Acute and chronic tirasemtiv treatment improves in vivo and in vitro muscle performance in actin-based nemaline myopathy mice

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Abstract

Nemaline myopathy, a disease of the actin-based thin filament, is one of the most frequent congenital myopathies. To date, no specific therapy is available to treat muscle weakness in nemaline myopathy. We tested the ability of tirasemtiv, a fast skeletal troponin activator that targets the thin filament, to augment muscle force—both in vivo and in vitro—in a nemaline myopathy mouse model with a mutation (H40Y) in Acta1. In Acta1H40Y mice, treatment with tirasemtiv increased the force response of muscles to submaximal stimulation frequencies. This resulted in a reduced energetic cost of force generation, which increases the force production during a fatigue protocol. The inotropic effects of tirasemtiv were present in locomotor muscles and, albeit to a lesser extent, in respiratory muscles, and they persisted during chronic treatment, an important finding as respiratory failure is the main cause of death in patients with congenital myopathy. Finally, translational studies on permeabilized muscle fibers isolated from a biopsy of a patient with the ACTA1H40Y mutation revealed that at physiological Ca2+ concentrations, tirasemtiv increased force generation to values that were close to those generated in muscle fibers of healthy subjects. These findings indicate the therapeutic potential of fast skeletal muscle troponin activators to improve muscle function in nemaline myopathy due to the ACTA1H40Y mutation, and future studies should assess their merit for other forms of nemaline myopathy and for other congenital myopathies.
Introduction

Congenital myopathies are a genetically heterogeneous group of early onset neuromuscular disorders characterized by distinct structural abnormalities in muscle fibers and by contractile weakness (1). The majority of these disorders are caused by defects either in the process of excitation–contraction coupling or in the assembly and interaction of proteins that make up the sarcomeres, the smallest contractile units in muscle. To date, no therapy is available to treat muscle weakness in congenital myopathies.

One of the most frequent congenital myopathies is nemaline myopathy (NEM; incidence ~1:50000 (2,3)). Currently, 12 genes have been implicated in NEM: alpha-actin 1 (ACTA1) (4), alpha- and beta-tropomyosin (TPM3 and TPM2) (5,6), nebulin (NEB) (7), leiomodin-3 (LMOD3) (8), troponin T (TNNT1) (9) and TNNT3) (10), coflin 2 (CFL2) (11), myopalladin (MYPN) (12), kelch family members 40 (KLHL40) and 41 (KLHL41) (13,14), and kelch repeat and BTB (POZ) domain containing 13 (KTBD13) (15). All of these genes encode proteins that are associated with the actin-based thin filament. Recent studies revealed that thin filament dysfunction is a major contributor to muscle weakness in NEM patients (16–23). Thus, thin-filament dysfunction is a therapeutic target in NEM.

Here, we aimed to test the ability of the small molecule, fast-twitch muscle troponin activator, tirasemtiv, to augment thin filament function in NEM. Tirasemtiv amplifies the response of the thin filament to calcium (Ca²⁺) in fast skeletal muscle fibers, leading to increased muscle force at submaximal rates of nerve stimulation (24). Thus, troponin activation is an appealing therapeutic approach in NEM. In our studies, we took advantage of an NEM mouse model with the heterozygous NM_009606.3(Acta1:p.His47Tyr mutation in Acta1 (25), one of the most frequently affected genes in NEM patients. Note that throughout the manuscript, this mutation is referred to as Acta1H40Y to be consistent with existing literature utilizing an older numbering scheme. We studied the acute and chronic in vitro and in vivo effects of tirasemtiv on skeletal muscle contractility and metabolism that included respiratory muscle, as respiratory failure is the main cause of death in NEM (1).

We found that acute and chronic treatment with tirasemtiv resulted in a profound increase in the force response to submaximal stimulation frequencies in Acta1H40Y mice. The energetic cost of force generation was reduced in muscle of tirasemtiv-treated Acta1H40Y mice. An interesting finding was that chronic treatment of Acta1H40Y mice with tirasemtiv also increased the force response to maximal stimulation, with no muscle mass increase. This suggests that muscle remodeling had occurred to improve contractility. Finally, studies on permeabilized muscle fibers isolated from a biopsy of a patient with the ACTA1H40Y mutation revealed that at physiological Ca²⁺ concentrations, tirasemtiv increased force generation to values that were close to those generated in muscle fibers of healthy subjects. Together, these findings indicate the therapeutic potential of fast skeletal troponin activators to alleviate muscle weakness in NEM.

Results

Baseline characteristics of the mouse model

Acta1H40Y mice had lower body weights and lower muscle mass, except for the soleus muscle which had significantly increased muscle weight (Fig. 1A). According to previous studies (24), tirasemtiv specifically affects the contractility of fast-twitch muscle fibers with fast skeletal muscle troponin, which express type 2 MHC isoforms. Therefore, we evaluated the MHC isoform composition of EDL and gastrocnemius muscles, the muscles selected for our contractility assays. As shown in Figure 1B, both EDL and gastrocnemius muscles contain mainly type 2B MHC isoforms, with EDL muscle also significant amounts of type 2X MHC isoforms. Minor differences in MHC isoform proportions were observed between Acta1(WT) and Acta1H40Y mice. The magnitude of the effect of tirasemtiv by type 2 MHC isoform is unknown. Therefore, we isolated permeabilized single muscle fibers from a WT mouse muscle, determined their calcium sensitivity of force in the presence and absence of tirasemtiv, and determined the MHC isoform in the fibers. As shown in Figure 1C, tirasemtiv increased the calcium sensitivity of force with a comparable magnitude in both type 2AX and 2B fibers (with, as expected, no effect in type 1 fibers). Thus, both EDL and gastrocnemius muscles are appropriate for testing the efficacy of tirasemtiv to improve muscle function in the Acta1H40Y mouse model. Further characterization of both muscles showed that the cross-sectional area (CSA) of individual muscle fibers was smaller in EDL and gastrocnemius of Acta1H40Y mice compared to WT mice (Fig. 1D). Similarly, the maximal force generating capacity was lower in EDL and gastrocnemius of Acta1H40Y mice compared to WT mice (Fig. 1E).

Thus, EDL and gastrocnemius muscles of Acta1H40Y mice display contractile weakness. Both muscle types contain a high proportion of fast-twitch muscle fibers, providing a large treatment window for testing the efficacy of acute and chronic tirasemtiv administration.

