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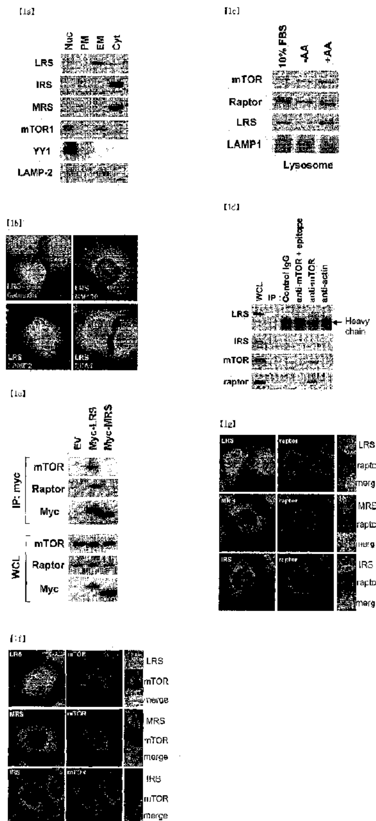
(54) Title: NOVEL USE OF LEUCYL TRNA SYNTHETASE



(57) Abstract: The present invention relates to novel use of leucyl tRNA synthetase and more particularly it relates a method of screening an agent for preventing or treating mTORCl mediated diseases by screening an test agent which inhibits its binding ability of LRS to RagD or RagD GTPases and a method of reducing cell size compared to the control group comprising inhibiting expression of intracellular LRS. The method of the present invention provides novel method of regulating cell size and further the screening method of screening can be used in developing novel reagent for treatment of disease such as cancer.

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【DESCRIPTION】

【Invention Title】

NOVEL USE OF LEUCYL TRNA SYNTHETASE

【Technical Field】

<1> This application claims priority from and the benefit of Korean Patent Application No. 10-2011-0095893 filed on September 22, 2011, which is hereby incorporated by reference for all purposes as if fully set forth herein.

<2>

<3> The present invention relates to novel use of leucyl tRNA synthetase and more particularly it relates a method of screening an agent for preventing or treating mTORC1 mediated diseases by screening an test agent which inhibits binding ability of LRS to RagD or RagD GTPases and a method of reducing cell size compared to the control group comprising inhibiting expression of intracellular LRS.

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<5>

【Background Art】

<6> A leucine is one of three branched chain amino acids. Unlike other amino acids, leucine and the other branched chain amino acids, isoleucine and valine, escape liver metabolism due to the defect of the branched chain amino acid aminotransferase and directly influence muscle protein synthesis. Leucine not only serves as a substrate for protein synthesis but also is recognized as a potent signal nutrient that regulates protein metabolism. Oral administration of leucine increases rates of skeletal muscle protein synthesis in rats (Crozier SJ, et. al., J Nutr. 135 (2005), 376382) and removal of leucine from a complete meal prevents stimulation of protein synthesis (Stipanuk MH., Nutr Rev. 65 (2007), 122129). Leucine-induced protein synthesis is mediated by the mammalian target of rapamycin (mTOR) complex 1 (mTORC1), which is composed of mTOR, regulatory associated protein of mammalian target of rapamycin (Raptor), G-protein βsubunit-like protein (GβL), and ras homolog enriched in brain (Rheb)(Bhaskar PT, et. al., Dev

Cell. 12 (2007), 487502). mTORC1 phosphorylates S6K and 4E-BP, the rate-limiting step in translation, resulting in the translation initiation of mRNAs displaying a 5' cap structure (Ma XM, Nat Rev Mol Cell Biol., 10(2009), 307-318; Holz MK, et. al., Cell 123(2005), pp 569-580).

<7>

<8> mTORC1 regulates translation and cell growth by coordinating several upstream inputs such as growth factors, intracellular energy status, and amino acid availability. The Tuberous Sclerosis Complex (TSC) 1 and TSC2 regulate GTP/GDP exchange of Ras-like GTPase, Rheb to transmit growth factor and intracellular energy signals to mTORC1. When bound to GTP, Rheb interacts with and activates mTORC1 (Tee AR, et. al., Curr Biol. 13 (2003), 12591268) and appears to be necessary for the activation of mTORC1 by all signals, including amino acid availability. In contrast, TSC1-TSC2 is dispensable for the regulation of mTORC1 by amino acids, and, in cells lacking TSC2, the mTORC1 pathway is sensitive to amino acid starvation but resistant to growth factor withdrawal (Roccio M, et. al., Oncogene. 25 (2006), 657-664).

<9>

<10> Recently, the Rag GTPases, which are also the members of the Ras family of GTP-binding proteins, were shown to be amino acid-specific regulators of the mTORC1 pathway (Sancak Y, et. al., Cell 141 (2010), 290-303). Mammals express four Rag proteins -RagA, RagB, RagC, and RagD- form heterodimers consisting of RagA or RagB with RagC or RagD. RagA and RagB, like RagC and RagD, are highly similar to each other and are functionally redundant (Schurmann A, et. al., J Biol Chem. 270 (1995), 28982-28988). Rag heterodimers containing GTP-bound RagB interact with mTORC1, and amino acids induce the mTORC1-Rag interaction by promoting the loading of RagB with GTP, which enables it to directly interact with the Raptor component of mTORC1 (Sancak Y, et. al., Cell 141 (2010), 290-303; Kim E, Nat Cell Biol. 10 (2008), 935-945). The activation of the mTORC1 pathway by amino acids correlates with the movement of mTORC1 from an undefined location to a compartment containing Rab7 (Sancak Y, et. al., Science 320 (2008), 1496-1501), a marker of both late endosomes and lysosomes (Bucci C, et. al., Mol

Biol Cell. ,11 (2000), 467-480). Recent report shows that amino acids induce the movement of mTORC1 to lysosome, where the Rag GTPases reside. Ragulator complex, which is composed of MAPKSP1, ROBLD3, and c11orf59 gene products, interacts with the Rag GTPases, recruits them to lysosomes and is essential for mTORC1 activation (Sancak Y, et. al., Cell 141 (2010), 290-303). However, how intracellular leucine is sensed for mTORC1 activation and how GTP/GDP cycles of Rag GTPases are regulated by amino acid for mTORC1 activation are unknown.

<11>

<12> Aminoacyl-tRNA synthetases (ARSs) are essential enzymes for cellular protein synthesis and viability that catalyze the ligation of specific amino acids to their cognate tRNAs. The enzyme reaction is separated into two steps: the ATP-PPi exchange reaction for amino acid activation and aminoacylation of tRNA (Park S, et. al., Trends Biochem Sci. 30 (2005), 569-574). Based on amino acid sequence alignments and structural features, ARSs have been divided into two classes (Eriani G, et. al., Nature 347 (1990), 203-206; Burbaum JJ, et. al., J Biol Chem. 266 (1991), 16965-16968). The class I synthetases share two consensus sequences, the HIGH (His-Ile-Gly-His) and KMSKS (Lys-Met-Ser-Lys-Ser) motifs, that form a nucleotide binding Rossmann fold (Arnez JG, et. al., Trends Biochem sci. 22 (1997), 211-216). In contrast, the class II synthetases do not contain the Rossmann fold, but share a very different catalytic domain (Cusack S, et. al., Nucl Acids Res. 19 (1991), 3489-3498). Leucyl-tRNA synthetase (LRS) is the class I enzyme, which is characterized by the HIGH and KMSKS motifs (Cusack S, et. al., EMBO J. 19 (2000), 2351-361). Structurally, LRS consists of the catalytic domain of bipartite Rossmann fold with a large insertion domain called CP1, a tRNA-binding anticodon domain, and a C-terminal extension domain (Cusack S, et. al., EMBO J. 19 (2000), 2351?361). In higher eukaryotic cells, LRS exists as a component of the ARS complex consisting of nine different tRNA synthetases and three non-enzymatic components, p18/AIMP3, p38/AIMP2, and p43/AIMP1 (Lee SW, et. al., J Cell Sci. 117 (2004), 3725-3734; Park S, et. al., Trends Biochem Sci. 30 (2005), 569-574; Park SG, et. al., Proc Natl Acad Sci USA 105

(2008), pp. 11043-11049). It has been shown that the C-terminal domain of LRS is crucial for the interaction with other components of the ARS complex (Ling C, et. al., J Biol Chem. 280 (2005), 34755-3463). Among the components of the complex, several different components are involved in various cell signaling processes (Lee YN, et. al., Immunity 20 (2004), 145?51; Park S, et. al., Trends Biochem Sci. 30 (2005), pp. 569-574; Park SG, et. al., Proc Natl Acad Sci USA 105 (2008), pp. 11043-11049). For instance, glutamyl-prolyl-tRNA synthetase (EPRS) suppresses translation of the target inflammatory mRNAs by forming an interferon gamma-activated inhibitor of translation (GAIT) complex (Sampath P, et. al., Cell. 119 (2004), 195-208). Lysyl-tRNA synthetase (KRS) and its product, Ap4A, function as signaling regulators in the immune response by regulating gene expression (Lee YN, et. al.,. Immunity 20 (2004), 145?51; Yannay-Cohen N, et. al., Mol Cell 34 (2009), 603-611). Methionyl-tRNA synthetase (MRS) and glutaminyl-tRNA synthetase (QRS) are involved in rRNA biogenesis (Ko et al., 2000) and anti-apoptotic signal regulation (Ko YG, et. al., J Cell Biol 149 (2000), 567-574), respectively. Besides, cytosolic LRS was reported to be potentially implicated in lung cancer growth (Shin SH, et. al., Exp Mol Med. 40 (2008), 229-236), and the mitochondrial LRS may be involved in diabetes ('t Hart LM, et. al., Diabetes. 54 (2005), 1892-1895; Li R., et. al., Mol Cell Biol. 30 (2010), 2147-154).

<13>

【Disclosure】

【Technical Problem】

<14> Accordingly, the inventors investigated non-canonical function of LRS apart from its catalytic function for protein synthesis. In this work, they found that LRS is an mTORC1-associated protein and plays an essential role for amino acid induced mTORC1 activation. In addition, ablation of leucine binding ability in LRS desensitized the mTORC1 pathway to amino acid. Among the components of mTORC1, they found that LRS directly interacts with Rag GTPase in amino acid-dependent manner and functions as a GTPase-activating protein (GAP) for Rag GTPase to activate mTORC1 and thereby completing the present invention.

<15>

<16> Accordingly, an object of the present invention is to provide a

<17> method for screening agent for preventing or treating of mTORC1-mediated disease comprising the steps of:

<18> (a) contacting LRS(Leucyl tRNA synthetase), RagD and a test agent with or without the test agent;

<19> (b) comparing the binding affinity between LRS and RagD with the test agent to the binding affinity between LRS and RagD without the test agent;

<20> (c) measuring a change between the binding affinity between LRS and RagD.

<21>

<22> Another object of the present invention is to provide a method of reducing cell size compared to the control group comprising inhibiting expression of intracellular LRS.

<23>

<24> Another object of the present invention is to provide method for screening agent for preventing or treating of mTORC1-mediated disease comprising the steps of:

<25> (a) contacting LRS(Leucyl tRNA synthetase), RagD and a test agent with or without the test agent;

<26> (b) comparing the binding affinity between LRS and RagD with the test agent to the binding affinity between LRS and RagD without the test agent;

<27> (c) indentifying the test agent inhibiting the binding affinity between LRS and RagD.

<28>

<29>

【Technical Solution】

<30> In order to accomplish the object, the present invention provides a method for screening agent for preventing or treating of mTORC1-mediated disease comprising the steps of:

<31> (a) contacting LRS(Leucyl tRNA synthetase), RagD and a test agent with or without the test agent;

<32> (b) comparing the binding affinity between LRS and RagD with the test agent to the binding affinity between LRS and RagD without the test agent;

<33> (c) measuring a change between the binding affinity between LRS and RagD.

<34>

<35> In order to accomplish another object, the present invention provides a method of reducing cell size compared to the control group comprising inhibiting expression of intracellular LRS.

<36>

<37> In order to accomplish another object, the present invention provides a method for screening agent for preventing or treating of mTORC1-mediated disease comprising the steps of:

<38> (a) contacting LRS(Leucyl tRNA synthetase), RagD and a test agent with or without the test agent;

<39> (b) comparing the binding affinity between LRS and RagD with the test agent to the binding affinity between LRS and RagD without the test agent;

<40> (c) indentifying the test agent inhibiting the binding affinity between LRS and RagD.

<41>

<42> Hereinafter, the present invention will be described in more detail.

<43>

<44> The present invention first identified that leucyltRNA synthetase (LRS) plays a critical role in amino acid-induced mTORC1 activation. That is, they identified that LRS of the present invention directly binds to Rag GTPase, the mediator of amino acid signaling to mTORC1, in amino acid-dependent manner and functions as a GTPase-activating protein (GAP) for Rag GTPase to activate mTORC1.

<45>

<46> The present inventors confirmed that leucyltRNA synthetase (LRS) plays a critical role in amino acid-induced mTORC1 activation and LRS senses intracellular leucine concentration and mediates leucine-induced mTORC1 activation. More particularly, they confirmed that LRS directly binds to Rag

GTPase, the mediator of amino acid signaling to mTORC1, in amino acid-dependent manner and functions as a GTPase-activating protein (GAP) for Rag GTPase to activate mTORC1.

<47>

<48> Definition

<49>

<50> Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention pertains. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOTY (2d ed. 1994); THE CAMBRIDGE DICTIONARY OF SCIENCE AND TECHNOLOGY (Walker ed., 1988); and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY. In addition, the following definitions are provided to assist the reader in the practice of the invention.

<51>

<52> An "expression", as used herein, refers to formation of protein or nucleic acid in cells.

<53>

<54> A "host cell," as used herein, refers to a prokaryotic or eukaryotic cell that contains heterologous DNA that has been introduced into the cell by any means, e.g., electroporation, calcium phosphate precipitation, microinjection, transformation, viral infection, and/or the like.

<55>

<56> The term "polypeptide" is used interchangeably herein with the terms "polypeptides" and "protein(s)", and refers to a polymer of amino acid residues, e.g., as typically found in proteins in nature.

<57>

<58> The term "LRS polypeptide," refers to a polypeptide known as leucyl tRNA synthetase. The said LRS polypeptide may be a polypeptide having an amino acid sequence of SEQ ID NO: 1(GenBank Accession No: NP_064502.9). And the inventive LRS includes functional equivalents thereof.

<59>

<60> The term "functional equivalents"refers to polypeptide comprising the amino acid sequence having at least 70% amino acid sequence homology(i.e., identity), preferably at least 80%, and more preferably at least 90%, for example, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and 100% amino acid sequence homology, that exhibit substantially identical physiological activity to the polypeptide of SEQ ID NO: 1. The "substantially identical physiological activity" means binding to RagD and functioning as a GTPase-activating protein (GAP) for Rag GTPase to activate mTORC1. The functional equivalents may include, for example peptides produced by as a result of addition, substitution or deletion of some amino acid of SEQ ID NO:1. Substitutions of the amino acids are preferably conservative substitutions. Examples of conservative substitutions of naturally occurring amino acids are as follows: aliphatic amino acids (Gly, Ala, Pro), hydrophobic amino acids (Ile, Leu, Val), aromatic amino acids (Phe, Tyr, Trp), acidic amino acids (Asp, Glu), basic amino acids (His, Lys, Arg, Gln, Asn) and sulfur-containing amino acids (Cys, Met). Furthermore, the functional equivalents also include variants with deletion of some of the amino acid sequence of the LRS of the present invention. Deletion or substitutions of the amino acids are preferably located at regions that are not directly involved in the physiological activity of the inventive polypeptide. And deletion of the amino acids is preferably located at regions that are not directly involved in the physiological activity of the LRS. In addition, the functional equivalents also include variants with addition of several amino acids in both terminal ends of the amino acid sequence of the LRS or in the sequence. Moreover, the inventive functional equivalents also include polypeptide derivatives which have modification of some of the chemical structure of the inventive polypeptide while maintaining the fundamental backbone and physiological activity of the inventive polypeptide. Examples of this modification include structural modifications for changing the stability, storage, volatility or solubility of the

inventive polypeptide.

<61>

<62> Sequence identity or homology is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with amino acid sequence of LRS (SEQ ID NO: 1), after aligning the sequences and introducing gaps, if necessary,to achieve the maximum percent sequence identity, and not considering any conservative substitutions (as described above) as part of the sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the amino acid sequence of LRS shall be construed as affecting sequence identity or homology. Thus, sequence identity can be determined by standard methods that are commonly used to compare the similarity in position of the amino acids of two polypeptides. Using a computer program such as BLAST or FASTA, two polypeptides are aligned for optimal matching of their respective amino acids (either along the full length of one or both sequences or along a predetermined portion of one or both sequences). The programs provide a default opening penalty and a default gap penalty, and a scoring matrix such as PAM 250 (a standard scoring matrix; see Dayhoff et al., in Atlas of Protein Sequence and Structure, vol. 5, supp. 3 (1978)) can be used in conjunction with the computer program. For example, the percent identity can be calculated as the follow. The total number of identical matches multiplied by 100 and then divided by the sum of the length of the longer sequence within the matched span and the number of gaps introduced into the longer sequences in order to align the two sequences.

<63>

<64> The polypeptide according to the present invention can be prepared by separating from nature materials or genetic engineering methods. For example, a DNA molecule encoding the LRS or its functional equivalents (ex: In case of LRS, SEQ ID NO: 2 (Genbank Accession No. NM_020117.9), and in case of RagD, SEQ ID NO: 3 (Genbank Accession No. NM_021244.4)) is constructed according to any conventional method. The DNA molecule may synthesize by performing PCR using suitable primers. Alternatively, the DNA molecule may also be

synthesized by a standard method known in the art, for example using an automatic DNA synthesizer (commercially available from Biosearch or Applied Biosystems). The constructed DNA molecule is inserted into a vector comprising at least one expression control sequence(ex: promoter, enhancer) that is operatively linked to the DNA sequence so as to control the expression of the DNA molecule, and host cells are transformed with the resulting recombinant expression vector. The transformed cells are cultured in a medium and condition suitable to express the DNA sequence, and a substantially pure polypeptide encoded by the DNA sequence is collected from the culture medium.The collection of the pure polypeptide may be performed using a method known in the art, for example, chromatography. In this regard, the term "substantially pure polypeptide" means the inventive polypeptide that does not substantially contain any other proteins derived from host cells. For the genetic engineering method for synthesizing the inventive polypeptide, the reader may refer to the following literatures: Maniatis et al., *Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory* 1982; Sambrook et al., *Molecular Cloning; A Laboratory Manual, Cold Spring Harbor Press*, N.Y., Second(1998) and Third(2000) Editions; *Gene Expression Technology, Method in Enzymology, Genetics and Molecular Biology, Method in Enzymology, Guthrie & Fink (eds.), Academic Press, San Diego, Calif.* 1991; and Hitzeman et al., *J. Biol. Chem.*, 255, 12073-12080 1990.

<65>

<66> Alternatively, the inventive polypeptide can be chemically synthesized easily according to any technique known in the art (Creighton, Proteins: Structures and Molecular Principles, W.H. Freeman and Co., NY, 1983). As a typical technique, they are not limited to, butinclude liquid or solid phase synthesis, fragment condensation, F-MOC or T-BOC chemistry (Chemical Approaches to the Synthesis of Peptides and Proteins, Williams et al., Eds., CRC Press, Boca Raton Florida, 1997; A Practical Approach, Atherton & Sheppard,Eds., IRL Press, Oxford, England, 1989).

<67>

<68> The terms "nucleic acid," "DNA sequence" or "polynucleotide" refer to a

deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in manner similar to naturally occurring nucleotides.

<69>

<70> The term "the nucleotide encoding LRS or functional equivalents thereof" may have a nucleic acid encoding a polypeptide having the amino acid sequence of SEQ ID NO: 1 or a polypeptide having the amino acid sequence homology of at least 70% to the polypeptide. The nucleic acid includes DNA, cDNA or RNA. The polynucleotide may have a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1 or an amino acid sequence homology of at least 70% to SEQ ID NO: 1. Preferably, the polynucleotide comprises the nucleotide sequence of SEQ ID NO. 2. The nucleic acid can be isolated from a natural source or be prepared by a genetic engineering method known in the art.

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<73> The term "analog" is used herein to refer to a molecule that structurally resembles a reference molecule but which has been modified in a targeted and controlled manner, by replacing a specific substituent of the reference molecule with an alternate substituent. Compared to the reference molecule, an analog would be expected, by one skilled in the art, to exhibit the same, similar, or improved utility. Synthesis and screening of analogs, to identify variants of known compounds having improved traits (such as higher binding affinity for a target molecule) is an approach that is well known in pharmaceutical chemistry.

<74> The term "homologous" when referring to proteins and/or protein sequences indicates that they are derived, naturally or artificially, from a common ancestral protein or protein sequence. Similarly, nucleic acids and/or nucleic acid sequences are homologous when they are derived, naturally or artificially, from a common ancestral nucleic acid or nucleic acid sequence.

<75>

<76> As used herein, "contacting" has its normal meaning and refers to combining two or more agents (e.g., two polypeptides) or combining agents and cells (e.g., a protein and a cell). Contacting can occur in vitro, e.g., combining two or more agents orcombining a test agent and a cell or a cell lysate in a test tube or other container. Contacting can also occur in a cell or in situ, e.g., contacting two polypeptides in a cell by coexpression in the cell of recombinant polynucleotides encoding the two polypeptides, or in a cell lysate.

