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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

5 This application claims priority to U.S. provisional patent application 60/470,675 filed 5/14/2003. The contents of the prior application are hereby incorporated in their entirety.

BACKGROUND OF THE INVENTION

10 Retinoblastoma, a pediatric eye tumor, has served as an important model for the 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
15 plays a key role in the etiology of several other primary malignancies.

 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-
20 330). RB regulates cell proliferation by restricting cell cycle progression at a specific 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
25 determined by the complex interaction of multiple kinases and their inhibitors that 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.

30 RB sequence is conserved in evolution, and exists in mouse (Bernards R et al (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*, and identified their human orthologs, hereinafter referred to as Modifiers 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
5 activity by, for example, binding to and inhibiting the respective nucleic acid (i.e. DNA or mRNA).

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
10 comprising an 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 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;
15 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, 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.

20 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 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
25 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 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
30 specifically binds an 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 mammalian animal predetermined to have a pathology associated with the RB pathway.

DETAILED DESCRIPTION OF THE INVENTION

The Rb 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. Modifiers of the Rbf pathway were identified. 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.

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 indirectly, for example, by affecting an MRB function such as enzymatic (e.g., catalytic) or 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

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), shown in Table 1 and in the appended sequence listing.

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 exhibits one or more functional activities associated with a full-length, wild-type MRB protein, such as antigenic or immunogenic activity, enzymatic 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 as further discussed below. In one embodiment, a functionally active MRB polypeptide is an 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

5 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 an 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%
10 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
15 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,
20 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
25 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
30 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
5 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
10 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.)
15 *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),
20 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
25 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
30 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
5 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,
10 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
15 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,
20 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
25 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
30 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).

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 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.*, 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 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.* (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 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).
10 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 cycle progression.

15

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 the methods are also part of the invention. Such agents are useful in a variety of
20 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 an MRB-interacting or -modulating agent.

25 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 extra-cellular activity. In a preferred embodiment, the MRB - modulating agent
30 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-modulating agent.

5 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 comprise other active ingredients, as in combination therapy, and/or suitable carriers or
10 excipients. Techniques for formulation and administration of the compounds may be found in "Remington's Pharmaceutical Sciences" Mack Publishing Co., Easton, PA, 19th edition.

Small molecule modulators

15 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 up to 10,000, preferably up to 5,000, more preferably up to 1,000, and most preferably up to 500 daltons. This class of modulators
20 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 natural products, particularly secondary metabolites from organisms such as plants or fungi, which can also
25 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).

Small molecule modulators identified from screening assays, as described below, can be used as lead compounds from which candidate clinical compounds may be
30 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.

5 **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
10 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
15 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
20 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
25 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
30 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.

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
5 dissecting the portions of the MRB pathway responsible for various cellular responses and 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,
10 monoclonal (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab 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
15 selecting antigenic regions of a protein (Hopp and Wood (1981), Proc. Nati. Acad. Sci. 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)
20 Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York; and 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
25 antigens and/or immunogens are coupled to carrier proteins that stimulate the immune 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.
30 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.

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
5 specificity from the murine fragment. Chimeric antibodies are produced by splicing 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
10 (CDRs) (Carlos, T. M., J. M. Harlan. 1994. Blood 84:2068-2101) of mouse antibodies 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,
15 and Queen C. 1991 Nature 351: 501-501; Morrison SL. 1992 Ann. Rev. Immun. 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
20 an amino acid bridge, can be produced by methods known in the art (U.S. Pat. No. 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
25 antibodies in phage or similar vectors (Huse et al., Science (1989) 246:1275-1281). As 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
30 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
5 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
10 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
15 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
20 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-
25 245 (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
30 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).

5 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.

10

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
15 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 an 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
20 will be directly tested in a secondary assay.

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
25 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.

30 **Primary Assays**

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

Primary assays for small molecule modulators

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
5 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 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
10 cellular extracts. Screening assays may detect a variety of molecular events, including protein-DNA interactions, protein-protein interactions (*e.g.*, receptor-ligand binding), 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
15 range of detection methods including fluorescent, radioactive, colorimetric, spectrophotometric, and amperometric methods, to provide a read-out for the particular 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
20 for generating recombinant proteins produce sufficient quantities of proteins that retain their relevant biological activities and are of sufficient purity to optimize activity and 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
25 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 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
30 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 an 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).

