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(54) Title: MRBS AS MODIFIERS OF THE RB PATHWAY AND METHODS OF USE

(57) Abstract: Human MRB genes are identified as modulators of the RB pathway, and thus are therapeutic targets for disorders associated with defective RB function. Methods for identifying modulators of RB, comprising screening for agents that modulate the activity of MRB are provided.



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MRBS AS MODIFIERS OF THE RB PATHWAY AND METHODS OF USE**REFERENCE TO RELATED APPLICATIONS**

This application claims priority to U.S. provisional patent applications 60/401,737
5 filed 8/7/2002 and 60/428,872 filed 11/25/2002. The contents of the prior applications are
hereby incorporated in their entirety.

BACKGROUND OF THE INVENTION

Retinoblastoma, a pediatric eye tumor, has served as an important model for the
10 heritable predisposition to cancer. The primary mechanism in the development of
retinoblastoma is loss or inactivation of both alleles of this gene (Murphree, A. L. and
Benedict, W. F. (1984) Science 223: 1028-1033). The high incidence of second primary
tumors among patients who inherit one retinoblastoma gene suggests that this cancer gene
plays a key role in the etiology of several other primary malignancies.

15 The retinoblastoma protein, RB, functions as a tumor suppressor by controlling
progression through the cell cycle which is achieved by sequestering a variety of nuclear
proteins involved in cellular growth. Thus, it acts as a signal transducer connecting the
cell cycle clock with the transcriptional machinery (Weinberg, R. A. (1995) Cell 81: 323-
330). RB regulates cell proliferation by restricting cell cycle progression at a specific
20 point in G1, by interaction with the E2F family of transcription factors to arrest cells in G1
(Goodrich, D. W. et al. (1991) Cell 67: 293-302; Zhang, H. S. et al. (1999) Cell 97: 53-
61).

RB function is regulated primarily by its phosphorylation state, which is
determined by the complex interaction of multiple kinases and their inhibitors that
25 together form the 'Rb pathway' (DeCaprio, J. A. et al (1989) Cell 58: 1085-1095;
Buchkovich, K. et al (1989) Cell 58: 1097-1105; Chen, P.-L. et al. (1989) Cell 58: 1193-
1198). This pathway has been found to be functionally inactivated in almost all types of
cancer.

RB sequence is conserved in evolution, and exists in mouse (Bernards R et al
30 (1989) Proc. Natl. Acad. Sci. U.S.A. 86:6474-6478), rat (Roy NK et al. (1993) Nucleic
Acids Res. 21:170-170), *Drosophila* (Du W et al (1996) Genes Dev 10:1206-18), and *C.*
elegans (The C. elegans Sequencing Consortium (1998) Science 282:2012-2018).

The ability to manipulate the genomes of model organisms such as *Drosophila*
provides a powerful means to analyze biochemical processes that, due to significant

evolutionary conservation, have direct relevance to more complex vertebrate organisms.

Due to a high level of gene and pathway conservation, the strong similarity of cellular processes, and the functional conservation of genes between these model organisms and mammals, identification of the involvement of novel genes in particular pathways and their functions in such model organisms can directly contribute to the understanding of the correlative pathways and methods of modulating them in mammals (see, for example, Mechler BM et al., 1985 EMBO J 4:1551-1557; Gateff E. 1982 Adv. Cancer Res. 37: 33-74; Watson KL., et al., 1994 J Cell Sci. 18: 19-33; Miklos GL, and Rubin GM. 1996 Cell 86:521-529; Wassarman DA, et al., 1995 Curr Opin Gen Dev 5: 44-50; and Booth DR. 1999 Cancer Metastasis Rev. 18: 261-284). For example, a genetic screen can be carried out in an invertebrate model organism or cell having underexpression (e.g. knockout) or overexpression of a gene (referred to as a “genetic entry point”) that yields a visible phenotype, such as altered cell growth. Additional genes are mutated in a random or targeted manner. When a gene mutation changes the original phenotype caused by the mutation in the genetic entry point, the gene is identified as a “modifier” involved in the same or overlapping pathway as the genetic entry point. When inactivation of either gene is not lethal, but inactivation of both genes results in reduced viability or death of the cell, tissue, or organism, the interaction is defined as “synthetic lethal” (Bender, A and Pringle J, (1991) Mol Cell Biol, 11:1295-1305; Hartman J et al, (2001) Science 291:1001-1004; US PAT No:6,489,127). In a synthetic lethal interaction, the modifier may also be identified as an “interactor”. When the genetic entry point is an ortholog of a human gene implicated in a disease pathway, such as RB, modifier genes can be identified that may be attractive candidate targets for novel therapeutics.

All references cited herein, including patents, patent applications, publications, and sequence information in referenced Genbank identifier numbers, are incorporated herein in their entireties.

SUMMARY OF THE INVENTION

We have discovered genes that modify the RB pathway in *Drosophila* cells, and identified their human orthologs, hereinafter referred to as modifier of RB (MRB). The invention provides methods for utilizing these RB modifier genes and polypeptides to identify MRB-modulating agents that are candidate therapeutic agents that can be used in the treatment of disorders associated with defective or impaired RB function and/or MRB function. Preferred MRB-modulating agents specifically bind to MRB polypeptides and

restore RB function. Other preferred MRB-modulating agents are nucleic acid modulators such as antisense oligomers and RNAi that repress MRB gene expression or product activity by, for example, binding to and inhibiting the respective nucleic acid (i.e. DNA or mRNA).

5 MRB modulating agents may be evaluated by any convenient *in vitro* or *in vivo* assay for molecular interaction with an MRB polypeptide or nucleic acid. In one embodiment, candidate MRB modulating agents are tested with an assay system comprising a MRB polypeptide or nucleic acid. Agents that produce a change in the activity of the assay system relative to controls are identified as candidate RB modulating
10 agents. The assay system may be cell-based or cell-free. MRB-modulating agents include MRB related proteins (e.g. dominant negative mutants, and biotherapeutics); MRB - specific antibodies; MRB -specific antisense oligomers and other nucleic acid modulators; and chemical agents that specifically bind to or interact with MRB or compete with MRB binding partner (e.g. by binding to an MRB binding partner). In one specific embodiment,
15 a small molecule modulator is identified using a binding assay. In specific embodiments, the screening assay system is selected from an apoptosis assay, a cell proliferation assay, an angiogenesis assay, and a hypoxic induction assay.

In another embodiment, candidate RB pathway modulating agents are further tested using a second assay system that detects changes in the RB pathway, such as
20 angiogenic, apoptotic, or cell proliferation changes produced by the originally identified candidate agent or an agent derived from the original agent. The second assay system may use cultured cells or non-human animals. In specific embodiments, the secondary assay system uses non-human animals, including animals predetermined to have a disease or disorder implicating the RB pathway, such as an angiogenic, apoptotic, or cell
25 proliferation disorder (e.g. cancer).

The invention further provides methods for modulating the MRB function and/or the RB pathway in a mammalian cell by contacting the mammalian cell with an agent that specifically binds a MRB polypeptide or nucleic acid. The agent may be a small molecule modulator, a nucleic acid modulator, or an antibody and may be administered to a
30 mammalian animal predetermined to have a pathology associated the RB pathway.

DETAILED DESCRIPTION OF THE INVENTION

The Rb co-RNAi synthetic lethal screen was designed to identify modifier genes that are synthetic lethal with the *Drosophila* Rbf gene (Du W et al (1996) *supra*), a

Drosophila homolog of the human retinoblastoma (RB) gene. In addition to identifying modifier genes with synthetic lethal interactions with Rbf, this screen identified modifier genes that, when inactivated, preferentially reduced the viability of Rbf-deficient cells relative to normal cells. Modifiers of the Rbf pathway were identified, followed by
5 identification of their orthologs. Accordingly, vertebrate orthologs of these modifiers, and preferably the human orthologs, MRB genes (i.e., nucleic acids and polypeptides) are attractive drug targets for the treatment of pathologies associated with a defective RB signaling pathway, such as cancer. Table 1 (Example II) lists the modifiers and their orthologs.

10 In vitro and in vivo methods of assessing MRB function are provided herein. Modulation of the MRB or their respective binding partners is useful for understanding the association of the RB pathway and its members in normal and disease conditions and for developing diagnostics and therapeutic modalities for RB related pathologies. MRB-modulating agents that act by inhibiting or enhancing MRB expression, directly or
15 indirectly, for example, by affecting an MRB function such as binding activity, can be identified using methods provided herein. MRB modulating agents are useful in diagnosis, therapy and pharmaceutical development.

Nucleic acids and polypeptides of the invention

20 Sequences related to MRB nucleic acids and polypeptides that can be used in the invention are disclosed in Genbank (referenced by Genbank identifier (GI) or RefSeq number), and shown in Table 1.

The term "MRB polypeptide" refers to a full-length MRB protein or a functionally active fragment or derivative thereof. A "functionally active" MRB fragment or derivative
25 exhibits one or more functional activities associated with a full-length, wild-type MRB protein, such as antigenic or immunogenic activity, ability to bind natural cellular substrates, etc. The functional activity of MRB proteins, derivatives and fragments can be assayed by various methods known to one skilled in the art (Current Protocols in Protein Science (1998) Coligan *et al.*, eds., John Wiley & Sons, Inc., Somerset, New Jersey) and
30 as further discussed below. In one embodiment, a functionally active MRB polypeptide is a MRB derivative capable of rescuing defective endogenous MRB activity, such as in cell based or animal assays; the rescuing derivative may be from the same or a different species. For purposes herein, functionally active fragments also include those fragments that comprise one or more structural domains of an MRB, such as a binding domain.

Protein domains can be identified using the PFAM program (Bateman A., et al., Nucleic Acids Res, 1999, 27:260-2). Methods for obtaining MRB polypeptides are also further described below. In some embodiments, preferred fragments are functionally active, domain-containing fragments comprising at least 25 contiguous amino acids, preferably at least 50, more preferably 75, and most preferably at least 100 contiguous amino acids of an MRB. In further preferred embodiments, the fragment comprises the entire functionally active domain.

The term "MRB nucleic acid" refers to a DNA or RNA molecule that encodes a MRB polypeptide. Preferably, the MRB polypeptide or nucleic acid or fragment thereof is from a human, but can also be an ortholog, or derivative thereof with at least 70% sequence identity, preferably at least 80%, more preferably 85%, still more preferably 90%, and most preferably at least 95% sequence identity with human MRB. Methods of identifying orthologs are known in the art. Normally, orthologs in different species retain the same function, due to presence of one or more protein motifs and/or 3-dimensional structures. Orthologs are generally identified by sequence homology analysis, such as BLAST analysis, usually using protein bait sequences. Sequences are assigned as a potential ortholog if the best hit sequence from the forward BLAST result retrieves the original query sequence in the reverse BLAST (Huynen MA and Bork P, Proc Natl Acad Sci (1998) 95:5849-5856; Huynen MA *et al.*, Genome Research (2000) 10:1204-1210). Programs for multiple sequence alignment, such as CLUSTAL (Thompson JD et al, 1994, Nucleic Acids Res 22:4673-4680) may be used to highlight conserved regions and/or residues of orthologous proteins and to generate phylogenetic trees. In a phylogenetic tree representing multiple homologous sequences from diverse species (e.g., retrieved through BLAST analysis), orthologous sequences from two species generally appear closest on the tree with respect to all other sequences from these two species. Structural threading or other analysis of protein folding (e.g., using software by ProCeryon, Biosciences, Salzburg, Austria) may also identify potential orthologs. In evolution, when a gene duplication event follows speciation, a single gene in one species, such as *Drosophila*, may correspond to multiple genes (paralogs) in another, such as human. As used herein, the term "orthologs" encompasses paralogs. As used herein, "percent (%) sequence identity" with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of nucleotides or amino acids in the candidate derivative sequence identical with the nucleotides or amino acids in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary

to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul *et al.*, J. Mol. Biol. (1997) 215:403-410) with all the search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. A % identity value is determined by the number of matching identical nucleotides or amino acids divided by the sequence length for which the percent identity is being reported. "Percent (%) amino acid sequence similarity" is determined by doing the same calculation as for determining % amino acid sequence identity, but including conservative amino acid substitutions in addition to identical amino acids in the computation.

A conservative amino acid substitution is one in which an amino acid is substituted for another amino acid having similar properties such that the folding or activity of the protein is not significantly affected. Aromatic amino acids that can be substituted for each other are phenylalanine, tryptophan, and tyrosine; interchangeable hydrophobic amino acids are leucine, isoleucine, methionine, and valine; interchangeable polar amino acids are glutamine and asparagine; interchangeable basic amino acids are arginine, lysine and histidine; interchangeable acidic amino acids are aspartic acid and glutamic acid; and interchangeable small amino acids are alanine, serine, threonine, cysteine and glycine.

Alternatively, an alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman (Smith and Waterman, 1981, Advances in Applied Mathematics 2:482-489; database: European Bioinformatics Institute; Smith and Waterman, 1981, J. of Molec.Biol., 147:195-197; Nicholas et al., 1998, "A Tutorial on Searching Sequence Databases and Sequence Scoring Methods" (www.psc.edu) and references cited therein.; W.R. Pearson, 1991, Genomics 11:635-650). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff (Dayhoff: Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA), and normalized by Gribskov (Gribskov 1986 Nucl. Acids Res. 14(6):6745-6763). The Smith-Waterman algorithm may be employed where default parameters are used for scoring (for example, gap open penalty of 12, gap extension penalty of two). From the data generated, the "Match" value reflects "sequence identity."

Derivative nucleic acid molecules of the subject nucleic acid molecules include sequences that hybridize to the nucleic acid sequence of an MRB. The stringency of

hybridization can be controlled by temperature, ionic strength, pH, and the presence of denaturing agents such as formamide during hybridization and washing. Conditions routinely used are set out in readily available procedure texts (*e.g.*, Current Protocol in Molecular Biology, Vol. 1, Chap. 2.10, John Wiley & Sons, Publishers (1994); Sambrook *et al.*, Molecular Cloning, Cold Spring Harbor (1989)). In some embodiments, a nucleic acid molecule of the invention is capable of hybridizing to a nucleic acid molecule containing the nucleotide sequence of an MRB under high stringency hybridization conditions that are: prehybridization of filters containing nucleic acid for 8 hours to overnight at 65° C in a solution comprising 6X single strength citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M Na citrate; pH 7.0), 5X Denhardt's solution, 0.05% sodium pyrophosphate and 100 µg/ml herring sperm DNA; hybridization for 18-20 hours at 65° C in a solution containing 6X SSC, 1X Denhardt's solution, 100 µg/ml yeast tRNA and 0.05% sodium pyrophosphate; and washing of filters at 65° C for 1h in a solution containing 0.1X SSC and 0.1% SDS (sodium dodecyl sulfate).

In other embodiments, moderately stringent hybridization conditions are used that are: pretreatment of filters containing nucleic acid for 6 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA; hybridization for 18-20h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, and 10% (wt/vol) dextran sulfate; followed by washing twice for 1 hour at 55° C in a solution containing 2X SSC and 0.1% SDS.

Alternatively, low stringency conditions can be used that are: incubation for 8 hours to overnight at 37° C in a solution comprising 20% formamide, 5 x SSC, 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured sheared salmon sperm DNA; hybridization in the same buffer for 18 to 20 hours; and washing of filters in 1 x SSC at about 37° C for 1 hour.

Isolation, Production, Expression, and Mis-expression of MRB Nucleic Acids and

Polypeptides

MRB nucleic acids and polypeptides, are useful for identifying and testing agents that modulate MRB function and for other applications related to the involvement of MRB in the RB pathway. MRB nucleic acids and derivatives and orthologs thereof may be obtained using any available method. For instance, techniques for isolating cDNA or

genomic DNA sequences of interest by screening DNA libraries or by using polymerase chain reaction (PCR) are well known in the art. In general, the particular use for the protein will dictate the particulars of expression, production, and purification methods.

For instance, production of proteins for use in screening for modulating agents may require methods that preserve specific biological activities of these proteins, whereas production of proteins for antibody generation may require structural integrity of particular epitopes. Expression of proteins to be purified for screening or antibody production may require the addition of specific tags (*e.g.*, generation of fusion proteins). Overexpression of an MRB protein for assays used to assess MRB function, such as involvement in cell cycle regulation or hypoxic response, may require expression in eukaryotic cell lines capable of these cellular activities. Techniques for the expression, production, and purification of proteins are well known in the art; any suitable means therefore may be used (*e.g.*, Higgins SJ and Hames BD (eds.) *Protein Expression: A Practical Approach*, Oxford University Press Inc., New York 1999; Stanbury PF et al., *Principles of Fermentation Technology*, 2nd edition, Elsevier Science, New York, 1995; Doonan S (ed.) *Protein Purification Protocols*, Humana Press, New Jersey, 1996; Coligan JE et al, *Current Protocols in Protein Science* (eds.), 1999, John Wiley & Sons, New York). In particular embodiments, recombinant MRB is expressed in a cell line known to have defective RB function (*e.g.* SAOS-2 osteoblasts, BT549 breast cancer cells, and C33A cervical cancer cells, among others, available from American Type Culture Collection (ATCC), Manassas, VA). The recombinant cells are used in cell-based screening assay systems of the invention, as described further below.

