Mechanism of disease and therapeutic rescue of Dok7 congenital myasthenia

Congenital myasthenia (CM) is a devastating neuromuscular disease, and mutations in DOK7, an adaptor protein that is crucial for forming and maintaining neuromuscular synapses, are a major cause of CM. The most common disease-causing mutation (DOK7<sup>3124,1127dup</sup>) truncates DOK7 and leads to the loss of two tyrosine residues that are phosphorylated and recruit CRK proteins, which are important for anchoring acetylcholine receptors at synapses. Here we describe a mouse model of this common form of CM (Dok7<sup>CM</sup> mice) and a mouse with point mutations in the two tyrosine residues (Dok7<sup>297</sup>). We show that Dok7<sup>CM</sup> mice had severe deficits in neuromuscular synapse formation that caused neonatal lethality. Unexpectedly, these deficits were due to a severe deficiency in phosphorylation and activation of muscle-specific kinase (MUSK) rather than a deficiency in DOK7 tyrosine phosphorylation. We developed agonist antibodies against MUSK and show that these antibodies restored neuromuscular synapse formation and prevented neonatal lethality and late-onset disease in Dok7<sup>CM</sup> mice. These findings identify an unexpected cause for disease and a potential therapy for both Dok7<sup>CM</sup> and other forms of CM caused by mutations in AGRIN, LRP4 or MUSK, and illustrate the potential of targeted therapy to rescue congenital lethality.

**Article**

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Congenital myasthenia is a group of diseases caused by mutations in genes that are important for the formation, function, and maintenance of neuromuscular synapses. Mostly, mutations in these genes are recessive and diminish gene activity, thereby causing synaptic deficits that lead to early onset structural and functional deficits in the neuromuscular synapse, which are responsible for muscle weakness throughout life.

The formation and maintenance of neuromuscular synapses requires the assembly of highly specialized presynaptic and postsynaptic membranes, which involves the coordinated action of several key molecules. AGRIN, which is released from motor nerve terminals, binds to the lipoprotein receptor-related protein 4 (LRP4) in muscle, stimulating the formation of a complex between LRP4 and muscle-specific kinase (MUSK), a receptor tyrosine kinase that acts as a master regulator of synaptic differentiation. LRP4, once clustered in the postsynaptic membrane as a consequence of MUSK activation, also signals directly back to motor axons to stimulate presynaptic differentiation. Mutations in AGRIN, LRP4 and MUSK, as well as in the genes that encode subunits of acetylcholine receptors (AChRs), also cause CM.

Activation of MUSK also depends on the adaptor protein DOK7. Mutations in DOK7 are responsible for 10–20% of all cases of CM. The disease is debilitating—causing weakness in limb, neck and facial muscles—and one-quarter of patients with DOK7 CM require non-invasive ventilation at some point during their lifetime. Few treatments abate the clinical symptoms. The N-terminal region of DOK7 contains pleckstrin homology (PH) and phosphotyrosine-binding (PTB) domains, which function to dimerize DOK7 and bind a phosphorylated tyrosine motif in the MUSK juxtamembrane (JM) region. A failure of DOK7 to bind MUSK leads to a failure of AGRIN to stimulate MUSK phosphorylation, demonstrating that DOK7 is essential to stabilize phosphorylation of MUSK, probably by promoting its dimerization. In addition, AGRIN-stimulated MUSK phosphorylation leads to phosphorylation of two tyrosine residues in the C-terminal region of DOK7, which triggers the recruitment of CRK and CRK-L-proteins that participate in the clustering of AChRs.

The most common cause of Dok7 CM is a four-base-pair duplication (residues 1124–1127, TGCC), which leads to a frameshift and premature termination of Dok7<sup>1127</sup>. Some individuals with Dok7 CM are homozygous for this mutant allele, whereas others carry this mutant allele in combination with a different mutant allele of Dok7. The truncated form of Dok7 retains the PH and PTB domains and binds to the tyrosine-phosphorylated JM region of MUSK, but lacks the two tyrosine residues that are phosphorylated and recruit CRK proteins, suggesting that the loss of these tyrosine residues is responsible for the synaptic deficits in this common form of Dok7 CM.

Synapse formation requires Dok7 C-terminal region

To study how loss of the C-terminal region of Dok7 leads to defects in the structure and function of neuromuscular synapses, we generated...
Fig. 1 | The C-terminal region of DOK7 is essential for synaptic differentiation and to sustain MUSK tyrosine phosphorylation.

a. Dok7<sup>CM</sup> (Dok7<sup>CM</sup>) leads to a frame-shift and premature termination, including loss of Y396 and Y406. b. Expected and observed numbers of progeny, and y<sup>2</sup> values, from intercrossing Dok7<sup>homozygous</sup> mutant mice.

c. d. Staining of AChRs (red) and axons and nerve terminals (green) in diaphragm muscles from wild-type and Dok7<sup>mutant</sup> mice. Scale bars, 10 μm.

Fig. 2 | Recruitment of CRK to the synapse and to the MUSK–DOK7 complex is impaired in Dok7<sup>CM/CM</sup> mice.

a. Staining for CRK-L (green) and ACHRs (red) in muscle sections from E18.5 mice. Scale bars, 5 μm. Representative images from three experiments. b. Top, co-immunoprecipitation of CRK with MUSK from muscles of E18.5 mice. Scale bars, 5 μm. Representative images from three experiments.

c. Amino acid sequence of the MUSK JM region showing binding site for DOK7 and potential binding site for CRK.

d. Left, affinity capture of DOK7 and CRK with phosphorylated and non-phosphorylated peptides detected by immunoblotting. The peptide sequences are shown. Right, quantification. Plots show individual data and mean ± s.e.m.; *P < 0.05, **P < 0.0005, ****P < 0.00005; two-sided Student’s t-test.

Dok7<sup>CM</sup> lowers DOK7 levels and MUSK phosphorylation

To determine how loss of the DOK7 C-terminal region caused the synaptic defects, we measured expression of Dok7 mRNA and truncated DOK7 protein in Dok7<sup>CM/CM</sup> mice using antibodies against the DOK7 PTB domain that detected the truncated and wild-type proteins equally (Extended Data Fig. 2). Dok7 mRNA levels were normal in muscle from Dok7<sup>CM/CM</sup> mice, whereas the truncated DOK7 protein was expressed at threefold lower levels than the wild-type DOK7 protein (Fig. 1e, Extended Data Fig. 3). By contrast, Dok7<sup>2YF</sup> was expressed at normal levels (Fig. 1f) and, as expected<sup>15,20</sup>, was not tyrosine phosphorylated (Extended Data Fig. 4).

Because DOK7 functions as a dimer to dimerize MUSK, thereby stabilizing MUSK tyrosine phosphorylation<sup>17</sup>, we determined whether MUSK tyrosine phosphorylation was diminished in Dok7<sup>CM/CM</sup> mice. MUSK phosphorylation was reduced sevenfold in Dok7<sup>CM/CM</sup> mice but was normal in Dok7<sup>2YF</sup> mice (Fig. 1g, h).

CRK proteins are recruited directly to MUSK

We anticipated that recruitment of CRK proteins to the synapse would be absent or severely reduced in both Dok7<sup>CM/CM</sup> and Dok7<sup>2YF</sup> mutant mice. Indeed, CRK recruitment to the synapse and to the MUSK complex was substantially diminished (2.8-fold in Dok7<sup>CM/CM</sup> mice (Fig. 2a, b), but to our surprise was only modestly reduced (by 28%) in Dok7<sup>2YF</sup> mice.

a mouse model of the most common form of Dok7<sup>CM/CM</sup> (Dok7<sup>CM</sup>) and a second mouse mutant (Dok7<sup>2YF</sup> mice), in which the two tyrosine residues in the C-terminal region are mutated to phenylalanine (Fig. 1a).

