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(57) Abstract: The present disclosure provides a method of detecting a single nucleotide polymorphism (SNP) associated with metabolic syndrome in an individual; and a method of detecting a predisposition of an individual to develop metabolic syndrome. The present disclosure further provides nucleic acid reagents and kits for determining a subject's genotype with respect to a single nucleotide polymorphism at rs11246020 in a sirtuin-3 (SIRT3) gene.



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COMPOSITIONS AND METHODS FOR DETERMINING PREDISPOSITION TO DEVELOPING METABOLIC SYNDROME

CROSS-REFERENCE

- [0001]** This application claims the benefit of U.S. Provisional Patent Application No. 61/375,585, filed August 20, 2010, which application is incorporated herein by reference in its entirety.

BACKGROUND

- [0002]** "Metabolic Syndrome," also called "Syndrome X," the "Insulin Resistance Syndrome," or the "Deadly Quartet," is characterized by an accumulation of risk factors for cardiovascular disease, stroke and/or diabetes mellitus type II. The risk factors that characterize Metabolic Syndrome include an increased amount of adipose tissue inside the abdominal cavity (abdominal obesity), insulin resistance with increased risk of developing diabetes, hyperinsulinemia, high levels of blood fats, increased blood pressure, and elevated serum lipids.

SUMMARY OF THE INVENTION

- [0003]** The present disclosure provides a method of detecting a single nucleotide polymorphism (SNP) associated with metabolic syndrome in an individual; and a method of detecting a predisposition of an individual to develop metabolic syndrome. The present disclosure further provides nucleic acid reagents and kits for determining a subject's genotype with respect to a single nucleotide polymorphism at rs11246020 in a sirtuin-3 (SIRT3) gene.

BRIEF DESCRIPTION OF THE DRAWINGS

- [0004]** Figures 1A-C depict the effect of a chronic high-fat diet feeding on global mitochondrial hyperacetylation and hepatic SIRT3.
- [0005]** Figures 2A-G depict diet-induced obesity and insulin resistance in mice lacking SIRT3.

- [0006] Figures 3A-E depict development of hepatic steatosis and inflammation in SIRT3KO mice fed a high-fat diet.
- [0007] Figures 4A-C depict high expression and activity of hepatic SCD1 in SIRT3KO mice.
- [0008] Figures 5A-D depict association of an SNP in the human SIRT3 gene with human metabolic syndrome.
- [0009] Figures 6A-E depict the effect of the rs11246020 SNP on SIRT3 enzymatic activity.
- [0010] Figure 7 depicts an amino acid sequence of human SIRT3. Val-208 is in bold and underlined.
- [0011] Figure 8 depicts genomic and cDNA nucleotide sequences in a human SIRT3 coding region. The codon encoding Val-208 is in bold and underlined.
- [0012] Figures 9A-L depict a SIRT3 human genomic nucleotide sequence.
- [0013] Figures 10A and 10B depict a human SIRT3 cDNA sequence.

DEFINITIONS

- [0014] The term "metabolic syndrome" is a term that is understood in the art, and refers to metabolic abnormalities, including central obesity, insulin resistance, hyperlipidemia, hyperglycemia, hypertension, and hepatic steatosis. The International Diabetes Foundation definition of metabolic syndrome is central obesity (body mass index $> 30 \text{ kg/m}^2$) and two or more of: 1) triglycerides $> 150 \text{ mg/dL}$; 2) high density lipoprotein (HDL) $< 40 \text{ mg/dL}$ in males, $< 50 \text{ mg/dL}$ in females, or specific treatment for low HDL; 3) elevated blood pressure, e.g., systolic BP $> 130 \text{ mm Hg}$ or diastolic BP $> 85 \text{ mm Hg}$, or treatment for elevated BP, or previous diagnosis of elevated BP; and 4) fasting blood glucose $> 100 \text{ mg/dL}$ or previous diagnosis of type 2 diabetes.
- [0015] A "nucleic acid," "polynucleotide," or "oligonucleotide" is a polymeric form of nucleotides of any length, may be DNA or RNA, and may be single- or double-stranded. Nucleic acids may include promoters or other regulatory sequences. Oligonucleotides are usually prepared by synthetic means. Nucleic acids include segments of DNA, or their complements spanning or flanking a polymorphic site in a SIRT3 gene, as described herein. The segments can be between 5 and 100 contiguous bases, and often range from a lower limit of 5, 10, 12, 15, 20, or 25 nucleotides to an upper limit of 10,

15, 20, 25, 30, 50 or 100 nucleotides (where the upper limit is greater than the lower limit). Nucleic acids between 5-10, 5-20, 10-20, 12-30, 15-30, 10-50, 20-50 or 20-100 bases are common. The polymorphic site can occur within any position of the segment. A reference to the sequence of one strand of a double-stranded nucleic acid defines the complementary sequence and except where otherwise clear from context, a reference to one strand of a nucleic acid also refers to its complement. For certain applications, nucleic acid (e.g., RNA) molecules may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the backbone of the molecule. Modified nucleic acids include peptide nucleic acids (PNAs) and nucleic acids with nontraditional bases such as inosine, queosine and wybutosine and acetyl-, methyl-, thio- and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases.

[0016] "Hybridization probes" are nucleic acids capable of binding in a base-specific manner to a complementary strand of nucleic acid. Such probes include nucleic acids and peptide nucleic acids. Hybridization may be performed under stringent conditions which are known in the art. For example, see, e.g., Berger and Kimmel (1987) *Methods In Enzymology*, Vol. 152: *Guide To Molecular Cloning Techniques*, San Diego: Academic Press, Inc.; Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Vols. 1-3, Cold Spring Harbor Laboratory; Sambook (2001) 3rd Edition; Rychlik, W. and Rhoads, R. E., 1989, *Nucl. Acids Res.* 17, 8543; Mueller, P. R. et al. (1993) In: *Current Protocols in Molecular Biology* 15.5, Greene Publishing Associates, Inc. and John Wiley and Sons, New York; and Anderson and Young, *Quantitative Filter Hybridization in Nucleic Acid Hybridization* (1985)). As used herein, the term "probe" includes primers. Probes and primers are sometimes referred to as "oligonucleotides."

[0017] The term "primer" refers to a single-stranded oligonucleotide capable of acting as a point of initiation of template-directed DNA synthesis under appropriate conditions, in an appropriate buffer and at a suitable temperature. The appropriate length of a primer depends on the intended use of the primer but typically ranges from 15 to 30 nucleotides. A primer sequence need not be exactly complementary to a template but must be sufficiently complementary to hybridize with a template. The term "primer site" refers to the area of the target DNA to which a primer hybridizes. The term "primer pair" means a

set of primers including a 5' upstream primer, which hybridizes to the 5' end of the DNA sequence to be amplified and a 3' downstream primer, which hybridizes to the complement of the 3' end of the sequence to be amplified.

[0018] Exemplary hybridization conditions for short probes and primers is about 5 to 12 degrees C below the calculated T_m . Formulas for calculating T_m are known and include: $T_m = 4^\circ \text{C} \times (\text{number of G's and C's in the primer}) + 2^\circ \text{C} \times (\text{number of A's and T's in the primer})$ for oligonucleotides ("oligos") <14 bases and assumes a reaction is carried out in the presence of 50 mM monovalent cations. For longer oligos, the following formula can be used: $T_m = 64.9^\circ \text{C} + 41^\circ \text{C} \times (\text{number of G's and C's in the primer} - 16.4)/N$, where N is the length of the primer. Another commonly used formula takes into account the salt concentration of the reaction (Rychlik, supra, Sambrook, supra, Mueller, supra.): $T_m = 81.5^\circ \text{C} + 16.6^\circ \text{C} \times (\log_{10}[\text{Na}^+] + [\text{K}^+]) + 0.41^\circ \text{C} \times (\% \text{ GC}) - 675/N$, where N is the number of nucleotides in the oligonucleotide. The aforementioned formulae provide a starting point for certain applications; however, the design of particular probes and primers may take into account additional or different factors. Methods for design of probes and primers for use in the methods of the invention are well known in the art.

[0019] As used herein, the terms "allele" and "allelic variant" refer to alternative forms of a gene including introns, exons, intron/exon junctions and 3' and/or 5' untranslated regions that are associated with a gene or portions thereof. Generally, alleles occupy the same locus or position on homologous chromosomes. When a subject has two identical alleles of a gene, the subject is said to be homozygous for the gene or allele. When a subject has two different alleles of a gene, the subject is said to be heterozygous for the gene.

[0020] As used herein, the term "isolated" as used herein with respect to a nucleic acid, refers to a nucleic acid separated from macromolecules or other contaminants that may be present in the natural source of the nucleic acid, or that may be present during recombinant or chemical synthesis of the nucleic acid. An isolated nucleic acid can be purified, e.g., at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or greater than 99%, pure.

[0021] Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of

describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0022] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0023] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0024] It must be noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “an oligonucleotide probe” includes a plurality of such oligonucleotide probes and reference to “the primer” includes reference to one or more primer and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as “solely,” “only” and the like in connection with the recitation of claim elements, or use of a “negative” limitation.

[0025] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DETAILED DESCRIPTION

- [0026] The present disclosure provides a method of detecting a single nucleotide polymorphism (SNP) associated with metabolic syndrome in an individual; and a method of detecting a predisposition of an individual to develop metabolic syndrome. The present disclosure further provides nucleic acid reagents and kits for determining a subject's genotype with respect to a single nucleotide polymorphism at rs11246020 in a sirtuin-3 (SIRT3) gene.
- [0027] A polymorphic site, rs11246020, has been identified in a human SIRT3 gene, in which a polymorphism is associated with a propensity to develop metabolic syndrome. In particular, a codon encoding Val-208 of human SIRT3, when mutated such that the codon encodes Ile, is associated with a propensity to develop metabolic syndrome. The codon encoding Val-208 of SIRT3 corresponds to nucleotides 3365-3367 of the genomic sequence and nucleotides 656-658 of the mRNA sequence, as depicted in Figure 8. The genomic sequence depicted in Figure 8 (and in Figures 9A-L) is from GenBank Accession No. NC_000011.9; the cDNA sequence depicted in Figure 8 (and in Figure 10) is from GenBank Accession No. NM_012239. The codon encoding Val-208 of SIRT3 is present within the exon encoded by nucleotides 3217-3449 of the SIRT3 genomic sequence depicted in Figures 9A-L and presented in GenBank Accession No. NC_000011.9.
- [0028] Where the polymorphic site rs11246020 comprises a SNP that is predictive of increased risk of developing metabolic syndrome, a G to A mutation occurs in the first nucleotide of the codon encoding Val-208 of human SIRT3. For example, the gtc codon underlined and in bold in Figure 8, which encodes Val-208, is mutated to atc, which encodes isoleucine. In other words, where nucleotide 3365 of the human genomic SIRT3 gene depicted in Figures 9A-L or depicted in Figure 8 is mutated from G to A in the genome of an individual, the individual has an increased risk of developing metabolic syndrome. Said another way, where nucleotide 3365 of the human genomic SIRT3 gene depicted in Figures 9A-L or depicted in Figure 8 is mutated from G to A in the genome of an individual, the individual has a propensity to develop metabolic syndrome.
- [0029] In some embodiments, the genome of an individual comprises a SIRT3 allele encoding a SIRT3 polypeptide having Val-208. In some embodiments, the genome of an

individual comprises a SIRT3 allele encoding a SIRT3 polypeptide having Ile-208. In some embodiments, the individual is homozygous for a SNP that is indicative of an increased propensity to develop metabolic syndrome. In some embodiments, the individual is heterozygous for a SNP that is indicative of an increased propensity to develop metabolic syndrome.

[0030] The presence of an SNP that is associated with a propensity in an individual to develop metabolic syndrome indicates that the individual has an at least about 25%, at least about 50%, at least about 2-fold, at least about 5-fold, or at least about 10-fold, increased risk of developing metabolic syndrome, compared to the risk that an individual who does not have the SNP will develop metabolic syndrome.

DETECTION OF SIRT3 POLYMORPHISMS ASSOCIATED WITH METABOLIC SYNDROME

[0031] The present disclosure provides a method of detecting a single nucleotide polymorphism (SNP) associated with metabolic syndrome in an individual. In some cases, the method involves analyzing a polynucleotide sample from the individual for the presence of a SNP at rs11246020 in a sirtuin-3 (SIRT3) gene, where the SNP is associated with metabolic syndrome, and where the presence of the polymorphism is indicative of a polymorphism associated with metabolic syndrome. In other cases, the method involves analyzing a sample from the individual for the presence of a SIRT3 polypeptide that has a V208I substitution; the presence of the V208I substitution is indicative of a polymorphism associated with metabolic syndrome. In other instances, the method involves analyzing a sample from the individual for enzymatic activity of a SIRT3 polypeptide in the sample; reduced SIRT3 enzymatic activity that is associated with a V208I substitution is indicative of a polymorphism associated with metabolic syndrome.

Detecting a SNP in a SIRT3 gene

[0032] A SNP in a SIRT3 gene can be detected by one or more of the following techniques: (a) restriction fragment length analysis; (b) sequencing; (c) a micro-sequencing assay; (d) hybridization; (e) an invader assay; (f) a gene chip hybridization assay; (g) an oligonucleotide ligation assay; (h) ligation rolling circle amplification; (i) a 5' nuclease assay; (j) a polymerase proofreading method; (k) an allele specific polymerase chain reaction; (l) matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectroscopy; (m) a ligase chain reaction assay; (n) enzyme-

amplified electronic transduction; (o) a single base pair extension assay; and (p) reading sequence data. Such methods are well known to those skilled in the art.

[0033] In some cases, the SNP is detected by hybridizing nucleic acid from an individual with a nucleic acid probe that includes the SNP. A nucleic acid probe that includes the SNP is also referred to as an allele-specific probe.

[0034] Polymorphisms are detected in a target nucleic acid isolated from an individual being assessed. Typically genomic DNA is analyzed. For assay of genomic DNA, virtually any biological sample containing genomic DNA or RNA, e.g., nucleated cells, is suitable. For example, genomic DNA can be obtained from peripheral blood leukocytes collected from a patient. Other suitable samples include saliva, cheek scrapings, organ biopsy samples, whole blood, buccal samples, tissue biopsy samples, and the like. Alternatively RNA or cDNA can be assayed. Methods for purification or partial purification of nucleic acids from patient samples for use in diagnostic or other assays are well known

[0035] Detection of a SNP that indicates a predisposition to developing metabolic syndrome can be carried out using any of a variety of well-known methods. Suitable methods include, but are not limited to, use of allele-specific probes; use of allele-specific primers; direct sequence analysis; denaturing gradient gel electrophoresis (DGGE) analysis; single-strand conformation polymorphism (SSCP) analysis; and denaturing high performance liquid chromatography (DHPLC) analysis. Other well known methods to detect polymorphisms in DNA include use of: Molecular Beacons technology (see, e.g., Piatek et al., 1998, Nat. Biotechnol. 16:359-63; Tyagi, and Kramer, 1996, Nat. Biotechnology 14:303-308; and Tyagi, et al., 1998, Nat. Biotechnol. 16:49-53), Invader technology (see, e.g., Neri et al., 2000, Advances in Nucleic Acid and Protein Analysis 3826:117-125 and U.S. Pat. No. 6,706,471), nucleic acid sequence based amplification (Nasba) (Compton, 1991), Scorpion technology (Thelwell et al., 2000, Nuc. Acids Res, 28:3752-3761 and Solinas et al., 2001, "Duplex Scorpion primers in SNP analysis and FRET applications" Nuc. Acids Res, 29:20.), restriction fragment length polymorphism (RFLP) analysis, and the like.

[0036] The design and use of allele-specific probes for analyzing polymorphisms are described by e.g., Saiki et al., 1986; Dattagupta, EP 235,726, Saiki, WO 89/11548. Briefly, allele-specific probes are designed to hybridize to a segment of target DNA

from one individual but not to the corresponding segment from another individual, if the two segments represent different polymorphic forms. Hybridization conditions are chosen that are sufficiently stringent so that a given probe essentially hybridizes to only one of two alleles. Typically, allele-specific probes are designed to hybridize to a segment of target DNA such that the polymorphic site aligns with a central position of the probe.

[0037] Allele-specific probes are often used in pairs, one member of a pair designed to hybridize to the reference allele of a target sequence and the other member designed to hybridize to the variant allele. Several pairs of probes can be immobilized on the same support for simultaneous analysis of multiple polymorphisms within the same target gene sequence.

[0038] Exemplary allele-specific probes for analyzing a SIRT3 SNP that is predictive of metabolic syndrome include:

[0039] Exemplary allele-specific probes comprising the “G” allele at nt 3365 of a human SIRT3 gene (nucleotide sequences corresponding to SEQ ID NO:4 given):

[0040] 1) 5'- aactacaagcccaac**gtc**actcactacttt -3' (SEQ ID NO:5; nt 3350-3379);

[0041] 2) 5'- caagcccaac**gtc**actcactacttttccg -3' (SEQ ID NO:6; nt 3355-3384);

[0042] 3) 5'- caagcccaac**gtc**actcactact -3' (SEQ ID NO:7; nt 3355-3377);

[0043] 4) 5'- tacaagcccaac**gtc**actcactactttctc -3' (SEQ ID NO:8; nt 3364-3382);

[0044] 5) 5'- taccctggaaactacaagcccaac**gtc**actcactacttttctccggctgct -3' (SEQ ID NO:9; nt 3341-3390); and

[0045] 6) 5'- actacaagcccaac**gtc**actcactactttc -3' (SEQ ID NO:10; nt 3351-3380).

[0046] Exemplary allele-specific probes comprising the minor “A” allele at nt 3365 of a human SIRT3 gene (nucleotide sequences corresponding to SEQ ID NO:4 given):

[0047] 1) 5'- aactacaagcccaac**atc**actcactactttt -3' (SEQ ID NO:11; nt 3350-3379);

[0048] 2) 5'- caagcccaac**atc**actcactacttttccg -3' (SEQ ID NO:12; nt 3355-3384);

[0049] 3) 5'- caagcccaac**atc**actcactact -3' (SEQ ID NO:13; nt 3355-3377);

[0050] 4) 5'- tacaagcccaac**atc**actcactactttctc -3' (SEQ ID NO:14; nt 3364-3382);

[0051] 5) 5'- taccctggaaactacaagcccaac**atc**actcactacttttctccggctgct -3' (SEQ ID NO:15; nt 3341-3390); and

[0052] 6) 5'- actacaagcccaac**atc**actcactactttc -3' (SEQ ID NO:16; nt 3351-3380).

[0053] The design and use of allele-specific primers for analyzing polymorphisms are described by, e.g., WO 93/22456. Briefly, allele-specific primers are designed to hybridize to a site on target DNA overlapping a polymorphism and to prime DNA amplification according to standard PCR protocols only when the primer exhibits perfect complementarity to the particular allelic form. A single-base mismatch prevents DNA amplification and no detectable PCR product is formed. The method works best when the polymorphic site is at the extreme 3'-end of the primer, because this position is most destabilizing to elongation from the primer.

[0054] Suitable allele-specific primers for analyzing a metabolic syndrome-associated SNP in human SIRT3 include:

[0055] 5'- taccctggaaactacaagcccaac~~at~~cact-3' (SEQ ID NO:17);

[0056] 5'- accctggaaactacaagcccaac~~at~~cac-3' (SEQ ID NO:18); and

[0057] 5'- taccctggaaactacaagcccaac~~at~~c-3' (SEQ ID NO:19);

[0058] where corresponding control primers for the "G" allele include:

[0059] 5'- taccctggaaactacaagcccaac~~gt~~cact-3' (SEQ ID NO:20);

[0060] 5'- accctggaaactacaagcccaac~~gt~~cac-3' (SEQ ID NO:21); and

[0061] 5'- taccctggaaactacaagcccaac~~gt~~c-3' (SEQ ID NO:22).

[0062] These primers are used in standard PCR protocols in conjunction with another common primer that hybridizes to the complementary strand of the SIRT3 gene at a specified location from the polymorphism. The common primers are chosen such that the resulting PCR products can vary from about 100 to about 300 bases in length, or about 150 to about 250 bases in length, although smaller (about 50 to about 100 bases in length) or larger (about 300 to about 500 bases in length) PCR products are possible. The length of the primers can vary from about 10 to 30 bases in length, or about 15 to 25 bases in length. The sequences of the common primers can be determined by inspection of the human SIRT3 genomic sequence, which is found under GenBank accession number NC_000011.9 and depicted in Figures 9A-L.

[0063] Suitable methods for detecting polymorphisms include those that involve amplifying DNA or RNA from target samples (e.g., amplifying the segments of the SIRT3 gene of an individual using SIRT3-specific primers) and analyzing the amplified gene. This can be accomplished by standard polymerase chain reaction (PCR and RT-PCR) protocols or other methods known in the art. The amplifying may result in the

generation of SIRT3 allele-specific oligonucleotides, which span the single nucleotide polymorphic sites in the SIRT3 gene. The SIRT3-specific primer sequences and SIRT3 allele-specific oligonucleotides may be derived from the coding (exons) or non-coding (promoter, 5' untranslated, introns or 3' untranslated) regions of the SIRT3 gene.

[0064] Exemplary, non-limiting, primer pairs that amplify a region including the rs11246020 polymorphic site include (where the nucleotides correspond to the numbering depicted in Figure 10A):

Forward primer	Reverse primer
5'-gtaccctggaaactacaagc-3' (nt 631-650) (SEQ ID NO:23)	5'-gcagccggagaaagtagtga-3' (reverse complement of nt 661-680) (SEQ ID NO:24)
5'-ccaaggagctgtaccctgga-3' (nt 621-640) (SEQ ID NO:25)	5'-ttgtcatgaagcagccggag-3' (reverse complement of nt 671-690) (SEQ ID NO:26)
5'-ttcactttggccaaggagct-3' (nt 611-630) (SEQ ID NO:27)	5'-aagcagccccttgatcatgaa-3' (reverse complement of nt 681-700) (SEQ ID NO:28)

[0065] Amplification products generated using PCR can be analyzed by the use of denaturing gradient gel electrophoresis (DGGE). Different alleles can be identified based on sequence-dependent melting properties and electrophoretic migration in solution. See Erlich, ed., PCR Technology, Principles and Applications for DNA Amplification, Chapter 7 (W.H. Freeman and Co, New York, 1992).

[0066] Alleles of target sequences can be differentiated using single-strand conformation polymorphism (SSCP) analysis. Different alleles can be identified based on sequence- and structure-dependent electrophoretic migration of single stranded PCR products. Amplified PCR products can be generated according to standard protocols, and heated or otherwise denatured to form single stranded products, which may refold or form secondary structures that are partially dependent on base sequence.

[0067] Alleles of target sequences can be differentiated using denaturing high performance liquid chromatography (DHPLC) analysis. Different alleles can be identified based on base differences by alteration in chromatographic migration of single stranded PCR products. Amplified PCR products can be generated according to standard protocols, and heated or otherwise denatured to form single stranded products, which

may refold or form secondary structures that are partially dependent on the base sequence.

[0068] Direct sequence analysis of polymorphisms can be accomplished using DNA sequencing procedures that are well-known in the art. See Sambrook et al., *Molecular Cloning, A Laboratory Manual* (2nd Ed., Cold Spring Harbor Press, New York 1989) and Zyskind et al., *Recombinant DNA Laboratory Manual* (Acad. Press, 1988).

[0069] A wide variety of other methods are known in the art for detecting polymorphisms in a biological sample. See, e.g., Ullman et al. "Methods for single nucleotide polymorphism detection" U.S. Pat. No. 6,632,606; Shi, 2002, "Technologies for individual genotyping: detection of genetic polymorphisms in drug targets and disease genes" *Am J Pharmacogenomics* 2:197-205; Kwok et al., 2003, "Detection of single nucleotide polymorphisms" *Curr Issues Biol.* 5:43-60).

Detecting a metabolic syndrome-associated SIRT3 variant

[0070] In some cases, a SIRT3 variant with a V208I substitution is detected. A SIRT3 variant that has a V208I substitution is also referred to herein as "SIRT3-V208I." A SIRT3-V208I polypeptide can be detected using an antibody that can distinguish between a SIRT3-V208I polypeptide and a wild-type human SIRT3 polypeptide, e.g., a SIRT3 polypeptide that has a valine at position 208. The antibody is one that can distinguish between a first and a second SIRT3 polypeptide, where the first and the second SIRT3 polypeptides differ in amino acid sequence only at amino acid 208, where the first SIRT3 polypeptide has a valine at amino acid 208; and the second SIRT3 polypeptide has an isoleucine at amino acid 208. For example, the antibody is one that can distinguish between a first and a second SIRT3 polypeptide, where the first SIRT3 polypeptide comprises the amino acid sequence as depicted in Figure 7, with a valine at amino acid 208 ("SIRT3-V208"); and the second SIRT3 polypeptide comprises the amino acid sequence as depicted in Figure 7, but has an isoleucine instead of a valine at amino acid 208 ("SIRT3-I208"). A suitable antibody can distinguish between SIRT3-V208 and SIRT3-I208, e.g., a suitable antibody binds SIRT3-I208 with an affinity of at least about 10^{-7} M, at least about 5×10^{-7} M, at least about 10^{-8} M, at least about 5×10^{-8} M, at least about 10^{-9} M, or greater than 10^{-9} M; and binds SIRT3-V208 with an affinity of less than 10^{-7} M (e.g., binds SIRT3-V208 with an affinity of 10^{-6} M, 10^{-5} M, 10^{-4} M, or less).