Peptidyl-prolyl isomerase (PPIase) proteins, which include cyclophilins, FK506 binding proteins and paravulins, catalyze the isomerization of *cis-trans* proline peptide bonds in oligopeptides and are thought to be essential for protein folding during protein synthesis in the cell. Spectrophotometric assays for PPIase activity can detect isomerization of labeled peptide substrates, either by direct measurement of isomer-specific absorbance, or by coupling isomerization to isomer-specific cleavage by chymotrypsin (Scholz C *et al.*, FEBS Lett (1997) 414:69-73; Janowski B *et al.*, Anal Biochem (1997) 252:299-307; Kullertz G *et al.*, Clin Chem (1998) 44:502-8). Alternative assays use the scintillation proximity or fluorescence polarization assay to screen for ligands of specific PPIases (Graziani F *et al.*, J Biolmol Screen (1999) 4:3-7; Dubowchik GM *et al.*, Bioorg Med Chem Lett (2000) 10:559-562). Assays for 3,2-trans-enoyl-CoA isomerase activity have also been described (Binstock, J. F., and Schulz, H. (1981) Methods Enzymol. 71:403-411; Geisbrecht BV *et al* (1999) J Biol Chem. 274:21797-803). These assays use 3-cis-octenoyl-CoA as a substrate, and reaction progress is

monitored spectrophotometrically using a coupled assay for the isomerization of 3-cis-octenoyl-CoA to 2-trans-octenoyl-CoA.

Transcription factors control gene transcription. Electrophoretic mobility shift assay (EMSA) or gel shift assay is one of the most powerful methods for studying protein-DNA interactions. High throughput gel shift assays for transcription factors may involve fluorescence (Cyano dye Cy5) labeled oligodeoxynucleotide duplexes as specific probes and an automatic DNA sequencer for analysis (Ruscher K, et al., (2000) J Biotechnol 78:163-70). Alternatively high throughput methods involve colorimetric assays (Renard P, et al. (2001) Nucleic Acids Res 29(4):E21), or homogeneous fluorescence assays for the detection and quantification of sequence-specific DNA-binding proteins (Heyduk T, and Heyduk E (2001) Nat Biotechnol 20:171-6.)

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-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 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 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. In some embodiments of the invention, an apoptosis assay may be used as a secondary
5 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 compared to wild type cells suggests that the MRB plays a direct role in the apoptotic
10 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 undergoing DNA synthesis by incorporation of BRDU into newly-synthesized DNA.
15 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
20 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,
25 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
30 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.

5 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
10 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-
15 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
20 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
25 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
30 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
5 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
10 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
15 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
20 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
25 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
30 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
5 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
10 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
15 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.

20 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
25 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
30 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

5 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

10 angiogenic factors such as TNF- α . 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- α , ephrin, etc.

Cell Migration. An invasion/migration assay (also called a migration assay) tests

15 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

20 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

25 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

30 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 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 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 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; 5 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 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 10 (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 test nucleic acid modulators.

15 **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 20 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 25 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 30 interacting pathways.

Cell-based assays

Cell based assays may use 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
5 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
10 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
15 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,
20 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®
25 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
30 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.

10 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
15 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
20 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
25 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
30 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.

5

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.

Kits for detecting expression of MRB in various samples, comprising at least one antibody specific to MRB, all reagents and/or devices suitable for the detection of antibodies, the immobilization of antibodies, and the like, and instructions for using such kits in diagnosis or therapy are also provided.

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

We performed a clonal screen in the fly eye to identify second-site mutations which are synthetically lethal with Rbf1 null ($Rbf1^-$) cells, but allow Rbf1 wild type ($Rbf1^+$) cells to survive. Using the FLP/FRT system driven by ey^-FLP , we simultaneously
 5 generated marked w^+ , $Rbf1^-$ homozygous clones and screened for their elimination by a second site mutation. Selectivity of these potential synthetic mutations for $Rbf1^-$ cells was demonstrated in a counterscreen on $Rbf1^+$ clones.

