Reducing dynamin 2 (DNM2) rescues DNM2-related dominant centronuclear myopathy

Suzie Buonoa,b,c,d, Jacob A. Rossf, Hichem Tasfaouta,b,c,d, Yotam Levyf, Christine Kretzb,c,d, Leigha Tayefehg, John Matsonb, Shuling Guo, Pascal Kellersa,b,c,d, Brett P. Monia, Marc Bitouni,j,d, Julien Ochala, Jocelyn Laporteb,c,d,1, and Belinda S. Cowlinge,b,c,d

a Institut de Génétique et de Biologie Moléculaire et Cellulaire, 67404 Illkirch, France; b Institut National de la Santé et de la Recherche Médicale, U1258, 67404 Illkirch, France; c Centre National de la Recherche Scientifique, UMR7104, Illkirch, 67404, France; d Strasbourg University, 67404 Illkirch, France; e Dynacure, 67400 Illkirch, France; f Centre of Human and Applied Physiological Sciences, School of Basic and Medical Biosciences, Faculty of Life Sciences and Medicine, King’s College London, 55, 1UL London, United Kingdom; g Ionis Pharmaceuticals Inc., Carlsbad, CA 92010; h UMRS 974, Sorbonne Université, F-75013 Paris, France; i INSERM UMR 974, Institute of Myology, F-75013 Paris, France; and j Centre of Research in Myology, Institute of Myology, F-75013 Paris, France

Centronuclear myopathies (CNM) are a group of severe muscle diseases for which no effective therapy is currently available. We have previously shown that reduction of the large GTPase DNM2 in a mouse model of the X-linked form, due to loss of myotubularin (MTM1), prevents the development of the skeletal muscle pathophysiology. As DNM2 is mutated in autosomal dominant forms, here we tested whether DNM2 reduction can rescue DNM2-related CNM in a knock-in mouse harboring the p. R465W mutation (Dnm2R465W+) and displaying a mild CNM phenotype similar to patients with the same mutation. A single intramuscular injection of adeno-associated virus-shRNA targeting Dnm2 resulted in reduction in protein levels 5 wk post injection, with a corresponding improvement in muscle mass and fiber size distribution, as well as an improvement in histopathological CNM features. To establish a systemic treatment, weekly i.p. injections of antisense oligonucleotides targeting Dnm2 were administered to Dnm2R465W+ mice for 5 wk. While muscle mass, histopathology, and muscle ultrastructure were perturbed in Dnm2R465W+ mice compared with wild-type mice, these features were indistinguishable from wild-type mice after reducing DNM2. Therefore, DNM2 knockdown via two different strategies can efficiently correct the myopathy due to DNM2 mutations, and it provides a common therapeutic strategy for several forms of centronuclear myopathy. Furthermore, we provide an example of treating a dominant disease by targeting both alleles, suggesting that this strategy may be applied to other dominant diseases.

Significance

Centronuclear myopathies are rare and severe congenital muscle diseases. Here we hypothesized that reducing dynamin 2 (DNM2) may rescue the pathophysiology observed in DNM2-related dominant centronuclear myopathy. The total DNM2 expression was reduced in a faithful murine model (Dnm2R465W+ mice) using two different methods targeting both mutated and wild-type Dnm2, adeno-associated virus-shRNA, or antisense oligonucleotides, leading to a restoration of muscle mass, histopathology, and muscle ultrastructural features to wild-type levels. This provides a therapeutic strategy for treating this disease. We also propose that targeting both alleles in dominant muscle diseases to a mutation in only one of the alleles can successfully rescue the phenotypes.
was developed using antisense oligonucleotides (ASO) that target the nuclear pre-mRNA of Dnm2. Repeated injections of ASO targeting Dnm2 into Mtm1−/− mice efficiently reduced DNM2 and improved lifespan while reducing disease pathology (23). Most recently, using adenovirus-associated virus to express shRNA targeting Dnm2 in Mtm1−/− mice, we demonstrated a robust DNM2 knockdown and disease rescue, providing an alternative strategy to reduce DNM2 (24). Therefore, targeting DNM2 was shown to be a valid potential therapy for MTM1-CNM using different approaches.

