(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 364 025 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
31.07.2013 Bulletin 2013/31

(21) Application number: **02711634.2**

(22) Date of filing: **31.01.2002**

(51) Int Cl.:
C12N 15/12 *(2006.01)* ***C12N 15/63*** *(2006.01)*
C07K 14/435 *(2006.01)* ***C07K 16/18*** *(2006.01)*
A61K 48/00 *(2006.01)* ***A61K 39/395*** *(2006.01)*

(86) International application number:
PCT/AU2002/000096

(87) International publication number:
WO 2002/061081 (08.08.2002 Gazette 2002/32)

(54) **A NOVEL GENE BNO1 MAPPING TO CHROMOSOME 16Q24.3**

NEUES, AUF CHROMOSOM 16Q24.3 KARTIERTES GEN BNO1

UNE NOUVELLE CARTOGRAPHIE DU GENE BNO1 AU CHROMOSOME 16Q24.3

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR

(30) Priority: **31.01.2001 AU PP278301**

(43) Date of publication of application:
26.11.2003 Bulletin 2003/48

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Printed by Jouve, 75001 PARIS (FR)

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Description

Technical Field

[0001] The present invention relates to a novel gene which has been identified at the distal tip of the long arm of chromosome 16 at 16q24.3. The BNO1 gene encodes a polypeptide that forms part of a ubiquitin-ligase complex involved in targeting proteins by ubiquitination for degradation by the proteasome. In view of the realisation that BNO1 is involved in ubiquitination and protein degradation, the invention is also concerned with the therapy of disorders associated with this process, such as cancer (in particular breast and prostate carcinoma), immune/inflammatory disease and neurological disease. In addition, the invention is concerned with the diagnosis of disorders associated with ubiquitination and the screening of drugs for therapeutic intervention in these disorders.

Background Art

[0002] The development of human carcinomas has been shown to arise from the accumulation of genetic changes involving both positive regulators of cell function (oncogenes) and negative regulators (tumour suppressor genes). For a normal somatic cell to evolve into a metastatic tumour it requires changes at the cellular level, such as immortalisation, loss of contact inhibition and invasive growth capacity, and changes at the tissue level, such as evasion of host immune responses and growth restraints imposed by surrounding cells, and the formation of a blood supply for the growing tumour.

[0003] Molecular genetic studies of colorectal carcinoma have provided substantial evidence that the generation of malignancy requires the sequential accumulation of a number of genetic changes within the same epithelial stem cell of the colon. For a normal colonic epithelial cell to become a benign adenoma, progress to intermediate and late adenomas, and finally become a malignant cell, inactivating mutations in tumour suppressor genes and activating mutations in proto-oncogenes are required (Fearon and Vogelstein,1990).

[0004] The employment of a number of techniques, such as loss of heterozygosity (LOH), comparative genomic hybridisation (CGH) and cytogenetic studies of cancerous tissue, all of which exploit chromosomal abnormalities associated with the affected cell, has aided in the identification of a number of tumour suppressor genes and oncogenes associated with a range of tumour types.

[0005] In one aspect, studies of cancers such as retinoblastoma and colon carcinoma have supported the model that LOH is a specific event in the pathogenesis of cancer and has provided a mechanism in which to identify the cancer causing genes. This model is further highlighted in Von Hippel-Lindau (VHL) syndrome, a rare disorder that predisposes individuals to a variety of tumours including clear cell carcinomas of the kidneys and islet cell tumours of the pancreas. Both sporadic and inherited cases of the syndrome show LOH for the short arm of chromosome 3 and somatic translocations involving 3p in sporadic tumours, and genetic linkage to the same region in affected families has also been observed. The VHL tumour suppressor gene has since been identified from this region of chromosome 3 and mutations in it have been detected in 100% of patients who carry a clinical diagnosis of VHL disease. In addition, the VHL gene is inactivated in approximately 50-80% of the more common sporadic form of renal clear cell carcinoma.

[0006] The genetic determinants involved in breast cancer are not as well defined as that of colon cancer due in part to the histological stages of breast cancer development being less well characterised. However, as with colon carcinoma, it is believed that a number of genes need to become involved in a stepwise progression during breast tumourigenesis.

[0007] Certain women appear to be at an increased risk of developing breast cancer. Genetic linkage analysis has shown that 5 to 10% of all breast cancers are due to at least two autosomal dominant susceptibility genes. Generally, women carrying a mutation in a susceptibility gene develop breast cancer at a younger age compared to the general population, often have bilateral breast tumours, and are at an increased risk of developing cancers in other organs, particularly carcinoma of the ovary.

[0008] Genetic linkage analysis on families showing a high incidence of early-onset breast cancer (before the age of 46) was successful in mapping the first susceptibility gene, *BRCA1,* to chromosome 17q21 (Hall *et al*., 1990). Subsequent to this, the *BRCA2* gene was mapped to chromosome 13q12-q13 (Wooster *et al*., 1994) with this gene conferring a higher incidence of male breast cancer and a lower incidence of ovarian cancer when compared to *BRCA1.*

[0009] Both BRCA1 and BRCA2 have since been cloned (Miki et al., 1994; Wooster et al., 1995) and numerous mutations have been identified in these genes in susceptible individuals with familial cases of breast cancer.

[0010] Additional inherited breast cancer syndromes exist, however they are rare. Inherited mutations in the *TP53* gene have been identified in individuals with Li-Fraumeni syndrome, a familial cancer resulting in epithelial neoplasms occurring at multiple sites including the breast. Similarly, germline mutations in the *MMC4C1*/*PTEN* gene involved in Cowden's disease and the ataxia telangiectasia (AT) gene have been shown to confer an increased risk of developing breast cancer, among other clinical manifestations, but together account for only a small percentage of families with an inherited predisposition to breast cancer.

[0011] Somatic mutations in the *TP53* gene have been shown to occur in a high percentage of individuals with sporadic

breast cancer. However, although LOH has been observed at the *BRCA1* and *BRCA2* loci at a frequency of 30 to 40% in sporadic cases (Cleton-Jansen *et al*., 1995; Saito *et al.,* 1993), there is virtually no sign of somatic mutations in the retained allele of these two genes in sporadic cancers (Futreal *et al*., 1994; Miki *et al.,* 1996). Recent data suggests that DNA methylation of the promoter sequence of these genes may be an important mechanism of down-regulation. The use of both restriction fragment length polymorphisms and small tandem repeat polymorphic markers has identified numerous regions of allelic imbalance in breast cancer suggesting the presence of additional genes, which may be implicated in breast cancer. Data compiled from more than 30 studies reveals the loss of DNA from at least 11 chromosome arms at a frequency of more than 25%, with regions such as 16q and 17p affected in more than 50% of tumours (Devilee and Cornelisse, 1994; Brenner and Aldaz, 1995). However only some of these regions are known to harbour tumour suppressor genes shown to be mutated in individuals with both sporadic *(TP53* and RB genes) and familial (*TP53, RB, BRCA1,* and *BRCA2* genes) forms of breast cancer.

[0012] Cytogenetic studies have implicated loss of the long arm of chromosome 16 as an early event in breast carcinogenesis since it is found in tumours with few or no other cytogenetic abnormalities. Alterations in chromosome 1 and 16 have also been seen in several cases of ductal carcinoma *in situ* (DCIS), the preinvasive stage of ductal breast carcinoma. In addition, LOH studies on DCIS samples identified loss of 16q markers in 29 to 89% of the cases tested (Chen et al., 1996; Radford et al., 1995). In addition, examination of tumours from other tissue types have indicated that 16q LOH is also frequently seen in prostate, liver, ovarian and primitive neuroectodermal carcinomas. Together, these findings suggest the presence of a gene mapping to the long arm of chromosome 16 that is critically involved in the early development of a large proportion of breast cancers as well as cancers from other tissue types, but to date no such gene has been identified.

Disclosure of the Invention

[0013] The present invention provides an isolated BNO1 nucleic acid molecule mapping to chromosome 16q24.3 comprising the nucleotide sequence set forth in SEQ ID Numbers: 1 or 3.

[0014] It also provides an isolated BNO1 nucleic acid molecule comprising the nucleotide sequence set forth in SEQ ID Numbers: 1 or 3 which encodes a polypeptide that forms part of a ubiquitin-ligase complex involved in targeting proteins by ubiquitination for degradation by the proteasome.

[0015] The invention also encompasses an isolated BNO1 nucleic acid molecule that is at least 95% identical to a DNA molecule consisting of the nucleotide sequence set forth in SEQ ID Numbers: 1 or 3 and which encodes a polypeptide that forms part of a ubiquitin-ligase complex involved in targeting proteins by ubiquitination for degradation by the proteasome.

[0016] Any one of the polynucleotide variants described above can encode an amino acid sequence, which contains at least one functional or structural characteristic of BNO1.

[0017] Typically, sequence identity is calculated using the BLASTN algorithm with the BLOSSUM62 default matrix.

[0018] The invention also encompasses an isolated BNO1 nucleic acid molecule that encodes a polypeptide that forms part of a ubiquitin-ligase complex involved in protein degradation through ubiquitination, and which hybridizes under stringent conditions with a DNA molecule consisting of the nucleotide sequence set forth in SEQ ID Numbers: 1 or 3.

[0019] Under stringent conditions, hybridization will most preferably occur at 42°C in 750 mM NaCl, 75 mM trisodium citrate, 2% SDS, 50% formamide, 1X Denhart's, 10% (w/v) dextran sulphate and 100 μg/ml denatured salmon sperm DNA. Useful variations on these conditions will be readily apparent to those skilled in the art. The washing steps which follow hybridization most preferably occur at 65°C in 15 mM NaCl, 1.5 mM trisodium citrate, and 1% SDS. Additional variations on these conditions will be readily apparent to those skilled in the art

[0020] The invention also provides an isolated BNO1 nucleic acid molecule which encodes a polypeptide having the amino acid sequence set forth in SEQ ID Numbers: 2 or 4.

[0021] Preferably, sequence identity is determined using the BLASTP algorithm with the BLOSSUM62 default matrix.

[0022] We also disclose an isolated nucleic acid molecule comprising exons 1 to 9 or exons 1, 2, 2.5, and 3 to 9 identified in the nucleotide sequences set forth in SEQ ID Numbers: 1 and 3 respectively.

[0023] Still further, there is provided an isolated nucleic acid molecule consisting of the nucleotide sequence set forth in SEQ ID Numbers: 1 or 3.

[0024] We also disclose an isolated nucleic acid molecule consisting of the nucleotide sequence set forth in SEQ ID NO: 1 from base 4 to base 1,621 or set forth in SEQ ID NO: 3 from base 4 to base 1,708.

[0025] We also disclose an isolated gene comprising the nucleotide sequence set forth in SEQ ID Numbers: 1 or 3 and **BNO1** control elements.

[0026] Preferably, the BNO1 control elements are those which mediate expression in breast, prostate, liver and ovarian tissue.

[0027] The nucleotide sequences of the present invention can be engineered using methods accepted in the art so as to alter BNO1-encoding sequences for a variety of purposes. These include, but are not limited to, modification of

the cloning, processing, and/or expression of the gene product. PCR reassembly of gene fragments and the use of synthetic oligonucleotides allow the engineering of BNO1 nucleotide sequences. For example, oligonucleotide-mediated site-directed mutagenesis can introduce mutations that create new restriction sites, alter glycosylation patterns and produce splice variants etc.

[0028] As a result of the degeneracy of the genetic code, a number of polynucleotide sequences encoding BNO1, some that may have minimal similarity to the polynucleotide sequences of any known and naturally occurring gene, may be produced. Thus, we also disclose each and every possible variation of polynucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the polynucleotide sequence of naturally occurring BNO1, and all such variations are to be considered as being specifically disclosed.

[0029] The polynucleotides of this invention include RNA, cDNA, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified, or may contain non-natural or derivatised nucleotide bases as will be appreciated by those skilled in the art. Such modifications include labels, methylation, intercalators, alkylators and modified linkages. In some instances it may be advantageous to produce nucleotide sequences encoding BNO1 or its derivatives possessing a substantially different codon usage than that of the naturally occurring BNO1. For example, codons may be selected to increase the rate of expression of the peptide in a particular prokaryotic or eukaryotic host corresponding with the frequency that particular codons are utilized by the host. Other reasons to alter the nucleotide sequence encoding BN01 and its derivatives without altering the encoded amino acid sequences include the production of RNA transcripts having more desirable properties, such as a greater half-life, than transcripts produced from the naturally occurring sequence.

[0030] The invention also encompasses production of DNA molecules, which encode BNO1 and its derivatives, or fragments thereof, entirely by synthetic chemistry. Synthetic sequences may be inserted into expression vectors and cell systems that contain the necessary elements for transcriptional and translational control of the inserted coding sequence in a suitable host. These elements may include regulatory sequences, promoters, 5' and 3' untranslated regions and specific initiation signals (such as an ATG initiation codon and Kozak consensus sequence) which allow more efficient translation of sequences encoding BNO1. In cases where the complete BNO1 coding sequence including its initiation codon and upstream regulatory sequences are inserted into the appropriate expression vector, additional control signals may not be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals as described above should be provided by the vector. Such signals may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers appropriate for the particular host cell system used (Scharf et al., 1994).

[0031] The present invention allows for the preparation of purified BN01 polypeptide or protein, from the polynucleotides of the present invention or variants thereof. In order to do this, host cells may be transfected with a DNA molecule as described above. Typically said host cells are transfected with an expression vector comprising a DNA molecule according to the invention. A variety of expression vector/host systems may be utilized to contain and express sequences encoding BNO1. These include, but are not limited to, microorganisms such as bacteria transformed with plasmid or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with viral expression vectors (e.g., baculovirus); or mouse or other animal or human tissue cell systems. Mammalian cells can also be used to express the BNO1 protein using various expression vectors including plasmid, cosmid and viral systems such as adenoviral, retroviral or vaccinia virus expression systems. The invention is not limited by the host cell employed.

[0032] The polynucleotide sequences, or variants thereof, of the present invention can be stably expressed in cell lines to allow long term production of recombinant proteins in mammalian systems. Sequences encoding BNO1 can be transformed into cell lines. using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. The selectable marker confers resistance to a selective agent, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be propagated using tissue culture techniques appropriate to the cell type.

[0033] The protein produced by a transformed cell may be secreted or retained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode BNO1 may be designed to contain signal sequences which direct secretion of BNO1 through a prokaryotic or eukaryotic cell membrane.

[0034] In addition, a host cell strain may be chosen for its ability to modulate expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, glycosylation, phosphorylation, and acylation. Post-translational cleavage of a "prepro" form of the protein may also be used to specify protein targeting, folding, and/or activity. Different host cells having specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO or HeLa cells), are available from the American Type Culture Collection (ATCC) and may be chosen to ensure the correct modification and processing of the foreign protein.

[0035] When large quantities of BNO1 are needed such as for antibody production, vectors which direct high levels of expression of BNO1 may be used such as those containing the T5 or T7 inducible bacteriophage promoter. The present invention also includes the use of the expression systems described above in generating and isolating fusion proteins which contain important functional domains of the protein. These fusion proteins are used for binding, structural and functional studies as well as for the generation of appropriate antibodies.

[0036] In order to express and purify the protein as a fusion protein, the appropriate BNO1 cDNA sequence is inserted into a vector which contains a nucleotide sequence encoding another peptide (for example, glutathionine succinyl transferase). The fusion protein is expressed and recovered from prokaryotic or eukaryotic cells. The fusion protein can then be purified by affinity chromatography based upon the fusion vector sequence and the BNO1 protein obtained by enzymatic cleavage of the fusion protein.

[0037] Fragments of BNO1 may also be produced by direct peptide synthesis using solid-phase techniques. Automated synthesis may be achieved by using the ABI 431A Peptide Synthesizer (Perkin-Elmer). Various fragments of BNO1 may be synthesized separately and then combined to produce the full-length molecule.

[0038] According to the invention there is provided an isolated polypeptide comprising the amino acid sequence set forth in SEQ ID Numbers: 2 or 4.

[0039] According to a still further aspect of the invention there is provided an isolated polypeptide, comprising the amino acid sequence set forth in SEQ ID Numbers: 2 or 4, that forms part of a ubiquitin-ligase complex involved in protein degradation through ubiquitination.

[0040] The invention also encompasses an isolated polypeptide that forms part of a ubiquitin-ligase complex involved in protein degradation through ubiquitination that has at least 95%, identity with the amino acid sequence set forth in SEQ ID Numbers: 2 or 4.

[0041] Preferably, sequence identity is determined using the BLASTP algorithm with the BLOSSUM62 default matrix.

[0042] Also envisaged is an isolated polypeptide consisting of the amino acid sequence set forth in SEQ ID Numbers: 2 or 4.

[0043] In a further aspect of the invention there is provided a method of preparing a polypeptide as described above, comprising the steps of:

(1) culturing the host cells under conditions effective for production of the polypeptide; and
(2) harvesting the polypeptide.

[0044] Substantially purified BNO1 protein or fragments thereof can then be used in further biochemical analyses to establish secondary and tertiary structure for example by x-ray crystallography of BNO1 protein or by nuclear magnetic resonance (NMR). Determination of structure allows for the rational design of pharmaceuticals to interact with the protein, alter protein charge configuration or charge interaction with other proteins, or to alter its function in the cell.