Homozygous Dok7<sup>CM/CM</sup> mice were present at the expected numbers at embryonic day 18.5 (E18.5), but were rarely found alive a day later, at birth, when neuromuscular synapses are essential for respiration and survival (Fig. 1b). We stained diaphragm muscles from E18.5 embryos with probes that allowed us to visualize presynaptic and postsynaptic differentiation and found fivefold fewer synapses in Dok7<sup>CM/CM</sup> than in wild-type mice (Fig. 1c, d). Moreover, the synapses that did form were immature, as both synaptic size and the density of synaptic AChRs were reduced fivefold (Fig. 1c, d, Extended Data Fig. 1a). By contrast, homozygous Dok7<sup>2YF</sup> mice were born at the expected frequency (Fig. 1b) and thrived as fertile adult mice. Moreover, their neuromuscular synapses appeared largely normal (Fig. 1c, d, Extended Data Fig. 1b). Thus, unexpectedly, loss of the two tyrosine residues in the C-terminal region of DOK7 is not the cause of the lethality and severe synaptic deficits in Dok7<sup>CM/CM</sup> mice.
phosphorylated after stimulation by AGRIN 12,18,22,23. We found that antibodies targeting MUSK 
recognized mouse but not human MUSK 25.

We investigated whether chronic dosing could lead to long-term survival. Repeated injections of X17 in the nine surviving Dok7CM mice at P24 and P44 led to survival of these mice for at least two months (Fig. 4a, b), at which point we assessed their motor performance and the structure of their neuromuscular synapses. X17 rescued synapse formation and maturation, as the neuromuscular synapses of these mice had developed the complex pretzel-like shape characteristic of fully mature mouse neuromuscular synapses (Fig. 4c). Moreover, X17 rescued the recruitment of CRK proteins to the neuromuscular synapse (Fig. 4d).

Antibody X17 rescued the motor function of Dok7CM mice, as assessed by grip strength and rotarod assays (Fig. 4e). Moreover, Dok7CM mice injected with X17 were fertile and produced offspring at the expected frequency. Together, these findings indicate that reduced MUSK tyrosine phosphorylation is central to disease in Dok7CM mice. Even if the C-terminal region of DOK7 has an additional role in synapse formation, this function can be overridden by stimulating MUSK.

Therapeutic reversal in adult Dok7CM mice

We next sought to determine whether X17 could reverse neuromuscular deficits that develop during adulthood, a question particularly relevant to developing a human therapy as DOK7 CM in humans would probably be treated during adult life. We treated Dok7CM mice with X17 either at P4, P24 and P44 or at P4 and P18 but then discontinued antibody treatment. Both groups of Dok7CM mice continued to maintain their weight and mobility for 2–3 months (Fig. 4f), indicating that the effects of the antibody lasted for longer than its lifetime in the blood. However, mice ultimately began to lose weight and display motor deficits (Fig. 4f, g; Supplementary Video 1). When the mice were losing weight at a rate of about 0.4 g per day, we injected X17 once again and monitored their weight and mobility. Two days after the resumption of X17 treatment, the Dok7CM mice began to regain weight (by about 0.4 g per day over the next week) (Fig. 4f). Within one week of reintroducing antibody treatment, the motor performance of the Dok7CM mice had been restored (Fig. 4g, Supplementary Video 1). The mice continued to gain weight and their motor performance continued to improve for at least one additional week after antibody treatment, when the experiment was ended (Fig. 4f, g).
Most previous studies of DOK7 have relied upon analysis of transfected muscle and non-muscle cells that overexpress DOK7. In this context, which bypasses the normal requirement for AGRIN and LRP4 to stimulate MUSK, the in vivo consequences of DOK7 mutations might have been masked by the overexpression of DOK7.

Inbred C57BL/6 mice containing the Dok7<sup>CM</sup> mutation showed more severe functional deficits than humans with the same mutation. The mutant phenotype was less severe in mice with a mixed genetic background, as outbred mice survived for up to three weeks postnatally, whereas inbred mutant mice died at birth. Modifiers in the hybrid strains may lessen disease severity, or C57BL/6 mice may contain genes that worsen the phenotype. In either case, the modestly prolonged lifespan of Dok7<sup>CM</sup> mice on the mixed background offers a mouse model that presents a longer temporal window in which to assess therapeutic approaches.

These experiments demonstrate full rescue from congenital lethality by targeted therapy. Our findings point to an unforeseen therapeutic approach, as this strategy does not directly target the mutant protein but rather targets a wild-type protein that has diminished activity caused by the mutation of an upstream gene, in this case Dok7. Epistatic rescue in this way could also provide therapy for CM caused by mutations in AGRIN, LRP4 or MUSK, in addition to DOK7, as well as for other neuromuscular diseases. Moreover, this strategy has the potential for widespread use to treat genetic disorders in humans for which the disease mechanism is understood and suitable targets have been identified.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-021-03672-3.

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**Discussion**

Stimulation of MUSK with an agonist antibody rescued synapse formation and motor function, prevented lethality and allowed Dok7<sup>CM</sup> mice to thrive postnatally as fertile adults. Moreover, the motor deficits that developed in adult Dok7<sup>CM</sup> mice after withdrawal of antibody treatment were readily reversed by reinitiating antibody treatment. Thus this therapeutic strategy, which avoids the complex requirements for gene therapy<sup>25</sup>, might be beneficial for humans with DOK7 CM or other neuromuscular diseases.
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Methods
Mice
To generate Dok7<sup>cm</sup> mice, we microinjected in vitro-transcribed single guide RNA (sgRNA; 5′ TGGCATTGCCACAGGCAG 3′) and in vitro-transcribed Cas9 RNA (10 ng/μl), together with a DNA repair template (5′ ATGCCGGCAGCAACCTGGAGAGCCCACCTGGGTGAGGA 3′; reverse: 5′ GCTTTACTTGAGTCCGCCACAGA 3′), 10 ng/μl) and the DNA repair template (5′ ATGCCGGCAATCTGGACGTCTGGCGGGCCGGTGAGGA 3′; reverse: 5′ GCGGCCTCGGCAGTTACAG 3′; reverse: 5′ GCTTTACCTTGAGTCCGCCACAGA 3′). We analysed 14 mice that were born from injected zygotes containing the TGCC duplication, into the pronuclei of C57BL/6 mouse embryos. Whole genomic DNA was isolated from tail DNA of these mice and a primer (forward: 5′ GCAGTTACAGGAGGTTGG 3′) was used to confirm sequence changes. DNA sequencing from these lines confirmed the sequence of the Dok7<sup>cm</sup> mutation. Mice were subsequently genotyped using primers (forward: 5′ GCAGTTACAGGAGGTTGG 3′; reverse: 5′ GCTTTACCTTGAGTCCGCCACAGA 3′). We analysed five genomic loci that scored the highest probability for off-target recognition (http://crispr.mit.edu). We found no evidence for mutations in these genes (Extended Data Table 2).

An earlier study described a similar mouse model, generated using classic embryonic stem cell gene targeting, for this common form of Dok7<sup>cm</sup> CM<sup>27</sup>. Although the lethality of these mutant mice could be rescued by an adenoviral-associated vector expressing wild-type Dok7, establishing a gene therapy approach to treat Dok7<sup>cm</sup> CM<sup>27</sup> this study did not examine the cause of disease in the Dok7<sup>cm</sup> CM<sup>27</sup> mouse model.

