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(54) Title: MAMMAL SESTRINS IN THE TREATMENT OF MUSCLE WASTING

(57) Abstract: The invention discloses a mammal sestrin product applicable in muscle atrophy, as well as any recombinant construction (vectors) comprising this mammal sestrin. A non-human animal model of muscle cell hypertrophy is also disclosed. In particular, the invention proposes tools for the treatment of atrophies of any aetiology, such as muscular dystrophies, cachexia, and atrophy related to malnutrition or to immobilization.



Mammal sestrins in the treatment of muscle wasting

The present invention is related to the field of medicine and, especially, the field of muscle disorders or myopathies. More particularly, the invention is related to new agents for the treatment of muscular atrophy or wasting.

## BACKGROUND ART

Muscle atrophy, also called muscle wasting, is defined as a decrease in the mass of the muscle; it can be a partial or complete wasting away of muscle. Atrophic muscle is a weak muscle, due to the fact that the ability to exert force is related to mass. Muscle wasting or atrophy refers then to the progressive loss of muscle mass and/or to the progressive weakening and degeneration of the skeletal or voluntary muscles, which control movement, or of the cardiac muscles, which control the heart (cardiomyopathies), and smooth muscles.

Physiologically, muscle atrophy occurs by an imbalance between protein synthesis and protein degradation. During atrophy, there is a down-regulation of protein synthesis pathways, and/or an activation of protein breakdown pathways. Muscle wasting is associated to a catabolic state. The term "catabolic state" means a pathological situation in which the catabolic pathways are activated, such as hepatic glycogenolysis, lipolysis, proteolysis and thermogenesis.

The particular protein degradation pathway which seems to be responsible for much of the muscle loss seen in a muscle undergoing atrophy is the ATP-dependent ubiquitin/proteasome pathway. In this system, specific proteins are targeted for destruction by the ligation of at least four copies of a small peptide called ubiquitin onto a substrate protein. When a substrate is thus "poly-ubiquitinated", it is targeted for destruction by the proteasome. Particular enzymes in the ubiquitin/proteasome pathway allow ubiquitination to be directed to some proteins but not others. A search for transcriptional markers of muscle atrophy led to the discovery of the E3 ubiquitin ligases MuRF1 and MAFbx (also named Atrogin-1). Each E3 ubiquitin ligase binds to a particular set of substrates, causing their ubiquitination.

Muscle atrophy is a highly debilitating response associated to a wide range of

diseases or physical conditions or states, including among others muscular dystrophies, such as Duchene's muscular dystrophy, Becker's muscular dystrophy, limb girdle muscular dystrophy, etc., post-polio muscle atrophy, cachexia, such as cardiac disease, AIDS and cancer associated cachexia, leprosy, diabetes, cancer, renal disease, obstructive pulmonary disease, emphysema, paraplegia, hemiplegia, and sarcopenia. Among those physical states or conditions leading also to muscle atrophy or wasting, malnutrition, immobilized or casted limb, long term hospitalization and non or reduced gravity situations as space trips are included.

Especially of interest is the cachexia, which is defined as wasting of lean body mass that cannot be nutritionally reversed, thus indicating a deregulation in the pathways maintaining body composition. Cachexia is observed as a co-lateral symptom in cancers, HIV-virus infections and malnutrition states. It is a positive risk factor for death, meaning that if the patient has cachexia, the chance of death from the underlying condition is dramatically increased.

Current palliative treatments of muscle atrophy associated disorders or physical conditions include the use of glucocorticoids, which have demonstrated a slow muscle strength declination in cases of Duchene's Muscular Dystrophy (DMD). In general, muscular dystrophy refers to a group of hereditary muscle diseases that weakens the muscles that move the human body. Muscular dystrophies are characterized by progressive skeletal muscle weakness, defects in muscle proteins, and the final death of muscle cells and tissue.

Focusing on the mechanisms involved in muscle dystrophies, some putative therapeutically approaches have been proposed. Rüegg et al., "Molecular Mechanisms and Treatment Options for Muscle Wasting Diseases", Annu. Rev. Pharmacol. Toxicol. - 2011, Vol. N° 51, pp.: 373-395, propose the use of Insulin-Like Growth Factor 1 (IGF-1) in muscle dystrophies. The proposal is based on the rationale that activation of the IGF-1 pathway not only leads to a compensatory hypertrophy, but also affects pathways whose function is lost in muscle dystrophies. One of these pathways is the one in which the FoxO induced muscle-specific ubiquitin ligases MuRF1 and MAFbx are involved.

The IGF-1-induced pathway can block the transcriptional upregulation of

MuRF1 and MAFbx via the phosphatidylinositol-3 kinase (PI3K)/AKT/FoxO pathway. MuRF1's substrates include several components of the sarcomeric thick filament, including myosin heavy chain. Thus, by blocking MuRF1, IGF-1 prevents the breakdown of the thick filament, particularly myosin heavy chain, which is asymmetrically lost in settings of cortisol-linked skeletal muscle atrophy. IGF-1/PI3K/AKT signalling also dominantly inhibits the effects of myostatin, which is a member of the transforming growth factor- $\beta$  family of proteins.

Nonetheless, Rüegg et al. (*supra*) also indicate that still many challenges have to be faced before using IGF-1 in muscular dystrophies. First of all, IGF-1 is expressed in several isoforms, and it is not clear whether all are equally potent in skeletal muscle. Further, IGF-1 released into the blood is bound to many proteins. IGF-1 is also involved in the control of cell and organ size systemically, making its use problematic for the patients who already have normal levels of IGF-1. Finally, high levels of IGF-1 can cause hypoglycaemia by stimulating the insulin receptor.

Despite that IGF-1/IGFR signaling is fundamental for muscle growth during development and regeneration after injury, its requirement for the positive effect of exercise and increased load on muscle mass has been recently challenged. Indeed, impaired IGF-1/IGFR signaling did not affect protein kinase B (AKT) and the mammalian target of rapamycin (mTOR) activation nor compensatory hypertrophy after muscle overloading in mice, suggesting the existence of alternative molecular activators of the AKT/mTOR pathway in adult muscle. Consistent with this, additional studies have also shown that myofiber-produced myokines regulate hypertrophic muscle growth, independently of the IGF-1/AKT-pathway. Conversely, evidence of mTORC1 activation in response to increasing workload independent of IGF-1 or AKT signaling have also been reported. These studies, taken together, strongly suggest that the components of the classically considered canonical IGF-1/AKT/mTOR signaling cascade may not be the unique regulators of hypertrophic growth of adult skeletal muscle.

In US 20060069049 it is indicated the use of agents capable of regulating the phosphorylation and activity of the transcription factor FoxO, which is a protein that upregulates MuRF1 and MAFbx.

On the other hand, those individuals suffering from cachexia (of any of the possible aetiologies) are currently treated with corticosteroids or drugs that mimic progesterone in order to increase the appetite and reverse weight loss.

5 Nonetheless, these compounds are not able to reverse muscle loss.

It is then clear from the above-mentioned therapeutically strategies to face atrophic or wasted muscles, that there is still a need for alternative therapies or more effective approaches to fight all those diseases and physical  
10 conditions involving muscular atrophy.

## SUMMARY OF THE INVENTION

It has been surprisingly found that increased expression of mammalian sestrin  
15 led to adult muscle hypertrophy and, more importantly, it prevented disuse-induced myofiber atrophy and preserved muscle force. Based on the results below, sestrin emerges as a new important molecule regulating AKT-dependent physiological muscle growth, acting through Glycogen synthase kinase 3 (GS3K) and FoxO pathway inhibition, independently of mTORC1  
20 regulation.

The inventors have identified sestrins as novel positive regulators of physiological muscle growth in mammals. The key and unexpected mechanism here is that sestrins induced an increase in muscle mass via  
25 activation of protein kinase B (AKT), independently of mTORC1 activity. These findings constitute novel and previously unknown activities of the Sestrin family of proteins on mammalian skeletal muscle.

By administering an adeno-associated vector comprising mammalian sestrins  
30 to mice, in which sestrins are then over-expressed, promotion of muscle cell hypertrophy rescued atrophic cells.

Thus, in a first aspect the invention provides a mammalian sestrin product for use in the treatment of muscle atrophy. This aspect can be formulated as the  
35 use of a mammalian sestrin product for the manufacture of a medicament for the treatment of muscular atrophy. The invention also provides a method for the treatment of muscular atrophy which comprises administering a

mammalian sestrin product to an animal, including a human, in need thereof.

In the sense of the present invention the term "treatment" is referred to the administration of an agent aimed to ameliorate the cause or the symptoms of muscular atrophy associated to diseases or physical conditions, wherein muscle cells are non-functional or partially deprived of function due to protein mutations, down-regulation of protein synthesis, or immobilization, but also to avoid the onset of such symptoms. Thus, for the scope of the present invention the term "treatment" also includes the concept of prevention.

Although experimental data below are performed by over-expressing mammalian sestrins in a host from a vector containing the DNA codifying for these proteins, external administration of appropriate doses of these proteins in a purified form will have the same effect.

The invention has the advantage that atrophy may be rescued, compensated or avoided by promoting cell hypertrophy, and in consequence recovering muscle mass. As above indicated, muscle weight loss leads to weakening states. The weakening states may cause death, due to the immobilization of critical muscles that control, for example, the pulmonary system.

