO:2) corresponding to positions 7218 and 1267, respectively, on the ATM

transcript; GenBank Accession No. NM_138292) were ligated into the vector. As a control an oligo corresponding to *E. coli* LacZ mRNA (5'-gatccccaaggccagacgcgaattatttcaagagaataattcgcgtctggccttttttttggaaa-3'; SEQ ID NO:3) was cloned into pRETRO-SUPER. HeLa or 293 cells transfected with an ecotropic receptor were co-infected with 293 packaged viral particles of RNAi's and selected with 10 μg/ml puromycin and 200 μg/ml hygromycin to generate the stable lines.

***Metabolic labeling*** - Metabolic labeling was carried out by pre-equilibrating cells for 3 hours in phosphate-free DMEM (Gibco) prior to the addition of 0.5 mCi/ml $^{32}$P-orthophosphate (NEN) for 1.5 hours. After labeling, cells were treated for 30 minutes with Etop, or remained untreated, following which the cells were lysed with RIPA buffer (150 mM NaCl, 1 % NP-40, 0.5 % deoxycholic acid, 0.1 % SDS, 50 mM Tris, pH 8.0) in the presence of cocktails of protease and phosphatase inhibitors (1:100 Cat No. P5726 Sigma). Radiolabelled BID was immunoprecipitated using anti-BID Abs (polyclonal anti-mBID Abs described in PCT/IL2006/000021) at a dilution of 1:1000).

***Alkaline or potato-acid phosphatase treatment*** - MEFs were treated with Etop for either 30 or 60 min, lysed in phosphatase buffer (150 mM NaCl, 1 % CHAPS, 10 mM HEPES, pH 7.5) and were either remained untreated or incubated for 30 minutes at 37 °C with either alkaline phosphatase (1 U/1 μg protein; Roche) or with potato-acid phosphatase (PAP; 1.5 U/30 μg protein; Sigma). The reaction with PAP was performed in a phosphatase buffer adjusted to pH 5.5. At the end of the reaction the lysates were analyzed by Western blot using anti-BID Abs (1:1000).

***In vitro kinase assay*** - 293T cells were transiently transfected with pcDNAIII-Flag ATM WT or kinase inactive (KI) (Canman C.E., et al., 1998, Science 281: 1677-1679) or pcDNAIII-Flag ATR WT or KI (K2327R) (Tibbetts R.S., et al., 1999, Genes Dev 13: 152-157.) or pcDNAIII vector alone using the ProFection kit (Promega). 24 hours post transfection, cells were treated with 10 Gy IR for 30 minutes and then harvested and lysed in TNE buffer [100 mM NaCl, 5 mM EDTA, 0.5 % NP40, 1 x protease inhibitor cocktail set III (Calbiochem), 10 mM β-glycerophosphate, phosphatase inhibitor cocktail I and II (Sigma), 1 mM sodium orthovanadate, 5 mM sodium fluoride, 0.5 mM EDTA, 50 mM Tris, pH 8.0]. Lysates were precleared with protein A Sepharose beads (Amersham) for 30 minutes at 4 °C mixing and Flag-

tagged proteins were immunoprecipitated with anti-Flag M2 Abs coupled to agarose beads (Sigma) for 2 hours at 4 °C mixing. Immunoprecipitates were washed twice with TNE buffer, once with 100 mM Tris (pH 8.0) containing 0.5 M LiCl, and twice with kinase buffer (10 mM β-glycerophosphate, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 10 μM ATP, 50 mM NaCl, 1 mM DTT, phosphatase inhibitor cocktail I and II, 10 mM HEPES, pH 7.5). Kinase assays were performed by adding 30 μl of kinase buffer containing 10 μCi [$\gamma$-$^{32}$P] ATP and 3 μg of recombinant mouse wtBID or BID-S61A/S78A per reaction and incubating for 30 minutes at 30 °C. Proteins were separated by 12 % SDS-PAGE and transferred to PVDF membrane (Immun-blot™, Bio-Rad) and phosphorylated proteins were visualized by autoradiography. Flag-tagged proteins were subjected to immunoblotting with anti-Flag M2 Abs (Sigma) and BID levels were monitored by coomassie staining. 6xHis-tagged mouse BID was produced and purified from bacteria as previously described (Oh K.J., et al., 2005, J Biol Chem 280: 753-767).

***Formaldehyde treatment and subcellular fractionation*** - Formaldehyde was added directly to the tissue culture media to a final concentration of 1 % and the cells were incubated for 10 minutes at room temperature. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M and incubation at room temperature for 5 minutes. Cells were then subfractionated as previously described (Eldar et al., 1992). Briefly, cells were rinsed with wash buffer (125 mM KCl, 5 mM magnesium acetate, 5 mM EGTA, 1 mM β-mercaptoethanol, 30 mM Tris-HCl, pH 7.5) at 4 °C, scraped from the plates, washed twice with the same buffer and allowed to swell for 10 minutes in 0.5 ml swelling buffer [same as wash buffer except that the KCl concentration was 10 mM and protease (set III; Calbiochem) and phosphatase (set I and II; Sigma) inhibitor cocktails were added]. The cells were then lysed in a 2-ml Wheaton Dounce glass homogenizer using 30 complete up and down cycles of a glass "B"-type pestle. The homogenate obtained was overlaid on an equal volume of swelling buffer containing 25 % glycerol and centrifuged (600 x g at 4 °C for 5 minutes). The upper layer of the supernatant was designated the cytosolic fraction. It should be noted that all organelle membranes (besides the nuclear membrane) and the plasma membrane are contained in this fraction. The nuclear pellet was washed once with swelling buffer containing 25 % glycerol and 0.1 % Triton X-100. Nuclei were

resuspended in sonication buffer (100 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM β-mercaptoethanol, 10 mM Tris, pH 9.0). At this stage both the cytosolic and nuclear samples were incubated at 65 °C for 4-5 hours to reverse formaldehyde cross-links. Nuclei were then disrupted by brief sonication. Aliquots of nuclear and cytosolic fractions were separated by 12 % or 15 % SDS-PAGE and transferred to PVDF membrane (Immun-blot™, Bio-Rad).

***Western blot analysis*** - Proteins were size-fractionated by SDS-PAGE and transferred to PVDF membranes (Immobilon-P, BioRad). Western blots were developed by use of the enhanced chemiluminescence reagent (NEN).

***Anti murine and anti human BID Abs*** - Purified recombinant Histidine-tagged murine BID (Zha, J. *et al.*, 2000. Science 290:1761-1765) was used as an immunogen to generate polyclonal anti-murine BID Abs. Protein A purified anti-murine BID Abs and anti-human BID Abs (R & D), anti-MEK Abs (Sigma), anti-BAX Abs (Santa Cruz), and anti-lamin B Abs (Santa Cruz) were used for Western blotting.

***BID-S61A, BID-S78A, and BID-S61A/S78A cDNAs*** - were obtained using the QuickChange site-directed mutagenesis kit (Stratagene). Wild-type BID (SEQ ID NO:6; GenBank Accession No. NM_007544) and the BID mutants (SEQ ID NOs:7-9) were cloned into the *Eco*R1 site of pcDNAIII (Invitrogen) and into the *Bam*H1 and *Eco*R1 sites of the pBABE retroviral expression vector.

***Generation of polyclonal phospho-specific antibodies*** - Anti-pS61 and anti-pS78 were generated in collaboration with Bethyl Laboratories, Inc. (Montgomery, TX). Briefly, phosphorylated synthetic peptides, which represent portions of mouse BID around either serine 61 or serine 78, were used as immunogens to immunize rabbits. Antibodies that were not phospho-specific were removed by solid phase absorption. Antibodies that were specific for either BID pSer61 (*i.e.*, phosphorylated Ser 61) or BID pSer78 (*i.e.*, phosphorylated Ser 78) were affinity-purified using the phosphopeptide immobilized on solid support.

***Generation of monoclonal phospho-specific antibodies*** – Anti-pS78 monoclonal antibodies are generated by immunizing mice with the following synthetic peptides which represent portions of mouse BID protein (SEQ ID NO:16; GenBank Accession No. P70444) around Serine 78: CGRIEPDSESQEE (SEQ ID

NO:10; underlined Serine being phosphorylated) and CEPDSESQEEIIH (SEQ ID NO:38; underlined Serine being phosphorylated).

***BrdU labeling and analysis*** - A total of 2 x $10^5$ cells were treated for 2 hours with 20 μM Etop, washed twice with PBS and incubated in fresh medium (10 % FBS) for 8 hours. The cells were then pulsed labeled for 30 minutes with 10 μM BrdU (Sigma; added to the medium), washed with PBS, fixed with cold 70 % ethanol and incubated overnight at -20 °C. The next day, cells were collected and resuspended in 2N HCl with 0.5 % Triton X-100 for 30 minutes at room temperature followed by neutralization with 0.1 M $Na_2B_4O_7$. Cells were then collected and incubated with anti-BrdU Abs (Becton-Dickinson) for 30 minutes in the dark at room temperature. The cells were washed with PBS, and stained with FITC labeled goat anti-mouse Abs (Jackson) for 30 minutes at room temperature in the dark. The cells were then resuspended in PBS containing PI (5 μg/ml) and analyzed by FACScan. To evaluate cells that were in S phase, cells were gated on the BrdU+ population and DNA content was evaluated by PI.

In the BrdU pulse-chase experiments (to follow the progress of cells through S phase), cells were treated for 2 hours with 20 μM Etop and labeled immediately with 10 μM BrdU for 30 minutes. Cells were then washed, incubated in fresh medium for the indicated time points, and fixed for BrdU analysis as described above. The percentage of BrdU positive cells in early S phase and in late S/$G_2$ phase was determined by PI counterstaining.

***Cell cycle assays*** - MEFs were treated for 2 hours with 20 μM Etop, rinsed, and released into drug-free medium. Eight or twenty-four hours after release, cells were collected for fixation in methanol. Following fixation, cells were washed and resuspended in PBS with 25 μg/ml propidium-iodide (PI) and 50 μg/ml RNAse a half hour before FACScan analysis. Analysis of the cell cycle results was performed using the ModFit LT program (Tripathi et al., 2003).

***Immunocytochemistry*** - For immunocytochemistry, MEFs or HeLa cells were grown on glass cover slips. At the designated time points, the cells were fixed for 10 minutes with 4 % paraformaldehyde in PBS and permeabilized for 5 minutes with 0.2 % Triton X-100 in PBS. For blocking, the cells were incubated for 1 hour at room temperature in PBS containing 0.1 % Triton and 3 % bovine serum albumin (BSA).

For immunostaining, cells were incubated overnight at 4 °C with either anti-murine BID Abs (diluted 1:200) or the anti-pS61/pS78 Abs (diluted 1:100) in blocking solution. After three washes with PBS containing 0.1 % Triton, the cells were stained for 1 hour at room temperature with Alexa 488-labeled goat anti-rabbit Abs (dilution 1:120, Molecular Probes), followed by 5 minutes incubation with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining (10 μg/ml). The coverslips were mounted with elvanol, and the cells were viewed under a Nikon fluorescence microscope at a magnification of 200x/400x. Images were captured using a 1310 digital camera (DVC).

## *EXAMPLE 1*

## *BID INVOLVES IN DNA DAMAGE-INDUCED APOPTOSIS VIA ATM KINASE*

To determine whether BID is required for DNA damage-induced apoptosis, hTERT-immortalized *BID*$^{+/+}$ and *BID*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were generated and their response to a variety of DNA-damaging agents was analyzed.

***Experimental Results***

***BID is important for DNA damage-induced apoptosis*** - The DNA damaging agents used included Etoposide (Etop; a specific inhibitor of topoisomerase II), cisplatin (Cis; forms covalent adducts with the DNA), ultraviolet radiation (UV; induces thymine dimers), and ionizing radiation (IR). As is shown in Figures 1a-b, the *BID*$^{-/-}$ MEFs were less susceptible to all four treatments than the *BID*$^{+/+}$ MEFs. These DNA-damaging reagents also induced less cell death in primary *BID*$^{-/-}$ MEFs than in primary *BID*$^{+/+}$ MEFs (data not shown), confirming that this decreased sensitivity is not due to hTERT immortalization. Splenocytes from *BID*$^{-/-}$ mice also display less susceptibility to Etop treatment (Figure 2a), indicating that the decreased death response to DNA damage is a general feature of BID deficiency, although it is more pronounced in MEFs.

To confirm that *BID*$^{-/-}$ MEFs are indeed less sensitive to DNA damage-induced cell death than *BID*$^{+/+}$ MEFs, clonogenic survival assays were performed with MEFs following DNA damage. The results demonstrate that *BID*$^{-/-}$ MEFs have increased clonogenic survival following IR and Etop treatment (Figure 3a and data not shown). In addition, the present inventors created MEFs that are more sensitive to

DNA damage-induced cell death by transforming primary *BID*$^{+/+}$ and *BID*$^{-/-}$ MEFs with the SV40 whole genome. Cell death assays with these cells using much lower levels of DNA-damaging reagents confirmed that BID loss reduces sensitivity to DNA damage-induced cell death (Figures 2b-c). Finally to confirm that the reduced susceptibility of *BID*$^{-/-}$ MEFs to DNA-damaging reagents was due to the absence of BID, *BID*$^{-/-}$ MEFs were infected with recombinant adenoviruses carrying the BID vector prior to treatment with Etop or IR. The results show that reintroduction of BID did not induce cell death on its own but fully restored susceptibility to Etop- (and partially to IR-) induced cell death (Figure 3b).

***DNA-damaging reagents causing double-strand breaks induce double electrophoretic mobility shift in BID*** – To explore whether BID was modified in response to DNA damage, hTERT-immortalized *BID*$^{+/+}$ MEFs treated with the DNA-damaging reagents described hereinabove and cell lysates were subjected to Western blot analysis using anti-BID antibodies. As is shown in Figures 4a-b, Etop and IR, which are known to induce double-strand breaks (DSBs) in DNA, unlike Cis or UV, induced a double electrophoretic mobility shift in BID. To further study the involvement of BID in apoptosis, MEFs were treated with several other apoptotic reagents: thapsigargin (Thaps; stress signaling from the ER, which inhibits the $Ca^{2+}$ adenosine triphosphate pump); TNFα together with actinomycin D; or with staurosporine (STS; a kinase inhibitor) and were further subjected to Western blot analysis using anti-BID antibodies. As is further shown in Figures 4a-b, none of the other apoptotic reagents affected the electrophoretic mobility of BID. Etop and IR induced a similar double electrophoretic mobility shift in BID also in primary *BID*$^{+/+}$ MEFs (data not shown), confirming that this shift is not due to hTERT immortalization. Similar mobility shifts have been associated with covalent modifications of proteins, for example, as a consequence of phosphorylation.

***The double electrophoretic mobility shift in BID is due to phosphorylation of BID*** - To define whether the double electrophoretic mobility shift in BID was due to phosphorylation, *BID*$^{+/+}$ MEFs were treated for 30 minutes with Etop, lysed and incubated for 30 minutes at 37 °C with alkaline phosphatase or remained untreated. Western blot analysis using anti-BID antibodies demonstrated that treatment with alkaline phosphatase abolished the electrophoretic mobility shifts in BID (Figure 4c),

indicating that these shifts are most likely due to phosphorylation. Taken together, these results strongly suggest that BID is rapidly phosphorylated in response to reagents that induce double-strand breaks in DNA.

To confirm that BID was phosphorylated, human cervical adenocarcinoma (HeLa) cells were transfected with mouse BID, labeled with $^{32}$P-orthophosphate, treated with Etop or remained untreated and were subjected to BID-immunoprecipitation. Exposure to Etop resulted in a marked increase in $^{32}$P-labeling of BID, which appeared as a doublet (Figure 4d). Western blot analysis of the same samples with anti-BID antibodies indicated that the two $^{32}$P-labeled bands correspond to the two slower-migrating forms of BID (Figure 4e).

***The phosphorylation of BID is mediated by the ATM kinase*** - The ATM kinase plays a pivotal role in the immediate response of cells to double-strand breaks. To determine whether ATM is involved in the phosphorylation of BID, mouse embryonic fibroblasts deficient in both ATM and the p19ARF tumor suppressor gene were utilized, since loss of ARF has been shown to reverse premature replicative arrest of Atm-null MEFs (Kamijo et al., 1999). Accordingly, Atm/Arf double knockout MEFs, as well as *Atm*$^{+/+}$*Arf*$^{-/-}$ MEFs, were treated with Etop or IR, and were subjected to Western blot analysis using anti-BID antibodies. Figures 5a-b show that following Etop or IR treatment, the slower migrating bands of BID do not appear in the ATM-deficient cells. Thus, the presence of the ATM kinase appears to play an essential role in the process by which etoposide and ionizing radiation induce phosphorylation of BID.

To corroborate these findings, a stable HeLa cell line carrying a siRNA against ATM (Elkon R., et al., 2005, Genome Biol 6, R43), in which the level of ATM was reduced by ~95 % was utilized along with a control HeLa cell line, which carried a siRNA against LacZ. Both HeLa cell lines were transfected with mouse BID, exposed to Etop treatment and were further subjected to Western blot analysis using anti-BID antibodies. Exposure of control HeLa cells to Etop induced a double electrophoretic mobility shift in BID that was absent in the ATM knocked down cells (Figures 6a-b). *BID*$^{-/-}$ MEFs were used as a specificity control (Figures 6a-b). These results further confirm that the presence of ATM is essential for BID phosphorylation.

Altogether, these results demonstrate that BID involves in DNA damage-induced apoptosis and that this involvement is mediated phosphorylation of BID by ATM.

## *EXAMPLE 2*

## *MOUSE AND HUMAN BID ARE PHOSPHORYLATED ON PIKK CONSENSUS SITES*

ATM is a member of the PIKK family. The common phosphorylation sites for PIKKs are serines or threonines followed by glutamine residues, a motif commonly dubbed "SQ/TQ" (Kim et al., 1999). Mouse BID carries two such motifs ($S^{61}Q$ and $S^{78}Q$), whereas human and rat BID carry only one ($S^{78}Q$) (Figure 7a). To determine whether mouse BID is phosphorylated on one or both of these sites, each of the serine residues were mutated to alanine residues and the effect of Etop was evaluated on the mutated BID proteins, as follows.

***Transfection of HeLa cells with the S61A BID mutant abolished the Etop – induced double electrophoretic mobility shift*** - HeLa cells were transfected with either wild-type (wt) BID or one of the BID mutants (*i.e.*, S61A or S78A), treated with Etop and subjected to Western blot analysis using anti-BID antibodies. As is shown in Figure 7b, treatment of the BID-wt – transfected HeLa cells with Etop resulted in a double electrophoretic mobility shift, which was abolished in HeLa cells transfected with the BID S61A mutant. In contrast, mutating the S78 site had no effect on the appearance of the two slower-migrating bands. Thus, BID phosphorylation on S61 is likely the cause for the electrophoretic mobility shift.

