

## rAAV6-microdystrophin preserves muscle function and extends lifespan in severely dystrophic mice

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**Mice carrying mutations in both the dystrophin and utrophin genes die prematurely as a consequence of severe muscular dystrophy. Here, we show that intravascular administration of recombinant adeno-associated viral (rAAV) vectors carrying a microdystrophin gene restores expression of dystrophin in the respiratory, cardiac and limb musculature of these mice, considerably reducing skeletal muscle pathology and extending lifespan. These findings suggest rAAV vector-mediated systemic gene transfer may be useful for treatment of serious neuromuscular disorders such as Duchenne muscular dystrophy.**

Miniaturized dystrophin-based proteins that restore sarcolemmal organization of the dystrophin-glycoprotein complex can be highly functional in transgenic mice<sup>1</sup>. But delivering potentially therapeutic microdystrophin constructs throughout the musculature of animals with muscular dystrophy using traditional methods has proven inefficient. Recently, we established that intravascular administration of rAAV vectors pseudotyped with the serotype-6 capsids (rAAV6) can transduce the striated musculature of mice<sup>2</sup>. This advance enables assessment of systemic microdystrophin delivery in animal models of disease. Historically, the dystrophin-deficient *mdx* mouse<sup>3</sup> has been used as the primary model of Duchenne muscular dystrophy (DMD), although this mouse does not experience the severe, body-wide dystrophy that shortens lifespan by 75% in humans<sup>4–6</sup>. The robustness of *mdx* mice is attributed to compensatory overexpression of the dystrophin-related protein utrophin, as knockout of both dystrophin and utrophin in mice causes progressive muscle wasting, impaired mobility and premature death<sup>5,6</sup>. Here, we tested the hypothesis that systemic administration of rAAV6-microdystrophin can ameliorate the pathology associated with severe muscular dystrophy in dystrophin-utrophin double-knockout mice.

We administered  $\sim 3 \times 10^{12}$  vector genomes of rAAV6-microdystrophin to 1-month-old double-knockout mice through the tail vein as described previously<sup>2</sup>, resulting in uniform, body-wide expression of dystrophin for at least 1 year (Supplementary Fig. 1 online).

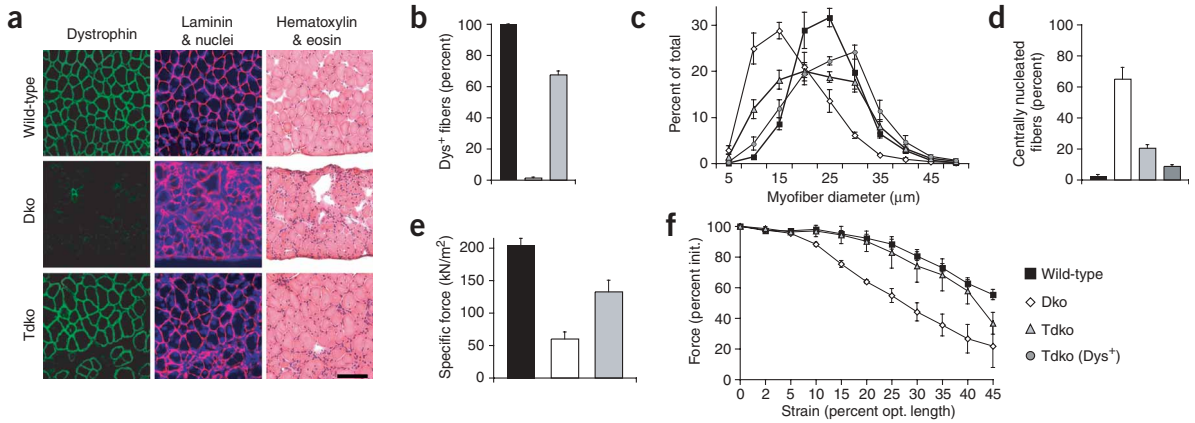
Because muscle deterioration leading to respiratory failure is the primary cause of death in humans with DMD<sup>7</sup>, we examined the effects of treatment upon mouse diaphragm muscles<sup>4</sup>. Expression of dystrophin in diaphragm muscles examined 18 weeks after treatment was widespread (Fig. 1a,b) and was associated with a considerable reduction in the prevalence of smaller muscle cells compared with untreated muscles (Fig. 1c). Treatment also reduced the frequency of centrally nucleated myofibers—a feature of muscle regeneration—by  $\sim 85\%$  compared with the diaphragm muscles of untreated mice (Fig. 1d). Notably, the diaphragm muscles of treated mice showed more than twofold increased normalized force-producing capacity compared with the muscles of untreated mice (Fig. 1e). Using a protocol we developed for subjecting muscles to progressively increased strain under contraction (Supplementary Methods online), we determined that the diaphragm muscles of treated mice showed improved resistance to contraction-induced injury (the principal mechanism of muscle pathology attributed to dystrophin deficiency<sup>1,8</sup>), which was essentially equal to that shown by the muscles of wild-type mice (Fig. 1f).