Sestrins are small, conserved proteins that accumulate in cells upon genotoxic, oxidative and metabolic stresses. Sestrins function as activators of AMP-activated protein kinase (AMPK) and inhibitors of target of rapamycin (TOR) complex 1 (TORC1). The inventors have now identified sestrins as novel positive regulators of physiological muscle growth in mammals. The key and unexpected mechanism here is that sestrins induced an increase in muscle mass via activation of protein kinase B (AKT), independently of mTORC1 activity. These findings constitute novel and previously unknown activities of the Sestrin family of proteins on mammalian skeletal muscle.

There are three isoforms of sestrin in mammals named sesn-1, sesn-2 and sesn-3. The three of them are accumulated under energy demand conditions, such as starvation, overloading, exercise or regeneration, as well as in genotoxic, oxidative and metabolic stresses.

In human, sestrin-1 (or sesn-1), also known as p53-regulated protein PA26, is

a protein that in humans is encoded by the *SESN1* gene. The amino acid sequence of sestrin-1 is the one under the UniProtKB/Swiss Prot database Q9Y6P5-1, version 2 from April 27, 2001. In the present disclosure is also referred by SEQ ID NO: 1.

5

The *SESN1* gene is conserved in chimpanzee, dog, cow, mouse, rat, chicken, and zebrafish.

10 Sestrin-2 (or *sesn-2*) also known as Hi95 is a protein that in humans is encoded by the *SESN2* gene. The amino acid sequence of sestrin-2 is the one under the UniProtKB/Swiss Prot database P58004, version 1 from April 27, 2001. In the present disclosure is also referred by SEQ ID NO: 2.

15 The *SESN2* gene is conserved in chimpanzee, dog, cow, and mouse.

15

In human, sestrin-3 (or *sesn-3*) has the amino acid sequence under the UniProtKB/Swiss Prot database P58005, sequence version of Jan 23, 2002. In the present disclosure is also referred by SEQ ID NO: 3.

20 In mice, sestrin-1, also known as p53-regulated protein PA26, is a protein that in mice is encoded by the *SESN1* gene. The amino acid sequence of sestrin-1 is the one under the UniProtKB/Swiss Prot database P58006, version 2 from July 5, 2005. In the present disclosure is also referred by SEQ ID NO: 4.

25 Mice sestrin-2 is a protein encoded by the *SESN2* gene. The amino acid sequence of sestrin-2 is the one under the UniProtKB/Swiss Prot database Q3TCT3, version 1 from October 11, 2005. In the present disclosure is also referred by SEQ ID NO: 5.

30 Sestrin-3 in mice is represented by the amino acid sequence under the UniProtKB/Swiss Prot database Q9CYP7, Version 1, last modified April 27, 2001. In the present disclosure is also referred by SEQ ID NO: 6.

35 As used herein, the term "mammalian sestrin product" means a product comprising a fragment or the entire polypeptide sequence of a mammalian sestrin. Suitable mammalian sestrin products include natural, synthetic, or recombinant biologically active polypeptide of mammalian sestrins; biologically

active polypeptide variants of mammalian sestrins or fragments thereof, including hybrid fusion proteins or dimers; or to biologically active polypeptide analogues of mammal sestrins or fragments thereof. Analogues include products where one or more amino acid residues have been replaced by a different amino acid. Conservative amino acid substitutions are preferred. The mammalian sestrin product may be directly and externally administered as a purified polypeptide. On the other hand, using the gene therapy, the mammalian sestrin product may be produced *in situ* in the treated mammalian (including human) by administering a suitable vector comprising the nucleotide sequence codifying for the mammalian sestrin product, said mammal sestrin product being functionally delivered into the mammal body after it has been duly expressed with the aid of the nucleotide constructs constituting the expression vector. Recombinant biologically active polypeptides of mammalian sestrins can be administered as purified polypeptides derived from an expression vector or, in the alternative in the form of vectors, as such.

Functionality of the administrated sestrin product can be easily assessed by administration to a cell line (such as the myoblast cell line C2C12) and taking microscopic images from the cultures. Treated cells having a size greater than the size of the controls (not sestrin product treatment) indicates a hypertrophic phenotype.

Sestrin products may be produced by isolation and purification from mammalian tissues. Alternatively, they can be produced by biotechnological methods including cells (microorganisms, cell lines, etc.) , which allow the obtention of the protein in the form of a recombinant protein. Said recombinant protein is derived from specialised expression nucleic acid vectors. The term "vector" in the present invention refers to a composition of matter which can be used to deliver a nucleic acid of interest to the interior of a cell. Non-limiting examples of vectors include an autonomously replicating plasmid or a virus. For purposes of this invention, the terms "expression construct," "expression vector," and "vector," are used interchangeably to demonstrate the application of the invention in a general, illustrative sense, and are not intended to limit the invention.

The findings shown in the present invention constitute novel and previously unknown activities of the Sestrin family of proteins on mammalian skeletal



muscle. Indeed, Sestrins had been associated to responses to genotoxic and oxidative stress in cultured cells.

In Hee Lee et al., "Sestrin as Feedback Inhibitor of TOR That Prevents Age-Related Pathologies", Science - 2010, Vol. 327, pp.: 1223-1228, sestrin is indicated as a natural inhibitor of age-related myopathies in *Drosophila* (which has a single sestrin; dSesn). Hee Lee et al. show that dSesn-null flies exhibited highly accelerated age-related skeletal and cardiac muscle degeneration that was preceded by accumulation of swollen mitochondria, ubiquitinated protein aggregates and reactive oxygen species (ROS), caused by defective autophagy. In *Drosophila* it is proved that sestrin prevents pathologies caused by chronic TOR activation that may result from diminished autophagic clearance of damaged mitochondria, protein aggregates, or lipids. The authors in Hee Lee et al. demonstrate that over-expression of dSesn reduced the size of the cells in larval wing discs and adult eyes, although the cell number of these areas remained unchanged. They concluded that Sesn inhibits cell growth without affecting cell proliferation and does so independently of its redox activity. Additionally, Hee Lee et al. demonstrate that the expression of exogenous dSesn prevented muscle degeneration due to the maintenance of the cell proliferation rate.

Thus, it can be said that in *drosophila* dSesn appears to control and restrain the aberrations caused by TOR hyperactivation linked to oxidative stress in age-associated pathologies, including loss of muscle mass and tissue degeneration. However, whether sestrins have a role in the regulation of muscle mass in physiological conditions remains unknown from the data shown by Hee Lee et al.

In *drosophila*, dSesn was shown to be induced upon chronic activation of TOR associated to age-related tissue wasting, acting as a negative feed-back regulator of TOR function through activation of AMPK. Thus, it appears that the function of sestrins, and their downstream effectors, in the induction of mammalian adult muscle growth are distinct from those associated to tissue aging and degeneration in *drosophila*.

On the other hand, Budanov et al., "p53 Target Genes Sestrin1 and Sestrin 2 Connect Genotoxic Stress and mTOR Signaling", Cell - 2008, Vol. 134, pp.:

451-460, discloses that both sestrins in HEK293 cells provoked cell size decrease.

Thus, apart of acting as agents to fight oxidative effects in cells, the teachings  
5 from Budanov et al., and Hee Lee et al. (*supra*) allow to affirm that sestrins have another effect: to diminish cell size.

Surprisingly, the inventors have now found that muscle in basal condition, in which sestrins are over-expressed, exhibits a phenotype of cell hypertrophy,  
10 or which is the same, a pattern of enlargement of cell size. These observations are well shown in the examples below. The induction or promotion of muscle cell hypertrophy implies also the advantage of increasing muscle mass. The examples below demonstrate moreover that sestrins can rescue atrophic muscle cells. Due to this rescue ability, mammal sestrins are  
15 interesting tools as agents for the treatment of those diseases ongoing with muscle atrophy, being the diseases from different causes. Indeed and as derivable from the examples below, mammalian sestrins give raise to greater mammalian cells in comparison with non-treated muscles; to a greater number of fibers in the muscle; and to an increased level of regenerating muscle cells.

20 So then, the present invention provides a mammalian sestrin product for use in the treatment of muscle atrophy.

Although the examples below are directed to skeletal mouse muscle, they can  
25 be extended to cardiac muscle since both are striated muscles. Striated muscle tissue comprises muscle fibers as individual components. The fibers are combined into parallel fibers.

Thus, in the sense of the present invention "muscle" refers to multiple bundles  
30 of striated muscle tissue (skeletal or cardiac), and the term "muscle cell" and "muscle fiber" are used as synonymous and refer to a cell unit into this tissue. In particular, muscle cells, also named myofibers (muscle fiber) are long, cylindrical, multinucleated cells composed of actin and myosin myofibrils repeated as a sarcomere, the basic functional unit of the muscle fiber and  
35 responsible for skeletal muscle's striated appearance and forming the basic machinery necessary for muscle contraction. The principal cytoplasmic proteins are myosin and actin (also known as "thick" and "thin" filaments,

respectively) which are arranged in a repeating unit called a sarcomere. The interaction of myosin and actin is responsible for muscle contraction.

5 In a preferred embodiment, the treatment of muscle atrophy by means of the use of the mammalian sestrin product, is achieved by the effect of hypertrophy of muscle cells including all those forms embedded in the group of striated muscle tissue or striated muscle cells.

10 In a preferred embodiment the striated muscle cells are skeletal muscle cells.

In another preferred embodiment the striated muscle cells are cardiac muscle cells.

