Cardiac $\alpha$-actin over-expression therapy in dominant ACTA1 disease

Gianina Ravenscroft¹, Elyshia McNamara¹, Lisa M. Griffiths², John M. Papadimitriou⁴, Edna C. Hardeman⁶, Anthony J. Bakker⁵, Kay E. Davies⁷, Nigel G. Laing¹,³ and Kristen J. Nowak¹,*

¹Western Australian Institute for Medical Research; Centre for Medical Research, The University of Western Australia, Nedlands, WA, Australia, ²Department of Neuropathology, PathWest Anatomical Pathology and ³Department of Anatomical Pathology, Royal Perth Hospital, Perth, WA, Australia, ⁴School of Pathology and Laboratory Medicine and ⁵School of Anatomy, Physiology and Human Biology, The University of Western Australia, Crawley, WA, Australia, ⁶School of Anatomy, Department of Medical Sciences, University of New South Wales, Sydney, NSW, Australia and ⁷Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK

More than 200 mutations in the skeletal muscle $\alpha$-actin gene (ACTA1) cause either dominant or recessive skeletal muscle disease. Currently, there are no specific therapies. Cardiac $\alpha$-actin is 99% identical to skeletal muscle $\alpha$-actin and the predominant actin isoform in fetal muscle. We previously showed cardiac $\alpha$-actin can substitute for skeletal muscle $\alpha$-actin, preventing the early postnatal death of Acta1 knock-out mice, which model recessive ACTA1 disease. Dominant ACTA1 disease is caused by the presence of ‘poison’ mutant actin protein. Experimental and anecdotal evidence nevertheless indicates that the severity of dominant ACTA1 disease is modulated by the relative amount of mutant skeletal muscle $\alpha$-actin protein present. Thus, we investigated whether transgenic over-expression of cardiac $\alpha$-actin in postnatal skeletal muscle could ameliorate the phenotype of mouse models of severe dominant ACTA1 disease. In one model, lethality of ACTA1D286G, Acta1¹/² mice was reduced from $\sim$59% before 30 days of age to $\sim$12%. In the other model, Acta1H40Y, in which $\sim$80% of male mice die by 5 months of age, the cardiac $\alpha$-actin transgene did not significantly improve survival. Hence cardiac $\alpha$-actin over-expression is likely to be therapeutic for at least some dominant ACTA1 mutations. The reason cardiac $\alpha$-actin was not effective in the Acta1H40Y mice is uncertain. We showed that the Acta1H40Y mice had endogenously elevated levels of cardiac $\alpha$-actin in skeletal muscles, a finding not reported in dominant ACTA1 patients.

INTRODUCTION

Skeletal muscle $\alpha$-actin is one of six actin proteins in higher eukaryotes and is the predominant isoform expressed in postnatal skeletal muscle, where it is fundamental to muscle contraction. Mutations in the ACTA1 gene encoding the human isoform of this protein can cause devastating diseases (1). All patients with ACTA1 disease have weakness and hypotonia leading to a reduced ability to move, breathe and swallow (2), apart from one exceptional patient described to have hypertonia (3). Around 50% of ACTA1 disease patients die within the first year of life (2). However, ACTA1 disease shows a spectrum of clinical phenotypes, with some patients having mild disease compatible with adulthood survival (4) and/or adult onset (5).

The pathological features identified in patients’ skeletal muscle biopsies may include excess actin filaments, nemaline bodies, intranuclear rods (6), fibre-type disproportion (7), cores (8), caps (9), zebra bodies (10) and dystrophic features (11,12), but also minimal change myopathy (8). Some biopsies have only one predominant pathology, but others show a combination of features (13,14). These findings support the hypothesis that the structural lesions comprise a pathological continuum rather than distinct, separate entities (1).

The majority of ACTA1 disease patients have only a single, mostly de novo, dominant mutation (2). Despite the presence of normal skeletal muscle $\alpha$-actin protein, the expression of the mutant isoform gives rise to disease, presumably through a ‘poison protein’ or ‘dominant gain of function’ mechanism.
About 10% of patients have recessive ACTA1 disease as a result of genetic or functional null mutations (2), with some recessive ACTA1 disease patients when tested having a complete absence of skeletal muscle α-actin protein (10).