Among DMD patients who use mechanical respiratory support, cardiac dysfunction attributed to dystrophin deficiency is an increasingly prevalent cause of death. Though prior reports suggest that double-knockout mice succumb to skeletal muscle dysfunction before developing life-threatening cardiac disease<sup>9</sup>, we evaluated transduction of cardiac tissue in treated double-knockout mice (Supplementary Fig. 2 online). Heart and body masses in untreated double-knockout mice were approximately one-half those of wild-type mice. Though the hearts of untreated double-knockout mice lacked dystrophin and were comprised of more small cardiomyocytes than nondystrophic hearts, the only indication of reduced cardiac function that we identified through echocardiography was an increased myocardial performance index—an inverse correlate of contractility<sup>10</sup>. In treated mice, expression of dystrophin was restored throughout the myocardium (Supplementary Fig. 2). Treated mice showed moderately increased cardiomyocyte size but considerably increased heart dimensions and mass, consistent with the approximately doubled body mass (Supplementary Fig. 2). Treatment did not significantly alter myocardial performance index. These data indicate that intravenous administration of rAAV6-microdystrophin can transduce the entire heart of an adult dystrophic mammal. As traditional methods cannot transduce the myocardium to any practical degree without invasive access<sup>11,12</sup>—a risk factor for frail patients—adaptation of this methodology may prove useful for the treatment of conditions associated with heart disease.

DMD patients experience incapacitating limb-muscle degeneration, so we assessed the properties of the tibialis anterior hindlimb muscles of treated mice. Restoration of dystrophin expression (Fig. 2a) increased mean myofiber size by  $\sim 85\%$  (Fig. 2b) and

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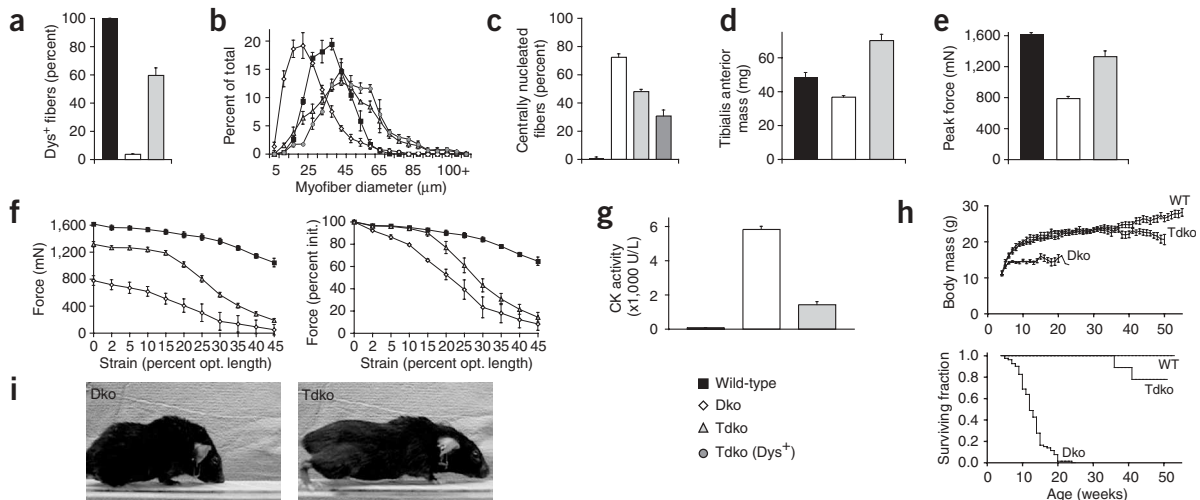
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**Figure 1** Systemic administration of rAAV6-microdystrophin enhances the structural and functional properties of respiratory muscles in dystrophic mice. (a) Restoration of dystrophin expression (green) throughout the diaphragm muscle of a treated mouse (Tdko) contributes to improved muscle fiber size and organization (red, laminin; blue, nuclei; pink, eosin; purple, hematoxylin), as compared with the muscles of an untreated dystrophic mouse (Dko) and a wild-type mouse (WT). Scale bar, 100  $\mu\text{m}$ . (b) Treatment restored expression of dystrophin in the vast majority of diaphragm myofibers of treated mice, in contrast with no expression in the muscles of untreated mice and comprehensive expression in the muscles of wild-type mice. (c) Dystrophin-positive myofibers of treated diaphragm muscles (Tdko (Dys<sup>+</sup>)) were less frequently small in size (mean myofiber diameter; wild-type,  $22.1 \pm 0.7$ ; dystrophic,  $14.9 \pm 0.7$ ; treated dystrophin-positive fibers,  $23.2 \pm 1 \mu\text{m}$ ) and (d) centrally nucleated compared with the myofibers in the muscles of untreated mice. (e) Treatment also improved the peak isometric force-producing capacity of diaphragm muscles as normalized for cross-sectional area. (f) Contractile performance after consecutive eccentric contractions of progressively increasing strain (identified as percent beyond optimal muscle length and expressed in terms of force relative to respective initial output) shows that treatment essentially corrects the susceptibility to contraction-induced injury otherwise observed in dystrophic diaphragm muscles. Error bars represent s.e.m.