The nucleotide sequence encoding an MRB polypeptide can be inserted into any appropriate expression vector. The necessary transcriptional and translational signals, including promoter/enhancer element, can derive from the native MRB gene and/or its flanking regions or can be heterologous. A variety of host-vector expression systems may be utilized, such as mammalian cell systems infected with virus (*e.g.* vaccinia virus, adenovirus, *etc.*); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, plasmid, or cosmid DNA. An isolated host cell strain that modulates the expression of, modifies, and/or specifically processes the gene product may be used.

To detect expression of the MRB gene product, the expression vector can comprise a promoter operably linked to an MRB gene nucleic acid, one or more origins of replication, and, one or more selectable markers (*e.g.* thymidine kinase activity, resistance

to antibiotics, *etc.*). Alternatively, recombinant expression vectors can be identified by assaying for the expression of the MRB gene product based on the physical or functional properties of the MRB protein in *in vitro* assay systems (*e.g.* immunoassays).

The MRB protein, fragment, or derivative may be optionally expressed as a fusion, or chimeric protein product (*i.e.* it is joined via a peptide bond to a heterologous protein sequence of a different protein), for example to facilitate purification or detection. A chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other using standard methods and expressing the chimeric product. A chimeric product may also be made by protein synthetic techniques, *e.g.* by use of a peptide synthesizer (Hunkapiller *et al.*, Nature (1984) 310:105-111).

Once a recombinant cell that expresses the MRB gene sequence is identified, the gene product can be isolated and purified using standard methods (*e.g.* ion exchange, affinity, and gel exclusion chromatography; centrifugation; differential solubility; electrophoresis). Alternatively, native MRB proteins can be purified from natural sources, by standard methods (*e.g.* immunoaffinity purification). Once a protein is obtained, it may be quantified and its activity measured by appropriate methods, such as immunoassay, bioassay, or other measurements of physical properties, such as crystallography.

The methods of this invention may also use cells that have been engineered for altered expression (mis-expression) of MRB or other genes associated with the RB pathway. As used herein, mis-expression encompasses ectopic expression, over-expression, under-expression, and non-expression (*e.g.* by gene knock-out or blocking expression that would otherwise normally occur).

Genetically modified animals

Animal models that have been genetically modified to alter MRB expression may be used in *in vivo* assays to test for activity of a candidate RB modulating agent, or to further assess the role of MRB in a RB pathway process such as apoptosis or cell proliferation. Preferably, the altered MRB expression results in a detectable phenotype, such as decreased or increased levels of cell proliferation, angiogenesis, or apoptosis compared to control animals having normal MRB expression. The genetically modified animal may additionally have altered RB expression (*e.g.* RB knockout). Preferred genetically modified animals are mammals such as primates, rodents (preferably mice or rats), among others. Preferred non-mammalian species include zebrafish, *C. elegans*, and *Drosophila*. Preferred genetically modified animals are transgenic animals having a

heterologous nucleic acid sequence present as an extrachromosomal element in a portion of its cells, i.e. mosaic animals (see, for example, techniques described by Jakobovits, 1994, *Curr. Biol.* 4:761-763.) or stably integrated into its germ line DNA (i.e., in the genomic sequence of most or all of its cells). Heterologous nucleic acid is introduced into the germ line of such transgenic animals by genetic manipulation of, for example, embryos or embryonic stem cells of the host animal.

Methods of making transgenic animals are well-known in the art (for transgenic mice see Brinster et al., *Proc. Nat. Acad. Sci. USA* 82: 4438-4442 (1985), U.S. Pat. Nos. 4,736,866 and 4,870,009, both by Leder et al., U.S. Pat. No. 4,873,191 by Wagner et al., and Hogan, B., *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); for particle bombardment see U.S. Pat. No., 4,945,050, by Sandford *et al.*; for transgenic *Drosophila* see Rubin and Spradling, *Science* (1982) 218:348-53 and U.S. Pat. No. 4,670,388; for transgenic insects see Berghammer A.J. *et al.*, A Universal Marker for Transgenic Insects (1999) *Nature* 402:370-371; for transgenic Zebrafish see Lin S., *Transgenic Zebrafish*, *Methods Mol Biol.* (2000);136:375-3830); for microinjection procedures for fish, amphibian eggs and birds see Houdebine and Chourrout, *Experientia* (1991) 47:897-905; for transgenic rats see Hammer *et al.*, *Cell* (1990) 63:1099-1112; and for culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection see, e.g., *Teratocarcinomas and Embryonic Stem Cells, A Practical Approach*, E. J. Robertson, ed., IRL Press (1987)). Clones of the nonhuman transgenic animals can be produced according to available methods (see Wilmut, I. *et al.* (1997) *Nature* 385:810-813; and PCT International Publication Nos. WO 97/07668 and WO 97/07669).

In one embodiment, the transgenic animal is a "knock-out" animal having a heterozygous or homozygous alteration in the sequence of an endogenous MRB gene that results in a decrease of MRB function, preferably such that MRB expression is undetectable or insignificant. Knock-out animals are typically generated by homologous recombination with a vector comprising a transgene having at least a portion of the gene to be knocked out. Typically a deletion, addition or substitution has been introduced into the transgene to functionally disrupt it. The transgene can be a human gene (e.g., from a human genomic clone) but more preferably is an ortholog of the human gene derived from the transgenic host species. For example, a mouse MRB gene is used to construct a homologous recombination vector suitable for altering an endogenous MRB gene in the

mouse genome. Detailed methodologies for homologous recombination in mice are available (see Capecchi, *Science* (1989) 244:1288-1292; Joyner *et al.*, *Nature* (1989) 338:153-156). Procedures for the production of non-rodent transgenic mammals and other animals are also available (Houdebine and Chourrout, *supra*; Pursel *et al.*, *Science* (1989) 244:1281-1288; Simms *et al.*, *Bio/Technology* (1988) 6:179-183). In a preferred embodiment, knock-out animals, such as mice harboring a knockout of a specific gene, may be used to produce antibodies against the human counterpart of the gene that has been knocked out (Claesson MH *et al.*, (1994) *Scan J Immunol* 40:257-264; Declerck PJ *et al.*, (1995) *J Biol Chem.* 270:8397-400).

10 In another embodiment, the transgenic animal is a "knock-in" animal having an alteration in its genome that results in altered expression (e.g., increased (including ectopic) or decreased expression) of the MRB gene, e.g., by introduction of additional copies of MRB, or by operatively inserting a regulatory sequence that provides for altered expression of an endogenous copy of the MRB gene. Such regulatory sequences include
15 inducible, tissue-specific, and constitutive promoters and enhancer elements. The knock-in can be homozygous or heterozygous.

Transgenic nonhuman animals can also be produced that contain selected systems allowing for regulated expression of the transgene. One example of such a system that may be produced is the cre/loxP recombinase system of bacteriophage P1 (Lakso *et al.*,
20 PNAS (1992) 89:6232-6236; U.S. Pat. No. 4,959,317). If a cre/loxP recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other
25 containing a transgene encoding a recombinase. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.* (1991) *Science* 251:1351-1355; U.S. Pat. No. 5,654,182). In a preferred embodiment, both Cre-LoxP and Flp-Frt are used in the same system to regulate expression of the transgene, and for sequential deletion of vector sequences in the same cell (Sun X *et al.*
30 (2000) *Nat Genet* 25:83-6).

The genetically modified animals can be used in genetic studies to further elucidate the RB pathway, as animal models of disease and disorders implicating defective RB function, and for *in vivo* testing of candidate therapeutic agents, such as those identified in screens described below. The candidate therapeutic agents are administered to a

genetically modified animal having altered MRB function and phenotypic changes are compared with appropriate control animals such as genetically modified animals that receive placebo treatment, and/or animals with unaltered MRB expression that receive candidate therapeutic agent.

5 In addition to the above-described genetically modified animals having altered MRB function, animal models having defective RB function (and otherwise normal MRB function), can be used in the methods of the present invention. For example, a mouse with defective RB function can be used to assess, *in vivo*, the activity of a candidate RB modulating agent identified in one of the *in vitro* assays described below. Transgenic
10 mice with defective RB function have been described in literature (Robanus-Maandag E et al. (1998) Genes Dev 12:1599-609; Windle, J. J. et al (1990) Nature 343: 665-669).

Preferably, the candidate RB modulating agent when administered to a model system with cells defective in RB function, produces a detectable phenotypic change in the model system indicating that the RB function is restored, i.e., the cells exhibit normal cell
15 cycle progression.

Modulating Agents

The invention provides methods to identify agents that interact with and/or modulate the function of MRB and/or the RB pathway. Modulating agents identified by
20 the methods are also part of the invention. Such agents are useful in a variety of diagnostic and therapeutic applications associated with the RB pathway, as well as in further analysis of the MRB protein and its contribution to the RB pathway. Accordingly, the invention also provides methods for modulating the RB pathway comprising the step of specifically modulating MRB activity by administering a MRB-interacting or -
25 modulating agent.

As used herein, an "MRB-modulating agent" is any agent that modulates MRB function, for example, an agent that interacts with MRB to inhibit or enhance MRB activity or otherwise affect normal MRB function. MRB function can be affected at any level, including transcription, protein expression, protein localization, and cellular or
30 extra-cellular activity. In a preferred embodiment, the MRB - modulating agent specifically modulates the function of the MRB. The phrases "specific modulating agent", "specifically modulates", etc., are used herein to refer to modulating agents that directly bind to the MRB polypeptide or nucleic acid, and preferably inhibit, enhance, or otherwise alter, the function of the MRB. These phrases also encompass modulating agents that alter

the interaction of the MRB with a binding partner, substrate, or cofactor (e.g. by binding to a binding partner of an MRB, or to a protein/binding partner complex, and altering MRB function). In a further preferred embodiment, the MRB- modulating agent is a modulator of the RB pathway (e.g. it restores and/or upregulates RB function) and thus is also a RB-
5 modulating agent.

Preferred MRB-modulating agents include small molecule compounds; MRB-interacting proteins, including antibodies and other biotherapeutics; and nucleic acid modulators such as antisense and RNA inhibitors. The modulating agents may be formulated in pharmaceutical compositions, for example, as compositions that may
10 comprise other active ingredients, as in combination therapy, and/or suitable carriers or excipients. Techniques for formulation and administration of the compounds may be found in "Remington's Pharmaceutical Sciences" Mack Publishing Co., Easton, PA, 19th edition.

15 **Small molecule modulators**

Small molecules are often preferred to modulate function of proteins with enzymatic function, and/or containing protein interaction domains. Chemical agents, referred to in the art as "small molecule" compounds are typically organic, non-peptide molecules, having a molecular weight less than 10,000, preferably less than 5,000, more
20 preferably less than 1,000, and most preferably less than 500 daltons. This class of modulators includes chemically synthesized molecules, for instance, compounds from combinatorial chemical libraries. Synthetic compounds may be rationally designed or identified based on known or inferred properties of the MRB protein or may be identified by screening compound libraries. Alternative appropriate modulators of this class are
25 natural products, particularly secondary metabolites from organisms such as plants or fungi, which can also be identified by screening compound libraries for MRB-modulating activity. Methods for generating and obtaining compounds are well known in the art (Schreiber SL, Science (2000) 151: 1964-1969; Radmann J and Gunther J, Science (2000) 151:1947-1948).

30 Small molecule modulators identified from screening assays, as described below, can be used as lead compounds from which candidate clinical compounds may be designed, optimized, and synthesized. Such clinical compounds may have utility in treating pathologies associated with the RB pathway. The activity of candidate small molecule modulating agents may be improved several-fold through iterative secondary

functional validation, as further described below, structure determination, and candidate modulator modification and testing. Additionally, candidate clinical compounds are generated with specific regard to clinical and pharmacological properties. For example, the reagents may be derivatized and re-screened using *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development.

Protein Modulators

Specific MRB-interacting proteins are useful in a variety of diagnostic and therapeutic applications related to the RB pathway and related disorders, as well as in validation assays for other MRB-modulating agents. In a preferred embodiment, MRB-interacting proteins affect normal MRB function, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In another embodiment, MRB-interacting proteins are useful in detecting and providing information about the function of MRB proteins, as is relevant to RB related disorders, such as cancer (e.g., for diagnostic means).

An MRB-interacting protein may be endogenous, i.e. one that naturally interacts genetically or biochemically with an MRB, such as a member of the MRB pathway that modulates MRB expression, localization, and/or activity. MRB-modulators include dominant negative forms of MRB-interacting proteins and of MRB proteins themselves. Yeast two-hybrid and variant screens offer preferred methods for identifying endogenous MRB-interacting proteins (Finley, R. L. et al. (1996) in DNA Cloning-Expression Systems: A Practical Approach, eds. Glover D. & Hames B. D (Oxford University Press, Oxford, England), pp. 169-203; Fashema SF et al., Gene (2000) 250:1-14; Drees BL Curr Opin Chem Biol (1999) 3:64-70; Vidal M and Legrain P Nucleic Acids Res (1999) 27:919-29; and U.S. Pat. No. 5,928,868). Mass spectrometry is an alternative preferred method for the elucidation of protein complexes (reviewed in, e.g., Pandley A and Mann M, Nature (2000) 405:837-846; Yates JR 3rd, Trends Genet (2000) 16:5-8).

An MRB-interacting protein may be an exogenous protein, such as an MRB-specific antibody or a T-cell antigen receptor (see, e.g., Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory; Harlow and Lane (1999) Using antibodies: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press). MRB antibodies are further discussed below.

In preferred embodiments, an MRB-interacting protein specifically binds an MRB protein. In alternative preferred embodiments, an MRB-modulating agent binds an MRB substrate, binding partner, or cofactor.

5 ***Antibodies***

In another embodiment, the protein modulator is an MRB specific antibody agonist or antagonist. The antibodies have therapeutic and diagnostic utilities, and can be used in screening assays to identify MRB modulators. The antibodies can also be used in dissecting the portions of the MRB pathway responsible for various cellular responses and
10 in the general processing and maturation of the MRB.

Antibodies that specifically bind MRB polypeptides can be generated using known methods. Preferably the antibody is specific to a mammalian ortholog of MRB polypeptide, and more preferably, to human MRB. Antibodies may be polyclonal, monoclonal (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab
15 fragments, F(ab')₂ fragments, fragments produced by a FAb expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Epitopes of MRB which are particularly antigenic can be selected, for example, by routine screening of MRB polypeptides for antigenicity or by applying a theoretical method for selecting antigenic regions of a protein (Hopp and Wood (1981), Proc. Natl. Acad. Sci.
20 U.S.A. 78:3824-28; Hopp and Wood, (1983) Mol. Immunol. 20:483-89; Sutcliffe et al., (1983) Science 219:660-66) to the amino acid sequence of an MRB. Monoclonal antibodies with affinities of 10^8 M^{-1} preferably 10^9 M^{-1} to 10^{10} M^{-1} , or stronger can be made by standard procedures as described (Harlow and Lane, *supra*; Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York; and
25 U.S. Pat. Nos. 4,381,292; 4,451,570; and 4,618,577). Antibodies may be generated against crude cell extracts of MRB or substantially purified fragments thereof. If MRB fragments are used, they preferably comprise at least 10, and more preferably, at least 20 contiguous amino acids of an MRB protein. In a particular embodiment, MRB-specific antigens and/or immunogens are coupled to carrier proteins that stimulate the immune
30 response. For example, the subject polypeptides are covalently coupled to the keyhole limpet hemocyanin (KLH) carrier, and the conjugate is emulsified in Freund's complete adjuvant, which enhances the immune response. An appropriate immune system such as a laboratory rabbit or mouse is immunized according to conventional protocols.

The presence of MRB-specific antibodies is assayed by an appropriate assay such as a solid phase enzyme-linked immunosorbant assay (ELISA) using immobilized corresponding MRB polypeptides. Other assays, such as radioimmunoassays or fluorescent assays might also be used.