We hypothesized that reducing DNM2 may also rescue the pathophysiology observed in DNM2-related CNM, as observed in the models of MTM1- and BIN1-CNM. In this study, we reduced total DNM2 expression in Dnm2R465W mice through several methodologies and analyzed the impact on CNM pathology and DNM2 function. We highlight here that targeting the total pool of a gene in a nonallele-specific manner efficiently rescued an autosomal dominant myopathy due to gain-of-function mutations.

Results

Reducing DNM2 by Intramuscular Adeno-Associated Virus-shRNA Delivery in Dnm2R465W Mice Improves Muscle Mass. Heterozygous R465W knock-in mice (Dnm2R465W/+ ) are viable with a normal lifespan and body weight and progressively develop a myopathic phenotype similar to patients (16), whereas the heterozygous knockout Dnm2−/− mice are viable and do not present with any obvious clinical phenotypes (22). To decrease DNM2 expression, we first crossed Dnm2+/− mice with Dnm2R465W/+ mice to produce Dnm2R465W−/− mice expressing only the mutated allele. A strong reduction in the proportion of pups with this genotype was noted (SI Appendix, Table S1; 3% in lieu of expected 25%), with only a few Dnm2R465W−/− mice surviving more than a week and dying before weaning at 3 wk. This indicates that selective loss of the wild-type Dnm2 allele does not rescue the myopathy in Dnm2R465W−/− mice and thus that a wild-type DNM2 protein is necessary for embryonic and perinatal survival.

We then aimed to reduce the total pool of DNM2 after birth. Adeno-associated virus (AAV) vectors have previously been used effectively to repress gene expression by encoding for shRNA complementary to the target mRNA (25). We previously screened several Dnm2 shRNA sequences that provide robust targeting of Dnm2 mRNA for degradation in vitro and in vivo (24). The best shRNA-Dnm2 sequence (AAV-sh) as well as a scrambled control sequence (AAV-C) were selected and evaluated here for their potential to knock down Dnm2 mRNA in the Dnm2R465W−/− mice. Intramuscular injections of AAV-sh or AAV-C were administered into the tibialis anterior (TA) muscles of 3-wk-old wild-type or Dnm2R465W−/− mice (1.2 × 10^5 viral genome per TA) (SI Appendix, Table S2). Mice were killed 5 wk post injection, and DNM2 protein expression was quantified from TA muscles. Of note, Dnm2R465W−/− muscle expresses Dnm2 mRNA and protein at the same level as wild-type littersmates (Fig. 1 A–C), suggesting that the mutation does not alter protein stability. In wild-type and Dnm2R465W−/− mice treated with AAV-sh, RT-qPCR detected a significant reduction of Dnm2 mRNA expression relative to AAV-C–treated animals (Fig. 1D). A corresponding 30–40% reduction in DNM2 protein expression was observed by Western blot (Fig. 1B) and densitometry (Fig. 1C). Muscle transduction was confirmed by GFP expression from the same AAV constructs (Fig. 1B).

To determine if reducing DNM2 has an impact on the phenotypes, muscle mass and force were analyzed. While wild-type muscles appeared similar in size regardless of the AAV construct injected (Fig. 1D, Left, and Fig. 1E), muscle mass was clearly reduced in Dnm2R465W−/− muscles (Fig. 1D, AAV-C, Right, and Fig. 1E), in accordance with previously published data (16). Analysis of the TA muscle mass/body-weight ratio showed that the reduction of TA muscle mass observed in Dnm2R465W−/− mice was rescued to a wild-type level by reducing DNM2 expression (Fig. 1 D and E). Absolute TA muscle force was significantly reduced in Dnm2R465W−/− mice (Fig. 1F). A trend toward improvement was observed when mice were treated with AAV-sh, although this was not statistically significant. No difference in specific muscle force (absolute force relative to muscle mass) or in time to fatigue was observed (Fig. 1G and SI Appendix, Table S3). These results indicate that shRNA can efficiently reduce Dnm2 mRNA and protein expression in vivo in Dnm2R465W−/− mice and show that reducing DNM2 after a single intramuscular injection of AAV-sh targeting Dnm2 efficiently improves muscle mass in this model of dominant CNM.