- [0071] *In vitro* methods for detection of a SIRT3-V208I polypeptide include, but are not limited to, enzyme linked immunosorbent assays (ELISAs), radioimmunoassays (RIA), Western blots, immunoprecipitation, immunofluorescence, and protein arrays/chips (e.g., arrays of antibodies or aptamers). For further information regarding immunoassays and related protein detection methods, see Current Protocols in Immunology, John Wiley & Sons, N.Y., and Hage, "Immunoassays", Anal Chem. 1999 Jun. 15; 71(12):294R-304R.
- [0072] An "antibody specific for a SIRT3-V208I polypeptide" includes both monoclonal antibodies and polyclonal antibodies, as well as antigen-reactive proteolytic fragments of such antibodies, such as Fab, F(ab)₂, and Fv fragments. In addition, an "antibody specific for a SIRT3-V208I polypeptide" further includes any of a variety of engineered antigen-binding molecules such as a chimeric antibody (U.S. Pat. Nos. 4,816,567 and 4,816,397; Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851, 1984; Neuberger et al., Nature 312:604, 1984), a humanized antibody (U.S. Pat. Nos. 5,693,762; 5,585,089; and 5,565,332), a single-chain Fv (U.S. Pat. No. 4,946,778; Ward et al., Nature 334:544, 1989), a bispecific antibody with two binding specificities (Segal et al., J. Immunol. Methods 248:1, 2001; Carter, J. Immunol. Methods 248:7, 2001), a diabody, a triabody, and a tetrabody (Todorovska et al., J. Immunol. Methods, 248:47, 2001), as well as a Fab conjugate (dimer or trimer), and a minibody.
- [0073] Many methods are known in the art for generating and/or identifying antibodies to a given target antigen (Harlow, Antibodies, Cold Spring Harbor Press, (1989)). In general, an isolated polypeptide (e.g., a SIRT3-V208I polypeptide), or a portion thereof (e.g., a fragment of a SIRT3-V208I polypeptide that includes amino acid 208) is used as an immunogen and is administered to a mammalian organism, such as a rat, rabbit, hamster or mouse. Either a full-length protein, an antigenic peptide fragment (e.g., a peptide fragment including amino acid 208), or a fusion protein can be used. A protein used as an immunogen may be naturally-occurring, synthetic or recombinantly produced, and may be administered in combination with an adjuvant, including but not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substance such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and the like.
- [0074] Monoclonal antibodies can be produced by hybridoma technology (Kohler and Milstein, Nature, 256:495, 1975), which immortalizes cells secreting a specific

monoclonal antibody. The immortalized cell lines can be created in vitro by fusing two different cell types, typically lymphocytes, and tumor cells. The hybridoma cells may be cultivated in vitro or in vivo. Additionally, fully human antibodies can be generated by transgenic animals (He et al., J. Immunol., 169:595, 2002). Fd phage and Fd phagemid technologies may be used to generate and select recombinant antibodies in vitro (Hoogenboom and Chames, Immunol. Today 21:371, 2000; Liu et al., J. Mol. Biol. 315:1063, 2002). The complementarity-determining regions of an antibody can be identified, and synthetic peptides corresponding to such regions may be used to mediate antigen binding (U.S. Pat. No. 5,637,677).

[0075] Antibodies can be prepared against regions or discrete fragments of a SIRT3-V208I polypeptide. Such fragments can be selected on a physical property, such as fragments corresponding to regions that are located on the surface of the protein, e.g., hydrophilic regions, or can be selected based on sequence uniqueness, but should include amino acid 208 (e.g., amino acid 208-I). An antigenic fragment will typically comprise at least about 8-10 contiguous amino acid residues in which at least one of the amino acid residues is an amino acid affected by a SNP disclosed herein (e.g., amino acid 208). The antigenic peptide can comprise, however, at least 12, 14, 16, 20, 25, 50, 100 (or any other number in-between) or more amino acid residues, provided that at least one amino acid is affected by a SNP disclosed herein.

[0076] Detection of an antibody specific for a SIRT3-V208I polypeptide (e.g., an antibody that can distinguish between two SIRT3 polypeptides that differ in amino acid sequence only at amino acid 208, such that a first SIRT3 polypeptide has a valine at amino acid 208, and the second SIRT3 polypeptide has an isoleucine at amino acid 208) can be facilitated by coupling (i.e., physically linking) the antibody or an antigen-reactive fragment thereof to a detectable substance. Detectable substances include, but are not limited to, various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, and luciferase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein; fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes

luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S , and ^3H .

[0077] Detection of a SIRT3-V208I polypeptide in a biological sample obtained from an individual can be carried out by: a) contacting the biological sample with an antibody, as described above, that can distinguish between a SIRT3-V208I polypeptide and a wild-type human SIRT3 polypeptide; and b) detecting binding of the antibody with a SIRT3 polypeptide present in the biological sample. Detection of binding indicates the presence in the sample of a SIRT3-V208I polypeptide, and indicates that the individual has a propensity to develop metabolic syndrome. In some instances, the antibody comprises a detectable label.

[0078] The detection method can include a positive control, e.g., an antibody that binds a SIRT3 epitope that is shared between a SIRT3-V208 polypeptide and a SIRT3-I208 polypeptide.

Detecting SIRT3 enzymatic activity

[0079] As noted above, in some cases, a subject method involves detecting SIRT3 enzymatic activity in a biological sample obtained from an individual.

[0080] For example, a biological sample can be contacted with an acetylated SIRT3 substrate in the presence of NAD^+ ; and the amount of released acetyl groups quantified. Suitable SIRT3 substrates include acetylated histone H4 N-terminal peptides (e.g., histone H4 amino acids 1-23, amino acids 1-24, amino acids 1-25, and the like). The substrate can include a radiolabelled acetyl group, such that, upon action of an enzymatically active SIRT3 polypeptide, the radioactive acetyl group is released and can be measured. See, e.g., Emiliani et al. (1998) *Proc. Natl. Acad. Sci. USA* 95:2795.

[0081] As one non-limiting example, deacetylase assays are performed in 100 μl of deacetylase buffer (4 mM, MgCl_2 , 0.2 mM dithiothreitol, 50 mM Tris-HCl, pH 8.5) containing 50 ng of recombinant SIRT3, NAD^+ and [^3H] histone H4 peptide substrate. The substrate can be prepared by *in vitro* acetylation of a histone H4 N-terminal peptide (amino acids 1-25; N-msgrgkggkglgkkgakrhrkvlrd-C; SEQ ID NO:29) with radiolabeled acetyl-CoA and recombinant P300/CBP-associated factor (PCAF; see, e.g., GenBank Accession Nos. NP_003875 and XP_001003318) (Heltweg et al. (2005) *Methods* 36:332). Deacetylation reactions can be conducted at 37 °C under gentle agitation, and stopped by adding 25 μl of stop solution (0.2 M HCl, 0.32 M acetic acid).

Radioactivity is extracted into 500 µl ethyl acetate by vortexing for 15 seconds. After centrifugation at 14,000g for 5 min, 450 µl of the ethyl acetate fraction is mixed with 5 ml of scintillation fluid, and the radioactivity was measured with a liquid scintillation counter.

[0082] A positive control can include a wild-type SIRT3 polypeptide, e.g., a SIRT3 polypeptide comprising a valine at amino acid 208, e.g., a SIRT3 polypeptide comprising the amino acid sequence depicted in Figure 7. A negative control can include a SIRT3 polypeptide that is catalytically inactive, e.g., SIRT3-H248Y.

[0083] SIRT3-V208I has enzymatic activity in deacetylating an acetylated H4 histone N-terminal peptide that is substantially lower than the enzymatic activity of SIRT3-V208. Thus, detection of SIRT3 enzymatic activity (catalytic efficiency) in a biological sample from an individual that is at least about 15%, at least about 20%, at least about 25%, at least about 30%, or at least about 35%, lower than the enzymatic activity of wild-type SIRT3 (SIRT3-V208) indicates that the individual could have a SIRT3 polymorphism associated with metabolic syndrome.

DETERMINING A PREDISPOSITION TO DEVELOPING METABOLIC SYNDROME

[0084] The present disclosure provides methods of determining a predisposition of an individual to develop metabolic syndrome. In some cases, the methods generally involving detecting in polynucleotide sample obtained from the individual the presence of a SNP in a SIRT3 gene at rs11246020, where the presence of the polymorphism is indicative of predisposition of the individual to develop metabolic syndrome. As described above, a SNP at rs11246020 that is indicative of a predisposition of an individual to develop metabolic syndrome includes a "A" instead of a "G" at nucleotide 3365 of SEQ ID NO:4 (or nucleotide 125 of SEQ ID NO:3). Such a SNP is also referred to herein as a "metabolic syndrome-associated SNP." In other cases, the method involves analyzing a sample from the individual for the presence of a SIRT3 polypeptide that has a V208I substitution; the presence of the V208I substitution is indicative of a predisposition of the individual to develop metabolic syndrome. In other instances, the method involves analyzing a sample from the individual for enzymatic activity of a SIRT3 polypeptide in the sample; reduced SIRT3 enzymatic activity that is associated with a V208I substitution is indicative of a predisposition of the individual to develop metabolic syndrome.

[0085] Where the polymorphic site rs11246020 comprises a SNP that is predictive of increased risk of developing metabolic syndrome, a G to A mutation occurs in the first nucleotide of the codon encoding Val-208 of human SIRT3. For example, the gtc codon underlined and in bold in Figure 8, which encodes Val-208, is mutated to atc, which encodes isoleucine. In other words, where nucleotide 3365 of the human genomic SIRT3 gene depicted in Figures 9A-L or depicted in Figure 8 is mutated from G to A in the genome of an individual, the individual has an increased risk of developing metabolic syndrome. Said another way, where nucleotide 3365 of the human genomic SIRT3 gene depicted in Figures 9A-L or depicted in Figure 8 is mutated from G to A in the genome of an individual, the individual has a propensity to develop metabolic syndrome.

[0086] In some embodiments, the genome of an individual comprises a SIRT3 allele encoding a SIRT3 polypeptide having Val-208. In some embodiments, the genome of an individual comprises a SIRT3 allele encoding a SIRT3 polypeptide having Ile-208. In some embodiments, the individual is homozygous for a SNP that is indicative of an increased propensity to develop metabolic syndrome. In some embodiments, the individual is heterozygous for a SNP that is indicative of an increased propensity to develop metabolic syndrome.

[0087] The presence of an SNP that is associated with a propensity in an individual to develop metabolic syndrome indicates that the individual has an at least about 25%, at least about 50%, at least about 2-fold, at least about 5-fold, or at least about 10-fold, increased risk of developing metabolic syndrome, compared to the risk that an individual who does not have the SNP will develop metabolic syndrome.

Detecting a metabolic syndrome-associated SNP

[0088] A nucleic acid sample obtained from an individual being tested for a predisposition to developing metabolic syndrome can be genotyped to determine which allele(s) is/are present at any given genetic region (e.g., SNP position) of interest by methods well known in the art. The neighboring sequence can be used to design SNP detection reagents such as oligonucleotide probes, which may optionally be implemented in a kit format. Exemplary suitable SNP genotyping methods are described in Chen et al., "Single nucleotide polymorphism genotyping: biochemistry, protocol, cost and throughput", Pharmacogenomics J. 2003; 3(2):77-96; Kwok et al., "Detection of single nucleotide polymorphisms", Curr Issues Mol. Biol. 2003 April; 5(2):43-60; Shi,

"Technologies for individual genotyping: detection of genetic polymorphisms in drug targets and disease genes", *Am J. Pharmacogenomics*. 2002; 2(3):197-205; and Kwok, "Methods for genotyping single nucleotide polymorphisms", *Annu Rev Genomics Hum Genet* 2001; 2:235-58. Exemplary techniques for high-throughput SNP genotyping are described in Marnellos, "High-throughput SNP analysis for genetic association studies", *Curr Opin Drug Discov Devel*. 2003 May; 6(3):317-21. Common SNP genotyping methods include, but are not limited to, TaqMan assays, molecular beacon assays, nucleic acid arrays, allele-specific primer extension, allele-specific PCR, arrayed primer extension, homogeneous primer extension assays, primer extension with detection by mass spectrometry, pyrosequencing, multiplex primer extension sorted on genetic arrays, ligation with rolling circle amplification, homogeneous ligation, oligonucleotide ligation assay (OLA) (U.S. Pat. No. 4,883,750; and U.S. Pat. No. 5,912,148), multiplex ligation reaction sorted on genetic arrays, restriction-fragment length polymorphism, single base extension-tag assays, and the Invader assay. Such methods may be used in combination with detection mechanisms such as, for example, luminescence or chemiluminescence detection, fluorescence detection, time-resolved fluorescence detection, fluorescence resonance energy transfer, fluorescence polarization, mass spectrometry, and electrical detection.

[0089] Various methods for detecting polymorphisms include, but are not limited to, methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA duplexes (Myers et al., *Science* 230:1242 (1985); Cotton et al., *PNAS* 85:4397 (1988); and Saleeba et al., *Meth. Enzymol.* 217:286-295 (1992)), comparison of the electrophoretic mobility of variant and wild type nucleic acid molecules (Orita et al., *PNAS* 86:2766 (1989); Cotton et al., *Mutat. Res.* 285:125-144 (1993); and Hayashi et al., *Genet. Anal. Tech. Appl.* 9:73-79 (1992)), and assaying the movement of polymorphic or wild-type fragments in polyacrylamide gels containing a gradient of denaturant using denaturing gradient gel electrophoresis (DGGE) (Myers et al., *Nature* 313:495 (1985)). Sequence variations at specific locations can also be assessed by nuclease protection assays such as RNase and S1 protection or chemical cleavage methods.

[0090] In one exemplary embodiment, SNP genotyping is performed using a TaqMan assay, which is also known as the 5' nuclease assay (U.S. Pat. Nos. 5,210,015 and

5,538,848). The TaqMan assay detects the accumulation of a specific amplified product during PCR. The TaqMan assay utilizes an oligonucleotide probe labeled with a fluorescent reporter dye and a quencher dye. The reporter dye is excited by irradiation at an appropriate wavelength, it transfers energy to the quencher dye in the same probe via a process called fluorescence resonance energy transfer (FRET). When attached to the probe, the excited reporter dye does not emit a signal. The proximity of the quencher dye to the reporter dye in the intact probe maintains a reduced fluorescence for the reporter. The reporter dye and quencher dye may be at the 5' most and the 3' most ends, respectively, or vice versa. Alternatively, the reporter dye may be at the 5' or 3' most end while the quencher dye is attached to an internal nucleotide, or vice versa. In yet another embodiment, both the reporter and the quencher may be attached to internal nucleotides at a distance from each other such that fluorescence of the reporter is reduced.

[0091] During PCR, the 5' nuclease activity of DNA polymerase cleaves the probe, thereby separating the reporter dye and the quencher dye and resulting in increased fluorescence of the reporter. Accumulation of PCR product is detected directly by monitoring the increase in fluorescence of the reporter dye. The DNA polymerase cleaves the probe between the reporter dye and the quencher dye only if the probe hybridizes to the target SNP-containing template which is amplified during PCR, and the probe is designed to hybridize to the target SNP site only if a particular SNP allele is present.

[0092] Suitable TaqMan primer and probe sequences can readily be determined using the SNP and associated nucleic acid sequence information provided herein. A number of computer programs, such as Primer Express (Applied Biosystems, Foster City, Calif.), can be used to rapidly obtain optimal primer/probe sets. Also suitable for use are modifications of the Taqman assay well known in the art such as the use of Molecular Beacon probes (U.S. Pat. Nos. 5,118,801 and 5,312,728) and other variant formats (U.S. Pat. Nos. 5,866,336 and 6,117,635).

[0093] Another exemplary method suitable for genotyping a metabolic syndrome-associated SNP is the use of two oligonucleotide probes in an OLA. In this method, one probe hybridizes to a segment of a target nucleic acid with its 3' most end aligned with the SNP site. A second probe hybridizes to an adjacent segment of the target nucleic acid molecule directly 3' to the first probe. The two juxtaposed probes hybridize to the target

nucleic acid molecule, and are ligated in the presence of a linking agent such as a ligase if there is perfect complementarity between the 3' most nucleotide of the first probe with the SNP site. If there is a mismatch, ligation would not occur. After the reaction, the ligated probes are separated from the target nucleic acid molecule, and detected as indicators of the presence of a SNP.

[0094] Another exemplary method suitable for genotyping a metabolic syndrome-associated SNP is based on mass spectrometry. Mass spectrometry takes advantage of the unique mass of each of the four nucleotides of DNA. SNPs can be unambiguously genotyped by mass spectrometry by measuring the differences in the mass of nucleic acids having alternative SNP alleles. MALDI-TOF (Matrix Assisted Laser Desorption Ionization--Time of Flight) mass spectrometry technology is preferred for extremely precise determinations of molecular mass, such as SNPs. Numerous approaches to SNP analysis have been developed based on mass spectrometry. Suitable mass spectrometry-based methods of SNP genotyping include primer extension assays, which can also be utilized in combination with other approaches, such as traditional gel-based formats and microarrays.

[0095] As a non-limiting example, a primer extension assay can involve designing and annealing a primer to a template PCR amplicon upstream (5') from a target SNP position. A mix of dideoxynucleotide triphosphates (ddNTPs) and/or deoxynucleotide triphosphates (dNTPs) are added to a reaction mixture containing template (e.g., a SNP-containing nucleic acid molecule which has typically been amplified, such as by PCR), primer, and DNA polymerase. Extension of the primer terminates at the first position in the template where a nucleotide complementary to one of the ddNTPs in the mix occurs. The primer can be either immediately adjacent (i.e., the nucleotide at the 3' end of the primer hybridizes to the nucleotide next to the target SNP site) or two or more nucleotides removed from the SNP position. If the primer is several nucleotides removed from the target SNP position, the only limitation is that the template sequence between the 3' end of the primer and the SNP position cannot contain a nucleotide of the same type as the one to be detected, or this will cause premature termination of the extension primer. Alternatively, if all four ddNTPs alone, with no dNTPs, are added to the reaction mixture, the primer will always be extended by only one nucleotide, corresponding to the target SNP position. In this instance, primers are designed to bind one nucleotide

upstream from the SNP position (i.e., the nucleotide at the 3' end of the primer hybridizes to the nucleotide that is immediately adjacent to the target SNP site on the 5' side of the target SNP site). Extension by only one nucleotide minimizes the overall mass of the extended primer, thereby increasing the resolution of mass differences between alternative SNP nucleotides. Furthermore, mass-tagged ddNTPs can be employed in the primer extension reactions in place of unmodified ddNTPs. This increases the mass difference between primers extended with these ddNTPs, thereby providing increased sensitivity and accuracy, and is particularly useful for typing heterozygous base positions. Mass-tagging also alleviates the need for intensive sample-preparation procedures and decreases the necessary resolving power of the mass spectrometer.

[0096] The extended primers can then be purified and analyzed by MALDI-TOF mass spectrometry to determine the identity of the nucleotide present at the target SNP position. In one method of analysis, the products from the primer extension reaction are combined with light absorbing crystals that form a matrix. The matrix is then hit with an energy source such as a laser to ionize and desorb the nucleic acid molecules into the gas-phase. The ionized molecules are then ejected into a flight tube and accelerated down the tube towards a detector. The time between the ionization event, such as a laser pulse, and collision of the molecule with the detector is the time of flight of that molecule. The time of flight is precisely correlated with the mass-to-charge ratio (m/z) of the ionized molecule. Ions with smaller m/z travel down the tube faster than ions with larger m/z and therefore the lighter ions reach the detector before the heavier ions. The time-of-flight is then converted into a corresponding, and highly precise, m/z . In this manner, SNPs can be identified based on the slight differences in mass, and the corresponding time of flight differences, inherent in nucleic acid molecules having different nucleotides at a single base position. For further information regarding the use of primer extension assays in conjunction with MALDI-TOF mass spectrometry for SNP genotyping, see, e.g., Wise et al., "A standard protocol for single nucleotide primer extension in the human genome using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry", *Rapid Commun Mass Spectrom.* 2003; 17(11):1195-202.

[0097] The following references provide further information describing mass spectrometry-based methods for SNP genotyping: Bocker, "SNP and mutation discovery

using base-specific cleavage and MALDI-TOF mass spectrometry", *Bioinformatics*. 2003 July; 19 Suppl 1: 144-153; Storm et al., "MALDI-TOF mass spectrometry-based SNP genotyping", *Methods Mol. Biol.* 2003; 212:241-62; Jurinke et al., "The use of Mass ARRAY technology for high throughput genotyping", *Adv Biochem Eng Biotechnol.* 2002; 77:57-74; and Jurinke et al., "Automated genotyping using the DNA MassArray technology", *Methods Mol. Biol.* 2002; 187:179-92.

[0098] A metabolic syndrome-associated SNP can also be detected by direct DNA sequencing. A variety of automated sequencing procedures can be utilized ((1995) *Biotechniques* 19:448), including sequencing by mass spectrometry (see, e.g., WO94/16101; Cohen et al., *Adv. Chromatogr.* 36:127-162 (1996); and Griffin et al., *Appl. Biochem. Biotechnol.* 38:147-159 (1993)). The nucleic acid sequences disclosed herein enable one of ordinary skill in the art to readily design sequencing primers for such automated sequencing procedures. Commercial instrumentation, such as the Applied Biosystems 377, 3100, 3700, 3730, and 3730.times.1 DNA Analyzers (Foster City, Calif.), is commonly used in the art for automated sequencing.

[0099] Other methods that can be used to genotype a metabolic syndrome-associated SNP include single-strand conformational polymorphism (SSCP), and denaturing gradient gel electrophoresis (DGGE) (Myers et al., *Nature* 313:495 (1985)). SSCP identifies base differences by alteration in electrophoretic migration of single stranded PCR products. Single-stranded PCR products can be generated by heating or otherwise denaturing double stranded PCR products. Single-stranded nucleic acids may refold or form secondary structures that are partially dependent on the base sequence. The different electrophoretic mobilities of single-stranded amplification products are related to base-sequence differences at SNP positions. DGGE differentiates SNP alleles based on the different sequence-dependent stabilities and melting properties inherent in polymorphic DNA and the corresponding differences in electrophoretic migration patterns in a denaturing gradient gel (Erlich, ed., *PCR Technology, Principles and Applications for DNA Amplification*, W.H. Freeman and Co, New York, 1992, Chapter 7).

[00100] Sequence-specific ribozymes (U.S. Pat. No. 5,498,531) can also be used to detect a metabolic syndrome-associated SNP based on the development or loss of a ribozyme cleavage site. Perfectly matched sequences can be distinguished from mismatched

sequences by nuclease cleavage digestion assays or by differences in melting temperature. If the SNP affects a restriction enzyme cleavage site, the SNP can be identified by alterations in restriction enzyme digestion patterns, and the corresponding changes in nucleic acid fragment lengths determined by gel electrophoresis.

[00101] SNP genotyping can include the steps of, for example, collecting a biological sample from a human subject (e.g., sample of tissues, cells, fluids, secretions, etc.), isolating nucleic acids (e.g., genomic DNA, mRNA or both) from the cells of the sample, contacting the nucleic acids with one or more primers which specifically hybridize to a region of the isolated nucleic acid containing a target SNP under conditions such that hybridization and amplification of the target nucleic acid region occurs, and determining the nucleotide present at the SNP position of interest, or, in some assays, detecting the presence or absence of an amplification product (assays can be designed so that hybridization and/or amplification will only occur if a particular SNP allele is present or absent). In some assays, the size of the amplification product is detected and compared to the length of a control sample; for example, deletions and insertions can be detected by a change in size of the amplified product compared to a normal genotype.

Detecting a metabolic syndrome-associated SIRT3 variant

[00102] In some cases, a SIRT3 variant with a V208I substitution is detected. In some cases, a SIRT3 variant with a V208I substitution is detected. A SIRT3 variant that has a V208I substitution is also referred to herein as "SIRT3-V208I." A SIRT3-V208I polypeptide can be detected using an antibody that can distinguish between a SIRT3-V208I polypeptide and a wild-type human SIRT3 polypeptide, e.g., a SIRT3 polypeptide that has a valine at position 208. The antibody is one that can distinguish between a first and a second SIRT3 polypeptide, where the first and the second SIRT3 polypeptides differ in amino acid sequence only at amino acid 208, where the first SIRT3 polypeptide has a valine at amino acid 208; and the second SIRT3 polypeptide has an isoleucine at amino acid 208. For example, the antibody is one that can distinguish between a first and a second SIRT3 polypeptide, where the first SIRT3 polypeptide comprises the amino acid sequence as depicted in Figure 7, with a valine at amino acid 208 ("SIRT3-V208"); and the second SIRT3 polypeptide comprises the amino acid sequence as depicted in Figure 7, but has an isoleucine instead of a valine at amino acid 208 ("SIRT3-I208"). A

suitable antibody can distinguish between SIRT3-V208 and SIRT3-I208, e.g., a suitable antibody binds SIRT3-I208 with an affinity of at least about 10^{-7} M, at least about 5×10^{-7} M, at least about 10^{-8} M, at least about 5×10^{-8} M, at least about 10^{-9} M, or greater than 10^{-9} M; and binds SIRT3-V208 with an affinity of less than 10^{-7} M.

[00103] Detection of a SIRT3-V208I polypeptide in a biological sample obtained from an individual can be carried out by: a) contacting the biological sample with an antibody, as described above, that can distinguish between a SIRT3-V208I polypeptide and a wild-type human SIRT3 polypeptide; and b) detecting binding of the antibody with a SIRT3 polypeptide present in the biological sample. Detection of binding indicates the presence in the sample of a SIRT3-V208I polypeptide, and indicates that the individual has a propensity to develop metabolic syndrome. In some instances, the antibody comprises a detectable label.

[00104] The detection method can include a positive control, e.g., an antibody that binds a SIRT3 epitope that is shared between a SIRT3-V208 polypeptide and a SIRT3-I208 polypeptide.

Detecting SIRT3 enzymatic activity

[00105] As noted above, in some cases, a subject method involves detecting SIRT3 enzymatic activity in a biological sample obtained from an individual. Detection of enzymatic activity can be carried out independently of, or in addition to, detection of a SIRT3-V208I polypeptide.

[00106] For example, a biological sample can be contacted with an acetylated SIRT3 substrate in the presence of NAD^+ ; and the amount of released acetyl groups quantified. Suitable SIRT3 substrates include acetylated histone H4 N-terminal peptides (e.g., histone H4 amino acids 1-23, amino acids 1-24, amino acids 1-25, and the like). The substrate can include a radiolabelled acetyl group, such that, upon action of an enzymatically active SIRT3 polypeptide, the radioactive acetyl group is released and can be measured. See, e.g., Emiliani et al. (1998) *Proc. Natl. Acad. Sci. USA* 95:2795.

[00107] As one non-limiting example, deacetylase assays are performed in 100 μl of deacetylase buffer (4 mM, MgCl_2 , 0.2 mM dithiothreitol, 50 mM Tris-HCl, pH 8.5) containing 50 ng of recombinant SIRT3, NAD^+ and [^3H] histone H4 peptide substrate. The substrate can be prepared by *in vitro* acetylation of a histone H4 N-terminal peptide (amino acids 1-25; N-msgrgkggkglgkkgakhrkvld-C; SEQ ID NO:29) with

radiolabeled acetyl-CoA and recombinant P300/CBP-associated factor (PCAF; see, e.g., GenBank Accession Nos. NP_003875 and XP_001003318) (Heltweg et al. (2005) *Methods* 36:332). Deacetylation reactions can be conducted at 37 °C under gentle agitation, and stopped by adding 25 µl of stop solution (0.2 M HCl, 0.32 M acetic acid). Radioactivity is extracted into 500 µl ethyl acetate by vortexing for 15 seconds. After centrifugation at 14,000g for 5 min, 450 µl of the ethyl acetate fraction is mixed with 5 ml of scintillation fluid, and the radioactivity was measured with a liquid scintillation counter.

