

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
27 December 2007 (27.12.2007)

PCT

(10) International Publication Number
WO 2007/147868 A2

(51) International Patent Classification:
C12N 15/11 (2006.01)

(21) International Application Number:
PCT/EP2007/056181

(22) International Filing Date: 21 June 2007 (21.06.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0612273.3 21 June 2006 (21.06.2006) GB

(71) Applicant (for all designated States except US): **ENS - ECOLE NORMALE SUPERIEURE DE LYON** [FR/FR]; 46 allée d'Italie, F-69007 Lyon (FR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **SCHAEFFER, Laurent** [FR/FR]; 30 rue des Iris, F-69630 Chaponost (FR). **RAMOND, Francis** [FR/FR]; Les Trois Croix, F-42660 Planfoy (FR). **KHOCHBIN, Saadi** [FR/FR]; 9 Allée des Etangs, F-38240 Meylan (FR).

(74) Agents: **TETAZ, Franck** et al.; Cabinet Regimbeau, 129 rue Servient, F-69326 Lyon Cedex 03 (FR).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:

— of inventorship (Rule 4.17(iv))

Published:

— without international search report and to be republished upon receipt of that report
— with sequence listing part of description published separately in electronic form and available upon request from the International Bureau

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PREVENTION OF MUSCLE ATROPHY

(57) Abstract: The present invention relates to a method of treating muscle atrophy, a pathological condition associated with many diseases. The invention also relates to the use of an agent in the manufacture of a medicament for treating muscle atrophy. There is also provided a method of identifying agents for potential use in treating muscle atrophy.



WO 2007/147868 A2

PREVENTION OF MUSCLE ATROPHY

Field of the Invention

The present invention relates to a method of treating muscle atrophy, a pathological
5 condition associated with many diseases. The invention also relates to the use of an agent
in the manufacture of a medicament for treating muscle atrophy. There is also provided a
method of identifying agents for potential use in treating muscle atrophy.

Background to the Invention

Skeletal muscle atrophy mainly results from the degradation of muscle proteins by
10 the ubiquitin/proteasome pathway (reviewed by e.g. Mitch and Goldberg, NEJM **335**,
1897-1905, Dec. 1996). The prevention of muscle wasting has been a major issue during
the last 30 years since muscle atrophy is associated with many pathologies and severely
impairs the daily life of the patients (Akio Innui, CA Cancer J Clin 2002; 52: 72-91).
Cancer cachexia is probably the first cause of muscle wasting. Muscle atrophy negatively
15 interferes with chemotherapies and is associated with 20% of cancer mortality. Muscle
atrophy also results from muscle inactivity or aging, and is associated with various
pathologies such as hyperthyroidism, severe burns, infections, starvation, or AIDS.

Few specific factors have been shown to be involved in the degradation of muscle
proteins during atrophy. The expression of atrogin-1/MAFbx, an F-box protein belonging
20 to E3 ubiquitin ligase complexes, is upregulated by FOXO transcription factors during
muscle atrophy (Sandri et al., 2004, Cell. 2004 Apr 30;117(3):399-412) and atrogin plays a
central role in this process since muscle wasting is significantly reduced in atrogin -/-
animals (Bodine et al., 2001, Science. 2001 Nov 23;294(5547):1704-8; Gomez et al.,
2001). The mechanisms through which atrogin induces muscle wasting are still largely
25 unknown. However, atrogin has been shown to be involved in the degradation of the
bHLH myogenic transcription factor MyoD (Tintignac et al., J Biol Chem. 2005 Jan 28;
280(4):2847-56). MyoD inactivation during muscle atrophy seems to be a central feature
since three pathways have been shown to lead to inactivation of this factor at different
levels: atrogin degrades MyoD, and myostatin as well as NFκB inhibit the expression and
30 the activity of MyoD (Langley et al., J Biol Chem. 2002 Dec 20; 277(51):49831-40. Epub
2002 Sep 18; Guttridge et al., Science. 2000 Sep 29; 289(5488):2363-6).

Mammalian HDACs have been classified in three subclasses according to their
homologies with the yeast HDACs. Class II HDACs are homologous to the yeast Hda1

and are characterized by the presence of a long N-terminal extension which confers them the ability to shuttle between the nucleus and the cytoplasm and to interact with a variety of proteins (reviewed in Yang & Gregoire, Mol Cell Biol. Apr;25(8):2873-84, 2005). HDAC6 is unique among HDACs since it contains an ubiquitin binding motif (Seigneurin-Berny et al., Mol Cell Biol. 2001 Dec; 21(23):8035-44), and two deacetylase domains, which can deacetylate α tubulin (Hubbert et al., Nature. 2002 May 23;417(6887):455-8; Matsuyama et al., EMBO J. 2002 Dec 16;21(24):6820-31; Zhang et al., EMBO J. **22** (5), 1168-1179, 2003). In addition, HDAC6 has been shown to interact with components of the ubiquitin proteasome pathway (Seigneurin-Berny et al., Mol Cell Biol. 2001 Dec; 21(23):8035-44; Hook et al., Proc Natl Acad Sci U S A. 2002 Oct 15; 99(21):13425-30).

It is amongst objects of the present invention to provide means to reduce and/or mitigate against muscle atrophy in a subject.

It is a further object of the present invention to provide a method of screening candidate agents for potential utility in treating conditions associated with muscle atrophy.

Summary of the Invention

The present invention is based in part on observations by the present inventors that expression of HDAC6 appears to be associated with skeletal muscle atrophy.

In summary, the present inventors have first observed that HDAC6 expression is upregulated during muscle atrophy, in several classical mouse models leading to muscle atrophy (for example, fasting or muscle denervation). They have also shown that HDAC6 interacted with Atrogin1/Mafbx, a known actor of muscle atrophy (Bodine et al., Science. 2001 Nov 23;294(5547):1704-8; Gomes et al., Proc Natl Acad Sci U S A. 2001 Dec 4;98(25):14440-5; Sandri et al. Cell. 2004 Apr 30;117(3):399-412). The inventors have next demonstrated that HDAC6 forms a ternary complex with Atrogin and MyoD, and that HDAC6 is required for the atrogin-dependent MyoD degradation. Altogether these *in vitro* results indicated that HDAC6 is an important factor involved in muscle atrophy. Confirmation of these conclusions *in vivo* was obtained using an approach based on (i) analysis of HDAC6 knockout mice and (ii) RNA interference (RNAi)-mediated HDAC6 gene knock-down. The present inventors have found out that HDAC-6 is involved in muscle atrophy and that the abolition or reduction of HDAC-6 expression leads to a decrease of muscle atrophy.

The present inventors have thus found out a new mechanism of action which can advantageously be exploited in the treatment and prevention skeletal muscle atrophy.

In a first aspect, the present invention thus provides a method of reducing and/or preventing muscle atrophy, the method comprising the step of administering to a subject, a therapeutically effective amount of an agent which is capable of reducing expression and/or activity of HDAC6 in the subject.

5 By muscle atrophy we mean a loss of skeletal muscle mass. Muscle atrophy can be evaluated by several well known methods, including for example the measure of the muscle wet weight, or muscle fiber diameter.

By reducing or preventing muscle atrophy we mean slow or reduce or halt the loss of muscle mass, or reduce the extent of or the likelihood of occurrence of muscle atrophy.

10 By subject, we mean any human or a animal in need thereof, i.e. a subject at risk or suffering from a disease or condition associated with muscle atrophy. Non limiting examples of which are given below.

Therapeutically effective amounts of an agent refer to the amount of agent capable of reducing the extent of muscle atrophy by at least 10%, or reducing the extent of muscle fiber loss by at least 1%, relative to a control subject (i.e. non treated with the said agent).

The method comprises administration of at least one dose of an agent. Muscle atrophy being often a long term process, chronic administration of doses of the agent can be envisaged.

20 The dose to be administered comprises in addition to a therapeutically effective amount of the agent a pharmaceutically acceptable excipient. Formulation of the agent used in the method of the invention can be carried out using methods of preparation that are standard in the art (see e.g., Remington 's pharmaceutical Sciences ed. A. Gennaro, Mark publishing Co. Easton, PA, 1990).

25 As a matter of example the dose can be formulated as a sterile aqueous solution which can be administered by for example subcutaneous, intramuscular or intradermal routes. Mucosal administration can also be contemplated such as oral or nasal administration.

It could also be envisaged to administer a dose of the agent directly in the muscle by electroporation. Various method of electroporation have been disclosed in the art see e.g. 30 US2006036210 or WO2004063350, to cite a few of them.