5 Chimeric antibodies specific to MRB polypeptides can be made that contain different portions from different animal species. For instance, a human immunoglobulin constant region may be linked to a variable region of a murine mAb, such that the antibody derives its biological activity from the human antibody, and its binding specificity from the murine fragment. Chimeric antibodies are produced by splicing
10 together genes that encode the appropriate regions from each species (Morrison et al., Proc. Natl. Acad. Sci. (1984) 81:6851-6855; Neuberger et al., Nature (1984) 312:604-608; Takeda et al., Nature (1985) 31:452-454). Humanized antibodies, which are a form of chimeric antibodies, can be generated by grafting complementary-determining regions (CDRs) (Carlos, T. M., J. M. Harlan. 1994. Blood 84:2068-2101) of mouse antibodies
15 into a background of human framework regions and constant regions by recombinant DNA technology (Riechmann LM, et al., 1988 Nature 323: 323-327). Humanized antibodies contain ~10% murine sequences and ~90% human sequences, and thus further reduce or eliminate immunogenicity, while retaining the antibody specificities (Co MS, and Queen C. 1991 Nature 351: 501-501; Morrison SL. 1992 Ann. Rev. Immun.
20 10:239-265). Humanized antibodies and methods of their production are well-known in the art (U.S. Pat. Nos. 5,530,101, 5,585,089, 5,693,762, and 6,180,370).

MRB-specific single chain antibodies which are recombinant, single chain polypeptides formed by linking the heavy and light chain fragments of the Fv regions via an amino acid bridge, can be produced by methods known in the art (U.S. Pat. No.
25 4,946,778; Bird, Science (1988) 242:423-426; Huston et al., Proc. Natl. Acad. Sci. USA (1988) 85:5879-5883; and Ward et al., Nature (1989) 334:544-546).

Other suitable techniques for antibody production involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors (Huse et al., Science (1989) 246:1275-1281). As
30 used herein, T-cell antigen receptors are included within the scope of antibody modulators (Harlow and Lane, 1988, *supra*).

The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, antibodies will be labeled by joining, either covalently or non-covalently, a substance that provides for a detectable signal, or that is toxic to cells

that express the targeted protein (Menard S, et al., Int J. Biol Markers (1989) 4:131-134).

A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, fluorescent emitting lanthanide metals, chemiluminescent moieties, bioluminescent moieties, magnetic particles, and the like (U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241). Also, recombinant immunoglobulins may be produced (U.S. Pat. No. 4,816,567). Antibodies to cytoplasmic polypeptides may be delivered and reach their targets by conjugation with membrane-penetrating toxin proteins (U.S. Pat. No. 6,086,900).

When used therapeutically in a patient, the antibodies of the subject invention are typically administered parenterally, when possible at the target site, or intravenously. The therapeutically effective dose and dosage regimen is determined by clinical studies. Typically, the amount of antibody administered is in the range of about 0.1 mg/kg –to about 10 mg/kg of patient weight. For parenteral administration, the antibodies are formulated in a unit dosage injectable form (e.g., solution, suspension, emulsion) in association with a pharmaceutically acceptable vehicle. Such vehicles are inherently nontoxic and non-therapeutic. Examples are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils, ethyl oleate, or liposome carriers may also be used. The vehicle may contain minor amounts of additives, such as buffers and preservatives, which enhance isotonicity and chemical stability or otherwise enhance therapeutic potential. The antibodies' concentrations in such vehicles are typically in the range of about 1 mg/ml to about 10 mg/ml. Immunotherapeutic methods are further described in the literature (US Pat. No. 5,859,206; WO0073469).

Nucleic Acid Modulators

Other preferred MRB-modulating agents comprise nucleic acid molecules, such as antisense oligomers or double stranded RNA (dsRNA), which generally inhibit MRB activity. Preferred nucleic acid modulators interfere with the function of the MRB nucleic acid such as DNA replication, transcription, translocation of the MRB RNA to the site of protein translation, translation of protein from the MRB RNA, splicing of the MRB RNA to yield one or more mRNA species, or catalytic activity which may be engaged in or facilitated by the MRB RNA.

In one embodiment, the antisense oligomer is an oligonucleotide that is sufficiently complementary to an MRB mRNA to bind to and prevent translation, preferably by binding to the 5' untranslated region. MRB-specific antisense oligonucleotides, preferably range from at least 6 to about 200 nucleotides. In some embodiments the oligonucleotide is preferably at least 10, 15, or 20 nucleotides in length. In other embodiments, the oligonucleotide is preferably less than 50, 40, or 30 nucleotides in length. The oligonucleotide can be DNA or RNA or a chimeric mixture or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, agents that facilitate transport across the cell membrane, hybridization-triggered cleavage agents, and intercalating agents.

In another embodiment, the antisense oligomer is a phosphothioate morpholino oligomer (PMO). PMOs are assembled from four different morpholino subunits, each of which contain one of four genetic bases (A, C, G, or T) linked to a six-membered morpholine ring. Polymers of these subunits are joined by non-ionic phosphodiamidate intersubunit linkages. Details of how to make and use PMOs and other antisense oligomers are well known in the art (e.g. see WO99/18193; Probst JC, Antisense Oligodeoxynucleotide and Ribozyme Design, Methods. (2000) 22(3):271-281; Summerton J, and Weller D. 1997 Antisense Nucleic Acid Drug Dev. :7:187-95; US Pat. No. 5,235,033; and US Pat No. 5,378,841).

Alternative preferred MRB nucleic acid modulators are double-stranded RNA species mediating RNA interference (RNAi). RNAi is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Methods relating to the use of RNAi to silence genes in *C. elegans*, *Drosophila*, plants, and humans are known in the art (Fire A, et al., 1998 Nature 391:806-811; Fire, A. Trends Genet. 15, 358-363 (1999); Sharp, P. A. RNA interference 2001. Genes Dev. 15, 485-490 (2001); Hammond, S. M., et al., Nature Rev. Genet. 2, 110-1119 (2001); Tuschl, T. Chem. Biochem. 2, 239-245 (2001); Hamilton, A. et al., Science 286, 950-952 (1999); Hammond, S. M., et al., Nature 404, 293-296 (2000); Zamore, P. D., et al., Cell 101, 25-33 (2000); Bernstein, E., et al., Nature 409, 363-366 (2001); Elbashir, S. M., et al., Genes Dev. 15, 188-200 (2001); WO0129058; WO9932619; Elbashir SM, et al., 2001 Nature 411:494-498).

Nucleic acid modulators are commonly used as research reagents, diagnostics, and therapeutics. For example, antisense oligonucleotides, which are able to inhibit gene

expression with exquisite specificity, are often used to elucidate the function of particular genes (see, for example, U.S. Pat. No. 6,165,790). Nucleic acid modulators are also used, for example, to distinguish between functions of various members of a biological pathway. For example, antisense oligomers have been employed as therapeutic moieties in the treatment of disease states in animals and man and have been demonstrated in numerous clinical trials to be safe and effective (Milligan JF, *et al*, Current Concepts in Antisense Drug Design, J Med Chem. (1993) 36:1923-1937; Tonkinson JL *et al.*, Antisense Oligodeoxynucleotides as Clinical Therapeutic Agents, Cancer Invest. (1996) 14:54-65). Accordingly, in one aspect of the invention, an MRB-specific nucleic acid modulator is used in an assay to further elucidate the role of the MRB in the RB pathway, and/or its relationship to other members of the pathway. In another aspect of the invention, an MRB-specific antisense oligomer is used as a therapeutic agent for treatment of RB-related disease states.

15 Assay Systems

The invention provides assay systems and screening methods for identifying specific modulators of MRB activity. As used herein, an "assay system" encompasses all the components required for performing and analyzing results of an assay that detects and/or measures a particular event. In general, primary assays are used to identify or confirm a modulator's specific biochemical or molecular effect with respect to the MRB nucleic acid or protein. In general, secondary assays further assess the activity of a MRB modulating agent identified by a primary assay and may confirm that the modulating agent affects MRB in a manner relevant to the RB pathway. In some cases, MRB modulators will be directly tested in a secondary assay.

25 In a preferred embodiment, the screening method comprises contacting a suitable assay system comprising an MRB polypeptide or nucleic acid with a candidate agent under conditions whereby, but for the presence of the agent, the system provides a reference activity (e.g. binding activity), which is based on the particular molecular event the screening method detects. A statistically significant difference between the agent-biased activity and the reference activity indicates that the candidate agent modulates MRB activity, and hence the RB pathway. The MRB polypeptide or nucleic acid used in the assay may comprise any of the nucleic acids or polypeptides described above.

Primary Assays

The type of modulator tested generally determines the type of primary assay.

Primary assays for small molecule modulators

5 For small molecule modulators, screening assays are used to identify candidate modulators. Screening assays may be cell-based or may use a cell-free system that recreates or retains the relevant biochemical reaction of the target protein (reviewed in Sittampalam GS *et al.*, Curr Opin Chem Biol (1997) 1:384-91 and accompanying references). As used herein the term "cell-based" refers to assays using live cells, dead
10 cells, or a particular cellular fraction, such as a membrane, endoplasmic reticulum, or mitochondrial fraction. The term "cell free" encompasses assays using substantially purified protein (either endogenous or recombinantly produced), partially purified or crude cellular extracts. Screening assays may detect a variety of molecular events, including protein-DNA interactions, protein-protein interactions (*e.g.*, receptor-ligand binding),
15 transcriptional activity (*e.g.*, using a reporter gene), enzymatic activity (*e.g.*, via a property of the substrate), activity of second messengers, immunogenicity and changes in cellular morphology or other cellular characteristics. Appropriate screening assays may use a wide range of detection methods including fluorescent, radioactive, colorimetric, spectrophotometric, and amperometric methods, to provide a read-out for the particular
20 molecular event detected.

Cell-based screening assays usually require systems for recombinant expression of MRB and any auxiliary proteins demanded by the particular assay. Appropriate methods for generating recombinant proteins produce sufficient quantities of proteins that retain their relevant biological activities and are of sufficient purity to optimize activity and
25 assure assay reproducibility. Yeast two-hybrid and variant screens, and mass spectrometry provide preferred methods for determining protein-protein interactions and elucidation of protein complexes. In certain applications, when MRB-interacting proteins are used in screens to identify small molecule modulators, the binding specificity of the interacting protein to the MRB protein may be assayed by various known methods such as substrate
30 processing (*e.g.* ability of the candidate MRB-specific binding agents to function as negative effectors in MRB-expressing cells), binding equilibrium constants (usually at least about 10^7 M^{-1} , preferably at least about 10^8 M^{-1} , more preferably at least about 10^9 M^{-1}), and immunogenicity (*e.g.* ability to elicit MRB specific antibody in a heterologous host

such as a mouse, rat, goat or rabbit). For enzymes and receptors, binding may be assayed by, respectively, substrate and ligand processing.

The screening assay may measure a candidate agent's ability to specifically bind to or modulate activity of a MRB polypeptide, a fusion protein thereof, or to cells or membranes bearing the polypeptide or fusion protein. The MRB polypeptide can be full length or a fragment thereof that retains functional MRB activity. The MRB polypeptide may be fused to another polypeptide, such as a peptide tag for detection or anchoring, or to another tag. The MRB polypeptide is preferably human MRB, or is an ortholog or derivative thereof as described above. In a preferred embodiment, the screening assay detects candidate agent-based modulation of MRB interaction with a binding target, such as an endogenous or exogenous protein or other substrate that has MRB-specific binding activity, and can be used to assess normal MRB gene function.

Suitable assay formats that may be adapted to screen for MRB modulators are known in the art. Preferred screening assays are high throughput or ultra high throughput and thus provide automated, cost-effective means of screening compound libraries for lead compounds (Fernandes PB, *Curr Opin Chem Biol* (1998) 2:597-603; Sundberg SA, *Curr Opin Biotechnol* 2000, 11:47-53). In one preferred embodiment, screening assays uses fluorescence technologies, including fluorescence polarization, time-resolved fluorescence, and fluorescence resonance energy transfer. These systems offer means to monitor protein-protein or DNA-protein interactions in which the intensity of the signal emitted from dye-labeled molecules depends upon their interactions with partner molecules (*e.g.*, Selvin PR, *Nat Struct Biol* (2000) 7:730-4; Fernandes PB, *supra*; Hertzberg RP and Pope AJ, *Curr Opin Chem Biol* (2000) 4:445-451).

A variety of suitable assay systems may be used to identify candidate MRB and RB pathway modulators (*e.g.* U.S. Pat. Nos. 5,550,019 and 6,133,437 (apoptosis assays); and U.S. Pat. Nos. 5,976,782, 6,225,118 and 6,444,434 (angiogenesis assays), among others). Specific preferred assays are described in more detail below.

Apoptosis assays. Assays for apoptosis may be performed by terminal deoxynucleotidyl transferase-mediated digoxigenin-11-dUTP nick end labeling (TUNEL) assay. The TUNEL assay is used to measure nuclear DNA fragmentation characteristic of apoptosis (Lazebnik *et al.*, 1994, *Nature* 371, 346), by following the incorporation of fluorescein-dUTP (Yonehara *et al.*, 1989, *J. Exp. Med.* 169, 1747). Apoptosis may further be assayed by acridine orange staining of tissue culture cells (Lucas, R., *et al.*, 1998, *Blood*

15:4730-41). Other cell-based apoptosis assays include the caspase-3/7 assay and the cell death nucleosome ELISA assay. The caspase 3/7 assay is based on the activation of the caspase cleavage activity as part of a cascade of events that occur during programmed cell death in many apoptotic pathways. In the caspase 3/7 assay (commercially available Apo-
5 ONE™ Homogeneous Caspase-3/7 assay from Promega, cat# 67790), lysis buffer and caspase substrate are mixed and added to cells. The caspase substrate becomes fluorescent when cleaved by active caspase 3/7. The nucleosome ELISA assay is a general cell death assay known to those skilled in the art, and available commercially (Roche, Cat# 1774425). This assay is a quantitative sandwich-enzyme-immunoassay which uses
10 monoclonal antibodies directed against DNA and histones respectively, thus specifically determining amount of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Mono and oligonucleosomes are enriched in the cytoplasm during apoptosis due to the fact that DNA fragmentation occurs several hours before the plasma membrane breaks down, allowing for accumulation in the cytoplasm. Nucleosomes are not present in
15 the cytoplasmic fraction of cells that are not undergoing apoptosis. An apoptosis assay system may comprise a cell that expresses an MRB, and that optionally has defective RB function (e.g. RB is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the apoptosis assay system and changes in induction of apoptosis relative to controls where no test agent is added, identify candidate RB modulating agents.
20 In some embodiments of the invention, an apoptosis assay may be used as a secondary assay to test a candidate RB modulating agents that is initially identified using a cell-free assay system. An apoptosis assay may also be used to test whether MRB function plays a direct role in apoptosis. For example, an apoptosis assay may be performed on cells that over- or under-express MRB relative to wild type cells. Differences in apoptotic response
25 compared to wild type cells suggests that the MRB plays a direct role in the apoptotic response. Apoptosis assays are described further in US Pat. No. 6,133,437.

Cell proliferation and cell cycle assays. Cell proliferation may be assayed via bromodeoxyuridine (BRDU) incorporation. This assay identifies a cell population
30 undergoing DNA synthesis by incorporation of BRDU into newly-synthesized DNA. Newly-synthesized DNA may then be detected using an anti-BRDU antibody (Hoshino *et al.*, 1986, *Int. J. Cancer* 38, 369; Campana *et al.*, 1988, *J. Immunol. Meth.* 107, 79), or by other means.

Cell proliferation is also assayed via phospho-histone H3 staining, which identifies a cell population undergoing mitosis by phosphorylation of histone H3. Phosphorylation of histone H3 at serine 10 is detected using an antibody specific to the phosphorylated form of the serine 10 residue of histone H3. (Chadlee, D.N. 1995, J. Biol. Chem 270:20098-

105). Cell Proliferation may also be examined using [³H]-thymidine incorporation (Chen, J., 1996, Oncogene 13:1395-403; Jeoung, J., 1995, J. Biol. Chem. 270:18367-73). This assay allows for quantitative characterization of S-phase DNA syntheses. In this assay, cells synthesizing DNA will incorporate [³H]-thymidine into newly synthesized DNA. Incorporation can then be measured by standard techniques such as by counting of radioisotope in a scintillation counter (e.g., Beckman LS 3800 Liquid Scintillation Counter). Another proliferation assay uses the dye Alamar Blue (available from Biosource International), which fluoresces when reduced in living cells and provides an indirect measurement of cell number (Voytik-Harbin SL et al., 1998, In Vitro Cell Dev Biol Anim 34:239-46). Yet another proliferation assay, the MTS assay, is based on in vitro cytotoxicity assessment of industrial chemicals, and uses the soluble tetrazolium salt, MTS. MTS assays are commercially available, for example, the Promega CellTiter 96[®] Aqueous Non-Radioactive Cell Proliferation Assay (Cat.# G5421).