[00108] A positive control can include a wild-type SIRT3 polypeptide, e.g., a SIRT3 polypeptide comprising a valine at amino acid 208, e.g., a SIRT3 polypeptide comprising the amino acid sequence depicted in Figure 7. A negative control can include a SIRT3 polypeptide that is catalytically inactive, e.g., SIRT3-H248Y.

[00109] SIRT3-V208I has enzymatic activity in deacetylating an acetylated H4 histone N-terminal peptide that is substantially lower than the enzymatic activity of SIRT3-V208. Thus, detection of SIRT3 enzymatic activity (catalytic efficiency) in a biological sample from an individual that is at least about 15%, at least about 20%, at least about 25%, at least about 30%, or at least about 35%, lower than the enzymatic activity of wild-type SIRT3 (SIRT3-V208) indicates that the individual has a propensity to develop metabolic syndrome.

Generating a report

[00110] The results of a test (e.g., an individual's risk for developing metabolic syndrome, based detection of a metabolic syndrome-associated SNP, as described above), and/or any other information pertaining to a test, can be provided in the form of a "report". A tangible report can optionally be generated as part of a testing process (which may be interchangeably referred to herein as "reporting", or as "providing" a report, "producing" a report, or "generating" a report). Examples of tangible reports may include, but are not limited to, reports on paper (such as computer-generated printouts of test results) or equivalent formats and reports stored on computer readable medium (such as a compact disk (CD), computer hard drive, or computer network server, etc.). Reports, e.g., those stored on computer readable medium, can be part of a database (such as a database of patient records, which may be a "secure database" that has security features that limit access to the report, such as to allow only the patient and the patient's medical

practitioner(s) to view the report, for example). In addition to, or as an alternative to, generating a tangible report, reports can also be displayed on a computer screen (or the display of another electronic device or instrument).

[00111] A report can further be "transmitted" or "communicated" (these terms may be used herein interchangeably), such as to the individual who was tested, a medical practitioner (e.g., a doctor, nurse, clinical laboratory practitioner, genetic counselor, etc.), a healthcare organization, a clinical laboratory, and/or any other party intended to view or possess the report. The act of "transmitting" or "communicating" a report can be by any means known in the art, based on the form of the report. Furthermore, "transmitting" or "communicating" a report can include delivering a report ("pushing") and/or retrieving ("pulling") a report. For example, reports can be transmitted/communicated by such means as being physically transferred between parties (such as for reports in paper format), such as by being physically delivered from one party to another, or by being transmitted electronically or in signal form (e.g., via e-mail or over the internet, by facsimile, and/or by any wired or wireless communication methods known in the art), such as by being retrieved from a database stored on a computer network server, etc.

Counseling and treatment

[00112] Based on the results of a subject method, an individual may be identified as predisposed to developing metabolic syndrome. In such instances, the individual may be counseled by a medical personnel to do one or more of the following: 1) modify the diet of the individual, e.g., to reduce caloric intake, to alter the quality and quantity of the diet, etc.; 2) prescribe an exercise regimen; and 3) undergo frequent monitoring to monitor one or more of: a) fasting blood glucose levels; b) triglyceride levels; c) HDL levels; d) blood pressure; and e) BMI. The individual may be advised to undergo treatment to ameliorate one or more manifestations of metabolic syndrome, e.g., reducing fasting blood glucose levels, reducing triglyceride levels, increasing HDL levels, reducing blood pressure, and reducing BMI.

REAGENTS, DEVICES, AND KITS

[00113] The present disclosure provides reagents, devices, and kits for detecting SIRT3 SNPs associated with increased risk of developing metabolic syndrome. Although particularly suited for screening for risk of developing metabolic syndrome in a subject,

it will be understood that in certain embodiments these reagents, devices and kits can be used for analysis of SIRT3 polymorphisms for any purpose, including research applications.

SNP detection reagents

[00114] The present disclosure provides SNP detection reagents for detecting a metabolic syndrome-associated SNP in a human SIRT3 gene. A subject SNP detection reagent includes an allele-specific probe, and allele-specific primer, and a primer pair that specifically amplifies a region in a human SIRT3 gene that contains a metabolic syndrome-associated SNP.

[00115] A subject SNP detection reagent can have a length of from about 15 nucleotides (nt) to about 250 nt, e.g., from about 15 nt to about 20 nt, from about 20 nt to about 25 nt, from about 25 nt to about 30 nt, from about 30 nt to about 40 nt, from about 40 nt to about 50 nt, from about 50 nt to about 75 nt, from about 75 nt to about 100 nt, from about 100 nt to about 150 nt, from about 150 nt to about 200 nt, or from about 200 nt to about 250 nt.

[00116] In some embodiments, a subject SNP detection reagent is an allele-specific probe.

[00117] Exemplary allele-specific probes comprising the "G" allele at nt 3365 of a human SIRT3 gene (nucleotide sequences corresponding to SEQ ID NO:4 given):

[00118] 1) 5'- aactacaagcccaac**gtc**actcactacttt -3' (SEQ ID NO:5; nt 3350-3379)

[00119] 2) 5'- caagcccaac**gtc**actcactactttctccg -3' (SEQ ID NO:6; nt 3355-3384)

[00120] 3) 5'- caagcccaac**gtc**actcactact -3' (SEQ ID NO:7; nt 3355-3377)

[00121] 4) 5'- tacaagcccaac**gtc**actcactactttctc -3' (SEQ ID NO:8; nt 3364-3382)

[00122] 5) 5'- taccctggaaactacaagcccaac**gtc**actcactactttctccggtgct -3' (SEQ ID NO:9; nt 3341-3390); and

[00123] 6) 5'- actacaagcccaac**gtc**actcactactttc -3' (SEQ ID NO:10; nt 3351-3380).

[00124] Exemplary allele-specific probes comprising the "A" allele at nt 3365 of a human SIRT3 gene (nucleotide sequences corresponding to SEQ ID NO:4 given):

[00125] 1) 5'- aactacaagcccaac**atc**actcactactttt -3' (SEQ ID NO:11; nt 3350-3379);

[00126] 2) 5'- caagcccaac**atc**actcactactttctccg -3' (SEQ ID NO:12; nt 3355-3384);

[00127] 3) 5'- caagcccaac**atc**actcactact -3' (SEQ ID NO:13; nt 3355-3377);

[00128] 4) 5'- tacaagcccaac**atc**actcactactttctc -3' (SEQ ID NO:14; nt 3364-3382);

- [00129] 5) 5'-taccctggaaactacaagcccaacatcactcactactttctccggctgct-3' (SEQ ID NO:15; nt 3341-3390); and
- [00130] 6) 5'-actacaagcccaacatcactcactactttc-3' (SEQ ID NO:16; nt 3351-3380).
- [00131] A subject SNP detection reagent can include a pair of allele-specific probes, e.g., where the first member (the "reference" member) of the pair of allele-specific probe includes a "G" at a position corresponding to nt 125 of SEQ ID NO:3, or nt 3365 of SEQ ID NO:4; and where the second member of the pair includes an "A" at a position corresponding to nt 125 of SEQ ID NO:3, or nt 3365 of SEQ ID NO:4.
- [00132] Exemplary, non-limiting allele-specific probe pairs include:
- [00133] Pair 1:
- [00134] 5'-aactacaagcccaacgtcactcactacttt-3' (SEQ ID NO:5; nt 3350-3379); and
- [00135] 5'-aactacaagcccaacatcactcactacttt-3' (SEQ ID NO:11);
- [00136] Pair 2:
- [00137] 5'-caagcccaacgtcactcactactttctccg-3' (SEQ ID NO:6; nt 3355-3384); and
- [00138] 5'-caagcccaacatcactcactactttctccg-3' (SEQ ID NO:12);
- [00139] Pair 3
- [00140] 5'-caagcccaacgtcactcactact-3' (SEQ ID NO:7; nt 3355-3377); and
- [00141] 5'-caagcccaacatcactcactact-3' (SEQ ID NO:13);
- [00142] Pair 4
- [00143] 5'-tacaagcccaacgtcactcactactttctc-3' (SEQ ID NO:8; nt 3364-3382); and
- [00144] 5'-tacaagcccaacatcactcactactttctc-3' (SEQ ID NO:14);
- [00145] Pair 5
- [00146] 5'-taccctggaaactacaagcccaacgtcactcactactttctccggctgct-3' (SEQ ID NO:9; nt 3341-3390); and
- [00147] 5'-taccctggaaactacaagcccaacatcactcactactttctccggctgct-3' (SEQ ID NO:15); and
- [00148] Pair 6
- [00149] 5'-actacaagcccaacgtcactcactactttc-3' (SEQ ID NO:10; nt 3351-3380); and
- [00150] 5'-actacaagcccaacatcactcactactttc-3' (SEQ ID NO:16).
- [00151] In some embodiments, a subject SNP detection reagent is an allele-specific primer. Exemplary, non-limiting allele-specific primers for analyzing a metabolic syndrome-associated SNP in human SIRT3 include:
- [00152] 5'-taccctggaaactacaagcccaacatcact-3' (SEQ ID NO:17);

- [00153] 5'- accctggaaactacaagcccaacatcac-3' (SEQ ID NO:18); and
- [00154] 5'- taccctggaaactacaagcccaacatc-3' (SEQ ID NO:19),
- [00155] or the complement of any of the forgoing;
- [00156] where corresponding control ("reference") primers for the "G" allele include:
- [00157] 5'- taccctggaaactacaagcccaacgtcact-3' (SEQ ID NO:20);
- [00158] 5'- accctggaaactacaagcccaacgtcac-3' (SEQ ID NO:21); and
- [00159] 5'- taccctggaaactacaagcccaacgtc-3' (SEQ ID NO:22),
- [00160] or the complement of any of the forgoing.
- [00161] In some embodiments, a subject SNP detection reagent comprises pairs of allele-specific primers, where the first member (the "reference" member) of the pair of allele-specific probe includes a "G" at a position corresponding to nt 125 of SEQ ID NO:3, or nt 3365 of SEQ ID NO:4; and where the second member of the pair includes an "A" at a position corresponding to nt 125 of SEQ ID NO:3, or nt 3365 of SEQ ID NO:4.
- [00162] In some embodiments, a subject SNP detection reagent comprises a pair of nucleic acids, where the first member of the pair is an allele-specific primer, as described above, and the second member of the pair hybridizes to a human SIRT3 gene at a location upstream or downstream of the location to which the first member hybridizes, such that, under standard polymerase chain reaction conditions, the first and the second members of the primer pair amplify a segment of the human SIRT3 gene comprising nucleotide 3365 of SEQ ID NO:4, e.g., the first and the second members of the primer pair amplify a segment of the human SIRT3 gene that includes from 1 nt to 300 nt (e.g., from 1 nt to 5 nt, from 5 nt to 10 nt, from 10 nt to 15 nt, from 15 nt to 20 nt from 20 nt to 25 nt, from 25 nt to 50 nt, from 50 nt to 100 nt, from 100 nt to 200 nt, from 100 nt to 300 nt) 5' of nucleotide 3365 of SEQ ID NO:5 and from 1 nt to 300 nt (e.g., from 1 nt to 5 nt, from 5 nt to 10 nt, from 10 nt to 15 nt, from 15 nt to 20 nt from 20 nt to 25 nt, from 25 nt to 50 nt, from 50 nt to 100 nt, from 100 nt to 200 nt, from 100 nt to 300 nt) 3' of nucleotide 3365 of SEQ ID NO:4. The PCR product thus produced has a length of from about 15 to about 600 nt.
- [00163] Exemplary primer pairs, where the forward primer is an allele-specific primer, include:

Forward primer	Reverse primer
5'- taccctggaaactacaagcccaac <u>gtc</u> act-	5'-gcagccggagaaagtagtga-3'

3' (SEQ ID NO:20)	(SEQ ID NO:24)
5'- accctggaaactacaagcccaac gtc ac-3' (SEQ ID NO:21)	5'-ttgtcatgaagcagccggag-3' (SEQ ID NO:26)
5'- taccctggaaactacaagcccaac gtc -3' (SEQ ID NO:22)	5'-aagcagccccttgtcatgaa-3' (SEQ ID NO:28)

[00164] In some embodiments, a subject SNP detection reagent comprises a pair of nucleic acids, where the first member of the primer pair hybridizes to a region that is 5' of nucleotide 3365 of the human SIRT3 gene as set forth in SEQ ID NO:4, where the second member of the primer pair hybridizes to a region that is 3' of nucleotide 3365 of the human SIRT3 gene as set forth in SEQ ID NO:4, where the such that, under standard polymerase chain reaction conditions, the first and the second members of the primer pair amplify a segment of the human SIRT3 gene comprising nucleotide 3365 of SEQ ID NO:4, e.g., the first and the second members of the primer pair amplify a segment of the human SIRT3 gene that includes from 5 nt to 300 nt 5' (e.g., from 5 nt to 10 nt, from 10 nt to 15 nt, from 15 nt to 20 nt from 20 nt to 25 nt, from 25 nt to 50 nt, from 50 nt to 100 nt, from 100 nt to 200 nt, from 100 nt to 300 nt) of nucleotide 3365 of SEQ ID NO:5 and from 5 nt to 300 nt (e.g., from 5 nt to 10 nt, from 10 nt to 15 nt, from 15 nt to 20 nt from 20 nt to 25 nt, from 25 nt to 50 nt, from 50 nt to 100 nt, from 100 nt to 200 nt, from 100 nt to 300 nt) 3' of nucleotide 3365 of SEQ ID NO:4. The PCR product thus produced has a length of from about 15 to about 600 nt.

[00165] Exemplary, non-limiting, primer pairs that amplify a region including the rs11246020 polymorphic site include (where the nucleotides correspond to the numbering depicted in Figure 10A):

Forward primer	Reverse primer
5'- gtaccctggaaactacaagc -3' (nt 631-650) (SEQ ID NO:23)	5'-gcagccggagaaagtagtga-3' (reverse complement of nt 661-680) (SEQ ID NO:24)
5'- ccaaggagctgtaccctgga-3' (nt 621-640) (SEQ ID NO:25)	5'-ttgtcatgaagcagccggag-3' (reverse complement of nt 671-690) (SEQ ID NO:26)
5'- ttcactttggccaaggagct-3' (nt 611-630) (SEQ ID NO:27)	5'-aagcagccccttgtcatgaa-3' (reverse complement of nt 681-700) (SEQ ID NO:28)

[00166] A subject SNP detection reagent can comprise a detectable label, e.g., a radiolabel, a fluorogenic dye, etc. For example, in some embodiments, a subject SNP detection reagent is labeled with a fluorogenic reporter dye that emits a detectable signal. Suitable reporter dyes include, but are not limited to, Acridine, AMCA, BODIPY, Cascade Blue, Cy2, Cy3, Cy5, Cy7, Dabcyl, Edans, Eosin, Erythrosin, Fluorescein, 6-Fam, Tet, Joe, Hex, Oregon Green, Rhodamine, Rhodol Green, Tamra, Rox, and Texas Red. Suitable fluorogenic dyes include, e.g., 5-carboxyfluorescein, 6-carboxyfluorescein, 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein, N,N,N',N'-tetramethyl-6-carboxy rhodamine, 6-carboxyrhodamine X, 4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein, 4,7,2',4',5',7'-hexachloro-5-carboxyfluorescein, 2',4',5',7'-tetrachloro-5-carboxyfluorescein, 4,7,2',7'-tetrachloro-6-carboxyfluorescein, 1',2',7',8'-dibenzo-4,7-dichloro-5-carboxyfluorescein, and 1',2',7',8'-dibenzo-4,7-dichloro-6-carboxyfluorescein.

[00167] A subject SNP detection reagent can be further labeled with a quencher dye such as Tamra, e.g., when the reagent is used as a self-quenching probe such as a TaqMan (U.S. Pat. Nos. 5,210,015 and 5,538,848) or Molecular Beacon probe (U.S. Pat. Nos. 5,118,801 and 5,312,728), or other stemless or linear beacon probe (Livak et al., 1995, PCR Method Appl. 4:357-362; Tyagi et al., 1996, Nature Biotechnology 14: 303-308; Nazarenko et al., 1997, Nucl. Acids Res. 25:2516-2521; U.S. Pat. Nos. 5,866,336 and 6,117,635).

[00168] A subject SNP detection reagent is in some embodiments immobilized on a substrate. Suitable substrates include, e.g., glass; plastic; paper, nylon, nitrocellulose, or other type of membrane (e.g., which membrane may be in the form of a test strip); a filter; a chip; or any other suitable solid support.

SNP detection kits

[00169] Subject reagents include, e.g., allele-specific primers, primer pairs for amplification, allele-specific probes, and combinations thereof. Such reagents may be contained in separate containers in a subject kit. In an embodiment, the kit contains a first container containing a probe, primer, or primer pair for a metabolic syndrome-associated SIRT3 gene SNP, as described above, and a second container containing a

reference probe, primer, or primer pair, e.g., for detecting the reference allele corresponding to the metabolic syndrome-associated SIRT3 gene SNP.

[00170] In one embodiment, the invention provides kits comprising an allele-specific oligonucleotide that hybridizes to a human SIRT3 gene comprising a metabolic syndrome-associated SIRT3 gene SNP. The kits may contain one or more pairs of SIRT3 allele-specific oligonucleotides hybridizing to different forms of a polymorphism. The SIRT3 allele-specific oligonucleotides may include sequences derived from the coding (exons) or non-coding (promoter, 5' untranslated, introns or 3' untranslated) region of the SIRT3 gene. The SIRT3 allele-specific oligonucleotides may be provided immobilized on a substrate.

[00171] A subject kit can include at least one SIRT3-specific primer that hybridizes to a region spanning or adjacent to a metabolic syndrome-related polymorphism in the human SIRT3 gene. The SIRT3-specific primers may include sequences derived from the coding (exons) or non-coding (promoter, 5' untranslated, introns or 3' untranslated) region of the SIRT3 gene. A subject kit can contain one or more pairs of SIRT3-specific primers that hybridize to opposite strands of nucleic acid adjacent to a metabolic syndrome-associated polymorphism in the SIRT3 gene. In the presence of appropriate buffers and enzymes, the SIRT3-specific primer pairs are useful in amplifying specific polymorphisms in the SIRT3 gene.

[00172] A subject kit can include, in addition to a SNP detection reagent, one or more biochemical reagents, containers, packages such as packaging intended for commercial sale, substrates to which SNP detection reagents are attached, electronic hardware components, etc.

[00173] In some embodiments, a subject SNP detection kit contains one or more detection reagents and other components (e.g., a buffer, enzymes such as DNA polymerases or ligases, chain extension nucleotides such as deoxynucleotide triphosphates, and in the case of Sanger-type DNA sequencing reactions, chain terminating nucleotides, positive control sequences, negative control sequences, and the like) necessary to carry out an assay or reaction, such as amplification and/or detection of a SNP-containing nucleic acid molecule. A subject kit can further contain means for determining the amount of a target nucleic acid, and means for comparing the amount

with a standard, and can comprise instructions for using the kit to detect the SNP-containing nucleic acid.

[00174] A subject SNP detection kit can include components that are used to prepare nucleic acids from a test sample for the subsequent amplification and/or detection of a SNP-containing nucleic acid molecule. Such sample preparation components can be used to produce nucleic acid extracts (including DNA and/or RNA) from any bodily fluids (such as blood, serum, plasma, urine, saliva, phlegm, gastric juices, semen, tears, sweat, etc.), skin, hair, cells (especially nucleated cells; e.g., peripheral blood mononuclear cells), biopsies, buccal swabs or tissue specimens. The test samples used in a subject method will vary based on such factors as the assay format, nature of the detection method, and the specific tissues, cells or extracts used as the test sample to be assayed. Methods of preparing nucleic acids and cell extracts are well known in the art and can be readily adapted to obtain a sample that is compatible with the system utilized. Automated sample preparation systems for extracting nucleic acids from a test sample are commercially available.

[00175] In some embodiments, a subject SNP detection kit is a compartmentalized kit. A compartmentalized kit includes any kit in which reagents are contained in separate containers. Such containers include, for example, small glass containers, plastic containers, strips of plastic, glass or paper, or arraying material such as silica. Such containers allow one to efficiently transfer reagents from one compartment to another compartment such that the test samples and reagents are not cross-contaminated, or from one container to another vessel not included in the kit, and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another or to another vessel. Such containers may include, for example, one or more containers which will accept the test sample, one or more containers which contain at least one probe or other SNP detection reagent for detecting a subject SNP, one or more containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, etc.), and one or more containers which contain the reagents used to reveal the presence of the bound probe or other SNP detection reagents. The kit can optionally further comprise compartments and/or reagents for, for example, nucleic acid amplification or other enzymatic reactions such as primer extension reactions, hybridization, ligation, electrophoresis (preferably capillary electrophoresis), mass spectrometry, and/or laser-

induced fluorescent detection. The kit may also include instructions for using the kit. Exemplary compartmentalized kits include microfluidic devices known in the art (see, e.g., Weigl et al., "Lab-on-a-chip for drug development", *Adv Drug Deliv Rev.* 2003 Feb. 24; 55(3):349-77). In such microfluidic devices, the containers may be referred to as, for example, microfluidic "compartments", "chambers", or "channels."

[00176] Microfluidic devices, which can also be referred to as "lab-on-a-chip" systems, biomedical micro-electro-mechanical systems (bioMEMs), or multicomponent integrated systems, are suitable for inclusion in a subject SNP detection kit. Such systems miniaturize and compartmentalize processes such as probe/target hybridization, nucleic acid amplification, and capillary electrophoresis reactions in a single functional device. Such microfluidic devices typically utilize detection reagents in at least one aspect of the system, and such detection reagents may be used to detect a subject SNP. One example of a microfluidic system is disclosed in U.S. Pat. No. 5,589,136, which describes the integration of PCR amplification and capillary electrophoresis in chips. Exemplary microfluidic systems comprise a pattern of microchannels designed onto a glass, silicon, quartz, or plastic wafer included on a microchip. The movements of the samples may be controlled by electric, electroosmotic or hydrostatic forces applied across different areas of the microchip to create functional microscopic valves and pumps with no moving parts. Varying the voltage can be used as a means to control the liquid flow at intersections between the micro-machined channels and to change the liquid flow rate for pumping across different sections of the microchip. See, for example, U.S. Pat. Nos. 6,153,073, and 6,156,181.

[00177] For genotyping SNPs, an exemplary microfluidic system may integrate, for example, nucleic acid amplification, primer extension, capillary electrophoresis, and a detection method such as laser induced fluorescence detection. In a first step of an exemplary process for using such an exemplary system, nucleic acid samples are amplified, e.g., by PCR. Then, the amplification products are subjected to automated primer extension reactions using ddNTPs (specific fluorescence for each ddNTP) and the appropriate oligonucleotide primers to carry out primer extension reactions which hybridize just upstream of the targeted SNP. Once the extension at the 3' end is completed, the primers are separated from the unincorporated fluorescent ddNTPs by capillary electrophoresis. The separation medium used in capillary electrophoresis can

be, for example, polyacrylamide, poly(ethylene glycol) or dextran. The incorporated ddNTPs in the single nucleotide primer extension products are identified by laser-induced fluorescence detection.

EXAMPLES

[00178] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); kb, kilobase(s); bp, base pair(s); nt, nucleotide(s); i.m., intramuscular(ly); i.p., intraperitoneal(ly); s.c., subcutaneous(ly); rt, room temperature; and the like.

Example 1

MATERIALS AND METHODS

Antibodies

[00179] Antibodies used were specific for ATPase subunit α and β (Invitrogen Molecular Probes, Carlsbad, CA), monoclonal and polyclonal acetyllysine (Cell Signaling Technology, Danvers, MA), SIRT3 [as described(3)], ETF and LCAD.

Animal Studies

[00180] All animal studies were performed according to IACUC-approved protocols. Studies used wt and SIRT3KO 129Sv (3), male 3-month old or 12-month old mice, maintained on a standard chow diet (5053 PicoLab diet, Ralston Purina Company, St. Louis, MO) or a high-fat 'Western diet' (TD.88137; Harlan Teklad, Indianapolis, IN). For metabolic measurements, body weight and composition were measured by dual-

energy X-ray absorptiometry (DEXA) scanning. The Comprehensive Lab Animal Monitoring System (CLAMS) method was used to measure activity level, food intake, volume of O₂ consumption, volume of CO₂ production (Oxymax OPTO-M3 system, Columbus Instruments, Columbus, OH). Mean VO₂ and VCO₂ were calculated for dark and light cycles and normalized to lean body mass. Mean activity and respiratory exchange ratio were calculated for dark and light cycles. For SCD1 mouse studies, wt (C57Bl/6), SIRT3KO (C57Bl/6-*Sirt3*^{-/-}), SCD1KO (B6.129-*Scd1*^{tm1Niam}/J, The Jackson Laboratory, Bar Harbor, ME), or dKO (C57Bl/6-*Scd1*^{-/-}*Sirt3*^{-/-}), mice were used. Mice were sacrificed at 7:00 h for fed mouse studies, or transferred to a new cage without food for 24 h from 7:00 h to 7:00 h, and then sacrificed for fasted mouse studies.

SIRT3 Adenovirus preparation and murine injection

[00181] Murine SIRT3 cDNA was cloned into pShuttle-IRES-GFP-1 vector (Stratagene/Agilent, Santa Clara, CA), or an empty vector as a negative control. Adenoviruses were recombined and produced using pAdeasy Adenoviral System (Stratagene/Agilent, Santa Clara, CA). After amplification with Ad-293 as packaging cell line, virus was purified using cesium chloride gradient ultra-centrifugation and dialyzed into PBS plus 10% glycerol as described (40). For injection, 3-month old male mice were injected via tail vein with adenovirus over-expression either green fluorescent protein (GFP) (control) or SIRT3 at dose of 5×10^9 plaque forming units (PFU)/g body weight, as described (41). The mice were monitored for signs of distress, and recovered under observation. On the sixth day after virus injection, the mice were sacrificed and livers were removed and measured for total lipids.

Glucose and Insulin Tolerance

[00182] Glucose and insulin tolerance tests were performed according to The Jackson Laboratory protocol. For glucose tolerance tests, a 200 mg/ml glucose solution was prepared. Mice were fasted 6hs from 7:00 h to 13:00 h, and then glucose injected into the intraperitoneal space (2g /kg body weight). For insulin tolerance tests, mice were fasted 6hs from 7:00 h to 13:00 h, and pre-warmed insulin was injected into the interaperitoneal space at a concentration of 1U insulin/kg body weight. Glucose was monitored at regular intervals up until 2h with a handheld personal glucose monitoring device (Freestyle, Abbott Park, IL).

