

**GENE EDITING SYSTEMS COMPRISING AN RNA GUIDE TARGETING
STATHMIN 2 (STMN2) AND USES THEREOF**

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 63/231,784, filed August 11, 2021, and U.S. Provisional Application No. 63/322,002, filed March 21, 2022, the contents of each of which are incorporated by reference herein in their entirety.

BACKGROUND

10 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) genes, collectively known as CRISPR-Cas or CRISPR/Cas systems, are adaptive immune systems in archaea and bacteria that defend particular species against foreign genetic elements.

SUMMARY OF THE PRESENT DISCLOSURE

15 The present disclosure is based, at least in part, on the development of a system for genetic editing of a stathmin 2 (STMN2) gene. The system involves a Cas12i polypeptide such as a Cas12i2 polypeptide and an RNA guide mediating cleavage at a genetic site within the
20 STMN2 gene by the CRISPR nuclease polypeptide. As reported herein, the gene editing system disclosed herein has achieved successful editing of STMN2 gene with high editing efficiency and accuracy.

25 Without being bound by theory, the gene editing system disclosed herein may exhibit one or more of the following advantageous features. Compared to SpCas9 and Cas12a, Cas12i effectors are smaller (1033 to 1093aa) which, in conjunction with their short mature crRNA (40-43 nt), is preferable in terms of delivery and cost of synthesis. Cas12i cleavage results in larger deletions compared to the small deletions and +1 insertions induced by Cas9 cleavage. Cas12i PAM sequences also differ from those of Cas9. Therefore, larger and different portions of genetic sites of interest can be disrupted with a Cas12i polypeptide and RNA guide compared to Cas9. Using an unbiased
30 approach of tagmentation-based tag integration site sequencing (TTISS), more potential off-target sites with a higher number of unique integration events were identified for SpCas9 compared to Cas12i2. *See* WO/2021/202800. Therefore, Cas12i such as Cas12i2 may be more specific than Cas9.

35 Accordingly, described herein are gene editing systems for editing a STMN2 gene, pharmaceutical compositions or kits comprising such, methods of using the gene editing systems to produce genetically modified cells, and the resultant cells thus produced. Also described herein

are uses of the gene editing systems disclosed herein, the pharmaceutical compositions and kits comprising such, and/or the genetically modified cells thus produced for treating neurodegenerative diseases (e.g., amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD)) in a subject.

5 In one aspect, the present disclosure provides a gene editing system for genetic editing of a stathmin 2 (STMN2) gene, comprising (i) a Cas12i2 polypeptide or a first nucleic acid encoding the Cas12i2 polypeptide, wherein the Cas12i2 polypeptide comprises an amino acid sequence at least 95% identical to SEQ ID NO: 448 and comprises one or more mutations relative to SEQ ID NO: 448; and (ii) an RNA guide or a second nucleic acid encoding the RNA
10 guide, wherein the RNA guide comprises a spacer sequence specific to a target sequence within an STMN2 gene, the target sequence being adjacent to a protospacer adjacent motif (PAM) comprising the motif of 5'-TTN-3', which is located 5' to the target sequence; wherein the Cas12i2 polypeptide comprises mutations at position D581, I926, and V1030 in SEQ ID NO: 448, which optionally are amino acid substitutions of D581R, I926R, and V1030G.

15 In another aspect, the present disclosure provides a gene editing system for genetic editing of a stathmin 2 (STMN2) gene, comprising (i) a Cas12i polypeptide or a first nucleic acid encoding the Cas12i polypeptide, optionally wherein the Cas12i polypeptide is a Cas12i2 polypeptide; and (ii) an RNA guide or a second nucleic acid encoding the RNA guide, wherein the RNA guide comprises a spacer sequence specific to a target sequence within exon 1, exon 2,
20 exon 3, exon 4, exon 5, exon 6, exon 7, or an intron of a STMN2 gene, the target sequence being adjacent to a protospacer adjacent motif (PAM) comprising the motif of 5'-TTN-3', which is located 5' to the target sequence; and wherein the target sequence is of any one of SEQ ID NOs: 4617, 4615, 4618, 4619, 4563-4573, 4612-4614, 4616, and 4620.

