

Hypertrophy and dietary tyrosine ameliorate the phenotypes of a mouse model of severe nemaline myopathy

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Nemaline myopathy, the most common congenital myopathy, is caused by mutations in genes encoding thin filament and thin filament-associated proteins in skeletal muscles. Severely affected patients fail to survive beyond the first year of life due to severe muscle weakness. There are no specific therapies to combat this muscle weakness. We have generated the first knock-in mouse model for severe nemaline myopathy by replacing a normal allele of the α -skeletal actin gene with a mutated form (H40Y), which causes severe nemaline myopathy in humans. The Acta1(H40Y) mouse has severe muscle weakness manifested as shortened lifespan, significant forearm and isolated muscle weakness and decreased mobility. Muscle pathologies present in the human patients (e.g. nemaline rods, fibre atrophy and increase in slow fibres) were detected in the Acta1(H40Y) mouse, indicating that it is an excellent model for severe nemaline myopathy. Mating of the Acta1(H40Y) mouse with hypertrophic four and a half LIM domains protein 1 and insulin-like growth factor-1 transgenic mice models increased forearm strength and mobility, and decreased nemaline pathologies. Dietary L-tyrosine supplements also alleviated the mobility deficit and decreased the chronic repair and nemaline rod pathologies. These results suggest that L-tyrosine may be an effective treatment for muscle weakness and immobility in nemaline myopathy.

Keywords: nemaline myopathy; animal models; tyrosine; treatments

Abbreviations: FHL1 = four and a half LIM domains protein 1; IGF-1 = insulin-like growth factor-1; PND = post-natal day

Introduction

The congenital muscle disease, nemaline myopathy, is a clinically and genetically heterogeneous condition caused by mutations in genes that encode skeletal muscle thin filament and thin filament-associated proteins (Laing *et al.*, 1995; Nowak *et al.*, 1999; Wallgren-Pettersson *et al.*, 1999; Sanoudou and Beggs, 2001). Patients with nemaline myopathy are clinically defined by skeletal muscle weakness of varying onset and severity and the presence of electron-dense nemaline rods in the skeletal muscles (Wallgren-Pettersson *et al.*, 1999; Sanoudou and Beggs, 2001; Nguyen and Hardeman, 2008). The large spectrum of histological features of this family also includes: aggregations of thin filaments; mitochondrial abnormalities; abnormal fibre sizes (either atrophy and/or hypertrophy); predominance of slow, oxidative fibres; and myofibre regions lacking myosin thick filaments (Wallgren-Pettersson *et al.*, 1988). A chronic focal repair process was identified as an additional pathology of nemaline myopathy (Sanoudou *et al.*, 2006). This repair is distinct from the classical form of muscle regeneration that occurs in the muscular dystrophies where there is extensive myonecrosis and large numbers of regenerating myofibres with centralized nuclei. The discovery of myofibre repair/regeneration as a general feature of nemaline myopathy suggests that therapies designed for dystrophies may also be applicable to nemaline myopathy.

Increasing the size of muscle fibres, hypertrophy, has been shown to improve muscle strength in the elderly (Dupler and Cortes, 1993) and in patients with neuromuscular diseases (Lindeman *et al.*, 1999). Indeed, there are a number of studies showing that ectopic expression of hypertrophic factors [e.g. myostatin and insulin-like growth factor-1 (IGF-1)] can reduce muscle weakness and disease pathology in dystrophic mice (Barton *et al.*, 2002; Bogdanovich *et al.*, 2002; Gregorevic *et al.*, 2002; Parsons *et al.*, 2006). There is some circumstantial evidence suggesting that fibre hypertrophy may also be beneficial for nemaline patients. This is mainly through the observations that nemaline patients with mild forms of the disease and the *TPM3*(M9R) mouse model, which displays mild muscle weakness, have large fast/glycolytic fibres (Wallgren-Pettersson, 1989; Corbett *et al.*, 2001). Thus, one of the aims of the present study was to examine the ability of hypertrophic factors [IGF-1, *c-Ski* and four and a half LIM domains protein 1 (FHL1)] to improve muscle weakness and disease pathology in a mouse model of severe nemaline myopathy.

Protein and amino acid supplements have long been used with exercise training to improve athletic performance and muscle strength (Crowe *et al.*, 2006; Campbell and Leidy, 2007). There are a few reports suggesting that patients with nemaline myopathy may also benefit from L-tyrosine supplements (Kalita, 1989; Wallgren-Pettersson and Laing, 2003; Ryan *et al.*, 2008). The first report described an adult male patient and his 7-year-old son who, when given daily L-tyrosine supplements (250–500 mg), improved whole body strength (in the father), decreased excessive pharyngeal secretions (in the son) and improved general stamina (both patients) (Kalita, 1989). Ten days of tyrosine withdrawal returned both patients to preclinical conditions, suggesting the

beneficial effect was specifically due to L-tyrosine administration. More recently, a clinical trial of L-tyrosine supplements was performed on a small ($n = 5$) cohort of patients with nemaline myopathy of various ages and disease severity (Ryan *et al.*, 2008). Within 72 h of treatment, four of the five patients reported a decrease in excessive saliva secretion and improvements in muscle strength and 'energy' levels, but no long-term improvements were noted. Unfortunately, the study was under-powered (only five patients with varying levels of disease severity) and uncontrolled (no placebo group, large age variability and variable disease mutations) and firm conclusions about the beneficial effect of L-tyrosine could not be made.

Previously, we generated the first animal model of nemaline myopathy by expressing the *TPM3*(Met9Arg) mutation in a transgenic mouse (Corbett *et al.*, 2001). This mouse had the features of patients with this mutation including cytoplasmic nemaline rods, fast-fibre (IIB) hypertrophy, increase in proportion of slow fibres and mild late-onset muscle weakness. In order to trial potential therapies for nemaline myopathy and other congenital myopathies, we have now generated a knock-in mouse with a mutation (H40Y) in the α -skeletal actin gene (*Acta1*) that causes a dominantly inherited severe form of the disease in humans (Nowak *et al.*, 1999). The *Acta1*(H40Y) mouse has the features of the human condition, including early lethality, severe muscle weakness, cytoplasmic and intranuclear rods, and focal muscle repair and regeneration. Mating the severely affected *Acta1*(H40Y) mice with hypertrophic IGF-1 (Musaro *et al.*, 2001) and FHL1 (Cowling *et al.*, 2008) transgenic mice or treatment with dietary L-tyrosine supplements alleviated muscle weakness, reduced muscle pathologies and increased mobility. These findings clearly suggest that nemaline patients may benefit from therapies that promote muscle hypertrophy. Furthermore, this is the first clear demonstration that L-tyrosine supplements can significantly reduce both the clinical and pathological features of nemaline myopathy.

Materials and methods

Mice

All studies were performed in accordance with the guidelines of the National Health and Medical Research Council of Australia and the institutional Animal Care and Ethics Committee.

Three skeletal muscle hypertrophic transgenic mouse lines were used: (i) a mouse line that expresses the muscle isoform of IGF-1 driven by the rat myosin light chain 1/3 fast intron enhancer (obtained from Nadia Rosenthal, EMBL Monterotondo, Italy) (Musaro *et al.*, 2001); (ii) a mouse line that expresses the chicken FB28c *c-ski* isoform under the control of the MSV-LTR promoter (Stephen Hughes, National Cancer Institute, MA, USA) and has hypertrophy only in the fast IIB fibres (Sutrave *et al.*, 1990); and (iii) a mouse line generated in our laboratory that expresses the four-and-a-half LIM domain protein, FHL1 driven by the human α -skeletal actin promoter and has hypertrophy in the majority of muscle fibres (Cowling *et al.*, 2008).

