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(54) Title: NOVEL THERAPEUTIC APPROACHES FOR BIRT-HOGG-DUBE (BHD) SYNDROME

(57) Abstract: The present invention relates to compositions and methods for treating BHD-based tumors in a mammal. The methods include administering to a mammal an effective amount of a therapeutic agent that promotes or induces TGF- β dependent apoptosis in the BHD-based tumor. The therapeutic agents of the present invention include BH3-only molecules, HDAC inhibitors, autophagy inhibitors, and expression vectors that encode BHD, BIM and components of the TGF- β signaling pathway.



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TITLE OF THE INVENTION

Novel Therapeutic Approaches For Birt-Hogg-Dubé (BHD) Syndrome

BACKGROUND OF THE INVENTION

Birt-Hogg-Dubé (BHD) syndrome is a rare, inherited cancer susceptibility disease characterized by benign hair follicle tumors and lung cysts in a majority of patients, and renal cell carcinoma (RCC) in approximately one-third of diagnosed BHD cases (Schmidt et al., 2005, *Am J Hum Genet* 76(6):1023-1033). Genetic analyses of families affected by BHD syndrome previously identified germline mutations in the *BHD* gene on chromosome 17p11.2, most of which were predicted to prematurely truncate the encoded protein (Nickerson et al., 2002, *Cancer Cell* 2(2):157-164; Toro et al., 2008, *J Med Genet* 45(6):321-331). Mutational analysis of BHD-related tumors from patients, as well as those from mouse and rat models of BHD syndrome, identified inactivating “second hits” in the remaining wild-type *BHD* allele, formally establishing BHD as a tumor suppressor (Okimoto *et al.*, 2004, *Proc Natl Acad Sci USA* 101(7):2023-2027; Vocke *et al.*, 2005, *J Natl Cancer Inst* 97(12):931-935; Hasumi *et al.*, 2009, *Proc Natl Acad Sci USA* 106(44):18722-18727). *BHD* inactivation has also been described in a subset of von Hippel-Lindau-independent RCC syndromes, in approximately one-third of sporadic renal cancers, and in 60% of idiopathic cystic lung disease cases (Khoo *et al.*, 2003, *Cancer Res* 63(15):4583-4587; Gunji *et al.*, 2007, *J Med Genet* 44(9):588-593; Woodward *et al.*, 2008, *Clin Cancer Res* 14(18):5925-5930). BHD syndrome results from loss-of-function mutations in the *BHD* gene. *BHD* is also inactivated in a significant fraction of patients with sporadic renal cancers and idiopathic cystic lung disease, and little is known about its mode of action.

While the genetic basis of BHD syndrome is well understood, the cellular and molecular mechanisms of the protein encoded by *BHD*, also called folliculin, remain unclear. Dissecting BHD's molecular functions is particularly challenging since the *BHD* gene product bears no sequence or functional homology to any known protein. Studies in *S. pombe* have suggested a role for the putative yeast BHD ortholog in amino acid homeostasis, while siRNA-mediated knockdown of *Drosophila* BHD in the fly has implicated it in male germline stem cell maintenance through Stat and/or BMP signaling (Singh *et al.*, 2006, *Oncogene* 25(44):5933-5941; van Slegtenhorst *et al.*, 2007, *J Biol Chem* 282(34):24583-24590). Finally, mammalian cell culture analyses and mouse models have implicated BHD in mTOR signaling, potentially mediated through an interaction with its binding partner,

called folliculin-interacting protein or FNIP1, and AMP-activated kinase (AMPK) (Baba *et al.*, 2006, Proc Natl Acad Sci USA 103(42):15552-15557). However, these studies depict a contradictory role for BHD's involvement in mTOR signaling, since BHD loss can result in either stimulation or inhibition of mTOR depending on the system examined (Baba *et al.*, 2006, Proc Natl Acad Sci USA 103(42):15552-15557; Baba *et al.*, 2008, J Natl Cancer Inst 100(2):140-154; Hartman *et al.*, 2009, Oncogene 28(13):1594-1604; Hudon *et al.*, 2009, J Med Genet. [Epub ahead of print]). In addition, the cause-effect relationship between these signaling aberrations and basic cellular and biological consequences has not yet been demonstrated.

Thus, there is a long felt need in the art for understanding the cellular and molecular mechanisms of the protein encoded by *BHD*, and its role in BHD Syndrome and tumor development. Also needed is a composition and method for treating BHD-based tumors, as well as sporadic cases of cystic lung disease and renal cell carcinoma. The present invention satisfies these needs.

BRIEF SUMMARY OF THE INVENTION

The invention includes a method of treating a BHD-based tumor in a mammal. The method comprises administering to a mammal an effective amount of a therapeutic agent that promotes or induces TGF- β dependent apoptosis in the BHD-based tumor.

In one embodiment, the mammal is a human. In another embodiment, the therapeutic agent is a BH3-only molecule. In yet another embodiment, the BH3-only molecule is a protein selected from the group consisting of BID, BIM, BAD and NOXA. In yet another embodiment, the BH3-only molecule is a mimetic. In yet another embodiment, the BH3-only mimetic is ABT-737. In yet another embodiment, the therapeutic agent is an HDAC inhibitor. In yet another embodiment, the HDAC inhibitor is Trichostatin. In yet another embodiment, the HDAC inhibitor is Vorinostat. In yet another embodiment, the therapeutic agent is an autophagy inhibitor. In yet another embodiment, the autophagy inhibitor is chloroquine. In yet another embodiment, the autophagy inhibitor is 3-methyl-adenine (3-MA). In yet another embodiment, the therapeutic agent is an expression vector encoding BHD. In yet another embodiment, the therapeutic agent is an expression vector encoding BIM.

The invention also includes a method for promoting or inducing TGF- β dependent apoptosis in a BHD-based tumor cell of a mammal. The method comprises contacting the cell with an effective amount of a therapeutic agent.

In one embodiment, the mammal is a human. In another embodiment, the therapeutic agent is selected from the group consisting of a BH3-only molecule, an HDAC inhibitor and an autophagy inhibitor. In yet another embodiment, the therapeutic agent is an expression vector encoding BHD. In yet another embodiment, the therapeutic agent is an expression vector encoding BIM.

The invention further includes a composition for promoting or inducing TGF- β dependent apoptosis in a BHD-based tumor cell of a mammal. The composition comprises a BH3-only molecule.

The invention also includes a composition for promoting or inducing TGF- β dependent apoptosis in a BHD-based tumor cell of a mammal. The composition comprises an HDAC inhibitor.

The invention further includes a composition for promoting or inducing TGF- β dependent apoptosis in a BHD-based tumor cell of a mammal. The composition comprises an autophagy inhibitor.

The invention also includes a composition for promoting or inducing TGF- β dependent apoptosis in a BHD-based tumor cell of a mammal. The composition comprises an expression vector encoding BHD.

The invention further includes a composition for promoting or inducing TGF- β dependent apoptosis in a BHD-based tumor cell of a mammal. The composition comprises an expression vector encoding BIM.

BRIEF DESCRIPTION OF THE DRAWINGS

For the purpose of illustrating the invention, there are depicted in the drawings certain embodiments of the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

Figure 1, comprising Figures 1A-1G, is a series of charts and images demonstrating that *Bhd*^{m/m} embryos are early embryonic lethal. Figure 1(A) is a schematic depicting the integration site of Bay Genomics PGTX2 gene trap vector in murine *Bhd* intron 8, the location of probe and restriction sites for Southern blot genotyping, and location of primers (P1, P2, and P3) for PCR-based genotyping. Figure 1(B) depicts a Southern blot of PvuII-digested genomic DNA. Figure 1(C) depicts PCR-based genotypes of *Bhd*^{+/+} or *Bhd*^{+/m} ES cells to discriminate wild-type (WT) from mutant (MUT) alleles, where *m* specifically designates the gene-trapped mutant *Bhd* allele from Bay Genomics. Figure 1(D) depicts relative *Bhd* mRNA levels in *Bhd*^{+/+} vs. *Bhd*^{+/m} ES cells assayed by qRT-PCR, using primers

spanning exons just downstream of the trap cassette (exons 9-10) or across the terminal exons of the *Bhd* gene (exons 12-13), ruling out the presence of aberrant transcripts generated from cryptic transcriptional start sites downstream of the trap cassette. Figure 1(E) depicts X-Gal staining, showing *Bhd*^{+/m} ES cells express the *Bhd*- β -galactosidase fusion product. Figure 1(F) is a table of progeny obtained from *Bhd*^{+/m} intercrosses, showing no *Bhd*^{-/-} embryos could be recovered after e6.5. In Figure 1(F), (*) indicates probable genotypes of embryos based on normal vs. abnormal phenotypic appearance at e6.5. Figure 1(G) depicts 20x images of H&E stained cross-sections of paraffin embedded e6.5 embryos from *Bhd*^{+/m} intercrosses. In Figure 1(G), (a) exemplifies normal morphology while (b) and (c) demonstrate gross abnormalities, probable *Bhd*^{m/m} genotypes.

Figure 2, comprising Figures 2A-2E, is a series of charts and images characterizing the German Gene Trap *Bhd* allele. Figure 2(A) is a schematic depicting the integration site of FlipRosageo trap vector in intron 1 of the murine *Bhd* gene, as well as the location of probe and restriction digest sites for Southern blot genotyping, as well as primer positions (P1, P2, P3) for PCR-based genotyping. Figure 2(B) depicts a Southern blot, and Figure 2(C) depicts PCR-based genotyping, of *Bhd*^{+/+} vs *Bhd*^{+/m} ES cells to discriminate wild-type (WT) from mutant (MUT) alleles. (-) denotes the gene-trapped mutant *Bhd* allele obtained from the German Gene Trap Consortium throughout the manuscript. Figure 2(D) depicts a qRT-PCR analysis of *Bhd* expression levels using primers just downstream (exons 1-2) or far downstream (exons 9-10, 12-13) from the trap integration site. Figure 2(E) depicts X-gal staining demonstrating *Bhd*^{+/m} ES cells express β -galactosidase from the trap cassette.

Figure 3, comprising Figures 3A-3H, is a series of charts and images demonstrating that *Bhd*^{-/-} ES cells do not have a proliferative or growth advantage, but are resistant to apoptosis. Figure 3(A) depicts PCR genotypes verifying loss of wild-type *Bhd* allele in three independent ES clones, designated as 1, 2 and 3, after step-up selection. Figure 3(B) depicts a Western blot demonstrating loss of BHD protein expression in three independent *Bhd*^{-/-} ES clones. Figure 3(C) depicts a growth curve showing *Bhd*^{-/-} ES cells proliferate similar to *Bhd*^{+/+} and *Bhd*^{+/m} counterparts, but have increased saturation density. Figure 3(D) depicts Coulter counter size measurements showing *Bhd*^{-/-} cells are significantly smaller than *Bhd*^{+/+} and *Bhd*^{+/m} ES cells (* p=.000623). Figure 3(E) depicts *Bhd*^{-/-} ES cells that are significantly resistant to apoptosis, as shown by a brightfield image of *Bhd*^{+/+} and *Bhd*^{-/-} ES cells starved of amino acids for one day. Figure 3(F) depicts a representative FACs plot showing *Bhd*^{-/-} ES cells have significantly less sub-G₁ DNA content following one day of amino acid starvation.

Figure 3(G) depicts a FACs analysis of cells containing sub-G₁ DNA content after one day serum, glucose and amino acid (AA) deprivation compared to *Bhd*^{+/+} and *Bhd*^{+/-} cells, but not by TNF α treatment (* p<.0000345). Figure 3(H) depicts a Western blot of caspase and parp cleavage with and without amino acid starvation in *Bhd*^{+/+} and *Bhd*^{+/-} ES cells. In Figure 3, all bar graphs represent averages of three independent experiments with error bars showing standard error of the mean (SEM).

Figure 4, comprising Figures 4A-4C, is a series of images demonstrating that *Bhd*^{+/-} ES cells maintain characteristics of pluripotency in culture. Figure 4(A) depicts a Western blot showing three independent *Bhd*^{+/-} ES clones maintain expression of pluripotency factors Oct-4, Nanog and Sox2. Figure 4(B) depicts a Western blot showing p-STAT3 (Y705) levels are unaffected even when cultured in non-saturating concentrations of LIF. Figure 4(C) depicts stain positive for alkaline phosphatase, demonstrating observed effects are not due to precocious differentiation of *Bhd*^{+/-} ES cells in culture.

Figure 5, comprising Figures 5A-5D, is a series of charts and images demonstrating that death resistance of *Bhd*^{+/-} cells is not due to increased intracellular amino acid levels but results from decreased Bim protein levels. Figure 5(A) depicts intracellular amino acid levels normalized to total protein in *Bhd*^{+/+} vs. *Bhd*^{+/-} ES cells as assessed by HPLC. The data in Figure 5A represents the average of three independent experiments and error bars reflect standard deviation. Figure 5(B) depicts a Western blot showing *Bhd*^{+/-} ES cells have decreased levels of several BH3-only proteins, most notably Bim. Figure 5(C) depicts the quantification of sub-G₁ populations by flow cytometry, where restoration of *Bhd* and *BimEL* expression, or treatment with ABT-737 (ABT), chloroquine (CQ) or 3-methyl-adenine (3MA) rescues the death resistance phenotype of *Bhd*^{+/-} ES cells in response to two-day amino acid-starvation, compared to untreated or vector-only (*MSCV*) infected controls. In Figure 5(C), the bar graphs represent three independent experiments, and error bars show SEM. Figure 5(D) depicts 20X images of paraffin-embedded sections of solid renal tumors (labeled T) from *Bhd*^{+/-} mice 18-21 months in age (a-c), or a human patient exhibiting loss of Bim expression, compared to normal adjacent tissue (labeled N). In Figure 5(D), (a) and (b) are hybrid clear cell-oncocytic lesions, and (c) is a solid papillary projection from a cyst, while (d) is a human chromophobe RCC.

Figure 6, comprising Figures 6A and 6B, demonstrate that *Bhd*^{+/-} ES cells also exhibit loss of Bim expression. Figure 6(A) depicts PCR genotyping of *Bhd*^{+/+}, parental *Bhd*^{+/-} (P) ES cells, two independent *Bhd*^{+/-} clones (1 and 2) that underwent step-up

selection without homozygosing the *m* allele, and three independent *Bhd^{m/m}* clones that homozygosed the *Bhd^m* allele after step-up selection (denoted as 1, 2 and 3). Figure 6(B) is a Western blot depicting loss of BHD protein in *Bhd^{m/m}* clones as well as loss of Bim protein expression. A non-specific band (N.S.) is shown for a loading control.

Figure 7, comprising Figures 7A-7D, is a series of charts and images demonstrating that Bim is transcriptionally downregulated in *Bhd^{-/-}* cells, but is not due to hyperactivation of mTORC1, mTORC2 or ERK hyperactivation. Figure 7(A) depicts a qRT-PCR analysis showing *Bim* mRNA is reduced in *Bhd^{-/-}* ES cells (* $p=1.44 \times 10^{-7}$). Figure 7(B) depicts a Western blot showing that mTORC1 (indicated by p-S6K1 (T389) and p-4E-BP1 (T70)), mTORC2 (indicated by p-Akt (S473) p-FoxO (FoxO1 (Thr24)/FoxO3a (Thr32)/FoxO4 (Thr28)), ERK (indicated by p-MEK (S217/271), p-ERK (T202/Y204), and p-p90RSK (T359/S363)) signaling pathways are hyperactivated in *Bhd^{-/-}* ES cells. Figure 7(C) depicts inhibition of hyperactivated mTORC2 by 5 μ M LY294002 (LY) or ERK by 10 μ M PD98059 (PD) or a combination of both, or inhibition of mTORC1 by 20 nM rapamycin (rapa) for 24 hours, does not restore Bim protein expression levels in *Bhd^{-/-}* cells by Western blot. Drug efficacy is shown by decreased p-FOXO levels with LY treatment and decreased p-ERK levels with PD treatment in *Bhd^{-/-}* cells. Decreased mobility shifts in S6K demonstrate effectiveness of both LY and rapa treatments. Doses were chosen based on their ability to bring activation levels of signaling components in *Bhd^{-/-}* cells to those seen in *Bhd^{+/+}* cells. Figure 7(D) depicts *Bhd^{-/-}* ES cells pre-treated for six hours with the same of panel inhibitors as 7(C), then starved of amino acids for two days with inhibitors being re-added after one day of starvation to maintain concentrations. Death was assessed by quantification of sub-G₁ populations by flow cytometry (upper, * $p < .0345$) and caspase and parp cleavage by Western blot (lower). In Figure 7, all bar graphs represent averages of three independent experiments with error bars reflecting SEM.

Figure 8, comprising Figures 8A and 8B, demonstrate that loss of BHD does not affect *Bim*-specific miRNAs or Bim proteolytic degradation. Figure 8(A) depicts a Western blot showing that *Bhd^{-/-}* ES cells treated with 10 μ M MG-132 for 4 hours does not restore Bim protein levels, though ubiquitinated forms of HIF-1 α accumulate. A non-specific band (N.S.) is shown as a loading control. Figure 8(B) depicts *Bhd^{-/-}* ES cells having similar levels of mir-19 and mir-92 compared to *Bhd^{+/+}* and *Bhd^{+/-}* counterparts as shown by Northern blot, with tryosyl-tRNA used a loading control.

Figure 9, comprising Figures S5A-S5D, is a series of images demonstrating that loss of BHD results in decreased amino-acid-sensitive eIF2 α phosphorylation but this does not account for Bim loss. Figure 9(A) depicts a Western blot showing basal p-eIF2 α (S51) levels are lower in *Bhd*^{-/-} ES cells. As depicted in Figure 9(B), though eIF2 α phosphorylation in response to amino acid (AA) deprivation over a time course is attenuated in *Bhd*^{-/-} ES cells, ATF4 and Chop accumulation is only slightly attenuated by examining nuclear extracts. As depicted in Figure 9(C), eIF2 α becomes phosphorylated normally in response to thapsigargin (TG). As depicted in Figure 9(D), overexpression of Chop in *Bhd*^{-/-} ES cells does not rescue expression of Bim protein levels as shown by Western blot compared to uninfected or vector control-infected cells (*MSCV*). Creb is shown as a nuclear extract loading control.

Figure 10, comprising Figures 10A-10G, is a series of charts and images demonstrating that *Bhd*^{-/-} ES cells exhibit phenotypes and transcriptional defects characteristic of TGF β -signaling components. Figure 10(A) depicts *Bhd*^{+/+} and *Bhd*^{-/-} ES cells cultured for 24 hours in N2B27 media, then stimulated with 10 ng/mL of activin. Bim mRNA levels were significantly induced in *Bhd*^{+/+} after stimulation but not in *Bhd*^{-/-} ES cells (* $p=0.00864$). Figure 10(B) depicts qRT-PCR analysis showing canonical TGF β target genes *Lefty1*, *PAI-1*, *Col2* and *Pitx2* are significantly downregulated in multiple *Bhd*^{-/-} ES cell clones (* $p<0.00829$). Figure 10(C) depicts X-gal stained 7x image of whole mount (a) or 20x image of sectioned (b) yolk sac taken from a e10.5 *Bhd*^{+/-} embryo exhibiting high *Bhd* expression in visceral endoderm of the yolk sac. Figure 10(D) depicts a 20X image of day 12 EBs. *Bhd*^{+/+} EBs form expanded cystic structures reminiscent of yolk sacs, while *Bhd*^{-/-} EBs fail to do so. Figure 10(E) depicts a qRT-PCR analysis on RNA from embryoid bodies, showing *Bhd*^{+/+} EBS significantly fail to express maximal levels of mature yolk sac markers α -feto-protein (*AFP*) and *trithioredoxin* (*TTR*). Averages represent maximal expression levels for each mRNA, which occurred between day 6 and 9 of EB development (* $p<1.0 \times 10^{-5}$ for both mRNAs). Figure 10(F) depicts the quantification of benzediene-positive EBs that express hemoglobin (Hb) at day 10 in methylcellulose cultures, demonstrating *Bhd*^{-/-} EBs fail to form erythroid lineages (* $p=2.32 \times 10^{-6}$). Data represents the average of three independent experiments with error bars showing standard deviation. Figure 10(G) depicts a qRT-PCR analysis of *Gata-1* and *CD34* mRNA expression in day 10 EBs in methylcellulose cultures, which is significantly reduced in *Bhd*^{-/-} EBs ($<4.49 \times 10^{-6}$).

Figure 11, comprising Figures 11A and 11B, demonstrate that *Bhd*^{-/-} ES cells have characteristics similar to TGFβ-signaling component mutants. Figure 11(A) depicts qRT-PCR analysis of additional canonical transcriptional targets of TGFβ family ligands, showing that *Lefty2*, *SnoN* and *p15* are also downregulated in *Bhd*^{-/-} ES cells. Figure 11(B) is a representative image of benzediene-stained day 10 EB cultures grown in methylcellulose, showing *Bhd*^{-/-} ES cells generate no hemoglobinized colonies.

Figure 12 depicts a series of Graphs showing mRNA levels of *Brachyury T*, *FGF-5* and *HNF4* in *Bhd*^{+/+} and *Bhd*^{-/-} EBs grown in suspension over a timecourse of 9 days, as measured by qRT-PCR. *Brachyury T*, a mesodermal marker is robustly induced in *Bhd*^{-/-} EBs, though in a delayed fashion while overall induction of ectodermal marker *Fgf-5* and early induction of endodermal marker *Hnf4* occur normally. Figure 12 demonstrates that *Bhd*^{-/-} EBs induce mesodermal, ectodermal and early endodermal markers normally.

Figure 13, comprising Figures 13A-13F, is a series of charts and images demonstrating loss of BHD results in HDAC-mediated silencing of TGFβ-transcriptional targets. Figure 13(A) depicts a Western blot of nuclear abstracts showing basal or (Figure 13(B)) induced levels after 30 minutes of 10 ng/mL activin stimulation of nuclear phospho-smad2, Smad2 and Smad4 accumulation is unaffected in *Bhd*^{-/-} ES cells. The non-specific (N.S.) band is shown as a loading control. Figure 13(C) depicts qRT-PCR using primers located in the *Lefty1* promoter on genomic DNA immunoprecipitated with acetyl-histone H3 antibody from ES cells stimulated with activin for 1 hour following 24 hour culture in N2B27. Figure 13(D) depicts a Western blot showing total levels of acetyl-H3 are unaffected in *Bhd*^{+/+} vs. *Bhd*^{-/-} cells with and without activin treatment. Figure 13(E) shows treatment of *Bhd*^{-/-} cells with trichostatin A (TSA) restores mRNA levels of *PAI-1*, *p15* and *Bim* as depicted by qRT-PCR analysis of mRNA expression levels (* p<0.0362). Figure 13(F) shows treatment of *Bhd*^{-/-} ES cells with TSA rescues the the *Bhd*^{-/-} cellular death resistance phenotype and *Bim* expression as depicted by analysis of sub-G₁ populations (above, * p=.000145) and Western blot for *Bim*, and caspase and parp cleavage (below).

Figure 14, comprising Figures 14A and 14B, illustrates the model that loss of BHD leads to increased HDAC-dependent repression of Smad-specific promoters. Figure 14(A) depicts that in the presence of BHD, Smad-dependent transcription is active, allowing transcription of anti-apoptotic genes like *Bim* and anti-proliferative genes like *p15* to promote tumor suppression. However, as depicted in Figure 14(B), when BHD is lost, Smad-

dependent transcription is repressed and apoptotic and anti-proliferative responses are lost, leading to tumor formation.

DETAILED DESCRIPTION

The present invention encompasses compositions and methods for treating BHD-based tumors, as well as sporadic cases of cystic lung disease and renal cell carcinoma, by correcting a defective apoptotic function that correlates with the absence of BHD, particularly the failure to undergo apoptosis as a result of misregulated TGF β -mediated chromatin modifications at target gene promoters. The present invention is based on the discovery that BHD-deficient cells exhibited defects in cell-intrinsic apoptosis that correlated with reduced expression of the BH3-only protein Bim. The present invention is also based on the discovery that Bim protein deficiency in *Bhd*^{-/-} cells is not a consequence of elevated mTOR or ERK activity, but results instead from reduced *Bim* transcription associated with altered TGF- β -mediated chromatin modifications. The compositions and methods of the invention are contemplated for use in a mammal, preferably, a human.

