TREATMENT OF FRAILTY SYNDROME AND SYMPTOMS THEREOF

Abstract:
Compositions and methods to improve energy metabolism; increase levels of ATP; reduce or delay insulin resistance; control the blood levels of glucose, insulin, triglycerides, free fatty acids, cholesterol, or combinations thereof; reduce weight gain; improve cognitive function and mental acuity; improve memory or retention of memories; increase physical activity; improve endurance; reduce or prevent an increase in the ratio of fibrous tissue to muscle (as also referred to as the fibrous tissue: muscle ratio); reduce or prevent an increase in inflammation, such as chronic or acute inflammation; reduce amyloid blockage of mitochondrial respiration, or combinations thereof in a subject are disclosed. Typically, the methods include administering a therapeutically effective amount of a pharmaceutical composition comprising a fusion polypeptide and a pharmaceutically acceptable carrier to a subject. The compositions and methods can be used to treat or prevent age-related disorders or a symptom or co-morbidity thereof.

FIGURE 42
### Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(a))
- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(ii))
- of inventorship (Rule 4.17(iv))

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TREATMENT OF FRAILTY SYNDROME 
AND SYMPTOMS THEREOF

FIELD OF THE INVENTION

The invention is generally directed to methods and compositions for 
treating or inhibiting age-related disorders, in particular, frailty syndrome.

BACKGROUND OF THE INVENTION

According to the U.S. Census Bureau, the elderly population 
increased eleven-fold between 1900 and 1994, while the nonelderly 
population increased only three-fold. About 1 in 8 Americans were elderly 
in 1994, and about 1 in 5 are predicted to be elderly by the year 2030 (Day, 
(1993)). Some of the socioeconomic consequences of the aging population 
include a rise in health care costs and an increased need for personal 
assistance (Medelson and Schwartz, Health Affairs, 12(1): 119-125 (1993)).

Medical conditions linked with aging and the increased health care 
costs associated with the aging population include, but are not limited to, 
metabolic disorders such as insulin resistance or diabetes, loss of physical 
activity, loss of endurance, depression, muscle loss, sarcopenia, frailty 
syndrome, inflammation, and neurological disorders such as Alzheimer's 
disease, and comorbidities thereof including, but not limited to, obesity or 
excessive weight gain, vascular disease, heart disease, and atherosclerosis.

One disorder of particular importance is frailty. Frailty is a syndrome 
that can be characterized by loss of reserve, feebleness, vulnerability, and 
failure of homeostasis (Chan, The Hong Kong Medical Diary, 13(9):7-9 
(2008)). The loss of reserve and resilience is part of a feed-forward loop 
inviting associated comorbidities leading to further decreasing reserve. It is 
believed that declines in the molecular, cellular and physiological systems of 
the aging body are the underlying mechanisms associated with the reduction 
in the effectiveness of muscle and bone as well as declines in the circulatory, 
hormonal, and immune systems that are typical of frail individuals (Fried, et 
and Chan, The Hong Kong Medical Diary, 13(9):7-9 (2008)). Frailty can be
the consequence of one or more additional underlying diseases, for example cachexia, immobilization, aging, chronic disease, or cancer.

Although frailty, like many other age-related diseases, is often associated with chronological age, not all elderly individuals are frail and not all frail individuals are elderly. Frail individuals are typically at an increased risk of disability and death from minor internal stresses such as anxiety and depression, or external stresses such as physical strain, infections, heat, and cold. For example, individuals suffering from frailty can exhibit one or more symptoms including sarcopenia, unintentional non-muscle weight loss greater than 10 lbs per week, decreased grip strength, low energy expenditure, weakness, fatigue, and decreased walking time. These factors can contribute to a progressive increase in disability, dependency, the need for long term care, and mortality in frail individuals over time (Chan, The Hong Kong Medical Diary, 13(9):7-9 (2008)).

Current treatments for many age-related disorders, including frailty are generally limited to treating the physical symptoms of the disease. For example, frail individuals may be encouraged to increase their amount of exercise and dietary intake, which can induce weight gain, increase mobility, enhance physical performance, improve gait, improve balance, increase bone mineral density, and increase general well-being (Espinoza and Walston, Cleveland Clinic Journal of Medicine, 72(12): 1105-1112 (2005)). Pharmaceutical treatments can include agents to improve appetite, analgesics, or hormone replacement therapy. Many of these traditional remedies are insufficient because they are limited to managing symptoms of the disease (such as pain), their efficacy is low, or they require the help or service of a caregiver (for example exercise or physical therapy).

Therefore, it is an object of the invention to provide compositions and methods for treating age-related disorders such as frailty or sarcopenia, or a co-morbidity thereof.

It is also an object of the invention to provide compositions and methods for treating metabolic disorders such as insulin resistance, diabetes, and hypercholesterolemia.
It is also an object of the invention to provide compositions and methods for treating or preventing excessive weight gain.

It is also an object of the invention to provide compositions and methods for increasing muscle retention, or increasing muscle regenerative capacity.

It is further object of the invention to compositions and methods to treat or prevent one or more symptoms of sacropenia.

It is also an object of the invention to provide compositions and methods for increasing muscle retention, or increasing muscle regenerative capacity.

It is also an object of the invention to provide compositions and methods for increasing stamina, endurance, physical activity or combinations thereof.

**SUMMARY OF THE INVENTION**

Compositions and methods to improve energy metabolism; increase levels of ATP; reduce or delay insulin resistance; control the blood levels of glucose, insulin, triglycerides, free fatty acids, cholesterol, or combinations thereof; reduce weight gain; improve cognitive function and mental acuity; improve memory or retention of memories; increase physical activity; improve endurance; reduce or prevent an increase in the ratio of fibrous tissue to muscle (as also referred to as the fibrous tissue:muscle ratio); reduce or prevent an increase in inflammation, such as chronic or acute inflammation; reduce amyloid blockage of mitochondrial respiration, or combinations thereof in a subject are disclosed. Typically, the methods include administering a therapeutically effective amount of a pharmaceutical composition including a mitochondrial transcription factor such as mitochondrial transcription factor A - mitochondrial (TFAM) or a functional fragment or fusion protein thereof, and a pharmaceutically acceptable carrier to a subject. The subject can be young or old, male or female, healthy or have one or more the diseases or disorders described herein.

Suitable compositions for use with the disclosed methods typically include a mitochondrial DNA-binding polypeptide, preferably a mitochondrial transcription factor or mitochondrial DNA-binding fragment or fusion protein thereof. Examples of mitochondrial DNA-binding
polypeptides include, but are not limited to, mitochondrial transcription factors such as transcription factor A, mitochondrial (TFAM), transcription factor B1, mitochondrial (TFB1M), transcription factor B2, mitochondrial (TFB2M), Polymerase (RNA) Mitochondrial (DNA directed) (POLRMT); and functional fragments, variants, and fusion polypeptides thereof.

Exemplary fusion polypeptides include a mitochondrial DNA binding polypeptide, a protein transduction domain, and a targeting signal or targeting domain. Suitable mitochondrial DNA-binding polypeptides include a mature transcription factor A - mitochondria (TFAM) polypeptide, or a mitochondrial DNA binding fragment thereof. In a preferred embodiment, the targeting signal is an amino acid sequence that targets the fusion protein to the mitochondria, for example a mitochondrial localization signal. In some embodiments the mitochondrial DNA binding polypeptide is TFAM polypeptide having at least 95% sequence identity to SEQ ID NO:26 or a mitochondrial DNA binding fragment thereof.

Diseases, disorder, and conditions that can be treated using the disclosed compositions and methods include age-related disorders or symptoms or co-morbidities thereof. Examples of age-related disorders include, but are not limited to, metabolic disorders such as insulin resistance or diabetes, loss of physical activity, loss of endurance, depression, muscle loss, sarcopenia, frailty syndrome, chronic or acute inflammation, neurological disorders such as Alzheimer’s disease, and comorbidities of age-related disorders including, but not limited to, obesity or excessive weight gain, vascular disease, heart disease, and atherosclerosis. In some embodiment a subject with an age-related disorder, or comorbidity thereof is elderly. In some embodiments a subject with an age-related disorder, or comorbidity thereof is not elderly.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a bar graph showing optical density measurements of brain lysates of vehicle control and rhTFAM treated (low = 0.5 mg/kg, and high = 1.5 mg/kg) mice subjected to SDS-PAGE and immune-blotted using an antibody cocktail for the various subunits of Complexes I-V electron transport chain protein subunits.
Figure 2 is a bar graph showing maximal enzymatic capacity (mOD/min) of C-I of the ETC following administration of a low dose (0.5 mg/kg) of rhTFAM, a high dose of rhTFAM (1.5 mg/kg) or vehicle control.

Figure 3 is a bar graph showing maximal enzymatic capacity (mOD/min) of C-IV of the ETC following administration of a low dose of rhTFAM, a high dose of rhTFAM or vehicle control (low = 0.5 mg/kg, and high = 1.5 mg/kg).

Figure 4 is a bar graph showing the level of ATP in the brain of db/db mice following administration of a low dose (0.2 mg/kg) of rhTFAM, a high dose (1.2 mg/kg) of rhTFAM or vehicle control. The rhTFAM doses are approximately 0.2, 0.4, 0.8 and 1.2 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 5 is a bar graph showing the blood glucose level (mg/dL) of control (saline) and rhTFAM treated ob/ob mice over time (Days). rhTFAM was administered in a dose of 50 µg, 75 µg, 100 µg, or 150 µg. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 6 is a bar graph showing the glycated serum proteins (GPS) (µmol/L) of control (saline) and rhTFAM treated ob/ob mice over time (Days). rhTFAM was administered in a dose of 50 µg, 75 µg, 100 µg, or 150 µg. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 7 is a line graph showing the blood glucose levels (mg/dL) of ob/ob mice over time (min) as measured in a glucose tolerance assay prior to initiation of rhTFAM treatment (Day 0).

Figure 8 is a line graph showing the blood glucose levels (mg/dL) of control (saline) and rhTFAM treated ob/ob mice over time (min) as measured in a glucose tolerance assay (min) following initiation of rhTFAM treatment (Day 28). rhTFAM was administered in a dose of 50 µg, 75 µg, 100 µg, or 150 µg. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg for an average mouse with a body weight of about 30 grams.

Figure 9 is a bar graph showing the triglyceride levels (mg/dL) of control (saline) and rhTFAM treated ob/ob mice over time (Days). rhTFAM...
was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 10 is a bar graph showing the free fatty acid (FFA) levels (mEq/L) of control (saline) and rhTFAM treated ob/ob mice over time (Days). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 11 is a bar graph showing total cholesterol (mg/dL) of control (saline) and rhTFAM treated ob/ob mice over time (Days). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 12 is a bar graph showing insulin (ng/ml) of control (saline) and rhTFAM treated ob/ob mice over time (Days). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 13 is a line graph showing the accumulated food intake (grams) of control (saline) and rhTFAM ob/ob mice over time (Days).

Figure 14 is a bar graph showing the weight (grams) of control (saline) and rhTFAM treated ob/ob mice over time (Days). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams. The bars, from left to right at each time point represent vehicle controls, rhTFAM dose of 50 µl, 75 µl, 100 µl, and 150 µl, respectively.

Figure 15 is a bar graph showing the blood glucose level (mg/dL) of C57BL/6 mice fed a regular diet (RD) or a high fat diet (HFD) prior to initiation of rhTFAM treatment (Day 0).

Figure 16 is a bar graph showing the blood glucose level (mg/dL) of C57BL/6 mice fed a regular diet (RD) or a high fat diet (HFD) after control treatment (vehicle) or treatment with rhTFAM (Day 28). rhTFAM was
administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 17 is a bar graph showing the blood glucose level (mg/dL) of C57BL/6 mice fed a regular diet (RD) or a high fat diet (HFD) after control treatment (vehicle) or treatment with rhTFAM (Day 28). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 18 is a line graph showing the blood glucose levels (mg/dL) of C57BL/6 mice fed a regular diet (RD) or a high fat diet (HFD) over time (min) as measured in a glucose tolerance assay prior to initiation of rhTFAM treatment (Day 0).

Figure 19 is a line graph showing the blood glucose levels (mg/dL) of vehicle and rhTFAM treated C57BL/6 mice fed a high fat diet (HFD) over time (min) as measured in a glucose tolerance assay (min) following initiation of rhTFAM treatment (Day 28). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 20 is a line graph showing the blood glucose levels (mg/dL) of vehicle and rhTFAM treated C57BL/6 mice fed a high fat diet (HFD) over time (min) as measured in a glucose tolerance assay (min) following initiation of rhTFAM treatment (Day 56). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 21 is a bar graph showing the insulin level (ng/ml) of C57BL/6 mice fed a regular diet (RD) or a high fat diet (HFD) prior to initiation of rhTFAM treatment (Day 0).

Figure 22 is a bar graph showing the insulin level (ng/ml) of C57BL/6 mice fed a high fat diet (HFD) after control treatment (vehicle) or treatment with rhTFAM (Day 28). rhTFAM was administered in a dose of
50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 23 is a bar graph showing the insulin level (ng/ml) of C57BL/6 mice fed a high fat diet (HFD) after control treatment (vehicle) or treatment with rhTFAM (Day 56). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 24 is a bar graph showing the level of triglycerides (mg/dL) of C57BL/6 mice fed a regular diet (RD) or a high fat diet (HFD) prior to initiation of rhTFAM treatment (Day 0).

Figure 25 is a bar graph showing the level of triglycerides (mg/dL) of C57BL/6 mice fed a high fat diet (HFD) after control treatment (vehicle) or treatment with rhTFAM (Day 28). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 26 is a bar graph showing the level of triglycerides (mg/dL) of C57BL/6 mice fed a high fat diet (HFD) after control treatment (vehicle) or treatment with rhTFAM (Day 56). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 27 is a bar graph showing total cholesterol (mg/dL) of C57BL/6 mice fed a regular diet (RD) or a high fat diet (HFD) prior to initiation of rhTFAM treatment (Day 0).

Figure 28 is a bar graph showing total cholesterol (mg/dL) of C57BL/6 mice fed a high fat diet (HFD) after control treatment (vehicle) or treatment with rhTFAM (Day 28). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.
Figure 29 is a bar graph showing total cholesterol (mg/dL) of C57BL/6 mice fed a high fat diet (HFD) after control treatment (vehicle) or treatment with rhTFAM (Day 56). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 30 is a bar graph showing the free fatty acid (FFA) levels (mmol/L) of C57BL/6 mice fed a regular diet (RD) or a high fat diet (HFD) prior to initiation of rhTFAM treatment (Day 0).

Figure 31 is a bar graph showing the free fatty acid (FFA) levels (mmol/L) of C57BL/6 mice fed a high fat diet (HFD) after control treatment (vehicle) or treatment with rhTFAM (Day 28). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 32 is a bar graph showing the free fatty acid (FFA) levels (mmol/L) of C57BL/6 mice fed a high fat diet (HFD) after control treatment (vehicle) or treatment with rhTFAM (Day 56). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 33 is a line graph showing the weight (grams) of C57BL/6 mice fed a high fat diet (HFD) after control treatment (vehicle) or treatment with rhTFAM over time (days). rhTFAM was administered in a dose of 50 µl, 75 µl, 100 µl, or 150 µl. The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

Figure 34A is a dot plot showing the latency period (sec) in an accelerating speed rotarod assay of control (vehicle treated) and rhTFAM (dose is 0.22 mg/kg for an average mouse with a body weight of about 30 grams) treated young C57BL/6J mice over increasing speed (RPM). The plot shows the raw data from 3 trials and 3 runs/trial (n=10/group).
Figure 34B is a bar graph showing the latency period (sec) in a accelerating speed rotarod assay of control (vehicle treated) and rhTFAM (dose is 0.22 mg/kg for an average mouse with a body weight of about 30 grams) treated young C57BL/6J mice on days 1, 2, and 3.

Figure 35A is a dot plot showing the latency period (sec) in a constant speed rotarod assay of control (vehicle treated) and rhTFAM (dose is 0.22 mg/kg for an average mouse with a body weight of about 30 grams) treated young C57BL/6J mice at 15 RPM across three trials. The plot shows the raw data from 3 trials and 3 runs/trial (n=10/group).

Figure 35B is a bar graph showing the latency period (sec) in a constant speed rotarod assay of control (vehicle treated) and rhTFAM (dose is 0.22 mg/kg for an average mouse with a body weight of about 30 grams) treated young C57BL/6J mice on days 1, 2, and 3.

Figure 36A and 36B are bar graphs showing total distance traveled (cm) (36A) and percent time in the peripheral zone (36B) of young C57BL/6J mice in a standardized open field test following administration of vehicle control or rhTFAM (dose is 0.22 mg/kg for an average mouse with a body weight of about 30 grams).

Figure 37 is a bar graph showing total resting time (seconds) of young C57BL/6J mice in a standardized open field test following administration of vehicle control or rhTFAM (dose is 0.22 mg/kg for an average mouse with a body weight of about 30 grams).

Figure 38 is a line graph showing the latency to platform (seconds) of young C57BL/6J mice in a Morris Water Maze test following administration of vehicle control or rhTFAM (dose is 0.22 mg/kg for an average mouse with a body weight of about 30 grams). The graph charts four trials, all on the same day.

Figure 39 is a line graph showing the latency to platform (seconds) of young C57BL/6J mice in a Morris Water Maze test following administration of vehicle control or rhTFAM. The graph charts four trials, one each on four consecutive days.

Figure 40 is a line graph showing the average latency to platform (seconds) of aged C57BL/6J mice in a Morris Water Maze test following
administration of vehicle control, or a low or high dosage regime of rhTFAM (dose is 0.5 mg/kg ("low") or 1.5 mg/kg ("high") for an average mouse with a body weight of about 30 grams). The graph charts average latency of runs per day over a seven day period. Error bars = SEM.

Figure 41A and 41B are bar graphs showing average time spent in platform zone (41A) and average platform zone entries (41B) of aged C57BL/6J mice in a standardized open field test following administration of vehicle control or a low or high dosage regime of rhTFAM (dose is 0.5 mg/kg ("low") or 1.5 mg/kg ("high") for an average mouse with a body weight of about 30 grams).

Figure 42 is a bar graph showing the ratio of collagen and fibrosis to muscle in B6; 129S7-Sod1tmILeb/J strain 2972 mice (ratio of "blue channel" (collagen) to "red channel" (muscle)) following treatment of vehicle control or rhTFAM (0.35 mg/kg).

Figure 43 is a bar graph showing the ratio of thiobarbituric acid reactive substances to total protein (TBARS/total protein (µM/µg) in a thiobarbituric acid reactive substances assay (TBARS assay) following treatment of vehicle control or rhTFAM (dose is 0.5 mg/kg ("low") or 1.5 mg/kg ("high") for an average mouse with a body weight of about 30 grams).

Figure 44 is a line graph showing the oxygen consumption rates (OCR) as a % deviation from control for control and abeta treated cells (oligomeric or fibrilar form) treated with vehicle control or rhTFAM at time point "compound." The uncoupler FCCP was used to stimulate maximal respiration at time point ("FCCP") followed by rotenone to inhibit respiration at time point ("rotenone").

Figure 45 is a line graph showing the oxygen consumption rates (OCR) as a % deviation from control for abeta treated cells (oligomeric or fibrilar form) treated with vehicle control or rhTFAM at time point "compound." The uncoupler FCCP was used to stimulate maximal respiration at time point ("FCCP") followed by rotenone to inhibit respiration at time point ("rotenone").

Figure 46 is a graph of the area under the curve in arbitrary units of lactate determined using magnetic resonance spectroscopy versus time in
hours of in a voxel of brain from mice treated with rhTFAM (arrows) or vehicle.

Figure 47 is a graph of the area under the curve in percent change of lactate determined using magnetic resonance spectroscopy versus time in hours of in a voxel of brain from mice treated with rhTFAM (arrows) or vehicle.

Figure 48 is a graph of the area under the curve in arbitrary units of N-Acetyl aspartate determined using magnetic resonance spectroscopy versus time in hours of in a voxel of brain from mice treated with rhTFAM (arrows) or vehicle.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

In describing and claiming the disclosed subject matter, the following terminology will be used in accordance with the definitions set forth below.

As used herein, "age-related disorder" includes any disease, disorder, or condition associated with aging or increased age, and includes, but is not limited to, all of the diseases, disorders and conditions described herein. A subject of any age can suffer from, or be diagnosed with an age-related disorder.

As used herein a human "newborn" is less than 1 month old, an "infant" is about 1 month to about 12 months old; a "child" is about 1 year to about 12 years old; an "adolescent" is about 13 years to about 17 years old; an "adult" is about 18 years to about 64 years old; and an "elder" or "elderly person" (also referred to collectively as "the elderly") is greater than about 64 years old.

As used herein "comorbidity" means one or more disorders or diseases in addition to the age-related disease or disorder of interest, or an effect of such additional disorders or diseases.
As used herein, "treat" means to prevent, reduce, decrease, or ameliorate one or more symptoms, characteristics or comorbidities of an age-related disease, disorder or condition; to reverse the progression of one or more symptoms, characteristics or comorbidities of an age-related disorder; to halt the progression of one or more symptoms, characteristics or comorbidities of an age-related disorder; to prevent the occurrence of one or more symptoms, characteristics or comorbidities of an age-related disorder; to inhibit the rate of development of one or more symptoms, characteristics or comorbidities or combinations thereof.

The terms "individual," "subject," and "patient" are used interchangeably herein, and refer to a mammal, including, but not limited to, rodents, simians, and humans.

The terms "reduce", "inhibit", "ameliorate" and "decrease" are used relative to a control. One of skill in the art would readily identify the appropriate control to use for each experiment. For example a decreased response in a subject or cell treated with a compound is compared to a response in subject or cell that is not treated with the compound.

The terms "increase", "induce", "activate" and "improve" are used relative to a control. One of skill in the art would readily identify the appropriate control to use for each experiment. For example an increased response in a subject or cell treated with a compound is compared to a response in subject or cell that is not treated with the compound.