[0045] The BNO1 gene has been identified from a region of restricted LOH seen in breast and prostate cancer and appears to be down regulated in its expression in cancer cell lines derived from these tissues. In addition, chemical and structural similarity in the context of sequences and motifs, exists between regions of BNO1 and F-box proteins. F-box proteins are the substrate recognition components of one class of ubiquitin-E3 ligases, the so called "SCF" class, which are involved in the degradation of proteins through ubiquitination and subsequent proteolysis carried out by the proteasome. To date, proteins shown to be regulated by this mechanism include oncogenes, tumour suppressor genes, transcription factors and other signalling molecules. These proteins influence many cellular processes such as modulation of the immune and inflammatory responses, development and differentiation, as well as processes that are involved in cancer development such as cell-cycle regulation and apoptosis. BNO1 has also been shown to interact with Skp1, an essential component of SCF ubiquitin-E3 ligases.

[0046] A strong precedent for a tumour suppressor protein belonging to the ubiquitin-proteasome degradation system has previously been provided by the VHL gene. This gene has been demonstrated to associate with elongin C, elongin B, and cullin-2 in a complex termed VCB-CUL-2. This multiprotein complex exhibits structural and functional similarity to SCF ubiquitin ligases and has been shown to be involved in the ubiquitination of VHL substrates.

[0047] Collectively, this information suggests BNO1 is involved in the processes that lead to cancer, particularly breast and prostate carcinoma, most likely through its role in the ubiquitination of proteins involved in important cellular functions such as cell cycle regulation. As BNO1 is expressed in many tissue types, alterations in BNO1 function may also cause pathologies in these tissues through consequential abnormalities in the ubiquitination process.

[0048] With the identification of the BNO1 nucleotide and protein sequence, probes and antibodies raised to the gene can be used in a variety of hybridisation and immunological assays to screen for and detect the presence of either a normal or mutated gene or gene product. In addition the nucleotide and protein sequence of the BNO1 gene provided in this invention enables therapeutic methods for the treatment of all diseases associated with abnormalities of BNO1 function, including cancer, immune/inflammatory disease and neurological disorders, and also enables methods for the diagnosis or prognosis of all diseases associated with abnormalities of BNO1 function.

[0049] Examples of such disorders include, but are not limited to, cancers, immune/inflammatory disorders and neurological disorders. Cancers include adenocarcinoma, leukemia, lymphoma, melanoma, myeloma, sarcoma, teratocarcinoma, and, in particular, cancers of the breast, prostate, liver, ovary, head and neck, heart, brain, pancreas, lung, skeletal muscle, kidney, colon, uterus, testis, adrenal gland, blood, germ cells, placenta, synovial membrane, tonsil, cervix, lymph tissue, skin, bladder, spinal cord, thyroid gland and stomach. Other cancers may include those of the bone, bone marrow, gall bladder, ganglia, gastrointestinal tract, parathyroid, penis, salivary glands, spleen and thymus. Immune/inflammatory disorders include acquired immunodeficiency syndrome (AIDS), Addison's disease, adult respiratory distress syndrome, allergies, ankylosing spondylitis, amyloidosis, anemia, asthma, atherosclerosis. autoimmune hemolytic anemia, autoimmune thyroiditis, autoimmune polyenodocrinopathy-candidiasis-ectodermal dystrophy (APECED), bronchitis, cholecystitis, contact dermatitis, Crohn's disease, cystic fibrosis, atopic dermatitis, dermatomyositis, diabetes mellitus, emphysema, episodic lymphopenia with lymphocytotoxins, erythroblastosis fetalis, erythema nodosum, atrophic gastritis, glomerulonephritis, Goodpasture's syndrome, gout, Graves' disease, Hashimoto's thyroiditis, hypereosinophilia, irritable bowel syndrome, multiple sclerosis, myasthenia gravis, myocardial or pericardial inflammation, osteoarthritis, osteoporosis, pancreatitis, polymyositis, psoriasis, Reiter's syndrome, rheumatoid arthritis, scleroderma, Sjogren's syndrome, systemic anaphylaxis, systemic lupus erythematosus, systemic sclerosis, thrombocytopenic purpura, ulcerative colitis, uveitis, Werner syndrome, complications of wound healing (eg scarring), cancer, hemodialysis, and extracorporeal. circulation, viral, bacterial, fungal, parasitic, protozoal, and helminthic infections, and trauma. Neurological disorders may include Parkinson's disease and Alzheimer's disease.

[0050] In the treatment of diseases associated with decreased BNO1 expression and/or activity, it is desirable to increase the expression and/or activity of BNO1. In the treatment of disorders associated with increased BNO1 expression and/or activity, it is desirable to decrease the expression. and/or activity of BNO1.

Enhancing BNO1 gene or protein function

[0051] Enhancing, stimulating or re-activating BNO1 gene or protein function can be achieved in a variety of ways. We also disclose the administration of an isolated DNA molecule, as described above, to a subject in need of such treatment may be initiated.

[0052] Typically, BNO1 is administered to a subject to treat or prevent a disorder associated with decreased activity and/or expression of BNO1.

[0053] In a further aspect, there is provided the use of an isolated DNA molecule, as described above, in the manufacture of a medicament for the treatment of a disorder associated with decreased activity and/or expression of BNO1.

[0054] Typically, a vector capable of expressing BNO1 or a fragment or derivative thereof may be administered to a subject to treat or prevent a disorder associated with decreased activity and/or expression of TSG18 including, but not limited to, those described above. Transducing retroviral vectors are often used for somatic cell gene therapy because of their high efficiency of infection and stable integration and expression. The full length BNO1 gene, or portions thereof, can be cloned into a retroviral vector and driven from its endogenous promoter or from the retroviral long terminal repeat or from a promoter specific for the target cell type of interest. Other viral vectors can be used and include, as is known in the art, adenoviruses, adeno-associated virus, vaccinia virus, papovaviruses, lentiviruses and retroviruses of avian, murine and human origin.

[0055] Gene therapy would be carried out according to established methods (Friedman, 1991; Culver, 1996). A vector containing a copy of the BNO1 gene linked to expression control elements and capable of replicating inside the cells is prepared. Alternatively the vector may be replication deficient and may require helper cells or helper virus for replication and virus production and use in gene therapy.

[0056] Gene transfer using non-viral methods of infection can also be used. These methods include direct injection of DNA, uptake of naked DNA in the presence of calcium phosphate, electroporation, protoplast fusion or liposome delivery. Gene transfer can also be achieved by delivery as a part of a human artificial chromosome or receptor-mediated gene transfer. This involves linking the DNA to a targeting molecule that will bind to specific cell-surface receptors to induce endocytosis and transfer of the DNA into mammalian cells. One such technique uses poly-L-lysine to link asialoglycoprotein to DNA. An adenovirus is also added to the complex to disrupt the lysosomes and thus allow the DNA to avoid degradation and move to the nucleus. Infusion of these particles intravenously has resulted in gene transfer into hepatocytes.

[0057] In affected subjects that express a mutated form of BNO1 it may be possible to prevent the disorder by introducing into the affected cells a wild-type copy of the gene such that it recombines with the mutant gene. This requires a double recombination event for the correction of the gene mutation. Vectors for the introduction of genes in these ways are known in the art, and any suitable vector may be used. Alternatively, introducing another copy of the gene bearing a second mutation in that gene may be employed so as to negate the original gene mutation and block any negative effect.

[0058] In affected subjects that have decreased expression of BNO1, a mechanism of down-regulation may be abnormal methylation of the CpG island present in the 5' end of the gene. Therefore, in an alternative approach to therapy,

administration of agents that remove BNO1 promoter methylation will reactivate BNO1 gene expression and may suppress the associated disease phenotype.

[0059] In a further aspect, a suitable agonist may also include a small molecule or peptide that can mimic the function of wild-type BNO1.

Inhibiting BNo1 gene or protein function

[0060] Inhibiting the function of a mutated gene or protein can be achieved in a variety of ways. We also disclose a method of treating a disorder associated with increased activity and/or expression of BNO1, comprising administering an antagonist of BNO1 to a subject in need of such treatment.

[0061] In still another aspect of the invention there is provided the use of an antagonist of BNO1 in the manufacture of a medicament for the treatment of a disorder associated with increased activity and/or expression of BNO1.

[0062] Such disorders may include, but are not limited to, those discussed above. In one aspect of the invention an isolated DNA molecule, which is the complement of any one of the DNA molecules described above and which encodes an RNA molecule that hybridises with the mRNA encoded by BNO1, may be administered to a subject in need of such treatment.

[0063] In a still further aspect of the invention there is provided the use of an isolated DNA molecule which is the complement of a DNA molecule of the invention and which encodes an RNA molecule that hybridises with the mRNA encoded by BNO1, in the manufacture of a medicament for the treatment of a disorder associated with increased activity and/or expression of BNO1.

[0064] Typically, a vector expressing the complement of the polynucleotide encoding BNO1 may be administered to a subject to treat or prevent a disorder associated with increased activity and/or expression of BNO1 including, but not limited to, those described above. Antisense strategies may use a variety of approaches including the use of antisense oligonucleotides, ribozymes, DNAzymes, injection of antisense RNA and transfection of antisense RNA expression vectors. Many methods for introducing vectors into cells or tissues are available and equally suitable for use *in vivo*, *in vitro,* and *ex vivo.* For *ex vivo* therapy, vectors may be introduced into stem cells except human embryonic stem cells taken from the patient and clonally propagated for autologous transplant back into that same patient. Delivery by transfection, by liposome injections, or by polycationic amino polymers may be achieved using methods which are well known in the art. (For example, see Goldman et *al.,* 1997).

[0065] We also disclose a method of treating a disorder associated with increased activity and/or expression of BNO1 comprising administering an antagonist of BNO1 to a subject in need of such treatment.

[0066] In still another aspect of the invention there is provided the use of an antagonist of BNO1 in the manufacture of a medicament for the treatment of a disorder associated with increased activity and/or expression of BNO1.

[0067] Such disorders may include, but are not limited to, those discussed above. In one aspect purified protein according to the invention may be used to produce antibodies which specifically bind BNO1. These antibodies may be used directly as an antagonist or indirectly as a targeting or delivery mechanism for bringing a pharmaceutical agent to cells or tissues that express BNO1. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric and single chain antibodies as would be understood by the person skilled in the art.

[0068] For the production of antibodies, various hosts including rabbits, rats, goats, mice, humans, and others may be immunized by injection with a protein of the invention or with any fragment or oligopeptide thereof, which has immunogenic properties. various adjuvants may be used to increase immunological response and include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface-active substances such as lysolecithin. Adjuvants used in humans include BCG (bacilli Calmette-Guerin) and Corynebacterium parvum.

[0069] It is preferred that the oligopeptides, peptides, or fragments used to induce antibodies to BNO1 have an amino acid sequence consisting of at least about 5 amino acids, and, more preferably, of at least about 10 amino acids. It is also preferable that these oligopeptides, peptides, or fragments are identical to a portion of the amino acid sequence of the natural protein and contain the entire amino acid sequence of a small, naturally occurring molecule. Short stretches of amino acids from these proteins may be fused with those of another protein, such as KLH, and antibodies to the chimeric molecule may be produced.

[0070] Monoclonal antibodies to BNO1 may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique. (For example, see Kohler et al., 1975; Kozbor *et al.,* 1985; Cote *et al.,* 1983; Cole *et al.,* 1984).

[0071] Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature. (For example, see Orlandi *et al.,* 1989; Winter *et al.,* 1991).

[0072] Antibody fragments which contain specific binding sites for BNO1 may also be generated. For example, such fragments include, F(ab')2 fragments produced by pepsin digestion of the antibody molecule and Fab fragments gen-

erated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity. (For example, see Huse *et al.,* 1989).

[0073] Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between a protein and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes is preferred, but a competitive binding assay may also be employed.

[0074] We also disclose a method for the treatment of a disorder shown to be associated with abnormal activity and/or expression of BNO1, comprising administering a nucleic acid molecule, antibody or compound as described above, to a subject in need of such treatment.

[0075] In another aspect the invention provides the use of a nucleic acid molecule, antibody or compound as described above, in the manufacture of a medicament for the treatment of a disorder shown to be associated with abnormal activity and/or expression of BNO1.

[0076] In a further aspect a pharmaceutical composition comprising a nucleic acid molecule, antibody or compound as described above, and a pharmaceutically acceptable carrier may be administered.

[0077] The pharmaceutical composition may be administered to a subject to treat or prevent a disorder associated with abnormal activity and/or expression of BNO1 including, but not limited to, those provided above. Pharmaceutical compositions in accordance with the present invention are prepared by mixing BNO1 or active fragments or variants thereof having the desired degree of purity, with acceptable carriers, excipients, or stabilizers which are well known. Acceptable carriers, excipients or stabilizers are nontoxic at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including absorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitrol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG).

[0078] In further embodiments, any of the genes, peptides, antagonists, antibodies, complementary sequences, or vectors of the invention may be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents may be made by those skilled in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents may act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, therapeutic efficacy with lower dosages of each agent may be possible, thus reducing the potential for adverse side effects.

Drug screening

[0079] According to still another aspect of the invention, peptides of the invention, particularly purified BNO1 polypeptides, and cells expressing these are useful for screening of candidate pharmaceutical agents in a variety of techniques for the treatment of disorders associated with BNO1 dysfunction. Such techniques include, but are not limited to, utilising eukaryotic or prokaryotic host cells that are stably transformed with recombinant molecules expressing the BNO1 polypeptide or fragment thereof, preferably in competitive binding assays. Binding assays will measure for the formation of complexes between the BNO1 polypeptide, or fragments thereof, and the agent being tested, or will measure the degree to which an agent being tested will interfere with the formation of a complex between the BNO1 polypeptide, or fragment thereof, and a known ligand, particularly other members of the SCF complex and BNO1 substrates targeted for ubiquitination.

[0080] Another technique for drug screening provides high-throughput screening for compounds having suitable binding affinity to the BNO1 polypeptide (see PCT published application WO84/03564). In this stated technique, large numbers of small peptide test compounds can be synthesised on a solid substrate and can be assayed through BNO1 polypeptide binding and washing. Bound BNO1 polypeptide is then detected by methods well known in the art. In a variation of this technique, purified polypeptides can be coated directly onto plates to identify interacting test compounds.

[0081] An additional method for drug screening involves the use of host eukaryotic cell lines which carry mutations in the BNO1 gene. The host cell lines are also defective at the polypeptide level. Other cell lines may be used where the gene expression of BNO1 can be switched off. The host cell lines or cells are grown in the presence of various drug compounds and the rate of growth of the host cells is measured to determine if the compound is capable of regulating the growth of defective cells.

[0082] BNO1 polypeptide may also be used for screening compounds developed as a result of combinatorial library technology. This provides a way to test a large number of different substances for their ability to modulate activity of a polypeptide. The use of peptide libraries is preferred (see patent WO97/02048) with such libraries and their use known

in the art.

[0083] A substance identified as a modulator of polypeptide function may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many in vivo pharmaceutical applications. In addition, a mimic or mimetic of the substance may be designed for pharmaceutical use. The design of mimetics based on a known pharmaceutically active compound ("lead" compound) is a common approach to the development of novel pharmaceuticals. This is often desirable where the original active compound is difficult or expensive to synthesise or where it provides an unsuitable method of administration. In the design of a mimetic, particular parts of the original active compound that are important in determining the target property are identified. These parts or residues constituting the active region of the compound are known as its pharmacophore. Once found, the pharmacophore structure is modelled according to its physical properties using data from a range of sources including x-ray diffraction data and NMR. A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be added. The selection can be made such that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, does not degrade *in vivo* and retains the biological activity of the lead compound. Further optimisation or modification can be carried out to select one or more final mimetics useful for *in vivo* or clinical testing.

[0084] It is also possible to isolate a target-specific antibody and then solve its crystal structure. In principle, this approach yields a pharmacophore upon which subsequent drug design can be based as described above. It may be possible to avoid protein crystallography altogether by generating anti-idiotypic antibodies (anti-ids) to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of the anti-ids would be expected to be an analogue of the original binding site. The anti-id could then be used to isolate peptides from chemically or biologically produced peptide banks.

[0085] Any of the therapeutic methods described above may be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

Diagnostic and prognostic applications

[0086] Polynucleotide sequences encoding BNO1 may be used for the in-vitro diagnosis or prognosis of disorders associated with BNO1 dysfunction, or a predisposition to such disorders. Examples of such disorders include, but are not limited to, cancers, immune/inflammatory disorders and neurological disorders. Cancers include adenocarcinoma, leukemia, lymphoma, melanoma, myeloma, sarcoma, teratocarcinoma, and, in particular, cancers of the breast, prostate, liver, ovary, head and neck, heart, brain, pancreas, lung, skeletal muscle, kidney, colon, uterus, testis, adrenal gland, blood, germ cells, placenta, synovial membrane, tonsil, cervix, lymph tissue, skin, bladder, spinal cord, thyroid gland and stomach. Other cancers may include those of the bone, bone marrow, gall bladder, ganglia, gastrointestinal tract, parathyroid, penis, salivary glands, spleen and thymus. Immune/inflammatory disorders include acquired immunodeficiency syndrome (AIDS), Addison's disease, adult respiratory distress syndrome, allergies, ankylosing spondylitis, amyloidosis, anemia, asthma, atherosclerosis. autoimmune hemolytic anemia, autoimmune thyroiditis, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), bronchitis, cholecystitis, contact dermatitis, Crohn's disease, cystic fibrosis, atopic dermatitis, dermatomyositis, diabetes mellitus, emphysema, episodic lymphopenia with lymphocytotoxins, erythroblastosis fetalis, erythema nodosum, atrophic gastritis, glomerulonephritis, Goodpasture's syndrome, gout, Graves' disease, Hashimoto's thyroiditis, hypereosinophilia, irritable bowel syndrome, multiple sclerosis, myasthenia gravis, myocardial or pericardial inflammation, osteoarthritis, osteoporosis, pancreatitis, polymyositis, psoriasis, Reiter's syndrome, rheumatoid arthritis, sclerodernna, Sjogren's syndrome, systemic anaphylaxis, systemic lupus erythematosus, systemic sclerosis, thrombocytopenic purpura, ulcerative colitis, uveitis, Werner syndrome, complications of wound healing (eg scarring), cancer, hemodialysis, and extracorporeal circulation, viral, bacterial, fungal, parasitic, protozoal, and helminthic infections, and trauma. Neurological disorders may include Parkinson's disease and Alzheimer's disease.

