(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 November 2014 (13.11.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/180908 A1

(51) International Patent Classification:
A61K 31/713 (2006.01) *C12Q 1/48* (2006.01)

(21) International Application Number:
PCT/EP2014/059344

(22) International Filing Date:
7 May 2014 (07.05.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
13 167017.6 8 May 2013 (08.05.2013) EP

(71) Applicants: **DEUTSCHES KREBSFORSCHUNG-SZENTRUM** [DE/DE]; Im Neuenheimer Feld 280, 69120 Heidelberg (DE). **RUPRECHT-KARLS-UNIVERSITÄT HEIDELBERG** [DE/DE]; Grabengasse 1, 691 17 Heidelberg (DE).

(72) Inventors: **ALGIRE, Carolyn;** Hildastrasse 3a, 691 15 Heidelberg (DE). **HERZIG, Stephan;** Im den Krautackern 5, 6925 1 Gaiberg (DE).

(74) Agent: **DICK, Alexander;** Herzog Fiesser & Partner, Patentanwalte PartG mbB, Dudenstrasse 46, 68167 Mannheim (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available):* AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, 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, IR, IS, JP, KE, KG, 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, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, 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, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2014/180908 A1

(54) Title: ACIP/PEPTIDE-BASED INHIBITION OF CANCER CACHEXIA

(57) Abstract: The present invention relates to an inhibitor of Adenosine-monophosphate-activated kinase (AMPK) inactivation and to said inhibitor of AMPK inactivation for use in preventing and/or treating disease, particularly a disease selected from the list consisting of cancer cachexia, non- tumor-cachexiae, lipodystrophy, metabolic syndrome, and diabetes type I. The present invention further relates to a polynucleotide encoding an inhibitor of AMPK inactivation and to a device and to a kit comprising the inhibitor of AMPK inactivation according to the present invention. The invention also relates to a use of an inhibitor of AMPK inactivation for preventing and/or treating cancer cachexia, a non-tumor-cachexia, lipodystrophy, metabolic syndrome, or diabetes type I; to a method for predicting and/or diagnosing cancer cachexia in a subject afflicted with cancer; to a method for identifying a compound for treating or preventing cancer cachexia; and to a pharmaceutical composition comprising the inhibitor of AMPK inactivation of the present invention.

ACIP/Peptide-based inhibition of Cancer Cachexia

The present invention relates to an inhibitor of Adenosine-monophosphate-activated kinase (AMPK) inactivation and to said inhibitor of AMPK inactivation for use in preventing and/or treating disease, particularly a disease selected from the list consisting of cancer cachexia, non-tumor-cachexiae, lipodystrophy, metabolic syndrome, and diabetes type I. The present invention further relates to a polynucleotide encoding an inhibitor of AMPK inactivation and to a device and to a kit comprising the inhibitor of AMPK inactivation according to the present invention. The invention also relates to a use of an inhibitor of AMPK inactivation for preventing and/or treating cancer cachexia, a non-tumor-cachexia, lipodystrophy, metabolic syndrome, or diabetes type I; to a method for predicting and/or diagnosing cancer cachexia in a subject afflicted with cancer; to a method for identifying a compound for treating or preventing cancer cachexia; and to a pharmaceutical composition comprising the inhibitor of AMPK inactivation of the present invention.

5'-adenosine monophosphate-activated protein kinase (AMPK) consists of one each of alpha, beta, and gamma subunits (AMPKalpha, AMPKbeta, and AMPKgamma, respectively), of which several isoforms may exist in a subject, respectively. The gamma subunit comprises cystathionine beta synthase (CBS) domains giving AMPK its ability to sensitively detect shifts in the AMP:ATP ratio. The alpha subunit comprises the kinase activity, which is activated if AMP is bound to the gamma subunit. The beta subunit apparently serves as an adaptor between the alpha and the gamma subunit. AMPK has a function as a metabolic master switch regulating cellular energy-generating pathways, including glucose uptake and beta-oxidation of fatty acids (Hardie et al. (2012) Nat Rev Mol Cell Biol 13: 251).

Cachexia relates to a syndrome having pathological loss of body mass as a symptom, which cannot be reversed nutritionally; i.e. even if a patient increases energy uptake, lean body mass continues to decrease. The specific form of cachexia related to cancer, i.e. cancer cachexia occurs in 30-70% of cancer patients and still represents an as-yet non-curable and fatal paraneoplastic syndrome in a variety of tumor entities, most notably in cancers of the colon, the pancreas, and the lung (Fearon KC, Glass DJ, Guttridge DC (2012) Cancer Cachexia: Mediators,

Signaling, and Metabolic Pathways. *Cell Metab 16: 153;* Tisdale MJ (2002) Cachexia in cancer patients. *Nat Rev Cancer* 2: 862). While white adipose tissue (WAT) has been described as an important endocrine organ controlling systemic energy metabolism (Galic et al, 2009), molecular mechanisms in tumor-induced WAT dysfunction have not been studied in detail. The importance of WAT lipid homeostasis as a critical determinant of body weight, insulin sensitivity, and peripheral energy handling in both mice and humans was described earlier (Yu and Ginsberg, 2005). However, means and methods for preventing, improving, or curing cancer cachexia and its deleterious effects are still largely missing.

There is, thus, a need in the art for means and methods to comply with the aforementioned needs. The technical problem underlying the invention can be seen as the provision of means and methods which allow for improved prevention and treatment of diseases related to lipid metabolism, especially cancer cachexia. The technical problem is solved by the embodiments characterized in the claims and herein below.

Accordingly, the present invention relates to an inhibitor of Adenosine-monophosphate-activated kinase (AMPK) inactivation.

As used herein, the terms "Adenosine-monophosphate-activated kinase" and "AMPK" relate to a heterotrimeric protein kinase activated by an increasing concentration of 5'-adenosine-monophosphate (AMP) in the vertebrate cell, which kinase is known to the skilled person. Preferably, AMPK is mouse AMPK (mAMPK) or human AMPK (hAMPK); more preferably, mAMPK comprises subunit beta-1 with Genbank Acc. No: Q9R078.2, GL22096265, or hAMPK comprises subunit beta-1 with Genbank Acc. No: CAA12024.1, GL2916800.

The terms "cell death-inducing DFFA-like effector a protein" and "Cidea" relate to the apoptosis inducing protein known under said designations to the skilled person. Preferably, the terms relate to the human Cidea (hCidea, isoform 1: Genbank Acc. No: NP_001270.1 GL4557465) and mouse Cidea (mCidea, Genbank Acc. No: NP_03 1728.2 GI: 162287227) and their isoforms, as well as their homologs in other species.

The term "AMPK inactivation", as used herein, relates to a complete or, preferably, partial removal of AMPK activity; more preferably, the term relates to a complete or partial inactivation of the AMPK molecules comprised in a cell, wherein the term partial inactivation may relate to a reduction of the catalytic activity of AMPK molecules and/or to an abolishment of activity of a fraction of AMPK molecules comprised in a cell. Preferably, AMPK inactivation is irreversible AMPK inactivation, i.e., more preferably, inactivation by destruction of the catalytic activity

and/or the structural integrity of AMPK. Most preferably, AMPK inactivation is degradation of at least one subunit of AMPK as specified herein. Preferably, AMPK inactivation is cachexia-induced AMPK inactivation, which is, e.g., prevalent in patients afflicted with tumor cachexia; AMPK inactivation can, however, also be induced and its inhibition can be tested in an experimental setting as described herein below.

It is understood by the skilled person that the term "at least one" relates to an amount or number of one or more and includes, preferably, an amount or number of two or more, three or more, four or more, or five or more.

The term "compound" refers to a chemical molecule, i.e. any organic or inorganic substance. The organic molecule may belong to any known chemical class of molecules. Preferably, organic molecules are lipids, fatty acids, purines, pyrimidines, alkaloids, amino acids, peptides, polypeptides, proteins, biogenic amines, isoprenoids or steroids.

According to this specification, the term "inhibitor" relates to a compound reducing the rate at which a specific process (the inhibited process) occurs or which prevents said process from progressing or from occurring.

Thus, an "inhibitor of AMPK inactivation" is a compound reducing the rate at which AMPK is inactivated, preferably irreversibly inactivated, or which prevents inactivation of AMPK, preferably irreversible inactivation of AMPK, from progressing or from occurring. From this, it is understood by the skilled person that an inhibitor of AMPK inactivation, preferably, is not an activator of AMPK. Preferably, the inhibitor of AMPK inactivation inhibits AMPK inactivation by at least 25%, more preferably by at least 50%>, still more preferably by at least 75%, or, most preferably, by at least 90%. Preferably, the inhibitor of AMPK inactivation is specific, i.e. specifically has the effect of inhibiting AMPK inactivation, more preferably without modulating cellular processes other than the ones described in the present specification to a detectable extent. Preferably, the inhibitor of AMPK inactivation inhibits AMPK inactivation when brought into contact with a cell. More preferably, the inhibitor of AMPK inactivation inhibits AMPK inactivation when provided in the medium surrounding a cell.

Preferably, the inhibitor of AMPK inactivation is an inhibitor of AMPKbeta degradation, i.e. preferably, a compound reducing the rate at which the beta subunit of AMPK is degraded, or which prevents degradation of the beta subunit of AMPK from progressing or from occurring.

More preferably, the inhibitor of AMPK inactivation is an inhibitor of an interaction between AMPK and an "AMPK inactivator", i.e. an interaction partner of AMPK mediating AMPK

inactivation. The inhibitor of an interaction between AMPK and an AMPK inactivator, preferably, is a molecule physically inhibiting the interaction of said two molecules, i.e. reducing the rate at which said interaction occurs or preventing said interaction from progressing or from occurring. It is understood that, preferably, said term relates to inhibiting the interaction of said molecules while they are present in the same cell and/or in the same compartment of a cell, more preferably, at normal. i.e. physiological, concentrations. Thus, it is understood that an inhibitor reducing the amount of AMPK or at least one of its subunits, and/or the amount of an AMPK inactivator within a cell, e.g. a siRNA, more preferably, is not an inhibitor of AMPK inactivation.

Most preferably, the inhibitor of AMPK inactivation is an inhibitor of AMPKbeta/cell death-inducing DFFA-like effector a protein (Cidea) interaction. Preferably, the "inhibitor of AMPK/Cidea interaction" is a compound specifically binding to the interaction site in AMPKbeta mediating binding of Cidea. More preferably, the inhibitor of AMPK/Cidea interaction is a compound specifically binding to the interaction site in Cidea mediating binding to AMPKbeta.

Preferably, the inhibitor of AMPK inactivation is selected from the list of molecule types consisting of an inhibitory peptide, an antibody, an aptamer, an anticalin, a Designed Ankyrin Repeat Protein (DARPin), and a low molecular weight compound. More preferably, the inhibitor of AMPK/Cidea interaction is an inhibitory peptide.

As used herein, the term "inhibitory peptide" relates to any chemical molecule comprising at least one peptide having the activity of inhibiting AMPK inactivation as specified herein above. Preferably, the inhibitory peptide comprises a peptide having an amino acid sequence corresponding to an amino acid sequence of at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, at least twelve, at least 13, at least 14, or at least 15 consecutive amino acids comprised in an AMPK polypeptide, preferably an AMPKbeta polypeptide, or in an AMPK inactivator, preferably the Cidea polypeptide. Preferably, the inhibitory peptide comprises a peptide having an amino acid sequence corresponding to an amino acid sequence of 5 to 200, more preferably 6 to 100, even more preferably 7 to 50, or, most preferably, 8 to 30 consecutive amino acids comprised in an AMPK polypeptide, preferably an AMPKbeta polypeptide, or in an AMPK inactivator, preferably the Cidea polypeptide. More preferably, said contiguous amino acids are derived from the C-terminal 50 amino acids of an AMPKbeta. Even more preferably, the inhibitory peptide comprises the amino acid sequence of SEQ ID NO:l (NHVMLNHLYALSIKDGV, corresponding to amino acid residues 232-248 of

mouse AMPKbeta-1, Genbank Acc. NO: Q9R078.2 GL22096265, and also corresponding to amino acid residues 232-248 of human AMPKbeta-1, Genbank Acc. NO: CAA12024.1 GL2916800). Most preferably, the inhibitory peptide has the amino acid sequence of SEQ ID NO:5 (MDYKDDDDKGGGGGASNNHVMLNHLYALSIKDGV).

Moreover, also encompassed are variants of the aforementioned inhibitory peptides. Such variants have at least the same essential biological activity as the specific inhibitory peptides. A preferred assay is described herein in the accompanying Examples. Moreover, it is to be understood that a variant as referred to in accordance with the present invention shall have an amino acid sequence which differs due to at least one amino acid substitution, deletion and/or addition, wherein the amino acid sequence of the variant is still, preferably, at least 50%, 60%, 70%, 80%, 85%, 90%, 92%, 95%, 97%, 98%, or 99% identical with the amino sequence of the specific inhibitory peptides. The degree of identity between two amino acid sequences can be determined by algorithms well known in the art. Preferably, the degree of identity is to be determined by comparing two optimally aligned sequences over a comparison window, where the fragment of amino acid sequence in the comparison window may comprise additions or deletions (e.g., gaps or overhangs) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment. The percentage is calculated by determining the number of positions at which the identical amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman (1981), by the homology alignment algorithm of Needleman and Wunsch (1970), by the search for similarity method of Pearson and Lipman (1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, PASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by visual inspection. Given that two sequences have been identified for comparison, GAP and BESTFIT are preferably employed to determine their optimal alignment and, thus, the degree of identity. Preferably, the default values of 5.00 for gap weight and 0.30 for gap weight length are used. Variants referred to above may be allelic variants or any other species specific homologs, paralogs, or orthologs. Moreover, the variants referred to herein include fragments of the specific inhibitory eptides or the aforementioned types of variants as long as these fragments and/or variants have the essential biological activity as referred to above. Such fragments may be or be derived from, e.g., degradation products or splice variants of the inhibitory peptides. Further

included are variants which differ due to posttranslational modifications such as phosphorylation, glycosylation, ubiquitinylation, sumoylation or myristylation.

Preferably, the inhibitory peptide comprises further amino acids which may serve e.g. as immunogens, as a tag for purification or detection or as a linker. In a preferred embodiment of the inhibitory peptide of the present invention, said inhibitory peptide further comprises an immunogenic peptide. The term "immunogenic peptide" refers to a stretch of amino acids which is added to or introduced into the inhibitory peptide of the invention. Preferably, the immunogenic peptide shall be added C- or N- terminally to the inhibitory peptide of the present invention. In another preferred embodiment of the inhibitory peptide of the present invention, said inhibitory peptide further comprises a detectable tag. The term "detectable tag" refers to a stretch of amino acids which are added to or introduced into the inhibitory peptide of the invention. Preferably, the tag shall be added C- or N- terminally to the inhibitory peptide of the present invention. The said stretch of amino acids shall allow for detection of the inhibitory peptide by an antibody which specifically recognizes the tag or it shall allow for forming a functional conformation, such as a chelator or it shall allow for visualization by fluorescent tags. Preferred tags are the Myc-tag, FLAG-tag, 6-His-tag, HA-tag, GST-tag or GFP-tag. These tags are all well known in the art. More preferably, the inhibitory peptide comprises further amino acids which may serve as mediators of cell entry, i.e., preferably, the inhibitory peptide further comprises at least one cell-penetrating peptide (CPP). CPPs are well known in the art and include, e.g., Penetratins, HIV-tat-related peptides, Transportans, and the like, see, e.g. Nasrollahi et al. (2012), Chem Biol Drug Des 80: 639-646.

The term inhibitory peptide also includes chemically modified peptides, e.g., containing modified amino acids or being biotinylated or coupled to fluorophores, such as fluorescin, or Cy 3, being conformationally restricted, e.g. by disulfide bridging or by stapling (Walensky 2004, Science 305(5689): 1466-1470). Such modifications may improve the biological properties of the inhibitory peptides, e.g., binding or stability, or may be used as detection labels.

As used herein, the term "antibody" relates to a soluble immunoglobulin from any of the classes IgA, IgD, IgE, IgG, or IgM binding to AMPK or to an AMPK inactivator, having the activity of inhibiting AMPK inactivation as specified herein above. Antibodies against the AMPK polypeptide, preferably the AMPKbeta polpeptide, or to an AMPK inactivator, e.g., preferably, the Cidea polypeptide, can be prepared by well known methods using a purified protein or a suitable fragment derived therefrom as an antigen. A fragment which is suitable as an antigen may be identified by antigenicity determining algorithms well known in the art. Such fragments

may be obtained either from one of the polypeptides of the invention by proteolytic digestion, may be a synthetic peptide, or may be recombinantly expressed. Preferably, the peptide used as an antigen is located at or close to the interaction site in the AMPK inactivator (e.g. Cidea) mediating binding to AMPKbeta; more preferably, the peptide used as an antigen is located at or close to the interaction site in AMPKbeta mediating binding of an AMPK inactivator, e.g. Cidea. More preferably, the antigen comprises the amino acid sequence of SEQ ID NO: 1 or any suitable subsequence, preferably identified as specified above, comprising, preferably at least five, more preferably at least six, even more preferably at least seven, or, most preferably at least eight contiguous amino acids of the amino acid sequence of SEQ ID NO:l. Suitability of an antibody thus generated as an inhibitor of AMPK inactivation can be tested by the assay as described herein in the Examples. Preferably, the antibody of the present invention is a monoclonal antibody, a polyclonal antibody, a human or humanized antibody or primatized, chimerized or fragment thereof. More preferably, the antibody is a single chain antibody. Also comprised as antibodies of the present invention are a bispecific antibody, a synthetic antibody, an antibody fragment, such as Fab, Fv or scFv fragments etc., or a chemically modified derivative of any of these. Preferably, the antibody of the present invention shall specifically bind (i.e. does not cross react with other polypeptides or peptides) to the AMPK polypeptide, preferably the AMPKbeta polpeptide, or the AMPK inactivator, preferably the Cidea polypeptide of the invention. Specific binding can be tested by various well known techniques. Antibodies or fragments thereof can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. Monoclonal antibodies can be prepared by the techniques originally described in Kohler and Milstein (1975), Nature 256, 495; and Galfre (1981), Meth. Enzymol. 73, 3, which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals.

In the context of this invention, an "aptamer" is an oligonucleic acid or a peptide specifically binding its interaction partner and having the activity of inhibiting AMPK inactivation as specified herein above. Peptide aptamers, preferably, are peptides comprising 8-80 amino acids, more preferably 10-50 amino acids, and most preferably 15-30 amino acids. They can e.g. be isolated from randomized peptide expression libraries in a suitable host system like baker's yeast (see, for example, Klenenz et al. (2002), Cell. Mol. Life Sci. 59, 1993 - 1998). Peptide aptamers, preferably, are used as free peptides; it is, however, also contemplated by the present invention that peptide aptamers are fused to proteins serving as "scaffolds", meaning that the covalent linking to said proteins serves to fix the three-dimensional structure of said peptide aptamer to one specific conformation. An RNA or DNA aptamer is an RNA or DNA molecule

that is able to specifically bind to the three-dimensional surface of a polypeptide and to inhibit the function of said polypeptide. RNA or DNA aptamers can be obtained e.g. by in vitro selection, e.g. systematic evolution of ligands by exponential enrichment (SELEX). Methods relating to the development and use of RNA and DNA aptamers are known in the art (see, for example, Ulrich (2006), Handb. Exp. Pharmacol. 173, 305 - 326 and Ulrich (2005), Med. Chem. 1(2), 199 - 208).

As used herein, the term "anticalin" relates to an artificial polypeptide derived from a lipocalin specifically binding its interaction partner. Similarly, a "Designed Ankyrin Repeat Protein" or "DARPin", as used herein, is an artificial polypeptide comprising several ankyrin repeat motifs and specifically binding its interaction partner. The anticalins and the DARPins of the present invention have the activity of inhibiting AMPK inactivation as specified herein above.

As used herein, the term "low molecular weight compound" relates to any compound as defined herein above having a molecular mass of less than 2500 g/mol. Preferably, the low molecular weight compound has a molecular mass of less than 2000 g/mol; more preferably, the low molecular weight compound has a molecular mass of less than 1500 g/mol; most preferably, the low molecular weight compound has a molecular mass of less than 1000 g/mol. Preferably, the low molecular weight compound is an organic compound, i.e. a compound comprising at least one C-C bond. More preferably, the low molecular weight compound is not a nucleic acid or peptide; most preferably, the low molecular weight compound is not a biological macromolecule.

The term "disease", as used herein, relates to a condition of a body part, organ, body system, or subject pathologically devating from normal and, preferably, characterized by a group of identifiable signs and/or symptoms. Preferably, the disease is a disease related to lipid metabolism. More preferably, the disease is a disease having pathological loss of body mass which cannot be reversed nutritionally as a symptom. Even more preferably, the disease is selected from the list consisting of cancer cachexia, non-tumor-cachexiae, lipodystrophy, metabolic syndrome, and diabetes type I. Most preferably, the disease is cancer cachexia.

The term "preventing" refers to retaining health with respect to the diseases or disorders referred to herein for a certain period of time in a subject. It will be understood that the said period of time is dependent on the amount of the drug compound which has been administered and individual factors of the subject discussed elsewhere in this specification. It is to be understood that prevention may not be effective in all subjects treated with the compound according to the present invention. However, the tern requires that a statistically significant portion of subjects of

a cohort or population are effectively prevented from suffering from a disease or disorder referred to herein or its accompanying symptoms. Preferably, a cohort or population of subjects is envisaged in this context which normally, i.e. without preventive measures according to the present invention, would develop a disease or disorder as referred to herein. Whether a portion is statistically significant can be determined without further ado by the person skilled in the art using various well known statistic evaluation tools, e.g., determination of confidence intervals, p-value determination, Student's t-test, Mann-Whitney test etc.. Preferred confidence intervals are at least 90%, at least 95%, at least 97%, at least 98% or at least 99 %. The p-values are, preferably, 0.1, 0.05, 0.01, 0.005, or 0.0001. Preferably, the treatment shall be effective for at least 60%>, at least 70%>, at least 80%>, or at least 90%> of the subjects of a given cohort or population.

The term "treating" refers to ameliorating the diseases or disorders referred to herein or the symptoms accompanied therewith to a significant extent. Said treating as used herein also includes an entire restoration of the health with respect to the diseases or disorders referred to herein. It is to be understood that treating as used in accordance with the present invention may not be effective in all subjects to be treated. However, the term shall require that a statistically significant portion of subjects suffering from a disease or disorder referred to herein can be successfully treated. Whether a portion is statistically significant can be determined without further ado by the person skilled in the art using various well known statistic evaluation tools discussed herein above.

Advantageously, it was found in the work underlying the present invention that inhibition of AMPK inactivation prevents cancer cachexia from occurring. Specifically, it was found that inhibition of AMPK/Cidea interaction leads to a reduced degradation of AMPK and that this inhibition also counteracts lipolysis in adipocytes driven by serum from a cachectic subject.

The definitions made above apply mutatis mutandis to the following. Additional definitions and explanations made further below also apply for all embodiments described in this specification mutatis mutandis.

In a further preferred embodiment, the present invention relates to a polynucleotide encoding an inhibitor of AMPK inactivation of the present invention.

The term "polynucleotide", as used herein, relates to a polynucleotide comprising a nucleic acid sequence which encodes an inhibitor of AMPK inactivation, preferably an inihibitory peptide, having the biological activity as described above. Preferably, the polynucleotide is a

polynucleotide comprising or having the nucleic acid sequence of SEQ ID NO:2. It is to be understood that a polypeptide or peptide having an amino acid sequence as detailed above may also be encoded due to the degenerated genetic code by more than one species of polynucleotide. Moreover, the term "polynucleotide" as used in accordance with the present invention further encompasses variants of the aforementioned specific polynucleotides. Said variants may represent orthologs, paralogs or other homologs of the polynucleotide of the present invention. The polynucleotide variants, preferably, comprise a nucleic acid sequence characterized in that the sequence can be derived from the aforementioned specific nucleic acid sequences by at least one nucleotide substitution, addition and/or deletion whereby the variant nucleic acid sequence shall still encode a polypeptide having the activity as specified above. Variants also encompass polynucleotides comprising a nucleic acid sequence which is capable of hybridizing to the aforementioned specific nucleic acid sequences, preferably, under stringent hybridization conditions. These stringent conditions are known to the skilled worker and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N. Y. (1989), 6.3.1-6.3.6. A preferred example for stringent hybridization conditions are hybridization conditions in 6 x sodium chloride/sodium citrate (= SSC) at approximately 45°C, followed by one or more wash steps in 0.2 x SSC, 0.1% SDS at 50 to 65°C. The skilled worker knows that these hybridization conditions differ depending on the type of nucleic acid and, for example when organic solvents are present, with regard to the temperature and concentration of the buffer. For example, under "standard hybridization conditions" the temperature differs depending on the type of nucleic acid between 42°C and 58°C in aqueous buffer with a concentration of 0.1 to 5 x SSC (pH 7.2). If organic solvent is present in the abovementioned buffer, for example 50% formamide, the temperature under standard conditions is approximately 42°C. The hybridization conditions for DNA:DNA hybrids are preferably for example 0.1 x SSC and 20°C to 45°C, preferably between 30°C and 45°C. The hybridization conditions for DNA:RNA hybrids are preferably, for example, 0.1 x SSC and 30°C to 55°C, preferably between 45°C and 55°C. The abovementioned hybridization temperatures are determined for example for a nucleic acid with approximately 100 bp (= base pairs) in length and a G + C content of 50% in the absence of formamide. The skilled worker knows how to determine the hybridization conditions required by referring to textbooks such as the textbook mentioned above.