The term "polypeptides" includes proteins and functional fragments thereof. Polypeptides are disclosed herein as amino acid residue sequences. Those sequences are written left to right in the direction from the amino to the carboxy terminus. In accordance with standard nomenclature, amino acid residue sequences are denominated by either a three letter or a single letter code as indicated as follows: Alanine (Ala, A), Arginine (Arg, R), Asparagine (Asn, N), Aspartic Acid (Asp, D), Cysteine (Cys, C), Glutamine (Gln, Q), Glutamic Acid (Glu, E), Glycine (Gly, G), Histidine (His, H), Isoleucine (Ile, I), Leucine (Leu, L), Lysine (Lys, K), Methionine (Met, M), Phenylalanine (Phe, F), Proline (Pro, P), Serine (Ser, S), Threonine (Thr, T), Tryptophan (Trp, W), Tyrosine (Tyr, Y), and Valine (Val, V).
The term "functional fragment" as used herein is a fragment of a full-length protein retaining one or more function properties of the full-length protein.

The term "functional fragment of TFAM" as used herein is a fragment of full-length TFAM that is when administered to a patient can improve energy metabolism; increase levels of ATP; reduce or delay insulin resistance; control the blood levels of glucose, insulin, triglycerides, free fatty acids, cholesterol, or combinations thereof; reduce weight gain; improve cognitive function and mental acuity; improve memory or retention of memories; increase physical activity; improve endurance; reduce or prevent an increase in the ratio of fibrous tissue to muscle (as also referred to as the fibrous tissue : muscle ratio); reduce or prevent an increase in inflammation, such as chronic or acute inflammation; reduce amyloid-mediated inhibition of mitochondrial respiration, or combinations thereof; or treat or improve one or more symptoms of an age-related disorder compared to a control.

"Variant" refers to a polypeptide or polynucleotide that differs from a reference polypeptide or polynucleotide, but retains essential properties. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more modifications (e.g., substitutions, additions, and/or deletions). A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polypeptide may be naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally.

Modifications and changes can be made in the structure of the polypeptides of in disclosure and still obtain a molecule having similar characteristics as the polypeptide (e.g., a conservative amino acid substitution). For example, certain amino acids can be substituted for other amino acids in a sequence without appreciable loss of activity. Because it is the interactive capacity and nature of a polypeptide that defines that
polypeptide's biological functional activity, certain amino acid sequence substitutions can be made in a polypeptide sequence and nevertheless obtain a polypeptide with like properties.

In making such changes, the hydrophobic index of amino acids can be considered. The importance of the hydrophobic amino acid index in conferring interactive biologic function on a polypeptide is generally understood in the art. It is known that certain amino acids can be substituted for other amino acids having a similar hydrophobic index or score and still result in a polypeptide with similar biological activity. Each amino acid has been assigned a hydrophobic index on the basis of its hydrophobicity and charge characteristics. Those indices are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cysteine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is believed that the relative hydrophobic character of the amino acid determines the secondary structure of the resultant polypeptide, which in turn defines the interaction of the polypeptide with other molecules, such as enzymes, substrates, receptors, antibodies, antigens, and the like. It is known in the art that an amino acid can be substituted by another amino acid having a similar hydrophobic index and still obtain a functionally equivalent polypeptide. In such changes, the substitution of amino acids whose hydrophobic indices are within ±2 is preferred, those within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

Substitution of like amino acids can also be made on the basis of hydrophilicity, particularly, where the biological functional equivalent polypeptide or peptide thereby created is intended for use in immunological embodiments. The following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); proline (-0.5 ± 1); threonine (-0.4); alanine (-0.5); histidine (-
0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent polypeptide. In such changes, the substitution of amino acids whose hydrophilicity values are within ± 2 is preferred, those within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include (original residue: exemplary substitution): (Ala: Gly, Ser), (Arg: Lys), (Asn: Gin, His), (Asp: Glu, Cys, Ser), (Gin: Asn), (Glu: Asp), (Gly: Ala), (His: Asn, Gin), (He: Leu, Val), (Leu: Ile, Val), (Lys: Arg), (Met: Leu, Tyr), (Ser: Thr), (Thr: Ser), (Tip: Tyr), (Tyr: Trp, Phe), and (Val: He, Leu). Embodiments of this disclosure thus contemplate functional or biological equivalents of a polypeptide as set forth above. In particular, embodiments of the polypeptides can include variants having about 50%, 60%, 70%, 80%, 90%, and 95% sequence identity to the polypeptide of interest.

"Identity," as known in the art, is a relationship between two or more polypeptide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide as determined by the match between strings of such sequences. "Identity" can also mean the degree of sequence relatedness of a polypeptide compared to the full-length of a reference polypeptide. "Identity" and "similarity" can be readily calculated by known methods, including, but not limited to, those described in (Computational Molecular Biology, Lesk, A. M., Ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., Ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., Eds., Humana Press, New Jersey, 1994; Sequence Analysis in

Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. The percent identity between two sequences can be determined by using analysis software (i.e., Sequence Analysis Software Package of the Genetics Computer Group, Madison Wis.) that incorporates the Needelman and Wunsch, (J. Mol. Biol, 48: 443-453, 1970) algorithm (e.g., NBLAST, and XBLAST). The default parameters are used to determine the identity for the polypeptides of the present disclosure.

By way of example, a polypeptide sequence may be identical to the reference sequence, that is be 100% identical, or it may include up to a certain integer number of amino acid alterations as compared to the reference sequence such that the % identity is less than 100%. Such alterations are selected from: at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence. The number of amino acid alterations for a given % identity is determined by multiplying the total number of amino acids in the reference polypeptide by the numerical percent of the respective percent identity (divided by 100) and then subtracting that product from said total number of amino acids in the reference polypeptide.

As used herein, the term "low stringency" refers to conditions that permit a polynucleotide or polypeptide to bind to another substance with little or no sequence specificity.

As used herein, the term "purified" and like terms relate to the isolation of a molecule or compound in a form that is substantially free (at
least 60% free, preferably 75% free, and most preferably 90% free) from
other components normally associated with the molecule or compound in a
native environment.

As used herein, the term "pharmaceutically acceptable carrier"
5 encompasses any of the standard pharmaceutical carriers, such as a
phosphate buffered saline solution, water and emulsions such as an oil/water
or water/oil emulsion, and various types of wetting agents.

"Operably linked" refers to a juxtaposition wherein the components
are configured so as to perform their usual function. For example, control
sequences or promoters operably linked to a coding sequence are capable of
effecting the expression of the coding sequence, and an organelle
localization sequence operably linked to protein will assist the linked protein
to be localized at the specific organelle.

"Localization Signal or Sequence or Domain" or "Targeting Signal or
15 Sequence or Domain" are used interchangeably and refer to a signal that
directs a molecule to a specific cell, tissue, organelle, intracellular region or
cell state. The signal can be polynucleotide, polypeptide, or carbohydrate
moiety or can be an organic or inorganic compound sufficient to direct an
attached molecule to a desired location. Exemplary targeting signals include
mitochondrial localization signals from the precursor proteins list in U.S.
Patent No. 8,039,587, and cell targeting signals known in the art such as
those in Wagner et al, Adv Gen, 53:333-354 (2005) the disclosures of which
are incorporated herein by reference in their entirety. It will be appreciated
that the entire sequence need not be included, and modifications including
truncations of these sequences are within the scope of the disclosure
provided the sequences operate to direct a linked molecule to a specific cell
type. Targeting signals of the present disclosure can have 80 to 100%
sequence identity to the mitochondrial localize signal or cell targeting signal
sequences. One class of suitable targeting signals include those that do not
interact with the targeted cell in a receptor:ligand mechanism. For example,
targeting signals include signals having or conferring a net charge, for
example a positive charge. Positively charged signals can be used to target
negatively charged cell types such as neurons and muscle. Negatively charged signals can be used to target positively charged cells.

"Cell surface marker" refers to any molecule such as moiety, peptide, protein, carbohydrate, nucleic acid, antibody, antigen, and/or metabolite presented on the surface or in the vicinity of a cell sufficient to identify the cell as unique in either type or state.

II. Methods of Treatment

It has been discovered that the compositions disclosed herein can improve energy metabolism; increase levels of ATP; reduce or delay insulin resistance; control the blood levels of glucose, insulin, triglycerides, free fatty acids, cholesterol, or combinations thereof; reduce weight gain; improve cognitive function and mental acuity; improve memory or retention of memories; increase physical activity; improve endurance; reduce or prevent an increase in the ratio of fibrous tissue to muscle (as also referred to as the fibrous tissue : muscle ratio); reduce or prevent an increase in inflammation, such as chronic or acute inflammation; reduce amyloid-mediated inhibition of mitochondrial respiration, or combinations thereof. Therefore, the disclosed compositions and methods can be used to improve energy improve energy metabolism; increase levels of ATP; reduce or delay insulin resistance; control the blood levels of glucose, insulin, triglycerides, free fatty acids, cholesterol, or combinations thereof; reduce weight gain; improve cognitive function and mental acuity; improve memory or retention of memories; increase physical activity; improve endurance; reduce or prevent an increase in the ratio of fibrous tissue to muscle (as also referred to as the fibrous tissue : muscle ratio); reduce or prevent an increase in inflammation, such as chronic or acute inflammation; reduce amyloid-mediated inhibition of mitochondrial respiration, or combinations thereof in a subject.

In some embodiments, the subject has been diagnosed or is likely to be diagnosed with one or more diseases, disorders, conditions or symptoms or comorbidities thereof associated with aging or increased age, such as the diseases and disorders described herein. In some embodiments the subject has not been diagnosed or is likely to be diagnosed with one or more
diseases, disorders, conditions or symptoms or comorbidities thereof associated with aging or increased age, such as the diseases and disorders described herein. In some embodiments, the subject is a normal or healthy.

The disclosed methods typically include administering to the subject an effective amount of one or more of the disclosed compositions to improve energy metabolism; increase levels of ATP; reduce or delay insulin resistance; control the blood levels of glucose, insulin, triglycerides, free fatty acids, cholesterol, or combinations thereof; reduce weight gain; improve cognitive function and mental acuity; improve memory or retention of memories; increase physical activity; improve endurance; reduce or prevent an increase in the ratio of fibrous tissue to muscle (as also referred to as the fibrous tissue : muscle ratio); reduce or prevent an increase in inflammation, such as chronic or acute inflammation; reduce amyloid-mediated inhibition of mitochondrial respiration, or combinations thereof in a subject.

As discussed in more detail below, the disclosed compositions and methods can be used to treat one or more age-related diseases, disorders, conditions, or symptoms or comorbidities thereof. In preferred embodiments, the subject is a human subject. The subject can be male or female.

Although the compositions and methods are useful for treating age-related diseases, subjects of any age can be treated. Age-related disorders typical occur or occur with higher frequency in adult or elderly subjects relative to younger age groups, however, newborns, infants, children, and adolescents can also suffer from or be diagnosed with age-related disorders. Therefore, newborns, infants, children, adolescents, adults, and elderly can be treated for an age-related disorder using the compositions and methods disclosed herein. In some embodiments the subject is elderly.

In some embodiments, the composition is administered in an effective amount to treat one or more one or more symptoms or comorbidities of an age-related disease, disorder or condition. The effects of the treatment can be measured relative to a control. Suitable controls are known or can be determined by one of skill in the art. For example, in some
embodiments, the disclosed compositions and methods improve energy metabolism; increase levels of ATP; reduce or delay insulin resistance; control the blood levels of glucose, insulin, triglycerides, free fatty acids, cholesterol, or combinations thereof; reduce weight gain; improve cognitive function and mental acuity; improve memory or retention of memories; increase physical activity; improve endurance; reduce or prevent an increase in the ratio of fibrous tissue to muscle (as also referred to as the fibrous tissue : muscle ratio); reduce or prevent an increase in inflammation, such as chronic or acute inflammation; reduce amyloid-mediated inhibition of mitochondrial respiration, or combinations thereof in a treated subject relative to an untreated subject.

In some embodiments, the compositions and methods prevent, reduce, decrease, or ameliorate one or more symptoms, characteristics or comorbidities of an age-related disease, disorder or condition; to reverse the progression of one or more symptoms, characteristics or comorbidities of an age related disorder; to halt the progression of one or more symptoms, characteristics or comorbidities of an age-related disorder; to prevent the occurrence of one or more symptoms, characteristics or comorbidities of an age-related disorder; to inhibit the rate of development of one or more symptoms, characteristics or comorbidities or combinations thereof in a treated subject relative to an untreated subject. The untreated subject can be the treated subject prior to initiation of treatment, or a matched subject not receiving the compound.

Suitable compositions for use with the disclosed methods are discussed in more detail below and typically include a mitochondrial DNA-binding polypeptide, preferably a mitochondrial transcription factor or mitochondrial DNA-binding fragment thereof. In some embodiments the mitochondrial DNA-binding polypeptide can be a recombinant fusion protein including a mitochondrial DNA-binding polypeptide, a protein transduction domain, and optionally one or more targeting signals. In a preferred embodiment the recombinant protein is a transcription factor A-mitochondrial (TFAM) fusion protein including a protein transduction domain, and a mitochondrial localization signal. Exemplary fusion proteins
containing a mitochondrial transcription factor polypeptide are disclosed in U.S. Patent Nos. 8,039,587, 8,062,891, 8,133,733, and U.S. Published Application Nos. 2009/0123468, 2009/0208478, and 2006/0211647 all of which are incorporated by reference in their entireties.

In some embodiments, the disclosed compositions inhibit or reduce mitochondrial dysfunction, cause an increase in mitochondrial number, increase mitochondrial respiration, increase mitochondrial Electron Transport Chain (ETC) activity, increased oxidative phosphorylation, increased oxygen consumption, increased ATP production, or combinations thereof relative to a control. In some embodiments, the disclosed compositions reduce oxidative stress or oxidative damage in the subject compared to a control.

A. Diseases to Be Treated

As discussed above, the compositions disclosed herein can be used to improve energy metabolism; increase levels of ATP; reduce or delay insulin resistance; control the blood levels of glucose, insulin, triglycerides, free fatty acids, cholesterol, or combinations thereof; reduce weight gain; improve cognitive function and mental acuity; improve memory or retention of memories; increase physical activity; improve endurance; reduce or prevent an increase in the ratio of fibrous tissue to muscle (as also referred to as the fibrous tissue : muscle ratio); reduce or prevent an increase in inflammation, such as chronic or acute inflammation; reduce amyloid-mediated inhibition of mitochondrial respiration, or combinations thereof in healthy or diseased subjects. The compositions are particularly useful for treating a subject having a disease, disorder, condition or symptom or comorbidity thereof associated with aging or increased age, including, but not limited to metabolic disorders such as insulin resistance or diabetes, vascular disease, heart disease, atherosclerosis, obesity or excessive weight gain, loss of physical activity, loss of endurance, depression, muscle loss, sarcopenia, frailty syndrome, inflammation, and neurological disorders such as Alzheimer's disease. The disclosed compositions and methods can reduce aging or pre-mature aging, increase longevity, increase lifespan, or combination thereof in a subject.
1. **Metabolic Disorders**

The disclosed compositions and methods are useful for treating one or more symptoms or comorbidities of a metabolic disorder, including, but not limited to, insulin resistance, Type 1 or 2 diabetes mellitus, insulin insensitivity, impaired fasting glycaemia, impaired glucose tolerance (IGT), dysglycemia, hypertriglyceridaemia, hyperglyceridaemia, dyslipoproteinemia, hyperlipidaemia, hypercholesterolemia, h ypolipoproteinemia, and metabolic syndrome.

As shown in the Examples below, the disclosed compositions can decrease or delay development or progression of insulin resistance, and increases in blood levels of insulin, triglyceride, cholesterol and free fatty acid, in two mouse models: ob/ob mice (Examples 4-5) and a diet induced obesity (DIO) mice (Examples 7-8). The ob/ob mouse is a model of obesity in which mice develop obesity, insulin resistance and hyperglycemia as a result of a mutated leptin gene (see, for example, Georgi, et al, *Microcirculation*, 18(3):238-251 (2011)). By four weeks of age, homozygous animals rapidly gain weight and exhibit hyperglycemia and elevated serum insulin. Diet Induced Obesity (DOB) mice are wildtype mice that are fed a high fat diet to induce obesity.

a. **Insulin Resistance and Diabetes**

In some embodiments the disclosed compositions are used to treat or prevent insulin resistance or diabetes. Insulin resistance and diabetes can be diagnosed using an oral glucose tolerance test (OGTT). Typically, a fasting patient takes a 75 gram oral dose of glucose. Blood glucose levels are then measured over the following 2 hours. After 2 hours a glycaemia less than 7.8 mmol/L (140 mg/dl) is considered normal, a glycaemia of between 7.8 to 11.0 mmol/dl (140 to 197 mg/dl) is considered as Impaired Glucose Tolerance (IGT) and a glycaemia of greater than or equal to 11.1 mmol/dl (200 mg/dl) is considered diabetes mellitus. An OGTT can be normal or mildly abnormal in simple insulin resistance. A fasting serum insulin level of greater than approximately 60 pmol/L is also considered evidence of insulin resistance. In some embodiments, the disclosed compositions reduce or decrease fasting blood glucose level, insulin level, or combinations
thereof, or to reduce, decrease, or delay a rise in fasting blood glucose level, insulin level, or combinations thereof over time. In some embodiments, the compositions disclosed herein delay a rise in fasting blood glucose level, insulin level, or combinations thereof that can occur over time in subjects with high fat diets, little or no exercise, hereditary mutations, hormone changes, advanced age (i.e., becoming elderly), increasing weight or other factors that put them at risk for insulin resistance or diabetes.

b. Blood Lipid Levels

High blood cholesterol and other blood lipids, such as triglycerides, are considered two major risk factors for heart disease. According to the American Heart Association, a total blood cholesterol level of less than 200 mg/dL is typically considered desirable; about 200 to about 239 mg/dL is considered borderline high; and about 240 mg/dL and above is considered high. An LDL blood cholesterol level of less than 100 mg/dL is typically considered optimal; about 100 to about 129 mg/dL is considered near or above optimal; about 130 to about 159 mg/dL is considered borderline high; about 160-189 mg/dL is considered high; and about 190 mg/dL and above is considered very high.

According to the America Heart Association, blood triglyceride levels of less than 150 mg/dL are considered normal; levels of about 150 to about 199 mg/dL are considered borderline high; and levels of about 200 mg/dL or more are considered high.

In some embodiments, the disclosed compositions are used to reduce or decrease total blood cholesterol level, LDL blood cholesterol level, blood triglyceride level or combinations thereof. The compositions reduce or decrease total blood cholesterol level, LDL blood cholesterol level, blood triglyceride level or combinations thereof by 1%, 5%, 10%, 15%, 20%, 25%, or more than 25% relative to a control.

In some embodiments, the disclosed compositions are used to reduce, decrease, or delay a rise in total blood cholesterol level, LDL blood cholesterol level, blood triglyceride level or combinations thereof over time. The disclosed compositions can reduce, decrease, or delay a rise in total blood cholesterol level, LDL blood cholesterol level, blood triglyceride level
or combinations thereof by 1%, 5%, 10%, 15%, 20%, 25%, or more than 25% relative to a control over time.

In some embodiments, the compositions disclosed herein delay a rise in total blood cholesterol level, LDL blood cholesterol level, blood triglyceride level or combinations thereof that can occur over time in subjects with high fat diets, little or no exercise, hereditary mutations, hormone changes, advanced age (i.e., becoming elderly), increasing weight or other factors for high cholesterol or high triglycerides.

Another embodiment provides a method of treating, preventing or inhibiting the progression of cardiovascular disease in a subject in need thereof by administering to the subject an effective amount of a protein having 95% or more sequence identity to SEQ ID NO:26.

c. Metabolic Syndrome

In some embodiments the metabolic disorder is metabolic syndrome, which typically includes a finding of at least two, preferably three or more of the following symptoms: blood pressure equal to or higher than 130/85 mmHg; fasting blood sugar (glucose) equal to or higher than 100 mg/dL; large waist circumference (length around the waist): Men - 40 inches or more, Women - 35 inches or more; low HDL cholesterol: Men - under 40 mg/dL, Women - under 50 mg/dL, Triglycerides equal to or higher than 150 mg/dL.

One embodiment provides a method for treating or inhibiting the progression of a metabolic disorder or disease in a subject in need thereof by administering to the subject an effective amount of a fusion protein, wherein the fusion polypeptide contains (a) a mitochondrial DNA binding polypeptide; (b) a protein transduction domain; and (c) a mitochondrial localization signal. The subject can display one or more symptoms selected from the group consisting of excessive appetite relative to healthy subjects, elevated blood glucose levels relative to healthy subjects, increased glucose sensitivity relative to healthy subjects, increased glycosylated protein levels relative to healthy subjects, elevated insulin levels relative to healthy subjects, decreased insulin sensitivity relative to healthy subjects, increased blood triglyceride levels relative to healthy subjects, increased blood
cholesterol levels relative to healthy subjects, increased blood free fatty acid levels relative to healthy subjects, or a combination thereof. The metabolic disorder or disease can be metabolic selected from the list consisting of prediabetes, impaired fasting glycaemia, impaired glucose tolerance (IGT), dysglycemia, insulin resistance, hypertriglyceridemia, hyperglyceridemia, stroke, arteriosclerotic vascular disease (ASVD), Dyslipoproteinemia, Hypolipoproteinemia, and Hyperlipidemia or Hypercholesterolemia. An exemplary fusion protein contains at least 95% sequence identity to SEQ ID NO:26.

Comorbidities of metabolic disorders include heart disease, vascular disease, atherosclerosis, diabetes, heart attack, kidney disease, nonalcoholic fatty liver disease, peripheral artery disease, and stroke.

In some embodiments, the disclosed compositions are used to prevent, improve, reduce, delay, or improve one or more symptoms or comorbidities of metabolic disorder.

Methods of treating a metabolic disorder can also include dietary modifications such as reduced fat, increased fruits, vegetables, and whole-grain products, increase fish or fish oils; increased exercise; weight loss; managing blood pressure and blood sugar; and not smoking. Combination therapies can include administration of the compositions disclosed herein in combination with a second therapeutic agent that is known in the art for treating insulin resistance, Type 1 or 2 diabetes mellitus, high cholesterol, high blood lipids, metabolic syndrome, or a symptom of comorbidity thereof. For example, the compositions can be administered in combination with insulin or a cholesterol-lowering drug. Cholesterol lowering drugs include, but are not limited to, statins such as atorvastatin (Lipitor), simvastatin (Zocor), lovastatin (Mevacor), pravastatin (Pravachol), and rosuvastatin (Crestor).

