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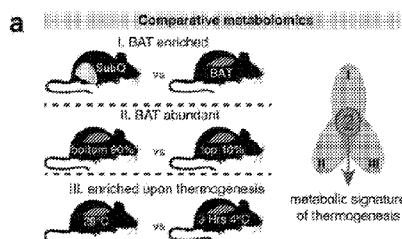
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(54) Title: METHODS FOR IDENTIFICATION, ASSESSMENT, PREVENTION, AND TREATMENT OF METABOLIC DISORDERS USING SUCCINATE

Figure 1



(57) Abstract: The present invention relates to methods for identifying, assessing, preventing, and treating metabolic disorders and modulating metabolic processes using succinate and derivatives thereof.



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METHODS FOR IDENTIFICATION, ASSESSMENT, PREVENTION, AND TREATMENT OF METABOLIC DISORDERS USING SUCCINATE

Cross-Reference to Related Applications

- 5 This application claims the benefit of U.S. Provisional Application No. 62/615,659, filed on 10 January 2018; the entire contents of said application are incorporated herein in their entirety by this reference.

Statement of Rights

- 10 This invention was made with government support under grant numbers DK103295, DK97441, DK112268, GM067945, and 5-P30-CA06516, awarded by the National Institutes of Health. The government has certain rights in the invention.

Background of the Invention

- 15 Metabolic disorders comprise a collection of health disorders or risks that increase the risk of morbidity and loss of quality of life. For example, diabetes, obesity, including central obesity (disproportionate fat tissue in and around the abdomen), atherogenic dyslipidemia (including a family of blood fat disorders, *e.g.*, high triglycerides, low HDL cholesterol, and high LDL cholesterol that can foster plaque buildups in the vascular
20 system, including artery walls), high blood pressure (130/85 mmHg or higher), insulin resistance or glucose intolerance (the inability to properly use insulin or blood sugar), a chronic prothrombotic state (*e.g.*, characterized by high fibrinogen or plasminogen activator inhibitor-1 levels in the blood), and a chronic proinflammatory state (*e.g.*, characterized by higher than normal levels of high-sensitivity C-reactive protein in the blood), are all
25 metabolic disorders collectively afflicting greater than 50 million people in the United States.

- Brown fat has attracted significant interest as an antidiabetic (*e.g.*, anti-type 2 diabetes) and anti-obesity tissue owing to its ability to dissipate energy as heat (Cannon and Nedergaard (2004) *Physiol. Rev.* 84:277-359; Harms and Seale (2013) *Nat. Med.* 19:1252-
30 1263). Activation of thermogenesis in brown and beige fat increases energy expenditure and can combat metabolic disease. However, in order to do so, brown and beige adipose tissue require extrinsic stimuli to activate thermogenesis. Adipocyte lipolysis through cyclic purine nucleotide signaling (*e.g.*, through cAMP/PKA signaling) via adrenergic stimulus and related pathways, are well known as upstream activators of adipose tissue

thermogenesis (Pfeifer *et al.* (2015) *Annu Rev Pharmacol Toxicol* 55, 207-227). Nevertheless, attempts to pharmacologically target thermogenic pathways have so far shown limited success (Cypess *et al.* (2012) *PNAS* 109, 10001-10005; Cypess *et al.* (2015) *Cell Metab* 21, 33-38; Carey *et al.* (2013) *Diabetologia* 56, 147-155). It is now recognized that at least two types of thermogenic fat cells exist – classical interscapular brown fat, as well as inducible brown-like adipocytes in white fat (also known as beige fat), which tends to be dispersed among white fat depots (Wu *et al.* (2012) *Cell* 150:366-376; Shinoda *et al.* (2015) *Nat. Med.* 4:389-394). BAT has high basal levels of UCP1, whereas beige fat has low basal levels that are highly inducible upon stimulation with cold or other agents (Wu *et al.* (2012) *Cell* 150:366-376). Despite their common ability to exhibit adaptive thermogenesis, brown and beige cells do not derive from the same lineage precursors (Seale *et al.* (2008) *Nature* 454:961-967) and express different molecular signatures (Wu *et al.* (2012) *Cell* 150:366-376; Harms and Seale (2013) *Nat. Med.* 19:1252-1263). Mouse models resistant to weight gain through enhanced brown and beige fat content or activity have demonstrated that activation of thermogenesis in fat can be a powerful strategy to improve metabolic health and prevent weight gain (Fisher *et al.* (2012) *Genes Dev.* 26:271-281; Vegiopoulos *et al.* (2010) *Science* 328:1158-1161; Ye *et al.* (2012) *Cell* 151:96-110).

Despite decades of scientific research, few effective therapies have emerged to treat metabolic disorders. Accordingly, there is a great need to identify regulators of metabolic disorders, especially those that regulate adipose tissue thermogenesis (*e.g.*, thermogenesis of brown and/or beige fat). Such regulators would also be useful in the generation of diagnostic, prognostic, and therapeutic agents to effectively control metabolic disorders in subjects.

25 **Summary of the Invention**

The present invention is based, in part, on the discovery that substantial and selective accumulation of succinate is a unique metabolic signature of thermogenic adipose tissue and has the ability to modulate many metabolic processes, including modulating adipose thermogenesis, oxygen consumption, energy expenditure, blunted weight gain, and glucose homeostasis. This accumulation occurs independently of the adrenergic cascade, and is sufficient to activate brown adipocyte thermogenic respiration *in vivo*. Moreover, selective accumulation is driven by a newly discovered capacity for fat cells such as brown/beige adipocytes to sequester elevated circulating succinate, accumulating in excess

of 100x more succinate compared to most cells which are generally not permeable to succinate. In addition, brown adipocyte can be initiated and thermogenic respiration activated by pharmacological elevation of circulating succinate to drive UCP1-dependent brown adipocyte thermogenesis *in vivo*, which stimulates protection against diet-induced obesity and improves glucose tolerance. Thus, succinate modulates adipose tissue homeostasis and glucose metabolism and has the therapeutic ability to treat metabolic disorders, especially obesity-induced metabolic disorders.

In one aspect, an agent that modulates succinate or a biologically active fragment thereof, in a subject for use in modulating a metabolic response in the subject, optionally wherein the agent is formulated in a pharmaceutically acceptable carrier, is provided.

Numerous embodiments are further provided that can be applied to any aspect of the present invention described herein. For example, in one embodiment, succinate is upregulated. In another embodiment, succinate is upregulated using an agent selected from the group consisting of succinic acid and salts thereof, and an agent that promotes muscle contraction. In still another embodiment, the medicament further comprises an additional agent that increases the metabolic response. In yet another embodiment, succinate is downregulated. In another embodiment, succinate is downregulated using an agent selected from the group consisting of a metabolizer of succinate, an antioxidant, a mitochondria-targeted antioxidant, an inhibitor of muscle shivering, an inhibitor of plasma membrane transport, an inhibitor of plasma membrane secondary active transport via the Na^+/K^+ -ATPase, an inhibitor of SLC25A10, and an inhibitor of ROS-dependent cysteine oxidation. In still another embodiment, the medicament further comprises an additional agent that decreases the metabolic response. In yet another embodiment, the metabolic response is selected from the group consisting of: a) modified expression of a marker selected from the group consisting of: cidea, adiponectin, adipsin, otopetrin, type II deiodinase, cig30, ppar gamma 2, pgc1 α , ucp1, elovl3, cAMP, Prdm16, cytochrome C, cox4i1, coxIII, cox5b, cox7a1, cox8b, glut4, atpase b2, cox II, atp5o, ndufb5, ap2, ndufs1, GRP109A, acylCoA-thioesterase 4, EARA1, claudin1, PEPCK, fgf21, acylCoA-thioesterase 3, dio2, fatty acid synthase (fas), leptin, resistin, and nuclear respiratory factor-1 (nrf1); b) modified thermogenesis in adipose cells; c) modified differentiation of adipose cells; d) modified insulin sensitivity of adipose cells; e) modified basal respiration, leak respiration, or uncoupled respiration; f) modified whole body oxygen consumption; g) modified obesity or appetite; h) modified insulin secretion of pancreatic beta cells; i)

modified glucose tolerance; and j) modified activity of UCP1 protein. In another embodiment, the metabolic response is upregulated. In still another embodiment, the metabolic response is downregulated.

In another aspect, a method for modulating a metabolic response comprising
 5 contacting a cell with an agent that modulates succinate, to thereby modulate the metabolic response, is provided.

As described above, certain embodiments are applicable to any method described herein. For example, in one embodiment succinate is upregulated. In another embodiment, succinate is upregulated using an agent selected from the group
 10 consisting of succinic acid and salts thereof, and an agent that promotes muscle contraction. In still another embodiment, the method further comprises contacting the cell with an additional agent that increases the metabolic response. In yet another embodiment, succinate is downregulated. In another embodiment, succinate is downregulated using an agent selected from the group consisting of a metabolizer of
 15 succinate, an antioxidant, a mitochondria-targeted antioxidant, an inhibitor of muscle shivering, an inhibitor of plasma membrane transport, an inhibitor of plasma membrane secondary active transport via the Na^+/K^+ -ATPase, an inhibitor of SLC25A10, and an inhibitor of ROS-dependent cysteine oxidation. In still another embodiment, the method further comprises contacting the cell with an additional agent
 20 that decreases the metabolic response. In yet another embodiment, the step of contacting occurs *in vivo*. In another embodiment, the step of contacting occurs *in vitro*. In still another embodiment, the cell is selected from the group consisting of fibroblasts, adipoblasts, preadipocytes, adipocytes, white adipocytes, brown adipocytes, and beige adipocytes. In yet another embodiment, the metabolic response
 25 is selected from the group consisting of: a) modified expression of a marker selected from the group consisting of: cidea, adiponectin, adipsin, otopetrin, type II deiodinase, cig30, ppar gamma 2, pgc1 α , ucp1, elov13, cAMP, Prdm16, cytochrome C, cox4i1, coxIII, cox5b, cox7a1, cox8b, glut4, atpase b2, cox II, atp5o, ndufb5, ap2, ndufs1, GRP109A, acylCoA-thioesterase 4, EARA1, claudin1, PEPCK, fgf21, acylCoA-thioesterase 3, dio2, fatty acid synthase (fas), leptin, resistin, and nuclear respiratory factor-1 (nrf1); b) modified thermogenesis in adipose cells; c) modified differentiation of adipose cells; d) modified insulin sensitivity of adipose cells; e) modified basal respiration, leak respiration, or uncoupled respiration; f) modified whole body oxygen
 30

consumption; g) modified obesity or appetite; h) modified insulin secretion of pancreatic beta cells; i) modified glucose tolerance; and j) modified activity of UCP1 protein. In another embodiment, the metabolic response is upregulated. In still another embodiment, the metabolic response is downregulated.

5 In still another aspect, a method of preventing or treating a metabolic disorder in a subject comprising administering to the subject an agent that promotes succinate in the subject, thereby preventing or treating the metabolic disorder in the subject, is provided. In one embodiment, the agent is selected from the group consisting of succinic acid and salts thereof, and an agent that promotes muscle contraction. In still
10 another embodiment, the agent is administered orally or systemically, optionally wherein the administration is in a solution comprising 1% to 2% succinate and/or is *ad libitum*. In yet another embodiment, the agent is administered in a pharmaceutically acceptable formulation. In another embodiment, the metabolic disorder is selected from the group consisting of pain, insulin resistance, hyperinsulinemia,
15 hypoinsulinemia, type II diabetes, hypertension, hyperhepatosteatosis, hyperuricemia, fatty liver, non-alcoholic fatty liver disease, polycystic ovarian syndrome, acanthosis nigricans, hyperphagia, endocrine abnormalities, triglyceride storage disease, Bardet-Biedl syndrome, Lawrence-Moon syndrome, and Prader-Labhart-Willi syndrome. In still another embodiment, the subject is a non-human animal or a human, optionally
20 wherein the non-human animal is an animal model of the metabolic disorder.

In yet another aspect, a method for preventing or treating a metabolic disorder in a subject comprising administering to the subject an agent that inhibits or reduces succinate in the subject, thereby preventing or treating the metabolic disorder in the subject, is provided. In one embodiment, the agent is selected from the group
25 consisting of a metabolizer of succinate, an antioxidant, a mitochondria-targeted antioxidant, an inhibitor of muscle shivering, an inhibitor of plasma membrane transport, an inhibitor of plasma membrane secondary active transport via the Na^+/K^+ -ATPase, an inhibitor of SLC25A10, and an inhibitor of ROS-dependent cysteine oxidation. In another embodiment, the agent is administered systemically. In still
30 another embodiment, the agent is administered in a pharmaceutically acceptable formulation. In yet another embodiment, the metabolic disorder is selected from the group consisting of obesity-associated cancer, anorexia, and cachexia. In another

embodiment, the subject is a non-human animal or a human, optionally wherein the non-human animal is an animal model of the metabolic disorder.

In another aspect, a cell-based assay for screening for agents that modulate a metabolic response in a cell by modulating succinate, comprising contacting the cell in the presence of succinate with a test agent that modulates succinate, and determining the ability of the test agent to modulate a metabolic response in the cell, is provided.

In still another aspect, a method for assessing the efficacy of an agent that modulates succinate uptakes, for modulating a metabolic response in a subject, comprising: a) detecting in a subject sample at a first point in time, the amount of succinate; b) repeating step a) during at least one subsequent point in time after administration of the agent; and c) comparing the amount detected in steps a) and b), wherein a significantly lower amount of succinate in the first subject sample relative to at least one subsequent subject sample, indicates that the agent increases the uptake of succinate in the subject and/or wherein a significantly higher amount of succinate in the first subject sample relative to at least one subsequent subject sample, indicates that the agent decreases the uptake of succinate in the subject. Optionally, expression and/or activity of a marker listed in Table 1 is detected in steps a) and b), wherein a significantly lower expression and/or activity of a marker listed in Table 1 in the first subject sample relative to at least one subsequent subject sample, indicates that the agent increases the metabolic response in the subject and/or wherein a significantly higher expression and/or activity of a marker listed in Table 1 in the first subject sample relative to at least one subsequent subject sample, indicates that the agent decreases the metabolic response in the subject. In yet a further option, a metabolic response, selected from a) modified thermogenesis in adipose cells; b) modified differentiation of adipose cells; c) modified insulin sensitivity of adipose cells; d) modified basal respiration, leak respiration, or uncoupled respiration; e) modified whole body oxygen consumption; f) modified obesity or appetite; g) modified insulin secretion of pancreatic beta cells; h) modified glucose tolerance; and i) modified activity of UCP1 protein, is detected in steps a) and b), wherein a significantly lower metabolic response in the first subject sample relative to at least one subsequent subject sample, indicates that the agent increases the metabolic response in the subject and/or wherein a significantly higher metabolic response in the first subject sample

relative to at least one subsequent subject sample, indicates that the agent decreases the metabolic response in the subject.

As described above, certain embodiments are applicable to any method described herein. For example, in one embodiment, succinate is upregulated. In another
5 embodiment, succinate is downregulated. In still another embodiment, the agent is selected from the group consisting of succinic acid and salts thereof, an agent that promotes muscle contraction a metabolizer of succinate, an antioxidant, a mitochondria-targeted antioxidant, an inhibitor of muscle shivering, an inhibitor of plasma membrane transport, an inhibitor of plasma membrane secondary active transport via the Na^+/K^+ -ATPase, an inhibitor of
10 SLC25A10, and an inhibitor of ROS-dependent cysteine oxidation. In yet another embodiment, the subject has undergone treatment for the metabolic disorder, has completed treatment for the metabolic disorder, and/or is in remission from the metabolic disorder between the first point in time and the subsequent point in time. In another embodiment, the first and/or at least one subsequent sample is selected from the group consisting of *ex vivo* and *in vivo* samples. In still another embodiment, the first and/or at least one
15 subsequent sample is obtained from an animal model of a metabolic disorder. In yet another embodiment, the first and/or at least one subsequent sample is selected from the group consisting of tissue, whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, urine, stool, and bone marrow. In another embodiment, the first and/or at least one
20 subsequent sample is a portion of a single sample or pooled samples obtained from the subject. In a further embodiment, modulation comprises upregulation by at least 25% relative to the second sample. In yet a further embodiment, modulation comprises downregulation by at least 25% relative to the second sample. In still another embodiment, a significantly higher expression and/or activity comprises upregulating the expression
25 and/or activity by at least 25% relative to the second sample. In yet another embodiment, a significantly lower expression and/or activity comprises downregulating the expression and/or activity by at least 25% relative to the second sample. In another embodiment, the amount of the marker is compared. In still another embodiment, the amount of the marker is determined by determining the level of protein expression of the marker. In yet another
30 embodiment, the presence of the protein is detected using a reagent which specifically binds with the protein. In another embodiment, the reagent is selected from the group consisting of an antibody, an antibody derivative, and an antibody fragment. In still another embodiment, the level of expression of the marker in the sample is assessed by detecting

the presence in the sample of a transcribed polynucleotide or portion thereof. In yet another embodiment, the transcribed polynucleotide is an mRNA or a cDNA. In another embodiment, the step of detecting further comprises amplifying the transcribed polynucleotide. In still another embodiment, the level of expression of the marker in the sample is assessed by detecting the presence in the sample of a transcribed polynucleotide which anneals with the marker or anneals with a portion of a polynucleotide under stringent hybridization conditions. In yet another embodiment, the metabolic response is selected from the group consisting of: a) modified expression of a marker selected from the group consisting of: cidea, adiponectin, adipsin, otopetrin, type II deiodinase, cig30, ppar gamma 2, pgc1 α , ucp1, elovl3, cAMP, Prdm16, cytochrome C, cox4i1, coxIII, cox5b, cox7a1, cox8b, glut4, atpase b2, cox II, atp5o, ndufb5, ap2, ndufs1, GRP109A, acylCoA-thioesterase 4, EARA1, claudin1, PEPCK, fgf21, acylCoA-thioesterase 3, dio2, fatty acid synthase (fas), leptin, resistin, and nuclear respiratory factor-1 (nrf1); b) modified thermogenesis in adipose cells; c) modified differentiation of adipose cells; d) modified insulin sensitivity of adipose cells; e) modified basal respiration, leak respiration, or uncoupled respiration; f) modified whole body oxygen consumption; g) modified obesity or appetite; h) modified insulin secretion of pancreatic beta cells; i) modified glucose tolerance; and j) modified expression of UCP1 protein. In another embodiment, the metabolic response is upregulated. In still another embodiment, the metabolic response is downregulated.

As described above, certain embodiments are applicable to any method described herein. For example, in one embodiment, the succinate is natural or synthetic. In still another embodiment, the succinate is a metabolite or a pro-drug. In yet another embodiment, the the succinate is monobasic or a dibasic salt. In a preferred embodiment, the succinate is a sodium salt of succinic acid.

Brief Description of Figures

Figure 1 includes 9 panels, identified as panels A, B, C, D, E, F, G, H, and I, which show the selective and substantial accumulation of succinate in adipose tissue thermogenesis. Panel A shows an illustrative diagram of the comparative metabolomics strategy used to identify conserved metabolic signatures of adipose tissue thermogenesis in mice. Panels B-D show the results from the comparative approach illustrating all annotated metabolites (grey), metabolites fulfilling each individual criterion (black), and metabolites

fulfilling all criteria (red). BAT-enriched metabolites in panel B were defined as those that were enriched more than fourfold ($-\log P > 4$) in BAT versus subcutaneous inguinal adipose tissue (SubQ; $n = 8$). Abundant BAT metabolites (in Panel C) were defined as those within the 10% most abundant annotated metabolite ion intensities ($n = 8$); although
 5 determination of abundance in this way is not absolute, as ion intensity can vary between species on the basis of factors other than abundance. BAT metabolites enriched upon activation of thermogenesis by exposure to 4°C for 3 hours (Hrs) in (Panel D) were defined as those that were enriched more than threefold ($-\log P > 4$) versus BAT in mice housed at 29°C ($n = 8$). Panel E and Panel I show a determination of absolute succinate content in
 10 mouse adipose tissues ($n = 8$; brain $n = 6$; heart $n = 6$; liver $n = 4$; kidney $n = 4$). Conditions: BAT, 3 h at 4 °C; SubQ and epididymal fat (Epi) 2 weeks at 4 °C. Panel F shows abundance profile of mitochondrial TCA cycle metabolites and metabolites proximal to TCA cycle in BAT at 29°C, 23°C, and following 3 Hrs exposure to at 4°C ($n = 8$). α KG, α -ketoglutarate; α GP, α -glycerophosphate. Panel G shows abundance of succinate in BAT,
 15 SubQ, and epididymal (Epi) adipose tissue comparing 29°C to 2 weeks 4°C exposure ($n = 8$; except 2 weeks 4°C BAT, $n=5$). Panel H shows abundance of succinate in BAT following 3-adrenoreceptor agonism by CL treatment ($n = 5$; 5 min & 30 min $n = 4$). Data are mean $\pm$ s.e.m. of at least four mouse replicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-tailed Student's t-test for pairwise comparisons, one-way ANOVA for multiple
 20 comparisons involving one independent variable).

Figure 2 includes 4 panels, identified as panels A, B, C and D, which show quality control of MS analysis of TCA cycle metabolites in thermogenic adipose tissue. Panel A shows a quality control determination of coefficient of variation for LC–MS quantification of succinate. Panel B shows a determination of linearity of the relationship between LC–
 25 MS succinate peak intensity and succinate concentration for absolute determination of succinate concentration. Panels C and D show quality control for mass-spectrometry analysis of succinate in thermogenic adipose tissue. Because of its unusual abundance in BAT, special consideration is required to determine the linearity of the relationship between LC–MS succinate peak intensity and succinate concentration for quantitative analysis.
 30 Succinate abundance is measured in extraction solution as described in the methods section. Absolute determination of succinate concentration is compared between succinate extracted from BAT (red) and the same samples following 100-fold dilution (green). Samples are analysed in parallel with defined amounts of [^{13}C]succinate (black) used at concentrations

that are within the established linear range of the mass spectrometer. Following 100-fold dilution of BAT extracts, succinate signals are within the linear range of detection. However, undiluted extracts are at concentrations that result in a nonlinear relationship and are therefore not appropriate for quantitative analysis (Panel C). Calculation of the apparent dilution factor reveals the effect of nonlinearity with apparent dilutions ranging from ~11–28-fold that are in fact 100-fold (Panel D).

Figure 3 includes 8 panels, identified as panels A, B, C, D, E, F, G, and H, which show the results of metabolite analysis of the acute response of BAT to β -adrenergic stimulus *in vivo* by CL. Panels A and E shows rapid depletion of BAT triacylglycerol (TAG) species following i.v. injection of 1 mg/kg CL. Panels B and F shows depletion of BAT diacylglycerol (DAG) species following i.v. injection of 1 mg/kg CL. Panels C and G shows accumulation of free fatty acid species and acyl-carnitine species in BAT following i.v. injection of CL. Panel D shows a profile of mitochondrial TCA cycle metabolites and related metabolites in BAT following i.v. injection of CL ($n = 5$; 5 min & 30 min $n = 4$). Data are mean $\pm$ s.e.m. of at least four mouse replicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-tailed Student's *t*-test for pairwise comparisons, one-way ANOVA for multiple comparisons involving one independent variable). Panel H shows abundance of TCA cycle metabolites in BAT following intravenous β -adrenoreceptor agonism with 1 mg/kg isoproterenol or 1 mg/kg CL ($n = 5$; CL, iso, $n = 4$). Panels C and G show one-way ANOVA; panel H shows two-sided *t*-test; data are mean $\pm$ s.e.m. of biologically independent samples.

Figure 4 includes 15 panels, identified as panels A, B, C, D, E, F, G, H, I, J, K, L, M, N, and O, which show selective accumulation of succinate via extracellular uptake in brown adipocytes. Panel A shows a schematic diagram illustrating potential inputs to succinate-directed flux by conventional BAT metabolism and using ^{13}C -metabolite labelling strategy. Panels B-C show the ^{13}C -isotopologue profile of TCA cycle metabolites succinate in mouse BAT at 29°C or 4°C following i.v. infusion of ^{13}C -glucose (Panel B) and ^{13}C -palmitate (Panel C) ($n = 5$) (N.D., not detected). Panel D shows a schematic diagram summarizing a model for extracellularly driven succinate accumulation in brown adipocytes and ^{13}C -succinate metabolic labelling strategy (FH, fumarate hydratase). Panel E shows abundance of succinate in mouse plasma comparing 29°C to acute 4°C exposure ($n = 6$). Panel F shows intracellular abundance of succinate in isolated brown adipocytes following addition of extracellular succinate (0 min, 60 min, $n = 4$; 10 min & 30 min $n = 5$).

Panel G shows ^{13}C -isotopologue ($m + 0$ and $m + 4$) profile of TCA cycle metabolites downstream of mitochondrial succinate oxidation in brown adipocytes following extracellular addition of ^{13}C -succinate ($n = 5$). Panel H shows ^{13}C -isotopologue profile of mitochondrial cataplerotic product aspartate in brown adipocytes following extracellular addition of ^{13}C -succinate ($n = 5$). Panel I shows ^{13}C -isotopologue ($m + 0$ and $m + 4$) profile of TCA cycle metabolites downstream of mitochondrial succinate oxidation in BAT following i.v. administration of ^{13}C -succinate ($n = 3$). Data are mean $\pm$ s.e.m. of at least three mouse replicates or three cell replicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-tailed Student's t-test for pairwise comparisons, one-way ANOVA for multiple comparisons involving one independent variable). Panel J shows abundance of intracellular ($m + 4$) [^{13}C] succinate 10 min after treatment with [^{13}C]succinate in brown ($n = 5$), white ($n = 4$), and pre-adipocytes (Pre) ($n = 4$). Panel K shows intracellular abundance of TCA cycle metabolites fumarate (Fum) ($n = 4$), α -ketoglutarate (αKG) ($n = 4$) and succinate (Suc) ($n = 5$) in brown adipocytes 10 min following extracellular addition. Panel L shows ^{13}C -isotopologue ($m + 0$ and $m + 4$) profile of metabolites downstream of mitochondrial succinate oxidation in brown adipocytes following extracellular addition of [^{13}C]succinate ($n = 5$). Panel M shows abundance of [^{13}C]succinate in mouse tissues 15 min after intravenous bolus injection of 100 mg kg $^{-1}$ of [^{13}C]succinate ($n = 4$; except BAT, $n = 3$). Panel N shows abundance of ($m + 4$) TCA cycle metabolites in BAT following intravenous bolus injection of 100 mg kg $^{-1}$ of [^{13}C]succinate ($n = 3$). Panel O shows abundance of ($m + 4$) [^{13}C]succinate and total succinate in plasma following intravenous bolus injection of 100 mg kg $^{-1}$ of [^{13}C]succinate ($n = 3$, except 15 min, $n = 4$).

Figure 5 includes 4 panels, identified as panels A, B, C, and D, which show the results of ^{13}C isotopologue labelling of glucose and TCA cycle metabolites in mouse BAT following i.v. ^{13}C -glucose at 29°C or 4°C. Panel A shows proportional isotopic labelling of BAT glucose. Panels B-D show proportional isotopic labelling profile of TCA cycle metabolites, citrate (Panel B), fumarate (Panel C), and malate (Panel D) in mouse BAT. Mice were administered [U- ^{13}C]-glucose i.v. at 29°C or 4°C followed by BAT harvesting and snap freezing for LC-MS analysis at indicated time-points ($n = 5$). Data are mean $\pm$ s.e.m. of at least five mouse replicates.

Figure 6 includes 5 panels, identified as panels A, B, C, D, E, F, and G, which show the results of ^{13}C isotopologue labelling of palmitate and TCA cycle metabolites in BAT following i.v. ^{13}C -glucose at 29°C or 4°C and analysis of succinate levels in isolated BAT

cells. Panel A shows proportional isotopic labelling of BAT palmitate. Panels B-D show proportional isotopic labelling profile of TCA cycle metabolites citrate (Panel B), fumarate (Panel C), and malate (Panel D) in BAT. Mice were administered [U-¹³C]-glucose i.v. at 29°C or 4°C followed by BAT harvesting and snap freezing for LC-MS analysis at indicated timepoints ($n = 5$). Panel E shows a comparison of succinate abundance in BAT *in vivo* versus differentiated BAT cells ($n = 8$; BAT cells $n = 7$). Data are mean $\pm$ s.e.m. of at least five replicates. Panels F and G show proportional isotopic labelling profile of glycolytic metabolites 3-phosphoglycerate (panel F) and lactate (panel G) in mouse BAT.

Figure 7 includes 12 panels, identified as A, B, C, D, E, F, G, H, I, J, K, and L, which show accumulation of succinate controlling brown adipocyte thermogenesis via SDH oxidation and ROS production. Panel A shows the representative oxygen consumption rate (OCR) experiment of brown adipocytes $\pm$ acute addition succinate to determine effects on respiration, $\pm$ oligomycin (oli) to determine leak respiration, $\pm$ 2,4-dinitrophenol (DNP) to determine chemically uncoupled maximal respiration, and rotenone + antimycin (r/a) to determine non-mitochondrial respiration (5 mM succinate $n = 7$; 1 mM succinate $n = 6$). Panels B-C show the effect of acute addition of increasing concentrations of extracellular succinate on brown adipocyte OCR (Panel B), leak respiration and chemically uncoupled maximal respiration (Max), (panel C; vehicle $n = 20$; 0.25 mM $n = 6$; 0.5 mM $n = 7$; 1 mM $n = 18$; 5 mM $n = 13$; 10 mM $n = 12$). Panel D shows the effect of acute addition of mitochondrial and cellular substrates on brown adipocyte OCR (1 mM, $n = 6$; 10 mM, $n = 7$; 10 mM glycerol 3-phosphate (G3P), $n = 6$; 10 mM α -ketoglutarate, 10 mM fumarate, $n = 5$). Panel E shows the effect of acute addition of succinate on cellular OCR across diverse cell types. immbrown adip., immortalized De2.3 brown adipocytes; myo, myoblasts; HEK, human embryonic kidney; hepato, HepG2 hepatocytes; osteo, MILO-Y4 osteocytes. (1 mM $n = 6$; 5 mM $n = 7$; 5 mM brown pre-adipocyte & SubQ adipocyte $n = 5$). Panel F shows the effect of succinate treatment on brown adipocyte ROS levels assessed by DHE oxidation ($n = 15$). Panel G shows the effect of succinate treatment on Prx3 cysteine sulfonylation status ($n = 4$; succinate $n = 3$). Panel H shows the inhibition of succinate-stimulated OCR by suppression of mitochondrial ROS with MitoQ or suppression of cysteine oxidation with NAC (MitoQ vehicle $n = 15$; 100 nM MitoQ $n = 18$; 500 nM MitoQ $n = 17$; NAC vehicle $n = 7$; 5 mM NAC $n = 6$; 10 mM NAC $n = 7$). Panel I is a diagram showing the potential pathways for succinate-driven thermogenic ROS in brown adipocytes and pathway inhibitors. (1), Malonate inhibits succinate oxidation by SDH

(Quastel and Wooldridge (1928) *Biochem J.* 22, 689-702); (2), Atpenin A5 (AA5) inhibits electron transfer between SDH and the ubiquinone pool (Miyadera *et al.* (2003) *PNAS* 100, 473-477); (3), S1Q2.2 inhibits ROS production from mitochondrial complex I specifically (Brand *et al.* (2016) *Cell Metab* 24, 582-592); and (4), iGP1 inhibits electron transfer between α GPDH and the ubiquinone pool (Orr *et al.* (2014) *PLoS One* 9, e89938). Panel J shows a representative OCR experiment demonstrating inhibition of succinate stimulated OCR by suppression of SDH oxidation with malonate (vehicle, $n = 6$; 1 mM malonate, $n = 6$; 5 mM malonate, $n = 5$). Panel K shows the effect on succinate-stimulated OCR by inhibition of pathways linked to succinate oxidation by SDH ($n = 12$; 5 mM malonate $n = 11$; Atpenin A5 (AA5) vehicle, $n = 11$; 10 nM AA5, 100 nM AA5, S1Q2.2 vehicle, S1Q2.2 1 μ M $n = 6$; S1Q2.2 10 μ M $n = 7$; iGP vehicle $n = 21$; iGP 10 μ M $n = 17$; iGP 100 μ M $n = 26$). G3P = glycerol-3-phosphate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-tailed Student's t -test for pairwise comparisons, one-way ANOVA for multiple comparisons involving one independent variable). Panel L shows the potential pathways of succinate-driven thermogenic ROS.

Figure 8 includes 23 panels, identified as panels A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, and W, which show representative OCR experiments of brown adipocytes and other cell types $\pm$ acute addition succinate and related substrates. Panels A-D show effects on respiration determined by acute addition, $\pm$ oligomycin (oli) to determine leak respiration, $\pm$ 2,4-dinitrophenol (DNP) to determine chemically uncoupled maximal respiration, and rotenone + antimycin (r/a) to determine non-mitochondrial respiration. Panel A shows effects of acute addition of cellular and mitochondrial respiratory substrates on brown adipocyte respiration (pyruvate: vehicle, $n = 7$; 1 mM, $n = 6$; 10 mM, $n = 7$; glucose: vehicle, $n = 7$; 1 mM, $n = 6$; 10 mM, $n = 7$; glutamine: vehicle, $n = 6$; 1 mM, $n = 6$; 10 mM, $n = 7$; G3P: vehicle, $n = 7$; 1 mM, $n = 6$; 10 mM, $n = 6$; α KG: vehicle, $n = 6$; 1 mM, $n = 6$; 10 mM, $n = 5$; fumarate: vehicle, $n = 6$; 1 mM, $n = 6$; 10 mM, $n = 5$; malate: vehicle, $n = 6$; 1 mM, $n = 6$; 10 mM, $n = 7$). Panel B shows a representative OCR trace monitoring effect of acute addition of succinate or noradrenaline in the De2.3 immortalized brown adipocyte cell line (Pan *et al.* (2009) *Cell* 137:73-86) (NE; vehicle $n = 7$; succinate $n = 6$; NE $n = 7$). Panel C shows representative OCR trace monitoring dose-dependent effects of acute addition of succinate in the De2.3 immortalized brown adipocyte cell line (vehicle $n = 7$; 1 mM succinate $n = 6$; 5 mM succinate $n = 7$). Panel D shows representative OCR trace monitoring dose-dependent effects of acute addition of succinate in various cell

types (subcutaneous white adipocytes: vehicle $n = 6$; 1 mM $n = 6$; 5 mM $n = 5$; myoblasts: vehicle $n = 7$; 1 mM $n = 6$; 5 mM $n = 7$; HEK $n = 7$; 1 mM $n = 6$; 5 mM $n = 7$; hepatocytes: vehicle $n = 7$; 1 mM $n = 6$; 5 mM $n = 7$; osteocytes: vehicle $n = 7$; 1 mM $n = 6$; 5 mM $n = 7$; A549 lung: vehicle $n = 7$; 1 mM $n = 6$; 5 mM $n = 7$; brown pre-adipocytes: vehicle $n = 7$; 1 mM $n = 6$; 5 mM $n = 7$). Panel E shows abundance of succinate in mouse plasma comparing 29 °C to acute 4 °C exposure ($n = 6$). Panel F shows comparison of succinate abundance in BAT in vivo ($n = 8$) versus brown adipocytes ($n = 7$). Panels G-J show full ^{13}C -isotopologue profile of fumarate (panel G), malate (panel H), citrate (panel I), and aspartate (panel J) in brown adipocytes following extracellular addition of [^{13}C]succinate ($n = 5$). Panel K shows ^{13}C -isotopologue ($m + 4$) profile of TCA metabolites downstream of mitochondrial succinate oxidation in BAT following intravenous administration of 100 mg kg^{-1} [^{13}C]succinate as a bolus ($n = 3$). Panel L shows time course of abundance of ($m + 4$) [^{13}C]succinate in plasma following 100 mg kg^{-1} intravenous [^{13}C]succinate ($n = 3$, except 15 min, $n = 4$). Panel M shows representative mouse nuchal muscle EMG traces at 29 °C and after acute cold exposure with or without curare (0.1 mg kg^{-1}). Panel N shows quantification of succinate in BAT at 29 °C and after acute cold exposure with or without inhibition of muscle shivering with curare (0.1 mg kg^{-1} ; $n = 5$). Panel O shows the effect of acute addition of palmitic acid on brown adipocyte respiration (vehicle, $n = 18$; palmitic acid, $n = 16$). Effects on respiration were determined by acute addition of oligomycin (oli) to determine leak respiration, 2,4-dinitrophenol (DNP) to determine chemically uncoupled maximal respiration, and rotenone plus antimycin (r/a) to determine non-mitochondrial respiration. In all cases basal respiration in these cells is measured in the presence of 1 mM pyruvate. One-way ANOVA (panels 8E and 8N); data are mean $\pm$ s.e.m. of biologically independent samples. Panel P shows the effect of acute addition of succinate on cellular OCR in human brown adipocytes (basal, $n = 6$; 1 mM, $n = 6$; 5 mM, $n = 7$). Panel Q shows representative OCR trace monitoring dose-dependent effect of acute addition of succinate in human immortalized brown adipocytes (vehicle, $n = 7$; 1 mM succinate, $n = 6$; 5 mM succinate, $n = 7$). Panel R shows inhibition of succinate-stimulated OCR in brown adipocytes by DTNB. Panel S shows the representative OCR experiment ($n = 7$; 0.1 mM DTNB, $n = 6$). Panels T and U show inhibition of succinate-stimulated OCR in brown adipocytes by DIDS. $n = 12$, except 1 mM DIDS, $n = 8$). Panels V and W show inhibition of succinate-stimulated OCR in brown adipocytes by treatment with the Na^+/K^+ ATPase inhibitor ouabain ($n = 5$). Effects on respiration were determined by acute addition of

oligomycin (oli) to determine leak respiration, 2,4-dinitrophenol (DNP) to determine chemically uncoupled maximal respiration, or rotenone plus antimycin (r/a) to determine non-mitochondrial respiration. In all cases basal respiration in these cells is measured in the presence of 1 mM pyruvate. Two-sided *t*-test (panel H); two-way ANOVA (panels J, L, and N); data are mean $\pm$ s.e.m. of biologically independent samples.

Figure 9 includes 15 panels, identified as panels A, B, C, D, E, F, G, H, I, J, K, L, M, N, and O, which show results examining mechanisms of succinate-driven thermogenesis in brown adipocytes. Panel A shows that succinate-induced respiration is intact in brown adipocytes lacking SUCNR1 ($n = 10$; except 1 mM succinate $n = 9$). Panel B shows measurement of cAMP levels in brown adipocytes 10 min following addition of succinate. Panel C shows immunoblot analysis results of PKA substrate phosphorylation following addition of succinate (30 min) or NE (5 min). Panel D shows glycerol release rate results from brown adipocytes as an index of lipolysis in response to succinate or NE ($n = 6$). Panel E shows representative high resolution microscopic images illustrating effect of acute (10 min) addition of succinate on DHE oxidation in brown adipocytes. Scale bars = 20 μ m. Panel F shows that acute addition of succinate drives rapid DHE oxidation in brown adipocytes ($n = 15$). Panel G shows the effect of succinate treatment on Prx family cysteine-thiol sulfonic acids (SO₃⁻) status (vehicle, $n = 4$; succinate $n = 3$). (two-tailed Student's *t*-test for pairwise comparisons, one-way ANOVA for multiple comparisons involving one independent variable). Panels H-J show the effect of acute addition of succinate in mitochondria isolated from BAT, monitoring effects on basal respiration rate (panel I), leak respiration (panel J), and chemically uncoupled maximal respiration (panel J). $n = 7$, except succinate, $n = 8$). Panel K shows the quantitation of SLC25A10 protein levels in mouse liver, brain, heart, and BAT ($n = 3$). Panels L and M show the inhibition of succinate-stimulated OCR in brown adipocytes by treatment with the SLC25A10 inhibitor diethyl butylmalonate (DEBM; $n = 11$). Data are mean $\pm$ s.e.m. of at least three replicates. Panel N shows the effect of succinate treatment on ROS levels in brown adipocyte assessed by DHE oxidation ($n = 15$). Panel O shows the effect of succinate treatment on Prx3 cysteine-thiol sulfonic acid formation (vehicle, $n = 4$; succinate, $n = 3$).

Figure 10 includes 12 panels, identified as panels A, B, C, D, E, F, G, H, I, J, K, and L, which show mechanisms of succinate-driven thermogenic ROS and respiration in brown adipocytes. Panel A shows representative OCR experiments of brown adipocytes $\pm$ acute addition succinate $\pm$ MitoQ or NAC. Effects on respiration were determined by acute

addition of oligomycin (oli) to determine leak respiration, 2,4-dinitrophenol (DNP) to determine chemically uncoupled maximal respiration, and rotenone + antimycin (r/a) to determine non-mitochondrial respiration (MitoQ: vehicle, $n = 6$; 100 mM $n = 6$; 500 mM $n = 5$; NAC: vehicle, $n = 7$; 5 mM $n = 6$; 10 mM $n = 7$). Panel B shows that treatment with malonate prevents succinate-driven DHE oxidation in brown adipocytes ($n = 30$). Panels C-E show representative OCR experiments of brown adipocytes $\pm$ acute addition succinate $\pm$ AA5 (vehicle, $n = 6$; 10 nM $n = 6$; 100 nM $n = 5$) (Panel C), S1Q2.2 (vehicle, $n = 6$; 10 μ M $n = 6$; 100 μ M $n = 7$) (Panel D), or iGP1 (vehicle, $n = 7$; 1 μ M $n = 6$; 100 μ M $n = 7$) (Panel E). Data are mean $\pm$ s.e.m. of at least three replicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-tailed Student's t-test for pairwise comparisons (panels G and B), one-way ANOVA (panels I and L) for multiple comparisons involving one independent variable, two-way ANOVA (panel J)). Panels F and G show the representative OCR experiments on brown adipocytes with and without acute addition of diamide. Vehicle, $n = 6$; 200 μ M diamide, $n = 5$. Panel H shows the potential pathways for succinate-driven thermogenic ROS in brown adipocytes via SDH or electron transfer via ubiquinol (QH2): (1) Malonate inhibits succinate oxidation by SDH19; (2) atpenin A5 (AA5) inhibits electron transfer between SDH and the ubiquinone pool20; (3) S1Q2.2 inhibits ROS production from mitochondrial complex I21; (4) iGP1 inhibits electron transfer between α GPDH and the ubiquinone pool23; and (5) S3Q3 inhibits ROS production from mitochondrial complex III22. Panel I shows that treatment of brown adipocytes with malonate results in rapid intracellular accumulation ($n = 4$) in brown adipocytes ($n = 30$). Panels J and K show the representative OCR experiments in brown adipocytes with or without acute addition of succinate, with or without S3Q3 (vehicle, $n = 13$; 1 μ M $n = 12$; 10 μ M $n = 13$). Panel L shows the treatment of brown adipocytes with S3Q3 has no effect on succinate-driven DHE oxidation in brown adipocytes ($n = 30$, except 1 μ M, $n = 15$).

Figure 11 includes 17 panels, identified as A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, and Q, which show that elevation of circulating succinate stimulates UCP1-dependent thermogenesis *in vivo* and protects against diet-induced obesity. Panel A shows effect of acute i.v. administration of succinate on interscapular temperature (vehicle, $n = 6$; succinate $n = 5$). Panel B shows acute effect of i.v. succinate on whole body oxygen consumption in WT and UCP1KO mice. Basal O_2 consumption rate determined as described in the methods section ($n = 10$; succinate $n = 7$; UCP1KO $n = 6$). Panel C shows acute oral administration of succinate by gavage drives elevation of circulating succinate ($n = 4$; 10%

30 min $n = 6$). Panel D shows change in body mass during high fat feeding $\pm$ intervention with 1% and 1.5% sodium succinate in drinking water (0%, $n = 35$; 1% $n = 26$; 1.5% $n = 18$). Panel E shows change in body mass during high fat feeding $\pm$ intervention with 2% succinate in drinking water including a vehicle group pair fed to the 2% succinate group (0%, $n = 24$; 2%, $n = 22$; 0% pair fed, $n = 18$). Panels F-G show percent body composition of mice following 4 weeks high fat feeding $\pm$ low succinate (Panel F) (0%, $n = 35$; 1% $n = 26$; 1.5% $n = 18$) and high succinate supplementation in drinking water (Panel F) (0%, $n = 24$; 2%, $n = 22$; 0% pair fed $n = 18$). Panels H-I show free living whole body energy expenditure of mice during 4 weeks high fat feeding $\pm$ low succinate (Panel H) ($n = 23$; 1% $n = 26$; 1.5% $n = 18$) and high succinate supplementation in drinking water (Panel I) ($n = 24$; pair fed $n = 18$). Panel J shows fasting blood glucose concentration in mice following high fat feeding $\pm$ 4 weeks succinate supplementation in drinking water ($n = 9$). Panel K shows i.p. glucose tolerance test in mice following high fat feeding $\pm$ 4 weeks succinate supplementation in drinking water ($n = 9$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-tailed Student's t -test for pairwise comparisons, one-way ANOVA for multiple comparisons involving one independent variable, two-way ANOVA for multiple comparisons involving two independent variables). Panel L shows mouse whole-body energy expenditure during 4 weeks high-fat feeding with or without low (0%, $n = 35$; 1%, $n = 26$; 1.5%, $n = 18$) or high succinate (0%, $n = 24$; 2%, $n = 22$; 0% pair fed, $n = 18$). Panels M and N show change in body mass (panel M) and body composition (panel N) during high-fat feeding in $Ucp1^{+/-}$ and $Ucp1^{-/-}$ mice with or without 1.5% sodium succinate ($Ucp1^{+/-}$ 0%, $n = 18$; $Ucp1^{+/-}$ 1.5%, $n = 17$; $Ucp1^{-/-}$ 0%, $n = 13$; $Ucp1^{-/-}$ 1.5%, $n = 15$). Two-way ANOVA (panels D and K (left), M); one-way ANOVA (panels F, G, L, and J); two-sided t -test (panel N); data are mean $\pm$ s.e.m. of biologically independent samples. Panel O shows the acute effect of intravenous succinate on whole body oxygen consumption in wild-type (WT) and UCP1(KO) mice. Basal O_2 consumption rate determined as described in the Methods (vehicle, $n = 10$; succinate, $n = 7$; UCP1(KO), $n = 9$). Panel P shows mouse interscapular temperature following acute exposure to 4 °C with or without acute intravenous administration of malonate ($n = 8$). Malonate was administered 10 min before transition to 4 °C. Panel Q shows that acute oral administration of succinate by gavage drives elevation of circulating succinate ($n = 4$, except 10% 30 min, $n = 6$). * $P < 0.05$, ** $P < 0.01$. Two-way ANOVA (panel A, O (left), P); one-way ANOVA (panel O (middle, right), Q); data are mean $\pm$ s.e.m. of biologically independent samples.

Figure 12 includes 8 panels, identified as panels A, B, C, D, E, F, G, H, I, J, K, and L, which show metabolic characterization of mice following systemic succinate administration. Panel A shows water consumption during high fat feeding $\pm$ intervention with 1% and 1.5% sodium succinate in drinking water indicates lack of aversion to succinate-containing water ($n = 23$; 1% $n = 26$; 1.5% $n = 18$). Panel B shows water consumption during high fat feeding $\pm$ intervention with 2% succinate in drinking water indicates lack of aversion to succinate-containing water ($n = 24$). Panel C shows body weights of high fat diet feeding mice prior to intervention with 1% and 1.5% sodium succinate in drinking water (vehicle, $n = 35$; 1%, $n = 26$; 1.5%, $n = 18$). Panel D shows body weights of high fat diet feeding mice prior to intervention with 2% sodium succinate in drinking water (vehicle, $n = 24$; 2%, $n = 22$; pair fed $n = 18$). Panel E shows kcal consumption during high fat feeding $\pm$ intervention with 1% and 1.5% sodium succinate in drinking water, including kcal consumed from food alone, as well as kcal consumed from both food and succinate-containing water. Consumption data indicate no effect of succinate on kcal consumed by food, demonstrating an increase in total kcal consumed from both food and succinate-containing water in 1% and 1.5% groups ($n = 23$; 1% $n = 26$; 1.5% $n = 18$). Panel F shows kcal consumption during high fat feeding $\pm$ intervention with 2% sodium succinate in drinking water, including kcal consumed from food alone, as well as kcal consumed from both food and succinate-containing water. Consumption data indicate no effect on total kcal consumed from both food and succinate-containing water in 2% groups ($n = 24$). Vehicle pair fed group was fed the same amount of food as the 2% succinate treated mice consumed *ad libitum*. Panels G-H show kcal absorption and energy assimilation during high fat feeding $\pm$ intervention with either 1.5% (Panel G) or 2% (Panel H) sodium succinate in drinking water. The proportion of energy assimilated from diet was determined by subtracting the total kcal remaining in mouse feces from the total kcal consumed in the same period ($n = 6$). Panel I shows that water consumption during high-fat feeding with or without intervention with 1% and 1.5% sodium succinate in drinking water indicates lack of aversion to succinate-containing water (vehicle, $n = 35$; 1%, $n = 26$; 1.5%, $n = 18$). Panel J shows that water consumption during high-fat feeding with or without intervention with 2% succinate in drinking water indicates lack of aversion to succinate-containing water (vehicle, $n = 24$; 2%, $n = 22$). Panel K shows the caloric consumption during high-fat feeding with or without intervention with 1% or 1.5% sodium succinate in drinking water (vehicle, $n = 35$; 1%, $n = 26$; 1.5%, $n = 18$). Panel L shows the caloric

consumption during high-fat feeding with or without 2% sodium succinate in drinking water, pair-fed mice in this experiment were fed the same number of calories as the 2% succinate group (vehicle, $n = 24$; 2%, $n = 22$; pair fed, $n = 18$). Two-sided t-test (panel G); data are mean $\pm$ s.e.m. of biologically independent samples.

5 **Figure 13** includes 9 panels, identified as panels A, B, C, D, E, F, G, H, and I, which show the assessment of morphologic effects of systemic succinate administration on mouse tissues. Panels A–F show the representative images of haematoxylin and eosin (panels A, B, and D–F) or Masson's trichrome (panel C) staining of indicated tissues harvested from mice following high-fat feeding with or without 4 weeks succinate
10 supplementation in drinking water. (A, C, E top panels: 4 $\times$ magnification; scale bars, 1 mm; A, C, E bottom panels, B, D, F: 40 $\times$ magnification, scale bars, 50 μ m). Panel D shows the cardiac morphometric analysis with or without 1.5% sodium succinate. Lower panels show representative images of cell width (40 $\times$ magnification; scale bars, 50 μ m). Bar charts show quantitative analysis of cardiomyocyte width and length and nuclear diameter ($n =$
15 15). Panel G shows the intraperitoneal glucose tolerance test in mice following high-fat feeding with or without 4 weeks succinate supplementation in drinking water, quantifying relative changes in glucose upon glucose challenge ($n = 9$). Panels H and I show mRNA expression of inflammatory (panel H) and anti-inflammatory (panel I) markers in the indicated tissues with or without 1.5% sodium succinate in wild-type and *Ucp1*^{-/-} mice ($n =$
20 3). Two-way ANOVA (panel G); one-way ANOVA (panel H); data are mean $\pm$ s.e.m. of biologically independent samples.

Figure 14 includes 9 panels, identified as panels A, B, C, D, E, and F, which show metabolic characterization of UCP1-deficient mice following systemic succinate administration. Panel A shows change in body mass in *Ucp1*^{+/-} ($n = 9$; 1.5% $n = 9$) and
25 *Ucp1*^{-/-} (UCP1KO, $n = 7$; 1.5% $n = 8$) male mice during high-fat feeding with or without 1.5% sodium succinate in drinking water. Panel B shows change in body mass in *Ucp1*^{+/-} (0%, $n = 9$; 1.5%, $n = 8$) and *Ucp1*^{-/-} (0%, $n = 6$; 1.5% $n = 7$) female mice during high-fat feeding with or without 1.5% sodium succinate in drinking water. Panel C shows body weights of high-fat diet feeding *Ucp1*^{+/-} (vehicle, $n = 18$; 1.5%, $n = 17$) and *Ucp1*^{-/-}
30 (vehicle, $n = 13$; 1.5%, $n = 15$) mice prior to intervention with 1.5% sodium succinate in drinking water. Panel D shows water consumption during high-fat feeding with or without intervention with 1.5% sodium succinate in drinking water in *Ucp1*^{+/-} (0%, $n = 18$; 1.5%, $n = 17$) and *Ucp1*^{-/-} (0%, $n = 13$; 1.5%, $n = 15$) mice indicates lack of aversion to succinate-

containing water. Panel E shows energy consumption during high-fat feeding with or without intervention with 1.5% sodium succinate in drinking water in *Ucp1^{+/-}* (0%, *n* = 18; 1.5%, *n* = 17) and *Ucp1^{-/-}* (0%, *n* = 13; 1.5%, *n* = 15) mice. Panel F shows Energy expenditure of *Ucp1^{+/-}* and *Ucp1^{-/-}* mice during 6 weeks high-fat feeding with or without 1.5% sodium succinate (*Ucp1^{+/-}* 0%, *n* = 18; *Ucp1^{+/-}* 1.5%, *n* = 17; *Ucp1^{-/-}* 0%, *n* = 13; *Ucp1^{-/-}* 1.5%, *n* = 15). Two-way ANOVA (panels A and B); one-way ANOVA (panel F); data are mean ± s.e.m. of biologically independent samples.

Note that for every figure containing a histogram, the bars from left to right for each discrete measurement correspond to the figure boxes from top to bottom in the figure legend as indicated.

Detailed Description of the Invention

The present invention is based in part on the discovery that succinate has the ability to modulate adipose thermogenesis and related metabolic activity (*e.g.*, modulate one or more biological activities of a) brown fat and/or beige fat, such as expression of a marker selected from the group consisting of: cidea, adiponectin, adipsin, otopetrin, type II deiodinase, cig30, ppar gamma 2, pgcl α , ucp1, elovl3, cAMP, Prdm16, cytochrome C, cox4i1, coxIII, cox5b, cox7a1, cox8b, glut4, atpase b2, cox II, atp5o, ndufb5, ap2, ndufs1, GRP109A, acylCoA-thioesterase 4, EARA1, claudin1, PEPCK, fgf21, acylCoA-thioesterase 3, dio2, fatty acid synthase (fas), leptin, resistin, and nuclear respiratory factor-1 (nrf1); b) thermogenesis in adipose cells; c) differentiation of adipose cells; d) insulin sensitivity of adipose cells; e) basal respiration, leak respiration, or uncoupled respiration; f) whole body oxygen consumption; g) obesity or appetite; h) insulin secretion of pancreatic beta cells; i) glucose tolerance; and j) modified activity of UCP1 protein).

It is demonstrated herein that succinate is selectively taken up by thermogenic fat cells (*e.g.*, beige and brown fat cells) and can act systemically on cells in culture and *in vivo* to activate brown adipocyte thermogenic respiration *in vivo*. This selective accumulation is driven by a newfound capacity for brown adipocytes to sequester elevated circulating succinate, and brown adipose tissue (BAT) thermogenesis can be activated by pharmacological elevation of circulating succinate to drive UCP1-dependent BAT thermogenesis *in vivo*, which stimulates robust protection against diet-induced obesity and improves glucose tolerance.

In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

The term “amino acid” is intended to embrace all molecules, whether natural or synthetic, which include both an amino functionality and an acid functionality and capable of being included in a polymer of naturally-occurring amino acids. Exemplary amino acids include naturally-occurring amino acids; analogs, derivatives and congeners thereof; amino acid analogs having variant side chains; and all stereoisomers of any of any of the foregoing. The names of the natural amino acids are abbreviated herein in accordance with the recommendations of IUPAC-IUB.

The term “antisense” nucleic acid refers to oligonucleotides which specifically hybridize (*e.g.*, bind) under cellular conditions with a gene sequence, such as at the cellular mRNA and/or genomic DNA level, so as to inhibit expression of that gene, *e.g.*, by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix.

The terms “beige fat” or “brite (brown in white) fat” or “iBAT (induced brown adipose tissue)” or “recruitable BAT (brown adipose tissue)” or “wBAT (white adipose BAT)” refer to clusters of UCP1-expressing adipocytes having thermogenic capacity that develop in white adipose tissue (WAT). Beige fat can develop in subcutaneous WAT, such as in inguinal WAT, or in intra-abdominal WAT such as in epididymal WAT. Similar to adipocytes in brown adipose tissue (BAT), beige cells are characterized by a) multilocular lipid droplet morphology, b), high mitochondrial content, and/or c) expression of a core set of brown fat-specific genes, such as *Ucp1*, *Cidea*, *Pgc1a*, and other listed in Table 1. BAT and beige fat both are able to undergo thermogenesis, but these are distinct cell types since beige cells do not derive from *Myf5* precursor cells like BAT cells, beige fat express thermogenic genes only in response to activators like beta-adrenergic receptor or PPARgamma agonists unlike constitutive expression in BAT cells (Harms and Seale (2013) *Nat. Med.* 19:1252-1263).