Only symptomatic treatment, e.g. respiratory support is currently available for ACTA1 disease patients, and although this brings significant benefit (16), there is a need to investigate therapeutic approaches that address the fundamental disease mechanisms.

A number of potential treatments have been indicated or investigated. For example, anecdotal evidence suggests endurance exercise training may be beneficial for at least some less severely affected patients with certain ACTA1 mutations (1,17). Nguyen et al. (18) experimentally investigated using genetic methodologies three factors that induce myofibre hypertrophy, and also tested oral l-tyrosine administration in a dominant ACTA1 disease mouse model (knock-in Acta1H40Y). They transgenically introduced three factors that cause muscle hypertrophy: four and a half LIM domains 1 (FHL1), insulin-like growth factor 1 (IGF1) and c-ski to the Acta1H40Y mouse model. All three factors induced muscle hypertrophy. FHL1 and IGF1, but not c-ski also increased muscle strength and returned mobility to normal levels, but none increased survival. Nguyen et al. (18) tested oral delivery of l-tyrosine, since oral l-tyrosine had been reported to be of benefit in a small number of nemaline myopathy patients (19,20). Four weeks of oral l-tyrosine increased muscle weight, forearm strength mobility, but an effect on survival was not tested (18).

Another method of therapy for genetic disorders, which has been used in clinical practice, is the upregulation of one member of a protein family to compensate for the lack of another member from that family. This has been investigated for muscle diseases, most notably for utrophin in dystrophin-deficiency (21–23). However, the most widely known and used example of such upregulation is upregulation of fetal hemo-globin to treat diseases associated with the adult hemoglobins (17,28), yet cardiac involvement in dominant ACTA1 disease patients is only rarely reported (29).

For dominant ACTA1 disease, multiple observations support the notion that changing the ratio of mutant skeletal muscle α-actin to total striated muscle α-actin (the sum of cardiac and skeletal muscle α-actins) may affect the severity of the disease. These include:

1. Mildly or seemingly unaffected somatic mosaic parents of severely affected children with dominant ACTA1 disease suggest a reduced proportion of mutant α-actin protein is associated with survival into adulthood and very mild clinical symptoms for multiple ACTA1 mutations (6,15).
2. Around 15% of α-actin in the heart of dominant ACTA1 disease patients should be mutant skeletal muscle α-actin (17,28), yet cardiac involvement in dominant ACTA1 disease patients is only rarely reported (29).
3. Extraocular muscles express similarly high levels of cardiac α-actin to the mature heart (28), and even severely affected ACTA1 disease patients do not have ophthalmoplegia (30).
4. The severity of disease in mouse models of dominant ACTA1 disease appears to correlate with the ratio of mutant to wild-type skeletal muscle α-actin protein (31).

RESULTS

ACTA1D286G.Acta1+/−.ACTC mice: bodyweight and survival

ACTA1D286G.Acta1+/−.ACTC mice (Fig. 1A), with only ~9% of ACTA1D286G.Acta1+/−.ACTC mice dying by postnatal day 18 and ~88% surviving to at least 60 days of age. All ACTA1D286G.Acta1+/−.ACTC mice (littermate controls for ACTA1D286G.Acta1+/−.ACTC mice) had a normal lifespan (data not shown), similar to TgACTA1D286G+/+ mice (31). As per previous findings (31), the body mass of ACTA1D286G.Acta1+/−.ACTC mice at postnatal day 12 was significantly lower than wild-type mice (Fig. 1B). Although the weights of ACTA1D286G.Acta1+/−.ACTC mice also varied significantly from wild-type mice, they were significantly heavier than ACTA1D286G.Acta1+/−.ACTC mice (Fig. 1B).

For dominant ACTA1 disease, multiple observations support the notion that changing the ratio of mutant skeletal muscle α-actin to total striated muscle α-actin (the sum of cardiac and skeletal muscle α-actins) may affect the severity of the disease. These include:

1. Mildly or seemingly unaffected somatic mosaic parents of severely affected children with dominant ACTA1 disease suggest a reduced proportion of mutant α-actin protein is associated with survival into adulthood and very mild clinical symptoms for multiple ACTA1 mutations (6,15).
2. Around 15% of α-actin in the heart of dominant ACTA1 disease patients should be mutant skeletal muscle α-actin (17,28), yet cardiac involvement in dominant ACTA1 disease patients is only rarely reported (29).
3. Extraocular muscles express similarly high levels of cardiac α-actin to the mature heart (28), and even severely affected ACTA1 disease patients do not have ophthalmoplegia (30).
4. The severity of disease in mouse models of dominant ACTA1 disease appears to correlate with the ratio of mutant to wild-type skeletal muscle α-actin protein (31).