Histology and Microscopy

[00183] For histology studies, mice were perfused or tissues were dissected and drop-fixed or in fresh 3% paraformaldehyde overnight. The following day, tissues were cryoprotected using a sucrose gradient (10% for 1 h, 20% for 2 h and 30% overnight). After cryoprotection, tissue was placed in OCT (Tissue Tek 4583) in a peel-away mold (Ted Pella, Redding, CA) and frozen using dry-ice-cooled isopentane (M32631, Sigma-Aldrich, St. Louis, MO), and stored at -80°C. Specimens were placed at -20°C for 1h to equilibrate and 8- μ m sections were cut onto charged slides (Snowcoat X-tra, Surgipath, Richmond, IL). Slides were dried at RT for 5 min then stored at -20°C. For ORO staining, slides were brought to room temperature, and washed in running water for 10 min, to remove OCT. They were placed in 50% isopropanol for 3 minutes, followed by a further 3 min in 100% isopropanol and into 0.5% ORO (O-0625, Sigma-Aldrich, St. Louis, MO) in 100% isopropanol (398039-2L, Sigma-Aldrich, St. Louis, MO) for 2 h. Specimens were then differentiated in three 3-min 85% isopropanol washes and running water for 10 min. Specimens were then counterstained with Mayer's hematoxylin (American Mastertech HXMMHGAL, Lodi, CA) for 10 sec, followed by bluing in running water for a further 10 min. Slides were mounted using crystal mount (American Mastertech MMC00168E, Lodi, CA) and left to dry overnight. Slides were imaged using an Axio observer Z1 microscope (Zeiss, Thornwood, NY) with a Plan Apo 63X/1.4 Oil DIC M27 objective, captured on an Axiocam MRM REV 3 camera, and processed using Axiovision software (Zeiss, Thornwood, NY).

Histological Grading

[00184] Livers sections from wild-type (wt) and SIRT3KO mice were fixed, stained with hematoxylin & eosin, Masson's trichrome, or reticulin, and blind-scored for inflammation, steatosis, ballooning, and fibrosis. Inflammation was graded based on the average number of inflammatory aggregates in 10X field: none, 0; minimal (<1 inflammatory aggregate), 1; mild (1 inflammatory aggregate), 2; moderate (2-3 inflammatory aggregates), 3; severe (>3 inflammatory aggregates), 4. Steatosis was graded based on the percent of overall fat in normal areas: none, 0; minimal (<5%), 1; mild (5-33%), 2; moderate (33-66%), 3; severe (66-100%), 4. Hepatocyte ballooning was graded based on the prevalence of cells: rare, 1; mild, 2; moderate, 3; severe, 4.

Fibrosis was graded based on the location and severity of collagen: centrizonal, 1; centrizonal and periportal, 2; bridging, 3; cirrhosis, 4.

Immunoassays

[00185] Mouse plasmas were measured for cytokines and hormones using electrochemiluminescent immunoassay technology from Meso Scale Discovery (MSD, Gaithersburg, MD). Measurements were performed with an MSD SI-2400 imager for the determination of multiplex panels of analytes in 96-well plates using reagent kits from MSD. Assays included a seven-plex mouse proinflammatory panel for IFN- γ , IL-10, IL-12p70, IL-1 β , IL-6, TNF- α , and KC chemokine; a biplex mouse metabolic panel for insulin and leptin; and single panels for mouse adiponectin and resistin. Additional measurements were performed by conventional spectrophotometric methodology on a Beckman-Coulter DxC600 autoanalyzer.

Metabolomics, Metabolite and Lipid Analysis from Tissue and Plasma

[00186] After hepatic protein precipitation with methanol, supernatants were dried, esterified with hot, acidic methanol (acylcarnitines) or *n*-butanol (amino acids), and then analyzed by tandem mass spectrometry (Quattro Micro, Waters Corporation, Milford, MA). Acylcarnitines were assayed by adapting described methods for analysis of amino acids in dried blood spots (42, 43). Organic acids were extracted in ethyl acetate, dried, and then converted to their trimethyl silyl esters using N,O-bis (trimethylsilyl) trifluoroacetamide, with protection of alpha-keto groups by oximation with ethoxyamine hydrochloride, followed by gas chromatography-MS (Trace DSQ, Thermo Fisher Scientific, Waltham, MA) (44). For lipid analysis, total lipids were extracted from tissue, cells, or plasma by the method of Folch-Lees (45) or Bligh-Dyer (46). Individual lipoproteins and lipid classes were separated by thin layer chromatography (TLC) on Silica Gel 60 A plates and visualized with rhodamine 6G. Lipid ester bands were scraped from the TLC plates and methylated using BF₃/methanol as described by Morrison and Smith (47). For acyl-CoA measurements, hepatic acyl CoA esters were extracted, analyzed and purified based on previously published methods (48-50). The acyl CoAs were analyzed by flow injection analysis using positive electrospray ionization on Quattro micro, triple quadrupole mass spectrometer (Waters, Milford, MA) employing methanol/water (80:20, v:v) containing 30 mM ammonium hydroxide as the mobile phase. Spectra were acquired in the multichannel acquisition mode monitoring the

neutral loss of 507 amu (phosphoadenosine diphosphate) and scanning from m/z 750-1060. Heptadecanoyl CoA and $^{13}\text{C}_3$ malonyl CoA were employed as internal standards for the long and short chain CoA esters, respectively. The endogenous CoAs were quantified using calibrators prepared by spiking liver homogenates with authentic CoAs (Sigma-Aldrich, St. Louis, MO) having saturated acyl chain lengths C_0 - C_{18} and unsaturated species of $\text{C}_{16:1}$, $\text{C}_{18:2}$, $\text{C}_{18:1}$ and $\text{C}_{20:4}$. Corrections for the heavy isotope effects, mainly ^{13}C , to the adjacent $m+2$ spectral peaks in a particular chain length cluster were made empirically by referring to the observed spectra for the analytical standards.

Microarrays and RT-PCR

[00187] Livers were removed from wt and SIRT3KO mice (3-months old, standard diet, fasted 24 h, $n=3/\text{genotype}$) and RNA was extracted in Trizol (Invitrogen, Carlsbad, CA) according to the manufactures instructions. The samples were prepared using Affymetrix WT cDNA Synthesis and Amplification Kits and WT Target Labeling and Control Reagents according to the manufacturer's instructions. Labeled cDNA samples were hybridized, stained, and scanned to Affymetrix Mouse Gene 1.0 ST arrays according to manufacturer's instructions. Raw intensities from the CEL files were analyzed using Affymetrix Power Tools (APT, version 1.10.1) to generate an RMA [robust multi-array average (51)] intensity on a log2 scale for each probe set and various quality metrics. The perfect match (PM) intensities per probe set were defined as (i) background corrected; (ii) quantile-normalized (to make the distribution of intensities the same for all arrays); and (iii) summarized for each probe set using a robust fit of linear models.

[00188] For reverse transcription-polymerase chain reaction (RT-PCR), RNA (2 μg) was reverse transcribed with SuperScript III reverse transcriptase and oligo(dT) primers (Invitrogen, Carlsbad, CA) to generate cDNA. Real-time quantitative polymerase chain reaction (PCR) was performed with a PerkinElmer ABI Prism 7700 (Applied Biosystems, Foster City, CA) and SYBR green detection of amplified products. Each 10 μl PCR reaction mix contained 0.6 μl of cDNA, 5 μl of 2 $\times$ SYBR green master mix (Qiagen, Germantown, MD), and 10 pmol of each primer. Relative mRNA abundance was normalized to the internal standard cyclophilin. Oligonucleotide primers were designed using qPrimerDepot, NCI, NIH. Primer sequences for various genes are listed in Table 1.

Table 1

Gene	Sequence
<i>Agpat4</i>	F: 5'- CATCTGGCCCATCAACAAG -3' (SEQ ID NO:30) R: 5'- GACCACCACTCCAGAAGCAT -3' (SEQ ID NO:31)
<i>Agpat7</i>	F: 5'- CTACAGAAGGCTGGGCTGTC -3' (SEQ ID NO:32) R: 5'- GTCTGAGGATCCGAGAGCTG -3' (SEQ ID NO:33)
<i>Cyclophilin</i>	F: 5'- TGGAAGAGCACCAAGACAGACA -3' (SEQ ID NO:34) R: 5'- TGCCGGAGTCGACAATGAT -3' (SEQ ID NO:35)
<i>Dgat1</i>	F: 5'- GACGGCTACTGGGATCTGA -3' (SEQ ID NO:36) R: 5'- TCACCACACACCAATTCAGG -3' (SEQ ID NO:37)
<i>Dgat2</i>	F: 5'- CGCAGCGAAAACAAGAATAA -3' (SEQ ID NO:38) R: 5'- GAAGATGTCTTGGAGGGCTG -3' (SEQ ID NO:39)
<i>Gpat1</i>	F: 5'- CCTCTTTTGCCACAACATCA -3' (SEQ ID NO:40) R: 5'- CCAAGCTTGTGAATCAAGG -3' (SEQ ID NO:41)
<i>Gpat2</i>	F: 5'- GTTGCCATTGCCTATGACCT -3' (SEQ ID NO:42) R: 5'- GTCTCCGAAAGACAGCCAAG -3' (SEQ ID NO:43)
<i>Leptin</i>	F: 5'- TCAAGACCATTTGTCACCAGG -3' (SEQ ID NO:44) R: 5'- TGAAGCCCAGGAATGAAGTC -3' (SEQ ID NO:45)
<i>Lipin1</i>	F: 5'- TTTTGCATACAAAGGCAGC -3' (SEQ ID NO:46) R: 5'- TTCACCGTCACAAACACCTG -3' (SEQ ID NO:47)

<i>Lipin2</i>	F: 5'- GGTTCAGGAAAGCTCGTTGA - 3' (SEQ ID NO:48) R: 5'- GCCCACATAATTCATGGTTTG -3' (SEQ ID NO:49)
<i>Lipin3</i>	F: 5'- AGCACTGCTGGGAAAAACAG - 3' (SEQ ID NO:50) R: 5'- CTACTGTGGGACCCTTGGAC -3' (SEQ ID NO:51)
<i>Mgat1</i>	F: 5'- GATCTTCAGGGAAGCGACAG - 3' (SEQ ID NO:52) R: 5'- GGTCCCCACACTAAGGGTTT -3' (SEQ ID NO:53)
<i>Scd1</i>	F1: 5'- GCTCTACACCTGCCTCTTCG - 3' (SEQ ID NO:54) R1: 5'- CAGCCGAGCCTTGTAAGTTC -3' (SEQ ID NO:55) F2: 5'- CCTCCTGCAAGCTCTACACC -3' (SEQ ID NO:56) R2: 5'- CAGCCGTGCCTTGTAAGTTC -3' (SEQ ID NO:57)
<i>Scd2</i>	F1: 5'- TCCTGCAAGCTCTACACCTG - 3' (SEQ ID NO:58) R1: 5'- TGCCTTGTATGTTCTGTGGC -3' (SEQ ID NO:59) F2: 5'- CTGACCTACCTCCACGGGTA -3' (SEQ ID NO:60) R2: 5'- ACGTCATCTGGGACATAGGC -3' (SEQ ID NO:61)
<i>SREBP1a</i>	F: 5'- GGCCGAGATGTGCGAACT -3' (SEQ ID NO:62) R: 5'- TTGTTGATGAGCTGGAGCATGT -3' (SEQ ID NO:63)
<i>SREBP1c</i>	F: 5'- GAACAGACACTGGCCGAGAT -3' (SEQ ID NO:64) R: 5'- GTTGTGATGAGCTGGAGCA -3' (SEQ ID NO:65)
<i>HSL</i>	F: 5'- ACCCATAGTCAAGAACCCCTTC -3' (SEQ ID NO:66) R: 5'- TCTACCACTTTCAGCGTCACCG -3' (SEQ ID NO:67)

ACC
 F: 5'- TGACAGACTGATCGCAGAGAAAG -3'
 (SEQ ID NO:68)
 R: 5'- TGGAGAGCCCCACACACA -3'
 (SEQ ID NO:69)

FAS
 F: 5'- GCTGCGGAAACTTCAGGAAAT -3'
 (SEQ ID NO:70)
 R: 5'- AGAGACGTGTCACTCCTGGACTT -3'
 (SEQ ID NO:71)

F: forward; R: reverse

Statistical Analyses

[00189] Results are given as the mean $\pm$ standard error. Statistical analyses represent a one-tailed Students *t*-test or a Wilcoxon rank-sum test and null hypotheses were rejected at 0.05. For microarray analyses, linear models were fit for each gene to estimate cell-type effects and associated significance using the limma package (R/Bioconductor, Smyth 2008). Moderated *t*-statistics and the associated *p*-values were calculated, as well as B-statistics (logOdds), the log posterior odds ratio. *P*-values were adjusted for multiple testing by controlling for false-discovery rate (FDR) using the Benjamini-Hochberg method (52) and controlling for family-wise error rate using the Bonferroni correction (adjP). Gene annotations were retrieved from Affymetrix [*Mus musculus* v9 assembly (July 2007) and NetAffx annotation release 27 (Nov 2008)]. Differential expression was defined as fold-change, *p*-value, and log odds.

Human Patient Liver Samples

[00190] Human liver samples were collected from donor livers or recipient livers during liver transplantation at the Liver Tissue Procurement and Distribution System, University of Minnesota. All of samples were collected and quickly frozen in liquid nitrogen. All samples were diagnosed as non-alcoholic steatohepatitis by histological analyses. Normal healthy liver samples were provided by the Liver Tissue Procurement and Distribution System, and collected from the part of donor livers that were not used for transplantation. The age, sex, AST levels, and NASH status of various subjects are presented in **Table 2**.

Table 2

Case #	Age	Sex	AST (U/L)	NASH
647	50	Female	13	Positive
801	44	Female	46	Positive
935	60	Female	30	Positive
970	62	Female	25	Positive
1098	59	Female	64	Positive

Protocol

[00191] The UCSF Committee on Human Research approved all protocols. Informed consent was obtained from all participants for DNA isolation and plasma collection.

Study Design

[00192] The design was a cross-sectional cohort study of nonalcoholic fatty liver disease. The Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN) was established by the National Institute of Diabetes & Digestive and Kidney Diseases (NIDDK) in 2002 to assess the natural history, pathogenesis, and therapy of NAFLD in the United States (53). The baseline demographic and clinical data were obtained from adult subjects enrolled in the NAFLD Database, an observational cohort study. These studies were conducted by 8 Clinical Centers and a central Data Coordinating Center. Both study protocols were approved by all participating center IRBs and an independent Data and Safety Monitoring Board. Each participant provided written informed consent. Participants for the current study were those with a biopsy-proven diagnosis of NAFLD. In order to limit genetic heterogeneity, the genetic association study was limited to non-Hispanic Caucasians adults at least 18 years of age (n=834).

Measurements

[00193] Demographic and clinical characteristics are presented in **Table 5**. Demographic information collected during screening interviews as part of the registration process included age, sex, waist circumference, weight and height. Body mass index (BMI) was calculated as the weight (kg) divided by the height (meters) squared. Comorbid conditions (i.e., hypertension, type 2 diabetes), was obtained from the medical record.

Smoking status (either current or ever) was determined by self-report. Baseline lipid measurements were obtained by peripheral venipuncture after a minimum of 10 hours of fasting. Serum was used to determine alanine aminotransferase (ALT, U/L), aspartate aminotransferase (AST, U/L), gamma glutamyl transferase (GGT, U/L), alkaline phosphatase (ALP, U/L), glucose (mg/dL), insulin (mg/dL), total plasma triglyceride (TG, mg/dL), total plasma cholesterol (TC, mg/dL), low density lipoprotein cholesterol (LDLC, mg/dL) and high density lipoprotein cholesterol (HDLC, mg/dL) using the clinical laboratories at each study site. Low-density lipoprotein cholesterol (LDL) was calculated using the Friedewald Formula.

[00194] Presence of the metabolic syndrome was established using two sets of criteria. The first was using the International Diabetes Foundation (IDF) criteria (54) where possessing any three of the following risk factors met the criteria for Metabolic Syndrome: TC >200mg/dl; LDLC >160mg/dl; HDLC <40mg/dl for men or <50mg/dl for women; TG >200mg/dl; blood glucose >126mg/dl or a diagnosis of type 2 diabetes; a blood pressure >140/90 mmHg or a diagnosis of hypertension; or a waist circumference of > 90cm for men or > 80cm for women. In order to decrease the heterogeneity in the genetic etiology of Metabolic Syndrome due to phenocopy from type 2 diabetes, the IDF criteria were refined by excluding participants with type 2 diabetes.

[00195] Histology data are presented in **Table 5**. The NASH CRN Pathology Committee developed and validated a feature-based histological scoring system that encompasses the spectrum of lesions of NAFLD (55). Liver biopsy slides from subjects were read centrally by the Pathology Committee during which biopsies were rigorously evaluated according to the published scoring system (55). Steatosis grade was scored according to amount (%) of biopsy occupied using a four-point scale. A diagnosis of NAFLD required the presence of $\geq 5\%$ steatosis. Fibrosis was staged from 0 to 4, with 0 = none; 1a = mild zone 3 (central) perisinusoidal fibrosis, 1b = moderate zone 3 perisinusoidal fibrosis, 1c = periportal and portal fibrosis (zone 1) only; 2 = both perisinusoidal and periportal or portal fibrosis; 3 = bridging fibrosis and 4 = cirrhosis. Diagnostic determinations of each biopsy were also assigned. Categories utilized were steatosis, steatohepatitis, and cirrhosis.

- [00196] Blood collection and genotyping: Blood samples were obtained by venipuncture and genomic DNA was extracted from peripheral blood lymphocytes (Invitrogen, Carlsbad, CA). Genotyping was performed blinded to clinical status; positive and negative controls were included. DNA samples were quantitated with a Nanodrop Spectrophotometer (ND-1000) and normalized to a concentration of 50 ng/ μ L (diluted in 10 mM Tris/1 mM EDTA). Samples were genotyped by TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA) and processed according to the standard protocol.
- [00197] Single nucleotide polymorphisms interrogating the SIRT3 gene were selected in two ways: 1) tagging SNPs (tagSNPs) were selected to capture a large portion of the variance in each candidate gene while reducing redundancy in SNP marker information (i.e., minor allele frequency > 5%, linkage disequilibrium [LD] >0.80) performed using Snagger (56) ($n_{\text{tagSNP}} = 10$); and 2) incorporation of non-synonymous SNPs reported in dbSNP (www.ncbi.nlm.nih.gov/entrez) ($n=3$). Potential functional roles of SNPs were examined using PUPASuite 2.0 (57), a comprehensive search engine that tests a series of functional effects (i.e., non-synonymous changes, altered transcription factor binding sites, exonic splicing enhancing or silencing, splice site alterations, microRNA target alterations). For those SNPs resulting in alterations in the amino acid sequence, the functional impact of the change was explored using PolyPhen (Polymorphism Phenotyping; [http://genetics\(dot\)bwh\(dot\)harvard\(dot\)edu/pph/](http://genetics(dot)bwh(dot)harvard(dot)edu/pph/)), a tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein using empirical rules applied to the sequence, in addition to phylogenetic and structural information characterizing the substitution.
- [00198] Rigorous quality control (QC) procedures were adopted to ensure high quality data for downstream analyses. The first QC step was to exclude SNPs with poor quality data. SNP call rate was set at 95%, resulting in the exclusion of one SNP (rs11246007). Given the goal of identifying common genetic risk alleles for NAFLD and/or the Metabolic Syndrome) and the allele frequency estimates deposited in the public databases used to select SNPs for study, the second QC step was to excluded SNPs with a minor allele frequency (MAF) less than 5% from subsequent analyses; 2 SNPs were excluded (rs3020901 and rs551570). The third QC step involved the inclusion of replicate samples. Three samples, a mother-father-offspring trio, were included on each

96-well sample plate used to genotype participant DNA samples. Genotype concordance for the three samples across the 96-well sample plates was determined to be 100% for all remaining SNPs. Finally, because deviations from Hardy-Weinberg expectations, a measure of quality control, is not valid as a quality control estimate in cohort studies of affected individuals, informative missingness in genotype data was evaluated; no evidence of informative missingness was detected. A summary of the SNPs is presented in Table 3.

Table 3

SIRT3 SNP Measurement and Quality Control Assessments

rsID	Position	SNP Call Rate	MAF	Functional Change
rs3825075	207140	99.2%	0.266	
rs3847648	208613	99.0%	0.036	
rs536715	208640	98.9%	0.119	
rs3020901	208906	98.9%	0.000	G369S
rs11246007	211584	89.1%	n/a	
rs3782115	213272	99.2%	0.286	
rs12222188	214063	0.00%	n/a	
rs551570	214088	98.8%	0.000	T320N
rs1023430	214393	99.3%	0.205	
rs7934919	214832	99.3%	0.218	
rs11246020	223067	99.4%	0.216	V208I
rs1056098	225619	98.9%	0.003	
rs28365927	226091	97.5%	0.147	R80W

Abbreviations: rsID, reference sequence identifier; Position, nucleotide position for the variation; SNP, single nucleotide polymorphism; MAF, minor allele frequency.

Statistical Analyses

[00199] Intercooled Stata 9.2 for Windows was used for statistical analysis. Descriptive statistics and frequency distributions were generated on the sample demographic and clinical characteristics, and biochemical measurements.

[00200] Allele and genotype frequencies were determined by gene counting. Measures of linkage disequilibrium, D' and r^2 , were computed from the genotypes with Haploview 4.2 (<http://www.broad.mit.edu/mpg/haploview/>). LD-based haplotype block definition was based on D' confidence interval (58). Gene structure for *SIRT3* was rendered with FancyGene 1.4 (genomic sequence accession NM_012239).

[00201] For association tests, four genetic models were assessed for each SNP: dominant, recessive, log additive, and codominant. Barring trivial improvements ($\Delta < 10\%$) the genetic model that best fit the data, by maximizing the significance of the p-value was selected for each SNP. The dominant model was generally found to fit the data best. Both un-adjusted and adjusted associations were calculated; logistic regression was used to control for age, sex, BMI, other relevant co-morbid conditions (i.e., Metabolic Syndrome, NAFLD). Genetic model fit and both unadjusted and adjusted odds ratios and 95% confidence intervals (95% CI) for the SNP were also calculated. Linear regression was used to model the relationship between genotype and continuous outcome variables while controlling for relevant covariates.

[00202] Permutation tests were used to adjust the type-I error rate against inflation due to testing of multiple SNPs. To account for multiple comparisons, outcome status was permuted 10,000 times to determine the likelihood that our findings were due to chance. Permutation analyses were done using Intercooled Stata 9.2 for Windows.

[00203] Four SNPs in SIRT3 were found to occur in the same haploblock (rs1023430, rs7934919, rs11246020, rs2836592); therefore, haplotype analyses were conducted in order to localize the association signal within the gene and to determine if haplotypes improved the strength of the association with the outcome. Haplotypes were constructed using the program PHASE version 2.1 (59) ([http://www\(dot\)stat\(dot\)washington\(dot\)edu/stephens/software.html](http://www(dot)stat(dot)washington(dot)edu/stephens/software.html)). In order to improve the stability of haplotype inference, the haplotype construction procedure was repeated 5 times using different seed numbers with each cycle; only haplotypes that were inferred with probability estimates greater than or equal to 0.9 were retained for downstream analyses. Haplotypes with frequency estimates of 1% or less were grouped into a single category.

Cell Biology, Immunoprecipitation and SIRT3 Activity Assay

[00204] Wild-type (Wt) and recombinant human SIRT3 was cloned into pTrcHis2 expression vectors, generated by standard PCR-based cloning strategies and verified by DNA sequencing. For immunoprecipitation experiments, murine liver mitochondria were prepared and purified as described (Graham (2001) *Current Protocols in Cell Biology*, Bonifacio et al., eds. Chapter 3, Units 3.3 and 3.4; and Hirschey et al. (2009) *Methods Enzymol.* 457:137). Briefly, mitochondria were lysed by sonication and

resuspended in a low-stringency immunoprecipitation (IP) buffer (0.05% non-ionic detergent NP-40, 50 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris-HCl, pH 7.4, 10 mM nicotinamide, 1 μ M trichostatin A, protease inhibitor cocktail (Roche)). For radioactive SIRT3 activity assays, recombinant SIRT3 proteins were expressed and purified as described (Hirschey et al. (2009) *Methods Enzymol.* 457:137). Deacetylase assays were performed in 100 μ l of deacetylase buffer (4 mM, MgCl_2 , 0.2 mM dithiothreitol, 50 mM Tris-HCl, pH 8.5) containing 50 ng of recombinant SIRT3, NAD^+ and [^3H] histone H4 peptide substrate. The substrate was prepared by *in vitro* acetylation of a histone H4 N-terminal peptide (amino acids 1–25) with radiolabeled acetyl-CoA and recombinant PCAF (Heltweg et al. (2005) *Methods* 36:332). Deacetylation reactions were conducted at 37 °C under gentle agitation, and stopped by adding 25 μ l of stop solution (0.2 M HCl, 0.32 M acetic acid). Radioactivity was extracted into 500 μ l ethyl acetate by vortexing for 15 s. After centrifugation at 14,000g for 5 min, 450 μ l of the ethyl acetate fraction was mixed with 5 ml of scintillation fluid (Perkin Elmer), and the radioactivity was measured with a liquid scintillation counter (Beckman LS6000).

RESULTS

[00205] Chronic high-fat diet feeding induced mitochondrial protein

hyperacetylation. Mitochondrial protein acetylation is regulated by high-fat diet feeding (Fig. 1A). Western blot analysis of hepatic mitochondrial extracts with an anti-acetyllysine antibody revealed that chronic high fat feeding (13 weeks) but not acute high fat feeding (1 week) induced global mitochondrial protein acetylation. High-fat diet feeding accelerates the development of metabolic abnormalities, including central obesity, insulin resistance, hyperlipidemia, hyperglycemia, hypertension, and hepatic steatosis, defined as the metabolic syndrome (10). Because high-fat diet feeding leads to mitochondrial protein hyperacetylation, the possibility that the major nicotinamide adenine dinucleotide (NAD^+)-dependent mitochondrial protein deacetylase, SIRT3 [reviewed in (11, 12)], might be implicated in the development of the metabolic syndrome associated with high-fat feeding was tested.

[00206] SIRT3 is reduced with chronic high-fat diet feeding. Of the seven mammalian sirtuins (SIRT1-SIRT7), SIRT3 is the primary regulator of mitochondrial protein acetylation and mice lacking SIRT3 have hyperacetylated mitochondrial proteins (3).

