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(54) Title: PAPSSs AS MODIFIERS OF THE AXIN PATHWAY AND METHODS OF USE

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



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## PAPSSs AS MODIFIERS OF THE AXIN PATHWAY AND METHODS OF USE

### REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. provisional patent application 60/401,534  
5 filed 8/6/2002. The content of the prior application is hereby incorporated in its entirety.

### BACKGROUND OF THE INVENTION

Deregulation of beta-catenin signaling is a frequent and early event in the development of a variety of human tumors, including colon cancer, melanoma, ovarian  
10 cancer, and prostate cancer. Activation of beta-catenin signaling can occur in tumor cells by loss-of-function mutations in the tumor suppressor genes Axin or APC, as well as by gain-of-function mutations in the oncogene beta-catenin itself. Axin normally functions as a scaffolding protein that binds beta-catenin, APC, and the serine/threonine kinase GSK3-beta. Assembly of this degradation complex allows GSK3-beta to phosphorylate beta-  
15 catenin, which leads to beta-catenin ubiquitination and degradation by the proteasome. In the absence of Axin activity, beta-catenin protein becomes stabilized and accumulates in the nucleus where it acts as a transcriptional co-activator with TCF for the induction of target genes, including the cell cycle regulators cyclin D1 and c-Myc.

The *C. elegans* gene *pry-1* is the structural and functional ortholog of vertebrate  
20 Axin (Korswagen HC et al. (2002) Genes Dev. 16:1291-302). PRY-1 is predicted to contain conserved RGS and DIX domains that, in Axin, bind APC and Dishevelled, respectively. Overexpression of the *C. elegans pry-1* gene in zebrafish can fully rescue the mutant phenotype of *masterblind*, the zebrafish Axin1 mutation. *pry-1* loss-of-function mutations produce several phenotypes that appear to result from increased beta-  
25 catenin signaling (Gleason JE et al. (2002) Genes Dev. 16:1281-90; Korswagen et al., *supra*).

Three-prime-phosphoadenosine 5-prime-phosphosulfate (PAPS) is the sulfate donor cosubstrate for all sulfotransferase (SULT) enzymes. SULTs catalyze the sulfate conjugation of many endogenous and exogenous compounds, including drugs and other  
30 xenobiotics. In humans, PAPS is synthesized from adenosine 5-prime triphosphate (ATP) and inorganic sulfate by 2 isoforms, phosphoadenosine-phosphosulfate synthetase 1 (PAPSS1) and phosphoadenosine-phosphosulfate synthetase 2 (PAPSS2). PAPSS enzymes have kinase and sulfurylase domains, and catalyze 2 sequential reactions to synthesize PAPS. These reactions are catalyzed by separate enzymes encoded by 2 or 3

genes in simpler organisms. Mutations in PAPSS2 may result in spondyloepimetaphyseal dysplasia (ul Haque, M. F (1998) Nat Genet 20:157-62), and PAPSS2 is expressed in metastatic and non-metastatic colon carcinoma cells (Franzon, V. L. et al (1999) Int J Biochem Cell Biol 31:613-26).

5            PAPSS genes are conserved through evolution and have orthologs in yeast and higher eukaryotes, including a marine worm, *Drosophila*, and mouse (Yanagisawa, K. et al (1998) Biosci. Biotech. Biochem. 62: 1037-1040).

          The ability to manipulate the genomes of model organisms such as *C. elegans* provides a powerful means to analyze biochemical processes that, due to significant  
10        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  
15        correlative pathways and methods of modulating them in mammals (see, for example Dulubova I, et al, J Neurochem 2001 Apr;77(1):229-38; Cai T, et al., Diabetologia 2001 Jan;44(1):81-8; Pasquinelli AE, et al., Nature. 2000 Nov 2;408(6808):37-8; Ivanov IP, et al., EMBO J 2000 Apr 17;19(8):1907-17; Vajo Z et al., Mamm Genome 1999 Oct;10(10):1000-4). For example, a genetic screen can be carried out in an invertebrate  
20        model organism having underexpression (e.g. knockout) or overexpression of a gene (referred to as a “genetic entry point”) that yields a visible phenotype. 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  
25        the genetic entry point is an ortholog of a human gene implicated in a disease pathway, such as AXIN, 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  
30        their entireties.

## SUMMARY OF THE INVENTION

          We have discovered genes that modify the AXIN pathway in *C. elegans*, and identified their human orthologs, hereinafter referred to as phosphoadenosine-

phosphosulfate synthetase (PAPSS). The invention provides methods for utilizing these AXIN modifier genes and polypeptides to identify PAPSS-modulating agents that are candidate therapeutic agents that can be used in the treatment of disorders associated with defective or impaired AXIN function and/or PAPSS function. Preferred PAPSS-

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

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

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

The invention further provides methods for modulating the PAPSS function and/or the AXIN pathway in a mammalian cell by contacting the mammalian cell with an agent that specifically binds a PAPSS 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 the AXIN pathway.

### DETAILED DESCRIPTION OF THE INVENTION

5 Genetic screens were designed to identify modifiers of the axin pathway in *C. elegans*, where a reduction of function *pry-1* (*axin*) mutant was used. Various specific genes were silenced by RNA inhibition (RNAi). The T14G10.1 gene was identified as a modifier of the AXIN pathway. Accordingly, vertebrate orthologs of these modifiers, and preferably the human orthologs, PAPSS genes (i.e., nucleic acids and polypeptides) are  
10 attractive drug targets for the treatment of pathologies associated with a defective AXIN signaling pathway, such as cancer.

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

20

#### **Nucleic acids and polypeptides of the invention**

Sequences related to PAPSS nucleic acids and polypeptides that can be used in the invention are disclosed in Genbank (referenced by Genbank identifier (GI) number) as GI#s 20127474 (SEQ ID NO:1), 15030251 (SEQ ID NO:2), 2853266 (SEQ ID NO:3),  
25 7211187 (SEQ ID NO:4), 2673861 (SEQ ID NO:5), 7211174 (SEQ ID NO:6), 4758879 (SEQ ID NO:7), 14602765 (SEQ ID NO:8), 5052074 (SEQ ID NO:9), and 3769609 (SEQ ID NO:10) for nucleic acid, and GI#s 20127475 (SEQ ID NO:11) and 4758880 (SEQ ID NO:12) for polypeptides.

The term "PAPSS polypeptide" refers to a full-length PAPSS protein or a  
30 functionally active fragment or derivative thereof. A "functionally active" PAPSS fragment or derivative exhibits one or more functional activities associated with a full-length, wild-type PAPSS protein, such as antigenic or immunogenic activity, enzymatic activity, ability to bind natural cellular substrates, etc. The functional activity of PAPSS 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 PAPSS polypeptide is a PAPSS derivative capable of rescuing defective endogenous PAPSS 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 a PAPSS, such as a kinase domain or a binding domain. Protein domains can be identified using the PFAM program (Bateman A., et al., Nucleic Acids Res, 1999, 27:260-2). For example, the APS kinase domain (PFAM 1583) of PAPSS from GI#s 20127475 4758880 (SEQ ID NOs:11 and 12, respectively) is located at approximately amino acid residues 51 to 209 and 41-199. Methods for obtaining PAPSS polypeptides are also further described below. In some embodiments, preferred fragments are functionally active, domain-containing fragments comprising at least 25 contiguous amino acids, preferably at least 50, more preferably 75, and most preferably at least 100 contiguous amino acids of any one of SEQ ID NOs:11 and 12 (a PAPSS). In further preferred embodiments, the fragment comprises the entire kinase (functionally active) domain.