We therefore tested experimentally whether over-expression of cardiac α-actin might ameliorate the phenotype of dominant skeletal muscle α-actin disease. We crossed the same TgACTC mouse line with two dominant skeletal muscle α-actin disease mouse models; TgACTA1D286G.KOActa1+/− (31) and KActa1H40Y (18). Approximately, 59% of TgACTA1D286G.KOActa1+/− mice present between postnatal day 12 and 17 with splayed legs, while ~80% of male KActa1H40Y mice die before 5 months of age. Our data show significant amelioration of phenotype for the TgACTA1D286G.KOActa1+/− line, but not the KActa1H40Y line. Thus over-expression of cardiac α-actin appears to be therapeutic in at least one mouse model of dominant ACTA1 disease.
Both ACTA1D286G.Acta1+/- and ACTA1D286G.Acta1+/+ ACTC mice have improved survival and body-weight. (A) Survival curves for ACTA1D286G.Acta1+/- ACTC (n = 184) and ACTA1D286G.Acta1+/+ ACTC (n = 242) mice reveal significant improvement for mice with the ACTC transgene (Log-rank Mantel-Cox test, P < 0.0001). (B) Body mass of ACTA1D286G.Acta1+/- ACTC mouse pups (n = 37) is significantly increased compared with ACTA1D286G.Acta1+/+ (n = 15) mouse pups, but still less than the body weights of wild-type mouse pups (WT; n = 30) (one-way ANOVA, P < 0.0001; all pups weighed at postnatal day 12).

ACTA1D286G.Acta1+/- ACTC mice: muscle morphology, histology and pathology

Genotype had a significant effect on mean gastrocnemius myofibre diameter of ACTA1D286G.Acta1+/- , ACTA1D286G.Acta1+/+ . ACTC and ACTA1D286G.Acta1+/- ACTC day 12 mouse pups (Fig. 2A). ACTA1D286G.Acta1+/- ACTC mice had myofibre diameters intermediate between ACTA1D286G.Acta1+/- and ACTA1D286G.Acta1+/+ .ACTC mice, indicating the ACTC transgene partially restored myofibre diameter (Fig. 2A).

Variation in myofibre sizes and structural lesions similar to those seen in ACTA1D286G.Acta1+/- muscles were apparent in ACTA1D286G.Acta1+/- .ACTC gastrocnemius muscle at day 12 (Fig. 2B). Gastrocnemius muscles of adult (3–4 months old) ACTA1D286G.Acta1+/+.ACTC mice had evident nemaline bodies (Fig. 2C), internally located nuclei, large variation in myofibre size and ringbinden (Fig. 2C and D).

ACTA1D286G.Acta1+/- ACTC mice: cardiac α-actin and mutant D286G skeletal muscle α-actin levels

Cardiac α-actin immunohistochemistry was performed to confirm the expression of the ACTC transgene in adult ACTA1D286G.Acta1+/- .ACTC mice. Cardiac α-actin was preferentially expressed in MHCIIB myofibres in ACTA1D286G.Acta1+/- .ACTC mice, while cardiac α-actin expression in age-matched wild-type mice was minimal, except for within intrafusal myofibres (arrow; Fig. 3A). Increased relative levels of cardiac α-actin in the skeletal muscles of both ACTA1D286G.Acta1+/- .ACTC and ACTA1D286G.Acta1+/+.ACTC mice when compared with wild-type mice were corroborated by western blotting (Fig. 3B and C).

The contribution of mutant D286G skeletal muscle α-actin to the total striated α-actin protein pool was determined by mass spectrometry (Fig. 3D). In ACTA1D286G.Acta1+/+.ACTC mouse pups, ~40% of the total striated α-actin pool was the mutant D286G protein, whereas in ACTA1D286G.Acta1+/- .ACTC mice it was ~20%, similar to mildly affected TgACTA1D286G.Acta1+/- mice (Fig. 3D) and (31). Thus the presence of the ACTC transgene was associated with reduced levels of mutant D286G protein.