2. Weight Gain and Obesity

The disclosed compositions and methods are useful for treating or preventing obesity or one or more symptoms or comorbidities thereof. As shown in the Examples below, the disclosed compositions reduce
undesirable weight gain over time in ob/ob mice (Example 6) and diet induced obesity mice (Example 9).

In some embodiments, the disclosed compositions are used to reduce or decrease, total body weight in a subject. The disclosed compositions can also be used to reduce, decrease, or delay a rise in total body weight over time. In some embodiments, the compositions disclosed herein delay a rise in total body weight that can occur over time in subjects with high fat diets, little or no exercise, hereditary mutations, hormone changes, advancing age (i.e., elderly), diabetes, high cholesterol or high triglycerides.

In some embodiments the subject is a healthy individual of normal weight, or is already overweight, and any additional weight gain could result in obesity or obesity-associate comorbidities. Body Mass Index is a standardized method of determining a subject's weight category using a calculus that is known in the art. A subject can be, for example, underweight: BMI of less 18.5; normal weight: BMI of 18.5-24.9; overweight: BMI of 25-29.9; or obese: BMI of 30 or greater. Therefore, in preferred embodiments, the disclosed compositions are useful for treating or preventing weight gain in a subject with a normal BMI, an overweight BMI, or an obese BMI. For example, the disclosed compositions can be used to treat or prevent weight gain in a subject with a BMI of about 25, 26, 27, 28, 29, 30, or more.

In some embodiments, the subject consumes less food, for example, 0.5%, 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, or 50% less food, over time while being administered the compositions.

Another embodiment provides a method of decreasing appetite in a subject in need thereof by administering an effective amount of a fusion protein to the subject to decrease the subject's appetite, wherein the fusion protein contains three domains selected from the group consisting of (1) a domain comprising a protein transduction domain, (2) a domain comprising a mitochondrial targeting signal, and (3) a domain comprising a mature mitochondrial transcription factor-A (TFAM).
3. **Endurance and Physical Activity**

The disclosed compositions and methods are useful for increasing or improving physical activity; increasing or improving endurance; or combinations thereof compared to, for example, a matched, untreated subject. As shown in the Examples below, the disclosed compositions improve endurance (Example 10) and increase activity (Example 11) in normal mice, compared to controls.

The terms "endurance" or "stamina" as used herein mean the ability or strength to continue or last, especially despite fatigue, stress, or other adverse conditions. Therefore, in some embodiments, a subject treated with the disclosed compositions continues or persists in a physical activity longer compared to a control. In some embodiments, a subject treated with disclosed compositions completes a physical activity faster compared to a control. Controls can include, for example, the subject prior to treatment, or an untreated subject. Physical activities include, but are not limited to, walking, jogging, running, biking, swimming, and lifting. Increased endurance can include an increase in the duration of the physical activity, or an increase in intensity of the physical activity over the same duration. In some embodiments, the disclosed compositions are used to reduce or delay a decline in endurance or physical activity over time, for example with increasing age or in an elderly subject.

In some embodiments, the disclosed compositions are used to reduce or decrease, fatigue in a subject. In some embodiments, the disclosed compositions are used to reduce, decrease, or delay fatigue in a subject over time. As used herein "fatigue" can also include weakness, exhaustion, lethargy, languidness, languor, lassitude and listlessness.

In some embodiments endurance, physical activity, or combinations thereof is improved in a subject without increasing anxiety in the subject. One embodiment provides a method of increasing physical stamina or endurance in an animal by administering to the animal an effective amount of a fusion protein comprising three domains selected from the group consisting of (1) a domain containing a protein transduction domain, (2) a domain containing a mitochondrial targeting signal, and (3) a domain
containing a mature mitochondrial transcription factor-A (TFAM). The animal is preferably a human.

Still another embodiment provides a method of increasing physical activity in a subject by administering to the subject an effective amount of a fusion protein having three domains selected from the group consisting of (1) a domain having a protein transduction domain, (2) a domain having a mitochondrial targeting signal, and (3) a domain having a mature mitochondrial transcription factor-A (TFAM). Typically the subject has one or more symptoms selected from the group consisting of weakness, fatigue, exhaustion, lethargy, languardness, languor, lassitude and listlessness.

4. Cognitive Function, Mental Acuity, and Memory Retention

The disclosed compositions and methods are useful for increasing or improving cognitive function, increasing or improving mental acuity, increasing or improving memory or retention of memories, increasing or improving learning, particularly spatial learning, or combinations thereof. As shown in the Examples below, the disclosed compositions improve spatial learning and retention of memories in normal (Example 12) and aged (Example 13) mice, compared to controls.

In some embodiments, a subject treated with the disclosed compositions continues or maintains a learned activity or memory longer when compared to a control. In some embodiments, a subject treated with the disclosed compositions completes a mental challenge faster compared to a control. Controls can include, for example, the subject prior to treatment, or an untreated subject. Mental activities can include, for example, spatial navigation, which also includes both mental and physical aspects.

In some embodiments, the disclosed compositions are used to reduce or delay memory loss or reduced learning capacity in a subject over time. In some embodiments, the disclosed compositions are used to reduce or delay a decline in spatial learning ability or memory retention over time, for example with increasing age or in an elderly subject.
5. **Depression**

Depression and depressive disorders are a group of illnesses that involves the body, mood, and thoughts. The compositions and methods disclosed herein can be used to treat depression, a depressive disorder, or a symptom thereof. Depression is typically characterized by having one, preferably two or more symptoms including, but not limited to, persistent sad, anxious, or empty mood; feelings of hopelessness or pessimism; feelings of guilt, worthlessness, or helplessness; loss of interest or pleasure in hobbies and activities that were once enjoyed, including sex; decreased energy; fatigue; difficulty concentrating, remembering, or making decisions; insomnia; early morning awakening or oversleeping; appetite and/or weight loss, or overeating and weight gain; thoughts of death or suicide; suicide attempts; restlessness; irritability; persistent physical symptoms that do not respond to treatment, such as headaches, digestive disorders and chronic pain. These symptoms often interfere with the ability to work, study, sleep, eat, and enjoy pleasurable activities.

Depressive disorders include major depressive disorder (MDD), unipolar depression, clinical depression, Major Depressive Disorder (single episode), Major Depressive Disorder (Recurrent), atypical depression, somnolence, hypsomnolence, leaden paralysis, melancholic depression, anhedonia, Psychotic Major Depression (PMD), psychotic depression, catatonic depression, Postpartum Depression (PPD), Seasonal Affective Disorder (SAD), winter depression, Dysthymia and Depressive Disorder Not Otherwise Specified (DD-NOS), Recurrent Brief Depression (RBD), minor depressive disorder, Bipolar Disorder (BD), Bipolar I, Bipolar II, Cyclothymia and Bipolar Disorder Not Otherwise Specified (BD-NOS).

In some embodiments the method for treating depression includes co-administration of the disclosed compositions in combinations with a second anti-depressant or anti-anxiety therapeutic agent.

6. **Muscle Loss**

The disclosed compositions and methods are useful for reducing collagen accumulation, fibrosis, increasing muscle regenerative capacity, increasing muscle retention, or combinations thereof. As shown in the
Examples below, the disclosed compositions reduce muscle cartilage formation (Example 14).

Over time, aging individuals can experience one or more of the following: (1) muscle fibers are reduced in number and shrink in size, (2) muscle tissue is replaced more slowly and lost muscle tissue is replaced with a tough, fibrous tissue, (3) changes in the nervous system cause muscles to have reduced tone and ability to contract. These changes can contribute to fatigue, weakness and reduced tolerance to exercise. The disclosed compositions can used to reduce or delay (1), (2), (3), or combinations thereof. For example, in a preferred embodiment, the disclosed compositions reduce or delay loss of muscle, build-up of fibrous tissue, or combinations thereof. The formation of fibrous tissue such as collagen or cartilage can be around the muscle or bone of a subject. In some embodiments, the disclosed compositions prevent, delay, reduce, or inhibit loss of muscle in a subject over time. In some embodiments, the disclosed compositions reduce the ratio of fibrous tissue to muscle in a subject. In some embodiments, the disclosed compositions reduce an increase in the ratio of fibrous tissue to muscle in a subject over time, for example in aging individuals. The ratio of fibrous tissue: muscle can be a reduction in the fibrous: muscle ratio throughout the body of a subject, or in a discrete location, such as around joints. Fibrous tissue includes, but is not limited to collagen, cartilage, and combinations thereof.

In some embodiments, the subject suffers from a disease or condition such as muscle atrophy, muscular dystrophy, sarcopenia, frailty, or combinations thereof. The disclosed compositions and methods can be used to treat or prevent muscle atrophy, muscular dystrophy, sarcopenia, frailty, combinations thereof, or one or more symptoms or comorbidities thereof. The muscle atrophy or sarcopenia can result from cachexia, immobilization, aging, chronic disease, cancer, or combinations thereof.

Sarcopenia typically refers to the loss of skeletal muscle mass associated with advancing age (Cruz-Jentoft, A. et al, Age and Aging, 39:412-423 (2010); Lang, T. et al, Osteoporosis Int, 21:543-559 (2010)).
Loss of skeletal muscle mass can also be unrelated to age. For example, loss of skeletal muscle mass occurs in subjects with cachexia.

Muscle is a dynamic tissue that responds to damage and loss throughout the entire life of an individual. For example, following a physiological stimuli such as exercise or injury the muscle tissue responses by mounting a regenerative response that restores the cytoarchitecture within about a two week period (Shi and Garry, et al, *Genes Dev.*, 20(13): 1692-708 (2006) citing Hawke and Garry, *J. Appl. Physiol*, 91:534-551 (2001); Cossu and Biressi, *Semin. Cell Dev. Biol*, 16:623-631 (2005); Dhawan and Rando, *Trends Cell Biol*, 15:666-673 (2005); Holtermann and Rudnicki, *Semin. Cell Dev. Biol*, 16:575-584 (2005)). It has been suggested that sacropenia results, at least in part, from the loss of muscle regenerative capacity. As used herein, muscle regenerative capacity can be affected by the rate of muscle regeneration, the rate of formation of fibrous tissue, or combinations thereof. For example, in some individuals, muscle regenerative capacity is low or reduced when muscle tissue is replaced more slowly and lost muscle tissue is replaced with a tough, fibrous tissue. Therefore, muscle regenerative capacity can be increased by increasing the rate of formation or amount of muscle mass, reducing the rate of formation or amount of fibrous tissue, or any combination thereof. In some embodiments, the disclosed compositions and methods are used to increase muscle regenerative capacity in a subject in need thereof. Certain embodiments provide methods for treating sacropenia in a subject by administering to the sacropenic subject an effective amount of one or more of the disclosed compositions to increase or promote muscle regenerative capacity in the subject. In some embodiments, an increase in muscle regenerative capacity is characterized by decreased or delay increase in fibrous tissue: muscle ratio.

Other factors that can contribute to the onset or progression of sacropenia include mitochondrial dysfunction (Lang, T. et al., *Osteoporosis Int*, 21:543-559 (2010)). The build-up of reactive oxygen species can cause mitochondrial dysfunction, which impairs muscle respiration and could contribute to muscle fiber deterioration. Thus, another embodiment provides a method for treating sacropenia in a subject by administering to the subject
an effective amount of the disclosed compositions to reduce or inhibit mitochondrial dysfunction in the subject. Still another embodiment provides treating sarcopenia in subject in need thereof by administering to the subject an effective amount of the disclosed compositions to reduce or inhibit mitochondrial dysfunction in the subject.

Methods for measuring changes in skeletal muscle mass are known in the art. See for example Lang, T. et al, *Osteoporosis Int*, 21:543-559 (2010). Briefly, changes in skeletal muscle mass with age can be determined using lean body mass measurements with dual X-ray absorptiometry (DXA) and with muscle cross-sectional areas quantified by three-dimensional imaging methods such as X-ray computed tomography (CT) or with magnetic resonance imaging (MRI). Leg lean muscle mass by DXA can be used as a marker for skeletal muscle mass. Thus, changes in leg lean muscle mass can be used to detect or assisting in the diagnosis of sarcopenia or to monitor the effectiveness of a treatment for sarcopenia.

7. **Frailty Syndrome**

The disclosed compositions and methods can also be used to treat or prevent frailty syndrome, or one or more symptoms or comorbidities thereof. Frailty syndrome is a syndrome of multiple co-existing conditions including weakness, immobility, and poor tolerance to physiological and psychological stressors. (Sara Espinoza and Jeremy Walston, *Cleveland Journal Clinical Medicine*, 72(12): 1105-1 112 (2005)). Individuals diagnosed with frailty syndrome are referred to as "frail." Frailty can exist in subjects regardless of age, disability, or disease.

A subject is diagnosed as having frailty syndrome when the subject is diagnosed with three or more of the following: sarcopenia, shrinking or unintentional non-muscle weight loss greater than 10 lbs per year, decreased grip strength, low energy expenditure, poor endurance and energy, and decreased walking time. Preferably, a subject is diagnosed with frailty syndrome when the subject is diagnosed with two or more of the following: weight loss of > 10 lbs in the last year; weak grip strength; exhaustion; slow gait speed; and low physical activity. Methods for measuring or quantifying weak grip strength; exhaustion; slow gait speed; and low physical activity

Exemplary symptoms and comorbidities of frailty syndrome include, but are not limited to sarcopenia, shrinking or unintentional non-muscle weight loss greater than 10 lbs per year, decreased grip strength, low energy expenditure, poor endurance, and energy, and decreased walking time. In some embodiments the subjects are elderly subjects.

One embodiment provides a method for treating frailty syndrome in a subject in need thereof by administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a fusion polypeptide and a pharmaceutically acceptable carrier to treat one or more symptoms of frailty syndrome, wherein the fusion polypeptide contains (a) a mitochondrial DNA binding polypeptide; (b) a protein transduction domain; and (c) a mitochondrial localization signal. The mitochondrial DNA-binding polypeptide can be a transcription factor A - mitochondria (TFAM) polypeptide containing an amino acid sequence having 95% or more sequence identity to SEQ ID NO:3, or a mitochondrial DNA binding fragment thereof.

8. **Inflammation**

The disclosed compositions and methods are useful for reducing inflammation, an inflammatory response, or combinations thereof. As shown in Example 15 below, the disclosed compositions reduce, or prevent an increase in inflammation in a mouse model compared to controls.

Inflammation is significant component of the aging process and has been linked to many age-associated processes including wrinkle formation, arthritis, heart disease, Alzheimer's disease and cancer. In some embodiments, the disclosed compositions reduce inflammation, an inflammatory response, or combinations thereof in a subject. The inflammation or inflammatory response can be acute inflammation or an acute inflammatory response. The inflammation or inflammatory response can be chronic inflammation or a chronic inflammatory response. The inflammation can be due in-part or in-total to reactive oxygen species, or the damage on cells and tissues caused by such reactive oxygen species.
disclosed compositions can reduce an increase in inflammation or an inflammatory response in a subject over time, for example in aging or elderly individuals. In some embodiments the disclosed compositions are used to treat, prevent, improve or ameliorate inflammation-associated disorders such as arthritis.

9. Neurological Disorders

The disclosed compositions and methods can be used to treat or prevent the development or progression of neurological disorders. It is believed that mitochondrial dysfunction and oxidative stress play a role in neurodegenerative disease pathogenesis, including, but not limited to, Alzheimer’s, Parkinson’s, Huntington’s, and Amyotrophic lateral sclerosis (ALS). As discussed above, the disclosed compositions and methods can reduce oxidative stress in a subject.

Some neurodegenerative diseases, known as proteinopathies are caused in part by the accumulation of protein aggregates either inside or outside the cell. For example, although the cause and progression of Alzheimer’s disease is not well understood, studies indicate that the disease is associated with plaques and tangles of amyloid-β polypeptides. Accumulation of amyloid-β can cause impairment of total respiration, and as a result, the respiratory control ratio together with ATP production are decreased in effected cells (Rhein, et al, *Cell. Mol. Neurobiol.*, 29(6-7): 1063-71 (2009)). Therefore, it is believed that chronic exposure to soluble amyloid-β protein can cause decreased respiratory capacity of mitochondrial electron transport chain, leading to impairment of energy homeostasis and ultimately increased neuronal cell death. The Example 16 below shows that the disclosed compositions can be used to increase maximal respiration in amyloid-β polypeptides cells compared to controls.

The disclosed compositions can increase respiration in subjects. The compositions are particularly useful for increasing or maintaining respiration in a subject with a proteinopathy such as Alzheimer’s disease that can cause a decrease in respiration over time. Therefore, the disclosed compositions can increase respiration, or prevent or delay a decline in respiration associated with a proteinopathy, such as Alzheimer’s disease. In some
In some embodiments the disclosed methods are used to treat Alzheimer's disease. In some embodiments, the disclosed compositions are used to treat, prevent, improve or ameliorate one or more symptoms of Alzheimer's disease, for example, a reduction in respiration, impairment of energy production, reduced levels of ATP, increase neuronal cell death, or combinations thereof.

In some embodiments the disclosed compositions are used to treat, prevent, improve or ameliorate one or more symptoms of Alzheimer's disease in an aging or elderly subject.

10. **Hypertension**

The disclosed compositions and methods can be used to treat or prevent the development or progression of high blood pressure or hypertension. Primary hypertension has no known cause. The methods and compositions disclosed herein can be combined with other methods of treating and preventing of hypertension including maintaining normal body weight, reducing dietary sodium intake, engaging in regular aerobic physical activity such as brisk walking, limiting alcohol consumption, consuming a diet rich in fruit and vegetables, consuming a diet with reduced content of saturated and total fat, and combinations thereof.

The disclosed compositions may aid, augment, replace or supplement such lifestyle changes such as increased physical activity, maintain or achieve normal body weight, and lower blood lipids in a similar way a changed diet may.

Insulin resistance and obesity are known risk factors of hypertension. Disclosed compositions may reduce weight increases and insulin resistance and may hence reduce the risk of developing hypertension, or may reduce blood pressure in a patient with hypertension, insulin resistance, obesity, or combinations thereof.

11. **Brain Metabolism**

It has been discovered that administering one or more mitochondrial DNA binding proteins or fusion proteins thereof to a subject can modulate the subject's brain metabolism. In particular the levels of N-acetylaspartate (NAA) can be increased and levels of lactate in the brain can be decreased by administering mitochondrial DNA binding proteins or fusion proteins
thereof. A preferred fusion protein has at least 95% sequence identity to SEQ ID NO:26. The compositions can also increase ATP levels in the brain. The modulation of levels of NAA, ATP or lactate are relative to levels of NAA, ATP, or lactate in the subject prior to administration or to levels in healthy subjects.

One embodiment provides a method of treating of modulating brain metabolism in a subject in need thereof by administering to the subject a fusion protein containing three domains selected from the group consisting of (1) a domain containing a protein transduction domain, (2) a domain containing a mitochondrial targeting signal, and (3) a domain containing a mature mitochondrial transcription factor-A (TFAM) in an amount effective to increase levels of ATP in the brain, to increase levels of N-Acetyl aspartate in the brain, or to reduce levels of lactate in the brain relative to subject's levels prior to administration of the fusion protein.

D. Administration

1. Methods of Administration

The composition can be administered prophylactically, therapeutically, or combinations thereof. Therefore, the composition can be administered during a period before, during, or after onset of one or more symptoms of an age-related disorder. In some embodiments, the composition is administered with one or more additional therapeutic agents as part of a co-therapy, one or more second treatments, or combinations thereof. Second treatments include, but are not limited to, diet and exercise regimes, nutrition supplementation, vitamin supplementation, incontinence management, rehabilitative therapy, cognitive therapy, and management of inadvertent adverse effects or complications resulting from medical treatment.

The composition and the second therapeutic agent or treatment can be administered to the subject together or separately. The composition and the additional therapeutic agent or treatment can be administered on the same day, on a different days, or combinations thereof.

For example, the subject can be administered the disclosed composition 0, 1, 2, 3, 4, 5, or more days before administration of or
exposure to the additional therapeutic agent or treatment. In some embodiments, the subject can be administered one or more doses of the composition every 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, 35, or 48 days prior to a first administration of or exposure to the additional therapeutic agent or treatment.

The subject can also be administered the composition for 0, 1, 2, 3, 4, 5, or more days after administration of or exposure to the additional therapeutic agent or treatment. The subject can also be administered the composition during administration of or exposure to the additional therapeutic agent or treatment. The composition can be administered on the same day as the antineoplastic agent, or on a different day. The subject can be administered one or more doses of the composition every 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, 35, or 48 days during or after administration of the additional therapeutic agent or treatment.

2. Formulations

The mitochondrial DNA-polypeptide compositions provided herein may be administered in a physiologically acceptable carrier to a host. Preferred methods of administration include systemic or direct administration to a cell. The compositions can be administered to a cell or patient, as is generally known in the art for protein therapies. One embodiment provides a pharmaceutical composition includes a recombinant mitochondrial DNA-binding protein, for example a fusion protein. The fusion protein preferably contains polynucleotide-binding domain of a mitochondrial DNA-binding protein, a targeting domain, and a protein transduction domain and a pharmaceutically acceptable carrier or excipient. Preferably the polynucleotide-binding polypeptide includes TFAM or a fragment thereof capable of binding a polynucleotide.

The compositions can be combined in admixture with a pharmaceutically acceptable carrier vehicle. Therapeutic formulations are prepared for storage by mixing the active ingredient having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 17th edition, Osol, A. Ed. (198)), in the form of lyophilized formulations or aqueous solutions.
Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween®, Pluronics® or PEG.

The compositions of the present disclosure can be administered parenterally. As used herein, “parenteral administration” is characterized by administering a pharmaceutical composition through a physical breach of a subject's tissue. Parenteral administration includes administering by injection, through a surgical incision, or through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration includes subcutaneous, intraperitoneal, intravenous, intraarterial, intramuscular, intrasternal injection, and kidney dialytic infusion techniques.