Alternatively, polynucleotide variants are obtainable by PCR-based techniques such as mixed oligonucleotide primer- based amplification of DNA, i.e. using degenerated primers against conserved domains of the polypeptides of the present invention. Conserved domains of the polypeptides of the present invention may be identified by a sequence comparison of the nucleic

acid sequence of the polynucleotide or of the amino acid sequence of the polypeptides as specified above. Suitable PCR conditions are well known in the art. As a template, DNA or cDNA from AAVs may be used. Further, variants include polynucleotides comprising nucleic acid sequences which are at least 70%, at least 75%, at least 80%>, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to the nucleic acid sequences detailed above. The percent identity values are, preferably, calculated over the entire amino acid or nucleic acid sequence region. A series of programs based on a variety of algorithms is available to the skilled worker for comparing different sequences as described herein above.

A polynucleotide comprising a fragment of any of the aforementioned nucleic acid sequences is also encompassed as a polynucleotide of the present invention. The fragment shall encode a polypeptide or peptide which still has the biological activity as specified above. Accordingly, the polypeptide may comprise or consist of the domains of the polypeptide of the present invention conferring the said biological activity. A fragment as meant herein, preferably, comprises at least 50, at least 100, at least 250 or at least 500 consecutive nucleotides of any one of the aforementioned nucleic acid sequences or encodes an amino acid sequence comprising at least 20, at least 30, at least 50, at least 80, at least 100 or at least 150 consecutive amino acids of any one of the aforementioned amino acid sequences.

The polynucleotide of the present invention shall be provided, preferably, either as an isolated polynucleotide (i.e. isolated from its natural context) or in genetically modified form. The polynucleotide, preferably, is DNA including cDNA, or RNA. The term encompasses single as well as double stranded polynucleotides. Moreover, comprised are also chemically modified polynucleotides including naturally occurring modified polynucleotides such as glycosylated or methylated polynucleotides or artificially modified ones such as biotinylated polynucleotides. The polynucleotides of the present invention either essentially consist of the aforementioned nucleic acid sequences or comprise the aforementioned nucleic acid sequences. Thus, they may contain further nucleic acid sequences as well.

Specifically, in a preferred embodiment, the present invention also relates to a vector comprising the polynucleotide of the present invention. .

The term "vector", preferably, encompasses phage, plasmid, viral or retroviral vectors as well as artificial chromosomes, such as bacterial or yeast artificial chromosomes. More preferably, the term relates to a vector derived from an AAV. Moreover, the term also relates to targeting constructs which allow for random or site- directed integration of the targeting construct into genomic DNA. Such targeting constructs, preferably, comprise DNA of sufficient length for

either homologous or heterologous recombination. The vector encompassing the polynucleotides of the present invention, preferably, further comprises selectable markers for propagation and/or selection in a host. The vector may be incorporated into a host cell by various techniques well known in the art. For example, a plasmid vector can be introduced in a precipitate such as a calcium phosphate precipitate or rubidium chloride precipitate, or in a complex with a charged lipid or in carbon-based clusters, such as fullerens. Alternatively, a plasmid vector may be introduced by heat shock or electroporation techniques. Should the vector be a virus, it may be packaged in vitro using an appropriate packaging cell line prior to application to host cells. Viral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host/cells.

More preferably, in the vector of the invention the polynucleotide is operatively linked to expression control sequences allowing expression in prokaryotic or eukaryotic cells or isolated fractions thereof. Expression of said polynucleotide comprises transcription of the polynucleotide, preferably into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic cells, preferably mammalian cells, are well known in the art. They, preferably, comprise regulatory sequences ensuring initiation of transcription and, optionally, poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the lac, trp or tac promoter in E. coli, and examples for regulatory elements permitting expression in eukaryotic host cells are the AOX1 or GALl promoter in yeast or the CMV-, SV40-, RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. Moreover, inducible expression control sequences may be used in an expression vector encompassed by the present invention. Such inducible vectors may, preferably, comprise tet or lac operator sequences or sequences inducible by heat shock or other environmental factors. Suitable expression control sequences are well known in the art. Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the polynucleotide. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDVl (Pharmacia), pBluescript (Stratagene), pCDM8, pRc/CMV, pcDNAl, pcDNA3 (InVitrogene) or pSPORTl (GIBCO BRL). Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of the polynucleotides or vector of the invention into targeted cell population. Methods which are well known to those skilled in the art

can be used to construct recombinant viral vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1994). Most preferably, the vector is a plasmid pLKO-puro FLAG having an insertion of hybridized oligonucleotides with SEQ ID NOs: 3 and 4, respectively, into the TaqI site of the plasmid, resulting in plasmid pLKO-puro FLAG-ACIP.

Further, the present invention relates to a host cell comprising an inhibitor of AMPK or a polynucleotide or vector encoding the same.

The term "host cell", as used herein, relates to any bacterial, archeal, or eukaryotic cell. Preferably the host cell is a cell naturally or artificially comprising AMPK and Cidea. Preferably, the host cell is a mammalian cell, more preferably a horse, pig, cow, sheep, goat, rat, mouse, dog, cat, or guinea pig cell. Most preferably, the host cell is a human cell. Preferably, the cell is an adipocyte.

Also, the present invention relates to a device comprising the inhibitor of AMPK inactivation of the present invention.

The term "device", as used herein, relates to a system of means comprising at least the means referred to in the claims or herein. More preferably, the device comprises all the means in one device. However, it is also contemplated that the means of the current invention may appear as separate devices in such an embodiment and are, preferably, packaged together as a kit. The person skilled in the art will realize how to link the means without further ado. Preferred devices are those which can be applied without the particular knowledge of a specialized technician.

The term "device comprising the inhibitor of AMPK inactivation" relates to a device comprising the inhibitor of AMPK inactivation and, preferably, a means of applying said inhibitor of AMPK inactivation to a subject. Means of applying the compounds of the present invention, including the polynucleotides and the inhibitory peptides, are well known to the skilled person and include, e.g. syringes, infusion sets, inhalers, and the like. Preferably, the aforesaid means are comprised by a single device.

The present invention further relates to a device for predicting and/or diagnosing cancer cachexia in a subject, comprising i) an analyzing unit for determining the amount of AMPK in a sample of said subject and ii) an evaluation unit having embedded an algorithm for comparing said amount to a reference amount and for predicting and/or diagnosing cancer cachexia in a subject.

The "device for predicting and/or diagnosing cancer cachexia" is a device comprising at least the aforementioned means. Said device for predicting and/or diagnosing cancer cachexia may accordingly include an analyzing unit for the measurement of the amount of the peptides or polypeptides in an applied sample and a data processing unit for processing the resulting data for the evaluation. Alternatively, where means such as test stripes are used for determining the amount of the peptides or polypeptides, the means for comparison may comprise control stripes or tables allocating the determined amount to a reference amount. The test stripes are, preferably, coupled to a ligand which specifically binds to the peptides or polypeptides referred to herein. The strip or device, preferably, comprises means for detection of the binding of said peptides or polypeptides to the said ligand. Preferred means for detection are disclosed in connection with embodiments relating to the method of the invention above. In such a case, the means are operatively linked in that the user of the system brings together the result of the determination of the amount and the diagnostic or prognostic value thereof due to the instructions and interpretations given in a manual. The means may appear as separate devices in such an embodiment and are, preferably, packaged together as a kit. The person skilled in the art will realize how to link the means without further ado. Preferred devices are those which can be applied without the particular knowledge of a specialized clinician, e.g., test stripes or electronic devices which merely require loading with a sample. The results may be given as output of raw data which need interpretation by the clinician. Preferably, the output of the device is, however, processed, i.e. evaluated, raw data the interpretation of which does not require a clinician. Further preferred devices comprise the analyzing units/devices (e.g., biosensors, arrays, solid supports coupled to ligands specifically recognizing the peptide, Plasmon surface resonance devices, NMR spectrometers, mass-spectrometers etc.) or evaluation units like, e.g. a computer or other data storage and/or processing device, in accordance with the method of the invention.

The present invention further relates to a kit comprising the inhibitor of AMPK inactivation of the present invention and an instruction manual.

The term "kit", as used herein, refers to a collection of the aforementioned components, preferably, provided separately or within a single container. The container, also preferably, comprises instructions for carrying out the method of the present invention. Examples for such the components of the kit as well as methods for their use have been given in this specification. The kit, preferably, contains the aforementioned components in a ready-to-use formulation. Preferably, the kit may additionally comprise instructions, e.g., a user's manual for applying the inhibitor of AMPK inactivation with respect to the applications provided by the methods of the present invention. Details are to be found elsewhere in this specification. Additionally, such

user's manual may provide instructions about correctly using the components of the kit. A user's manual may be provided in paper or electronic form, e.g., stored on CD or CD ROM. The present invention also relates to the use of said kit in any of the methods according to the present invention.

The present invention also relates to the use of an inhibitor of AMPK inactivation according to the present invention for preventing and/or treating cancer cachexia, a non-tumor-cachexia, lipodystrophy, metabolic syndrome, or diabetes type I.

The present invention further relates to a method for predicting and/or diagnosing cancer cachexia in a subject afflicted with cancer, comprising a) determining the amount of AMPKbeta in a sample of said subject, b) comparing the amount of AMPKbeta determined in step a) to a reference, and thereby predicting and/or diagnosing cancer cachexia in said subject afflicted with cancer.

The method for predicting and/or diagnosing cancer cachexia of the present invention, preferably, is an in vitro method. Moreover, it may comprise steps in addition to those explicitly mentioned above. For example, further steps may relate, e.g., to obtaining a sample for step a), or obtaining reference values in step b). Moreover, one or more of said steps may be performed by automated equipment.

The term "subject", as used herein, relates to a mammal, preferably a horse, pig, cow, sheep, goat, rat, mouse, dog, cat, or guinea pig cell. More preferably, the subject is a human. Accordingly, the term "subject afflicted with cancer" relates to a subject suspected or, preferably, having been diagnosed to be afflicted with cancer. More preferably, the subject afflicted with cancer is a subject afflicted with colon cancer, pancreas cancer, and/or lung cancer.

The term "sample" preferably refers to a sample of a body fluid or to a sample of wash/rinse fluid obtained from an outer or inner body surface or, more preferably, to a sample from a tissue or an organ. The sample preferably comprises cells, more preferably adipocytes, most preferably adipocyte-derived polypeptides. Samples of blood, plasma, serum, urine, saliva, or lacrimal fluid are encompassed by the method of the present invention. Such samples can be obtained by use of brushes, (cotton) swabs, spatula, rinse/wash fluids, punch biopsy devices, puncture of cavities with needles or surgical instrumentation. Cells may be obtained from the body fluids or the tissues or organs by separating techniques such as filtration or centrifugation. Preferably, samples are obtained from body tissues known to comprise AMPK, i.e., preferably, adipose tissue or the like. It is, however, also envisaged by the present invention that the sample is a

tumor sample. It is to be understood that the sample may be further processed in order to carry out the method of the present invention. Particularly, cells might be enriched from the obtained sample by methods and means known in the art. Moreover, polypeptides might be extracted and/or purified from the obtained sample by methods and means known in the art. Thus, the term sample also may relate to polypeptides purified and/or extracted from any sample as mentioned above.

The term "determining" relates to the quantification of the amount of AMPK present in a sample, i.e. measuring the amount or concentration of said AMPK, preferably semi-quantitatively or quantitatively. Measuring can be done directly or indirectly. The determining of the amount of AMPK can be accomplished in a variety of ways known to the skilled person, e.g. by western blotting, co-immunoprecipitation, or the like. Preferably, the amount of AMPK is determined by the methods described in the examples herein below.

In accordance with the present invention, determining the amount of the AMPK can be achieved by all known means for determining the amount of a polypeptide or peptide in a sample, provided that they are adapted to specifically detect the AMPK of the present invention. Preferably, detection agents are to be used which specifically bind to and, thus, allow for the detection of the AMPK. Detection agents, preferably, encompass antibodies or fragments thereof that specifically bind to the AMPK, aptameres, anticalins, or Designed Ankyrin Repeat Proteins (DARPins) that specifically bind to the AMPK. Preferably, double-specificity immunoassays are applied, i.e. assays wherein the presence or the intensity of a signal will depend on the presence of two epitopes comprised in AMPK. Said means comprise immunoassay devices and methods which may utilize labeled molecules in various sandwich, competition, or other assay formats. Said assays will develop a signal which is indicative of the presence or absence of AMPK. Moreover, the signal strength can, preferably, be correlated directly or indirectly (e.g. reverse-proportional) to the amount of AMPK present in a sample. Said methods comprise, preferably, biosensors, optical devices coupled to immunoassays, biochips, analytical devices such as mass-spectrometers, NMR-analyzers, or chromatography devices. Further, methods include micro-plate ELISA-based methods, fully-automated or robotic immunoassays (available for example on multi parameter biochip platforms or ElecsysTM analyzers), CBA (an enzymatic Cobalt Binding Assay, available for example on Roche-HitachiTM analyzers), and latex agglutination assays (available for example on Roche-HitachiTM analyzers).

The term "amount" as used herein encompasses the absolute amount of AMPK referred to herein, the relative amount or concentration of AMPK referred to herein as well as any value or

parameter which correlates thereto. Such values or parameters comprise intensity signal values from all specific physical or chemical properties obtained from AMPK referred to herein by measurements, e.g., expression levels determined from biological read out systems in response to the polypeptides referred to herein or intensity signals obtained from specifically bound ligands. It is to be understood that values correlating to the aforementioned amounts or parameters can also be obtained by all standard mathematical operations.

"Comparing" as used herein encompasses comparing the amount of AMPK referred to herein which is comprised by the sample to be analyzed with an amount of AMPK in a suitable reference sample as specified elsewhere herein in this description. Also encompassed is comparing the ratio of the amount of the AMPK to a suitable control polypeptide in the sample to a suitable reference ratio. It is to be understood that comparing as used herein refers to a comparison of corresponding parameters or values, e.g., an absolute amount of AMPK is compared to an absolute reference amount of AMPK; a concentration of AMPK is compared to a reference concentration of AMPK; an intensity signal obtained from AMPK in a test sample is compared to the same type of intensity signal of said AMPK in a reference sample; or a ratio of the amount of AMPK to the amount of a control polypeptide is compared to a corresponding reference ratio. The comparison referred to in the methods of the present invention may be carried out manually or computer assisted. For a computer assisted comparison, the value of the determined amount or ratio may be compared to values corresponding to suitable references which are stored in a database by a computer program. The computer program may further evaluate the result of the comparison by means of an expert system. Accordingly, the result of the identification referred to herein may be automatically provided in a suitable output format.

The terms "reference value", "reference amount", and "reference" as used herein refer to an amount of AMPK which allows assessing if being afflicted with cancer cachexia or not being afflicted with cancer cachexia is to be assumed for the subject from which the sample is derived. A suitable reference value may be determined from a reference sample to be analyzed together, i.e. simultaneously or subsequently, with the sample.

Reference amounts can, in principle, be calculated for a group or cohort of subjects as specified herein based on the average or mean values for AMPK by applying standard methods of statistics. In particular, accuracy of a test such as a method aiming to diagnose an event, or not, is best described by its receiver-operating characteristics (ROC) (see especially Zweig 1993, Clin. Chem. 39:561-577). The ROC graph is a plot of all of the sensitivity versus specificity pairs resulting from continuously varying the decision threshold over the entire range of data

observed. The clinical performance of a diagnostic method depends on its accuracy, i.e. its ability to correctly allocate subjects to a certain prognosis or diagnosis. The ROC plot indicates the overlap between the two distributions by plotting the sensitivity versus 1-specificity for the complete range of thresholds suitable for making a distinction. On the y-axis is sensitivity, or the true-positive fraction, which is defined as the ratio of number of true-positive test results to the product of number of true-positive and number of false-negative test results. This has also been referred to as positivity in the presence of a disease or condition. It is calculated solely from the affected subgroup. On the x-axis is the false-positive fraction, or 1-specificity, which is defined as the ratio of number of false-positive results to the product of number of true-negative and number of false-positive results. It is an index of specificity and is calculated entirely from the unaffected subgroup. Because the true- and false-positive fractions are calculated entirely separately, by using the test results from two different subgroups, the ROC plot is independent of the prevalence of the event in the cohort. Each point on the ROC plot represents a sensitivity/-specificity pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the two distributions of results) has an ROC plot that passes through the upper left corner, where the true-positive fraction is 1.0, or 100% (perfect sensitivity), and the false-positive fraction is 0 (perfect specificity). The theoretical plot for a test with no discrimination (identical distributions of results for the two groups) is a 45° diagonal line from the lower left corner to the upper right corner. Most plots fall in between these two extremes. If the ROC plot falls completely below the 45° diagonal, this is easily remedied by reversing the criterion for "positivity" from "greater than" to "less than" or vice versa. Qualitatively, the closer the plot is to the upper left corner, the higher the overall accuracy of the test. Dependent on a desired confidence interval, a threshold can be derived from the ROC curve allowing for the diagnosis or prediction for a given event with a proper balance of sensitivity and specificity, respectively. Accordingly, the reference to be used for the methods of the present invention can be generated, preferably, by establishing a ROC for said cohort as described above and deriving a threshold amount there from. Dependent on a desired sensitivity and specificity for a diagnostic method, the ROC plot allows deriving suitable thresholds.

Preferably, the reference amount as used herein is derived from samples of subjects for which it is known if their donors were afflicted with cancer cachexia or not. This reference amount level may be a discrete figure or may be a range of figures. Evidently, the reference level or amount may vary between individual subunits of AMPK. The measuring system therefore, preferably, is calibrated with a sample or with a series of samples comprising known amounts of AMPK or of the AMPK subunit to be determined. More preferably, the system is calibrated with a series of

mixtures comprising defined amounts of AMPK. It is understood by the skilled person that in such case the amount of AMPK will preferably be expressed as arbitrary units (AU). Thus, preferably, the amount of AMPK is determined by comparing the signal obtained from the sample to signals comprised in a calibration curve. It is, however, understood by the skilled person that a reference amount may preferably also be obtained from a population of subjects for whom it is unknown if they are afflicted with cancer cachexia or not, provided that the cohort of subjects is large enough to be statistically representative of the average population, since the proportion of subjects afflicted with cancer cachexia in an average population is low.

The reference amount applicable for an individual subject may vary depending on various physiological parameters such as age, gender, or subpopulation. Thus, a suitable reference amount may be determined by the methods of the present invention from a reference sample to be analyzed together, i.e. simultaneously or subsequently, with the test sample. Moreover, a threshold amount can be preferably used as a reference amount. Preferably, a reference amount is obtained from one or more individual(s) known not to be afflicted with cancer cachexia and an amount of AMPK which is below the reference amount is indicative of cancer cachexia; and an amount of AMPK which is equal or above the reference amount will be indicative of the absence of cancer cachexia. More preferably, a reference amount is obtained from one or more individual(s) known to be afflicted with cancer cachexia and an amount of AMPK which is higher than the reference amount is indicative of the absence of cancer cachexia, whereas an amount of AMPK which is equal to or lower than the reference amount will be indicative of cancer cachexia being present. It is to be understood that the aforementioned amounts may vary due to statistics and errors of measurement. A decrease or an increase of AMPK amounts referred to herein is, preferably, a statistically significant decrease or increase.

Moreover, the present invention relates to a method for identifying an inhibitor of AMPK inactivation, comprising a) contacting a host cell comprising AMPK with a compound suspected of inhibiting AMPK inactivation, b) detecting inhibition of AMPK inactivation, and c) thereby identifying a compound for treating cancer cachexia.

The method for identifying an inhibitor of AMPK inactivation of the present invention, preferably, is an in vitro method. Moreover, it may comprise steps in addition to those explicitly mentioned above. For example, further steps may relate, e.g., to identifying a compound suspected of inhibiting AMPK inactivation for step a), or determining the amount of AMPK present in a host cell for step b). Moreover, one or more of said steps may be performed by automated equipment. Preferably, the method for identifying a compound for treating or

preventing cancer cachexia is adapted for high-throughput screening (HTS) of one or more compound libraries.

The term "inhibitor of AMPK inactivation" has been defined herein above. Preferably, the inhibitor of AMPK inactivation is a compound for treating or preventing cancer cachexia. The term "compound for treating or preventing cancer cachexia" relates to an inhibitor of AMPK inactivation having suitable pharmacological properties to enable application of said compound in a subject. Preferably, the compound for treating or preventing cancer cachexia is an inhibitor of AMPK inactivation with low or absent toxicity.

The term "compound suspected of inhibiting AMPK inactivation" relates to any compound as defined herein above for which it has not been excluded that it has the capacity of inhibiting AMPK inactivation. Preferably, a compound suspected of inhibiting AMPK inactivation is a compound for which a suspicion, or, more preferably, an expectation exists that it inhibits AMPK inactivation. Such suspicion or expectation may, e.g., come from molecular modeling, from the fact that said compound is a derivative of a known inhibitor of AMPK inactivation, or from the fact that said compound has been shown to have an impact on lipid metabolism in a subject or in cells.

It is understood by the skilled person that "detecting inhibition of AMPK inactivation" includes all detection methods detecting inhibition of any one of the AMPK inactivation modi as detailed herein above. E.g., preferably, detecting inhibition of AMPK inactivation relates to detecting the amount of AMPK present in a host cell and comparing said amount to a suitable control. In said case, detecting inhibition of AMPK inactivation, more preferably relates to determining the amount of AMPK in a host cell contacted with serum from a cachectic individual and comparing said amount to an amount determined in a host cell contacted with a control serum. More preferably, detecting inhibition of AMPK inactivation relates to detecting an inhibition of a molecular interaction between AMPK and Cidea. In said case, preferably, said interaction may be detected by, e.g. co-immunoprecipitation or, most preferably, by inverse two-hybrid screening. The term two-hybrid screening is well known in the art. The term "inverse two-hybrid screening", as used herein, relates to a method using AMPK, preferably AMPKbeta, and Cidea as bait and prey polypeptides, respectively, causing the interaction assay to be positive in the absence of an inhibitor. In the presence of an inhibitor of AMPK/Cidea interaction, the interaction assay is negative, thus identifying the inhibitor of AMPK/Cidea interaction and, thus, an inhibitor of AMPK inactivation.

The term "serum from a cachectic individual" is understood by the skilled person and relates to serum obtained from a subject afflicted with cachexia, more preferably, with cancer cachexia. As described in the Examples herein, cachexia is, preferably, experimentally induced in experimental animals by implanting an appropriate tumor or appropriate tumor cells, e.g., more preferably, C-26 cells. It is understood that, preferably, other body fluids, including blood, plasma, urine may be used as serum from a cachectic individual as well.

The present invention also relates to a compound identified by the method for identifying a compound for treating cancer cachexia for use in preventing and/or treating cancer cachexia, a non-tumor-cachexia, lipodystrophy, metabolic syndrome, or diabetes type I.

Moreover, the present invention relates to a pharmaceutical composition comprising the inhibitor of AMPK inactivation according to the present invention and a pharmaceutically acceptable carrier.

The term "pharmaceutical composition" as used herein telates to the compounds of the present invention and optionally one or more pharmaceutically acceptable carrier. The compounds of the present invention can be formulated as pharmaceutically acceptable salts. Acceptable salts comprise acetate, methylester, HC1, sulfate, chloride and the like. The pharmaceutical compositions are, preferably, administered topically or systemically. Suitable routes of administration conventionally used for drug administration are oral, intravenous, or parenteral administration as well as inhalation. However, depending on the nature and mode of action of a compound, the pharmaceutical compositions may be administered by other routes as well. For example, polynucleotide compounds may be administered in a gene therapy approach by using viral vectors or viruses or liposomes.

Moreover, the compounds can be administered in combination with other drugs either in a common pharmaceutical composition or as separated pharmaceutical compositions wherein said separated pharmaceutical compositions may be provided in form of a kit of parts.

The compounds are, preferably, administered in conventional dosage forms prepared by combining the drugs with standard pharmaceutical carriers according to conventional procedures. These procedures may involve mixing, granulating and compressing or dissolving the ingredients as appropriate to the desired preparation. It will be appreciated that the form and character of the pharmaceutically acceptable carrier or diluent is dictated by the amount of active ingredient with which it is to be combined, the route of administration and other well-known variables.

The carrier(s) must be acceptable in the sense of being compatible with the other ingredients of the formulation and being not deleterious to the recipient thereof. The pharmaceutical carrier employed may be, for example, either a solid, a gel or a liquid. Exemplary of solid carriers are lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate, stearic acid and the like. Exemplary of liquid carriers are phosphate buffered saline solution, syrup, oil such as peanut oil and olive oil, water, emulsions, various types of wetting agents, sterile solutions and the like. Similarly, the carrier or diluent may include time delay material well known to the art, such as glyceryl mono-stearate or glyceryl distearate alone or with a wax. Said suitable carriers comprise those mentioned above and others well known in the art, see, e.g., Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania.