Definitions

As used herein, each of the following terms has the meaning associated with it in this section.

The articles “a” and “an” are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

The term “about” will be understood by persons of ordinary skill in the art and will vary to some extent on the context in which it is used.

“Allogeneic” refers to a graft derived from a different animal of the same species.

“Alloantigen” is an antigen that differs from an antigen expressed by the recipient.

The term “antibody” as used herein, refers to an immunoglobulin molecule, which is able to specifically bind to a specific epitope on an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoactive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and

F(ab)₂, as well as single chain antibodies and humanized antibodies (Harlow et al., 1988; Houston et al., 1988; Bird et al., 1988).

The term “antigen” or “Ag” as used herein is defined as a molecule that provokes an immune response. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. The skilled artisan will understand that any macromolecule, including virtually all proteins or peptides, can serve as an antigen. Furthermore, antigens can be derived from recombinant or genomic DNA. A skilled artisan will understand that any DNA, which comprises a nucleotide sequences or a partial nucleotide sequence encoding a protein that elicits an immune response therefore encodes an “antigen” as that term is used herein. Furthermore, one skilled in the art will understand that an antigen need not be encoded solely by a full length nucleotide sequence of a gene. It is readily apparent that the present invention includes, but is not limited to, the use of partial nucleotide sequences of more than one gene and that these nucleotide sequences are arranged in various combinations to elicit the desired immune response. Moreover, a skilled artisan will understand that an antigen need not be encoded by a “gene” at all. It is readily apparent that an antigen can be generated synthesized or can be derived from a biological sample. Such a biological sample can include, but is not limited to a tissue sample, a tumor sample, a cell or a biological fluid.

“Antisense” refers particularly to the nucleic acid sequence of the non-coding strand of a double stranded DNA molecule encoding a polypeptide, or to a sequence which is substantially homologous to the non-coding strand. As defined herein, an antisense sequence is complementary to the sequence of a double stranded DNA molecule encoding a polypeptide. It is not necessary that the antisense sequence be complementary solely to the coding portion of the coding strand of the DNA molecule. The antisense sequence may be complementary to regulatory sequences specified on the coding strand of a DNA molecule encoding a polypeptide, which regulatory sequences control expression of the coding sequences.

As used herein, the term “autologous” is meant to refer to any material derived from the same individual to which it is later to be re-introduced into the individual.

The term “DNA” as used herein is defined as deoxyribonucleic acid.

“Encoding” refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the

biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

As used herein "endogenous" refers to any material from or produced inside an organism, cell, tissue or system.

As used herein, the term "exogenous" refers to any material introduced from or produced outside an organism, cell, tissue or system.

The term "expression" as used herein is defined as the transcription and/or translation of a particular nucleotide sequence driven by its promoter.

The term "expression vector" as used herein refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules, microRNA, siRNA, ribozymes, and the like. Expression vectors can contain a variety of control sequences, which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operatively linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well.

The term "heterologous" as used herein is defined as DNA or RNA sequences or proteins that are derived from the different species.

"Homologous" as used herein, refers to the subunit sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, e.g., two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, e.g., if half (e.g., five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, e.g., 9 of 10, are matched or

homologous, the two sequences share 90% homology. By way of example, the DNA sequences 5'ATTGCC3' and 5'TATGGC3' share 50% homology.

As used herein, "homology" is used synonymously with "identity."

An "isolated nucleic acid" refers to a nucleic acid segment or fragment which has been separated from sequences which flank it in a naturally occurring state, i.e., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, i.e., the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid, i.e., RNA or DNA or proteins, which naturally accompany it in the cell. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (i.e., as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence. It also includes a nucleic acid that have been removed from its native environment and placed in another, typically artificial, environment.

As used herein, the term "modulate" is meant to refer to any change in biological state, i.e. increasing, decreasing, and the like. For example, the term "modulate" refers to the ability to regulate positively or negatively the expression or activity of BHD, Bim and/or other components of the TGF β - signaling pathway in a cell.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or an RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

The term "polynucleotide" as used herein is defined as a chain of nucleotides. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the general knowledge that nucleic acids are polynucleotides, which can be hydrolyzed into the monomeric "nucleotides." The monomeric nucleotides can be hydrolyzed into nucleosides. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any means available in the art, including, without limitation,

recombinant means, i.e., the cloning of nucleic acid sequences from a recombinant library or a cell genome, using ordinary cloning technology and PCR™, and the like, and by synthetic means.

The term “polypeptide” as used herein is defined as a chain of amino acid residues, usually having a defined sequence. As used herein the term polypeptide is mutually inclusive of the terms “peptide” and “protein”.

“Proliferation” is used herein to refer to the reproduction or multiplication of similar forms of entities, for example proliferation of a cell. That is, proliferation encompasses production of a greater number of cells, and can be measured by, among other things, simply counting the numbers of cells, measuring incorporation of ³H-thymidine into the cell, and the like.

The term “promoter” as used herein is defined as a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence.

As used herein, the term “promoter/regulatory sequence” means a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

A “constitutive” promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell under most or all physiological conditions of the cell.

An “inducible” promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell substantially only when an inducer which corresponds to the promoter is present in the cell.

A “tissue-specific” promoter is a nucleotide sequence which, when operably linked with a polynucleotide encodes or specified by a gene, causes the gene product to be produced in a cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

The term “RNA” as used herein is defined as ribonucleic acid.

The term “recombinant DNA” as used herein is defined as DNA produced by joining pieces of DNA from different sources.

The term “recombinant polypeptide” as used herein is defined as a polypeptide produced by using recombinant DNA methods.

“Fragment” as the term is used herein, is a nucleic acid sequence that differs in length (i.e., in the number of nucleotides) from the length of a reference nucleic acid sequence, but retains essential properties of the reference molecule. Similarly, a protein fragment can exist that is a part of a larger parent protein. One example of a retained essential property would be the ability of the fragment nucleic acid to hybridize to a particular target mRNA, much like the reference nucleic acid sequence, and thereby diminish expression. A fragment of a nucleic acid can be a naturally occurring or can be a fragment that is not known to occur naturally. Non-naturally occurring fragments of nucleic acids may be made by mutagenesis techniques or by direct synthesis. Preferably, the fragment is at least about 25% of the length of the reference nucleic acid sequence. More preferably, the fragment is at least about 35% of the length of the reference nucleic acid sequence. Even more preferably, the fragment is at least about 45% of the length of the reference nucleic acid sequence.

“Variant” as the term is used herein, is a nucleic acid sequence that differs in sequence from a reference nucleic acid sequence, but retains essential properties of the reference molecule. One example of a retained essential property would be the ability of the variant nucleic acid to hybridize to a particular target mRNA, much like the reference nucleic acid sequence, and thereby diminish expression. A variant of a nucleic acid can be a naturally occurring such as an allelic variant, or can be a variant that is not known to occur naturally. Non-naturally occurring variants of nucleic acids may be made by mutagenesis techniques or by direct synthesis. Preferably, the variant shares at least about 80% homology with the reference nucleic acid sequence. More preferably, the variant shares at least about 90% homology with the reference nucleic acid sequence. Even more preferably, the variant shares at least about 95% homology with the reference nucleic acid sequence.

As used herein, a “substantially purified” cell is a cell that is essentially free of other cell types. A substantially purified cell also refers to a cell which has been separated from other cell types with which it is normally associated in its naturally occurring state. In some instances, a population of substantially purified cells refers to a homogenous population of cells. In other instances, this term refers simply to cell that have been separated from the cells with which they are naturally associated in their natural state. In some embodiments, the cells are cultured *in vitro*. In other embodiments, the cells are not cultured *in vitro*.

As used herein, a “therapeutically effective amount” is the amount of a therapeutic composition sufficient to provide a beneficial effect to a mammal to which the composition is administered.

The term “transfected” or “transformed” or “transduced” as used herein refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A “transfected” or “transformed” or “transduced” cell is one which has been transfected, transformed or transduced with exogenous nucleic acid. The cell includes the primary subject cell and its progeny.

The phrase “under transcriptional control” or “operatively linked” as used herein means that the promoter is in the correct location and orientation in relation to a polynucleotide to control the initiation of transcription by RNA polymerase and expression of the polynucleotide.

A “vector” is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term “vector” includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

The term “virus” as used herein is defined as a particle consisting of nucleic acid (RNA or DNA) enclosed in a protein coat, with or without an outer lipid envelope, which is capable of replicating within a whole cell.

“Xenogeneic” refers to a graft derived from an animal of a different species.

A “conservative substitution” is the substitution of an amino acid with another amino acid with similar physical and chemical properties. In contrast, a “nonconservative substitution” is the substitution of an amino acid with another amino acid with dissimilar physical and chemical properties.

As used herein, the terms “gene” and “recombinant gene” refer to nucleic acid molecules comprising an open reading frame encoding a polypeptide.

As used herein, the term “genetically engineered” refers to a modification of the inherent genetic material of a microorganism (e.g., one or more of the deletion such as a gene knockout, addition, or mutation of one or more nucleic acid residues within the genetic

material), addition of exogenous genetic material to a microorganism (e.g., transgene, stable plasmid, integrating plasmid, naked genetic material, among other things), causing the microorganism to alter its genetic response due to external or internal signaling (e.g., environmental pressures, chemical pressures, among other things), or any combination of these or similar techniques for altering the overall genetic makeup of the organism.

"Mutants," "derivatives," and "variants" of a polypeptide (or of the DNA encoding the same) are polypeptides which may be modified or altered in one or more amino acids (or in one or more nucleotides) such that the peptide (or the nucleic acid) is not identical to the wild-type sequence, but has homology to the wild type polypeptide (or the nucleic acid).

A "mutation" of a polypeptide (or of the DNA encoding the same) is a modification or alteration of one or more amino acids (or in one or more nucleotides) such that the peptide (or nucleic acid) is not identical to the sequences recited herein, but has homology to the wild type polypeptide (or the nucleic acid).

As used herein, a "mutant form" of a gene is a gene which has been altered, either naturally or artificially, changing the base sequence of the gene, which results in a change in the amino acid sequence of an encoded polypeptide. The change in the base sequence may be of several different types, including changes of one or more bases for different bases, small deletions, and small insertions. Mutations may also include transposon insertions that lead to attenuated activity, *i.e.*, by resulting in expression of a truncated protein. By contrast, a normal form of a gene is a form commonly found in a natural population of an organism. Commonly a single form of a gene will predominate in natural populations. In general, such a gene is suitable as a normal form of a gene; however, other forms which provide similar functional characteristics may also be used as a normal gene.

The term "engineer" refers to any manipulation of a cell that result in a detectable change in the cell, wherein the manipulation includes but is not limited to inserting a polynucleotide and/or polypeptide heterologous to the cell and mutating a polynucleotide and/or polypeptide native to the cell. A polynucleotide or polypeptide is "heterologous" to a cell if it is not part of the polynucleotides and polypeptides expressed in the cell as it exists in nature, *i.e.*, it is not part of the wild-type of that cell. A polynucleotide or polypeptide is instead "native" to a cell if it is part of the polynucleotides and polypeptides expressed in the cell as it exists in nature, *i.e.*, it is part of the wild-type of that cell.

A "disease" is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate. In contrast, a "disorder" in an animal is a state of health in which the animal is

able to maintain homeostasis, but in which the animal's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal's state of health. A disease or disorder is "alleviated" if the severity of a symptom of the disease or disorder, the frequency with which such a symptom is experienced by a patient, or both, are reduced.

The term "therapeutic treatment" as used herein is a treatment administered to a subject in need thereof.

The expressions "treat" and "treatment" mean cause, or the act of causing, a postponement of development of a disorder and/or a reduction in the severity of symptoms that will or are expected to develop. The terms further include ameliorating existing symptoms, preventing additional symptoms, and ameliorating or preventing the underlying metabolic causes of symptoms.

The expression "effective amount", when used to describe therapy to an individual, refers to the amount of a compound that results in a therapeutically useful effect.

As used herein, "individual" (as in the subject of the treatment) means mammals, particularly non-human primates, e.g. apes and monkeys, and most particularly humans.

As used herein, a "BHD-based tumor" is a tumor in which BHD protein is absent, and/or is insufficient and/or ineffective in its role in TGF- β dependent apoptosis prior to therapeutic treatment of the tumor cells.

As used herein, "TGF- β " refers to transforming growth factor beta protein family.

As used herein, the "TGF- β signaling pathway" refers to the biochemical signaling mechanism involved in cellular processes such as cell growth, cell differentiation, apoptosis, cellular homeostasis and other cellular functions. The TGF- β signaling pathway functions in both the adult organisms and the developing embryos.

Description

It is demonstrated herein that tumor formation in BHD patients results, at least in part, from a lack of TGF β -mediated *Bim* transcription and consequent apoptotic resistance. BHD may also regulate other tumor suppressive target genes such as *p15* and *PAI-1* (Hannon *et al.*, 1994, Nature 371(6494):257-261; Kortlever *et al.*, 2006, Nat Cell Biol 8(8):877-884), which inhibit cell cycle and drive replicative senescence respectively, could drive tumor formation in BHD patients.

The apoptotic resistance caused by reduced Bim expression is independent of mTOR or ERK hyperactivation. It is further demonstrated that BHD is required for normal yolk sac and hematopoietic development, similar to TGF β receptor and ligand mutants, and also for normal expression of a broad spectrum of Smad target genes. Further, apoptotic resistance and Bim downregulation observed in *Bhd*^{-/-} cells is attributable to hypo-acetylation of TGF β target gene promoters.

By identifying a specific tumor suppressive mechanism for BHD in regulating TGF- β dependent apoptosis, the present invention contemplates a number of targeted therapies that have a corrective effect on apoptosis-resistant cells. Thus, the present invention encompasses compositions and methods for treating BHD-based tumors, as well as sporadic cases of cystic lung disease and renal cell carcinoma, by correcting a defective apoptotic function that correlates with the absence of BHD, particularly the failure to undergo apoptosis as a result of misregulated TGF β -mediated chromatin modifications at target gene promoters. The methods of the invention are contemplated for use in a mammal, preferably, a human.

Gene Therapy and Therapeutic Compositions

The present invention includes a gene therapy approach, and generally the use of nucleic acid compositions, for treating BHD-based tumors, as well as sporadic cases of cystic lung disease and renal cell carcinoma, and associated symptoms.

In one embodiment, the full-length BHD gene, cDNA or fragments thereof, can be cloned into a vector for use as a therapeutic agent in gene therapy. Nucleotide sequences for BHD are known in the art and can be obtained from patent publications and public databases containing nucleic acid sequences. In another embodiment, the full-length Bim gene, cDNA or fragments thereof, can be cloned into a vector for use in gene therapy. Nucleotide sequences for Bim are known in the art and can be obtained from patent publications and public databases containing nucleic acid sequences. In yet another embodiment, components of the TGF- β signaling pathway can be cloned into vectors for use in gene therapy.

Nucleotide sequences for components of the TGF- β signaling pathway are also known in the art and can be obtained from patent publications and public databases containing nucleic acid sequences.

In another exemplary embodiment, inhibitors of components of the TGF- β signaling pathway, as well as components of TGF- β dependent apoptosis, can be used to inhibit such components, such that inhibition of the component leads to the promotion of apoptosis in the

targeted cells. Exemplary inhibitors can include, by non-limiting example, small interfering RNA (siRNA), a microRNA, an antisense nucleic acid, a ribozyme, an expression vector encoding a transdominant negative mutant, an intracellular antibody, a peptide and a small molecule.

One skilled in the art will appreciate, based on the disclosure provided herein, that one way to decrease the mRNA and/or protein levels of a cell component is by reducing or inhibiting expression of the nucleic acid encoding the component. Thus, the protein level of the component in a cell can also be decreased using a molecule or compound that inhibits or reduces gene expression such as, for example, an antisense molecule or a ribozyme.

In an exemplary embodiment, the modulating sequence is an antisense nucleic acid sequence which is expressed by a plasmid vector. The antisense expressing vector is used to transfect a mammalian cell or the mammal itself, thereby causing reduced endogenous expression of a desired inhibitor of the component in the cell. However, the invention should not be construed to be limited to inhibiting expression of a component by transfection of cells with antisense molecules. Rather, the invention encompasses other methods known in the art for inhibiting expression or activity of a protein in the cell including, but not limited to, the use of a ribozyme, the expression of a non-functional component (i.e. transdominant negative mutant) and use of an intracellular antibody.

Antisense molecules and their use for inhibiting gene expression are well known in the art (*see, e.g.*, Cohen, 1989, In: Oligodeoxyribonucleotides, Antisense Inhibitors of Gene Expression, CRC Press). Antisense nucleic acids are DNA or RNA molecules that are complementary, as that term is defined elsewhere herein, to at least a portion of a specific mRNA molecule (Weintraub, 1990, Scientific American 262:40). In the cell, antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule thereby inhibiting the translation of genes.

The use of antisense methods to inhibit the translation of genes is known in the art, and is described, for example, in Marcus-Sakura (1988, Anal. Biochem. 172:289). Such antisense molecules may be provided to the cell via genetic expression using DNA encoding the antisense molecule as taught by Inoue, 1993, U.S. Patent No. 5,190,931.

Alternatively, antisense molecules of the invention may be made synthetically and then provided to the cell. Antisense oligomers of between about 10 to about 30, and more preferably about 15 nucleotides, are preferred, since they are easily synthesized and introduced into a target cell. Synthetic antisense molecules contemplated by the invention

include oligonucleotide derivatives known in the art which have improved biological activity compared to unmodified oligonucleotides (*see* U.S. Patent No. 5,023,243).

Ribozymes and their use for inhibiting gene expression are also well known in the art (*see, e.g.,* Cech et al., 1992, J. Biol. Chem. 267:17479-17482; Hampel et al., 1989, Biochemistry 28:4929-4933; Eckstein et al., International Publication No. WO 92/07065; Altman et al., U.S. Patent No. 5,168,053). Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences encoding these RNAs, molecules can be engineered to recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, 1988, J. Amer. Med. Assn. 260:3030). A major advantage of this approach is the fact that ribozymes are sequence-specific.

There are two basic types of ribozymes, namely, tetrahymena-type (Hasselhoff, 1988, Nature 334:585) and hammerhead-type. Tetrahymena-type ribozymes recognize sequences which are four bases in length, while hammerhead-type ribozymes recognize base sequences 11-18 bases in length. The longer the sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to tetrahymena-type ribozymes for inactivating specific mRNA species, and 18-base recognition sequences are preferable to shorter recognition sequences which may occur randomly within various unrelated mRNA molecules.

Ribozymes useful for inhibiting the expression of a component may be designed by incorporating target sequences into the basic ribozyme structure which are complementary to the mRNA sequence of the desired component targeted by the present invention. Ribozymes targeting the desired component may be synthesized using commercially available reagents (Applied Biosystems, Inc., Foster City, CA) or they may be genetically expressed from DNA encoding them.

In another aspect of the invention, the component can be inhibited by way of inactivating and/or sequestering the component. As such, inhibiting the effects of a component can be accomplished by using a transdominant negative mutant. Alternatively an intracellular antibody specific for the desired component, otherwise known as an antagonist to the component, may be used. In one embodiment, the antagonist is a protein and/or compound having the desirable property of interacting with a binding partner of the cytokine signaling regulator and thereby competing with the corresponding wild-type component. In another embodiment, the antagonist is a protein and/or compound having the desirable property of interacting with the component and thereby sequestering the component.

A small interfering RNA (siRNA) is an RNA molecule comprising a set of nucleotides that is targeted to a gene or polynucleotide of interest. As used herein, the term "siRNA" encompasses all forms of siRNA including, but not limited to (i) a double stranded RNA polynucleotide, (ii) a single stranded polynucleotide, and (iii) a polynucleotide of either (i) or (ii) wherein such a polynucleotide, has one, two, three, four or more nucleotide alterations or substitutions therein.

An siRNA in the form of a double stranded polynucleotide comprises about 18 base pairs, about 19 base pairs, about 20 base pairs, about 21 base pairs, about 22 base pairs, about 23 base pairs, about 24 base pairs, about 25 base pairs, about 26 base pairs, about 27 base pairs, about 28 base pairs, about 29 base pairs or about 30 base pairs in length. The double stranded siRNA capable of interfering with the expression and/or the activity of a targeted component as explained herein throughout.

A single stranded siRNA comprises a portion of an RNA polynucleotide sequence that is targeted to a gene or polynucleotide of interest. A single stranded siRNA comprises a polynucleotide of about 18 nucleotides, about 19 nucleotides, about 20 nucleotides, about 21 nucleotides, about 22 nucleotides, about 23 nucleotides, about 24 nucleotides, about 25 nucleotides, about 26 nucleotides, about 27 nucleotides, about 28 nucleotides, about 29 nucleotides or about 30 nucleotides in length. The single stranded siRNA is capable of interfering with expression and/or activity of a target polynucleotide, or a variant thereof. The single strand siRNA is also capable of annealing to a complementary sequence to result in a dsRNA that is capable of interfering with the expression and/or the activity of a component.

In yet another aspect, the siRNA comprises a polynucleotide comprising either a double stranded or a single stranded polynucleotide, wherein the siRNA has one, two, three, four or more nucleotide alterations or substitutions therein.

An siRNA polynucleotide is an RNA nucleic acid molecule that interferes with RNA activity that is generally considered to occur via a post-transcriptional gene silencing mechanism. An siRNA polynucleotide preferably comprises a double-stranded RNA (dsRNA) but is not intended to be so limited and may comprise a single-stranded RNA (see, e.g., Martinez et al., 2002 Cell 110:563-74). The siRNA polynucleotide included in the invention may comprise other naturally occurring, recombinant, or synthetic single-stranded or double-stranded polymers of nucleotides (ribonucleotides or deoxyribonucleotides or a combination of both) and/or nucleotide analogues as provided herein (e.g., an oligonucleotide

or polynucleotide or the like, typically in 5' to 3' phosphodiester linkage). Accordingly it will be appreciated that certain exemplary sequences disclosed herein as DNA sequences capable of directing the transcription of the siRNA polynucleotides are also intended to describe the corresponding RNA sequences and their complements, given the well established principles of complementary nucleotide base-pairing.

An siRNA may be transcribed using as a template a DNA (genomic, cDNA, or synthetic) that contains a promoter for an RNA polymerase promoter. For example, the promoter can be the U6 promoter or the H1 RNA polymerase III promoter. Alternatively, the siRNA may be a synthetically derived RNA molecule. In certain embodiments, the siRNA polynucleotide may have blunt ends. In certain other embodiments, at least one strand of the siRNA polynucleotide has at least one, and preferably two nucleotides that "overhang" (i.e., that do not base pair with a complementary base in the opposing strand) at the 3' end of either strand of the siRNA polynucleotide. In a preferred embodiment of the invention, each strand of the siRNA polynucleotide duplex has a two-nucleotide overhang at the 3' end. The two-nucleotide overhang is preferably a thymidine dinucleotide (TT) but may also comprise other bases, for example, a TC dinucleotide or a TG dinucleotide, or any other dinucleotide. The overhang dinucleotide may also be complementary to the two nucleotides at the 5' end of the sequence of the polynucleotide that is targeted for interference. For a discussion of 3' ends of siRNA polynucleotides see, e.g., WO 01/75164.

Preferred siRNA polynucleotides comprise double-stranded polynucleotides of about 18-30 nucleotide base pairs, preferably about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, or about 27 base pairs, and in other preferred embodiments about 19, about 20, about 21, about 22 or about 23 base pairs, or about 27 base pairs, whereby the use of "about" indicates that in certain embodiments and under certain conditions the processive cleavage steps that may give rise to functional siRNA polynucleotides that are capable of interfering with expression of a selected polypeptide may not be absolutely efficient. Hence, siRNA polynucleotides, may include one or more siRNA polynucleotide molecules that may differ (e.g., by nucleotide insertion or deletion) in length by one, two, three, four or more base pairs as a consequence of the variability in processing, in biosynthesis, or in artificial synthesis of the siRNA. The siRNA polynucleotide of the present invention may also comprise a polynucleotide sequence that exhibits variability by differing (e.g., by nucleotide substitution, including transition or transversion) at one, two, three or four nucleotides from a particular sequence. These differences can occur at any of the nucleotide positions of a particular siRNA polynucleotide sequence, depending on the

length of the molecule, whether situated in a sense or in an antisense strand of the double-stranded polynucleotide. The nucleotide difference may be found on one strand of a double-stranded polynucleotide, where the complementary nucleotide with which the substitute nucleotide would typically form hydrogen bond base pairing, may not necessarily be correspondingly substituted. In preferred embodiments, the siRNA polynucleotides are homogeneous with respect to a specific nucleotide sequence.