Cell proliferation may also be assayed by colony formation in soft agar (Sambrook et al., Molecular Cloning, Cold Spring Harbor (1989)). For example, cells transformed with MRB are seeded in soft agar plates, and colonies are measured and counted after two weeks incubation.

Cell proliferation may also be assayed by measuring ATP levels as indicator of metabolically active cells. Such assays are commercially available, for example Cell Titer-Glo[™], which is a luminescent homogeneous assay available from Promega.

Involvement of a gene in the cell cycle may be assayed by flow cytometry (Gray JW et al. (1986) Int J Radiat Biol Relat Stud Phys Chem Med 49:237-55). Cells transfected with an MRB may be stained with propidium iodide and evaluated in a flow cytometer (available from Becton Dickinson), which indicates accumulation of cells in different stages of the cell cycle.

Accordingly, a cell proliferation or cell cycle assay system may comprise a cell that expresses an MRB, and that optionally has defective RB function (e.g. RB is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the assay system and changes in cell proliferation or cell cycle relative to controls where no test agent is added, identify candidate RB modulating agents. In some embodiments of the

invention, the cell proliferation or cell cycle assay may be used as a secondary assay to test a candidate RB modulating agents that is initially identified using another assay system such as a cell-free assay system. A cell proliferation assay may also be used to test whether MRB function plays a direct role in cell proliferation or cell cycle. For example, a cell proliferation or cell cycle assay may be performed on cells that over- or under-express MRB relative to wild type cells. Differences in proliferation or cell cycle compared to wild type cells suggests that the MRB plays a direct role in cell proliferation or cell cycle.

Angiogenesis. Angiogenesis may be assayed using various human endothelial cell systems, such as umbilical vein, coronary artery, or dermal cells. Suitable assays include Alamar Blue based assays (available from Biosource International) to measure proliferation; migration assays using fluorescent molecules, such as the use of Becton Dickinson Falcon HTS FluoroBlock cell culture inserts to measure migration of cells through membranes in presence or absence of angiogenesis enhancer or suppressors; and tubule formation assays based on the formation of tubular structures by endothelial cells on Matrigel® (Becton Dickinson). Accordingly, an angiogenesis assay system may comprise a cell that expresses an MRB, and that optionally has defective RB function (e.g. RB is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the angiogenesis assay system and changes in angiogenesis relative to controls where no test agent is added, identify candidate RB modulating agents. In some embodiments of the invention, the angiogenesis assay may be used as a secondary assay to test a candidate RB modulating agents that is initially identified using another assay system. An angiogenesis assay may also be used to test whether MRB function plays a direct role in cell proliferation. For example, an angiogenesis assay may be performed on cells that over- or under-express MRB relative to wild type cells. Differences in angiogenesis compared to wild type cells suggests that the MRB plays a direct role in angiogenesis. U.S. Pat. Nos. 5,976,782, 6,225,118 and 6,444,434, among others, describe various angiogenesis assays.

Hypoxic induction. The alpha subunit of the transcription factor, hypoxia inducible factor-1 (HIF-1), is upregulated in tumor cells following exposure to hypoxia in vitro. Under hypoxic conditions, HIF-1 stimulates the expression of genes known to be important in tumour cell survival, such as those encoding glycolytic enzymes and VEGF.

Induction of such genes by hypoxic conditions may be assayed by growing cells transfected with MRB in hypoxic conditions (such as with 0.1% O₂, 5% CO₂, and balance N₂, generated in a Napco 7001 incubator (Precision Scientific)) and normoxic conditions, followed by assessment of gene activity or expression by Taqman®. For example, a hypoxic induction assay system may comprise a cell that expresses an MRB, and that optionally has defective RB function (e.g. RB is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the hypoxic induction assay system and changes in hypoxic response relative to controls where no test agent is added, identify candidate RB modulating agents. In some embodiments of the invention, the hypoxic induction assay may be used as a secondary assay to test a candidate RB modulating agents that is initially identified using another assay system. A hypoxic induction assay may also be used to test whether MRB function plays a direct role in the hypoxic response. For example, a hypoxic induction assay may be performed on cells that over- or under-express MRB relative to wild type cells. Differences in hypoxic response compared to wild type cells suggests that the MRB plays a direct role in hypoxic induction.

Cell adhesion. Cell adhesion assays measure adhesion of cells to purified adhesion proteins, or adhesion of cells to each other, in presence or absence of candidate modulating agents. Cell-protein adhesion assays measure the ability of agents to modulate the adhesion of cells to purified proteins. For example, recombinant proteins are produced, diluted to 2.5g/mL in PBS, and used to coat the wells of a microtiter plate. The wells used for negative control are not coated. Coated wells are then washed, blocked with 1% BSA, and washed again. Compounds are diluted to 2× final test concentration and added to the blocked, coated wells. Cells are then added to the wells, and the unbound cells are washed off. Retained cells are labeled directly on the plate by adding a membrane-permeable fluorescent dye, such as calcein-AM, and the signal is quantified in a fluorescent microplate reader.

Cell-cell adhesion assays measure the ability of agents to modulate binding of cell adhesion proteins with their native ligands. These assays use cells that naturally or recombinantly express the adhesion protein of choice. In an exemplary assay, cells expressing the cell adhesion protein are plated in wells of a multiwell plate. Cells expressing the ligand are labeled with a membrane-permeable fluorescent dye, such as BCECF, and allowed to adhere to the monolayers in the presence of candidate agents.

Unbound cells are washed off, and bound cells are detected using a fluorescence plate reader.

High-throughput cell adhesion assays have also been described. In one such assay, small molecule ligands and peptides are bound to the surface of microscope slides using a microarray spotter, intact cells are then contacted with the slides, and unbound cells are washed off. In this assay, not only the binding specificity of the peptides and modulators against cell lines are determined, but also the functional cell signaling of attached cells using immunofluorescence techniques in situ on the microchip is measured (Falsey JR et al., *Bioconjug Chem.* 2001 May-Jun;12(3):346-53).

Tubulogenesis. Tubulogenesis assays monitor the ability of cultured cells, generally endothelial cells, to form tubular structures on a matrix substrate, which generally simulates the environment of the extracellular matrix. Exemplary substrates include MatrigelTM (Becton Dickinson), an extract of basement membrane proteins containing laminin, collagen IV, and heparin sulfate proteoglycan, which is liquid at 4° C and forms a solid gel at 37° C. Other suitable matrices comprise extracellular components such as collagen, fibronectin, and/or fibrin. Cells are stimulated with a pro-angiogenic stimulant, and their ability to form tubules is detected by imaging. Tubules can generally be detected after an overnight incubation with stimuli, but longer or shorter time frames may also be used. Tube formation assays are well known in the art (e.g., Jones MK et al., 1999, *Nature Medicine* 5:1418-1423). These assays have traditionally involved stimulation with serum or with the growth factors FGF or VEGF. Serum represents an undefined source of growth factors. In a preferred embodiment, the assay is performed with cells cultured in serum free medium, in order to control which process or pathway a candidate agent modulates. Moreover, we have found that different target genes respond differently to stimulation with different pro-angiogenic agents, including inflammatory angiogenic factors such as TNF-alpha. Thus, in a further preferred embodiment, a tubulogenesis assay system comprises testing an MRB's response to a variety of factors, such as FGF, VEGF, phorbol myristate acetate (PMA), TNF-alpha, ephrin, etc.

Cell Migration. An invasion/migration assay (also called a migration assay) tests the ability of cells to overcome a physical barrier and to migrate towards pro-angiogenic signals. Migration assays are known in the art (e.g., Paik JH et al., 2001, *J Biol Chem* 276:11830-11837). In a typical experimental set-up, cultured endothelial cells are seeded

onto a matrix-coated porous lamina, with pore sizes generally smaller than typical cell size. The matrix generally simulates the environment of the extracellular matrix, as described above. The lamina is typically a membrane, such as the transwell polycarbonate membrane (Corning Costar Corporation, Cambridge, MA), and is generally part of an upper chamber that is in fluid contact with a lower chamber containing pro-angiogenic stimuli. Migration is generally assayed after an overnight incubation with stimuli, but longer or shorter time frames may also be used. Migration is assessed as the number of cells that crossed the lamina, and may be detected by staining cells with hemotoxylin solution (VWR Scientific, South San Francisco, CA), or by any other method for determining cell number. In another exemplary set up, cells are fluorescently labeled and migration is detected using fluorescent readings, for instance using the Falcon HTS FluoroBlok (Becton Dickinson). While some migration is observed in the absence of stimulus, migration is greatly increased in response to pro-angiogenic factors. As described above, a preferred assay system for migration/invasion assays comprises testing an MRB's response to a variety of pro-angiogenic factors, including tumor angiogenic and inflammatory angiogenic agents, and culturing the cells in serum free medium.

Sprouting assay. A sprouting assay is a three-dimensional *in vitro* angiogenesis assay that uses a cell-number defined spheroid aggregation of endothelial cells ("spheroid"), embedded in a collagen gel-based matrix. The spheroid can serve as a starting point for the sprouting of capillary-like structures by invasion into the extracellular matrix (termed "cell sprouting") and the subsequent formation of complex anastomosing networks (Korff and Augustin, 1999, J Cell Sci 112:3249-58). In an exemplary experimental set-up, spheroids are prepared by pipetting 400 human umbilical vein endothelial cells into individual wells of a nonadhesive 96-well plates to allow overnight spheroidal aggregation (Korff and Augustin: J Cell Biol 143: 1341-52, 1998). Spheroids are harvested and seeded in 900 μ l of methocel-collagen solution and pipetted into individual wells of a 24 well plate to allow collagen gel polymerization. Test agents are added after 30 min by pipetting 100 μ l of 10-fold concentrated working dilution of the test substances on top of the gel. Plates are incubated at 37°C for 24h. Dishes are fixed at the end of the experimental incubation period by addition of paraformaldehyde. Sprouting intensity of endothelial cells can be quantitated by an automated image analysis system to determine the cumulative sprout length per spheroid.

Primary assays for antibody modulators

For antibody modulators, appropriate primary assays test is a binding assay that tests the antibody's affinity to and specificity for the MRB protein. Methods for testing antibody affinity and specificity are well known in the art (Harlow and Lane, 1988, 1999, *supra*). The enzyme-linked immunosorbant assay (ELISA) is a preferred method for
5 detecting MRB-specific antibodies; others include FACS assays, radioimmunoassays, and fluorescent assays.

In some cases, screening assays described for small molecule modulators may also be used to test antibody modulators.

Primary assays for nucleic acid modulators

For nucleic acid modulators, primary assays may test the ability of the nucleic acid modulator to inhibit or enhance MRB gene expression, preferably mRNA expression. In general, expression analysis comprises comparing MRB expression in like populations of
15 cells (*e.g.*, two pools of cells that endogenously or recombinantly express MRB) in the presence and absence of the nucleic acid modulator. Methods for analyzing mRNA and protein expression are well known in the art. For instance, Northern blotting, slot blotting, ribonuclease protection, quantitative RT-PCR (*e.g.*, using the TaqMan®, PE Applied Biosystems), or microarray analysis may be used to confirm that MRB mRNA expression
20 is reduced in cells treated with the nucleic acid modulator (*e.g.*, Current Protocols in Molecular Biology (1994) Ausubel FM *et al.*, eds., John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm DH and Guiseppi-Elie, A Curr Opin Biotechnol 2001, 12:41-47). Protein expression may also be monitored. Proteins are most commonly detected with
25 specific antibodies or antisera directed against either the MRB protein or specific peptides. A variety of means including Western blotting, ELISA, or in situ detection, are available (Harlow E and Lane D, 1988 and 1999, *supra*).

In some cases, screening assays described for small molecule modulators, particularly in assay systems that involve MRB mRNA expression, may also be used to
30 test nucleic acid modulators.

Secondary Assays

Secondary assays may be used to further assess the activity of MRB-modulating agent identified by any of the above methods to confirm that the modulating agent affects

MRB in a manner relevant to the RB pathway. As used herein, MRB-modulating agents encompass candidate clinical compounds or other agents derived from previously identified modulating agent. Secondary assays can also be used to test the activity of a modulating agent on a particular genetic or biochemical pathway or to test the specificity of the modulating agent's interaction with MRB.

Secondary assays generally compare like populations of cells or animals (*e.g.*, two pools of cells or animals that endogenously or recombinantly express MRB) in the presence and absence of the candidate modulator. In general, such assays test whether treatment of cells or animals with a candidate MRB-modulating agent results in changes in the RB pathway in comparison to untreated (or mock- or placebo-treated) cells or animals. Certain assays use "sensitized genetic backgrounds", which, as used herein, describe cells or animals engineered for altered expression of genes in the RB or interacting pathways.

Cell-based assays

Cell based assays may use a variety of mammalian cell lines known to have defective RB function (*e.g.* SAOS-2 osteoblasts, BT549 breast cancer cells, and C33A cervical cancer cells, among others, available from American Type Culture Collection (ATCC), Manassas, VA). Cell based assays may detect endogenous RB pathway activity or may rely on recombinant expression of RB pathway components. Any of the aforementioned assays may be used in this cell-based format. Candidate modulators are typically added to the cell media but may also be injected into cells or delivered by any other efficacious means.

Animal Assays

A variety of non-human animal models of normal or defective RB pathway may be used to test candidate MRB modulators. Models for defective RB pathway typically use genetically modified animals that have been engineered to mis-express (*e.g.*, over-express or lack expression in) genes involved in the RB pathway. Assays generally require systemic delivery of the candidate modulators, such as by oral administration, injection, etc.

In a preferred embodiment, RB pathway activity is assessed by monitoring neovascularization and angiogenesis. Animal models with defective and normal RB are used to test the candidate modulator's affect on MRB in Matrigel® assays. Matrigel® is

an extract of basement membrane proteins, and is composed primarily of laminin, collagen IV, and heparin sulfate proteoglycan. It is provided as a sterile liquid at 4° C, but rapidly forms a solid gel at 37° C. Liquid Matrigel® is mixed with various angiogenic agents, such as bFGF and VEGF, or with human tumor cells which over-express the MRB. The mixture is then injected subcutaneously(SC) into female athymic nude mice (Taconic, Germantown, NY) to support an intense vascular response. Mice with Matrigel® pellets may be dosed via oral (PO), intraperitoneal (IP), or intravenous (IV) routes with the candidate modulator. Mice are euthanized 5 - 12 days post-injection, and the Matrigel® pellet is harvested for hemoglobin analysis (Sigma plasma hemoglobin kit). Hemoglobin content of the gel is found to correlate the degree of neovascularization in the gel.

In another preferred embodiment, the effect of the candidate modulator on MRB is assessed via tumorigenicity assays. Tumor xenograft assays are known in the art (see, e.g., Ogawa K et al., 2000, *Oncogene* 19:6043-6052). Xenografts are typically implanted SC into female athymic mice, 6-7 week old, as single cell suspensions either from a pre-existing tumor or from *in vitro* culture. The tumors which express the MRB endogenously are injected in the flank, 1×10^5 to 1×10^7 cells per mouse in a volume of 100 μ L using a 27gauge needle. Mice are then ear tagged and tumors are measured twice weekly. Candidate modulator treatment is initiated on the day the mean tumor weight reaches 100 mg. Candidate modulator is delivered IV, SC, IP, or PO by bolus administration.

Depending upon the pharmacokinetics of each unique candidate modulator, dosing can be performed multiple times per day. The tumor weight is assessed by measuring perpendicular diameters with a caliper and calculated by multiplying the measurements of diameters in two dimensions. At the end of the experiment, the excised tumors may be utilized for biomarker identification or further analyses. For immunohistochemistry staining, xenograft tumors are fixed in 4% paraformaldehyde, 0.1M phosphate, pH 7.2, for 6 hours at 4°C, immersed in 30% sucrose in PBS, and rapidly frozen in isopentane cooled with liquid nitrogen.

In another preferred embodiment, tumorigenicity is monitored using a hollow fiber assay, which is described in U.S. Pat No. US 5,698,413. Briefly, the method comprises implanting into a laboratory animal a biocompatible, semi-permeable encapsulation device containing target cells, treating the laboratory animal with a candidate modulating agent, and evaluating the target cells for reaction to the candidate modulator. Implanted cells are generally human cells from a pre-existing tumor or a tumor cell line. After an appropriate period of time, generally around six days, the implanted samples are harvested for

evaluation of the candidate modulator. Tumorigenicity and modulator efficacy may be evaluated by assaying the quantity of viable cells present in the macrocapsule, which can be determined by tests known in the art, for example, MTT dye conversion assay, neutral red dye uptake, trypan blue staining, viable cell counts, the number of colonies formed in soft agar, the capacity of the cells to recover and replicate in vitro, etc.