***Mouse BID is phosphorylated on both Serine 61 and Serine 78 in response to Etop treatment*** - To confirm the results presented above and to establish whether serine 78 is also phosphorylated in mouse BID in response to Etop, phospho-specific antibodies to serine 61 (anti-pS61) and serine 78 (anti-pS78) were generated. To define whether these antibodies recognize the phosphorylated form of BID, $BID^{+/+}$ MEFs were treated for 30 minutes with Etop or remained untreated, lysed, and incubated for 30 minutes at 37 °C with potato-acid phosphatase or remained untreated. Western blot analysis using the anti-pS61 antibodies demonstrated that these antibodies recognize a band of the expected size of BID in Etop-treated cells,

and that treatment with potato-acid phosphatase abolishes this recognition (Figure 7d; note that these antibodies recognize an additional ~30 kDa protein that shares antigenicity with pS61-BID). Western blot analysis using the anti-pS78 antibodies demonstrated that these antibodies recognize three bands (one strong band and two very faint bands) in Etop-treated cells and that treatment with potato-acid phosphatase abolishes all three bands (Figure 7f). The bands identified with both antibodies corresponded to BID, since they were not identified in *BID*$^{-/-}$ MEFs (Figures 7d and f).

***Mutation of serine to alanine prevents the recognition of mouse BID by the pS61 and pS78 antibodies*** – To determine whether mutation of either serine 61 or serine 78 to alanine abolishes the recognition of mouse BID by the phospho-specific antibodies, HeLa cells transfected with either wt-BID, BID-S61A mutant, or BID-S78A mutant and the transfected cells were treated with Etop and subjected to Western blot analysis using the phospho-specific antibodies. As is shown in Figures 8a-d, the anti-pS61 and anti-pS78 antibodies recognized BID in cells expressing wt-BID and treated with Etop, but not in cells expressing the BID-S61A or BID-S78A mutant, respectively.

***ATM phosphorylates BID in vitro at S61 and S78*** - To determine if ATM (and possibly ATR) could directly phosphorylate BID, *in vitro* kinase assays were performed in 293T cells using flag-tagged human ATM and ATR and the kinase-inactive forms thereof, and purified recombinant mouse wt-BID and the BID-S61A/S78A double mutant. The results demonstrate that wild type ATM and ATR, but not the kinase-inactive forms, efficiently phosphorylate recombinant wt-BID *in vitro* (Figures 9a-c), whereas mutating both S61 and S78 completely abolished BID phosphorylation (Figures 9d-e). Taken together, these data indicate that BID is a substrate of both ATM and ATR *in vitro*, and that S61 and/or S78 are the sites phosphorylated by these kinases.

***BID is phosphorylated on both S61 and S78 in mouse splenocytes*** - The phosphorylation status of BID in mouse splenocytes was tested by treating cells with Etop and subjecting the cell lysates to Western blot analyses using anti-BID, anti pS61 or anti-pS78 antibodies. As is shown in Figures 10a-d, Etop treatment induced the phosphorylation of BID on both S61 and S78 in splenocytes.

***human BID is phosphorylated on S78 in response to Etop*** - As mentioned above, human BID carries only one PIKK consensus site (S78; Figure 7a). To determine whether endogenous human BID is phosphorylated on S78, Western blot analysis was performed with anti-human BID and anti-pS78 antibodies on lysates of HEK293 cells following Etop treatment. As is shown in Figures 11a-c, the anti-pS78 antibodies recognized a band of the size of human BID only in cells treated with Etop. To confirm these results, human BID was cloned and WT-human BID or BID-S78A mutant were expressed HeLa cells which were subject to Etop treatment. Western blot analysis using anti-pS78 antibodies showed an increase in the intensity of the band that represents phosphorylated BID in cells transfected with wild-type human BID but not in cells transfected with the BID-S78A mutant (Figures 12a-c). Thus, human BID is phosphorylated on S78 in response to Etop.

***Phosphorylation of serine 61 and serine 78 is transient, ATM-dependent, and only occurs in response to reagents that induce double-strand breaks*** - To determine the time course of endogenous mouse BID phosphorylation, $BID^{+/+}$ MEFs were treated with Etop and were subjected to Western blot analysis using anti-BID, anti-pS61, or anti-pS78 antibodies. As is shown in Figures 13a-d, phosphorylation of S61 was detected by 15 minutes (the first time point analyzed), reached a peak at 1 hour, and was reduced by 3 hours post Etop treatment. Phosphorylation of S78 was also transient (peak at 2-3 hours), though was somewhat delayed, compared to phosphorylation of the S61 site.

***Phosphorylation of BID occurs hours prior to onset of apoptosis*** – To determine when apoptosis begins in MEFs treated with Etop, $BID^{+/+}$ MEFs were treated with 100 μM Etop and cell death was determined following 2, 4, 8, 12 and 22 hours of treatment. As is shown in Figure 14, the onset of apoptosis occurred between 8 and 12 hours following Etop treatment, which is hours after BID phosphorylation.

***Endogenous mouse BID is phosphorylated on S61 and S78 only in $Atm^{+/+}Arf^{-/-}$ MEFs treated with Etop*** - To determine whether endogenous mouse BID is phosphorylated on S61 and S78 in an ATM-dependent manner, the phospho-specific antibodies were utilized in Western blot analysis of Atm/Arf double knockout and $Atm^{+/+}Arf^{-/-}$ mouse embryonic fibroblasts following Etop treatment. As is shown

in Figures 15a-d, both anti-pS61 and anti-pS78 recognized phosphorylated BID only in $Atm^{+/+}Arf^{-/-}$ MEFs which were treated with Etop.

***Mouse BID is phosphorylated on S61 only in response to reagents that induce double-strand breaks*** - To show that phosphorylation of mouse BID was specific for reagents inducing double-strand breaks, mouse embryonic fibroblasts were treated with several DNA-damaging and other apoptotic reagents (previously described with respect to Figures 4a-b). Post-treatment, cells were lysed, and the phosphorylation of endogenous mouse BID was examined by Western blot analysis using anti-pS61 antibodies. These results demonstrated that mouse BID is phosphorylated on S61 only in response to Etop and IR treatments which are known to induce double-strand breaks (Figures 16a-b).

***Human BID is phosphorylated in an ATM-dependent manner in response to reagents which induce double-strand DNA breaks*** - To define whether phosphorylation of human BID was also ATM-dependent and occurred only in response to reagents that induce double-strand breaks, stable HEK293 cell line in which ATM was knocked down by siRNA [these cells were generated like the HeLa ATM knocked down cells (Elkon et al., 2005)] or control cells, which carried a siRNA against LacZ, were exposed to Etop, IR, UV, or STS, and were subjected to Western blot analysis using anti-pS78 antibodies. Exposure of LacZ knocked down cells to Etop or IR, but not to UV or STS, induced phosphorylation of endogenous human BID on serine 78, which did not occur in the ATM knocked down cells (Figures 17a-b).

Altogether, these results demonstrate that BID phosphorylation on serine 61 and serine 78 is transient, ATM-dependent, and only occurs in response to reagents that induce double-strand breaks. In addition, phosphorylation of BID occurs hours prior to onset of apoptosis.

## *EXAMPLE 3*

## *CELLULAR BID LOCALIZES AND PHOSPHORYLATED IN THE NUCLEUS*

The cellular location of BID and its phosphorylated form were analyzed in healthy cells by immunofluoresence of $BID^{+/+}$ and $BID^{-/-}$ MEFs using anti-BID antibodies.

***BID is localized to cells and nuclei of MEFs*** - Surprisingly, immunofluorescence analysis using the anti-BID antibodies revealed positive staining of BID also in the nucleus (Figure 18a). These antibodies specifically recognized BID, since very low staining was present in *BID*$^{-/-}$ MEFs (Figure 18b). To determine whether Etop treatment leads to a change in the staining pattern of BID, *BID*$^{+/+}$ and *BID*$^{-/-}$ MEFs were treated for 3 hours with Etop prior to fixation. These studies have indicated that Etop did not change the staining pattern of BID (Figures 18e and f).

***BID partially localizes to the nuclear fraction*** - To assess the cellular localization of BID, MEFs of BID+/+ were subjected to subcellular fractionations followed by Western blot analysis using anti-BID antibodies. In these experiments cellular BID was detected only in the soluble/cytoplasmic fraction (Figure 19a, lanes 1 and 2). MEK and BAX (cytosolic proteins) and lamin B (a nuclear protein) were used as markers to confirm the purity of the nuclear/cytoplasmic fractions (Figures 19b-d, lanes 1 and 2). To further examine the possibility that BID is loosely associated with the cell nuclei, MEFs were treated with formaldehyde as a cross-linker prior to cellular disruption and the cellular fractions were further subjected to Western blot analysis using anti BID antibodies. As is further shown in Figures 19a-d, a small fraction of cellular BID was localized to the nuclear fraction (Figure 19a, lanes 3 and 4). These results together with the immunofluoresence results demonstrate that BID might be loosely associated with the nuclear fraction, and that cellular disruption leads to its dissociation from this fraction. In addition, these results suggest that the association of BID to the nuclear fraction might be via the interaction with another protein(s), and that cross-linking is required to preserve this interaction.

***Etop treatment does not change the level of BID associated with the nuclear fraction*** - To determine whether Etop treatment leads to a change in the levels of nuclear BID, cells were treated for 1 and 3 hours with Etop, and then treated with formaldehyde followed by subcellular fractionation. The results demonstrate that Etop treatment did not change the levels of BID associated with the nuclear fraction (data not shown).

***BID is phosphorylated in the nucleus*** - To determine the cellular location of the phosphorylated forms of BID, immunofluoresence studies were performed with the phospho-specific antibodies using *BID*$^{+/+}$ and *BID*$^{-/-}$ MEFs following 30 minutes

of Etop treatment. These antibodies detected an increase in nuclear fluorescence in *BID*$^{+/+}$ MEFs treated with Etop, however a similar increase was detected in *BID*$^{-/-}$ MEFs (data not shown). Thus, these antibodies cross-react with other phospho-S/T-Q proteins. Nonetheless, the fact that BID partially localizes to the nucleus, and that all currently identified substrates of ATM are nuclear proteins, suggests that BID is phosphorylated in the nucleus.

To assess whether BID might be involved in the immediate cellular response to DNA damage, the earliest point following exposure to ionizing radiation at which BID is phosphorylated was determined in mouse embryonic fibroblasts. Phosphorylation of serine 61 was detectable immediately after exposing cells to 50 Gy IR (Figures 20a-c). Since phosphorylation of BID on both serine 61 and serine 78 occurs several hours prior to the onset of apoptosis (Figures 13a-d), the present inventors have speculated that phosphorylation might also occur in response to extremely low levels of ionizing radiation which do not result in apoptosis. Indeed, a 25-fold lower dose of IR (0.2 Gy) was sufficient to induce phosphorylation of BID (Figures 21a-c).

Altogether, these results demonstrate that BID partially localizes to and is phosphorylated in the nucleus.

## *EXAMPLE 4*

## *BID INVOLVEMENT IN THE ACCUMULATION IN THE S AND G2 PHASES OF CELL CYCLE FOLLOWING ETOPOSIDE TREATMENT IS PHOSPHORYLATION-DEPENDENT*

The functional consequences of certain ATM phosphorylation events include activation of cell cycle checkpoints, which result in temporary arrest of cell cycle progression to enable DNA repair (Iliakis et al., 2003). The best-documented, damage-induced cell cycle checkpoints operate in the $G_1$/S boundary, and at the S and $G_2$ phases. To examine the possible involvement of BID in cell cycle arrest following DNA double-strand breaks, the present inventors performed cell cycle analyses on *BID*$^{+/+}$ and *BID*$^{-/-}$ mouse embryonic fibroblasts following etoposide treatment, as follows.

***BID$^{-/-}$ mouse embryonic fibroblasts fail to accumulate in the S and $G_2$ phases of the cell cycle following etoposide treatment*** - Etop induced accumulation of *BID*$^{+/+}$ MEFs in the S and $G_2$ phases of the cell cycle (as measured 8 hours after release into drug-free medium), whereas such an accumulation was not observed in the *BID*$^{-/-}$ MEFs (Figures 22a-i). These results suggest that BID is important for the accumulation of cells in the S and $G_2$ phases of the cell cycle following double-strand break DNA damage.

To determine whether BID is required for S phase arrest following DNA damage, double labeling experiments were performed with BrdU and PI to determine the level of DNA synthesis and to follow the progress of cells through S phase after Etop treatment. The results demonstrate that *BID*$^{+/+}$ MEFs show a decrease in DNA synthesis (less BrdU positive cells), whereas *BID*$^{-/-}$ MEFs fail to show a decrease in DNA synthesis (Figure 23a). Moreover, *BID*$^{-/-}$ MEFs are not delayed in their progression form S to $G_2$/M (Figure 23c) as compared to *BID*$^{+/+}$ MEFs (Figure 23b). Taken together, these experiments prove that *BID*$^{-/-}$ MEFs fail to arrest in S phase following Etop treatment.

***BID$^{-/-}$ mouse embryonic fibroblasts expressing a non-phosphorylatable BID mutant (S61A/S78A) do not accumulate in the S phase following etoposide treatment*** - To assess whether ATM-mediated phosphorylation regulates BID's function in this process, *BID*$^{-/-}$ single stable clones expressing either wt-BID or a non-phosphorylatable BID mutant (S61A/S78A) were generated. Initial analysis showed that the wt-BID (in the wt-BID clones) was phosphorylated on S61 and S78 in response to Etop, and that BID-S61A/S78A (in the mutant BID clones) was not phosphorylated (Figures 24a-f). Next, cell cycle analysis was performed on two of the wt-BID and two of the BID-S61A/S78A stable clones. As shown in Figures 25a-i, Etop induced accumulation of the wt-BID clones in the S and $G_2$ phases of the cell cycle (as measured 8 hours after release into drug-free medium), whereas the mutant BID clones bypassed accumulation in the S phase and rapidly accumulated in the $G_2$ phase. Thus, the mutant BID cells were found to be impaired in their ability to temporarily arrest in S phase following double-strand break DNA damage.

***BID$^{-/-}$ mouse embryonic fibroblasts expressing BID-S61A/S78A are more susceptible to Etop-induced apoptosis than those expressing wild-type BID*** - To explore a possible connection between BID phosphorylation, cell cycle progression, and apoptosis, the levels of apoptosis was assessed in the two wt-BID and in the two BID-S61A/S78A clones following Etop treatment. The results indicate that Etop induced a significantly higher rate of apoptosis in *BID$^{-/-}$* clones expressing BID-S61A/S78A than in the *BID$^{-/-}$* clones expressing wt-BID (Figure 26a). It is interesting that UV or TNFα treatment did not induce increased apoptosis in the mutant BID clones (Figure 26a). Western blot analysis using anti-BID antibodies indicated that the increase in apoptosis seen in the mutant BID clones in response to Etop was not due to either higher levels of expression of mutant BID, or to its enhanced cleavage to tBID (Figures 26b-c). Thus, the mutant BID clones were found to be more susceptible to apoptosis induced solely by a reagent that leads to DNA double-strand breaks.

Altogether, these results demonstrate that BID involves in the accumulation of cells in the S and $G_2$ phases following etoposide treatment and that this involvement is dependent on the ability of BID to become phosphorylated. In addition, cells expressing an unphosphorylatable BID variant (S61A/S78A) were found more susceptible to Etop-induced apoptosis than WT cells expressing phosphorylatable BID.

## *EXAMPLE 5*

## *WHOLE ANIMAL IRRADIATION INDUCES PROMINENT PHOSPHORYLATION OF BID IN LYMPHOID ORGANS*

To assess the *in vivo* relevance of BID phosphorylation in response to DNA damaging agents mice were exposed to ionizing radiation and the level of expression and phosphorylation state of BID was determined in various organs, as follows.

***BID is phosphorylated in vivo in response to ionizing radiation*** – Seven week old BID$^{+/+}$ or BID$^{-/-}$ mice were subjected to a sub-lethal dose of IR (3 Gy) and one hour after exposure the phosphorylation status of BID and its expression level were studied in a variety of tissues using the anti-pS78 antibodies and antibodies to unmodified BID. As is shown in Figures 27a-b, BID was mainly phosphorylated in

the thymus, spleen, bone marrow, and kidney, and these were also the organs in which BID was mainly expressed.

***Phosphorylation of BID in the thymus is transient and increases in an ionizing radiation dose-dependent manner*** – The present inventors have uncovered that phosphorylation of BID is transient and is increased in an ionizing radiation dose-dependent manner in MEFs (See Figures 21a-b and Example 3 hereinabove). To determine whether a similar picture appears *in vivo*, mice were exposed to two doses of IR (3 or 6 Gy), sacrificed 1 or 4 hours later, and the phosphorylation status of BID was analyzed in the thymus. Figures 28a-b show that the phosphorylation of BID in the thymus is also transient (high at 1 hour, and reduced by 4 hours post IR), and it also increases in an ionizing radiation dose-dependent manner.

Altogether, these results demonstrate that BID is phosphorylated in vivo in response to ionizing radiation and that BID phosphorylation in the thymus is transient and increases in an ionizing radiation dose-dependent manner.

## *EXAMPLE 6*

## *CONSTRUCTION OF A NON-PHOSPHORYLATABLE BID MOUSE*

To test the hypothesis that BID and its ATM-dependent phosphorylation reflect an additional novel, pro-survival activity of BID *in vivo*, the present inventors have generated mice in which the endogenous BID gene has been replaced ("knock-in") with a gene that drives the expression of a BID protein carrying mutations in the ATM phosphorylation sites [*i.e.*, BID S61A/S78A, in which both of the serine residues phosphorylation sites (S61 and S78) are converted to alanine residues], as follows.

***Materials and Experimental Methods***

***Generation and analysis of BID knock-in mice*** - A targeting vector in which mutations in the S61 and S78 serine residues are introduced into the genomic BID sequence was constructed (Figure 29a). The mutations were linked to silent restriction fragment length polymorphisms (RFLPs) in order to facilitate genotyping. This targeting vector (pRapidflirt) contains a positive selection marker flanked by recognition sites for the Cre recombinase (LoxP-Neo-LoxP), which is used for selection of 129 embryonic stem (ES) cells. Transient expression of the Cre recombinase in ES cells allows excision of the neomycin gene flanked by the two

LoxP sites. Excision of the neomycin gene was confirmed by Southern blotting (Figures 29b and c) to detect the presence or absence of the LoxP-Neo-LoxP cassette, and a PCR-based assay was performed to detect the RFLPs linked to the BID serine-to-alanine mutations (Figures 30a-b). Targeted 129 ES clones aggregated with morrulae from CD1 mice, which is an outbred strain, so the chimeric mice was on a mixed 129/CD1 background. The BID S61A/S78A/+ chimeric animals were then crossed to obtain the homozygotes for the mutant BID gene (BID S61A/S78A).

***Experimental Results***

***Generation of BID S61A/S78A knock-in (KI) mice*** – KI mice in which endogenous BID is no longer capable of being phosphorylated on serine residues 61 and 78 were generated, as follows. A 7-kb 5' fragment and a 2-kb 3' fragment [in which mutations in these two serine residues have been introduced and linked to silent restriction fragment length polymorphisms (RFLPs) to facilitate genotyping] were cloned into the pRapidflirt gene-replacement vector (Figure 29a). This vector contains a neomycin-resistant gene (neo) flanked by recognition sites for the Cre recombinase (LoxP-Neo-LoxP). The targeting vector was transfected into R1 embryonic stem (ES) cells (derived from 129/ola mice), and neomycin resistant clones were selected. Southern-blot analysis was performed using two probes (probe-L and probe-S; Figures 29b-c). Two homologous recombinant R1 clones were identified and aggregated with tetraploid embryos and implanted into white-coated ICR foster mothers. The first generation of black-coated mice, were born, bred again to white ICR mice, and the second generation of BID S61A/S78A/+ animals were obtained. The heterozygous mutant BID S61A/S78A/+ animals are crossed to a general deleter strain, PGK-Cre to excise the neo cassette, which is flanked by two LoxP sites (illustrated by 2 rectangles in Figure 29a). Intercross of BID S61A/S78A/+ animals results in offspring homozygous for the mutant BID gene (BID S61A/S78A) (Figures 30a-b).