Because increased hepatic mitochondrial protein acetylation during high-fat diet feeding, was observed, the possibility that SIRT3 expression might be suppressed in the liver of wt mice fed a high-fat diet was tested. Hepatic SIRT3 expression was initially increased in response to a one-week high-fat diet feeding in wt mice (Fig. 1B) and mitochondria protein acetylation was unchanged (Fig. 1B). However, hepatic SIRT3 was suppressed with chronic high-fat diet feeding (13 weeks) compared to a standard diet (Fig. 1B). Additionally, the acetylation level of a specific SIRT3 target, LCAD, was significantly increased in wt mice fed chronically on a high-fat diet. To test whether this downregulation of SIRT3 expression also occurs in humans, hepatic SIRT3 protein expression was measured in humans liver samples from patients with non-alcoholic steatohepatitis, a condition frequently associated with the Western diet in humans. Measurement of SIRT3 expression revealed a severe reduction (3.5-fold) in hepatic SIRT3 expression in human fatty livers compared to normal liver controls (Fig. 1C).

[00207] **SIRT3KO mice develop diet-induced obesity and insulin resistance.** To test the possible role of SIRT3 and chronic mitochondrial protein hyperacetylation during high fat feeding, SIRT3KO mice, which show constitutive mitochondrial protein hyperacetylation (3, 4), were placed on a high-fat diet. No early differences in weight were noted between wt and SIRT3KO mice (Fig. 2A). However, SIRT3KO mice developed diet-induced obesity at an accelerated rate when maintained on a high-fat diet, (Fig. 2A). SIRT3KO mice weighed 7% more than wt mice ($p=0.075$) by 18 weeks, 10% more by 33 weeks and 15% more by 52 weeks (p -values: 0.042 and 0.018, respectively). Dual energy X-ray absorptiometry (DEXA) analyses showed that the increased weight in SIRT3KO mice was due to increased adiposity.

[00208] To determine the relative contributions of energy intake (food consumption) and energy expenditure (activity, respiration) to increased obesity, metabolic cage analysis was performed in 3-month-old wt and SIRT3KO mice. Oxygen consumption (VO_2) was 12% lower in SIRT3KO mice during light ($p=0.018$) and 9% lower during dark ($p=0.043$) cycles (Fig. 2B). Additionally, SIRT3KO mice had lower CO_2 exhalation (VCO_2) during both light and dark cycles (14% lower during each cycle, $p=0.041$ and 0.026, respectively) (Fig. 2C). However, no significant differences were observed in the respiratory exchange ratio (RER), food intake, or spontaneous activity. Thus, it was

concluded that diet-induced obesity in SIRT3KO mice was due to lower energy expenditure.

[00209] Insulin resistance is a hallmark of obesity and the metabolic syndrome (10, 13, 14). Glucose tolerance and insulin sensitivity were measured in wt and SIRT3KO mice. Obese 12-month-old SIRT3KO mice fed a high-fat diet exhibited hyperglycemia during glucose-tolerance testing and were insulin resistant by insulin tolerance testing (Fig. 2D, 2E). To assess the role of SIRT3 on insulin resistance in the absence of obesity, glucose and insulin tolerance were measured in non-obese 12-month old standard diet fed wt and SIRT3KO mice. Marked hyperglycemia was observed in SIRT3KO mice upon intraperitoneal glucose injection (52% increase in area under the curve) and marked insulin resistance upon intraperitoneal insulin injection (34% increase in area under the curve, Fig. 2F and 2G, respectively). These data demonstrate lack of SIRT3 and mitochondrial protein hyperacetylation lead to disrupted insulin signaling and insulin resistance, even in the absence of obesity. Furthermore, high-fat diet feeding accelerates the development of insulin resistance and glucose intolerance both in wt and SIRT3KO mice.

[00210] **Hepatic steatosis and non-alcoholic steatohepatitis in SIRT3KO mice.**

Abnormal hepatic lipid accumulation has been proposed as possible mechanism for the development of insulin resistance in the metabolic syndrome (10). Lipids were measured in wt and SIRT3KO mice fed a high-fat diet. Staining of liver sections for total lipids with Oil Red O showed high lipid levels in wt mice fed a high-fat diet but even higher levels in SIRT3KO mice fed the same diet (Fig. 3A, 3D and S4). Direct measurement of lipids in liver tissue homogenates showed that 3-month old SIRT3KO mice had 38% more hepatic triglycerides than wt mice, and 41% more hepatic cholesterol esters (Fig. 3B). Metabolomic analyses of 3-month old wt and SIRT3KO mice fed a high-fat diet revealed increased accumulation of hepatic long-chain acylcarnitine species in SIRT3KO mice, but not organic acids or amino acids. These results are consistent with the previously demonstrated reduced fatty acid oxidation in SIRT3KO mouse livers (4).

[00211] Because SIRT3 deficiency reduces fatty acid oxidation and results in accumulation of hepatic lipids, an effort was made to determine if SIRT3 overexpression is protective against hepatic lipid accumulation. Recombinant adenoviruses containing the cDNA encoding *Sirt3*, or green fluorescent protein (GFP) as a control, were injected

into the tail veins of wt mice, and hepatic tissue homogenates were assessed for total lipid levels. Hepatic triglyceride levels were 50% lower in wt mice injected with SIRT3-expressing adenovirus than in mice injected with the GFP-expressing virus (Fig. 3C). These data demonstrate that SIRT3 overexpression reduces hepatic lipids and suggest a possible therapeutic role for enhanced SIRT3 expression in the management of the metabolic syndrome.

[00212] Histological analysis of liver sections from 12-month old mice fed a high-fat diet showed higher lipid levels in SIRT3KO mice than in wt mice (see above) with features consistent with steatohepatitis. Aged SIRT3KO mice had more macrovesicular steatosis, which was evaluated based on the percentage of fat in a histologic section of the liver parenchyma. Strikingly, 12-month-old SIRT3KO mice also had more lobular lymphoplasmacytic inflammation (Fig. 3D), as determined by histological scoring of the average number of lymphoplasmacytic aggregates in five 100X fields. Additionally, a trend toward more hepatocyte ballooning degeneration and hepatic fibrosis was observed in the SIRT3KO mice than in wt mice (Fig. 3D).

[00213] Because signs of hepatic inflammation, which has been linked to obesity and metabolic dysfunction (15), were observed, serum inflammatory cytokines were measured from wt and SIRT3KO mice fed a high-fat diet. It was found that aged SIRT3KO mice had markedly higher levels of inflammatory cytokines than wt mice (Fig. 3E). In particular, interferon- γ (3-fold), IL-10 (3-fold), IL-12p70 (12-fold), IL-6 (10-fold), TNF- α (1.7-fold), and CXCL1 (1.2-fold) were all significantly higher in obese 12-month-old SIRT3KO mice fed a high-fat diet compared to wt mice (Fig. 3E), but were unchanged in non-obese 3-month-old SIRT3KO mice. These data show that lack of SIRT3 and the accompanying mitochondrial protein hyperacetylation is linked to increased cytokines and the development of steatohepatitis in aged, obese mice.

[00214] **SIRT3KO mice develop hyperlipidemias with high-fat diet feeding.** Obesity, insulin resistance, and the metabolic syndrome often coincide with lipid abnormalities, including hypertriglyceridemia, hypercholesterolemia, and other dyslipidemias (10). Serum lipid measurements from 12-month-old SIRT3KO mice fed a high-fat diet revealed higher levels of triglyceride (97% increase) and cholesterol (141% increase) than in wt mice (Table 4). Higher levels of low-density lipoproteins (LDL, 60% increase) and very-low-density lipoproteins (VLDL, 100% increase) were also found in

SIRT3KO mice (**Table 4**). No differences in HDL levels were detected (Table 1). Consistent with the insulin resistance data discussed above, fasting insulin levels were increased in 12 month-old SIRT3KO mice (285% increase), but levels of leptin, adiponectin, and resistin were unchanged (**Table 4**).

Table 4

Metabolic Parameter	Wildtype	SIRT3 KO	P Value
Triglyceride (mg/dl)	108.4 ± 5.7	213.2 ± 60.1	< 0.05
Cholesterol (mg/dl)	119.4 ± 11.3	287.2 ± 47.1	< 0.05
HDL (mg/dl)	65.0 ± 1.9	60.0 ± 4.3	n.s.
LDL (mg/dl)	112.7 ± 10.8	179.9 ± 39.4	< 0.05
VLDL (mg/dl)	21.6 ± 1.1	42.6 ± 12.0	< 0.05
Glucose (mg/dl)	101.2 ± 6.3	95.2 ± 1.9	n.s.
Insulin (ng/ml)	0.7 ± 0.2	2.7 ± 0.9	< 0.05
Leptin (ng/ml)	100.2 ± 43.9	156.2 ± 43.8	n.s.
Adiponectin (µg/ml)	15.6 ± 0.4	14.9 ± 0.9	n.s.
Resistin (µg/ml)	8.6 ± 1.6	8.8 ± 2.1	n.s.

[00215] **Table 4.** Metabolic parameters in wt and SIRT3KO mice. Serum triglyceride, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), glucose, insulin, leptin, adiponectin, and resistin concentrations were determined in 12-month-old wt and SIRT3KO mice fed a high-fat diet ad libitum (fasted 24 h, n=5 mice/genotype).

[00216] **Increased SCD1 expression in SIRT3KO mice contributes to the pathogenicity.** To characterize possible changes in nuclear gene expression that occur as a consequence of SIRT3 deficiency, microarray analyses were performed on livers of wt and SIRT3KO mice. Because high-fat diet feeding is associated with large changes in gene transcription (16), and to identify gene expression changes unique to SIRT3 deficiency, gene expression was compared in wt and SIRT3KO mice fed a standard diet. Of 28,800 genes tested, 18 were significantly upregulated (increased greater than 1.2-fold, $p < 0.001$), and nine were significantly downregulated (decreased greater than 1.2-fold, $p < 0.001$) in liver from SIRT3KO mice. Interestingly, the most highly induced gene (1.7-fold increase) was stearoyl-CoA desaturase 1 (SCD1). SCD1 is a fatty acid

synthesis enzyme that catalyzes the biosynthesis of monounsaturated long-chain acyl CoAs from saturated long-chain acyl CoAs (17). SCD1 has been implicated in the pathogenesis of the metabolic syndrome in mice (18) and humans (19). Increased SCD1 mRNA abundance was independently validated using quantitative RT-PCR and a five-fold increase in mRNA was detected in SIRT3KO mice in comparison to wt mice (Fig. 4A). Surprisingly, SCD1 was the only mRNA for lipogenic genes whose expression was increased (Fig. 4A).

[00217] To determine if increased SCD1 expression correlated with increased SCD1 activity, the plasma desaturation index was measured. This index represents the ratio of serum palmitoleate:palmitate (16:1/16:0) or oleate:stearate (18:1/18:0) and is a well-documented marker for SCD1 activity (18). In the plasma of SIRT3KO mice fed a standard diet, the free fatty acid desaturation indexes for C16:1/C16:0 and C18:1/C18:0 were increased (213% and 62%, respectively) (Fig. 4B). Triglyceride desaturation indices were also increased (66% for triglyceride C16:1/C16:0) (Fig. 4B).

[00218] Because SCD1 has been implicated in the pathogenesis of the metabolic syndrome (18, 19) and mice lacking SCD1 (SCD1KO) are protected from hepatic steatosis (20), mice lacking both SIRT3 and SCD1 (dKO) were generated. Under standard-diet fed conditions, no differences in hepatic lipids were observed between wt, SIRT3KO, SCD1KO or dKO mice (Fig. 4C). However, high-fat diet-induced hepatic steatosis was observed in wt mice and was exacerbated in SIRT3KO mice as described above. Hepatic steatosis was absent in SCD1KO mice fed a high-fat diet, as previously reported (21). Furthermore, mice lacking both SIRT3 and SCD1 showed markedly reduced hepatic triglycerides with high-fat diet feeding (Fig. 4C), demonstrating loss of SCD1 ameliorates hepatic steatosis induced by SIRT3 deficiency and mitochondrial protein hyperacetylation.

[00219] **Genetic association of a single nucleotide polymorphism (SNP) in *SIRT3* with the metabolic syndrome in humans.** Because mice lacking SIRT3 developed the metabolic syndrome at an accelerated pace compared to wt mice fed a high-fat diet, tests were conducted to determine whether variability in the *SIRT3* gene was correlated to increased susceptibility for developing the metabolic syndrome in humans. Single nucleotide polymorphisms (SNPs) from the *SIRT3* gene were interrogated in DNA samples from a cross-sectional cohort of patients with nonalcoholic fatty liver disease

[obtained from the Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN), **Table 5**]. Genotype frequencies for 13 *SIRT3* SNPs were measured, seven of which passed all quality control criteria (**Table 3**). Two SNPs in near-perfect linkage disequilibrium (rs7934919 and rs11246020; D' : 1.0, $r^2 = 0.98$, Fig. 5A) showed an association with a diagnosis of the metabolic syndrome (MetSyn) and non-alcoholic fatty liver disease (NAFLD). The strength of these associations was virtually unchanged following adjustment for age, sex, and BMI.

Table 5**Demographic, Clinical, and Histological Characteristics of Participants**

Characteristics	Mean $\pm$ SD or Frequency (%)
Age (years)	48.9 $\pm$ 10.83
Sex (% female)	64.0%
Body Mass Index, BMI (kg/m ²)	34.6 $\pm$ 6.53
Waist circumference (cm)	109.7 $\pm$ 14.03
Alanine aminotransferase, ALT (U/L)	65.3 $\pm$ 44.80
Aspartate aminotransferase, AST (U/L)	49.1 $\pm$ 32.00
Gamma glutamyl transferase, GGT (U/L)	71.7 $\pm$ 89.73
Alkaline phosphatase, ALP (U/L)	89.5 $\pm$ 35.02
Glucose (mg/dL)	107.0 $\pm$ 36.45
Insulin (mg/dL)	22.5 $\pm$ 23.27
HOMA-IR	6.4 $\pm$ 8.48
Total plasma triglyceride, TG (mg/dL)	176.9 $\pm$ 115.10
Total plasma cholesterol, TC (mg/dL)	193.5 $\pm$ 41.59
Low density lipoprotein cholesterol, LDLC (mg/dL)	116.8 $\pm$ 35.96
High density lipoprotein cholesterol, HDLC (mg/dL)	44.5 $\pm$ 12.98

Nonalcoholic fatty liver disease (NAFLD) diagnosis	
Steatosis	32.3%
Steatohepatitis	42.4%
Cirrhosis	10.6%
NAFLD Activity Score, NAS	
0–4	54.3%
5–8	45.7%
Steatosis grade	
0–1	45.6%
2–3	54.4%
Fibrosis stage	
0–1	49.7%
2–4	50.3%
Lobular inflammation	
0	0.7%
1	54.8%
2	34.5%
3	9.9%
Ballooning	
0	34.5%
1	24.3%
2	41.2%
Metabolic Syndrome	
IFC criteria (% meeting criteria)	48.6%
IFC criteria, excluding T2D (% meeting criteria)	47.7% [†]
Hypertension (% with diagnosis)	48.0%
Diabetes (% with diagnosis)	30.6%
Current Smoker (% yes)	
Current	9.6%
Ever	39.4%

[†] Based on 766 participants.

Abbreviations: U, units; HOMA-IR, homeostasis model assessment of insulin resistance

[00220] Next, haplotype analysis of the haplotype block was performed to refine the association signal and to determine if either SNP was a surrogate of the other. However, the four-SNP haplotypes did not improve the association signal (Fig. 5B). Additionally, rs7934919 appears to be a surrogate of rs11246020 given the associations detected were strongest for rs11246020, and adjusting for rs7934919 in multivariable analyses completely attenuated the association signal. Regression analysis of the inferred diplotypes for the 4-SNP haplotype provided additional evidence that rs11246020 underlies the association signal between SIRT3 gene variation and NAFLD ($p=0.003$) and MetSyn ($p=0.007$), based on the observation that the association signal did not

improve substantially in comparison with analysis of rs11246020 alone (Fig. 5B, shaded row).

[00221] Thus, the single rs11246020 “G” minor allele was associated with increased odds for metabolic syndrome (odds ratio [OR]: 1.5, 95% Confidence Interval [CI]: 1.07—2.02, $p=0.017$), after adjustment for age, sex, and BMI (Fig. 5C, middle column). In contrast, the same minor allele was associated with decreased odds of steatohepatitis (OR: 0.7; 95% CI: 0.49—0.94; $p=0.022$) compared to steatosis alone after adjustment for age, sex, and BMI (Fig. 5C, middle column). Additionally, the precision of the point estimate improved when NAFLD was controlled for in the model examining the relationship between rs11246020 and metabolic syndrome (Fig. 5C, right column, OR: 1.5, 95% CI: 1.12, 2.13, $p=0.008$). A similar association was observed with steatohepatitis (Fig. 5C, right column, OR: 0.6, 95% CI: 0.43, 0.85, $p=0.004$) and cirrhosis (Fig. 5C, right column, OR: 0.6, 95% CI: 0.32, 0.95, $p=0.031$) when controlled for the Metabolic Syndrome.

[00222] **Fig. 1.** Chronic high-fat diet feeding results in global mitochondrial hyperacetylation and reduces hepatic SIRT3. (A) Mitochondria were isolated from livers of wt mice fed a standard or high-fat diet for 1 week or 13 weeks (Jackson Laboratory) and analyzed for mitochondrial protein acetylation by western blot analysis with an antiserum specific anti-acetyllysine; electron transfer flavoprotein was used as a reference, $n=3$ mice/condition. (B) Mitochondria were isolated from livers of wt mice fed a standard or high-fat diet for 1 week, 5 weeks or 13 weeks (Jackson Laboratory) and analyzed for SIRT3 expression by western blot analysis with an antiserum specific for anti-SIRT3; electron transfer flavoprotein was used as a reference. Integrated density values were calculated for standard diet and high-fat diet fed wt mice; data represented in arbitrary units (AU); $n=3$ mice/condition, $*p<0.05$; (C) Hepatic tissue samples were collected from normal liver transplant donors, or fatty liver transplant recipients and analyzed for SIRT3 expression by western blot analysis with an antiserum specific for human SIRT3; actin was used as a reference, $n=5$ samples/condition.

[00223] **Fig. 2.** Mice lacking SIRT3 develop diet-induced obesity and insulin resistance. (A) Body weight measurements were recorded from wt and SIRT3KO mice weaned onto and maintained on a high-fat diet ($n=20$ /genotype); (B, C) Wt and SIRT3KO mice were assessed for the volume of oxygen consumption (VO_2) (B) and carbon dioxide

exhalation (VCO_2) (C), in metabolic cages, $n=5$ /genotype, data collected over 48 h and normalized to lean body mass, averages were totaled for dark and light cycles. (D, E) 12-month old SIRT3KO and wt mice fed a high-fat diet were tested for glucose (D) and insulin tolerance (E) and measured for blood glucose levels; inset data represent area under the curve (AUC). (F, G) 12-month old SIRT3KO and wt mice fed a standard diet were tested for glucose (F) and insulin tolerance (G) and measured for blood glucose levels; inset data represent AUC, ($n=5$ /genotype, fasted 6 h, standard diet).

[00224] Fig. 3. SIRT3KO mice fed a high-fat diet develop hepatic steatosis and inflammation. (A) Histological analysis of livers from high-fat diet fed wt and SIRT3KO mice with Oil Red O Stain (fed or fasted 24 h). (B) Livers extracts from wt and SIRT3KO mice fed a high-fat diet were analyzed for total phospholipids, triglycerides and cholesterol esters ($n=5$ /genotype, fasted 24 h). (C) Hepatic lipids were measured in wt mice 1 week after injection with adenovirus expression vectors for GFP or SIRT3. (D) Hepatic sections from 12-month-old wt and SIRT3KO mice fed a high-fat diet were fixed, stained with hematoxylin & eosin (H&E), Masson's trichrome, or reticulin, and scored for inflammation, steatosis, ballooning, and fibrosis, ($n=15$ /genotype, Wilcoxon rank-sum test, $*p<0.05$). (E) Cytokine analyses were conducted on serum obtained from 12-month-old wt and SIRT3KO mice maintained on a high-fat diet ($n=5$ /genotype).

[00225] Fig. 4. SIRT3KO mice have high expression and activity of hepatic SCD1. (A) mRNA transcript levels were quantified by qPCR from wt and SIRT3KO mice, ($*p<0.05$, $n=3$ /genotype, 3-month old mice, standard diet). (B) Plasma samples from 3-month old SIRT3KO and wt mice were analyzed for desaturation indices in triglyceride (TG) phospholipids (PL) and free fatty acids (FFA) by measuring palmitate (C16), palmitoleate (C16:1), stearate (C18) and oleate (C18:1) ($*p<0.05$, $n=5$ /genotype, 3-month-old mice, standard diet). (C) Hepatic triglycerides from wt, SCD1KO, SIRT3KO, and SCD1KO/SIRT3KO (dKO) mice fed a standard (SD) or high-fat (HF) diet were measured ($n=5$ /genotype, fed or fasted 24 h).

[00226] Fig. 5. A unique SNP in the human SIRT3 gene is associated with human metabolic syndrome (A) Heat map detailing the pairwise LD among the 7 SNPs spanning the SIRT3 coding region; the pairwise correlations (D') are rendered within each diamond with greater LD reflected by darker shades of gray; SIRT3 gene structure is depicted above the LD heat map: exons are depicted in gray boxes, introns as

connecting black lines, and untranslated regions as smaller boxes shades in pink; approximately 10 kbp of flanking DNA sequence was included in the tagSNP selection procedure; tagSNPs the promoter region is included in the diagram to the right of the transcription start site indicated by the arrow found directly above the SIRT3 gene structure diagram; SNPs rendered in bold are included in the haploblock outlined in the black triangle; the two SNPs that encode for nonsynonymous polymorphisms (rs11246020 and rs28365927) are rendered in green font color. **(B)** Haplotype frequency estimates, inferred from rs1023430, rs7934919, rs11246020, and rs28365927, reading from left to right in the “haplotype” column. **(C)** Differences in clinical phenotypes by SIRT3 rs11246020 genotype. Abbreviations: P, *p*-value for the test statistic; OR (95% CI), the odds ratio and 95% confidence interval for the test statistic; UNADJ, unadjusted; ADJ, adjusted for age, sex, and BMI unless otherwise indicated; NAS, NAFLD Activity Score; **p*-value for the overall model (i.e. steatosis, steatohepatitis, cirrhosis) †Metabolic syndrome criteria based on the IDF guidelines, excluding the presence of type 2 diabetes **(D)** Working model. SIRT3 functions to deacetylate mitochondrial proteins, and increase fatty acid oxidation and energy production. In SIRT3KO mice or mice fed a high-fat diet, mitochondrial proteins are hyperacetylated, resulting in reduced energy expenditure and less fatty acid oxidation, which contributes to insulin resistance, obesity, and increased inflammation. Similarly, humans with fatty liver disease have reduced SIRT3 production and a unique SNP in the SIRT3 gene is associated with the metabolic syndrome.

[00227] Abbreviations for Figure 5C: rs, reference sequence for a single nucleotide polymorphism; OR_{UNADJ} (95% CI), the odds ration and 95% confidence interval for the bivariate test statistic; P_{UNADJ}, *p*-value for the unadjusted test statistic; OR_{ADJ} (95% CI), the odds ratio and 95% confidence interval for the test statistic adjusted either for age, sex, and BMI for metabolic syndrome for NAFLD; P_{ADJ}, *p*-value for the adjusted test statistic; †, metabolic syndrome criteria based on IDF guidelines, excluding the presence of type 2 diabetes; ‡, model adjusted for age and sex; *, model adjusted for age, sex, and metabolic syndrome; ¥, model adjusted for age, sex, and NADLD.

Functional SNP in SIRT3 causes a point mutation and reduces enzymatic activity.

[00228] The non-synonymous point mutation encoded by rs11246020 results in a change of valine to isoleucine at residue 208 of the SIRT3 polypeptide. The V208I

polymorphism lies within the conserved catalytic deacetylase domain of SIRT3 (Figure 6A and Frye et al. (2000) *Biochem. Biophys. Res. Commun.* 273:793) and could therefore affect its enzymatic activity. To test this possibility, recombinant wt SIRT3, SIRT3-V208I and catalytically-inactive SIRT3-H248Y were expressed in *E. coli*; and their deacetylase activity *in vitro* was tested. A steady-state kinetic analysis of SIRT3 activity was performed, and the initial rates of radioactive release were measured as a function of NAD^+ concentration. The resulting saturation curves were fitted to the Michaelis-Menten equation and the $V_{\max}$ and K_M kinetic parameters were compared among wt SIRT3, SIRT3-V208I and catalytically-inactive SIRT3-H248Y. An 18% increase in the K_M for NAD^+ was observed in SIRT3-V208I, compared to wt SIRT3 (Figures 6B–D), indicating that more NAD^+ is required in SIRT3-V208I deacetylation reaction. Coincident with the increase in K_M , a 19% reduction in $V_{\max}$ for NAD^+ by SIRT3-V208I, compared to wt SIRT3, was observed (Figure 6B). Additionally, a 28% reduction in SIRT3-V208I $V_{\max}$ was observed for the peptide substrate, compared to wt SIRT3, but no change in the K_M for the peptide substrate (Figure 6C). These results indicate that the SIRT3-V208I mutation reduces the catalytic efficiency by 34% compared to wt SIRT3, in three independent enzyme preparations (Figure 6D, E). These observations demonstrate the mutant SIRT3-V208I has reduced enzyme efficiency and could partially explain how human patients with rs11246020 have increased susceptibility to developing the metabolic syndrome.

[00229] Figure 6. The rs11246020 SNP in the human *SIRT3* gene encodes a point-mutation and reduces SIRT3 enzymatic activity. **(A)** Schematic of SIRT3 protein; mitochondrial targeting sequence (MTS), mitochondrial processing peptidase (MPP) site. **(B–D)** Steady-state kinetic analyses of SIRT3 activity; rates of activity were measured as a function of $[\text{NAD}^+]$ (B) or $[^3\text{H}\text{-histone H4 peptide}]$ (C), as measured by organic-soluble radioactive signal; (D) summary table of kinetic parameters, $n=3$ independent measurements/sample, $\pm$ standard error. **(E)** Protein levels of wt, V208I, and H248Y SIRT3 were assessed for three independent preparations (Prep1-3) to measure enzyme kinetic parameters.