The term “binding” or “interacting” refers to an association, which may be a stable association, between two molecules, *e.g.*, between a polypeptide of the invention and a binding partner, due to, for example, electrostatic, hydrophobic, ionic and/or hydrogen-bond interactions under physiological conditions. Exemplary interactions include protein-

protein, protein-nucleic acid, protein-small molecule, and small molecule-nucleic acid interactions.

The term “biological sample” when used in reference to a diagnostic assay is intended to include tissues, cells and biological fluids isolated from a subject, as well as
5 tissues, cells and fluids present within a subject.

The term “isolated polypeptide” refers to a polypeptide, in certain embodiments prepared from recombinant DNA or RNA, or of synthetic origin, or some combination thereof, which (1) is not associated with proteins that it is normally found within nature, (2) is isolated from the cell in which it normally occurs, (3) is isolated free of other proteins
10 from the same cellular source, (4) is expressed by a cell from a different species, or (5) does not occur in nature.

The terms “label” or “labeled” refer to incorporation or attachment, optionally covalently or non-covalently, of a detectable marker into a molecule, such as a polypeptide. Various methods of labeling polypeptides are known in the art and may be used. Examples
15 of labels for polypeptides include, but are not limited to, the following: radioisotopes, fluorescent labels, heavy atoms, enzymatic labels or reporter genes, chemiluminescent groups, biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (*e.g.*, leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). Examples and use of such labels are described in more
20 detail below. In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

The terms “metabolic disorder” and “obesity related disorders” are used interchangeably herein and include a disorder, disease or condition which is caused or characterized by an abnormal or unwanted metabolism (*i.e.*, the chemical changes in living
25 cells by which energy is provided for vital processes and activities) in a subject. Metabolic disorders include diseases, disorders, or conditions associated with aberrant or unwanted (higher or lower) thermogenesis or aberrant or unwanted levels (high or low) adipose cell (*e.g.*, brown or white adipose cell) content or function. Metabolic disorders can be characterized by a misregulation (*e.g.*, downregulation or upregulation) of PGC-1 activity.
30 Metabolic disorders can detrimentally affect cellular functions such as cellular proliferation, growth, differentiation, or migration, cellular regulation of homeostasis, inter- or intra-cellular communication; tissue function, such as liver function, muscle function, or adipocyte function; systemic responses in an organism, such as hormonal responses (*e.g.*,

insulin response). Examples of metabolic disorders include obesity, insulin resistance, type II diabetes, hypertension, hyperuricemia, fatty liver, non-alcoholic fatty liver disease, polycystic ovarian syndrome, acanthosis nigricans, hyperphagia, endocrine abnormalities, triglyceride storage disease, Bardet-Biedl syndrome, Lawrence-Moon syndrome, Prader-Labhart-Willi syndrome, anorexia, and cachexia.

In some embodiments, “pain” is included within the term “metabolic disorder.” Pain is a sensation and a perception that is comprised of a complex series of mechanisms. Pain can be experienced both acutely and chronically. Acute pain is the instantaneous onset of a painful sensation in response to a noxious stimulus. It is considered to be adaptive because it can prevent an organism from damaging itself in some instances. Unlike acute pain (*e.g.*, the transient protective physiology pain), persistent pain (also called chronic pain) usually has a delayed onset but can last for hours to days, or even months or years. Persistent pain may involve an amalgamation of physical, social, and psychologic factors. Persistent pain occurs in a variety of forms including, but not limited to, spontaneous pain (painful sensation without an external stimulus), allodynia (painful sensation in response to a normally innocuous stimulus) and hyperalgesia (strong painful sensation to a mildly painful stimulus). Persistent pain can be caused by many different factors. For example, persistent pain can be caused by conditions that accompany the aging process (*e.g.*, conditions that may affect bones and joints in ways that cause persistent pain). In some embodiments, persistent pain can be caused by inflammation or nerve injury (for example, damage to or malfunction of the nervous system). In some embodiments, persistent pain can be inflammatory pain or neuropathic pain (for example, peripheral neuropathic pain and central neuropathic pain). In some embodiments, persistent pain is mediated by hyper-excitable pain-processing neurons in peripheral and central nervous system (*e.g.*, peripheral sensitization or central sensitization). Surrogate indicators of pain are well-known in the art and can be assayed using routine methods, such as hot plate or tail immersion assays to determine thermally-induced pain, electronic von Frey apparatus assays to determine mechanically-induced pain, acetic acid assays to determine chemically-induced pain, adjuvant injection assays to determine inflammatory pain, and the like.

As used herein, “obesity” refers to a body mass index (BMI) of 30 kg/m² or more (National Institute of Health, Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults (1998)). However, the present invention is also intended to include a disease, disorder, or condition that is characterized by a body

mass index (BMI) of 25 kg/m² or more, 26 kg/m² or more, 27 kg/m² or more, 28 kg/m² or more, 29 kg/m² or more, 29.5 kg/m² or more, or 29.9 kg/m² or more, all of which are typically referred to as overweight (National Institute of Health, Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults (1998)).

5 The obesity described herein may be due to any cause, whether genetic or environmental. Examples of disorders that may result in obesity or be the cause of obesity include overeating and bulimia, polycystic ovarian disease, craniopharyngioma, the Prader-Willi Syndrome, Frohlich's syndrome, Type II diabetics, GH-deficient subjects, normal variant short stature, Turner's syndrome, and other pathological conditions showing reduced
10 metabolic activity or a decrease in resting energy expenditure as a percentage of total fat-free mass, e.g., children with acute lymphoblastic leukemia.

As used herein, the term "succinate" includes the anion form taken by succinic acid in an aqueous solution, such as in living organisms in particular, and is also intended to include succinic acid, succinic acid salts, and derivatives thereof unless otherwise specified.

15 In some embodiments, what is meant by succinate, succinic acid, and/or succinic acid salt is a monobasic succinic acid salt in which one of the two carboxylic acid groups is converted to a salt, thus containing, for example, one free carboxylic acid group and one metal carboxylate group in its molecular structure. In other embodiments, what is meant by succinate, succinic acid, and/or succinic acid salt is a dibasic succinic acid salt in which
20 both carboxylic acid groups are converted to salt groups, thus containing, for example, two metal carboxylate groups in its molecular structure.

The term "salts thereof" may refer to relatively non-toxic, inorganic and organic base addition salts of succinate and/or succinic acid. These salts can likewise be prepared in situ during the final isolation and purification of succinate, or by separately reacting the
25 purified succinate in its free acid form with a suitable base, such as the hydroxide, carbonate or bicarbonate of a metal cation, with ammonia, or with an organic primary, secondary or tertiary amine. Representative alkali or alkaline earth salts include the lithium, sodium, potassium, calcium, magnesium, and aluminum salts and the like. Representative organic amines useful for the formation of base addition salts include
30 ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine and the like.

In other embodiments, succinate can be administered to subjects. Gut epithelial cells are permeable to succinate. Accordingly, in some embodiments, succinate, succinic

acid salts, and derivatives thereof, can be prepared for oral administration, for example, aqueous or non-aqueous solutions or suspensions, preferably as an aqueous formulation to be given *ad libitum*. In yet other embodiments the aqueous formulation comprises 0.5% to 4% succinate or absolute equivalent amounts thereof in a relevant formulation, such as a solid dosage form. Succinate in the aqueous formulations disclosed herein can be at a concentration of about 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1.0%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, 2.0%, 2.1%, 2.2%, 2.3%, 2.4%, 2.5%, 2.6%, 2.7%, 2.8%, 2.9%, 3.0%, 3.1%, 3.2%, 3.3%, 3.4%, 3.5%, 3.6%, 3.7%, 3.8%, 3.9%, or 4.0%, or any range in between, such as at a concentration of 1.0% to 1.5% inclusive. In preferred embodiments, the aqueous solution comprises 1% to 2% succinate or absolute equivalent amounts thereof. In yet further preferred embodiments, the aqueous solution comprises 1.5% succinate or absolute equivalent amounts thereof. Absolute equivalent amounts can be determined according to well-known metrics, such as mg/kg/day. For example, absolute equivalent amounts for the aqueous formulations described above can be 50 mg/kg/day, 100 mg/kg/day, 200 mg/kg/day, 300 mg/kg/day, 400 mg/kg/day, 500 mg/kg/day, 600 mg/kg/day, 700 mg/kg/day, 800 mg/kg/day, 900 mg/kg/day, 1,000 mg/kg/day, 1,100 mg/kg/day, 1,200 mg/kg/day, 1,300 mg/kg/day, 1,400 mg/kg/day, 1,500 mg/kg/day, 1,600 mg/kg/day, 1,700 mg/kg/day, 1,800 mg/kg/day, 1,900 mg/kg/day, 2,000 mg/kg/day, 2,100 mg/kg/day, 2,200 mg/kg/day, 2,300 mg/kg/day, 2,400 mg/kg/day, 2,500 mg/kg/day, 2,600 mg/kg/day, 2,700 mg/kg/day, 2,800 mg/kg/day, 2,900 mg/kg/day, 3,000 mg/kg/day, or any range in between, such as 1,000 mg/kg/day to 1,800 mg/kg/day.

In some embodiments, succinic acid salts, and derivatives thereof, can be produced and administered which have enhanced biological properties (*e.g.*, pro-drugs). In addition, the succinate can be modified according to well-known pharmacological methods in the art (*e.g.*, pegylation, glycosylation, oligomerization, etc.) in order to further enhance desirable biological activities, such as increased bioavailability and decreased proteolytic degradation. In preferred embodiments, succinate is not a substituted succinate, for example dimethyl- or diethyl- succinate. In some embodiments, the succinate can be modified to be an inhibitor, such as a competitive inhibitor that prevents or impedes uptake of extracellular succinate.

It will be appreciated that specific sequence identifiers (SEQ ID NOs) have been referenced throughout the specification for purposes of illustration and should therefore not be construed to be limiting. Any marker of the invention, including, but not limited to, the

markers described in the specification and markers described herein (*e.g.*, *cidea*,
adiponectin (*adipoq*), *adipsin*, *otopetrin*, *type II deiodinase*, *cig30*, *ppar gamma 2*, *pgc1 α* ,
ucp1, *elovl3*, *cAMP*, *Prdm16*, *cytochrome C*, *cox4i1*, *coxIII*, *cox5b*, *cox7a1*, *cox8b*, *glut4*,
atpase b2, *cox II*, *atp5o*, *ndufb5*, *ap2*, *ndufs1*, *GRP109A*, *acylCoA-thioesterase 4*, *EARA1*,
5 *claudin1*, *PEPCK*, *fgf21*, *acylCoA-thioesterase 3*, *dio2*, *fatty acid synthase (fas)*, *leptin*,
resistin, and *nuclear respiratory factor-1 (nrf1)*), are well-known in the art and can be used
in the embodiments of the invention.

There is a known and definite correspondence between the amino acid sequence of a
particular protein and the nucleotide sequences that can code for the protein, as defined by
10 the genetic code (shown below). Likewise, there is a known and definite correspondence
between the nucleotide sequence of a particular nucleic acid and the amino acid sequence
encoded by that nucleic acid, as defined by the genetic code.

GENETIC CODE

15	Alanine (Ala, A)	GCA, GCC, GCG, GCT
	Arginine (Arg, R)	AGA, ACG, CGA, CGC, CGG, CGT
	Asparagine (Asn, N)	AAC, AAT
	Aspartic acid (Asp, D)	GAC, GAT
	Cysteine (Cys, C)	TGC, TGT
20	Glutamic acid (Glu, E)	GAA, GAG
	Glutamine (Gln, Q)	CAA, CAG
	Glycine (Gly, G)	GGA, GGC, GGG, GGT
	HISTIDINE (HIS, H)	CAC, CAT
	ISOLEUCINE (ILE, I)	ATA, ATC, ATT
25	LEUCINE (LEU, L)	CTA, CTC, CTG, CTT, TTA, TTG
	LYSINE (LYS, K)	AAA, AAG
	METHIONINE (MET, M)	ATG
	PHENYLALANINE (PHE, F)	TTC, TTT
	PROLINE (PRO, P)	CCA, CCC, CCG, CCT
30	SERINE (SER, S)	AGC, AGT, TCA, TCC, TCG, TCT
	THREONINE (THR, T)	ACA, ACC, ACG, ACT
	TRYPTOPHAN (TRP, W)	TGG
	TYROSINE (TYR, Y)	TAC, TAT
	VALINE (VAL, V)	GTA, GTC, GTG, GTT

TERMINATION SIGNAL (END) TAA, TAG, TGA

An important and well-known feature of the genetic code is its redundancy, whereby, for most of the amino acids used to make proteins, more than one coding nucleotide triplet may be employed (illustrated above). Therefore, a number of different
5 nucleotide sequences may code for a given amino acid sequence. Such nucleotide sequences are considered functionally equivalent since they result in the production of the same amino acid sequence in all organisms (although certain organisms may translate some sequences more efficiently than they do others). Moreover, occasionally, a methylated
10 methylations do not affect the coding relationship between the trinucleotide codon and the corresponding amino acid.

In view of the foregoing, the nucleotide sequence of a DNA or RNA coding for a fusion protein or polypeptide of the present invention (or any portion thereof) can be used to derive the fusion protein or polypeptide amino acid sequence, using the genetic code to
15 translate the DNA or RNA into an amino acid sequence. Likewise, for a fusion protein or polypeptide amino acid sequence, corresponding nucleotide sequences that can encode the fusion protein or polypeptide can be deduced from the genetic code (which, because of its redundancy, will produce multiple nucleic acid sequences for any given amino acid sequence). Thus, description and/or disclosure herein of a nucleotide sequence which
20 encodes a fusion protein or polypeptide should be considered to also include description and/or disclosure of the amino acid sequence encoded by the nucleotide sequence. Similarly, description and/or disclosure of a fusion protein or polypeptide amino acid sequence herein should be considered to also include description and/or disclosure of all possible nucleotide sequences that can encode the amino acid sequence.

25

I. Nucleic Acids

Also provided herein are compositions comprising one or more nucleic acids comprising or capable of expressing at least 1, 2, 3, 4, 5, 10, 20 or more small nucleic acids or antisense oligonucleotides or derivatives thereof, wherein said small nucleic acids or
30 antisense oligonucleotides or derivatives thereof in a cell specifically hybridize (*e.g.*, bind) under cellular conditions, with cellular nucleic acids (*e.g.*, small non-coding RNAs such as miRNAs, pre-miRNAs, pri-miRNAs, miRNA*, piwiRNA, anti-miRNA, a miRNA binding site, a variant and/or functional variant thereof, cellular mRNAs or a fragments thereof).

Such nucleic acids are useful, for example, in modulating the expression of brown/beige fat genes, especially those associated with succinate, such as SDH. In one embodiment, expression of the small nucleic acids or antisense oligonucleotides or derivatives thereof in a cell can enhance or upregulate one or more biological activities associated with the

5 corresponding wild-type, naturally occurring, or synthetic small nucleic acids. In another embodiment, expression of the small nucleic acids or antisense oligonucleotides or derivatives thereof in a cell can inhibit expression or biological activity of cellular nucleic acids and/or proteins, *e.g.*, by inhibiting transcription, translation and/or small nucleic acid processing of, for example, one or more biomarkers of the present invention, including one

10 or more biomarkers listed in Table 1, the Figures, and the Examples, or fragment(s) thereof. In one embodiment, the small nucleic acids or antisense oligonucleotides or derivatives thereof are small RNAs (*e.g.*, microRNAs) or complements of small RNAs. In another embodiment, the small nucleic acids or antisense oligonucleotides or derivatives thereof can be single or double stranded and are at least six nucleotides in length and are less than about

15 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 50, 40, 30, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, or 10 nucleotides in length. In another embodiment, a composition may comprise a library of nucleic acids comprising or capable of expressing small nucleic acids or antisense oligonucleotides or derivatives thereof, or pools of said small nucleic acids or antisense oligonucleotides or derivatives thereof. A pool of nucleic acids may comprise

20 about 2-5, 5-10, 10-20, 10-30 or more nucleic acids comprising or capable of expressing small nucleic acids or antisense oligonucleotides or derivatives thereof.

In one embodiment, binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, “antisense” refers to the range of techniques

25 generally employed in the art, and includes any process that relies on specific binding to oligonucleotide sequences.

It is well-known in the art that modifications can be made to the sequence of a miRNA or a pre-miRNA without disrupting miRNA activity. As used herein, the term “functional variant” of a miRNA sequence refers to an oligonucleotide sequence that varies

30 from the natural miRNA sequence, but retains one or more functional characteristics of the miRNA. In some embodiments, a functional variant of a miRNA sequence retains all of the functional characteristics of the miRNA. In certain embodiments, a functional variant of a miRNA has a nucleobase sequence that is at least about 60%, 65%, 70%, 75%, 80%, 85%,

90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the miRNA or precursor thereof over a region of about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more nucleobases, or that the functional variant hybridizes to the complement of the miRNA or precursor thereof under stringent hybridization conditions. Accordingly, in certain embodiments the nucleobase sequence of a functional variant is capable of hybridizing to one or more target sequences of the miRNA.

miRNAs and their corresponding stem-loop sequences described herein may be found in miRBase, an online searchable database of miRNA sequences and annotation, found on the world wide web at microrna.sanger.ac.uk. Entries in the miRBase Sequence database represent a predicted hairpin portion of a miRNA transcript (the stem-loop), with information on the location and sequence of the mature miRNA sequence. The miRNA stem-loop sequences in the database are not strictly precursor miRNAs (pre-miRNAs), and may in some instances include the pre-miRNA and some flanking sequence from the presumed primary transcript. The miRNA nucleobase sequences described herein encompass any version of the miRNA, including the sequences described in Release 10.0 of the miRBase sequence database and sequences described in any earlier Release of the miRBase sequence database. A sequence database release may result in the re-naming of certain miRNAs. A sequence database release may result in a variation of a mature miRNA sequence.

In some embodiments, miRNA sequences of the present invention may be associated with a second RNA sequence that may be located on the same RNA molecule or on a separate RNA molecule as the miRNA sequence. In such cases, the miRNA sequence may be referred to as the active strand, while the second RNA sequence, which is at least partially complementary to the miRNA sequence, may be referred to as the complementary strand. The active and complementary strands are hybridized to create a double-stranded RNA that is similar to a naturally occurring miRNA precursor. The activity of a miRNA may be optimized by maximizing uptake of the active strand and minimizing uptake of the complementary strand by the miRNA protein complex that regulates gene translation. This can be done through modification and/or design of the complementary strand.

In some embodiments, the complementary strand is modified so that a chemical group other than a phosphate or hydroxyl at its 5' terminus. The presence of the 5' modification apparently eliminates uptake of the complementary strand and subsequently

favors uptake of the active strand by the miRNA protein complex. The 5' modification can be any of a variety of molecules known in the art, including NH₂, NHCOCH₃, and biotin.

In another embodiment, the uptake of the complementary strand by the miRNA pathway is reduced by incorporating nucleotides with sugar modifications in the first 2-6
5 nucleotides of the complementary strand. It should be noted that such sugar modifications can be combined with the 5' terminal modifications described above to further enhance miRNA activities.

In some embodiments, the complementary strand is designed so that nucleotides in the 3' end of the complementary strand are not complementary to the active strand. This
10 results in double-strand hybrid RNAs that are stable at the 3' end of the active strand but relatively unstable at the 5' end of the active strand. This difference in stability enhances the uptake of the active strand by the miRNA pathway, while reducing uptake of the complementary strand, thereby enhancing miRNA activity.

Small nucleic acid and/or antisense constructs of the methods and compositions
15 presented herein can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of cellular nucleic acids (*e.g.*, small RNAs, mRNA, and/or genomic DNA). Alternatively, the small nucleic acid molecules can produce RNA which encodes mRNA, miRNA, pre-miRNA, pri-miRNA, miRNA*, piwiRNA, anti-miRNA, or a miRNA binding site, or a
20 variant thereof. For example, selection of plasmids suitable for expressing the miRNAs, methods for inserting nucleic acid sequences into the plasmid, and methods of delivering the recombinant plasmid to the cells of interest are within the skill in the art. See, for example, Zeng *et al.* (2002), *Molecular Cell* 9:1327-1333; Tuschl (2002), *Nat. Biotechnol.* 20:446-448; Brummelkamp *et al.* (2002), *Science* 296:550-553; Miyagishi *et al.* (2002),
25 *Nat. Biotechnol.* 20:497-500; Paddison *et al.* (2002), *Genes Dev.* 16:948-958; Lee *et al.* (2002), *Nat. Biotechnol.* 20:500-505; and Paul *et al.* (2002), *Nat. Biotechnol.* 20:505-508, the entire disclosures of which are herein incorporated by reference.

Alternatively, small nucleic acids and/or antisense constructs are oligonucleotide probes that are generated *ex vivo* and which, when introduced into the cell, results in
30 hybridization with cellular nucleic acids. Such oligonucleotide probes are preferably modified oligonucleotides that are resistant to endogenous nucleases, *e.g.*, exonucleases and/or endonucleases, and are therefore stable *in vivo*. Exemplary nucleic acid molecules for use as small nucleic acids and/or antisense oligonucleotides are phosphoramidate,

phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol *et al.* (1988) *BioTechniques* 6:958-976; and Stein *et al.* (1988) *Cancer Res* 48:2659-2668.

5 Antisense approaches may involve the design of oligonucleotides (either DNA or RNA) that are complementary to cellular nucleic acids (*e.g.*, complementary to biomarkers listed in Table 1, the Figures, and the Examples,). Absolute complementarity is not required. In the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to
10 hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with a nucleic acid (*e.g.*, RNA) it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

15 Oligonucleotides that are complementary to the 5' end of the mRNA, *e.g.*, the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have recently been shown to be effective at inhibiting translation of mRNAs as well (Wagner, R. (1994) *Nature* 372:333). Therefore,
20 oligonucleotides complementary to either the 5' or 3' untranslated, non-coding regions of genes could be used in an antisense approach to inhibit translation of endogenous mRNAs. Oligonucleotides complementary to the 5' untranslated region of the mRNA may include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could also be used in
25 accordance with the methods and compositions presented herein. Whether designed to hybridize to the 5', 3' or coding region of cellular mRNAs, small nucleic acids and/or antisense nucleic acids should be at least six nucleotides in length, and can be less than about 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 50, 40, 30, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, or 10 nucleotides in length.

30 Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit gene expression. In one embodiment these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. In another

embodiment these studies compare levels of the target nucleic acid or protein with that of an internal control nucleic acid or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

Small nucleic acids and/or antisense oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. Small nucleic acids and/or antisense oligonucleotides can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc., and may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.* (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:6553-6556; Lemaitre *et al.* (1987) *Proc. Natl. Acad. Sci.* 84:648-652; PCT Publication No. W088/09810, published December 15, 1988) or the blood-brain barrier (see, *e.g.*, PCT Publication No. W089/10134, published April 25, 1988), hybridization-triggered cleavage agents. (See, *e.g.*, Krol *et al.* (1988) *BioTechniques* 6:958-976) or intercalating agents. (See, *e.g.*, Zon (1988), *Pharm. Res.* 5:539-549). To this end, small nucleic acids and/or antisense oligonucleotides may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

Small nucleic acids and/or antisense oligonucleotides may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxyethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-

oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Small nucleic acids and/or antisense oligonucleotides may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and
5 hexose.

In certain embodiments, a compound comprises an oligonucleotide (*e.g.*, a miRNA or miRNA encoding oligonucleotide) conjugated to one or more moieties which enhance the activity, cellular distribution or cellular uptake of the resulting oligonucleotide. In certain such embodiments, the moiety is a cholesterol moiety (*e.g.*, antagomirs) or a lipid
10 moiety or liposome conjugate. Additional moieties for conjugation include carbohydrates, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. In certain embodiments, a conjugate group is attached directly to the oligonucleotide. In certain embodiments, a conjugate group is attached to the oligonucleotide by a linking moiety selected from amino, hydroxyl,
15 carboxylic acid, thiol, unsaturations (*e.g.*, double or triple bonds), 8-amino-3,6-dioxaoctanoic acid (ADO), succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), 6-aminohexanoic acid (AHEx or AHA), substituted C1-C10 alkyl, substituted or unsubstituted C2-C10 alkenyl, and substituted or unsubstituted C2-C10 alkynyl. In certain such embodiments, a substituent group is selected from hydroxyl,
20 amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl.

In certain such embodiments, the compound comprises the oligonucleotide having one or more stabilizing groups that are attached to one or both termini of the oligonucleotide to enhance properties such as, for example, nuclease stability. Included in
25 stabilizing groups are cap structures. These terminal modifications protect the oligonucleotide from exonuclease degradation, and can help in delivery and/or localization within a cell. The cap can be present at the 5'-terminus (5'-cap), or at the 3'-terminus (3'-cap), or can be present on both termini. Cap structures include, for example, inverted deoxy abasic caps.

30 Suitable cap structures include a 4',5'-methylene nucleotide, a 1-(beta-D-erythrofuransyl) nucleotide, a 4'-thio nucleotide, a carbocyclic nucleotide, a 1,5-anhydrohexitol nucleotide, an L-nucleotide, an alpha-nucleotide, a modified base nucleotide, a phosphorodithioate linkage, a threo-pentofuransyl nucleotide, an acyclic

3',4'-seco nucleotide, an acyclic 3,4-dihydroxybutyl nucleotide, an acyclic 3,5-dihydroxypentyl nucleotide, a 3'-3'-inverted nucleotide moiety, a 3'-3'-inverted abasic moiety, a 3'-2'-inverted nucleotide moiety, a 3'-2'-inverted abasic moiety, a 1,4-butanediol phosphate, a 3'-phosphoramidate, a hexylphosphate, an aminohexyl phosphate, a 3'-phosphate, a 3'-phosphorothioate, a phosphorodithioate, a bridging methylphosphonate moiety, and a non-bridging methylphosphonate moiety 5'-amino-alkyl phosphate, a 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate, a 6-aminohexyl phosphate, a 1,2-aminododecyl phosphate, a hydroxypropyl phosphate, a 5'-5'-inverted nucleotide moiety, a 5'-5'-inverted abasic moiety, a 5'-phosphoramidate, a 5'-phosphorothioate, a 5'-amino, a bridging and/or non-bridging 5'-phosphoramidate, a phosphorothioate, and a 5'-mercapto moiety.

It is to be understood that additional well-known nucleic acid architecture or chemistry can be applied. Different modifications can be placed at different positions to prevent the oligonucleotide from activating RNase H and/or being capable of recruiting the RNAi machinery. In another embodiment, they may be placed such as to allow RNase H activation and/or recruitment of the RNAi machinery. The modifications can be non-natural bases, e.g. universal bases. It may be modifications on the backbone sugar or phosphate, e.g., 2'-O-modifications including LNA or phosphorothioate linkages. As used herein, it makes no difference whether the modifications are present on the nucleotide before incorporation into the oligonucleotide or whether the oligonucleotide is modified after synthesis.

Preferred modifications are those that increase the affinity of the oligonucleotide for complementary sequences, i.e. increases the t_m (melting temperature) of the oligonucleotide base paired to a complementary sequence. Such modifications include 2'-O-fluoro, 2'-O-methyl, 2'-O-methoxyethyl. The use of LNA (locked nucleic acid) units, phosphoramidate, PNA (peptide nucleic acid) units or INA (intercalating nucleic acid) units is preferred. For shorter oligonucleotides, it is preferred that a higher percentage of affinity increasing modifications are present. If the oligonucleotide is less than 12 or 10 units long, it may be composed entirely of LNA units. A wide range of other non-natural units may also be build into the oligonucleotide, e.g., morpholino, 2'-deoxy-2'-fluoro-arabinonucleic acid (FANA) and arabinonucleic acid (ANA). In a preferred embodiment, the fraction of units modified at either the base or sugar relatively to the units not modified at either the base or sugar is selected from the group consisting of less than 99%, 95%, less

than 90%, less than 85% or less than 75%, less than 70%, less than 65%, less than 60%, less than 50%, less than 45%, less than 40%, less than 35%, less than 30%, less than 25%, less than 20%, less than 15%, less than 10%, and less than 5%, less than 1%, more than 99%, more than 95%, more than 90%, more than 85% or more than 75%, more than 70%,
 5 more than 65%, more than 60%, more than 50%, more than 45%, more than 40%, more than 35%, more than 30%, more than 25%, more than 20%, more than 15%, more than 10%, and more than 5% and more than 1%.

Small nucleic acids and/or antisense oligonucleotides can also contain a neutral peptide-like backbone. Such molecules are termed peptide nucleic acid (PNA)-oligomers
 10 and are described, *e.g.*, in Perry-O'Keefe *et al.* (1996) Proc. Natl. Acad. Sci. U.S.A. 93:14670 and in Eglom *et al.* (1993) Nature 365:566. One advantage of PNA oligomers is their capability to bind to complementary DNA essentially independently from the ionic strength of the medium due to the neutral backbone of the DNA. In yet another embodiment, small nucleic acids and/or antisense oligonucleotides comprises at least one
 15 modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In a further embodiment, small nucleic acids and/or antisense oligonucleotides are α -anomeric oligonucleotides. An α -anomeric oligonucleotide forms specific double-
 20 stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier *et al.* (1987) Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue *et al.* (1987) Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue *et al.* (1987) FEBS Lett. 215:327-330).

25 Small nucleic acids and/or antisense oligonucleotides of the methods and compositions presented herein may be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein *et al.* (1988) Nucl. Acids Res. 16:3209,
 30 methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin *et al.* (1988) Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc. For example, an isolated miRNA can be chemically synthesized or recombinantly produced using methods known in the art. In some instances, miRNA are chemically synthesized

using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. Commercial suppliers of synthetic RNA molecules or synthesis reagents include, *e.g.*, Proligo (Hamburg, Germany), Dharmacon Research (Lafayette, Colo., USA), Pierce Chemical (part of Perbio Science, Rockford, Ill., USA), Glen Research (Sterling, Va., USA), ChemGenes (Ashland, Mass., USA), Cruachem (Glasgow, UK), and Exiqon (Vedbaek, Denmark).

Small nucleic acids and/or antisense oligonucleotides can be delivered to cells *in vivo*. A number of methods have been developed for delivering small nucleic acids and/or antisense oligonucleotides DNA or RNA to cells; *e.g.*, antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (*e.g.*, antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systematically.

In one embodiment, small nucleic acids and/or antisense oligonucleotides may comprise or be generated from double stranded small interfering RNAs (siRNAs), in which sequences fully complementary to cellular nucleic acids (*e.g.*, mRNAs) sequences mediate degradation or in which sequences incompletely complementary to cellular nucleic acids (*e.g.*, mRNAs) mediate translational repression when expressed within cells. In another embodiment, double stranded siRNAs can be processed into single stranded antisense RNAs that bind single stranded cellular RNAs (*e.g.*, microRNAs) and inhibit their expression. RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. *in vivo*, long dsRNA is cleaved by ribonuclease III to generate 21- and 22-nucleotide siRNAs. It has been shown that 21-nucleotide siRNA duplexes specifically suppress expression of endogenous and heterologous genes in different mammalian cell lines, including human embryonic kidney (293) and HeLa cells (Elbashir *et al.* (2001) *Nature* 411:494-498). Accordingly, translation of a gene in a cell can be inhibited by contacting the cell with short double stranded RNAs having a length of about 15 to 30 nucleotides or of about 18 to 21 nucleotides or of about 19 to 21 nucleotides. Alternatively, a vector encoding for such siRNAs or short hairpin RNAs (shRNAs) that are metabolized into siRNAs can be introduced into a target cell (see, *e.g.*, McManus *et al.* (2002) *RNA* 8:842; Xia *et al.* (2002) *Nature Biotechnology* 20:1006; and Brummelkamp *et al.* (2002) *Science* 296:550). Vectors that can be used are commercially available, *e.g.*, from OligoEngine under the name pSuper RNAi System™.

Ribozyme molecules designed to catalytically cleave cellular mRNA transcripts can also be used to prevent translation of cellular mRNAs and expression of cellular polypeptides, or both (See, *e.g.*, PCT International Publication WO90/11364, published October 4, 1990; Sarver *et al.* (1990) Science 247:1222-1225 and U.S. Patent No. 5,093,246). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy cellular mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well-known in the art and is described more fully in Haseloff and Gerlach (1988) Nature 334:585-591. The ribozyme may be engineered so that the cleavage recognition site is located near the 5' end of cellular mRNAs; *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the methods and compositions presented herein also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug, *et al.* (1984) Science 224:574-578; Zaug, *et al.* (1986) Science 231:470-475; Zaug, *et al.* (1986) Nature 324:429-433; published International patent application No. WO88/04300 by University Patents Inc.; Been, *et al.* (1986) Cell 47:207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The methods and compositions presented herein encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in cellular genes.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.*, for improved stability, targeting, etc.). A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous cellular messages and inhibit translation. Because ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription of cellular genes are preferably single stranded and composed of deoxyribonucleotides. The base composition of these oligonucleotides should promote triple helix formation via Hoogsteen base pairing rules, which generally require sizable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in CGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizable stretch of either purines or pyrimidines to be present on one strand of a duplex.

Small nucleic acids (*e.g.*, miRNAs, pre-miRNAs, pri-miRNAs, miRNA*, piwiRNA, anti-miRNA, or a miRNA binding site, or a variant thereof), antisense oligonucleotides, ribozymes, and triple helix molecules of the methods and compositions presented herein may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well-known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Moreover, various well-known modifications to nucleic acid molecules may be introduced as a means of increasing intracellular stability and half-life. One of skill in the

art will readily understand that polypeptides, small nucleic acids, and antisense oligonucleotides can be further linked to another peptide or polypeptide (*e.g.*, a heterologous peptide), *e.g.*, that serves as a means of protein detection. Non-limiting examples of label peptide or polypeptide moieties useful for detection in the invention
5 include, without limitation, suitable enzymes such as horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; epitope tags, such as FLAG, MYC, HA, or HIS tags; fluorophores such as green fluorescent protein; dyes; radioisotopes; digoxigenin; biotin; antibodies; polymers; as well as others known in the art, for example, in Principles of Fluorescence Spectroscopy, Joseph R. Lakowicz (Editor), Plenum Pub
10 Corp, 2nd edition (July 1999).

In addition, other nucleic acid forms are well-known in the art, such as mRNAs, cDNAs, and products thereof, and other modulatory agents (*e.g.*, antibodies, small molecules, peptides, fusion proteins, or small nucleic acids) are contemplated and they can be incorporated into pharmaceutical compositions and administered to a subject *in vivo*.
15 The compositions may contain a single such molecule or agent or any combination of agents described herein. Based on the genetic pathway analyses described herein, it is believed that such combinations of agents is especially effective in diagnosing, prognosing, preventing, and treating a metabolic disorder. Thus, “single active agents” described herein can be combined with other pharmacologically active compounds (“second active agents”) known in the art according to the methods and compositions provided herein. It is believed
20 that certain combinations work synergistically in the treatment of particular types of metabolic disorders. Second active agents can be large molecules (*e.g.*, proteins) or small molecules (*e.g.*, synthetic inorganic, organometallic, or organic molecules). For example, agents that modulate (*e.g.*, promote) brown fat cell-like development and/or activity can be provided as combination agents. Exemplary agents include, without limitation, PRDM16
25 (U.S. Pat. Publ. 2011/0059051), C/EBP β (U.S. Pat. Publ. 2012/0022500), FNDC5/Irisin (U.S. Pat. 8,969,519 and PCT Publ. No. WO 2013/039996), Meteorin/Meteorin-like (PCT Publ. No. WO 2014/116556), respiration uncoupling agents (*e.g.*, dinitrophenol, CCCP, and FCCP), and the like.

30

II. Identification of Compounds that Modulate Succinate

The succinate and agents described herein may be used to identify modulators of one or more of biological activities of succinate. In particular, information useful for the

design of therapeutic and diagnostic molecules, including, for example, succinate and derivatives thereof is now available or attainable using the methods described herein.

In one aspect, modulators, inhibitors, or antagonists against the succinate and agents of the invention, biological complexes containing them, or orthologues thereof, may be used to treat any disease or other treatable condition of a patient (including humans and animals), including, for example, metabolic disorders.

Modulators of succinate may be identified and developed as set forth below using techniques and methods known to those of skill in the art. The modulators of the invention can be used, for example, to inhibit and treat succinate and/or thermogenesis-mediated diseases or disorders. The modulators of the invention may elicit a change in one or more of the following activities: (a) a change in the level and/or rate of formation of succinate-protein complex or product (*e.g.*, naturally occurring or synthetic), such as UCPI, (b) a change in the uptake of succinate, (c) a change in the stability of succinate, (d) a change in the metabolism of a succinate, or (e) a change in the activity of at least one polypeptide contained in a succinate complex or substrate/enzyme configuration. A number of methods for identifying a molecule which modulates a succinate are known in the art. For example, in one such method, succinate is contacted with a test compound, and the activity of succinate is determined in the presence of the test compound, wherein a change in the activity of the succinate in the presence of the compound as compared to the activity in the absence of the compound (or in the presence of a control compound) indicates that the test compound modulates the activity of the succinate. Included within the term succinate modulation is succinate uptake.

Compounds to be tested for their ability to act as modulators of succinate can be produced, for example, by bacteria, yeast or other organisms (*e.g.* natural products), produced chemically (*e.g.* small molecules, including peptidomimetics), or produced recombinantly. Compounds for use with the above-described methods may be selected from the group of compounds consisting of lipids, carbohydrates, polypeptides, peptidomimetics, peptide-nucleic acids (PNAs), small molecules, natural products, aptamers and polynucleotides. In certain embodiments, the compound is a polynucleotide. In some embodiments, said polynucleotide is an antisense nucleic acid. In other embodiments, said polynucleotide is an siRNA. In certain embodiments, the compound comprises an analogue of succinate (*e.g.*, a dominant negative form that binds to, but does not activate, succinate-induced activity). In other embodiments, the compound promotes

(*e.g.*, increases enzymatic activity, such as a substrate having a structure requiring a lower activation energy) or inhibits (*e.g.*, decreases succinate activity, such as a reversible or irreversible inhibitor, like a covalent inhibitor).

A variety of assay formats will suffice and, in light of the present disclosure, those
5 not expressly described herein may nevertheless be comprehended by one of ordinary skill in the art based on the teachings herein. Assay formats for analyzing succinate-substrate complex formation and/or activity of succinate may be generated in many different forms, and include assays based on cell-free systems, *e.g.* purified proteins or cell lysates, as well as cell-based assays, which utilize intact cells, such as to determine cellular uptake, cellular
10 metabolism, and the like, of succinate. Simple binding assays can also be used to detect agents which modulate succinate, for example, by enhancing the formation of succinate metabolites, by enhancing the rate of succinate metabolism, and/or by enhancing the binding of succinate to a polypeptide or polypeptide complex. Another example of an assay useful for identifying a modulator of succinate is a competitive assay that combines
15 succinate with a potential modulator, such as, for example, polypeptides, nucleic acids, natural substrates or ligands, or substrate or ligand mimetics, under appropriate conditions for a competitive inhibition assay. Succinate can be labeled, such as by radioactivity or a colorimetric compound, such that succinate complex formation and/or activity can be determined accurately to assess the effectiveness of the potential modulator.

20 Assays may employ kinetic or thermodynamic methodology using a wide variety of techniques including, but not limited to, microcalorimetry, circular dichroism, capillary zone electrophoresis, nuclear magnetic resonance spectroscopy, fluorescence spectroscopy, and combinations thereof. Assays may also employ any of the methods for isolating, preparing and detecting succinate, succinate-complexes, polypeptide complexes comprising
25 succinate, and the like, as described above.

Complex formation between succinate and a binding partner (*e.g.*, a succinate-binding enzyme or a metabolizer) may be detected by a variety of methods. Modulation of the complex's formation may be quantified using, for example, detectably labeled proteins such as radiolabeled, fluorescently labeled, or enzymatically labeled polypeptides or
30 binding partners, by immunoassay, or by chromatographic detection. Methods of isolating and identifying succinate-complexes described above may be incorporated into the detection methods.

In certain embodiments, it may be desirable to immobilize a succinate-binding polypeptide to facilitate separation of succinate complexes from uncomplexed forms, as well as to accommodate automation of the assay. Binding of a succinate-binding polypeptide to a binding partner may be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein may be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/polypeptide (GST/polypeptide) fusion proteins may be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtitre plates, which are then combined with the binding partner, *e.g.* an ^{35}S -labeled binding partner, and the test compound, and the mixture incubated under conditions conducive to complex formation, *e.g.* at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound label, and the matrix immobilized and radiolabel determined directly (*e.g.* beads placed in scintillant), or in the supernatant after the complexes are subsequently dissociated. Alternatively, the complexes may be dissociated from the matrix, separated by SDS-PAGE, and the level of succinate-binding polypeptides found in the bead fraction quantified from the gel using standard electrophoretic techniques such as described in the appended examples.

Other techniques for immobilizing proteins on matrices are also available for use in the subject assay. For instance, succinate-binding polypeptide may be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated polypeptide molecules may be prepared from biotin-NHS(N-hydroxy-succinimide) using techniques well-known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, Ill.), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with the polypeptide may be derivatized to the wells of the plate, and polypeptide trapped in the wells by antibody conjugation. As above, preparations of a binding partner and a test compound are incubated in the polypeptide presenting wells of the plate, and the amount of complex trapped in the well may be quantified. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the binding partner, or which are reactive with the succinate-binding polypeptide and compete with the binding partner; as well as enzyme-

linked assays which rely on detecting an enzymatic activity associated with the binding partner, either intrinsic or extrinsic activity. In the instance of the latter, the enzyme may be chemically conjugated or provided as a fusion protein with the binding partner. To illustrate, the binding partner may be chemically cross-linked or genetically fused with horseradish peroxidase, and the amount of succinate trapped in the succinate complex may be assessed with a chromogenic substrate of the enzyme, *e.g.* 3,3'-diamino-benzadine tetrahydrochloride or 4-chloro-1-naphthol. Likewise, a fusion protein comprising a polypeptide and glutathione-S-transferase may be provided, and succinate complex formation quantified by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) J Biol Chem 249:7130).

Antibodies against the succinate-binding polypeptide can be used for immunodetection purposes. Alternatively, the succinate-binding polypeptide to be detected may be "epitope-tagged" in the form of a fusion protein that includes, in addition to the polypeptide sequence, a second polypeptide for which antibodies are readily available (*e.g.*, from commercial sources). For instance, the GST fusion proteins described above may also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include myc-epitopes (*e.g.*, see Ellison *et al.* (1991) *J. Biol. Chem.* 266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharmacia, N.J.).

In certain *in vitro* embodiments of the present assay, the protein or the set of proteins engaged in a protein-protein, protein-substrate, or protein-nucleic acid interaction comprises a reconstituted protein mixture of at least semi-purified proteins. By semi-purified, it is meant that the proteins utilized in the reconstituted mixture have been previously separated from other cellular or viral proteins. For instance, in contrast to cell lysates, the proteins involved in a protein-substrate, protein-protein or nucleic acid-protein interaction are present in the mixture to at least 50% purity relative to all other proteins in the mixture, and more preferably are present at 90-95% purity. In certain embodiments of the subject method, the reconstituted protein mixture is derived by mixing highly purified proteins such that the reconstituted mixture substantially lacks other proteins (such as of cellular or viral origin) which might interfere with or otherwise alter the ability to measure activity resulting from the given protein-substrate, protein-protein interaction, or nucleic acid-protein interaction.

In one embodiment, the use of reconstituted protein mixtures allows more careful control of the protein-substrate, protein-protein, or nucleic acid-protein interaction conditions. Moreover, the system may be derived to favor discovery of modulators of particular intermediate states of the protein-protein interaction. For instance, a
5 reconstituted protein assay may be carried out both in the presence and absence of a candidate agent, thereby allowing detection of a modulator of a given protein-substrate, protein-protein, or nucleic acid-protein interaction.

Assaying biological activity resulting from a given protein-substrate, protein-protein or nucleic acid-protein interaction, in the presence and absence of a candidate modulator,
10 may be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes.

In yet another embodiment, a succinate-binding polypeptide may be used to generate a two-hybrid or interaction trap assay (see also, U.S. Pat. No. 5,283,317; Zervos et al. (1993) Cell 72:223-232; Madura et al. (1993) J. Biol Chem 268:12046-12054; Bartel et
15 al. (1993) Biotechniques 14:920-924; and Iwabuchi et al. (1993) Oncogene 8:1693-1696), for subsequently detecting agents which disrupt binding of the interaction components to one another.

In particular, the method makes use of chimeric genes which express hybrid proteins. To illustrate, a first hybrid gene comprises the coding sequence for a binding
20 domain of a transcriptional activator may be fused in frame to the coding sequence for a “bait” protein, *e.g.*, a succinate-binding polypeptide of sufficient length to bind to a potential interacting protein. The second hybrid protein encodes a transcriptional activation domain fused in frame to a gene encoding a “fish” protein, *e.g.*, a potential interacting protein of sufficient length to interact with the protein-protein interaction component
25 polypeptide portion of the bait fusion protein. If the bait and fish proteins are able to interact, *e.g.*, form a protein-protein interaction component complex, they bring into close proximity the two domains of the transcriptional activator. This proximity causes transcription of a reporter gene which is operably linked to a transcriptional regulatory site responsive to the transcriptional activator, and expression of the reporter gene may be
30 detected and used to score for the interaction of the bait and fish proteins. The host cell also contains a first chimeric gene which is capable of being expressed in the host cell. The gene encodes a chimeric protein, which comprises (a) a binding domain that recognizes the responsive element on the reporter gene in the host cell, and (b) a bait protein (*e.g.*, a

succinate-binding polypeptide). A second chimeric gene is also provided which is capable of being expressed in the host cell, and encodes the “fish” fusion protein. In one embodiment, both the first and the second chimeric genes are introduced into the host cell in the form of plasmids. Preferably, however, the first chimeric gene is present in a chromosome of the host cell and the second chimeric gene is introduced into the host cell as part of a plasmid.

The binding domain of the first hybrid protein and the transcriptional activation domain of the second hybrid protein may be derived from transcriptional activators having separable binding and transcriptional activation domains. For instance, these separate binding and transcriptional activation domains are known to be found in the yeast GAL4 protein, and are known to be found in the yeast GCN4 and ADR1 proteins. Many other proteins involved in transcription also have separable binding and transcriptional activation domains which make them useful for the present invention, and include, for example, the LexA and VP16 proteins. It will be understood that other (substantially) transcriptionally-inert binding domains may be used in the subject constructs; such as domains of ACE1, λ cI, lac repressor, jun or fos. In another embodiment, the binding domain and the transcriptional activation domain may be from different proteins. The use of a LexA DNA binding domain provides certain advantages. For example, in yeast, the LexA moiety contains no activation function and has no known affect on transcription of yeast genes. In addition, use of LexA allows control over the sensitivity of the assay to the level of interaction (see, for example, PCT Publ. No. WO 1994/10300).

In certain embodiments, any enzymatic activity associated with the bait or fish proteins is inactivated, *e.g.*, dominant negative or other mutants of a protein-protein interaction component can be used.

Continuing with the illustrative example, formation of a complex between the bait and fish fusion proteins in the host cell, causes the activation domain to activate transcription of the reporter gene. The method is carried out by introducing the first chimeric gene and the second chimeric gene into the host cell, and subjecting that cell to conditions under which the bait and fish fusion proteins and are expressed in sufficient quantity for the reporter gene to be activated. The formation of a complex results in a detectable signal produced by the expression of the reporter gene.

In still further embodiments, the succinate, or substrate-complex or protein-complex thereof, of interest may be generated in whole cells, taking advantage of cell culture

techniques to support the subject assay. For example, the succinate, or complex thereof, may be constituted in a prokaryotic or eukaryotic cell culture system. Advantages to generating the succinate complex, in an intact cell includes the ability to screen for modulators of the level and/or activity of succinate, or complex thereof, which are
5 functional in an environment more closely approximating that which therapeutic use of the modulator would require, including the ability of the agent to gain entry into the cell. Furthermore, certain of the *in vivo* embodiments of the assay are amenable to high throughput analysis of candidate agents.

The succinate can be endogenous to the cell selected to support the assay.
10 Alternatively, some or all of the components can be derived from exogenous sources. For instance, fusion proteins can be introduced into the cell by recombinant techniques (such as through the use of an expression vector), as well as by microinjecting the fusion protein itself or mRNA encoding the fusion protein. Moreover, in the whole cell embodiments of the subject assay, the reporter gene construct can provide, upon expression, a selectable
15 marker. Such embodiments of the subject assay are particularly amenable to high throughput analysis in that proliferation of the cell can provide a simple measure of the protein-protein interaction.

The amount of transcription from the reporter gene may be measured using any method known to those of skill in the art to be suitable. For example, specific mRNA
20 expression may be detected using Northern blots or specific protein product may be identified by a characteristic stain, western blots or an intrinsic activity. In certain embodiments, the product of the reporter gene is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detection signal based on color, fluorescence, or
25 luminescence.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays of the present invention which are performed in cell-free systems, such as may be derived with purified or semi-purified
30 proteins or with lysates, are often preferred as “primary” screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro*

system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with other proteins or changes in enzymatic properties of the molecular target. Accordingly, potential modulators of succinate may be detected in a cell-free assay generated by constitution of succinate in a cell lysate. In an alternate format, the assay can be derived as a reconstituted protein mixture which, as described below, offers a number of benefits over lysate-based assays.

The activity of succinate or a succinate-binding polypeptide may be identified and/or assayed using a variety of methods well-known to the skilled artisan. For example, the activity of succinate may be determined by assaying for the level of expression of RNA and/or protein molecules. Transcription levels may be determined, for example, using Northern blots, hybridization to an oligonucleotide array or by assaying for the level of a resulting protein product. Translation levels may be determined, for example, using Western blotting or by identifying a detectable signal produced by a protein product (*e.g.*, fluorescence, luminescence, enzymatic activity, *etc.*). Depending on the particular situation, it may be desirable to detect the level of transcription and/or translation of a single gene or of multiple genes.

In other embodiments, the biological activity of succinate may be assessed by monitoring changes in the phenotype of a targeted cell. For example, the detection means can include a reporter gene construct which includes a transcriptional regulatory element that is dependent in some form on the level and/or activity of succinate. Accordingly, the level of expression of the reporter gene will vary with the level of succinate.

Similarly, succinate activity can be assessed using well-known enzymatic analysis methods. For example, the rate or amount of succinate catalysis, enzyme association, enzyme dissociation, product biosynthesis, product catalysis (*e.g.*, breakdown), and the like can be analyzed.

Moreover, in the whole cell embodiments of the subject assay, the reporter gene construct can provide, upon expression, a selectable marker. A reporter gene includes any gene that expresses a detectable gene product, which may be RNA or protein. Preferred reporter genes are those that are readily detectable. The reporter gene may also be included in the construct in the form of a fusion gene with a gene that includes desired transcriptional regulatory sequences or exhibits other desirable properties. For instance, the product of the reporter gene can be an enzyme which confers resistance to an antibiotic or other drug, or an enzyme which complements a deficiency in the host cell (*i.e.* thymidine kinase or

dihydrofolate reductase). To illustrate, the aminoglycoside phosphotransferase encoded by the bacterial transposon gene Tn5 neo can be placed under transcriptional control of a promoter element responsive to the level of a succinate present in the cell. Such embodiments of the subject assay are particularly amenable to high through-put analysis in that proliferation of the cell can provide a simple measure of inhibition of succinate.

Similarly, individual cells or analyses of phenotypes in organisms can be formed to determine effects of test agents on the modulation (*e.g.*, upregulation) of one or more of the following succinate-mediated biological activities: a) brown fat and/or beige fat gene expression, such as expression of a marker selected from the group consisting of: cidea, adiponectin, adipsin, otopetrin, type II deiodinase, cig30, ppar gamma 2, pgc1 α , ucp1, elovl3, cAMP, Prdm16, cytochrome C, cox4i1, coxIII, cox5b, cox7a1, cox8b, glut4, atpase b2, cox II, atp5o, ndufb5, ap2, ndufs1, GRP109A, acylCoA-thioesterase 4, EARA1, claudin1, PEPCCK, fgf21, acylCoA-thioesterase 3, dio2, fatty acid synthase (fas), leptin, resistin, and nuclear respiratory factor-1 (nrf1); b) thermogenesis in adipose cells; c) differentiation of adipose cells; d) insulin sensitivity of adipose cells; e) basal respiration, leak respiration, or uncoupled respiration; f) whole body oxygen consumption; g) obesity or appetite; h) insulin secretion of pancreatic beta cells; i) glucose tolerance; j) modified phosphorylation of EGFR, ERK, AMPK, protein kinase A (PKA) substrates having an RRX(S/T) motif, wherein the X is any amino acid and the (S/T) residue is a serine or threonine, HSL; k) modified activity of UCP1 protein; and l) growth and effects of metabolic disorders, such as obesity-associated cancer, cachexia, anorexia, diabetes, and obesity.

III. Methods of the Invention

One aspect of the present invention relates to methods of using and/or selecting agents (*e.g.*, antibodies, fusion constructs, peptides, small molecules, small nucleic acids, and the like) which bind to, upregulate, downregulate, or modulate succinate and/or one or more biomarkers of the present invention listed in Table 1, the Figures, and the Examples, and/or a metabolic disorder. Such methods can use screening assays, including cell-based and non-cell based assays.

In any method described herein, such as a diagnostic method, prognostic method, therapeutic method, or combination thereof, all steps of the method can be performed by a single actor or, alternatively, by more than one actor. For example, diagnosis can be

performed directly by the actor providing therapeutic treatment. Alternatively, a person providing a therapeutic agent can request that a diagnostic assay be performed. The diagnostician and/or the therapeutic interventionist can interpret the diagnostic assay results to determine a therapeutic strategy. Similarly, such alternative processes can apply to other assays, such as prognostic assays.

In one embodiment, the invention relates to assays for screening candidate or test compounds which bind to or modulate the expression or activity level of, one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment or ortholog thereof. Such compounds include, without limitation, antibodies, proteins, fusion proteins, nucleic acid molecules, and small molecules.

As described in detail in the section above, in one embodiment, an assay is a cell-based assay, comprising contacting a cell expressing one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof, with a test compound and determining the ability of the test compound to modulate (*e.g.*, stimulate or inhibit) the level of interaction between the biomarker and its natural binding partners as measured by direct binding or by measuring a parameter related to a metabolic disorder.

For example, in a direct binding assay, the biomarker polypeptide, a binding partner polypeptide of the biomarker, or a fragment(s) thereof, can be coupled with a radioisotope or enzymatic label such that binding of the biomarker polypeptide or a fragment thereof to its natural (or synthetic) binding partner(s) or a fragment(s) thereof can be determined by detecting the labeled molecule in a complex. For example, the biomarker polypeptide, a binding partner polypeptide of the biomarker, or a fragment(s) thereof, can be labeled with ^{125}I , ^{35}S , ^{14}C , or ^3H , either directly or indirectly, and the radioisotope detected by direct counting of radioemission or by scintillation counting. Alternatively, the polypeptides of interest can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

It is also within the scope of this invention to determine the ability of a compound to modulate the interactions between one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof, and its natural (or synthetic) binding

partner(s) (e.g., naturally occurring or synthetic SDH) or a fragment(s) thereof, without the labeling of any of the interactants (e.g., using a microphysiometer as described in McConnell, H. M. *et al.* (1992) *Science* 257:1906-1912). As used herein, a “microphysiometer” (e.g., Cytosensor) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between compound and receptor.

In a preferred embodiment, determining the ability of blocking agents (e.g., antibodies, fusion proteins, peptides, nucleic acid molecules, and small molecules) to antagonize the interaction between a given set of nucleic acid molecules and/or polypeptides can be accomplished by determining the activity of one or more members of the set of interacting molecules. For example, the activity of one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof, can be determined by detecting induction of metabolic response, detecting catalytic/enzymatic activity of an appropriate substrate, detecting the induction of a reporter gene (comprising a target-responsive regulatory element operatively linked to a nucleic acid encoding a detectable marker, e.g., chloramphenicol acetyl transferase), or detecting a cellular response regulated by the biomarker or a fragment thereof (e.g., modulations of biological pathways identified herein, such as modulated cellular respiration, brown/beige fat gene expression, mitochondrial biosynthesis, and the like).

In yet another embodiment, an assay of the present invention is a cell-free assay in which one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof, e.g., a biologically active fragment thereof, is contacted with a test compound, and the ability of the test compound to bind to the polypeptide, or biologically active portion thereof, is determined. Binding of the test compound to the biomarker or a fragment thereof, can be determined either directly or indirectly as described above. Determining the ability of the biomarker or a fragment thereof to bind to its natural (or synthetic) binding partner(s) or a fragment(s) thereof can also be accomplished using a technology such as real-time Biomolecular Interaction Analysis (BIA) (Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345 and Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705). As used herein, “BIA” is a technology for studying biospecific

interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore). Changes in the optical phenomenon of surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological polypeptides. One or more biomarkers polypeptide or a fragment thereof can be immobilized on a BIAcore chip and multiple
5 agents, *e.g.*, blocking antibodies, fusion proteins, peptides, small molecules, and the like, can be tested for binding to the immobilized biomarker polypeptide or fragment thereof. An example of using the BIA technology is described by Fitz *et al.* (1997) *Oncogene* 15:613.