Generation of the Acta1(H40Y) knock-in mouse line

The detailed description of the generation of the knock-in targeting construct is contained in the online Supplementary material. Briefly, the *Acta1*(H40Y) knock-in fragment was designed to contain a PGKneo cassette that is flanked upstream by a 5.1-kb genomic DNA sequence that extends from 1.84 kb upstream of exon 1 to 0.28 kb downstream of exon 7. Downstream of the PGKneo cassette is a 2.3-kb fragment of *Acta1* genomic DNA. This fragment contains additional sequences downstream of the 3'-end of the *Acta1* gene (Supplementary Fig. 1A). The location of the PGKneo cassette was chosen on the basis that the position was beyond the end of the *Acta1* gene 3'-UTR and did not impinge on any other known genes. The construct also contains a PGK–diphtheria toxin A chain (PGK–DTA) cassette that was used to select against embryonic stem cells containing random insertions of the knock-in construct.

The entire knock-in fragment was sequenced, linearized with SacII and then electroporated into R129/Sv embryonic stem cells. Positive clones were detected using Southern blotting, polymerase chain reaction screening as well as sequencing across the mutation site. Two positive clones were injected into ARC or ARC/Balb/c blastocysts and the resulting chimaeric males were mated with either R1–129 or C57BL/6Jarc females to generate mice on an inbred R1–129 or on a mixed R1–129xC57BL/6Arc background. Both polymerase chain reaction and Southern screening, together with DNA sequencing were used to ensure correct insertion of the mutation into the mouse genome (Supplementary Fig. 1B–D). Mass spectrometry confirmed the presence of the mutant α -skeletal actin protein in muscles from the knock-in mice (Supplementary Fig. 1E).

Electron microscopy

Transmission electron microscopy on muscles was performed as previously described (Joya *et al.*, 2004).

Immunohistochemical analyses of rod pathology

Mouse muscles were tissue collected and prepared for frozen sectioning as previously described (Corbett *et al.*, 2001). Nematine rods were detected immunohistochemically with an anti- α -actinin primary antibody (Joya *et al.*, 2004). The percentage of fibres with rods and the percentage of rod-containing nuclei in each muscle section were scored using the Image-Pro Plus software (Media Cybernetics).

Determining the percentage of fibres with internal nuclei

Mouse muscles were collected and prepared for frozen sectioning as previously described (Corbett *et al.*, 2001). Mid-belly transverse muscle sections (5 μ m) were fixed in 2% paraformaldehyde for 2 min and then stained with haematoxylin and eosin as described by Joya *et al.* (2004). Images of the entire muscle were collaged using Adobe Photoshop software and the percentage of fibres with internal nuclei in each muscle section were counted using Image-Pro Plus software.

Myosin heavy chain fibre type and diameter analysis and NADH staining of oxidative fibres

The percentage of MyHC2A and MyHC1 fibres in 8-week-old knock-in and wild-type soleus muscles, and the percentage of NADH-positive (oxidative) fibres in the flexor digitorum profundus muscle were determined as previously described (Nair-Shalliker *et al.*, 2004).

Assessing eye phenotype

The right eyes of 6- to 8-week-old knock-in and wild-type littermates were photographed using a Sony Handycam digital video camera (Sony Central). The vertical and horizontal eyelid openings were measured using the Image-Pro Plus software.

Forearm grip strength

The forearm grip strength of mice was measured using a dynamometer (Columbus Instruments) as previously described (Chang *et al.*, 2008). In short, the mouse was allowed to grasp a horizontal bar with its forepaws while suspended horizontally by the tail. The mouse was then gradually pulled away from the bar by its tail until the mouse released the bar. The maximum force (N) achieved before release was recorded by a force transducer. The mean force over 10 consecutive trials was used as a measurement of forearm grip strength for each mouse.

In vitro testing of single muscle contractile properties

Contractile properties of isolated extensor digitorum longus and soleus muscles were measured as described by Vlahovich *et al.* (2008). Optimal muscle length was determined with digital callipers during a series of isometric twitch contractions. Maximum isometric force (tetanic force) was determined from the plateau of the frequency–force curve (1–200 Hz) and expressed as force/cross-sectional area. Cross-sectional area was determined as described previously (Vlahovich *et al.*, 2008).

Mobility assessment

Mouse mobility was measured in a 1 m² open field apparatus (San Diego Instruments). Mobility was detected with photobeam detectors arranged around the perimeter of the field. The number of ambulatory movements (in X-Y-Z directions) in a 20-min interval was recorded. All experiments were performed between 9 am and 11 am.

Dietary L-tyrosine administration

The L-tyrosine (Sigma/Aldrich) supplement was individually prepared for each mouse (25 mg suspended in 60 μ l of distilled water). The tyrosine solution was dispensed with a sterile 1 ml hypodermic syringe into the mouth of the mouse taking care that all L-tyrosine solution was swallowed. Mice were treated with L-tyrosine for 4 weeks from 4 to 8 weeks of age.

Statistical analysis

Statistical significance was tested at $P < 0.05$ levels using a Students two-tailed unpaired *t*-test assuming unequal variances (PASW Statistics 18). All data were shown to be normally distributed using D'Agostino and Perron's test for Gaussian distribution (PASW Statistics 18).

Results

The *Acta1*(H40Y) nemaline mouse displays early lethality and severe muscle weakness

In keeping with the severity of the disease in patients with the *ACTA1*(H40Y) mutation, the *Acta1*(H40Y) knock-in mice experience early lethality; 52–61% of knock-in males and 3–5% of knock-in females died by 13 weeks of age (Fig. 1A). This occurred in knock-in mice on the inbred, R1–129 and the mixed, R1–129x57BL/6JArc backgrounds. All littermate wild-type control mice had a normal lifespan, living beyond 12 months of age. Patients with nemaline myopathy experience decreased mobility and can be wheelchair dependant due to muscle weakness. To examine the mobility in the knock-in mice, an open field apparatus was used. Spontaneous movements of mice at various ages were measured and the number of ambulations in 20 min is shown in Fig. 1B. Compared with wild-type mice, there was a significant decrease in the mobility of knock-in mice at weaning [post-natal day (PND) 21] and at various ages up until maturity (12 weeks of age).

To assess whether muscle weakness was a potential cause of premature death and poor movement, forearm grip strength was measured from early after weaning (PND15) until 8 weeks of age. A marked decrease in forearm grip strength was detected in the knock-in mice at PND15 and they remained weaker than wild-type littermates throughout their life (Fig. 1C). Although female knock-in mice did not die from muscle weakness, we detected a significant 49–54% deficit in forearm grip strength in knock-in females across all ages examined (PND15 to 8 weeks of age) (Fig. 1C).

To ascertain whether the whole limb weakness was intrinsic to skeletal muscle, we next measured force output of isolated extensor digitorum longus muscles from 8-week-old knock-in and wild-type littermates. Both absolute twitch and tetanic (200 Hz stimulation frequency) forces were markedly reduced in muscles from the knock-in mice; twitch and tetanic force in male and female knock-in mice were 30–40% of wild-type littermates (Supplementary Fig. 2A and B). Individual muscle weight (Supplementary Fig. 2F and G) and myofibre size (Fig. 2H) were also smaller in the knock-in mice compared with littermate controls. However, the lower force in the knock-in muscles is not simply due to myofibre atrophy as specific twitch and tetanic force (force/cross-sectional area) were also lower (2-fold) in the knock-in muscle (Fig. 1D and E, respectively). Force outputs in the slow-twitch soleus muscle were also significantly lower for the knock-in mice (Supplementary Fig. 2C and D). Thus, the

presence of the *Acta1*(H40Y) mutation leads to a marked loss of intrinsic muscle strength, which is presumably responsible for the decrease in mobility in the knock-in mice.

Distinct eye and facial phenotypes were also observed in the knock-in mice. Atrophy of facial muscles was evident in some knock-in mice. In addition, we observed that most knock-in mice had watery eyes and ptosis (drooping eyelids) (Fig. 1F). To assess the degree of eyelid abnormalities, the right eyes of 6- to 8-week-old mice were photographed and the palpebral fissure dimensions (vertical and horizontal separations between the upper and lower eyelids) were measured. The ratio of vertical-to-horizontal eyelid separation was used as an index of eyelid opening. A significant 39 and 51% deficit in eyelid opening was detected in knock-in female and male mice, respectively. This indicates that in the knock-in mice there is a loss of facial and eyelid muscle strength.