***BID S61A/S78A mice (BID KI mice) exhibit endogenous mutated BID which is incapable of being phosphorylated at the ATM regulatory sites*** - To confirm that endogenous BID in these animals is no longer capable of being phosphorylated on serines 61 and 78, $BID^{+/+}$, BID S61A/S78A/+, and BID S61A/S78A animals were exposed to a sub-lethal dose of IR and the phosphorylation status of BID and its level of expression in tissues were examined. Western blot

analysis using antibodies to unmodified BID revealed that the amounts of BID protein expressed in spleen and thymi from all three types of animals were similar (Figure 31b). In contrast, Western blot analysis using the anti-pS78 antibodies detected S-78 phosphorylated BID in wild-type and heterozygous tissues, but failed to detect phosphorylated BID in tissues obtained from BID S61A/S78A mice (Figure 31a). Similar results were obtained using the anti-pS61 antibodies (data not shown), indicating that the endogenous BID in the KI animals is no longer capable of being phosphorylated at the ATM regulatory sites (serines 61 and 78), but its level and expression pattern are normal.

Altogether, these results demonstrate the generation of a BID S61A/S78A knock-in (KI) mice which exhibit endogenous unphosphorylatable BID at the ATM regulatory sites.

## *EXAMPLE 7*

## *TRAPPING BID IN THE NUCLEUS INHIBITS ITS FUNCTION IN THE DOUBLE STRAND BREAK (DSB) DNA DAMAGE PATHWAY*

***BID carries a nuclear export signal (NES)*** - As is shown in Figures 18a-h and 19a-d and described in Example 3, hereinabove, BID is partially localized to the nucleus. This localization suggests that BID might carry a nuclear localization signal (NLS). Examination of the human, rat and mouse BID sequences revealed that they do not carry a classic NLS but do carry a classic nuclear export signal (NES) (Figure 32a). Thus, the nuclear localization of BID is likely to be governed on the basis of regulated export. The Rev type NES sequence is comprised of an 8 to 12 amino acid peptide in which mutations of critical leucines abolish the export activity of the protein (Pemberton, L.F., et al., Curr. Opin. Cell Biol, 10: 392-399, 1998).

***BID NES regulates the "shuttling" of BID from the nucleus to the cytosol*** - To determine whether the putative BID NES sequence was indeed regulating the "shuttling" of BID from the nucleus to the cytosol, the present inventors have mutated all three of the conserved leucine residues in the BID NES to alanine residues (L35A/L38A/L42A) by PCR. Wild-type BID and the triple mutant (BID-3LA) were transiently transfected into HeLa cells and the localization of BID was assessed by immunofloursence. These studies have shown that mutating the BID NES did not seem to increase the nuclear levels of BID (data not shown). Moreover, treating the

cells with leptomycin B (LMB), a specific inhibitor of CMR1 that inhibits the nuclear export of Rev-type proteins (Wolff, B., et al., Chem Biol, 4: 139-147, 1997), also had no effect on the nuclear levels of BID. A possible explanation to these results is that the nuclear import rate of BID is very low and therefore inhibiting its nuclear export does not show a detectable effect. To examine this possibility, a strong NLS [the NLS of the SV40 large-T antigen (Kalderon, D., et al., Nature, 311: 33-38, 1984)] was fused to the BID-3LA mutant and assessed its cellular location by immunofloursence. The NLS-BID-3LA variant is expected to be "trapped" in the nucleus due to increased import and decreased export. Indeed, Figures 32b-e show that NLS-BID-3LA is more confined to the nucleus than wt-BID. It is important to note that fusing the same NLS to wt-BID did not increase the nuclear levels of wtBID (data not shown). To further assess whether fusing the NLS indeed increased the nuclear levels of BID-3LA, the formaldehyde/subcellular fractionation assay was employed. As is shown in Figures 32f-g most of the NLS-BID-3LA was localized to the nuclear fraction.

***BID export from the nucleus is required for the accumulation of cells in the S phase following Etop treatment*** - To further examine the effect of the NES signal on BID involvement in cell cycle arrest following inducement of DNA damage, stable $BID^{-/-}$ MEFs clones expressing the wt-BID or NLS-BID-3LA were generated. No difference was found in the phosphorylation of wt-BID and NLS-BID-3LA in the stable clones in terms of level and kinetics of phosphorylation following Etop treatment (data not shown). In addition, cycle analysis on two of the wt-BID and two of the NLS-BID-3LA stable clones revealed that while Etop induced accumulation of the wt-BID clones in the S phase of the cell cycle (as measured 8 hours after Etop treatment), the mutant BID clones (*i.e.*, the NLS-BID-3LA mutants which are more confined to the nucleus) bypassed accumulation in the S phase and rapidly accumulated in the $G_2$ phase (Figures 33a-c). Thus, the NLS-BID-3LA cells were found to be impaired in their ability to arrest in S phase following DSB DNA damage.

***BID export from the nucleus is needed for Etop – induced apoptosis*** - Moreover, the levels of apoptosis in the two wt-BID and in the two NLS-BID-3LA clones were assessed following Etop treatment. As is shown in Figure 34, Etop induced a significantly lower rate of apoptosis in $BID^{-/-}$ clones expressing NLS-BID-3LA than in the $BID^{-/-}$ clones expressing wt-BID. Western blot analysis using anti-

BID antibodies indicated that the decrease in apoptosis seen in the NLS-BID-3LA clones in response to Etop was not due to either lower levels of expression of NLS-BID-3LA, or less cleavage to tBID (data not shown).

Altogether, these results demonstrate that BID carries a nuclear export signal (NES) and that this NES regulates the "shuttling" of BID from the nucleus to the cytosol. In addition, the results demonstrate that BID export from the nucleus is required for the accumulation of cells in the S phase and for apoptosis induction following Etop treatment.

Taken together, these results suggest that nucleo-cytoplasmic shuttling of phosphorylated BID is important for its S phase arrest/checkpoint function and apoptotic function in the DSB pathway. These results suggest that BID acts both inside and outside of the nucleus to execute its functions in this pathway.

In addition, the paraformaldehyde/cellular fractionation experiments present a tool that can be used to test whether BID's activities in the DSB DNA damage pathway require nuclear localization, and to purify proteins that bind to nuclear BID.

***Analysis and Discussion***

The findings presented hereinabove demonstrate that double-strand break DNA-damaging reagents induce an immediate and transient phosphorylation of BID; BID phosphorylation is mediated by the ATM kinase; $BID^{-/-}$ mouse embryonic fibroblasts fail to delay the cell cycle in response to etoposide; introduction of a non-phosphorylatable BID mutant restore delay in the G2 but not in the S phase; and, in response to etoposide, the mutant BID cells entered apoptosis more readily than the wild-type BID cells.

Prior studies performed by the present inventors suggested that full-length BID is a player in the DNA damage pathway, since a caspase-8 non-cleavable BID mutant sensitized $BID^{-/-}$ mouse embryonic fibroblasts to DNA damage-induced apoptosis (Sarig et al., 2003). The current findings demonstrate that in MEFs BID is important for apoptosis induced by a variety of DNA-damaging reagents (Figures 1a-d). Splenocytes from $BID^{-/-}$ mice also display less susceptibility to DNA damage (Figures 2a-c), indicating that the decreased death response to DNA damage is a general feature of BID deficiency.

As mentioned above, $BID^{-/-}$ MEFs are less susceptible than $BID^{+/+}$ MEFs to reagents that induce different forms of DNA damage. On the other hand, BID

phosphorylation occurs only in response to reagents that induce double-strand breaks in DNA. Thus, phosphorylation of BID does not seem to be critical for its pro-apoptotic activity. However, lack of BID phosphorylation does effect the susceptibility of cells to double-strand break DNA damage since $BID^{-/-}$ MEFs expressing the BID-S61A/S78A mutant are more susceptible than $BID^{-/-}$ MEFs expressing wild-type BID to etoposide-induced apoptosis (Figures 26a-c). Thus, ATM-mediated BID phosphorylation might serve as a mechanism to inhibit BID's apoptotic activity or alternatively as a mechanism to activate a pro-survival activity of BID.

If phosphorylation was inhibiting the apoptotic activity of BID, then what would likely be the molecular basis of such inhibition? The only known apoptotic function of BID lies in its ability to induce the release of pro-apoptotic factors from the mitochondria (e.g., cytochrome c). Caspase truncated BID (tBID) is much more efficient than full-length BID in inducing cytochrome c release; it would therefore be expected that ATM-mediated BID phosphorylation inhibits the cleavage of BID, since casein kinase 1 and/or 2-mediated phosphorylation of BID was demonstrated to inhibit its cleavage (Desagher et al., 2001). However, the findings of the present invention suggest that ATM-mediated phosphorylation of BID is not related to BID cleavage, for the following reasons: Phosphorylation occurs many hours before the activation of caspases and the onset of apoptosis (Figures 13-14 and data not shown). A relatively small amount of BID is phosphorylated (Figures 4a-e), and phosphorylation occurs also in response to extremely low, non-apoptotic levels of DNA damage (Figures 20-21). Moreover, the BID-S61A/S78A mutant was not found to be more susceptible to cleavage than wt-BID (Figures 26b-c). Lastly, though TNFα relies on the generation of tBID to induce/enhance apoptosis (Yin et al., 1999; Zhao et al., 2001), the BID-S61A/S78A mutant clones are not more susceptible to TNFα-induced apoptosis than the wtBID clones (Figure 26a). Thus, BID phosphorylation does not seem to serve as a mechanism to inhibit BID's apoptotic activity.

The results presented in Figures 22-25 suggest that ATM-mediated phosphorylation of BID regulates a novel, pro-survival function of BID related to cell cycle arrest following DNA double-strand breaks. Etoposide treatment induced

accumulation of BID+/+ mouse embryonic fibroblasts in the S and G2 phases of the cell cycle, whereas such an accumulation was not observed in the BID-/- MEFs (Figures 22a-i). Moreover, using BrdU labeling the present inventors demonstrated that BID is specifically required for S phase arrest. In addition, when wild-type BID was reintroduced into $BID^{-/-}$ cells it restored the ability to accumulate in the S and G2 phases of the cell cycle. On the other hand, introducing the non-phosphorylatable BID mutant into BID-/- cells, restored accumulation only in the G2 phase (Figures 24-25). These findings imply that BID phosphorylation plays an important role in S phase arrest.

To demonstrate the BID-dependent S phase checkpoint the present inventors used relatively high levels of etoposide, suggesting that this function of BID is associated with high levels of genotoxic stress in fibroblasts. On the other hand, in myeloid and in activated T cells this function of BID is associated with lower levels of genotoxic stress (Zinkel SS., et al., 2005, Cell 122: 579-591). Hematopoietic cells are primed to undergo cell cycle arrest and apoptosis following treatment with DNA-damaging reagents, whereas fibroblasts are relatively resistant to DNA-damaging reagents and prevent proliferation of mutations by entering into long-term G1 or G2 arrest (Baus F., et al., 2003, Embo J 22, 3992-4002; Di Leonardo A., et al., 1994, Genes Dev 8, 2540-2551). Thus, the activation threshold and the biological impact of the BID-dependent S phase checkpoint function may vary largely among different cell types.

How might the involvement of BID in S phase arrest be related to apoptosis? Following DNA double-strand breaks, the cell may decide to activate a survival system (mainly through the ATM kinase, which induces cell cycle arrest and DNA repair), or in the face of extensive or irreparable damage, the cell may activate the apoptotic machinery. The results presented in Figures 26a-c demonstrate that $BID^{-/-}$ MEFs expressing the BID-S61A/S78A mutant are more susceptible than $BID^{-/-}$ MEFs expressing wild-type BID to etoposide-induced apoptosis, but not to UV- or to TNFα-induced apoptosis. Thus, it appears that the non-phosphorylatable BID mutant sensitizes $BID^{-/-}$ MEFs to apoptosis induced only by reagents that induce double-strand breaks in DNA. These results, together with the cell cycle results (Figures 22-25) suggest that the impaired ability of the mutant BID cells to induce cell cycle arrest results in increased sensitivity to double-strand break DNA damage.

If BID is indeed capable of playing a pro-survival role in the response of cells to DNA double-strand breaks, then why are the $BID^{-/-}$ MEFs less sensitive than $BID^{+/+}$ MEFs to apoptosis induced by etoposide and ionizing radiation? BID may play both a pro-survival and a pro-apoptotic role in this pathway. Based on the finding that the phosphorylation of BID occurs in response to extremely low levels of ionizing radiation, and increases in an ionizing radiation dose-dependent manner (Figures 20-21), the present inventors propose that BID acts as a sentinel of DNA double-strand breaks. BID might translate the damage into either cell cycle arrest/DNA repair processes (at low levels of damage) or apoptosis (at high levels of damage).

The whole-animal radiation studies revealed that sub-lethal doses of IR induce prominent phosphorylation of BID in lymphoid organs (Figures 27-28). The fact that lymphoid populations are highly sensitive to radiation, and that mice and humans lacking ATM develop acute lymphoid leukemias and lymphomas, suggests that BID's balancing act between cell survival and cell death might be required for the survival and suppression of tumorigenesis in these populations following DNA damage. Having a mouse model in which the endogenous BID gene has been replaced (knock-in) with a gene that drives the expression of a BID protein carrying mutations in the ATM phosphorylation sites (BID S61A/S78A) (Figures 29-31) is instrumental in addressing the issues described above.

Finally, the present findings demonstrate that BID carries a classical NES, which regulates the "shuttling" of BID from the nucleus to the cytosol; BID export from the nucleus is required for the accumulation of cells in the S phase and for apoptosis induction following Etop treatment (Figures 32-34). These results suggest that BID acts both inside and outside of the nucleus to execute its functions in the DSB DNA damage pathway.

In summary, this study raises the novel possibility that the BH3-only BID protein, a molecule that was previously considered to be active only as a pro-apoptotic factor, also plays a pro-survival role (Figure 35). Without being bound to any theory, BID, which plays both a pro-apoptotic and a pro-survival function in the DNA damage pathway, is an excellent candidate to coordinate/balance between genotoxic stress responses and apoptotic cell death.

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.

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. 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 that such reference is available as prior art to the present invention.

## *REFERENCES*

### *(Additional references are cited in text)*

Brummelkamp, T. R., Bernards, R., and Agami, R. (2002). Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell 2, 243-247.

Desagher, S., Osen-Sand, A., Montessuit, S., Magnenat, E., Vilbois, F., Hochmann, A., Journot, L., Antonsson, B., and Martinou, J. C. (2001). Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8. Mol Cell 8, 601-611.

Graham, F. L., and van der Eb, A. J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52, 456-467.

Iliakis, G., Wang, Y., Guan, J., and Wang, H. (2003). DNA damage checkpoint control in cells exposed to ionizing radiation. Oncogene 22, 5834-5847.

Kamijo, T., van de Kamp, E., Chong, M. J., Zindy, F., Diehl, J. A., Sherr, C. J., and McKinnon, P. J. (1999). Loss of the ARF tumor suppressor reverses premature replicative arrest but not radiation hypersensitivity arising from disabled atm function. Cancer Res 59, 2464-2469.

Kim, S. T., Lim, D. S., Canman, C. E., and Kastan, M. B. (1999). Substrate specificities and identification of putative substrates of ATM kinase family members. J Biol Chem 274, 37538-37543.

Sarig, R., Zaltsman, Y., Marcellus, R. C., Flavell, R., Mak, T. W., and Gross, A. (2003). BID-D59A is a potent inducer of apoptosis in primary embryonic fibroblasts. J Biol Chem 278, 10707-10715.

Sax, J. K., Fei, P., Murphy, M. E., Bernhard, E., Korsmeyer, S. J., and El-Deiry, W. S. (2002). BID regulation by p53 contributes to chemosensitivity. Nat Cell Biol 4, 842-849.

Tripathi, A. K., Chaturvedi, R., Ahmad, R., Asim, M., Sawlani, K. K., Singh, M. K., Tripathi, P., and Tekwani, B. L. (2003). Flow cytometric analysis of aneuploidy and S-phase fraction in chronic myeloid leukemia patients: role in early detection of accelerated phase. Leuk Res 27, 899-902.

Wood, L. D., Halvorsen, T. L., Dhar, S., Baur, J. A., Pandita, R. K., Wright, W. E., Hande, M. P., Calaf, G., Hei, T. K., Levine, F., et al. (2001). Characterization of ataxia telangiectasia fibroblasts with extended life-span through telomerase expression. Oncogene 20, 278-288.

Yin, X. M., Wang, K., Gross, A., Zhao, Y., Zinkel, S., Klocke, B., Roth, K. A., and Korsmeyer, S. J. (1999). Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature 400, 886-891.

Zhao, Y., Li, S., Childs, E. E., Kuharsky, D. K., and Yin, X. M. (2001). Activation of pro-death Bcl-2 family proteins and mitochondria apoptosis pathway in tumor necrosis factor-alpha-induced liver injury. J Biol Chem 276, 27432-27440.

Zinkel, S. S., Ong, C. C., Ferguson, D. O., Iwasaki, H., Akashi, K., Bronson, R. T., Kutok, J. L., Alt, F. W., and Korsmeyer, S. J. (2003). Proapoptotic BID is required for myeloid homeostasis and tumor suppression. Genes Dev 17, 229-239.

WHAT IS CLAIMED IS:

1. An isolated polypeptide comprising a BID amino acid sequence having a mutation in a PIKK phosphorylation site.

2. An isolated polypeptide comprising a BID amino acid sequence having a PIKK phosphorylation site and being capable of inhibiting PIKK-mediated phosphorylation of endogenous BID.

3. An isolated polypeptide comprising a BID amino acid sequence which comprises a phosphorylated PIKK phosphorylation site.

4. The isolated polypeptide of claim 1, wherein said mutation abolishes phosphorylation at said phosphorylation site.

5. The isolated polypeptide of claim 1, wherein said mutation mimics phosphorylation at said phosphorylation site.

6. The isolated polypeptide of claims 3 and/or 5, wherein said BID amino acid sequence does not exceed 20 amino acids in length.

7. The isolated polypeptide of claim 2, 4, and/or 6, wherein the polypeptide is capable of inducing apoptosis.

8. An isolated polypeptide comprising a BID amino acid sequence wherein the isolated polypeptide being incapable of inducing apoptosis.

9. An isolated polypeptide comprising a BID amino acid sequence attached to a nuclear targeting moiety.

10. The isolated polypeptide of claim 9, wherein said nuclear targeting moiety is an NLS.

11. The isolated polypeptide of claims 3, 5, 9 and/or 10, wherein said BID amino acid sequence is at least 20 amino acids long.