The diluent(s) is/are selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, physiological saline, Ringer's solutions, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants, or nontoxic, nontherapeutic, nonimmunogenic stabilizers and the like.

A therapeutically effective dose refers to an amount of the compounds to be used in a pharmaceutical composition of the present invention which prevents, ameliorates or treats the symptoms accompanying a disease or condition referred to in this specification. Therapeutic efficacy and toxicity of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50.

The dosage regimen will be determined by the attending physician and other clinical factors; preferably in accordance with any one of the above described methods. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Progress can be monitored by periodic assessment. A typical dose can be, for example, in the range of 1 to 1000 µg; however, doses below or above this exemplary range are envisioned, especially considering the aforementioned factors. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 µg to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1 µg to 10 mg units per kilogram of

body weight per minute, respectively. Progress can be monitored by periodic assessment. However, depending on the subject and the mode of administration, the quantity of substance administration may vary over a wide range to provide from about 0.01 mg per kg body mass to about 10 mg per kg body mass, preferably.

The pharmaceutical compositions and formulations referred to herein are administered at least once in order to treat or ameliorate or prevent a disease or condition recited in this specification. However, the said pharmaceutical compositions may be administered more than one time, for example from one to four times daily up to a non-limited number of days.

Specific pharmaceutical compositions are prepared in a manner well known in the pharmaceutical art and comprise at least one active compound referred to herein above in admixture or otherwise associated with a pharmaceutically acceptable carrier or diluent. For making those specific pharmaceutical compositions, the active compound(s) will usually be mixed with a carrier or the diluent, or enclosed or encapsulated in a capsule, sachet, cachet, paper or other suitable containers or vehicles. The resulting formulations are to be adopted to the mode of administration, i.e. in the forms of tablets, capsules, suppositories, solutions, suspensions or the like. Dosage recommendations shall be indicated in the prescribers or users instructions in order to anticipate dose adjustments depending on the considered recipient.

The present invention also relates to a method of preventing and/or treating cancer cachexia in a subject afflicted with cancer, comprising applying an effective amount of an inhibitor of AMPK inactivation according to the invention to said subject, and thereby preventing and/or treating cancer cachexia in said subject afflicted with cancer.

Moreover, the present invention relates to a use of an inhibitor of AMPK inactivation of the present invention or of a polynucleotide encoding said inhibitor of AMPK inactivation for the manufacture of a medicament.

The present invention also relates to a use of an inhibitor of AMPK inactivation according to the present invention or of an polynucleotide encoding said inhibitor of AMPK inactivation for the manufacture of a medicament for the treatment of cancer cachexia.

All references cited in this specification are herewith incorporated by reference with respect to their entire disclosure content and the disclosure content specifically mentioned in this specification.

Figure Legends

Fig. 1: Cancer cachexia induces remodeling of white adipose tissue **(A)** Histology showing uncoupling protein (UCP)-l and Voltage-dependent Anion Channel (VDAC) staining in inguinal white adipose tissue (iWAT) and brown adipose tissue (BAT) for control (ctrl), pair fed (PF) and C-26 cachectic animals. **(B)** Photographs indicating the 'browning' (darkening) of the iWAT and BAT (arrows) in cachectic (C-26) vs. control (ctrl) animals. **(C)** Average tissue weights (TW) of inguinal (iWAT), brown (BAT) and abdominal (aWAT) adipose tissue depots and gastrocnemius skeletal muscle (GC), *p <0.05 ctrl vs. C-26 ; #p<0.05 PF vs. C-26 (ctrl and PF n=5, C-26 n=10). Error bars represent SEM. **(D)** mRNA levels in the iWAT of control (ctrl), pair fed (PF) or cachectic (C-26) animals. *p <0.05 ctrl vs. C-26; #p<0.05 PF vs. C-26. rel. ex. (ordinate): relative expression.

Fig. 2: WAT remodeling and cachexia are independent of brown adipose tissue (BAT) activity and norepinephrine signaling. **(A)** hematoxylin and eosin (H&E) staining of iWAT and BAT from mice housed at thermoneutrality. **(B)** Body fat content and lean mass of control, pair fed (PF) and cachectic (C-26) mice measured by EchoMRI one day prior to sacrifice. Abbreviations: ch (%): change in %; fm: fat mass; lm: lean mass; *p=0.02 ctrl. vs. C-26 (ctrl and PF n=5, C-26 n=10) **(C)** Serum levels of non-esterified fatty acids (sN) *p<0.05 ctrl. vs. C-26. **(D)** mRNA levels (rel. ex.: relative expression) of uncoupling protein (UCP)-l, Cidea, and Carnitine O-Palmitoyltransferase (CPT) 1b in the inguinal white adipose tissue (iWAT) of control (ctrl), pair fed (PF) and cachectic (C-26) animals. Cidea: *p<0.04 ctrl. vs. C-26. **(E)** Average heat production per mouse as measured by indirect calorimetry showing no increase in heat production in cachectic mice at 30 degrees. aH3p (ordinate): average H3 production/24 hours **(F)** Serum levels of non-esterified fatty acids (sN) *p<0.05 ctrl vs. C-26; #p<0.05 60HDA vs. 60HDA C-26. **(G)** mRNA levels in the iWAT of ctrl and cachectic C-26 animals, injected with saline (control) and 60HDA, respectively. Cidea: *p<0.04 ctrl vs. C-26; #p<0.04 60HDA vs. 60HDA C-26. rel.ex. (ordinate): relative expression.

Fig. 3: Cidea-AMPK interaction and cachexia induced lipolysis **(A)** Immunoblots of the lipolysis and AMPK signaling pathways in iWAT and BAT of control and cachectic animals. β-Actin is shown as a loading control. ACC, Acetyl CoA Carboxylase; HSL, hormone-sensitive lipase; Ser, Serine **(B)** Immunoprecipitation (IP) of AMPKa and blotting for AMPKβI. S6K was used as mock IP pulldown and β-Actin of 5% input is shown as a loading control. inp: input **(C)** Non-esterified fatty acid (NEFA) levels in the supernatants of primary adipocytes differentiated in the presence of either control or C-26 mouse serum +/- norepinephrine (NE). NEFA levels were

normalized to protein content. *p=0.01 Ctrl. vs. C-26; Abbreviations: l°ac: 1° adipocytes; NEFA:NEFA expressed as mmol/l/mg **(D)** mRNA expression in primary adipocytes in control or C-26 serum. *p<0.05 , rel.ex: relative expression **(E)** V5 tag IP and blot for AMPKβI in wildtype MEFs or AMPKβ-/- MEFs transfected with V5-Cidea +/- control peptide (CP) or AMPK-Cidea interfering peptide (ACIP). 5% input was run to measure V5 transfection and β-Actin as loading control. **(F)** Flag IP and blot for Cidea in primary adipocytes differentiated in C-26 serum. Flag and β-Actin were measured in 5% input as controls. **(G)** IP AMPKa and blot for AMPKβ in primary adipocytes differentiated in control or C-26 serum and transfected with CP or ACIP. S6K was used as mock. Membrane was stripped and re-blotted for AMPKa total. **(H)** (Top) NEFA levels (in mmol/l/mg) in the supernatants of primary adipocytes differentiated in either control of C-26 serum, +/- control peptide (CP) or ACIP. NEFA concentration was normalized to protein concentration in the well. (Bottom) Immunoblots of key AMPK phosphorylation target sites in the same cells as above: ACC, Acetyl Co Carboxylase; HSL, Hormone-sensitive lipase; NEFA: *p<0.05 Ctrl vs. C-26; #p<0.004 C-26+CP vs. C-26+ACIP; **(I)** IP AMPKa and blot for AMPKβI in primary adipocytes differentiated in control or C 26 serum and transfected with CP or ACIP. HA-antibody instead of AMPKa was used for pull-down as mock IP. Membrane was stripped and re-blotted for AMPKa total. **(K)** Immunoblots from primary adipocytes differentiated in C26 serum, transfected with control peptide (CP) or inhibitory peptide (ACIP). **(L)** NEFA and glycerol levels in the supernatants of primary adipocytes differentiated in either control or C26 serum, +/- CP or ACIP. Levels were normalized to protein concentration in the well. Data are means ± SEM, *p<0.05 Ctrl vs. C26, #p<0.05 CP vs. ACIP (n=4). (M) AMPK beta 1 protein levels by immunoblot relative to Ponceau staining from 3T3-L1 cells treated with ImM cycloheximide for 0, 1, 2, 4, 8 and 12 hrs. n=3.

Fig. 4: (A) Change of body weight, body fat and lean mass in Ctrl and C26 mice injected with 60HDA or NaCl, measured by ECHO-MRI body composition analysis. **(B)** Serum NEFA levels, **(C)** tumor weights, **(D)** tissue weights, **(E)** iWAT CIDEA mRNA expression and **(F)** iWAT, aWAT and BAT norepinephrine (NE) levels of the same animals. Data are means ± SEM, *p<0.05/**p<0.01/***p<0.001 Ctrl vs. C26 (Ctrl n=5, C26 n=8). **(G)** UCP1 mRNA levels in iWAT of male Balb/c mice injected with NaCl or 60HDA into the inguinal fat pads and housed at 22°C or 4°C for 24 hrs. Data are means ± SEM, ***p<0.001 24°C vs. 4°C, #p<0.05 NaCl vs. 60HDA. n=5.

Fig. 5: Cancer cachexia induces lipolysis and remodeling of white adipose tissue **(A)** Change of body weight, body fat and lean mass in control (Ctrl), pair-fed (PF) and C26-induced cachectic (C26) mice measured by ECHO-MRI body composition analysis. **(B)** Average tissue weights of gastrocnemius skeletal muscle (GC), inguinal white (iWAT), abdominal white (aWAT) and brown (BAT) adipose tissue depots of the same animals. **(C)** H&E staining of iWAT of the same animals, housed at 22°C or 30°C during the whole experiment. Scale bar 20 μιη. **(D)** Serum non-esterified fatty acid (NEFA) levels of the same animals. **(E)** NEFA and glycerol levels in the media of primary adipocytes differentiated in the presence of either control or C26 mouse serum. Levels were normalized to protein content. **(F)** Average tissue weights of GC, iWAT, aWAT and BAT in APC delta580 heterozygous ($APC^{580/+}$) cachectic animals. **(G)** Serum NEFA and glycerol levels of the same animals. Data are means ± SEM, *p<0.05/**p<0.01/***p<0.001 Ctrl vs. cachectic ; #p<0.05/##p<0.01/###p<0.001 PF vs. cachectic (Ctrl, PF, $APC^{580/+}$ n=5, C26 n=10).

Fig. 6: Cancer cachexia leads to reduced adipose tissue AMPK. **(A)** mRNA levels in the iWAT of Ctrl or C26 cachectic animals. **(B)** Immunoblots of the lipolysis and AMPK signaling pathways in iWAT of C26 cachectic animals. **(C)** Immunoprecipitation (IP) of AMPKa and blotting (IB) for AMPKβI . HA was used as mock IP pulldown and β-Actin of 5% input is shown as a loading control. **(D)** AMPKal and a2 activity in aWAT, iWAT and BAT of C26 cachectic animals. **(E)** Immunoblots of the lipolysis and AMPK signaling pathways in iWAT and BAT of $APC^{580/+}$ cachectic animals. All mice were housed at 22°C during the whole experiment. Data are means ± SEM, *p<0.05/**p<0.01 Ctrl vs. C26 (n=6-8).

Fig. 7: Interfering with AMPK-CIDEA interaction reduces WAT remodeling in cachexia. **(A)** Scheme of flag-ACIP expressing adeno-associated virus. ITR (inverted terminal repeats), miPvl22 (miRNA122 binding site), SV40pA (simian virus poly A) **(B)** flag-ACIP mRNA expression in iWAT, aWAT and gastrocnemius muscle (GC) of mice injected with $3*10^9$ ifu Ctrl AAV (C) into the left inguinal fat pad and $3*10^9$ ifu flag-ACIP AAV (ACIP) into the right inguinal fat pad of the same animal. **(C)** iWAT weights of PBS or C26 cell injected mice, injected with Ctrl or flag-ACIP AAV into the left and right inguinal fat pad, respectively. **(D)** H&E stainings of iWAT of the same C26 animals; scale bar 20 μιη. **(E)** Immunoblots from iWAT of the same animals. Data are means ± SEM, *p<0.05 (PBS n=6, C26 n=10).

Fig. 8: ACIP reduces WAT wasting in cachexia and ameliorates tumor-induced weight loss. **(A-C)** Body weight, fat mass and lean mass change of control (PBS) or cachectic (C26) animals injected intravenously with $5*10^{11}$ ifu AAV without or with flag-ACIP (C, ACIP) or with IOOμI

NaCl (Ctrl) as measured by ECHO-MRI body composition analysis. (D) Tissue weights of the same animals. **(E)** IP AMPKa and blot for AMPKβI in iWAT of PBS or C26 cell injected animals +/- C or ACIP AAV. Membrane was stripped and re-blotted for AMPKa total. AMPKa and AMPKβ were measured in 5% input as control. **(F)** Immunoblot from iWAT of the same animals. **(G)** Kaplan-Meier curve showing the percentage of event-free time (event meaning >10% body weight loss) in C or ACIP AAV injected animals with C26 tumors. Data are means ± SEM, $^{a}p<0.05$ (on Ctrl PBS), $^{b}p<0.05$ ($_{on}$ C PBS), $^{c}p<0.05$ (on ACIP PBS), $^{d}p<0.05$ ($_{on}$ C C26) (PBS n=4, C26 n=12-15).

The following Examples shall merely illustrate the invention. They shall not be construed, whatsoever, to limit the scope of the invention.

Example 1

Cachexia occurs in 30-70% of cancer patients and still represents an as-yet non-curable and fatal paraneoplastic syndrome in a variety of tumor entities, most notably in cancers of the colon, the pancreas, and the lung (Fearon et al., 2012). While white adipose tissue (WAT) has been described as an important endocrine organ controlling systemic energy metabolism (Galic et al., 2009), molecular mechanisms in tumor-induced WAT dysfunction have not been studied in detail. The importance of WAT lipid homeostasis as a critical determinant of body weight, insulin sensitivity, and peripheral energy handling, in both mice and humans (Yu and Ginsberg, 2005), initially prompted us to investigate WAT biology in the tumor bearing state.

To this end, we subcutaneously transplanted Colon (C) 26 tumor cells into wild-type mice. Consistent with our previous studies (Berriel Diaz et al, 2008; Jones et al, 2013), C26 tumor cell implantation caused a massive reduction in body weight, skeletal muscle as well as WAT mass after 21 days (Fig. 1C). Remarkably, histological examination of WAT depots revealed a massive remodeling of this tissue, characterized by the appearance of smaller multi-locular adipocytes, increased mitochondrial content as well as enhanced expression of brown adipocyte marker genes, including uncoupling protein (UCP) 1, Cidea, CPTlbeta, and PGC-1 alpha, thereby resembling so-called brown-into-white (brite)/beige adipose tissue (Fig. 1A, IB, ID) (Algire et al, 2013). Consistent with the dramatic reduction in adipose tissue depot sizes and smaller lipid droplet size in beige/brite depots, tumor-bearing animals displayed elevated levels of circulating free fatty acids (FFA) and enhanced basal energy expenditure determined by indirect calorimetry as compared with healthy control littermates.

Activation of classical, constitutive brown adipose tissue (BAT) has been correlated with cancer cachexia in other studies (Tsoli et al, 2012), and signs of mild BAT activation were also noted in

our own experiments as indicated by induction of UCP1 mRNA levels in BAT from C26 tumor-bearing mice. This prompted us to test for the potential contribution of BAT activity to the cancer cachexia phenotype in this model by placing the animals at thermoneutral 30 C housing temperature which effectively abrogates sympathetic BAT activation. Importantly, housing of experimental animals at thermoneutral conditions throughout the tumor growth period did not alter the observed WAT remodeling nor did it abrogate the loss of WAT upon tumor growth, induction of FFA levels or thermogenic gene expression in WAT (Fig. 2A, 2B, 2C, 2D), indicating that classical ("constitutive") BAT was not of major importance for the cachectic phenotype under these conditions, and arguing for non-BAT wasting energetic cycles in the tumor bearing state.

Notably, brite cell recruitment and WAT remodeling was not observed in pair-fed, non-tumor carrying animals (Fig. 2A), suggesting that the tissue remodeling/wasting phenotype was not conferred through simple starvation and/or reduction in food intake but rather relied on the presence of the tumor *per se.* Indeed, chemical blockade of sympathetic nerve endings by depot-specific injection of 60HDA into inguinal fat pads of tumor-bearing and control littermates had no influence on systemic energy expenditure, WAT wasting and remodeling, i.e. induction of circulating FFA levels, in cachectic animals (Fig. 2E, 2F, 2G) but significantly blunted cold-induced brite cell recruitment in WAT depots. Thus far, these data overall supported the notion that tumor-borne signals rather than the classical beta-sympathetic innervation pathways to BAT or WAT confer brite cell recruitment, WAT remodeling and exaggerated energy expenditure and lipid mobilization in cancer cachexia.

Example 2

To next define the molecular events leading to cachectic WAT remodeling and lipid removal, we explored the status of the main lipolytic cascade in WAT. Consistent with the elevation of circulating FFA and glycerol levels in tumor-bearing mice, levels of ATGL were found to be elevated by 3-4-fold, and phosphorylation of protein kinase A (PKA) targets, indicative of lipolytic activation, was found to be enhanced in cachectic animals as demonstrated by Western Blot analysis. Also, the inhibitory HSL phosphorylation at Ser 565 was completely lost in WAT from tumor-bearing animals (Fig. 3A), suggesting that tumor growth abolishes a major brake in lipid mobilization in WAT depots, thereby promoting loss of energy stores under these conditions.

Enhanced lipolysis in cancer cachexia was independent of classical beta-sympathetic innervation pathways of WAT, as demonstrated by chemical blockade of sympathetic nerve endings of

tumor-bearing and control littermates. Depot-specific injection of 60HDA (6-hydroxydopamine) into inguinal fat pads, which effectively blocks adrenergic signaling (Thureson-Klein et al, 1976), had no influence on WAT wasting and remodeling in cachectic animals (Fig. 4 A-C). In contrast, 60HDA injection significantly reduced norepinephrine levels specifically in iWAT and blunted cold-induced white-to-brown adipocyte conversion in iWAT depots (Fig. 4F, 4G), demonstrating the principal effectiveness of this treatment. Consistent with the absence of a beta sympathetic impact on cachectic lipolysis, massive WAT remodeling and lipolytic drive were not observed in pair-fed, non-tumor carrying animals (Fig. 5A-D), suggesting that the WAT remodeling/lipolytic phenotype was not conferred through simple starvation and/or reduction in food intake but rather relied on the presence of the tumor per se.

In line with this assumption, incubation of primary white adipocytes with serum from C26 tumor-carrying mice significantly stimulated NEFA and glycerol release as compared with serum from healthy controls (Fig. 5E). Also, 3T3-L1 adipocytes treated with C26 cell supernatants displayed elevated NEFA release, while this effect was lost by boiling the supernatants. Together, these data demonstrate the cell autonomy of the observed pro-lipolytic effect of cancer cachectic conditions.

To further confirm the above findings in an independent genetic model of colon cancer-induced wasting metabolism, we employed animals carrying an APC mutation thereby developing numerous intestinal lesions at 5-6 month of age (Kuraguchi et al, 2006). Lesion appearance triggered a significant reduction in body weight, skeletal muscle and AT mass (Fig. 5F), thereby mimicking the observed cachectic features in the C26 transplantation model. Importantly, APC mutant cachectic animals also displayed elevated NEFA and glycerol levels in serum (Fig. 5G), validating WAT remodeling and enhanced lipolytic activity in an independent experimental setting.

Example 3

AMP-activated kinase (AMPK) has been described as a major negative regulator of lipolysis in WAT (Djouder et al, 2010), prompting us to explore AMPK activity status under these conditions. Consistent with the loss of HSL Ser565 phosphorylation (Fig. 3A), p-ACC was also reduced and AMPK subunit beta protein expression was virtually absent from cachectic WAT stores (Fig. 3A, 3B), while AMPK subunit alpha levels remained unaffected, suggesting that tumor-borne signals disrupt the functional activity of AMPK by interfering with AMPK alpha-

beta subunit interaction. Indeed, AMPK beta could not be detected in immunoprecipitates of AMPK alpha from WAT of cachectic animals, while AMPK beta was readily recovered in healthy control depots (Fig. 3B).

To test whether this effect is mediated by the tumor environment in a cell autonomous fashion, we incubated primary adipocytes derived from the WAT stromo-vascular fraction of wild-type mice to serum from cachectic or non-cachectic animals. Exposure of primary white adipocytes to "cachectic" serum induced the expression of the brown-like thermogenic program (Fig. 3D), elevated basal lipolysis (Fig. 3C), and led to the abrogation of AMPK beta expression as demonstrated by Western analysis. Of note, incubation of primary white adipocytes with C26 tumor cell supernatant failed to provoke the described cellular response, indicating that a complex interaction between tumor- and host-derived mediators drives WAT remodeling and lipid mobilization upon cancer cachexia.

Example 4

Apart from the induction of UCP1, Cidea represents a prominent key marker gene for the brown/brite adipocyte lineage (Vegiopoulos et al., 2010). As shown above, Cidea was indeed strongly up-regulated in both WAT depots from cachectic animals as well as in isolated primary adipocytes upon exposure to "cachectic" serum (Fig. 3D). Intriguingly, Cidea was found to be also upregulated in WAT from human patients suffering from colon cancer. Interestingly, Cidea has recently been shown to trigger the ubiquitin-dependent proteasomal degradation of AMPK beta, conferred through direct protein-protein interaction with the beta subunit (Qi et al., 2008). Based on the concomitant induction of Cidea and the ablation of AMPK expression in cachectic WAT depots, we hypothesized that the Cidea-AMPKbeta interaction may represent a conserved molecular node in tumor-induced WAT remodeling and energy loss. To test this assumption, we designed a peptide targeting the Cidea-AMPKbeta interaction surface to block inter-molecular interaction at this site. The designed peptide was indeed able to efficiently bind to Cidea in cellular transfection assays (Fig. 3E, 3F). Importantly, co-transfection of the inhibitory peptide with tagged versions of Cidea and AMPK beta led to the almost complete loss of Cidea-AMPKbeta interaction as shown in co-immunoprecipiation assays using MEFs (Fig. 3E). Furthermore, delivery of the inhibitory peptide into primary white adipocytes was able to prevent the interaction of endogenous Cidea and AMPK beta (Fig. 3G), thereby leading to stabilization of AMPK beta subunit expression, AMPK alpha/beta subunit interaction and counteracting lipolysis (Fig. 3H) in these cells driven by "cachectic" serum. Moreover, delivery of the

inhibitory peptide into these cells was able to restore the AMPK alpha-beta interaction (Fig. 31), thereby leading to the reconstitution of AMPK substrate phosphorylation, most notably HSL Ser 565 (Fig. 3K). In congruence with the molecular rescue of anti-lipolytic AMPK activity, ACIP delivery also prevented both NEFA and glycerol release from C26 mouse serum treated primary white adipocytes (Fig. 3L), overall demonstrating that the ACIP peptide was able to counteract tumor-induced lipid breakdown in adipocytes by preserving AMPK anti-lipolytic activity. Moreover, transfection of 3T3-L1 cells with ACIP led to the stabilization of AMPK beta protein independent of treatment with C26 cell supernatant (Fig. 3M).

Example 5: ***AMPK complexformation and activity is impaired in cancer cachectic WAT***

To define the molecular events leading to cachectic WAT remodeling and lipid mobilization, we performed expression profiling of genes involved in lipid metabolism in WAT of C26 tumor cachectic and healthy control animals (Fig. 6A). While mRNA levels of main lipolytic enzymes and regulators (hormone sensitive lipase, HSL; adipocyte triglyceride lipase, ATGL; lipoprotein lipase, LPL; perilipin A, PLIN1) remained unchanged, both mRNA and protein levels of the lipid droplet associated protein cell death-inducing DNA fragmentation 45-like effector (Cide) a, (CIDEA) was specifically enhanced in tumor-bearing/cachectic animals (Fig. 6A, B). Consistent with the elevation of lipolytic flux and the proportional enhancement of NEFA re-esterification under these conditions (Vaughan, 1962), genes in the NEFA-TG re-esterification pathway were found to be induced in the cancer cachectic state, most notably phosphoenolpyruvate carboxykinase (PEPCK), the key glyceroneogenic enzyme in WAT, and pyruvate dehydrogenase kinase (PDK) 4, which enables pyruvate to be used for glyceroneogenesis in face of low glucose levels (Flachs et al, 2013). Gene expression of UCP 1 was down-regulated in WAT of tumor-bearing mice, excluding possible involvement of energy dissipation based on mitochondrial uncoupling in the WAT loss.