Parenteral formulations can include the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules or in multi-dose containers containing a preservative. Parenteral administration formulations include suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, reconstitutable dry (i.e. powder or granular) formulations, and implantable sustained-release or biodegradable formulations. Such formulations may also include one or more additional ingredients including suspending, stabilizing, or dispersing agents. Parenteral formulations may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. Parenteral formulations may also include dispersing agents, wetting agents, or
suspending agents described herein. Methods for preparing these types of formulations are known. Sterile injectable formulations may be prepared using non-toxic parenterally-acceptable diluents or solvents, such as water, 1,3-butane diol, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic monoglycerides or diglycerides. Other parentally-administrable formulations include microcrystalline forms, liposomal preparations, and biodegradable polymer systems. Compositions for sustained release or implantation may include pharmaceutically acceptable polymeric or hydrophobic materials such as emulsions, ion exchange resins, sparingly soluble polymers, and sparingly soluble salts.

Pharmaceutical compositions may be prepared, packaged, or sold in a buccal formulation. Such formulations may be in the form of tablets, powders, aerosols, atomized solutions, suspensions, or lozenges made using known methods, and may contain from about 0.1% to about 20% (w/w) active ingredient with the balance of the formulation containing an orally dissolvable or degradable composition and/or one or more additional ingredients as described herein. Preferably, powdered or aerosolized formulations have an average particle or droplet size ranging from about 0.1 nanometers to about 200 nanometers when dispersed.

The composition can include one or more additional ingredients. As used herein, "additional ingredients" include: excipients, surface active agents, dispersing agents, inert diluents, granulating agents, disintegrating agents, binding agents, lubricating agents, sweetening agents, flavoring agents, coloring agents, preservatives, physiologically degradable compositions (e.g., gelatin), aqueous vehicles, aqueous solvents, oily vehicles and oily solvents, suspending agents, dispersing agents, wetting agents, emulsifying agents, demulcents, buffers, salts, thickening agents, fillers, emulsifying agents, antioxidants, antibiotics, antifungal agents, stabilizing agents, and pharmaceutically acceptable polymeric or hydrophobic materials. Other additional ingredients which may be included in the pharmaceutical compositions are known. Suitable additional ingredients are described in Remington's Pharmaceutical Sciences, 17th ed. Mack Publishing Co., Genaro, ed., Easton, Pa. (1985).
Dosages and desired concentrations of the polynucleotide-binding polypeptide disclosed herein in pharmaceutical compositions of the present disclosure may vary depending on the particular use envisioned. The determination of the appropriate dosage or route of administration is well within the skill of an ordinary physician. Animal experiments provide reliable guidance for the determination of effective doses for human therapy. Interspecies scaling of effective doses can be performed following the principles laid down by Mordenti, J. and Chappell, W. "The use of interspecies scaling in toxicokinetics" In Toxicokinetics and New Drug Development, Yacobi et al, Eds., Pergamon Press, New York 1989, pp. 42-96.

The composition can be administered intravenously in a wide dosing range from about 0.01 milligram per kilogram body weight (mg/kg) to about 10 mg/kg, alternatively about 0.01 milligram per kilogram body weight (mg/kg) to about 1.0 mg/kg, depending on patient's age and physical state, as well as dosing regimen and schedule.

The dose can be administered in separate administrations of 2, 3, 4, 5 or 6 doses. The dose can be administered every day, every two days, every three days, every four days, every five days, every six days, every seven days, once every two weeks, or once a month.

In some embodiments the composition is lyophilized in 20 mM glutamate, 10 mg/mL trehalose, 30 mg/mL mannitol, pH 4.5 and reconstituted in sterile water prior to use. In another embodiment the composition is lyophilized in 20 mM histidine, 10 mg/mL trehalose, 30 mg/mL mannitol, pH 6.5 and reconstituted in sterile water prior to use. In yet another embodiment, the composition is dissolved in 20mM histidine, 150mM NaCl pH 6.5 and kept frozen prior to use.

III. Compositions

Compositions that improve energy metabolism; increase levels of ATP; reduce or delay insulin resistance; control the blood levels of glucose, insulin, triglycerides, free fatty acids, cholesterol, or combinations thereof; reduce weight gain; improve cognitive function and mental acuity; improve memory or retention of memories; increase physical activity; improve
endurance; reduce or prevent an increase in the ratio of fibrous tissue to muscle (as also referred to as the fibrous tissue : muscle ratio); reduce or prevent an increase in inflammation, such as chronic or acute inflammation; reduce amyloid-mediated inhibition of mitochondrial respiration, or combinations thereof or treat or improve one or more symptoms of an age-related disorder are provided. The composition typically includes an effective amount of a mitochondrial DNA-binding polypeptide. Examples of a mitochondrial DNA-binding polypeptides include, but are not limited to, mitochondrial transcription factors such as transcription factor A, mitochondrial (TFAM) having GenBank Accession No. NM_003201; transcription factor Bl, mitochondrial (TFB1M) having GenBank Accession No. AF151833; transcription factor B2, mitochondrial (TFB2M) having GenBank Accession No. AK026835; Polymerase (RNA) Mitochondrial (DNA directed) (POLRMT) having GenBank Accession No. NM_005035; and functional fragments, variants, and fusion polypeptides thereof.

In preferred embodiments the composition includes a recombinant fusion protein including a mitochondrial DNA-binding polypeptide, a protein transduction domain, and optionally one or more targeting signals. In some embodiments, the disclosed compositions inhibit or reduce mitochondrial dysfunction. In still other embodiments, the compositions induce or promote muscle regeneration capacity. The compositions can also cause an increase in mitochondrial number, an increase in mitochondrial respiration, an increase mitochondrial Electron Transport Chain (ETC) activity, increased oxidative phosphorylation, increased oxygen consumption, increased ATP production, or combinations thereof relative to a control. In preferred embodiments the composition reduces oxidative stress.

Exemplary fusion proteins containing a mitochondrial transcription factor polypeptide are disclosed in U.S. Patent Nos. 8,039,587, 8,062,891, 8,133,733, and U.S. Published Application Nos. 2009/0123468, 2009/0208478, and 2006/0211647 all of which are specifically incorporated by reference herein in their entireties.
A. Polypeptides

1. Polynucleotide binding domain

The compositions typically include an effective amount of a mitochondrial DNA-binding polypeptide optionally having a PTD and optionally having one or more targeting signals or domains. In certain embodiments, the mitochondrial DNA-binding polypeptide is a polypeptide known to bind or package a mtDNA. Preferably, the mitochondrial DNA-binding polypeptide is a recombinant polypeptide. The recombinant polypeptide can be used as a therapeutic agent either alone or in combination with a polynucleotide, or any other active agent. In preferred embodiments the polynucleotide-binding domain includes mature TFAM, a functional fragment of TFAM, or a variant thereof. In certain embodiments, the polynucleotide-binding polypeptide includes at least a portion of a member of the high mobility group (HMG) of proteins effective to bind a polynucleotide, for example an HMG box domain.

"Mature TFAM" refers to TFAM after it has been post-translationally modified and is in the form that is active in the mitochondrion. For example, a mature TFAM is one in which the endogenous mitochondrial signal sequence has been cleaved.

a. Transcription Factor A, Mitochondria (TFAM)

One embodiment provides a non-histone polynucleotide-binding polypeptide, for example mitochondrial transcription factor A (TFAM) polypeptide, for functional fragment, or a variant thereof. Variant TFAM can have 80%, 85%, 90%, 95%, 99% or greater sequence identity with a reference TFAM, for example naturally occurring TFAM having GenBank Accession No. NM_003201. In certain embodiments, the variant TFAM has 80%, 85%, 90%, 95%, 99% or greater sequence identity with a reference TFAM. In certain embodiments, the variant TFAM has 80%, 85%, 90%, 95%, 99% or greater sequence identity over the full-length of mature human TFAM.

TFAM is a member of the high mobility group (HMG) of proteins having two HMG-box domains. TFAM as well as other HMG proteins bind,
wrap, bend, and unwind DNA. Thus, embodiments of the present disclosure include polynucleotide binding polypeptides including one or more polynucleotide binding regions of the HMG family of proteins, and optionally induce a structural change in the polynucleotide when the polypeptide binds or becomes associated with the polynucleotide.

In some embodiment, the polynucleotide-binding polypeptide is full-length TFAM polypeptide, or variant therefore. For example, a preferred TFAM polypeptide has at least 80, 85, 90, 95, 99, or 100 percent sequence identity to the full-length TFAM precursor.

Many nuclear encoded mitochondrial proteins destined for the mitochondrial matrix are translated as a "preprotein." The preprotein sequence includes a signal peptide as known as an "amino-terminal signal", or a "presequence" that facilitates translocation from the cytosol through the mitochondrial translocation machinery in the outer membrane called the Tom complex (Translocator outer membrane) as well as the machinery in the inner membrane called the Tim complex (Translocator Inner Membrane). Once the preprotein enters the inner mitochondrial matrix, the signal sequence is cleaved by a protease such as MPP. A mitochondrial protein with the signal sequence cleaved or removed can be referred to as a "mature" protein. Therefore, in some embodiments, the polynucleotide-binding polypeptide is a mature TFAM polypeptide, or variant thereof. For example, in some embodiments, the cleavable mitochondrial targeting sequence of a TFAM preprotein is amino acid residue 1 of SEQ ID NO: 1 to amino acid residue 42 of SEQ ID NO: 1.
In certain embodiments, a preferred TFAM polypeptide has at least 80, 85, 90, 95, 99, or 100 percent sequence identity to the mature TFAM sequence:

SSVLASCPKK PVSSYLRSFK EQLPIFKAQN PDAKTELIR
5 RIAQRWREL P DSKKKIYQDA YRAEWQYKE EISRFKEQLT
PSQIMSLEKE IMDKHLKRKA MTKKELTLL GKPKRPRSAY
NVYVAERFQE A KGDSPQEKL KTVKENKNDL SDSEKELYIQ
HAKEDETRYH NEMKSWEEQM IEVGRKDLLR RTIKQKRYG AEEC (SEQ ID NO:3).

In some embodiments, the polynucleotide-binding polypeptide is a functional fragment of TFAM, or variant thereof. Functional fragments can be effective when administered alone, or can be effective when administered in combination with a polynucleotide. Functional fragments of TFAM can include, but are not limited to, a fragment of full-length TFAM sufficient to bind non-specifically to a polynucleotide, a fragment of full-length TFAM sufficient to bind specifically to the mtDNA light strand promoter (LSP), the mtDNA heavy strand promoter 1 (HSP1), the mtDNA heavy strand promoter 2 (HSP2), or combinations thereof, a fragment of full-length TFAM sufficient to induce mitochondrial transcription, a fragment of full-length TFAM sufficient to induce oxidative phosphorylation, a fragment of full-length TFAM sufficient to induce mitochondrial biogenesis, and combinations thereof.

From N-terminus to C-terminus, mature TFAM includes four domains, a first HMG box (also referred to herein as HMG box 1), followed by a linker region (also referred to herein as linker), followed by a second HMG box (also referred to herein as HMG box 2), followed by a C-terminal tail. Functional fragments of TFAM typically include one or more domains of mature TFAM, or a variant thereof. For example, in some embodiments, the functional fragment includes one or more HMG box 1 domains of TFAM, one or more linker domains of TFAM, one or more HMG box 2 domains of TFAM, one or more C-terminal tail domains of TFAM, or combinations thereof. The domains can be arranged in the same orientation of the domains of endogenous TFAM, or they can be rearranged so they are...
in a different order or orientation than the domains found in endogenous TFAM protein. In certain embodiments the functional fragment includes a first HMG box domain, and second HMG box domain linked to the first HMG box domain with a linker, typically a peptide linker. The linker can be the endogenous linker domain of TFAM, or a heterologous linker that allows the first and the second HMG box domains to maintain their functional activity. Deletion studies characterizing the activity of different domains and hybrid constructs of TFAM are known in the art and characterized for example in Dairaghi, et al, J. Mol. Biol, 249:1 1-28 (1995), Matsushima, et al, J. Biol. Chem., 278(33):3 1149-3 1158 (2003), and Gangeloff, et al., Nucl. Acid. Res., 37(10):3 153-3 164 (2009), all of which are specifically incorporated by reference herein in their entireties.

In certain embodiments a functional fragment is one or more domains of TFAM according to SEQ ID NO: 3. For example, an HMG box 1 of TFAM can be a polypeptide including the sequence from amino acid residue 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 of SEQ ID NO: 3 to amino acid residue 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, or 95 of SEQ ID NO: 3, or a variant thereof with 80, 85, 90, 95, 99, or greater than 99 percent sequence identity to the corresponding fragment of SEQ ID NO: 3.

A linker region of TFAM can be a polypeptide including the sequence from amino acid residue 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, or 85 of SEQ ID NO: 3 to amino acid residue 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, or 115 of SEQ ID NO: 3, or a variant thereof with 80, 85, 90, 95, 99, or greater than 99 percent sequence identity to the corresponding fragment of SEQ ID NO: 3.

An HMG box 2 of TFAM can be a polypeptide including the sequence from amino acid residue 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, or 115 of SEQ ID NO: 3 to amino acid residue 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, or 187 of SEQ ID NO: 3, or a variant thereof with 80, 85, 90, 95, 99, or greater than 99 percent sequence identity to the corresponding fragment of SEQ ID NO: 3.
A C-terminal tail of TFAM can be a polypeptide including the sequence from amino acid residue 172, 173, 174, 175, 176, or 187 of SEQ ID NO: 3 to amino acid residue 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, or 204 of SEQ ID NO: 3, or a variant thereof with 80, 85, 90, 95, 99, or greater than 99 percent sequence identity to the corresponding fragment of SEQ ID NO: 3.

Variants of TFAM and functional fragments of TFAM are also provided. Typically, the variants of TFAM and functional fragments of TFAM include one or more conservative amino acid substitutions relative to the corresponding reference sequence, for example SEQ ID NO:3, or a fragment thereof. One embodiment provides a TFAM polypeptide having one or more serine residues at positions 1, 2 and 13 SEQ ID NO:3 substituted with an alanine or threonine residue. A preferred embodiment provides a TFAM polypeptide having serine 13 of SEQ ID NO:3 substituted for an alanine or threonine. The variant TFAM polypeptides have improved mtDNA binding in the presence of glucose or elevated glucose levels.

Selected model organisms that have TFAM sequences that are useful in the compositions and methods disclosed herein include, but are not limited to those disclosed in Table 1:

**Table 1: Organism, Protein And Percent Identity And Length Of Aligned Region**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Protein Description</th>
<th>Percent Identity</th>
<th>Length (aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. sapiens</em></td>
<td>sp:Q00059 - MTTL_HUMAN Transcription factor 1, mitochondrial precursor (MTTF1)</td>
<td>100 %</td>
<td>246</td>
</tr>
<tr>
<td><em>M. musculus</em></td>
<td>ref:NP_033386.1 - transcription factor A, mitochondrial [Mus musculus]</td>
<td>63 %</td>
<td>237</td>
</tr>
<tr>
<td><em>R. norvegicus</em></td>
<td>ref:NP_112616.1 - transcription factor A, mitochondrial [Rattus norvegicus]</td>
<td>64 %</td>
<td>237</td>
</tr>
<tr>
<td><em>A. thaliana</em></td>
<td>ref:NP_192846.1 - 98b like protein [Arabidopsis thaliana]</td>
<td>27 %</td>
<td>189</td>
</tr>
<tr>
<td><em>C. elegans</em></td>
<td>ref:NP_501245.1 - F45E4.9.p [Caenorhabditis elegans]</td>
<td>27 %</td>
<td>189</td>
</tr>
<tr>
<td><em>D. melanogaster</em></td>
<td>ref:NP_524415.1 - mitochondrial transcription factor A [Drosophila melanogaster]</td>
<td>34 %</td>
<td>183</td>
</tr>
</tbody>
</table>
b. **Transcription Factor Bl, Mitochondrial (TFBIM)**

The polynucleotide-binding polypeptide can be transcription factor Bl, mitochondrial (TFBIM). A preferred TFBIM has GenBank Accession No. AF151833. TFB1 is part of the complex involved in mitochondrial transcription. The process of transcription initiation in mitochondria involves three types of proteins: the mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor A (TFAM), and mitochondrial transcription factors B1 and B2 (TFB1M, TFB2M). POLRMT, TFAM, and TFBIM or TFB2M assemble at the mitochondrial promoters and begin transcription. TFBIM has about 1/10 the transcriptional activity of TFB2M, and both TFBs are also related to rRNA methyltransferases and TFBIM can bind S-adenosylmethionine and methylate mitochondrial 12S rRNA. Additionally, TFBIM and TFB2M can bind single-stranded nucleic acids.

A preferred TFBIM polypeptide has at least 80, 85, 90, 95, 99, or 100 percent sequence identity to MAASGKLSTC RLPLPTIRE IIKLLRLQAA NELSQNFLLD LRLTDKIVRK AGNLTNAYVV EVGPGFGGIT RSILNADVAE LLVVEKDTIRF IPGLQLMSDA AGKLRIVHGV DLTVFKVEKA FSESJRPWE DDPNVHIIG NLPFSVSTPL IIKWLENISC RDGPVFYVGR TQMTLFQKEV AERLAANTGS KQRSLVSA VAQLCNVRHIF TIPQAFVPK PEVDVGVYHF TPLIQPKIEQ PFKLVENVQ NVFQQRRKYC HRGLMLFPE AQRLESTGRL LELADIDPTL RPRQLSISHF KSLCDVYRKHM CDEDPQLFAY NLFREEKRK SKNEEKEEDD AENYRL (SEQ ID NO:4).

c. **Transcription Factor B2, Mitochondrial (TFB2M)**

In still another embodiment, the polynucleotide-binding polypeptide includes TFB2M. In a preferred embodiment the TFB2M polypeptide has GenBank Accession No. AK026835. TFB2M also possesses a Rossmann-fold making it part of the NAD-binding protein family. TFB2M levels modulate mtDNA copy number and levels of mitochondrial transcripts as
would be expected of a mitochondrial transcription factor. It is appreciated by those skilled in the art that increased activity of mitochondria causes an increase in mitochondrial biogenesis.

A preferred TFB2M polypeptide has at least 80, 85, 90, 95, 99, or 100 percent sequence identity to:

```
MVI PWGLPR   RLRLSALAGA   GRFLCLGSEA   ATRKHLPAR
HCGLS D S P Q   LWPEPFRNP   PRKASKASLD   FKRKYTDRL
AETLAQI YLG   KPSRPPHLLL   ECNPGPGI   LT   QALLEAGAKV
VALES DKTFI   PHLES LGKNL   DGKLRVI   HCD   FFKLDPRSGG
VIKPPAMS SR   GLFKNLGIEA   VPWTADI PLK   VVGMFPSRGE
KRALWKLAYD   LYSCST SIYKF   GRIEVNMFI   G   EKEFQKLMAD
PGNPDLYHL   SVIWQLACE I   KVLHMEPWS S   FDIYTRKGPL
ENPKRRELLD   QLQQKLYLIQ   MI PRQNLFTK   NLTLMNYNI F
FHLLKHC FGR   RSATVI DHLR   SLTPLDARD I   LMQI GQDE
KVVMNHIPQDF   KTLFET IERS   KDCAYKWLYD   ETLED
```

(SEQ ID NO:5).

d. Polymerase (RNA) Mitochondrial (DNA directed) (POLRMT)

Still another polynucleotide-binding polypeptide that can be used to modulate mitochondrial biological activity is POLRMT. In a preferred embodiment, the POLRMT polypeptide has GenBank Accession No. NM_005035. POLRMT is a mitochondrial RNA polymerase similar in structure to phage RNA polymerases. Unlike phage polymerases, POLRMT contains two pentatricopeptide repeat (PPR) domains involved in regulating mitochondrial transcripts. It is appreciated by those skilled in the art that deletion of regulatory domains enables constitutive function.

A preferred POLRMT polypeptide has at least 80, 85, 90, 95, 99, or 100 percent sequence identity to:

```
MSALCWGRGA   AGLKRALRPC   GRPGLPKGEK   TAGGVCGPRR
SS SAS PQEOQD   QDRRKDWHGV   ELLEVLQARV   RQLQAESVSE
VVVNRVVARV   LPECGSGDGGS   LQPBRKVQMG   AKDATPVC
RWA KI LEDK   RTQQRMRQRL   KAKLQMPQFS   GE FDLATRRL
QVE PRLLSKQ   MAGCLEDCTR   QAPE S PEEQ   LARLLQEPAG
```
In some embodiments, the polynucleotide-binding polypeptide is a non-TFAM HMG domain. Generally, the HMG domain includes a global fold of three helices stabilized in an 'L-shaped' configuration by two hydrophobic cores. The high mobility group chromosomal proteins HMG1 or HMG2, which are common to all eukaryotes, bind DNA in a non-
sequence-specific fashion, for example to promote chromatin function and gene regulation. They can interact directly with nucleosomes and are believed to be modulators of chromatin structure. They are also important in activating a number of regulators of gene expression, including p53, Hox transcription factors and steroid hormone receptors, by increasing their affinity for DNA. HMG proteins include HMG-1/2, HMG-I(Y) and HMG-14/17.

The HMG-1/2-box proteins can be further distinguished into three subfamilies according to the number of HMG domains present in the protein, their specific of sequence recognition and their evolutionary relationship. The first group contains chromosomal proteins bound to DNA with no sequence specificity (class I, HMG1 and HMG2), the second contains ribosomal and mitochondrial transcription factors which show sequence specificity in the presence of another associating factor when bound with DNA (class II, yeast ARS binding protein ABF-2, UBF and mitochondrial transcription factor mtTF-1), and the third contains gene-specific transcription factors which show sequence specific DNA binding (class III, lymphoid enhancer-binding factors LEF-1 and TCF-1; the mammalian sex-determining factor SRY, and the closely related SOX proteins; and the fungal regulatory proteins Mat-MC, Mat-al, Stel 1 and Roxl). The HMG1/2-box DNA binding domain is about 75 to about 80 amino acids and contains highly conserved proline, aromatic and basic residues. Common properties of HMG domain proteins include interaction with the minor groove of the DNA helix, binding to irregular DNA structure, and the capacity to modulate DNA structure by bending.