The cell-free assays of the present invention are amenable to use of both soluble
10 and/or membrane-bound forms of proteins. In the case of cell-free assays in which a membrane-bound form protein is used it may be desirable to utilize a solubilizing agent such that the membrane-bound form of the protein is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-
15 methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether)_n, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

In one or more embodiments of the above described assay methods, it may be
20 desirable to immobilize either the biomarker nucleic acid and/or polypeptide, the natural (or synthetic) binding partner(s) of the biomarker, or fragments thereof, to facilitate separation of complexed from uncomplexed forms of the reactants, as well as to accommodate automation of the assay. Binding of a test compound in the assay can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include
25 microtiter plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase-base fusion proteins, can be adsorbed onto glutathione Sepharose® beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtiter plates, which are then combined with the test compound,
30 and the mixture incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described

above. Alternatively, the complexes can be dissociated from the matrix, and the level of binding or activity determined using standard techniques.

In an alternative embodiment, determining the ability of the test compound to modulate the activity of one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof, or of natural (or synthetic) binding partner(s) thereof can be accomplished by determining the ability of the test compound to modulate the expression or activity of a gene, *e.g.*, nucleic acid, or gene product, *e.g.*, polypeptide, that functions downstream of the interaction. For example, cellular migration or invasion can be determined by monitoring cellular movement, matrigel assays, induction of invasion-related gene expression, and the like, as described further herein.

In another embodiment, modulators of one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof, are identified in a method wherein a cell is contacted with a candidate compound and the expression or activity level of the biomarker is determined. The level of expression of biomarker RNA or polypeptide or fragments thereof in the presence of the candidate compound is compared to the level of expression of biomarker RNA or polypeptide or fragments thereof in the absence of the candidate compound. The candidate compound can then be identified as a modulator of biomarker expression based on this comparison. For example, when expression of biomarker RNA or polypeptide or fragments thereof is greater (statistically significantly greater) in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of biomarker expression. Alternatively, when expression of biomarker RNA or polypeptide or fragments thereof is reduced (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of biomarker expression. The expression level of biomarker RNA or polypeptide or fragments, or products thereof such as enzyme catalyzed products, thereof in the cells or produced by the cells can be determined by methods described herein for detecting biomarker mRNA or polypeptide or fragments thereof.

In yet another aspect of the present invention, a biomarker of the present invention, including one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof, can be used as "bait" in a two-hybrid assay or three-hybrid assay (see,

e.g., U.S. Pat. No. 5,283,317; Zervos *et al.* (1993) Cell 72:223-232; Madura *et al.* (1993) J. Biol. Chem. 268:12046-12054; Bartel *et al.* (1993) Biotechniques 14:920-924; Iwabuchi *et al.* (1993) Oncogene 8:1693-1696; and Brent W094/10300), to identify other nucleic acids and/or polypeptides which bind to or interact with the biomarker or fragments thereof and are involved in activity of the biomarkers. Such biomarker-binding proteins are also likely to be involved in the propagation of signals by the biomarker polypeptides or biomarker natural (or synthetic) binding partner(s) as, for example, downstream elements of one or more biomarkers -mediated signaling pathway.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for one or more biomarkers polypeptide is fused to a gene encoding the DNA binding domain of a known transcription factor (e.g., GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified polypeptide ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" polypeptides are able to interact, *in vivo*, forming one or more biomarkers -dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the polypeptide which interacts with one or more biomarkers polypeptide of the present invention, including one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof.

In another aspect, the invention pertains to a combination of two or more of the assays described herein. For example, a modulating agent can be identified using a cell-based or a cell-free assay, and the ability of the agent to modulate the activity of one or more biomarkers polypeptide or a fragment thereof can be confirmed *in vivo*, e.g., in an animal such as an animal model for cellular transformation and/or tumorigenesis.

This invention further pertains to novel agents identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use an agent identified as described herein in an appropriate animal model. For example, an agent identified as described herein can be used in an animal model to determine the efficacy,

toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

5 In other aspects of the present invention, the biomarkers described herein, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or fragments thereof, can be used in one or more of the following methods: a) screening assays; b) predictive medicine (*e.g.*, diagnostic assays, prognostic assays, and monitoring of clinical trials); and c) methods of treatment (*e.g.*,
10 therapeutic and prophylactic, *e.g.*, by up- or down-modulating the copy number, level of expression, and/or level of activity of the one or more biomarkers and/or modulating relevant endpoints such as succinate uptake, cellular respiration, glucose homeostasis, etc.).

The biomarkers described herein or agents that modulate the expression and/or activity of such biomarkers can be used, for example, to (a) express one or more biomarkers
15 of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof (*e.g.*, via a recombinant expression vector in a host cell in gene therapy applications or synthetic nucleic acid molecule), (b) detect biomarker RNA or a fragment thereof (*e.g.*, in a biological sample) or a genetic alteration in one or more biomarkers gene, and/or (c)
20 modulate biomarker activity, as described further below. The biomarkers or modulatory agents thereof can be used to treat conditions or disorders characterized by insufficient or excessive production of one or more biomarkers polypeptide or fragment thereof or production of biomarker polypeptide inhibitors. In addition, the biomarker polypeptides or fragments thereof can be used to screen for naturally occurring biomarker binding
25 partner(s), to screen for drugs or compounds which modulate biomarker activity, as well as to treat conditions or disorders characterized by insufficient or excessive production of biomarker polypeptide or a fragment thereof or production of biomarker polypeptide forms which have decreased, aberrant or unwanted activity compared to biomarker wild-type polypeptides or fragments thereof (*e.g.*, amounts in metabolic disorder samples as
30 compared to control samples).

A. Screening Assays

In one aspect, the present invention relates to a method for preventing in a subject, a disease or condition associated with an unwanted, more than desirable, or less than

desirable, expression and/or activity of one or more biomarkers described herein. Subjects at risk for a disease that would benefit from treatment with the claimed agents or methods can be identified, for example, by any one or combination of diagnostic or prognostic assays known in the art and described herein (see, for example, agents and assays described above in the section describing methods of selecting agents and compositions).

B. Predictive Medicine

The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, and monitoring of clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. Accordingly, one aspect of the present invention relates to diagnostic assays for determining the expression and/or activity level of biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or fragments thereof, in the context of a biological sample (*e.g.*, blood, serum, cells, or tissue) to thereby determine whether an individual is afflicted with a disease or disorder, or is at risk of developing a disorder, associated with aberrant or unwanted biomarker expression or activity. The present invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing a disorder associated with biomarker expression or activity. For example, mutations in one or more biomarkers gene can be assayed in a biological sample.

Such assays can be used for prognostic or predictive purpose to thereby prophylactically treat an individual prior to the onset of a disorder characterized by or associated with biomarker expression or activity. For example, succinate is associated with increased thermogenesis and metabolism such that upregulation of succinate predicts treatment of metabolic disorders, either alone or in combination with additional agents. Downregulation and/or reduced activity of succinate indicates reduced thermogenesis and metabolism.

Another aspect of the present invention pertains to monitoring the influence of agents (*e.g.*, drugs, compounds, small nucleic acid-based molecules, and the like) on the expression or activity of biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or fragments thereof, in clinical trials. These and other agents are described in further detail in the following sections.

The term “altered amount” of a marker or “altered level” of a marker refers to increased or decreased copy number of the marker and/or increased or decreased expression level of a particular marker gene or genes in a test sample, as compared to the expression level or copy number of the marker in a control sample. The term “altered amount” of a marker also includes an increased or decreased protein level of a marker in a sample, *e.g.*, a metabolic disorder sample, as compared to the protein level of the marker in a normal, control sample.

The “amount” of a marker, *e.g.*, expression or copy number of a marker, or protein level of a marker, in a subject is “significantly” higher or lower than the normal amount of a marker, if the amount of the marker is greater or less, respectively, than the normal level by an amount greater than the standard error of the assay employed to assess amount, and preferably at least twice, and more preferably three, four, five, ten or more times that amount. Alternately, the amount of the marker in the subject can be considered “significantly” higher or lower than the normal amount if the amount is at least about two, and preferably at least about three, four, or five times, higher or lower, respectively, than the normal amount of the marker. In some embodiments, the amount of the marker in the subject can be considered “significantly” higher or lower than the normal amount if the amount is 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50% or more, higher or lower, respectively, than the normal amount of the marker.

The term “altered level of expression” of a marker refers to an expression level or copy number of a marker in a test sample *e.g.*, a sample derived from a subject suffering from a metabolic disorder, that is greater or less than the standard error of the assay employed to assess expression or copy number, and is preferably at least twice, and more preferably three, four, five or ten or more times the expression level or copy number of the marker or chromosomal region in a control sample (*e.g.*, sample from a healthy subject not having the associated disease) and preferably, the average expression level or copy number of the marker or chromosomal region in several control samples. The altered level of expression is greater or less than the standard error of the assay employed to assess expression or copy number, and is preferably at least twice, and more preferably three, four, five or ten or more times the expression level or copy number of the marker in a control sample (*e.g.*, sample from a healthy subject not having the associated disease) and

preferably, the average expression level or copy number of the marker in several control samples.

The term “altered activity” of a marker refers to an activity of a marker which is increased or decreased in a disease state, *e.g.*, in a metabolic disorder sample, as compared to the activity of the marker in a normal, control sample. Altered activity of a marker may be the result of, for example, altered expression of the marker, altered protein level of the marker, altered structure of the marker, or, *e.g.*, an altered interaction with other proteins involved in the same or different pathway as the marker, or altered interaction with transcriptional activators or inhibitors.

The term “altered structure” of a marker refers to the presence of mutations or allelic variants within the marker gene or marker protein, *e.g.*, mutations which affect expression or activity of the marker, as compared to the normal or wild-type gene or protein. For example, mutations include, but are not limited to substitutions, deletions, or addition mutations. Mutations may be present in the coding or non-coding region of the marker.

The term “altered cellular localization” of a marker refers to the mislocalization of the marker within a cell relative to the normal localization within the cell *e.g.*, within a healthy and/or wild-type cell. An indication of normal localization of the marker can be determined through an analysis of cellular localization motifs known in the field that are harbored by marker polypeptides. For example, SLNCR is a nuclear transcription factor coordinator and naturally functions to present combinations of nuclear transcription factors within the nucleus such that function is abrogated if nuclear import and/or export is inhibited.

The term “body fluid” refers to fluids that are excreted or secreted from the body as well as fluids that are normally not (*e.g.*, amniotic fluid, aqueous humor, bile, blood and blood plasma, cerebrospinal fluid, cerumen and earwax, cowper’s fluid or pre-ejaculatory fluid, chyle, chyme, stool, female ejaculate, interstitial fluid, intracellular fluid, lymph, menses, breast milk, mucus, pleural fluid, peritoneal fluid, pus, saliva, sebum, semen, serum, sweat, synovial fluid, tears, urine, vaginal lubrication, vitreous humor, vomit). In a preferred embodiment, body fluids are restricted to blood-related fluids, including whole blood, serum, plasma, and the like.

The term “classifying” includes “to associate” or “to categorize” a sample with a disease state. In certain instances, “classifying” is based on statistical evidence, empirical

evidence, or both. In certain embodiments, the methods and systems of classifying use of a so-called training set of samples having known disease states. Once established, the training data set serves as a basis, model, or template against which the features of an unknown sample are compared, in order to classify the unknown disease state of the sample. In certain instances, classifying the sample is akin to diagnosing the disease state of the sample. In certain other instances, classifying the sample is akin to differentiating the disease state of the sample from another disease state.

The term “control” refers to any reference standard suitable to provide a comparison to the expression products in the test sample. In one embodiment, the control comprises obtaining a “control sample” from which expression product levels are detected and compared to the expression product levels from the test sample. Such a control sample may comprise any suitable sample, including but not limited to a sample from a control patient (can be stored sample or previous sample measurement) with a known outcome; normal tissue or cells isolated from a subject, such as a normal patient or the patient in need of metabolism modulation, cultured primary cells/tissues isolated from a subject such as a normal subject or the patient in need of metabolism modulation, adjacent normal cells/tissues obtained from the same organ or body location of the patient in need of metabolism modulation, a tissue or cell sample isolated from a normal subject, or a primary cells/tissues obtained from a depository. In another preferred embodiment, the control may comprise a reference standard expression product level from any suitable source, including but not limited to housekeeping genes, an expression product level range from normal tissue (or other previously analyzed control sample), a previously determined expression product level range within a test sample from a group of patients, or a set of patients with a certain outcome (for example, survival for one, two, three, four years, etc.) or receiving a certain treatment. It will be understood by those of skill in the art that such control samples and reference standard expression product levels can be used in combination as controls in the methods of the present invention. In one embodiment, the control may comprise normal or non-metabolic disorder cell/tissue sample. In another preferred embodiment, the control may comprise an expression level for a set of patients, such as a set of metabolic disorder patients, or for a set of metabolic disorder patients receiving a certain treatment, or for a set of patients with one outcome versus another outcome. In the former case, the specific expression product level of each patient can be assigned to a percentile level of expression, or expressed as either higher or lower than the mean or average of the reference standard

expression level. In another preferred embodiment, the control may comprise normal cells, cells from patients treated with a therapeutic and cells from patients having modulated metabolism. In another embodiment, the control may also comprise a measured value for example, average level of expression of a particular gene in a population compared to the level of expression of a housekeeping gene in the same population. Such a population may comprise normal subjects, metabolic disorder patients who have not undergone any treatment (*i.e.*, treatment naive), or metabolic disorder patients undergoing therapy. In another preferred embodiment, the control comprises a ratio transformation of expression product levels, including but not limited to determining a ratio of expression product levels of two genes in the test sample and comparing it to any suitable ratio of the same two genes in a reference standard; determining expression product levels of the two or more genes in the test sample and determining a difference in expression product levels in any suitable control; and determining expression product levels of the two or more genes in the test sample, normalizing their expression to expression of housekeeping genes in the test sample, and comparing to any suitable control. In particularly preferred embodiments, the control comprises a control sample which is of the same lineage and/or type as the test sample. In another embodiment, the control may comprise expression product levels grouped as percentiles within or based on a set of patient samples, such as all patients with a metabolic disorder. In one embodiment a control expression product level is established wherein higher or lower levels of expression product relative to, for instance, a particular percentile, are used as the basis for predicting outcome. In another preferred embodiment, a control expression product level is established using expression product levels from metabolic disorder control patients with a known outcome, and the expression product levels from the test sample are compared to the control expression product level as the basis for predicting outcome. As demonstrated by the data below, the methods of the present invention are not limited to use of a specific cut-point in comparing the level of expression product in the test sample to the control.

The term “pre-determined” biomarker amount and/or activity measurement(s) may be a biomarker amount and/or activity measurement(s) used to, by way of example only, evaluate a subject that may be selected for a particular treatment, evaluate a response to a treatment such as an anti-immune checkpoint inhibitor therapy, and/or evaluate the disease state. A pre-determined biomarker amount and/or activity measurement(s) may be determined in populations of patients with or without a metabolic disorder. The pre-

determined biomarker amount and/or activity measurement(s) can be a single number, equally applicable to every patient, or the pre-determined biomarker amount and/or activity measurement(s) can vary according to specific subpopulations of patients. Age, weight, height, and other factors of a subject may affect the pre-determined biomarker amount and/or activity measurement(s) of the individual. Furthermore, the pre-determined biomarker amount and/or activity can be determined for each subject individually. In one embodiment, the amounts determined and/or compared in a method described herein are based on absolute measurements. In another embodiment, the amounts determined and/or compared in a method described herein are based on relative measurements, such as ratios (e.g., serum biomarker normalized to the expression of a housekeeping or otherwise generally constant biomarker). The pre-determined biomarker amount and/or activity measurement(s) can be any suitable standard. For example, the pre-determined biomarker amount and/or activity measurement(s) can be obtained from the same or a different human for whom a patient selection is being assessed. In one embodiment, the pre-determined biomarker amount and/or activity measurement(s) can be obtained from a previous assessment of the same patient. In such a manner, the progress of the selection of the patient can be monitored over time. In addition, the control can be obtained from an assessment of another human or multiple humans, e.g., selected groups of humans, if the subject is a human. In such a manner, the extent of the selection of the human for whom selection is being assessed can be compared to suitable other humans, e.g., other humans who are in a similar situation to the human of interest, such as those suffering from similar or the same condition(s) and/or of the same ethnic group.

Outcome measures, such as overall survival, increased thermogenesis, and weight loss can be monitored over a period of time for subjects following therapy for whom the measurement values are known. In certain embodiments, the same doses of therapeutic agents are administered to each subject. In related embodiments, the doses administered are standard doses known in the art for therapeutic agents. The period of time for which subjects are monitored can vary. For example, subjects may be monitored for at least 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 55, or 60 months or longer. Biomarker threshold values that correlate to outcome of a therapy can be determined using methods such as those described in the Examples section. Outcomes can also be measured in terms of a “hazard ratio” (the ratio of death rates for one patient group to another; provides likelihood of death at a certain time point), “overall survival” (OS), and/or “progression

free survival.” In certain embodiments, the prognosis comprises likelihood of overall survival rate at 1 year, 2 years, 3 years, 4 years, or any other suitable time point. The significance associated with the prognosis of poor outcome in all aspects of the present invention is measured by techniques known in the art. For example, significance may be measured with calculation of odds ratio. In a further embodiment, the significance is measured by a percentage. In one embodiment, a significant risk of poor outcome is measured as odds ratio of 0.8 or less or at least about 1.2, including by not limited to: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.5, 3.0, 4.0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0 and 40.0. In a further embodiment, a significant increase or reduction in risk is at least about 20%, including but not limited to about 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% and 98%. In a further embodiment, a significant increase in risk is at least about 50%. Thus, the present invention further provides methods for making a treatment decision for a patient in need of modulated metabolism, comprising carrying out the methods for prognosing a patient according to the different aspects and embodiments of the present invention, and then weighing the results in light of other known clinical and pathological risk factors, in determining a course of treatment for the patient in need of modulated metabolism.

A “kit” is any manufacture (*e.g.*, a package or container) comprising at least one reagent, *e.g.*, a probe, for specifically detecting or modulating the expression of a marker of the present invention. The kit may be promoted, distributed, or sold as a unit for performing the methods of the present invention. Kits comprising compositions described herein are encompassed within the present invention.

1. Diagnostic Assays

The present invention provides, in part, methods, systems, and code for accurately classifying whether a biological sample is associated with a metabolic disorder or a clinical subtype thereof. In some embodiments, the present invention is useful for classifying a sample (*e.g.*, from a subject) as a sample that will respond to metabolic intervention using a statistical algorithm and/or empirical data (*e.g.*, the presence or level of one or biomarkers described herein).

An exemplary method for detecting the level of expression or activity of one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or fragments

thereof, and thus useful for classifying whether a sample is associated with a metabolic disorder, involves obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of detecting the biomarker (*e.g.*, polypeptide or nucleic acid that encodes the biomarker or fragments thereof) such that the level of expression or activity of the biomarker is detected in the biological sample. In some embodiments, the presence or level of at least one, two, three, four, five, six, seven, eight, nine, ten, fifty, hundred, or more biomarkers of the present invention are determined in the individual's sample. In certain instances, the statistical algorithm is a single learning statistical classifier system. Exemplary statistical analyses are presented in the Examples and can be used in certain embodiments. In other embodiments, a single learning statistical classifier system can be used to classify a sample as a metabolic disorder sample, a metabolic disorder subtype sample, or a non-metabolic disorder sample based upon a prediction or probability value and the presence or level of one or more biomarkers described herein. The use of a single learning statistical classifier system typically classifies the sample as a metabolic disorder sample with a sensitivity, specificity, positive predictive value, negative predictive value, and/or overall accuracy of at least about 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

Other suitable statistical algorithms are well-known to those of skill in the art. For example, learning statistical classifier systems include a machine learning algorithmic technique capable of adapting to complex data sets (*e.g.*, panel of markers of interest) and making decisions based upon such data sets. In some embodiments, a single learning statistical classifier system such as a classification tree (*e.g.*, random forest) is used. In other embodiments, a combination of 2, 3, 4, 5, 6, 7, 8, 9, 10, or more learning statistical classifier systems are used, preferably in tandem. Examples of learning statistical classifier systems include, but are not limited to, those using inductive learning (*e.g.*, decision/classification trees such as random forests, classification and regression trees (C&RT), boosted trees, etc.), Probably Approximately Correct (PAC) learning, connectionist learning (*e.g.*, neural networks (NN), artificial neural networks (ANN), neuro fuzzy networks (NFN), network structures, perceptrons such as multi-layer perceptrons, multi-layer feed-forward networks, applications of neural networks, Bayesian learning in belief networks, etc.), reinforcement learning (*e.g.*, passive learning in a known environment such as naive learning, adaptive dynamic learning, and temporal difference

learning, passive learning in an unknown environment, active learning in an unknown environment, learning action-value functions, applications of reinforcement learning, etc.), and genetic algorithms and evolutionary programming. Other learning statistical classifier systems include support vector machines (*e.g.*, Kernel methods), multivariate adaptive regression splines (MARS), Levenberg-Marquardt algorithms, Gauss-Newton algorithms, mixtures of Gaussians, gradient descent algorithms, and learning vector quantization (LVQ). In certain embodiments, the method of the present invention further comprises sending the metabolic disorder classification results to a clinician, *e.g.*, an endocrinologist, cardiologist, or hematologist.

10 In another embodiment, the method of the present invention further provides a diagnosis in the form of a probability that the individual has a metabolic disorder or a clinical subtype thereof. For example, the individual can have about a 0%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or greater probability of having a metabolic disorder or a clinical subtype thereof. In yet another embodiment, the method of the present invention further provides a prognosis of a metabolic disorder in the individual. For example, the prognosis can be surgery, development or progression of a metabolic disorder or a clinical subtype thereof, development of one or more symptoms, or recovery from the metabolic disorder. In some instances, the method of classifying a sample as a metabolic disorder sample is further based on the symptoms (*e.g.*, clinical factors) of the individual from which the sample is obtained. The symptoms or group of symptoms can be, for example, those associated with the metabolic disorder. In some embodiments, the diagnosis of an individual as having a metabolic disorder of interest or a clinical subtype thereof is followed by administering to the individual a therapeutically effective amount of a drug useful for treating one or more symptoms associated with the metabolic disorder or a clinical subtype thereof.

25 In some embodiments, an agent for detecting biomarker RNA, genomic DNA, or fragments thereof is a labeled nucleic acid probe capable of hybridizing to biomarker RNA, genomic DNA, or fragments thereof. The nucleic acid probe can be, for example, full-length biomarker nucleic acid, or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions well-known to a skilled artisan to biomarker mRNA or genomic DNA. Other suitable probes for use in the diagnostic assays of the present invention are described

herein. In some embodiments, the nucleic acid probe is designed to detect transcript variants (*i.e.*, different splice forms) of a gene.

In some embodiments, succinate and derivatives thereof can be analyzed using physical separation techniques. Separation and purification in the present invention may include any procedure known in the art, such as capillary electrophoresis (*e.g.*, in capillary or on-chip) or chromatography (*e.g.*, in capillary, column or on a chip). Electrophoresis is a method which can be used to separate ionic molecules under the influence of an electric field. Electrophoresis can be conducted in a gel, capillary, or in a microchannel on a chip. Examples of gels used for electrophoresis include starch, acrylamide, polyethylene oxides, agarose, or combinations thereof. A gel can be modified by its cross-linking, addition of detergents, or denaturants, immobilization of enzymes or antibodies (affinity electrophoresis) or substrates (zymography) and incorporation of a pH gradient. Examples of capillaries used for electrophoresis include capillaries that interface with an electrospray. Capillary electrophoresis (CE) is preferred for separating complex hydrophilic molecules and highly charged solutes. CE technology can also be implemented on microfluidic chips. Depending on the types of capillary and buffers used, CE can be further segmented into separation techniques such as capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), capillary isotachophoresis (cITP) and capillary electrochromatography (CEC). An embodiment to couple CE techniques to electrospray ionization involves the use of volatile solutions, for example, aqueous mixtures containing a volatile acid and/or base and an organic such as an alcohol or acetonitrile. Capillary isotachophoresis (cITP) is a technique in which the analytes move through the capillary at a constant speed but are nevertheless separated by their respective mobilities. Capillary zone electrophoresis (CZE), also known as free-solution CE (FSCE), is based on differences in the electrophoretic mobility of the species, determined by the charge on the molecule, and the frictional resistance the molecule encounters during migration which is often directly proportional to the size of the molecule. Capillary isoelectric focusing (CIEF) allows weakly-ionizable amphoteric molecules, to be separated by electrophoresis in a pH gradient. CEC is a hybrid technique between traditional high performance liquid chromatography (HPLC) and CE. Separation and purification techniques used in the present invention include any chromatography procedures known in the art. Chromatography can be based on the differential adsorption and elution of certain analytes or partitioning of analytes between mobile and stationary phases. Different examples of chromatography include, but not

limited to, liquid chromatography (LC), gas chromatography (GC), high performance liquid chromatography (HPLC), etc.

Biomarker metabolites, such as those shown in Table 1 or Figure 1 can be detected in numerous ways according to well-known techniques. For example, such metabolites, as
5 well as biomarker proteins, can be detected using mass spectrometry methods, such as MALDI/TOF (time-of-flight), SELDI/TOF, liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography-mass spectrometry (HPLC-MS), capillary electrophoresis-mass spectrometry, nuclear magnetic resonance spectrometry, or tandem mass spectrometry (*e.g.*,
10 MS/MS, MS/MS/MS, ESI-MS/MS, etc.). See for example, U.S. Patent Application Nos: 20030199001, 20030134304, 20030077616, which are herein incorporated by reference.

Mass spectrometry methods are well known in the art and have been used to quantify and/or identify biomolecules, such as chemical metabolites and proteins (see, *e.g.*, Li et al. (2000) *Tibtech* 18, 151-160; Rowley *et al.* (2000) *Methods* 20, 383-397; Kuster and
15 Mann (1998) *Curr. Opin. Structural Biol.* 8, 393-400). Further, mass spectrometric techniques have been developed that permit at least partial *de novo* sequencing of isolated proteins (see, *e.g.*, Chait et al. (1993) *Science* 262, 89-92; Keough et al. (1999) *Proc. Natl. Acad. Sci. USA.* 96, 7131-7136; reviewed in Bergman (2000) *EXS* 88, 133-44).

In certain embodiments, a gas phase ion spectrophotometer is used. In other
20 embodiments, laser-desorption/ionization mass spectrometry is used to analyze the sample. Modern laser desorption/ionization mass spectrometry ("LDI-MS") can be practiced in two main variations: matrix assisted laser desorption/ionization ("MALDI") mass spectrometry and surface-enhanced laser desorption/ionization ("SELDI"). In MALDI, the analyte is mixed with a solution containing a matrix, and a drop of the liquid is placed on the surface
25 of a substrate. The matrix solution then co-crystallizes with the biological molecules. The substrate is inserted into the mass spectrometer. Laser energy is directed to the substrate surface where it desorbs and ionizes the biological molecules without significantly fragmenting them. However, MALDI has limitations as an analytical tool. It does not provide means for fractionating the sample, and the matrix material can interfere with
30 detection, especially for low molecular weight analytes (see, *e.g.*, Hellenkamp et al., U.S. Pat. No. 5,118,937 and Beavis and Chait, U.S. Pat. No. 5,045,694).

In SELDI, the substrate surface is modified so that it is an active participant in the desorption process. In one variant, the surface is derivatized with adsorbent and/or capture

reagents that selectively bind the protein of interest. In another variant, the surface is derivatized with energy absorbing molecules that are not desorbed when struck with the laser. In another variant, the surface is derivatized with molecules that bind the protein of interest and that contain a photolytic bond that is broken upon application of the laser. In each of these methods, the derivatizing agent generally is localized to a specific location on the substrate surface where the sample is applied (see, *e.g.*, Hutchens and Yip, U.S. Pat. No. 5,719,060 and Hutchens and Yip, WO 98/59361). The two methods can be combined by, for example, using a SELDI affinity surface to capture an analyte and adding matrix-containing liquid to the captured analyte to provide the energy absorbing material.

For additional information regarding mass spectrometers, see, *e.g.*, Principles of Instrumental Analysis, 3rd edition., Skoog, Saunders College Publishing, Philadelphia, 1985; and Kirk-Othmer Encyclopedia of Chemical Technology, 4.sup.th ed. Vol. 15 (John Wiley & Sons, New York 1995), pp. 1071-1094.

Detection of the presence of a marker or other substances will typically involve detection of signal intensity. For example, in certain embodiments, the signal strength of peak values from spectra of a first sample and a second sample can be compared (*e.g.*, visually or by computer analysis) to determine the relative amounts of particular biomolecules. Software programs such as the Biomarker Wizard program (CIPHERGEN Biosystems, Inc., Fremont, Calif.) can be used to aid in analyzing mass spectra. The mass spectrometers and their techniques are well known to those of skill in the art.

Any person skilled in the art understands, any of the components of a mass spectrometer (*e.g.*, desorption source, mass analyzer, detect, etc.) and varied sample preparations can be combined with other suitable components or preparations described herein, or to those known in the art. For example, in some embodiments a control sample may contain heavy atoms (*e.g.* ^{13}C) thereby permitting the test sample to be mixed with the known control sample in the same mass spectrometry run. In some embodiments, internal controls, such as phenylalanine-d8 and/or valine-d8 can be run with the samples.

In one embodiment, a laser desorption time-of-flight (TOF) mass spectrometer is used. In laser desorption mass spectrometry, a substrate with a bound marker is introduced into an inlet system. The marker is desorbed and ionized into the gas phase by laser from the ionization source. The ions generated are collected by an ion optic assembly, and then in a time-of-flight mass analyzer, ions are accelerated through a short high voltage field and let drift into a high vacuum chamber. At the far end of the high vacuum chamber, the

accelerated ions strike a sensitive detector surface at a different time. Since the time-of-flight is a function of the mass of the ions, the elapsed time between ion formation and ion detector impact can be used to identify the presence or absence of molecules of specific mass to charge ratio.

5 In some embodiments the relative amounts of one or more biomolecules present in a first or second sample is determined, in part, by executing an algorithm with a programmable digital computer. The algorithm identifies at least one peak value in the first mass spectrum and the second mass spectrum. The algorithm then compares the signal strength of the peak value of the first mass spectrum to the signal strength of the peak value
10 of the second mass spectrum of the mass spectrum. The relative signal strengths are an indication of the amount of the biomolecule that is present in the first and second samples. A standard containing a known amount of a biomolecule can be analyzed as the second sample to provide better quantification of the amount of the biomolecule present in the first sample. In certain embodiments, the identity of the biomolecules in the first and second
15 sample can also be determined.

Another preferred agent for detecting succinate biomarkers in complex with biomarker proteins is an antibody capable of binding to the biomarker, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (*e.g.*, Fab or F(ab')₂) can be used.
20 The term “labeled”, with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody
25 and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term “biological sample” is intended to include tissues, cells, and biological fluids isolated from a subject, as well as tissues, cells, and fluids present within a subject. That is, the detection method of the present invention can be used to detect biomarker mRNA, polypeptide, genomic DNA, or fragments thereof, in a biological sample
30 *in vitro* as well as *in vivo*. For example, *in vitro* techniques for detection of biomarker mRNA or a fragment thereof include Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection of biomarker polypeptide include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and

immunofluorescence. *In vitro* techniques for detection of biomarker genomic DNA or a fragment thereof include Southern hybridizations. Furthermore, *in vivo* techniques for detection of one or more biomarkers polypeptide or a fragment thereof include introducing into a subject a labeled anti- biomarker antibody. For example, the antibody can be labeled
 5 with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

In one embodiment, the biological sample contains polypeptide molecules from the test subject. Alternatively, the biological sample can contain RNA molecules from the test subject or genomic DNA molecules from the test subject. A preferred biological sample is
 10 a hematological tissue (*e.g.*, a sample comprising blood, plasma, B cell, bone marrow, *etc.*) sample isolated by conventional means from a subject.

In another embodiment, the methods further involve obtaining a control biological sample from a control subject, contacting the control sample with a compound or agent capable of detecting the desired biomarker, such as succinate or succinate derivative and/or
 15 polypeptide, RNA, cDNA, small RNAs, mature miRNA, pre-miRNA, pri-miRNA, miRNA*, piwiRNA, anti-miRNA, or a miRNA binding site, or a variant thereof, genomic DNA, or fragments thereof of one or more biomarkers listed in Table 1, the Figures, and the Examples, such that the presence of biomarker polypeptide, RNA, genomic DNA, or fragments thereof, is detected in the biological sample, and comparing the presence of
 20 biomarker polypeptide, RNA, cDNA, small RNAs, mature miRNA, pre-miRNA, pri-miRNA, miRNA*, piwiRNA, anti-miRNA, or a miRNA binding site, or a variant thereof, genomic DNA, or fragments thereof in the control sample with the presence of biomarker polypeptide, RNA, cDNA, small RNAs, mature miRNA, pre-miRNA, pri-miRNA, miRNA*, piwiRNA, piwiRNA, anti-miRNA, or a miRNA binding site, or a variant thereof,
 25 genomic DNA, or fragments thereof in the test sample.

The invention also encompasses kits for detecting the presence of a biomarker of interest, such as succinate, succinate derivative, *etc.* such as by using a polypeptide, RNA, cDNA, small RNAs, mature miRNA, pre-miRNA, pri-miRNA, miRNA*, piwiRNA, anti-miRNA, or a miRNA binding site, or a variant thereof, genomic DNA, or fragments
 30 thereof, of one or more biomarkers listed in Table 1, the Figures, and the Examples, in a biological sample. For example, the kit can comprise a labeled compound or agent capable of detecting one or more biomarkers polypeptide, RNA, cDNA, small RNAs, mature miRNA, pre-miRNA, pri-miRNA, miRNA*, piwiRNA, anti-miRNA, or a miRNA binding

site, or a variant thereof, genomic DNA, or fragments thereof, in a biological sample; means for determining the amount of the biomarker polypeptide, RNA, cDNA, small RNAs, mature miRNA, pre-miRNA, pri-miRNA, miRNA*, piwiRNA, anti-miRNA, or a miRNA binding site, or a variant thereof, genomic DNA, or fragments thereof, in the sample; and means for comparing the amount of the biomarker polypeptide, RNA, cDNA, small RNAs, mature miRNA, pre-miRNA, pri-miRNA, miRNA*, piwiRNA, anti-miRNA, or a miRNA binding site, or a variant thereof, genomic DNA, or fragments thereof, in the sample with a standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect the biomarker polypeptide, RNA, cDNA, small RNAs, mature miRNA, pre-miRNA, pri-miRNA, miRNA*, piwiRNA, anti-miRNA, or a miRNA binding site, or a variant thereof, genomic DNA, or fragments thereof.

In some embodiments, therapies tailored to treat stratified patient populations based on the described diagnostic assays are further administered, such as metabolic disorder standards of treatment, immune therapy, and combinations thereof described herein.

2. Prognostic Assays

The diagnostic methods described herein can furthermore be utilized to identify subjects having or at risk of developing a disease or disorder associated with aberrant expression or activity of one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof. As used herein, the term “aberrant” includes biomarker expression or activity levels which deviates from the normal expression or activity in a control.

The assays described herein, such as the preceding diagnostic assays or the following assays, can be used to identify a subject that would benefit from metabolic interventions (*e.g.*, low levels of plasma succinate indicate that succinate administration would be differentially beneficial). Alternatively, the prognostic assays can be used to identify a subject having or at risk for developing a disorder associated with a misregulation of biomarker activity or expression. Thus, the present invention provides a method for identifying and/or classifying a disease associated with aberrant expression or activity of one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples.

Furthermore, the prognostic assays described herein can be used to determine whether a subject can be administered an agent (*e.g.*, an agonist, antagonist, peptidomimetic, polypeptide, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with aberrant biomarker expression or activity. For example, such methods can be used to determine whether a subject can be effectively treated with an agent for a metabolic disorder. Thus, the present invention provides methods for determining whether a subject can be effectively treated with an agent for a disease associated with aberrant biomarker expression or activity in which a test sample is obtained and biomarker polypeptide or nucleic acid expression or activity is detected (*e.g.*, wherein a significant increase or decrease in biomarker polypeptide or nucleic acid expression or activity relative to a control is diagnostic for a subject that can be administered the agent to treat a disorder associated with aberrant biomarker expression or activity). In some embodiments, significant increase or decrease in biomarker expression or activity comprises at least 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 times or more higher or lower, respectively, than the expression activity or level of the marker in a control sample.

The methods of the present invention can also be used to detect genetic alterations in one or more biomarkers of the present invention, including one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof, thereby determining if a subject with the altered biomarker is at risk for a metabolic disorder characterized by aberrant biomarker activity or expression levels. In preferred embodiments, the methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic alteration characterized by at least one alteration affecting the integrity of a gene encoding one or more biomarkers, or the mis-expression of the biomarker (*e.g.*, mutations and/or splice variants). For example, such genetic alterations can be detected by ascertaining the existence of at least one of 1) a deletion of one or more nucleotides from one or more biomarkers gene, 2) an addition of one or more nucleotides to one or more biomarkers gene, 3) a substitution of one or more nucleotides of one or more biomarkers gene, 4) a chromosomal rearrangement of one or more biomarkers gene, 5) an alteration in the level of a messenger RNA transcript of one or more biomarkers gene, 6) aberrant modification of one or more biomarkers gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of an RNA transcript of one or more

biomarkers gene, 8) a non-wild type level of one or more biomarkers polypeptide, 9) allelic loss of one or more biomarkers gene, and 10) inappropriate post-translational modification of one or more biomarkers polypeptide. As described herein, there are a large number of assays known in the art which can be used for detecting alterations in one or more biomarkers gene. A preferred biological sample is a tissue or serum sample isolated by conventional means from a subject.

In certain embodiments, detection of the alteration involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, *e.g.*, U.S. Patents 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, *e.g.*, Landegran *et al.* (1988) *Science* 241:1077-1080; and Nakazawa *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:360-364), the latter of which can be particularly useful for detecting point mutations in one or more biomarkers gene (see Abravaya *et al.* (1995) *Nucleic Acids Res.* 23:675-682). This method can include the steps of collecting a sample of cells from a subject, isolating nucleic acid (*e.g.*, genomic DNA, mRNA, cDNA, small RNA, mature miRNA, pre-miRNA, pri-miRNA, miRNA*, piwiRNA, anti-miRNA, or a miRNA binding site, or a variant thereof) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to one or more biomarkers gene of the present invention, including the biomarker genes listed in Table 1, the Figures, and the Examples, or fragments thereof, under conditions such that hybridization and amplification of the biomarker gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Alternative amplification methods include: self-sustained sequence replication (Guatelli, J. C. *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:1874-1878), transcriptional amplification system (Kwoh, D. Y. *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:1173-1177), Q-Beta Replicase (Lizardi, P. M. *et al.* (1988) *Bio-Technology* 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well-known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In an alternative embodiment, mutations in one or more biomarkers gene of the present invention, including one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof, from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Patent 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In other embodiments, genetic mutations in one or more biomarkers gene of the present invention, including a gene listed in Table 1, the Figures, and the Examples, or a fragment thereof, can be identified by hybridizing a sample and control nucleic acids, *e.g.*, DNA, RNA, mRNA, small RNA, cDNA, mature miRNA, pre-miRNA, pri-miRNA, miRNA*, piwiRNA, anti-miRNA, or a miRNA binding site, or a variant thereof, to high density arrays containing hundreds or thousands of oligonucleotide probes (Cronin, M. T. *et al.* (1996) Hum. Mutat. 7:244-255; Kozal, M. J. *et al.* (1996) Nat. Med. 2:753-759). For example, genetic mutations in one or more biomarkers can be identified in two dimensional arrays containing light-generated DNA probes as described in Cronin *et al.* (1996) *supra*. Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential, overlapping probes. This step allows the identification of point mutations. This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence one or more biomarkers gene of the present invention, including a gene listed in Table 1, the Figures, and the Examples, or a fragment thereof, and detect mutations by comparing the sequence of the sample biomarker gene with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxam and Gilbert (1977) Proc. Natl. Acad. Sci.

USA 74:560 or Sanger (1977) Proc. Natl. Acad. Sci. USA 74:5463. It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays (Naeve, C. W. (1995) Biotechniques 19:448-53), including sequencing by mass spectrometry (see, *e.g.*, PCT International Publication No. WO 94/16101; Cohen *et al.* (1996) Adv. Chromatogr. 36:127-162; and Griffin *et al.* (1993) Appl. Biochem. Biotechnol. 38:147-159).

Other methods for detecting mutations in one or more biomarkers gene of the present invention, including a gene listed in Table 1, the Figures, and the Examples, or fragments thereof, include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al.* (1985) Science 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes formed by hybridizing (labeled) RNA or DNA containing the wild-type sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to base pair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with SI nuclease to enzymatically digest the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation. See, for example, Cotton *et al.* (1988) Proc. Natl. Acad. Sci. USA 85:4397 and Saleeba *et al.* (1992) Methods Enzymol. 217:286-295. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in biomarker genes of the present invention, including genes listed in Table 1, the Figures, and the Examples, or fragments thereof, obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu *et al.* (1994) Carcinogenesis 15:1657-1662). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage

products, if any, can be detected from electrophoresis protocols or the like. See, for example, U.S. Patent 5,459,039.

In other embodiments, alterations in electrophoretic mobility will be used to identify mutations in biomarker genes of the present invention, including genes listed in Table 1, the
5 Figures, and the Examples, or fragments thereof. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita *et al.* (1989) Proc Natl. Acad. Sci USA 86:2766; see also Cotton (1993) Mutat. Res. 285:125-144 and Hayashi (1992) Genet. Anal. Tech. Appl. 9:73-79). Single-stranded DNA fragments of sample and control nucleic acids
10 will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a
15 change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen *et al.* (1991) Trends Genet. 7:5).

In yet another embodiment the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing
20 gradient gel electrophoresis (DGGE) (Myers *et al.* (1985) Nature 313:495). When DGGE is used as the method of analysis, DNA will be modified to ensure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample
25 DNA (Rosenbaum and Reissner (1987) Biophys. Chem. 265:12753).

Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions
30 which permit hybridization only if a perfect match is found (Saiki *et al.* (1986) Nature 324:163; Saiki *et al.* (1989) Proc. Natl. Acad. Sci. USA 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and

hybridized with labeled target DNA. In some embodiments, the hybridization reactions can occur using biochips, microarrays, etc., or other array technology that are well-known in the art.

Alternatively, allele specific amplification technology which depends on selective
5 PCR amplification may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs *et al.* (1989) *Nucleic Acids Res.* 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension
10 (Prossner (1993) *Tibtech* 11:238). In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini *et al.* (1992) *Mol. Cell Probes* 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany (1991) *Proc. Natl. Acad. Sci USA* 88:189). In such cases, ligation will occur only if there is a perfect match at the 3'
15 end of the 5' sequence making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid or antibody reagent described herein, which may be conveniently used, *e.g.*, in clinical settings to diagnose
20 patients exhibiting symptoms or family history of a disease or illness involving one or more biomarkers of the present invention, including one or more biomarkers listed in Table 1, the Figures, and the Examples, or fragments thereof.

3. Monitoring of Effects During Clinical Trials

25 Monitoring the influence of agents (*e.g.*, drugs) on the expression or activity of one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof (*e.g.*, the modulation of a metabolic state) can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent
30 determined by a screening assay as described herein to increase expression and/or activity of one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof, can be monitored in clinical trials of subjects exhibiting decreased

expression and/or activity of one or more biomarkers of the present invention, including one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof, relative to a control reference. Alternatively, the effectiveness of an agent
5 determined by a screening assay to decrease expression and/or activity of one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof, can be monitored in clinical trials of subjects exhibiting decreased expression and/or activity of the biomarker of the present invention, such as succinate, a succinate derivative,
10 and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof relative to a control reference. In such clinical trials, the expression and/or activity of the biomarker can be used as a “read out” or marker of the phenotype of a particular cell.

In some embodiments, the present invention provides a method for monitoring the
15 effectiveness of treatment of a subject with an agent (*e.g.*, succinate or succinate derivative, an agonist, antagonist, peptidomimetic, polypeptide, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) including the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression and/or activity of one or more biomarkers of
20 the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or fragments thereof in the pre-administration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the biomarker in the post-administration samples (such as circulating level, intracellular level, cellular uptake, metabolism, etc.); (v) comparing the level of expression or activity of the biomarker or
25 fragments thereof in the pre-administration sample with the that of the biomarker in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression or activity of one or more biomarkers to higher levels than detected (*e.g.*, to increase the effectiveness of the agent.) Alternatively, decreased
30 administration of the agent may be desirable to decrease expression or activity of the biomarker to lower levels than detected (*e.g.*, to decrease the effectiveness of the agent). According to such an embodiment, biomarker expression or activity may be used as an

indicator of the effectiveness of an agent, even in the absence of an observable phenotypic response.

C. Methods of Treatment

- 5 The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder characterized by insufficient or excessive production of biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or fragments thereof, which have aberrant expression or activity compared to a control.
- 10 Moreover, agents of the present invention described herein can be used to detect and isolate the biomarkers or fragments thereof, regulate the bioavailability of the biomarkers or fragments thereof, and modulate biomarker expression levels or activity.

1. Prophylactic Methods

- In one aspect, the present invention provides a method for preventing in a subject, a
- 15 disease or condition associated with an aberrant expression or activity of one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof, by administering to the subject an agent which modulates biomarker expression or at least one activity of the biomarker. Subjects at risk for a disease or disorder which is caused or
- 20 contributed to by aberrant biomarker expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the biomarker expression or activity aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression.

25 2. Therapeutic Methods

- Another aspect of the present invention pertains to methods of modulating the expression or activity of, or interaction with natural (or synthetic) binding partner(s) of, one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or
- 30 fragments thereof, for therapeutic purposes. The biomarkers of the present invention have been demonstrated to correlate with adipose tissue thermogenesis and modulation of metabolism. Accordingly, the activity and/or expression of the biomarker, as well as the interaction between one or more biomarkers or a fragment thereof and its natural (or

synthetic) binding partner(s) or a fragment(s) thereof can be modulated in order to modulate the immune response.

Modulatory methods of the present invention involve contacting a cell with one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof or agent that modulates one or more of the activities of biomarker activity associated with the cell or produced by the cell. An agent that modulates biomarker activity can be an agent as described herein, such as a nucleic acid or a polypeptide, a naturally-occurring binding partner of the biomarker, an antibody against the biomarker, a combination of antibodies against the biomarker and antibodies against other immune related targets, one or more biomarkers agonist or antagonist, a peptidomimetic of one or more biomarkers agonist or antagonist, one or more biomarkers peptidomimetic, other small molecule, or small RNA directed against or a mimic of one or more biomarkers nucleic acid gene expression product.

An agent that modulates the expression of one or more biomarkers of the present invention, including one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof includes small molecules (*e.g.*, succinate, succinate derivatives, succinate modulators), as well as a nucleic acid molecule described herein, *e.g.*, an antisense nucleic acid molecule, RNAi molecule, shRNA, mature miRNA, pre-miRNA, pri-miRNA, miRNA*, piwiRNA, anti-miRNA, or a miRNA binding site, or a variant thereof, or other small RNA molecule, triplex oligonucleotide, ribozyme, or recombinant vector for expression of one or more biomarkers polypeptide. For example, an oligonucleotide complementary to the area around one or more biomarkers polypeptide translation initiation site can be synthesized. One or more antisense oligonucleotides can be added to cell media, typically at 200 µg/ml, or administered to a patient to prevent the synthesis of one or more biomarkers polypeptide. The antisense oligonucleotide is taken up by cells and hybridizes to one or more biomarkers mRNA to prevent translation. Alternatively, an oligonucleotide which binds double-stranded DNA to form a triplex construct to prevent DNA unwinding and transcription can be used. As a result of either, synthesis of biomarker polypeptide is blocked. When biomarker expression is modulated, preferably, such modulation occurs by a means other than by knocking out the biomarker gene.

Agents which modulate expression, by virtue of the fact that they control the amount of biomarker in a cell, also modulate the total amount of biomarker activity in a cell.

In one embodiment, the agent stimulates one or more activities of one or more biomarkers of the present invention, including one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof. Examples of such stimulatory agents include active biomarker polypeptides, or a fragment thereof, such as succinate binding partners, and/or a nucleic acid molecule encoding the biomarker or a fragment thereof that has been introduced into the cell (*e.g.*, cDNA, mRNA, shRNAs, siRNAs, small RNAs, mature miRNA, pre-miRNA, pri-miRNA, miRNA*, piwiRNA, anti-miRNA, or a miRNA binding site, or a variant thereof, or other functionally equivalent molecule known to a skilled artisan). In another embodiment, the agent inhibits one or more biomarker activities. In one embodiment, the agent inhibits or enhances the interaction of the biomarker with its natural (or synthetic) binding partner(s). Examples of such inhibitory agents include antisense nucleic acid molecules, anti-biomarker antibodies, biomarker inhibitors, and compounds identified in the screening assays described herein.

These modulatory methods can be performed *in vitro* (*e.g.*, by contacting the cell with the agent) or, alternatively, by contacting an agent with cells *in vivo* (*e.g.*, by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a condition or disorder that would benefit from up- or down-modulation of one or more biomarkers of the present invention, such as succinate, a succinate derivative, and/or one or more biomarkers listed in Table 1, the Figures, and the Examples, or a fragment thereof, *e.g.*, a disorder characterized by unwanted, insufficient, or aberrant expression or activity of the biomarker or fragments thereof. In one embodiment, the method involves administering an agent (*e.g.*, an agent identified by a screening assay described herein), or combination of agents that modulates (*e.g.*, upregulates or downregulates) biomarker expression or activity. In another embodiment, the method involves administering one or more biomarkers polypeptide or nucleic acid molecule as therapy to compensate for reduced, aberrant, or unwanted biomarker expression or activity.

Stimulation of biomarker activity is desirable in situations in which the biomarker is abnormally downregulated and/or in which increased biomarker activity is likely to have a beneficial effect. Likewise, inhibition of biomarker activity is desirable in situations in

which biomarker is abnormally upregulated and/or in which decreased biomarker activity is likely to have a beneficial effect.

In addition, these modulatory agents can also be administered in combination therapy with, *e.g.*, metabolism enhancing agents, such as transplanted brown and/or beige fat cells, hormones, and the like. The preceding treatment methods can be administered in conjunction with other forms of conventional therapy (*e.g.*, standard-of-care treatments for metabolic disorders are well-known to the skilled artisan), either consecutively with, pre- or post-conventional therapy. For example, these modulatory agents can be administered with a therapeutically effective dose of a metabolism modulatory agent.

The methods of the present invention relate to succinate sufficient to modulate (*e.g.*, induce or repress) brown and/or beige fat cell differentiation and/or activity, wherein increases in differentiated brown and/or beige fat cells or activity increase energy expenditure and favorably affect other metabolic processes and can therefore be used to treat metabolic disorders such as obesity, diabetes, decreased thermogenesis and subjects in need of more exercise; and, wherein decreases in differentiated brown and/or beige fat cells or activity decrease energy expenditure and can therefore be used to treat the effects of such conditions as cachexia, anorexia, and obesity-associated cancer.

The invention also relates to methods for increasing energy expenditure in a mammal comprising inducing expression and/or activity of succinate sufficient to activate brown and/or beige fat cell differentiation or activity in the mammal, wherein the differentiated and/or more active brown fat and/or beige fat cells promote energy expenditure thereby increasing energy expenditure in the mammal.

The term “sufficient to activate” is intended to encompass any increase in amount and/or activity of succinate that promotes, activates, stimulates, enhances, or results in brown fat and/or beige fat differentiation or activity.

In another aspect, the invention relates to methods for treating metabolic disorders in a subject comprising administering to the subject an agent that induces promotion and/or activity of succinate, wherein promotion and/or activity of succinate increases respiration and energy expenditure to thereby treat the metabolic disorder. In one embodiment, total respiration is increased following increase and/or upregulation of succinate. In another embodiment, uncoupled respiration is increased following increase and/or upregulation of succinate. Uncoupled respiration dissipates heat and thereby increases energy expenditure in the subject.

As used herein, the term “agent” and “therapeutic agent” is defined broadly as anything that cells from a subject having a metabolic disorder may be exposed to in a therapeutic protocol. In one embodiment, the agent is succinate. In another embodiment, the agent is a succinic acid salt.

5 The term “administering” is intended to include routes of administration which allow the agent to perform its intended function of modulating (*e.g.*, increasing or decreasing) succinate. Examples of routes of administration which can be used include injection (subcutaneous, intravenous, parenterally, intraperitoneally, intrathecal, *etc.*, such as in a subcutaneous injection into white, brown, and/or beige fat depots), oral, inhalation, 10 and transdermal. The injection can be bolus injections or can be continuous infusion. Depending on the route of administration, the agent can be coated with or disposed in a selected material to protect it from natural conditions which may detrimentally affect its ability to perform its intended function. The agent may be administered alone, or in conjunction with a pharmaceutically acceptable carrier. Further the agent may be 15 coadministered with a pharmaceutically acceptable carrier. The agent also may be administered as a prodrug, which is converted to its active form *in vivo*. The agent may also be administered in combination with one or more additional therapeutic agent(s) (*e.g.*, before, after or simultaneously therewith).

 The term “effective amount” of an agent that induces modulation of succinate is that 20 amount necessary or sufficient to modulate (*e.g.*, increase or decrease) succinate in the subject or population of subjects. The effective amount can vary depending on such factors as the type of therapeutic agent(s) employed, the size of the subject, or the severity of the disorder.

 It will be appreciated that individual dosages may be varied depending upon the 25 requirements of the subject in the judgment of the attending clinician, the severity of the condition being treated and the particular compound being employed. In determining the therapeutically effective amount or dose, a number of additional factors may be considered by the attending clinician, including, but not limited to: the pharmacodynamic characteristics of the particular agent and its mode and route of administration; the desired 30 time course of treatment; the species of mammal; its size, age, and general health; the specific disease involved; the degree of or involvement or the severity of the disease; the response of the individual subject; the particular compound administered; the mode of

administration; the bioavailability characteristics of the preparation administered; the dose regimen selected; the kind of concurrent treatment; and other relevant circumstances.

Treatment can be initiated with smaller dosages which are less than the effective dose of the compound. Thereafter, in one embodiment, the dosage should be increased by small increments until the optimum effect under the circumstances is reached. For convenience, the total daily dosage may be divided and administered in portions during the day if desired.

The effectiveness of any particular agent to treat a metabolic disorder can be monitored by comparing two or more samples obtained from a subject undergoing anti-metabolic disorder or metabolic disorder-related disorder treatment. In general, it is preferable to obtain a first sample from the subject prior to beginning therapy and one or more samples during treatment. In such a use, a baseline of expression of cells from subjects with obesity or obesity-related disorders prior to therapy is determined and then changes in the baseline state of expression of cells from subjects with obesity or obesity-related disorders is monitored during the course of therapy. Alternatively, two or more successive samples obtained during treatment can be used without the need of a pre-treatment baseline sample. In such a use, the first sample obtained from the subject is used as a baseline for determining whether the expression of cells from subjects with obesity or obesity-related disorders is increasing or decreasing.

Another aspect of the invention relates to a method for inducing brown fat and/or beige fat cell differentiation and/or activity in a mammal comprising modulating succinate in a mammal and, optionally, monitoring the differentiation of brown fat cells in the mammal. Increased brown and/or beige adipose tissue in the mammal will warm up the body and blood of the mammal resulting in an increased energy expenditure from the cells. The increased energy expenditure will increase the metabolic rate of the subject and may be used for the treatment and/or prevention of obesity and obesity related disorders. The induction of brown fat cells may be monitored by analyzing a) brown fat and/or beige fat gene expression, such as expression of a marker selected from the group consisting of: cidea, adiponectin, adipsin, otopetrin, type II deiodinase, cig30, ppar gamma 2, pgc1 α , ucp1, elovl3, cAMP, Prdm16, cytochrome C, cox4i1, coxIII, cox5b, cox7a1, cox8b, glut4, atpase b2, cox II, atp5o, ndufb5, ap2, ndufs1, GRP109A, acylCoA-thioesterase 4, EARA1, claudin1, PEPCK, fgf21, acylCoA-thioesterase 3, dio2, fatty acid synthase (fas), leptin, resistin, and nuclear respiratory factor-1 (nrf1); b) thermogenesis in adipose cells; c)

differentiation of adipose cells; d) insulin sensitivity of adipose cells; e) basal respiration, leak respiration, or uncoupled respiration; f) whole body oxygen consumption; g) obesity or appetite; h) insulin secretion of pancreatic beta cells; i) glucose tolerance; j) modified phosphorylation of EGFR, ERK, AMPK, protein kinase A (PKA) substrates having an
5 RRX(S/T) motif, wherein the X is any amino acid and the (S/T) residue is a serine or threonine, HSL; k) modified activity of UCP1 protein; and l) growth and effects of metabolic disorders, such as obesity-associated cancer, cachexia, anorexia, diabetes, and obesity.