<77>

<78> The term "agent" or "test agent" includes any substance, molecule, element, compound, entity, or a combination thereof. It includes, but is not limited to, e.g., protein, polypeptide, small organic molecule, polysaccharide, polynucleotide, and the like. It can be a natural product, a synthetic compound, or a chemical compound, or a combination of two or more substances. Unless otherwise specified, the terms "agent", "substance", and "compound" can be used interchangeably.

<79> More specifically, test agents that can be identified with methods of the present invention include polypeptides, beta-turn mimetics, polysaccharides, phospholipids, hormones, prostaglandins, steroids, aromatic compounds, heterocyclic compounds, benzodiazepines, oligomeric N-substituted glycines, oligocarbamates, polypeptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Some test agents are synthetic molecules, and others natural molecules. Test agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. Combinatorial libraries can be produced for many types of compound that can be synthesized in a step-by-step fashion. Large combinatorial libraries of compounds can be constructed by the encoded synthetic libraries (ESL) method described in WO 95/12608, WO 93/06121, WO 94/08051, WO 95/35503 and WO 95/30642. Peptide libraries can also be generated by phage display methods (see, e.g., Devlin, WO 91/18980). Libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts can be obtained from commercial sources or collected in the

field. Known pharmacological agents can be subject to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

<80>

<81> The test agents can be naturally occurring proteins or their fragments. Such test agents can be obtained from a natural source, e.g., a cell or tissue lysate. Libraries of polypeptide agents can also be prepared, e.g., from a cDNA library commercially available or generated with routine methods. The test agents can also be peptides, e.g., peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred and from about 7 to about 15 being particularly preferred. The peptides can be digests of naturally occurring proteins, random peptides, or "biased" random peptides.

<82>

<83> The test agents can also be "nucleic acids". Nucleic acid test agents can be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of prokaryotic or eukaryotic genomes can be similarly used as described above for proteins.

<84>

<85> In some preferred methods, the test agents are small molecules (e.g., molecules with a molecular weight of not more than about 1,000). Preferably, high throughput assays are adapted and used to screen for such small molecules. A number of assays are available for such screening, e.g., as described in Schultz (1998) Bioorg Med Chem Lett 8:2409-2414; The present inventorsller (1997) Mol Divers. 3:61-70; Fernandes (1998) Curr Opin Chem Biol 2:597-603; and Sittampalam (1997) Curr Opin Chem Biol 1:384-91.

<86>

<87> The library of the inventive method of screening test agent may be prepared based on the structural research of LRS or a fragment or analog thereof. These structural research makes it possible to identify test agent which may bind to LRS.

<88>

<89> The three-dimensional structures of the LRS can be studied in a number of ways, e.g., crystal structure and molecular modeling. Methods of studying protein structures using x-ray crystallography are well known in the literature. See Physical Bio-chemistry, Van Holde, K. E. (Prentice-Hall, New Jersey 1971), pp. 221-239, and Physical Chemistry with Applications to the Life Sciences, D. Eisenberg & D. C. Crothers (Benjamin Cummings, Menlo Park 1979). Computer modeling of structures of LRS provides another means for designing test agents for screening LRS. Methods of molecular modeling have been described in the literature, e.g., U.S. Pat. No. 5,612,894 entitled "System and method for molecular modeling utilizing a sensitivity factor", and U.S. Pat. No. 5,583,973 entitled"Molecular modeling method and system". In addition, protein structures can also be determined by neutron diffraction and nuclear magnetic resonance (NMR). See, e.g., Physical Chemistry, 4th Ed. Moore, W. J. (Prentice-Hall, New Jersey 1972), and NMR of Proteins and Nucleic Acids, K. Wuthrich (Wiley-Interrscience, New York 1986).

<90>

<91> Hereinafter, the present invention will be described in detail.

<92>

<93> The present invention provides a method for screening agent for preventing or treating of mTORC1-mediated disease comprising the steps of:

<94> (a) contacting LRS(Leucyl tRNA synthetase), RagD and a test agent with or without the test agent;

<95> (b) comparing the binding affinity between LRS and RagD with the test agent to the binding affinity between LRS and RagD without the test agent;

<96> (c) measuring a change between the binding affinity between LRS and RagD.

<97>

<98> In the present invention, Rag protein belongs to Rag subfamily of Ras small GTPase and there are four kinds of Rag, RagA, RagB, RagC, RagD. Among them, A and B are ortholog of Gtr1p GTPase of yeast and C and D are ortholog of Gtr2p GTPase of yeast. RagD binds to A or B to form a dimer and mediates activation of the mTORC1 pathway by amino acids. (Trends in Biochemiccal

Sciences, 33: 565-568, 2008). Preferably, Rag may be RagD.

<99>

<100> It is known that mTOR (mammalian target of rapamycin) is related to cancer, rejection of transplant, autoimmune diseases, diabetes, obesity, cardiovascular diseases, disorders in nerve system, aging and the like (Drug Discovery Today, 12;112-124, 2007). Accordingly, mTOR mediated diseases of the present invention may be cancer, autoimmune diseases, diabetes, obesity, cardiovascular diseases.

<101>

<102> More particularly, the cancer comprises, but are not limited to, malignant melanoma, leukaemia, colon cancer, lung cancer, liver cancer, stomach cancer, esophagus cancer, pancreatic cancer, gall bladder cancer, kidney cancer, bladder cancer, prostate cancer, testis cancer, cervical cancer, endometrial carcinoma, choriocarcinoma, ovarian cancer, breast cancer, thyroid cancer, brain tumor, head or neck cancer, skin cancer, lymphoma, and it also comprises B-cell neoplasms such as precursor B-cell neoplasm, T-cell and NK-cell neoplasm such as precursor T-cell neoplasm and Hodgkin lymphoma (Hodgkin disease) such as Classical Hodgkin lymphoma.

<103>

<104> Various biochemical and molecular biology techniques or assays well known in the art can be employed to practice the present invention. Such techniques are described in, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, N.Y., Second (1989) and Third (2000) Editions; and Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York (1987-1999).

<105>

<106> Preferably, the test agent is first assayed for their ability to modulate a biological activity of LRS (the first assay step). Particularly, in the first step, modulating agents that modulate a biological activity of an the said polypeptide may be identified by assaying a biological activity of isolated LRS in the presence of a test agent. More preferably, the present invention may comprise:

<107> (a) contacting test agents with LRS in the presence of a test agent; and

<108> (b) measuring activity of LRS and selecting a testing agent which changes activity of LRS.

<109>

<110> More preferably, the present invention may comprise:

<111> (a) contacting LRS(Leucyl tRNA synthetase), RagD and a test agent with or without the test agent;

<112> (b) measuing a binding affinity between LRS and RagD with or without the test agent;

<113> (c) comparing the binding affinity between LRS and RagD with the test agent to the binding affinity between LRS and RagD without the test agent;

<114> (d) measuring a change between the binding affinity between LRS and RagD with the test agent to the binding affinity between LRS and RagD without the test agent.

<115>

<116> Modulation of different biological activities of LRS can be assayed in the first step. For example, a test agent can be assayed for activity to modulate expression level of LRS, e.g., transcription or translation. The test agent can also be assayed for activities in modulating cellular level or stability of LRS, e.g., post-translational modification or proteolysis.

<117> Test agents that increase a biological activity of LRS by the first assay step are identified, the test agents are then subject to further testing whether the test agents have ability to bind Rag, more exactly RagD (or RagD GTPase), in the presence of LRS (the second testing step).

<118>

<119> In both the first step and the second step, an intact LRS and subunits or their fragments, analogs, or functional derivatives can be used. The fragments that can be employed in these assays usually retain one or more of the biological activities of LRS. And fusion proteins containing such fragments or analogs can also be used for the screening of test agents. Functional derivatives of LRS usually have amino acid deletions and/or

insertions and/or substitutions while maintaining one or more of the bioactivities and therefore can also be used in practicing the screening methods of the present invention.

<120>

<121> A variety of the well-known techniques can be used to identify test agents that modulate LRS. Preferably, the test agents are screened with a cell based assay system. For example, in a typical cell based assay (i.e., the second screening step), activity of the reporter gene (i.e., enzyme activity) is measured in the presence of test agent, and then compared the activity of the reporter gene in the absence of test agent. The reporter gene can encode any detectable polypeptide (response or reporter polypeptide) known in the art, e.g., detectable by fluorescence or phosphorescence or by virtue of its possessing an enzymatic activity. The detectable response polypeptide can be, e.g., luciferase, alpha-glucuronidase, alpha-galactosidase, chloramphenicol acetyl transferase, green fluorescent protein, enhanced green fluorescent protein, and the human secreted alkaline phosphatase.

<122>

<123> In the cell-based assays, the test agent (e.g., a peptide or a polypeptide) can also be expressed from a different vector that is also present in the host cell. In some methods, a library of test agents is encoded by a library of such vectors (e.g., a cDNA library; see the Example below). Such libraries can be generated using methods well known in the art (see, e.g., Sambrook et al. and Ausubel et al., supra) or obtained from a variety of commercial sources.

<124>

<125> In addition to cell based assays described above, it can also be screened with non-cell based methods. These methods include, e.g., mobility shift DNA-binding assays, methylation and uracil interference assays, DNase and hydroxy radical footprinting analysis, fluorescence polarization, and UV crosslinking or chemical cross-linkers. For a general overview, see, e.g., Ausubel et al., supra (chapter 12, DNA-Protein Interactions). One technique

for isolating co-associating proteins, including nucleic acid and DNA/RNA binding proteins, includes use of UV crosslinking or chemical cross-linkers, including e.g., cleavable cross-linkers dithiobis (succinimidylpropionate) and 3,3'-dithiobis (sulfosuccinimidyl-propionate); see, e.g., McLaughlin, Am. J. Hum. Genet., 59:561-569, 1996; Tang, Biochemistry, 35:8216-8225, 1996; Lingner, Proc. Natl. Acad. Sci. U.S.A., 93:10712, 1996; and Chodosh, Mol. Cell. Biol., 6:4723-4733, 1986.

<126>

<127> **Fist assay step: Screening test agents that modulate LRS(Optional)**

<128>

<129> A number of assay systems can be employed to screen test agents for modulators of LRS. As noted above, the screening can utilize an in vitro assay system or a cell-based assay system. In this screening step, test agents can be screened for binding to LRS, altering cellular level of LRS, or modulating other biological activities of LRS.

<130>

<131> 1) Screening of test agents that bind LRS

<132> In the first screening step some methods, binding of a test agent to LRS is determined. For example, it can be assayed by a number of methods including e.g., labeled in vitro protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, functional assays (phosphorylation assays, etc.), and the like. See, e.g., U.S. Pat. Nos. 4,366,241; 4,376,110; 4,517,288; and 4,837,168; and also Bevan et al., Trends in Biotechnology 13:115-122, 1995; Ecker et al., Bio/Technology 13:351-360, 1995; and Hodgson, Bio/Technology 10:973-980, 1992. The test agent can be identified by detecting a direct binding to LRS, e.g., co-immunoprecipitation with LRS by an antibody directed to LRS. The test agent can also be identified by detecting a signal that indicates that the agent binds to LRS, e.g., fluorescence quenching.

<133>

<134> Competition assays provide a suitable format for identifying test agents that specifically bind to LRS. In such formats, test agents are

screened in competition with a compound already known to bind to LRS. The known binding compound can be a synthetic compound. It can also be an antibody, which specifically recognizes LRS polypeptide, e.g., a monoclonal antibody directed against LRS. If the test agent inhibits binding of the compound known to bind LRS, then the test agent also binds LRS.

<135> Numerous types of competitive binding assays are known, for example: solid phase direct or indirect radioimmunoassay (RIA), solid phase direct or indirect enzyme immunoassay (EIA), sandwich competition assay (see Stahli et al., Methods in Enzymology 9:242-253 (1983)); solid phase direct biotin-avidin EIA (see Kirkland et al., J. Immunol. 137:3614-3619 (1986)); solid phase direct labeled assay, solid phase direct labeled sandwich assay (see Harlow and Lane, "Antibodies, A Laboratory Manual," Cold Spring Harbor Press (1988)); solid phase direct label RIA using .sup.125I label (see Morel et al., Mol. Immunol. 25(1):7-15 (1988)); solid phase direct biotin-avidin EIA (Cheung et al., Virology 176:546-552 (1990)); and direct labeled RIA (Moldenhauer et al., Scand. J. Immunol. 32:77-82 (1990)). Typically, such an assay involves the use of purified polypeptide bound to a solid surface or cells bearing either of these, an unlabelled test agent and a labeled reference compound. Competitive inhibition is measured by determining the amount of label bound to the solid surface or cells in the presence of the test agent. Usually the test agent is present in excess. Modulating agents identified by competition assay include agents binding to the same epitope as the reference compound and agents binding to an adjacent epitope sufficiently proximal to the epitope bound by the reference compound for steric hindrance to occur. Usually, when a competing agent is present in excess, it will inhibit specific binding of a reference compound to a common target polypeptide by at least 50 or 75%.

<136>

<137> The screening assays can be either in insoluble or soluble formats. One example of the insoluble assays is to immobilize LRS or its fragments onto a solid phase matrix. The solid phase matrix is then put in contact with test agents, for an interval sufficient to allow the test agents to bind.

Following washing away any unbound material from the solid phase matrix, the presence of the agent bound to the solid phase allows identification of the agent. The methods can further include the step of eluting the bound agent from the solid phase matrix, thereby isolating the agent. Alternatively, other than immobilizing LRS, the test agents are bound to the solid matrix and the LRS is then added.

<138> Soluble assays include some of the combinatory libraries screening methods described above. Under the soluble assay formats, neither the test agents nor LRS are bound to a solid support. Binding of LRS or fragment thereof to a test agent can be determined by, e.g., changes in fluorescence of either LRS or the test agents, or both. Fluorescence may be intrinsic or conferred by labeling either component with a fluorophor.

<139> In some binding assays, either LRS, the test agent, or a third molecule (e.g., an antibody against LRS) can be provided as labeled entities, i.e., covalently attached or linked to a detectable label or group, or cross-linkable group, to facilitate identification, detection and quantification of the polypeptide in a given situation. These detectable groups can comprise a detectable polypeptide group, e.g., an assayable enzyme or antibody epitope. Alternatively, the detectable group can be selected from a variety of other detectable groups or labels, such as radiolabels (e.g., 125I, 32P, 35S) or a chemiluminescent or fluorescent group. Similarly, the detectable group can be a substrate, cofactor, inhibitor or affinity ligand.

<140>

<141> 2) Screening test agents modulating other activities of LRS

<142> Binding of a test agent to LRS provides an indication that the agent can be a modulator of LRS. It also suggests that the agent may modulate biological activity of Rag, preferably RagD or RagD GTPase. Thus, a test agent that binds to LRS can be further tested for ability to modulate activity of laminin receptor.

<143> Alternatively, a test agent that binds to LRS can be further examined to determine its activity on LRS. The existence, nature, and extent of such activity can be tested by an activity assay. Such an activity assay can

confirm that the test agent binding to LRS indeed has a modulatory activity on LRS. More often, such activity assays can be used independently to identify test agents that modulate activities of LRS (i.e., without first assaying their ability to bind to LRS). In general, such methods involve adding a test agent to a sample containing LRS in the presence or absence of other molecules or reagents which are necessary to test a biological activity of LRS and determining an alteration in the biological activity of LRS. In addition to assays for screening agents that modulate enzymatic or other biological activities of LRS, the activity assays also encompass in vitro screening and in vivo screening for alterations in expression or cellular level of LRS.

<144>

<145> Second test step : Screening agents that modulate mTORC1 activity by Rag

<146> Once a modulating agent has been identified to bind to KRS and/or to modulate a biological activity (including cellular level) of LRS, it can be tested for ability to modulate mTORC1 activity by Rag or further be tested whether there is ability of preventing or treating mTORC1 mediated diseases such as cancer. Modulation of the modulating agent is typically tested in the presence of LRS. When a cell-based screening system is employed, LRS can be expressed from an expression vector that has been introduced into a host cell. Alternatively, LRS can be supplied endogenously by the host cell in the screening system.

<147>

<148> Meanwhile, the present invention provides a method of reducing cell size compared to the control group comprising inhibiting expression of intracellular LRS.

<149>

<150> mTORC1 is known to regulate cell size (Fingar DC, Salama S, Tsou C, Harlow E, Blenis J. Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev. **16 (2002), pp. 1472-1487**). According to the relationship between LRS and mTORC1 which is

identified by the present inventors, the present inventor confirmed that suppression of LRS expression results reduction of cell size. As a result, it is confirmed that the cells suppressed LRS showed smaller in size compared to those of the control group (Fig. 2e upper and Fig. 2f).

<151>

<152> The suppression of intracellular LRS is regulated with various well known methods in the art. The suppression of intracellular LRS may be controlled, but not limited thereto, through transforming the cells with the vectors comprising polyncleotides encoding antisense RNA or interference RNA of LRS operably linked to a promoter.

<153>

<154>

<155> The "promoter" means a DNA sequence regulating the expression of nucleic acid sequence operably linked to the promoter in a specific host cell, and the term "operably linked" means that one nucleic acid fragment is linked to other nucleic acid fragment so that the function or expression thereof is affected by the other nucleic acid fragment. Additionally, the promoter may further comprise an operator sequence for controlling transcription, a sequence encoding a suitable mRNA ribosome-binding site, and sequences controlling the termination transcription and translation. The promoter may be constitutive promoter which constitutively induces the expression of a target gene, or inducible promoter which induces the expression of a target gene at a specific site and a specific time.

<156>

<157> The cells of the present invention may be cells having signal transduction system mediated by mTORC1.

<158>

<159> The present invention also provides a method for screening agent for preventing or treating of mTORC1-mediated disease comprising the steps of:

<160> (a) contacting LRS(Leucyl tRNA synthetase), RagD and a test agent with or without the test agent;

<161> (b) comparing the binding affinity between LRS and RagD with the test

agent to the binding affinity between LRS and RagD without the test agent;

<162> (c) indentifying the test agent inhibiting the binding affinity between LRS and RagD.

<163>

<164> More preferably, the present invention may comprise:

<165> (a) contacting LRS(Leucyl tRNA synthetase), RagD and a test agent with or without the test agent;

<166> (b) measuing a binding affinity between LRS and RagD with or without the test agent;

<167> (c) comparing the binding affinity between LRS and RagD with the test agent to the binding affinity between LRS and RagD without the test agent;

<168> (d) indentifying the test agent inhibiting the binding affinity between LRS and RagD.

<169>

<170> As mentioned above, the screening method can be performed using various methods known in the art, including labeled invitroprotein-protein binding assays(invitrofull-down assays), EMSA(electrophoretic mobility shift assays), immuno ssays for protein binding, functional assays(phosphorylation assays,etc.), yeast-2hybrid assay, assays of non-immune immunoprecipitations, immunoprecipitation/Westernblot assays, immuno-co-localization assays, and the like.

<171> For example, yeast two hybrid assays can be performed using yeast expressing LRS and RagD, or parts or homologues of these proteins, fused with the DNA-binding domain of bacteria repressor LexA or yeast GAL4 and the transactivation domain of yeast GAL4 protein, respectively (KIM, M. J. et al., Nat. Gent., 34:330-336, 2003). The binding between LRS and RagD reconstitutes a transactivator that induces the expression of a reporter gene under the control by a promoter having a regulatory sequence bound to the DNA-binding domain of LexA protein or GAL4.

<172> As described above, the reporter gene may be any gene known in the art, which encodes a detectable polypeptide. For example, chloramphenicol acetyltransferase (CAT), luciferase, β-galactosidase, β-glucosidase,

alkaline phosphatase, green fluorescent protein (GFP), etc. may be used. If the level of binding between LRS and RagD, or parts or homologues of these proteins is stimulated or enhanced by a test agent, the expression of the reporter gene will be increased compared to that under a normal condition. Conversely, if the level of the binding is suppressed or attenuated by a test agent, the reporter gene will not be expressed or expressed less than that under a normal condition.

<173> Further, a reporter gene encoding a protein which enables growth of yeast (i.e., when the reporter gene is not expressed, the growth of yeast is inhibited) may be selected. For example, the reporter genes may be auxotropic genes encoding enzymes involved in a biosynthesis pathway for amino acids or nitrogenous bases (e.g., yeast genes such as ADE3, HIS3, etc. or equivalent genes from other species). If the interaction between LRS and RagD, or parts or homologues of these proteins, expressed in this system, is inhibited or attenuated by a test agent, the reporter gene will not be expressed.

<174> Accordingly, the growth of yeast is arrested or becomes slowed down under the above conditions. This effect caused by the expression of the reporter gene may be observed by the naked eye or a device (e.g., a microscope).

<175>

<176> Hereafter, the figures of the present invention will be described.

<177>

<178> Figure 1 shows that Leucyl-tRNA synthetase (LRS) is an mTOR-associated protein. (A) Subcellular fractionation of LRS. Each fraction was subjected to immunoblotting with anti-LRS, IRS, MRS, and mTOR antibodies. YY1 and LAMP2 were used as nucleus and endomembrane markers, respectively. Nuc, nucleus; PM, plasma membrane; EM, endomembrane; Cyt, cytosol. (B) Immunofluorescence staining of LRS in HeLa cells. HeLa cells were reacted with anti-LRS, anti-calnexin (ER marker), anti-GM130 (Golgi marker), anti-LAMP2 (lysosome marker), or anti-EEA1 (endosome marker) antibodies and visualized with alexa 488-conjugated and alexa 594-conjugated secondary antibodies, respectively.