Given the absence of major changes in lipase gene expression (Fig. 6A) we next probed for the lipase activation status by monitoring critical phosphorylation events in cachectic and non-cachectic WAT. In line with a minor influence of beta-sympathetic signaling on cachexia-dependent WAT lipid loss, ATGL-activating perilipin A and HSL-activating Ser 660 phosphorylation (Zechner et al, 2012) were reduced rather than increased in iWAT of cachectic animals. However, Western Blot analysis demonstrated that the inhibitory HSL phosphorylation at Ser 565 (Daval et al, 2005) was severely blunted by almost 50% in WAT from tumor-bearing

animals (Fig. 6B), suggesting that tumor growth abolishes a major brake in lipid mobilization in WAT depots, thereby promoting loss of energy stores under these conditions.

HSL Ser 565 has previously been identified as a substrate for AMP-activated protein kinase (AMPK) which in turn has been described as a major negative feed-back regulator of lipolysis in WAT in response to energy costly, futile NEFA re-esterification (Daval et al, 2005; Sullivan et al, 1994); (Djouder et al, 2010). These findings prompted us to further explore the AMPK activity status under tumor cachectic conditions. Consistent with the loss of HSL Ser 565 phosphorylation, prototypical AMPK-dependent Acetyl-CoA carboxylase Ser 79 phosphorylation (Fullerton et al, 2013) was diminished (Fig. 6B). Also, AMPK subunit alpha/beta protein expression was significantly reduced in cachectic WAT stores (Fig. 6B) and in primary mouse adipocytes exposed to serum from cachectic animals, suggesting that cancer cachexia disrupts the functional activity of AMPK.

Indeed, AMPK beta could hardly be detected in immunoprecipitates of AMPK alpha from WAT and BAT of cachectic animals, while AMPK beta was readily recovered in healthy control WAT and BAT depots (Fig. 6C). Consequently, AMPK enzymatic activity was substantially lower in cachectic WAT and BAT depots as compared with healthy controls in ex vivo substrate utilization assays, reflecting a change in the activities of both AMPKal and AMPKa2 isoforms (Fig. 6D).

Importantly, impairment of AMPK activity, reduced expression of AMPK alpha/beta, and loss of HSL inhibition could also be verified in both WAT and BAT depots of cachectic APC mutant animals as compared with controls (Fig. 6E), overall supporting the notion that loss of WAT AMPK alpha/beta subunit interaction and the subsequent impairment of AMPK activity during cancer cachexia promotes unchecked lipolysis via loss of inhibitory HSL Ser 565 phosphorylation.

Example 6: Local ACIP administration protects WAT depots against tumor-induced weight loss

Our results thus far promoted the notion that the prevention of CIDEA-AMPK beta interaction by ACIP may represent a molecular therapeutic approach to counteract tumor-induced WAT depletion. To explore this possibility, we generated an adeno-associated virus (AAV) carrying ACIP under the control of the adipose tissue-specific adiponectin promoter (Fig. 7A). By

microinjection directly into the inguinal fat pads, ACIP-carrying and empty control AAVs were injected into the right and left iWAT depots of wild-type mice, respectively. Following a 2-week recovery period, the animals were rendered cachectic by C26 tumor cell implantation. Expression analysis demonstrated that the ACIP peptide could specifically be detected only locally in the injected iWAT depots but not in the control-injected iWAT nor in non-injected aWAT and gastrocnemius muscle from the same animal (Fig. 7B). Consistent with data from isolated adipocytes and in contrast to control-injected fat depots, ACIP expression led to an average 30 % increase in iWAT depot weight under tumor-bearing conditions, correlating with an increase in adipocyte lipid droplet sizes as demonstrated by histological examination (Fig. 7C, D). In contrast, no major effect by ACIP was observed in iWAT depots from healthy control animals (Fig. 7C). In congruence with an AMPK and thus lipid preserving effect, local ACIP delivery rescued the inhibitory HSL Ser 565 phosphorylation in cachectic animals, and elevated both AMPK alpha and beta levels as compared with control-treated, tumor-bearing animals (Fig. 7E), demonstrating that the inhibitory ACIP peptide efficiently counteracts main cachexia-driven WAT phenotypes upon local administration *in vivo.*

Example 7: Systemic ACIP delivery counteracts WAT tissue depletion and prolongs survival in cancer-cachectic animals

To explore the potency of the ACIP peptide at the systemic level and circumvent the depot restrictions of the site-directed injection approach, we employed the ACIP AAV vector system in a systemic delivery mode.

Wild-type mice were injected by the ACIP-carrying or control AAV via tail vein injection and after a 15 day recovery period, C26 tumor cells were implanted. 21 days after tumor cell implantation, control AAV-injected animals displayed all signs of cancer cachexia, including a substantial loss of body weight, fat pad and skeletal muscle mass (Fig. 8A-C). Remarkably, injection of ACIP-carrying AAV significantly reduced all main signs of tumor-induced cachexia in WAT depots (Fig. 8A-C), most notably including a significant amelioration of iWAT and aWAT loss upon tumor growth while leaving the actual tumor size unaffected (Fig. 8D). Consistent with these physiological effects, ACIP delivery restored AMPK alpha-beta interaction in WAT of cancer cachectic animals (Fig. 8E), and rescued Ser 565 phosphorylation of HSL in this tissue (Fig. 8F). Importantly, while all control-injected animals had to be sacrificed for ethical reasons due to a substantial 10-15 % weight loss after 21 days of tumor growth, roughly 50 % of ACIP-treated mice had not reached this degree of cachexia at this time point, overall demonstrating that the ACIP-mediated restoration of anti-lipolytic AMPK activity in WAT

promotes the survival of tumor-bearing animals and efficiently counteracts systemic energy depletion under cancer cachectic conditions.

Results of Examples above and shown in the figures were obtained using the following experimental procedures:

Animal experiments: 9-10-week-old BALB/c male mice were obtained from Charles River Laboratories (CRL, Brussels, Belgium). Mice were injected with $1.5xl0^6$ cells of C-26 colon carcinoma. Tumor growth, body weight and body composition were measured for -20 days, post C-26 cell injection. Food was weighed daily and a pair feeding group was established when C-26 mice displayed symptoms of anorexia. The pair fed mice were then paired to a cachectic mouse such that the pair fed mouse was given the same amount of food, by weight, as was consumed by the tumor bearing mouse on the previous day.

All mice were maintained on a 12 hrs light-dark cycle at 24°C with regular unrestricted diet unless stated otherwise. Organs including tumor, heart, liver, fat pads, and gastrocnemius muscles were collected, snap frozen and used for further analysis. Total body fat content was determined by an Echo magnetic resonance imaging (ECHO-MRI) body composition analyzer (Echo Medical Systems, Houston, TX). For thermoneutrality, mice were housed in temperature controlled housing at 30 degrees. The mice were acclimatized to the temperature for 10 days prior to C-26 cell injection. Indirect calorimetry was performed in PhenoMaster cages (TSE Systems, Bad Homburg, Germany) with individually housed mice at 30°C, 22°C or 4°C. Calculations of resting metabolic rate (RMR) and body weight-adjusted oxygen consumption were performed as described (Tschop et al, 201 1). All mice were maintained on a 12 hrs light-dark cycle at 24°C with regular unrestricted diet unless stated otherwise.

6-Hydroxy dopamine (Sigma, Munich, Germany) was dissolved in saline solution (B. Braun, Melsungen, Germany) with 1% ascorbic acid (Sigma, Munich, Germany). While under anesthesia of isofluorane, mice underwent surgery where each inguinal white adipose tissue depot was injected 14 times (2μL per injection) with lOmg/mL 60HDA or saline solution. The mice were allowed to recover for a minimum of 10 days prior to C-26 injection or PBS. For the cold exposure experiment, mice were left to recover for 10 days before being placed in the TSE Phenomaster for 24 hours at 22° followed by 24 hours at 4°C. Tissue norepinephrine levels were determined using commercially available ELISA kits (Abnova, Heidelberg).

APC delta580 mice were obtained from NCI Frederick and housed until heterozygous animals developed multiple intestinal neoplasia and cachexia at 5-6 months of age (Kuraguchi et al, 2006).

Recombinant viruses. AMPKβI amino acids 232-248 and n-terminal flag tag were cloned into puc57-pAdiponectin-miR122. Recombinant adeno-associated viruses (AAV) were produced by co-transfection of HEK 293 cells with puc57 and pDP8.ape plasmids (Plasmid factory, Bielefeld) and purified by VectorBiolabs (Philadelphia). Control AAV represents an empty virus without peptide. Mice were injected at $3*10^9$ ifu/fat pad by microinjection into the inguinal fat pad (12 injections at different sites with 4 µï each) as described above. In a separate experiment, mice were injected with $5*10^{11}$ ifu into the tail vein. Experiments were initiated 2-3 weeks following virus infection.

Histology: Tissues were collected and immediately stored in 4% paraformaldehyde for 24 hrs at 4°C. Paraffin embedded tissues were cut in 4µιη sections and mounted. Sections were stained with anti-UCP-1 or anti-VDAC-1 (Abeam, Cambridge, UK) at 1:100 and were counter-stained with hematoxylin by standard procedures. Stainings were imaged under 40X magnification (Zeiss, Jena, Germany). Hematoxylin stained nuclei were counted from each tissue section using ImageJ.

Quantitative Taqman RT-PCR. Total RNA was extracted from frozen organ samples or cultured adipocytes using QIAzol and the RNeasy (Qiagen, Hilden) kit. cDNA was prepared by reverse transcription using M-MuLV enzyme and Oligo dT primer (Fermentas, St. Leon-Rot). cDNAs were amplified using assay-on-demand kits and an ABI StepOnePlus sequence detector (Applied Biosystems, Darmstadt). RNA expression data were quantified according to the delta Ct method as described (Livak and Schmittgen, 2001) (Jones et al., 2013) and normalized to levels of TATA-box binding protein RNA (TBP) for tissue and cultured adipocytes.

Primary adipocytes isolation and differentiation: 6-7 week old male NMRI mice were obtained from Charles River (Brussels, Belgium). Inguinal white adipose tissue was dissected and primary adipocytes were isolated as previously described (Vegiopoulos et al, Science 2010). Primary adipocytes were differentiated in standard differentiation cocktails unless otherwise stated. Serum of either control or cachectic mice from completed experiments was pooled and added to the differentiation cocktail at a concentration of 1% for both the differentiation induction and the differentiation maturation. Fetal Bovine Serum (Gibco) was added at a concentration of 9% or 4% for induction and maturation, respectively. Media was replaced every other day. For indicated experiments, norepinephrine (Sigma, Munich, Germany) was

added to the culture media on Day 8 at a final concentration of 0.5μM, for two hours. For C-26 and MC38 conditioned media experiments, C-26 and MC38 cells were plated in 15cm plates and the media collected 48 hours later. Media from 15cm plates with no cells was used as control media for "white adipose tissue" (WAT) control differentiation. Media was filtered and was used 3:1 with fresh media and changed every other day for the duration of the adipocyte differentiation protocol.

For indicated experiments, primary adipocytes were transfected with control peptide + Flag (CP) or AMPK-Cidea Interfering Peptide+Flag (ACIP), 48 hours post-isolation and 48 hours prior to the induction of differentiation, using Turbofect transfection reagent (Thermo Scientific) as per the manufacturer's instructions.

Cell culture: Wildtype MEFs or AMPKβ1β2-/- MEFs, generously given by Dr. Gregory Steinberg (McMaster University, Hamilton, Canada), were cultured in 5g/L DMEM (Gibco), 10% FCS and 1% Pen/Strep. Cells were transfected (Turbofect, Thermoscientific) with a V5 tagged Cidea overexpression construct +/- CP or ACIP for 48 hours. Cells were lysed and analyzed by co-immunoprecipitation for interaction between Cidea and AMPKβI.

CP and ACIP: Oligos coding for AMPKβI amino acids 232-248 were cloned into pLKO-puro FLAG (Addgene, Cambridge, MA). Control peptide was pLKO-puro HA-FLAG.

NEFA quantification: Serum was taken from animals immediately following sacrifice. Non-esterified free fatty acids were quantified using 4μL serum in the NEFA Detection Kit (Wako Chemical GmBh, Neuss, Germany). For the lipolysis assay, primary adipocytes were cultured in Kreb's Ringer (KR) supplemented with 3% BSA (Sigma) and NEFAs were quantified with 4uL of conditioned KR, following 2 hours of incubation, and normalized to protein concentration by BCA assay (Thermo Scientific). Where indicated, 0.5μM nor-epinephrine was added to the KR for 2 hours.

Protein analysis. Proteins were extracted from frozen organ samples or cultured adipocytes following lysis in ice cold lysis buffer (50mM Tris pH 7.2, 150mM NaCl, 1% NP-40, 0.5 % Triton X, ImM EDTA, ImM Na3V04, ImM NaF, ^g/mL pepstatin A and ImM DTT) and extracts were separated on 10-15% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Western blot assays were performed using antibodies specific for p-ACC, ACC total, p-HSL Ser565, HSL total, AMPKa, PKA substrates (Cell Signaling, Danvers), AMPKβI, V5, Cidea, perilipin total (Abeam, Cambridge, UK).

For immunoprecipitation, 500µg protein was pre-washed in 40µL protein A/G agrose (Santa Cruz) and 400µL lysis buffer (see above) for 1 hour at 4° degrees. After spinning (13.000 rpm, 4°C, 1 min) supernatants were collected and incubated with 7µL of the respective antibody over night at 4°C on a rotating wheel. The next day, 40µL of protein A/G agarose was added to the samples and rotated for an additional 2 hours. Protein-bound beads were washed five times with lysis buffer, proteins were eluted by the addition of SDS sample buffer and subjected to immunoblotting.

AMPK activity assay. Samples of adipose tissue (BAT, aWAT, iWAT) were collected by using freeze-clamping with liquid nitrogen. Frozen tissues were crushed and mixed with lysis buffer (50mM Tris, pH 7.4; ImM EDTA; 0.15M NaCl; lmM benzamidine; ImM dithiothreitol; 50mM sodium fluoride; 5mM pyrophosphate tetrasodium; ImM phenylmethylsulfonylfiuoride; 0.2% Triton X-100; 1% glycerol; lOmg/ml aprotonin; lOmg/ml pepstatin; lOmg/ml leupeptin and Phosphatase Inhibitor-Mix I, (Serva, Heidelberg) under liquid nitrogen. Tissue lysates were sonicated and centrifuged at 18,000 g for 10 min at 4°C. Protein concentration in the supernatants was measured by BCA assay (Thermo Scientific).

Activities of a1 and a2 AMPK isoforms were measured as before (Jelenik et al, 2010) using a peptide substrate (Hardie et al., 2000). Briefly, AMPKal and AMPKa2 isoforms were separately immunoprecipitated from the tissue extracts by using specific sheep antibodies (gift of Dr. D. Grahame Hardie) bound to Protein G Sepharose (GE Healthcare Bio-Sciences AB, Sweden). The activity of precipitated protein was determined in a HEPES-Brij buffer containing AMP (ImM), [γ-^{32}P]ATP (ImM) and AMARA peptide (ImM; Vidia Prague, Czech Republic). After 15 min at 30°C, the reaction was stopped by washing with 1% phosphoric acid solution using paper filters. The activity of lU/mg protein corresponds to lnmol ^{32}P-AMARA peptide/mg protein per min.

Statistical analysis. Statistical analyses were performed in SigmaPlot 12.0 using one-way and two-way ANOVA or Student t test, where appropriate. $p<0.05$ was considered statistically significant. Error bars in figures represent standard error of the mean (SEM).

References

1. Algire, C , Medrikova, D., and Herzig, S. (2013). White and brown adipose stem cells: From signaling to clinical implications. Biochim Biophys Acta 1831, 896-904.
2. Berriel Diaz, M., Krones-Herzig, A., Metzger, D., Ziegler, A., Vegiopoulos, A., Klingenspor, M., Muller-Decker, K., and Herzig, S. (2008). Nuclear receptor cofactor

receptor interacting protein 140 controls hepatic triglyceride metabolism during wasting in mice. Hepatology 48, 782-791.

3. Daval, M., Diot-Dupuy, F., Bazin, R., Hainault, I., Viollet, B., Vaulont, S., Hajduch, E., Ferre, P., and Foufelle, F. (2005). Anti-lipolytic action of AMP-activated protein kinase in rodent adipocytes. J Biol Chem 280, 25250-25257.
4. Djouder, N., Tuerk, R.D., Suter, M., Salvioni, P., Thali, R.F., Scholz, R., Vaahtomeri, K., Auchli, Y., Rechsteiner, H., Brunisholz, R.A., Viollet, B., Makela, T.P., Wallimann, T., Neumann, D., and Krek, W. (2010). PKA phosphorylates and inactivates AMPKalpha to promote efficient lipolysis. EMBO J 29, 469-481.
5. Fearon, K.C., Glass, D.J., and Guttridge, D.C. (2012). Cancer cachexia: mediators, signaling, and metabolic pathways. Cell Metab 16, 153-166.
6. Flachs, P., Rossmeisl, M., Kuda, O., and Kopecky, J. (2013). Stimulation of mitochondrial oxidative capacity in white fat independent of UCP1: a key to lean phenotype. Biochim Biophys Acta 1831, 986-1003.
7. Fullerton, M.D., Galic, S., Marcinko, K., Sikkema, S., Pulinilkunnil, T., Chen, Z.P., O'Neill, H.M., Ford, R.J., Palanivel, R., O'Brien, M., et al. (2013). Single phosphorylation sites in Accl and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. Nat Med 19, 1649-1654.
8. Galic, S., Oakhill, J.S., and Steinberg, G.R. (2009). Adipose tissue as an endocrine organ. Mol Cell Endocrinol 316, 129-139.
9. Jelenik, T., Rossmeisl, M., Kuda, O., Jilkova, Z.M., Medrikova, D., Kus, V., Hensler, M., Janovska, P., Miksik, I., Baranowski, M., et al. (2010). AMP-activated protein kinase alpha2 subunit is required for the preservation of hepatic insulin sensitivity by n-3 polyunsaturated fatty acids. Diabetes 59, 2737-2746.
10. Jones, A., Friedrich, K., Rohm, M., Schafer, M., Algire, C , Kulozik, P., Seibert, O., Muller-Decker, K., Sijmonsma, T., Strzoda, D., Sticht, C , Gretz, N., Dallinga-Thie, G.M., Leuchs, B., Kogl, M., Stremmel, W., Diaz, M.B., and Herzig, S. (2013). TSC22D4 is a molecular output of hepatic wasting metabolism. EMBO Mol Med 5, 294-308.
11. Kuraguchi, M., Wang, X.P., Bronson, R.T., Rothenberg, R., Ohene-Baah, N.Y., Lund, J.J., Kucherlapati, M., Maas, R.L., and Kucherlapati, R. (2006). Adenomatous polyposis coli (APC) is required for normal development of skin and thymus. PLoS genetics 2, el46.
12. Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

13. Qi, J., Gong, J., Zhao, T., Zhao, J., Lam, P., Ye, J., Li, J.Z., Wu, J., Zhou, H.M., and Li, P. (2008). Downregulation of AMP-activated protein kinase by Cidea-mediated ubiquitination and degradation in brown adipose tissue. EMBO J 27, 1537-1548.

14. Sullivan, J.E., Brocklehurst, K.J., Marley, A.E., Carey, F., Carling, D., and Beri, R.K. (1994). Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMP-activated protein kinase. FEBS Lett 353, 33-36.

15. Thureson-Klein, A., Lagercrantz, FL, and Barnard, T. (1976). Chemical sympathectomy of interscapular brown adipose tissue. Acta physiologica Scandinavica 98, 8-18.

16. Tschop, M.H., Speakman, J.R., Arch, J.R., Auwerx, J., Bruning, J.C., Chan, L., Eckel, R.H., Farese, R.V., Jr., Galgani, J.E., Hambly, C , Herman, M.A., Horvath, T.L., Kahn, B.B., Kozma, S.C., Maratos-Flier, E., Muller, T.D., Munzberg, FL, Pfluger, P.T., Plum, L., Reitman, M.L., Rahmouni, K., Shulman, G.I., Thomas, G., Kahn, C.R., and Ravussin, E. (201 1). A guide to analysis of mouse energy metabolism. Nat Methods 9, 57-63.

17. Tsoli, M., Moore, M., Burg, D., Painter, A., Taylor, R., Lockie, S.H., Turner, N., Warren, A., Cooney, G., Oldfield, B., Clarke, S., and Robertson, G. (2012). Activation of thermogenesis in brown adipose tissue and dysregulated lipid metabolism associated with cancer cachexia in mice. Cancer Res 72, 4372-4382.

18. Vaughan, M. (1962). The production and release of glycerol by adipose tissue incubated in vitro. J Biol Chem 237, 3354-3358.

19. Vegiopoulos, A., Muller-Decker, K., Strzoda, D., Schmitt, I., Chichelnitskiy, E., Ostertag, A., Berriel Diaz, M., Rozman, J., Hrabe de Angelis, M., Nusing, R.M., Meyer, C.W., Wahli, W., Klingenspor, M., and Herzig, S. (2010). Cyclooxygenase-2 controls energy homeostasis in mice by de novo recruitment of brown adipocytes. Science 328, 1158-1 161.

20. Yu, Y.H., and Ginsberg, H.N. (2005). Adipocyte signaling and lipid homeostasis: sequelae of insulin-resistant adipose tissue. Circ Res 96, 1042-1052.

21. Zechner, R., Zimmermann, R., Eichmann, T.O., Kohlwein, S.D., Haemmerle, G., Lass, A., and Madeo, F. (2012). FAT SIGNALS—lipases and lipolysis in lipid metabolism and signaling. Cell Metab 15, 279-291.

Claims

1. An inhibitor of Adenosine-monophosphate-activated kinase (AMPK) inactivation.

2. The inhibitor of AMPK inactivation according to claim 1 for use in preventing and/or treating disease.

3. The inhibitor of AMPK inactivation according to claim 2, wherein the disease is selected from the list consisting of cancer cachexia, non-tumor-cachexiae, lipodystrophy, metabolic syndrome, and diabetes type I.

4. The inhibitor of AMPK inactivation according to any one of claims 1 to 3, wherein the inhibitor of AMPK inactivation is an inhibitor of AMPKbeta degradation.

5. The inhibitor of AMPK inactivation according to any one of claims 1 to 4, wherein the inhibitor of AMPK inactivation is an inhibitor of AMPKbeta/cell death-inducing DFFA-like effector a (Cidea) interaction.

6. The inhibitor of AMPK inactivation according to claim 5, wherein the inhibitor of AMPKbeta/Cidea interaction is a compound i) specifically binding to the interaction site in Cidea mediating binding to AMPKbeta, or ii) specifically binding to the interaction site in AMPKbeta mediating binding of Cidea, wherein said compound is selected from the list consisting of an inhibitory peptide, an antibody, an aptamer, an anticalin, a Designed Ankyrin Repeat Protein (DARPin), and a low molecular weight compound.

7. The inhibitor of AMPK inactivation according to claim 6, wherein said inhibitory peptide comprises the amino acid sequence of SEQ ID NO:l.

8. A polynucleotide encoding an inhibitor of AMPK inactivation according to any one of claims 1 to 7.

9. A device comprising the inhibitor of AMPK inactivation according to any one of claims 1 to 7.

10. A kit comprising the inhibitor of AMPK inactivation according to any one of claims 1 to 7 and an instruction manual.

11. Use of an inhibitor of AMPK inactivation according to any one of claims 1 to 7 for preventing and/or treating cancer cachexia, a non-tumor-cachexia, lipodystrophy, metabolic syndrome, or diabetes type I.

12. A method for predicting and/or diagnosing cancer cachexia in a subject afflicted with cancer, comprising:

a) determining the amount of AMPKbeta in a sample of said subject,

b) comparing the amount of AMPKbeta determined in step a) to a reference, and thereby predicting and/or diagnosing cancer cachexia in said subject afflicted with cancer.

13. The method of claim 12, wherein said sample is a tumor sample or an adipose tissue sample.

14. A method for identifying a compound for treating or preventing cancer cachexia, comprising:

a) contacting a host cell comprising AMPK with a compound suspected of inhibiting AMPK degradation,

b) further contacting said host cell with serum from a cachectic individual,

c) detecting inhibition of AMPK inactivation, and

d) thereby identifying a compound for treating cancer cachexia.

15. A pharmaceutical composition comprising the inhibitor of AMPK inactivation according to any one of claims 1 to 7 and a pharmaceutically acceptable carrier.