Reducing DNM2 by AAV-shRNA in Dnm2R465W Mice Corrects Histological Defects. To determine if the functional improvement in Dnm2R465W−/− muscle force was linked to an amelioration of muscle histology, transversal TA sections were stained with hematoxylin and eosin (H&E) and succinate dehydrogenase (SDH) (Fig. 2A, Upper and Lower, respectively). A significant reduction in fiber size was observed in Dnm2R465W−/− muscles compared with wild type (Fig. 2 A and B), with a reduction in the size heterogeneity observed in TA fibers (2 C and D). Fiber size and distribution
were restored to wild-type levels by treatment with AAV-sh targeting Dnm2. Unlike patients, no mislocalization of nuclei was observed in Dnm2RW+ mice (Fig. 2E), as described previously (16), and the absence of myofiber degeneration and centralized nuclei confirms that AAV treatment was not toxic to the muscles. The most striking histological abnormality previously observed in these mice was the abnormal central accumulation of SDH (labeling mainly mitochondria oxidative activity) within fibers. Here we confirm that observation, with ~5% of fibers with abnormal SDH staining in Dnm2RW+ muscles injected with AAV-C (Fig. 24, arrows; SI Appendix, Table S4). This was dramatically improved in Dnm2RW+ mice with reduced DNM2, with almost all fibers displaying SDH staining akin to wild-type muscle. Therefore, reducing DNM2 by AAV-shRNA intramuscular injection in Dnm2RW+ mice ameliorates fiber size and distribution of oxidative activity to wild-type levels. Taken together with the improvement in muscle mass and function, reducing DNM2 by intramuscular injections of AAV-shRNA was able to fully rescue

Fig. 2. Reducing DNM2 by AAV-shRNA in Dnm2RW+ mice corrects histological features of disease. (A) Transverse muscle sections from wild-type and DNM2 R465W knock-in (Dnm2RW+) mice, treated with AAV-shRNA targeting Dnm2 (AAV-sh) or AAV-scrambled control (AAV-C), stained with H&E or SDH. Arrows indicate abnormal central accumulation of oxidative staining. (Scale bar: 50 μm.) (B) Fiber size average and (C and D) fiber size distribution were quantified from H&E images in A. (E) Fibers with internal or centralized nuclei (“abnormal nuclei position”) were quantified from H&E images in A. n = 7–8 mice per group. (B–E) More than 500 fibers per mouse were analyzed. Graphs represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 3. Reducing DNM2 by systemic ASO injection in Dnm2RW+ mice improves muscle mass. (A) Dnm2 mRNA expression quantified by RT-qPCR analysis, relative to Hprt expression, in wild-type and DNM2 R465W knock-in (Dnm2RW+) mice, treated with ASO-1–targeting Dnm2 (ASO-1) or ASO-control (ASO-C). (B) Immunoblot for protein expression of DNM2 and GAPDH (protein loading control). (C) DNM2 protein expression quantified relative to GAPDH loading control. (D) TA muscle mass from wild-type and Dnm2RW+ mice, treated with ASO-1 or ASO-C, represented as a ratio to body weight. (E) Absolute maximum force (Po) and (F) specific maximum force (sPo) (relative to TA muscle weight). Each point represents one mouse; n ≥ 5 per group. Graphs represent mean ± SEM. *P < 0.05, ***P < 0.001.
Reducing DNM2 by systemic ASO injection in Dnm2<sup>RW/+</sup> mice corrects the CNM phenotype in TA muscles from the Dnm2<sup>RW/+</sup> mouse model.