To generate Dok7<sup>cm</sup> mice, we injected an sgRNA (5′ TTCGAGGTGTGGCTCTGCTCAGTCTGCCTGCCCCCTGGAGCCAGCGCACCTGAGcccagactgtgtgcctgccaccacctggggcgcccgagtagt 3′, 10 ng/μl) together with the DNA repair template (5′ ATGCCGGCAATCTGGACGTCTGGCGGGCCGGTGAGGA 3′; reverse: 5′ GCTTTACTTGAGTCCGCCACAGA 3′) and in vitro-transcribed Cas9 RNA (30 ng/μl), together with the DNA repair template (5′ ATGCCGGCAATCTGGACGTCTGGCGGGCCGGTGAGGA 3′; reverse: 5′ GCTTTACTTGAGTCCGCCACAGA 3′) and in vitro-transcribed Cas9 RNA (30 ng/μl), together with the DNA repair template (5′ ATGCCGGCAATCTGGACGTCTGGCGGGCCGGTGAGGA 3′; reverse: 5′ GCTTTACTTGAGTCCGCCACAGA 3′) and in vitro-transcribed Cas9 RNA (30 ng/μl), together with the DNA repair template (5′ ATGCCGGCAATCTGGACGTCTGGCGGGCCGGTGAGGA 3′; reverse: 5′ GCTTTACTTGAGTCCGCCACAGA 3′) and in vitro-transcribed Cas9 RNA (30 ng/μl), together with the DNA repair template (5′ ATGCCGGCAATCTGGACGTCTGGCGGGCCGGTGAGGA 3′; reverse: 5′ GCTTTACTTGAGTCCGCCACAGA 3′) and in vitro-transcribed Cas9 RNA (30 ng/μl), together with the DNA repair template (5′ ATGCCGGCAATCTGGACGTCTGGCGGGCCGGTGAGGA 3′; reverse: 5′ GCTTTACTTGAGTCCGCCACAGA 3′). Together, these experiments generated a mouse model.

Growth of cultured cells
C2C12 mouse muscle cells, purchased from and authenticated by ATCC (ATCC Cat CRL-1772), were grown at 37 °C in growth medium (GM): Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/l glucose, 1 mM glutamine and sodium pyruvate (Corning cellgro), supplemented with 10% fetal bovine serum (FBS; GemCell). Myoblast fusion and myotube formation were induced by adding 1% horse serum. Immortalized myoblasts were isolated from wild-type and DOK7<sup>−/−</sup> heterozygote and DOK7<sup>−/−</sup> homozygote C2C12 mouse muscle cells, purchased from and authenticated by ATCC (ATCC Cat CRL-1573), were grown at 37 °C in the same medium as described above for C2C12 myoblasts and transfected using Lipofectamine 3000 Transfection Reagent Kit (Thermofisher Scientific). All cell lines tested negative for mycoplasma contamination using the e-MycO Plus PCR detection kit.

Antibody treatment of C2 myotubes
Three days after C2C12 myotubes had formed, the cultures were treated for 30 min with 10 nM biotinylated Fabs in complex with 2.5 nM streptavidin, 10 nM IgGs, or 0.5 nM recombinant neural AGRIN-B8 (R&D Systems). Myotubes were homogenized at 4 °C in lysis buffer (50 mM sodium chloride, 30 mM triethanolamine, pH 7.5, 50 mM sodium fluoride, 5 mM EDTA, 5 mM EGTA, 2 mM sodium orthovanadate, 1 mM N-ethylmaleimide, 1 mM sodium tetrathionate, 10 mM pepstatin, plus complete protease inhibitor mix (Roche)). NP-40 was added to a final concentration of 1%, and the extract was incubated with rocking for 30 min at 4 °C. Insoluble proteins were removed by centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatant was pre-cleared for 1 h at 4 °C with Protein G-agarose beads (Sigma-Aldrich) before incubation overnight at 4 °C with antibodies against MUSK (MUSK 1A) or goat anti-DOK7 (R&D Systems, AF562), followed by incubation for 4 h with Protein G-agarose beads. The beads were subsequently washed (three times for 9 min) in lysis buffer containing 1% NP-40. Proteins were eluted from the beads with 1% SDS in lysis buffer.

Isolation of MUSK and DOK7 from muscle
Whole leg muscles or cultured muscle cells were homogenized at 4 °C in lysis buffer (50 mM sodium chloride, 30 mM triethanolamine pH 7.5, 50 mM sodium fluoride, 5 mM EDTA, 5 mM EGTA, 2 mM sodium orthovanadate, 1 mM N-ethylmaleimide, 1 mM sodium tetrathionate, 10 μM pepstatin, plus complete protease inhibitor mix (Roche)). NP-40 was added to a final concentration of 1%, and the extract was incubated with rocking for 30 min at 4 °C. Insoluble proteins were removed by centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatant was pre-cleared for 1 h at 4 °C with Protein G-agarose beads (Sigma-Aldrich) before overnight incubation at 4 °C with antibodies against MUSK (MUSK 1A) or goat anti-DOK7 (R&D Systems, AF 6398), followed by incubation for 4 h with Protein G-agarose beads. The beads were subsequently washed (three times for 9 min) in lysis buffer containing 1% NP-40. Proteins were eluted from the beads with 1% SDS in lysis buffer.

Western blotting
Proteins were fractionated by SDS–PAGE and transferred to PVDF membranes. Blots were probed with antibodies against MUSK (R&D Systems, AF562), phosphotyrosine (Millipore, 05-3221) or DOK7 (BD Bioscience, 530831) and chemiluminescence was detected using the ECL western blotting detection kit (Amersham). Blots were probed with antibodies against CRK (BD Bioscience, 610035) and CRK-L (Santa Cruz Biotechnology, sc-636092) and quantified using FIJI/ImageJ software, as described previously<sup>28,29</sup>. Antibodies against CRK (BD Bioscience, 610035) and CRK-L (Santa Cruz Biotechnology, sc-636092) were quantified using BandQuant software (2014) as previously<sup>28,29</sup>. The graphs show the mean values from at least three separate experiments. A two-sided Student’s t-test was used to determine statistical significance and was conducted using GraphPad Prism 9.0 software.

Whole-mount muscle immunohistochemistry
Diaphragm muscles were dissected from E18.5 embryos and postnatally in oxygenated L-15 medium. The muscles were pinned onto Sylgard-coated dissection dishes, fixed for 1.5 h at 1% PFA and blocked for 1 h in PBS with 3% BSA (Sigma IgG free) and 0.5% Triton X-100 (PBST). Diaphragm muscles were stained with Alexa 488-conjugated anti-BGT (Invitrogen) to label AChRs and with antibodies against neurofilament-L (Neuromics, NF-L) or with antibodies against synapsin 1/2 (Synaptic Systems, 106002). The antibodies were force-pipetted into the muscles, and the muscles were incubated overnight at 4 °C with Protein G-agarose beads (Sigma-Aldrich) before overnight incubation at 4 °C with antibodies against MUSK (MUSK 1A) or goat anti-DOK7 (R&D Systems, AF 6398), followed by incubation for 4 h with Protein G-agarose beads. The beads were subsequently washed (three times for 9 min) in lysis buffer containing 1% NP-40. Proteins were eluted from the beads with 1% SDS in lysis buffer.
**Staining single muscle fibres**

Tibialis anterior muscles were dissected in oxygenated L-15 medium, pinned to a Sylgard-coated dish and fixed in 2% PFA (in PBS) for 2 h. After several rinses in PBS, one to three myofibres were manually teased with fine forceps. Fixed myofibres were blocked for 2 h at room temperature in PBS containing 5% BSA, 1% normal goat serum, and 0.04% saponin. Fibres were then incubated with primary antibodies overnight at 4 °C, washed three times for 5 min with PBS containing 0.04% saponin, incubated with secondary antibodies for 2 h at room temperature, washed again, and mounted in Vectashield (Vector Laboratories). Antibodies against CRK-L (Santa Cruz Biotechnology, sc-365092) were used and the postsynaptic membrane was visualized by staining with Alexa Fluor 488–anti-BGT (Invitrogen).