SOX (SRY-type HMG box) proteins have critical functions in a number of developmental processes, including sex determination, skeleton formation, pre-B and T cell development and neural induction. SOX9 plays a direct role during chondrogenesis by binding and activating the chondrocyte-specific enhancer of the Col2al gene. Loss of SOX9 gene function leads to the genetic condition known as Campomelic Dysplasia (CD), a form of dwarfism characterized by extreme skeletal malformation, and one in which three-quarters of XY individual are either intersexes or exhibit male to
female sex reversal. There are more than 20 members cloned in SOX family. All of which contain an HMG domain, which can bind specifically to the double strand DNA motif and shares >50% identity with the HMG domain of SRY, the human testis-determining factor. The preferred DNA-binding site of SOX9 have been defined to be AGAACAATGG (SEQ ID NO: 6), which contains the SOX core-binding element (SCBE), AACAAT, flanking 5' AG and 3' GG nucleotides enhance binding by SOX9.

In one embodiment, the recombinant polynucleotide-binding polypeptide has at least one HMG box domain, generally at least two, more particularly 2-5 HMG box domains. The HMG box domain can bind to an AT rich DNA sequence, for example, using a large surface on the concave face of the protein, to bind the minor groove of the DNA. This binding bends the DNA helix axis away from the site of contact. The first and second helices contact the DNA, their N-termini fitting into the minor groove whereas helix 3 is primarily exposed to solvent. Partial intercalation of aliphatic and aromatic residues in helix 2 occurs in the minor groove.

In other embodiments, the polynucleotide-binding polypeptide can have at least one polynucleotide binding domain, typically two or more polynucleotide binding domains. The polynucleotide binding domains can be the same or different. For example, the polynucleotide-binding polypeptide can include at least one HMG box in combination with one or more DNA binding domains selected from the group consisting of an HMG box, homeodomain and POU domain; zinc finger domain such as C2H2 and C2C2; amphipathic helix domain such as leucine zipper and helix-loop-helix domains; and histone folds. The polynucleotide binding domain can be specific for a specific polynucleotide sequence, or preferably non-specifically binds to a polynucleotide. Alternatively, the polynucleotide-binding polypeptide can have more a combination of at least one polynucleotide binding domain that binds in a sequence specific manner and at least one polynucleotide binding-domain that binds DNA non-specifically.

f. **Helix-turn-helix**

Certain embodiments provide polynucleotide-binding polypeptides having a helix-turn-helix motif or at least a polynucleotide binding region of
a helix-turn-helix protein. Helix-turn-helix proteins have a similar structure to bacterial regulatory proteins such as the lac repressor and cro proteins, the lac repressor and so on which bind as dimers and their binding sites are palindromic. They contain 3 helical regions separated by short turns which is why they are called helix-turn-helix proteins. One protein helix (helix 3) in each subunit of the dimer occupies the major groove of two successive turns of the DNA helix. Thus, in another embodiment, the disclosed polynucleotide-binding polypeptides can form dimers or other multi-component complexes, and have 1 to 3 helices.

**g. Homeodomain**

In yet another embodiment, the polynucleotide-binding polypeptide includes a homeodomain or a portion of a homeodomain protein. Homeodomain proteins bind to a sequence of 180 base pairs initially identified in a group of genes called homeotic genes. Accordingly, the sequence was called the homeobox. The 180 bp corresponds to 60 amino acids in the corresponding protein. This protein domain is called the homeodomain. Homeodomain-containing proteins have since been identified in a wide range of organisms including vertebrates and plants. The homeodomain shows a high degree of sequence conservation. The homeodomain contains 4 a helical regions. Helices II and III are connected by 3 amino acids comprising a turn. This region has a very similar structure to helices II and III of bacterial DNA binding proteins.

**h. Zinc Finger**

Yet another embodiment provides a modified polynucleotide-binding polypeptide having a zinc finger domain or at least a portion of a zinc finger protein. Zinc finger proteins have a domain with the general structure: Phe (sometimes Tyr) - Cys - 2 to 4 amino acids - Cys - 3 amino acids - Phe (sometimes Tyr) - 5 amino acids - Leu - 2 amino acids - His - 3 amino acids - His. The phenylalanine or tyrosine residues which occur at invariant positions are required for DNA binding. Similar sequences have been found in a range of other DNA binding proteins though the number of fingers varies. For example, the SP1 transcription factor which binds to the GC box found in the promoter proximal region of a number of genes has 3 fingers.
This type of zinc finger which has 2 cysteines and 2 histidines is called a $\text{C}_2\text{H}_2$ zinc finger.

Another type of zinc finger which binds zinc between 2 pairs of cysteines has been found in a range of DNA binding proteins. The general structure of this type of zinc finger is: Cys - 2 amino acids - Cys - 13 amino acids - Cys - 2 amino acids - Cys. This is called a $\text{C}_2\text{C}_2$ zinc finger. It is found in a group of proteins known as the steroid receptor superfamily, each of which has 2 $\text{C}_2\text{C}_2$ zinc fingers.

i. **Leucine zipper**

Another embodiment provides a modified polynucleotide-binding polypeptide having a leucine zipper or at least a portion of a leucine zipper protein. The first leucine zipper protein was identified from extracts of liver cells, and it was called C/EBP because it is an enhancer binding protein and it was originally thought to bind to the CAAT promoter proximal sequence. C/EBP will only bind to DNA as a dimer. The region of the protein where the two monomers join to make the dimer is called the dimerization domain. This lies towards the C-terminal end of the protein. When the amino acid sequence was examined it was found that a leucine residue occurs every seventh amino acid over a stretch of 35 amino acids. If this region were to form an a helix then all of these leucines would align on one face of the helix.

Because leucine has a hydrophobic side chain, one face of the helix is very hydrophobic. The opposite face has amino acids with charged side chains which are hydrophilic. The combination of hydrophobic and hydrophilic characteristics gives the molecule is amphipathic moniker. Adjacent to the leucine zipper region is a region of 20-30 amino acids which is rich in the basic (positively charged) amino acids lysine and arginine. This is the DNA binding domain - often referred to as the bZIP domain - the basic region of the leucine zipper. C/EBP is thought to bind to DNA by these bZIP regions wrapping round the DNA helix.

The leucine zipper - bZIP structure has been found in a range of other proteins including the products of the $\text{jun}$ and $\text{fas}$ oncogenes. Whereas C/EBP binds to DNA as a homodimer of identical subunits, fos cannot form
homodimers at all and jun/jun homodimers tend to be unstable. However fos/jun heterodimers are much more stable. These fos/jun heterodimers correspond to a general transcription factor called AP1 which binds to a variety of promoters and enhancers and activates transcription. The consensus API binding site is TGACTCA which is palindromic.

j. Helix-loop-helix

Another embodiment provides a modified polynucleotide-binding polypeptide having helix-loop-helix domain or a polynucleotide binding portion of a helix-loop-helix protein. Helix-loop-helix proteins are similar to leucine zippers in that they form dimers via amphipathic helices. They were first discovered as a class of proteins when a region of similarity was noticed between two enhancer binding proteins called E47 and E12. This conserved region has the potential to form two amphipathic separated by a loop hence helix-loop-helix. Next to the dimerization domain is a DNA binding domain, again rich in basic amino acids and referred to as the bHLH domain. These structures are also found in a number of genes required for development of the Drosophila nervous system - the Achaete-scute complex, and in a protein called MyoD which is required for mammalian muscle differentiation.

k. Histone Fold

In still another embodiment, the modified polynucleotide-binding polypeptide includes a histone polypeptide, a fragment of a histone polypeptide, or at least one histone fold. Histone folds exist in histone polypeptides monomers assembled into dimers. Histone polypeptides include H2A, H2B, H3, and H4 which can form heterodimers H2A-2B and H3-H4. It will be appreciated that histone-like polypeptides can also be used in the disclosed compositions and methods. Histone-like polypeptides include, but are not limited to, HMf or the histone from Methanothermous fervidus, other archaeal histones known in the art, and histone-fold containing polypeptides such as MJ1647, CBF, TAFII or transcription factor IID, SPT3, and Drl-DRAP (Sanderman, K., et al., Cell. Mol. Life Sci. 54:1350-1364 (1998), which is specifically incorporated by reference herein in its entirety).
2. Protein Transduction Domain

In some embodiments, the polynucleotide-binding polypeptide is fusion protein modified to include a protein transduction domain (PTD). As used herein, a "protein transduction domain" or PTD refers to a polypeptide, polynucleotide, carbohydrate, organic or inorganic compound that facilitates traversing a lipid bilayer, micelle, cell membrane, organelle membrane, or vesicle membrane. A PTD attached to another molecule facilitates the molecule traversing membranes, for example going from extracellular space to intracellular space, or cytosol to within an organelle.

In preferred embodiments, the protein transduction domain is a polypeptide. A protein transduction domain can be a polypeptide including positively charged amino acids. Thus, some embodiments include PTDs that are cationic or amphipathic. Protein transduction domains (PTD), also known as a cell penetrating peptides (CPP), are typically polypeptides including positively charged amino acids. PTDs are known in the art, and include but are not limited to small regions of proteins that are able to cross a cell membrane in a receptor-independent mechanism (Kabouridis, P., Trends in Biotechnology (11):498-503 (2003)). Although several PTDs have been documented, the two most commonly employed PTDs are derived from TAT (Frankel and Pabo, Cell, 55(6): 1189-93(1988)) protein of HIV and Antennapedia transcription factor from Drosophila, whose PTD is known as Penetratin (Derossi et al, J Biol Chem., 269(14): 10444-50 (1994)).

Exemplary protein transduction domains include polypeptides with 11 Arginine residues, or positively charged polypeptides or polynucleotides having 8-15 residues, preferably 9-11 residues.

The Antennapedia homeodomain is 68 amino acid residues long and contains four alpha helices. Penetratin is an active domain of this protein which consists of a 16 amino acid sequence derived from the third helix of Antennapedia. TAT protein consists of 86 amino acids and is involved in the replication of HIV-1. The TAT PTD consists of an 11 amino acid sequence domain (residues 47 to 57: YGRKKRRQRRR (SEQ ID NO:7)) of the parent protein that appears to be critical for uptake. Additionally, the basic domain Tat(49-57) or RKKRRQRRRR (SEQ ID NO:8) has been shown to be a PTD. In
the current literature TAT has been favored for fusion to proteins of interest
for cellular import. Several modifications to TAT, including substitutions of
Glutamine to Alanine, i.e., Q → A, have demonstrated an increase in cellular
uptake anywhere from 90% (Wender et al, *Proc Natl Acad Sci USA*,
97(24):13003-8 (2000)) to up to 33 fold in mammalian cells. (Ho et al,
*Cancer Res.*, 61(2):474-7 (2001)).

The most efficient uptake of modified proteins was revealed by
mutagenesis experiments of TAT-PTD, showing that an 11 arginine stretch
was several orders of magnitude more efficient as an intercellular delivery
vehicle. Therefore, PTDs can include a sequence of multiple arginine
residues, referred to herein as poly-arginine or poly-ARG. In some
embodiments the sequence of arginine residues is consecutive. In some
embodiments the sequence of arginine residues is non-consecutive. A poly-
ARG can include at least 7 arginine residues, more preferably at least 8
arginine residues, most preferably at least 11 arginine residues. In some
embodiments, the poly-ARG includes between 7 and 15 arginine residues,
more preferably between 8 and 15 arginine residues. In some embodiments
the poly-ARG includes between 7 and 15, more preferably between 8 and 15
consecutive arginine residues. An example of a poly-ARG is RRRRRRR.

(SEQ ID NO:9). Additional exemplary PTDs include but are not limited to;
RRQRTSKLM KR (SEQ ID NO: 10);
GWTLNSAGYL LGKINLKALA ALAKKIL (SEQ ID NO: 11);
WEAKLAKALA KALAKHLAKA LAKALKCEA (SEQ ID NO: 12); and
RQIKIWFQNRI MKWKK (SEQ ID NO: 13).

Without being bound by theory, it is believed that following an initial
ionic cell-surface interaction, some polypeptides containing a protein
transduction domain are rapidly internalized by cells via lipid raft-dependent
macropinocytosis. For example, transduction of a TAT-fusion protein was
found to be independent of interleukin-2 receptor/raft-, caveolar- and
48(II):1453-60 (2000)). Therefore, in some embodiments the
polynucleotide-binding polypeptide includes an endosomal escape sequence
that enhances escape of the polypeptide-binding protein from macropinosomes. The some embodiments the endosomal escape sequence is part of, or consecutive with, the protein transduction domain. In some embodiments, the endosomal escape sequence is non-consecutive with the protein transduction domain. In some embodiments the endosomal escape sequence includes a portion of the hemagglutinin peptide from influenza (HA). One example of an endosomal escape sequence includes GDIMG EW GNEIFG AIAG FLGGE (SEQ ID NO: 14).

In one embodiment a protein transduction domain including an endosomal escape sequence includes the amino acid sequence RRRRRRRRRR RGE GDIMG EW GNEIFG AIAG FLGGE (SEQ ID NO: 15).

3. **Targeting Signal or Domain**

In some embodiments the polynucleotide-binding polypeptide is modified to include one or more targeting signals or domains. The targeting signal can include a sequence of monomers that facilitates *in vivo* localization of the molecule. The monomers can be amino acids, nucleotide or nucleoside bases, or sugar groups such as glucose, galactose, and the like which form carbohydrate targeting signals. Targeting signals or sequences can be specific for a host, tissue, organ, cell, organelle, non-nuclear organelle, or cellular compartment. For example, in some embodiments the polynucleotide-binding polypeptide includes both a cell-specific targeting domain and an organelle specific targeting domain to enhance delivery of the polypeptide to a subcellular organelle of a specific cells type.

i. **Organelle Targeting**

In some embodiments, the polynucleotide-binding polypeptide is modified to target a subcellular organelle. Targeting of the disclosed polypeptides to organelles can be accomplished by modifying the disclosed compositions to contain specific organelle targeting signals. These sequences can target organelles, either specifically or non-specifically. In some embodiments the interaction of the targeting signal with the organelle does not occur through a traditional receptor:ligand interaction.

The eukaryotic cell comprises a number of discrete membrane bound compartments, or organelles. The structure and function of each organelle is
largely determined by its unique complement of constituent polypeptides. However, the vast majority of these polypeptides begin their synthesis in the cytoplasm. Thus organelle biogenesis and upkeep require that newly synthesized proteins can be accurately targeted to their appropriate compartment. This is often accomplished by amino-terminal signaling sequences, as well as post-translational modifications and secondary structure.

Organelles can have single or multiple membranes and exist in both plant and animal cells. Depending on the function of the organelle, the organelle can consist of specific components such as proteins and cofactors. The polypeptides delivered to the organelle can enhance or contribute to the functioning of the organelle. Some organelles, such as mitochondria and chloroplasts, contain their own genome. Nucleic acids are replicated, transcribed, and translated within these organelles. Proteins are imported and metabolites are exported. Thus, there is an exchange of material across the membranes of organelles. Exemplary organelles include the nucleus, mitochondrion, chloroplast, lysosome, peroxisome, Golgi, endoplasmic reticulum, and nucleolus. Synthetic organelles can be formed from lipids and can contain specific proteins within the lipid membranes. Additionally, the content of synthetic organelles can be manipulated to contain components for the translation of nucleic acids.

**Targeting the Mitochondria**

In certain embodiments polynucleotide-binding polypeptides are disclosed that specifically target mitochondria. Mitochondria contain the molecular machinery for the conversion of energy from the breakdown of glucose into adenosine triphosphate (ATP). The energy stored in the high energy phosphate bonds of ATP is then available to power cellular functions. Mitochondria are mostly protein, but some lipid, DNA and RNA are present. These generally spherical organelles have an outer membrane surrounding an inner membrane that folds (cristae) into a scaffolding for oxidative phosphorylation and electron transport enzymes. Most mitochondria have flat shelf-like cristae, but those in steroid secreting cells may have tubular
cristae. The mitochondrial matrix contains the enzymes of the citric acid cycle, fatty acid oxidation and mitochondrial nucleic acids.

Mitochondrial DNA is double stranded and circular. Mitochondrial RNA comes in the three standard varieties; ribosomal, messenger and transfer, but each is specific to the mitochondria. Some protein synthesis occurs in the mitochondria on mitochondrial ribosomes that are different than cytoplasmic ribosomes. Other mitochondrial proteins are made on cytoplasmic ribosomes with a signal peptide that directs them to the mitochondria. The metabolic activity of the cell is related to the number of cristae and the number of mitochondria within a cell. Cells with high metabolic activity, such as heart muscle, have many well developed mitochondria. New mitochondria are formed from preexisting mitochondria when they grow and divide.

The inner membranes of mitochondria contain a family of proteins of related sequence and structure that transport various metabolites across the membrane. Their amino acid sequences have a tripartite structure, made up of three related sequences about 100 amino acids in length. The repeats of one carrier are related to those present in the others and several characteristic sequence features are conserved throughout the family.

Mitochondrial targeting agents generally consist of a leader sequence of highly positively charged amino acids. This allows the protein to be targeted to the highly negatively charged mitochondria. Unlike receptor:ligand approaches that rely upon stochastic Brownian motion for the ligand to approach the receptor, the mitochondrial localization signal of some embodiments is drawn to mitochondria because of charge. Therefore, in some embodiments, the mitochondrial targeting agent is a protein transduction domain including but not limited to the protein transduction domains discussed in detail above.

Mitochondrial targeting agents also include short peptide sequences (Yousif, et al, Chembiochem., 10(13):2131 (2009)), for example mitochondrial transporters-synthetic cell-permeable peptides, also known as mitochondria-penetrating peptides (MPPs), that are able to enter mitochondria. MPPs are typically cationic, but also lipophilic; this
combination of characteristics facilitates permeation of the hydrophobic mitochondrial membrane. For example, MPPs can include alternating cationic and hydrophobic residues (Horton, et al, Chem Biol, 15(4):375-82 (2008)). Some MPPs include delocalized lipophilic cations (DLCs) in the peptide sequence instead of, or in addition to natural cationic amino acids (Kelley, et al, Pharm. Res., 2011 Aug 11 [Epub ahead of print]). Other variants can be based on an oligomeric carbohydrate scaffold, for example attaching guanidinium moieties due to their delocalized cationic form (Yousif, et al, ChemBiochem, 10(13):2131 (2009).

Mitochondrial targeting agents also include mitochondrial localization signals or mitochondrial targeting signals. Many mitochondrial proteins are synthesized as cytosolic precursor proteins containing a leader sequence, also known as a presequence, or peptide signal sequence. Typically, cytosolic chaperones deliver the precursor protein to mitochondrial receptors and the General Import Pore (GIP) (Receptors and GIP are collectively known as Translocase of Outer Membrane or TOM) at the outer membrane. Typically, the precursor protein is translocated through TOM, and the intermembrane space by small TIMs to the TIM23 or 22 (Translocase of Inner Membrane) at the inner membrane. Within the mitochondrial matrix the targeting sequence is cleaved off by mtHsp70.

As discussed above, in order to enter the mitochondria, a protein generally must interact with the mitochondrial import machinery, consisting of the Tim and Tom complexes (Translocase of the Inner/Outer Mitochondrial Membrane). With regard to the mitochondrial targeting signal, the positive charge draws the linked protein to the complexes and continues to draw the protein into the mitochondria. The Tim and Tom complexes allow the proteins to cross the membranes. Accordingly, one embodiment of the present disclosure delivers compositions of the present disclosure to the inner mitochondrial space utilizing a positively charged targeting signal and the mitochondrial import machinery. In another embodiment, PTD-linked compounds containing a mitochondrial localization signal do not seem to utilize the TOM/TIM complex for entry into the mitochondrial matrix, see Del Gaizo et al. Mol Genet Metab. 80(1-
The N-terminal region of the proteins can be used to target molecules to the mitochondrion. The sequences are known in the art, see for example, U.S. Patent No. 8,039,587, which is specifically incorporated by reference herein in its entirety. The identification of the specific sequences necessary for translocation of a linked compound into a mitochondrion can be determined using predictive software known to those skilled in the art, including the tools located at http://ihg.gsf.de/ihg/mitoprot.html. Using the software the predicted sequence from Eta that can be used to target the disclosed composition is

MFRAAAPGQL RRAASLLRF (SEQ ID NO: 16).

The predicted mitochondrial targeting signal from Did is

MQSWSRVYCS LAKRGFNRI SHGLQGLSAV PLRTY (SEQ ID NO: 17).

In certain embodiments, the mitochondrial targeting agent is the mitochondrial localization signal of a mangano-superoxide dismutase (also referred to herein as "SOD2" and "Mn-SOD" and "superoxide dismutase (Mn)) precursor protein. Several mitochondrial localization signals for SOD2 are known in the art. In some embodiments the mitochondrial targeting signal includes the amino acid sequence

MLSRAVCGTS RQLAPVLGYL GSRQ (SEQ ID NO: 18)

or SEQ ID NO: 18 without the N-terminal methionine

LSRAVCGLTS QLAPVLGYLG SRQ (SEQ ID NO: 19).

In another embodiment the mitochondrial targeting signal includes the amino acid sequence

MLSRAVCGLTS RQLAPVLGYL GSRQ (SEQ ID NO:20 );

or SEQ ID NO:20 without the N-terminal methionine

LSRAVCGLTS QLAPVLGYLG SRQ (SEQ ID NO:21).

In some embodiments, the composition is preferentially delivered to the mitochondrial using a mitochondrial delivery vehicle, such as a lipid raft, mitochondrially targeted nanoparticle, or mitochondriotropic liposome. In such cases, one or more polynucleotide-binding polypeptides can be associated with, encapsulated within, dispersed in or on, or covalently attached to the mitochondrial delivery vehicle.
In certain embodiments, polynucleotide-binding polypeptides are encapsulated, coupled to, or otherwise associated with mitochondriotropic liposomes. Mitochondriotropic liposomes are cationic liposomes that can be used to deliver an encapsulated agent to the mitochondria of a cell. Mitochondriotropic liposomes are known in the art. See, for example, U.S. Patent Application Publication No. US 2008/0095834 to Weissig, et al, which is specifically incorporated by reference herein in its entirety. Mitochondriotropic liposomes are liposomes which contain a hydrophobized amphiphilic delocalized cation, such as a triphenylphosphonium or a quinolinium moiety, incorporated into or conjugate to the lipid membrane of the liposome. As a result, the liposomes can be used to deliver compounds incorporated within them to the mitochondria.