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[00230] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

CLAIMS

What is claimed is:

1. A method of detecting a predisposition of an individual to develop metabolic syndrome, the method comprising detecting in polynucleotide sample obtained from the individual the presence of a single nucleotide polymorphism in a sirtuin-3 (SIRT3) gene at rs11246020, wherein the presence of said polymorphism is indicative of predisposition of the individual to develop metabolic syndrome.
2. The method of claim 1, wherein said detecting step comprises a method selected from: a) allele specific oligonucleotide hybridization; b) size analysis; c) sequencing; d) hybridization; e) 5' nuclease digestion; f) single-stranded conformation polymorphism; g) allele specific hybridization; h) primer specific extension; i) oligonucleotide ligation assay; and j) a polymerase chain reaction assay.
3. The method of claim 1, wherein the polymorphism is detected with an oligonucleotide that distinguishes between at least two alternative alleles of the polymorphism.
4. The method of claim 3, wherein the oligonucleotide is detectably-labeled.
5. The method of claim 4, wherein the oligonucleotide is detectably-labeled with a fluorescent moiety.
6. The method of claim 5, wherein the oligonucleotide further comprises a quencher moiety that quenches the fluorescent moiety when the oligonucleotide is intact.
7. A method of detecting a single nucleotide polymorphism (SNP) associated with metabolic syndrome in an individual, comprising: analyzing a nucleic acid sample from said individual for the presence of a SNP at rs11246020 in a sirtuin-3 (SIRT3) gene, wherein said SNP is associated with metabolic syndrome, and wherein the presence of said polymorphism is indicative of a polymorphism associated with metabolic syndrome.

8. The method of claim 7, wherein the SNP is detected by one or more of the following techniques: (a) restriction fragment length analysis; (b) sequencing; (c) micro-sequencing assay; (d) hybridization; (e) invader assay; (f) a gene chip hybridization assay; (g) oligonucleotide ligation assay; (h) ligation rolling circle amplification; (i) 5' nuclease assay; (j) a polymerase proofreading method; (k) an allele specific polymerase chain reaction; (l) matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectroscopy; (m) a ligase chain reaction assay; (n) enzyme-amplified electronic transduction; (o) single base pair extension assay; and (p) reading sequence data.

9. The method of claim 7, wherein rs11246020 is represented by position 3365 of SEQ ID NO:4 or its complement.

10. The method of claim 9, wherein the presence of A at position 3365 of SEQ ID NO:4 or its complement indicates that the individual has an increased risk for developing metabolic syndrome.

11. The method of claim 7, wherein said nucleic acid is a nucleic acid extract from a biological sample from said individual.

12. The method of claim 11, wherein said biological sample is blood, saliva, or buccal cells.

13. The method of claim 7, wherein said analyzing comprises nucleic acid amplification

14. The method of claim 13, wherein said nucleic acid amplification is carried out by polymerase chain reaction.

15. The method of claim 7, wherein said analyzing is performed using sequencing, 5' nuclease digestion, molecular beacon assay, oligonucleotide ligation assay, size analysis, single-

stranded conformation polymorphism analysis, or denaturing gradient gel electrophoresis (DGGE).

16. The method of claim 7, wherein said analyzing is performed using an allele-specific method.

17. The method of claim 16, wherein said allele-specific method is allele-specific probe hybridization, allele-specific primer extension, or allele-specific amplification.

18. An isolated polynucleotide consisting of from 18 consecutive bases to about 100 consecutive bases of SEQ ID NOs:3 or 4, or a complement thereof, wherein said isolated polynucleotide includes a single nucleotide polymorphism at position 125 of SEQ ID NO:3 or at position 3365 of SEQ ID NO:4.

19. The isolated polynucleotide of claim 18, wherein the polynucleotide comprises a detectable label.

20. The isolated polynucleotide of claim 19, wherein the detectable label is a fluorogenic dye.

21. The isolated polynucleotide of claim 18, wherein the polynucleotide is immobilized on a substrate.

22. A nucleic acid primer pair that amplifies a segment of a human SIRT3 gene comprising a single nucleotide polymorphism at position 125 of SEQ ID NO:3 or at position 3365 of SEQ ID NO:4, wherein said primer pair comprises a first member and a second member.

23. The nucleic acid primer pair of claim 22, wherein said first member is an allele-specific primer.

24. A kit comprising:

- a) a nucleic acid reagent for determining a subject's genotype with respect to a single nucleotide polymorphism at rs11246020 in a sirtuin-3 (SIRT3) gene;
- b) instructions for determining the subject's genotype with respect to the SNP at rs11246020 in the SIRT3 gene.

25. The kit of claim 24, wherein the nucleic acid reagent is:

- a) an allele-specific probe;
- b) an allele-specific primer;
- c) a first allele-specific probe that detects a "G" nucleotide at position 125 of SEQ ID NO:3 or at position 3365 of SEQ ID NO:4; and a second allele-specific probe that detects an "A" nucleotide at position 125 of SEQ ID NO:3 or at position 3365 of SEQ ID NO:4;
- d) a nucleic acid primer pair that amplifies a segment of a human SIRT3 gene that includes nucleotide 3365 of SEQ ID NO:4.

26. The kit of claim 24, further comprising one or more of:

- a) a buffer;
- b) a DNA polymerase; and
- c) deoxyribonucleotides.

27. A kit for detecting predisposition to developing metabolic syndrome in a subject, said kit comprising a primer oligonucleotide that hybridizes 5' or 3' to a SIRT3 rs11246020 allele.

28. The kit of claim 27, further comprising a second primer oligonucleotide that hybridizes either 3' or 5' respectively to the allele, such that the allele can be amplified.

29. The kit of claim 27, wherein said primer hybridizes to a region in the range of between about 50 and about 1000 base pairs.

30. The kit of claim 27, further comprising one or more reagents for detecting nucleic acid hybridization.

31. The kit of claim 27, further comprising one or more reagents for nucleic acid amplification.

32. The kit of claim 27, further comprising a control nucleic acid reagent that provides for detection of an allele at rs11246020 that is not associated with metabolic syndrome.

33. A method of detecting a predisposition of an individual to develop metabolic syndrome, the method comprising detecting in biological sample obtained from the individual the presence of a V208I substitution in a sirtuin-3 (SIRT3) polypeptide, wherein the presence of said V208I is indicative of predisposition of the individual to develop metabolic syndrome.

34. The method of claim 33, wherein said detecting comprises contacting the sample with an antibody that distinguishes between SIRT3-V208 and SIRT3-I208.

35. The method of claim 34, wherein said antibody comprises a detectable label.

36. A method of detecting a polymorphism associated with metabolic syndrome in an individual, the method comprising analyzing a biological sample from said individual for the presence of a V208I substitution in a sirtuin-3 (SIRT3) polypeptide, wherein said V208I substitution is associated with metabolic syndrome, and wherein the presence of said V208I substitution is indicative of a polymorphism associated with metabolic syndrome.

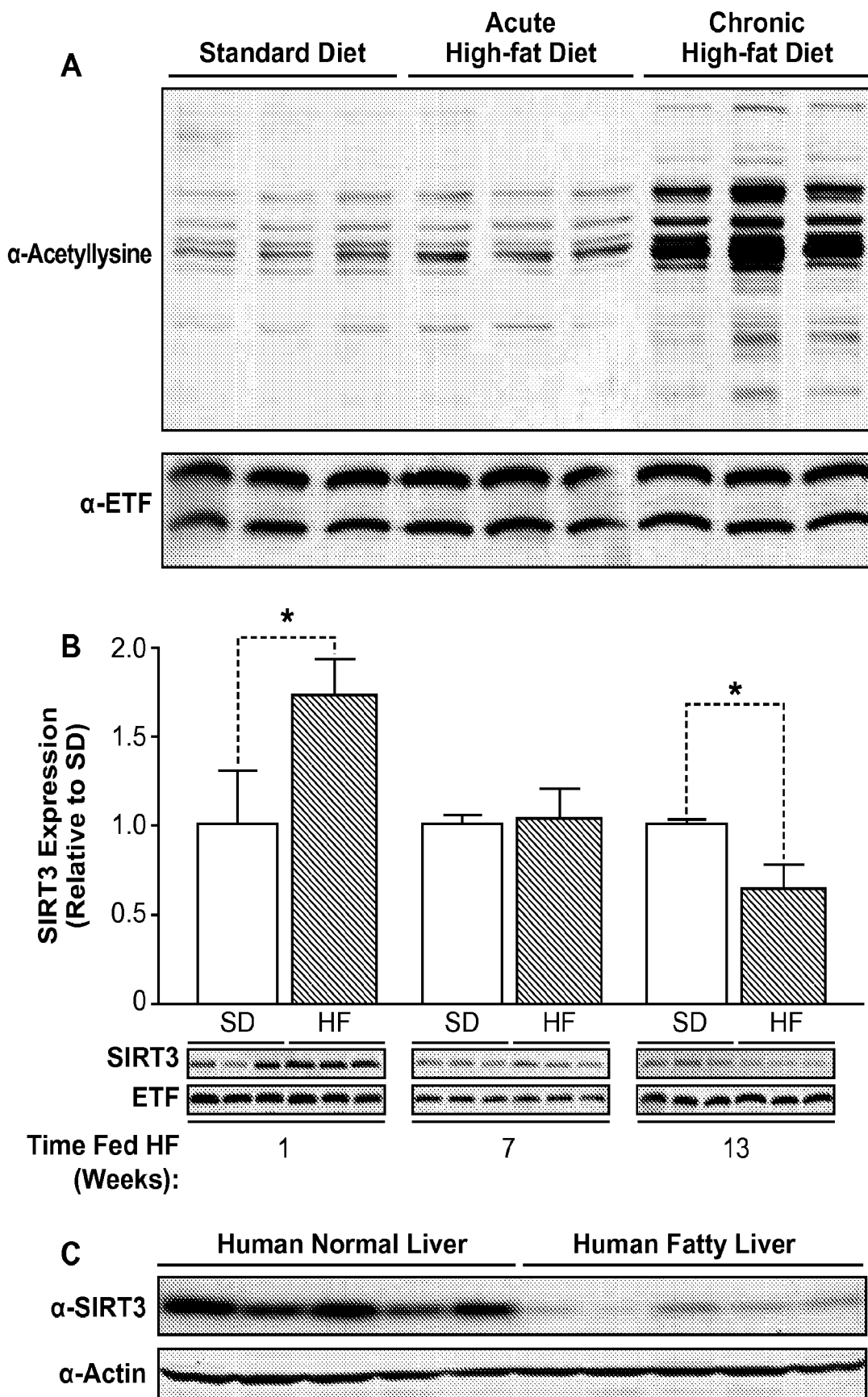


FIG. 1

SUBSTITUTE SHEET (RULE 26)

+

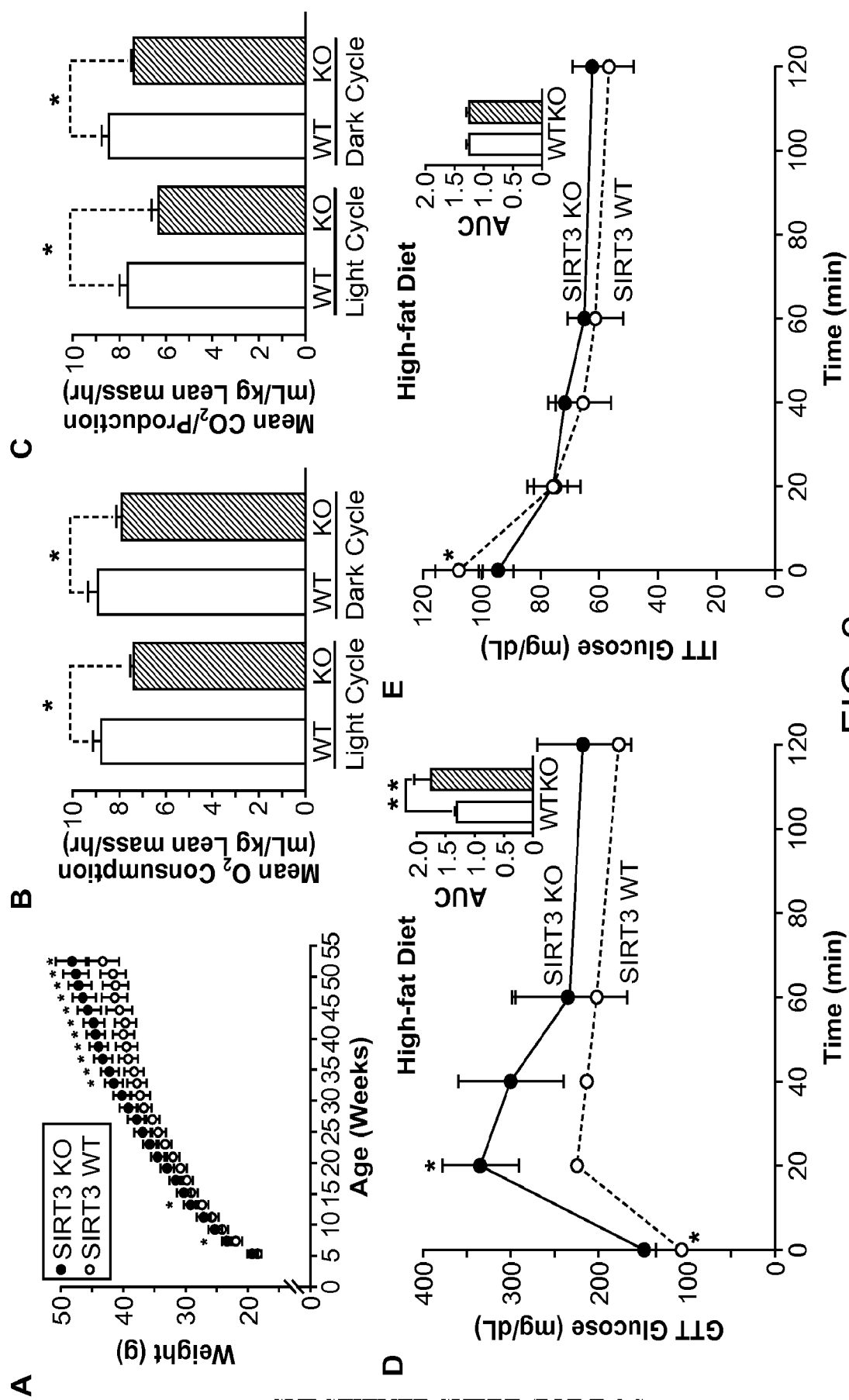


FIG. 2

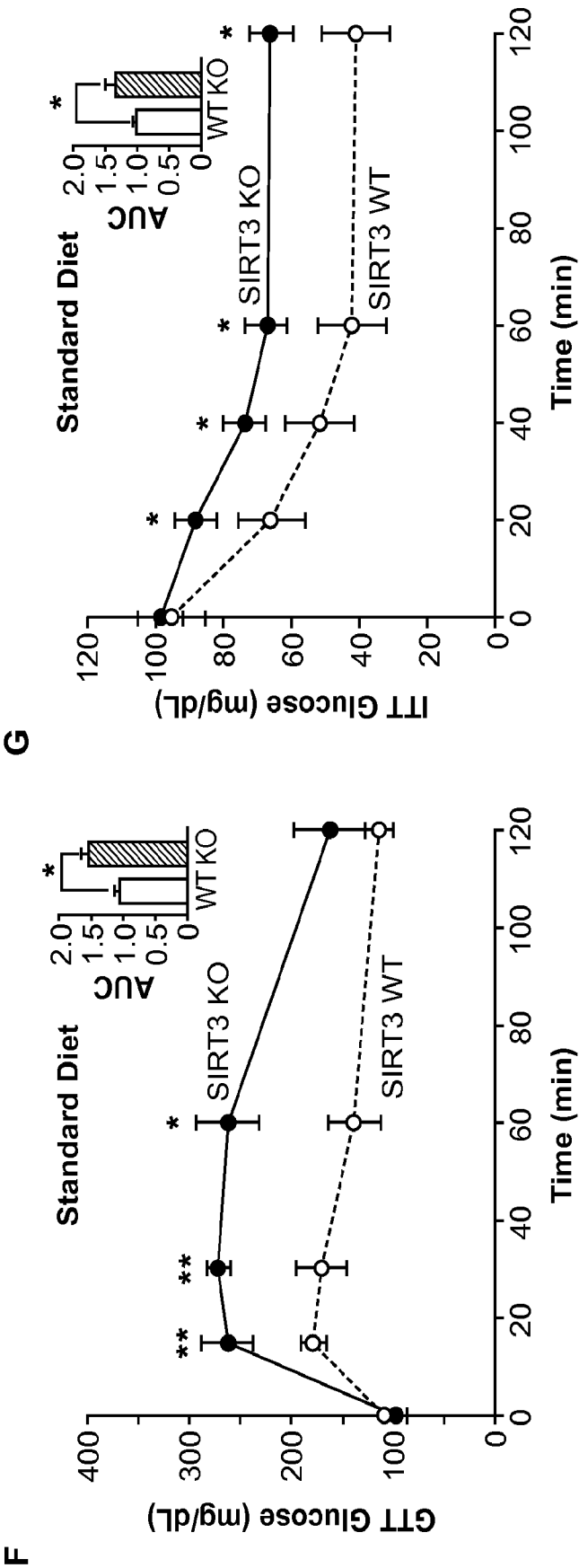
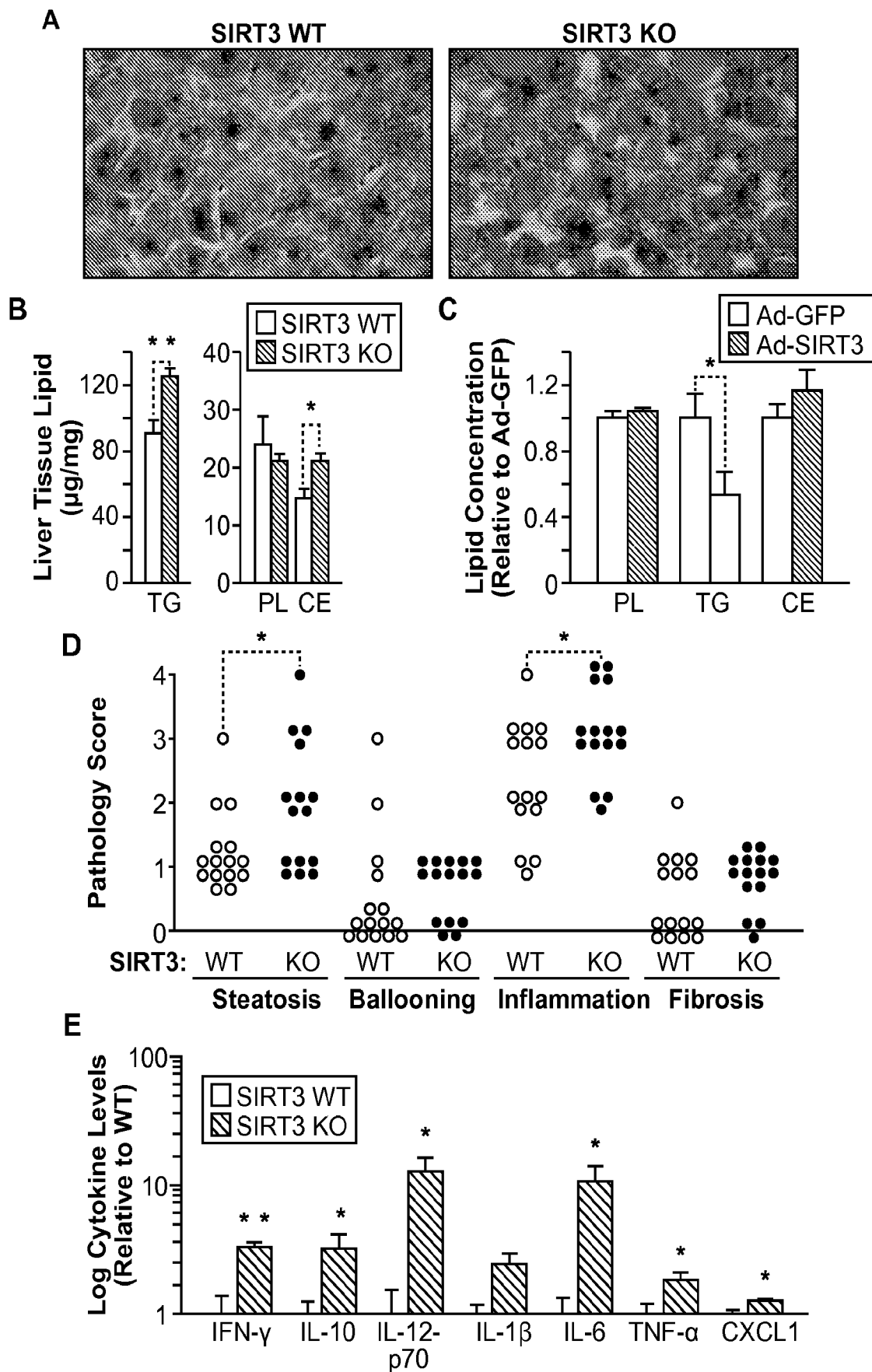


FIG. 2 (Cont.)

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+

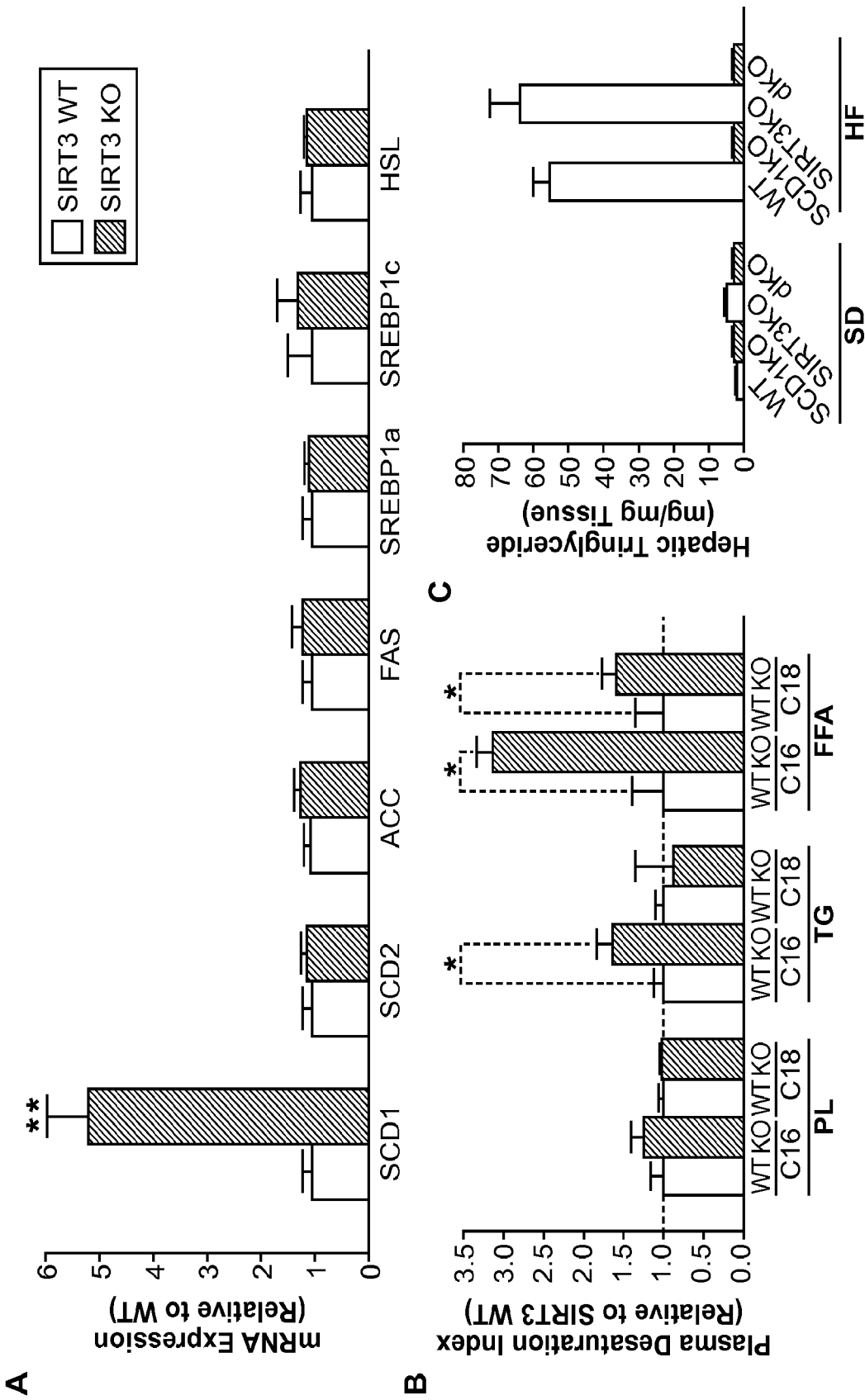


FIG. 4

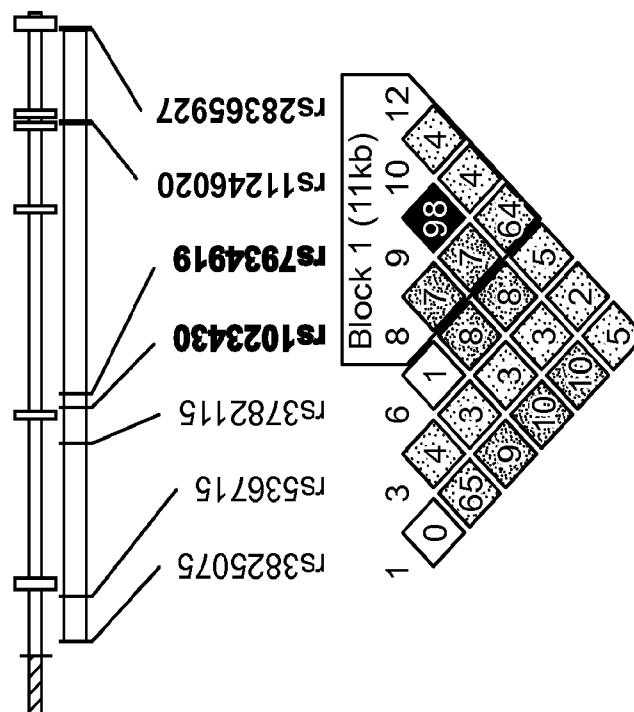


FIG. 5A

Haplotype*	Estimated Frequency	Standard Error	P Value
ACAG	0.575	0.0009	n.s.
ATGG	0.216	0.0005	<0.05
GCAA	0.146	0.0011	n.s.
GCAG	0.060	0.0010	n.s.
ACAA	0.002	0.0004	-
ATAG	0.002	0.0001	-
GTGG	<0.000	0.0002	-
GTGA	<0.000	0.0001	-

