SUMMARY

[0006] The present disclosure provides, among other things, improved methods and compositions for the treatment of DMD based on administration of a recombinant follistatin-Fc fusion protein. The disclosure encompasses, *inter alia*, the unexpected observation that certain amino acid modifications in the follistatin polypeptide result in improved follistatin protein that specifically targets myostatin and activin A with high affinity and does not bind to non-target BMPs or heparin with meaningful affinity. It is contemplated that activation of Smad2/3 pathway by myostatin and activin A leads to inhibition of myogenic protein expression and as a result, myoblasts do not differentiate into muscle. Therefore, myostatin and activin are viable targets for stimulation of muscle regeneration. However, myostatin and activin antagonists including follistatin can bind bone morphogenetic proteins (BMPs) due to certain structural similarities. BMPs, especially, BMP-9 and BMP-10, are pivotal morphogenetic signals, orchestrating tissue architecture throughout the body. Inhibition of such BMPs may lead to undesired pathological conditions. Follistatin also binds to cell surface heparan-sulfate proteoglycans through a basic heparin-binding sequence (HBS) in the first of three FS domains. It is contemplated that inactivation, reduction or modulation of heparin binding may increase *in vivo* exposure and/or half-life of follistatin. Thus, the present disclosure provides improved follistatin that has longer half-life and is more potent for effective treatment of DMD.

[0007] In one aspect, the present disclosure provides recombinant follistatin polypeptides comprising an amino acid sequence at least 80% identical to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5, wherein the recombinant follistatin protein has a heparin binding domain (HBS), and wherein one or more amino acids within the HBS is substituted with an amino acid having a less positive charge in comparison to the substituted amino acid. In one embodiment, the one or more amino acids within the HBS are substituted with an amino acid having a neutral charge. In one embodiment, the one or more amino acids within the HBS are substituted with an amino acid having a negative charge. In one embodiment, the one or more comprises at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids. In one embodiment, the one or more comprises 3 amino acids. In one embodiment, the recombinant polypeptide has decreased heparin binding affinity in comparison to naturally occurring follistatin. In one embodiment, the recombinant follistatin protein does not bind to BMP-9 or BMP-10. In one embodiment, the recombinant follistatin protein has a sequence at least 80% identical to any one of SEQ ID NO: 12-40 or SEQ ID NO: 101-106.

[0008] Disclosed are recombinant follistatin polypeptides comprising an amino acid sequence at least 80% identical to SEQ ID NO:2, SEQ NO:4 or SEQ ID NO:5

and wherein the amino acids corresponding to positions 66 to 88 of SEQ ID NO:2, SEQ NO:4 or SEQ ID NO:5 are identical to any one of SEQ ID NO:42-67 or SEQ ID NO:111-116. In some embodiments, the amino acid sequence corresponding to positions 66 to 88 of SEQ ID NO: 2, SEQ ID NO: 4, or SEQ ID NO: 5 are identical to any one of SEQ ID NO: 58-67 or SEQ ID NO: 111-113. In some embodiments, the recombinant follistatin polypeptide is a hyperglycosylation mutant. In some embodiments, the amino acid sequence of the recombinant follistatin polypeptide is at least 90%, identical to SEQ ID NO:2, SEQ NO:4 or SEQ ID NO:5. In some embodiments, the amino acid sequence of the recombinant follistatin polypeptide is at least 95%, identical to SEQ ID NO:2, SEQ NO:4 or SEQ ID NO:5. In some embodiments, the amino acid sequence of the recombinant follistatin polypeptide is at least 98%, identical to SEQ ID NO:2, SEQ NO:4 or SEQ ID NO:5. In some embodiments, the amino acid sequence is of the recombinant follistatin polypeptide is 100% identical to SEQ ID NO:2, SEQ NO:4 or SEQ ID NO:5.

[0009] In one aspect, the present dislosure provides recombinant follistatin polypeptides comprising an amino acid sequence at least 80% identical to SEQ ID NO:2, SEQ NO:4 or SEQ ID NO:5 and comprising any one of the amino acid variations selected from the group consisting of C66S, C66A, G74N, K75E, K75N, K76A, K76D, K76S, K76E, C77S, C77T, R78E, R78N, N80T, K81A, K81D, K82A, K82D, K81E, K82T, K82E, K84E, P85T, R86N, V88E and V88T, or combinations thereof. In some embodiments, the amino acid sequence of the recombinant follistatin polypeptide is at least 90%, identical to SEQ ID NO:2, SEQ NO:4 or SEQ ID NO:5. In some embodiments, the amino acid sequence of the recombinant follistatin polypeptide is at least 95%, identical to SEQ ID NO:2, SEQ NO:4 or SEQ ID NO:5. In some embodiments, the amino acid sequence of the recombinant follistatin polypeptide is at least 98%, identical to SEQ ID NO:2, SEQ NO:4 or SEQ ID NO:5. In some embodiments, the amino acid sequence of the recombinant follistatin polypeptide is 100% identical to SEQ ID NO:2, SEQ NO:4 or SEQ ID NO:5.