In another preferred embodiment, a tumorigenicity assay use a transgenic animal, usually a mouse, carrying a dominant oncogene or tumor suppressor gene knockout under the control of tissue specific regulatory sequences; these assays are generally referred to as transgenic tumor assays. In a preferred application, tumor development in the transgenic model is well characterized or is controlled. In an exemplary model, the "RIP1-Tag2" transgene, comprising the SV40 large T-antigen oncogene under control of the insulin gene regulatory regions is expressed in pancreatic beta cells and results in islet cell carcinomas (Hanahan D, 1985, Nature 315:115-122; Parangi S et al, 1996, Proc Natl Acad Sci USA 93: 2002-2007; Bergers G et al, 1999, Science 284:808-812). An "angiogenic switch," occurs at approximately five weeks, as normally quiescent capillaries in a subset of hyperproliferative islets become angiogenic. The RIP1-TAG2 mice die by age 14 weeks. Candidate modulators may be administered at a variety of stages, including just prior to the angiogenic switch (e.g., for a model of tumor prevention), during the growth of small tumors (e.g., for a model of intervention), or during the growth of large and/or invasive tumors (e.g., for a model of regression). Tumorigenicity and modulator efficacy can be evaluating life-span extension and/or tumor characteristics, including number of tumors, tumor size, tumor morphology, vessel density, apoptotic index, etc.

Diagnostic and therapeutic uses

Specific MRB-modulating agents are useful in a variety of diagnostic and therapeutic applications where disease or disease prognosis is related to defects in the RB pathway, such as angiogenic, apoptotic, or cell proliferation disorders. Accordingly, the invention also provides methods for modulating the RB pathway in a cell, preferably a cell pre-determined to have defective or impaired RB function (e.g. due to overexpression, underexpression, or misexpression of RB, or due to gene mutations), comprising the step of administering an agent to the cell that specifically modulates MRB activity. Preferably, the modulating agent produces a detectable phenotypic change in the cell indicating that the RB function is restored. The phrase "function is restored", and equivalents, as used herein, means that the desired phenotype is achieved, or is brought closer to normal

compared to untreated cells. For example, with restored RB function, cell proliferation and/or progression through cell cycle may normalize, or be brought closer to normal relative to untreated cells. The invention also provides methods for treating disorders or disease associated with impaired RB function by administering a therapeutically effective amount of an MRB -modulating agent that modulates the RB pathway. The invention further provides methods for modulating MRB function in a cell, preferably a cell pre-determined to have defective or impaired MRB function, by administering an MRB -modulating agent. Additionally, the invention provides a method for treating disorders or disease associated with impaired MRB function by administering a therapeutically effective amount of an MRB -modulating agent.

The discovery that MRB is implicated in RB pathway provides for a variety of methods that can be employed for the diagnostic and prognostic evaluation of diseases and disorders involving defects in the RB pathway and for the identification of subjects having a predisposition to such diseases and disorders.

Various expression analysis methods can be used to diagnose whether MRB expression occurs in a particular sample, including Northern blotting, slot blotting, ribonuclease protection, quantitative RT-PCR, and microarray analysis. (*e.g.*, Current Protocols in Molecular Biology (1994) Ausubel FM *et al.*, eds., John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm and Guiseppi-Elie, Curr Opin Biotechnol 2001, 12:41-47). Tissues having a disease or disorder implicating defective RB signaling that express an MRB, are identified as amenable to treatment with an MRB modulating agent. In a preferred application, the RB defective tissue overexpresses an MRB relative to normal tissue. For example, a Northern blot analysis of mRNA from tumor and normal cell lines, or from tumor and matching normal tissue samples from the same patient, using full or partial MRB cDNA sequences as probes, can determine whether particular tumors express or overexpress MRB. Alternatively, the TaqMan® is used for quantitative RT-PCR analysis of MRB expression in cell lines, normal tissues and tumor samples (PE Applied Biosystems).

Various other diagnostic methods may be performed, for example, utilizing reagents such as the MRB oligonucleotides, and antibodies directed against an MRB, as described above for: (1) the detection of the presence of MRB gene mutations, or the detection of either over- or under-expression of MRB mRNA relative to the non-disorder state; (2) the detection of either an over- or an under-abundance of MRB gene product

relative to the non-disorder state; and (3) the detection of perturbations or abnormalities in the signal transduction pathway mediated by MRB.

Thus, in a specific embodiment, the invention is drawn to a method for diagnosing a disease or disorder in a patient that is associated with alterations in MRB expression, the method comprising: a) obtaining a biological sample from the patient; b) contacting the sample with a probe for MRB expression; c) comparing results from step (b) with a control; and d) determining whether step (c) indicates a likelihood of the disease or disorder. Preferably, the disease is cancer. The probe may be either DNA or protein, including an antibody.

EXAMPLES

The following experimental section and examples are offered by way of illustration and not by way of limitation.

I. Drosophila RB screen

RNA interference (RNAi) was used to create Rbf-deficient cultured *Drosophila* cells (Schneider S2 cells (Schneider, I. (1972) J. Embryol. Exp. Morph. 27, 363), adapted to serum-free media, from Invitrogen Corp., Carlsbad, CA). Cells were treated for 3 days with Rbf double stranded RNA (dsRNA) or a control dsRNA representing sequences from an EGFP luciferase cDNA. Following pretreatment with Rbf or control dsRNA, cells were plated in 96-well format and dsRNA representing approximately 6000 different *Drosophila* genes were added to individual wells. A cell proliferation assay (ProCheck™ assay – Serological Corporation, Norcross, GA) was used to quantify cell viability after a 96-hour incubation. For each of the greater than 6000 dsRNA sequences tested in this manner, cell viability data was obtained on Rbf-deficient cells (Rbf dsRNA-treated) and control cells (EGFP luciferase dsRNA-treated). Comparison of this data for each dsRNA identified dsRNA sequences that preferentially reduced the viability of Rbf-deficient cells.

II. Analysis of Table 1

BLAST analysis (Altschul et al., *supra*) was employed to identify orthologs of *Drosophila* modifiers. The columns “MRB symbol”, and “MRB name aliases” provide a symbol and the known name abbreviations for the Targets, where available, from Genbank. “MRB RefSeq_NA or GI_NA”, “MRB GI_AA”, “MRB NAME”, and “MRB Description” provide the reference DNA sequences for the MRBs as available from

National Center for Biology Information (NCBI), MRB protein Genbank identifier number (GI#), MRB name, and MRB description, all available from Genbank, respectively. The length of each amino acid is in the "MRB Protein Length" column.

Names and Protein sequences of *Drosophila* modifiers of RB from screen

- 5 (Example I), are represented in the "Modifier Name" and "Modifier GI_AA" column by GI#, respectively.

Table 1

MRB symbol	MRB name aliases	MRB RefSeq_NA or GI_AA	NA SEQ ID NO	MRB GI_AA	AA SEQ ID NO	MRB NAME	MRB DESCRIPTION	MRB PROTE IN LENGT H	MOD IFIER NAM E	MODIFIE R GI_AA
LOC2 05865	similar to 40S ribosomal protein S6 (Phosphoprotein NP33)	XM_116191 .1	1	20533870	17	similar to 40S ribosomal protein S6 (Phosphoprotein NP33)	na	177	RpS6	17737290
LOC1 37397	similar to Rim2 protein	XM_070395 .1	2	17448285	18	similar to Rim2 protein	na	299	RpS6	17737290
LOC1 20318	similar to 40S ribosomal protein S6 (Phosphoprotein NP33)	XM_061988 .1	3	17458725	19	similar to 40S ribosomal protein S6 (Phosphoprotein NP33)	na	248	RpS6	17737290
LOC1 60369	similar to ribosomal protein S6	XM_090253 .1	4	18579097	20	similar to ribosomal protein S6	na	240	RpS6	17737290

RPS6	phosphoprotein NP33, 40S ribosomal protein S6, ribosomal protein S6	NM_001010.2	5	15342049	21	ribosomal protein S6	RNA binding; structural constituent of ribosome; structural constituent of ribosome; structural constituent of ribosome	249	RpS6	17737290
LOC121253	similar to 40S ribosomal protein S6 (Phosphoprotein NP33)	XM_062542.1	6	17474556	22	similar to 40S ribosomal protein S6 (Phosphoprotein NP33)	na	249	RpS6	17737290
LOC196391	similar to 40S ribosomal protein S6 (Phosphoprotein NP33)	XM_115132.1	7	20552076	23	similar to 40S ribosomal protein S6 (Phosphoprotein NP33)	na	100	RpS6	17737290
LOC133354	LOC133354 similar to chaperonin containing TCP1, subunit 6A (zeta 1); chaperonin containing T-complex subunit 6 na	XM_068312.2	8	20540233	24	similar to chaperonin containing TCP1, subunit 6A (zeta 1); chaperonin containing T-complex subunit 6	na	531	CG8231	18859933
KIAA0107	KIAA0107 KIAA0107 gene product	NM_014814.1	9	7661914	25	KIAA0107 gene product	Protein containing a PCI (proteasome, COP9-complex and eIF3) or PINT (Proteasome, Int-6, Nip-1 and TRIP-15) domain, which are found in multi-protein	389	Rpn7	21355773

							complexes, has moderate similarity to S. cerevisiae Rpn7p, which functions in transcription elongation			
PSMB4	PSMB4 HN3 HsN3 PROS26 proteasome chain 3 macropain beta chain proteasome beta chain proteasome subunit HsN3 proteasome subunit, beta type, 4 multicatalytic endopeptidase complex beta chain proteasome (prosome, macropain) subunit, beta type, 4	NM_002796.2	10	22538467	26	proteasome (prosome, macropain) subunit, beta type, 4	Proteasome subunit, beta type, 4, a subunit of the catalytic core component of the 26S proteasome, involved in trypsin-like and peptidylglutamyl-peptide cleavage activities of ubiquitinated proteins, binds human immunodeficiency virus type 1 Nef protein	264	CG12000	21430236

PSM B7	PSMB7 Z macropain chain Z proteasom e subunit Z proteasom e subunit alpha proteasom e subunit beta 7 proteasom e catalytic subunit 2 multicatal ytic endopepti dase complex chain Z proteasom e (prosome, macropain) subunit, beta type, 7	NM_002799 .2	11	4506203	27	proteaso me (prosom e, macropa in) subunit, beta type, 7	Proteasome (prosome, macropain) subunit beta 7, a subunit of the 26S proteasome, replaced by PSMB10 upon interferon gamma (IFNG) stimulation	277	Prosb eta2	15292263
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PSM B10	PSMB10 LMP10 MECL1 MGC1665 proteasom e MECL-1 macropain subunit MECL-1 proteasom e subunit MECL1 proteasom e subunit beta 7i proteasom e catalytic subunit 2i multicatal ytic endopepti dase complex subunit MECL-1 proteasom e (prosome, macropain) subunit, beta type, 10	NM_002801 .2	12	4506191	28	proteaso me (prosom e, macropa in) subunit, beta type, 10	Proteasome (prosome, macropain) subunit beta type 10, beta type subunit of the proteasome protease, which is involved in protein degradation and the generation of peptides presented by MHC class I molecules	273	Prosb eta2	15292263
LOC1 31037	LOC1310 37 similar to proteasom e (prosome, macropain) 26S subunit, ATPase na	XM_067168 .5	13	22043073	29	similar to proteaso me (prosom e, macropa in) 26S subunit, ATPase	na	318	Rpt1	17137738

SF3A3	SF3A3 PRP9 SAP61 SF3a60 splicing factor 3a, subunit 3, 60kD pre-mRNA splicing factor SF3a (60kD), similar to S. cerevisiae PRP9 (spliceosome-associated protein 61) splicing factor 3a, subunit 3, 60kDa	NM_006802.1	14	5803167	30	splicing factor 3a, subunit 3, 60kDa	Spliceosome-associated protein 61, a subunit of the heterotrimeric splicing factor SF3a, involved in the formation of the 17S U2 snRNP and assembly of the prespliceosome, contains a C2H2 zinc finger	501	noi	17137118
LOC219819879	LOC219819879 similar to Splicing factor 3A subunit 3 (Spliceosome associated protein 61) (SAP61) (SF3a60) na	XM_010539.5	15	18578493	31	similar to Splicing factor 3A subunit 3 (Spliceosome associated protein 61) (SAP61) (SF3a60)	na	258	noi	17137118

SUPT 6H	SUPT6H SPT6 SPT6H emb-5 KIAA016 2 suppressor of Ty (S.cerevisi ae) 6 homolog suppressor of Ty 6 homolog (S. cerevisiae)	NM_003170 1 XM_0170 37.3	16	11321573	32	suppress or of Ty 6 homolo g (S. cerevisi ae)	Suppressor of Ty 6 homolog, a putative modulator of chromatin structure that is able to bind histone H3, has a highly acidic amino terminus, a degenerate SH2 domain, and a predicted leucine zipper	1603	Spt6	21356229
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III. High-Throughput In Vitro Fluorescence Polarization Assay

Fluorescently-labeled MRB peptide/substrate are added to each well of a 96-well microtiter plate, along with a test agent in a test buffer (10 mM HEPES, 10 mM NaCl, 6 mM magnesium chloride, pH 7.6). Changes in fluorescence polarization, determined by using a Fluorolite FPM-2 Fluorescence Polarization Microtiter System (Dynatech Laboratories, Inc), relative to control values indicates the test compound is a candidate modifier of MRB activity.

IV. High-Throughput In Vitro Binding Assay.

³³P-labeled MRB peptide is added in an assay buffer (100 mM KCl, 20 mM HEPES pH 7.6, 1 mM MgCl₂, 1% glycerol, 0.5% NP-40, 50 mM beta-mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors) along with a test agent to the wells of a Neutralite-avidin coated assay plate and incubated at 25°C for 1 hour. Biotinylated substrate is then added to each well and incubated for 1 hour. Reactions are stopped by washing with PBS, and counted in a scintillation counter. Test agents that cause a difference in activity relative to control without test agent are identified as candidate RB modulating agents.

V. Immunoprecipitations and Immunoblotting

For coprecipitation of transfected proteins, 3×10^6 appropriate recombinant cells containing the MRB proteins are plated on 10-cm dishes and transfected on the following day with expression constructs. The total amount of DNA is kept constant in each transfection by adding empty vector. After 24 h, cells are collected, washed once with phosphate-buffered saline and lysed for 20 min on ice in 1 ml of lysis buffer containing 50

mM Hepes, pH 7.9, 250 mM NaCl, 20 mM -glycerophosphate, 1 mM sodium orthovanadate, 5 mM p-nitrophenyl phosphate, 2 mM dithiothreitol, protease inhibitors (complete, Roche Molecular Biochemicals), and 1% Nonidet P-40. Cellular debris is removed by centrifugation twice at $15,000 \times g$ for 15 min. The cell lysate is incubated
5 with 25 μ l of M2 beads (Sigma) for 2 h at 4 °C with gentle rocking.

After extensive washing with lysis buffer, proteins bound to the beads are solubilized by boiling in SDS sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and blotted with the indicated antibodies. The reactive bands are visualized with horseradish peroxidase
10 coupled to the appropriate secondary antibodies and the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech).

VI. Expression analysis

All cell lines used in the following experiments are NCI (National Cancer Institute)
15 lines, and are available from ATCC (American Type Culture Collection, Manassas, VA 20110-2209). Normal and tumor tissues are obtained from Impath, UC Davis, Clontech, Stratagene, Ardis, Genome Collaborative, and Ambion.

TaqMan analysis is used to assess expression levels of the disclosed genes in various samples.

20 RNA is extracted from each tissue sample using Qiagen (Valencia, CA) RNeasy kits, following manufacturer's protocols, to a final concentration of 50ng/ μ l. Single stranded cDNA is then synthesized by reverse transcribing the RNA samples using random hexamers and 500ng of total RNA per reaction, following protocol 4304965 of Applied Biosystems (Foster City, CA).

25 Primers for expression analysis using TaqMan assay (Applied Biosystems, Foster City, CA) are prepared according to the TaqMan protocols, and the following criteria: a) primer pairs are designed to span introns to eliminate genomic contamination, and b) each primer pair produced only one product. Expression analysis is performed using a 7900HT instrument.

30 Taqman reactions are carried out following manufacturer's protocols, in 25 μ l total volume for 96-well plates and 10 μ l total volume for 384-well plates, using 300nM primer and 250 nM probe, and approximately 25ng of cDNA. The standard curve for result analysis is prepared using a universal pool of human cDNA samples, which is a mixture of cDNAs from a wide variety of tissues so that the chance that a target will be present in

appreciable amounts is good. The raw data are normalized using 18S rRNA (universally expressed in all tissues and cells).

