Nystagmus Associated With the Absence of MYOD Expression Across the Lifespan in Extraocular and Limb Muscles

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Purpose. The extraocular muscles (EOMs) undergo significant levels of continuous myonuclear turnover and myofiber remodeling throughout life, in contrast to limb skeletal muscles. Activation of the myogenic pathway in muscle precursor cells is controlled by myogenic transcription factors, such as MYOD. Limb muscles from *MyoD*$^{-/-}$ mice develop normally but have a regeneration defect, and these mice develop nystagmus. We examined *MyoD*$^{-/-}$ mice to determine if they have an aging phenotype.

Methods. Eye movements of aging *MyoD*$^{-/-}$ mice and littermate controls (wild type) were examined using optokinetic nystagmus (OKN). We assessed limb muscle function, changes to myofiber number, mean cross-sectional area, and abundance of the PAX7 and PITX2 populations of myogenic precursor cells.

Results. Aging did not significantly affect limb muscle function despite decreased mean cross-sectional areas at 18+ months. Aging wild type mice had normal OKN responses; all aging *MyoD*$^{-/-}$ mice had nystagmus. With OKN stimulus present, the *MyoD*$^{-/-}$ mice at all ages had shorter slow phase durations compared to wild type age matched controls. In the dark, the *MyoD*$^{-/-}$ mice had a shorter slow phase duration with age. This correlated with significantly decreased fiber numbers and cross-sectional areas. The EOM in *MyoD*$^{-/-}$ mice had increased numbers of PAX7-positive satellite cells and significantly decreased PITX2-positive myonuclei.

Conclusions. The absence of MYOD expression in aging mice causes a decrease in ongoing myofiber remodeling, EOM fiber size, and number, and is associated with the development of spontaneous nystagmus. These results suggest that muscle-specific mutations can result in nystagmus, with increasing aging-related changes in the *MyoD*$^{-/-}$ EOM.

Keywords: extraocular muscles (EOMs), skeletal muscle, muscle precursor cells, satellite cells

Skeletal muscle has a tremendous capacity to regenerate and repair after injury or in disease due to myogenic precursor cells that reside in the muscles. In the event of muscle disease or injury, these myogenic precursor cells activate, divide, and can fuse into existing myofibers or form new myofibers.[1–3] All skeletal muscles also undergo myofiber remodeling to some extent, and in some at quite significant levels.[4–6] MYOD is a part of a family of basic helix-loop-helix transcription factors that can induce muscle-specific gene expression, and it normally functions in the commitment of a myogenic precursor cell to differentiate.[7] Previous studies on *MyoD* knockout mice showed that they had normal skeletal muscle, but were regeneration deficient.[8,9] In addition, the muscle precursor cells in these *MyoD* knockout mice were differentiation deficient[10,11] and fusion deficient.[11,12] Due to the very high level of myonuclear turnover in the extraocular muscles (EOMs), we hypothesized that their structure and function would be significantly affected in *MyoD*$^{-/-}$ mice. We recorded the eye movements in these mice and compared them to age-matched controls by analyzing their optokinetic nystagmus reflex. The *MyoD*$^{-/-}$ mice developed spontaneous nystagmus, manifested as uncontrolled oscillatory movements, and these abnormal eye movements correlated with a reduction in myofiber size and number.[13] The waveforms in the young *MyoD*$^{-/-}$ mice were similar to those that have been described in human subjects with infantile nystagmus syndrome.[14]

Aging results in changes to limb and body skeletal muscle function and structure over time, with losses in muscle contractile force, muscle fiber size, and muscle fiber number.[15,16] The EOMs, however, are resistant to aging-related changes.[17–19] From a functional standpoint, most studies of eye movements in aging humans showed normal

saccade accuracy and binocular coordination.[20] Other studies showed some increase in saccade latency at distance,[21] as well as the development of age-related distance esotropia.[22] These changes in eye position and function were linked to central nervous system pathology not seen in the normal aging population,[23] and/or anatomic changes in the orbital connective tissue elements that alter EOM paths.[24] There is evidence for decreased levels of microtubule-associated protein 1A/1B-light chain 3 (LC3) in a rat model of aging[25] and in cytochrome c oxidase levels in aging human EOMs.[26] The lack of development of sarcopenia and muscle weakness in the EOMs in aging suggests that despite the decreased cytochrome oxidase or LC3 levels[25,26] in the normal aging population, normal function is maintained. However, looking specifically at the normal optokinetic nystagmus in young compared to old human subjects, a number of studies showed a significant effect of aging on aspects of these reflex movements, including increased gain and decreased low phase velocity,[27–31] as well as loss of the torsional optokinetic nystagmus response.[32] Thus, an effect by this muscle-specific mutation in *MyoD* on nystagmus during aging was predicted.

The elevated numbers of myogenic precursor cells in the EOMs compared to limb and body skeletal muscles,[33,34] the resistance to aging of the myogenic precursor cell populations,[17,19] as well as the innervation to the EOMs[18,19] provide a mechanism by which the EOMs are able to maintain their normalcy over time. We hypothesized that in the absence of MYOD expression in the aging mice, measurable loss in muscle structure and function would be greater in the EOMs than in the control age-matched wild type mice due to their high rate of myonuclear addition throughout life.[4,5,35,36] We hypothesized that in the $MyoD^{-/-}$ mice, the EOMs would display worsening of their spontaneous abnormal eye movements, and that aging of the limb skeletal muscles in the $MyoD^{-/-}$ mice would cause functional and structural alterations. Functional assays for the limb muscles were grip strength and rotarod performance, and the EOMs were tested using optokinetic nystagmus. MYOD is a critical transcription factor for differentiation along the myogenic lineage.[7,8] As both PAX7 and PITX2-positive myogenic precursor cells turn on the MYOD pathway as they differentiate, we investigated how the absence of MYOD affected these two myogenic precursor lineages to see if there was a correlation between changes in these myogenic precursor cells and changes in muscle structure and function over time. The potential role of these changes within the EOMs was examined to determine a potential mechanism.

Methods

Mice were housed and maintained by Research Animal Resources. $MyoD^{+/-}$ mice[8] were used to produce $MyoD^{+/+}$, $MyoD^{+/-}$, and $MyoD^{-/-}$ littermates. Mice were genotyped using PCR, and the primers used were: 5′-GCGAATAGCAA GGATAACAGA-3′ (common), 5′-CTTGGGTATCTGCAACAGG TT-3′ (wild type specific), and 5′-GCGCCAACTGCCAGCG GGGCT-3′ (mutant specific). Primers were produced by Integrated DNA Technologies (Coralville, IA, USA). Mice for all experiments in this study ranged from 3 months up to 22 months of age. The Institutional Animal Care and Use Committee at the University of Minnesota approved all experiments. All experiments followed the animal use recommendations of the National Institutes of Health and the Association for Research in Vision and Ophthalmology. Animals were maintained in a 12-hour light/dark cycle with food and water ad libitum.

At each of five ages, 3 months, 6 months, 12 months, 18 months, and 19+ months, the limbs and EOMs were tested for functional and morphological changes over time. Functional assays included grip strength and rotarod walking time for the limb muscles and optokinetic nystagmus recordings for the EOMs. Morphological changes assessed in tibialis anterior muscles included myofiber mean cross-sectional area and numbers of PAX7-positive satellite cells. Morphological changes assessed in EOMs included myofiber mean cross-sectional area, total myofiber number, percent of fibers expressing the slow twitch myosin isoform (*myh7*), numbers of PAX7-positive satellite cells, and numbers of PITX2-positive myogenic precursor cells and PITX2-positive myonuclei.

Functional Assays

Forelimb grip strength was determined using a force gauge (Columbus Instruments, Columbus, OH, USA). The mice were lifted to the rectangular metal grid in the horizontal plane and were pulled steadily backward until they could not hold on any longer. This was repeated 3 times per mouse with a 1-minute reset between measurements for each of the ages tested, and the forces produced were averaged for each mouse. The mice were weighed, and force in Newtons (N) per gram of body weight was calculated for each mouse. For both the $MyoD^{-/-}$ and wild type mice, the mean values from individual mice were averaged at each of the five age groups tested, with a minimum of six mice per age and genotype.

Walking time was determined for each mouse in all five age groups using a Rota-Rod RS (Letica Scientific Instruments, Rochester Hills, MI, USA), with a minimum of three mice per age and genotype. Mice were trained to use the Rota-Rod prior to their testing day. Mice were placed individually on the Rota-Rod, allowed to walk starting at a speed of 4 revolutions per minute (rpm) with ramping up to 40 rpm, with an increase of 1 rpm every 8 seconds, for up to 5 minutes until they walked for 30 consecutive seconds. Every mouse was given 3 trials during training days with a 20-minute rest between each trial. On testing days, mice were measured using the acceleration feature of the Rota-Rod. Mice were placed on the Rota-Rod starting at 4 rpm with rpms increasing by 1 rpm every 8 seconds until a speed of 40 rpm was reached. The amount of time they walked during 3 trials was measured with a 20-minute break in between each trial. Averages were calculated from the three trials for each of the $MyoD^{-/-}$ and wild type mice, and the average walking time per age group was calculated from the averaged values for each mouse.

