SUMMARY

In one aspect of the present disclosure, there is provided a fusion protein, comprising:

(a) a protein of interest to be delivered to mitochondria in a cell;

a cell penetrating peptide (CPP); and

a target enhancing sequence (TES), wherein the TES comprises a protease sensitive peptide;

wherein the CPP is located at the N-terminus of the fusion protein and wherein the TES is fused at the C-terminus of the CPP; or wherein the CPP is located at the C-terminus of the fusion protein and wherein the TES is fused at the N-terminus of the CPP; or

(b) a protein of interest to be delivered to mitochondria in a cell;

a cell penetrating peptide (CPP); and

a target enhancing sequence (TES), wherein the TES comprises a nuclear export signal peptide;

wherein the protein of interest is delivered to mitochondria.

In another aspect of the present disclosure, there is provided a nucleic acid encoding the fusion protein disclosed herein.

In a further aspect of the present disclosure, there is provided an expression vector for introducing a fusion protein into a cell comprising the nucleic acid of disclosed herein.

In another aspect of the present disclosure, there is provided a conjugate for intracellular delivery of proteins to mitochondria comprising the fusion protein disclosed herein and a moiety linked to said fusion protein, wherein said moiety is selected from a group consisting of a radioactive label, a fluorescent label, a small molecule, and a polymeric molecule.

In a further aspect of the present disclosure, there is provided a cell comprising the fusion protein disclosed herein, the nucleic acid disclosed herein, the expression vector disclosed herein, the conjugate disclosed herein, or a combination thereof.

In another aspect of the present disclosure, there is provided a pharmaceutical composition comprising the fusion protein disclosed herein, a conjugate disclosed herein, or a combination thereof and a pharmaceutically acceptable diluent, carrier, additive or excipient.

In a further aspect of the present disclosure, there is provided a fusion protein disclosed herein, a nucleic acid disclosed herein, a vector disclosed herein or a conjugate disclosed herein, for delivery of a protein of interest to a cell.

In another aspect of the present disclosure, there is provided a fusion protein disclosed herein, a nucleic acid disclosed herein, a vector of disclosed herein or a conjugate disclosed herein, for delivery of a protein of interest to mitochondria in a cell. In a further aspect of the present disclosure, there is provided a use of the fusion protein disclosed herein, the nucleic acid disclosed herein, the vector disclosed herein, the conjugate disclosed herein or the pharmaceutical composition disclosed herein, in the manufacture of a medicament for treating a mitochondria associated disorder in a subject in need thereof.

In another aspect of the present disclosure, there is provided a modified fusion protein for increasing an amount of a protein of interest delivered to mitochondria in a cell, wherein:

the modified fusion protein is produced by modifying a sequence of a fusion protein comprising the protein of interest and a cell penetrating peptide (CPP) by introducing into the fusion protein a target enhancing sequence (TES); and

wherein a cell is to be contacted with the modified fusion protein,

thereby increasing the amount of the protein of interest delivered to mitochondria in the cell relative to the amount of the protein of interest delivered to the cell by the fusion protein lacking the TES;

wherein the TES comprises a nuclear export signal peptide; or

wherein the TES comprises a protease sensitive peptide; wherein the CPP is located at the N-terminus of the modified fusion protein and the TES is fused at the C-terminus of the CPP; or wherein the CPP is located at the C-terminus of the modified fusion protein and the TES is fused at the N-terminus of the CPP; and

wherein the protein of interest is delivered to mitochondria.

In a further aspect of the present disclosure, there is provided a modified fusion protein for use in increasing an amount of a protein of interest delivered to mitochondria in a cell, wherein:

the modified fusion protein is produced by modifying a sequence of a fusion protein comprising the protein of interest and a cell penetrating peptide (CPP) by introducing into the fusion protein a target enhancing sequence (TES); and

wherein a cell is to be contacted with the modified fusion protein,

thereby increasing the amount of the protein of interest delivered to mitochondria in the cell relative to the amount of the protein of interest delivered to the cell by the fusion protein lacking the TES;

wherein the TES comprises a nuclear export signal peptide; or

wherein the TES comprises a protease sensitive peptide; wherein the CPP is located at the N-terminus of the modified fusion protein and the TES is fused at the C-terminus of the CPP; or wherein the CPP is located at the C-terminus of the modified fusion protein and the TES is fused at the N-terminus of the CPP; and

wherein the protein of interest is delivered to mitochondria.

In a further aspect of the present disclosure, there is provided a fusion protein disclosed herein, a nucleic acid disclosed herein, a vector disclosed herein, a conjugate disclosed herein, or a pharmaceutical composition disclosed herein, for use in therapy.