Males ($yw;ey^-FLP$) were treated with the mutagen 5mM EMS and crossed to females ($w,Rbf^-, pExp\{3.5ey^-FLP; FRT2.1[rbf^+, w^+]\}; P\{ry[+t7.2]=neoFRT\}42D; ry[605]$
 10 $FRT 42-1, M53 pi-M /CyO$). Using FRT mediated recombination we induced marked homozygous clones in the eye for the mutant chromosome and scored their survival in $Rbf1^-$ cells. Mutant chromosomes causing death of the Rbf^- cells were maintained in fly stocks and were then counterscreened by testing induced clones against Rbf^+ cells. Mutations that failed to kill clones of Rbf^+ but not Rbf^- cells were designated as synthetic
 15 lethals.

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
 20 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,
 25 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 (Example I), are represented in the "Modifier Name" and "Modifier GI_AA" column by GI#, respectively.

30

Table 1

MRB symbol	MRB name aliases	MRB RefSeq_NA or GI_NA	NA SEQ ID NO	MRB GI_AA	AA SEQ ID NO	MRB NAME	MRB Description	MRB Protein Length	Modifier Name	Modifier GI_AA
ELL	ELL ELL gene (11-19 lysine-rich leukemia gene)	NM_006532	1	5729812	6	ELL gene (11-19 lysine-rich leukemia gene)	transcription co-repressor; positive transcription elongation factor; transcription initiation factor antagonist	621	Ell	24667059
ELL2	ELL2 ELL-related RNA polymerase II, elongation factor	NM_012081	2	6912354	7	ELL-related RNA polymerase II, elongation factor	na	640	Ell	24667059
FLJ22637	FLJ22637 hypothetical protein FLJ22637	NM_025165	3	13376768	8	hypothetical protein FLJ22637	Pol II transcription elongation factor	397	Ell	24667059
LOC284674	LOC284674 similar to RNA polymerase II elongation factor ELL2 na	XM_208235	4	27480831	9	similar to RNA polymerase II elongation factor ELL2	na	190	Ell	24667059
KIAA0073	KIAA0073 KIAA0073 protein	NM_015342	5	24308049	10	KIAA0073 protein	cis-trans isomerase; peptidyl-prolyl cis-trans isomerase	646	CG3511	19922912

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.

10

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 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 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) 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, Ardaïs, Genome Collaborative, and Ambion.

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

RNA is extracted from each tissue sample using Qiagen (Valencia, CA) RNeasy kits, following manufacturer's protocols, to a final concentration of 50ng/μl. Single
5 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).

Primers for expression analysis using TaqMan® assay (Applied Biosystems, Foster City, CA) are prepared according to the TaqMan® protocols, and the following
10 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.

TaqMan® reactions are carried out following manufacturer's protocols, in 25 μl total volume for 96-well plates and 10 μl total volume for 384-well plates, using 300nM
15 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).

20 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 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
25 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 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
30 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 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 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 an 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 an MRB polypeptide, and the candidate test agent is a small molecule modulator.
- 20 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 of an apoptosis assay system, a cell proliferation assay system, an angiogenesis assay
- 25 system, and a hypoxic induction assay system.
7. The method of claim 1 wherein the assay system includes a binding assay comprising an 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 an 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:
- (d) administering the candidate RB pathway modulating agent identified in (c) to a
5 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 an 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.
15. The method of claim 13 wherein the candidate modulator is selected from the group
20 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,
wherein a difference between the agent-biased activity and the reference activity of
30 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.
- 5 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 an MRB polypeptide or nucleic acid.
- 10 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.
- 15 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.
- 20 24. The method of claim 23 wherein said disease is cancer.

25

SEQUENCE LISTING

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

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aaaagtgcag attctgatcg agatgttttt aatgagaaac cttctaaaga agaagtcatg 1440
gcagctactc aagctgaagg acctaaacga gtttcggaca gtgccattat ccacaccagc 1500
atgggagaca ttcacaccaa actttttcct gttgagtgcc ctaagacagt ggaaaacttc 1560
tgtgttcaca gcagaaatgg ttattataat gggcatacat ttcaccgtat aattaagggc 1620
tttatgattc agactggaga tccaacaggt actggtatgg gaggagaaag catatgggga 1680
ggagaatttg aagatgaatt tcattcaaca ttacgacatg acaggccgta cacactcagc 1740
atggctaacg cgggatcaaa tactaatgga tcccagtttt tcataacggt agtaccaacg 1800
ccttggcttg ataataagca tacagtattt ggacgagtga ctaaaggaat ggaagttgta 1860
cagaggatct ccaacgtcaa agtcaatccc aaaacagata agccctatga ggatgtcagc 1920
atcataaata ttactgtcaa gtaaaataag atttgtttta atgtacttgc aaataaaaaat 1980
acaatattaa acagattatt ttacattagg aagcttagga cttgctgaat atacagatca 2040
tgtttcaaag atacagtatt ttgtatttt ttattaaagg ctatttttta aaaaaaaaaa 2100
aaaaaaaaaa 2110