Optokinetic Nystagmus Testing

All mice were examined for normal eye movements using optokinetic nystagmus testing.[13] To stabilize the head during testing, all mice were given head posts. A scalp incision was made, and two small self-tapping screws were placed through the cranium. Geristore dental cement (DenMat LLC, Lompoc, CA, USA) was placed over the screws, and nylon posts were embedded vertically in the cement. After 2 weeks, each mouse was placed on a stage in the optokinetic nystagmus testing chamber, heads stabilized using the headposts, where a video camera was focused on the pupil. Using

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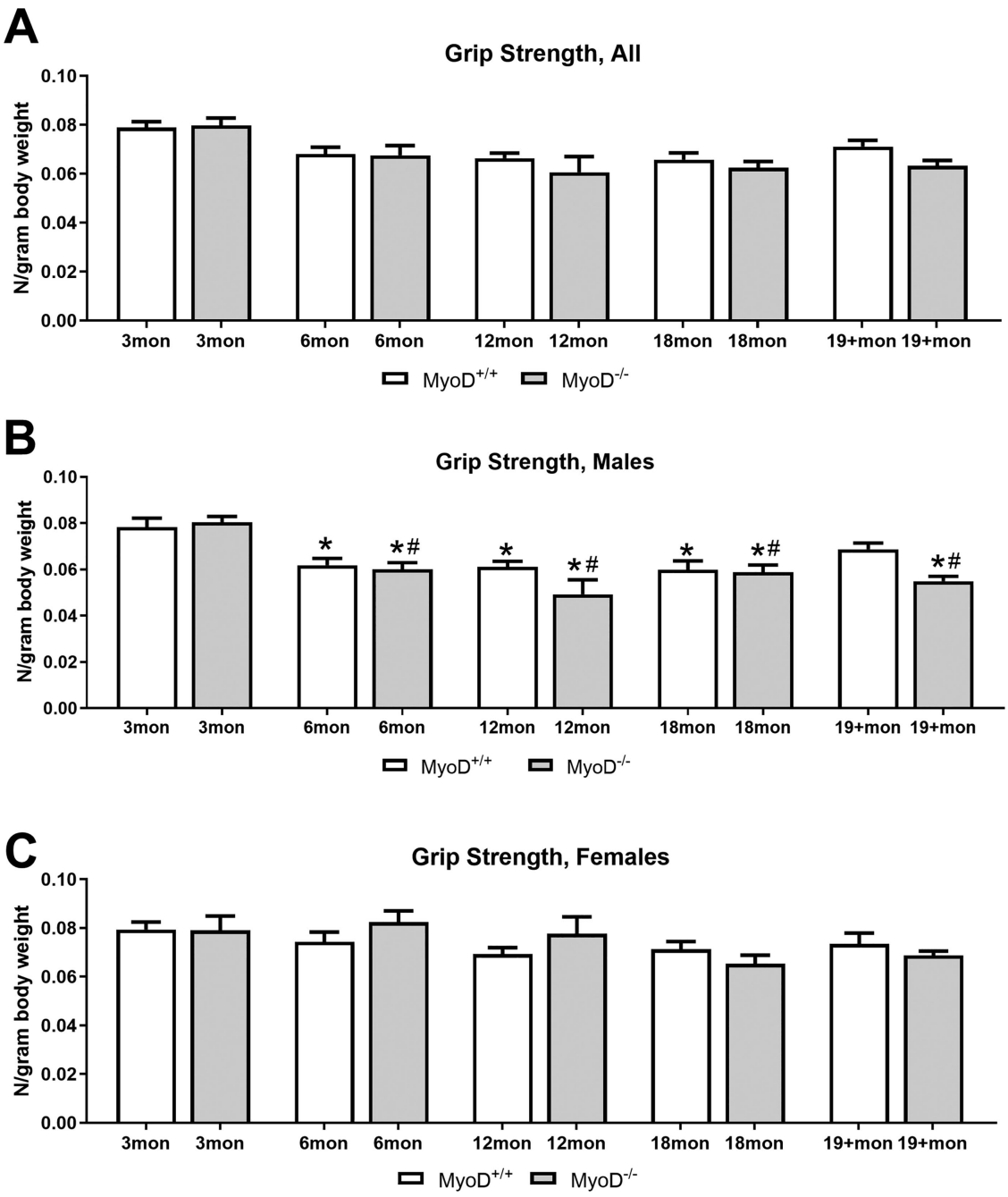

Figure 1. Grip strength varies based on sex in aging MyoD$^{-/-}$ mice. Grip strength analysis examined in (**A**) all mice ($N = 7$), (**B**) male mice ($N = 3$), and (**C**) female mice ($N = 4$) *MyoD*$^{-/-}$ and littermate wild type mice at 3, 6, 12, 18, and 19+ months. * Indicates significant difference from 3 month control mice. # Indicates significant difference from 3 month *MyoD*$^{-/-}$ mice.

the ISCAN eye tracking program (ISCAN Inc., Woburn, MA, USA), movement of the pupil based on corneal reflection compared to a reference point was recorded both in the absence of stimuli and in the presence of the optokinetic nystagmus (OKN) stimulus moving in the horizontal plane for 86 seconds. There was one stimulus velocity for all eye movement analyses, 0.5 cycles/second, which is in line with others in the field. Two full series were given to the mice, one with varying spatial frequencies of: 0.06, 0.08, 0.10, 0.12, and 0.15 cycles/degree. There was then a rest period and a recording of the eyes without stimuli. A second series with varying contrast ratios of 10, 25, 50, 75, 100, 200, and 400 were recorded. There was no randomization in the series; each mouse followed the same pattern of contrasts and spatial frequencies. For the graphs in the figures, 20 second segments of each trace with the least background noise (consisting mainly of blinks) were used. For the figures, we are reporting a spatial frequency of the black and white bars that were the OKN stimulus was set at 0.12 and contrast at 100. Recordings were taken by alternating periods of stimuli and no stimuli. Data were analyzed using custom R programs (R Foundation for Statistical Computing, Vienna,

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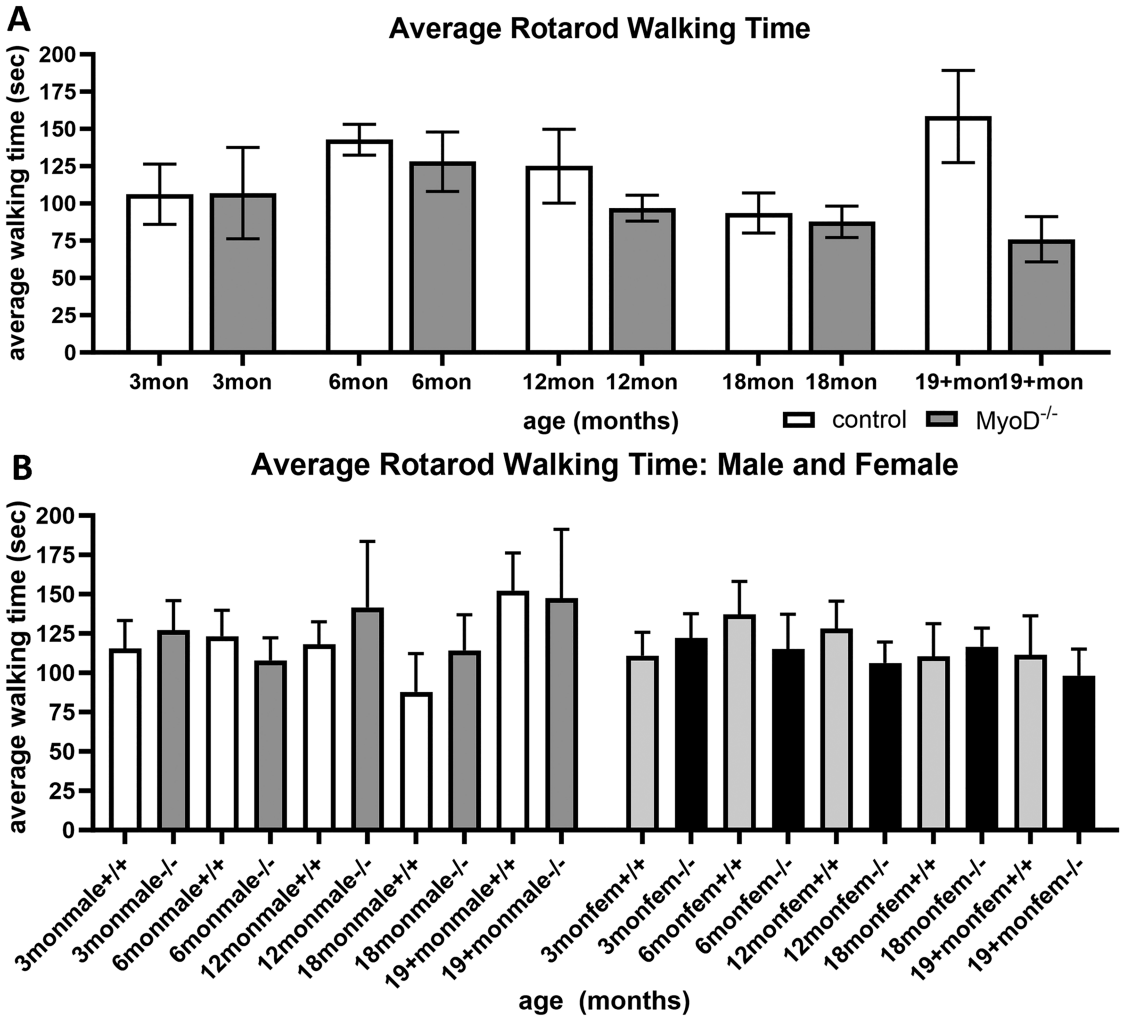

Figure 2. Average rotarod duration is unchanged in aging MyoD$^{-/-}$ mice. (**A**) Average rotarod walking time in *MyoD*$^{-/-}$ and littermate wild type mice at 3, 6, 12, 18, and 19+ months. No significant differences were seen between genotypes at any of the ages examined or between genotypes. (**B**) There were no significant differences in average walking time on the rotarod between male mice and female mice between all ages and all genotypes. Even in aged mice, the loss of MyoD expression did not cause a significant loss in walking duration of the mice.