(C) Lysosomal localization of LRS. 293T cells were starved for amino acids for 1 hour and re-stimulated with amino acids for 5 min. Cells were fractionated with lysosome isolation kit (Sigma-Aldrich). Lysosomal proteins were immunoblotted with anti-mTOR, anti-Raptor, anti-LRS, and anti-LAMP1 antibodies. (D) 293T cell lysates were immunoprecipitated with anti-mTOR antibody and the co-precipitated LRS and Raptor were determined by immunoblotting. Goat IgG, anti-mTOR antibody plus blocking epitope peptide and anti-actin antibody were used as negative controls. (E) 293T cells were transfected with control plasmid (EV), myc-tagged LRS, or MRS. Cell lysates were immunoprecipitated with anti-myc antibody and the co-precipitated mTOR and Raptor were determined by immunoblotting. (F) Co-localization of LRS with mTOR in HeLa cells. Cells were reacted with anti-LRS, anti-MRS, anti-IRS, and anti-mTOR antibodies and visualized with alexa 488-conjugated and alexa 594-conjugated secondary antibodies, respectively. (G) Co-localization of LRS with Raptor in HeLa cells. Cells were reacted with anti-LRS, anti-MRS, anti-IRS, and anti-Raptor antibodies and visualized with alexa 488-conjugated and alexa 594-conjugated secondary antibodies, respectively.

<179>

<180> Figure 2 shows the effect of LRS on mTORC1 activation and lysosomal localization, cell size, and autophagy. (A) 293T cells were transfected with 6 kinds of LRS siRNA for 48 hours, and amino acid dependent S6K phosphorylation was determined by immunoblotting. (B) 293T cells were transfected with control, mTOR, LRS, IRS, MRS, or VRS siRNA for 48 hours, and amino acid-dependent S6K phosphorylation was determined by immunoblotting. (C) 293T cells were transfected with control, LRS, IRS, MRS, or VRS siRNA for 48 hours, and leucine-dependent S6K phosphorylation was determined by immunoblotting. (D) 293T cells were transfected with control or LRS siRNA for 48 hours, and cells were starved for amino acids for 1 hour and re-stimulated with amino acids for 5 min. Cells were fractionated with lysosome isolation kit (Sigma-Aldrich). Lysosomal proteins were immunoblotted with anti-mTOR, anti-Raptor, anti-LRS, and anti-LAMP2 antibodies (E) Cell size distributions of cells transfected with control, LRS, IRS, VRS, or MRS siRNA. (F) Cell size

distributions (FSC of G1 cells) from (E) were quantified. ($n=3$ and $p<0.0001$). (G) Effects of LRS downregulation on LC3 cleavage. 293T cells were transfected with the indicated siRNAs for 48 hours and starved for leucine for 2 hours. Cell lysates were prepared and LC3-I and -II were determined by immunoblotting with anti-LC3 antibody. Autophagy induction is indicated by the ratio of LC3 II/LC3 I. (H) After co-transfection of EGFP-LC3 with the indicated siRNAs, Cells were starved for leucine and serum for 2 hours. Accumulation of EGFP-LC3 in puncta was monitored. (I) Quantitative analysis of EGFP-LC3 puncta from (F). At least eight cells were analyzed per sample. The data represents mean±S.D. LRS siRNA and mTOR siRNA-transfected cells showed statistically significant increase of LC3 puncta per cell compared to control siRNA-transfected cells ($p=0.0005$ and $p<0.0001$, respectively).

<181>

<182> Figure 3 shows direct interaction of LRS with RagD GTPase. (A) Purified GST-LRS were incubated with protein extracts from 293T cells transfected with HA-tagged RagA, RagB, RagC, RagD, Rheb1, GβL, Raptor, or mTOR and the co-precipitation of HA-tagged proteins were determined by immunoblotting with anti-HA antibody. Inputs are the amount of 10% protein extract used. (B) 293T cells were transfected with the indicated cDNAs in expression vectors. Cell lysates were prepared, and cell lysates and HA-tagged immunoprecipitates were analyzed by immunoblotting with anti-myc or anti-HA antibodies. WCL means whole cell lysate. (C) After co-transfection of HA-tagged RagD with myc-tagged LRS, IRS, MRS, or EPRS, cell lysates were immunoprecipitated with anti-HA antibody and the co-precipitated myc-tagged protein was determined by immunoblotting with anti-myc antibody. (D)293T cells were transfected with the indicated cDNAs in expression vectors. Cell lysates were prepared, and cell lysates and myc-tagged immunoprecipitates were analyzed by immunoblotting with anti-FLAG, anti-myc, or anti-HA antibodies. (E) 293T cells were transfected with control or myc-RagD/HA-RagB. Cell lysates were immunoprecipitated with anti-myc antibody and the myc-tagged immunoprecipitates were analyzed by immunoblotting with anti-HA, anti-LRS or anti-Raptor antibodies. (F) Each of the functional domains of RagD GTPase was

expressed as GST fusion protein. Purified GST-RagD proteins were incubated with myc-tagged LRS, and the co-precipitation of myc-LRS was determined by immunoblotting with anti-myc antibody. (G) After co-transfection of FLAG tagged LRS with HA-tagged RagB, and myc-tagged WT or mutated RagD, cell lysates were immunoprecipitated with anti-myc antibody and the co-precipitated LRS and RagB were determined by immunoblotting with anti-FLAG and anti-HA antibodies. (H) Each of the C-terminal fragments of LRS was expressed as GST fusion protein. Purified GST-LRS proteins were incubated with HARagD-transfected cell lysates, and the co-precipitation of HA-RagD was determined by immunoblotting with anti-HA antibody. (I) After co-transfection of HA-tagged RagD with myc-tagged WT or mutated LRS, cell lysates were immunoprecipitated with anti-HA antibody and the coprecipitated LRS was determined by immunoblotting with anti-myc antibody.

<183>

<184> Figure 4 shows that LRS forms a molecular complex with RagD and Raptor in amino acid-dependent manner. (A) Amino acid-stimulated interaction of LRS with RagD and Raptor. 293T cells were starved for amino acids for 1 hour and re-stimulated with amino acids for 5 min. Cell lysates were immunoprecipitated with anti-Raptor antibody and the co-precipitated LRS and RagD were determined by immunoblotting with anti-LRS and anti-RagD antibody. (B) 293T cells were transfected with the indicated cDNAs in expression vectors. Cells were starved for leucine for 1 hour and restimulated with leucine for 5 min. Cell lysates and myc-tagged immunoprecipitaes were analyzed by immunoblotting with anti-FLAG and anti-myc antibodies. (C) 293T cells were transfected with the indicated cDNAs in expression vectors. Cells were starved for amino acids for 1 hour and restimulated with amino acids for 5 min. Cell lysates and HA-tagged immunoprecipitaes were analyzed by immunoblotting with anti-myc, anti-FLAG, and anti-HA antibodies. (D) LRS is necessary for the complex formation of RagD with Raptor. 293T cells were transfected with control or LRS siRNAs for 48 hours. Cells were starved for amino acids for 1 hour and re-stimulated with amino acids for 5 min. Cell lysates were immunoprecipitated with anti-Raptor antibody and the

precipitates were analyzed by immunoblotting with anti-LRS and anti-RagD antibodies.

<185>

<186> Figure 5 shows LRS functions as a leucine receptor for mTORC1 signaling. (A) Primary sequence alignment of N-terminal region of several species leucyl-tRNA synthetases. The class 1a conserved HIGH motif, which is important to ATP binding, is boxed in grey. (B) Leucylations by LRS WT and mutants (F50A/Y52A, F50A, and Y52A) were carried out by using 4 μM tRNALeu and 50 nM enzymes. (C) 293T cells were transfected with LRS WT or F50A/Y52A mutant for 24 hours and then starved for amino acids for 1 hour and re-stimulated with amino acids for 5 min. Leucine-dependent S6K phosphorylation was determined by immunoblotting. (D) After co-transfection of HA-RagD/myc-RagB with myc-tagged WT or mutated LRS, cell lysates were immunoprecipitated with anti-HA antibody, and the co-precipitated LRS was determined by immunoblotting with anti-myc antibody. (E) 293T cells were transfected with the indicated cDNAs in expression vectors. Cell lysates were immunoprecipitated with anti-HA antibody, and the co-precipitated LRS and Raptor were determined by immunoblotting with anti-myc antibody.

<187>

<188> Figure 6 shows interaction of LRS with RagD in a manner that depends on the nucleotide binding state of RagD. Effects of expressing the indicated proteins on the phosphorylation of S6K in response to starvation and stimulation with (A) amino acids or (B) leucine. Cell lystaes were prepared from 293T cells starved for 1 hour of (A) amino acids or (B) leucine and then stimulated with amino acids or leucine for 5 min. (C) Purified GST or GST-LRS protein was incubated with HA-RagD transfected cell lysates in the presence of GDPβS or GTPγS. The co-precipitated RagD was determined by immunoblotting with anti-HA antibody. (D) Purified GST or GST-LRS protein was incubated with myc-tagged RagD WT, S77L (GDP), or Q121L (GTP) transfected cell lysates. The coprecipitated RagD was determined by immunoblotting with anti-myc antibody. (E) After co-transfection of FLAG-tagged LRS with myc-tagged WT or mutated RagD, cell lysates were immunoprecipitated with anti-myc

antibody and the co-precipitated LRS and RagD were determined by immunoblotting with anti-FLAG and anti-myc antibodies. (F) 293T cells were transfected with the indicated cDNAs in expression vectors. Cell lysates were prepared, and cell lysates and myc-tagged immunoprecipitates were analyzed by immunoblotting with anti-FLAG or anti-myc antibody. (G) 293T cells were transfected with the indicated cDNAs in expression vectors. Cell lysates were prepared, and cell lysates and myc-tagged immunoprecipitates were analyzed by immunoblotting with anti-FLAG, anti-HA, or anti-myc antibodies.

<189>

<190> Figure 7 shows that LRS acts as a GTPase-activating protein for RagD. (A) The indicated amounts of Histagged LRS (759-1176 a.a) fragment were incubated with 0.15 μM RagD for 20 min at 37oC. The error bars represent mean S.D. (n = 3). (B) His-tagged LRS fragment (0.3 μM) were incubated with RagD for the indicated times. The error bars represent mean S.D. (n = 3). (C) Sequence alignment of putative GAP motif of LRS with several species ADP-ribosylation factor-GAPs (ARF-GAPs). Conserved residues are black. h, hydrophobic; s, Gly or Ala; x, any residue. hs, Homo sapiens; rn, Rattus norvegicus; dm, Drosophila melanogaster; sc, Saccharomyces cerevisiae; ss, Sus scrofa. (D) Effects of LRS WT and mutants on GTP hydrolysis of RagD. Purified LRS WT (759-1176 a.a) fragment or mutants (H844A, R845A) fragments were incubated with RagD for 20 min at 37 ° C. The error bars represent mean S.D. (n = 3). (E) 293T cells were transfected with LRS WT or GAP mutants (H844A, R845A) for 24 hours and then starved for amino acids for 1 hour and re-stimulated with amino acids for 5 min. Leucine-dependent S6K phosphorylation was determined by immunoblotting. (F) Schematic representation for role of LRS in amino acid signaling to mTORC1.

<191>

<192> Figure 8 shows that Time-lapse confocal live cell imaging of lysosomal localization of LRS. 293T cells were transfected with EGFP-LRS (A) or EGFP control (B) expression vector for 36 hours and then stained with LysoTracker Red DND-99 (Molecular Probes) for 30min. Cells were starved for leucine for 50 min and re-stimulated with 0.8 mM leucine for 12 min. During leucine

starvation, cells were monitored at 10 min intervals and then monitored at 1 min intervals after re-stimulation with leucine. (C) Quantitative analysis showing leucine-dependent lysosomal localization of LRS.

<193>

<194> Figure 9 shows that The effect of LRS knockdown on leucine- or leucine analogues-stimulated S6K phosphorylation. (A) The effect of leucine analogues on leucine-stimulated S6K phosphorylation. 293T cells were starved for 1 hour of leucine and preincubated with either 0.8 or 8 mM leucinol or leucine amide. After 5 min, 0.8 mM leucine was added. After 5min incubation, cells were harvested and S6K phosphorylation was determined by immunoblotting. (B) HeLa cells were starved for 1 hour of leucine and preincubated with either 0.8 or 8 mM leucinol or leucine amide. After 5 min, 0.8 mM leucine was added. After 5min incubation, cells were harvested and S6K phosphorylation was determined by immunoblotting. (C) 293T cells were transfected with control or LRS siRNAs for 48 hours and leucine- or leucine analogues-stimulated S6K phosphorylation was determined by immunoblotting. Concentration of L-leucine and leucine amide was 0.8 mM and concentration of D-leucine, norleucine, and leucinol was 8 mM. (D) HeLa cells were transfected with control or LRS siRNAs for 48 hours and leucine- or leucine analogues-stimulated S6K phosphorylation was determined by immunoblotting. Concentration of L-leucine and leucine amide was 0.8 mM and concentration of D-leucine, norleucine, and leucinol was 8 mM.

<195>

<196> Figure 10 shows that LRS is involved in mTORC1 activation in tRNA-independent manner. (A) Effect of tRNA on in vitro LRS-RagD binding. 293T cell lysates were incubated with purified GST or GST-fused LRS in the presence of the combinations of leucine (0.1 mM), ATP (0.1 mM), and $tRNA^{Leu}$ (25 μg). The precipitated RagD was determined by immunoblotting with anti-RagD antibody. (B) Primary sequence alignment of several species leucyl-tRNA synthetases. The class 1a conserved KMSKS motif, which is important to tRNA binding, is boxed in black. (C) Leucylation and ATP-PPi exchange activities by LRS K716A/K719A mutant were carried out. (D) Effect of K716A/K719A mutant

on RagD binding. 293T cells were transfected with myc-tagged LRS WT or mutant, and HA-tagged RagD for 24 hours. Cell lysates were immunoprecipitated with anti-HA antibody and the co-precipitated LRS and RagD were determined by immunoblotting with anti-myc and anti-myc antibodies. (E) 293T cells were transfected with the indicated cDNAs for 24 hours and leucine-dependent S6K phosphorylation was determined by immunoblotting.

<197>

<198> Figure 11 shows that Leucine activates mTORC1 in dose-dependent manner. (A) 293T cells were treated with leucine at the indicated concentration for 5 min. Leucine-dependent S6K phosphorylation was determined by immunoblotting. (B) Quantitation of p-S6K band in (A). EC50 of leucine stimulation of mTORC1 was about 80mM. (C) Kinetic parameters for leucylation and ATP-PPi exchange activity of WT LRS.

<199>

<200> Figure 12 shows that Effect of amino acid stimulation on GTP/GDP status of RagD. Wild type and mutant forms of myc-RagD were transfected into 293T cells and the cells were labeled with 32P-phosphate. Myv-RagD was immunoprecipitated and the bound nucleotides were eluted and analyzed by TLC. EV means control empty vector.

<201>

<202> Figure 13 shows ELISA assay result for measuring binding affinity between LRS and RagD. (A) ELISA result showing that RagD protein bind to LRS-(1-1176) in a dose dependent manner(96 well plate is coated with LRS-(1-1176)(500ng/ml in carbonate buffer). GST is negative control. RagD protein is used as GST-RagD form. Primary antibody is anti-GST antibody(Z-5, 1:100 dilution). Secondary antibody is HRP-conjugated anti-rabbit antibody(1:5000 dilution)). (B) The comparison result of binding affinity for control(GST), RagD and RagD+LRS-(759-1176) on 96 well plate coated with LRS-(1-1176). LRS-(759-1176) decrease the binding affinity of RagD on plate coated with LRS-(1-1176).

<203>

【Advantageous Effects】

<205> The present invention relates to novel use of LRS and provides a method of screening an agent for preventing or treating mTORC1 mediated diseases nd a method of reducing cell size compared to the control group. Accordingly, the screening methods can be used for developing novel treating agent for diseases such as cancer.

<206>

【Description of Drawings】

<207> Figure 1 shows that Leucyl-tRNA synthetase (LRS) is an mTOR-associated protein.

<208> Figure 2 shows the effect of LRS on mTORC1 activation and lysosomal localization, cell size, and autophagy.

<209> Figure 3 shows direct interaction of LRS with RagD GTPase.

<210> Figure 4 shows that LRS forms a molecular complex with RagD and Raptor in amino acid-dependent manner.

<211> Figure 5 shows LRS functions as a leucine receptor for mTORC1 signaling.

<212> Figure 6 shows interaction of LRS with RagD in a manner that depends on the nucleotide binding state of RagD.

<213> Figure 7 shows that LRS acts as a GTPase-activating protein for RagD.

<214> Figure 8 shows that Time-lapse confocal live cell imaging of lysosomal localization of LRS.

<215> Figure 9 shows that The effect of LRS knockdown on leucine- or leucine analogues-stimulated S6K phosphorylation.

<216> Figure 10 shows that LRS is involved in mTORC1 activation in tRNA-independent manner.

<217> Figure 11 shows that Leucine activates mTORC1 in dose-dependent manner.

<218> Figure 12 shows that Effect of amino acid stimulation on GTP/GDP status of RagD.

<219> Figure 13 shows ELISA assay result for measuring binding affinity between LRS and RagD.

<220>

【Mode for Invention】

<221> **<Methods>**

<222> **1. Cell Culture and Reagents**

<223> HEK293T cells and HeLa cells were grown in DMEM (Hyclone) containing 10% fetal bovine serum and antibiotics. CHO-tSH1 cells were kind gift from Dr. Mike Clemens. CHO-tSH1 cells were grown in

<224> in Dulbecco' s modified Eagle' s medium/Nutrient Mixture Ham' s F-12 (Sigma) supplemented with 9% (v/v) fetal bovine serum, 100 mg/ml streptomycin sulfate, and 100 units/ml penicillin G at 34 ° C. The tsH1 line contains a temperature-sensitive leucyl-tRNA synthetase that is active at 34 ° C but defective at 39.5 ° C. Amino acid deprivation and addition experiments were performed using DMEM (+AA) and DPBS containg 25mM glucose, 1mM sodium pyruvate, 1x MEM vitamins (Invitrogen) (-AA). L-Leucine, D-Leucine, L-Leucinamide, Leucinol, Norleucine (Sigma Aldrich) were dissolved in PBS [pH7.6] and treated at the concentration of 0.8mM or 8mM. [^{32}P]pyrophosphate (80.70mCi/mL) was obtained from PerkinElmer Life Sciences. [^{3}H]Leucine was obtained from American Radiolabeled Chemicals. RagC and RagD siRNAs were from Invitrogen.

<225>

<226> **2. Amino acids or Leucine starvation and stimulation of cells**

<227> For leucine depletion, cells were rinsed with leucine-free DMEM twice, and incubated in leucine-free DMEM for 60minutes, and stimulated with 52mg/ml leucine for 5-60 minutes. For amino acid starvation, Cells were rinsed with and incubated in DPBS containing containg 25mM glucose, 1mM sodium pyruvate, 1x MEM vitamins for 60minutes, and replaced with and incubated in DMEM for 5-60 minutes.

<228>

<229> **3. Antibodies and plasmids**

<230> Antibodies were obtained from the following sources: anti-mTOR blocking peptide, antibodies to mTOR (for IP), HA, c-MYC, laminA, as well as HRP-labeled anti-mouse, anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, phospho-S473 Akt/PKB,

phospho-T308 Akt, S6K1, Akt, LC3, RagC, RagD from Cell Signaling Technology; antibodies to LAMP2 (H4B4), mTOR (Y391), Raptor from Abcam (for Western) antibodies to Raptor, mTOR, FLAG from Invitrogen (for Western, IF, IP) mouse monoclonal antibody to mTOR, clone 2ID8.2 from Millipore (for Western) LysoTracker Red DND-99 from Molecular Probe; monoclonal mouse anti-calnexin antibody from BD Pharmigen; HA-tagged mTORC1 components constructs including RagA, RagB, RagC, RagD, mTOR, Raptor, GbL, Rheb1 were generously provided by Dr. D-H. Kim (University of Minnesota) and Dr. E.J. Kim (Catholic University of Daegu). All other DNA constructs including LRS, IRS, MRS, and EPRS were laboratory stocks. Transfection was performed using Geneporter system (Gene Therapy System).

<231>

<232> **4. Preparation of Cell Lysate and Immunoprecipitation**

<233> Cells were dissolved in the lysis buffer containing 1% Triton X-100, 40 mM HEPES (pH7.4), 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate and protease inhibitor cocktail, and the lysates were centrifuged at 13,000rpm for 30minutes. We then fractionated 20 μgof the extracted proteins by SDS-PAGE. For immunoprecipitations, the cells were lysed (50 mM Tris-HCL (pH 7.4), 10 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM $MgCl_2$, 0.1% CHAPS, and 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) and the primary antibodies were added to the lysates and incubated with rotation for 2hr at 4° C. 50% slurry of protein agarose G-sepharose was then added and the incubation continued for an additional 4hr. After washing three times with the ice-cold lysis buffer, the precipitates were dissolved in the SDS sample buffer and separated by SDS-PAGE.

<234>

<235> **5. Immunofluorescence Staining**

<236> Cells were seeded onto cover slips and fixed with 100% aceton for 5min at -20°C. After incubation with PBS blocking buffer containing 2% BSA, the cells were incubated with the primary antibody (1:100) for 2h and Alexa488- or Alexa595-conjugated secondary antibody (1:1,000) in blocking buffer containing 2% BSA and 10% fetal bovine serum for 1h. Nuclei were stained with

DAPI. After wasing with PBS, the cells were mounted and observed via confocal laser scanning microscope (Nikon A1R).

<237>

<238> **6. Mutations of LRS and RagD**

<239> Point mutations in LRS and RagD were generated via site-directed mutagenesis by using a QuikChange kit (Stratagene), and the mutants were confirmed by DNA sequencing.