Polynucleotides that comprise the siRNA polynucleotides of the present invention may in certain embodiments be derived from a single-stranded polynucleotide that comprises a single-stranded oligonucleotide fragment (e.g., of about 18-30 nucleotides) and its reverse complement, typically separated by a spacer sequence. According to certain such embodiments, cleavage of the spacer provides the single-stranded oligonucleotide fragment and its reverse complement, such that they may anneal to form, optionally with additional processing steps that may result in addition or removal of one, two, three or more nucleotides from the 3' end and/or the 5' end of either or both strands, the double-stranded siRNA polynucleotide of the present invention. In certain embodiments the spacer is of a length that permits the fragment and its reverse complement to anneal and form a double-stranded structure (e.g., like a hairpin polynucleotide) prior to cleavage of the spacer, and optionally, subsequent processing steps that may result in addition or removal of one, two, three, four, or more nucleotides from the 3' end and/or the 5' end of either or both strands. A spacer sequence may therefore be any polynucleotide sequence as provided herein that is situated between two complementary polynucleotide sequence regions which, when annealed into a double-stranded nucleic acid, result in an siRNA polynucleotide. Preferably, the spacer sequence comprises at least 4 nucleotides. In certain embodiments, the spacer may comprise 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21-25, 26-30, 31-40, 41-50, 51-70, 71-90, 91-110, 111-150, 151-200 or more nucleotides. Examples of siRNA polynucleotides derived from a single nucleotide strand comprising two complementary nucleotide sequences separated by a spacer have been described (e.g., Brummelkamp et al., 2002 *Science* 296:550; Paddison et al., 2002 *Genes Develop.* 16:948; Paul et al., 2002 *Nat. Biotechnol.* 20:505-508; Grabarek et al., 2003 *BioTechniques* 34:734-44).

Polynucleotide variants may contain one or more substitutions, additions, deletions, and/or insertions such that the activity of the siRNA polynucleotide is not substantially diminished. The effect of any such alterations in nucleotide content on the activity of the siRNA polynucleotide may generally be assessed as described elsewhere herein. Variants preferably exhibit at least about 75%, 78%, 80%, 85%, 87%, 88% or 89% identity and more

preferably at least about 90%, 92%, 95%, 96%, or 97% identity to a portion of a polynucleotide sequence that encodes a native protein component. The percent identity may be readily determined by comparing sequences of the polynucleotides to the corresponding portion of the target polynucleotide, using any method including using computer algorithms well known to those having ordinary skill in the art. These include the Align or the BLAST algorithm (Altschul, 1991 J. Mol. Biol. 219:555-565; Henikoff and Henikoff, 1992, Proc. Natl. Acad. Sci. USA 89:10915-10919).

Certain siRNA polynucleotide variants can be substantially homologous to a portion of a polynucleotide encoding a target polypeptide. Single-stranded polynucleotides derived from these polynucleotide variants are capable of hybridizing under moderately stringent conditions to a naturally occurring DNA or RNA sequence encoding the target polypeptide. An siRNA polynucleotide that detectably hybridizes to the polynucleotide sequence encoding the target polypeptide under moderately stringent conditions may have a nucleotide sequence that includes at least 10 consecutive nucleotides, more preferably 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 consecutive nucleotides that are complementary to a particular target polynucleotide. In certain preferred embodiments, such an siRNA sequence (or its complement) will be unique to a single particular polynucleotide encoding the target polypeptide for which interference with expression is desired. In certain other embodiments, the sequence (or its complement) may be shared by two or more related polynucleotides encoding the target polypeptide for which interference with polypeptide expression is desired.

Suitable moderate stringent conditions include, for example, pre-washing the polynucleotide in a solution of 5X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing the polynucleotide at 50°C-70°C, 5X SSC for 1-16 hours (e.g., overnight); followed by washing the polynucleotide once or twice at 22-65°C for 20-40 minutes with one or more each of 2X, 0.5X and 0.2X SSC containing 0.05-0.1% SDS. For additional stringency, hybridization conditions may include an additional wash in 0.1X SSC and 0.1% SDS at 50-60°C for 15-40 minutes. Those of ordinary skill in the art will understand that, variations in stringency of hybridization conditions may be achieved by altering the time, temperature, and/or concentration of the solutions used for the pre-hybridization, hybridization, and wash steps. Suitable conditions may also depend in part on the particular nucleotide sequences of the probe used, and of the blotted, proband nucleic acid sample. Accordingly, it will be appreciated that suitably stringent conditions can be readily selected, without undue experimentation, when a desired selectivity of the polynucleotide is identified, based on its

ability to hybridize to one or more certain proband sequences while not hybridizing to certain other proband sequences.

Sequence specific siRNA polynucleotides of the present invention may be designed using one or more of several criteria. For example, to design an siRNA polynucleotide that has about 21 consecutive nucleotides identical to a sequence encoding a polypeptide of interest, the open reading frame of the polynucleotide sequence may be scanned for about 21-base sequences length that have one or more of the following characteristics: (1) an A+T/G+C ratio of approximately 1:1 but no greater than 2:1 or 1:2; (2) an AA dinucleotide or a CA dinucleotide at the 5' end; (3) an internal hairpin loop melting temperature less than 55°C; (4) a homodimer melting temperature of less than 37°C (melting temperature calculations as described in (3) and (4) can be determined using computer software known to those skilled in the art); (5) a sequence of at least 16 consecutive nucleotides not identified as being present in any other known polynucleotide sequence. Alternatively, an siRNA polynucleotide sequence may be designed and chosen using a computer software available commercially from various vendors, e.g., OligoEngine.TM. (Seattle, Wash.); Dharmacon, Inc. (Lafayette, Colo.); Ambion Inc. (Austin, Tex.); and QIAGEN, Inc. (Valencia, Calif.)). See also Elbashir et al., 2000 *Genes & Development* 15:188-200; Elbashir et al., 2001 *Nature* 411:494-98. The siRNA polynucleotide may then be tested for the ability to interfere with the expression of the target polypeptide according to methods known in the art and described elsewhere herein. The determination of the effectiveness of an siRNA polynucleotide includes not only consideration of its ability to interfere with the expression of the target polypeptide, but also whether the siRNA polynucleotide is toxic to the host cell. For example, a desirable siRNA would exhibit an RNA interference activity and would also not exhibit an unwanted biological consequence.

Based on the present disclosure, it should be appreciated that the siRNAs may effect silencing of the target polypeptide expression to different degrees. The siRNAs thus must first be tested for their effectiveness. Selection of siRNAs are made therefrom based on the ability of a given siRNA to interfere with or modulate the expression of the target polypeptide. Accordingly, identification of specific siRNA polynucleotide sequences that are capable of interfering with expression of a desired target polypeptide requires production and testing of each siRNA.

One skilled in the art will readily appreciate that as a result of the degeneracy of the genetic code, many different nucleotide sequences may encode the same polypeptide. That is, an amino acid may be encoded by one of several different codons, and a person skilled in

the art can readily determine that while one particular nucleotide sequence may differ from another, the polynucleotides may in fact encode polypeptides with identical amino acid sequences. As such, polynucleotides that vary due to differences in codon usage are specifically contemplated by the present invention.

Polynucleotides of the siRNA may be prepared using any of a variety of techniques, which are useful for the preparation of specifically desired siRNA polynucleotides. For example, a polynucleotide may be amplified from a cDNA prepared from a suitable cell or tissue type. Such a polynucleotide may be amplified via polymerase chain reaction (PCR). Using this approach, sequence-specific primers are designed based on the sequences provided herein, and may be purchased or synthesized directly. An amplified portion of the primer may be used to isolate a full-length gene, or a desired portion thereof, from a suitable DNA library using well known techniques. A library (cDNA or genomic) is screened using one or more polynucleotide probes or primers suitable for amplification. Preferably, the library is size-selected to include larger polynucleotide sequences. Random primed libraries may also be preferred in order to identify 5' and other upstream regions of the genes. Genomic libraries are preferred for obtaining introns and extending 5' sequences. The siRNA polynucleotide contemplated by the present invention may also be selected from a library of siRNA polynucleotide sequences.

For hybridization techniques, a partial polynucleotide sequence may be labeled (e.g., by nick-translation or end-labeling with ³²P) using well known techniques. A bacterial or bacteriophage library may then be screened by hybridization to filters containing denatured bacterial colonies (or lawns containing phage plaques) with the labeled probe (see, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 2001). Hybridizing colonies or plaques are selected and expanded, and the DNA is isolated for further analysis.

Alternatively, numerous amplification techniques are known in the art for obtaining a full-length coding sequence from a partial cDNA sequence. Within such techniques, amplification is generally performed via PCR. One such technique is known as "rapid amplification of cDNA ends" or RACE (see, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 2001). siRNA polynucleotides may generally be prepared by any method known in the art, including, for example, solid phase chemical synthesis. Modifications in a polynucleotide sequence may also be introduced using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis. Further, siRNAs may be chemically

modified or conjugated with other molecules to improve their stability and/or delivery properties. Included as one aspect of the invention are siRNAs as described herein, wherein one or more ribose sugars has been removed therefrom.

Alternatively, siRNA polynucleotide molecules may be generated by *in vitro* or *in vivo* transcription of suitable DNA sequences (e.g., polynucleotide sequences encoding a target polypeptide, or a desired portion thereof), provided that the DNA is incorporated into a vector with a suitable RNA polymerase promoter (such as for example, T7, U6, H1, or SP6 although other promoters may be equally useful). In addition, an siRNA polynucleotide may be administered to a mammal, as may be a DNA sequence (e.g., a recombinant nucleic acid construct as provided herein) that supports transcription (and optionally appropriate processing steps) such that a desired siRNA is generated *in vivo*.

In one embodiment, an siRNA polynucleotide, wherein the siRNA polynucleotide is capable of interfering with expression of a target polypeptide can be used to generate a silenced cell for the targeted polypeptide. Any siRNA polynucleotide that, when contacted with a biological source for a period of time, results in a significant decrease in the expression of the target polypeptide is included in the invention. Preferably the decrease is greater than about 10%, more preferably greater than about 20%, more preferably greater than about 30%, more preferably greater than about 40%, about 50%, about 60%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95% or about 98% relative to the expression level of the target polypeptide detected in the absence of the siRNA.

In another embodiment, the siRNA polynucleotide that, when contacted with a biological source for a period of time, results in a significant decrease in the expression of the target polypeptide. Preferably the decrease is about 10%-20%, more preferably about 20%-30%, more preferably about 30%-40%, more preferably about 40%-50%, more preferably about 50%-60%, more preferably about 60%-70%, more preferably about 70%-80%, more preferably about 80%-90%, more preferably about 90%-95%, more preferably about 95%-98% relative to the expression level of the target polypeptide detected in the absence of the siRNA.

In yet another embodiment, the siRNA polynucleotide that, when contacted with a biological source for a period of time, results in a significant decrease in the expression of the target polypeptide. Preferably the decrease is about 10% or more, more preferably about 20% or more, more preferably about 30% or more, more preferably about 40% or more, more preferably about 50% or more, more preferably about 60% or more, more preferably about 70% or more, more preferably about 80% or more, more preferably about 90% or more, more

preferably about 95 % or more, more preferably about 98% or more relative to the expression level of the target polypeptide detected in the absence of the siRNA.

Following the generation of the siRNA polynucleotide, a skilled artisan will understand that the siRNA polynucleotide will have certain characteristics that can be modified to improve the siRNA as a therapeutic compound. Therefore, the siRNA polynucleotide may be further designed to resist degradation by modifying it to include phosphorothioate, or other linkages, methylphosphonate, sulfone, sulfate, ketyl, phosphorodithioate, phosphoramidate, phosphate esters, and the like (see, e.g., Agrwal et al., 1987 Tetrahedron Lett. 28:3539-3542; Stec et al., 1985 Tetrahedron Lett. 26:2191-2194; Moody et al., 1989 Nucleic Acids Res. 12:4769-4782; Eckstein, 1989 Trends Biol. Sci. 14:97-100; Stein, In: Oligodeoxynucleotides. Antisense Inhibitors of Gene Expression, Cohen, ed., Macmillan Press, London, pp. 97-117 (1989)).

Any polynucleotide may be further modified to increase its stability *in vivo*. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends; the use of phosphorothioate or 2' O-methyl rather than phosphodiester linkages in the backbone; and/or the inclusion of nontraditional bases such as inosine, queosine, and wybutosine and the like, as well as acetyl- methyl-, thio- and other modified forms of adenine, cytidine, guanine, thymine, and uridine.

In one exemplary embodiment, retroviruses can be used as a vector for gene therapy, for a high efficiency of infection and stable integration and expression (Orkin et al., Prog. Med. Genet. 7:130-142, 1988). In further embodiments, other viral transfection systems may be utilized, including (without limitation) adenovirus, adeno-associated virus (AAV) (McLaughlin et al., J. Virol. 62:1963-1973, 1988), Vaccinia virus (Moss et al., Annu. Rev. Immunol. 5:305-324, 1987), Bovine Papilloma virus (Rasmussen et al., Methods Enzymol. 139:642-654, 1987) or members of the herpesvirus group such as Epstein-Barr virus (Margolskee et al., Mol. Cell. Biol. 8:2837-2847, 1988).

In another embodiment, techniques including RNA-DNA hybrid oligonucleotides, as described by Cole-Strauss, et al. (Science 273:1386-1389, 1996) can be used. Such techniques may allow for site-specific integration of cloned sequences, thereby permitting accurately targeted gene replacement.

In another embodiment, non-infectious methods of delivery can be used, such as lipidic and liposome-mediated gene delivery for transfection with various genes (for reviews, see Templeton and Lasic, Mol. Biotechnol. 11:175-180, 1999; Lee and Huang, Crit. Rev. Ther. Drug Carrier Syst. 14:173-206; and Cooper, Semin. Oncol. 23:172-187, 1996). For

example, cationic liposomes have been analyzed for their ability to transfect monocytic leukemia cells, and shown to be a viable alternative to using viral vectors (de Lima et al., Mol. Membr. Biol. 16:103-109, 1999). Such cationic liposomes can also be targeted to specific cells through the inclusion of, for instance, monoclonal antibodies or other appropriate targeting ligands (Kao et al., Cancer Gene Ther. 3:250-256, 1996).

The present invention also includes compositions including therapeutic agents that promote and/or induce apoptosis in BHD-based tumors, cystic lung disease and renal cell carcinoma, and associated disease. In one exemplary embodiment, the compositions include a therapeutically effective amount of a BH3-only peptide or protein, or a BH3-only mimetic. BH3-only proteins connect various stress stimuli to the Bcl-2-family-regulated, common mechanism of apoptosis. Non-limiting examples of BH3-only peptides or proteins can include BID, BIM, BAD and/or NOXA, or fragments thereof, while non-limiting examples of BH3-only mimetics can include ABT-737.

In another embodiment, the compositions can include a therapeutically effective amount of a histone deacetylase (HDAC) inhibitor. HDACs function as part of large multiprotein complexes, which are tethered to the promoter and repress transcription, thereby reducing uncontrolled cell proliferation. By non-limiting example, HDAC inhibitors of the present invention include Trichostatin and Vorinostat.

In yet another exemplary embodiment, the compositions may include autophagy inhibitors, such as chloroquine and 3-MA.

Therapeutic applications

The present invention is based, in part, on the novel discovery that tumor formation in BHD patients results, at least in part, from a lack of TGF β -mediated *Bim* transcription and consequent apoptotic resistance. Particularly, apoptotic resistance and Bim downregulation is attributable to hypo-acetylation of TGF β target gene promoters.

As demonstrated by the data disclosed herein, the present invention contemplates a number of targeted therapies that can be used as a treatment for a BHD-based tumor in a mammal. In all instances, whether treating or diagnosing a BHD-based tumor, the most preferred mammal is a human.

One skilled in the art would appreciate, based on the present disclosure, that promoting and/or inducing TGF- β dependent apoptosis provides an important and novel

therapeutic for the treatment of BHD-based tumors, as well as sporadic cases of cystic lung disease and renal cell carcinoma.

A therapeutic composition, as described above, is administered to a mammal, thereby promoting or inducing apoptosis in the targeted cells and providing a therapeutic benefit. The skilled artisan would appreciate, based upon the disclosure provided herein, that the therapeutic compositions can promote or induce apoptosis using a wide range of techniques known or to be developed in the future. That is, the invention encompasses promoting and/or inducing TGF- β dependent apoptosis in a mammal, and thereby preventing the progression and invasiveness of a BHD-based tumor. The present invention discloses methods for promoting and/or inducing TGF- β dependent apoptosis in a mammal, e.g. BH3-only molecules, HDAC inhibitors, autophagy inhibitors, and expressing or administering a gene therapy vectors encoding BHD, Bim or a component of the TGF- β signaling pathway. This is because, as demonstrated by the data disclosed herein, affecting TGF- β dependent apoptosis mediates a variety of effects, including, but not limited to decreased tumor size and increased survival time in mammals afflicted with BHD-based tumors, and thereby provides a novel and powerful therapeutic.

Ex vivo procedures are well known in the art and are discussed more fully below. Briefly, cells are isolated from a mammal (preferably a human) and modified to promote and/or induce TGF- β dependent apoptosis according to the methods of the invention. For example, the cell is modified to have a component of BHD or Bim activated, expressed or upregulated, a component of the TGF- β signaling pathway activated or inactivated, or any combinations thereof. The heightened cell can be administered to a mammalian recipient to provide a therapeutic benefit. The mammalian recipient may be a human and the cell so modified can be autologous with respect to the recipient. Alternatively, the cells can be allogeneic, syngeneic or xenogeneic with respect to the recipient. The procedure for *ex vivo* expansion is known and understood by those skilled in the art, and therefore the present invention is not limited to any particular method of *ex vivo* expansion of the cells.

In addition to using a cell-based therapy in terms of *ex vivo* therapy, the present invention also provides compositions and methods for *in vivo* therapy to promote and/or induce TGF- β dependent apoptosis. With respect to *in vivo* therapy, the present invention provides a component of BHD or Bim activated, expressed or upregulated, a component of the TGF- β signaling pathway activated or inactivated, or any combinations thereof. As such, the cell-based therapy used for *in vivo* immunization comprises an inhibitor component, an

activator component, or any combination thereof, wherein the cell-based therapy is able to promote and/or induce TGF- β dependent apoptosis.

One skilled in the art recognizes that different methods of delivery may be utilized to administer a vector into a cell in gene therapy. Examples include: (1) methods utilizing physical means, such as electroporation (electricity), a gene gun (physical force) or applying large volumes of a liquid (pressure); and (2) methods wherein said vector is complexed to another entity, such as a nanoparticle including but not limited to a liposome, an aggregated protein or a transporter molecule.

Cells containing the desired nucleic acid may also contain a suicide gene i.e., a gene which encodes a product that can be used to destroy the cell. In many gene therapy situations, it is desirable to be able to express a gene for therapeutic purposes in a host, cell but also to have the capacity to destroy the host cell at will. The nucleic acid sequence corresponding to the inhibitor and/or activator of the invention can be linked to a suicide gene, whose expression is not activated in the absence of a suicide gene activator compound. When death of the cell in which both the inhibitor/activator and the suicide gene have been introduced is desired, the suicide gene activator compound is administered to the cell thereby activating expression of the suicide gene and killing the cell. Examples of suicide gene/prodrug combinations which may be used are herpes simplex virus-thymidine kinase (HSV-tk) and ganciclovir, acyclovir; oxidoreductase and cycloheximide; cytosine deaminase and 5-fluorocytosine; thymidine kinase thymidilate kinase (Tdk::Tmk) and AZT; and deoxycytidine kinase and cytosine arabinoside.

In another embodiment, the compounds of the present invention may be used in combination with existing therapeutic agents used to treat BHD syndrome, particularly BHD-based tumors, as well as sporadic cases of cystic lung disease and renal cell carcinoma. In some instances, the compounds of the invention may be used in combination with these therapeutic agents to enhance the therapeutic effect of the therapeutic agent.

In order to evaluate potential therapeutic efficacy of the compounds of the invention in combination with the therapeutics described elsewhere herein, these combinations may be tested for therapeutic activity according to methods known in the art.

In some embodiments, an effective amount of a compound of the invention and an existing therapeutic agent is a synergistic amount. As used herein, a "synergistic combination" or a "synergistic amount" of a compound of the invention and an existing therapeutic agent is a combination or amount that is more effective in the therapeutic or prophylactic treatment of a disease than the incremental improvement in treatment outcome

that could be predicted or expected from a merely additive combination of (i) the therapeutic or prophylactic benefit of the compound of the invention when administered at that same dosage as a monotherapy and (ii) the therapeutic or prophylactic benefit of the therapeutic agent when administered at the same dosage as a monotherapy.

Dosage and Formulation (Pharmaceutical compositions)

The present invention envisions treating a disease, such as cancer, cystic lung disease and the like, in a mammal by the administration of a therapeutic agent, as described herein. Administration of the therapeutic agent in accordance with the present invention may be continuous or intermittent, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of the agents of the invention may be essentially continuous over a preselected period of time or may be in a series of spaced doses. Both local and systemic administration is contemplated. The amount administered will vary depending on various factors including, but not limited to, the composition chosen, the particular disease, the weight, the physical condition, and the age of the mammal, and whether prevention or treatment is to be achieved. Such factors can be readily determined by the clinician employing animal models or other test systems which are well known to the art. Pharmaceutical formulations, dosages and routes of administration for nucleic acids are generally disclosed, for example, in Felgner et al., supra.

One or more suitable unit dosage forms having the therapeutic agent(s) of the invention, which, as discussed below, may optionally be formulated for sustained release (for example using microencapsulation, see WO 94/07529, and U.S. Pat. No. 4,962,091 the disclosures of which are incorporated by reference herein), can be administered by a variety of routes including parenteral, including by intravenous and intramuscular routes, as well as by direct injection into the diseased tissue. For example, the therapeutic agent may be directly injected into the tumor. The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods well known to pharmacy. Such methods may include the step of bringing into association the therapeutic agent with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the product into the desired delivery system.

When the therapeutic agents of the invention are prepared for administration, they are preferably combined with a pharmaceutically acceptable carrier, diluent or excipient to form

a pharmaceutical formulation, or unit dosage form. The total active ingredients in such formulations include from 0.1 to 99.9% by weight of the formulation. A "pharmaceutically acceptable" is a carrier, diluent, excipient, and/or salt that is compatible with the other ingredients of the formulation, and not deleterious to the recipient thereof. The active ingredient for administration may be present as a powder or as granules; as a solution, a suspension or an emulsion.

Pharmaceutical formulations containing the therapeutic agents of the invention can be prepared by procedures known in the art using well known and readily available ingredients. The therapeutic agents of the invention can also be formulated as solutions appropriate for parenteral administration, for instance by intramuscular, subcutaneous or intravenous routes.

The pharmaceutical formulations of the therapeutic agents of the invention can also take the form of an aqueous or anhydrous solution or dispersion, or alternatively the form of an emulsion or suspension.

Thus, the therapeutic agent may be formulated for parenteral administration (e.g., by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampules, pre-filled syringes, small volume infusion containers or in multi-dose containers with an added preservative. The active ingredients may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredients may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

It should be appreciated that the unit content of active ingredient or ingredients contained in an individual aerosol dose of each dosage form need not in itself constitute an effective amount for treating the particular indication or disease since the necessary effective amount can be reached by administration of a plurality of dosage units. Moreover, the effective amount may be achieved using less than the dose in the dosage form, either individually, or in a series of administrations.

The pharmaceutical formulations of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are well-known in the art. Specific non-limiting examples of the carriers and/or diluents that are useful in the pharmaceutical formulations of the present invention include water and physiologically acceptable buffered saline solutions, such as phosphate buffered saline solutions pH 7.0-8.0.