**Systemic Reduction of DNM2 by ASO Improves Muscle Mass in Dnm2<sup>RW/+</sup> Mice.** As shRNA targeting Dnm2 was able to locally rescue the CNM phenotype, we next tested a second approach to reduce DNMT2, to confirm its therapeutic potential as a therapeutic target for DNM2-related CNM and validate a systemic treatment. ASO-mediated knockdown represents another promising therapeutic approach for neuromuscular diseases. We recently found that i.p. injections of ASO targeting Dnm2 in Mtm1<sup>-/-</sup> mice result in improved lifespan, muscle force, and histology (23). Here we applied a similar approach to Dnm2<sup>RW/+</sup> mice. To assess if systemic ASO delivery could rescue the CNM phenotype, weekly injections of 25 mg/kg of ASO targeting Dnm2 (ASO-1) were administered to wild-type or Dnm2<sup>RW/+</sup> mice from 3 to 7 wk of age by i.p. injections, and these were compared with littermate mice treated with 25 mg/kg of ASO control (not targeting any known mouse genes, ASO-C) (SI Appendix, Table S2). Of note, we targeted a different sequence and exon compared with the shRNA approach as a way to control for potential off-target effects. We observed a similar concentration of ASO uptake in wild-type and Dnm2<sup>RW/+</sup> mouse muscle tissue, indicating efficient delivery to the target tissue (SI Appendix, Table S5). The genotypes did not alter the ASO uptake. Notably, and similarly to the AAV-shRNA intramuscular approach, ASO systemic administration resulted in an ~50% reduction in Dnm2 RNA and protein expression in muscle (Fig. 3 A–C). A significant improvement in TA muscle mass relative to body weight, and a nonsignificant improvement in absolute muscle force (P = 0.051), was observed in ASO-1–treated Dnm2<sup>RW/+</sup> compared with mice treated with ASO-C (Fig. 3 D and E). Importantly, these values were indistinguishable from wild-type mice treated with ASO-1. Again, no significant difference in specific maximal force or relaxation time was observed between groups (Fig. 3F and SI Appendix, Table S3). Therefore, ASO administration can efficiently reduce Dnm2 mRNA and protein expression in vivo in Dnm2<sup>RW/+</sup> mice, and this correlated with normalization of muscle mass.

**Systemic ASO Treatment in Dnm2<sup>RW/+</sup> Mice Corrects Fiber Size and Histological Abnormalities.** To determine if an amelioration in muscle histopathology was also observed by systemic ASO-1 administration, transversal TA sections were stained with H&E and SDH (Fig. 4A, Upper and Lower, respectively). A reduced mean fiber size was observed in Dnm2<sup>RW/+</sup> muscles compared with wild-type and was not corrected by administration of ASO-1 targeting Dnm2 (Fig. 4 A and B). However, the fiber size distribution was clearly restored to the wild-type distribution by administration of ASO-1 targeting Dnm2 (Fig. 4 C and D). This is explained by the fact that the Dnm2<sup>RW/+</sup> muscles have an abnormal enrichment of middle-sized fibers while the wild-type and ASO-1–treated Dnm2<sup>RW/+</sup> muscles display the typical heterogeneous fiber distribution with small, medium, and large fibers; indeed, the absence of large fibers in the Dnm2<sup>RW/+</sup> muscle was strongly rescued by lowering DNM2 (Fig. 4 C and D). Nuclei position was unaffected in ASO-1 treated mice (Fig. 4E). The abnormal accumulation of SDH centrally within Dnm2<sup>RW/+</sup> fibers was confirmed, and this was completely rescued in Dnm2<sup>RW/+</sup> mice with reduced Dnm2 (Fig. 4A, Lower, and SI Appendix, Table S4). Therefore, reducing DNM2 by systemic ASO injection rescues the physiological and histological defects due to the Dnm2 mutation to wild-type levels.

**Ultrastructure Morphology of Dnm2<sup>RW/+</sup> Muscles After DNM2 Reduction by ASO.** We next analyzed TA muscles at the ultrastructural level by transmission electron microscopy. Longitudinal images of Dnm2<sup>RW/+</sup> muscles presented overall well-organized fibers, with a notable reduction in myofibril width (Fig. 5, asterisks; SI Appendix, Fig. S14), as observed previously (16). Measurement of sarcomere length and myofibril width confirmed a tendency for the Dnm2<sup>RW/+</sup> muscles to have thinner myofibrils (SI Appendix, Fig. S1 B and