Effect of acute tirasemtiv administration

First, we evaluated the effect of acute administration of 3 μm tirasemtiv on the in vitro contractility of EDL muscle in 9-month-old Acta1(WT) and Acta1H40Y mice. This concentration was selected based on previous studies showing a maximal effect on contractility at 3 μm (without significantly slowing the rate of muscle relaxation) (26). Tirasemtiv induced a leftward shift of the force-stimulation frequency curve in both Acta1(WT) and Acta1H40Y EDL muscle (Fig. 2A, left panels). Consequently, the force generated at 40 Hz (normalized to maximal force at 200 Hz) increased by ∼50% in Acta1(WT) muscle and by ∼100% in Acta1H40Y mice (Fig. 2A, middle panels). Tirasemtiv did not affect the maximal force generated by EDL muscle (Fig. 2A, right panels). Absolute force values are shown in Table 1.

Second, we evaluated the effect of acute administration of 3 mg/kg tirasemtiv on the in vivo contractility of gastrocnemius muscle in 9-month-old mice. This treatment resulted in comparable tirasemtiv plasma concentrations in Acta1(WT) and Acta1H40Y mice (Acta1(WT): 11.7 ± 1.1 μM; Acta1H40Y: 9.8 ± 0.9 μM; assessed nocturnally, the time of day at which the contractility experiments were performed). Tirasemtiv increased the force generated at 20 Hz (normalized to maximal force at 150 Hz) by ~20% in Acta1(WT) muscle and by ~26% in Acta1H40Y mice (Fig. 2B, left panels). Tirasemtiv did not affect the maximal force generated by gastrocnemius muscle (Fig. 2B, right panels). Absolute force values are shown in Table 2.

Thus, these findings show that acute administration of tirasemtiv increased in vitro and in vivo submaximal force generation of Acta1H40Y EDL and gastrocnemius mouse muscles.

Effect of chronic tirasemtiv administration

Five-month-old Acta1(WT) and Acta1H40Y mice were fed chow enriched with tirasemtiv for 4 weeks. This treatment resulted
in comparable tirasemtiv plasma concentrations in Acta1(WT) and Acta1H40Y mice (Acta1(WT): 29 ± 4 μM; Acta1H40Y: 22 ± 3 μM; assessed nocturnally, the time of day at which the contractility experiments were performed). After 4 weeks, no effect of tirasemtiv on muscle mass was observed in Acta1(WT) and in Acta1H40Y mice (Fig. 3A). In line with this finding, no effect of chronic tirasemtiv administration on fiber CSA in gastrocnemius muscle was observed (Fig. 3B). Furthermore, chronic tirasemtiv administration did not affect the proportion of fast-twitch muscle fibers (Fig. 3C), an important finding as this indicates that the number of fibers sensitive to tirasemtiv was not affected by chronic administration.

First, we evaluated the effect of chronic administration of tirasemtiv on the in vitro contractility of EDL of Acta1(WT) and Acta1H40Y mice. Note that after excision of the muscles from the mouse leg and prior to the contractility assay, muscles were bathed for ~20 min in tirasemtiv-free Ringer solution. Previous work indicated that this time-frame is sufficient to completely remove tirasemtiv from the muscles. Interestingly, despite the removal of tirasemtiv, the chronic administration of tirasemtiv induced a small, but significant leftward shift of the force-stimulation frequency curve in both Acta1(WT) and Acta1H40Y EDL muscle (Fig. 4A, left panel). However, post-hoc analysis showed that the force generated at 40 Hz (normalized to maximal force at 200 Hz) was not significantly different between tirasemtiv-treated and vehicle-treated Acta1(WT) mice (Fig. 4A, middle panel). Note that the absolute force at 40 Hz was higher in tirasemtiv-treated than in vehicle-treated Acta1(WT) mice (Table 3). The Acta1H40Y mice had a similar response (Fig. 4A, left and middle panels).

Interestingly, chronic administration of tirasemtiv significantly increased the maximal force generated by EDL muscle by ~15% in Acta1(WT) mice and by ~43% in Acta1H40Y mice (Fig. 4A, right panels). Absolute force values are shown in Table 3.

Next, we evaluated the effect of chronic administration of tirasemtiv on the in vivo contractility of gastrocnemius muscle in Acta1H40Y mice. Note that during these in vivo assays, tirasemtiv was present in the muscles. After 4 weeks, Tirasesmtiv increased the force generated at 20 Hz (normalized to maximal force at 150 Hz) by ~25% in Acta1H40Y muscle (Fig. 4B, top panel). Tirasesmtiv also increased the maximal force (normalized to muscle...
Figure 2. Effects of acute tiraemtiv (Tira) administration on in vitro (A) and in vivo (B) muscle function in Acta1(WT) and Acta1H40Y mice. (A) Left panels: Force-stimulation frequency relation of EDL muscle. Middle panels: The force at 40 Hz stimulation relative to that at 200 Hz stimulation. Right panels: The force at maximal stimulation (200 Hz). (B) Left panels: The force at 20 Hz stimulation relative to that at 150 Hz stimulation in gastrocnemius muscle. Right panels: The force at maximal stimulation (150 Hz). Note that all data are presented relative to the vehicle (V) treated group.

Table 1. In vitro muscle mechanics—acute treatment

| Frequency | Diaphragm | EDL |
|-----------|-----------|-----|
|           | 20 Hz     | 40 Hz | 150 Hz | 200 Hz |
| Acta1(WT)—Vehicle | - | - | 70 ± 10 | 200 ± 20 |
| Absolute force (mN) | - | - | 125 ± 10 | 377 ± 18 |
| Normalized force (mN/mm²) | 57 ± 8 | 143 ± 13 | 32 ± 2 | 100 ± 0 |
| Relative force (% of maximum) | 43 ± 4 | 100 ± 0.1 | 100 ± 0 | 100 ± 0 |
| Acta1(WT)—Tiraemtiv | - | - | 100 ± 10b | 180 ± 4 |
| Absolute force (mN) | - | - | 160 ± 23b | 385 ± 27 |
| Normalized force (mN/mm²) | 64 ± 5b | 99 ± 0.4 | 47 ± 5b | 100 ± 0.2 |
| Relative force (% of maximum) | 76 ± 14b | 126 ± 14 | 160 ± 23b | 100 ± 0.2 |
| Acta1H40Y—Vehicle | - | - | 4 ± 1b | 10 ± 2b |
| Absolute force (mN) | - | - | 37 ± 7 | 81 ± 10b |
| Normalized force (mN/mm²) | 41 ± 1 | 100 ± 0.1 | 13 ± 3b | 34 ± 7b |
| Relative force (% of maximum) | - | - | 81 ± 10b | 100 ± 0.1 |
| Acta1H40Y—Tiraemtiv | - | - | 20 ± 3b | 22 ± 5 |
| Absolute force (mN) | - | - | 54 ± 11b | 29 ± 5b |
| Normalized force (mN/mm²) | 63 ± 5b | 100 ± 0.2 | 66 ± 6b | 93 ± 3 |
| Relative force (% of maximum) | - | - | 78 ± 13 | 39 ± 4 |

*aVehicle versus Tiraemtiv (P < 0.05)  
bActa1(WT) versus Acta1H40Y (P < 0.05)

Subsequently, the gastrocnemius muscle was subjected to repetitive stimulations at 20 Hz to induce fatigue. As shown in Figure 4B (lower panel, left), in tiraemtiv-treated Acta1H40Y mice the force generated was higher than in vehicle-treated mice at all time points. The fatigue index was comparable between tiraemtiv-treated and vehicle-treated Acta1H40Y mice. Interestingly, the reduction in phosphocreatine (PCr; Fig. 4B, lower panel, right) and pH (Vehicle: from 7.08 ± 0.02 to 6.98 ± 0.01; tiraemtiv: from 7.13 ± 0.03 to 6.96 ± 0.05) during the fatigue protocol was comparable between tiraemtiv-treated and vehicle-treated Acta1H40Y mice.