Austria), in order to filter the raw pupil trace by subtracting the raw corneal reflection trace. Average horizontal positions were determined for each OKN recording session. These were graphed with the horizontal trace set arbitrarily at 0. Using a second custom R program, the timing between onsets of the slow phase movements of the OKN recordings were calculated to determine the duration of the slow phase for the wild type and duration of the oscillations in the *MyoD*$^{-/-}$ mice. Statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA, USA).

Morphometric Analyses

Both wild type littermate controls and *MyoD*$^{-/-}$ mice at 3, 6, 12, 18, and 19+ months of age were euthanized using an overdose of carbon dioxide. The eyes were removed with all rectus muscles intact on the globe, as well as one tibialis anterior muscle per mouse. These specimens were embedded in tragacanth gum and snap-frozen. The blocks were stored at -80°C until sectioned at 12 µm. For all studies, sections midway between insertion site and the end of the globe were examined. We analyzed three separate slides per animal and measured all muscles. These were averaged for each individual mouse, and the averages were used for all analyses.

One series of slides from each block was stained with hematoxylin and eosin for fiber area and fiber number counts. A second series of slides from each block was immunostained for expression of PAX7 by our standard methods.[33] Briefly, slides were blocked in 10% horse serum in phosphate buffered saline (PBS) containing 1% Triton-X 100 (antibody buffer), followed by an hour incubation at room temperature with an antibody to PAX7 (1:50 in antibody buffer; Hybridoma Bank PAX7c; University of Iowa, Iowa City, IA, USA). After rinsing in PBS, the slides were incubated using the Vector Elite mouse IgG kit (PK-1062; Vector Laboratories, Burlingame, CA, USA) following the kit instructions. They were reacted in with 3,3′-diaminobenzidine tetrahydrochloride (DAB; D5905; Sigma, St. Louis, MO, USA) to intensify the stain. These slides were dehydrated, followed by treatment in xylene, and

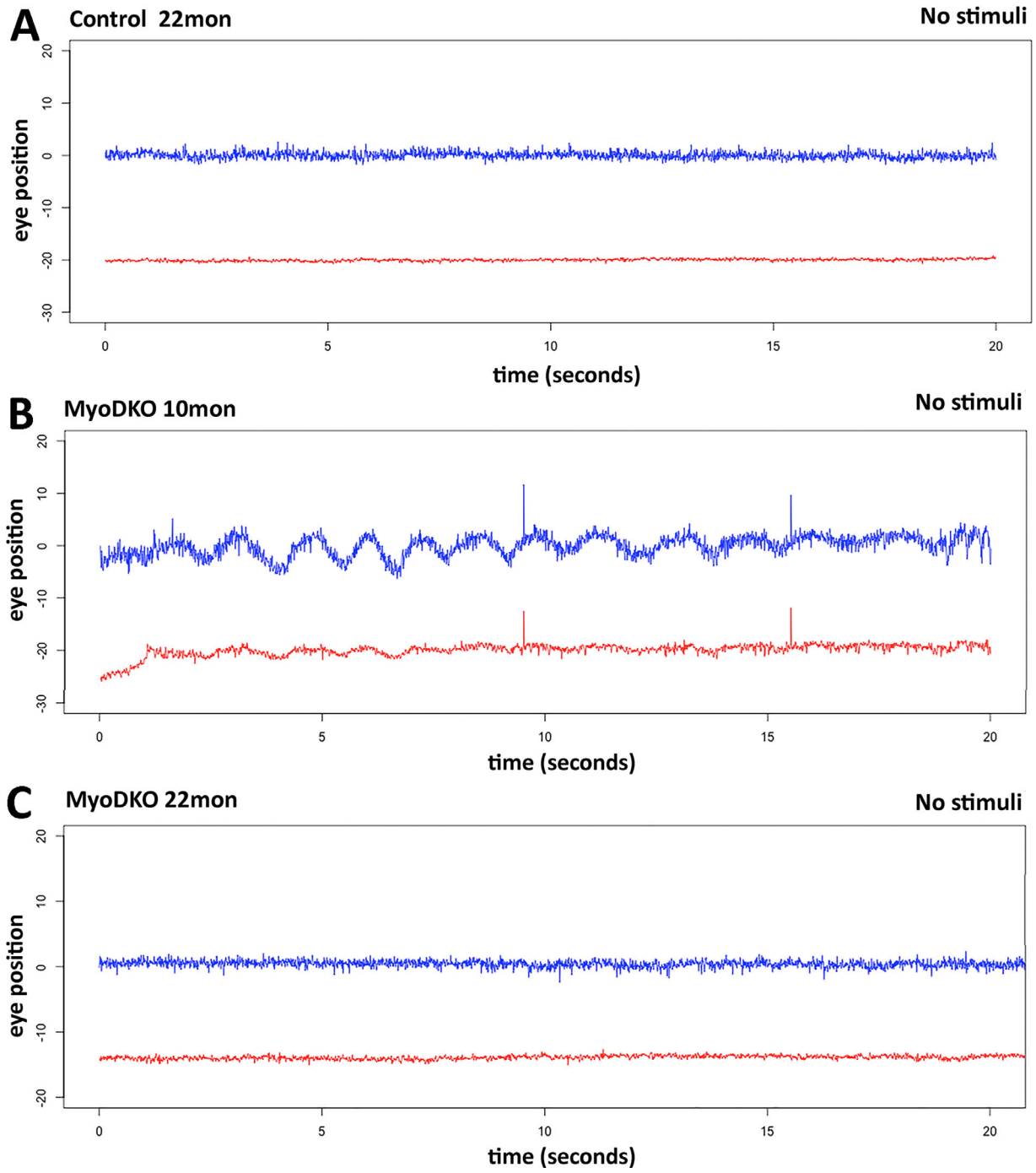

Figure 3. Optokinetic nystagmus is variable in the absence of stimuli. Eye movement recordings in the absence of the optokinetic stimulus from (**A**) a wild type mouse at 22 months of age and (**B**) a *MyoD*$^{-/-}$ mouse at 10 months of age, and (**C**) a *MyoD*$^{-/-}$ mouse at 22 months of age. In the wild type mouse, no eye movements are seen in the absence of stimuli. Only one mouse over 12 months showed spontaneous pendular waveforms typical of nystagmus. All the other *MyoD*$^{-/-}$ mice examined at 18 and 19+ months showed no eye movements in the absence of stimuli.

coverslipped using Permount mounting medium (Fisher Scientific, Waltham, MA, USA).

PITX2 immunohistochemistry followed our standard published procedures.[33] Briefly, slides were incubated in 20% goat serum/0.2% bovine serum albumin (BSA) in antibody buffer, followed by 1 hour incubation in an antibody to PITX2 (1:100; Capra Science, Ängelholm, Sweden; 1:500, Innovagen, Stockholm, Sweden). After a PBS rinse, they were incubated in goat-anti-rabbit-Alexa Fluor 488 (1:2000; Jackson ImmunoResearch, West Grove, PA, USA) in antibody buffer, followed by incubation with an antibody to dystrophin (1:75, abcam ab1527; Abcam, Waltham, MA, USA) for 1 hour at room temperature. After a PBS rinse, slides were blocked with the 10% goat serum with 0.2% BSA in antibody buffer for 30 minutes, followed by incubation in goat-anti-rabbit Rhodamine Red (1:1000, Jackson

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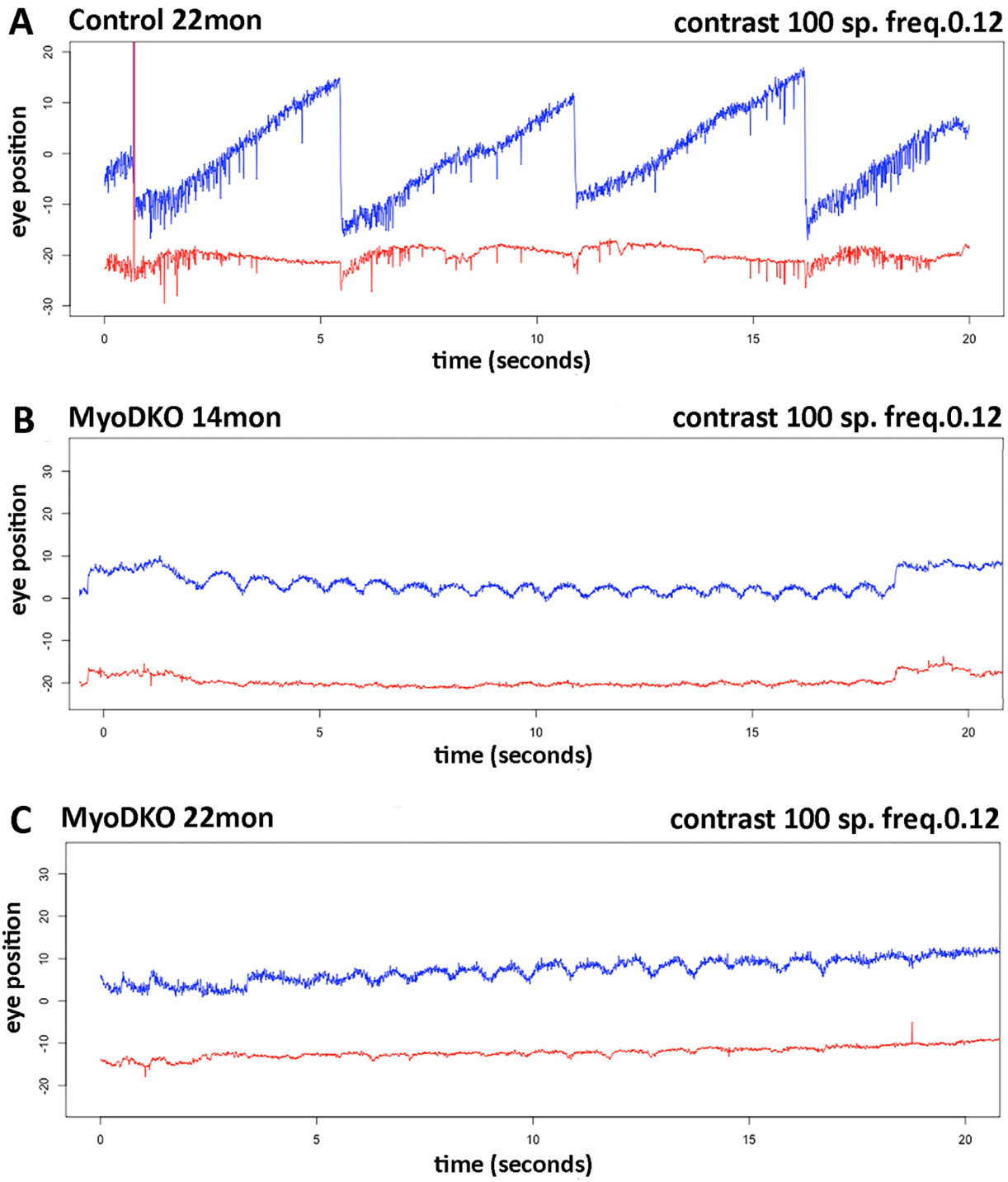

