CLAIMS

1. A bifunctional protein construct comprising a first binding moiety and a second binding moiety, wherein the first binding moiety specifically binds to a muscle-specific molecule, and wherein the second binding moiety specifically binds to a Notch receptor and activates the Notch receptor.

2. The bifunctional protein construct of claim 1, wherein:

(1) the muscle-specific molecule is a target antigen on the sarcolemma, between the sarcolemma and the basal lamina, or in the basal lamina, optionally wherein:

(a) the target antigen is selected from the group consisting of laminin, agrin, nidogen, perlecan, and M-cadherin (CDH15); and/or

(b) the target antigen is a component of the dystrophin-associated glycoprotein complex (DGC), optionally wherein the target antigen is selected from the group consisting of α-dystroglycan (α-DG), β-DG, laminin-211, perlecan, collagen, α-sarcoglycan, β-sarcoglycan, γ-sarcoglycan, δ-sarcoglycan, ε-sarcoglycan, ζ-sarcoglycan, biglycan, sarcospan, and matriglycan on α-DG; and/or

(c) the target antigen is laminin subunit alpha-2 (LAMA2) of laminin-211, CDH15, α-DG, or matriglycan on α-DG; and/or

(2) (a) the first binding moiety comprises an antibody moiety specifically binding to the muscle-specific molecule, optionally wherein the antibody moiety is selected from the group consisting of a full-length antibody, a Fab, a Fab', a $F(ab')_2$, an scFv, and an sdAb, further optionally wherein:

(i) the antibody moiety specifically binds to LAMA2 (anti-LAMA2 antibody moiety), optionally wherein:

a) the anti-LAMA2 antibody moiety comprises an HC-CDR1 comprising the amino acid sequence of SEQ ID NO: 1, an HC-CDR2 comprising the amino acid sequence of SEQ ID NO: 2, an HC-CDR3 comprising the amino acid sequence of SEQ ID NO: 3, an LC-CDR1 comprising the amino acid sequence of SEQ ID NO: 4, an LC-CDR2 comprising the amino acid sequence of SEQ ID NO: 5, and an LC-CDR3 comprising the amino acid sequence of SEQ ID NO: 6, optionally wherein the anti-LAMA2 antibody moiety comprises a VH

comprising the amino acid sequence of SEQ ID NO: 49, and a VL comprising the amino acid sequence of SEQ ID NO: 50; or

b) the anti-LAMA2 antibody moiety comprises an HC-CDR1 comprising the amino acid sequence of SEQ ID NO: 7, an HC-CDR2 comprising the amino acid sequence of SEQ ID NO: 8, an HC-CDR3 comprising the amino acid sequence of SEQ ID NO: 9, an LC-CDR1 comprising the amino acid sequence of SEQ ID NO: 10, an LC-CDR2 comprising the amino acid sequence of SEQ ID NO: 11, and an LC-CDR3 comprising the amino acid sequence of SEQ ID NO: 12, optionally wherein the anti-LAMA2 antibody moiety comprises a VH comprising the amino acid sequence of SEQ ID NO: 54, and a VL comprising the amino acid sequence of SEQ ID NO: 65; or

(ii) the antibody moiety specifically binds to matriglycan on α-DG (anti-matriglycan antibody moiety), optionally wherein the anti-matriglycan antibody moiety comprises an HC-CDR1 comprising the amino acid sequence of SEQ ID NO: 74, an HC-CDR2 comprising the amino acid sequence of SEQ ID NO: 75, an HC-CDR3 comprising the amino acid sequence of SEQ ID NO: 76, an LC-CDR1 comprising the amino acid sequence of SEQ ID NO: 83, an LC-CDR2 comprising the amino acid sequence of SEQ ID NO: 84, and an LC-CDR3 comprising the amino acid sequence of SEQ ID NO: 85, further optionally wherein the anti-matriglycan antibody moiety comprises a VH comprising the amino acid sequence of SEQ ID NO: 90, and a VL comprising the amino acid sequence of SEQ ID NO: 95; or