For each expression analysis, tumor tissue samples are compared with matched normal tissues from the same patient. A gene is considered overexpressed in a tumor
5 when the level of expression of the gene is 2 fold or higher in the tumor compared with its matched normal sample. In cases where normal tissue is not available, a universal pool of cDNA samples is used instead. In these cases, a gene is considered overexpressed in a tumor sample when the difference of expression levels between a tumor sample and the average of all normal samples from the same tissue type is greater than 2 times the
10 standard deviation of all normal samples (i.e., $\text{Tumor} - \text{average}(\text{all normal samples}) > 2 \times \text{STDEV}(\text{all normal samples})$).

A modulator identified by an assay described herein can be further validated for therapeutic effect by administration to a tumor in which the gene is overexpressed. A decrease in tumor growth confirms therapeutic utility of the modulator. Prior to treating a
15 patient with the modulator, the likelihood that the patient will respond to treatment can be diagnosed by obtaining a tumor sample from the patient, and assaying for expression of the gene targeted by the modulator. The expression data for the gene(s) can also be used as a diagnostic marker for disease progression. The assay can be performed by expression analysis as described above, by antibody directed to the gene target, or by any other
20 available detection method.

WHAT IS CLAIMED IS:

1. A method of identifying a candidate RB pathway modulating agent, said method comprising the steps of:

- 5 (a) providing an assay system comprising a MRB polypeptide or nucleic acid;
 (b) contacting the assay system with a test agent under conditions whereby, but for the presence of the test agent, the system provides a reference activity; and
 (c) detecting a test agent-biased activity of the assay system, wherein a difference between the test agent-biased activity and the reference activity identifies the test agent as
10 a candidate RB pathway modulating agent.

2. The method of Claim 1 wherein the assay system comprises cultured cells that express the MRB polypeptide.

- 15 3. The method of Claim 2 wherein the cultured cells additionally have defective RB function.

4. The method of Claim 1 wherein the assay system includes a screening assay comprising a MRB polypeptide, and the candidate test agent is a small molecule
20 modulator.

5. The method of Claim 4 wherein the assay is a binding assay.

6. The method of Claim 1 wherein the assay system is selected from the group consisting
25 of an apoptosis assay system, a cell proliferation assay system, an angiogenesis assay system, and a hypoxic induction assay system.

7. The method of Claim 1 wherein the assay system includes a binding assay comprising a MRB polypeptide and the candidate test agent is an antibody.

30

8. The method of Claim 1 wherein the assay system includes an expression assay comprising a MRB nucleic acid and the candidate test agent is a nucleic acid modulator.

9. The method of Claim 8 wherein the nucleic acid modulator is an antisense oligomer.

10. The method of Claim 8 wherein the nucleic acid modulator is a PMO.

11. The method of Claim 1 additionally comprising:

5 (d) administering the candidate RB pathway modulating agent identified in (c) to a model system comprising cells defective in RB function and, detecting a phenotypic change in the model system that indicates that the RB function is restored.

12. The method of Claim 11 wherein the model system is a mouse model with defective RB function.

10

13. A method for modulating a RB pathway of a cell comprising contacting a cell defective in RB function with a candidate modulator that specifically binds to a MRB polypeptide, whereby RB function is restored.

15 14. The method of Claim 13 wherein the candidate modulator is administered to a vertebrate animal predetermined to have a disease or disorder resulting from a defect in RB function.

20 15. The method of Claim 13 wherein the candidate modulator is selected from the group consisting of an antibody and a small molecule.

16. The method of Claim 1, comprising the additional steps of:

(e) providing a secondary assay system comprising cultured cells or a non-human animal expressing MRB ,

25 (f) contacting the secondary assay system with the test agent of (b) or an agent derived therefrom under conditions whereby, but for the presence of the test agent or agent derived therefrom, the system provides a reference activity; and

(g) detecting an agent-biased activity of the second assay system,

30 wherein a difference between the agent-biased activity and the reference activity of the second assay system confirms the test agent or agent derived therefrom as a candidate RB pathway modulating agent,

and wherein the second assay detects an agent-biased change in the RB pathway.

17. The method of Claim 16 wherein the secondary assay system comprises cultured cells.
18. The method of Claim 16 wherein the secondary assay system comprises a non-human animal.
19. The method of Claim 18 wherein the non-human animal mis-expresses a RB pathway gene.
20. A method of modulating RB pathway in a mammalian cell comprising contacting the cell with an agent that specifically binds a MRB polypeptide or nucleic acid.
21. The method of Claim 20 wherein the agent is administered to a mammalian animal predetermined to have a pathology associated with the RB pathway.
22. The method of Claim 20 wherein the agent is a small molecule modulator, a nucleic acid modulator, or an antibody.
23. A method for diagnosing a disease in a patient comprising:
- (a) obtaining a biological sample from the patient;
 - (b) contacting the sample with a probe for MRB expression;
 - (c) comparing results from step (b) with a control;
 - (d) determining whether step (c) indicates a likelihood of disease.
24. The method of Claim 23 wherein said disease is cancer.

SEQUENCE LISTING

<110> EXELIXIS, INC.

<120> MRBS AS MODIFIERS OF THE RB PATHWAY AND METHODS OF USE

<130> EX03-052C-PC

<150> US 60/401,737

<151> 2002-08-07

<150> US 60/428,872

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<213> Homo sapiens

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20             25             30

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Ala Thr Glu Ile Ala Ala Tyr Ala Leu Gly Glu Glu Trp Lys Gly Tyr

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 Ile Gln His Leu Val Thr Pro Arg Val Leu Gln His Lys Gln Gln Pro
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 Ile Ala Leu Lys Lys Gln Cys Thr Lys Lys Asn Arg Glu Glu Ala Ala
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 Thr Lys Phe Ala Asp Gly Cys Gly His Asn Cys Ser Tyr Cys Gln Thr
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Lys Phe Cys Ala Arg Cys Gly Gly Arg Val Ser Leu Arg Ser Asn Lys
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 Val Met Trp Val Cys Asn Leu Cys Arg Lys Gln Gln Glu Ile Leu Thr
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 Lys Ser Gly Ala Trp Phe Tyr Asn Ser Gly Ser Asn Thr Pro Gln Gln
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 Pro Asp Gln Lys Val Leu Arg Gly Leu Arg Asn Glu Glu Ala Pro Gln
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 Glu Lys Lys Pro Lys Leu His Glu Gln Thr Gln Phe Gln Gly Pro Ser
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 Cys Thr Phe Tyr Glu Lys His Met Ala Thr Glu Val Ala Ala Asp Ala
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 180 185 190
 Asp Lys Gln Trp Phe Pro Met Lys Arg Gly Val Leu Ile His Gly Arg
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 Val Arg Leu Leu Met Arg Asp Lys Asn Ile Pro Gly Leu Thr Asp Thr
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 Thr Met Pro His His Leu Gly Pro Lys Arg Ala Ser Arg Ile His Lys
 225 230 235 240
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 245 250 255
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<210> 19
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<212> PRT

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Thr Glu Val Ala Ala Asp Ala Leu Gly Lys Glu Trp Lys Gly Tyr Val
 35 40 45

Gly Val Leu Thr His Gly His Val His Leu Leu Leu Asn Lys Arg His
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Ala Cys Tyr Arg Pro Arg Arg Thr Gly Lys Arg Lys Gln Lys Ser Ala
 65 70 75 80

Arg Gly Cys Ile Val Asp Ala Asn Leu Ser Ile Leu Asn Leu Val Ile
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Val Lys Lys Gly Glu Lys Asp Ile Pro Gly Leu Thr Asp Thr Met Val
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Pro Cys His Leu Gly Pro Lys Arg Ala Ser Arg Ile Cys Lys Leu Phe
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Ser Leu Ser Glu Asp Asp Asp Val His Gln Cys Val Val Arg Lys Ala
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Leu Asn Lys Glu Gly Lys Lys Pro Arg Thr Lys Ala Pro Thr Thr Lys
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Tyr Leu Val Thr Pro His Val Leu Gln His Glu Trp Trp His Ile Ala
 165 170 175

Leu Lys Lys Gln Arg Ile Lys Glu Asn Lys Glu Val Ala Ala Glu Tyr
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Ala Lys Leu Leu Ala Lys Arg Met Lys Glu Ala Lys Glu Lys His Gln
 195 200 205

Glu Gln Ile Ala Lys Arg Arg Arg Leu Ser Ser Leu Cys Leu Trp Val
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Lys Asn Asn Leu Cys Leu Glu Gln Arg His Leu Ile Ala Thr Gly Ala

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 Asn Phe Cys Ile Thr Val Trp Thr
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 Ala Thr Glu Val Ala Ala Asp Thr Leu Gly Glu Glu Trp Lys Gly Tyr
 35 40 45

 Val Val Arg Ile Ser Gly Gly Asn Asn Lys Gln Gly Phe Pro Met Lys
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 Gln Gly Val Leu Thr His Gly Arg Val His Leu Leu Leu Ser Lys Gly
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 His Ser Cys Tyr Arg Pro Arg Arg Thr Gly Glu Arg Lys Arg Lys Ser
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 Val Arg Gly Cys Ile Val Asp Ala Asn Leu Ser Ile Leu Asn Leu Ile
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 Ile Val Lys Lys Lys Lys Lys Val Lys Lys Asp Ile Pro Gly Leu Thr
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 Asp Thr Met Val Pro Cys Arg Leu Gly Pro Lys Lys Ala Ser Arg Ile
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 Cys Lys Leu Ser Asn Leu Ser Glu Glu Asp Asp Val Arg Gln Tyr Val
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 Val Arg Lys Pro Ser Asn Lys Gly Gly Lys Lys Pro Arg Thr Lys Ala
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 Pro Lys Ile Gln His Leu Val Thr Pro His Phe Leu Gln His Lys Gly
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Val Val Arg Ile Ser Gly Gly Asn Asp Lys Gln Gly Phe Pro Met Lys
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Ile Val Lys Lys Gly Glu Lys Asp Ile Pro Gly Leu Thr Asp Thr Thr
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Pro Leu Asn Lys Glu Gly Lys Lys Pro Arg Thr Arg Ala Pro Lys Ile
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Ala Leu Lys Lys Gln Arg Thr Lys Lys Asn Lys Glu Glu Ala Ala Glu
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Tyr Ala Lys Leu Leu Ala Lys Arg Met Lys Glu Ala Lys Glu Lys Arg
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Gln Glu Gln Ile Ala Lys Arg Arg Arg Leu Ser Ser Leu Arg Ala Ser
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 35 40 45

Val Val Arg Ile Ser Gly Gly Asn Asp Lys Gln Gly Phe Pro Met Lys
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Gln Gly Val Leu Thr His Gly Arg Val Arg Leu Leu Leu Ser Lys Gly
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His Ser Cys Tyr Arg Pro Arg Arg Thr Gly Glu Arg Lys Arg Lys Ser
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Val Arg Gly Cys Ile Val Asp Ala Asn Leu Ser Val Leu Asn Leu Val
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Ile Val Lys Lys Gly Glu Lys Asp Ile Pro Gly Leu Thr Asp Thr Thr
 115 120 125