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<210> 6
 <211> 621
 <212> PRT
 <213> Homo sapiens

<400> 6

```

Met Ala Ala Leu Lys Glu Asp Arg Ser Tyr Gly Leu Ser Cys Gly Arg
1          5          10          15

```

```

Val Ser Asp Gly Ser Lys Val Ser Val Phe His Val Lys Leu Thr Asp
          20          25          30

```

```

Ser Ala Leu Arg Ala Phe Glu Ser Tyr Arg Ala Arg Gln Asp Ser Val
          35          40          45

```

```

Ser Leu Arg Pro Ser Ile Arg Phe Gln Gly Ser Gln Gly His Ile Ser
50          55          60

```

```

Ile Pro Gln Pro Asp Cys Pro Ala Glu Ala Arg Thr Phe Ser Phe Tyr
65          70          75          80

```

```

Leu Ser Asn Ile Gly Arg Asp Asn Pro Gln Gly Ser Phe Asp Cys Ile
          85          90          95

```

```

Gln Gln Tyr Val Ser Ser His Gly Glu Val His Leu Asp Cys Leu Gly

```

100					105					110					
Ser	Ile	Gln	Asp	Lys	Ile	Thr	Val	Cys	Ala	Thr	Asp	Asp	Ser	Tyr	Gln
		115					120					125			
Lys	Ala	Arg	Gln	Ser	Met	Ala	Gln	Ala	Glu	Glu	Glu	Thr	Arg	Ser	Arg
	130					135					140				
Ser	Ala	Ile	Val	Ile	Lys	Ala	Gly	Gly	Arg	Tyr	Leu	Gly	Lys	Lys	Val
145						150					155				160
Gln	Phe	Arg	Lys	Pro	Ala	Pro	Gly	Ala	Thr	Asp	Ala	Val	Pro	Ser	Arg
				165					170					175	
Lys	Arg	Ala	Thr	Pro	Ile	Asn	Leu	Ala	Ser	Ala	Ile	Arg	Lys	Ser	Gly
			180					185					190		
Ala	Ser	Ala	Val	Ser	Gly	Gly	Ser	Gly	Val	Ser	Gln	Arg	Pro	Phe	Arg
		195					200					205			
Asp	Arg	Val	Leu	His	Leu	Leu	Ala	Leu	Arg	Pro	Tyr	Arg	Lys	Ala	Glu
	210					215					220				
Leu	Leu	Leu	Arg	Leu	Gln	Lys	Asp	Gly	Leu	Thr	Gln	Ala	Asp	Lys	Asp
225						230					235				240
Ala	Leu	Asp	Gly	Leu	Leu	Gln	Gln	Val	Ala	Asn	Met	Ser	Ala	Lys	Asp
				245					250					255	
Gly	Thr	Cys	Thr	Leu	Gln	Asp	Cys	Met	Tyr	Lys	Asp	Val	Gln	Lys	Asp
			260					265					270		
Trp	Pro	Gly	Tyr	Ser	Glu	Gly	Asp	Gln	Gln	Leu	Leu	Lys	Arg	Val	Leu
		275					280					285			
Val	Arg	Lys	Leu	Cys	Gln	Pro	Gln	Ser	Thr	Gly	Ser	Leu	Leu	Gly	Asp
		290				295					300				
Pro	Ala	Ala	Ser	Ser	Pro	Pro	Gly	Glu	Arg	Gly	Arg	Ser	Ala	Ser	Pro
305						310					315				320
Pro	Gln	Lys	Arg	Leu	Gln	Pro	Pro	Asp	Phe	Ile	Asp	Pro	Leu	Ala	Asn
				325					330					335	
Lys	Lys	Pro	Arg	Ile	Ser	His	Phe	Thr	Gln	Arg	Ala	Gln	Pro	Ala	Val
			340					345					350		