---

**Fig. 4.** Reducing DNM2 by systemic ASO injection in Dnm2<sup>RW/+</sup> mice corrects histological features of disease. (A) Transverse muscle sections from wild-type and DNM2<sup>RW/+</sup> Knock-in (Dnm2<sup>RW/+</sup>) mice, treated with ASO-1 targeting Dnm2 (ASO-1) or ASO-control (ASO-C), stained with H&E or SDH. Arrows indicate abnormal central accumulation of oxidative staining. (Scale bar: 50 μm.) (B) Fiber size average and (C and D) fiber size distribution were quantified from H&E images in A. (E) Fibers with internal or centralized nuclei (“abnormal nuclei position”) were quantified from H&E images in A. n = 5–6 mice per group. (B–E) More than 500 fibers per mouse were analyzed. Graphs represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
mice, treated with ASO (ASO-1) or ASO-control (ASO-C), were isolated, skinned, and stained for actin (phalloidin, red) and nuclei (DAPI, blue) (Fig. 6A). Individual fiber size and force were analyzed. No difference in specific force produced from isolated fibers relative to the cross-sectional area was observed (Table S6). Images are representative of two mice per genotype. Asterisks (*) indicate abnormally small myofibrils that were not observed in Dnm2RW/+ after ASO-1 administration.

**Discussion**

In this study, we demonstrate that DNM2 knockdown, through either AAV intramuscular or ASO systemic delivery, can correct the CNM phenotype observed in Dnm2RW/+ mice by restoring muscle mass and structure. These results provide insights on the impact of DNM2 mutations in CNM and support a therapeutic application.

Dominant mutations in the DNM2 gene lead to centronuclear myopathy in patients and in the mouse model (4, 16). Approximately 30% of ADCNM patients with DNM2 mutations present with the single-point p.R465W mutation in exon 11 (c.1393 A > T, p.R465W). While DNM2 expression is not increased in Dnm2RW/+ mice (Fig. 1), in vitro experiments showed that several DNM2-CNM mutations, including p.R465W, increase oligomerization and GTPase activity (12, 13). However, based on these in vitro data, it was unclear if these alterations lead to a gain-of-function or to a final impaired activity of the protein. In vivo work demonstrated that increased expression of wild-type DNM2 in mice promotes a CNM-like phenotype with muscle defects and centralization of nuclei, supporting that DNM2-CM mutations are, at least in part, gain-of-function (14, 15). Based on this rationale, we targeted the overall DNM2 expression by two different approaches: shRNA and ASO-mediated knockdown. Importantly, we achieved knockdown of DNM2 to ~50% at the RNA and protein levels by targeting both the mutated and wild-type allele, leading to a rescue in the CNM phenotype. These results support the hypothesis of a gain-of-function mechanism in the p.R465W DNM2-related CNM, as overall reduction of DNM2 is sufficient to rescue CNM features in mice. However, as knockout of wild-type Dnm2 in Dnm2RW/+ mice is not viable (SI Appendix, Table S1), this suggests that the pathomechanisms may be more complex and may vary for different DNM2 mutations or that some wild-type DNM2 protein is needed for embryogenesis. We cannot also exclude an additive mechanism between a gain-of-function and a partial dominant-negative effect of CNM mutations. The role of DNM2 in muscle and the pathological mechanism of CNM mutations are not fully understood. Here, we provide data suggesting that DNM2 is important for fiber hypertrophy, most probably during postnatal development, as we noted a strong reduction in large fibers in the Dnm2RW/+ muscles, associated with a decrease in absolute force, when analyzing the mice at the end of the muscle postnatal development (8 wk). This is confirmed by the fact that decreasing DNM2 in a time window from 3 to 8 wk, corresponding to the period of postnatal muscle hypertrophy, rescued these phenotypes. However, the finding that specific force of isolated fibers and entire muscles is normal in Dnm2RW/+ mice suggests that the DNM2 mutation impairs myofiber hypertrophy but not muscle contraction, correlating with the rather mild myopathy associated with this mutation in patients, which could then be due mainly to fiber atrophy rather than contractile defect.