In summary, the present inventors have observed that HDAC6 expression is upregulated during muscle atrophy, in several classical conditions leading to muscle atrophy (for example, fasting or muscle denervation). They have next shown that HDAC6

interacted with Atrogin1/Mafbx, a known actor of muscle atrophy (Bodine et al., Science. 2001 Nov 23;294(5547):1704-8; Gomes et al., Proc Natl Acad Sci U S A. 2001 Dec 4;98(25):14440-5; Sandri et al. Cell. 2004 Apr 30;117(3):399-412). Atrogin has been shown to induce the degradation of MyoD (Tintignac et al., J Biol Chem. 2005 Jan 28;280(4):2847-56). The inventors have shown that HDAC6 forms a ternary complex with Atrogin and MyoD, and that HDAC6 is required for the atrogin-dependent MyoD degradation. Altogether these results indicated that HDAC6 was probably involved in muscle atrophy. This was next demonstrated in vivo using an approach based on (i) analysis of HDAC6 knockout mice and (ii) RNA interference (RNAi)-mediated HDAC6 gene knock-down. The results obtained showed that complete elimination or even reduction in HDAC6 expression reduced muscle atrophy.

In a further aspect there is provided use of an agent which is capable of reducing expression and/or activity of HDAC6 in a subject, for the manufacture of a medicament for treating or preventing muscle atrophy.

As used herein, agent capable of reducing expression and/or activity of HDAC-6 (or inhibitor of HDAC-6) means a compound that inhibits the expression and/or translation of the HDAC6 gene itself and/or prevents or disrupts binding of HDAC6 protein to atrogin-1 and/or MyoD and/or ubiquitin and/or ubiquitinated proteins. According to a particular embodiment of the invention the said agent or inhibitor is specific of HDAC-6 i.e. does not inhibit other class I HDAC proteins, preferably does not inhibit other HDAC class II proteins.

By inhibition or prevention is meant total or at least partial reduction (at least 30%, 40%, preferably at least 50%, in particular at least 60, 70, 80 or 90 %) of the contemplated activity. Such inhibition can be easily shown and quantified using the method of screening disclosed in the present application.

Diseases or conditions which are associated with muscle atrophy and may therefore benefit from preventing and/or treating muscle atrophy include cachexia associated with cancer, cerebrovascular injury, peripheral nerve injury, osteoarthritis, rheumatoid arthritis, prolonged corticosteroid therapy, diabetes, burns, poliomyelitis, amyotrophic lateral sclerosis, Guillain-Barre syndrome, muscular dystrophy, congenital myotonia, myotonic dystrophy and myopathy, myasthenia gravis, congenital myasthenia, as well as muscle atrophy which may occur due to old age and prolonged immobilisation or weightlessness.

Limiting muscle atrophy would also be beneficial to astronauts staying for long period in space.

The agent may have an effect on the expression of the HDAC6 gene itself and/or regulate activity of the HDAC6 protein, such as regulating its binding to other proteins, including atrogen, ubiquitin or ubiquitinated proteins and/or MyoD.

With respect to expression, the agent may down-regulate expression of the HDAC6 gene. Down-regulation of the HDAC6 gene may be achieved by way of an RNA interference (RNAi) approach, using one or more small interfering RNAs (siRNA), or small hairpin RNA (shRNA) constructs designed to target distinct regions of the HDAC6 gene. RNAi is well known to the skilled addressee, but for a review of the subject, the reader is directed to, for example, Nature, vol. 431, no. 7006. An exemplar RNAi sequence designed to target a distinct regions of the HDAC6 gene is GAAGUGUGCUGUACCCAC. This sequence can be used to generate a small hairpin RNA construct. Other siRNA molecules suitable for use in down-regulating HDAC6 may be designed using the BIOPREDsi algorithm that computationally predicts 21-nt siRNA sequences that have an optional knockdown effect for a given gene (www.biopredsi.org). BIOPREDsi can be used either by uploading a particular sequence or an identifier for a given gene. The software then will design a user-specified number of siRNA sequences that are predicted to have an optimal knockdown effect on the target gene. For every siRNA sequence a score is returned, which reflects the predicted potential of the siRNA to decrease the expression levels of a target mRNA.

Alternatively down-regulation of HDAC6 expression may be achieved using antisense oligonucleotides designed to bind to HDAC6 nucleic acid, such as mRNA and prevent and/or reduce transcription and/or translation to HDAC6 protein. Again, the use of antisense is well known to the skilled addressee. However the skilled reader is directed to a brief review on the subject by Stein & Dias (Molecular Cancer Therapeutics, Vol. 1, 437 – 355, 2002).

Agents which act to regulate activity of the HDAC6 protein include antibodies or chemical agents which may be capable of, for example, preventing or inhibiting binding of HDAC6 to atrogen, ubiquitin and/or MyoD. In addition, DNA or RNA or peptide aptamers that can tightly bind to HDAC6 and prevent its interaction with ubiquitin, atrogen and/or MyoD could also be developed and used.

Suitable antibodies may be monoclonal or polyclonal antibodies or fragments thereof which specifically bind HDAC6, ubiquitin, atrogin or MyoD and which inhibit the formation of a complex between HDAC6, ubiquitin, atrogin, MyoD and combinations thereof which comprise HDAC6, such as HDAC6 and atrogin and HDAC6, atrogin and
5 MyoD and HDAC6 and ubiquitin.

The term "antibody" as referred to herein includes whole antibodies, including those of the IgG, IgM and IgA isotypes, and any antigen binding fragment (i.e., "antigen-binding portion") or single chain thereof. An "antibody" refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by
10 disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. The IgG heavy chain constant region is comprised of four domains, C_{H1} , hinge, C_{H2} and C_{H3} . Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The light chain constant region is comprised of one
15 domain, C_L . The V_H and V_L regions can be further subdivided into regions of hyper variability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the
20 heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the classical complement system.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"),
25 as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., HDAC6, atrogin or MyoD and prevent complex formation with one or more of the other proteins). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of
30 an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L , V_H , C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a

single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341: 544-546), which consists of a V_H domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker
5 that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242 : 423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85: 5879-5883) or via other means such as the use of disulphide bonds or through dimerization motifs. Such single chain antibodies are also intended to be encompassed within the term
10 "antigen-binding portion" or an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies. Other known antibody fragments include nanobodiesTM, the technology surrounding which is licensed to Ablynx in Belgium.

15 The antibodies or fragments of the present invention may also be humanised. An antibody can be humanized by any method, which is capable of replacing at least a portion of a CDR of a human antibody with a CDR derived from a nonhuman antibody. Winter describes a method which may be used to prepare the humanized antibodies of the present invention (UK Patent Application GB 2188638A, filed on March 26, 1987), the contents of
20 which is expressly incorporated by reference. The human CDRs may be replaced with nonhuman CDRs, for example using oligonucleotide site-directed mutagenesis as described in International Application WO 94/10332 entitled, *Humanized Antibodies to Fc Receptors for Immunoglobulin G on Human Mononuclear-Phagocytes*.

Also within the scope of the invention are chimeric and humanized antibodies in
25 which specific amino acids have been substituted, deleted or added. In particular, preferred humanized antibodies have amino acid substitutions in the framework region, such as to improve binding to the antigen. For example, in a humanized antibody having mouse CDRs, amino acids located in the human framework region can be replaced with the amino acids located at the corresponding positions in the mouse antibody. Such
30 substitutions are known to improve binding of humanized antibodies to the antigen in some instances. Antibodies in which amino acids have been added, deleted, or substituted are referred to herein as modified antibodies or altered antibodies.

The present invention further embraces variants and equivalents which are substantially homologous to the humanized antibodies and antibody fragments set forth herein. These may contain, e.g., conservative substitutions, i.e. the substitution of one or more amino acids by similar amino acids. For example, conservative substitution refers to
5 the substitution of an amino acid with another within the same general class, e.g., one acidic amino acid with another acidic amino acid, one basic amino acid with another basic amino acid, or one neutral amino acid by another neutral amino acid. What is intended by conservative amino acid substitution is well known in the art.

As used herein, "specific binding" refers to antibody binding to a predetermined
10 antigen. Typically, the antibody binds with a dissociation constant (K_D) of 10^{-7} M or less, and binds to the predetermined antigen with a K_D that is at least two-fold less than its K_D for binding to a non-specific antigen (e.g., BSA, casein) other than the predetermined antigen or a closely-related antigen.