 In some examples, the present disclosure features system for genetic editing of a stathmin
25 2 (STMN2) gene, comprising (i) a Cas12i polypeptide or a first nucleic acid encoding the Cas12i polypeptide, and (ii) an RNA guide or a second nucleic acid encoding the RNA guide. The RNA guide comprises a spacer sequence specific to a target sequence within an STMN2 gene, the target sequence being adjacent to a protospacer adjacent motif (PAM) comprising the motif of 5'-TTN-3', which is located 5' to the target sequence.

30 In some examples, the Cas12i is a Cas12i2 polypeptide. In other examples, the Cas12i is a Cas12i4 polypeptide.

 In some examples, the Cas12i polypeptide is a Cas12i2 polypeptide comprising an amino acid sequence at least 95% identical to SEQ ID NO: 448. In some instances, the Cas12i2 polypeptide may comprise one or more mutations relative to SEQ ID NO: 448. In some
35 examples, the one or more mutations in the Cas12i2 polypeptide are at positions D581, G624,

F626, P868, I926, V1030, E1035, and/or S1046 of SEQ ID NO: 448. In some examples, the one or more mutations are amino acid substitutions, which optionally is D581R, G624R, F626R, P868T, I926R, V1030G, E1035R, S1046G, or a combination thereof.

5 In one example, the Cas12i2 polypeptide comprises mutations at positions D581, D911, I926, and V1030 (*e.g.*, amino acid substitutions of D581R, D911R, I926R, and V1030G). In another example, the Cas12i2 polypeptide comprises mutations at positions D581, I926, and V1030 (*e.g.*, amino acid substitutions of D581R, I926R, and V1030G). In yet another example, the Cas12i2 polypeptide comprises mutations at positions D581, I926, V1030, and S1046 (*e.g.*, amino acid substitutions of D581R, I926R, V1030G, and S1046G). In still another example, the
10 Cas12i2 polypeptide comprises mutations at positions D581, G624, F626, I926, V1030, E1035, and S1046 (*e.g.*, amino acid substitutions of D581R, G624R, F626R, I926R, V1030G, E1035R, and S1046G). In another example, the Cas12i2 polypeptide comprises mutations at positions D581, G624, F626, P868, I926, V1030, E1035, and S1046 (*e.g.*, amino acid substitutions of D581R, G624R, F626R, P868T, I926R, V1030G, E1035R, and S1046G).

15 Exemplary Cas12i2 polypeptides for use in any of the gene editing systems disclosed herein may comprise the amino acid sequence of any one of SEQ ID NOs: 449-453. In one example, the exemplary Cas12i2 polypeptide for use in any of the gene editing systems disclosed herein comprises the amino acid sequence of SEQ ID NO: 450. In another example, the

vector. In some examples, the first and/or second vector is a viral vector. In some specific examples, the first and second vectors are the same vector.

In some examples, any of the systems described herein may comprise one or more lipid nanoparticles (LNPs), which encompass the Cas12i polypeptide (e.g., the Cas12i2 polypeptide) or the first nucleic acid encoding the Cas12i polypeptide, the RNA guide or the second nucleic acid encoding the RNA guide, or both.

In some examples, the system described herein may comprise an LNP, which encompasses the Cas12i polypeptide (e.g., the Cas12i2 polypeptide) or the first nucleic acid encoding the Cas12i polypeptide, and a viral vector comprising the second nucleic acid encoding the RNA guide. In some examples, the viral vector is an AAV vector. In other examples, the system described herein may comprise an LNP, which encompasses the RNA guide or the second nucleic acid encoding the RNA guide, and a viral vector comprising the first nucleic acid encoding the Cas12i polypeptide. In some examples, the viral vector is an AAV vector.

In some aspects, the present disclosure also refers to a pharmaceutical composition comprising any of the gene editing systems disclosed herein, and a kit comprising the components of the gene editing system.