The active ingredients of this invention, including compounds, expression vectors, transduced cells, polynucleotides and polypeptides, can be formulated and administered to treat a variety of disease states by any means that produces contact of the active ingredient with the agent's site of action in the body of the organism. They can be administered by any conventional means available for use in conjunction with pharmaceuticals, either as individual therapeutic active ingredients or in a combination of therapeutic active ingredients. They can be administered alone, but are generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

In general, water, suitable oil, saline, aqueous dextrose (glucose), and related sugar solutions and glycols such as propylene glycol or polyethylene glycols are suitable carriers for parenteral solutions. Solutions for parenteral administration contain the active ingredient, suitable stabilizing agents and, if necessary, buffer substances. Antioxidizing agents such as sodium bisulfate, sodium sulfite or ascorbic acid, either alone or combined, are suitable stabilizing agents. Also used are citric acid and its salts and sodium Ethylenediaminetetraacetic acid (EDTA). In addition, parenteral solutions can contain preservatives such as benzalkonium chloride, methyl- or propyl-paraben and chlorobutanol. Suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences, a standard reference text in this field.

The active ingredients of the invention may be formulated to be suspended in a pharmaceutically acceptable composition suitable for use in mammals and in particular, in humans. Such formulations include the use of adjuvants such as muramyl dipeptide derivatives (MDP) or analogs that are described in U.S. Patent Nos. 4,082,735; 4,082,736; 4,101,536; 4,185,089; 4,235,771; and 4,406,890. Other adjuvants, which are useful, include alum (Pierce Chemical Co.), lipid A, trehalose dimycolate and dimethyldioctadecylammonium bromide (DDA), Freund's adjuvant, and IL-12. Other components may include a polyoxypropylene-polyoxyethylene block polymer (Pluronic®), a non-ionic surfactant, and a metabolizable oil such as squalene (U.S. Patent No. 4,606,918).

Additionally, standard pharmaceutical methods can be employed to control the duration of action. These are well known in the art and include control release preparations and can include appropriate macromolecules, for example polymers, polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methyl cellulose, carboxymethyl cellulose or protamine sulfate. The concentration of macromolecules as well as the methods of incorporation can be adjusted in order to control release. Additionally, the agent can be

incorporated into particles of polymeric materials such as polyesters, polyamino acids, hydrogels, poly (lactic acid) or ethylenevinylacetate copolymers. In addition to being incorporated, these agents can also be used to trap the compound in microcapsules.

Accordingly, the pharmaceutical composition of the present invention may be delivered via various routes and to various sites in an mammal body to achieve a particular effect (see, e.g., Rosenfeld et al., 1991; Rosenfeld et al., 1991a; Jaffe et al., *supra*; Berkner, *supra*). One skilled in the art will recognize that although more than one route can be used for administration, a particular route can provide a more immediate and more effective reaction than another route. Local or systemic delivery can be accomplished by administration comprising application or instillation of the formulation into body cavities, inhalation or insufflation of an aerosol, or by parenteral introduction, comprising intramuscular, intravenous, peritoneal, subcutaneous, intradermal, as well as topical administration.

The active ingredients of the present invention can be provided in unit dosage form wherein each dosage unit, e.g., a teaspoonful, tablet, solution, or suppository, contains a predetermined amount of the composition, alone or in appropriate combination with other active agents. The term "unit dosage form" as used herein refers to physically discrete units suitable as unitary dosages for human and mammal subjects, each unit containing a predetermined quantity of the compositions of the present invention, alone or in combination with other active agents, calculated in an amount sufficient to produce the desired effect, in association with a pharmaceutically acceptable diluent, carrier, or vehicle, where appropriate. The specifications for the unit dosage forms of the present invention depend on the particular effect to be achieved and the particular pharmacodynamics associated with the pharmaceutical composition in the particular host.

The methods described herein are by no means all-inclusive, and further methods to suit the specific application will be apparent to the ordinary skilled artisan. Moreover, the effective amount of the compositions can be further approximated through analogy to compounds known to exert the desired effect.

EXAMPLES

The invention is now described with reference to the following Examples. These Examples are provided for the purpose of illustration only, and the invention is not limited to these Examples, but rather encompasses all variations which are evident as a result of the teachings provided herein.

The experiments disclosed herein were conducted to explore the cellular and molecular mechanisms of the protein encoded by *BHD*, and its role in BHD Syndrome and tumor development. The results disclosed herein demonstrate that the major biological outcome of BHD deficiency is resistance to cell-intrinsic apoptosis. Given that evasion of cell death is one of the “essential alterations” that dictate all tumor development (Hanahan *et al.*, 2000, *Cell* 100(1):57-70), this property represents one of BHD’s major tumor suppressive activities. It has also been demonstrated herein that BHD’s role in the apoptotic response is dependent on expression of the BH3-only protein Bim, which is lost in BHD-related tumors from both human patients and mouse models. Intriguingly, previous studies have shown that both BHD and Bim are expressed in renal proximal tubules, where cysts are thought to arise in the *Bhd* gene trap knock-out model, and Bim expression is lost in a majority of human clear cell RCCs and modulates apoptotic sensitivity in RCC cell lines *in vitro* (O’Reilly *et al.*, 2000, *Am J Pathol* 157(2):449-461; Zantl *et al.*, 2007, *Oncogene* 26(49):7038-7048; Guo *et al.*, 2009, *Oncogene* 28(16):1864-1874; Hudon *et al.*, 2009, *J Med Genet.* [Epub ahead of print]). Additionally, Bim and BHD have each been shown to play roles in polycystic kidney disease in independent mouse knock-out models (Bouillet *et al.*, 2001, *Dev Cell* 1(5):645-653; Baba *et al.*, 2008, *J Natl Cancer Inst* 100(2):140-154).

Having established Bim as a major effector of BHD-dependent apoptosis, it was next investigated what signaling and transcriptional networks might mediate this effect. The mTORC1, mTORC2 and ERK pathways were eliminated as being essential, since inhibition of these hyperactivated pathways in *Bhd*^{-/-} cells did not rescue Bim expression levels, nor the death resistance phenotype. Although the observed upregulation of the mTOR and ERK pathways as described herein is consistent with previous observations in late-stage murine cysts and tumors (Baba *et al.*, 2008, *J Natl Cancer Inst* 100(2):140-154; Hasumi *et al.*, 2009, *Proc Natl Acad Sci USA* 106(44):18722-18727), the molecular basis of this phenomenon is unclear. It is believed that chronic loss of signaling from the TGF- β pathway (or others) may result in compensatory activation of the mTOR pathway, as crosstalk between the two has been previously reported (Cobbold *et al.*, 2009, *Proc Natl Acad Sci USA* 106(29):12055-12060; Wu *et al.*, 2009, *Dev Cell* 17(1):35-48). Upregulation of the mTOR and ERK pathways observed in the studies by Baba *et al.* and Hasumi *et al.* could additionally be explained by mutational accumulation due to a selective advantage for tumor growth *in vivo*. This explanation is supported by findings in the *Bhd* gene-trap mouse model where cystic and solid lesions exhibit a variable mixture of upregulated and downregulated mTOR activation profiles, suggesting this event is not required for tumor formation but rather stochastic

(Hartman *et al.*, 2009, *Oncogene* 28(13):1594-1604; Hudon *et al.*, 2009, *J Med Genet.* [Epub ahead of print]). Collectively, these findings suggest that although some BHD-related renal lesions exhibit mTOR and ERK hyperactivation (which could contribute secondarily to tumor progression), this may not represent a direct consequence of BHD loss nor be a primary driver of tumor-promoting processes such as apoptotic resistance.

Also explored was whether BHD regulates apoptosis through interaction with the TGF β pathway, which is an especially attractive candidate pathway for several reasons. First, the BHD fly ortholog has been suggested to act as a positive effector downstream of the TGF β family member *Decapentaplegic (Dpp)* in *Drosophila* (Singh *et al.*, 2006, *Oncogene* 25(44):5933-5941). Second, multiple reports have demonstrated that TGF β signaling regulates *Bim* expression in diverse cell types (Willey *et al.*, 2003, *J Biol Chem* 278(20):18069-18077; Ramjaun *et al.*, 2007, *Oncogene* 26(7):970-981; Ramesh *et al.*, 2008, *EMBO Rep* 9(10):990-997; Yu *et al.*, 2008, *J Cell Physiol* 215(2):422-433; Houde *et al.*, 2009, *J Biol Chem* 284(35):23397-23404). Finally, TGF β signaling components appear to be downregulated in many human cancers. For example, in humans, *Smad4* germline mutations are associated with juvenile polyposis syndrome which predisposes individuals to gastrointestinal cancers, and *TGF β RI*, *TGF β RII* and *activin receptor II* are mutated or downregulated in a broad spectrum of sporadic cancers (Levy *et al.*, 2006, *Cytokine Growth Factor Rev* 17(1-2):41-58). Epigenetic silencing of genes involved in TGF β signaling have also specifically been observed in renal cell carcinomas (McRonalld *et al.*, 2009, *Mol Cancer* 8:31). Genetic ablation of these signaling components in mice have confirmed the tumor suppressive function of this pathway in models of colon, mammary and pancreatic cancer (Massague, 2008, *Cell* 134(2):215-230).

It was observed that many molecular and phenotypic defects in *Bhd*^{-/-} ES cells are similar to TGF β receptor and ligand mutants, including decreased expression of many canonical Smad targets as well as *in vitro* hematopoietic and yolk sac defects. It is also probable that the reduced size of *Bhd*^{-/-} cells is due to loss of TGF β -mediated transcriptional effects on cellular hypertrophy as the TGF β pathway has previously been reported to drive cell growth (Wu *et al.*, 2009, *Dev Cell* 17(1):35-48). Clearly, the yolk sac and hematopoietic defects observed in *Bhd*^{-/-} EBs cannot explain the early lethality of *Bhd*^{n/m} embryos. It is therefore possible that the *in vitro* EB conditions employed bypassed essential BHD functions operating early in embryogenesis, thereby revealing a role for BHD role in subsequent yolk sac and hematopoietic development. The *in vivo* developmental failure of *Bhd*^{n/m} embryos is

likely a result of failed cavitation, gastrulation and/or patterning defects characteristic of *Smad2* or the TGF β -related ligand *Nodal* mutants, which also die around e6.5 (Zhou *et al.*, 1993, Nature 361(6412):543-547; Weinstein *et al.*, 1998, Proc Natl Acad Sci USA 95(16):9378-9383).

Mechanistically, BHD appears to be required for Smad-mediated histone modifications downstream of phosphorylated-Smad nuclear accumulation, as demonstrated herein that the well-characterized *Lefty1* promoter does not become acetylated in response to activin in *Bhd*^{-/-} cells, despite normal nuclear Smad translocation. Since the HDAC inhibitor TSA rescues Smad target gene expression, it is believed that this effect is mediated through a Smad-specific, HDAC-containing, repressional complex, as depicted in Figure 7, many of which have been well-described in the literature (Luo, 2004. Curr Opin Genet Dev 14(1):65-70. Importantly, HDAC inhibitor further reversed the death resistance phenotype of *Bhd*^{-/-} cells concomitant with restored *Bim* expression, supporting the belief that failed TGF β -mediated transcriptional response of *Bhd*^{-/-} cells is responsible for the observed apoptotic defect. The data presented herein indicates that tumor formation in BHD patients results, at least in part, from a lack of TGF β -mediated *Bim* transcription and consequent apoptotic resistance. BHD may also regulate other tumor suppressive target genes such as *p15* and *PAI-1* (Hannon *et al.*, 1994, Nature 371(6494):257-261; Kortlever *et al.*, 2006, Nat Cell Biol 8(8):877-884), which inhibit cell cycle and drive replicative senescence respectively, could drive tumor formation in BHD patients.

The materials and methods employed in the experiments and examples disclosed herein are now described.

Embryonic stem cell culture and treatments: For *in vitro* studies, ES cells were adapted to gelatin, maintained in DMEM supplemented with 15% FBS, LIF, BME, non-essential amino acids and L-glutamine, split at a maximal dilution of 1:7, and all experiments were performed on low passage number cell lines. To generate *Bhd*^{-/-} and *Bhd*^{m/m} ES cell clones, *Bhd*^{+/-} cells were selected on 1 mg/mL and *Bhd*^{+/-m} on 8 mg/mL G418 for two weeks, after which clones were picked, genotyped and expanded. For serum deprivation, cells were deprived of FBS as well as LIF for 24 hours. For glucose or amino acid withdrawal, cells were either deprived of all glucose or all amino acids respectively in the presence of dialyzed serum and LIF for indicated times. For TNF α treatment, cells were cultured in 50 ng/mL TNF α in combination with 10 μ M cycloheximide for 24 hours. To assess the effect of inhibitors on cell death, 5 μ M LY294002, 10 μ M PD98059, 20nM rapamycin or 10 nM TSA

were all administered for 6 hours prior to amino acid deprivation, then readministered 24 hours later, to maintain constant concentrations until harvesting. For activin treatments, cells were cultured in N2B27 media as previously described (Ying *et al.*, 2003, Cell 115(3):281-292), then treated with 10 ng/mL recombinant activin for 30 minutes to assess Smad phosphorylation, one hour to assess acetyl-H3 by ChIP or two hours to assess *Bim* mRNA induction.

Characterization of gene-trapped ES cells and mice: *Bhd*^{+/*m*} ES cells were obtained from Bay Genomics and *Bhd*^{+/-} ES cells were obtained from the German Gene Trap Consortium, with isogenic parental *Bhd*^{+/+} lines being obtained from each respective source. Gene trap insertions were mapped by direct PCR amplification of the respective intronic-trap cassette boundary using primers in the exon just upstream from the insertion and within the 5' end of the trap cassette. Southern blots were carried out on either PvuII or BamHI-digested DNA on *Bhd*^{+/*m*} or *Bhd*^{+/-} ES cell respectively, with 5' end-directed probes being generated by PCR amplification from genomic DNA and subsequent TOPO-cloning. *Bhd*^{+/*m*} mice were generated as previously described (Hartman *et al.*, 2009, Oncogene 28(13):1594-1604) and *Bhd*^{+/*m*} intercrosses and embryo collection were performed as previously described (Covello *et al.*, 2006, Genes Dev 20(5):557-570).

X-Gal staining of tissues and embryos: X-gal staining was carried out as previously described for both cells and whole mount tissues fixed in 4% paraformaldehyde, according to the Sanger Institute gene trap protocol. X-gal stained yolk sac sections came from tissue snap-frozen in O.C.T.

RNA isolation and quantitative real-time PCR: RNA was extracted using Qiagen RNeasy columns with DNAase treatment, and cDNA was written off approximately 1 µg of RNA per reaction using the ABI one-step RT-PCR Master Mix. qRT-PCR reactions were run in triplicate on an ABI 7900HT machine for each experiment, utilizing either SYBR green or Taqman primer probe sets as noted in the Supplementary Materials. All target mRNA levels were normalized to *β-actin* or *18s* expression levels. All qRT-PCR data reflects average mRNA levels from three independent RNA extractions and reverse transcription reactions with error bars showing standard error of the mean.

Alkaline phosphatase staining: Cells were stained using the Alkaline Phosphatase Detection Kit from Millipore according to manufacturer's instructions.

Protein extraction and western blotting: Whole cell lysates were prepared using a buffer containing 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM

glycerophosphate, 50 mM NaF, 1% Triton and freshly added protease inhibitor cocktail tablets from Roche and 2 mM orthovanadate. Nuclear extracts were prepared using the Active Motif Nuclear Extraction kit, with 500 mM NaCl added after nuclease digestion followed by sonication of nuclear extracts. Lysates were run on polyacrylamide gels, transferred to nitrocellulose, blotted using standard protocols, and visualized on film using HRP-conjugated antibodies and ECL reagent. Rabbit polyclonal anti-BHD raised against the carboxy-terminal 20 amino acids of murine BHD was generated and affinity purified by Quality Control Biosystems. Caspase 3, Parp, BclXL, Puma, Bad, Bid, BMF, Bik, p-S6K1, S6K1, p-4E-BP1, 4E-BP1, p-Akt, Akt, p-FoxO, FoxO1, FoxO3, FoxO4, p-MEK, MEK, p-ERK, ERK, p-p90RSK, p90-RSK, p-Smad2 and Smad2 antibodies were all obtained from Cell Signaling Technology; Bax, Noxa, Chop and ATF4 antibodies were from Santa Cruz, and Bak antibody from Upstate Biotechnology.

Cell size measurements: Cell size was measured using a Z2 Coulter particle size analyzer. At least 10,000 cells were measured for each experiment.

Proliferation assay: 5×10^4 cells were plated in a gelatinized 12 well plate and counted using the Countess automatic counter from Invitrogen.

Sub-G₁ quantification: Cells were fixed and stained as previously described, then fluorescence was measured by flow cytometry (Riccardi *et al.*, 2006, Nat Protoc 1(3):1458-1461). At least 10,000 cells were counted for each experiment and data was analyzed using FlowJo software.

Measurement of intracellular amino acids: Cells were lysed in 4% perchloric acid containing 40 nM 6 amino-caproic acid as an internal standard and centrifuged to pellet precipitated protein which was dissolved in 1M NaOH and quantified by BCA. The supernatant was neutralized using 1M KOH and 1M KHCO₃ after which the perchlorate was allowed to precipitate on ice for one hour, then spun down. Amino acids were subsequently derivatized by *o*-phthalaldehyde and intracellular concentrations determined by column chromatography as previously described.

Patient material: Paraffin-embedded patient samples were obtained from the Pathology archives at the University of Pennsylvania and patient identifiers were removed before analysis. Protocols were approved by the University of Pennsylvania Institutional Review Board.

Immunohistochemistry: Paraffin-embedded tissue was immunostained using a rabbit polyclonal anti-Bim antibody from Novus following antigen retrieval in sodium citrate solution and developed using diaminobenzidine.

Embryoid bodies: For suspension cultures, 8×10^6 ES cells were plated in 10 cm bacterial dishes in DMEM containing 10% FBS and BME. Media was changed every other day, with EBs being split 1:2 on day 3. Methylcellulose cultures and benzidene staining were performed as previously described (Cooper *et al.*, 1974, Proc Natl Acad Sci USA 71(5):1677-1680; Adelman *et al.*, 1999, Genes Dev 13(19):2478-2483).

Northern blotting: Northern blots for miRNAs were done as previously described (Gruber *et al.*, 2009, Cell 138(2):328-339).

Chromatin Immunoprecipitation: 1×10^6 ES cells were fixed for 10 minutes in 1% formaldehyde for 10 minutes at room temperature. Crosslinking was quenched with 125 mM glycine for 5 minutes, then cells were lysed and sonicated to shear DNA for 3 minutes with on/off pulses using a Fisher Sonic Dismembrator Model 500 at 20% power. Chromatin immunoprecipitation was carried using the Imgenex QuikChIP kit according to manufacturer's instructions using a pan acetyl-H3 antibody from Millipore. Eluted protein/DNA complexes were purified by phenol/chloroform extraction and ethanol precipitation.

Statistics: All *p* values were generated using a two-tailed Student's T test.

The results of the experiments presented herein are now described.

Example 1: Homozygous mutant *Bhd* embryos die during early embryogenesis

To define the basic molecular functions of BHD, it was initially attempted to establish mouse embryonic fibroblasts (MEFs) from a gene-trap mouse model, derived from ES cells obtained from Bay Genomics. In order to fully characterize this mutant *Bhd* allele (denoted as *m*), the insertional position of the trap cassette was mapped to intron 8 of the murine *Bhd* gene, and established Southern and PCR-based genotyping assays to discriminate wild-type from the *m* allele, as depicted in Figures 1A -1C. Using multiple RT-PCR probes, it was observed that a 40-50% reduction in *Bhd* mRNA expression in *Bhd*^{+/*m*} ES cells compared to *Bhd*^{+/+} controls by qRT-PCR, as depicted in Figure 1D, and confirmed expression of the *Bhd*-*βgeo* fusion product by staining the ES cells with X-Gal, as depicted in Figure 1E.

Bhd^{+/*m*} intercrosses yielded no viable *Bhd*^{*m/m*} embryos after e7.5. Although the e6.5 embryos could not be genotyped, it was observed that approximately 25% of these were severely abnormal or partially resorbed upon dissection, as depicted in Figure 1F. As depicted in Figure 1G, cross sections of these defective embryos revealed grossly atrophied

and disorganized egg cylinders, containing distorted visceral endoderm and lack of cavitation. This phenotype is consistent with a recently-reported embryonic lethal phenotype for an independent mutant *Bhd* allele (Hasumi *et al.*, 2009, Proc Natl Acad Sci USA 106(44):18722-18727).

Example 2: Loss of BHD does not affect proliferation, but results in decreased cellular size and resistance to cell-intrinsic apoptosis

Since the early embryonic lethality of the Bay Genomics mutant allele precluded the development of differentiated cell-types, an ES cell model was generated for *in vitro* studies. To do this, an independent gene-trapped mutant *Bhd* allele from the German Gene Trap Consortium was used. This allele lacked expression of the entire *Bhd* open reading frame (ORF), in contrast to the Bay Genomics allele which could encode a truncated protein. The null allele (denoted as “-”) was characterized by mapping the trap cassette insertion to intron 1 of the murine *Bhd* gene and designing Southern and PCR-based genotyping assays to discriminate wild-type from null allele, as depicted in Figures 2A-2C. Again, *Bhd* mRNA levels were approximately 40-50% of those in *Bhd*^{+/+} cells, as shown by qRT-PCR using primers spanning multiple *Bhd* exons (Figure 2D). *β-geo* expression driven off the trap cassette was also verified in *Bhd*^{+/+} cells by X-Gal staining, as depicted in Figure 2E.

To generate homozygous *Bhd*^{-/-} cells, *Bhd*^{+/+} cells were plated in high concentrations of G418, and several independent cell lines were isolated, denoted as “1”, “2” and “3”. PCR genotyping verified genomic loss of the wild-type allele in these lines, and Western blot confirmed complete loss of BHD protein expression, as depicted in Figures 3A and 3B. To confirm that loss of BHD did not affect the undifferentiated state of *Bhd*^{-/-} ES cells, levels of Oct-4, Nanog and Sox2 pluripotency factors were analyzed by Western blot and equivalent expression in *Bhd*^{+/+}, *Bhd*^{+/-} and *Bhd*^{-/-} cell lines were observed, as depicted in Figure 4A. Phosphorylated Stat3, an additional ES cell pluripotency marker, was also unaffected in *Bhd*^{-/-} ES cells compared to *Bhd*^{+/+} cells, even at sub-saturating concentrations of leukemia-inhibitory factor (LIF) (Figure 4B). Finally, *Bhd*^{+/+}, *Bhd*^{+/-} and *Bhd*^{-/-} ES cell lines were stained for alkaline phosphatase activity, another pluripotency marker of ES cells in culture, and no differences between the three genotypes were observed, as depicted in Figure 4C. Taken together, these findings show that loss of BHD does not affect ES cell pluripotency in culture, allowing for cellular and molecular comparisons in *in vitro* studies.

To gain insight into BHD's tumor suppressive properties, *Bhd*^{+/+}, *Bhd*^{+/-} and *Bhd*^{-/-} ES cell lines were assessed for proliferation, cell growth and apoptotic responses. *Bhd*^{-/-} ES cells did not proliferate significantly faster at days 1-3 after plating, but confluent cultures analyzed at day 4 were found to contain 15-20% more cells compared to *Bhd*^{+/+} and *Bhd*^{+/-} counterparts, as depicted in Figure 3C. This difference was attributed to a 15-20% decrease in cellular volume in *Bhd*^{-/-} cells, as depicted in Figure 3D. While this reduced size phenotype is interesting and statistically significant, it appears inconsistent with a role for BHD in tumor suppression. For example, loss of the tumor suppressive cell growth regulators TSC2 and LKB1 results in the opposite phenotype (Inoki *et al.*, 2003, Cell 115(5):577-590; Shackelford *et al.*, 2009, Nat Rev Cancer 9(8):563-575).

Finally, the response of *Bhd*^{-/-} ES cells to cell-intrinsic apoptotic stimuli was assessed by starving cells of serum, glucose or amino acids for 24 hours. *Bhd*^{-/-} ES cells clearly demonstrated a clear resistance to all three apoptosis-inducing stresses, as revealed by light microscopy (depicted in Figure 3E), and confirmed by flow cytometric assessment of sub-G1 populations. In addition, cleavage of caspase-3 and parp was also significantly reduced in *Bhd*^{-/-} cells, following nutrient starvation compared to *Bhd*^{+/+} controls, as depicted in Figure 3H. This effect was specific to the intrinsic apoptotic pathway, as *Bhd*^{-/-} cells showed no differential response to the death-receptor ligand TNF α , as depicted in Figures 3F and 3G.