<240>

<241> **7. Subcellular Fractionation**

<242> Cells were seeded and cultivated to 70% confluence. The cells were washed by amino-acid free media 3times and normal media added for 5min. And then lysosome fraction was extracted using lysosome isolation kit (SIGMA-ALDRICH) following the manufacturer's instruction. Briefly, extraction buffer added to the cells, cells were broken using Dounce homogenizer with 20 strokes. After centrifugation the sample at 1,000 g for 10min, the supernatant was further centrifuged at 20,000 g for 20min. The pellet was re-suspended in extraction buffer (lysosomal fraction).

<243>

<244> **8. Time-Lapse Live Cell Imaging**

<245> Cell imaging was performed using a confocal laser scanning microscope (Nikon A1R). All images were captured with a CFI Plan Apochromat VC objective lens (60x/1.40 Oil) at a resolution of 512x512 using digital zooming. All images were stored as ND or JPG2000 files, which are standard formats for Nikon A1Rsi confocal microscope.

<246>

<247> **9. Image Analysis**

<248> Cell images were used for quantitative analysis. This process was performed with Nikon imaging software NIS-element AR 64-bit version 3.00. Image file formats were transferred from ND or JPG2000 files to ICS or TIFF formats using NIS-element software. Quantitative analysis of lysosomal colocalization was performed using the "Time-measurement" tool for "Region Of Intensity"(ROI) in the NIS-element software. After ROIs were defined

according to localization of LysoTracker, localization of other components was measured using the defined ROIs. RelativeFluorescence Units (RFU) was normalized against the initial intensity of ROI, and then plotted using OriginPro 7.5.For the quantitative analysis of colocalization, we also used ImageJ colocalization finder plugin. The index of co-localization corresponds to the mean±S.D of the overlap coefficient (R)*100 obtained for more than 10 cells for each co-labeling. The ratio between green and red signals is ranged between 0.8 and 1.2.

<249>

<250> **10. *In Vitro* Pull-down Assay**

<251> Recombinant LRS or RagD fragment proteins were expressed as GSTfusion proteins and purified by glutathione sepharose. The interactions between RagD fragments and myc-LRS overexpressed cell lysates or between LRS fragments and HA-RagD overexpressed cell lysates were tested using *in vitro* binding assays. Binding assay was conducted in 25 mM Tris-HCl buffer (pH7.4) containing 10 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM EGTA, and 0.5% Triton X-100.

<252>

<253> **11. Cell Size Determinations**

<254> For measurement of cell size using forward scatter units (FSC) with unfixed cells. 293T cells were plated, washed once with PBS, and resuspended in PBS containing 0.1% serum, 5mM EDTA, 5ng/ml propidium iodide (PI; Sigma). Samples were analyzed by FACS analysis (FACS caliber; Becton Dickinson) for cell size (FSC).The mean of FSC of G1 phase cells was determined.

<255>

<256> **12. ATP-PPi exchange assay**

<257> The ATP-PPi exchange reaction was performed in a reaction mixture containing 2mM [^{32}P]pyrophosphate (PPi) (80.70mCi/mL) , 50mM HEPES-KOH (pH7.6), 2mM $MgCl_2$, 8mM KF, 4mM ATP, various concentration of leucine and 25nM of LRS. Reaction were initiated with enzyme and conducted in a 37° C heat block. Aliquots (10ul) were taken at different time points and the reactions were stopped using 1ml of quenching buffer (50mM NaPPi, 3.5% $HClO_4$, 2%

activated charcoal). The charcoal suspension was filtered through a Whatman GF/A filter, washed four times with 5ml of water and rinsed with 10ml of 100% ethanol. The charcoal powder on the filters was dried, and the synthesized [^{32}P]ATP was counted using a scintillation counter (Beckman Coulter).

<258>

<259> **13. Leucylation assay**

<260> The leucylation assay was carried out in a buffer containing 1mM spermine, 50mM HEPES-KOH (pH7.6), 25mM KCl, 5mM $MgCl_2$, 4mM ATP, 2mg/ml bovine liver $tRNA^{Leu}$, various concentration of [^{3}H]Leu (60Ci/mmol) and 10-100nM of LRS. Reactions were initiated with enzyme and conducted in a 37° C heat block. Aliquots (10ul) were taken at different time points and quenched on Whatman filter pads that were presoaked with 5% trichloroacetic acid (TCA). The pads were washed three times for 10min each with cold 5% TCA, once with cold 100% ethanol. The washed pads were then dried. Radioactivity was quantified in a scintillation counter (Beckman Coulter)

<261>

<262> **14.In Vitro GTPase Assay**

<263> GTPase assays were conducted in assay buffer (20 mM piperazine-*N,N9*-bis(2-ethanesulfonic acid), 20 mM HEPES, 5 mM $MgCl_2$, 125 mM NaCl, 5 mM KCl at pH 7.0, 0.5 mM GTP) containing 0.1% bovine serum albumin in a final volume of 200 ml using GTPase assay kit (Innova Biosciences) according to manufacturer ' s instruction.

<264>

<265> **15. In vivo GTPase Assay**

<266> 293T cells were washed with phosphate-free DMEM and incubated with 1 ml of phosphate-free DMEM for 60 min. Cells were then incubated with 100 μCi of [^{32}P]phosphate/ml for 8 hr. After labeling, cells were lysed with prechilled lysis buffer (0.5% NP-40, 50 mM Tris [pH 7.5], 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol(DTT), 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/ml, 10 μg of aprotinin/ml) for 30 min on ice. The lysates were

then centrifuged at 12,000 X g for 15 min at 4° C. The supernatant (160 μl) was transferred to a fresh tube and 16 μl of NaCl (500 mM) was added to inhibit GAP activity. Myc-RagD was then immunoprecipitated with anti-Myc antibody and protein-G sepharose bead for 1 hr at 4° C. The beads were washed with wash buffer 1 (50 mM Tris [pH 8.0], 500 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5% Triton X-100) three times at 4° C and then washed with wash buffer 2 (50 mM Tris [pH 8.0], 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100) three times at 4° C. The myc-RagD-bound nucleotideswere eluted with 20 μl of elution buffer (2 mM EDTA, 0.2% sodium dodecyl sulfate, 1 mM GDP, 1 mM GTP) at 68° C for 10 min. The eluted nucleotides were applied onto polyethyleneimine cellulose plates (Baker-flex) and developed in 0.75 M KH_2PO_4[pH 3.4] solution. GTP and GDP was visualized and quantified by a phosphoimager.

<267>

<268> **16. RT-PCR**

<269> RNAs were extracted from the cultivated cells using RNA extraction kit (RNeasy Mini). Total RNA (1 mg) was used for reverse transcription with 1 ml dNTP (2.5 mM each), 1 ml random hexamer (5 mM) and 200 unit of MMLV reverse transcriptase in 20 ml reaction. After 1: 4 dilution of the cDNA solution, 1 ml was used for PCR reaction (Takara).

<270> RagC, Sense : 5' -TCGGCTACGGCGTGGAGGAG-3' (SEQ ID NO: 19),

<271> RagC, Antisense : 5' -CGCCCCCCGGACCACAGCCA-3' (SEQ ID NO: 20),

<272> RagD, Sense : 5' -TGAGCTGGTGGGGCTAGCGG-3' (SEQ ID NO: 21),

<273> RagD, Antisense : 5' -GGGTCACTGAAGTCCAGAACTC-3' (SEQ ID NO: 22).

<274>

<275> **17. ELISA assay for measuring binding affinity between LRS and RagD.**

<276> In order to examine whether the LRS and RagD proteins bind to each other, primers represented by SEQ ID NOs: 1 to 8 were prepared.

<277>

<278> 【Table 1】

<279> Primer set used for LRS fragment synthesis

<280>

Primers	Sequence	Seq. No.
LRS-(1-1176) sense	GGA ATT CCA TAT GGC GGA AAG AAA AGG AAC AGC CAA AGT	4
LRS-(1-1176) antisense	CGG GAT CCT TAA TGA ACC AGA TAG ATT ATT GTA TCG	5
LRS-(759-1176) sense	GGA ATT CCA TAT GGC AGA TGC AGG TAT TCT CCG	6
RagD-(1-400) sense	CGG GAT CCA TGA GCC AGG TGC TGG GGA AG	7
RagD-(1-400) antisense	CGC TCG AGC TAC AGC AGC ACT CTA GGG GTC	8

<281> First, each fragment of LRS was synthesized by PCR amplification using the cDNA of LRS as a template and a primer set (Table 1) specific for each fragment. The PCR amplification was performed under the following conditions: predenaturation of the template DNA at 95 ℃ for 2 min; and then 30 cycles, each consisting of 30 sec at 95 ℃, 30 sec at 56 ℃, and 3 min and 30 sec at 72 ℃; followed by final extension at 72 ℃ for 10 min. Each of the PCR products was digested with NdeI and BamHI and ligated into a pET16a vector (Novagen) digested with the same restriction enzymes. E.coli BL21(DE3) cells were transformed with the vector and cultured to induce the expression of peptides. Each peptide expressed as a His-tag fusion protein was purified by Ni-chelating agarose. To remove lipopolysaccharide, the protein solution was dialyzed in pyrogen-free buffer (10mM potassium phosphate buffer, pH 6.0, 100 mM sodium chloride). After dialysis, the protein was loaded to polymyxin resin (Bio-Rad) equilibrated with the same buffer, after which it was incubated for 20 minutes, and then eluted, thereby preparing each fragment of LRS.

<282> A fragment of RagD was synthesized by PCR amplification using the cDNA of LRS as a template and a primer set (Table 1) specific for each fragment. The PCR amplification was performed under the following conditions: predenaturation of the template DNA at 95 ℃ for 2 min; and then 30 cycles, each consisting of 30 sec at 95 ℃, 30 sec at 56 ℃, and 1 min and 30 sec at 72 ℃; followed by final extension at 72 ℃ for 5 min. Each of the PCR products was digested with BamHI and XhoI and ligated into a pGEX4T3 vector

(GE healthcare) digested with the same restriction enzymes. E. coli BL21 cells were transformed with the vector and cultured to induce the expression of peptides. Each peptide expressed as a GST-tag fusion protein was purified by GSH agarose. To remove lipopolysaccharide, the protein solution was dialyzed in pyrogen-free buffer (10mM potassium phosphate buffer, pH 6.0, 100 mM sodium chloride). After dialysis, the protein was loaded to polymyxin resin (Bio-Rad) equilibrated with the same buffer, after which it was incubated for 20 minutes, and then eluted, thereby preparing each fragment of RagD.

<283> Purified GST-RagD was incubated with His-LRS-(1-1176) coated on a plate at various concentrations and was subjected to ELISA using anti-GST antibody(Z-5, 1:100 dilution) and HRP-conjugated anti-rabbit antibody(1:5000 dilution), and the results of the ELISA are shown in FIG. 13.

<284>

<285> **<Results>**

<286> **1. Identification of LRS as an mTOR-associated protein**

<287> To investigate whether LRS has an additional activity, separated from its catalytic role within the ARS complex, we first examined its subcellular distribution. Cell fractionation analysis showed that large amount of LRS was localized to endomembrane fraction with mTOR as well as cytosol where IRS and MRS were mainly found (Figure 1a). Immunofluorescence analysis showed that LRS was well co-localized with the ER marker, calnexin, and the endosome marker, EEA1. Minor amount of LRS showed co-localization with lysosome marker, LAMP2, but little with the Golgi marker, GM130 (Figure 1b). Previous report showed that amino acids induce the movement of mTORC1 to lysosomal membranes (Sancak et al., 2010). Thus, we examined the lysosomal localization of LRS upon amino acid stimulation (Figure 1c and figure 8). We biochemically analyzed the lysosomal localization of LRS using sucrose gradient fractionation. Amino acid depletion decreased lysosomal mTOR, Raptor, and LRS, but amino acid supplement clearly induced lysosomal translocation of LRS as well as mTOR and Raptor (Figure 1c). Quantitative analysis of lysosomal colocalization was performed using time lapse confocal live cell imaging.

Leucine depletion decreased colocalization of EGFP-LRS with lysosomal marker, LysoTracker. However, leucine supplementation recovered the colocalization of

<288> EGFP-LRS with lysosome within 10 min (Figure 8a). Colocalization of control EGFP with LysoTracker showed little change by the depletion or addition of leucine (Figure 8b). The intensity of colocalization was quantified by NIS-element software. Lysosomal localization of LRS gradually decreased for 50 min after leucine depletion, but lysosomal localization of LRS was rapidly induced within 10 min after leucine supplementation (Figure 8c). These results suggest that leucine induces the movement of LRS to lysosomal fraction as well as mTORC1.

<289>

<290> We investigated whether LRS would form a complex with mTORC1. HEK293T cell lysates were immunoprecipitated with anti-goat IgG, anti-mTOR, and anti-actin antibodies and then the immunoprecipitates were analyzed with anti-LRS, anti-IRS, anti-mTOR, and anti-Raptor antibodies. LRS was coprecipitated with mTOR and Raptor only in mTOR immunoprecipitates (Figure 1d). The intensity of LRS band decreased significantly when competitive epitope peptide against mTOR antibody was added after cell lysis, indicating that LRS specifically interacted with mTORC1. Interestingly, isoleucyl-tRNA syntheatse (IRS) was not detected within mTOR immunoprecipitates, consistent with Figure 1a, implying that LRS in mTORC1 should be different from that within ARS complex. To examine this possibility, HEK293T cells were transfected with myc-tagged LRS or methionyl-tRNA synthetase (MRS) and cell lysates were immunoprecipitated with anti-myc antibody and then the immunoprecipitates were analyzed with anti-mTOR and anti-Raptor antibodies. mTOR and Raptor were only detected in LRS immunoprecipitates (Figure 1e). Furthermore, LRS was well co-localized with mTOR (Figure 1f) and with Raptor (Figure 1g). Consistent with Figure 1d and E, MRS and IRS were not co-localized with mTORC1 (Figure 1f and G). These results suggest that LRS in mTORC1 is different from that bound to ARS complex.

<291>

<292> **2. Effect of LRS on mTORC1 activation, lysosomal localization, cell**

size, and autophagy

<293> To see the importance of LRS in the control of mTORC1 activation, we monitored the effect of LRS knockdown on mTORC1 activation using 6 different kinds of LRS siRNA (Table 2). All siRNAs significantly suppressed the expression of LRS and inhibited amino acid-induced S6K phosphorylation (Figure 2a). Next, we monitored the specific involvement of LRS on mTORC1 activation. Knockdown of mTOR and LRS, but not of IRS, MRS, or valyl-tRNA synthetase (VRS), significantly inhibited amino acid-induced S6K phosphorylation (Table 3, Figure 2b and 2c). Also, LRS specifically mediated leucine-induced S6K phosphorylation (Figure 2c). These results suggest that endogenous LRS is involved in amino acid-induced mTORC1 activation pathway. Next, we monitored the effect of LRS knockdown on amino acid-induced lysosomal localization of mTORC1. While amino acid supplement induced lysosomal localization of mTOR and Raptor in si-controltransfected cells, lysosomal localization of mTOR and Raptor was not observed in si-LRS-transfected cells (Figure 2d). Therefore, this result suggests that LRS mediates amino acid-induced lysosomal localization of mTORC1.

<294>

<295> mTORC1 is known to control cell size (Fingar DC, Salama S, Tsou C, Harlow E, Blenis J. Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev. **16 (2002), pp 1472-1487**). If mTORC1 pathway is inhibited, it leads to a reduction in cell size. Consistent with LRS being a mediator of amino acid signaling to mTORC1, LRS-suppressed cells were smaller in size than control cells (Figure 2e upper panel). However, IRS, VRS, and MRS knockdown had no effect on cell size (Figure 2e lower panel). Quantitative analysis of cell size regulation was performed and we confirmed that rapamycin treatment or LRS knockdown specifically reduced cell size (Figure 2f). In addition, autophagy, a process normally inhibited by mTORC1 pathway, was activated in LRS downregulated cells, as detected by the increase of LC3-II/LC-3-I ratio (Figure 2g). Also, downregulation of endogenous LRS specifically activate autophagy, as detected by an increase compared to in control cells in the size and the number of

GFP-LC3-II puncta (Figure 2g and 2i). These results suggest the specific role of LRS in the regulation of mTORC1 pathway.

<296>

<297> 【Table 2】

<298> siRNA sequences targeting LRS

<299>

Location	siRNA sequence (5' to 3')	Seq. No.
105 (5' UTR)	CAGCAGGUGUGAAGCGUGUGCUUUA	9
195 (5' UTR)	CCAGGGUCAUUGUCGUGGAUUUGCA	10
396 (CDS)	CAUAUAUGAAUGGACGCCUUCAUUU	11
792 (CDS)	CGCCACUGGCUAUUCAGGAUUUAAA	12
1312 (CDS)	UGGUGCAUCACUUUCUGCACCUUUA	13
3844 (5' UTR)	CAGAACCUUAGGCUGGACCUAAAUA	14

<300> 【Table 3】

<301> siRNA sequences targeting mTOR, IRS, VRS, and MRS

<302>

Target	siRNA sequence (5' to 3')	Seq. No.
mTOR	GGAAGUACCCUACUUUGCUUGAGGU	15
IRS	GGAAGCCAGAUUGUCAGCCCUCUAU	16
VRS	AGAAGAGGAUGUCAUGACCGGUCUC	17
MRS	CUACCGCUGGUUUAACAUUUCGUUU	18

<303> **3. LRS directly interacts with RagD GTPase**

<304> We investigated whether LRS would interact with the key components of mTORC1 pathway. The interaction was tested by *in vitro* pull-down assay using GST-LRS and HA-tagged mTORC1 molecules. GST-LRS specifically co-precipitated with HA-RagD, but not with others (Figure 3a), indicating their specific and direct interaction. Co-immunoprecipitation assay also showed the specific interaction of LRS with RagD, but not with RagA, RagB, and RagC (Figure 3b), despite its sequence homology with RagC (81.1% identity) (Sekiguchi T, Hirose E, Nakashima N, Ii M, Nishimoto T. Novel G proteins, Rag C and Rag D, interact with GTP-binding proteins, Rag A and Rag B. J Biol. Chem. **276 (2001), pp7246-7257**). Next, we examined specific interaction between LRS and RagD. RagD only interacted with LRS, but not with IRS, MRS, or EPRS (Figure 3c). Since Rag GTPases form a heterodimer for mTORC1 activation, we examined which heterodimer of Rag GTPases is a specific binding partner for LRS. Consistently, LRS showed specific interaction with RagD heterodimer, but not

with RagC heterodimer (Figure 3d). Interestingly, RagB/RagD heterodimer showed higher affinity for LRS than RagA/RagD heterodimer (Figure 3d). We also investigated whether endogenous LRS can form a complex with Rag B/RagD heterodimer and found that endogenous LRS also can form a complex with Raptor and RagB/RagD heterodimer (Figure 3e). Next, we determined the peptide region of RagD that is involved in the interaction with LRS by *in vitro* pull-down assay. The myc-tagged LRS was precipitated with GST-RagD fragments. The peptides spanning aa 1-400 and 230-400 of RagD interacted with LRS (Figure 3f). As shown in Figure 3b, LRS interacted with RagD, but not with RagC in spite of sequence homology. Therefore, we hypothesized that the C-terminal 230-400 region of RagD may confer the binding specificity for LRS. Within 230-400 region of RagD, 371-400 region of RagD only has sequence variability compared with that of RagC. To confirm this hypothesis, we prepared different point mutants of RagD at the amino acid position of 379, 383, 385, 388, and 389, and tested whether these mutations would affect the interaction with RagB or LRS. While the RagD mutants at 379, 383, and 389 were coimmunoprecipitated with LRS, the mutants at 385 and 388 lost their binding capability (Figure 3g), further confirming the interaction of the C-terminal region of RagD with LRS. In contrast, all the mutants retained the binding ability to RagB, indicating that RagD has different binding sites for RagB and LRS. Conversely, we determined the peptide region of LRS that is involved in the interaction with RagD by *in vitro* pull-down assay. The HA-tagged RagD-transfected cell lysates were pull-downed with GST-fused LRS fragments. The peptide spanning aa 951-1176 of LRS interacted with RagD (data not shown), implying the C-terminal region of LRS for the interaction with RagD. To further confirm the RagD-binding site of LRS, we prepared different deletion mutants of LRS, incubated them with HA-tagged RagD and tested which deletion would affect the interaction with RagD. While the peptides spanning 759-1120, 759-1176, and 951-1176 of LRS bound to RagD, the peptide spanning 971-1176 lost its binding capability (Figure 3h), implying the peptide region spanning 951-971 of LRS for the interaction with RagD. To confirm this conclusion, we also prepared alanine substitutions at S953/V954, R956/K957, and N969/K970

located in the RagD binding region of LRS. We then tested whether any of these mutations would affect the interaction with RagD. In the immunoprecipitation assay, while the two mutants (S953A/V954A and R956A/K957A) showed interaction with RagB/RagD heterodimer, N969A/K970A mutant lost its binding capability (Figure 3i).

<305>

<306>

<307> **4. LRS forms molecular complex with RagD and Raptor in amino acid-dependent manner.**

<308> We investigated whether Raptor would interact with RagD and LRS in amino acid-dependent manner. 293T cells were treated with amino acid for 5min and protein lysates were prepared to perform co-immunoprecipitation of Raptor with LRS and RagD. The interaction of Raptor with RagD and LRS increased after amino acid supplementation (Figure 4a). Next, we examined whether LRS would interact with RagD in amino acid-dependent manner. While RagA/RagD heterodimer formation would not be affected by amino acid, LRS interacted with RagA/RagD heterodimer in amino acid-dependent manner (Figure 4b), implying that LRS-RagD interaction is also amino acid-dependent.