In another aspect, the present disclosure refers to an *in vitro* method for editing a stathmin 2 (STMN2) gene in a cell, the method comprising contacting a host cell with the gene editing system for editing the STMN2 gene set forth herein to genetically edit the STMN2 gene in the host cell; wherein the host cell is cultured *in vitro*. In another aspect, the present disclosure provides a use of a gene editing system in the manufacture of a medicament for editing a stathmin 2 (STMN2) gene in a subject having a neurodegenerative disease, wherein the gene editing system is set forth herein. In another aspect, the present disclosure provides the gene editing system as disclosed herein for use in therapy.

In other examples, the present disclosure also features a method for editing a stathmin 2 (STMN2) gene in a cell, the method comprising contacting a host cell with any of the systems disclosed herein to genetically edit the STMN2 gene in the host cell. In some examples, the host cell is cultured *in vitro*. In other examples, the contacting step is performed by administering the system for editing the STMN2 gene to a subject comprising the host cell.

Also within the scope of the present disclosure is a cell comprising a disrupted a stathmin 2 (STMN2) gene, which can be produced by contacting a host cell with the system disclosed herein genetically edit the STMN2 gene in the host cell.

Still in other examples, the present disclosure provides a method for treating neurodegenerative diseases (e.g., amyotrophic lateral sclerosis (ALS) or frontotemporal dementia

(FTD)) in a subject. The method may comprise administering to a subject in need thereof any of the systems for editing a stathmin 2 (STMN2) gene or any of the cells disclosed herein.

In another aspect, the present disclosure refers to an RNA guide, comprising (i) a spacer sequence as disclosed herein that is specific to a target sequence in a stathmin 2 (STMN2) gene,
5 wherein the target sequence is adjacent to a protospacer adjacent motif (PAM) comprising the motif of 5'-TTN-3', which is located 5' to the target sequence; and (ii) a direct repeat sequence.

In some examples, the spacer may be 20-30-nucleotides in length. In some examples, the spacer is 20-nucleotides in length.

In some examples, the direct repeat sequence may be 23-36-nucleotides in length. In some examples, the direct repeat sequence is 23-nucleotides in length.

In some examples, the target sequence is within exon 1, exon 2, exon 3, exon 4, exon 5, exon 6, exon 7, or an intron of the STMN2 gene.

5 In some examples, the RNA guide comprises the sequence of any one of SEQ ID NOs: 4508, 4512, 4559, and 4561, or the second nucleic acid encodes an RNA guide comprising any one of SEQ ID NOs: 4508, 4512, 4559, and 4561.

In some examples, the RNA guide comprises the sequence of any one of SEQ ID NOs: 4505, 4506, 4507, 4508, 4509, 4510, 4511, 4512, 4513, 4514, 4515, 4554, 4555, 4556, 4557,
10 4558, 4559, 4560, 4561, and 4562, or the second nucleic acid encodes an RNA guide comprising any one of SEQ ID NOs: 4505, 4506, 4507, 4508, 4509, 4510, 4511, 4512, 4513, 4514, 4515, 4554, 4555, 4556, 4557, 4558, 4559, 4560, 4561, and 4562.

In some examples, the direct repeat sequence may be at least 90% identical to any one of SEQ ID NOs: 1-10 or a fragment thereof that is at least 23-nucleotides in length. In some
15 examples, the direct repeat sequence is any one of SEQ ID NOs: 1-10, or a fragment thereof that is at least 23-nucleotides in length. By way of non-limiting example, the direct repeat sequence is 5'-AGAAAUCCGUCUUUCAUUGACGG-3' (SEQ ID NO: 10).

Also described herein are any of the gene editing systems disclosed herein, pharmaceutical compositions or kits comprising such, or genetically modified cells generated by the gene editing
20 system for use in treating neurodegenerative disease (e.g., amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD)) in a subject, as well as uses of the gene editing systems disclosed herein, pharmaceutical compositions or kits comprising such, or genetically modified cells generated by the gene editing system for manufacturing a medicament for treatment of neurodegenerative disease (e.g., amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD)) in a subject.