Thus, chronic administration of tiraemtiv increased submaximal force generation. Chronic administration also increased maximal force generation, suggesting that chronic administration of tiraemtiv induces muscle remodeling to improve contractility. The force generated during the development of fatigue was
Figure 3. Effects of chronic tirasemtiv (Tira) administration on muscle mass, fiber size and fiber type distribution. (A) Body and wet muscle weights, normalized over tibia length, in Acta1(WT) (left) and Acta1H40Y (right) mice. Data are presented relative to the vehicle (V) treated group. (B) Fiber cross-sectional area of gastrocnemius muscle. Right panel shows representative cryosections stained with wheat germ agglutinin to demarcate muscle fibers. Data are presented relative to the data from the vehicle (V) treated group. (C) The effect of chronic tirasemtiv administration on fiber type composition in gastrocnemius muscle.

Figure 4. Effects of chronic tirasemtiv (Tira) administration on in vitro (A) and in vivo (B) muscle function. (A) Left panels: Force-stimulation frequency relation of EDL muscle. Middle panels: The force at 40 Hz stimulation relative to that at 200 Hz stimulation. Right panels: The force at maximal stimulation (200 Hz). Note that data are presented relative to the vehicle (V) treated group. (B) Top left panel: The force at 20 Hz stimulation relative to that at 150 Hz stimulation in Acta1H40Y gastrocnemius muscle. Top right panel: The force at maximal stimulation (150 Hz) in Acta1H40Y gastrocnemius muscle. Note that data are presented relative to the vehicle (V) treated group. Bottom left panel: Force of gastrocnemius muscle in Acta1H40Y mice during a fatigue protocol. Bottom right panel: Phosphocreatine (PCr) levels in gastrocnemius muscle in Acta1H40Y mice during the fatigue protocol.
higher in tirasemtiv-treated mice, while the metabolic changes
were comparable. This indicates a lower energetic cost of
force generation in muscle of tirasemtiv-treated Acta1H40Y
mice.

Effect of tirasemtiv on the respiratory muscles

Diaphragm muscle of Acta1H40Y mice had a significantly larger
CSA of slow-twitch fibers than Acta1(WT) mice (Fig. 5A).
Tirasemtiv affects the contractility of fast-twitch muscle fibers,
which express type 2 MHC isoforms. Therefore, we evaluated
the MHC isoform composition of diaphragm muscles. As shown
in Figure 5B, diaphragm contains ∼5% type 1 MHC, a percentage
that was comparable between Acta1(WT) and Acta1H40Y mice.
Thus, the vast majority of fibers expressed type 2 MHC, with
about 2A MHC the most abundant one. In Acta1H40Y mice,
there was a significant increase in fibers expressing type 2A MHC at
the expense of type 2B and type 2X MHC. The maximal tetanic
force generation in muscle of Acta1H40Y −treated mice, while the metabolic changes
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Thus, the vast majority of fibers expressed type 2 MHC, with
type 2A MHC the most abundant one. In Acta1H40Y mice,
there was a significant increase in fibers expressing type 2A MHC at
the expense of type 2B and type 2X MHC. The maximal tetanic
tension (150 Hz) generated by isolated diaphragm strips was
lower in Acta1H40Y than in Acta1(WT) mice (Fig. 5C, left panel).
The force-stimulation frequency curve was comparable between
mice (Fig. 5C, right panel). Finally, we applied plethysmography
to determine the in vivo functioning of the respiratory muscles.
As shown in Figure 5D, tidal volume was higher in Acta1H40Y mice
than in Acta1(WT) mice, and respiratory frequency was reduced in
Acta1H40Y mice.

We evaluated the effect of acute administration of 3 μm
tirasemtiv on the in vitro contractility of diaphragm strips of
Acta1(WT) and Acta1H40Y mice. Tirasemtiv induced a leftward
shift of the force-stimulation frequency relation in both Acta1(WT)
and Acta1H40Y diaphragm (Fig. 6A, left panel). Consequently,
the force generated at 20 Hz (normalized to maximal force
at 150 Hz) was increased by 47% in the force generated at 20 Hz (normalized to maximal force at 150 Hz) was comparable (Fig. 6E, lower left panel), resulting in a higher force generated at 20 Hz (normalized to maximal force at 150 Hz; Fig. 6E, lower right panel). Chronic administration of tirasemtiv did not affect the maximal force generated by the diaphragm in Acta1(WT) and Acta1H40Y mice (Fig. 6F). Absolute force values are shown in Table 3.

We also assessed the effect of chronic tirasemtiv administra-
tion on in vivo respiratory muscle function using plethysmogra-
phy. Note that during these assays, tirasemtiv was present in the
respiratory muscles. Chronic administration of tirasemtiv did not
affect tidal volume and respiratory frequency during exposure
to 5% CO2 in both Acta1(WT) and Acta1H40Y mice (Fig. 6G and H;
Table 6). The data collected at room air (normal CO2), as well as
the absolute data, are shown in Table 6.

Effect of chronic tirasemtiv administration on protein
expression in gastrocnemius

We determined, in gastrocnemius muscle, the effect of chronic
tirasemtiv administration on the expression of several markers
of intracellular pathways involved in the regulation of muscle
atrophy/hypertrophy, oxidative stress and mitochondrial struc-
ture and function (see Supplementary Material, Figs S1 and S2).
Chronic administration of tirasemtiv increased Muf1 and Mfn2 levels in Acta1H40Y muscles, but decreased Mfn1 levels in Acta1(WT) muscles. tirasemtiv increased Opa1 levels in Acta1(WT) muscles, but not in Acta1H40Y muscles.