[0087] diagnosis or prognosis may be used to determine the severity, type or stage of the disease state in order to initiate an appropriate therapeutic intervention.

[0088] In another embodiment of the invention, the polynucleotides that may be used for diagnostic or prognostic purposes include oligonucleotide sequences, genomic DNA and complementary RNA and DNA molecules. The polynucleotides may be used to detect and quantitate gene expression in biopsied tissues in which mutations in BNO1 or abnormal expression of BNO1 may be correlated with disease. Genomic DNA used for the diagnosis or prognosis may be obtained from body cells, such as those present in the blood, tissue biopsy, surgical specimen, or autopsy material. The DNA may be isolated and used directly for detection of a specific sequence or may be amplified by the polymerase chain reaction (PCR) prior to analysis. Similarly, RNA or cDNA may also be used, with or without PCR amplification. To detect a specific nucleic acid sequence, direct nucleotide sequencing, reverse transcriptase PCR (RT-PCR), hybridization using specific oligonucleotides, restriction enzyme digest and mapping, PCR mapping, RNAse protection, and various other methods may be employed. Oligonucleotides specific to particular sequences can be chemically synthesized and labelled radioactively or non-radioactively and hybridised to individual samples immobilized on membranes or other

solid-supports or in solution. The presence, absence or excess expression of BNO1 may then be visualized using methods such as autoradiography, fluorometry, or colorimetry.

[0089] In a particular aspect, the nucleotide sequences encoding BNO1 may be useful in assays that detect the presence of associated disorders, particularly those mentioned previously. The nucleotide sequences encoding BNO1 may be labelled by standard methods and added to a fluid or tissue sample from a patient under conditions suitable for the formation of hybridization complexes. After a suitable incubation period, the sample is washed and the signal is quantitated and compared with a standard value. If the amount of signal in the patient sample is significantly altered in comparison to a control sample then the presence of altered levels of nucleotide sequences encoding BNO1 in the sample indicates the presence of the associated disorder. Such assays may also be used to evaluate the efficacy of at particular therapeutic treatment regimen in animal studies, in clinical trials, or to monitor the treatment of an individual patient.

[0090] In order to provide a basis for the diagnosis or prognosis of a disorder shown to be associated with a mutation in BNO1, the nucleotide sequence of the BNO1 gene can be compared between normal tissue and diseased tissue in order to establish whether the patient expresses a mutant gene.

[0091] In order to provide a basis for the diagnosis or prognosis of a disorder shown to be associated with abnormal expression of BNO1, a normal or standard profile for expression is established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, encoding BNO1, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the values obtained from normal subjects with values from an experiment in which a known amount of a substantially purified polynucleotide is used. Another method to identify a normal or standard profile for expression of BNO1 is through quantitative RT-PCR studies. RNA isolated from body cells of a normal individual, particularly RNA isolated from tumour cells, is reverse transcribed and real-time PCR using oligonucleotides specific for the BNO1 gene is conducted to establish a normal level of expression of the gene.

[0092] Standard values obtained in both these examples may be compared with values obtained from samples from patients who are symptomatic for a disorder. Deviation from standard values is used to establish the presence of a disorder.

[0093] Once the presence of a disorder is established and a treatment protocol is initiated, hybridization assays or quantitative RT-PCR studies may be repeated on a regular basis to determine if the level of expression in the patient begins to approximate that which is observed in the normal subject. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several days to months.

[0094] In one aspect, hybridization with PCR probes which are capable of detecting polynucleotide sequences, including genomic sequences, encoding BNO1 or closely related molecules may be used to identify nucleic acid sequences which encode BNO1. The specificity of the probe, whether it is made from a highly specific region, e.g., the 5' regulatory region, or from a less specific region, e.g., a conserved motif, and the stringency of the hybridization or amplification will determine whether the probe identifies only naturally occurring sequences encoding BNO1, allelic variants, or related sequences.

[0095] Probes may also be used for the detection of related sequences, and should preferably have at least 50% sequence identity to any of the BNO1 encoding sequences. The hybridization probes of the subject invention may be DNA or RNA and may be derived from the sequence of SEQ ID Numbers: 1 or 3 or from genomic sequences including promoters, enhancers, and introns of the BNO1 gene (SEQ ID Numbers: 5-11).

[0096] Means for producing specific hybridization probes for DNAs encoding BNO1 include the cloning of polynucleotide sequences encoding BNO1 or BNO1 derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, and are commercially available. Hybridization probes may be labelled by radionuclides such as ^{32}P or ^{35}S, or by enzymatic labels, such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, or other methods known in the art.

[0097] According to a further aspect of the invention there is provided the use of a polypeptide as described above in the diagnosis or prognosis of a disorder shown to be associated with BNO1, or a predisposition to such disorders.

[0098] When a diagnostic or prognostic assay is to be based upon the BNO1 protein, a variety of approaches are possible. For example, diagnosis or prognosis can be achieved by monitoring differences in the electrophoretic mobility of normal and mutant proteins. Such an approach will be particularly useful in identifying mutants in which charge substitutions are present, or in which insertions, deletions or substitutions have resulted in a significant change in the electrophoretic migration of the resultant protein. Alternatively, diagnosis may be based upon differences in the proteolytic cleavage patterns of normal and mutant proteins, differences in molar ratios of the various amino acid residues, or by functional assays demonstrating altered function of the gene products.

[0099] In another aspect, antibodies that specifically bind BNO1 may be used for the diagnosis or prognosis of disorders characterized by abnormal expression of BNO1, or in assays to monitor patients being treated with BNO1 or agonists, antagonists, or inhibitors of BNO1. Antibodies useful for diagnostic purposes may be prepared in the same manner as described above for therapeutics. Diagnostic or prognostic assays for BNO1 include methods that utilize the antibody

and a label to detect BNO1 in human body fluids or in extracts of cells or tissues. The antibodies may be used with or without modification, and may be labelled by covalent or non-covalent attachment of a reporter molecule.

[0100] A variety of protocols for measuring BNO1, including ELISAs, RIAs, and FACS, are known in the art and provide a basis for diagnosing altered or abnormal levels of BNO1 expression. Normal or standard values for BNO1 expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to BNO1 under conditions suitable for complex formation. The amount of standard complex formation may be quantitated by various methods, preferably by photometric means. Quantities of BNO1 expressed in subject, control, and disease samples from biopsied tissues are compared with the standard values. Deviation between standard and subject values establishes the parameters for diagnosing disease.

[0101] Once an individual has been diagnosed with a disorder, effective treatments can be initiated. These may include administering a selective agonist to the mutant BNO1 so as to restore its function to a normal level or introduction of wild-type BNO1, particularly through gene therapy approaches as described above. Typically, a vector capable of expressing the appropriate full-length BNO1 gene or a fragment or derivative thereof may be administered. In an alternative approach to therapy, substantially purified BNO1 polypeptide and a pharmaceutically acceptable carrier may be administered as described above or drugs which can replace the function of, or mimic the action of BNO1 may be administered.

[0102] In the treatment of disorders shown to be associated with increased BNO1 expression and/or activity, the affected individual may be treated with a selective antagonist such as an antibody to the relevant protein or an antisense (complement) probe to the corresponding gene as described above, or through the use of drugs which may block the action of BNO1.

Microarray

[0103] In further embodiment, complete cDNAs, oligonucleotides or longer fragments derived from any of the polynucleotide sequences described herein may be used as targets in a microarray. The microarray can be used to monitor the expression level of large numbers of genes simultaneously and to identify genetic variants, mutations, and polymorphisms. This information may be used to determine gene function, to understand the genetic basis of a disorder, to diagnose or prognose a disorder, and to develop and monitor the activities of therapeutic agents. Microarrays may be prepared, used, and analyzed using methods known in the art. (For example, see Schena et al., 1996; Heller et al., 1997).

Transformed hosts

[0104] The present invention also provides for the production of genetically modified (knock-out, knock-in and transgenic), non-human animal models transformed with the DNA molecules of the invention. These animals are useful for the study of the BNO1 gene function, to study the mechanisms of disease as related to the BNO1 gene, for the screening of candidate pharmaceutical compounds, for the creation of explanted mammalian cell cultures which express the protein or mutant protein and for the evaluation of potential therapeutic interventions.

[0105] The BNO1. gene may have been inactivated by knock-out deletion, and knock-out genetically modified non-human animals are therefore provided.

[0106] Animal species which are suitable for use in the animal models of the present invention are rats, mice, hamsters; guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates such as monkeys and chimpanzees. For initial studies, genetically modified mice and rats are highly desirable due to their relative ease of maintenance and shorter life spans. For certain studies, transgenic yeast or invertebrates may be suitable and preferred because they allow for rapid screening and provide for much easier handling. For longer term studies, non-human primates may be desired due to their similarity with humans.

[0107] To create an animal model for mutated BNO1 several methods can be employed. These include generation of a specific mutation in a homologous animal gene, insertion of a wild type human gene and/or a humanized animal gene by homologous recombination, insertion of a mutant (single or multiple) human gene as genomic or minigene cDNA constructs using wild type or mutant or artificial promoter elements or insertion of artificially modified fragments of the endogenous gene by homologous recombination. The modifications include insertion of mutant stop codons, the deletion of DNA sequences, or the inclusion of recombination elements (lox p sites) recognized by enzymes such as Cre recombinase.

[0108] To create a transgenic mouse, which is preferred, a mutant version of BNO1 can be inserted into a mouse germ line using standard techniques of oocyte microinjection or transfection or microinjection into embryonic stem cells. Alternatively, if it is desired to inactivate or replace the endogenous BNO1 gene, homologous recombination using marine embryonic stem cells may be applied.

[0109] For oocyte injection, one or more copies of the mutant or wild type BNO1 gene can be inserted into the pronucleus of a just-fertilized mouse oocyte. This oocyte is then reimplanted into a pseudo-pregnant foster mother. The liveborn mice can then be screened for integrants using analysis of tail DNA for the presence of human BNO1 gene sequences.

The transgene can be either a complete genomic sequence injected as a YAC, BAC, PAC or other chromosome DNA fragment, a cDNA with ether the natural promoter or a heterologous promoter, or a minigene containing all of the coding region and other elements found to be necessary for optimum expression.

[0110] According to still another aspect of the invention there is provided the use of genetically modified non-human animals as described above for the screening of candidate pharmaceutical compounds.

[0111] The identification of the nucleotide and amino acid sequence of both isoforms of BNO1 enables the identification of BNO1-specific protein substrates using protein interaction studies such as the yeast two-hybrid analysis as would be understood by those skilled in the art. These protein substrates would be targets for degradation via ubiquitination mediated by the BNO1-containing ubiquitin-E3 ligase. Each isoform of BNO1 may share common protein substrates or may interact with isoform-specific substrates.

[0112] We also disclose a complex of wild-type BNO1 and a BNO1-specific substrate that is targeted for degradation by ubiquitination.

[0113] We also disclose a complex of BNO1 and proteins of the ubiquitin-E3 ligase complex.

[0114] We also disclose a complex of wild-type BNO1 and the Skp1 protein.

[0115] We also disclose a nucleic acid encoding a mutant BNO1 polypeptide which cannot form a complex with wild-type proteins with which wild-type BNO1 does form a complex. Typically one of these proteins is Skp1 while others are BNO1-specific protein substrates targeted for degradation by ubiquitination.

[0116] We also disclose a mutant BNO1 polypeptide which cannot form a complex with wild-type proteins with which wild-type BNO1 does form a complex. Typically one of these proteins is Skp1 while others are BNO1-specific protein substrates targeted for degradation by ubiquitination.

[0117] We also disclose the use of complexes as described above in screening for candidate pharmaceutical compounds. One may also screen for a drug which replaces the activity of BNO1 in a patient deficient in BNO1.

[0118] It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art, in Australia or in any other country. Throughout this specification and the claims, the words "comprise", "comprises" and "comprising" are used in a non-exclusive sense, except where the context requires otherwise.

Brief Description of the Drawings

[0119]

Figure 1. Schematic representation of tumours with interstitial and terminal allelic loss on chromosome arm 16q in the two series of tumour samples. Polymorphic markers are listed according to their order on 16q from centromere to telomere and the markers used for each series are indicated by X. Tumour identification numbers are shown at the top of each column. At the right of the figure, the three smallest regions of loss of heterozygosity are indicated.

Figure 2. Northern blot analysis of the BNO1 gene. The size of the BNO1 gene in kilobases is indicated by an arrow on the left of the Northern. The blot contained RNA from the following tissues: 1: Mammary gland; 2: Bone marrow; 3: Testis; 4: Ovary; 5: Uterus; 6: Prostate; 7: Stomach; 8: Bladder; 9: Spinal cord; 10: Brain; 11: Pancreas; 12: Thyroid. A single band of approximately 3.6 Kb was seen in all tissues except bone marrow. Strongest expression of the gene was seen in the brain.

Figure 3. BNO1 F-box sequence alignment compared with the F-box consensus sequence as reported by Kipreos and Pagano, (2000). The single letter amino-acid code is used. Bold capital letters indicate residues found in over 40% of F-box sequences; non-bold capital letters indicate residues found in 20-40% of F-box sequences; bold, lower case letters indicate residues found in 15-19% of the F-boxes; non-bold lower case letters indicate residues found in 10-14% of F-boxes. The top line represents the F-box motif of BNO1 indicating a high degree of homology with the consensus.

Figure 4. Quantitative RT-PCR expression analysis of the BNO1 gene in breast cancer cell lines. BNO1 copy numbers in normalized normal mammary gland (breast) cDNA were arbitrarily set to a 'baseline' of 1.0e+06 (empty bar). Breast cancer cell lines and other normal tissue cDNA copy numbers were calculated relative to the 'baseline'. Grey filled bars represent amplicon fold expression down-regulation compared to the baseline reference, while black filled bars represent amplicon fold expression up-regulation from the baseline reference. Note: replicate cell lines (a and b) represent independent cell cultures, total RNA isolation and reverse transcription reactions. Replicates served as another level of control to monitor the variability in gene expression resulting from differences in cell confluency, total RNA integrity and reverse transcription efficiencies.

Modes for performing the invention

EXAMPLE 1: Collection of breast cancer patient material

[0120] Two series of breast cancer patients were analysed for this study. Histopathological classification of each tumour specimen was carried out by our collaborators according to World Health Organisation criteria (WHO, 1981). Patients were graded histopathologically according to the modified Bloom and Richardson method (Elston and Ellis, 1990) and patient material was obtained upon approval of local Medical Ethics Committees. Tumour tissue DNA and peripheral blood DNA from the same individual was isolated as previously described (Devilee et al., 1991) using standard laboratory protocols.

[0121] Series 1 consisted of 189 patients operated on between 1986 and 1993 in three Dutch hospitals, a Dutch University and two peripheral centres. Tumour tissue was snap frozen within a few hours of resection. For DNA isolation, a tissue block was selected only if it contained at least 50% of tumour cells following examination of haematoxilin and eosin stained tissue sections by a pathologist. Tissue blocks that contained fewer than 50% of tumour cells were omitted from further analysis.

[0122] Series 2 consisted of 123 patients operated on between 1987 and 1997 at the Flinders Medical Centre in Adelaide, Australia. Of these, 87 were collected as fresh specimens within a few hours of surgical resection, confirmed as malignant tissue by pathological analysis, snap frozen in liquid nitrogen, and stored at -70°C. The remaining 36 tumour tissue samples were obtained from archival paraffin embedded tumour blocks. Prior to DNA isolation, tumour cells were microdissected from tissue sections mounted on glass slides so as to yield at least 80% tumour cells. In some instances, no peripheral blood was available such that pathologically identified paraffin embedded non-malignant lymph node tissue was used instead.

EXAMPLE 2: LOH analysis of chromosome 16q markers in breast cancer samples.

[0123] In order to identify the location of genes associated with breast cancer, LOH analysis of tumour samples was conducted. A total of 45 genetic markers mapping to chromosome 16 were used for the LOH analysis of the breast tumour and matched normal DNA samples collected for this study. Figure 1 indicates for which tumour series they were used and their cytogenetic location. Details regarding all markers can be obtained from the Genome Database (GDB) at http://www.gdb.org. The physical order of markers with respect to each other was determined from a combination of information in GDB, by mapping on a chromosome 16 somatic cell hybrid map (Callen et al., 1995) and by genomic sequence information.

[0124] Four alternative methods were used for the LOH analysis:

1) For RFLP and VNTR markers, Southern blotting was used to test for allelic imbalance. These markers were used on only a subset of samples. Methods used were as previously described (Devilee et al., 1991).