The term "PAPSS nucleic acid" refers to a DNA or RNA molecule that encodes a PAPSS polypeptide. Preferably, the PAPSS polypeptide or nucleic acid or fragment thereof is from a human, but can also be an ortholog, or derivative thereof with at least 70% sequence identity, preferably at least 80%, more preferably 85%, still more preferably 90%, and most preferably at least 95% sequence identity with human PAPSS. Methods of identifying orthologs are known in the art. Normally, orthologs in different species retain the same function, due to presence of one or more protein motifs and/or 3-dimensional structures. Orthologs are generally identified by sequence homology analysis, such as BLAST analysis, usually using protein bait sequences. Sequences are assigned as a potential ortholog if the best hit sequence from the forward BLAST result retrieves the original query sequence in the reverse BLAST (Huynen MA and Bork P, Proc Natl Acad Sci (1998) 95:5849-5856; Huynen MA *et al.*, Genome Research (2000) 10:1204-1210). Programs for multiple sequence alignment, such as CLUSTAL (Thompson JD et al, 1994, Nucleic Acids Res 22:4673-4680) may be used to highlight conserved regions and/or residues of orthologous proteins and to generate phylogenetic trees. In a phylogenetic tree representing multiple homologous sequences from diverse species (e.g., retrieved through BLAST analysis), orthologous sequences from two species

generally appear closest on the tree with respect to all other sequences from these two species. Structural threading or other analysis of protein folding (e.g., using software by ProCeryon, Biosciences, Salzburg, Austria) may also identify potential orthologs. In evolution, when a gene duplication event follows speciation, a single gene in one species, such as *C. elegans*, may correspond to multiple genes (paralogs) in another, such as human. As used herein, the term "orthologs" encompasses paralogs. As used herein, "percent (%) sequence identity" with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of nucleotides or amino acids in the candidate derivative sequence identical with the nucleotides or amino acids in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul *et al.*, J. Mol. Biol. (1997) 215:403-410) with all the search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. A % identity value is determined by the number of matching identical nucleotides or amino acids divided by the sequence length for which the percent identity is being reported. "Percent (%) amino acid sequence similarity" is determined by doing the same calculation as for determining % amino acid sequence identity, but including conservative amino acid substitutions in addition to identical amino acids in the computation.

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

Alternatively, an alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman (Smith and Waterman, 1981, Advances in Applied Mathematics 2:482-489; database: European Bioinformatics Institute; Smith and Waterman, 1981, J. of Molec.Biol., 147:195-197; Nicholas *et al.*, 1998, "A Tutorial on Searching Sequence Databases and Sequence Scoring Methods" ([www.psc.edu](http://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  
5 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  
10 sequences that hybridize to the nucleic acid sequence of any of SEQ ID NOs:1-10. 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);  
15 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 any one of SEQ ID NOs:1-10 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  
20 (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).

25 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),  
30 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  $\mu$ g/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.

5 **Isolation, Production, Expression, and Mis-expression of PAPSS Nucleic Acids and Polypeptides**

PAPSS nucleic acids and polypeptides, are useful for identifying and testing agents that modulate PAPSS function and for other applications related to the involvement of PAPSS in the AXIN pathway. PAPSS nucleic acids and derivatives and orthologs thereof  
10 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  
15 require methods that preserve specific biological activities of these proteins, whereas production of proteins for antibody generation may require structural integrity of particular epitopes. Expression of proteins to be purified for screening or antibody production may require the addition of specific tags (*e.g.*, generation of fusion proteins). Overexpression of a PAPSS protein for assays used to assess PAPSS function, such as involvement in cell  
20 cycle regulation or hypoxic response, may require expression in eukaryotic cell lines capable of these cellular activities. Techniques for the expression, production, and purification of proteins are well known in the art; any suitable means therefore may be used (*e.g.*, Higgins SJ and Hames BD (eds.) Protein Expression: A Practical Approach, Oxford University Press Inc., New York 1999; Stanbury PF et al., Principles of  
25 Fermentation Technology, 2<sup>nd</sup> edition, Elsevier Science, New York, 1995; Doonan S (ed.) Protein Purification Protocols, Humana Press, New Jersey, 1996; Coligan JE et al, Current Protocols in Protein Science (eds.), 1999, John Wiley & Sons, New York). In particular embodiments, recombinant PAPSS is expressed in a cell line known to have defective AXIN function. The recombinant cells are used in cell-based screening assay systems of  
30 the invention, as described further below.

The nucleotide sequence encoding a PAPSS polypeptide can be inserted into any appropriate expression vector. The necessary transcriptional and translational signals, including promoter/enhancer element, can derive from the native PAPSS gene and/or its flanking regions or can be heterologous. A variety of host-vector expression systems may

be utilized, such as mammalian cell systems infected with virus (*e.g.* vaccinia virus, adenovirus, *etc.*); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, plasmid, or cosmid DNA. An isolated host cell strain that modulates the expression of, modifies, and/or specifically processes the gene product may be used.

To detect expression of the PAPSS gene product, the expression vector can comprise a promoter operably linked to a PAPSS 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 PAPSS gene product based on the physical or functional properties of the PAPSS protein in *in vitro* assay systems (*e.g.* immunoassays).

The PAPSS 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 PAPSS 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 PAPSS 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 PAPSS or other genes associated with the AXIN 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 PAPSS expression may be used in *in vivo* assays to test for activity of a candidate AXIN modulating agent, or to further assess the role of PAPSS in a AXIN pathway process such as apoptosis or cell proliferation. Preferably, the altered PAPSS expression results in a detectable phenotype, such as decreased or increased levels of cell proliferation, angiogenesis, or apoptosis compared to control animals having normal PAPSS expression. The genetically modified animal may additionally have altered AXIN expression (e.g. AXIN knockout). Preferred genetically modified animals are mammals such as primates, rodents (preferably mice or rats), among others. Preferred non-mammalian species include zebrafish, *C. elegans*, and *Drosophila*. Preferred genetically modified animals are transgenic animals having a heterologous nucleic acid sequence present as an extrachromosomal element in a portion of its cells, i.e. mosaic animals (see, for example, techniques described by Jakobovits, 1994, Curr. Biol. 4:761-763.) or stably integrated into its germ line DNA (i.e., in the genomic sequence of most or all of its cells). Heterologous nucleic acid is introduced into the germ line of such transgenic animals by genetic manipulation of, for example, embryos or embryonic stem cells of the host animal.

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

according to available methods (see Wilmut, I. *et al.* (1997) *Nature* 385:810-813; and PCT International Publication Nos. WO 97/07668 and WO 97/07669).

In one embodiment, the transgenic animal is a "knock-out" animal having a heterozygous or homozygous alteration in the sequence of an endogenous PAPSS gene that results in a decrease of PAPSS function, preferably such that PAPSS expression is undetectable or insignificant. Knock-out animals are typically generated by homologous recombination with a vector comprising a transgene having at least a portion of the gene to be knocked out. Typically a deletion, addition or substitution has been introduced into the transgene to functionally disrupt it. The transgene can be a human gene (e.g., from a human genomic clone) but more preferably is an ortholog of the human gene derived from the transgenic host species. For example, a mouse PAPSS gene is used to construct a homologous recombination vector suitable for altering an endogenous PAPSS 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 PAPSS gene, e.g., by introduction of additional copies of PAPSS, or by operatively inserting a regulatory sequence that provides for altered expression of an endogenous copy of the PAPSS 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. 5 (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 10 the AXIN pathway, as animal models of disease and disorders implicating defective AXIN 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 PAPSS function and phenotypic changes are compared with appropriate control animals such as genetically modified animals that 15 receive placebo treatment, and/or animals with unaltered PAPSS expression that receive candidate therapeutic agent.

In addition to the above-described genetically modified animals having altered PAPSS function, animal models having defective AXIN function (and otherwise normal PAPSS function), can be used in the methods of the present invention. For example, a 20 AXIN knockout mouse can be used to assess, *in vivo*, the activity of a candidate AXIN modulating agent identified in one of the *in vitro* assays described below. Preferably, the candidate AXIN modulating agent when administered to a model system with cells defective in AXIN function, produces a detectable phenotypic change in the model system indicating that the AXIN function is restored, i.e., the cells exhibit normal cell cycle 25 progression.

### **Modulating Agents**

The invention provides methods to identify agents that interact with and/or modulate the function of PAPSS and/or the AXIN pathway. Modulating agents identified 30 by the methods are also part of the invention. Such agents are useful in a variety of diagnostic and therapeutic applications associated with the AXIN pathway, as well as in further analysis of the PAPSS protein and its contribution to the AXIN pathway. Accordingly, the invention also provides methods for modulating the AXIN pathway

comprising the step of specifically modulating PAPSS activity by administering a PAPSS-interacting or -modulating agent.

As used herein, a "PAPSS-modulating agent" is any agent that modulates PAPSS function, for example, an agent that interacts with PAPSS to inhibit or enhance PAPSS activity or otherwise affect normal PAPSS function. PAPSS function can be affected at any level, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In a preferred embodiment, the PAPSS - modulating agent specifically modulates the function of the PAPSS. The phrases "specific modulating agent", "specifically modulates", etc., are used herein to refer to modulating agents that directly bind to the PAPSS polypeptide or nucleic acid, and preferably inhibit, enhance, or otherwise alter, the function of the PAPSS. These phrases also encompass modulating agents that alter the interaction of the PAPSS with a binding partner, substrate, or cofactor (e.g. by binding to a binding partner of a PAPSS, or to a protein/binding partner complex, and altering PAPSS function). In a further preferred embodiment, the PAPSS- modulating agent is a modulator of the AXIN pathway (e.g. it restores and/or upregulates AXIN function) and thus is also a AXIN-modulating agent.