* rs1023430, rs7934919, rs11246020, and rs28365927

FIG. 5B

	rs11246020		Unadjusted		Adjusted (Age, Sex, BMI)		Adjusted (MetSyn or NAFLD)	
	AA	AG/GG	OR_{UNADJ}(95%CI)	P_{UNADJ}	OR_{ADJ}(95%CI)	P_{ADJ}	OR_{ADJ}(95%CI)	P_{ADJ}
Steatosis grade	0-1	210	-	-	-	-	-	-
	2-3	240	1.1 (0.83-1.52)	0.487	1.1 (0.84-1.56)	0.392	1.1 (0.83-1.57)*	0.4867
Fibrosis stage	0-1	220	-	-	-	-	-	-
	2-4	226	0.9 (0.70-1.28)	0.757	0.9 (0.69-1.29)	0.701	0.95 (0.69-1.31)*	0.7567
NAFLD diagnosis	Steatosis	151	-	0.038	-	0.011	-	0.003
	Steatohepatitis	230	0.7 (0.49-0.95)	0.025	0.7 (0.49-0.94)	0.022	0.6 (0.43-0.85)*	0.004
	Cirrhosis	59	0.6 (0.37-1.02)	0.061	0.6 (0.35-1.02)	0.057	0.6 (0.32-0.99)*	0.031
NAS	0-4	240	-	-	-	-	-	-
	5-8	212	0.9 (0.67-1.22)	-	0.9 (0.67-1.24)	0.548	0.8 (0.61-1.16)*	-
MetSyn	No	282	-	-	-	-	-	-
	Yes	238	1.4 (1.03-1.82)	0.031	1.3 (0.96-1.76)†	0.095	1.4 (0.99-1.84)¥	0.056
MetSyn†	No	247	-	-	-	-	-	-
	Yes	226	1.6 (1.15-2.09)	0.004	1.5 (1.07-2.02)†	0.017	1.5 (1.12-2.13)¥	0.008

FIG. 5C

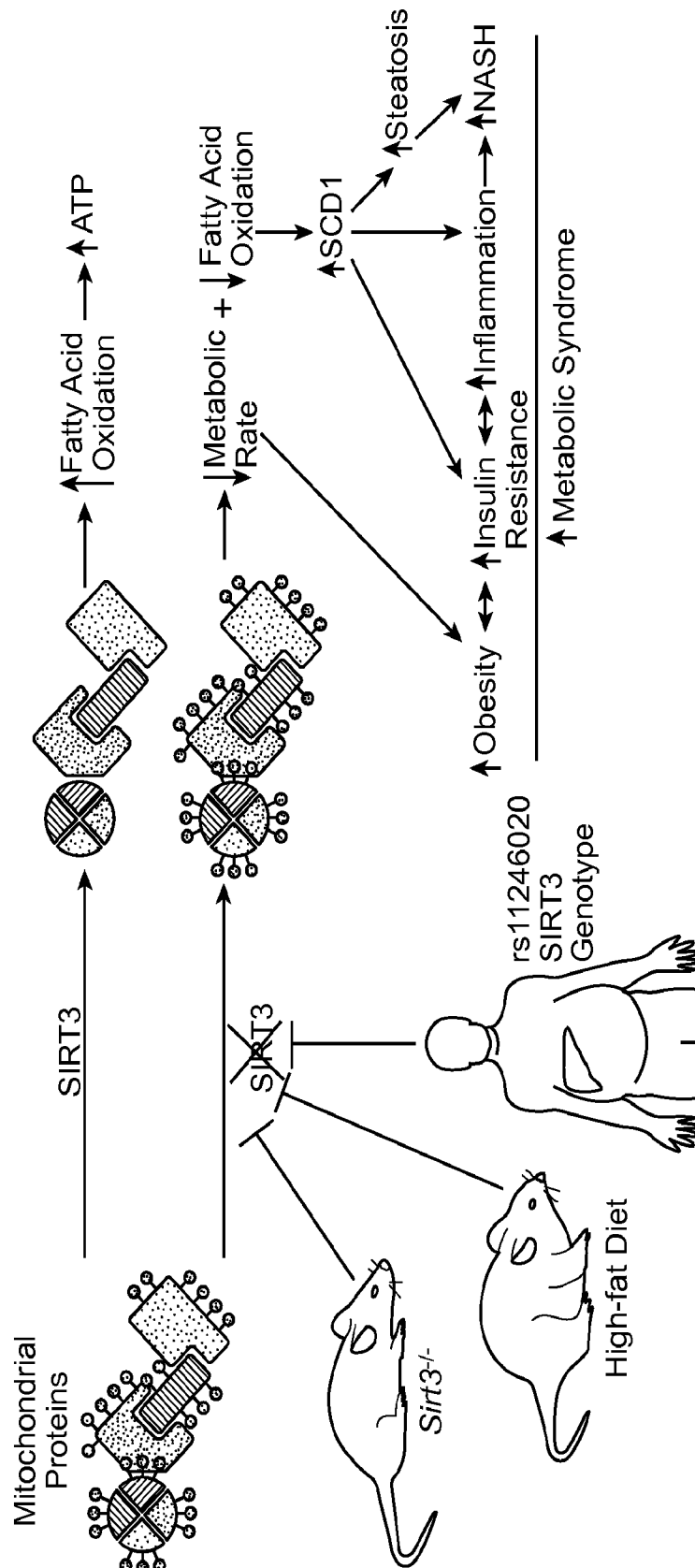


FIG. 5D

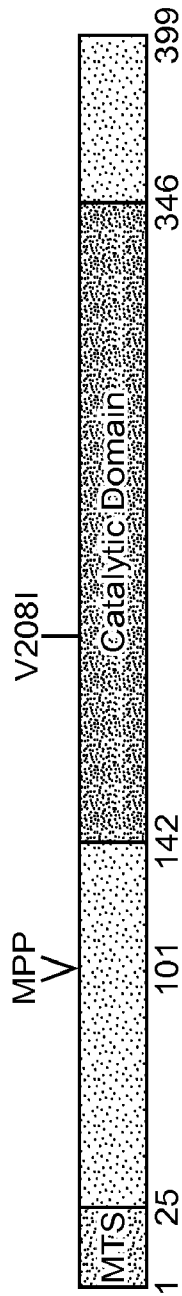


FIG. 6A

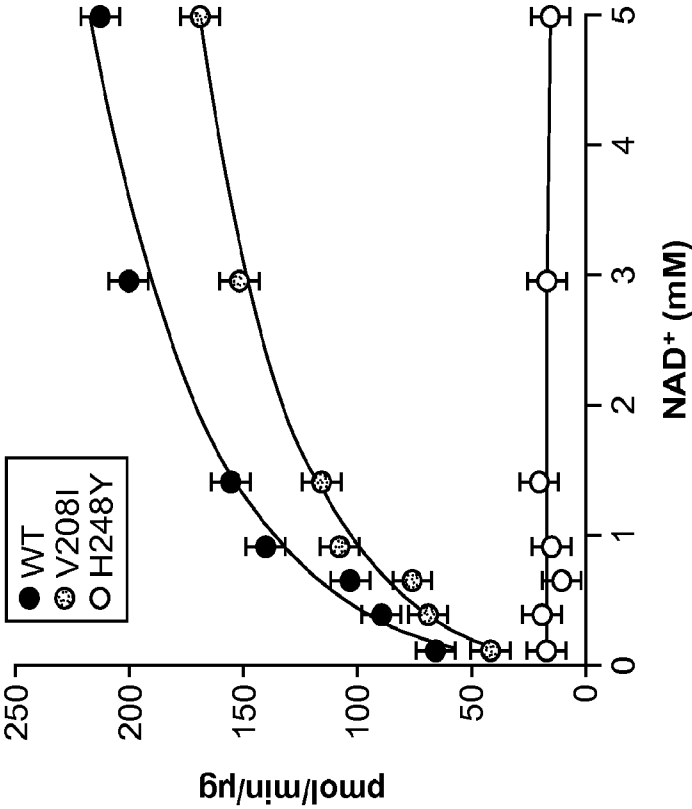
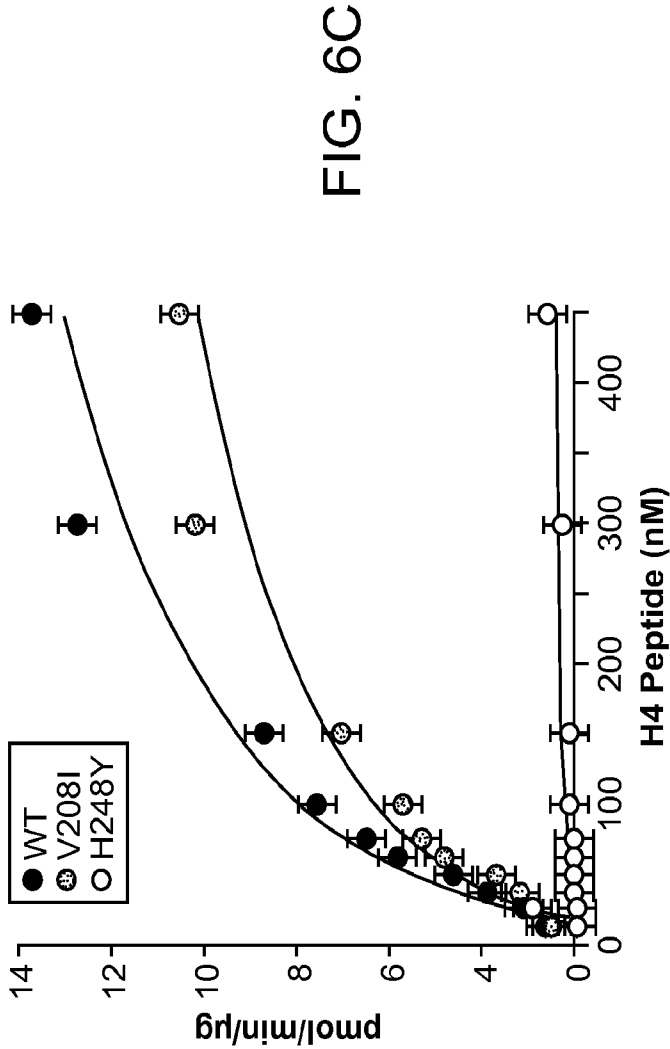


FIG. 6B



		SIRT3		T Test
		WT	V208I	(WT vs V208I)
NAD ⁺	V _{max} (pmol/min/mg)	15.9±0.01	13.5±0.40	0.0037
	K _M (mM)	0.88±0.01	1.07±0.03	0.0045
Catalytic Efficiency (V _{max} /K _M)		19.04	12.62	
Peptide	V _{max} (pmol/min/mg)	16.5±0.79	11.8±1.15	0.0299
	K _M (nM)	146.9±14.8	153.4±19.6	0.8042
Catalytic Efficiency (V _{max} /K _M)		0.09	0.06	
			0.02	

FIG. 6D

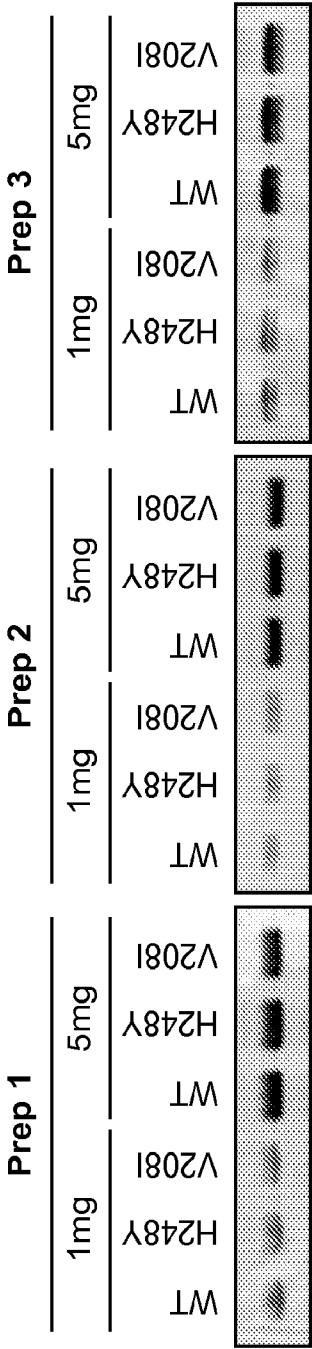


FIG. 6E

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GenBank Accession No. NP_036371
Homo sapiens
SIRT3

1 mafwgwraaa alrlwgrvve rveagggvgp fqacgcrllv ggrddvsagl rgshgargep
61 ldparplqrp prpevpfrafr rqprraaapsf ffssikggrr sisfsvgass vvgsggssdk
121 gklslqdvae lilaracqrv vvmvgagist psgipdfrsp gsglysnlqq ydlpyeaiif
181 elpfffhnpk pfftlakely pgnykpn^vth yflrllhdkg lllrltqni dglervsgip
241 asklveahgt fasatctvcq rpfpgedira dvmadruprc pvctgvvcpd ivffgeplpq
301 rflhvvdfp madllilgt slevepfasl teavrsvpr llinrdlvvp lawhprsrdrv
361 aqlgdvvhgv eslvellgwt eemrdlvqre tdkldgpd (SEQ ID NO:1)

FIG. 7

3241 caacctccag cagtagcatc tccgtaccc cgaggccatt tttgaactcc cattcttctt
532 caacctccag cagtagcatc tccgtaccc cgaggccatt tttgaactcc cattcttctt

3301 tcacaaaccc aagccctttt tcaactttggc caaggagctg taccctggaa actacaagcc
592 tcacaaaccc aagccctttt tcaactttggc caaggagctg taccctggaa actacaagcc

3361 caacgtcact cactactttc tccggctgct tcatgacaag gggctgcttc tgcggctcta
652 caacgtcact cactactttc tccggctgct tcatgacaag gggctgcttc tgcggctcta

3421 cacgcagaac atcgatgggc ttgagagagg tgagccctct gtgagtcgca cacattcttt
712 cacgcagaac atcgatgggc ttgagagag (SEQ ID NO:2)

3481 ttctcccacg gaggtcggg tgccctgtttc tcccaggag gctcgggtgc ctgtttctca
(SEQ ID NO:3)

FIG. 8



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GenBank NC_000011.9
Homo sapiens
 Genomic - SIRT3

```

1  cggaggcggg  gctggggcgc  cgggggcggg  ggtgggaggg  ggaggcgggg  ccggggcgcc
61  gggggcgggg  cgagtcggga  ggactccttg  gactgcgcgg  aacatggcgt  tctggggttg
121  gcgcgccggc  gcagccctcc  ggctgtgggg  ccgggtagtt  gaacgggtcg  aggccggggg
181  aggcgtgggg  ccgtttcagg  cctgcggtg  tcggctgggtg  cttggcggca  gggacgatgt
241  gagtgcgggg  ctgagaggca  gccatggggc  ccgcggtgag  ccttggacc  cggcgcgccc
301  cttgcagagg  cctccagac  ccgagtgcc  cagggcattc  cggaggcagc  cgagggcagc
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481  gagtttacgt  gcgttccaac  accggggcct  ccgtctttat  gctgatctgt  ggacgtcact
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601  tatgtgcat  catgctgtgg  tgaataacct  tgttggcacc  tcaactgtttg  cataaattcc
661  tagtactggg  attgctaggt  ccaaaagtat  atgcatttaa  cgctttaatg  atccctcata
721  aatgttgtgc  ggttaatact  cccactggca  tgtgatocta  cccgtctcca  tagtaattgc
781  atctaagaac  tgaggctaca  taggaaaaaa  tcttaagggtc  catggttgtt  aggtatcaga
841  tgccctataa  tggatgaata  tttaagtaaa  taatgggcaca  ttgacttgct  gtctgacaca
901  actgctgaaa  atgttggtga  aaagtgaatc  tcgaagtgg  agctcacact  tgtaatacca
961  gcactttcgg  aggtgagac  ggggatcgt  cgaggccagg  agtcaagag  cagcctgggc
1021  aacataacga  gaccccatc  tctacaaaa  atttaaaat  tagccgggca  tgggtggcgca
1081  agcctgttgt  ccagttact  cctgagacta  aggcaagagg  atcccttgag  tccaggagt
1141  caaggctgca  gtgagctatg  attgagccac  tgcactccag  cctgggtgac  agagcaagac
1201  tcttatctac  cttgtggaaa  aaaaaagaa  aaaagttaa  tagatcctga  agagtggcc
1261  gggcgcggt  gctcacgct  gtaatcccag  cactttggga  ggatgagacg  ggtggataac
1321  gaggtcagga  gttcgagacc  agctgggcca  acatggtgaa  acccatttc  tactgaaatt
1381  acaaaaatta  gccgggcgtg  gtggcgggcg  cctgtaatcc  tagctactca  ggaggcggag
1441  gcaggagaat  cacttgaacc  cggaaggcag  aggttgca  gagccagat  ggcaccatg

```

FIG. 9A

```

1501 cactccagcc cgggcgaaaag agcaaaaact ccgtctcaaa aaaaaacaaa gatcccgaag
1561 agtcagtcgt tcattccaaga agccctgact gagctcttat tctgtgccag actctgttct
1621 aggtgctatg gatggagcag ggaccaggac ctacaaacac gtggagcgca ttctaataac
1681 tgtgatactc tgcaaaactg tlaaaacaaa tccttgcggc cgggcgcggt ggctcacgcc
1741 tgtaattcca gcaactttgga aggccgagac gggcggatca cgaggtcagg agatcgagac
1801 catcctggct aacacagtga aaccctgtct ttactaaaaa tacaaggaaa ttagccaggc
1861 gtggtggcgg gtgcctgtag tcccagctac tcgggaggct gaggcaggag aatggcgtga
1921 acctgggagg cggagcttgc agtgagccga gatcgcgaca ctgcactcca gcctgggcaa
1981 cagagcgaga ctccgtctca aaacaaaaca aacaaaaat ccttgctacc aacaaaaat
2041 actggcccta aagcaggagg cataaggctg cgtgtgagga gtagtccatt gcatgggctt
2101 cctccagttt ggctttggat ttttgagctc tctgaatttg tgttttgtgt cctttgcaca
2161 gtgtccctcc caggttgcac ccattgcctgc cctcagggt ccccaactcc cagtgaattt
2221 cacaccatgc agcatgtact ttaagccata catcttacct atgttccaga catttggtaa
2281 aacgccgttt atgtgtttt cagtgactca caaacathtt atttacgtaa atgaggaatg
2341 tactcaggca cccttcgtga taccttgccc tcagcaggca ctcaacagca ataataactt
2401 tcatttcctt tgtgcttctc ttaacagttc gtgttttagta ggagcacagt agctgtttgg
2461 ggaacgacct taagtgaata gatgtctgaa atgctgtgtg aagtggggcg ctaataaggt
2521 ggtgagatgg ttttagaacc aggttttcc ctctttgctt cttgactcct taaccagttt
2581 tcattaggag accctttgg aggtttgcag tcagtcagag aacctgaatt ttttgctccc
2641 ctgaattatg aatgagcac agttcccat taccocaata gtctctaaag aaaggtgatt
2701 tgattttctga ttatgactg ctttttgttg taacctcoga ggttggcgggt ggtgcagagg
2761 attttgagag gcctggggag acacacatgc tcattctcga tatctgtttc ctttgctgct
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2881 gtgttttttt ctgtcagtat taaagtgga agaaggtcca agctttccct gcaggatgta
2941 tcaagtgttg ttggaagtgg aggcagcagt gacaagggga gcatggtggg gcccggcac
3001 gctgagctga ttccggccag agcctgccag aggttggttg tcatggtggg gcccggcac
3061 agcacaccca gtggcatcc agacttcagg tactgcccac agtcccacct gccttctgcg
3121 ctccccccc ctgactcctg ccctccccac tgcccagttt attcacatc cctgtcctct
3181 tccaaaggcc tcagagcctt tcttctctcc ctgcagatcg ccggggagtg gcctgtacag
3241 caacctccag cagtacgac tcccgtaccc cgaggccatt ttggaactcc cattcttctt

```

FIG. 9B

3301 tcacaaacccc agccctttt tcaactttggc caaggagctg taccctggaa actacaagcc
 3361 caacgtccact cactactttc tccggctgct tcacgacaag tccatgacaag tggctgtctc tgcggctcta
 3421 caccgagaac atcgatgggc ttgagagagg ttgagccctct tggagccctct tggagtcgca cacatctctt
 3481 ttctccacg gaggctcggg tgccctgttc tcccaggag tcccaggag tccatgctgc ctgttctca
 3541 caggggatgc tcgggtgcct gtttctggg aactagttag ggctttgttc ccaaggaata tgtcctctgg
 3601 ctgggactt ttttgagcc gccactgctt gttctcctgc atacctgcag tgatctctt tcttttaaga
 3661 gtgtttaag gttttgccaa tctggtgtga tgagcacaca caaagagagc caagagagc accgtcctgt
 3721 agtgacatt gttttgccc gaaattttt agcacacagc cagttcttcc tgcttctca tgcgtcctc
 3781 agtgtattat ggaattttt gaaacaaatc tctcacatca tgaatttaaat tgcgtcctca tgcgtcctc
 3841 cctcatcacc agcacacagc agacacacagc cagttcttcc tgagcacaca caaagagagc caaagagagc
 3901 agattatttt gatgcttaga tagctctaga gaatacttta aaaacaatc tttgggaggg
 3961 catgaatgga tagctctaga tagctctaga gaatacttta aaaacaatc tttgggaggg
 4021 ggtgtgggat cacacctgta atccagcat tttgggaggg cgtggcaaaa cgtggcaaaa
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 4261 cactccagcc tgggcgacag agggagattc tctgatatgtg attcacactg aggttgcatg
 4321 aattaaaaaa taaaaaacac tctgatatgtg attcacactg aggttgcatg aggttgcatg
 4381 taatatggtc acacttagtc aggttgtgac aacttctggc cgtgcaccta cgtgcaccta
 4441 agcgattcac aggttgtgac aggttgtgac aacttctggc cgtgcaccta cgtgcaccta
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 4561 gctccgggag agactgctga tgcagacagg tgcagacagg tgcagacagg tgcagacagg
 4621 aatgtgttcc ctgcccagaa ggatccccctg acttggaaac acttggaaac acttggaaac
 4681 cccatgttgg tgggctcttc tcccagaggt gcagcagggc gcagcagggc gcagcagggc
 4741 agctcctggc tgtgagattc agcactgctg agttagggca agttagggca agttagggca
 4801 ctccctgcagc tgggacctg gctgtgccac cgcagcctt cgcagcctt cgcagcctt
 4861 acaactttca taagaagtca ctttttgtct gtttctacca gtttctacca gtttctacca
 4921 tgatatattat atcctcattt taacttggtg tctgtagaat tctgtagaat tctgtagaat
 4981 ttagatataa atttttgaga caggtctctg gctctgttgc gctctgttgc gctctgttgc
 5041 atgatcatag ctcaatatag cctgaaactct tgggcccgaag tgggcccgaag tgggcccgaag

FIG. 9C

5101 ctggaatagc tgggactacg ggcattgcact accatgcctg gctaataaaa aaaatttttt
 5161 aagacaagtc tcaatatgtt gctgaggctg gtctcaaaact cctggcctca agtgatcctc
 5221 ctgccttggc ctccaaagc actgggattta caggcatgag ccactgcacc cagcctggta
 5281 ttaggtgtaa tttttttttt ttctttttttt ttctttttttt gctctcttac ccaactgga
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 6061 ctcgaaggga cagttgaaa gtaaaaaat ttaagacaat atagaaatat tttttttttt
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 6301 aaggaaattt tccagctgca tccagctgca cactttttttt ggtcactgc ggtcactgc
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 6481 gcaccacagt tggtttgttt gtacatggat ggtgtgttgg ggtgtgttgg ggtgtgttgg
 6541 ttgtgtatag ttctgctgtg gagcatatgc aagtattcgt aagtattcgt aagtattcgt
 6601 ttctttttgag tatgatacct acaagtattg ctgggtaact ctgggtaact ctgggtaact
 6661 ttattttatt tttagagac agtcttacc agtcttacc agtcttacc agtcttacc
 6721 tttcggctca atacaacct tgcctcccg gctcccgcc gctcccgcc gctcccgcc
 6781 gagaagctgg gattacaggt gctggccacc atgcccagct atgcccagct atgcccagct
 6841 tagagatggg gtttcaccaa gttggccagg ctgctcttgc ctgctcttgc ctgctcttgc
 6901 caccgcctc ggcctcccaa agtgctaaaga ttacaggcgt ttacaggcgt ttacaggcgt
 6961 tgtttaactt ttttgttgtt ttgtttttg acggagtcta acggagtcta acggagtcta