Any means for the introduction of a therapeutic agent into mammals, human or non-
10 human, or cells thereof may be adapted to the practice of this invention for the delivery of the various constructs of the invention into the intended recipient. In one embodiment of the invention, small molecules and/or nucleic acids are delivered to cells by transfection, *e.g.*, by delivery of “naked” small molecules and/or DNA using uptake machinery by cells or in a complex that does not require cellular machinery for uptake. A colloidal system
15 includes macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a lipid-complexed or liposome-formulated DNA. In the former approach, prior to formulation of DNA, *e.g.*, with lipid, a plasmid containing a transgene bearing the desired DNA constructs may first be experimentally
20 optimized for expression (*e.g.*, inclusion of an intron in the 5' untranslated region and elimination of unnecessary sequences (Felgner, et al., Ann NY Acad Sci 126-139, 1995). Formulation of DNA, *e.g.* with various lipid or liposome materials, may then be effected using known methods and materials and delivered to the recipient mammal. See, *e.g.*, Canonico et al, Am J Respir Cell Mol Biol 10:24-29, 1994; Tsan et al, Am J Physiol 268;
25 Alton et al., Nat Genet. 5:135-142, 1993 and U.S. patent No. 5,679,647 by Carson et al.

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of
30 liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs, which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in

order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand. Naked DNA or DNA associated with a delivery vehicle, *e.g.*, liposomes, can be administered to several sites in a subject (see below).

Nucleic acids can be delivered in any desired vector. These include viral or non-viral vectors, including adenovirus vectors, adeno-associated virus vectors, retrovirus vectors, lentivirus vectors, and plasmid vectors. Exemplary types of viruses include HSV (herpes simplex virus), AAV (adeno associated virus), HIV (human immunodeficiency virus), BIV (bovine immunodeficiency virus), and MLV (murine leukemia virus). Nucleic acids can be administered in any desired format that provides sufficiently efficient delivery levels, including in virus particles, in liposomes, in nanoparticles, and complexed to polymers.

The nucleic acids encoding a protein or nucleic acid of interest may be in a plasmid or viral vector, or other vector as is known in the art. Such vectors are well-known and any can be selected for a particular application. In one embodiment of the invention, the gene delivery vehicle comprises a promoter and a demethylase coding sequence. Preferred promoters are tissue-specific promoters and promoters which are activated by cellular proliferation, such as the thymidine kinase and thymidylate synthase promoters. Other preferred promoters include promoters which are activatable by infection with a virus, such as the α - and β -interferon promoters, and promoters which are activatable by a hormone, such as estrogen. Other promoters which can be used include the Moloney virus LTR, the CMV promoter, and the mouse albumin promoter. A promoter may be constitutive or inducible.

In another embodiment, naked polynucleotide molecules are used as gene delivery vehicles, as described in WO 90/11092 and U.S. Patent 5,580,859. Such gene delivery vehicles can be either growth factor DNA or RNA and, in certain embodiments, are linked to killed adenovirus. Curiel et al., *Hum. Gene. Ther.* 3:147-154, 1992. Other vehicles which can optionally be used include DNA-ligand (Wu et al., *J. Biol. Chem.* 264:16985-16987, 1989), lipid-DNA combinations (Felgner et al., *Proc. Natl. Acad. Sci.*

USA 84:7413 7417, 1989), liposomes (Wang et al., Proc. Natl. Acad. Sci. 84:7851-7855, 1987) and microprojectiles (Williams et al., Proc. Natl. Acad. Sci. 88:2726-2730, 1991).

A gene delivery vehicle can optionally comprise viral sequences such as a viral origin of replication or packaging signal. These viral sequences can be selected from
 5 viruses such as astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, poxvirus, retrovirus, togavirus or adenovirus. In a preferred embodiment, the growth factor gene delivery vehicle is a recombinant retroviral vector. Recombinant retroviruses and various uses thereof have been described in numerous references including, for example, Mann et al., Cell 33:153, 1983, Cane and Mulligan,
 10 Proc. Nat'l. Acad. Sci. USA 81:6349, 1984, Miller et al., Human Gene Therapy 1:5-14, 1990, U.S. Patent Nos. 4,405,712, 4,861,719, and 4,980,289, and PCT Application Nos. WO 89/02,468, WO 89/05,349, and WO 90/02,806. Numerous retroviral gene delivery vehicles can be utilized in the present invention, including for example those described in EP 0,415,731; WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent
 15 No. 5,219,740; WO 9311230; WO 9310218; Vile and Hart, Cancer Res. 53:3860-3864, 1993; Vile and Hart, Cancer Res. 53:962-967, 1993; Ram et al., Cancer Res. 53:83-88, 1993; Takamiya et al., J. Neurosci. Res. 33:493-503, 1992; Baba et al., J. Neurosurg. 79:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805).

20 Other viral vector systems that can be used to deliver a polynucleotide of the invention have been derived from herpes virus, *e.g.*, Herpes Simplex Virus (U.S. Patent No. 5,631,236 by Woo et al., issued May 20, 1997 and WO 00/08191 by Neurovex), vaccinia virus (Ridgeway (1988) Ridgeway, "Mammalian expression vectors," In: Rodriguez R L, Denhardt D T, ed. Vectors: A survey of molecular cloning vectors and their uses.
 25 Stoneham: Butterworth,; Baichwal and Sugden (1986) "Vectors for gene transfer derived from animal DNA viruses: Transient and stable expression of transferred genes," In: Kucherlapati R, ed. Gene transfer. New York: Plenum Press; Coupar et al. (1988) Gene, 68:1-10), and several RNA viruses. Preferred viruses include an alphavirus, a poxvirus, an arena virus, a vaccinia virus, a polio virus, and the like. They offer several attractive
 30 features for various mammalian cells (Friedmann (1989) Science, 244:1275-1281; Ridgeway, 1988, *supra*; Baichwal and Sugden, 1986, *supra*; Coupar et al., 1988; Horwich et al. (1990) J. Virol., 64:642-650).

In other embodiments, target DNA in the genome can be manipulated using well-known methods in the art. For example, the target DNA in the genome can be manipulated by deletion, insertion, and/or mutation are retroviral insertion, artificial chromosome techniques, gene insertion, random insertion with tissue specific promoters, gene targeting, transposable elements and/or any other method for introducing foreign DNA or producing modified DNA/modified nuclear DNA. Other modification techniques include deleting DNA sequences from a genome and/or altering nuclear DNA sequences. Nuclear DNA sequences, for example, may be altered by site-directed mutagenesis.

10 IV. Pharmaceutical Compositions

In another aspect, the present invention provides pharmaceutically acceptable compositions which comprise a therapeutically-effective amount of an agent that modulates (*e.g.*, increases or decreases) succinate, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. As described in detail below, the pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; (4) intravaginally or intrarectally, for example, as a pessary, cream or foam; or (5) aerosol, for example, as an aqueous aerosol, liposomal preparation or solid particles containing the compound.

The phrase “therapeutically-effective amount” as used herein means that amount of an agent that modulates (*e.g.*, enhances) succinate, or expression and/or activity of a succinate enzyme complex, or composition comprising an agent that modulates (*e.g.*, enhances) succinate, or expression and/or activity of the complex, which is effective for producing some desired therapeutic effect, *e.g.*, weight loss, at a reasonable benefit/risk ratio.

The phrase “pharmaceutically acceptable” is employed herein to refer to those agents, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals

without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase “pharmaceutically-acceptable carrier” as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid
5 filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject chemical from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be “acceptable” in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1)
10 sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols,
15 such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in
20 pharmaceutical formulations.

The term “pharmaceutically-acceptable salts” refers to the relatively non-toxic, inorganic and organic acid addition salts of the agents that modulates (*e.g.*, enhances) succinate, or expression and/or activity of the complex encompassed by the invention. These salts can be prepared in situ during the final isolation and purification of the agents,
25 or by separately reacting a purified agent in its free base form with a suitable organic or inorganic acid, and isolating the salt thus formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, phosphate, nitrate, acetate, valerate, oleate, palmitate, stearate, laurate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphthylate, mesylate, glucoheptonate, lactobionate, and laurylsulphonate
30 salts and the like (See, for example, Berge *et al.* (1977) “Pharmaceutical Salts”, *J. Pharm. Sci.* 66:1-19).

In other cases, the agents useful in the methods of the present invention may contain one or more acidic functional groups and, thus, are capable of forming pharmaceutically-

acceptable salts with pharmaceutically-acceptable bases. The term “pharmaceutically-acceptable salts” in these instances refers to the relatively non-toxic, inorganic and organic base addition salts of agents that modulates (*e.g.*, enhances) succinate, or expression and/or activity of the complex. These salts can likewise be prepared in situ during the final
5 isolation and purification of the agents, or by separately reacting the purified agent in its free acid form with a suitable base, such as the hydroxide, carbonate or bicarbonate of a pharmaceutically-acceptable metal cation, with ammonia, or with a pharmaceutically-acceptable organic primary, secondary or tertiary amine. Representative alkali or alkaline earth salts include the lithium, sodium, potassium, calcium, magnesium, and aluminum salts
10 and the like. Representative organic amines useful for the formation of base addition salts include ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine and the like (see, for example, Berge *et al.*, *supra*).

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening,
15 flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl
20 palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Formulations useful in the methods of the present invention include those suitable
25 for oral, nasal, topical (including buccal and sublingual), rectal, vaginal, aerosol and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well-known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of
30 administration. The amount of active ingredient, which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 1 per cent to about ninety-nine percent of active ingredient,

preferably from about 5 per cent to about 70 per cent, most preferably from about 10 per cent to about 30 per cent.

Methods of preparing these formulations or compositions include the step of bringing into association an agent that modulates (*e.g.*, increases or decreases) succinate
5 with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a agent with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations suitable for oral administration may be in the form of capsules,
10 cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a agent as
15 an active ingredient. A compound may also be administered as a bolus, electuary or paste.

In solid dosage forms for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically-acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose,
20 mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7)
25 wetting agents, such as, for example, acetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a
30 similar type may also be employed as fillers in soft and hard-filled gelatin capsules using

such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, 5 gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered peptide or peptidomimetic moistened with an inert liquid diluent.

10 Tablets, and other solid dosage forms, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well-known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired 15 release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions, which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they 20 release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions, which can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

25 Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl 30 benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut,

corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring,
5 perfuming and preservative agents.

Suspensions, in addition to the active agent may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

10 Formulations for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more agents with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the
15 active agent.

Formulations which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

Dosage forms for the topical or transdermal administration of an agent that
20 modulates (*e.g.*, increases or decreases) succinate include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active component may be mixed under sterile conditions with a pharmaceutically-acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The ointments, pastes, creams and gels may contain, in addition to a agent,
25 excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to an agent that modulates (*e.g.*, increases or decreases) succinate, excipients such as lactose, talc, silicic acid, aluminum
30 hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays

can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

The agent that modulates (*e.g.*, increases or decreases) succinate, can be alternatively administered by aerosol. This is accomplished by preparing an aqueous aerosol, liposomal preparation or solid particles containing the compound. A nonaqueous (*e.g.*, fluorocarbon propellant) suspension could be used. Sonic nebulizers are preferred because they minimize exposing the agent to shear, which can result in degradation of the compound.

Ordinarily, an aqueous aerosol is made by formulating an aqueous solution or suspension of the agent together with conventional pharmaceutically acceptable carriers and stabilizers. The carriers and stabilizers vary with the requirements of the particular compound, but typically include nonionic surfactants (Tweens, Pluronics, or polyethylene glycol), innocuous proteins like serum albumin, sorbitan esters, oleic acid, lecithin, amino acids such as glycine, buffers, salts, sugars or sugar alcohols. Aerosols generally are prepared from isotonic solutions.

Transdermal patches have the added advantage of providing controlled delivery of a agent to the body. Such dosage forms can be made by dissolving or dispersing the agent in the proper medium. Absorption enhancers can also be used to increase the flux of the peptidomimetic across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the peptidomimetic in a polymer matrix or gel.

Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this invention.

Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more agents in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper

fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting
5 agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may
10 be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having
15 poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution, which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of an agent
20 that modulates (*e.g.*, increases or decreases) succinate, in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions,
25 which are compatible with body tissue.

When the agents of the present invention are administered as pharmaceuticals, to humans and animals, they can be given *per se* or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

30 Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be determined by the methods of the present invention so as to obtain an amount of the active ingredient, which is effective to achieve the desired therapeutic

response for a particular subject, composition, and mode of administration, without being toxic to the subject.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Pat. No. 5,328,470) or by stereotactic injection (see *e.g.*, Chen et al. (1994) Proc. Natl. Acad. Sci. USA 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

Exemplification

This invention is further illustrated by the following examples, which should not be construed as limiting.

Example 1: Materials and Methods for Examples 2-5

A. Animal Procedures

Animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center. Unless otherwise stated, mice used were male C57BL/6J (8–12 weeks of age; Jackson Laboratories), and housed in a temperature-controlled (23 °C) room on a 12 hour (h) light/dark cycle. Both male and female UCP1(KO) (B6.129-Ucp1tm1Kz/J) and littermate matched heterozygotes were used.

B. Body temperature and cold exposure

Interscapular body temperature was assessed using implantable electronic ID transponders (Bio Medic Data Systems, Inc). When studying acute activation of thermogenesis, mice were housed from birth at 23°C to allow for recruitment of thermogenic adipose tissue (Cannon and Nedergaard (2011) *J. Exp. Biol.* 214:242-253). Before individual housing at 4 °C or any experiments involving acute activation of thermogenesis, mice were placed at thermoneutrality (29°C) for 1 day which allows both for maintenance BAT UCP1 protein content (Gospodarska *et al.* (2015) *J. Biol. Chem.* 290:8243-8255) and for measurement of induction of BAT thermogenesis by acute intervention.

C. Metabolite analyses by MS

Metabolites were profiled using an LC-MS system comprised of a Nexera X2 U-HPLC (Shimadzu Scientific Instruments; Marlborough, MA) coupled to a Q Exactive Plus orbitrap mass spectrometers (Thermo Fisher Scientific; Waltham, MA). Tissues were rapidly isolated and homogenized in extraction solution of 80% methanol containing inosine-¹⁵N₄, thymine-d₄ and glycocholate-d₄ internal standards (Cambridge Isotope Laboratories; Andover, MA) at 4:1 volume to wet weight ratio. Thirty µL of each homogenate was diluted in a further 120 µL extraction solution. The samples were centrifuged (10 min, 9,000 x g, 4°C) and the supernatants were injected directly onto a 150 x 2.0 496 mm Luna NH₂ column (Phenomenex; Torrance, CA). For analysis of TCA cycle metabolites in BAT, an additional 100× dilution was performed in extraction solution to render succinate abundance within the linear quantification range of the instrument, as described in Figures 2C and 2D. The column was eluted at a flow rate of 400 µL/min with initial conditions of 10% mobile phase A (20 mM ammonium acetate and 20 mM ammonium hydroxide in water) and 90% mobile phase B (10 mM ammonium hydroxide in 75:25 v/v acetonitrile/methanol) followed by a 10 min linear gradient to 100% mobile phase A. MS analyses were carried out using electrospray ionization in the negative ion mode using full scan analysis over m/z 70-750 at 70,000 resolution and 3 Hz data acquisition rate. Additional MS settings were: ion spray voltage, -3.0 kV; capillary temperature, 350°C; probe heater temperature, 325 °C; sheath gas, 55; auxiliary gas, 10; and S-lens RF level 50. Raw data were processed using Progenesis QI software version 1.0 (NonLinear Dynamics) for feature alignment, nontargeted signal detection, and signal integration. Targeted processing of a subset of known metabolites and isotopologues was conducted using TraceFinder software 4.1 (Thermo Fisher Scientific; Waltham, MA). Compound identities were confirmed using reference standards. Succinate was quantified using a targeted LC-MS method operated on a ACQUITY UPLC (Waters Corp.; Milford, MA) coupled to a 5500 QTRAP mass spectrometer (SCIEX; Framingham, MA) as described previously in Chouchani *et al.* (2016) *Nature* 532:112-116 and Townsend *et al.* (2013) *Clin. Chem.* 59:1657-1667. For succinate analysis of mouse serum for shivering and curare experiments samples were analysed using reverse phase ion-pairing chromatography coupled to tandem mass spectrometry (Agilent LC-MS). Analytes were eluted in buffer A (97% H₂O, 3% MeOH, 10 mM tributylamine, 15 mM glacial acetic acid, pH 5.5) and buffer B (10 mM tributylamine, 15 mM glacial acetic acid in 100% MeOH). Samples were

run on a ZORBAX Extend-C18, 2.1×150 mm, $1.8 \mu\text{m}$ (Agilent) with a flow rate of 0.25 ml/min for 2.5 min of buffer A, followed by a linear gradient (100% buffer A to 80% buffer A) for 5 min, followed by a linear gradient (80% buffer A to 55% buffer A) for 5.5 min, followed by a linear gradient (55% buffer A to 1% buffer A) for 7 min, followed by 4 min with (1% buffer A). Samples were ionized using Agilent Jet Spray ionization; nebulizer 45 psi, capillary -2000 V, nozzle voltage: 500 V, sheath gas temperature 325°C , and sheath gas flow 12 l/min. An Agilent 6470 Triple Quadrupole mass spectrometer was used for mass detection with a targeted method. Peaks were integrated in Mass Hunter (Agilent).

D. ^{13}C Carbon metabolite tracing

[U- ^{13}C]-glucose (2.4 g/kg), [U- ^{13}C]-palmitate (80 mg/kg) or [U- ^{13}C]-succinate (100 or 500 mg/kg) (all from Cambridge Isotope Laboratories) were administered by tail vein injection and mice were individually housed at 4°C or 29°C for the indicated times prior to tissue harvest. [U- ^{13}C]-palmitate was conjugated to 1% BSA prior to injection. All injections were performed as a bolus over 20 s. For *in vitro* studies [U- ^{13}C]-succinate was added to BAT cells for the indicated times at a final concentration of 5 mM. Cells were washed and lysed directly in metabolite extraction buffer, snap frozen in liquid nitrogen and stored at -80°C until MS analysis was performed.

E. Primary brown adipocyte preparation and differentiation

Interscapular brown adipose stromal vascular fraction was obtained from 2- to 6-day-old pups as described previously in Kir *et al.* (2014) *Nature* 513:100-104. Interscapular brown adipose was dissected, washed in PBS, minced, and digested for 45 min at 37°C in PBS containing 1.5 mg ml^{-1} collagenase B, 123 mM NaCl, 5 mM KCl, 1.3 mM CaCl_2 , 5 mM glucose, 100 mM HEPES, and 4% essentially fatty-acid-free BSA. Tissue suspension was filtered through a $40 \mu\text{m}$ cell strainer and centrifuged at $600 \times g$ for 5 min to pellet the SVF. The cell pellet was resuspended in adipocyte culture medium and plated. Cells were maintained at 37°C in 10% CO_2 . Primary brown pre-adipocytes were counted and plated in the evening, 12 h before differentiation at 15,000 cells per well of a Seahorse plate. Pre-adipocyte plating was scaled according to surface area. The following morning, brown pre-adipocytes were induced to differentiate for 2 days with an adipogenic cocktail (1 μM rosiglitazone, 0.5 mM IBMX, 5 μM dexamethasone, $0.114 \mu\text{g ml}^{-1}$ insulin, 1 nM T3, and 125 μM Indomethacin) in adipocyte culture medium. Two days after induction, cells were re-fed every 48 h with adipocyte culture medium containing 1 μM

rosiglitazone, 1 nM T₃, and 0.5 µg ml⁻¹ insulin. Cells were fully differentiated by day 7 after induction.

F. Primary white adipocyte preparation and differentiation

Inguinal white adipose stromal vascular fraction was obtained from 2- to 6-day-old pups as described previously in Kazak *et al.* (2015) *Cell* 163:643-655. Adipose tissue was dissected, washed in PBS, minced, and digested for 45 min at 37°C in HBSS containing collagenase D (10 mg/ml), dispase II (3 U/ml) and CaCl₂ (10 mM). Tissue suspension was filtered through a 40 µm cell strainer and centrifuged at 600g for 5 min to pellet the SVF. The cell pellet was resuspended in adipocyte culture medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) and plated. Cells were maintained at 37°C in 10% CO₂. Primary white pre-adipocytes were counted and plated in the evening, 12 h before differentiation at 15,000 cells per well of a Seahorse plate. Pre-adipocyte plating was scaled according to surface area. The following morning, white pre-adipocytes were induced to differentiate for 2 days with an adipogenic cocktail (rosiglitazone (1 µM), IBMX (0.5 mM), dexamethasone (1 µM), insulin (5 µg/ml)) in adipocyte culture medium. Two days after induction, cells were re-fed every 48 h with adipocyte culture medium containing rosiglitazone (1 µM) and insulin (5 µg/ml). Cells were fully differentiated by day 6 after induction.

G. Maintenance of cell lines

All cell lines were grown in DMEM supplemented with 10% FBS and 1% P/S. Cells were detached using 0.05% trypsin and subcultured every other day.

H. Cellular respirometry of primary adipocytes

Cellular OCR of primary brown or white adipocytes was determined using a Seahorse XF24 Extracellular Flux Analyzer. Adipocytes were plated and differentiated in XF24 V7 cell culture microplates. Prior to analysis adipocyte culture medium was changed to respiration medium consisting of DMEM lacking NaHCO₃ (Sigma), NaCl (1.85 g/L), phenol red (3 mg/L), 2% fatty acid free BSA, and sodium pyruvate (1 mM), pH 7.4. Basal respiration was determined to be the OCR in the presence of substrate (1 mM sodium pyruvate) alone. Respiration uncoupled from ATP synthesis was determined following addition of oligomycin (4.16 µM). Maximal respiration was determined following addition of DNP (2 mM). Rotenone (3 µM) and antimycin (3 µM) were used to abolish mitochondrial respiration. Leak respiration was calculated as OCR following rotenone/antimycin treatment subtracted from OCR following oligomycin treatment.

I. Cellular respirometry of cell lines

Cellular OCR of cells lines was determined using a Seahorse XF24 563

Extracellular Flux Analyzer as described above with a few minor changes. All cell lines, except DE cells and immortalized human brown adipocytes, were plated at a density of 50,000 cells/well and a final concentration of 0.2% fatty acid free BSA was used. De2.3 cells and immortalized human brown adipocytes were plated at 5,000 cells/well and 3,000 cells/well, respectively, and a final concentration of 2% fatty acid free BSA was used. Respiration uncoupled from ATP synthesis was determined following addition of oligomycin (1.25 μ M). Rotenone (3 μ M) and antimycin (3 μ M) were used to abolish mitochondrial respiration.

J. Imaging of brown adipocytes

ROS production was estimated by oxidation of DHE and ratiometric assessment.

Cells were grown on 35 mm glass bottom No. 1.5 coverslips (MatTek). Ten minutes prior to imaging cells were loaded with dihydroethidium (DHE, 5 μ M, Sigma) in imaging buffer (NaCl, 156 mM; KCl, 3 mM; MgCl₂ 2 mM; KH₂PO₄, 1.25 mM; HEPES, 10 mM; sodium pyruvate, 1 mM). Cell culture dishes were mounted in a Tokai Hit INU microscope stage top incubator (37°C and 5% CO₂). Oxidized DHE was excited at 500 nm and the emitted signal was acquired at 632 nm. Reduced DHE was excited at 380 nm and the emitted signal was acquired at 460 nm. A time-lapse was performed in which cells were imaged every 20 s using an exposure time of 30 ms, 4×4 camera binning and the ND4 filter in. Four images were acquired prior to acute treatment with compounds of interest and imaged for 10 min. For experiments including inhibitors of succinate-dependent ROS induction these compounds were added in the initial 10 min incubation prior to imaging. All images were collected with an Inverted Nikon Ti fluorescence microscope equipped with 10x SF objective lens using MetaMorph 7.2 acquisition software and the Perfect Focus System for maintenance of focus over time. All measured cell parameters were analysed with Fiji image processing software. Briefly, 5 cells were selected from each image acquired with 10x objective, background removal was performed and a ratio of oxidized DHE over reduced DHE was calculated. For high resolution images a 40X SF objective lens with a layer of mineral oil on top of the media was used and cells were imaged at one time-point, 10 mins post-treatment. Images were processed using the Fiji ratio plus plugin with background correction for each channels and multiplication factors set to 1. The

fluorescence images are displayed using the same setting and were pseudocolored using the fire LUT. The transmitted light images are displayed using the autoscale LUT gray scale.

K. Assessment of protein thiol sulfonic acids

Samples were homogenized in 50 mM Tris base, containing 100 mM NaCl, 100 μ M DTPA, 0.1% SDS, 0.5% sodium deoxycholate, 0.5% Triton-X 100, 10 mM TCEP and 50 mM iodoacetamide. Following incubation for 15 min, SDS was added to a final concentration of 1% and samples were incubated for a further 15 min.

L. Protein digestion

Protein pellets were dried and resuspended in 8 M urea containing 50 mM HEPES (pH 8.5). Protein concentrations were measured by BCA assay (Thermo Scientific) before protease digestion. Protein lysates were diluted to 4 M urea and digested with LysC (Wako, Japan) in a 1/100 enzyme/protein ratio overnight. Protein extracts were diluted further to a 1.0 M urea concentration, and trypsin (Promega) was added to a final 1/200 enzyme/protein ratio for 6 h at 37 °C. Digests were acidified with 20 μ l of 20% formic acid (FA) to a pH ~2, and subjected to C18 solid-phase extraction (50 mg Sep-Pak, Waters).

M. LC-MS/MS parameters for targeted Prx cysteine peptide analysis

All spectra were acquired using an Orbitrap Fusion mass spectrometer (Thermo Fisher) in line with an Easy-nLC 1000 (Thermo Fisher Scientific) ultra-high pressure liquid chromatography pump. Peptides were separated onto a 100 μ M inner diameter column containing 1 cm of Magic C4 resin (5 μ m, 100Å, MichromBioresources) followed by 30cm of Sepax Technologies GP-C18 resin (1.8 μ m, 120 Å) with a gradient consisting of 9–30% (ACN, 0.125% FA) over 180 min at ~250 nl min⁻¹. For all LC-MS/MS experiments, the mass spectrometer was operated in the data-dependent mode. MS1 spectra were collected at a resolution of 120,000 with an AGC 611 target of 150,000 and a maximum injection time of 100 ms. The ten most intense ions were selected for MS2 (excluding 1 Z-ions). MS1 precursor ions were excluded using a dynamic window (75 s $\pm$ 10 ppm). The MS2 precursors were isolated with a quadrupole mass filter set to a width of 0.5 Th. For the MS3 based TMT quantitation, MS2 spectra were collected at an AGC of 4,000, maximum injection time of 200 ms, and CID collision energy of 35%. MS3 spectra were acquired with the same Orbitrap parameters as the MS2 method except HCD collision energy was increased to 55%. Synchronous- precursor-selection was enabled to include up to six MS2 fragment ions for the MS3 spectrum.

N. Data processing and MS2 spectra assignment

A compilation of in-house software was used to convert .raw files to mzXML format, as well as to adjust monoisotopic m/z measurements and erroneous peptide charge state assignments. Assignment of MS2 spectra was performed using the SEQUEST algorithm (Eng *et al* (1994) *J. Am. Sc. Mass Spectrom.* 5:976-989). All experiments used the Mouse UniProt database (downloaded 10 April 2014) where reversed protein sequences and known contaminants, such as human keratins, were appended. SEQUEST searches were performed using a 20 ppm precursor ion tolerance, while requiring each peptide's amino/carboxy (N/C) terminus to have trypsin protease specificity and allowing up to two missed cleavages. For targeted assessment of Prx cysteine sulfonylation, TMT tags on lysine residues and peptide N termini (+229.16293 Da), iodoacetamide on cysteine residues (+57.0214637 Da) were set as static modifications and oxidation of methionine residues (+15.99492 Da) and sulfonylation on cysteine residues (+9.036719 Da versus iodoacetamide) as variable modifications. Determination of sulfonylation status of the Prx peptides was determined by comparing TMT reporter ion abundance of sulfonylated peptides normalized to the unmodified (iodoacetamide-labelled) forms. An MS2 spectra assignment false discovery rate of less than 1% was achieved by applying the target-decoy database search strategy (Elias and Gygi (2007) *Nat. Methods* 4:207-214). Protein filtering was performed using an in-house linear discrimination analysis algorithm to create one combined filter parameter from the following peptide ion and MS2 spectra metrics: XCorr, ΔC_n score, peptide ion mass accuracy, peptide length and missed-cleavages (Huttlin *et al.* (2010) *Cell* 134:1174-1189). Linear discrimination scores were used to assign probabilities to each MS2 spectrum for being assigned correctly, and these probabilities were further used to filter the data set to a 1% protein-level false discovery rate.

O. Western Blotting

Samples were isolated in 50 mM Tris, pH 7.4, 500 mM NaCl, 1% NP40, 20% glycerol, 5 mM EDTA and 1 mM phenylmethylsulphonyl fluoride, supplemented with a cocktail of protease inhibitors (Roche). Homogenates were centrifuged at 16,000 g x 10 min at 4°C, and the supernatants were used for subsequent analyses. Protein concentration was determined using the bicinchoninic acid assay (Pierce). Protein lysates were denatured in Laemmli buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue, 100 mM DTT), resolved by 4%–12% NuPAGE Bis-Tris SDS–PAGE (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane. Primary antibodies (pPKA substrate (CST 9624 s); Tubulin (Abcam AB44928)) were diluted in TBS containing 0.05%

Tween (TBS-T), 5% BSA and 0.02% NaN₃ (Huttlin *et al.* (2010) *Cell* 134:1174-1189). Membranes were incubated overnight with primary antibodies at 4°C. For secondary antibody incubation, anti-rabbit HRP (Promega) was diluted in TBS-T containing 5% milk. Results were visualized with enhanced chemiluminescence (ECL) western blotting substrates (Pierce).

P. Glycerol release

Adipocytes were incubated in respiration medium and treated as indicated before collection of medium and quantification of glycerol using free glycerol reagent (Sigma-Aldrich) relative to glycerol standard.

Q. Metabolic phenotyping

Whole-body energy metabolism was evaluated using a Comprehensive Lab Animal Monitoring System (CLAMS, Columbia Instruments). Mice were acclimated in the metabolic chambers and acclimated with mock injection procedures to minimize contribution of stress to the metabolic phenotype. O₂ levels were collected every 60s. Basal O₂ consumption rate was determined to be the average of the lowest three consecutive readings determined prior to intervention. Maximum O₂ consumption rate was determined as the mean of three highest rates recorded post-intervention.

R. Determination of free living whole body total energy expenditure

Whole body energy expenditure in mice was determined using the energy balance method, otherwise known as the law of energy conservation, which accounts for caloric intake, change in body weight, and change in lean and fat mass throughout dietary intervention, as described previously in Ravussin *et al.* (2013) *Int. J. Obes.* 37:399-403; Goldgof *et al.* (2014) *J. Biol. Chem.* 289:19341-19350; and Guo and Hall (2011) *PLoS One* 6:e15961. Briefly, individual mouse body weight and body composition were determined prior to dietary intervention $\pm$ sodium succinate. Throughout the four-week intervention period, mouse kcal intake was measured, as well as changes in body weight and body composition (fat mass and fat-free mass). Kilocalorie intake was determined on the basis of the energy density of high fat diet (5.24 kcal/gram) and the energy density of ingested succinate (2.99 kcal/gram). Energy density of accumulated fat mass in mice was 9.4 kcal/gram and fat-free mass was 1.8 kcal/gram (Guo and Hall (2011) *PLoS One* 6:e1596140). Based on bomb calorimetry of feces during the experiment, which found no differences in kcal absorption between interventions, it was confirmed that the calculated

metabolizable energy intake based on kcal intake measurements adequately accounted for any differences due to digestion.

S. High fat feeding

All mouse high fat feeding experiments were performed with age-matched littermate controls. At eight weeks of age, mice were switched to high fat diet (OpenSource Diets, D12492) with 60% kcal from fat, 20% kcal from carbohydrate, and 20% kcal from protein. Following initiation of high fat feeding, mice provided with succinate in drinking water had it supplemented to the indicated level using sodium succinate. Succinate-containing drinking water was freshly prepared and replaced every two days.

10 T. Body composition analysis

Body composition was examined with Echo MRI (Echo Medical Systems, Houston, Texas) using the 3-in-1 Echo MRI Composition Analyzer.

U. GTT

Mice were fasted for 6 hrs. Glucose (1 g/kg) was administered i.p., and blood glucose levels were measured at 0, 15, 30, 45, 60, 75, 90 and 120 minutes using a glucometer.

V. Bomb calorimetry of feces

Calorimetry was conducted using a Parr 6725EA Semimicro Calorimeter and 1107 Oxygen Bomb. During dietary intervention, fecal specimens from mice were collected over a 48 h period. Collected samples and baked at 60°C for 48 h to remove water content. Fecal samples were combusted and the energy content of the fecal matter was measured as heat of combustion (kcal/g).

W. Statistical analyses

Data were expressed as mean $\pm$ s.e.m. and P values were calculated using two-tailed Student's t-test for pairwise comparison of variables, one-way ANOVA for multiple comparison of variables, and two-way ANOVA for multiple comparisons involving two independent variables. ANOVA analyses were subjected to Bonferroni's post hoc test. Sample sizes were determined on the basis of previous experiments using similar methodologies. For all experiments, all stated replicates are biological replicates. For *in vivo* studies, mice were randomly assigned to treatment groups. For MS analyses, samples were processed in random order and experimenters were blinded to experimental conditions.

X. Representative brown and beige fat markers

Table 1 below provides representative gene expression markers for brown and/or beige fat. In addition, assays for analyzing quantitative RT-PCR, mitochondrial biogenesis, oxygen consumption, glucose uptake, energy intake, energy expenditure, weight loss, multilocular lipid droplet morphology, mitochondrial content, and the like modulated by succinate and exhibited by brown and/or beige fat cells are well-known in the art (see, at least Harms and Seale (2013) *Nat. Med.* 19:1252-1263 and U.S. Pat. Publ. 2013/0074199).

Table 1

Gene Symbol	Gene Name	GenBank Gene Accession Number	GenBank Protein Accession Number	Gene ID
adipsin	complement factor D	<i>e.g.</i> , NM_013459.2 and NM_001928.2	<i>e.g.</i> , NP_038487.1 and NP_001919.2	<i>e.g.</i> , 11537 and 1675
fatty acid transporter cd36	fatty acid transporter/cd36	<i>e.g.</i> , NM_007643.3 and NM_000072.3 and NM_001001547.2 and NM_001001548.2 and NM_001127443.1 and NM_001127444.1	<i>e.g.</i> , NP_031669.2 and NP_000063.2 and NP_001001547.1 and NP_001001548.1 and NP_001120915.1 and NP_001120916.1	<i>e.g.</i> , 12491 and 948
adiponectin	adiponectin	<i>e.g.</i> , NM_009605.4 and NM_004797.2	<i>e.g.</i> , NP_0033735.3 and NP_004788.1	<i>e.g.</i> , 11450 and 9370
UCP-1	uncoupling protein 1	<i>e.g.</i> , NM_009463.3 and NM_021833.4	<i>e.g.</i> , NP_033489.1 and NP_068605.1	<i>e.g.</i> , 22227 and 7350
cidea	cell death-inducing DFFA-like effector a	<i>e.g.</i> , NM_007702.2 and NM_001279.3 and NM_198289.2	<i>e.g.</i> , NP_031728.1 and NP_001270.1 and NP_938031.1	<i>e.g.</i> , 12683 and 1149
PGC1a	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	<i>e.g.</i> , NM_008904.2 and NM_013261.3	<i>e.g.</i> , NP_032930.1 and NP_037393.1	<i>e.g.</i> , 19017 and 10891
Elovl3	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3	<i>e.g.</i> , NM_007703.2 and NM_152310.1	<i>e.g.</i> , NP_031729.1 and NP_689523.1	<i>e.g.</i> , 12686 and 83401
C/EBPbeta	CCAAT/enhancer binding protein beta	<i>e.g.</i> , NM_009883.3 and NM_005194.2	<i>e.g.</i> , NP_034013.1 and NP_005185.2	<i>e.g.</i> , 12608 and 1051
Cox7a1	cytochrome c oxidase subunit VIIa polypeptide 1	<i>e.g.</i> , NM_009944.3 and NM_001864.2	<i>e.g.</i> , NP_034074.1 and NP_001855.1	<i>e.g.</i> , 12865 and 1346
Otopetrin	Otopetrin 1	<i>e.g.</i> , NM_172709.3 and NM_177998.1	<i>e.g.</i> , NP_766297.2 and NP_819056.1	<i>e.g.</i> , 21906 and 133060

Type II deiodinase	Deiodinase, iodothyronine, type II	<i>e.g.</i> , NM_010050.2 and NM_000793.4 and NM_001007023.2 and NM_013989.3	<i>e.g.</i> , NP_034180.1 and NP_000784.2 and NP_001007024.1 and NP_054644.1	<i>e.g.</i> , 13371 and 1734
cytochrome C	cytochrome c	<i>e.g.</i> , NM_009989.2 and NM_018947.4	<i>e.g.</i> , NP_034119.1 and NP_061820.1	<i>e.g.</i> , 13067 and 54205
cox4i1	cytochrome c oxidase subunit IV isoform 1	<i>e.g.</i> , NM_009941.2 and NM_001861.2	<i>e.g.</i> , NP_034071.1 and NP_001852.1	<i>e.g.</i> , 12857 and 1327
coxIII	mitochondrially encoded cytochrome c oxidase III	<i>e.g.</i> , NC_005089.1 and ENST00000362079	<i>e.g.</i> , NP_904334.1 and ENSP00000354982	<i>e.g.</i> , 17705 and 4514
cox5b	cytochrome c oxidase subunit Vb	<i>e.g.</i> , NM_009942.2 and NM_001862.2	<i>e.g.</i> , NP_034072.2 and NP_001853.2	<i>e.g.</i> , 12859 and 1329
cox8b	cytochrome c oxidase subunit 8B, mitochondrial precursor	<i>e.g.</i> , NM_007751.3	<i>e.g.</i> , NP_031777.1	<i>e.g.</i> , 12869 and 404544
glut4	solute carrier family 2 (facilitated glucose transporter), member 4	<i>e.g.</i> , NM_009204.2 and NM_001042.2	<i>e.g.</i> , NP_033230.2 and NP_001033.1	<i>e.g.</i> , 20528 and 6517
atpase b2	ATPase, H ⁺ transporting, lysosomal 56/58kDa, V1 subunit B2	<i>e.g.</i> , NM_057213.2 and NM_001693.3	<i>e.g.</i> , NP_476561.1 and NP_001684.2	<i>e.g.</i> , 117596 and 526
coxII	mitochondrially encoded cytochrome c oxidase II	<i>e.g.</i> , NC_005089.1 and ENST00000361739	<i>e.g.</i> , NP_904331 and ENSP00000354876	<i>e.g.</i> , 17709 and 4513
atp5o	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit	<i>e.g.</i> , NM_138597.2 and NM_001697.2	<i>e.g.</i> , NP_613063.1 and NP_001688.1	<i>e.g.</i> , 28080 and 539
ndufb5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16kDa	<i>e.g.</i> , NM_025316.2 and NM_002492.2	<i>e.g.</i> , NP_079592.2 and NP_002483.1	<i>e.g.</i> , 66046 and 4711
Rarres2	retinoic acid receptor responder (tazarotene induced) 2	<i>e.g.</i> , NM_027852.2 and NM_002889.3	<i>e.g.</i> , NP_082128.1 and NP_002880.1	<i>e.g.</i> , 71660 and 5919
Car3	carbonic anhydrase 3	<i>e.g.</i> , NM_007606.3 and NM_005181.3	<i>e.g.</i> , NP_031632.2 and NP_005172.1	<i>e.g.</i> , 12350 and 761
Peg10	paternally expressed 10	<i>e.g.</i> , NM_001040611.1 and NM_001040152.1 and NM_001172437.1 and NM_001172438.1 and NM_015068.3	<i>e.g.</i> , NP_001035701.1 and NP_001035242.1 and NP_001165908.1 and NP_001165909.1 and NP_055883.2	<i>e.g.</i> , 170676 and 23089
Cidec	Cidec cell death-inducing DFFA-like effector c	<i>e.g.</i> , NM_178373.3 and NM_022094.2	<i>e.g.</i> , NP_848460.1 and NP_071377.2	<i>e.g.</i> , 14311 and 63924
Cd24a	CD24a antigen	<i>e.g.</i> , NM_009846.2 and NM_013230.2	<i>e.g.</i> , NP_033976.1 and NP_037362.1	<i>e.g.</i> , 12484 and 100133941
Nr1d2	nuclear receptor subfamily 1, group D, member 2	<i>e.g.</i> , NM_011584.4 and NM_001145425.1 and NM_005126.4	<i>e.g.</i> , NP_035714.3 and NP_001138897.1 and NP_005117.3	<i>e.g.</i> , 353187 and 9975

Ddx17	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	<i>e.g.</i> , NM_001040187.1 and NM_001098504.1 and NM_001098505.1 and NM_006386.4 and NM_030881.3	<i>e.g.</i> , NP_001035277.1 and NP_001091974.1 and NP_001091975.1 and NP_006377.2 and NP_112020.1	<i>e.g.</i> , 67040 and 10521
Aplp2	amyloid beta (A4) precursor-like protein 2	<i>e.g.</i> , NM_001102455.1 and NM_001142276.1 and NM_001142277.1 and NM_001142278.1 and NM_001642.2	<i>e.g.</i> , NP_001095925.1 and NP_001135748.1 and NP_001135749.1 and NP_001135750.1 and NP_001633.1	<i>e.g.</i> , 11804 and 334
Nr3c1	nuclear receptor subfamily 3, group C, member 1	<i>e.g.</i> , NM_008173.3 and NM_000176.2 and NM_001018074.1 and NM_001018075.1 and NM_001018076.1 and NM_001018077.1 and NM_001020825.1 and NM_001024094.1	<i>e.g.</i> , NP_032199.3 and NP_000167.1 and NP_001018084.1 and NP_001018085.1 and NP_001018086.1 and NP_001018087.1 and NP_001018661.1 and NP_001019265.1	<i>e.g.</i> , 14815 and 2908
Rybp	RING1 and YY1 binding protein	<i>e.g.</i> , NM_019743.3 and NM_012234.4	<i>e.g.</i> , NP_062717.2 and NP_036366.3	<i>e.g.</i> , 56353 and 23429
Txnip	thioredoxin interacting protein	<i>e.g.</i> , NM_001009935.2 and NM_006472.3	<i>e.g.</i> , NP_001009935.1 and NP_006463.3	<i>e.g.</i> , 56338 and 10628
Cig30	Elongation of very long chain fatty acids-like 3	<i>e.g.</i> , NM_152310.1 and NM_007703.1 ¹	<i>e.g.</i> , NP_689523.1 and NP_031729.1 ¹	<i>e.g.</i> , 83401 and 12686
Ppar gamma 2	Peroxisome proliferator-activated receptor gamma 2	<i>e.g.</i> , NM_015869.4 and NM_011146.2 ¹	<i>e.g.</i> , NP_056953 and NP_035276.1 ¹	<i>e.g.</i> , 5468 and 19016
Prdm16	PR domain containing 16 protein	<i>e.g.</i> , NM_022114.3 and NM_199454.2 and NM_027504.3	<i>e.g.</i> , NP_071397.3 and NP_955533.2 and NP_081780.3	<i>e.g.</i> , 63976 and 70673
Ap2	Fatty acid binding protein 4	<i>e.g.</i> , NM_0011442.2 and NM_024406.1	<i>e.g.</i> , NP_001433.1 and NP_077717.1	<i>e.g.</i> , 2167 and 11770
Ndufs2	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase	<i>e.g.</i> , NM_001166159.1 and NM_004550.4 and NM_153064.4	<i>e.g.</i> , NP_001159631.1 and NP_004541.1 and NP_694704.1	<i>e.g.</i> , 4720 and 226646
Grp109A	Hydroxycarboxylic acid receptor 2	<i>e.g.</i> , NM_177551 and NM_030701.3	<i>e.g.</i> , NP_808219 and NP_109626.1	<i>e.g.</i> , 338442 and 80885
AcylCoA-thioesterase 4	Acyl-coenzyme A thioesterase 4	<i>e.g.</i> , NM_152331 and NM_134247.3	<i>e.g.</i> , NP_689544 and NP_599008.3	<i>e.g.</i> , 122970 and 171282
Claudin1	Claudin1	<i>e.g.</i> , NM_021101.4 and NM_016674.4	<i>e.g.</i> , NP_066924.1 and NP_057883.1	<i>e.g.</i> , 9076 and 12737
PEPCK	Phosphoenolpyruvate carboxykinase (mitochondrial)	<i>e.g.</i> , NM_001018073.1 and NM_004563.2 and NM_028994.2	<i>e.g.</i> , NP_001018083.1 and NP_004554.2 and NP_083270.1	<i>e.g.</i> , 5106 and 74551
Fgf21	Fibroblast growth factor 21	<i>e.g.</i> , NM_019113 and NM_020013.4	<i>e.g.</i> , NP_061986 and NP_064397.1	<i>e.g.</i> , 26291 and 56636
AcylCoA-thioesterase 3	Acyl-coenzyme A thioesterase 4	<i>e.g.</i> , NM_001037161.1 and NM_134246.3	<i>e.g.</i> , NP_001032238.1 and NP_599007.1	<i>e.g.</i> , 641371 and 171281
Dio2	Type II iodothyronine deiodinase	<i>e.g.</i> , NM_00793.5 and NM_010050.2	<i>e.g.</i> , NP_000784.2 and NP_034180.1	<i>e.g.</i> , 1734 and 13371

Y. Immortalized human brown pre-adipocyte line differentiation

Immortalized human brown preadipocytes (Shinoda *et al.* (2015) *Nat. Med.* 4:389-394) were cultured with animal component-free medium (Stem Cell Technologies; #05449). Brown adipocyte differentiation was induced by treating confluent preadipocytes with animal component-free adipogenic differentiation medium (Stem Cell Technologies; #05412) supplemented with T3 (1 nM) and rosiglitazone (0.5 μ M). Cells were fully differentiated 2 weeks after induction.

Z. Assessment of respiration in isolated mitochondria

BAT mitochondria were isolated as described previously³⁷. Using freshly isolated mitochondria, basal respiration was measured in the presence of 10 mM pyruvate and 10 mM malate in the presence of 3 mM GDP in 50 mM KCl, 10 mM TES, 1mM EGTA medium containing 0.4% (w/v) fatty acid-free bovine serum albumin, 1 mM KH_2PO_4 , 2 mM MgCl_2 and 0.46 mM TCAI_2 . OCR was monitored in a Seahorse XF24 instrument at 2.5 μ g mitochondrial protein per well. Succinate was added acutely at 5 mM following determination of basal respiration, leak was determined using 1 $\mu\text{g ml}^{-1}$ oligomycin. 0.1 mM DNP was applied to determine chemically uncoupled respiration.

AA. Histological analysis

Tissues were extracted and placed in tissue clamps in 10% neutral buffer formalin (NBF) overnight. The following day, samples were rinsed twice in PBS and stored in 70% ethanol. Tissue fragments were embedded in paraffin, sectioned and mounted on glass slides. For histological and morphometric studies, the sections were stained with haematoxylin and eosin or Masson's trichrome. Digital images were collected with a Nikon Ti2 motorized inverted microscope equipped with a 4 $\times$ or 40 $\times$ objective lens. Images were acquired with a Nikon DS-Fi1 colour camera controlled with NIS-Elements image acquisition software. The quantitative analysis of cardiomyocyte cross-sectional height and width and nuclear diameter were measured using Fiji image processing software.

AB. Gene expression analysis

Total RNA was extracted from frozen tissue using TRIzol (Invitrogen), purified with a PureLink® RNA Mini Kit (Invitrogen) and quantified using a Nanodrop 2000 UV-visible spectrophotometer. cDNA was prepared using 1 μ g total RNA by a reverse transcription-polymerase chain reaction (RT-PCR) using a high capacity cDNA reverse transcription kit (Applied Biosystems), according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed on cDNA using SYBR Green probes. qPCR

was performed on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) using GoTaq® qPCR Master Mix (Promega). Reactions were performed in a 384-well format using an ABI PRISM® 7900HT real time PCR system (Applied Biosystems). Fold changes in expression were calculated by the $\Delta\Delta C_t$ method using mouse cyclophilin A as an endogenous control for mRNA expression. All fold changes are expressed normalized to the vehicle control. SYBR primer pair sequences were as follows.

Il1b: forward, 5'-TGGCAACTGTTCTG-3';
reverse, 5'-GGAAGCAGCCCTTCATCTTT-3';
Il10: forward, 5'-AGGCGCTGTCATCG-ATTT-3';
reverse, 5'-TCACTTGGTCTTGGAGCTTAT-3';
Tnf α : forward, 5'-GCCTCTTCT-CATTCCTGCTT-3';
reverse, 5'-TGGGAACCTTCTCATCCCTTTG-3';
Arg1: forward, 5'-GATTATCGGAGCGCCTTCT-3';
reverse, 5'-TGGTCTCTTCAGTCATACTCT-3';
Nos2: forward, 5'-CCAAGCCCTTCACTACTTCC-3';
reverse, 5'-CTCTGAGGGCTGATCAAAGG-3';
Il6: forward, 5'-ACAAAGCCAGAGTCCTTCAGAGAG-3';
reverse, 5'-TTGGATGGTCTTGGTCCTTAGCCA-3';
Mrc1: forward, 5'-GGCGAGCATCAAGAGTAAAGA-3';
reverse, 5'-CATAGGTCAGTCCCAACCAAA-3';
cyclophilin (*Ppia*): forward, 5'-GGAGATGGTCAAGGAGGAA-3';
reverse, 5'-GCCCGTAGTGCTTCAGCTT-3'.

Example 2: Succinate is a distinct metabolic signature of adipose tissue thermogenesis

A comparative metabolomics approach was applied to identify conserved metabolic signatures of adipose tissue thermogenesis (Figure 1A). Liquid chromatography tandem mass spectrometry (LC-MS)-based metabolomic analysis of mouse adipose tissues were gated on three criteria: i) metabolite enrichment in thermogenic adipose tissue (BAT vs subcutaneous white adipose tissue; Figure 1B); ii) metabolite abundance in thermogenic adipose tissue (estimated as the 10% most abundant annotated metabolite ion intensities; Figure 1C); iii) increased metabolite abundance upon acute activation of BAT thermogenesis by exposure to 4°C versus 29 °C (Figure 1D). The comparative analysis revealed that only two metabolites fulfilled all these criteria for a thermogenic signature

(Figures 1A-1D and Tables 2–4). One of these was the mitochondrial tricarboxylic acid (TCA) cycle intermediate succinate, and the other was AMP.

Table 2: Quantification of metabolites identified by targeted LC-MS metabolomics.