Asn Gly Lys Leu Gly Val Pro Asn Gly Arg Glu Ala Leu Leu Pro Thr
 355 360 365

Pro Gly Pro Pro Ala Ser Thr Asp Thr Leu Ser Ser Ser Thr His Leu
 370 375 380

Pro Pro Arg Leu Glu Pro Pro Arg Ala His Asp Pro Leu Ala Asp Val
 385 390 395 400

Ser Asn Asp Leu Gly His Ser Gly Arg Asp Cys Glu His Gly Glu Ala
 405 410 415

Ala Ala Pro Ala Pro Thr Val Arg Leu Gly Leu Pro Leu Leu Thr Asp
 420 425 430

Cys Ala Gln Pro Ser Arg Pro His Gly Ser Pro Ser Arg Ser Lys Pro
 435 440 445

Lys Lys Lys Ser Lys Lys His Lys Asp Lys Glu Arg Ala Ala Glu Asp
 450 455 460

Lys Pro Arg Ala Gln Leu Pro Asp Cys Ala Pro Ala Thr His Ala Thr
 465 470 475 480

Pro Gly Ala Pro Ala Asp Thr Pro Gly Leu Asn Gly Thr Cys Ser Val
 485 490 495

Ser Ser Val Pro Thr Ser Thr Ser Glu Thr Pro Asp Tyr Leu Leu Lys
 500 505 510

Tyr Ala Ala Ile Ser Ser Ser Glu Gln Arg Gln Ser Tyr Lys Asn Asp
 515 520 525

Phe Asn Ala Glu Tyr Ser Glu Tyr Arg Asp Leu His Ala Arg Ile Glu
 530 535 540

Arg Ile Thr Arg Arg Phe Thr Gln Leu Asp Ala Gln Leu Arg Gln Leu
 545 550 555 560

Ser Gln Gly Ser Glu Glu Tyr Glu Thr Thr Arg Gly Gln Ile Leu Gln
 565 570 575

Glu Tyr Arg Lys Ile Lys Lys Thr Asn Thr Asn Tyr Ser Gln Glu Lys
 580 585 590

His Arg Cys Glu Tyr Leu His Ser Lys Leu Ala His Ile Lys Arg Leu

12

Arg Lys Thr His Ser Ser Ser Thr Ile Ser Gln Arg Pro Tyr Arg Asp
 195 200 205

Arg Val Ile His Leu Leu Ala Leu Lys Ala Tyr Lys Lys Pro Glu Leu
 210 215 220

Leu Ala Arg Leu Gln Lys Asp Gly Val Asn Gln Lys Asp Lys Asn Ser
 225 230 235 240

Leu Gly Ala Ile Leu Gln Gln Val Ala Asn Leu Asn Ser Lys Asp Leu
 245 250 255

Ser Tyr Thr Leu Lys Asp Tyr Val Phe Lys Glu Leu Gln Arg Asp Trp
 260 265 270

Pro Gly Tyr Ser Glu Ile Asp Arg Arg Ser Leu Glu Ser Val Leu Ser
 275 280 285

Arg Lys Leu Asn Pro Ser Gln Asn Ala Thr Gly Thr Ser Arg Ser Glu
 290 295 300

Ser Pro Val Cys Ser Ser Arg Asp Ala Val Ser Ser Pro Gln Lys Arg
 305 310 315 320

Leu Leu Asp Ser Glu Phe Ile Asp Pro Leu Met Asn Lys Lys Ala Arg
 325 330 335

Ile Ser His Leu Thr Asn Arg Val Pro Pro Thr Leu Asn Gly His Leu
 340 345 350

Asn Pro Thr Ser Glu Lys Ser Ala Ala Gly Leu Pro Leu Pro Pro Ala
 355 360 365

Ala Ala Ala Ile Pro Thr Pro Pro Pro Leu Pro Ser Thr Tyr Leu Pro
 370 375 380

Ile Ser His Pro Pro Gln Ile Val Asn Ser Asn Ser Asn Ser Pro Ser
 385 390 395 400

Thr Pro Glu Gly Arg Gly Thr Gln Asp Leu Pro Val Asp Ser Phe Ser
 405 410 415

Gln Asn Asp Ser Ile Tyr Glu Asp Gln Gln Asp Lys Tyr Thr Ser Arg
 420 425 430

Thr Ser Leu Glu Thr Leu Pro Pro Gly Ser Val Leu Leu Lys Cys Pro
 435 440 445

Lys Pro Met Glu Glu Asn His Ser Met Ser His Lys Lys Ser Lys Lys
 450 455 460

Lys Ser Lys Lys His Lys Glu Lys Asp Gln Ile Lys Lys His Asp Ile
 465 470 475 480

Glu Thr Ile Glu Glu Lys Glu Glu Asp Leu Lys Arg Glu Glu Glu Ile