The *Acta1*(H40Y) mouse has the pathological features of patients with nemaline myopathy

One of the distinguishing features of patients with nemaline myopathy with the *ACTA1*(H40Y) mutation is the presence of nemaline rods in both the nuclei and the cytoplasm of myofibres (Nowak *et al.*, 1999). In the *Acta1*(H40Y) knock-in mouse, nemaline rods were also detected in the cytoplasm and the nucleus of the muscle fibres (Fig. 2A and B, respectively). Rods were observed in all muscles examined in the knock-in mouse and there was significant variation in the number of rod-containing fibres and rod-containing nuclei between muscles (Fig. 2C and D), as have been observed in human patients with nemaline myopathy and the *TPM3*(M9R) mouse model (Shafiq *et al.*, 1967; Wallgren-Pettersson *et al.*, 1999, 2003; Corbett *et al.*, 2001). Cytoplasmic rods were also detected in cardiomyocytes of knock-in hearts (data not shown). Another inclusion observed in the knock-in mouse muscle was electron-dense and -light areas replacing the sarcomeric filament arrays (Fig. 2E). On closer examination, the electron dense areas appear to be composed of thick filaments and organelles such as mitochondria; whereas, electron-light areas appear to contain thin filaments (Fig. 2E).

Focal myofibre damage, chronic regeneration and repair have been observed in nemaline myopathy (Shimomura and Nonaka, 1989; Gyure *et al.*, 1997; Ryan *et al.*, 2003), and recently these were confirmed as features of nemaline myopathy using array analyses (Sanoudou *et al.*, 2003, 2006). In muscle from the *Acta1*(H40Y) mouse, areas of myofibre degeneration (myofibres lacking eosin staining), infiltrating mononucleated cells and fibres with internal nuclei were detected (Fig. 2F) indicating that there was ongoing chronic repair or regeneration in this nemaline myopathy mouse model as well. To quantify the degree of chronic repair/regeneration, we counted the percentage of fibres with internal nuclei in soleus, extensor digitorum longus and flexor digitorum profundus muscles (Fig. 2G). In adult wild-type muscles internal nuclei were present in 1.2–3.5% of fibres. In the knock-in muscles, however, there was a much greater number of fibres

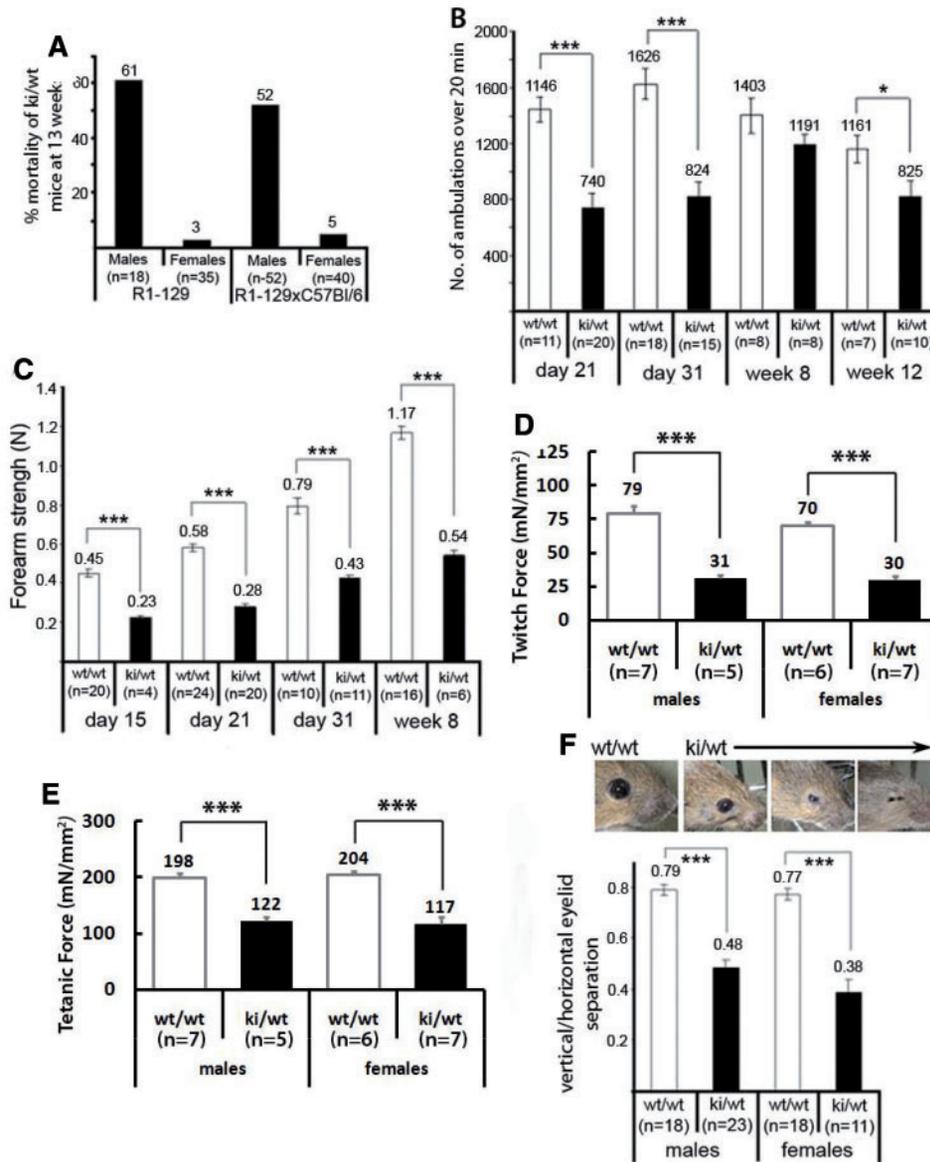


Figure 1 *Acta1*(H40Y) mice die early, have limb and eyelid muscle weakness and decreased ambulation. (A) The percentage of knock-in mice from the inbred (R1–129) and the mixed (R1–129xC57Bl/6) backgrounds that died by 13 weeks of age. (B) Total animal movements over 20 min in an open field apparatus. The number of ambulations on PNDs 21, 31 and 8 and at 12 weeks of age was significantly less in the knock-in (ki/wt) versus wild-type (wt/wt) female mice. (C) Forearm grip strength of female knock-in mice was significantly decreased compared with wild-type mice at PNDs 15, 21, 31 and at 8 weeks of age. (D and E) Isolated *in vitro* force output of extensor digitorum longus muscles from knock-in and wild-type mice. Specific (force/cross-sectional area) twitch (D) and tetanic force (E) were markedly decreased in knock-in versus wild-type mice. (F) The eyelid openings of knock-in mice are smaller than wild-type mice. *Top*: Photographs of 6- to 12-week-old mice showing the variation in the severity of the eyelid opening defect in knock-in versus wild-type mice. *Bottom*: The ratio of vertical-to-horizontal eyelid separation was measured to quantify the eyelid opening defect. Values are mean \pm SE. Statistical significance is indicated by: *** $P \leq 0.001$, * $P \leq 0.05$.

with internal nuclei in extensor digitorum longus, soleus and flexor digitorum profundus muscles compared with wild-type mice (26-, 6- and 4-fold increase, respectively) (Fig. 2G).

Myofibre atrophy is a feature of many congenital muscle diseases including nemaline myopathy (North *et al.*, 1997; Sanoudou and Beggs, 2001). Whole body and individual muscle weights were significantly lower in knock-in mice compared with wild-type

littermate controls (Supplementary Fig. 2E–G). To determine whether the lower muscle weight is due to myofibre atrophy, myofibre diameters of extensor digitorum longus muscles were measured (Fig. 2H). Significant fibre atrophy was present in the knock-in muscles, indicated by a 42% increase in the percentage fibres with diameters $<30 \mu\text{m}$ and a 41% decrease in fibres with diameters $>40 \mu\text{m}$ (Fig. 2H).