(iii) the antibody moiety specifically binds to CDH15 (anti-CDH15 antibody moiety), optionally wherein the anti-CDH15 antibody moiety comprises an HC-CDR1 comprising the amino acid sequence of SEQ ID NO: 102, an HC-CDR2 comprising the amino acid sequence of SEQ ID NO: 103, an HC-CDR3 comprising the amino acid sequence of SEQ ID NO: 104, an LC-CDR1 comprising the amino acid sequence of SEQ ID NO: 105, an LC-CDR2 comprising the amino acid sequence of SEQ ID NO: 106, and an LC-CDR3 comprising the amino acid sequence of SEQ ID NO: 107, further optionally wherein the anti-CDH15 antibody moiety comprises a VH comprising the amino acid sequence of SEQ ID NO: 108, and a VL comprising the amino acid sequence of SEQ ID NO: 109; or

(b) the first binding moiety comprises a non-antibody moiety specifically binding to the muscle-specific molecule, optionally wherein the non-antibody moiety comprises a protein domain selected from the group consisting of a laminin G-like domain (LG domain) of laminin, an LG domain of agrin, an LG domain of nidogen, an LG domain of perlecan, the laminin coiled-coil binding domain of agrin, and the laminin γ binding domain of nidogen, further optionally wherein the non-antibody moiety comprises an LG domain of laminin, further optionally wherein the non-antibody moiety comprises LG4-5 domains of LAMA2 (LAMA2 LG4-5), further optionally wherein LAMA2 LG4-5 comprises the amino acid sequence of SEQ ID NO: 118 or 119.

3. The bifunctional protein construct of any one of claims 1-2, wherein:
(1) the second binding moiety comprises an extracellular domain (ECD) or a variant thereof of a Notch ligand selected from the group consisting of Delta-like 1 (DLL1), DLL3, DLL4, Jagged1 (Jag1), and Jag2, optionally wherein the second binding moiety comprises:

(a) a DLL4 ECD or a variant thereof, wherein the DLL4 ECD comprises the amino acid sequence of SEQ ID NO: 125, and wherein the variant DLL4 ECD comprises the amino acid sequence of any of SEQ ID NOs: 126-129 and 260-267;

(b) a DLL1 ECD or a variant thereof, and wherein the DLL1 ECD comprises the amino acid sequence of SEQ ID NO: 130;

(c) a DLL3 ECD or a variant thereof, and wherein the DLL3 ECD comprises the amino acid sequence of SEQ ID NO: 131;

(d) a Jag1 ECD or a variant thereof, wherein the Jag1 ECD comprises the amino acid sequence of SEQ ID NO: 132, and wherein the variant Jag1 ECD comprises the amino acid sequence of SEQ ID NO: 133 or 134; or

(e) a Jag2 ECD or a variant thereof, and wherein the Jag2 ECD comprises the amino acid sequence of SEQ ID NO: 135; and/or

(2) (a) the first binding moiety is fused to the second binding moiety via an optional linker, optionally wherein:

(i) the second binding moiety comprises a variant DLL4 ECD comprising the amino acid sequence of any of SEQ ID NOs: 126-129 and 260-267;

(ii) the second binding moiety comprises a DLL1 ECD comprising the amino acid sequence of SEQ ID NO: 130;

(iii) the second binding moiety comprises a DLL3 comprising the amino acid sequence of SEQ ID NO: 131;

(iv) the second binding moiety comprises a variant Jag1 ECD comprising the amino acid sequence of SEQ ID NO: 133 or 134; or

(v) wherein the second binding moiety comprises a Jag2 ECD comprising the amino acid sequence of SEQ ID NO: 135,

further optionally wherein: (A) the bifunctional protein construct comprises the amino acid sequence of any of SEQ ID NOs: 136-144; or (B) the first binding moiety comprises a Fab specifically binding to a muscle-specific molecule, optionally wherein the second binding moiety is fused to the N-terminus of the VL of the Fab via an optional linker; or

(b) the bifunctional protein construct further comprises an Fc domain comprising a first subunit and a second subunit, optionally wherein:

(i) a) the Fc domain is derived from human IgG1 comprising the amino acid sequence of SEQ ID NO: 187;

b) each subunit of the Fc domain comprises an L234A/L235A mutation (EU numbering);

c) each subunit of the Fc domain comprises an H435A mutation (EU numbering);

d) each subunit of the Fc domain comprises an P329G mutation (EU numbering);

e) each subunit of the Fc domain comprises the amino acid sequence of SEQ ID NO: 188 or 277;

f) either the first subunit of the Fc domain or the second subunit of the Fc domain comprises an H435R/Y436F mutation (EU numbering); and/or

g) the Fc domain comprises knob-into-hole mutations, and wherein:

i) the first subunit of the Fc domain comprises a knob mutation, and the second subunit of the Fc domain comprises a hole mutation; or

ii) the second subunit of the Fc domain comprises a knob mutation, and the first subunit of the Fc domain comprises a hole mutation,

further optionally wherein the knob mutation is T366W (EU numbering), and the hole mutation is T366S/L368A/Y407V (EU numbering); and/or

(ii) a) the bifunctional protein construct comprises: i) a first unit of a second binding moiety specifically binding to a first Notch receptor, and ii) a second unit of a second binding moiety specifically binding to a second Notch receptor, and wherein the first unit of the second binding moiety and the second unit of the second binding moiety each independently comprises an ECD or a variant thereof of a Notch ligand selected from the group consisting of DLL1, DLL3, DLL4, Jag1, and Jag2; and/or

b) the bifunctional protein construct comprises: i) a first unit of a first binding moiety specifically binding to a first muscle-specific molecule, and ii) a second unit of a first binding moiety specifically binding to a second muscle-specific molecule; and/or

(iii) the first unit of the first binding moiety comprises a first antibody moiety specifically binding to the first muscle-specific molecule, the second unit of the first binding moiety comprises a second antibody moiety specifically binding to the second muscle-specific molecule, and wherein the first antibody moiety and the second antibody moiety are each independently selected from the group consisting of a Fab, an scFv, and an sdAb.

4. The bifunctional protein construct of claim 3(2)(b)(iii), wherein

(a) the first antibody moiety is a first Fab (Fab1), and the second antibody moiety is a second Fab (Fab2);

wherein the bifunctional protein construct comprises:

(i) a first polypeptide comprising from N' to C': a first unit of a second binding moiety specifically binding to a first Notch receptor – optional linker – the first subunit of the Fc domain – optional linker – VH1-(H1-CH1);

(ii) a second polypeptide comprising from N' to C': a second unit of a second binding moiety specifically binding to a second Notch receptor – optional linker – the second subunit of the Fc domain – optional linker – VH2-(H2-CH1);

(iii) a third polypeptide comprising from N' to C': VL1-(L1-CL); and

(iv) a fourth polypeptide comprising from N' to C': VL2-(L2-CL); and

wherein VL1-(L1-CL) and VH1-(H1-CH1) form Fab1, and VH2-(H2-CH1) and VL2-(L2-CL) form Fab2, optionally wherein Fab1 and Fab2 both specifically bind to LAMA2, and wherein anti-LAMA2 Fab1 and/or anti-LAMA2 Fab2 comprises a first

polypeptide comprising the amino acid sequence of SEQ ID NO: 165, and a second polypeptide comprising the amino acid sequence of SEQ ID NO: 166; or

(b) the first antibody moiety is a first Fab (Fab1), and the second antibody moiety is a second Fab (Fab2);

wherein the bifunctional protein construct comprises:

(i) a first polypeptide comprising from N' to C': a first unit of a second binding moiety specifically binding to a first Notch receptor – optional linker – the first subunit of the Fc domain – optional linker – VL1-(L1-CL);

(ii) a second polypeptide comprising from N' to C': a second unit of a second binding moiety specifically binding to a second Notch receptor – optional linker – the second subunit of the Fc domain – optional linker – VL2-(L2-CL);