<309>

<310> To assess the importance of LRS for the interaction of RagD with Raptor, we compared the RagD-Raptor interaction in the presence or absence of exogenous LRS. In the absence of exogenous LRS, RagD slightly interacted with Raptor upon amino acid supplementation. However, overexpression of LRS significantly enhanced amino acid-induced RagD-Raptor binding (Figure 4c). Conversely, downregulation of endogenous LRS weakened amino acid-induced RagD-Raptor binding (Figure 4d), suggesting that LRS augments RagD-Raptor binding.

<311>

<312> **5. LRS functions as a leucine sensor for mTORC1 signaling**

<313> LRS is a class I aminoacyl-tRNA synthetase and has a conserved HIGH motif, which serves as an ATP-binding site (Figure 5a). Previous structural study demonstrated that the large hydrophobic pocket to accommodate the

substrate leucine side chain is formed by Phe50 and Tyr52, highly conserved residues among leucyl-tRNA synthetases of different species (Figure 5a) (Cusack S, Yaremchuk A, Tukalo M. The 2Å crystal structure of leucyl-tRNA synthetase and its complex with a leucyl-adenylate analogue. EMBO J. **19(2000), pp.2351-2361**). The α-amino group of leucine makes hydrogen bond to the carbonyl oxygen of Phe50 and the sulfate in leucyl-adenylate hydrogen bonds to the main chain of Tyr52 (Cusack et al., 2000). Alanine substitution of these conserved Phe50 and Tyr52 significantly suppressed leucylation activity of LRS due to the increased Km for leucine (Figure 5b and Table 4). To access the importance of leucine binding of LRS for the activation of mTORC1 and for the complex formation with RagD and Raptor, we investigated the effect of F50A/Y52A mutant of LRS on them. Leucine-induced S6K phosphorylation was enhanced by the introduction of WT LRS, but not of the F50A/Y52A mutant (Figure 5c). Also, F50A/Y52A mutant lost the ability to bind RagB/RagD heterodimer (Figure 5d), and could not mediate the association of RagB/RagD heterodimer and Raptor (Figure 5d). These results clearly show that leucine sensing by LRS is critical for mTORC1 activation.

<314>

<315> 【Table 4】

<316>

Substrates	Constants	LRS WT	LRS F50A/Y52
		Leucylation	Leucylation
Leucine	K_m(mM)	0.0159±0.0004	0.536±0.063
	$K_{cat}(S^{-1})$	0.368±0.009	0.2±0.04
	$K_{cat}/Km(S^{-1}mM^{-1})$	22.9±0.57	2.71±0.232

<317> Previous efforts to identify leucine sensor for mTORC1 activation were analyzed by examining the effects of leucine analogues and emperature-sensitive leucyl-tRNA synthetase mutant (Lynch CJ, Fox HL, Vary TC, Jefferson LS, Kimball SR. Regulation of amino acid-sensitive TOR signaling by leucine analogues in adipocytes. J Cell Biochem. **77 (2000), pp.234-251**; Wang RC, Levine B. Autophagy in cellular growth control. FEBS Lett. **584(2010), pp1417-1426**; Xin Y, Li W, First EA. The 'KMSKS' motif in Tyrosyl-tRNA synthetase participates in the initial binding of tRNA. Tyr. Biochemistry **39(2002), pp.**

340-347).

<318>

<319> Through the structure-activity relationship studies, leucine analogues with a modified amino group, a modified carboxylic group, charged R groups, or bulkier aliphatic R groups lost mTORC1 agonist activity (Lynch CJ, Fox HL, Vary TC, Jefferson LS, Kimball SR. Regulation of amino acid-sensitive TOR signaling by leucine analogues in adipocytes. J Cell Biochem. **77 (2000), pp.234-251**). However, since the effects of leucine analogues on leucylation or ATP-PPi exchange activity of LRS were not clearly determined and leucine analogues have different effects on mTORC1, further investigations are needed. In this study, we analyzed the effect of leucine analogues, leucinol and leucinamide, on leucine-induced S6K phosphorylation. The leucine analogue, leucinol, is a competitior against leucine (Lynch CJ, Fox HL, Vary TC, Jefferson LS, Kimball SR. Regulation of amino acid-sensitive TOR signaling by leucine analogues in adipocytes. J Cell Biochem. **77 (2000), pp.234-251**), thereby inhibiting leucylation (Vaughan MH, Hansen BS. Control of initiation of protein synthesis in human cells. Evidence for a role of uncharged transfer rebonucleic acid, J Biol Chem. **248 (1973), pp. 7087-7096**). Interestingly, leucinol itself had no effect on S6K phosphorylation, but inhibited leucine-induced S6K phosphorylation in dose-dependent manner in two

<320> different cell types (Figure 9a and 9b). In contrast, leucinamide itself significantly induced S6K phosphorylation and these effects were further increased in the presence of L-leucine (Figure 9a and 9b). Although the effects of leucine analogues varied, their effects were commonly disappeared by suppression of LRS (Figure 9c and 9d), further illustrating the significance of LRS for amino acid signaling.

<321>

<322> To investigate whether tRNA charging activity of LRS is involved in RagD binding and mTORC1 activation, we performed *in vitro* competition assay using LRS substrates, leucine, ATP, and $tRNA^{Leu}$.

<323> Interestingly, $tRNA^{Leu}$, but not ATP, significantly competed with RagD for LRS binding (Figure 10a), suggesting that RagD and tRNA show exclusive

access to LRS *in vitro*. To prove that interaction between LRS and RagD is independent of the leucylation activity, we made alanine mutant (K716A/K719A) of the conserved KMSKS motif which is an important for tRNA binding (Figure 10b)(Hountondji C. Dessen P, Blanquet S. Sequence similarities among the family of aminoacyl-tRNA synthetases. biochimie **69 (1986), pp.1071-1078**; Xin Y, Li W, First EA. The 'KMSKS' motif in Tyrosyl-tRNA synthetase participates in the initial binding of tRNA. Tyr. Biochemistry **39(2002), pp. 340-347**). Although this mutant showed little leucylation activity, it retained the ATP-PPi exchange activity (Figure 10c). K716A/K719A mutant of LRS showed no difference from the wild type LRS its effect on RagB/RagD heterodimer formation (Figure 10d) and leucine-induced mTORC1 activation (Figure 10e), suggesting that the tRNA charging activity of LRS is not involved in mTORC1 activation.

<324>

<325> Comparison between the K_m value of leucine for LRS and the EC_{50} for leucine stimulation of mTORC1 can give a clue to the involvement of LRS in mTORC1 signaling. The EC_{50} for leucine stimulation of mTORC1 was about 80 μM in 293T cells (Figure 11a and 11b) and in HeLa cells (data not shown). In the absence of $tRNA^{Leu}$ (ATP-PPi exchange), the K_m of leucine for LRS was 143± 61 μM although the K_m of leucine for LRS was 15.9± 0.4 μM in the presence of $tRNA^{Leu}$ (leucylation) (Figure 11c). Therefore, the K_m of leucine for LRS in ATP-PPi exchange reaction is comparable to the EC_{50} for leucine stimulation of mTORC1. These results clearly support that leucine, but not tRNA, binding to LRS is involved in RagD binding and mTORC1 activation.

<326>

<327> **6. LRS interacts with GTP form of RagD**

<328> Since Rag GTPases are Ras family GTP-binding proteins, mTORC1 activation and LRS binding can be affected by the GTP/GDP binding status of Rag GTPases. Indeed, it is known that heterodimers of GTP-bound RagA or B and GDP-bound RagC or D show strong binding to mTORC1 (Sancak Y, Peterson TR,

Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* **320 (2008), pp. 1496-1501**; Kim E, Goraksha-Hicks P, Li L, Neufeld TP, Guan KL. Regulation of TORC1 by Rag GTPases in nutrient response. *Nat Cell Biol*. **10 (2008), pp. 935-945**). Among heterodimers of Rag GTPases, heterodimer of GTPbound RagB and GDP-bound RagD, which interacted strongly with mTORC1, not only activate mTORC1 pathway, but also make it insensitive to deprivation of leucine or amino acids (Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* **320 (2008), pp. 1496-1501**). Consistently, we also observed that the combination of GTP-bound RagB and GDP-bound RagD showed the highest effect on S6K phosphorylation in response to leucine and amino acids (Figure 6a and 6b).

<329>

<330> We examined whether the GTP/GDP status of RagD can affect LRS binding. HA-RagD transfected 293T cell lysates were incubated with GST or GST-LRS in the presence of GDPβS or GTPγS, followed by immunoblot analysis. GDPβS, but not GTPγS, significantly reduced the binding affinity of LRS to RagD (Figure 6c). We again performed *in vitro* binding assay using myc-tagged RagD WT, GTP-bound form (Q121L), or GDP-bound form (S77L)-transfected cells. Consistently, S77L mutant of RagD showed lower affinity for LRS (Figure 6d), indicating that interaction between LRS and RagD is controlled by the GTP/GDP cycle of RagD. Since intracellular concentration of GTP is higher than GDP (Lowy, 1993), the GTP-form (Q121L) of RagD might show the binding affinity to LRS comparable with RagD WT. We investigated whether the binding of LRS to RagD would be affected by GTP/GDP status. Different forms of Myc-RagD (WT, GTP, and GDP forms) were expressed with FLAG-LRS in 293T cells and their binding to LRS was compared by coimmunoprecipitation of LRS with RagD. RagD Q121L showed higher affinity for LRS than RagD WT, but RagD S77L had very weak binding to LRS (Figure 6e). Next, we monitored the interaction between LRS and heterodimers of RagA/B and RagD upon GTP/GDP status of Rag GTPases.

<331> Interestingly, the interaction between LRS and RagA/D or RagB/D

heterodimer was determined by GTP/GDP status of RagD, but not of RagA or RagB (Figure 6f and 6g). GTP-bound RagD, but not GDP-bound RagD, tightly interacted with LRS. These results suggest that LRS has no effect on GTP/GDP cycle of RagA or RagB and that LRS dynamically associated with GTP-bound RagD and then dissociate from GDP-bound RagD when the bound GTP is changed into GDP due to its intrinsic GTPase activity. Since GTP form of RagD is inhibitory for mTORC1 activation, LRS appears to bind the inactive Rag heterodimer in order to facilitate GTP to GDP transition, and dissociate from the active Rag heterodimer for mTORC1 activation.

<332>

<333> **7. LRS acts as GTPase-activating protein for RagD GTPase**

<334> Since LRS interacts with GTP form of RagD, but not with GDP form of RagD, we investigated whether LRS had GTPase-activating protein (GAP) function for RagD GTPase to activate mTORC1 pathway. First, we confirmed the amino acid-induced GTP/GDP status of RagD. Consistent with previous model, amino acid stimulation of cells increased the GDP form of RagD (Figure 12). In *in vitro* GTPase assay, addition of WT LRS fragment (759-1176 a.a) enhanced GTP hydrolysis by RagD GTPase in dose- and time-dependent manner (Figure 7a and 7b), indicating that LRS has intrinsic GAP activity for RagD GTPase. Through amino acid sequence alignment, we found that LRS had putative GAP motif, which was found in several Arf-GAP proteins (Figure 7c). To prove that this motif of LRS is indeed important for the GAP activity, we made alanine mutants (H844A and R845A) of putative LRS GAP motif. In *in vitro* GTPase assay, H844A and R845A mutants lost their GAP activity while WT LRS showed GAP activity (Figure 7d). Next, we examined the effect of H844A or R845A mutation on leucine-induced mTORC1 activation. While WT LRS enhanced leucine-induced S6K phosphorylation, H884A and R845A mutant lost their activities. These results clearly indicate that LRS functions as a GAP for RagD GTPase to activate mTORC1 activation. Combined together, the binding of LRS to ARS complex in the cytoplasm and to RagD GTPase in the lysosome may take place independently. Lysosomal LRS interacts with RagD and facilitates the conversion of the inactive heterodimer of Rag GTPases into the active form,

leading to the activation of mTORC1(Figure 7f).

<335>

<336> **8. ELISA assay for measuring binding affinity between LRS and RagD.**

<337> Purified GST-RagD was incubated with His-LRS-(1-1176) coated on a plate at various concentrations and was subjected to ELISA using anti-GST antibody, and the results of the ELISA are shown in FIG. 13.

<338> As a result, it was shown that RagD was bound to LRS.

<339> In addition, when His-LRS-(759-1176) was added during the process of incubating GST-RagD with His-LRS-(1-1176) coated on the plate, the binding between RagD and LRS was inhibited, suggesting that a region of residues 759-1176 of LRS is involved in binding with RagD to inhibit the binding between LRS-(1-1176) and RagD. In addition, it could be seen that such results of ELISA by the protein overlay method are results with specificity.

<340>

<341>

<342> **[Application Example 1]**

<343> **Anticancer and antitumor**

<344> It was found that a large number of human cancers (lymphoma, melanoma, breast cancer, ovarian cancer, prostate cancer, stomach cancer, and head and neck cancers) have mutant genes translated in the mTOR pathway (Guertin, D. et al., An expanding role for mTOR in cancer. Trends Mol. Med. 11(2005), pp 353-361). The test agent of the present invention, which inhibits the binding between LRS and RagD, can be used as an anticancer agent which inhibits the mTOR pathway to inhibit the occurrence of a mutant gene caused by translation in the mTOR pathway.

<345>

<346> **[Application Example 2]**

<347> **Anti-obesity**

<348> The mTOR pathway is known to be involved in lipid metabolisms, and it was found that the excessive production of lipids by the over-expression of preadipocytes leads to an increase in the activity of the mTOR pathway (Kim, J. E and Chen, J, Regulation of peroxisome proliferator-activated receptor-γ

activity by mammalian target of rapamycin and amino acids in adipogenesis, Diabetes, 52(2004), pp 1748-1756; Cho, H. et al., Regulation of adipocyte differentiation and insulin action with rapamycin. Biochem. biophys. Res. Cmmun., 321(2004), pp 942-948)). Particularly, it was found that the mTOR pathway is highly involved in the accumulation of lipids and the growth of adipocytes (Um, S. et al., Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity, Nature, 431(2004), pp 200-205). The test agent of the present invention inhibits the activity of the mTOR pathway, suggesting it is effective against excessive lipid production and lipid accumulation, which influence obesity.

<349>

<350>

【Industrial Applicability】

<351> As can be seen foregoing, the present invention provides method of screening an agent for preventing or treating mTORC1 mediated diseases and a method of reducing cell size. The method of the present invention provides novel method of regulating cell size and further the screening method of screening can be used in developing novel reagent for treatment of disease such as cancer.

【CLAIMS】

【Claim 1】

<353> Method for screening agent for preventing or treating of mTORC1-mediated disease comprising the steps of:

<354> (a) contacting LRS(Leucyl tRNA synthetase), RagD and a test agent with or without the test agent;

<355> (b) comparing the binding affinity between LRS and RagD with the test agent to the binding affinity between LRS and RagD without the test agent;

<356> (c) measuring a change between the binding affinity between LRS and RagD.

<357>

【Claim 2】

<358> The method according to claim 1, the mTORC1-mediated disease is selected from the group consisting of cancer, autoimmune disease, diabetes, obesity and cardiovascular disease.

<359>

【Claim 3】

<360> Method for decreasing size of a cell compared with size of a control or normal cell comprising the step of inhibiting LRS expression

<361>

【Claim 4】

<362> The method according to claim 3, the inhibition is performed by transforming the cell with a expression vector comprising promoter and antisense RNA or interference RNA for LRS, which is operably linked to the promoter.

<363>

【Claim 5】

<364> Method for screening agent for preventing or treating of mTORC1-mediated disease comprising the steps of:

<365> (a) contacting LRS(Leucyl tRNA synthetase), RagD and a test agent with or without the test agent;

<366> (b) comparing the binding affinity between LRS and RagD with the test agent to the binding affinity between LRS and RagD without the test agent;

<367> (c) indentifying the test agent inhibiting the binding affinity between LRS and RagD.

<368>

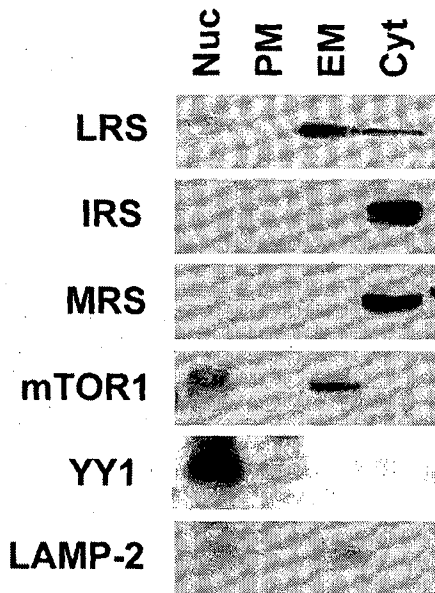
【Claim 6】

<369> The method according to claim 5, the mTORC1-mediated disease is selected from the group consisting of cancer, autoimmune disease, diabetes, obesity and cardiovascular disease.

<370>

【DRAWINGS】

【1a】



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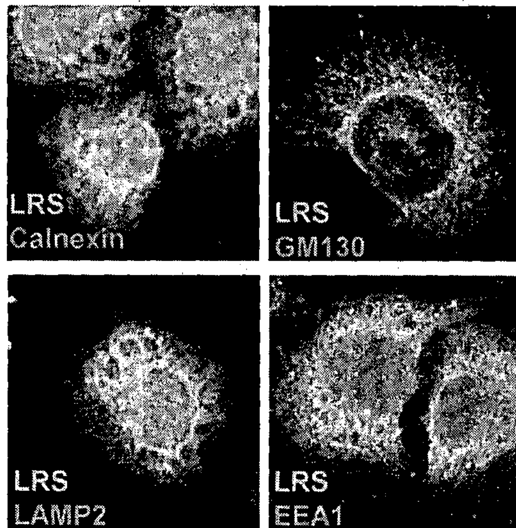

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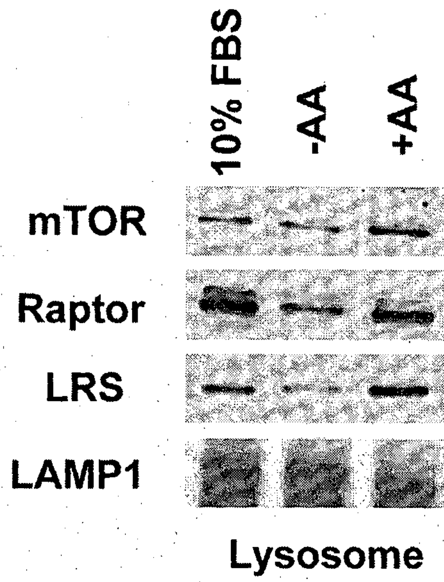

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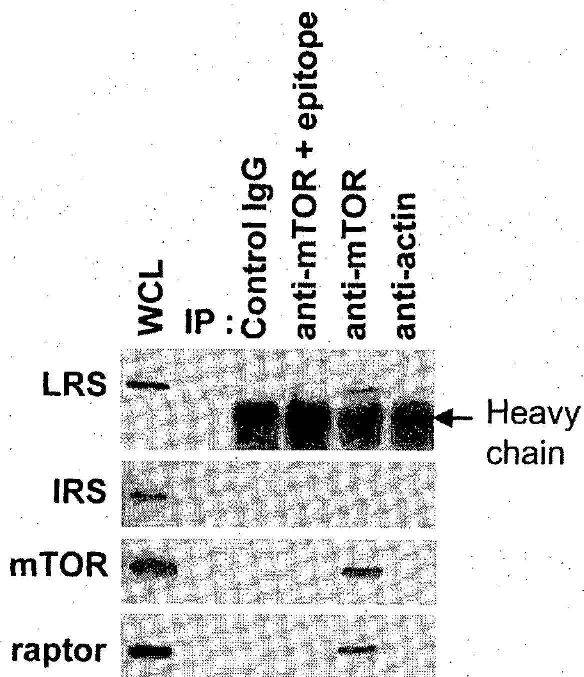

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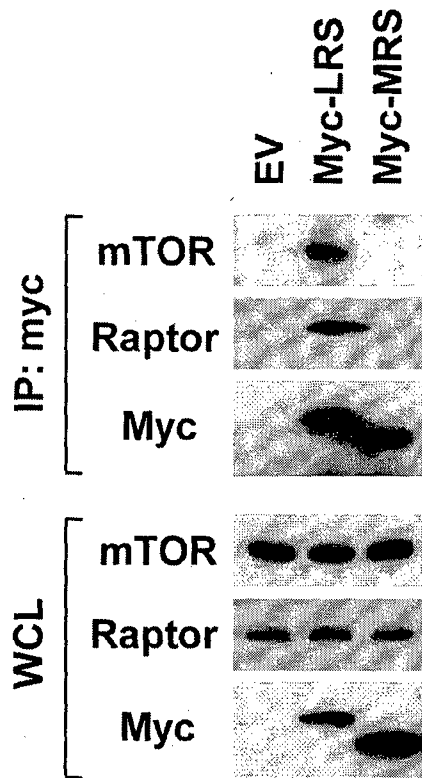