ACTC mice exhibit pathological features. (A) Morphometry of gastrocnemius myofibres at postnatal day 12–14 (one-way ANOVA, P = 0.0018) showing the mean myofibre diameter in ACTA1D286G.Acta1+/- .ACTC and ACTA1D286G.Acta1+/- .ACTC day 12 mouse pups. Gastrocnemius muscles of adult (4 months old) ACTA1D286G.Acta1+/- .ACTC gastrocnemius muscles revealing the presence of nemaline bodies (patches of dark blue/purple staining, arrowheads), internally located nuclei, a large variation in myofibre size and ringbinden (arrows), Phalloidin-FITC and Hoechst staining confirming the large variation in fibre size, increased number of internally located nuclei and ringbinden (arrows) in ACTA1D286G.Acta1+/- .ACTC gastrocnemius muscle (4 months old). Scale bars: 50 μm.
**ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC mice: voluntary running wheel activity**

Voluntary running wheel activity at 1 month could not be determined for ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC mice as the majority die before this age. There was no statistically significant difference between ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC and ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC mice, which have similar levels of mutant D286G protein (Fig. 3D), for the total distance run per day (Fig. 4A), the average speed (Fig. 4B) and maximum speed (Fig. 4C).

**Acta1<sup>H40Y</sup> ACTC mice: bodyweight, survival and breeding**

On an R1-129 genetic background, 52–61% of male Acta1<sup>H40Y+/−</sup> mice die by 13 weeks of age, whereas only 3–5% of female Acta1<sup>H40Y+/−</sup> mice die by this time (18). Additionally both male and female KI Acta1<sup>H40Y+/−</sup> mice had significantly decreased bodyweights compared with their littermate controls (18). Thus the male mice model moderately severe human dominant disease (2).

We compared survival of male Acta1<sup>H40Y</sup> ACTC and Acta1<sup>H40Y</sup> ACTC mice on the CBB6F1 background (Fig. 5A). Both groups had only ~20% survival by 6 months of age (Fig. 5A), with no increase in survival associated with the presence of the ACTC transgene. There was no increased mortality of female Acta1<sup>H40Y+/−</sup> and Acta1<sup>H40Y</sup> ACTC mice compared with wild-type mice (data not shown).

Both male and female Acta1<sup>H40Y</sup> ACTC and Acta1<sup>H40Y</sup> male mice weighed significantly less than sex-matched wild-type mice on the CBB6F1 genetic background (Fig. 5B).

We were unable to produce homozygous Acta1<sup>H40Y+/+</sup> mice and in fact never derived offspring from KI Acta1<sup>H40Y+/−</sup> male mice, even when they were mated with wild-type females, suggesting these mice were infertile.

---

**Figure 3.** ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC mice have elevated cardiac α-actin expression. (A) Immunohistochemistry highlights preferential expression of cardiac α-actin in MHCIIB myofibres of ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC muscle; WT shown as comparison (3- to 4-months-old mice; scale bars: 50 μm). Arrows highlight cardiac α-actin-positive intratrusal myofibres of a muscle spindle in both ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC muscle and WT muscle. (B–C) Western blotting and quantification relative to myosin heavy chain (MHC) shows increased expression of cardiac α-actin in ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC muscle (n = 10) compared with WT (n = 2, P < 0.05); ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC muscle (n = 2, P < 0.05) and ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC muscle (n = 6, P < 0.01) mice (all aged between postnatal day 10–17, one-way ANOVA). The difference in cardiac α-actin abundance between ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC (n = 10) and ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC (n = 3) muscle was not significant. *P < 0.05, **P < 0.01. (D) Contribution of mutant D286G skeletal muscle α-actin to the total striated muscle α-actin protein pool, as determined by mass spectrometry on gastrocnemius muscle lysates (all aged between postnatal day 10–17), was significantly lower in ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC (n = 10) muscle compared with ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> muscle (n = 5) (one-way ANOVA, P < 0.0001). D286G abundance was significantly greater in ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> mice compared with WT (P < 0.0001) and ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC (n = 5) mice (P < 0.001); and in ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC (P < 0.001) and ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> mice compared with WT mice, but was not significantly different between ACTA1<sup>D286G</sup> Acta<sup>1+/−</sup> ACTC and ACTA1<sup>D286G</sup> ACTC (P > 0.05). Legend same as for Figure 3C.
Acta1H40Y.ACTC mice: muscle morphology, histology and pathology