Effect of tirasemtiv on permeabilized quadriceps fibers of
a patient with the ACTA1H40Y mutation

Finally, we studied the ability of tirasemtiv to restore the force
generated at submaximal calcium levels in muscle fibers iso-
lated from muscle of a patient harboring the ACTA1H40Y
mutation. First, we performed histological assays in the muscle biopsy and observed in NADH-stained cryosections a relatively high proportion of fast-twitch fibers (Fig. 7A). Electron microscopy showed muscle fibers with severely damaged myofibrillar structure, nemaline rods (a hallmark feature of NEM) and nuclear rods, but also fibers with normal structure (Fig. 7B). Small bundles were permeabilized, exposed to solutions with incremental Ca2+ concentration, and the force generated was determined. The myosin heavy chain (MHC) composition of these small bundles consists of 33% type 1 and 67% type 2 fibers. Experiments were performed in the presence and absence of 10 μm tirasemtiv. As shown in Figure 7C, tirasemtiv induced a profound leftward shift of the force-pCa relation in fast-twitch fibers, indicating increased Ca2+ sensitivity of force. This shift was illustrated by an increase in the pCa50 (Fig. 7C, inset). Importantly, at low, yet physiological Ca2+ concentrations, tirasemtiv increased active tension in ACTA1H40Y fibers to values that were close to, or even exceeded those generated in fibers of healthy subjects (Fig. 7D). These findings show the promise of tirasemtiv.
Table 3. In vitro muscle mechanics—chronic treatment

| Frequency   | Diaphragm | EDL |
|-------------|-----------|-----|
|             | 20 Hz     | 150 Hz | 40 Hz   | 200 Hz |
| Acta1(WT)—Vehicle |          |       |          |         |
| Absolute force (mN) | -        | -      | 90 ± 10  | 270 ± 10 |
|Normalized force (mN/mm²) | 116 ± 13 | 212 ± 11 | 136 ± 9  | 409 ± 20  |
|Relative force (% of maximum) | 53 ± 4  | 100 ± 0.4 | 33 ± 1  | 100 ± 0  |
|Acta1(WT)—Tirasemtiv |          |       |          |         |
| Absolute force (mN) | -        | -      | 130 ± 10° | 310 ± 10° |
|Normalized force (mN/mm²) | 113 ± 11 | 216 ± 15 | 199 ± 14° | 482 ± 10° |
|Relative force (% of maximum) | 54 ± 2  | 100 ± 0.1 | 41 ± 3°  | 100 ± 0  |
|Acta1 H40Y—Vehicle |          |       |          |         |
| Absolute force (mN) | -        | -      | 30 ± 4b  | 80 ± 10b |
|Normalized force (mN/mm²) | 63 ± 12b | 119 ± 16 | 60 ± 8b  | 164 ± 18b |
|Relative force (% of maximum) | 50 ± 3  | 100 ± 0.2 | 36 ± 2  | 98 ± 1  |
|Acta1 H40Y—Tirasemtiv |          |       |          |         |
| Absolute force (mN) | -        | -      | 50 ± 5c  | 110 ± 10c |
|Normalized force (mN/mm²) | 78 ± 13  | 126 ± 18 | 91 ± 9c  | 211 ± 13 |
|Relative force (% of maximum) | 60 ± 3c | 99 ± 0.3 | 42 ± 2c  | 99 ± 0.3 |

Table 4. In vivo muscle mechanics—chronic treatment

| Frequency | Week 0 | Week 4 |
|-----------|--------|--------|
|           | 20 Hz  | 150 Hz | 20 Hz  | 150 Hz |
| Acta1H40Y—Vehicle |        |        |        |        |
| Absolute force (mN) | 39 ± 3  | 179 ± 13 | 34 ± 3  | 149 ± 8c |
|Normalized force (mN/mg) | -      | -      | 0.5 ± 0.04 | 2 ± 0.1 |
|Acta1H40Y—Tirasemtiv |        |        |        |        |
| Absolute force (mN) | 40 ± 5  | 177 ± 9  | 46 ± 4a  | 165 ± 6a |
|Normalized force (mN/mg) | -      | -      | 0.8 ± 0.1b | 3 ± 0.2a |

Table 5. Plethysmography—acute treatment

|         | Vehicle | Tirasemtiv |
|---------|---------|------------|
|         | Rest    | 5% CO₂  | Rest | 5% CO₂  |
| Acta1(WT) |        |          |      |         |
| Breathing frequency (per min) | 150 ± 5  | 214 ± 5  | 156 ± 3 | 211 ± 2  |
| Tidal volume (ml/kg) | 10 ± 0.2 | 14 ± 0.3 | 10 ± 0.3 | 15 ± 0.3a |
| Minute volume (ml/kg/min) | 1444 ± 49 | 2921 ± 114 | 1551 ± 48 | 3191 ± 60 |
| Acta1H40Y |        |          |      |         |
| Breathing frequency (per min) | 145 ± 4  | 209 ± 3  | 144 ± 2b | 203 ± 5  |
| Tidal volume (ml/kg) | 12 ± 0.3b | 17 ± 0.5b | 12 ± 0.2b | 18 ± 0.4a,b |
| Minute volume (ml/kg/min) | 1666 ± 61b | 3466 ± 118b | 1708 ± 50 | 3735 ± 116b |

Discussion

In skeletal muscles of mice with the Acta1H40Y mutation, acute in vitro or in vivo treatment with tirasemtiv increased the force response to submaximal stimulation frequencies. The increase in muscle force at submaximal stimulation persisted following chronic treatment of Acta1H40Y mice with tirasemtiv. Furthermore, 31P-MRS revealed that the increased force generation during treatment with tirasemtiv did not increase muscle energy consumption, indicating that the energetic cost of force generation was reduced in muscle of tirasemtiv-treated Acta1H40Y mice. Chronic treatment of Acta1H40Y mice with tirasemtiv also increased the force response to maximal stimulation, with no muscle mass increase. This suggests that muscle remodeling had occurred to improve contractility. Finally, studies on muscle
Figure 5. Characterization of respiratory muscles in the Acta1H40Y mouse model. (A) Diaphragm fiber cross sectional area; left panel shows typical diaphragm cryosections stained to identify slow-twitch muscle fibers. (B) MHC isoform composition in the diaphragm muscle as determined by SDS-PAGE. Inset: a typical SDS-PAGE result showing separation of the four isoforms. (C) Left panel: In vitro maximal tension of diaphragm strips stimulated with 150 Hz. Right panel: the force-stimulation frequency relation of diaphragm strips. (D) In vivo respiratory function, as determined by whole body plethysmography. Left panel shows tidal volume and right panel shows respiratory rate.