15 In another preferred embodiment, the mammal sestrin product for use as disclosed above, is a human sestrin. Preferably the human sestrin product is selected from the group consisting of sestrin-1 (SEQ ID NO: 1), sestrin-2 (SEQ ID NO: 2), sestrin-3 (SEQ ID NO: 3), and mixtures thereof.

20 In a more preferred embodiment, the sestrin product is a human sestrin-1 corresponding to SEQ ID NO: 1.

In another preferred embodiment, the sestrin product is a human sestrin-2 corresponding to SEQ ID NO: 2.

25 With the sestrin product for the use according to the invention, muscle atrophy which is associated to several diseases may be treated. In particular it may be treated muscle atrophy associated to a disease selected from the group consisting of muscle dystrophy, post-polio muscle atrophy, cachexia, leprosy, diabetes, cancer, renal disease, obstructive pulmonary disease, emphysema, 30 paraplegia, hemiplegia, and sarcopenia.

Thus, the present invention provides a mammalian sestrin product for use also in the treatment of muscle dystrophy.

35 Examples of muscle muscular dystrophy refer to Duchenne muscular dystrophy, Becker's muscular dystrophy, limb girdle muscular dystrophy, congenital muscular dystrophy, facioscapulohumeral muscular dystrophy,

myotonic muscular dystrophy, oculopharyngeal muscular dystrophy, distal muscular dystrophy, and Emery-Dreifuss muscular dystrophy.

5 Among cachexias to be treated with the sestrin product, those from different aetiologies can be faced, such as cardiac disease, AIDS and cancer associated cachexia.

10 In another preferred embodiment of the first aspect of the invention, muscle atrophy is associated to a physical condition selected from the group consisting of malnutrition, immobilized or casted limb, and long term hospitalization.

15 A second aspect the invention relates to an adeno-associated vector comprising a polynucleotide sequence codifying for a mammal sestrin product. Especially suitable vectors are the adeno-associated virus encompassing the protein of interest or a fragment thereof functionally active. These vectors are specially adapted to be used in the treatment of muscle atrophy.

20 In general, these expression vectors usually comprise a promoter operably linked to a polynucleotide encoding the inhibitory oligonucleotide. As used herein, the terms "operably linked" or "under transcriptional control" as referred to an oligonucleotide mean that a promoter is in the correct location and orientation in relation to the oligonucleotide to control the initiation of transcription by RNA polymerase and expression of said oligonucleotide. The  
25 term "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of an oligonucleotide.

30 When mammalian sestrin products are used derived from an expression vector, for example by administering the referred vector to an individual (mammal including human), preferred adeno-associated vectors are those which comprise a human sestrin selected from the group consisting of sestrin-1, sestrin-2, sestrin-3, and mixtures thereof. Preferably, the human sestrin in the adeno-associated vector is human sestrin-1 corresponding to SEQ ID NO:  
35 1. Also preferred, the human sestrin in the adeno-associated vector is human sestrin-2 corresponding to SEQ ID NO: 2. This adeno-associated vector is, therefore, a suitable tool for the treatment of muscle atrophy, by administering

it to an animal, including a human, in need thereof. This can also be formulated as the adeno-associated vector for use in the treatment of muscle atrophy. This preferred aspect may also be formulated as the use of the adeno-associated vector comprising the sequence codifying for a mammal  
5 sestrin product for the preparation of a medicament for the treatment of muscle atrophy.

The mammalian sestrin product, purified from a natural source, in the form of a recombinant protein or in the form of an expression vector can be  
10 administered as a pharmaceutical composition. In any of these forms the sestrin product is adapted to be used in the treatment of muscle atrophy, and in particular muscle dystrophies.

Therefore, a third aspect of the invention is a pharmaceutical composition  
15 comprising an adeno-associated vector, as defined above, along with pharmaceutically acceptable excipients and carriers.

When administered *in vivo*, the mammal sestrin product gives raise to a phenotype of muscle cell hypertrophy, in other words, of cell size  
20 enlargement. In mice this capacity leads to animals with an increase in the volume of an organ or tissue due to this enlargement of the cells.

The pharmaceutical composition according to the invention comprising an adeno-associated vector as defined above is preferably for use in the  
25 treatment of muscle atrophy. Preferably human sestrins are employed and more preferably human sestrin-1 corresponding to SEQ ID NO: 1 and/or human sestrin-2 corresponding to SEQ ID NO: 2

The mammalian sestrin product is administered by any method known to  
30 those in the art suitable for delivery to the targeted organ, tissue, or cell type. For example, in certain embodiments of the invention, the composition may be administered by parenteral administration, such as intravenous injection, intra-arterial injection, intrapericardial injection, or subcutaneous injection, or by direct injection into the tissue (e.g., intramuscular). In other embodiments, the  
35 pharmaceutical composition that comprises a mammal sestrin product or an adeno-associated virus comprising it, may be administered by oral, transdermal, intraperitoneal, subcutaneous, sustained release, controlled

release, delayed release, suppository, or sublingual routes of administration. The composition of the invention may also be administered by a catheter system.

- 5 In a preferred embodiment, the pharmaceutical composition of the invention is administered intramuscularly, and is thus formulated in a form suitable for intra-muscular administration. Suitable formulations for intra-muscular administration include solutions, suspensions, dispersions, emulsions, oils and the like.

10

The pharmaceutical compositions of the invention are formulated in conjunction with pharmaceutically acceptable excipients and/or carriers. The term "pharmaceutically acceptable" as used herein refers to compounds, materials, compositions, and/or dosage forms which are, within the scope of  
15 sound medical judgement, suitable for being administered to an animal, including a human, without undue toxicity, incompatibility, instability and allergic response, among others. Each pharmaceutically acceptable carrier, excipients, etc., must also be acceptable in the sense of being compatible with the other ingredients of the pharmaceutical formulation.

20

Pharmaceutically acceptable excipients and/or carriers used in the composition of the present invention are specifically suitable for gene therapy and preferably designed to enhance the stability and bioavailability of the mammal sestrin products.

25

In one embodiment, the composition of the invention comprises excipients and/or vehicles suitable for parental, preferably, intramuscular administration. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Intravenous  
30 vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Examples are sterile liquids such as water and oils, with or without the addition of a surfactant and other pharmaceutically acceptable adjuvants. In general, water, saline, aqueous dextrose and related sugar solutions, and glycols such as propylene glycols or  
35 polyethylene glycol are preferred liquid carriers, particularly for injectable solutions.

The pharmaceutical compositions provided herewith may be controlled-release compositions, i.e. compositions in which the active compound is released over a period of time after administration. For example, the agent may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. Alternatively, the composition is an immediate-release composition, i.e. a composition in which of the active compound is released immediately after administration.

- 10 The compositions also include, in another embodiment, incorporation of the active material into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts.) Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance. Liposomes including the mammal sestrin product or adeno-associated vector comprising it, are particularly suitable for the delivery of these products for use in the treatment of muscle atrophy according to the present invention.
- 20 In one embodiment, the present invention comprises the use of the mammal sestrin product or adeno-associated vector comprising it, as a sole active ingredient for the treatment of muscle atrophy. However, also encompassed within the scope of the present invention is the use of these compounds in combination with one or more additional therapeutic agents. In a particular embodiment, these additional agents are appropriate for treating muscle atrophy. As will be apparent to the skilled in the art, the combination of a mammal sestrin product or adeno-associated vector comprising it, with one or more additional active ingredients, may be effective not only when the active ingredients are used in a single pharmaceutical composition, but also when used in two different compositions, either administered simultaneously, sequentially or separately after a certain period of time.

- 35 In a further aspect, the invention relates to a non-human animal model of muscle cell hypertrophy obtainable by administering to said animal an external amount of a mammal sestrin product or an adeno-associated vector, both as defined above, wherein said mammal has a phenotype of muscle cell hypertrophy.

In a preferred embodiment of the non-human animal model of muscle cell hypertrophy, the mammal sestrins are over-expressed when administered by means of an adeno-associated vector comprising them. Preferably, the non-human animal is a mouse, wherein in a period between 20 and 40 days after the administration of the mammal sestrin product, clear hypertrophic muscles are observed.

Throughout the description and claims the word "comprise" and variations of the word, are not intended to exclude other technical features, additives, components, or steps. Furthermore the word "comprise" and its variations encompasses the expression "consisting of". Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. The following examples and drawings are provided by way of illustration, and they are not intended to be limiting of the present invention. Furthermore, the present invention covers all possible combinations of particular and preferred embodiments described herein.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1, related to results 1, is an agarose gel electrophoresis image showing the levels of cDNA from transcripts (mRNA) of sestrin-1 gene (sesn1), sestrin-2 gene (sesn2) and sestrin-3 gene (sesn3). D means days post-denervation.  $\beta$ -actin is the positive electrophoresis control.

FIG. 2, related to results 1, is also an agarose gel electrophoresis image showing the levels of cDNA from transcripts (mRNA) of sestrin-1 gene (sesn1), sestrin-2 gene (sesn2) and sestrin-3 gene (sesn3) after 24 hours of starvation.  $\beta$ -actin is the positive electrophoresis control. C means control mice; ST means starved mice.

FIG. 3, related to results 2, is an image taken by optical microscopy (microscope DMR6000B (Leica) equipped with a DFC300FX camera) from the myoblast cell line called C2C12 in fed and starved conditions. F means fed; and ST means starved.