2) Microsatellite markers were amplified from tumour and normal DNA using the polymerase chain reaction (PCR) incorporating standard methodologies (Weber and May, 1989; Sambrook et al., 1989). A typical reaction consisted of 12 μl and contained 100 ng of template, 5 pmol of both primers, 0.2 mM of each dNTP, 1 μCurie [α-^{32}P]dCTP, 1.5 mM $MgCl_2$, 1.2 μl Supertaq buffer and 0.06 units of Supertaq (HT biotechnologies). A Phosphor Imager type 445 SI (Molecular Dynamics, Sunnyvale, CA) was used to quantify ambiguous results. In these cases, the Allelic Imbalance Factor (AIF) was determined as the quotient of the peak height ratios from the normal and tumour DNA pair. The threshold for allelic imbalance was defined as a 40% reduction of one allele, agreeing with an AIF of ≥ 1.7 or ≤ 0.59. This threshold is in accordance with the selection of tumour tissue blocks containing at least 50% tumour cells with a 10% error-range. The threshold for retention has been previously determined to range from 0.76 to 1.3 (Devilee et al., 1994). This leaves a range of AIFs (0.58 - 0.75 and 1.31 - 1.69) for which no definite decision has been made. This "grey area" is indicated by grey boxes in Figure 1 and tumours with only "grey area" values were discarded completely from the analysis.

3) The third method for determining allelic imbalance was similar to the second method above, however radioactively labelled dCTP was omitted. Instead, PCR of polymorphic microsatellite markers was done with one of the PCR primers labelled fluorescently with FAM, TET or HEX. Analysis of PCR products generated was on an ABI 377 automatic sequencer (PE Biosystems) using 6% polyacrylamide gels containing 8M urea. Peak height values and peak sizes were analysed with the GeneScan programme (PE Biosystems). The same thresholds for allelic imbalance, retention and grey areas were used as for the radioactive analysis.

4) An alternative fluorescent based system was also used. In this instance PCR primers were labelled with fluorescein or hexachlorofluorescein. PCR reaction volumes were 20 μl and included 100 ng of template, 100 ng of each primer, 0.2 mM of each dNTP, 1-2 mM $MgCl_2$, 1X AmpliTaq Gold buffer and 0.8 units AmpliTaq Gold enzyme (Perkin Elmer). Cycling conditions were 10 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, followed by 25

cycles of 94°C 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, with a final extension of 72°C for 10 minutes. PCR amplimers were analysed on an ABI 373 automated sequencer (PE Biosystems) using the GeneScan programme (PE Biosystems). The threshold range of AIF for allele retention was defined as 0.61 - 1.69, allelic loss as ≤0.5 or ≥2.0, or the "grey area" as 051 - 0.6 or 1.7 - 1.99.

[0125] The first three methods were applied to the first tumour series while the last method was adopted for the second series of tumour samples. For statistical analysis, a comparison of allelic imbalance data for validation of the different detection methods and of the different tumour series was done using the Chi-square test.

[0126] The identification of the smallest region of overlap (SRO) involved in LOH is instrumental for narrowing down the location of the gene targeted by LOH. Figure 1 shows the LOH results for tumour samples, which displayed small regions of loss (ie interstitial and telomeric LOH) and does not include samples that showed complex LOH (alternating loss and retention of markers). When comparing the two sample sets at least three consistent regions emerge with two being at the telomere in band 16q24.3 and one at 16q22.1. The region at 16q22.1 is defined by the markers D16S398 and D16S301 and is based on the interstitial LOH events seen in three tumours from series 1 (239/335/478) and one tumour from series 2 (237). At the telomere (16q24.2 - 16q24.3), the first region is defined by the markers D16S498 and D16S3407 and is based on four tumours from series 2 (443/75/631/408) while the second region (16q24.3) extends from D16S3407 to the telomere and is based on one tumour from series 1 (559) and three from series 2 (97/240/466). LOH limited to the telomere but involving both of the regions identified at this site could be found in an additional 17 tumour samples.

[0127] Other studies have shown that the long arm of chromosome 16 is also a target for LOH in prostate, lung, hepatocellular, ovarian, rhabdomyosarcoma and Wilms' tumours. Detailed analysis of prostate carcinomas has revealed an overlap in the smallest regions of LOH seen in this cancer to that seen with breast cancer which suggests that 16q harbours a gene implicated in many tumour types.

EXAMPLE 3: Construction of a physical map of 16q24.3

[0128] To identify novel candidate breast cancer genes mapping to the smallest regions of overlap at 16q24.3, a clone based physical map contig covering this region was needed. At the start of this phase of the project the most commonly used and readily accessible cloned genomic DNA fragments were contained in lambda, cosmid or YAC vectors. During the construction of whole chromosome 16 physical maps, clones from a number of YAC libraries were incorporated into the map (Doggett *et al*., 1995). These included clones from a flow-sorted chromosome 16-specific YAC-library (McCormick *et al*., 1993), from the CEPH Mark I and MegaYAC libraries and from a half-telomere YAC library (Riethman *et al*., 1989). Detailed STS and Southern. analysis of YAC clones mapping at 16q24.3 established that very few were localised between the CY2/CY3 somatic cell hybrid breakpoint and the long arm telomere. However, those that were located in this region gave inconsistent mapping results and were suspected to be rearranged or deleted. Coupled with the fact that YAC clones make poor sequencing substrates, and the difficulty in isolating the cloned human DNA, a physical map based on cosmid clones was the initial preferred option.

[0129] A flow-sorted chromosome 16 specific cosmid library had previously been constructed (Longmire et al., 1993), with individual cosmid clones gridded in high-density arrays onto nylon membranes. These filters collectively contained ~15,000 clones representing an approximately 5.5 fold coverage of chromosome 16. Individual cosmids mapping to the critical regions at 16q24.3 were identified by the hybridisation of these membranes with markers identified by this and previous studies to map to the region. The strategy to align overlapping cosmid clones was based on their STS content and restriction endonuclease digestion pattern. Those clones extending furthest within each initial contig were then used to walk along the chromosome by the hybridisation of the ends of these cosmids back to the high-density cosmid grids. This process continued until all initial contigs were linked and therefore the region defining the location of the breast cancer tumour suppressor genes would be contained within the map. Individual cosmid clones representing a minimum tiling path in the contig were then used for the identification of transcribed sequences by exon trapping, and for genomic sequencing.

[0130] Chromosome 16 was sorted from the mouse/human somatic cell hybrid CY18, which contains this chromosome as the only human DNA, and *Sau*3A partially digested CY18 DNA was ligated into the *Bam*HI cloning site of the cosmid sCOS-1 vector. All grids were hybridised and washed using methods described in Longmire et al. (1993). Briefly, the 10 filters were pre-hybridised in 2 large bottles for at least 2 hours in 20 ml of a solution containing 6X SSC; 10 mM EDTA (pH8.0); 10X Denhardt's; 1% SDS and 100 μg/ml denatured fragmented salmon sperm DNA at 65°C. Overnight hybridisations with [α-^{32}P]dCTP labelled probes were performed in 20 ml of fresh hybridisation solution at 65°C. Filters were washed sequentially in solutions of 2X SSC; 0.1% SDS (rinse at room temperature), 2X SSC; 0.1% SDS (room temperature for 15 minutes), 0.1X SSC; 0.1% SDS (room temperature for 15 minutes), and 0.1X SSC; 0.1% SDS (twice for 30 minutes at 50°C if needed). Membranes were exposed at -70°C for between 1 to 7 days.

[0131] Initial markers used for, cosmid grid screening were those known to be located below the somatic cell hybrid

breakpoints CY2/CY3 and the long arm telomere (Callen *et al*., 1995). These included three genes, *CMAR, DPEP1,* and *MC1R*; the microsatellite marker D16S303; an end fragment from the cosmid 317E5, which contains the *BBC1* gene; and four cDNA clones, yc81e09, yh09a04,. D16S532E, and ScDNA-C113. The IMAGE consortium cDNA clone, yc81e09, was obtained through screening an arrayed normalised infant brain oligo-dT primed cDNA library (Soares *et al*., 1994), with the insert from cDNA clone ScDNA-A55. Both the ScDNA-A55 and ScDNA-C113 clones were originally isolated from a hexamer primed heteronuclear cDNA library constructed from the mouse/human somatic cell hybrid CY18 (Whitmore *et al*., 1994). The IMAGE cDNA clone yh09a04 was identified from direct cDNA selection of the cosmid 37B2 which was previously shown to map between the CY18A(D2) breakpoint and the 16q telomere. The EST, D16S532E, was also mapped to the same region. Subsequent to these initial screenings, restriction fragments representing the ends of cosmids were used to identify additional overlapping clones.

[0132] Contig assembly was based on methods previously described (Whitmore et al., 1998). Later during the physical map construction, genomic libraries cloned into BAC or PAC vectors (Genome Systems or Rosewell Park Cancer. Institute) became available. These libraries were screened to aid in chromosome walking or when gaps that could not be bridged by using the cosmid filters were encountered. All BAC and PAC filters were hybridised and washed according to manufacturers recommendations. Initially, membrane were individually pre-hybridised in large glass bottles for at least 2 hours in 20 ml of 6X SSC; 0.5% SDS; 5X Denhardt's; 100 μg/ml denatured salmon sperm DNA at 65°C. Overnight hybridisations with [α-^{32}P]dCTP labelled probes were performed at 65°C in 20 ml of a solution containing 6X SSC; 0.5% SDS; 100 μg/ml denatured salmon sperm DNA. Filters were washed sequentially in solutions of 2X SSC; 0.5% SDS (room temperature 5 minutes), 2X SSC; 0.1% SDS (room temperature 15 minutes) and 0.1X SSC; 0.5% SDS (37°C 1 hour if needed). PAC or BAC clones identified were aligned to the existing contig based on their restriction enzyme pattern or formed unique contigs which were extended by additional filter screens.

[0133] As the microsatellite D16S303 was known to be the most telomeric marker in the 16q24.3 region (Callen *et al*., 1995), fluorescence *in situ* hybridisation (FISH) to normal metaphase chromosomes using whole cosmids mapping in the vicinity of this marker, was used to define the telomeric limit for the contig. Whole cosmid DNA was nick translated with biotin-14-dATP and hybridised *in situ* at a final concentration of 20 ng/μl to metaphases from 2 normal males. The FISH method had been modified from that previously described (Callen *et al*., 1990). Chromosomes were stained before analysis with both propidium iodide (as counter-stain) and DAPI (for chromosome identification). Images of metaphase preparations were captured by a cooled CCD camera using the CytoVision Ultra image collection and enhancement system (Applied Imagine Int. Ltd.). The cosmid 369E1 showed clear fluorescent signals at the telomere of the long arm of chromosome 16. However, this probe also gave clear signal at the telomeres of chromosomal arms 3q, 7p, 9q, 11p, and 17p. Conversely, the cosmid 439G8, which mapped proximal to D16S303, gave fluorescent signals only at 16qter with no consistent signal detected at other telomeres. These results enabled us to establish the microsatellite marker D16S303 as the boundary of the transition from euchromatin to the subtelomeric repeats, providing a telomeric limit to the contig (Whitmore et al., 1998).

[0134] A high-density physical map consisting of cosmid, BAC and PAC clones has been established, which extends approximately 3 Mb from the telomere of the long arm of chromosome 16. This contig extends beyond the CY2/CY3 somatic cell hybrid breakpoint and includes the 2 regions of minimal LOH identified at the 16q24.3 region in breast cancer samples. To date, a single gap of unknown size exists in the contig and will be closed by additional contig extension experiments. The depth of coverage has allowed the identification of a minimal tiling path of clones which were subsequently used as templates for gene identification methods such as exon trapping and genomic DNA sequencing.

EXAMPLE 4: Identification of candidate breast cancer genes by analysis of genomic DNA sequence.

[0135] Selected minimal overlapping BAC and PAC clones from the physical map contig were sequenced in order to aid in the identification of candidate breast cancer genes. DNA was prepared from selected clones using a large scale DNA isolation kit (Qiagen). Approximately 25-50 ug of DNA was then sheared by nebulisation (10psi for 45 seconds) and blunt ended using standard methodologies (Sambrook et al., 1989). Samples were then run on an agarose gel in order to isolate DNA in the 2-4 Kb size range. These fragments were cleaned from the agarose using QIAquick columns (Qiagen), ligated into puc18 and used to transform competent DH10B or DH5a *E. coli* cells. DNA was isolated from transformed clones and was sequenced using vector specific primers on an ABI377 sequencer. Analysis of genomic sequence was performed using PHRED, PHRAP and GAP4 software on a SUN workstation. To assist in the generation of large contigs of genomic sequence, information present in the high-throughput genomic sequence (htgs) database at NCBI was incorporated into the assembly phase of the sequence analysis. The resultant genomic sequence contigs were masked for repeats and analysed using the BLAST algorithm (Altschul et al., 1997) to identify nucleotide and protein homology to sequences in the GenBank non-redundant and EST databases at NCBI. The genomic sequence was also analysed for predicted gene structure using the GENSCAN program.

[0136] Homologous IMAGE Consortium cDNA clones were purchased from Genome Systems and were sequenced.

These longer stretches of sequence were then compared to known genes by nucleotide and amino acid sequence comparisons using the above procedures. Any sequences that are expressed in the breast are considered to be candidate breast cancer genes. Those genes whose function could implicate them in the tumourigenic process, as predicted from homology searches with known proteins, were treated as the most likely candidates. Evidence that a particular candidate is the responsible gene comes from the identification of defective alleles of the gene in affected individuals or from analysis of the expression levels of a particular candidate gene in breast cancer samples compared with normal control tissues.

EXAMPLE 5: Identification of the BNO1 sequence

Genomic Sequence Analysis

[0137] Sequences from BAC clones mapping close to the CY2/CY3 breakpoint were assembled and used in BLASTN homology searches of the dbEST database at NCBI. (http://www.acbi.nlm.nih.gov). A large number of cDNA clones were identified to be part of the sequence in this region and these could be further characterised into distinct UniGene clusters.

[0138] The human IMAGE cDNA clone 46795, corresponding to the UniGene cluster Hs.7970, was sequenced and used in further database homology searches. This identified an overlapping cDNA clone present in the non-redundant database (GenBank accession number AL117444) that extended the sequence of clone 46795 further 5'. As this additional 5' sequence was also present in the genomic sequence located 5' to the 46795 clone sequence, it confirmed that AL117444 most likely belonged to the Hs.7970 transcript. To verify this fact, RT-PCR was done.

[0139] Briefly, polyA+ mRNA from normal mammary gland (Clontech) was initially primed with an oligo-dT primer and reverse transcribed using the OmniScript RT kit (Qiagen) according to manufacturers conditions. Control reactions were included for each RNA template which omitted reverse transcriptase from the cDNA synthesis step. This was to determine the presence of any genomic DNA contamination in the RNA samples. The resulting first strand cDNA was PCR amplified using primers AL-1 (specific for AL117444; SEQ ID NO: 20) and 7970-1 (specific for the 3' end of Hs.7970; SEQ ID NO: 21) using the HotStarTaq kit (Qiagen) in a 10 ul reaction volume for 35 cycles. Initially, primers to the control house-keeping gene Esterase D (SEQ ID Numbers: 22 and 23) were used in a separate reaction to confirm the presence of cDNA templates for each reverse transcription reaction. Primer sequences are shown in Table 1. These experiments confirmed that the AL117444 and IMAGE cDNA clone 46795 belonged to the Hs.7970 transcript.

Northern Analysis

[0140] To determine the size of the gene corresponding to Hs.7970, a polyA$^+$ Northern blot obtained from Clontech was probed with a portion of the gene which was generated by PCR using primers BNO1-2 (SEQ ID NO: 24) and BNO1-3 (SEQ ID NO: 25). Table 1 lists the primer sequences used. Hybridisations were conducted in 10 ml of ExpressHyb solution (Clontech) overnight at 65°C. Filters were washed, according to manufacturers conditions. Figure 2 shows the results of the hybridisation. A single band of approximately 3.6 kb was detected in the mammary gland, testis, ovary, uterus, prostate; stomach, bladder, spinal cord, brain, pancreas and thyroid. Strongest expression of the gene was seen in the brain. The size of the mRNA corresponding to Hs.7970 as determined by the Northern hybridisation indicated that additional 5' sequence needed to be obtained for the gene.

5' Sequence Identification

[0141] To identify additional 5' sequence for the His.7970 transcript, cDNA sequences present in dbEST corresponding to the mouse orthologue were utilised. The furthest 5' extending mouse clone (AU080856) included a putative translation start site. Alignment of AU080856 with the human genomic sequence containing Hs.7970 delineated the corresponding human sequence of this transcript up to an identical translation start site. Additional RT-PCR experiments were conducted which confirmed the presence of this 5' sequence in the human Hs.7970 transcript. In addition, further dbEST blast searches identified human cDNA clones containing the 5' end of the gene (eg IMAGE clone 3958783).

[0142] The RT-PCR experiments also indicated that Hs.7970 exists as an alternatively spliced isoform. This variant is due to the inclusion of an additional in-frame exon (exon 2.5) located between exons 2 and 3.