(iii) a third polypeptide comprising from N' to C': VH1-(H1-CH1); and

(iv) a fourth polypeptide comprising from N' to C': VH2-(H2-CH1); and

wherein VL1-(L1-CL) and VH1-(H1-CH1) form Fab1, and VH2-(H2-CH1) and VL2-(L2-CL) form Fab2, optionally wherein Fab1 and Fab2 both specifically bind to LAMA2, and wherein anti-LAMA2 Fab1 and/or anti-LAMA2 Fab2 comprises a first polypeptide comprising the amino acid sequence of SEQ ID NO: 165, and a second polypeptide comprising the amino acid sequence of SEQ ID NO: 166; or

(c) the first antibody moiety is a first sdAb (sdAb1), and the second antibody moiety is a second sdAb (sdAb2);

wherein the bifunctional protein construct comprises:

(i) a first polypeptide comprising from N' to C': a first unit of a second binding moiety specifically binding to a first Notch receptor – optional linker – the first subunit of the Fc domain – optional linker – sdAb1; and

(ii) a second polypeptide comprising from N' to C': a second unit of a second binding moiety specifically binding to a second Notch receptor – optional linker – the second subunit of the Fc domain – optional linker – sdAb2;

optionally wherein:

(i) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a variant DLL4 ECD comprising the amino acid sequence of any of SEQ ID NOs: 126-129 and 260-267;

(ii) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a DLL1 ECD comprising the amino acid sequence of SEQ ID NO: 130;

(iii) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a DLL3 ECD comprising the amino acid sequence of SEQ ID NO: 131;

(iv) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a variant Jag1 ECD comprising the amino acid sequence of SEQ ID NO: 133 or 134; or

(v) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a Jag2 ECD comprising the amino acid sequence of SEQ ID NO: 135,

further optionally wherein the bifunctional protein construct comprises a first polypeptide and a second polypeptide each comprising the amino acid sequence of any of SEQ ID NOs: 223-231 and 269-276; or

(d) the first antibody moiety is a first scFv (scFv1), and the second antibody moiety is a second scFv (scFv2);

wherein the bifunctional protein construct comprises:

i) a first polypeptide comprising from N' to C': a first unit of a second binding moiety specifically binding to a first Notch receptor – optional linker – the first subunit of the Fc domain – optional linker – scFv1; and

ii) a second polypeptide comprising from N' to C': a second unit of a second binding moiety specifically binding to a second Notch receptor – optional linker – the second subunit of the Fc domain – optional linker – scFv2,

optionally wherein scFv1 and scFv2 both specifically bind to LAMA2, and wherein anti-LAMA2 scFv1 and/or anti-LAMA2 scFv2 comprises the amino acid sequence of SEQ ID NO: 145 or 268,

further optionally wherein:

(i) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a variant DLL4 ECD comprising the amino acid sequence of any of SEQ ID NOs: 126-129 and 260-267;

(ii) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a DLL1 ECD comprising the amino acid sequence of SEQ ID NO: 130;

(iii) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a DLL3 ECD comprising the amino acid sequence of SEQ ID NO: 131;

(iv) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a variant Jag1 ECD comprising the amino acid sequence of SEQ ID NO: 133 or 134; or

(v) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a Jag2 ECD comprising the amino acid sequence of SEQ ID NO: 135,

further optionally wherein the bifunctional protein construct comprises a first polypeptide and a second polypeptide each comprising the amino acid sequence of any of SEQ ID NOs: 223-231 and 269-276.

5. The bifunctional protein construct of any one of claims 3(2)(b)(ii), 3(2)(b)(iii), 4(a) and 4(b), wherein:

(a) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a variant DLL4 ECD, and wherein the variant DLL4 ECD comprises the amino acid sequence of any of SEQ ID NOs: 126-129 and 260-267;

(b) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a DLL1 ECD, and wherein the DLL1 ECD comprises the amino acid sequence of SEQ ID NO: 130;

(c) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a DLL3 ECD, and wherein the DLL3 ECD comprises the amino acid sequence of SEQ ID NO: 131;

(d) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a variant Jag1 ECD, and wherein the variant Jag1 ECD comprises the amino acid sequence of SEQ ID NO: 133 or 134; or

(e) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a Jag2 ECD, and wherein the Jag2 ECD comprises the amino acid sequence of SEQ ID NO: 135.