Table 6. Plethysmography—chronic treatment

|                     | Week 0                              | Week 4                              |
|---------------------|-------------------------------------|-------------------------------------|
|                     | Rest 5% CO2                         | Rest 5% CO2                         |
| Acta1(WT)—Vehicle   |                                     |                                     |
| Breathing frequency (per min) | 174 ± 3 249 ± 3                      | 166 ± 4 249 ± 2                      |
| Tidal volume (ml/kg) | 11 ± 0.2 16 ± 0.3                    | 10 ± 0.2c 16 ± 0.2                   |
| Minute volume (ml/kg/min) | 1846 ± 50 4090 ± 108                 | 1618 ± 42c 3934 ± 67                 |
| Acta1(WT)—Tirasemtiv |                                     |                                     |
| Breathing frequency (per min) | 168 ± 4 261 ± 4                      | 159 ± 3 244 ± 3                      |
| Tidal volume (ml/kg) | 11 ± 0.3 17 ± 0.3                    | 10 ± 0.2 16 ± 0.2                    |
| Minute volume (ml/kg/min) | 1797 ± 71 4282 ± 108                 | 1656 ± 57 4014 ± 98c                 |
| Acta1H40Y—Vehicle    |                                     |                                     |
| Breathing frequency (per min) | 157 ± 5b 247 ± 5                     | 144 ± 3bc 237 ± 5b                   |
| Tidal volume (ml/kg) | 13 ± 0.3b 20 ± 0.5b                  | 12 ± 0.4b 19 ± 0.6b                  |
| Minute volume (ml/kg/min) | 2015 ± 76 4894 ± 198b               | 1767 ± 71c 4551 ± 189b               |
| Acta1H40Y—Tirasemtiv |                                     |                                     |
| Breathing frequency (per min) | 157 ± 5 245 ± 5                      | 141 ± 4e 234 ± 4e                    |
| Tidal volume (ml/kg) | 12 ± 0.3 19 ± 0.3                    | 12 ± 0.4 20 ± 0.4bc                  |
| Minute volume (ml/kg/min) | 1879 ± 86 4530 ± 171                 | 1677 ± 59 4527 ± 117                 |

*aVehicle versus Tirasemtiv (P < 0.05)
*bActa1(WT) versus Acta1H40Y (P < 0.00)
*cWeek 0 versus Week 4 (P < 0.05)
fibers from a patient with the ACTA1*H40Y* mutation revealed that, at physiological Ca^{2+} concentrations, *tirasemtiv* increased force generation to values that were close to those generated in muscle fibers of healthy subjects. Together, these findings indicate the therapeutic potential of fast skeletal troponin activators to alleviate muscle weakness in patients with the ACTA1*H40Y* mutation.

The effect of *tirasemtiv* persists during chronic treatment of Acta1*H40Y* mice

NEM is a congenital myopathy, which, in the majority of cases, is caused by mutations in genes encoding proteins of the skeletal muscle thin filament (16). The most severely affected patients fail to survive beyond the first year of life due to severe muscle weakness. The mechanisms underlying muscle weakness have gained widespread attention during the past years. These include disturbed interactions between the thin and thick filaments, reduced length of the thin filaments, muscle fiber hypertrophy and myofibrillar disarray (17–19, 27–29). To date, no specific therapies are available for NEM patients. Considering the central role of the thin filament in the NEM pathology, targeting the functioning of this sarcomeric microstructure might prove an effective approach (1). Therefore, we tested the ability of *tirasemtiv*, a fast skeletal muscle troponin activator that specifically targets the troponin complex on the thin filament (24), to alleviate muscle weakness in NEM. We hypothesized that fast skeletal troponin activation would augment the force response to submaximal stimulation frequencies. These are clinically relevant frequencies considering that during daily life activities the force levels of muscle range between 10% and 65% of its maximal level (30). To test this hypothesis, we made use of the Acta1*H40Y* mouse model, a knock-in mouse with a mutation (p.His42Tyr) in the α-skeletal actin gene that causes a dominantly inherited severe form of the disease in humans (25). In line with our hypothesis, we found that acute administration of *tirasemtiv* markedly increases force generation at submaximal stimulation frequencies, with a more than 25% increase of force in gastrocnemius muscle *in vivo* (Fig. 2B). This effect is comparable to that observed after 4 weeks of chronic *tirasemtiv* treatment via chow (Fig. 4B), which is an important finding as it indicates that long-term administration of *tirasemtiv* does not desensitize the muscles to its effect. In addition to the persistent positive inotropic effect of chronic *tirasemtiv* treatment at submaximal stimulation frequencies, both EDL and gastrocnemius muscles showed increased force at maximal
Figure 7. Effect of tirsemtiv on the contractility of permeabilized quadriceps fibers of a patient with ACTA1H40Y mutation. (A) NADH stained cryosection of the patient’s muscle biopsy. The cryosection shows both slow-twitch (dark; *) and fast-twitch (light; #) muscle fibers. Bar: 100 μm. (B) electron microscopy image showing a fiber with severely damaged myofilibrar structure, nemaline rods (*) and a nuclear rod (arrow), and a fiber with preserved ultrastructure (#). (C) The relative force-pCa relation of permeabilized muscle fibers isolated from the patient’s biopsy (inset at top shows a typical bundle of 5–10 fibers used for the contractility assays; left bar graph shows the MHC composition of the muscle bundles; right bar graph shows the tirsemtiv-induced shift in the pCa50, i.e. the pCa required to generate 50% of maximal force). (D) The tension-pCa relation of treated and untreated permeabilized muscle fibers from the patient, compared to the tension-pCa relation of muscle fibers from healthy subjects. The yellow bar indicates the physiological calcium concentration in muscle fibers during normal contractility.

stimulation frequencies (increase in EDL: ~43%, in gastrocnemius: ~35%; Fig. 4). This finding is not explained by the direct effects of tirsemtiv, as at saturating calcium concentrations tirsemtiv does not augment force generation (24). Indeed, this inotropic effect was not observed during acute treatment with tirsemtiv (Fig. 2). Thus, this finding indicates that, during chronic tirsemtiv treatment, the EDL and gastrocnemius muscles adapt by structural remodeling.

The nature of this remodeling is not clear (it does not include increased muscle fiber size or muscle mass, or changes in fiber type proportion; Fig 3). It is tempting to speculate that the positive inotropic effects of tirsemtiv improved the myofilibrar structure in muscle fibers. Damaged myofilibrar proteins are labeled by chains of ubiquitin molecules, which mark them for degradation by the proteasome (31). Several major myofilibrar proteins, including myosin, are ubiquitinated by MuRF1 (32). We observed that in the Acta1H40Y mice, chronic tirsemtiv treatment increased the levels of MuRF1 (Supplementary Material, Fig. S2). This increase in MuRF1 protein might have facilitated the degradation of damaged proteins in Acta1H40Y mice, thereby improving the myofilibrar ultrastructure and force generation. Another mechanism via which tirsemtiv might have increased the force generating capacity of muscle following chronic treatment could be based on post-translational modifications of myofilibrar proteins, such as oxidation and nitrosylation. Both of them have been shown to be able to affect force production (33,34). Our results rule out the possibility that a tirsemtiv-induced decrease in the oxidation of myosin or other proteins is involved (Supplementary Material, Fig. S1), but leave open the possibility that other post-translational modifications play a role.