**Cryosection immunohistochemistry**

Limb muscles were embedded in optimal cutting temperature (OCT) medium and frozen on a dry-ice platform. Ten-micrometre sections, collected onto poly-L-lysine-coated glass slides, were fixed in 1–4% PFA for 10 min, washed in PBS with 3% BSA (PB) three times for 5 min, permeabilized with PB + 0.5% X-Triton (PB/T) for 10 min, washed in PB and incubated overnight at 4 °C with primary antibodies against CRK-L (Santa Cruz Biotechnology, sc-365092) in PB/T in a humidified chamber. Sections were washed in PB three times for 5 min before overnight incubation at 4 °C with secondary antibodies and Alexa Fluor 488–anti-BGT (Invitrogen), diluted in PBS, in a humidified chamber. Sections were washed three times for 5 min in PB, then PBS, before mounting in Vectashield anti-fade mounting medium.

**Behaviour**

All-limb grip strength was measured using a grip-strength apparatus (Bioseb). Mice were allowed to grip the grid with both forelimbs and hindlimbs, and the mouse was pulled back steadily, until the mouse lost grip on the grid. The grip strength meter digitally displayed the maximum force applied (in grams) as the grasp was released. The mean measurement from six consecutive trials was taken as an index of all-limb grip strength. Mice were given an interval of 10–15 s between trials. Body weight was determined after all grip-strength measurements to analyse for potential co-variability. To enhance the robustness of all-limb grip strength, mice were given an interval of 10–15 s between trials. Body weight was determined after all grip-strength measurements to analyse for potential co-variability. To enhance the robustness of all-limb grip strength, mice were given an interval of 10–15 s between trials.

**Development of synthetic antibodies**

The full-length extracellular region (E22 to T494 of mouse MUSK and E22 to T495 of human MUSK), including the Fz domain and the C-terminal flanking sequence (D307 to T494 of mouse MUSK and K314 to T495 of human MUSK) were expressed as a C-terminal fusion with the Avi tag, using a secretion signal sequence of mouse IgkVIII in EXP293 cells with the Expf ectamite 293 Transfection kit (Thermo Fisher Scientific) using standard procedures provided by the vendor. The proteins were purified from the filtered culture supernatant using a HiTrap Nickel column (GE Healthcare) and biotinylated in vitro using the biA enzyme in the presence of 0.5 mM biotin and 10 mM ATP. The biotinylated proteins were further purified using a Superdex S75 10/300 column (GE Healthcare).

Sorting of an antibody phage-display library was performed as described previously34. In brief, a phage-display library was first sorted with all four antigens at 100 nM in the first round, followed by sorting with a single antigen at 100, 50 and 20 nM in the second, third and fourth rounds, respectively. To enrich for clones that bind to both human and mouse Fz domains, we used multiple sorting strategies in which alternate antigens were used in successive rounds (for example, human Fz; mouse ECD; human ECD). Individual clones were screened using phage enzyme-linked immunosorbent assay (ELISA) with the four antigens34, and the DNA sequences of clones bound to all of the antigens were determined.

The Fab proteins with the Avi tag at the C-terminus of the heavy chain of selected clones were produced from Escherichia coli and biotinylated as described previously34. The mouse IgG2a-LALAPG sample of clone X17 was produced using a modified version of the pFUSE-mlgG2a-Fc vector (InvivoGen) containing the LALAPG mutations in the Fc region and human CH1 domain and the pFUSE-CLig vector (InvivoGen). This chimeric antibody consisted of a human Fab and mouse Fc sequences. In addition, we exchanged the mouse Fc sequences with those from human IgG1, containing LALAPG mutations, to generate hlgG1-X17, hlgG1-X2 and hlgG1-X3 antibodies.

**Affinity measurements**

The affinities of antibody clones in the Fab and IgG formats were measured using a bead-binding assay35–37. A biotinylated human antigen protein was immobilized on Dynabeads M280 streptavidin beads (Thermo Fisher Scientific) by rapidly mixing 100 μl of tenfold diluted beads in PBSS (PBS containing 0.5% bovine serum albumin (BSA, GeminBiO)) and 100 μl of 50 nM protein. The beads were then blocked with 2 μM biotin, washed twice with PBS and resuspended in 1 ml PBSB. This reaction was appropriately scaled for the number of measurements when necessary. Five microlitres of the diluted beads and 20 μl of an antibody sample were mixed in a well of a 96-well polypropylene plate (Greiner Bio-One, catalogue number 650261) and incubated at room temperature for 30 min with gentle shaking. Samples were transferred to the wells of a 96-well filter plate (Millipore MultiScreen HTS HV, 0.45 mm, Thermo Fisher); the liquid was removed using a vacuum manifold and the wells were washed three times with 200 μl ice-cold PBSB using the vacuum manifold. The beads were stained with anti-human Fab antibody labelled with Alexa Fluor 647 (Jackson Immuno Research, Alexa Fluor 647 AffiniPure Goat Anti-Human IgG, F(ab’), fragment specific, 109-605-097). Following washing, the beads were suspended in 70 μl PBSB and analysed using an iQue screener (Sartorius) or an Intellicyt HTFC system. The resulting titration curves were analysed by nonlinear least-squares fitting of a 1:1 binding model using the GraphPad Prism software.

**Half-life of antibody in blood**

Mice were injected intraperitoneally with antibodies. Mouse blood samples were centrifuged, and supernatants were diluted 2,000-fold in PBSS. Antibody levels were measured using the bead assay described above except that the binding reaction was performed at 4 °C. The half-life was determined by nonlinear least squares fitting of the median fluorescence intensities with a single exponential curve.

**Phosphopeptide pull-down assay**

HEK 293 cells were transfected with plasmids encoding HA-tagged DOK7 and HA-tagged CRKL at 37 °C for 48 h (Lipofectamine 3000, Thermofisher Scientific). After 48 h, the transfected cells were homogenized at 4 °C in lysis buffer; NP-40 was added to a final concentration of 1%, and the extract was incubated with rocking for 30 min at 4 °C. Insoluble proteins were removed by centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatants were precleared for 1 h at 4 °C with streptavidin-agarose beads (Sigma-Aldrich).

Four biotinylated phosphopeptides ((1) ELLLDRLHPNPMp(Y)QRMPLLLN, (2) ELLLDRLHPNPMp(Y)RMPLLLN, (3) ELLLDRLHPAPMp(Y)QRMLPLLNN, and (4) ELLLDRLHPAPMp(Y)AAAPLLLN) (Thermofisher Scientific) were immobilized on streptavidin-agarose beads and
incubated overnight at 4 °C in lysis buffer (50 mM sodium chloride, 30 mM triethanolamine, pH 7.5, 50 mM sodium fluoride, 5 mM EDTA, 5 mM EGTA, 2 mM sodium orthovanadate, 1 mM β-mercaptoethanol, 1 mM sodium tetrathionate, and 10 μM pepstatin, plus complete protease inhibitor mix (Roche)), containing 1% NP-40. The cell extracts, pre-cleared on streptavidin-agarose beads, were incubated overnight at 4 °C with biotinylated phosphopeptides immobilized on streptavidin-agarose beads. The beads were subsequently washed (three times for 9 min) in lysis buffer containing 1% NP-40. Proteins were eluted from the beads with 1% SDS in lysis buffer. Western blotting was performed using antibodies against HA tag (Abcam, ab49969).