Other chemical agents which can prevent or disrupt complex interaction or complex
15 formation between HDAC6, ubiquitin, atrogin and/or MyoD include small chemical molecules or competitive peptides of HDAC6, ubiquitin, atrogin or MyoD which are capable of competing with the binding of the natural HDAC6 protein with ubiquitin, atrogin and/or MyoD. Such chemicals or peptides can easily be tested by the skilled addressee for their ability to prevent or disrupt complex formation between HDAC6 and
20 atrogin and/or MyoD. One such chemical may be tubacin, which is known to target HDAC6 (Haggarty et al., PNAS, 2003, 100(8): 4389-4394). The provision of candidate chemicals for use in the present invention are well known to those skilled in the art. For example libraries of compounds can be easily synthesised and tested. This is well described for example in: Applications of combinatorial technologies to drug discovery.
25 2. Combinatorial organic synthesis, library screening techniques, and future direction, J. Med. Chem., 1994, 37, 1385-1401. Also peptides can easily be synthesised and tested using techniques well known to the skilled addressee. RNA or DNA aptamers can be selected in vitro by iterative cycles of the SELEX procedure with the goal of identifying such molecules that are able to prevent or disrupt the interaction of HDAC6 and atrogin
30 and/or MyoD and/or ubiquitin. A review on aptamers can be found for example in (Nimjee SM et al., 2005).

Inhibitors of HDAC are known in the art, including WO2005/034880, EP1362914, EP1297815 and references cited herein (inter alia AU 9,013,101; AU 9,013,201; AU

9,013,401; AU 6,794,700; EP 1,233,958; EP 1,208,086; EP 1,174,438; EP 1,173,562; EP 1,170,008; EP 1,123,111; JP 2001/348340; U.S. 2002/103192; U.S. 2002/65282; U.S. 2002/61860; WO 02/51842; WO 02/50285; WO 02/46144; WO 02/46129; WO 02/30879; WO 02/26703; WO 02/26696; WO 01/70675; WO 01/42437; WO 01/38322; WO 01/18045; WO 01/14581; Furumai et al. (2002); Hinnebusch et al. (2002); Mai et al. (2002); Vigushin et al. (2002); Gottlicher et al. (2001); Jung (2001); Komatsu et al. (2001); Su et al. (2000).). Inhibitors of HDAC include depudecin (Kwon HJ et al.; PNAS 1998), Trapoxins A and B (Kijima M et al.; J. Biol. Chem. 1993), Cyclic hydroxamic-acid containing peptide (CHAPs) (Komatsu Y et al.; Cancer Res. 2001), Apicidins A, C, D1, D2 and D3 (Singh et al.; J. Org. Chem. 2002), Oxamflatin (Kim YB et al.; Oncogene 1999), synthetic hydroxamic acid-containing HDAC inhibitors (Monneret C; European Journal of Medicinal Chemistry 2005), and synthetic benzamide-containing HDAC inhibitors (Monneret C; European Journal of Medicinal Chemistry 2005). Selection of inhibitors specific to HDAC-6 can be made in screening these inhibitors using the method of screening disclosed in the present application.

Peptide aptamers may also be used. Peptide aptamers are proteins that are designed to interfere with other protein interactions inside cells. Peptide aptamers consists of a variable peptide loop attached at both ends to a protein scaffold. The variable loop length is typically comprised of 10 to 20 amino acids and the scaffold may be any protein which has good solubility and capacity properties e.g. thioredoxin-A. For a review see Hoppe-Seyler et al., Curr Mol Med 2004, Aug 4(5): 529-38.

Specifically, inhibitors of HDAC-6 have also been disclosed in the art, such as Paclitaxel and Lonafarnib (Marcus et al. ; Cancer Res. 2005), Tubacin and Paclitaxel 1 μ M (Marcus et al. ; Cancer Res. 2005), Tubacin (Haggarty et al. ; PNAS 2003), Trichostatin A (TSA) (Wong et al. ; Journal of American Chemical Society 2003) and Suberoylanilide hydroxamic acid (SAHA) (Wong et al.; JACS 2003).

All inhibitors of HDAC were known in the art as regulators of protein acetylation, mainly histone acetylation, and as regulators of cellular processes such as apoptosis, gene expression and tubulin acetylation to be used in the treatment of diseases such as cancer, cardiac hypertrophy (cf. EP 1 297 851), or neurological disorders (for example, sodium valproate is used to treat epilepsy and bipolar disorders : Bioorg Med Chem Lett. 2007 Jun 6, PMID: 17566732). None of these references identified any relationship between HDAC-

6 and muscle atrophy. None of them suggests using inhibitors of HDAC in the prevention and treatment of muscle atrophy.

Thus, in a further aspect the present invention provides an agent for use in preventing or disrupting interaction or complex formation between HDAC6 and ubiquitin, atrogin and/or MyoD for use in treating muscle atrophy. The agent may be any of the
5 aforementioned agents, such as an RNAi molecule, antibody or fragment thereof, small chemical molecule or aptamer.

The present invention also provides a pharmaceutical composition comprising an agent capable of preventing or disrupting interaction or complex formation between
10 HDAC6 and ubiquitin, atrogin and/or MyoD and a pharmaceutically acceptable excipient therefore.

In a further aspect, there is provided a screening assay for identifying an agent capable of preventing or disrupting interaction or complex formation between HDAC6 and atrogin and/or MyoD and/or ubiquitin, comprising the steps of:

- 15 a) providing HDAC6 and atrogin and/or MyoD and/or ubiquitin;
- b) contacting a candidate agent with HDAC6 and atrogin and/or MyoD and/or ubiquitin;
- c) allowing HDAC6 and atrogin and/or MyoD and/or ubiquitin to come into contact with one another under conditions which are suitable to allow complex formation
20 between HDAC6 and atrogin and/or MyoD in the absence of the candidate agent; and
- d) detecting whether or not the candidate agent is capable of disrupting complex formation.

HDAC6, atrogin and MyoD and/or ubiquitin may all be provided as purified proteins obtained by, for example, recombinant expression of the proteins, or as purified
25 extracts from appropriate cells. Enzymatically active HDAC6 can be expressed in insect cells from baculovirus expression vector (Zhang et al., J Biol Chem. 2006 Feb 3;281(5):2401-4). All such techniques are well known to the skilled addressee.

As will be appreciated such as a screening assay is conducted *in vitro* and may be carried out in any appropriate container, such as in a well of a multi-well plate, or the like.

30 Detection of complex formation can be carried out in a number of suitable ways. For example, HDAC6, ubiquitin, atrogin and/or MyoD, as used in the assay may be directly labelled in some manner, for example, chemically, fluorescently, radiometrically or the like, with one of the other proteins bound to the surface of the receptacle. In this

manner, an assay can be set up such that a detectable signal is obtained when the labelled protein complexes with the substrate bound protein.

Alternatively, a protein complex may be detected, by way of, for example, an antibody which is capable of specifically binding the complex. Such an antibody may be
5 detected by way of a label bound to the antibody or by way of a further labelled antibody designed to bind to the first antibody.

In a further aspect, there is provided a screening assay for selecting *in vivo* an agent capable of preventing or disrupting interaction or complex formation between HDAC6 and atrogen and/or MyoD and/or ubiquitin (HDAC6 inhibitor), for reducing and/or preventing
10 muscle atrophy comprising the steps of:

- inducing muscle atrophy in an animal model, particularly a mice,
- quantifying muscle atrophy prior administering a sample of HDAC6 inhibitor
- administering an appropriate amount of HDAC6 inhibitor by appropriate means
- quantifying muscle atrophy after administration of the HDAC6 inhibitor and
15 - comparing the results obtained.

The present invention will now be further described by way of example and with reference to the Figures which show:

Figure 1: HDAC6 is upregulated during muscle atrophy and interacts with Atrogen

HDAC6 is activated in two different models of muscular atrophy. **a** Relative expression
20 levels of *atrogin-1*, *FOXO3*, and *HDAC6* were assayed by quantitative RTPCR in the *tibialis anterior* muscle of 48 hours-fasted mice and pair-fed littermates. Data are means $\pm$ sem, n = 4 mice. **b** The same assay was performed on a time course of 0, 3, 7, and 14 days-denervated or contralateral innervated muscles.