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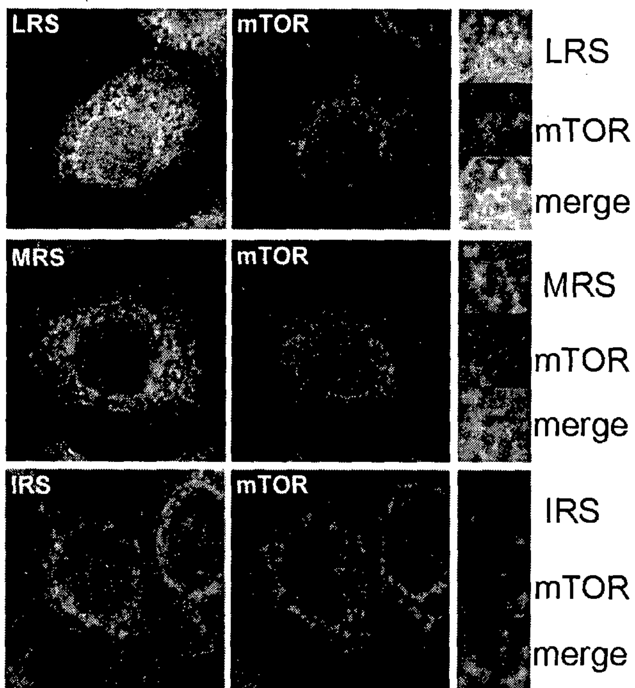


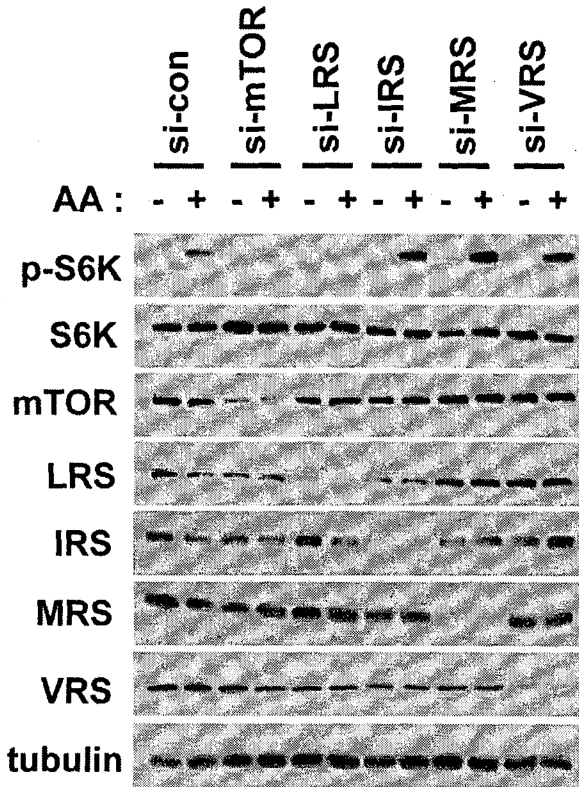
【1g】

LRS
raptor
LRS
raptor
merge
MRS
raptor
MRS
raptor
merge
IRS
raptor
IRS
raptor
merge

【2a】

- AA
+ AA
si-control
si-control
si-LRS (105)
si-LRS (195)
si-LRS (392)
si-LRS (795)
si-LRS (1312)
si-LRS (3844)
p-S6K
S6K
p-Akt
Akt
LRS
tubulin

【2b】



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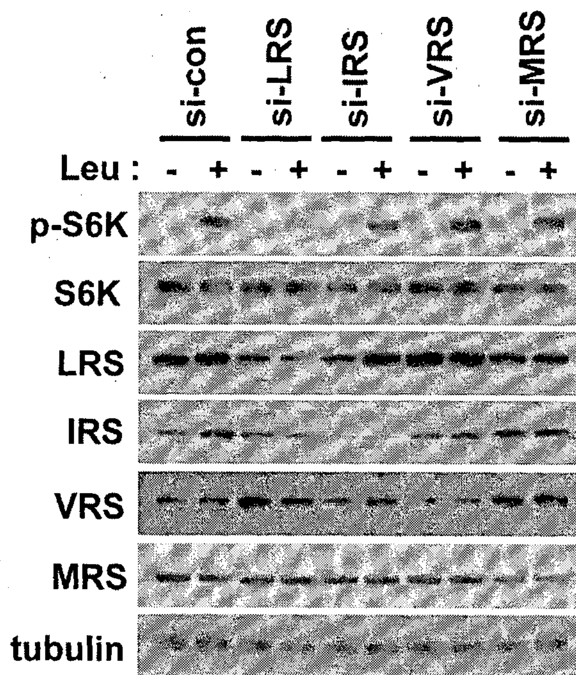

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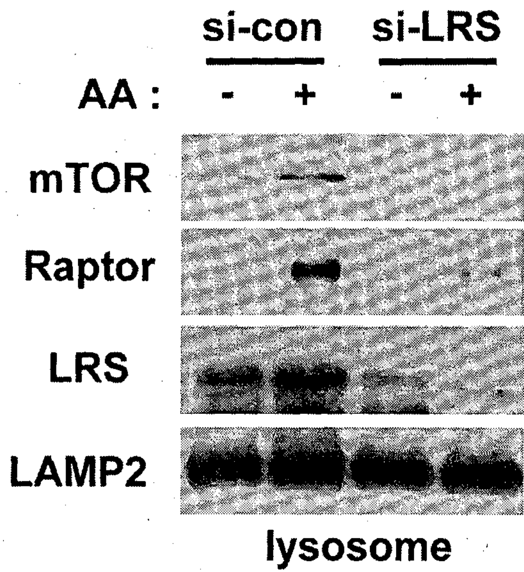

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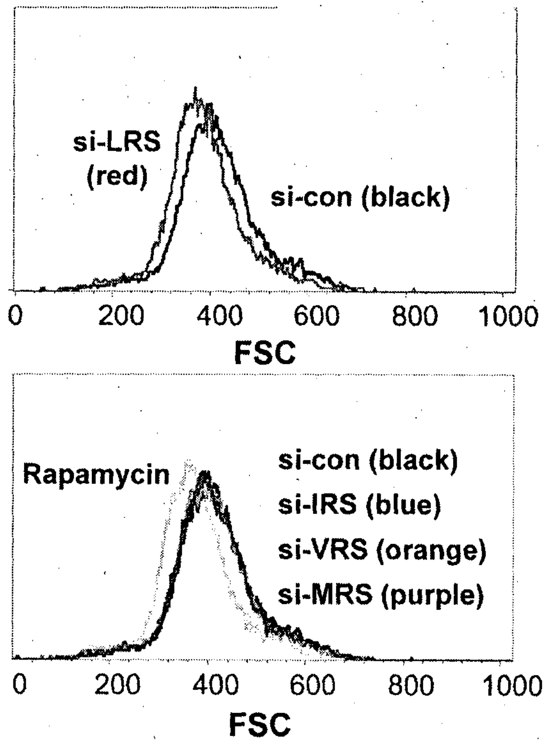

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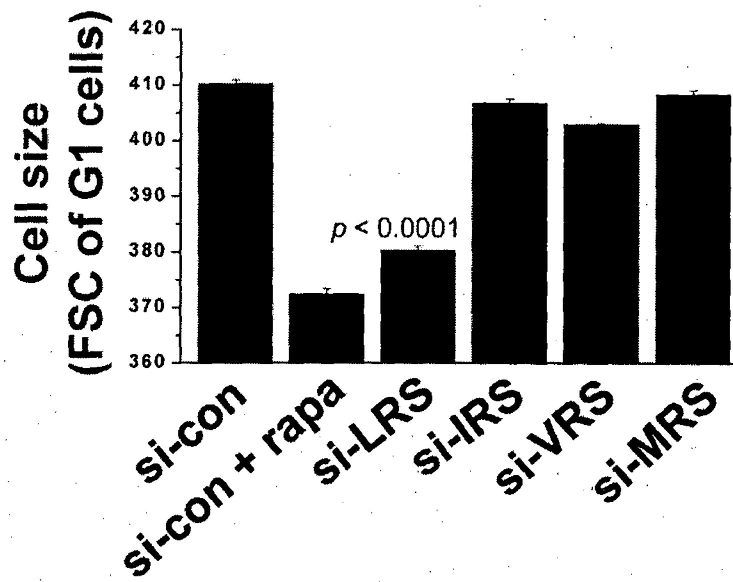

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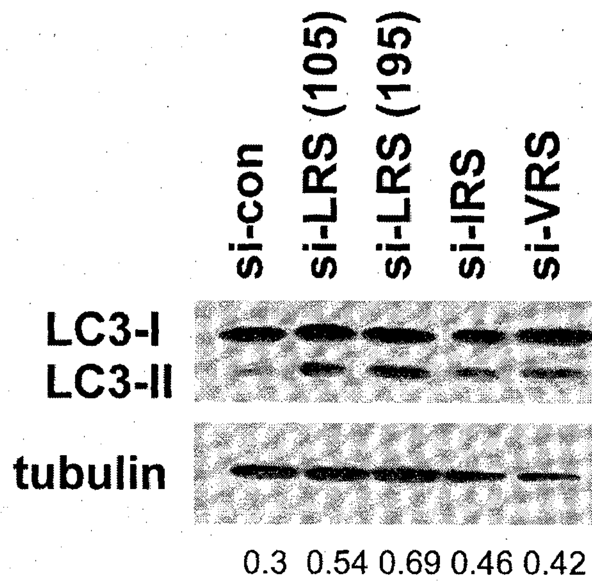

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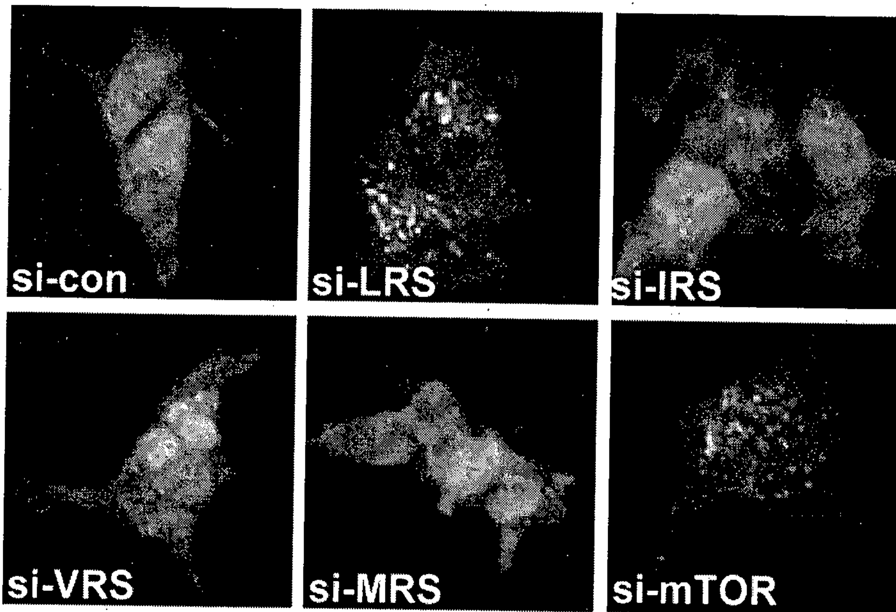

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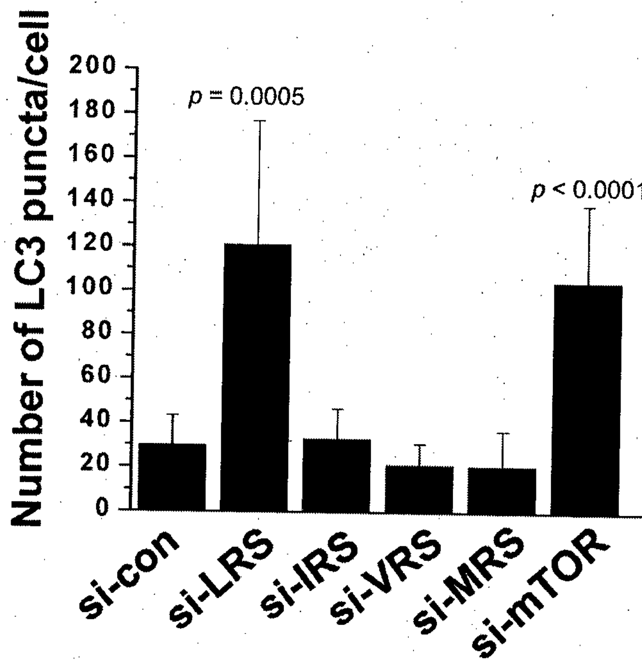
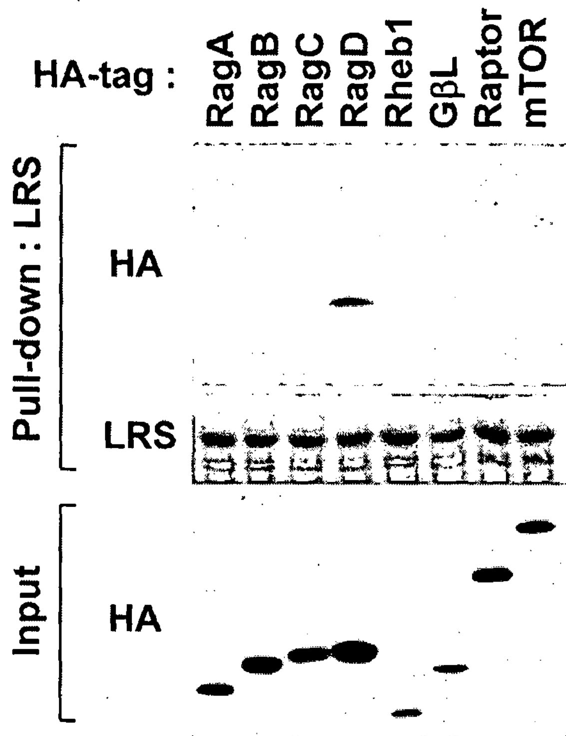


【3a】



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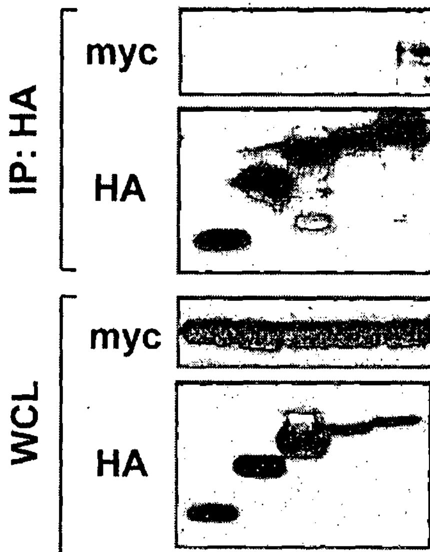

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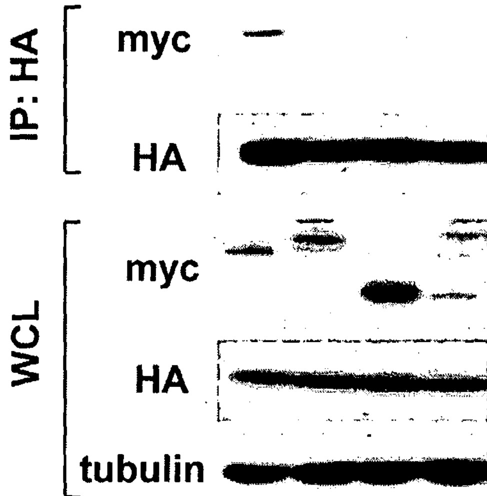

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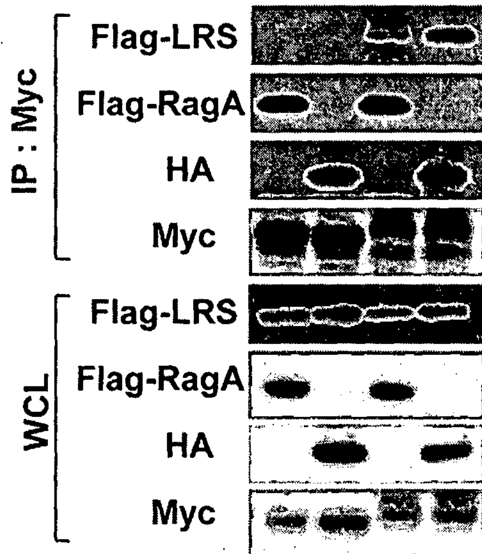

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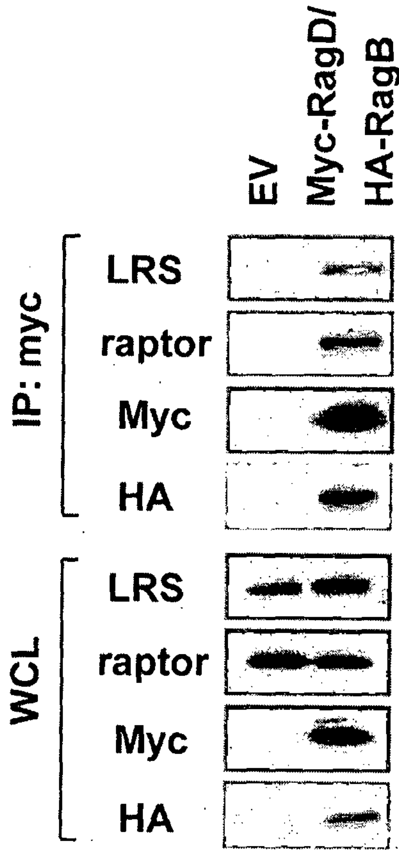

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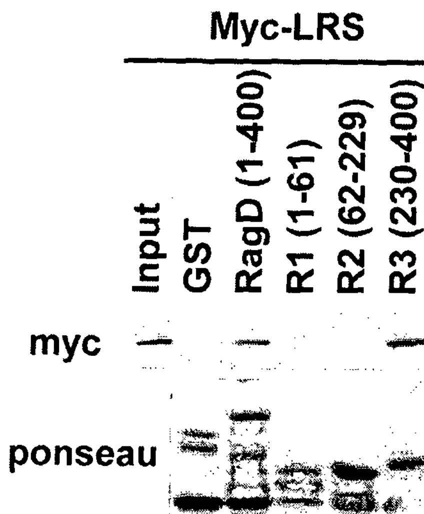
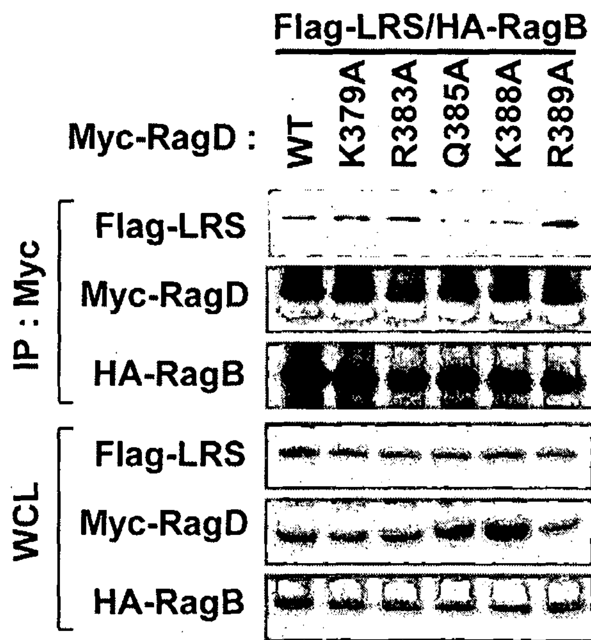
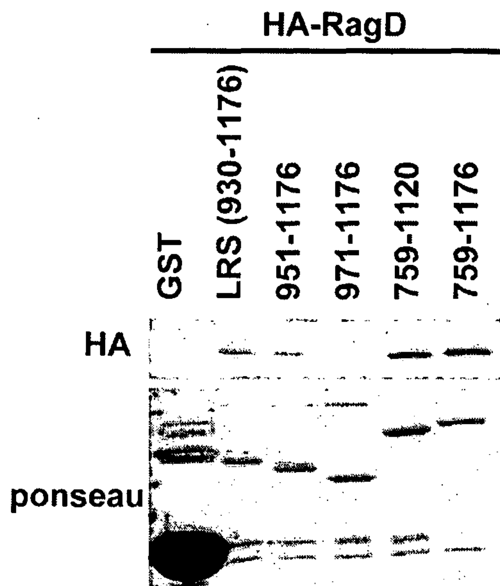