12. The isolated polypeptide of claims 8 and/or 11, wherein the polypeptide is capable of inducing cell cycle arrest.

13. An isolated polynucleotide encoding the polypeptide of any of claims 1-12.

14. A transformed cell expressing the polypeptide of any of claims 1-12.

15. The isolated polypeptide of claims 1 and/or 3, wherein said PIKK phosphorylation site comprises a residue corresponding to a serine residue at position 78 of a BID polypeptide set forth by SEQ ID NO:15.

16. The isolated polypeptide of claims 1, 2 and/or 3, wherein said PIKK is an ATM.

17. The isolated polypeptide of claim 1, 2 and/or 3, wherein said PIKK is an ATR.

18. The isolated polypeptide of claim 12, wherein said cell cycle arrest is induced following DSB in a DNA.

19. The isolated polypeptide of claim 12, wherein said cell cycle arrest occurs at an S phase.

20. A method of inducing cell cycle arrest comprising introducing into-, or expressing in the cell a polypeptide comprising a BID amino acid sequence incapable of inducing apoptosis, thereby inducing cell cycle arrest.

21. A method of treating a disease associated with a genomic instability comprising introducing into-, or expressing in cells of an individual in need thereof a

polypeptide comprising a BID amino acid sequence incapable of inducing apoptosis, thereby inducing cell cycle arrest and treating the disease associated with the genomic instability.

22. The method of claim 20 or 21, wherein said polypeptide is the isolated polypeptide of claims 8 and/or 12.

23. A method of inducing apoptosis, comprising introducing into-, or expressing in a cell a polypeptide comprising a BID amino acid sequence incapable of inducing cell cycle arrest, thereby inducing apoptosis.

24. A method of treating cancer, comprising introducing into-, or expressing in cells of an individual in need thereof a polypeptide comprising a BID amino acid sequence incapable of inducing cell cycle arrest, thereby inducing apoptosis and treating the cancer.

25. The method of claim 23 or 24, wherein said polypeptide is the isolated polypeptide of claim 7.

26. The isolated polypeptide of claim 3, wherein the isolated polypeptide is as set forth in SEQ ID NOs:20-22.

27. The isolated polypeptide of claim 3, wherein the isolated polypeptide is as set forth in SEQ ID NOs:32-34.

28. The method of claim 21, wherein said disease associated with genomic instability is associated with an abnormal S phase checkpoint.

29. The method of claim 21, wherein said disease associated with genomic instability is selected from the group consisting of Ataxia-Telangiectasia, Fanconi anemia, Bloom's syndrome, hereditary breast and ovarian cancer syndromes involving BRCA1 and Nijmegen breakage syndrome (NBS1).

30. The method of claim 24, wherein the cancer is of a lymphoid origin.

31. An antibody comprising an antigen recognition domain capable of specifically binding a BID polypeptide phosphorylated on a serine residue corresponding to amino acid 78 of human BID as set forth in SEQ ID NO:15 but does not bind said BID polypeptide when not phosphorylated on this respective position.

32. An antibody comprising an antigen recognition domain capable of specifically binding a BID polypeptide phosphorylated on a serine residue corresponding to amino acid 78 of mouse BID as set forth in SEQ ID NO:16 but does not bind said BID polypeptide when not phosphorylated on this respective position.

33. An antibody comprising an antigen recognition domain capable of specifically binding a BID polypeptide phosphorylated on a serine residue corresponding to amino acid 61 of mouse BID as set forth in SEQ ID NO:16 but does not bind said BID polypeptide when not phosphorylated on this respective position.

34. A kit for detecting a presence and/or level of a PIKK-mediated phosphorylated BID comprising the antibody of claims 31, 32 and/or 33.

35. A method of detecting cellular exposure to DNA damaging agents, comprising detecting in a biological sample a presence and/or level of a PIKK-mediated phosphorylated BID, thereby detecting the cellular exposure to the DNA damaging agents.

36. A kit for detecting cellular exposure to DNA damaging agents, comprising at least one reagent capable of detecting a presence and/or level of a PIKK-mediated phosphorylated BID.

37. A method of diagnosing a disease associated with an abnormal PIKK-mediated phosphorylation of BID, comprising detecting in cells of an individual in need thereof a presence and/or level of a PIKK-mediated phosphorylated BID,

thereby diagnosing the disease associated with the abnormal PIKK-mediated phosphorylation of BID.

38. A kit for diagnosing a disease associated with an abnormal PIKK-mediated phosphorylation of BID, comprising at least one reagent capable of detecting a presence and/or level of a PIKK-mediated phosphorylated BID.

39. The method of claim 37, wherein said disease associated with the abnormal PIKK-mediated phosphorylation of BID is selected from the group consisting of Ataxia-Telangiectasia, Fanconi anemia, Bloom's syndrome, hereditary breast and ovarian cancer syndromes involving BRCA1 and Nijmegen breakage syndrome (NBS1).

40. The method of claims 35 and/or 37, wherein said detecting in the cells said presence and/or level of said PIKK-mediated phosphorylated BID comprises using the antibody of claims 31, 32 and/or 33.

41. The kit of claims 36 and/or 38, wherein said at least one reagent comprises the antibody of claims 31, 32 and/or 33.

42. A method of inducing apoptosis comprising contacting a cell with the antibody of claim 31, thereby inducing apoptosis in the cell.

43. A pharmaceutical composition comprising as an active ingredient the antibody of claim 31 and a pharmaceutically acceptable carrier.

44. A method of identifying an agent capable of inducing cell cycle arrest, comprising:

(a) contacting a plurality of cells with a plurality of molecules;

(b) identifying at least one molecule from said plurality of molecules capable of increasing a level of a phosphorylated BID polypeptide, said at least one molecule being the agent capable of inducing cell cycle arrest.

45. The method of claim 44, wherein said phosphorylated BID polypeptide is present in a cell nucleus.

46. The method of claim 44, wherein said phosphorylated BID polypeptide being phosphorylated on a PIKK phosphorylation site.

47. A multicellular organism comprising a genome which comprises a genetically modified BID gene which comprises a mutation in a PIKK phosphorylation site.

48. The multicellular organism of claim 47, wherein said mutation abolishes phosphorylation at said PIKK phosphorylation site.

49. The multicellular organism of claim 47, wherein said mutation comprises a substitution of a serine residue with an alanine residue at a position corresponding to amino acid residue 78 of a BID polypeptide set forth by SEQ ID NO:16.

50. The multicellular organism of claim 47, wherein said mutation comprises a substitution of a serine residue with an alanine residue at a position corresponding to amino acid residue 61 of a BID polypeptide set forth by SEQ ID NO:16.

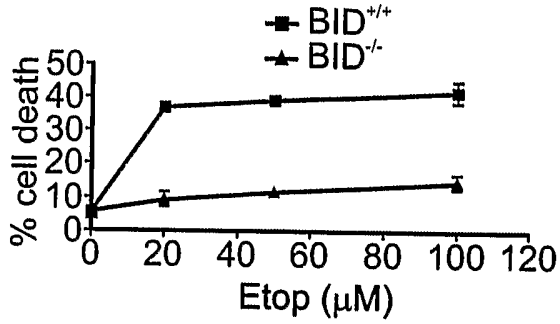



Fig. 1a

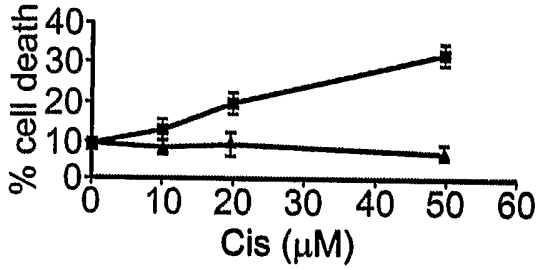



Fig. 1b

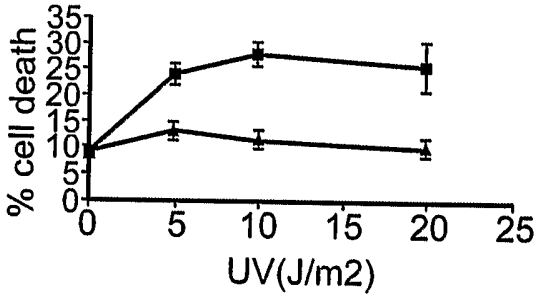



Fig. 1c

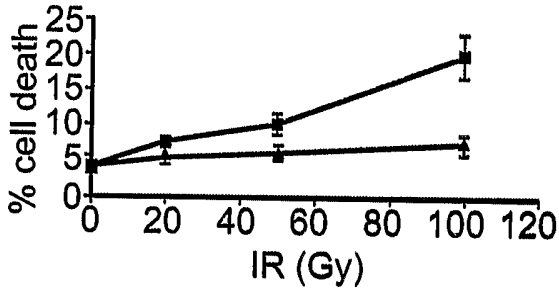



Fig. 1d

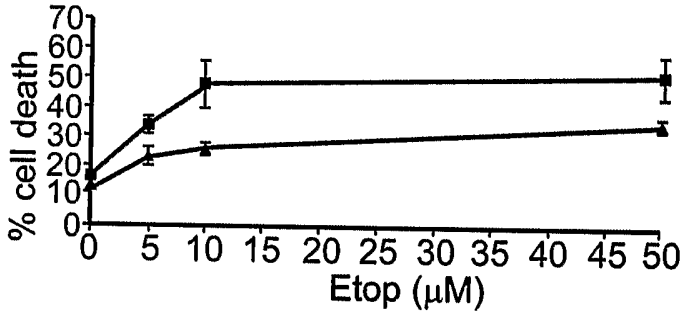



Fig. 2a

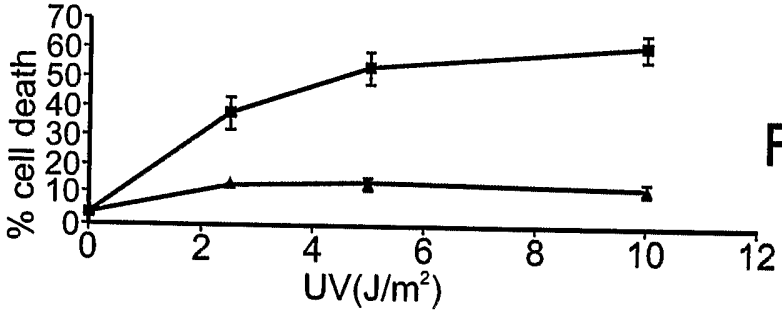



Fig. 2b

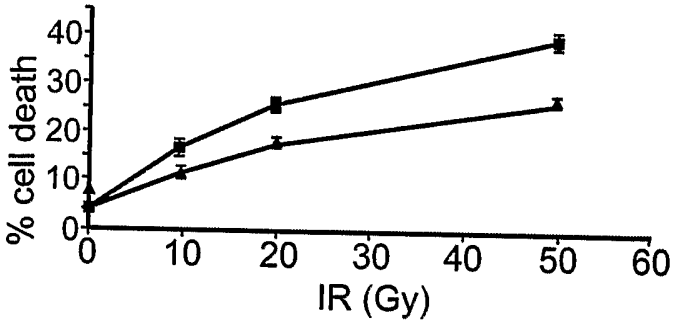



Fig. 2c

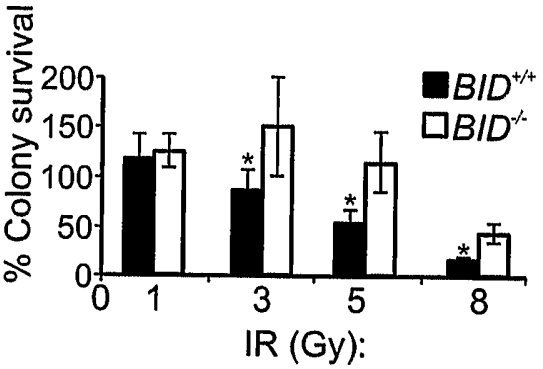



Fig. 3a

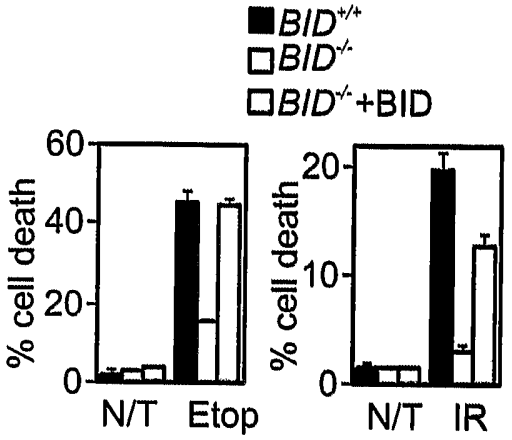



Fig. 3b Fig. 3c

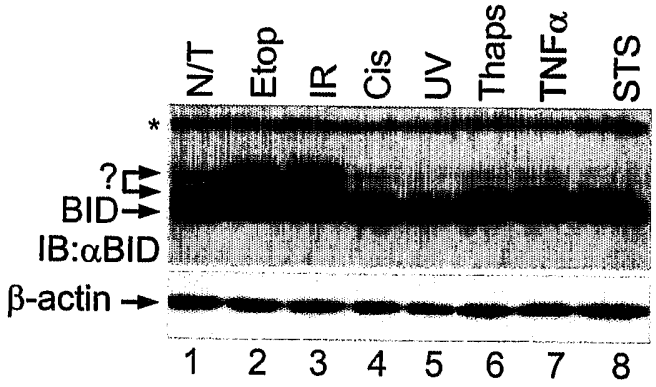



Fig. 4a

Fig. 4b

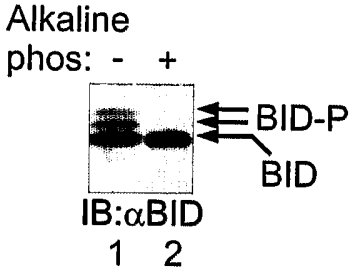



Fig. 4c

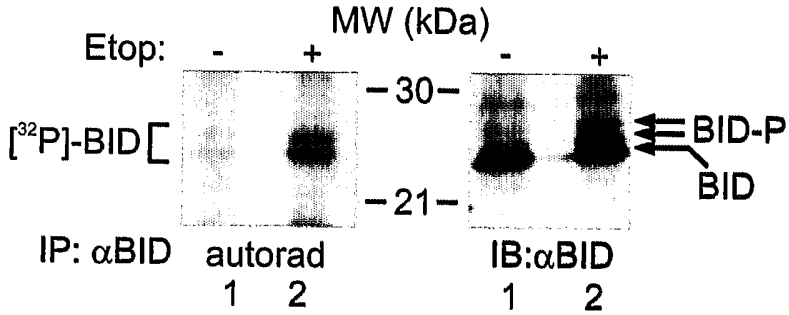



Fig. 4d Fig. 4e

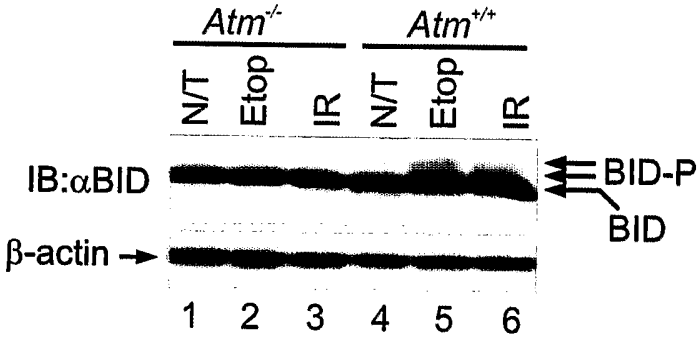



Fig. 5a

Fig. 5b

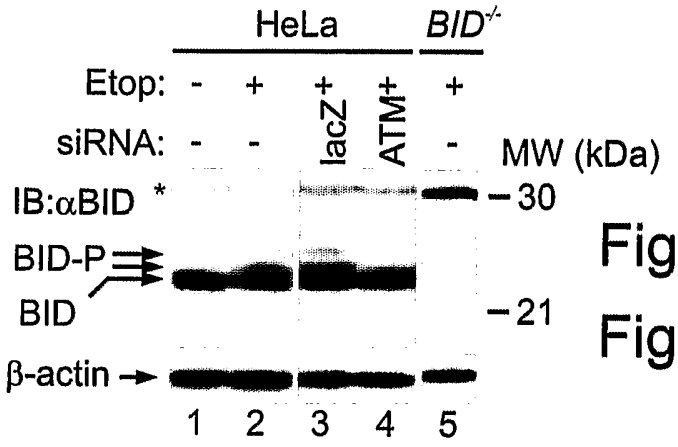



Fig. 6a

Fig. 6b

| | | 61 | | | | | | | | | | | | | | | 78 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BID_mouse | 58 | - | T | D | G | S | Q | A | S | R | - | - | - | - | - | - | I | E | P | D | S | E | S | Q | E | E | I | - | 82 |
| BID_human | 59 | - | T | D | G | N | R | S | S | H | - | - | - | - | - | - | I | E | P | D | S | E | S | Q | E | D | I | - | 82 |
| BID_rat | 59 | - | T | D | G | S | R | A | S | R | - | - | - | - | - | - | I | E | P | D | S | E | S | Q | D | E | V | - | 83 |