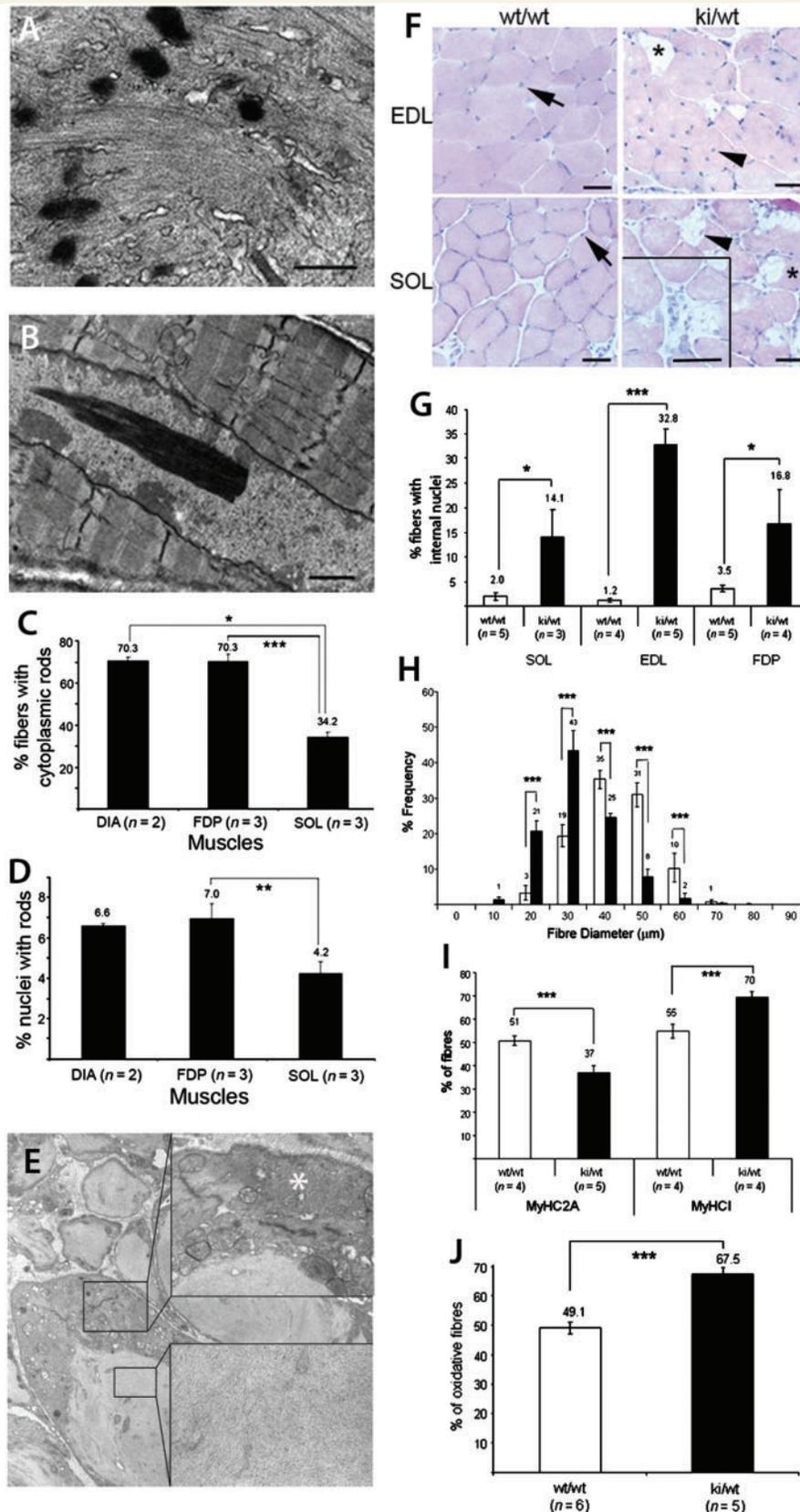


Figure 2 Muscle pathologies in the *Acta1*(H40Y) mouse. (A and B) Representative electron micrographs of cytoplasmic (A) and intranuclear (B) rods in female knock-in flexor digitorum profundus (FDP) and diaphragm (DIA) muscles, respectively. (C and D) Graphs show the percentage of fibres with cytoplasmic rods (C) and the percentage of nuclei with rods (D). Nemaline rods were detected on muscle sections with an anti- α -actinin antibody and co-stained with DAPI to detect nuclei. The number of fibres with cytoplasmic rods and

Increase in the number slow oxidative fibres is another feature of patients with nemaline myopathy (Volpe *et al.*, 1982). In normal mouse adult soleus muscle, ~50% of fibres express the fast myosin heavy-chain 2A isoform and the remaining fibres express the slow myosin heavy-chain 1 isoform (Fig. 2I). In the knock-in soleus, however, compared with wild-type mice, there was a 27% increase in type 1 fibres and a concomitant decrease in type 2A fibres (Fig. 2I). In keeping with shift towards slow fibre types, there was also an increase in the number of oxidative (NADH positive) fibres in muscles from the knock-in mice compared with wild-type mice (Fig. 2J). Thus, collectively the data show that the *Acta1*(H40Y) mouse has the features of the human disease, severe muscle weakness, cytoplasmic and nuclear nemaline rods, localized degeneration, chronic repair/regeneration, fibre atrophy and increase in the number of slow oxidative fibres.

IGF-1- and FHL1-induced muscle hypertrophy ameliorates the muscle weakness and immobility of the *Acta1*(H40Y) mice

Induction of muscle hypertrophy has been shown to improve muscle strength and reduce disease pathology in a number of different models of muscular dystrophy (Barton *et al.*, 2002; Bogdanovich *et al.*, 2002; Gregorevic *et al.*, 2002; Parsons *et al.*, 2006). To determine whether muscle hypertrophy could improve the muscle strength and mobility of the nemaline mice, we mated *Acta1*(H40Y) female mice with the following hypertrophic transgenic lines: IGF-1 (Musaro *et al.*, 2001), FHL1 (Cowling *et al.*, 2008) and *c-ski* (Sutrave *et al.*, 1990).

Crossing the knock-in mice with the three hypertrophic mouse lines had no impact on survival of the knock-in mice (data not shown). However, crossing with the hypertrophic mouse lines did lead to a significant increase in muscle weight (Supplementary Fig. 2F and G) and myofibre size (data not shown). This was associated with an improvement in forearm grip strength (Fig. 3A and B) and mobility (Fig. 3C and D) in the nemaline mice overexpressing FHL1 and IGF-1. FHL1 improved the forearm strength of knock-in female mice by 67% (Fig. 3A), while IGF-1 improved forearm strength by 25% (Fig. 3B). The improvement in

forearm strength of the knock-in mice was evident at PND21 for FHL1 and at PND31 for IGF-1 and was still apparent at 8 weeks of age (last time-point examined). The mobility of knock-in/IGF-1 and knock-in/FHL1 mice was similar to control wild-type mice (Fig. 3C and D). In contrast, despite the profound muscle hypertrophy in the *c-Ski* expressing knock-in mice, there was no improvement in forearm strength or mobility (Supplementary Fig. 2H and I). Thus, overexpression of IGF-1 and FHL1 eliminated the mobility defect of the knock-in mice, while *c-Ski* was without effect.

FHL1- and IGF-1-induced hypertrophy decreases nemaline rod pathologies

To determine whether FHL1 and IGF-1 overexpression in the knock-in mice reduces nemaline rod pathology, the number of rods was determined in flexor digitorum profundus, extensor digitorum longus, soleus and diaphragm muscles. Both FHL1 and IGF-1 reduced rod pathologies (Fig. 4), while *c-Ski* had no effect (data not shown). FHL1 reduced the percentage of fibres with cytoplasmic rods by 48–64% (Fig. 4A) and the percentage of nuclei with rods by 56–77% (Fig. 4B). IGF-1 was less effective at reducing cytoplasmic rod pathology, but still reduced the percentage of rods in knock-in extensor digitorum longus by 25% (Fig. 4C). There was also a clear trend towards a reduction (38–57% decrease in mean values) in intranuclear rods with IGF-1 but the differences were not statistically significant (Fig. 4D).