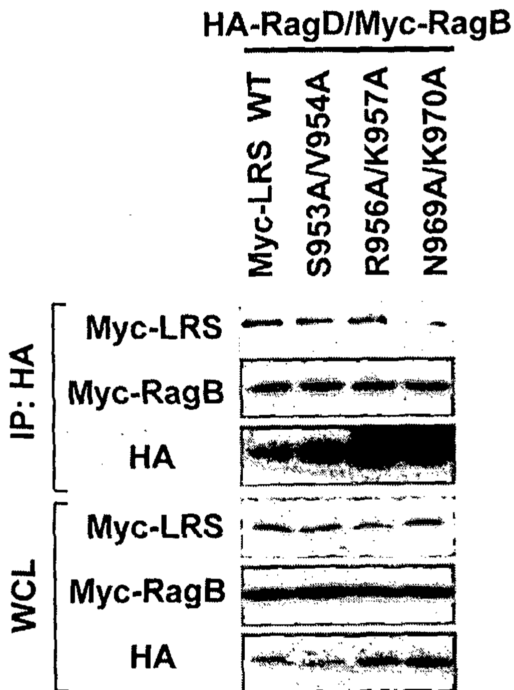
【3g】



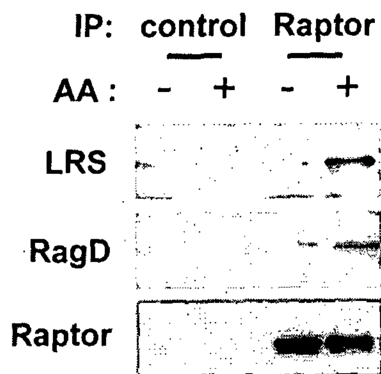
【3h】



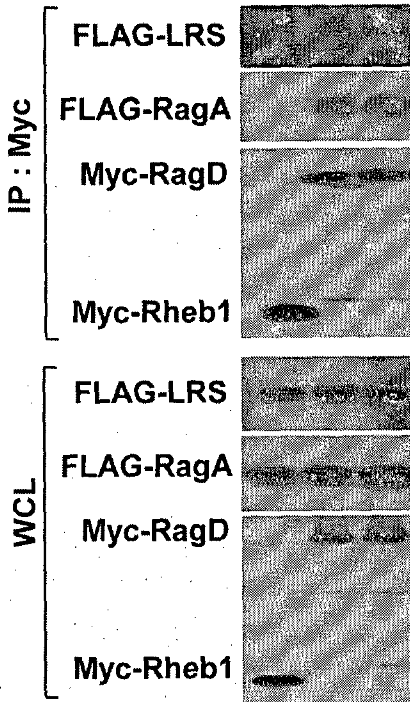
【3i】



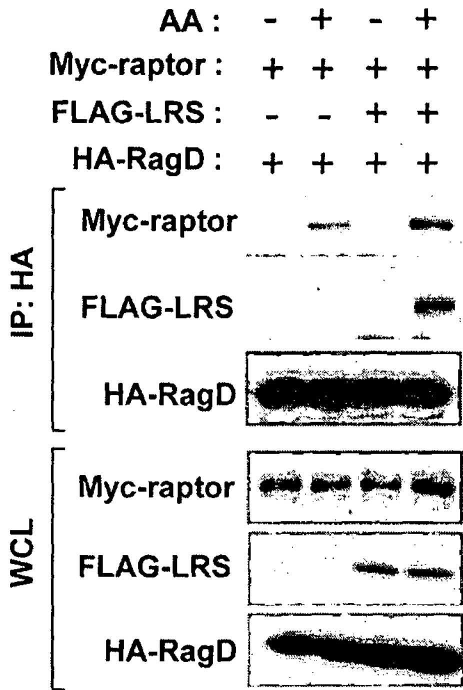
【4a】



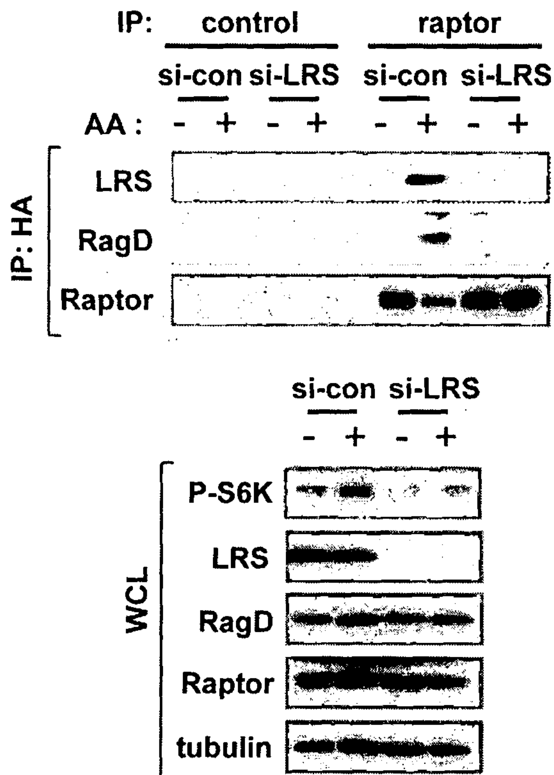
【4b】



【4c】



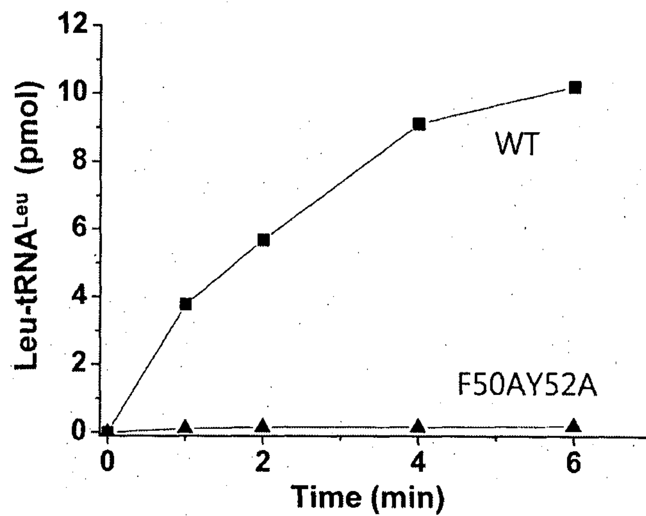
【4d】



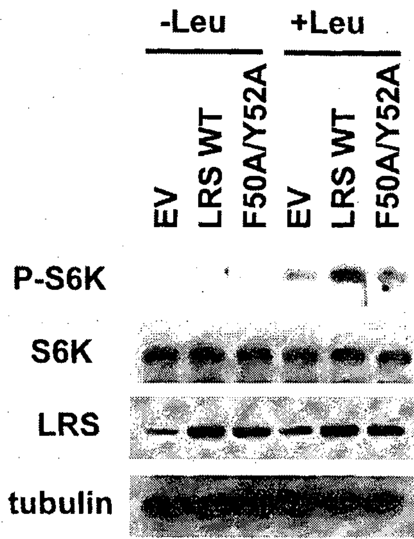
【5a】

```
                                       31                       50          HIGH motif       78
Homo sapiens                           EVNASNLEK--QTSKGKYFVTFPYPYMNGRLHLGHTFSLSKCEFAVGY
Mus musculus                           EVSASRLEKQKQSSKGKYFVTFPYPYMNGRLHLGHTFSLSKCEFAVGY
Rattus norvegicus                      EVSASSLEKQKQSSKGKYFVTFPYPYMNGRLHLGHTFSLSKCEFAVGY
Bos Taurus                             EVNACNLEK--QTSKDKYFVTFPYPYMNGRLHLGHTFSLSKCEFAVGY
Xenopus laevis                         EVNASDPQA--QNSKGKYLVTFPYPYMNGRLHLGHTFSLSKCEFSVGY
Streptococcus thermophilus LMG 18311   KTG-TDASK----PKFYALDMFPYPSGAG-LHVGHPEGYTATDILSRF
Thermus thermophilus HB27              KAKDLPGGR----GKQYVLVMFPYPSGD--LHMGHLKNYTMGDVLARF
```

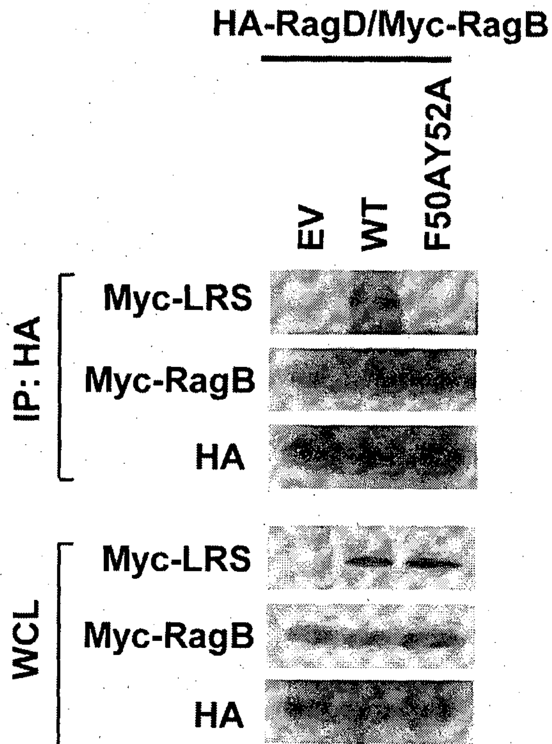
【5b】



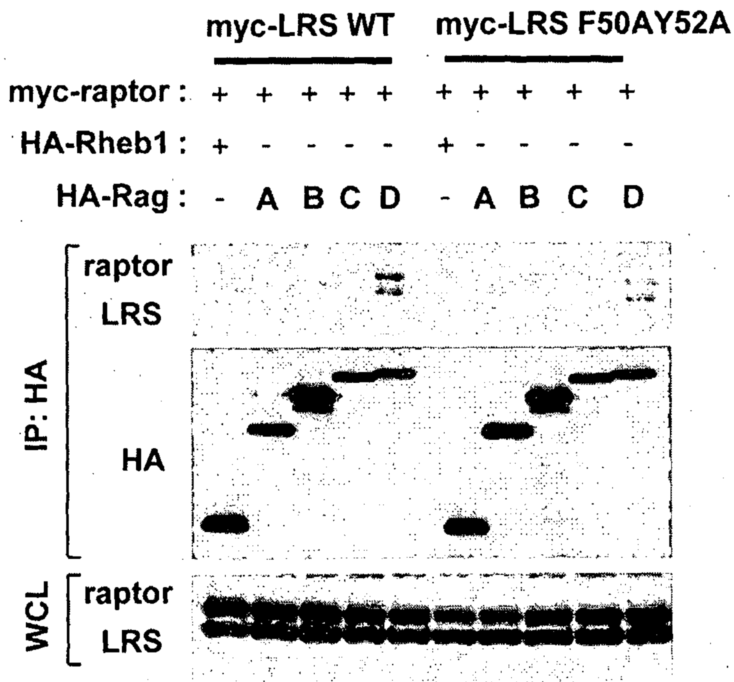
【5c】



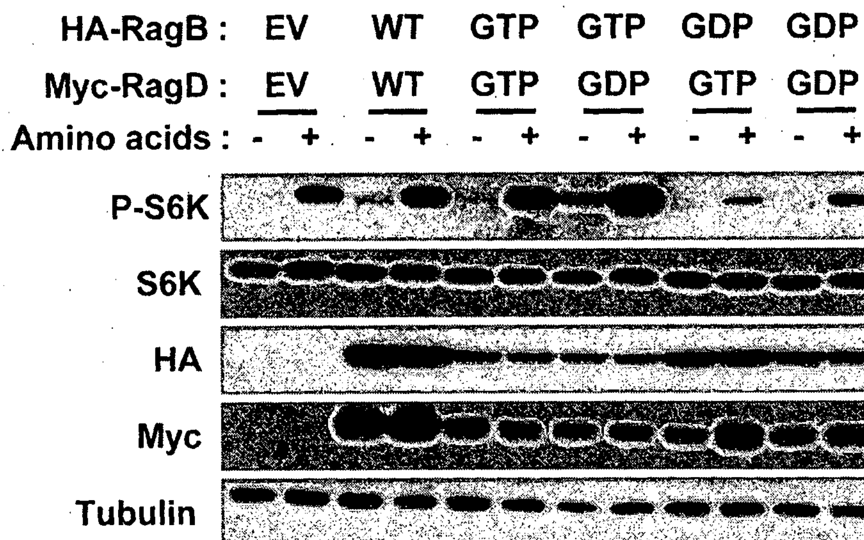
【5d】



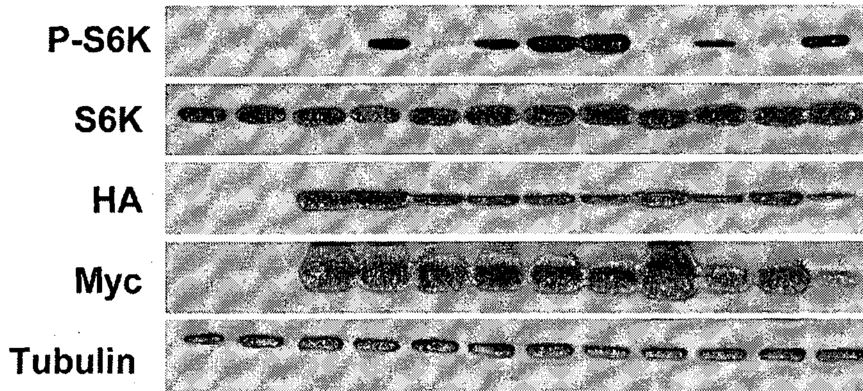
【5e】



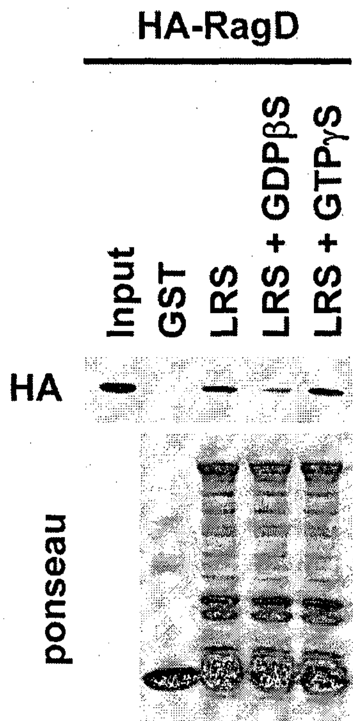
【6a】



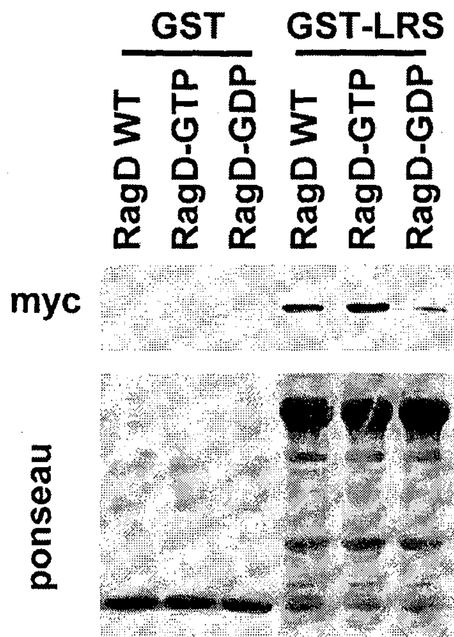
【6b】



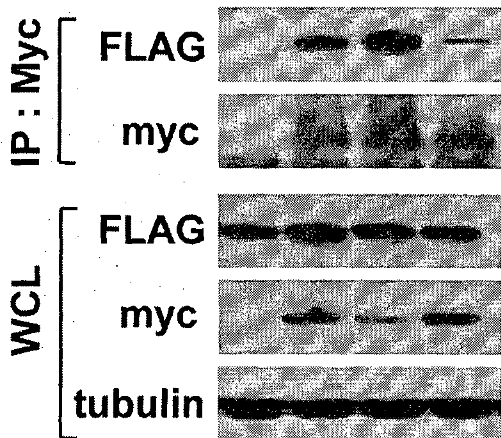
【6c】



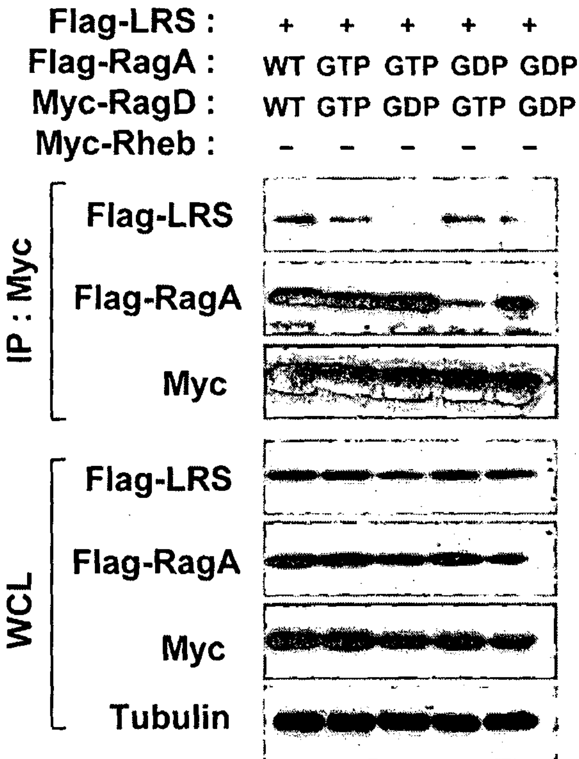
【6d】



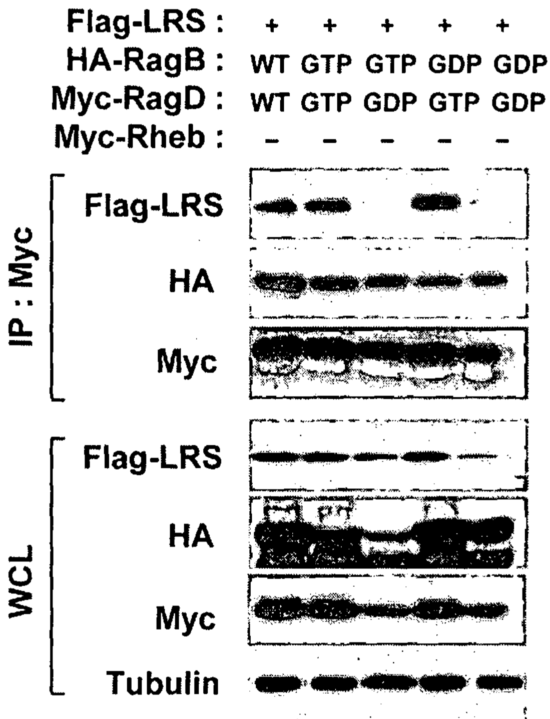
【6e】



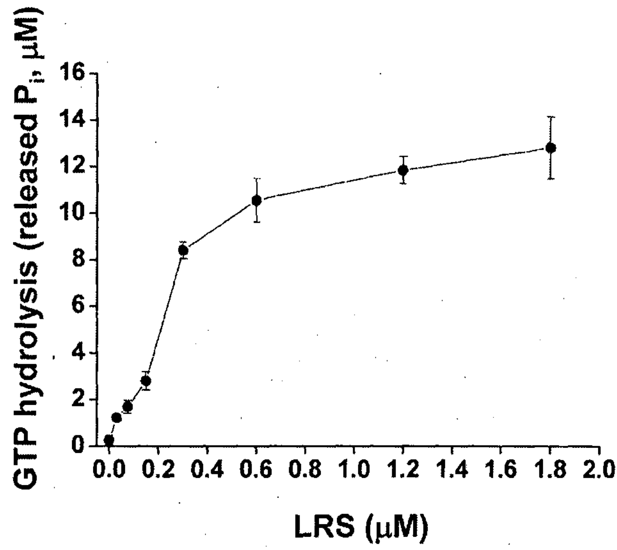
【6f】



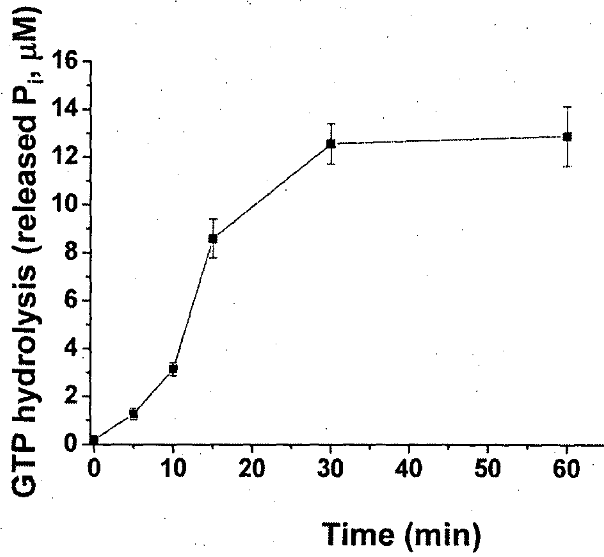
【6g】



【7a】



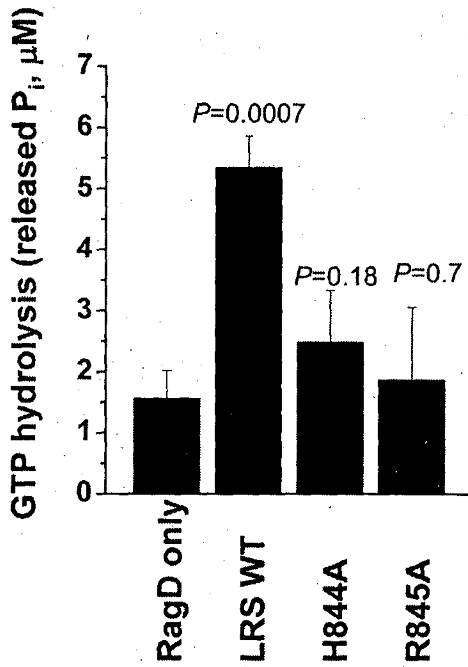
【7b】



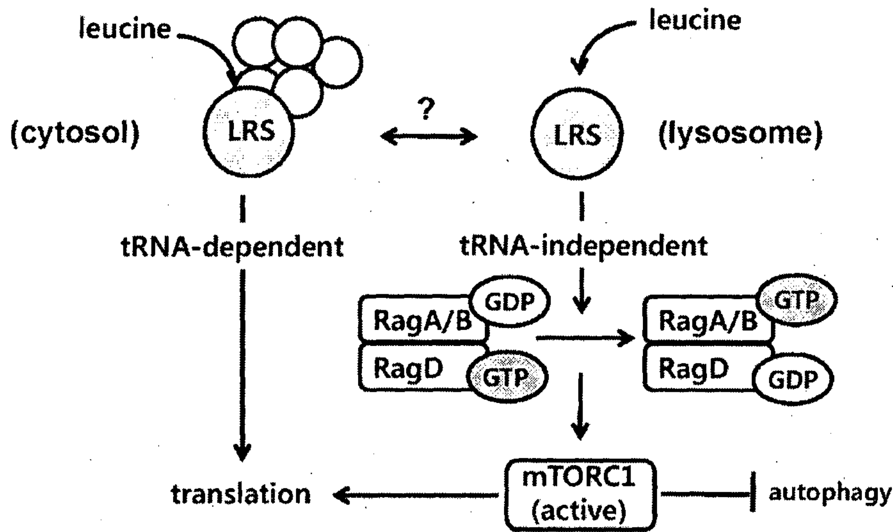
【7c】

ArfGAPs	Consensus shHRxhx
*hs*ARD1	AKHRRVP
*rn*ArfGAP	GRHRGLG
*dm*ArfGAP	GKHRSLG
*sc*Gcs1	GIHRGLG
*sc*Sps18	NLLRGMG
*sc*Glo3	AVHRNMG
*rn*Centaurin	GIHRNIP
*ss*p42IP4	GIHRNIP
*hs*LRS	GMHRELV
	842 848