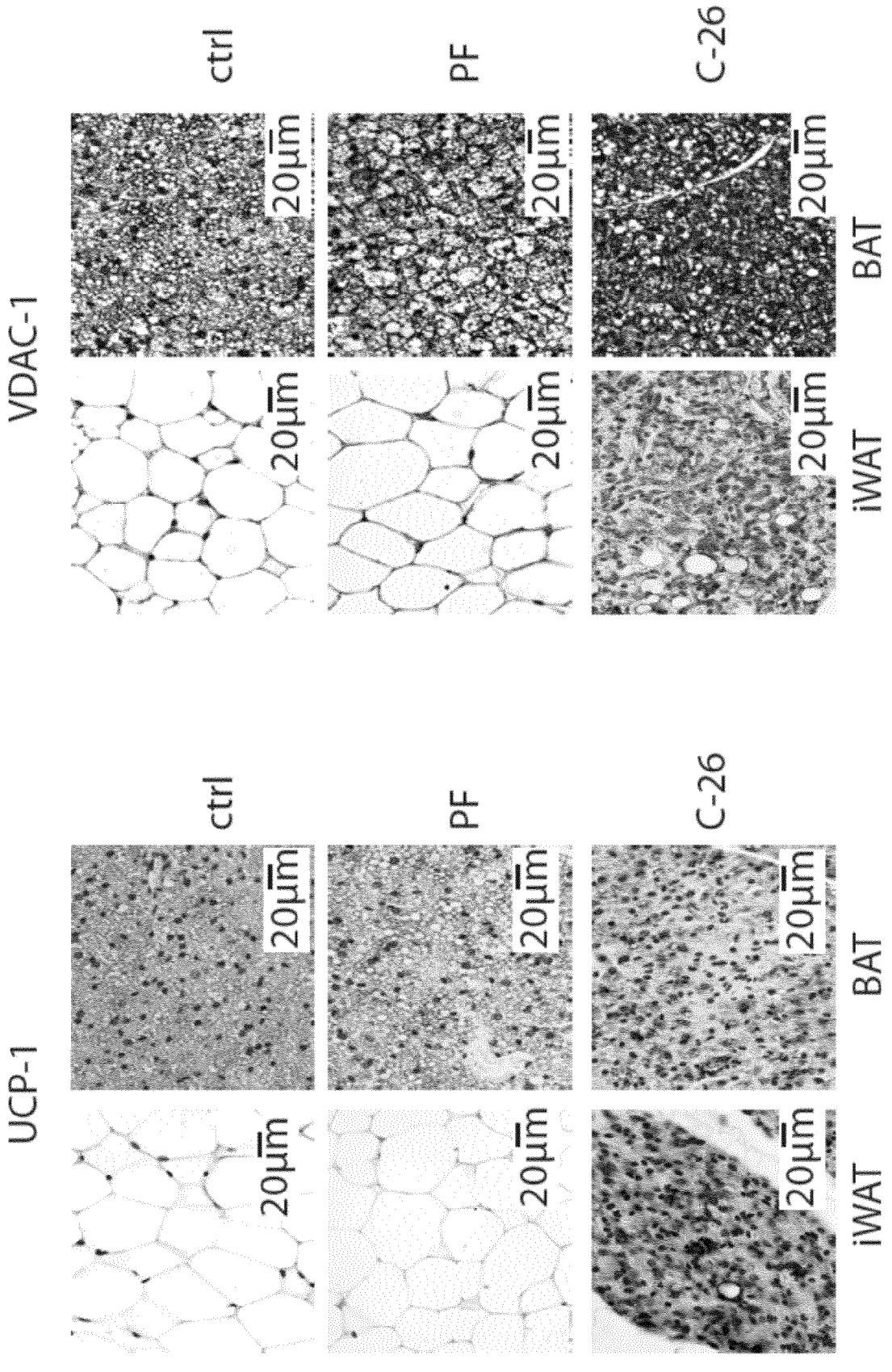


Fig. 1 A)

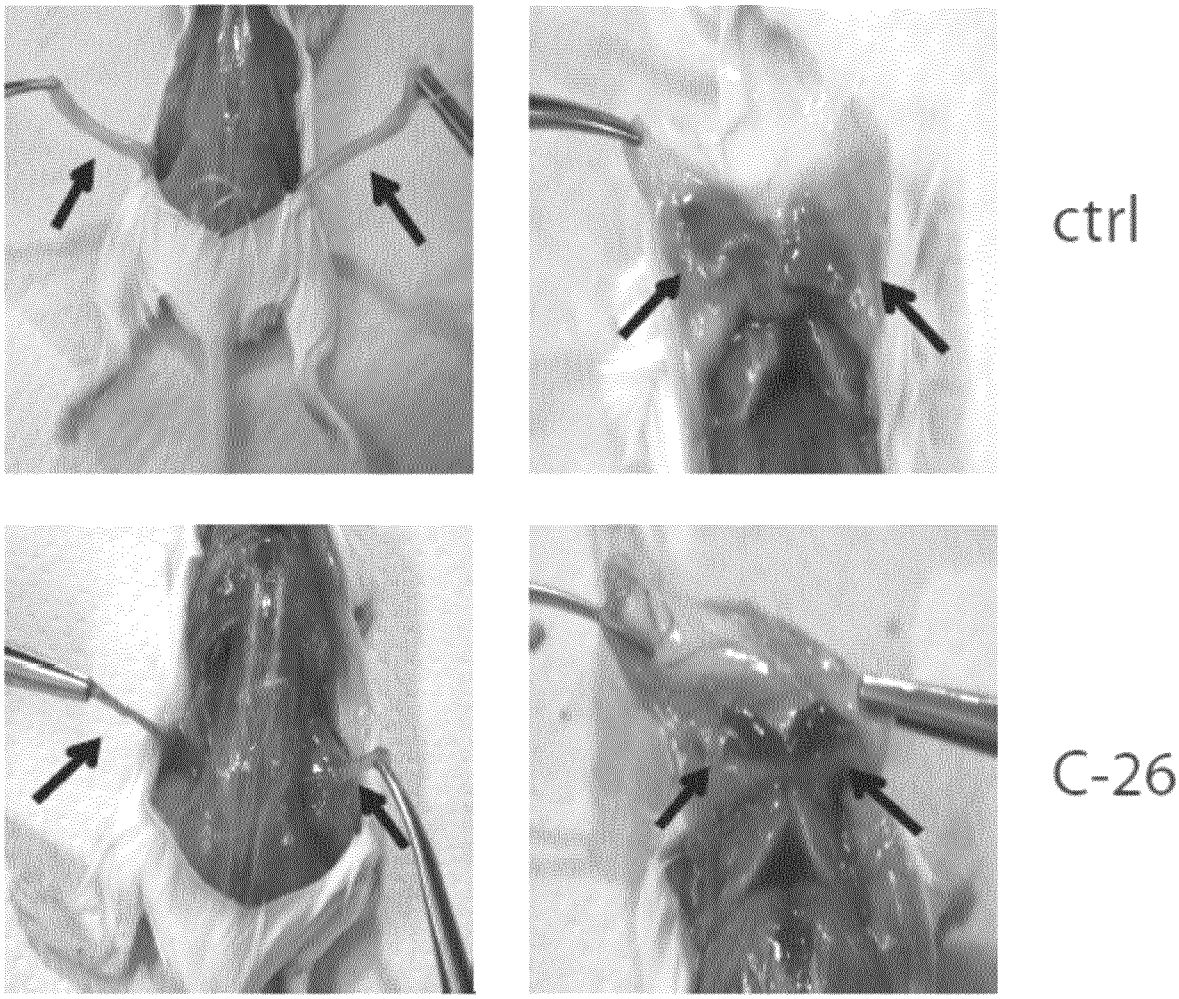


Fig. 1 B)

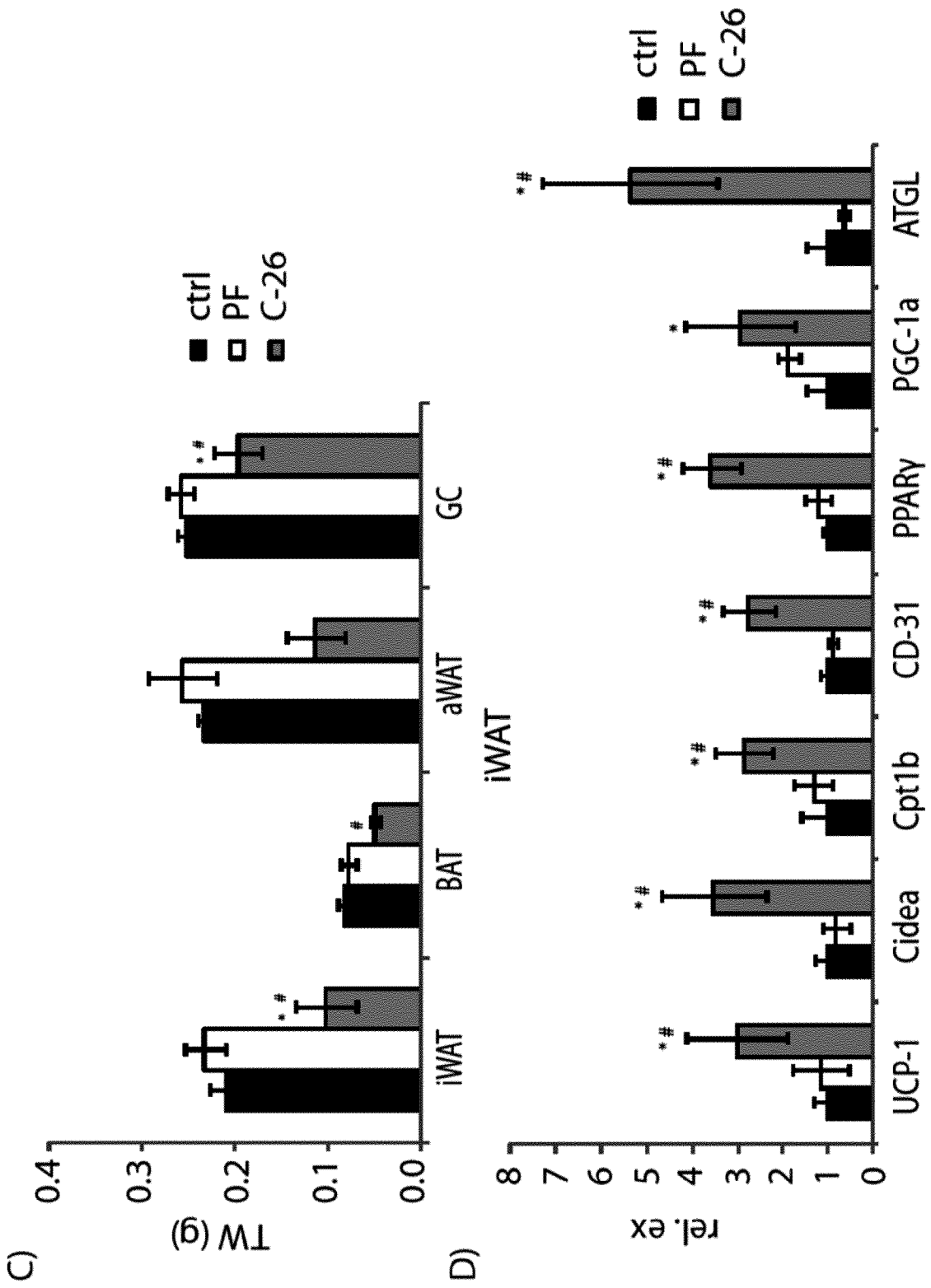
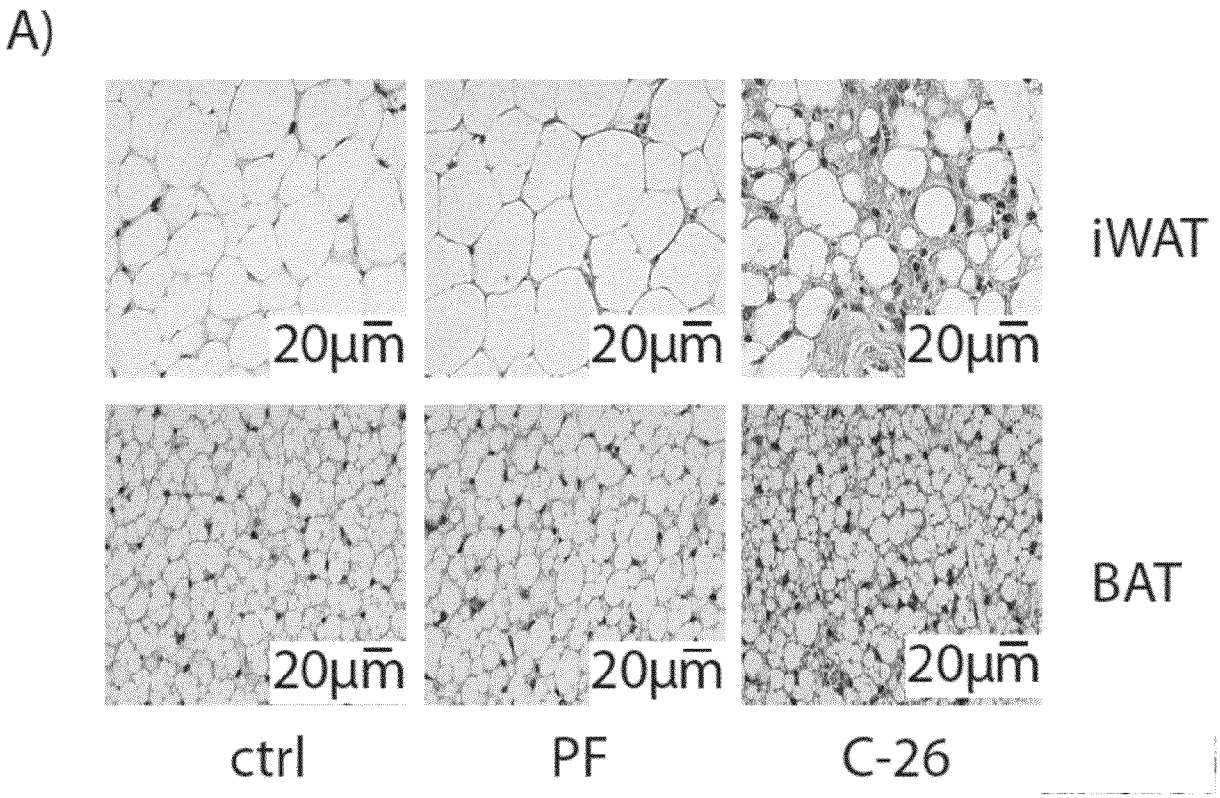

C)
TW (g)
0.4
0.3
0.2
0.1
0.0
ctrl
PF
C-26
iWAT
BAT
aWAT
GC
D)
rel. ex
8
7
6
5
4
3
2
1
0
iWAT
UCP-1
Cidea
Cpt1b
CD-31
PPARγ
PGC-1a
ATGL

Fig. 1 C), D)


A)
20µm
20µm
20µm
iWAT
20µm
20µm
20µm
BAT
ctrl
PF
C-26

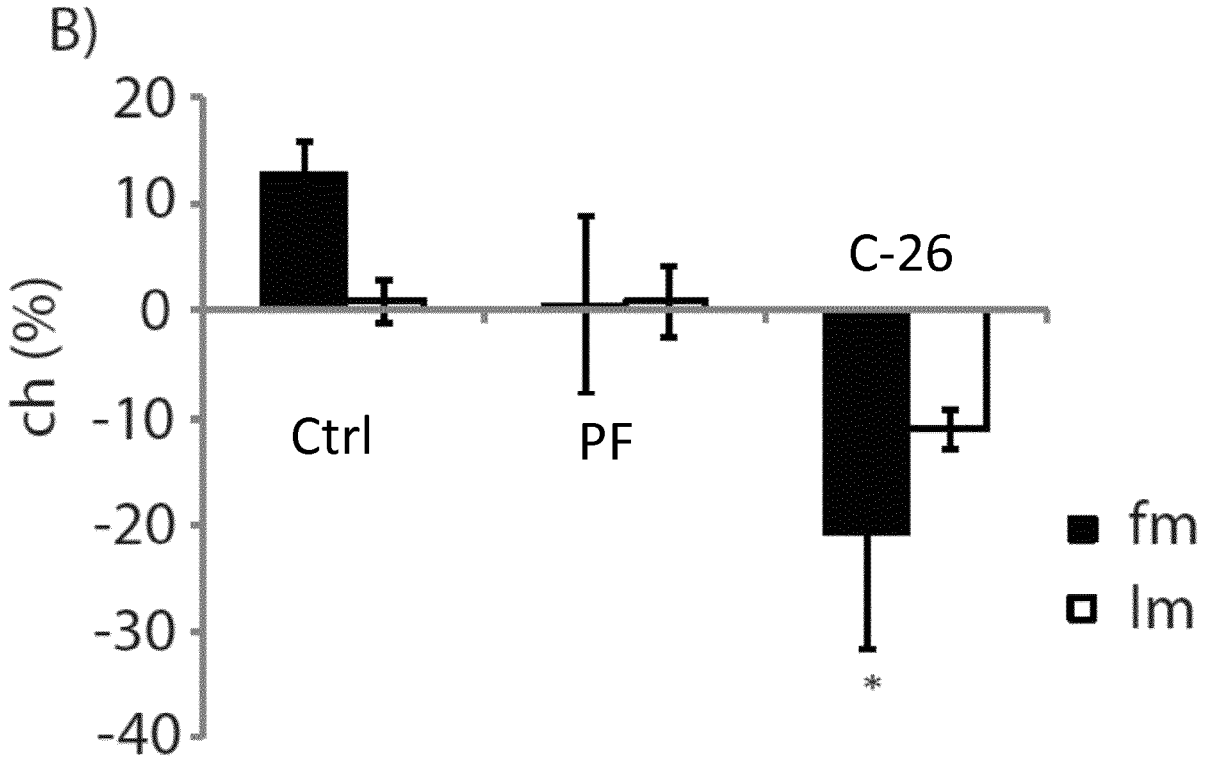

B)
20
10
0
-10
-20
-30
-40
ch (%)
Ctrl
PF
C-26
*
fm
lm

Fig. 2 A), B)

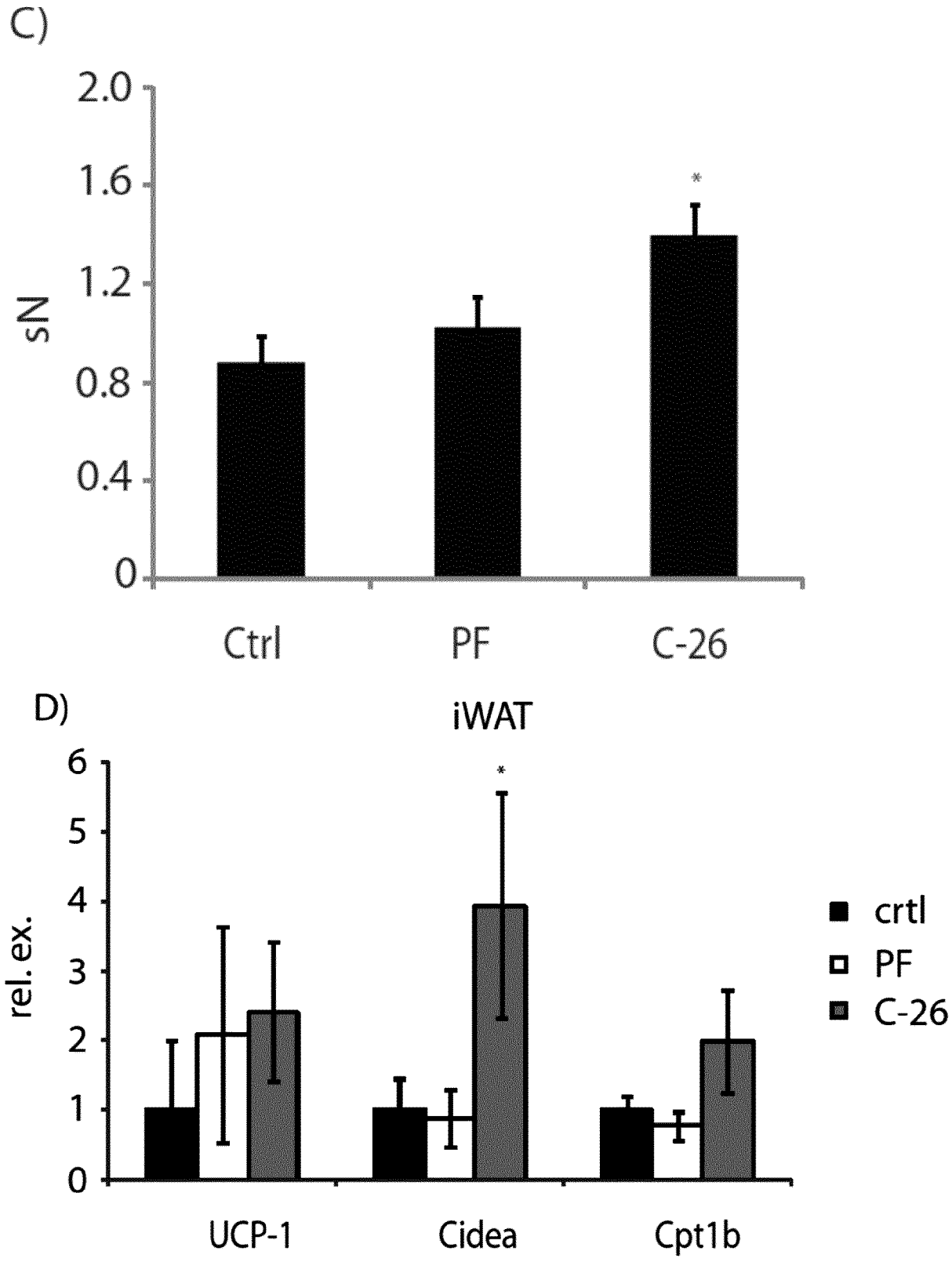

C)
2.0
1.6
1.2
0.8
0.4
0
sN
*
Ctrl
PF
C-26
D)
iWAT
6
5
4
3
2
1
0
rel. ex.
*
crtl
PF
C-26
UCP-1
Cidea
Cpt1b

Fig. 2 C), D)

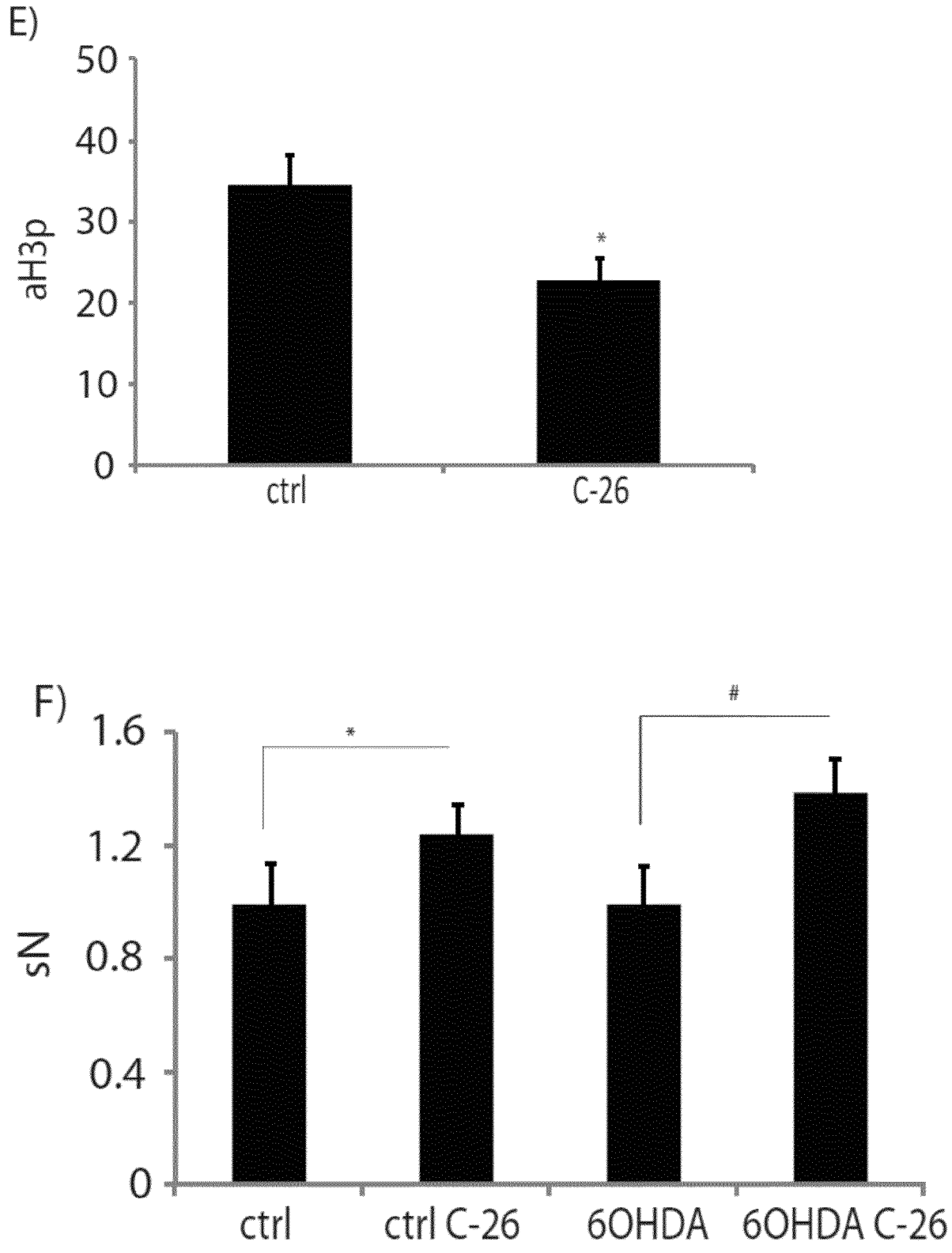

E)
50
40
30
20
10
0
aH3p
*
ctrl
C-26
F)
1.6
1.2
0.8
0.4
0
sN
*
#
ctrl
ctrl C-26
6OHDA
6OHDA C-26