HDAC6 interacts with *Atrogen-1* *via* its deacetylase domain 1. **c** HA-tagged *HDAC6* and
25 Flag-tagged atrogen were expressed in Cos cells. Extracts were then used to immunoprecipitate Flag-atrogen and the coimmunoprecipitation of HA-*HDAC6* was monitored by an anti-HA antibody. + and - represents extracts from cells expressing only *HDAC6* (-) or *HDAC6* and *Atrogen* (+). The arrow shows the *HDAC6* co-immunoprecipitated with atrogen. *: Ig heavy chain (anti-Flag). **d** The indicated fragments
30 of *HDAC6* were fused to GST, expressed in bacteria and immobilized on glutathione beads (shown on the coomassie panel). Extracts from Cos cells over-expressing Flag-atrogen were incubated with glutathione beads covered with GST or GST-*HDAC6* fragments. Beads were then washed and the retained atrogen visualized by a western blot. *HDAC6*

fragments are : N-ter is the N-terminal non catalytic domain, HDD1 and HDD2 respectively correspond to the catalytic domains 1 and 2 of HDAC6, and C-ter is the ubiquitin-binding domain of HDAC6 (Seigneurin-Berny et al., Mol Cell Biol. 2001 December; 21(23): 8035–8044.).

5 Figure 2: HDAC6, Atrogin-1, and MyoD, form a functional ternary complex.

a The indicated tagged proteins were expressed in Cos cells (input panel). Flag-Atrogin (A) was then immunoprecipitated and the co-immunoprecipitated HA-MyoD (M) and HA-HDAC6 (H) were detected by western blot using an anti-HA antibody (IP panel). **b** Cos cells were expressed as in **a** except that extracts were used to monitor protein binding to
 10 ubiquitin beads. Proteins retained on Ub-beads were then detected using the anti-HA (HDAC6 and MyoD) and anti-Flag (atrogin). I, input; B, bound. **c** The indicated proteins were expressed in Cos cells together with His-tagged ubiquitin. Ubiquitinated proteins were then captured under denaturing conditions using Ni-beads and analyzed by western blot. Note that the capture of HDAC6 is probably due to its ability to bind Ni through its
 15 Histidine/Cysteine rich C-ter domain.

Figure 3: HDAC6 regulates MyoD levels

HDAC6 is required for the Atrogin-dependent degradation of MyoD. **a** C2C12 cells were cotransfected with vectors expressing HA-MyoD (M), Atrogin-1 (A), and the HDAC6-shRNA (sh) as indicated. The cells were incubated with cycloheximide (CHX) 24
 20 hours after transfection, and total proteins harvested at the indicated times of treatment for analysis by Western blotting with anti-HA or anti α -tubulin. **b** Nuclear MyoD and Myogenin are increased in the absence of HDAC6 in C2C12 differentiating cells. Western blot showing endogenous MyoD (upper panel) and Myogenin (lower panel) in nuclear (N), membranes (P), and cytoplasmic (C) fractions of C2C12 cells transfected with either the
 25 HDAC6-shRNA or a control vector, upon the indicated times of differentiation. Arrows indicate the cytoplasmic (40kDa) and the nuclear phosphorylated (50kDa) forms of MyoD. **c** Atrogin-dependent inactivation of MyoD requires HDAC6. Luciferase activity of a reporter vector under control of the *myogenin* promotor was assayed 24h after transfection of various constructs as indicated, in C2C12 cells. Data are means $\pm$ sem, n = 3 wells.

30 Figure 4: HDAC6 inactivation reduces muscle atrophy *in vivo*.

HDAC6 $-/-$ (KO) mice show reduced atrophy upon denervation. **a** Loss of mass and cross-sectional surface were measured in 14 days-denervated *tibialis anterior* muscles from wild-type and KO mice. Results are means $\pm$ sem, n = 5 mice, plotted as a percent of

innervated control contralateral muscles. Cryosections were performed, and distribution and mean value of muscle fibers areas were plotted as a frequency histogram for WT and KO muscles (**b**), as well as for muscles electroporated with the HDAC6-shRNA expression vector, or mock treated (**d**). **c,e** Representative pictures of KO and WT muscles cryosections (**c**) and of electroporated muscles (**e**, *: GFP-positive, shRNA-transfected fibres). **f** Expression levels of *MCK* were assayed by quantitative RTPCR in the *tibialis anterior* muscle of WT compared to KO littermates. Data are means $\pm$ sem, n = 5 mice.

Material and methods

Plasmids

10 The HDAC6-shRNA plasmid was constructed by cloning double stranded oligonucleotides (Eurogentec, Belgium), into the BamH1/HindIII sites of the pRNAT-H1.1/Neo vector (Genescript Corporation, USA) expressing the cytoplasmic GFP protein.

The sequences of the oligonucleotides were as follows:

HDAC6-shRNA plus strand (SEQ ID NO1)

15 gatcccGAAGTGTGCTGTACCCACtcaagagaGTGGGGTACAGCACACTTCtttttccaaa

HDAC6-shRNA minus strand (SEQ ID NO2)

agcttttggaaaaaaGAAGTGTGCTGTACCCACtctcttgaaGTGGGGTACAGCACACTTCg,

with the shRNA sequences in sense and antisense orientations underlined.

Electroporation and denervation

20 Four to five week old Swiss Webster mice were anesthetized with 100 μ l of 0.05% Xylazine- 1.7% Ketamine in NaCl 0.9%. 5 μ g of empty or HDAC6-shRNA vector or empty control pRNAT vector were injected transcutaneously into the *tibialis anterior* (TA) muscle using a sterile 1ml syringe with a 27 gauge needle. Caliper electrode plates (Q-biogen, France) were immediately applied on each side of the muscle, and a series of
25 8 electrical pulses (2Hz, 20 ms each) was delivered with a standard square-wave electroporator (ECM 830, Q-biogen). Electrical contact was ensured by shaving the leg of the animal and by conductive gel application.

Two days later, atrophy was induced in the left TA muscle of the previously electroporated animals, by sciatic nerve transection under anesthesia. The TA of the right
30 leg of the same animal was left innervated, and used as a control.

Fourteen days after denervation, TA were dissected, weighted, fixed 1 hour in 4% paraformaldehyde at 4°C, washed briefly in PBS, and incubated 30 min in 0.1M Glycine in PBS at room temperature. The effectiveness of plasmid expression was monitored

under fluorescence microscope by the presence of GFP expressing fibers. The muscles were then incubated 1h at 4°C in 30% sucrose, and finally embedded with liquid-nitrogen freezed Cryomatrix resin (Thermo Shandon, USA) before conservation at -20°C. Twenty-five µm thick transversal cryosections of the muscles were then realized, and immediately mounted on glass slides in a fluorescence medium (Dako, Denmark).

Images were acquired on an Axioplan fluorescence microscope (Zeiss) with a Coolsnap digital camera (Nikon), and the surface measurement of the GFP-expressing fibers performed with the AnalySIS software (Olympus).

Quantitative RT-PCR analysis.

Total RNA was extracted from dissected muscle fibers 1 week after electroporation using the Nucleospin RNA II kit (Macherey-Nagel, France). First strand cDNA was synthesized from 200 ng of total RNA using the Superscript II reverse-transcriptase as described by the manufacturer (Life-Technologies, France). Primer sequences were chosen using the primer 3 input software (www-genome.wi.mit.edu), with Tm fixed at 60°C and amplicon length between 100 and 150 bp. Primer sequences were as follows:

β-actin: forward (SEQ ID NO3) 5'-CCCTGTATGCCTCTGGTCG-3',
reverse (SEQ ID NO4) 5'-ATGGCGTGAGGGAGAGCAT-3';
mHDAC6: forward (SEQ ID NO5) : 5'-AAGTGGAAGAAGCCGTGCTA-3',
reverse (SEQ ID NO6) 5'-CTCCAGGTGACACATGATGC-3';
mfoxo3a: forward (SEQ ID NO7) 5'-AGCCGTGTACTGTGGAGCTT-3',
reverse (SEQ ID NO8) 5'-TCTTGGCGGTATATGGGAAG-3';
mMAFbx: forward (SEQ ID NO9) 5'-CAGACCTGCATGTGCTCAGT-3',
reverse (SEQ ID NO10) 5'-CCAGGAGAGAATGTGGCAGT-3';
MCK forward (SEQ ID NO11) 5'-GACCTCAGCAAGCACAACAA-3',
reverse (SEQ ID NO12) 5'-CAGGTCCTTGAAGACCGTGT-3'.

Real-time quantitative RT-PCR were performed according to the manufacturer's instructions with the Roche SYBRGreen Master DNA detection kit in a Light-Cycler (Roche Molecular Diagnostic). Results were normalized to β-actin expression.

Cell culture

The mouse skeletal muscle cell line C2C12 and COS cells were cultivated in a proliferating medium (DMEM, Invitrogen) supplemented with 12% and 10% of foetal bovine serum (Perbio), respectively. Differentiation of the C2C12 cells was induced by

switching the cells into low serum medium (DMEM+ 5% horse serum (Bio Media, France)).