[0143] In combination, these experiments have established that the Hs.7970 transcript, termed BNO1, exists as two alternatively spliced isoforms. One isoform is 3,574 bp in length (SEQ ID NO: 1) and is composed of 9 exons that span approximately 55 Kb of genomic DNA, while the second form of BNO1, which contains exon 2.5, is 3,661 bp in length (SEQ ID NO: 3). Table 2 shows the genomic structure of the gene indicating the size of introns and exons. Analysis of the BNO1 isoforms indicates that isoform 1 (minus exon 2.5) has an open reading frame of 1,617 nucleotides which codes for a protein of 539 amino acids (SEQ ID NO: 2). Isoform 2 (plus exon 2.5) of BNO1 has an open reading frame

of 1,704 bp in length and codes for a protein of 568 amino acids (SEQ ID NO: 4). Partial genomic DNA sequences indicating exon/intron junctions for BNO1 are set forth in SEQ ID Numbers: 5-11.

EXAMPLE 6: Characteristics of the BNO1 Sequence

Nucleotide Sequence

[0144] A large number of human cDNA clones are present in dbEST which represent the BNO1 gene. An observation of the tissues these cDNA clones were derived from indicates that the gene is also expressed in the adrenal gland, blood, colon, germ cells, heart, kidney, liver, lung, muscle, placenta, synovial membrane, tonsil, cervix, lymph tissue and the skin. These tissues are in addition to those shown to express BNO1 from Northern analysis (eg mammary gland, testis, ovary, uterus, prostate, stomach, bladder, spinal cord, brain, pancreas and thyroid) and RT-PCR procedures (eg human mammary gland).

[0145] The human BNO1 nucleotide sequence also detects a large number of mouse cDNA clones as previously mentioned. *In silico* BLAST analysis of mouse genomic DNA sequence in the htgs database at NCBI using the human BNO1 nucleotide sequence was successful in identifying the mouse BNO1 nucleotide (SEQ ID NO: 12) and corresponding amino acid sequence (SEQ ID NO: 13). The amino acid homology between the two genes is as high as 95% (from amino acid 76 in exon 1 to amino acid 369 in exon 8) which suggests that the gene is highly conserved between the two species.

[0146] Analysis of the human genomic sequence located 3' to the BNO1 gene identified the presence of a number of additional UniGene clusters (Hs.130367, Hs.227170 and Hs.87068) running in the same orientation. RT-PCR experiments using a Hs.130367 (130367-1; SEQ ID NO: 26) and Hs.87068 (87068-1; SEQ ID NO: 27) specific primer (see Table 1 for primer sequences) indicated that these two UniGene clusters could be linked. Sequencing of the RT-PCR product also identified the presence of the Hs.227170 cluster. Additional RT-PCR experiments using a BNO1 specific primer (BNO1-1; SEQ ID NO: 28) in combination with a Hs.130367 specific primer (130367-2; SEQ ID NO: 29) established that Hs.130367 could also be linked to the BNO1 gene (see Table 1 for primer sequences). Therefore, the three UniGene clusters lying 3' to BNO1 most likely represent variants of this gene that contain additional 3' UTR sequences. The absence of Northern bands corresponding to the size of these BNO1 variants suggests that they are rare forms of the gene. SEQ ID Numbers:14-19 represent the nucleotide sequences of these variants.

Amino Acid Sequence

[0147] The amino acid sequence of BNO1 was used for *in silico* analysis to identify homologous proteins in order to establish the function of the gene product. Analysis of the BNO1 protein against the Prosite and PfScan databases (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html), showed that both splice isoforms of this protein (SEQ ID Numbers: 2 and 4) contain an F-box domain at the amino terminal end with a highly significant expectation value of 5.6e-10. Figure 3 shows the sequence of the F-box of BNO1 compared to the consensus F-box sequence.

[0148] The F-box is a protein motif of approximately 50 amino acids that defines an expanding family of eukaryotic proteins. F-box containing proteins are the substrate-recognition components of the SCF ubiquitin-ligase complexes. These complexes contain four components: Skp1, Cullin, Rbx/Roc1/Hrt1, and an F-box protein. The F-box motif tethers the F-box protein to other components of the SCF complex by binding the core SCF component, Skp1. This motif is generally found in the amino half of the proteins and is often coupled with other protein domains in the variable carboxy terminus of the protein. The most common carboxy terminal domains include leucine-rich repeats (LRRs) and WD-40 domains. There are currently three subdivisions of the F-box protein family based on the type of carboxy terminal motifs present in the protein sequences. Following the pattern proposed by Cenciarelli et al (1999) and Winston et al (1999), the nomenclature adopted by the Human Genome Organisation denotes F-boxes that contain LRRs as FBXL, those containing WD repeats as FBXW, and those lacking all known protein-interaction domains FBXO. Analysis of the BNO1 sequence failed to identify additional protein motifs present in the gene indicating that BNO1 forms part of the FBXO class of F-box proteins.

[0149] The ubiquitin-dependant proteasome degradation pathway is an important mechanism for regulating protein abundance in eukaryotes. A wide variety of proteins have been shown to be regulated by this mechanism and include oncogenes, tumour suppressor genes, transcription factors and other signalling molecules (Hershko and Ciechanover, 1998; Baumeister et al., 1998). These proteins influence a number of important cellular processes such as cell-cycle regulation and apoptosis, modulation of the immune and inflammatory responses, development and differentiation. The diverse range of proteins and processes that are regulated by ubiquitination suggests that pathologies arising from a disruption of the ubiquitination process will also be diverse. For example there is precedence for this in neurodegenerative disorders. Parkin, a protein mutated in inherited forms of Parkinson's disease, is an E3 ubiquitin ligase (Shimura et al., 2000) and in Alzheimer's disease defective ubiquitination of cerebral proteins has been identified (Lopez Salon et al., 2000).

[0150] The ubiquitination process begins with the addition of ubiquitin moieties (ubiquitination) to target proteins and follows a multi-step process, the end point of which is the proteolysis of polyubiquitinated substrates by a 26S multi-protein complex (Haas and Siepmann, 1997; Hochstrasser, 1996). Ubiquitination of substrates targeted for degradation requires 3 classes of enzyme: the ubiquitin-activating enzymes (E1), the ubiquitin-conjugating enzymes (E2) and the ubiquitin ligases (E3). The E3 proteins play an integral role in cell cycle progression. SCF complexes (a class of E3 ligases) have been shown to regulate the G1-S phase transition (reviewed in Peters, 1998). A wide variety of SCF targets have been reported that include G1-phase cyclins, cyclin-dependant kinase inhibitors, DNA replication factors, transcription factors that promote cell-cycle progression and other important cellular proteins. The sequences present in the variable carboxy terminal region of the F-box proteins therefore allow recruitment of specific substrates for ubiquitination and subsequent degradation.

[0151] Recent studies of the Von Hippel-Lindau (VHL) tumour suppressor protein have shown that it is part of a complex that functions as a ubiquitin-protein ligase E3 (Zaibo et al., 2001). The VHL protein links the ligase complex to target proteins which include HIFα (hypoxia inducible factor) (Ohh et al., 2000; Cockman et al., 2000) and VDU1 (VHL interacting deubiquitinating enzyme 1) (Zaibo et al., 2001). HIFα has been shown to regulate genes involved in angiogenesis, a process critical for the growth of tumours (Wang et al., 1995; Semenza, 2000), while VDU1 has deubiquitinating activity.

[0152] The predicted role of BNO1, based on the presence of the F-box domain, indicates that the gene may be involved in a diverse range of cellular processes including cell-cycle regulation. Combined with the fact that BNO1 lies in a region of LOH seen in breast and other tumour types suggests BNO1 is an ideal candidate breast cancer gene.

EXAMPLE 7: Examination of the expression level of BNO1 in breast cancer cell lines

[0153] To investigate a potential role of BNO1 in breast cancer, the level of expression of the gene was compared in breast cancer cell lines with normal tissue controls. Examination of the genomic sequence surrounding BNO1 shows that the 5' end including exon 1 is extremely G-C rich suggesting the presence of a CpG island. While not wishing to be bound by theory, this raises the possibility that epigenetic mechanisms to inactivate BNO1 function may exist. Abnormal methylation at this site may result in a down-regulation of BNO1 transcription of the remaining copy of the gene. Recent studies have shown that this mechanism has been responsible for the inactivation of other tumour suppressor genes such as RB1 (Ohtani-Fujita et al., 1997), VHL (Prowse et al., 1997), MLH1 (Herman et al., 1998) and BRCA1 (Esteller et al., 2000).

[0154] To detect the level of expression of BNO1 in cancer samples compared with normal controls, quantitative RT-PCR using BNO1 specific primers was done. This initially involved the isolation of RNA from breast cancer cell lines along with appropriate cell line controls.

Breast/Prostate Cancer Cell Lines and RNA Extraction

[0155] Cancer cell lines were purchased from ATCC (USA) and grown in the recommended tissue culture medium. Breast cancer cell lines were chosen for RT-PCR analysis that demonstrated homozygosity for a number of markers mapping to chromosome 16q indicating potential LOH for this chromosomal arm. Cells were harvested from confluent cultures and total RNA was extracted using the RNAeasy kit (Qiagen). Breast cancer cell lines obtained for RNA extraction were BT549, MDA-MB-468, CAMA-1, ZR75-30, MDA-MB-157, ZR75-1, SKBR3, MDA-MB-231, T47D, and MDA-MB-436. The normal breast epithelial cell line MCF12A and the prostate cancer cell line PC3 were also purchased. PolyA$^+$ mRNA was subsequently isolated from all sources using the Oligotex bead system (Qiagen). PolyA$^+$ mRNA from normal mammary gland, prostate, ovary and liver was purchased commercially (Clontech, USA).

Reverse Transcription

[0156] PolyA$^+$ mRNA was primed with oligo-dT primers and reverse transcribed using the Omniscript RT kit (Qiagen) according to manufacturers conditions. Control reactions were included for each RNA template which omitted reverse transcriptase from the cDNA synthesis step. This was to determine the presence of any genomic DNA contamination in the RNA samples.

cDNA Normalisation

[0157] Internal standard curve amplicons were generated from a mixed pool of normal tissue cDNA using the HotStarTaq™ DNA Polymerase kit (Qiagen). A reaction mix sufficient to generate >1 ug of amplicon cDNA contained 10 ul of 10$\times$ PCR buffer (containing 15 mM $MgCl_2$), 2 ul of 10 mM dNTP mix, 0.5 uM of each primer, 0.5 ul of 2.5 units HotStarTaq polymerase (Qiagen), 100 ng of cDNA template and DEPC treated water to 100 ul. Amplification cycling was performed as follows: 94°C for 10 minutes followed by 35 cycles at 93°C for 20 seconds, 60°C for 30 seconds and

70°C for 30 seconds with a final extension at 72°C for 4 minutes. Amplicons were purified using the QIAquick gel extraction kit (Qiagen) according to manufacturers conditions and concentrations were measured at A_{260}. Purified amplicons were serially diluted 10-fold from 10 ng/ul to 1 *f*g/ul. These dilutions served as internal standards of known concentration for real-time analysis of BNO1 specific amplicons as described below.

Real-time PCR

[0158] All cDNA templates were amplified using the SYBR Green I PCR Master Mix kit (PE Biosystems, USA). PCR reactions were in a volume of 25 ul and included 12.5 ul of SYBR Green I PCR Master mix, 0.5 uM of each primer, 2 ul normalised cDNA template (see below) and 9.5 ul of water. Real-time PCR analysis was performed using the Rotor-Gene™2000 (Corbett Research, AUS) with the following amplification cycling conditions: 94°C for 10 minutes followed by 45 cycles of 93°C for 20 sec, 60°C for 30 sec and 70°C for 30 sec. Fluorescence data was acquired at 510 nm during the 72°C extension phase. Melt curve analyses were performed with an initial 99-50°C cycling followed by fluorescence monitoring during heating at 0.2°C/second to 99°C. Prior to real-time quantification, product size and specificity was confirmed by ethidium bromide staining of 2.5% agarose gels following electrophoresis of completed PCRs. Control and BNO1 specific primers used for all real-time PCR applications are listed in Table 1 and are represented by the SEQ ID Numbers: 30-41.

Real-time PCR Quantification

[0159] Quantification analyses were performed on the Rotor-Gene™ DNA sample analysis system (Version 4.2, Build 96). Standard curves were generated by amplifying 10-fold serial dilutions (1 ul of 10 ρg/ul down to 1 ul of 1 fg/ul in triplicate) of the internal standard amplicon during real-time PCR of BNO1 amplicons from normal tissues and breast cancer cell lines. Internal standard amplicon concentrations were arbitrarily set to 1.0e+12 copies for 10 pg standards to 1.0e+08 copies for 1 fg standards. C_T (cycle threshold) coefficients of variation for all internal standard dilutions averaged 2% between triplicate samples within the same and different runs. The Rotor-Gene™ quantification software generated a line of best-fit at the parameter C_T and determined unknown normal tissue and breast cancer cell line BNO1 amplicon copy numbers by interpolating the noise-band intercept of BNO1 amplicons against the internal standards with known copy numbers.

Normalization and relative expression of data

[0160] To account for variation in sample-to-sample starting template concentrations, RiboGreen™ RNA quantitation (Molecular Probes) was used to accurately assay 1 ug of normal tissue and breast cancer cell line RNA for cDNA synthesis. Selected housekeeping gene expression levels were then analyzed in all samples to determine the most accurate endogenous control for data normalization. Housekeeping amplicons included Esterase D (Accession Number M13450), Cyclophilin (Accession Number X52851), APRT (Accession Number M16446) and RNA Polymerase II (Accession Number Z47727). As Cyclophilin displayed the least variable expression profile, calculated BNO1 copy numbers were divided by the respective Cyclophilin amplicon copy number for each breast cancer cell line and normal tissue analyzed. BNO1 copy numbers in normalized normal breast cDNA were arbitrarily set to a 'baseline' of 1.0e+06 copies. Breast cancer cell lines and other normal tissue cDNA copy numbers were calculated relative to the 'baseline'. Data was expressed as log relative mRNA copy number. Figure 4 shows the results from these experiments.

[0161] The degree of variation in mRNA expression levels for Cyclophilin, RNA polymerase II subunit and APRT were relatively uniform between the normal tissues and cancer cell lines. Three-way combinations for normalization between Cyclophilin, RNA polymerase II subunit and APRT demonstrated a mean 7-fold and maximum 50-fold variance in mRNA expression level between samples. The significance of variable mRNA expression levels within a gene of interest may therefore reasonably be evaluated based on these normalization results. A predicted aberrant decrease in gene of interest mRNA copy number of ~100 fold in breast cancer cell lines relative to a 'baseline' normal breast expression level was therefore considered to be significantly abnormal.

[0162] Figure 4 indicates that BNO1 amplicons specific for exon 5-7 and isoform 1 (minus exon 2.5) show a consistent pattern of mRNA expression among normal tissues and breast cancer cell lines. For both amplicons analyzed, the breast cancer cell lines MDA-MB-468, SK-BR3, MDA-MB-231 and the prostate cancer cell line PC3 all display low-level mRNA expression with respect to the 'baseline' normal breast tissue. A significant 725-fold reduction in BNO1 exon 5-7 mRNA expression was detected in SK-BR3 with respect to the normal breast tissue expression (equivalent to an approximately 350,000-480,000 down-regulation in mRNA molecule expression). Similar results were obtained for isoform 1 of BNO1 (minus exon 2.5), with a 248-fold reduction in mRNA expression in SK-BR3 (equivalent to an approximately 300,000-1,000,000 down-regulation in mRNA molecule expression). BNO1 isoform 2 (plus exon 2.5) displayed significantly low mRNA expression in the cell lines MDA-MB-468, CAMA-1, SK-BR3 and MDA-MB-231, with no expression

detected in ZR75-30. These results indicate that both isoforms of the BNO1 gene are down-regulated in certain breast cancer cell lines as well as a prostate cancer cell line. The exact mechanism of this down-regulation is not known at this stage but may result from mechanisms such as mutation or promoter methylation. From these expression studies we propose that BNO1 is a protein responsible for the development of breast and prostate cancer. Due to its broad tissue expression pattern, BNO1 may also be implicated in cancers originating from other tissues.

[0163] Other methods to detect BNO1 expression levels may be used. These include the generation of polyclonal or monoclonal antibodies, which are able to detect relative amounts of both normal and mutant forms of BNO1 using various immunoassays such as ELISA assays (See Example 11 and 12).

EXAMPLE 8: Analysis of tumours and cell lines for BNO1 mutations

[0164] The BNO1 gene was screened by SSCP analysis in DNA isolated from tumours from series 1 as well as a subset of series 2 tumours (not shown in Figure 1) that displayed loss of the whole long arm of chromosome 16. These samples from series 2 were used due to larger amounts of DNA being available. In total 45 primary breast tumours with 16q LOH were examined for mutations.

[0165] A number of cell lines were also screened for mutations. These included 22 breast cancer cell lines (BT20, BT474, BT483, BT549, CAMA-1, DU4475, Hs578T, MCF7, MB157, MB231, MB361, MB415, MB436, MB453, MB468, SKBR3, T47D, UACC893, ZR75-1, ZR75-30, MB134 and MB175), 2 prostate cancer cell lines (LNCAP and PC3), 2 gastric carcinoma cell lines (AGS and KATO), 1 liver cancer cell line (HEP2) and 2 normal breast epithelial cell lines (HBL100 and MCF12A). All cell lines were purchased from ATCC, grown according to manufacturers conditions, and DNA isolated from cultured cells using standard protocols (Wyman and White, 1980; Sambrook et al., 1989).