At 1 month of age, the extensor digitorum longus (EDL), gastrocnemius and quadriceps muscles of both Acta1H40Y and Acta1H40Y.ACTC mice (of both sexes) showed nemaline bodies and large regions of thin filament aggregates, but these were not visible in soleus muscles (Fig. 6A). Gomori trichrome and hematoxylin and eosin staining of tibialis anterior muscle showed paler regions correlating with thin filament accumulations (Fig. 6B). The presence of nemaline bodies and thin filament accumulations (actin lakes) was confirmed in EDL and gastrocnemius muscles from 4-week-old Acta1H40Y and Acta1H40Y.ACTC (of both sexes) mice. Similarly, male Acta1H40Y.P<0.05 and Acta1H40Y.ACTC (P<0.05) mice are significantly smaller than male WT mice, but there is no significant difference between male Acta1H40Y and Acta1H40Y.ACTC mice. Similarly, female Acta1H40Y (P<0.05) and Acta1H40Y.ACTC (P<0.01) mice are significantly smaller than female WT mice, but there is no significant difference between Acta1H40Y and Acta1H40Y.ACTC female mice (one-way ANOVA). *P<0.05, **P<0.01 relative to WT.

Acta1H40Y.ACTC mice: voluntary running wheel activity

Analysis of voluntary running wheel activity of both male and female 1-month-old Acta1H40Y mice demonstrated a significant decrease in distance covered, average speed and maximum speed compared with wild-type mice (Fig. 8A, B and C). The decreased performance was not improved by the presence of the ACTC transgene in Acta1H40Y.ACTC mice.

DISCUSSION

In this study, we attempted to reduce the severity of disease in two mouse models of dominant skeletal muscle α-actin (ACTA1) disease by transgenically increasing the levels of cardiac α-actin in the skeletal muscle of these mice. The severity of the more severe dominant ACTA1 disease mouse model studied here, Acta1H40Y, Acta1H40Y.ACTC mice, was significantly improved by delivering cardiac α-actin, but the severity of the second model, the Acta1H40Y mouse, was not.
continued presence of \( \text{ACTA1} \) disease-related pathological features. Survival of the \( \text{ACTA1}^{D286G}.\text{Acta1}\)^{+/-} mice, despite abundant nemaline bodies, ringbinden, internal nuclei and variation in myofibre size corresponds with the lack of correlation between muscle pathology and disease severity reported in patients (5). These data also suggest caution concerning the relevance of measuring the frequency of pathological features in animal models of \( \text{ACTA1} \) and related diseases when evaluating the efficacy of treatments.

Western blotting confirmed increased relative levels of cardiac \( \alpha \)-actin in the skeletal muscles of both \( \text{ACTA1}^{D286G}.\text{Acta1}\)^{+/-} \( \text{ACTC} \) and \( \text{ACTA1}^{D286G}.\text{Acta1}\)^{+/-} \( \text{ACTC} \) mice (Fig. 3B and C), which correlated with the desired outcome of decreasing the proportion of mutant \( D286G \) skeletal muscle \( \alpha \)-actin protein (Fig. 3D). Transgenic cardiac \( \alpha \)-actin over-expression reduced the mutant \( D286G \) skeletal muscle \( \alpha \)-actin load from \(~\)40% in severely affected \( \text{ACTA1}^{D286G}.\text{Acta1}\)^{+/-} mice, to \(~\)20% in the \( \text{ACTA1}^{D286G}.\text{Acta1}\)^{+/-} \( \text{ACTC} \) mice with a significantly improved phenotype, a level akin to that detected in the only mildly affected \( \text{ACTA1}^{D286G}+/- \) mice (31).

**Acta1H40Y.\text{ACTC} \) mice**

In contrast to the findings achieved with the severe \( \text{ACTA1}^{D286G}.\text{Acta1}\)^{+/-} mouse model, an amelioration of disease phenotype was not detected after introduction of the \( \text{ACTC} \) transgene to the \( \text{Acta1H40Y} \) model. The survival rate of 20% of male \( \text{Acta1H40Y} \) mice on the CBB6F1 background by 6 months of age was not improved (Fig. 5A). The lower body-weight of male and female \( \text{Acta1H40Y} \) mice was also not counteracted by \( \text{ACTC} \) transgenic expression (Fig. 5B).