Thus, BHD deficiency does not confer an obvious increase in cellular proliferation or growth, but does reduce cell-intrinsic apoptosis, thereby identifying a plausible cellular role for BHD as a tumor suppressor.

Example 3: Bim expression is lost in *Bhd*^{-/-} cells and BHD-related tumors and contributes to apoptotic resistance

Apoptotic resistance of *Bhd*^{-/-} cells can either be due to increased intracellular nutrient stores, allowing them to withstand starvation, or result from a defect in the cell-intrinsic death machinery. To assess the former possibility, levels of thirteen different intracellular amino acids were evaluated by high performance liquid chromatography in *Bhd*^{+/+} and *Bhd*^{-/-} ES cells, as mutation of *S. pombe* BHD resulted in increased intracellular stores of glutamate, citrulline and ornithine (van Slegtenhorst *et al.*, 2007, J Biol Chem 282(34):24583-24590). *Bhd*^{+/+} and *Bhd*^{-/-} ES cells had comparable levels of all intracellular amino acids tested, including arginine and glutamate, suggesting that nutrient homeostasis was not playing a role in the apoptotic resistance phenotype, as depicted in Figure 5A.

To determine if the cell-intrinsic death machinery might be impaired in *Bhd*^{-/-} ES cells, protein levels of the major pro-survival and pro-apoptotic Bcl2 family members were examined, as well as several BH3-only pro-apoptotic proteins. Amongst the major Bcl2 family members, ES cells were found to most highly express Bak and BclXL, the levels of which were unaltered in *Bhd*^{-/-} ES cells. Several BH3-only proteins, however, were misregulated in *Bhd*^{-/-} ES cells compared to *Bhd*^{+/+} and *Bhd*^{+/-} controls. Both extra-long (EL) and long (L) isoforms of Bim, as well as Puma α and Bad were significantly downregulated in *Bhd*^{-/-} ES cells, whereas Bid and BMF levels were upregulated, and Bik, Noxa and Puma β were unchanged, as depicted in Figure 5B. Similar results were obtained using three independent BHD-deficient ES clones homozygous for the *Bhd*^m allele, which displayed decreased BimEL protein levels compared to *Bhd*^{+/+} and *Bhd*^{+/-m} controls, as depicted in Figures 6A and 6B. Bim regulation in subsequent experiments was focused on, given its dominant role amongst BH3-only proteins in mediating the cell-intrinsic death response and frequent loss in renal cell carcinoma (Kim *et al.*, 2006, Nat Cell Biol 8(12):1348-1358; Zantl *et al.*, 2007, Oncogene 26(49):7038-7048; Guo *et al.*, 2009, Oncogene 28(16):1864-1874). Additionally, Bim has been shown to play an important role in murine models of polycystic kidney disease (Bouillet *et al.*, 2001, Dev Cell 1(5):645-653), the most prominent phenotype observed in a kidney-specific *Bhd* knock-out mouse model (Baba *et al.*, 2008, J Natl Cancer Inst 100(2):140-154).

To test whether the apoptotic resistance of *Bhd*^{-/-} ES cells was Bim-dependent, *BimEL* or *Bhd* expression was restored by retroviral transduction in *Bhd*^{-/-} ES cells. Restoration of *BimEL* or *Bhd* expression, or treatment of cells with the BH3-only chemical mimetic ABT-737, rescued the apoptotic response of *Bhd*^{-/-} ES cells to amino acid deprivation, as assessed by FACs analysis and depicted in Figure 5C (upper), and Parp and caspase-3 cleavage (lower). Importantly, *Bhd* reconstitution was sufficient to restore Bim protein levels. Interestingly, autophagy inhibitors 3-methyl-adenine (3-MA) and chloroquine rescued the apoptotic response in *Bhd*^{-/-} cells, also depicted in Figure 5C, suggesting that *Bhd*^{-/-} cells depended on autophagy for survival under nutrient deprivation, similar to apoptosis-deficient *Bax*^{-/-}, *Bak*^{-/-} double knock-out cells (Lum *et al.*, 2005, Cell 120(2):237-248). None of the treatments described had an effect on cell viability under non-starvation conditions, suggesting their cell death effects were not a consequence of general cellular toxicity (data not shown).

To determine whether loss of BHD results Bim deficiency *in vivo*, immunohistochemistry on BHD-related tumors was performed from both human BHD patients and from aged *Bhd*^{+/-} mice. In 3/3 human-derived tumors (one fibrofolliculoma, one chromophobe-clear cell hybrid RCC, and one chromophobe RCC) and 5/5 solid murine renal tumors (4 hybrid oncocytic and one papillary mass projecting from a cyst), absence or significantly reduced Bim expression was observed, as depicted in Figure 5D. This confirms that loss of Bim expression is a common event in the *in vivo* formation of BHD-related tumors of diverse histologies, which likely contributes to the pathogenesis of BHD syndrome.

Example 4: *Bim* is transcriptionally downregulated in *Bhd*^{-/-} cells independent of mTORC1, mTORC2, ERK, or GCN2-eIF2 α pathway misregulation

Bim mRNA is regulated transcriptionally by several well-characterized signal transduction pathways, translationally by miRNAs, and on the level of protein stability (Dijkers *et al.*, 2000, Curr Biol 10(19):1201-1204; Dehan *et al.*, 2009, Mol Cell 33(1):109-116; Su *et al.*, 2009, Genes Dev 23(3):304-317). *Bim* mRNA was initially assessed and found to be downregulated by about 80% in *Bhd*^{-/-} ES cells compared to *Bhd*^{+/+} and *Bhd*^{+/-} cells, as depicted in Figure 7A. To investigate a possible additional role for enhanced proteolytic degradation of Bim, *Bhd*^{-/-} ES cells were treated with the proteasome inhibitor MG-132, but no increase in BimEL and BimL protein was observed, despite accumulation of ubiquitinated HIF-1 α under the same treatment, as depicted in Figure 8A (Dehan *et al.*, 2009, Mol Cell 33(1):109-116). Levels of *mir-19* and *mir-92*, miRNAs previously shown to regulate *Bim* translation in ES cells (Su *et al.*, 2009, Genes Dev 23(3):304-317) were also examined, but no differences between *Bhd*^{-/-} and control cells were observed. Collectively, these experiments suggest that reduced transcription may be a primary mechanism causing Bim deficiency in *Bhd*^{-/-} cells, as opposed to altered Bim protein stability or *mir-19* or *mir-92* function, as depicted in Figure 8B.

As *Bim* transcription appeared to be misregulated in *Bhd*^{-/-} ES cells, it was determined which transcriptional networks and upstream signaling pathways were involved in this misregulation. mTOR and ERK pathways have been linked to *Bim* regulation (Dijkers *et al.*, 2000, Curr Biol 10(19):1201-1204; Dehan *et al.*, 2009, Mol Cell 33(1):109-116), and a previous report demonstrated mTOR and ERK hyperactivation in late-stage cysts and tumors derived from a mutant *Bhd* mouse model (Baba *et al.*, 2008, J Natl Cancer Inst 100(2):140-154). Western blot analysis of *Bhd*^{+/+}, *Bhd*^{+/-} and *Bhd*^{-/-} ES cells revealed that *Bhd*^{-/-} ES cells

also exhibited hyper-phosphorylation of S6K1 and 4E-BP1 (indicative of increased mTORC1 activity), hyperphosphorylation of Akt and the FoxO family of proteins (indicative of mTORC2 hyperactivation) and the hyperactivation of the MEK-ERK-p90RSK cascade, as depicted in Figure 7B.

To determine if these hyperactivated pathways were playing a role in decreased *Bim* levels in *Bhd*^{-/-} cells, cells were treated with inhibitors of each of these pathways at doses that reduced signaling in *Bhd*^{-/-} cells to levels observed in *Bhd*^{+/+} cells. It was found that effective inhibition of PI3K-dependent mTORC2 activity, MEK activity, or mTORC1 activity, either singly or in combination, was insufficient to restore Bim protein levels in *Bhd*^{-/-} cells, as depicted in Figure 7C. More importantly, treatment of *Bhd*^{-/-} ES cells with the same panel of inhibitors was unable to rescue the death resistance phenotype, as assessed by sub-G1 population quantification, depicted in Figure 7D (upper), or caspase and parp cleavage following two-day amino acid starvation (lower).

Bim expression is known to be regulated by Chop, a transcription factor activated by ER stress as part of the unfolded protein response (UPR) (Puthalakath *et al.*, 2007, Cell 129(7):1337-1349). Serum and amino acid withdrawal stimulate eIF2 α kinase activity through the nutrient-sensing GCN2 pathway, which also regulates Chop activity (Berlanga *et al.*, 1999, Eur J Biochem 265(2):754-762; Harding *et al.*, 2000, Mol Cell 6(5):1099-1108). Western blot analysis revealed that basal eIF2 α phosphorylation was significantly reduced in *Bhd*^{-/-} ES cells relative to *Bhd*^{+/+} and *Bhd*^{+/-} controls (Figure 9A). Moreover, amino acid withdrawal-induced phosphorylation of eIF2 α was attenuated in *Bhd*^{-/-} cells, as depicted in Figure 9B, whereas thapsigargin had no obvious effect, as depicted in Figure 9C. Interestingly, attenuated eIF2 α phosphorylation appeared to have little effect on downstream ER stress effectors, as ATF4 and Chop accumulation were only slightly attenuated in *Bhd*^{-/-} cells under amino acid starvation, and induction of their cognate target genes *Asparagine synthetase*, *Trb3*, and *ATF3* was unaffected, as depicted in Figures 9B and 9C (other data not shown). Finally, it was demonstrated that ectopically expressed Chop did not restore Bim protein levels in *Bhd*^{-/-} ES cells, as depicted in Figure S5D. Collectively, these data suggest that although the mTOR, ERK and GCN2 pathways are detectably altered in *Bhd*^{-/-} cells, these alterations do contribute significantly to the observed defects in Bim expression and apoptotic response.

Example 5: *Bhd*^{-/-} ES cells show many phenotypic and molecular defects characteristic of TGFβ-pathway mutants

Having excluded the mTOR, ERK and GCN2 pathways from contributing to the death resistance and *Bim* downregulation observed in *Bhd*^{-/-} ES cells, the TGFβ pathway was considered, since many reports had previously shown it to regulate *Bim* transcription in diverse cell types (Wildevy *et al.*, 2003, J Biol Chem 278(20):18069-18077; Ramjaun *et al.*, 2007, Oncogene 26(7):970-981; Ramesh *et al.*, 2008, EMBO Rep 9(10):990-997; Yu *et al.*, 2008, J Cell Physiol 215(2):422-433; Houde *et al.*, 2009, J Biol Chem 284(35):23397-23404) and Smad downstream effectors regulate a large number of target genes in ES cells (Ogawa *et al.*, 2007, J Cell Sci 120(Pt 1):55-65; Guzman-Ayala *et al.*, 2009, PLoS One 4(1):e4268). First, it was verified that the TGFβ ligand family regulates *Bim* mRNA in ES cells by treating them with activin, a TGFβ family ligand to which ES cells are particularly responsive. A significant increase in *Bim* mRNA within 2 hours in *Bhd*^{+/+} ES cells was observed, which did not occur in *Bhd*^{-/-} ES cells. To ascertain whether TGFβ-mediated transcriptional downregulation was a general phenomenon in *Bhd*^{-/-} ES cells, the basal mRNA levels of a number of several well-described, canonical TGFβ transcriptional targets, including *Lefty1*, *PAI-1*, *Cola2*, *Pitx2*, *Lefty2*, *SnoN*, and *p15*, were examined. Strikingly, transcript levels for all targets examined were significantly lower in each *Bhd*^{-/-} ES cell clone tested, compared to *Bhd*^{+/+} and *Bhd*^{+/-} controls, as depicted in Figures 10B and 11A. Similar results were observed for activin-mediated stimulation of these mRNA targets (data not shown).

TGFβ1 ligand, as well as the receptors TGFβRI and TGFβRII have been shown to be necessary for yolk sac vasculogenesis and normal embryonic hematopoiesis *in vivo*, and in ES cell-derived cystic embryoid body (EB) cultures, an *in vitro* model of yolk sac development (Dickson *et al.*, 1995, Development 121(6):1845-1854; Oshima *et al.*, 1996, Dev Biol 179(1):297-302; Goumans *et al.*, 1999, Development 126(16):3473-3483; Larsson *et al.*, 2001, Embo J 20(7):1663-1673). Interestingly, strong BHD expression was observed in yolk sac visceral endoderm, as revealed by X-Gal staining of E10.5 embryos heterozygous for the Bay Genomics *Bhd*^m allele, as depicted in Figure 10C. To investigate whether BHD is required for normal yolk sac development, day 12 EBs from *Bhd*^{+/+} ES cells and two independent *Bhd*^{-/-} ES cell clones were generated, and it was observed that *Bhd*^{-/-} ES cells failed to form expanded cystic EBs, as depicted in Figure 10D (Doetschman *et al.*, 1985, J Embryol Exp Morphol 87:27-45). mRNA levels of yolk sac markers *α-fetoprotein* (*Afp*) and

trithioredoxin (Ttr) in differentiated *Bhd*^{+/+} and *Bhd*^{-/-} EBs were assessed, and it was found that maximal levels of both transcripts were severely reduced in *Bhd*^{-/-} EBs, as depicted in Figure 10E. To further evaluate the role of BHD in embryonic hematopoietic stem cell development, *Bhd*^{+/+} and *Bhd*^{-/-} EBs were generated in methylcellulose cultures which promotes the differentiation of hematopoietic lineages. *Bhd*^{-/-} EBs failed to form hemoglobinized colonies at day 10 in culture, as depicted in Figures 10F and 11B, with severely reduced mRNA expression levels of the erythroid marker *Gata-1* and *CD34* (a marker of both hematopoietic and endothelial lineages), as depicted in Figure 10G. These *in vitro* defects are unlikely to be a consequence of failed mesoderm formation, as the mesodermal marker *Brachyury T* was induced robustly in *Bhd*^{-/-} EBs, though in a delayed fashion. *Bhd*^{-/-} EBs were also able to strongly induce early visceral endoderm marker *Hnf4* at early timepoints, as well as primitive ectoderm marker *Fgf-5*, as depicted in Figure 12. Hence, *Bhd*^{-/-} ES cells exhibit molecular and developmental deficits related to yolk sac and hematopoietic differentiation that mimic those of TGFβ ligand and receptor mutants.

Example 6: TGFβ-mediated transcription is downregulated due to hypo-acetylation of target gene promoters

Because BHD deficiency resulted in molecular and phenotypic defects synonymous with TGFβ receptor and ligand mutants, it was next investigated whether the transcriptional defects were occurring at the level of receptor-mediated phosphorylation and nuclear translocation of Smad transcriptional regulators. To assess the former possibility, phosphorylated and total nuclear levels of the receptor-regulated Smad2, and of the common partner Smad4, which form a complex and translocate to the nucleus upon cellular engagement of TGFβ ligands (Zhang *et al.*, 1996, Nature 383(6596):168-172), were examined. Western blot analysis did not reveal any differences in basal or activin-induced nuclear accumulation of phospho-Smad2, total Smad2, or total Smad4 in *Bhd*^{+/+} versus *Bhd*^{-/-} ES cells, suggesting TGFβ receptor-mediated phosphorylation and translocation of Smads was not compromised in *Bhd*^{-/-} cells, as depicted in Figures 13A and 13B. It therefore seemed likely that the transcriptional defects detected in *Bhd*^{-/-} cells were due to inhibition of transcriptional activity at target gene promoters themselves. Because Smads have been suggested to induce transcription via acetylation-dependent histone modifications, chromatin immunoprecipitations were performed to assess levels of acetylated histone H3 at the *Lefty1* promoter in response to activin in *Bhd*^{+/+} versus *Bhd*^{-/-} ES cells. Activin-induced acetyl-H3 at

this specific genomic location had been demonstrated previously in P19 embryonal carcinoma cells, which share many common features with ES cells (Ross *et al.*, 2006, *Embo J* 25(19):4490-4502). Whereas *Bhd*^{+/+} ES cells demonstrated a significant increase in relative levels of acetylated histone H3 at the *Lefty1* promoter after one hour of activin treatment, *Bhd*^{-/-} ES cells exhibited no response, as depicted in Figure 13C. This difference in acetyl-H3 at the *Lefty1* promoter was not due to a global reduction of acetyl-H3 levels, as these were unchanged by Western blot in *Bhd*^{+/+} versus *Bhd*^{-/-} ES cells with and without activin, as depicted in Figure 13D. To determine whether restoration of acetylated histone levels in *Bhd*^{-/-} cells could rescue downregulated target gene expression, cells were treated with a low dose of the histone deacetylase (HDAC) inhibitor trichostatin A (TSA). It was observed that TSA treatment of *Bhd*^{-/-} ES cells restored mRNA levels of several TGF β target genes, such as *PAI-1*, *p15* and *Bim*, to levels seen in *Bhd*^{+/+} cells, as depicted in Figure 13E. Importantly, it was also observed that TSA treatment rescued the death resistance phenotype of *Bhd*^{-/-} ES cells in response to amino acid deprivation, as assessed by FACs analysis and depicted in Figure 6F (upper), and caspase and parp cleavage by Western blot along with a concomitant restoration of Bim protein expression (lower). Treatment of cells with TSA under non-starvation conditions did not induce cell death, eliminating the possibility that non-specific toxic effects of TSA mediated this effect (data not shown). Collectively, these data support a model whereby loss of BHD inhibits TGF β ligand-mediated transcriptional activity, which in turn results in apoptotic resistance due to downregulation of Bim. This loss of transcriptional activity is most likely due to increased recruitment of an HDAC-containing repressor to Smad target genes, given the ability of the HDAC inhibitor TSA to rescue target gene expression and the apoptotic resistance of *Bhd*^{-/-} cells, as depicted in Figure 14.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed:

1. A method of treating a BHD-based tumor in a mammal, the method comprising administering to a mammal an effective amount of a therapeutic agent that promotes or induces TGF- β dependent apoptosis in the BHD-based tumor.
2. The method of claim 1, wherein the mammal is a human.
3. The method of claim 1, wherein the therapeutic agent is a BH3-only molecule.
4. The method of claim 1, wherein the BH3-only molecule is a protein selected from the group consisting of BID, BIM, BAD and NOXA.
5. The method of claim 3, wherein the BH3-only molecule is a mimetic.
6. The method of claim 5, wherein the BH3-only mimetic is ABT-737.
7. The method of claim 1, wherein the therapeutic agent is an HDAC inhibitor.
8. The method of claim 7, wherein the HDAC inhibitor is Trichostatin.
9. The method of claim 7, wherein the HDAC inhibitor is Vorinostat.
10. The method of claim 1, wherein the therapeutic agent is an autophagy inhibitor.
11. The method of claim 10, wherein the autophagy inhibitor is chloroquine.
12. The method of claim 10, wherein the autophagy inhibitor is 3-methyl-adenine (3-MA).
13. The method of claim 1, wherein the therapeutic agent is an expression vector encoding BHD.

14. The method of claim 1, wherein the therapeutic agent is an expression vector encoding BIM.
15. A method for promoting or inducing TGF- β dependent apoptosis in a BHD-based tumor cell of a mammal, the method comprising contacting the cell with an effective amount of a therapeutic agent.
16. The method of claim 15, wherein the mammal is a human.
17. The method of claim 15, wherein the therapeutic agent is selected from the group consisting of a BH3-only molecule, an HDAC inhibitor and an autophagy inhibitor.
18. The method of claim 15, wherein the therapeutic agent is an expression vector encoding BHD.
19. The method of claim 15, wherein the therapeutic agent is an expression vector encoding BIM.
20. A composition for promoting or inducing TGF- β dependent apoptosis in a BHD-based tumor cell of a mammal, the composition comprising a BH3-only molecule.
21. A composition for promoting or inducing TGF- β dependent apoptosis in a BHD-based tumor cell of a mammal, the composition comprising an HDAC inhibitor.
22. A composition for promoting or inducing TGF- β dependent apoptosis in a BHD-based tumor cell of a mammal, the composition comprising an autophagy inhibitor.
23. A composition for promoting or inducing TGF- β dependent apoptosis in a BHD-based tumor cell of a mammal, the composition comprising an expression vector encoding BHD.
24. A composition for promoting or inducing TGF- β dependent apoptosis in a BHD-based tumor cell of a mammal, the composition comprising an expression vector encoding BIM.