Fig. 2 E), F)

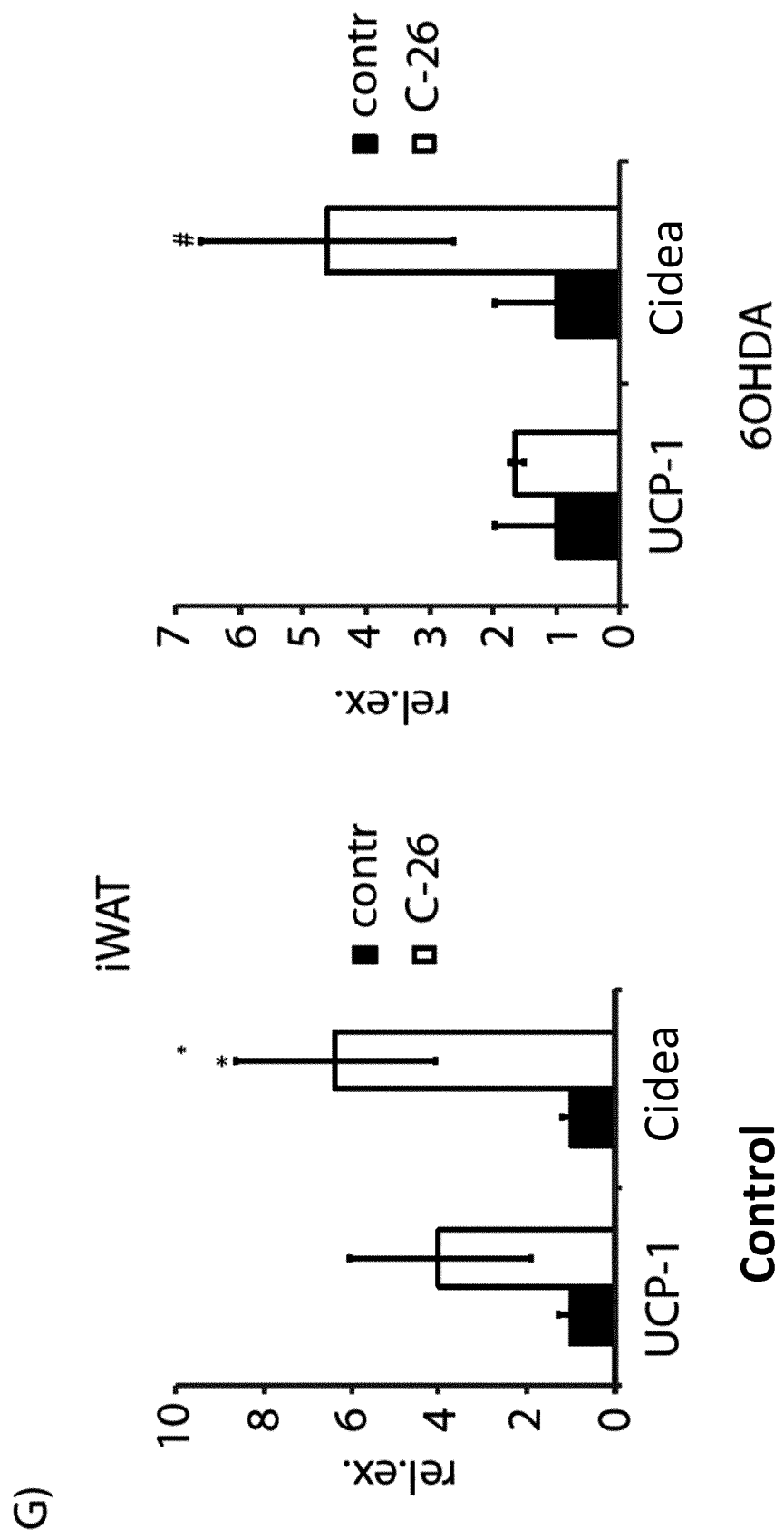

G)
iWAT
rel.ex.
10
8
6
4
2
0
*
*
contr
C-26
UCP-1
Cidea
Control
6OHDA
rel.ex.
7
6
5
4
3
2
1
0
#
contr
C-26
UCP-1
Cidea

Fig. 2 G)

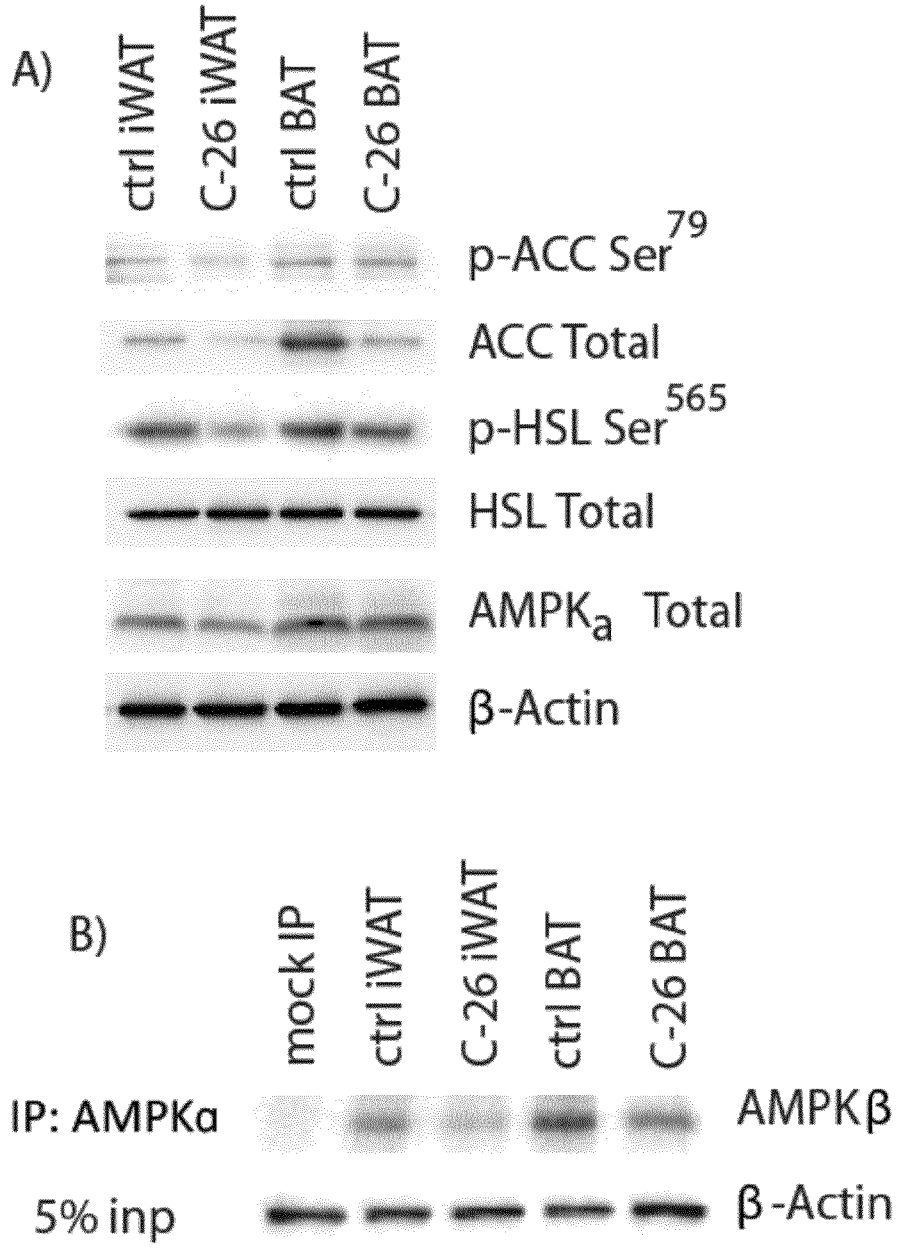

A)
ctrl iWAT
C-26 iWAT
ctrl BAT
C-26 BAT
p-ACC Ser79
ACC Total
p-HSL Ser565
HSL Total
AMPKa Total
β-Actin
B)
mock IP
ctrl iWAT
C-26 iWAT
ctrl BAT
C-26 BAT
IP: AMPKα
AMPKβ
5% inp
β-Actin

Fig. 3 A), B)

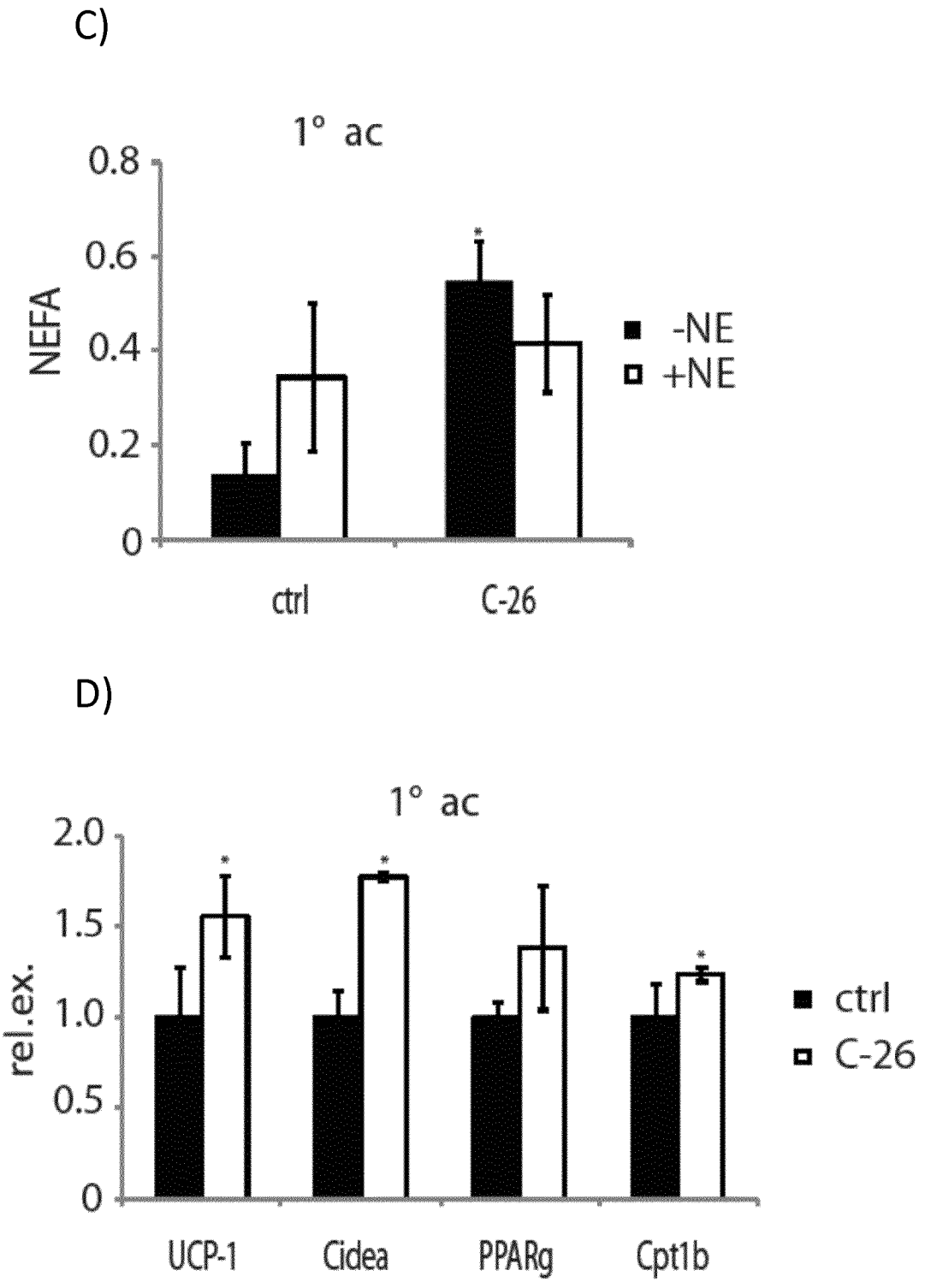
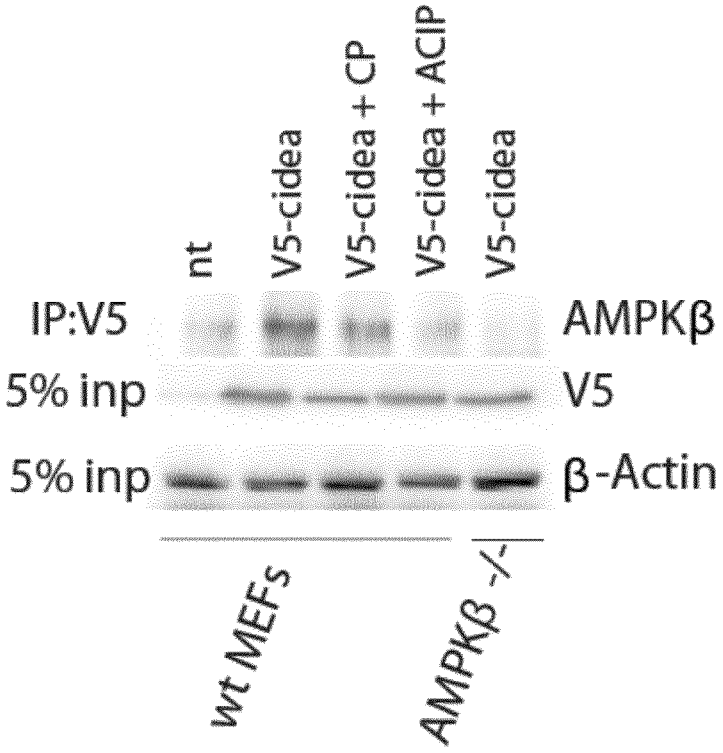

C)
1° ac
NEFA
0.8
0.6
0.4
0.2
0
-NE
+NE
ctrl
C-26
D)
1° ac
rel.ex.
2.0
1.5
1.0
0.5
0
ctrl
C-26
UCP-1
Cidea
PPARg
Cpt1b

Fig. 3 C), D)

E)


nt
V5-cidea
V5-cidea + CP
V5-cidea + ACIP
V5-cidea
IP:V5
AMPKβ
5% inp
V5
5% inp
β-Actin
wt MEFs
AMPKβ -/-

F)

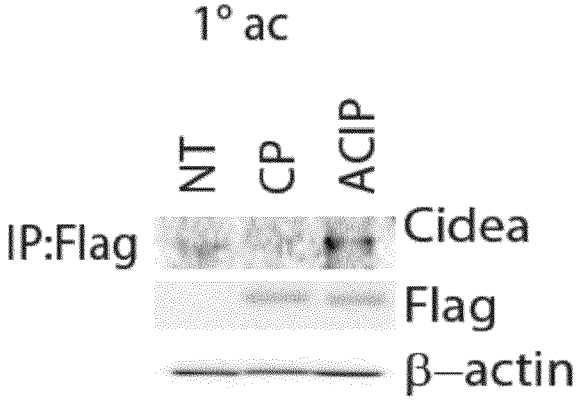
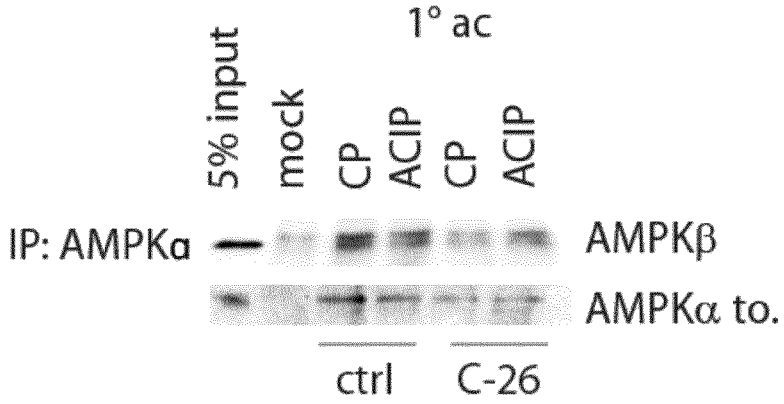

1° ac
NT
CP
ACIP
IP:Flag
Cidea
Flag
β–actin

Fig. 3 E), F)

G)


1° ac
5% input
mock
CP
ACIP
CP
ACIP
IP: AMPKα
AMPKβ
AMPKα to.
ctrl
C-26

H)

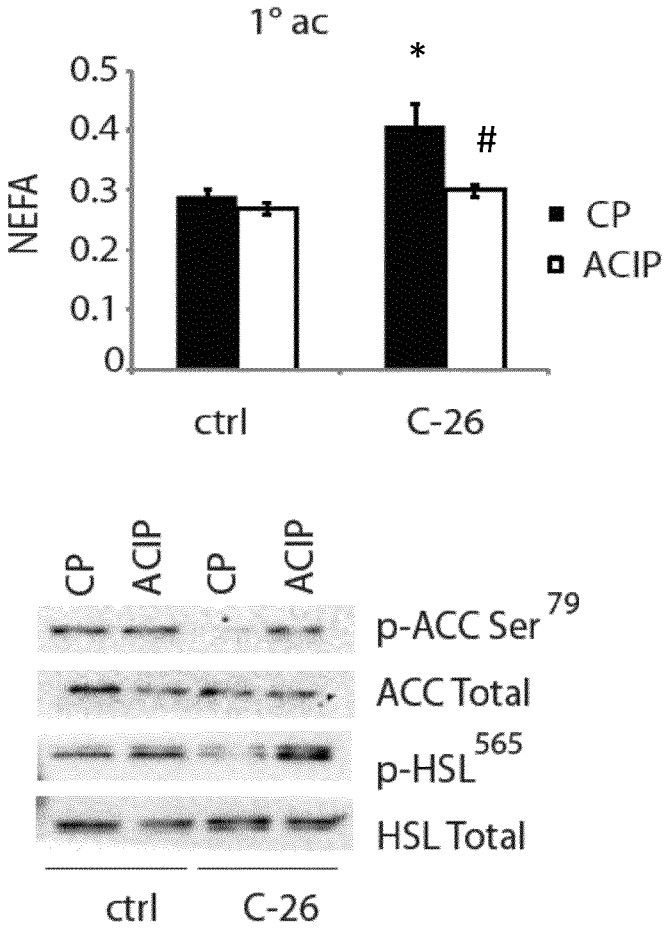

1° ac
*
#
0.5
0.4
0.3
0.2
0.1
0
NEFA
CP
ACIP
ctrl
C-26
CP
ACIP
CP
ACIP
p-ACC Ser 79
ACC Total
p-HSL 565
HSL Total
ctrl
C-26

Fig. 3 G), H)

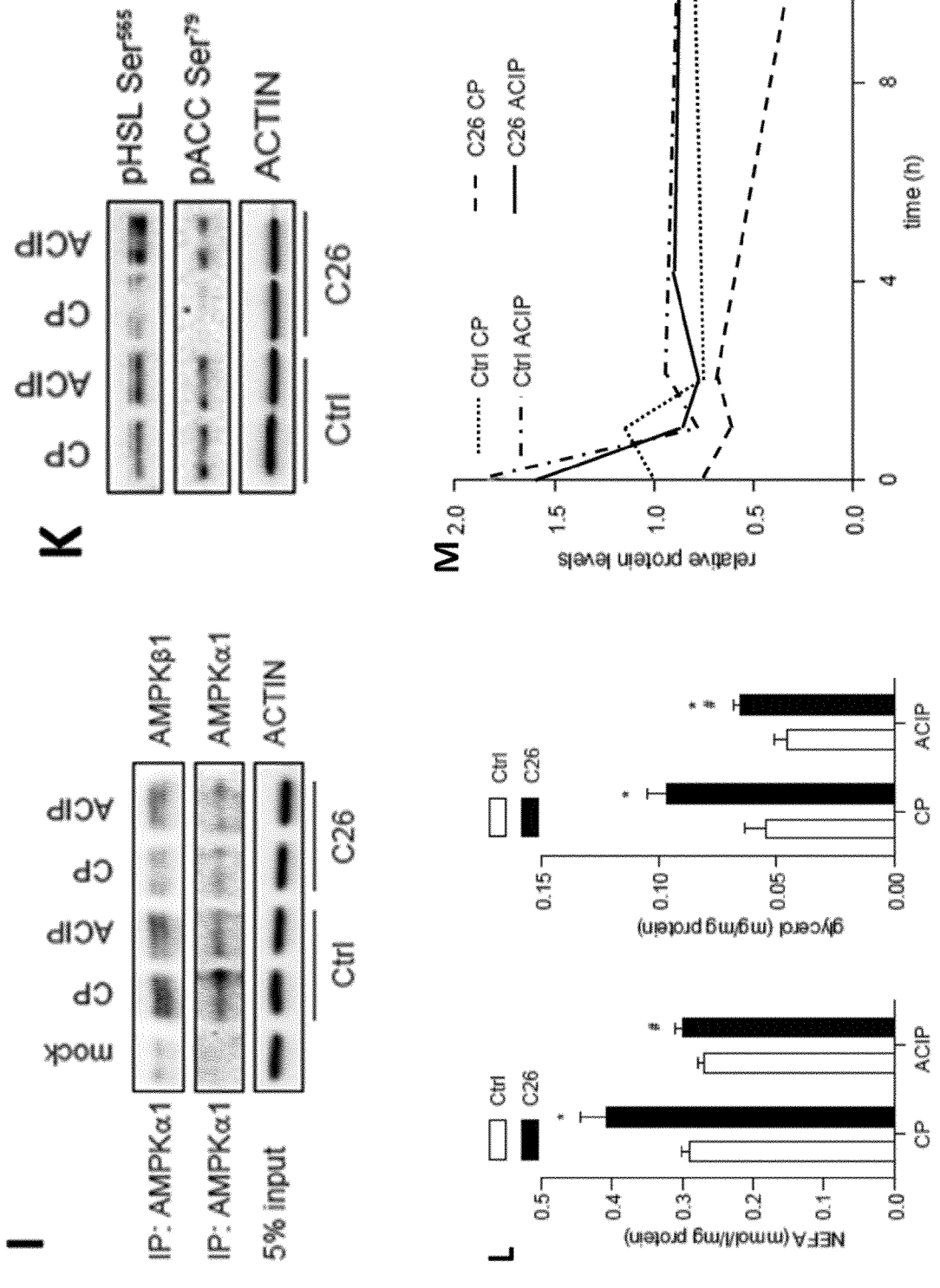

I
IP: AMPKα1
IP: AMPKα1
5% input
mock
CP
ACIP
CP
ACIP
AMPKβ1
AMPKα1
ACTIN
Ctrl
C26
K
CP
ACIP
CP
ACIP
pHSL Ser⁶⁵⁵
pACC Ser⁷⁹
ACTIN
Ctrl
C26
M
Ctrl CP
Ctrl ACIP
C26 CP
C26 ACIP
relative protein levels
time (h)
L
Ctrl
C26
NEFA (mmol/l/mg protein)
glycerol (mg/mg protein)
CP
ACIP

Fig. 3 I) - M)

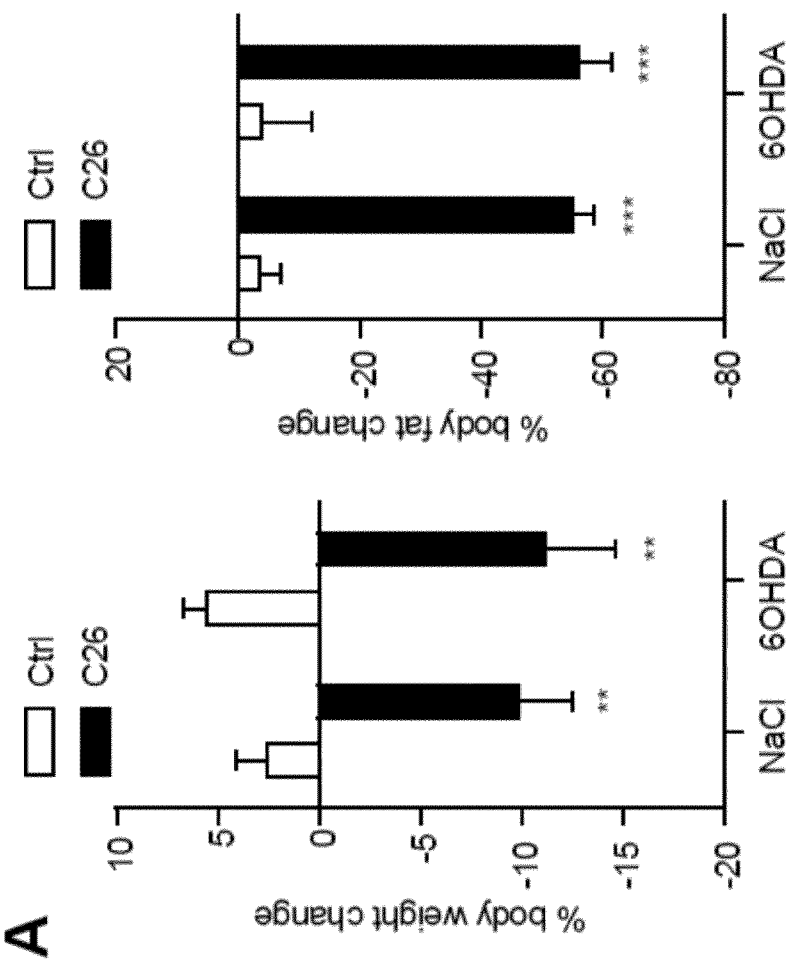

A
% body weight change
% body fat change
% lean mass change
Ctrl
C26
NaCl
6OHDA
**
***