The present disclosure provides fusion proteins that are capable of being efficiently delivered to cells, *e.g.*, in greater levels than previously achieved. The present disclosure further provides fusion proteins that are capable of efficiently delivering a therapeutic protein of interest to cells and achieving and maintaining the proper subcellular localization and/or function of the therapeutic protein of interest. For example, the present disclosure provides fusion proteins that

are capable of being efficiently delivered to non-nuclear organelles. An exemplary fusion protein provided by the present disclosure comprises, in addition to a protein of interest to be delivered to a cell, a cell penetrating peptide (CPP); and/or a target enhancing sequence (TES). In the fusion protein of the present disclosure, the CPP is capable of interference with delivery of the protein of interest to the protein's proper subcellular localization and/or with its function, and the TES prevents this interference by CPP. Another exemplary fusion protein provided by the present disclosure comprises, in addition to a protein of interest to be delivered to a non-nuclear organelle, an organelle targeting sequence (OTS); a cell penetrating peptide (CPP); and/or a target enhancing sequence (TES). In said fusion protein of the present disclosure, the CPP is capable of interference with delivery of the protein of interest to the non-nuclear organelle, and the TES prevents this interference by CPP.

The present disclosure also is based, at least on part, on the surprising discovery that including certain amino acid sequences (*i.e.*, TES) in a fusion protein that also comprises a protein of interest and a CPP, facilitated effective delivery of the protein of interest to the cell, *e.g.*, resulted in increased levels of the protein of interest in cells treated with the fusion protein, and allowed for proper subcellular localization and/or function of the protein of interest. In particular, the present inventors have discovered that cells treated with fusion proteins comprising a protein of interest, CPP and TES contained significantly higher amounts of the protein of interest than cells treated with a fusion protein comprising a protein of interest and CPP, but lacking TES. Thus, the present inventors have surprisingly discovered that introducing TES into a fusion protein comprising CPP and a protein of interest can significantly increase the amount of protein of interest in a cell that has been contacted with the fusion protein. The present inventors also surprisingly discovered that fusion proteins comprising a protein of interest, CPP and TES were correctly processed by the cellular machinery, achieved proper cellular localization, *e.g.*, were targeted to a non-nuclear organelle, and possessed the desired activity of the protein of interest when delivered to cells.

CLAIMS

1. A fusion protein, comprising:

(a) a protein of interest to be delivered to mitochondria in a cell;

a cell penetrating peptide (CPP); and

a target enhancing sequence (TES), wherein the TES comprises a protease sensitive peptide;

wherein the CPP is located at the N-terminus of the fusion protein and wherein the TES is fused at the C-terminus of the CPP; or wherein the CPP is located at the C-terminus of the fusion protein and wherein the TES is fused at the N-terminus of the CPP; or

(b) a protein of interest to be delivered to mitochondria in a cell;

a cell penetrating peptide (CPP); and

a target enhancing sequence (TES), wherein the TES comprises a nuclear export signal peptide;

wherein the protein of interest is delivered to mitochondria.

2. The fusion protein of claim 1, wherein the fusion protein comprises or consists of, starting at the N-terminus:

CPP;

TES; and

a protein of interest; or

wherein the fusion protein comprises or consists of, starting at the C-terminus:

a protein of interest;

TES; and

CPP.

3. The fusion protein of claim 1 or 2, wherein the fusion protein further comprises a mitochondrial targeting sequence (MTS).

4. The fusion protein of claim 3, wherein the MTS is heterologous to the protein of interest; or wherein the MTS is endogenous to the protein of interest.

5. The fusion protein of claim 1, wherein the protein of interest is selected from the group consisting of Frataxin (FXN), Tafazin, Pyruvate Dehydrogenase (PDH), SLIRP, LRPPC, PARK2 protein, PARK6 protein, PARK7 protein, Phospholipase A2, Group VI and a variant or derivative thereof.

6. The fusion protein of claim 5, wherein the protein of interest comprises Frataxin (FXN) or PARK2 protein, or a variant or derivative thereof.

7. The fusion protein of any one of claims 1-6, wherein the CPP comprises a peptide selected from the group of CPPs listed in the Database of Cell-Penetrating Peptides CPPsite 2.0; or wherein the CPP comprises a peptide selected from the group consisting of HIV-TAT, galanin, mastoparan, transportan, penetratin, polyarginine, VP22, and a variant or derivative thereof.

8. The fusion protein of claim 7, wherein the CPP comprises HIV-TAT or a variant or derivative thereof.

9. The fusion protein of claim 1, wherein the nuclear export signal peptide comprises a sequence having at least 85% sequence identity to any one of SEQ ID NOs. 36-43.

10. The fusion protein of claim 9, wherein the nuclear export signal peptide comprises a peptide selected from the group consisting of NES1 and NES2, or a variant or derivative thereof.