 485 490 495

Ala Lys Leu Asn Asn Ser Ser Pro Asn Ser Ser Gly Gly Val Lys Glu
 500 505 510

Asp Cys Thr Ala Ser Met Glu Pro Ser Ala Ile Glu Leu Pro Asp Tyr
 515 520 525

Leu Ile Lys Tyr Ile Ala Ile Val Ser Tyr Glu Gln Arg Gln Asn Tyr
 530 535 540

Lys Asp Asp Phe Asn Ala Glu Tyr Asp Glu Tyr Arg Ala Leu His Ala
 545 550 555 560

Arg Met Glu Thr Val Ala Arg Arg Phe Ile Lys Leu Asp Ala Gln Arg
 565 570 575

Lys Arg Leu Ser Pro Gly Ser Lys Glu Tyr Gln Asn Val His Glu Glu
 580 585 590

Val Leu Gln Glu Tyr Gln Lys Ile Lys Gln Ser Ser Pro Asn Tyr His
 595 600 605

Glu Glu Lys Tyr Arg Cys Glu Tyr Leu His Asn Lys Leu Ala His Ile
 610 615 620

Lys Arg Leu Ile Gly Glu Phe Asp Gln Gln Gln Ala Glu Ser Trp Ser
 625 630 635 640

<210> 8
 <211> 397
 <212> PRT
 <213> Homo sapiens

<400> 8

Met Glu Glu Leu Gln Glu Pro Leu Arg Gly Gln Leu Arg Leu Cys Phe
 1 5 10 15

Thr Gln Ala Ala Arg Thr Ser Leu Leu Leu Leu Arg Leu Asn Asp Ala

20										25					30															
Ala	Leu	Arg	Ala	Leu	Gln	Glu	Cys	Gln	Arg	Gln	Gln	Val	Arg	Pro	Val															
		35					40					45																		
Ile	Ala	Phe	Gln	Gly	His	Arg	Gly	Tyr	Leu	Arg	Leu	Pro	Gly	Pro	Gly															
	50					55					60																			
Trp	Ser	Cys	Leu	Phe	Ser	Phe	Ile	Val	Ser	Gln	Cys	Cys	Gln	Glu	Gly															
65					70					75					80															
Ala	Gly	Gly	Ser	Leu	Asp	Leu	Val	Cys	Gln	Arg	Phe	Leu	Arg	Ser	Gly															
				85					90					95																
Pro	Asn	Ser	Leu	His	Cys	Leu	Gly	Ser	Leu	Arg	Glu	Arg	Leu	Ile	Ile															
			100					105						110																
Trp	Ala	Ala	Met	Asp	Ser	Ile	Pro	Ala	Pro	Ser	Ser	Val	Gln	Gly	His															
	115						120						125																	
Asn	Leu	Thr	Glu	Asp	Ala	Arg	His	Pro	Glu	Ser	Trp	Gln	Asn	Thr	Gly															
	130					135					140																			
Gly	Tyr	Ser	Glu	Gly	Asp	Ala	Val	Ser	Gln	Pro	Gln	Met	Ala	Leu	Glu															
145					150					155					160															
Glu	Val	Ser	Val	Ser	Asp	Pro	Leu	Ala	Ser	Asn	Gln	Gly	Gln	Ser	Leu															
				165					170					175																
Pro	Gly	Ser	Ser	Arg	Glu	His	Met	Ala	Gln	Trp	Glu	Val	Arg	Ser	Gln															
			180					185					190																	
Thr	His	Val	Pro	Asn	Arg	Glu	Pro	Val	Gln	Ala	Leu	Pro	Ser	Ser	Ala															
		195					200					205																		
Ser	Arg	Lys	Arg	Leu	Asp	Lys	Lys	Arg	Ser	Val	Pro	Val	Ala	Thr	Val															
	210					215					220																			
Glu	Leu	Glu	Glu	Lys	Arg	Phe	Arg	Thr	Leu	Pro	Leu	Val	Pro	Ser	Pro															
225					230					235					240															
Leu	Gln	Gly	Leu	Thr	Asn	Gln	Asp	Leu	Gln	Glu	Gly	Glu	Asp	Trp	Glu															
				245					250					255																
Gln	Glu	Asp	Glu	Asp	Met	Asp	Pro	Arg	Leu	Glu	His	Ser	Ser	Ser	Val															
			260					265					270																	