25 The details of one or more examples of the disclosure are set forth in the description below. Other features or advantages of the present disclosure will be apparent from the following drawings and detailed description of several examples, and also from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

30 The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure, which can be better understood by reference to the drawing in combination with the detailed description of specific embodiments presented herein.

FIG. 1 shows editing percentage of STMN2 intron target sequences by the indicated
35 guides, as described in Example 1.

WHAT IS CLAIMED IS:

1. A gene editing system for genetic editing of a stathmin 2 (STMN2) gene, comprising

5 (i) a Cas12i2 polypeptide or a first nucleic acid encoding the Cas12i2 polypeptide, wherein the Cas12i2 polypeptide comprises an amino acid sequence at least 95% identical to SEQ ID NO: 448 and comprises one or more mutations relative to SEQ ID NO: 448; and

(ii) an RNA guide or a second nucleic acid encoding the RNA guide, wherein the RNA guide comprises a spacer sequence specific to a target sequence within an STMN2 gene, the target sequence being adjacent to a protospacer adjacent motif (PAM) comprising the motif of 5'-TTN-3', which is located 5' to the target sequence;

10 wherein the Cas12i2 polypeptide comprises mutations at position D581, I926, and V1030 in SEQ ID NO: 448, which optionally are amino acid substitutions of D581R, I926R, and V1030G.

15 2. The gene editing system of claim 1, wherein the Cas12i2 polypeptide further comprises one or more mutations in the Cas12i2 polypeptide at positions G624, F626, P868, E1035, and/or S1046 of SEQ ID NO: 448; optionally wherein the further mutations are amino acid substitutions G624R, F626R, P868T, E1035R, S1046G, or a combination thereof.

20 3. The gene editing system of claim 2, wherein the Cas12i2 polypeptide comprises: (i) mutations at positions D581, D911, I926, and V1030, which optionally are amino acid substitutions of D581R, D911R, I926R, and V1030G;

(ii) mutations at positions D581, I926, V1030, and S1046, which optionally are amino acid substitutions of D581R, I926R, V1030G, and S1046G;

25 (iii) mutations at positions D581, G624, F626, I926, V1030, E1035, and S1046, which optionally are amino acid substitutions of D581R, G624R, F626R, I926R, V1030G, E1035R, and S1046G; or

(iv) mutations at positions D581, G624, F626, P868, I926, V1030, E1035, and S1046, which optionally are amino acid substitutions of D581R, G624R, F626R, P868T, I926R, V1030G, E1035R, and S1046G.

4. The gene editing system of claim 1, wherein the Cas12i2 polypeptide comprises an amino acid sequence at least 98% identical to SEQ ID NO: 448 and comprises one or more mutations relative to SEQ ID NO: 448, wherein the Cas12i2 polypeptide comprises mutations at position D581, I926, and V1030 in SEQ ID NO: 448, which optionally are amino acid substitutions of D581R, I926R, and V1030G.

5. The gene editing system of claim 1, wherein the Cas12i2 polypeptide comprises the amino acid sequence of SEQ ID NO: 449, 450, 451, 452, or 453, optionally wherein the Cas12i2 polypeptide comprises the amino acid sequence of SEQ ID NO: 450 or 453.

6. A gene editing system for genetic editing of a stathmin 2 (STMN2) gene, comprising

(i) a Cas12i polypeptide or a first nucleic acid encoding the Cas12i polypeptide, optionally wherein the Cas12i polypeptide is a Cas12i2 polypeptide; and
(ii) an RNA guide or a second nucleic acid encoding the RNA guide, wherein the RNA guide comprises a spacer sequence specific to a target sequence within exon 1, exon 2, exon 3, exon 4, exon 5, exon 6, exon 7, or an intron of a STMN2 gene, the target sequence being adjacent to a protospacer adjacent motif (PAM) comprising the motif of 5'-TTN-3', which is located 5' to the target sequence; and

wherein the target sequence is of any one of SEQ ID NOs: 4617, 4615, 4618, 4619, 4563-4573, 4612-4614, 4616, and 4620.

7. The gene editing system of any one of claims 1-6, which comprises the first nucleic acid encoding the Cas12i2 polypeptide.