11. The fusion protein of claim 1, wherein the protease sensitive peptide comprises a ubiquitin-like modifier.

12. The fusion protein of claim 11, wherein the protease sensitive peptide comprises a sequence having at least 85% sequence identity to any one of SEQ ID NOs. 18-31.

13. The fusion protein of claim 1, wherein the protease sensitive peptide comprises a peptide selected from the group consisting of ubiquitin, a caspase cleavage domain, a calpain cleavage

domain, SUMO1, SUMO2, SUMO3, SUMO4, ISG15, Atg8, Atg12, NEDD8, and a variant or derivative thereof.

14. The fusion protein of claim 1, wherein the fusion protein comprises an amino acid sequence having at least 85%, 90%, or 95% sequence identity to any of SEQ ID NOs. 55-61, 69-71, and 81.

15. The fusion protein of any one of claims 1-14, further comprising a secretion signal (SS) and/or an extracellular proteolytic site (EPS).

16. A nucleic acid encoding the fusion protein of any one of claims 1-15.

17. An expression vector for introducing a fusion protein into a cell comprising the nucleic acid of claim 16.

18. A conjugate for intracellular delivery of proteins to mitochondria comprising the fusion protein of any one of claims 1-15 and a moiety linked to said fusion protein, wherein said moiety is selected from a group consisting of a radioactive label, a fluorescent label, a small molecule, and a polymeric molecule.

19. A cell comprising the fusion protein of any one of claims 1-15, the nucleic acid of claim 16, the expression vector of claim 17, the conjugate of claim 18, or a combination thereof.

20. A pharmaceutical composition comprising the fusion protein of any one of claims 1-15, a conjugate of claim 18, or a combination thereof and a pharmaceutically acceptable diluent, carrier, additive or excipient.

21. The fusion protein of any one of claims 1-15, the nucleic acid of claim 16, the vector of claim 17 or the conjugate of claim 18, for delivery of a protein of interest to a cell.

22. The fusion protein of any one of claims 1-15, the nucleic acid of claim 16, the vector of claim 17 or the conjugate of claim 18, for delivery of a protein of interest to mitochondria in a cell.

23. Use of the fusion protein of any one of claims 1-15, the nucleic acid of claim 16, the vector of claim 17, the conjugate of claim 18 or the pharmaceutical composition of claim 20, in the manufacture of a medicament for treating a mitochondria associated disorder in a subject in need thereof.

24. The use of claim 23, wherein the mitochondria associated disorder is selected from the group consisting of Friedreich's ataxia (FDRA), Barth Syndrome, Parkinson's Disease, Wilson's Disease, Leigh Syndrome and Fibrosis.

25. The use of claim 24, wherein the mitochondria associated disorder is FDRA or Parkinson's Disease.

26. The use of any one of claims 23-25, wherein said subject is a human.

27. A modified fusion protein for increasing an amount of a protein of interest delivered to mitochondria in a cell, wherein:

the modified fusion protein is produced by modifying a sequence of a fusion protein comprising the protein of interest and a cell penetrating peptide (CPP) by introducing into the fusion protein a target enhancing sequence (TES); and

wherein a cell is to be contacted with the modified fusion protein,

thereby increasing the amount of the protein of interest delivered to mitochondria in the cell relative to the amount of the protein of interest delivered to the cell by the fusion protein lacking the TES;

wherein the TES comprises a nuclear export signal peptide; or

wherein the TES comprises a protease sensitive peptide; wherein the CPP is located at the N-terminus of the modified fusion protein and the TES is fused at the C-terminus of the CPP; or wherein the CPP is located at the C-terminus of the modified fusion protein and the TES is fused at the N-terminus of the CPP; and

wherein the protein of interest is delivered to mitochondria.

28. A modified fusion protein for use in increasing an amount of a protein of interest delivered to mitochondria in a cell, wherein:

the modified fusion protein is produced by modifying a sequence of a fusion protein comprising the protein of interest and a cell penetrating peptide (CPP) by introducing into the fusion protein a target enhancing sequence (TES); and

wherein a cell is to be contacted with the modified fusion protein,

thereby increasing the amount of the protein of interest delivered to mitochondria in the cell relative to the amount of the protein of interest delivered to the cell by the fusion protein lacking the TES;

wherein the TES comprises a nuclear export signal peptide; or

wherein the TES comprises a protease sensitive peptide; wherein the CPP is located at the N-terminus of the modified fusion protein and the TES is fused at the C-terminus of the CPP; or wherein the CPP is located at the C-terminus of the modified fusion protein and the TES is fused at the N-terminus of the CPP; and

wherein the protein of interest is delivered to mitochondria.

29. The fusion protein of any one of claims 1-15, the nucleic acid of claim 16, the vector of claim 17, the conjugate of claim 18, or the pharmaceutical composition of claim 20, for use in therapy.