[0166] BNO1 exons were amplified by PCR using flanking intronic primers, which were labeled at their 5' ends with HEX. An exception was made for exon 1 and 8, as due to their size had to be split into 2 overlapping amplimers. Table 3 lists the sequences of all primers used for the SSCP analysis, the expected amplimer size and the $MgCl_2$ concentration used in the PCR reaction. Typical PCR reactions were performed in 96-well plates in a volume of 10 ul using 30 ng of template DNA. Cycling conditions were an initial denaturation step at 94°C for 3 minutes followed by 35 cycles of 94°C for 30 seconds, 60°C for $1^{1/2}$ minutes and 72°C for $1^{1/2}$ minutes. A final extension step of 72°C for 10 minutes followed. Twenty ul of loading dye comprising 50% (v/v) formamide, 12.5 mM EDTA and 0.02% (w/v) bromophenol blue were added to completed reactions which were subsequently run on 4% polyacrylamide gels and analysed on the GelScan 2000 system (Corbett Research, AUS) according to manufacturers specifications.

[0167] Of all 12 amplicons tested, only 2 identified SSCP bandshifts. In exon 2.5, identical bandshifts were seen in 2 tumour samples from series 1 (380 and 355) and the breast cancer cell line MCF7. SSCP analysis of the corresponding normal DNA from sample 380 and 355 identified the same bandshift indicating the change was most likely not causative for the disease. Sequence analysis of this bandshift in all samples showed that a single nucleotide base change (-5T→C) was responsible for this bandshift. This change does not affect the consensus splice acceptor site score for this exon and hence most likely represents a polymorphism. The incidence of this change in the general population has not been examined as yet. In exon 8b, a bandshift was identified in only a single cancer cell line (KATO). Sequencing of this bandshift indicated a C→T change at position +10 of this amplicon which is located in the splice donor site (5' splice site). This base change occurs outside the splice junction consensus sequence and it is envisaged that the mutation has no effect on splicing of this exon.

EXAMPLE 9: Immunoprecipitation of BNO1 and Skp1

[0168] To test if BNO1 contained a functional F-box motif, a co-immunoprecipitation assay was employed. This involved cloning of the full-length Myc-tagged open reading frame of BNO1 into the SalI/ClaI sites of the retroviral expression vector LNCX2 (Clontech) using standard techniques (Sambrook et al., 1989). Following this, 10^7 293T cells were transfected with 10 ug of the BNO1-LNCX2 construct or separately with LCNX2 vector alone as a control using Lipofectamine 2000 (Invitrogen) according to manufacturers instructions. Cells were harvested 24 hours post-transfection and lysed in 2 ml of lysis buffer (50mM Tris-HCL [pH 7.5], 150mM NaCl, 0.5% Nonidet P-40 supplemented with 1mM PMSF and 5μg/ml leupeptin, antipain and aprotenin). Following this, 0.5 ml of the cell lysate was incubated with 2 ug of anti-Myc monoclonal antibody (Roche) or anti-$p19^{Skp1}$ rabbit polyclonal antibody (Neo Markers, Fremont, CA) for 1 hour and protein A-Sepharose for 1 hour at 4°C. Immune complexes were washed three times with 1 ml of lysis buffer followed by separation on 10% SDS-PAGE and immunoblotting according to standard techniques (Sambrook et al., 1989).

[0169] Results from these experiments indicated that BNO1 specifically co-precipitated with endogenous Skp1, confirming both an association between these two proteins and the presence of a functional F-box within BNO1. This interaction indicates that BNO1 belongs to a novel E3-ubiquitin ligase complex that may be critical for the controlled degradation of BNO1 specific substrates.

EXAMPLE 10: Analysis of the BNO1 gene

[0170] The following methods are used to determine the structure and function of BNO1.

Biological studies

[0171] Mammalian expression vectors containing BNO1 cDNA (representing both isoforms of BNO1) can be transfected into breast, prostate or other carcinoma cell lines that have lesions in the gene. Phenotypic reversion in cultures (eg cell morphology, growth of transformants in soft-agar, growth rate) and in non human animals (eg tumourigenicity in nude mice) is examined. These studies can utilise wild-type or mutant forms of BNO1. Deletion and missense mutants of BNO1 can be constructed by in vitro mutagenesis.

Molecular biological studies

[0172] The ability of both isoforms of the BNO1 protein to bind known and unknown proteins can be examined. Procedures such as the yeast two-hybrid system are used to discover and identify any functional partners, particularly BNO1 specific substrates or isoform-specific substrates that are targeted for degradation by ubiquitination. The principle behind the yeast two-hybrid procedure is that many eukaryotic transcriptional activators, including those in yeast, consist of two discrete modular domains. The first is a DNA-binding domain that binds to a specific promoter sequence and the second is an activation domain that directs the RNA polymerase II complex to transcribe the gene downstream of the DNA binding site. Both domains are required for transcriptional activation as neither domain can activate transcription on its own. In the yeast two-hybrid procedure, the gene of interest or parts thereof (BAIT), is cloned in such a way that it is expressed as a fusion to a peptide that has a DNA binding domain. A second gene, or number of genes, such as those from a cDNA library (TARGET), is cloned so that it is expressed as a fusion to an activation domain. Interaction of the protein of interest with its binding partner brings the DNA-binding peptide together with the activation domain and initiates transcription of the reporter genes. The first reporter gene will select for yeast cells that contain interacting proteins (this reporter is usually a nutritional gene required for growth on selective media). The second reporter is used for confirmation and while being expressed in response to interacting proteins it is usually not required for growth.

[0173] The nature of the BNO1 interacting genes and proteins can also be studied such that these partners can also be targets for drug discovery. Of particular interest are those BNO1-interacting proteins that are targeted for ubiquitination and subsequent degradation by the BNO1-containing ubiquitin-E3 ligase.

Structural studies

[0174] BNO1 recombinant proteins can be produced in bacterial, yeast, insect and/or mammalian cells and used in crystallographical and NMR studies. Together with molecular modeling of the protein, structure-driven drug design can be facilitated.

EXAMPLE 11: Generation of polyclonal antibodies against BNO1

[0175] The knowledge of the nucleotide and amino acid sequence of BNO1 allows for the production of antibodies, which selectively bind to BNO1 protein or fragments thereof. Following the identification of mutations in the gene, antibodies can also be made to selectively bind and distinguish mutant from normal protein. Antibodies specific for mutagenised epitopes are especially useful in cell culture assays to screen for malignant cells at different stages of malignant development. These antibodies may also be used to screen malignant cells, which have been treated with pharmaceutical agents to evaluate the therapeutic potential of the agent.

[0176] To prepare polyclonal antibodies, short peptides can be designed homologous to the BNO1 amino acid sequence. Such peptides are typically 10 to 15 amino acids in length. These peptides should be designed in regions of least homology to the mouse orthologue to avoid cross species interactions in further down-stream experiments such as monoclonal antibody production. Synthetic peptides can then be conjugated to biotin (Sulfo-NHS-LC Biotin) using standard protocols supplied with commercially available kits such as the PIERCE™ kit (PIERCE). Biotinylated peptides are subsequently complexed with avidin in solution and for each peptide complex, 2 rabbits are immunized with 4 doses of antigen (200 μg per dose) in intervals of three weeks between doses. The initial dose is mixed with Freund's Complete adjuvant while subsequent doses are combined with Freund's Immuno-adjuvant. After completion of the immunization, rabbits are test bled and reactivity of sera assayed by dot blot with serial dilutions of the original peptides. If rabbits show significant reactivity compared with pre-immune sera, they are then sacrificed and the blood collected such that immune sera can separated for further experiments.

EXAMPLE 12: Generation of monoclonal antibodies specific for BNO1

[0177] Monoclonal antibodies can be prepared for BNO1 in the following manner. Immunogen comprising intact BNO1 protein or BNO1 peptides (wild type or mutant) is injected in Freund's adjuvant into mice with each mouse receiving four injections of 10 to 100 ug of immunogen. After the fourth injection blood samples taken from the mice are examined for the presence of antibody to the immunogen. Immune mice are sacrificed, their spleens removed and single cell suspensions are prepared (Harlow and Lane, 1988). The spleen cells serve as a source of lymphocytes, which are then fused with a permanently growing myeloma partner cell (Kohler and Milstein, 1975). Cells are plated at a density of $2X10^5$ cells/well in 96 well plates and individual wells are examined for growth. These wells are then tested for the presence of BNO1 specific antibodies by ELISA or RIA using wild type or mutant BNO1 target protein. Cells in positive wells are expanded and subcloned to establish and confirm monoclonality. Clones with the desired specificity are expanded and grown as ascites in mice followed by purification using affinity chromatography using Protein A Sepharose, ion-exchange chromatography or variations and combinations of these techniques.

Industrial Applicability

[0178] The BNO1 gene is implicated in cancer and based on its role in the ubiquitination process, BNO1 may also be implicated in cellular mechanisms which are regulated by this process. The novel DNA molecules of the present invention are therefore useful in methods for the early detection of disease susceptible individuals as well as in therapeutic procedures associated with these disease states.

TABLE 1

Primers Used for Analysis of BNO1

Primer Name	Primer Sequence (5' → 3')
AL-1	GTG AAG AAG GAT GAG TTC TCC
7970-1	AGC TGA GCA TCA CAA TCT CC
ESTD-F	GGA GCT TCC CCA ACT CAT AAA TGC C
ESTD-R	GCA TGA TGT CTG ATG TGG TCA GTA A
BNO1-2	TGC GAA GCT GCT TCA CCG AT
BNO1-3	GGC CGT ACA TGC ACT CCA CTG
130367-1	GAG AAC CTG CAG TTG TGC TG
87068-1	ATG GTG CTG CTT GTA GCA AG
BNO1-1	TGC CCA TAT GAG ATG ACG AGG
130367-2	ACA CTC AGC AGT GGA CAC TTG
Cyclophilin-F[1]	GGC AAA TGC TGG ACC CAA CAC AAA
Cyclophilin-R[1]	CTA GGC ATG GGA GGG AAC AAG GAA
APRT-F[1]	GAC TGG GCT GCG TGC TCA TCC
APRT-R[1]	AGG CCC TGT GGT CAC TCA TAC TGC
RNA Polymerase II-F[1]	AGG GGC TAA CAA TGG ACA CC
RNA Polymerase II-R[1]	CCG AAG ATA AGG GGG AAC TAC T
BNO1 (Exon 5-7)-F[1]	CCG GCG GGA GGC AGG AGG AGT
BNO1 (Exon 5-7)-R[1]	GCG GCG GTA GGT CAG GCA GTT GTC
BNO1 (Isoform 1)-F[1]	TGC GAA GCT GCT TCA CCG AT
BNO1 (Isoform 1)-R[1]	GGC CGT ACA TGC ACT CCA CTG
BNO1 (Isoform 2)-F[1]	GTG AAG TCG GGA CGT TTT GTG A
BNO1 (Isoform 2)-R[1]	CCG TGG TGG GGC CCT TTG TGG

Note: [1]These primers were labeled at their 5' ends with HEX. Isoform 1 of BNO1 lacks exon 2.5 (SEQ ID NO:1). Isoform 2 of BNO1 contains exon 2.5 (SEQ ID NO:3).

TABLE 2

Splice Sites of the *BNO1* Gene

Exon	Size (bp)	3' Splice site (intron/exon)	Consensus shength (%)	5' Splice site (exon/ intron)	Consensus strength (%)	Intron size (bp)
1	343	5'UTR		TGCCGTGAGG/ **gt**gagcgcgc	83.03	23042
2	72	cttgttac**ag**/ AGTATGGTGT	94.28	TATGCGAAGC / **gt**gagtgaat	75.36	1797
2.5	87	gtctgttc**ag**/ GTATAAACCC	90.0	TACACCTGCC/ **gt**atgtacct	66.97	11160
3	77	cctcctgt**ag**/ TGCTTCACCG	78.70	GAACGTGGTG/ **gt**aagtcccg	92.15	3408
4	168	cctcctgt**ag**/ GTGGACGGCC	84.95	CCACATCCAG/ **gt**gtgtgcag	85.40	646
5	75	aacactga**ag**/ ATTGTGAAGA	63.39	GAGGCAGGAG /**gt**gagcccac	90.87	6612
6	110	cttttgga**ag**/ GAGTTTCGGA	85.65	GTCAGTACGA/ **gt**gagtgcgg	76.46	697
7	154	ctccccac**ag**/ CAACTGCCTG	85.32	CAAGATCACG / **gt**gagtggcg	88.50	1017
8	401	tgctccac**ag**/ GGCGACCCCA	89.22	GCAGGATGTG / **gt**aaggatg	87.59	2375
9	2174	ttctgctc**ag**/ TTTTTATGGC	90.62	3'UTR		

TABLE 3

Primers used for the SSCP analysis of *BNO1*

Exo n	Primer 1 (5' → 3')	Primer 2 (5' → 3')	[$MgCl_s$]	Product Size (bp)
1a	GCGCTGGAGCGTGCGCACA	AGCTCGGGCGGCAGCTCCA	2.0 mM	269
1b	GGTCGGGGGCGGCTTGTG	GCCTCCACCTGGCAGGGA	2.0 mM	252
2	CTGTCGCGTTATGAGTTGTTG	GTACAAAGTTAATCATGGATGGT	2.0 mM	168
2.5	AGGCATTGGGTCGTATTCAC	AGAAGCCAAAGCTCGCAGGA	1.5 mM	198
3	GGCACGCTGGGTCTAACAC	CCTGCCCGTGCACAGACCT	1.5 mM	167
4	CTCATGGACCTTTGCCCATCT	GTCTGCAGCTGAGAATAGCAC	1.0 mM	290
5	GTGATGGACTCTGTTCCTCAC	AGGTCCGCACCATATGAACAC	2.0 mM	170
6	CACAGCCTCCTGTCATATGGA	ACCCCAGCACCGAGCAGGA	1.5 mM	187
7	GGCGTTCTCAGTCCTGCCT	CCCTGACTCCACAGCCCAC	1.5 mM	284
8a	CTGGCCTGAGCCCTGCTGA	ACCCTCTCGCGCACCTCCA	1.0 mM	171
8b	CAATGAGCTCTCCCGCATC	CCATGCTGTCCCACCTTCA	1.5 mM	354
9	AGAATGCTGTACGTGGCGTG	AGGAGGTGAGGGACTGAATG	1.0 mM	292

Note: All primes were labelled at their 5' ends with HEX.

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[0179] References cited herein are listed on the following pages, and are incorporated herein by this reference.

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Claims

1. An isolated BNO1 nucleic acid molecule mapping to human chromosome 16q24.3 and comprising the nucleotide sequence set forth in SEQ ID Numbers: 1 or 3.

2. An isolated BNO1 nucleic acid molecule comprising the nucleotide sequence set forth in SEQ ID Numbers: 1 or 3, which encodes a polypeptide capable of forming part of a ubiquitin-ligase complex involved in targeting proteins by ubiquitination for degradation by the proteasome.

3. An isolated BNO1 nucleic acid molecule that is at least 95% identical to a DNA molecule consisting of the nucleotide sequence set forth in SEQ ID Numbers: 1 or 3 and which encodes a polypeptide capable of forming part of a ubiquitin-ligase complex involved in targeting proteins by ubiquitination for degradation by the proteasome.

4. An isolated BNO1 nucleic acid molecule which encodes a polypeptide having the amino acid sequence set forth in SEQ ID Numbers: 2 or 4.

5. An isolated nucleic acid molecule consisting of the nucleotide sequence set forth in SEQ ID Numbers: 1 or 3.

6. An expression vector which comprises a nucleic acid molecule as defined in any one of claims 1 to 5 operably linked to suitable control elements.

7. An isolated cell transformed with the expression vector of claim 6.

8. A cell as claimed in claim 7 in which recombinant BNO1 expression may be switched off.

9. A cell as claimed in any one of claims 7 or 8 which is an eukaryotic cell.

10. A method of preparing a polypeptide encoded by any of the nucleic acids of claims 1 to 5, comprising the steps of:

    (1) culturing a cell as defined in claim 7 or 9 under conditions effective for production of the polypeptide; and
    (2) harvesting the polypeptide.

11. An isolated BNO1 polypeptide comprising the amino acid sequence set forth in SEQ ID Numbers: 2 or 4.

12. An isolated BNO1 polypeptide, comprising the amino acid sequence set forth in SEQ ID Numbers: 2 or 4, which is capable of forming part of a ubiquitin-ligase complex involved in protein degradation through ubiquitination.

13. An isolated BNO1 polypeptide capable of forming part of a ubiquitin-ligase complex involved in protein degradation

through ubiquitination that has at least 95% identity with the amino acid sequence set forth in SEQ ID Numbers: 2 or 4.

14. An isolated BNO1 polypeptide consisting of the amino acid sequence set forth in SEQ ID Numbers: 2 or 4.

15. An antibody for detection fo BNO1 which is immunologically reactive with a polypeptide as defined in any one of claims 11 to 14, preferably a monoclonal antibody, a humanised antibody, a chimaeric antibody or an antibody fragment including a Fab fragment, $F(ab')_2$ fragment, Fv fragment, single chain antibodies and single domain antibodies.

16. The use of a nucleic acid molecule as claimed in any one of claims 1 to 5 in the manufacture of a medicament for the treatment of disorder associated with decreased expression or activity of BNO1.

17. The use as claimed in claim 16 wherein the nucleic acid molecule is a part of an expression vector which also includes suitable control elements.

18. The use of an antagonist of BNO1 in the manufacture of a medicament for the treatment of a disorder associated with increased expression or activity of BN01, wherein said antagonist is an antibody as claimed in claim 15.