6. The bifunctional protein construct of claim 3(2)(b)(ii) or 3(2)(b)(iii), wherein the first antibody moiety is a first Fab (Fab1), and the second antibody moiety is a second Fab (Fab2);

wherein the bifunctional protein construct comprises:

(a) a first polypeptide comprising from N' to C': a first unit of a second binding moiety specifically binding to a first Notch receptor – optional linker – VH1-(H1-CH1) – optional linker – the first subunit of the Fc domain;

(b) a second polypeptide comprising from N' to C': a second unit of a second binding moiety specifically binding to a second Notch receptor – optional linker – VH2-(H2-CH1) – optional linker – the second subunit of the Fc domain;

(c) a third polypeptide comprising from N' to C': a third unit of a second binding moiety specifically binding to a third Notch receptor – optional linker – VL1-(L1-CL); and

(d) a fourth polypeptide comprising from N' to C': a fourth unit of a second binding moiety specifically binding to a fourth Notch receptor – optional linker – VL2-(L2-CL); and

wherein VL1-(L1-CL) and VH1-(H1-CH1) form Fab1, and VH2-(H2-CH1) and VL2-(L2-CL) form Fab2.

7. The bifunctional protein construct of claim 3(2)(b)(i) or 3(2)(b)(ii), wherein the first unit of the first binding moiety comprises a first non-antibody moiety specifically binding to the first muscle-specific molecule, the second unit of the first binding moiety comprises a second non-antibody moiety specifically binding to the second muscle-specific molecule, and wherein the first non-antibody moiety and the second non-antibody moiety are each independently selected from the group consisting of an LG domain of laminin, an LG domain of agrin, an LG domain of nidogen, and an LG domain of perlecan, optionally wherein:

(1) the first non-antibody moiety and/or the second non-antibody moiety comprises LAMA2 LG4-5 comprising the amino acid sequence of SEQ ID NO: 118 or 119; and/or

(2) the bifunctional protein construct comprises:

(a) (i) a first polypeptide comprising from N' to C': the first unit of the first binding moiety – optional linker – the first subunit of the Fc domain – optional linker – a first unit of a second binding moiety specifically binding to a first Notch receptor; and

(ii) a second polypeptide comprising from N' to C': the second unit of the first binding moiety – optional linker – the second subunit of the Fc domain – optional linker – a second unit of a second binding moiety specifically binding to a second Notch receptor; or

(b) (i) a first polypeptide comprising from N' to C': a first unit of a second binding moiety specifically binding to a first Notch receptor – optional linker

– the first subunit of the Fc domain – optional linker – the first unit of the first binding moiety; and

(ii) a second polypeptide comprising from N' to C': a second unit of a second binding moiety specifically binding to a second Notch receptor – optional linker – the second subunit of the Fc domain – optional linker – the second unit of the first binding moiety; and/or

(3) (a) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a variant DLL4 ECD, and wherein the variant DLL4 ECD comprises the amino acid sequence of any of SEQ ID NOs: 126-129 and 260-267;

(b) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a DLL1 ECD, and wherein the DLL1 ECD comprises the amino acid sequence of SEQ ID NO: 130;

(c) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a DLL3 ECD, and wherein the DLL3 ECD comprises the amino acid sequence of SEQ ID NO: 131;

(d) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a variant Jag1 ECD, and wherein the variant Jag1 ECD comprises the amino acid sequence of SEQ ID NO: 133 or 134; or

(e) the first unit of the second binding moiety and/or the second unit of the second binding moiety comprises a Jag2 ECD, and wherein the Jag2 ECD comprises the amino acid sequence of SEQ ID NO: 135.