Oral L-tyrosine improved forearm and facial muscle weakness and immobility of the *Acta1*(H40Y) mice

L-Tyrosine was recently reported to provide beneficial effects on a small cohort of patients with nemaline myopathy ($n = 5$) (Ryan *et al.*, 2008). These patients were of variable ages and the causative mutations in the majority of these were not known. The effect of L-tyrosine in this study on muscle strength and clinical parameters was not convincing. We have used the *Acta1*(H40Y) mice to more rigorously investigate the effects of oral L-tyrosine on the clinical and pathological features of nemaline

Figure 2 Continued

number of nuclei with rods were counted from entire muscle sections. (E) Electron microscopy images showing segregated filament pathology in female knock-in (ki/wt) flexor digitorum profundus muscle. At higher magnification, the electron dense areas appear to be composed of thick filaments and organelles such as mitochondria (area marked by asterisk, *top*); whereas, electron-light areas appear to contain thin filaments (*bottom*). (F) Transverse section (5 μm) of extensor digitorum longus (EDL) and soleus (SOL) muscles from 8-week-old female wild-type and knock-in mice stained with haematoxylin and eosin. Wild-type (wt/wt) muscles have peripheral nuclei (arrows in extensor digitorum longus and soleus) whereas knock-in muscles have peripheral and internal nuclei (arrowhead in extensor digitorum longus). Degraded fibres with areas lacking eosin stained contractile proteins (asterisks in ki/wt extensor digitorum longus and soleus) and areas of infiltrating mononucleated cells (see high magnification insert in ki/wt soleus) were also detected in knock-in muscles. (G) There was an increase in the percentage of fibres with internal nuclei in 8-week-old female knock-in soleus, extensor digitorum longus and flexor digitorum profundus muscles. (H) There was a decrease in fibre size in the female knock-in muscle. Shown is the frequency of fibres with increasing 10 μm diameter intervals in the extensor digitorum longus muscles of knock-in (ki/wt) (black bars) and wild-type (wt/wt) (white bars) female mice ($n = 3/\text{group}$). (I) There was an increase in the percentages of myosin heavy chain (MyHC) 2A and 1 fibres in the soleus muscles from female knock-in mice (8-week-old). (J) Oxidative (NADH positive) fibre number was also increased in the flexor digitorum profundus muscles from female knock-in mice. Values in the graphs are mean \pm SE. Statistical significance is indicated by: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$. Scale bars = 500 nm (A), 1 μm (B) and 40 μm (F).

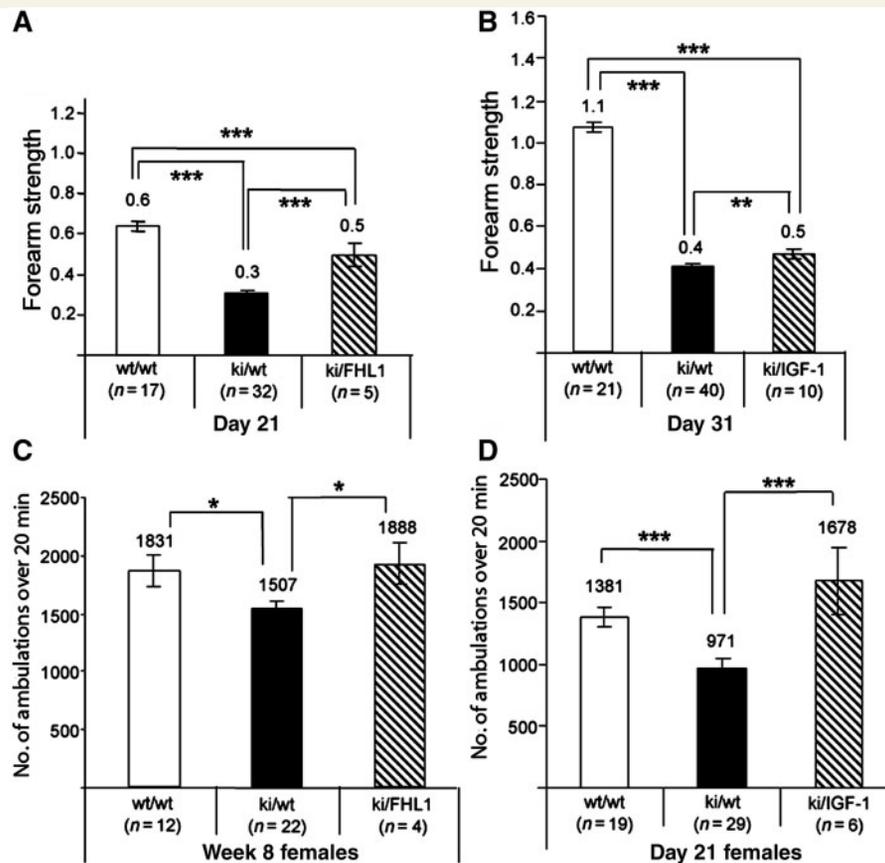


Figure 3 FHL1- and IGF-1-induced hypertrophy increases the forearm strength and mobility of nemaline mice. FHL1 and IGF-1 transgenic mice were mated with *Acta1*(H40Y) mice and muscle strength was measured. (A and B) Crossing knock-in mice with FHL1 (A) and IGF-1 (B) transgenic mice improved forearm grip strength of knock-in mice at PND 21 (for FHL1) and PND 31 (for IGF-1) (data for females are shown). (C and D) Both FHL1 (C) and IGF-1 (D) increased the total number of ambulations in knock-in female mice. This occurred at 3 weeks of age for knock-in/IGF-1 (C) and 8 weeks of age for knock-in/FHL1 mice (D). Values are mean \pm SE. Statistical significance is indicated by: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

myopathy. Knock-in and wild-type littermates were given oral L-tyrosine (25 mg/day) for 4 weeks commencing at PND20. The forearm strength and mobility of treated and untreated mice were assessed after 10 days and 4 weeks of treatment.

Ten days of L-tyrosine supplementation was sufficient to improve the mobility of knock-in animals. Activity was increased (by 64%) back to wild-type levels at this time-point (Fig. 5A). However, this length of L-tyrosine treatment had no effect on forearm grip strength or muscle size (data not shown), but 4 weeks of treatment did increase muscle weight (data not shown) and improved forearm strength by 40% (Fig. 5B). This length of treatment also reduced eyelid weakness as indicated by an 18% increase in vertical-to-horizontal eyelid opening (Fig. 5C).

Oral L-tyrosine reduced myofibre degeneration, repair and rod pathologies

We next determined whether L-tyrosine supplementation could improve muscle pathologies in the knock-in mice. As shown

above, myofibre degeneration and repair were evident in non-tyrosine treated knock-in muscles; presence of fibres with internal nuclei and areas of myofibre degeneration and areas of infiltrated mononucleated cells (Figs 2F and 6A). Four weeks of L-tyrosine treatment substantially reduced the pathological appearance of the knock-in muscles. In all muscles examined from the treated mice, there were fewer disrupted fibres; the cytoplasm of myofibres were homogeneously stained with eosin and virtually all myonuclei were at the fibre periphery (Fig. 6A). To confirm the reduction in chronic myofibre repair/regeneration with L-tyrosine treatment, the number of internal nuclei was counted in untreated wild-type and knock-in/wild-type muscles and treated knock-in/wild-type muscles (Fig. 6B). L-Tyrosine treatment significantly reduced the percentage of fibres with internal nuclei in all muscles examined (Fig. 6B). In the soleus and flexor digitorum profundus muscles, the number of fibres with internal nuclei approached wild-type levels. L-Tyrosine treatment also reduced the number of nemaline rods. There was a 50% reduction in the percentage of fibres with cytoplasmic rods in the soleus muscle and a non-significant reduction in fibres with rods in two other muscles examined (Fig. 6C). Thus, oral L-tyrosine supplementation had a major impact on major pathologies associated with nemaline