- 5 Relative abundance is expressed as BAT abundance relative to subcutaneous white adipose tissue abundance. Log2FC = log2 of fold change between BAT and subcutaneous abundance. Negative log10(pvalue) = negative log10 of the P value

Metabolite	Log2FC	negative LOG10(pvalue)
dTMP	6.33	5.31
C18:1-OH carnitine	6.09	7.05
C16-OH carnitine	5.76	8.06
C9 carnitine	5.23	3.11
C3-DC carnitine	5.11	7.10
adenvlosuccinate	4.92	2.63
C4-OH carnitine	4.63	2.32
alpha-glycerophosphate	4.43	4.43
8-hydroxy-deoxyguanosine	4.18	7.78
C10 carnitine	4.08	3.25
C8 carnitine	4.07	3.65
C7 carnitine	4.07	3.22
C14:2 carnitine	3.98	1.54
C3-DC-CH3 carnitine	3.92	2.93
C4 carnitine	3.90	3.91
NADP	3.81	4.31
NAD	3.68	8.78
C18:1 carnitine	3.67	4.87
C18:2 carnitine	3.58	4.99
C16 carnitine	3.55	5.23
C14:1 carnitine	3.52	2.44
C20 carnitine	3.49	2.57
DHAP/glyceraldehyde 3P	3.46	4.27
C12:1 carnitine	3.42	5.22
C12 carnitine	3.41	3.25
IMP	3.41	5.32
glutathione oxidized	3.36	2.84
xanthine	3.33	7.93
succinate	3.21	5.32
C14 carnitine	3.17	3.28
PEP	3.17	2.07
C6 carnitine	3.15	2.62
C5:1 carnitine	3.13	2.20
C18 carnitine	3.07	4.81
anserine	2.98	3.02
trimethyllysine	2.97	7.99
UDP-glucuronate	2.91	1.01
6-phosphogluconate	2.79	3.01
adenine	2.72	7.14
serotonin	2.67	9.68
malate	2.63	4.36
C20:4 carnitine	2.63	5.08
5-hydroxytryptophan	2.58	10.32
C2 carnitine	2.57	7.08
C22:6 LPE	2.56	7.40
AMP	2.55	6.82
C36:3 PS plasmalogen	2.43	0.67
carnosine	2.41	5.66
cotinine N-oxide	2.41	8.03

fumarate/maleate	2.39	4.49
alpha-glycerophosphocholine	2.32	6.64
C16:1 LPC plasmalogen	2.31	6.47
C3 carnitine	2.28	6.85
leucylglycine	2.27	4.57
hypotaurine	2.26	2.17
GMP	2.23	6.82
niacinamide	2.17	5.63
creatine	2.14	7.12
C20:4 LPE	2.10	6.09
butyrobetaine	2.08	7.36
carnitine	2.07	7.17
MDA	2.03	7.37
pentose monophosphate	2.02	5.60
C5-DC carnitine	1.95	2.83
beta-guanidinopropionic acid	1.93	6.02
C18:2 LPE	1.87	5.64
C14:0 LPC	1.85	5.26
hypoxanthine	1.79	5.50
pterin	1.78	4.95
xanthosine	1.77	4.13
6,8-dihydroxypurine	1.77	5.23
betaine	1.76	3.95
D-Alany-D-alanine	1.73	2.53
UMP	1.72	6.60
C5 carnitine	1.70	2.56
cAMP	1.69	6.17
2,1-deoxycortisol	1.68	2.64
methythioadenosine	1.67	5.27
C18:1 LPE	1.66	6.31
lactose	1.65	0.78
alanine	1.64	3.61
propionate	1.64	5.61
CMP	1.64	4.86
3-phosphoglycerate	1.62	2.67
C16:1 LPC	1.60	5.63
choline	1.60	5.61
hexose diphosphate	1.59	1.47
indole-3-propionate	1.54	0.69
phosphocreatine	1.49	2.00
corticosterone	1.47	2.24
spermidine	1.43	2.71
erythronate	1.42	8.37
inositol	1.40	6.41
C20:1 LPE	1.38	3.80
2-phosphoglycerate	1.33	2.33
guanosine	1.25	4.04
s-adenosylmethionine	1.24	3.25
inosine	1.24	2.88
5-Hydroxytryptophol	1.23	3.99
Ne,Ne dimethyllysine	1.21	3.29
linoleoyl ethanolamide	1.20	2.23
SDMA	1.20	3.98
C32:2 PC	1.17	3.06
hydroxyproline	1.16	1.97
C18:0 LPE B	1.13	2.87
proline betaine	1.12	3.05
creatinine	1.11	4.34
3-methylhistidine	1.10	2.69
N-acetylmethionine	1.09	3.23
pantothenate	1.07	2.57
cytidine	1.06	3.61
acetyl-galactosamine	1.05	2.44
pyruvate	1.05	0.84

trimethylamine-N-oxide	1.04	6.43
C16:0 LPC B	1.03	3.68
histidine	1.01	2.53
UDP-galactose/UDP-glucose	1.00	3.40
uridine	0.99	3.79
taurine	0.98	5.10
ATP	0.96	0.65
arginine	0.94	3.61
GDP	0.92	1.16
C18:1 LPC	0.90	3.17
C18:2 LPC	0.90	2.53
ADP	0.90	1.67
glutamine	0.90	4.70
adenosine	0.89	2.90
uric acid	0.87	1.89
citrulline	0.86	2.23
NMMA	0.82	1.38
adipate	0.81	3.19
C20:0 LPE	0.81	2.63
fucose	0.81	3.81
homoarginine	0.81	1.48
pipecolic acid	0.81	3.00
thiamine	0.80	2.17
N-acetylhistamine	0.79	1.48
tryptophan	0.78	2.65
N-acetylmethionine	0.78	1.42
glycine	0.75	1.88
methionine	0.72	1.66
N-acetyltryptophan	0.71	1.32
alpha-hydroxybutyrate	0.70	1.52
1-methylhistamine	0.70	1.44
dimethylglycine	0.70	2.26
ADMA	0.66	1.57
lysine	0.66	1.09
taurocholate	0.65	0.37
alloisoleucine	0.62	2.38
valine	0.62	1.49
phenylalanine	0.61	1.37
C20:4 LPC	0.58	1.27
sphingosine	0.57	0.54
C18:0 LPC	0.57	1.91
1-methylnicotinamide	0.57	1.55
C16:0 LPE	0.57	1.63
sphingosine A	0.56	0.52
isoleucine	0.56	1.24
pyruvaldehyde	0.56	1.62
citrate/isocitrate	0.55	1.55
tyrosine	0.55	1.53
C22:6 LPC	0.53	1.28
aconitate	0.53	0.90
N6-acetyl-L-lysine	0.53	1.48
C20:3 LPC A	0.52	1.48
thymine	0.52	1.49
leucine	0.52	1.43
N-acetylgalactosamine	0.51	1.98
4-hydroxybenzaldehyde	0.51	1.42
acetylalanine	0.51	2.67
methylvguanidine	0.51	0.56
proline	0.50	0.65
allantoin	0.48	1.22
caproate	0.48	0.20
threonine	0.47	1.09
suberate	0.47	1.70
N6-acetyllysine	0.46	1.09

2-aminoadipate	0.46	1.49
5-methylcytidine	0.45	0.99
asparagine	0.44	0.81
isovalerate	0.44	0.50
C22:5 LPC	0.44	0.75
pseudouridine	0.44	1.03
acetaminophen	0.43	3.05
C18:3 LPC	0.42	0.88
C20:3 LPC	0.42	0.89
N1-Methyl-2-pyridone-5-carboxamide	0.42	1.07
azelate	0.41	1.59
S-methyl-L-cysteine-S-oxide	0.39	0.65
sphingosine B	0.39	0.35
N-methylproline	0.38	0.97
2-hydroxy-3-methylpentanoate	0.38	2.03
C20:5 LPC	0.38	0.63
imidazole propionate	0.38	0.25
fructose/glucose/galactose	0.37	1.09
glutamate	0.35	1.04
N-acetyl-leucine	0.30	0.71
3-methyladipate/pimelate	0.30	0.73
sebacate	0.30	0.24
C18:3 LPE	0.28	0.51
sorbitol	0.27	1.00
N-lauroylglycine	0.27	0.46
pyroglutamic acid	0.25	0.37
C34:3 PC plasmalogen	0.24	0.37
phenylacetyl-glycine	0.24	0.27
caprylate	0.24	0.13
serine	0.22	0.36
methionine sulfoxide	0.22	0.20
methyl N-methylanthranilate	0.21	0.55
biotin	0.20	0.21
ornithine	0.18	0.15
phytosphingosine	0.17	0.15
ribothymidine	0.16	0.33
C38:6 PE	0.15	0.31
4-guanidinobutanoic acid	0.14	0.30
beta-alanine	0.13	0.18
1-methyladenosine	0.13	0.32
N1-acetylspermidine	0.12	0.13
N-acetylspermidine	0.12	0.13
pyridoxamine	0.11	0.14
guanidoacetic acid	0.10	0.20
C18:1 LPC plasmalogen	0.10	0.10
C18:1 LPC plasmalogen minor B	0.10	0.10
putrescine	0.10	0.12
2-deoxyadenosine	0.09	0.03
N-acetylaspartic acid	0.09	0.05
7-Methylguanine	0.07	0.11
glutathione reduced	0.04	0.11
C20:1 LPC	0.02	0.03
myristoleate	0.01	0.03
anthranilic acid	0.01	0.04
C18:0 LPE	0.01	0.01
5-methylcytosine	0.00	0.01
C34:3 PC	-0.03	0.05
cyclohexylamine	-0.04	1.21
urate	-0.05	0.14
phosphocholine	-0.07	0.07
aspartate	-0.07	0.16
N-acetylglutamine	-0.08	0.10
eicosapentaenoate	-0.09	0.09
4-hydroxymandelate/homogentisate	-0.13	0.15

malonate	-0.13	0.34
L-a-glutamyl-L-Lysine	-0.15	0.12
N-acetylhistidine	-0.15	0.19
N4-acetylcytidine	-0.16	0.26
1-methylguanine	-0.18	0.42
indolelactate	-0.19	0.25
UDP	-0.19	0.22
C40:6 PE	-0.20	0.35
N-carbamoyl-beta-alanine	-0.20	0.24
1-methylguanosine	-0.21	0.35
L-alpha-glutamyl-L-lysine	-0.22	0.18
histamine	-0.24	0.36
N-acetylputrescine	-0.25	0.49
orotate	-0.26	0.29
1,2,4-trimethylbenzene	-0.29	0.36
oleoyl glycine	-0.29	0.13
gabapentin	-0.35	1.71
homovanillate	-0.35	0.95
glucuronate	-0.36	1.30
cytosine	-0.38	0.64
C30:0 PC	-0.38	0.72
biliverdin	-0.39	0.10
N2,N2-dimethylguanosine	-0.43	0.42
C38:7 PC plasmalogen	-0.44	1.13
ectoine	-0.45	0.43
guanine	-0.45	0.64
NH4 C46:2 TAG	-0.46	0.72
gentisate	-0.48	0.69
2-deoxycytidine	-0.49	0.83
CDP	-0.49	0.47
DMGV	-0.50	0.76
alpha-ketoglutarate	-0.54	2.53
biopterin	-0.55	1.83
C36:4 PE	-0.58	2.43
cotinine	-0.59	0.19
N-alpha-acetyarginine	-0.62	1.21
NH4 C44:1 TAG	-0.65	0.67
kynurenic acid	-0.65	0.65
indoxylsulfate	-0.67	0.85
hippurate	-0.67	0.43
N-alpha-acetylarginine	-0.68	1.29
Pantothenol	-0.69	0.25
trigonelline	-0.70	1.05
GABA	-0.70	1.08
imidazoleacetic acid	-0.72	1.20
2-hydroxyglutarate	-0.75	2.31
sphinganine	-0.94	0.80
4-Hydroxy-3-methylacetophenone	-0.96	0.74
methylimidazole acetic acid	-0.96	1.96
phosphoethanolamine	-1.10	1.97
alpha-ketoisovalerate	-1.11	4.02
C38:4 PE	-1.22	3.76
xanthurenate	-1.24	0.85
levulinate	-1.24	1.06
sphingosine C	-1.25	1.57
L-threo-Sphingosine	-1.30	1.58
quinate	-1.30	2.41
C34:2 PE	-1.30	3.83
C36:5 PC plasmalogen-B	-1.36	4.74
C18:2 SM	-1.40	2.72
C14:0 SM	-1.46	3.08
NH4 C44:2 TAG	-1.53	1.07
C34:3 PE plasmalogen	-1.63	2.92
C38:7 PE plasmalogen	-1.70	3.79

C16:1 SM	-1.81	4.76
C16:0 SM	-1.81	3.43
C18:0 SM	-1.82	3.19
C36:2 PE	-1.92	3.80
C36:2 PC	-1.94	5.48
NH4 C46:3 TAG	-1.98	0.37
4-pyridoxate	-2.15	2.32
NH4 C36:3 DAG	-2.24	3.56
C20:0 SM	-2.25	2.93
NH4 C34:2 DAG	-2.34	3.70
C36:5 PE plasmalogen	-2.45	4.90
urocanic acid	-2.52	2.31
NH4 C36:2 DAG	-2.61	2.83
C38:6 PC plasmalogen	-2.64	5.01
acetyltyrosine	-2.71	1.02
C40:7 PE plasmalogen	-2.78	3.78
C34:0 PE	-2.89	5.05
NH4 C36:4 DAG	-2.97	3.68
phenyllactate	-3.14	0.96
NH4 C34:3 DAG	-3.14	3.70
C18:1 SM	-3.17	7.65
cystathionine	-3.19	1.26
C22:0 SM	-3.39	2.88
C24:0 LPC	-4.03	3.97
salicylurate	-4.03	1.89
C34:2 PE plasmalogen	-4.20	3.28
4-Hydroxystyrene	-4.58	3.75
C16:0 Ceramide (d18:1)	-4.73	2.64
NH4 C34:1 DAG	-5.10	1.05
quinolate	-5.77	4.45

Table 3: Peak ion intensity of metabolites identified by targeted LC-MS metabolomics in BAT

Metabolite	Peak ion intensity
inositol	3427821778.03
fructose/glucose/galactose	413919272.55
alanine	360888119.67
glycine	46193381.44
taurine	27033439.33
carnitine	15316834.88
adenine	15110027.97
C2 carnitine	13286857.56
citrate/isocitrate	10922461.49
AMP	10335874.89
phenylalanine	9335869.88
alpha-glycerophosphocholine	8697745.56
valine	8381334.13
glutamate	7260690.87
leucine	7187379.44
glutathione reduced	6394809.23
malate	5822206.64
ribothymidine	5249899.20
uridine	5071605.06
tryptophan	5016118.69
isoleucine	4155528.63
C18:3 LPC	4121016.81
creatine	3617224.81
C36:2 PC	3412489.13
succinate	3373055.93
acetylalanine	3299993.53
ADMA	3165508.69

C16:0 LPC B	2987221.31
erythronate	2862144.51
propionate	2700274.36
betaine	2642735.69
aspartate	2641145.00
tyrosine	2329459.88
MDA	2281532.02
N1-acetylspermidine	2224885.88
niacinamide	2188310.75
pantothenate	2094375.56
UMP	1831415.27
fucose	1797386.30
adenosine	1709185.63
glutamine	1647743.48
fumarate/maleate	1642292.87
histidine	1637336.37
NAD	1557515.35
C18:1 carnitine	1461674.94
C4 carnitine	1392106.88
eicosapentaenoate	1340125.88
2-phosphoglycerate	1335414.29
IMP	1276743.40
xanthine	1191531.81
sphingosine C	1151908.75
choline	1147215.13
sphingosine A	1122340.00
beta-alanine	1058896.94
C18:0 LPC	1052551.19
ADP	1049877.47
alpha-glycerophosphate	974885.99
2-hydroxyglutarate	936237.18
C16 carnitine	918839.94
SDMA	873514.81
caprylate	872065.50
methionine	845078.77
aconitate	789280.12
lactose	760805.53
glutathione oxidized	751790.76
sorbitol	738948.60
salicylurate	716232.73
guanine	709033.24
C4-OH carnitine	705642.25
homoarginine	700211.88
GMP	619057.74
alloisoleucine	609470.00
4-hydroxymandelate/homogentisate	557368.15
DMGV	521021.25
C18:2 LPC	504789.88
proline	496701.74
C18:2 carnitine	491143.94
2-furoylglycine	471017.69
UDP-galactose/UDP-glucose	461335.15
histamine	459852.63
phenyllactate	455084.74
phosphocholine	454417.13
urate	451942.94
arginine	421041.00
C34:3 PC	414499.44
lysine	393638.37
3-4-dihydroxyphenylglycol	393608.84
isovalerate	379923.94
C16:0 SM	375074.81
methylimidazole acetic acid	365344.25
C3 carnitine	361642.13

serine	360526.91
NADP	359472.43
butyrobetaine	347875.38
urocanic acid	332223.63
alpha-ketoglutarate	318817.96
threonine	299663.65
cystathionine	298800.60
creatinine	297123.81
N6-acetyl-L-lysine	285675.31
caproate	282295.22
C18:2 SM	281054.50
C18:1 LPC	277166.31
N6-acetyllysine	269515.80
hypoxanthine	268548.50
C14 carnitine	259562.81
alpha-hydroxybutyrate	244079.88
acetyltyrosine	238399.18
uric acid	230695.44
N-acetylgalactosamine	220675.29
C20:3 LPC	212310.13
C18 carnitine	211059.75
thymine	207478.13
methylthioadenosine	205392.75
4-methylcatechol	199174.47
glucuronate	196186.03
N-alpha-acetylarginine	196168.56
C38:6 PE	188943.38
2-aminoadipate	178513.31
inosine	178016.55
trimethyllysine	176184.31
dCMP	160773.67
C14:1 carnitine	151730.25
pyruvate	140756.09
asparagine	140311.51
C5 carnitine	136405.88
UDP	127259.66
taurodeoxycholate/taurochenodeoxycholate	125808.46
L-a-glutamyl-L-Lysine	125701.00
5-methyl-THF	125043.29
proline betaine	124535.94
C38:7 PE plasmalogen	124459.56
cytidine	117565.88
C32:2 PC	115912.00
ATP	114501.28
C38:4 PE	108835.00
C6 carnitine	107164.50
mevalonate 5-phosphate	106931.35
C34:2 PE	105097.75
indoxylsulfate	104178.50
C36:5 PE plasmalogen	99624.31
CMP	99073.33
FMN	92722.61
C36:4 PE	91555.00
C30:0 PC	81691.00
C20:4 LPC	76556.19
N4-acetylcytidine	72269.86
C12 carnitine	71869.38
linoleoyl ethanolamide	70871.07
spermidine	69204.44
C22:6 LPC	67655.38
anserine	65894.00
C40:6 PE	62908.19
C36:5 PC plasmalogen-B	60152.31
DHAP/glyceraldehyde 3P	59862.93

C36:2 PE	59228.13
C18:1-OH carnitine	58299.31
C16:1 LPC	58243.19
citrulline	58156.74
phosphoethanolamine	57568.94
phosphocreatine	55885.53
C20:3 LPC A	55815.75
pentose monophosphate	55505.96
C22:5 LPC	54407.19
C3-DC-CH3 carnitine	52922.06
C38:7 PC plasmalogen	49819.75
C8 carnitine	49230.19
pyruvaldehyde	47508.88
C18:0 SM	46333.81
3-phosphoglycerate	46284.39
1-methylnicotinamide	45678.13
C20:4 carnitine	44743.81
C3-DC carnitine	43648.88
C20:5 LPC	42829.19
4-hydroxybenzaldehyde	41839.36
hypotaurine	41538.50
corticosterone	40905.01
guanosine	40697.66
C10 carnitine	39455.50
C14:2 carnitine	37483.44
adenylosuccinate	34959.87
C16-OH carnitine	34608.38
C16:0 LPE	33807.75
alpha-ketoisovalerate	33128.38
C20:0 LPE	32768.81
GDP	32169.39
N2,N2-dimethylguanosine	31910.31
s-adenosylmethionine	31870.38
trigonelline	31256.38
hydroxyproline	28982.50
C18:0 LPE	28356.00
cyclohexylamine	28231.38
Pantothenol	27955.94
C22:6 LPE	26894.31
5-hydroxytryptophan	26487.38
serotonin	26055.88
trimethylamine-N-oxide	25694.75
C16:1 SM	25387.00
cytosine	25168.50
homovanillate	24558.99
L-threo-Sphingosine	24506.44
hexose diphosphate	23852.87
sphingosine	23728.06
xanthurenate	23649.38
malonate	21600.84
C40:7 PE plasmalogen	21438.94
C18:1 LPE	21303.56
N-acetylspermidine	21092.13
azelate	20763.97
C38:6 PC plasmalogen	19707.75
C34:3 PC plasmalogen	18489.19
hippurate	18183.79
C18:2 LPE	18175.56
adipate	17836.57
quininate	17497.72
1-methylhistamine	16907.44
C20:4 LPE	16482.75
C12:1 carnitine	16375.25
sebacate	15787.54

2-hydroxy-3-methylpentanoate	15746.58
sphingosine B	14892.38
N1-Methyl-2-pyridone-5-carboxamide	14522.94
C34:0 PE	14439.73
guanidoacetic acid	12944.75
cAMP	12692.97
C18:1 SM	11662.13
S-methyl-L-cysteine-S-oxide	11571.50
C34:3 PE plasmalogen	11562.75
ornithine	11304.50
thiamine	11055.94
7-Methylguanine	10529.06
2-deoxycytidine	10439.88
1-methyladenosine	10290.19
C5:1 carnitine	10212.50
allantoin	10108.25
N-carbamoyl-beta-alanine	9749.56
orotate	9665.38
C18:0 LPE B	9567.69
Ne,Ne dimethyllysine	9515.63
3-methylhistidine	9208.81
taurocholate	9159.86
myristoleate	8576.50
N-acetylmethionine	8419.75
C20:0 SM	8376.80
UDP-glucuronate	7942.57
C22:0 SM	7918.13
4-pyridoxate	7845.84
gentisate	7608.83
4-guanidinobutanoic acid	7537.63
pipecolic acid	7425.56
dimethylglycine	7282.63
C14:0 LPC	7248.25
3-methyladipate/pimelate	7247.27
8-hydroxy-deoxyguanosine	7132.44
quinolinate	7010.17
beta-guanidinopropionic acid	6945.94
NH4 C36:3 DAG	6796.88
sphinganine	6769.06
NMMA	6684.88
putrescine	6600.25
NH4 C36:4 DAG	6312.13
C5-DC carnitine	6151.50
imidazole propionate	6144.88
C20 carnitine	5818.44
C34:2 PE plasmalogen	5406.36
biotin	5220.19
CDP	5196.16
NH4 C34:2 DAG	5144.00
suberate	5072.52
phenylacetyl glycine	4694.13
2,1-deoxycortisol	4549.06
C20:1 LPC	4335.19
C14:0 SM	4283.06
pyridoxamine	4179.56
NH4 C34:3 DAG	4133.87
levulinate	4046.69
D-Alany-D-alanine	3786.81
NH4 C46:2 TAG	3718.31
N-acetyl leucine	3532.31
NH4 C44:1 TAG	3517.75
1-methylguanosine	3396.50
indolelactate	3372.04
5-methylcytidine	3345.69

N-acetylhistamine	3343.06
C16:1 LPC plasmalogen	3137.81
C7 carnitine	3117.44
N-acetyltryptophan	2909.94
C18:1 LPC plasmalogen	2798.06
GABA	2718.25
C18:3 LPE	2713.13
N-methylproline	2667.75
dTMP	2486.06
C18:1 LPC plasmalogen minor B	2236.13
ectoine	2187.13
N-alpha-acetylgarginine	2109.50
C20:1 LPE	1926.38
pseudouridine	1902.00
N-lauroylglycine	1889.31
5-methylcytosine	1857.00
N-acetylputrescine	1711.44
C24:0 LPC	1695.64
kynurenic acid	1680.00
acetyl-galactosamine	1677.38
6-phosphogluconate	1617.31
biliverdin	1560.64
L-alpha-glutamyl-L-lysine	1405.63
indole-3-propionate	1399.04
acetaminophen	1337.94
leucylglycine	1283.31
PEP	1266.03
methionine sulfoxide	1263.13
phytosphingosine	1168.80
oleoyl glycine	1143.50
biopterin	1120.69
N-acetylaspatic acid	1064.69
4-Hydroxystyrene	1031.11
NH4 C36:2 DAG	1016.07
cotinine N-oxide	1014.13
pterin	965.94
N-acetylhistidine	907.13
1-methylguanine	844.81
xanthosine	838.40
2-deoxyadenosine	835.56
5-Hydroxytryptophol	825.13
C16:0 Ceramide (d18:1)	695.60
1,2,4-trimethylbenzene	643.63
C9 carnitine	602.25
N-acetylglutamine	578.06
methyl N-methylanthranilate	576.75
imidazoleacetic acid	571.19
methylguanidine	535.38
N-acetylmethionine	510.13
anthranilic acid	507.25
4-Hydroxy-3-methylacetophenone	506.75
NH4 C44:2 TAG	482.44
gabapentin	459.00
6,8-dihydroxypurine	361.73
carosine	332.15
cotinine	317.20
C24:1 Ceramide (d18:1)	276.00
XMP	271.87
NH4 C34:1 DAG	257.25
NH4 C46:3 TAG	198.50
pyroglutamic acid	142.81
C36:3 PS plasmalogen	74.82

Table 4: Quantification of metabolites identified by targeted LC-MS metabolomics.

Relative abundance is expressed as BAT abundance following 3 Hrs exposure of mice to 4 degrees relative to BAT from mice exposed to thermoneutrality. Log2FC = log2 of fold change between BAT from cold exposed mice versus thermoneutrality. Negative

5 log10(pvalue) = negative log10 of the P value

Metabolite	Log2FC	negative LOG10(pvalue)
UDP-glucuronate	5.98	4.22
C20 carnitine	5.29	3.51
FMN	4.88	7.04
dCMP	4.66	7.72
XMP	4.26	1.98
C9 carnitine	4.05	5.38
C14:2 carnitine	3.93	4.34
leucylglycine	3.71	4.88
carosine	3.68	5.44
C10 carnitine	3.54	4.80
D-Alany-D-alanine	3.53	4.81
N-acetylaspartic acid	3.45	3.89
C14:1 carnitine	3.45	4.57
C12 carnitine	3.38	5.37
NADP	3.19	5.19
xanthosine	3.14	3.80
C3-DC-CH3 carnitine	3.08	6.13
C8 carnitine	3.08	5.36
C14 carnitine	2.99	5.60
CDP	2.63	4.07
adenylosuccinate	2.61	3.59
N-acetylmethionine	2.61	7.47
C7 carnitine	2.52	4.07
3-4-dihydroxyphenylglycol	2.51	5.25
N-carbamoyl-beta-alanine	2.49	7.30
6,8-dihydroxypurine	2.47	3.37
C3-DC carnitine	2.45	7.17
C12:1 carnitine	2.45	4.39
phosphocholine	2.42	6.64
hippurate	2.35	1.32
PEP	2.31	1.79
2,1-deoxycortisol	2.28	2.48
acetyl-galactosamine	2.27	5.48
uric acid	2.24	5.11
glutathione oxidized	2.20	5.79
C6 carnitine	2.19	4.71
2-deoxvadenosine	2.18	4.17
beta-alanine	2.13	7.21
C18 carnitine	2.05	4.86
N-acetyl-galactosamine	2.02	5.33
C5-DC carnitine	1.98	8.60
C20:4 carnitine	1.94	4.28
N-acetylglutamine	1.93	3.10
ATP	1.92	2.43
N4-acetylcytidine	1.91	4.16
cAMP	1.90	7.50
N2,N2-dimethylguanosine	1.86	4.41
betaine	1.86	8.12
ADP	1.80	2.08
succinate	1.77	6.72
ADMA	1.77	3.09
C5 carnitine	1.76	2.55
C4 carnitine	1.73	5.92

trimethylamine-N-oxide	1.73	5.15
anserine	1.70	1.56
linoleoyl ethanolamide	1.70	5.17
AMP	1.68	7.06
N1-Methyl-2-pyridone-5-carboxamide	1.65	8.70
adenosine	1.64	9.19
xanthurenate	1.61	1.57
N-acetylleucine	1.60	5.19
guanine	1.59	3.32
guanosine	1.58	3.79
Ne,Ne dimethyllysine	1.58	3.44
1-methylguanosine	1.56	5.46
C5:1 carnitine	1.56	1.64
acetyltyrosine	1.56	0.62
SDMA	1.53	3.00
GDP	1.53	1.86
corticosterone	1.52	1.87
N-acetylspermidine	1.49	4.69
N1-acetylspermidine	1.49	4.72
thiamine	1.47	4.77
DMGV	1.45	7.71
N6-acetyl-L-lysine	1.42	5.98
urocanic acid	1.42	2.82
cystathionine	1.40	1.85
pyroglutamic acid	1.39	3.78
UDP	1.38	2.38
CMP	1.38	6.40
hexose diphosphate	1.37	1.07
C18:1 carnitine	1.35	3.22
cytidine	1.34	4.89
butyrobetaine	1.34	5.68
N-alpha-acetylarginine	1.32	5.92
C16 carnitine	1.30	3.29
adenine	1.30	7.31
1-methylguanine	1.29	4.20
N-alpha-acetylarginine	1.29	5.85
spermidine	1.28	4.08
leucine	1.28	3.78
kynurenic acid	1.25	3.27
mevalonate 5-phosphate	1.24	1.72
biotin	1.20	2.02
valine	1.19	4.80
UMP	1.18	7.31
C18:1-OH carnitine	1.17	5.70
isoleucine/leucine	1.15	4.43
guanidoacetic acid	1.12	4.35
alpha-hydroxybutyrate	1.10	3.44
1-methyladenosine	1.10	3.98
6-phosphogluconate	1.09	1.44
5-methylcytosine	1.08	2.04
inosine	1.08	3.07
7-Methylguanine	1.06	4.96
8-hydroxy-deoxyguanosine	1.06	5.43
taurine	1.05	8.48
aconitate	1.05	2.84
N6-acetyllysine	1.04	3.63
allantoin	1.02	5.31
pseudouridine	1.02	3.67
dTMP	1.02	2.09
NMMA	1.01	1.82
homoarginine	1.00	1.92
C16-OH carnitine	1.00	4.76
NAD	0.99	5.56
isoleucine	0.99	3.48

hydroxyproline	0.99	2.02
hypotaurine	0.96	0.57
5-methylcytidine	0.95	5.08
urate	0.95	3.93
dimethylglycine	0.95	4.26
glutamine	0.95	1.70
taurocholate	0.94	0.17
alloisoleucine	0.94	4.01
pentose monophosphate	0.93	3.80
choline	0.90	4.83
N-acetylhistidine	0.90	3.43
4-hydroxybenzaldehyde	0.88	1.86
C20:4 LPE	0.87	4.83
taurodeoxycholate/taurochenodeoxycholate	0.87	0.16
proline betaine	0.87	4.01
glutamate	0.86	2.84
L-α-glutamyl-L-Lysine	0.86	3.04
ribothymidine	0.86	3.75
2-hydroxyglutarate	0.86	2.06
β-guanidinopropionic acid	0.85	2.73
serine	0.84	2.39
fructose/glucose/galactose	0.84	4.72
C3 carnitine	0.84	4.13
acetylalanine	0.81	7.40
sebacate	0.77	1.55
phosphoethanolamine	0.74	2.96
caprylate	0.74	1.24
uridine	0.73	3.89
cytosine	0.70	1.17
L-α-glutamyl-L-lysine	0.70	2.41
methyl N-methylanthranilate	0.69	1.15
niacinamide	0.69	3.76
methylthioadenosine	0.69	3.25
s-adenosylmethionine	0.68	2.24
carnitine	0.67	4.77
creatine	0.66	2.64
thymine	0.65	2.72
2-deoxycytidine	0.65	0.89
biopterin	0.65	1.79
C18:2 LPE	0.64	3.70
C2 carnitine	0.63	5.00
1-methylhistamine	0.63	2.27
fucose	0.60	1.83
sphingosine	0.60	0.42
1,2,4-trimethylbenzene	0.60	0.85
sorbitol	0.58	3.16
sphingosine A	0.57	0.41
oleoyl glycine	0.57	0.24
N-acetylmethionine	0.57	2.38
histidine	0.55	1.83
aspartate	0.52	1.53
trigonelline	0.51	0.79
lysine	0.51	0.94
histamine	0.50	2.25
trimethyllysine	0.50	1.67
pipecolic acid	0.49	3.09
xanthine	0.48	1.42
citrate/isocitrate	0.48	1.27
alanine	0.45	1.76
GMP	0.42	1.75
glutathione reduced	0.42	3.14
α-glycerophosphate	0.42	1.77
serotonin	0.41	1.63
C18:0 LPE B	0.41	3.08

sphingosine B	0.41	0.27
IMP	0.37	0.84
glycine	0.37	1.31
imidazoleacetic acid	0.35	0.49
C18:2 carnitine	0.34	0.53
GABA	0.34	0.72
citrulline	0.32	0.56
methionine sulfoxide	0.30	0.42
hypoxanthine	0.30	0.50
phenylacetyl glycine	0.29	0.47
5-hydroxytryptophan	0.28	0.99
tyrosine	0.28	1.73
N-acetyltryptophan	0.28	0.50
asparagine	0.27	0.65
C4-OH carnitine	0.26	0.32
pterin	0.23	0.31
creatinine	0.23	0.47
N-lauroyl glycine	0.22	0.43
proline	0.19	0.48
arginine	0.19	0.71
UDP-galactose/UDP-glucose	0.19	0.58
azelate	0.14	0.17
suberate	0.12	0.24
4-hydroxymandelate/homogentisate	0.12	0.15
cotinine	0.11	0.06
phenylalanine	0.10	0.26
pyridoxamine	0.09	0.15
tryptophan	0.06	0.25
4-guanidinobutanoic acid	0.06	0.08
acetaminophen	0.06	0.20
methionine	0.05	0.10
C22:6 LPE	0.04	0.10
threonine	0.04	0.09
N-methylproline	0.04	0.08
N-acetylhistamine	0.03	0.08
cyclohexylamine	-0.01	0.36
C14:0 LPC	-0.01	0.03
anthranilic acid	-0.03	0.06
sphingosine C	-0.04	0.04
gentisate	-0.04	0.06
L-threo-Sphingosine	-0.05	0.06
ornithine	-0.06	0.11
NH4 C44:2 TAG	-0.06	0.03
NH4 C46:2 TAG	-0.08	0.06
4-pyridoxate	-0.09	0.03
Pantothenol	-0.11	0.05
C16:0 LPC B	-0.11	0.79
2-hydroxy-3-methylpentanoate	-0.15	0.58
lactose	-0.16	0.14
phytosphingosine	-0.16	0.19
3-methylhistidine	-0.17	0.33
C18:1 LPC plasmalogen	-0.21	0.80
myristoleate	-0.26	0.43
indoxylsulfate	-0.27	0.21
1-methylnicotinamide	-0.28	1.08
NH4 C44:1 TAG	-0.33	0.31
NH4 C46:3 TAG	-0.34	0.11
pyruvaldehyde	-0.37	1.48
fumarate/maleate	-0.40	1.79
malate	-0.40	1.68
orotate	-0.41	0.38
alpha-ketoglutarate	-0.44	0.70
homovanillate	-0.47	0.90
putrescine	-0.49	1.34

methylguanidine	-0.53	0.40
C18:1 LPE	-0.55	2.80
N-acetylputrescine	-0.56	1.52
inositol	-0.56	7.47
S-methyl-L-cysteine-S-oxide	-0.57	1.09
C18:2 LPC	-0.60	5.86
C20:3 LPC	-0.63	5.63
C18:3 LPC	-0.65	5.59
3-phosphoglycerate	-0.72	3.18
imidazole propionate	-0.72	0.56
3-methyladipate/pimelate	-0.73	1.37
2-aminoadipate	-0.74	1.52
erythronate	-0.76	3.81
glucuronate	-0.77	5.70
isovalerate	-0.81	1.26
C16:1 LPC	-0.81	3.37
C20:0 LPE	-0.83	6.09
quinat	-0.85	2.47
cotinine N-oxide	-0.85	2.22
C18:0 LPC	-0.87	5.58
malonate	-0.88	3.71
pantothenate	-0.89	2.81
2-phosphoglycerate	-0.92	3.48
C16:0 LPE	-0.99	6.13
alpha-ketoisovalerate	-1.00	1.88
methylimidazole acetic acid	-1.01	3.08
alpha-glycerophosphocholine	-1.05	5.81
C20:4 LPC	-1.06	3.60
DHAP/glyceraldehyde 3P	-1.07	3.08
C22:6 LPC	-1.08	3.98
C20:5 LPC	-1.08	8.33
sphinganine	-1.14	3.35
C36:4 PE	-1.15	2.94
caproate	-1.20	0.42
MDA	-1.20	5.86
inosine-15N4	-1.21	5.03
propionate	-1.23	3.99
levulinate	-1.28	1.38
gabapentin	-1.34	6.70
quinolinate	-1.37	1.46
C18:1 LPC	-1.40	7.59
indole-3-propionate	-1.46	3.50
phosphocreatine	-1.47	1.70
C18:1 LPC plasmalogen minor B	-1.50	3.39
eicosapentaenoate	-1.54	3.33
adipate	-1.68	3.47
5-Hydroxytryptophol	-1.72	2.25
C22:5 LPC	-1.73	5.98
C18:0 LPE	-1.77	7.31
C34:2 PE	-1.78	4.32
C20:3 LPC A	-1.83	5.97
C18:3 LPE	-1.84	7.79
NH4 C34:1 DAG	-1.87	0.45
C38:4 PE	-1.95	3.16
4-Hydroxy-3-methylacetophenone	-1.99	1.00
NH4 C36:2 DAG	-2.00	0.98
C38:6 PE	-2.06	5.30
ectoine	-2.17	2.29
pyruvate	-2.27	3.01
C36:2 PE	-2.43	3.75
C16:0 Ceramide (d18:1)	-2.52	0.84
C20:1 LPE	-2.72	9.05
C16:1 LPC plasmalogen	-2.82	5.60
C36:2 PC	-2.89	4.63

indolelactate	-2.98	4.21
C18:2 SM	-3.12	9.61
C16:0 SM	-3.21	6.95
4-Hydroxystyrene	-3.41	2.56
C34:3 PC	-3.46	6.40
C32:2 PC	-3.73	5.84
C40:6 PE	-4.03	4.67
C36:5 PE plasmalogen	-4.48	5.20
NH4 C36:3 DAG	-4.74	2.95
C16:1 SM	-4.76	8.55
C18:0 SM	-4.78	5.94
NH4 C34:2 DAG	-4.88	3.66
C38:7 PE plasmalogen	-5.18	5.13
C20:1 LPC	-5.75	6.18
C30:0 PC	-5.94	6.38
NH4 C34:3 DAG	-6.42	2.98
NH4 C36:4 DAG	-6.75	3.96
C14:0 SM	-6.91	6.58
C36:5 PC plasmalogen-B	-7.34	4.62
C20:0 SM	-7.48	3.33
C38:7 PC plasmalogen	-7.49	4.49
C38:6 PC plasmalogen	-7.68	4.61

Absolute succinate concentrations in 4°C-activated BAT were over 100x that found in heart and over 10x that found in kidney (Figures 1E and 2A-2B), making it by far the most succinate-enriched tissue observed. Moreover, succinate accumulation upon activation of thermogenesis in BAT was highly selective compared to other TCA cycle metabolites (Figures 1E, 1F, 2C, and 2D). Additionally, increasing thermogenic beige adipocyte content in subcutaneous adipose tissue by chronic exposure to 4°C significantly increased succinate concentration (Figure 1G).

10 **Example 3: Selective and substantial accumulation of succinate in brown adipocytes through extracellular uptake**

To investigate the role of succinate in BAT thermogenesis, the mechanisms responsible for driving its selective accumulation were explored. Whereas pharmacological β -adrenergic stimulation of BAT was sufficient to drive triglyceride lipolysis (Figures 3E-3G), stimulation of β 1, β 2 or β 3-adrenergic receptors had no effect on succinate levels in BAT (Figure 3H). Therefore, selective and substantial accumulation of succinate is a signature of adipose tissue thermogenesis upon cold exposure, but is independent of β -adrenergic signaling. As expected, pharmacological β -adrenergic stimulation of BAT using CL-316,243 (CL) was sufficient to drive triglyceride lipolysis (Figures 3A-3C), yet had no effect on BAT succinate levels (Figures 1H and 3D).

To assess the contribution of established metabolic inputs to the build-up of succinate upon cold-induced BAT thermogenesis an array of ^{13}C -isotopologue labelling

experiments *in vivo* followed by LC-MS analyses was performed. In mammalian cells succinate is generated by the TCA cycle, and BAT TCA metabolism is driven by oxidation of carbons from glucose and fatty acids (Figure 4A). As expected, following intravenous (i.v.) bolus injection at thermoneutrality [U-¹³C]-glucose was taken up by BAT (Figure 5A) and oxidized by the TCA cycle (Figures 4B, 5B-5D, and 6F-6G), as indicated by the diagnostic ($m + 2$) and ($m + 4$) isotopologues of the TCA intermediates (Figures 4 and 5B-5D). Labelled $m + 3$ lactate was increased in comparison to other glycolytic intermediates (Figure 6G). However, the contribution of [U-¹³C]-glucose to succinate was unchanged in BAT following exposure to 4°C and was comparable to other TCA cycle metabolites (Figure 4B). I.V.-administered [U-¹³C]-palmitate was also readily taken up by BAT (Figure 6A) and TCA cycle intermediates were enriched in ¹³C-carbons derived from palmitate oxidation (Figures 4C and 6B-6D). Following exposure to 4°C, the contribution of [U-¹³C]-palmitate to succinate remained unchanged and was comparable to that in other TCA cycle metabolites (Figure 4C). Together these data demonstrate that the major carbon sources for the BAT TCA do not contribute to succinate build-up upon activation of thermogenesis.

Lack of conventional TCA cycle involvement (Figures 4B-4C, 5A-5D, 6A-6D, and 6F-6G), and the unusual selectivity of succinate accumulation upon activation of thermogenesis (Figures 1E-1F), indicated BAT can sequester succinate from circulation (Figure 4D). It is well established that most mammalian cell membranes are impermeable to succinate (Hem *et al.* (1968) *Biochem. J.* 107:807-815; Ehinger *et al.* (2016) *Nat. Commun.* 7:12317; MacDonald *et al.* (1989) *Arch. Biochem. Biophys.* 269:400-406). However, circulating succinate levels are substantial and dynamic, and reported extracellular concentrations range from low micromolar to millimolar (Hochachka and Dressendorfer (1976) *Eur. J. Appl. Physiol. Occup. Physiol.* 35:235-242; Sadagopan *et al.* (2007) *Am. J. Hypertens.* 20:1209-1215; Correa *et al.* (2007) *J. Hepatol.* 47:262-269). Exposure of mice to 4°C led to substantial elevation in circulating succinate (Figures 4E and 8E), indicating the extracellular pool of succinate contributes to accumulation in BAT. Differentiated primary brown adipocytes were monitored in standard medium, which lacks extracellular succinate. Adipocyte succinate concentrations were substantially reduced compared to *in vivo* BAT (Figures 6E and 8F). Brown adipocytes were then supplemented with [U-¹³C]-succinate. Unlike most mammalian cells, which are impermeable to extracellular succinate (Hem *et al.* (1968) *Biochem. J.* 107:807-815; Ehinger *et al.* (2016)

Nat. Commun. 7:12317; MacDonald *et al.* (1989) *Arch. Biochem. Biophys.* 269:400-406), brown adipocytes rapidly internalized [U-¹³C]-succinate leading to substantial (~45x) elevation in intracellular succinate levels (Figure 4F). This capacity for succinate internalization was not observed in differentiated white adipocytes or pre-adipocytes isolated from BAT (Figure 4J). Moreover, brown adipocytes did not display comparable capacity to internalize the structurally similar mitochondrial dicarboxylates fumarate and α-ketoglutarate (Figure 4K). Succinate internalization by brown adipocytes resulted in accumulation of labelled (m + 4) isotopologues of TCA cycle metabolites downstream of mitochondrial succinate oxidation (Figures 4G and 8G-8I). TCA cycle utilization of extracellular succinate was additionally demonstrated by rapid mitochondrial cataplerosis and accumulation of the (m + 4) isotopologue of aspartate and m + 2 and m + 3 citrate (Figures 4H, 4L, 8I, and 8J). Intravenous [U-¹³C]-succinate was also readily taken up by BAT *in vivo* resulting in increased abundance of the endogenous pool (Figures 4I and 4M), coinciding with metabolism of succinate to downstream TCA cycle metabolites (Figures 4I, 4N, and 8L) and clearance of [U-¹³C]succinate from blood plasma (Figures 4O and 8L). The results indicated that brown adipocytes have the distinct capacity to accumulate and oxidize succinate by sequestering it from the extracellular milieu.

BAT accumulates increased circulating succinate (Fig. 4M), the levels of which increase upon exposure to 4 °C (Figure 8E). This indicates that upon exposure to cold, peripheral tissues supply succinate to BAT via the circulation. Interventions that drive muscle contraction, such as exercise, are also known to result in increased circulating succinate (Hochachka and Dressendorfer (1976) *Eur. J. Appl. Physiol. Occup. Physiol.* 35:235-242). Since muscle shivering is an early response to exposure to environmental cold, it was believed that this contractile activity could drive succinate release from muscle to supply BAT accumulation. Upon exposure of mice to 4 °C for 30 min, extensive shivering by electromyography (EMG) was observed (Figure 8M). The shivering could be inhibited using the nicotinic acetylcholine receptor inhibitor curare (Figure 8M); this significantly blunted cold-dependent accumulation of succinate in BAT (Figure 8N). These findings indicate that shivering muscle can be a source of succinate, although effects of curare on respiratory function can also contribute.

Example 4: Brown adipocyte thermogenic leak respiration is controlled by intracellular accumulation of succinate

The newfound ability for brown adipocytes to rapidly internalize extracellular succinate was used to investigate the potential role of succinate accumulation in brown adipocyte thermogenesis (Figure 4F). Upon succinate addition, thermogenic respiration in primary brown adipocytes was directly monitored using cellular respirometry adipocytes (Li *et al.* (2014) *EMBO Rep.* 15:1069-1076; Chouchani *et al.* (2016) *Nature* 532:112-116). Succinate addition resulted in acute and robust concentration-dependent stimulation of brown adipocyte respiration (Figures 7A-7B). Succinate-stimulated respiration was specifically attributable to thermogenic leak respiration, which is UCP1-dependent in brown adipocytes (Li *et al.* (2014) *EMBO Rep.* 15:1069-1076; Chouchani *et al.* (2016) *Nature* 532:112-116) (Figure 7C). Succinate utilization had no effect on chemically-induced maximal respiration, indicating that its effects were not attributable to increased mitochondrial substrate supply (Brand and Nicholls (2011) *Biochem. J.* 435:297-312) (Figure 7C). Moreover, the capacity for succinate to trigger thermogenic leak respiration was unique, with other mitochondrial or cellular substrates having no effect (Figures 7D 8A, and 8O). Testing of a comprehensive panel of cell types revealed succinate-stimulated leak respiration to be distinct to mature primary brown adipocytes and immortalized brown adipocytes and immortalized human brown adipocytes (Figures 7E, 8B-8D, and 8P-8Q). Capacity for internalization of extracellular succinate to trigger thermogenic leak respiration is a distinct feature of brown adipocytes. Notably, broad pharmacological inhibition of plasma membrane transport or inhibition of plasma membrane secondary active transport via the Na⁺/K⁺-ATPase inhibited succinate-stimulated respiration (Figures 8R-8W).

Succinate-dependent respiration did not require ligation of its cognate G-protein coupled receptor, succinate receptor 1 (SUCNR1; Figure 9A) (He *et al.* (2004) *Nature* 429:188-193). Moreover, succinate-dependent thermogenesis did not involve elevation of cAMP levels (Figure 9B), activation of protein kinase A (PKA) signaling (Figure 9C), or elevated lipolysis (Figure 9D). Therefore, succinate-dependent thermogenesis is independent of SUCNR1 signalling and the lipolytic cascade.

Mitochondrial succinate oxidation can drive extensive ROS formation under certain conditions (Murphy (2009) *Biochem. J.* 417:1-13). Elevation of ROS levels in brown adipocytes can support thermogenesis (Chouchani *et al.* (2016) *Nature* 532:112-116; Han *et*

al. (2016) *Diabetes* 65:2639-2651; Chouchani *et al.* (2017) *J. Biol. Chem.* 292:16810-16816), but the mechanisms that control thermogenic ROS production are unknown. It was found that the thermogenic effects of succinate were recapitulated in isolated BAT mitochondria (Figures 9H-9J), and that the mitochondrial dicarboxylate carrier SLC25A10 is highly expressed in BAT (Figure 9K). SLC25A10 mediates rapid equilibration of mitochondrial and cytosolic succinate pools, indicating that mitochondria in brown adipocytes can access extracellular succinate. Indeed, chemical inhibition of SLC25A10 blunted succinate-driven respiration (Figures 9L and 9M). Furthermore, addition of succinate to brown adipocytes resulted in a rapid and robust increase in ROS production (Figures 7F, 9E-9F, and 9N), indicating that it is a potent molecular source of ROS in these cells. Since protein cysteine residues are the principal signaling targets of thermogenic ROS (Quastel and Wooldridge (1928) *Biochem. J.* 22:689-702), the oxidation status of catalytic cysteines in the peroxiredoxin (Prx) family was profiled using MS. Prx cysteines are major cysteine targets of ROS, exhibiting hyperoxidation to sulfonic acid (SO₃⁻) in response to elevated ROS levels. Due to the selective compartmentalization of Prx family members, assessment of Prx redox state is indicative of local changes in ROS signaling. Prx3, the only Prx isoform expressed exclusively in the mitochondrial matrix, uniquely exhibited elevation of cysteine hyperoxidation to SO₃⁻ following succinate treatment, whereas Prx1, Prx2, Prx4 and Prx5 did not (Figures 7G, 9O, and 9G). Succinate-induced ROS production shifts thiol redox status in the mitochondrial matrix specifically. To examine the relevance of succinate-driven ROS for thermogenesis the mitochondria-targeted antioxidant MitoQ20 was used to deplete succinate-induced mitochondrial ROS levels, or inhibited ROS-dependent cysteine oxidation using N-acetyl cysteine (NAC) (Atkuri *et al.* (2007) *Curr. Opin. Pharmacol.* 7:355-359). Suppression of both processes substantially inhibited succinate-dependent respiration (Figures 7H and 10A). By contrast, increasing the cysteine oxidation state with diamide was sufficient to drive respiration in brown adipocytes (Figures 10F and 10G).

Succinate can control ROS levels by fueling superoxide production through several proximal electron circuits in the mitochondrial respiratory chain (Figures 7I, 7L, and 10H). All of which require succinate oxidation by the flavin site on SDH (Figures 7I and 7L). Treatment of brown adipocytes with malonate, a competitive inhibitor of succinate oxidation by the SDH flavin (Figures 7I, 7L, and 10H) (Quastel and Wooldridge (1928) *Biochem. J.* 22:689-702), abrogated both succinate-dependent ROS production and

succinate-dependent thermogenic respiration (Figures 7J-7K, 10B, and 10I). Systematic manipulation of the downstream superoxide producing sites linked to succinate oxidation (Figure 10H) revealed electron transfer between SDH and ubiquinone was required, since inhibition at the SDH Q-site (Miyadera *et al.* (2003) *PNAS* 100, 473-477) fully inhibited succinate-dependent thermogenesis (Figures 7K and 10C).

Inhibition of ROS production through mitochondrial complex I (Brand *et al.* (2016) *Cell Metab.* 24, 582-592), mitochondrial complex III (Orr *et al.* (2015) *Nat. Chem. Biol.* 11:834-836, or α -glycerophosphate dehydrogenase (Orr *et al.* (2014) *PLoS One* 9, e89938) (α GPDH) did not have as substantial effects on succinate-stimulated respiration (Figures 7K and 10D, 10E, 10J-10L), but these sites can be relevant and are more fully explored.

Taken together, the data provide a model for an activation pathway for adipocyte thermogenic respiration, wherein BAT possesses a distinct capacity to sequester elevated circulating succinate (Figure 4), rapidly oxidizing it at SDH to drive Q pool reduction, ROS production, and thermogenesis (Figure 7). Such a model is believed to clarify many unexplained aspects of BAT thermogenesis, such as the importance of ROS in supporting BAT heat production (Chouchani *et al.* (2016) *Nature* 532:112-116; Han *et al.* (2016) *Diabetes* 65:2639-2651; Chouchani *et al.* (2017) *J. Biol. Chem.* 292:16810-16816), and the observation that adrenergic stimulus does not fully recapitulate the acute thermogenic effect of exposure to 4°C (Pfeifer *et al.* (2015) *Annu. Rev. Pharmacol. Toxicol.* 55:207-227; Cypess *et al.* (2015) *Cell Metab.* 21:33-38; Carey *et al.* (2013) *Diabetologia* 56:147-155; Ravussin *et al.* (2014) *PLoS One* 9:e85876; Hanssen *et al.* (2015) *Nature Med.* 21:863-865). Moreover, it provides an integrating paradigm for the longstanding and unexplained observation of variable and substantial concentrations of circulating succinate (Hochachka and Dressendorfer (1976) *Eur. J. Appl. Physiol. Occup. Physiol.* 35:235-242; Sadagopan *et al.* (2007) *Am. J. Hypertens.* 20:1209-1215; Correa *et al.* (2007) *J. Hepatol.* 47:262-269).

Example 5: Elevated circulating succinate stimulates UCP1-dependent thermogenesis *in vivo* and protects against diet-induced obesity

This model predicts that acute increase of systemic succinate should be sufficient to drive BAT thermogenesis *in vivo*. Intravenous injection of succinate resulted in a rapid increase in interscapular BAT temperature (Figure 11A) and whole-body oxygen consumption (Figures 11B and 11O). The effects peaked at the time of maximal utilization of circulating succinate by BAT (Figures 4I and 4N). Importantly, in mice genetically lacking UCP1 (UCP1 KO) the thermogenic effects of succinate were abrogated (Figures

11B and 11O). Conversely, thermogenesis initiated by cold exposure was suppressed by co-administration of the SDH inhibitor malonate (Figure 11P). Together, these findings indicate that BAT utilization of circulating succinate drives a robust increase in UCP1-dependent whole-body energy expenditure.

5 The effect of elevating circulating succinate resulting in UCP1-dependent thermogenesis *in vivo* prompted the investigation of the role of this pathway in modulation of whole-body energy expenditure in diet-induced obesity. Dietary succinate is well tolerated and safe at substantial concentrations (Maekawa *et al.* (1990) *Chem. Toxicol.* 28:235-241) and acute oral administration resulted in elevation of circulating succinate
10 (Figures 11C and 11Q) (Browne *et al.* (1978) *Proc. R. Soc. Lond. B Biol. Sci.* 200:117-135). *Ad libitum* high-fat diet fed mice were provided drinking water supplemented with sodium succinate at doses ranging from 1% to 2%. No aversion to succinate was observed (Figures 12A-12B and 12I-12J), and mice exhibited a robust dose-dependent suppression and reversal of weight gain induced by high fat feeding over four weeks (Figures 11D-11E
15 and 12C-12D) due to diminution of fat mass (Figures 11F-11G). Several lines of evidence indicate that this robust protection against diet-induced obesity was attributable at least partly attributable to an increase in energy expenditure based on several lines of evidence. First, succinate administration did not inhibit *ad libitum* caloric intake (Figures 12E-12F and 12K-12L). Moreover, controlled pair feeding recapitulated the metabolic phenotype
20 observed under *ad libitum* conditions (Figures 11F-11G and 12D). Succinate supplementation did not inhibit caloric absorption or energy assimilation (Figures 12G-12H). Most importantly, the energy balance method was applied to determine free-living whole-body energy expenditure which controls for caloric intake, caloric absorption, and changes in body weight and body composition over the course of an experiment (Ravussin
25 *et al.* (2013) *Int. J. Obes. Lond.* 37:399-403; Goldgof *et al.* (2014) *J. Biol. Chem.* 289:19341-19350). This analysis revealed that succinate supplementation drove a robust elevation in whole-body energy expenditure over the four-week period (Figures 11I and 11L). These systemic effects of dietary succinate resulted in brown, subcutaneous and epididymal adipose depots exhibiting smaller adipocyte size and reduced lipid
30 accumulation (Figure 13A). Additionally, livers of mice receiving dietary succinate exhibited reduced lipid deposition, whereas heart and kidneys were morphologically indistinguishable from controls (Figures 13B-13F).

Succinate supplementation lowered fasting circulating glucose levels (Figure 11K), and reversed glucose intolerance induced by high fat feeding (Figures 11K and 13G). Moreover, there was no evidence of increased inflammatory or anti-inflammatory markers in adipose tissues; and in some cases, such as for interleukin-1 β , there was a decrease in expression (Figures 13H and 13I). These findings are noteworthy, since in certain physiological settings, circulating succinate can engage immune cell recruitment and activation (Peruzzotti-Jametti *et al* (2018) *Cell Stem Cell* 22:355-368; Littlewood-Evans *et al* (2016) *J. Exp. Med.* 213:1655-1662). The lack of immunogenic signalling by succinate here can be explained by the function of thermogenic adipose tissue as a sink for succinate (Figure 4M), rapidly clearing circulating succinate (Figures 4N and 4O) that would otherwise be predicted to antagonize immunogenic signaling (Peruzzotti-Jametti *et al* (2018) *Cell Stem Cell* 22:355-368; Littlewood-Evans *et al* (2016) *J. Exp. Med.* 213:1655-1662). This interpretation is supported by the observation that dietary succinate potentiated inflammatory signalling in adipose depots of UCP1(KO) mice but not those of wild-type mice (Figure 13H), indicating that competent thermogenic adipose tissue antagonizes inflammatory signalling by succinate in a dominant fashion. Finally, to determine whether the robust weight loss and energy expenditure effects of dietary succinate required adipose tissue thermogenesis, the physiological effect of succinate supplementation in UCP1(KO) mice, a genetic model lacking thermogenically competent adipose tissue was examined (Kazak *et al* (2017) *Proc. Nat. Acad. Sci.* 114:7981-7986). The inhibitory effects of succinate supplementation on weight gain (Figures 11M, 11N and 14A-14E) and stimulatory effects on energy expenditure (Figure 14F) of succinate supplementation did not occur in UCP1(KO) mice.

Based on the foregoing, these data identify an unexpected mechanism for activation of BAT thermogenesis through utilization of a systemic pool of the TCA cycle intermediate succinate. This pathway operates independently from the canonical BAT lipolytic cascade. Succinate exerts acute control over UCP1-dependent thermogenesis by triggering mitochondrial ROS production via SDH oxidation. Succinate-dependent thermogenesis is systemically integrated by the newfound capacity for brown adipocytes to sequester extracellular succinate. Therefore, as well as identifying a molecular pathway for activation of adipocyte thermogenesis, these results demonstrate that succinate acts as a previously unappreciated systemic redox signal, and exerts profound effects on whole-body metabolism. This distinct faculty can be leveraged through pharmacological elevation of

circulating succinate to drive BAT thermogenesis *in vivo*. Dietary interventions that involve acute elevation of systemic succinate levels can protect against metabolic disease in individuals with a sufficient endowment of thermogenic adipose tissue.

5 **Incorporation by Reference**

All publications, patents, and patent applications mentioned herein are hereby incorporated by reference in their entirety as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

10 Also incorporated by reference in their entirety are any polynucleotide and polypeptide sequences which reference an accession number correlating to an entry in a public database, such as those maintained by The Institute for Genomic Research (TIGR) on the World Wide Web and/or the National Center for Biotechnology Information (NCBI) on the World Wide Web.

15

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following

20 claims.

What is claimed:

1. An agent that modulates succinate in a subject, for use in modulating a metabolic response in the subject, optionally wherein the agent is formulated with a pharmaceutically acceptable carrier.
2. The agent of claim 1, wherein succinate is upregulated.
3. The agent of claim 2, wherein the succinate is upregulated using an agent selected from the group consisting of succinic acid and salts thereof, and an agent that promotes muscle contraction.
4. The agent of claim 2 or 3, wherein the medicament further comprises an additional agent that increases the metabolic response.
5. The agent of claim 2, wherein the succinate is downregulated.
6. The agent of claim 5, wherein the succinate is downregulated using an agent selected from the group consisting of a metabolizer of succinate, an antioxidant, a mitochondria-targeted antioxidant, an inhibitor of muscle shivering, an inhibitor of plasma membrane transport, an inhibitor of plasma membrane secondary active transport via the Na⁺/K⁺-ATPase, an inhibitor of SLC25A10, and an inhibitor of ROS-dependent cysteine oxidation.
7. The agent of any one of claims 1-6, wherein the medicament further comprises an additional agent that decreases the metabolic response.
8. The agent of any one of claims 1-7, wherein the metabolic response is selected from the group consisting of:
 - a) modified expression of a marker selected from the group consisting of: cidea, adiponectin, adipsin, otopetrin, type II deiodinase, cig30, ppar gamma 2, pgc1 α , ucpl1, elovl3, cAMP, Prdm16, cytochrome C, cox4i1, coxIII, cox5b, cox7a1, cox8b, glut4, atpase b2, cox II, atp5o, ndufb5, ap2, ndufs1, GRP109A, acylCoA-thioesterase 4, EARA1, claudin1, PEPCK, fgf21, acylCoA-thioesterase 3, dio2, fatty acid synthase (fas), leptin, resistin, and nuclear respiratory factor-1 (nrf1);
 - b) modified thermogenesis in adipose cells;

- c) modified differentiation of adipose cells;
 - d) modified insulin sensitivity of adipose cells;
 - e) modified basal respiration, leak respiration, or uncoupled respiration;
 - f) modified whole body oxygen consumption;
 - g) modified obesity or appetite;
 - h) modified insulin secretion of pancreatic beta cells;
 - i) modified glucose tolerance; and
 - j) modified activity of UCP1 protein.
9. The agent of any one of claims 1-8, wherein the metabolic response is upregulated.
10. The agent of any one of claims 1-8, wherein the metabolic response is downregulated.
11. A method for modulating a metabolic response comprising contacting a cell with an agent that modulates succinate, to thereby modulate the metabolic response.
12. The method of claim 11, wherein the succinate is upregulated.
13. The method of claim 12, wherein the succinate is upregulated using an agent selected from the group consisting of succinic acid and salts thereof, and an agent that promotes muscle contraction.
14. The method of any one of claims 11-13, further comprising contacting the cell with an additional agent that increases the metabolic response.
15. The method of claim 11, wherein the succinate is downregulated.
16. The method of claim 15, wherein the succinate is downregulated using an agent selected from the group consisting of a metabolizer of succinate, an antioxidant, a mitochondria-targeted antioxidant, an inhibitor of muscle shivering, an inhibitor of plasma membrane transport, an inhibitor of plasma membrane secondary active transport via the Na⁺/K⁺-ATPase, an inhibitor of SLC25A10, and an inhibitor of ROS-dependent cysteine oxidation.
17. The method of any one of claims 11, 15, and 16, further comprising contacting the cell with an additional agent that decreases the metabolic response.