Figure 4. Optokinetic nystagmus recordings in the presence of stimuli. Eye movement recordings showing optokinetic responses in the presence of the OKN stimulus from (**A**) a wild type mouse at 20 months of age. In (**B**) a *MyoD*$^{-/-}$ mouse at 14 months of age and (**C**) a *MyoD*$^{-/-}$ mouse at 22 months of age, eye movements were recorded and all showed pendular nystagmus. Representative eye movement recordings at a spatial frequency of 0.12 and at a contrast of 100 are shown. *Blue* represents the horizontal and *red* represents the vertical. Even in aging mice, nystagmus wave forms were present during OKN stimulus presentation.

ImmunoResearch) for 1 hour at room temperature. The slides were rinsed in PBS, and coverslipped with VectaShield (Vector Labs., Burlingame, CA, USA). The slides were examined and photographed using a Leica DM4000B microscope.

Another set of sections midway between insertion site and the end of the globe were immunostained for the slow type 1 myosin heavy chain isoform (MyHC). Slides were incubated in blocking serum, followed by incubation in avidin/biotin blocking reagents (Vector; SP-2001). The sections were incubated in primary antibody against type 1 MyHC (1:50; *myh7*; Novus NBP2-94079) overnight at 4°C. The next day, after three rinses in PBS, the slides

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were incubated in blocking serum, followed by incubation in secondary antibody using the Vector Elite ABC kit (PK-61-1) following the kit directions. After rinses in PBS, the slides were reacted with 3,3′-diaminobenzidine tetrahydrochloride (DAB; D5905; Sigma) and heavy metals. They were dehydrated and coverslipped in Permount mounting medium.

All analyses were performed on 3 muscle sections, 3 to 4 different fields per section, from a minimum of 3 animals per genotype for both muscles and both genotypes at 3, 6, 12, 18, and 19+ months. These were averaged to determine the mean for each mouse, and the averages were used for the statistical analyses.

Mean Myofiber Cross-Sectional Areas

The mean cross-sectional areas for the tibialis anterior and the EOM were manually traced using the Bioquant Analysis System (Bioquant, Nashville, TN, USA). Total fiber numbers were manually determined in cross-sections of the EOMs. A minimum of 200 fibers were traced per muscle fiber layer in the EOM, if possible, based on overall number of fibers in the cross-section, and in the tibialis anterior, with 3 to 4 separate fields in 3 sections per mouse. The numbers per field were averaged, and the averages per muscle from each of the three sections were averaged and used to determine the overall mean myofiber cross-sectional area per animal for each age range. In addition, total fiber number was determined by manual counting using the Bioquant Analysis System (Bioquant) using a similar strategy, with three sections analyzed per animal for each genotype and at each of the five time points. These were used to determine the average total fiber number. All myofibers were counted in each section. No sampling was performed.

Myogenic Precursor Number Morphometry

PAX7-positive cells were counted in individual non-overlapping fields such that a minimum of 200 myofibers were included in the counts, if possible. In each field, the total myofiber number was determined in order to calculate the number of positive cells as a percent of fiber number. PITX2-positive nuclei were analyzed as being inside the dystrophin ring or outside of the dystrophin ring. As dystrophin lines the inner side of the sarcolemma,[37] this allowed differentiation of PITX2-positive nuclei that were myonuclei from those external to the sarcolemma. These were quantified based on total fiber number per counted field and expressed as a percent.

Statistical Analyses

Using GraphPad Prism software (GraphPad), 2-way ANOVAs were performed on all morphometric data analyses. If significant differences were found, Tukey's or Dunnett's post hoc multiple comparison tests were performed to determine significant differences based on genotype and age, with significance at $P < 0.05$.

Results

Effects of Loss of MYOD Expression on Limb Muscle Function

Grip Strength. Grip strength was determined for the *MyoD*$^{-/-}$ and wild type mice for each of the five postnatal time points. When all mice of a given age were pooled, there were no significant differences between the genotypes (Fig. 1A). When male and female mice were examined separately, the wild type male mice were significantly weaker than the 3-month-old wild type mice at 6, 12, and 18 months, with decreases of 21.1% ($P = 0.0171$), 22% ($P = 0.0085$), and 23.6% ($P = 0.0044$) in the older wild type mice, respectively. In the *MyoD*$^{-/-}$ male mice, grip strength showed significant decreases at 6, 12, 18, and 19+ months compared to the 3-month control mice, at 23.3% ($P = 0.013$), 37.11% ($P = 0.0033$), 25% ($P = 0.0017$), and 29.9% ($P = 0.0005$), respectively. The *MyoD*$^{-/-}$ mice at all ages examined were also significantly weaker compared to the 3-month-old *MyoD*$^{-/-}$ mice, with decreases of 25.3% ($P = 0.005$), 38.7% ($P = 0.0001$), 27% ($P = 0.0058$), and 31.7% ($P = 0.0005$),

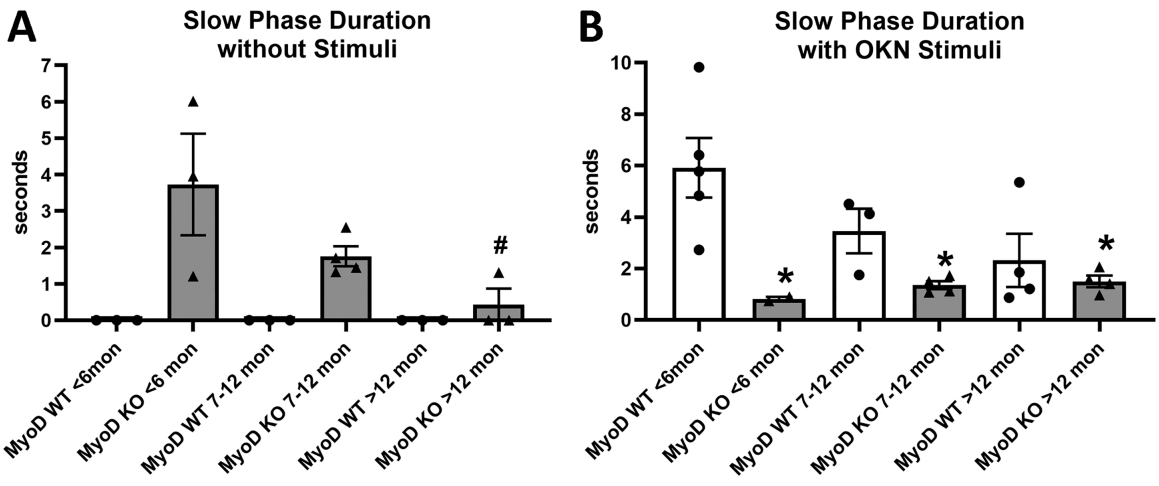

Figure 5. Time of slow phase changes. Calculation of the time between beginning and end of each full pendular excursion of the eye movement responses in wild type and *MyoD*$^{-/-}$ mice (**A**) in the absence of stimuli, and (**B**) in the presence of the OKN stimulus. Duration time of the troughs of the pendular waveforms of the *MyoD*$^{-/-}$ mice significantly decreased compared to the 3 month control values only in the presence of stimuli, with a 86.1% decrease at 3 months ($P = 0.022$), 77% decrease at 6 months ($P = 0.01$), and a 74.7% decrease at greater than 12 months ($P = 0.013$). The waveforms stayed remarkably stable in the *MyoD*$^{-/-}$ mouse over time, with no significant differences seen. * Indicates significant difference from the 3 month wild type mice.