Preferred PAPSS-modulating agents include small molecule compounds; PAPSS-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 excipients. Techniques for formulation and administration of the compounds may be found in "Remington's Pharmaceutical Sciences" Mack Publishing Co., Easton, PA, 19<sup>th</sup> edition.

25

### **Small molecule modulators**

Small molecules are often preferred to modulate function of proteins with enzymatic function, and/or containing protein interaction domains. Chemical agents, referred to in the art as "small molecule" compounds are typically organic, non-peptide molecules, having a molecular weight less than 10,000, preferably less than 5,000, more preferably less than 1,000, and most preferably less than 500 daltons. This class of modulators includes chemically synthesized molecules, for instance, compounds from combinatorial chemical libraries. Synthetic compounds may be rationally designed or identified based on known or inferred properties of the PAPSS 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 be identified by screening compound libraries for PAPSS-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 designed, optimized, and synthesized. Such clinical compounds may have utility in treating pathologies associated with the AXIN pathway. The activity of candidate small molecule modulating agents may be improved several-fold through iterative secondary functional validation, as further described below, structure determination, and candidate modulator modification and testing. Additionally, candidate clinical compounds are generated with specific regard to clinical and pharmacological properties. For example, the reagents may be derivatized and re-screened using *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development.

### Protein Modulators

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

A PAPSS-interacting protein may be endogenous, i.e. one that naturally interacts genetically or biochemically with a PAPSS, such as a member of the PAPSS pathway that modulates PAPSS expression, localization, and/or activity. PAPSS-modulators include dominant negative forms of PAPSS-interacting proteins and of PAPSS proteins themselves. Yeast two-hybrid and variant screens offer preferred methods for identifying endogenous PAPSS-interacting proteins (Finley, R. L. et al. (1996) in DNA Cloning-Expression Systems: A Practical Approach, eds. Glover D. & Hames B. D (Oxford University Press, Oxford, England), pp. 169-203; Fashema SF et al., Gene (2000) 250:1-

14; Drees BL Curr Opin Chem Biol (1999) 3:64-70; Vidal M and Legrain P Nucleic Acids Res (1999) 27:919-29; and U.S. Pat. No. 5,928,868). Mass spectrometry is an alternative preferred method for the elucidation of protein complexes (reviewed in, e.g., Pandley A and Mann M, Nature (2000) 405:837-846; Yates JR 3<sup>rd</sup>, Trends Genet (2000) 16:5-8).

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

10 In preferred embodiments, a PAPSS-interacting protein specifically binds a PAPSS protein. In alternative preferred embodiments, a PAPSS-modulating agent binds a PAPSS substrate, binding partner, or cofactor.

### *Antibodies*

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

20 Antibodies that specifically bind PAPSS polypeptides can be generated using known methods. Preferably the antibody is specific to a mammalian ortholog of PAPSS polypeptide, and more preferably, to human PAPSS. Antibodies may be polyclonal, monoclonal (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')<sub>2</sub> fragments, fragments produced by a FAb expression library, anti-  
25 idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Epitopes of PAPSS which are particularly antigenic can be selected, for example, by routine screening of PAPSS polypeptides for antigenicity or by applying a theoretical method for selecting antigenic regions of a protein (Hopp and Wood (1981), Proc. Natl. Acad. Sci. U.S.A. 78:3824-28; Hopp and Wood, (1983) Mol. Immunol. 20:483-89;  
30 Sutcliffe et al., (1983) Science 219:660-66) to the amino acid sequence shown in SEQ ID NOs:11 or 12. Monoclonal antibodies with affinities of  $10^8 \text{ M}^{-1}$  preferably  $10^9 \text{ M}^{-1}$  to  $10^{10} \text{ M}^{-1}$ , or stronger can be made by standard procedures as described (Harlow and Lane, *supra*; Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York; and U.S. Pat. Nos. 4,381,292; 4,451,570; and 4,618,577). Antibodies

may be generated against crude cell extracts of PAPSS or substantially purified fragments thereof. If PAPSS fragments are used, they preferably comprise at least 10, and more preferably, at least 20 contiguous amino acids of a PAPSS protein. In a particular embodiment, PAPSS-specific 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.

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

          Chimeric antibodies specific to PAPSS polypeptides can be made that contain different portions from different animal species. For instance, a human immunoglobulin constant region may be linked to a variable region of a murine mAb, such that the antibody derives its biological activity from the human antibody, and its binding specificity from the murine fragment. Chimeric antibodies are produced by splicing together genes that encode the appropriate regions from each species (Morrison et al., 15   Proc. Natl. Acad. Sci. (1984) 81:6851-6855; Neuberger et al., Nature (1984) 312:604-608; Takeda et al., Nature (1985) 31:452-454). Humanized antibodies, which are a form of chimeric antibodies, can be generated by grafting complementary-determining regions (CDRs) (Carlos, T. M., J. M. Harlan. 1994. Blood 84:2068-2101) of mouse antibodies into a background of human framework regions and constant regions by recombinant DNA technology (Riechmann LM, et al., 1988 Nature 323: 323-327). Humanized 25   antibodies contain ~10% murine sequences and ~90% human sequences, and thus further reduce or eliminate immunogenicity, while retaining the antibody specificities (Co MS, and Queen C. 1991 Nature 351: 501-501; Morrison SL. 1992 Ann. Rev. Immun. 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). 30

          PAPSS-specific single chain antibodies which are recombinant, single chain polypeptides formed by linking the heavy and light chain fragments of the Fv regions via an amino acid bridge, can be produced by methods known in the art (U.S. Pat. No.

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  
5 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  
10 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  
15 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  
20 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  
25 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  
30 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**

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

In one embodiment, the antisense oligomer is an oligonucleotide that is sufficiently complementary to a PAPSS mRNA to bind to and prevent translation, preferably by binding to the 5' untranslated region. PAPSS-specific antisense oligonucleotides,  
15 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  
20 modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, agents that facilitate transport across the cell membrane, hybridization-triggered cleavage agents, and intercalating agents.

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

Alternative preferred PAPSS nucleic acid modulators are double-stranded RNA species mediating RNA interference (RNAi). RNAi is the process of sequence-specific,

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

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

### Assay Systems

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

modulating agent affects PAPSS in a manner relevant to the AXIN pathway. In some cases, PAPSS modulators will be directly tested in a secondary assay.

In a preferred embodiment, the screening method comprises contacting a suitable assay system comprising a PAPSS 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. kinase activity), which is based on the particular molecular event the screening method detects. A statistically significant difference between the agent-biased activity and the reference activity indicates that the candidate agent modulates PAPSS activity, and hence the AXIN pathway. The PAPSS polypeptide or nucleic acid used in the assay may comprise any of the nucleic acids or polypeptides described above.

### **Primary Assays**

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

#### ***Primary assays for small molecule modulators***

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

Cell-based screening assays usually require systems for recombinant expression of PAPSS and any auxiliary proteins demanded by the particular assay. Appropriate methods for generating recombinant proteins produce sufficient quantities of proteins that retain

their relevant biological activities and are of sufficient purity to optimize activity and 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 PAPSS-interacting proteins are used in screens to identify small molecule modulators, the binding specificity of the interacting protein to the PAPSS protein may be assayed by various known methods such as substrate processing (e.g. ability of the candidate PAPSS-specific binding agents to function as negative effectors in PAPSS-expressing cells), binding equilibrium constants (usually at least about  $10^7 \text{ M}^{-1}$ , preferably at least about  $10^8 \text{ M}^{-1}$ , more preferably at least about  $10^9 \text{ M}^{-1}$ ), and immunogenicity (e.g. ability to elicit PAPSS specific antibody in a heterologous host such as a mouse, rat, goat or rabbit). For enzymes and receptors, binding may be assayed by, respectively, substrate and ligand processing.

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

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

A variety of suitable assay systems may be used to identify candidate PAPSS and AXIN pathway modulators (e.g. U.S. Pat. No. 6,165,992 (kinase assays); U.S. Pat. Nos. 5,550,019 and 6,133,437 (apoptosis assays); and U.S. Pat. Nos. 5,976,782, 6,225,118 and 6,444,434 (angiogenesis assays), among others). Specific preferred assays are described in more detail below.