Fig. 7a

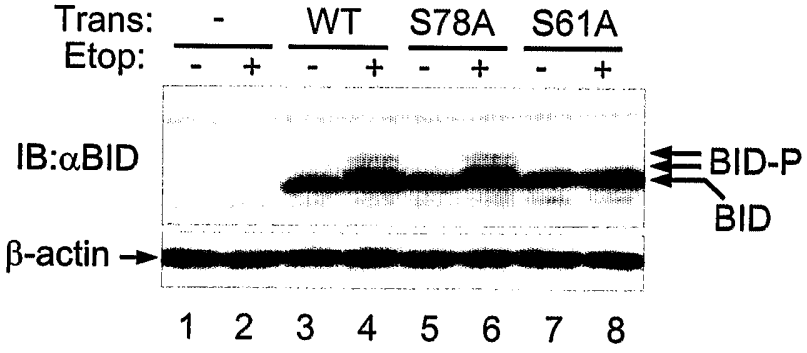



Fig. 7b

Fig. 7c

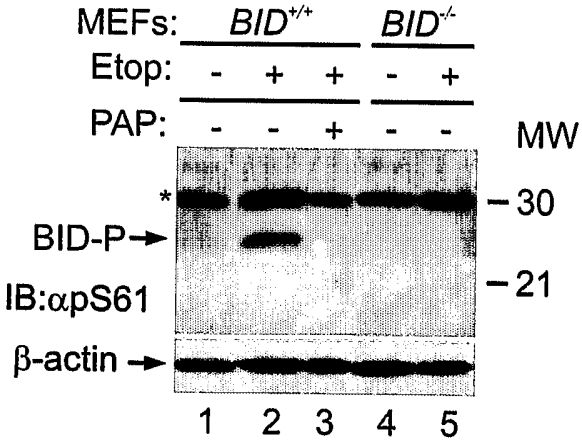



Fig. 7d

Fig. 7e

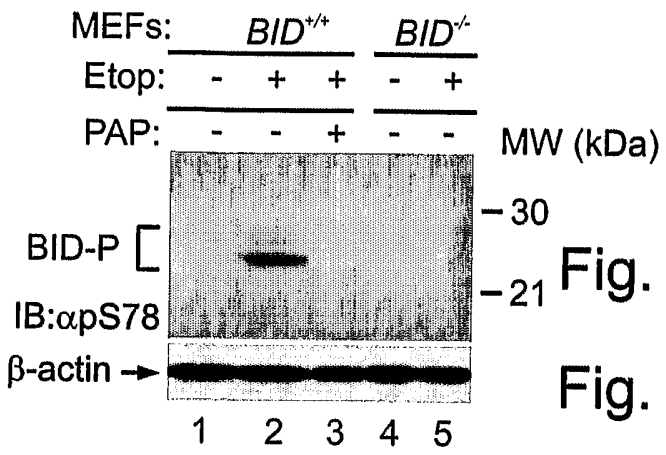



Fig. 7f

Fig. 7g

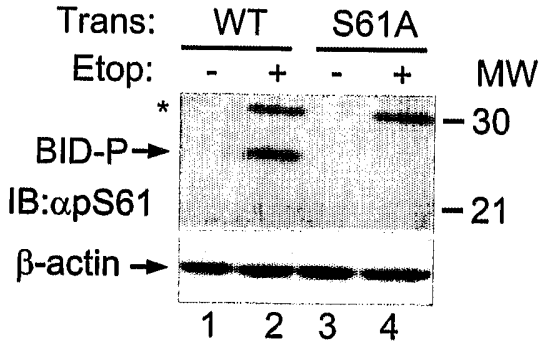



Fig. 8a

Fig. 8b

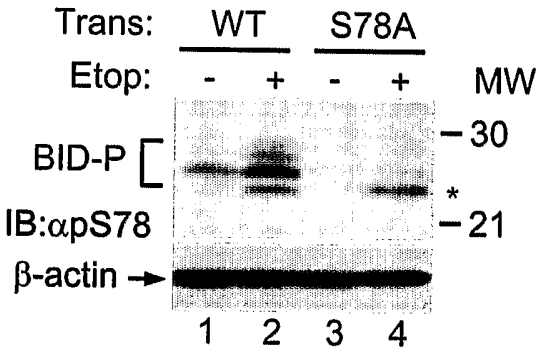



Fig. 8c

Fig. 8d

Fig. 9a

Fig. 9b

Fig. 9c

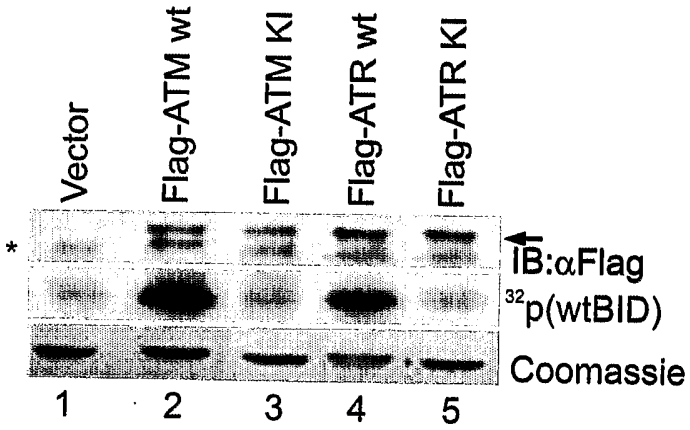


Fig. 9d
Fig. 9e

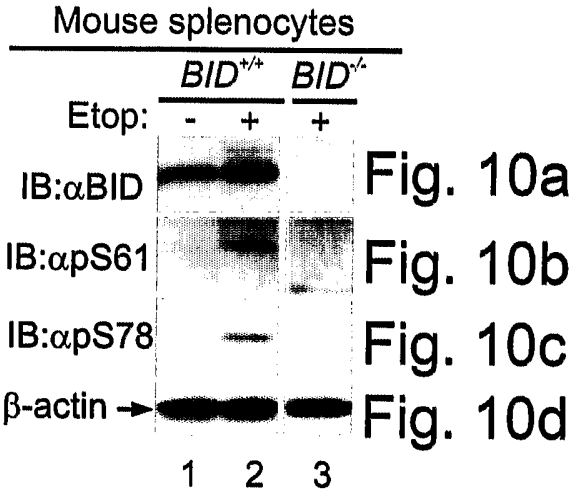




Fig. 10a
Fig. 10b
Fig. 10c
Fig. 10d

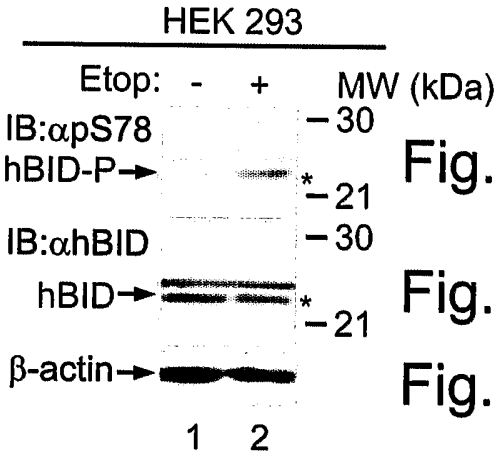
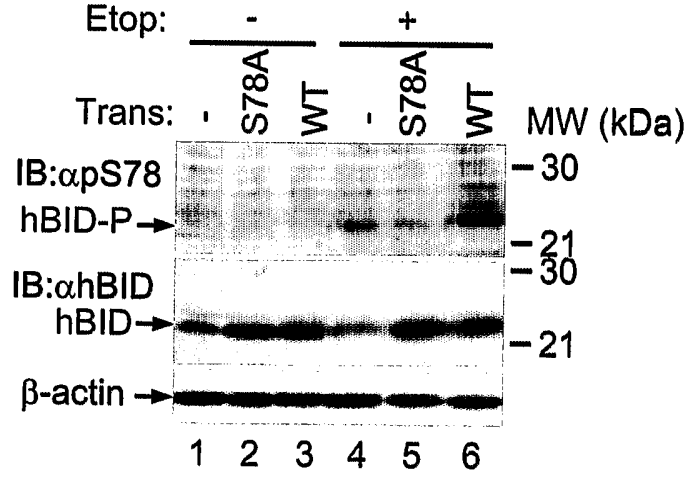