19. The use of an isolated nucleic acid molecule which is the complement of a nucleic acid molecule as defined in any one of claims 1 to 5, the transcription product of which is a mRNA that hybridizes with the mRNA encoded by BNO1, in the manufacture of a medicament for the treatment of a disorder associated with increased activity or expression of BNO1.

20. A method for screening for a compound capable of modulating the activity of BNO1 comprising combining a peptide as claimed in any one of claims 11 to 14 and a candidate compound, and determining the binding of said candidate compound to said peptide.

21. A method of screening for drug candidates comprising the steps of:

(1) providing a cell as claimed in any one of claims 7 or 9;
(2) adding a drug candidate to said cell; and
(3) determining the effect of said drug candidate on the expression of BNO1 by said cell.

22. The use of a nucleic acid as claimed in any one of claims 1 to 5 in screening for drug candidates.

23. The in vitro use of a nucleic acid as claimed in any one of claims 1 to 5 for the diagnosis or prognosis of disorders associated with BNO dysfunction, or a predisposition to such disorders.

24. The in vitro use of a polypeptide as claimed in any of claims 11 to 14 for the diagnosis or prognosis of disorders associated with BNO1 dysfunction, or a predisposition to such disorders.

25. The in vitro use of an antibody as defined in claim 15 in the diagnosis or prognosis of a disorder associated with BNO1, or a predisposition to such disorders.

26. An in vitro method for the diagnosis or prognosis of a disorder associated with mutations in BNO1, or a predisposition to such disorders in a patient, comprising the steps of:

comparing BNO1 or a nucleic acid which codes for BNO1 from a sample to be obtained from a patient with wild-type BNO1 or a nucleic acid which codes for it in order to establish whether the person expresses a mutant BNO1.

27. A method as claimed in claim 26 wherein the nucleotide sequence of DNA from the patient is compared to the sequence of DNA encoding wild-type BNO1.

28. An in vitro method for the diagnosis or prognosis of a disorder associated with abnormal expression or activity of BNO1, or a predisposition to such disorders, comprising the steps of:

(1) establishing a profile for normal expression of BNO1 in unaffected subjects;
(2) measuring the level of expression of BNO1 in a person suspected of abnormal expression or activity of

BNO1; and
(3) comparing the measured level of expression with the profile for normal expression.

29. A method as claimed in claim 28 wherein reverse transcriptase PCR is employed to measure levels of expression.

30. A method as claimed in claim 28 wherein a hybridisation assay using a probe derived from BNO1, or a fragment thereof, is employed to measure levels of expression.

31. A method as claimed in claim 30 wherein the probe has at least 50% sequence identity to a nucleotide sequence encoding BNO1, or a fragment thereof.

32. An in vitro method for the diagnosis or prognosis of a disorder associated with BNO1, or a predisposition to such disorders, comprising the steps of:

(1) establishing a physical property of wild-type BNO1;
(2) measuring the property for a BNO1 expressed by a person suspected of an abnormality of BNO1; and,
(3) comparing it to the established property for wild-type BNO1 in order to establish whether the person expresses a mutant BNO1.

33. A method as claimed in claim 32 wherein the property is the electrophoretic mobility.

34. A method as claimed in claim. 32 wherein the property is the proteolytic cleavage pattern.

35. A genetically modified non-human animal selected from the group consisting of rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs and non-human primates such as monkeys and chimpanzees, transformed with an isolated nucleic acid molecule as defined in any one of claims 1 to 5.

36. A genetically modified non-human animal selected from the group consisting of rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs and non-human primates such as monkeys and chimpanzees, in which homologous BNO1 gene and gene function has been knocked out.

37. The use of a genetically modified non-human animal as defined in either one of claims 35 or 36 in screening for candidate pharmaceutical compounds.

38. A microarray for detecting od BN01 comprising a nucleic acid encoding either isoform of BN01 or, a fragment thereof, or nucleic acids encoding both isoforms of BN01, or fragments thereof.

Patentansprüche

1. Isoliertes BNO1-Nucleinsäuremolekül, das auf dem menschlichen Chromosom 16q24.3 kartiert und die in SEQ ID Nr.: 1 oder 3 dargestellte Nucleotidsequenz aufweist.

2. Isoliertes, die in SEQ ID Nr.: 1 oder 3 dargestellte Nucleotidsequenz aufweisendes BNO1-Nucleinsäuremolekül, das für ein Polypeptid codiert, das einen Teil eines Ubiquitin-Ligase-Komplexes bilden kann, der an der Adressierung von Proteinen durch Ubiquitinierung zum Abbau durch das Proteasom beteiligt ist.

3. Isoliertes BNO1-Nucleinsäuremolekül, das zu mindestens 95% identisch mit einem DNA-Molekül ist, das aus der in SEQ ID Nr.: 1 oder 3 dargestellten Nucleotidsequenz besteht und für ein Polypeptid codiert, das einen Teil eines Ubiquitin-Ligase-Komplexes bilden kann, der an der Adressierung von Proteinen durch Ubiquitinierung zum Abbau durch das Proteasom beteiligt ist.

4. Isoliertes BNO1-Nucleinsäuremolekül, das für ein Polypeptid mit der in SEQ ID Nr.: 2 oder 4 dargestellten Aminosäuresequenz codiert.

5. Isoliertes Nucleinsäuremolekül, das aus der in SEQ ID Nr.: 1 oder 3 dargestellten Nucleotidsequenz besteht.

6. Expressionsvektor, der ein in einem der Ansprüche 1 bis 5 definiertes Nucleinsäuremolekül aufweist, das funktionell

mit geeigneten Kontrollelementen verbunden ist.

7. Isolierte Zelle, die mit einem Expressionsvektor nach Anspruch 6 transformiert ist.

8. Zelle nach Anspruch 7, in der eine rekombinante BNO1-Expression abgeschaltet werden kann.

9. Zelle nach einem der Ansprüche 7 oder 8, die eine eukaryotische Zelle ist.

10. Verfahren zur Herstellung eines Polypeptids, das durch eine der Nucleinsäuren nach einem der Ansprüche 1 bis 5 codiert wird, wobei das Verfahren die folgenden Schritte aufweist:

(1) Kultivieren einer Zelle gemäß der Definition in Anspruch 7 oder 9 unter Bedingungen, die für die Produktion des Polypeptids wirksam sind; und
(2) Ernten des Polypeptids.

11. Isoliertes BNO1-Polypeptid, das die in SEQ ID Nr.: 2 oder 4 dargestellte Aminosäuresequenz aufweist.

12. Isoliertes, die in SEQ ID Nr.: 2 oder 4 dargestellte Aminosäuresequenz aufweisendes BNO1-Polypeptid, das einen Teil eines Ubiquitin-Ligase-Komplexes bilden kann, der am Proteinabbau durch Ubiquitinierung beteiligt ist.

13. Isoliertes BNO1-Polypeptid, das einen Teil eines am Proteinabbau durch Ubiquitinierung beteiligten Ubiquitin-Ligase-Komplexes bilden kann, wobei das Polypeptid zu mindestens 95% identisch mit der in SEQ ID Nr.: 2 oder 4 dargestellten Aminosäuresequenz ist.

14. Isoliertes BNO1-Polypeptid, das aus der in SEQ ID Nr.: 2 oder 4 dargestellten Aminosäuresequenz besteht.

15. Antikörper zum Nachweis von BNO1, der mit einem in einem der Ansprüche 11 bis 14 definierten Polypeptid immunologisch reaktiv ist, vorzugsweise ein monoklonaler Antikörper, ein humanisierter Antikörper, ein chimärer Antikörper oder ein Antikörperfragment, das ein Fab-Fragment, $F(ab')_2$-Fragment, Fv-Fragment einschließt, einkettige Antikörper und Einzeldomänen-Antikörper.

16. Verwendung eines Nucleinsäuremoleküls nach einem der Ansprüche 1 bis 5 bei der Herstellung eines Medikaments zur Behandlung einer mit verminderter Expression oder Aktivität von BNO1 assoziierten Erkrankung.

17. Verwendung nach Anspruch 16, wobei das Nucleinsäuremolekül ein Teil eines Expressionsvektors ist, der außerdem geeignete Kontrollelemente enthält.

18. Verwendung eines Antagonisten von BNO1 bei der Herstellung eines Medikaments zur Behandlung einer Erkrankung, die mit erhöhter Expression oder Aktivität von BNO1 assoziiert ist, wobei der Antagonist ein Antikörper nach Anspruch 15 ist.

19. Verwendung eines isolierten Nucleinsäuremoleküls, das ein Komplement eines in einem der Ansprüche 1 bis 5 definierten Nucleinsäuremoleküls ist, dessen Transkriptionsprodukt eine mRNA ist, die mit der durch BNO1 codierten mRNA hybridisiert, bei der Herstellung eines Medikaments zur Behandlung einer mit erhöhter Aktivität oder Expression von BNO1 assoziierten Erkrankung.

20. Verfahren zum Durchmustern auf eine Verbindung, welche die Aktivität von BNO1 modulieren kann, wobei das Verfahren aufweist: Vereinigen eines Peptids nach einem der Ansprüche 11 bis 14 mit einer Kandidatenverbindung und Bestimmen der Bindung der Kandidatenverbindung an das Peptid.

21. Durchmusterungsverfahren für Wirkstoffkandidaten, das die folgenden Schritte aufweist:

(1) Bereitstellen einer Zelle nach einem der Ansprüche 7 oder 9;
(2) Zusatz eines Wirkstoffkandidaten zu der Zelle; und
(3) Bestimmen der Wirkung des Wirkstoffkandidaten auf die Expression von BNO1 durch die Zelle.

22. Verwendung einer Nucleinsäure nach einem der Ansprüche 1 bis 5 beim Durchmustern auf Wirkstoffkandidaten.

23. In-vitro-Verwendung einer Nucleinsäure nach einem der Ansprüche 1 bis 5 für die Diagnose oder Prognose von mit BNO1-Dysfunktion assoziierten Erkrankungen oder einer Prädisposition für solche Erkrankungen.

24. In-vitro-Verwendung eines Polypeptids nach einem der Ansprüche 11 bis 14 für die Diagnose oder Prognose von mit BNO1-Dysfunktion assoziierten Erkrankungen oder einer Prädisposition für solche Erkrankungen.

25. In-vitro-Verwendung eines Antikörpers gemäß der Definition in Anspruch 15 bei der Diagnose oder Prognose von mit BN01 assoziierten Erkrankungen oder einer Prädisposition für solche Erkrankungen.

26. In-vitro-Verfahren für die Diagnose oder Prognose einer mit Mutationen in BNO1 assoziierten Erkrankung oder einer Prädisposition für solche Erkrankungen bei einem Patienten, wobei das Verfahren die folgenden Schritte aufweist:

Vergleich von BNO1 oder einer für BNO1 codierenden Nucleinsäure aus einer von einem Patienten zu entnehmenden Probe mit Wildtyp-BNO1 oder einer dafür codierenden Nucleinsäure, um festzustellen, ob der Patient ein mutiertes BNO1 exprimiert.

27. Verfahren nach Anspruch 26, wobei die Nucleotidsequenz der DNA von dem Patienten mit der für Wildtyp-BNO1 codierenden DNA-Sequenz verglichen wird.

28. In-vitro-Verfahren für die Diagnose oder Prognose einer mit anomaler Expression oder Aktivität von BNO1 assoziierten Erkrankung oder einer Prädisposition für solche Erkrankungen, wobei das Verfahren die folgenden Schritte aufweist:

(1) Ermitteln eines Profils für normale Expression von BNO1 bei nicht betroffenen Probanden;
(2) Messen des Expressionsgrads von BNO1 bei einem Probanden mit Verdacht auf anomale Expression oder Aktivität von BNO1; und
(3) Vergleich des gemessenen Expressionsgrads mit dem Profil für normale Expression.

29. Verfahren nach Anspruch 28, wobei zur Messung von Expressionsgraden Revertase-PCR angewandt wird.

30. Verfahren nach Anspruch 28, wobei zur Messung von Expressionsgraden ein Hybridisierungsassay mit einer von BNO1 oder einem Fragment davon abgeleiteten Sonde angewandt wird.

31. Verfahren nach Anspruch 30, wobei die Sonde eine Sequenzidentität von mindestens 50% mit einer für BNO1 oder ein Fragment davon codierenden Nucleotidsequenz aufweist.

32. In-vitro-Verfahren für die Diagnose oder Prognose einer mit BNO1 assoziierten Erkrankung oder einer Prädisposition für solche Erkrankungen, wobei das Verfahren die folgenden Schritte aufweist:

(1) Ermitteln einer physikalischen Eigenschaft von Wildtyp-BNO1;
(2) Messen der Eigenschaft für ein BNO1, das durch einen Probanden mit Verdacht auf eine Anomalität von BNO1 exprimiert wird; und
(3) Vergleich der Eigenschaft mit der ermittelten Eigenschaft für Wildtyp-BNO1, um festzustellen, ob der Proband ein mutiertes BNO1 exprimiert.

33. Verfahren nach Anspruch 32, wobei die Eigenschaft die elektrophoretische Beweglichkeit ist.

34. Verfahren nach Anspruch 32, wobei die Eigenschaft das proteolytische Spaltungsmuster ist.

35. Genetisch modifiziertes nichtmenschliches Tier, das aus der Gruppe ausgewählt ist, die aus Ratten, Mäusen, Hamstern, Meerschweinchen, Kaninchen, Hunden, Katzen, Ziegen, Schafen, Schweinen und nichtmenschlichen Primaten, wie z. B. kleineren Affen oder Schimpansen besteht, wobei das Tier mit einem isolierten Nucleinsäuremolekül gemäß der Definition in einem der Ansprüche 1 bis 5 transformiert ist.

36. Genetisch modifiziertes nichtmenschliches Tier, das aus der Gruppe ausgewählt ist, die aus Ratten, Mäusen, Hamstern, Meerschweinchen, Kaninchen, Hunden, Katzen, Ziegen, Schafen, Schweinen und nichtmenschlichen Primaten, wie z. B. kleineren Affen oder Schimpansen besteht, in denen das homologe BNO1-Gen und die Genfunktion inaktiviert worden sind.

37. Verwendung eines genetisch modifizierten nichtmenschlichen Tiers, wie in einem der Ansprüche 35 oder 36 definiert, beim Durchmustern auf pharmazeutische Kandidatenverbindungen.

38. Mikroarray zum Nachweis von BNO1, der eine für die eine oder andere Isoform von BNO1 oder einem Fragment davon codierende Nucleinsäure oder Nucleinsäuren aufweist, die für beide Isoformen von BNO1 oder Fragmente davon codieren.

Revendications

1. Cartographie de la molécule d'acide nucléique BNO1 isolée au chromosome humain 16q24.3 et comprenant la séquence de nucléotides présentée dans la SEQ ID numéro: 1 ou 3.

2. Molécule d'acide nucléique BNO1 isolée comprenant la séquence de nucléotides présentée dans la SEQ ID numéro: 1 ou 3, qui code pour un polypeptide capable de former une partie d'un complexe d'ubiquitine-ligase impliqué dans le ciblage de protéines par ubiquitination pour une dégradation par le protéasome.

3. Molécule d'acide nucléique BNO1 isolée qui est au moins 95% identique à une molécule d'ADN constituée de la séquence de nucléotides présentée dans la SEQ ID numéro: 1 ou 3 et qui code pour un polypeptide capable de former une partie d'un complexe d'ubiquitine-ligase impliqué dans le ciblage de protéines par ubiquitination pour une dégradation par le protéasome.

4. Molécule d'acide nucléique BNO1 isolée qui code pour un polypeptide possédant la séquence d'acides aminés présentée dans la SEQ ID numéro: 2 ou 4.

5. Molécule d'acide nucléique isolée constituée de la séquence de nucléotides présentée dans la SEQ ID numéro: 1 ou 3.

6. Vecteur d'expression qui comprend une molécule d'acide nucléique telle que définie dans l'une quelconque des revendications 1 à 5 liée de manière fonctionnelle à des éléments de contrôle appropriés.

7. Cellule isolée transformée avec le vecteur d'expression selon la revendication 6.

8. Cellule selon la revendication 7, dans laquelle l'expression de BNO1 recombinant peut être interrompue.

9. Cellule selon l'une quelconque des revendications 7 et 8, qui est une cellule eucaryote.

10. Procédé pour la préparation d'un polypeptide codé par l'un quelconque des acides nucléiques selon les revendications 1 à 5, comprenant les étapes:

    (1) de culture d'une cellule telle que définie dans la revendication 7 ou 9 dans des conditions efficaces pour la production du polypeptide; et
    (2) de récolte du polypeptide.

11. Polypeptide BNO1 isolé comprenant la séquence d'acides aminés présentée dans la SEQ ID numéro: 2 ou 4.

12. Polypeptide BNO1 isolé, comprenant la séquence d'acides aminés présentée dans la SEQ ID numéro: 2 ou 4, qui est capable de former une partie d'un complexe d'ubiquitine-ligase impliqué dans une dégradation de protéines par ubiquitination.

13. Polypeptide BNO1 isolé capable de former une partie d'un complexe d'ubiquitine-ligase impliqué dans une dégradation de protéines par ubiquitination qui possède au moins 95% d'identité avec la séquence d'acides aminés présentée dans la SEQ ID numéro: 2 ou 4.