FIG. 9D

```

7021 gtgcagtggc gcaatctcgg ctactgtac catcgcctc ctgggtcaa gcgattctcc
7081 tgcctcagct tcctgagtag ctgggactac aggcacacac caccacgcc ggctaatttt
7141 tgtatttagt agagtagttt caccatgttg gccaggatgt ctcgatctct tgacctcgtg
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7381 ttaattacag ctatcccagt ggtgggagg tggtaaccca ctgtagcact gattggcatt
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7681 ttttgatgaa atctagtgtg tctatttatt ttctactct ttgggaatca tatctgagaa
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8101 tatacgtatt attgttttgg tcaactcatt cattgaaatga ccaatttatt caacaaatag
8161 tttagtcctc tcagtgccag gccctttgct gggttctgtg gatgtgaata gaaatlaaaa
8221 taaggtggag tcataccaac gttacaactg ggtgccatag gaatttact ataatatgt
8281 actggacact aactgactc agctgtagga acagagtagg ctgggaagca cctttgagat
8341 gaggccgtct ggatgtaaga gtctgtttct ggcagcagag ggagggcatt tgggaggcct
8401 gcttgctctg ttagggtttg aactgtatcc tgacgatcat gggagggcatt ggaggatttt
8461 taggcaggaa atgatgtga agggatttgc atttaaaaga gattatttg gactgggcac
8521 agtggctcac gcctgtaatt ccagcacttt gggaggccta gacagcgagg tcacctgagg
8581 tgggaagctt gagagcagcc tgccaaaatg gtgaaaatccc atctctacta aaatatcaaa
8641 aatagccag gtgtgtgtgt gcacctgtaa tccagctac tcaggaggct gaggcagaag
8701 aattgcttga acctgggagg ggaggttgtc gtgagctgag atgcacccac tgcactccag
8761 cctgggtgac agagtgagac tcatctcaa aacaaaataa ataaataaat aagagaataa
8821 aatagataat ttgggcaaca ggtgatgtt aattcccac ctttgttct ctccttcagt

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FIG. 9E

8881 actgtgttgg ctggtctgaa tcttttggct tttcataaa atttctgaaa ctgttgctat
 8941 ctgttctctg cctgttttat ttttgttgtt gttgtttgga ttttttgttt ttttgagata
 9001 gagtcttctg ctgttgccca ggctggagtg cagtggtagt atctcagctc attgcaacct
 9061 cccctctca ggttcaagcg attctcttgc ctcatcctcc ctatcctctc ggattgcagg
 9121 catgcccac catgcccagc taatttttgt atttttagta gagatggggg ttcactatgt
 9181 tggtagggct ggtgtcgaa tctgacctc atgatccacc cgcctcggcc tcccaagtg
 9241 ctgggatgac aggcattgag caccgtgtct ggcctctctg cctgtttttt aagacttttg
 9301 tctttggagt tttgcggttt ctccactgtg gttttccata tgaattttct tttatttctt
 9361 ctgcttaaaa ttgttaggc agttggtcat ggtggctcac gccgtaac ccagtaactt
 9421 gggaggccgg gtgggtggat cgctgaggt caggagtgtg agaccagcct gaccaacatg
 9481 gagaaacccc atatctacta aaaatacaaa attagccggg cgtgatgggt catgcctata
 9541 atctcagata ctgggaggc tgaggcaggc gaattgcttg aaccagggag gcggaggttg
 9601 cgtgagctg agattaggc attgcactcc agcctgggaa agccttctct taagaaaaat
 9661 caaaaaaaa ggcggtggc tctgcctgt aatccagta ctttgggaag ctgagcggg
 9721 tctaggctgg tggatcacct gagttaggag tttagagacca gcctggccaa cccgtctct
 9781 tggatcacct gagttaggag tttagagacca gcctggccaa cccgtctct
 9841 actaaaaata caaatgag caggaatgt gctggaaccc aggagcaga gctactctg
 9901 gaggctgagg actccagcct ggtgacaga gcgagatgcc agtgtagtg aaccgagatc
 9961 atgccaactg tctaagccct gcaaccaact gaatggagcc ctcccctgg tcaagggaac
 10021 aaaataaaat ccttggaagc ctctccctcc tgaagtagca ggagaggagg tcaagcgcg
 10081 cccagagaaa cctccctcc tgaagtagca ggagaggagg tcaagcgcg
 10141 ctcaactgtac cctccctcc tgaagtagca ggagaggagg tcaagcgcg
 10201 accagccctt tcaaaagact ctacactgct aatgtggatt actggtttat ctcccagat
 10261 acagattaaa gacaagatga gattaatggt tccctcacc ttccctgaga ggtctgcta
 10321 gttctcttcc tctgtttcct tctatcttag gtaaaatgta catttactgg gacttactg
 10381 aagtctcaca agtacgtaac cactcactc cactcactc acagccacc caagaccaca
 10441 tgtaattact aaacctcctg agaacctctt gggacagaaa ggcctcactt ggtgagatc
 10501 ctcaatgtttt tccgttgggc accctcaggc tggctcagta tggctcactt gcttgagatc
 10561 ctgaatctgc ggttggcgt ctttcatggt tgctggatca tgctgtcttc
 10621 ggatatgagt tgcccctagc ccactatctg gccctctct ttagggacct ctgatbgaac
 10681 atacattaga ccttctctct cctgctgttc ctgatcactc tctgtcctg taattgctgc
 10741 ttcttgtgct gcattctggg taatttcttc tggctctttt tccagtttac taatatgtga

FIG. 9F

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10801 ttctgtttata aggtccttcc attgagtgtt tctttctttc tttttccca aacagcaact
10861 caaaacagac atlgagtttt aattttgggt cctgtacttt ttatttctag aagttctatt
10921 tggttatttt tcaagtctgc tacattatta cataattttt taataatttt atatctttaa
10981 tagatagttt ttgtgtttgt ttgtttttgt ttgttttttt cagctcacag tagctgggat tacaggcacg catcaccacg
11041 tgcccaggct ggagtgcagt ccctgcctca gcctcccaag ttagtagaga cagggtttca ccatgtggc caagacagtc
11101 caagtgattc cctggctaatt tttttgtgtt ttgtcccacc ttgcccctcc cgggcctccc gaagtcctgg gattacagag
11161 cctggctaatt tttttgtgtt ttgtcccacc ttgcccctcc cgggcctccc atttcaagtt ttttaaat ttgtgttcc
11221 tcgattctct ctgagccact tttttgctat accccatgcc tccaaagataa gaatatctga atcttttctt gggctctttt
11281 ctgagccact tttttgctat accccatgcc tccaaagataa gaatatctga atcttttctt gggctctttt cagttttttt
11341 ttaatttttt aaatataata cagttttact ttgtatctgt tgccttttgc tgggctgagc ctgcttaaaa caagccccgt
11401 aaatataata cagttttact ttgtatctgt tgccttttgc tgggctgagc ctgcttaaaa caagccccgt ctgtaactct
11461 ctgtgtcttt tagctgtaaa ggtttacctt agaaagtcag tcttgacgca cagtgttgt tactccta atgcccagc
11521 tagctgtaaa ggtttacctt agaaagtcag tcttgacgca cagtgttgt tactccta atgcccagc aatggcatga
11581 catctgctcc aagtcattta ttatatataa tcttgacgca cagtgttgt tactccta atgcccagc aatggcatga
11641 cacttgctcc aagtcattta ttatatataa tcttgacgca cagtgttgt tactccta atgcccagc aatggcatga
11701 agtgagttaa gctgacctga atgcccctca cgtgttttgg cagtgttgt tactccta atgcccagc aatggcatga
11761 tcaaacccaga gacagtttca ctttctgac cgtgttttgg cagtgttgt tactccta atgcccagc aatggcatga
11821 gcagactact gtttgaggac tgggtgtgtc tgggtgtgtc tgggtgtgtc tgggtgtgtc tgggtgtgtc tgggtgtgtc
11881 tctggctgcc atccaggga tagagcatgg tgggtgtgtc tgggtgtgtc tgggtgtgtc tgggtgtgtc tgggtgtgtc
11941 attatgggtg aaacatatga tgtccaaagt tgggtgtgtc tgggtgtgtc tgggtgtgtc tgggtgtgtc tgggtgtgtc
12001 agattccgga gggctgtgaa acaattagct tgggtgtgtc tgggtgtgtc tgggtgtgtc tgggtgtgtc tgggtgtgtc
12061 catgggggtt catgatccct ctctcacctt ttacacaggt gatggcagac aggttcccc gagccgctgc cccagaggtt
12121 agaacttgtt gccttttctt ttacacaggt gatggcagac aggttcccc gagccgctgc cccagaggtt ctgtgtgcat
12181 tctgttttcc aggtgacgt gatggcagac aggttcccc gagccgctgc cccagaggtt ctgtgtgcat cccagaggtt
12241 gttgtgaagc ccgacattgt gtcttttggg gatggcagac aggttcccc gagccgctgc cccagaggtt ctgtgtgcat
12301 gtggttgatt tcccattggc agatctgctg gaggtgtggg ggcaggggaa aggttcccc gagccgctgc cccagaggtt
12361 cagcaggcct gtgcaggag gtgcaggag gtgcaggag gtgcaggag gtgcaggag gtgcaggag gtgcaggag gtgcaggag
12421 ctcatgggct gtgcaggag gtgcaggag gtgcaggag gtgcaggag gtgcaggag gtgcaggag gtgcaggag gtgcaggag
12481 tacaggagtc acctcatggg ctgtcggcag ctgtcggcag ctgtcggcag ctgtcggcag ctgtcggcag ctgtcggcag
12541 ggaaggaggg ggtacaggag ggtacaggag ggtacaggag ggtacaggag ggtacaggag ggtacaggag ggtacaggag
12601 ctggagggca ggggaaggga ggggtacagg ggggtacagg ggggtacagg ggggtacagg ggggtacagg ggggtacagg
12661 gccactttgg gaaggtgaat ggattcagct ggggttccag ggggttccag ggggttccag ggggttccag ggggttccag

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FIG. 9G

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12721 tgcaggagac aggaagggtgg gaagctgctg ggcttttgag gtggtatagg tgtgggtggg
12781 gcaccacgtg tgaggagaag ggagaaggat gggggtgtgg gggcgaggtg gaggggcagg
12841 gcctcccagg tcagatcagg aatactgatg ctgcagtggg aggagtgata aatccacagg
12901 gactggcccg gggacgtagc tgcacccttg gggtcaggga ggcctttggg gagtggagag
12961 tggagcaagc agtgccagg agtgccagg ggctggctct ggagaagtg ggagagggc
13021 atggcgagga ggggactgg actgcagtc actgcagtc tggggacgtg agaaggaggc
13081 gtctggcttg gtctgtaag gtctgtaag tcagggcact tgctgggtgag gcgtgattg ggaggagga
13141 tgcccagctc ggttctgtgt gttctgtgag gtgtcggaaa tgcgccctgg atgtgttcaa
13201 gcagctgttc tgcgtctgcg tctgcagtc tctgcagtc aggaatctc tgactggatg gatgagaga
13261 agaaatgaga ccatgaagtc agtgtgtgcg agtgtggatg taggcaccga ggacagaagg
13321 gccagaaaga cctgtgctca gaggggccca gggcagccac gggggcccag agcatgtgtt
13381 tgttcaggca ttcattgtgt gctgggccct gctccagtgc tftggacata cacggtcaga
13441 tgtcagtgta agaaattata tagtttgtta ggagatgatt ggagctgc aaccagctgc aggttcaagt caggcaggtg
13501 aagtaggata gaaaggctag aagggttggg aaccagctgc aggttcaagt caggcaggtg
13561 gagtggatc taagagtta ccgaggtgag gagggtctgg gagggtcac cagcaggtg
13621 acaagcagca aggaggttg ggcgaagtca gcatgtatg gcatgtatg gaggcagaga
13681 agaaggtgg gcacaagtgt catctcatg gaggctggcg gaggctcaga aggtgaatg
13741 gttcagcttg ggttccaggc cagagtctga agtgtgagag agtgtgagag cagcagaccc
13801 gtgagccac catgggggca agactgggag cagcagaccc cagcagaccc ttgtaggtaa
13861 tcaccggctg gcactggctg ggggccaggt aaagccagca gaggtgagg ggtgctggag
13921 tctgggtgtg tgtgaacgca gagtgggtac gctggcacag gcatggggag gccgtggatt
13981 cccaaggacc ccagggctgt ggctggagca gctggaaaga gctggaaaga gcttcctgag
14041 atggagaagc ctggtagggtg ggtttggggg agctcaggaa agctcaggaa tttccacgga
14101 tttgaggatg tgctgcggac tggaggtgtt gaggaggtgg gaggaggtct ggagtcagg
14161 aaggcccaga ggcaggagct ggggtgctgg agtgagcaga agtgagagcc cagggtgtgg
14221 acagacatga gaatgccatg ctaggcctag gatggagga gatggagga gagacacagg aggagcagtc
14281 agaggggcca tgggtggtcag cacagtgtgg gctgagacca gctgaccgtg ctggccacca
14341 ggtgtggaca ctbgggccac cagccatctt tattgttgaa gtgaaatgaa aatggcctt
14401 ttcctgatta taagtgtatc atgtgtgtct ttgaaaagat tcgtacatta tataaagata
14461 taaagcaaa gatcaacttt ttaatccac caactgttag cattttattc tgtgtcctct
14521 cagctttctt tttttgtac aaatgcatac gtacagatgg taattttaa ctaaaagtgtg
14581 gacagcactg tacattctat tctgcagcct gctgttttca cgtaacaatg tgagtcocatg

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FIG. 9H

14641 gagatgtgct gcggtgttaa tgccaacagt ttcccttaca cggatatgag gtcccaactg
 14701 agaaacccat gtctgtaagg ctggtgccat catgaactga atgagctgggt gtcacgcaca
 14761 gactctatct catgtgcttt gtggacgaaa tgttaatgtg tttgactgtg gactaccact
 14821 ccagatcccc tttaggtgtg ctgaaacata tttaggtgtg ggatgtaaac catatgatct tttctaaaat
 14881 ctcaaaaatt ttcaaaaatt atgtaagcaa ttcttactg tttaggtgtg agagctttaa catagatctt gtggatttaa
 14941 tttacattaa tgggaggcca aggcaggaa abtgctgaa atggacaatg atgtaagcaa atgtaagcaa
 15001 tttacattaa tgggaggcca aggcaggaa abtgctgaa atggacaatg atgtaagcaa atgtaagcaa
 15061 tgggaggcca aggcaggaa abtgctgaa atggacaatg atggacaatg atggacaatg atggacaatg
 15121 tagtcagacc tcgtctctac aaaaataaaa agggaggatt gcttgagccc aagatagcca gcttaggcatt
 15181 agctatttga gaggctgagg atgccactgc cttatctact catccaagta taccatgca tgaatatccc
 15241 aactatgac atgctatgac gctgtaacat cctgtgtat cctgtgtat cctgtgtat cctgtgtat
 15301 gaatatccct tagtgaatat cctgtgtat cctgtgtat cctgtgtat cctgtgtat cctgtgtat
 15361 tagtgaatat cctgtgtat cctgtgtat cctgtgtat cctgtgtat cctgtgtat cctgtgtat
 15421 gctgataaat actgagaact actgagaact actgagaact actgagaact actgagaact actgagaact
 15481 ccatgcaagt actcctgaa actcctgaa actcctgaa actcctgaa actcctgaa actcctgaa
 15541 ctacccatgc aagtaactct aagtaactct aagtaactct aagtaactct aagtaactct aagtaactct
 15601 gtgaataatc ctgtgtgtca ctgtgtgtca ctgtgtgtca ctgtgtgtca ctgtgtgtca ctgtgtgtca
 15661 tgtggtgaat atccctgtgt atccctgtgt atccctgtgt atccctgtgt atccctgtgt atccctgtgt
 15721 atgctgataa atattgagaa atattgagaa atattgagaa atattgagaa atattgagaa atattgagaa
 15781 ctgtgttttt atgtagccat atgtagccat atgtagccat atgtagccat atgtagccat atgtagccat
 15841 attggttaaa aatgcatgt aatgcatgt aatgcatgt aatgcatgt aatgcatgt aatgcatgt
 15901 aaagaatgaa gtatcatctt gtatcatctt gtatcatctt gtatcatctt gtatcatctt gtatcatctt
 15961 atatttgtac ctcttgacca ctcttgacca ctcttgacca ctcttgacca ctcttgacca ctcttgacca
 16021 ttttaatttt ttttaatttt ttttaatttt ttttaatttt ttttaatttt ttttaatttt ttttaatttt
 16081 cttttttttt cttttttttt cttttttttt cttttttttt cttttttttt cttttttttt cttttttttt
 16141 gtggcgcgat ctgggctcac ctgggctcac ctgggctcac ctgggctcac ctgggctcac ctgggctcac
 16201 caacctccc agtagctcg agtagctcg agtagctcg agtagctcg agtagctcg agtagctcg
 16261 ttttagtaga gacgtgggtt cactatgttg cactatgttg cactatgttg cactatgttg cactatgttg
 16321 ctgatctgcc cgcctcgcc tcccaaaagt tcccaaaagt tcccaaaagt tcccaaaagt tcccaaaagt
 16381 agctgttaat ataaatttca ataaatttca ataaatttca ataaatttca ataaatttca ataaatttca
 16441 ttataaatga gtttatcaat tagcaatata tagcaatata tagcaatata tagcaatata tagcaatata
 16501 cagtttatga tgatgagagg ttttttaaaa ttttttaaaa ttttttaaaa ttttttaaaa ttttttaaaa

FIG. 9I

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16561 agataccgag ataatagtat ttacatagtc agatctgtca ttttcccttg ccttatctga
16621 cttcagtttt tacgcctaga acgttcttcc ctatccttac ttgtaaatcc ttagttttct
16681 tcaagaactg ttttgtttac attgaaacct ttaatccatt tgaatttttt tcagtgggag
16741 gtaggaatgg agtattttcc tccagataa tgagccagtt ttaatgocat ttattaatgt
16801 atccattttt ttaccttgtt cctcttaaaa gaagccttga gagctgtatg aagaagcctg
16861 atggcaccca gctaggcctc ctgggggca ccagctcgtc ggggtgggaa gacatggag agacgtaaca
16921 gtaactcctg ctgggggca gctagccaga ggagatggac tgggtgcccg atgctggtgg tggctgtcag
16981 agcaagcagg gctagccaga gctagccaga gaggacagg agtctgtgc tgtgtccct ctgctcatg
17041 ctgggagaca gctagccaga gctagccaga gaggacagg agtctgtgc tgtgtccct ctgctcatg
17101 ggaaggttgg atccctgatg agccttgaa gaggagtggg agaacagaac gtgctcaca agaggaggca
17161 aacagcaaga agtgggagag agagagagag agccttgaa cggaggtggg gaaaagcaag gatgtgtgga
17221 agcggcagga gctcagctga agaaatgaga ttggtggtgg gcggagggga gcggagggcca
17281 tctgactgcc aatctgctgc tctccaaagg cctcgggtggc ttggtgcatga aacatcctga
17341 ggagcttgtt aagtggacac tgtgcccctc acgtttggcc cattttccag gtggagcctt
17401 ttgccagctt gaccgaggcc gtgcggagct cagtcccccg actgctcatc aaccgggact
17461 tgggtggggcc cttggcttgg catcctcgca gcagggaactg gcccagctg ggggacgtgg
17521 ttcacggcgt gaaaagccta gtggagcttc tgggctggac agaagatg cgggaccttg
17581 tgcagcggga aactgggaag gtacagactg ctgagggagg ctgcccagg ggtgccctt
17641 ctcactgctc taggtatgg tccacctgat atggagcccc atggagctga ctggacctga ctgggtgtta
17701 taatagctga tgatgctgtg aatcctgcac caggcaactac acagatgcta acaggccaag cagggacacg
17761 ctttcaatac ctccatttag agatgaggaa tctttaacag actataggcg ttaccttcac
17821 gtccagacac tcagggctga tgagtctgta tcttaacag tctaaggcct tctaagatga
17881 tccctaaaaa aagcagcca acagtgtccc tctaaggcct tctaaggcct tctaagatga
17941 tacgggttaa agaacttga gaagctaaag ttctctgcat ctatgtggga tgaaggctac
18001 atgagtgatg cttttcggaa gtaattggta tgagagccat tgatcaatta taatccgctg
18061 aggaaagcca aagagccttg atgctttcta agagatttca accgcctat aaacaagtga
18121 agttacagaa aatgcagttt ccataaagaa tcatgtagca tgtgtagcct tgatatgaag
18181 ggttttcaat gggaggttgt agcattaaag atttaagtc agtgtcatg attacaggca
18241 ggtgtaacac tgtccaaagg ggatttcacc agtgtattag agtgcctctt ggtgcctcat
18301 aaaggcatac ctgagactgg gtaatttata aaggaaagag gtttaatgga ctcacagttc
18361 catatggctg tgggggcctc acaatcatag tggaaagcaa agcaggagca aagtactgtc
18421 ttacaaaggtg gcaggcaaga ggacgtgtgc ggggggaactg cctttataa aactatcaga

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FIG. 9J

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18481 tcttgtgaga cttattcaact atcagaagaa gagtgtggga aagaccgcgc cccatgattc
18541 agttacttcc acttgcccc acacagccaa accatatcaa catggggatg attacaattc
18601 ttgggtgggg gccttccaag gcatgtgtct ctagggcca gttgcaggct caattcatta
18661 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
18721 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
18781 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
18841 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
18901 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
18961 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19021 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19081 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19141 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19201 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19261 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19321 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19381 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19441 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19501 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19561 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19621 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19681 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19741 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19801 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19861 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19921 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
19981 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
20041 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
20101 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
20161 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
20221 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
20281 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag
20341 tgcctcatgg ccaacctctg gaacccagct aacaatgag aacatcatgt cctccaccag

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FIG. 9K

20401 cggcccagct cctccctctg aagggtggtca cattctgttg actctccata ctgagcctct
 20461 cctccagaaa cagatctgtt ccagaacatt ccagcacttt ccactctggc tccttgtccc
 20521 cacactacgc cccccacc gaaggcatta tgcacttaaa ttcctctagt tgcactaatct
 20581 ctgagacagg gaaggcatta tgcacttaaa tgcacttaaa tgcacttaaa tgcacttaaa
 20641 ttgtgacata ggaggtggaa tgcacttaaa tgcacttaaa tgcacttaaa tgcacttaaa
 20701 ggctgtaccc agggccagcc cactgcaca tccctctcc tgcacttaaa tgcacttaaa
 20761 ggctctcacc cactgcaca tccctctcc tgcacttaaa tgcacttaaa tgcacttaaa
 20821 acaaaaagcac tccctctcc tgcacttaaa tgcacttaaa tgcacttaaa tgcacttaaa
 20881 ttaatgacag tgactcccag gaagggggtc ctgtgacttt ctgtgacttt ctgtgacttt
 20941 ggtggaattg gtgacctagg aaaactgttg aattctaaaa aattctaaaa aattctaaaa
 21001 cctagtttaa tttccctttt ttattttttg agtcttgccc agtcttgccc agtcttgccc
 21061 ggtgttatga ttcagctca ttcagctca ttcagctca ttcagctca ttcagctca
 21121 tagctgggat tacaggtgtg tccaccaca ctagacat ctagacat ctagacat
 21181 caaggttttg ctatgttggc caggctggtc caggctggtc caggctggtc caggctggtc
 21241 cctcgccctc ccaaatgtgt ccaaatgtgt ccaaatgtgt ccaaatgtgt ccaaatgtgt
 21301 tgataattta cacatacat tttgtccaaa actctctttt actctctttt actctctttt
 21361 tggctatgtg tggatgtgg tataaatctc aatctatggt ca (SEQ ID NO:4)

FIG. 9L

GenBank Accession No. NM_012239
Homo sapiens
 SIRT3

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1  gcgagtcagg aggaactcctt ggactgcgcg gaacatgggc ttctgggggtt ggcgcgccgc
61  ggcagccctc cggctgtggg gccgggtagt tgaacgggtc tgaacgggtc gaggcgtggg
121  gccgtttcag gcctgcggct gtccgctggt gcttggcggc gcttggcggc agggacgatg tgagtgcggg
181  gctgagaggc agccatgggg ccgcgggtga cccttggac gccctggac cggcgcgcc ccttgcagag
241  gcctcccaga ccgaggtgc tcgagtatta aagtggaag ccgagggcag cagctcccag
301  tttcttcttt tgcagtatta aagtggaag ccgagggcag tcttttctg tgggtgcttc
361  aagtgttgtt ggaagtggag gcagcagtga caaggggaag ctctccctg aggatgtagc
421  tgagctgatt cgggccagag cctgccagag ggtggtggtc atggtggggg ccggcatcag
481  cacaccagtt ggcattccag acttcagatc gccggggagt gccctgtaca gcaacctcca
541  gcagtaagat ctccgtacc ccgaggccat ttttgaactc ccattcttct ttcacaaacc
601  caagcccttt ttcactttgg ccaaggagct gtaccctgga aactacaagc ccaacgtcac
661  tcaactactt ctccggctgc ttcatgacaa ggggctgctt ctgcggctct acacgcagaa
721  catcgatggg cttgagagag tgtcgggcat ccctgcctca aagctggttg aagctcatgg
781  aacctttgcc tctgccacct gcacagtctg ccaaagaccc ttcccagggg aggacattcg
841  ggctgacgtg atggcagaca gggttccccg ctgcccggtc tgcaccggcg ttgtgaagcc
901  cgacatttgt ttctttgggg agccgctgcc ccagaggttc ttgctgcatt tggttgattt
961  ccccatggca gatctgctgc tcctccttgg gacctccctg gaggtggagc cttttgccag
1021  cttgaccgag gccgtgcgga gctcagttcc ccgactgctc atcaaccggg acttgggtggg
1081  gcccttggct tggcatcctc gcagcaggga cgtggcccag ctgggggagc tggttcacgg
1141  cgtggaaagc ctagtggagc ttctgggctg gacagaagag atgcgggacc ttgtgcagcg
1201  ggaactgggg aagcttgatg gaccagacaa ataggatgat ggctgcccc acacaataaa
1261  tggtaacata ggagacatcc acatcccaat tctgacaaga cctcatgcct gaagacagct
1321  tgggcagggtg aaaccagaat atgtgaactg agtggacacc cgaggctgcc actggaatgt
1381  ctcttcaggc catgagctgc agtgactggt agggctgtgt ttacagtcag ggcaccccg
1441  tcacatatac aaaggagctg cctgcctgtt tgctgtgttg aactcttcac tctgctgaag

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FIG. 10A

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1501 ctccctaattg aaaaagcttt cttctgactg tgacctctct gaactgaatc agaccaactg
1561 gaatcccaga ccgagctctgc ttctctgtgcc tagttgaacg gcaagctcgg catctgttgg
1621 ttacaagatc cagacttggg ccgagcggtc ccagccctc ttcatgttcc gaagtgtagt
1681 cttgaggccc tggcgccgca ctctagcat gttggtctcc tttagtggg ctatttttaa
1741 tgagagaaaa tctgttcttt ccagcatgaa atacatttag tctcctcaaa gggactgcag
1801 gtgttgacat gagtggaaa ggaacccctg ggatacgtgg cgtccctct attggaacag
1861 tctgaggact gaaggcattt gtccctggat ttattggaga cggcccagct cctccctctg
1921 aagggtgtca cattctgttg actctccata ctacgcctct cctccagaaa cagatctgtt
1981 ccagaacatt ccagcacttt ctatctggcc tcttctctcc tccactacgc cccccacc
2041 tcgccagggc ttcccttagt gacactgtta gagctaattc ctgagacagg gaaggcata
2101 ctcaactaaa acccaggctg agtccctggc acctgctgga ttgtgacata ggaggtggaa
2161 tccactgaac tgctacttct gcacaggctc ctctccttgg ggctgtaccc aggccagcc
2221 ctgatggctc accctgtcag gcaccagctg ctccctcctg ggctctcacc cacctgcaca
2281 tcctccctcc tagcatcaca ttacctgcgt gtttccccag ttaataagcac tccccattct
2341 tgaaccttgc ctacctggg ctgagctgac ggcaatagat ttaatgacag tgactcccag
2401 gaagggggtc ctgtgacttt gcgccttaat aagaacaaaa ggtggaattg gtgacctagg
2461 aaactgttg aattctaaaa agaatgaagt tagtttctaa cctagttaa tgttcctttt
2521 ttattttttg agtcttgccc tgtcactcag ggtggagtgc tagtattatga tctcagctca
2581 ctgcaacttc cgcctccccg gtttaagcga ttctcctggg tagctgggat tacaggtgtg
2641 tcccaccaca cctagcacat gggcatattt gtaatagaga caagggtttg ctatgtttgg
2701 caggctggtc tcgaaactcct ggcttcaagt gatccacca cctcggcctc ccaaatgtct
2761 gggattacag gcatgagcca ctgtgcctgg cccctttatt tgataattta cacatacat
2821 ttgtccaaa actcttcttt atttcaagat gatgtttctg tggctatgtg tggtatgtgg
2881 tataaatctc aatctatggt caaaaaaaaaa (SEQ ID NO:5)

FIG. 10B