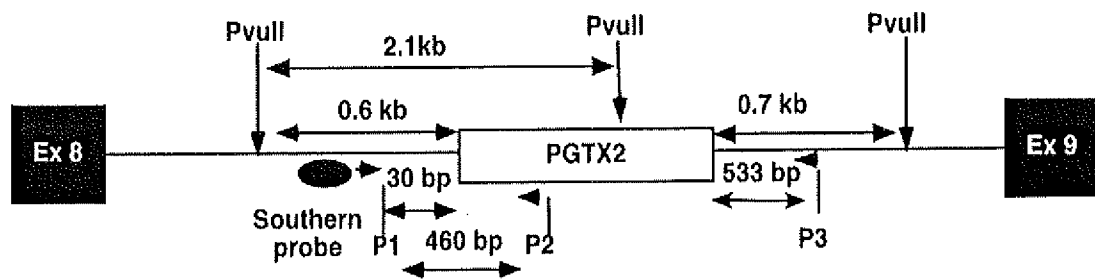
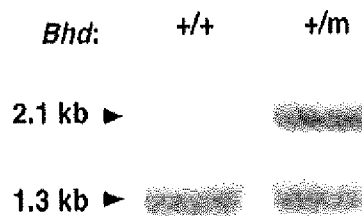
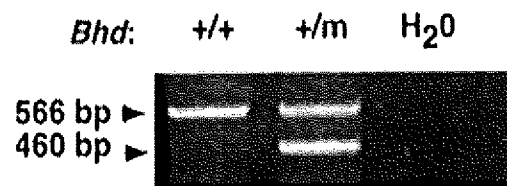
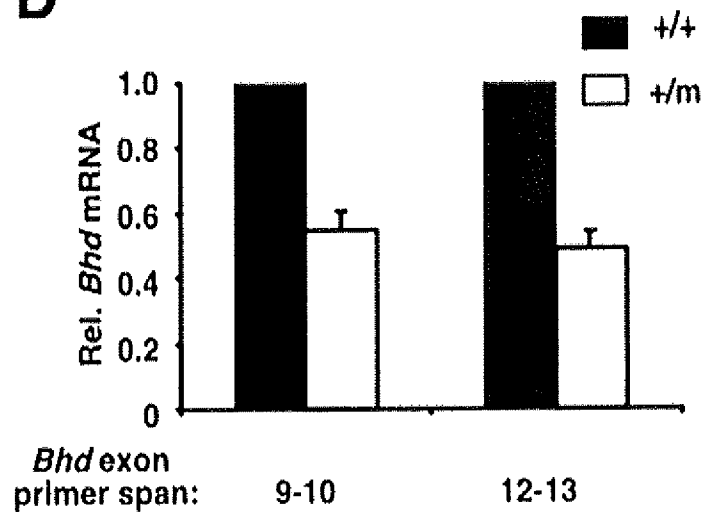
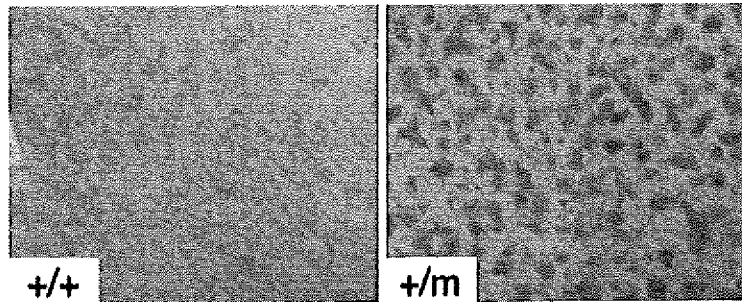
1/23**A****B****C****D**

Figure 1A-1D

Replacement Sheet

2/23**E****F**

	+/+	+/m	Abnormal
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e7.5	19%	62%	19%
e6.5	76%*		24%*

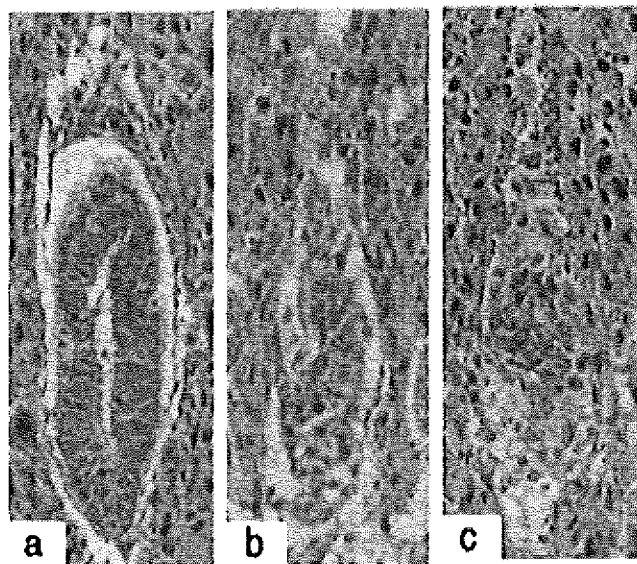
G

Figure 1E-1G

Replacement Sheet

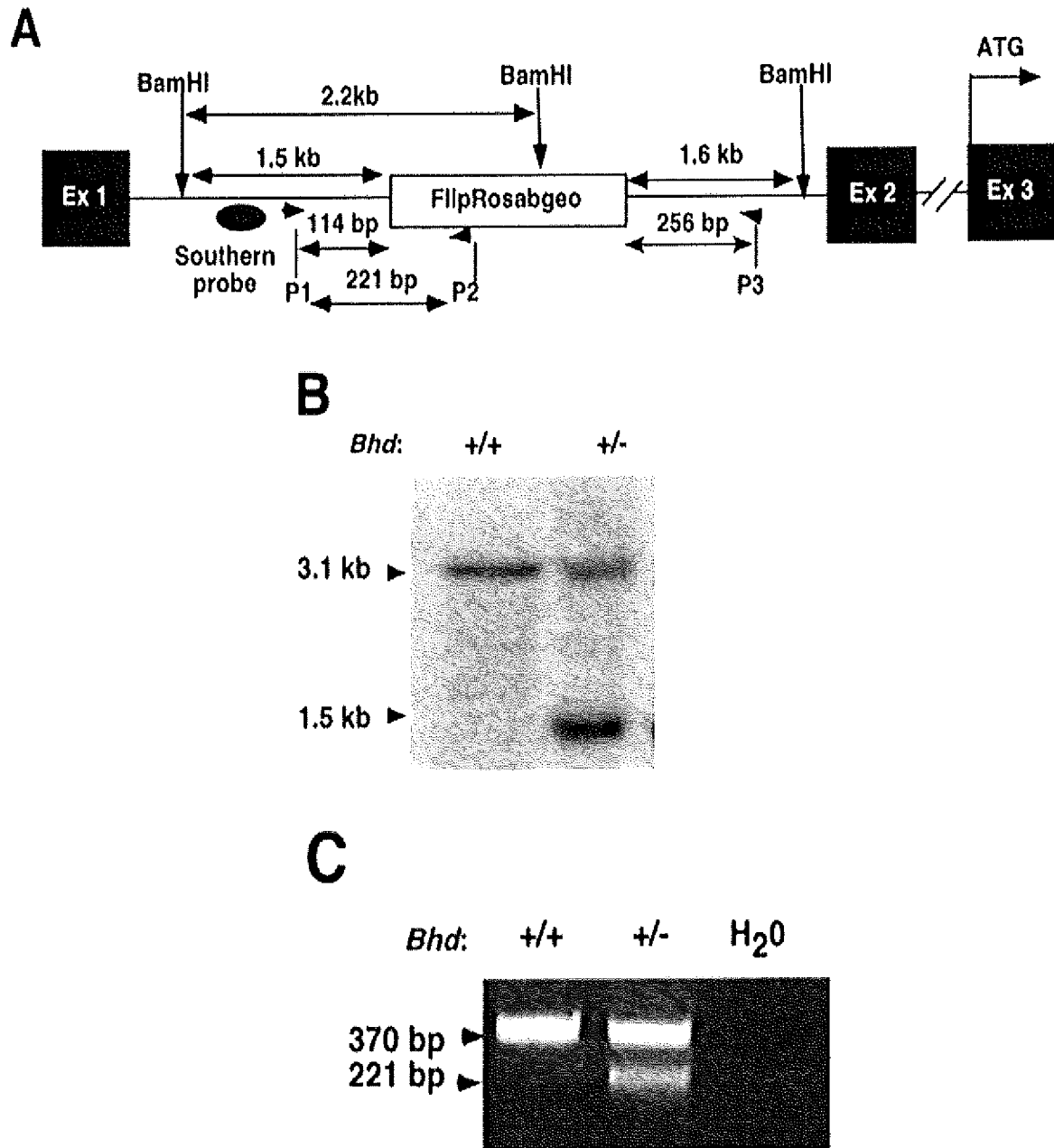
3/23

Figure 2A-2C

Replacement Sheet

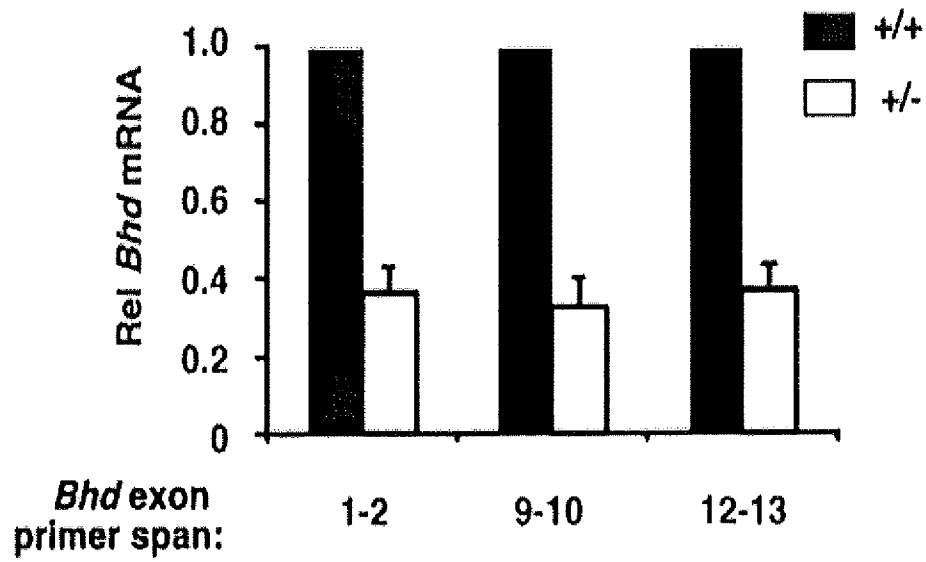
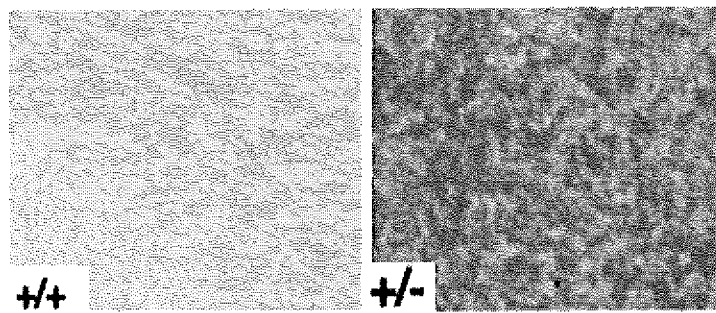
4/23**D****E**

Figure 2D-2E

Replacement Sheet

5/23

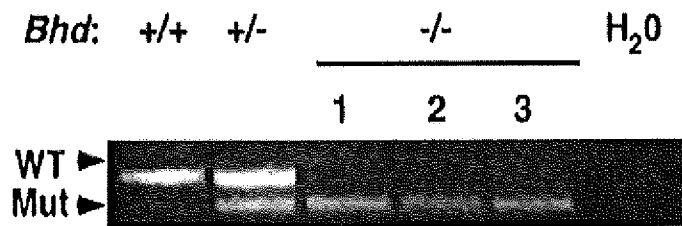
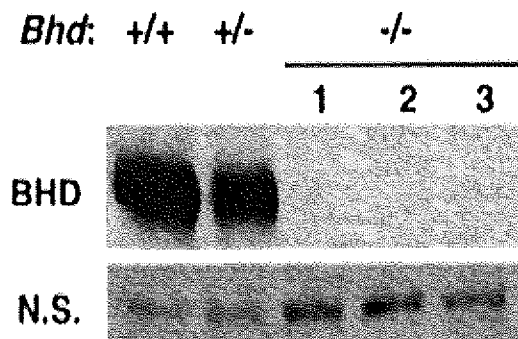
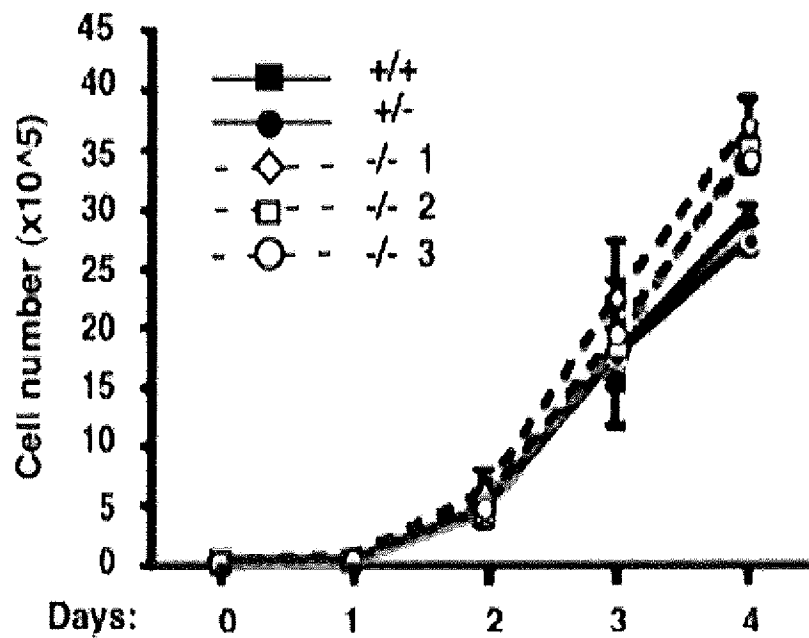
A**B****C**

Figure 3A-3C

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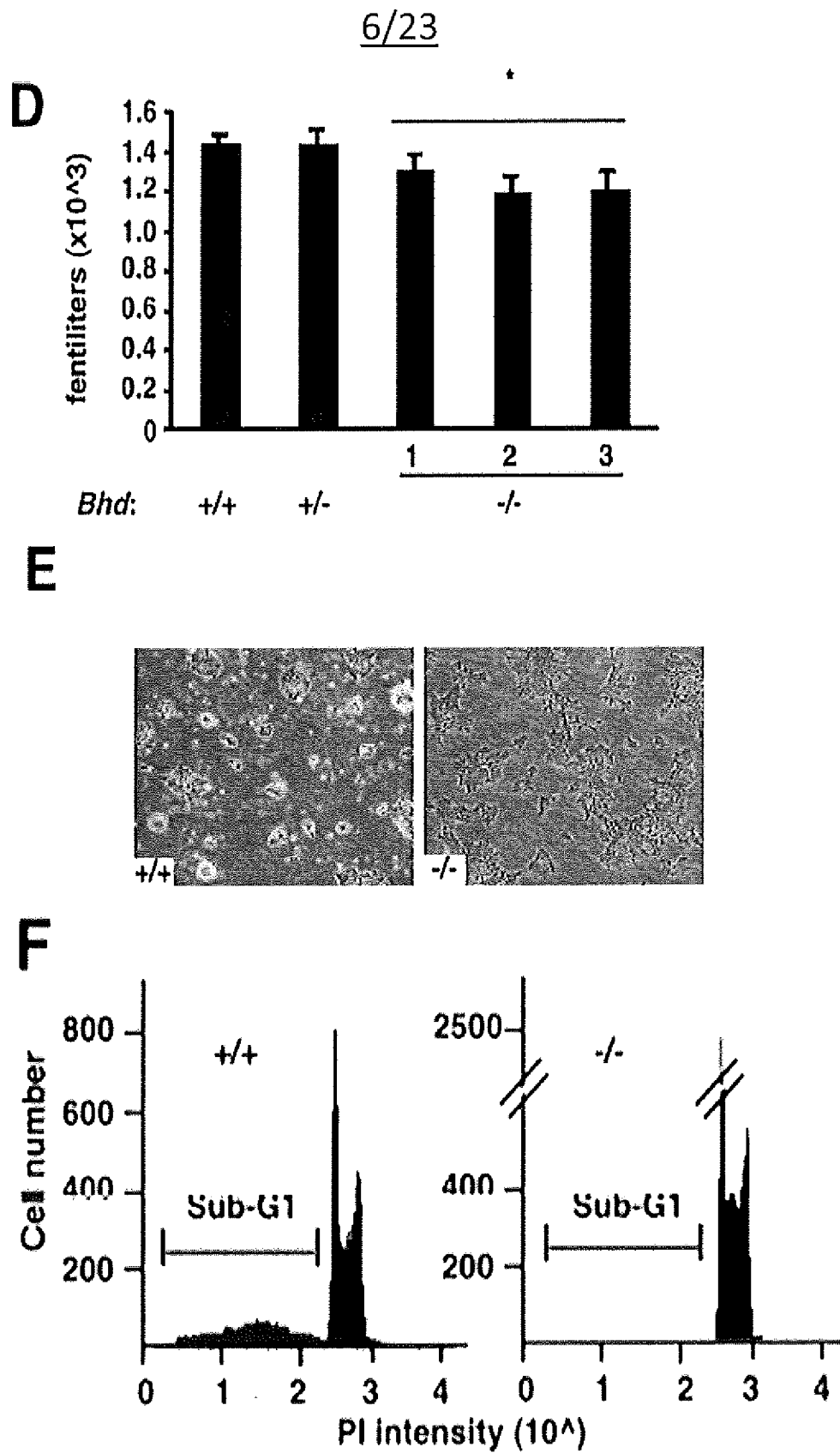


Figure 3D-3F

Replacement Sheet

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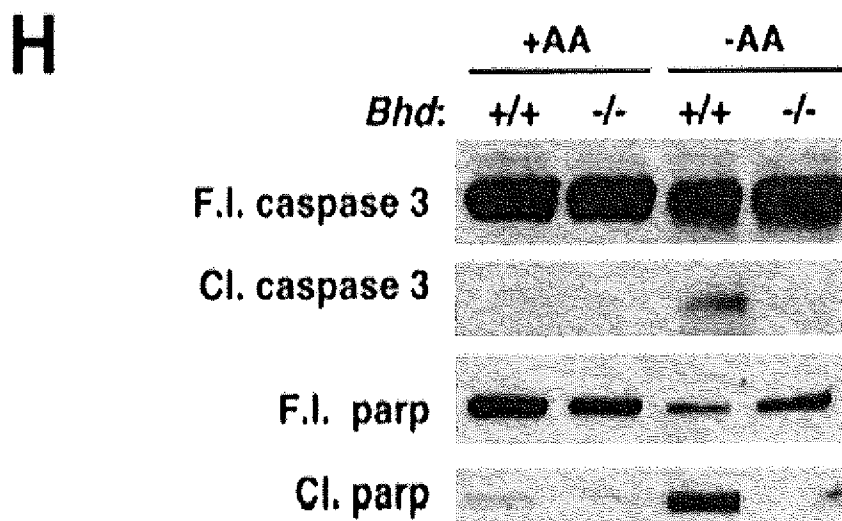
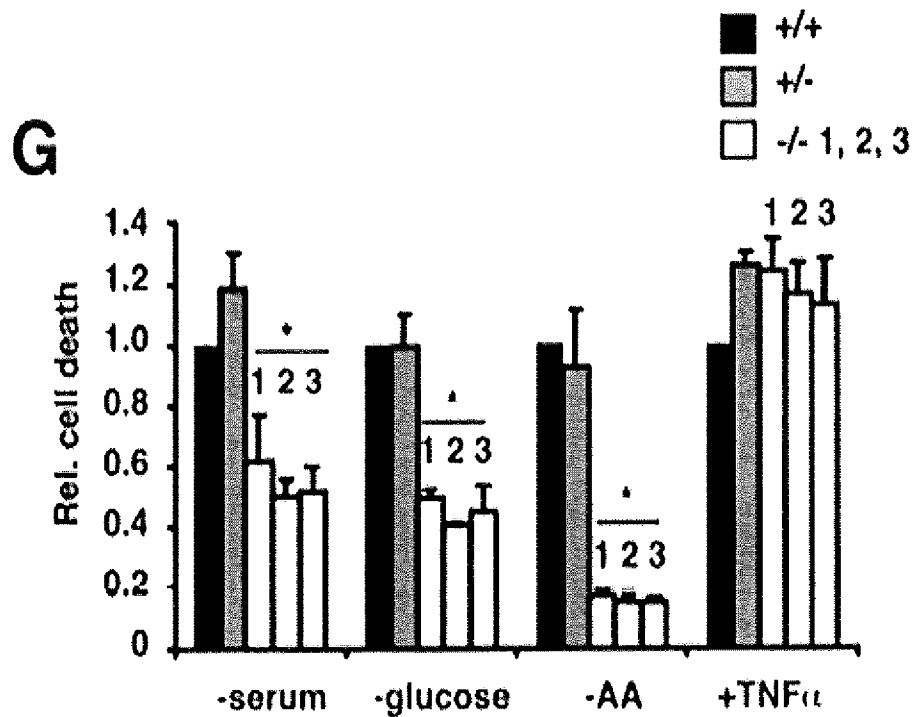


Figure 3G-3H

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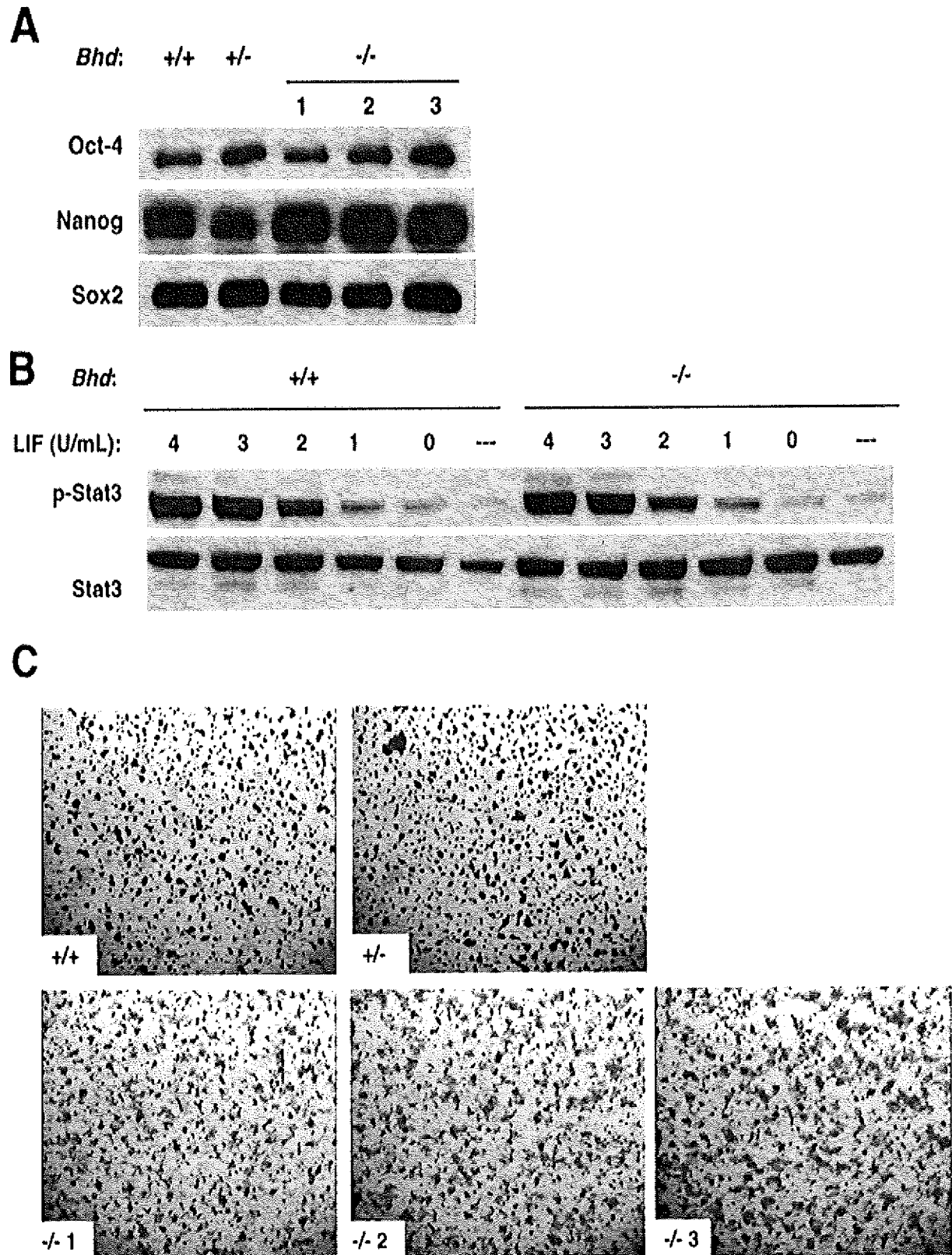


Figure 4

Replacement Sheet

9/23

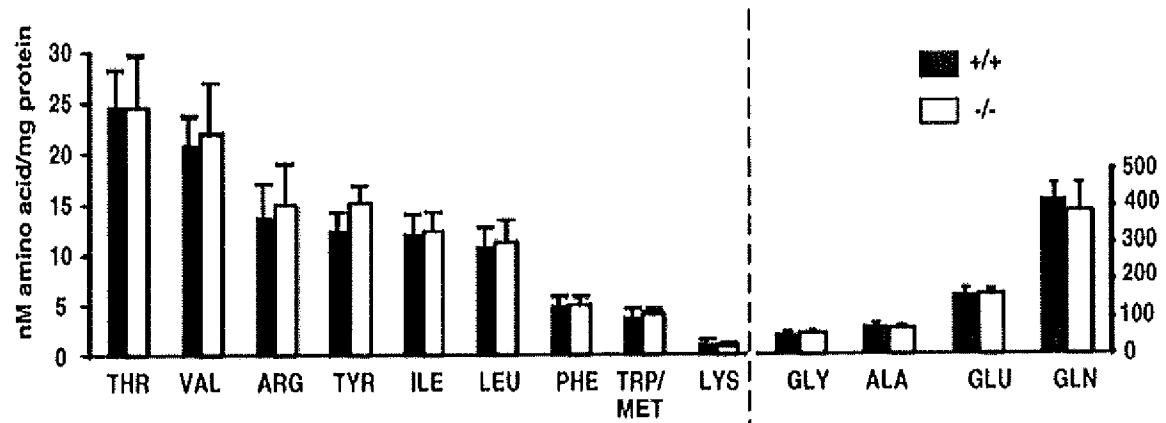
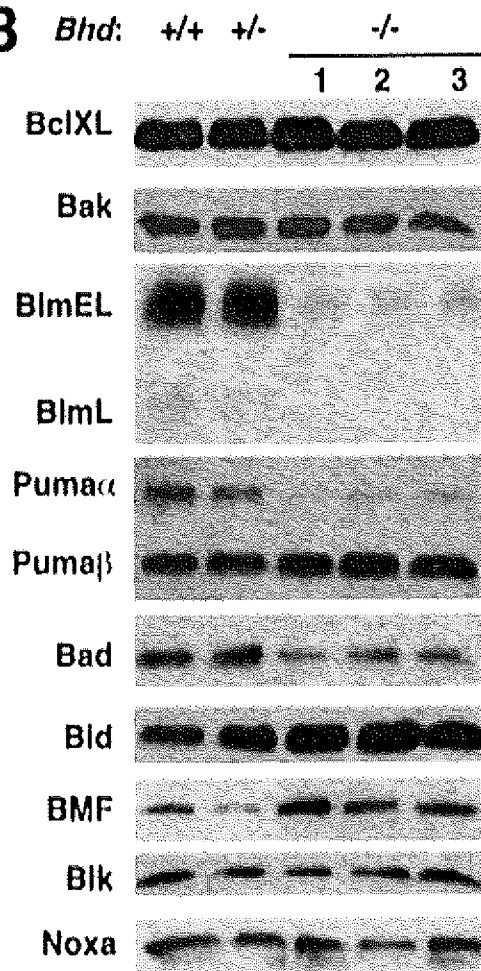
A**B**