Nemaline bodies and large ‘actin lakes’ were seen in 1-month-old EDL, gastrocnemius and quadriceps muscles from both male and female \( \text{Acta1H40Y.\text{ACTC}} \) and \( \text{Acta1H40Y} \) mice, but not in soleus muscles (Fig. 6A and B). Similarly, electron microscopy demonstrated the presence of nemaline bodies and the thin filament accumulations in the predominantly fast twitch EDL and gastrocnemius muscles (Fig. 6C). This finding of pathological lesions only within the predominantly fast twitch muscles, similar to expression of transgenes by skeletal muscle promoters (21,32,33), was unexpected since endogenous skeletal muscle \( \alpha \)-actin is expressed in both slow and fast twitch myofibres. Slow-twitch predominant myofibres may be selectively spared in mouse models of nemaline myopathy.

The few surviving 6-months-old male \( \text{Acta1H40Y} \) \( \text{ACTC} \) mice showed striking skeletal muscle pathology, such as nemaline bodies and variation in myofibre size (Fig. 6D) similar to surviving 6-months-old \( \text{Acta1H40Y} \) male and female mice. Therefore the presence of the \( \text{ACTC} \) transgene did not appear to influence the severity of pathological features not unlike the \( \text{ACTA1}^{D286G}.\text{Acta1}\)^{+/-} \( \text{ACTC} \) mice described earlier.

**Increased levels of cardiac \( \alpha \)-actin in Acta1H40Y mice**

Unexpectedly, cardiac \( \alpha \)-actin levels were increased in \( \text{Acta1H40Y} \) mice, that do not have the \( \text{ACTC} \) transgene and to similarly high levels to \( \text{Acta1H40Y.\text{ACTC}} \) mice with the \( \text{ACTC} \) transgene (Fig. 7A to D). This suggests the \( \text{ACTC} \) transgene had little effect in increasing the levels of cardiac \( \alpha \)-actin in this model beyond endogenous levels. As with the \( \text{ACTC} \)
transgene driven by the HSA promoter in Acta1H40Y ACTC mice, cardiac α-actin expression in Acta1H40Y mice without the transgene was predominantly in MHCIIIA myofibres (Fig.7C), with MHCIIA myofibres negative for cardiac α-actin (Fig. 7A–C). In Acta1H40Y muscle, only very occasional small myofibres showed double labelling for developmental MHC and cardiac α-actin, indicating that the majority of cardiac α-actin was being expressed in mature myofibres (Fig. 7A) and was not due to the presence of regenerating, immature myofibres.

How the presence of the Acta1H40Y knock-in allele evokes increased cardiac α-actin in MHCIIIA myofibres is unclear. It could be hypothesized that the phenotype of the Acta1H40Y mice would be more severe without the elevated levels of endogenously cardiac α-actin. Increased cardiac α-actin levels have not been reported for any patients with dominant ACTA1 disease (5), unlike patients with recessive ACTA1 disease who show upregulated cardiac α-actin levels (5,10). However, to our knowledge, muscle biopsies from patients with the p.H40Y ACTA1 mutation have not been tested for cardiac α-actin, so it is possible that increased cardiac α-actin levels may also occur in patients with the p.H40Y ACTA1 mutation.

The sex difference in severity in the Acta1H40Y mice also remains unexplained. Sex difference in severity is not generally considered to occur in patients with dominant ACTA1 disease (2). At the time Nguyen et al. published their study, two patients were known with p.H40Y ACTA1 mutations—a male who died at 2 months of age (6) and a female patient still alive at 51 years of age (18). This at the time matched the phenotype in the Acta1H40Y mice. However, one of two additional cases now in the ACTA1 locus-specific database (http://www.dmd.nl/nmdb2/home.php?Select_db=ACTA1) is a severely affected female, which does not fit with the sex difference exhibited by the mouse model.