- FIG. 4, related to results 3, corresponds to Digital images of the cells extracted from *tibialis* acquired with a microscope DMR6000B (Leica) equipped with a DFC300FX camera for immunohistochemical color pictures, using Plan Fluo 10x/0.3 and 20x/0.75 objective lens and maximum optical sections. Images were composed and edited in Photoshop CS4 (Adobe). Panel A is the control and corresponds to the cells of a control mouse. Panel B corresponds to the state of cells in a mouse 28 days after having received an injection of adeno-associated virus carrying the gene codifying for human sestrin-1 (AAV-Sesn1). Panel C corresponds to the state of cells in a mouse 28 days after having received an injection of adeno-associated virus carrying the gene codifying for human sestrin-2 (AAV-Sesn2). Augmented fragments in dashed lines in the right of FIG. 4 are all equally augmented in order to be comparable.
- FIG. 5, related to results 3, is a bar diagram showing the size (area) of muscle fibers in control (C, AAV-Control) and tested or injected mice (AAV-Sesn1) at 28 days post-injection.
- FIG. 6, also relating to results 3, is a diagram bar that shows the number of nucleus per fiber (N/F) in muscles of control (C or white bars) and of injected mice with AAV-Sesn1 (AAV-Sesn1 or black bars). Y-axis shows the number of cells.
- FIG. 7 (A), related to results 4, is another diagram bar that shows the relative mRNA expression of Atrogin (At) and of the E3 ubiquitin ligase MuRF1 (MuRF), both being markers of an atrophic tissue. White bars are the control (C); Black bars (DEN 7d) correspond to denervated mouse muscle at 7 days post-denervation; and dashed bars (DENN + AAV-hSesn1) correspond to the denervated mouse muscle mice which further received the treatment with AAV-Sesn1. FIG. 7(B), relates to results 4, and shows the Fiber size ( $\mu\text{m}^2$ ), Maximum Force (mN) and Specific Force ( $\text{mN}/\text{mm}^2$ ) of immobilized mice. White bars are the control (C) and correspond to immobilized mouse muscle at 7 days post-immobilization with AAV-Control (without Sesn1); dashed bars are basal (B) values; and Black bars (IMM + AAV-Sesn1) correspond to the immobilized mouse muscle mice which further received the treatment with AAV-Sesn1.

FIG. 8, related to results 5 is a diagram bar that shows the effect of depleting sestrin-1 expression. The effects are represented by determining the levels of expression of MuRF1 labeled with luciferase (MuRF1 RLU is relative luciferase units to detect MuRF1 levels). C means control and corresponds to mice in which no small-hairpin RNA (shRNA) was electroporated (injected). sh-sesn1 corresponds to the group of mice receiving a small-hairpin RNA depleting the transcript of sestrin 1. White bars are fed animals and black bars are fasted (24 h starvation) animals.

## 10 EXAMPLES

The following examples are provided by way of illustration, and are not intended to be limiting of the present invention.

### 15 Materials and methods

#### 1. Animal model.

Wild-type (C57BL/6) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in the animal facility of the PRBB Barcelona under conventional conditions with constant temperature and humidity and fed a standard diet. Male C57BL/6 mice, aged 8-12 weeks, were used for AAV injections, starvation and denervation experiments. Starvation experiments were done by fasting one group of mice for 24 hours before sacrifice.

Denervation was produced by cutting the sciatic nerve in another group of mice high in the thigh after the every mouse had been anesthetized with ketamine/xylazine (80/10 mg/kg).

All animal experiments were approved by the Catalan Government Animal Care Committee.

#### 2. Preparation of adeno-associated virus comprising sestrin mammal products and *in vivo* administration.

AAV2/1-CMV-GFP, AAV2/1-CMV-Sesn1-IRES-GFP and AAV2/1-CMV-Sesn2-IRES-GFP were produced in the Centre for Animal Biotechnology and Gene Therapy of the Universitat Autònoma de Barcelona, (CBATEG-UAB). GFP means Green Fluorescent Protein; CMV is an enhancer derived from cytomegalovirus; IRES means internal ribosome entry site.

AAV vector construction, production and titration for AAV2/1-CMV-Sesn1-IRES-GFP, AAV2/1-CMV-Sesn2-IRES-GFP and their control AAV2/1-sh(-)-IRES-GFP:

Throughout the disclosure the vector constructions AAV2/1-CMV-Sesn1-IRES-GFP, AAV2/1-CMV-Sesn2-IRES-GFP and AAV2/1-sh(-)-IRES-GFP are mentioned in a simplified mode as AAV-Sesn1, AAV-Sesn2, and control (C), respectively.

cDNA corresponding to SEQ ID NO: 1 (human sestrin-1) or to SEQ ID NO: 2 (human sestrin-2) were cloned into XhoI and BamHI sites between the internal terminal repeats of adeno-associated virus serotype 2 (AAV2), under the regulation of the chicken  $\beta$ -actin promoter and the enhancer (CAG) of cytomegalovirus (CMV). The woodchuck hepatitis virus responsive element was added at the 3' end to stabilize mRNA expression, as disclosed in Loeb JE et al, "Enhanced expression of transgenes from adeno-associated virus vectors with the woodchuck hepatitis virus posttranscriptional regulatory element: implications for gene therapy". Hum Gene Ther-1999, Vol. 10, pp.: 2295–2305.

AAV2/1-CMV-Sesn1-IRES-GFP, AAV2/1-CMV-Sesn2-IRES-GFP and AAV2/1-sh(-)-IRES-GFP were generated as previously described by Zolotukhin S et al., "Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield", Gene Therapy -1999, Vol. 6, pp.: 973–985. Briefly, the vectors were generated by triple transfection of HEK 293-AAV cells (Stratagene, Carlsbad, CA, USA) with branched polyethylenimine (Sigma, St Louis, MO, USA), with the plasmid containing the internal terminal repeats of AAV2, the AAV-helper plasmid for each serotype (kindly provided by JM Wilson, University of Pennsylvania, Philadelphia, PA, USA), and the pXX6 plasmid containing helper adenoviral genes. The technology using pXX6 plasmid is disclosed and directly applicable from Xiao

X, Li J, Samulski RJ. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. J Virol -1998, Vol. 72, pp.: 2224-2232.

- 5 Vectors were purified by CsCl or iodixanol gradients. Encapsidated DNA was quantified by a PicoGreen assay (Invitrogen, Carlsbad, CA, USA) following denaturation of the AAV particles and the titers were calculated as  $\mu\text{g}$  per ml. Titters for adeno-associated viruses encompassing GFP (rAAVGFP vectors, or which is the same: AAV2/1-CMV-Sesn1-IRES-GFP, AAV2/1-CMV-Sesn2-IRES-GFP and AAV2/1-sh(-)-IRES-GFP) in international units (IU) per ml  
10 were measured in QBI-HEK 293A cells (Q-Biogene, Carlsbad, CA, USA) by counting transduction events 72 h after vector exposure.

- Small skin incisions were made in the targeted muscle areas of health mice  
15 (Wild-type (C57BL/6)), after every mouse had been anesthetized. The corresponding vector was injected into the *tibialis* (40  $\mu\text{l}$ ,  $5\text{E}+12$  gc/ml) and *soleus* (25  $\mu\text{l}$ ,  $5\text{E}+12$  gc/ml) of each leg using a Hamilton syringe. Gc/ml are genome copies per millilitre.

- 20 3. *In vivo* transfection of shRNA and luciferase assays.

- Adult health mice muscles were transfected by intramuscular injection of small-hairpin RNA (shRNA), 20  $\mu\text{g}$  total, followed by electroporation to increase gene transfer efficiency. Electric pulses were applied by two stainless  
25 steel spatula electrodes connected to an ECM830 BTX porator (Genetronics, San Diego, CA, USA) with the following settings: 5 pulses of 20 ms each and 200 ms interval, the voltage was adjusted according to the thickness of the muscle ( $220\text{ V cm}^{-1}$ ). Muscles were analyzed 8 days later (with or without a 24 hour fasting period). Muscles were collected and luciferase activity was  
30 determined with a luciferase assay kit (Promega). Activity results were normalized by dividing luciferase activity by  $\beta$ -galactosidase activity, this later analysed by any of the known methods, such as the colorimetric assay with the substrate ortho-Nitrophenyl- $\beta$ -galactoside (ONPG).

- 35 4. RNA isolation and quantitative real time PCR (qPCR).

Total RNA was isolated from muscle tissue using miRNeasy Mini Kit (Qiagen). For qPCR experiments (when required), DNase digestion of 10 mg of RNA was performed using 2 U DNase (Turbo DNA-free, Ambion). Complementary DNA was synthesized from 2 mg of total RNA using the First-Strand cDNA  
5 Synthesis kit (Amersham Biosciences). For qPCR, reactions were performed on a LightCycler 480 System using a Light Cyclyer 480 SYBR Green I Master (Roche Diagnostic Corporation) and specific primers. Thermocycling conditions were as follow: initial step of 10 min at 95 °C, then 50 cycles of 15 s denaturation at 94 °C, 10 s annealing at 60 °C and 15 s extension at 72 °C.  
10 Reactions were run in triplicate, and automatically detected threshold cycle (Ct) values were compared between samples. Transcripts of the ribosomal protein L7 gene were used as endogenous control, with each unknown sample normalized to L7 content.

15 5. Western blotting.