Val Pro Arg Arg Leu Gly Pro Lys Arg Ala Ser Arg Ile Arg Lys Leu
 130 135 140

Phe Asn Leu Ser Lys Glu Asp Asp Val Arg Gln Tyr Val Val Arg Lys
 145 150 155 160

Pro Leu Asn Lys Glu Gly Lys Lys Pro Arg Thr Lys Ala Pro Lys Ile
 165 170 175

Gln Arg Leu Val Thr Pro Arg Val Leu Gln His Lys Arg Arg Arg Ile
 180 185 190

Ala Leu Lys Lys Gln Arg Thr Lys Lys Asn Lys Glu Glu Ala Ala Glu
 195 200 205

Tyr Ala Lys Leu Leu Ala Lys Arg Met Lys Glu Ala Lys Glu Lys Arg
 210 215 220

Gln Glu Gln Ile Ala Lys Arg Arg Arg Leu Ser Ser Leu Arg Ala Ser
 225 230 235 240

Thr Ser Lys Ser Glu Ser Ser Gln Lys
 245

<210> 23
 <211> 100
 <212> PRT
 <213> Homo sapiens

<400> 23

Met Lys Leu Asn Ile Phe Pro Ala Ala Gly Cys Gln Lys Leu Ile Ala
 1 5 10 15

Val Asp Asp Glu His Lys Leu Arg Ser Leu Tyr Glu Met Pro Val Ala
 20 25 30

Thr Glu Val Ala Ala Asp Ala Leu Val Lys His Gly Arg Val Leu Trp
 35 40 45

Ser Gly Ser Val Val Gly Thr Thr Asn Lys Pro Ser His Glu Ala Gly
 50 55 60

Arg Ala Ser Arg Ile Arg Arg Leu Phe Asn Val Ser Lys Glu Asp Asp
 65 70 75 80

Val Phe Phe Arg Arg Arg Arg Arg Arg Leu Asn Val Glu Met Leu Lys
 85 90 95

Cys Phe Lys Cys

100

<210> 24

<211> 323

<212> PRT

<213> Homo sapiens

<400> 24

Met Ala Val Val Lys Thr Leu Asn Pro Lys Ala Glu Val Ala Arg Thr
 1 5 10 15

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

Val Leu Gly Thr Asn Leu Gly Pro Lys Gly Thr Met Asn Met Leu Val
 35 40 45

Ser Gly Ala Gly Asp Ile Lys Leu Thr Lys Lys Met Ala Ile Ile Ile
 50 55 60

Thr Glu Gly Phe Glu Ala Ala Lys Glu Lys Ala Leu Arg Phe Leu Glu
 65 70 75 80

Glu Val Lys Ile Arg Lys Glu Met Asp Arg Glu Thr Leu Ile Asn Val
 85 90 95

Ala Arg Thr Ser Leu His Thr Lys Val His Ala Glu Leu Ala Asp Ala
 100 105 110

Leu Thr Glu Ala Val Val Asp Ser Ile Leu Ala Ile Lys Arg Gln Asp
 115 120 125

Glu Pro Ile Asp Leu Phe Met Val Val Ile Met Glu Met Lys His Lys
 130 135 140

Ser Glu Thr Asp Thr Ser Leu Ile Arg Gly Leu Val Leu Asp His Gly
 145 150 155 160

Ala Trp His Pro Asp Met Lys Lys Arg Asp Gln Ile Ser Thr His Ser
 165 170 175

Leu Ser Ser Lys Asp Ala Val Arg Asp Gly Leu Arg Ala Val Lys Asn
 180 185 190

Pro Ile Asp Glu Gly Cys Val Phe Pro Gly Ala Gly Ala Val Glu Val
 195 200 205

Ala Met Ala Glu Ala Leu Ile Lys Tyr Lys Pro Ser Val Lys Gly Arg
 210 215 220

Ala Gln Leu Gly Val Gln Ala Phe Ala Asp Val Leu Leu Ile Ile Pro
 225 230 235 240

Lys Val Leu Asp Gln Asn Ser Gly Phe Asp Leu Gln Glu Thr Leu Val
 245 250 255

Lys Ile Gln Ala Glu Cys Ser Glu Ser Gly Gln Leu Val Gly Val Asp
 260 265 270

Leu Asp Thr Gly Glu Pro Met Val Ala Ala Glu Val Gly Ile Trp Asp
 275 280 285

Asn Tyr Cys Val Lys Lys Gln Leu Leu His Ser Cys Thr Val Ile Ala
 290 295 300

Thr Asn Ile Leu Leu Val Asp Glu Ile Met Gln Ala Gly Met Phe Ser
 305 310 315 320

Leu Lys Gly

<210> 25
 <211> 389
 <212> PRT
 <213> Homo sapiens

<400> 25

Met Pro Leu Glu Asn Leu Glu Glu Glu Gly Leu Pro Lys Asn Pro Asp
 1 5 10 15

Leu Arg Ile Ala Gln Leu Arg Phe Leu Leu Ser Leu Pro Glu His Arg
 20 25 30

Gly Asp Ala Ala Val Arg Asp Glu Leu Met Ala Ala Val Arg Asp Asn
 35 40 45

Asn Met Ala Pro Tyr Tyr Glu Ala Leu Cys Lys Ser Leu Asp Trp Gln
 50 55 60

Ile Asp Val Asp Leu Leu Asn Lys Met Lys Lys Ala Asn Glu Asp Glu
 65 70 75 80

Leu Lys Arg Leu Asp Glu Glu Leu Glu Asp Ala Glu Lys Asn Leu Gly
 85 90 95

Glu Ser Glu Ile Arg Asp Ala Met Met Ala Lys Ala Glu Tyr Leu Cys
 100 105 110

Arg Ile Gly Asp Lys Glu Gly Ala Leu Thr Ala Phe Arg Lys Thr Tyr
 115 120 125

Asp Lys Thr Val Ala Leu Gly His Arg Leu Asp Ile Val Phe Tyr Leu
 130 135 140

Leu Arg Ile Gly Leu Phe Tyr Met Asp Asn Asp Leu Ile Thr Arg Asn
 145 150 155 160

Thr Glu Lys Ala Lys Ser Leu Ile Glu Glu Gly Gly Asp Trp Asp Arg
 165 170 175

Arg Asn Arg Leu Lys Val Tyr Gln Gly Leu Tyr Cys Val Ala Ile Arg
 180 185 190

Asp Phe Lys Gln Ala Ala Glu Leu Phe Leu Asp Thr Val Ser Thr Phe
 195 200 205

Thr Ser Tyr Glu Leu Met Asp Tyr Lys Thr Phe Val Thr Tyr Thr Val
 210 215 220

Tyr Val Ser Met Ile Ala Leu Glu Arg Pro Asp Leu Arg Glu Lys Val
 225 230 235 240

Ile Lys Gly Ala Glu Ile Leu Glu Val Leu His Ser Leu Pro Ala Val
 245 250 255

Arg Gln Tyr Leu Phe Ser Leu Tyr Glu Cys Arg Tyr Ser Val Phe Phe
 260 265 270

Gln Ser Leu Ala Val Val Glu Gln Glu Met Lys Lys Asp Trp Leu Phe
 275 280 285

Ala Pro His Tyr Arg Tyr Tyr Val Arg Glu Met Arg Ile His Ala Tyr
 290 295 300

Ser Gln Leu Leu Glu Ser Tyr Arg Ser Leu Thr Leu Gly Tyr Met Ala
 305 310 315 320

Glu Ala Phe Gly Val Gly Val Glu Phe Ile Asp Gln Glu Leu Ser Arg
 325 330 335

Phe Ile Ala Ala Gly Arg Leu His Cys Lys Ile Asp Lys Val Asn Glu

340 345 350
 Ile Val Glu Thr Asn Arg Pro Asp Ser Lys Asn Trp Gln Tyr Gln Glu
 355 360 365
 Thr Ile Lys Lys Gly Asp Leu Leu Leu Asn Arg Val Gln Lys Leu Ser
 370 375 380
 Arg Val Ile Asn Met
 385
 <210> 26
 <211> 264
 <212> PRT
 <213> Homo sapiens
 <400> 26
 Met Glu Ala Phe Leu Gly Ser Arg Ser Gly Leu Trp Ala Gly Gly Pro
 1 5 10 15
 Ala Pro Gly Gln Phe Tyr Arg Ile Pro Ser Thr Pro Asp Ser Phe Met
 20 25 30
 Asp Pro Ala Ser Ala Leu Tyr Arg Gly Pro Ile Thr Arg Thr Gln Asn
 35 40 45
 Pro Met Val Thr Gly Thr Ser Val Leu Gly Val Lys Phe Glu Gly Gly
 50 55 60
 Val Val Ile Ala Ala Asp Met Leu Gly Ser Tyr Gly Ser Leu Ala Arg
 65 70 75 80
 Phe Arg Asn Ile Ser Arg Ile Met Arg Val Asn Asn Ser Thr Met Leu
 85 90 95
 Gly Ala Ser Gly Asp Tyr Ala Asp Phe Gln Tyr Leu Lys Gln Val Leu
 100 105 110
 Gly Gln Met Val Ile Asp Glu Glu Leu Leu Gly Asp Gly His Ser Tyr
 115 120 125
 Ser Pro Arg Ala Ile His Ser Trp Leu Thr Arg Ala Met Tyr Ser Arg
 130 135 140
 Arg Ser Lys Met Asn Pro Leu Trp Asn Thr Met Val Ile Gly Gly Tyr
 145 150 155 160

Ala Asp Gly Glu Ser Phe Leu Gly Tyr Val Asp Met Leu Gly Val Ala
 165 170 175

Tyr Glu Ala Pro Ser Leu Ala Thr Gly Tyr Gly Ala Tyr Leu Ala Gln
 180 185 190

Pro Leu Leu Arg Glu Val Leu Glu Lys Gln Pro Val Leu Ser Gln Thr
 195 200 205

Glu Ala Arg Asp Leu Val Glu Arg Cys Met Arg Val Leu Tyr Tyr Arg
 210 215 220

Asp Ala Arg Ser Tyr Asn Arg Phe Gln Ile Ala Thr Val Thr Glu Lys
 225 230 235 240

Gly Val Glu Ile Glu Gly Pro Leu Ser Thr Glu Thr Asn Trp Asp Ile
 245 250 255

Ala His Met Ile Ser Gly Phe Glu
 260

<210> 27
 <211> 277
 <212> PRT
 <213> Homo sapiens

<400> 27

Met Ala Ala Val Ser Val Tyr Ala Pro Pro Val Gly Gly Phe Ser Phe
 1 5 10 15

Asp Asn Cys Arg Arg Asn Ala Val Leu Glu Ala Asp Phe Ala Lys Arg
 20 25 30

Gly Tyr Lys Leu Pro Lys Val Arg Lys Thr Gly Thr Thr Ile Ala Gly
 35 40 45

Val Val Tyr Lys Asp Gly Ile Val Leu Gly Ala Asp Thr Arg Ala Thr
 50 55 60

Glu Gly Met Val Val Ala Asp Lys Asn Cys Ser Lys Ile His Phe Ile
 65 70 75 80

Ser Pro Asn Ile Tyr Cys Cys Gly Ala Gly Thr Ala Ala Asp Thr Asp
 85 90 95

Met Thr Thr Gln Leu Ile Ser Ser Asn Leu Glu Leu His Ser Leu Ser
 100 105 110

Thr Gly Arg Leu Pro Arg Val Val Thr Ala Asn Arg Met Leu Lys Gln
 115 120 125

Met Leu Phe Arg Tyr Gln Gly Tyr Ile Gly Ala Ala Leu Val Leu Gly
 130 135 140

Gly Val Asp Val Thr Gly Pro His Leu Tyr Ser Ile Tyr Pro His Gly
 145 150 155 160

Ser Thr Asp Lys Leu Pro Tyr Val Thr Met Gly Ser Gly Ser Leu Ala
 165 170 175

Ala Met Ala Val Phe Glu Asp Lys Phe Arg Pro Asp Met Glu Glu Glu
 180 185 190

Glu Ala Lys Asn Leu Val Ser Glu Ala Ile Ala Ala Gly Ile Phe Asn
 195 200 205

Asp Leu Gly Ser Gly Ser Asn Ile Asp Leu Cys Val Ile Ser Lys Asn
 210 215 220

Lys Leu Asp Phe Leu Arg Pro Tyr Thr Val Pro Asn Lys Lys Gly Thr
 225 230 235 240

Arg Leu Gly Arg Tyr Arg Cys Glu Lys Gly Thr Thr Ala Val Leu Thr
 245 250 255

Glu Lys Ile Thr Pro Leu Glu Ile Glu Val Leu Glu Glu Thr Val Gln
 260 265 270

Thr Met Asp Thr Ser
 275

<210> 28
 <211> 273
 <212> PRT
 <213> Homo sapiens

<400> 28

Met Leu Lys Pro Ala Leu Glu Pro Arg Gly Gly Phe Ser Phe Glu Asn
 1 5 10 15

Cys Gln Arg Asn Ala Ser Leu Glu Arg Val Leu Pro Gly Leu Lys Val
 20 25 30

Pro His Ala Arg Lys Thr Gly Thr Thr Ile Ala Gly Leu Val Phe Gln
 35 40 45

Asp Gly Val Ile Leu Gly Ala Asp Thr Arg Ala Thr Asn Asp Ser Val
 50 55 60
 Val Ala Asp Lys Ser Cys Glu Lys Ile His Phe Ile Ala Pro Lys Ile
 65 70 75 80
 Tyr Cys Cys Gly Ala Gly Val Ala Ala Asp Ala Glu Met Thr Thr Arg
 85 90 95
 Met Val Ala Ser Lys Met Glu Leu His Ala Leu Ser Thr Gly Arg Glu
 100 105 110
 Pro Arg Val Ala Thr Val Thr Arg Ile Leu Arg Gln Thr Leu Phe Arg
 115 120 125
 Tyr Gln Gly His Val Gly Ala Ser Leu Ile Val Gly Gly Val Asp Leu
 130 135 140
 Thr Gly Pro Gln Leu Tyr Gly Val His Pro His Gly Ser Tyr Ser Arg
 145 150 155 160
 Leu Pro Phe Thr Ala Leu Gly Ser Gly Gln Asp Ala Ala Leu Ala Val
 165 170 175
 Leu Glu Asp Arg Phe Gln Pro Asn Met Thr Leu Glu Ala Ala Gln Gly
 180 185 190
 Leu Leu Val Glu Ala Val Thr Ala Gly Ile Leu Gly Asp Leu Gly Ser
 195 200 205
 Gly Gly Asn Val Asp Ala Cys Val Ile Thr Lys Thr Gly Ala Lys Leu
 210 215 220
 Leu Arg Thr Leu Ser Ser Pro Thr Glu Pro Val Lys Arg Ser Gly Arg
 225 230 235 240
 Tyr His Phe Val Pro Gly Thr Thr Ala Val Leu Thr Gln Thr Val Lys
 245 250 255
 Pro Leu Thr Leu Glu Leu Val Glu Glu Thr Val Gln Ala Met Glu Val
 260 265 270
 Glu