Fig. 11a
Fig. 11b
Fig. 11c

Fig. 12a
Fig. 12b
Fig. 12c



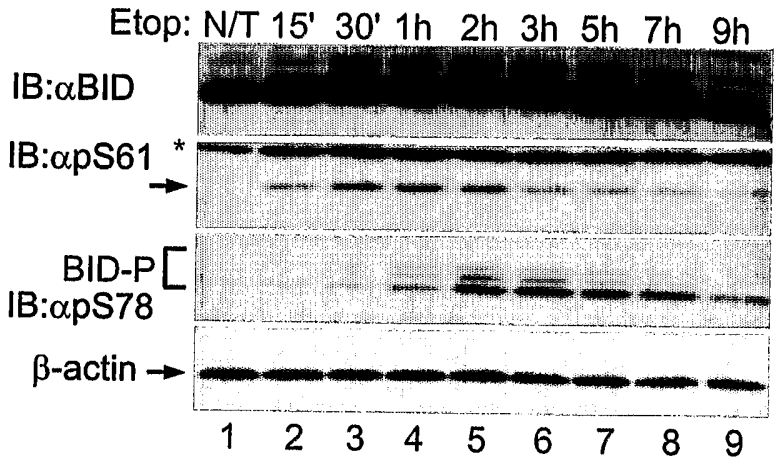



Fig. 13a

Fig. 13b

Fig. 13c

Fig. 13d

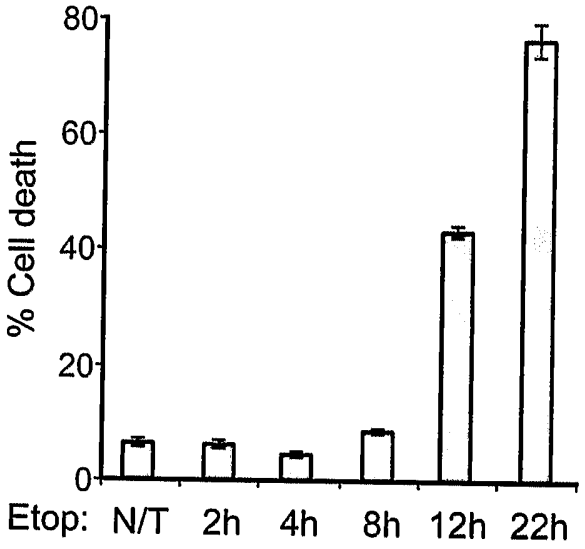



Fig. 14

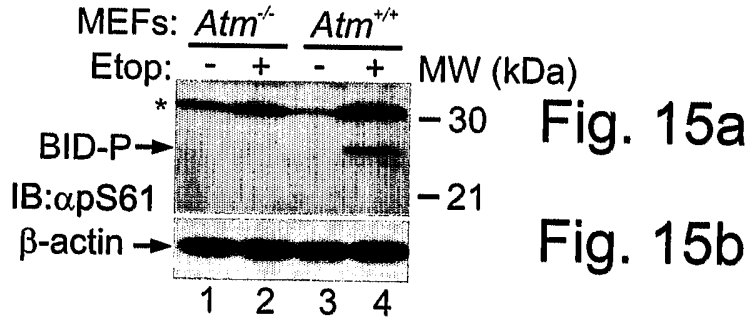



Fig. 15a

Fig. 15b

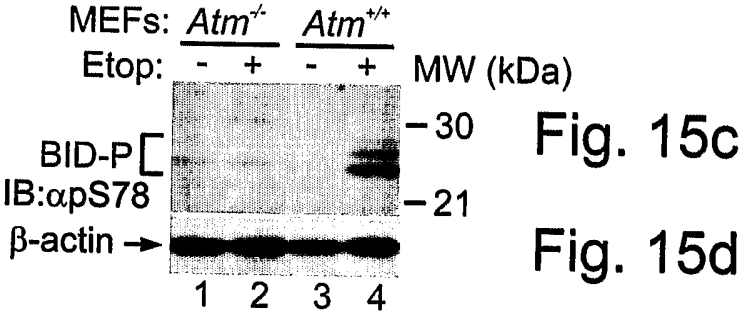



Fig. 15c

Fig. 15d

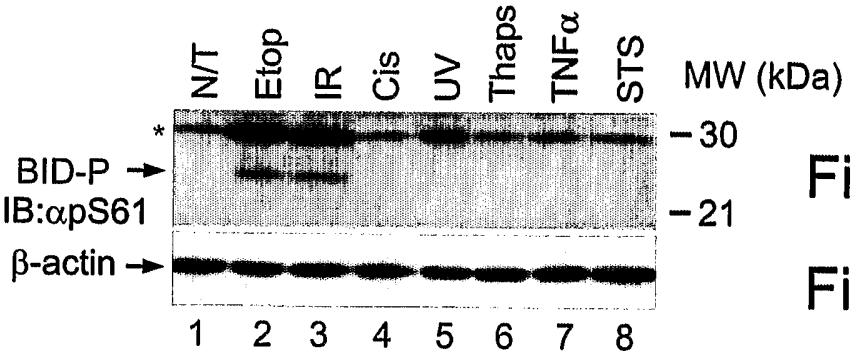



Fig. 16a

Fig. 16b

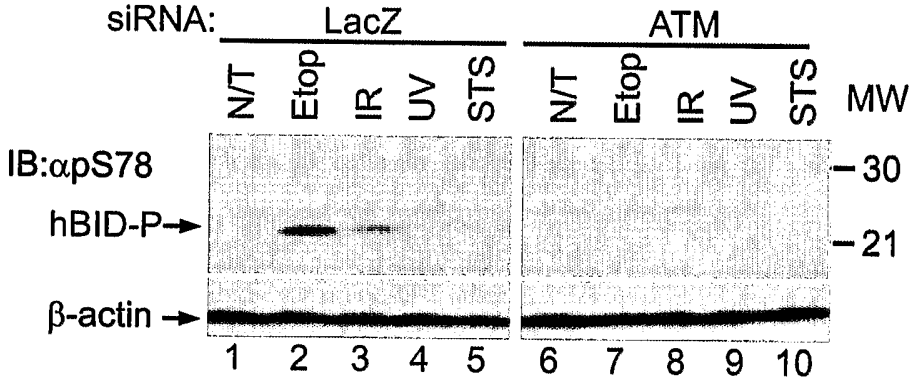



Fig. 17a

Fig. 17b

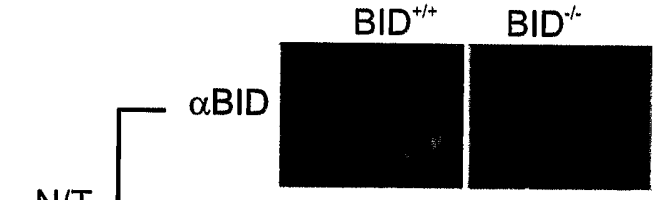



Fig. 18a Fig. 18b


Fig. 18c Fig. 18d

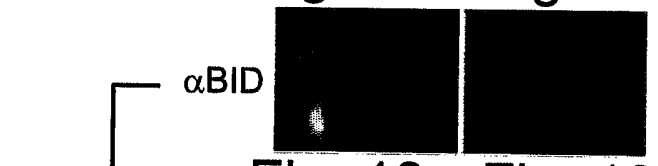



Fig. 18e Fig. 18f

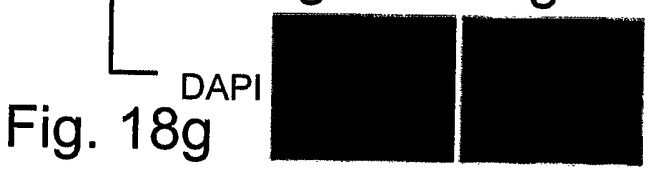



Fig. 18g

Fig. 18h

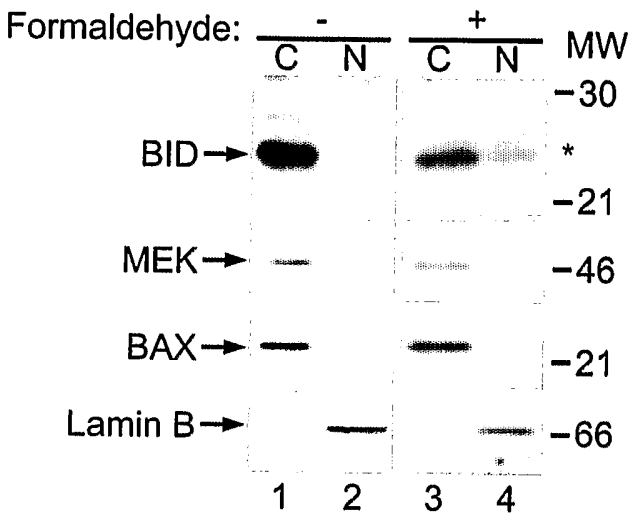



Fig. 19a

Fig. 19b

Fig. 19c

Fig. 19d

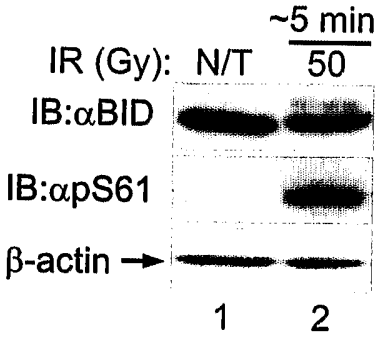



Fig. 20a

Fig. 20b

Fig. 20c

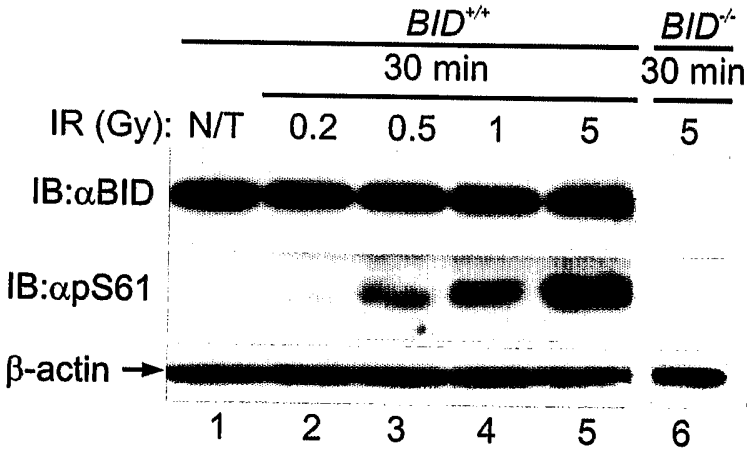



Fig. 21a

Fig. 21b

Fig. 21c

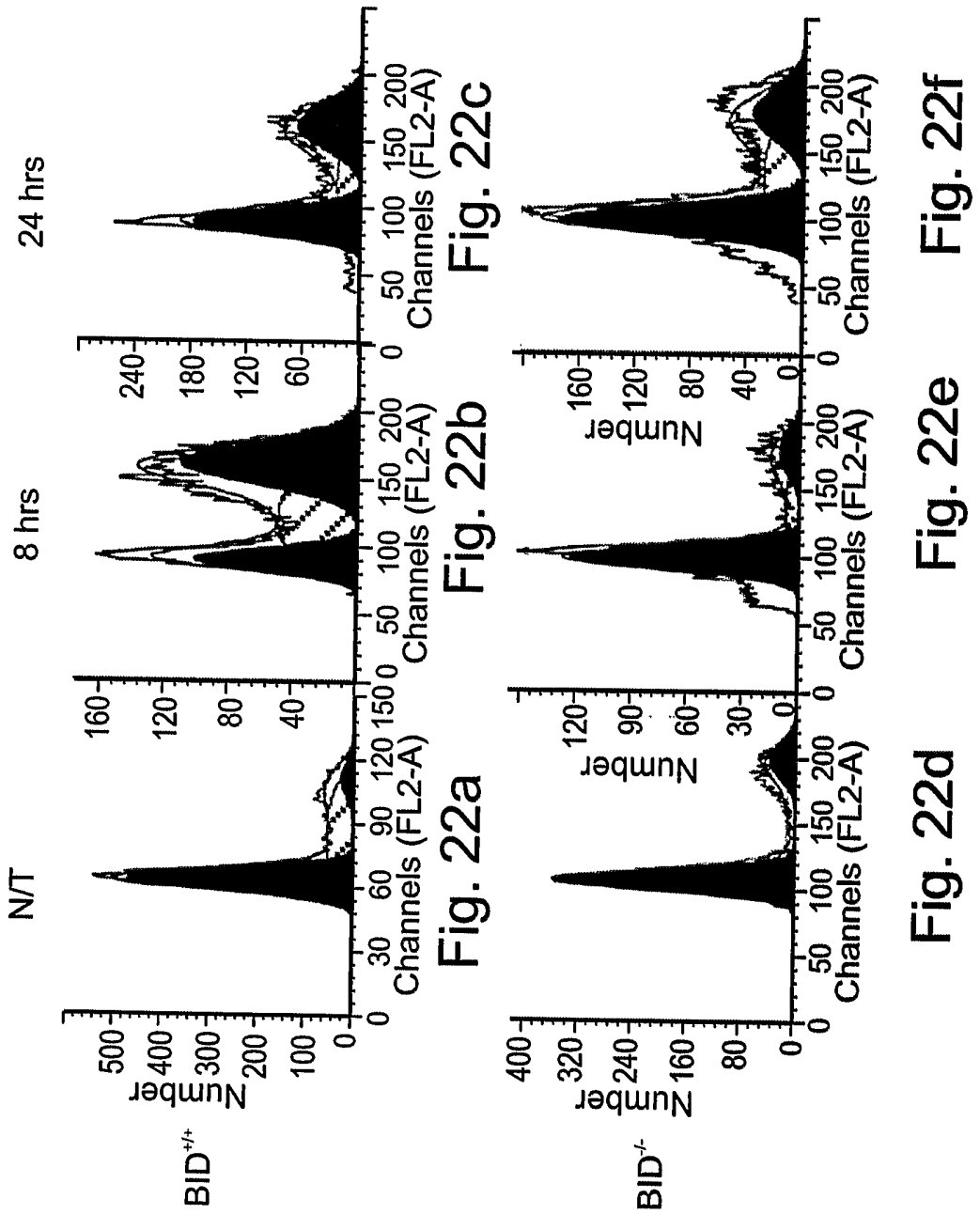



Fig. 22a Fig. 22b Fig. 22c

Fig. 22d Fig. 22e Fig. 22f

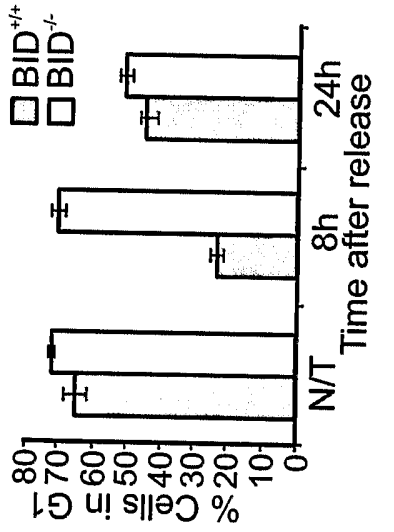



Fig. 22g

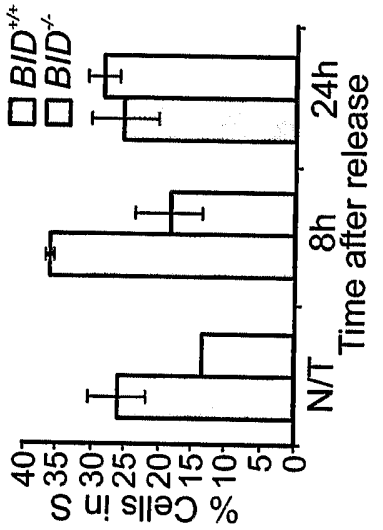



Fig. 22h

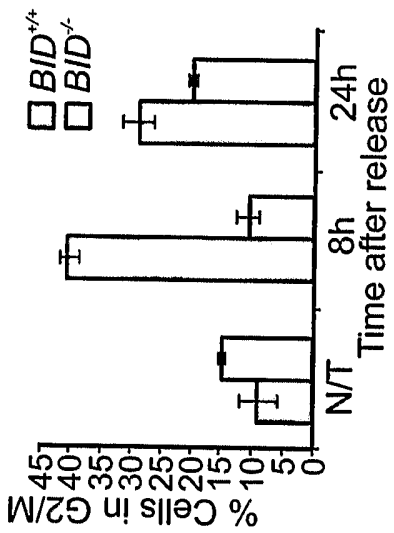



Fig. 22i

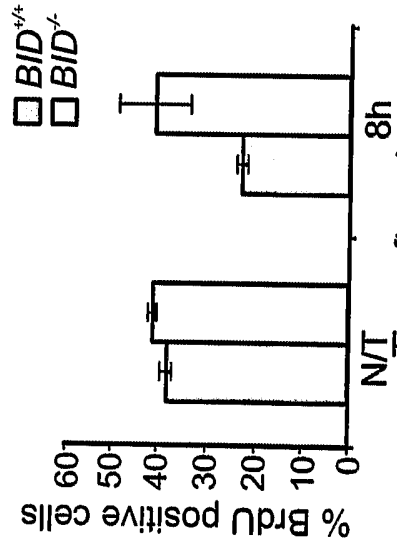



Fig. 23b

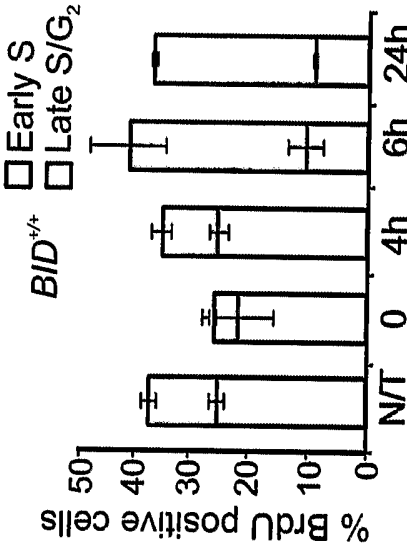



Fig. 23c

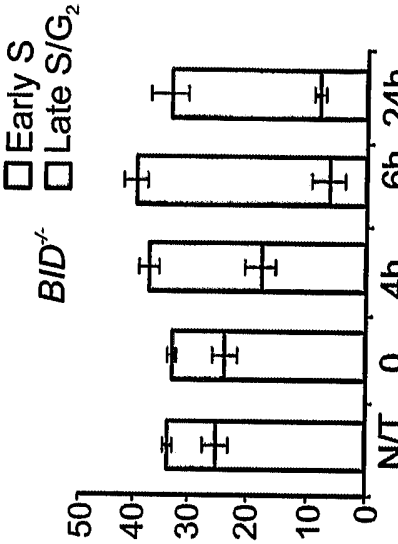



Fig. 23a

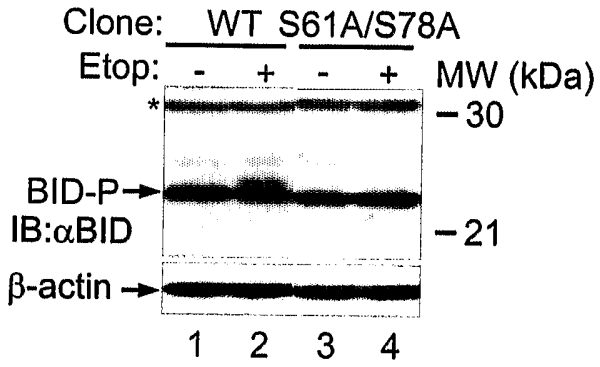



Fig. 24a

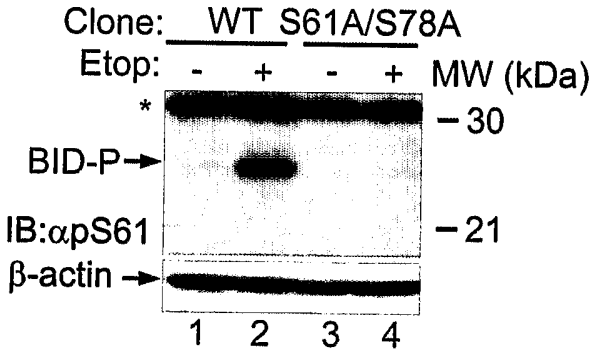



Fig. 24b

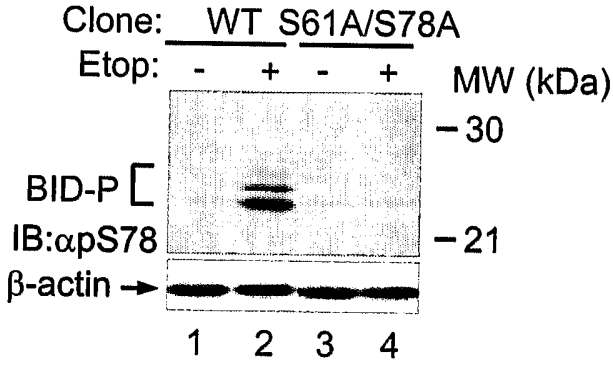



Fig. 24c

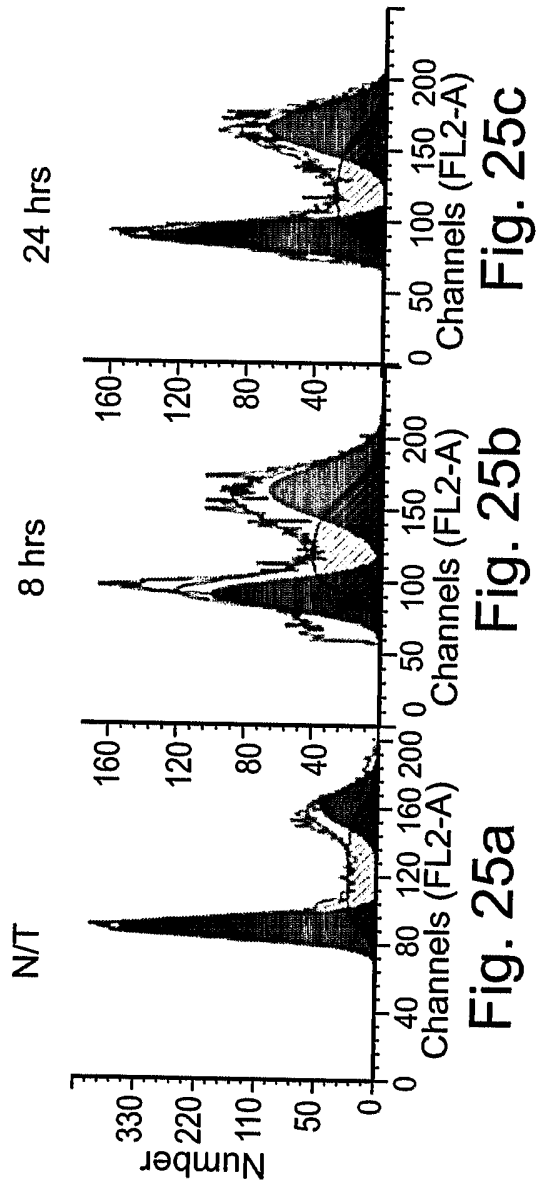



Fig. 25a Fig. 25b Fig. 25c

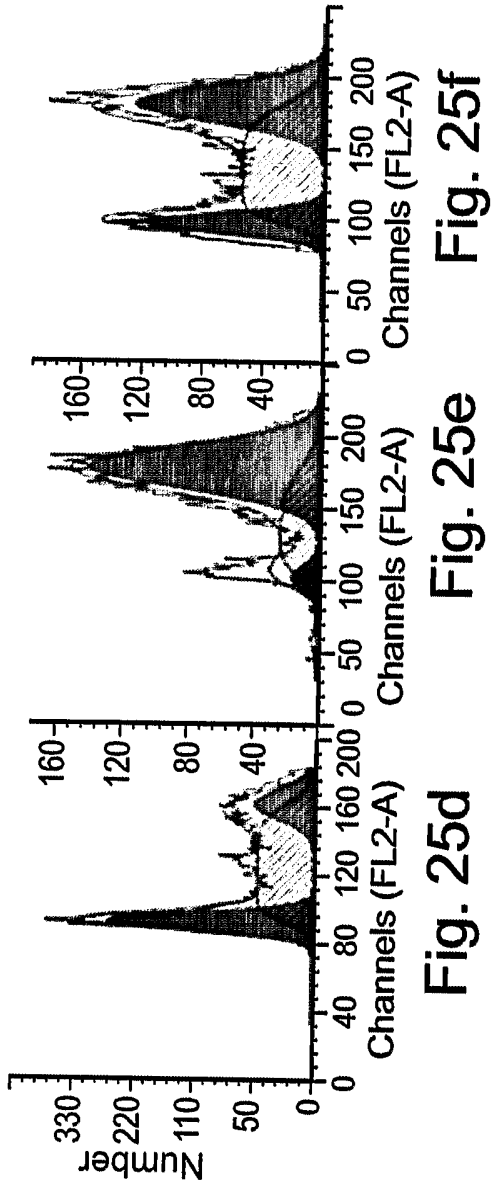



Fig. 25d Fig. 25e Fig. 25f

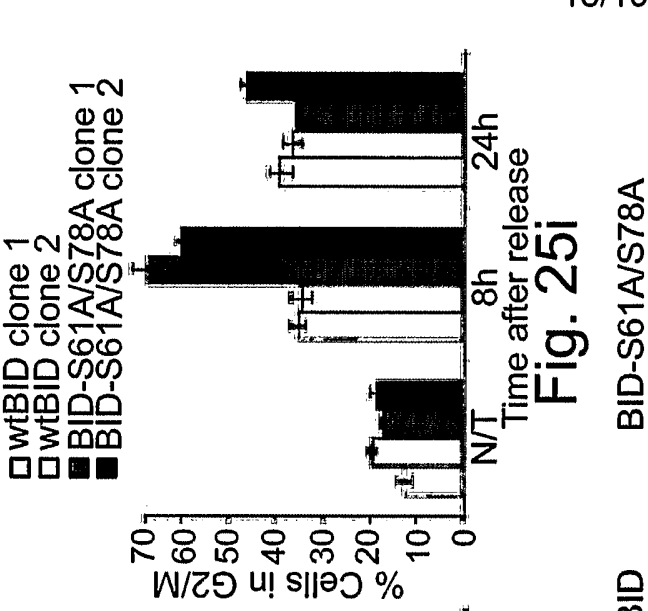



Fig. 25i

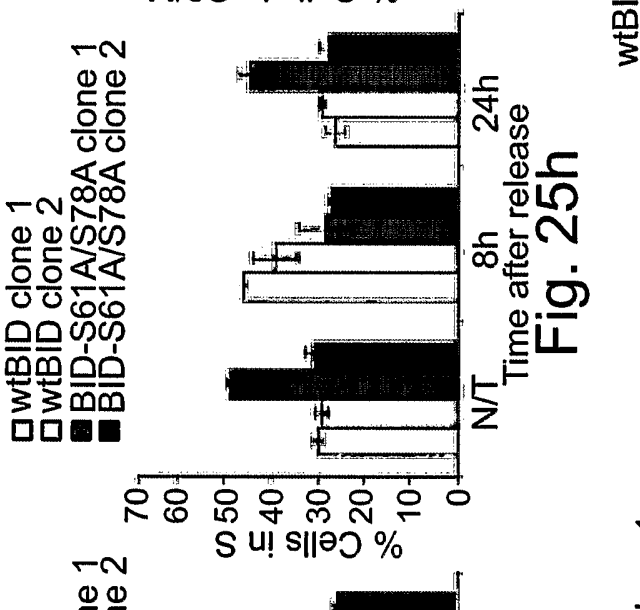



Fig. 25h

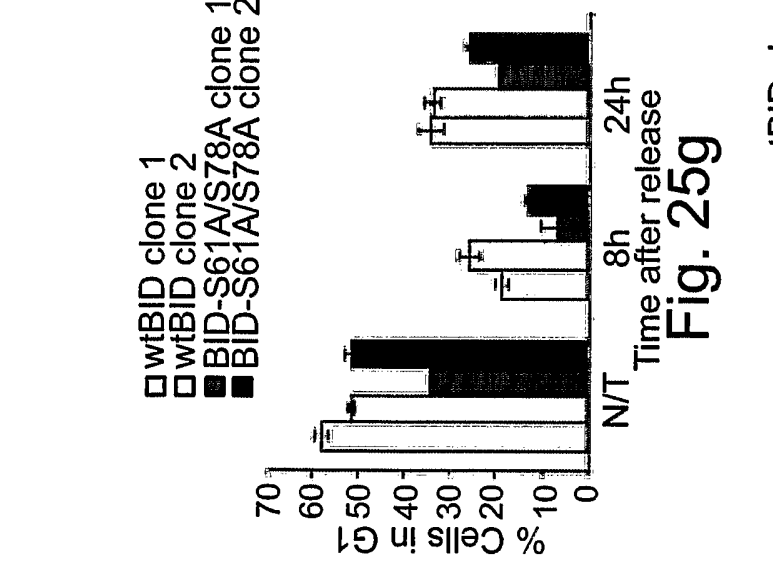



Fig. 25g




Fig. 26a

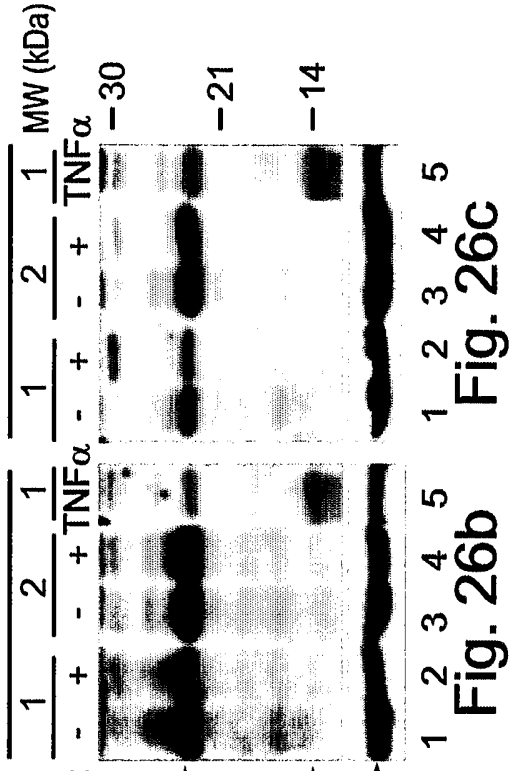



Fig. 26b

Fig. 26c

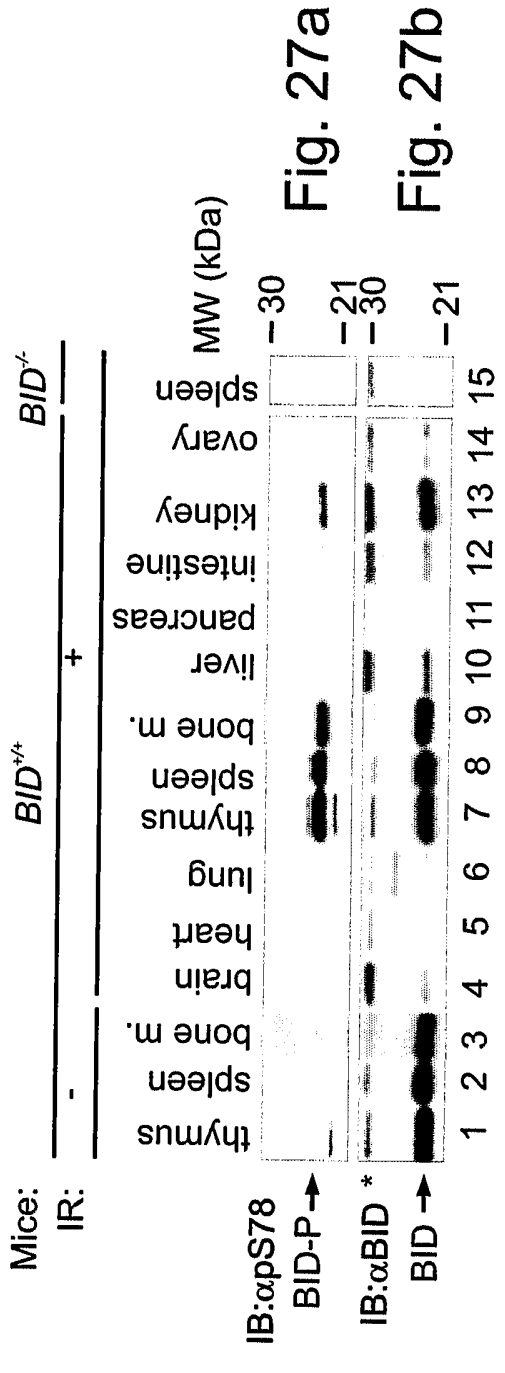



Fig. 27a

Fig. 27b




Fig. 28a

Fig. 28b

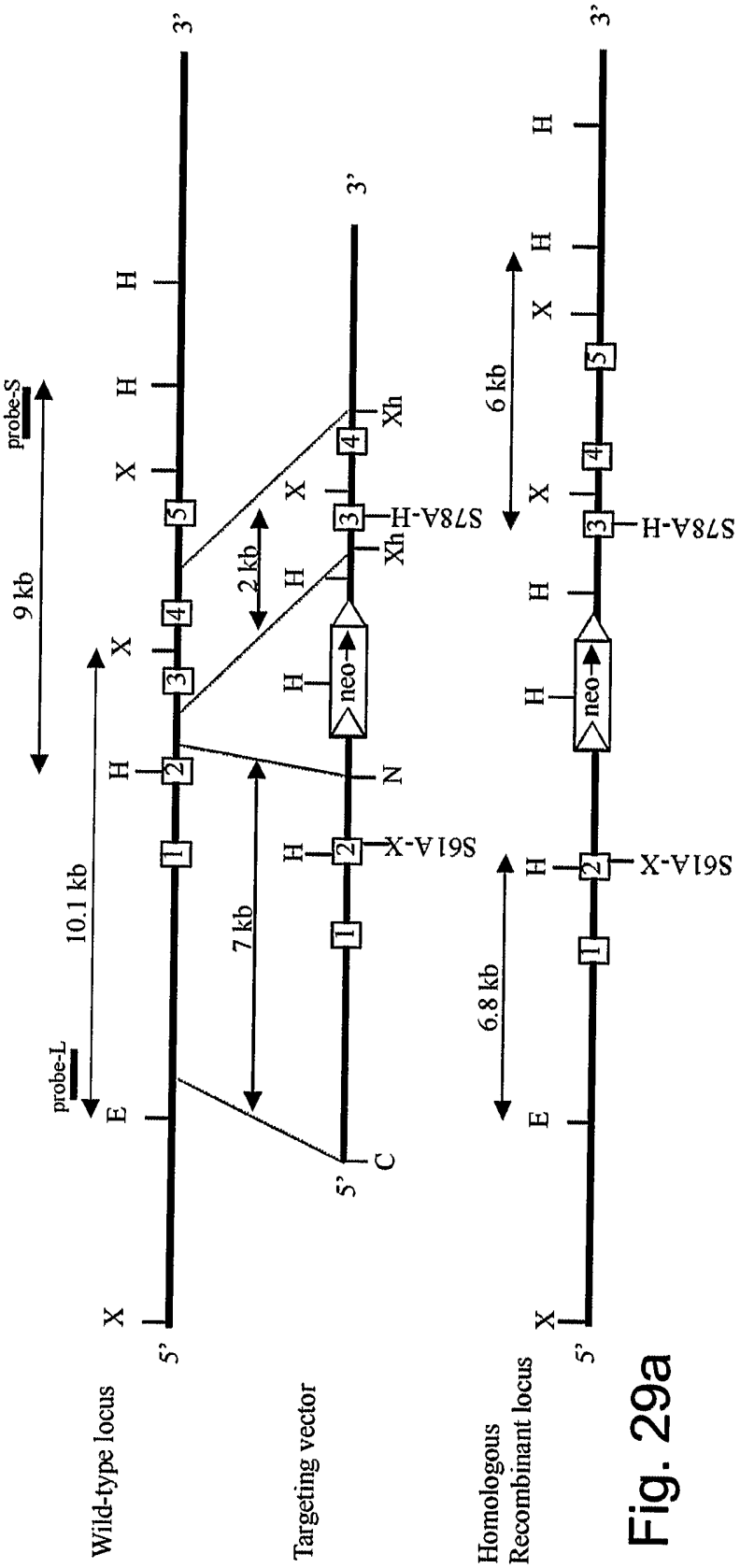



Fig. 29a

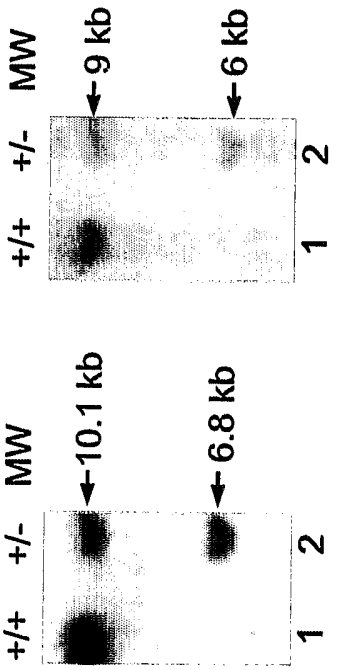



Fig. 29c




Fig. 29b

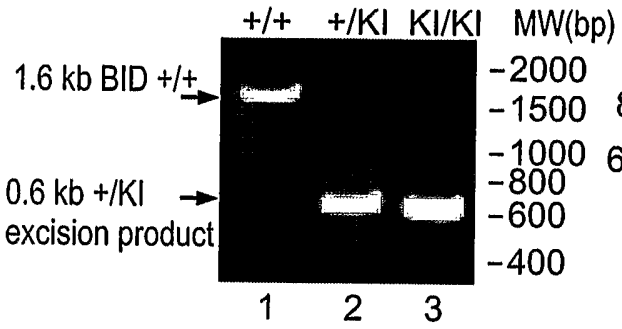



Fig. 30a

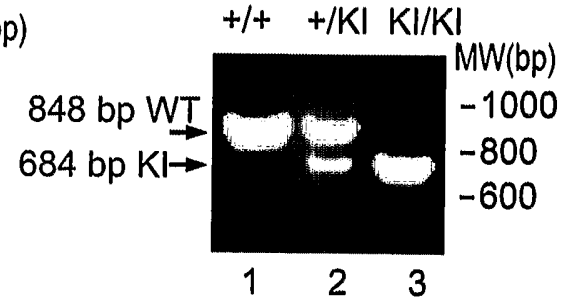



Fig. 30b

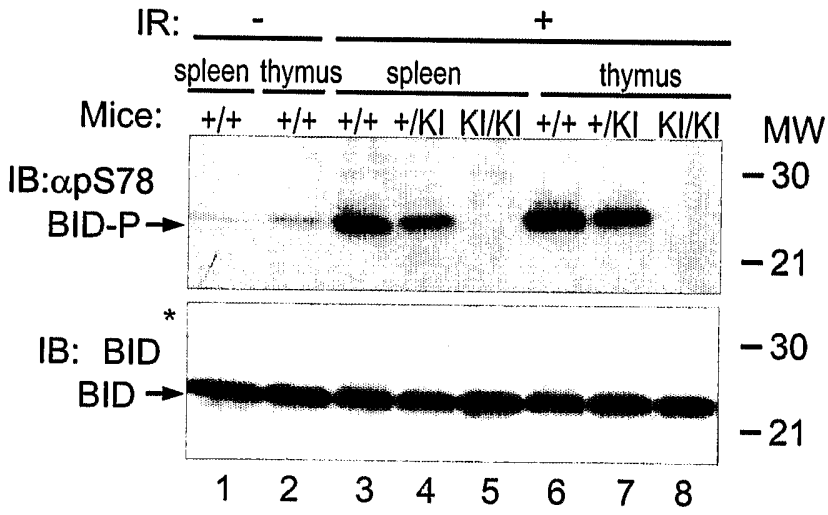



Fig. 31a

Fig. 31b

| | | |
|---|---|---|
| BID_mouse | 33 - Q E L E V L G R E L P V | - 44 |
| BID_human | 35 - R E L D A L G H E L P V | - 46 |
| BID_rat | 34 - Q E L E V L G Q E L P V | - 45 |
| *NES* consensus: | $L X_{(1-3)} L X_{(2-3)} L X L$ | |