Gln Glu Asp Ser Glu Ser Pro Ser Pro Glu Asp Ile Pro Asp Tyr Leu
275 280 285

Leu Gln Tyr Arg Ala Ile His Ser Ala Glu Gln Gln His Ala Tyr Glu
290 295 300

Gln Asp Phe Glu Thr Asp Tyr Ala Glu Tyr Arg Ile Leu His Ala Arg
305 310 315 320

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

Arg Val Arg Arg Gly Thr Pro Glu Tyr Lys Val Leu Glu Asp Lys Ile
340 345 350

Ile Gln Glu Tyr Lys Lys Phe Arg Lys Gln Tyr Pro Ser Tyr Arg Glu
355 360 365

Glu Lys Arg Arg Cys Glu Tyr Leu His Gln Lys Leu Ser His Ile Lys
370 375 380

Gly Leu Ile Leu Glu Phe Glu Glu Lys Asn Arg Gly Ser
385 390 395

<210> 9
<211> 190
<212> PRT
<213> Homo sapiens

<400> 9

Met Glu Glu Asn His Ser Val Ser His Lys Lys Ser Lys Lys Lys Ser
1 5 10 15

Lys Lys His Lys Glu Lys Asp Gln Ile Lys Lys His Asp Ile Glu Thr
20 25 30

Ile Glu Glu Lys Glu Lys Asp Leu Lys Arg Glu Glu Glu Ile Ala Lys
35 40 45

Leu Asn Asn Ser Ser Pro Asn Ser Ser Gly Gly Ala Lys Glu Asp Cys
50 55 60

Thr Ala Ser Met Glu Pro Ser Ala Ile Glu Leu Pro Asp Tyr Leu Ile
65 70 75 80

Lys Tyr Ile Ala Ile Ile Ser Tyr Glu Gln Arg Arg Asn Tyr Lys Asp
85 90 95

Asp Val Ser Ala Glu Tyr Asp Glu Tyr Arg Ala Leu His Ala Arg Met
 100 105 110

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

Leu Ser Pro Gly Ser Lys Glu Tyr Gln Asn Val His Glu Glu Val Leu
 130 135 140

Gln Glu Tyr Arg Lys Ile Lys Gln Ser Ser Pro Asn Tyr His Glu Glu
 145 150 155 160

Lys Tyr Arg Cys Glu Tyr Leu His Asn Lys Leu Ala His Ile Lys Arg
 165 170 175

Leu Leu Gly Glu Phe Asp Gln Gln Gln Ala Glu Ser Trp Pro
 180 185 190

<210> 10
 <211> 646
 <212> PRT
 <213> Homo sapiens

<400> 10

Met Ala Ala Glu Ser Gly Ser Asp Phe Gln Gln Arg Arg Arg Arg Arg
 1 5 10 15

Arg Asp Pro Glu Glu Pro Glu Lys Thr Glu Leu Ser Glu Arg Glu Leu
 20 25 30

Ala Val Ala Val Ala Val Ser Gln Glu Asn Asp Glu Glu Asn Glu Glu
 35 40 45

Arg Trp Val Gly Pro Leu Pro Val Glu Ala Thr Leu Ala Lys Lys Arg
 50 55 60

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

Ser Met Tyr Glu Arg Ser Tyr Met His Arg Asp Val Ile Thr His Val
 85 90 95

Val Cys Thr Lys Thr Asp Phe Ile Ile Thr Ala Ser His Asp Gly His
 100 105 110

Val Lys Phe Trp Lys Lys Ile Glu Glu Gly Ile Glu Phe Val Lys His

115	120	125
Phe Arg Ser His Leu Gly Val Ile Glu Ser Ile Ala Val Ser Ser Glu		
130	135	140