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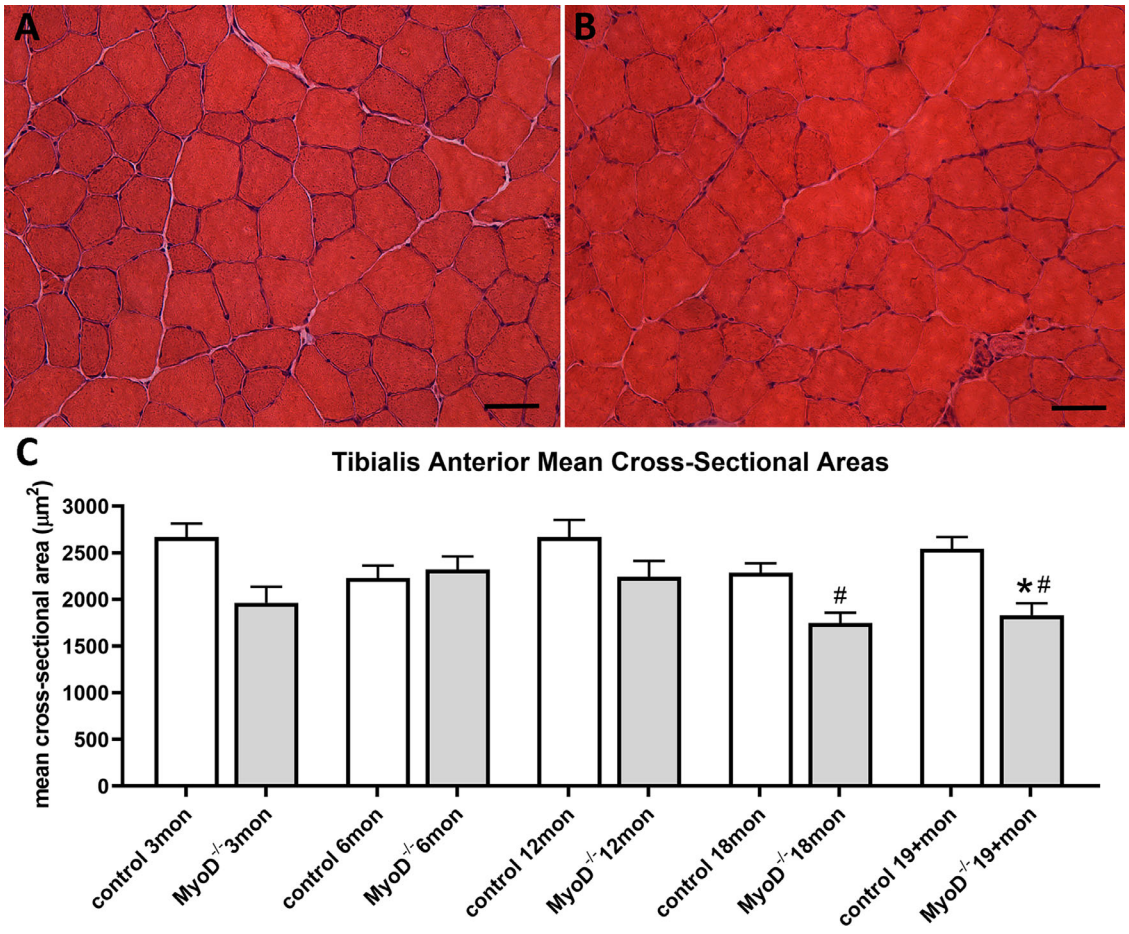

Figure 6. Tibialis anterior mean myofiber cross-sectional areas are unchanged. Photomicrograph of tibialis anterior muscle from a (**A**) wild type control mouse and a (**B**) *MyoD*$^{-/-}$ mouse. Scale bar is 50 µm. (**C**) Mean cross-sectional areas from the tibialis anterior muscles from wild type and *MyoD*$^{-/-}$ mice at 3, 6, 12, 18, and 19+ months. There were no significant differences in mean cross-sectional areas between the aged-matched tibialis anterior muscles until 19+ months. There were no significant differences between any of the mean cross-sectional areas of the tibialis anterior muscle fibers from the *MyoD*$^{-/-}$ mice except at 18 months, and at 19+ months the mean cross-sectional areas of the *MyoD*$^{-/-}$ muscles were significantly smaller than the 3 month control muscles. Only in advanced aged mice was there a measurable effect on myofiber cross-sectional area. There were five mice for all ages and for each genotype. This represents measurements in all extraocular muscles on three slides each for each animal, which are then averaged for each animal. * Indicates significant difference from age-matched wild type mice. # Indicates significant difference from wild type mice at 3 months.

respectively (see Fig. 1B). There were no significant differences based on genotype or age for the female mice (see Fig. 1C).

Rotarod Walking Time. There was a great deal of inter-animal variation in the results of the rotarod testing (Fig. 2A). ANOVA analysis indicated significance ($P = 0.02$), thus post hoc Dunnett's multiple comparison tests were performed. The post hoc *t*-tests showed there were no significant differences based on genotype or age for any of the mice tested (see Fig. 2A). The data were then re-examined based on sex, and ANOVA analysis indicated that the data were not significantly different based on sex for either male mice ($P = 0.78$) or female mice ($P = 0.94$) or between male mice and female mice ($N = 4$ at each age, sex, and genotype; $P = 0.97$; Fig. 2B).

Effect of Loss of MyoD Expression on EOM Function

In the absence of OKN stimuli, control mice showed no spontaneous movements, except an occasional saccade (not shown; Fig. 3A). In the absence of OKN stimuli, the *MyoD*$^{-/-}$ mice at 10 months showed signs of pendular nystagmus, but in aged mice (22 months) these movements ceased (Figs. 3B, 3C).

For the wild type mice up to 22 months of age, there were normal optokinetic reflex responses in the presence of the OKN stimulus (Fig. 4A). As shown previously for *MyoD*$^{-/-}$ mice up to 12 months of age,[13] in the absence of MYOD expression, nystagmus in the presence of the OKN stimulus was present in the aged *MyoD*$^{-/-}$ mice (Figs. 4B, 4C). In these aged mice, the nystagmus waveforms in the presence of the OKN stimulus were pendular; however, jerk and pendular spontaneous nystagmus waveforms were seen in the *MyoD*$^{-/-}$ mice at the younger ages, as previously reported.[13] The retinas of all mice appeared histologically normal (Supplementary Fig. S1).

Durations of the slow phases of the nystagmus, as measured from trough to trough, and normal waveforms in the presence or absence of the OKN stimulus were determined. In the absence of stimuli, no eye movements were present in any of the normal wild type mice; however, the *MyoD*$^{-/-}$ mice had the spontaneous pendular waveforms

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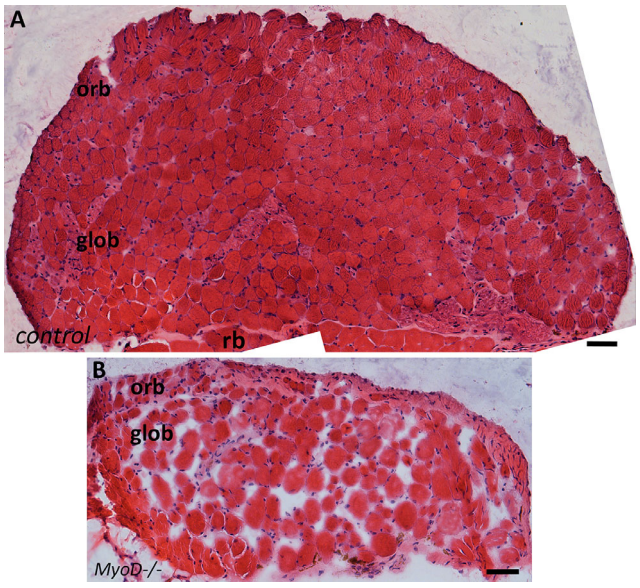

Figure 7. Myofiber loss is apparent in EOM in aging *MyoD*$^{-/-}$ mice. Photomicrographs of lateral rectus muscles from (**A**) wild type litter-mate control (wild type), and (**B**) a *MyoD*$^{-/-}$ mouse at 18 months. Note the significant loss of muscle fibers in the absence of MYOD expression. Orb, orbital layer; glob, global layers; rb, retractor bulbi, present in mice. Scale bar is 50 µm.

characteristic of nystagmus (see Fig. 4A). The *MyoD*$^{-/-}$ mice aged 6 months and younger had apparent longer slow phase tracking times than those aged 7 months and older, but was not statistically significant. Only the waveforms at greater than 12 months were significantly different from the youngest group of mice, with an 88.3% decrease ($P = 0.007$). There was a 52.9% difference in the 6 to 12-month-old mice, but this was not statistically different ($P = 0.19$; Fig. 5A). In the presence of the OKN stimulus, all the wild type mice had normal eye movement responses, showing normal slow and fast phase movements. The 7 to 12 month wild type mice had 41.5% shorter durations than the young mice (<6 months), and the old mice (>12 months) had a 60.8% reduction in length of slow phase duration compared to the young wild type mice; neither of these reductions were statistically different (Fig. 5B). The slow phase durations for all the *MyoD*$^{-/-}$ mice were significantly shorter than the durations in the wild type mice at < 6 months, with decreases of 86.1% ($P = 0.022$) at <6 months, 77% ($P = 0.01$) at 6 to 12 months, and 74.7% ($P = 0.013$) at >12 months of age (see Fig. 5B). There were no significant differences between stimulus-induced slow phase durations between any of the ages of *MyoD*$^{-/-}$ mice.

Effect of Loss of MyoD Expression on Mean Cross-Sectional Areas

The mean myofiber cross-sectional areas for the tibialis anterior muscle in *MyoD*$^{-/-}$ and littermate wild type mice were examined at ages 3, 6, 12, 18, and 19+ months of age (Fig. 6). ANOVA analysis indicated significance ($P = 0.0001$), thus post hoc Tukey's multiple comparison tests were performed. Tibialis anterior mean myofiber cross-sectional areas were significantly different between the *MyoD*$^{-/-}$ and 3-month-old wild type mice at 18 months ($P = 0.0011$) and 19+ months ($P = 0.025$), and significantly different between wild type wild type at 19+ months compared to wild type from *MyoD*$^{-/-}$ mice at 19+ months ($P = 0.041$). In the post hoc multiple comparison tests, there were no statistically significant differences between the mean myofiber cross-sectional areas of the muscles of the *MyoD*$^{-/-}$ and wild type for a given set of ages, except for *MyoD*$^{-/-}$ and wild type mice at 19+ months, where there was a 27.6% decrease in the area ($P = 0.041$). In addition, there were significant differences in mean cross-sectional areas when mean cross-sectional areas at 18 and 19+ months in the *MyoD*$^{-/-}$ muscles were compared to 3-month wild type muscles (34.5% decrease, $P = 0.001$; 31.4% decrease, $P = 0.025$, respectively; see Fig. 6). All these parameters were examined comparing male and female mice, and there were no significant differences seen based on sex (data not shown).