**Kinase assays.** In some preferred embodiments the screening assay detects the ability of the test agent to modulate the kinase activity of a PAPSS polypeptide. In further embodiments, a cell-free kinase assay system is used to identify a candidate AXIN modulating agent, and a secondary, cell-based assay, such as an apoptosis or hypoxic induction assay (described below), may be used to further characterize the candidate AXIN modulating agent. Many different assays for kinases have been reported in the literature and are well known to those skilled in the art (e.g. U.S. Pat. No. 6,165,992; Zhu et al., *Nature Genetics* (2000) 26:283-289; and WO0073469). Radioassays, which monitor the transfer of a gamma phosphate are frequently used. For instance, a scintillation assay for p56 (lck) kinase activity monitors the transfer of the gamma phosphate from gamma -<sup>33</sup>P ATP to a biotinylated peptide substrate; the substrate is captured on a streptavidin coated bead that transmits the signal (Beveridge M *et al.*, *J Biomol Screen* (2000) 5:205-212). This assay uses the scintillation proximity assay (SPA), in which only radio-ligand bound to receptors tethered to the surface of an SPA bead are detected by the scintillant immobilized within it, allowing binding to be measured without separation of bound from free ligand.

Other assays for protein kinase activity may use antibodies that specifically recognize phosphorylated substrates. For instance, the kinase receptor activation (KIRA) assay measures receptor tyrosine kinase activity by ligand stimulating the intact receptor in cultured cells, then capturing solubilized receptor with specific antibodies and quantifying phosphorylation via phosphotyrosine ELISA (Sadick MD, *Dev Biol Stand* (1999) 97:121-133).

Another example of antibody based assays for protein kinase activity is TRF (time-resolved fluorometry). This method utilizes europium chelate-labeled anti-phosphotyrosine antibodies to detect phosphate transfer to a polymeric substrate coated onto microtiter plate wells. The amount of phosphorylation is then detected using time-resolved, dissociation-enhanced fluorescence (Braunwalder AF, et al., *Anal Biochem* 1996 Jul 1;238(2):159-64).

**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 a PAPSS, and that optionally has defective AXIN function (e.g. AXIN 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 AXIN modulating agents. In some embodiments of the invention, an apoptosis assay may be used as a secondary assay to test a candidate AXIN modulating agents that is initially identified using a cell-free assay system. An apoptosis assay may also be used to test whether PAPSS function plays a direct role in apoptosis. For example, an apoptosis assay may be performed on cells that over- or under-express PAPSS relative to wild type cells. Differences in apoptotic response compared to wild type cells suggests that the PAPSS plays a direct role in the apoptotic response. Apoptosis assays are described further in US Pat. No. 6,133,437.

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

Cell proliferation is also assayed via phospho-histone H3 staining, which identifies a cell population undergoing mitosis by phosphorylation of histone H3. Phosphorylation of histone H3 at serine 10 is detected using an antibody specific to the phosphorylated form of the serine 10 residue of histone H3. (Chadlee, D.N. 1995, *J. Biol. Chem* 270:20098-105). Cell Proliferation may also be examined using [<sup>3</sup>H]-thymidine incorporation (Chen, J., 1996, *Oncogene* 13:1395-403; Jeoung, J., 1995, *J. Biol. Chem.* 270:18367-73). This assay allows for quantitative characterization of S-phase DNA syntheses. In this assay, cells synthesizing DNA will incorporate [<sup>3</sup>H]-thymidine into newly synthesized DNA. Incorporation can then be measured by standard techniques such as by counting of radioisotope in a scintillation counter (e.g., Beckman LS 3800 Liquid Scintillation Counter). Another proliferation assay uses the dye Alamar Blue (available from Biosource International), which fluoresces when reduced in living cells and provides an indirect measurement of cell number (Voytik-Harbin SL *et al.*, 1998, *In Vitro Cell Dev Biol Anim* 34:239-46). Yet another proliferation assay, the MTS assay, is based on in vitro cytotoxicity assessment of industrial chemicals, and uses the soluble tetrazolium salt, MTS. MTS assays are commercially available, for example, the Promega CellTiter 96<sup>®</sup> 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 PAPSS are seeded in soft agar plates, and colonies are measured and counted after two weeks incubation.

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

Involvement of a gene in the cell cycle may be assayed by flow cytometry (Gray JW *et al.* (1986) *Int J Radiat Biol Relat Stud Phys Chem Med* 49:237-55). Cells transfected with a PAPSS 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 a PAPSS, and that optionally has defective AXIN function (e.g. AXIN is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the assay system and changes in cell proliferation or cell cycle relative to controls where no test agent is added, identify candidate AXIN 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 AXIN modulating agents that is initially identified using another assay system such as a cell-free assay system. A cell proliferation assay may also be used to test whether PAPSS 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 PAPSS relative to wild type cells. Differences in proliferation or cell cycle compared to wild type cells suggests that the PAPSS plays a direct role in cell proliferation or cell cycle.

**Angiogenesis.** Angiogenesis may be assayed using various human endothelial cell systems, such as umbilical vein, coronary artery, or dermal cells. Suitable assays include Alamar Blue based assays (available from Biosource International) to measure proliferation; migration assays using fluorescent molecules, such as the use of Becton Dickinson Falcon HTS FluoroBlock cell culture inserts to measure migration of cells through membranes in presence or absence of angiogenesis enhancer or suppressors; and tubule formation assays based on the formation of tubular structures by endothelial cells on Matrigel® (Becton Dickinson). Accordingly, an angiogenesis assay system may comprise a cell that expresses a PAPSS, and that optionally has defective AXIN function (e.g. AXIN 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 AXIN modulating agents. In some embodiments of the invention, the angiogenesis assay may be used as a secondary assay to test a candidate AXIN modulating agents that is initially identified using another assay system. An angiogenesis assay may also be used to test whether PAPSS function plays a direct role in cell proliferation. For example, an angiogenesis assay may be performed on cells that over- or under-express PAPSS relative to wild type cells. Differences in angiogenesis compared to wild type cells suggests that the PAPSS plays a

direct role in angiogenesis. U.S. Pat. Nos. 5,976,782, 6,225,118 and 6,444,434, among others, describe various angiogenesis assays.

**Hypoxic induction.** The alpha subunit of the transcription factor, hypoxia inducible factor-1 (HIF-1), is upregulated in tumor cells following exposure to hypoxia in vitro. Under hypoxic conditions, HIF-1 stimulates the expression of genes known to be important in tumour cell survival, such as those encoding glycolytic enzymes and VEGF. Induction of such genes by hypoxic conditions may be assayed by growing cells transfected with PAPSS in hypoxic conditions (such as with 0.1% O<sub>2</sub>, 5% CO<sub>2</sub>, and balance N<sub>2</sub>, 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 a PAPSS, and that optionally has defective AXIN function (e.g. AXIN is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the hypoxic induction assay system and changes in hypoxic response relative to controls where no test agent is added, identify candidate AXIN modulating agents. In some embodiments of the invention, the hypoxic induction assay may be used as a secondary assay to test a candidate AXIN modulating agents that is initially identified using another assay system. A hypoxic induction assay may also be used to test whether PAPSS function plays a direct role in the hypoxic response. For example, a hypoxic induction assay may be performed on cells that over- or under-express PAPSS relative to wild type cells. Differences in hypoxic response compared to wild type cells suggests that the PAPSS plays a direct role in hypoxic induction.

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

membrane-permeable fluorescent dye, such as calcein-AM, and the signal is quantified in a fluorescent microplate reader.

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

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

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

angiogenic factors such as TNF- $\alpha$ . Thus, in a further preferred embodiment, a tubulogenesis assay system comprises testing a PAPSS's response to a variety of factors, such as FGF, VEGF, phorbol myristate acetate (PMA), TNF- $\alpha$ , ephrin, etc.