Transfection experiments were performed with Fugene6 (Roche) according to the manufacturer's recommendations. $5 \cdot 10^5$ C2C12 or COS cells were plated on 35mm wells,
5 and transfected with 1.5µg of total plasmid DNA.

For protein half-life measurement, cells were treated for the indicated times by adding 15mg/ml of cycloheximide (Sigma) into the proliferation medium, to inhibit protein synthesis.

Protein extractions were done by using either 1X Passive Lysis Buffer (Promega)
10 for luciferase activity measurements, or a standard lysis buffer (25mM tris-HCL, 50mM NaCl, 0.5% Deoxycholic acid, 2% NP40, 0.2% SDS) supplemented with a protease inhibitor cocktail (Complete,Roche).

Cell fractionation

For fractionation experiments, cells were lysed in HES buffer (20 mM HEPES-
15 NaOH [pH 7.4], 1 mM EDTA, 250 mM sucrose, protease inhibitors [Complete; Roche Molecular Biochemicals], 50 mM NaF, 10 mM β-glycerophosphate, and 6 mM Na₃VO₄) by trituration through a 25-gauge needle 25 times on ice. The nuclei were removed by centrifugation at 1,500 x g for 10 min at 4°C, and the P100 and S100 fractions were obtained by centrifugation at 100,000 x g for 30 min at 4°C. Supernatants (S100) were
20 removed, and P100 fractions were resuspended in HES buffer containing 0.1% NP-40 and incubated at 4°C for 1 h. Protein concentration was measured (DC kit; BioRad), and equal amounts of each fraction were analyzed by western blotting.

Immunoprecipitations, pull-downs, and ubiquitylation assays

Immunoprecipitations and ubiquitin pull down were performed as described in ¹⁵.
25 GST-pull down as described in ¹¹, except that cell extracts over-expressing atrogin were used instead of reticulocyte lysates. In vivo ubiquitination was performed as described by Col et al., 2005.

Effect of HDAC6 inhibitors on muscle atrophy *in vivo*

30 **Animals** : five female OF1 outbred mice of 24 to 30 grams per condition.

Tested molecules can be administered at different frequencies, before and/or after induction of muscle atrophy, according to the biodisponibility of the product.

Protocol : Under anesthesia (Ketamine+Xylazine), the sciatic nerve of the left hindlimb of the mouse is cut twice, at 5 mm of interval to prevent nerve regeneration.

14 and 21 days following denervation, the animals are sacrificed and the right (intact) and left (atrophied) Tibialis anterior muscles are collected and fixed for 30 minutes at room temperature in 1X PBS + 3.7% formalin. Muscles are next rinsed in 1x PBS and bathed in PBS 1X + 30% sucrose for 2 hours at 4°C. Muscles are coated with hardening liquid for histological preparations and frozen in liquid nitrogen. Thereafter, and before sectioning, the muscles are conserved at -80°C.

With a cryostat, 10 transverse sections are realized in the median part of each muscle, and set up for visualization. Sections are observed with a microscope and the images are acquired with a digital camera. The « Metamorph » software is used for measuring the surface of the cross section of each muscle fiber.

Atrophy is quantified by comparing the average surface of the cross sections of innervated fibers to that of denervated fibers for each animal. The effect of the molecule is evaluated by comparing the levels of muscle atrophy measured in test and control animal pools.

Muscle atrophy can also be evaluated by comparing the weight of the control and denervated muscles, normalized to the weight or to the length of the femur of each animal.

20

Results and discussion

HDAC6 expression in skeletal muscle was reported previously¹⁸. We have used quantitative RT PCR to compare the expression of *HDAC6* in healthy muscle to its expression in denervated muscle, or in muscles from food-deprived animals, two situations leading to muscle wasting. In both cases, the expression of *HDAC6* increased during muscle atrophy, with a kinetic reminiscent of the up-regulation of *atrogin-1* (Fig.1a,b).

HDAC6 has previously been shown to interact with components of the ubiquitin proteasome pathway¹⁵. Since Atrogin plays a central role in muscle wasting and since it is upregulated simultaneously with *HDAC6* during muscle atrophy, we hypothesized that HDAC6 may be involved in the Atrogin-dependent protein degradation during muscle atrophy.

In order to investigate the possibility of a functional link between HDAC6 and Atrogin, the capacity of the two proteins to interact was analysed by immunoprecipitation

after their expression in Cos cells. Figure 1c shows that a small but significant fraction of the two proteins is present in a complex. This interaction was then confirmed in vitro using different fragments of HDAC6 fused to GST in a GST pull-down assay. Figure 1d shows a strong interaction between Atrogin and the first catalytic domain of HDAC6. The catalytic domain 1 of HDAC6 also interacted better than the full length protein with Atrogin in vivo, after their expression in Cos cells and immunoprecipitation (not shown). These data suggest that the conformation of the full-length HDAC6 may somehow interfere with its efficient binding to Atrogin.

10 Atrogin substrates are still largely unknown, but MyoD was recently identified as a target of Atrogin. It therefore appeared interesting to know whether HDAC6 could influence the Atrogin-dependent ubiquitination and degradation of MyoD.

Different combinations of HDAC6, Atrogin and MyoD were expressed in Cos cells and the interaction between the three proteins was monitored by immunoprecipitation. Figure 2a first confirmed the already published interaction between MyoD and Atrogin¹⁶ and further showed that the expression of MyoD significantly enhanced the Atrogin-HDAC6 interaction; in contrast no interaction was found between MyoD and HDAC6 alone. This experiment also suggested that the three proteins could form a ternary complex.

In order to confirm this hypothesis, and also to monitor the capacity of HDAC6 to bind Ubiquitin when interacting with Atrogin/MyoD, a Ubiquitin pull-down experiment was performed. Different combinations of HDAC6, Atrogin and MyoD were expressed in Cos cells, and the cellular extracts were incubated with Ubiquitin beads (Figure 2b). As shown in the figure, only HDAC6 could bind Ubiquitin alone, whereas neither Atrogin nor MyoD nor a combination of these two proteins could bind Ubiquitin. Consistently with Figure 1c, HDAC6 allowed the retention of Atrogin on Ubiquitin beads. Most interestingly, Atrogin and MyoD were both retained on Ubiquitin beads when HDAC6 was present, suggesting that HDAC6 creates a platform allowing the recruitment of MyoD to Atrogin, to proceed to its ubiquitination. Moreover, the presence of MyoD strongly enhanced the amount of Atrogin retained on Ub-beads, thus confirming the role of MyoD in stimulating HDAC6-Atrogin interaction. This experiment also showed that these three proteins indeed form a ternary complex.

Finally, HDAC6 was shown to enhance Atrogin-dependent MyoD ubiquitination. His-tagged Ubiquitin was expressed in Cos cells together with various combinations of

HDAC6, Atrogin and MyoD, and the cellular extracts were loaded on Nickel columns (Figure 2c). Atrogin alone did not induce a significant ubiquitination of MyoD, whereas in the presence of HDAC6, MyoD was efficiently ubiquitinated.

HDAC6, Atrogin and MyoD thus form a ternary complex, in which HDAC6 is
5 required to bring Atrogin and MyoD together, leading to efficient MyoD ubiquitination.

Since Atrogin has been shown to promote the degradation of MyoD in muscle cells¹⁶, shRNA expression vectors directed against HDAC6 were introduced into C2C12 muscle cells to evaluate the role of HDAC6 in Atrogin-mediated MyoD degradation. The stability of MyoD was assayed by western blot at different time points after the addition of cycloheximide (Fig. 3a). In the presence of Atrogin, MyoD half life was significantly
10 reduced. Conversely, when HDAC6 expression was diminished by the shRNA, Atrogin did not induce a significant degradation of MyoD, and the stability of MyoD was even increased in comparison to control MyoD-transfected cells. HDAC6 is thus required for MyoD degradation in muscle cells.

15 In muscle cells, MyoD can be localized either in the nucleus to activate transcription or in the cytoplasm. Upon myoblastic differentiation, MyoD is upregulated and a slow migrating phosphorylated form accumulates in the nucleus¹⁷. MyoD protein levels and localization were monitored in control and HDAC6-shRNA expressing C2C12 cells placed in differentiation medium. Figure 3b shows that when HDAC6 levels are
20 reduced by shRNA expression, differentiating myoblasts accumulate high levels of phosphorylated MyoD in the nucleus. Noticeably, the levels of cytoplasmic non phosphorylated MyoD were also significantly increased in HDAC6 shRNA expressing cells. The increase of MyoD protein levels in cells with impaired HDAC6 expression is consistent with a role for HDAC6 in MyoD degradation.