Cell lysates were prepared with IP buffer (50 mM Tris HCL pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EGTA, 5 mM EDTA, 20 mM NaF, 25 mM  $\beta$ -glycerophosphate, 0.1 mM sodium vanadate, 1 mM PMSF) supplemented  
20 with protease and phosphatase inhibitors (Complete Mini, Roche Diagnostic Corporation; phosphatase inhibitor cocktail, Sigma). Cleared lysates containing approximately 50  $\mu$ g of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes.

25 6. Histology and immunohistochemistry.

*Tibialis* muscles were frozen in isopentane cooled with liquid nitrogen, and stored at -80 °C until analysis. Cryosections (10  $\mu$ m thickness) were stained with Hematoxilin/eosin (H/E, SIGMA) or Sirius Red (Sigma). Digital images  
30 were acquired with a microscope DMR6000B (Leica) equipped with a DFC300FX camera for immunohistochemical color pictures, using Plan Fluo 10x/0.3 and 20x/0.75 objective lens and maximum optical sections. Images were composed and edited in Photoshop CS4 (Adobe), where background was reduced using brightness and contrast adjustments applied to the whole  
35 image. Image analysis of hypertrophied tissue was performed with the public domain computer-assisted image analysis software ImageJ (NIH, USA). Fiber size (area) measurements were performed as described in- Suelves M, et al.

"uPA deficiency exacerbates muscular dystrophy in MDX mice". J Cell Biol 2007, vol. 178, p. 1039-1051). .

#### 7. Cell culture for biochemical analysis.

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The C2C12 cell line was cultured in DMEM containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.001% Fungizone, (GM growth medium). To induce differentiation in C2C12 myoblasts, GM was replaced by differentiation medium DM (DMEM supplemented with 2% horse serum, 2 mM Lglutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.001% Fungizone) at myoblast subconfluence. To induce starvation in C2C12 myotubes, C2C12 cells were left in DM for 4 days and substituted for Hank's Balanced Salt Solution (HBSS) for the indicated time before samples were collected.

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#### 8. Statistical analysis.

Graphpad Prism software was used for all statistical analyses. Results from corresponding time points of each group were averaged and used to calculate descriptive statistics. One-way ANOVA and Tukey post-hoc test, or Dunnett's post-hoc test were used on multiple comparisons and all possible pair wise comparisons among groups at each time point. Data are mean ± s.e.m. Significance was accepted at  $P \leq 0.05$ .

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### 25 Results

The following results demonstrate that mammal sestrins are involved in the regulation of the muscle mass in atrophic processes by means of the induction of cell hypertrophy.

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#### 1. Denervation and starvation produce alterations of sestrin levels in mice.

Denervated mice and starved mice showed a biochemical pattern wherein the levels of different mice sestrin types (sestrin-1, 2 and 3) were affected by the denervation and starvation processes. To the best of the applicant's knowledge, this is the first time that sestrin levels in such a situation have been analyzed in mice.

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FIG. 1 corresponds to an image of an electrophoresis in agarose gel of the cDNA derived from mice muscle (*tibialis*) cell lysates analyzed after denervation. As can be seen, sestrin levels were significantly reduced from day 0 to day 28 post-denervation, with the exception of sestrin-3 levels. It is known that denervation produce muscle atrophy. Sestrin-1 levels decreased progressively. On the other hand sestrin-2 levels decreased drastically. Finally, sestrin-3 levels were progressively increased.

On the other hand, the starvation process, which results are depicted in FIG. 2, show that the levels of mRNA (cDNA) of sestrin-1 and sestrin-2 were increased in starved versus control mice. Levels of sestrin-3 remained unchanged.  $\beta$ -actin is the positive electrophoresis control. Without being bound to the theory, the inventors hypothesize that sestrin-1 levels in starved mice are increased as a protection cell mechanism. Probably, a prolonged starvation will progress to a decrease in the levels of the protein.

For the analysis of sestrin levels, both in starvation and denervation assays, total RNA was isolated from muscle tissue using miRNeasy Mini Kit (Qiagen). For qPCR experiments, DNase digestion of 10 mg of RNA was performed using 2 U DNase (Turbo DNA-free, Ambion). Complementary DNA was synthesized from 2 mg of total RNA using the First-Strand cDNA Synthesis kit (Amersham Biosciences). As positive control of the electrophoresis  $\beta$ -actin cDNA was detected. For the detection of the different sestrin types and the respective levels, ethidium bromide was used and the gel was visualized.

## 2. Starved myoblasts show atrophic phenotype

As can be seen in FIG. 3, starved cells (C2C12) showed an atrophic phenotype defined as longer and thinner shape, meanwhile fed cells were in a health state.

## 3. *In vivo* administration of AAV-Sesn1 or AAV-2.

Adeno-associated virus with the gene codifying for human sestrin-1, corresponding to SEQ ID NO: 1 (AAV-Sesn1) or SEQ ID NO: 2 (AAV-Sesn2) were prepared and administered (single injection) to health (Wild-type

(C57BL/6)) mice as indicated in methods 2. Control mice (C) were injected with the adeno-associated virus without the gene for sestrin-1 or sestrin-2 (AAV2/1-sh(-)-IRES-GFP).

- 5 28 days later, a sample of the *tibialis* of control and injected mice were extracted and processed as indicated in methods 6.

FIG. 4, panels A, B, and C show that in injected mouse, muscle fibers were greater than those of the control, thus showing a hypertrophic phenotype.

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FIG. 5 is the quantification of FIG.4, showing the size of AAV-Sesn1 injected myofibers. This parameter was calculated as exposed in methods 6. As can be seen the size of fibers in injected mice (AAV-Sesn1) (N=4) at day 28 post-injection was significantly greater than in control (C) mice.

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The same results (data not depicted in FIG. 5) were obtained for AAV-2 injected myofibers. That is, the size of fibers in injected mice (AAV-Sesn2) (N=4) at day 28 post-injection was significantly greater than in control (C) mice.

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This observation also conforms that the tissue in injected mice was hypertrophied.

25 The inventors also determined the number of nucleus per fiber (N/F) of muscle cells in injected (AAV-Sesn1), and control (C) mice. This parameter indirectly reveals the number of regenerating cells existing in a determined tissue, in such a way that the greater the number of nucleus, the greater the number of cells allowing to regenerated damaged or normal tissue.

30 Thus, as can be derived from FIG. 6, the number of nucleus per fiber was greater in mice which previously (28 days before) received the adeno-associated vector comprising the human gene of sestrin-1. These data allow deducing that a greater proportion of regenerating cells is present in mice treated with AAV-Sesn1 in comparison with control mice.

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All these observations lead to the conclusion that mammalian sestrins administered to mice promote tissue hypertrophy. Therefore, it is confirmed



that mammalian sestrins are useful in the treatment of muscle atrophy, since they are able to induce or promote muscle cell hypertrophy.

4. Mammalian sestrins are able to rescue atrophic tissue.

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The inventors also tested models of atrophied tissue (immobilization or denervation) in order to finally confirm that the administration of mammal sestrins were able to revert this situation due to its ability of inducing hypertrophy when over-expressed in mice.

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Thus, they proceed to perform denervation as indicated in methods 1 to mice (DEN) and 7 days post-denervation (or 7 days post immobilization) they detected the levels of relative mRNA expression of two atrophic markers: Atrogin (At) and the E3 ubiquitin ligase MuRF1 (MuRF). After this, denervated or immobilized mice received and injection of AAV-Sesn1 (adeno-associated virus with the human sestrin-1 gene; DEN + AAV-Sesn1; or Immobilized + AAV-Sesn1). The levels of At and MuRF were detected again. As control (C), mice without denervation (or immobilization) and without AAV-Sesn1 administration were used.

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As can be seen in FIG. 7, the levels of relative mRNA expression of two Atrogin (At) and the E3 ubiquitin ligase MuRF1 (MuRF) were highly increased after denervation was done. Interestingly, after the administration of AAV-Sesn1 the levels of both atrophic markers were highly reduced, coming back to control (C) values, thus to normal levels. Moreover, the reduction of fiber size and muscle force (both maximal and specific force) which is observed after immobilization is prevented by the administration of AAV-Sesn1. Thus mammalian sestrins exogenously administered are able to rescue atrophic tissue.

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mRNA levels were measured as indicated in methods 4.

All these experiments as a whole allow concluding that mammalian sestrins are useful tools for the treatment of muscle atrophy, said muscle atrophy being associated to a disease selected from the group consisting of muscular dystrophy, post-polio muscle atrophy, cachexia, leprosy, diabetes, cancer, renal disease, obstructive pulmonary disease, emphysema, paraplegia,

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hemiplegia, and sarcopenia. In the same way, they are useful tools for the treatment of muscle atrophy derived from a physical condition such as a malnutrition state, the atrophy derived from an immobilized limb, or the atrophy of muscle mass due to a long term hospitalization.

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5. Sestrin in mammals is related with atrophic patterns.

The inventors, also with the aim of resolving the possible role of mammal sestrins in atrophy, tested if fed and starved mice showed an atrophied phenotype once mouse sestrin-1 was kidnapped.

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They used a small-hairpin RNA (sh-RNA) corresponding to SEQ ID NO: 7 (gttcaagcactctgagaaggt) to deplete mouse Sesn1 transcript.