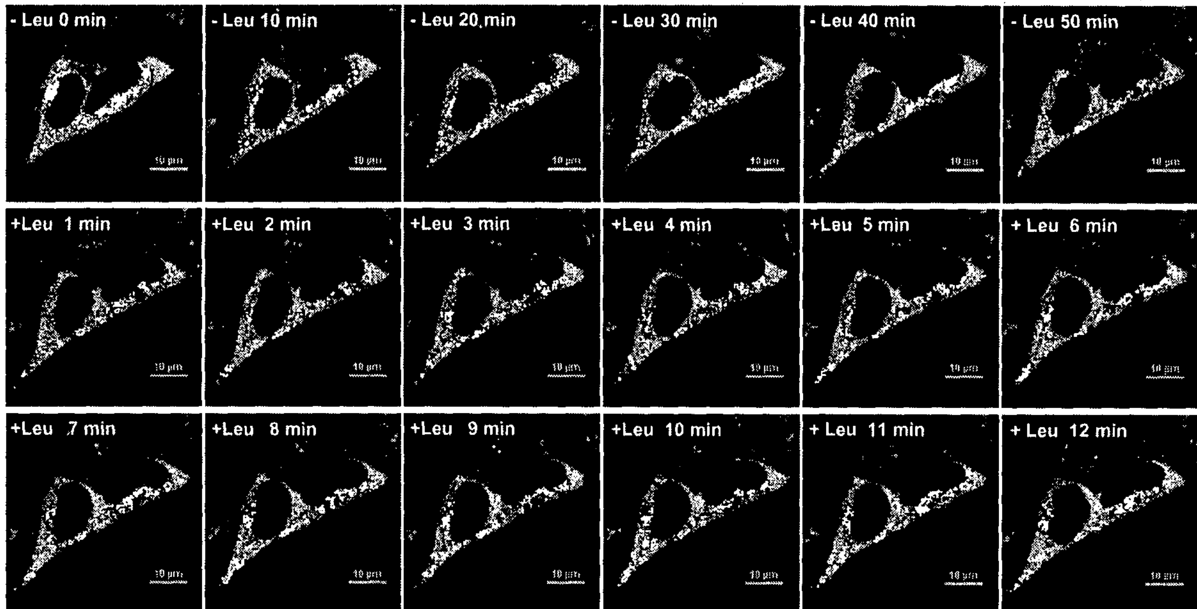
【7d】



【7f】

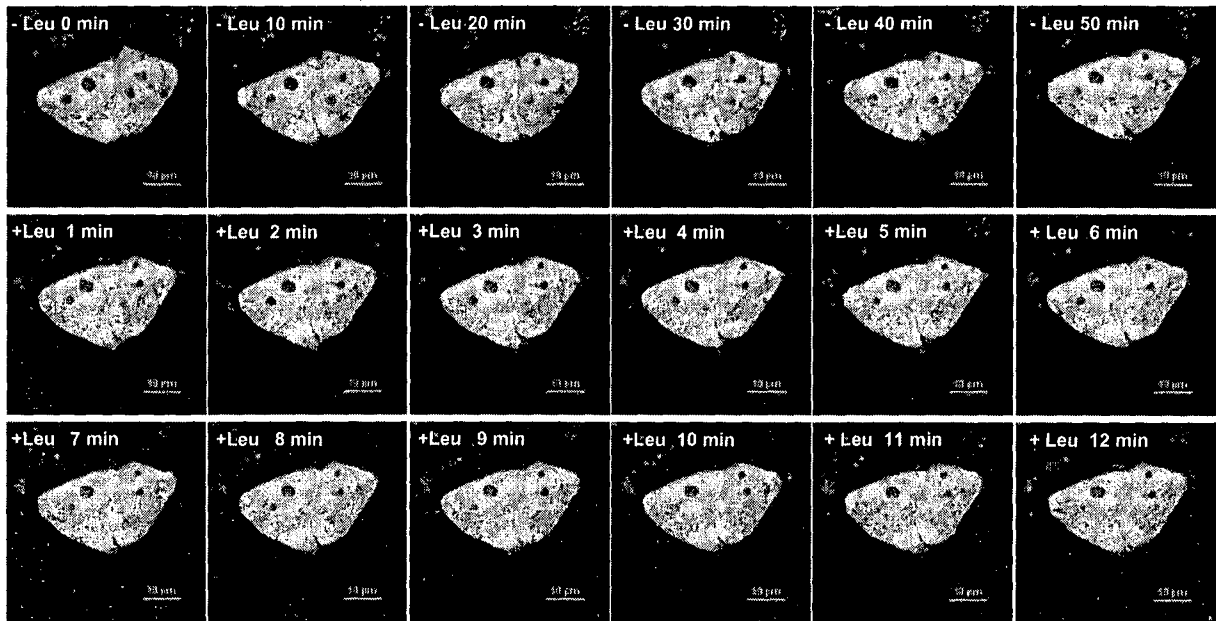


【8a】



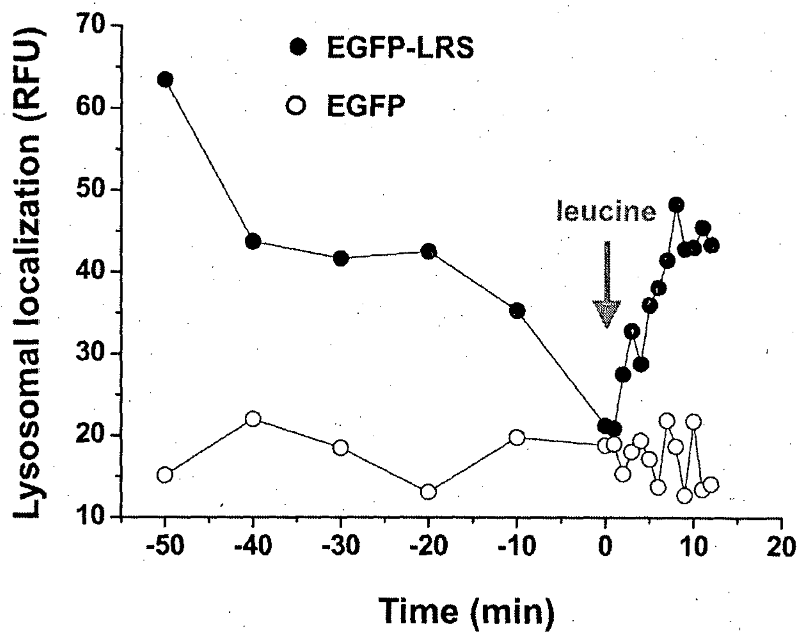
Green : EGFP-LRS Red : LysoTracker

【8b】



Green : EGFP Red : LysoTracker

【8c】



【9a】

Leucinol
Leucine amide
Leucinol
Leucine amide

Leu - - - - - + + + + +

p-S6K

S6K

LRS

tubulin

【9b】

Leucinol
Leucine amide
Leucinol
Leucine amide

Leu - - - - - + + + + +

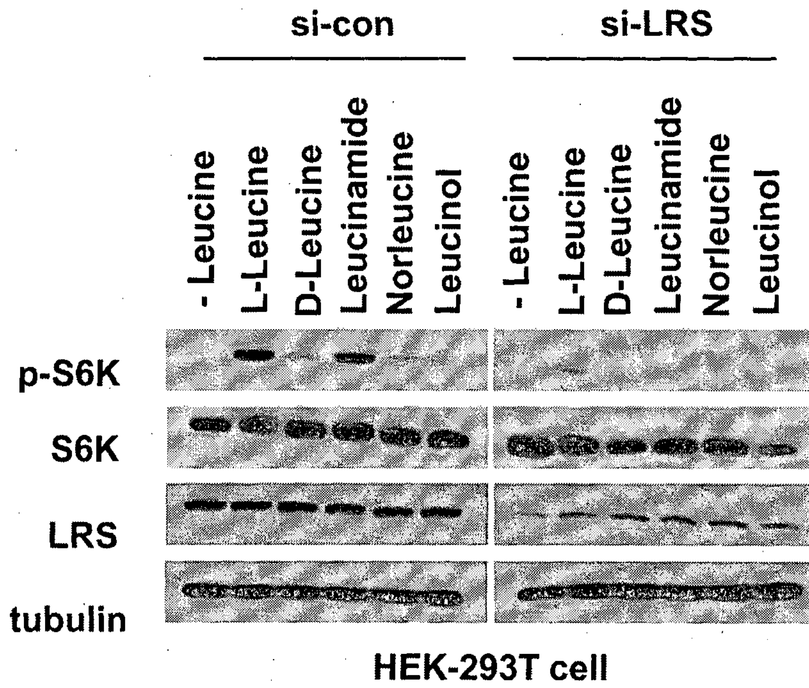
p-S6K

S6K

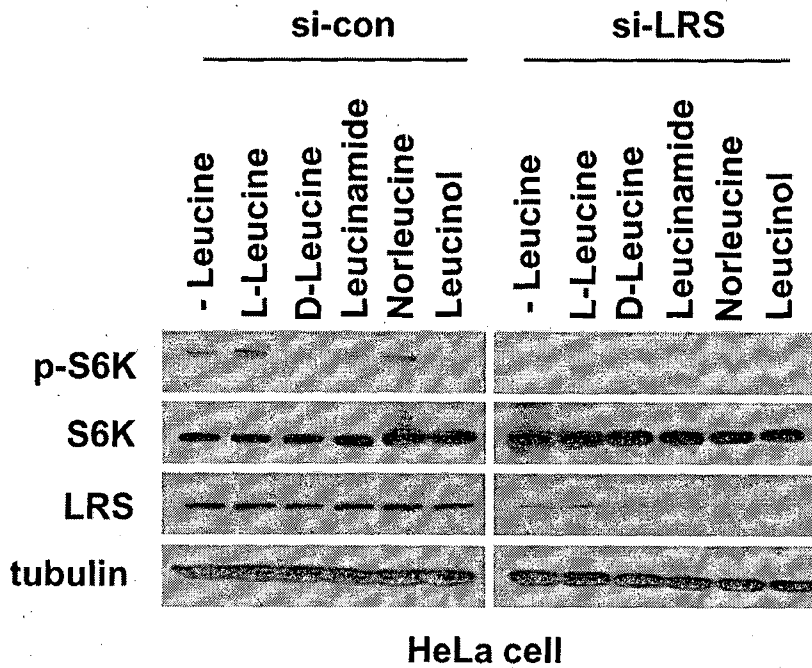
LRS

tubulin

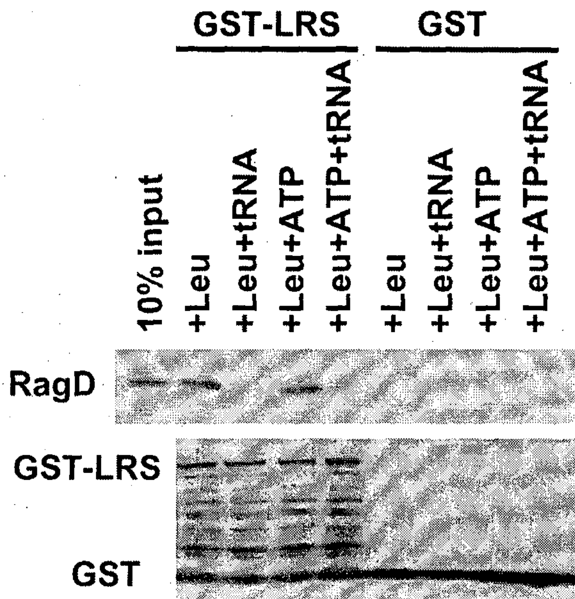
【9c】



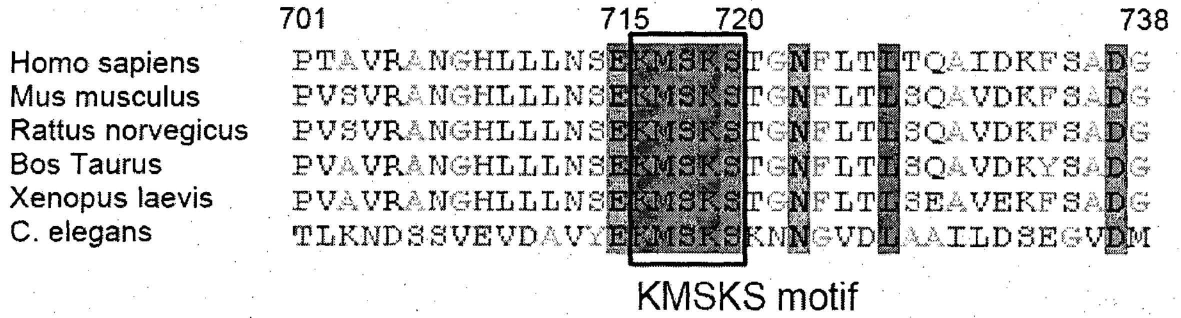
【9d】



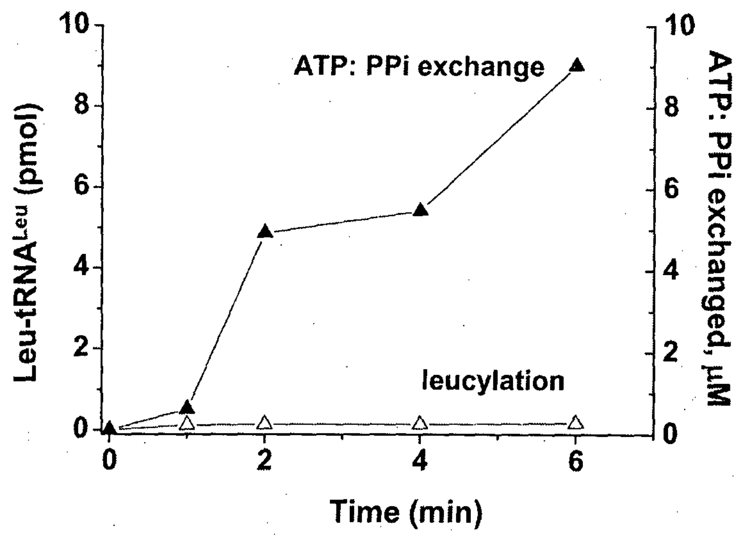
【10a】



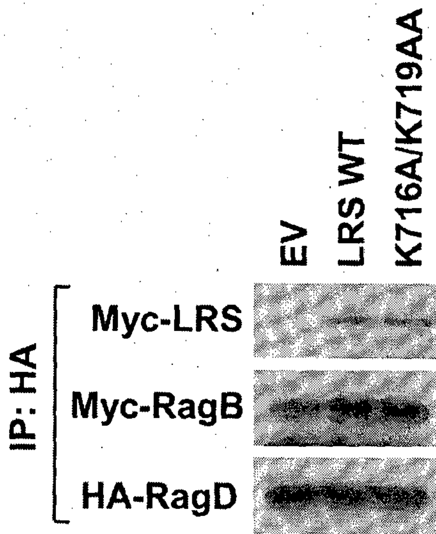
【10b】



【10c】



【10d】



【10e】

+Leu

EV

LRS WT

K716A/K719AA

P-S6K

S6K

Myc-LRS

tubulin

【11a】

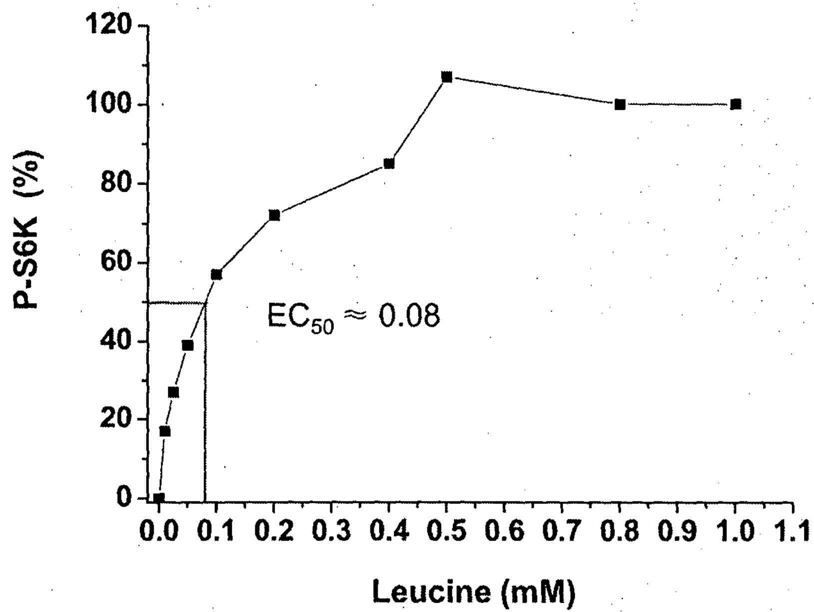
Leucine (mM) 0 0.01 0.025 0.05 0.1 0.2 0.4 0.5 0.8 1

P-S6K

S6K

Tubulin

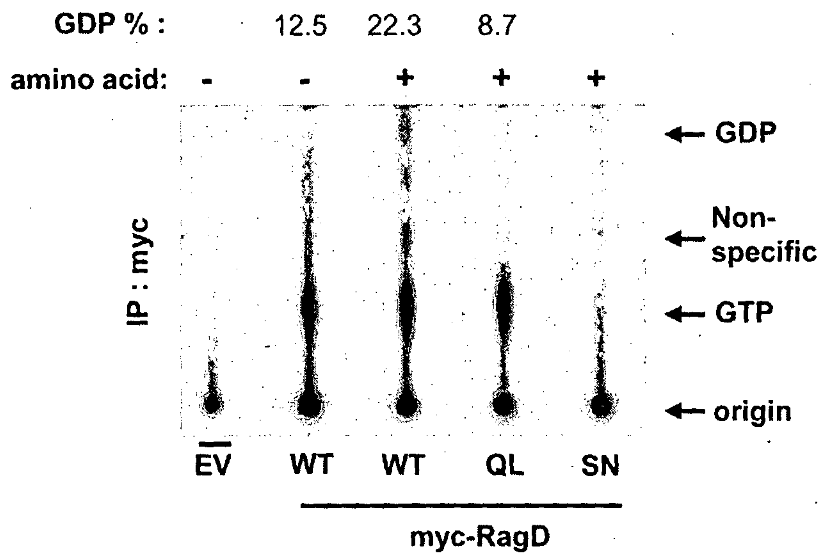
【11b】



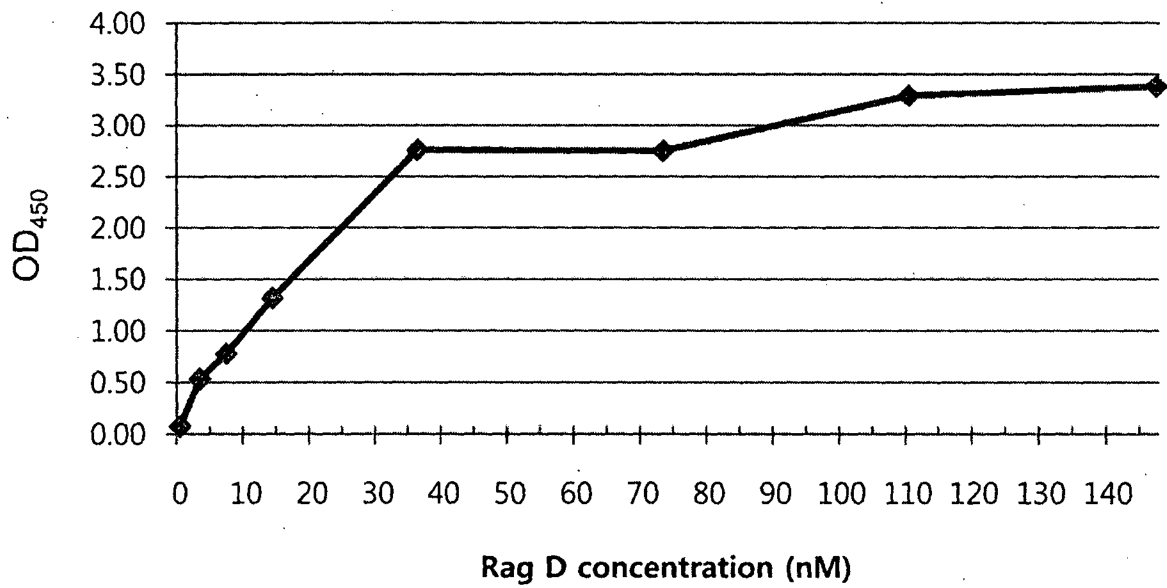
【11c】

Substrates	Constants	LRS WT	
		ATP-PPi exchange	Leucylation
Leucine	K_m (mM)	0.143 ± 0.061	0.0159 ± 0.0004
	k_{cat} (S^{-1})	0.16 ± 0.07	0.368 ± 0.009
	k_{cat}/K_m ($S^{-1}mM^{-1}$)	1.16 ± 0.49	22.9 ± 0.57

【12】



【13a】



【13b】