Fig. 4 A)

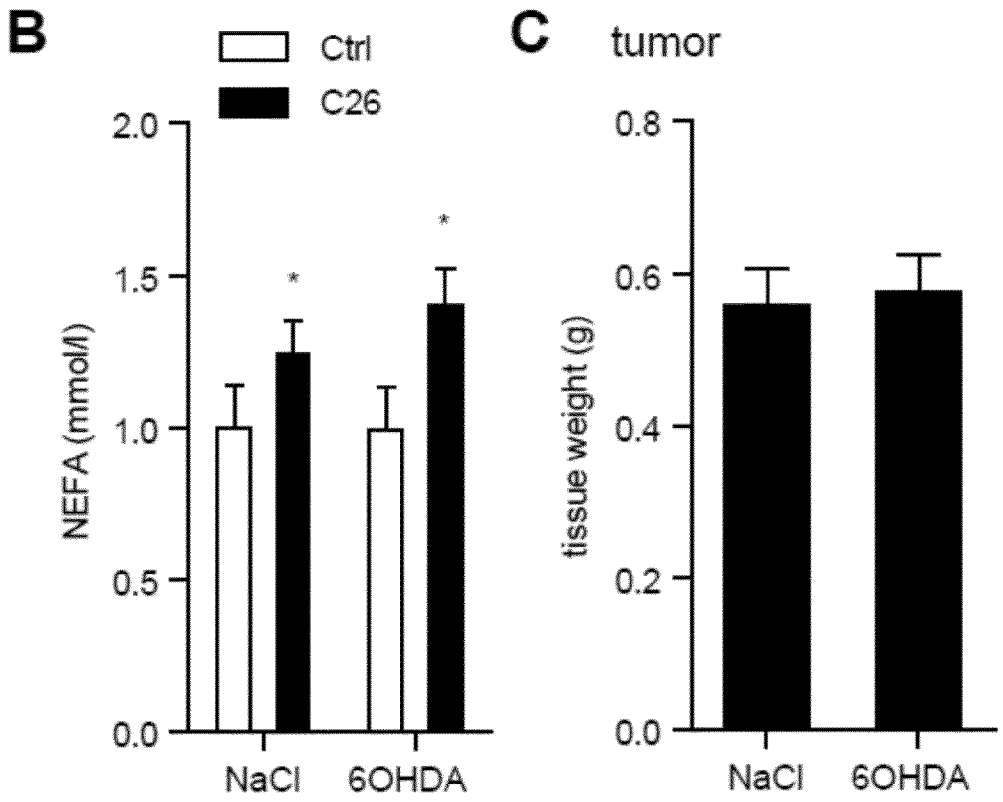

B
Ctrl
C26
2.0
1.5
1.0
0.5
0.0
NEFA (mmol/l)
*
*
NaCl
6OHDA
C
tumor
0.8
0.6
0.4
0.2
0.0
tissue weight (g)
NaCl
6OHDA

Fig. 4 B), C)

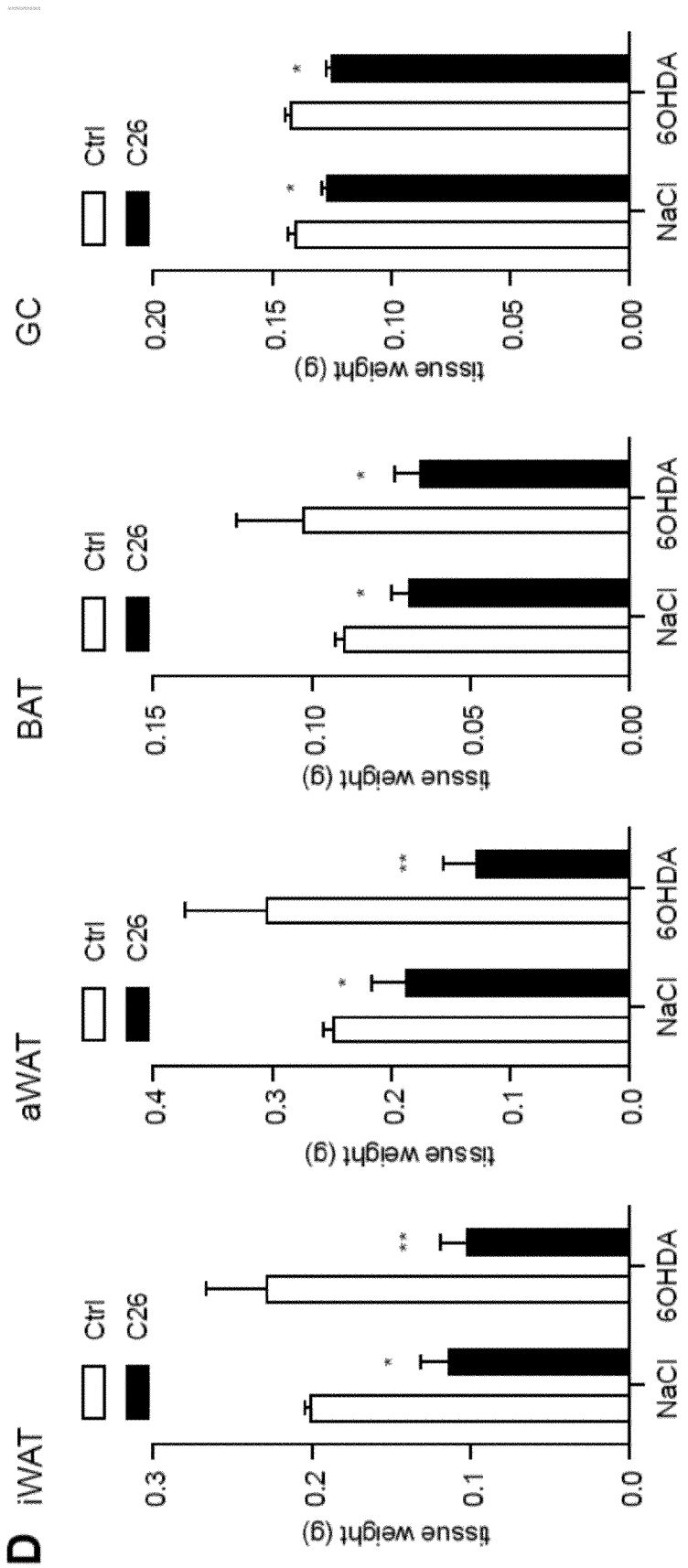


Fig. 4 D)

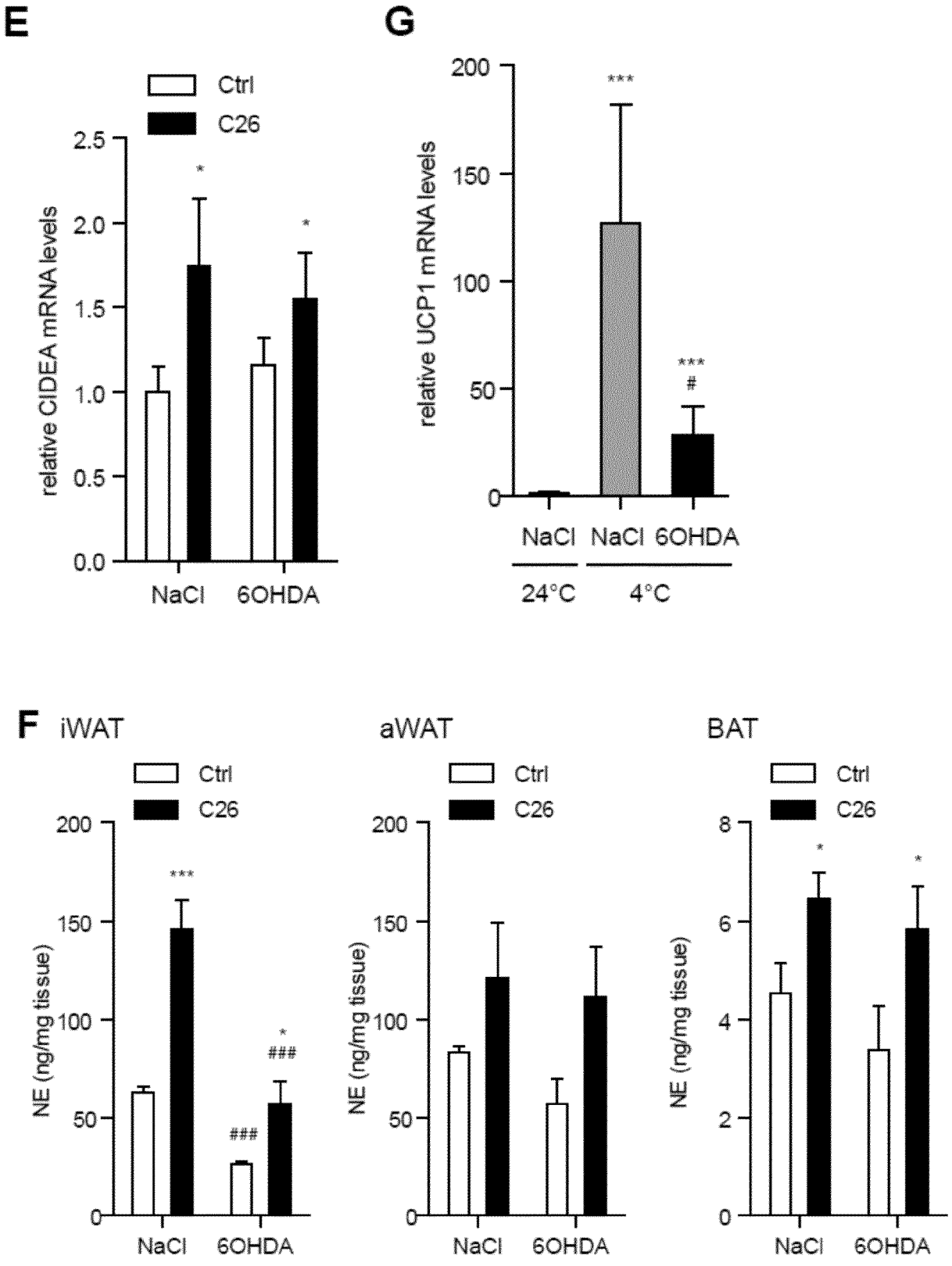

E
Ctrl
C26
relative CIDEA mRNA levels
2.5
2.0
1.5
1.0
0.5
0.0
*
*
NaCl
6OHDA
G
relative UCP1 mRNA levels
200
150
100
50
0
***
***
#
NaCl
NaCl
6OHDA
24°C
4°C
F
iWAT
aWAT
BAT
Ctrl
C26
NE (ng/mg tissue)
200
150
100
50
0
***
*
###
###
8
6
4
2
NaCl
6OHDA

Fig. 4 E) - G)

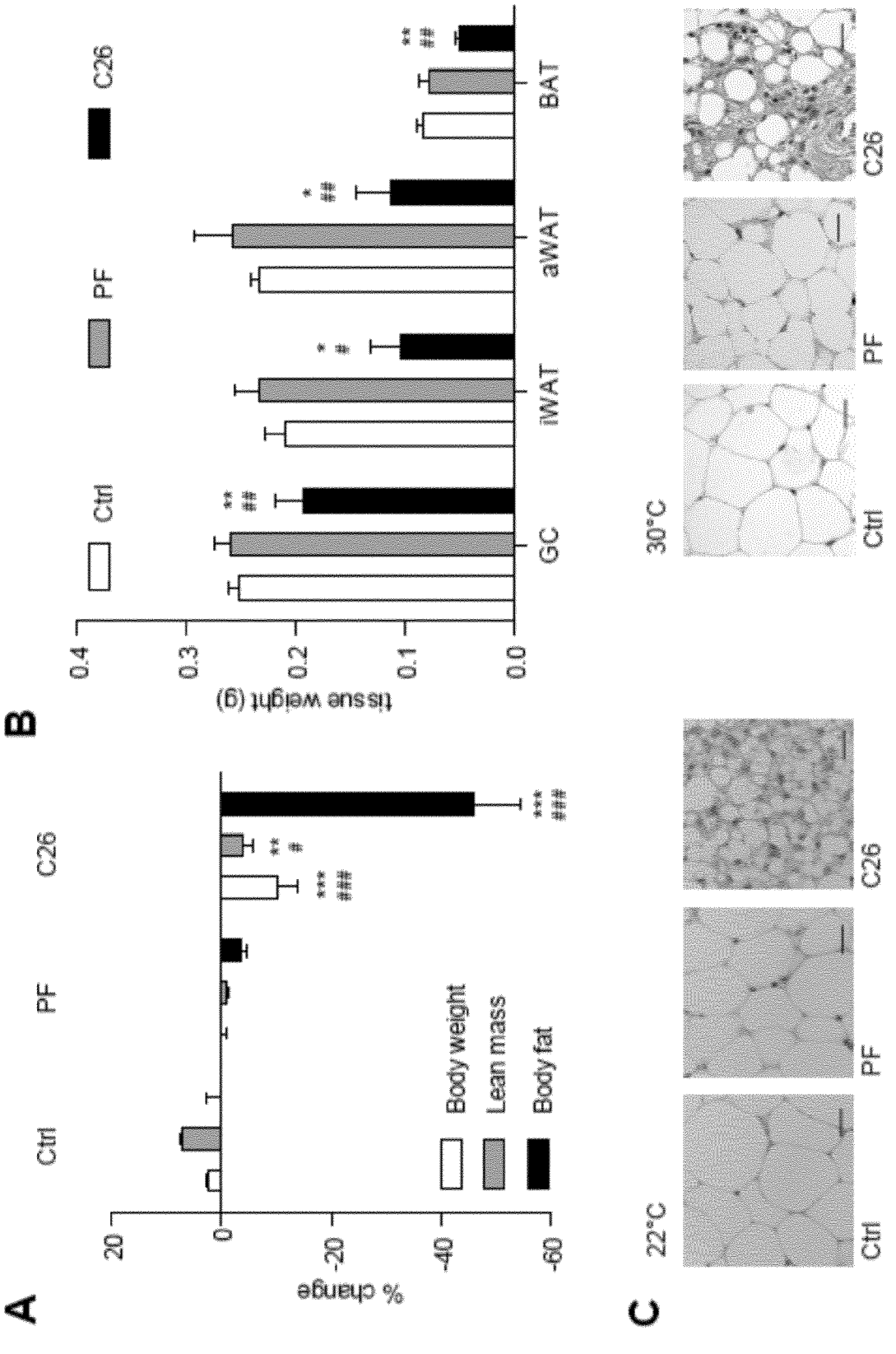

A
Ctrl
PF
C26
% change
20
0
-20
-40
-60
Body weight
Lean mass
Body fat
***
###
**
#
***
###
B
Ctrl
PF
C26
tissue weight (g)
0.4
0.3
0.2
0.1
0.0
GC
iWAT
aWAT
BAT
**
##
*
#
*
##
**
##
C
22°C
30°C
Ctrl
PF
C26

Fig. 5 A) - C)

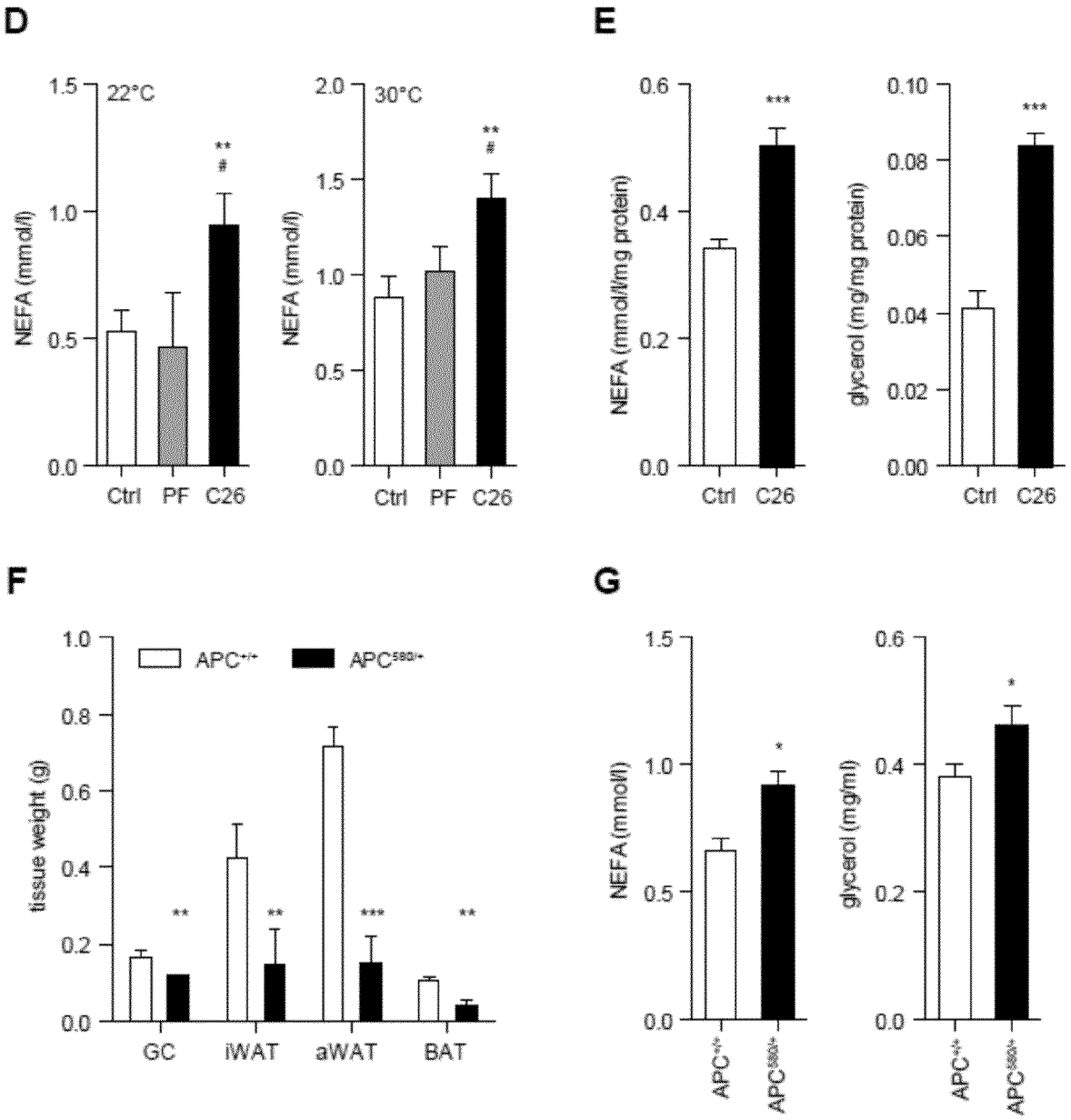

D
22°C
30°C
NEFA (mmol/l)
Ctrl
PF
C26
E
NEFA (mmol/mg protein)
glycerol (mg/mg protein)
F
APC+/+
APC580/+
tissue weight (g)
GC
iWAT
aWAT
BAT
G
NEFA (mmol/l)
glycerol (mg/ml)

Fig. 5 D) - G)

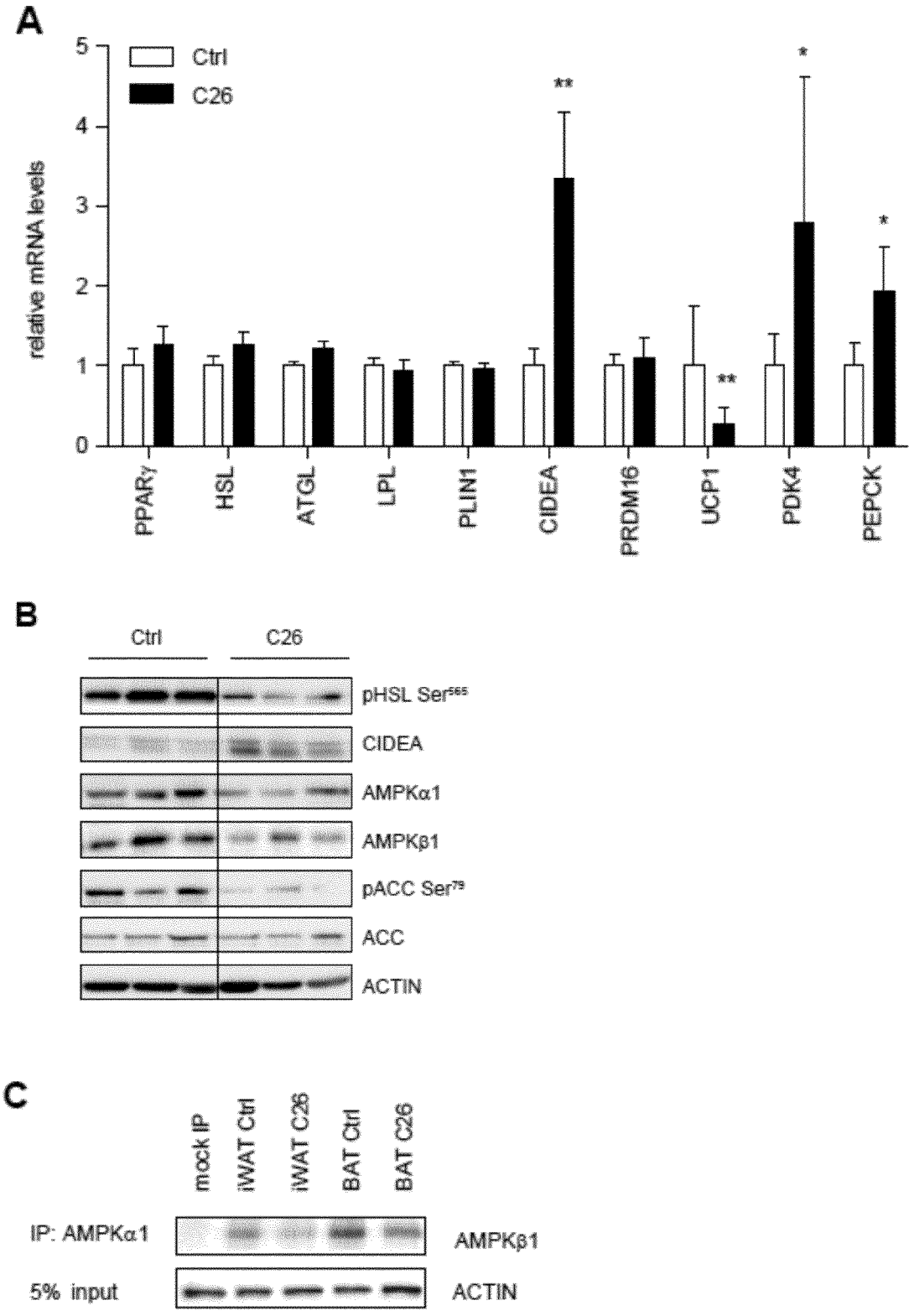

A
Ctrl
C26
relative mRNA levels
0
1
2
3
4
5
**
*
**
*
PPARγ
HSL
ATGL
LPL
PLIN1
CIDEA
PRDM16
UCP1
PDK4
PEPCK
B
Ctrl
C26
pHSL Ser565
CIDEA
AMPKα1
AMPKβ1
pACC Ser79
ACC
ACTIN
C
mock IP
iWAT Ctrl
iWAT C26
BAT Ctrl
BAT C26
IP: AMPKα1
AMPKβ1
5% input
ACTIN

Fig. 6 A) - C)