As indicated in methods 3, adult mice muscles were transfected by intramuscular injection of the small-hairpin RNAs followed by electroporation to increase gene transfer efficiency. Electric pulses were applied by two stainless steel spatula electrodes connected to an ECM830 BTX porator (Genetronics, San Diego, CA, USA) with the following settings: 5 pulses of 20ms each and 200 ms interval, the voltage was adjusted according to the thickness of the muscle ( $220 \text{ V cm}^{-1}$ ). Muscles were analyzed 8 days later (with or without a 24 hour fasting period). Muscles were collected and luciferase activity was determined with a luciferase assay kit (Promega). Activity results were normalized by dividing luciferase activity by  $\beta$ -galactosidase activity.

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As can be seen in FIG. 8 fed (white bars) and fasted (black bars) showed an increase in the levels of MuRF1 once sestrin-1 had been kidnapped or (in the control group), once starvation was induced. Thus, inhibition of sestrin-1 promotes atrophied tissue.

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FIG. 8 also shows that in mice in which a small-hairpin RNA for sestrin-1 (sh-Sesn) was electroporated the levels of the atrophic marker were greater in fed as well as in starved conditions.

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Thus, without being bound to theory, the inventors propose that in mammals sestrins play a crucial role in the control of muscle mass and in the

maintenance of normal size cells, since when they are depleted (i.e.: by kidnapping) muscle cells *in vivo* showed an atrophic phenotype.

It is also of great interest to mention that the proposed non-human animal model of muscle cell hypertrophy is a good model to study not only the pathways and processes involved in hypertrophy, but also a useful tool to test putative hypertrophy inhibitors. The hypertrophy model is a good approach, since it is a clean model generated from normal animals and avoids background from any pathology.

6. Characterization of sestrins role during physiological skeletal muscle growth.

Young and adult mice were subjected to a three-week intensive exercise training protocol, known to increase muscle mass. Further it was determined by qPCR the levels of sestrin-1 and sestrin-3. It was observed (data not shown) that both sestrins were up-regulated in the exercised muscles, and this correlated with increased myofiber size.

7. Sestrin-1 expression is reduced in muscle wasting-associated pathologies in humans, while its overexpression protects mice from disuse-induced muscle atrophy.

Because Sestrin-1 was increased during adult muscle growth and had a potent hypertrophic effect, the inventors wondered whether its expression would be deregulated in conditions coursing with muscle atrophy. Analysis of muscle biopsies of patients suffering from myopathies characterized by progressive muscle weakness and decreased muscle mass, including myotilinopathy, desminopathy and inclusion body myositis, showed that in all three cases hSesn-1 mRNA expression was reduced by an average of 75% with respect to a control group of healthy individuals (data not shown). Consistent with this, it was found that Sesn-1 expression decreased in atrophying muscle of adult mice after limb immobilization for one week, correlating with a significant reduction in myofiber size and increased levels of the muscle-specific ubiquitin ligase Atrogin (FIG. 7), thus suggesting that Sens-1 might help to preserve mass in disused muscle. More importantly, over-expression of hSesn1 via AAV delivery (but not infection with AAV) was capable of preventing both the immobilization-induced myofiber atrophy,

as indicated by the maintenance of fiber size and the loss of muscle force. These results demonstrate that Sestrins not only induce hypertrophic adult muscle growth but also functionally block muscle atrophy.

5     8. Sestrin-1 and Sestrin-2 induces adult muscle growth through activation of AKT signaling

In adult skeletal muscle, activation of AKT is considered the central event responsible for the increase in muscle mass. Accordingly, the activation of AKT in  
10     resting adult muscles overexpressing human Sestrin-1 (hSesn1) was analyzed. Analysis of protein extracts from muscles of adult mice infected with AAV-Sesn1 (SEQ ID NO: 1, AAV-hSesn1) or AAV-Control showed a significant increase of phosphorylated protein kinase B AKT (P-AKT) levels at 28 days after infection (data not shown) correlating with an increased myofiber size (FIG. 4). Likewise,  
15     overexpression of hSesn2 resulted in increased levels of P-AKT (data not shown) when determined by Western Blot, indicating the activation of this pathway in response to increased levels of mammalian sestrins. Interestingly, although no changes in fiber size were yet found at 7 days after hSesn1 overexpression (not shown), the activation of AKT was already detected at this time-point suggesting  
20     that the Sestrin-induced activation of the AKT growth-promoting pathway precedes the increase in muscle mass. Importantly, treatment of mice with AKT pathway inhibitor wortmannin reduced significantly the Sesn1-induced hypertrophic effect on myofibers, detectable by the decrease in the mean myofiber size and the lower frequency of very large myofibers (data not shown). Consistent with this,  
25     transfection of a dominant negative for of AKT in adult resting mouse myofibers reduced the hypertrophy induced by AAV-hSesn1 overexpression. Confirming these in vivo results, treatment of Sesn1-overexpressing myotubes with wortmannin abrogated the ability of Sesn1 to induce hypertrophy. These results uncover Sestrin as a novel upstream activator of AKT, thereby regulating  
30     physiological muscle growth.

9. Sestrin-1 induces myofiber hypertrophy independent of mammalian target of rapamycin complex 1 (mTORC1) activity.

35     A canonical pathway downstream of AKT activation associated with the positive regulation of protein synthesis is that of mTOR signaling (and in particular, mTORC1). Surprisingly, the data of the present invention revealed that mTORC1

was not implicated in the hypertrophic effect of mammal sestrins (Sesn1), based on the lack of induction of the phosphorylated forms of ribosomal protein S6 (P-S6) and E4-Binding Protein 1 (P-E4-BP1) in adult muscles infected with AAV-Sesn1 compared to AAV-Control (not shown). Consistent with this, treatment of mice with the mTORC1 inhibitor rapamycin did not prevent the Sesn1-induced hypertrophic effect, as evidenced by the similar mean size and frequency of very large myofibers in AAV-Sesn1 infected muscles with or without a 28 day rapamycin treatment. Similar results were obtained upon hSesn2 overexpression *in vivo*. Furthermore, treatment of the Sesn1-overexpressing myotubes with rapamycin did not block the ability of Sesn1 to induce myotube hypertrophy.

These results are in contrast with the reported function of sestrins in cultured cells exposed to genomic stress and in drosophila tissues undergoing oxidative stress, which has been associated with mTOR inhibition through AMPK dependent mechanisms, strongly suggesting that Sestrin exerts distinct effects and operates through distinct effector pathways in stress and in physiological conditions. Consistent with this idea, no significant increase in the activation of AMPK $\alpha$  was observed when hSesn1 was over-expressed in intact muscle. Since AKT activation is the central hypertrophic event underlying physiological muscle growth (i.e. exercise or compensatory hypertrophy), these results suggest that mammal Sestrins are novel regulators of this growth-promoting signalling cascade, which, in turn, may be acting through downstream pathways independent of mTORC1.

In addition to mTORC1, AKT can promote gain of muscle mass through the direct phosphorylation and consequent inhibition of the glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), resulting in increased protein synthesis. AKT also supports maintenance of muscle mass by preventing protein degradation through the phosphorylation and repression of transcription factors of the FoxO family. Indeed, physiological adult muscle growth is regulated by the net balance between protein synthesis and degradation. The inventors observed that the levels of phosphorylated GSK3 $\beta$  (P-GSK3) and FoxO1 (P-FoxO1) were significantly increased in resting muscles transduced with AAV-Sesn1 with respect to AAV-Control infection (data not shown), suggesting a possible involvement of these two branches of the AKT pathway in mediating Sestrin-induced muscle growth. Supporting these *in vivo* results, hSesn1-overexpressing myotubes exhibited increased levels of P-GSK3 and P-FoxO1 (data not shown), correlating with increased myotube size. Finally, it was investigated the possibility that Sestrins were activating the AKT pathway

thought upregulating IGF-1 expression and IGFR activation. Indeed, the involvement the IGF-1/IGFR axis in AKT activation and adult muscle growth in response to exercise and overloading is currently a matter of debate. It was found that the expression of IGF-1 and IGF-2, as well as the phosphorylation of IGFR (P-IGFR), were not altered by overexpression of sh-Sesn1 in resting adult muscle. Similar results were obtained in myotubes overexpressing hSesn1, strongly suggesting that mammal sestrins induce hypertrophy through AKT activation, independent of IGF-1/IGFR activity. Interestingly, it was found that Sesn-1-induced myofiber hypertrophy was significantly attenuated by overexpression of a shRNA specific for Rictor, which is known to inhibit mTORC2 complex formation. Since mTORC2 is known to activate AKT, taken together, the results support the idea that mammal sestrins induce adult skeletal muscle growth by activating the central hypertrophy regulator AKT, through an axis involving mTORC2-and GSK3, independently of mTORC1, and the concomitant repression of FoxO.

Indeed, the inventors demonstrate that mammal sestrins (in particular human Sesn1 (sestrin-1) and human Sesn2 (sestrin-2)) can directly induce hypertrophy in resting adult myofibers, and more importantly, Sesn1 (and Sesn2) overexpression preserved muscle mass from disuse-induced atrophy. Of note, increased levels of Sesn1 also protected mice from loss of muscle force, thus suggesting that mammal sestrins are new regulators of muscle function in the adult.