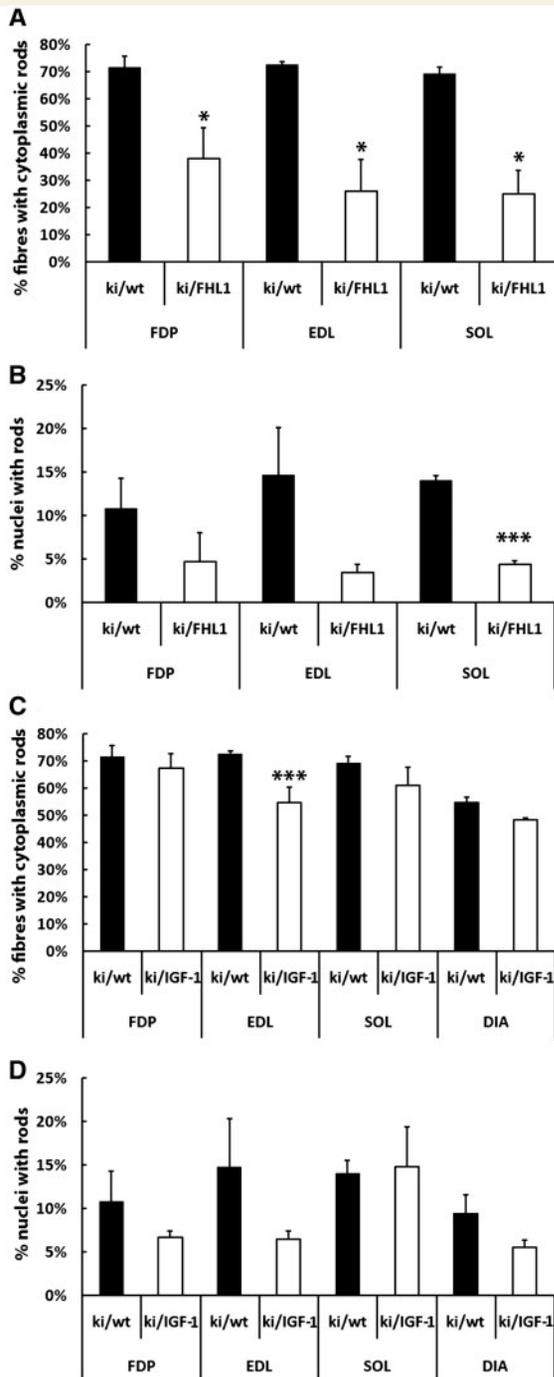


Figure 4 FHL1 and IGF-1 reduces rod pathology of the *Acta1*(H40Y) mice. (A and B) Overexpression of FHL in the knock-in mice (8-weeks-old) reduces the percentage of fibres with cytoplasmic rods (A) and percentage of nuclei with rods (B). (C) Overexpression of IGF-1 in the knock-in mice (8 week) also reduced the percentage of fibres with cytoplasmic nemaline rods. (D) There was also a trend towards a decrease in percentage of fibres with nuclear rods in the knock-in mice over-expressing IGF-1, but the effect was not statistically significant for any muscle ($P \geq 0.0972$). Values are mean \pm SE for 2–4 mice/group. Data are for female mice. Statistical significance is indicated by: *** $P \leq 0.001$, * $P \leq 0.05$. DIA = diaphragm; EDL = extensor digitorum longus; FDP = flexor digitorum profundus; SOL = soleus.

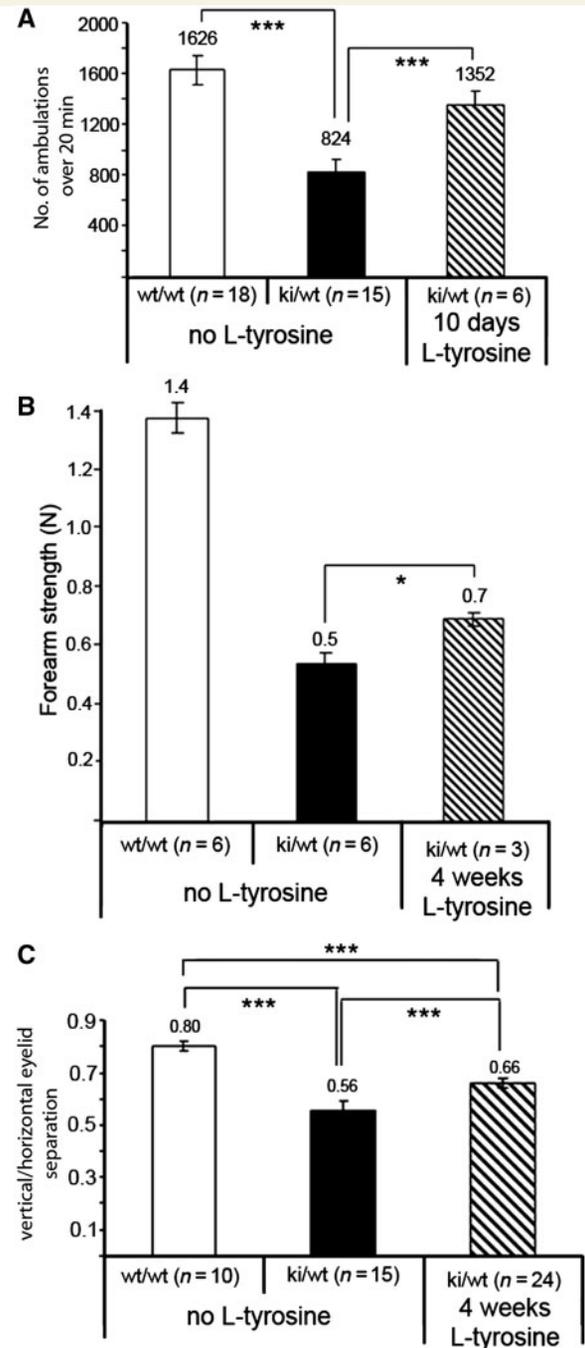


Figure 5 Dietary L-tyrosine increases ambulations and forearm and eyelid muscle strength of the *Acta1*(H40Y) mice. (A) L-tyrosine (25 mg/day for 10 days) treatment of the knock-in (ki/wt) mice increased the total number of ambulations to levels approaching the wild-type (wt/wt) mice. (B) Forearm grip strength was also significantly increased in knock-in (ki/wt) mice with L-tyrosine treatment. (C) Eyelid opening (indicator of eyelid muscle strength) was also significantly increased by L-tyrosine treatment. Shown is the ratio of eyelid vertical-to-horizontal separation. Values are mean \pm SE for 8- to 12-week-old female mice. Statistical significance is indicated by: *** $P \leq 0.001$, * $P \leq 0.05$.