Fig. 32a

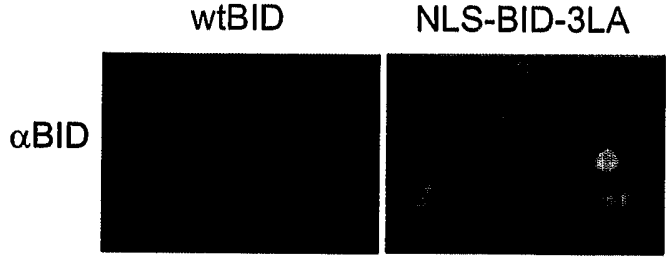



Fig. 32b Fig. 32c

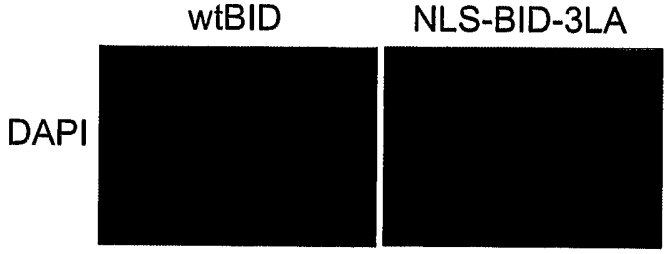



Fig. 32d Fig. 32e

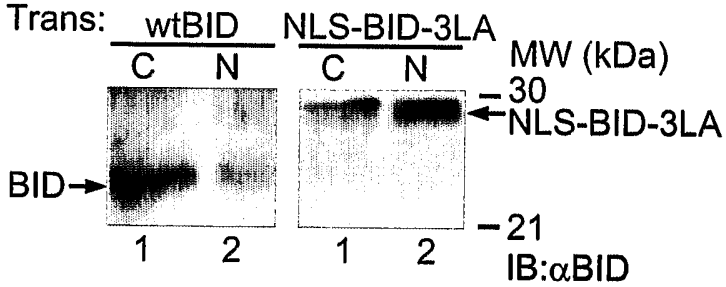



Fig. 32f Fig. 32g

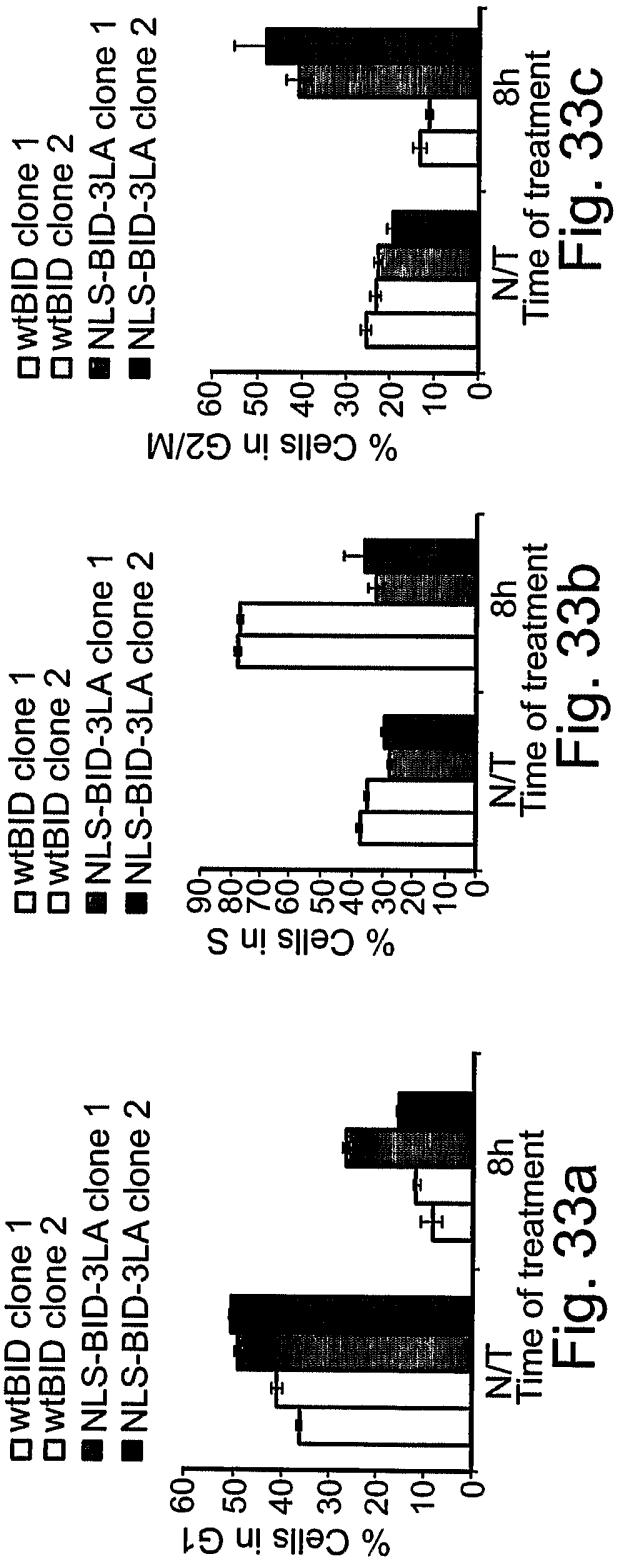



Fig. 33a




Fig. 33b




Fig. 33c




Fig. 34

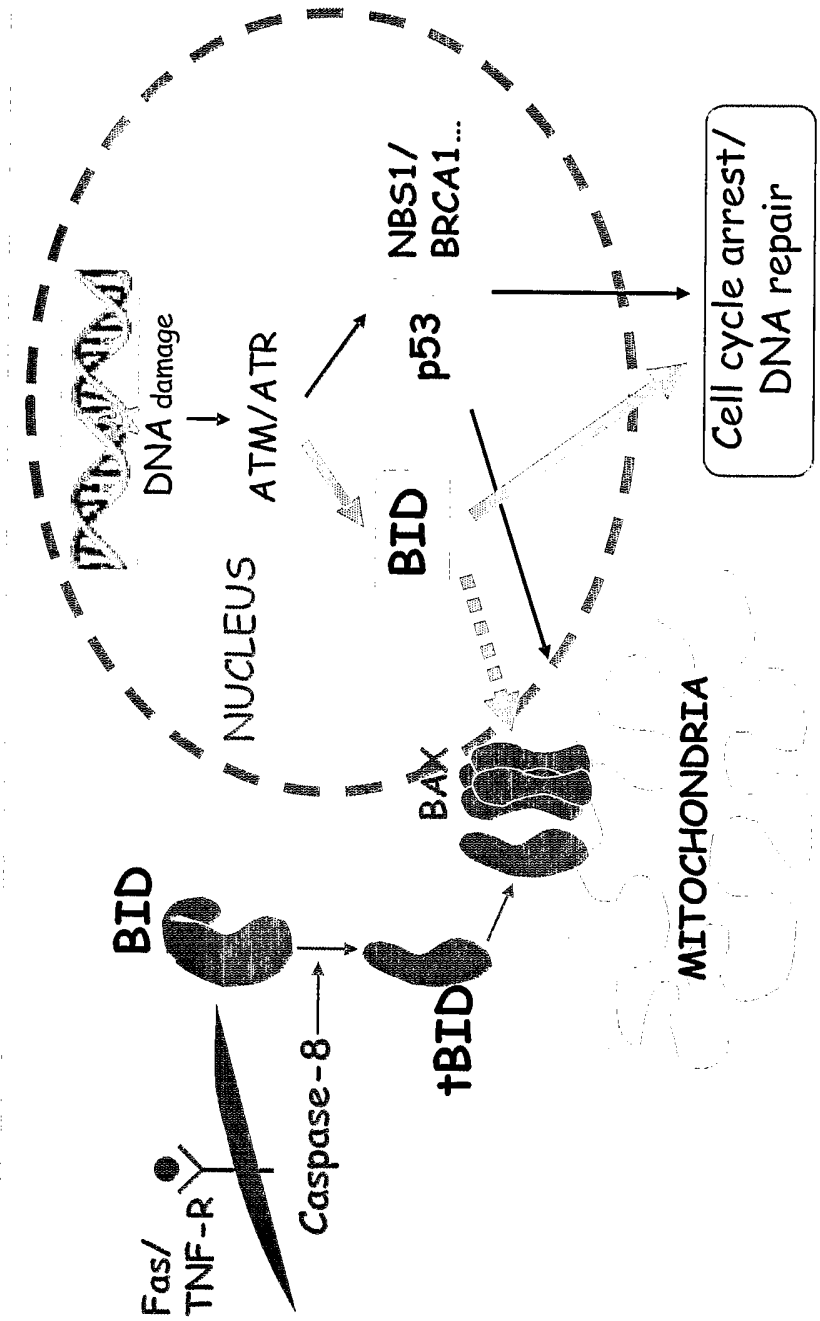

Fas/
TNF-R
Caspase-8
BID
tBID
BAX
MITOCHONDRIA
Apoptosis
NUCLEUS
DNA damage
ATM/ATR
BID
p53
NBS1/
BRCA1...
Cell cycle arrest/
DNA repair

Fig. 35

SEQUENCE LISTING

<110> Yeda Research And Development Co. Ltd.
Gross, Atan
Sarig, Rachel
Kamer, Iris

<120> ISOLATED BID POLYPEPTIDES, POLYNUCLEOTIDES ENCODING SAME AND ANTIBODIES DIRECTED THEREAGAINST AND METHODS OF USING SAME FOR INDUCING CELL CYCLE ARREST OR APOPTOSIS

<130> 31484

<160> 38

<170> PatentIn version 3.3

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<213> Artificial sequence

<220>
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<223> S78A mutant

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Phe Arg Arg Glu Leu Asp Ala Leu Gly His Glu Leu Pro Val Leu Ala
35 40 45

Pro Gln Trp Glu Gly Tyr Asp Glu Leu Gln Thr Asp Gly Asn Arg Ser
50 55 60

Ser His Ser Arg Leu Gly Arg Ile Glu Ala Asp Ser Glu Ala Gln Glu
65 70 75 80

Asp Ile Ile Arg Asn Ile Ala Arg His Leu Ala Gln Val Gly Asp Ser
85 90 95

Met Asp Arg Ser Ile Pro Pro Gly Leu Val Asn Gly Leu Ala Leu Gln
100 105 110

Leu Arg Asn Thr Ser Arg Ser Glu Glu Asp Arg Asn Arg Asp Leu Ala
115 120 125

Thr Ala Leu Glu Gln Leu Leu Gln Ala Tyr Pro Arg Asp Met Glu Lys
130 135 140

Glu Lys Thr Met Leu Val Leu Ala Leu Leu Leu Ala Lys Lys Val Ala
145 150 155 160

Ser His Thr Pro Ser Leu Leu Arg Asp Val Phe His Thr Thr Val Asn
165 170 175

Phe Ile Asn Gln Asn Leu Arg Thr Tyr Val Arg Ser Leu Ala Arg Asn
180 185 190

Gly Met Asp
195

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<212> DNA
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<223> S61A mutant

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actgtcaact ttattaacca gaacctattc tcctatgtga ggaacttggt tagaaacgag 720

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agagtttacg ccggctcctg catccacacc atgtaccttt gtcctatcag ctgtatgggt 960

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<223> S78A mutant

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<223> S61A and S78A mutant

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<223> Peptide containing phosphorylated S78 for immunization of mice