#### REFERENCES CITED IN THE APPLICATION

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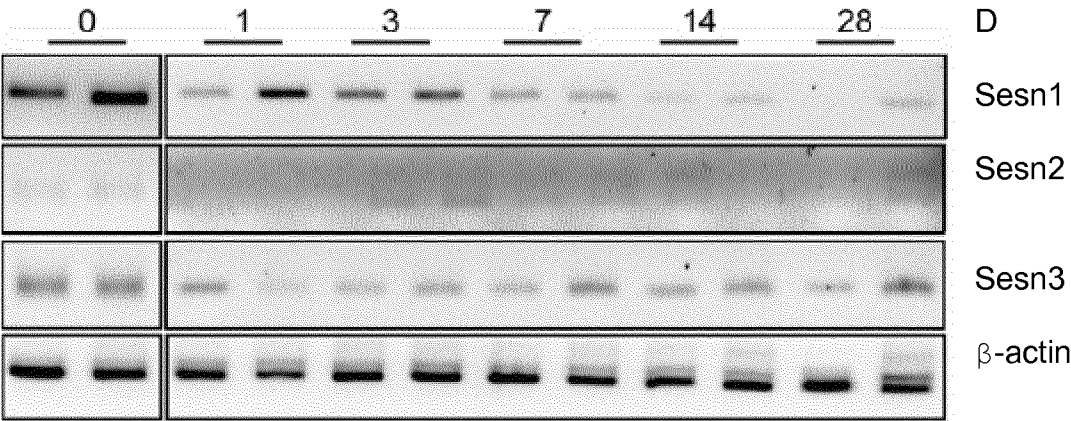
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## CLAIMS

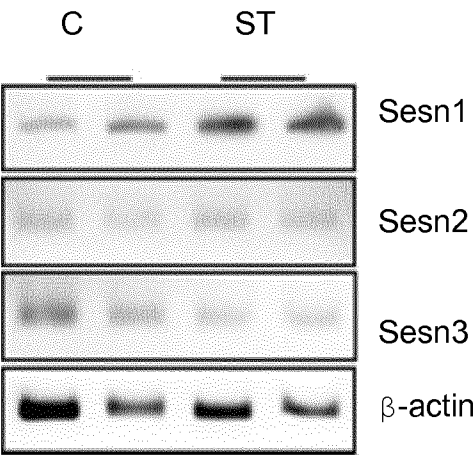
1. A mammal sestrin product for use in the treatment of muscle atrophy.
- 5
2. The mammal sestrin product for use according to claim 1, wherein the treatment comprises the hypertrophy of striated cell muscles.
3. The mammal sestrin product for use according to any of the claims 1-2,
- 10 wherein the sestrin is a human sestrin.
4. The mammal sestrin product for use according to claim 3, wherein the sestrin product is selected from the group consisting of sestrin-1, sestrin-2, sestrin-3, and mixtures thereof.
- 15
5. The mammal sestrin product for use according to claim 4, wherein the sestrin product is selected from the group consisting of the human sestrin-1 corresponding to SEQ ID NO: 1 and the human sestrin-2 corresponding to SEQ ID NO: 2, and mixtures thereof.
- 20
6. The mammal sestrin product for use according to any of the claims 1-5, wherein the muscle atrophy is associated to a disease selected from the group consisting of muscular dystrophy, post-polio muscle atrophy, cachexia, leprosy, diabetes, cancer, renal disease, obstructive pulmonary disease,
- 25 emphysema, paraplegia, hemiplegia, and sarcopenia.
7. The mammal sestrin product for use according to any of the claims 1-5, wherein the muscle atrophy is associated to a physical condition selected from the group consisting of malnutrition, immobilized or casted limb, and long term
- 30 hospitalization.
8. An adeno-associated vector comprising a polynucleotide sequence codifying for a mammal sestrin product.
- 35
9. The adeno-associated vector according to claim 8, wherein the sestrin product is a human sestrin product.



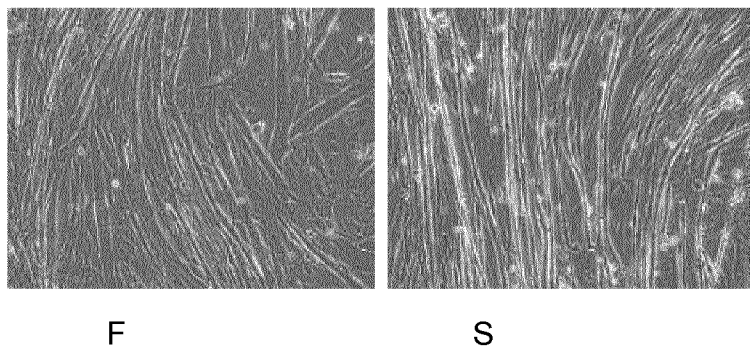
10. The adeno-associated vector according to claim 9, wherein the human sestrin product is selected from the group consisting of sestrin-1, sestrin-2, sestrin-3, and mixtures thereof.
- 5 11. The adeno-associated vector according to claim 10, wherein the sestrin product is selected from the group consisting of the human sestrin-1 corresponding to SEQ ID NO: 1 and the human sestrin-2 corresponding to SEQ ID NO: 2, and mixtures thereof.
- 10 12. The adeno-associated vector according to any of the claims 8-11, for use in the treatment of muscle atrophy.
13. A pharmaceutical composition comprising an adeno-associated vector as defined in any of the claims 8-12 along with pharmaceutically acceptable  
15 excipients and carriers.
14. The pharmaceutical composition according to claim 13, for use in the treatment of muscle atrophy.
- 20 15. The pharmaceutical composition according to any of the claims 12-13, which is for parenteral administration.