The fact that one p.H40Y ACTA1 patient is alive in her 50s indicates the H40Y mutation need not always be associated with a severe disease phenotype and suggests the existence of
of disease severity for other modifying factors. In the mice, obviously one factor is sex, but there must be other factors since some male mice also survive modifying factors. In the mice, obviously one factor is sex, but

Both the TgACTA1D286G+/+, KO Acta1+/− and KI Acta1H40Y/− line were bred with the transgenic cardiac α-actin mouse line (26) (‘Coco’, TgACTCΔco, CBβ6F1Arc mouse line), which over-expresses cardiac α-actin in predominantly myosin heavy chain (MHC) IIb containing myofibres. This resulted in TgACTA1D286G+/, KO Acta1+/−, TgACTCΔco and Acta1H40Y−/−, TgACTCΔco mice, respectively.

As the ACTCΔco transgene is inserted into the X chromosome, all males were Acta1+/−, and we bred all females to homozygosity (ACTA1+/−). For analysis, TgACTA1D286G+/+, KO Acta1+/−, TgACTCΔco individuals (hereafter denoted as Acta1+/−, Acta1+/−, ACTCΔco) either TgACTA1D286G+/+, KO Acta1+/−, TgACTCΔco or wild-type mice (on a CBβ6F1ArcXFVB/n background) were used as controls (hereafter denoted as Acta1+/−, Acta1+/−, ACTCΔco or wild-type, respectively). To study KI Acta1H40Y−/− mice, we compared them with either wild-type (CBβ6F1) or KI Acta1H40Y−/−, ACTCΔco mice (hereafter described as Acta1H40Y−/−, wild-type and Acta1H40Y−/−, ACTCΔco, respectively).

Body mass and survival of mice

The body mass of Acta1D286G−/−, Acta1+/−, Acta1D286G−/−, Acta1+/−, ACTCΔco and wild-type mice was determined at postnatal day 12, whereas for both male and female Acta1H40Y−/−, Acta1H40Y−/−, ACTCΔco and wild-type mice, the body mass was measured at 1 month of age.

Kaplan–Meier survival curves and log-rank tests were performed for the Acta1D286G−/−, Acta1+/− strain versus the Acta1D286G−/−, Acta1+/−, ACTCΔco strain, and male mice for the Acta1H40Y−/− versus the KI Acta1H40Y−/−, ACTCΔco strain.

Histology and immunohistochemistry

Freshly excised skeletal muscles were frozen in optimum cutting temperature medium using liquid nitrogen cooled isopentane. Gomori trichrome and hematoxylin and eosin staining were performed on 10 μm sections according to standard procedures (35). Morphometry was investigated by determining mean fibre diameter on gastrocnemius myofibres at day 12–14 for Acta1D286G−/−, Acta1+/−, Acta1D286G−/−, Acta1+/−, ACTCΔco and

example, it would be interesting to determine in the future whether a Drosophila model of the p.H40Y ACTA1 mutation could be rescued by either wild-type skeletal muscle or cardiac α-actin.
Ac1-20.4.2, diluted 1:20, Sigma) were conjugated to Zenon male

Acta1H40Y and 10% fetal calf serum (FCS) in phosphate buffered saline

room temperature for 1 h in 1% bovine serum albumin (BSA)

Previously (26). Briefly, 10

series of ethanols, embedded in araldite resin and cured for

1 h, and they were then dehydrated through an ascending

7.4. Osmium tetroxide (2%) was used to post-fix samples for

1 h, and after three 5 min washes in PBS

Excised skeletal muscles were cut into very thin strips before im-

mersion in 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH

488 secondary antibody (incubated 1:1000). After final

washes in PBS for all sections, they were mounted in Hydromount (National Diagnostics). Images were taken with an

Olympus IX-71 fluorescent microscope using an Olympus DP-71 camera.

Following fixation with 2% paraformaldehyde, muscle sec-

tions were stained with a 1:1000 dilution of phalloidin-FITC to

label filamentous (F-)actin, and the nuclei counterstained with

Hoechst.

Electron microscopy (EM)

Excised skeletal muscles were cut into very thin strips before im-

mersion in 2.5% glutaraldehyde in 0.1 m cacodylate buffer, pH

7.4. Osmium tetroxide (2%) was used to post-fix samples for

1 h, and they were then dehydrated through an ascending

series of ethanols, embedded in araldite resin and cured for

24 h or more at 70°C. An LKB 8800 ultramicrotome was utilized to

cut ultrathin sections (70 nm), and a Philips CM-10 transmission

EM to view grids. Grids were stained in saturated acidified uranyl acetate and lead citrate. Original negative film images

captured were scanned to a digital format and saved as a jpeg file.