25 If HDAC6 and Atrogin are required for MyoD degradation, their upregulation during muscle atrophy should limit the activation of MyoD-target genes during this process, and this would prevent the compensation of protein degradation by the activation of gene expression. Various combinations of MyoD, Atrogin and HDAC6 shRNA expression vectors were thus transfected into C2C12 muscle cells together with a reporter
30 gene controlled by the myogenin promoter, which is strongly dependent on MyoD. Under these conditions, MyoD activated the expression of the reporter gene by more than thirteen folds and the coexpression of Atrogin substantially reduced the transactivation by MyoD.

HDAC6 played a central role in this process, since HDAC6 downregulation almost completely abolished the inhibition of MyoD by Atrogin (figure 3c).

MyoD is crucial for C2C12 cells differentiation since it activates several downstream genes which allow differentiation to proceed. *Myogenin*, which is required to
5 activate the genes involved in muscle late differentiation, is one of MyoD best characterized target gene (reviewed in ¹). We have shown that in differentiating C2C12 cells, the reduction of HDAC6 level induced the accumulation of MyoD in the nucleus (Figure 3b). Since *myogenin* expression upon differentiation is activated by MyoD, the endogenous levels of Myogenin were monitored in differentiating C2C12 cells in the
10 presence or absence of HDAC6. As shown in figure 3b, and consistently with the increase in nuclear MyoD, cells expressing the HDAC6 shRNA contained higher levels of myogenin than control cells. HDAC6 is thus involved in the control of both MyoD and MyoD target genes. The upregulation of HDAC6 during muscle atrophy could therefore participate to the downregulation of MyoD activity.

15 Altogether, our results strongly suggest that HDAC6 could have a functional implication in muscle atrophy. In order to demonstrate *in vivo* the involvement of HDAC6 in this process, muscle wasting was evaluated in HDAC6 ^{-/-} animals. Mice lacking HDAC6 are viable and fertile, and present no obvious phenotype. Muscle atrophy was induced in control and HDAC6 ^{-/-} mice by denervation. The reduction of the muscle mass
20 15 days after denervation was of 49% in wild type control animals, whereas it was only of 34% in HDAC6 ^{-/-} animals (Fig. 4a). Similarly, the measure of both individual fibers and total *tibialis anterior* areas on muscle cross sections, revealed that it remained higher in HDAC6 ^{-/-} animals than in wild type controls upon denervation-induced atrophy (Fig. 4a, b, c). In the absence of HDAC6, muscle wasting is thus reduced.

25 To further confirm the involvement of HDAC6 in muscle atrophy, the shRNA expression vector directed against HDAC6 was electroporated in muscle and atrophy was induced by denervation. Similarly to HDAC6 ^{-/-} animals, the analysis of muscle cross sections showed reduced atrophy in fibers electroporated with the shRNA expression vector (siHDAC6, Fig. 4d, e). In addition, since the electroporation specifically affected
30 muscle fibers and was performed shortly before atrophy was induced, the effect of HDAC6 on muscle atrophy could not result from an indirect effect generated by the absence of HDAC6 during development or from its role in non muscle cells. Furthermore, the inhibition of muscle atrophy was even more pronounced with the shRNA than in the

HDAC6 $-/-$ animals, suggesting that some compensatory mechanism may have taken place in these animals to minimize the consequences of the absence of HDAC6.

Altogether, our results demonstrate that HDAC6 participates to muscle atrophy. The role of HDAC6 in muscle wasting is probably linked to atrogen, since HDAC6 directly
5 interacts with atrogen and allows it to recruit its substrate MyoD, to induce its ubiquitination and degradation. It is plausible that the beneficial effect of HDAC6 inactivation on muscle wasting results from the sparing of MyoD degradation, which would allow to retain expression of downstream protective genes. The *muscle creatine kinase* (MCK) which is involved in energy production and is well known to be activated by
10 bHLH myogenic factors could be such a gene. Indeed, the quantification of MCK expression in the muscle of HDAC6 $-/-$ and control mice by real time RT PCR indeed reveals an increase of more than two fold in the absence of HDAC6 (figure 4f). In addition to this HDAC6 and Atrogen dependent degradation, two other pathways involved in muscle wasting have been shown to lead to MyoD: Myostatin inhibits the expression of
15 MyoD, whereas NFkB reduces its activity^{4,10}. The convergence of three different pathways to the inactivation of MyoD suggests that it could be a central feature in muscle atrophy.

Moreover, it is possible that reduced degradation of other proteins than MyoD participates to muscle sparing observed in HDAC6 KO animals. Indeed, although they are
20 not yet characterized, several proteins in addition to MyoD are probably processed via Atrogen and HDAC6.

Muscle wasting is a debilitating affliction which affects millions of persons throughout the world. The design of treatments that could limit muscle atrophy would be of great benefit to patients affected by cachexia, but could also improve the daily life of
25 aged persons affected by muscle wasting, or limit muscle atrophy in astronauts staying for long period in space and in immobilized individuals. The discovery of the involvement of HDAC6 in muscle atrophy will probably open new avenues of therapeutic investigations, since it could constitute a valuable pharmacological target. For instance, Tubacin, a drug specifically affecting HDAC6 tubulin deacetylase activity has already been developed⁵.
30 Since HDAC6 interacts with Atrogen through its deacetylase domain, it will be interesting to test whether tubacin affects muscle atrophy.

All publication, patent applications, patents and other reference material mentioned are incorporated by reference in their entirety.

CLAIMS

1. A method of reducing and/or preventing muscle atrophy, the method comprising
5 the step of administering to a subject, an agent which is capable of reducing expression and/or activity of HDAC6 in the subject.

2. Use of an agent which is capable of reducing expression and/or activity of HDAC6 in a subject, for the manufacture of a medicament for treating or preventing muscle atrophy.

10 3. The method or use according to either of claims 1 or 2 wherein a disease or condition which is associated with muscle atrophy and may therefore benefit from preventing and/or treating muscle atrophy includes cachexia associated with cancer, cerebrovascular injury, peripheral nerve injury, osteoarthritis, rheumatoid arthritis, prolonged corticosteroid therapy, diabetes, burns, poliomyelitis, amyotrophic lateral
15 sclerosis, Guillain-Barre syndrome, muscular dystrophy, congenital myotonia, myotonic dystrophy and myopathy, as well as muscle atrophy which may occur due to old age and prolonged immobilisation or weightlessness.

4. The method or use according to any preceding claim wherein the agent has an effect on the expression of the HDAC6 gene itself and/or regulates activity of the
20 HDAC6 protein, such as regulating its binding to other proteins, including atrogen, ubiquitin or ubiquitinated proteins and/or MyoD.

5. The method or use according to claim 4 wherein the agent may down-regulates expression of the HDAC6 gene.

6. The method or use according to claim 5 wherein down-regulation of the
25 HDAC6 gene is achieved by way of an RNA interference (RNAi) approach, using one or more small interfering RNAs (siRNA), or small hairpin RNA (shRNA) constructs designed to target distinct regions of the HDAC6 gene.

7. The method or use according to claim 6 wherein the siRNA or shRNA molecule comprises the sequence GAAGUGUGCUGUACCCAC.

8. The method or use according to claim 5 wherein down-regulation of HDAC6 expression is achieved using one or more antisense oligonucleotides designed to bind to HDAC6 nucleic acid, such as mRNA and prevent and/or reduce transcription and/or translation to HDAC6 protein.

5 9. The method or use according to acclaims 1 – 4 wherein the agent which acts to regulate activity of the HDAC6 protein is an antibody or chemical agent which is capable of, preventing or inhibiting binding of HDAC6 to atrogen, ubiquitin and/or MyoD.

10 10. The method or use according to claims 1 – 4 wherein the agent which acts to regulate activity of the HDAC6 protein is a DNA, RNA or peptide aptamer that can tightly bind to HDAC6 and prevent its interaction with ubiquitin, atrogen and/or MyoD.

15 11. The method or use according to claim 9, wherein the agent is a monoclonal or polyclonal antibody or fragments thereof which specifically bind HDAC6, ubiquitin, atrogen or MyoD and which inhibit the formation of a complex between HDAC6, ubiquitin, atrogen, MyoD and combinations thereof which comprise HDAC6, such as HDAC6 and atrogen and HDAC6, atrogen and MyoD and HDAC6 and ubiquitin.

12. The method or use according to claim 11 wherein the monoclonal or polyclonal antibody or fragment thereof is humanised.