8. The gene editing system of claim 7, wherein the first nucleic acid is a messenger RNA (mRNA), or

wherein the first nucleic acid is included in a viral vector, which optionally is an adeno-associated viral (AAV) vector.

9. The gene editing system of any one of claims 1-5, 7, and 8, wherein the target sequence is within exon 1, exon 2, exon 3, exon 4, exon 5, exon 6, exon 7, or an intron of the STMN2 gene.

10. The gene editing system of claim 9, wherein the target sequence is of any one of SEQ ID NOs: 4617, 4615, 4618, 4619, 4563-4573, 4612-4614, 4616, and 4620.

11. The gene editing system of any one of claims 1-10, wherein the RNA guide comprises the sequence of any one of SEQ ID NOs: 4559, 4557, 4559, 4560, 4505-4515, 4554-4556, 4558, and 4562, or the second nucleic acid encodes the RNA guide.

12. The gene editing system of any one of claims 1-11, wherein the spacer sequence is 20-30-nucleotides in length, optionally wherein the spacer sequence is 20-nucleotides in length.

13. The gene editing system of any one of claims 1-12, wherein the RNA guide comprises the spacer sequence and a direct repeat sequence.

14. The gene editing system of claim 13, wherein the direct repeat sequence is 23-36-nucleotides in length.

15. The gene editing system of claim 14, wherein the direct repeat sequence is at least 90% identical to any one of SEQ ID NOs: 1-10 or a fragment thereof that is at least 23-nucleotides in length.

16. The gene editing system of claim 15, wherein the direct repeat sequence is any one of SEQ ID NOs: 1-10, or a fragment thereof that is at least 23-nucleotides in length.

17. The gene editing system of claim 16, wherein the direct repeat sequence is 5'-AGAAAUCCGUCUUUCAUUGACGG-3' (SEQ ID NO: 10).

18. The gene editing system of any one of claims 1-17, wherein the system comprises the second nucleic acid encoding the RNA guide.

5 19. The gene editing system of claim 18, wherein the second nucleic acid encoding the RNA guide is located in a viral vector.

10 20. The gene editing system of any one of claims 7-19, wherein the viral vector comprises the both the first nucleic acid encoding the Cas12i2 polypeptide and the second nucleic acid encoding the RNA guide.

15 21. The gene editing system of any one of claims 1-19, wherein the system comprises the first nucleic acid encoding the Cas12i2 polypeptide, which is located on a first vector, and wherein the system comprises the second nucleic acid encoding the RNA guide, which is located on a second vector.

22. The gene editing system of claim 21, wherein the first and second vector are the same vector.

20 23. A pharmaceutical composition comprising the gene editing system set forth in any one of claims 1-22.

24. A kit comprising the elements (i) and (ii) of the gene editing system set forth in any one of claims 1-22.

25 25. An *in vitro* method for editing a stathmin 2 (STMN2) gene in a cell, the method comprising contacting a host cell with the gene editing system for editing the STMN2 gene set forth in any one of claims 1-22 to genetically edit the STMN2 gene in the host cell; wherein the host cell is cultured *in vitro*.

30 26. Use of a gene editing system in the manufacture of a medicament for editing a stathmin 2 (STMN2) gene in a subject having a neurodegenerative disease, wherein the gene

editing system is set forth in any one of claims 1-22; wherein the neurodegenerative disease is amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD); and wherein the target sequence corresponding to the RNA guide of the gene editing system is located in intron 1.

5 27. The use of claim 26, wherein the subject is a human patient having the neurodegenerative disease.

 28. An RNA guide, comprising (i) a spacer sequence that is specific to a target sequence in a stathmin 2 (STMN2) gene, wherein the target sequence is adjacent to a protospacer
10 adjacent motif (PAM) comprising the motif of 5'-TTN-3', which is located 5' to the target sequence; and (ii) a direct repeat sequence.

 29. The RNA guide of claim 28, wherein the RNA guide is set forth in any one of claims 9-17.

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 30. The gene editing system of any one of claims 1-22 for use in therapy, wherein the target sequence corresponding to the RNA guide of the gene editing system is located in intron 1.