14. Polypeptide BNO1 isolé constitué de la séquence d'acides aminés présentée dans la SEQ ID numéro: 2 ou 4.

15. Anticorps pour la détection de BNO1 qui est immunologiquement réactif avec un polypeptide tel que défini dans l'une quelconque des revendications 11 à 14, de préférence un anticorps monoclonal, un anticorps humanisé, un

anticorps chimérique ou un fragment d'anticorps incluant un fragment Fab, un fragment $F(ab')_2$, un fragment Fv, des anticorps à une seule chaîne et des anticorps à un seul domaine.

16. Utilisation d'une molécule d'acide nucléique selon l'une quelconque des revendications 1 à 5 dans la fabrication d'un médicament pour le traitement d'un trouble associé à une expression ou une activité diminuée de BNO1.

17. Utilisation selon la revendication 16, dans laquelle la molécule d'acide nucléique est une partie d'un vecteur d'expression qui inclut également des éléments de contrôle appropriés.

18. Utilisation d'un antagoniste de BNO1 dans la fabrication d'un médicament pour le traitement d'un trouble associé à une expression ou une activité accrue de BNO1, dans laquelle ledit antagoniste est un anticorps selon la revendication 15.

19. Utilisation d'une molécule d'acide nucléique isolée qui est le complément d'une molécule d'acide nucléique telle que définie dans l'une quelconque des revendications 1 à 5, dont le produit de transcription est un ARNm qui s'hybride avec l'ARNm codé par BNO1, dans la fabrication d'un médicament pour le traitement d'un trouble associé à une activité ou une expression accrue de BNO1.

20. Procédé pour le criblage d'un composé capable de moduler l'activité de BNO1 comprenant la combinaison d'un peptide selon l'une quelconque des revendications 11 à 14 et d'un composé candidat, et la détermination de la liaison dudit composé candidat avec ledit peptide.

21. Procédé pour le criblage de médicaments candidats comprenant les étapes:

(1) de fourniture d'une cellule selon l'une quelconque des revendications 7 et 9;
(2) d'ajout d'un médicament candidat à ladite cellule; et
(3) de détermination de l'effet dudit médicament candidat sur l'expression de BNO1 par ladite cellule.

22. Utilisation d'un acide nucléique selon l'une quelconque des revendications 1 à 5 dans le criblage de médicaments candidats.

23. Utilisation in vitro d'un acide nucléique selon l'une quelconque des revendications 1 à 5 pour le diagnostic ou le pronostic de troubles associés à un dysfonctionnement de BNO1 ou d'une prédisposition pour ces troubles.

24. Utilisation in vitro d'un polypeptide selon l'une quelconque des revendications 11 à 14 pour le diagnostic ou le pronostic de troubles associés à un dysfonctionnement de BNO1 ou d'une prédisposition pour ces troubles.

25. Utilisation in vitro d'un anticorps selon la revendication 15 dans le diagnostic ou le pronostic d'un trouble associé à BNO1 ou d'une prédisposition pour ces troubles.

26. Procédé in vitro pour le diagnostic ou le pronostic d'un trouble associé à des mutations dans BNO1 ou d'une prédisposition pour ces troubles chez un patient, comprenant les étapes:

de comparaison de BNO1 ou d'un acide nucléique qui code pour BNO1 avec un échantillon devant être obtenu à partir d'un patient avec un BNO1 de type sauvage ou un acide nucléique qui code pour celui-ci dans le but d'établir si la personne exprime un BNO1 mutant.

27. Procédé selon la revendication 26, dans lequel la séquence de nucléotides de l'ADN provenant du patient est comparée à la séquence d'ADN codant pour un BNO1 de type sauvage.

28. Procédé in vitro pour le diagnostic ou le pronostic d'un trouble associé à une expression ou une activité anormale de BNO1 ou d'une prédisposition pour ces troubles, comprenant les étapes:

(1) d'établissement d'un profil pour une expression normale de BNO1 chez des sujets non affectés;
(2) de mesure du niveau d'expression de BNO1 chez une personne suspectée d'une expression ou d'une activité anormale de BNO1; et
(3) de comparaison du niveau d'expression mesuré avec le profil pour une expression normale.

29. Procédé selon la revendication 28, dans lequel une transcriptase inverse-PCR est employée pour mesurer les niveaux d'expression.

30. Procédé selon la revendication 28, dans lequel un dosage d'hybridation utilisant une sonde dérivée de BNO1, ou un fragment de celui-ci, est employé pour mesurer les niveaux d'expression.

31. Procédé selon la revendication 30, dans lequel la sonde possède au moins 50% d'identité de séquence avec une séquence de nucléotides codant pour BNO1 ou un fragment de celui-ci.

32. Procédé in vitro pour le diagnostic ou le pronostic d'un trouble associé à BNO1 ou d'une prédisposition pour ces troubles, comprenant les étapes:

(1) d'établissement d'une propriété physique d'un BNO1 de type sauvage;
(2) de mesure de la propriété pour un BNO1 exprimé par une personne suspectée d'un BNO1 anormal; et
(3) de comparaison de celle-ci avec la propriété établie pour un BNO1 de type sauvage dans le but d'établir si la personne exprime un BNO1 mutant.

33. Procédé selon la revendication 32, dans lequel la propriété est la mobilité électrophorétique.

34. Procédé selon la revendication 32, dans lequel la propriété est le modèle de clivage protéolytique.

35. Animal non humain génétiquement modifié choisi dans le groupe constitué de rats, de souris, d'hamsters, de cobayes, de lapins, de chiens, de chats, de chèvres, de moutons, de cochons et de primates non humains tels que des singes et des chimpanzés, transformé avec une molécule d'acide nucléique isolée telle que définie dans l'une quelconque des revendications 1 à 5.

36. Animal non humain génétiquement modifié choisi dans le groupe constitué de rats, de souris, d'hamsters, de cobayes, de lapins, de chiens, de chats, de chèvres, de moutons, de cochons et de primates non humains tels que des singes et des chimpanzés, dans lequel un gène BNO1 homologue et une fonction de gène ont été éliminés.

37. Utilisation d'un animal non humain génétiquement modifié tel que défini dans l'une ou l'autre des revendications 35 et 36 dans le criblage de composés pharmaceutiques candidats.

38. Micro-réseau pour la détection de BNO1 comprenant un acide nucléique codant pour l'une ou l'autre isoforme de BNO1, ou un fragment de celui-ci, ou des acides nucléiques codant pour les deux isoformes de BNO1, ou des fragments de celui-ci.

Figure 1

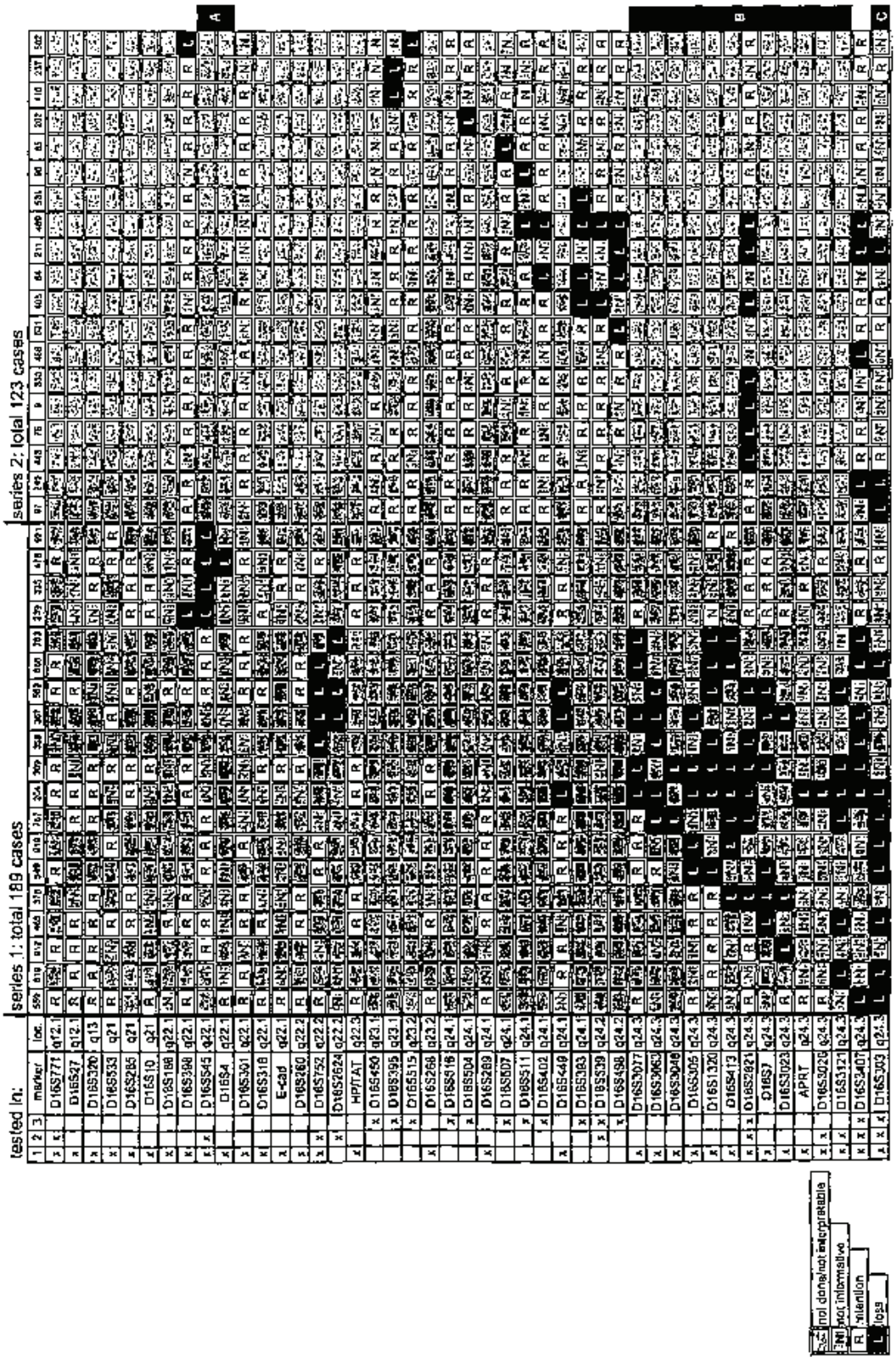

tested in:
series 1: total 189 cases
series 2: total 123 cases
marker
loc.
not done/not interpretable
not informative
retention
loss

Figure 2

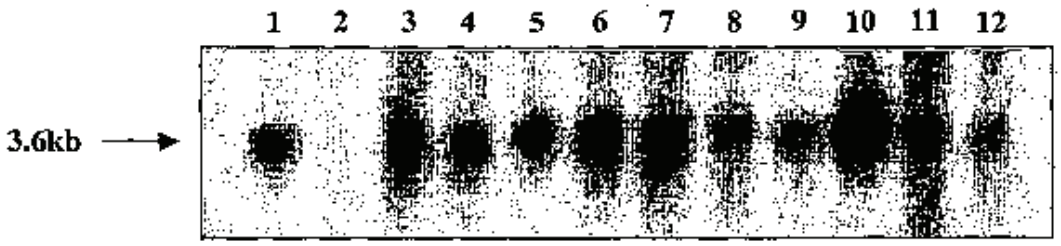
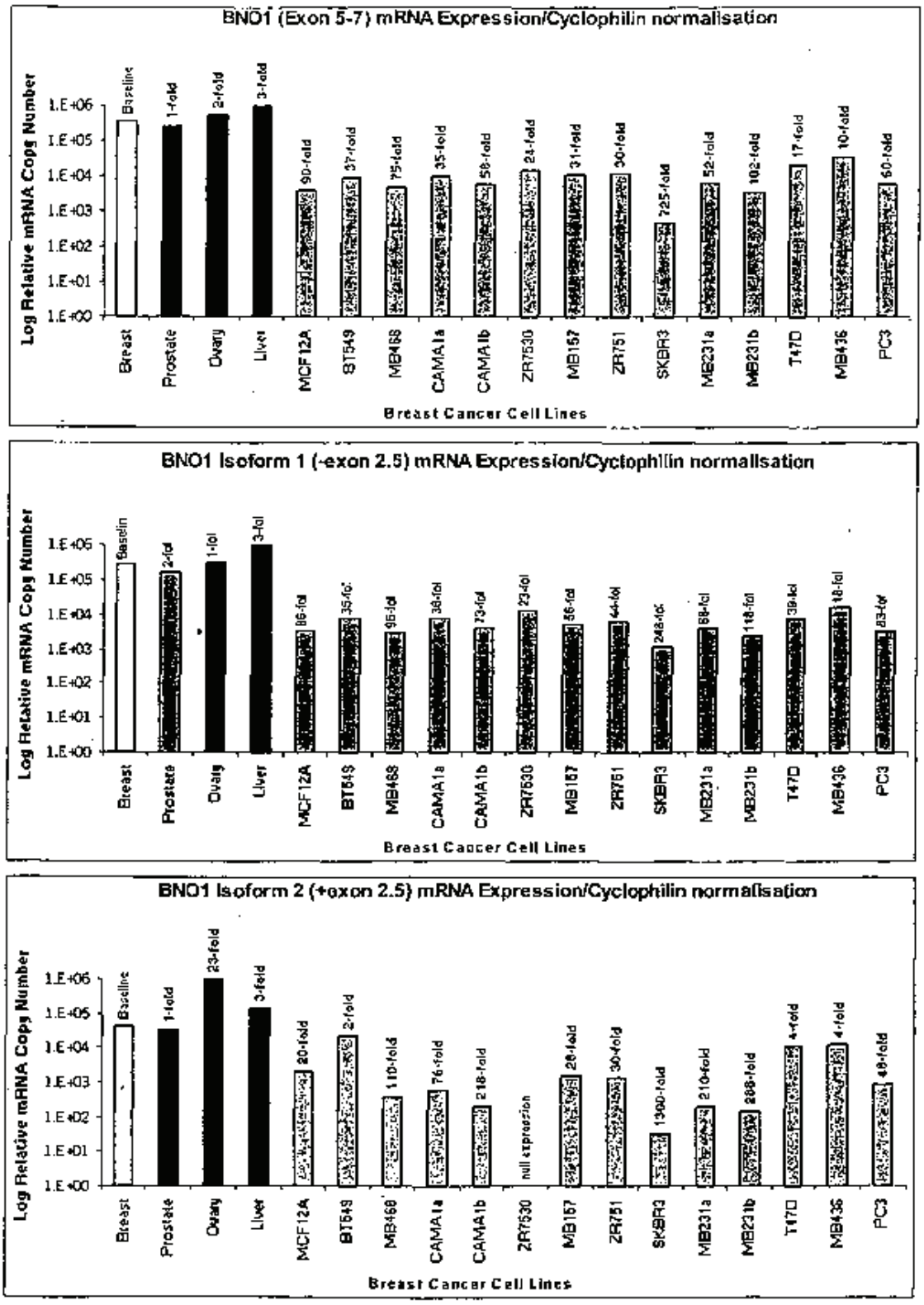


Figure 3

```
BNO1  - R C S L L E L P P E L L V E I F A S L P G T D L P S L A Q V C T K F R R I L H T D T I W R R R - -
        k P P P L L R L P e E I L r K I L e k I D P i D L L r L R K V S K K W R s L V D s l n i w f k f I e
        s s   s I s d m   l K l i k e V f k h M p f k E R f n F S l t C R R F K r i i k k   k f k i r k L l
              r f   n     i d v     n   i r r   s l     i k k     f l     n l   q   l r d     l f k d
                          a           s y   e I       v s             t                   r
                                                  q
```

Figure 4


BNO1 (Exon 5-7) mRNA Expression/Cyclophilin normalisation
Log Relative mRNA Copy Number
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
Baseline
1-fold
2-fold
3-fold
90-fold
37-fold
75-fold
35-fold
58-fold
24-fold
31-fold
30-fold
225-fold
52-fold
102-fold
17-fold
10-fold
60-fold
Breast
Prostate
Ovary
Liver
MCF12A
BT549
MB468
CAMA1a
CAMA1b
ZR7530
MB157
ZR751
SKBR3
MB231a
MB231b
T47D
MB436
PC3
Breast Cancer Cell Lines
BNO1 Isoform 1 (-exon 2.5) mRNA Expression/Cyclophilin normalisation
Log Relative mRNA Copy Number
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
Baselin
2-fol
1-fol
3-fol
86-fol
35-fo
95-fol
38-fol
73-fol
23-fol
56-fol
44-fol
248-fo
88-fol
118-fol
39-fol
18-fol
83-fol
Breast
Prostate
Ovary
Liver
MCF12A
BT549
MB468
CAMA1a
CAMA1b
ZR7530
MB157
ZR751
SKBR3
MB231a
MB231b
T47D
MB436
PC3
Breast Cancer Cell Lines
BNO1 Isoform 2 (+exon 2.5) mRNA Expression/Cyclophilin normalisation
Log Relative mRNA Copy Number
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
Baseline
1-fold
23-fold
3-fold
20-fold
2-fold
110-fold
76-fold
218-fold
null expression
28-fold
30-fold
1300-fold
210-fold
268-fold
4-fold
4-fold
48-fold
Breast
Prostate
Ovary
Liver
MCF12A
BT549
MB468
CAMA1a
CAMA1b
ZR7530
MB157
ZR751
SKBR3
MB231a
MB231b
T47D
MB436
PC3
Breast Cancer Cell Lines

REFERENCES CITED IN THE DESCRIPTION

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