**Tirasemtiv reduces the energetic cost of contraction in Acta1H40Y mice**

The benefit from fast skeletal troponin activators, such as tirsemtiv, involves both increased force development and lower energy cost of contraction. In the present study, we showed, using in vivo 31P-MRS in Acta1H40Y mice, that chronic treatment with tirsemtiv increased force generation of gastrocnemius muscles during a fatigue protocol at a stimulation frequency of 20 Hz. Such an increase is very likely due to tirsemtiv reducing the off-rate of Ca2+ from fast skeletal muscle troponin, with no effect on Ca2+ release and cytosolic Ca2+ concentrations (35). Importantly, the tirsemtiv-induced increase in force was not accompanied by a faster decay in muscle PCr concentration, suggesting a similar energy consumption rate in treated and untreated muscles (Fig. 4B). As we assume that cytosolic Ca2+ content did not vary (35)—note that force was measured at set stimulation frequencies—the energy required by SERCA to
re-uptake Ca\textsuperscript{2+} should be comparable in treated and untreated muscles. Energy utilization of SERCA pumps accounts for a relevant portion, 30-40%, of total ATP consumption during contraction (36). Therefore, similar SERCA ATP utilization in the presence of higher force could play a significant role in the similar PCR depletion between treated and untreated muscles, and therefore in the lower energy cost of contraction in treated muscles. In line with our reasoning, it has been previously shown that CK-2066260, a fast skeletal muscle troponin activator similar to tirasemtiv, decreases ATP utilization and glycogen consumption in contractions developing the same force, rendering the muscle more efficient and fatigue-resistant (35).

Another mechanism via which tirasemtiv might have enabled more force production with similar PCR consumption is by enhancing mitochondrial fusion (upregulation of MFN1 and MFN2, Supplementary Material, Fig. S2), which in turn could improve mitochondrial function and ATP production. Whether this mechanism plays a role requires further studies.

Thus, tirasemtiv ameliorates the energy cost of contraction in muscles from Acta1\textsuperscript{H40Y} mice, possibly through optimization of ATP consumption of SERCA pumps and/or through the production of more ATP by mitochondria. This effect is very important for daily-life activities of NEM patients, considering that muscle fatigue is one of their major complaints.

**Tirasemtiv improves diaphragm contractility**

NEM patients have reduced spirometric values, and consequently some patients may suffer from the sensation of dyspnea (37-39) and die from respiratory failure. Thus, weakness of the respiratory muscles is prominent in NEM (note that the contractility of the diaphragm was significantly impaired in Acta1\textsuperscript{H40Y} mice; Fig. 5C) and augmenting respiratory muscle contractility can be of great benefit to patients. Therefore, in our work we also studied the effect of tirasemtiv on the respiratory muscles. Acute treatment with tirasemtiv improved the force response of the diaphragm to submaximal stimulation frequencies (Fig. 6B). Furthermore, using unrestrained whole body plethysmography, we found that tirasemtiv increased tidal volume (and decreased respiratory frequency to maintain minute volume; Fig. 6C and D). These effects on tidal volume are in line with previous work on a mouse model for amyotrophic lateral sclerosis (40). However, unlike the persisting effect of tirasemtiv on leg muscle contractility from chronic treatment (Fig. 4), the positive inotropic effect on respiratory muscle function did not persist following chronic treatment (except for a 20% increase in the force response to submaximal stimulation; Fig. 6E). We speculate that since tidal volume was not reduced in Acta1\textsuperscript{H40Y} mice—even slightly increased (Fig. 5)—there was no physiological need to modulate tidal volume. Thus, as tidal volume was maintained, the energy cost of contraction of the respiratory muscles must have been reduced in the tirasemtiv-treated mice, an important benefit which in patients could attenuate the development of respiratory failure. Unfortunately, we could not assess energy consumption in the diaphragm using 31P-MRS. We cannot explain why tirasemtiv increased tidal volume during acute treatment (Fig. 6C). We speculate that this increase was transient, and that the monitoring time (30 min) might not have been sufficiently long for the mice to adapt their respiratory mechanics to the positive inotropic effects of tirasemtiv on the inspiratory muscles.

**Clinical perspective**

The positive inotropic effect of tirasemtiv in the Acta1\textsuperscript{H40Y} mouse model was mimicked in a patient biopsy with the ACTA1(H40Y) mutation. At physiological calcium concentrations, the force generated by the patient’s fibers nearly doubled in the presence of tirasemtiv (Fig. 7), and reached values that were close to those of healthy subjects. Although, recently, tirasemtiv did not meet its primary endpoint in a phase 3 clinical trial in ALS patients in part due to its side effect profile, including dizziness (41), our findings illustrate the great therapeutic promise of fast skeletal muscle troponin activation. Reldesemtiv, a newer fast skeletal muscle troponin activator, does not appear to exhibit the same side effect profile as tirasemtiv and is currently being developed and tested for efficacy in clinical trials (35,42-45). Fast skeletal muscle troponin activators target fast-twitch muscle fibers. The improved contractility of these fibers improves strenuous muscle exercise as well as coughing and airway clearance—both major challenges for NEM patients—as these maneuvers are accomplished through the recruitment of fast-twitch fibers. It is possible that in patients, the magnitude of the effect of tirasemtiv is blunted by slow-twitch fiber predominance, a common feature of NEM (1). The patient’s fiber bundles studied here consisted of a mix of slow- and fast-twitch fibers, with predominance of fast-twitch fibers. Consequently, the effect of tirasemtiv was large (Fig. 7). Clearly, the effect size will decrease with slow-twitch fiber predominance. However, although NEM patients display a heterogeneous phenotype, fatigue and dyspnea are common complaints. Thus, even a modest improvement of diaphragm muscle function would benefit NEM patients and attenuate the development of dyspnea and respiratory failure, the major cause of death in NEM. Clearly, NEM patients might also benefit from the recruitment of slow-twitch fibers. During normal breathing and low intensity exercises, these fibers are first recruited. An additional advantage of recruiting slow-twitch fibers is the above-mentioned slow-twitch fiber predominance in NEM patients. Levosimendan is a calcium sensitizer that is approved for human use by the European Medicines Agency for acutely decompensated severe chronic heart failure. It exerts its effect through binding to slow skeletal/cardiactroponin C, which is also the dominant troponin C isoform in slow-twitch skeletal muscle fibers. However, previous studies showed no inotropic effect of levosimendan on slow-twitch fibers of NEM patients (46). Furthermore, a disadvantage of compounds that target slow skeletal/cardiactroponin C might be that they also affect cardiac function. To date, no activators specific for TnC in slow-twitch skeletal muscle fibers—with no effect on cardiomyocytes—have been developed. Alternatively, future studies might address the development of activators that target slow skeletal TnI, which is exclusively expressed in skeletal muscle.