**FIG. 1**

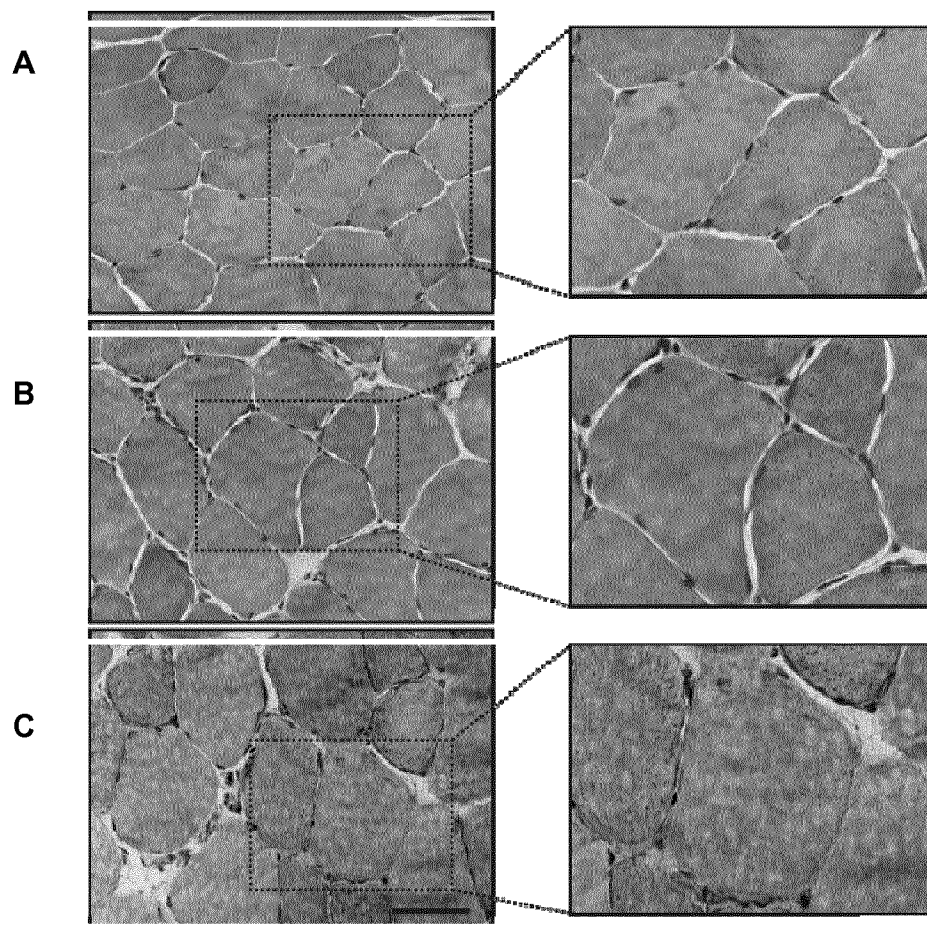


**FIG. 2**

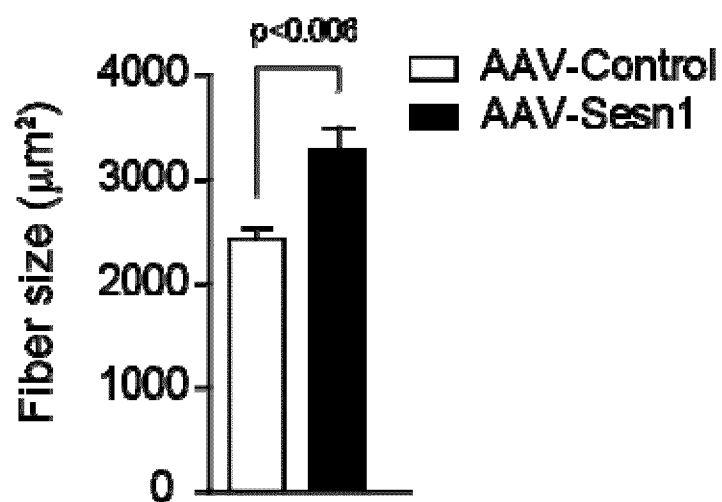


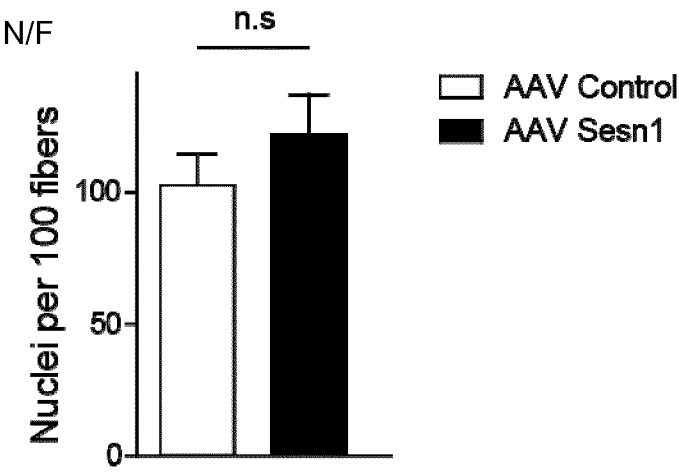
**FIG. 3**

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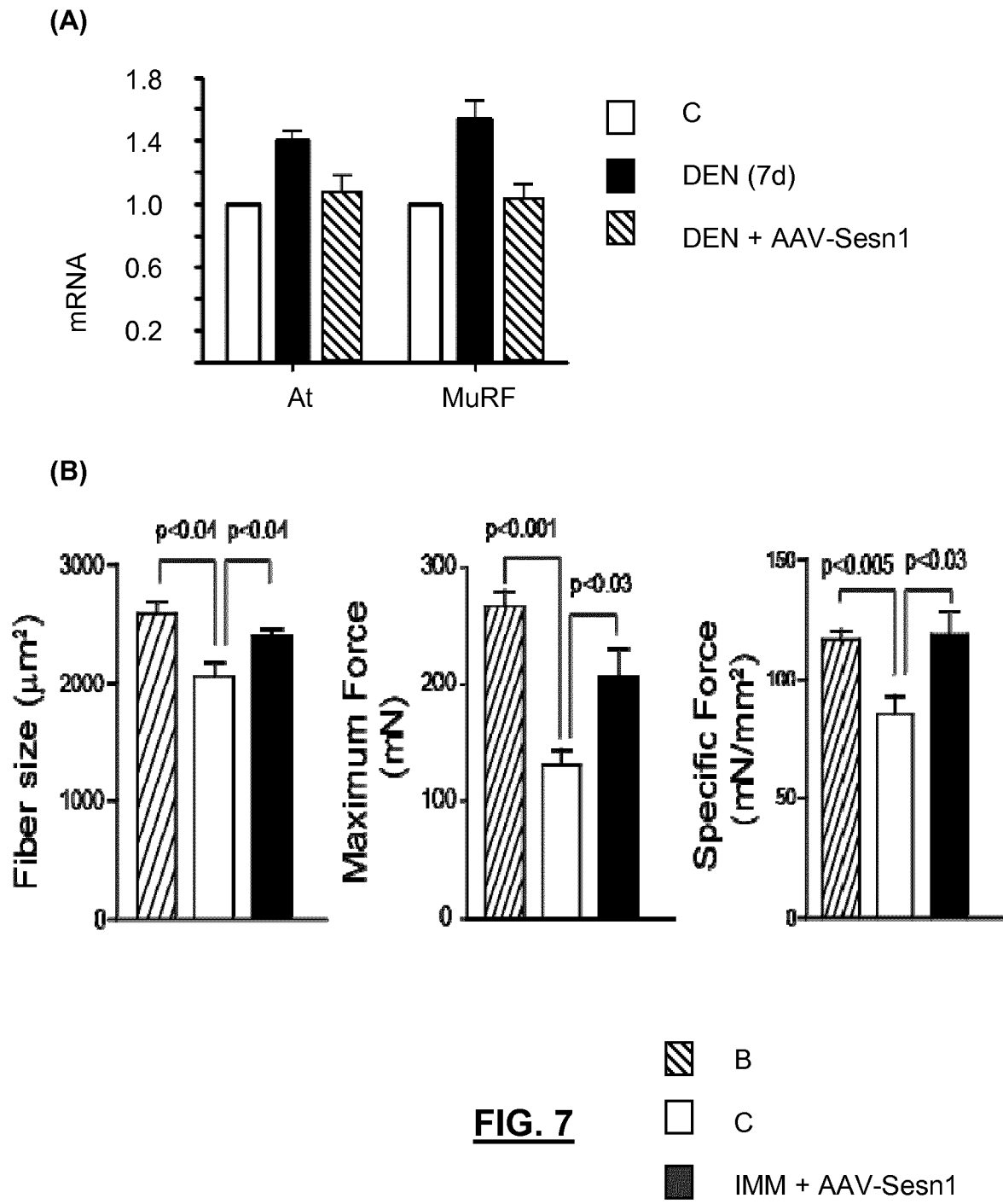
**FIG. 4**

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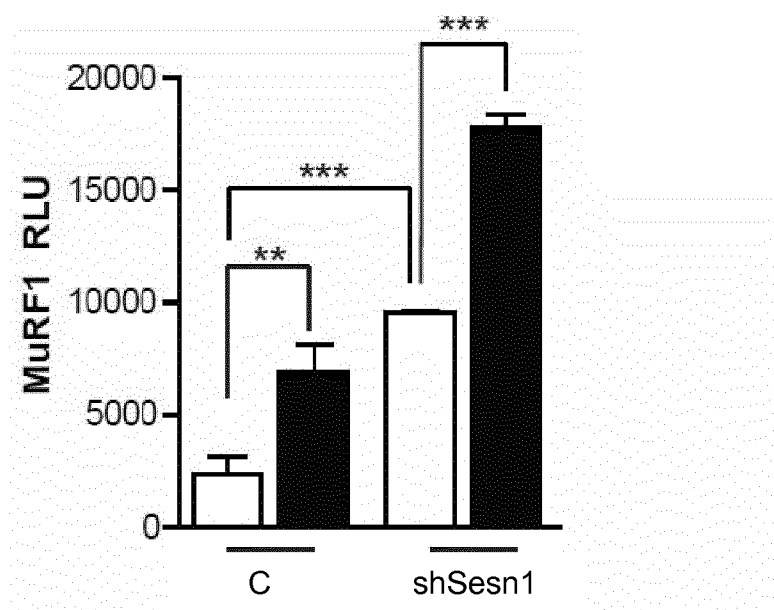
**FIG. 5**



**FIG. 6**



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**FIG. 8**



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2012/065192

### Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
    - a. (means)

☐

 on paper
    - ☒

 in electronic form
  - b. (time)

☒

 in the international application as filed
  - ☐

 together with the international application in electronic form
  - ☐

 subsequently to this Authority for the purpose of search
2. 

☐

 In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2012/065192

## A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/17 A61P21/06 C12N15/864  
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                         | Relevant to claim No. |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Y         | <p>J. H. LEE ET AL: "Sestrin as a Feedback Inhibitor of TOR That Prevents Age-Related Pathologies",<br/>SCIENCE,<br/>vol. 327, no. 5970,<br/>5 March 2010 (2010-03-05), pages<br/>1223-1228, XP055012738,<br/>ISSN: 0036-8075, DOI:<br/>10.1126/science.1182228<br/>abstract<br/>page 1227, left-hand column, paragraph 3 -<br/>middle column, paragraph 2; figure 6<br/>-----<br/>-/-</p> | 1-15                  |



Further documents are listed in the continuation of Box C.



See patent family annex.

## \* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

23 October 2012

Date of mailing of the international search report

30/10/2012

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2  
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Authorized officer

Böhmerova, Eva

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International application No  
PCT/EP2012/065192

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                                                           |                       |
|------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                        | Relevant to claim No. |
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| Y                                                    | -----<br>ANDREI V. BUDANOV ET AL: "p53 Target Genes Sestrin1 and Sestrin2 Connect Genotoxic Stress and mTOR Signaling", CELL, vol. 134, no. 3, 1 August 2008 (2008-08-01), pages 451-460, XP055012741, ISSN: 0092-8674, DOI: 10.1016/j.cell.2008.06.028 abstract figure 5                                                                                                                                 | 1-15                  |
| Y                                                    | -----<br>WO 2009/086526 A2 (SALK INST FOR BIOLOGICAL STUDI [US]; EVANS ROLAND M [US]; NARKAR VIHAN) 9 July 2009 (2009-07-09) paragraphs [0006] - [0011], [0040] - [0044], [0056], [0099] - [0101], [0112], [0113]; claims 47,51                                                                                                                                                                           | 1-15                  |
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|                                                      | -----<br>-/--                                                                                                                                                                                                                                                                                                                                                                                             |                       |

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2012/065192

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                        |                       |
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| A                                                    | <p>MARKUS A. RÜEGG ET AL: "Molecular Mechanisms and Treatment Options for Muscle Wasting Diseases", ANNUAL REVIEW OF PHARMACOLOGY AND TOXICOLOGY, vol. 51, no. 1, 10 February 2011 (2011-02-10), pages 373-395, XP055012732, ISSN: 0362-1642, DOI: 10.1146/annurev-pharmtox-010510-100537 cited in the application the whole document</p> <p>-----</p> | 1-15                  |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/065192

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s)                                                                                                      | Publication<br>date                                                                            |
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