For the EOM data, ANOVA analysis indicated significance ($P = 0.0001$), thus post hoc Tukey's multiple comparison tests were performed. The mean cross-sectional areas in the EOMs in the *MyoD*$^{-/-}$ mice were significantly different ($P = 0.0001$), and post hoc tests showed they were significantly smaller than the littermate wild type EOM myofibers at 12, 18, and 19+ months (26.8%, $P = 0.004$; 28.6%, $P = 0.0037$; and 31.2%, $P = 0.03$), respectively (Figs. 7, 8A). In addition, the mean cross-sectional area of the EOMs of the *MyoD*$^{-/-}$ mice at 6, 12, 18, and 19+ months were all significantly smaller than the wild type mean cross-sectional areas at 12, 18, and 19+ months (not specifically identified on Fig. 8A).

Effect of Loss of MYOD Expression on Total Fiber Number in the EOM

For the EOM data, ANOVA analysis indicated significance ($P = 0.0001$), thus post hoc Tukey's multiple comparison tests were performed. There were significantly fewer myofibers in the *MyoD*$^{-/-}$ mice compared to the wild type mice ($P < 0.0001$). Post hoc multiple comparison tests demonstrated that these differences were significant at all ages examined (3 months: 36% decrease, $P = 0.0001$; 6 months: 44.4% decrease, $P = 0.0001$; 12 months: 46.7% decrease, $P = 0.0001$; 18 months: 42.9% decrease, $P = 0.0001$; and 19+ months: 58.6% decrease, $P = 0.0001$; Fig. 8B).

Slow Myosin Heavy Chain Isoform Expression

For the EOM data, ANOVA analysis indicated significance ($P = 0.022$), thus post hoc Tukey's multiple comparison tests were performed. The percent of EOM myofibers that expressed type 1 MyHC was determined for *MyoD*$^{-/-}$ mice and wild type mice (Fig. 9). MyHC expression was significantly higher in the EOM orbital layer of 18 month and 19+ months old MyoD$^{-/-}$ mice compared to age-matched wild type mice, a 166.6% increase ($P = 0.037$) and 128.5% increase ($P = 0.039$), respectively (see Fig. 9).

Myogenic Precursor Cells

PAX7-Positive Cells. ANOVA analysis indicated significance ($P = 0.0001$), thus post hoc Tukey's multiple comparison tests were performed. The numbers of PAX7-positive nuclei in the tibialis anterior muscles of the *MyoD*$^{-/-}$ mice differed from that of age-matched wild type controls only at 3 and 6 months of age, where the number of these nuclei

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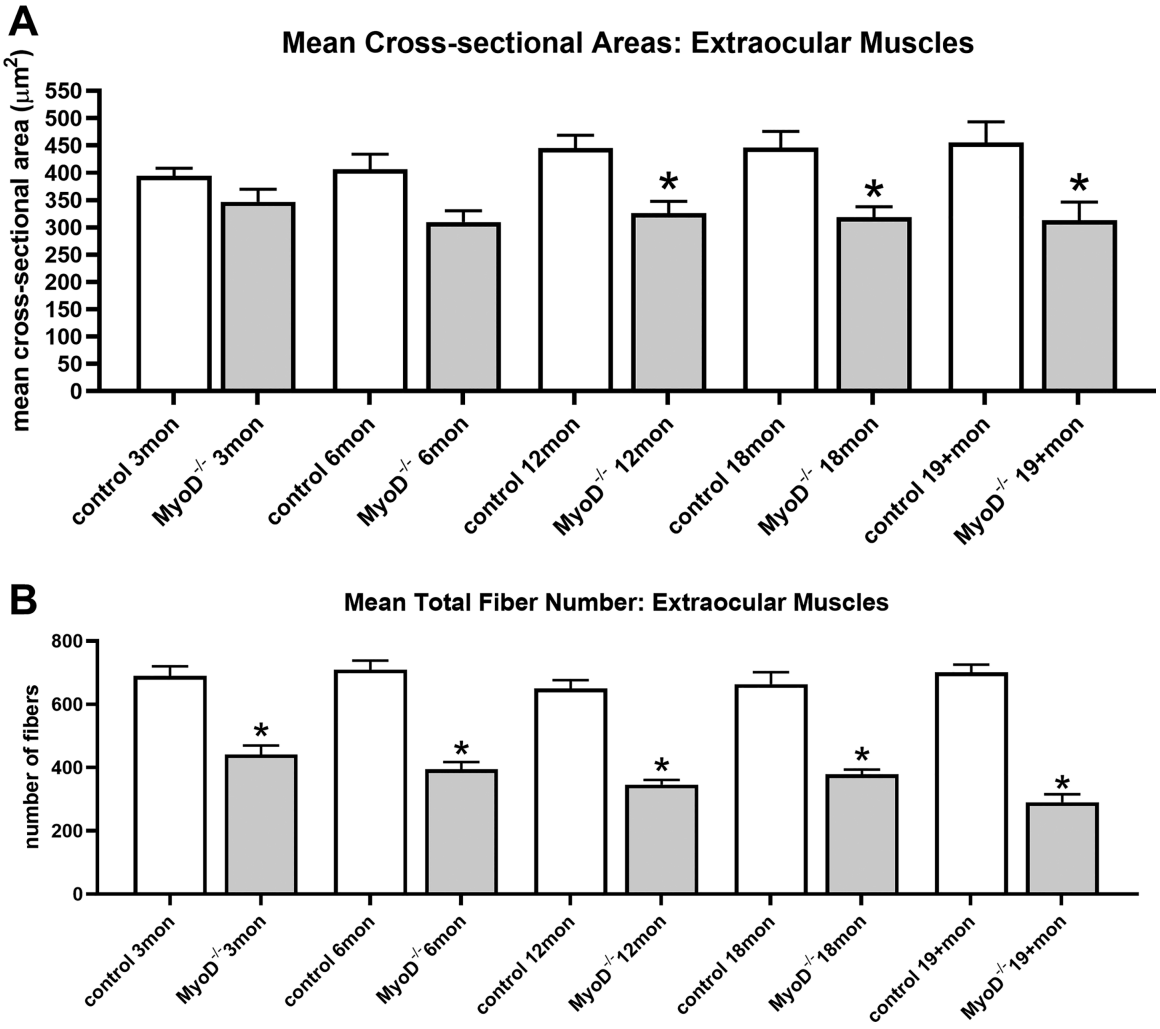

Figure 8. Myofiber mean-cross-sectional area and fiber number in the EOM is significantly decreased in aging *MyoD*$^{-/-}$ mice. (**A**) Morphometric analysis of mean myofiber cross-sectional area in wild type mice (*white bars*) and *MyoD*$^{-/-}$ (*gray bars*) mice at 3, 6, 12, 18, and 19+ months. There was a significant decrease in myofiber cross-sectional area compared to age-matched controls at 12 months, 18 months, and 19 months and older. (**B**) Morphometric analysis of total myofiber number in wild type (*white bars*) and *MyoD*$^{-/-}$ (*gray bars*) rectus muscles at 3, 6, 12, 18, and 19+ months. There were significantly fewer myofibers in the muscles from the *MyoD*$^{-/-}$ mice than the age-matched controls at every age. There were five mice for all ages and for each genotype. This represents measurements in all extraocular muscles on three slides each for each animal, which are then averaged for each animal. * Indicates significant difference from age-matched wild type mice.

were increased significantly (Fig. 10). At 3 and 6 months, the increases were 257% ($P < 0.0001$) and 260% ($P < 0.0001$), respectively, compared to the age-matched controls. There were no significant differences in the number of PAX7-positive nuclei in any of the other tibialis anterior muscles as the mice aged, and they also were not significantly different from the number in the wild type mouse tibialis anterior muscles at 3 months of age.

For the EOM data, ANOVA analysis indicated significance ($P = 0.002$), thus post hoc Tukey's multiple comparison tests were performed. In contrast to the pattern of PAX7-positive cells during aging in the tibialis anterior muscles, in the EOM, the number of PAX7-positive nuclei were increased in the *MyoD*$^{-/-}$ mice compared to the age-matched controls at 12 months ($P < 0.016$), 18 months ($P < 0.003$), and 19+ months ($P < 0.038$; see Fig. 10).

PITX2-Positive Cells. ANOVA analysis indicated significance ($P = 0.0001$), thus post hoc Tukey's multiple comparison tests were performed. The numbers of PITX2-positive nuclei were analyzed in the EOM based on their location inside or outside of immunostaining for dystrophin, which lines the internal surface of the sarcolemma (Fig. 11). There was a significant difference in the location of the PITX2-positive nuclei, from being inside the dystrophin ring and therefore myonuclei, to outside the dystrophin ring, in the *MyoD*$^{-/-}$ mice compared to the age-matched wild type mice (Fig. 12). For each of the 5 ages of mice, there were fewer numbers of PITX2-positive myonuclei in EOM from the *MyoD*$^{-/-}$ mice, with decreases of 33.2% ($P = 0.017$) 98.7% ($P = 0.02$), 81.8% ($P = 0.01$), 89.2% ($P = 0.008$), and 94.7% ($P = 0.004$), respectively. These correlated with increased numbers of nuclei outside the myofibers in the *MyoD*$^{-/-}$ mice. These were significantly increased compared to wild type mice at 3, 6, and 12 months of age, with increases of 235.4% ($P = 0.0001$), 270% ($P = 0.0001$), and 95.7% ($P = 0.02$), respectively. In the EOM from the mice 18 months and 19+ months of age, there was a 42.6% ($P = 0.28$) and 10.2% ($P = 0.96$) difference