Gly Ala Leu Phe Cys Ser Val Gly Asp Asp Lys Ala Met Lys Val Phe		
145	150	155
Asp Val Val Asn Phe Asp Met Ile Asn Met Leu Lys Leu Gly Tyr Phe		
	165	170
Pro Gly Gln Cys Glu Trp Ile Tyr Cys Pro Gly Asp Ala Ile Ser Ser		
	180	185
Val Ala Ala Ser Glu Lys Ser Thr Gly Lys Ile Phe Ile Tyr Asp Gly		
	195	200
Arg Gly Asp Asn Gln Pro Leu His Ile Phe Asp Lys Leu His Thr Ser		
	210	215
Pro Leu Thr Gln Ile Arg Leu Asn Pro Val Tyr Lys Ala Val Val Ser		
225	230	235
Ser Asp Lys Ser Gly Met Ile Glu Tyr Trp Thr Gly Pro Pro His Glu		
	245	250
Tyr Lys Phe Pro Lys Asn Val Asn Trp Glu Tyr Lys Thr Asp Thr Asp		
	260	265
Leu Tyr Glu Phe Ala Lys Cys Lys Ala Tyr Pro Thr Ser Val Cys Phe		
	275	280
Ser Pro Asp Gly Lys Lys Ile Ala Thr Ile Gly Ser Asp Arg Lys Val		
	290	295
Arg Ile Phe Arg Phe Val Thr Gly Lys Leu Met Arg Val Phe Asp Glu		
305	310	315
Ser Leu Ser Met Phe Thr Glu Leu Gln Gln Met Arg Gln Gln Leu Pro		
	325	330
Asp Met Glu Phe Gly Arg Arg Met Ala Val Glu Arg Glu Leu Glu Lys		
	340	345
Val Asp Ala Val Arg Leu Ile Asn Ile Val Phe Asp Glu Thr Gly His		
	355	360
		365

Phe Val Leu Tyr Gly Thr Met Leu Gly Ile Lys Val Ile Asn Val Glu
 370 375 380

Thr Asn Arg Cys Val Arg Ile Leu Gly Lys Gln Glu Asn Ile Arg Val
 385 390 395 400

Met Gln Leu Ala Leu Phe Gln Gly Ile Ala Lys Lys His Arg Ala Ala
 405 410 415

Thr Thr Ile Glu Met Lys Ala Ser Glu Asn Pro Val Leu Gln Asn Ile
 420 425 430

Gln Ala Asp Pro Thr Ile Val Cys Thr Ser Phe Lys Lys Asn Arg Phe
 435 440 445

Tyr Met Phe Thr Lys Arg Glu Pro Glu Asp Thr Lys Ser Ala Asp Ser
 450 455 460

Asp Arg Asp Val Phe Asn Glu Lys Pro Ser Lys Glu Glu Val Met Ala
 465 470 475 480

Ala Thr Gln Ala Glu Gly Pro Lys Arg Val Ser Asp Ser Ala Ile Ile
 485 490 495

His Thr Ser Met Gly Asp Ile His Thr Lys Leu Phe Pro Val Glu Cys
 500 505 510

Pro Lys Thr Val Glu Asn Phe Cys Val His Ser Arg Asn Gly Tyr Tyr
 515 520 525

Asn Gly His Thr Phe His Arg Ile Ile Lys Gly Phe Met Ile Gln Thr
 530 535 540

Gly Asp Pro Thr Gly Thr Gly Met Gly Gly Glu Ser Ile Trp Gly Gly
 545 550 555 560

Glu Phe Glu Asp Glu Phe His Ser Thr Leu Arg His Asp Arg Pro Tyr
 565 570 575

Thr Leu Ser Met Ala Asn Ala Gly Ser Asn Thr Asn Gly Ser Gln Phe
 580 585 590

Phe Ile Thr Val Val Pro Thr Pro Trp Leu Asp Asn Lys His Thr Val
 595 600 605

Phe Gly Arg Val Thr Lys Gly Met Glu Val Val Gln Arg Ile Ser Asn

[illegible]