While complete loss of Dnm2 is embryonically lethal, Dnm2 heterozygous knockout mice do not present any obvious clinical phenotype (22, 26), supporting reducing total levels of DNM2 as a therapeutic strategy without deleterious impact. Recently, allele-specific knockdown targeting the p.R465W point mutation...
has shown therapeutic potential in the Dnm2<sup>RW/+</sup> model (27). As only one allele is targeted in this approach, it may reduce potential effects of a massive inhibition of the DNM2 pool; however, it would require sufficient muscle targeting, and whether the allele specificity can be maintained in a clinical setting is unknown. Here we propose two approaches, adeno-associated virus or antisense oligonucleotides, to achieve reduction of the total DNM2 pool. DNM2 reduction by ASO allows injection and dosing to be adapted during the treatment. As ASOs from the same platform have now been accepted for use clinically for familial hypercholesterolemia [e.g., mipomersen (Kynamro)] and for spinal muscular atrophy [Nursinseren (Spinraza)], this approach has clear advantages for clinical development. Alternatively, AAV provides good muscle tropism and long-term expression following a single injection. Several challenges remain, as enough vector or ASO must be produced and delivered to skeletal muscles without on-target toxicity or off-target effects, with the long-term benefit outweighing potential risks.

Interestingly, DNM2 protein expression is increased in X-linked CNM biopsies from patient muscles and in muscles from the Mmtn<sup>−/−</sup> mouse model, and reducing Dnm2 also rescued disease features in this model (22, 23). Albeit a clear link to altered DNM2 expression in patients with BIN1 mutations has not yet been established, down-regulation of DNM2 level to 50% through a genetic cross in the Bin<sup>−/−</sup> mouse also rescued the lifespan and muscle symptoms (20). Based on these different results and the present study, we hypothesize that the increase in DNM2 expression or function may be a key factor leading to the CNM phenotype in muscle in all these CNM forms, independent of the mutated genes. Our data validate the importance of DNM2 expression in CNM, and we propose that the form of centronuclear myopathy due to mutations in DNM2 may be treated by targeting the total pool of DNM2. DNM2 reduction is a strategy that may have therapeutic potential for several forms of centronuclear myopathies. Targeting both the wild-type and mutated allele without distinction, as was successfully done here, represents a different paradigm from the allele-specific therapies that were preferred to date in dominant diseases and may be tested in other diseases.


Materials and Methods

Materials. Primary antibodies used were against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, MA8374; Chemicon), rabbit anti-DNM2 (R2865) (14), and mouse anti-GFP antibodies made onsite at the antibody facilities of Institut de Génétique et de Biologie Moleculaire et Cellulaire (IGBMC). Secondary antibodies against mouse and rabbit IgG, conjugated with horseradish peroxidase, were purchased from Jackson ImmunoResearch Laboratories. Rhodamine phallolidin, TRITC-conjugated R415, and DAPI nuclear stain (D3571) were purchased from Molecular Probes. An ECL chemiluminescent reaction kit was purchased from Pierce. Tritol reagent was purchased from MRGCENE. Supernatant II reverse transcriptase was purchased from Invitrogen.

Generation of Dnm2<sup>RW/+</sup> Mice. This mouse line was created at the Mouse Clinical Institute (www.ics-mci.fr), as described previously (16). Further details can be found in SI Appendix, Supplementary Methods.

Animal Experiments. Animals were housed in a temperature-controlled room (19–22 °C) with a 12:12-h light:dark cycle with free access to food. Animal experimentation was approved by the institutional ethical committee Com’Eth IGBMC-Institut Clinique de la Souris for 2017–5453 (ASO work), 2012–132/2017–5640 (AAV work), and 2013–034/01594.02 (Aurora in situ muscle contractile properties). Mice were humanely killed when required according to national and European legislations on animal experimentation. Male mice were analyzed in this study.

Statistics. Statistical analysis was performed using a two-way ANOVA followed by post hoc Bonferroni unless otherwise stated. P values of <0.05 were considered significant.

Additional methods can be found in SI Appendix.

ACKNOWLEDGMENTS. We thank Arnaud Ferry and Nadia Messaddeq for excellent technical assistance; and the animal house, histology platform, and imaging center of the IGBMC for support. This study was supported by the Grants ANR-10-LABX-0030-INRT, a French State fund managed by the Agence Nationale de la Recherche under the frame program Investissements d’Avenir ANR-10-IDEX-0002-02. This study was also supported by INSERM, CNRS, the University of Strasbourg, the Agence Nationale de la Recherche (14-CE12-0009), and Société d’Accélération du Transfert de Technologies (SATT) Conexus Alsace (2014) (J.L. and B.S.C.), and grants from the Medical Research Council (MRN002768/1) (to J.O.).