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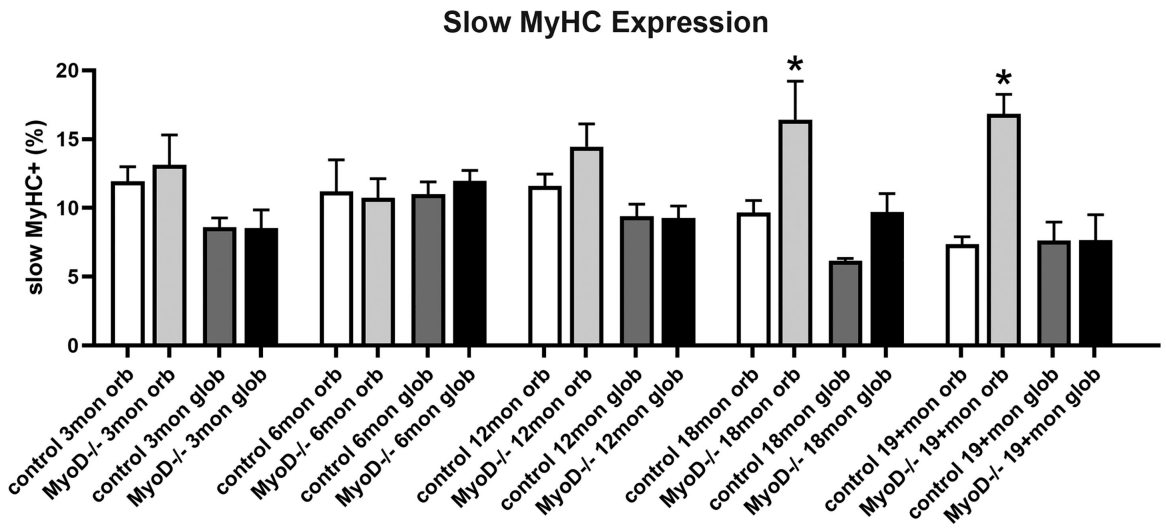

Figure 9. Increase of slow myofibers is significant in aging *MyoD*$^{-/-}$ mice. Percentage of myofibers expressing type 1 (slow) MyHC as a percentage of all myofibers in the both orbital and global layers of extraocular muscles from wild type mice (*white* and *dark gray*, respectively) and *MyoD*$^{-/-}$ (*light gray* and *black*, respectively) mice. There were no significant differences in the number of type 1 slow twitch muscle fibers except in the orbital layers of the *MyoD*$^{-/-}$ at 18 months and at 19+ months compared to their age-matched controls. There were four mice for all ages and for each genotype. This represents measurements in all extraocular muscles on three slides each for each animal, which were then averaged for each animal. * Indicates significant difference from age matched control mice.

over the control levels, but these were not significantly different.

Discussion

Due to the redundancy of *MyoD* and *Myf-5* in skeletal muscle formation, constitutive inactivation of *MyoD* results in relatively normal limb and body muscle development.[8] In our aging mice, from a functional viewpoint, there were no statistically significant differences between grip strength or average rotarod walking time in the *MyoD*$^{-/-}$ mice compared to controls. Despite some myonuclear addition that occurs in limb muscles over time,[4] the absence of MYOD expression in these aging mice had little apparent effect on limb muscle function over time. Only in grip strength measurements did we see a significant effect of aging and was seen in both the *MyoD*$^{-/-}$ and wild type mice, strongly suggesting that it is a function of sex and not related to the loss of MYOD expression. These results also suggest that this alteration in forelimb strength is also not directly attributable to aging, but may reflect the fact that the 3-month-old mouse, while sexually mature, has not achieved the adult equilibrium related to fiber size and myogenic precursor cell density.[38] These differences were not seen in rotarod walking duration. The EOMs, however, undergo extensive myonuclear turnover, even in aging.[4,5,35,36] This process requires MYOD expression,[39] and in its absence, we hypothesized that EOM function would be compromised. This was evident in the development of spontaneous nystagmus in the *MyoD*$^{-/-}$ mice as early as 3 months,[13] and the spontaneous nystagmus in the dark was not maintained in the oldest *MyoD*$^{-/-}$ mice. It is interesting to note that diaphragm muscle, which has a similarly high level of myonuclear addition, also showed functional deficits in *MyoD*$^{-/-}$ mice.[40] There was a reduced slow phase duration of eye movements in the oldest *MyoD*$^{-/-}$ mice in the absence of the OKN stimulus, and in the presence of the OKN stimulus, a reduced slow phase duration of the eye movements in all the *MyoD*$^{-/-}$ mice compared to wild type age matched controls. Based on the human studies of normal OKN responses with aging, which showed either decreased gain[30] or decreased slow phase velocity,[27–29,31] the changes with aging in the spontaneous nystagmus waveforms in our *MyoD*$^{-/-}$ mice were expected. In mouse age and human age equivalency charts, 3 to 6-month-old mice are the equivalent of 20 years old, at 1 year, they are the equivalent of humans at 50 years, at 18 months, they are the equivalent of 65 years, and by 19 months and older, they are the equivalent of 80 year old humans.[41]

As predicted by the maintenance of muscle function in the limbs of the *MyoD*$^{-/-}$ mice until 18 months and 19+ months, in limb muscle myofiber cross-sectional areas were only decreased compared to age matched wild type controls at the most advanced ages. In the EOMs, however, where nystagmus was evident throughout the lifespan, there were significant decreases in total myofiber number starting at 1 year, but significant decreases in mean cross-sectional areas even at 3 months of age. These decreases correlate with the loss of PITX2-positive myonuclei and concomitant increase in PITX2-positive cells outside the sarcolemma – suggesting an inability of these myogenic precursor cells to move down the differentiation pathway.[11,39,42]

In the leg muscles of the *MyoD*$^{-/-}$ mouse at 3 and 6 months of age, the number of PAX7-positive cells increased compared to the wild type mouse. These early time points mirror in vivo and in vitro studies showing the inability of PAX7-positive cells to fuse and differentiate in the absence of MYOD expression.[10,11,43] There were no significant changes in the number of PAX7-positive cells in the aging control limb muscles. Ultimately in the aging *MyoD*$^{-/-}$ mouse limb muscle, cells expressing PAX7 were significantly decreased and resembled levels in the control muscles. This low level of PAX7-positive cells correlated with the loss of myofiber size in the limb muscles at 18 months and older. It should be noted, however, that this did not translate into a significant loss of muscle function based on grip and

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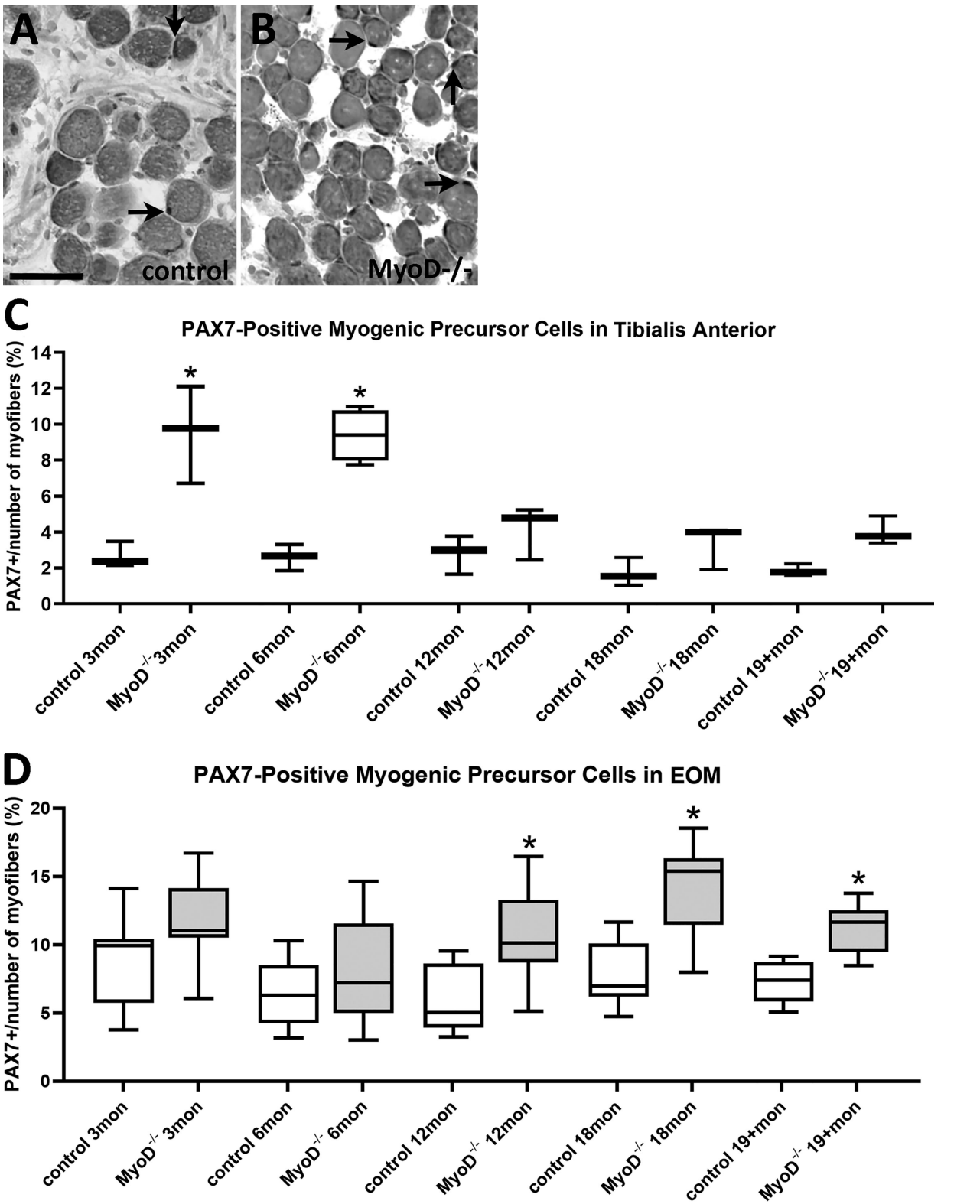