<210> 29
 <211> 318
 <212> PRT
 <213> Homo sapiens

<400> 29

Met Pro Asp Tyr Leu Gly Ala His Gln Arg Lys Thr Lys Asp Asp Glu
 1 5 10 15

Lys Asp Asp Lys Pro Ile Gln Val Leu Asp Glu Ala Asp Ile Ala Leu
 20 25 30

Leu Lys Thr Tyr Gly His Ser Thr Tyr Phe Arg Gln Ile Lys Gln Val
 35 40 45

Glu Asp Gly Val Gln Gln Leu Leu Lys Lys Ile Asn Glu Leu Thr Gly
 50 55 60

Ile Lys Glu Ser Asp Thr Gly Leu Ala Pro Pro Ala Leu Trp Asp Leu
 65 70 75 80

Ala Ala Asp Lys Gln Thr Leu Gln Ser Lys Gln Pro Leu Gln Val Ala
 85 90 95

Arg Cys Thr Lys Ile Ile Ser Ala Asp Ser Glu Asp Pro Lys Tyr Ile
 100 105 110

Val Asn Val Lys Gln Phe Ala Lys Phe Val Met Asp Phe Ser Asp Gln
 115 120 125

Val Ala Pro Thr Asp Ile Glu Glu Gly Met Arg Val Gly Val Asp Arg
 130 135 140

Asn Lys Tyr Gln Ile His Ile Pro Leu Pro Pro Lys Thr His Pro Thr
 145 150 155 160

Val Thr Met Met Gln Val Glu Glu Lys Pro Val Thr Tyr Ser Asp Val
 165 170 175

Gly Asp Cys Lys Glu Gln Ile Glu Lys Leu Arg Glu Val Val Glu Thr
 180 185 190

Pro Leu Leu His Pro Glu Arg Phe Val Asn Leu Gly Ile Glu Pro Pro
 195 200 205

Lys Gly Met Leu Leu Phe Asn Pro Pro Ser Thr Gly Arg His Ser Val
 210 215 220

Cys Gly Gln Leu Leu Ile Arg Leu Met Arg Ala Ser Phe Glu Leu Leu
225 230 235 240

Asp Leu Ser Cys Thr Glu Ile Ser Leu Met Arg Pro Gly Arg Leu Asp
245 250 255

Arg Lys Ile Glu Phe Ser Leu Pro Asp Leu Glu Gly Arg Thr His Ile
260 265 270

Phe Lys Ile His Ala His Ser Met Ser Val Glu Arg Tyr Ile Arg Phe
275 280 285

Glu Leu Leu Ala Arg Leu Cys Pro Asn Gly Thr Ala Ala Glu Ile Arg
290 295 300

Ser Val Cys Thr Glu Ala Gly Met Phe Ala Ile Arg Ala Arg
305 310 315

<210> 30

<211> 501

<212> PRT

<213> Homo sapiens

<400> 30

Met Glu Thr Ile Leu Glu Gln Gln Arg Arg Tyr His Glu Glu Lys Glu
1 5 10 15

Arg Leu Met Asp Val Met Ala Lys Glu Met Leu Thr Lys Lys Ser Thr
20 25 30

Leu Arg Asp Gln Ile Asn Ser Asp His Arg Thr Arg Ala Met Gln Asp
35 40 45

Arg Tyr Met Glu Val Ser Gly Asn Leu Arg Asp Leu Tyr Asp Asp Lys
50 55 60

Asp Gly Leu Arg Lys Glu Glu Leu Asn Ala Ile Ser Gly Pro Asn Glu
65 70 75 80

Phe Ala Glu Phe Tyr Asn Arg Leu Lys Gln Ile Lys Glu Phe His Arg
85 90 95

Lys His Pro Asn Glu Ile Cys Val Pro Met Ser Val Glu Phe Glu Glu
100 105 110

Leu Leu Lys Ala Arg Glu Asn Pro Ser Glu Glu Ala Gln Asn Leu Val
115 120 125

Glu Phe Thr Asp Glu Glu Gly Tyr Gly Arg Tyr Leu Asp Leu His Asp
 130 135 140

Cys Tyr Leu Lys Tyr Ile Asn Leu Lys Ala Ser Glu Lys Leu Asp Tyr
 145 150 155 160

Ile Thr Tyr Leu Ser Ile Phe Asp Gln Leu Phe Asp Ile Pro Lys Glu
 165 170 175

Arg Lys Asn Ala Glu Tyr Lys Arg Tyr Leu Glu Met Leu Leu Glu Tyr
 180 185 190

Leu Gln Asp Tyr Thr Asp Arg Val Lys Pro Leu Gln Asp Gln Asn Glu
 195 200 205

Leu Phe Gly Lys Ile Gln Ala Glu Phe Glu Lys Lys Trp Glu Asn Gly
 210 215 220

Thr Phe Pro Gly Trp Pro Lys Glu Thr Ser Ser Ala Leu Thr His Ala
 225 230 235 240

Gly Ala His Leu Asp Leu Ser Ala Phe Ser Ser Trp Glu Glu Leu Ala
 245 250 255

Ser Leu Gly Leu Asp Arg Leu Lys Ser Ala Leu Leu Ala Leu Gly Leu
 260 265 270

Lys Cys Gly Gly Thr Leu Glu Glu Arg Ala Gln Arg Leu Phe Ser Thr
 275 280 285

Lys Gly Lys Ser Leu Glu Ser Leu Asp Thr Ser Leu Phe Ala Lys Asn
 290 295 300

Pro Lys Ser Lys Gly Thr Lys Arg Asp Thr Glu Arg Asn Lys Asp Ile
 305 310 315 320

Ala Phe Leu Glu Ala Gln Ile Tyr Glu Tyr Val Glu Ile Leu Gly Glu
 325 330 335

Gln Arg His Leu Thr His Glu Asn Val Gln Arg Lys Gln Ala Arg Thr
 340 345 350

Gly Glu Glu Arg Glu Glu Glu Glu Glu Glu Gln Ile Ser Glu Ser Glu
 355 360 365

Ser Glu Asp Glu Glu Asn Glu Ile Ile Tyr Asn Pro Lys Asn Leu Pro
 370 375 380

Leu Gly Trp Asp Gly Lys Pro Ile Pro Tyr Trp Leu Tyr Lys Leu His
 385 390 395 400

Gly Leu Asn Ile Asn Tyr Asn Cys Glu Ile Cys Gly Asn Tyr Thr Tyr
 405 410 415

Arg Gly Pro Lys Ala Phe Gln Arg His Phe Ala Glu Trp Arg His Ala
 420 425 430

His Gly Met Arg Cys Leu Gly Ile Pro Asn Thr Ala His Phe Ala Asn
 435 440 445

Val Thr Gln Ile Glu Asp Ala Val Ser Leu Trp Ala Lys Leu Lys Leu
 450 455 460

Gln Lys Ala Ser Glu Arg Trp Gln Pro Asp Thr Glu Glu Glu Tyr Glu
 465 470 475 480

Asp Ser Ser Gly Asn Val Val Asn Lys Lys Thr Tyr Glu Asp Leu Lys
 485 490 495

Arg Gln Gly Leu Leu
 500

<210> 31
 <211> 258
 <212> PRT
 <213> Homo sapiens

<400> 31

Met Glu Thr Ile Leu Glu Gln Gln Gln Cys Tyr His Glu Glu Lys Glu
 1 5 10 15

Trp Leu Met Asp Val Met Ala Lys Glu Met Leu Thr Lys Lys Ser Met
 20 25 30

Leu Trp Asp Gln Ile Asn Ser Asp His Cys Thr Arg Ala Thr Gln Asp
 35 40 45

Ile Tyr Val Ala Met Ser Val Glu Phe Glu Glu Leu Leu Lys Ala Arg
 50 55 60

Glu Asn Pro Ser Glu Glu Ala Gln Asn Ser Val Glu Phe Thr Asp Glu
 65 70 75 80

Glu Gly Tyr Gly Arg Tyr Leu Asp Leu His Asp Cys Cys Leu Lys Tyr
85 90 95

Ile Asn Leu Lys Ala Ser Glu Lys Leu Asp Tyr Ile Thr Tyr Leu Ser
100 105 110

Ile Leu Asp Gln Leu Phe Asp Ile Pro Lys Asp Arg Arg Asn Ala Glu
115 120 125

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

Asp Arg Val Lys Pro Leu Gln Asp Gln Asn Glu Leu Ser Gly Lys Ile
145 150 155 160

Gln Ala Glu Phe Glu Lys Lys Trp Glu Asn Gly Ile Phe Pro Gly Trp
165 170 175

Pro Lys Glu Thr Ser Ser Ala Leu Thr Gln Ala Gly Ala His Leu Asp
180 185 190

Leu Ser Ala Phe Ser Ser Trp Glu Glu Leu Ala Ser Leu Gly Leu Asp
195 200 205

Arg Leu Lys Ser Ala Leu Leu Ala Leu Gly Leu Lys Cys Gly Arg Ile
210 215 220

Pro Glu Glu Arg Ala Gln Arg Leu Phe Ser Thr Lys Gly Lys Ser Leu
225 230 235 240

Glu Ser Leu Asp Thr Ser Leu Phe Ala Lys Asn Pro Lys Ser Lys Gly
245 250 255

Thr Lys

<210> 32

<211> 1603

<212> PRT

<213> Homo sapiens

<400> 32

Met Ser Asp Asp Glu Asp Asp Asp Glu Glu Glu Tyr Gly Lys Glu Glu
1 5 10 15

His Glu Lys Glu Ala Ile Ala Glu Glu Ile Phe Gln Asp Gly Glu Gly
20 25 30

Glu Glu Gly Gln Glu Ala Met Glu Ala Pro Met Ala Pro Pro Glu Glu
 35 40 45
 Glu Glu Glu Asp Asp Glu Glu Ser Asp Ile Asp Asp Phe Ile Val Asp
 50 55 60
 Asp Asp Gly Gln Pro Leu Lys Lys Pro Lys Trp Arg Lys Lys Leu Pro
 65 70 75 80
 Gly Tyr Thr Asp Ala Ala Leu Gln Glu Ala Gln Glu Ile Phe Gly Val
 85 90 95
 Asp Phe Asp Tyr Asp Glu Phe Glu Lys Tyr Asn Glu Tyr Asp Glu Glu
 100 105 110
 Leu Glu Glu Glu Tyr Glu Tyr Glu Asp Asp Glu Ala Glu Gly Glu Ile
 115 120 125
 Arg Val Arg Pro Lys Lys Thr Thr Lys Lys Arg Val Ser Arg Arg Ser
 130 135 140
 Ile Phe Glu Met Tyr Glu Pro Ser Glu Leu Glu Ser Ser His Leu Thr
 145 150 155 160
 Asp Gln Asp Asn Glu Ile Arg Ala Thr Asp Leu Pro Glu Arg Phe Gln
 165 170 175
 Leu Arg Ser Ile Pro Val Lys Gly Ala Glu Asp Asp Glu Leu Glu Glu
 180 185 190
 Glu Ala Asp Trp Ile Tyr Arg Asn Ala Phe Ala Thr Pro Thr Ile Ser
 195 200 205
 Leu Gln Glu Ser Cys Asp Tyr Leu Asp Arg Gly Gln Pro Ala Ser Ser
 210 215 220
 Phe Ser Arg Lys Gly Pro Ser Thr Ile Gln Lys Ile Lys Glu Ala Leu
 225 230 235 240
 Gly Phe Met Arg Asn Gln His Phe Glu Val Pro Phe Ile Ala Phe Tyr
 245 250 255
 Arg Lys Glu Tyr Val Glu Pro Glu Leu His Ile Asn Asp Leu Trp Arg
 260 265 270

Val Trp Gln Trp Asp Glu Lys Trp Thr Gln Leu Arg Ile Arg Lys Glu
 275 280 285
 Asn Leu Thr Arg Leu Phe Glu Lys Met Gln Ala Tyr Gln Tyr Glu Gln
 290 295 300
 Ile Ser Ala Asp Pro Asp Lys Pro Leu Ala Asp Gly Ile Arg Ala Leu
 305 310 315 320
 Asp Thr Thr Asp Met Glu Arg Leu Lys Asp Val Gln Ser Met Asp Glu
 325 330 335
 Leu Lys Asp Val Tyr Asn His Phe Leu Leu Tyr Tyr Gly Arg Asp Ile
 340 345 350
 Pro Lys Met Gln Asn Ala Ala Lys Ala Ser Arg Lys Lys Leu Lys Arg
 355 360 365
 Val Arg Glu Glu Gly Asp Glu Glu Gly Glu Gly Asp Glu Ala Glu Asp
 370 375 380
 Glu Glu Gln Arg Gly Pro Glu Leu Lys Gln Ala Ser Arg Arg Asp Met
 385 390 395 400
 Tyr Thr Ile Cys Gln Ser Ala Gly Leu Asp Gly Leu Ala Lys Lys Phe
 405 410 415
 Gly Leu Thr Pro Glu Gln Phe Gly Glu Asn Leu Arg Asp Ser Tyr Gln
 420 425 430
 Arg His Glu Thr Glu Gln Phe Pro Ala Glu Pro Leu Glu Leu Ala Lys
 435 440 445
 Asp Tyr Val Cys Ser Gln Phe Pro Thr Pro Glu Ala Val Leu Glu Gly
 450 455 460
 Ala Arg Tyr Met Val Ala Leu Gln Ile Ala Arg Glu Pro Leu Val Arg
 465 470 475 480
 Gln Val Leu Arg Gln Thr Phe Gln Glu Arg Ala Lys Leu Asn Ile Thr
 485 490 495
 Pro Thr Lys Lys Gly Arg Lys Asp Val Asp Glu Ala His Tyr Ala Tyr
 500 505 510
 Ser Phe Lys Tyr Leu Lys Asn Lys Pro Val Lys Glu Leu Arg Asp Asp
 515 520 525

Gln Phe Leu Lys Ile Cys Leu Ala Glu Asp Glu Gly Leu Leu Thr Thr
 530 535 540

Asp Ile Ser Ile Asp Leu Lys Gly Val Glu Gly Tyr Gly Asn Asp Gln
 545 550 555 560

Thr Tyr Phe Glu Glu Ile Lys Gln Phe Tyr Tyr Arg Asp Glu Phe Ser
 565 570 575

His Gln Val Gln Glu Trp Asn Arg Gln Arg Thr Met Ala Ile Glu Arg
 580 585 590

Ala Leu Gln Gln Phe Leu Tyr Val Gln Met Ala Lys Glu Leu Lys Asn
 595 600 605

Lys Leu Leu Ala Glu Ala Lys Glu Tyr Val Ile Lys Ala Cys Ser Arg
 610 615 620

Lys Leu Tyr Asn Trp Leu Arg Val Ala Pro Tyr Arg Pro Asp Gln Gln
 625 630 635 640

Val Glu Glu Asp Asp Asp Phe Met Asp Glu Asn Gln Gly Lys Gly Ile
 645 650 655

Arg Val Leu Gly Ile Ala Phe Ser Ser Ala Arg Asp His Pro Val Phe
 660 665 670

Cys Ala Leu Val Asn Gly Glu Gly Glu Val Thr Asp Phe Leu Arg Leu
 675 680 685

Pro His Phe Thr Lys Arg Arg Thr Ala Trp Arg Glu Glu Glu Arg Glu
 690 695 700

Lys Lys Ala Gln Asp Ile Glu Thr Leu Lys Lys Phe Leu Leu Asn Lys
 705 710 715 720

Lys Pro His Val Val Thr Val Ala Gly Glu Asn Arg Asp Ala Gln Met
 725 730 735

Leu Ile Glu Asp Val Lys Arg Ile Val His Glu Leu Asp Gln Gly Gln
 740 745 750

Gln Leu Ser Ser Ile Gly Val Glu Leu Val Asp Asn Glu Leu Ala Ile
 755 760 765

Leu Tyr Met Asn Ser Lys Lys Ser Glu Ala Glu Phe Arg Asp Tyr Pro
 770 775 780

Pro Val Leu Arg Gln Ala Val Ser Leu Ala Arg Arg Ile Gln Asp Pro
 785 790 795 800

Leu Ile Glu Phe Ala Gln Val Cys Ser Ser Asp Glu Asp Ile Leu Cys
 805 810 815

Leu Lys Phe His Pro Leu Gln Glu His Val Val Lys Glu Glu Leu Leu
 820 825 830

Asn Ala Leu Tyr Cys Glu Phe Ile Asn Arg Val Asn Glu Val Gly Val
 835 840 845

Asp Val Asn Arg Ala Ile Ala His Pro Tyr Ser Gln Ala Leu Ile Gln
 850 855 860

Tyr Val Cys Gly Leu Gly Pro Arg Lys Gly Thr His Leu Leu Lys Ile
 865 870 875 880

Leu Lys Gln Asn Asn Thr Arg Leu Glu Ser Arg Thr Gln Leu Val Thr
 885 890 895

Met Cys His Met Gly Pro Lys Val Phe Met Asn Cys Ala Gly Phe Leu
 900 905 910

Lys Ile Asp Thr Ala Ser Leu Gly Asp Ser Thr Asp Ser Tyr Ile Glu
 915 920 925

Val Leu Asp Gly Ser Arg Val His Pro Glu Thr Tyr Glu Trp Ala Arg
 930 935 940

Lys Met Ala Val Asp Ala Leu Glu Tyr Asp Glu Ser Ala Glu Asp Ala
 945 950 955 960

Asn Pro Ala Gly Ala Leu Glu Glu Ile Leu Glu Asn Pro Glu Arg Leu
 965 970 975

Lys Asp Leu Asp Leu Asp Ala Phe Ala Glu Glu Leu Glu Arg Gln Gly
 980 985 990

Tyr Gly Asp Lys His Ile Thr Leu Tyr Asp Ile Arg Ala Glu Leu Ser
 995 1000 1005

Cys Arg Tyr Lys Asp Leu Arg Thr Ala Tyr Arg Ser Pro Asn Thr
 1010 1015 1020

Glu	Glu	Ile	Phe	Asn	Met	Leu	Thr	Lys	Glu	Thr	Pro	Glu	Thr	Phe
1025						1030					1035			
Tyr	Ile	Gly	Lys	Leu	Ile	Ile	Cys	Asn	Val	Thr	Gly	Ile	Ala	His
1040						1045					1050			
Arg	Arg	Pro	Gln	Gly	Glu	Ser	Tyr	Asp	Gln	Ala	Ile	Arg	Asn	Asp
1055						1060					1065			
Glu	Thr	Gly	Leu	Trp	Gln	Cys	Pro	Phe	Cys	Gln	Gln	Asp	Asn	Phe
1070						1075					1080			
Pro	Glu	Leu	Ser	Glu	Val	Trp	Asn	His	Phe	Asp	Ser	Gly	Ser	Cys
1085						1090					1095			
Pro	Gly	Gln	Ala	Ile	Gly	Val	Lys	Thr	Arg	Leu	Asp	Asn	Gly	Val
1100						1105					1110			
Thr	Gly	Phe	Ile	Pro	Thr	Lys	Phe	Leu	Ser	Asp	Lys	Val	Val	Lys
1115						1120					1125			
Arg	Pro	Glu	Glu	Arg	Val	Lys	Val	Gly	Met	Thr	Val	His	Cys	Arg
1130						1135					1140			
Ile	Met	Lys	Ile	Asp	Ile	Glu	Lys	Phe	Ser	Ala	Asp	Leu	Thr	Cys
1145						1150					1155			
Arg	Thr	Ser	Asp	Leu	Met	Asp	Arg	Asn	Asn	Glu	Trp	Lys	Leu	Pro
1160						1165					1170			
Lys	Asp	Thr	Tyr	Tyr	Asp	Phe	Asp	Ala	Glu	Ala	Ala	Asp	His	Lys
1175						1180					1185			
Gln	Glu	Glu	Asp	Met	Lys	Arg	Lys	Gln	Gln	Arg	Thr	Thr	Tyr	Ile
1190						1195					1200			
Lys	Arg	Val	Ile	Ala	His	Pro	Ser	Phe	His	Asn	Ile	Asn	Phe	Lys
1205						1210					1215			
Gln	Ala	Glu	Lys	Met	Met	Glu	Thr	Met	Asp	Gln	Gly	Asp	Val	Ile
1220						1225					1230			
Ile	Arg	Pro	Ser	Ser	Lys	Gly	Glu	Asn	His	Leu	Thr	Val	Thr	Trp
1235						1240					1245			

Lys Val	Ser Asp Gly Ile Tyr	Gln His Val Asp Val	Arg Glu Glu
1250	1255	1260	
Gly Lys	Glu Asn Ala Phe Ser	Leu Gly Ala Thr Leu	Trp Ile Asn
1265	1270	1275	
Ser Glu	Glu Phe Glu Asp Leu	Asp Glu Ile Val Ala	Arg Tyr Val
1280	1285	1290	
Gln Pro	Met Ala Ser Phe Ala	Arg Asp Leu Leu Asn	His Lys Tyr
1295	1300	1305	
Tyr Gln	Asp Cys Ser Gly Gly	Asp Arg Lys Lys Leu	Glu Glu Leu
1310	1315	1320	
Leu Ile	Lys Thr Lys Lys Glu	Lys Pro Thr Phe Ile	Pro Tyr Phe
1325	1330	1335	
Ile Cys	Ala Cys Lys Glu Leu	Pro Gly Lys Phe Leu	Leu Gly Tyr
1340	1345	1350	
Gln Pro	Arg Gly Lys Pro Arg	Ile Glu Tyr Val Thr	Val Thr Pro
1355	1360	1365	
Glu Gly	Phe Arg Tyr Arg Gly	Gln Ile Phe Pro Thr	Val Asn Gly
1370	1375	1380	
Leu Phe	Arg Trp Phe Lys Asp	His Tyr Gln Asp Pro	Val Pro Gly
1385	1390	1395	
Ile Thr	Pro Ser Ser Ser Ser	Arg Thr Arg Thr Pro	Ala Ser Ile
1400	1405	1410	
Asn Ala	Thr Pro Ala Asn Ile	Asn Leu Ala Asp Leu	Thr Arg Ala
1415	1420	1425	
Val Asn	Ala Leu Pro Gln Asn	Met Thr Ser Gln Met	Phe Ser Ala
1430	1435	1440	
Ile Ala	Ala Val Thr Gly Gln	Gly Gln Asn Pro Asn	Ala Thr Pro
1445	1450	1455	
Ala Gln	Trp Ala Ser Ser Gln	Tyr Gly Tyr Gly Gly	Ser Gly Gly
1460	1465	1470	
Gly Ser	Ser Ala Tyr His Val	Phe Pro Thr Pro Ala	Gln Gln Pro
1475	1480	1485	

Val Ala Thr Pro Leu Met Thr Pro Ser Tyr Ser Tyr Thr Thr Pro
1490 1495 1500

Ser Gln Pro Ile Thr Thr Pro Gln Tyr His Gln Leu Gln Ala Ser
1505 1510 1515

Thr Thr Pro Gln Ser Ala Gln Ala Gln Pro Gln Pro Ser Ser Ser
1520 1525 1530

Ser Arg Gln Arg Gln Gln Gln Pro Lys Ser Asn Ser His Ala Ala
1535 1540 1545

Ile Asp Trp Gly Lys Met Ala Glu Gln Trp Leu Gln Glu Lys Glu
1550 1555 1560

Ala Glu Arg Arg Lys Gln Lys Gln Arg Leu Thr Pro Arg Pro Ser
1565 1570 1575

Pro Ser Pro Met Ile Glu Ser Thr Pro Met Ser Ile Ala Gly Asp
1580 1585 1590

Ala Thr Pro Leu Leu Asp Glu Met Asp Arg
1595 1600