Quantitative PCR with reverse transcription (RT–qPCR)
Total RNA was isolated from muscles of E18.5 wild-type and Dok7\textsuperscript{f/f} embryos using TRIZOL reagent (Invitrogen) and reverse transcribed with Superscript-III First strand kit (Invitrogen). Real-time qPCR was performed on a LightCycler 480 (Roche) using SYBR Green Master kit (Roche). PCRs were performed using primer pairs: 5′-CTGGTGAAAAGGACCTCTCGAAG-3′ and 5′-ATTTCTCATCTAAAGCAGAAG-3′ for Hprt, 5′-TCAGCC TCAGAAGGCGTGTTG-3′ and 5′-GCCTACCCAGAAGGAACTGGATG-3′ for Dok7. Samples were run in triplicate and Dok7 expression level was normalized to Hprt expression.

Statistics and reproducibility
No statistical method was used to predetermine sample size. No data were excluded from the analyses. The experiments were not randomized. The investigators were not blinded to the genotype of the mice with the exception of the motor performance experiments.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability
Raw data generated from this study are available upon a reasonable request. Source data are provided with this paper.

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29. Smith, C. L., Mittaud, P., Prescott, E. D., Fuhrer, C. & Burden, S. J. Src, Fyn, and Yes are not required for neuromuscular synapse formation but are necessary for stabilization of agrin-induced clusters of acetylcholine receptors. J. Neurosci. 21, 3151-3160 (2001).
Extended Data Fig. 1 | Characterization of neuromuscular synapses in Dok7 mutant mice. a, b, Left, diaphragm muscles from E18.5 (a) and P135 (b) wild-type and Dok7 mutant mice were stained with Alexa 488–anti-BGT to label AChRs (red) and antibodies against neurofilament and synapsin to label motor axons and nerve terminals (green). Scale bars, 50 μm (a), 10 μm (b). a, Right, endplate width, denervation and co-localization of synapses in wild-type, Dok7CM/CM and Dok72YF/2YF mice. The width of the endplate band (dashed lines) was increased by 45% in Dok7CM/CM mice but was normal in Dok72YF/2YF mice. In Dok7CM/CM mice, 17% of AChR clusters were completely unopposed by nerve terminals, indicating denervated myofibres. Many synapses in Dok7CM/CM mice were partially innervated, as nearly half of the AChR-rich area at synapses was not juxtaposed by nerve terminals. b, Right, in Dok72YF/2YF mice, synapses mature from a plaque-like to a complex, pretzel-like shape, characteristic of mature mouse neuromuscular synapses. Synapses in Dok72YF/2YF mice, however, often appeared elongated. The number of synapses and the density of synaptic AChRs were similar in wild-type and Dok72YF/2YF mice. Synaptic size was increased by 20% in Dok72YF/2YF mice when compared with wild-type mice. Data shown as mean ± s.e.m. from 3 mice (>50 synapses per mouse). n.s., not significant; *P < 0.05; ****P < 0.00005; two-sided Student’s t-test.
Extended Data Fig. 2 | Wild-type and truncated DOK7 are detected with similar efficiency by antibodies against the PH and PTB domains in DOK7.

a, HEK 293 cells were transiently transfected with a plasmid expressing either HA-tagged DOK7 or HA-tagged truncated DOK7 encoded by Dok7 1124_1127 TGCC dup. Proteins in cell lysates (triplicates) were separated by SDS–PAGE, and western blots were probed with either a rabbit antibody against the PTB domain in DOK7 or a monoclonal antibody against HA (left). We measured the grey levels of the bands for wild-type and truncated DOK7 proteins and normalized the level detected by western blotting with the rabbit antibody against DOK7 with the level detected by western blotting with the antibody against HA. The ratio for wild-type DOK7 was equivalent to the ratio for truncated DOK7 (left), indicating that the rabbit antibody against DOK7 detected wild-type and truncated DOK7 proteins with similar efficiency by western blotting.

b, Wild-type and truncated DOK7 were immunoprecipitated with similar efficiency by a goat antibody against the PTB domain in DOK7. HEK 293 cells were transiently co-transfected with plasmids expressing HA-tagged DOK7 and HA-tagged truncated DOK7 encoded by Dok7 1124_1127 TGCC dup. DOK7 proteins were immunoprecipitated from cell lysates (triplicates) with either a monoclonal antibody against HA or a goat antibody against the PTB domain in DOK7, and western blots were probed with the monoclonal antibody against HA (left). We measured the grey levels, subtracted the level for the background band in the control, non-transfected samples, and normalized the value for each protein immunoprecipitated with the goat antibody against DOK7 to the value for the same protein immunoprecipitated with the antibody against HA. This ratio was equivalent for wild-type and truncated DOK7 proteins, indicating that the goat antibody against DOK7 immunoprecipitated wild-type and truncated proteins with similar efficiency (right). Plots show individual values from three and four experiments for a and b, respectively, and the mean ± s.e.m. (n.s., not significant); two-sided Student’s t-test.
Extended Data Fig. 3 | Dok7 RNA expression is normal in Dok7<sup>CM/CM</sup> mice.

**a**, RT–PCR amplification of Dok7 RNA shows that Dok7 mRNA levels are similar in muscle from E18.5 wild-type and Dok7<sup>CM/CM</sup> mice. GAPDH was used as a loading control. **b**, Dok7 mRNA levels were quantified by qPCR, which showed that Dok7 mRNA levels are normal in Dok7<sup>CM/CM</sup> mice (n = 3 mice). **c**, DOK7 was immunoprecipitated from muscles of E18.5 wild-type and Dok7<sup>CM/CM</sup> mice, and the blots were probed with antibodies against DOK7 (left). Truncated DOK7, encoded by Dok7<sup>CM/CM</sup>, migrates at the predicted size, but is expressed at threefold lower levels than wild-type DOK7 (right; n = 10 mice). Data shown as individual data points and mean ± s.e.m.; n.s., not significant; ****P < 0.00005; two-sided Student’s t-test.
Extended Data Fig. 4 | Y396 and Y406 are the main, if not sole, tyrosine residues in DOK7 that are phosphorylated by AGRIN stimulation. We generated muscle cell lines from wild-type and Dok7^{2YF/2YF} mice and treated the cultured myotubes with AGRIN for 30 min. MUSK was immunoprecipitated, and western blots were probed with antibodies against MUSK or phosphotyrosine (pTyr). AGRIN stimulates DOK7 tyrosine phosphorylation in wild-type but not Dok7^{2YF/2YF} myotubes (data from two experiments).
Extended Data Fig. 5 | MUSK antibody clones. a, Amino acid sequences of the complementarity-determining regions (CDRs) of antibodies against MUSK developed in this study. CDR definitions are based on Wu and Kabat, except that CDR-H1 includes four additional residues at the N terminus to show diversified positions. b, Amino acid sequences of the VL and VH domains of clone X17. c, d, Binding titration of antibodies against MUSK in the Fab format to immobilized hFz, hECD, mFz and mECD, as tested using a bead-based binding assay. Curves show the best fit of the 1:1 binding model. The table lists apparent $K_d$ values (mean ± s.d., $n=3$). The datasets in c and d were obtained from different instruments, which resulted in different signal ranges. e, Binding titration of antibodies against MUSK in the IgG format, as in c.
Extended Data Fig. 6 | Chronic injection of MUSK agonist antibody X17 in wild-type mice has no effect on the organization of neuromuscular synapses, weight gain or motor behaviour. a, Blood half-life measurements of X17-mIgG2a-LALAPG. Nonlinear least-squares fitting of the median fluorescence intensities with a single exponential curve for three mice is shown. The half-life was determined to be 4.9 ± 0.2 days. b, Wild-type mice on a C57BL/6-CBA mixed background, injected at P4, P24 and P44 with X17 (n = 4), survived until P60, when the experiment was ended. The scatter plot shows the survival time for nine non-injected wild-type mice and four wild-type mice injected with X17 with mean ± s.e.m. (n.s., not significant). c, Wild-type mice injected with X17 (n = 4) gained weight like uninjected wild-type mice (n = 9). d, Left, diaphragm muscles from P60 wild-type mice and wild-type mice injected with X17 were stained with Alexa 488–anti-BGT to label AChRs (red) and antibodies against neurofilament and synapsin to label motor axons and nerve terminals (green). In wild-type mice treated with X17, synapses matured from a simple, plaque-like shape to a complex, pretzel-like shape, characteristic of mature mouse neuromuscular synapses. Scale bar, 10 μm. Right, injection of X17 in wild-type mice had no effect on synapse number, size or AChR density. We analysed more than 50 synapses per diaphragm muscle from two mice in each category. e, Motor performance of wild-type mice injected with X17, as assessed by grip strength and the latency to fall from a rotating rotarod, was similar to that of non-injected wild-type mice. The scatter plots show the values for 18 wild-type mice and 4 wild-type mice injected with X17 and the mean ± s.e.m; two-sided Student’s t-test.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | The C-terminal region of DOK7 is essential for complete differentiation and maturation of the neuromuscular synapse in Dok7<sup>CM/CM</sup> mice on a mixed genetic background.