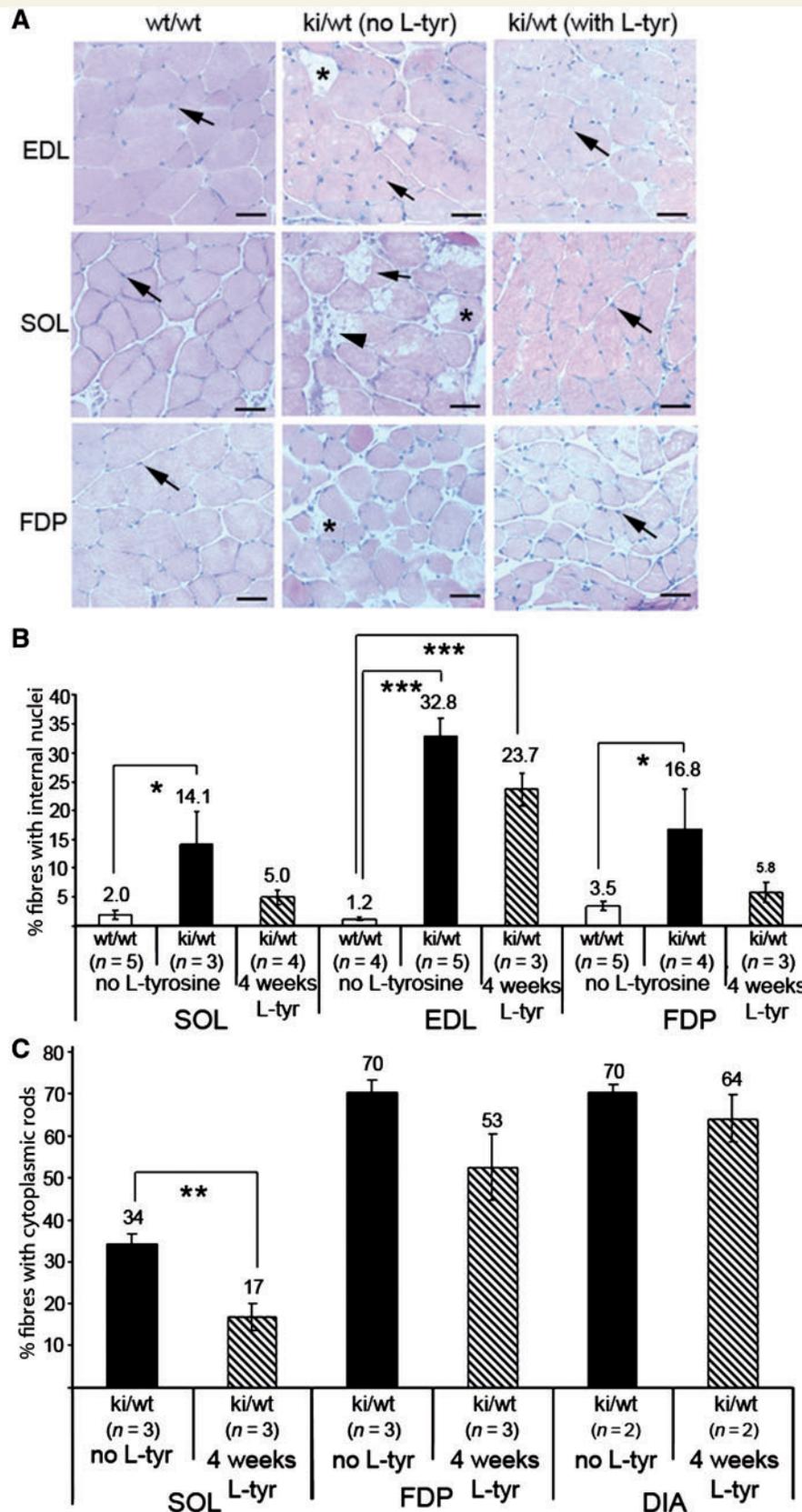


Figure 6 L-tyrosine treatment of *Acta1*(H40Y) mice ameliorates nemaline pathologies. (A) Haematoxylin and eosin stained sections (5 μm) of extensor digitorum longus (EDL), soleus (SOL) and flexor digitorum profundus (FDP) muscles from 8-week-old untreated wild-type (wt/wt), untreated knock-in (ki/wt) and L-tyrosine (L-tyr) treated (25 mg/day for 4 weeks) knock-in female mice. Untreated knock-in muscles have numerous fibres with internal nuclei (arrows), fibres lacking eosin staining (asterisks) and the presence of infiltrated

myopathy, myofibre degeneration/repair and the abundance of nemaline rods.

Discussion

We report the generation of an *Acta1*(H40Y) mutant mouse model for nemaline myopathy, which displays the clinical and pathological features of patients with nemaline myopathy with this mutation, including early onset of muscle weakness, increased mortality, decreased mobility and cytoplasmic and nuclear rods. Evidence of myofibre repair/regeneration was also detected in this mouse supporting the recent data obtained in patients (Sanoudou *et al.*, 2003) and the *TPM3*(M9R) nemaline myopathy mouse (Sanoudou *et al.*, 2006) that this is a common feature of nemaline myopathy. Recently, Ravenscroft *et al.* (2011) described a transgenic mouse model of the *Acta1*(D286G) mutation, however, unlike the human patients with this mutation, this mouse was not severely affected due to low level of expression of the mutant protein (25% of wild-type allele); crossing this mouse with hemizygous *Acta1* knockout mice increased the mutant protein load to 50% and resulted in a more severe phenotype (Ravenscroft *et al.*, 2011). Using the knock-in approach, we have created a monogenetic dominant negative severe *Acta1* mouse model for nemaline myopathy, which closely phenocopies the condition of patients with this mutation.

The *Acta1*(H40Y) mutation is a potent mutation with 50–60% of the males dying by 13 weeks of age. Although only a small percentage of female mice died early (3–5%), the effect on body and muscle weight, forearm strength and mobility was still significant. The deaths of knock-in mice may be due to respiratory muscle weakness since cytoplasmic and intranuclear rods and other pathologies were found to be particularly severe in the knock-in diaphragm muscle. This is also a common cause of death in severely affected patients with nemaline myopathy (Shimomura and Nonaka, 1989; Rifai *et al.*, 1993; Barohn *et al.*, 1994; Sasaki *et al.*, 1997; Nowak *et al.*, 1999; Ilkovski *et al.*, 2001). However, heart function also may be impaired in the knock-in mice since cytoplasmic rods were detected in cardiomyocytes of knock-in hearts (data not shown).

Cardiomyopathy is a rare occurrence in patients with nemaline myopathy (Ryan *et al.*, 2001) and was not reported in patients with the *Acta1*(H40Y) mutation (Nowak *et al.*, 1999). This is thought to be due largely to the low levels of α -skeletal actin expressed in human cardiac tissue (Ilkovski *et al.*, 2005). Mice also express a relatively low amount of skeletal actin compared with cardiac actin (5%:95%, respectively) (Vandekerckhove *et al.*, 1986). Therefore, the presence of rods in the heart of the

Acta1(His40Tyr) mouse may reflect that this is a very potent dominant negative protein.

Unexpectedly, the H40Y mutation was more potent in males with only 3–5% of female knock-in/wild-type mice dying by 13 weeks of age. The majority of male knock-in mice die at the onset of sexual maturity (6–8 weeks of age), which suggests that this mutation may disrupt the normal maturation of muscles critical for life (e.g. respiratory muscles). It is possible that this is more severe in male mice as their rate of post-natal growth is more rapid than female mice. However, it is interesting to note that of the two patients with this mutation, the most severe was a male who died at 2 months of age (Nowak *et al.*, 1999) while the female patient is still alive at 51 years of age (Wallgren-Pettersson, personal communication). However, no evidence for sexual dimorphism of disease severity has been reported for patients with nemaline myopathy with other mutations, and there are too few patients to tell whether disease severity is truly greater in males with the H40Y mutation. Elucidation of the reason for the enhanced potency of this mutation in the male knock-in mice may provide important insights into the mechanisms of muscle weakness in patients with this and other *ACTA1* mutations.

A defect in the opening of the eye lids (ptosis) was a novel feature of the knock-in mice. This is presumably due to weakness in the eyelid muscles and muscles surrounding the eye. Ptosis has not been reported in nemaline patients. However, selective facial muscle weakness is a characteristic feature of nemaline myopathy leading to tenting of the mouth and altered facial appearance (elongated face) (North *et al.*, 1997; Sanoudou and Beggs, 2001). As has been observed in many mouse models of muscle disease, the effects of a disease-causing mutation in the human are not always phenocopied precisely in the mouse.