18. The method of any one of claims 11-17, wherein the step of contacting occurs *in vivo*.
19. The method of any one of claims 11-17, wherein the step of contacting occurs *in vitro*.
20. The method of any one of claims 11-19, wherein the cell is selected from the group consisting of fibroblasts, adipoblasts, preadipocytes, adipocytes, white adipocytes, brown adipocytes, and beige adipocytes.
21. The method of any one of claims 11-20, wherein the metabolic response is selected from the group consisting of:
- a) modified expression of a marker selected from the group consisting of: cidea, adiponectin, adipsin, otopetrin, type II deiodinase, cig30, ppar gamma 2, pgc1 α , ucp1, elovl3, cAMP, Prdm16, cytochrome C, cox4i1, coxIII, cox5b, cox7a1, cox8b, glut4, atpase b2, cox II, atp5o, ndufb5, ap2, ndufs1, GRP109A, acylCoA-thioesterase 4, EARA1, claudin1, PEPCK, fgf21, acylCoA-thioesterase 3, dio2, fatty acid synthase (fas), leptin, resistin, and nuclear respiratory factor-1 (nrf1);
 - b) modified thermogenesis in adipose cells;
 - c) modified differentiation of adipose cells;
 - d) modified insulin sensitivity of adipose cells;
 - e) modified basal respiration, leak respiration, or uncoupled respiration;
 - f) modified whole body oxygen consumption;
 - g) modified obesity or appetite;
 - h) modified insulin secretion of pancreatic beta cells;
 - i) modified glucose tolerance; and
 - j) modified activity of UCP1 protein.
22. The method of any one of claims 11-21, wherein the metabolic response is upregulated.
23. The method of any one of claims 11-21, wherein the metabolic response is downregulated.
24. A method of preventing or treating a metabolic disorder in a subject comprising administering to the subject an agent that promotes succinate in the subject, thereby preventing or treating the metabolic disorder in the subject.

25. The method of claim 24, wherein the agent is selected from the group consisting of succinic acid and salts thereof, and an agent that promotes muscle contraction.
26. The method of claim 24 or 25, wherein the agent is administered orally or systemically, optionally wherein the administration is in a solution comprising 1% to 2% succinate and/or is *ad libitum*.
27. The method of any one of claims 24-26, wherein the agent is administered in a pharmaceutically acceptable formulation.
28. The method of any one of claims 24-27, wherein the metabolic disorder is selected from the group consisting of pain, insulin resistance, hyperinsulinemia, hypoinsulinemia, type II diabetes, hypertension, hyperhepatosteatosis, hyperuricemia, fatty liver, non-alcoholic fatty liver disease, polycystic ovarian syndrome, acanthosis nigricans, hyperphagia, endocrine abnormalities, triglyceride storage disease, Bardet-Biedl syndrome, Lawrence-Moon syndrome, and Prader-Labhart-Willi syndrome.
29. The method of any one of claims 24-28, wherein the subject is a non-human animal or a human, optionally wherein the non-human animal is an animal model of the metabolic disorder.
30. A method for preventing or treating a metabolic disorder in a subject comprising administering to the subject an agent that inhibits or reduces succinate in the subject, thereby preventing or treating the metabolic disorder in the subject.
31. The method of claim 30, wherein the agent is selected from the group consisting of a metabolizer of succinate, an antioxidant, a mitochondria-targeted antioxidant, an inhibitor of muscle shivering, an inhibitor of plasma membrane transport, an inhibitor of plasma membrane secondary active transport via the Na^+/K^+ -ATPase, an inhibitor of SLC25A10, and an inhibitor of ROS-dependent cysteine oxidation.
32. The method of claim 30 or 31, wherein the agent is administered systemically.
33. The method of any one of claims 30-32, wherein the agent is administered in a pharmaceutically acceptable formulation.

34. The method of any one of claims 30-33, wherein the metabolic disorder is selected from the group consisting of obesity-associated cancer, anorexia, and cachexia.
35. The method of any one of claims 30-34, wherein the subject is a non-human animal or a human, optionally wherein the non-human animal is an animal model of the metabolic disorder.
36. A cell-based assay for screening for agents that modulate a metabolic response in a cell by modulating succinate, comprising contacting the cell in the presence of succinate with a test agent that modulates succinate, and determining the ability of the test agent to modulate a metabolic response in the cell.
37. A method for assessing the efficacy of an agent that modulates succinate uptake, for modulating a metabolic response in a subject, comprising:
- a) detecting in a subject sample at a first point in time, the amount of succinate;
 - b) repeating step a) during at least one subsequent point in time after administration of the agent; and
 - c) comparing the amount detected in steps a) and b),
- wherein a significantly lower amount of succinate in the first subject sample relative to at least one subsequent subject sample, indicates that the test agent increases the uptake of succinate in the subject and/or
- wherein a significantly higher amount of succinate in the first subject sample relative to at least one subsequent subject sample, indicates that the test agent decreases the uptake of succinate in the subject;
- optionally wherein expression and/or activity of a marker listed in Table 1 is further detected in steps a) and b), and comparing the expression and/or activity of the marker,
- wherein a significantly lower expression and/or activity of a marker listed in Table 1 in the first subject sample relative to at least one subsequent subject sample, indicates that the test agent increases the metabolic response in the subject and/or wherein a significantly higher expression and/or activity of a marker listed in Table 1 in the first subject sample relative to at least one subsequent subject sample, indicates that the test agent decreases the metabolic response in the subject.
38. The assay or method of claim 36 or 37, wherein the succinate uptake is upregulated.

39. The assay or method of claim 36 or 37, wherein the succinate uptake is downregulated.
40. The assay or method of any one of claims 36-39, wherein the agent is selected from the group consisting of succinic acid and salts thereof, an agent that promotes muscle contraction, a metabolizer of succinate, an antioxidant, a mitochondria-targeted antioxidant, an inhibitor of muscle shivering, an inhibitor of plasma membrane transport, an inhibitor of plasma membrane secondary active transport via the Na^+/K^+ -ATPase, an inhibitor of SLC25A10, and an inhibitor of ROS-dependent cysteine oxidation .
41. The assay or method of any one of claims 36-40, wherein between the first point in time and the subsequent point in time, the subject has undergone treatment for the metabolic disorder, has completed treatment for the metabolic disorder, and/or is in remission from the metabolic disorder.
42. The assay or method of any one of claims 36-41, wherein the first and/or at least one subsequent sample is selected from the group consisting of *ex vivo* and *in vivo* samples.
43. The assay or method of any one of claims 36-42, wherein the first and/or at least one subsequent sample is obtained from an animal model of a metabolic disorder.
44. The assay or method of any one of claims 36-43, wherein the first and/or at least one subsequent sample is selected from the group consisting of tissue, whole blood, serum, plasma, buccal scrape, saliva, cerebrospinal fluid, urine, stool, and bone marrow.
45. The assay or method of claim 44, wherein the tissue comprises fibroblasts, adipoblasts, preadipocytes, adipocytes, white adipocytes, brown adipocytes, and/or beige adipocytes.
46. The method of any one of claims 36-45, wherein the first and/or at least one subsequent sample is a portion of a single sample or pooled samples obtained from the subject.
47. The assay or method of any one of claims 36-46, wherein modulation comprises upregulation by at least 25% relative to the second sample.

48. The assay or method of any one of claims 36-46, wherein modulation comprises downregulation by at least 25% relative to the second sample.
49. The assay or method of any one of claims 36-49, wherein a significantly higher expression and/or activity comprises upregulating the expression and/or activity by at least 25% relative to the second sample.
50. The assay or method of any one of claims 36-49, wherein the metabolic response is selected from the group consisting of:
- a) modified expression of a marker selected from the group consisting of: cidea, adiponectin, adipsin, otopetrin, type II deiodinase, cig30, ppar gamma 2, pgc1 α , ucp1, elovl3, cAMP, Prdm16, cytochrome C, cox4i1, coxIII, cox5b, cox7a1, cox8b, glut4, atpase b2, cox II, atp5o, ndufb5, ap2, ndufs1, GRP109A, acylCoA-thioesterase 4, EARA1, claudin1, PEPCCK, fgf21, acylCoA-thioesterase 3, dio2, fatty acid synthase (fas), leptin, resistin, and nuclear respiratory factor-1 (nrf1);
 - b) modified thermogenesis in adipose cells;
 - c) modified differentiation of adipose cells;
 - d) modified insulin sensitivity of adipose cells;
 - e) modified basal respiration, leak respiration, or uncoupled respiration;
 - f) modified whole body oxygen consumption;
 - g) modified obesity or appetite;
 - h) modified insulin secretion of pancreatic beta cells;
 - i) modified glucose tolerance; and
 - j) modified expression activity of UCP1 protein.
51. The assay or method of any one of claims 36-50, wherein a significantly lower expression and/or activity comprises downregulating the expression and/or activity by at least 25% relative to the second sample.
52. The assay or method of any one of claims 36-51, wherein the amount of the marker is compared.
53. The assay or method of claim 52, wherein the amount of the marker is determined by determining the level of protein expression of the marker.

54. The assay or method of claim 53, wherein the presence of the protein is detected using a reagent which specifically binds with the protein.
55. The assay or method of claim 54, wherein the reagent is selected from the group consisting of an antibody, an antibody derivative, and an antibody fragment.
56. The assay or method of claim 53, wherein the level of expression of the marker in the sample is assessed by detecting the presence in the sample of a transcribed polynucleotide or portion thereof.
57. The assay or method of claim 56, wherein the transcribed polynucleotide is an mRNA or a cDNA.
58. The assay or method of claim 56 or 57, wherein the step of detecting further comprises amplifying the transcribed polynucleotide.
59. The assay or method of claim 53, wherein the level of expression of the marker in the sample is assessed by detecting the presence in the sample of a transcribed polynucleotide which anneals with the marker or anneals with a portion of a polynucleotide under stringent hybridization conditions.
60. The assay or method of any one of claims 36-59, wherein the metabolic response is upregulated.
61. The assay or method of any one of claims 36-59, wherein the metabolic response is downregulated.
62. The agent, assay, or method of any one of claims 1-58, wherein the succinate is natural or synthetic.
63. The agent, assay, or method of any one of claims 1-60, wherein the succinate is a metabolite or a pro-drug.
64. The agent, assay, or method of any one of claims 1-60, wherein the succinate is a monobasic salt.

65. The agent, assay, or method of any one of claims 1-60, wherein the succinate is a dibasic salt.

66. The agent, assay, or method of any one of claims 62 or 63, wherein the succinate is a sodium salt of succinic acid.

Figure 1

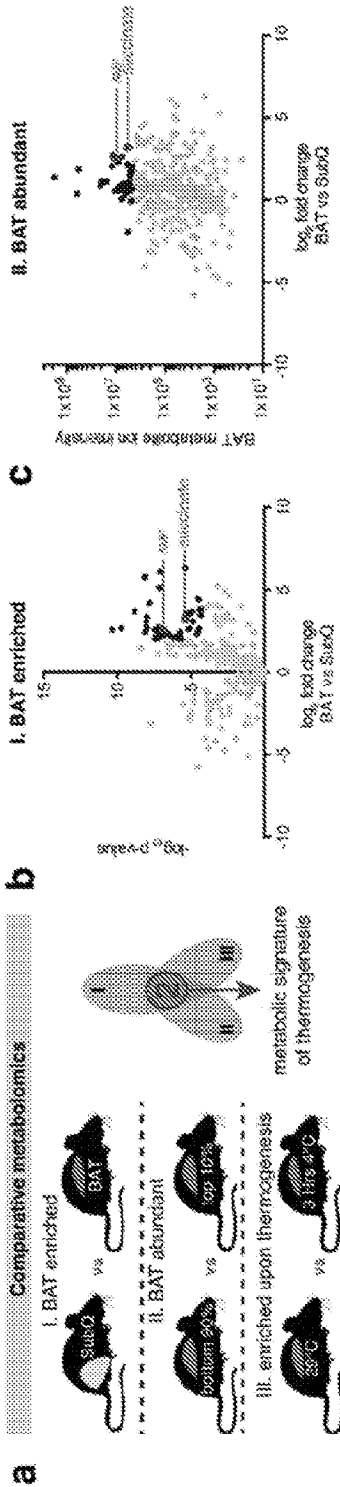
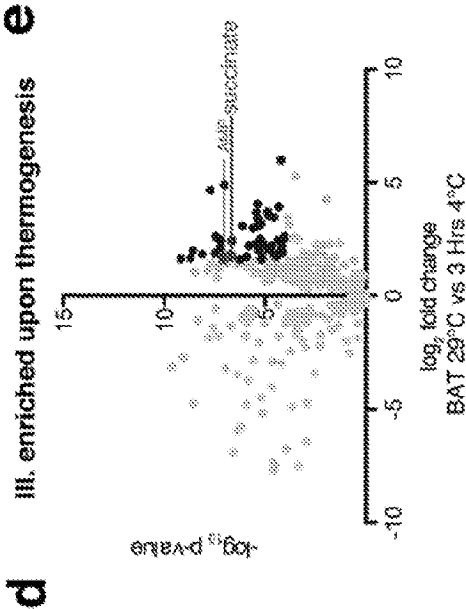


Figure 1 (cont.)



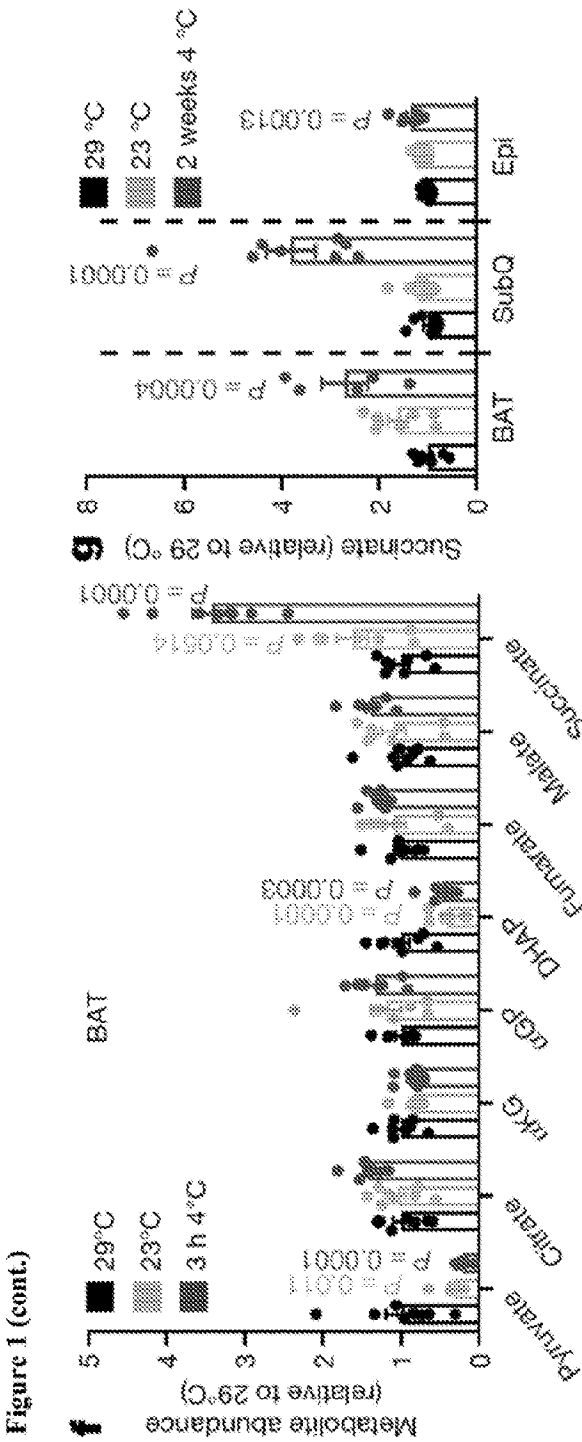


Figure 1 (cont.)

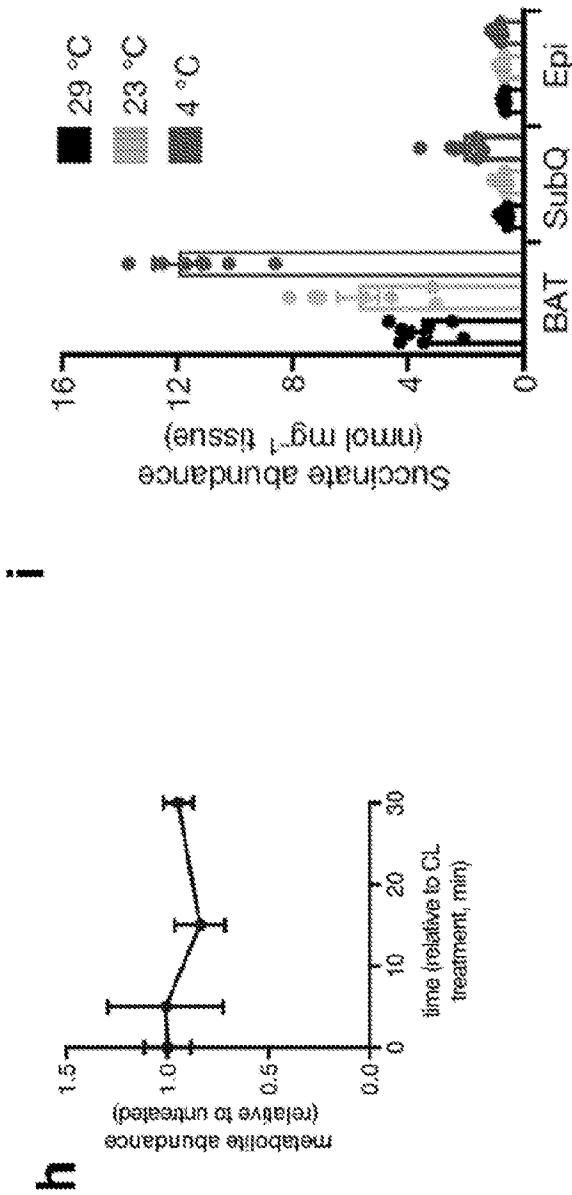


Figure 2

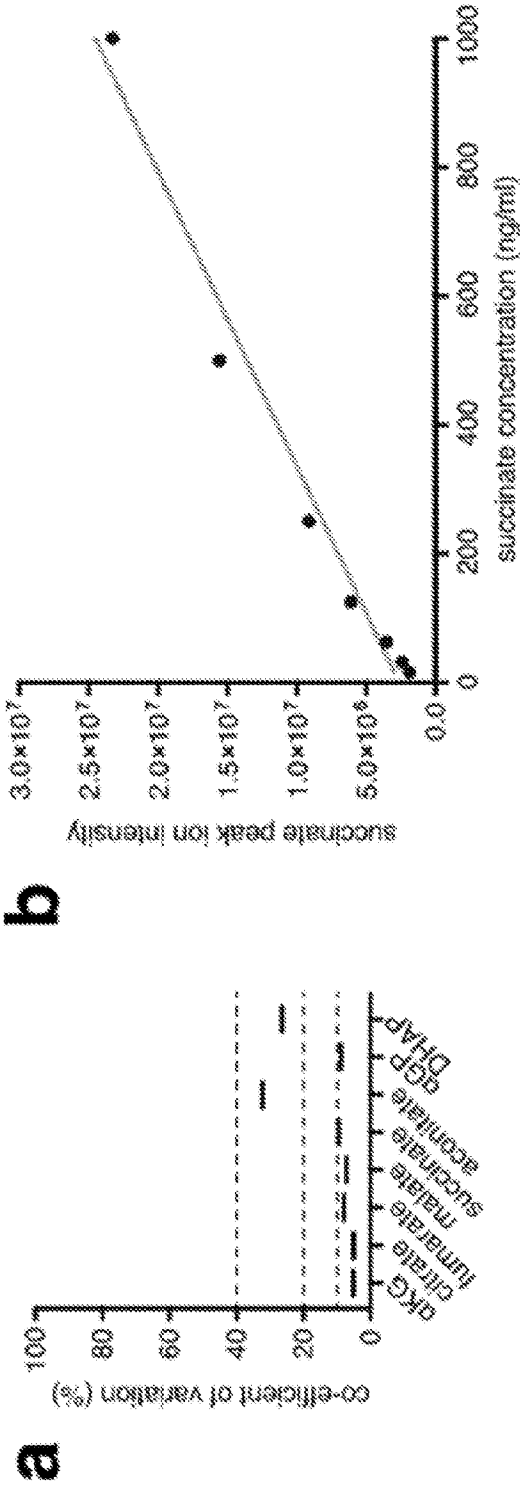
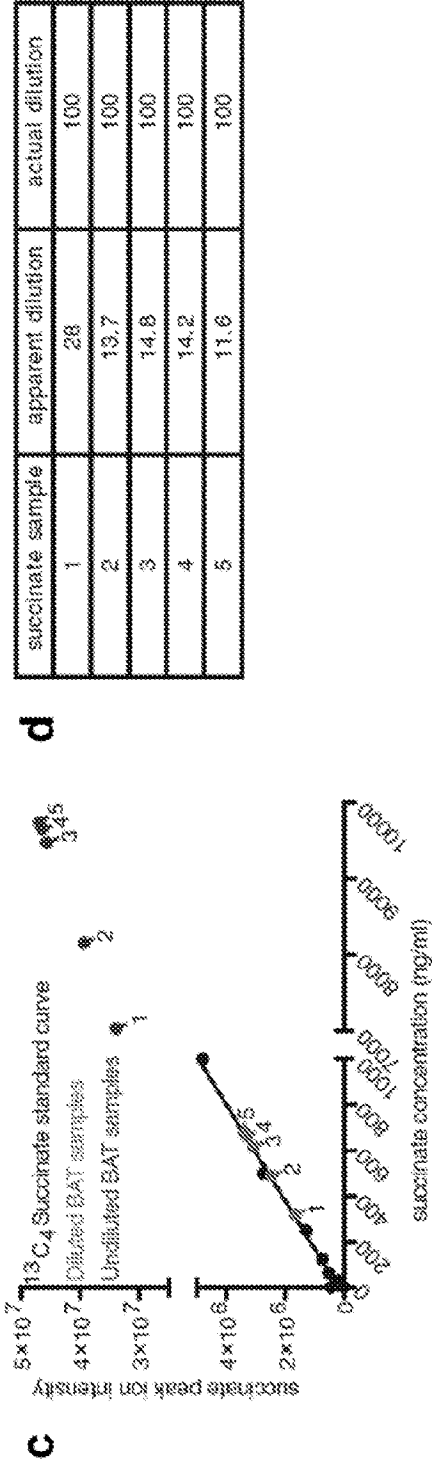


Figure 2 (cont.)



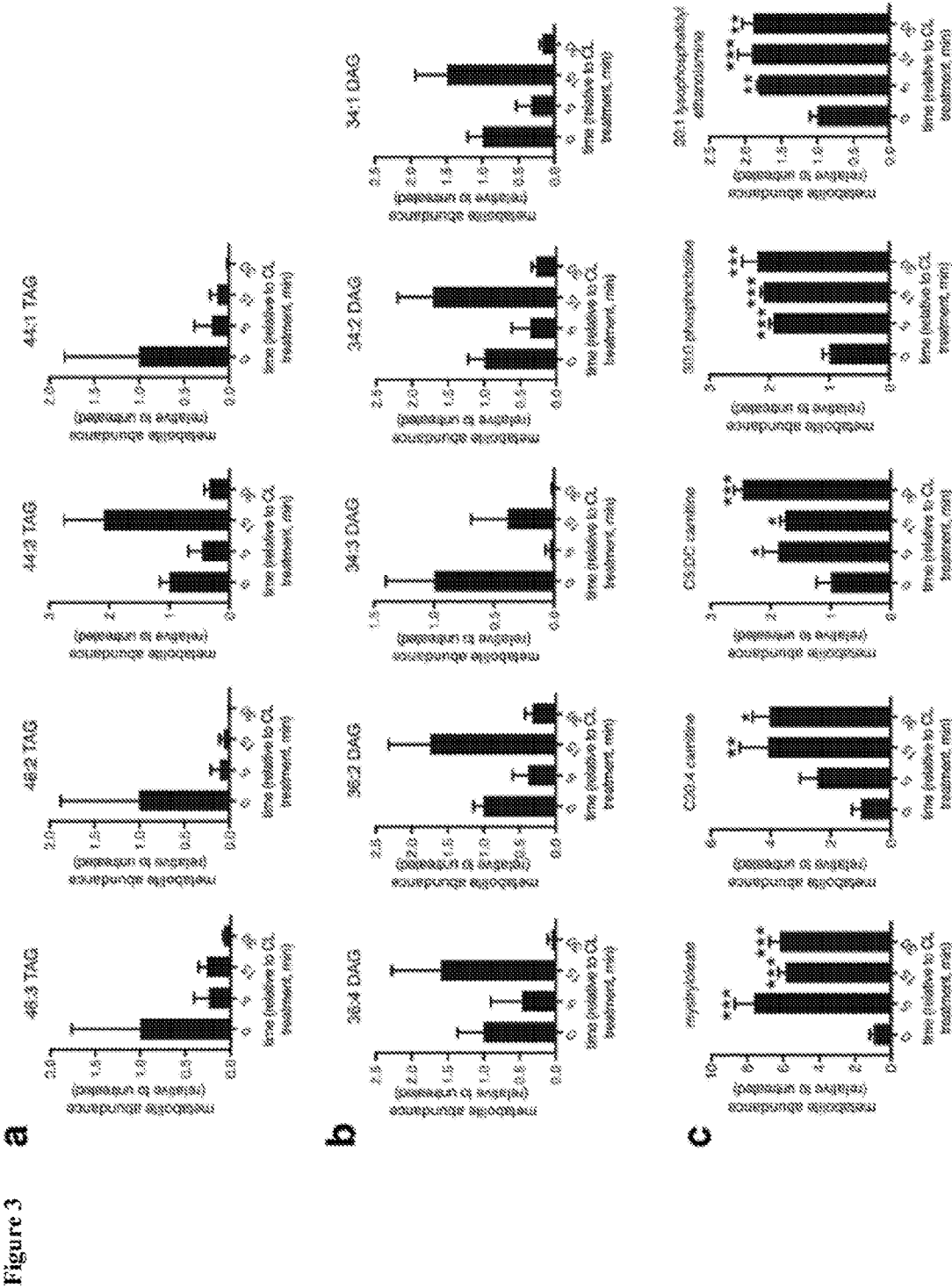


Figure 3 (cont.)

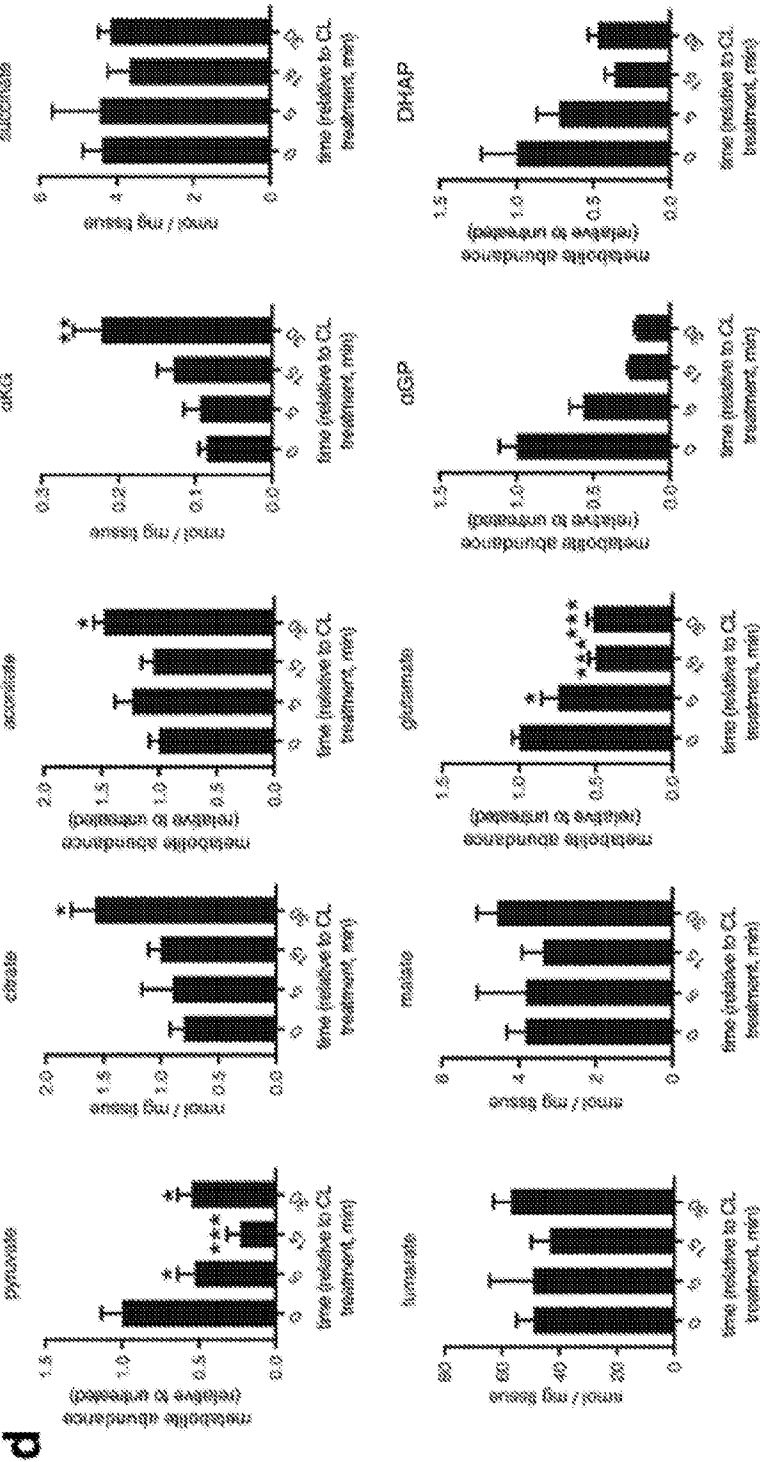
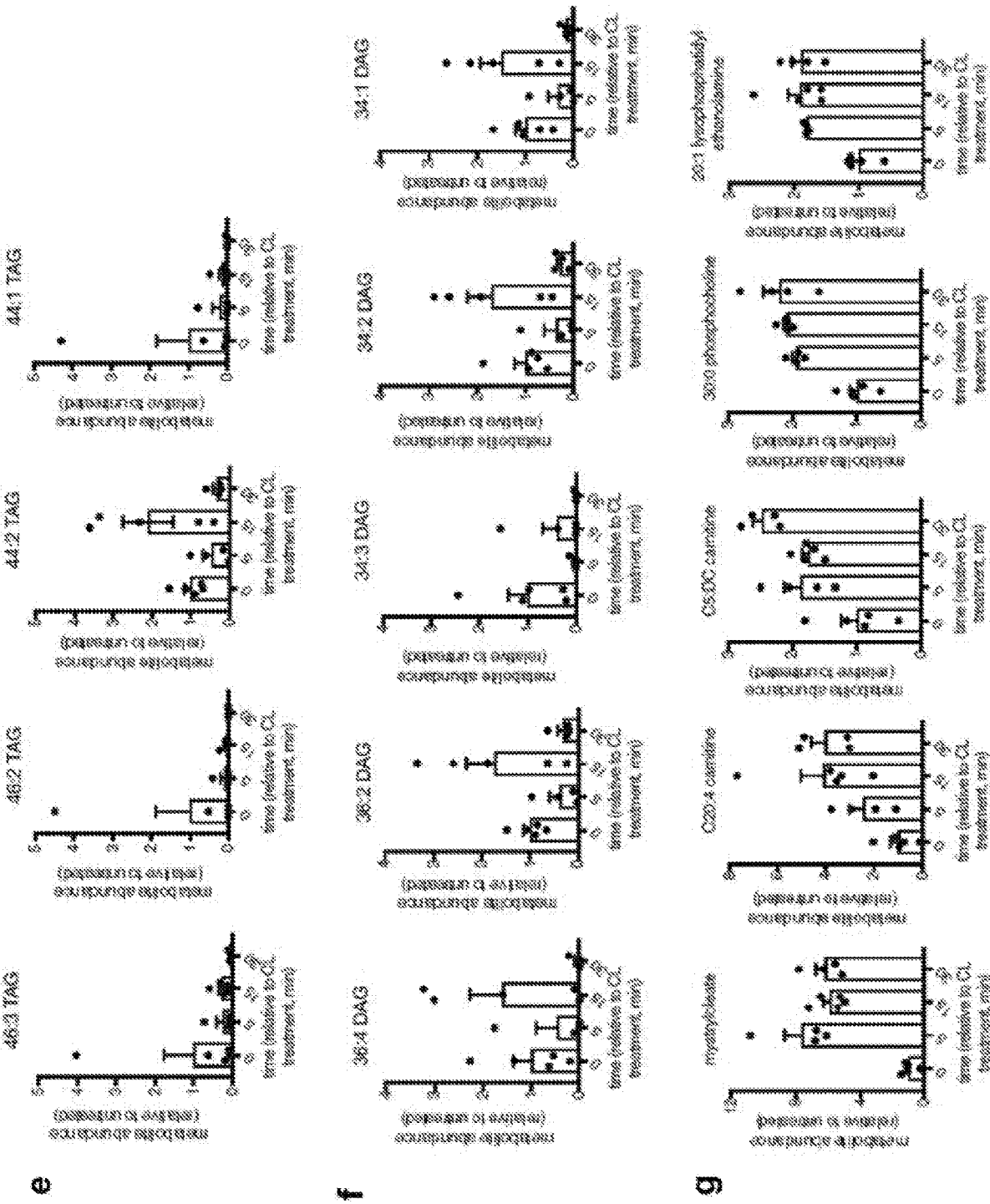


Figure 3 (cont.)



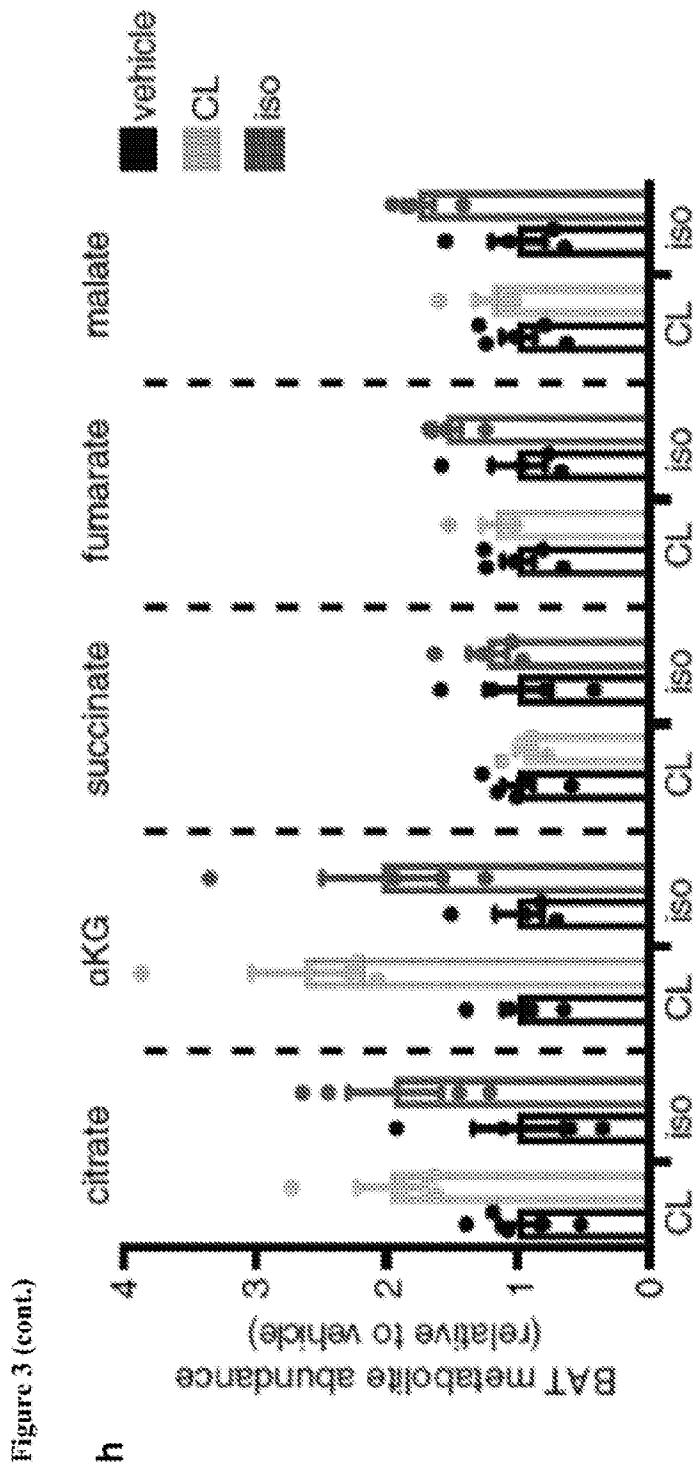


Figure 4

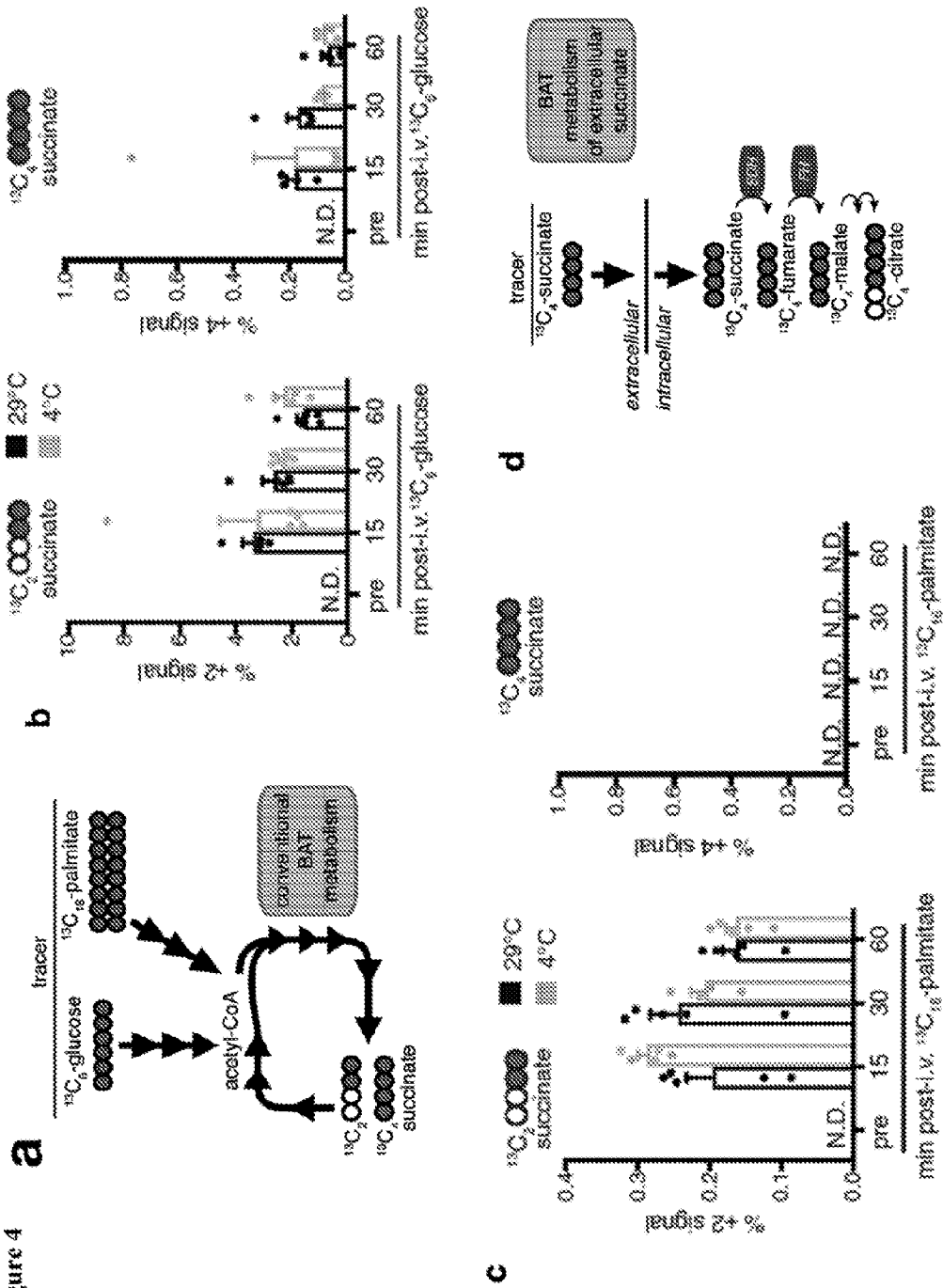
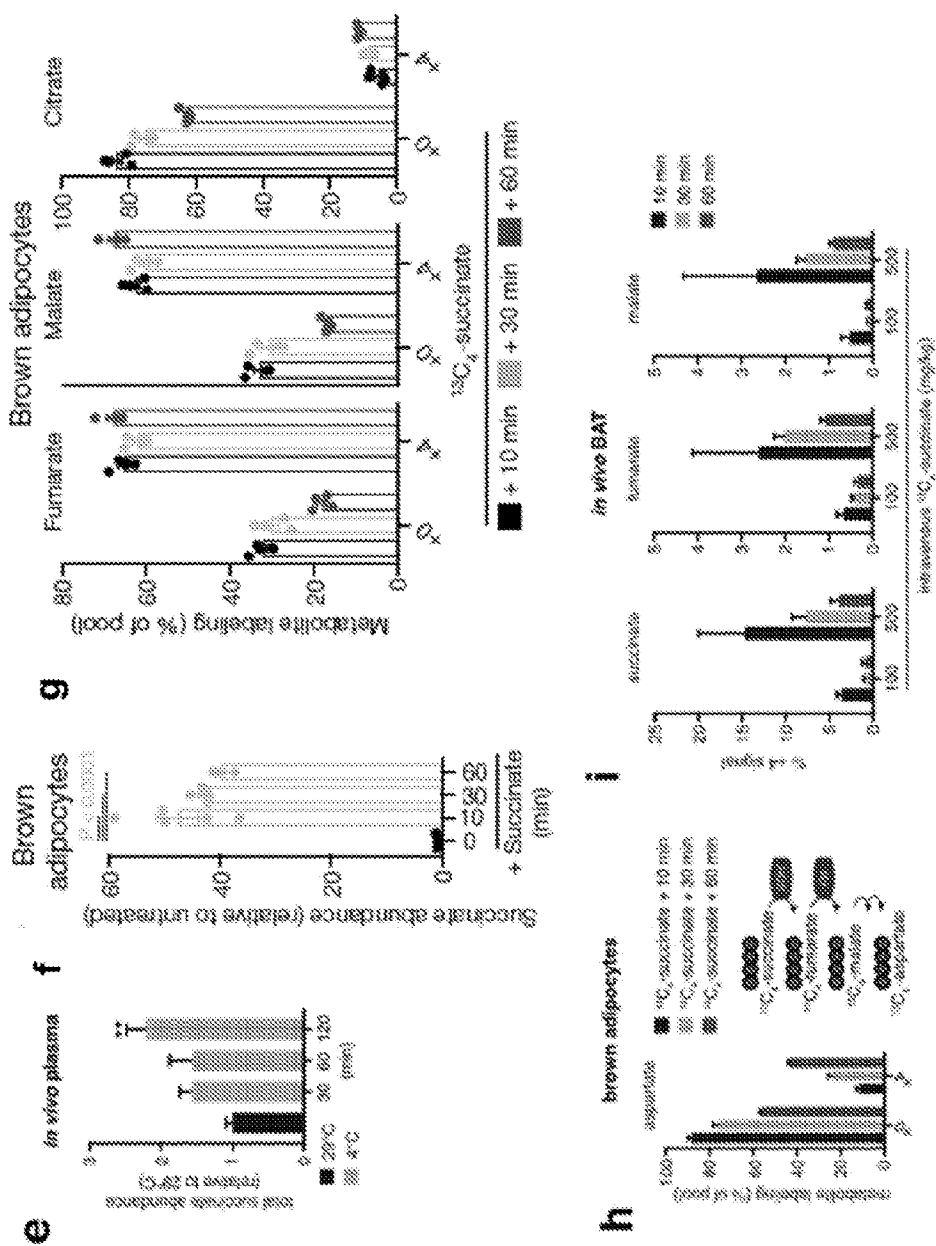


Figure 4 (cont.)



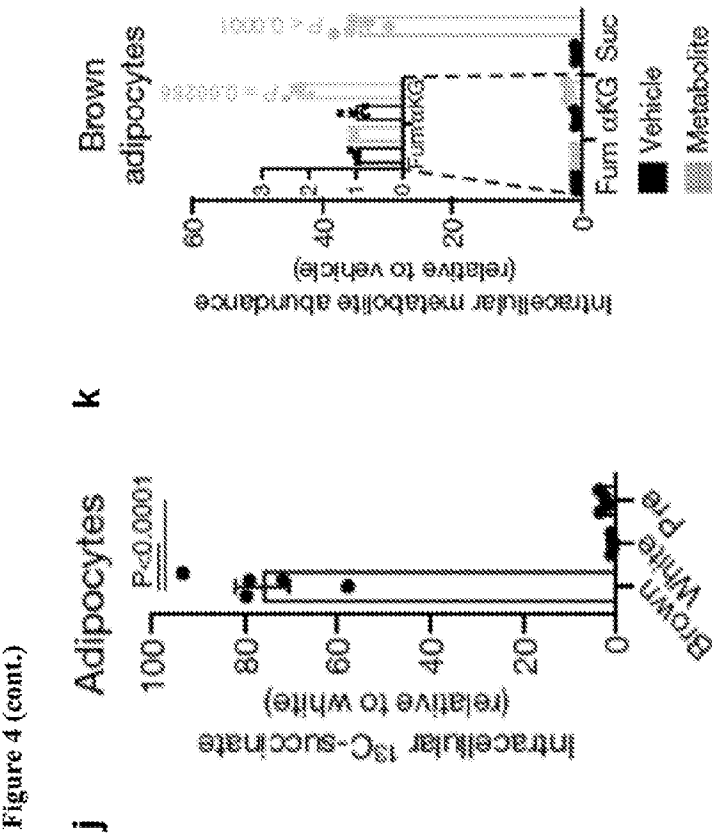


Figure 4 (cont.)

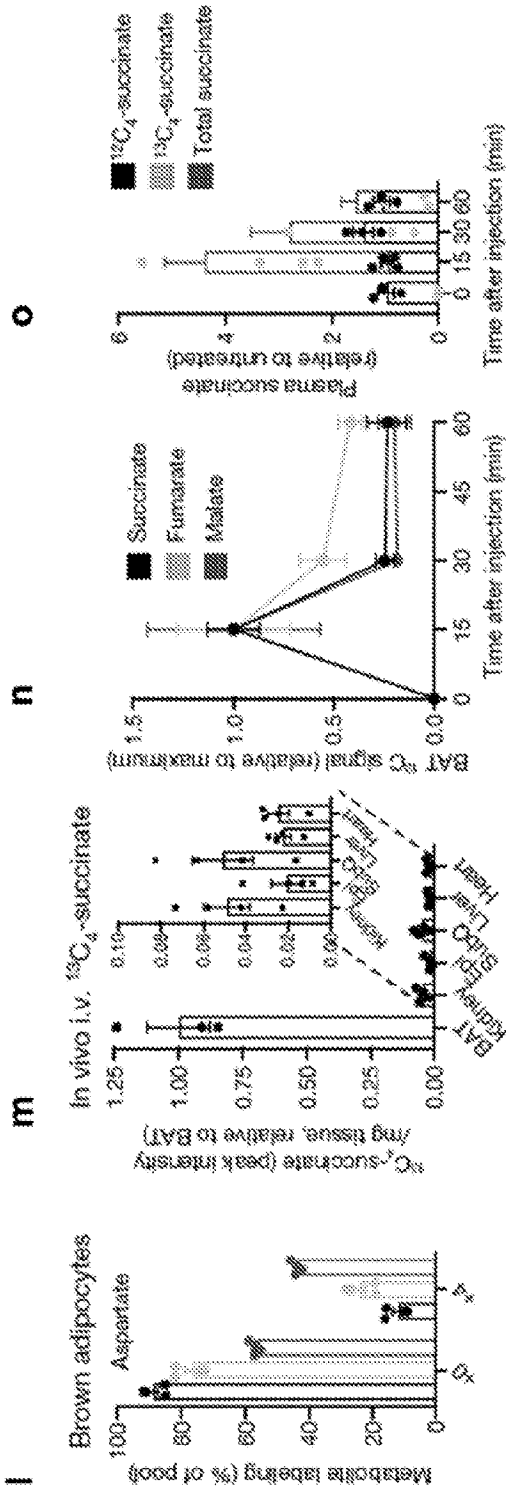


Figure 5

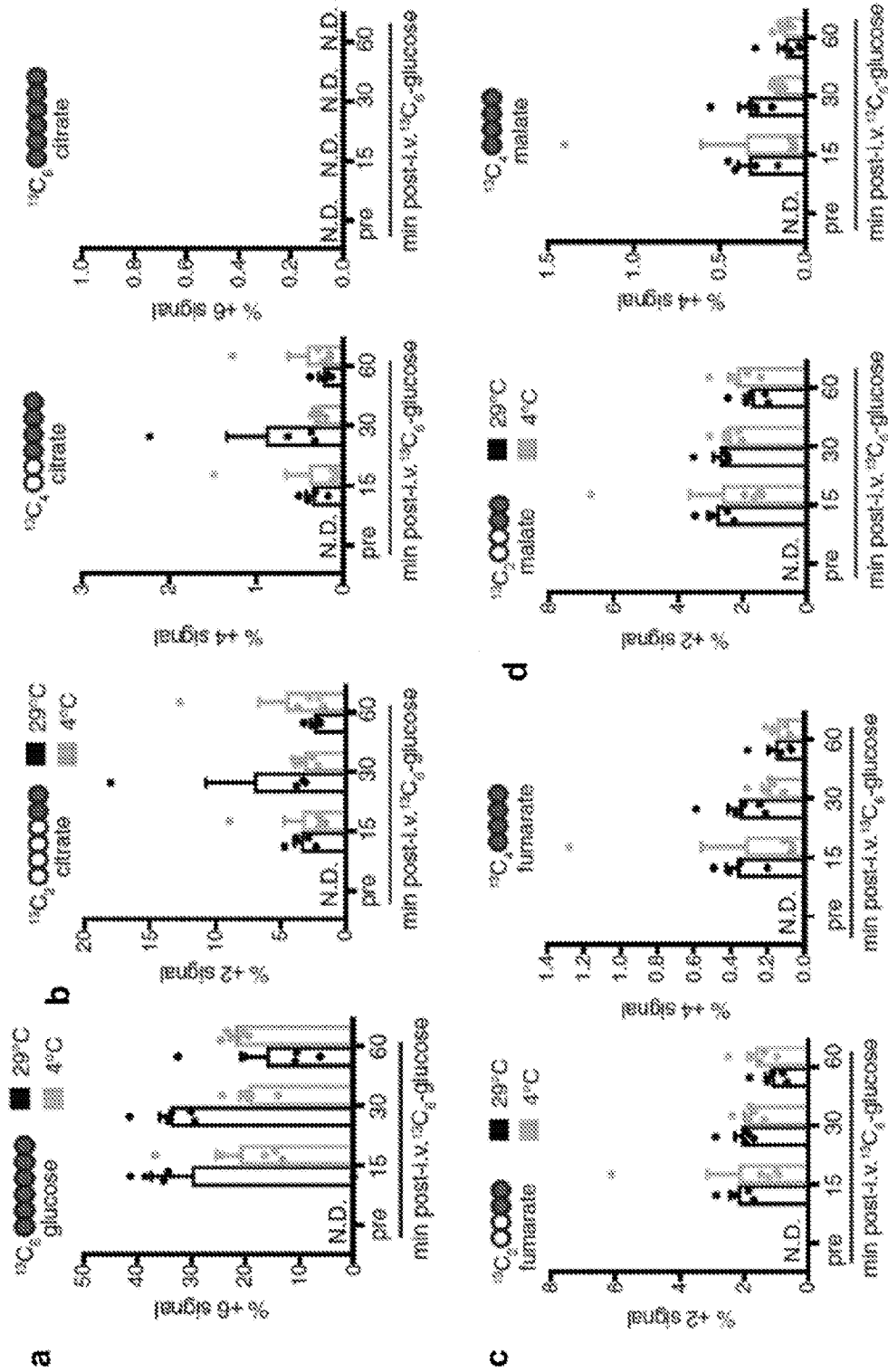
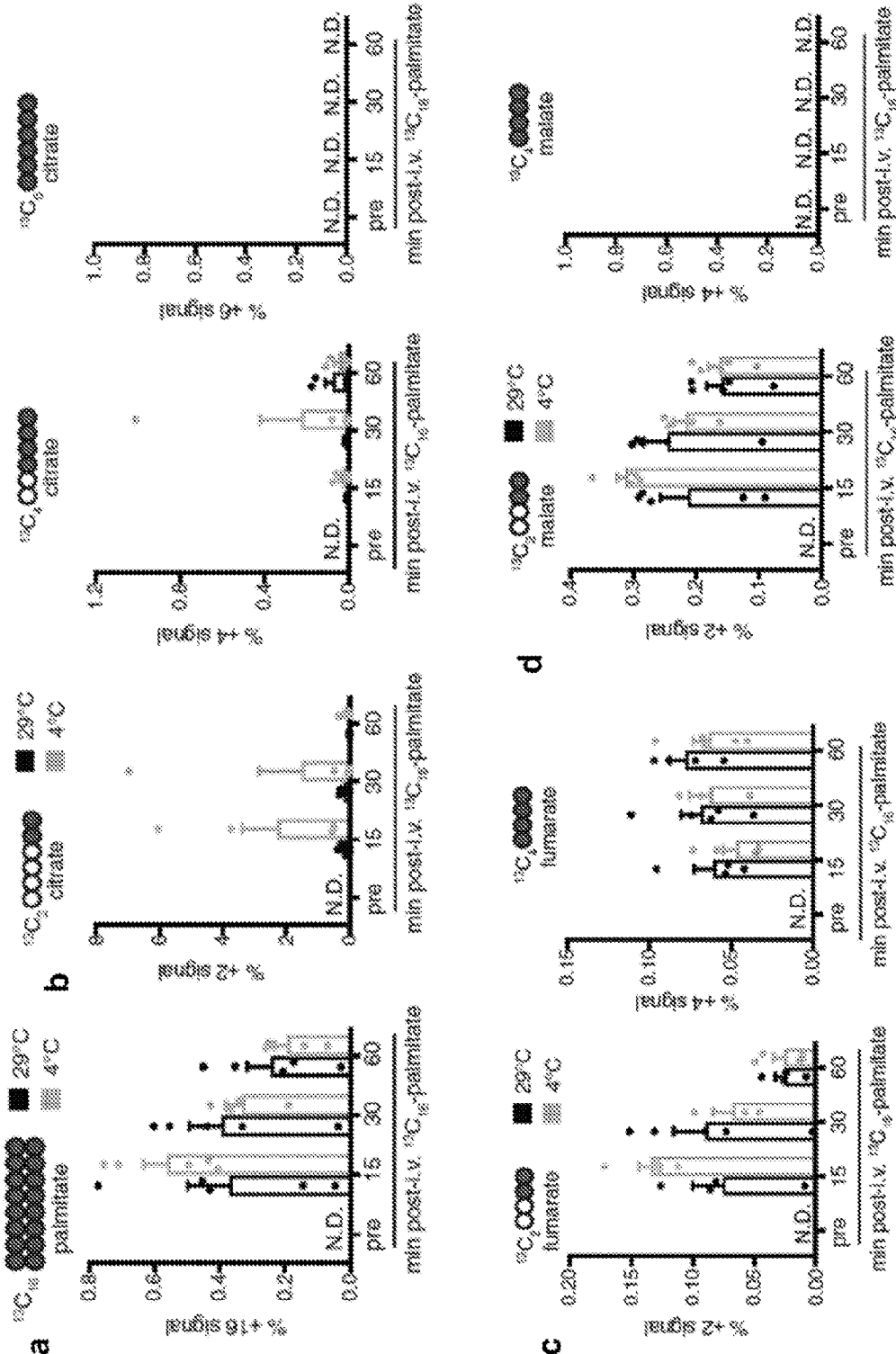


Figure 6



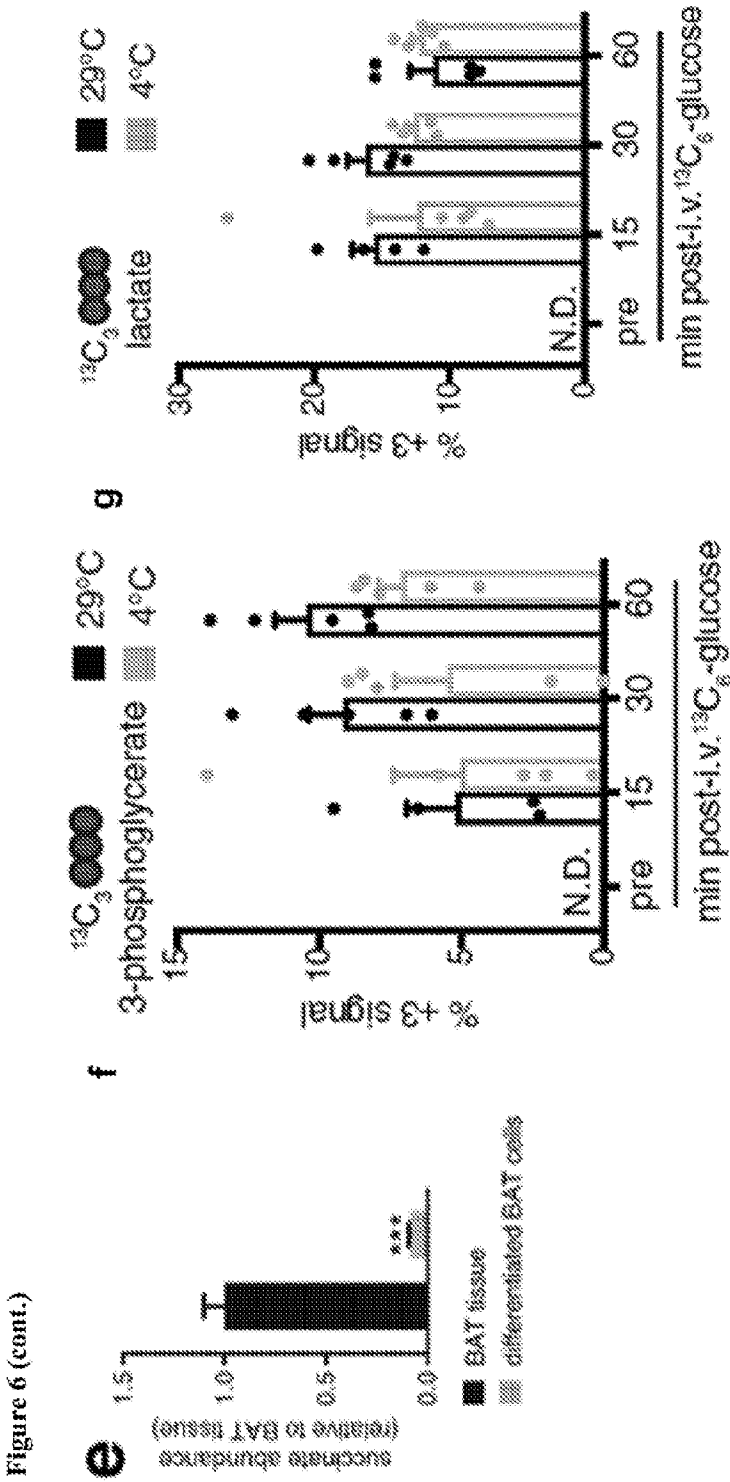


Figure 6 (cont.)