a–c, Left, diaphragm muscles from wild-type and Dok7<sup>CM/CM</sup> mice on a C57BL/6·CBA mixed background at E18.5 and P10 were stained with Alexa 488·anti-BGT to label AChRs (red) and antibodies against neurofilament and synapsin to label motor axons and nerve terminals (green). Scale bars, 50 μm (a), 10 μm (b, c).

a, Right, at E18.5, the endplate band (dashed white lines on left) is 30% wider in Dok7<sup>CM/CM</sup> than wild-type mice. Moreover, nerve terminals were absent from 15% of AChR clusters and the colocalization index (synapsin/AChR) was reduced 3.5-fold in Dok7<sup>CM/CM</sup> mice. n = 3 mice.

b, Right, the number of synapses, synaptic size and density of synaptic AChRs were reduced 3.2-, 4.5- and 8-fold, respectively, in E18.5 Dok7<sup>CM/CM</sup> mice. n = 3 mice (>50 synapses per mouse).

c, Right, at P10, the number of synapses, synaptic size and density of synaptic AChRs were reduced over tenfold in Dok7<sup>CM/CM</sup> mice. In addition, nerve terminals were absent from 20% of the AChR clusters in Dok7<sup>CM/CM</sup> mice. n = 3 mice (>50 synapses per mouse).

d, DOK7 was immunoprecipitated from muscles of E18.5 wild-type and Dok7<sup>CM/CM</sup> mice, and the blots were probed with antibodies against DOK7 (left). Truncated DOK7, encoded by Dok7<sup>CM/CM</sup>, migrated at the predicted size, but was expressed at threefold lower levels than wild-type DOK7 (right). Because DOK7 expression and MUSK phosphorylation were diminished to the same extent in the C57BL/6·CBA mixed breed and C57BL/6 inbred mice, other factors presumably led to increased survival in the mixed genetic background. n = 8 mice per genotype.

e, MUSK was immunoprecipitated from muscles of E18.5 wild-type and Dok7<sup>CM/CM</sup> mice, and the blots were probed with antibodies against MUSK, phosphotyrosine, and CRK (left). The levels of phosphotyrosine and CRK that co-isolated with the MUSK complex were normalized to MUSK expression (right). CRK association with the MUSK complex was 2.8-fold lower in Dok7<sup>CM/CM</sup> mice than wild-type mice. MUSK tyrosine phosphorylation was fivefold lower in Dok7<sup>CM/CM</sup> mice than wild-type mice. n = 3 mice per genotype. Scatter plots show individual data points and mean ± s.e.m.; *P < 0.05, **P < 0.005, ***P < 0.0005, ****P < 0.00005; two-sided Student’s t-test.
Extended Data Fig. 8 | Antibodies X2 and X3, like X17, rescue Dok\textsuperscript{CM/CM} mice from early lethality. 

**a**, Dok\textsuperscript{CM/CM} mice on a C57BL/6-CBA mixed background were injected at P4 with 10 mg kg\textsuperscript{-1} mlG2a-X3. At this dose, X3 failed to rescue the mice from lethality. 

**b**, By contrast, dosing with 20 mg kg\textsuperscript{-1} mlG2a-X3 at P4 rescued the mice from early lethality. These mice were subsequently injected with 10 mg kg\textsuperscript{-1} mlG2a-X3 at P18, which led to survival until P60, when the experiment was ended. 

**c**, Injecting Dok\textsuperscript{CM/CM} mice with 20 mg kg\textsuperscript{-1} hlG1-X2 at P4 likewise rescued Dok\textsuperscript{CM/CM} mice from early lethality; subsequent injection of 10 mg kg\textsuperscript{-1} hlG1-X2 at P18 led to survival of Dok\textsuperscript{CM/CM} mice until P60, when the experiment was ended.
Extended Data Table 1 | Dok7<sup>CM/CM</sup> mice on a mixed genetic background survive for approximately two weeks postnatally

We used a mixed genetic background of mice to analyse the survival of Dok7<sup>CM/CM</sup> mice. Dok7<sup>CM/+</sup> mice on a C57BL/6 background were crossed to wild-type CBA, 129sv1, FVB, or BALB/c mice. Heterozygous F1 progeny were then intercrossed to produce Dok7<sup>CM/CM</sup> mice on a mixed background. We determined the genotype of the progeny at P5–P10 or post-mortem. a, χ² analysis of F2 mice shows that the occurrence of genotypes is unlikely to occur by chance, indicating that homozygous Dok7<sup>CM/CM</sup> mice in each mixed genetic background survive postnatally. b, The table shows the average and maximum survival time (days) of homozygous Dok7<sup>CM/CM</sup> mice on a mixed genetic background. Despite surviving for up to three weeks after birth, DOK7 expression, MUSK phosphorylation and the organization of nerve terminals and AChRs were similar in E18.5 inbred C57BL/6 and mixed breed C57BL/6-CBA mice carrying the same Dok7 mutation.

| Strain     | Maximum survival | Average survival | SEM  | N  |
|------------|------------------|------------------|------|----|
| C57BL/6    | 5                | 0                | 0    | 1  |
| C57BL/6-CBA| 21               | 11.2             | 1.43 | 14 |
| C57BL/6-FVB| 17               | 11.6             | 1.31 | 7  |
| C57BL/6-129sv1| 17              | 15.2             | 1.32 | 5  |
| C57BL/6-BalbC| 15              | 12.3             | 1.45 | 3  |
Extended Data Table 2 | Sequence analysis of potential off-target sites failed to identify mutations in these genes

The top-ranked potential off-target genes in Dok7<sup>CM</sup> and Dok7<sup>2YF</sup> mice are indicated.