Figure 5A-5B

Replacement Sheet

10/23

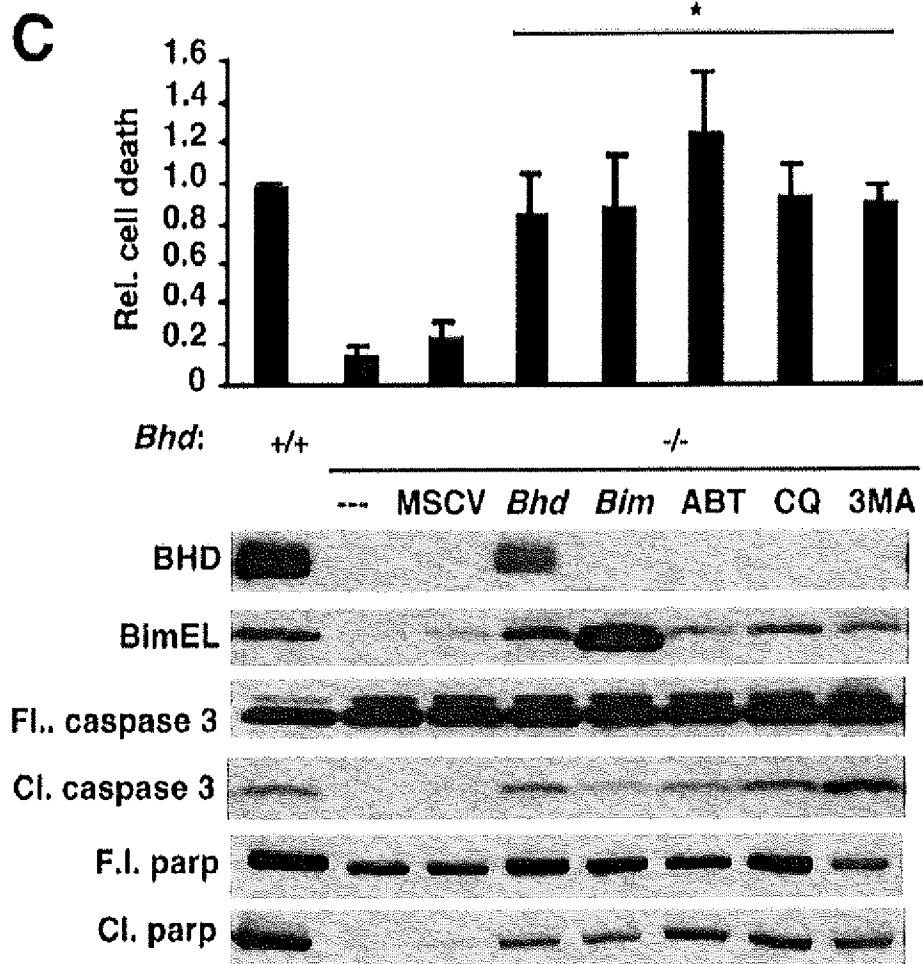


Figure 5C-5D

Replacement Sheet

11/23

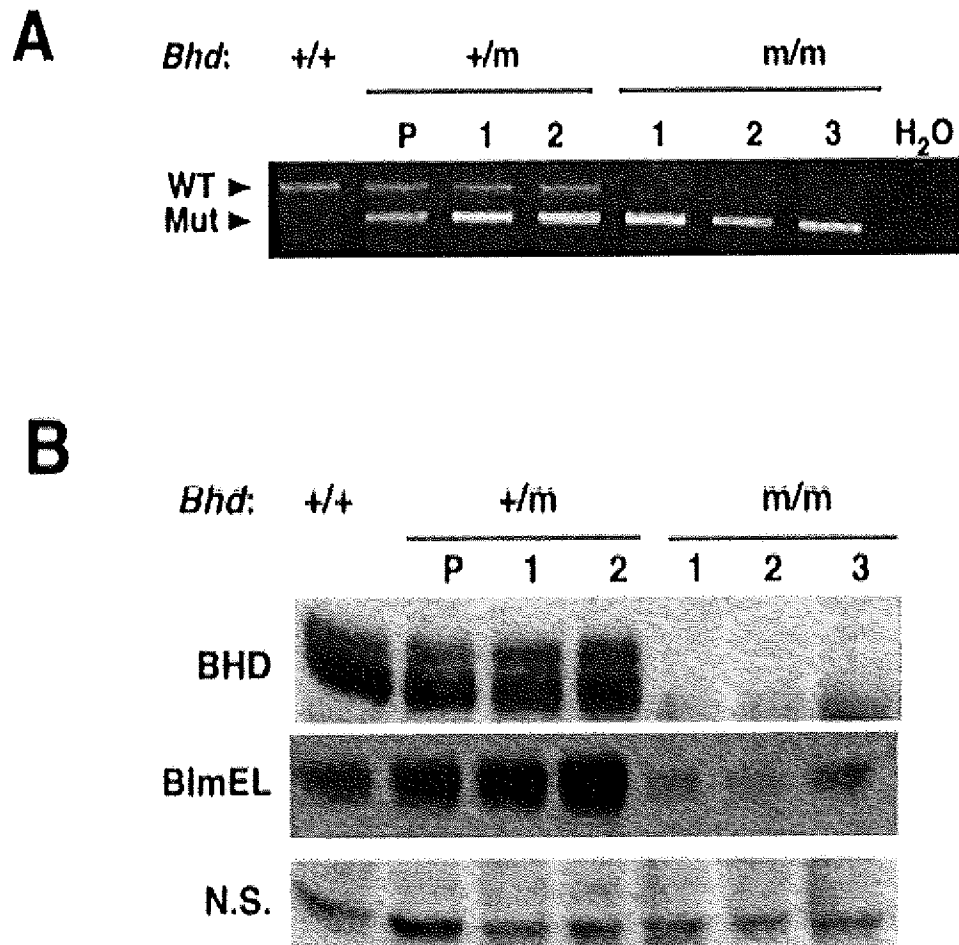


Figure 6

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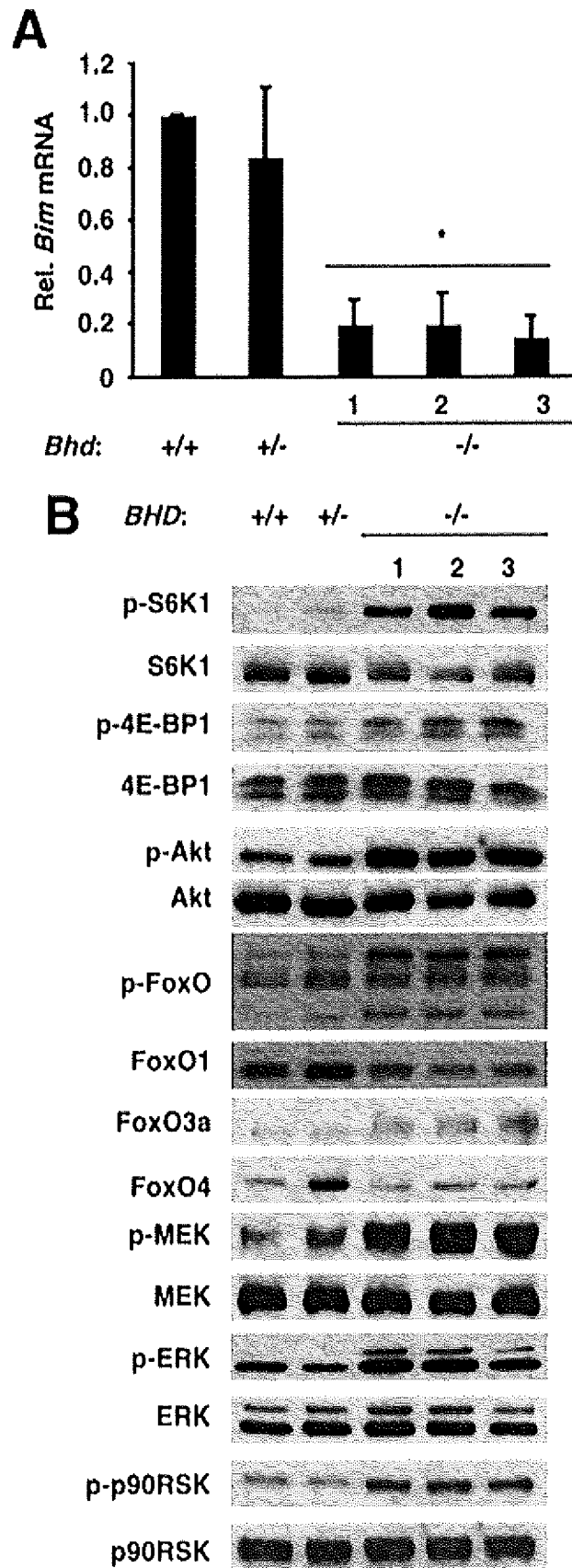
12/23

Figure 7A-7B

Replacement Sheet

13/23

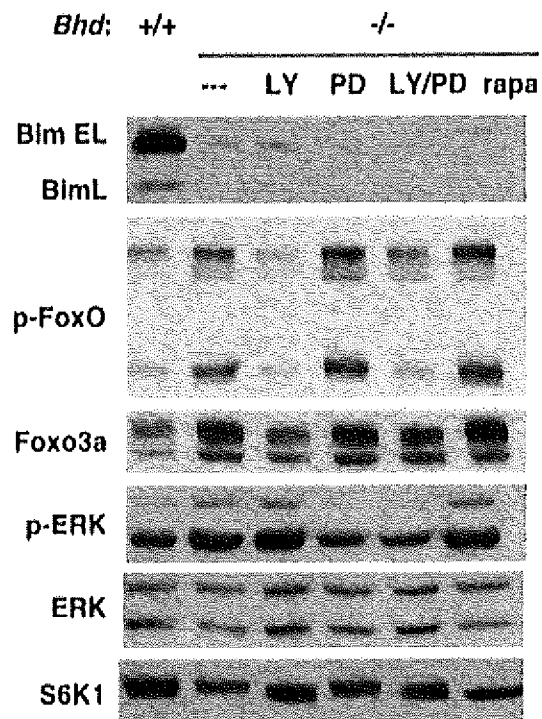
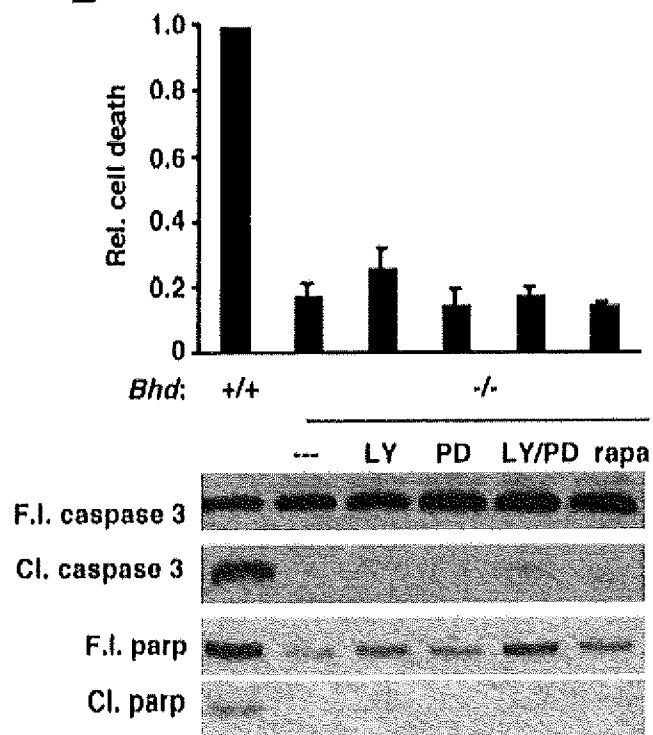
C**D**

Figure 7C-7D

Replacement Sheet

14/23

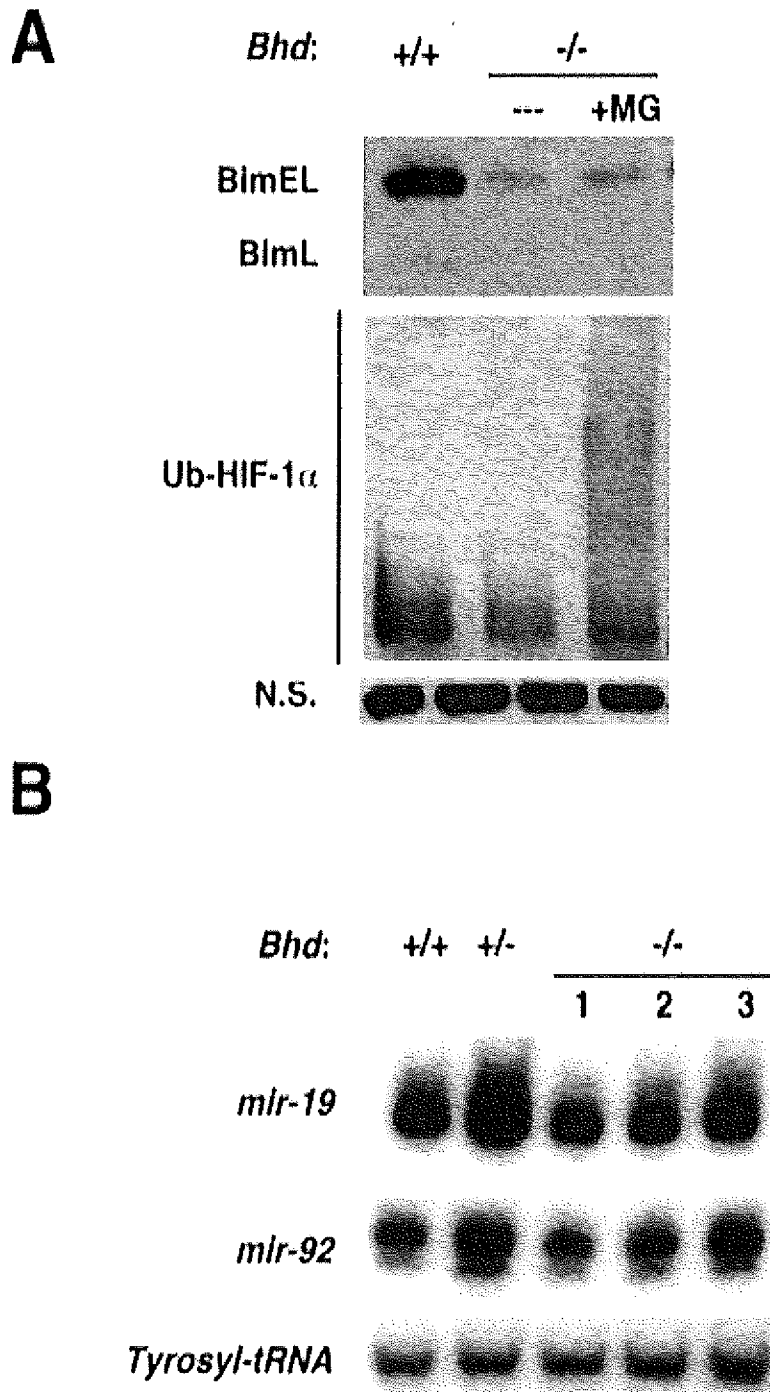
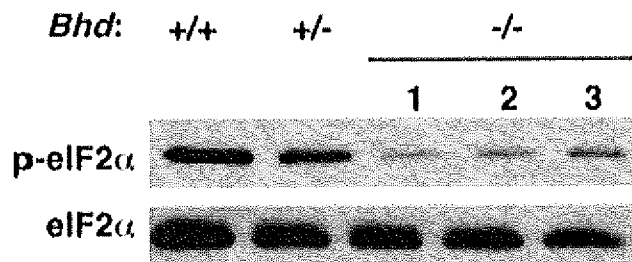
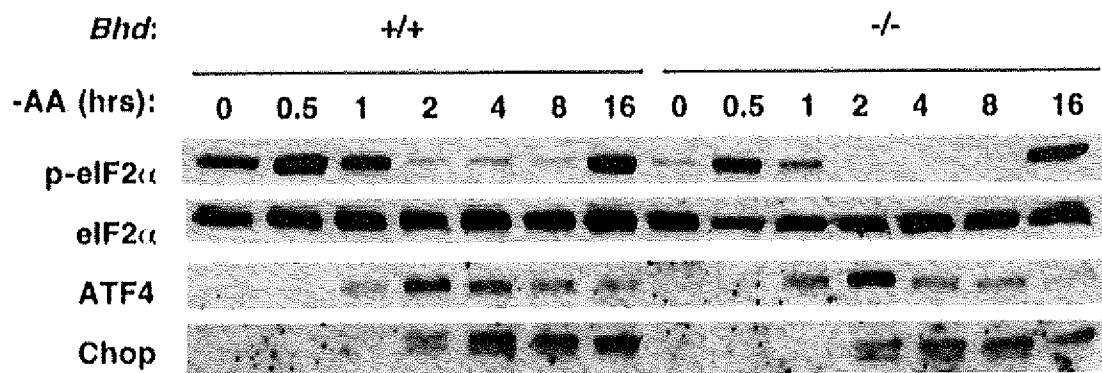


Figure 8

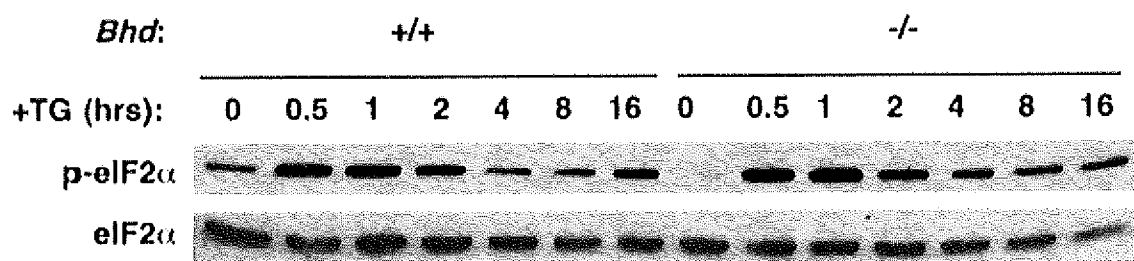
Replacement Sheet

15/23

A

**B**

C



D

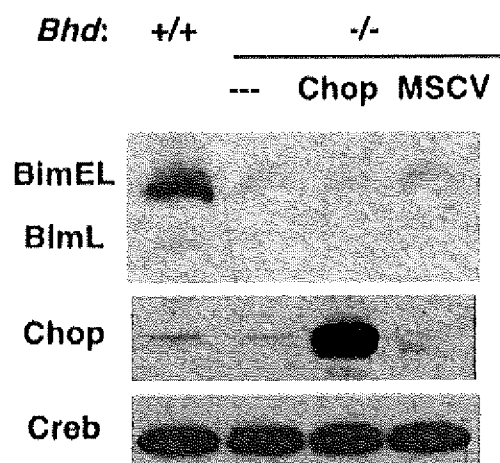


Figure 9

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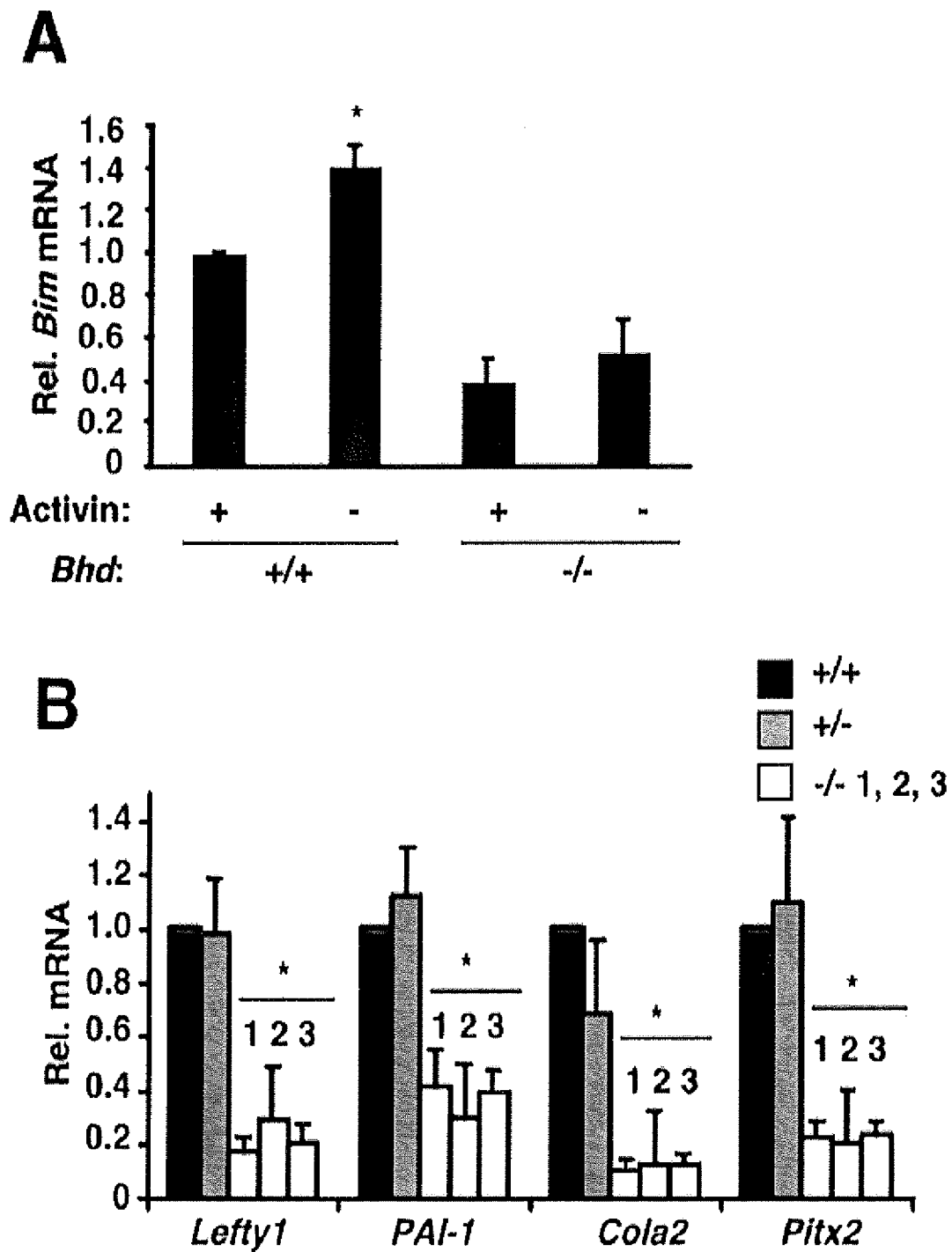
16/23

Figure 10A-10B

Replacement Sheet

SUBSTITUTE SHEET (RULE 26)