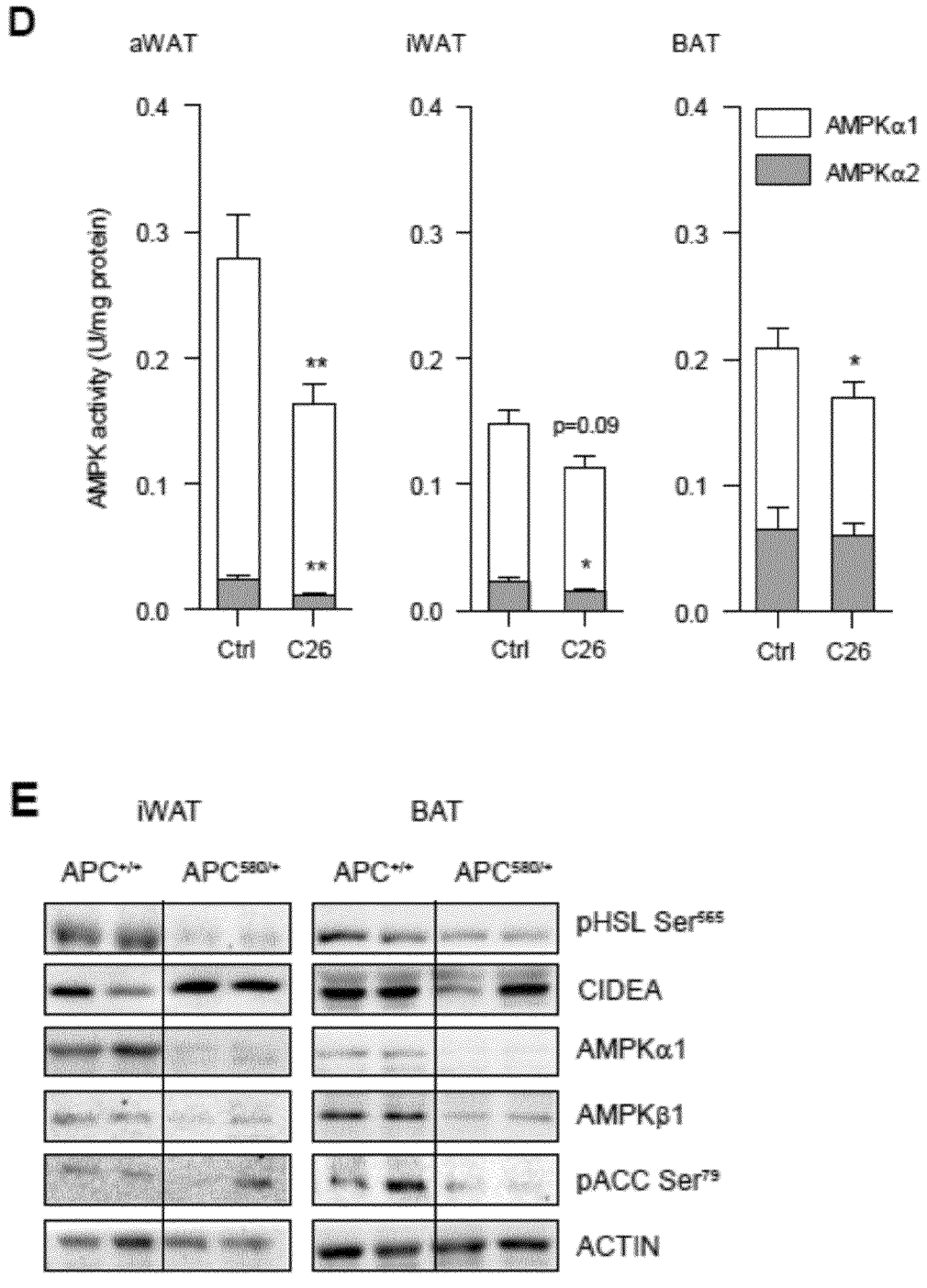

D
aWAT
iWAT
BAT
AMPK activity (U/mg protein)
0.4
0.3
0.2
0.1
0.0
AMPKα1
AMPKα2
**
**
p=0.09
*
*
Ctrl
C26
E
iWAT
BAT
APC+/+
APC580/+
APC+/+
APC580/+
pHSL Ser565
CIDEA
AMPKα1
AMPKβ1
pACC Ser79
ACTIN

Fig. 6 D) - E)

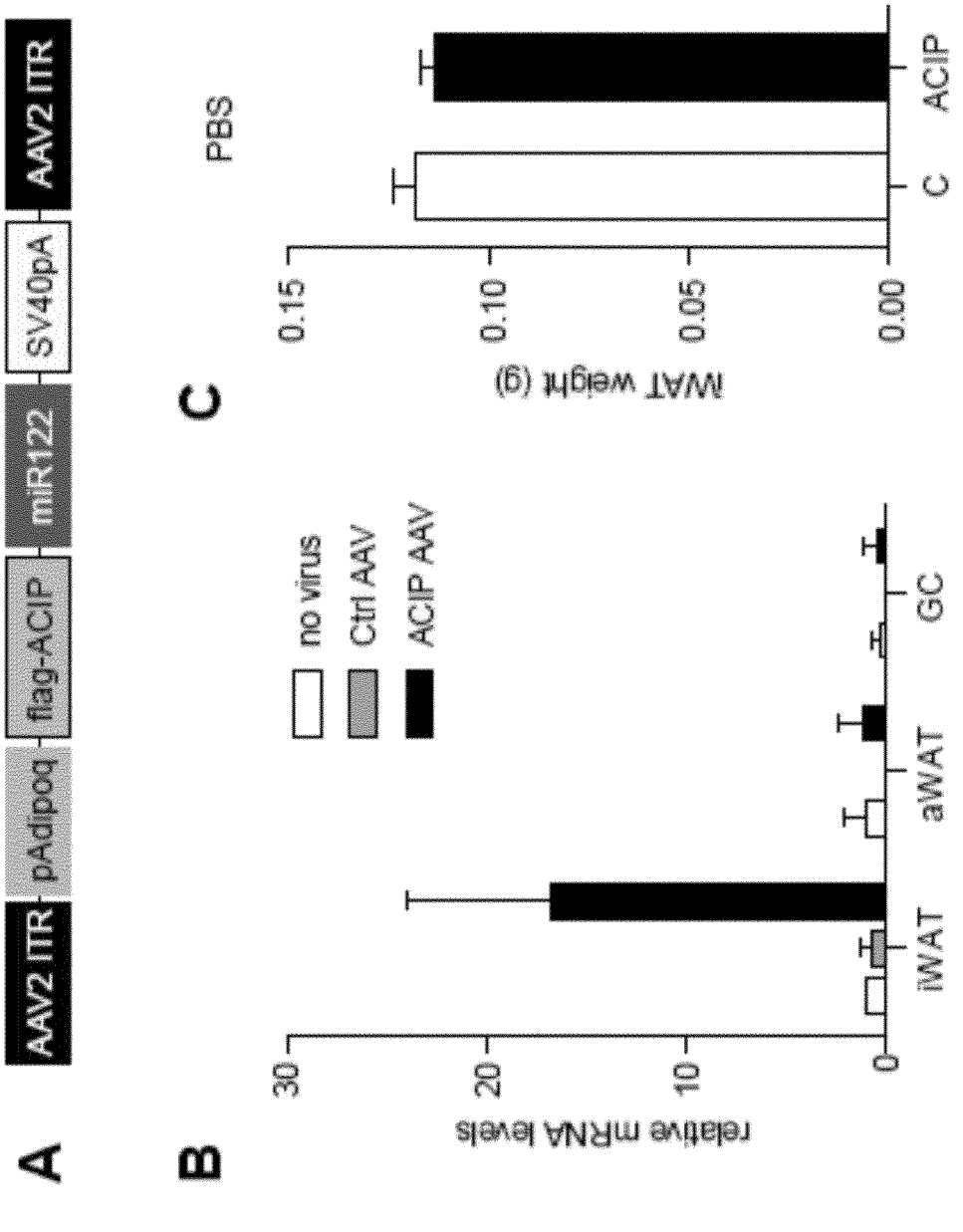

A
AAV2 ITR
pAdipoq
flag-ACIP
miR122
SV40pA
AAV2 ITR
B
relative mRNA levels
30
20
10
0
no virus
Ctrl AAV
ACIP AAV
iWAT
aWAT
GC
C
PBS
C26
iWAT weight (g)
0.15
0.10
0.05
0.00
C
ACIP
*

Fig. 7 A) - C)

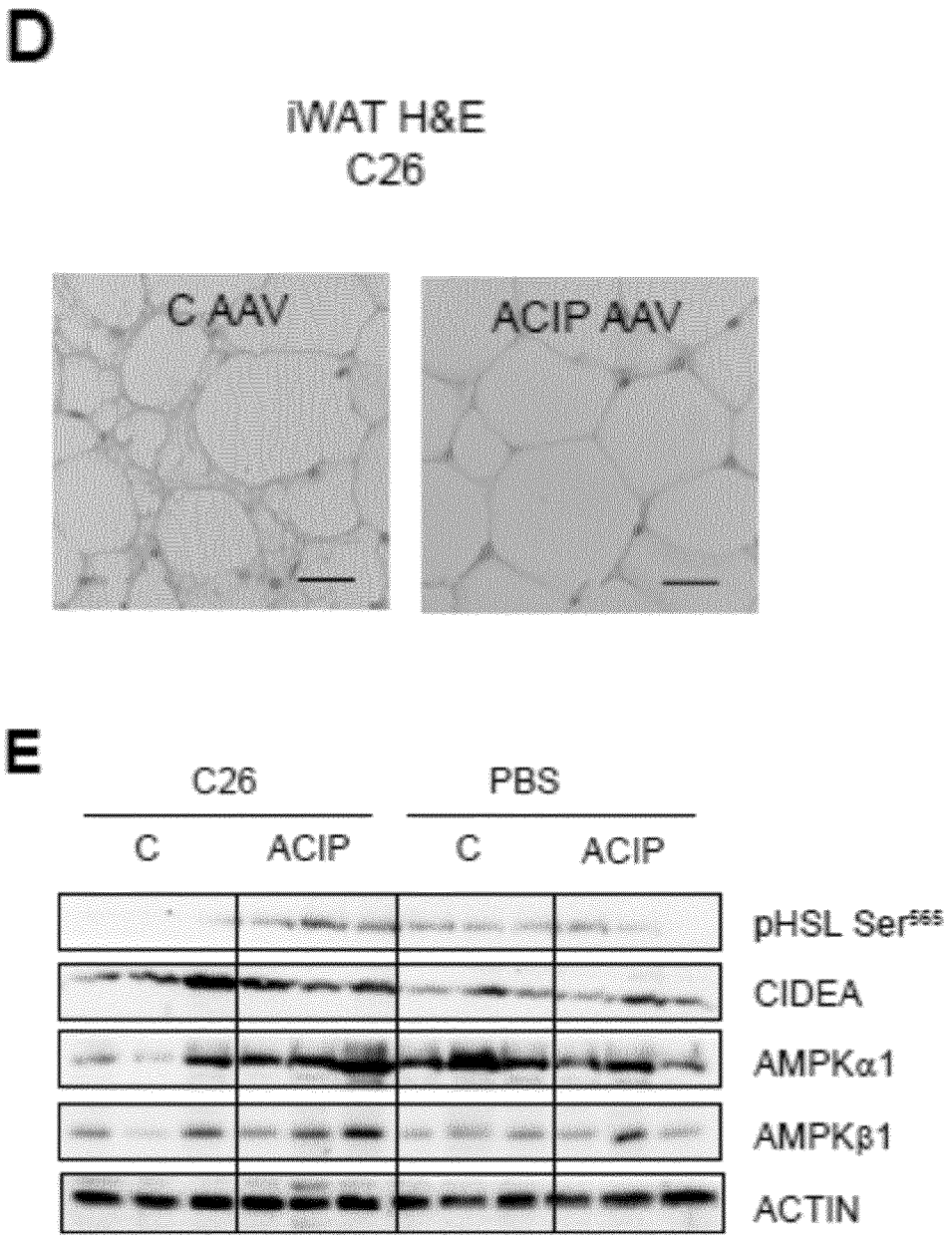

D
iWAT H&E
C26
C AAV
ACIP AAV
E
C26
PBS
C
ACIP
C
ACIP
pHSL Ser565
CIDEA
AMPKα1
AMPKβ1
ACTIN

Fig. 7 D), E)

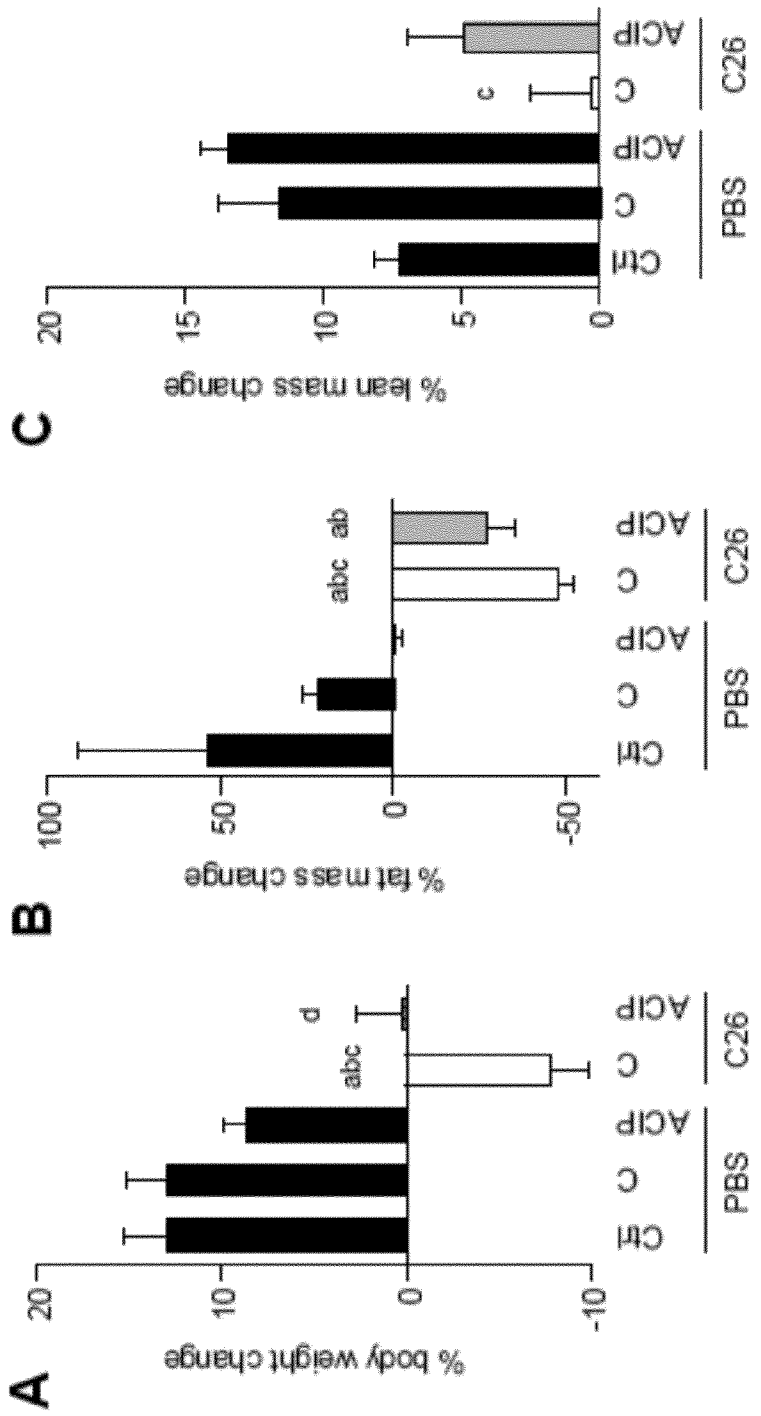

A
% body weight change
20
10
0
-10
Ctl
C
ACIP
PBS
abc
d
C
ACIP
C26
B
% fat mass change
100
50
0
-50
Ctl
C
ACIP
PBS
abc
ab
C
ACIP
C26
C
% lean mass change
20
15
10
5
0
Ctl
C
ACIP
PBS
c
C
ACIP
C26

Fig. 8 A) - C)

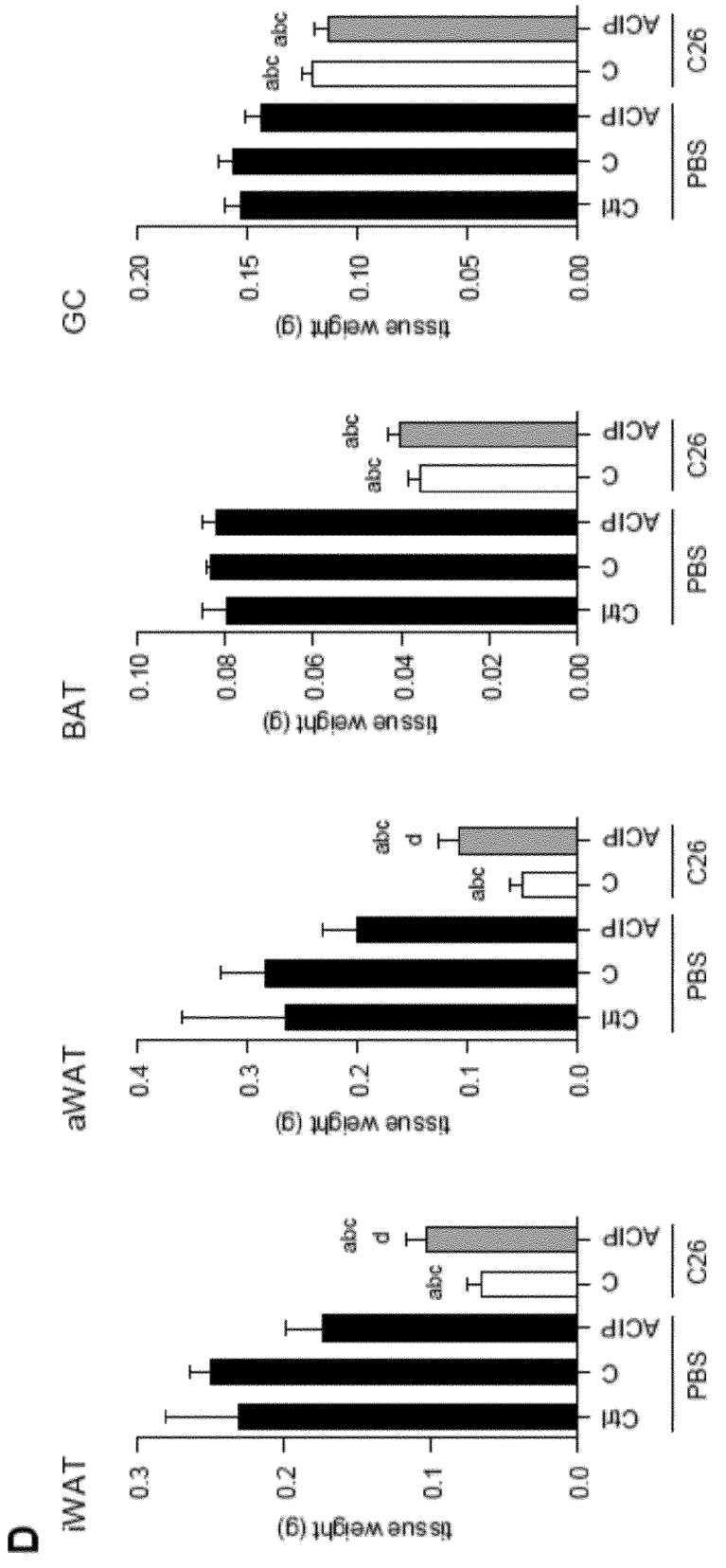


Fig. 8D)

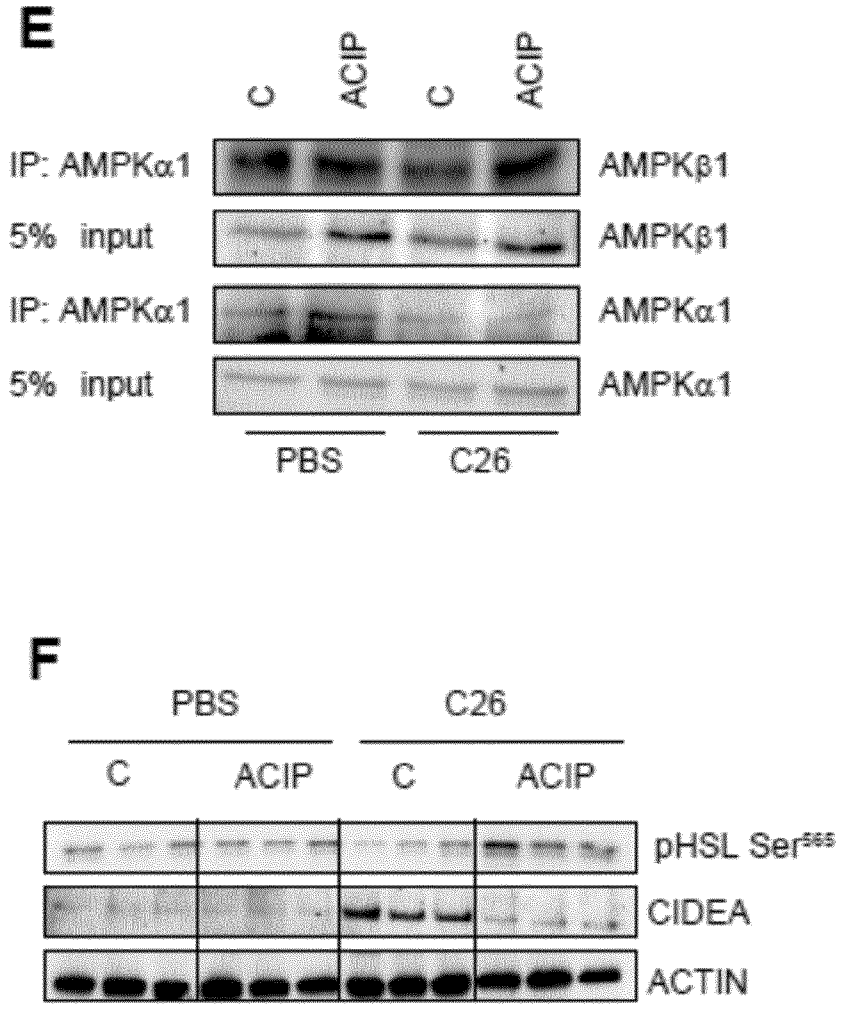

E
C
ACIP
C
ACIP
IP: AMPKα1
AMPKβ1
5% input
AMPKβ1
IP: AMPKα1
AMPKα1
5% input
AMPKα1
PBS
C26
F
PBS
C26
C
ACIP
C
ACIP
pHSL Ser565
CIDEA
ACTIN

Fig. 8 E), F)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/059344

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/713 C12Q1/48
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 C12Q

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 , WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97/25341 Al (ST VINCENTS INST MED RES [AU] ; DARTMOUTH COLLEGE [US] ; KEMP BRUCE E [A) 17 July 1997 (1997-07-17) whol e document esp. pages 4-5 and seq i d no 65	1-11
X	WO 2005/065667 A2 (HARVARD COLLEGE [US] ; GEN HOSPITAL CORP [US] ; SINCLAI R DAVID A [US] ; A) 21 July 2005 (2005-07-21)	1-9 , 11 , 15
Y	whol e document esp. pages 64-65	1-15
X	W0 2004/050898 A2 (ELIXI R PHARMACEUTICALS INC [US] ; APFELD JAVI ER [US] ; O' CONNOR GREG [US) 17 June 2004 (2004-06-17) whol e document esp seq i d no 14	1-15

-/- ·

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" documentwhich may throw doubts on priority claim(s) orwhich is cited to establish the publication date of another citation or other special reason (as specified)

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

"P" document published prior to the international filing date but later than the priority date claimed

"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

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"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	Date of mailing of the international search report
10 July 2014	19/08/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bruck, Mari anne

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/033562 A2 (XCOVERY INC [US] ; LIANG CONGXIN [US] ; GAO SHU [CN] ; LI ZHIGANG [CN]) 20 March 2008 (2008-03-20) whole document esp. claims 12 , 19-20	1 , 2
Y	WO 2006/004360 A1 (MD BIOALPHA CO LTD [KR] ; HA JOOHUN [KR] ; PARK MYUNGGYU [KR]) 12 January 2006 (2006-01-12) whole document esp. paragraphs [16, 18-20]	1-15
X	WO 2013/042137 A1 (AURIGENE DISCOVERY TECHNOLOGIES LTD [IN] ; ANIMA BORUAH [IN] ; HOSAHALLI) 28 March 2013 (2013-03-28) whole document esp pages 11	1 , 2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/059344

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9725341 A1	17-07-1997	AU 714905 B2	13-01-2000
		AU 1693697 A	01-08-1997
		CA 2241786 A1	17-07-1997
		DE 69734817 T2	29-06-2006
		EP 0873354 A1	28-10-1998
		ES 2251015 T3	16-04-2006
		JP 4448201 B2	07-04-2010
		JP 2000503202 A	21-03-2000
		WO 9725341 A1	17-07-1997
WO 2005065667 A2	21-07-2005	AU 2004312072 A1	21-07-2005
		CA 2548671 A1	21-07-2005
		CN 101247793 A	20-08-2008
		EP 1708689 A2	11-10-2006
		JP 2007527418 A	27-09-2007
		US 2005171027 A1	04-08-2005
		US 2010316679 A1	16-12-2010
		WO 2005065667 A2	21-07-2005
WO 2004050898 A2	17-06-2004	AT 423218 T	15-03-2009
		AU 2003300818 A1	23-06-2004
		CA 2508236 A1	17-06-2004
		EP 1576180 A2	21-09-2005
		WO 2004050898 A2	17-06-2004
WO 2008033562 A2	20-03-2008	AU 2007294686 A1	20-03-2008
		CA 2662902 A1	20-03-2008
		CN 101553482 A	07-10-2009
		EP 2079727 A2	22-07-2009
		JP 2010503686 A	04-02-2010
		KR 20090058563 A	09-06-2009
		US 2009076005 A1	19-03-2009
		US 2010261665 A1	14-10-2010
		US 2011224212 A1	15-09-2011
		US 2012115866 A1	10-05-2012
		WO 2008033562 A2	20-03-2008
WO 2006004360 A1	12-01-2006	EP 1765393 A1	28-03-2007
		KR 20060066610 A	16-06-2006
		WO 2006004360 A1	12-01-2006
WO 2013042137 A1	28-03-2013	NONE	