### Potential off-targets in Dok7<sup>CM</sup> mice

| Off-target | Sequence | Score | Mismatches | Sequence ID | Locus     |
|------------|----------|-------|------------|-------------|-----------|
| 1          | GCCCTGCACAGTCTGCCCTCTGG | 2.6   | 3MMs [1:3-8] | AL645994.7  | Chr11:+9049672 |
| 2          | GCACTGCAAGTCTGCCCTCTGG | 2.6   | 3MMs [1:3-8] | AC129606.4  | Chr8:+97387576 |
| 3          | TTTATGCTGCTGTCGGCCCTCAAG | 2.5   | 3MMs [2-4:10] | AC103939.9  | Chr18:+28855866 |
| 4          | GCCTGCTCAGTCTGCCCTCTGG | 2.3   | 3MMs [1:2-3] | AC154126.2  | Chr7:+36061330 |
| 5          | TCAGTCCTCAGTCTGCCCTCTGG | 1.4   | 3MMs [3-4:6]  | CT033750.18 | Chr17:-15692194 |

### Potential off-targets in Dok7<sup>2YF</sup> mice

| Off-target | Sequence | Score | Mismatches | Sequence ID | Locus     |
|------------|----------|-------|------------|-------------|-----------|
| 1          | GAATTCTAGGTGTCTAGCAGG | 1.7   | 3MMs [2-3:7] | AC113509.14 | Chr1:+183488722 |
| 2          | GGATTCTAGGTGTCTAGGCGG | 0.7   | 3MMs [3-7:19] | AC120877.15 | Chr6:+16848270 |
| 3          | GGTTCAATTTGTCTAGCAGG | 0.5   | 4MMs [3-7:4:10] | AC129777.4  | Chr8:+117950828 |
| 4          | AGCTGCTAGGTATGCTAGTGG | 0.5   | 4MMs [1-5:7:12] | AL596263.8  | Chr2:+161115283 |
| 5          | CACCTGGAGGGGCTAGAGG | 0.5   | 4MMs [1-2:6:11] | AL772268.6  | Chr11:-55192181 |
Reporting Summary

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- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection: ChemiDoc imaging system (Biorad), Zeiss ZEN Software (Zeiss Blue), Grip Strength apparatus (Bioseb), Rotarod (AccuRorot four-channel), Omnitech Electronics, Inc), LightCycler 480 (Roche), iQue screener (Sartorius). All softwares are from third party developers.

Data analysis: ImageJ v1.52P, Graphpad Prism 9.0, iQue screener (Sartorius), FlowJo V9.9.6. (BD), LightCycler 480 (Roche). All softwares are from third party developers.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

- **Sample size**: No statistical method was used to predetermine sample size. The initial sample size for any assay was estimated from past experiments, allowing us to select a sample size that had low variance among the group and ensuring reproducibility.
- **Data exclusions**: No data were excluded in the study.
- **Replication**: All experiments were performed as triplicate at minimum unless otherwise stated. All attempts at replication were successful.
- **Randomization**: The experiments were not randomized.
- **Blinding**: The investigators were not blinded to the genotype of the mice except for the motor performance experiments. Animals were allocated to groups according to their genotype.

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We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒ | Antibodies |
| ☒ | Eukaryotic cell lines |
| ☒ | Palaeontology and archaeology |
| ☒ | Animals and other organisms |
| ☒ | Human research participants |
| ☒ | Clinical data |
| ☒ | Dual use research of concern |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒ | ChIP-seq |
| ☒ | Flow cytometry |
| ☒ | MRI-based neuroimaging |

### Antibodies

**Antibodies used**

- Human anti-MuSK (MuSK 1A) (gift from Dr. Kevin O’Connor). Immunoprecipitation 2ug/mg of protein lysate
- Goat anti-Dok7 (R&D Systems, AF 6398). Immunoprecipitation 2ug/mg of protein lysate
- Goat anti-MuSK (R&D Systems, AF562). Western Blots 1/200
- Mouse anti-Phosphotyrosine (Millipore, 05-321). Western Blots 1/1000
- Rabbit anti-Dok7 (Homemade, #1916). Western Blots 1/400
- Mouse anti-Crk (BD Bioscience, 610035). Western Blots 1/1000
- Mouse anti-Crk-L (Santa Cruz Biotechnology, sc-365092). Immunofluorescence 1/100
- Rabbit anti-Neurofilament (Synaptic Systems, 302302). Immunofluorescence 1/3000
- Rabbit anti-Synapsin 1/2 (Synaptic Systems, 160002). Immunofluorescence 1/200
- Mouse anti-HA tag (ab49969, Abcam). Western Blots 1/5000
- Bungarotoxin, Alexa-488 conjugate (Thermofisher Scientific, B13422). Immunofluorescence 1/1000
- Alexa Fluor 647 AffiniPure Goat anti-Human IgG, Fab fragment specific (Jackson ImmunoResearch, 109-605-097). Immunofluorescence 1/200

**Validation**

Human anti-MuSK is an antibody for the detection of MuSK protein. Validation statement and citation is provided with Takata K, et al. Characterization of pathogenic monoclonal autoantibodies derived from muscle-specific kinase myasthenia gravis patients. ICI Insight. 2019;4(12):e127167. Published 2019 Jun 20. doi:10.1172/jci.insight.127167.

Goat anti-Dok7 (R&D Systems, AF 6398) is a polyclonal antibody for the detection of human, mouse, and rat Dok7 in immunoprecipitation and Western Blots. Citations are provided with 1) Sarcoglycan Alpha Mitigates Neuromuscular Junction Decline in Aged Mice by Stabilizing LRP4 (Authors: K Zhao, C Shen, L Li, H Wu, G Xing, Z Dong, H Jing, W Chen, H Zhang, Z Tan, J Pan, L Xiong, H...
Goat anti-MuSK (R&D Systems, AF562) is a polyclonal antibody for the detection of mouse and rat MuSK in ELISAs and western blots. Citations are provided with 1) Characterization of pathogenic monoclonal autoantibodies derived from muscle-specific kinase myasthenia gravis patients (Authors: K Takata, P Stathopoulou, M Cao, M Mané-Damas, ML Fichtner, ES Benotti, L Jacobson, P Waters, SR Irani, P Martinez-M, D Beeson, M Mosen, A Vincent, RJ Nowak, KC O’Connor JCI Insight, 2019;4(12)).

Mouse anti-HA tag (ab49969, Abcam) is a monoclonal antibody for the detection of the HA sequence in ELISAs, immunofluorescence, and western blot. Citations are provided with 1) Differential a2A- and a2C-adenosine receptor protein expression in presynaptic and postsynaptic density fractions of postmortem human prefrontal cortex (Authors: Erdos-Lam AM, Brosco-Mosquera I, Gabilondo AM, Meana JJ, Callado LF. Journal of psychopharmacology (Oxford, England) 2018; 32(6): 657-667), 2) Reduction in presynaptic a3 adrenoceptors during NMDA receptor activation (Authors: Badu-Nkansah KA et al. 2020 Mol. Biol. Cell 31(11):1140-1153).