Figure 10. There are different effects of aging in the PAX7 population in EOM in wild type and *MyoD*$^{-/-}$ mice. (**A**) PAX7-positive cells in cross-sections of wild type extraocular muscles and in (**B**) *MyoD*$^{-/-}$ extraocular muscles. *Arrows* indicate PAX7-positive cells. Note that there appears to be a higher density of myofibers with a PAX7-positive nucleus associated with myofibers in the extraocular muscles from the *MyoD*$^{-/-}$ mice. Scale bar is 50 µm. (**C**) Morphometric analysis of PAX7-positive cell populations in *MyoD*$^{-/-}$ and wild type muscles at 3, 6, 12, 18, and 19+ months from tibialis anterior. In the short-term, there was an increase in PAX7-positive cells, but the number then decreased to extremely low levels as the animals age. (**D**) Morphometric analysis of PAX7-positive cell populations in *MyoD*$^{-/-}$ and wild type muscles at 3, 6, 12, 18, and 19+ months from extraocular muscles. As the animals age, there was a significant increase in the number of PAX7-positive cells in the extraocular muscles of the *MyoD*$^{-/-}$ mice. There were four mice for all ages and for each genotype. This represents measurements in all extraocular muscles on three slides each for each animal, which are then averaged for each animal. * Indicates significant difference from the age-matched controls.

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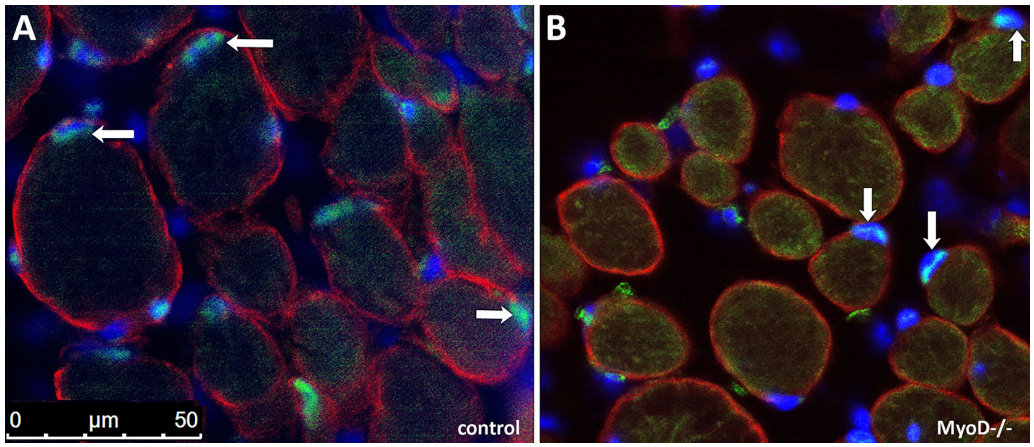

Figure 11. There are apparent changes to the number of PITX2 cells in *MyoD*$^{-/-}$ mice. Immunofluorescent staining of (**A**) 20 month wild type control extraocular muscles and (**B**) 20 month *MyoD*$^{-/-}$ extraocular muscles stained for PITX2 (*green*), dystrophin (*red*), and Hoechst (*blue*). If the nucleus is inside of the dystrophin staining of the sarcolemma, they are myonuclei, if outside the dystrophin ring, they are defined as PITX2-positive myogenic precursor cells if *green*. Hoechst identifies all nuclei within the section.

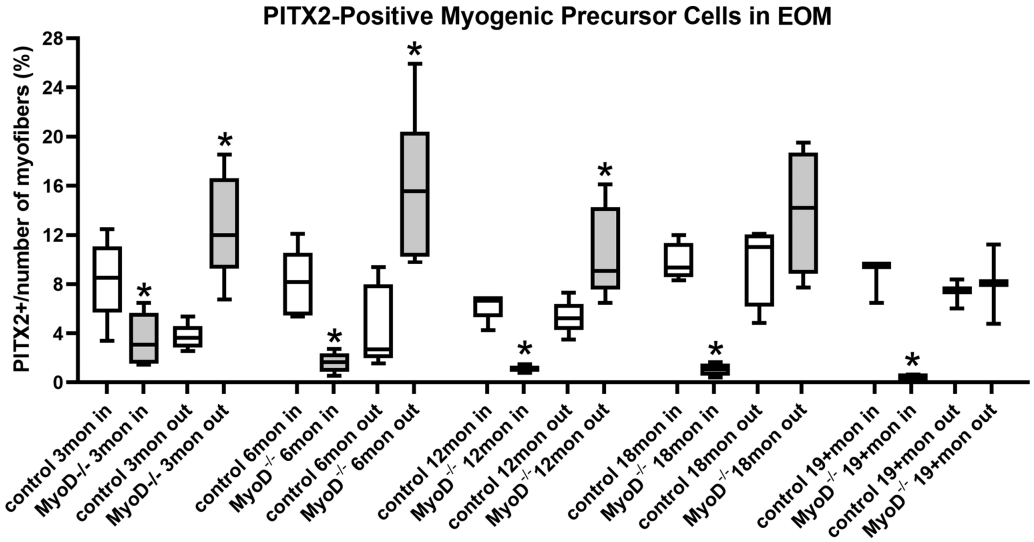

Figure 12. There is a significant change in location of the PITX2 cells in EOM between wild type and *MyoD*$^{-/-}$ mice with age. Morphometric analysis of PITX2-positive cell nuclei in wild type (*white bars*) and *MyoD*$^{-/-}$ (gray bars) extraocular muscles at 3, 6, 12, 18, and 19+ months. At all ages, there was a significant decrease in PITX2-positive myonuclei, and a concomitant increase in PITX2-positive cells outside the sarcolemma. This was significant up through 1 year, where the aging control extraocular muscles also saw an apparent increase in this population of PITX2-positive cells. There were three mice for all ages and for each genotype. This represents measurements in all extraocular muscles on three slides each for each animal, which are then averaged for each animal. * Indicates significant difference from the age-matched control for location of the labeled cells.

rotarod testing in the aging mice, although trended in that direction.

PAX7-positive cells are more abundant in the EOM compared to limb skeletal muscle.[33] On top of this already increased number in the EOMs, there was a significant increase in the PAX7-positive population of quiescent satellite cells at 1 year of age and older compared to the age-matched controls. Although increased numbers of PAX7-positive cells were only seen in young limb skeletal muscle, in contrast, in the EOMs, their number continued to increase over time. This is presumably due to their inability to effectively differentiate and fuse with existing myofibers, as was shown in a number of in vitro studies.[10,11,43] As there are high levels of myonuclear addition throughout the lifetime in EOMs, this increase in PAX7-positive cells supports the view that they were unable, in the absence of MYOD expression, to begin to differentiate and fuse into the EOMs.[4,5,35,36] One potential explanation for this process in the normal adult and aging EOMs is that they – at all ages – contain significant levels of neurotrophic factors, such as insulin-like growth factor-1.[44,45]

We previously showed that PITX2-positive cells represent a large proportion of the myogenic precursor cell population in the adult EOMs.[33] The numbers of PITX2-positive cells and PITX2-positive myonuclei relative to myofiber number stayed relatively unchanged in the normal EOM even in the oldest mice examined. However, even by 3 months in *MyoD*$^{-/-}$ mice, there were significantly decreased numbers of PITX2-positive myonuclei and increased numbers of PITX2-positive cells outside the sarcolemma. These signif-

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icantly decreased numbers of PITX2-positive myonuclei in the EOMs from the *MyoD*$^{-/-}$ mice became more significant as the mice aged. The inability to differentiate and fuse into existing myofibers from the lack of MYOD expression thus resulted in a large increase in these PITX2-positive cells within the *MyoD*$^{-/-}$ EOMs. Apparently, the lack of MYOD expression in the mutant mice resulted in the inability of either the PAX7-positive, PITX2-positive, or both cell types to differentiate and fuse into existing myofibers with the result that over time there was a significant loss of both myofiber number and myofiber mean cross-sectional areas. We hypothesize that the imbalance created by differential effects on the six EOMs[13] resulted in the presence of nystagmus in the *MyoD*$^{-/-}$ mice.

Our previous examination of surgical waste specimens from children with infantile nystagmus syndrome demonstrated that the EOMs had an increase in central nucleation,[46] usually associated with processes of regeneration in myofibers, or processes of denervation/reinnervation. In addition, these EOMs showed the presence of neuromuscular junctions that were smaller than controls and had increased numbers of neuromuscular junctions expressing the immature gamma isoform of the acetylcholine receptor.[47] These muscles from individuals with infantile nystagmus syndrome also showed increased numbers of myofibers positive for the type 1 (slow) MyHC isoform.[46] These changes were subsequently shown to be present during development of an animal model of infantile nystagmus syndrome.[48] These studies support the view that there are intrinsic changes within the EOMs in individuals with nystagmus, and based on the developmental study, may be a primary part of the development of nystagmus, as they are present before the eyes open.[48]

There are several limitations to our study. Due to our method of providing a stimulus that induces optokinetic nystagmus eye movements in normal mice, we cannot test our mice using a vertical stimulus. In addition, due to the nature of having to move the distances between mouse and camera for each individual mouse, we were not able to get measurements such as velocity that are possible in humans in a more fixed and controlled environment.

Collectively, these data support the view that mutations that affect the EOMs and their innervation can result in nystagmus in response to OKN stimuli, with spontaneous nystagmus waveforms in the dark present only in mice 12 months or younger. We hypothesize that the changes in the muscles would result in aberrant responses in the muscles, which we hypothesize are critical for the maintenance of the normal ocular motor function of eye movements.

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