[0010] In one aspect, the present disclosure provides recombinant follistatin polypeptides comprising an amino acid sequence selected from the group consisting of SEQ NO:12, SEQ ID NO:17-30 and SEQ ID NO:32-40.

[0011] Disclosed are recombinant follistatin fusion proteins comprising a recombinant follistatin polypeptide and an IgG Fc domain. In one aspect, the present disclosure provides a recombinant follistatin fusion protein comprising a follistatin polypeptide and an IgG Fc domain.

[0012] In one aspect, the present disclosure provides recombinant follistatin fusion proteins comprising a follistatin polypeptide and a human IgG Fc domain, wherein the recombinant follistatin polypeptide comprises an amino acid sequence at least 80% identical to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:5 and wherein the amino acids corresponding to positions 66 to 88 of SEQ ID

NO:2, SEQ ID NO:4 or SEQ ID NO:5 are identical to SEQ ID NO:41, 42, 43 or 58. In some embodiments, the recombinant follistatin polypeptide comprises an amino acid sequence that is at least 90% identical to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:5. In some embodiments, the recombinant follistatin polypeptide comprises an amino acid sequence that is at least 95% identical to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:5. In some embodiments, the recombinant follistatin polypeptide comprises an amino acid sequence that is at least 98% identical to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:5. In some embodiments, the recombinant follistatin polypeptide comprises an amino acid sequence that is 100% identical to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:5.

[0013] In one aspect, the present disclosure provides recombinant follistatin fusion proteins comprising a follistatin polypeptide and an IgG Fc domain, wherein the follistatin polypeptide comprises an amino acid sequence selected from any one of the group consisting of SEQ ID NO:12, SEQ ID NO:13, and SEQ ID NO:15 to SEQ ID NO:40.

[0014] In some embodiments, the IgG Fc domain comprises an amino acid substitution wherein the amino acid substitution is selected from the group consisting of L234A, L235A, H433K, N434F, and combinations thereof, according to EU numbering.

[0015] In some embodiments, the IgG Fc domain comprises an amino acid sequence of SEQ ID NO:6 and wherein the amino acid sequence comprises an amino acid substitution selected from the group consisting of L234A, L235A, H433K, N434F, and combinations thereof, according to EU numbering.

[0016] In some embodiments, the IgG Fc domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO:7 to SEQ ID NO:11. In some embodiments, the IgG Fc domain is a human IgG Fc domain. In some embodiments, the IgG Fc domain is an IgG1, IgG2, IgG3 or IgG4 Fc domain.

[0017] Disclosed are recombinant follistatin fusion proteins comprising an amino acid sequence of any one of SEQ ID NO:73 to SEQ ID NO:100. In one aspect, the present disclosure provides a recombinant follistatin fusion protein comprising an amino acid sequence of any one of SEQ ID NO:73 to SEQ ID NO:100, SEQ ID NO: 117, or SEQ ID NO: 118.

[0018] In some embodiments, the recombinant follistatin fusion protein binds to myostatin with an affinity dissociation constant (K_D) of 1 to 100 pM. In some embodiments, the recombinant follistatin fusion protein binds activin A with an affinity dissociation constant (K_D) of 1 to 100 pM. In some embodiments, the recombinant follistatin fusion protein does not bind to bone morphogenic protein-9 (BMP-9) and/or bone morphogenic protein-10 (BMP-10) in the range of 0.2 nM to 25 nM. In some embodiments, the recombinant follistatin fusion protein binds to heparin with an affinity dissociation constant (K_D) of 0.1 to 200 nM. In some embodiments, the recombinant

follistatin fusion protein binds to the Fc receptor with an affinity dissociation constant (K_D) of 25 to 400 nM.

[0019] In some embodiments, the recombinant follistatin fusion protein inhibits myostatin at an IC_{50} of 0.1 to 10 nM. In some embodiments, the recombinant follistatin fusion protein inhibits activin at an IC_{50} of 0.1 to 10 nM.