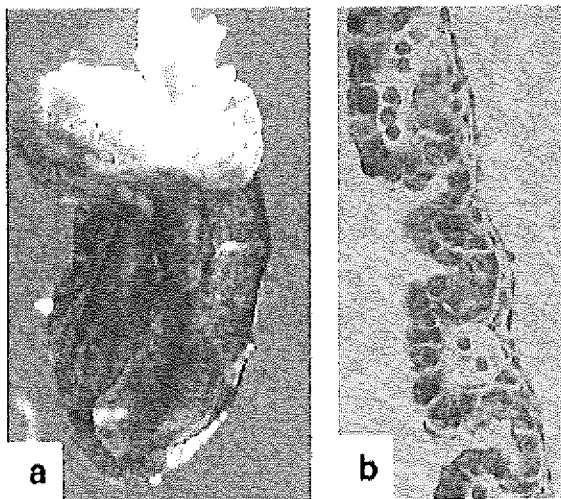
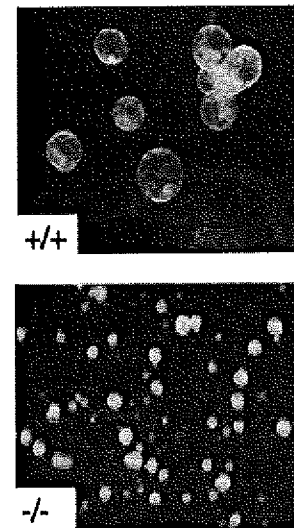
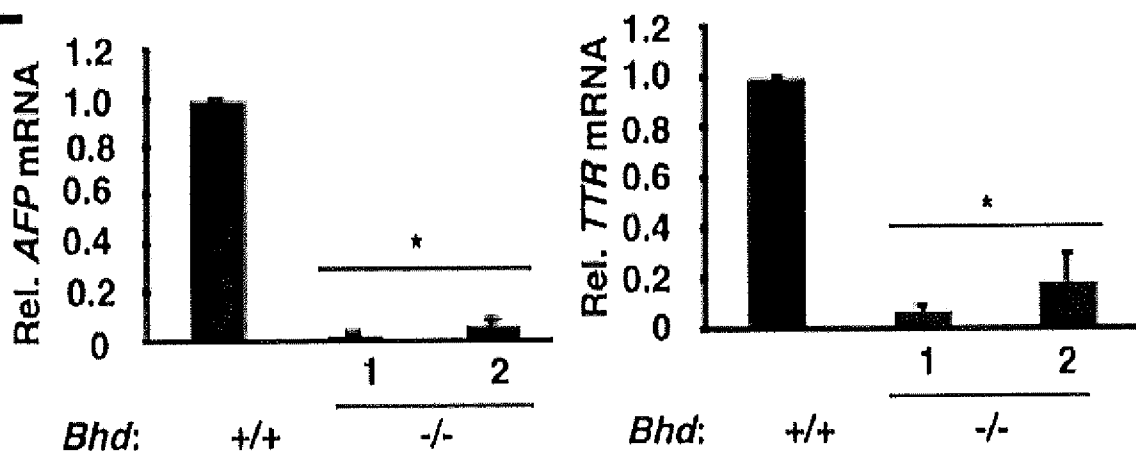
17/23**C****D****E**

Figure 10C-10E

Replacement Sheet

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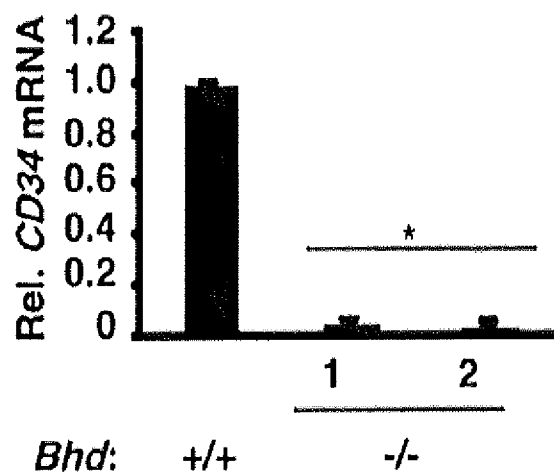
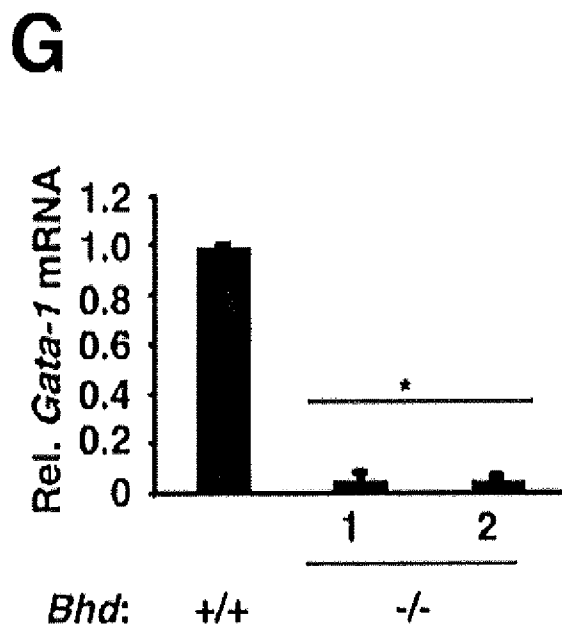
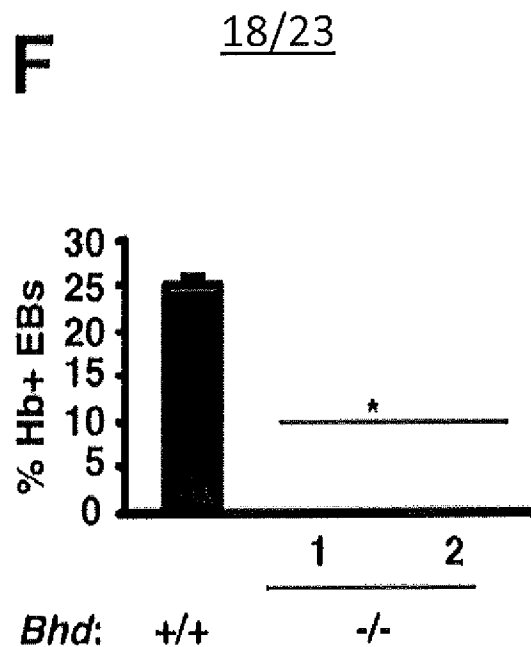


Figure 10F-10G

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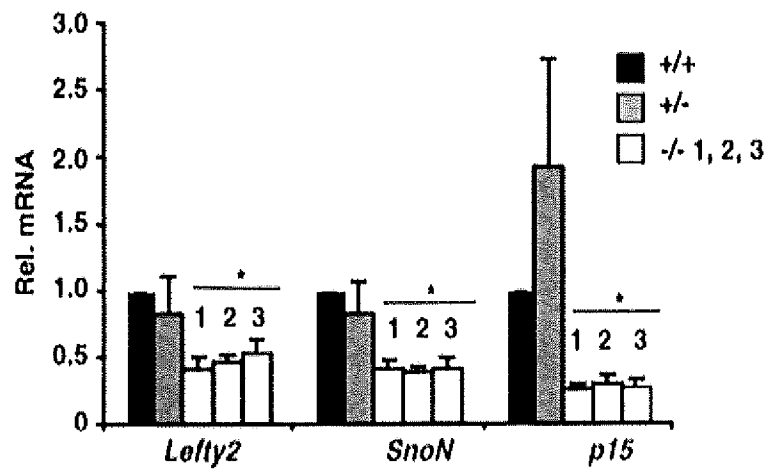
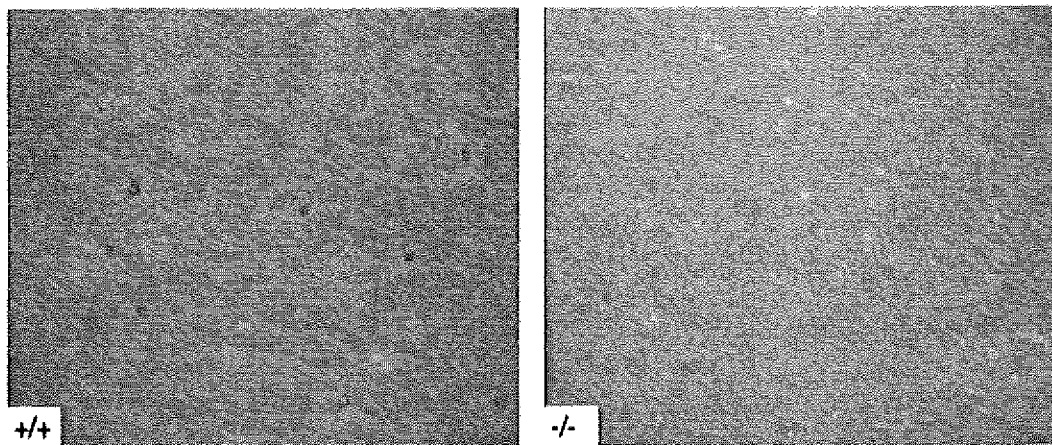
19/23**A****B**

Figure 11

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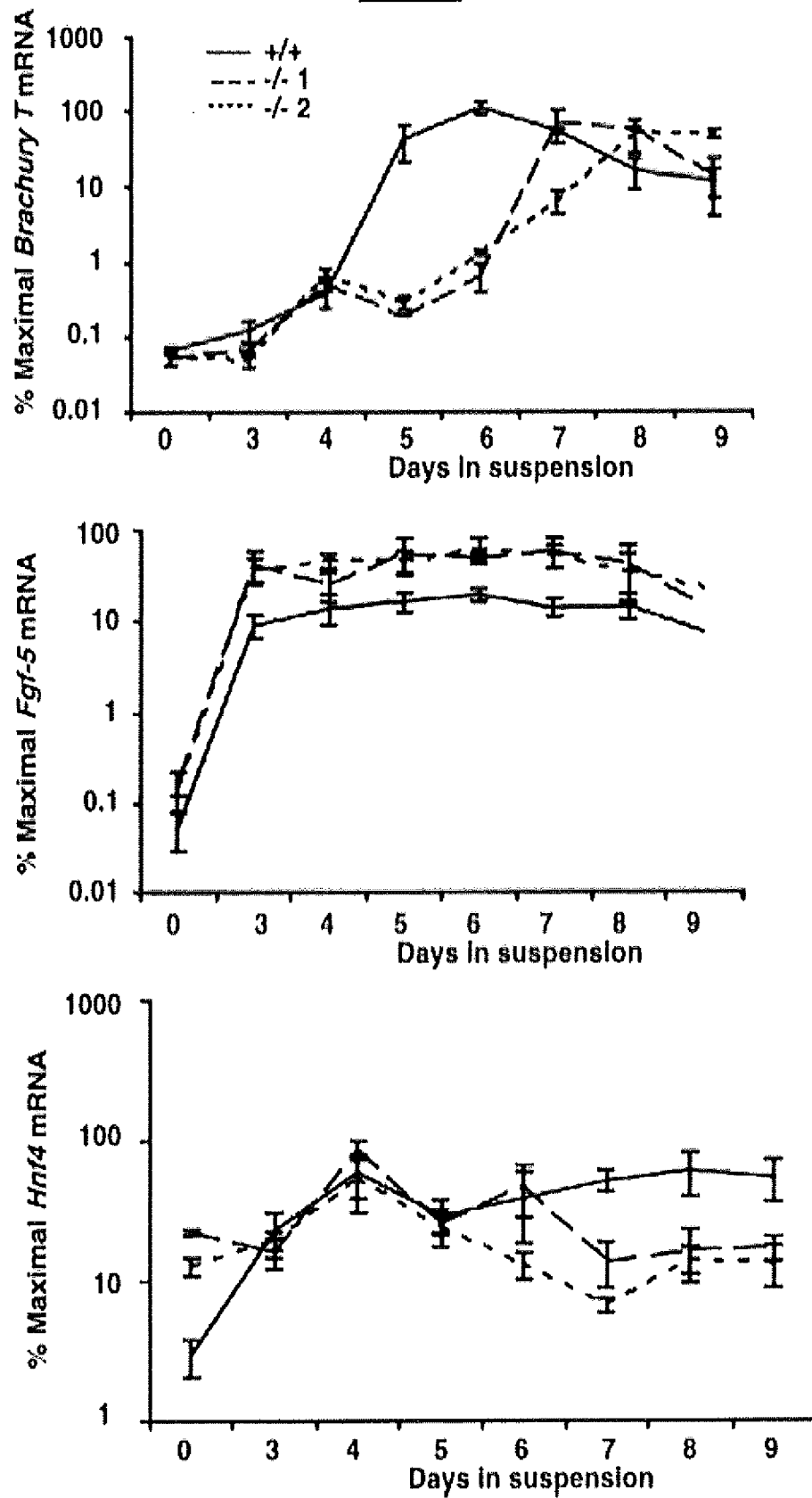
20/23

Figure 12

Replacement Sheet

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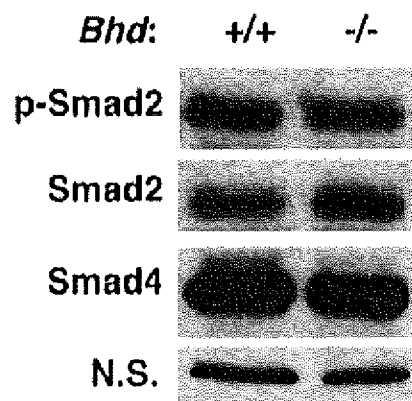
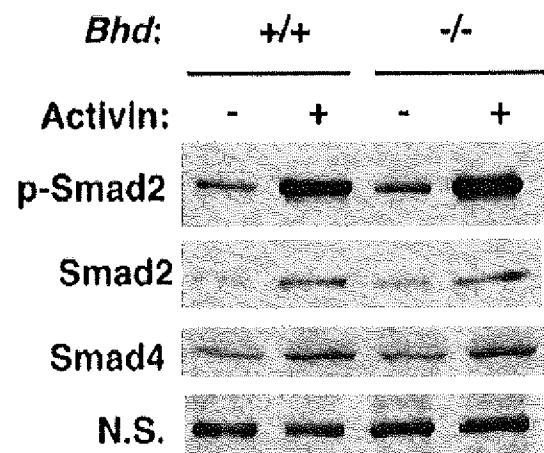
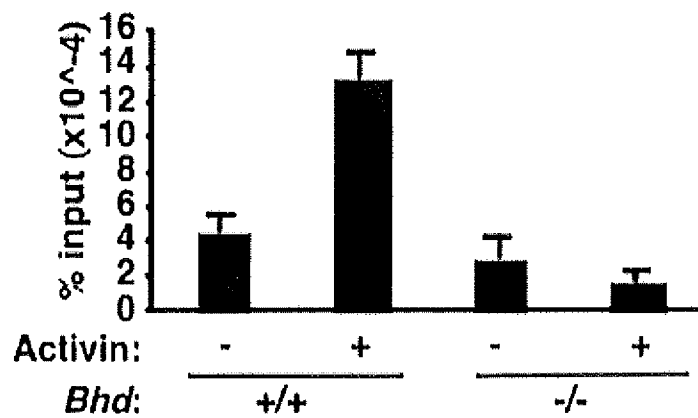
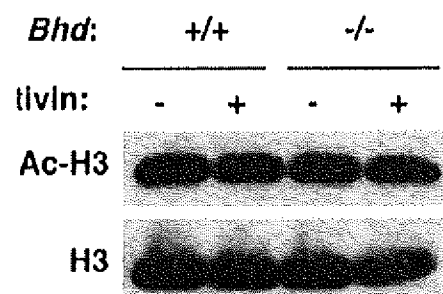
A**B****C****D**

Figure 13A-13D

Replacement Sheet

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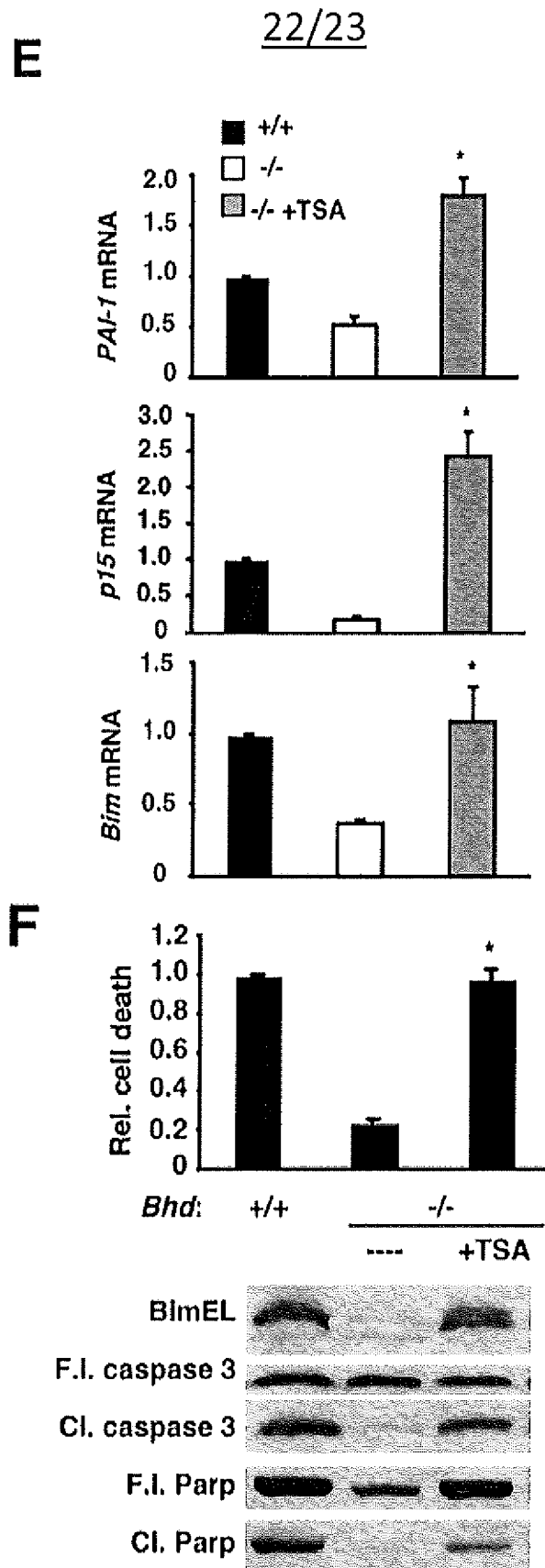


Figure 13E-13F

Replacement Sheet

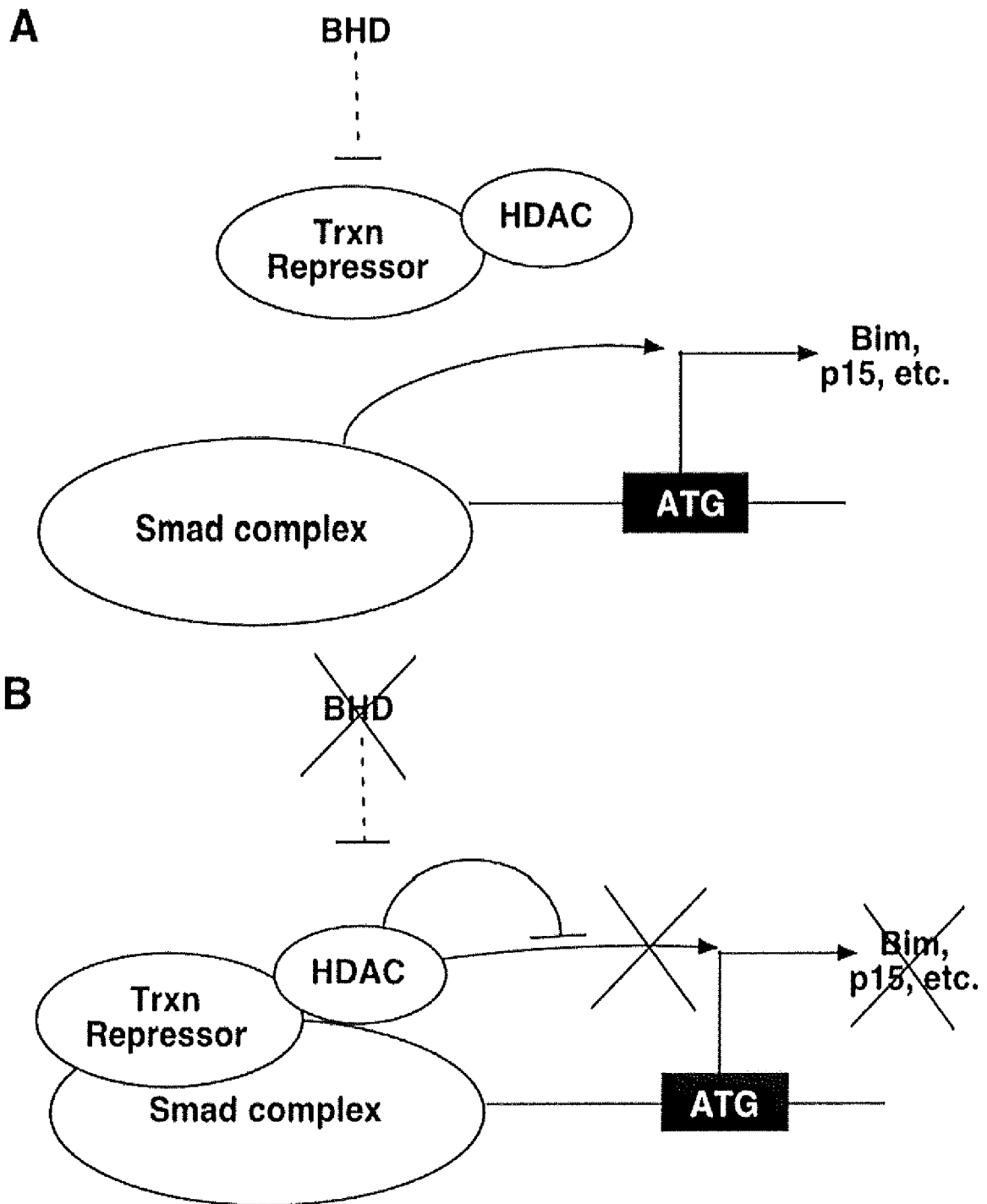
23/23

Figure 14

Replacement Sheet

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 11/27507

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/17

USPC - 514/19.2

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC: 514/19.2

IPC: A61K 38/17

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

USPC: 514/19.3 ; 514/8.9

IPC: A61K038/16 ; A61K038/00 ; A61K038/45 ; C07K014/495

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

PubWest (PGPB,USPT,USOC,EPAB,JPAB) (cancer or carcinoma or tumor) ; (renal or kidney or bladder) ; (apoptosis) (programmed cell death) ; (TGF? or (transforming growth factor))

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Hudon et al., "Renal tumour suppressor function of the Birt-Hogg-Dub? syndrome gene product folliculin" J Med Genet. 2010 Mar;47(3):182-9. Epub 2009 Oct 19. (pg 184, col 2, para 1, Fig 4c) ; (pg 189, col 1, para 1-2)	1-24
Y	Willey et al., "Smad3 potentiates transforming growth factor beta (TGFbeta)-induced apoptosis and expression of the BH3-only protein Bim in WEHI 231 B lymphocytes" J Biol Chem. 2003 May 16;278(20):18069-77. Epub 2003 Mar 13. (pg 18070, col 1, para 2) ; (pg 18072, col 1-2)	1-24
Y	CASH "Molecular mechanisms of the Birt-Hogg-Dube tumor suppressor" (January 1, 2010); dissertation; University of Pennsylvania; Abstract only. [online] downloaded from: http://repository.upenn.edu/dissertations/AA13429199/ on 21 May 2011	1-24
Y	Oltersdorf et al., "An inhibitor of Bcl-2 family proteins induces regression of solid tumours" Nature. 2005 Jun 2;435(7042):677-81. Epub 2005 May 15. (pg 677, col 2, para 3; Fig1d)	5-6
Y	Ammanamanchi et al., "Restoration of transforming growth factor-beta signaling through receptor RI induction by histone deacetylase activity inhibition in breast cancer cells" J Biol Chem. 2004 Jul 30;279(31):32620-5. Epub 2004 May 20. (pg 32621, col 2, para 3)	7-9, 21
Y	Bellodi et al., "Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells" J Clin Invest. 2009 May;119(5):1109-23. doi: 10.1172/JCI35660. Epub 2009 Apr 13. (pg 2, col 1, para 1-3) ; (pg 6, col 1, para 1)	10-12, 22

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

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

21 May 2011 (21.05.2011)

Date of mailing of the international search report

01 JUN 2011

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 11/27507

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Zhang J et al., "Different gene expression profiles of AD293 and HEK293 cell lines that show contrasting susceptibility to apoptosis induced by overexpression of Bim L" Acta Biochim Pol. 2006;53(3):525-30. Epub 2006 Sep 2. (pg 528, col 2, para 1).	14, 19, 24
A,P	Hong et al. "Tumor suppressor FLCN inhibits tumorigenesis of a FLCN-null renal cancer cell line and regulates expression of key molecules in TGF-Beta signaling" Molecular Cancer 2010, 9:160	1-24