Mouse anti-Phosphoryrosyne (Millipore, 04-321) is a monoclonal antibody clone 4G10 that detects tyrosine phosphorylated proteins in all species. This antibody is validated for use in immunocytochemistry, immunohistochemistry, immunoprecipitation, and western blot. Citations are provided with 1) ATP synthase promotes germ cell differentiation independent of oxidative phosphorylation. (Authors: Teixeira, FK; Sanchez, CG, Hurd, TR; Seiffert, JR; Czech, B; Preall, JB; Hannon, GJ; Lehmann, R. Nature cell biology 2017 689-96 2015), 2) Hyperosmotic stress activates the expression of members of the mR15/107 family and induces downregulation of anti-apoptotic genes in rat liver (Authors: Santos, D; Castoldi, M; Paluschinski, M; Sommerfeld, A; Häussinger, D. Scientific reports 2015 5:12292 2015), 3) The tyrosine phosphatase SHP-1 regulates hypoxia inducible factor-1α (HIF-1α) protein levels in endothelial cells under hypoxia (Authors: Alig, SK; Stampnik, Y; Pincher, J; Rotter, R; Gaitzsch, E; Ribeiro, A; Wörnle, M; Krötz, F; Mannelli, H. PloS one 2015 10: e0121113 2015), 4) Phospho-tyrosine dependent protein-protein interaction network (Authors: Grossmann, A; Benlaser, N; Birth, P; Hegele, A; Wachsmuth, F; Apert, L; Stelzl, U. Molecular systems biology 2015 11: 794-9 2015), 5) HSP90 inhibitor AUY922 induces cell death by disruption of the Bcr-Abl, Jak2 and HSP90 signaling network complex in leukemia cells. (Authors: Tao, W; Chakraborty, SN; Leng, X; Ma, H; Arlinghaus, RB. Genes & cancer 2015 6: 19-29 2015).

Rabbit anti-Dok7 (Homemade, #1916) is a polyclonal antibody that detects the PTB domain of Mouse Dok7 in western blots.

Mouse anti-Crk (BD Bioscience, 610035) is a polyclonal antibody for the detection of mouse Crk in western blots. Citations are provided with 1) Purification of pseudopodia from polarized cells reveals redistribution and activation of Rac through assembly of a CAS/Crk scaffold. (Authors: Cho SY, Klemke RL. J Cell Biol. 2002; 156(4):725-736), 2) Cbl-transforming variants trigger a cascade of molecular alterations that lead to epithelial mesenchymal conversion. (Authors: Fourrier TM, Lamorte L, Maroun CR, et al. Mol Biol Cell. 2000; 11(10):3397-3410), 3) A direct interaction between JNK1 and CrkII is critical for Rac1-induced JNK activation. (Authors: Cho SY, Klemke RL. J Cell Biol. 2002; 156(4):725-736), 4) CAS/Crk scaffold. (Authors: Cho SY, Klemke RL. J Cell Biol. 2002; 156(4):725-736), 5) Cbl-transforming variants trigger a cascade of molecular alterations that lead to epithelial mesenchymal conversion. (Authors: Fourrier TM, Lamorte L, Maroun CR, et al. Mol Biol Cell. 2000; 11(10):3397-3410).

Mouse anti-Crk (Santa Cruz Biotechnology, sc-365092) is a monoclonal antibody for the detection of Crk-L of mouse, rat and human origin by western blot, immunoprecipitation, immunofluorescence, and ELISA. Citations are provided with 1) Crk Adaptor Proteins Regulate NK Cell Expansion and Differentiation during Mouse Cytomegalovirus Infection (Authors: Nabekura, T. et al. 2018. J. Immunol. 200: 3420-3428), 2) KSHV-TK is a virus kinase that disrupts focal adhesions and induces Rho-mediated cell contraction (Authors: Gill, MB. et al. 2015. The EMBO journal. 34: 448-65), 3) A Novel Micropeptide Encoded by Y-Linked LINC00278 Links Cigarette Smoking and AR Signaling in Male ESophageal Squamous Cell Carcinoma (Authors: Wu S. et al. 2020 Cancer Res. 80(13): 3790-3803), 4) Proteomic analysis of desmosomes reveals novel components required for epidermal integrity (Authors: Badu-Nkansah KA et al. 2020 Mol. Biol. Cell 31(11):1140-1153).

Rabbit anti-Neurofilament L (Synaptic Systems, 171002) is a polyclonal antibody for the detection of human NF-L in western blots, immunoprecipitation, immunofluorescence, and ELISA. Citations are provided with 1) Impaired Neurofilament Integrity and Neuronal Morphology in Different Models of Focal Cerebral Ischemia and Human Stroke Tissue. (Authors: Magee B, Aleithe S, Altmann S, Bleitz A, Nitzsche B, Barthel H, Horn AKE, Hobusch C, Härtig W, Krueger M, Michalski D, et al. Frontiers in cellular neuroscience (2018) 12: 161), 2) Conditional deletion of L1CAM in human neurons impairs both axonal and dendritic arborization and action potential generation. (Authors: Patzke C, Acuna C, Giam LR, Wernig M, Sudhof TC. The Journal of experimental medicine (2016) 2134: 499-515), 3) Loss of CrkL expression results in impaired AMPAR-mediated plasticity and socio-cognitive deficits in mice. (Authors: Prieto M, Folci A, Poupon G, Schiavi S, Buzzelli V, Pronot M, François U, Pousina P, Lattuada N, Abelanet S, Castagnola S, et al. Nature communications (2021) 121: 1557), 4) The Actin Nucleator Cobl Is Critical for Centriolar Positioning, Postnatal Planar Cell Polarity Refinement, and Function of the Coelchea. (Authors: Haag N, Schüler S, Nitzsche B, Barthel H, Horn AKE, Hobusch C, Härtig W, Krueger M, Michalski D, et al).
Deubiquitinases Maintain Protein Homeostasis and Survival of Cancer Cells upon Glutathione Depletion (Authors: Harris IS et al. Cell Metab 29:1166-1181.e6 (2019)).

Bungarotoxin, Alexa-488 conjugate (Thermofisher Scientific, B13422). Fluorescent α-bungarotoxin conjugates can be used to facilitate identification of nicotinic AChRs and to localize neuromuscular junctions. Citations are provided with 1) A comparative assessment of lengthening followed by end-to-end repair and isograft repair of chronically injured peripheral nerves (Authors: Howarth HM, Orozco E, Lovering RM, Shah SB. Exp Neurol 2020; (331): 113328-113328), 2) A- and B-utrophin have different expression patterns and are differentially up-regulated in mdx muscle (Authors: Weir AP, Burton EA, Harrod G, Davies KE. J Biol Chem (2002) 277:45285-45290), 3) Acetylcholinesterase dynamics at the neuromuscular junction of live animals (Authors: Krejci E, Martinez-Pena y Valenzuela I, Ameziane R, Akaaboune M. J Biol Chem (2006) 281:10347-10354).

Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | C2C12 (ATCC Cat# CRL-1772), HEK-293 (ATCC Cat# CRL-1573), Immortalized myoblasts, methods described in Journal of Neuroscience 1 May 2001, 21 (9) 3151-3160; DOI: 10.1523/JNEUROSCI.21-09-03151.2001 |
|---------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Authentication      | C2C12, and HEK-293 cell lines were directly purchased from and authenticated by ATCC. Immortalized myoblasts were generated and authenticated in the Burden lab (see reference for methods, Journal of Neuroscience 1 May 2001, 21 (9) 3151-3160; DOI: 10.1523/JNEUROSCI.21-09-03151.2001) |
| Mycoplasma contamination | All cell lines were tested for mycoplasma contamination using the e-Myco Plus PCR detection kit, and cultured in the presence of antimycotic/antibiotic unless otherwise stated. |
| Commonly misidentified lines (See ICLAC register) | No misidentified cell lines were used. |

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Mouse, strains: C57BL/6, CBA, FVB, 129sv1, Balb/C males and females from Birth to 5 months old |
| Wild animals       | The study did not involve wild animals. |
| Field-collected samples | The study did not involve field-collected samples. |
| Ethics oversight   | The study was approved by IACUC of NYU Langone under the protocol number of IA16-00080. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.