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<221> MOD_RES
<222> (10)..(10)
<223> PHOSPHORYLATION

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<223> Single strand DNA oligonucleotide

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<212> DNA
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<223> Single strand DNA oligonucleotide

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<210> 13
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> Single strand DNA oligonucleotide

<400> 13
gggcaaagta gtggcttcag 20

<210> 14
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<220>
<223> Single strand DNA oligonucleotide

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ggagttcgcc acaagagaag 20

<210> 15
<211> 195
<212> PRT
<213> Homo sapiens

<400> 15

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20 25 30

Phe Arg Arg Glu Leu Asp Ala Leu Gly His Glu Leu Pro Val Leu Ala
35 40 45

Pro Gln Trp Glu Gly Tyr Asp Glu Leu Gln Thr Asp Gly Asn Arg Ser
50 55 60

Ser His Ser Arg Leu Gly Arg Ile Glu Ala Asp Ser Glu Ser Gln Glu
65 70 75 80

Asp Ile Ile Arg Asn Ile Ala Arg His Leu Ala Gln Val Gly Asp Ser
85 90 95

Met Asp Arg Ser Ile Pro Pro Gly Leu Val Asn Gly Leu Ala Leu Gln
100 105 110

Leu Arg Asn Thr Ser Arg Ser Glu Glu Asp Arg Asn Arg Asp Leu Ala
115 120 125

Thr Ala Leu Glu Gln Leu Leu Gln Ala Tyr Pro Arg Asp Met Glu Lys
130 135 140

Glu Lys Thr Met Leu Val Leu Ala Leu Leu Leu Ala Lys Lys Val Ala
145 150 155 160

Ser His Thr Pro Ser Leu Leu Arg Asp Val Phe His Thr Thr Val Asn
165 170 175

Phe Ile Asn Gln Asn Leu Arg Thr Tyr Val Arg Ser Leu Ala Arg Asn
180 185 190

Gly Met Asp
195

<210> 16
<211> 195
<212> PRT
<213> Mus musculus

<400> 16

Met Asp Ser Glu Val Ser Asn Gly Ser Gly Leu Gly Ala Glu His Ile
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Thr Asp Leu Leu Val Phe Gly Phe Leu Gln Ser Ser Gly Cys Thr Arg
20 25 30

Gln Glu Leu Glu Val Leu Gly Arg Glu Leu Pro Val Gln Ala Tyr Trp
35 40 45

Glu Ala Asp Leu Glu Asp Glu Leu Gln Thr Asp Gly Ser Gln Ala Ser
50 55 60

Arg Ser Phe Asn Gln Gly Arg Ile Glu Pro Asp Ser Glu Ser Gln Glu
65 70 75 80

Glu Ile Ile His Asn Ile Ala Arg His Leu Ala Gln Ile Gly Asp Glu
85 90 95

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Met Asp His Asn Ile Gln Pro Thr Leu Val Arg Gln Leu Ala Ala Gln
            100                 105                 110

Phe Met Asn Gly Ser Leu Ser Glu Glu Asp Lys Arg Asn Cys Leu Ala
        115                 120                 125

Lys Ala Leu Asp Glu Val Lys Thr Ala Phe Pro Arg Asp Met Glu Asn
    130                 135                 140

Asp Lys Ala Met Leu Ile Met Thr Met Leu Leu Ala Lys Lys Val Ala
145                 150                 155                 160

Ser His Ala Pro Ser Leu Leu Arg Asp Val Phe His Thr Thr Val Asn
                165                 170                 175

Phe Ile Asn Gln Asn Leu Phe Ser Tyr Val Arg Asn Leu Val Arg Asn
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Glu Met Asp
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<210> 17
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<212> PRT
<213> Homo sapiens

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<222> (78)..(78)
<223> PHOSPHORYLATION

<400> 17

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            20                  25                  30

Phe Arg Arg Glu Leu Asp Ala Leu Gly His Glu Leu Pro Val Leu Ala
        35                  40                  45

Pro Gln Trp Glu Gly Tyr Asp Glu Leu Gln Thr Asp Gly Asn Arg Ser
    50                  55                  60

Ser His Ser Arg Leu Gly Arg Ile Glu Ala Asp Ser Glu Ser Gln Glu
65                  70                  75                  80

Asp Ile Ile Arg Asn Ile Ala Arg His Leu Ala Gln Val Gly Asp Ser
                85                  90                  95

Met Asp Arg Ser Ile Pro Pro Gly Leu Val Asn Gly Leu Ala Leu Gln
            100                 105                 110

Leu Arg Asn Thr Ser Arg Ser Glu Glu Asp Arg Asn Arg Asp Leu Ala
        115                 120                 125
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Thr Ala Leu Glu Gln Leu Leu Gln Ala Tyr Pro Arg Asp Met Glu Lys
    130                 135                 140

Glu Lys Thr Met Leu Val Leu Ala Leu Leu Leu Ala Lys Lys Val Ala
145                 150                 155                 160

Ser His Thr Pro Ser Leu Leu Arg Asp Val Phe His Thr Thr Val Asn
                165                 170                 175

Phe Ile Asn Gln Asn Leu Arg Thr Tyr Val Arg Ser Leu Ala Arg Asn
            180                 185                 190

Gly Met Asp
        195
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<210> 18
<211> 195
<212> PRT
<213> Homo sapiens

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<221> misc_feature
<223> S78D mutant

<400> 18

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1               5                   10                  15

Thr Asn Leu Leu Val Phe Gly Phe Leu Gln Ser Cys Ser Asp Asn Ser
            20                  25                  30

Phe Arg Arg Glu Leu Asp Ala Leu Gly His Glu Leu Pro Val Leu Ala
        35                  40                  45

Pro Gln Trp Glu Gly Tyr Asp Glu Leu Gln Thr Asp Gly Asn Arg Ser
    50                  55                  60

Ser His Ser Arg Leu Gly Arg Ile Glu Ala Asp Ser Glu Asp Gln Glu
65                  70                  75                  80

Asp Ile Ile Arg Asn Ile Ala Arg His Leu Ala Gln Val Gly Asp Ser
                85                  90                  95

Met Asp Arg Ser Ile Pro Pro Gly Leu Val Asn Gly Leu Ala Leu Gln
            100                 105                 110

Leu Arg Asn Thr Ser Arg Ser Glu Glu Asp Arg Asn Arg Asp Leu Ala
        115                 120                 125

Thr Ala Leu Glu Gln Leu Leu Gln Ala Tyr Pro Arg Asp Met Glu Lys
    130                 135                 140

Glu Lys Thr Met Leu Val Leu Ala Leu Leu Leu Ala Lys Lys Val Ala
145                 150                 155                 160

Ser His Thr Pro Ser Leu Leu Arg Asp Val Phe His Thr Thr Val Asn
                165                 170                 175
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Phe Ile Asn Gln Asn Leu Arg Thr Tyr Val Arg Ser Leu Ala Arg Asn
180 185 190

Gly Met Asp
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<211> 7
<212> PRT
<213> Artificial sequence

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<223> Nuclear localization signal peptide (NLS)

<400> 19

Lys Lys Lys Arg Lys Val Glu
1 5

<210> 20
<211> 63
<212> PRT
<213> Artificial sequence

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<223> Recombinant polypeptide derived from WT Human Bid

<220>
<221> MOD_RES
<222> (4)..(4)
<223> PHOSPHORYLATION

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Ala Gln Val Gly Asp Ser Met Asp Arg Ser Ile Pro Pro Gly Leu Val
20 25 30

Asn Gly Leu Ala Leu Gln Leu Arg Asn Thr Ser Arg Ser Glu Glu Asp
35 40 45

Arg Asn Arg Asp Leu Ala Thr Ala Leu Glu Gln Leu Leu Gln Ala
50 55 60

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<223> Recombinant polypeptide derived from WT Human Bid

<220>
<221> MOD_RES
<222> (2)..(2)
<223> PHOSPHORYLATION

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1 5 10 15

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Val Gly Asp Ser Met Asp Arg Ser Ile Pro Pro Gly Leu Val Asn Gly
            20                  25                  30

Leu Ala Leu Gln Leu Arg Asn Thr Ser Arg Ser Glu Glu Asp Arg Asn
        35                  40                  45

Arg Asp Leu Ala Thr Ala Leu Glu Gln Leu Leu Gln Ala Tyr Pro Arg
    50                  55                  60

Asp Met Glu Lys Glu Lys Thr Met Leu Val Leu Ala Leu Leu Leu Ala
65                  70                  75                  80

Lys Lys Val Ala Ser His Thr Pro Ser Leu Leu Arg Asp Val Phe His
                85                  90                  95

Thr Thr Val Asn Phe Ile Asn Gln Asn Leu Arg Thr Tyr Val Arg Ser
            100                 105                 110

Leu Ala Arg Asn Gly Met Asp
        115
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<210> 22
<211> 61
<212> PRT
<213> Artificial sequence

<220>
<223> Recombinant polypeptide derived from WT Human Bid

<220>
<221> MOD_RES
<222> (2)..(2)
<223> PHOSPHORYLATION

<400> 22

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Glu Ser Gln Glu Asp Ile Ile Arg Asn Ile Ala Arg His Leu Ala Gln
1               5                   10                  15

Val Gly Asp Ser Met Asp Arg Ser Ile Pro Pro Gly Leu Val Asn Gly
            20                  25                  30

Leu Ala Leu Gln Leu Arg Asn Thr Ser Arg Ser Glu Glu Asp Arg Asn
        35                  40                  45

Arg Asp Leu Ala Thr Ala Leu Glu Gln Leu Leu Gln Ala
    50                  55                  60
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<210> 23
<211> 63
<212> PRT
<213> Artificial sequence

<220>
<223> Human Bid derived recombinant polypeptide, S78D mutated

<400> 23

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Asp Ser Glu Asp Gln Glu Asp Ile Ile Arg Asn Ile Ala Arg His Leu
1               5                   10                  15
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Ala Gln Val Gly Asp Ser Met Asp Arg Ser Ile Pro Pro Gly Leu Val
20 25 30

Asn Gly Leu Ala Leu Gln Leu Arg Asn Thr Ser Arg Ser Glu Glu Asp
35 40 45

Arg Asn Arg Asp Leu Ala Thr Ala Leu Glu Gln Leu Leu Gln Ala
50 55 60

<210> 24
<211> 119
<212> PRT
<213> Artificial sequence

<220>
<223> Human Bid derived recombinant polypeptide, S78D mutated

<400> 24

Glu Asp Gln Glu Asp Ile Ile Arg Asn Ile Ala Arg His Leu Ala Gln
1 5 10 15

Val Gly Asp Ser Met Asp Arg Ser Ile Pro Pro Gly Leu Val Asn Gly
20 25 30

Leu Ala Leu Gln Leu Arg Asn Thr Ser Arg Ser Glu Glu Asp Arg Asn
35 40 45

Arg Asp Leu Ala Thr Ala Leu Glu Gln Leu Leu Gln Ala Tyr Pro Arg
50 55 60

Asp Met Glu Lys Glu Lys Thr Met Leu Val Leu Ala Leu Leu Leu Ala
65 70 75 80

Lys Lys Val Ala Ser His Thr Pro Ser Leu Leu Arg Asp Val Phe His
85 90 95

Thr Thr Val Asn Phe Ile Asn Gln Asn Leu Arg Thr Tyr Val Arg Ser
100 105 110

Leu Ala Arg Asn Gly Met Asp
115

<210> 25
<211> 61
<212> PRT
<213> Artificial sequence

<220>
<223> Human Bid derived recombinant polypeptide, S78D mutated

<400> 25

Glu Asp Gln Glu Asp Ile Ile Arg Asn Ile Ala Arg His Leu Ala Gln
1 5 10 15

Val Gly Asp Ser Met Asp Arg Ser Ile Pro Pro Gly Leu Val Asn Gly
20 25 30

Leu Ala Leu Gln Leu Arg Asn Thr Ser Arg Ser Glu Glu Asp Arg Asn
35 40 45

Arg Asp Leu Ala Thr Ala Leu Glu Gln Leu Leu Gln Ala
50 55 60

<210> 26
<211> 70
<212> PRT
<213> Artificial sequence

<220>
<223> Recombinant polypeptide derived from WT Human Bid, fused to NLS

<400> 26

Lys Lys Lys Arg Lys Val Glu Asp Ser Glu Ser Gln Glu Asp Ile Ile
1 5 10 15

Arg Asn Ile Ala Arg His Leu Ala Gln Val Gly Asp Ser Met Asp Arg
20 25 30

Ser Ile Pro Pro Gly Leu Val Asn Gly Leu Ala Leu Gln Leu Arg Asn
35 40 45

Thr Ser Arg Ser Glu Glu Asp Arg Asn Arg Asp Leu Ala Thr Ala Leu
50 55 60

Glu Gln Leu Leu Gln Ala
65 70

<210> 27
<211> 126
<212> PRT
<213> Artificial sequence

<220>
<223> Recombinant polypeptide derived from WT Human Bid, fused to NLS

<400> 27

Lys Lys Lys Arg Lys Val Glu Glu Ser Gln Glu Asp Ile Ile Arg Asn
1 5 10 15

Ile Ala Arg His Leu Ala Gln Val Gly Asp Ser Met Asp Arg Ser Ile
20 25 30

Pro Pro Gly Leu Val Asn Gly Leu Ala Leu Gln Leu Arg Asn Thr Ser
35 40 45

Arg Ser Glu Glu Asp Arg Asn Arg Asp Leu Ala Thr Ala Leu Glu Gln
50 55 60

Leu Leu Gln Ala Tyr Pro Arg Asp Met Glu Lys Glu Lys Thr Met Leu
65 70 75 80

Val Leu Ala Leu Leu Leu Ala Lys Lys Val Ala Ser His Thr Pro Ser
85 90 95

Leu Leu Arg Asp Val Phe His Thr Thr Val Asn Phe Ile Asn Gln Asn
100 105 110

Leu Arg Thr Tyr Val Arg Ser Leu Ala Arg Asn Gly Met Asp
115 120 125

<210> 28
<211> 202
<212> PRT
<213> Artificial sequence

<220>
<223> Recombinant polypeptide derived from WT Human Bid, fused to NLS

<400> 28

Lys Lys Lys Arg Lys Val Glu Met Asp Cys Glu Val Asn Asn Gly Ser
1 5 10 15

Ser Leu Arg Asp Glu Cys Ile Thr Asn Leu Leu Val Phe Gly Phe Leu
20 25 30

Gln Ser Cys Ser Asp Asn Ser Phe Arg Arg Glu Leu Asp Ala Leu Gly
35 40 45

His Glu Leu Pro Val Leu Ala Pro Gln Trp Glu Gly Tyr Asp Glu Leu
50 55 60

Gln Thr Asp Gly Asn Arg Ser Ser His Ser Arg Leu Gly Arg Ile Glu
65 70 75 80

Ala Asp Ser Glu Ser Gln Glu Asp Ile Ile Arg Asn Ile Ala Arg His
85 90 95

Leu Ala Gln Val Gly Asp Ser Met Asp Arg Ser Ile Pro Pro Gly Leu
100 105 110

Val Asn Gly Leu Ala Leu Gln Leu Arg Asn Thr Ser Arg Ser Glu Glu
115 120 125

Asp Arg Asn Arg Asp Leu Ala Thr Ala Leu Glu Gln Leu Leu Gln Ala
130 135 140

Tyr Pro Arg Asp Met Glu Lys Glu Lys Thr Met Leu Val Leu Ala Leu
145 150 155 160

Leu Leu Ala Lys Lys Val Ala Ser His Thr Pro Ser Leu Leu Arg Asp
165 170 175

Val Phe His Thr Thr Val Asn Phe Ile Asn Gln Asn Leu Arg Thr Tyr
180 185 190

Val Arg Ser Leu Ala Arg Asn Gly Met Asp
195 200

<210> 29
<211> 21
<212> PRT
<213> Artificial sequence

<220>

<223> Synthetic polypeptide derived from WT Human Bid

<400> 29

Arg Leu Gly Arg Ile Glu Ala Asp Ser Glu Ser Gln Glu Asp Ile Ile
1 5 10 15

Arg Asn Ile Ala Arg
20

<210> 30
<211> 25
<212> PRT
<213> Artificial sequence

<220>
<223> Synthetic polypeptide derived from WT Human Bid

<400> 30

Asp Gly Asn Arg Ser Ser His Ser Arg Leu Gly Arg Ile Glu Ala Asp
1 5 10 15

Ser Glu Ser Gln Glu Asp Ile Ile Arg
20 25

<210> 31
<211> 25
<212> PRT
<213> Artificial sequence

<220>
<223> Synthetic polypeptide derived from WT Human Bid

<400> 31

Gly Arg Ile Glu Ala Asp Ser Glu Ser Gln Glu Asp Ile Ile Arg Asn
1 5 10 15

Ile Ala Arg His Leu Ala Gln Val Gly
20 25

<210> 32
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Synthetic polypeptide derived from WT Human Bid

<220>
<221> MOD_RES
<222> (11)..(11)
<223> PHOSPHORYLATION

<400> 32

Arg Leu Gly Arg Ile Glu Ala Asp Ser Glu Ser Gln Glu Asp Ile Ile
1 5 10 15

Arg Asn Ile Ala
20

<210> 33
<211> 20

<212> PRT
<213> Artificial sequence

<220>
<223> Synthetic polypeptide derived from WT Human Bid

<220>
<221> MOD_RES
<222> (9)..(9)
<223> PHOSPHORYLATION

<400> 33

Gly Arg Ile Glu Ala Asp Ser Glu Ser Gln Glu Asp Ile Ile Arg Asn
1 5 10 15

Ile Ala Arg His
20

<210> 34
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Synthetic polypeptide derived from WT Human Bid

<220>
<221> MOD_RES
<222> (14)..(14)
<223> PHOSPHORYLATION

<400> 34

Ser His Ser Arg Leu Gly Arg Ile Glu Ala Asp Ser Glu Ser Gln Glu
1 5 10 15

Asp Ile Ile Arg
20

<210> 35
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Human Bid derived synthetic polypeptide, S78D mutated

<400> 35

Arg Leu Gly Arg Ile Glu Ala Asp Ser Glu Asp Gln Glu Asp Ile Ile
1 5 10 15

Arg Asn Ile Ala
20

<210> 36
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Human Bid derived synthetic polypeptide, S78D mutated

<400> 36

Gly Arg Ile Glu Ala Asp Ser Glu Asp Gln Glu Asp Ile Ile Arg Asn
1 5 10 15

Ile Ala Arg His
20

<210> 37
<211> 20
<212> PRT
<213> Artificial sequence

<220>
<223> Human Bid derived synthetic polypeptide, S78D mutated

<400> 37

Ser His Ser Arg Leu Gly Arg Ile Glu Ala Asp Ser Glu Asp Gln Glu
1 5 10 15

Asp Ile Ile Arg
20

<210> 38
<211> 13
<212> PRT
<213> Artificial sequence

<220>
<223> Peptide containing phosphorylated S78 for immunization of mice

<220>
<221> MOD_RES
<222> (7)..(7)
<223> PHOSPHORYLATION

<400> 38

Cys Glu Pro Asp Ser Glu Ser Gln Glu Glu Ile Ile His
1 5 10