5           **Cell Migration.** An invasion/migration assay (also called a migration assay) tests the ability of cells to overcome a physical barrier and to migrate towards pro-angiogenic signals. Migration assays are known in the art (e.g., Paik JH et al., 2001, J Biol Chem 276:11830-11837). In a typical experimental set-up, cultured endothelial cells are seeded  
10 onto a matrix-coated porous lamina, with pore sizes generally smaller than typical cell size. The matrix generally simulates the environment of the extracellular matrix, as described above. The lamina is typically a membrane, such as the transwell polycarbonate membrane (Corning Costar Corporation, Cambridge, MA), and is generally part of an upper chamber that is in fluid contact with a lower chamber containing pro-angiogenic stimuli. Migration is generally assayed after an overnight incubation with stimuli, but  
15 longer or shorter time frames may also be used. Migration is assessed as the number of cells that crossed the lamina, and may be detected by staining cells with hemotoxylin solution (VWR Scientific, South San Francisco, CA), or by any other method for determining cell number. In another exemplary set up, cells are fluorescently labeled and migration is detected using fluorescent readings, for instance using the Falcon HTS  
20 FluoroBlok (Becton Dickinson). While some migration is observed in the absence of stimulus, migration is greatly increased in response to pro-angiogenic factors. As described above, a preferred assay system for migration/invasion assays comprises testing a PAPSS's response to a variety of pro-angiogenic factors, including tumor angiogenic and inflammatory angiogenic agents, and culturing the cells in serum free medium.

25

**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  
30 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 $\mu$ 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  $\mu$ 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 PAPSS 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 PAPSS-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 PAPSS gene expression, preferably mRNA expression. In general, expression analysis comprises comparing PAPSS expression in like populations of cells (*e.g.*, two pools of cells that endogenously or recombinantly express PAPSS) 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 PAPSS 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; 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 PAPSS protein or

specific peptides. A variety of means including Western blotting, ELISA, or in situ detection, are available (Harlow E and Lane D, 1988 and 1999, *supra*).

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

### Secondary Assays

Secondary assays may be used to further assess the activity of PAPSS-modulating agent identified by any of the above methods to confirm that the modulating agent affects PAPSS in a manner relevant to the AXIN pathway. As used herein, PAPSS-modulating agents encompass candidate clinical compounds or other agents derived from previously identified modulating agent. Secondary assays can also be used to test the activity of a modulating agent on a particular genetic or biochemical pathway or to test the specificity of the modulating agent's interaction with PAPSS.

Secondary assays generally compare like populations of cells or animals (*e.g.*, two pools of cells or animals that endogenously or recombinantly express PAPSS) in the presence and absence of the candidate modulator. In general, such assays test whether treatment of cells or animals with a candidate PAPSS-modulating agent results in changes in the AXIN 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 AXIN or interacting pathways.

### *Cell-based assays*

Cell based assays may detect endogenous AXIN pathway activity or may rely on recombinant expression of AXIN pathway components. Any of the aforementioned assays may be used in this cell-based format. Candidate modulators are typically added to the cell media but may also be injected into cells or delivered by any other efficacious means.

### *Animal Assays*

A variety of non-human animal models of normal or defective AXIN pathway may be used to test candidate PAPSS modulators. Models for defective AXIN pathway typically use genetically modified animals that have been engineered to mis-express (*e.g.*,

over-express or lack expression in) genes involved in the AXIN pathway. Assays generally require systemic delivery of the candidate modulators, such as by oral administration, injection, etc.

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

In another preferred embodiment, the effect of the candidate modulator on PAPSS is assessed via tumorigenicity assays. Tumor xenograft assays are known in the art (see, e.g., Ogawa K et al., 2000, *Oncogene* 19:6043-6052). Xenografts are typically implanted SC into female athymic mice, 6-7 week old, as single cell suspensions either from a pre-existing tumor or from *in vitro* culture. The tumors which express the PAPSS endogenously are injected in the flank,  $1 \times 10^5$  to  $1 \times 10^7$  cells per mouse in a volume of 100  $\mu$ 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 maybe utilized for biomarker identification or further analyses. For immunohistochemistry staining, xenograft tumors are fixed in 4% paraformaldehyde, 0.1M phosphate, pH 7.2, for 6 hours at 4°C, immersed in 30% sucrose in PBS, and rapidly frozen in isopentane cooled with liquid nitrogen.

In another preferred embodiment, tumorigenicity is monitored using a hollow fiber assay, which is described in U.S. Pat No. US 5,698,413. Briefly, the method comprises implanting into a laboratory animal a biocompatible, semi-permeable encapsulation device containing target cells, treating the laboratory animal with a candidate modulating agent, and evaluating the target cells for reaction to the candidate modulator. Implanted cells are generally human cells from a pre-existing tumor or a tumor cell line. After an appropriate period of time, generally around six days, the implanted samples are harvested for evaluation of the candidate modulator. Tumorigenicity and modulator efficacy may be evaluated by assaying the quantity of viable cells present in the macrocapsule, which can be determined by tests known in the art, for example, MTT dye conversion assay, neutral red dye uptake, trypan blue staining, viable cell counts, the number of colonies formed in soft agar, the capacity of the cells to recover and replicate in vitro, etc.

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

#### **Diagnostic and therapeutic uses**

Specific PAPSS-modulating agents are useful in a variety of diagnostic and therapeutic applications where disease or disease prognosis is related to defects in the AXIN pathway, such as angiogenic, apoptotic, or cell proliferation disorders.

Accordingly, the invention also provides methods for modulating the AXIN pathway in a cell, preferably a cell pre-determined to have defective or impaired AXIN function (e.g. due to overexpression, underexpression, or misexpression of AXIN, or due to gene mutations), comprising the step of administering an agent to the cell that specifically modulates PAPSS activity. Preferably, the modulating agent produces a detectable phenotypic change in the cell indicating that the AXIN 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 AXIN 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 AXIN function by administering a therapeutically effective amount of a PAPSS -modulating agent that modulates the AXIN pathway. The invention further provides methods for modulating PAPSS function in a cell, preferably a cell pre-determined to have defective or impaired PAPSS function, by administering a PAPSS -modulating agent. Additionally, the invention provides a method for treating disorders or disease associated with impaired PAPSS function by administering a therapeutically effective amount of a PAPSS -modulating agent.

The discovery that PAPSS is implicated in AXIN 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 AXIN 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 PAPSS 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 AXIN signaling that express a PAPSS, are identified as amenable to treatment with a PAPSS modulating agent. In a preferred application, the AXIN defective tissue overexpresses a PAPSS 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 PAPSS cDNA sequences as probes, can determine whether particular tumors

express or overexpress PAPSS. Alternatively, the TaqMan® is used for quantitative RT-PCR analysis of PAPSS expression in cell lines, normal tissues and tumor samples (PE Applied Biosystems).

Various other diagnostic methods may be performed, for example, utilizing  
5 reagents such as the PAPSS oligonucleotides, and antibodies directed against a PAPSS, as described above for: (1) the detection of the presence of PAPSS gene mutations, or the detection of either over- or under-expression of PAPSS mRNA relative to the non-disorder state; (2) the detection of either an over- or an under-abundance of PAPSS gene product relative to the non-disorder state; and (3) the detection of perturbations or abnormalities in  
10 the signal transduction pathway mediated by PAPSS.

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 PAPSS expression, the method comprising: a) obtaining a biological sample from the patient; b) contacting the sample with a probe for PAPSS expression; c) comparing results from step (b) with a  
15 control; and d) determining whether step (c) indicates a likelihood of the disease or disorder. Preferably, the disease is cancer, most preferably a cancer as shown in TABLE 1. The probe may be either DNA or protein, including an antibody.

## **EXAMPLES**

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

### **I. C. elegans AXIN screen**

We have found that the temperature-sensitive, reduction-of-function *pry-1* mutant  
25 *mu38* grown at 15°C produces a ruptured vulva (Rvl) phenotype by which about 95% of animals become eviscerated and die at the L4 molt. The *pry-1* Rvl mutant phenotype is suppressed by loss-of-function mutations in the beta-catenin ortholog *bar-1* and the TCF ortholog *pop-1*. The Rvl phenotype can also be generated by gain-of-function mutations in *bar-1*/beta-catenin that eliminate the consensus GSK3-beta phosphorylation sites and  
30 are predicted to prevent Axin-mediated degradation of BAR-1.

We designed a genetic screen to identify genes in addition to *bar-1*/beta-catenin and *pop-1*/TCF that act positively in beta-catenin signaling and, when inactivated, suppress the Rvl mutant phenotype of *pry-1*/Axin. The function of individual genes was inactivated by RNAi in *pry-1* (*mu38*) L1 larvae, and suppression of the Rvl phenotype was

scored as a statistically significant increase in the proportion of larvae that survived to adulthood without rupturing. Suppressor genes were subsequently counterscreened to eliminate those that appeared to suppress the *pry-1* mutant non-specifically, rather than those that specifically functioned in beta-catenin signaling. Suppressor genes that did not  
5 block vulva formation in a wildtype background, and that did not suppress the Rvl phenotype of two mutations in genes unrelated to beta-catenin signaling (*lin-1*/Ets and *daf-18*/PTEN) were considered to be specific *pry-1*/Axin suppressors. These suppressor genes, when inactivated, likely suppress beta-catenin's inappropriate transcriptional activation of target genes and, therefore, may be relevant for cancer therapy.