**Materials and Methods**

**Acta1\textsuperscript{H40Y} knock-in mouse model**

Mice with the heterozygous NM_009606.3(Acta1):p.His42Tyr mutation in Acta1 (referred to as Acta1\textsuperscript{H40Y} to be consistent with existing literature utilizing an older numbering scheme) and wild-type (WT) littermates were used for the experiments, a well-established NEM knock-in mouse model (25,47,48). Experiments were conducted in agreement with the French and Dutch guidelines for animal care. All animal experiments were approved by the Institutional Animal Care Committee of Aix-Marseille University (#15-14052012) and by the local animal ethics committee at VU University (AVD14002016501).
Experiments were only performed on females given that the majority of males typically die within the first 6-8 weeks after birth (25). Mice were housed in an environment-controlled facility (12-12 h light-dark cycle, 22°C), received water and standard food ad libitum. Mice were identified through PCR genotyping from mouse tail DNA.

**Tirasemtiv treatment**

For the studies in which the acute effects of tirasemtiv were studied, mice were I.P. injected with vehicle or 3 mg/kg tirasemtiv (40). Experiments were performed ~30 min after injection. For the experiments in which muscle contractility was studied in vitro, 10 μM tirasemtiv was added to the experimental solutions.

For the studies in which the chronic effects of tirasemtiv were studied, mice were first fed for 1 week with custom-made mouse pellets (BioServ). After 1 week, mice were switched to the same pellets containing tirasemtiv (600 ppm), or the same pellets without tirasemtiv. Mice were kept on the chow for 4 weeks. Mice were tested before treatment and after 4 weeks of tirasemtiv-enriched diet or regular diet. After 4 weeks, mice were euthanized and tissues were collected, i.e. tibialis anterior (TA), extensor digitorum longus (EDL), soleus (SOL), gastrocnemius and diaphragm (DIA).

**In vitro characterization of intact muscle**

In vitro characterization of intact muscle was performed as described previously (20,49). The experimental protocols consisted of a full tetanus at 150 Hz and a force-frequency protocol.

For the force-frequency protocol, the muscle was stimulated with incremental stimulation frequencies (diaphragm: 1, 5, 10, 20, 30, 40, 60, 80, 100, 150 Hz; EDL: 1, 5, 10, 20, 40, 60, 80, 100, 150, 200 Hz). Data were discarded when stimulation at 150 Hz rendered a force that was less than 95% of the force generated during the first stimulation at 150 Hz. Stimuli were applied with a train duration of 600 ms. The resting interval was 30 s between the stimulations at 1 and 10 Hz; 60 s after stimulation at 20 Hz; 90 s after stimulation at 30 Hz; and 120 s between stimulations at 50, 70, 100 and 150 Hz.

After completion of the contractility measurements, the length and weight of the muscles were determined. CSA (in mm²) was calculated by dividing muscle weight (g) by muscle length (mm) multiplied by specific density (1.056 g/ml) × 100.

**Plethysmography**

Mice were placed in unrestrained whole body plethysmography chambers for 30 min of acclimation. After acclimation, tidal volume, respiratory frequency and minute ventilation were monitored for 15 min at room air. After 15 min, mice were exposed to a 5% CO₂ gas mixture for 30 min and monitored. After the 5% CO₂ exposure, mice were re-exposed to room air for 15 min and monitored (40).

**In vivo investigations of the plantar flexor muscles**

Animal preparation: Mice were anaesthetized and individually placed supine in a home-built cradle specially designed for the strictly non-invasive functional investigation of the left hindlimb muscles as described previously (50).

Force output measurements: Non-invasive transcutaneous electrical stimulation was first elicited with square-wave pulses (0.5 ms duration) on the plantar flexor muscles. The individual maximal stimulation intensity was determined by progressively increasing the stimulus intensity until there was no further peak twitch force increase. Plantar flexion force was assessed in response to incremental frequencies (1–150 Hz; train duration = 0.75–1 s) and during a fatigue protocol (80 contractions; 20 Hz; 1.5 s on, 6 s off).

The peak force of each contraction was measured. Regarding the fatigue protocol, the corresponding tetanic force was averaged every five contractions. A fatigue index corresponding to the ratio between the last five and the first five contractions was determined. For chronic experiments, the resulting force was divided by the soleus and gastrocnemius muscle weight in order to obtain specific force (in mN/mg).

Experiments were performed in a 4.7-Tesla (T) horizontal superconducting magnet (47/30 Biospec Avance, Bruker, Ettlingen, Germany) equipped with a Bruker 120 mm BGA12SL (200 mT/m) gradient insert.

Metabolic changes were investigated using 31P-Nuclear Magnetic Resonance Spectroscopy (13P-MRS) at rest and during the fatiguing protocol. Spectra from the gastrocnemius muscle region were continuously acquired at rest and throughout the fatigue protocol. A total of 495 free induction decays (FID) were acquired (TR = 2 s).

Data were processed using proprietary software developed using IDL (Interactive Data Language, Research System, Inc., Boulder, CO, USA). The first 180 FID were acquired at rest and summed together (n = 1, time resolution = 6 min). The next 315 FID were acquired during the stimulation period and summed by blocks of 105 (n = 3, time resolution = 3.5 min). Relative concentrations of high-energy phosphate metabolites (phosphocreatine (PCr) and inorganic phosphate (Pi)) were obtained by a time-domain fitting routine using the AMARES-MRUI Fortran code and appropriate prior knowledge of the ATP multiples. Intracellular pH (pHᵢ) was calculated from the chemical shift of the Pi signal relative to PCr (51).

**Muscle fiber cross-sectional area analysis**

Muscle fibre CSA was determined in the mid-belly region of gastrocnemius and EDL muscles and in a portion of diaphragm muscle (52). Briefly, muscle serial transverse sections (10 μm thick) were obtained from each muscle and were immunostained with monoclonal antibodies against MHC isoforms (BA-F8 against MHC-1 and SC-71 against MHC-2A). The cryosections were incubated with primary antibody for 1 h at 37°C, rinsed with PBS buffer and incubated in a secondary rabbit anti-mouse antibody conjugated with peroxidase (DAKO, Denmark) for 1 h at 37°C. After washing in PBS buffer, the stain was visualized by using a DAB (3,3-Diaminobenzidine) solution. Images of the stained sections were captured from a light microscope (LeicaDMLS) equipped with a camera (Leica DFC280). Fibre CSA was measured with Image J analysis software (NIH, Bethesda, MD, USA) and expressed in micrometers squared.