20 13. The method or use according to claims 1 – 4 wherein the agent is a competitive peptide of HDAC6, ubiquitin, atrogen and/or MyoD which is capable of competing with the binding of the natural HDAC6 with ubiquitin, atrogen and/or MyoD.

14. An agent for use in disrupting interaction or complex formation between HDAC6 and ubiquitin, atrogen and/or MyoD for use in treating muscle atrophy.

15. The agent according to claim 14 which is an RNAi molecule, antibody or fragment thereof, small chemical molecule or aptamer.

25 16. A pharmaceutical composition comprising an agent capable of disrupting interaction or complex formation between HDAC6 and ubiquitin, atrogen and/or MyoD and a pharmaceutically acceptable excipient therefore.

17. The pharmaceutical composition according to claim 16, wherein the agent is RNAi molecule, antibody or fragment thereof, small chemical molecule or aptamer.

18. A screening assay for identifying an agent capable of disrupting complex formation between HDAC6 and atrogen and/or MyoD and/or ubiquitin, comprising the steps of:

- a) providing HDAC6 and atrogen and/or MyoD and/or ubiquitin;
- 5 b) contacting a candidate agent with HDAC6 and atrogen and/or MyoD and/or ubiquitin;
- c) allowing HDAC6 and atrogen and/or MyoD and/or ubiquitin to come into contact with one another under conditions which are suitable to allow complex formation between HDAC6 and atrogen and/or MyoD and/or ubiquitin in the absence of the candidate
- 10 agent; and
- d) detecting whether or not the candidate agent is capable of disrupting complex formation.

19. The screening assay according to claim 18 wherein HDAC6, atrogen, ubiquitin and/or MyoD is directly labelled by chemical, fluorescent or radiometric means.

15 20. The screening assay according to claim 18 wherein detection is carried out by way of an antibody which is capable of binding said complex.