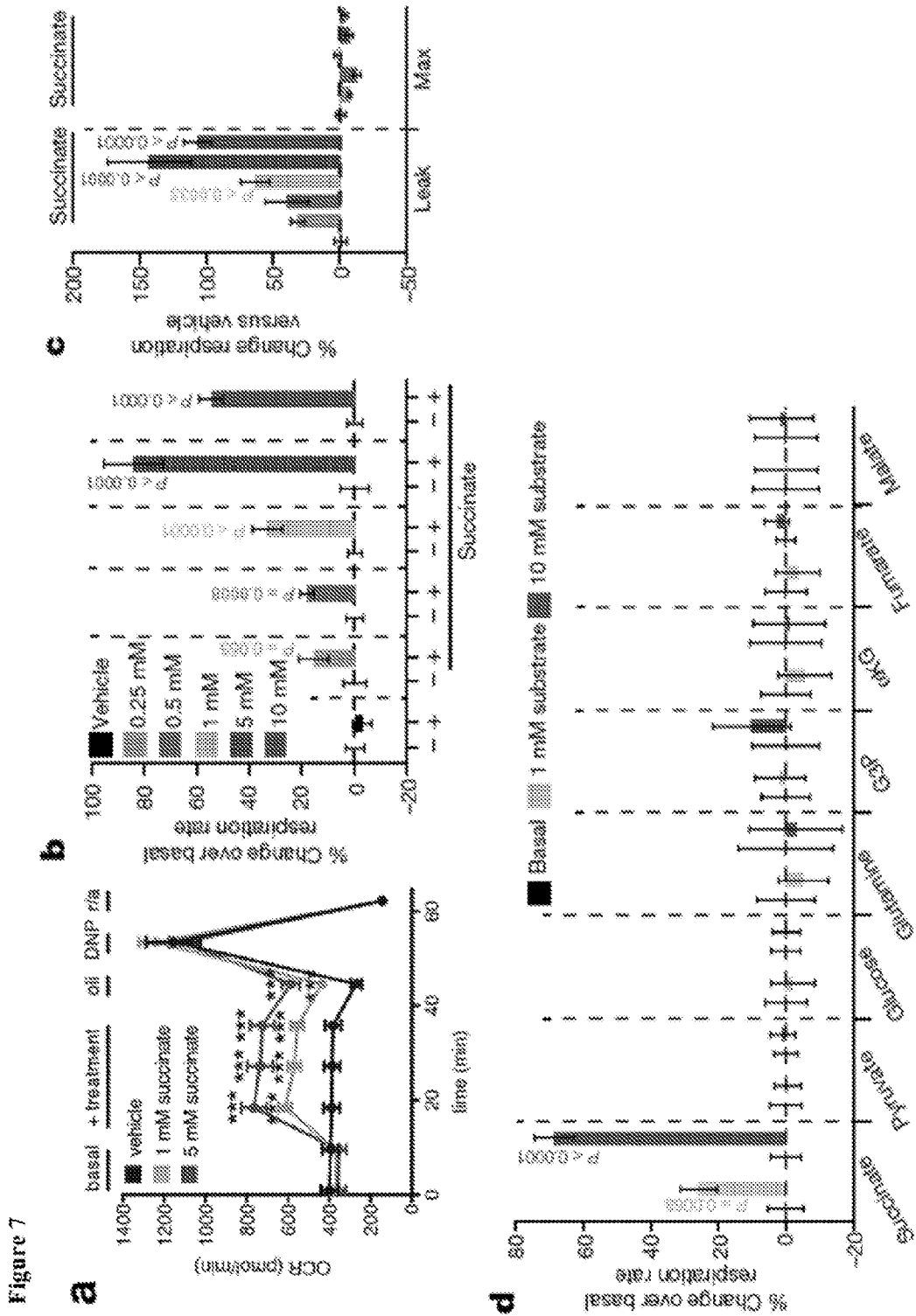


Figure 7 (cont.)

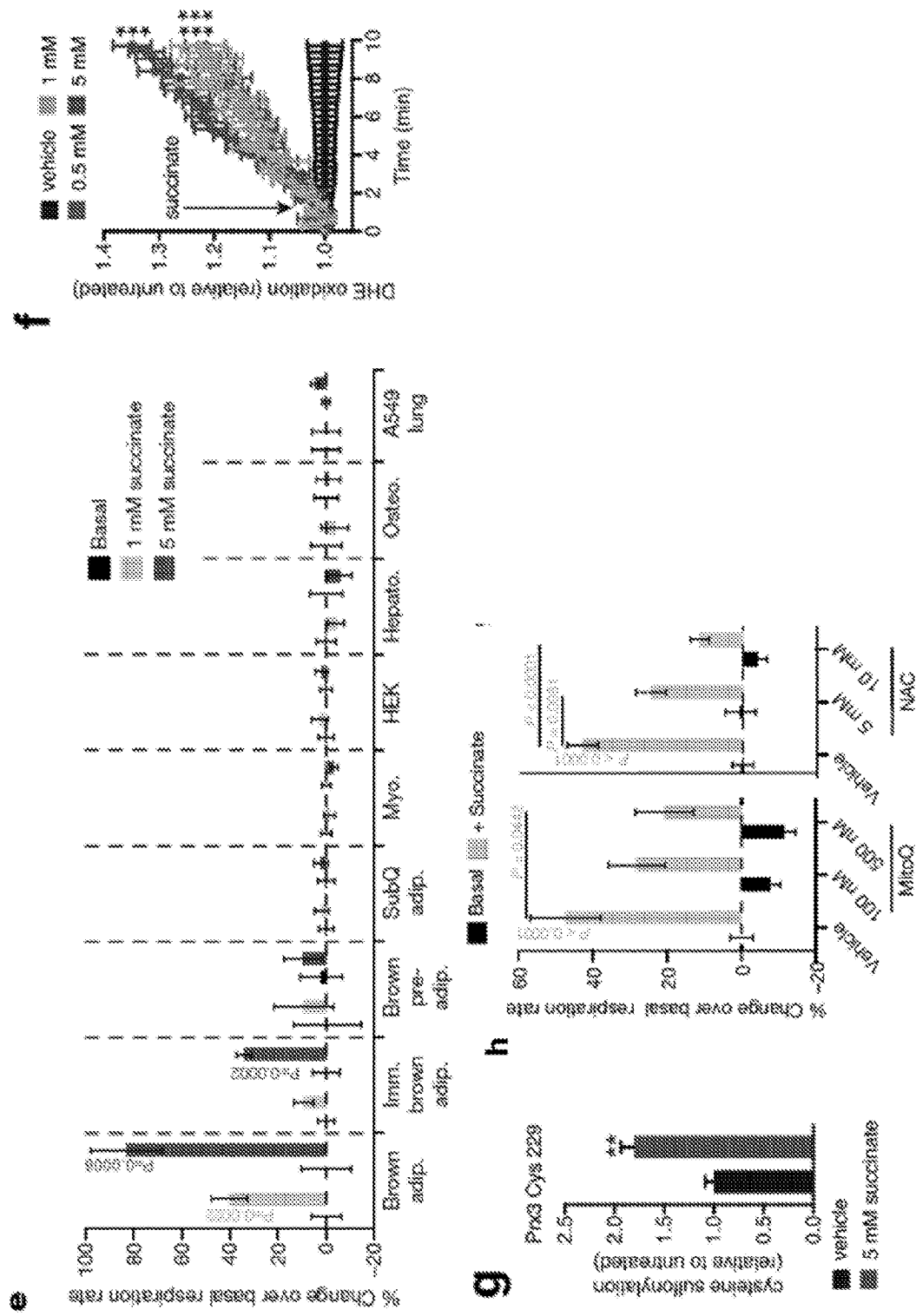


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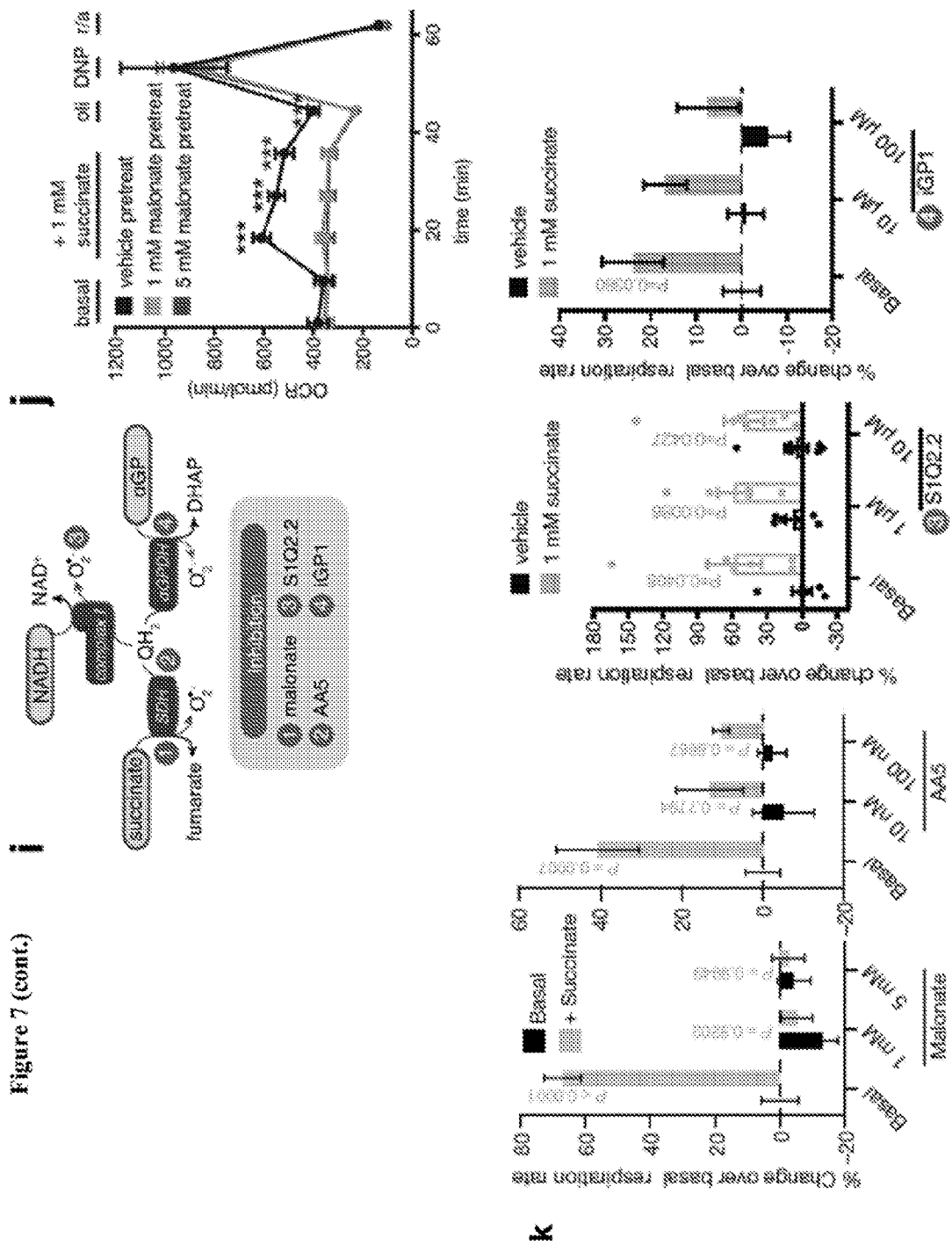


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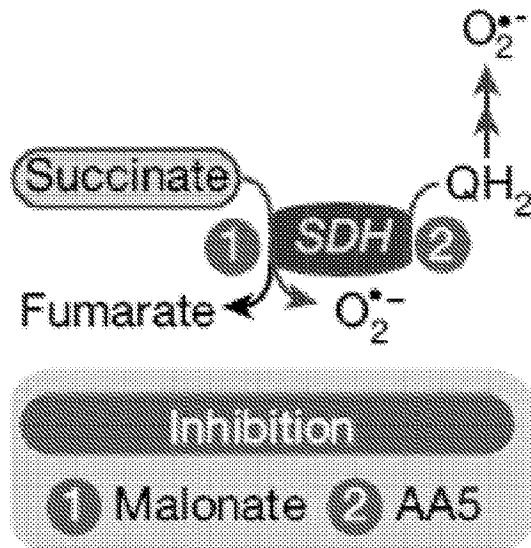


Figure 8

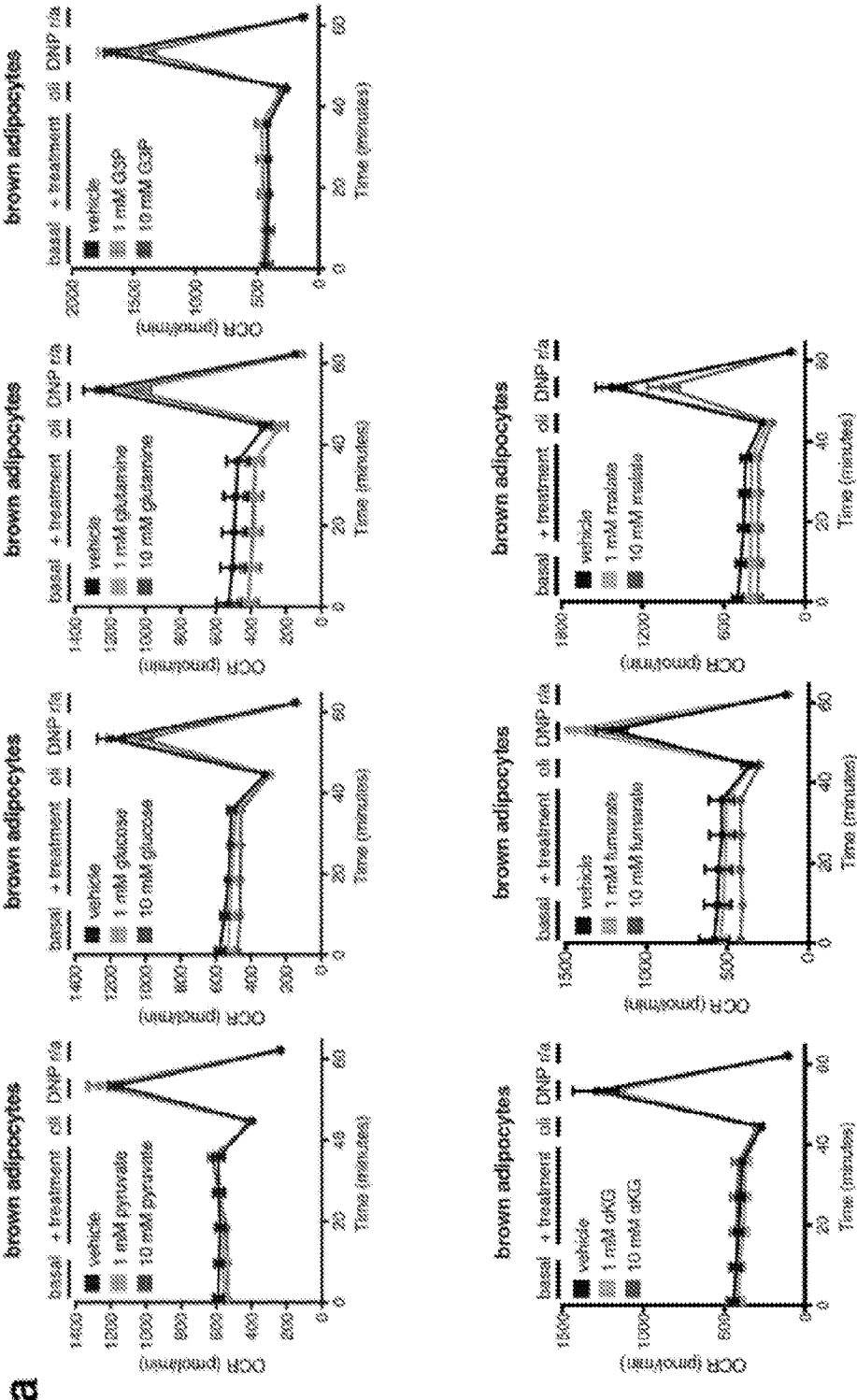


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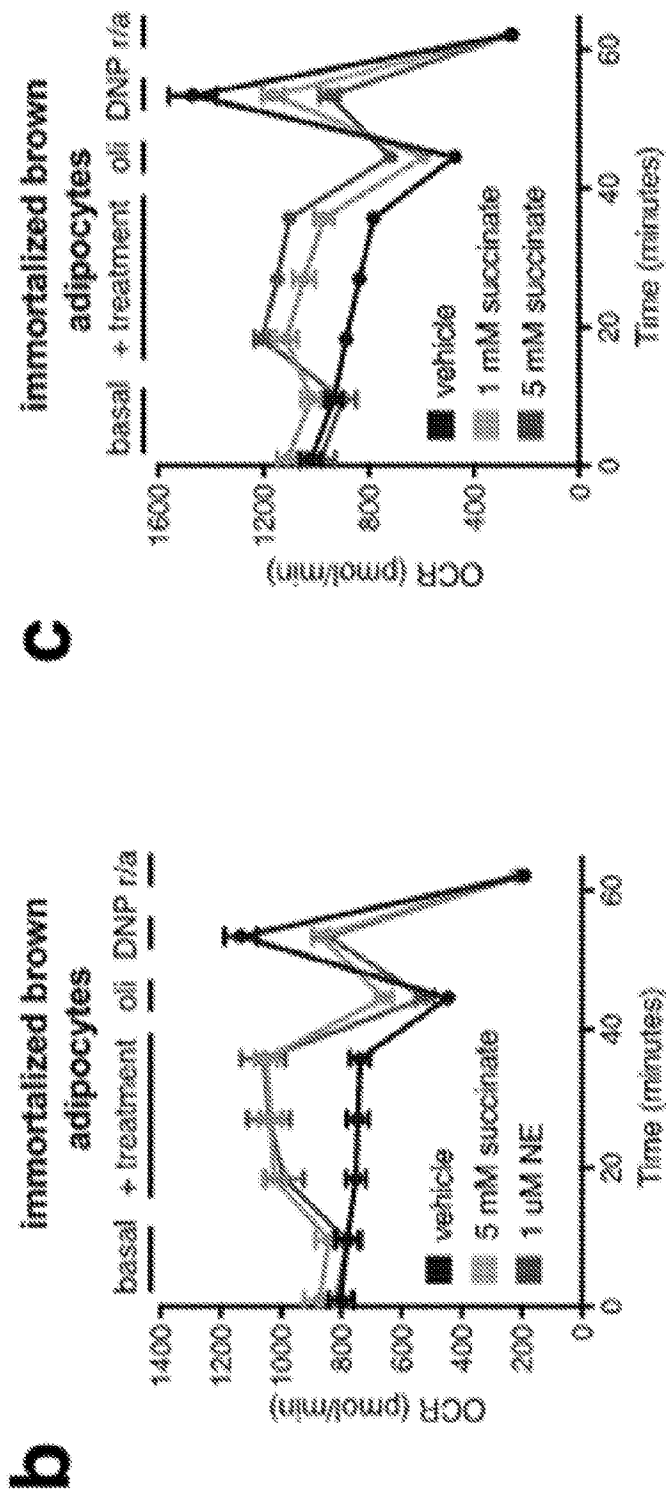
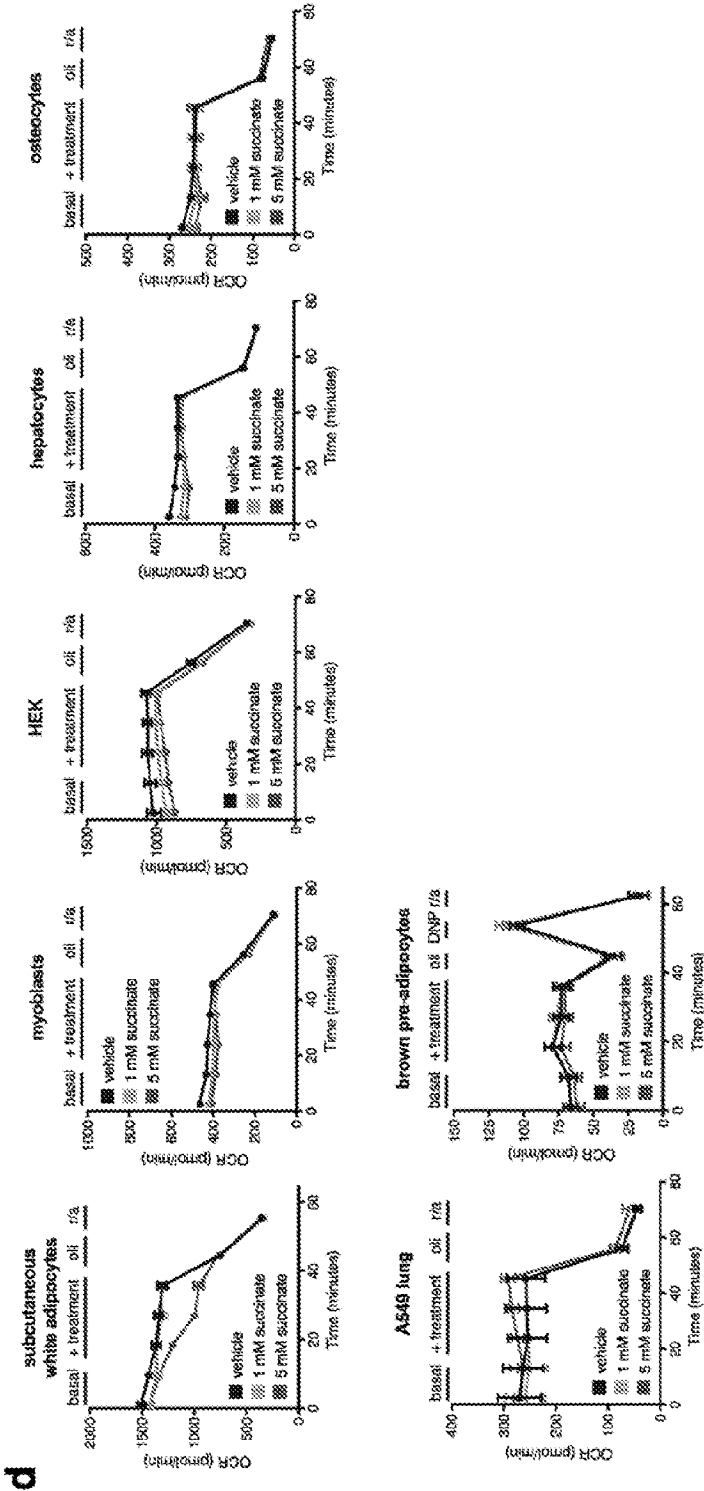


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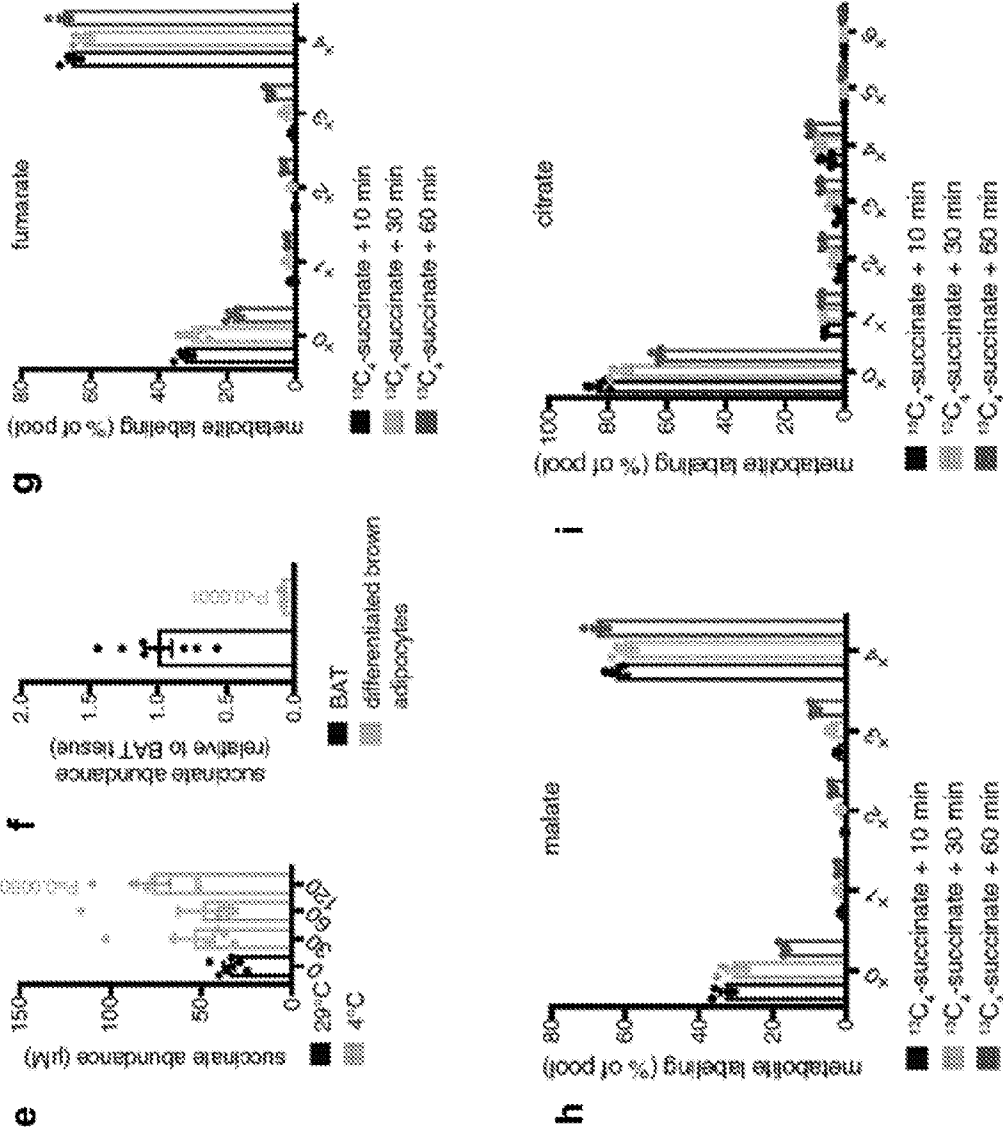
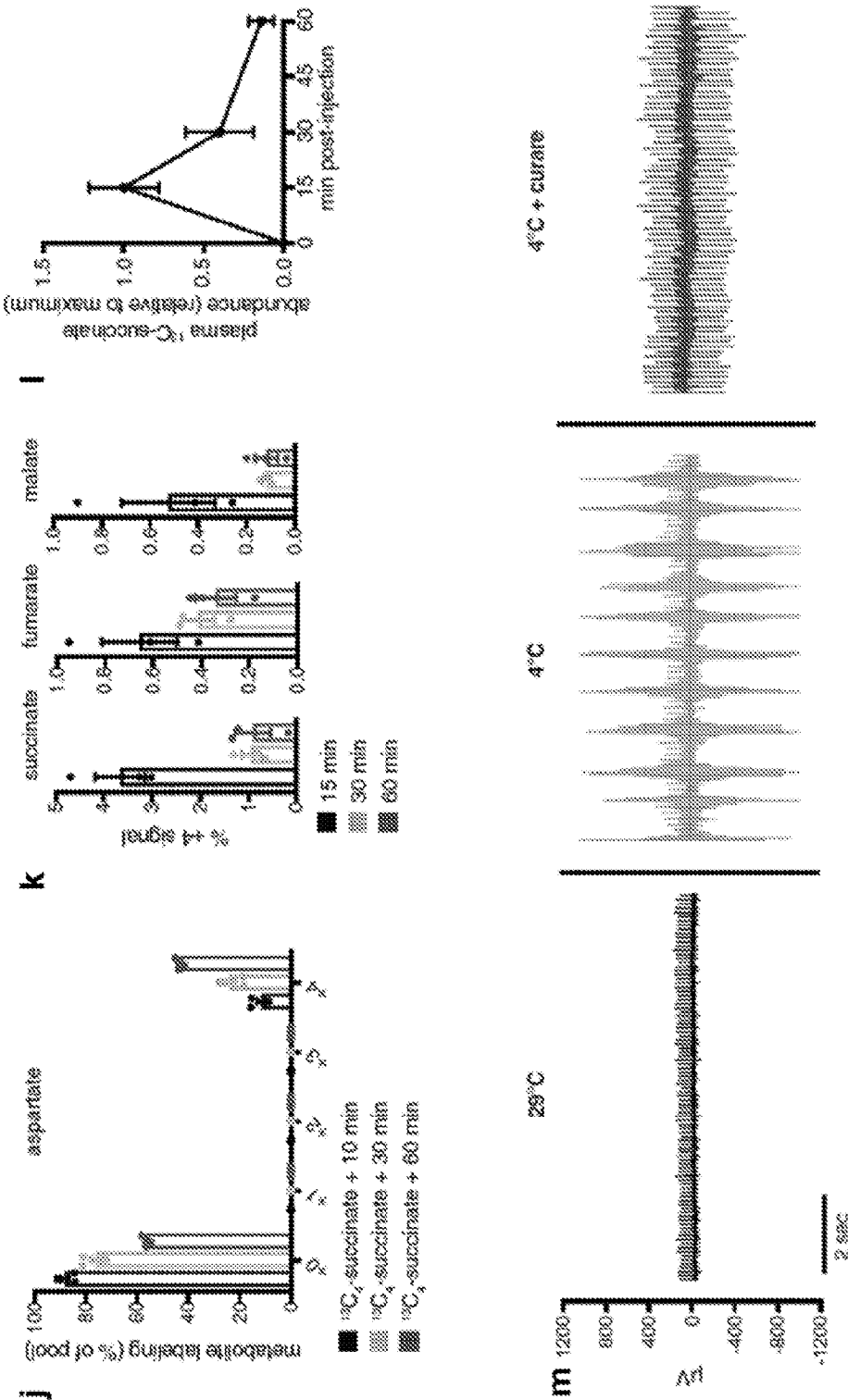


Figure 8 (cont.)

Figure 8 (cont.)



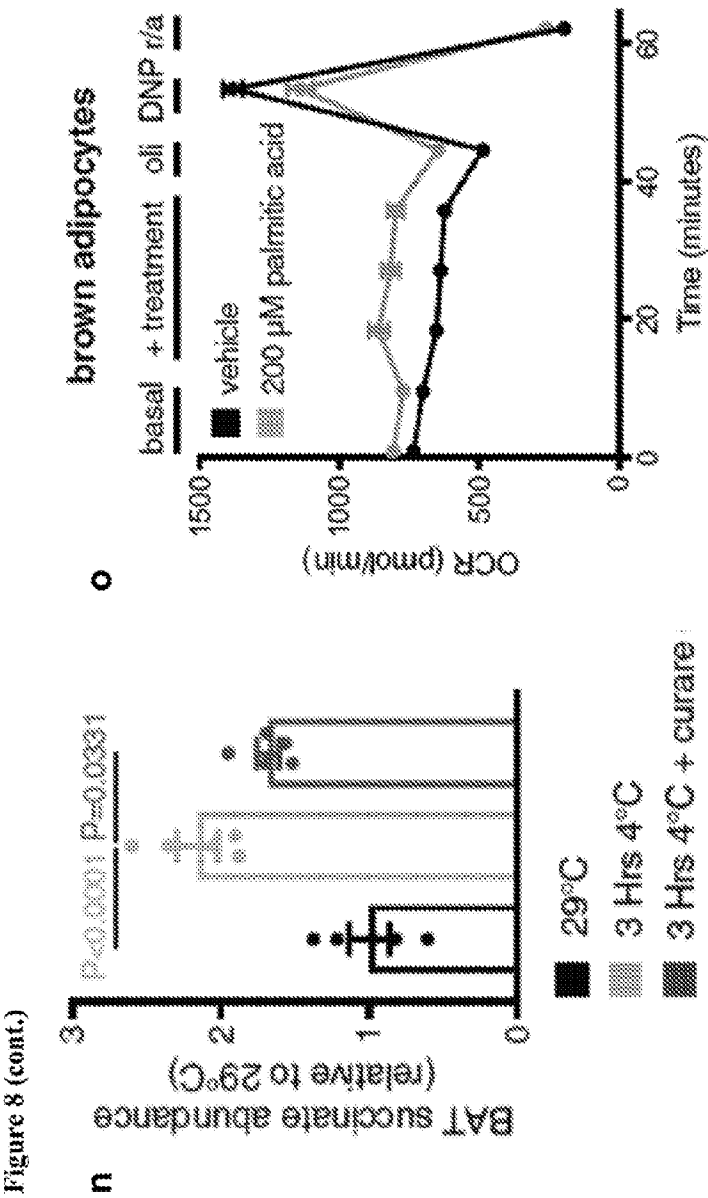


Figure 8 (cont.)

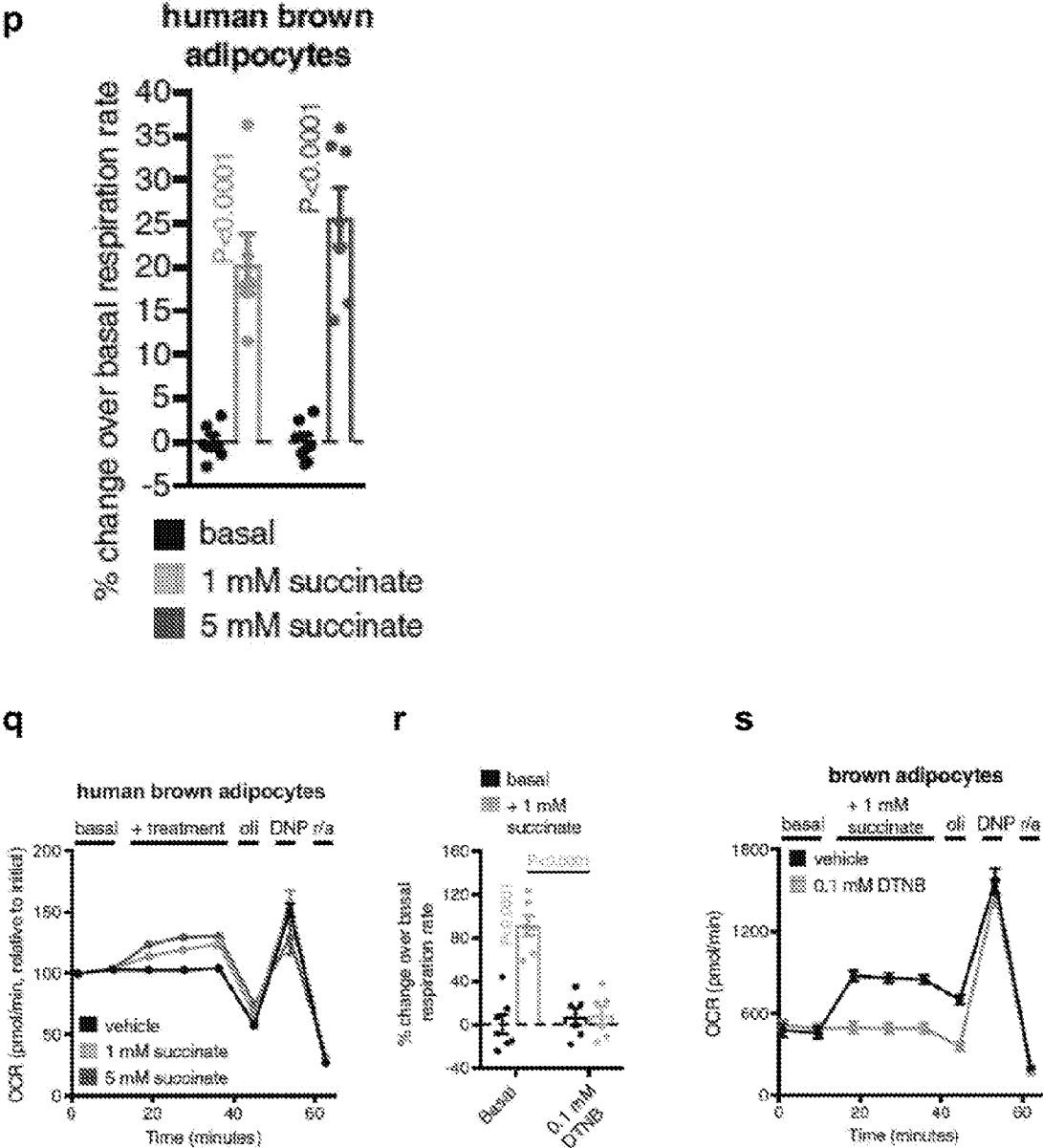
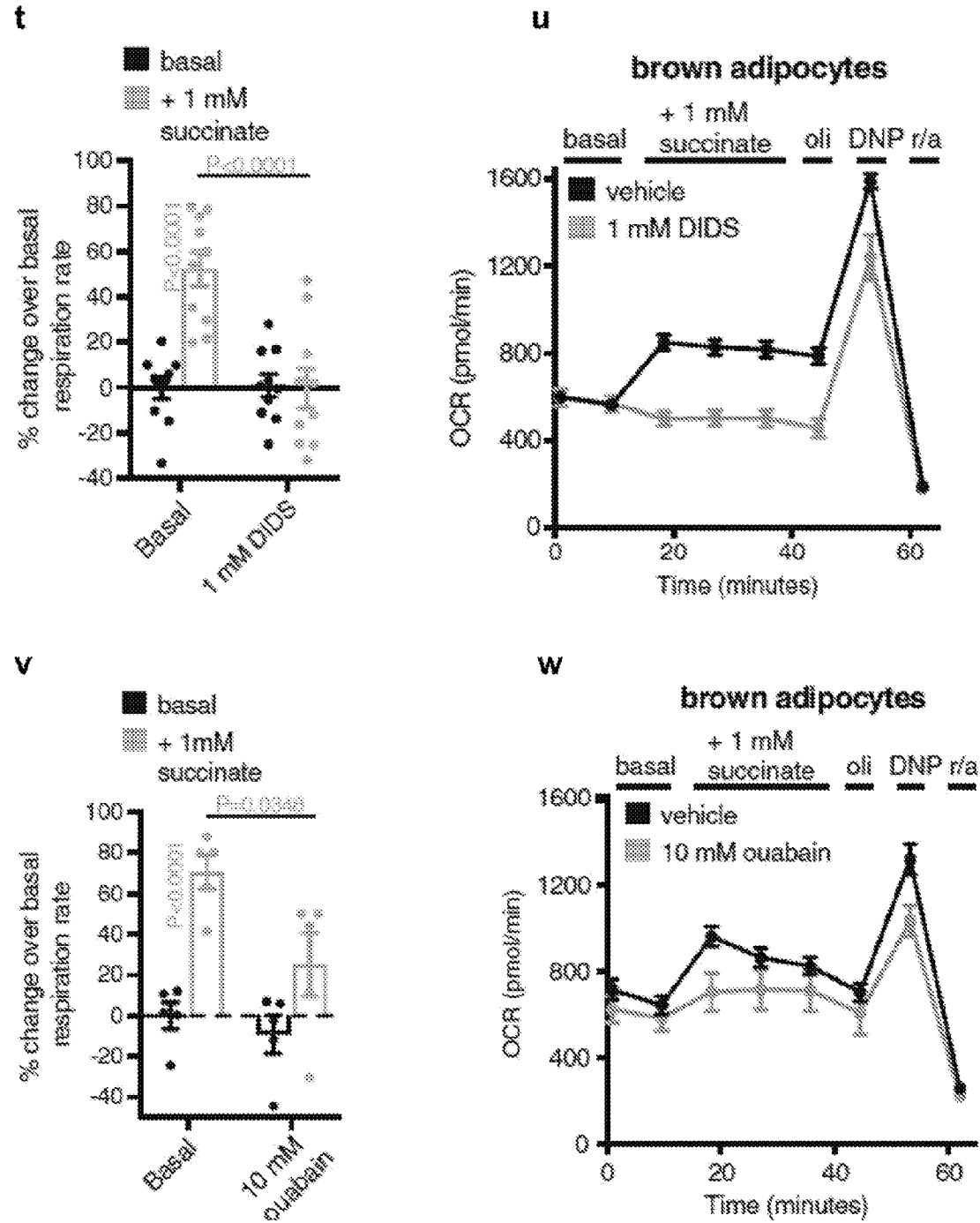


Figure 8 (cont.)



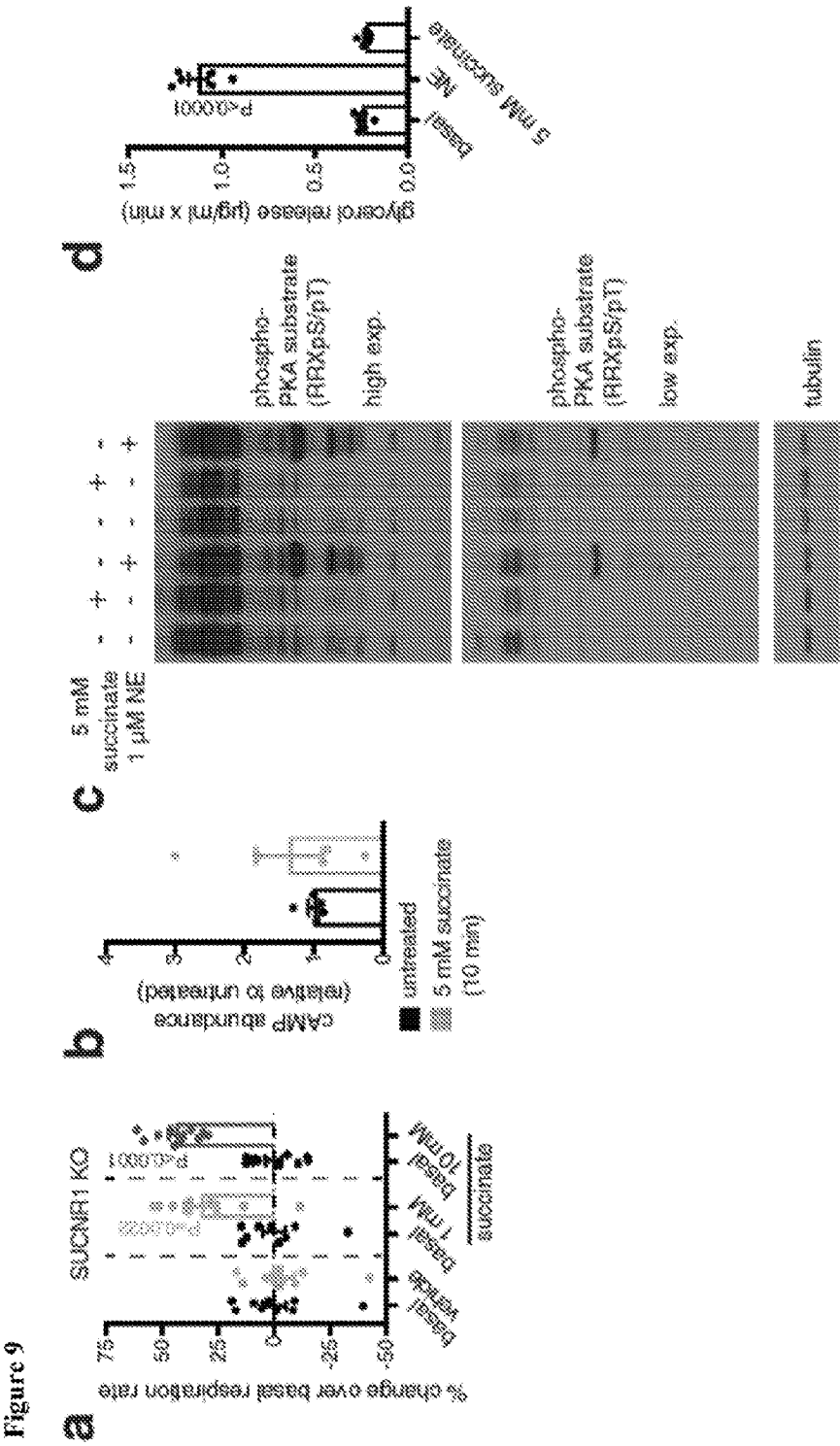


Figure 9 (cont.)

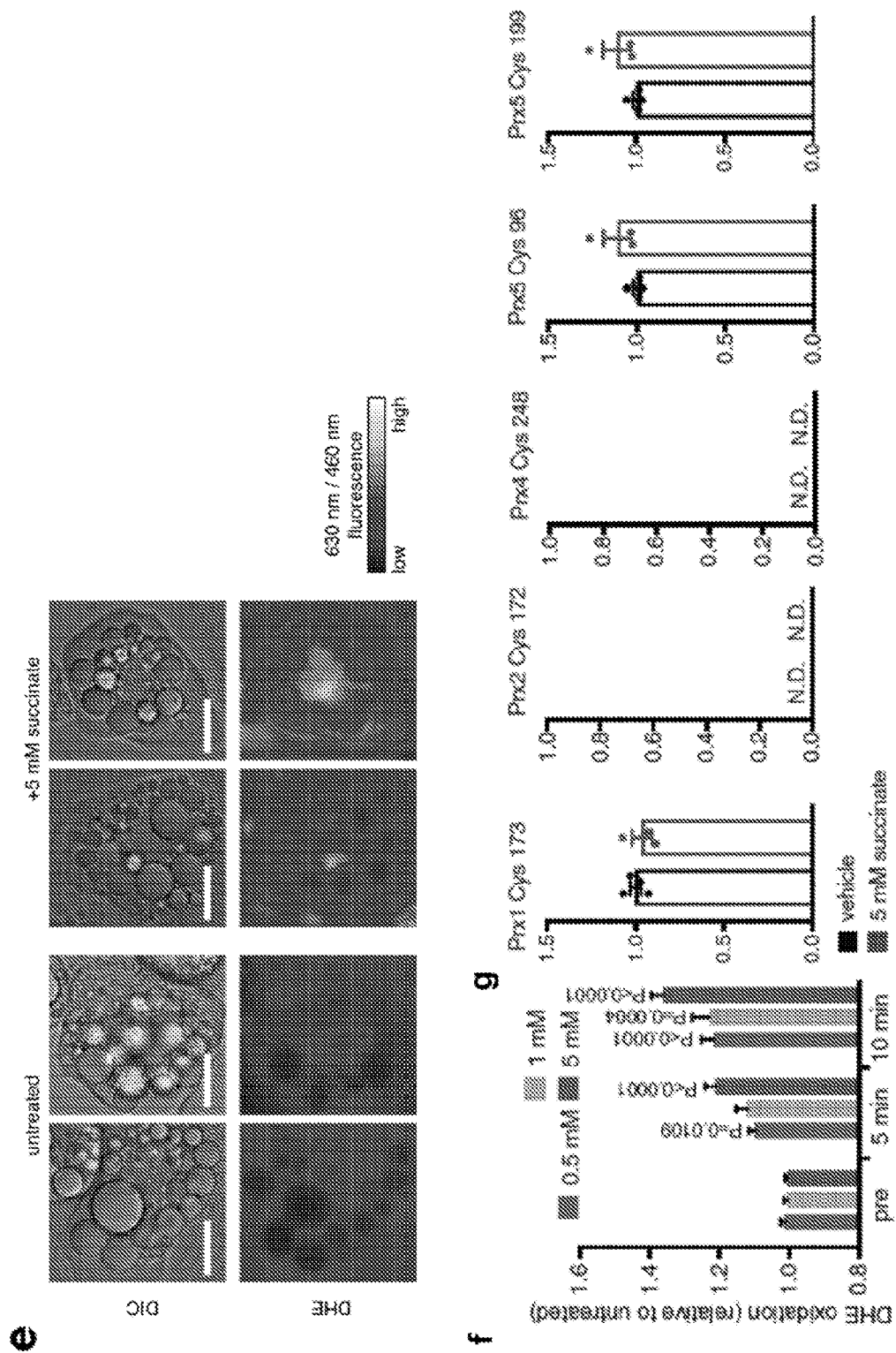


Figure 9 (cont.)