**MHC isoforms composition**

Frozen muscles were pulverized in a steel mortar with liquid nitrogen to obtain a powder that was immediately resuspended in Laemmli solution (53). The samples were incubated in ice for 20 min and finally spun at 18,000g for 30 min. Protein concentration in the dissolved samples was determined with a protein assay kit (RC DC Biorad). About 10 μg of proteins for each sample were loaded on 8% SDS-PAGE polyacrylamide gels and the electrophoresis was run overnight at 250 V. Following
A Coomassie stain, four bands corresponding to MHC isoforms were separated and their densitometric analysis was performed to assess the relative proportion of MHC isoforms (MHC-1, MHC-2A and MHC-2X) in the samples (54).

**Western blot analysis**

Frozen muscle samples were pulverized and immediately re-suspended in a lysis buffer (20 mM Tris–HCl, 1% Triton X100, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 100 mM NaF and 2 mM NaPPi supplemented with 1% protease, phosphatase inhibitors (Sigma-Aldrich) and 1 mM PMSF). The homogenate obtained was kept on ice for 20 min and then centrifuged at 18,000g for 20 min at 4°C. The supernatant was stored at −80°C until ready to use. Protein concentration was evaluated for each sample and equal amounts of muscle samples were loaded on gradient prestain gels purchased from Bio-Rad (AnyKd; Hercules, CA, USA). After the gel run, proteins were electro-transferred to PVDF membranes purchased from Bio-Rad (AnyKd Biorad gels) followed by western blotting. Proteins were separated by polyacrylamide gel electrophoresis (4%, 5%, 7.5%, 12%) in the presence/absence of 2,4-dinitrophenylhydrazone (DNP) by reaction with 2,4-dinitrophenylhydrazine (DNPH). In detail, 10 μg of proteins for each muscle sample were denatured with SDS solution at a final concentration of 6%. The DNPH solution was added to obtain the derivation; the reaction was stopped after 10 min of incubation at room temperature. The DNP-derivatized protein samples were separated by polyacrylamide gel electrophoresis (AnykD Biorad gels) followed by western blotting. Proteins were transferred to nitrocellulose membranes at 100 V for 2 h, stained with Ponceau Red (Sigma) and then scanned. The membranes were blocked by incubation with 3% bovine serum albumin (BSA) for 1 h; then incubated with rabbit anti-DNP antibody overnight at 4°C and subsequently with a horseradish peroxidase-antibody conjugate (goat anti-rabbit IgG). The positive bands were visualized by using a chemiluminescent reagent (ECL advance as described previously (55). The total protein carbonylation level and the MHC carbonylation level were analyzed quantitatively by comparison of the signal intensity of immune-positive proteins normalized on total proteins amount loaded on gels (Ponceau staining signal) (55).

**Gene expression analysis**

Total RNA was extracted from gastrocnemius muscles using an SV Total RNA isolation kit (Promega, Madison, WI, USA). The RNA concentration was measured using a Nano Drop instrument (ThermoScientific, Waltham, MA, USA) and 400 ng was used to generate cDNA with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA was analyzed by quantitative RT-PCR (Applied Biosystems AB7500) using a SYBR Green PCR kit (Applied Biosystems, Foster City, CA, USA) and the data were normalized to GAPDH content. Oligonucleotide primers were provided by Sigma Aldrich and were: MuRF-1 (FP: ACCTGCTGTGTTGAAAAACATC, RP: ACCTGCTGTGTTGAAAA-CATC) and Atrogin-1 (FP: GCAAAACTGTCACATTCTCTC, RP: CTTGAGGGAAAAATGAGAC). Differentially expressed genes were determined using a default threshold of 0.6. The difference between Ct (cycle threshold) values was calculated for each mRNA by determining the brightness–area product of the protein band (55).

**Patient muscle biopsies**

Quadriiceps muscle specimens, remaining from diagnostic procedures, were collected from a patient with the NM_001100.3 (ACTA1)His427Tyr mutation in ACTA1 (ACTA114607, case 86-1 from Agrawal and coworkers (56)). Ethical approval was obtained from the Human Research Ethics Committees of the Boston Children’s Hospital Institutional Review Board. Quadriiceps biopsies from six adult control subjects with no medical history were obtained. All biopsies were collected following informed consent supervised by the Radboud University Institutional Review Board (20). All biopsies were stored frozen and unfixed at −80°C until use.

**Permeabilized muscle fiber mechanics in patient biopsy**

Small strips were dissected from the muscle biopsies, permeabilized overnight and mechanical experiments were performed as described previously (18–20). As the contractile properties of muscle fibers are influenced by the MHC composition of the muscle fibers, we used a specialized SDS-PAGE technique to analyze the MHC isoform composition in the muscle fibers used in contractility experiments (57). In brief, muscles fibers were denatured by boiling for 2 min in SDS sample buffer. The stacking gel contained a 4% acrylamide concentration (pH 6.7), and the separating gel contained 7% acrylamide (pH 8.7) with 30% glycerol (v/v). The gels were run for 24 h at 15°C and a constant voltage of 275 volt. Finally, the gels were silver-stained, scanned, and analyzed with ImageQuant TL (GE Healthcare, Chicago, IL) software.

To test the effect of tirasemtiv on the calcium sensitivity of force, patient fibers were exposed to solutions with varying pCa’s (protocol as described above) and in the presence/absence of tirasemtiv (10 μM; concentration based on previous studies (42); tirasemtiv dissolved in 1% dimethylsulfoxide). Note that...
1% dimethylsulfoxide did not affect muscle fiber contractility (data not shown). After completion of the experiments, the MHC composition of the fibers was determined as described above.

**Effect of tirasemtiv on murine muscle fiber types**

Tirasemtiv is a fast skeletal muscle troponin activator, but the effect of tirasemtiv on individual murine type 2A, 2X and 2B fast muscle fibers is not known. Thus, to test the effect of tirasemtiv on the various muscle fiber types in mouse muscles, individual fibers were isolated from EDL and permeabilized as described above. The contractility of the permeabilized fibers was determined in the presence/absence of 10 μM tirasemtiv (protocol as described above). After completion of the experiments, the MHC isoform composition of the fibers was determined. The resolution of our SDS-PAGE allowed for identification of type 1 and 2B MHC isoforms; type 2A and 2X isoforms appeared as one band and these fibers were therefore grouped.

**Statistics**

Data are presented as mean ± standard error of the mean. For statistical analyses, one-way ANOVA’s, two-way ANOVA’s with Sidak’s multiple comparison tests and two-tailed statistical analyses, one-way ANOVA’s, two-way ANOVA’s with Sidak’s multiple comparison tests and two-tailed statistical analyses were used. A probability value < 0.05 was considered statistically significant.

**Supplementary Material**

Supplementary Material is available at HMG online.

Conflict of Interest statement. D.T.H. and F.I.M. are employees of Cytokinetics and were financially compensated for their work.

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