In other embodiments, polynucleotide-binding polypeptides are encapsulated within, dispersed in, associated with, or conjugated to a nanoparticle functionalized with one or more mitochondrial targeting agents. For example, the nanoparticle may contain one or be functionalized with one or more lipophilic cations or polypeptide targeting agents.

The nanoparticles may be formed from one or more polymers, copolymers, or polymer blends. In some embodiments, the one or more polymers, copolymers, or polymer blends are biodegradable. Examples of suitable polymers include, but are not limited to, polyhydroxyacids such as polylactic acid, polyglycolic acid, and poly(lactic acid-co-glycolic acids); polycaprolactones; poly(orthoesters); polyanhydrides; poly(phosphazenes); poly(hydroxyalkanoates); poly(lactide-co-caprolactones); polycarbonates such as tyrosine polycarbonates; polyamides (including synthetic and natural polyamides), polypeptides, and poly(amino acids); poly(esteramides); polyesters; poly(dioxanones); poly(alkylene alkylates); hydrophobic polyethers; polyurethanes; polyetheresters; polyacetals; polycyanoacrylates; polyacrylates; polymethylmethacrylates; polysiloxanes; poly(oxethylene)/poly(oxypolypropylene) copolymers; polyketals; polyphosphates; polyhydroxyvalerates; polyalkylene oxalates; polyalkylene succinates; poly(maleic acids), poly(alkylene glycols) such as polyethylene glycol (PEG), poly(propylene glycol) (PPG), and copolymers of ethylene
glycol and propylene glycol, poly(oxyethylated polyol), poly(olefinic alcohol), polyvinylpyrrolidone, poly(hydroxy alkylmethacrylamide), poly(hydroxyalkylmethacrylate), poly(saccharides), poly(vinyl alcohol), as well as blends and copolymers thereof. Techniques for preparing suitable polymeric nanoparticles are known in the art, and include solvent evaporation, hot melt particle formation, solvent removal, spray drying, phase inversion, coacervation, and low temperature casting. In some cases, the mitochondrial targeting agents are polypeptides that are covalently linked to the surface of the nanoparticle after particle formulation. In other cases, the mitochondrial targeting agents are lipophilic cations that are covalently bound to the particle surface. In some cases, a cationic polymer is incorporated into the particle to target the particle to the mitochondrion.

Polynucleotide-binding polypeptides can also be targeted to the mitochondria using lipid rafts or other synthetic vesicle compositions. See, for example, U.S. Patent Application Publication No. US 2007/0275924 to Khan, et al. which is specifically incorporated herein by reference herein in its entirety. The lipid raft compositions can include cholesterol, and one or more lipids selected from the group consisting of sphingomylein, gangliosides, phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, phosphatidylinositol, and a mitochondrial targeting agent. In certain embodiments, a polypeptide targeting agent is inserted into the lipid raft to target the raft to the mitochondria. The lipid rafts can be prepared and loaded with one or more polynucleotide-binding polypeptides using methods known in the art. See, for example, U.S. Patent No. 6,156,337 to Barenholz, et al.

A preferred polynucleotide-binding polypeptide that targets mitochondria has at least 80, 85, 90, 95, 99 or 100 percent sequence identity to

```
MARRRRRRRR RRRMAFLRSM WGVLSALGRS GAELCTGCGS
RLRS FVS FYV LPRWFS SVLA SCPKKPVS SY LRFSEQLPI
FKAQNPDAKT TELIRRIAQR WRELPDSKKK IYQDAYRAEW
QVYKEEI SRF KEQLT PSQIM SLEKE IMDKH LKRKAMTKKK
ELTLGLKPKR PRSAYNYVYA ERFQEA KGDS PQEKLKTVKE
```
Another embodiment provides a nucleic acid encoding the polypeptide according to SEQ ID NO:22 is

```
ATGGCGCGTC GTCGTCGTCG TCGTCGTCGT CGTCGTCGTA
TGGCGTTTCT CCGAACGATG TGGGGCGTCG TGAGTGCCCT
GGGAAGGTCT GGAGCAGAGC TGTGCACCGG CTGTGGAAGT
CGACTGCGCT CCCCTTCAG TTTTGTGTAT TTACCGAGGT
GGTTTTCATC TGTCCTGGCA AGTTGTCCAA AGAAACCTGT
AAGTCTTTAC CTTCGATTTT CTAAGAACA ACTACCATATA
TTAAAAAGCTC AGAACCCAGA TGCAAAAACT ACAGAACTAA
TTAGAAGAAT TGCCCAGCGT TGGAGGGAAC TTCCTGATTC
AAAGAAAAAA ATATATCAAG ATGCCTTATAG GCGGGAGTGG
CAGGTATATA AAGAAGAGAT AAGCAGATTT AAAGAAGACG
TAACTCCAG TCGATTATG TCTTTGAAAA AAGAAATCAT
GGACAAACAT TTAAAAAGGA AAGCTATGAC AAAAAAAAA
GGGTGATTCA CGGCAGGAAA AGCTGAAGAC TGTAAAGGAA
AACTGGAAAA ATCTGTCTGA CCTCTAAAAG GAATTATATA
TTCAGCATGC TAAAGAGGAC GAAACTCGTT ATCATAATGA
AATGAAGTCT TGGGAAGAAC AAATGATTGA AGTTGGACGA
AAGGATCTTC TACGTCGCAC AATAAAGAAA CAACGAAAAT
ATGGTGCTGA GGAGTGTTAA (SEQ ID NO: 24).
```
The sequence encoding the protein transduction domain is underlined, and
the sequence encoding the mitochondrial localization signal is double
underline. Still another embodiment provides a nucleic acid having at least
80, 85, 90, 95, 99 or more percent sequence identity to SEQ ID NO:24

Another preferred polynucleotide-binding polypeptides that targets
mitochondria has at least 80, 85, 90, 97, 99, or 100 percent sequence
identity to

| MRRRRRRRRR RRGEGDIMGE WGNEIPGAIA GFLGGEMLSR
| AVCGTSLQLP PVLYLGSRO SSVLASCPPK PVSSYLFSK
| EQLPIFKAQNP DAKTELIR RIAQRWRELDP DSKKKIYQDA
| YRAEWQVYKE EISRFKEQLTP SQIMSLKE IMDKHVRKRA
| MTKKELTLL GKPKRPRSAY NVYVAERFQE AGDSPQEKEL
| KTVKENWKNL SDSEKELIYQ HAKEDERYH NMDSWEEQM
| IEVGRKDLLR RITIKQRTYGA AEEC (SEQ ID NO:25),

or SEQ ID NO:25 without the N-terminal methionine

| RRRRRRRRRR RRGEDIMGWE GNEIFGAIAG FLGGEMLSRA
| VCGTSLQLPP VLYLGRSHA SVLASCPPK PVSSYLFSKE
| QLPIFKAQNP DAKTELIR RIAQRWRELDP DSKKKIYQDAY
| RAEWQVYKEE ISRFKEQLTP SQIMSLKEE MDKHLKRKAM
| TKKKELTLLL KPKRPRSAYN VYVAERFQEA KDSPQEKSLK
| TVKENWKNLS DSEKELIYQH AKEEDERYHN EMDSWEEQMI
| EVGRKDLLRR TIKQRTYGA EEC (SEQ ID NO:26)

In another embodiment, the recombinant polypeptide is encoded by a
nucleic acid having at least 80, 85, 90, 97, 99, or 100% sequence identity
to

| ATGCGGCGAC GCAGACGTCG TCGTGCGCGG CGTCGCGCGG
| AGGTTGATAT TATGAGTGA A TGGGGAACG AAATACGAG
| AGCGATCGCT GGTTTCTCG GTGGAGAATT GTTATCTACGC
| GCGGTAGTGA GCACCCAGCAG GCAGTCGCTT CCAGCTGCTT
| GCTATCTGGG TTCGCGCAG TCATTGTGT TAGGATCATG
| TCCGAAAAA CCTGTCGTCT GTTACCTGGG CTTCTCAGAG
| GAGGAGCTGG CTGGATTAAA AGCGAAAT CGCTCCTGCT
| AAACGACTGA ACTGATTGCG CGCATTGCAC AACGCTGGCG
Preferably the mitochondrial targeting signal, domain, or agent does not permanently damage the mitochondrion, for example the mitochondrial membrane, or otherwise impair mitochondrial function.

ii. **Cell targeting**

The proteins of interest disclosed herein can be modified to target a specific cell type or population of cells.

For example, the proteins of interest can be modified with galactosyl-terminating macromolecules to target the polypeptide of interest to the liver or to liver cells. The modified polypeptide of interest selectively enters hepatocytes after interaction of the carrier galactose residues with the asialoglycoprotein receptor present in large amounts and high affinity only on these cells.

In one embodiment, the targeting signal binds to its ligand or receptor which is located on the surface of a target cell such as to bring the composition and cell membranes sufficiently close to each other to allow penetration of the composition into the cell.

In a preferred embodiment, the targeting molecule is selected from the group consisting of an antibody or antigen binding fragment thereof, an antibody domain, an antigen, a T-cell receptor, a cell surface receptor, a cell surface adhesion molecule, a major histocompatibility locus protein, a viral
envelope protein and a peptide selected by phage display that binds specifically to a defined cell.

Targeting a polypeptide of interest to specific cells can be accomplished by modifying the polypeptide of interest to express specific cell and tissue targeting signals. These sequences target specific cells and tissues. In some embodiments the interaction of the targeting signal with the cell does not occur through a traditional receptor:ligand interaction. The eukaryotic cell comprises a number of distinct cell surface molecules. The structure and function of each molecule can be specific to the origin, expression, character and structure of the cell. Determining the unique cell surface complement of molecules of a specific cell type can be determined using techniques well known in the art.

One skilled in the art will appreciate that the tropism of the proteins of interest described can be altered by changing the targeting signal. In one specific embodiment, compositions are provided that enable the addition of cell surface antigen specific antibodies to the composition for targeting the delivery of polynucleotide-binding polypeptide. Exemplary cell surface antigens are disclosed in Wagner et al, Adv Gen, 53:333-354 (2005) which is specifically incorporated by reference herein in its entirety.

It is known in the art that nearly every cell type in a tissue in a mammalian organism possesses some unique cell surface receptor or antigen. Thus, it is possible to incorporate nearly any ligand for the cell surface receptor or antigen as a targeting signal. For example, peptidyl hormones can be used a targeting moieties to target delivery to those cells which possess receptors for such hormones. Chemokines and cytokines can similarly be employed as targeting signals to target delivery of the complex to their target cells. A variety of technologies have been developed to identify genes that are preferentially expressed in certain cells or cell states and one of skill in the art can employ such technology to identify targeting signals which are preferentially or uniquely expressed on the target tissue of interest

a. Brain Targeting

In one embodiment, the targeting signal is directed to cells of the nervous system, including the brain and peripheral nervous system. Cells in
the brain include several types and states and possess unique cell surface molecules specific for the type. Furthermore, cell types and states can be further characterized and grouped by the presentation of common cell surface molecules.

In one embodiment, the targeting signal is directed to specific neurotransmitter receptors expressed on the surface of cells of the nervous system. The distribution of neurotransmitter receptors is well known in the art and one so skilled can direct the compositions described by using neurotransmitter receptor specific antibodies as targeting signals.

Furthermore, given the tropism of neurotransmitters for their receptors, in one embodiment the targeting signal consists of a neurotransmitter or ligand capable of specifically binding to a neurotransmitter receptor.

In one embodiment, the targeting signal is specific to cells of the nervous system which may include astrocytes, microglia, neurons, oligodendrites and Schwann cells. These cells can be further divided by their function, location, shape, neurotransmitter class and pathological state. Cells of the nervous system can also be identified by their state of differentiation, for example stem cells. Exemplary markers specific for these cell types and states are well known in the art and include, but are not limited to CD 133 and Neurosphere.

b. **Muscle Targeting**

In one embodiment, the targeting signal is directed to cells of the musculoskeletal system. Muscle cells include several types and possess unique cell surface molecules specific for the type and state. Furthermore, cell types and states can be further characterized and grouped by the presentation of common cell surface molecules.

In one embodiment, the targeting signal is directed to specific neurotransmitter receptors expressed on the surface of muscle cells. The distribution of neurotransmitter receptors is well known in the art and one so skilled can direct the compositions described by using neurotransmitter receptor specific antibodies as targeting signals. Furthermore, given the tropism of neurotransmitters for their receptors, in one embodiment the targeting signal consists of a neurotransmitter. Exemplary neurotransmitters
expressed on muscle cells that can be targeted include but are not limited to acetycholine and norepinephrine.

In one embodiment, the targeting signal is specific to muscle cells which consist of two major groupings, Type I and Type II. These cells can be further divided by their function, location, shape, myoglobin content and pathological state. Muscle cells can also be identified by their state of differentiation, for example muscle stem cells. Exemplary markers specific for these cell types and states are well known in the art include, but are not limited to MyoD, Pax7 and MR4.

c. Antibodies

Another embodiment provides an antibody or antigen binding fragment thereof bound to the disclosed proteins of interest acting as the targeting signal. The antibodies or antigen binding fragment thereof are useful for directing the vector to a cell type or cell state. In one embodiment, the polypeptide of interest possesses an antibody binding domain, for example from proteins known to bind antibodies such as Protein A and Protein G from Staphylococcus aureus. For example, some embodiments include the amino acids sequence

HDEAQNAFYQVLNMPNLNA DQRNGFIQSL KDDPSQSANV

LGEAHDAAQQ NAFAVVQVLAQNLNADQRNGF IQSLKDDPSQ SANVLGEAE (SEQ ID NO:28) or

HDEAQQNAFYQVLNMPNLNA DQRNGFIQSL KDDPSQSANV
LGEAHDAAQQ NAFAVVQVLAQNLNADQRNGF IQSLKDDPSQ SANVLGEAGE G

(SEQ ID NO:29), both of which include the tandem domain B of Protein A.

In a preferred embodiment, the polynucleotide-binding protein has at least 80, 85, 90, 95, 99, or 100 percent sequence identity to

MRARRRRRRR RRGEGDIMG WGNIEIAGA GFLGGEHDEA
QQNAYQVLN MPNLNADQNRN GFIQSLKDDP SGSANVLGEA

HDEAQQAQYQVLNMPNLNA DQRNGFIQSL KDDPSQSANV
LGEAGEGSVV LASCPKPVVS SYLRSKEQL PIFKAQNPDA
KTTELIRRIA QRRRELPSK KKIYQDAYRA EWQYKKEEIS
RFKEQLTPSQ IMSLEKEIMD KHLKRKAMTK KKEILLLGKP
KRPRSAYNVY VAERFQEAKG DSPQEKLKTV KENWKNLSDS
EKELYIQHAK EDETRYHNEM KSWEQMIEV GRKDLLRRTI
KKQRKYGAEE C
(SEQ ID NO:30), or
5 SEQ ID NO: 30 without the N-terminal methionine
RRRRRRRRRR RGEQDGIMGEW GNEIGGAIAG FLGGEHDEAQ
QNAFYQVLNM PNLADQRNG FIQSLKDDPS QSANVLGEAH
DEAQNAYQ VLNPNLNAQ QRNGFIQSLK DDPSQSANVL
GEAGEGSSVL ASCPKPVSS YLRSFKEQLP IFKAQNPDAK
10 TTELIRRIAQ RWRELPSKK KIYQDAYRAE WQVYKEEISR
FKEQLTPSQI MSLEKEIMDK HLRKRKATKK KELTLLGPK
RPRSAVNYY AERFQEAKGD SPQKLTQVI KENWKNLSDSE
KELYIQHAKE DETRYHNEMK SWEQMIEVG RKDRLRRTIK
KQRKYGAEEC (SEQ ID NO:31).
15 Other domains known to bind antibodies are known in the art and can
be substituted. In certain embodiments, the antibody is polyclonal,
monoclonal, linear, humanized, chimeric or a fragment thereof.
Representative antibody fragments are those fragments that bind the
antibody binding portion of the non-viral vector and include Fab, Fab',
F(ab'), Fv diabodies, linear antibodies, single chain antibodies and bispecific
antibodies known in the art.
20 In some embodiments, the targeting domain includes all or part of an
antibody that directs the vector to the desired target cell type or cell state.
Antibodies can be monoclonal or polyclonal, but are preferably monoclonal.
For human gene therapy purposes, antibodies are derived from human genes
and are specific for cell surface markers, and are produced to reduce
potential immunogenicity to a human host as is known in the art. For
example, transgenic mice which contain the entire human immunoglobulin
gene cluster are capable of producing "human" antibodies can be utilized. In
one embodiment, fragments of such human antibodies are employed as
targeting signals. In a preferred embodiment, single chain antibodies
modeled on human antibodies are prepared in prokaryotic culture.
In preferred embodiments the polypeptide of interest is itself a fusion protein. The fusion protein can include, for example, a polynucleotide-binding polypeptide, a protein transduction domain, and optionally one or more targeting signals. A preferred polypeptide of interest is SEQ ID NO:26. Other exemplary fusion proteins containing a mitochondrial transcription factor polypeptide that are suitable for use as a polypeptide of interest are disclosed in U.S. Patent Nos. 8,039,587, 8,062,891, 8,133,733, and U.S. Published Application Nos. 2009/0123468, 2009/0208478, and 2006/0211647 all of which are specifically incorporated by reference herein in their entireties.

4. Additional Sequences

The fusion protein can optionally include additional sequences or moieties, including, but not limited to linkers and purification tags.

In a preferred embodiment the purification tag is a polypeptide. Polypeptide purification tags are known in the art and include, but are not limited to His tags which typically include six or more, typically consecutive, histidine residues; FLAG tags, which typically include the sequence DYKDDDDK (SEQ ID NO:32); haemagglutinin (HA) for example, YPYDVP (SEQ ID NO:33); MYC tag for example ILKKATAYIL (SEQ ID NO:34) or EQKLISEEDL (SEQ ID NO:35). Methods of using purification tags to facilitate protein purification are known in the art and include, for example, a chromatography step wherein the tag reversibly binds to a chromatography resin.

Purifications tags can be N-terminal or C-terminal to the fusion protein. The purification tags N-terminal to the fusion protein are typically separated from the polypeptide of interest at the time of the cleavage in vivo. Therefore, purification tags N-terminal to the fusion protein can be used to remove the fusion protein from a cellular lysate following expression and extraction of the expression or solubility enhancing amino acid sequence, but cannot be used to remove the polypeptide of interest. Purification tags C-terminal to the fusion protein can be used to remove the polypeptide of interest from a cellular lysate following expression of the fusion protein, but cannot be used to remove the expression or solubility enhancing amino acid
sequence. Purification tags that are C-terminal to the expression or solubility enhancing amino acid sequence can be N-terminal to, C-terminal to, or incorporated within the sequence of the polypeptide of interest.

In some embodiments, to fusion protein includes one or more linkers or spacers. In some embodiments linker or spacer is one or more polypeptides. In some embodiments, the linker includes a glycine-glutamic acid di-amino acid sequence. The linkers can be used to link or connect two domains, regions, or sequences of the fusion protein.

5 5. **Protein Expression**

Molecular biology techniques have developed so that therapeutic proteins can be genetically engineered to be expressed by microorganisms. The gram negative bacterium, *Escherichia coli*, is a versatile and valuable organism for the expression of therapeutic proteins. Although many proteins with therapeutic or commercial uses can be produced by recombinant organisms, the yield and quality of the expressed protein are variable due to many factors. For example, heterologous protein expression by genetically engineered organisms can be affected by the size and source of the protein to be expressed, the presence of an affinity tag linked to the protein to be expressed, codon biasing, the strain of the microorganism, the culture conditions of microorganism, and the *in vivo* degradation of the expressed protein. Some of these problems can be mitigated by fusing the protein of interest to an expression or solubility enhancing amino acid sequence. Exemplary expression or solubility enhancing amino acid sequences include maltose-binding protein (MBP), glutathione S-transferase (GST), thioredoxin (TRX), NUS A, ubiquitin (Ub), and a small ubiquitin-related modifier (SUMO).

In some embodiments, the compositions disclosed herein include expression or solubility enhancing amino acid sequence. In some embodiments, the expression or solubility enhancing amino acid sequence is cleaved prior administration of the composition to a subject in need thereof. The expression or solubility enhancing amino acid sequence can be cleaved in the recombinant expression system, or after the expressed protein in purified. In some embodiments, the expression or solubility enhancing is a
ULP1 or SUMO sequence. Recombinant protein expression systems that incorporate the SUMO protein ("SUMO fusion systems") have been shown to increase efficiency and reduce defective expression of recombinant proteins in *E. coli*, see for example Malakhov, et al, *J. Struct. Genet.* 5 Genomics, 5: 75-86 (2004), U.S. Patent No. 7,060,461, and U.S. Patent No. 6,872,551. SUMO fusion systems enhance expression and solubility of certain proteins, including severe acute respiratory syndrome coronavirus (SARS-CoV) 3CL protease, nucleocapsid, and membrane proteins (Zuo et al., *J. Struct. Funct. Genomics*, 6:103-111 (2005)).

**B. Combination Therapies**

In some embodiments the compositions including an effective amount of a mitochondrial DNA-binding polypeptide disclosed herein are administered in combination with one or more additional therapeutic agents. For example, the composition itself can include a combination of a mitochondrial DNA-binding polypeptide and one or more additional therapeutic agents. In another embodiment, a first composition including a mitochondrial DNA-binding polypeptide is separately co-administered with one or more additional compositions including one or more additional therapeutic agents. Examples of additional therapeutics include, but are not limited to, anti-inflammatories, anabolic agents, psycho-stimulants, selective androgen-receptor modulators, anti-depressant medications, anti-anxiety medications, analgesics, a myostatin inhibitor, insulin-like growth factor 1 (IGF-1), mecasermin, and combinations thereof (Chan, *The Hong Kong Medical Diary*, 13(9):7-9 (2008)). The compositions can be administered in combination or as part of a regime include physical exercise, in particular resistance exercise.