10 T14G10.1 was a suppressor. Orthologs of the modifiers are referred to herein as PAPSS.

BLAST analysis (Altschul et al., *supra*) was employed to identify orthologs of Drosophila modifiers. For example, representative sequences from PAPSS, GI#s20127475 and 4758880 (SEQ ID NOs:11 and 12), share 58% and 57% amino acid  
15 identity, respectively, with the *C.elegans* T14G10.1.

Various domains, signals, and functional subunits in proteins were analyzed using the PSORT (Nakai K., and Horton P., Trends Biochem Sci, 1999, 24:34-6; Kenta Nakai, Protein sorting signals and prediction of subcellular localization, Adv. Protein Chem. 54, 277-344 (2000)), PFAM (Bateman A., et al., Nucleic Acids Res, 1999, 27:260-2),  
20 SMART (Ponting CP, et al., SMART: identification and annotation of domains from signaling and extracellular protein sequences. Nucleic Acids Res. 1999 Jan 1;27(1):229-32), TM-HMM (Erik L.L. Sonnhammer, Gunnar von Heijne, and Anders Krogh: A hidden Markov model for predicting transmembrane helices in protein sequences. In Proc. of Sixth Int. Conf. on Intelligent Systems for Molecular Biology, p 175-182 Ed J. Glasgow,  
25 T. Littlejohn, F. Major, R. Lathrop, D. Sankoff, and C. Sensen Menlo Park, CA: AAAI Press, 1998), and clust (Remm M, and Sonnhammer E. Classification of transmembrane protein families in the Caenorhabditis elegans genome and identification of human orthologs. Genome Res. 2000 Nov;10(11):1679-89) programs. For example, the APS kinase domain (PFAM 1583) of PAPSS from GI#s 20127475 4758880 (SEQ ID NOs:11  
30 and 12, respectively) is located at approximately amino acid residues 51 to 209 and 41-199.

## II. High-Throughput In Vitro Fluorescence Polarization Assay

Fluorescently-labeled PAPSS 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  
5 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 PAPSS activity.

## III. High-Throughput In Vitro Binding Assay.

10 <sup>33</sup>P-labeled PAPSS peptide is added in an assay buffer (100 mM KCl, 20 mM HEPES pH 7.6, 1 mM MgCl<sub>2</sub>, 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  
15 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 AXIN modulating agents.

## IV. Immunoprecipitations and Immunoblotting

20 For coprecipitation of transfected proteins,  $3 \times 10^6$  appropriate recombinant cells containing the PAPSS 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  
25 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  $\mu$ l of M2 beads (Sigma) for 2 h at 4 °C with gentle rocking.  
30 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).

#### V. Kinase assay

5 A purified or partially purified PAPSS is diluted in a suitable reaction buffer, e.g., 50 mM Hepes, pH 7.5, containing magnesium chloride or manganese chloride (1-20 mM) and a peptide or polypeptide substrate, such as myelin basic protein or casein (1-10  $\mu\text{g/ml}$ ). The final concentration of the kinase is 1-20 nM. The enzyme reaction is conducted in microtiter plates to facilitate optimization of reaction conditions by increasing assay throughput. A 96-well microtiter plate is employed using a final volume 10 30-100  $\mu\text{l}$ . The reaction is initiated by the addition of  $^{33}\text{P}$ -gamma-ATP (0.5  $\mu\text{Ci/ml}$ ) and incubated for 0.5 to 3 hours at room temperature. Negative controls are provided by the addition of EDTA, which chelates the divalent cation ( $\text{Mg}^{2+}$  or  $\text{Mn}^{2+}$ ) required for enzymatic activity. Following the incubation, the enzyme reaction is quenched using 15 EDTA. Samples of the reaction are transferred to a 96-well glass fiber filter plate (MultiScreen, Millipore). The filters are subsequently washed with phosphate-buffered saline, dilute phosphoric acid (0.5%) or other suitable medium to remove excess radiolabeled ATP. Scintillation cocktail is added to the filter plate and the incorporated radioactivity is quantitated by scintillation counting (Wallac/Perkin Elmer). Activity is 20 defined by the amount of radioactivity detected following subtraction of the negative control reaction value (EDTA quench).

#### VI. Expression analysis

All cell lines used in the following experiments are NCI (National Cancer Institute) 25 lines, and are available from ATCC (American Type Culture Collection, Manassas, VA 20110-2209). Normal and tumor tissues were obtained from Impath, UC Davis, Clontech, Stratagene, Ardais, Genome Collaborative, and Ambion.

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

30 RNA was extracted from each tissue sample using Qiagen (Valencia, CA) RNeasy kits, following manufacturer's protocols, to a final concentration of 50ng/ $\mu\text{l}$ . Single stranded cDNA was 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) were prepared according to the TaqMan protocols, and the following criteria: a) primer pairs were designed to span introns to eliminate genomic contamination, and b) each primer pair produced only one product. Expression analysis was performed using a  
5 7900HT instrument.

Taqman reactions were carried out following manufacturer's protocols, in 25  $\mu$ l total volume for 96-well plates and 10  $\mu$ l total volume for 384-well plates, using 300nM primer and 250 nM probe, and approximately 25ng of cDNA. The standard curve for result analysis was prepared using a universal pool of human cDNA samples, which is a  
10 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 were normalized using 18S rRNA (universally expressed in all tissues and cells).

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

Results are shown in Table 1. Number of pairs of tumor samples and matched normal tissue from the same patient are shown for each tumor type. Percentage of the samples with at least two-fold overexpression for each tumor type is provided. A  
25 modulator identified by an assay described herein can be further validated for therapeutic effect by administration to a tumor in which the gene is overexpressed. A decrease in tumor growth confirms therapeutic utility of the modulator. Prior to treating a 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  
30 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.

Table 1

|                      |     |     |
|----------------------|-----|-----|
| <b>Seq ID NO:</b>    | 5   | 9   |
| <b>Breast</b>        | 10% | 10% |
| <b># of Pairs</b>    | 21  | 21  |
| <b>Colon</b>         | 22% | 8%  |
| <b># of Pairs</b>    | 36  | 36  |
| <b>Head And Neck</b> | 25% | 38% |
| <b># of Pairs</b>    | 8   | 8   |
| <b>Kidney</b>        | 0%  | 12% |
| <b># of Pairs</b>    | 24  | 24  |
| <b>Lung</b>          | 0%  | 8%  |
| <b># of Pairs</b>    | 24  | 24  |
| <b>Ovary</b>         | 18% | 9%  |
| <b># of Pairs</b>    | 11  | 11  |
| <b>Prostate</b>      | 8%  | 8%  |
| <b># of Pairs</b>    | 13  | 13  |
| <b>Skin</b>          | 0%  | 33% |
| <b># of Pairs</b>    | 3   | 3   |
| <b>Uterus</b>        | 26% | 0%  |
| <b># of Pairs</b>    | 19  | 19  |

## VII. PAPSS functional assays

RNAi experiments were carried out to knock down expression of PAPSS in various cell lines using small interfering RNAs (siRNA, Elbashir et al, *supra*).

Effect of PAPSS RNAi on cell proliferation. BrdU and Cell Titer-Glo™ assays, as described above, were employed to study the effects of decreased PAPSS expression on cell proliferation. RNAi of PAPSS of SEQ ID NO:6 decreased proliferation in LX-1 lung cancer cells and in 231T breast cancer cells. RNAi of PAPSS of SEQ ID NO:10 decreased proliferation in LX-1 lung cancer cells. MTS cell proliferation assay, as described above, was also employed to study the effects of decreased PAPSS expression on cell proliferation. RNAi of PAPSS of SEQ ID NO:6 decreased proliferation in A549 lung cancer cells, LX1 cells, HCT116 colon cancer cells, LNCAP prostate cancer cells, and SKBR3 breast cancer cells. RNAi of PAPSS of SEQ ID NO:10 decreased proliferation in

Effect of PAPSS RNAi on apoptosis. Nucleosome ELISA apoptosis assay, as described above, was employed to study the effects of decreased PAPSS expression on apoptosis. RNAi of PAPSS of SEQ ID NO:6 increased apoptosis in LX1 and A549 cells, whereas RNAi of PAPSS of SEQ ID NO:10 had no affect on apoptosis

Effect of PAPSS RNAi on cell cycle. Propidium iodide (PI) cell cycle assay, as described above, was employed to study the effects of decreased PAPSS expression on cell cycle. RNAi of PAPSS of SEQ ID NO:6 caused a sub G1 induction in LX1, SKBR3, and A549 cells. RNAi of PAPSS of SEQ ID NO:10 caused a sub G1 induction in SKBR3  
5 and LNCAP cells.