reduced the frequency of centrally nucleated muscle fibers by  $\sim 60\%$  (Fig. 2c) compared with the tibialis anterior muscles of untreated mice. As a consequence of these effects, tibialis anterior muscle mass was increased by more than 90% in treated dystrophic

mice (Fig. 2d). Treatment corrected 65% of the deficit in force-producing capacity otherwise shown by the tibialis anterior muscles of untreated mice (Fig. 2e). Furthermore, treated tibialis anterior muscles also showed improved resistance to



**Figure 2** Administration of rAAV6-microdystrophin improves limb muscle function and extends the lifespan of dystrophic mice. (a) Expression of dystrophin was restored in the majority of the myofibers comprising the tibialis anterior muscles of mice examined 18 weeks after treatment, compared with the muscles of untreated dystrophic and wild-type mice. (b) Dystrophin-positive myofibers in the muscles of treated mice (Tdko (Dys<sup>+</sup>)) were larger in diameter and (c) less frequently centrally nucleated than cells in the muscles of untreated mice. (d) Treatment was associated with increased tibialis anterior muscle mass and (e) peak isometric force-producing capacity compared with untreated muscles (though unchanged specific force; wild-type,  $240 \pm 11$ ; double-knockout,  $149 \pm 5$ ; treated double-knockout,  $145 \pm 6 \text{ kN/m}^2$ ). (f) Contractile performance expressed in terms of absolute force (left) and performance relative to initial output (right) indicates that the muscles of treated mice show improved force output and resistance to contraction-induced injury when subjected to consecutive eccentric contractions of progressively increasing strain (identified as percent beyond optimal muscle length). (g) Serum creatine kinase (CK) activity was markedly reduced in treated mice compared with untreated mice, yet remained moderately elevated compared with nondystrophic mice. (h) Body mass (top) of treated mice is increased compared with untreated mice, and comparable with the mass of wild-type mice. In cohorts monitored for lifespan (bottom), treated mice ( $n = 8$ ) lived significantly longer than untreated mice ( $n = 70$ ). (i) Untreated mice (top) typically show considerable muscle wasting and kyphotic posture by 16 weeks of age, whereas treated mice (bottom) retain considerably greater muscularity. Error bars represent s.e.m.

contraction-induced injury that was similar to that shown by wild-type muscles within the physiological range<sup>13</sup> (up to 15% strain, **Fig. 2f**).

Having studied specific striated muscles that influence physical condition, we considered whole-body indices of disease. Treatment corrected more than 75% of the discrepancy in serum creatine kinase levels (a measure of myocellular degeneration) normally observed between untreated and nondystrophic mice (**Fig. 2g**). Where untreated double-knockout mice had reduced body mass and died prematurely (80% mortality at 15 weeks of age) compared with wild-type mice, treated littermates had increased body mass beyond 2 weeks after treatment and considerably extended life span (cohort mean age greater than 1 year, **Fig. 2h**). The progressive muscle wasting observed in untreated dystrophic mice impaired posture and ambulation (**Fig. 2i** and **Supplementary Video 1** online), whereas treated mice retained sufficient muscularity and mobility to readily use a voluntary running wheel and untreated mice could not (**Fig. 2i** and **Supplementary Video 2** online).

This is the first study to show that an intervention can restore expression of dystrophin in the respiratory, cardiac and limb musculature of dystrophin-utrophin double-knockout mice, resulting in improved muscle function and an extended lifespan. The widespread and persistent transduction achieved without serious pathogenic effects after administration of rAAV6 vectors in this manner suggests that systemic gene replacement strategies may be beneficial for treatment of serious neuromuscular disorders such as DMD.

All experimental manipulation of mice was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Washington.

*Note: Supplementary information is available on the Nature Medicine website.*

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#### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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