**Examples**

As used herein, rhTFAM is a fusion protein including a protein transduction domain, a mitochondrial localization signal, and a mature TFAM polypeptide.
Example 1: rhTFAM increases mitochondrial electron transport chain (ETC) subunits in aged mice

Methods and Materials

Animals

This study was conducted with C57BL/6J mice that were received from the Charles River Institute for the National Institute of Aging. Forty five (45) approximately 20 month old C57BL/6J mice were transferred from the National Institute of Aging to Charlies River in vivo research laboratory in Sacramento, CA. The mice were ear notched for identification. Mice were housed 1-4 per cage in individually and positively ventilated polycarbonate cages with HEPA filtered air. Bed-o’cobs corn cob bedding was provided and was changed every two weeks or as needed. The animal room was lighted entirely with artificial fluorescent lighting on controlled 12 hr light/dark cycle (7 am to 7 pm light). The normal temperature and relative humidity range in the animal rooms was maintained at 22 ±4°C and 50 ±15%, respectively. The animal room was set for 15 air exchanges per hour. Filtered tap water acidified to a pH of 2.8 to 3.1, and LabDiet 5K16 was provided ad libitum. Following a 1 week acclimation, the mice were grouped by mean body weight per cage, and were dosed weekly via IV for a total of 4 doses. Dosing did not exceed 1% v/bw, and corresponds to approximately 0.5mg/kg for "low dose" and 1.5mg/kg for "high" dose in mice that weigh about 30 grams.

Table 2: Treatment Protocol

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Treatment</th>
<th>Dose</th>
<th>Dosage Route/Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>Vehicle control (50% Sorbitol in 2x PBS)</td>
<td>n/a</td>
<td>IV, once weekly, for 4 weeks.</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>GNC003 dose 1 (Low Dose)</td>
<td>20ug</td>
<td>IV, once weekly, for 4 weeks.</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>GNC003 dose 2 (High Dose)</td>
<td>60ug</td>
<td>IV, once weekly, for 4 weeks.</td>
</tr>
</tbody>
</table>
Body Weight

Body weights and clinical observations were recorded weekly at the time of dosing. Cage side observations were made daily.

Morris water maze testing

During Week 5, Morris water maze (MWM) training initiated. Water temperature was 22°C. Mice that sank or floated were removed from the study.

Mice were habituated to the water maze by placing each subject into the apparatus for 60 seconds with no opportunity to escape.

Commencing on the following day, the mice were trained in the hidden platform version of the maze. The platform was submerged below the surface of the water, and the mouse was placed in the water and given 60 seconds to locate the platform. Once on the platform, mice were allowed to remain for 15 seconds before being returned to their cage. For mice that did not locate the platform in 60 seconds, they were guided to the platform and allowed to remain on the platform for 15 seconds before being returned to their cage. Mice were padded dry between MWM trials and before being returned to the cage. Each mouse underwent four successive trials a day, with 10 minute intervals, for 7 days. The sequence of water entering points differed for each of the 4 trials each day, but the location of the platform remained constant throughout the study.

To measure the strength of spatial memory retention, on the 8th day, a 'probe test' was conducted. Mice were placed in the water to swim freely for 60 seconds without the platform and the time spent in the quadrant that had contained the platform, and the number of crosses over the previous position of the platform, was recorded.

The probe trial was repeated 15 days later.

Tissue processing

Twenty four hours following the final probe trial, the mice were sacrificed. Blood was collected and processed to serum. Brain, heart, kidneys, liver, and skeletal muscle were harvested, weighed and snap frozen.
Results
This study was to assess the effect of recombinant human transcription factor A mitochondrial (rhTFAM) on parameters of aging in aged mice. Optical density measurements show a significant increase of 60% in electron transport chain protein subunits in rhTFAM treated aged mice at the 0.3 mg/kg dose (Figure 1).

Example 2: rhTFAM increases ETC activity in aged mice

Methods and Materials
For details on treatment of animals, see Example 1.

Mitochondrial complex activity
To determine the activity of mitochondrial complex I, the microplate assay kit for complex I activity (MitoSciences, MS141) was used. Mitochondrial proteins (5.5 mg/mL) were extracted by adding 1/10 volume of lauryl maltoside, and 50 µg of proteins were used for the assay. Complex I was immunocaptured on microplates, and the activity was determined by the oxidation of NADH to NAD+. Complex I activity was measured by the increase in absorbance at 450 nm and expressed as the change in absorbance per minute per microgram of protein.

Mitochondrial complex IV activity was determined with the microplate assay kit for complex IV activity (MitoSciences, MS444). Mitochondrial proteins (5.5 mg/mL) were extracted by adding 1/10 volume of lauryl maltoside, and 75 µg of proteins were used for the assay. Complex IV was immunocaptured on microplate, and activity was determined by following the oxidation of reduced cytochrome C as revealed by a change in absorbance at 550 nm. Complex IV activity was expressed as the change in absorbance per minute per microgram of protein.

Results
Maximal enzymatic capacity of C-I and C-IV of the electron transport chain (ETC) was determined in the low dose, high dose and control groups. Complex IV showed a markedly higher activity in both treatment groups, whereas Complex I increase only reached significance in the high dose group (Figures 2 and 3). These results exhibit a clear dose dependent response, and the administrated doses achieve a pharmacologically measurable effect.
These results paired with improved cognition data (discussed below) are indications that rhTFAM crosses the blood-brain barrier (BBB). The results also indicate that the increases observed in these crude lysates are not due to increases in endothelial cell metabolism, but indeed in neurons.

5 Example 3: rhTFAM increases brain ATP in aged db/db mice

Materials and Methods

Animals

Fifty (50) 10 week old BKS.Cg-Dock7m +/- Leprdb/J (642) (referred to herein as db/db) male mice were ear notched for identification using the standard mouse ID format. Mice were housed at a density of 5 per cage in polycarbonate cages which were both individually and positively ventilated. Bed-o’cobs® corn cob bedding was provided and was changed every two weeks or as needed. The animal room was lighted entirely with artificial fluorescent lighting on controlled 12 hr light/dark cycle (6 a.m. to 6 p.m. light). The normal temperature and relative humidity ranges in the animal rooms were maintained at 22 ±4°C and 50 ±15%, respectively. The animal room was set for 15 air exchanges per hour. Filtered tap water acidified to a pH of 2.8 to 3.2 was provided ad libitum. LabDiet 5K0Q was provided ad libitum.

After a 2 week acclimation, the mice were grouped by mean body weight per cage.

Mice were dosed at 100uL via IV tail vein once a week for 12 weeks according to Table 3:

<table>
<thead>
<tr>
<th>Gp</th>
<th>n</th>
<th>Treatment</th>
<th>Dose (mg/mL)</th>
<th>Dose Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>Vehicle</td>
<td>n/a</td>
<td>IV tail vein, once a week for 12 weeks.</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>low dose hTFAM</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>Mid1 dose hTFAM</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>Mid2 dose hTFAM</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>High dose hTFAM</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

At the end of study, six mice from each group were sacrificed, and brain, heart, liver, kidney and skeletal muscle were flash frozen.
ATP Assay

The CellTiter-Glo® Luminescent Cell Viability Assay kit was used for ATP assay following the manufacturer's instruction. Briefly, the assay buffer and substrate were equilibrated to room temperature, and the buffer was transferred to and gently mixed with the substrate to obtain a homogeneous solution. After a 30 min equilibration of the plate to room temperature, 100 µl of the assay reagent was added into each well containing proteins and the content was mixed for 2 min. After 10 min incubation at room temperature, the luminescence was read on a PHERAstar FS Reader (BMG Labtech Cary, NC).

Results

Db/db mice are commonly used to study diabetes (Georgi, et al, Microcirculation, 18(3):238-251 (2011)). However, db/db mice also display tau hyperphosporylation (Kim, et al, Endocrinology, 150(12):5294-301 (2009), which is a hallmark of Alzheimer's disease. To measure energy production and availability in the brains of these mice, the brain tissue was harvested, and ATP was measured as described. As shown in Figure 4, brain ATP was increased by as much as 30% over vehicle treated animals.

Example 4: rhTFAM lowers fasting glucose levels and improves glucose tolerance of ob/ob mice

Materials and Methods

Animals

Male C57BL/6J ob/ob mice were obtained from Jackson Laboratories (Bar Harbor, ME). Upon arrival, mice were 7 weeks of age. The mice were housed in a room with a photocycle of 12 hours of light and 12 hours of dark and an ambient temperature of 22-25°C, fed diet #2018 containing 6.5%fat obtained from Teklad, Harlan and was given water ad libitum. Thirty mice, 8 weeks of age, selected for the study had body weight ranging from 47-49 g. Mice were assigned to five groups of 6 mice per group, to achieve similar group mean body weight at the start of the study.

After seven days of acclimation, mice were assigned to different groups according to their body weight. There were five groups, 50 µl, 75 µl,
100 µl, 150 µl and vehicle. The summary of the study design is outlined in Table 4.

**Table 4:** Treatment Protocol

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Test Article</th>
<th>Route of Administration</th>
<th>Dose</th>
<th>Dosing Volume</th>
<th>Dosing Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Test Protein</td>
<td>IV</td>
<td>Low (50 µL)</td>
<td>200 µL</td>
<td>Every 4 days</td>
</tr>
<tr>
<td>2</td>
<td>Test Protein</td>
<td>IV</td>
<td>Mid 1 (75 µL)</td>
<td>200 µL</td>
<td>Every 4 days</td>
</tr>
<tr>
<td>3</td>
<td>Test Protein</td>
<td>IV</td>
<td>Mid 2 (100 µL)</td>
<td>200 µL</td>
<td>Every 4 days</td>
</tr>
<tr>
<td>4</td>
<td>Test Protein</td>
<td>IV</td>
<td>High (150 µL)</td>
<td>200 µL</td>
<td>Every 4 days</td>
</tr>
<tr>
<td>5</td>
<td>Saline</td>
<td>IV</td>
<td>............</td>
<td>200 µL</td>
<td>Every 4 days</td>
</tr>
</tbody>
</table>

The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

**Body Weight**

Body weights were measured prior to dosing and twice a week thereafter. Food intake was measured twice a week by providing measured amount of food and food remaining.

**Blood Collection & Serum Processing**

Mice were fasted overnight and blood was collected in serum separator tubes by tail cut and allow to stand at room temperature for 30 minutes. The samples were then centrifuged. The serum supernatant was pippeted out into 0.5 ml eppendorf tubes and stored at -70°C.

At the end of the study mice were euthanized via carbon dioxide asphyxiation and liver, adipose and skeleton muscles were collected and flash frozen at -70 degree C.

Blood glucose, Triglycerides and Cholesterol were measured on day 0, 14, 21 and 28 utilizing a specific and distinct kits from Wako Chemicals USA, Inc. Richmond, VA

**Oral Glucose Tolerance Test**

Mice are fasted overnight on Day 0 and Day 28. Before beginning the oral glucose tolerance test (OGTT), the blood glucose was measured as
time 0 minutes. Then each mouse was given a single dose of glucose solution consisting of 2 g/kg body weight of D-(-)-Glucose (G7528, Sigma) solubilized in deionized water at a volume of 250 µl administered as oral gavage. The blood glucose levels were then measured periodically with One-Touch Glucose Meter manufactured by LifeScan Inc. Milpitas, CA, at thirty-minute intervals to observe for glucose clearance.

**Results**

As shown in Figure 5, 6, and 8, vehicle treated ob/ob mice showed increasing fasting blood glucose levels during the study. In all values from treatment groups indicated lower glucose levels in TFAM treated animals, with a tendency towards dose dependent response. The conclusion that TFAM treated animals had overall lower blood glucose levels and improved glucose control was further substantiated by the finding that treated cohorts exhibited lower levels of glycated serum proteins (GPS), see Figure 6. As shown in Figure 7 and 8, mock treated animals became less glucose tolerant over the course of the study, and this phenomenon was completely abolished by TFAM treatment. Overall, TFAM treated animals exhibited a markedly higher glucose tolerance than untreated animals.

**Example 5: rhTFAM reduces blood lipids of ob/ob mice**

**Materials and Methods**

See Example 4 for animal groups and general protocol. Blood glucose, Triglycerides and Cholesterol were measured on day 0, 14, 21 and 28 utilizing kits from Wako Chemicals USA, Inc. Richmond, VA. Insulin was measured by 1-2-3 Mouse Insulin Elisa kit from Alpco Diagnostics, Salem, NH. Glycated Serum Proteins were measured with a kit from Diazyme Laboratories, Poway, CA.

**Results**

The data presented in Figure 9 shows that rhTFAM stabilizes and reduces blood lipids. The difference between high dose and control group was significant at all three measuring points (Day 14, 21 and 28) with p>0.005 in a paired student t-Test.

Figure 10 shows that serum concentrations of free fatty acid of rhTFAM treated ob/ob mice were reduced compared to vehicle control over
a 28 day time course. Statistically significant (p>0.05 in a student t-test) reduction of free fatty acids (FFA) were observed in three of the four doses (50, 100 and 150ul) on day 28, compared to levels on day 0. A dose dependent response trend was also observed.

Figure 11 shows that total cholesterol levels trended to increase less during the study period than in untreated animals.

Figure 12 shows that the measured insulin trended downwards in all samples from TFAM treated mice. The highest concentration treatment group showed a difference (p=0.07) from at day 28 as compared to vehicle control. Take together with lower glucose levels and higher glucose tolerance; the TFAM treated animals indicate a higher sensitivity towards insulin. Insulin unresponsiveness is one of the hallmarks of type II diabetes mellitus. Significance (Student t-Test p>0.05) at day 28 as compared to beginning of the study.

Example 6: rhTFAM reduces food intake in ob/ob mice

Materials and Methods
See Example 4 for animal groups and general protocol.

Results
Accumulated food intake in untreated animals was higher than in TFAM treated animals (Figure 13). The ob/ob mice increased bodyweight throughout the study, however, the ob/ob treated with rhTFAM gained less weight then mice treated with vehicle control (Figure 14). The median mouse weight during this study was 48.0g at day 0 and 56.6g at day 28. Excessive food intake and appetite is a well-known symptom of diabetes. It was noted that the diminished food intake was observed in a population with increasing bodyweight.

Example 7 rhTFAM treatment improves glucose tolerance in mice with Diet Induced Obesity (DIO)

Materials and Methods

Mice

Male C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Upon arrival, mice were 5 weeks of age. The mice were housed in a room with a photocycle of 12 hours of light and 12 hours of dark
and an ambient temperature of 22-25°C, fed on regular diet (RD) for seven days followed by High fat diet (HFD), Teklad TD 06414 (Approx. 60% of total calories come from fat) obtained from Harlan laboratories, Indianapolis, and was given with water *ad libitum* for eight weeks.

The materials and kits used for biochemical analysis were: Glucose Accu Check Glucose Meter from Roche, CA for OGTT measurements, Glucose kits from Wako Chemicals USA, Inc. Richmond, VA for Glucose measurements on day 0, 28, and 56.

After eight weeks on high fat diet (60% KCal) mice were assigned to five groups according to the average body weight. There were ten mice per group and average body weight was ranging from 46-49 g. The summary of the study design is outlined in Table 5.

**Table 5**: Treatment Protocol

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Test Protein</th>
<th>ROA</th>
<th>Dose</th>
<th>Dosing Volume</th>
<th>Dosing Frequency</th>
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<tbody>
<tr>
<td>1</td>
<td>rhTFAM</td>
<td>IV</td>
<td>50 µL</td>
<td>100 µL</td>
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<tr>
<td>2</td>
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<td>IV</td>
<td>75 µL</td>
<td>100 µL</td>
<td>Every 4 days</td>
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<tr>
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<td>100 µL</td>
<td>100 µL</td>
<td>Every 4 days</td>
</tr>
<tr>
<td>4</td>
<td>rhTFAM</td>
<td>IV</td>
<td>150 µL</td>
<td>100 µL</td>
<td>Every 4 days</td>
</tr>
<tr>
<td>5</td>
<td>Vehicle</td>
<td>IV</td>
<td>2×PBS+Sorbitol</td>
<td>100 µL</td>
<td>Every 4 days</td>
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The doses are approximately 0.22, 0.33, 0.44 and 0.66 mg/kg respectively for an average mouse with a body weight of about 30 grams.

*Oral Glucose Tolerance*

Mice were fasted overnight on Day 0, Day 28 and Day 56. Before beginning the oral glucose tolerance test (OGTT), the blood glucose was measured as time 0 minutes, then each mouse was given a single dose of glucose solution consisting of 2 g/kg body weight of D-(+) Glucose (G7528, Sigma) solubilized in deionized water at a volume of 250 µl administered as oral gavage. The blood glucose levels were then measured periodically at thirty-minute intervals to observe for glucose clearance.
**Body Weight**

Body weights were measured prior to dosing and twice a week thereafter.

Food and water intakes were measured twice a week by providing measured amount of food and water and by subtracting the leftover food and or water.

**Blood Collection & Serum Processing**

On Day 0, 28 and 56, mice were fasted overnight and blood was collected from each mouse in a serum separator tubes by tail cut and allow to stand at room temperature for 30 minutes. The samples were then centrifuged. The serum supernatant was pippeted out into 0.5 ml eppendorf tubes and stored at -70°C.

On Day 28 urine samples were collected from individually caged mice during 24-h in metabolic cages. Mice had free access to food and water. At the end of the urine collection, blood samples were collected from tail vein in a lithium heparinized tube and processed for plasma by centrifugation.

Urine and plasma samples collected were analyzed for Albumin and Creatinine using commercially available assay kit.

All analysis was carried out as per manufacturer's instructions. Glucose, Triglyceride, Cholesterol, and Free Fatty Acid concentrations were measured using Wako kits (Wako Chemicals USA, Inc. Richmond, VA). Insulin levels were measured using Ultra-sensitive Mouse Insulin Elisa kit from Crystal Chem. Albumin concentration in urine and plasma samples were analyzed using Mouse Albumin ELISA assay kit (Immunology Consultants Laboratory, Inc.). Creatinine levels in urine and plasma samples were determined by using calorimetric assay kit (Assay Design and Bioassay Systems), respectively.

At the end of the study mice were euthanized via carbon dioxide asphyxiation and liver, adipose, skeletal muscle and kidneys were collected and flash frozen at -70 degree C.
Results

As shown in Figures 15-20, blood glucose was increased in mice fed high fat diet (HFD) compared to mice fed regular diet (RD). Blood glucose levels measured in mice after overnight fasting was lower in mice receiving rhTFAM compared to untreated control. This effect reached statistical significance in most treatment groups compared alone to untreated control. Combining all treatment groups and comparing them to control with a paired student T test, it becomes clear that the effect of rhTFAM increased over time (p=0.016 day 28 and p=0.008 day 56). Mice treated with rhTFAM displayed a lower glucose sensitivity as measured by the AccuCheck in an oral glucose tolerance test (OGTT). The glucose levels were not elevated to the same degree as in untreated animals after the glucose challenge, and the blood glucose levels 60 minutes after Glucose challenge were significantly lower in all treatment groups compared to non-treated animals (p<0.05 in 50ul treatment group, p<0.01 in 75ul, 100ul and 150ul treatment groups). In summary rhTFAM decreased basal glucose in DIO mice, and increased glucose tolerance in all four treatment groups.

Example 8 rhTFAM treatment decreases insulin, triglyceride, cholesterol and free fatty acid levels in mice with Diet Induced Obesity (DIO)

Materials and Methods

See Example 7 for Materials and Methods.

Results

Diet Induced Obesity (DIO) were assayed for insulin levels (Figures 21-23), triglyceride levels (Figures 24-26), cholesterol levels (Figures 27-29), and free fatty acid levels (Figures 30-32) with or without rhTFAM treatment. As shown in Figure 21, HFD had a large impact on blood insulin levels. Mice treated with TFAM displayed somewhat lower blood insulin levels compared to the non-treated cohort, this difference reached significance in a few treatment groups. Combining all treatment groups for statistical analysis, the trend of long term TFAM treatment to lower insulin levels was observed (p=0.058 day 28, p=0.036 day 56). Triglyceride and Cholesterol exhibited a marked difference in all treatment groups compared
to control, with all treatment groups on both day 28 and day 56 having a significant difference p>0.02. These effects were more pronounced after prolonged treatment, although large changes in both triglycerides and cholesterol were observed at the first measurement day 28. Free fatty acid levels were also lowered by TFAM treatment (p=0.033), whereas this effect could only be observed in the highest treatment group on day 28 (p=0.038).

Example 9 rhTFAM treatment delays or reduces weight gain in mice with Diet Induced Obesity (DIO)

Methods
See Example 7 for Materials and Methods.

Results
As shown in Figure 33, rhTFAM treated mice did not gain weight as rapidly as control, vehicle treated animals. A clear dose dependency was observed, with the two high dose level groups gaining the least weight. No difference in food intake was observed between control and TFAM treated groups.

Example 10: rhTFAM improves endurance in normal mice

Methods and Materials

Animals
Forty (40) 8 to 10 week old male C57BL/6J mice were ear notched for identification and housed in individually and positively ventilated polycarbonate cages with HEPA filtered air at a density of 5 mice per cage. Bed-o-cob corn cob bedding was used and cages were changed every two weeks. The animal room was lighted entirely with artificial fluorescent lighting, with a controlled 12 h light/dark cycle (6 am to 6 pm light). The normal temperature and relative humidity ranges in the animal rooms were 22 ± 4°C and 50 ± 15%, respectively. The animal rooms were set to have 15 air exchanges per hour. Filtered tap water, acidified to a pH of 2.8 to 3.2, and LabDiet 5LL4 was provided ad libitum.
After a 1 week acclimation:

1. Mice were assigned to treatment groups:
   a. Saline Control (n=20)
   b. rhTFAM (n=20) at a dose of 6.6ug per mouse per dose

(approximately 0.22mg / kg for an average mouse with a body weight of about 30 grams).

2. The mice were dosed IV tail vein weekly for 4 weeks.

3. At the end of the dosing period the following tests were executed
   a. Grip strength was measured on day 1.
   b. Locomotor open field testing was carried out on day 1.
   c. Rotarod training on day 2 and day 3 and rotarod testing were carried out on day 4.
   d. Morris water maze training was carried out on days 5 through 9 with a 14 day break followed by a probe trial test.