[0020] In one aspect, the present disclosure provides pharmaceutical compositions comprising the recombinant follistatin fusion protein and a pharmaceutically acceptable carrier.

[0021] In one aspect, the present disclosure provides a polynucleotide comprising a nucleotide sequence encoding the recombinant follistatin polypeptide.

[0022] In one aspect, the present disclosure provides a polynucleotide comprising a nucleotide sequence encoding the recombinant follistatin fusion protein. In one aspect, an expression vector comprising the polynucleotide is provided. In one aspect, a host cell comprising the polynucleotide or the expression vector is provided.

[0023] Disclosed is a method of making a recombinant follistatin fusion protein that specifically binds to myostatin and activin A by culturing the host cell. In one aspect, the present disclosure provides a method of making a recombinant follistatin fusion protein that specifically binds to myostatin comprising culturing the host cell.

[0024] In one aspect, the present disclosure provides a hybridoma cell producing the recombinant follistatin polypeptide or the recombinant follistatin fusion protein.

[0025] Disclosed is a method of treating Duchenne Muscular Dystrophy (DMD), the method comprising administering to a subject who is suffering from or susceptible to DMD an effective amount of the recombinant follistatin fusion protein or a pharmaceutical composition comprising the recombinant follistatin fusion protein, such that at least one symptom or feature of DMD is reduced in intensity, severity, or frequency, or has delayed onset.

[0026] In some embodiments, the method further comprises administering to the subject one or more additional therapeutic agents. In some embodiments the one or more additional therapeutic agents are selected from the group consisting of an anti-Flt-1 antibody or fragment thereof, edasalonexent, pamrevlumab prednisone, deflazacort, RNA modulating therapeutics, exon-skipping therapeutics and gene therapy.

[0027] In some embodiments, an effective amount of the recombinant follistatin fusion protein is administered parenterally. In some embodiments, the parenteral administration is selected from the group consisting of intravenous, intradermal, intrathecal, inhalation, transdermal

(topical), intraocular, intramuscular, subcutaneous, transmucosal administration, or combinations thereof. In some embodiments, the parenteral administration is intravenous administration. In some embodiments, the parenteral administration is subcutaneous administration. In some embodiments, the recombinant follistatin fusion protein is administered daily, twice weekly, weekly, monthly or bimonthly. In some embodiments the recombinant follistatin fusion protein is administered twice weekly.

[0028] In some embodiments, the recombinant follistatin fusion protein is delivered to one or more skeletal muscles selected from Table 1. In some embodiments, the administration of the recombinant follistatin fusion protein results in an increase in the mass of a muscle relative to a control. In some embodiments, the muscle is one or more skeletal muscles selected from Table 1. In some embodiments, the muscle is selected from the group consisting of diaphragm, triceps, soleus, tibialis anterior, gastrocnemius, extensor digitorum longus, rectus abdominus, quadriceps, and combinations thereof. In some embodiments, the muscle is the gastrocnemius muscle. In some embodiments, the increase in the mass of the muscle is an increase of at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200% or 500% relative to a control.

[0029] In some embodiments, the administration of the recombinant follistatin fusion protein results in muscle regeneration, increased muscle strength, increased flexibility, increased range of motion, increased stamina, reduced fatigability, increased blood flow, improved cognition, improved pulmonary function, inflammation inhibition, reduced muscle fibrosis, and/or reduced muscle necrosis.

[0030] In some embodiments, the at least one symptom or feature of DMD is selected from the group consisting of muscle wasting, muscle weakness, muscle fragility, muscle necrosis, muscle fibrosis, joint contracture, skeletal deformation, cardiomyopathy, impaired swallowing, impaired bowel and bladder function, muscle ischemia, cognitive impairment, behavioral dysfunction, socialization impairment, scoliosis, and impaired respiratory function.

[0030a] In one aspect, the present disclosure provides use of the recombinant follistatin fusion protein or the pharmaceutical composition, in the manufacture of a medicament for treating Duchenne Muscular Dystrophy (DMD) in a subject who is suffering from or susceptible to DMD such that at least one symptom or feature of DMD is reduced in intensity, severity, or frequency, or has delayed onset.

[0031] Disclosed are methods for inhibiting myostatin in a subject, the method comprising administering to the muscle of a subject a composition comprising an effective amount of the recombinant follistatin fusion protein.