Liquid chromatography mass spectrometry-multiple

reaction monitoring

The amount of D286G skeletal muscle α-actin protein was deter-

mined as described in Ravenscroft et al. (31). The mass spec-

trometry technique in Nguyen et al. (18) was attempted for
determining the amount of H40Y skeletal muscle α-actin protein,

but in the current study was not efficacious at quantifying

wild-type and H40Y skeletal muscle α-actin proteins.

Western blots and densitometry

Western blots were performed as per Nowak et al. (26), with
densitometry conducted using Image J.

Voluntary running wheel experiments

The voluntary activity of 1-month-old mice was monitored over

24 h periods for 7 consecutive days. Mice were housed singly in
cages containing a standard mouse running wheel connected to a

speedometer that recorded distance travelled, time spent

moving, average and maximum speeds.

Statistics

GraphPad Prism 4 was used to conduct two-tailed Student’s

t-tests with Welch’s correction for all the statistical analyses of
two data sets and one-way ANOVAs for comparing more than
two data sets. Where significance was obtained, Neuman–

Keuls multiple comparisons tests were employed. All data are

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Background strain</th>
<th>Definition</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>CBB6F1 or CBB6F1 × FVB/n</td>
<td>Non-genetically modified mice from the same mixed background strains as the genetically modified mice.</td>
<td>WT</td>
</tr>
<tr>
<td>Tg4ACTA1D286G+/+ .KO.Acta1+/− (26)</td>
<td>CBB6F1</td>
<td>Transgenically expresses ACTC using HSA promoter.</td>
<td>ACTC</td>
</tr>
<tr>
<td>Tg4ACTA1D286G+/+ .KO.Acta1+/− .Tg4ACTA1C (31)</td>
<td>CBB6F1</td>
<td>Transgenically expresses mutant ACTA1 (D286G) using HSA promoter. Only one endogenous Acta1 allele expressed.</td>
<td>ACTA1D286G.Acta1+/−</td>
</tr>
<tr>
<td>Tg4ACTA1D286G+/+ .KO.Acta1+/− .Tg4ACTA1C (18)</td>
<td>CBB6F1</td>
<td>Transgenically expresses mutant ACTA1 (D286G), and WT ACTC (both using HSA promoter). Only one endogenous Acta1 allele expressed.</td>
<td>ACTA1D286G.Acta1+/−.ACTC</td>
</tr>
<tr>
<td>KIAceta1H40Y+/− (18)</td>
<td>CBB6F1</td>
<td>Expresses mutant Acta1 (H40Y) from the knocked-in allele, and WT Acta1 from the one remaining endogenous allele.</td>
<td>Acta1H40Y</td>
</tr>
<tr>
<td>KIAceta1H40Y+/− .Tg4ACTA1C</td>
<td>CBB6F1</td>
<td>Expresses mutant Acta1 (H40Y) from the knocked-in allele, and WT Acta1 from the one remaining endogenous allele. Transgenically expresses WT ACTC using HSA promoter.</td>
<td>Acta1H40Y. ACTC</td>
</tr>
</tbody>
</table>

ACTA1 = human, Acta1 = mouse, which are identical at the protein level; HSA, human skeletal muscle actin.
presented as the mean ± standard error of the mean, with n in parenthesis.

**Conflict of Interest statement.** None declared.

**FUNDING**

This research was funded by Australian National Health and Medical Research Council (NH&MRC) project grant (APP1026933), L'Association Française contre les Myopathies (dossier 14970), National Health and Medical Research Council Future Fellowship (FT100100734), G.R. by NH&MRC Early Career Fellowship (APP1035955) and Research Council Future Fellowship (FT100100734), G.R. by Infrastructure Fund. K.J.N. was supported by Australian Medical Research Council (NH&MRC) project grant (MDA4067), A Foundation Building Strength and The Western dossier 14970, the Muscular Dystrophy Association of the USA (APP1026933), L’Association Franc¸aise contre les Myopathies.

**REFERENCES**