Results

Two different sets of experiments were conducted. In one set of experiments, mice were put on an accelerating rotarod (4rpm/min). In a second set of experiments, the mice were challenged with a constant speed at 15rpm. The time until the mouse fell off was recorded, and the trial was repeated 3 times for each condition, with at least 15 minutes of recovery time between each set, and with access to food and water. As shown in Figures 34A-B and 35A-B, mice treated with rhTFAM exhibited higher endurance than non-treated animals. Statistical difference between control and TFAM treated groups was observed in the fixed speed testing as (p=0.035 trial 1, p=0.053 trial 2 and p=0.086 trial 3; rhTFAM treatment significantly improved rotarod latency p=0.003).

Significance between the two groups was reached on the second day of the accelerated speed tests (p<0.05) and over the entirety of the study, rhTFAM treatment effect was significant (p=0.014).
Example 11: rhTFAM improves activity in normal mice

**Methods and Materials**
See Example 10 for Materials and Methods.

**Results**
Mice were subjected to a standardized open field test, where movement for each mouse was recorded for 900 seconds. The TFAM treated mice showed a significantly longer distance traveled (p=0.043) (Figure 36) and less resting time (p=0.017) (Figure 37), whereas no difference was observed in time spent in peripheral zone vs. central zone, indicating that TFAM did not alter anxiety levels of the mice. In summary, these results indicate a higher basal level of activity in TFAM treated mice, not likely due to a heightened level of anxiety.

Example 12: rhTFAM improves learning in normal mice

**Methods and Materials**
See Example 10 for Materials and Methods.

**Results**
Mice were subjected to a standardized Morris Water Maze trial (MWM) with a hidden platform. Time was recorded as an indication how quickly mice learned to find the platform. TFAM treated animals appeared to learn faster than untreated animals, as shown in Figures 38 and 39.

Example 13: rhTFAM increases learning and memory retention in aged mice

**Materials and Methods**
See Example 1 for Materials and Methods.

**Results**
Both TFAM treated groups outperformed the vehicle treated cohort in finding the hidden platform test, and retained their knowledge better 15 days post training (Figure 41). These experiments were performed in week 5, 6 and 7, whereas the treatment was completed in week 4, indicating that TFAM treatment provide long lasting cognitive benefits in aged mice.
Example 14 rhTFAM treatment of SOD1 knockout mice decrease muscle cartilage formation

Materials and Methods

Animals

A total of 49 (26 male, 23 female; B6;129S7-Sod1 tm1L.ey/J strain 2972 mice were aged to 9 months old. Mice were housed at a density of 4 per cage in polycarbonate cages which were both individually and positively ventilated. Bed-o'cobs ® corn cob bedding was provided and was changed every two weeks or as needed. The animal room was lighted entirely with artificial fluorescent lighting on controlled 12 hr light/dark cycle (6 a.m. to 6 p.m. light). The normal temperature and relative humidity ranges in the animal rooms were maintained at 22 ±4°C and 50 ±15%, respectively. The animal room was set for 15 air exchanges per hour. Filtered tap water acidified to a pH of 2.8 to 3.2 was provided ad libitum. Standard rodent chow was provided ad libitum. At 9 months of age, mice underwent the following procedures:

The mice were grouped by mean body weight per cage, and were gender matched prior to initiation of dosing. Dosing was via intravenous (IV) weekly for eight weeks. Dosing volume did not exceed 1% v/bw.

Table 5: Treatment Protocol

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<tr>
<th>Gp</th>
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<td>1</td>
<td>11</td>
<td>Vehicle</td>
<td>n/a</td>
<td>IV, once weekly for 8 weeks</td>
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<td>2</td>
<td>13</td>
<td>rhTFAM</td>
<td>0.35</td>
<td>IV once weekly for 8 weeks</td>
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Mice were sacrificed and muscles were formalin fixed.

Results

Percent collagen in aging skeletal muscle is shown in Figure 42.

Muscle sections were stained using Masson's Trichrome. Slides were scanned and RGB pixel image information was generated. Collagen and fibrosis were measured using the blue channel. Muscle was measured using the red channel. Total amount of collagen was measured for each muscle and
expressed as a ratio to total muscle. Ratio of collagen to muscle is a given tissue section was calculated as the ratio of Blue/Red channel (Figure 42). The difference between treated and non-treated was significant (p>0.0001).

**Example 15: rhTFAM decreases the level of inflammation in aging mouse tissue**

**Methods and Materials**

For details on treatment of animals, see Example 1.

**TBARS assay**

Plasma concentrations of thiobarbituric acid reactive substances (TBARS) are an index of lipid peroxidation and oxidative stress. The TBARS assay is a well-established method of assessing the level of inflammatory response in a subject. For TBARS measurements, a kit from Cayman Chemical Company, Ann Arbor, MI was utilized following the manufacturer's instructions on serum and brain tissues.

**Results**

As shown in Figure 43 the difference between rhTFAM treated mice and their control counterparts reached significance comparing their brain tissues three weeks after the last rhTFAM injection. The TBARS assay indicated a trend towards lower levels of inflammation, although that difference did not reach statistical significance at the chosen time point. In summary these results indicate a lowering of the inflammatory response in aged mice upon rhTFAM treatment.

**Example 16 rhTFAM reduces amyloid-mediated inhibition of mitochondrial maximal respiration**

**Materials and Methods**

Vehicle or rhTFAM was added to cells in culture and oxygen consumption rates (OCR) were measured for 56 minutes. Amyloid-beta (Abeta) (Sigma Cat. # A9810 - Amyloid β Protein Fragment 1-42) in oligomeric or fibrilar form (10 µM) was added to cells and oxygen consumption rates (OCR) were measured for 56 minutes. The uncoupler carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (300 nM) was used to stimulate maximal respiration followed by rotenone (100µM) to inhibit respiration.
Oligomycin was used in some experiments at a concentration of 1 µg/mL. Astrocytes, Pan 02 and HepG2 cell lines were tested.

**Results**

Both forms of amyloid tested inhibited FCCP stimulated maximal respiration. rhTFAM was added to Abeta challenged cells approximately an hour prior to FCCP treatment, and OCR was again measured. Initially, no suppression of basal oxygen consumption by the amyloid was observed after adding Abeta in either fibrillar or oligomeric forms. This is evidenced in Figures 44 and 45 which that both experimental and control cells show little or no deviation in oxygen consumption rates (OCR) from control (i.e., "0" indicates no change from control, -100 indicates no oxygen consumption, and 100 equals a doubling of oxygen consumption). Inhibition of maximal respiration was reversed by rhTFAM (Figure 44 and 45).

This data supports amelioration of the mitochondrial defect in Alzheimer's disease by rhTFAM treatment. Cells used in Figures 44 and 45 are mouse hippocampal neurons, (MN-h, ScienCell Research Laboratories, Carlsbad, California). Similar results were obtained in Pan 02 pancreatic adenocarcinoma cells, HepG2 human liver carcinoma cell line and in primary mouse astrocytes.

**Example 17: Modulation of Brain Metabolites**

**Materials and Methods**

Magnetic resonance spectroscopy (MRS) experiments were conducted to detect the modulation of brain metabolites following treatment with rhTFAM in naive rats. A Varian 7T spectrometer was used for all experiments.

**Animal Handling**

Twenty Sprague Dawly rats were used in this study and all animal handling protocols were approved by the Molecular Imaging IACUC, where animals were housed and all experiments performed. Animals received either 1 dose of saline (vehicle, N=10) or 1 dose of rhTFAM (1.0 mg/kg, N=10) intravenously after the Baseline imaging time point and before the 4hr and 24hr time points (hours post dosing).
Imaging Protocol

3iP and iH experiments were performed independently. A single resonance transmit and receive surface coil (1.2cm ID, tuned to 121Mhz for 3iPhosphorous nuclei was used for phosphorous MRS and a traditional volume resonator was used for proton MRS. 31P MRS acquisition parameters were: TR=3s, 640 averages, using a single hard pulse for excitation. iH MRS acquisition parameters were: TR=2.5s, 640 averages, TE=1.2 ms, using a PRESS sequence and a single voxel of volume 2x2x3 mm.

Data Analysis

All data were analyzed using custom code developed in Matlab. Raw FID data were zero-meaned and apodized using an exponential filter and Fourier transformed for spectral fitting in the frequency domain. A limited model (fitting select peaks and baseline components of the FFTs) was created using the generator function:

\[ Y_n = \sum_{k=1}^{n} \alpha_k e^{j\phi_k} e^{(-d_k-n\pi f_k t_n + 2j\pi f_k t_n)} \]

where: k = component number; n = time point; d = damping factor; g = 1; f = component frequency; j =V-1. Fits were optimized using a non-linear least squares approach with manual intervention when required.

Results

N-acetylaspartate, or NAA, is the second most abundant metabolite in the human central nervous system (CNS). NAA levels measured by magnetic resonance spectroscopy are changed in a wide array of CNS disorders. Magnetic resonance studies of human brain disorders have invariably detected decreases in brain NAA concentrations when neuronal loss or dysfunction are involved, with one major exception. The autosomal genetic disease, Canavan disease, involves the accumulation of NAA in the brain due to the lack of degradative enzyme activity. Virtually all other neurological disorders involving neuronal loss or dysfunction result in reductions in brain NAA levels including Alzheimer disease, epilepsy,
Amyotrophic lateral sclerosis, schizophrenia, multiple sclerosis, AIDS, traumatic brain injury, stroke and non-neuronal brain tumors such as glioma.

Lactate is another brain metabolite measurable by non-invasive magnetic resonance imaging that has clinical significance in the diagnosis of central nervous system diseases including but not limited to gliomas, depression, schizophrenia, Alzheimer's disease, Parkinson's, Multiple Sclerosis, ischemia and others.

The data in Figures 46-48 indicate that TFAM has a measurable, and statistically significant impact on these biomarkers. Figure 46 shows that brain lactate levels in mice treated with 1.0 mg/kg of rhTFAM were lower at 24 hours post administration compared to control mice treated with vehicle. Figure 47 shows percent change in brain lactate levels lactate. Figure 48 shows increases in brain levels of NAA in mice treated with rhTFAM compared to mice treated with vehicle at 4 hours post administration.
I claim:

1. A method for treating frailty syndrome in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a fusion polypeptide and a pharmaceutically acceptable carrier to treat one or more symptoms of frailty syndrome,
   wherein the fusion polypeptide comprises
   (a) a mitochondrial DNA binding polypeptide;
   (b) a protein transduction domain; and
   (c) a mitochondrial localization signal.

2. The method of claim 1, wherein the mitochondrial DNA-binding polypeptide comprises a transcription factor A - mitochondria (TFAM) polypeptide comprising an amino acid sequence having 95% or more sequence identity to SEQ ID NO:3, or a mitochondrial DNA binding fragment thereof.

3. The method as in claim 1, wherein the one or more symptoms of frailty syndrome are selected from the group consisting of sarcopenia, unintentional non-muscle weight loss greater than 10 lbs per year, decreased grip strength, low energy expenditure, weakness, decreased physical activity, fatigue, and decreased walking time.

4. The method of claim 1, wherein the subject is greater than 64 years of age.

5. A method of treating or inhibiting the progression of a metabolic disorder or disease in a subject in need thereof comprising administering to the subject an effective amount of a fusion protein wherein the fusion polypeptide comprises
   (a) a mitochondrial DNA binding polypeptide;
   (b) a protein transduction domain; and
   (c) a mitochondrial localization signal.
6. The method of claim 5, wherein the subject displays one or more symptoms selected from the group consisting of excessive appetite relative to healthy subjects, elevated blood glucose levels relative to healthy subjects, increased glucose sensitivity relative to healthy subjects, increased glycosylated protein levels relative to healthy subjects, elevated insulin levels relative to healthy subjects, decreased insulin sensitivity relative to healthy subjects, increased blood triglyceride levels relative to healthy subjects, increased blood cholesterol levels relative to healthy subjects, increased blood free fatty acid levels relative to healthy subjects, or a combination thereof.

7. The method of claim 5, wherein the metabolic disorder or disease is selected from the list consisting of prediabetes, impaired fasting glycaemia, impaired glucose tolerance (IGT), dysglycemia, insulin resistance, hypertriglyceridemia, hyperglycemia, stroke, arteriosclerotic vascular disease (ASVD), Dyslipoproteinemia, Hypolipoproteinemia, and Hyperlipidemia or Hypercholesterolemia.

8. The method of claim 5, wherein the fusion protein comprises at least 95% sequence identity to SEQ ID NO:26.

9. The method of claim 5, wherein the metabolic disorder or disease is decreased insulin sensitivity as compared to a healthy subject.

10. The method of claim 5, wherein the metabolic disorder or disease is elevated insulin levels as compared to a healthy subject.

11. The method of claim 5, wherein the metabolic disorder or disease is an excessive appetite relative to a healthy subject.

12. The method of claim 5, wherein the metabolic disorder or disease is elevated blood glucose level relative to a healthy subject.

13. The method of claim 12, wherein blood glucose levels are measured after overnight fasting.

14. The method of claim 12, wherein the elevated blood glucose levels are determined by detecting elevated glycosylated protein levels relative to a healthy subject.

15. The method of claim 5, wherein the disease or condition is elevated glycosylated protein levels as compared to a healthy subject.
16. The method of claim 5, wherein the disease or condition is increased glucose sensitivity.
17. The method of claim 5, wherein the disease or condition is obesity.
18. A method of decreasing appetite in a subject in need thereof comprising administering an effective amount of a fusion protein to the subject to decrease the subject’s appetite, wherein the fusion protein comprises three domains selected from the group consisting of (1) a domain comprising a protein transduction domain, (2) a domain comprising a mitochondrial targeting signal, and (3) a domain comprising a mature mitochondrial transcription factor-A (TFAM).
19. The method of claim 18, wherein the subject has an elevated blood glucose level relative to a healthy subject.
20. A method of treating, preventing or inhibiting the progression of cardiovascular disease in a subject in need thereof comprising administering to the subject an effective amount of a protein having 95% or more sequence identity to SEQ ID NO:26.
21. The method of claim 20, wherein the subject has increased blood cholesterol levels relative to a healthy subject.
22. The method of claim 20, wherein the subject has increased blood triglyceride levels relative to a healthy subject.
23. The method of claim 20, wherein the subject has increased blood free fatty acids relative to a healthy subject.
24. The method of claim 20, wherein the subject has increased blood glycosylated proteins relative to a healthy subject.
25. The method of claim 20, wherein the subject has increased oxidized blood proteins relative to a healthy subject.
26. A method of increasing physical stamina in an animal comprising administering to the animal an effective amount of a fusion protein comprising three domains selected from the group consisting of (1) a domain comprising a protein transduction domain, (2) a domain comprising a mitochondrial targeting signal, and (3) a domain comprising a mature mitochondrial transcription factor-A (TFAM).
27. The method of claim 26, where said animal is a human.
28. A method of increasing physical activity in a subject comprising administering to the subject an effective amount of a fusion protein comprising three domains selected from the group consisting of (1) a domain comprising a protein transduction domain, (2) a domain comprising a mitochondrial targeting signal, and (3) a domain comprising a mature mitochondrial transcription factor-A (TFAM).
29. The method of claim 28, wherein the patient has one or more symptoms selected from the group consisting of weakness, fatigue, exhaustion, lethargy, languidness, languor, lassitude and listlessness.
30. A method of modulating brain metabolism in a subject in need thereof comprising administering to the subject a fusion protein comprising three domains selected from the group consisting of (1) a domain comprising a protein transduction domain, (2) a domain comprising a mitochondrial targeting signal, and (3) a domain comprising a mature mitochondrial transcription factor-A (TFAM) in an amount effective to increase levels of ATP in the brain, in an effective amount to increase levels of N-Acetyl aspartate in the brain, or to reduce levels of lactate in the brain relative to the subject's levels prior to administration of the fusion protein.
31. The method of claim 30, wherein the fusion protein has a sequence that is at least 95% identical to SEQ ID NO:26.
**FIGURE 14**

- db/db mouse body weight

- HTFAM treatment reduces body weight in db/db mice (p=0.007)

**FIGURE 15**

- Glucose day 0

- RD vs HFD
Glucose Day 28

FIGURE 16

Glucose Day 56

FIGURE 17
OGTT-Day0

![OGTT-Day0 Graph](image)

FIGURE 18

OGTT-Day28

![OGTT-Day28 Graph](image)

FIGURE 19
FIGURE 26

Triglyceride Day 56

FIGURE 27

Cholesterol Day 0
Cholesterol Day 28

![Graph showing cholesterol levels on Day 28.]

**FIGURE 28**

Cholesterol Day 56

![Graph showing cholesterol levels on Day 56.]

**FIGURE 29**
Free Fatty Acid Day 56

Treatment Dose

FIGURE 32

Body Weight

Percent Change in Body Weight

Days of Treatment

FIGURE 33
Accelerating Rotarod in Young Mice
Raw Data from 3 Trials and 3 Runs/Trial (n=10/Group)

There is a statistically significant effect of rhTFAM on rotarod (p = 0.014)

FIGURE 34A

Latency on Rotarod in seconds

FIGURE 34B
Constant Speed (15 RPM) Rotarod in Young Mice
Data from 3 Trials and 3 Runs/Trial (n=10/Group)

There is a statistically significant effect of rhTFAM on rotarod (p=.003)

FIGURE 35A

Latency on Rotarod in seconds

FIGURE 35B
FIGURE 36A AND 36B

FIGURE 37
FIGURE 40
Morris Water Maze Probe Trial

FIGURE 41A AND 41B
rhTFAM reduces collagen and fibrosis in sarcopenic skeletal muscle from aged SOD-1/0 mice

**FIGURE 42**

**FIGURE 43**
AUC of NAA

FIGURE 48
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/17 A61P25/28

ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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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

Date of the actual completion of the international search

4 March 2013

Date of mailing of the international search report

03/06/2013

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

Authorized officer

Fayos, Cecile

Form PCT/ISA/210 (second sheet) (April 2005)
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<td>THOMAS RAVINDAR R ET AL: &quot;RhTFAM treatment stimulates mitochondrial oxidative metabolism and improves memory in aged mice &quot;, AGING SEP 2012, vol. 4, no. 9, September 2012 (2012-09), pages 620-635, XP002692780, ISSN: 1945-4589 the whole document</td>
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<td>WO 2008/072781 Al (UNIV KYUSHU NAT UNIV CORP [JP]; IDE T0M0MI [JP]; YAMATO MAYUMI [JP]; K) 19 June 2008 (2008-06-19) the whole document</td>
<td>1-4</td>
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<tr>
<td>Category</td>
<td>Citation of document, with indication, where appropriate, of the relevant passages</td>
<td>Relevant to claim No.</td>
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<td>GIUSEPPE PASSARINO ET AL: &quot;Mitochondrial functions, mitochondrial DNA and aging: a reappraisal&quot;, BIOGERONTOLOGY, KLUWER ACADEMIC PUBLISHERS, DO, vol. 11, no. 5, 3 July 2010 (2010-07-03), pages 575-588, XP019813306, ISSN: 1573-6768 the whole document page 581, left-hand column, last paragraph - right-hand column, paragraph 1</td>
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<td>Y, P</td>
<td>GOMEZ-CABRERA M C ET AL: &quot;Mitochondrial sources and targets of damage in cellular aging&quot;, CLINICAL CHEMISTRY AND LABORATORY MEDICINE, WALTER DE Gruyter &amp; Co, BERLIN, NEW YORK, vol. 50, no. 8, 1 February 2012 (2012-02-01), pages 1287-1295, XP009167450, ISSN: 1434-6621, DOI: 10.1515/CCLM-2011-0795 the whole document page 1289, left-hand column, last paragraph - right-hand column, paragraph 1</td>
<td>1-4</td>
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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.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

see additional sheet

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 an 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. X 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.:

   1-4

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.
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<td>US 2009208478 A1</td>
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This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-4

A method for treating frailty syndrome in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a fusion peptide de and a pharmaceutical caly acceptably carried to treat one or more symptoms of frailty syndrome, wherein the fusion peptide comprises: (a) a mitochondrial domain; (b) a protease domain; and (c) a mitochondrial localization signal.

2. Claims: 5-17

A method of treating or inhibiting the progression on of a metabolic disorder or disease in a subject in need thereof comprising administering to the subject an effective amount of a fusion peptide wherein the fusion peptide comprises: (a) a mitochondrial domain; (b) a protease domain; and (c) a mitochondrial localization signal.

3. Claims: 18, 19

A method of decreasing appetite in a subject in need thereof comprising administering to the subject to decrease the subject's appetite, wherein the fusion peptide comprises three domains selected from the group consisting of: (1) a domain comprising a protease domain; (2) a domain comprising a mitochondrial domain; and (3) a domain comprising a mature mitochondrial transcribed on factor-A (TFAM).


A method of treating, preventing or inhibiting the progression of cardiovascular disease in a subject in need thereof comprising administering to the subject an effective amount of a protease having 95% or more sequence identity to SEQ ID NO:26.

5. Claims: 26, 27

A method of increasing physical stamina in an animal comprising administering to the animal an effective amount of a fusion peptide comprising three domains selected from the group consisting of: (1) a domain comprising a protease domain; (2) a domain comprising a mitochondrial domain; and (3) a domain comprising a mitochondrial localization signal.
targeting signal, and (3) a domain comprising a mature mitochondrial translation factor-A (TFAM).

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6. claims: 28, 29

A method of increasing physical activity in a subject comprising administering to the subject an effective amount of a fusion protein comprising three domains selected from the group consisting of (1) a domain comprising a protein transductin domain, (2) a domain comprising a mitochondrial targeting signal, and (3) a domain comprising a mature mitochondrial translation factor-A (TFAM).

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7. claims: 30, 31

A method of modulating brain metabolism in a subject in need thereof comprising administering to the subject a fusion protein comprising three domains selected from the group consisting of (1) a domain comprising a protein transductin domain, (2) a domain comprising a mitochondrial targeting signal, and (3) a domain comprising a mature mitochondrial translation factor-A (TFAM) in an amount effective to increase levels of ATP in the brain, in an effective amount to increase levels of N-Acetyl aspartate in the brain, or to reduce levels of lactate in the brain relative to the subject's levels prior to administration of the fusion protein.

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