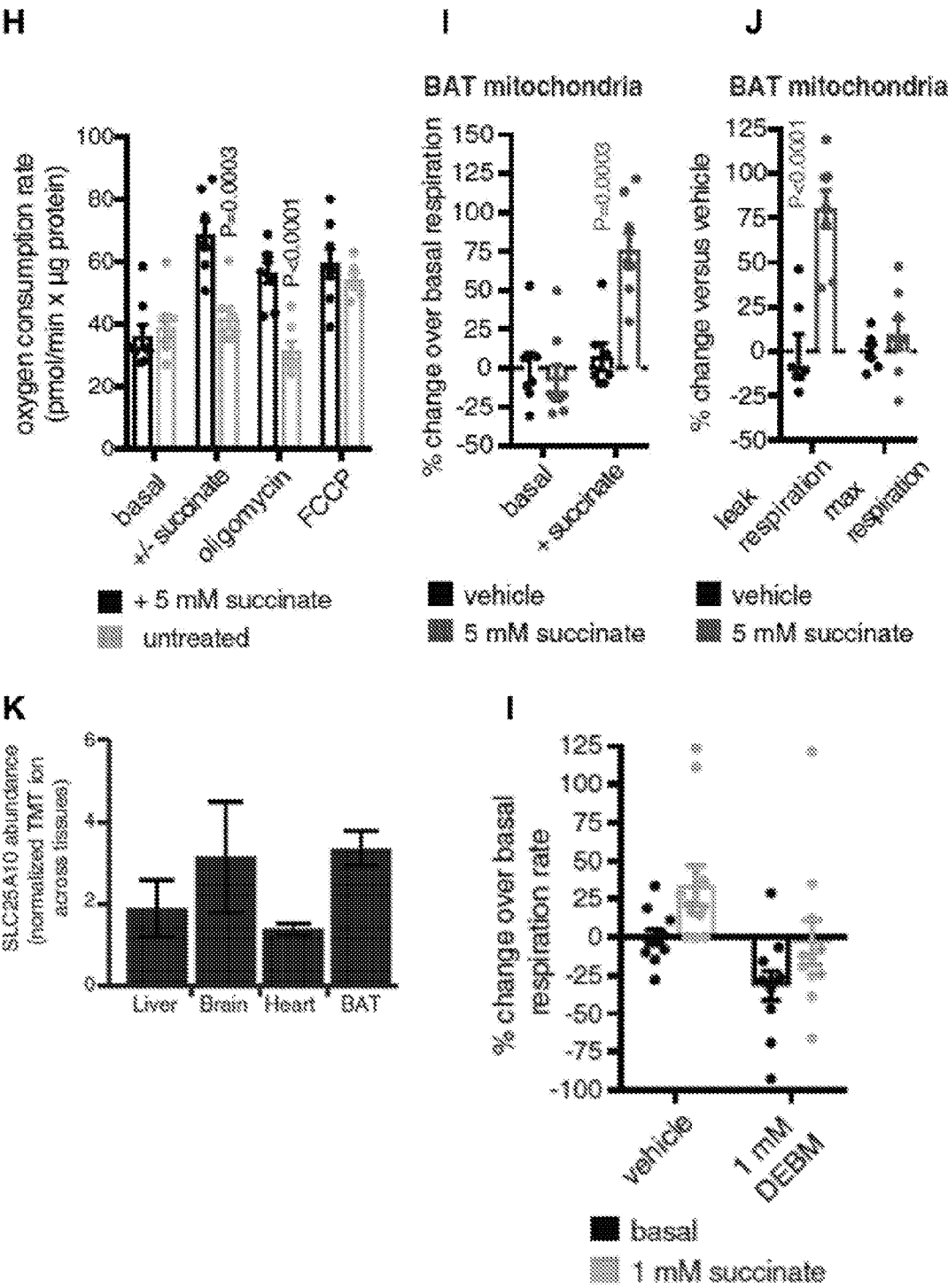
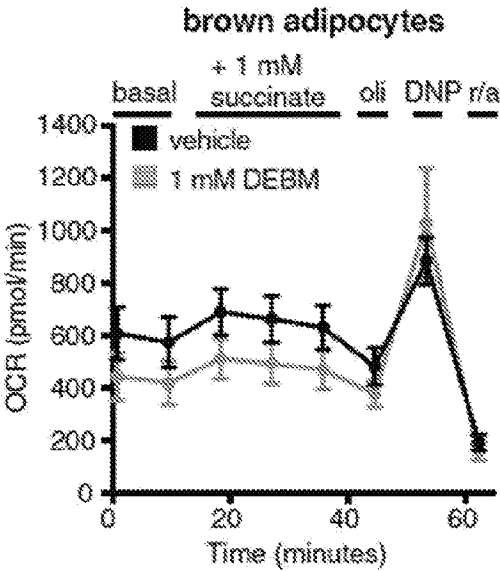
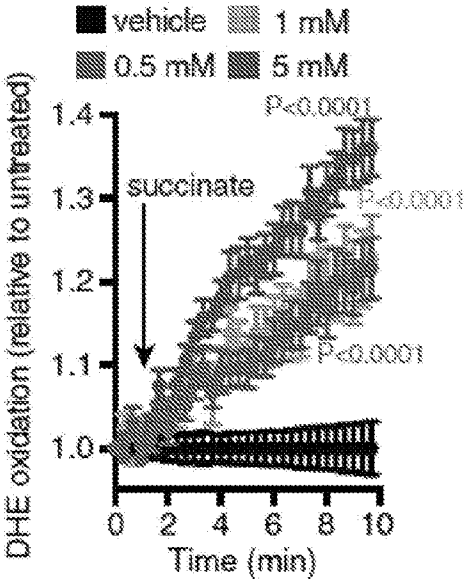


Figure 9 (cont.)

m



n



o

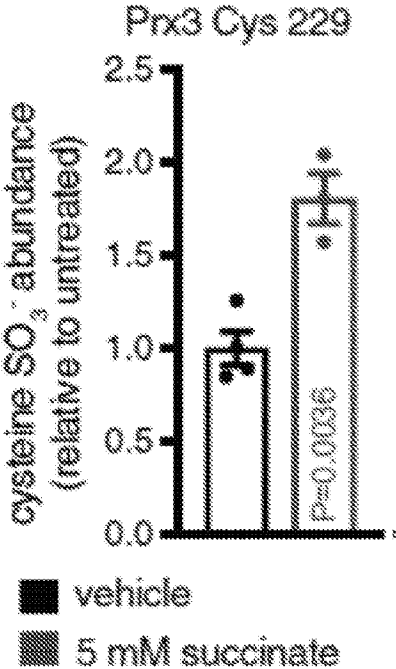
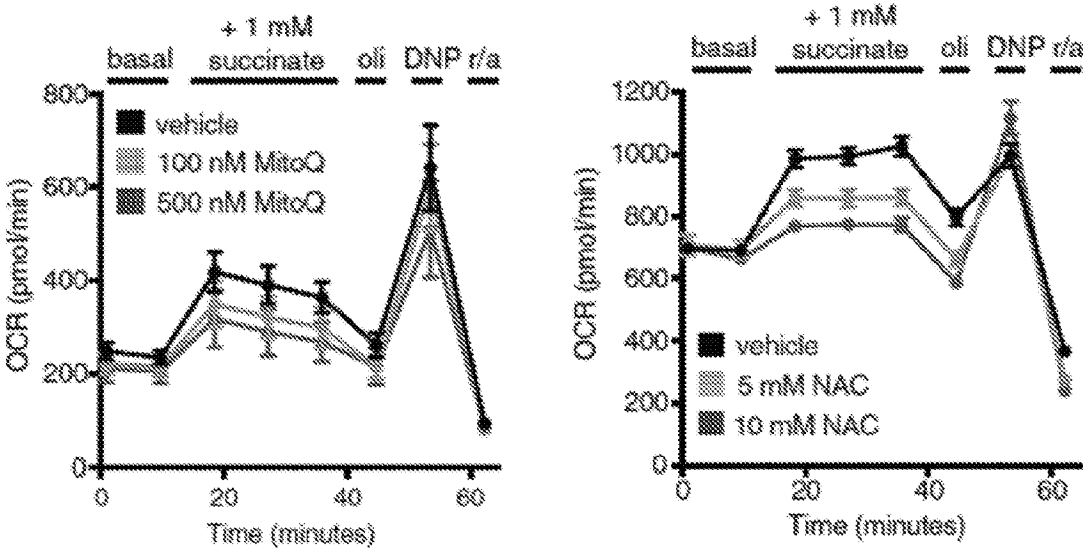
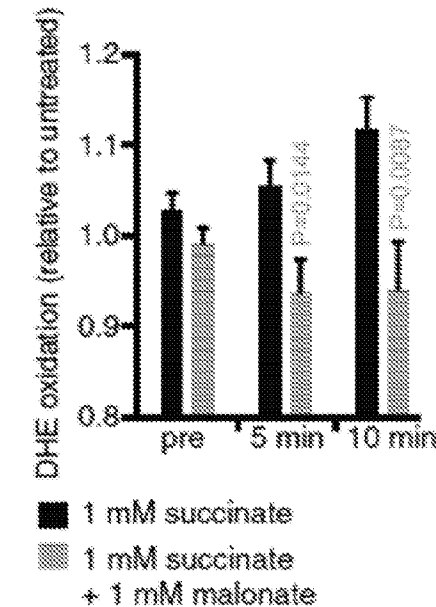


Figure 10

a



b



c

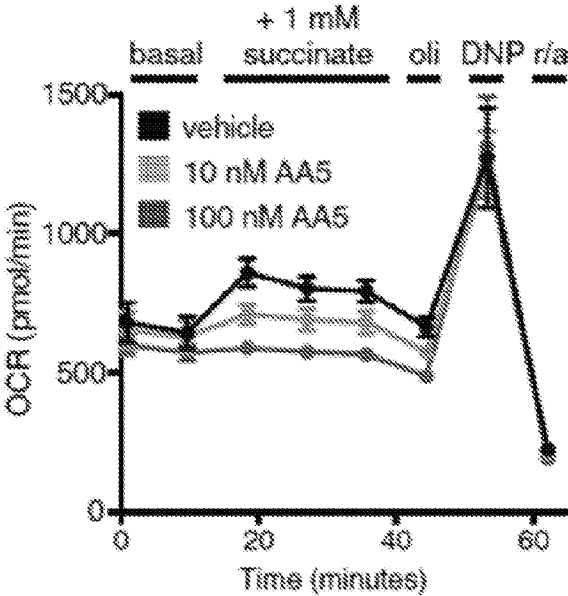
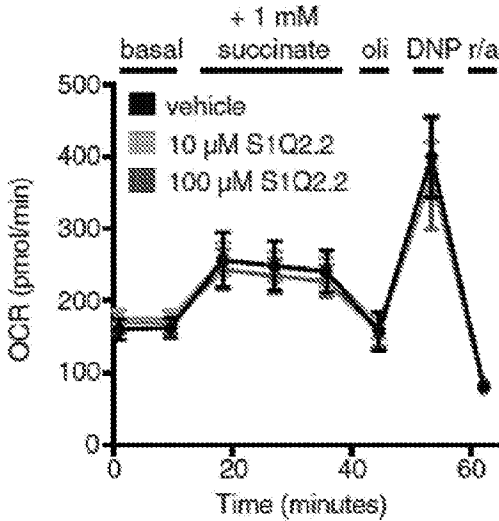
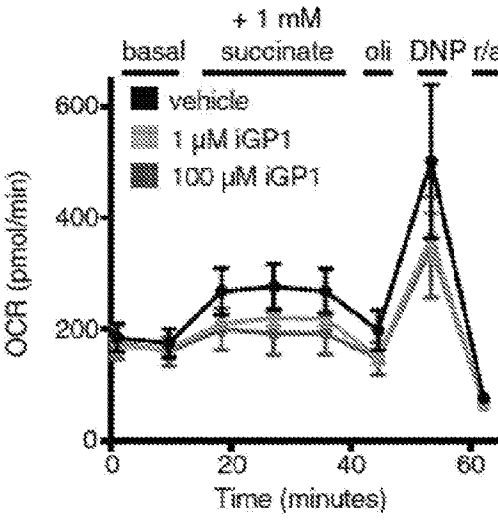


Figure 10 (cont.)

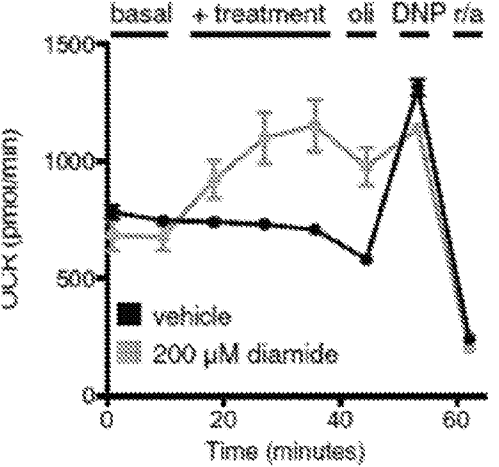
d



e



f



g

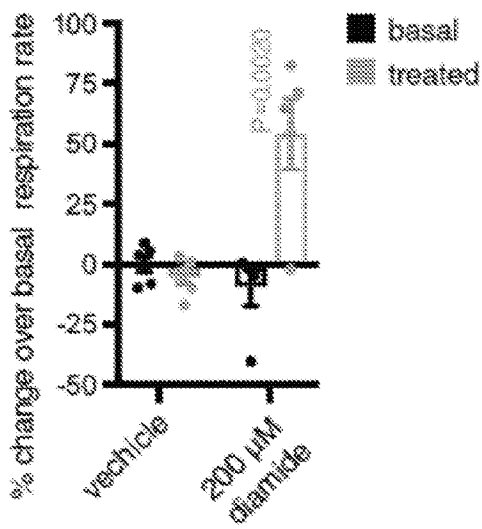


Figure 10 (cont.)

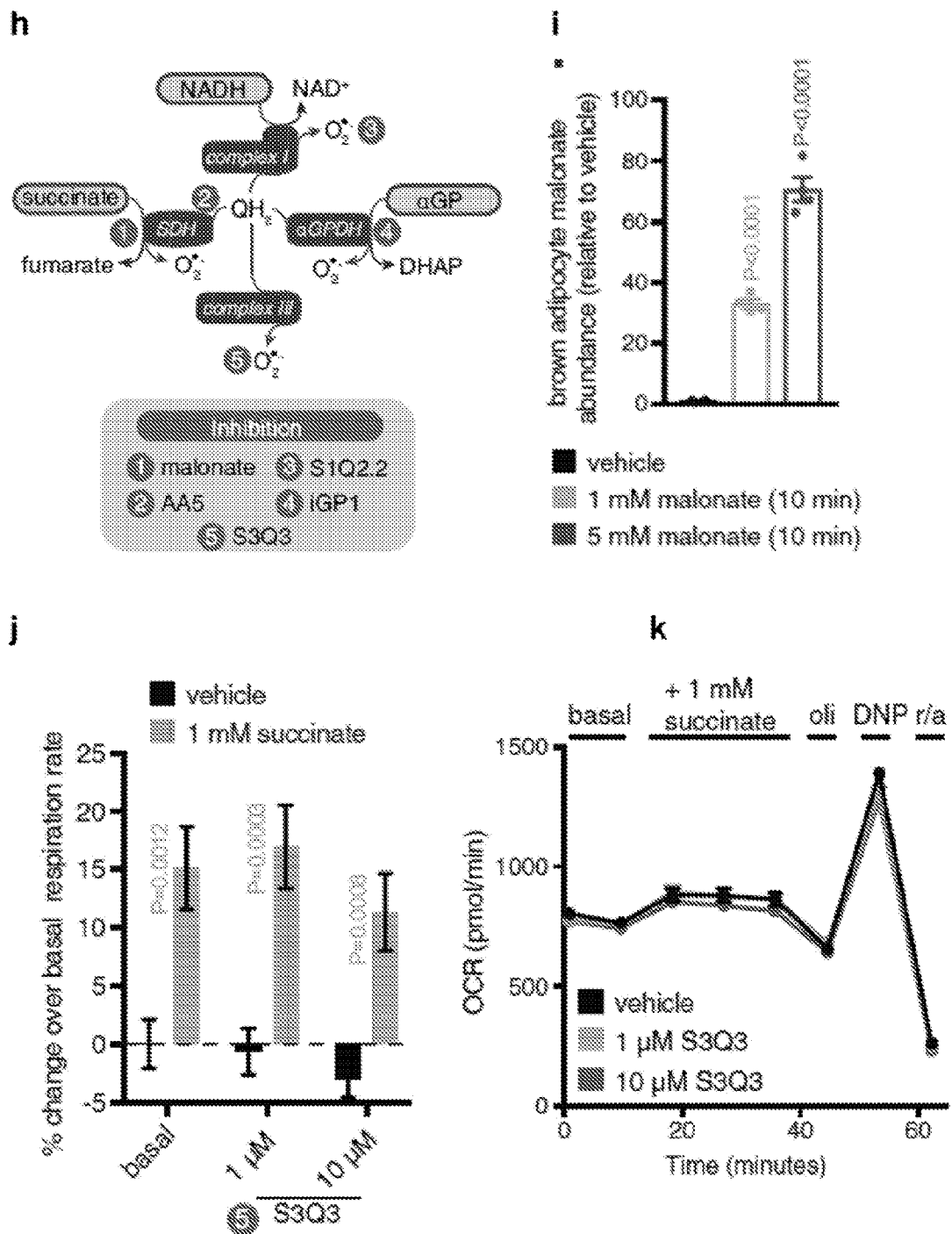


Figure 10 (cont.)

I

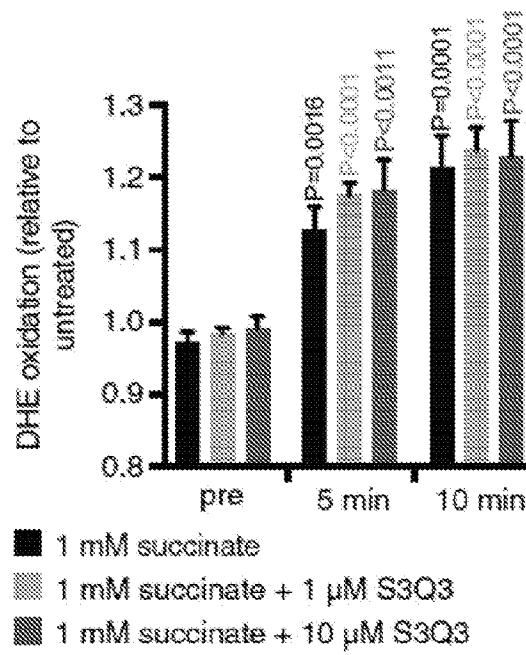


Figure 11

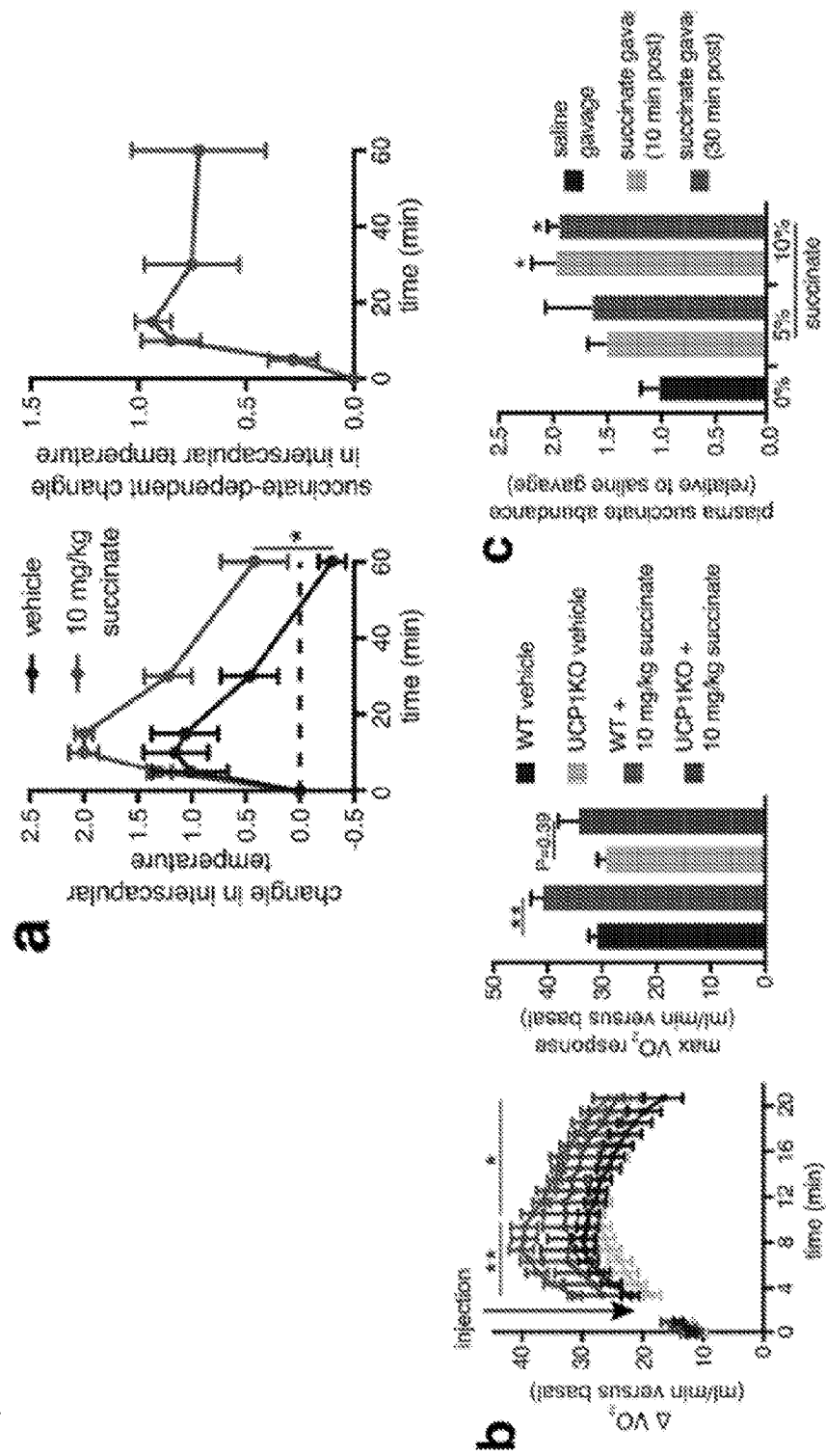


Figure 11 (cont.)

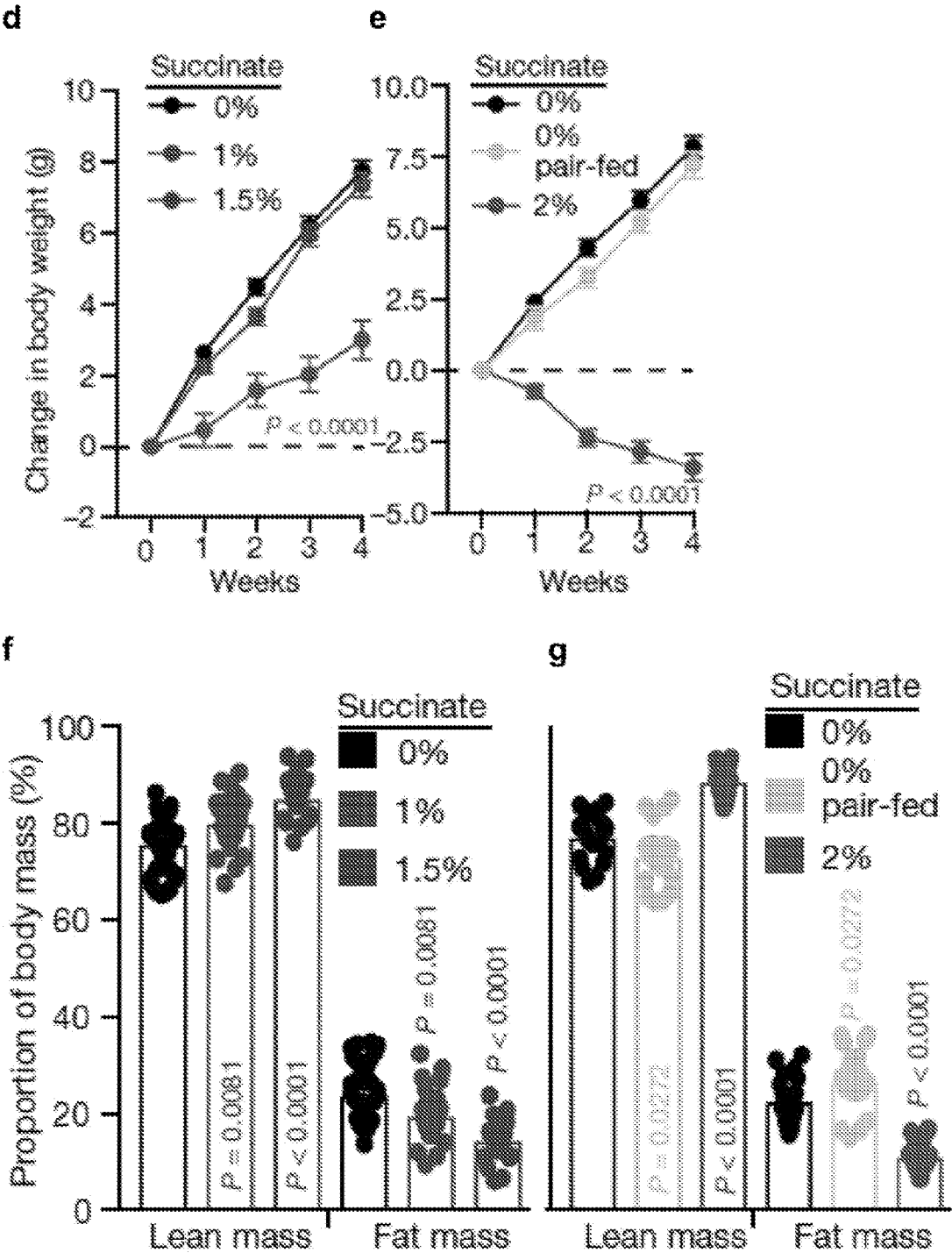


Figure 11 (cont.)

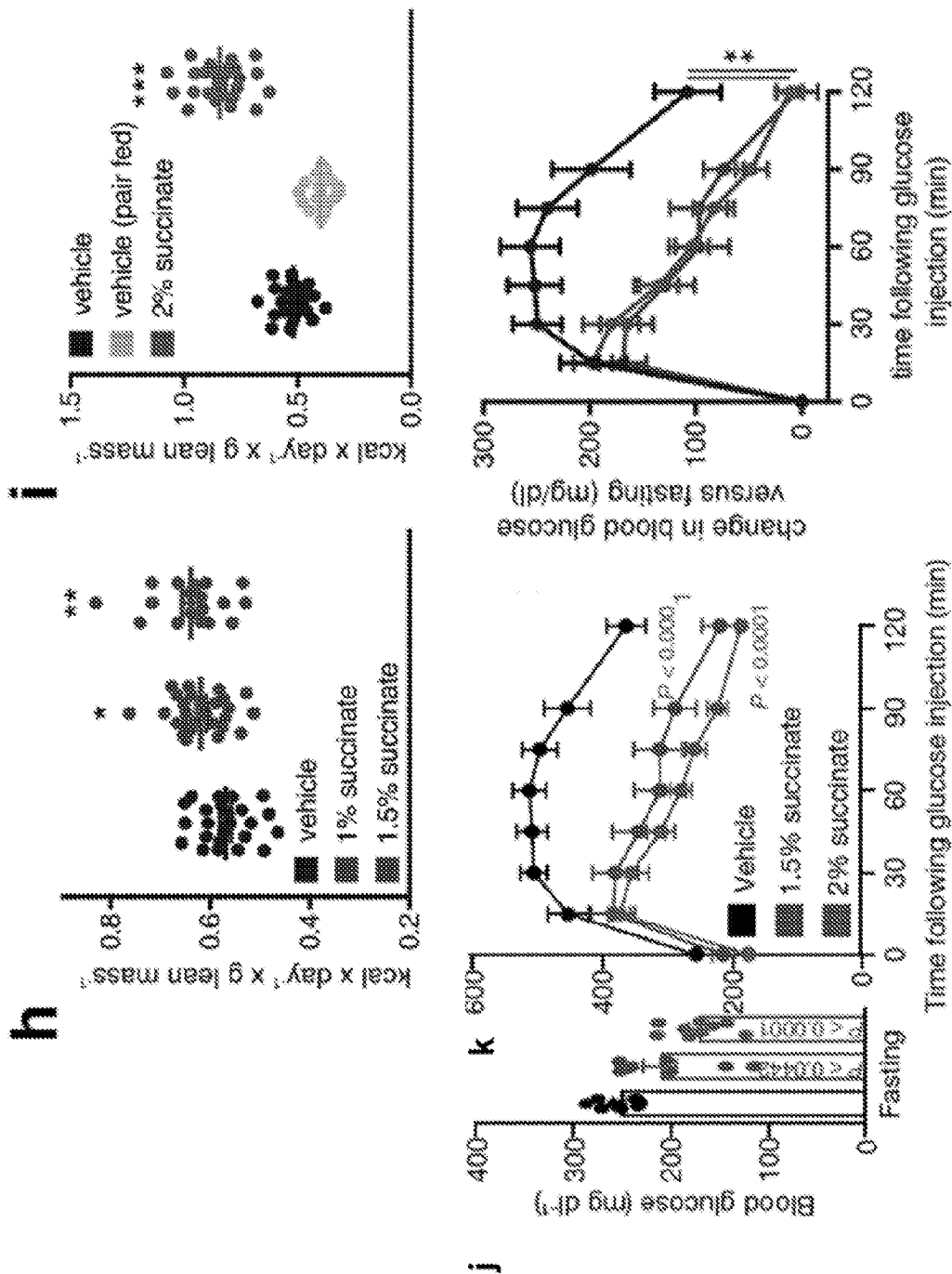
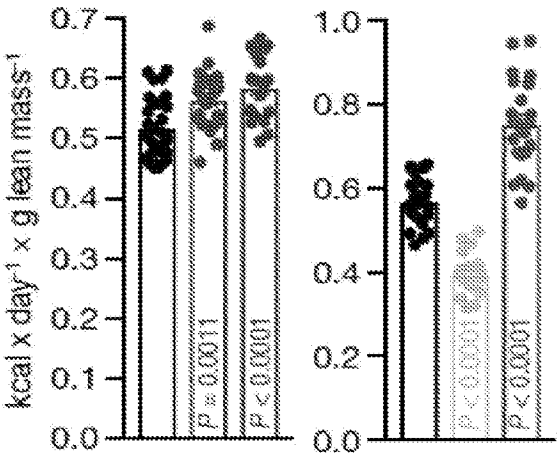
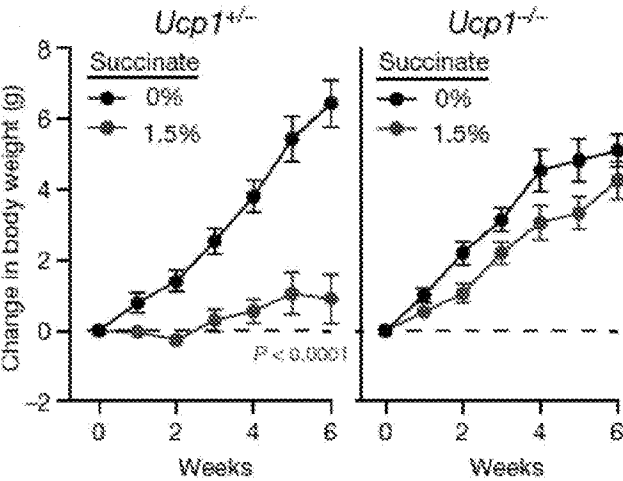


Figure 11 (cont.)

l



m



n

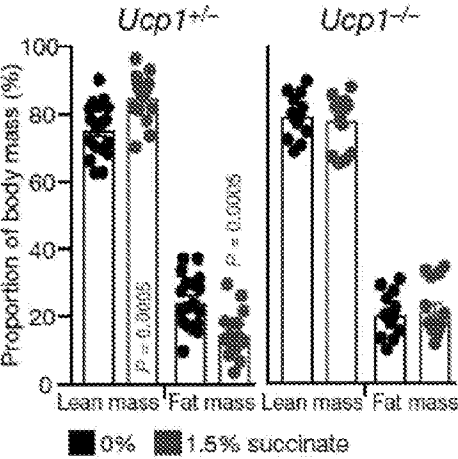
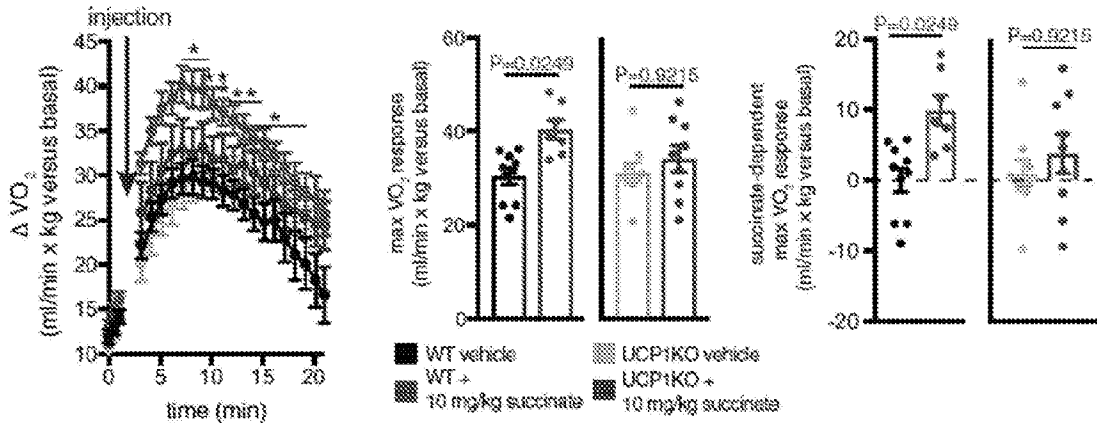
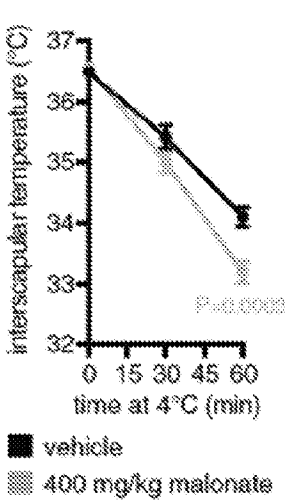


Figure 11 (cont.)

o



p



q

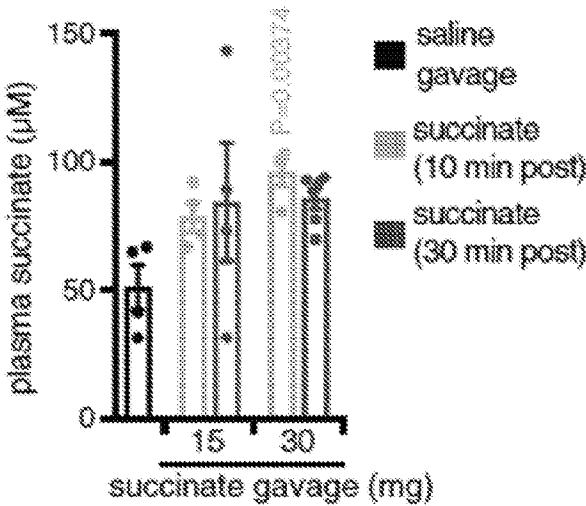


Figure 12

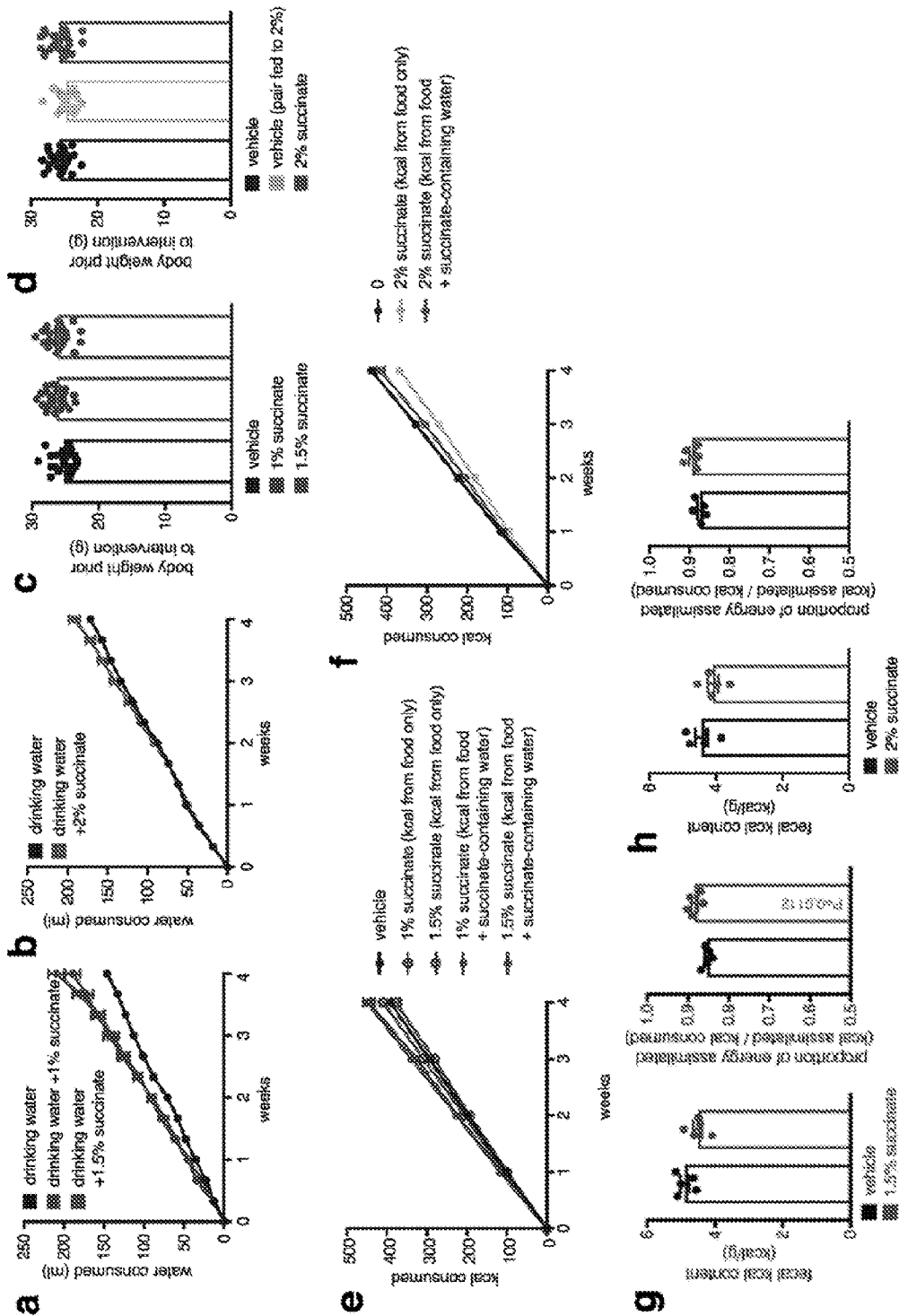
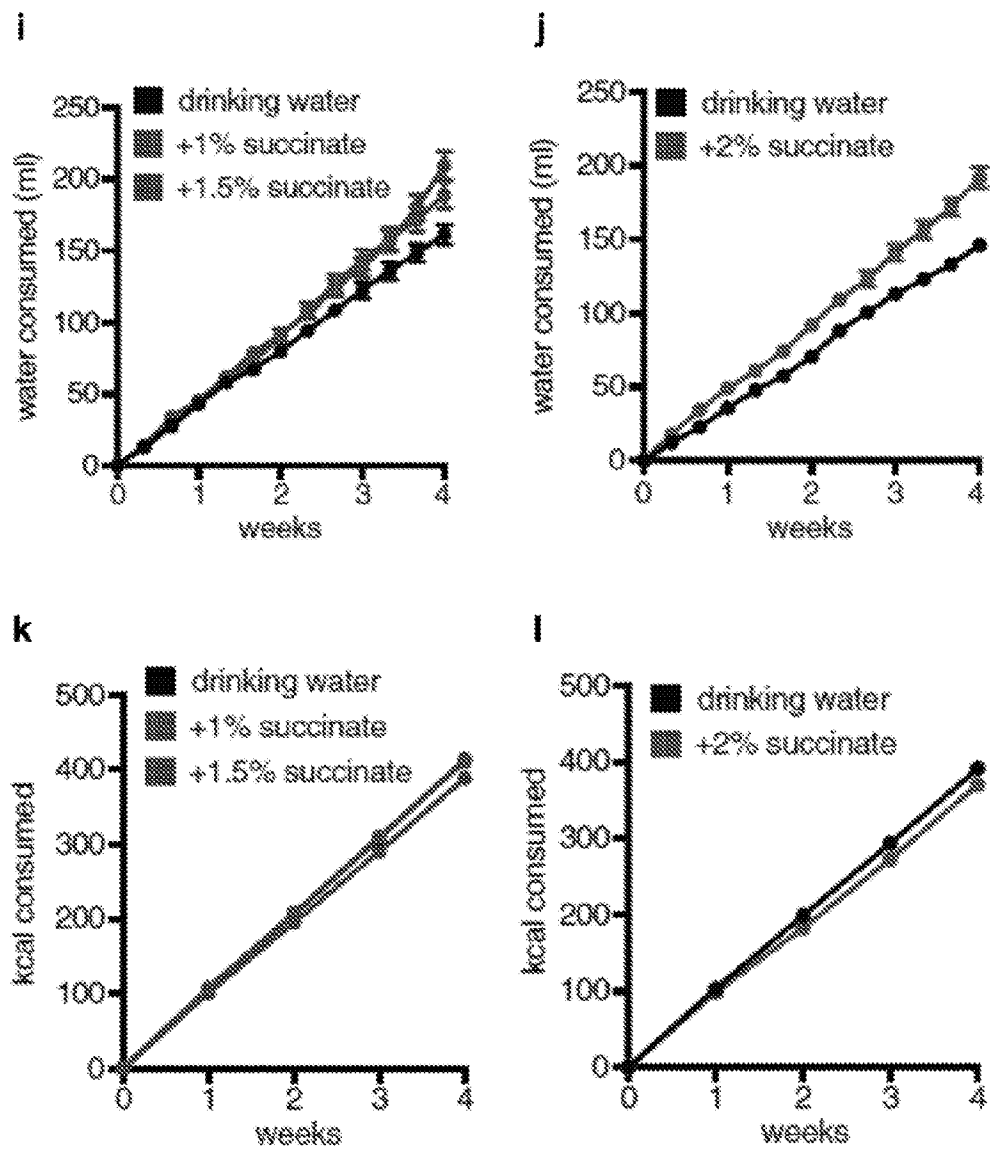


Figure 12 (cont.)



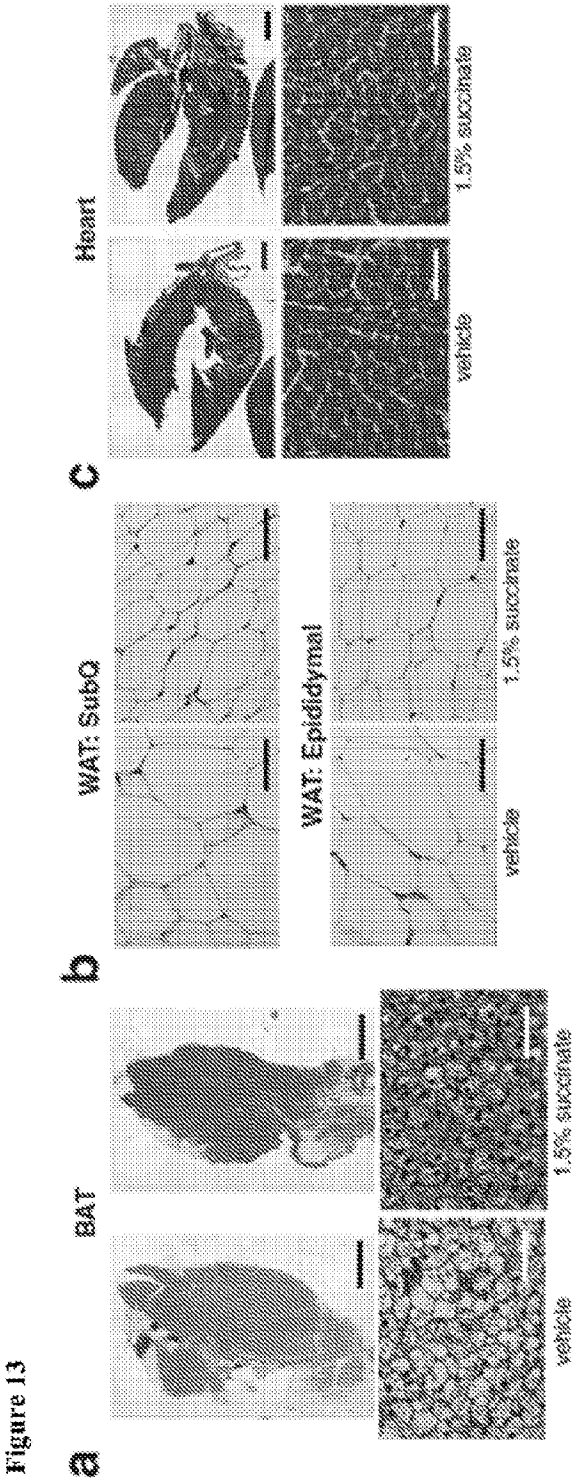
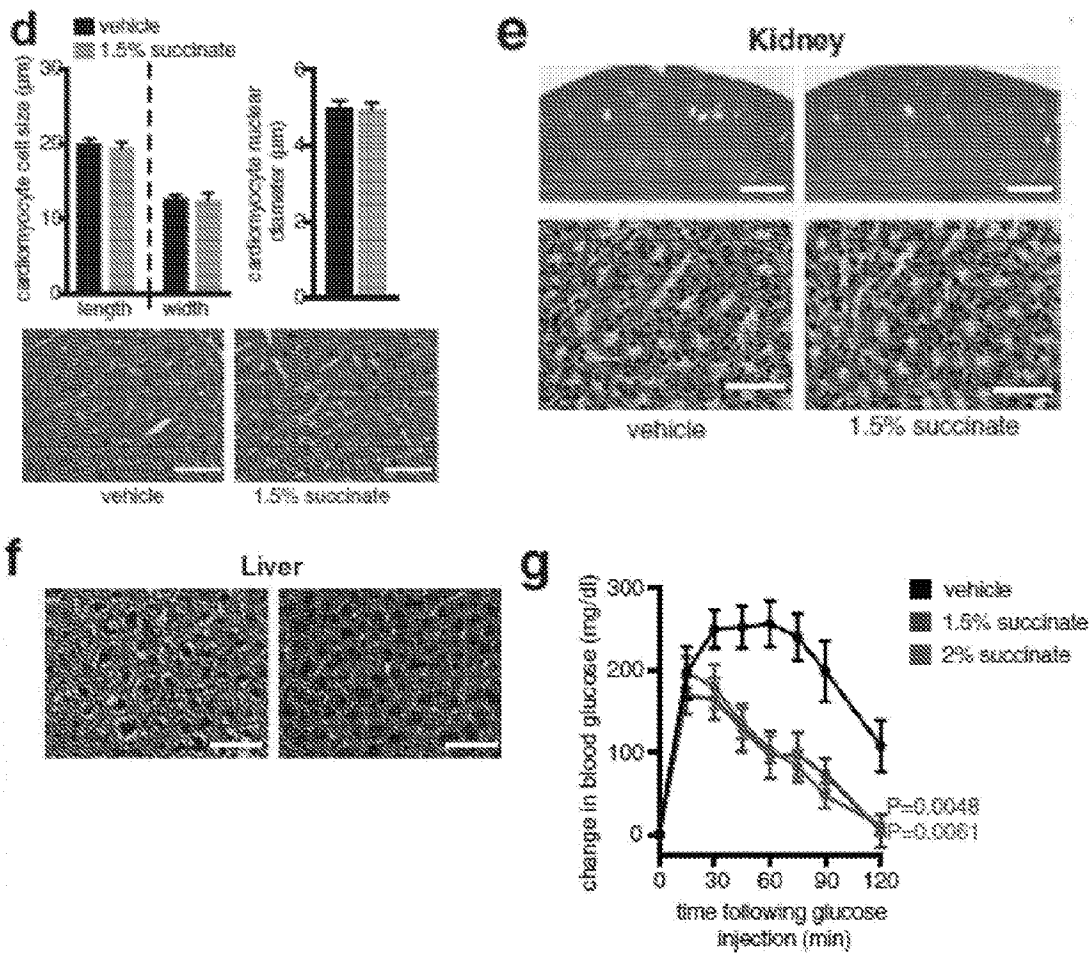


Figure 13 (cont.)



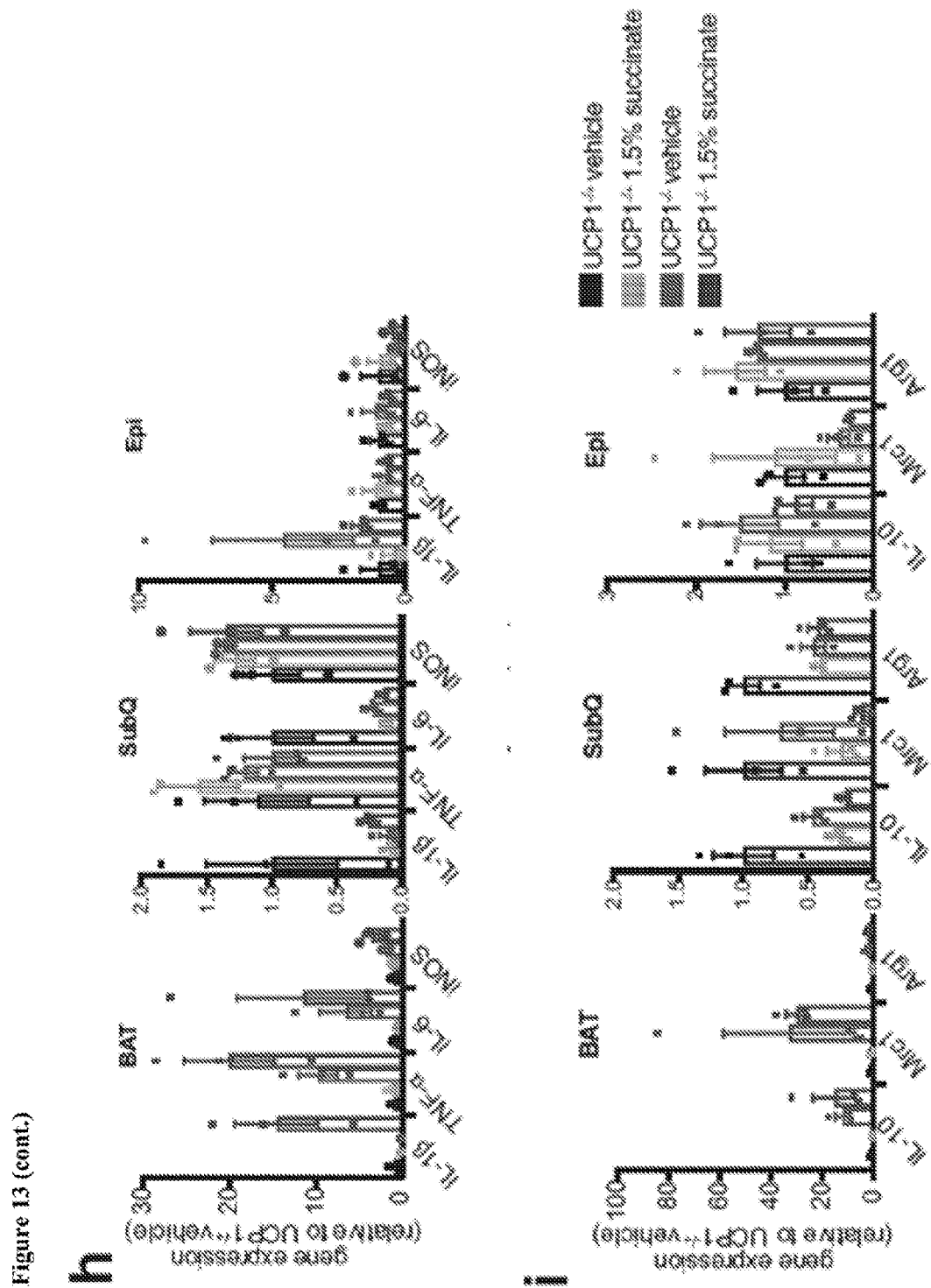


Figure 14

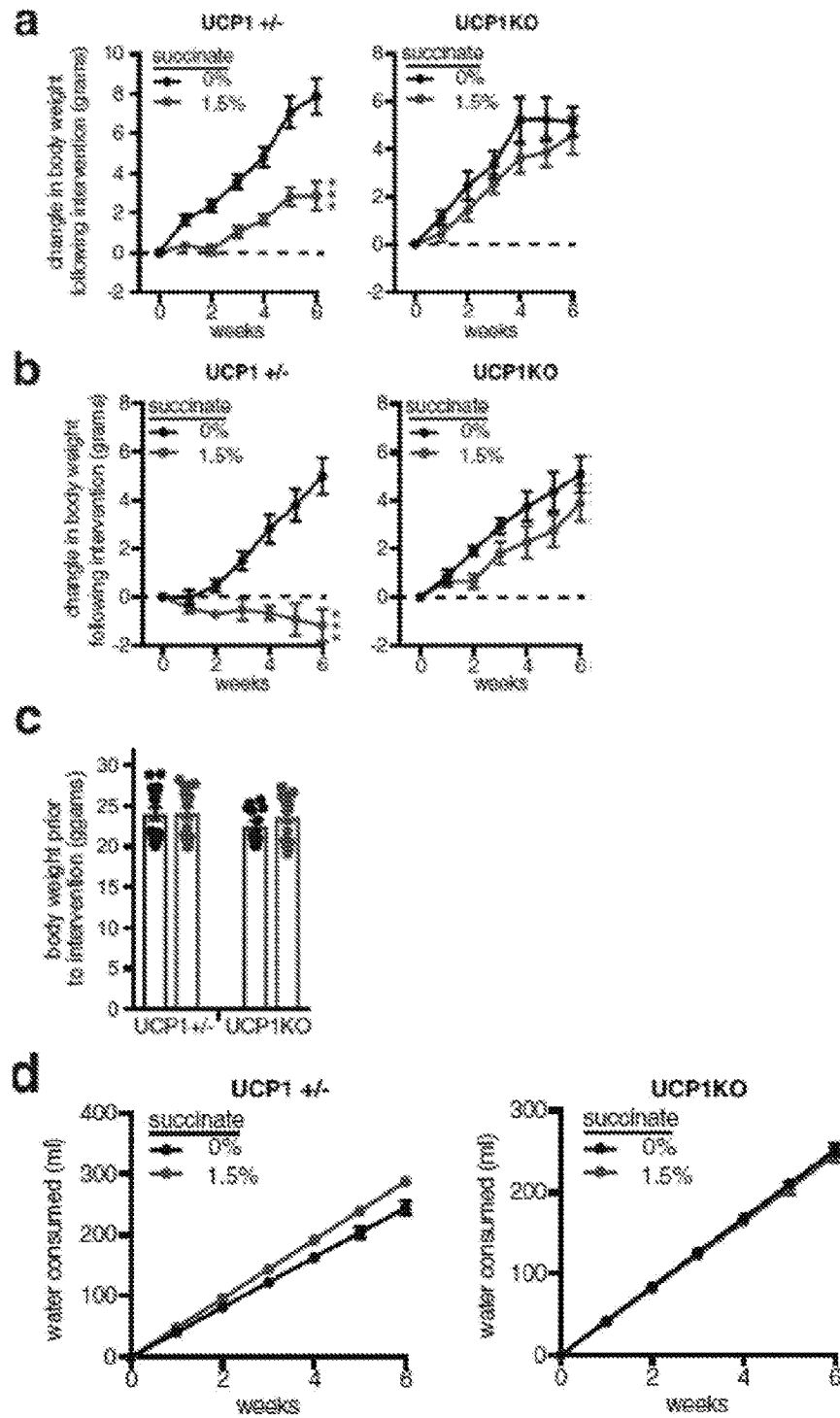
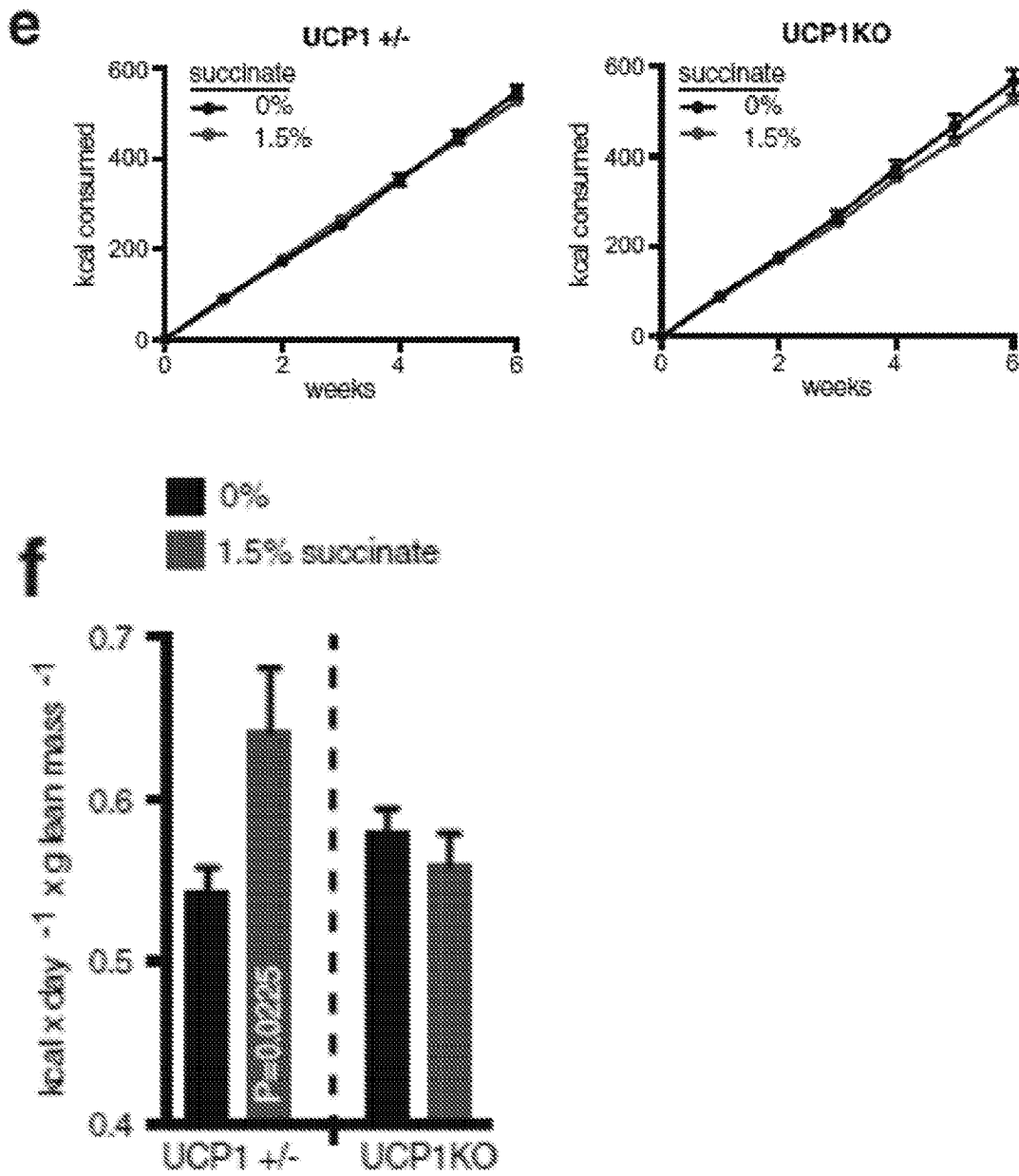


Figure 14



INTERNATIONAL SEARCH REPORT

019/012283 24.04.2019

International application No.

PCT/US2019/012283

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/19; A61K 31/194; A61K 31/225; A61P 35/00; A61P 43/00; C12N 1/19 (2019.01)

CPC - A61K 31/19; A61K 31/194; A61K 31/225; C07K 2319/00 (2019.02)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

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

USPC - 435/183; 435/6.15 (keyword delimited)

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2017/0022475 A1 (MEMORIAL SLOAN KETTERING CANCER CENTER et al) 26 January 2017 (26.01.2017) entire document	1-4, 11-14, 17, 24-26
X	US 2015/0025043 A1 (SKULACHEV et al) 22 January 2015 (22.01.2015) entire document	1, 5, 6, 11, 15, 16, 30-32
X	WO 2017/142855 A1 (ARTYOMOV et al) 24 August 2017 (24.08.2017) entire document	36
Y	US 2005/0095240 A1 (GANAPATHY et al) 05 May 2005 (05.05.2005) entire document	37-39
Y	CHUN et al. "Serial Plasma Metabolites Following Hypoxic-Ischemic Encephalopathy in a Nonhuman Primate Model," Developmental Neuroscience, 27 February 2015 (27.02.2015), Vol. 37, No. 2, Pgs. 161-171. entire document	37-39
P, X	MILLS et al. "Accumulation of Succinate Controls Activation of Adipose Tissue Thermogenesis," Nature, 18 July 2018 (18.07.2018), Vol. 560, No. 7716, Pgs. 102-106. entire document	1-6, 11-17, 24-26, 30-32, 36-39
A	US 2017/0135977 A1 (MEDICAL RESEARCH COUNCIL et al) 18 May 2017 (18.05.2017) entire document	1-6, 11-17, 24-26, 30-32, 36-39
A	PACKER et al. "Alpha-Lipoic Acid as a Biological Antioxidant," Free Radical Biology and Medicine, 01 August 1995 (01.08.1995), Vol. 19, Iss. 2, Pgs. 227-250. entire document	1-6, 11-17, 24-26, 30-32, 36-39
A	RIBE et al. "Endofacial Competitive Inhibition of Glucose Transporter-4 Intrinsic Activity by the MitogenActivated Protein Kinase Inhibitor SB203580," Endocrinology, 20 January 2005 (20.01.2005), Vol. 146, Iss. 4, Pgs. 1713-1717. entire document	1-6, 11-17, 24-26, 30-32, 36-39

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

17 March 2019

Date of mailing of the international search report

24 APR 2019

Name and mailing address of the ISA/US

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P.O. Box 1450, Alexandria, VA 22313-1450
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Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

PCT/US2019/012283 24.04.2019

International application No.

PCT/US2019/012283

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 7-10, 18-23, 27-29, 33-35, 40-66
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.