8. The bifunctional protein construct of claim 3(2)(b), wherein:

(a) the first binding moiety is fused to the N-terminus of the first subunit of the Fc domain via an optional first linker, and the second binding moiety is fused to the N-terminus of the second subunit of the Fc domain via an optional second linker; or

(b) the first binding moiety is fused to the N-terminus of the second subunit of the Fc domain via an optional first linker, and the second binding moiety is fused to the N-terminus of the first subunit of the Fc domain via an optional second linker.

9. One or more isolated nucleic acids encoding the bifunctional protein construct of any one of claims 1-8, or one or more vectors comprising the one or more isolated nucleic acids, optionally wherein the one or more vectors is a viral vector.

10. A host cell expressing the bifunctional protein construct of any one of claims 1-8, comprising the one or more isolated nucleic acids of claim 9, or comprising the one or more vectors of claim 9.

11. A pharmaceutical composition comprising: i) the bifunctional protein construct of any one of claims 1-8, the one or more isolated nucleic acids of claim 9, or the one or more vectors of claim 9; and ii) a pharmaceutically acceptable excipient.

12. A method of producing a bifunctional protein construct, comprising:

(a) culturing a host cell comprising the one or more isolated nucleic acids of claim 9 or the one or more vectors of claim 9, or the host cell of claim 10, under a condition suitable for the expression of the bifunctional protein construct; and

(b) recovering the expressed bifunctional protein construct from the cultured host cell.

13. Use of the bifunctional protein construct of any one of claims 1-8 or the pharmaceutical composition of claim 11 in the manufacture of a medicament for treating a muscle-related disease in an individual, optionally wherein:

(a) the muscle-related disease is selected from the group consisting of Pompe disease, centronuclear myopathy, fibrodysplasia ossificans progressive (FOP), Friedreich's ataxia (FRDA), familial hypertrophic cardiomyopathy, Laing distal myopathy, myofibrillar myopathy, and muscular dystrophy, optionally wherein the muscle disease is muscular dystrophy, further optionally wherein the muscular dystrophy comprises one or more of: Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), limb-girdle muscular dystrophy (LGMD), congenital muscular dystrophy (CMD), facioscapulohumeral muscular dystrophy (FSHD), myotonic dystrophy (DM), oculopharyngeal muscular dystrophy (OPMD), distal muscular dystrophy (DD), congenital myopathy, Charcot-Marie-Tooth (CMT) disorder, and Emery-Dreifuss muscular dystrophy (EDMD); and/or

(b) the bifunctional protein construct or the pharmaceutical composition is to be administered intravenously, subcutaneously, or intramuscularly.

14. An engineered DLL4 extracellular domain (ECD), wherein

(a) the engineered DLL4 ECD comprises a mutation selected from the group consisting of T52N and T135N, and wherein the amino acid position is in reference to

a reference DLL4 ECD comprising the amino acid sequence of SEQ ID NO: 126, optionally wherein the engineered DLL4 ECD further comprises a mutation at one or more amino acid positions selected from the group consisting of G2, E14, R66, P80, F81, H168, Q220, N231, and N260, further optionally wherein the further mutation is selected from the group consisting of G2S, E14H, R66S, R66T, P80L, F81L, H168Y, Q220H, N231D, and N260D; and/or

(b) the engineered DLL4 ECD comprises a mutation selected from the group consisting of:

(i) T52N and T135N;

(ii) T52N, R66S, and T135N;

(iii) E14H, T52N, R66T, P80L, T135N, and N231D;

(iv) T52N, R66T, P80L, T135N, Q220H, and N260D;

(v) G2S, T52N, F81L, T135N, and H168Y;

(vi) G2S, T52N, F81L, R66S, T135N, and H168Y;

(vii) G2S, E14H, T52N, F81L, R66T, P80L, T135N, H168Y, and N231D; and

(viii) G2S, T52N, R66T, P80L, F81L, T135N, H168Y, Q220H, and N260D,

optionally wherein the engineered DLL4 ECD comprises an amino acid sequence selected from the group consisting of any one of SEQ ID NOs: 261-264.

15. A protein construct comprising the engineered DLL4 ECD of claim 14, optionally wherein the protein construct further comprises a binding moiety that specifically binds to a muscle-specific molecule.