Colony formation assays, as described above were employed to study effects of RNAi of PAPSS on colony growth. RNAi of PAPSS of SEQ ID NO:6 decreased colony growth in LX1 cells.

RNAi of PAPSS of both SEQ ID NO:6 and SEQ ID NO:10 also decreased beta-  
10 catenin pathway signaling.

Overexpression of PAPSS of SEQ ID NO:5 along with RAS oncogene increased transformation of 3T3 cells as compared with RAS alone or SEQ ID NO:5 alone.

Overexpression of PAPSS of SEQ ID NO:9 modulated expression of the following transcription factors: AP2, E2F, TFIID, BM3, and STAT3.

15

## WHAT IS CLAIMED IS:

1. A method of identifying a candidate AXIN pathway modulating agent, said method comprising the steps of:
  - 5 (a) providing an assay system comprising a PAPSS 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 AXIN pathway modulating agent.
2. The method of Claim 1 wherein the assay system comprises cultured cells that express the PAPSS polypeptide.
- 15 3. The method of Claim 2 wherein the cultured cells additionally have defective AXIN function.
4. The method of Claim 1 wherein the assay system includes a screening assay comprising a PAPSS polypeptide, and the candidate test agent is a small molecule
- 20 modulator.
5. The method of Claim 4 wherein the assay is a kinase assay.
6. The method of Claim 1 wherein the assay system is selected from the group consisting
- 25 of an apoptosis assay system, a cell proliferation assay system, an angiogenesis assay system, and a hypoxic induction assay system.
7. The method of Claim 1 wherein the assay system includes a binding assay comprising a PAPSS polypeptide and the candidate test agent is an antibody.
- 30 8. The method of Claim 1 wherein the assay system includes an expression assay comprising a PAPSS nucleic acid and the candidate test agent is a nucleic acid modulator.
9. The method of Claim 8 wherein the nucleic acid modulator is an antisense oligomer.

10. The method of Claim 8 wherein the nucleic acid modulator is a PMO.
11. The method of Claim 1 additionally comprising:
- 5 (d) administering the candidate AXIN pathway modulating agent identified in (c) to a model system comprising cells defective in AXIN function and, detecting a phenotypic change in the model system that indicates that the AXIN function is restored.
12. The method of Claim 11 wherein the model system is a mouse model with defective AXIN function.
- 10 13. A method for modulating a AXIN pathway of a cell comprising contacting a cell defective in AXIN function with a candidate modulator that specifically binds to a PAPSS polypeptide, whereby AXIN 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 AXIN function.
- 15 15. The method of Claim 13 wherein the candidate modulator is selected from the group consisting of an antibody and a small molecule.
- 20 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 PAPSS ,
- 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 AXIN pathway modulating agent,
- and wherein the second assay detects an agent-biased change in the AXIN 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  
5 animal.
19. The method of Claim 18 wherein the non-human animal mis-expresses a AXIN pathway gene.
- 10 20. A method of modulating AXIN pathway in a mammalian cell comprising contacting the cell with an agent that specifically binds a PAPSS polypeptide or nucleic acid.
21. The method of Claim 20 wherein the agent is administered to a mammalian animal predetermined to have a pathology associated with the AXIN pathway.
- 15 22. The method of Claim 20 wherein the agent is a small molecule modulator, a nucleic acid modulator, or an antibody.
23. A method for diagnosing a disease in a patient comprising:  
20 (a) obtaining a biological sample from the patient;  
(b) contacting the sample with a probe for PAPSS expression;  
(c) comparing results from step (b) with a control;  
(d) determining whether step (c) indicates a likelihood of disease.
- 25 24. The method of Claim 23 wherein said disease is cancer.
25. The method according to Claim 24, wherein said cancer is a cancer as shown in Table 1 as having >25% expression level.
- 30

## SEQUENCE LISTING

&lt;110&gt; EXELIXIS INC.

&lt;120&gt; PAPSSs AS MOFIFIERS OF THE AXIN PATHWAY AND METHODS OF USE

&lt;130&gt; EX03-058C-PC

&lt;150&gt; US 60/401,534

&lt;151&gt; 2002-08-07

&lt;160&gt; 12

&lt;170&gt; PatentIn version 3.2

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 aaatacatga atcagcaggg ctgccattct ttgaaatatt tgtagatgca cctctaaata 540  
 tttgtgaaag cagagacgta aaaggcctct ataaaagggc cagagctggg gagattaaag 600  
 gatttacagg tattgattct gattatgaga aacctgaaac tcctgagcgt gtgcttaaaa 660  
 ccaatttgtc cacagtgagt gactgtgtcc accaggtagt ggaacttctg caagagcaga 720  
 acattgtacc ctatactata atcaaagata tccacgaact ctttgtgccg gaaaacaac 780  
 ttgaccacgt ccgagctgag gctgaaactc tcccttcatt atcaattact aagctggatc 840  
 tccagtgggt ccaggttttg agcgaaggct gggccactcc cctcaaagggt ttcattgcggg 900  
 agaaggagta cttacagggt atgcactttg acaccctgct agatgatggc gtgatcaaca 960  
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 gcaagtttgt cctggcacat ggtggacgga gggtagctat cttacgagac gctgaattct 1080  
 atgaacacag aaaagaggaa cgctgttccc gtgtttgggg gacaacatgt acaaaacacc 1140  
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agtcacccat tgttgccatc tttccgtctc ccatgttata tgctggcccc acagaggtcc 1560
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acttcatctc aggaactcga atgaggaagc tcgcccggga aggagagaat ccccagatg 1860
gcttcatggc ccccaaagca tggaagggtc tgacagatta ttacagggtc ctggagaaga 1920
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<212> PRT
<213> Homo sapiens

<400> 11

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Met Glu Ile Pro Gly Ser Leu Cys Lys Lys Val Lys Leu Ser Asn Asn
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Ala Gln Asn Trp Gly Met Gln Arg Ala Thr Asn Val Thr Tyr Gln Ala
20 25 30

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```

His His Val Ser Arg Asn Lys Arg Gly Gln Val Val Gly Thr Arg Gly
35 40 45

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Gly Phe Arg Gly Cys Thr Val Trp Leu Thr Gly Leu Ser Gly Ala Gly
50 55 60

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Lys Thr Thr Val Ser Met Ala Leu Glu Glu Tyr Leu Val Cys His Gly
65 70 75 80

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Ile Pro Cys Tyr Thr Leu Asp Gly Asp Asn Ile Arg Gln Gly Leu Asn
85 90 95

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Lys Asn Leu Gly Phe Ser Pro Glu Asp Arg Glu Glu Asn Val Arg Arg
100 105 110

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Ile Ala Glu Val Ala Lys Leu Phe Ala Asp Ala Gly Leu Val Cys Ile
115 120 125

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Thr Ser Phe Ile Ser Pro Tyr Thr Gln Asp Arg Asn Asn Ala Arg Gln
130 135 140

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

Ala Arg Ala Gly Glu Ile Lys Gly Phe Thr Gly Ile Asp Ser Glu Tyr  
 180 185 190

Glu Lys Pro Glu Ala Pro Glu Leu Val Leu Lys Thr Asp Ser Cys Asp  
 195 200 205

Val Asn Asp Cys Val Gln Gln Val Val Glu Leu Leu Gln Glu Arg Asp  
 210 215 220

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

Glu Asn Lys Leu His Leu Ala Lys Thr Asp Ala Glu Thr Leu Pro Ala  
 245 250 255

Leu Lys Ile Asn Lys Val Asp Met Gln Trp Val Gln Val Leu Ala Glu  
 260 265 270

Gly Trp Ala Thr Pro Leu Asn Gly Phe Met Arg Glu Arg Glu Tyr Leu  
 275 280 285

Gln Cys Leu His Phe Asp Cys Leu Leu Asp Gly Gly Val Ile Asn Leu  
 290 295 300

Ser Val Pro Ile Val Leu Thr Ala Thr His Glu Asp Lys Glu Arg Leu  
 305 310 315 320