Induction of hypertrophy reduces nemaline myopathy disease phenotype

The presence of fibre hypertrophy in the muscles of patients with nemaline myopathy (Laing *et al.*, 1995) and the *TPM3*(M9R) mouse model correlates with a milder form of the disease (Corbett *et al.*, 2001). Therefore, we investigated whether inducing hypertrophy in the *Acta1*(H40Y) mouse could improve muscle strength and reduce disease pathology. The approach taken was to cross the knock-in mice with a number of transgenic mouse lines that have hypertrophy specifically in skeletal muscle (IGF-1, FHL1 and *c-Ski*) (Sutrave *et al.*, 1990; Musaro *et al.*, 2001; Cowling *et al.*, 2008). All three crosses led to significant skeletal muscle hypertrophy, but only IGF-1 and FHL1 significantly improved muscle strength and mobility of the nemaline myopathy

Figure 6 Continued

mononucleated cells (arrow head). Remarkably after L-tyrosine treatment, knock-in muscles histomorphology approach the wild-type muscle; there are very few degenerating fibres or infiltrating mononucleated cells and almost all myonuclei are at the periphery (arrows). (B) There is a significant decrease in the percentage of fibres with internal nuclei in knock-in muscle after treatment with dietary L-tyrosine. (C) There was also a significant decrease in the number of cytoplasmic rods in the soleus muscle of knock-in mice (8-week-old) with L-tyrosine treatment. There was also a decrease in the mean percentage fibres with rods in the flexor digitorum profundus and diaphragm muscle but the differences were not statistically significant. The data were obtained from entire muscle sections. Values are mean \pm SEM from female mice. Statistical significance is indicated by: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$. Scale bars: A = 40 μ m; mean \pm SE.

mice. IGF-1 and FHL1 also reduced disease pathology (fibre degeneration and number of rods) contributing to the improved muscle strength and mobility of the knock-in mice. The reduction in disease pathology with these factors may be due to stimulation of muscle repair as IGF-1 is known to induce satellite cell proliferation and differentiation, and FHL1 acts through GATA-2 (Cowling *et al.*, 2008), which is important for fusion of myoblasts into mature myofibres (Michel *et al.*, 2004). The improved strength and mobility of the knock-in mice with IGF-1 and FHL1 is an important finding as it suggests that therapies that stimulate muscle hypertrophy may improve the mobility and hence the quality of life of patients with nemaline myopathy.

It is interesting that although overexpression of *c-Ski* produced the greatest hypertrophic effect of the three factors tested (3-fold increase in weight of the fast-twitch muscles), it had no effect on muscle strength or disease phenotype. This may be because *c-Ski* does not induce hypertrophy through the normal regulated increase in non-sarcomeric and sarcomeric components. In this mouse, the hypertrophy is associated with an increase in the amount of non-sarcomeric (sarcoplasmic reticulum membranes, cytosol and mitochondria) versus sarcomeric material (Bruusgaard *et al.*, 2005). This leads to areas devoid of myofibrils and a reduction in intrinsic myofibre force (force/myofibre size) capacity (Charge *et al.*, 2002; Bruusgaard *et al.*, 2005). Thus, it is apparent that not all hypertrophic factors are as equally effective at improving muscle strength and mobility.

A particularly dramatic effect of IGF-1 and FHL1 was a decrease in the number of rods in the knock-in mice. FHL1 had a greater effect than IGF-1, the former producing a decrease in cytoplasmic and nuclear rods in a number of muscles while IGF-1 only decreased the percentage of cytoplasmic rods in the extensor digitorum longus muscle. The reason for the decrease in rods is unclear. However, in a previous study, we showed that exercise can decrease the percentage of rod-containing fibres in the *TPM3(M9R)* nemaline myopathy mouse model (Joya *et al.*, 2004). Thus, the increase in activity with FHL1 and IGF-1 overexpression in the knock-in mice may be responsible for the decrease in the number of rod-containing fibres. However, IGF-1 had a greater effect on mobility than FHL1 and yet it had a lesser effect on rod pathologies. This suggests that exercise may not be the only mechanism for the significant improvement in cytoplasmic rod pathologies in the knock-in/FHL1 mice.

L-Tyrosine supplement reduces disease phenotypes of *Acta1(H40Y)* mice

The isolated reports of beneficial effects of L-tyrosine in nemaline patients (Kalita, 1989) have been difficult to confirm due to the rarity of the condition and the variability of disease presentation (Ryan *et al.*, 2008). We show here that L-tyrosine supplementation significantly improved the clinical features and the muscle pathologies of the *Acta1(H40Y)* nemaline myopathy mouse model. L-Tyrosine treatment increased body weight and mobility, and decreased the amount of muscle degeneration, muscle repair/regeneration and rod pathologies of knock-in mice. This study also detected a remarkable 40% improvement in forearm strength with

L-tyrosine treatment. As discussed for IGF-1 and FHL1, improved strength with L-tyrosine may also be due to increased mobility. However, L-tyrosine may also have exercise-independent effects as it also improved the watery eyes, facial atrophy and ptosis in knock-in mice, the latter indicating that it led to an increase in the strength of the skeletal muscles responsible for correct positioning of the eyelid. Thus, L-tyrosine has a clear and widespread impact on the limb and facial muscle weakness in this model of severe nemaline myopathy.

L-Tyrosine is a non-essential amino acid, readily synthesized from phenylalanine by phenylalanine hydroxylase. Its main fate is incorporation into proteins. However, a small portion of circulating tyrosine crosses the blood–brain barrier and is converted into L-dopa, the rate-limiting step for the generation of the catecholamine neurotransmitters dopamine, norepinephrine and epinephrine (Nagatsu *et al.*, 1964; Molinoff and Axelrod, 1971; Acworth *et al.*, 1988; During *et al.*, 1989; Kumer and Vrana, 1996). These molecules are involved in numerous pathways including motor coordination (Jordan *et al.*, 2008), learning (Tanaka, 2002), memory (Tanaka, 2002) and the sleep–wake cycle (Monti and Monti, 2007). There are several studies showing the beneficial effects of L-tyrosine on mobility. In one study, L-tyrosine improved mobility in older, mobility impaired mice (Thurmond and Brown, 1984). L-Tyrosine supplementation has also been shown to improve maze performance in diet restricted rats (Avraham *et al.*, 1996, 2001a, b).

The beneficial effects of L-tyrosine in the knock-in mice may be due to a number of different actions of L-tyrosine. L-Tyrosine improved the mobility of knock-in mice as early as 24 h after treatment (data not shown). The rapidity of this effect indicates that it is not due to a resolution of muscle pathology or intrinsic muscle strength. One possibility is an increase in L-tyrosine-derived catecholamines as activation of noradrenergic and dopaminergic receptors are known to improve locomotion in mammals (Jordan *et al.*, 2008). This is supported by studies showing that suppression of the dopamine pathway (blocking dopamine synthesis or transport) in mice decreases locomotor activity (Nishii *et al.*, 1998; Andersson *et al.*, 2006). Whether the improvement in muscle pathology and muscle strength with L-tyrosine is solely due to increased activity or to some other action of L-tyrosine requires investigation.

L-Tyrosine is readily available, easy to administer and our data suggest that long-term L-tyrosine supplementation is relatively safe since no overt adverse effects were apparent. We did not detect any muscle pathologies in long-term-treated wild-type mice and there was no adverse impact on overall muscle strength and mobility (data not shown). This agrees with human studies where high doses of L-tyrosine (100–300 mg/kg/day) have been well tolerated in children and adults with Rett Syndrome and phenylketonuria (Growdon *et al.*, 1982; Lykkelund *et al.*, 1988; Nielsen *et al.*, 1990; Mazzocco *et al.*, 1992; Pietz *et al.*, 1995; Kalsner *et al.*, 2001).

Conclusions

We have generated a knock-in [*Acta1(H40Y)*] mouse model of a severe form of nemaline myopathy, which closely

phenocopies the human condition. The muscle pathologies detected in the *Acta1*(H40Y) mouse, together with the early onset of muscle weakness and decreased mobility, suggest that this is a good candidate model for trialling potential therapies for nemaline myopathy. Indeed, induction of hypertrophy in this mouse led to significant improvement in muscle strength, nemaline pathologies and mobility. Remarkably, dietary L-tyrosine supplementation also improved mobility and reduced the severity of most of the nemaline pathologies, including muscle degeneration and myofibre repair. This suggests that L-tyrosine may also be beneficial for patients with dystrophy and other muscle degenerative conditions. The rapid and dramatic impact of L-tyrosine on mobility in the nemaline myopathy mice raises the possibility that L-tyrosine may be beneficial for a wide range of patients with impaired mobility.

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Supplementary material

Supplementary material is available at *Brain* online.

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