[0031a] In one aspect, the present disclosure provides use of the recombinant follistatin fusion protein in the manufacture of a medicament for inhibiting myostatin in a subject, wherein the medicament is to be administered to the muscle of the subject.

BRIEF DESCRIPTION OF THE DRAWING

[0032] The drawings are for illustration purposes only, not for limitation.

37. The recombinant follistatin fusion protein of any one of claims 18-30, wherein protein inhibits activin A at an IC_{50} of 0.1 to 10 nM.

38. A pharmaceutical composition comprising the recombinant follistatin fusion protein of any one of claims 18-37 and a pharmaceutically acceptable carrier.

39. A polynucleotide comprising a nucleotide sequence encoding the recombinant follistatin polypeptide of any one of claims 1-17.

40. A polynucleotide comprising a nucleotide sequence encoding the recombinant follistatin fusion protein of any one of claims 18-30.

41. An expression vector comprising the polynucleotide of claim 39 or 40.

42. A host cell comprising a polynucleotide of claim 39 or 40 or an expression vector of claim 30.

43. A method of making a recombinant follistatin fusion protein that specifically binds to myostatin comprising culturing the host cell of claim 42.

44. A hybridoma cell producing a recombinant follistatin polypeptide of any one of claims 1-17 or a recombinant follistatin fusion protein of any one of claims 18-30.

45. Use of the recombinant follistatin fusion protein of any one of claims 18-37 or the pharmaceutical composition of claim 38, in the manufacture of a medicament for treating Duchenne Muscular Dystrophy (DMD) in a subject who is suffering from or susceptible to

DMD such that at least one symptom or feature of DMD is reduced in intensity, severity, or frequency, or has delayed onset.

46. The use of claim 45, wherein the medicament is to be administered with one or more additional therapeutic agents.

47. The use of claim 46, wherein the one or more additional therapeutic agents are selected from the group consisting of an anti-Flt-1 antibody or fragment thereof, edasalonexent, pamrevlumab, prednisone, deflazacort, RNA modulating therapeutics, exon-skipping therapeutics and gene therapy.

48. The use of any one of claims 45-47, wherein the medicament is to be administered parenterally.

49. The use of claim 48, wherein the parenteral administration is selected from the group consisting of intravenous, intradermal, intrathecal, inhalation, transdermal (topical), intraocular, intramuscular, subcutaneous, transmucosal administration, or combinations thereof.

50. The use of claim 49, wherein the parenteral administration is intravenous administration.

51. The use of claim 49, wherein the parenteral administration is subcutaneous administration.

52. The use of any one of claims 45-51, wherein the medicament is to be administered daily, twice weekly, weekly, monthly or bimonthly.

53. The use of claim 52, wherein the medicament is to be administered twice weekly.

54. The use of any one of claims 45-53, wherein the medicament is to be delivered to one or more skeletal muscles selected from Table 1.

55. The use of any one of claims 45-54, wherein the administration of the medicament results in an increase in the mass of a muscle relative to a control.

56. The use of claim 55, wherein the muscle is one or more skeletal muscles selected from Table 1.

57. The use of claim 56, wherein the muscle is selected from the group consisting of diaphragm, triceps, soleus, tibialis anterior, gastrocnemius, extensor digitorum longus, rectus abdominus, quadriceps, and combinations thereof.

58. The use of claim 57, wherein the muscle is the gastrocnemius muscle.

59. The use of any one of claims 55-58, wherein the increase in the mass of the muscle is an increase of at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200% or 500% relative to a control.

60. The use of any one of claims 45-59, wherein the administration of the medicament results in muscle regeneration, increased muscle strength, increased flexibility, increased range of motion, increased stamina, reduced fatigability, increased blood flow, improved cognition, improved pulmonary function, inflammation inhibition, reduced muscle fibrosis, and/or reduced muscle necrosis.

61. The use of any one of claims 45-59, wherein the at least one symptom or feature of DMD is selected from the group consisting of muscle wasting, muscle weakness, muscle fragility, muscle necrosis, muscle fibrosis, joint contracture, skeletal deformation, cardiomyopathy, impaired swallowing, impaired bowel and bladder function, muscle ischemia, cognitive impairment, behavioral dysfunction, socialization impairment, scoliosis, and impaired respiratory function.

62. Use of the recombinant follistatin fusion protein of any one of claims 18-30 in the manufacture of a medicament for inhibiting myostatin in a subject, wherein the medicament is to be administered to the muscle of the subject.