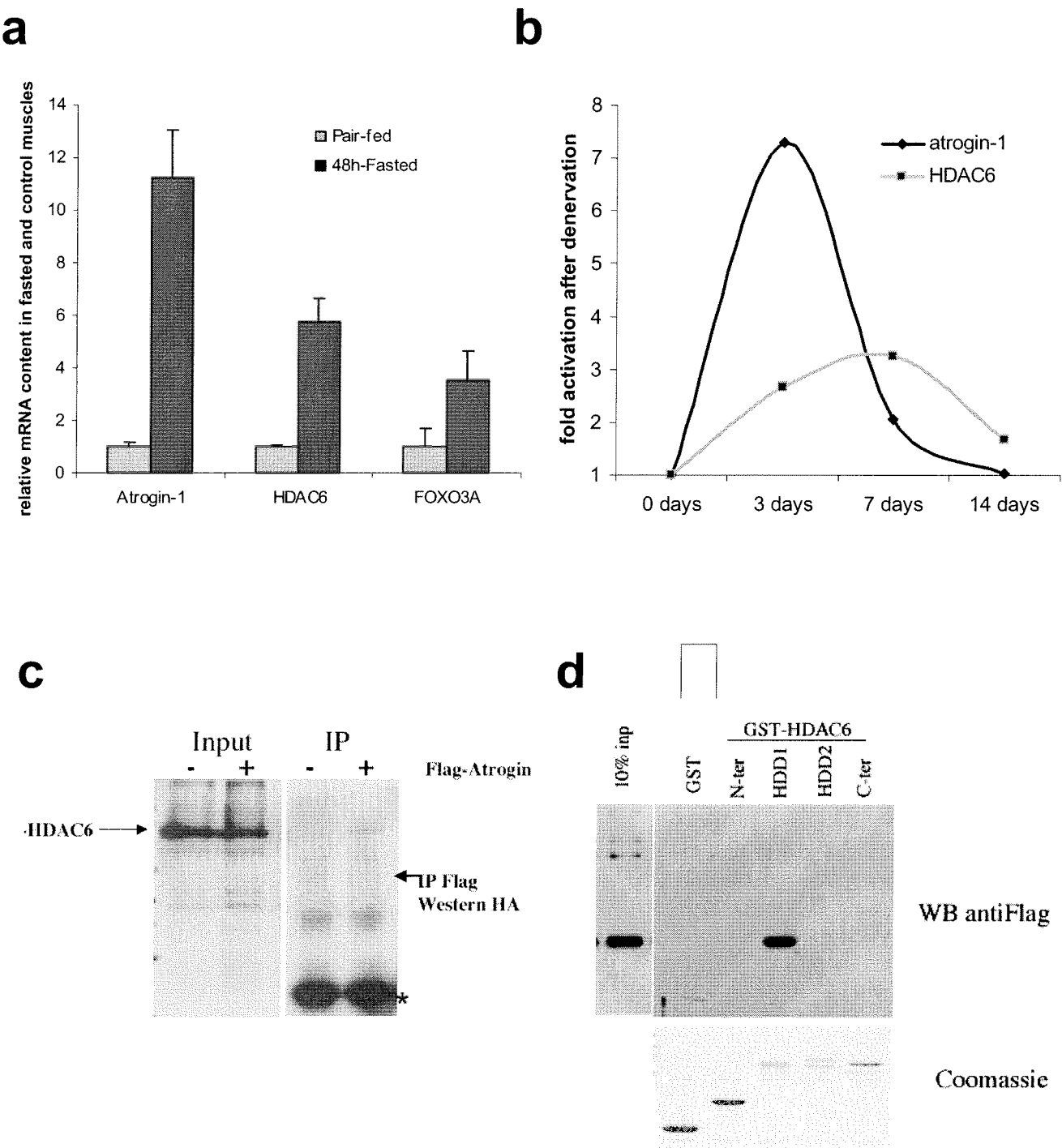


Figure 1

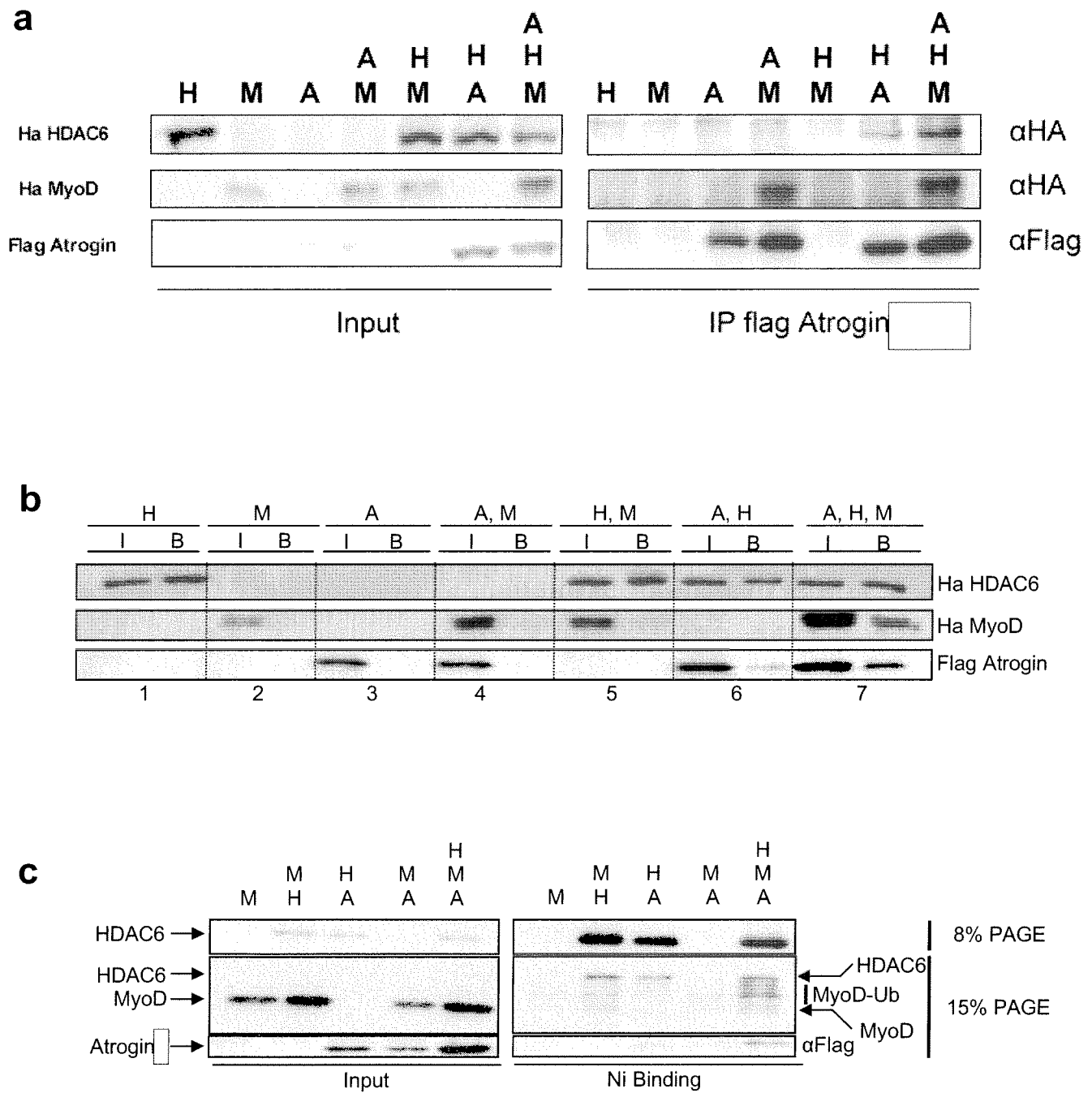


Figure 2

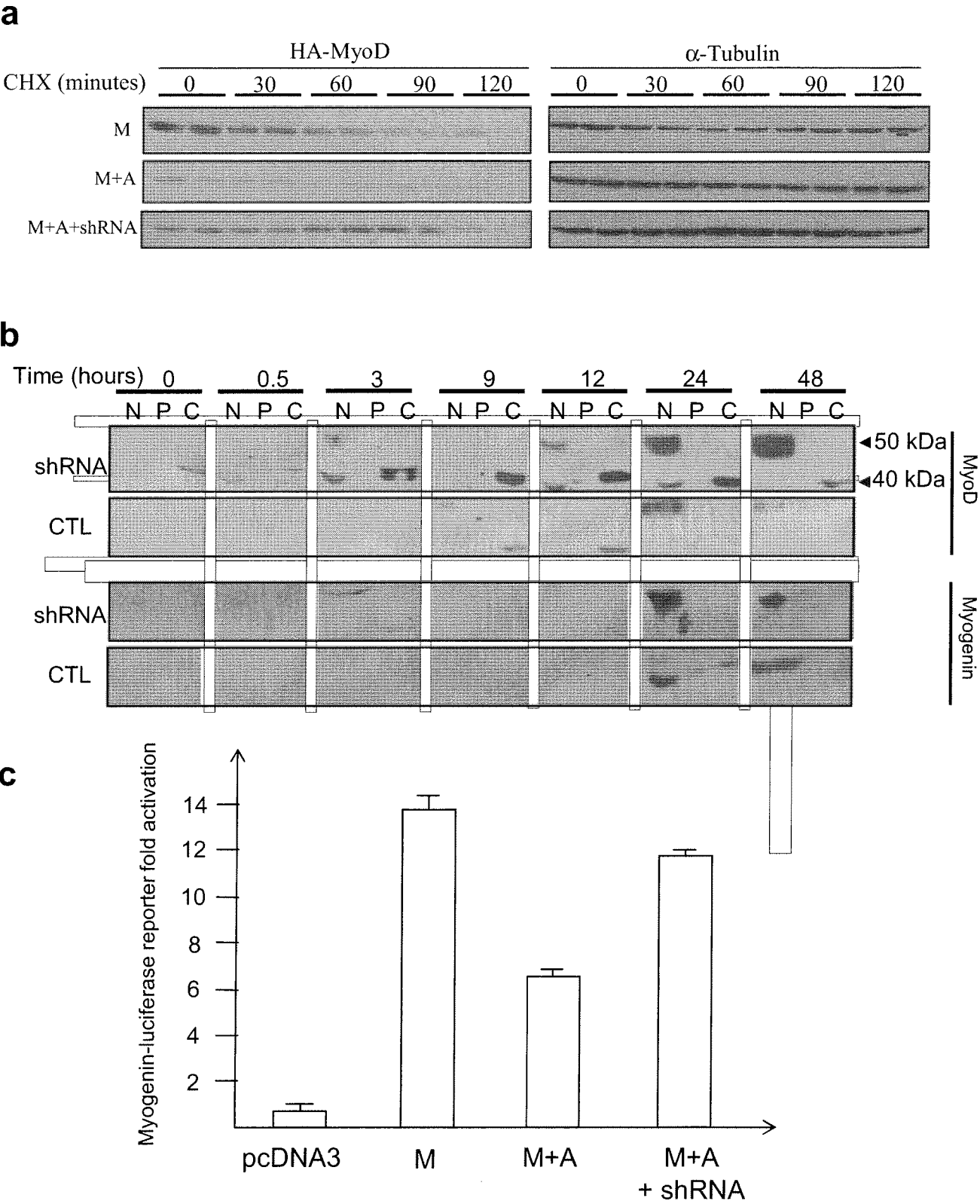


Figure 3

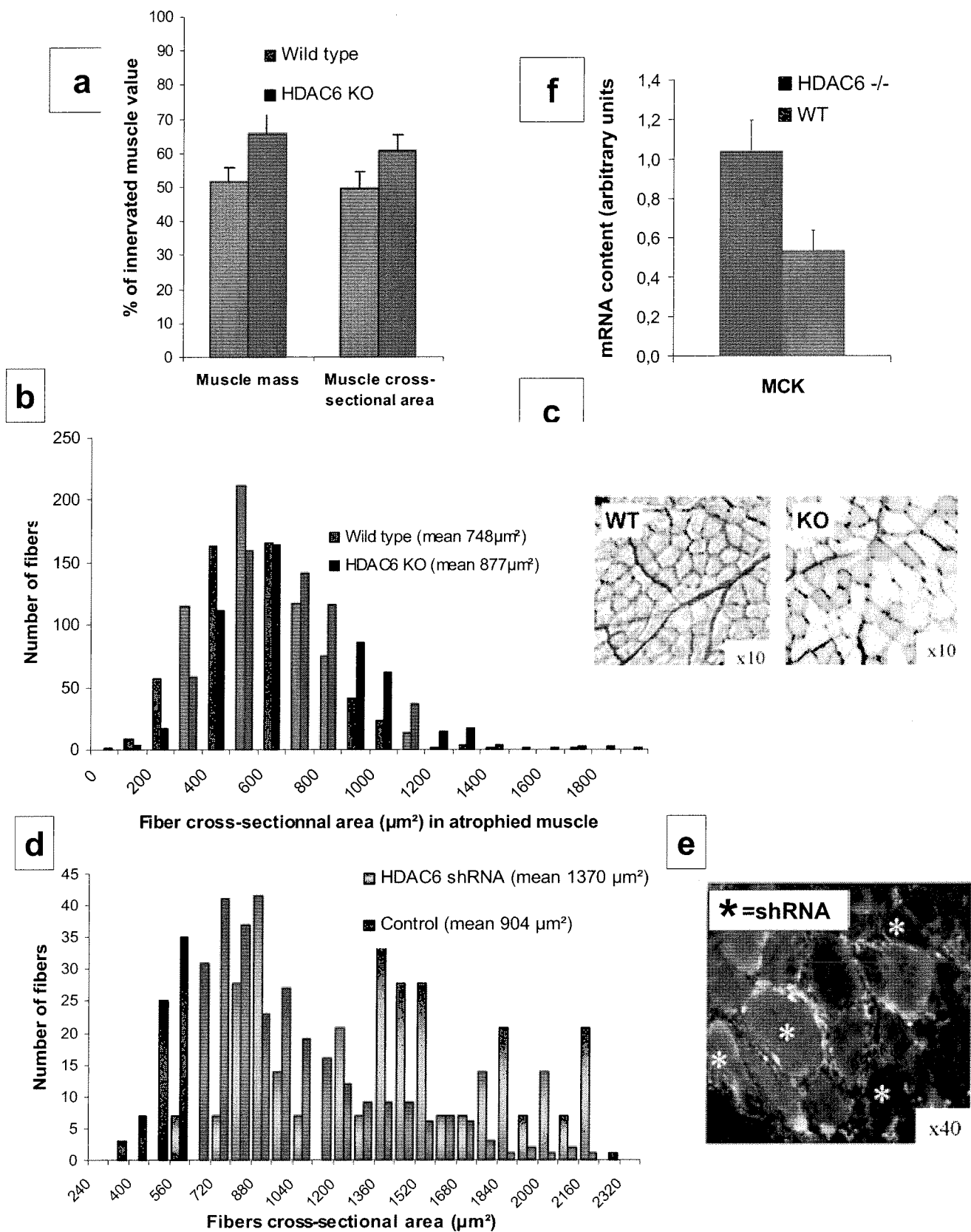


Figure 4