Asp Gly Cys Thr Ala Phe Ala Leu Met Tyr Glu Gly Arg Arg Val Ala  
 325 330 335

Ile Leu Arg Asn Pro Glu Phe Phe Glu His Arg Lys Glu Glu Arg Cys  
 340 345 350

Ala Arg Gln Trp Gly Thr Thr Cys Lys Asn His Pro Tyr Ile Lys Met  
 355 360 365

Val Met Glu Gln Gly Asp Trp Leu Ile Gly Gly Asp Leu Gln Val Leu  
 370 375 380

Asp Arg Val Tyr Trp Asn Asp Gly Leu Asp Gln Tyr Arg Leu Thr Pro  
 385 390 395 400

Thr Glu Leu Lys Gln Lys Phe Lys Asp Met Asn Ala Asp Ala Val Phe  
 405 410 415  
 Ala Phe Gln Leu Arg Asn Pro Val His Asn Gly His Ala Leu Leu Met  
 420 425 430  
 Gln Asp Thr His Lys Gln Leu Leu Glu Arg Gly Tyr Arg Arg Pro Val  
 435 440 445  
 Leu Leu Leu His Pro Leu Gly Gly Trp Thr Lys Asp Asp Asp Val Pro  
 450 455 460  
 Leu Met Trp Arg Met Lys Gln His Ala Ala Val Leu Glu Glu Gly Val  
 465 470 475 480  
 Leu Asn Pro Glu Thr Thr Val Val Ala Ile Phe Pro Ser Pro Met Met  
 485 490 495  
 Tyr Ala Gly Pro Thr Glu Val Gln Trp His Cys Arg Ala Arg Met Val  
 500 505 510  
 Ala Gly Ala Asn Phe Tyr Ile Val Gly Arg Asp Pro Ala Gly Met Pro  
 515 520 525  
 His Pro Glu Thr Gly Lys Asp Leu Tyr Glu Pro Ser His Gly Ala Lys  
 530 535 540  
 Val Leu Thr Met Ala Pro Gly Leu Ile Thr Leu Glu Ile Val Pro Phe  
 545 550 555 560  
 Arg Val Ala Ala Tyr Asn Lys Lys Lys Lys Arg Met Asp Tyr Tyr Asp  
 565 570 575  
 Ser Glu His His Glu Asp Phe Glu Phe Ile Ser Gly Thr Arg Met Arg  
 580 585 590  
 Lys Leu Ala Arg Glu Gly Gln Lys Pro Pro Glu Gly Phe Met Ala Pro  
 595 600 605  
 Lys Ala Trp Thr Val Leu Thr Glu Tyr Tyr Lys Ser Leu Glu Lys Ala  
 610 615 620

<210> 12  
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 <212> PRT  
 <213> Homo sapiens

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

Gly Leu Ser Gly Ala Gly Lys Thr Thr Ile Ser Phe Ala Leu Glu Glu  
 50 55 60

Tyr Leu Val Ser His Ala Ile Pro Cys Tyr Ser Leu Asp Gly Asp Asn  
 65 70 75 80

Val Arg His Gly Leu Asn Arg Asn Leu Gly Phe Ser Pro Gly Asp Arg  
 85 90 95

Glu Glu Asn Ile Arg Arg Ile Ala Glu Val Ala Lys Leu Phe Ala Asp  
 100 105 110

Ala Gly Leu Val Cys Ile Thr Ser Phe Ile Ser Pro Phe Ala Lys Asp  
 115 120 125

Arg Glu Asn Ala Arg Lys Ile His Glu Ser Ala Gly Leu Pro Phe Phe  
 130 135 140

Glu Ile Phe Val Asp Ala Pro Leu Asn Ile Cys Glu Ser Arg Asp Val  
 145 150 155 160

Lys Gly Leu Tyr Lys Arg Ala Arg Ala Gly Glu Ile Lys Gly Phe Thr  
 165 170 175

Gly Ile Asp Ser Asp Tyr Glu Lys Pro Glu Thr Pro Glu Arg Val Leu  
 180 185 190

Lys Thr Asn Leu Ser Thr Val Ser Asp Cys Val His Gln Val Val Glu  
 195 200 205

Leu Leu Gln Glu Gln Asn Ile Val Pro Tyr Thr Ile Ile Lys Asp Ile  
 210 215 220

His Glu Leu Phe Val Pro Glu Asn Lys Leu Asp His Val Arg Ala Glu  
 225 230 235 240

Ala Glu Thr Leu Pro Ser Leu Ser Ile Thr Lys Leu Asp Leu Gln Trp  
245 250 255

Val Gln Val Leu Ser Glu Gly Trp Ala Thr Pro Leu Lys Gly Phe Met  
260 265 270

Arg Glu Lys Glu Tyr Leu Gln Val Met His Phe Asp Thr Leu Leu Asp  
275 280 285

Asp Gly Val Ile Asn Met Ser Ile Pro Ile Val Leu Pro Val Ser Ala  
290 295 300

Glu Asp Lys Thr Arg Leu Glu Gly Cys Ser Lys Phe Val Leu Ala His  
305 310 315 320

Gly Gly Arg Arg Val Ala Ile Leu Arg Asp Ala Glu Phe Tyr Glu His  
325 330 335

Arg Lys Glu Glu Arg Cys Ser Arg Val Trp Gly Thr Thr Cys Thr Lys  
340 345 350

His Pro His Ile Lys Met Val Met Glu Ser Gly Asp Trp Leu Val Gly  
355 360 365

Gly Asp Leu Gln Val Leu Glu Lys Ile Arg Trp Asn Asp Gly Leu Asp  
370 375 380

Gln Tyr Arg Leu Thr Pro Leu Glu Leu Lys Gln Lys Cys Lys Glu Met  
385 390 395 400

Asn Ala Asp Ala Val Phe Ala Phe Gln Leu Arg Asn Pro Val His Asn  
405 410 415

Gly His Ala Leu Leu Met Gln Asp Thr Cys Arg Arg Leu Leu Glu Arg  
420 425 430

Gly Tyr Lys His Pro Val Leu Leu Leu His Pro Leu Gly Gly Trp Thr  
435 440 445

Lys Asp Asp Asp Val Pro Leu Asp Trp Arg Met Lys Gln His Ala Ala  
450 455 460

Val Leu Glu Glu Gly Val Leu Asp Pro Lys Ser Thr Ile Val Ala Ile  
465 470 475 480

Phe Pro Ser Pro Met Leu Tyr Ala Gly Pro Thr Glu Val Gln Trp His

|                                                                 | 485 |  | 490 |  | 495 |
|-----------------------------------------------------------------|-----|--|-----|--|-----|
| Cys Arg Ser Arg Met Ile Ala Gly Ala Asn Phe Tyr Ile Val Gly Arg |     |  |     |  |     |
|                                                                 | 500 |  | 505 |  | 510 |
| Asp Pro Ala Gly Met Pro His Pro Glu Thr Lys Lys Asp Leu Tyr Glu |     |  |     |  |     |
|                                                                 | 515 |  | 520 |  | 525 |
| Pro Thr His Gly Gly Lys Val Leu Ser Met Ala Pro Gly Leu Thr Ser |     |  |     |  |     |
|                                                                 | 530 |  | 535 |  | 540 |
| Val Glu Ile Ile Pro Phe Arg Val Ala Ala Tyr Asn Lys Ala Lys Lys |     |  |     |  |     |
|                                                                 | 545 |  | 550 |  | 555 |
|                                                                 |     |  |     |  | 560 |
| Ala Met Asp Phe Tyr Asp Pro Ala Arg His Asn Glu Phe Asp Phe Ile |     |  |     |  |     |
|                                                                 |     |  |     |  |     |
|                                                                 | 565 |  | 570 |  | 575 |
| Ser Gly Thr Arg Met Arg Lys Leu Ala Arg Glu Gly Glu Asn Pro Pro |     |  |     |  |     |
|                                                                 | 580 |  | 585 |  | 590 |
| Asp Gly Phe Met Ala Pro Lys Ala Trp Lys Val Leu Thr Asp Tyr Tyr |     |  |     |  |     |
|                                                                 | 595 |  | 600 |  | 605 |
| Arg Ser Leu Glu Lys Asn                                         |     |  |     |  |     |
|                                                                 | 610 |  |     |  |     |